LSM 510 / LSM 510 *DuoScan*
LSM 510 META / LSM 510 META *DuoScan*

Release 4.0

March 2006

Knowledge of this manual is required for the operation of the instrument. Would you therefore please make yourself familiar with the contents of this manual and pay special attention to hints concerning the safe operation of the instrument.

The specifications are subject to change; the manual is not covered by an update service.

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How to make best use of the LSM 510 and LSM 510 META operating instructions:

This operating manual also includes the instructions for the LSM 510 META *DuoScan* and the LSM 510 *DuoScan* systems.

A few symbols in these operating instructions will help you to recognize the nature and purpose of information immediately:

The WARNING symbol warns against hazards for the user that might arise when operating the laser.

This WARNING symbol warns against hazards from dangerously high voltages.

The CAUTION symbol warns against faults and hazards that might arise during operation and which might cause damage to the unit.

The NOTE symbol will help you to optimally solve your work problem. It represents a practical tip which will help you to find out which settings and methods are capable of improving or accelerating a procedure.

The HOT SURFACE symbol warns against hazards for the user that might arise when touching the lamp housing during operation.

The MAINS PLUG symbol remembers service personal to pull the mains plug before opening the device housing.

Depending on the problem, these operating instructions will supply you with various possibilities:

- If you want to know where to find certain general areas of information, refer to the following outline of sections to get a general overview.
- You will find a detailed table of contents at the start of every chapter. There you will see at a glance what topics are dealt with in detail.

Always remember: **The time you invest in getting acquainted with the product will pay for itself many times over in your application task.**

Contents

1 Notes on Device Safety

This section contains general notes on device safety, safe operation, and possible hazards caused by failure to observe the instructions.

2 LSM 510 and LSM 510 META - Setup Requirements

The Setup Requirements section outlines the installation and supply requirements of the LSM 510 and LSM 510 META Microscope Systems, together with the relevant specifications.

3 Introduction to Laser Scanning Microscopy

Here you will find an introduction to Laser Scanning Microscopy, with an explanation of the principles of confocal imaging. The section also outlines the ways to present LSM image series in three dimensions, and introduces you to the performance features of your LSM 510 or LSM 510 META.

4 Operation in Expert Mode

In the Operation section you will find the most important steps and procedures of the LSM menu structure. The step-by-step description how to get an image will be shown by typical application examples including the WINDOWS XP graphic user environment.

5 VBA Programming for LSM

6 Routine Mode and Tools

This section contains a description of the Routine Mode for scanning images using the LSM scanning module and the use of the tools for setting the microscope.

7 3D for LSM 510 and LSM 510 META

This section contains a description of the LSM 3D software package (basic program and add-ons). At the same time, all functions and settings are presented in a systematic form and in the order in which they can be reached from the basic menu via sub-menus and dialog boxes.

8 Annex

The annex contains the Application-specific Configurations, special notes and information for using the LSM microscope and the brochure The confocal Laser Scanning Microscopy.

9 Multiphoton Laser Scanning Microscopy - Using the Zeiss LSM 510 META NLO

10 Certification

11 Brief Operating Manual

This section contains the brief instructions of the LSM 510 and LSM 510 META Microscope Systems.

12 Laser safety warning labels

CHAPTER 1 NOTES ON DEVICE SAFETY

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1 NOTES ON DEVICE SAFETY

1.1 General

The LSM 510 and LSM 510 META laser scanning microscope, including its original accessories and compatible accessories from other manufacturers, may only be used for the purpose of microscopic techniques.

Laser Scanning Microscopes (LSM) are intended for high resolution imaging of biological or material samples, whereby in contrast to wide field microscopy the specimen is illuminated raster-fashion with a focused laser beam and the optical arrangement prevents light from out-of-focus regions of the specimen contributing to image formation.

☞ Installation and commissioning of the LSM 510 and LSM 510 META system must be performed by authorized Carl Zeiss service staff. The system should not be used prior to instruction by a Carl Zeiss representative.

The manufacturer will not assume liability for any malfunction or damage caused by anything other than the intended use of the LSM 510 or LSM 510 META or individual modules or parts of it, nor by any repair or other service operation performed or attempted by persons other than duly authorized service staff. Any such action will invalidate any claim under warranty, including parts not directly affected by such action. This also includes the modification of the system computer with new cards, etc. by the user. The use of a camera at the base port of Axiovert 200 M Combi stands is not allowed for reasons of laser safety. Any manipulation will result in the loss of warranty of laser safety.

Please read also the notes on device safety and manuals of the microscope, the HBO, the HAL and additional optional devices, if ordered, as the UV Laser, the piezo focusing device, the heating inserts and the Ti:Sa Laser.

⇒ As the system is largely operated via menus on a computer, you should be familiar with the principles of the operating system and its WINDOWS, WINDOWS 2000 or Windows XP graphical user interface. The respective manuals are supplied together with the programs.

The LSM 510 and LSM 510 META are devices that belong to laser hazard class 3B. The systems are equipped with safety interlocks that comply with laser hazard class 3B and 4. If equipped with a Ti:Sa Laser (see list in section 1.6), the LSM 510 and LSM 510 META are devices that belong to laser hazard class 4. WHO recommendations concerning health and industrial protection when handling laser devices must be observed. The operator of the unit must also observe all and any relevant statutory accident prevention regulations. The user is referred to the safety data sheet provided together with the manual.

1.2 Warning and Information Labels

The warning and information labels attached on the LSM 510 and LSM 510 META must be observed. Check whether all of the labels shown below are provided on your instrument, and contact Carl Zeiss Germany or one of the service agencies if you should discover that any of the labels should be missing. You will receive a free replacement.

Description of labels

Caution: Faults and hazards that might arise during operation which might cause damage to the unit or injury to the user.

Attention: Laser irradiation hazards possible when operating the system.

Attention: High voltage.

Pull the mains plug before opening the device housing.

Caution: Hot surface.

Caution: UV radiation.

Caution: Fingers can be caught.

The arrow points to the opening where laser light comes out during operation of the system.

Other labels on the system include one of the above depicted symbols and a detailed description of the handling instructions. See also the following drawings of the system parts.

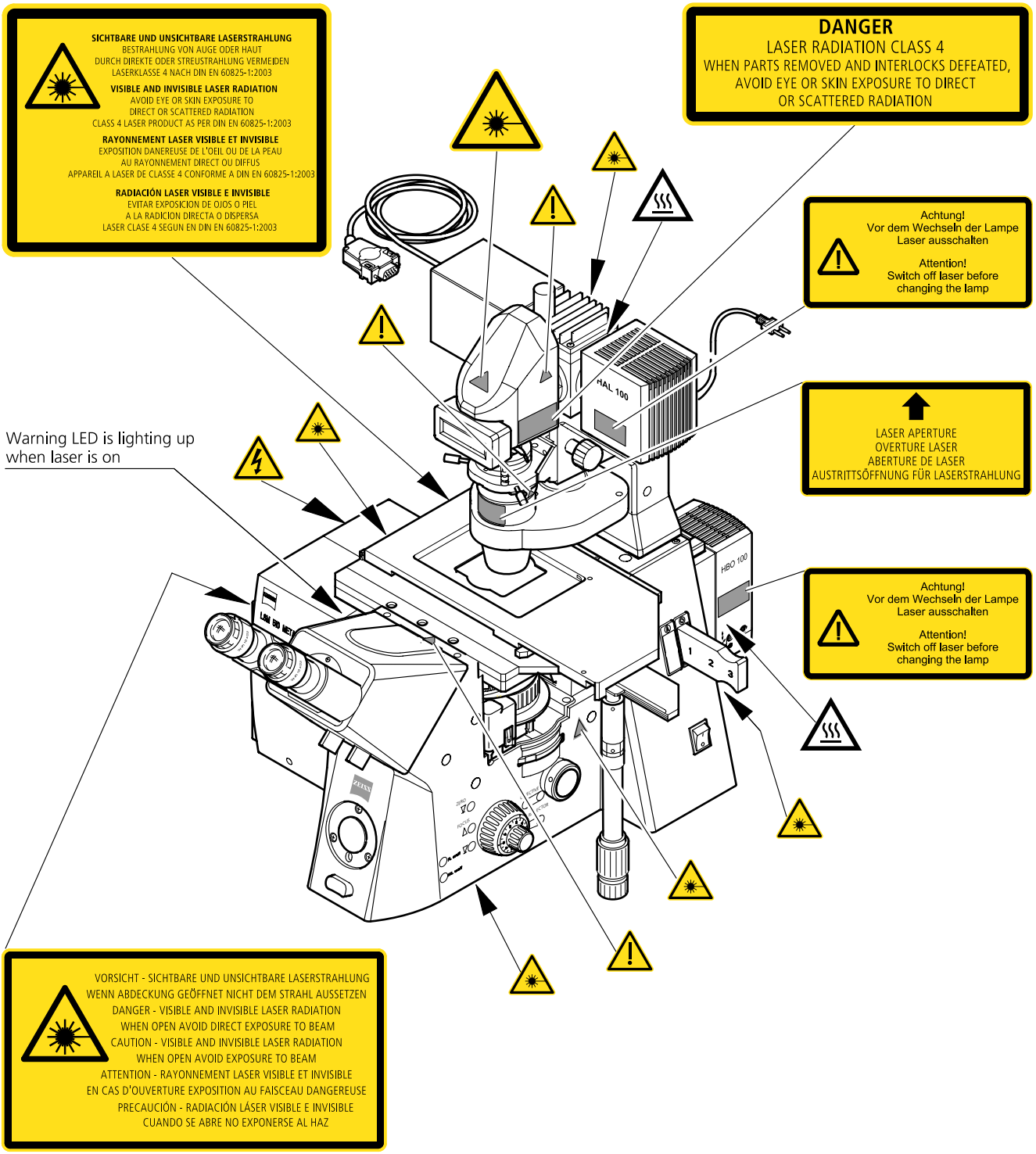

Fig. 1-1 Warning and information labels on the Axiovert 200 M microscope with the LSM 510 META scanning module

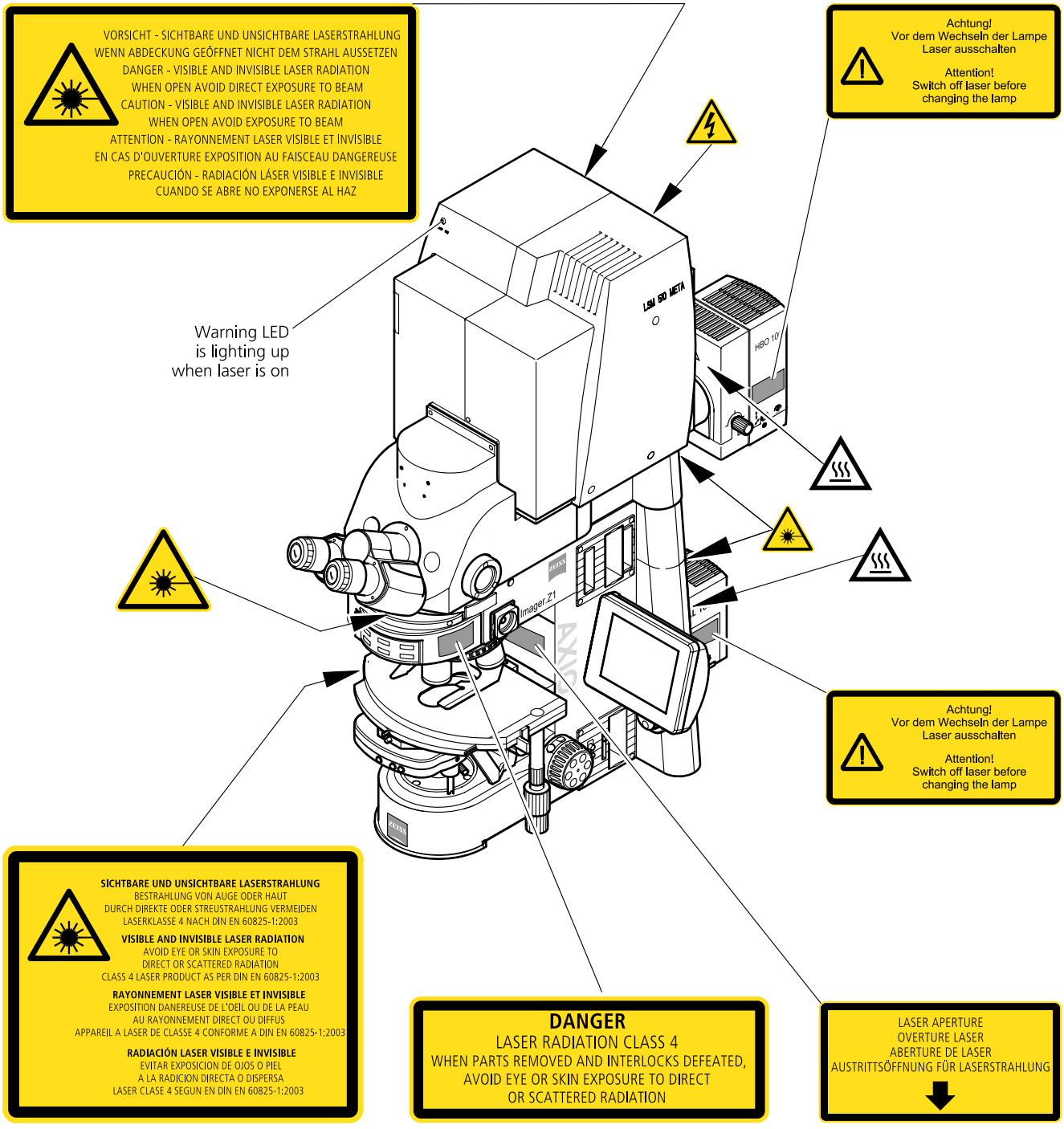

Fig. 1-2 **Warning and information labels on the Axio Imager.Z1 microscope with LSM 510 META scanning module**

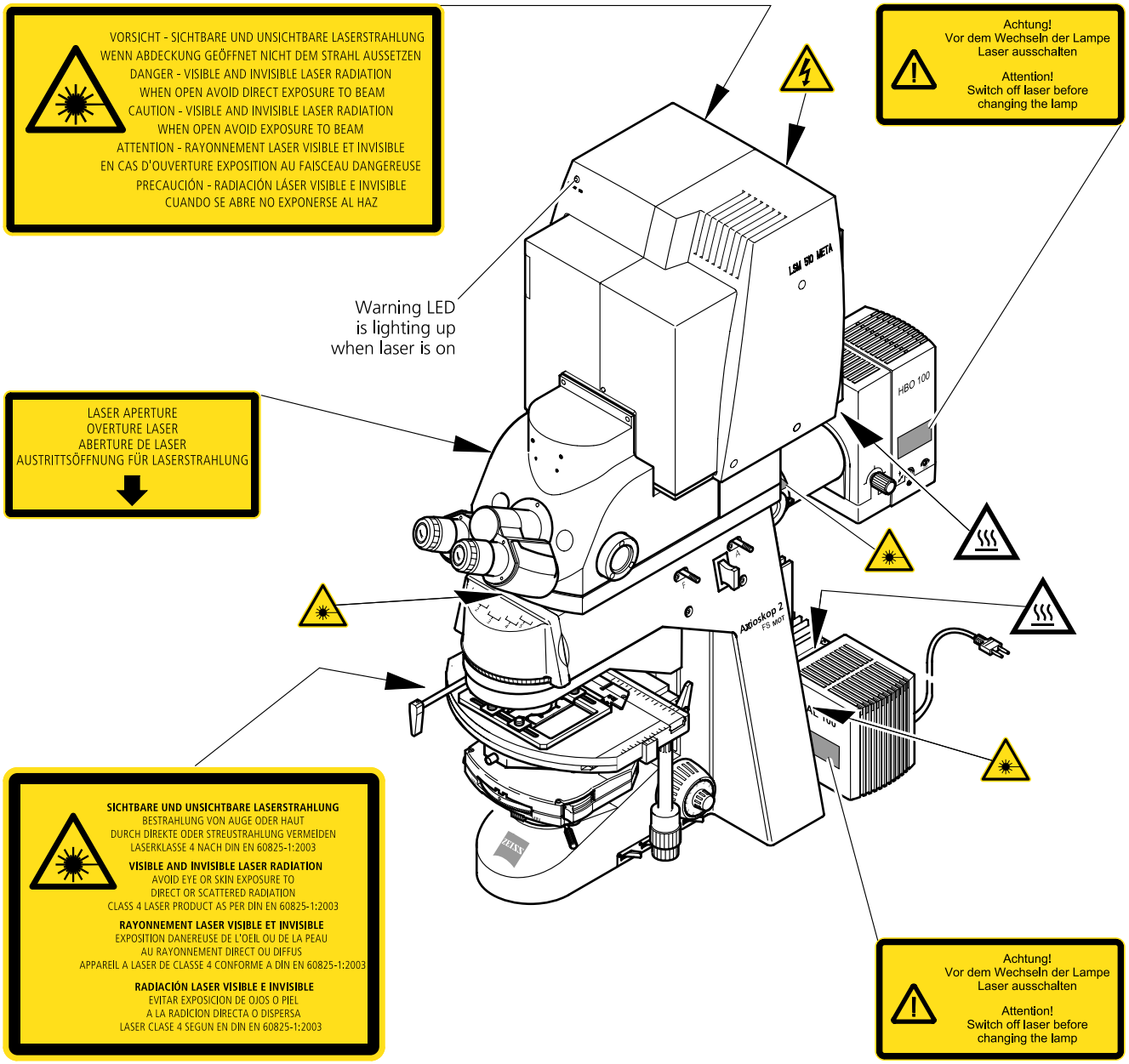

Fig. 1-3 Warning and information labels on the Axioskop 2 FS MOT microscope with the LSM 510 META scanning module

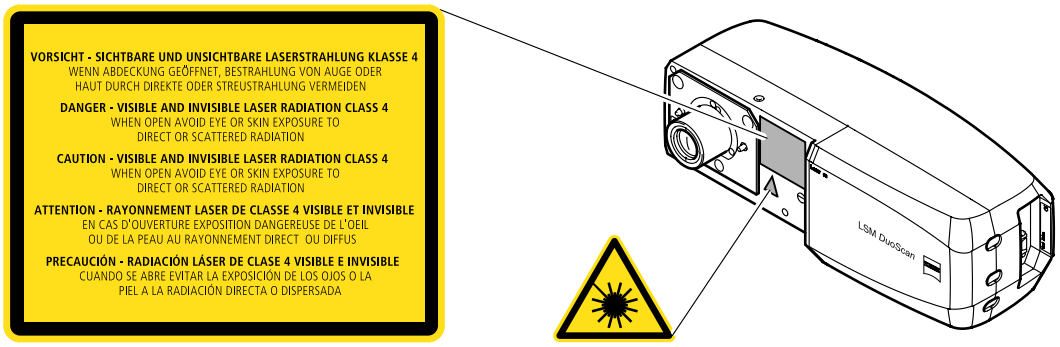

Fig. 1-4 Warning and information labels on LSM *DuoScan* (systems LSM 510 *DuoScan* or LSM 510 META *DuoScan* only)

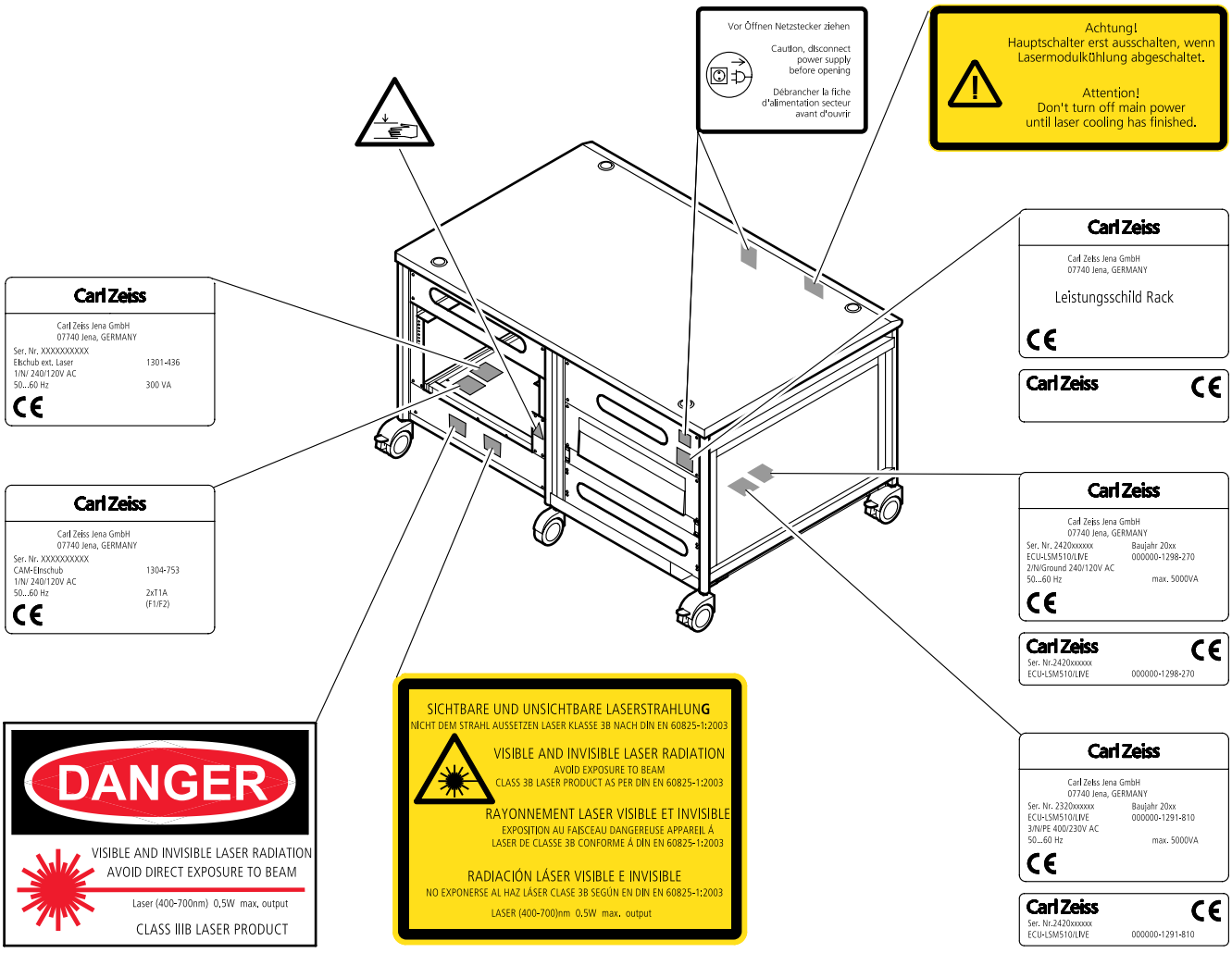

Fig. 1-5 Warning and information labels on the system electronic rack

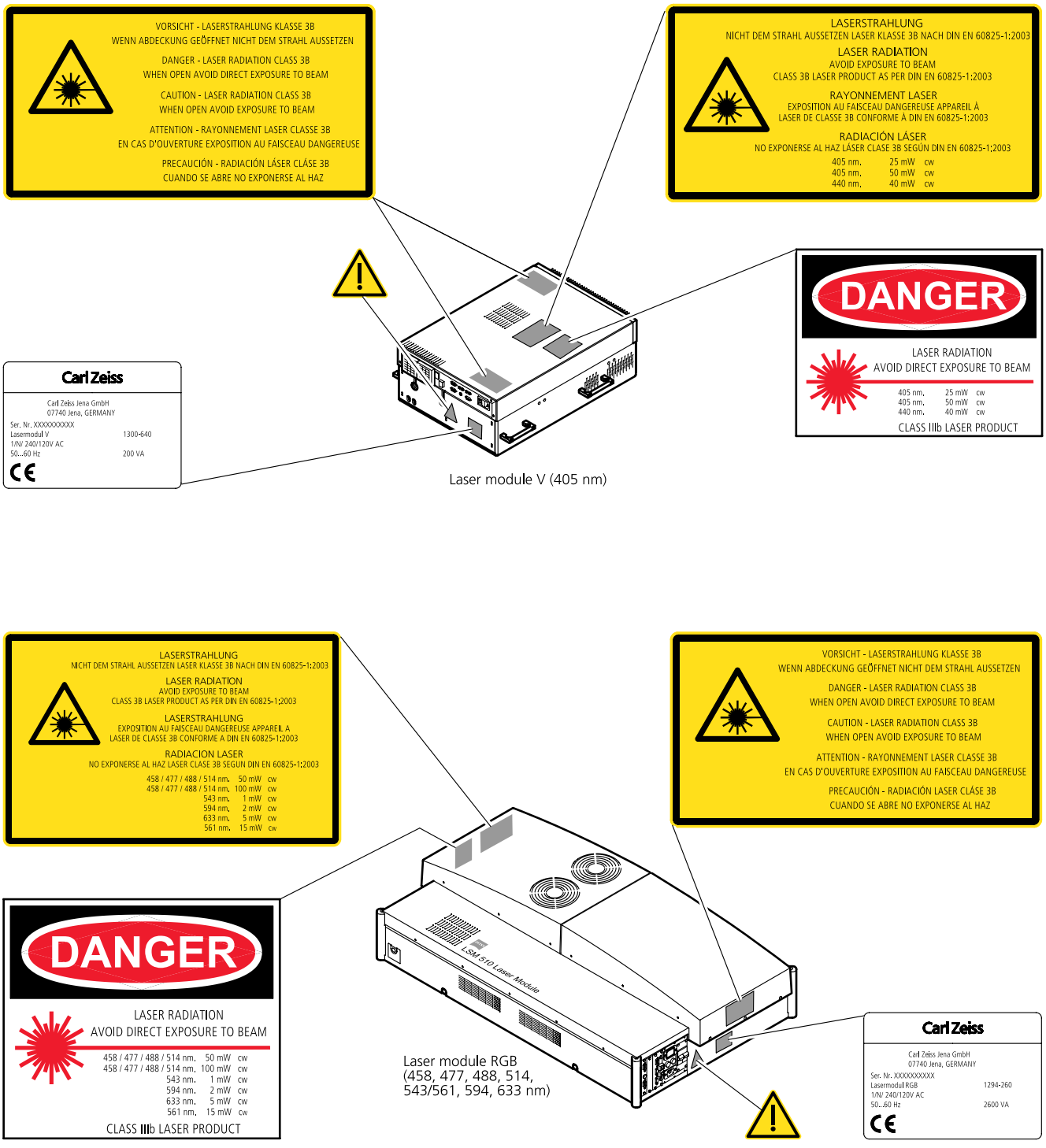

Fig. 1-6 Warning and information labels on laser components (page 1)

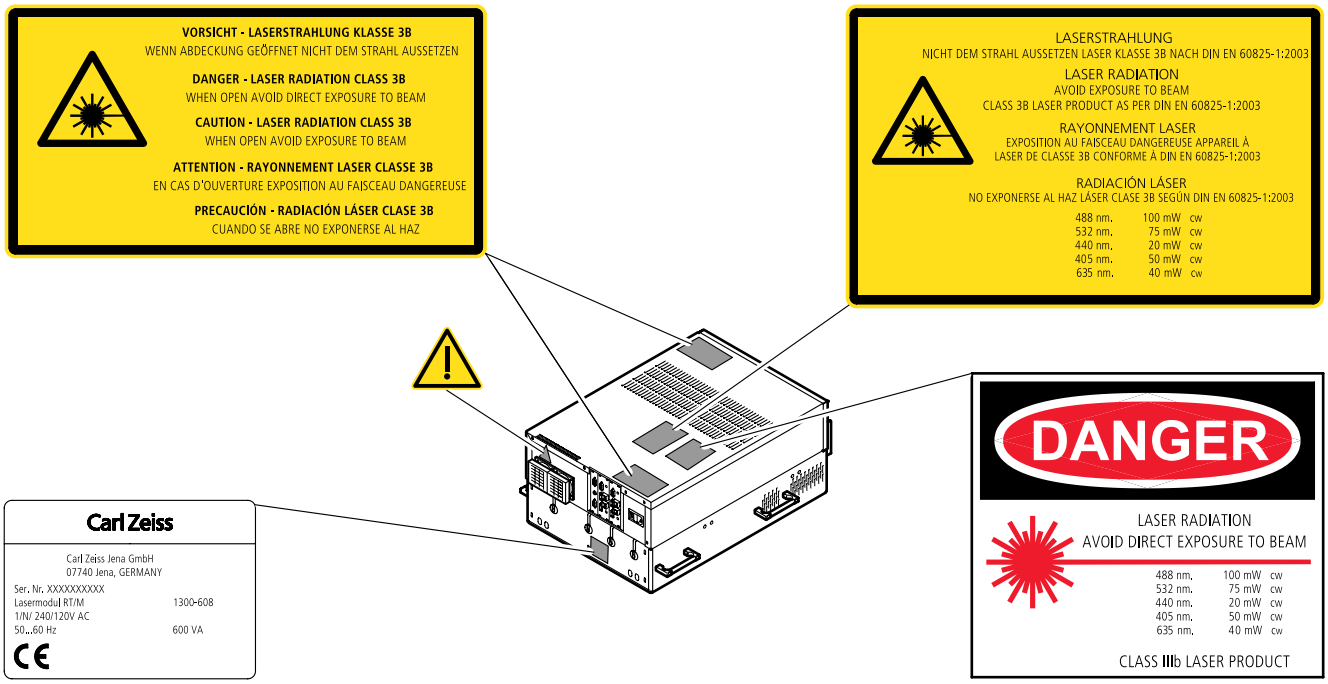

Fig. 1-7 Warning and information labels on laser module LSM 5 *LIVE*

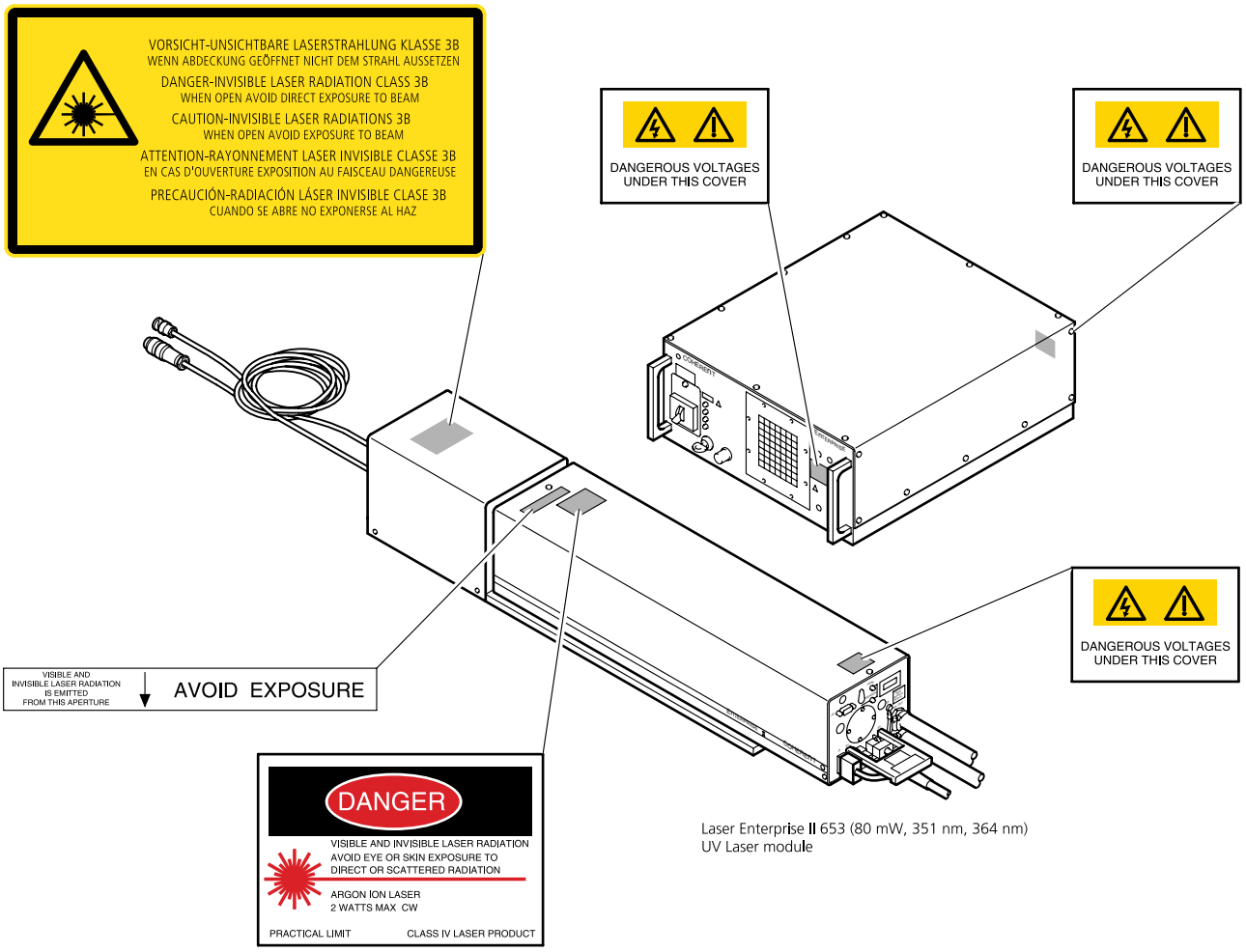

Fig. 1-8 Warning and information labels on laser components (page 2)

1.3 Regulations

Extensive knowledge of the hardware/the system is indispensable for safe operation of the LSM 510 and LSM 510 META.

☞ Read these operating instructions and all device publications belonging to the system conscientiously **before** operating the LSM 510 or LSM 510 META! You can obtain additional information on the hardware configuration delivered and on optional system extensions from the manufacturer or via the service hotline.

⇒ The LSM 510 and LSM 510 META have been designed, built and tested in conformity with the following regulations and guidelines:

DIN EN 61010-1 (IEC 601010-1) "Safety requirements for electrical equipment for measurement, control and laboratory use"

DIN EN 60825-1 (IEC publication 60825-1) "Safety of laser equipment", taking relevant CSA and UL specifications into account

DIN EN 61326: "Electrical equipment for control technology and laboratory use – EMC-requirements"

Low voltage directive: 73/23/EWG

EMC directive: 89/336/EWG

⇒ The company works according to a certified Environment Management System according to ISO 14001.

The Product was developed, tested and produced in accordance with the valid regulations and guidelines for environmental law of the European Union.

The product and its accessories have been classified as instrument category 9 (laboratory equipment or comparable standard). The product and its accessories agree with the EU-regulations 2002/95/EG (RoHS) and 2002/96/EG (WEEE) , if applicable for the product.

Carl Zeiss has installed a process for taking back and recycling the instruments within the member states of the European Union, which takes care of the appropriate utilization according to the said EU guidelines.

For details on the disposal and recycling please refer to your relevant Carl Zeiss sales or service organization.

The product must not be disposed in the household waste or through the municipal disposal organizations. In case of resale the seller is obliged to inform the buyer, that the product has to be disposed according to the said regulations.

1.4 Notes on Setting up the Microscope System

☞ Installation and commissioning of the LSM 510 and LSM 510 META system must be performed by authorized Carl Zeiss service staff. The system should not be used prior to instruction by a Carl Zeiss representative.

The LSM 510 or LSM 510 META laser scanning microscope is delivered in several crates.

The LSM 510 and LSM 510 META must be set up so as to ensure that the minimum clearance between the wall and the rear of the system is no less than 0.5 m. This clearance is needed for adjustment and maintenance operations.

Do not set up the unit in the proximity of heat sources such as radiators or direct sunlight. To avoid heat build-ups, the ventilation slots on the microscope system must not be covered up.

The system must not be set up in areas with potential danger by explosives.

The unit must be connected to a properly installed socket outlet with earthing contact by means of the mains cables supplied. Continuity of PE connection must not be affected by the use of extension leads.

The system contains components with dangerous voltage. The system must not be opened by anybody else than authorized Carl Zeiss Service staff. Before opening the main plug has to be disconnected.

Before connecting the mains cables, please check whether your mains voltage corresponds to the voltage specified on the rating plate of the laser module.

For reasons of laser safety, all ports must either be equipped with the corresponding device (scan head, camera, HBO lamp etc.) or covered with the counterpart of the laser safety kit provided.

Maintenance, repair, modification, removal or exchange of components, or other interference with the equipment beyond the operations described in this manual may only be carried out by the manufacturer Carl Zeiss or by persons expressly authorized by Carl Zeiss to do so.
This applies especially to the microscope system, the laser scanning module, lasers, the PC system, the power supply units, cable connections and other system components.

Please note that the LSM 510 and LSM 510 META are high-precision opto-electronic instruments. Inexpert handling may easily impair their function or even damage them.

The openings for ventilation must not be covered.

There are hot surfaces on the HBO and HAL lamp.

When sliding the compact Lasermodule V in and out of the System electronic rack take care not to catch your fingers.

After installation or conversion of the LSM system, authorized specialized staff must carefully check that it is in a proper condition and, particularly, that covers protecting against laser radiation are provided.

Tube openings or other unused mounts should always be protected against dust and moisture with the corresponding device components or with termination covers/blind plugs.

By establishing a corresponding workplace environment, please ensure that the formation of electrostatic charges of electronic components is avoided.

To avoid vibrations during operation, the LSM 510 and LSM 510 META should only be operated in conjunction with the system table (vibration damping).

1.5 Power Requirements

☞ The LSM 510 and LSM 510 META comes with a mains power supply cord and plug, either CEE red (3/N/PE 400/230V/16A), or NEMA L 14-30P (2/N/Ground 120/240V/30A), and with the matching mains socket outlet.
A ground wire (AWG10 green/yellow) is supplied because it is necessary to ground the system. The connecting part on both ends of the cable is a cable eye with 8 mm inner diameter.
A suitable grounding point must be installed in the room.
For systems (220 ... 240 V AC) equipped with X-Cite 120 the mains socket outlet must be equipped with a fuse having minimum tripping characteristic C according to IEC/EN 60898.

Line voltage	220 ... 240 V AC (±10 %)	100 ... 125 V AC (±10 %)
Line frequency	50...60 Hz	50...60 Hz
LSM incl. VIS laser		
– Max. current	3 phases at 16 A	2 phases at 25 A
– Power	Phase 1 = 1.9 kVA max. Phase 2 = 1.5 kVA max. Phase 3 = 2.6 kVA max.	Phase 1 = 3.2 kVA max. Phase 2 = 2.8 kVA max.
– Power consumption	5000 VA max.	5000 VA max.
Argon UV laser		
– Line Voltage	208...240 V AC (±10 %) 50 / 60 Hz	208...240 VAC (±10 %) 50 / 60 Hz
– Max. current	1 phase at 63 A Note: For Line Voltage 220 V the connector and power plug are rated for 63 Amps, However wiring and fuse should be rated for 32 Amps.	1 phase at: 208 V: 34 Amps 230 V: 31 Amps 240 V: 29 Amps
– Power consumption	7000 VA max.	7000 VA max.
Class of protection	I	I
Type of protection	IP 20	IP 20
Overvoltage category	II	II
Pollution degree	2	2

1.6 Notes on Handling the Laser Components

The LSM 510 and LSM 510 META are laser hazard class 3B instruments. If equipped with a Ti:Sa Laser, the LSM 510 and LSM 510 META are devices that belong to laser hazard class 4.
This moderate and high-risk class embrace medium-power and high power lasers. You must take care not to expose yourself to the radiation of such lasers. In particular, never look into the laser beam! Only personnel which has been instructed on laser safety is allowed to operate the system.

The following laser types are currently intended for use in the LSM 510 and LSM 510 META. The use of any other lasers as the ones listed below is not authorized.

Laser	Class	Power
1 Ar 351/364 nm (UV)	4*	80 mW
2 Diode laser 405 nm	3B	30 mW
3 Ar/ML 458/477/488/514 nm	3B	30 mW
4 HeNe 543 nm	3B	1 mW
5 DPSS 561 nm	3B	10 mW
6 HeNe 594 nm	3B	2 mW
7 HeNe 633 nm	3B	5 mW
8 Titanium:Sapphire Laser Mai Tai (Spectra Physics) 710-990 nm (depending on the model)	4	2 W
9 Titanium:Sapphire Laser Chameleon (Coherent) 710-980 (depending on the model)	4	2 W
10 Diode laser 405 nm	3B	50 mW
11 OPSS laser 488 nm	3B	100 mW
12 DPSS laser 532 nm	3B	75 mW

* Laser type class 4, if mounted on laser module with fiber output class 3B.

☞ Please note that for the maintenance of the UV Laser it is recommended to run the laser at maximum power once a day if the laser is not used frequently or only at low power levels. This enables the Autofill process which keeps up the correct tube gas pressure. This operation prolongs the life time of the tube and prevents complete tube failure if the laser is not used for a prolonged period of time. For details please refer to the Operator's Manual of the UV laser.

☞ Please contact Carl Zeiss if you intend to use a different laser other than the ones above.

If used properly, the LSM 510 and LSM 510 META will not pose any laser radiation risks for operating staff. Nevertheless, you should observe the following warnings:

- If necessary - insofar as specified by law - inform the laser protection officer before commissioning the laser.
- The laser modules are equipped with a key-interlock.
- Always store keys for laser key switches and, if applicable, keys for further laser power supply units, where they are inaccessible to persons not authorized to operate the laser.
- A red LED on the front of the scan head lights up when one or all of the lasers are switched on.
- Do not place any reflecting objects into the beam path.
- Never open any covers or panels.
- Never look into the laser beam, not even to simply view the specimen, whether with the aid of optical instruments or without. Otherwise you risk going blind!
- Do not leave any empty objective positions of the nosepiece uncovered.
- If a class 4 laser is attached to the system, already stray light can impose danger to the operator.
- With class 4 lasers take special care of fire protection requirements. Do not use or store flammable or explosive solids, fluids or gases in the vicinity of the system.
- Class 4 lasers can inflame also flammable materials like cloth or paper. Do not put such materials into the beam path.
- Do not reach into the process beam inside the sample area whenever the Class 4 laser is active!

Suitable protective measures must be taken if gases, dust or vapors hazardous to health, secondary radiation or explosive objects should arise on the specimen as a result of laser radiation.

For NLO systems equipped with a specific push and click filter for NDD imaging be aware that the NDD reflector cube in the reflector turret leads to a strong back reflection of HBO light into the specimen plane and the eyepiece. When observing the specimen through the ocular lens the use of the NDD reflector cube should be avoided. The light flash is not harmful but unpleasant. The reflex of closing the eyelid is sufficiently protective. To completely avoid this situation an additional filter (#1261-345) can be mounted into the NDD reflector cube which prevents the back reflection of the HBO light in the ocular plane.

1.7 Physical Dimensions

	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Large system table	150	80	78	100
Small system table	65	80	78	60
Passively damped anti-vibration table	130	100	75	137
Active anti-vibration table (NLO) for Mai Tai Laser or Chameleon	120	140	75	200
Active anti-vibration table (NLO) for Verdi Mira or Millenia Tsunami Laser	180	140	75	400
Scanning Module LSM 510	25	20	25	15
Scanning Module LSM 510 META	28	27	30.5	13
Scanning Module LSM *DuoScan*	40	15	13	8
Microscope	50	35	50	20
Laser Module RGB	110	70	28	95
Laser Module, UV	140	20	20	60
Laser Module V (405 nm)	66	52	22	30
Plug-in unit external laser	66	52	22	9
System Electronic Rack	110	70	58	90
Power supply for Ar (UV)	50	50	30	30
Cooling unit for Ar (UV)	80	45	50	30
Laser Module *LIVE*	66	52	22	58

1.8 Environmental Requirements

1. Operation, specified performance	T = 22 °C ± 3 °C without interruption (24 h a day independently whether system is operated or switched-off)
2. Operation, reduced performance	T = 10 °C to 35 °C, any conditions different from 1. and 5.
3. Storage, less than 16 h	T = -40 °C to 55 °C
4. Storage, less than 6 h	T = -55 °C to 70 °C
5. Temperature gradient	± 0.5 °C/h
6. Warm up time	1 h, for high-precision and/or long-term measurements ≥ 3 h
7. Relative humidity	< 65 % at 30 °C
8. Operation altitude	max. 2000 m
9. Loss of heat	4 kW

☞ These requirements do not include the requirements for high precision measurements. Please refer to the Operator's Manual of the microscope for these requirements.

1.9 Notes on Handling the Computer and Data Media

The computer used as standard in your LSM system is an IBM-compatible high-end Pentium computer with WINDOWS XP operating system.

☞ Do make sure, though, that you receive your LSM system with the operating system installed, with initialization and start files set up and with the LSM program also installed.

☞ When working with the hard disk, it is important to know that the more data it contains, the slower its operation will become. Therefore, data that you do not need permanently should be stored on other external devices.

⚠ When handling diskettes and USB sticks, avoid data losses by protecting them against extreme temperatures, moisture and magnetic fields. The data on a diskette is stored in the form of magnetic signals. To some extent, monitors, telephones or even lamps generate magnetic fields that might destroy this data. Also, never open the metal cover on diskette cases. A diskette's surface can also be destroyed by touching it.

⚠ When handling CDs, CD ROMs or DVDs, do not touch the data side of the disc (the side of the disc with no label or printing).

Do not apply paper labels or write on any part of the disc, data side or label side. If dust or fingerprints get on the disc, wipe it with a soft cloth from the center to the edge, but do not use benzine, paint thinner, record cleaner, or static repellent. This can damage the disc.

Do not place the disc in any place where it is exposed to direct sunlight or high temperatures.

Backup your data on a regular basis.

Do not install any other software without talking to your Carl Zeiss representative.

⚠ Never turn your computer off before you have terminated the LSM program and run down the WINDOWS XP operating system. Otherwise, the program and/or data files may get lost.

1.10 Notes on Care, Maintenance and Service

The manufacturer of the unit cannot be held liable for damage resulting from operating errors, negligence or unauthorized tampering with the device system, particularly as the result of removal or replacement of parts of the unit or as the result of the use of unsuitable accessories from other manufacturers.
Any such action will render all warranty claims null and void and also laser safety is no longer warranted.

You are well advised to arrange a service agreement with your nearest Carl Zeiss representative to guarantee perfect functioning of the microscope system in the long term.

Modifications and conversion work on the components of the system must only be carried out by the manufacturer, by the service agency or by persons authorized and trained for this purpose by the manufacturer.

Damaged units or parts may only be repaired or maintained by the responsible service agency.

During maintenance or repair carried out by the service personnel the customer is requested to stand aside and wear a pair of laser safety goggles if needed.

Before opening the housing of the halogen lamp switch off all laser units.

Care operations that may be carried out by operating staff are limited to cleaning painted and glass surfaces.

- Before cleaning the instrument make sure the main power supply is disconnected.
- Cleaning painted surfaces
  To do this, use a clean cloth that has been moistened in a mixture of water and some detergent; do not use any solvent, however. Dry with a lint-free cloth.
- Cleaning glass surfaces
  Glass surfaces that have become soiled or which are marked with fingerprints may be rubbed with a clean optical cleaning cloth.
  If soiling is persistent, dip the optical cleaning cloth into a mixture of distilled water and a small quantity of detergent.
  To complete cleaning, lightly breathe on the glass surface and rub it dry with a clean cloth. Lint or dust is best removed with a clean brush.
- Make sure that no cleaning liquid penetrates into the system.
- Dust filters in the ventilation entries of the system electronic rack have to be replaced every 6 month. For replacement please contact your local service representative.

1.11 User Interface

All user interface ports are equipped with a safety interlock system which warrants laser safety. These interlock devices must not be manipulated. Other interfaces which are not described here are service interfaces and are only to be operated by authorized Carl Zeiss service personnel. The following devices can be mounted and dismounted by the user:

- Halogen and HBO lamp
- Transmission PMT
- Switching Mirror
- Scan head

1.11.1 Mounting and Dismounting Lamps, TPMT and Switching Mirror

The ports of the lamps, the switching mirror and the transmission PMT are equipped with hardware interlock devices. At most ports (all ports of the Axioskop 2 FS MOT and the Axio Imager.Z1 as well as the HBO port of the Axiovert 200 M) the following interlock devices are present and have to be operated in the following way:

Interlock with sensor ring and contact ring:

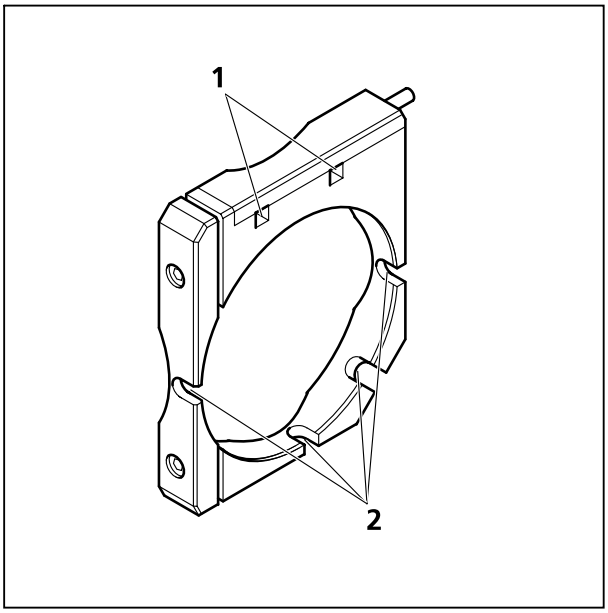

Fig. 1-9 Sensor ring mounted to the interface ports on the microscope side

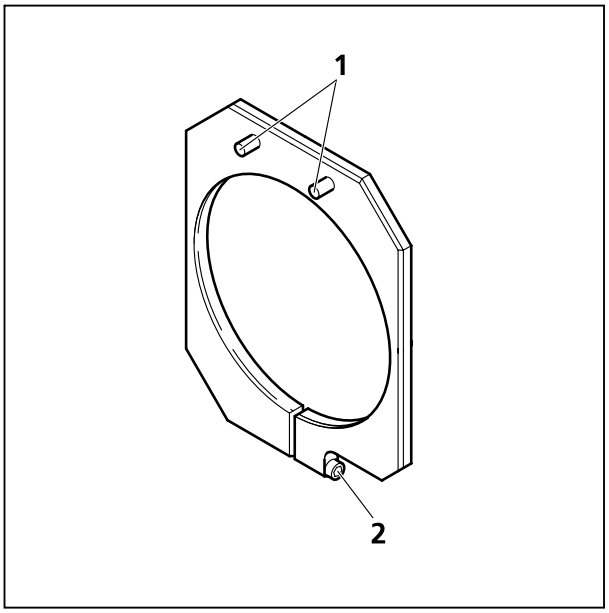

Fig. 1-10 Contact ring mounted to the lamp, TPMT or switching mirror

The interlock is working when the sensors of the sensor ring (Fig. 1-9/**1**) are depressed by the pins on the contact ring (Fig. 1-10/**1**). Whenever this is not the case, for example if the distance between the two devices is too large, the laser will be blocked and the system cannot be used.

☞ In case the system is not operating following the removal or attachment of any device on a port with safety interlock check again the connection of the Contact ring to the Sensor ring.

For dismounting the lamps, TPMT or switching mirror slightly unscrew the contact ring first (Fig. 1-10/**2** or Fig. 1-11/**2**) so it can be moved away from the sensor ring (Fig. 1-11/**1**). Then unscrew the lamp, TPMT or switching mirror (main screw
Fig. 1-11/**3**) which is in one of the recesses of the sensor ring (Fig. 1-9/**2**). Hold the device to be dismounted with one hand while unscrewing to keep it from dropping. The now empty port has to be closed with the blind cap to restore the functionality of the system. Use the main screw of the port to fix the cap. Make sure the pins of the cap depress the sensors of the sensor ring.

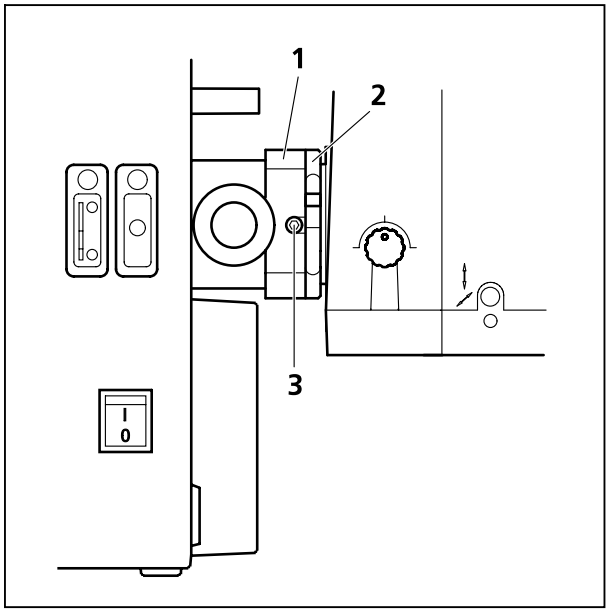

Fig. 1-11 HBO lamp mounted to Axiovert 200 M with sensor ring and contact ring for laser safety

☞ Do not remove the sensor ring from the microscope. This might result in failure of laser safety and a non operating system.

For mounting any lamp, TPMT or the switching mirror back onto the microscope reverse the steps for dismounting the device. Be careful not to bend the pins on the contact ring when screwing the device onto the microscope port.

For the Axiovert 200 M transmission port and the two ports available on the motorized switching mirror no sensor ring is present. Instead the sensors are directly installed at the Axiovert 200 M transmission port or the two ports of the motorized switching mirror.

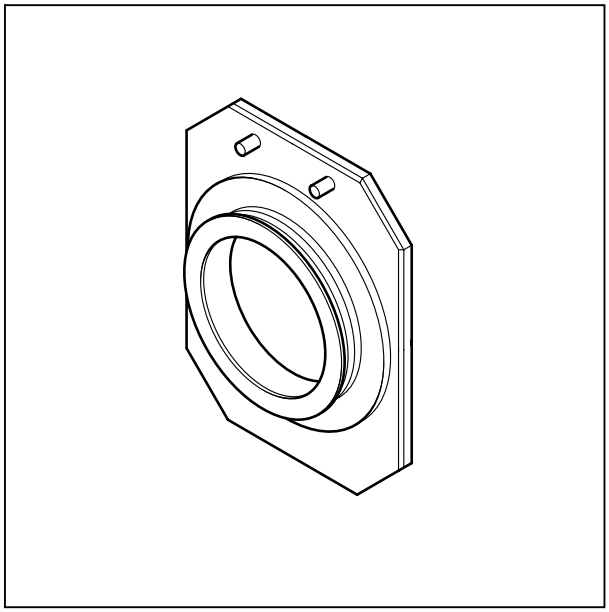

Fig. 1-12 Blind cap for closing any port equipped with an interlock device

1.11.2 Mounting and Dismounting the Scan Heads

The scan heads are connected to the microscope via an integrated safety interlock. They can be moved between two microscopes. Make sure the system is shut off completely before starting the following procedure:

☞ Be aware that the scan head weights up to 15 kg.

Moving the scan heads between Axiovert 200 M, Axioskop 2FS MOT and Axio Imager.Z1:

- LSM 510 META scan head: Remove the blue cover from the back of the scan head (Fig. 1-13 and Fig. 1-14).

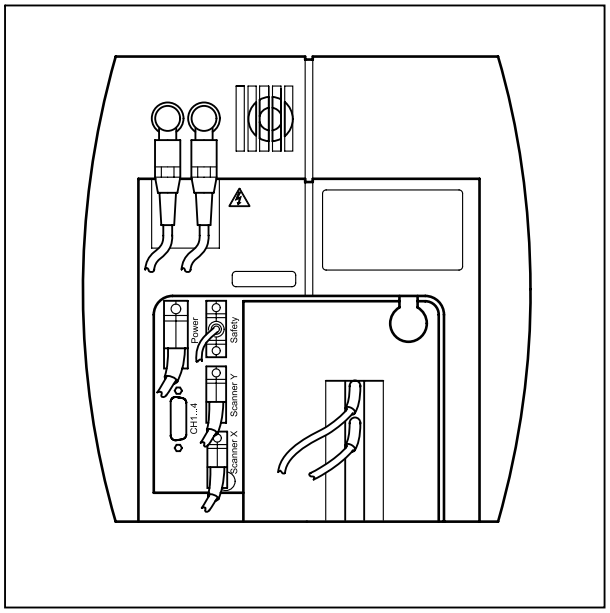

Fig. 1-13 Rear view of META scan head

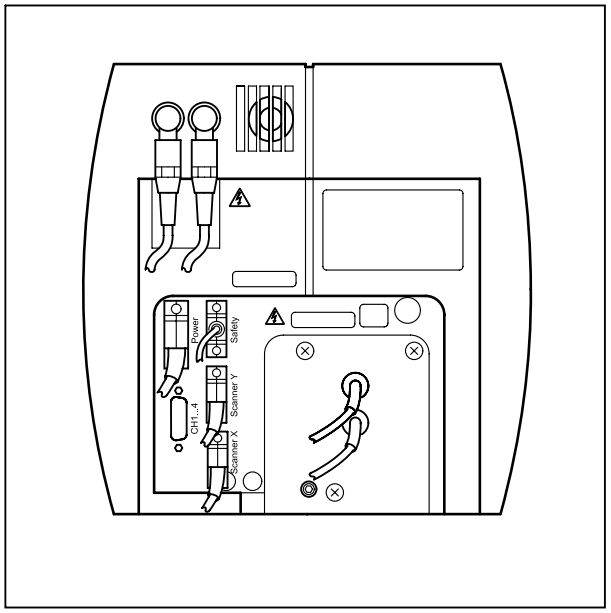

Fig. 1-14 Rear view of META scan head with blue cover removed

- Loosen the screw on the lower end of the scan head (Fig. 1-15/**1** and Fig. 1-16/**1**)

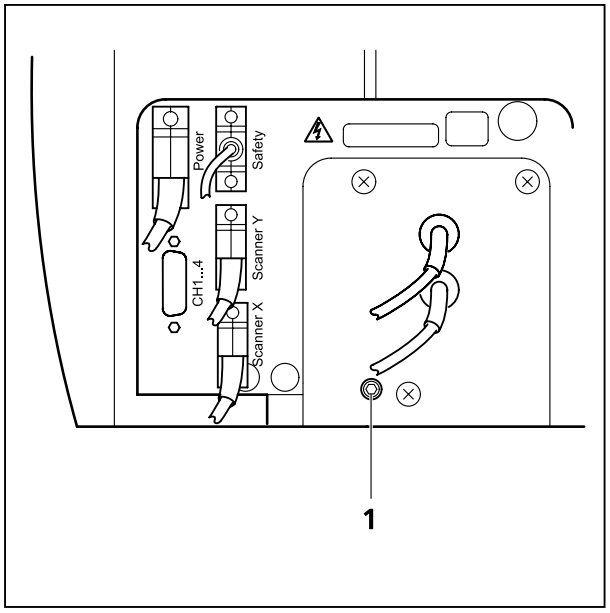

Fig. 1-15 Position of the screw on the LSM 510 META scan head

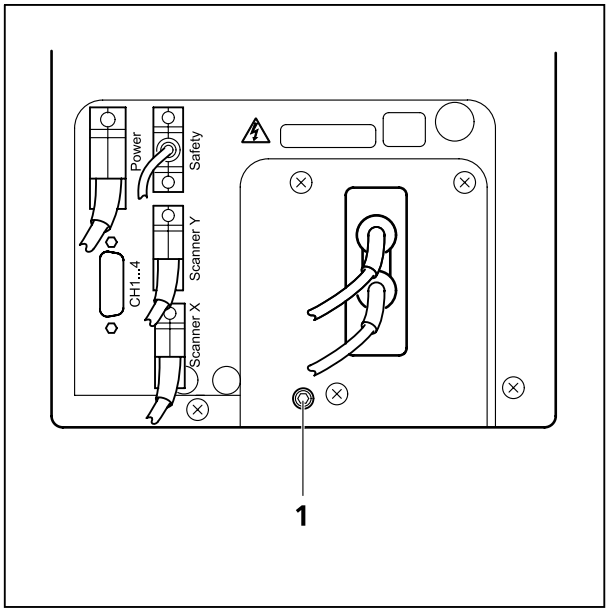

Fig. 1-16 Position of the screw on the LSM 510 scan head

- Loosen the screws on the port to the microscope for Axiovert 200 M (Fig. 1-17/**1**) or to the tube on Axio Imager.Z1 and Axioskop 2 FS MOT (Fig. 1-18/**1**).

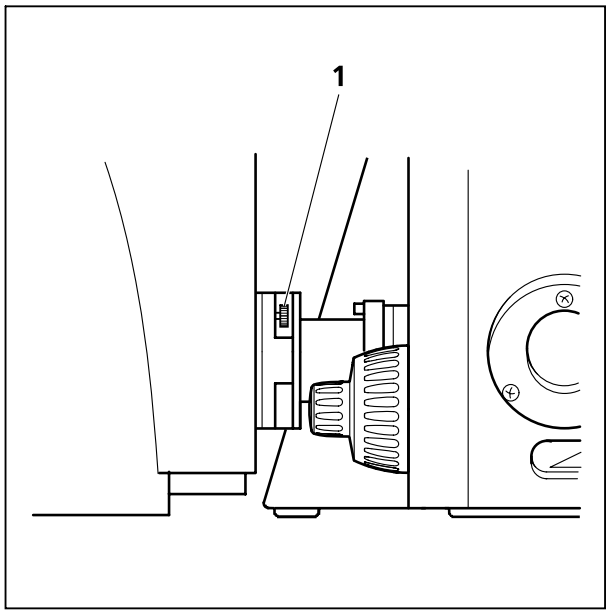

Fig. 1-17 Port connection between LSM and Axiovert 200 M

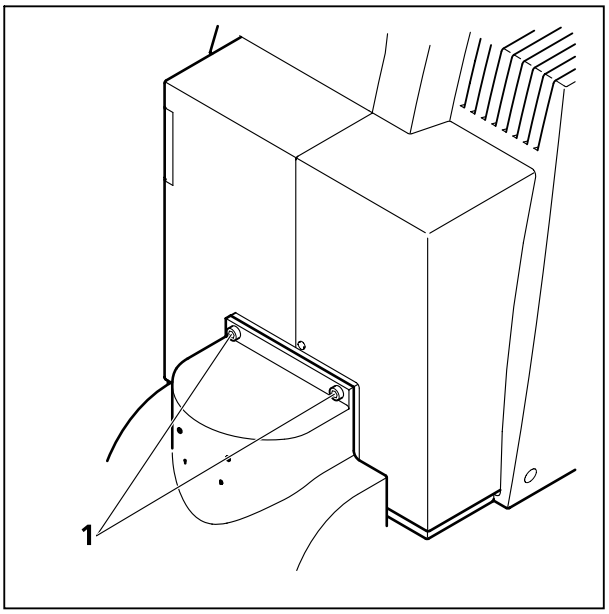

Fig. 1-18 Fastening screws of the scan head at the front of the tube on Axioskop 2 FS MOT and Axio Imager.Z1

- Slowly pull the scan head away from the microscope port or the tube. For mounting the scan head onto a microscope, make sure the pins and the electronic connections of the safety interface match closely. Fasten the screws on the front of the tube (Fig. 1-18/**1**) or at the microscope port (Fig. 1-17/**1**) followed by fastening the screw on the back of the scan head (Fig. 1-15/**1** and Fig. 1-16/**1**).
- Use the three fastening screws (Fig. 1-19/**1**) to attach or detach the LSM *DuoScan* to or from the microscope stand.

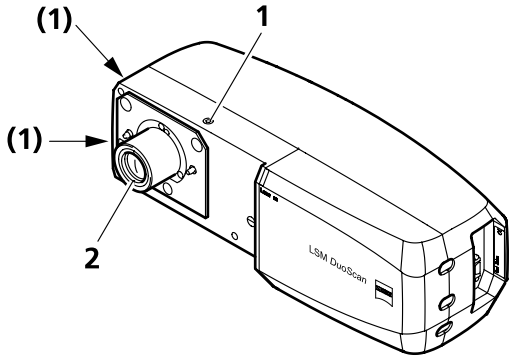

Fig. 1-19 Fastening screws and connections on LSM DuoScan

For NLO systems being coupled to a Chameleon or Verdi Mira Laser from Coherent using the beam housing kit designed for LSM 510, the beam tube connecting the periscope and the scan head has to be removed before the scan head can be dismounted. Perform the following steps for the removal of the tube:

- Unfasten the screw that clamps the outer tube to the inner tube (Fig. 1-21/**3**).
- Pull out the tube from the periscope cover by sliding the inner tube into the outer tube (Fig. 1-21/**6**).
- Turn the outer tube counter clockwise to unscrew the tube (Fig. 1-21/**4**) from the small black tube which is fixed to the scan head.
- Now the tube can be removed and you can proceed as described above.
- For rearranging the beam housing, first screw the tube to the scan head (Fig. 1-21/**4**). It should not be fastened completely to allow the inner tube to fit the side pin into the recess of the ring on the periscope (Fig. 1-21/**1**) and the recess of the outer tube (Fig. 1-21/**2**).
- Open the shutter in the periscope using an Allen key (Fig. 1-20/**1**) and slide in the tube completely, this will hold the shutter open.

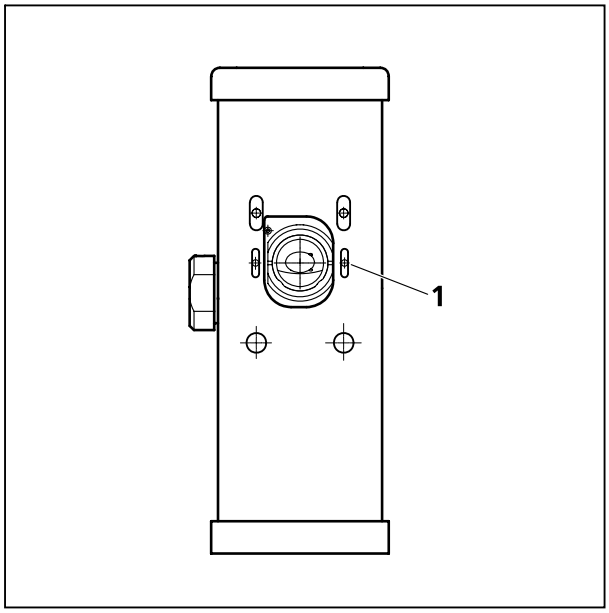

Fig. 1-20 Shutter opening on the periscope. Also applies for the large periscope for upright microscopes.

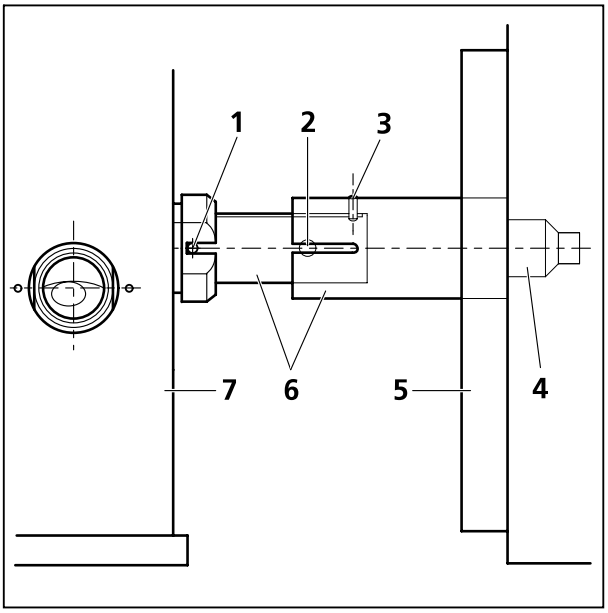

Fig. 1-21 Tube connection from the periscope housing (7) to the scan head (5). Applies for upright and inverted microscopes.

To ensure functioning of the system and laser safety the following connections have to be changed:

1. The connection of the microscope to the safety interface of the system is located either on the additional Safety-Box (Axioskop 2 FS MOT, Fig. 1-22/**1**) or on the rear side of the microscope (Axiovert 200 M or Axio Imager.Z1; Fig. 1-23/**1** and Fig. 1-24/**1**). This connection has to be unplugged from the microscope which is not in use and plugged into the microscope to be used following the exchange of the scan head.

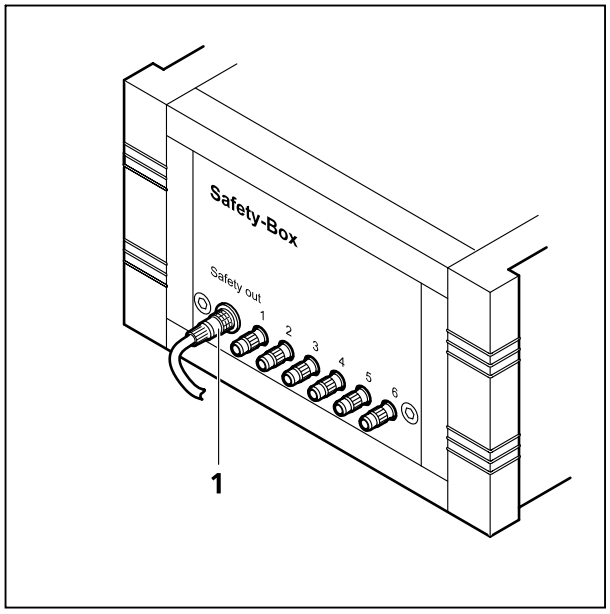

Fig. 1-22 Safety-Box of Axioskop 2 FS MOT with main connection to safety interface (1)

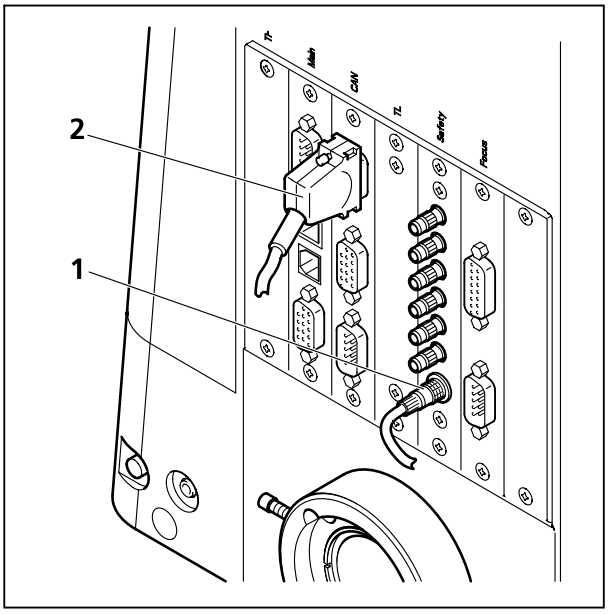

Fig. 1-23 Connection of Axio Imager.Z1 to safety interface (1) and electronics (2)

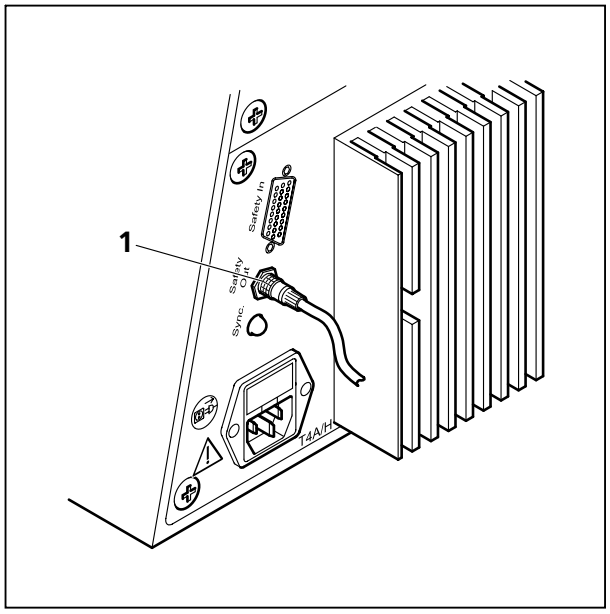

Fig. 1-24 Connection of Axiovert 200 M to safety interface (1)

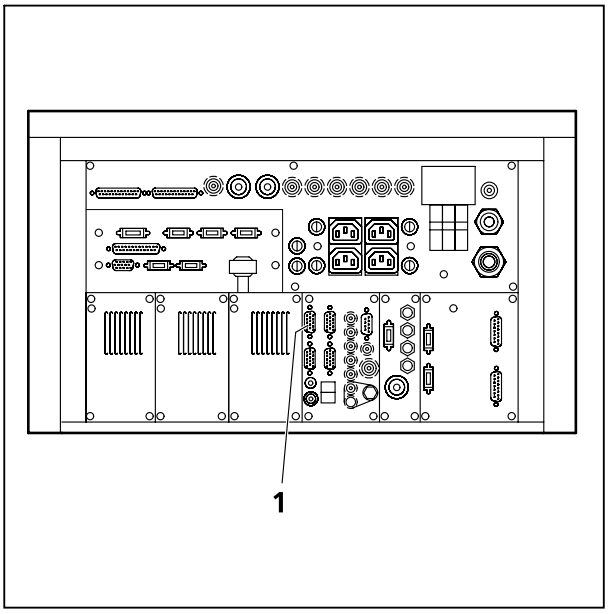

Fig. 1-25 Four CAN connections (1) are available on the rear side of the electronics module. The microscope in use has to be connected to one of them

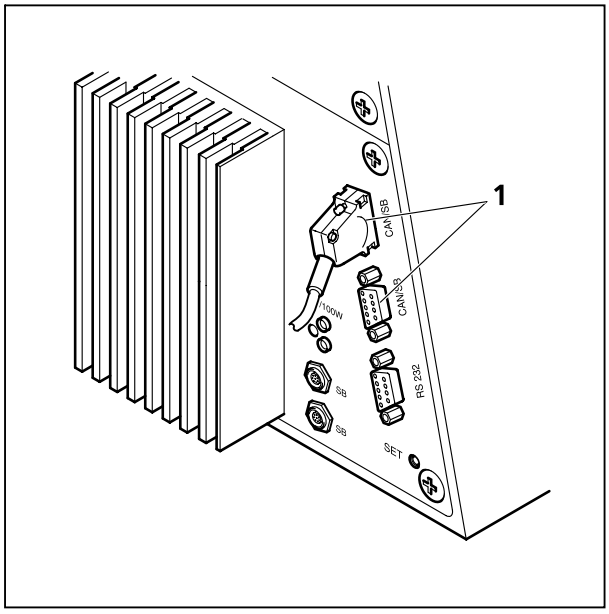

Fig. 1-26 CAN connections on the Axiovert 200 M (1). One of the plugs is occupied with the connection to the electronics

2. The plug for the main connection of the microscope to the electronics is situated on the rear side of the electronics rack. It is either of the four CAN connections shown in Fig. 1-25. Only ONE microscope can be connected to the electronics at a time. The connection has to be plugged in and unplugged at the electronics rack. The corresponding connections on the microscopes are shown in Fig. 1-23/**2**, Fig. 1-26/**1** and Fig. 1-27/**1**. Remove the connection of the microscope no longer in use at the electronics rack and plug in the connection of the microscope which should be used instead.

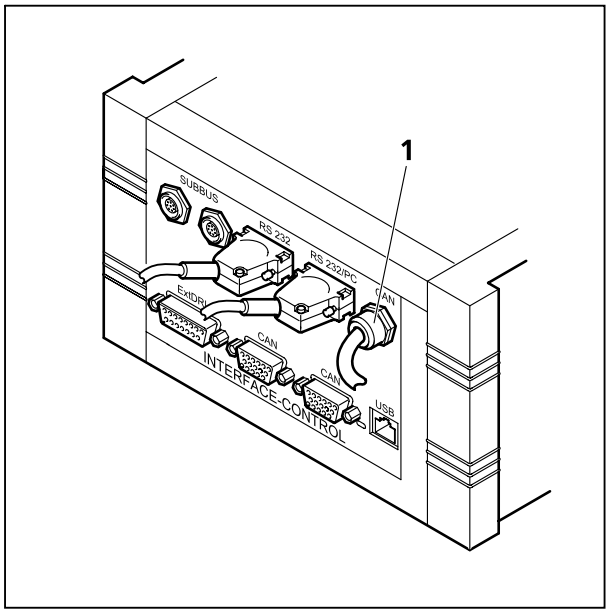

Fig. 1-27 The Axioskop 2 FS MOT is connected to the electronics via the Interface Control. The CAN connection is fixed (1) to the control box

3. After the connections of the non used microscope are disconnected and the ones of the used microscope are connected, the system can be switched on again. Before initializing the system with the LSM Software make sure to use the right database according to the microscope in use. It can be chosen via the icon Stand Select.

CHAPTER 2 LSM 510 AND LSM 510 META - SETUP REQUIREMENTS

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2 LSM 510 AND LSM 510 META - SETUP REQUIREMENTS

2.1 Space Requirements

2.1.1 LSM on large system table (one microscope, upright or inverted): 2 m × 3,50 m

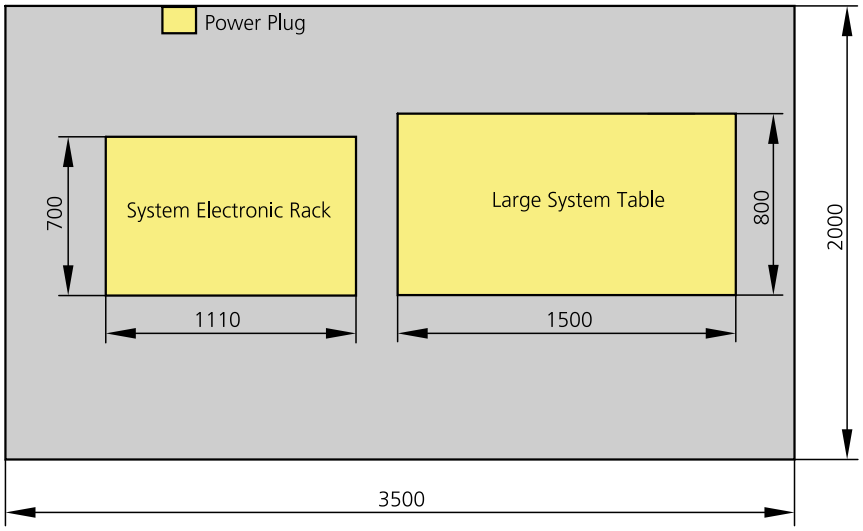


Fig. 2-1 Space requirements for LSM on large system table (measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the LSM system, the power supply for the microscope, the scanning unit, the Laser Module V and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack.

☞ On this table the extension of the system with LSM *DuoScan* is not possible!

2.1.2 LSM on small system table (one microscope, upright or inverted): 2 m x 3,5 m

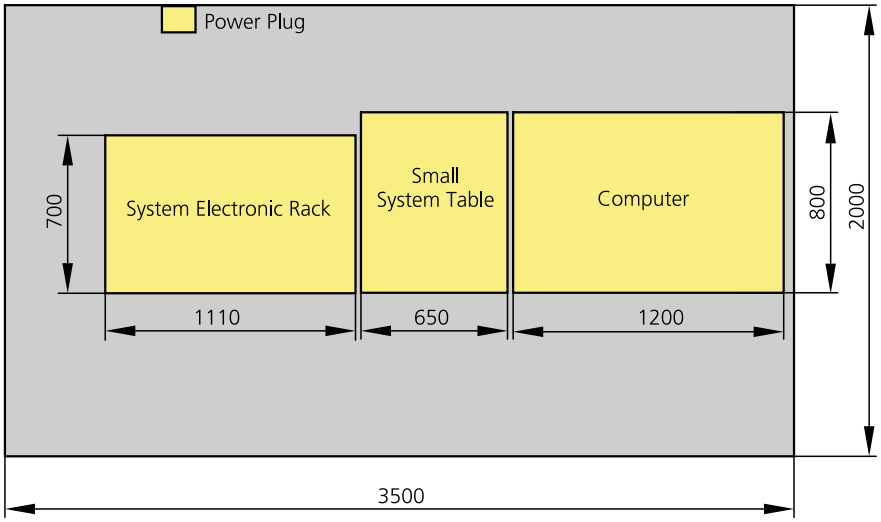


Fig. 2-2 Space requirements for LSM on small system table (measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the LSM system, the power supply for the microscope, the scanning unit, the Laser Module V and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack.

☞ On this table the extension of the system with LSM *DuoScan* is not possible!

2.1.3 LSM on passive anti vibration table (one microscope, upright or inverted): 2,2 m x 4,2 m

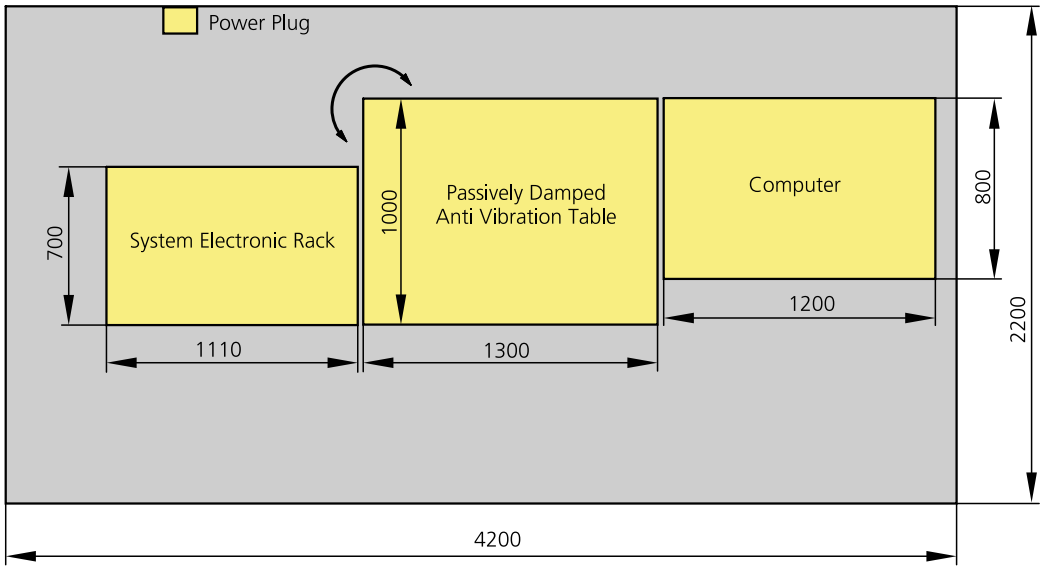


Fig. 2-3 Space requirements for LSM on Passively Damped Anti Vibration Table (system table with breadboard).
Depending on the ordered table it might be turned by 90°.
(measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the LSM system, the power supply for the microscope, the scanning unit, the Laser Module V and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack. With the extension of LSM *DuoScan* (VIS and/or 405 nm) the Laser Module *LIVE* is situated in the System Electronic Rack. It substitutes the Laser module V. With the extension of LSM *DuoScan* UV the system has to be set up according to chapter 2.1.5

2.1.4 LSM with Ar UV Laser

☞ We recommend placing the cooling unit of the Ar laser (UV) in a separate room to prevent heat accumulation and vibration. Length of the water hose: 400 cm

One microscope: 2,5 m x 3 m

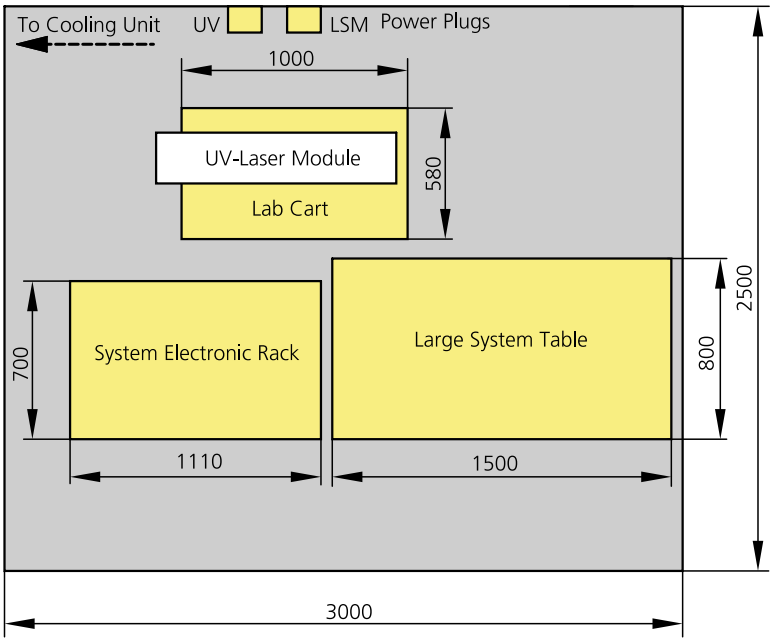


Fig. 2-4 Space requirements for LSM using one microscope and AR UV Laser (measurements in mm)

Two microscopes: 3 m x 3 m

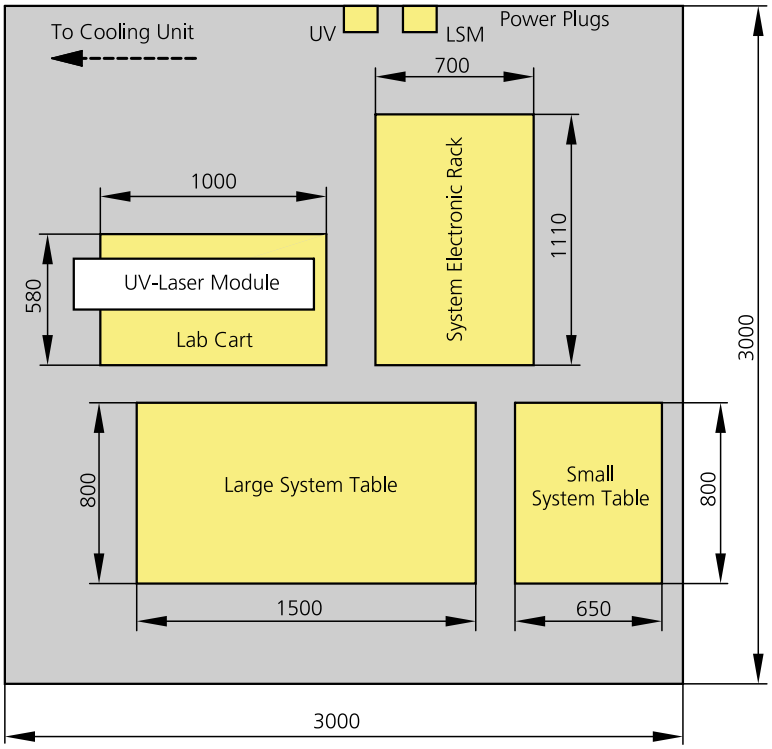


Fig. 2-5 Space requirements for LSM with two microscopes and AR UV Laser (measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the system, the power supply for the microscope, the scanning unit and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack. The lab cart holds the power supply for the UV laser and the UV Laser module.

☞ On this table the extension of the system with LSM *DuoScan* is not possible (see chapter 2.1.5)!

2.1.5 LSM 510 or LSM 510 META equipped with LSM *DuoScan* UV

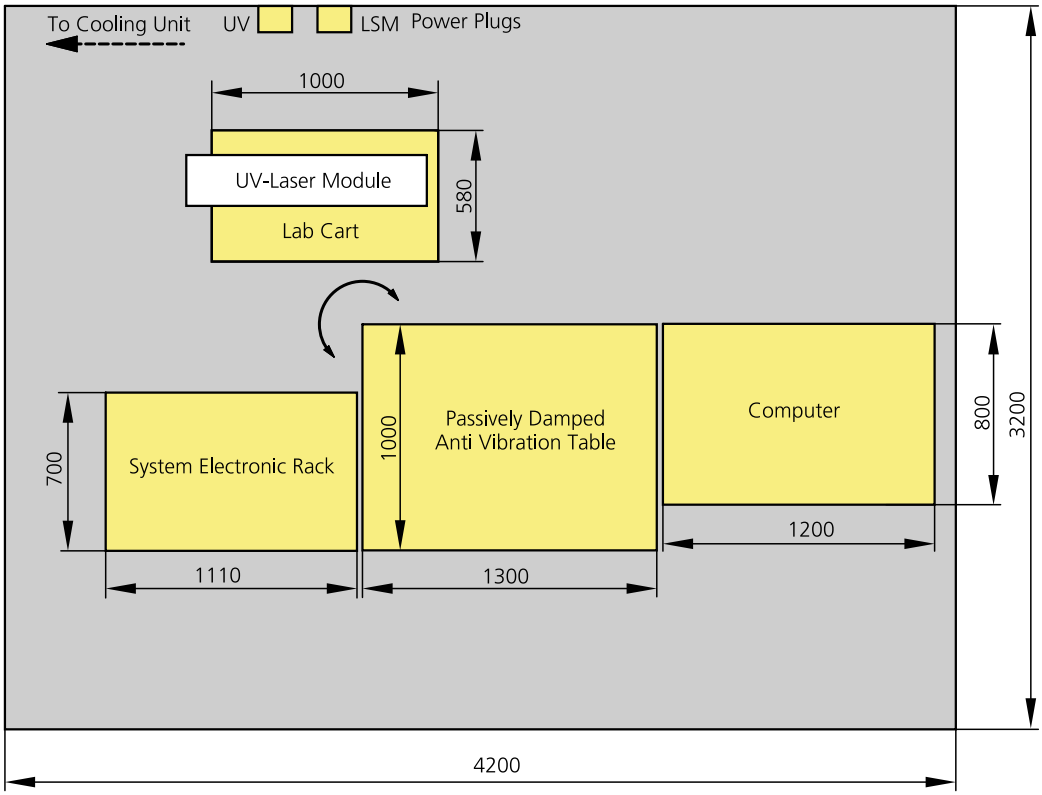


Fig. 2-6 Space requirements for LSM equipped with LSM *DuoScan* and AR UV Laser (measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the LSM system, the power supply for the microscope, the scanning unit and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack.

An additional lab cart is recommended which then contains the plug in unit for the UV laser and on top of it the UV laser itself.

2.1.6 LSM prepared for Two Photon Laser (NLO)

2.1.6.1 Coherent "Chameleon" or Spectra Physics “Mai Tai”. Directly-coupled to upright or inverted Microscope: 2,5 m x 4 m

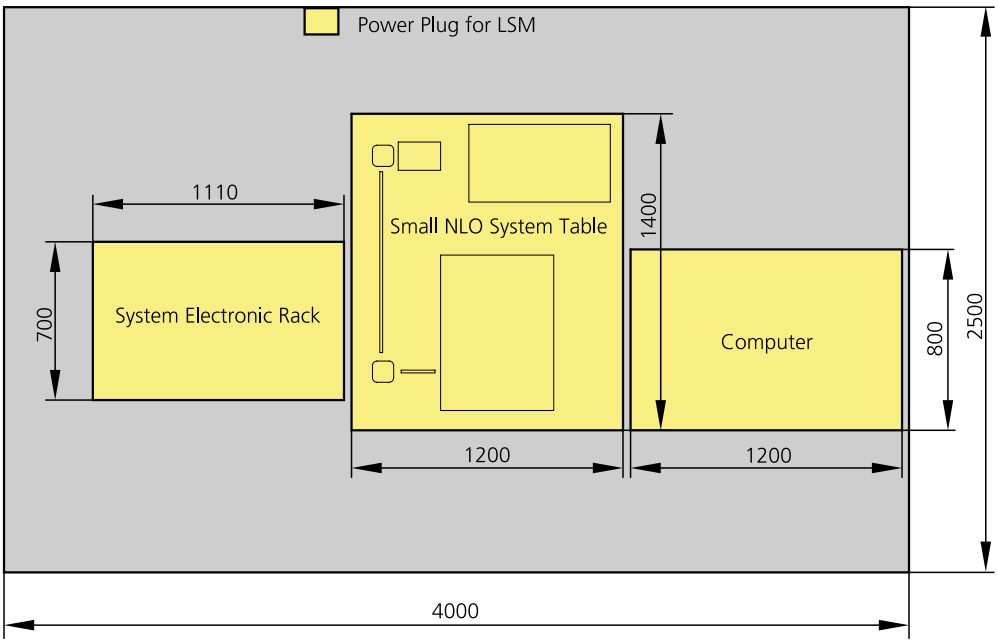


Fig. 2-7 Space requirements for Two Photon Laser and Small NLO System Table (measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the system, the power supply for the microscope, the scanning unit and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack. Power supply and cooling unit of the NLO Laser can be stored under the system table. With the extension of LSM *DuoScan* (VIS and/or 405 nm) the Laser Module LIVE is situated in the System Electronic Rack. The electronics for the NLO laser have to be set aside the System Electronic Rack. An additional lab cart (000000-0465-515) is recommended. With the extension of the LSM *DuoScan* UV only additional space is required to set up the UV laser (see chapter 2.1.4).

2.1.6.2 Coherent "Verdi Mira" or Spectra Physics “Millenia Tsunami”. Directly-coupled with upright or inverted Microscope: 3,5 m x 3,5 m

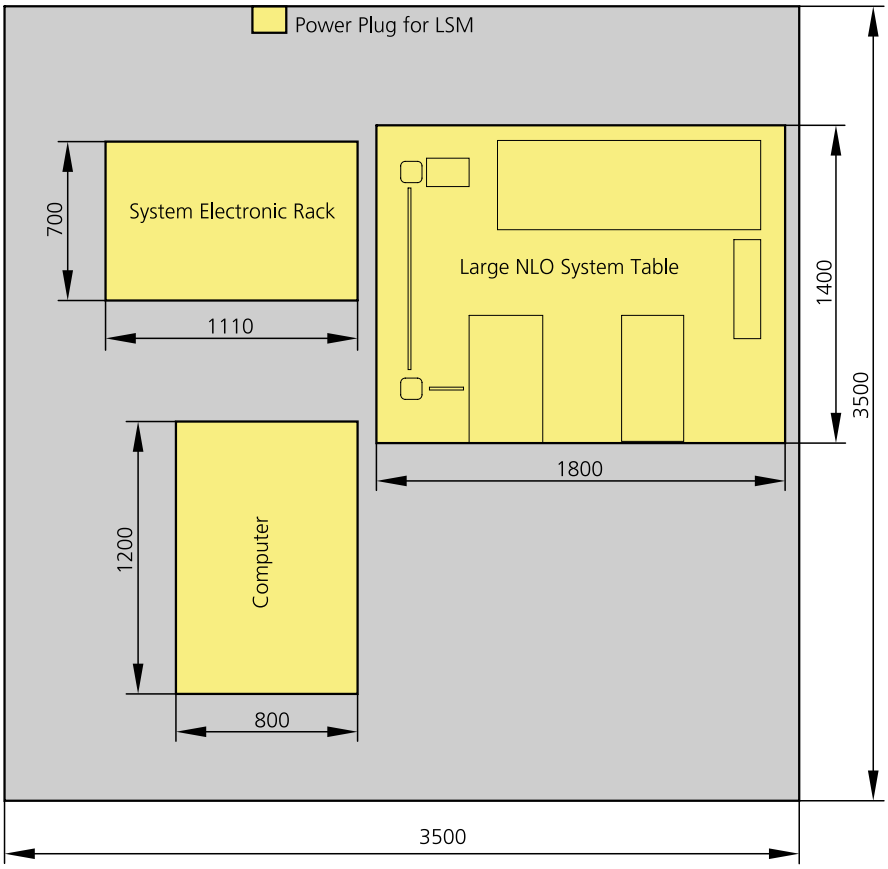


Fig. 2-8 Space requirements for Two Photon Laser and Large NLO System Table. Set up of one LSM system with two microscopes (measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the system, the power supply for the microscope, the scanning unit and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack. Power supply and cooling unit of the NLO Laser can be stored under the system table. This set up can hold two microscopes. Coherent “Chameleon” or Spectra Physics “Mai Tai” lasers can also be used. With the extension of LSM DuoScan (VIS and/or 405 nm) the Laser Module LIVE is situated in the System Electronic Rack. The electronics for the NLO laser have to be set aside the System Electronic Rack. An additional lab cart (000000-0465-515) is recommended. With the extension of the LSM DuoScan UV only additional space is required to set up the UV laser (see chapter 2.1.4).

2.2 Power Requirements

☞ The LSM 510 and LSM 510 META comes with a mains power supply cord and plug, either CEE red (3/N/PE 400/230V/16A), or NEMA L 14-30P (2/N/Ground 120/240V/30A), and with the matching mains socket outlet.
A ground wire (AWG10 green/yellow) is supplied because it is necessary to ground the system. The connecting part on both ends of the cable is a cable eye with 8 mm inner diameter.
A suitable grounding point must be installed in the room. For systems (220 ... 240 V AC) equipped with X-Cite 120 the mains socket outlet must be equipped with a fuse having minimum tripping characteristic C according to IEC/EN 60898.

Line voltage	220 … 240 V AC (±10 %)	100 … 125 V AC (±10 %)
Line frequency	50...60 Hz	50...60 Hz
LSM incl. VIS laser		
– Max. current	3 phases at 16 A	2 phases at 25 A
– Power	Phase 1 = 1.9 kVA max. Phase 2 = 1.5 kVA max. Phase 3 = 2.6 kVA max.	Phase 1 = 3.2 kVA max. Phase 2 = 2.8 kVA max.
– Power consumption	5000 VA max.	5000 VA max.
Argon UV laser		
– Line Voltage	208...240 V AC (±10 %) 50 / 60 Hz	208...240 VAC (±10 %) 50 / 60 Hz
– Max. current	1 phase at 63 A Note: For Line Voltage 220 V the connector and power plug are rated for 63 Amps, However wiring and fuse should be rated for 32 Amps.	1 phase at: 208 V: 34 Amps 230 V: 31 Amps 240 V: 29 Amps
– Power consumption	7000 VA max.	7000 VA max.
Class of protection	I	I
Type of protection	IP 20	IP 20
Overvoltage category	II	II
Pollution degree	2	2

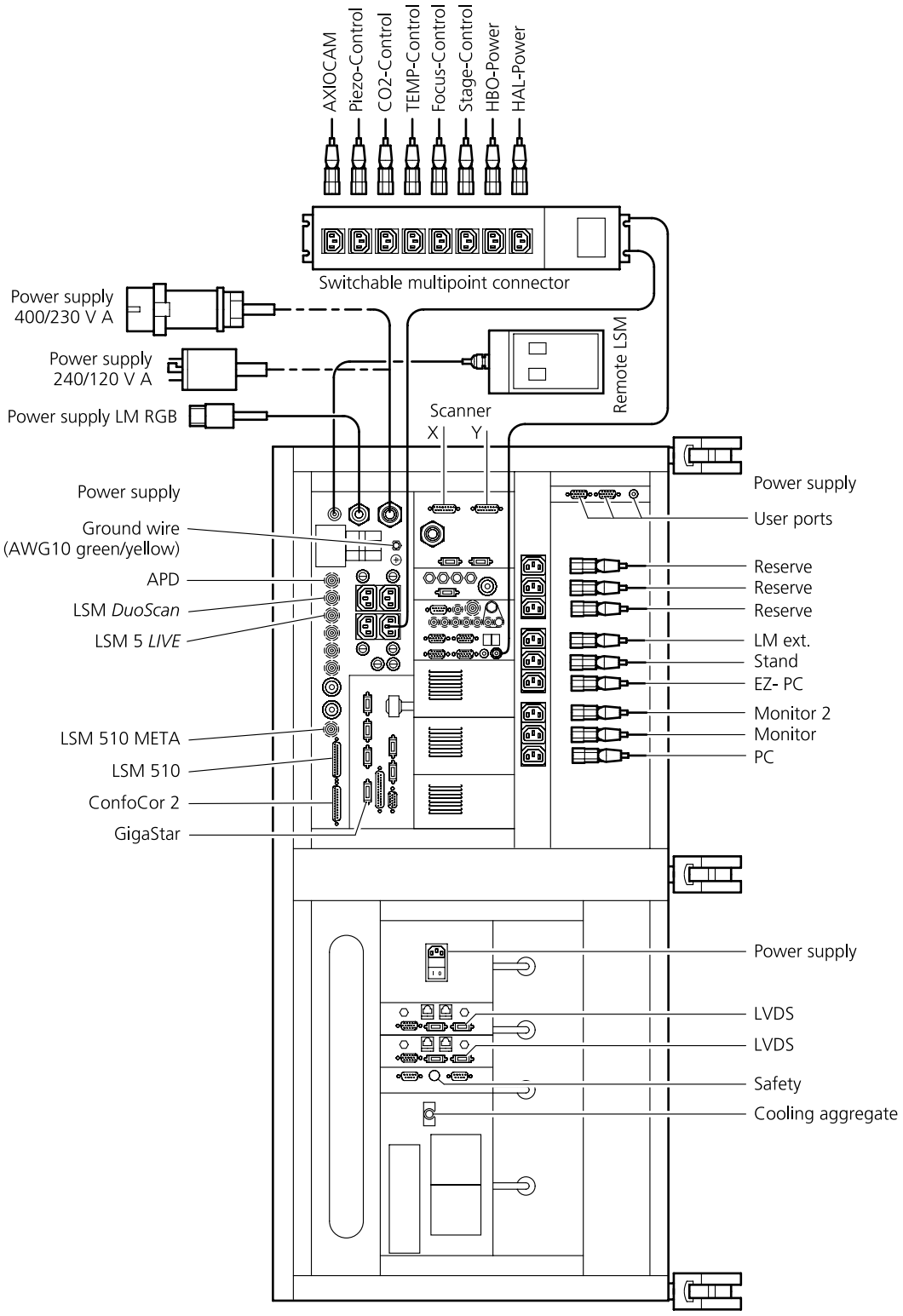


Fig. 2-9 **Power connector for LSM 510 / LSM 510 META and Laser Module V. Free/reserve outlets may be used to supply power to additional equipment. No more than 1 A can be provided by each outlet. (Scheme is turned 90° to real system.)**

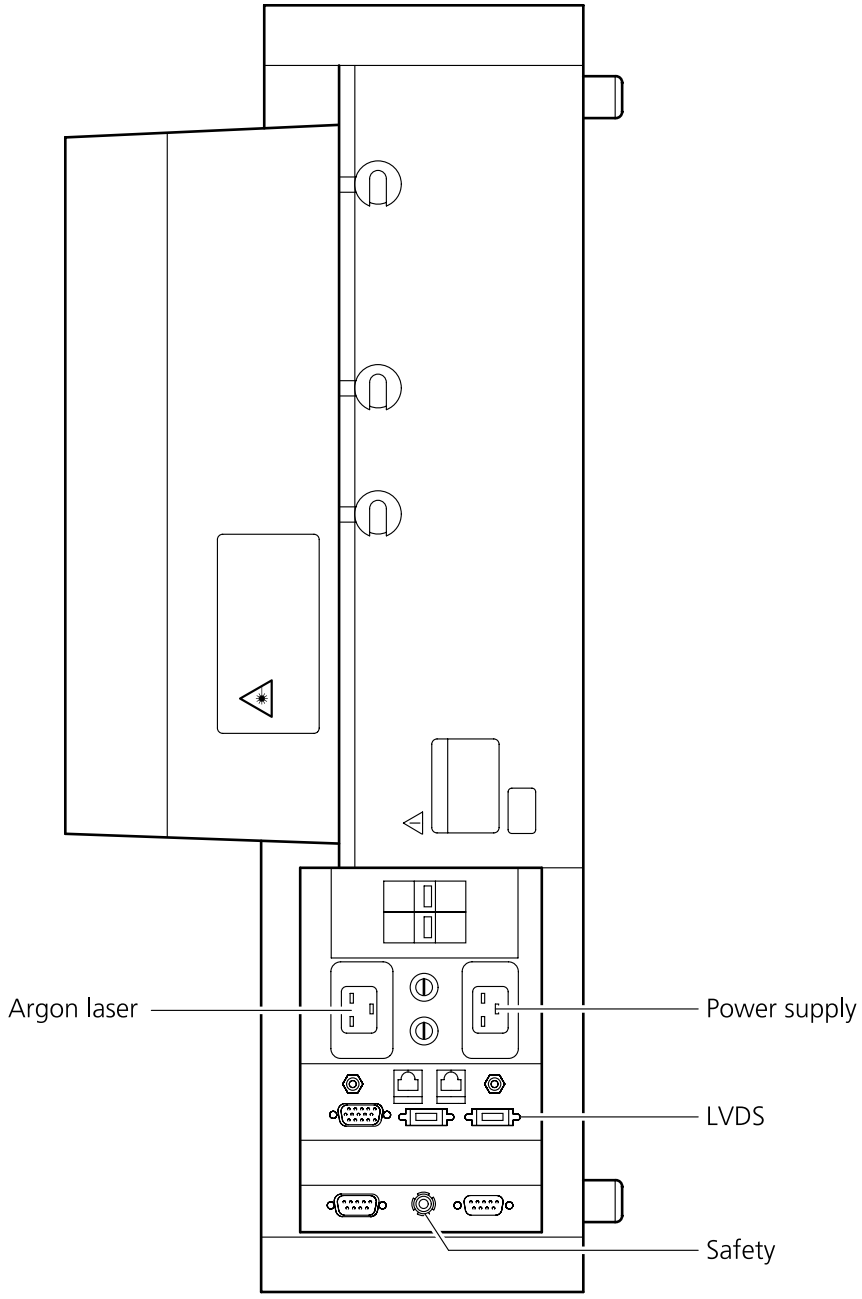
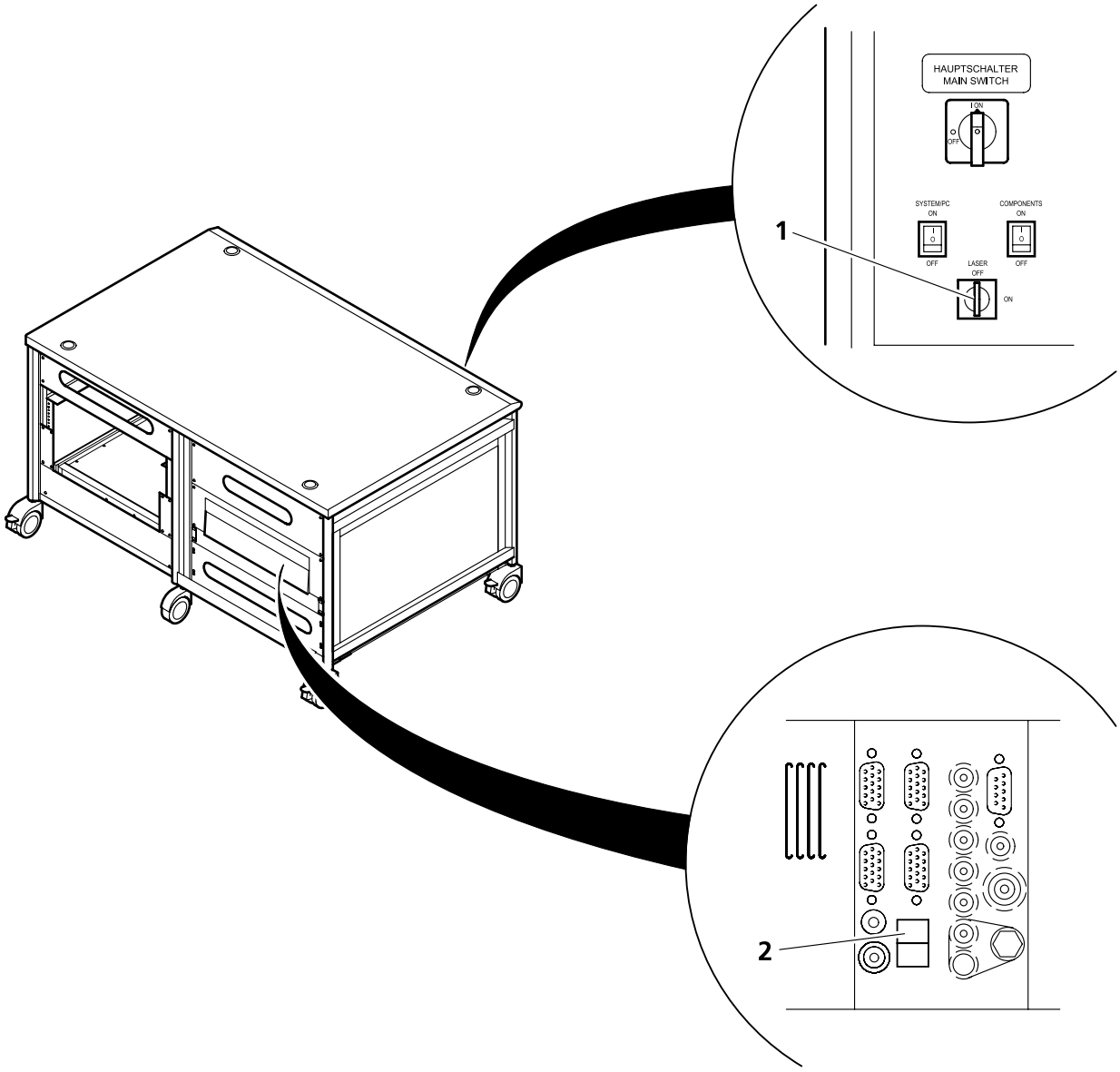


Fig. 2-10 Power connector for Laser Module RGB (Scheme is turned 90° to real system.)



1 Key-interlock Laser ON/OFF
2 Door interlock interface

Fig. 2-11 Key-interlock Laser ON/OFF and interface for connection of door interlock

The door interlock interface is covered with a green plug to bypass a door interlock.

- To use the interface remove the top of the green plug and the bypass wire.
- Then connect the wires of the door interlock at the same position.

Two door interlocks can be connected.

2.3 Physical Dimensions

	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Large system table	150	80	78	100
Small system table	65	80	78	60
Passively damped anti-vibration table	130	100	75	137
Active anti-vibration table (NLO) for Mai Tai Laser or Chameleon	120	140	75	200
Active anti-vibration table (NLO) for Verdi Mira or Millenia Tsunami Laser	180	140	75	400
Scanning Module LSM 510	25	20	25	15
Scanning Module LSM 510 META	28	27	30.5	13
Scanning Module LSM *DuoScan*	40	15	13	8
Microscope	50	35	50	20
Laser Module RGB	110	70	28	95
Laser Module, UV	140	20	20	60
Laser Module V (405 nm)	66	52	22	30
Laser Module *LIVE*	66	52	22	58
Plug-in unit external laser	66	52	22	9
System Electronic Rack	110	70	58	90
Power supply for Ar (UV)	50	50	30	30
Cooling unit for Ar (UV)	80	45	50	30
Water hose for Ar (UV)	700			
Fiber optic cable, VIS(ible)	300			
Fiber optic cable, UV	300			
Fiber optic cables at NLO systems	400			
Cables	350			
SCSI cable	350			

2.4 Dimension of Shipment Crates

Crate containing	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Large system table	160	85	95	120
Small system table	90	75	80	80
Passively damped anti-vibration table	145	115	115	150
Active anti-vibration table (NLO) for Mai Tai Laser or Chameleon	145	160	110	330
Active anti-vibration table (NLO) for Mira or Tsunami Laser	200	160	110	460
System Electronic Rack and Laser module	135	90	100	300
LSM, Microscope, Computer	135	90	100	150
Additional Hardware Components	135	90	61	100
UV laser unit	125	55	50	100
UV cooling unit	120	60	90	50

2.5 Environmental Requirements

1. Operation, specified performance	T = 22 °C ± 3 °C without interruption (24 h a day independently whether system is operated or switched-off)
2. Operation, reduced performance	T = 10 °C to 35 °C, any conditions different from 1. and 5.
3. Storage, less than 16 h	T = -40 °C to 55 °C
4. Storage, less than 6 h	T = -55 °C to 70 °C
5. Temperature gradient	± 0.5 °C/h
6. Warm up time	1 h, for high-precision and/or long-term measurements ≥ 2 h
7. Relative humidity	< 65 % at 30 °C
8. Operation altitude	max. 2000 m
9. Loss of heat	4 kW

2.6 Vibrations

Vibrations under operation conditions (with system table)	**Shipping shock (LSM 510 box)**
5 µm pp at 5 Hz 10 µm pp at 10 Hz 10 µm pp at 20 Hz	3 g

2.7 Microscopes

Inverted Axiovert 200 M BP or SP

Upright Axioskop 2 FS MOT

Upright Axio Imager.Z1

Upright Axio Imager.M1

All ICS objectives from Carl Zeiss and their accessories can be accommodated.

Z motor

DC servomotor, opto-electronically coded

Least Z interval: 50 nm (Axiovert 200 M BP or SP)
100 nm (Axioskop 2 FS MOT)
10 nm (Axio Imager.Z1)
25 nm (Axio Imager.M1)

Piezo Objective focus

Piezo-driven single objective drive

Max. travel 250 µm; resolution 10 nm

In the unlikely case of extreme fluctuations of the external power net or electromagnetic radiation, the piezo crystal will vary and disturbance in the image is visible. Note that this is not a defect and the piezo drive will not be damaged.

2.8 Scanning Module LSM 510 and LSM 510 META

Scanners	2 individually driven galvanometric scanners
Scanning speed	Up to ~5 frames/sec (512 × 512 pixels)
Field resolution	Max. 2048 × 2048 pixels (individually adjustable for each axis)
Field of view	10 × 10 mm² with a 1.25× objective
Zoom	1× ... 40×, continuous control
Channels	a) Up to 4 confocal reflection/fluorescence channels (PMT) simultaneously **or** b) 2 confocal reflection/fluorescence channels (PMT) and 1 META detector 1 transmitted light channel (PMT) and 2 NDD **or** 3 - 4 NDD (Non descanned detectors, PMT) 1 reference monitor diode Fiber-optic adaptation of external detectors
Dynamic range	12-bit DAC for each detection channel
Pinholes	4 individual variable pinholes (one per confocal channel or META detector) Computer controlled automatic alignment

2.9 Scanning Module LSM *DuoScan*

Scanners	2 individually driven galvanometric scanners
Scanning speed	Up to ~5 frames/sec (512 × 512 pixels)
Field of view	10 × 10 mm² with a 1.25× objective

2.10 Laser Module RGB (458, 477, 488, 514, 543 or 561,594, 633 nm)

Single-mode polarization preserving fiber
Laser beam attenuation for all lasers by VIS-AOTF
HeNe laser (543 nm, 1 mW)
HeNe laser (594 nm, 2 mW)
HeNe laser (633 nm, 5 mW)
DPSS laser (561 nm, 10 mW)
Ar laser (458, 477, 488, 514 nm, 30 mW)

2.11 Laser Module V (405 nm)

Single-mode polarization preserving fiber
Laser beam attenuation by UV-AOTF
Diode laser (405 nm, 30 mW)

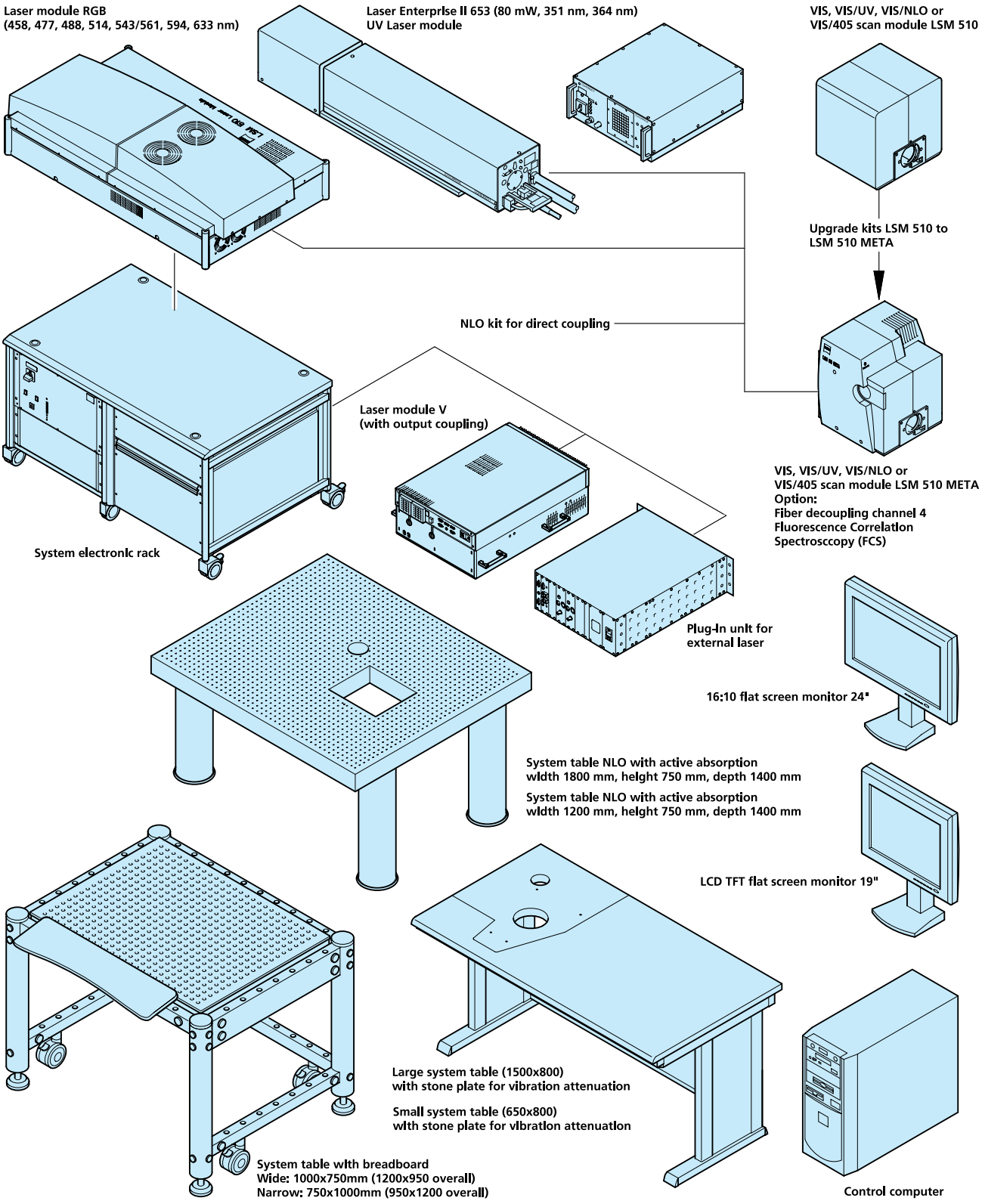
2.12 Laser Module UV (351, 364 nm)

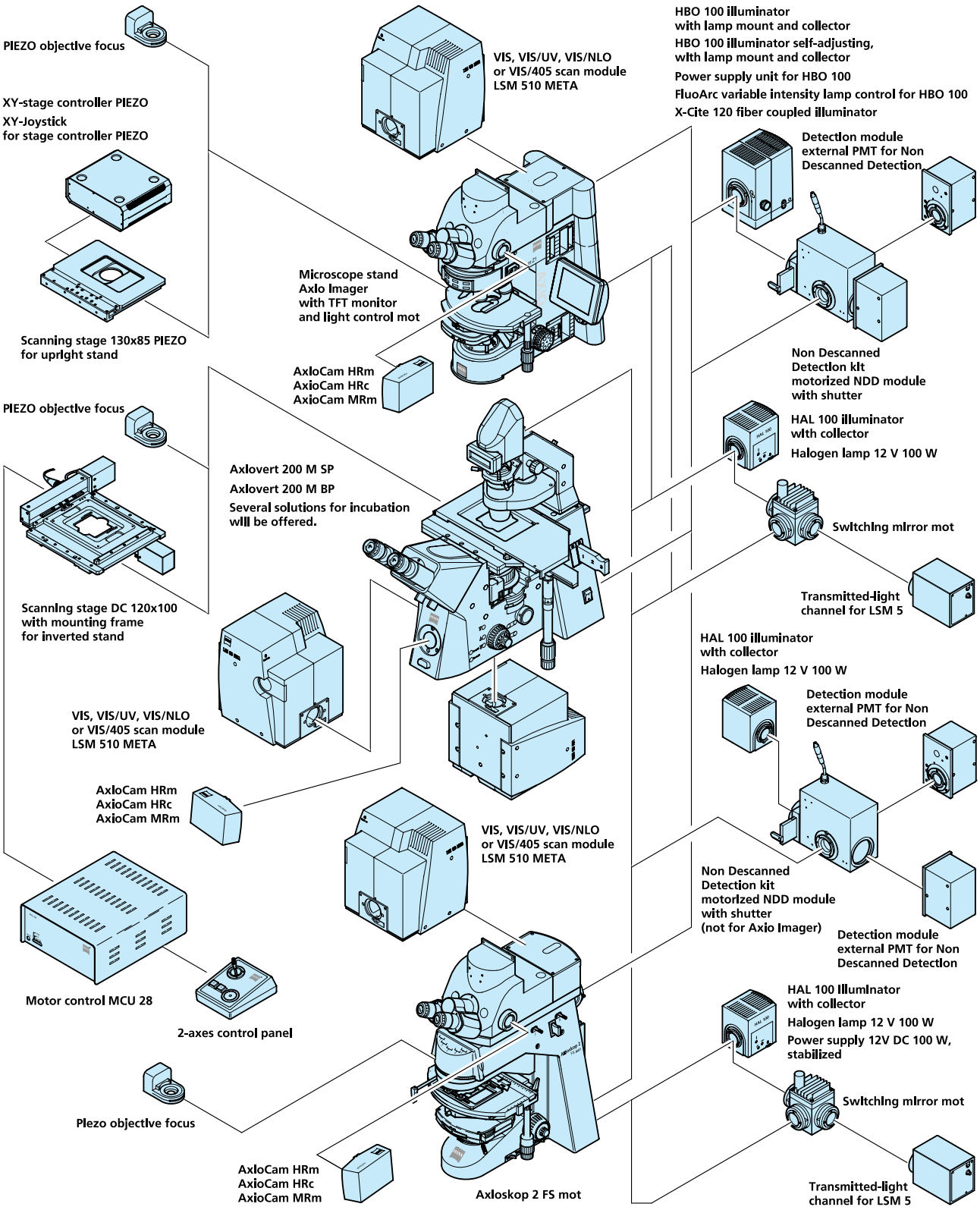
Single-mode polarization preserving fiber
Laser beam attenuation for all lasers by UV-AOTF
Ar laser (351, 364 nm, 80 mW)

2.13 Laser Module *LIVE* for LSM *DuoScan*

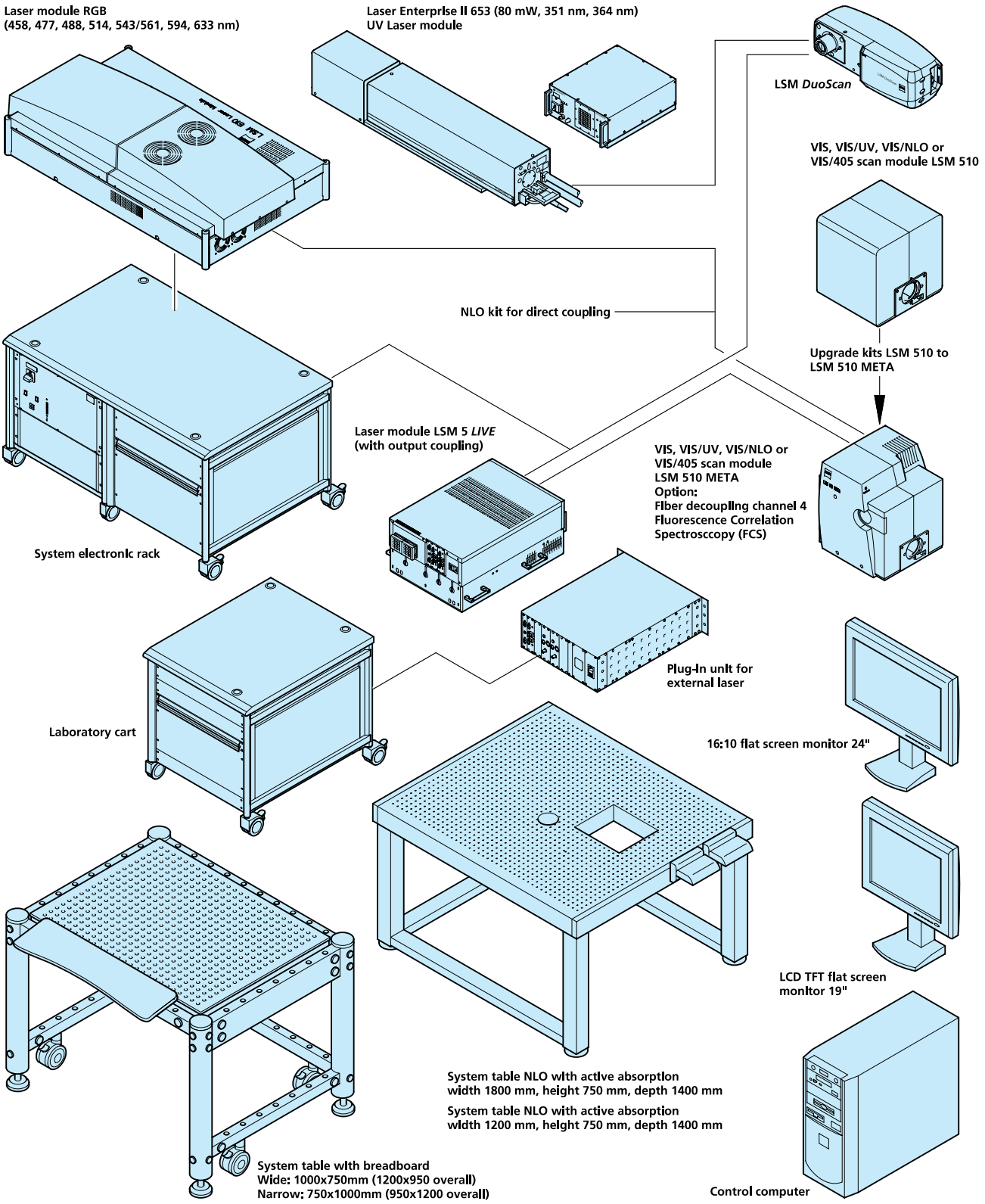
Single-mode polarization preserving fiber
Laser beam attenuation (AOTF) for all lasers
Diode laser (405 nm, 50 mW)
OPSS laser (488, 100 mW)
DPSS laser (532 nm, 75 mW)

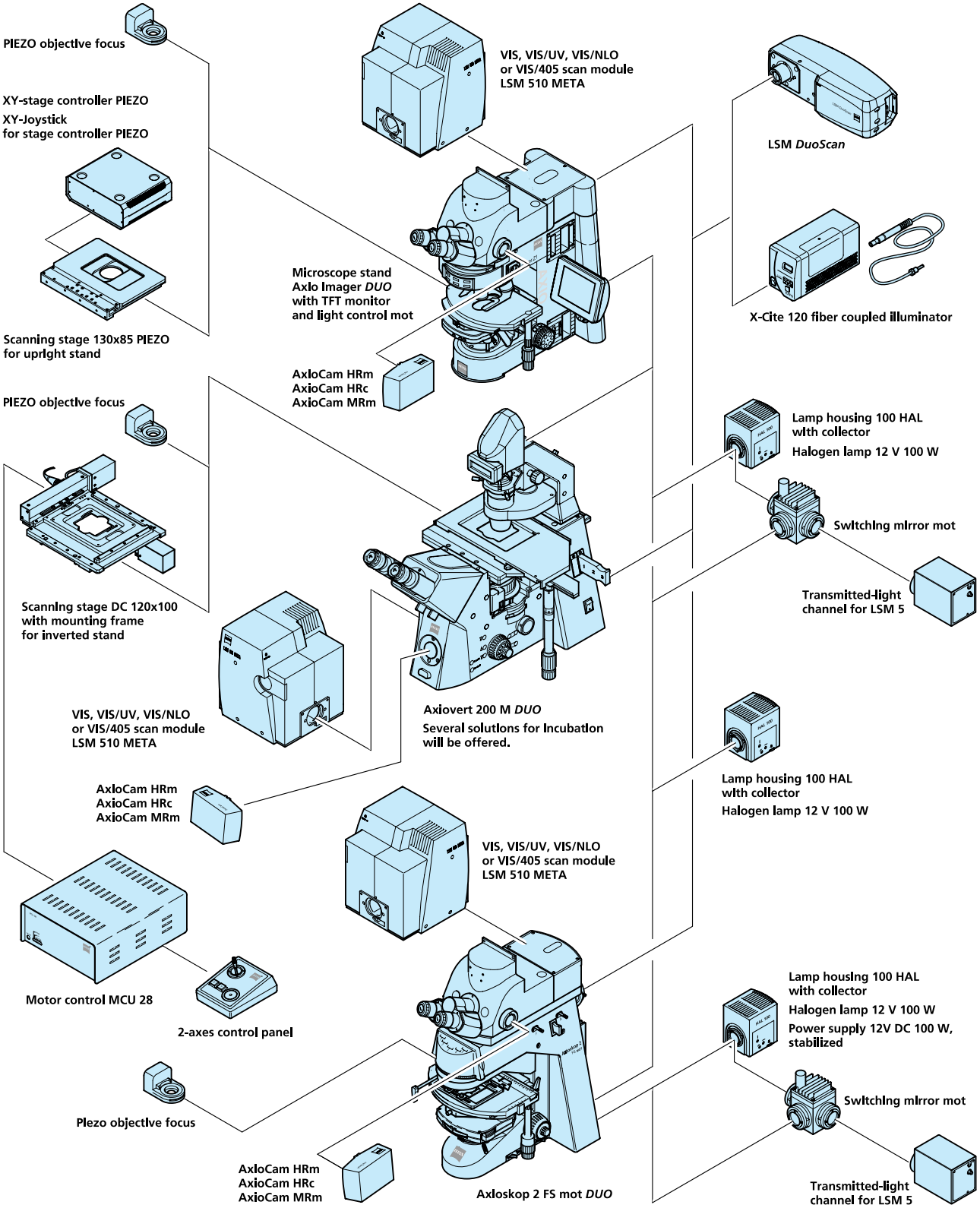
2.14 System Overview LSM 510 META




PIEZO objective focus
XY-stage controller PIEZO
XY-Joystick
for stage controller PIEZO
Scanning stage 130x85 PIEZO
for upright stand
VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META
HBO 100 illuminator
with lamp mount and collector
HBO 100 illuminator self-adjusting,
with lamp mount and collector
Power supply unit for HBO 100
FluoArc variable intensity lamp control for HBO 100
X-Cite 120 fiber coupled illuminator
Detection module
external PMT for Non
Descanned Detection
Microscope stand
Axio Imager
with TFT monitor
and light control mot
AxioCam HRm
AxioCam HRc
AxioCam MRm
Non Descanned
Detection kit
motorized NDD module
with shutter
HAL 100 illuminator
with collector
Halogen lamp 12 V 100 W
Switching mirror mot
Transmitted-light
channel for LSM 5
PIEZO objective focus
Axiovert 200 M SP
Axiovert 200 M BP
Several solutions for incubation
will be offered.
Scanning stage DC 120x100
with mounting frame
for inverted stand
VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META
AxioCam HRm
AxioCam HRc
AxioCam MRm
HAL 100 illuminator
with collector
Halogen lamp 12 V 100 W
Detection module
external PMT for Non
Descanned Detection
VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META
Non Descanned
Detection kit
motorized NDD module
with shutter
(not for Axio Imager)
Detection module
external PMT for Non
Descanned Detection
Motor control MCU 28
2-axes control panel
HAL 100 illuminator
with collector
Halogen lamp 12 V 100 W
Power supply 12V DC 100 W,
stabilized
Switching mirror mot
Piezo objective focus
AxioCam HRm
AxioCam HRc
AxioCam MRm
Axioskop 2 FS mot
Transmitted-light
channel for LSM 5

2.15 System Overview LSM 510 META *DuoScan*




PIEZO objective focus
XY-stage controller PIEZO
XY-Joystick
for stage controller PIEZO
Scanning stage 130x85 PIEZO
for upright stand
VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META
LSM *DuoScan*
Microscope stand
Axio Imager *DUO*
with TFT monitor
and light control mot
X-Cite 120 fiber coupled illuminator
AxioCam HRm
AxioCam HRc
AxioCam MRm
PIEZO objective focus
Lamp housing 100 HAL
with collector
Halogen lamp 12 V 100 W
Switching mirror mot
Transmitted-light
channel for LSM 5
Scanning stage DC 120x100
with mounting frame
for inverted stand
VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META
Axiovert 200 M *DUO*
Several solutions for Incubation
will be offered.
Lamp housing 100 HAL
with collector
Halogen lamp 12 V 100 W
AxioCam HRm
AxioCam HRc
AxioCam MRm
VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 META
Motor control MCU 28
2-axes control panel
Lamp housing 100 HAL
with collector
Halogen lamp 12 V 100 W
Power supply 12V DC 100 W,
stabilized
Switching mirror mot
Plezo objective focus
AxioCam HRm
AxioCam HRc
AxioCam MRm
Axioskop 2 FS mot *DUO*
Transmitted-light
channel for LSM 5

CHAPTER 3 INTRODUCTION TO LASER SCANNING MICROSCOPY

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3.4 Performance Features of the LSM 510 and LSM 510 META 3-5
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3 INTRODUCTION TO LASER SCANNING MICROSCOPY

3.1 Principle of Laser Scanning Microscopy

To yield information on their inner structure by conventional transmitted-light microscopy, specimens have to be very thin and translucent; otherwise image definition will be poor. In many cases it is a problem to satisfy these requirements.

The essential considerations have led to trailblazing changes in conventional microscopy and supplied a successful solution to the above problem.

- Unlike the practice of even illumination in conventional microscopy, the LSM technique projects the light of a point light source (a laser) through a high-NA objective onto a certain object plane of interest as a nearly diffraction-limited focus. However, if not for another "trick", the stray light produced outside the object plane, or the fluorescence of fluorescent specimens, would disturb the in-focus image of object point of interest, resulting in a blurred image of poor contrast. The problem therefore is how to capture only the light coming immediately from the object point in focus, while obstructing the light coming from out-of-focus areas of the specimen.

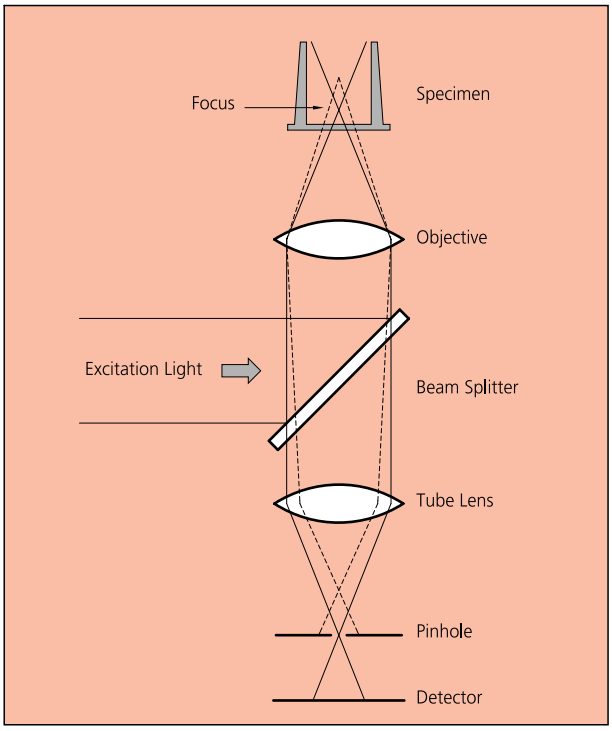

Fig 3-1 Principle of confocal imaging

- The light reflected, or the fluorescence light produced, at the focus of the high-NA objective is projected onto a variable pinhole diaphragm by the same objective and a tube lens. The focus inside the specimen and the pinhole are situated at optically conjugate points (**confocal imaging**)**.** The decisive advantage of this arrangement is the fact that essentially no other light than that coming from the object plane of interest can pass the narrow pinhole and be registered by a detector. Unwanted light coming from other specimen areas is focused outside the pinhole, which passes only a small fraction of it. The smaller the pinhole, the less stray light or fluorescence from out-of-focus areas will get on the detector. The image point thus generated is largely free from blur caused by unwanted light.
- In order to obtain an image of the selected object plane as a whole, it is necessary to scan the object plane in a point-by-point, line-by-line raster by means of an XY light deflection system. The detectors - as a rule, photomultipliers - convert the optical information into electric signals. This allows the image of any object plane to be generated and stored within less than a second. By a defined focusing (Z axis) movement it is possible to look at any object plane of interest. By scanning a succession of object planes in a specimen, a stack of slice images can be produced.

This way, the LSM technique in conjunction with ICS optics (Infinity Color-Corrected System) has brought decisive improvements over conventional microscopy in terms of resolving power and confocal depth contrast:

Object features in the order of 0.2 µm can be resolved, and height differences of less than 0.1 µm made visible, without the use of interference methods.

3.2 Three-Dimensional Presentations of LSM Image Stacks

One of the advantages of the LSM technique is that it can present structures in three dimensions. This opens up many ways to process images. Outlined below are some of the possible methods to extract spatial information from stacks of slice images.

- **Gallery**
  The simplest presentation of 3D information is a gallery showing the individual slice images (sections) of a stack arranged side by side, with each slice apart from the next by a defined, selectable interval on the Z axis.
- **Virtually infinite depth of focus**
  The entire set of data can be imaged as a single projection. The computer establishes an image composed of all in-focus optical sections. The image produced by this so-called composite method has a virtually infinite depth of focus, since the result is made up of information from in-focus planes only.
- **Rotary animation**
  A sequence of projections is computed, with the specimen being apparently rotated by a certain angle from image to image, for example by a full turn about an axis. If such a sequence is displayed on the monitor screen in rapid succession, the visual effect is that of a rotating three-dimensional object.
- **Stereo image pairs**
  The computer establishes a pair of images corresponding to those we see with the right and the left eye, respectively. The two images forming the stereo pair can be shown on the monitor side by side. They can be seen as a 3D image with suitable optical aids. Another possibility is to present both images in registration, with one image in the red channel and the other in the green one (anaglyph). Viewed through red and green color filters in a spectacle frame, which only pass the image intended for the respective eye, the two images form a 3D image in the brain
- **Color-coded height slices**
  Each level, i.e. each slice is assigned a different color. For direct evaluation, a color scale is shown, indicating the actual height above the bottom slice.
- **Orthogonal sections**
  This computation produces a triplet of mutually perpendicular sectional images.
- **Oblique sections**
  A section through the stack is made along an oblique plane defined by the selection of five coordinates, i.e. X, Y, Z, angle of rotation, and angle of tilt.
- **Topography** (option)
  A computing program for surface topography presentations (as required in materials research) is available.
- **Physiology** (option)
  With a special software, kinetic processes can be tracked, which is especially of interest to physiology.
- **Image VisArt** (option)
  Three-dimensional display of floating transparent structures (cells) or opaque structures (material)
- **3D Deconvolution** (option)

3.3 Optical Diagram of the LSM 510 and LSM 510 META (Schematic)

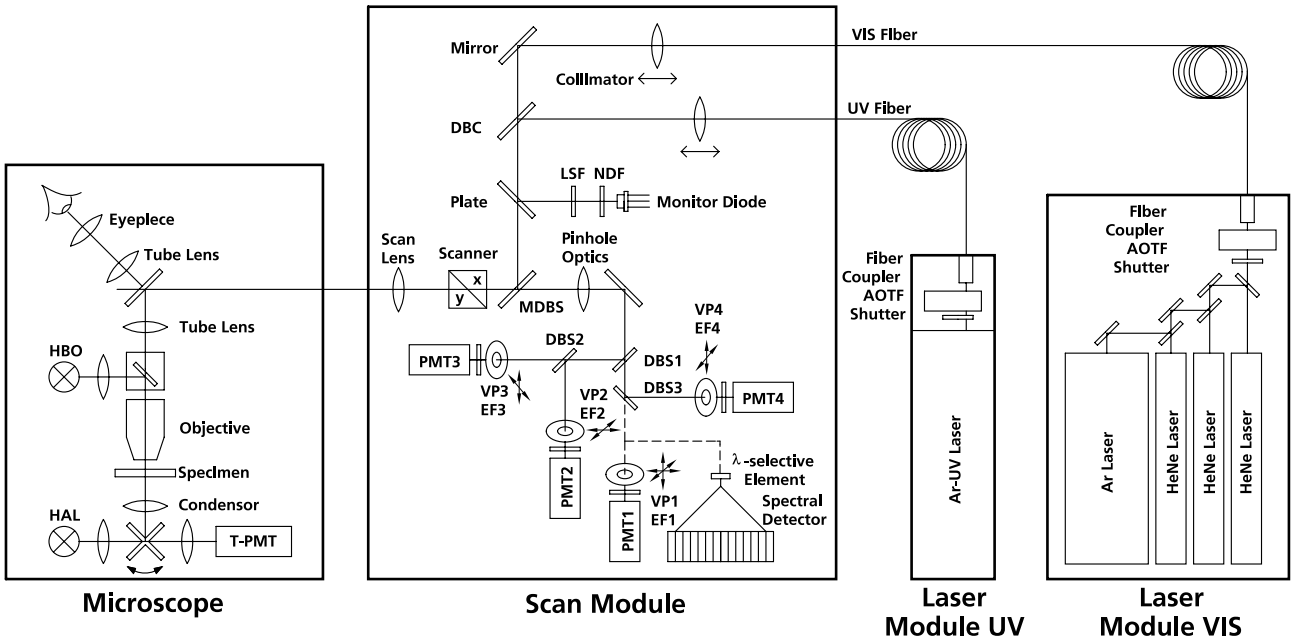

Fig. 3-2 Optical path, schematic (4-channel configuration)

AOTF	Acousto Optical Tunable Filter
DBC	Dichroic Beam Combiner
DBS	Dichroic Beam Splitter
EF	Emission Filter
HAL	Halogen Lamp
HBO	Mercury Vapor Short-Arc Lamp
LSF	Line Selection Filter
MDBS	Main Dichroic Beam Splitter
NDF	Neutral Density Filter
VP	Variable Pinhole
PMT	Photomultiplier
T-PMT	Transmission-Photomultiplier

The diagram above is a schematic representation of the LSM system.

Laser light is focused onto the specimen through an objective in a diffraction-limited mode. Light emitted at the focal plane and at planes below and above it is directed via an XY scanner onto a main dichroic beam splitter (MDBS), which separates the emissions from the excitation light. The fluorescences are separated from each other by a series of dichroic beam splitters (DBS1 ... maximally DBS4) and directed to individual photomultipliers (PMT1 ... maximally PMT4).

3.4 Performance Features of the LSM 510 and LSM 510 META

3.4.1 Optical and Mechanical Aspects

The highly integrated system design makes for the shortest possible optical paths, top-grade optical precision and high stability. The compact scanning module can be fitted to an inverted (Axiovert 200 M BP or SP) or upright (Axio Imager.Z1, Axio Imager.M1 or Axioskop 2 FS MOT) microscope in less than three minutes. On the Axiovert 200 M, the scanning module may be mounted either to the base port directly below the microscope or to the side port.

The spectral range available extends from the UV to the IR region.

For the VIS (visible-light) Laser Module, the user can select from up to six lasers with wavelengths of 633, 594, 561, 543, 514, 488, 477, 458 and 405 nm. The UV Laser Module provides wavelengths of 351 and 364 nm. A Ti:Sa Laser provides pulsed laser light from 690 to 1040 nm for Multiphoton imaging (NLO). Coupling of the laser light is through polarization-preserving single-mode optical fibers. One variable beam collimator each for the UV or NLO and visible ranges provides optimum adaptation of the respective laser wavelength to the objective used and, thus, optimum correction for Z aberrations.

Acousto-optical tunable filters (AOTF) adjust the necessary brightness for up to 8 laser lines within microseconds.

A monitor diode permanently registers the laser output; it can be used for the on-line checking of the intensity of the exciting light. This check is also possible selectively for the different wavelengths if a line selection filter is inserted.

The four internal image acquisition channels, usable for reflection or fluorescence, and an additional transmitted-light channel are ideal for the investigation of multiple fluorescence specimens. Separately in each of the four channels, the diameters of the pinholes and their XY positions can be optimized, and the desired emission filter placed into the beam path, by servo-motor control. In the case of pinhole VP1, this adjustment also includes positioning along Z. In the simultaneous registration of multiple fluorescence, identical optical sections can be obtained in each confocal channel. This is of importance, e.g., with the FISH method (fluorescence in-situ hybridization) used for genome analysis in cytogenetic studies.

The microscope's transmitted-light channel is equipped with a photomultiplier, too. It is therefore possible to superimpose a multiple fluorescence image on a brightfield, differential interference or phase image.

A fiber-optic cable connection to external special detectors, such as cooled PMTs or spectrometers, is also available. It substitutes one internal detection channel.

In addition to the emission filters for all standard and special applications, available in motor-controlled filter wheels, the user can easily install his own emission filters in two of the channels.

The high-NA C-APOCHROMAT objectives specially developed for the LSM technique reach the physical limit in resolving power, and can be used throughout the 350...800 nm spectral range with the same high quality, producing brilliant images.

A two-mirror scanner system, controlled by a real time electronics, offers several advantages. The large deflection angle of the scanning mirrors allows a wide area to be scanned. With a 1.25× objective, the object area scanned is 10 × 10 mm².

The scanning field size can be freely selected between 4 × 1 and 2048 × 2048 pixels.

It is possible to rotate the XY scanning field through 360° and carry out XY scans without having to rotate the specimen itself under laser radiation load.

Selection of the specimen detail of interest for zooming is fast and convenient, and the zoomed image is automatically centered. This saves the job of specimen centration with the microscope stage.
Using a bi-directional scanning facility will double the scanning rate to approx. 5 frames/sec (at 512 × 512 pixels); if two different laser wavelengths are used for the two scanning directions (wavelength 1 for left-to-right, and wavelength 2 for right-to-left scanning), two fluorochrome dyes can be viewed and documented in a quasi-simultaneous mode. This will prevent cross talk between detection channels.
The LSM 510 and LSM 510 META can be combined with a second scanner, the LSM *DuoScan*. Any of the above mentioned microscopes can be used. The *LSM DuoScan* can be equipped either with a UV Laser or a 405 nm laser both also together with a 488 and/or 532 nm laser. The software allows to simultaneously image and bleach/manipulate a sample. The usefulness of this option is dependent on the sample.

3.4.2 Microscope Equipment of the LSM 510 and LSM 510 META System

The LSM 510 or LSM 510 META system is equipped either with the inverted Axiovert 200 M BP or SP microscope or with the upright Axio Imager.Z1, Axio Imager.M1 or Axioskop 2 FS MOT microscopes.

Only the differences from the delivered operating manual "Axiovert 200 M" will be explained here.

(1) Stand

a) The motorized objective nosepiece 5× H DIC is firmly fixed to the stand, where no operating elements can be found for the nosepiece. Operation will be performed via LSM 5 software control. The "Restriction of the nosepiece height to protect the objectives during motorized objective change" is inactivated. The nosepiece will be moved down automatically before each motorized objective change.

b) The reflector mount is motorized and provided with the Axiovert 200 M reflector turret. The reflector turret has 5 positions: One transmitting light position, which is identical to the LSM position, and four further positions for fluorescence filter sets (reflector modules). If you want to use more than five conventional fluorescence filter sets, it is advisable to use a further reflector turret. When changing the reflector turret position you must make sure that the turret will click into position, since otherwise the image area will be cut. If the system is equipped with the LSM *DuoScan* up to three positions (depending on the laser equipment of the LSM *DuoScan*) are equipped with fixed beam combiners. Two positions of the reflector turrent can be equipped with conventional fluorescence filter sets.

c) The stand has a motorized focusing drive (fine coarse). Sensitivity of the focusing drive is adjusted to the delivered objectives by the manufacturer. If you want to use other objectives, sensitivity and parfocality can be adjusted via the Axioset program.

d) The stand features an integrated power supply for the internal motors and stand electronics. The power supply can be switched on at the right side of the stand. External power supply units will be used for the mercury vapor short arc lamp.

e) The analyzer slider for conventional DIC methods will be operated from the right side and is located just below the nosepiece.
When the rod is pushed in, the analyzer is located in the beam path. In the LSM-mode, the analyzer must **not** be located in the beam path, and the analyzer rod must be pulled out.

(2) Specimen stages and fine focus drives

a) Mechanical stage
The stage with coaxial drive must be mounted on the right side of the stand.

b) Scanning stage

c) Piezo objective focus drive

(3) Transmitted-light illumination

a) The illuminator support contains a security circuit which activates a shutter preventing laser light from reaching the stand when the support is moved to the back. A complementary shutter built in the stand prevents laser light from reaching the eyepieces during the scanning mode.

b) The illuminator support is equipped with a rotary polarizer. The Axiovert 200 M description contains the adjustment for the DIC mode during conventional observation.
For scanning in the transmitted-light DIC mode, the polarizer in the transmitted light support works like an analyzer and must be adjusted in such a manner that direct laser light will be blocked.
The conventional analyzer slider in the stand must not be located in the beam path because the laser light is already polarized.

c) A fully motorized, LSM 5 software-controlled switching mirror is mounted on the illuminator support. Alternatively, the light is directed to the LSM 5 transmitted-light detector or enables conventional transmitted-light observation.

d) The focusing screen for conventional transmitted-light is located in a support in front of the halogen lamp housing.

e) Further information on the halogen lamp and the condensers is provided in the Axiovert 200 M operating manual.

(4) Reflected light fluorescence

With the exception of the reflector slider, all the Axiovert 200 M fluorescence accessories can be used.
Further information is provided in the Axiovert 200 M operation manual.

(5) Imaging optics

Optovar sliders cannot be used.
The analyzer for the conventional DIC mode will be operated from the right side and is located just below the nosepiece.
Use of sliders with auxiliary objects (473704/14-0000-000) is not possible.

(6) Photo equipment

The stand does not feature an integrated SLR-port, but microscope cameras as described in the Axiovert 200 M and LSM 510 / LSM 510 META operation manual can be used.

(7) TV adaptation

The TV port at the side and the tubes can be used as described in the Axiovert 200 M operation manual.

The TV interface side port can be used with TV adapters 60 N or LSM adapters.

3.4.3 Computer Hardware and Software

The LSM 510 and LSM 510 META is controlled via a standard high-end Pentium PC. Linking to the electronic control system is made via Gigabit Ethernet interface. The PC comes with the WINDOWS XP operating system.

The instrument is fully motorized, permitting fast change-over between methods as well as automatic operation. Parameters once set or complex examination sequences once established can be saved and reproduced; therefore, complete application programs can be loaded and performed by pushbutton control.

The software of the LSM 510 and LSM 510 META have two levels. On the simple operator interface level, a result will be achieved after a few prompts; graphical prompting of the user in conjunction with automatic setting of many parameters is an ideal tool for daily routine jobs. The expert level offers perfect facilities for individual settings of functions and parameters.

Conversion of the light signals into a digital image is effected by means of four 12-bit A/D converters, each of which can generate 4096 brightness levels.

The software provides an enormously wide range of image processing functions, including all standard 2D/3D (stereo, projection) functions identical to sophisticated 3D reconstruction capabilities (surface and alpha rendering), digital processing of voxels and 3D measurement functions (surface areas, volumes).

As all files and images are recorded in MS Access databases, elegant image database editing is just as easy as transferring the records to other programs.

IMPORTANT NOTES FOR CHAPTER 4

Bleach series	When doing bleach series on LSM 510 *DuoScan* systems, the time stamps and bleach markers indicated in the images will depend on the start position of the scanners in the image frame. Since these are not identical for the imaging and the photomanipulation scanners, sometimes imaging seems to start too early after a bleach event. Ignore the last two digits in the time stamps and bleach markers in this case.
Macro	When using the modify images macro to convert time series data into Z stacks, an arbitrary section step of 1 µm is applied to the stack. This will also lead to an arbitrary time stamp when reconverting the Z stack into a time series. The order of the images however is preserved under all conditions.
Piezo objective focus units	The piezo objective focus units 1325-210, 1325-212 and 1325-213 with an increased travel range of 250 µm are controlled by a 14 bit logic. Due to the increased travel range, this implies a smallest step of 15 nm, in contrast to former fine focus solutions with a smaller travel range but a smallest step size of <10 nm.

CHAPTER 4 OPERATION IN EXPERT MODE

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4 OPERATION IN EXPERT MODE

4.1 Purpose

This chapter describes the operation of the LSM 510 and LSM 510 META Laser Scanning Microscopes, also when equipped with LSM *DuoScan*, exemplified by typical applications in conjunction with the LSM 5 software and its graphic user environment.

When starting up and operating the microscope system, mind the operating instruction manuals for the Axio Imager.Z1, Axio Imager.M1, Axiovert 200 M and Axioskop 2 FS microscopes:

- B 46-0046 Axio Imager, Operating Manual
- B 40-080 Axiovert 200 M, Operating Manual
- B 40-076 Axioskop 2 FS MOT, Operating Manual

4.1.1 Software

The LSM 5 software, Version 4.0:

- controls the microscope, the scanning and laser modules, tools (filters, stand, Axioset) and the image acquisition process
- displays and analyzes the images

It is based on the network-capable graphic 32-bit Microsoft ® WINDOWS XP operating system.

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☞ The installation of the software for the LSM 510 and the basic settings of the equipment components are carried out by Carl Zeiss service staff. This job includes the creation of a customized software configuration in line with the specific hardware components of the customer's microscope system.

The LSM 5 software is menu-controlled and normally uses its own windows for the activation of the various functions; within these windows, further submenus (panels) can be displayed and removed.

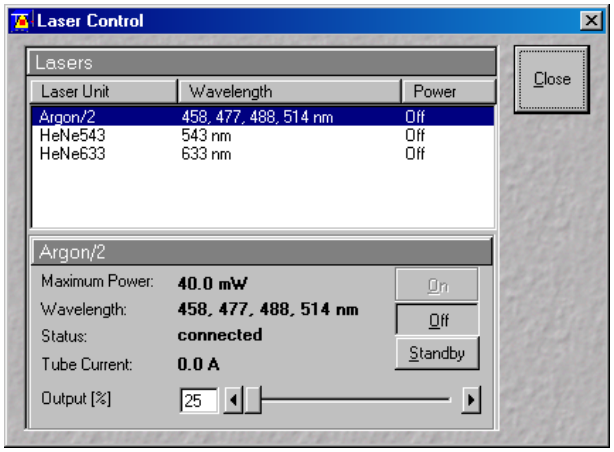
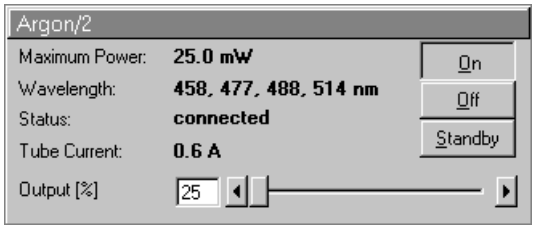
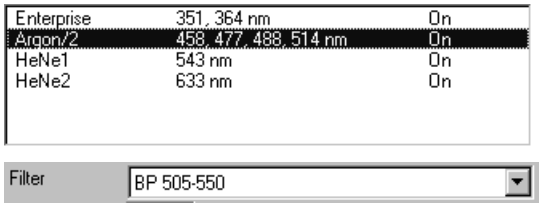
☞ The screen layout used in this manual is based on the former Windows ® standard layout and may vary from yours.

Images of the specimens to be examined, created by scanning, are displayed in separate **Image Display** windows.

Theoretically, the number of simultaneously opened windows for software operation or image display is unlimited, but should not be too excessive so that an overview is still possible.

Identical functions, e.g. **Laser Control**, can be performed in several software windows. Changes made by the software are recorded immediately and are automatically transferred to all the other windows concerned.

4.1.2 Windows and Window Elements

Window element	Description / Explanation
	Window (e.g.: **Laser Control** window) – Window displayed after activation of a function button (e.g.: **Laser** button in the toolbar of the Expert Mode).
	Panel (e.g.: **Argon** panel) – Limited function range within a window
	List box or selection box – Selection of one of the displayed options at a click of the mouse. – Open the box by clicking on the arrow button.
	Input box – Input of text or numeric values via the keyboard.
	Scrollbar with slider – Setting of numbers in the relevant input box by moving the slider or clicking on the arrow buttons or clicking on the slider and moving via the arrow keys of the keyboard. Press the **Shift** or **Ctrl** key while clicking on the arrow button to change the numeric values in coarse or fine steps.
	Check box – Activates / deactivates setting options.
	Button – Selection / performance of a function via mouse click.

4.1.3 Convention for the Text in this Manual

All the originally used terms of the software interface, e.g.

- names of windows,
- panels,
- input boxes,
- list / selection boxes,
- check boxes,
- menu items,
- names of buttons and
- keyboard keys,

are displayed in **bold letters** to allow easier identification.

4.1.4 Backup

System backup

- A complete backup is contained on the enclosed backup CD-ROM.

User files backup

The following user-generated files need to be included in a backup procedure (keep directory structure):

- Image database files: *.mdb (but not system_configuration_*.mdb)
- LSM Image files: *.lsm
- Exported images: *.* (*.Tiff, *.LSM-Tiff, *.BMP, …)
- Palette files: AIM \ Palette \ *.lut
- Filter files: AIM \ Filter \ *.krn
- Pinhole setting files: AIM \ PH*.pos
- Log files: AIM \ *.log

The following files generated during the system integration should also be included in a backup procedure:

- Parameter file for pinhole setting: AIM \ *.set
- Parameter file after pinhole adjustment: AIM \ *.adj
- Scanner files: AIM \ bin \ *.bin
- Microscope stand database: AIM \ database \ system_configuration_*.mdb

4.1.5 Software Operation

The LSM 5 software can be operated using the mouse, the PC keyboard, or both.

The operation of the mouse and the keyboard is identical to that of the Microsoft ® WINDOWS operating system and is therefore not dealt with in detail in this manual.

If required, see the Microsoft manual or online help for relevant information.

4.2 Switching on the System

The LSM system is equipped with a main power switch located at the front of the System Electronic Rack and two further switches labeled **System/PC** and **Components**. The main switch has to be set to **ON** to be able to switch on and off the system using the **System/PC** and **Components** switches. The main switch can be used to switch off the complete system with one switch only. The electronics to run the computer and the microscope are switched on with the **System/PC** switch. The laser module and the scan head are switched on with the **Components** switch. These switches are also accessible via the remote power control switch (Fig. 4-1). The "Enterprise" UV laser has to be switched on separately. Refer to the operating manual and CHAPTER ANNEX of the printed manual for handling of any Titanium-Sapphier-Laser used for Multiphoton (NLO) Microscopy.

☞ If the UV laser shall be used, it can be switched on after the start of the WINDOWS ® XP operating system - but must always be switched on before the LSM 5 software is started.

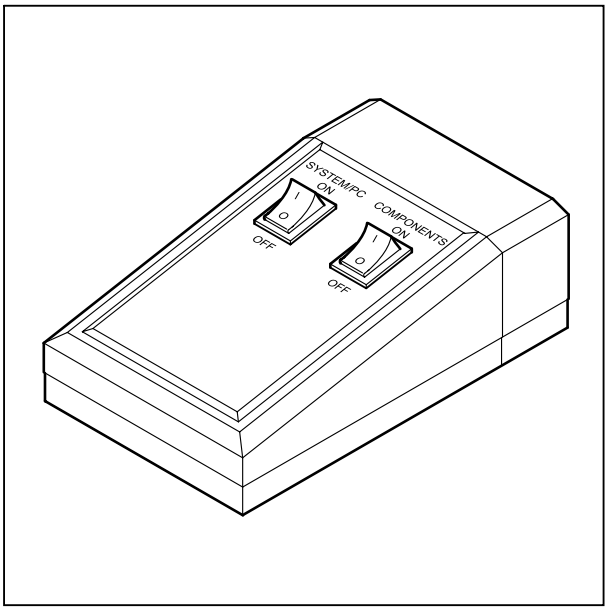


Fig. 4-1 REMOTE CONTROL switch

- When set to **ON** the **REMOTE CONTROL** switch labeled **System/PC** provides power to the microscope and the computer. This allows to use the microscope and the computer without running the LSM Software.

☞ The drives for floppy discs and CD/DVD of the computer must not contain any data storage item.

- To switch on the system completely put the **Components** switch also to **ON**. Now the complete system is ready to be initialized with the LSM Software.
- After switching on the computer type in the user name and password to log on to the computer.
- After entries, confirm by clicking the **OK** button or **Enter**.
  - The WINDOWS XP operating system desktop appears on the screen, showing a number of icons.

Change Filters

Change Filters icon

The **Change Filters** tool is used to update the filter data in the software after a change of filters in the reflector turret. See printed manual CHAPTER ROUTINE MODE AND TOOLS.

Stand Select

Stand Select icon

The **Stand Select** tool permits a new or updated database to be assigned to the LSM 5 software program. This function should preferably be performed by authorized service personnel. See printed manual CHAPTER ROUTINE MODE AND TOOLS.

LSM 510

LSM 510 icon

Start the LSM 5 software program for the LSM 510 *DuoScan* and LSM 510 META *DuoScan* laser scanning microscope.

4.2.1 Switching on the Enterprise UV Laser

- If the UV laser is required, switch it on via the toggle switch (Fig. 4-2/**1**) of the power supply.
  - It will be ready for operation after a few seconds.

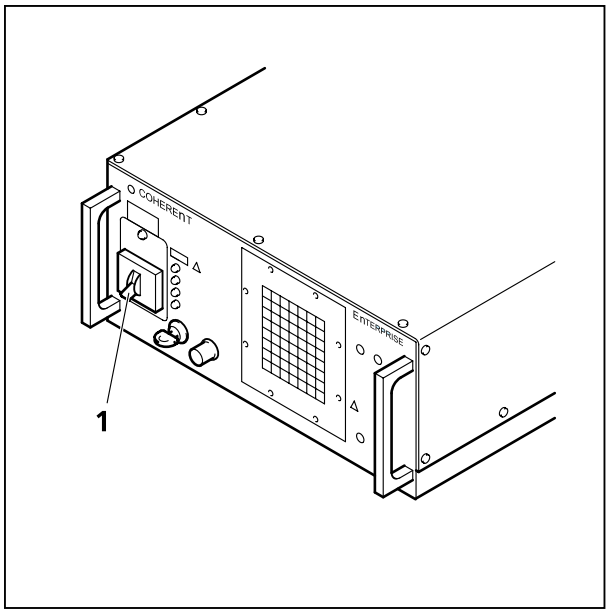


Fig. 4-2 Power supply of UV-Ar laser

Fig. 4-3 Starting the LSM 5 software

4.2.2 Starting the LSM 5 Program

The LSM 5 software program can be operated in two different modes (with or without connected instrument system).

- In the on-line mode, the entire program package (image recording and analysis) is available, while only a part of the software functions (image analysis only of already stored images)
- In the off-line mode no hardware functions are available. Of course, the off-line mode can also be started when the instrument system is connected. In that case, it is not necessary that the lasers and the microscope are switched on.

- Double-click on the **LSM 510** icon on the desktop of WINDOWS to start the LSM 5 software program (Fig. 4-3).
  - The **LSM 510 Switchboard** window appears on the screen (Fig. 4-4).

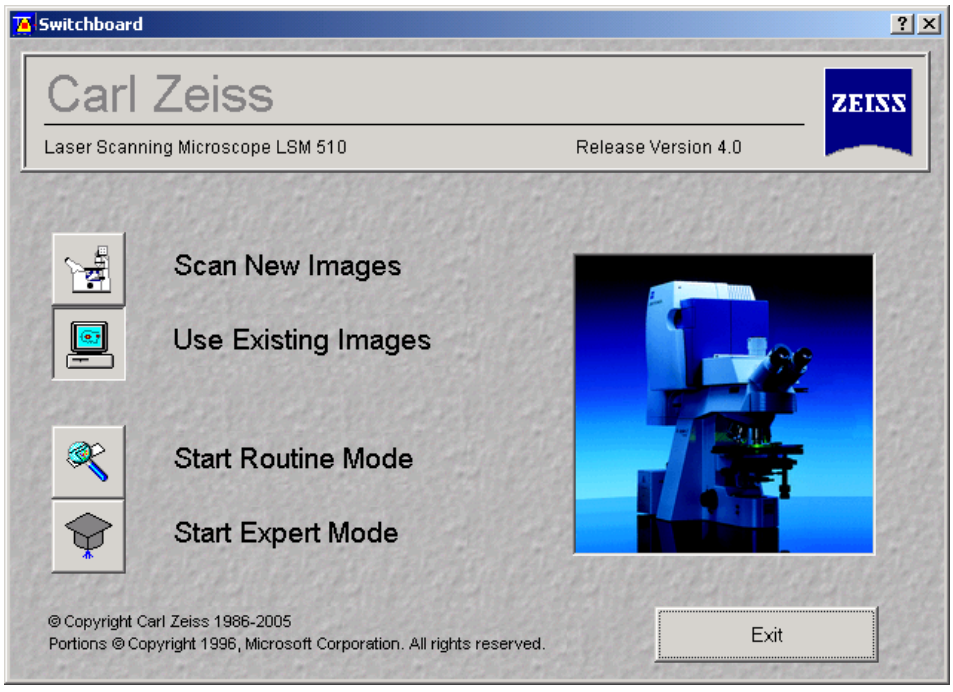


Fig. 4-4 LSM 510 Switchboard menu

Scan New Images Clicking on this button activates the complete LSM hardware (on-line mode).

☞ Please note that the **Scan New Images** button must be activated before setting up the Routine Mode or the Expert Mode. Otherwise, the hardware can not be controlled by the LSM 5 software.

Use Existing Images This item allows you to process and analyze previously acquired images with the LSM 5 software. In this mode, control of the hardware (laser module ...) is not possible (off-line mode).

Start Routine Mode Click on this button if you want to work with pre-configured system settings (typical applications).

Start Expert Mode Use of this mode requires to be thoroughly familiar with the exact microscope procedures and interrelations.

☞ In the Expert mode you need to set all parameters and functions upon your own decision; this mode therefore provides you with the greatest flexibility of operation. It is also possible, however, to call up stored configurations and to modify the parameters / settings if necessary.

After the start of the **Expert Mode** or the **Routine Mode**, instrument initialization is performed and can be monitored in the **Initialization** window and interrupted with a click on the **Cancel** button, if required.

Depending on the selected option (**Scan New Images** or **Use existing Images**), initialization is performed in the offline or online mode.

☞ Existing images can only be loaded and processed in the **Expert Mode**.
If you want to change from the **Expert Mode** to the **Routine Mode** and vice versa, close all the windows first.

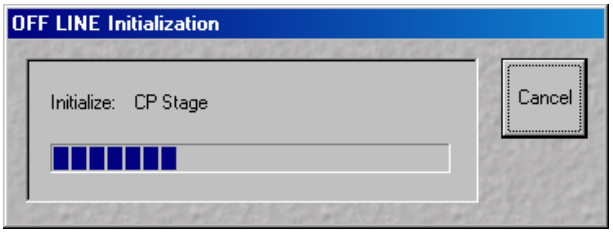


Fig. 4-5 OFF LINE Initialization window

4.3 Main Menu

The major functions can be selected in the **Main** menu of the Expert Mode either via the pull-down menus in the menu bar or via the **Main** menu toolbar which can be displayed or removed as required.

Further subordinate toolbars are available below this toolbar, depending on which button has just been pressed (**File**, **Acquire**, etc.).

In the standard setting of the LSM 5 software, the toolbars are automatically displayed after the start of the Expert Mode.

☞ However, since the LSM 5 software is operated more conveniently with the help of the toolbars, only this method of function activation will be described in the following.

- Click on the **Start Expert Mode** button in the **LSM 510 Switchboard** window.
  - The LSM 510 - Expert Mode **Main** menu appears on the screen.

The **Acquire** button is active automatically, and the submenus selectable in it are shown in the second (bottom) toolbar.

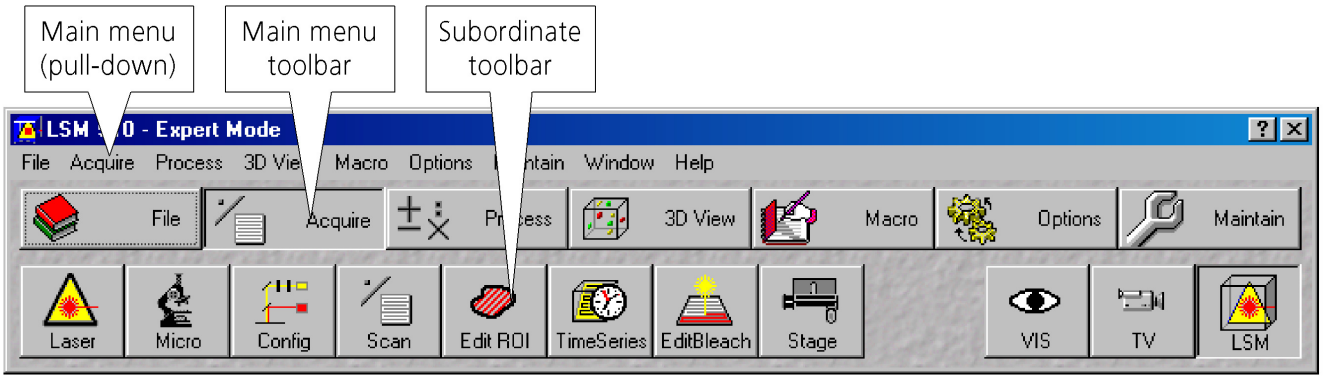
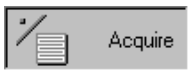


Fig. 4-6 **LSM 510 - Expert Mode Main menu**

File button — Open, save, import and export of image data. Printing individual or several images on one page. Ending (Exit) the Expert Mode.



Acquire button — Calling up and setting the necessary operating parameters. During the preparation for and execution of laser scan image acquisition, this menu item is used as the working dialog between the computer and the microscope.

Process button — Used for processing of acquired images.

3D View button — Used for three-dimensional reconstruction.

Macro button

Makes it possible for the user to store frequently used processes (Macro recorder) and to run them automatically (Macro play). It is possible to write new macros or to edit existing ones.

Options button

For custom-configuration of software and hardware options, and for exporting system operating sequences to the Routine Mode.

This menu item enables access to the coloring table.

In the **Settings for User** window you can specify essential operating modes and informative help, organized by tabs, which have an effect on the user interface.

Maintain button

Service mode for adjustment and setting of other parameters (e.g. objectives).

4.4 File Menu

The functions of the **File** menu permit images and the relevant information to be managed and handled completely in a database system. You can also create your own databases. The databases allow images to be stored, loaded and deleted. The additional functions **Import** and **Export** permit images from other systems to be made available to the LSM 5 software, or the export of images to other software packages. The **Print** function allows individual or several images to be arranged on a print page for printout. The **Expert Mode** can be ended via the **Exit** function.

In the pull-down Main menu **File** two additional functions not displayed in the Main Menu Toolbar are available.

Clicking **Recent Database** opens up a choice of the last 5 databases opened by the program. Clicking the appropriate line will open up that database. A similar menu exists for **Recent Image Files.**

- In the **Main** menu toolbar, click on **File**.
  - This opens another, subordinate toolbar in the **Main** menu.

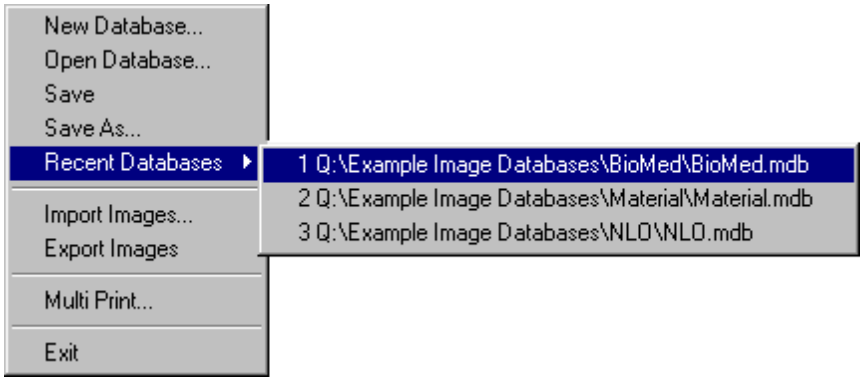


Fig. 4-7 File menu

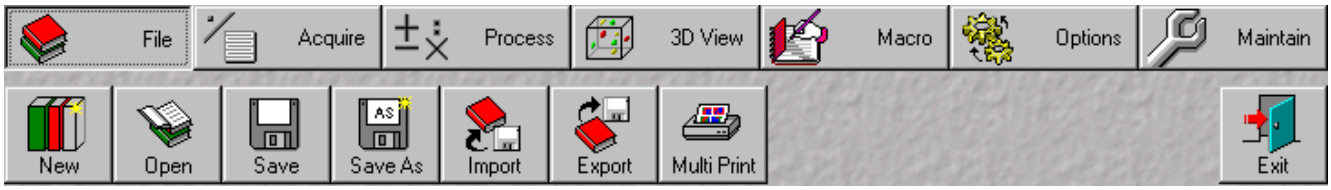


Fig. 4-8 Subordinate File menu toolbar

4.4.1 Create New Image Database

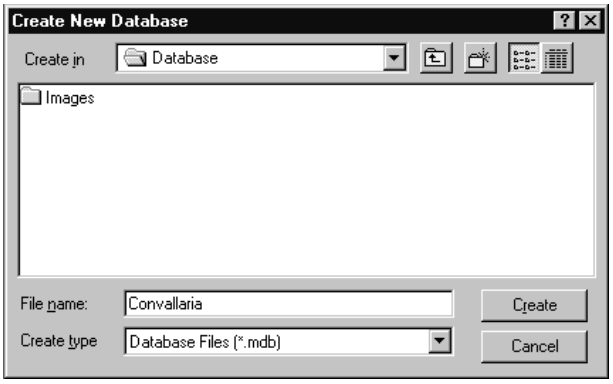


Fig. 4-9 Create New Database window

The **New** function permits the creation of a new image database.

- Click on the **New** button in the **File** subordinate toolbar of the **Main** menu.
  - This opens the **Create New Database** window for the selection of drives, directories and subdirectories.
- Enter the name of the image database you want to create in the **File name** text box, e. g. **Convallaria**.
- If you want to create the image database in a certain folder (drive / directory), click on the arrow button next to the **Create in** box.
  - This opens a drop-down list box showing all folders available for selection.

☞ To move up one layer of folders, click on the button. **Cancel** allows you to stop the process.

- If you want to create a new folder, click on the button.
- Click on the **Create** button.
  - This creates the new image database in the selected drive and directory.
  - The database files of the LSM 5 software have the filename extension ***.mdb**.

  The **Convallaria.mdb** window appears, presenting the opened image database with 0 of 0 image entries (see **Image Database window**, page 4-20).

The new image database can be used to store an acquired or processed image (see **Saving an Image**, page 4-30).

☞ The start settings and the extent of the parameters displayed in the image database window can be changed as required via the **Settings** function in the **Options** subordinate toolbar (see **Settings Function**, page 4-216).

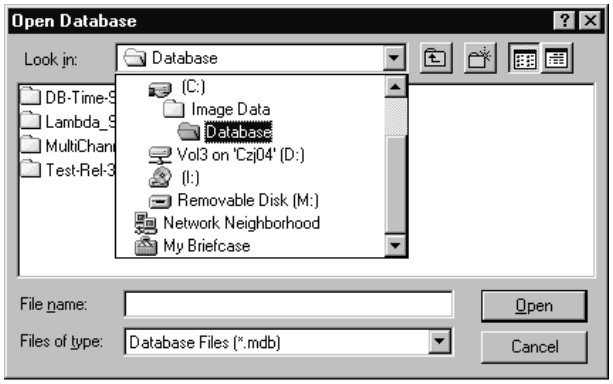


Fig. 4-10 Open Database window

4.4.2 Open Existing Image Database

The **Open** function allows one or several databases to be opened. The images stored in the database(s) are displayed in thumbnail form; they can be selected and loaded into the **Image Display** window (see page 4-247).

- Click on the **Open** button in the **File** subordinate toolbar of the **Main** menu.
  - This opens the **Open Database** window for selection of image databases.
- If you want to load an image database from another folder (drive / directory), click on the arrow button to the right of the **Look in** box.
  - This opens a drop-down list box in which you can select from all available folders.

The window displays the various image databases with the file extension *.mdb.

- Open the image database by a double click on the respective key icon (e.g. **Convallaria.mdb**), or click on the name of the image database for selection and open it by clicking on the **Open** button.
  - This opens the image database window, e.g. **Convallaria.mdb - AIM**, in which you can select from a variety of options.
- Click on the ☒ button in the title bar of the **Database** window to close this window and make no selection.

4.4.3 Image Database Window

The **Image Database** window allows you to choose one of three different display modes:

- Form
- Gallery
- Table

To choose the required mode, activate the relevant button on the right-hand side of the **Image Database** window. Loading of images into the **Image Display** window is possible in every display mode.

The buttons on the right have the following functions:

Form button — Show image database in form display mode.

Gallery button — Show image database in gallery display mode.

Table button — Show image database in table display mode.

	Load button	Load image and parameter from image database to **Image Display** window.
	Subset button	Load image and parameter with size reduction from image database to **Image Display** window.
	Reuse button	Reuse scan parameters of the selected image without loading the image.
	Refresh button	Refresh **Image Database** window.
	Copy button	Copy selected images to clipboard.
	Paste button	Paste images from clipboard into image database.
	Filter button	Select or edit search filter from image in the image database.
	On Filter button	Switch search filter on or off.
	Delete button	Delete selected images from the image database.

The status line, which displays the following current parameters, is at the bottom of the **Image Database** window:

Total: … Display of storage volume of the entire image database

Selected: … Display of storage volume of the selected image(s)

Current: … Display of storage volume of the current image

Clipboard: … Display of storage volume of the image(s) contained in the clipboard

4.4.3.1 Form Display Mode

When a image database is opened, the **Form** display mode is used, if no other settings were made under **Settings** in the **Options** subordinate toolbar.

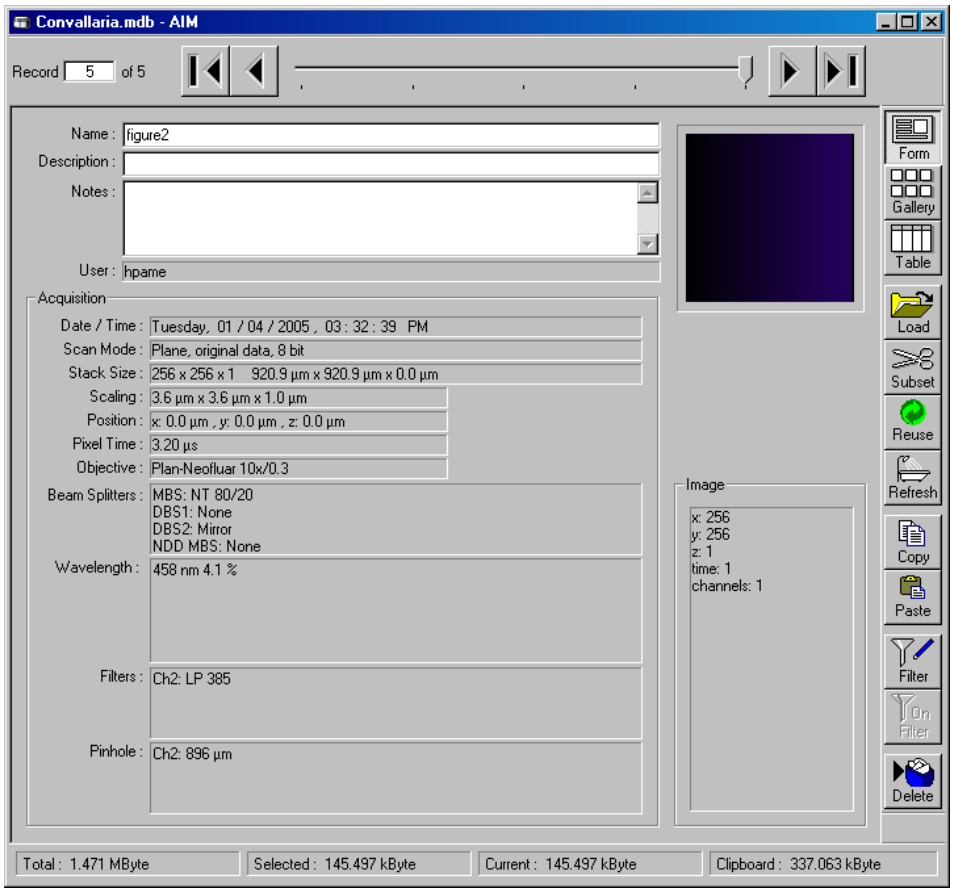


Fig. 4-11 Image Database window (Form display mode)

In the **Options** menu in the function **Settings** it is possible to define

- the start mode of the image database (Form, Gallery, Table)
- the **Recordset** displayed (first, last, middle) and
- the parameters shown.

The number of the image currently displayed from a set of images is indicated in the **Recordset** text box.

- From the image database you can display images using the recording slider, or in one of the following ways:

  show the next image

  show the previous image

  show the last image of the image database

  show the first image of the image database

The currently selected image is displayed as thumbnail in the **Image Display** window on the right. In the case of Z Stacks or time series, the **Slice** slider appears on the screen beside the **Image Display** window.

- You can browse through the series by dragging the **Slice** slider using the mouse.
- Click on the **Load** button to open the selected image.

☞ A double-click on the **Image Display** window of the database will also load the selected image.

For a description of the toolbars **Select** and **Display** see **Display and Analysis of Images**, page 4-247.

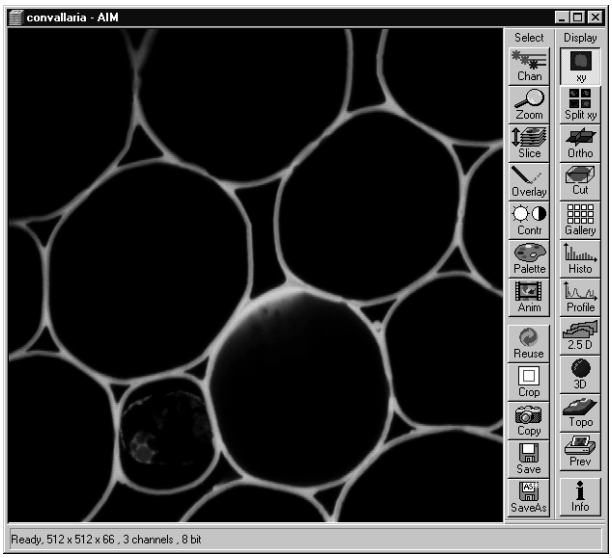


Fig. 4-12 Opened image displayed in the Image Display window

The name of the image is displayed in the **Name** input box of the **Image Database** window.

- If you want to change the name, click on the input box and enter the new name directly via the keyboard.
- The **Description** and **Notes** input boxes allow you to subsequently add descriptions or special notes on the recorded image via the keyboard.

The acquisition parameter settings of the image are displayed in the **Acquisition** panel.

Changes to an original scan image are automatically recorded in the **Processing** panel under **History**. If, for example, the image was added to the database via the clipboard, the entry **Imported file** will be shown under **History**.

Under **Image Size**, the size of the image in pixels for XY(Z) and the number of used channels are displayed.

4.4.3.2 Gallery Display Mode

- Click on the **Gallery** button. All images of the image database, e.g. **Convallaria.mdb**, (image series) are shown in a tiled arrangement of thumbnails on the screen.

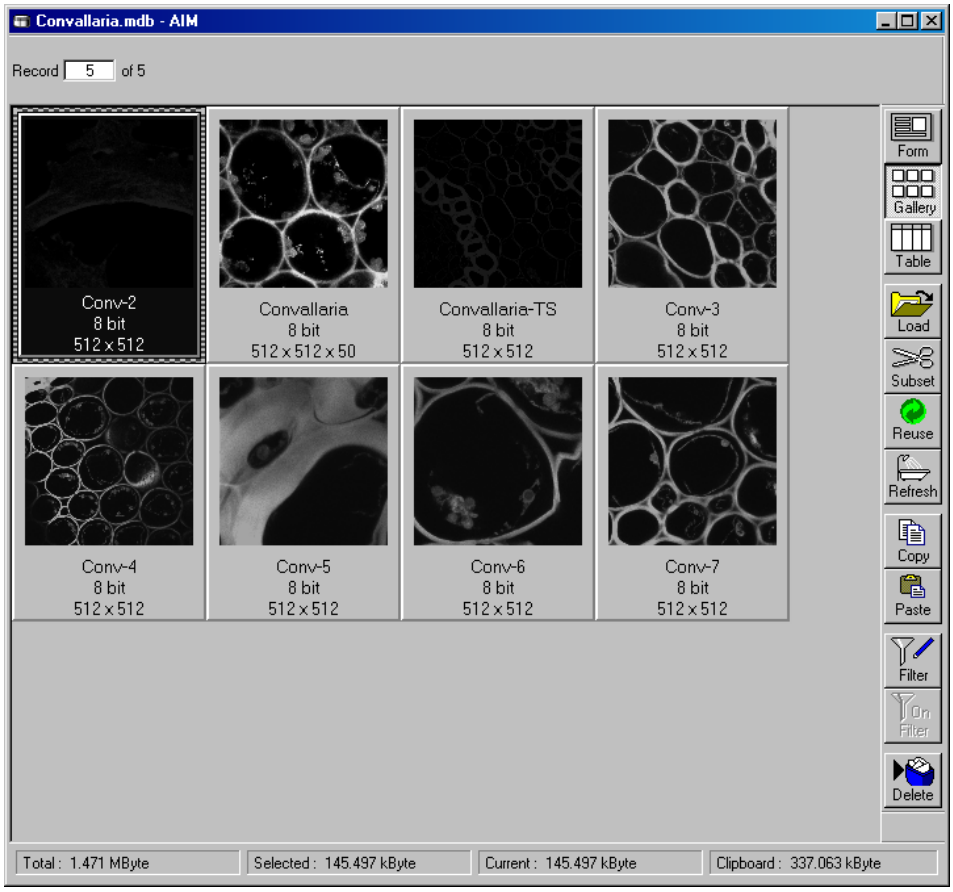


Fig. 4-13 Database window (Gallery display mode)

In the **Options** menu in the function **Settings** it is possible to define

- the start mode of the image database (Form, Gallery, Table)
- the recordset displayed (first, last, middle) and
- the parameters shown.

- To select **one** of the images of the database for normal-size presentation, double-click on the desired image. The same can be achieved by clicking on the desired image in the gallery and then clicking on the **Load** button.
- To select several single images press & hold down the **Ctrl**-key and select each desired image by a click of the mouse. If several images have been selected, they will all be opened and displayed one after the other.
- To select a number of consecutive images press & hold down the **Shift**-key, click on the first and the last image to be selected. All the images between these two will also be included in the selection. If several images have been selected, they will all be opened and displayed one after the other.

Selected images are highlighted in blue. Furthermore, the current image selected last receives a patterned frame.

4.4.3.3 Table Display Mode

- Click on the **Table** button.
  - All images of the image database, e.g. **Convallaria.mdb**, (image series) are shown in **Table** display mode on the screen.

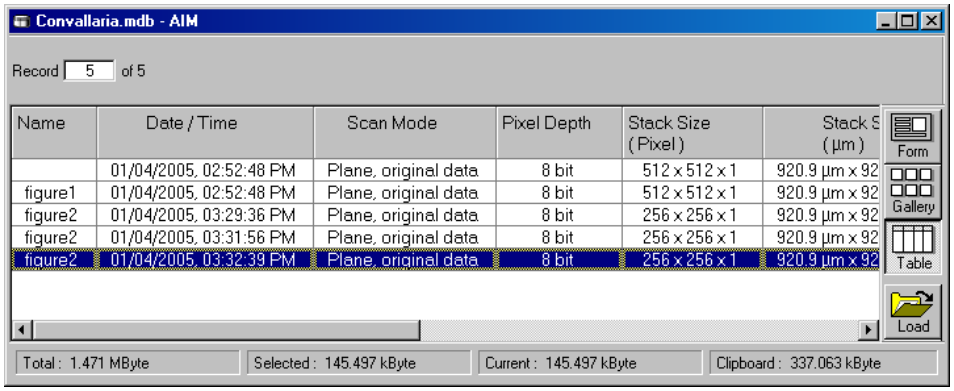


Fig. 4-14 Database window (Table display mode)

In the **Options** menu in the function **Settings** it is possible to define

- the start mode of the image database (Form, Gallery, Table)
- the recordset displayed (first, last, middle) and
- the parameters shown.

- To select one of the images of the database for normal-size presentation, double-click on the desired line. The same can be achieved by clicking on the desired image in the table and then clicking on the **Load** button. If several images have been selected, they will all be opened and displayed one after the other.

☞ The selection of several images is performed in the same way as in the **Gallery** mode, i.e. by pressing the **Shift** and **Ctrl** keys.

4.4.3.4 Load an Image into the Image Display Window

- Click on the **Load** button to load the selected images into the **Image Display** window or double click on the appropriate image in the database window.

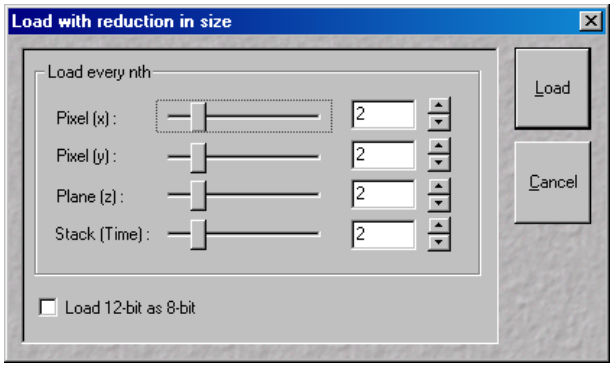


Fig. 4-15 Load with reduction in size window

4.4.3.5 Load Image with Reduced Resolution (Subset)

The **Subset** function allows images to be loaded with reduced resolution. For this purpose, the image pixels in XY(Z) are reduced. It is also possible to reduce the number of slices (in stacks and time series).

- Click on the **Subset** button to open the **Load with reduction in size** window.
- Enter one value each for **n** under **Pixel (x)**, **Pixel (y)**, **Pixel (z)** and **Stack (Time)** in the **Load every nth panel**.
- If required, turn on the ☑ **Load 12 bit as 8 bit** check box.
- Click on the **Load** button to load the selected images with reduction in size, time slices and stack slices.
- Use **Cancel** to exit the window without any selection.

4.4.3.6 Update Image Database Display (Refresh)

- Click on the **Refresh** button to update the **Image Database** window.

4.4.3.7 Copy / Paste Image(s) to the Clipboard

- Select the image(s) to be copied. You can use the **Shift** and **Ctrl** keys for multiple selection.
- Click on the **Copy** button.
  - The image(s) is(are) copied to the clipboard and can be inserted in either the same or another database or in other software packages.
- Click on the **Paste** button to insert the image in the current database.

Identical to the WINDOWS function "Drag & Drop", one or several images can be copied or moved from one database to another.

The **Form** mode allows only one image to be copied or moved. The **Gallery** and **Table** modes permit several images to be copied or moved simultaneously by multiple selection (keeping the Shift key pressed on clicking).

- Open the relevant databases and position both windows side to side.
- Select the required images (multiple selection by keeping the **Shift** key pressed) from one database.
- Click on a selected image and keep the mouse button pressed, move the mouse button to the window of the other database (a small rectangular appears near the mouse button) and release the mouse button again (Drag & Drop).

The images are now being moved to the other image database, i.e. they are deleted from the first image database and are then only available in the second image database.

- If the images shall only be copied, also press the **Ctrl** key during the Drag & Drop procedure (in addition to the rectangular, a "+" sign will also appear near the mouse button).

The images will then be available in both image databases.

4.4.3.8 Display Images with Certain Features (Filter)

The **Filter** function permits the display of the database to be modified in such a way that only images with certain features are displayed. This requires the definition and following activation of a relevant filter. Defined filters can be stored, reloaded and also deleted.

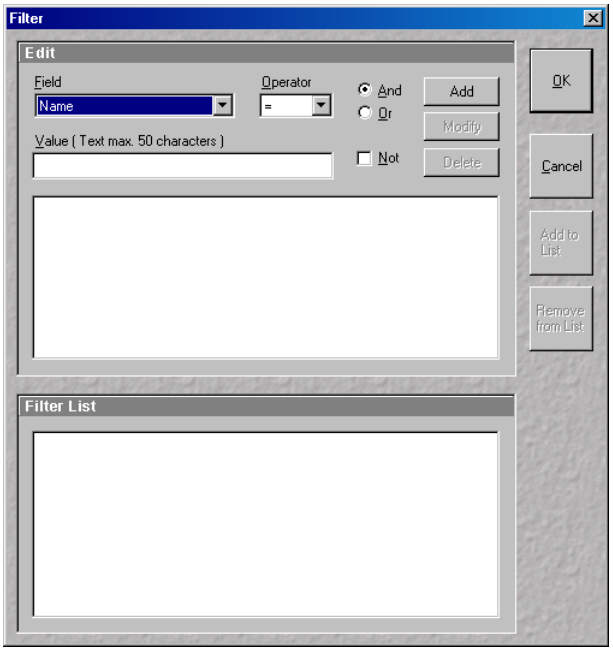


Fig. 4-16 Filter window

Edit panel

The following features can be used as filter functions under **Field**:

Name	Words or row of letters from the name of the image
Description	Words or row of letters from the description of the image
Scan Mode	Scan Mode in which the image was created: Stack or Plane
Date / Time	Date / Time of image acquisition
# Planes (z)	Pixel size of the image in the Z-direction (e.g.: 10)
# Lines (y)	Pixel size of the image in the Y-direction (e.g.: 512)
Samples (x)	Pixel size of the image in the X-direction (e.g.: 512)
Z-Step	Distance of Z Slices in a Z Stack in µm
User	Name of the user as entered in the WINDOWS NT login
Time series	Selection of time series

- Open the **Field** list box and select the filter feature (e.g.: **Scan Mode**).

The following operator signs can be activated under **Operator**:

=	equals
>	larger
<	smaller
>=	larger, equals
<=	smaller, equals
<>	smaller, larger

- Select the relevant operator sign (e.g.: **=**) from the **Operator** list box.

The relevant value or a combination of characters for the filter function (**Field**) is entered under **Value** via the keyboard:

- Enter the relevant text or value (e.g.: **Stack**).
- Click on **Add**. The defined filter feature is displayed in the work box of the **Edit** panel and is therefore activated (e.g.: **Scan Mode = stack)**.

If a further filter feature is to be linked with the already defined one, proceed as follows:

- Activate the relevant entries under **Field** and **Operator** and enter a value or text (e.g.: **Name = Convallaria)** under **Value**.

☞ If groups of letters shall be searched, the ***** sign can be entered for undefined letters (e.g.: if you search for the letter row Conv, enter **Conv***).

- Activate the required link type **And**, **Or** or **Not** with a click of the mouse (e.g.: **And**).
- Click on the **Add** button. The created filter feature is added to the work box of the **Edit** panel (e.g.: **AND Name = Convallaria**).

The **Modify** button enables you to edit an already defined filter feature:

- Activate the required feature on the work box.
- Make the necessary changes under **Field**, **Operator** and **Value**. Select the link type **And**, **Or** or **Not**.
- Click on **Modify**. The filter feature will be changed accordingly.

The **Delete** button enables you to delete a defined filter feature:

- Activate the required feature in the work box.
- Click on **Delete**. The filter feature will be deleted from the work box.
- Clicking on **OK** will activate the filter (the entire set of filter features) displayed in the work box and close the **Filter** window. **On Filter** is activated right on in the **Database** window and the filter function will be performed. Only those images which fulfill with the defined filter features will then be displayed. The procedure is interrupted via **Cancel**.

If required, the filter features displayed in the work box can be stored.

- Click on the **Add to List** button. The **Add to List** window will be opened.
- Enter a name for the filter and click on **OK**. The filter will be included in the **Filter List** panel.

Fig. 4-17 Add Filter to List window

Filter List panel

All the stored filters are displayed in the **Filter List** panel and can be activated any time at a click of the mouse.

- Click on the name of the required filter. The linked filter features will be displayed in the work box.

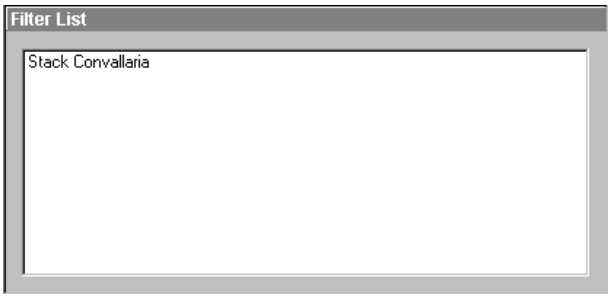


Fig. 4-18 Filter List panel

Filters which are no longer needed can be deleted.

- Click on the filter to be deleted in the **Filter List** panel.
- Click on **Remove from List**. The filter will be removed.

4.4.3.9 On Filter Function

The **On Filter** function is a toggle switch to activate or deactivate selected filter settings.

4.4.3.10 Delete Function

- Select the images to be deleted from the image database.
- Click on the **Delete** button. Confirm the safety inquiry then displayed by pressing **OK**.
  - The images and the acquisition parameters will be removed from the image database.

4.4.4 Save an Image to the Image Database

The **Save** function allows to store an image together with the acquisition parameters (and processing information) to be stored in an image database.

In the **Options** menu in the function **Settings** it is possible to define an **Autosave** function. When **Autosave** is off, the **Save** dialogue is the **Save As** dialogue.

Proceed as follows to save an acquired or an edited / processed image:

- Click on the **Save** or **Save As** button in the **File** subordinate toolbar of the **Main** menu.
  - The **Save Image and Parameter As** window appears on the screen.

☞ **Save**
Stores a newly created or changed image. Newly created images must be given a name and assigned to an existing or new database.

Save As
Stores a previously stored and called up image under a different name. If images are called up and stored again, the original data and time display will be retained.
Clicking on either of these buttons opens the **Save As** window to create and open an image database.
When the **Compress Files** ☑ check box is activated, the images are stored in a compressed form.

- If necessary, enter a description of the image or comments on it in the appropriate text boxes.
- The default display in the **User** text box is the name of the logged-on user. If you want, you can enter a different user name for the current image.
- Click on the **Open MDB** button if you want to open an existing image database in which you want to save the current image. Click on the **New MDB** button if you want to create a new database to save the current image.

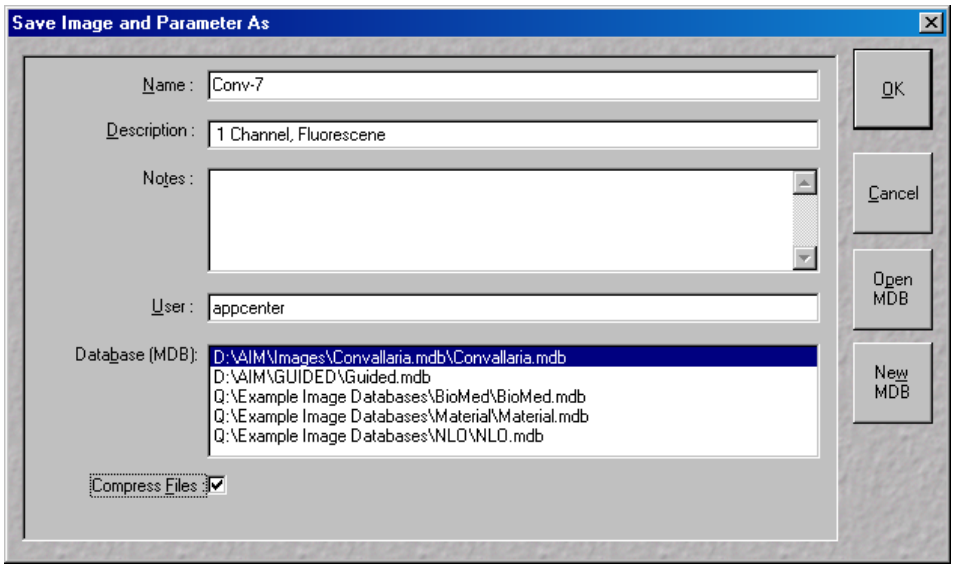


Fig. 4-19 Save Image and Parameter As window

- Enter the name of the image in the **Name** text box, e.g. **Conv-7**.

- Click on the **New MDB** button.
  - This opens the **Create New Database** window in which you can create a new image database, e. g. **Projections**.mdb (see **Create New Image Database**, page 4-19).
  - After creating the new database **Projections.mdb - AIM** window appears.

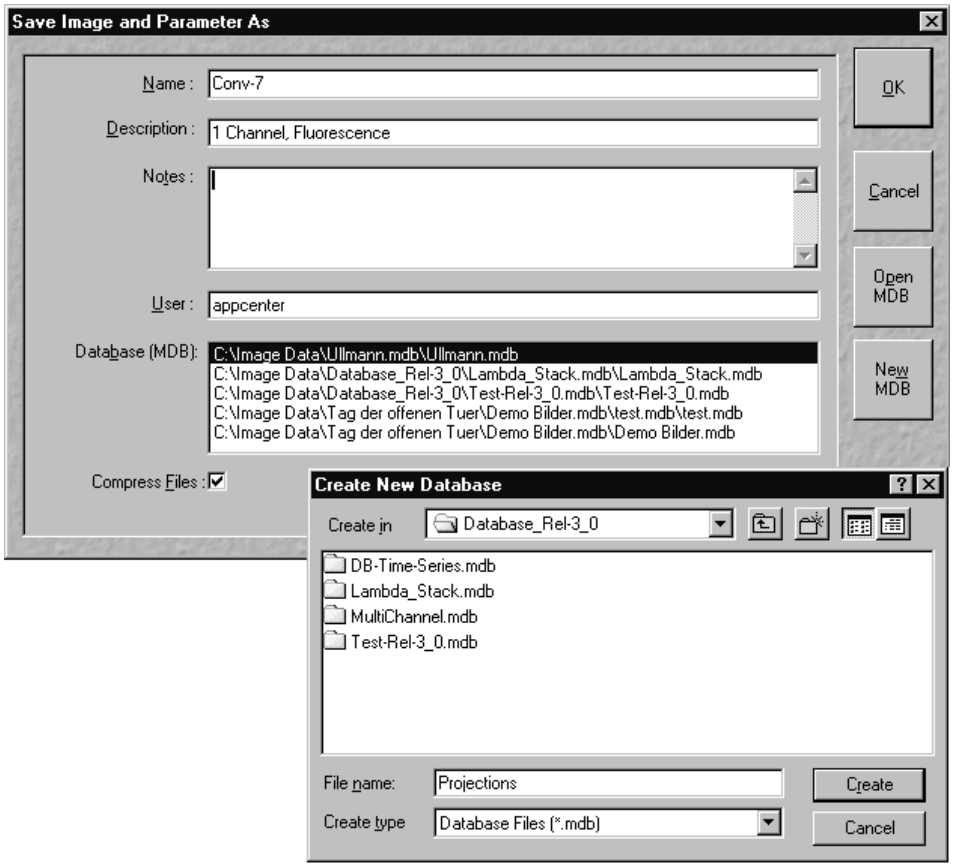


Fig. 4-20 Save Image and Parameter As window and Create New Database window

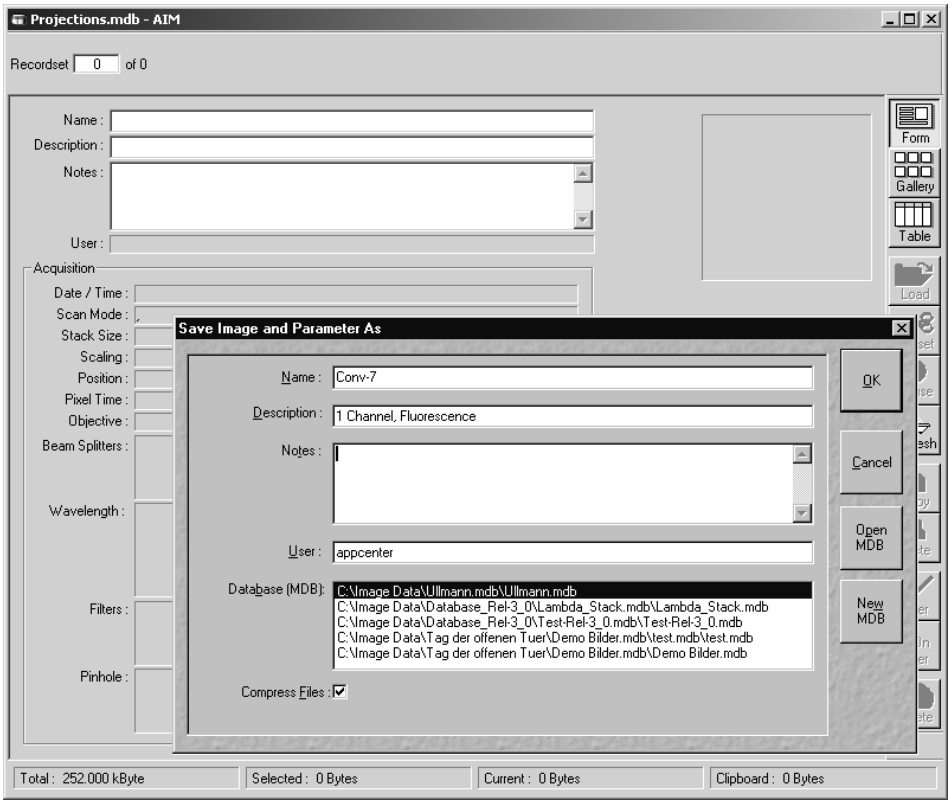


Fig. 4-21 Database window

- Click on the **OK** button in the **Save Image and Parameter As** window.
  - The **Projections.mdb - AIM** window now shows the saved image.
  - The **Recordset** box indicates the current number of the image in the image series contained in this database.
- In the **Description** text box you can enter, for example, the configuration of the image.
- In the **Notes** text box you can enter further information about the image content.

4.4.5 Import of Images

The **Import** function enables the import of externally created images into the **Image Display** window and the image database of the LSM 5 software.

- Click on the **Import** button in the **File** subordinate toolbar of the **Main** menu.
  - This opens the **Import Images** window.

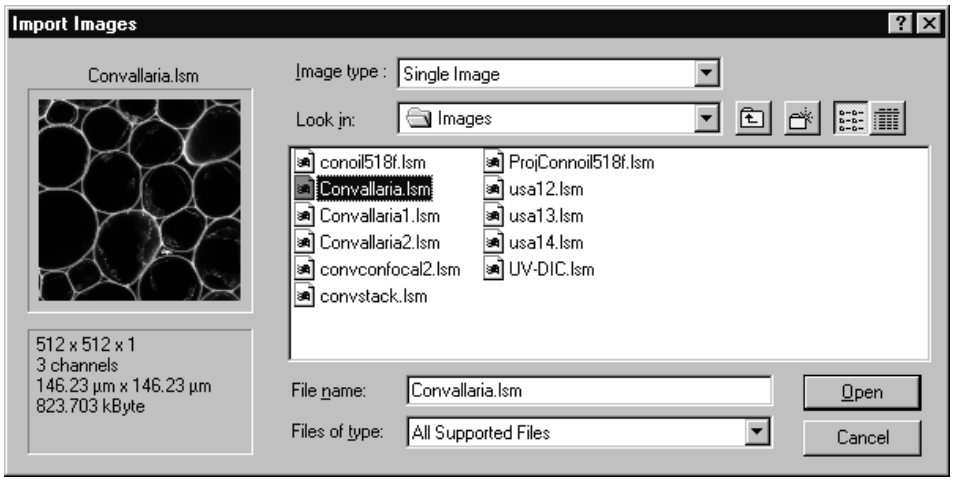


Fig. 4-22 Import Images window

- Select the data medium and the directory where the relevant image is contained in the **Look in** selection box.
- Select the image file by clicking on it.
  - The selected image will be shown for checking in the **Image Display** window (on the left) together with the relevant details (size, channels, storage volume).
- Select the image type (**Single Image** or **Image Series)** in the **Image type** selection box.
- Click on **Open**.
  - The image is displayed in a new **Image Display** window.

All the usual image and movie formats (e.g. **.tif**, **.jpg**, **.bmp**, **.pcx**, **.avi**, **.mov** etc.) are supported.

☞ When importing series, please make sure to select the first image for the representation of the entire series and to select the **Image Series** option under **Image type**.

- Finally, save the image in the desired image database via the **Save As** function.
- In **Processing History** this file is marked as imported file.

4.4.6 Export of Images

The **Export** function allows the export of acquired images and images loaded from the image database.

- Select the image to be exported.
- Click on the **Export** button in the **File** subordinate toolbar of the **Main** menu.
  - This opens the **Export Images and Data** window.
- Under **Save in**, select the data medium and the directory to which the image is to be exported.
- Enter a name for the image under **File name**.
- Select the image format into which the image is to be exported under **Image type** (**Single Image with raw data**, **Contents of the Image Display window**, **Full resolution**).
- Chose a compression level. For some file formats lossless compression or various other compression levels are available. The degree of losses for the image quality are listed according to the type of compression.
- Click on the **Save** button.
  - The image is stored on the relevant data medium / directory.

All the usual image and movie formats (e.g. **.tif**, **.jpg**, **.bmp**, **.pcx**, **.avi**, **.mov** etc.) are supported.

☞ When stacks or time series are exported, each frame is stored as an individual image.

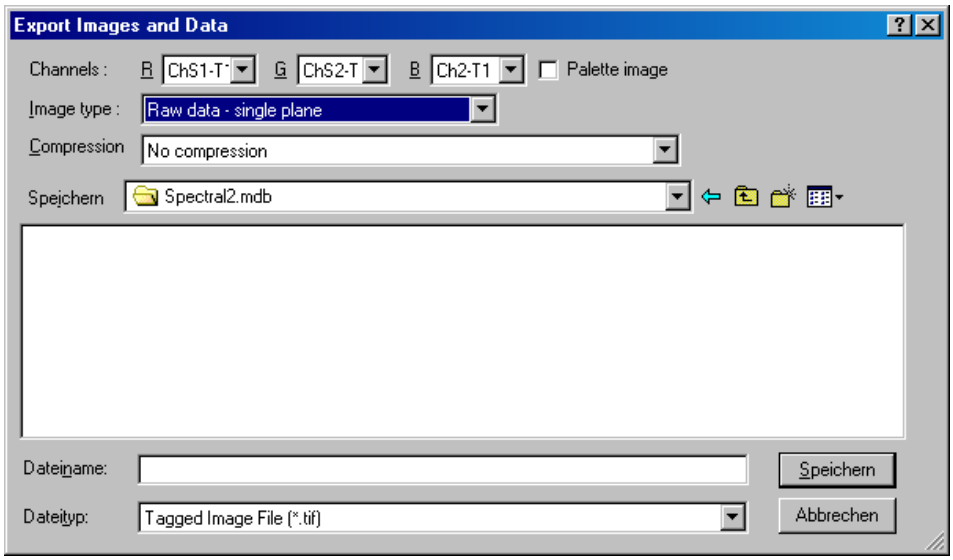


Fig. 4-23 Export Images and Data window

4.4.7 Multi Print

This function permits you to arrange several images on one print page and to print them out together.

- Click on the **Multi Print** button in the **File** subordinate toolbar of the **Main** menu.
  - This opens the **Print - AIM** window.

The main area of the **Print – AIM** window is used for the display of the print page in the selected paper orientation and for the arrangement of the images to be printed.

The **Print** toolbar with the following buttons is displayed on the right-hand side of the window:

Paste Paste from clipboard to sheet.

Delete Delete marked image.

Print Start printing.

Setup Printer setup.

Landsc. Landscape paper orientation.

Portrait Portrait paper orientation.

Zoom slider Zoom function for page preview.

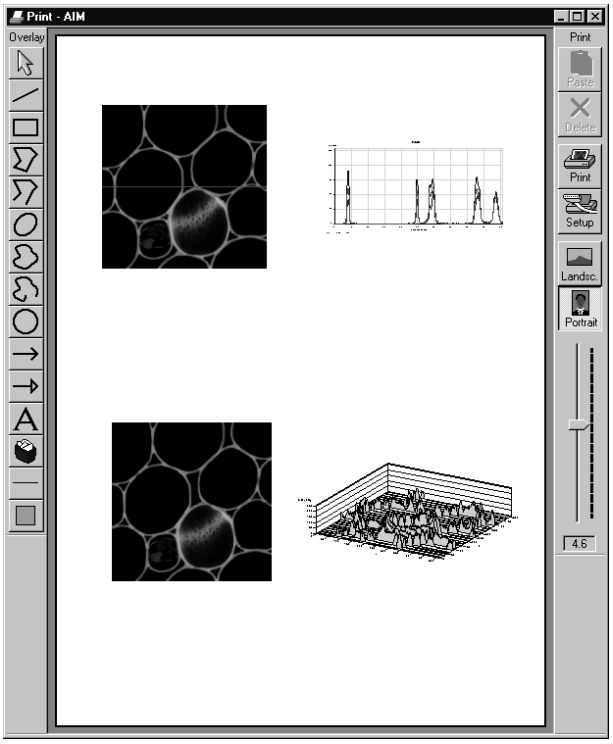


Fig. 4-24 Print - AIM window

4.4.7.1 Overlay Toolbar

The following functions can be performed on activation of the buttons in the **Overlay** toolbar (left-hand side):

Arrow (selection) button: Activation of the mouse button for selection, resizing or movement of an overlay element in the **Image Display** window.

RESIZING: Click on the handle and hold down the mouse button, drag the handle, release the mouse button.

MOVEMENT: Click on the line and hold down the mouse button, move the entire element, release the mouse button.

Line button: Creation of a straight line in the **Image Display** window.
Click and hold down the mouse button, draw a line in any required direction, release the mouse button to end the procedure.

Rectangle button: Creation of a rectangle in the **Image Display** window.

Click and hold down the mouse button, draw a rectangle in any required direction, release the mouse button to end the procedure.

Closed polyline button: Creation of a closed polyline figure in the **Image Display** window.

The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Open polyline button: Creation of an open polyline figure in the **Image Display** window.

The first click sets the starting point, each additional click adds a further line, a click with the right mouse button ends the procedure.

Ellipse button: Creation of an ellipse in the **Image Display** window.

The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and rotation direction can then be determined, the third click sets the second dimension and direction and ends the procedure.

Closed free-shape curve button: Creation of a closed Bezier figure in the **Image Display** window.

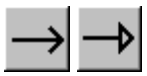
The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Open free-shape curve button: Creation of an open Bezier figure in the **Image Display** window.

The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Circle button: Creation of a circle in the **Image Display** window.

Clicking and holding down the mouse button sets the center point, drag the diameter and release the mouse button to end the procedure.



Line with arrow button: Creation of a line with arrow in the **Image Display** window.

Click and hold down the mouse button, drag the line in any required direction, release the mouse button to end the procedure.

A (Text) button: Creation of a text box in the **Image Display** window.

After clicking on **A**, the **Text** window will be displayed, and text can be entered via the keyboard. The **Font ...** button enables you to select the font style and size in the **Font** window. The entered text will be displayed in the left upper corner of the **Image Display** window after clicking on **OK** and can be moved to the required position using the mouse.

The **Text** window can also be activated with a double-click on a created text box, and the entered text can be edited subsequently.

Insert opens up a further window which allows you to annotate coordinates, time and Z-position with either automatic or user definable units and precision. This annotation is updated during image acquisition and can be exported with the image. The annotation can be stamped into already existing images.

Recycle bin button: All the overlay elements and dimensions dragged to the scanned image are deleted. If one overlay element was marked before, this element is now deleted from the scanned image.

Line button:

This button allows you to determine the line thickness of the area outline.

Color button: After clicking the **Color** button, the **Color** selection box will be opened. The colors displayed in the **Color** selection box can be assigned to the overlay elements with a click of the mouse. The currently selected color is displayed in the **Color** button. A selected color is automatically assigned to the currently selected overlay element and then to all the elements created afterwards.

4.4.7.2 Printing Images

To print several images on one page, proceed as follows:

- Use the **Overlay** functions to additionally illustrate the graphics and images to be printed.
- Select the paper orientation by clicking on the **Landsc.** or **Portrait** button.
- Open the image to be printed or select it from the relevant image database.
- Click on the **Copy** button. The image is copied to the clipboard.
- In the **Print - AIM** window, click on the **Paste** button.

The copied image appears in the work area of the **Print - AIM** window. You can click on it with the mouse and move it to any position on the print page or you can adapt the image size.

- Proceed in the same way with all other images you want to print.
- Finally, arrange all images on the print page as required.
- Click on the **Print** button to start the printout.
- Close the **Print - AIM** window by clicking on the ☒ button.

4.4.8 Exit the Expert Mode

- Make sure to save all required images in the image database or export them.
- Close all open windows of the LSM program by clicking on the closing icon ☒ in the top right corner of each window.
- Click on the **Exit** button in the **File** subordinate toolbar of the **Main** menu.
  - The LSM 510 - Expert Mode **Main** menu will be closed and the **LSM 510 Switchboard** menu appears on the screen.

4.5 Acquire Menu

For preparing and acquiring a scanning image, it is recommended to call up and use the tools of the subordinate toolbar in the following order:

- Conventional microscope setting.
- Laser setting.
- Configuring the optical system for the Scanning Mode.
- Setting of scan parameters.
- **EditROI** permits up to 99 regions within a frame to be defined and scanned.
- **TimeSeries** permits user-specific time series to be selected for the scan procedure.
- The **EditBleach** function is used to bleach a defined, freely selectable area within the scanning field.
- Upon selecting **Stage** you can set the focus (Z coordinate) and the Z step size between successive slices. If the optional, motorized X/Y-stage is connected, the X and Y-positions of the sample can also be selected.
- The **VIS**, **TV** and **LSM** buttons switch the beam path and indicate which beam path has been set in the binocular tube of the microscope (VIS for viewing, TV for camera observation, LSM for laser operation with monitor observation).

☞ For the scanning process, the **LSM** button in the toolbar subordinate to the **Acquire** item must be activated.

- In the **Main** menu toolbar, click on **Acquire**.
  - This opens another, subordinate toolbar in the **Main** menu.

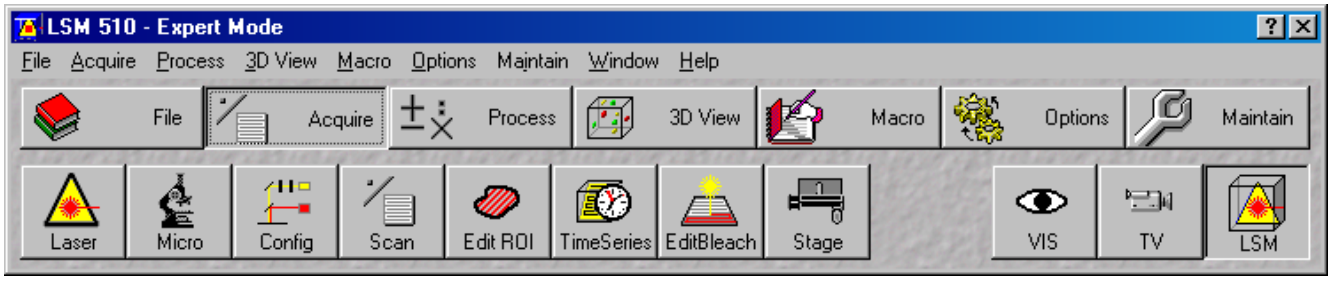


Fig. 4-25 Acquire menu

4.5.1 Laser Control

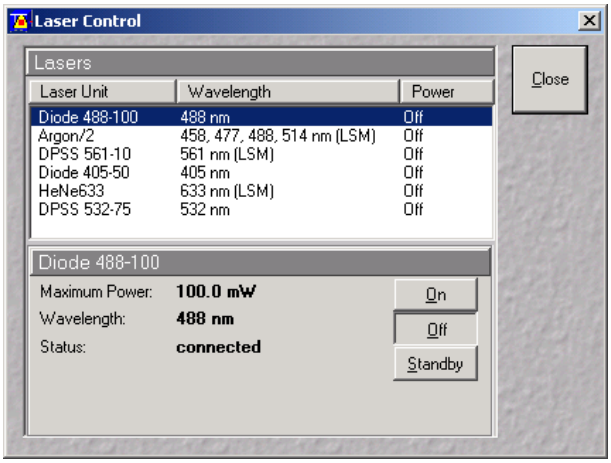


Fig. 4-26 Laser Control window

The **Lasers** panel shows the types, excitation wavelengths and operating status of all lasers available. The addition **(LSM)** indicates the lasers which are provided for imaging and manipulation through the LSM 510 or LSM 510 META scan head. All other lasers are only available for manipulation using the LSM *DuoScan.*

The subordinate laser settings panel shows the relevant and currently set **Maximum Power**, **Wavelength**, **Status**, **Tube Current** and **Output [%]** values of the current laser. The buttons **On**, **Off** and **Standby** permit the current laser to be set in the required status, and the laser intensity (**Output**) can be set using the slider or the input box. The name of the selected laser (Enterprise, Argon, HeNe1, HeNe2 etc.) is displayed in the headline of this setting panel for checking.

4.5.1.1 Opening / Closing the Laser Control Window

- Click on the **Laser** button in the **Acquire** subordinate toolbar.
  - This opens the **Laser Control** window, which shows all lasers connected to the system.

When the setting of the required lasers has been finished, the **Laser Control** window can be closed again.

- Click on the **Close** button to close the **Laser Control** window.
  - The **Laser Control** window will be closed.

4.5.1.2 Function Description

Lasers panel (upper)

List of available lasers, including the display of relevant wavelengths and switching status.

Selection of the laser to be switched on / off and setting of the laser output is performed in the subordinate setting panel.

Laser Settings panel (lower)

Switch on / off the required laser or set Standby operation.

Display Maximum Power, Wavelength, Status and Tube Current (only Enterprise and Argon) of the relevant laser.

Set the laser output for Enterprise and Argon.

4.5.1.3 Settings

- Click on the desired laser on the (upper) **Lasers** panel.
  - This highlights the selected laser.

On the lower panel of the **Laser Control** window, activate the laser as follows:

This applies to the Coherent UV-laser 653 II (Enterprise) and the Ar-multiline laser

- Click on the **Standby** button.
  - Wait for the laser to heat up, until the **Status ready - Standby** message appears (approx. two minutes).
- Click on the **On** button.
  - Status ready - On appears.
- Use the **Output [%]** slider to set the laser power which is ideal for the measurement job.

Thus, the laser needed for image acquisition is available.

Argon: Set output between 25 and 100 % of the maximum tube current. Optimum operation is at 8 A (lowest laser noise). However, the laser life is reduced if the laser is constantly operated at 8 A. Therefore, 8 A should be used only if this is absolutely necessary.

Enterprise: Set output between 50 and 100 % of the maximum tube current. Optimum operation is at 20 A (Tube Current; lowest noise). However, the laser life is reduced if the laser is constantly operated at 20 A. Therefore, 20 A should be used only if this is absolutely necessary.

To switch on the Enterprise laser, proceed as follows:

- The internal water cooling LP 5 is running.
- Start the PC, wait until NT system is booted.
- Switch on the power supply of the Enterprise laser, power potentiometer turned to maximum.
- Start the LSM 5 software.

☞ Please bear in mind that a cooling phase of at least 5 minutes is required between switching off of the laser via the software and switching off of the entire system via the REMOTE CONTROL main switch or the Power Supply switch of the Enterprise UV laser.

If the LSM 5 software is already running and you want to use the UV laser, do the following:

- Close the LSM 5 software.
- Switch on the power supply of the Enterprise, power potentiometer turned to maximum.
- Start the LSM 5 software again.

This applies to HeNe and Diode lasers:

- After selecting the laser, click on the **On** button.
  - The required laser for image acquisition is now available.

☞ After switching on the lasers in the laser control window and their status ready the system can be used for imaging. However, for quantitative imaging it is recommended to let the system warm up for 2 to 3 hours.

4.5.2 Microscope Control

The **Microscope Control** (**Micro** button) window permits motorized functions (objective and reflector change, condensor, filter and diaphragm settings) and the illumination mode (transmitted or reflected light) of the connected microscope to be controlled via the software.

Without any difference to software control, these microscope functions can also be operated directly on the stand via the relevant controls. In that case, any changes are recorded by the software and displayed in the relevant windows / panels.

☞ If you are using **Axioskop 2 FS MOT** as the basic microscope, the microscope functions cannot be controlled by the software as the instrument is not motorized except to the z drive. There is no **Micro** button in case of Axioskop 2 FS MOT.

4.5.2.1 Open the Microscope Control Window

- Click on the **Micro** button.
  - This opens the **Microscope Control** window on the screen.

After conclusion of the conventional setting of the connected microscope, the **Microscope Control** window can be closed again.

- Click on the **Close** button in the **Microscope Control** window.
  - The **Microscope Control** window will be closed.

4.5.2.2 Microscope Control Window for Axio Imager.Z1

- Click on the **Micro** button in the main menu.
- The microscope window opens in the last saved configuration.
- By clicking on the **More / Less** button the microscope window is displayed with or without detailed microscope beampath panel.

Reflected Light button	The shutter is switched on and off. When using the X-Cite 120 additional controls are available. See below for description.
Reflector button	Push and click reflector cube can be selected via graphical pop-up menu.
Objective button	Objective can be selected via graphical pop-up menu.
Condensor button	Numerical aperture of the condensor is set via input box or slider. Turret position selected from graphical pop-up menu (only for motorized condensors). By clicking on the **Close** button the value is stored and the **Condensor** frame is closed.
Field Stop button	Opening of luminous-field diaphragm (transmitted and reflected light) can be set via input box or slider. By clicking on the **Close** button the value is stored and the **Field Stop** frame is closed.
Filter button	Transmission values for attenuation filter (transmitted and reflected light) is set via input box or slider for the front or rear filter wheel in accordance with the available filter steps. By clicking on the **Close** button the value is stored and the **Filter** frame is closed.
Aperture Diaphragm button	**Diaphragm opening is** set via input box or slider. By clicking on the **Close** button the value is stored and the **Aperture Diaphragm** frame is closed.
Transmitted Light button	Transmitted light is switched on / off via **ON** button in the **Transmitted Light** frame, setting of light intensity can be varied via input box or slider. 3200 K color temperature for photo documentation can be switched on via **3200 K** button in the Transmitted Light frame. By clicking on the **Close** button the **Transmitted Light** frame is closed.

Recording of microscope settings

The upper part of the **Microscope Control** window shows the recording functionality of microscope configurations.

Complete microscope configurations can be created and applied.

The **Store** button permits existing microscope configurations to be stored under any name.

The **Apply** button permits existing stored microscope configurations to be loaded.

The **Delete** button permits existing microscope configurations to be deleted.

The **Assign** button permits the assignment of a microscope configuration to a button.

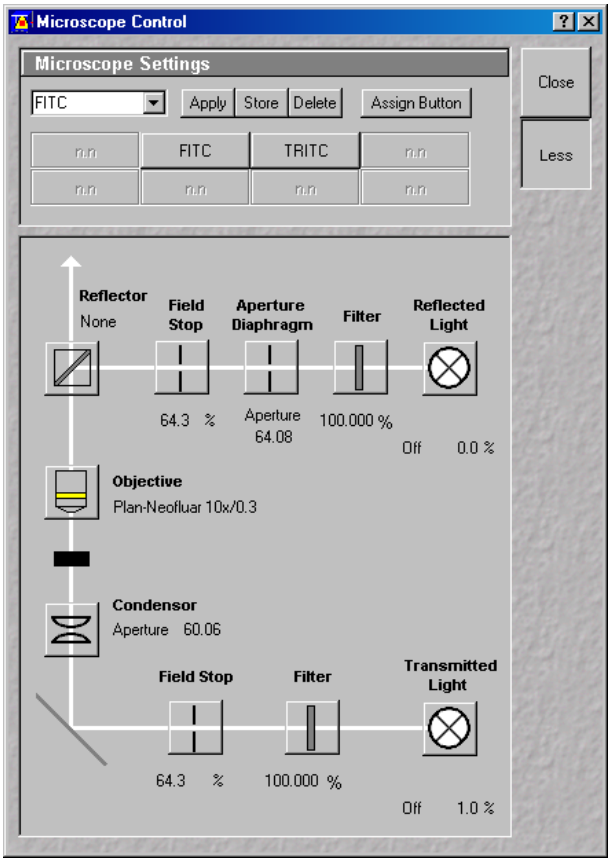


Fig. 4-27 Axio Imager.Z1 Control window

Load a microscope configuration

An existing microscope configuration can be loaded as follows:

- Click on the **arrow** button.
  - This opens a list box of all stored microscope configurations.
- Browse through the microscope configurations by clicking, or use the scroll bar at the side of the list box.
- Click on the desired microscope configuration.
  - The selected microscope configuration is shown in the first line of the **Microscope Configurations** list box.
- Click on the **Apply** button.
- Click on the **Close** button to close the microscope window.

☞ Only those microscope settings which are encoded and motorized can be reloaded.

Store a Microscope Configuration

A newly created or changed microscope configuration can be stored under a new name as follows:

- Enter the desired name in the first line of the microscope setting list box.
- Click on the **Store** button.
- The actual configuration with the chosen name is added to the microscope settings list.
- Click on the **Close** button to close the microscope window.

Delete a Microscope Configuration

A no longer required microscope configuration can be deleted as follows:

- Select the microscope configuration to be deleted from the microscope configuration list box.
- Click on the **Delete** button.
- Click on the **Close** button to close the microscope window.

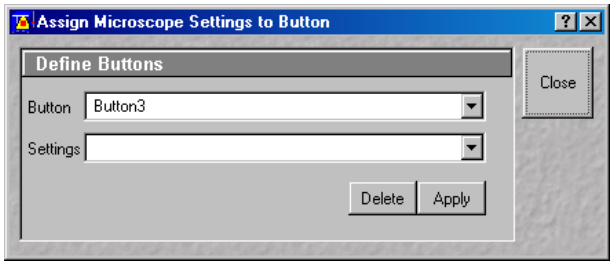


Fig. 4-28 **Assign-Microscope-Settings-To-Button** window

Assignment of a microscope configuration to a button

A microscope configuration can be assigned to a button as follows:

- Click on the **Assign** button.
- This opens the **Assign-Microscope-Settings-To-Button** window.
- Click on the arrow in the **Button** list box and select a button out of the list.

☞ With increasing numbers the buttons are arranged from the upper to the lower row from left-hand side to right-hand side.

- Click on the arrow in the **Settings** list box and select a microscope configuration.
- Click on the **Apply** button. A new button with the name of the selected microscope configuration has been created.
- Click on the **Close** button to close the **Assign-Microscope-Settings-To-Button** window.
- Click on the **Close** button to close the microscope window.

Conventional setting of the microscope Axio Imager

- Click on the **VIS** button in the **Acquire** subordinate toolbar.
- Place specimen on microscope stage.
  - The cover slip must be facing up.
- Click on the **Micro** button to open the **Microscope Control** window.
- Via the **Objective** button, select the required objective as follows:
  - Open the graphical pop-up menu by clicking on the **Objective** button.
  - Click on the objective you want to select.
  - The selected objective will automatically move into the beam path.
- Use the focusing drive (4-29/**5**) to focus the required object plane.
- Select specimen detail by moving the stage in X and Y using the XY stage fine motion control (4-29/**6** and **7**).

1 Switch HBO 100 power supply
2 RL button - reflected-light shutter ON/OFF with indicator
3 TL button - transmitted-light shutter ON/OFF with indicator
4 Light intensity control
5 Focusing drive
6 Control knob for X travel of mechanical stage
7 Control knob for Y travel of mechanical stage
8 Buttons for switching the beam path

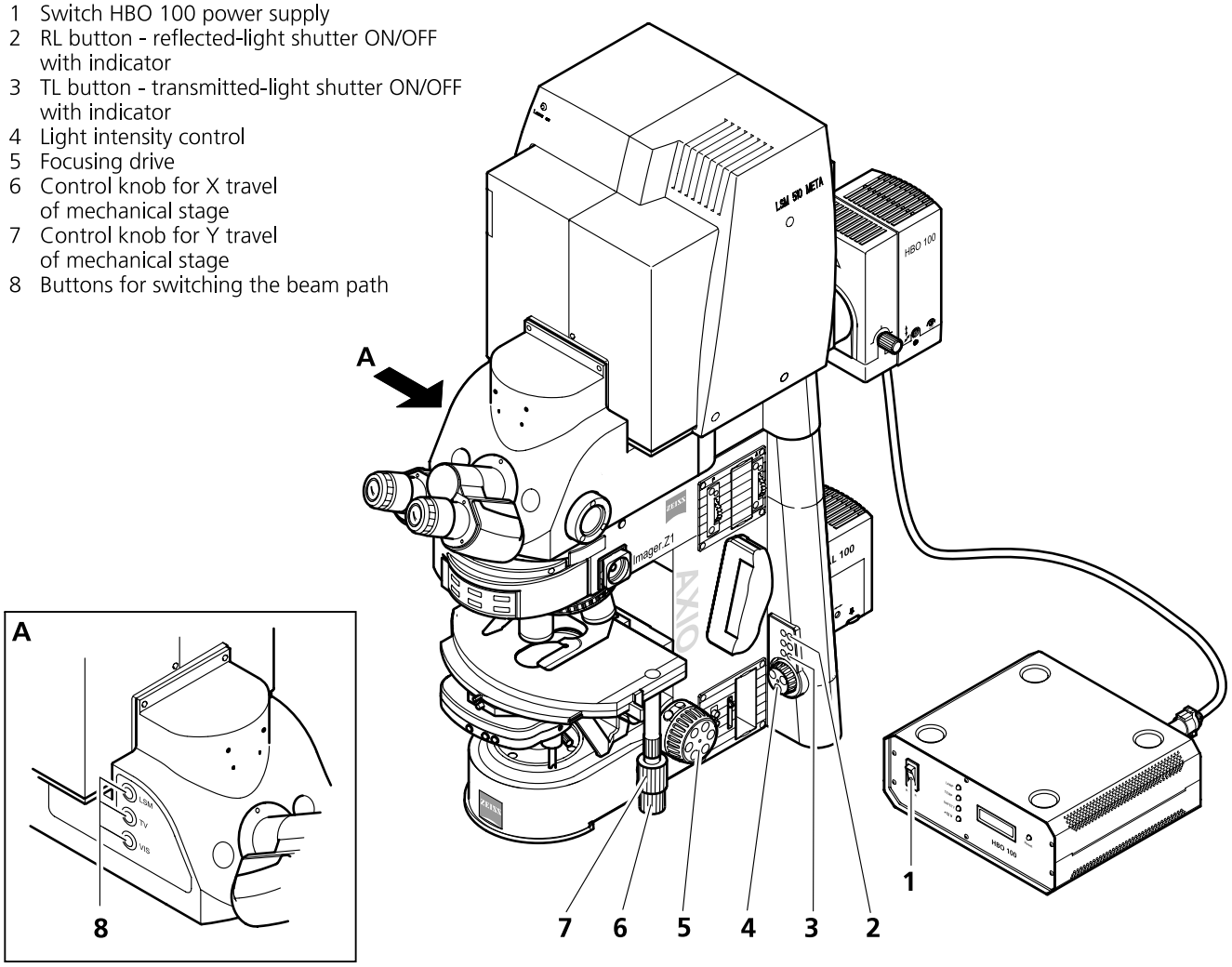


Fig. 4-29 LSM 510 with Axio Imager.Z1

Microscope settings on Axio Imager for transmitted-light observation

- Set the reflector turret position to **None** and click on the **On** button for transmitted light.
- Control the brightness of the halogen lamp with the potentiometer (4-29/**4**) or the **Intensity %** slider in the **Transmitted Light** panel.
- Set the required transmission value of the gray filters in the **Filter** frame.
- Set the condensor and the luminous-field diaphragm for KÖHLER illumination.

With **Transmitted Light** activated (**On**), the halogen lamp is automatically occluded in the laser scanning mode.

Microscope settings on Axio Imager for reflected-light observation (Epi-fluorescence)

- Turn on the HBO 100 W power supply with switch (4-29/**1**).
- Click on the RL reflected light button**.** The shutter opens.

☞ To avoid excessive bleaching of biological samples, expose the specimen to the minimum possible irradiation, i.e. keep the irradiation time as short as possible. For this, close the field stop to the necessary field of illumination and use additional filters if available.

- By clicking on the reflector turret button, select the reflector module (filter sets) to suit the type of fluorescence excitation. Proceed as follows:
- Click on the reflector turret button.
- Click on the desired reflector module.
  – The reflector turret moves the selected reflector module into the beam path.

☞ The FITC filter set consists of an excitation filter for the 450 - 490 nm spectral range, an FT color splitter for 510 nm and an LP long pass filter, which passes emission light wavelengths greater than 510 nm (FSET 09 = FITC, FSET 15 = Rhodamine, FSET 01 = DAPI).
Other filter sets:

DAPI: BP 365 FSET01
FT 395
LP 397

Rhodamin: BP 546 FSET15
FT 580
LP 590

☞ The filter sets described in this section are included in the standard configuration, but other sets are available on request.

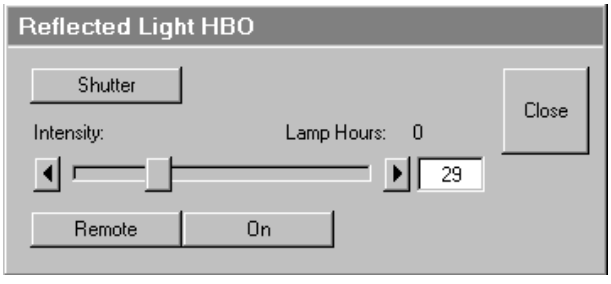


Fig. 4-30 X-Cite 120 control panel

☞ If the X-Cite 120 fiber coupled lamp for reflected-light illumination is used a further dialog is opened when clicking the Reflected light button. The lamp internal shutter and the attenuation function can be controlled via the LSM Software. The lamp has to be switched on first using the main switch on the lamp housing.

Remote Enables the attenuation control via LSM Software. This is also enabled when clicking the **ON** button.

ON Opens the shutter in the reflected light path and the internal shutter of the lamp. Both shutters are closed automatically when switching to LSM mode.

Shutter Opens and closes the shutter in the reflected light path independent of the status of the internal lamp shutter and the attenuation.

Intensity Sets the light via input box or slider.

Lamp Hours Indicates the total time the lamp has been in use.

☞ The aperture setting on the condensor of the Axio Imager.Z1 is performed in fixed steps.

4.5.2.3 Microscope Control Window for Axiovert 200 M

- Click on the **Micro** button in the main menu.
- The microscope window opens in the last saved configuration.
- By clicking on the **More / Less** button the microscope window is displayed with or without detailed microscope beampath panel.

Transmitted Light button Transmitted light is switched on / off via **ON** button in the **Transmitted Light** frame, setting of light intensity can be varied via input box or slider. 3200 K color temperature for photo documentation can be switched on via **3200 K** button in the Transmitted Light frame. The transmission light control potentiometer on the stand is disabled via the **Remote** button. By clicking on the **Close** button the **Transmitted Light** frame is closed.

Condensor button Numerical aperture of the condensor is set via input box or slider. Turret position selected from graphical pop-up menu (only for motorized condensors). By clicking on the **Close** button the **Condensor** frame is closed.

Objective button Objective can be selected via graphical pop-up menu.

Reflector button Push and click, reflector cube can be selected via graphical pop-up menu.

Tube Lens button Push and click, tube lens can be selected via graphical pop-up menu.

Reflected Light button The shutter is switched on and off. When using the X-Cite 120 additional controls are available. See below for description.

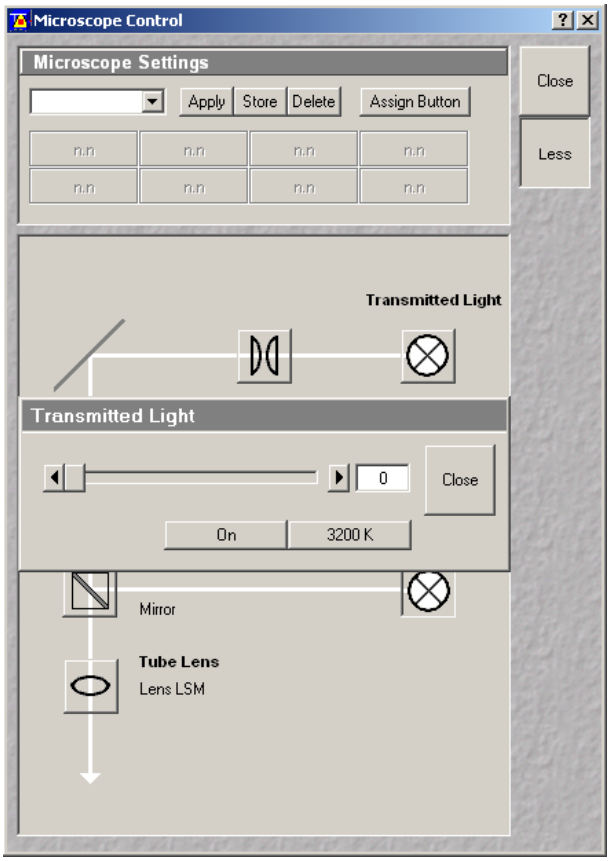


Fig. 4-31 **Axiovert 200 Control window**

Recording of microscope settings

The upper part of the **Microscope Control** window shows the recording functionality of microscope configurations.

Complete microscope configurations can be created and applied.

The **Store** button permits existing microscope configurations to be stored under any name.

The **Apply** button permits existing stored microscope configurations to be loaded.

The **Delete** button permits existing microscope configurations to be deleted.

The **Assign** button permits the assignment of a microscope configuration to a button.

Load a microscope configuration

An existing microscope configuration can be loaded as follows:

- Click on the **arrow** button.
  - This opens a list box of all stored microscope configurations.
- Browse through the microscope configurations by clicking, or use the scroll bar at the side of the list box.
- Click on the desired microscope configuration.
  - The selected microscope configuration is shown in the first line of the **Microscope Configurations** list box.
- Click on the **Apply** button.
- Click on the **Close** button to close the microscope window.

☞ Only those microscope settings which are encoded and motorized can be reloaded.

Store a microscope configuration

A newly created or changed microscope configuration can be stored under a new name as follows:

- Enter the desired name in the first line of the microscope setting list box.
- Click on the **Store** button.
- The actual configuration with the chosen name is added to the microscope settings list.
- Click on the **Close** button to close the microscope window.

Delete a microscope configuration

A no longer required microscope configuration can be deleted as follows:

- Select the microscope configuration to be deleted from the microscope configuration list box.
- Click on the **Delete** button.
- Click on the **Close** button to close the microscope window.

Assignment of a microscope configuration to a button

A microscope configuration can be assigned to a button as follows:

- Click on the **Assign** button.
- This opens the **Assign-Microscope-Settings-To-Button** window.
- Click on the arrow in the **Button** list box and select a button out of the list.

☞ With increasing numbers the buttons are arranged from the upper to the lower row from left-hand side to right-hand side.

- Click on the arrow in the **Settings** list box and select a microscope configuration.
- Click on the **Apply** button. A new button with the name of the selected microscope configuration has been created.
- Click on the **Close** button to close the **Assign-Microscope-Settings-To-Button** window.
- Click on the **Close** button to close the microscope window.

Conventional setting of the microscope Axiovert 200 M

For the conventional setting of the Axiovert 200 M, proceed as follows:

- Click on the **VIS** button in the **Acquire** subordinate toolbar.
- Place specimen on microscope stage.
  - The cover slip must be facing down.
- In the **Objective** list box, select the required objective.
- Use the focusing drive (4-32/**4**) to focus the required specimen plane.
- Select specimen detail by moving the stage in X and Y via the XY stage fine motion control (4-32/**3** and **2**).

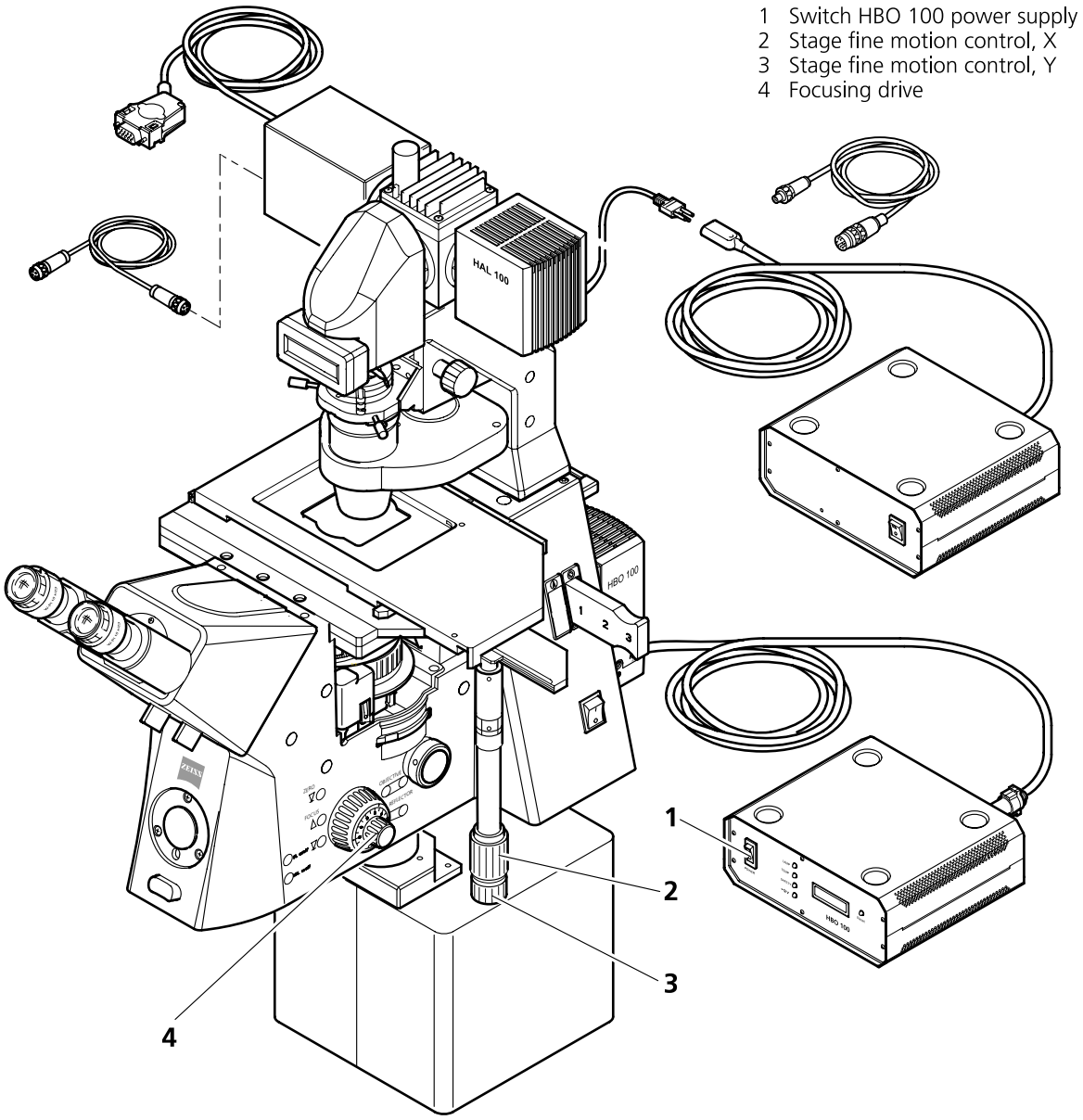


Fig. 4-32 LSM 510 with Axiovert 200 M BP

Microscope settings on Axiovert for transmitted-light observation

- Click on the **Reflected Light** button and set the shutter to the **Closed** position.
- Click on the **Transmitted light** button. Click on the **On** button in the **Transmitted Light** panel and set the transmitted light intensity via the slider or click on **3200 K**. Click on **Close** to close the **Transmitted Light** panel.
- Click on the **Condensor** button and set the aperture via the slider in the **Condensor** panel. Set the filter in the **Filter** selection box. Click on **Close**.
- Click on the **Objective** button and select the objective by clicking on it.
- Click on the **Reflector** button and select the **None**.

Microscope settings on Axiovert for reflected-light observation (Epi-fluorescence)

- Turn on the HBO 100 power supply switch (4-32/**1**).
- Click on the **Reflected Light** button and set the shutter in the **Open** position.
- Click on the **Reflector** button and select the desired filter set by clicking on it.
- The filter is automatically moved into the beam path to enable observation in epi-fluorescence.
- Click on the **Tube Lens** button and select the tube lens.
- Click on the **Objective** button and select the objective.

☞ If the X-Cite 120 fiber coupled lamp for reflected-light illumination is used a further dialog is opened when clicking the Reflected light button. The lamp internal shutter and the attenuation function can be controlled via the LSM Software. The lamp has to be switched on first using the main switch on the lamp housing.

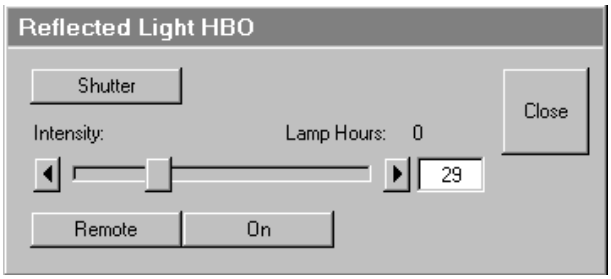


Fig. 4-33 X-Cite 120 control panel

Remote	Enables the attenuation control via LSM Software. This is also enabled when clicking the **ON** button.
ON	Opens the shutter in the reflected light path and the internal shutter of the lamp. Both shutters are closed automatically when switching to LSM mode.
Shutter	Opens and closes the shutter in the reflected light path independent of the status of the internal lamp shutter and the attenuation.
Intensity	Sets the light via input box or slider.
Lamp Hours	Indicates the total time the lamp has been in use.

4.5.2.4 Working in LSM Mode

Switchover to the scanning mode is then required.

- Click on the **LSM** button in the **Acquire** subordinate toolbar.

4.5.2.5 Microscope Control for Axioskop 2 FS MOT

For setting the Axioskop 2 FS MOT, proceed in the same way as with Axio Imager.Z1 and Axiovert 200 M.

Since the Axioskop 2 FS MOT is not motorized (except the external Z drive and the tube), all microscope settings have to be made manually.

Especially, the change of objectives is made manually. The used objective must be set in the **Scan Control** window.

4.5.3 Configuration Control

The setting of the beam path for the scanning procedure, i.e. the definition of channels (single detector, META detector) and tracks and the setting of the Acousto-Optical Tunable Filters (AOTF) of the various laser lines is performed in the **Configuration Control** window.

☞ A track is:
- a set of parameters for the detection channels and for illumination (wavelength and intensity)
- scanned simultaneously and identified and handled by the system with one name

The **Configuration Control** window has a different appearance, depending on which selection button has been activated (**Channel**,**Lambda Mode or Online Fingerprinting Mode**).

The **Lambda Mode and Online Fingerprinting Mode** are only available if the system contains the META detector. Only in the **Channel Mode** a subordinate toolbar exist which contains a **Single Track**, **Multi Track** or **Ratio** button. In the **Channel** mode use the **Single Track** and **Multi Track** buttons to toggle between the two image acquisition modes **single tracking** and **multitracking**. In addition, you can activate the **Ratio** button for the activation of up to two **Ratio** channels.

Performed settings can be stored as **Track Configurations** for **single tracking**. The number of traditional channels to be defined in one track is limited to 8 (incl. monitor diode, transmission and ratio channels), depending on the configuration. The number of channels to be defined in one track for LSM 510 META *DuoScan* systems is 8 (including monitor diode and transmission detector). Furthermore, 2 ratio channels can be used.

In case the number of available channels is not sufficient for the scanning procedure, further tracks can be added and configured. The combination of these tracks can also be stored as **Recording Configurations** for **multitracking**. A recording configuration may contain the maximum of 4 tracks. Regardless of the number of included tracks, the maximum of 8 channels (incl. monitor diode, transmission and ratio channels) can be used in a recording configuration in **multitracking**.

If several tracks have been activated, they are processed one after the other during the scan procedure.

If the maximum number of channels to be used in a **Single Track** or a **Multi Track** has already been achieved, it is no longer possible to add further channels or tracks.

If a second track or further tracks are used, the scan parameters can be changed as required. This avoids "cross-talk" from one channel to another when different tracks are used.

4.5.3.1 Open / Close the Configuration Control Window

- Click on the **Config** button in the **Acquire** subordinate toolbar.
  - The **Configuration Control** window is opened with the display last selected.

☞ The **Beam Path and Channel Assignment** panel differs according to the hardware configuration supplied.

- Click on the **Close** button to quit the **Configuration Control** window.

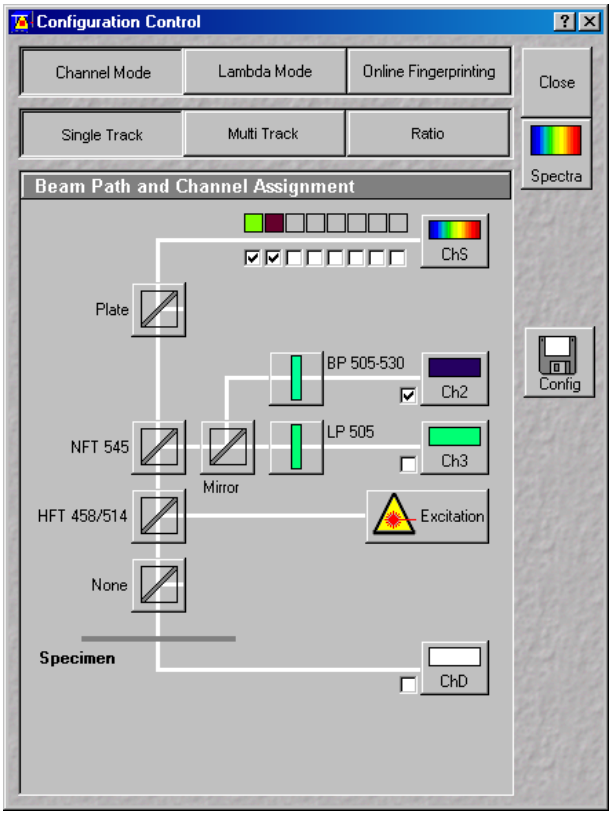


Fig. 4-34 **Configuration Control window, Single Track activated**

4.5.3.2 Spectra Button

The **Spectra** button opens the **Detection Spectra & Laser Lines** window. This window displays the laser wavelengths activated for excitation (as colored vertical lines) and the activated channels (as colored horizontal bars).

The color of the bar corresponds to the one assigned to the relevant channel. Non-active channels receive a gray bar over the entire spectral range.

The length and position of the bar corresponds to the emitted spectral range which is overlaid with the filter and beam splitters selected in the **Configuration Control** window or number of selected channels of the META detector.

- Click on the **Spectra** button to open the **Detection Spectra & Laser Lines** window and to check the settings you made. The window is closed via **Close**.

All amendments made in the **Configuration Control** or **Laser Control** window are updated on-line in the **Detection Spectra & Laser Lines** window.

- A click on the **Laser** button enables you to open the **Laser Control** window, switch lasers on and off, if required, and control the laser output.

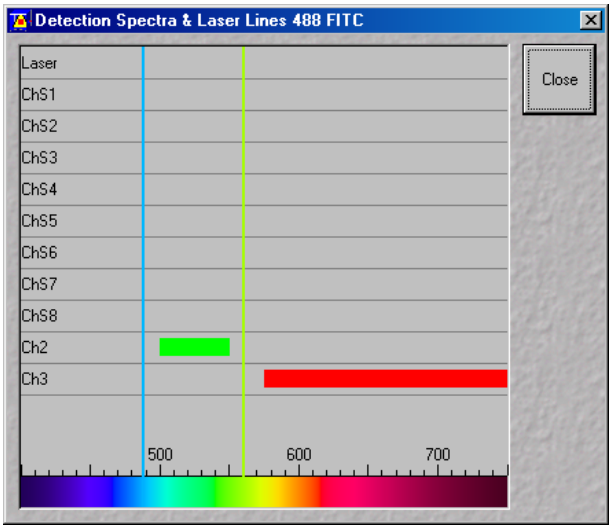


Fig. 4-35 **Detection Spectra & Laser Lines window**

4.5.3.3 Laserline Button

The **Laserline** button opens the Wavelength Switch Control window.

If more than 8 excitation laser lines (wavelengths) are available from the connected lasers, these can no longer be completely displayed in the **Line Active** column of the **Excitation** window. In such a case, this button is active and the required laser lines (if not displayed) must be allocated.

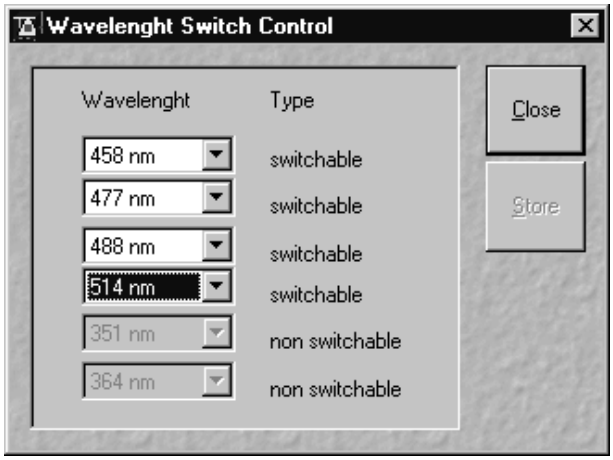


Fig. 4-36 Wavelength & Switch Control window

- For this purpose, click on the **Laser Line** button. The **Wavelength Switch Control** window is opened.
- Select the required laser lines in the selection boxes and confirm the selection with a click on **Store**.
- Click on the **Close** button of the **Wavelength Switch Control** window (**Close** is used to close the window without accepting the changes).

The laser lines are now available in the **Line active** column of the **Excitation** window.

If the laser lines 351 nm, 364 nm and / or 405 nm have been switched on via the relevant lasers, they are automatically entered into the **Line Active** column as **non switchable**.

- Click on the **Close** button to conclude the laser settings procedure.

Fig. 4-37 Track panel

4.5.3.4 Config Button

The **Config** button permits existing track configurations to be loaded, stored under any name, or deleted.

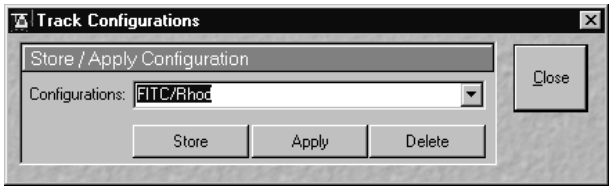


Fig. 4-38 Track Configurations window

(1) Load a track configuration

A configuration stored in the system, whether factory-supplied or user-created, can be accepted or selected for active operation as follows:

- Click on the **Config** button, the **Track Configurations** window appears on the screen.
- On the **Store / Apply Configuration** panel, click on the arrow button .
  - This opens a list box of all stored track configurations.
- Browse through the configurations by clicking, or use the scroll bar at the side of the list box.

- Click on the desired configuration.
  - The selected configuration is shown in the first line of the **Configurations** list box (e.g.: FITC/Rhod).
- Click on the **Apply** button.
  - This results in the stored instrument parameters being taken over for active use. The track configuration selected before is overwritten.

☞ The optical diagram of the configuration selected appears on the **Beam Path and Channel Assignment** panel. The newly loaded track has been automatically activated for the scanning procedure. The **Track Configurations** window is closed automatically.
In the **Options** menu in the function **Settings** it is possible to define the parameters to be used when applying a track configuration.

(2) Store a track configuration

A newly created or changed track configuration can be stored under a new name as follows:

- Click on the **Config** button, the **Track Configurations** window appears on the screen.
- Enter the desired name in the first line of the **Configurations** list box.
- Click on the **Store** button.
- Close the window by clicking on **Close**.

During storage via the **Store/Apply** function, all the data of the **Beam Path and Channel Assignment** and the Detector Gain, Ampl. Offset, Ampl. Gain and Data Depth (8 / 12 Bit) scan parameters of the current track (single tracking) will be stored.

(3) Delete a track configuration

A no longer required track configuration can be deleted as follows:

- Click on the **Config** button, the **Track Configurations** window appears on the screen.
- Select the configuration to be deleted from the **Configurations** list box.
- Click on the **Delete** button.
- Close the window by clicking on **Close**.

4.5.3.5 Settings for Single Track in the Channel Mode

The settings of the beam path for the scanning procedure with regard to the main dichroic beam splitter (HFT), secondary dichroic beam splitter (NFT), emission filters (EM) to be used and the assignment of channels, excitation wavelengths and laser intensities are performed in the **Beam Path and Channel Assignment** panel.

The setting can be performed manually or by using existing track configurations.

- Click on the **Single Track** button, unless it has already been activated.
  - The **Configuration Control** window for single tracking is displayed.
- Click on the **Descanned** button, unless it has already been activated.

(1) Beam Path and Channel Assignment panel

The **Beam Path and Channel Assignment** panel displays the selected track configuration which is used for the scan procedure.

You can change the settings of this panel using the following function elements.

Activation / deactivation of the excitation wavelengths (check box) and setting of excitation intensities (slider). Open the **Laser Control** window via the **Laser** button.

Selection of the main dichroic beam splitter (HFT) or secondary dichroic beam splitter (NFT) position through selection from the relevant list box.

Selection of an emission filter through selection from the relevant list box.

Activation / deactivation of the selected channel (Ch 1-4, monitor diode ChM, META detectors ChS1-8, transmission ChD) for the scanning procedure by assigning an existing color icon or defining a new one. Deactivation of the channel via deactivation of the check box.

The **Fiber Out** port of the LSM 510 permits connection to another detector.

For the configuration of the beam path, please refer to the application-specific configurations depending on the used dyes and markers and the existing instrument configuration (e.g.: module LSM 510 - Config. 26) listed in the annex.

The assignment of the numbered emission filters (1-4), NFT secondary dichroic beam splitters (1-3) and HFT main dichroic beam splitters in the **Beam Path and Channel Assignment** panel is shown in the **Configuration Control** window (Fig. 4-39). The numbers of the emission filters correspond to those of the channels lying behind (PMT photomultipliers).

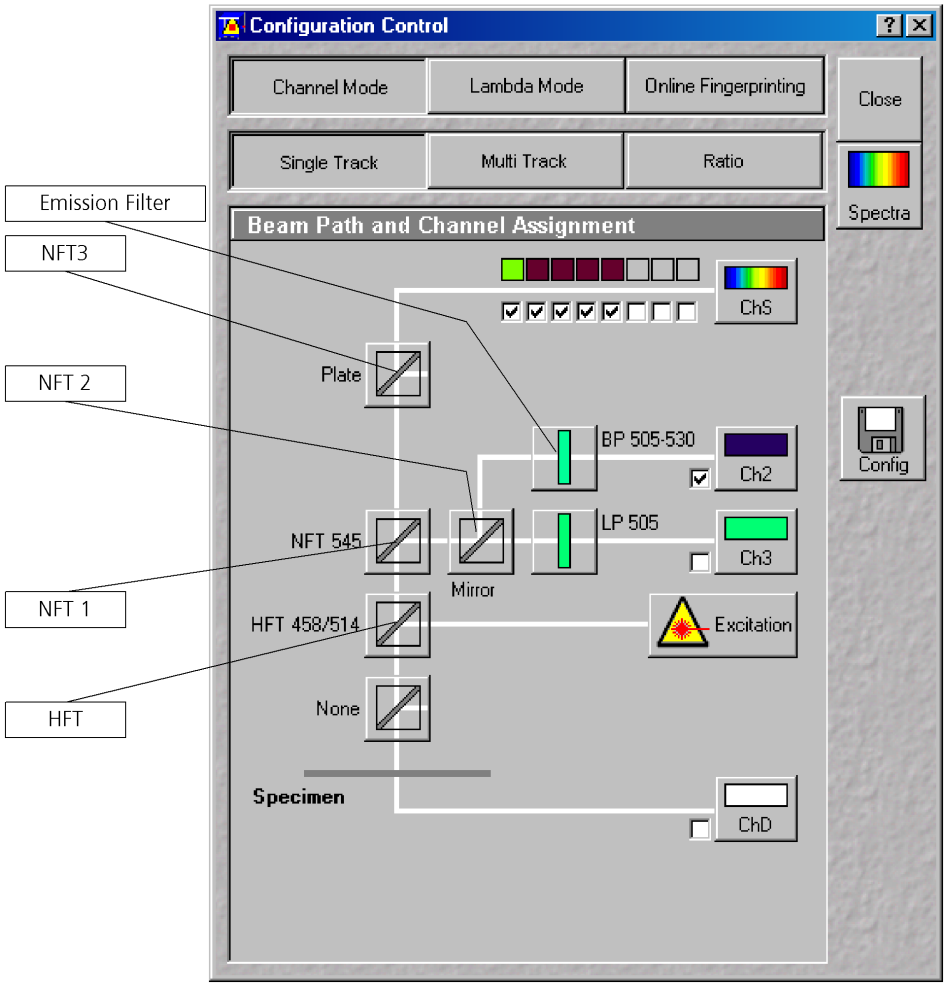


Fig. 4-39 Configuration Control window

(2) Beam path - HFT main dichroic beam splitters and NFT secondary dichroic beam splitters

- On the **Beam Path and Channel Assignment** panel, click on the HFT main dichroic beam splitters (see Fig. 4-39).
  - This opens a graphical pop-up window of all beam splitters available.
- To select a beam splitter, click on the respective line of the list.
  - The selected beam splitter moves into the beam path.
- Proceed accordingly to configure the NFT secondary dichroic beam splitters.

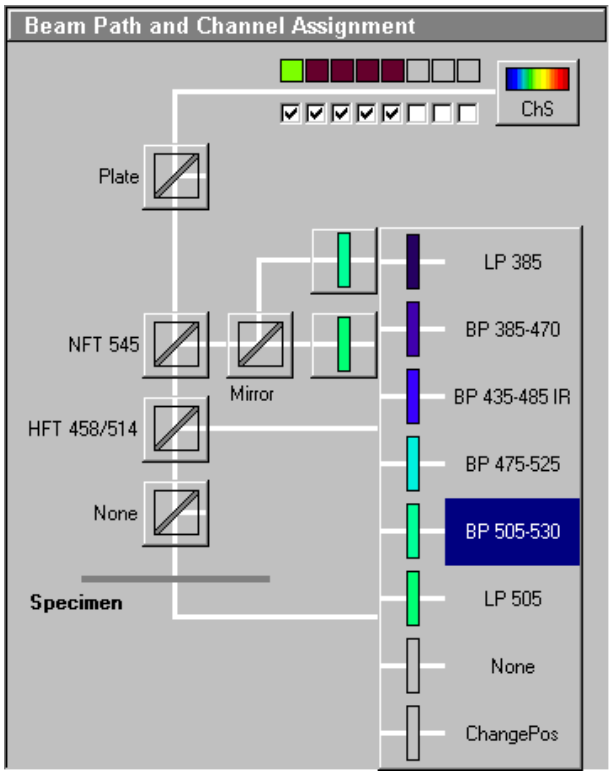


Fig. 4-40 Configuration Control window

(3) Beam path - Emission filter

- On the **Beam Path and Channel Assignment** panel, click on the emission filter symbol.
  - This opens a graphical pop-up window of all available emission filters (e.g. BP for band pass, or LP for long pass) with their wavelengths.
- To select an emission filter, click on the respective filter in the pop-up window.
  - The emission filter selected moves into the beam path in front of the PMT photomultiplier.
- Depending on the application, it may be necessary to insert additional mirrors, secondary dichroic beam splitters or neutral glass filters between the HFT main dichroic beam splitter and the PMT photomultiplier. To select these components, click on the respective symbols.

☞ For channels 1 and 2, it is possible to change the filters directly on the LSM 510 scan module (see CHAPTER ANNEX: Filter Change in the Detection Beam Path of Channels 1 and 2 of the printed manual).

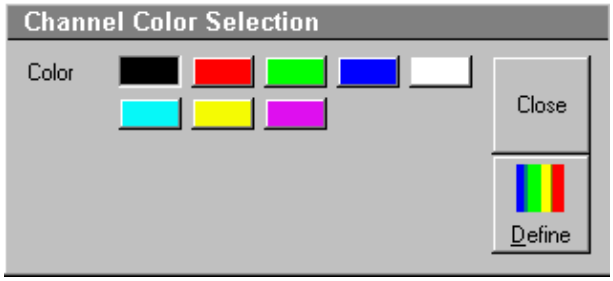


Fig. 4-41 Channel Color Selection window

(4) Beam path - Activation / Deactivation of Channels and Channel Color Assignment

- On the **Beam Path and Channel Assignment** panel, click on the channel symbols, e.g. .
  - This opens the **Channel Color Selection** window on the Beam Path and Channel Assignment panel.
- Click on the desired color bar.

This changes the color of the channel symbol.

- To close the **Channel Color Selection** box, click on the **Close** button.

Further colors for the corresponding channel can be produced as follows:

- Clicking on the **Define** button will open a further **Channel Colors** window.

All the available colors are shown as buttons in the **Current Set of Channel Colors** panel.

- Via a reticule in the **Define Color** panel, any desired color can be produced.
- Clicking on the **Add** button allows the color to be used for further channel coloring.
- Choose the desired color with the reticule (the reticule is in the left corner at the bottom of the color range).
- Define the brightness by use of the scroll bar.
- Use the **Add** button to add the color to the color range.
- To delete a defined color, click on the relevant color button and then on the **Remove** button.

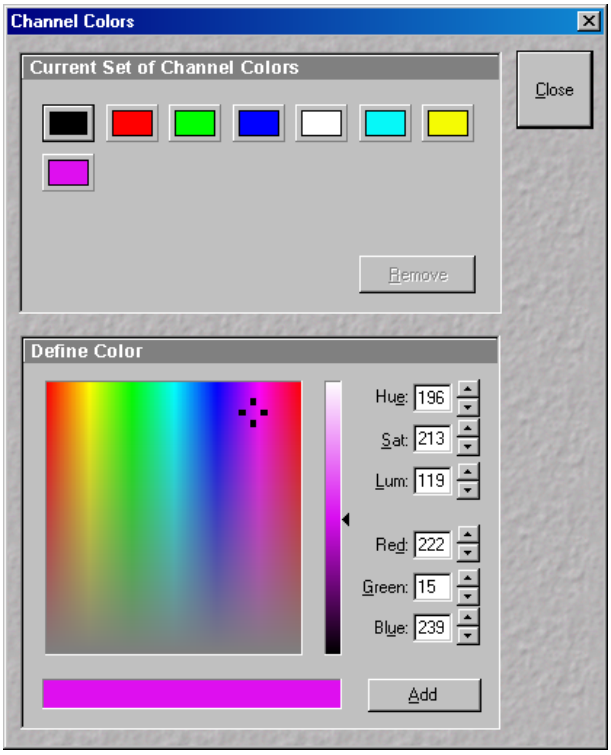


Fig. 4-42 Channel Colors window

☞ Standard colors (black for OFF, red, green, blue and white) cannot be removed.

- Click on the **Close** button to close the **Channel Colors** window.
  - Newly defined colors are accepted and displayed in the **Channel Color Selection** window. They can then be used in the same way as standard colors.

The PMT1 photomultiplier is activated / deactivated by the check box.

- Proceed in the same way for the other PMT photomultipliers.

☞ The

symbols for the transmitted-light PMT photomultiplier (ChD Transmission) and monitor diode (ChM) can be activated in the same way as the photomultipliers of channels 1 to 4. The use of the monitor diode function is described in detail in the annex of this manual.
When changing from the NFT 1 secondary dichroic beam splitter, the appropriate setting of the NFT 3 secondary dichroic beam splitter is performed automatically.

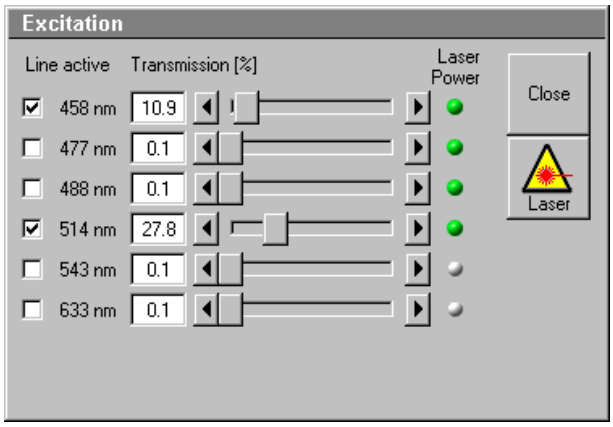


Fig. 4-43 Configuration Control window

(5) Beam path - Laser attenuation

- On the **Beam Path and Channel Assignment** panel, click on the **Excitation** button.
  - This opens a dialog box of all available lasers with their wavelengths and their usable Acousto-Optical Tunable Filters (AOTF) attenuation.
- To select the desired laser line, activate the check box for **Line Active**.
- Use the **Transmission [%]** slider to set the utilizable laser intensity (recommendation: start at 10 %).
  - The transmittance of the Acousto-Optical Tunable Filter (AOTF) changes accordingly.
- This allows you to adapt the laser intensity very sensitively to the job. Activate the check box for **Line Active**.
  - This activates the selected laser power for use. This is indicated by the **Laser Power** display lamps (status display green / gray).

☞ By clicking on the **Excitation** button you can check at any time which lasers are available for active operation.

If you deactivate **Line Active**, the laser wavelengths are deselected by means of the Acousto-Optical Tunable Filters (AOTF), i.e. these lasers change into standby status.

If you interrupt your work with the LSM for a break, it is recommended not to switch the Enterprise and argon lasers off by hardware action, but to put them into standby status as described.

Excitation filters, emission filters, HFT main dichroic beam splitters and NFT secondary dichroic beam splitters can be switched online, channels (PMT photomultipliers) only off-line.

4.5.3.6 Settings for Multi Track in the Channel Mode

The **Multi Track** function permit several tracks to be defined as one configuration (**Recording Configuration**) for the scan procedure, to be stored under any name, reloaded or deleted.

The maximum of four tracks with up to 8 channels can be defined simultaneously and then scanned one after the other. Each track is a separate unit and can be configured independently of the other tracks with regard to channels, Acousto-Optical Tunable Filters (AOTF), emission filters and dichroic beam splitters.

- Click on the **Multi Track** button.
  - The **Configuration Control** window for multitracking appears, which means that the **List of Tracks** panel is additionally displayed.

The tracks required for multitracking can either be configured manually one after the other (identical to single tracking) and then stored as recording configuration, or already existing recording configurations can be used and changed as required.

It is also possible to load already stored track configurations (single tracking) in a recording configuration.

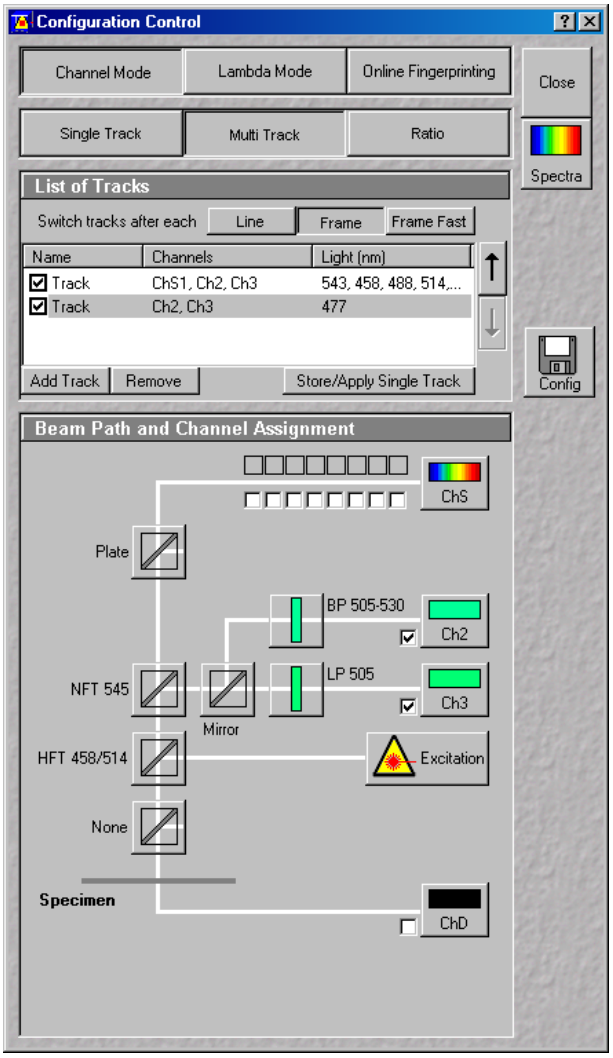


Fig. 4-44 **Configuration Control window, Multi Track activated**

(1) Beam Path and Channel Assignment panel

The **Beam Path and Channel Assignment** panel displays the track configuration of the track currently selected in the **List of Tracks** panel (highlighted in blue or gray).

The settings for this panel are performed separately for each track, in the same way as for single tracking. To do this, select the track to be configured from the **List of Tracks** panel (see the following description of the **List of Tracks** panel).

(2) List of Tracks panel

In the **List of Tracks** panel, the available tracks are displayed with names, activated channels and laser lines.

The **Line**, **Frame** and **Fast Switch** buttons are used to determine in which way switching between tracks is made during the scan procedure.

Furthermore, the sequence of tracks to be processed can be changed for the scan procedure.

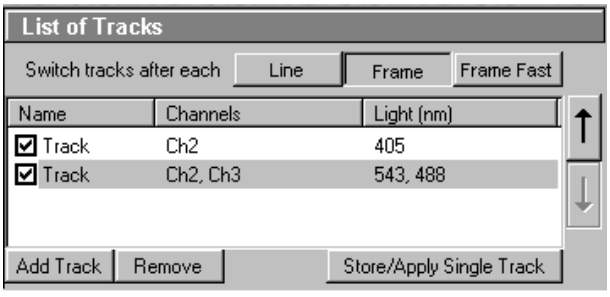


Fig. 4-45 **List of Tracks panel**

The **Add Track**, **Store/Apply Single Track** and **Remove** buttons permit individual tracks to be added, saved or deleted.

In addition, this panel is used to activate / deactivate the tracks for the scan procedure.

- To activate or deactivate one or several tracks for the scan procedure, activate / deactivate the check box of the relevant tracks.

  The configuration of the selected track is displayed in the **Beam Path and Channel Assignment - ...** panel.
- To select a track for the display of the beam path configuration, click on its name.
  - The selected track is highlighted in gray or blue.

☞ When you switch from multitracking to single tracking, the track selected in the multitracking mode (highlighted in blue or gray) is always transferred and automatically activated for the scan procedure. All other tracks are deactivated, and they remain deactivated when you switch back to the multitracking mode afterwards.

The following functions are available in the **List of Tracks** panel.

Modes

Switch tracks after each

Line button — Tracks are switched during scanning line by line. The following settings can be changed between tracks: Acousto-Optical Tunable Filters (AOTF) and the **Amplifier Offset**.

Switch tracks after each

Frame button — Tracks are switched during scanning frame by frame. The following settings can be changed between tracks: Acousto-Optical Tunable Filters (AOTF), the emission filters, the dichroic beam splitters, the channels, the settings of the pinhole position in XY(Z)-direction and pinhole diameter and **Gain** and **Amplifier Offset**.

Frame Fast button — The scanning procedure can be made faster. Only the Acousto-Optical Tunable Filters (AOTF) for the selected laser line and the **Amplifier Offset** are switched, and no other hardware components. The tracks are all matched to the current track with regard to emission filter, dichroic beam splitter, setting of Detector Gain, pinhole position and diameter. When **Line** button is selected, the same rules apply as for **Frame Fast**.

Settings

Add Track button	An additional track is added to the configuration list. The maximum of four tracks can be added. One track each with basic configuration is added, i.e.: one Ch 1 channel is activated, all laser lines are switched off, emission filters and dichroic beam splitters are set in accordance with the configuration last used.
Remove button	The single track previously marked in the **List of Tracks** panel in the Name column is deleted.
Store/Apply button	Opens the **Track Configurations** window. A selected track defined in a Recording Configuration can also be stored as a single track for single tracking applications. Also, it's possible to load a single track in a multitracking configuration.
	A click on this arrow button will move the selected track (highlighted in blue) one position upwards in the list box.
	A click on this arrow button will move the selected track (highlighted in blue) one position downwards in the list box.

When adding new tracks, the following sequence should be followed:

- Add a track by clicking on the **Add Track** button.
- Determine the configuration of the track in the **Beam Path and Channel Assignment** panel or select an existing one via the **Store/Apply Single Track** button of the **List of Tracks** panel.
- Store the name of a track configuration defined via the **Store/Apply** button of the **List of Tracks** panel. The new track name will then be displayed in the **List of Tracks** panel.

If this way of storing is performed, the created track will also be available as a single track and can therefore also be activated individually.

- Add the next track via the **Add Track** button and then configure and store it again.

The name of a track can also be changed directly in the **List of Tracks** panel. In that case, however, the edited track is not available as a single track configuration, but only within the recording configuration.

To edit a track name within **Recording Configurations,** proceed as follows:

- To select the track, click on the relevant track name in the **List of Tracks** panel. Then click on the name again to open the text editing field.
- Change the track name via the keyboard. Use **Esc** to undo the procedure.
- Click once in the area outside the text editing box to close this box.

☞ The channels of the individual tracks with the relevant scan parameters can be displayed in the **Scan Control** window after activation of the **Channels** button. The description of channel 1 in Track 1, for example, is Ch1-T1.

(3) Config button in Multi Track mode

The **Config** button in the **Multi Track** mode permits all tracks to be loaded, stored under any name, or deleted.

(a) Load a Channel Mode Configuration

An existing recording configuration can be loaded as follows:

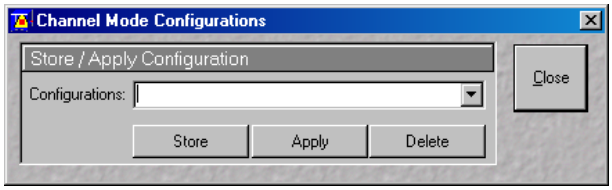


Fig. 4-46 Channel Mode Configurations window

- Click on the **Config** button, the **Channel Mode Configurations** window appears on the screen.
- On the **Store / Apply Configuration** panel, click on the arrow button .
  - This opens a list box of all stored recording configurations.
- Browse through the configurations by clicking, or use the scroll bar at the side of the list box.
- Click on the desired configuration.
  - The selected configuration is shown in the first line of the **Configurations** list box (e.g.: DAPI).
- Click on the **Apply** button.
  - The program loads those parameters of the selected **Channel Mode Configuration** which have been activated in the **Options** menu under **Settings / Recording Configuration** (see **Settings Function**, page 4-216). The **Channel Mode Configurations** window is automatically closed.

☞ The optical diagram of the configuration selected appears on the **Beam Path and Channel Assignment** panel. The entire recording configuration has been activated for the scanning procedure.

(b) Store a Channel Mode Configuration

A newly created or changed recording configuration can be stored under a new name as follows:

- Click on the **Config** button, the **Channel Mode Configurations** window appears on the screen.
- Enter the desired name in the first line of the **Configurations** list box.
- Click on the **Store** button.
- Close the window by clicking on **Close**.

During storage via the **Config button**, all the data of **Beam Path and Channel Assignment** and the Detector Gain, Ampl. Offset, Ampl. Gain and Data Depth (8 / 12 Bit) scan parameters of all the defined tracks (multitracking) are stored. Furthermore, the used objective, the **Frame Size**, **Zoom, Rotation & Offset** and **Scan Direction** parameters and the bleach parameters are stored.

(c) Delete a Channel Mode configuration

A no longer required recording configuration can be deleted as follows:

- Click on the **Config** button, the **Recording Configurations** window appears on the screen.
- Select the configuration to be deleted from the **Configurations** list box.
- Click on the **Delete** button.
- Close the window by clicking on **Close**.

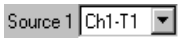
4.5.3.7 Ratio Settings Panel

The **Ratio Settings** panel permits you to activate two additional **Ratio** channels.

- Click on the **Ratio** button.
  - The **Ratio Settings** panel is displayed at the bottom of the **Configuration Control** window. The settings of the selected tracking mode (Single Track / Multi Track) remain unchanged.
- The **Ratio Settings** panel is only available in the **Single Track** and **Multi Track** mode.

The following function elements are provided in the **Ratio Settings** panel:

Activation of the **Ratio** channel (R1, R2) through assignment of an existing color or definition of a new one. Activation / deactivation of the **Ratio** channel via the check box.



Selection of the channels of which the ratio is to be formed from the relevant list box. **Source 2** in ratio settings includes the option to select "1st Image" for R1 and/or R2 (e.g. to calculate F/F_0 for single wavelength dyes).

A suitable color can be assigned to each of the two **Ratio Channels R1** and **R2**, in the same way as for the photomultiplier channels.

The channels of which a ratio will be formed are selected via the **Source 1** and **Source 2** list boxes.

- Click on the arrow button to select the required channel for **Source 1** and **2** from the list box now opened.

☞ The ratio to be formed between the selected channels can be defined more precisely using one of the four preset formulas in the **Scan Control** window after activation of the **Channels** button and a click on the relevant ratio button (e.g.: R1) for online display of radiometric or single wavelength dyes. The Set by min/max function (in Scan Control window - Channel mode) allows the definition of the display scaling according to the expected minimal and maximal values

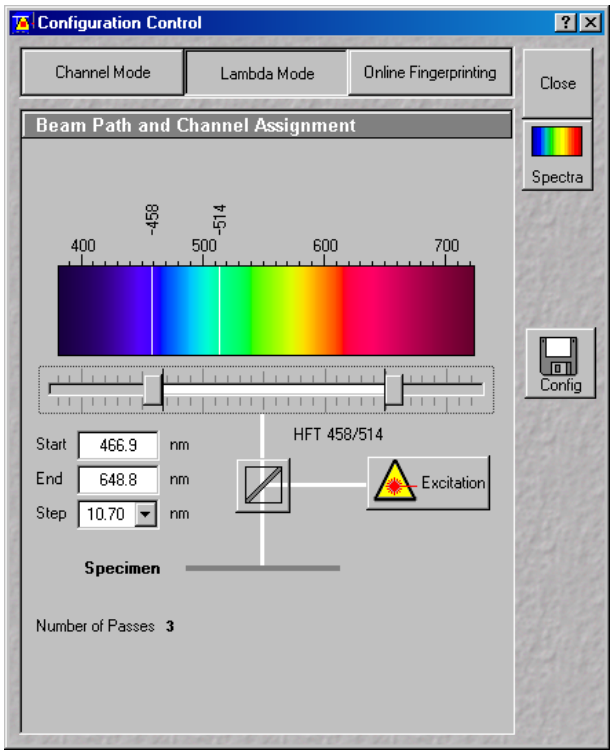


Fig. 4-47 **Configuration Control window; Lambda Mode activated**

4.5.3.8 Settings in the Lambda Mode

The **Lambda Mode** settings are only available if the LSM 510 contains the META detector. With this spectral module the overall emission from the sample is directed onto a wavelength-dispersive element and is imaged on a 32 channel detector. All 32 photomultipliers of the detector cover a spectral width of approximately 340 nm, a single PMT covers a spectral range of 10.7 nm. In the **Lambda Mode,** images, image stacks or time series can be recorded in a wavelength selective way.

This new experiment is called Lambda Stack. For the acquisition of a Lambda Stack, the fluorescence signal of 8 PMTs out of the 32 can be read out at once. The settings of the beam path for the **Lambda Mode** scanning procedure with regard to the main dichroic beam splitter and the META detector settings are performed in the **Beam Path and Channel Assignment** panel.

- Click on the **Config** Button in the **Acquire** Subordinate toolbar of the main menu.
  - The **Configuration Control** window opens.
- Click on the **Lambda Mode** button, unless it has already been activated.
  - The **Beam Path and Channel Assignment** panel for the **Lambda Mode** is opened.

(1) Beam Path and Channel Assignment panel

The **Beam Path and Channel Assignment** panel displays the configuration of laser lines, the main (dichroic) beam splitter and the spectral range of the META detector to be covered.

You can change the settings of this panel using the following function elements:

Beam Path configuration

Activation / deactivation of the excitation wavelengths (check box) and setting of excitation intensities (slider). Open the **Laser Control** window via the **Laser** button. The active laser lines are automatically displayed in the wavelength color bar.

Selection of the main dichroic beam splitter (HFT) through selection from the relevant list box.

META detector slider — Definition of number of PMTs out of the 32 PMT Detectors to fit the required spectral range.

Control buttons

Close Closes the **Configuration Control** window.

Spectra The **Spectra** button opens the **Detection Spectra & Laser Lines** window (see page 4-53).

Laserline The **Laserline** button opens the **Wavelength Switch Control** window (see page 4-54).

Config The **Config** button permits existing track configurations to be loaded, stored under any name, or deleted (see page 4-54).

(2) Beam path - Excitation

- On the **Beam Path and Channel Assignment** panel, click on the **Excitation** button.
  - This opens a dialog box of all available lasers with their wavelengths and their usable Acousto-Optical Tunable Filters (AOTF) attenuation.
- To select the desired laser line, activate the check box for **Line Active**.
- Use the **Transmission [%]** slider to set the utilizable laser intensity (recommendation: start at 50 %).
  - The transmittance of the Acousto-Optical Tunable Filter (AOTF) changes accordingly.
- This allows you to adapt the laser intensity very sensitively to the job. Activate the check box for **Line Active**.
  - This activates the selected laser power for use. This is indicated by the **Laser Power** displaying lamps (status display green / gray).

☞ By clicking on the **Excitation** button you can check at any time which lasers are available for active operation.
If you deactivate **Line Active**, the laser wavelengths are deselected by means of the Acousto-Optical Tunable Filters (AOTF), i.e. these lasers change into standby status.
If you interrupt your work with the LSM for a break, it is recommended not to switch the Enterprise and argon lasers off by hardware action, but to put them into standby status as described.
Excitation filters, emission filters, HFT main dichroic beam splitters and NFT secondary dichroic beam splitters can be switched online, channels (PMT photomultipliers) only off-line.

(3) Beam path - HFT main (dichroic) beam splitter

- On the **Beam Path and Channel Assignment** panel, click on the HFT main dichroic beam splitters .
  - This opens a graphical pop-up window of all beam splitters available.
- To select a beam splitter, click on the respective line of the list.
  - The selected beam splitter moves into the beam path.

(4) Beam path - META detector settings

On the Beam Path and Channel Assignment panel use the two sliders to define the spectral detection range of the META module. The current position of the slider is displayed in a white box when keeping the left mouse button pressed.

Both slider values are updated in the **Start** and **End** Input box.

The wavelength values can also be typed directly into the Start and End input boxes. The position of the sliders are updated after the next mouse click. The **Number of Passes** displays the number of successive scans to be performed in order to cover the required spectral range.

Step check box: Step size can be increased to 21.4 nm, 32.10 nm or 42.80 nm by binning of 2, 4 or 8 adjacent channels, respectively.

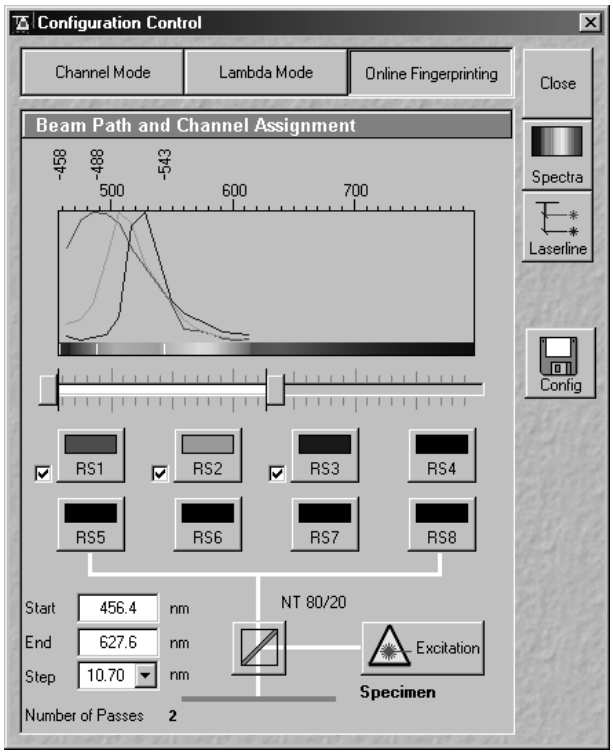


Fig. 4-48 Configuration Control window; Online Fingerprinting Mode activated

4.5.3.9 Settings in the Online Fingerprinting Mode

The use of this function permits the selection of reference spectra together with the excitation settings, to allow an immediate display of the unmixed images while scanning.

- Click on the **Config** button in the **Acquire** subordinate toolbar of the main menu.
  - The **Configuration Control** window opens.
- Click on the **Online Fingerprinting** button.
  - The **Beam Path and Channel Assignment** panel for the **Online Fingerprinting** mode is opened.

Beam Path configuration

Activation / deactivation of the excitation wavelengths (check box) and setting of excitation intensities (slider). Open the **Laser Control** window via the **Laser** button. The active laser lines are automatically displayed in the wavelength color bar.

Selection of the main dichroic beam splitter (HFT) through selection from

Control buttons for reference spectra

RS1 ... 8 Menu for selecting a display color and a reference spectra (reference spectra derive from earlier experiments via mean of ROI or ACE tools); selected spectra and colors appear in the wavelength chart. Unmixed results will be displayed during scanning, lambda stack will neither be displayed nor stored.

META detector slider Definition of number of PMTs out of the 32 PMT Detectors to fit the required spectral range. To optimize acquisition speed, a range detected in 1 or 2 passes is recommended.

Start box Start value of required spectral range.

End box End value of required spectral range.

Step box Step width in spectral range (10.70 recommended).

4.5.3.10 Non Descanned Panel

☞ The functions of the **Non Descanned** panel are described in CHAPTER ANNEX of the printed manual.

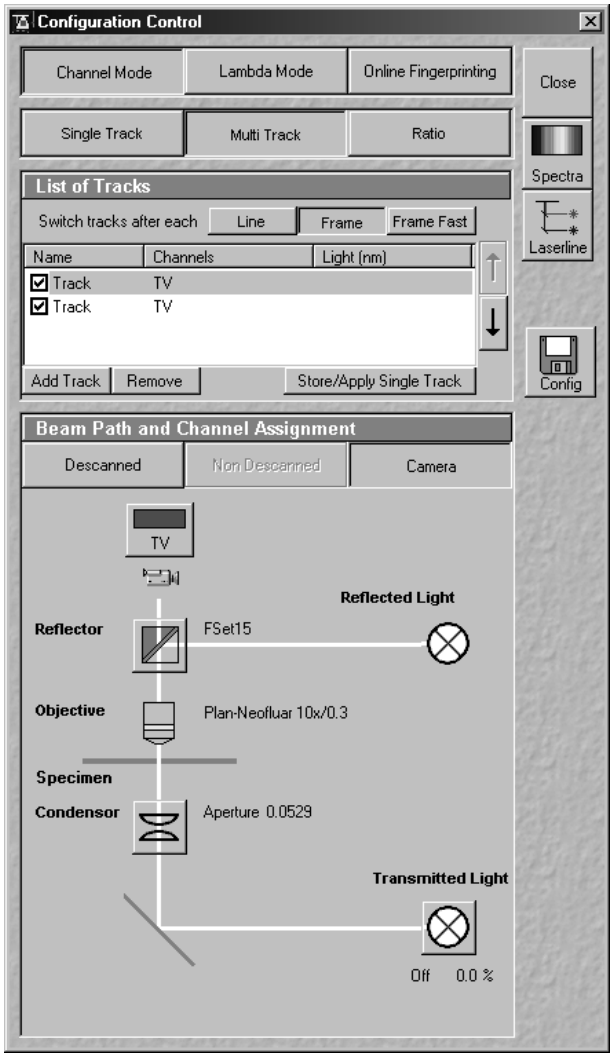


Fig. 4-49 Configuration Control window; camera detecting activated

4.5.3.11 Camera Detection Panel

The use of this function permits the use of a Zeiss AxioCam (HRm, HRc MRm) camera as an alternative external detector.

- Click on the **Config** button in the **Acquire** subordinate toolbar of the main menu.
  - The **Configuration Control** window opens.
- Activate one of the **Single Track** or **Multi Track** buttons and click on the **Camera** button.
  - The **Beam Path and Channel Assignment** panel for camera detection is opened.

Control buttons

TV Menu for selecting a display color for the camera image.

Reflector Selects a beam splitter for the excitation/emission.

Add Track Adds a second track to the acquisition in **Multi Track** mode, e.g. a different fluorescence filter cube or transmitted light.

☞ If TV and LSM tracks are mixed, the active detection port of the microscope has to be set according to the first track.

4.5.4 Scan Control

The scan parameters for image acquisition are set in the **Scan Control** window.

The microscope must be in the LSM mode, i.e. the **LSM** button on the tube of the microscope stand must be pushed or the **LSM** button in the **Acquire** subordinate toolbar must be activated to set the LSM mode.

The scanning actions are started via the buttons on the right-hand side of the **Scan Control** window, and the scan parameters are set in the main part of the window.

An acquired image is displayed in a separate **Image Display** window. If an **Image Display** window is not yet available, a new **Image Display** window is automatically opened during the acquisition.

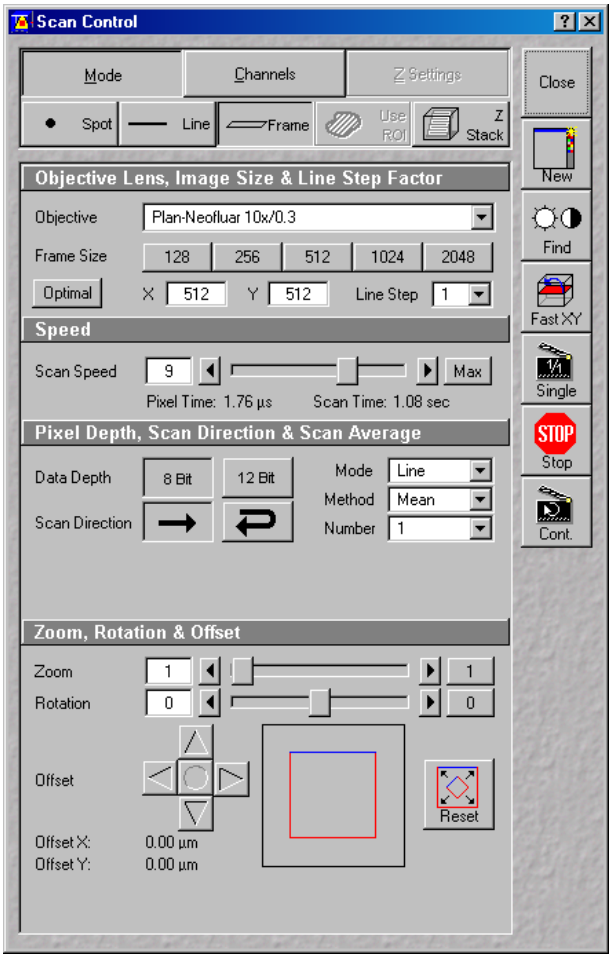


Fig. 4-50 Scan Control window

The following scanning modes can be performed:

Spot

- scanning of a spot (Spot + Time Series)

Line

- scanning of a line in the XY-plane (Line, Line + Time Series)
- scanning of a line with different Z-values (Line + Z Stack, Line + Z Stack + Time Series)

Frame

- scanning of an XY frame (Frame, Frame + Time Series)
- scanning of XY frames with different Z-values (Frame + Z Stack, Frame + Z Stack + Time Series)
- scanning of XY frames in defined ROIs (Frame + Use ROI + Time Series)
- scanning of XY frames with different Z-values in defined ROIs (Frame + Z Stack + Use ROI + Time Series)

4.5.4.1 Open / Close the Scan Control Window

- Click on the **Scan** button in the **Acquire** subordinate toolbar of the **Main** menu.
  - This opens the **Scan Control** window, which shows all lasers connected to the system.
- Click on the **Close** button to quit the **Scan Control** window.

The following main function buttons are available in the **Scan Control** window:

Generally available buttons

Mode button	When the button is activated, the following panels are available for the setting of the scanning parameters for the line and frame modes: **Objective Lens, Image Size & Line Step Factor**, **Speed**, **Pixel Depth, Scan Direction & Scan Average** and **Zoom, Rotation & Offset**.
Channels button	When the button is activated, the **Channel Settings** and **Excitation of Track ...** panels are available for the setting of the channels and the laser excitation.
Spot button	Activates the **Spot** scan mode.
Line button	Activates the **Line** scan mode.
Frame button	Activates the **Frame** scan mode.
Use ROI button	Activates the scanning procedure only within a **ROI** (region of interest) to be defined first.
Z Stack button	Activates the **Z Stack** scan mode, display of additional buttons on the right-hand side of the **Scan Control** window.
Z Settings button	When the button is activated, the **Z Settings** panel is available for the Z-scan parameter definition. The **Z Stack** scan mode must be active.
Close button	Closes the **Scan Control** window.
New button	Opens a new **Image Display** window.
Find button	Automatic optimization of image brightness and contrast. The settings for the **Find** function can be varied as required using the **Maintain** menu, **Set Find** (see page 4-242).
Fast XY button	Continuous scan with high speed. This function should be used to a limited extent and only for a short period of time. Fast XY switches temporarily to 512 x 512 frame size.
Single button	Single scan (named **Start** in the **Z Stack** mode).
Stop button	Stops the current scan procedure, no matter in which window the button is pressed (also see the **Time Series Control** and **Bleach Control** windows).
Cont. button / **Finish** button	Continuous scan (not available in **Spot Scan** mode and **Z Stack** mode). If you select the option **Frame** for **Mode** and the option **Continuous** for **Number** in the **Pixel Depth, Scan Direction & Scan Average** panel, the **Finish** button is displayed instead of the **Cont.** button. In this case, continuous averaging is performed when you have started the scan. If you click on the **Finish** button, the scan/averaging process is stopped after the scan of the current image has been completed.

Additional button in the Spot mode

Spot Sel button — Scans one image and displays a cross hair which can be freely positioned within the image window to define the spot for imaging.

Additional button in the Line mode

Line Sel button — Automatically defines a line in the center of the Image Display window (Frame) for creation of the intensity profile; using the mouse, the line for the intensity profile can then be positioned anywhere in the Image Display window.

Additional buttons in the Z Stack mode

Start button — Triggers the scan of a stack.

XYscan button — Triggers a single XY-scan

XYcont button — Triggers continuous XY-scan.

Line Sel button — To prepare the **Range** function, a cutline is created in the scanned XY-frame to determine the position at which the XZ-scan through the specimen is to be produced. Using the mouse, the line for the XZ-scan can be positioned anywhere in the scan frame. The cutline can be defined either as a straight line or free shape curve.

Range button — Produces an XZ-scan through the specimen within the limits determined in **Num Slices** and **Interval;** the cutline is determined via the **Line Sel** function.

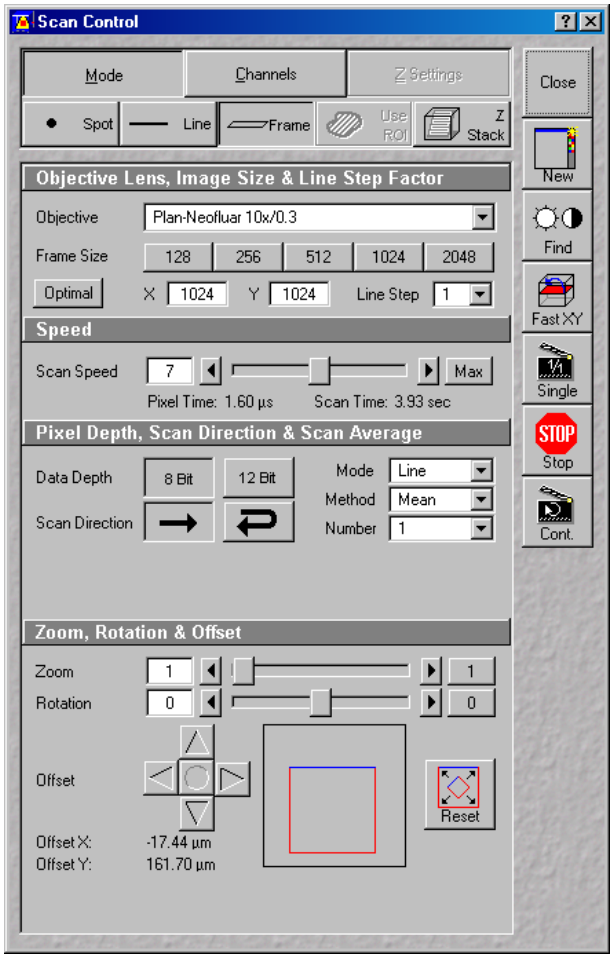


Fig. 4-51 **Scan Control window - Mode/Frame**

4.5.4.2 Frame

When the **Frame** button is activated, a frame of variable size is scanned pixel by pixel and line by line. The laser beam is moved over the specimen line by line.

The scan parameters and the channels (single detector, META detector) are set via the **Mode** and **Channels** buttons, and the laser settings can be checked again or changed.

(1) Mode

When the **Mode** button is activated, the **Objective Lens, Image Size & Line Step Factor**, **Speed**, **Pixel Depth, Scan Direction & Scan Average** and **Zoom, Rotation & Offset** panels are displayed in the **Scan Control** window.

Objective Lens, Image Size & Line Step Factor panel

- Open the **Objective** list box and select the objective to be used via a click of the mouse (identical to Microscope Control). When using immersion oil objectives, make sure to perform immersion as required.
- Select the **Frame Size** from the default sizes via the buttons **128**, **256**, **512**, **1024**, **2048,** or enter the required values via the keyboard. Recommended setting to start with: 512 x 512 pixels.

– It is also possible to enter different values for X and Y. The value for **Y** is freely selectable between 1 and 2048 pixels (integers). The value for **X** must always be an integral multiple of 4. The maximum value for **X** is also 2048 pixels.

– The Optimal button

sets the image resolution to an optimal value corresponding to the optical magnification (objective) the zoom and the emission range detected. This provides an image where no information is lost as optimal sampling is achieved.

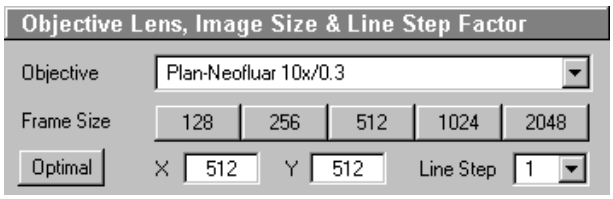


Fig. 4-52 **Objective Lens, Image Size & Line Step Factor panel**

- Select the Line Step size between 1 and 10. Only every n-th line is scanned. The lines in between are interpolated. This fast scan mode is called Step Scan.

Speed panel

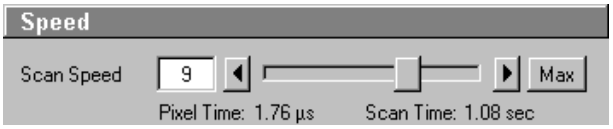


Fig. 4-53 Speed panel

- Select the **Scan Speed** from the 13 preset steps via slider or input box. Recommended: 7 for the first scan. A click on the **Max** button sets the maximum speed for the current zoom.
  - The Scan Speed determines the Pixel Time. In the case of different image formats, the Pixel Time is constant for the same Scan Speed, but the Scan Time is different.
  - Pixel Time dwell time of the laser beam on the pixel
  - Scan Time duration of the acquisition for the entire frame

☞ Fast XY only for fast image acquisition during parameter setup.
Pixel time and scan time will be shown. Fast XY = speeds 8 – 13 (depending on zoom), average = 1, max. resolution: 512 x 512 pixels.

Speed:	1 ... 8	9	10	11	12	13
Zoom:	0.7	1.0	1.4	2.5	3.6	5.7

Pixel Depth, Scan Direction & Scan Average panel

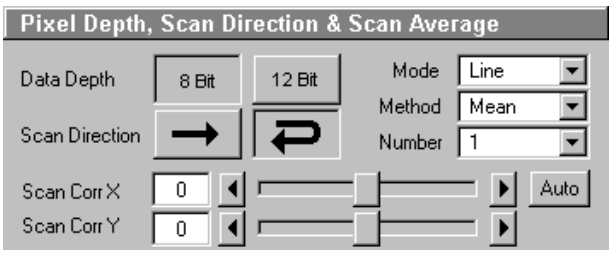


Fig. 4-54 Pixel Depth, Scan Direction & Scan Average panel

- Select **8 Bit** or **12 Bit** Data Depth, i.e. 256 or 4096 gray values.
- Select the **Unidirectional** or **Bi-directional** Scan Direction.
  - → Unidirectional: The laser scans in one direction only, then moves back with beam blanked and scans the next line.
  - ⮌ Bi-directional: The laser also scans when moving backwards, i.e. the Scan Time is halved.
  - The pixel shift between forward and backward movement (double image) resulting from bi-directional scanning must be corrected via the **Scan Corr X** and **Y** sliders. Zero° rotation requires correction in the X-direction, 90° rotation must be corrected in the Y-direction. If the image was rotated, correction is required in both coordinates. Correction is performed on-line in the continuous scan mode (**Cont.** button). The size of the shift depends on the Scan Speed. For automatic scan correction, click on the **Auto** button.
- Select the **Line** or **Frame** mode for averaging.
- Select the desired scan average method **Mean** or **Sum** in the **Method** selection box.
- Select the desired scan average from the available values **2**, **4**, **8** and **16** in the **Number** selection box or **Continues** (only for **Frame** average mode).

☞ The greater the number of averages selected for **Mean** average **Method**, the better the image quality will be; the scanning time will be prolonged accordingly.

Averaging can be performed in different ways, depending on whether the **Mean** or **Sum** method has been activated.

If you are using the **Mean** method, the image information is generated by adding up all scans pixel by pixel and then calculating the mean value.

In the **Sum** method, the pixel values of all scans are only added up, without a mean value being calculated.

To create the image information using the **Line** average mode, each line (depending on the setting) is scanned 2, 4, 8 or 16 times during Scan Average, and then the average value per pixel is calculated. This minimizes noise interference during the scanning procedure.

If the **Frame** average mode is used to create the image information, the complete frame is scanned 2, 4, 8 or 16 times, depending on the setting. The average value is recalculated after each frame scan.

The **Frame** average mode also permits continuous averaging.

For this, select the **Continuous** option in the **Number** selection box.

If you have selected the **Continuous** option, the **Finish** button for ending continuous averaging is displayed instead of the **Cont.** button. Use the **Single** button in this case to start continuous scanning. When you click on the **Finish** button, the scan currently in progress will be completed before the process is stopped.

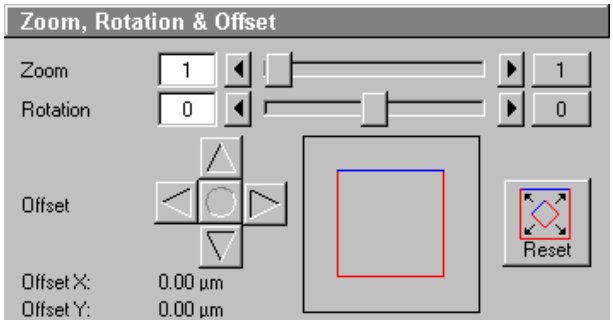


Fig. 4-55 **Zoom, Rotation & Offset panel**

Zoom, Rotation & Offset panel

In this panel, the scan range is set for zoom, rotation and offset in relation to the field of view of the microscope. The diagonals of the outer square on the right-hand side correspond to the field of view of the microscope.

The inner square contained in it (rectangle in the case of differently set frame size) represents the scan range and immediately shows the changes made to zoom, rotation and offset.

The blue line at the top of the scan range is helpful for orientation when the scan range is rotated in the direction of the field of view.

- Set the desired zoom factor via the slider (**Zoom**) or by clicking on the arrow buttons.
  - The zoom factor can be set continuously in the range from 0.7 to the maximum of 40, and is displayed in the relevant input box. The value 0.7 corresponds to factor 1, and value 40 to factor 52, related to the field of view. From zoom factor 5.6, the magnification will be empty, and the zoom factors will be displayed in red in that case. Clicking on button **1** enables immediate resetting to the zoom factor 1.
  - Recommended setting to start with: Zoom 1.
- To rotate the scan area, use the slider (**Rotation**) or click on the arrow buttons.
  - Clicking on button **0** enables immediate resetting to 0°.
  - Recommended setting to start with: Rotation 0°.
- Move the scan area by clicking on the 4 arrow buttons (**Offset**).
  - The offset of the scan area from the center of the field of view is displayed online in µm for X and Y.

– A click on the center button will recenter the scan area to the field of view.
– Clicking, holding and drawing the rectangle with the mouse permits the scan area to be moved directly within the field of view.
– Recommended setting to start with: Offset X = 0, Y = 0

☞ During the scan procedure, the functions **Objective change**, **Speed**, **Scan Corr**, **Zoom**, **Rotation** and **Offset** can be influenced online.

By clicking on the **Reset** button the scan zoom is set to 1 and the XY offsets are set to the zero position and the ratio angle is set to 0°.

(2) Channels

If the **Channels** button is activated, the **Channel Settings** and **Excitation of Track ...** panels are displayed in the **Scan Control** window.

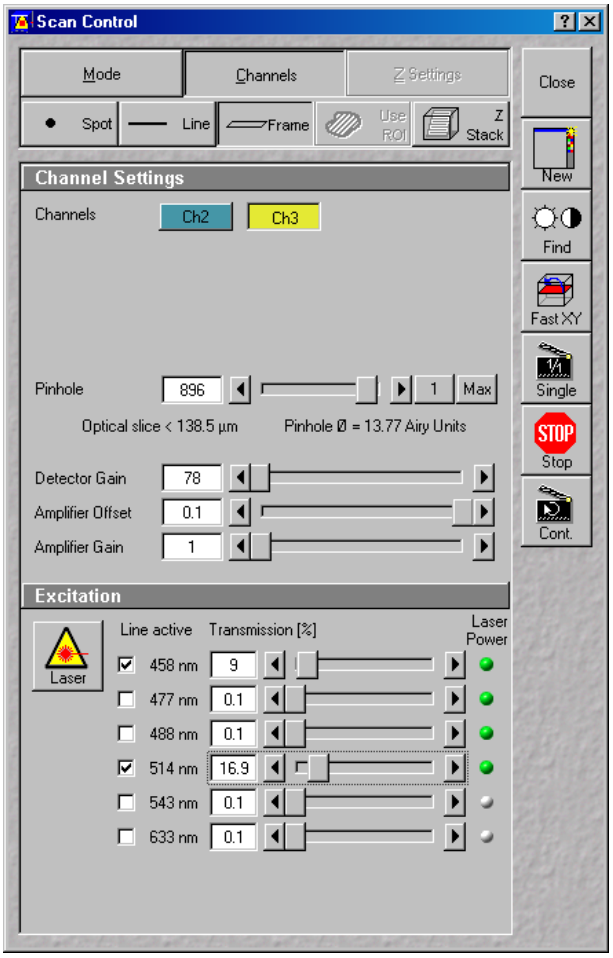


Fig. 4-56 Scan Control window – Channels

Channel Settings panel

In the **Channel Settings** panel, the channels (incl. META channels if present and ratio channels) defined in the **Configuration Control** window are listed track by track as selectable buttons.

Depending on the selected **Channels b**utton (e.g. **ChS1-T1**), the currently used settings of Pinhole, Detector Gain, Amplifier Offset and Amplifier Gain are displayed.

- The slider near **Pinhole** enables you to change the pinhole diameter of the relevant channel.
  – The pinhole diameter is indicated in **µm**, **Optical Slice** and **Airy Units**. The Airy value depends on the aperture of the objective, excitations and the emission wavelength.
  – A small pinhole diameter will increase the depth of focus, but reduce the light intensity received by the PMT photomultiplier.
  – When you vary the pinhole diameter, an Optical Slice value is displayed. For optimum depth resolution, Airy values should be small, but in fluorescence applications not below 1.0 to keep the intensity loss within a reasonable limit.
  – A click on the **1** button sets the pinhole to a diameter of 1 Airy unit. A click on the **Max** button sets the pinhole diameter to the maximum.

- The sliders (and the relevant arrow buttons) near **Detector Gain**, **Ampl. Offset** and **Ampl. Gain** enable you to set the photomultiplier of the selected channel during continuous scanning.
  - Detector Gain: Setting of the high voltage of the PMT photomultiplier - setting of image contrast and brightness (values available between 80 and 1250)
  - Amplifier Offset: Setting of the electronic offset - background of the image can be set (values available between -2 and 0.1)
  - Amplifier Gain: Amplification factor (values available between 1 and 3)

☞ The parameters **Detector Gain**, **Ampl. Offset** and **Ampl. Gain** are described in section **Pinhole / Detector Gain / Ampl. Offset / Ampl. Gain** (see page 4-349) in the context of image optimization. In case the **Lambda Mode** has been chosen in the **Configuration Control** window only the META channel **ChS** is displayed in the **Channel** settings of the **Scan Control** window.

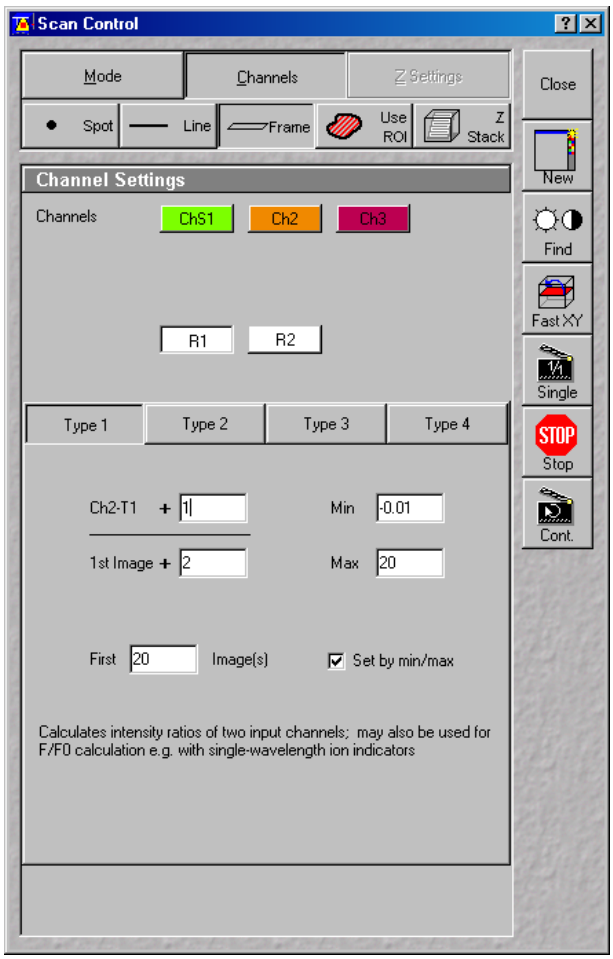


Fig. 4-57 **Channel Settings panel of a Ratio Channel**

The parameters of a ratio channel are set in a separate dialog box.

- Click on the button of a ratio channel (e.g. **R1**). The dialog box for the setting of the ratio parameters is displayed.

Clicking on the required tabs enables you to choose from four formulas (**Type 1** to **4**) for ratio calculation. The relevant decimal values can be entered in the input boxes via the keyboard. The entered values remain unchanged even after switchover to another formula and can be reactivated any time.

The formula type activated last is always used for ratio formation during the scan procedure. If the input box does not contain any value at all or no suitable value, the useful value last used will be activated.

The ratio channels are displayed in the **Image Display** window (see Fig. 4-60).

- Select the required formula and enter the relevant values.

Letters can be entered into the formula fields which will be valued as 1; it is also possible to make no entry, which will also be valued as 1, but will not be displayed.

Set by min/max (in Scan Control window - **Channels** mode) allows the definition of the display scaling according to the expected minimal and maximal values.

Excitation panel

- In the **Excitation** panel you can select other lasers and vary laser intensities (in the same way as in the **Laser Control** or **Configuration Control** window) and you can program the AOTF for different laser lines.

If more than 8 laser lines are available the AOTF can be programmed for various lines. By clicking on the **Laserline** button the **Wavelength Switch Control** window opens.

- Select the required laser lines in the selection boxes and confirm the selection with a click on **Store**.
- Click on the **Close** button to close the **Wavelength Switch Control** window.

☞ All parameters under Channels can be varied online.

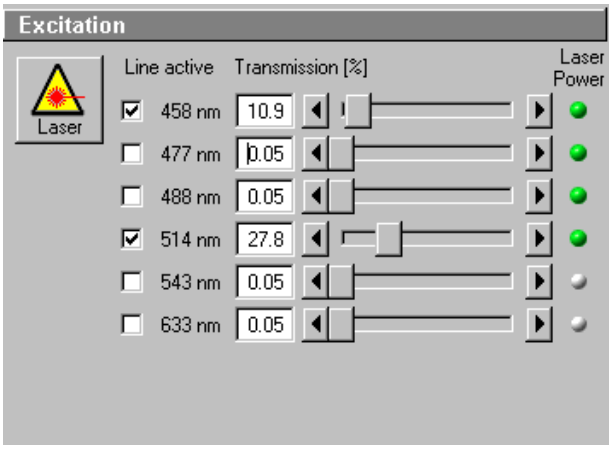


Fig. 4-58 Excitation of Track ... panel

Acquisition of a frame

Once you have set up your parameter as defined in the above section, you can acquire a frame image of your specimen.

- Click on the **Single** button in the **Scan Control** window. The system will automatically start the acquisition of a frame. The individual channels and the overlay image can be viewed by changing to the **Split xy** mode. This button is located on the right-hand side of the **Image Display** window.

The following scan image shows the result with two defined tracks plus the **Ratio** channel and the overlay (see Fig. 4-60). See the appropriate **Channel Settings** panel in the **Scan Control** window (Fig. 4-59).

☞ A similar array of images is displayed when choosing Online Fingerprinting Mode with five reference spectra used for online unmixing and one ratio channel being defined.

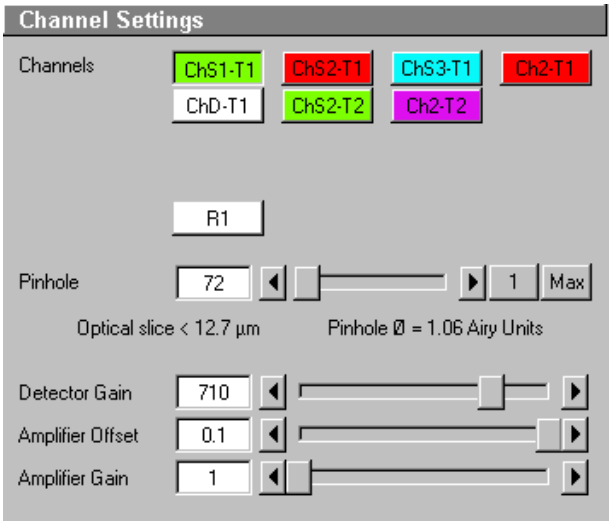


Fig. 4-59 Channel Settings panel for two defined tracks plus Ratio channel

In case the **Lambda Mode** has been chosen in the **Configuration Control** window, the following scan image shows the result of the Lambda Stack.

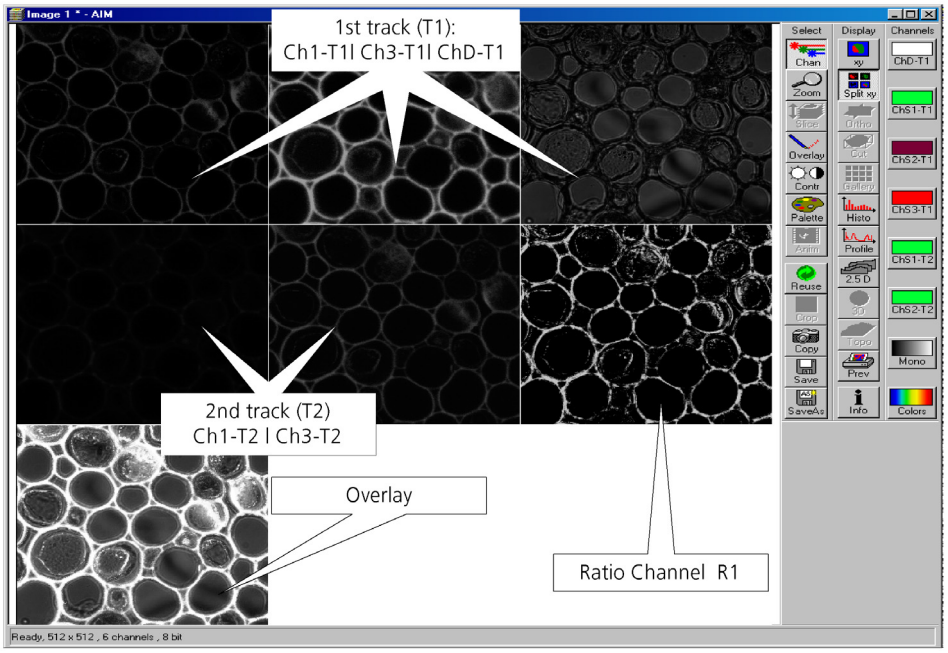


Fig. 4-60 **Image Display window with two tracks plus ratio track (Split xy mode)**

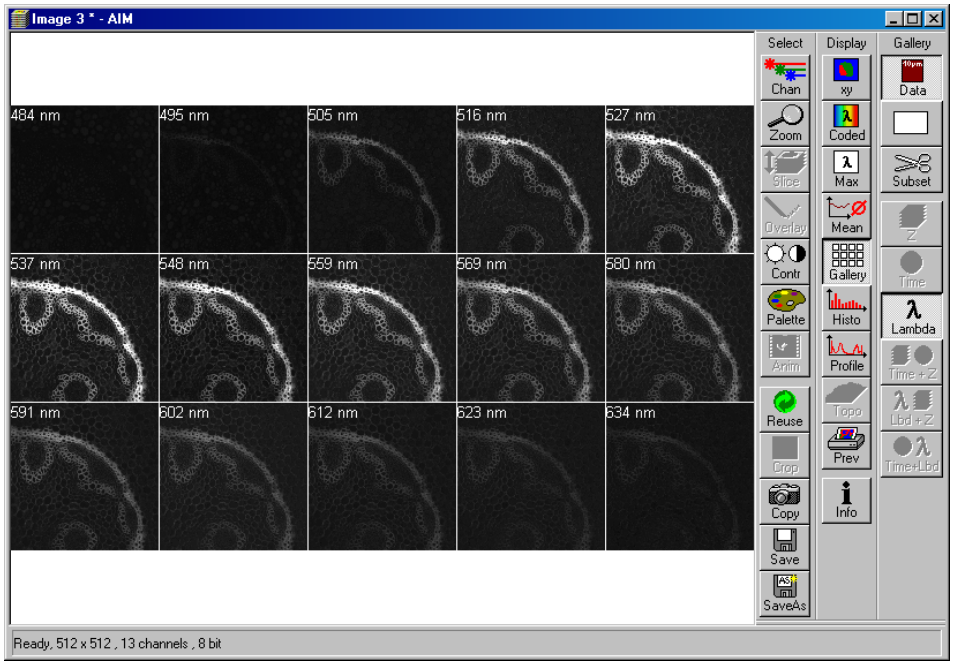


Fig. 4-61 **Image Display window with a Lambda Stack**

(3) Z Stack

This function permits a series of XY-images to be produced in different focus positions (Z slices).

When the **Z Stack** button is pressed, the **Z Settings** button is automatically activated and the **Z Settings** panel is displayed in the **Scan Control** window. However, it is possible at all times to switch over to setting / changing the scan parameters or the PMT photomultipliers and lasers via the **Channels** and **Mode** buttons.

The additional **XYscan**, **XYcont**, **Line Sel and Range buttons are available on the right-hand side of the Scan Control** window, and the labeling of the **Single** button changes to **Start**.

The **Z Stack** function is deactivated by clicking again on the **Z Stack** button.

***Z Settings** panel - overview*

The parameters of the Z Stack to be created are defined and displayed online in the **Z Settings** panel.

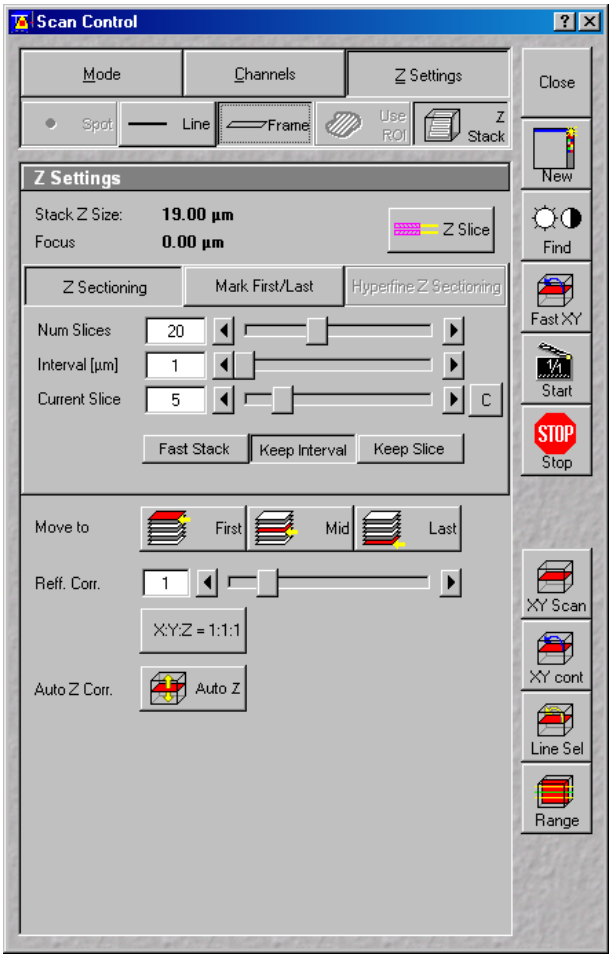


Fig. 4-62 Scan Control window - Z Settings

Stack Z Size: The dimension of the Z Stack in µm. The stage (nosepiece) is moved in such a way that the stack size, dependent on the refractive index, is achieved optically.

Focus Position: The current Z position. If the refractive index (Refr. Corr.) changes, the value of the focus position in relation to the "0" also changes (online).

Z Slice: Opens the **Optical Slice** window (Fig. 4-63). The **Optical Slice** window contains three buttons (**Optimal Interval: ... µm**, **Optimal Pinhole Diameter and Undo**) to allow the setting of the optimal interval and the optimal pinhole diameter of fluorescence stacks. Both values influence each other and depend on the objective used.

In the case of a fixed pinhole diameter, half the value of the smallest pinhole diameter used is taken to determine the optimum interval. Accordingly, the pinhole diameter to be used in the case of a preset interval is determined by doubling the value of the selected interval. Undo resets the just before altered values.

The **Optical Slice** window displays the following information:

Black: Stack Z Size (µm) = intervals x (number of slices - 1)

Optimal Interval = depending on the objective used and the pinhole diameter setting

Red and other colors: Presentation of the actual data set by the operator helps to optimize stack creation.

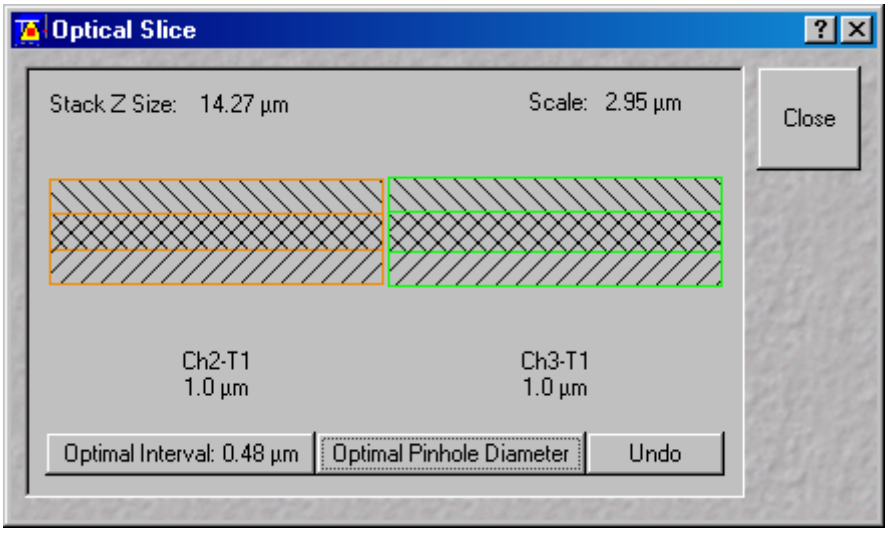


Fig. 4-63 Optical Slice window

Tabs

Z Sectioning: Tab for setting of **Number of Slices**, **Interval** and **Current Slice** via slider / arrow button.

Mark First/Last: Tab for determination of the Z-value for the first and last XY-image of the stack, combined with manual focusing or **Stage control.**

Hyperfine Z Sectioning: Tab for production of a Z Stack using the optional Piezo focus for objectives.

First: Scanning / Display of the beginning (first XY-image) of the stack.

Mid: Scanning / Display of the center (XY-image in the center) of the stack.

Last: Scanning / Display of the end (last XY-image) of the stack.

Refr. Corr.: Considers the different refractive index between the immersion medium of the objective (n') and the embedding medium of the specimen (n), which can be set between 0.5 and 3 via the slider / arrow buttons

$$\text{Ratio} = \frac{n}{n'}$$

X:Y:Z=1:1:1 Clicking on this button will set the Z-interval in such a way that the voxel has identical dimensions in the X-,Y- and Z-directions (cube).

Auto Z Corr. This function permits the set values of the scan parameters **Detector Gain**, **AOTF**, **Ampl. Offset** and **Ampl. Gain** (as measure for the brightness level) to be varied between two freely selectable slices of a stack to be recorded. During the scan procedure, the interim values of these three parameters are automatically linearly interpolated between the initial and end values (see page 4-88).

The parameters of a Z Stack can be defined using the **Z Sectioning** tab, the **Mark First/Last** tab or - if the optional Piezo focus for objectives is connected - the **Hyperfine Z Sectioning** tab.

Z Sectioning tab

Num Slices: Entry of the number of sections (single XY-images) to be recorded with the stack via the slider / arrow buttons. The entry does not influence the interval.

Interval: Entry of the step width (Z-distance between the single XY-images) via slider / arrow buttons. The entry has no influence on **Num Slices**.

Current Slice: Display of the current position of the slice within the stack. Change of position via slider / arrow keys. Reset of the current slice position in the center of the stack by clicking on the **C** button. Of course, the borders of the stack are also changed if the current slice position is changed.

Keep Interval: The interval remains constant when the stack limits or number of slices are changed.

Keep Slices: The number of slices remains constant when the stack limits or interval are changed.

☞ The Stack Z Size is slightly adjusted to hold the number of slices with no interval changes or the interval size if the number of slices is varied.

Fast Z Line: Not available for frame mode. Fast Z scan for overviews (only for **Line** scan mode). The stack size is retained; the interval is adapted depending on the scan speed.

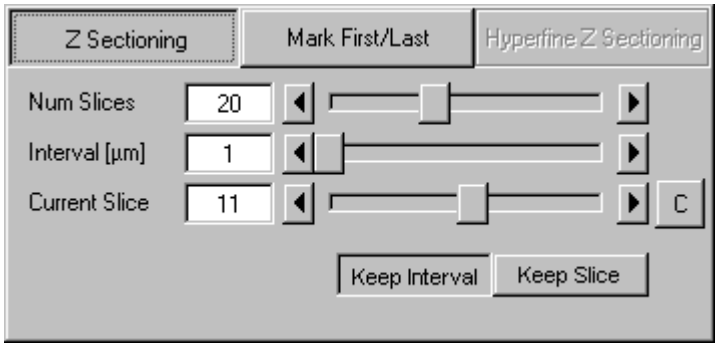


Fig. 4-64 Scan Control window - Z Sectioning tab activated

Determining the optimum stack size with the help of the Line Sel and Range function

- Click on the **Line Sel** button.
  - An XY-scan of the current slice is performed. The cutline is displayed in the image center. The **Line** toolbar is displayed on the right-hand side of the **Image Display** window.

The **Line** toolbar permits you to define the position, shape, width and color of the cutline in the **Image Display** window.

The following function buttons are available:

Arrow selection button: Activates the mouse pointer for the selection and positioning of the cutline in the **Image Display** window and for changing its length.

Length change: Click on the drag point and keep the mouse button pressed. Drag the point and release the mouse button.

Shifting: Click on the line and keep the mouse button pressed. Shift the complete line and release the mouse button.

Line arrow button: Generation of a straight cutline in any direction in the **Image Display** window.

Opened free shape curve button: Generation of an open, free shape curve (spline) in the **Image Display** window. The first click sets the starting point, each further click adds a line segment. A click with the right mouse button ends the process.

Line button: Selecting the line width of the cutline.

Color button: Selecting the color of the cutline.

- Click on the **Line arrow** button or the **Opened free shape curve** button in the **Line** toolbar.
- Define a straight line or a free shape curve (spline) as the cutline for the XZ scan.

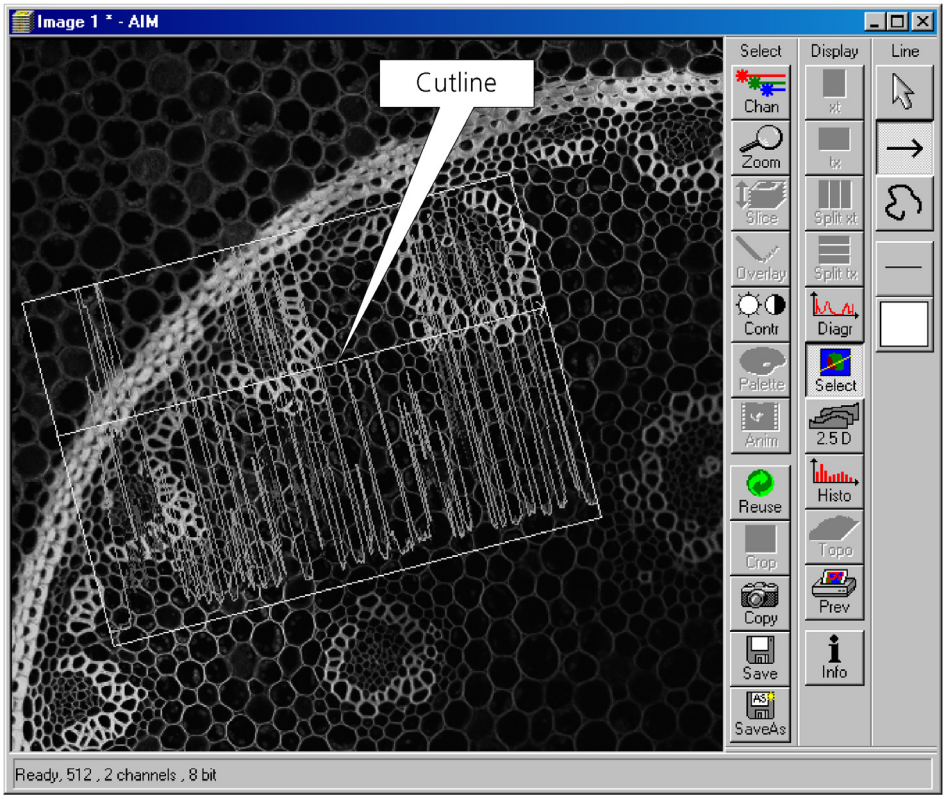


Fig. 4-65 Image Display window with cutline displayed

- Then click on the **Range** button.
  - The XZ-scan will be performed and displayed in the **Image Display** window. At the same time, the position of the current slice is shown with a green line and the positions of the first and last slice with two red lines.

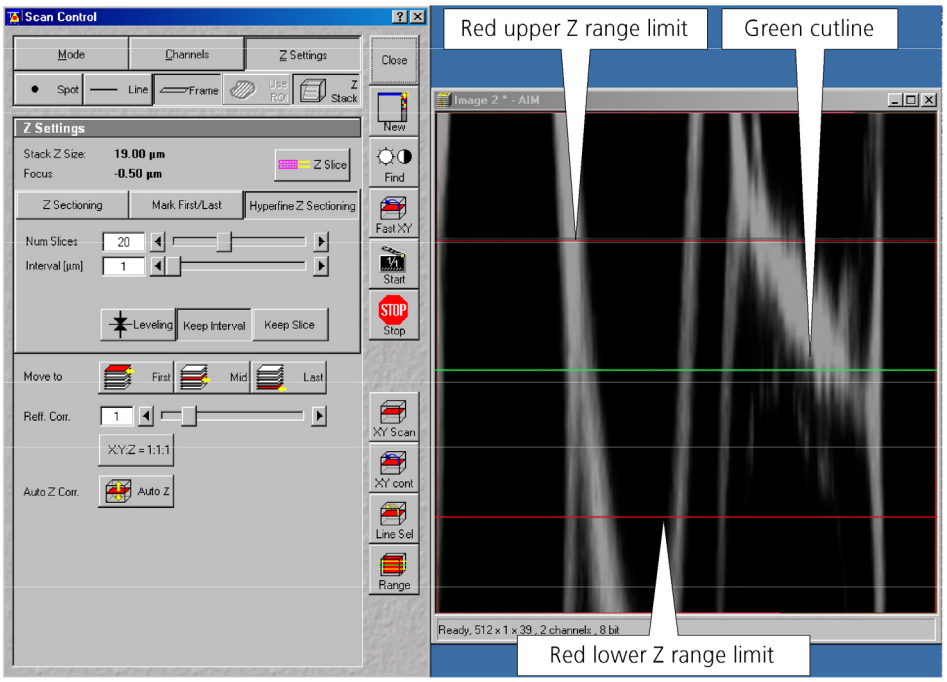


Fig. 4-66 Scan Control window and Image Display window

- Moving the green line (current slice) enables you to change the current focus position (moving the stage or nosepiece in the process). The stack limits are also changed, while interval and Num Slice remain unchanged.
- Shifting one of the red lines enables you to change the stack size; in that case, the interval size is matched, and the Num Slice remains constant.
  - Changing the values of **Num Slice**, **Interval** and **Current Slice** in the **Z Sectioning** tab will, of course, also change the positions of the red and green lines in the **Image Display** window.
- A click on the **Start** button will start the recording of the Z Stack.
  - The settings of the entire **Scan Control** window (**Mode**, **Channels, Z settings**) will be used when the stack is produced.

☞ In Frame mode the stack is acquired as a stack of images in xyz. In Line mode the stack is displayed by a xz image. Continuous imaging of the xz stack is also possible.

Mark First/Last *tab*

The determination of the optimum stack size is performed here via focusing during a continuous scan.

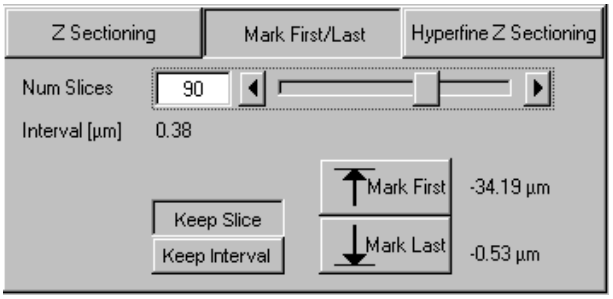


Fig. 4-67 Mark First/Last tab

- Click on the **XYcont** button.
  - A continuous XY-scan of the set focus position will be performed.
  - If you have reduced the scan speed or have set image averaging, you should use the fast scanning mode to find the lowest and highest points of focus. These settings are made under **Mode** in the **Scan Control** menu, or directly via the **FAST XY** button.
- Use the manual focusing drive or the **Stage and Focus Control** window (see **Stage**, page 4-122) to focus on the upper position of the specimen area where the Z Stack is to start.
- Click on the **Mark First** button to set the upper position of the Z Stack.
- Then focus on the lower specimen area where the recording of the Z Stack is to end.
- Click on the **Mark Last** button to set this lower position.
- **Keep Slice** enables the **Num Slices** slider which allows to vary the number of slices. The limits of the Z Stack remain constant, the interval is matched accordingly.
- **Keep Interval** enables the **Interval [µm]** slider which allows to vary the interval between the slices. The number of slices is matched accordingly, the limit of the Z Stack is adjusted.
- Click on the **Start** button to start the recording of the Z Stack.

In case the upper and lower limits of the stack have been switched round, automatic matching will be performed by the software, since the stage of the Axio Imager.Z1 always moves from bottom to top and the nosepiece of the Axiovert 200 M always moves from top to bottom.

☞ Setting via **Range** is not possible via the **Mark First/Last** function, i.e. the lines cannot be shifted.
The **Fast Z Line** functions is not available in frame mode.
When you change from **Mark First/Last** to **Z Sectioning** or vice versa, the values are updated in the **Z Sectioning** tab.

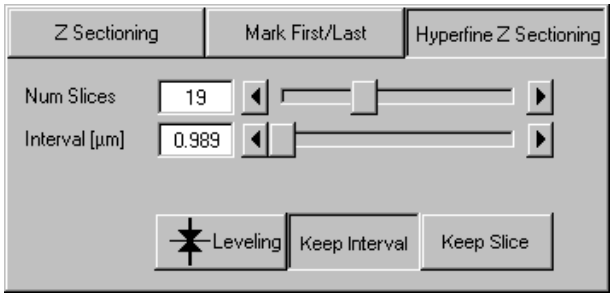


Fig. 4-68 Hyperfine Z Sectioning tab

Hyperfine Z Sectioning *tab*

Activation of this tab is only possible if the piezo objective focusing device has been connected.

The piezo objective focusing device can be controlled via software (see **Stage**, page 4-122).

The accuracy of the piezo objective focusing device regarding the step width in the Z-direction lies in the range of 60 nm.

The piezo objective focusing device allows stacks to be produced considerably quicker than via the focus of the microscope stand.

The focus position remains unchanged.

- Clicking on the **Leveling** button moves the piezo objective focus to the zero position, while the motor focus moves into the opposite direction at the same time, i.e. the position of the object in relation to the objective remains unchanged. This function is used to set defined initial conditions.
- Use the slider or the arrow keys to set the number of slices for the Z Stack.
- Use the slider or the arrow keys to set the size of the interval.

Num Slices and **Interval** can be varied independently of each other within the piezo objective focus work range of ±250 µm. When change is made to **Z Sectioning,** or vice versa, values are also taken over, provided they are within the piezo objective focus work range.

If a larger range is set for the Z Stack under **Z Sectioning** or **Mark First/Last**, the **Interval** is matched accordingly when changing to **Hyperfine Z Sectioning**, while **Num Slice** remains constant.

- Use **XYcont**, **Line Sel** and **Range** to determine the parameters of the Z Stack (identical to Z Sectioning).

If the green line (Current Slice) is shifted after the creation of **Range**, the focus position will change (the piezo objective focusing device remains in the center position). The red lines (stack limits) can only be changed symmetrically to the Current-Slice position within the piezo objective focusing device work range.

☞ Since the piezo objective focusing device moves from bottom to top during the creation of the Z Stack, top and bottom of the Axiovert 200 M have been switched round.

Auto Z Corr.

The function **Auto Z Correction** allows a linear variation of Detector Gain, Ampl. Offset, and Ampl. Gain values between the different slices of a stack.

- Click on the **Auto Z** button, the **Auto Z Brightness Correction** panel opens.

The buttons **Set A** and **Set B** permit definition of two distinct gain / offset / AOTF settings at two different Z positions A and B.

Pressing the **Move A** and Move **B** buttons permits the defined Z-position to be directly approached.

The **Enable test** check box permits simulation of the value changes for **Detector Gain**, **Ampl. Offset**, **Ampl. Gain** and **Attenuation** in the **Scan Control** window without the scanners being in operation.

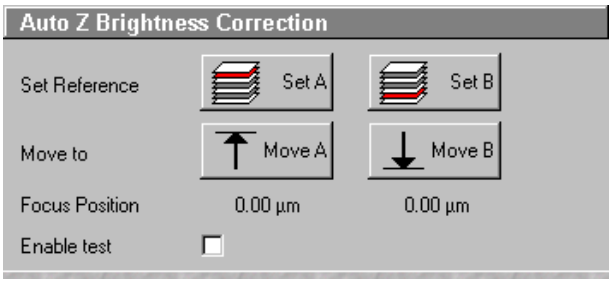


Fig. 4-69 Z Brightness level control panel

☞ If a Z Stack is performed and the **Auto Z Brightness Correction** window is opened this correction is automatically performed equal whether the **Enable test** box is enabled or disabled.

- Use the focusing drive to set the Z-position where the brightness level correction is to be started.
- In the **Scan Control** window, set the initial values for **Detector Gain**, **Ampl. Offset** and **Ampl. Gain.** If required, start the continuous scan procedure for this purpose. Click on the **Set A** button.
- Use the focusing drive to set the Z-position where the brightness level correction is to be ended.
- Set the end value for **Detector Gain**, **Ampl. Offset** and **Ampl. Gain** in the **Scan Control** window**.** Click on the **Set B** button.
- If required, check the change of the set values by activating **Enable test.**

After the start of the scan procedure, the brightness level values are linearly interpolated between the defined references A and B.

☞ Note that the total Z range where the interpolation takes place can exceed the Z reference A and B.

Acquisition of a Z Stack

Once you have set up your image as defined in the above section, you can collect a series of confocal images through the different focal planes of your specimen.

- Click on the **Start** button on the **Scan Control** window. The system will automatically start the creation of a Z Stack. Be careful not to bump the air table or the microscope until Z sectioning is completed. Each successive Z Slice can be viewed by changing to the **Gallery** mode. This button is located on the right-hand side of the image.

A black bar will be shown under the image and will move from left to right, showing that the LSM 510 is in the process. The laser will automatically stop scanning when the Z Stack is completed.

The entire stack of images can be saved using the **Save** or **Save As** buttons on the right-hand side of the image.

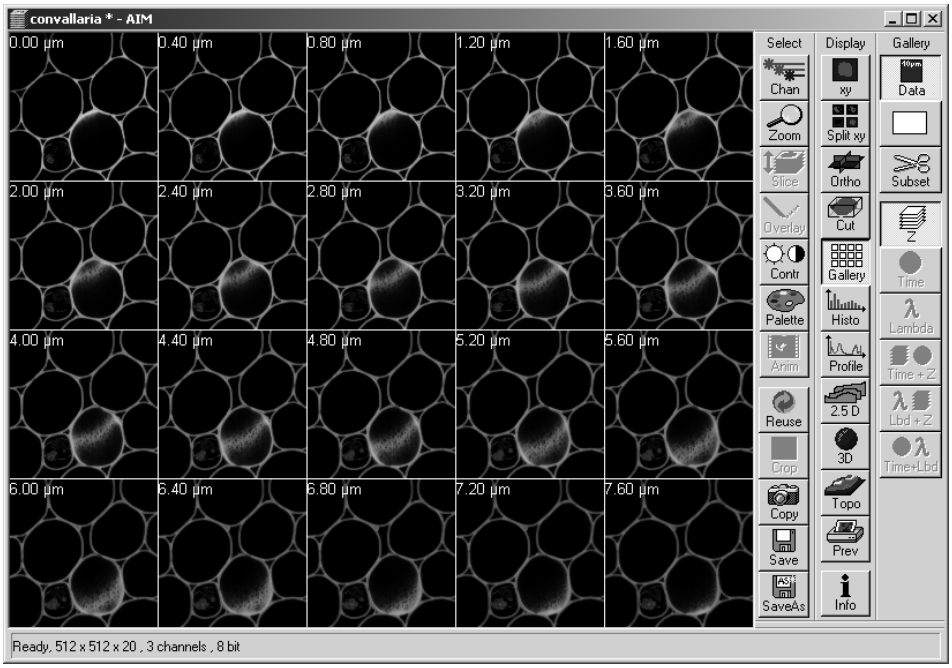


Fig. 4-70 Image Display window of a Z Stack

(4) Use ROI (Region Of Interest)

Performance of the **Frame** and **Z Stack** scan modes can be limited to one or several freely definable sections within the **Image Display** window using the **Use ROI** function.

The laser scans the entire line length, but is limited in the Y-direction by the ROIs. The Scan Time is therefore reduced.

Definition and activation of the ROIs to be used is performed via the **Edit ROI** function (**Acquire** subordinate toolbar).

If no ROI has been activated, the **Use ROI** button is not available.

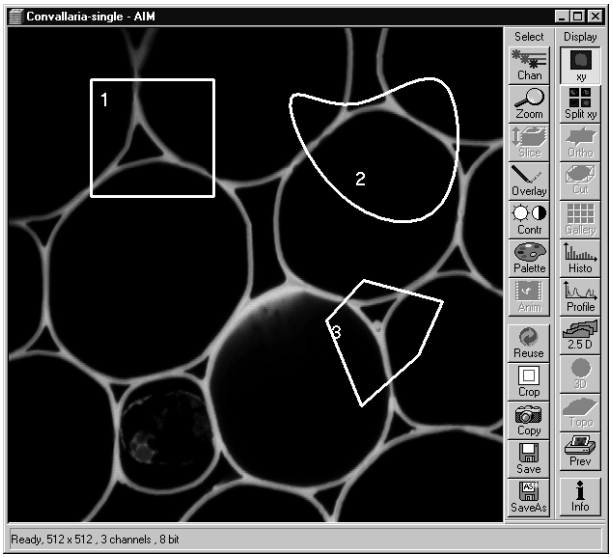


Fig. 4-71 **Image Display window created via the Use ROI function**

- Click on the **Edit ROI** button in the **Acquire** subordinate toolbar to open the **Edit ROI** window.
- Define one or several ROIs as required or select an existing ROI from the **ROI Lists** panel (see **Edit ROI**, page 4-100).
- The selected ROI is automatically activated when the **Edit ROI** window is closed with a click on **Close**.
- Click on the **Use ROI** button in the **Scan Control** window to perform the scan procedure in the defined ROI exclusively.

Only the regions of interest defined before are visible in the new scanning image, the other areas remain dark.

☞ The Scan Time is updated when ROIs are used.

- Clicking on the **Use ROI** button again will deactivate the function.

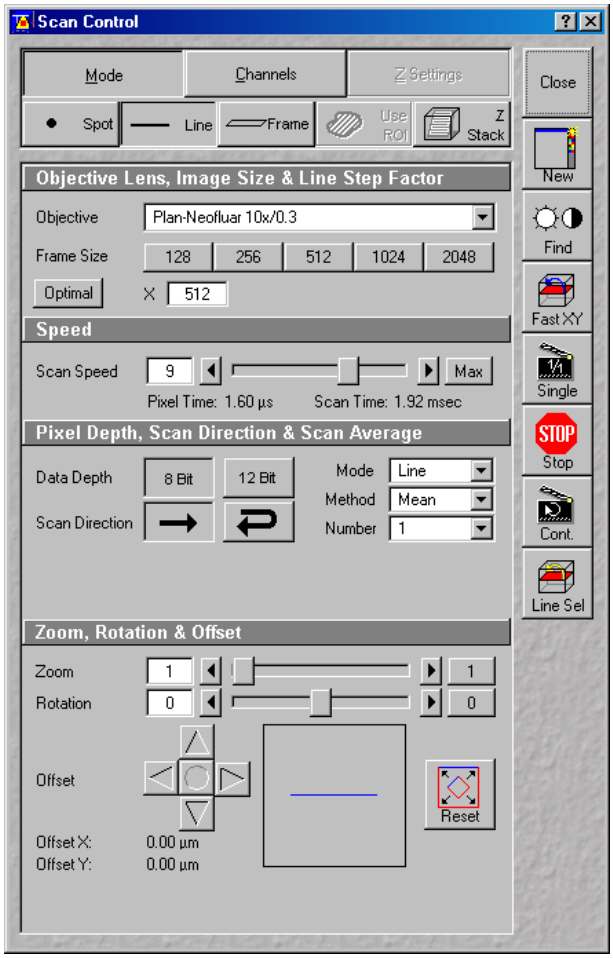


Fig. 4-72 **Scan Control window - Mode/Line**

4.5.4.3 Line

In the **Line** mode, fluorescent or reflected light along a freely definable line is displayed in the form of an intensity profile.

All the possibilities of creating an image (Frame, Z Stack) are also available in the **Line** mode.

The **Line** and **Frame** buttons are activated alternately and exclude each other.

If the **Line** button has been selected, the **Line Sel** (selection) button also appears on the right-hand side of the **Scan Control** window. It permits positioning of the line to be scanned as required within the **Image Display** window (Frame in XY-plane).

- Set all the parameters for the Scan procedure (**Mode** and **Channels** or **Z Settings**) in the same way as for the scanning of a frame or a Z Stack.
- Then click on the **Line Sel** button.
  - A frame will be scanned and the currently selected scan line and its intensity profile will be displayed. The **Line** toolbar is displayed on the right-hand side of the **Image Display** window.

The **Line** toolbar permits you to define the position, shape, width and color of the scan line in the **Image Display** window.

The scan line can be defined either as a straight line or a free shape curve (spline).

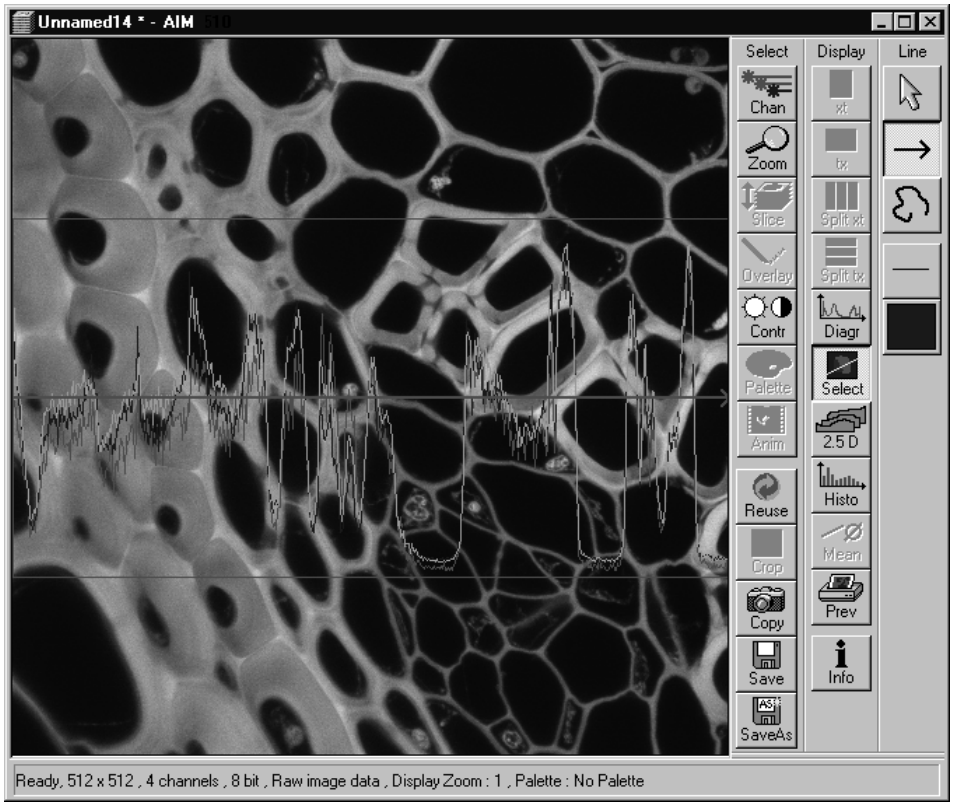


Fig. 4-73 Image Display window after activation of the Line Sel button

Buttons in the Line toolbar

The following function buttons are available in the **Line** toolbar:

Arrow selection button: Activates the mouse pointer for the selection and positioning of the scan line in the **Image Display** window and for changing its length.

Length change: Click on the drag point and keep the mouse button pressed. Drag the point and release the mouse button.

Shifting: Click on the line and keep the mouse button pressed. Shift the complete line and release the mouse button.

Line arrow button: Generation of a straight scan line in any direction in the **Image Display** window.

Opened free shape curve button: Generation of an open, free shape curve (spline) in the **Image Display** window. The first click sets the starting point, each further click adds a line segment. A click with the right mouse button ends the process.

Line button: Selecting the line width of the scan line.

Color button: Selecting the color of the scan line.

(1) Defining a straight line as the scan line

- Activate the **Line arrow** button of the **Line** toolbar. Click on the spot in the frame at which the line is to start and keep the mouse button pressed.
- Then drag the line to its desired end position and let go off the mouse button again.

The position of the line in the image can be changed as follows:

- Activate the **Arrow selection** button. To change the position in the X/Y-direction, click on the line and keep the mouse button pressed.
- Then move the lines to the desired position and let go off the mouse button again.
- To change the rotation direction or the length of the line, click on the start or end point of the line and keep the mouse button pressed.
- Change the rotation direction and / or the length of the line as required and let go off the mouse button again.

The intensity profile for the defined line is displayed on-line.

After release of the mouse button, the relevant intensity profile along the drawn line will be displayed. In the **Zoom, Rotation & Offset** panel, the current, changed angle and the offset in X and Y are displayed.

- When the **Line Sel** button is pressed again, a frame will be scanned in such a way that the selected line lies exactly in the center of the Y-axis again and is parallel to the X-axis.

☞ The position of the Line (rotation and offset) can also be changed directly in the **Zoom, Rotation & Offset** panel of the **Scan Control** window.
In the **Line** mode, Line Stacks can also be recorded over a defined period of time (see **Time Series**, page 4-104).

(2) Defining a free shape curve (spline) as the scan line

- Activate the **Free shape curve** button of the **Line** toolbar.
- Draw the your shape curve (spline) in the **Image Display** window using the mouse. The first click sets the starting point, each further click adds a line segment. A click with the right mouse button ends the line definition.

The scanner represented by a white line immediately begins with the on-line tracing of the defined free shape curve. The laser excitation remains inactive in this process.

If the defined free shape curve becomes too complicated or the selected **Scan Speed** is too high, the following message appears in the status bar of the **Image Display** windows:

Maximum scanner speed exceeded!

- In this case, reduce the **Scan Speed** set in the **Scan Control** window.
- If the generated contour and the line traced by the scanner are not in coincidence, reduce the **Scan Speed** by a further amount.

If no sufficient coincidence of the two lines can be achieved by the reduction of the scan speed, you have to calibrate the scanner position signal.

- Click on the **Maintain** button in the **Main** menu and then on the **Spline** button.
  - The **Calibrate Spline Scan** window is opened.
- Bring the generated contour and the scanner line to coincidence by varying the amplitude or offset values for X and Y.
- If necessary, match the free shape curve to the scanner line.
- Then click on the **Single** or **Cont.** button to execute the scan process, with the laser activated.

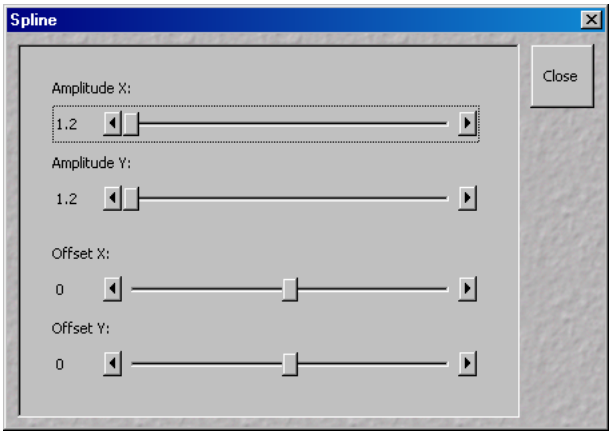


Fig. 4-74 Calibrate Spline Scan

A **Line** scan is performed along the defined freehand shape curve, and the intensity profile is displayed at the bottom of the **Image Display** window.

Irrespective of the length of the defined freehand shape curve, the intensity profile is always calculated (by interpolation) and displayed in accordance with the pixel value set under **Line Length** (2048 pixels maximum).

- Click on the **Stop** button to terminate the scan procedure.

☞ As soon as the free shape curve is modified, the laser excitation is deactivated and the scanner again starts to trace the newly generated free shape curve.

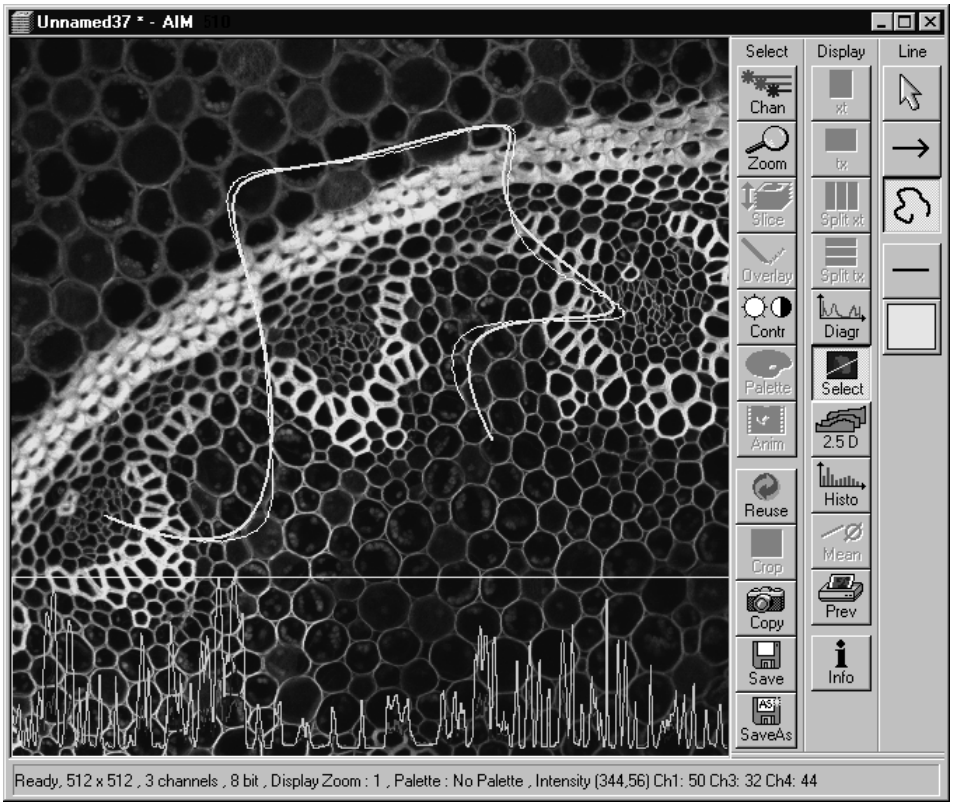


Fig. 4-75 **Image Display window after definition of a freehand shape curve for the line scan process**

(3) Selecting the width and color of the scan line

- Line color and width can be set via the **Line** and **Color** buttons of the **Line** toolbar.

(4) Line Stack

The intensity profile of a defined straight line or free shape curve can also be recorded as a Z Stack. To do this, proceed in the same way as for the Frame Stack.

4.5.4.4 Spot

In the **Spot** mode fluorescent or reflected light occurring from a single voxel xyz is detected. In this mode a spot can be defined by two perpendicular lines in the **Image Display** window.

In the **Spot** mode the **Z Stack** button is not available. After definition of the spot position the only possible scan mode is a time series of a spot.

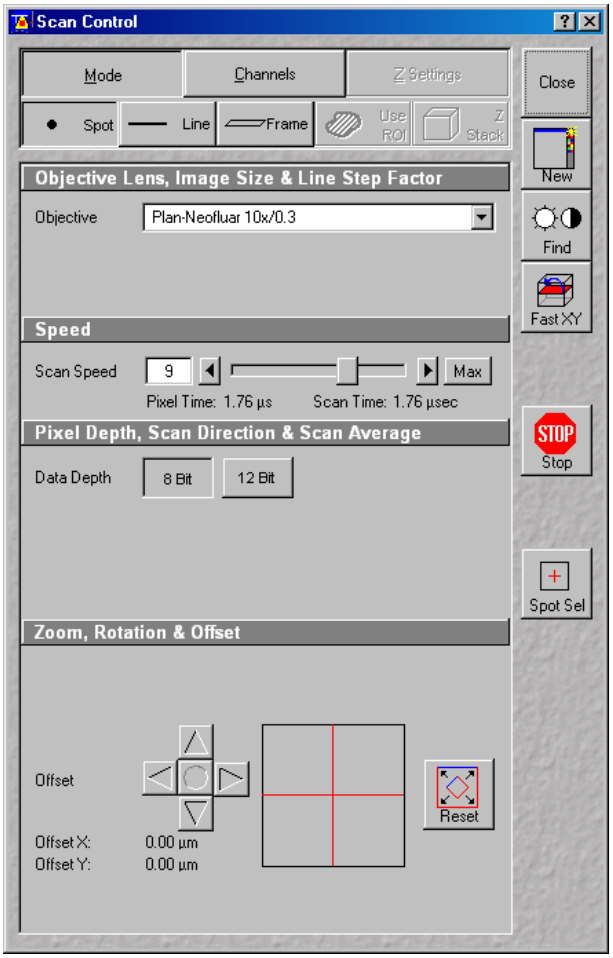


Fig. 4-76 Scan Control window - Mode/Spot

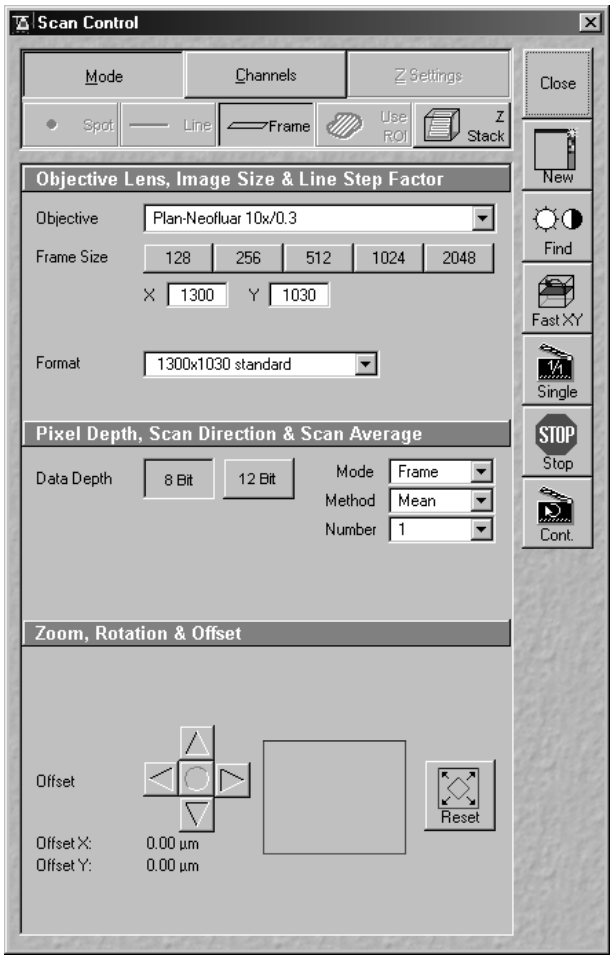


Fig. 4-77 **Scan Control window - Mode, settings for camera control**

4.5.4.5 Camera Control

The use of this function permits the control of the external CCD-camera settings.

(1) Open / Close the Scan control window for camera control

- In the **Configuration Control** window, activate the **Camera** button.
- Click on the **Scan** button in the **Acquire** subordinate toolbar of the main menu.
- Click on the **Close** button.

(2) Function description

Mode button — Displays the selected objective, frame size and pixel depth.

Frame Size — Selects between square formats or free defined frame sizes.

Format — Selects between a range of default camera resolutions. The 5x5 binning mode can be used for focusing without delay of the image display.

Data Depth — Sets the pixel depth.

Zoom/Offset — Shifts a subregion in the frame.

Reset — Resets the frame/subregion to default value selected in **Format**.

Channels button — Displays the activated channels and possible settings.

Exposure time — Sets the exposure time of the camera.

Find Starts a prescan and sets the exposure time automatically.

In case of a camera multitracking, only one channel should be selected in Configuration Control in order to speed up the find function.

Fast X/Y Starts a fast online scan mode, e.g. for focusing. Also, the 5x5 binning mode can be used (to be set in **Mode** / **Format**).

Single Starts a single image acquisition (The **Image Display** window appears.).

Cont. Starts acquisition of a series of images (The **Image Display** window appears.).

Crop Defines a ROI for camera acquisition in the **Image Display** window. Note that this is just a **Crop** function, while the whole sample is illuminated. Rotation of the ROI is not possible.

Info button Shows the acquisition parameters in the **Image Display** window.

Close Close the **Scan Control** window.

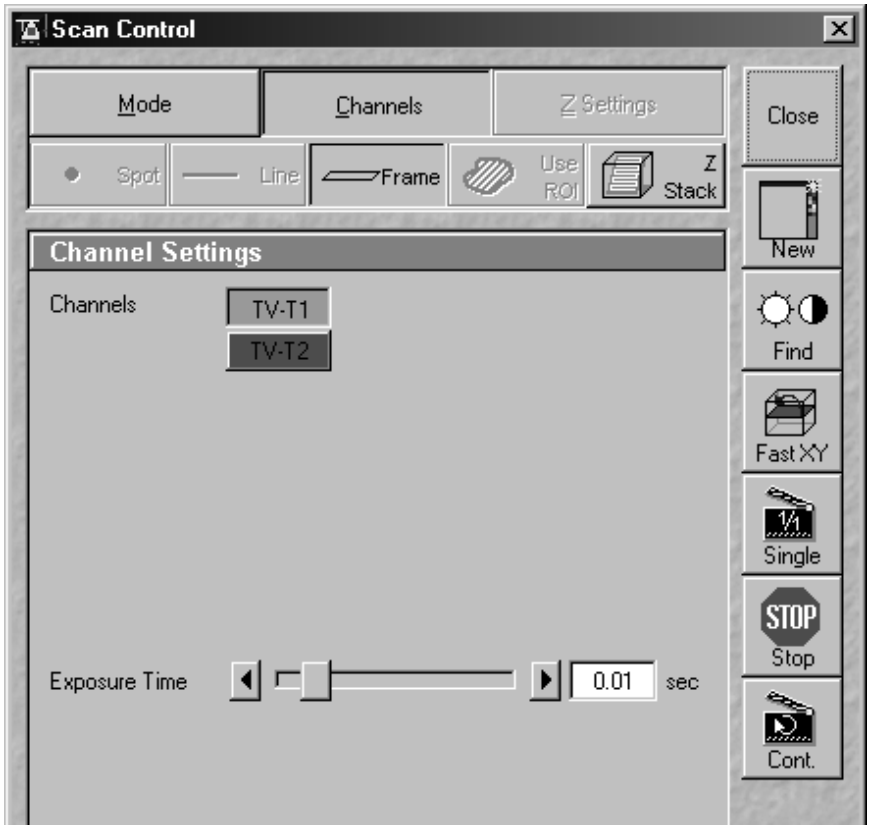


Fig. 4-78 Scan Control window - Channels, settings for camera control

4.5.5 Edit ROI (Region Of Interest)

A scan image allows certain areas (ROIs) to be defined. Only these areas of interest will be scanned. The laser beam will be switched on only in these areas via Acousto-Optical Tunable Filters (AOTF). Definition and activation of the ROIs for the scan procedure is performed in the **Edit ROI** window.

4.5.5.1 Open / Close the Edit ROI Window

- Click on the **Edit ROI** button in the **Acquire** subordinate toolbar of the **Main** menu. The **Edit ROI** window appears on the screen and the ROIs defined last are visible in the **Image Display** window.
- Click on the **Close** button in the **Edit ROI** window. The **Edit ROI** window is closed and the ROIs disappear from the **Image Display** window.

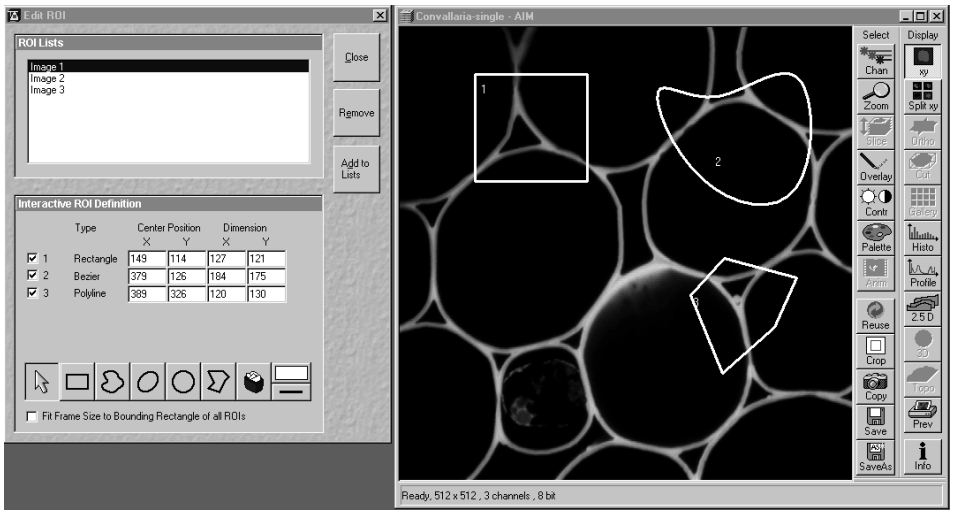


Fig. 4-79 Edit ROI window and Image Display window with ROIs

The **Use ROI** status display (button) in the **Scan Control** window shows whether the ROI mode is activated or not. If ROIs shall not be taken in consideration during scanning, the **Use ROI** button must be deactivated prior to the scanning procedure.

When **Edit ROI** is activated and the first ROI is drawn in the **Image Display** window, the **Use ROI** is activated automatically.

4.5.5.2 Function Description

The following functions are available on the right-hand side of the **Edit ROI** window:

Close button — The **Edit ROI** window is closed.

Remove button — An entry marked in **ROI Lists** (stored ROI configuration) is deleted.

Add to Lists button — The **Add ROI List** window is opened.

(1) ROI Lists panel

In the **ROI Lists** panel, all the currently defined and stored ROI configurations are shown.

- Click on the ROI configuration which you want to use for the scan procedure.
  - The selected ROI configuration is highlighted in blue and displayed in the opened **Image Display** window.
- To produce a new ROI configuration, an already stored configuration can be activated, changed and stored under a new name using the **Add to List** button.
- To delete a stored ROI configuration from the list, click on its name first (highlighted in blue) and then on the **Remove** button.

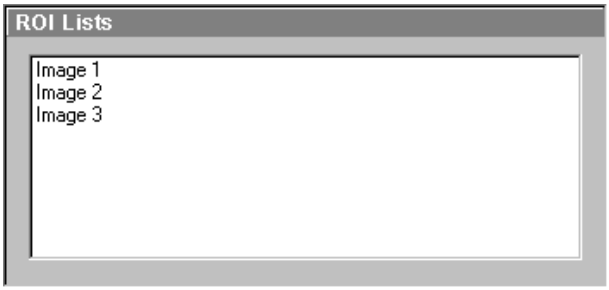


Fig. 4-80 ROI Lists panel

(2) Interactive ROI Definition panel

In the **Interactive ROI Definition** panel, the parameters of the ROI configuration just selected from the **ROI Lists** panel are displayed. Furthermore, it contains all the functions required for the creation of ROIs.

The X and Y values for **Center Position** and **Dimension** can be edited.

- Activate the relevant text box with a mouse click and enter the new value via the keyboard.
- If you click outside the edited text box, the new value will be taken over and the ROI figure be shifted to the new position.

The upper part of the panel gives an overview of all the individual figures stored under the selected name according to type, position within the **Image Display** window (in pixels) and greatest dimension in X and Y (in pixels). The origin of the position indication lies in the left top corner of the **Image Display** window.

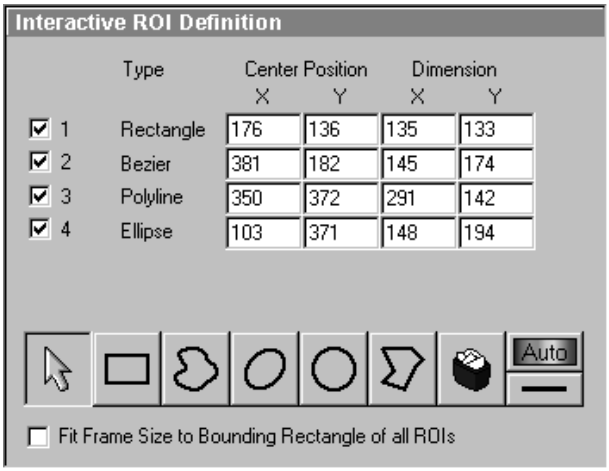


Fig. 4-81 Interactive ROI Definition panel

☑ Check box (e.g.: 1 - 4): Clicking on this check box allows a ROI to be deactivated. The tick disappears from the check box, as does the relevant marked area from the scanning image. Clicking on the check box again will reactivate the ROI.

Arrow button: Activation of the mouse button to change the size or move the ROIs in the **Image Display** window.

Rectangle button: Draw of a rectangle in the **Image Display** window; click and keep mouse button pressed, drag the rectangle in any direction, let go off the mouse button to end the procedure.

Bezier button: Draw of a bezier figure in the **Image Display** window; first click sets the starting point, each additional click adds a line, double-click on the starting point closes the figure and ends the procedure.

Ellipse button: Draw of an ellipse in the **Image Display** window; first click sets the center point, displayed line permits determination of the extension, second click sets the first dimension, then the second dimension and the rotation direction can be determined, third click sets the second dimension and direction and ends the procedure.

Circle button: Draw of a circle in the **Image Display** window; click and keep the mouse button pressed to set the center point, drag the diameter, let go off mouse button again to end the procedure.

Polyline button: Draw of a polyline figure in the **Image Display** window; first click sets the starting point, each further click adds a line, double-click on the starting point closes the figure and ends the procedure.

Recycle bin button: All the ROIs dragged to the scanning image are deleted. If an area outline was marked before, this area is now deleted in the scanning image.

Auto / Color button: A defined color from the list of colors can be assigned to the ROIs. In that case, the same color is assigned to all the individual figures. In the **Auto** position, the outlines of the dragged ROIs are automatically colored differently.

Line button: This button allows you to determine the line thickness of the area outline. This is for display purposes only. The scanned line is not effected.

☑ **Fit Frame Size to bounding Rectangle of all ROIs** check box: If this check box is ticked, the scan procedure is displayed only within a rectangle which is defined by the greatest extension in X and Y of all the individual figures together, i.e. the pixel number and the data quantity of the **Image Display** window are reduced.

- In the toolbar of the **Interactive ROI Definition** panel, click on the symbol of the area you want to use to mark the region of interest in the scanning image. Five different area symbols are available in the form of buttons.

- Click on the marking area and keep the mouse button pressed to drag the area into the region of interest in the scanning image. The marking area will be numbered automatically and entered in the **Interactive ROI Definition** panel with its position and dimension parameters and the appropriate number.
- The dragged marking area is marked by clicking on its outline; its size can be changed by clicking on the marking points. Clicking on the area edge beside the marking points allows repositioning of the area on the scanning image.

☞ The digits of the ROIs can be shifted independently of the contours of the figure.

- If you have framed all the required ROIs in accordance with steps 2 to 4, you can store these ROIs under any required name via the **Add to Lists** button.
- The **Add ROI List** window will appear. Enter any required name to store the ROIs and click on the **OK** button.
- This stored ROI configuration appears in the **ROI Lists** panel of the **Edit ROI** window.

Fig. 4-82 Add ROI List window

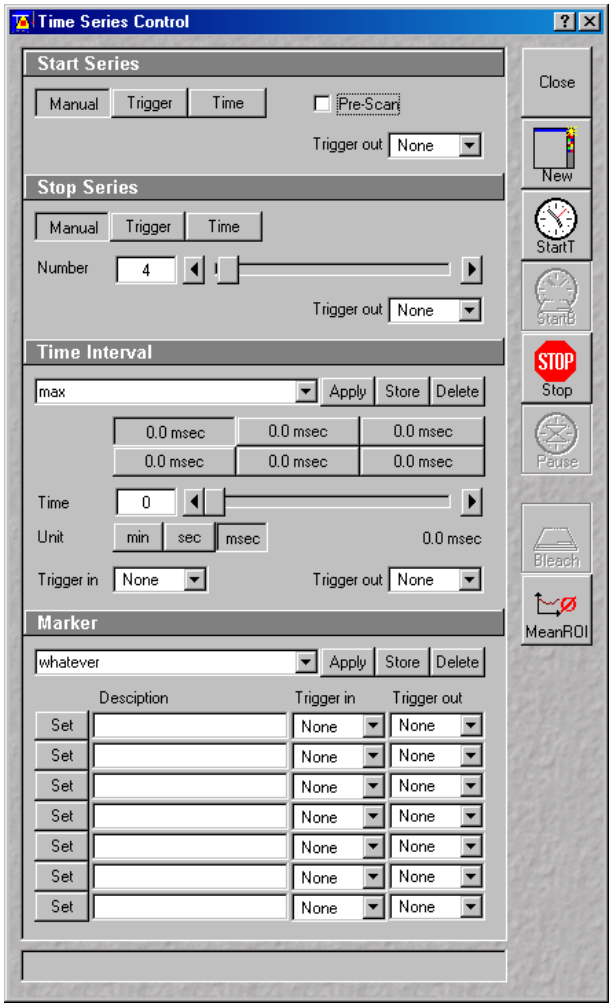


Fig. 4-83 **Time Series Control window**

4.5.6 Time Series

The **Time Series Control** window allows the definition of parameters for time series.

The **Time Series** function offers the following options for the creation of image series:

- Definition of break times between 0.1 ms and 10 hours.
- Determination of the number of steps from 1 to 10,000 for one scanning procedure.
- Setting of markers.
- Interruption of time control via pause function, and resume of the time series function.
- Triggering of time series via:
  - numeric input
  - external trigger pulses
  - time (of the PC)

4.5.6.1 Open / Close the Time Series Control Window

- Click on the **Time Series** button in the **Acquire** subordinate toolbar of the **Main** menu.

The **Time Series Control** window appears on the screen.

- Click on the **Close** button to close the **Time Series Control** window.

The following functions are available on the right-hand side of the **Time Series Control** window:

Close button — Closes the **Time Series Control** window.

New button — Opens a new **Image Display** window.

Start T button — Starts the Time Series.

Start B button — Starts the Time Series in combination with a bleach procedure. Bleach procedure must be defined first in the **Bleach Control** window.

Stop button — Stops the entire Time Series. A current scan is interrupted.

Pause button — Interrupts the Time Series. Button labeling is changed to **Resume**. A current scan is performed until the end. When the button is pressed again, the Time Series is immediately continued with the next scan procedure.

Bleach button — Starts a Bleach procedure without a Time Series. Bleach procedure must be defined first in the **Bleach Control** window.

Mean ROI button — Creates a Time Series with the intensity values of the Frame or the default ROIs. An average value is formed of the intensity values of the Frame or the ROIs determined in the relevant scan procedure. These average values are displayed in an extended **Image Display** window as a function of the time which has passed.

The status line, in which the phases of the current Time Series or notes for the user are displayed, is in the lower part of the **Time Series Control** window.

4.5.6.2 Start Series Panel

In this panel, the parameters for the start of the time series are set.

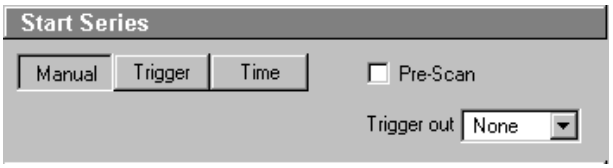


Fig. 4-84 Start Series panel

The following functions are available:

Manual button — The time series is started manually with a click on the **Start T** or **Start B** button.

Trigger button — The time series is started via a trigger signal from Trigger Control.

Time button — The time series is started when the set time is reached. The internal computer time applies.

Time input box — Input of the time for the start of the time series (**Time** button activated).

Trigger in list box — Selection of the trigger key (1-4) with which the start is to be triggered (**Trigger** button activated).

Trigger out list box — Selection of the trigger keys (1-4) for the out signal.

Pre-Scan — If this box is checked before starting the Time Series a continuous scan is started but no images are acquired until the button **Go** , which then appears on the lower right hand side of the control window, is clicked.

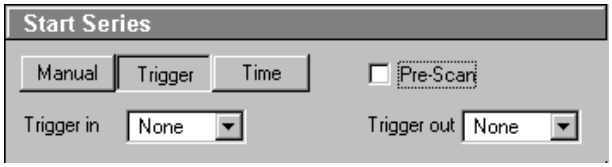


Fig. 4-85 Start Series panel

(1) Start via Trigger

For the start via trigger control (**Trigger** button activated), first determine the trigger key which is to trigger the start of the Time Series.

- Open the **Trigger in** list box with a click on the arrow button.
- Choose one of the trigger keys 1 to 4 (e.g. **Trigger1**).

It is also possible to trigger an out signal via trigger control.

- Open the **Trigger out** list box with a click on the arrow button.
- Choose one of the trigger keys 1 to 4 (e.g. **Trigger1**).

In this example, the scan procedure is triggered on pressing key **1** of the trigger control, and an out signal is given at the same time.

☞ When starting a Time Series via Trigger, the **Start T** or **Start B** button must be pressed first. **Waiting for Trigger** will then be displayed in the status line.
Then the relevant trigger key on the Trigger Control must be pressed to start the first scan procedure of the Time Series.

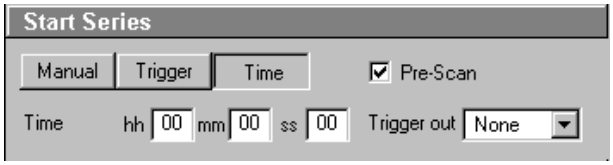


Fig. 4-86 Start Series panel

(2) Start via Time

For the start via the time set on the PC (**Time** button activated), the start time must be entered first in the **Time** input box.

- Click in the **Time** input box to open it.
- Enter a start time via the keyboard. Then click outside the input box once to close it again.

☞ When starting a Time Series via the time, the **Start T** or **Start B** button must also be pressed in this case. **Waiting for Start Time** will be displayed in the status line.
The Time Series is started when the starting time has been reached.
The starting time for the Time Series can be changed online.

(3) Pre-Scan

The **Pre-Scan** allows to image the sample continuously before actually starting the image acquisition.

☞ This function should only be used for starting the time series manually. After clicking the Start or Start B button, the button **Go** appears on the lower right hand side of the control window. By clicking this button the actual image acquisition is started.

4.5.6.3 Stop Series Panel

In this panel, the parameters for the end of the time series are set and the number of cycles is determined.

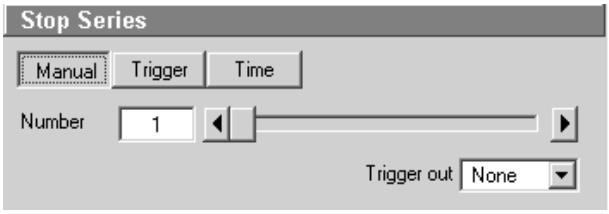


Fig. 4-87 Stop Series panel

The following functions are available:

Manual button — The time series is finished manually with a click on the **Stop** button.

Trigger button — The time series is finished via a trigger signal.

Time button — The time series is finished when the set time has been reached. The internal computer time applies as the set time.

Number input box / arrow keys / slider — Determination of the number of images acquired or image stacks for the time series.

Time input box — Input of the time for the end of the time series (**Time** button activated).

Trigger in list box — Selection of the trigger keys (1-4) with which the end is to be triggered (**Trigger** button activated).

Trigger out list box — Selection of the trigger keys (1-4) for the out signal.

- Use the slider near **Number** to select the images or image stacks for the time series.

(1) Stop via Trigger

To end the Time Series via Trigger Control (**Trigger** button activated), first determine the trigger key which is to end the Time Series.

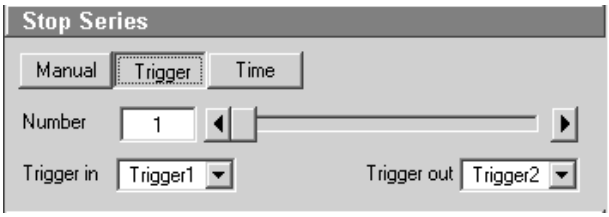


Fig. 4-88 Stop Series panel

- Open the **Trigger in** list box with a click on the arrow button.
- Choose one of the trigger keys 1 to 4 (e.g. **Trigger2**).

It is also possible to trigger an out signal via Trigger Control.

- Open the **Trigger out** list box with a click on the arrow button.
- Choose one of the trigger keys 1 to 4 (e.g. **Trigger2**).

In this example, the Time Series is ended on pressing key **2** of the Trigger Control, and an out signal is given at the same time.

☞ If the entered number of cycles has been processed without a trigger impulse having been given to end the procedure, the Time Series is finished.
If a trigger signal is given before the cycles have been processed, the Time Series will only be interrupted. **Waiting for Trigger** will be displayed in the status line. The Time Series can now be continued via a new trigger signal or ended via **Stop**.

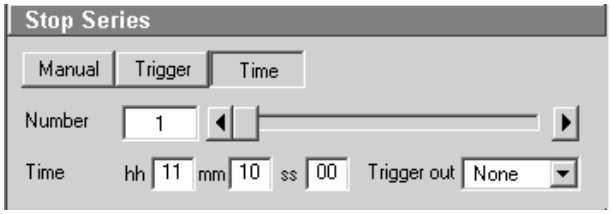


Fig. 4-89 Stop Series panel

(2) Stop via Time

To end the Time Series via the time set on the PC (**Time** button activated), the end time must first be entered in the **Time** input box.

- Click on the **Time** input box to open it.
- Enter the end time via the keyboard. Then click outside the input box once to close the box.

☞ The Time Series is interrupted when the end time has been reached.
If the entered **Number** of cycles has been processed, the Time Series is finished.
If the number of cycles has not yet been processed, the Time Series is only interrupted. **Waiting for Start Time** is displayed in the status line. The Time Series can now be continued by entering a new start time, or finished via **Stop**.
The end time for the Time Series can be changed online.

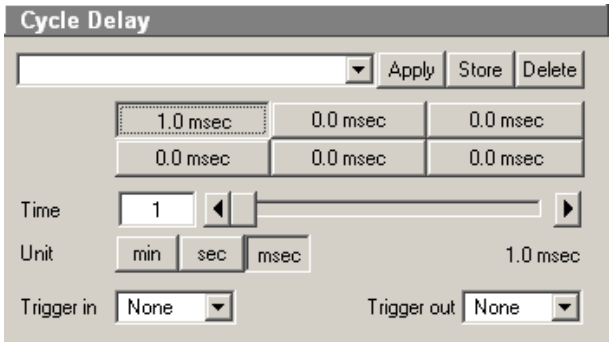


Fig. 4-90 Cycle Delay panel

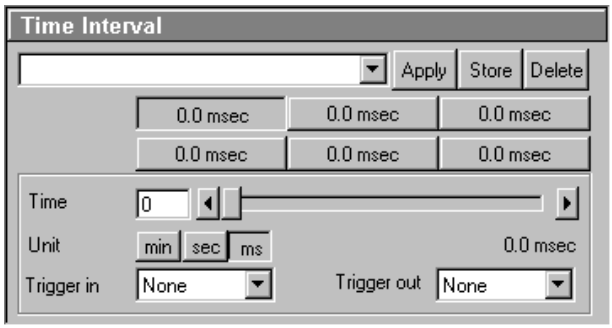


Fig. 4-91 Time Interval panel

4.5.6.4 Cycle Delay / Time Interval Panels

Depending on the settings in the **Time Series** tab (see **Options** menu, **Settings**), the time series interval is defined either as a **Cycle Delay** or **Time Interval**. Accordingly, either the **Cycle Delay** panel or the **Time Interval** panel is displayed in the **Time Series Control** window.

Cycle Delay is the interval between the end of one scan process and the beginning of the next.

Time Interval is the interval between the beginning of one scan process and the beginning of the next.

The **Time Delay** (or **Time Interval)** panel permits the intervals to be activated and changed.

Function description

Time delay or **Time Interval** list box	List of the stored sets of time delays or Time Intervals for time series.
Apply button	Application of the sets of delays for time series selected in the list box.
Store button	Storage of sets of delays for time series.
Delete button	Deletion of sets of delays for time series from the list box.
Time buttons	Activation of the time for the time series set for the relevant button.
Time input box / arrow buttons / slider	Determination of the cycle time for the currently activated **Time** button.
Unit buttons	Selection of time units: **min**, **sec** or **ms**.
Trigger in list box	Selection of the trigger key (1-4) to be used to activate the **Time** button for the delay time.
Trigger out list box	Selection of the trigger key (1-4) for the out signal.

Setting delay time or time interval

- The delay time or time interval to be used during the Time Series is set to a default value by activating a **Time** button.

For this purpose, the relevant time must be assigned to the **Time** button first.

- Activate a **Time** button with a click of the mouse.
- Set the required delay time or time interval via the slider (arrow keys or input box) near **Time**. The set time is displayed online on the button. Select the time unit by clicking on the relevant button near **Unit**.

You can assign different times to all the six **Time** buttons and store this assignment either as a set of delays or of time intervals.

- Enter a name in the **Time Delay** list box or **Time Interval** list box and click on **Store** to store the set of delays.

Activating delay time or time interval

If required, a set of delays or time intervals can be activated again quickly.

- Open the list box with a click on the arrow button and select the required set with a click of the mouse.
- Then click on the **Apply** button to activate the set. The stored delays are assigned to the **Time** buttons.

Sets of delays or Sets of time intervals which are no longer required can be deleted.

- Open the list box and select the required set.

- Click on the **Delete** button. The set will be removed.

The **Time** buttons can also be activated via keys 1 to 4 of the Trigger Control.

- Click on the required **Time** button.
- Open the **Trigger in** list box with a click on the arrow button.
- Choose one of the trigger keys 1 to 4 (e.g. **Trigger3**).

It is also possible to trigger an out signal via Trigger Control.

- Click on the required **Time** button.
- Open the **Trigger out** list box with a click on the arrow button.
- Choose one of the trigger keys 1 to 4 (e.g. **Trigger3**).

In this example, the relevant **Time** button is activated on pressing key **3** of the Trigger Control, and an out signal is given at the same time.

☞ The delays or time intervals can be changed online with a click on another **Time** button. The new delay will be applied immediately.
A change of the delay during a Time Series is displayed in the **Image Display** window if the **Gallery** button (**Display** toolbar) is activated.

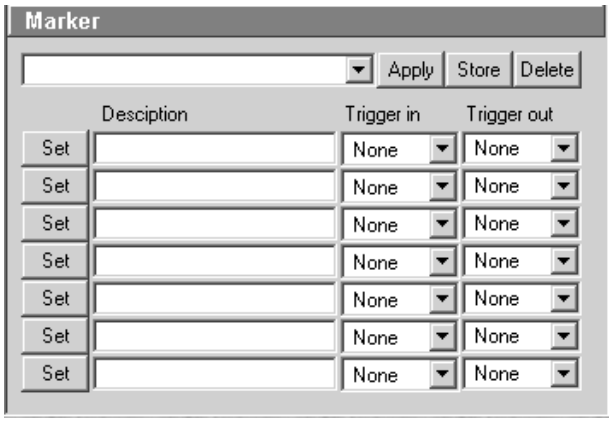


Fig. 4-92 Marker panel

4.5.6.5 Marker Panel

The setting of a marker permits information about the moment in the current time series and any required comment to be assigned to the current scan. The time indication is set automatically, while comments must be defined before.

The markers (red squares) are visible in the **Image Display** window if the **Gallery** button (**Display** toolbar) is activated.

On storage of the image, all the markers, including the time indication and the comments, are stored along with the image contents.

Function description

Marker list box	List of the stored combinations of markers.
Apply button	Application of the marker combinations selected from the list box.
Store button	Storage of a combination of markers.
Delete button	Deletion of a combination of markers from the Marker list box.

Set 1-7 button	Setting of a marker during the scan procedure.
Description input box (1-7)	Entry of the comments for the marker.
Trigger in list box (1-7)	Selection of the trigger key (1-4) with which the marker is to be set.
Trigger out list box (1-7)	Selection of the trigger key (1-4) for the out signal.

Setting marker for the current scan

- A marker for the current scan is set by clicking on one of the **Set 1** to **7** marker buttons.

The assignment of any required comment for the marker must be performed as follows:

- Click in the **Edit Text** box of the required marker key (e.g.: **Set 1**) to open the editing box.
- Enter the comments via the keyboard. Then click outside the editing box to close this box again.

You can assign comments of any required length to all the seven **Set** buttons and store this assignment as a combination of marker keys.

- Enter a name in the Marker list box and click on **Store** to store the combination.

  If required, a combination of markers can be activated again quickly.
- Open the Marker list box with a click on the arrow button and select the required combination with a click of the mouse.
- Then click on the **Apply** button to activate the combination. The relevant comments are displayed in the **Edit Text** boxes of the **Set** buttons.

Combinations which are no longer required can be deleted.

- Open the Marker list box and select the required combination.
- Click on the **Delete** button. The combination will be removed.

The marker buttons can also be activated via keys 1 to 4 of the Trigger Control.

- Click on the required **Set** button.
- Open the **Trigger in** list box with a click on the arrow button.
- Select one of the trigger keys 1 to 4 (e.g. **Trigger4**).

It is also possible to trigger an out signal via Trigger Control.

- Click on the required **Set** button.
- Open the **Trigger out** list box with a click on the arrow button.
- Select one of the trigger keys 1 to 4 (e.g. **Trigger4**).

In this example, the relevant **Set** button is activated on pressing key **4** of the Trigger Control, a marker is set in the Scan and an out signal given at the same time.

4.5.6.6 Time Series of a Frame

- Set the relevant parameters for time control in the **Start Series**, **End Series** and **Time Delay** panels.
- Start the Time Series with a click on the **Start T** or **Start B** button.
- If you use Trigger Control, confirm the relevant Trigger key to start the Time Series with the first scan procedure.
- Use the **Set 1** to **Set 7** buttons to set markers during the scanning procedure which will allow you to evaluate interesting scanning images later.

☞ Time end will finish time series even if you have created a program which would exceed the time end.
Bleach times will be added. No break is possible during bleaching.

☞ If you want to integrate a bleaching procedure in a time series, start must be triggered via **Start B**. The bleaching procedure must be defined first in the **Bleach Control** window (see page 4-118).
If a time series is interrupted before its programmed end, the programmed number of images will be taken over in the database. However, only those images are stored which were created before interruption of the time series. This is due to the fact that the original image parameters are to be taken over via the **Reuse** function.
If a stop time for time series is entered via the **Trigger** button or the **Time** button, the recording of the series will not be definitely finished. It is possible to either continue the series via new settings of **Trigger** and **Time** or to definitely finish the time series via the **Stop** key.

The following example of a scanning image was taken using the **Time Series** function. Both the time and the markers set during the scanning procedure are projected in the image series in different colors.

If the cursor is moved to a marker position in the scanning image, the relevant information on the image detail is automatically provided in an additional window.

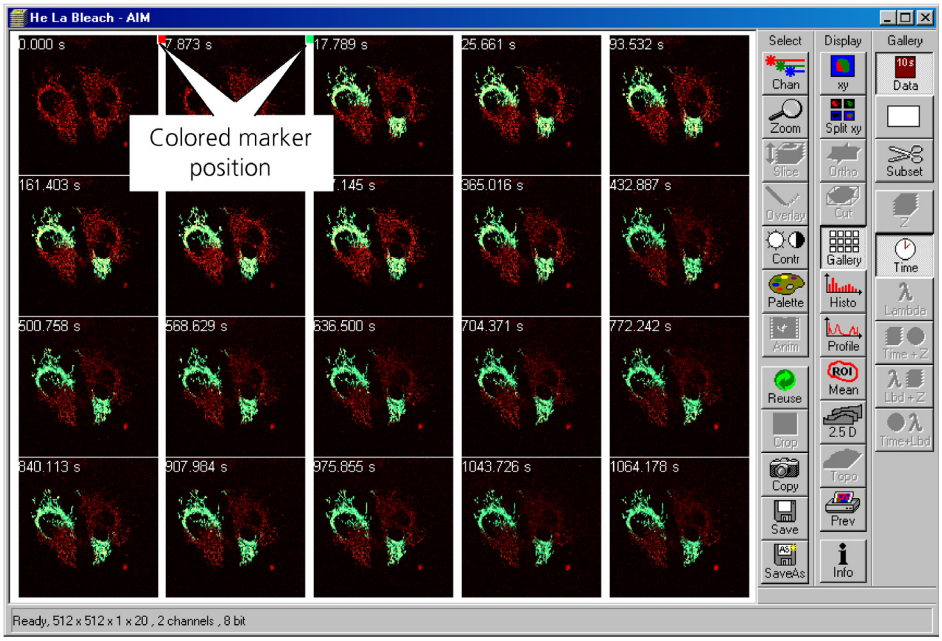


Fig. 4-93 Image Display window of a Time Series Scan

The image markers have different colors with the following meaning:

- **red**: manually set marker with time indication and comments
- **blue**: automatically set marker with change of delay
- **green**: automatically set marker at the beginning and at the end of a bleaching procedure

4.5.6.7 Time Series of a Frame over Z Stack

- First, set all parameters required for recording a Z Stack in the **Scan Control** window.
- Then set the parameters required for recording the time series in the **Time Series Control** window (identical procedure as for the time series of a frame).
- Start the time series by clicking on **Start T**.
  - Complete stacks are now recorded at the defined time intervals. The result is displayed in the form of the combined **Image Display** window of the stack and time series (4D).

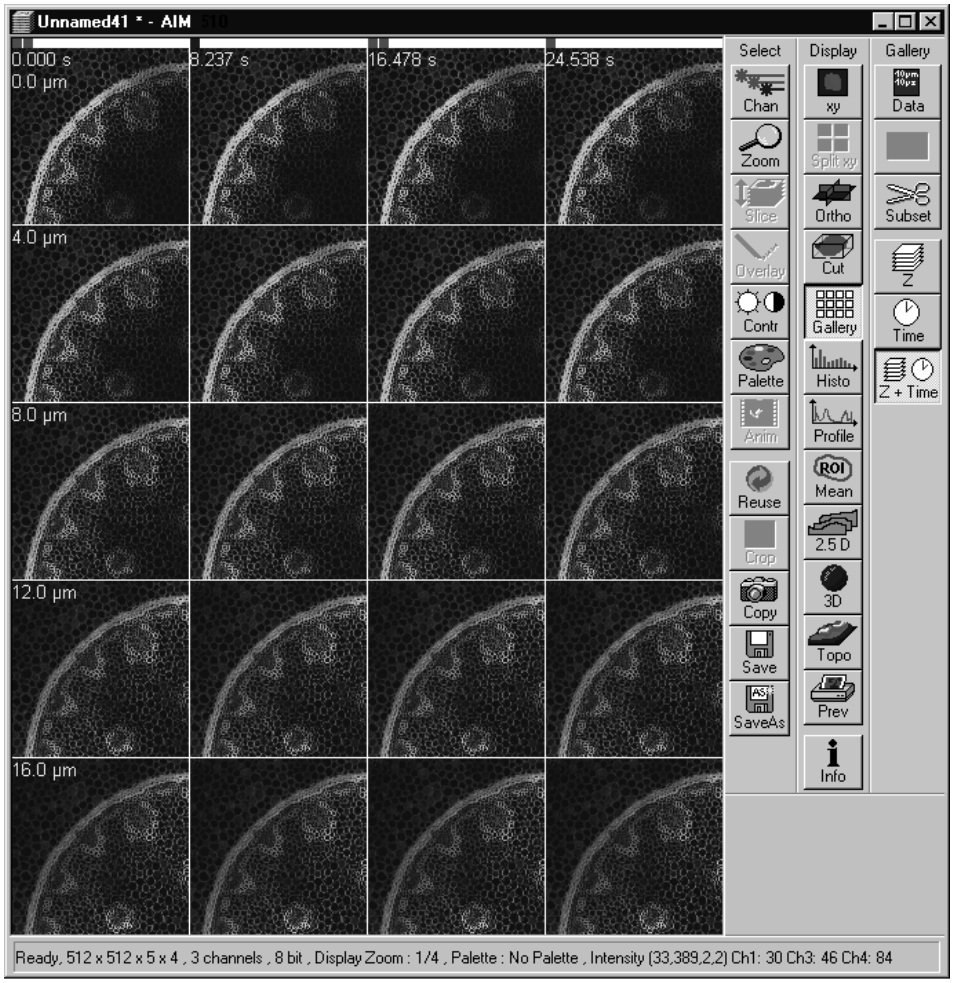


Fig. 4-94 Image Display window of a Z Stack and a Time Series Scan

The additional **Z**, **Time** and **Z + Time** buttons are available in the **Gallery** toolbar of the **Image Display** window.

When you click on the **Z** button, the individual frames of the Z Stack are displayed for the selected Time Slice. When you click on the **Time** button, the individual frames of the time series are displayed for the selected Z Slice.

For Z Stacks over the time (4D) following offline functions will be enlarged:

- Slice (**Z** slider and **Time** slider)
- Gallery (**Z**, **Time** and **Z + Time** buttons)
- 3D (slider for single time index)

To select the Z or Time Slices, use the appropriate sliders which are displayed if the **Slice** button in the **Image Display** window has been activated.

When you click on the **Z + Time** button, all individual frames will be displayed.

☞ Working in **Online Fingerprinting** mode when acquiring a **Z Stack** results in a similar image window with the same display functionalities.

4.5.6.8 Time Series of a Frame over a Z Stack over Lambda

- Activate the **META** mode sheet in the configuration control and set the relevant parameters.
- Set the relevant parameters for the **Start Series**, **End Series** and **Time Delay** panels.
- Start the time series with a click on the **Start T** or **Start B** button.

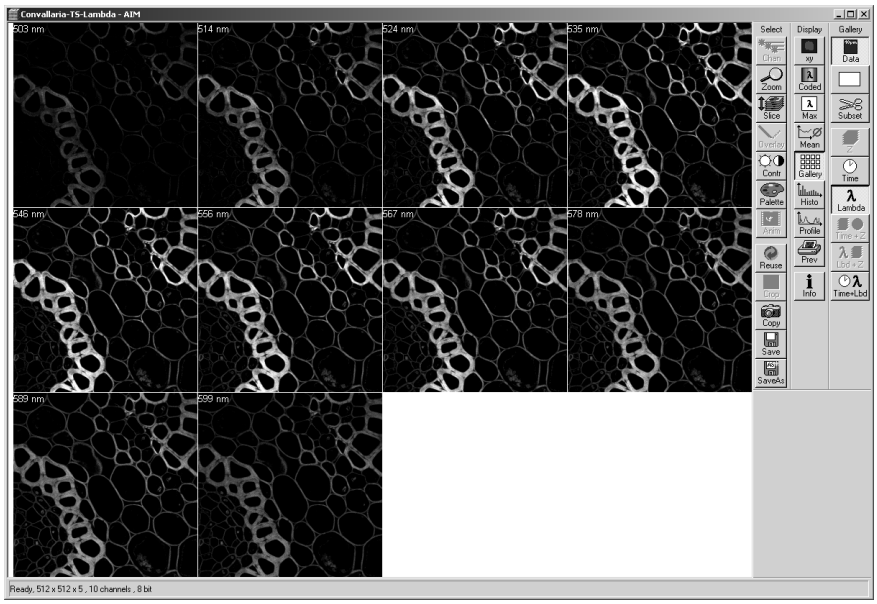


Fig. 4-95 Image Display window of a Time Series over Lambda

4.5.6.9 Time Series with Mean ROI

- Set all the parameters in the same way as for Time Series of a frame.
- Then click on the **Mean ROI** button in the time series frame.

A mean intensity profile of the defined ROIs (To be defined using the **Edit ROI**, see page 4-100) is created as a function of time.

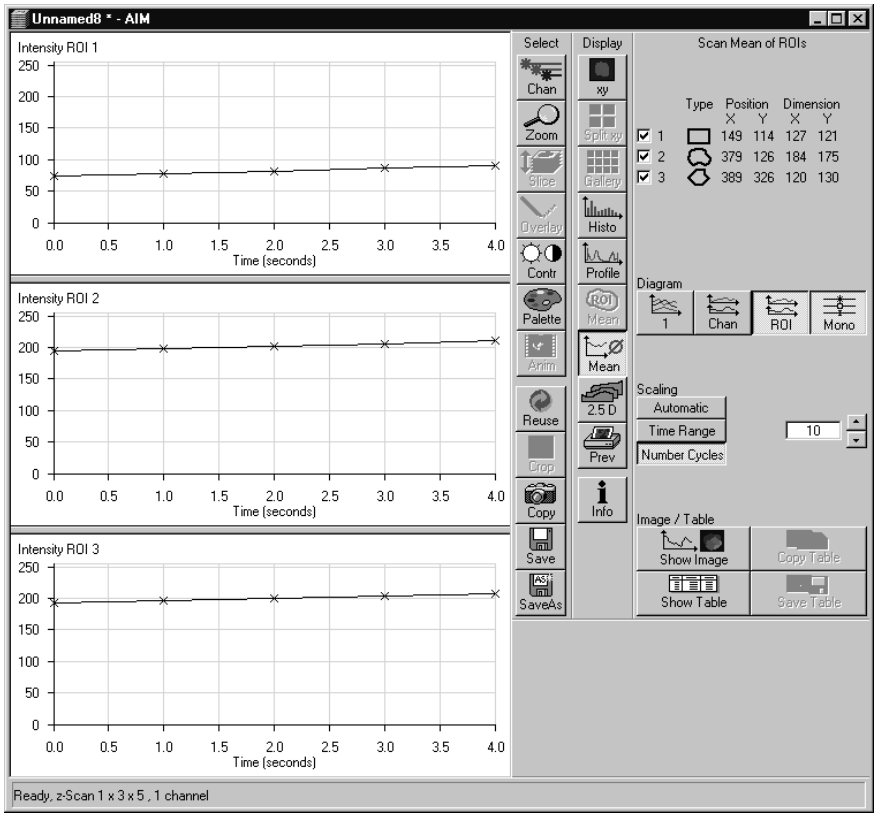


Fig. 4-96 Image Display window of a Time Series with Mean ROI

The **Image Display** window of the **Mean ROI** function is structured differently than that of a frame.

On the left-hand side of the **Image Display** window, the intensity time profiles per ROI are displayed graphically.

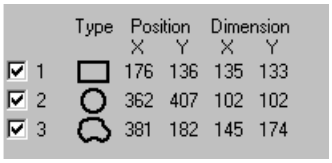
The **Select** and **Display** toolbars, which are also available in the standard **Image Display** window, are positioned in the center.

The **Scan Mean of ROIs** toolbar with further function elements is additionally displayed on the right-hand side. The major purpose of these function elements is to vary the display of the recorded Mean ROI.

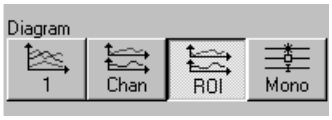
By selecting the appropriate options (see **Options** menu, **Settings** – **Scan Mean of ROIs)** you can activate the following additional functions:

- Display of the live image in the **Image Display** window of the **Mean ROI** function (used ROIs only)
- Scan of the complete image (if **Live Image** has been activated)
- Saving of the complete time series (if **Live Image** has been activated)

The following functions are available:

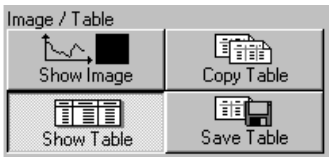


Display of the data of the ROIs used for the creation of the **MeanROI** (identical to the **Edit ROI** window). If the check box of a ROI is deactivated, the ROI's intensity values are no longer displayed in the Intensity-Time diagram.



1 button: Intensity values for ROI and Channels are displayed in a diagram. **Chan** button: Intensity values are displayed separately for each channel used. **ROI** button: Intensity values are displayed separately for each ROI used. **Mono** button: Switches between color and monochromic display of intensity profiles.

Automatic button: Automatic scaling of the display of Intensity-Time diagrams. **Time Range** button: Display of Intensity-Time diagrams is scaled depending on the Time Range set in the input box shown on the left. **Number Cycles** button: Display of Intensity-Time diagrams is scaled depending on the Number Cycle set in the input box shown on the left.



Show Image button: Shows the scan image in the **Image Display** window to the side of the intensity diagram. This button is active only if the **Live Image** option is activated. **Copy Table** button: The table of intensity values is copied to the clipboard. **Show Table** button: The table of intensity values is displayed at the bottom left of the **Image Display** window. **Save Table** button: The table of intensity values can be stored as a text file.

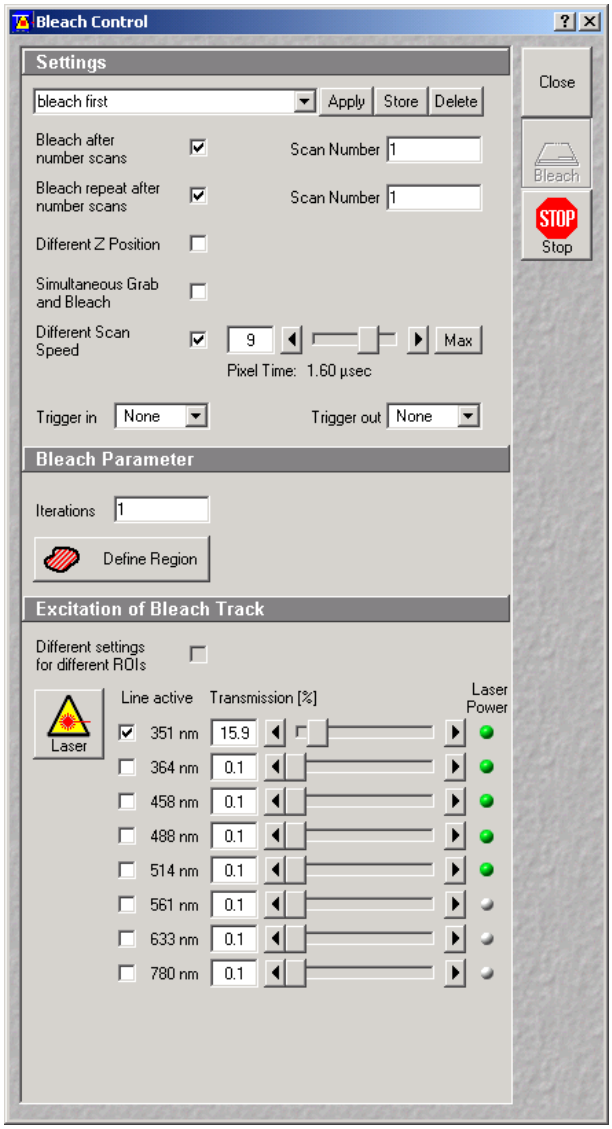


Fig. 4-97 Bleach Control window

4.5.7 Edit Bleach

The use of this function permits the setting of bleaching parameters for spot, line or frame bleaching.

4.5.7.1 Open / Close the Edit Bleach Window

- Click on the **Edit Bleach** button in the **Acquire** subordinate toolbar of the **Main** menu. The **Bleach Control** window appears on the screen.
- Click on the **Close** button to close the **Bleach Control** window.

The following functions are available on the right-hand side of the **Bleach Control** window:

Close button	The **Bleach Control** window is closed.
Bleach button	Starts the bleaching procedure. This button is only active when a region for bleaching has been defined.
Stop button	Ends the bleaching procedure.

4.5.7.2 Settings Panel

The **Settings** panel allows you to determine when, where and how the bleaching process shall be done (only works in connection with time series).

Furthermore, all the settings of the **Bleach Control** window can be stored, reactivated or deleted in this panel.

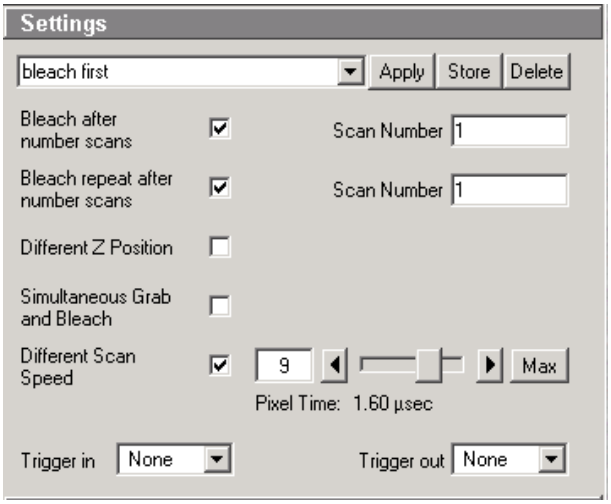


Fig. 4-98 Settings panel

Bleach after number scans: If this check box is ticked ☑, the bleaching procedure is automatically performed in combination with a time series. Under **Scan Number,** you must enter after how many scanning procedures bleaching is to be performed.

Scan Number: Number of Scans in a time series, after performance of which the bleaching procedure shall be started.

Bleach repeat after number scans: If this check box is ticked ☑, the bleaching procedure is automatically performed in combination with a time series. Under **Scan Number,** you must enter after how many scanning procedures bleaching is to be repeated.

Scan Number: Number of Scans in a time series, after performance of which the bleaching procedure shall be repeated.

Different Z Position: If this check box is ticked ☑, you can set the current stage position as the one in which the bleaching will be done by clicking the **Mark Position Z** button. This function is only available using the **Line** or **Frame** scanning mode.

Simultaneous Grab and Bleach (Only available at systems equipped with LSM *DuoScan*.): If this check box is ticked ☑, only the laser lines form the LSM *DuoScan* are available and the bleach procedure will be done simultaneously to the imaging procedure that is set up in the scan control window. For the imaging the configuration set up in the configuration control menu is used. The bleach procedure is performed according to the set up in Bleach Control window. It is not possible to chose a different Z-Position for bleaching when this function is activated.

Different Scan Speed: If this check box is ticked ☑, the speed for scanning during the bleach process can be defined independently of the scan speed during image acquisition. A lower speed results in a longer pixel dwell time which increases the efficiency of bleaching.

Different XY Spot Bleach Position: If this check box is ticked ☑, you can set a different XY position for spot bleaching. This function is only available using the **Spot** scanning mode. Click on the **Spot Select** button in the **Scan Control** window. A new image is produced and two crosshairs appear in the image. The red crosshair marks the spot that will be imaged. The green crosshair marks the spot that will be bleached. Move the center of the crosshairs to the desired positions and perform bleaching.

Store settings of the Bleach Control

Proceed as follows to store the entire settings of the **Bleach Control** window:

- Enter a name in the Settings list box and click on **Store** to store the settings.

If required, stored settings for the bleaching procedure can be reactivated quickly.

- Open the Settings list box with a click on the arrow button and select the required name with a click of the mouse.
- Then click on the **Apply** button to activate these settings. The **Bleach Control** window will be updated accordingly.

Delete settings of the Bleach Control

Settings which are no longer required can be deleted.

- Open the Settings list box and select the required name.
- Click on the **Delete** button. This stored setting will be removed.

The bleaching procedure can also be activated via keys 1 to 4 of the Trigger Control.

- Open the **Trigger in** list box with a click on the arrow button.
- Select one of the trigger keys 1 to 4 (e.g. **Trigger4**).

It is also possible to trigger an out signal via trigger control.

Fig. 4-99 Bleach Parameter panel

4.5.7.3 Bleach Parameter Panel

The **Bleach Parameter** panel allows you to determine how often the bleaching process shall be performed, and to select the area for bleaching in the scan image via the **Define Region** button.

- Enter the number of iterations of the bleaching procedure in the **Iterations** input box.
- Click on the **Define Region** button.
  - The **Bleach Regions** window appears.

The definition of bleach regions corresponds to the **Edit ROI** function and is performed in the same way (see **Edit ROI**, page 4-100).

ROIs already defined with **Edit ROI** are also available in the **Bleach Regions** window. They can be activated directly, modified - if required - and stored under a new name.

☞ ROIs newly defined in **the Bleach Regions** window will then also be available in the **Edit ROI** window.

- Define the required bleach regions in the scan image or use an existing ROI.

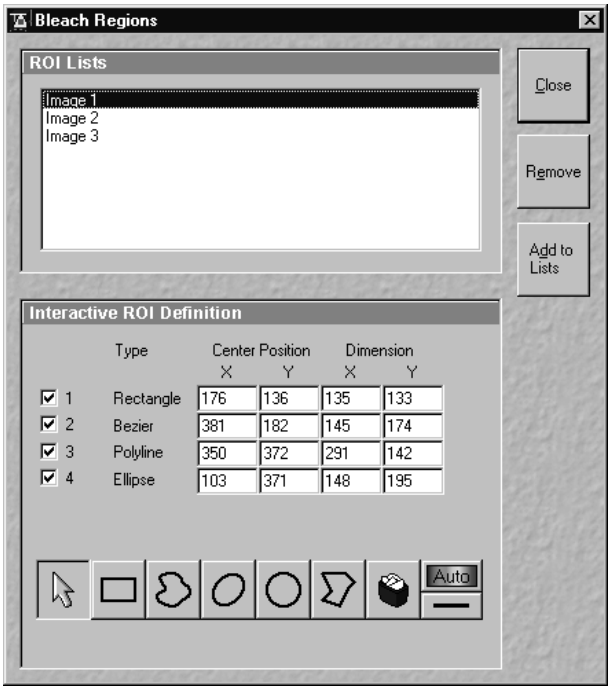


Fig. 4-100 Bleach Regions window

4.5.7.4 Excitation of Bleach Track Panel

In the **Excitation of Bleach Track** panel you can select the lasers and laser intensities for bleaching.

☞ If the system is equipped with LSM *DuoScan* and the check box **Simultaneous Grab and Bleach** is ticked, only those lasers are displayed which are available through the LSM *DuoScan*.

The setting of the lasers for the bleaching procedure corresponds to that for the scanning procedure and must be performed accordingly (see **Laser Control**, **Configuration Control** and **Scan Control**).

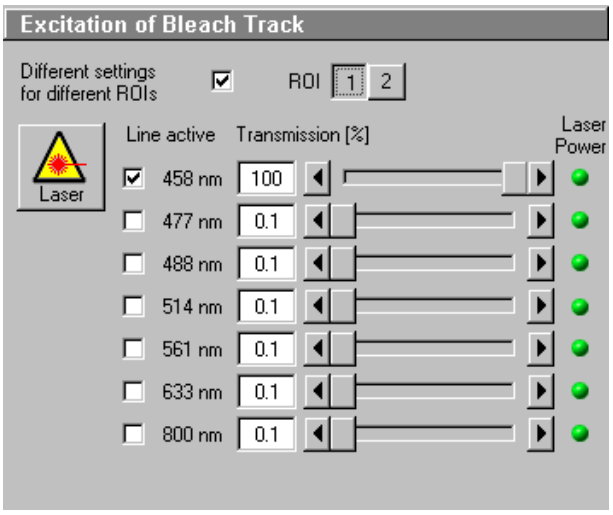


Fig. 4-101 Excitation of Bleach Track panel

- Select the required laser wavelength and its intensity under **Excitation**.
- If required, switch the relevant laser to **On** (**Laser** button).
- If more than one ROI has been defined under Bleach Parameters, a different laser intensity and laser line can be defined for each region. Toggle between the Buttons indicating the number of the ROI and check the laser line and intensity for each ROI.

4.5.7.5 Start / End a Bleaching Procedure

- The bleaching process will be started via the **Bleach** button. Only the bleach protocol set up will be executed but no image is displayed. It is also possible to start the bleaching process via the **Bleach** button in the **Time Series Control** window or to combine it with a time series and hence with imaging. To perform the latter it is necessary to define the number of images to be acquired before bleaching and to define the region for bleaching.

When a trigger key is activated to start the bleaching procedure, the **Waiting for Trigger** message first appears in the status line of the **Bleach Control** window. In that case, the bleaching procedure is started after activation of the relevant trigger key.

- The bleaching process can be finished via **Stop** in the **Bleach Control** window.

☞ **Stop** does not only stop the bleaching process, but the entire scanning process.

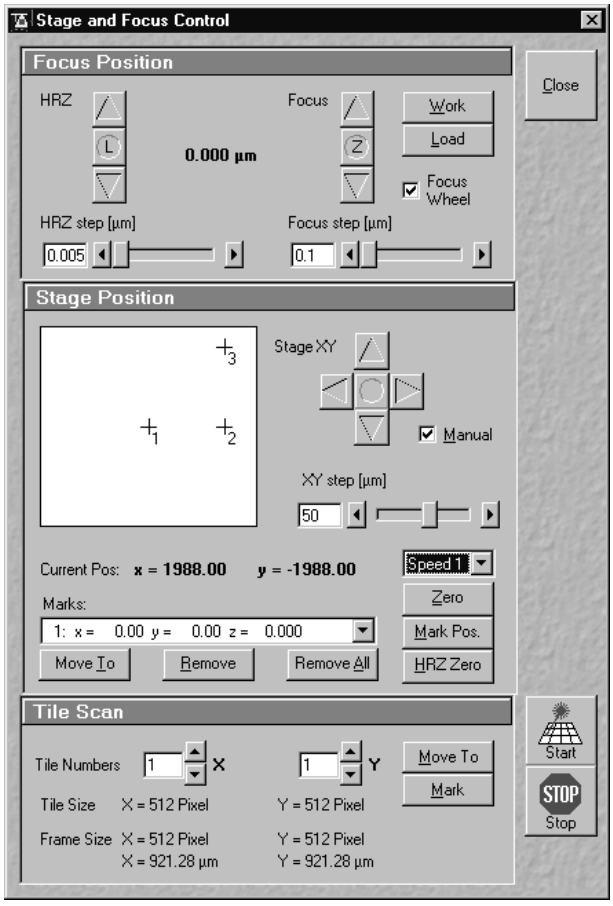


Fig. 4-102 Stage and Focus Control window

4.5.8 Stage

The following software description applies to systems which are equipped with a motorized stage.

This window enables you to activate both the motor focus and the scanning stage.

The **Focus Position** and **Stage Position** panels include the function keys for the performance of defined moves and the display of the current Z and X, Y positions.

By use of an LSM 510 META scanhead on an Axiovert 200 M sideport system care should be taken when moving the motorized XY scanning stage to the maximum positions, so that fingers are not bruised between scan head and stage.

4.5.8.1 Open / Close the Stage and Focus Control Window

- Click on the **Stage** button in the **Acquire** subordinate toolbar of the **Main** menu.
  - The **Stage and Focus Control** window appears on the screen.
- Click on the **Close** button in the **Stage and Focus Control** window to close this window.

The following functions are available on the right-hand side of the **Stage and Focus Control** window:

Close button — The **Stage and Focus Control** window is closed.

Start button — Starts the tile scanning procedure.

Stop button — Ends the scanning procedure.

4.5.8.2 Focus Position Panel

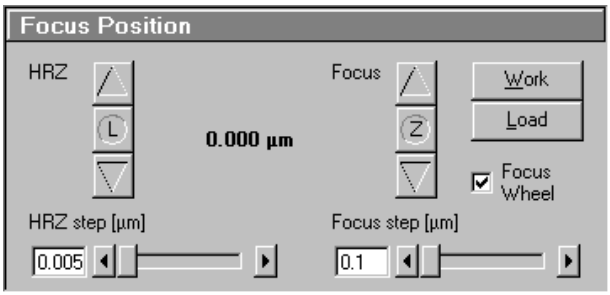


Fig. 4-103 Focus Position panel

Focus buttons (Z Moves)

Clicking on the ▲ button moves the specimen stage / nosepiece upwards.

Clicking on the **Z** button sets the current Z-position to zero.

Clicking on the ▼ button moves the specimen stage / nosepiece downwards.

Focus Step slider

0.1 µm is the smallest value which can be set, and 100 µm the highest.

- Clicking on the arrow keys changes the step size by 1 µm.
- Pressing the **CTRL** key and clicking changes the step size by 0.05 µm.
- Pressing the **Shift** key and clicking changes the step size by 10 µm.

Work button

Pressing the **Work** button moves the specimen stage / nosepiece back to the Work position. This is the position last set before the **Load** button was pressed.

Load button

Clicking on the **Load** button lowers the specimen stage / nosepiece to make it easier for you to change the specimen (or objective).

Focus Wheel check box

Clicking on this check box activates / deactivates the focus wheel of the microscope.

Use of the optional piezo objective focusing device

The **HRZ Step** slider is used to set the step width of the fine focusing device.

- Use the arrows ▲ and ▼ of **HRZ** to move the fine focusing device upwards or downwards in steps.

As soon as the focus position is changed (via handwheel or software), the piezo objective focus is automatically leveled.

- A click on the **L** button moves the piezo objective focus in the center position of its travel range and the focus position is reset accordingly. Therefore, the same Z-level remains visible (the current position is not set to zero).

The motor focus of the stand is operated in the same way via the relevant buttons. Moving into the **Work** or **Load** position is always performed via the motor focus and not via the piezo objective focus.

☞ Please see CHAPTER ANNEX of the printed manual for further information on the piezo objective focus.

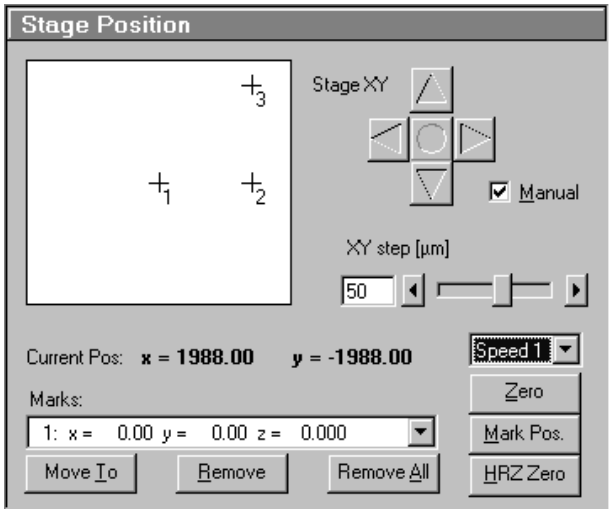


Fig. 4-104 Stage Position panel

4.5.8.3 Stage Position Panel

The **Stage Position** panel shows a symbolic specimen carrier in the left upper.

The buttons for moving to a position and mark it are below or on its right.

The **Current Position** display for X and Y is below.

Below that, you will find the **Marks** selection box of marked positions and the possibility to activate and delete them.

Moving the scanning stage

The scanning stage can be moved using the joystick, or software-controlled using the **Stage XY** buttons, or manually.

Stage XY buttons

- Clicking on the arrow buttons moves the stage in X or Y direction.
- Clicking on the **Center** button moves the stage in the XY = 0 position.

XY Step slider

1 µm is the smallest value which can be set for XY movement, and 100 µm the highest.

Manual check box

This check box activates / deactivates the motor control of the stage and the joystick, if available.

If **Manual** is active, the scanning stage can be moved manually via the knurled screws. The **Move To** and **Center** function buttons in **Stage Position** are without a function. The **Current Position** is updated. You can zero the display via **ZERO** and mark manually set positions (**Mark pos.**).

The scanning stage cannot be moved via the software or the joystick.

If **Manual** is deactivated, the scanning stage can be moved via the software or the joystick. All the functions of the **Stage Position** window are available.

Current Pos(ition) field

Current Pos displays the currently set stage position in relation to the zero position.

Marks selection box

Clicking on the arrow button displays the table of the session-related marked specimen areas. The table includes the ordinal number, the X-position and the Y-position. Click on the appropriate mark to select it for operation.

Move To button

Clicking on the **Move To** button moves the stage to the position selected before from the **Marks** selection box.

Remove

The **Remove** command enables a selected position to be deleted from the table. The position then also disappears from the specimen carrier display.

☞ The selected position is deleted, the position with the next number in sequence moves up one number.

Remove All

The **Remove All** command deletes all the entries marked in the current session.

Speed selection box

Clicking on the arrow key displays the table of the available speeds for stage movement. Click on the appropriate speed to select it for operation.

Zero button

Zeros the **Current Position** display and thus sets the currently set stage position to 0 in relation to X and Y. The already marked object areas thus receive new X and Y-coordinates.

Mark Pos. button

Mark Pos. allows the **Current Position** to be marked. This marked position is then stored in the **Marks** selection box in sequence. The marked position is shown on the specimen carrier with a cross and its ordinal number.

HRZ Zero button

Zeros the **Current Position** display and thus sets the currently set stage position to 0 in relation to X and Y. The already marked object areas thus receive new X and Y-coordinates.

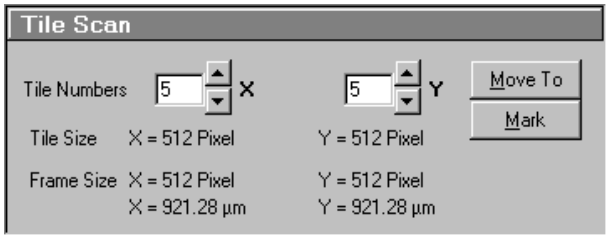


Fig. 4-105 Tile Scan window

4.5.8.4 Tile Scan Panel

This function permits a frame to be created as an overview image of the specimen with a maximum size of 4096 x 4096 pixels. According to settings, such a frame is divided in XY-tiles of 1 x 1 to the maximum of 15 x 15. A tile of special interest (target) can then be selected for scanning.

The application of the **Tile Scan** function requires an objective with a minimum magnification factor of 2.5x.

Tiles Numbers X / Y input box

Input of the number of tiles for **X** or **Y** from which the frame is to be composed.

Tile Size X / Y display

Display of the size of a single tile in µm (corresponds to the value selected in the **Scan Control** window).

Frame Size X / Y display

Display of the frame size of the tile scan for **X** or **Y**. Specification in pixels and µm.

Move To button

If the **Move To** button is activated, a rectangle with a target allowing the selection of the region of interest is positioned in the center of the scanned frame. Click and hold down the left mouse button to drag the rectangle to the required specimen area. When you release the mouse button, the stage moves to the selected position.

Mark button

If the **Mark** button is activated, marks previously set in the Tile Scan image are displayed, and further marks can be added at spots of special interest by a mouse click in the Tile Scan image. By activating the **Move To** button, the stage can be moved to the individual marks set in Tile Scan in the same way as it is moved to the marks set in the **Stage Position** panel.

Creating an overview image

- Set the number of tiles for the frame in the **Tiles Numbers X / Y** input boxes of the **Tile Scan** window.
  - The resulting frame size is displayed on-line.

- Click on **Start**.
  - The overview frame is scanned and displayed on the screen in a new **Image Display** window.

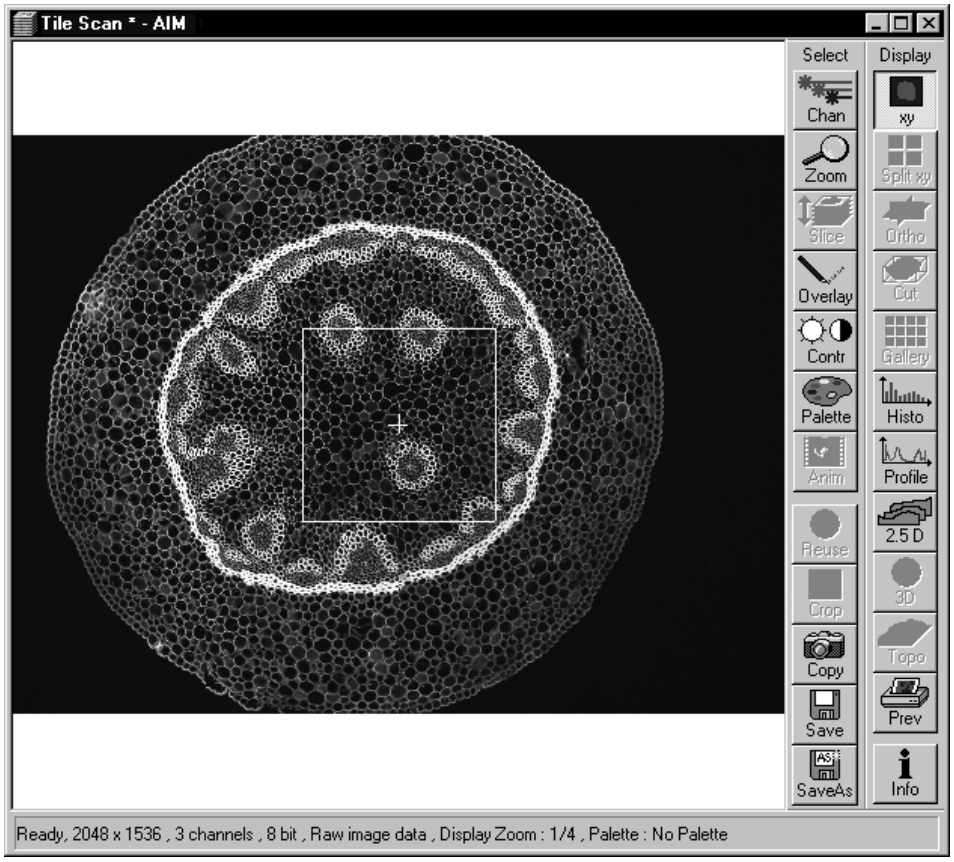


Fig. 4-106 Image Display window of a Tile Scan

- Activate the **Move To** button.
- In the tile scan image, move the target to the required spot of the frame (dragging with the mouse).
  - The microscope stage then travels to the selected position.

Or:

- Activate the **Mark** button.
- Set a mark at the spot of interest by clicking with the mouse in the Tile scan image. A cross with the consecutive number of the mark is displayed in the Tile Scan image. The new mark is also displayed in the specimen carrier (**Stage Position** panel) and included in the **Marks** selection box.
- Select the mark in the **Marks** selection box and click on the **Move To** button in the **Stage Position** panel. The stage moves to the selected position.
- Then click on the **Single** button in the **Scan Control** window to scan the selected area as a single image.
  - The single image is scanned and displayed in a new **Image Display** window.

☞ **Overlay** functions cannot be activated in the **Tile Scan Image Display** window.
The created overview frame can then be stored like any other scan image. If a stored overview frame is opened again, the rectangle with target will appear again. However, it can be deleted using the **Overlay** function.

4.5.9 VIS, TV and LSM Buttons

The **VIS**, **TV** and **LSM** buttons are included in the **Acquire** subordinate toolbar of the **Main** menu.

They switch the beam path and indicate which beam path has been set in the binocular tube of the microscope:

- **VIS**: observation via the eyepieces of the binocular tube, lasers are off
- **TV**: camera observation (if connected) via camera adapter of the binocular tube
- **LSM**: screen observation via laser excitation using the LSM 510 and software evaluation

☞ If the beam path of the microscope is changed manually via buttons on the tube this is recorded by the software and the relevant button is activated automatically.

Vice versa, beam path can be "switched" via activation of the appropriate buttons in the software.

4.6 Process Menu

The functions of the **Process** menu permit already stored scan images to be subsequently linked and processed using mathematical functions and algorithms.

- In the **Main** menu toolbar, click on **Process**.
  - This opens another, subordinate toolbar in the **Main** menu.

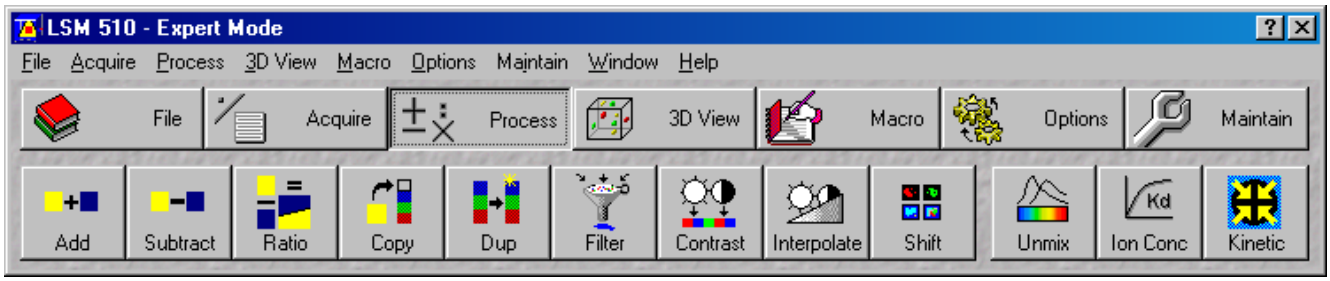


Fig. 4-107 Process menu

4.6.1 Add

The **Add** function links two channels each of one or two images into a new channel through addition. The channel created in this way can be stored via the **Save As** function. (This also works with extracted META channels.)

4.6.1.1 Open / Close the Add Window

- Click on the **Add** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Add** window.
- Click on the **Close** button to quit the **Add** window.

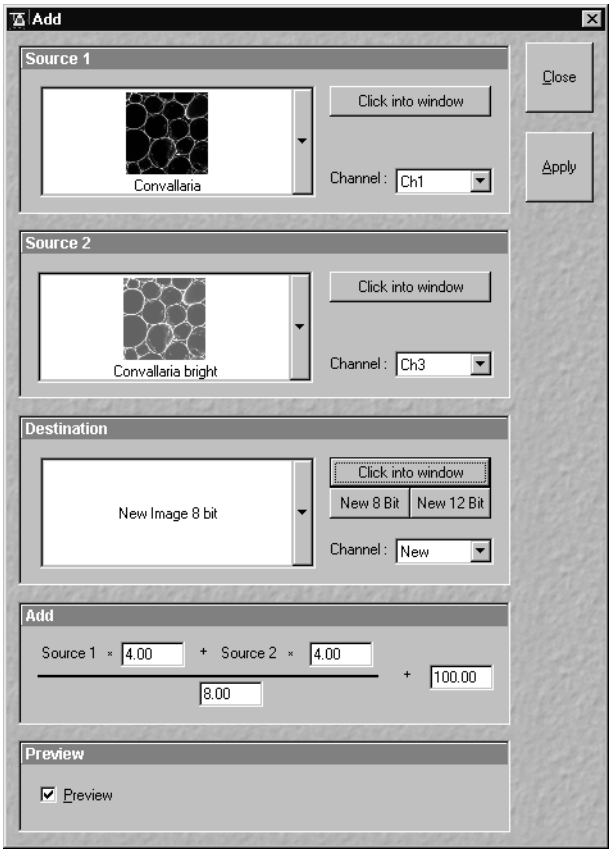


Fig. 4-108 Add window

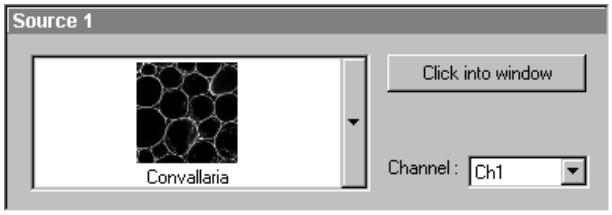


Fig. 4-109 Source 1 panel

4.6.1.2 Source Panel

In the **Source 1** panel, the first image source for the addition process is determined. The current image is displayed in the display box of the image selection box.

Proceed as follows to select an image via the image selection box:

- Click on the arrow button. The image selection box is opened and all the currently loaded images are displayed in a minimized form.
- Click on the required image. This image will then appear in the display box of the image selection box and has been selected as Source 1.

Use the **Click into window** button to directly select the opened image:

- Click on the **Click into window** button first and then double-click on the relevant **Image Display** window. The selected image will then be displayed in the display box of the image selection box and has been activated as Source 1.

The channel which is to be used for the **Add** operation is selected via the **Channel** selection box:

- Click on the arrow button. The **Channel** selection box is opened and shows all the recorded channels of the relevant image.
- Click on the required channel to activate it.

In the **Source 2** panel, the second image source for the addition process is determined. The procedure is identical to that for Source 1.

- Select the image for Source 2 and the relevant channel.

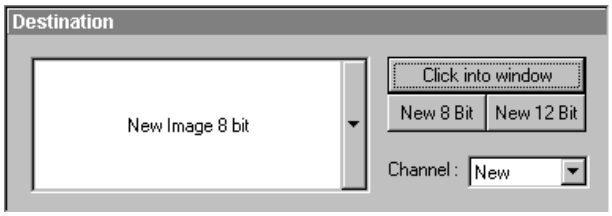


Fig. 4-110 Destination panel

4.6.1.3 Destination Panel

In the **Destination** panel, it is determined in which **Image Display** window the **Add** operation is performed, and the data format which the newly created image shall have.

The **Add** operation can be performed in an already opened window or in a new **Image Display** window.

- Click on the arrow button of the image selection box to open this box.
- Click on the relevant image if the **Add** operation shall be performed in an existing **Image Display** window.

  or

- Click on New Image 8 bit or New Image 12 bit to use a new Image Display window.

☞ You can also use the **Click into window** button for image selection.
Clicking on the **New 8 bit** or **New 12 Bit** button enables you to determine directly and quickly whether the new image is to be created in the 8-bit or 12-bit format.

If an existing **Image Display** window is used to perform the Add function, you must determine whether an existing channel shall be overwritten with the Add operation or whether a new channel shall be added.

- In the **Channel** selection box, click on the channel which shall be overwritten, or click on **New** for a new channel.

4.6.1.4 Add Panel

In the **Add** panel, the currently set formula for the **Add** operation is displayed. The editable input boxes permit the formula to be changed with any numeric values.

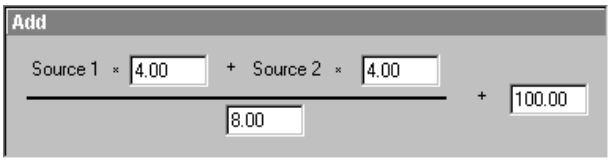


Fig. 4-111 Add panel

- Click in the required input box and enter the relevant value.
- Click on the **Apply** button to perform the operation in the activated window or a new **Image Display** window.
- The new image can then be stored via the **Save As** function.

4.6.1.5 Preview Panel

The Preview function enables you to preview the result of the defined **Add** operation in a preview window.

Fig. 4-112 Preview panel

- Activate the ☑ **Preview** check box with a click of the mouse. The **Add - Preview Image Display** window is displayed with the operation result.
- Deactivate the **Preview** check box to close the **Add - Preview Image Display** window.

☞ After a change of the formula in the **Add** panel, click in the **Add - Preview Image Display** window for an update.

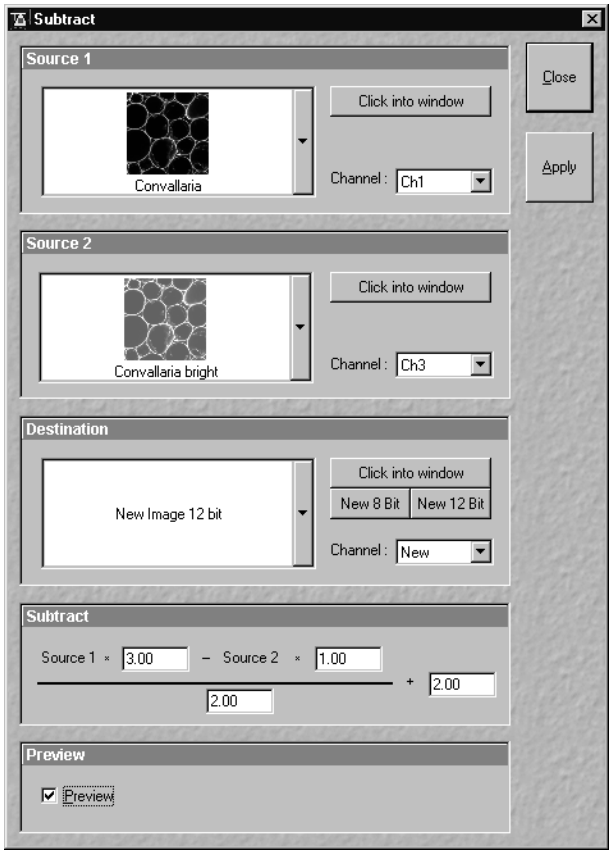


Fig. 4-113 Subtract window

4.6.2 Subtract

The **Subtract** function links two channels each of one or two images into a new channel by subtraction. The channel created in this way can be stored via the **Save As** function.

4.6.2.1 Open / Close the Subtract Window

- Click on the **Subtract** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Subtract** window.
- Click on the **Close** button to quit the **Subtract** window.

4.6.2.2 Performance of the Subtract Function

This function is performed in the same way as the **Add** function (see **Add**, page 4-129). The only difference is that the mathematical formula is based on subtraction.

4.6.3 Multiply

The **Multiply** function permits two channels each to be linked into a new channel by multiplication. The channel created in this way can be stored via the **Save As** function.

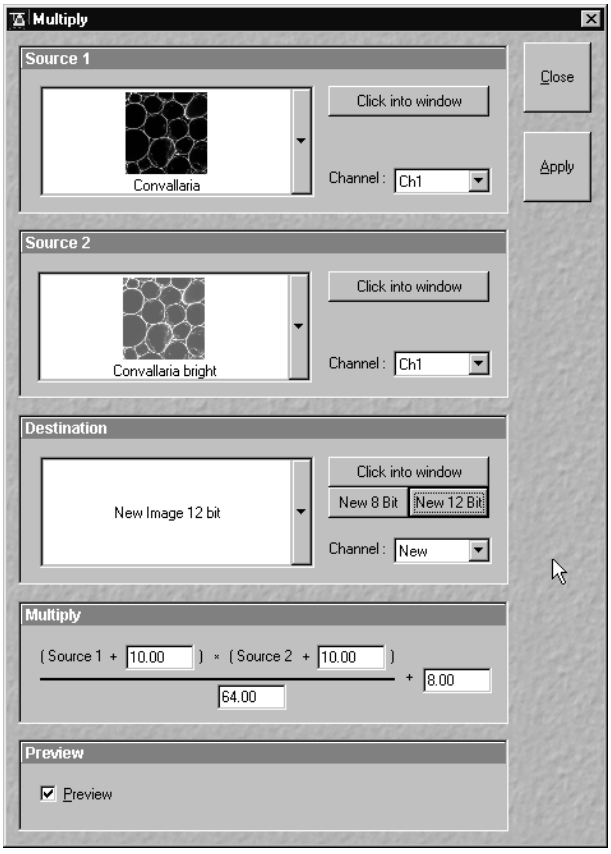


Fig. 4-114 Multiply window

4.6.3.1 Open / Close the Multiply Window

- Click on **Multiply…** in the **Process** pull down menu of the **Main** menu.
  - This opens the **Multiply** window.
- Click on the **Close** button to quit the **Multiply** window.

4.6.3.2 Performance of the Multiply Function

This function is performed in the same way as the **Add** function (see **Add**, page 4-129). The only difference is that the mathematical formula is based on multiplication.

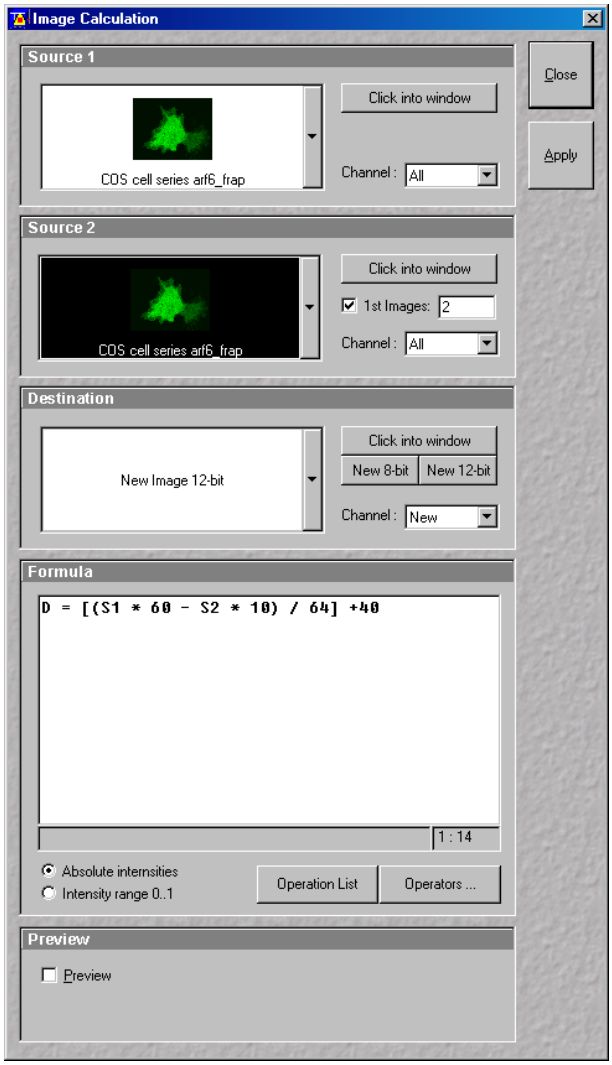


Fig. 4-115 Image Calculation window

4.6.4 Ratio

The **Ratio** function permits to create a new image or image series by offsetting two images or image series against each other. The channel created in this way can be stored via the **Save As** function.

4.6.4.1 Open / Close the Ratio Window

- Click on the **Ratio** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Ratio** window.
- Click on the **Close** button to quit the **Ratio** window.

4.6.4.2 Performance of the Ratio Function

This function is performed in the same way as the **Add** function (see **Add**, page 4-129).

However, self edited formulas can be used for image calculation.

4.6.4.3 Formula Panel

The formulas can be typed in using the keyboard.

- Click the **Operators** button to open the permissible operators list.
- Select the operator(s) from this list (Fig. 4-116) and click the **Insert** button.

  The operators will be inserted into the formula at the cursor position. By highlighting the selected operator a description of the function of this operator is displayed in the lower part of the operators list.

The calculation works pixel by pixel and starts with the upper left pixel irregardless of the image size of the two images or image series.

☞ When choosing images of different data depth the check box next to **Intensity range 1…0** should be marked. The image intensity for all images will then be normalized to values between 1 and 0.

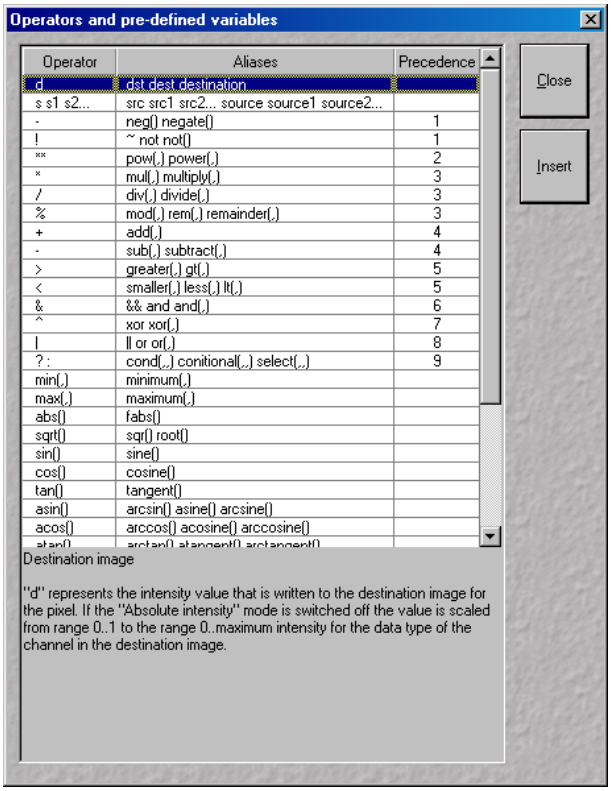


Fig. 4-116 Operators window

4.6.5 Copy (Channel)

The **Copy** function permits one channel each of an existing image to be copied and stored as a new image.

The selection of Source, Channel and Destination is made in the same way as in the **Add** function (see **Add**, page 4-129).

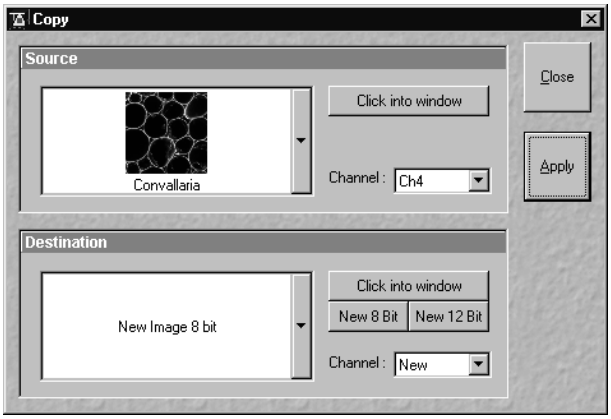


Fig. 4-117 Copy window

4.6.5.1 Open / Close the Copy Window

- Click on the **Copy** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Copy** window.
- Click on the **Close** button to quit the **Copy** window.

4.6.5.2 Performance of the Copy Function

- Select Source, Channel and Destination and then click on the **Apply** button.

– The image of the copied channel is then displayed in a new window or in the **Image Display** window activated for it.

- The new image can be stored via the **Save As** function.

☞ For Z Stacks or Time Series, the entire series of the selected channel is copied.

4.6.6 Duplication (Image)

This function permits images (including Z Stacks and Time Series) to be duplicated completely.

- If several images have been opened, select the image to be duplicated.
- Click on the **Dup** button in the **Process** subordinate toolbar of the **Main** menu.
  – The selected image is duplicated and displayed in a new **Image Display** window.
- Use the **Save As** function to store the image under a new name.

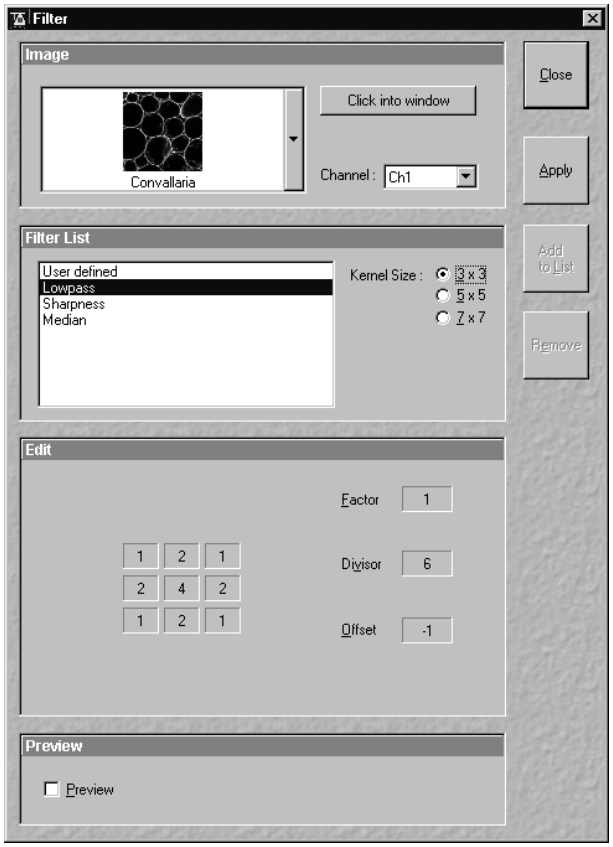


Fig. 4-118 Filter window

4.6.7 Filter

The filter function permits the subsequent processing of scanned images via the integrated **Lowpass**, **Sharpness** and **Median** filters. Furthermore, **User-defined** filters can be installed by the user. User-defined filters can be stored, reloaded and removed.

4.6.7.1 Open / Close the Filter Window

- Click on the **Filter** button in the **Process** subordinate toolbar of the **Main** menu.
  – This opens the **Filter** window.
- Click on the **Close** button to quit the **Filter** window.

4.6.7.2 Image Panel

In the **Image** panel, the image or channel to be processed is selected.

The currently selected image is displayed in the image selection box.

Proceed as follows to select an image via the image selection box:

- Click on the arrow button. The image selection box is opened and all the currently loaded images are displayed in a minimized form.
- Click on the required image, which will then appear in the display box of the image selection box and will be available for filtering.

☞ You can also use the **Click into window** button to select the image.

- Open the **Channel** selection box with a click on the arrow button and select the channel to be processed.

4.6.7.3 Filter List and Edit Panel

In the **Filter List** panel, the filters and the matrix size (**Kernel Size**) are selected.

The matrix of the selected filter and the set filter parameters **Factor**, **Divisor** and **Offset** are displayed in the **Edit** panel.

(1) Kernel Size

The size of the filter matrix can be modified here. The effect of a filter increases along with the matrix size. However, this also increases the time required for filtering.

- Select the required matrix size by clicking on one of the selection buttons **3 x 3**, **5 x 5** or **7 x 7**.

(2) Lowpass filter

With the lowpass filter, the gray value of each center pixel is replaced with the average value of the surrounding neighbor pixels. The viewed neighbor pixels are defined by a square. The modified pixel now is the center pixel of the filter matrix.

Image noise will be reduced by the application of the lowpass filter. The cutoff of regions will blur. Local maxima will be flattened. The dynamic range will be reduced considerably.

This filter permits the matrix size to be modified only in the 3 preset steps.

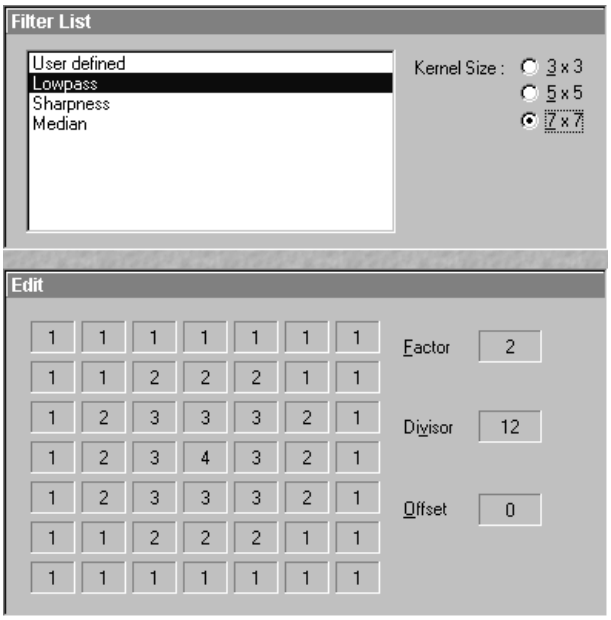


Fig. 4-119 Filter List and Edit panel (Lowpass)

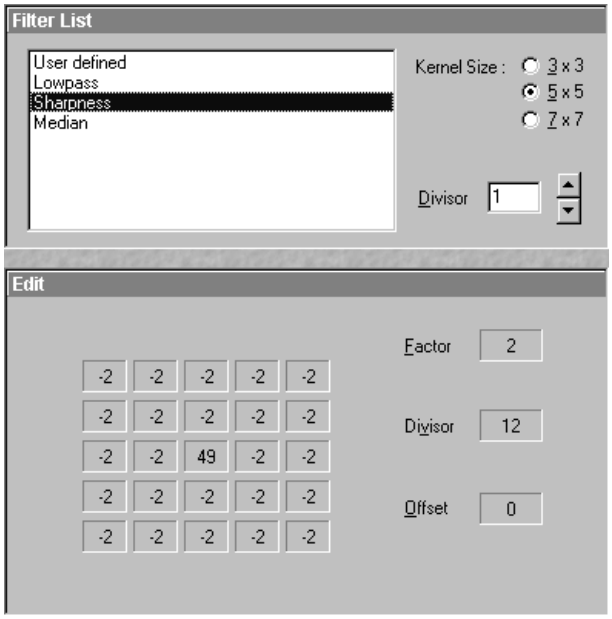


Fig. 4-120 Filter List and Edit panel (Sharpness)

(3) Sharpness filter

With the sharpness filter, the original image is filtered with a lowpass filter first. The result of this filtering is then subtracted from the original image.

This will improve image sharpness.

The matrix size can be modified in the 3 preset steps.

Furthermore, divisor values ranging from **1** to **78** can be entered. The higher the divisor value, the lower the image sharpness.

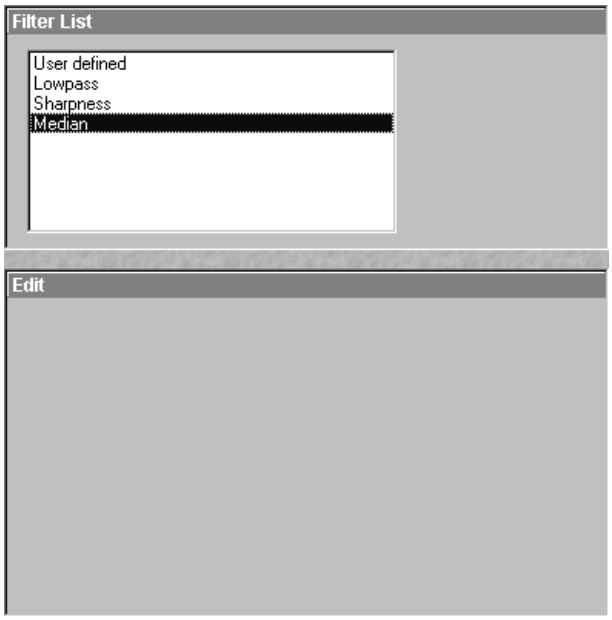


Fig. 4-121 Filter List and Edit panel (Median)

(4) Median filter

With the median filter, the gray value of each center pixel is replaced with the median value of the surrounding neighbor pixels. The viewed neighbor pixels are defined by a square. The modified pixel now is the center pixel of the filter matrix.

The median value is defined as the middle value (not average) of all the gray values sorted in ascending order within a matrix.

Image noise will be reduced by the application of the median filter. The cutoff of regions will slightly blur. Local maxima will be flattened. The dynamic range will be reduced considerably.

The settings of this filter can not be modified.

(5) User-defined filter

The **User-defined** function permits you to create your own filters. In addition to the **Kernel Size**, the parameters **Factor**, **Divisor** and **Offset** can be modified here.

The filter result can be subtracted from the original image via the **Subtract from Source** check box.

Proceed as follows to store **User-defined** filters:

- Click on the **Add To List** button and enter a name in the **Add Filter To List** window. The name will be included in the **Filter List**.

Proceed as follows to activate stored, **User-defined** filters:

- Click on the name of the filter in the **Filter List**. The filter will then be activated immediately.

Proceed as follows to delete **User-defined** filters:

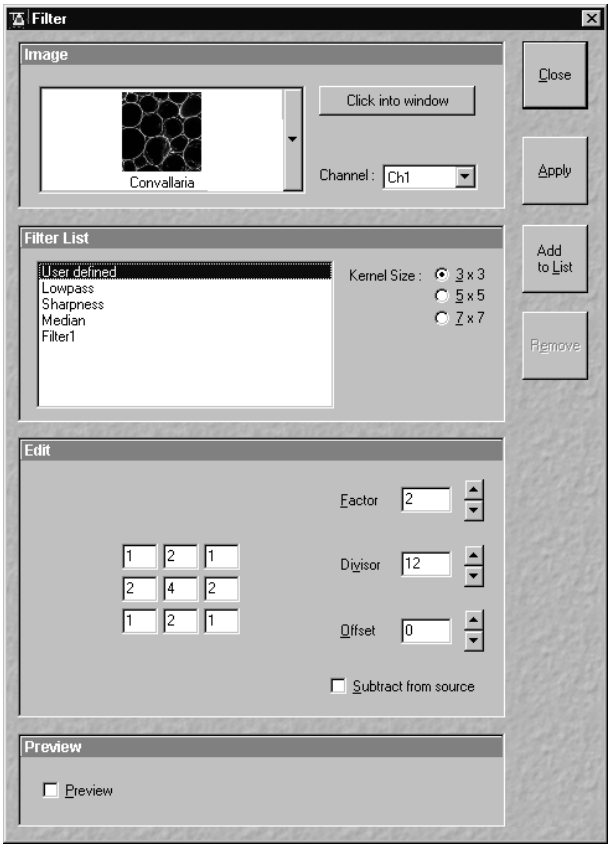


Fig. 4-122 Filter window (User-defined filter)

- Click on the name of the filter in the **Filter List** and then on the **Remove** button. The filter will be deleted.

- After selection of the required filter, click on the **Apply** button to start the filter procedure.
  - Filtering will be performed and displayed in the current **Image Display** window.
- In the case of images with several channels, activate the **xy** button in the **Display** image toolbar to display all the channels. Each channel must be filtered separately.
- Use the **Save As** function to store the newly created image.

Fig. 4-123 Add Filter to List panel

Fig. 4-124 Preview panel

4.6.7.4 Preview Panel

The Preview function allows you to have the result of the **Filter** operation displayed as a preview image.

- Activate the ☑ **Preview** check box with a click of the mouse. The **Filter - Preview Image Display** window with the filter result will be displayed.
- Deactivate the **Preview** check box to close the **Filter - Preview Image Display** window.

☞ After a change of the filter settings, click in the **Filter - Preview Image Display** window once to update it.

4.6.8 Contrast

The **Contrast** function permits the subsequent modification of contrast and brightness of the stored image.

- Open the image to be processed and click on the **Contrast** button.
  - The function is performed with firmly set parameters and the result is displayed in a new **Image Display** window. The procedure can be repeated as often as required.
- The newly created image can be stored using the **Save As** function.

4.6.9 Interpolate

This function permits the continuous contrast and brightness change in a Z- stack or Z- stacks over time through interpolation between the starting and end values. This permits the subsequent compensation of signal loss when imaging further into deeper tissue regions where the excitation efficiency decreases but also the detection efficiency. Interpolation can be defined for the entire image or only for individual channels.

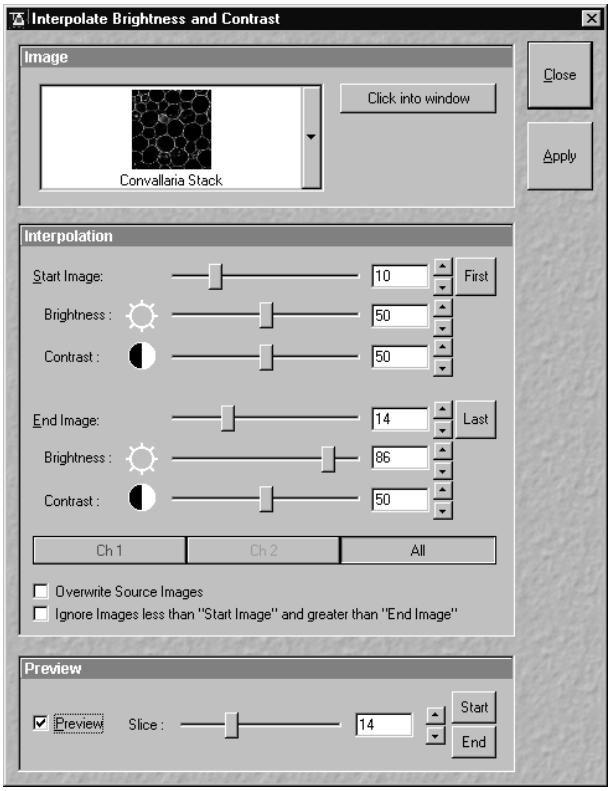


Fig. 4-125 Interpolate Brightness and Contrast window

4.6.9.1 Open / Close the Interpolate Brightness and Contrast Window

- Click on the **Interpolate** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Interpolate Brightness and Contrast** window.
- Click on the **Close** button to quit the window.

4.6.9.2 Image Panel

The image to be processed is selected in the **Image** panel.

The currently selected image is shown in the display box of the image selection box.

Proceed as follows to select a series via the image selection box:

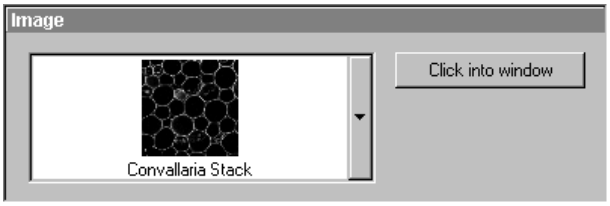


Fig. 4-126 Image panel

- Click on the arrow button. The image selection box will be opened and all the currently loaded images will be displayed in a minimized form.
- Click on the required image, which will then appear in the display box of the image selection box and has been selected for the interpolation procedure.

☞ You can also use the Click into window button for image selection.

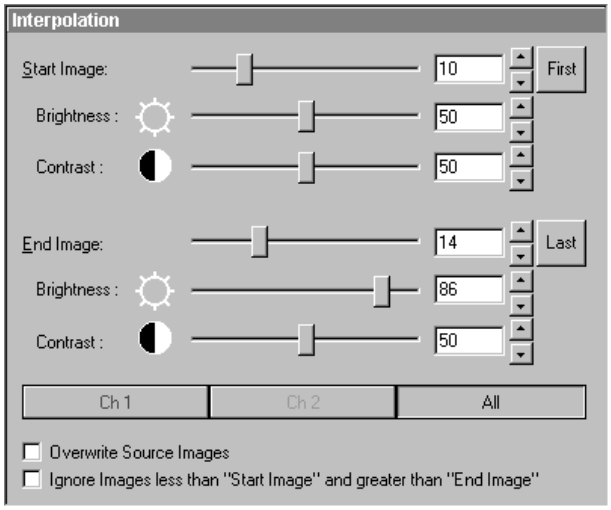


Fig. 4-127 Interpolation panel

4.6.9.3 Interpolation Panel

In the **Interpolation** panel, the parameters for the interpolation procedure are set.

- Use the **Start Image** slider to select the slice at which the interpolation procedure shall start. Clicking on the **First** button permits the fast selection of the first slice in the series.
- Use the **Brightness** and **Contrast** sliders to set the image brightness and contrast for the first slice (**Start Image**).
- Use the **End Image** slider to select the slice at which the interpolation procedure shall end. Clicking on the **Last** button permits the fast selection of the last slice in a series.
- Use the **Brightness** and **Contrast** sliders to set the image brightness and contrast for the last slice (**End Image**).
- Use the available Channel buttons (e.g.: **Ch1**) to select the channel for interpolation or click on the **All** button if the entire image is to be interpolated.
- Having set the parameters, click on the **Apply** button. Interpolation will be performed in a new **Image Display** window.
- The newly created image (series) can be stored using the **Save As** function.

☞ If you activate the **Overwrite Source Images** check box, interpolation will be performed in the current **Image Display** window.
If you activate the **Ignore Images less than "Start Image" and greater than "End Image"** check box, only the slices lying between Start Image and End Image will be taken into consideration for interpolation. Otherwise, brightness and contrast will also be changed for the other slices.

4.6.9.4 Preview Panel

The **Preview** function enables you to see the result of interpolation for one slice each in a preview window.

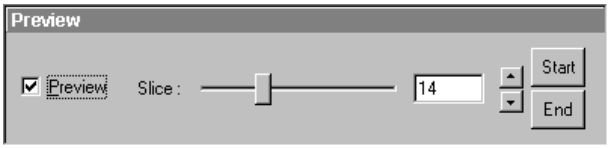


Fig. 4-128 Preview panel

- Activate the ☑ **Preview** check box with a click of the mouse.
  - The **Interpolate C&B - Preview Image Display** window will be displayed. At the same time, the **Slice** slider with the relevant input box and arrow keys and the two buttons **Start** and **End** are displayed in the **Preview** panel.
- Use the slider or input box / arrow keys to set the slice which shall be displayed in the preview window.
- Clicking on the **Start** or **End** button permits the fast activation of the **Start Image** or **End Image** for previewing.
- Deactivate the **Preview** check box to close the **Interpolate C&B - Preview Image Display** window.

4.6.10 Channel Shift

The **Channel Shift** function is used to produce a congruent image with relation to the pixels of the various channels.

This pixel correction function is particularly important in UV applications.

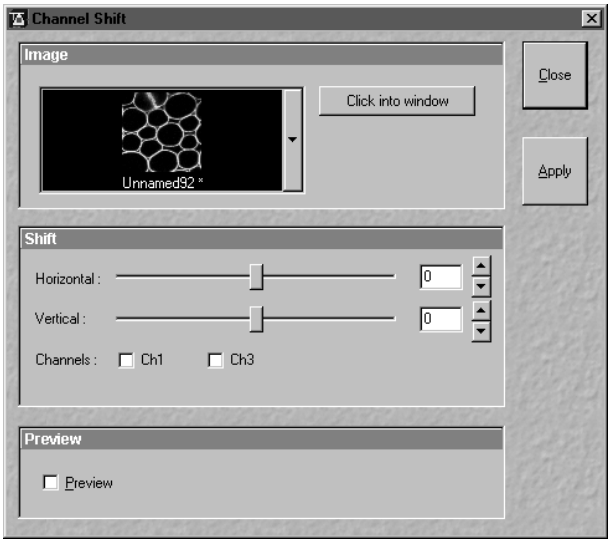


Fig. 4-129 Channel Shift window

4.6.10.1 Open / Close the Channel Shift Window

- Click on the **Shift** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Channel Shift** window.
- Click on the **Close** button to quit the window.

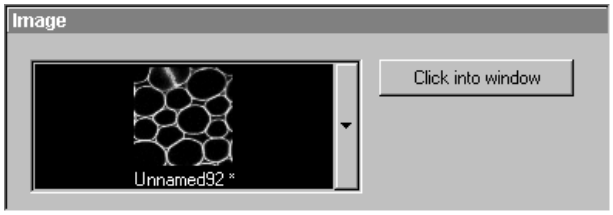


Fig. 4-130 Image panel

4.6.10.2 Image Panel

- Click on the arrow button. The image selection box will be opened and all the currently loaded images are displayed in a minimized form.
- Click on the required image, which will then appear in the display box of the image selection box and has been selected for the **Shift** function.

☞ You can also use the **Click into window** button for image selection.

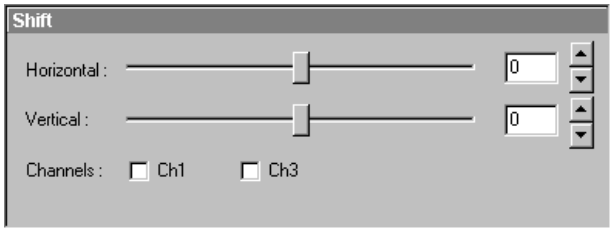


Fig. 4-131 Shift panel

4.6.10.3 Shift Panel

- Select the channels required for processing in the **Shift** panel by clicking on the **Ch1** or **Ch3** buttons. A tick ☑ will appear in the button when the channels are activated.
- Use the scrollbar or the ▲ and ▼ buttons to select the pixel shift in the horizontal and vertical direction.
- Click on the **Apply** button to activate the setting.

4.6.10.4 Preview Panel

- If ☑ **Preview** is activated, a preview of the shift is shown in a separate **Image Display** window.

Fig. 4-132 Preview panel

The following image shows the result of a pixel shift via the **Shift** function. This image change can be stored in the image database via the **Save** or **Save As** buttons.

For applications requiring 3- or 4-channel scanning, proceed in the same way as described for the 1- or 2-channel mode.

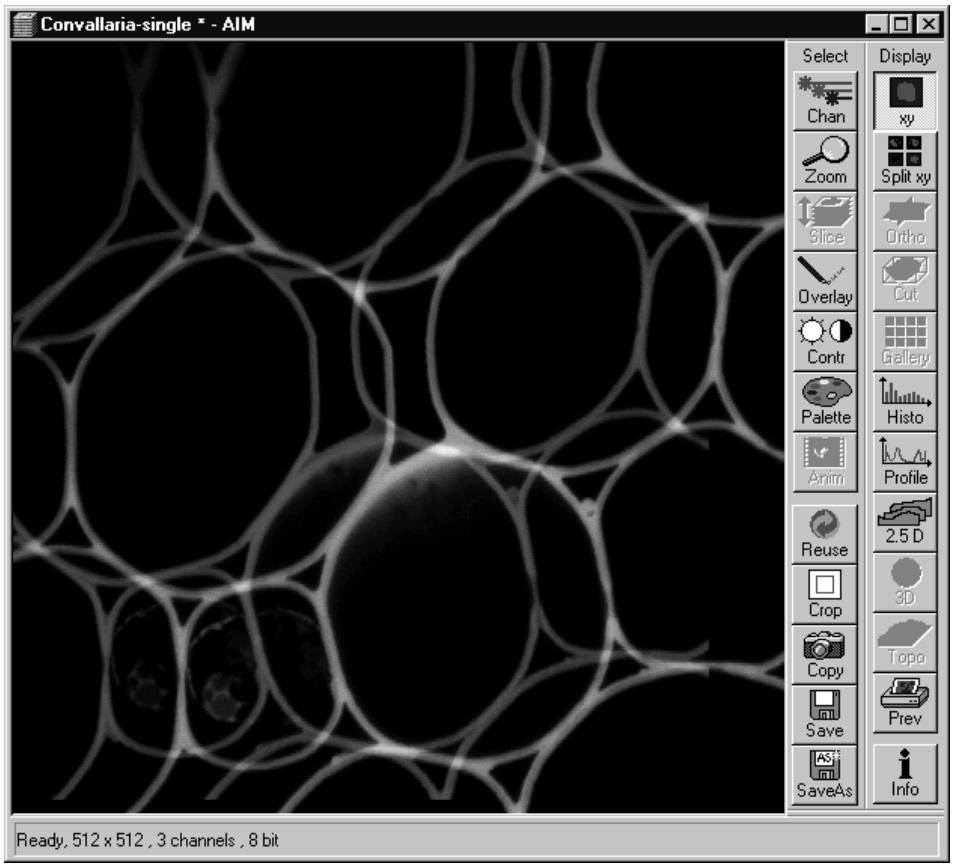


Fig. 4-133 Image Display window with channel shift

4.6.11 Unmix

The **Unmix** functionality permits to extract the emission of single fluorescence dyes (e.g. GFP only, YFP only etc.) from the overall emission band of strongly overlapping multifluorescence signal intensities by a pixelwise linear unmixing procedure .

Mathematically, experimental fluorescence spectra of monolabelled samples are taken as an external reference. Up to 8 different reference signals can be varied in this least-square-fit based algorithm to produce an 8 channel multifluorescence stack without any partial overlap between the channels.

4.6.11.1 Open / Close the Unmix Window

- Click on the **Unmix** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Linear Unmixing** window.
- Click on the **Close** button to quit the **Unmix** window.

4.6.11.2 Source Panel

In the **Source** panel the image source for the linear unmixing process has to be defined.

This has to be a Lambda Stack, a Lambda Stack Z series or a Lambda Stack T series.

Proceed as follows to select an image via the image selection box:

- Click on the arrow button. The image selection box is opened and all the currently loaded images, stacks, time series with a Lambda dimension are displayed in a minimized form.
- Click on the required image. This image will then appear in the display box of the image selection box and has been selected.

4.6.11.3 Definition of Channels Panel

In the **Channels** panel the number of reference spectra (number of fluorescence channels) can be selected from the channel selection boxes.

- Select the references fluorescence dye spectra which are present in the sample with the check boxes.
- If necessary change the colors of the relevant fluorescence channel.
- If no predefined reference spectra exist, please define reference signals via the **Save to Spectra DB** button in the **Display** - **Mean** functionality (see page 4-338).
- After definition of the required reference spectra set click on the **Apply** button.
- A new window with the resulting channels of the unmixing procedure opens immediately.

There are several settings that can be chosen for linear unmixing.

Autoscale balances the intensity of the unmixed images.

Generate channels with Residuals shows the difference between the offered spectrum and the resulting image in intensity values. The higher the intensity in this additional channel the worse the fit of the spectra to the dataset. This occurs for example when pixels are saturated and indicates to repeat the image acquisition with different settings to avoid overexposure or in extreme cases to chose different reference spectra.

Ask for Background ROI, when this is checked a window appears before the calculation starts which asks for the spectrum of the background which is then subtracted from the images prior to unmixing.

Advanced Linear Unmixing restarts the unmixing process again if negative values are generated. The negative values are set to zero and ignored for the next unmixing calculation.

Ignore Overexposed Pixels and **Ignore Underexposed Pixels** will exclude this pixels for calculation of the unmixed images.

Multichannel Unmixing allows to apply the unmixing algorithm to a multichannel image (up to 8 channels) without the use of reference spectra. The calculation of residuals and the subtraction of background based on a background spectrum are not available when this procedure is chosen. It will not work for heavily overlapping signals.

☞ Try to avoid saturation of fluorescence signals in the stack to be unmixed. This will generate a high signal in the residuals channel.
To get the highest quality unmixing results, please define an extra background channel, if possible.

Handling of Reference Spectra

- **Reference Spectra** used for **Linear Unmixing** are stored with result images.
- ReUsable and storable via **Load from Image** and **Write to DB** buttons.
- New file type *.umx for **Save** and **Load** of combinations (configurations) of **Reference Spectra.**

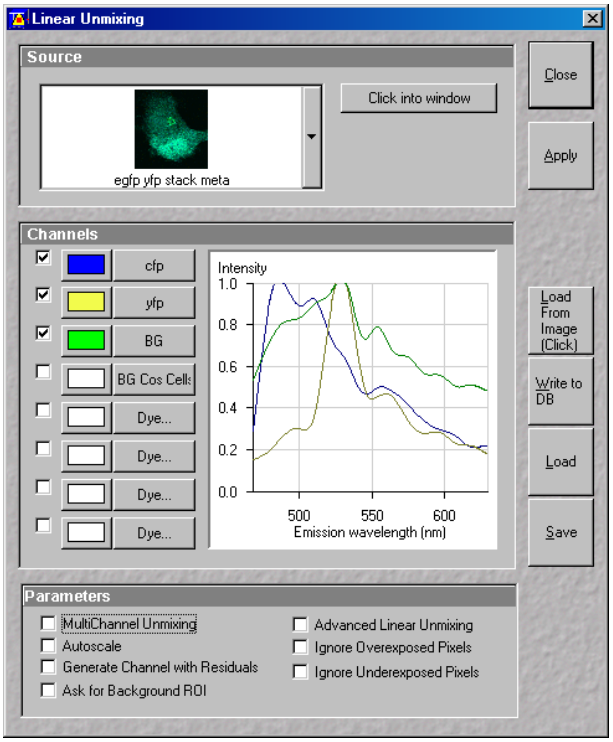


Fig. 4-134 Handling of Reference Spectra

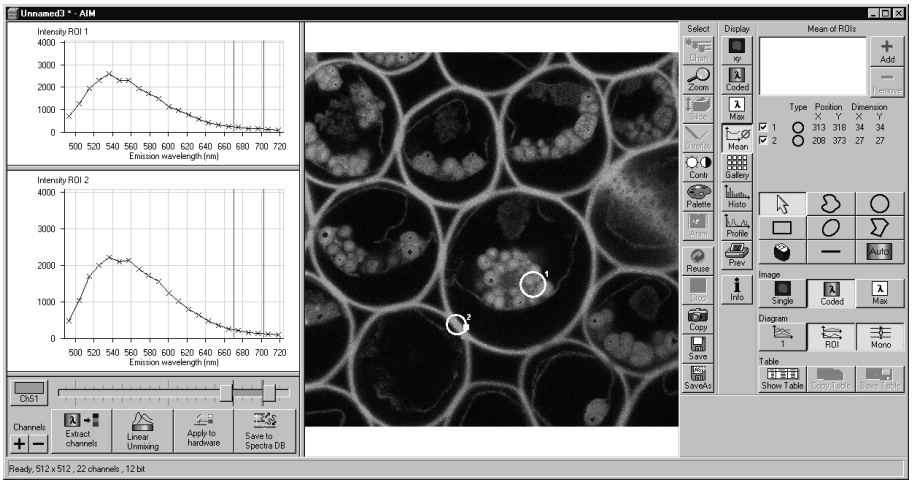


Fig. 4-135 Image Display window before unmixing

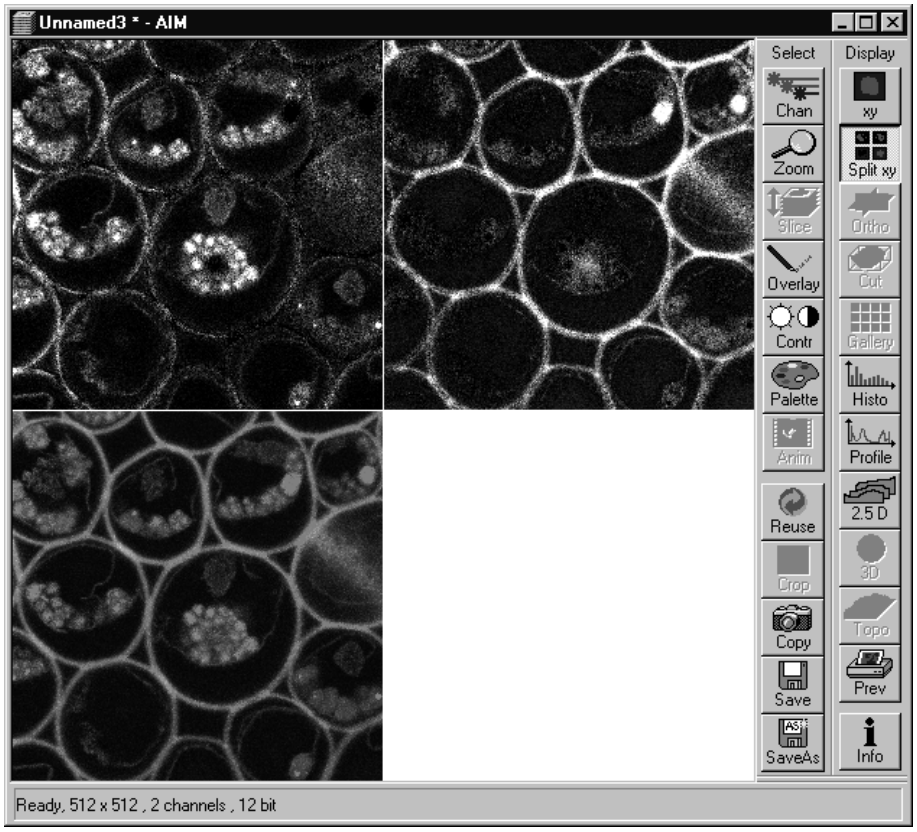


Fig. 4-136 Image Display window after unmixing

4.6.12 Ion Concentration

The use of this function (option) permits the calibration of ion concentrations in physiological experiments.

4.6.12.1 Open / Close the Ion Concentration Window

- Click on the **Ion Conc** button in the **Process** subordinate toolbar of the main menu.
  - This opens the **Ion Concentration** window.
- Click on the **Close** button.

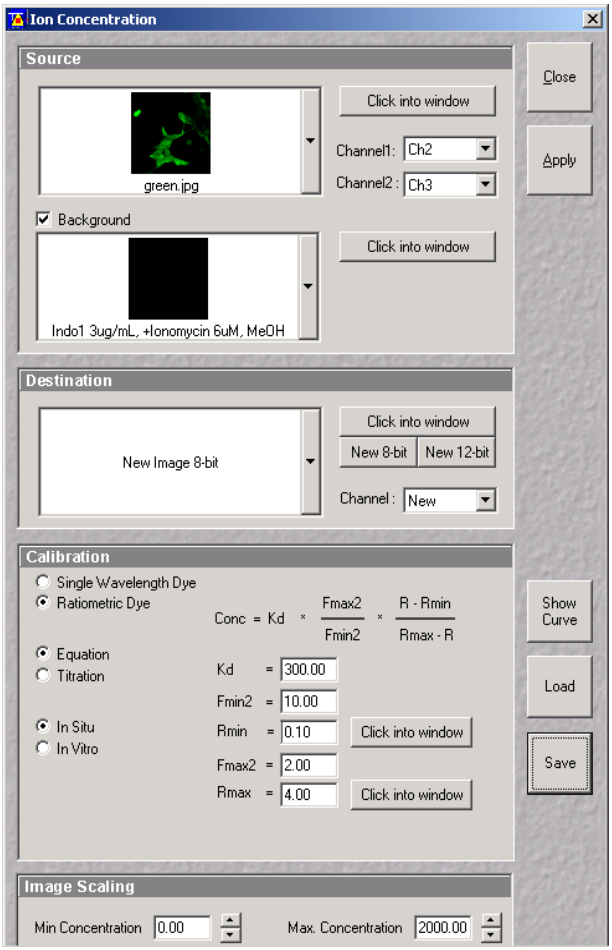


Fig. 4-137 Ion Concentration window

4.6.12.2 Function Description

Ion Conc button	Activates the **Ion Concentration** window.
Source window	Selects input of images to be processed.
Destination window	Select output and pixel depth of processed image.
Calibration window	Sets the six different calibration options, according to the dyes used (single wavelength, ratiometric) and required method.
Show Curve button	Shows resulting calibration curve.
Image scaling window	Sets min. and max. concentration.
Preview window	Activates **Preview** function.

4.6.12.3 Single Wavelength Dyes – Offline Calibration

- Subtract background/autofluorescence image from raw images to obtain.
- Perform equation- or titration calibration (compare F with a calibration curve → titration calibration or put F values in calibration formula).

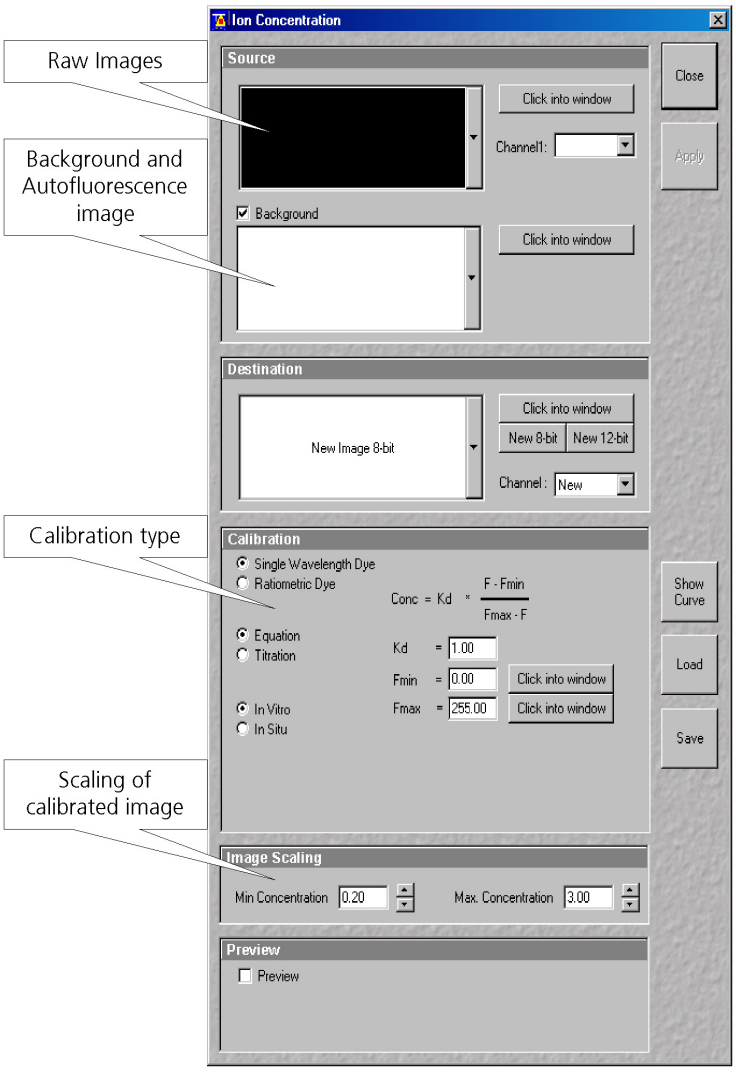


Fig. 4-138 Ion Concentration window

4.6.12.4 Ratiometric Dyes

- Fura-2, Indo-, SNARF, Cameleon, Ratiometric Pericam, Phluorin,...
- Display fluorescence ratio R over time
- Display fluorescence ratio R corrected for background/autofluorescence over time
- Calculate absolute ion concentrations (pixel by pixel) via titration calibration (known ion concentrations applied to the cells – in situ – or in solutions – in vitro or equation calibration where possible [Fura-2, Indo-, SNARF]
- Calculation of R eliminates artifacts and uncertainties caused by
  - inhomogenous dye distribution
  - photobleaching
  - may be applied with moving cells

4.6.12.5 Ratiometric Dyes - Online Ratio

R(t1) = F1(t1) / F2(t1), R(t2) = F1(t2) / F2(t2) ...

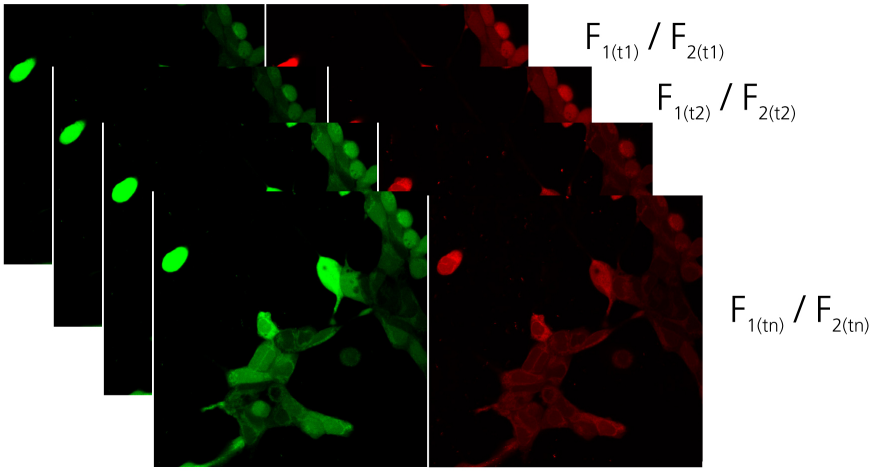


Fig. 4-139 Ratiometric Dyes – Online ratio

4.6.12.6 Ratiometric Dyes - Calibration

- Subtract background/autofluorescence images from raw images to obtain R_{korr} [=F_1-$F_{1Background}$)/(F_2-$F_{2Background}$)] when calibration reference is not obtained with the experimental sample (in situ)
- Calculate ratio R
- Perform equation- or titration calibration (compare R with a calibration curve -> titration calibration or put R values in calibration formula)

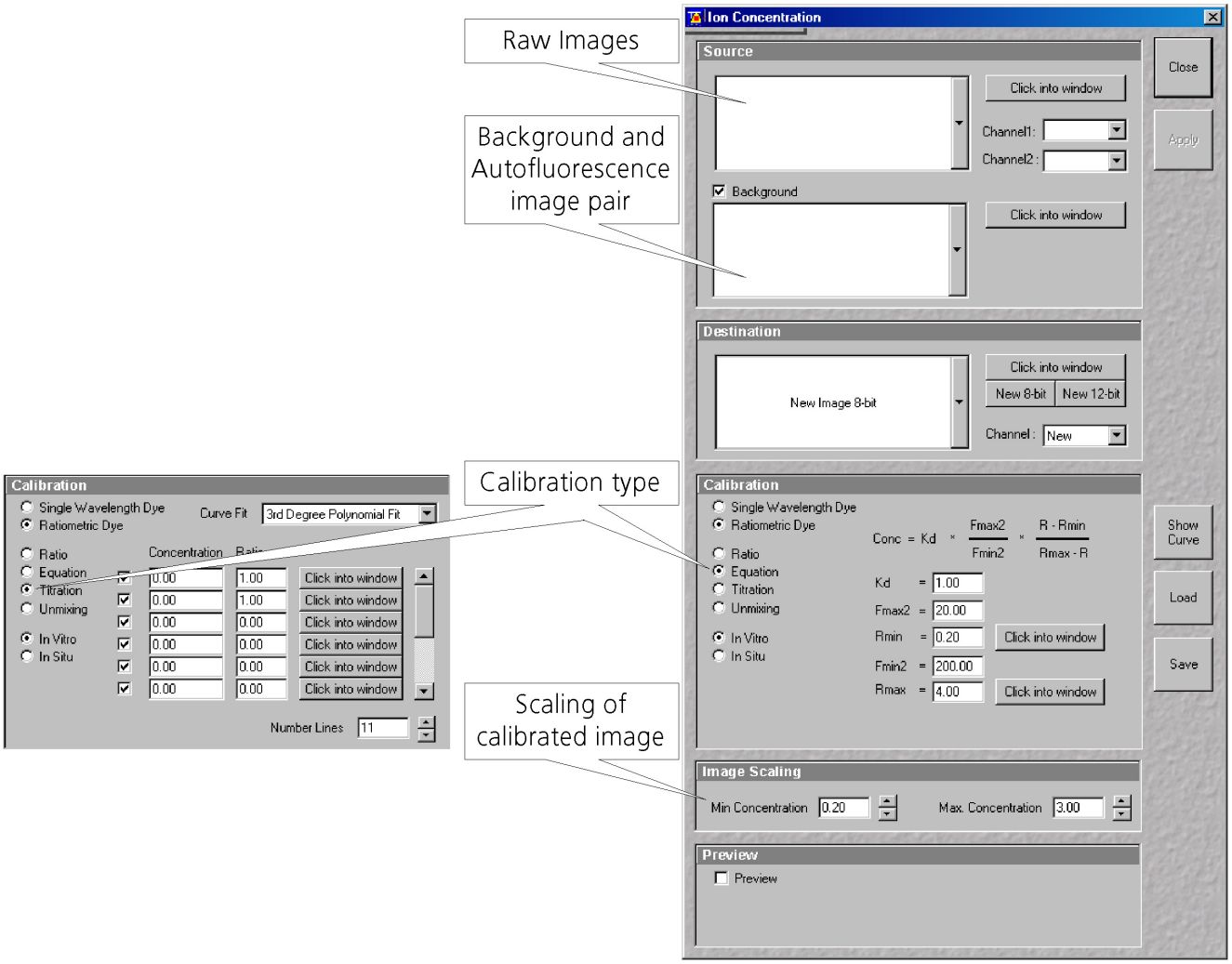


Fig. 4-140 Ratiometric Dyes - Calibration

4.6.12.7 Ratiometric Dyes - Equation Calibration (Grynkiewicz)

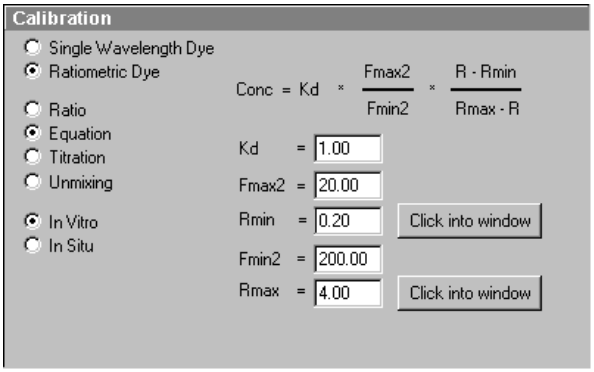


Fig. 4-141 Ion Concentration button

Fura-2, Indo-1,..

K_D (dissociation constant) taken from literature

R_{min}: derived from ion-free state of the dye (e.g. 0 Ca^{2+})

R_{max}: derived from ion-bound state of the dye (e.g. saturated with Ca^{2+})

F_{min2} and F_{max2} are the minimum and maximum fluorescence intensities at wavelength 2

R_{min}, R_{max}, F_{min2} and F_{max2} may be determined in the cells under investigation (in situ) or in solutions (in vitro)

Calibration parameters may be saved and reloaded (*.cal)

4.6.12.8 Options for Calibration Image Selection (Equation- or Titration Calibration)

- Click into image window.
- Select source channel(s).
- Optional background subtraction
- Optional calculation of parameters from overlay region(s)

4.6.13 Kinetic

The use of this function (option) permits

– a correction of FRAP data for bleaching and background

– the fitting of the FRAP data to a mono exponential or double exponential model for intensity recovery.

4.6.13.1 Open / Close the Kinetic Analysis window

- Click on the **Kinetic** button in the **Process** subordinate toolbar of the main menu.
  – The **Kinetic Analysis** window opens.
- Click on the **Close** button.

4.6.13.2 Function Description

Click into window Allows to select the image series of the FRAP experiment to be analyzed by clicking into the appropriate image window.

Channel
pull down menu Allows to select single channels or all channels for analysis.

Kinetic Model
pull down menu Allows to select the mathematical model (mono or double exponential model) for fitting the data.

Background Region
checkbox Displays a button **Select (Click)** that allows to chose the region of interest which represents the mean background intensity that should be used to correct the data. First Click Select (Click), than click into the ROI that has been drawn using the drawing tools in the Mean ROI image display. If a region has been selected a cross appears next to the button.

Reference Region
checkbox Displays a button **Select (Click)** that allows to chose the region of interest which represents the fluorescence intensity of a reference cell which has not been bleached. The mean intensity within that region is used to correct the data at each time point for any bleaching artifact that occurred during the imaging process. First Click Select (Click), than click into the ROI that has been drawn using the drawing tools in the Mean ROI image display. If a region has been selected a cross appears next to the button.

Combine Regions
checkbox Opens a further dialogue with the following options: **Add Region**, **New Group, Remove Group** and **Remove All**. It provides the possibility to chose more than one ROI for analysis and also to group them together according to the experimental set up. First click **Add Region**, than click into the ROI that has been drawn using the drawing tools in the Mean ROI image display. For each added ROI a cross is displayed in the line. Click Add Region for each ROI to be added. Clicking New Group puts the cursor in the next line and further ROIs or Group of ROIs can be added for analysis.

Apply Performs the calculations and the fitting of the data.

4.6.13.3 Example: FRAP Performed in a Nucleus Expressing GFP Labeled Proteins

Display of the image series in the **Mean ROI** display mode: The drawing tools are used to define the ROI to be analyzed (ROI 1), the background ROI (ROI 2), and the reference ROI (ROI 3). The reference ROI must be a neighboring cell which has been imaged with the same laser intensity over time identical to the cell, which has been bleached to induce FRAP. Make sure the whole cell or cell compartment of interest is imaged and therefore illuminated.

☞ Use the **Slice** submenu in the **Select** toolbar of the image window to display the image right after the bleach event. This makes it easy to chose the ROI for analysis. The region should be slightly smaller than the original region that has been bleached. The latter is listed in the Mean of ROIs list in the image window.

In the **ROI Mean Display** three ROIs are defined. See **Additional Display Mode in Time Series**, page 4-333 for further details.

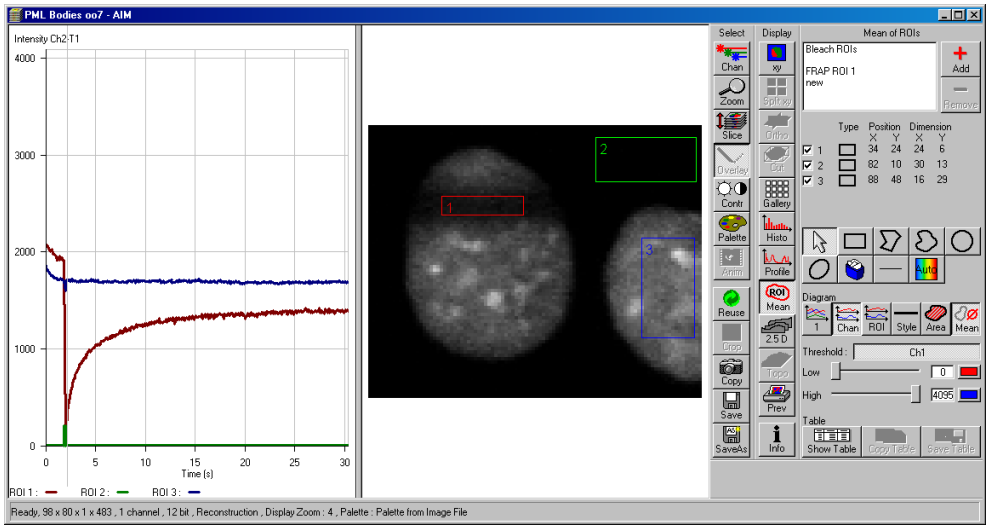


Fig. 4-142 Image window displaying the first bleached image of a images series

- Open the **Kinetic** dialogue and chose the image series for analysis.
- Mark the checkboxes for background and reference region.
- Press **Select (Click)** for the Background Region, than click into the ROI number 2 (Background region) in the image. The value of the mean intensity of this region will be subtracted from the intensity values within the ROI to be analyzed.
- Press **Select (Click)** for the Reference Region. The intensity values of the ROI to be analyzed will be corrected for changes in intensity calculated for the reference region. The correction is done for each time point taking the actual intensity difference in the reference region into account.

  If no other option is chosen the remaining ROI or ROIs are used for analysis. Each ROI is analyzed separately.
- Chose the **Kinetic Model** in the pull down list, then press **Apply**.
  - The result of the fit is displayed in the table. The result can be copied to the clipboard (Copy **Results**) or saved as a text file (**Save Results**). The following values are calculated and shown:
  - The final signal intensity in the analyzed ROIs following recovery **I0** (of the fitted curve).

- The amplitude of the fitted curve (which equals the mobile fraction) **I1 mobile fraction**.
- The fitted parameter **T1** (seconds).
- The rate constant for the exchange of molecules between the bleached region and the surrounding area **K** (per second).
- The part of the immobile fraction of the protein **I delta immobile fraction.**

Show Table opens a further table beneath the results. It shows all intensity values being corrected for background intensity and intensity loss of the reference region.

The values can be saved as a text file (**Save Table**) or copied into Excel via clipboard (**Copy Table**).

The data can be normalized optionally when marking the checkbox **Normalize** in the Kinetic Analysis Diagram toolbar.

☞ The calculation of the parameters is based on the same ROIs unless other ROIs or moved ROIs are selected again. The Kinetic Display is always available once the analysis has been performed. The analysis is not stored with the image.

☞ Note that this modeling is a very basic approach to your experiment. It offers a first hint on the possible presence of only one or, in case of a bad fit, more than one mobile fractions of the labeled protein within the cell or cell compartment examined.

☞ The half time of the recovery can be calculated using the following formula: $t_{half} = \frac{\ln 0.5}{-T1}$

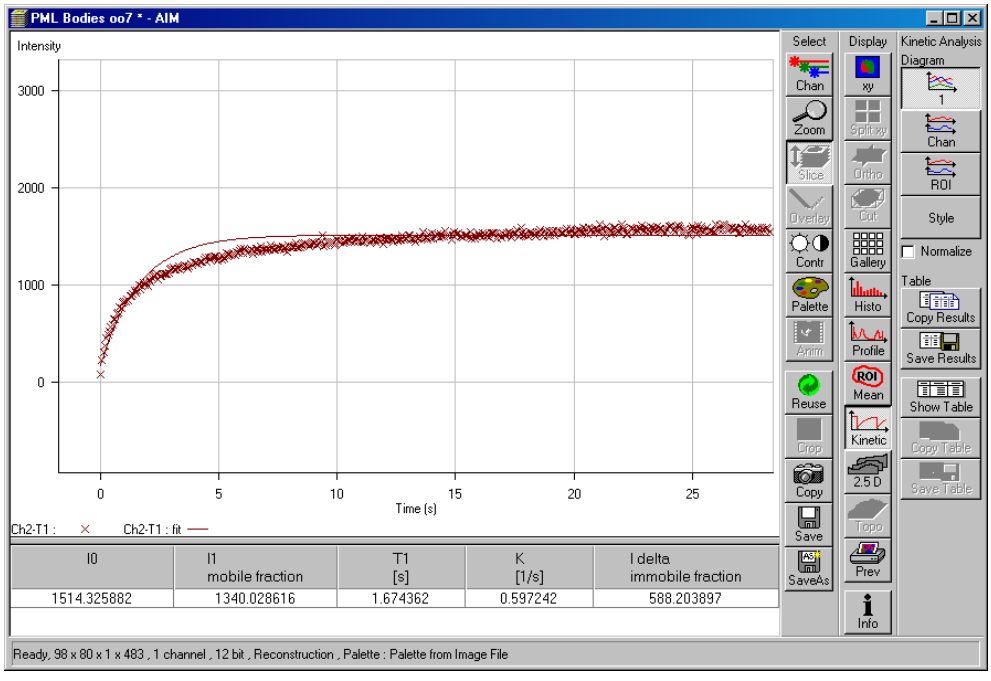


Fig. 4-143 Image window displaying the analysis of FRAP data using a mono exponential fit

If the analysis is done using the double exponential fit the fitted curve displays the mean of the fitted values for the two different mobile fractions. The table shows the following additional parameters:

- The amplitude of the two curves, displayed as one (which equals each part of the mobile fractions) **I1** and **I2**.
- The fitted parameters **T1** and **T2** (seconds) for each mobile fraction.
- The rate constant for the exchange of molecules between the bleached region and the surrounding area **K1** and **K2** (per second) for each mobile fraction.

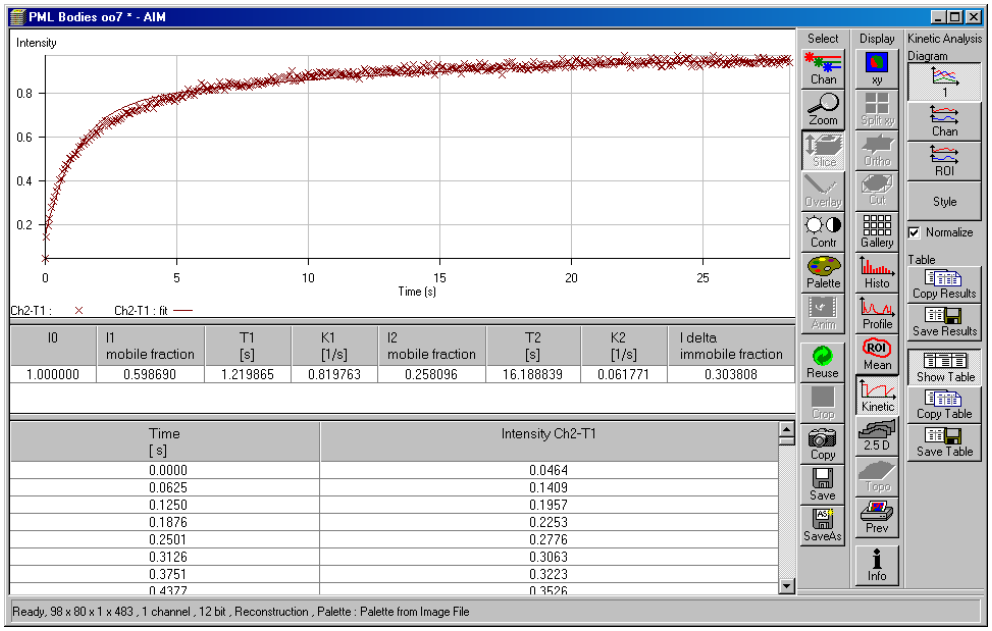


Fig. 4-144 Image window displaying the analysis of FRAP data using a double exponential fit

☞ The raw data of the experiment can be exported for further analysis using the Mean ROI display mode and, within this dialogue, the table display of the results.

☞ Note that this modeling is also a very basic approach to your experiment. It offers a first hint on the possible presence of two mobile fractions of the labeled protein within the cell or cell compartment examined.

Please refer to relevant scientific literature or the website of the EAMNET (http://www.embl.de/eamnet) for further information on how to set up and perform FRAP experiments. A schematic curve marking the data points that are calculated performing the Kinetic Analysis is shown in Fig. 4-145. Please note, that the naming of the data points is not consistent with the information on the website.

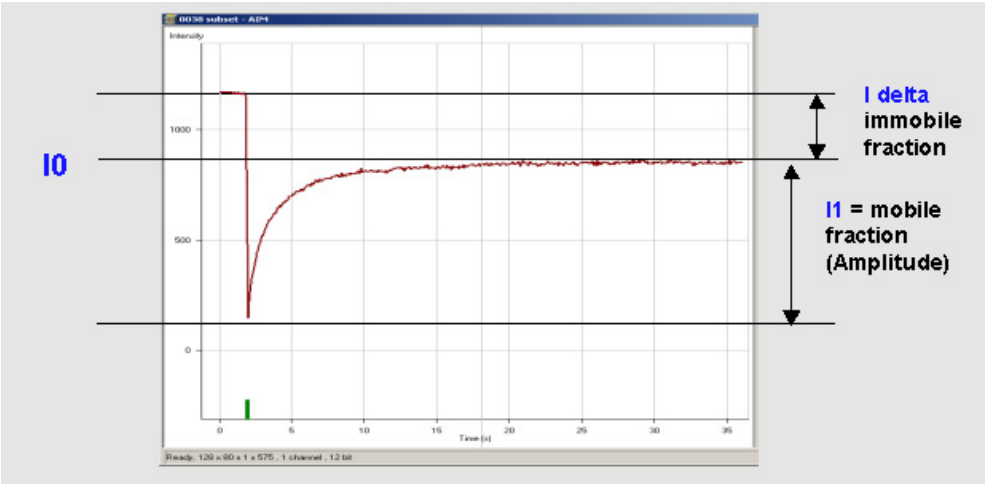


Fig. 4-145 **Schematic FRAP curve with marks at the relevant data points. I0, I1 and I delta which are calculated performing the Kinetic Analysis**

4.6.14 Image Scaling

The use of this function permits the scaling of a imported image in voxel dimensions. Setting the values will provide the voxel dimensions in the X-,Y- and Z-directions (Z-direction not for 2D images).

4.6.15 Copy Window Contents

The use of this function permits to duplicate the displayed image.

4.6.16 Copy Full Resolution

The use of this function permits to display the current image with full resolution in a new Image Display window.

4.6.17 Paste Bitmap

This function allows to paste bitmap images from the clipboard into a Image Display window.

Images can be incorporated from other programs such as MS Excel, MS Powerpoint or MS Word.

4.7 3D View Menu

The 3D View functions serve to record and play back series of images for 3D display of microscopic structures.

- In the **Main** menu toolbar, click on **3D View**.
  - This opens another, subordinate toolbar in the **Main** menu.

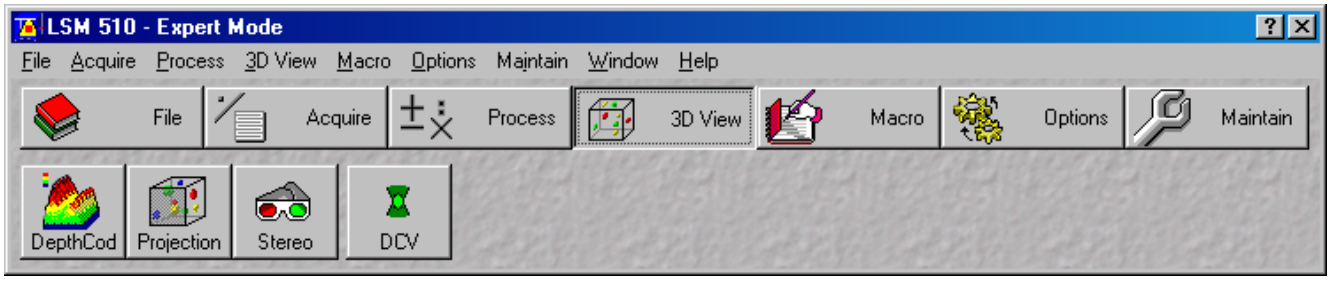


Fig. 4-146 3D View menu

4.7.1 3D DepthCod (Color Coded Depth Map)

By means of the **Depth Coding** function, the depth information contained in a sequence can be colored with the colors of the rainbow, in which case "blue " stands for front and "red" stands for rear.

A stack of images must be available.

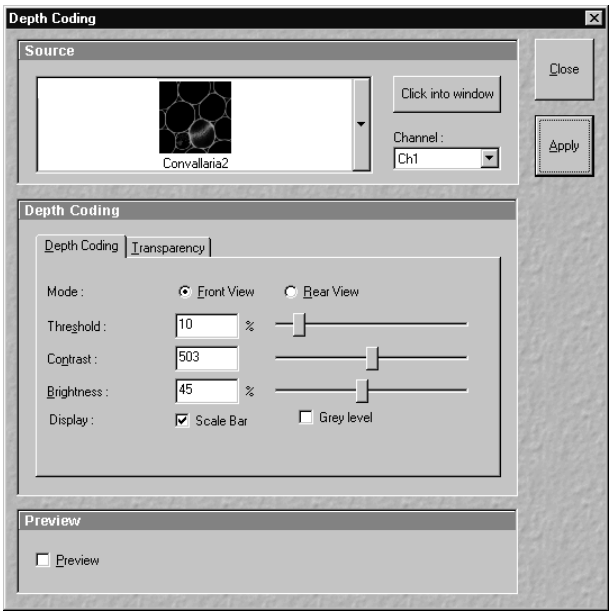


Fig. 4-147 Depth Coding window

4.7.1.1 Open / Close the Depth Coding Window

- Click on the **DepthCod** button in the **3D View** subordinate toolbar of the **Main** menu.
  - This opens the **Depth Coding** window.
- Click on the **Close** button to quit the window.

4.7.1.2 Source Panel

In the **Source** panel, the image source is selected. The currently selected image is displayed in the display box of the image selection box. Proceed as follows to select an image via the image selection box:

- Click on the arrow button. The image selection box will be opened and all the currently loaded images are displayed in a minimized form.
- Click on the required image, which will then be shown in the display field of the image selection box and be available for the following operation.

The **Click into window** button enables you to select the opened image directly:

- Click on the **Click into window** button first and then double-click on the relevant **Image Display** window. The selected image will then be shown in the display box of the image selection box.

Select the channels to be processed via the **Channel** selection box:

- Click on the arrow button to open the selection box. Click on the required channel to activate it.

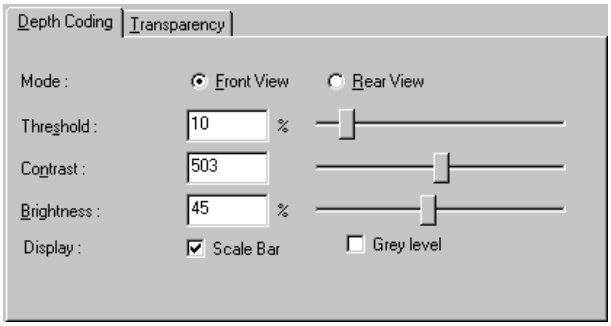


Fig. 4-148 Depth Coding tab

4.7.1.3 Depth Coding Panel

- On the **Depth Coding** panel you can set the desired parameters. Activate the **Scale Bar** check box ☑ if you want a color scale to be shown.

(1) Depth Coding tab

Mode **Front View**: The image is viewed from the front / above when this option is activated.

Mode **Rear View**: The image is viewed from the rear / below when this option is activated.

Threshold: All brightness values below the Threshold (range: 0 to 255) are ignored or treated like 0 when determining the depth and the display.

Contrast: Defines the factor with which the contrast of the overlaid series affects the contrast of the depth-coded color.

Brightness: Defines the factor with which the brightness of the overlaid series affects the brightness of the depth-coded color.

Display Scale Bar: Displays a colored scale in the image.

Display Grey level: The depth information is displayed in gray levels.

(2) Transparency tab

Mode **Maximum**: The color is defined by the z position of the brightness value.

Mode **Transparent**: The transparent projection is built up from the rear to the front. The color is defined by the Z position at which the original was last higher than or equal to Threshold.

Mode **Keep Maximum**: Activating this option modifies the specification governing calculation of the projection.

Threshold: Pixel value at which the ramp rises (variable from 0 to 100 %).

Ramp: Slope of the ramp (variable from 0 to 100 %; 0 % corresponds to a vertical rise).

Maximum Opacity: Degree of visibility at the top corner of the ramp (variable from 0 to 100 %; 0 corresponds to the bottom edge in the diagram).

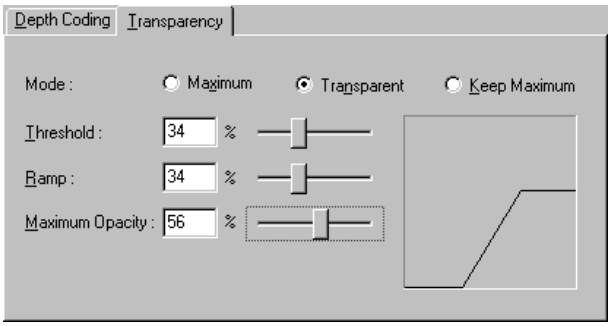


Fig. 4-149 Transparency tab

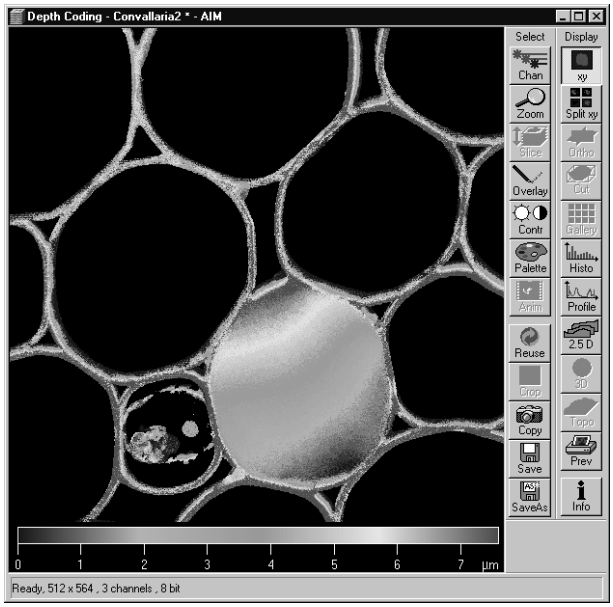


Fig. 4-150 Depth Coding image

4.7.1.4 Preview Panel

The **Preview** function permits you to regard the influence of parameter changes in an **Image Display** window.

- After finding the optimum adjustment using the **Preview** function, you have to generate the final version of the image using the **Apply** button.
  - The system then generates a color-coded depth map for the selected channel.

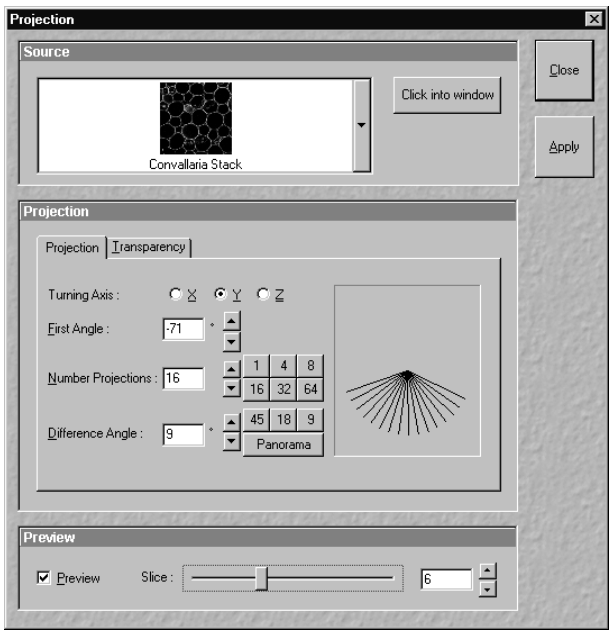


Fig. 4-151 Projection window

4.7.2 Projection

By means of the **Projection** function, one single projection or a series of projections can be calculated after rotation of the data package about the X, Y or Z axis.

A stack of images must be available.

4.7.2.1 Open / Close the Projection Window

- Click on the **Projection** button in the **3D View** subordinate toolbar of the **Main** menu.
  - This opens the **Projection** window.
- Click on the **Close** button to quit the window.

4.7.2.2 Source Panel

- Select the image for the projection operation from the image selection box.

4.7.2.3 Projection Panel

- On the **Projection** panel, set the parameters needed for the animation: **Turning Axis**, **First Angle**, **Number Projections** and **Difference Angle** in the **Projection** tab and the **Mode** parameters in the **Transparency** tab.

(1) Projection tab

Turning Axis X/Y/Z: Selects the axis about which the data package is to be rotated.

First Angle: Rotation angle in degrees.

Number Projections: Number of projections for a sequence (variable from 0 to 100).

Difference Angle: Angle increment of a sequence.

☞ The number keys permit the direct selection of preset values for **Number Images** and **Difference Angle**. If the **Panorama** button is pressed, a panorama sequence of the image series is computed.

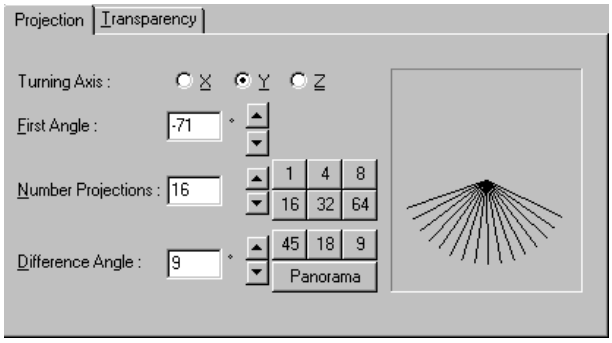


Fig. 4-152 Projection tab

(2) Transparency tab

Mode **Maximum**: The color is defined by the z position of the brightness value.

Mode **Transparent**: The transparent projection is built up from the rear to the front. The color is defined by the z position at which the original was last higher than or equal to Threshold.

Mode **Keep Maximum**: Activating this option modifies the specification governing calculation of the projection.

Threshold: Pixel value at which the ramp rises (variable from 0 to 100 %).

Ramp: Slope of the ramp (variable from 0 to 100 %; 0 % corresponds to a vertical rise).

Maximum Opacity: Degree of visibility at the top corner of the ramp (variable from 0 to 100 %; 0 % corresponds to the bottom edge in the diagram).

Brightness: The image can be brightened again by modifying the value (from 0.2 to 5).

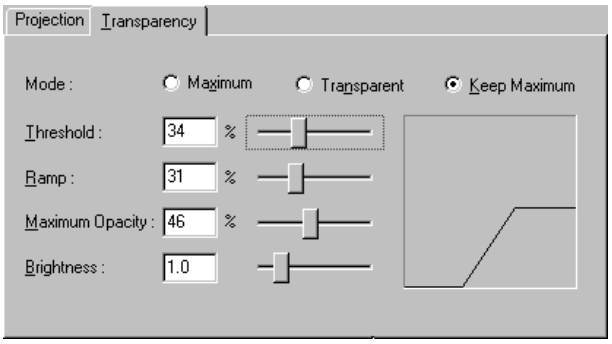


Fig. 4-153 Transparency tab

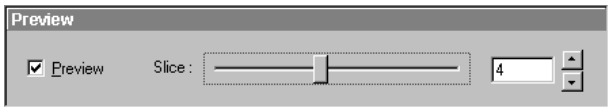


Fig. 4-154 Preview panel

4.7.2.4 Preview Panel

The **Preview** function permits you to regard the influence of parameter changes in an **Image Display** window.

The **Slice** slider enables you to select the slice which shall be displayed in the **Preview Image Display** window.

- After finding the optimum adjustment using the **Preview** function, you have to generate the final version of the image using the **Apply** button.
  - The projection appears. The computation can be followed in the image or by the progress bar.

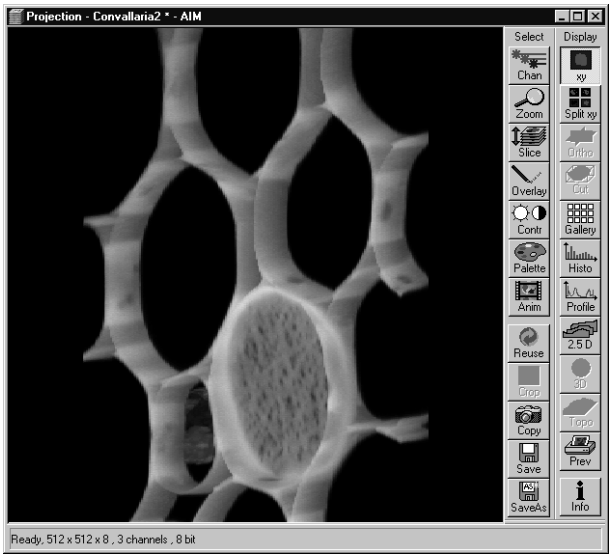


Fig. 4-155 Projection image

☞ The computed 3D sequence can be animated with the **Anim** button in the **Select** toolbar.
In addition, the **Animate** window appears, in which you can influence the direction and speed of 3D image rotation (see **Animate**, page 4-262).

You can browse through the rotation sequence manually with the **Slice** button in the **Select** toolbar and the **Slice** slider.

- To view the computed 3D sequence as a gallery on the screen, click on the **Gallery** button in the **Display** toolbar.

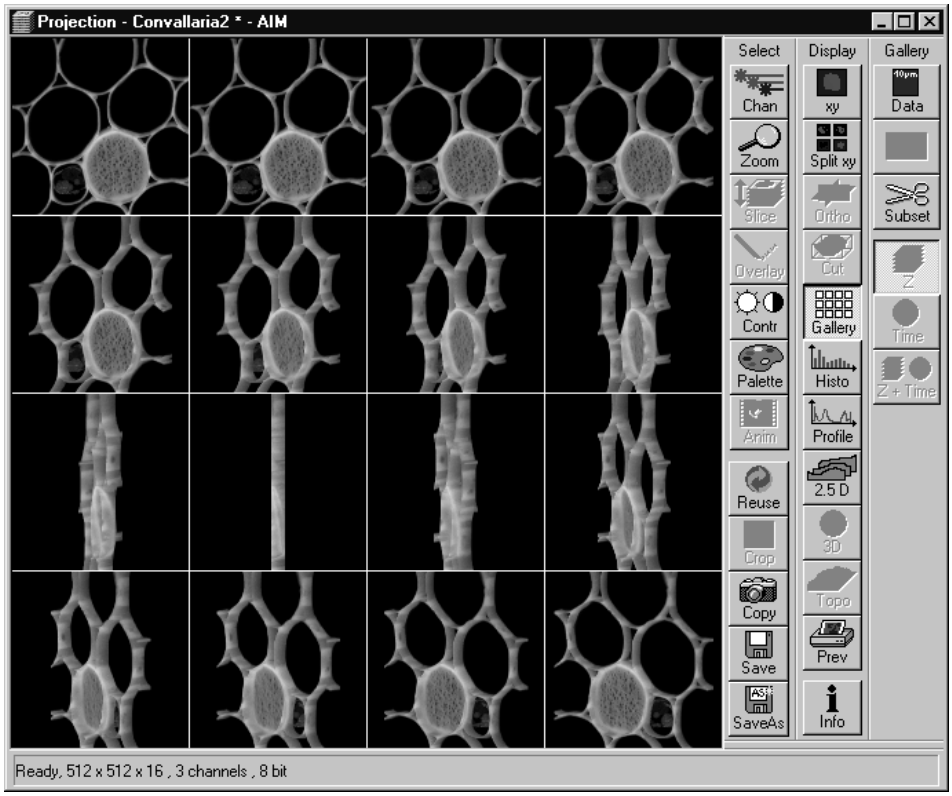


Fig. 4-156 Projection image (Gallery)

4.7.3 Stereo

Stereoscopic images can be generated in a variety of ways by means of the **Stereo** function.

A stack of images must be available.

4.7.3.1 Open / Close the Stereo Images Window

- Click on the **Stereo** button in the **3D View** subordinate toolbar of the **Main** menu.
  - This opens the **Stereo Images** window.
- Click on the **Close** button to quit the window.

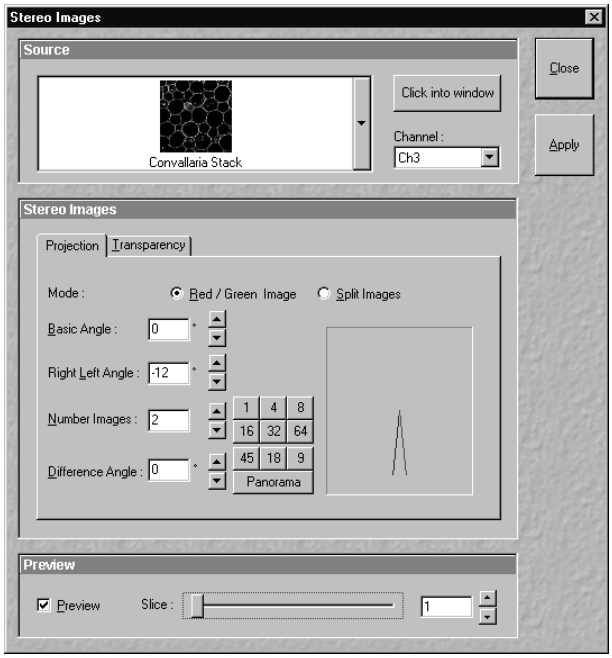


Fig. 4-157 Stereo Images window

4.7.3.2 Source Panel

- Select the image for the projection operation from the image selection box.
- Select the channel to be used from the **Channel** selection box.

4.7.3.3 Stereo Images Panel

- In the **Stereo Images** panel, set the parameters needed for stereoscopic viewing: **Mode**, **Basic Angle**, **Right Left Angle**, **Number Images** and **Difference Angle** in the **Projection** tab and the **Mode** parameters in the **Transparency** tab.

(1) Projection tab

Mode **Red / Green Image**: This displays a stereo image for red / green anaglyph observation using red / green spectacles.

Mode **Split Images**: This displays a pair of stereo images for observation through a stereoscope. Colored stereo images are also possible.

Basic Angle: Direction angle at which the specimen is viewed; 0° from the front, 180° from the rear.

Right Left Angle: Angle between right and left (red and green) image.

Number Images: Number of 3D images (slices).

Difference Angle: Angle increment of a sequence.

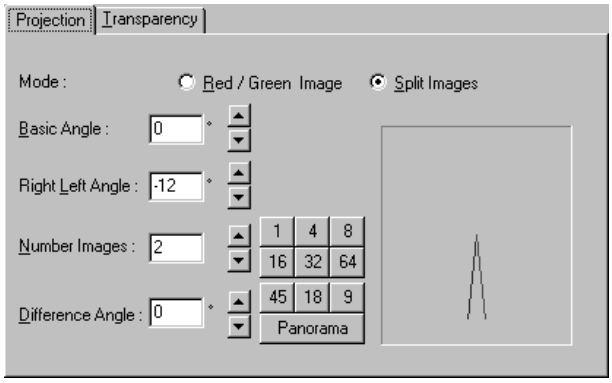


Fig. 4-158 Projection tab

☞ The number keys permit the direct selection of preset values for **Number Images** and **Difference Angle**. If the **Panorama** button is pressed, a panorama sequence of the image series is computed.

(2) Transparency tab

Mode **Maximum**: The color is defined by the z position of the brightness value.

Mode **Transparent**: The transparent projection is built up from the rear to the front. The color is defined by the z position at which the original was last higher than or equal to Threshold.

Mode **Keep Maximum**: Activating this option modifies the specification governing calculation of the projection.

Threshold: Pixel value at which the ramp rises (variable from 0 to 100 %).

Ramp: Slope of the ramp (variable from 0 to 100 %; 0 % corresponds to a vertical rise).

Maximum Opacity: Degree of visibility at the top corner of the ramp (variable from 0 to 100 %; 0 corresponds to the bottom edge in the diagram).

Brightness: The image can be brightened again by modifying the value (from 0.2 to 5).

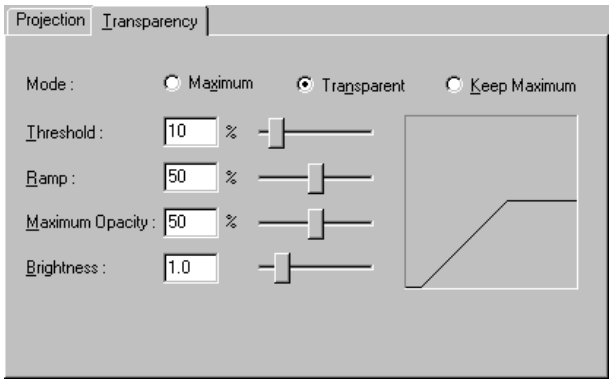


Fig. 4-159 Transparency tab

4.7.3.4 Preview Panel

The **Preview** function permits you to regard the influence of parameter changes in an **Image Display** window.

The **Slice** slider enables you to select the slice which shall be displayed in the **Preview Image Display** window.

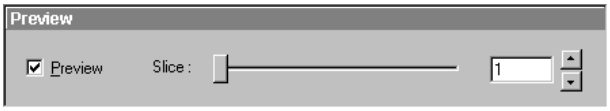


Fig. 4-160 Preview panel

- To start computation of the stereoscopic image, click on the **Apply** button.
  - The image is built up twice (once each for the red and green colors), resulting in a stereoscopic image.

☞ The stereoscopic effect can only be seen with the aid of red / green 3D goggles. The red lens is to be used for the right eye and the green lens for the left eye.
The presentation can be modified by selecting the **Split Images** (Mode) option in the **Projection** tab of the **Stereo Images** panel.

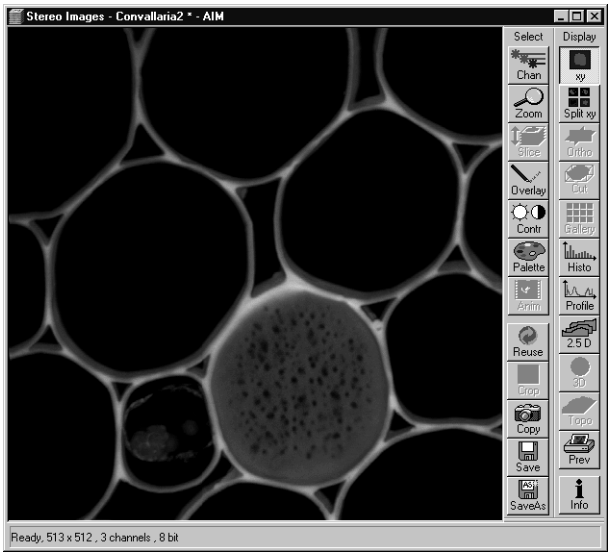


Fig. 4-161 Stereoscopic image (red / green)

- By clicking on the **Apply** button, the two stereo pairs are presented side by side and can be viewed without red / green 3D goggles.

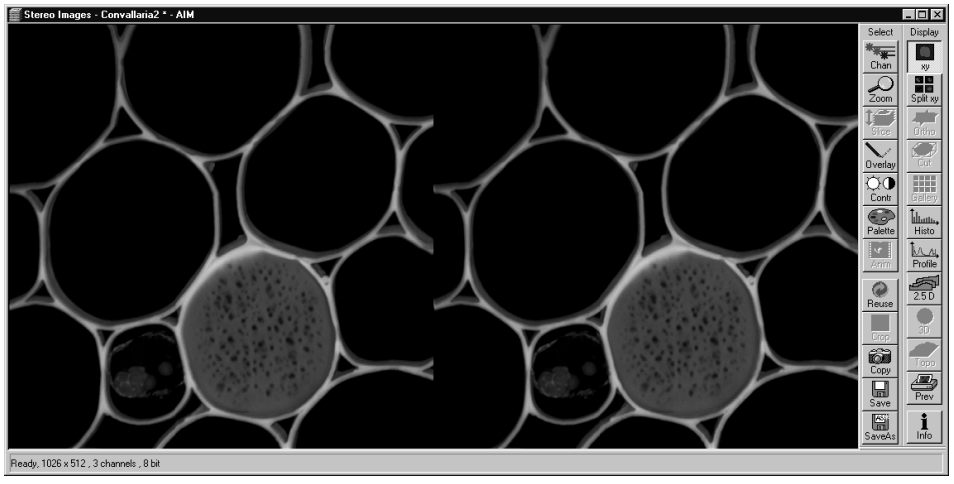


Fig. 4-162 Stereoscopic image (split)

4.7.4 3D DeConVolution (DCV)

The 3D Deconvolution option is used for the resolution enhancement of fluorescence image stacks.

When a three-dimensional object is reproduced by an optical system the resulting image of the object does not correspond exactly to the object's actual form. The image of the object is "distorted" as it passes through the optical system. In physical terms the actual object is convolved by the optical system's **Point Spread Function** (PSF).

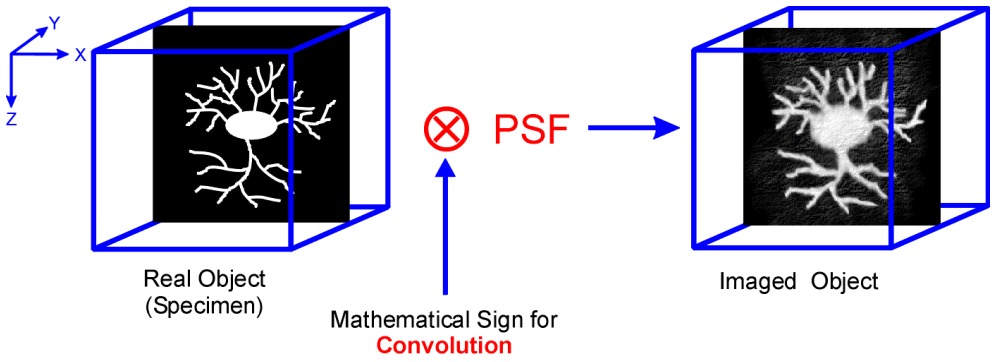


Fig. 4-163 Point Spread Function (PSF)

The **Point Spread Function** describes how the light of a point object is distorted by the optical system. This "convolution" makes the image appear grainy and structures in the image seem blurred. This effect is most prominent in the axial (Z-)direction as each lens is optimized for the two-dimensional image of the object.

If the PSF is known it is possible to use mathematical algorithms to undo this distortion. The image of the object is deconvolved using the PSF and the actual form is reconstructed:

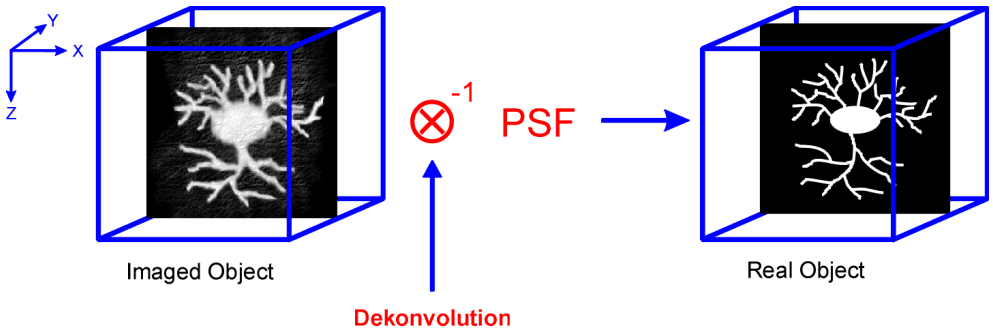


Fig. 4-164 Point Spread Function (PSF)

The effect of 3D deconvolution can be demonstrated impressively on objects with a known form. As a rule fluorescent beads are used for this purpose. The following figure shows the 3D deconvolution of an image stack with a fluorescent bead with a diameter of 1 µm.

As the resolution of an optical system is significantly lower in the axial direction than in the lateral (X/Y-)direction, the greatest improvement in resolution can be achieved in the Z-direction.

The Z Stack must meet the following requirements:

- At least two-fold oversampling in xyz (z: half of optimal interval button)
- High signal-to-noise ratio
- Detector gain < 500 V

Calculation is either made for one channel of the opened image which must first be selected accordingly, or for all channels of a stack.

Calculation is started via **Apply** and can be stopped using the **ESC** key, if required.

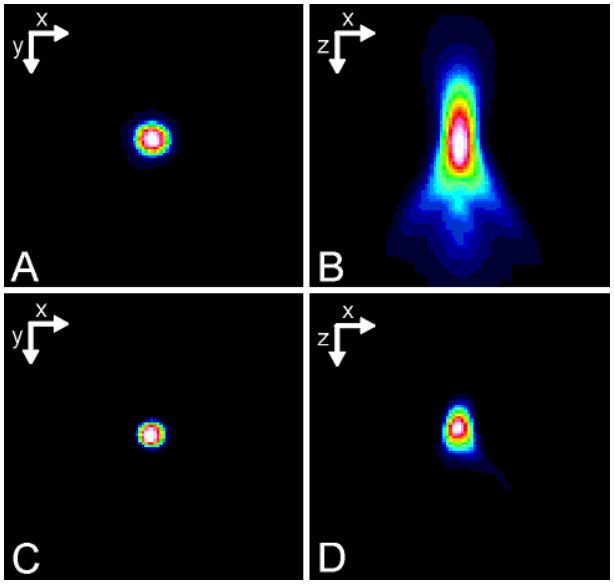


Fig. 4-165 Image of a fluorescent bead with a diameter of 1µm before deconvolution (A,B) and after deconvolution (C,D)

4.7.4.1 Open / Close the 3D Deconvolution Window

- Click on the **DCV** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **3D Deconvolution** window.
- Click on the **Close** button to quit the window.

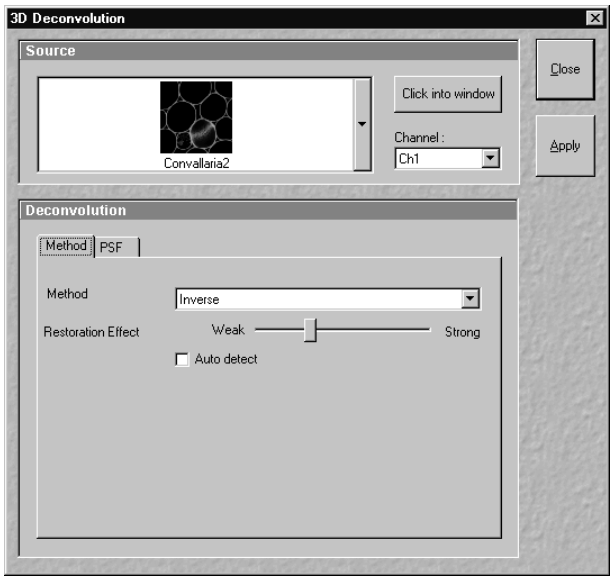


Fig. 4-166 3D Deconvolution window

Fig. 4-167 Source panel

4.7.4.2 Source Panel

The image to be processed is selected in the **Source** panel. The currently selected image is shown in the display box of the image selection box. Proceed as follows to select a image via the image selection box:

- Click on the arrow button. The image selection box will be opened and all the currently loaded images will be displayed in a minimized form.
- Click on the required image, which will then appear in the display box of the image selection box and has been selected for the interpolation procedure.

☞ You can also use the **Click into window** button for image selection.

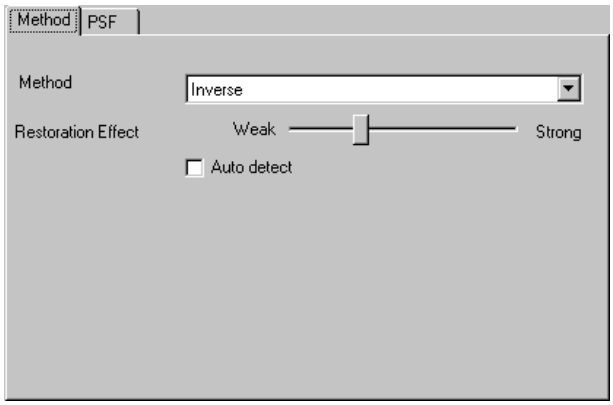


Fig. 4-168 Method tab

4.7.4.3 Deconvolution Panel

The **Deconvolution** panel contains the two tabs **Method** and **PSF**.

(1) Method tab

The **Method** tab permits selection between the calculation methods **Nearest Neighbour**, **Inverse** and **Iterative**.

(a) Nearest Neighbor

The Nearest Neighbor method is the simplest and fastest algorithm which in principle corresponds to a 3D sharpness filter.

(b) Inverse Filter

The regularized inverse filter generally achieves better results than the Nearest Neighbor algorithm. It is well suited to process several image stacks for a pre-selection of images for the use of the iterative high-end methods.

(c) Constrained Iterative

The best image quality is achieved using the Constrained Iterative Maximum Likelihood Algorithm. Increasing the resolution in the image, especially in the Z-direction, is only possible with this method. Due to the complex mathematical method, depending on the image size and the PC being used the calculation can take up to several hours.

In the **Inverse** method, the **Restoration Effect** slider permits the noise-to-signal ratio to be selected between the settings **Weak** (low noise) and **Strong** (pronounced noise).

Activation of the **Auto detect** check box will start a routine for the automatic determination of the noise level in the entire image part of the Z Stack (not available in the **Nearest Neighbour** method). If **Auto detect** is enabled, the **Restoration Effect** slider is disabled.

The **Iterative** method permits (in addition to the parameters of the **Inverse** method) the maximum number of iterations to be entered between 1 and 200 under **Maximum Iterations** and the **Auto Stop** function to be activated / deactivated. The **Auto Stop** function interrupts the calculation depending on the set image improvement (delta between last but one and last cycle in %), no matter whether the value under **Maximum Iterations** has been achieved or not.

The **Nearest Neighbour** method permits entry of the **Number of Neighbours** and the **Sharpness in Focus** value in addition to the **Restoration Effect**.

(2) PSF tab

In the 3D Deconvolution option a theoretical point spread function (PSF) is calculated from the systems settings (objective data, wavelengths, pinhole diameter).

The PSF data are displayed in the **PSF** tab. In the case of wavelengths above 700 nm, the **NLO** button is automatically enabled.

The displayed values are always taken over by the system data, but can be edited subsequently for simulation purposes.

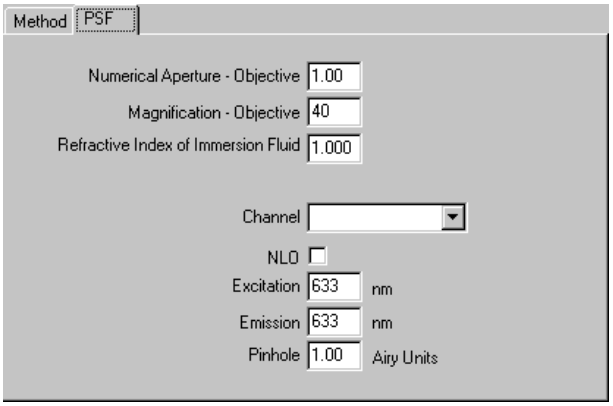


Fig. 4-169 PSF tab

4.8 Macro Menu

The macro function permits the recording, running and editing of command sequences and their allocation to buttons in the **Macro** menu.

- In the **Main** menu toolbar, click on **Macro**.
  - This opens another, subordinate toolbar in the **Main** menu.

Using the **Macro** Button (optional) opens the dialogue for editing and recording Macros based on VBA programming. VBA programming is described in CHAPTER VBA PROGRAMMING FOR LSM of the printed manual.

Using the **Visual** Button (optional) allows to construct automated work flows using the arrangement of symbols which depict the single steps within a work flow. See **Visual Macro Editor**, page 4-203 for further details.

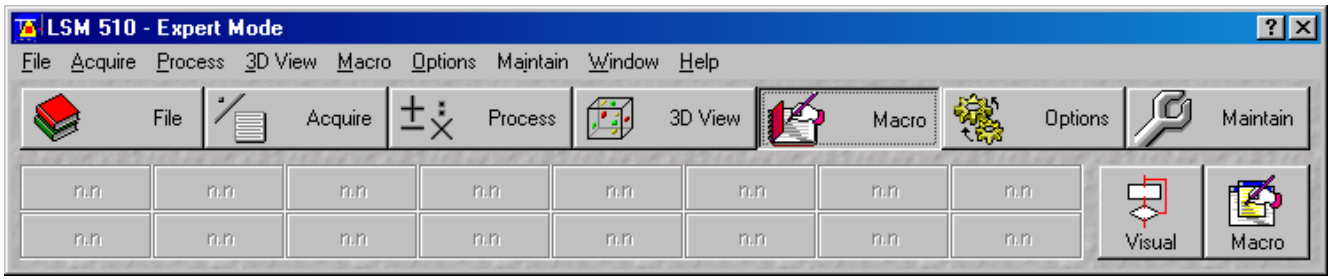


Fig. 4-170 Macro menu

4.8.1 Macro Language

"Visual Basic for Applications", called VBA in the following, is used as the Macro language. This language is well known through its widespread use as Macro language in the "Microsoft Word for Windows" and "Microsoft Excel for Windows" products. Experience with "Microsoft Visual Basic" would also be beneficial for macro-programming of the LSM 510.

An Integrated Development Environment, called IDE in the following, is available for the editing and debugging of macros. IDE includes an "online help program" where the VBA language is described in detail.

Macros are stored in project files. One project file can include several macros.

4.8.2 Macro Control

4.8.2.1 Open / Close the Macro Control Window

- Click on the **Macro** button in the **Macro** subordinate toolbar of the **Main** menu.
  - This opens the **Macro Control** window.
- Click on the **Close** button to quit the window.

4.8.2.2 Edit Macro Function

This function allows you to manage project data. Macros can be recorded, stored, performed, edited and, if required, deleted.

- Press the **Edit Macro** button to switch to the **Macro** and **Recording** panels.
- Click on the **Close** button to quit the window.

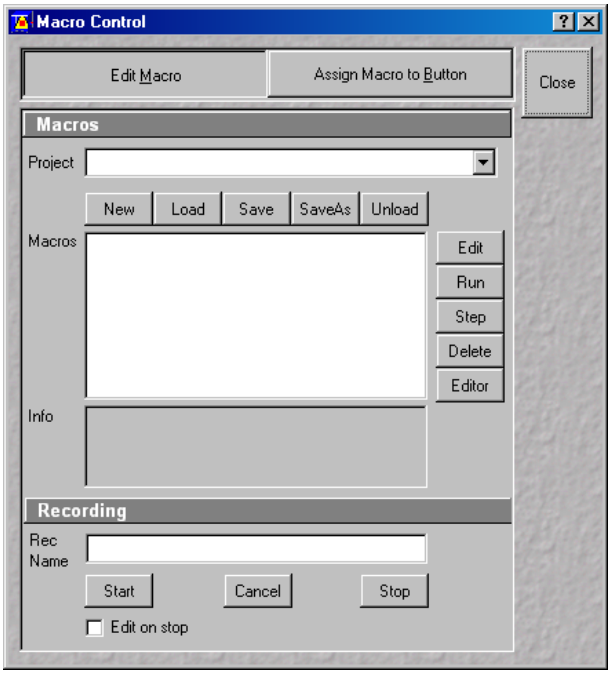


Fig. 4-171 Macro Control window

(1) Macros panel

New button: Creates a new project.

Load button: Opens an existing project.

Save button: Stores the project on the hard disk.

Save As button: Stores an existing project under a new name.

Unload button: Removes the selected macro from the **Macros** list.

Edit button: Allows macros to be edited and debugged. The editor (**Microsoft Visual Basic**) is automatically located at the beginning of the relevant macro.

Run button: Runs a macro.

Step button: Opens the editor for line-by-line editing / debugging.

Delete button: Deletes the selected macro.

Editor button: Opens the editor. Displays the processed area of the macro edited last.

Fig. 4-172 Macro panel

Macros are stored and managed in project files (*.lvb). Before you can record or edit a macro, you have to create a project as follows:

- Press the **New** button to create a project file.
  - A new project is created and displayed in the **Project** selection box (e.g.: **LSM 150503**). The project name is automatically default, but can be edited afterwards.

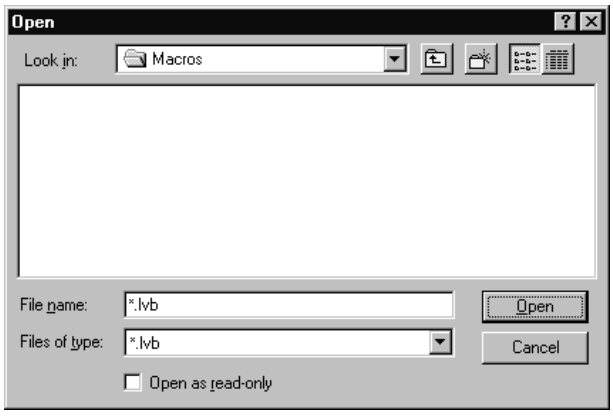


Fig. 4-173 Open window

To activate an existing project, proceed as follows:

- Press the **Load** button.
  - The **Open** window will be opened.
- Select the relevant project file (data extension: ***.lvb**) from the **Macros** list box. Click on the **Open** button.
  - The project file will be opened and the macros contained in it are displayed in the **Macros** selection box of the **Macro Control** window.

Recorded macros are stored in main memory first. Before the macros can be assigned to the buttons in the **Macro** submenu, the project must be stored on the hard disk.

- Press the **Save** button under the project name in the **Macro Control** window and determine the file name in the **Project** selection box, if required.

(2) Recording panel

Before recording a command sequence, you can enter the name for the macro to be created in the **Rec Name** input box of the **Recording** panel.

Start button: Starts recording.

Cancel button: Cancels the recording procedure.

Stop button: Stops recording.

☑ **Edit On Stop**: On stopping the recording procedure, the macro editor is automatically opened at the relevant position.

Proceed as follows to record a macro:

- Enter a name for the macro to be created under **Rec Name** in the **Recording** panel.
- Click on the **Start** button to start recording the macro.
- Then perform the operations to be stored, e.g.:
  - Click on the **Find** button in the **Scan Control** window. A Find scan will be performed.
  - Click on the **New** button in the **Scan Control** window. A new **Image Display** window will be opened.
  - Click on the **Single** button in the **Scan Control** window. A **Single** scan will be performed.
- Then click on the **Stop** button to end the recording. (**Cancel** enables you to cancel recording)
  - If recording was successful, the entered **Rec Name** will then also be available in the **Macros** list box of the **Macro** panel. The new macro is automatically assigned to the current project. It is possible to assign as many macros as required to a project.
- Click on the **Save** button to store the new macro.

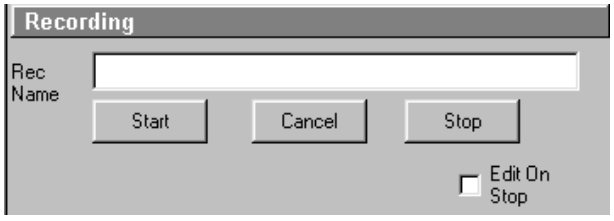


Fig. 4-174 Recording panel

Proceed as follows to perform a macro:

- Select the required macro from the **Macros** list box of the **Recording** panel.
- Click on the **Run** button to start performing the macro.

☞ Provided that a macro is linked to a button in the **Macro** subordinate toolbar, you only need to click on this button to perform the macro.

Proceed as follows to delete a macro:

- Select the required macro from the **Macros** list box of the **Recording** panel.
- Click on the **Delete** button. The macro will be removed from the list.

Proceed as follows to edit a macro:

- Select the required macro from the **Macros** list box of the **Recording** panel.
- Click on the **Edit** button. The Microsoft Visual Basic editing window will be opened.
- Make the required changes (also see the notes on page 4-179).

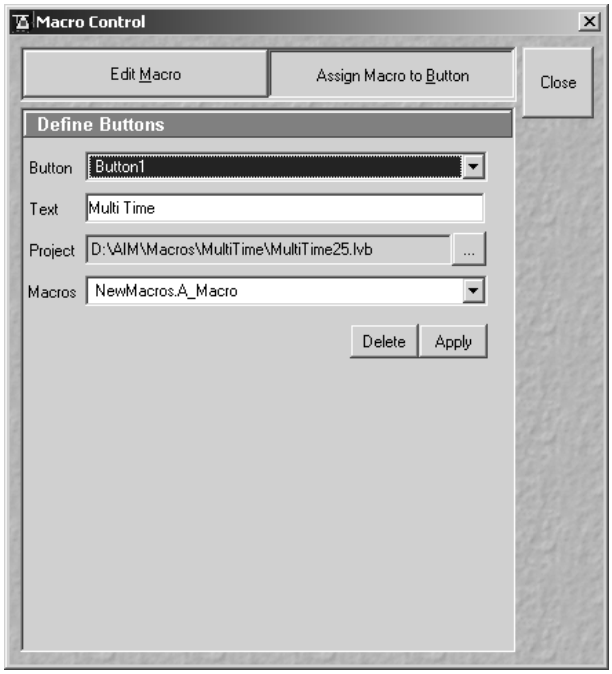


Fig. 4-175 Macro Control window

4.8.2.3 Assign Macro to Button Function

This function permits stored macros to be linked with one button each in the **Macro** subordinate toolbar.

- Press the **Assign Macro to Button** button to switch to the **Define Buttons** panel.

Define Buttons panel

Proceed as follows to link a macro to a button of the **Macro** subordinate toolbar:

- Select the button number from the **Button** selection box.
- Enter the button labelling in the **Text** editing box.
- Select the name of the project file from the **Project** box using the **...** button
- Select the macro name from the **Macros** box.
- Press the **Apply** button to assign the relevant macro to the specified button in the **Macro** toolbar.

Delete the linking of a button in the Macro subordinate toolbar

Proceed as follows to delete the linking between a button in the **Macro** subordinate toolbar and a macro:

- Select the button number from the **Button** selection box.
- Press the **Delete** button to delete the linking.

4.8.2.4 Editing and Debugging of Macros

The **Edit** button activates IDE (Integrated Development Environment) which allows macros to be edited and debugged. Under the **Help - Contents and Index** menu item, IDE contains detailed "online" help on its operation and on the VBA macro language. Therefore, only a few hints are provided in the following:

You should activate the required toolbars. We would recommend you to activate the **Debug** toolbar via the **View - Toolbars -Debug** menu item.

The following buttons in the toolbar can help you when debugging macros:

Starts running the command lines.

Stops running the command lines.

Interrupts processing of the command lines (pause).

Sets a breakpoint in the line with the text cursor.

Processes a command line and steps into subprocedures.

Processes a command line and steps over subprocedures.

Exits the subprocedure (step out).

Displays the value of the marked expression (Watch). If nothing is marked, the value of the variable above the text cursor is displayed.

Activates the Watch window in which values of variables and expressions can be displayed. For this, text is marked in the code window and dragged into the Watch window. Variables can be modified in the Watch window.

In the left-hand edge of the code window you will find an arrow beside the current command line. A new current command line can be determined by moving the arrow via the mouse. This makes it possible to skip command lines or to process command lines several times.

4.8.3 Overview of Available Macros

Documentation files (*.rtf, *.doc) of advanced macros will be located in the macro directory.

Name	**Description**
AOTFfitlin.lvb	Linearize laser attenuation (AOTF or mechanical)
Autofocus.lvb	Automatic focusing according to a set configuration
Bleach.lvb	Bleaching of a rectangular area or a line; combines old macros BleachRectangle.lvb, BleachLine.lvb and Spot.lvb (useful for LSM 5 PASCAL)
Centerv.lvb	Centers the field of view around the position marked with the cross tool;
ConcatenateImages.lvb	Enables the combination of all kinds of images irregardless of the size and data depth to be presented in a time series. Imported images can also be included. Type of concatenation (fitted to the smallest frame or ROI, fixed size or fitted to the largest frame) can be defined by the user. Description see also chapter 4.8.4.6
CopyPasteOverlays.lvb	Copies actual overlay drawing into the clipboard and pastes the drawing into a selected image window
CopyPasteRoi.lvb	Copies drawing element of overlay into clipboard and pastes it into other selected windows
Distance.lvb	Example macro for measurement
DivideThroughReferenceImage.lvb	- divide complete time series through a single image/part of the series - duplicate a single image or part of a time series to this series.
ExcitationFingerprinting.lvb	Automated generation of excitation lambda stacks for generating excitation spectra which can be used for linear unmixing to separate overlapping dyes. Any detector can be used, also NDDs. Description see chapter 4.8.4.5.
FastModeSwitch.lvb	Store settings from "Scan-Control" and reuse.
FileExport.lvb	Exports one or more selected images according to the set file format in one go; Exports image intensity values in ASCI format;
FRET plus.lvb	(Option) Enables the user to perform FRET experiments based on sensitized emission or acceptor photo bleaching (depending on the type of LSM) including commonly accepted analysis algorithms.
HotKey.lvb	Shift focus with a button and start Single-Scan
KSPlastv.lvb	KS software macro
Lambdatrans.lvb	Time series alternating between lambda and transmission mode
LsmHWAdmin.lvb (also Button in Maintain)	Direct service hardware access (password protected)
LsmHWAdminEx.lvb	Calibration service macro (password protected)

Name	Description
LsmTime.lvb	Triggered Time scan Macro
MetaExport.lvb	Export of META image files including all channels as tif, bmp, ...
ModifySeries.lvb	Modifies Z Stacks and Time Stacks like Rotation of the stacks, being mirrored, Conversion of time stacks into z-stacks and vice versa;
StitchArtPlus.lvb	StitchArt Plus macro (**Option**)
MultiTime.lvb	Set up of time series experiments including repeated imaging, bleaching and autofocusing with defined configurations at multiple locations and for various views at each location (**Software option**)
OptimizeGDC.lvb	Optimize the max. peak power of fiber coupled Ti:Sa lasers
OptimizeLIVE.lvb	Temporarily adjusts the confocal aperture position of a LSM 5 LIVE without changing the general calibration values
Pixel.lvb	Displays and stores the mean intensity values of each line of one or more channels of one or more images; data from each line are stored as a txt file in the current folder;
Profile.lvb	Displays the pixel values along a line
RecoveryChameleon.lvb	Imitates recovery of chameleon NLO laser in case the laser is falling into CW lasing and does not get to mode lock anymore.
ResetLIVEServos.lvb	Reinitializes all servo motors of a LSM 5 LIVE to overcome possible warmup deviations from default positions
Scalebar.lvb	Indication of self defined intensity levels assigned to a ROI as scale bar in the image; also attaches tick marks and concentration values to the grayscale/color wedge.
SphericObjectiveCorrection.lvb	Calibrates the spheric component of objective error by automatic acquisition of a Z stack on a flat surface and a spherical fit of the topographic data.
TestGrid.lvb	Projects a testgrid into the image window (password protected).
TileScanRotation.lvb	Defines rotation angle of scanners during tile scan
TimeSeriesShutter.Lvb	Close laser shutter in time series on LSM 5 PASCAL
Trigger.lvb	Trigger test

☞ During installation, default macros will be installed according to their type either in AIM\, AIM\HWT or AIM\Macros\. Self generated Macros will be in AIM\Macros.

In case of a new installation, old macros will be stored in AIM\Macros\BackupMacros or AIM\Backup\, to avoid problems with identical names of existing and new macros.

4.8.4 Sample Macros

The LSM 5 software package includes e. g. the **Distance**, **Profile**, **Spot**, **Center** and **Multiple Time Series** sample macros.

They can be easily executed by clicking on the relevant button in the **Macros** subordinate toolbar.

During the execution of a macro, the **Stop Macros** window is always displayed on the screen. This enables a macro to be stopped any time by pressing the **Stop** button.

The functions of the sample macros are explained below.

4.8.4.1 ScanfieldTransform Macro

The Macro enables to match the scan fields of the LSM 510 or LSM 510 META with the LSM DuoScan. The procedure is necessary to make sure that the LSM DuoScan can also be used for bleaching, activation or uncaging of a selected ROI. If the scan fields do not match the manipulation will not be performed within the selected ROI but with some offset to its position when using the LSM DuoScan. A specific slide (#1105-820) is provided with the system to perform the scan field match.

Function Description

- The checkboxes **LSM** and **DuoScan** allow to chose which scanner is used for scan field adjustment.
- **Offset X** and **Offset Y** shift the image in X and y direction
- **Amplitude** adjusted the amplitude of the X scanner.
- **Rotation** rotates the scan field
- If the **Test grid** checkbox is ticked a test grid is projected into the top most image window.

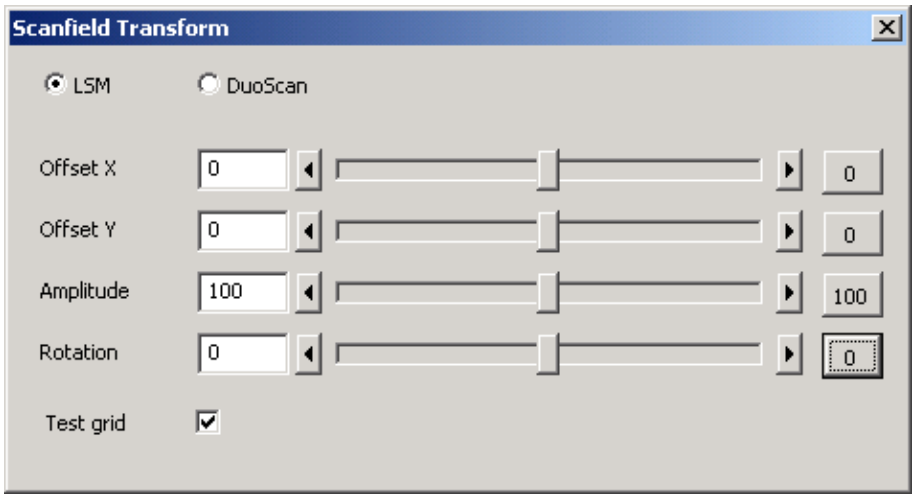


Fig. 4-176 Scanfield Transform Macro

To match the scan fields perform the following steps:

- Use the specific slide provided and focus onto the grid structure.
- Start the ScanfieldTransform Macro.
- Chose a scanner by ticking the corresponding check box.
- Tick the checkbox for the Test grid.
- Set up a track for reflection imaging with the scanner chosen in the Macro.

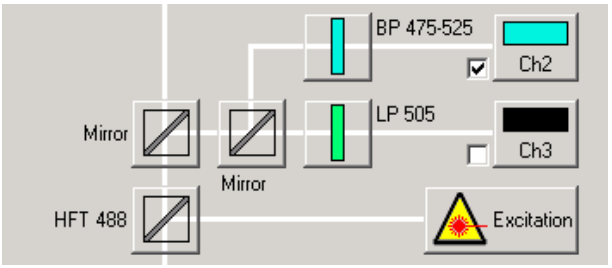


Fig. 4-177 Sample Configuration for imaging the test slide in reflection using the Argon Laser of the LSM 510

- Start a continuous scan and position the center of the grid in the center of the image window.
- If necessary refocus onto the test slide. The projection of the Test grid into the image can be selected and deselected while scanning.

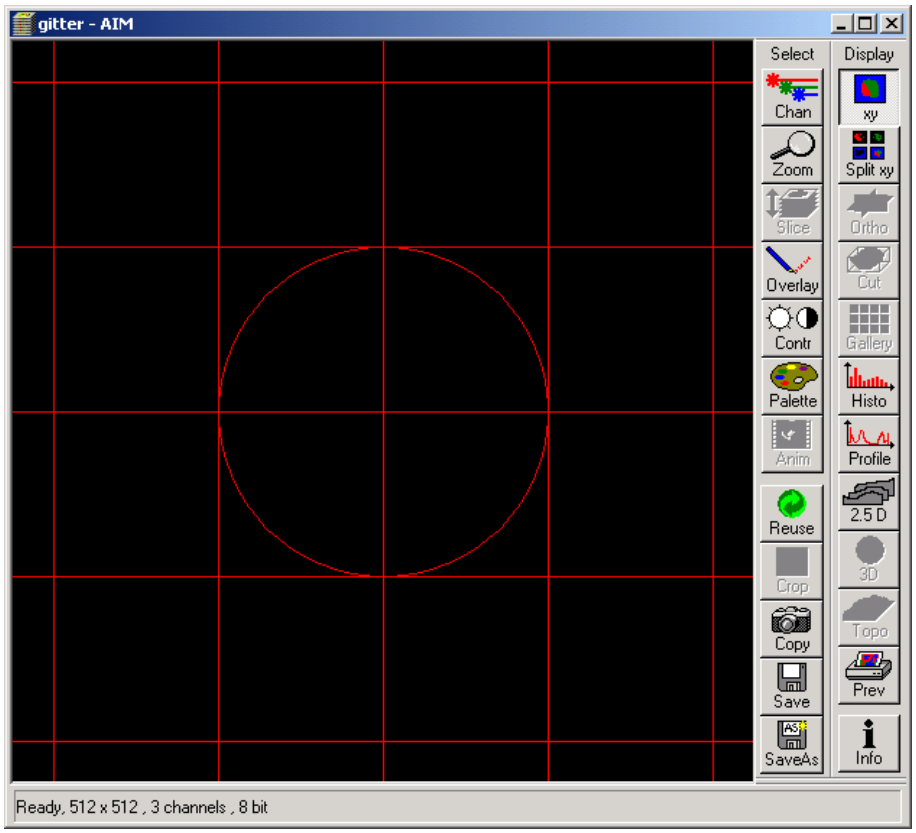


Fig. 4-178 Projection of the Test grid into the image window

- Position the test slide such that the center point position matches the center point position of the Test grid projection.
- Use the sliders for Offset, Amplitude and Rotation to adjust the scanned image to the projected Test grid.

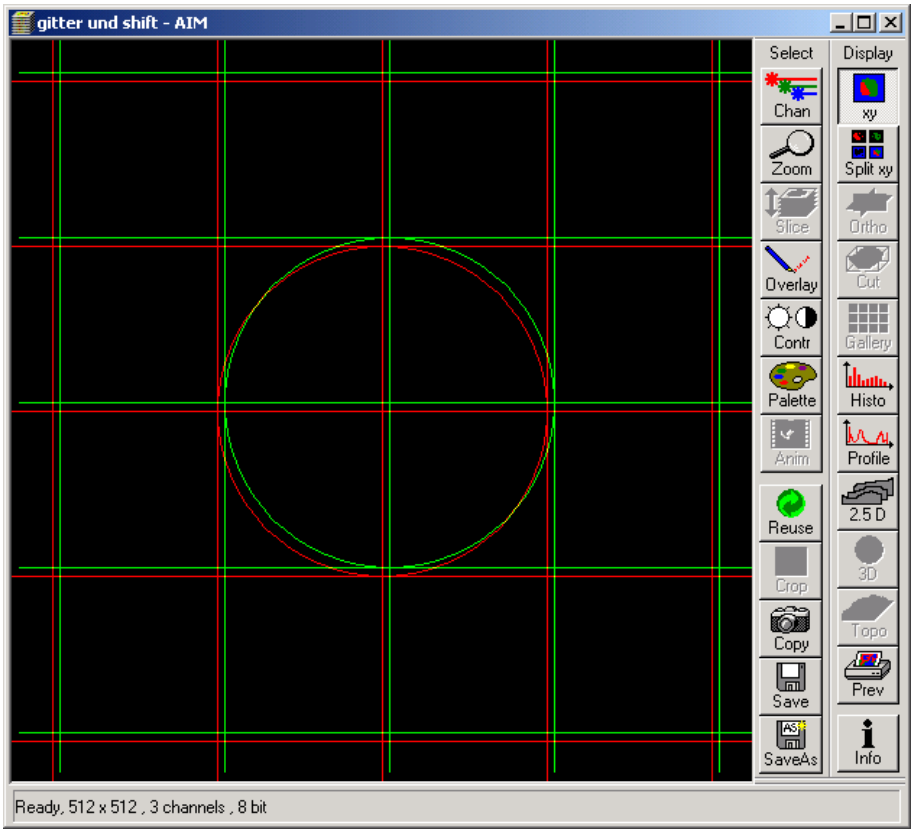


Fig. 4-179 **Projection of the Test grid and the scanned image of the test slide into the image window**

☞ Red represents the projection of the Test grid. Green represents the scanned image of the test slide. Using the functions within the macro allows to shift the scanned image such that is matches the projection.

- Stop scanning, chose the other scanner, set up a corresponding configuration in reflection and repeat the adjustment of the scanned image to the projected Test grid in the image window.
- When both images match the Test grid projection the scan fields also match each other.

4.8.4.2 Distance Macro - Distance.lvb

This macro permits measurement of the distance of a line created in the scan image.

- Click on the **Distance** button in the **Macro** subordinate toolbar.
  - An XY scanning image of the specimen is recorded and displayed. At the same time, the **Mouse position test** window appears on the screen.
- Then draw a line over the distance to be measured by clicking and holding down the mouse button. The click of the mouse sets the starting point, releasing the mouse sets the end point of the line.
  - After release of the mouse button, the length of the line in the scanning image is displayed (in µm).
  - Any required number of lines can be defined in the image. The previous line is deleted.
- A click on the **Exit** button in the **Mouse position test** window will end the macro.

4.8.4.3 Profile Macro – Profile.lvb

This macro permits the gray values of a line created in the scanning image to be determined pixel by pixel.

- Click on the **Profile** button in the **Macro** subordinate toolbar.
  - An XY scanning image of the specimen is recorded and displayed. The **Profile** window is shown on the screen.
- Then click and hold down the mouse button to draw a line in the scanning image for which the gray values shall be determined.
  - The current numbers of the pixels of the created line to which the relevant gray value is assigned now appear in the **Profile** window.
  - At the same time, the distance of the created line is displayed in µm for checking.
  - Any required number of lines can be defined in the image. The previous line is deleted.
- A click on **Cancel** will end the macro.

4.8.4.4 Bleach Macro – Bleach.lvb

This macro permits the specimen to be excited with the laser as required along a line created in the scanning image. Useful for systems LSM 5 PASCAL which do not have the Bleach functionality in the main software.

- Click on the **Bleach** button in the **Macro** subordinate toolbar.
  - An XY scanning image of the specimen is recorded and displayed. The **Spot Scan** window is shown on the screen.
- Click on the **Select Excitation Line** button.
- Create a free-hand line (spline) in the scanning image over the area to be excited by clicking and holding down the mouse button.
- Then determine the duration of the excitation by moving the Exposure Time slider.
- Click on the **Excite** button to trigger the excitation procedure.
- A click on **Exit** will end the macro.

4.8.4.5 Excitation Fingerprinting

***System requirements*:**

- LSM 510 NLO or LSM 510 Meta NLO system in combination with a software tunable ultra fast laser, software release 3.2 or higher.

With the LSM 510 NLO and LSM 510 META NLO in combination with a software tunable ultra fast laser (multiphoton lasers such as Chameleon from Coherent or Mai Tai from Spectra Physics) one can acquire excitation spectra and use these spectra to perform linear unmixing. This process is called Excitation Fingerprinting.

To use the functionality, a macro is provided to perform the necessary steps for the calibration of the system and the image acquisition.

☞ The calibration of the system ensures a constant laser power at the specimen plane by adjusting the AOM transmission in coordination with the applied wavelength. This has to be done for each set up in the field where the laser is also available.

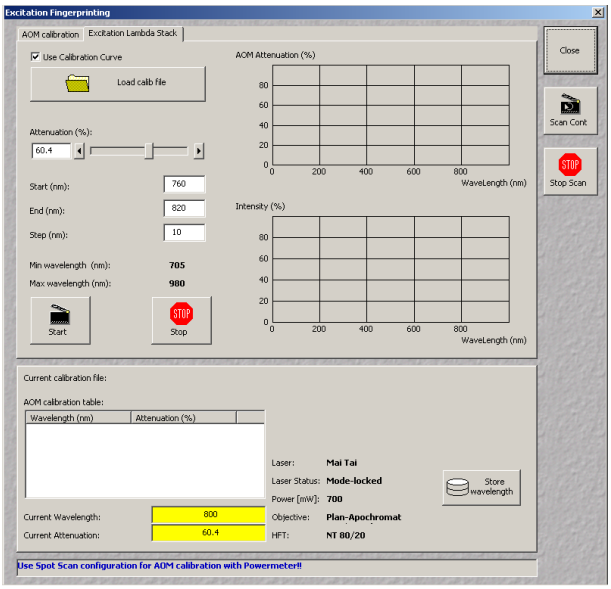


Fig. 4-180 Excitation Fingerprinting macro window

(1) Set up the following configuration for calibration and image acquisition:

- Use the KP 650 as main dichroic and the KP 685 as emission filter.
- Select excitation laser line and set transmission values mode.
- To perform the AOM calibration put the meter in the specimen plane using the objective and main dichroic you will use for image acquisition later on.
- Select Frame as the scanning mode.
- Use the bidirectional scanning mode.
- Adjust the zoom to the maximal value (40) by moving the slider to the far right position.
- Set the scan speed to Max
- Start the Macro
- Start scanning with **Scan Cont**.
- Change view to **AOM calibration**.
- Chose lowest wavelength available.
- Set AOM Transmission to a value around 8.
- Activate the **Auto+** option checkbox (Auto-is chosen when starting with the highest wavelength available).
- By pressing **Add and + 10 steps** the AOM Value will be added to the list and the laser is tuned to the next wavelength: 10 nm further.

- Adjust the AOM Transmission to keep the power indicated at the power meter constant, then press **Add and + 10 steps** again.
- Repeat this procedure until you have acquired the AOM settings for all wavelengths; 10 nm steps are sufficient.

☞ The step size for tuning can be edited. Choosing **Manual** (checkbox) requires to perform the tuning using the slider or typing in a value. Single values or the whole list can be deleted.

Normalize list will set the highest value to 100 and adjust the others accordingly. Recommended for all data. Mean intensity (%) refers to the overall image intensity value; The value is displayed as % of the maximum intensity value at chosen data depth (8 or 12 bit). Calibration files are stored as *.aom files in a folder of choice. Scanning can be started and stopped using the buttons **Scan Cont** and **Stop Scan** on the right side of the Macro Window.

☞ It is recommended to use a power meter with sub-mW resolution to obtain best results.

(2) Using a Mai Tai Laser:

In the lower part of the window the calibration table is shown together with the file currently used, the laser status, objective and main dichroic; The latter will influence the laser power in the sample -> use calibration curves acquired with the same settings as image acquisition is performed.

The store wavelength button will update the laser wavelength in the main SW.

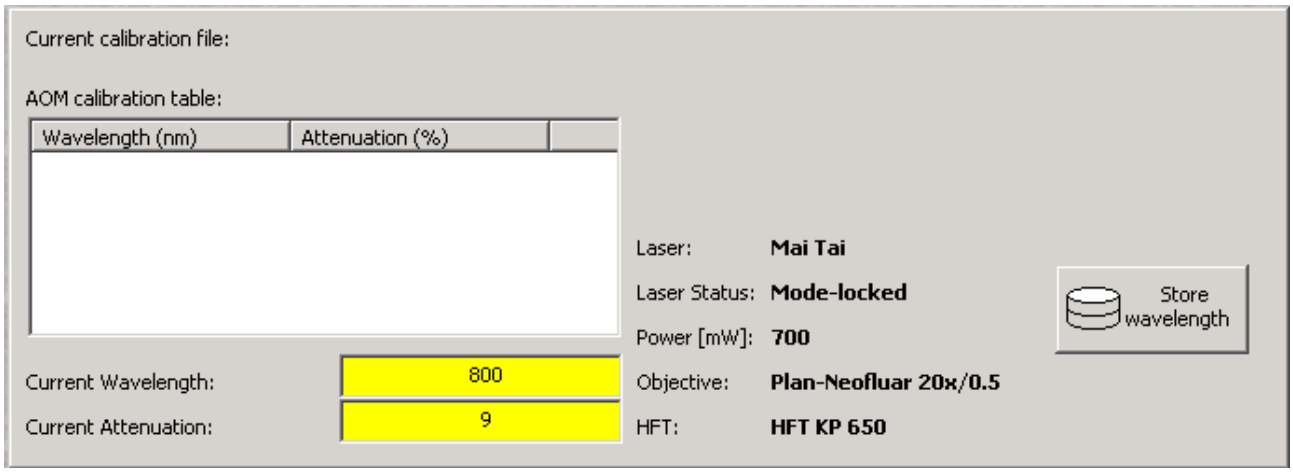


Fig. 4-181 Example using a Mai Tai Laser

(3) Using a Chameleon Laser:

In the lower part of the window the calibration table is shown together with the file currently used, the laser status, objective and main dichroic; The latter will influence the laser power at the sample -> use calibration curves acquired with the same settings as image acquisition is performed. When tuning at whatever process you are performing the option Wait for optimization can be set as desired.

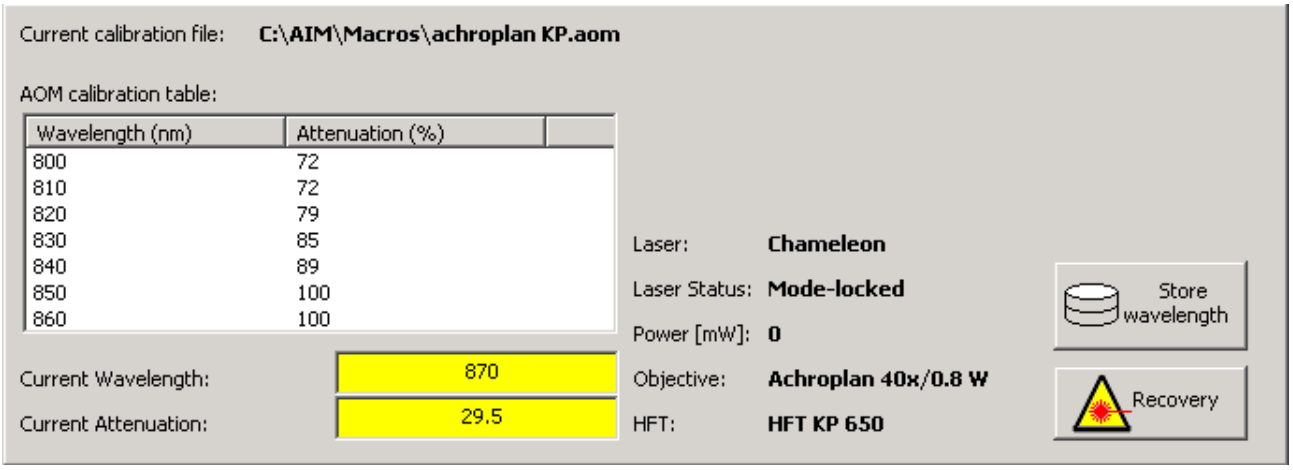


Fig. 4-182 Example using a Chameleon Laser

☞ If the laser does not mode lock after several minutes press Recovery and wait for mode locking (takes three to five minutes). Do not tune to a new wavelength if the laser status is indicated as busy! If the tuning has been done during the status "busy" the LSM software might crash. Restart the software and use the Macro "RecoverChameleon" to reset the laser and the database.

(4) Acquisition of an Excitation Lambda Stack to generate the Excitation Spectra

Excitation Fingerprinting can be performed using predefined Excitation Spectra or using ACE (Automatic Component Extraction). The most convenient and best working method varies depending on the labeling of the specimen. If the different fluorophores show no or only moderate spatial overlap in the specimen, ACE will work well. If the fluorochromes are strongly co-localized, it is recommended to use predefined Excitation Spectra of the individual fluorochromes as reference spectra for linear unmixing.

- After focusing the specimen and setting the AOM transmission such that a good image is generated at the chosen wavelength switch to Excitation Lambda Stack
- Load the calibration file for the AOM required for the configuration of Main Dichroic and Objective used
- Indicate the wavelength range to be used and the step size (this does no have to correspond with the range or step size of the calibration curve)
- Start the image acquisition; A lambda stack will be acquired. The **Data** button (Gallery Mode) displays the wavelengths used for exitation of each image.

(5) Linear Unmixing

- Use the **Mean** button to display the dialog needed to perform Excitation Fingerprinting.The Spectra can be defined manually using the drawing tools to define the appropriate region in the image or by applying ACE (Automatic component extraction).
- Click on the **Linear Unmixing**. The system then performs the unmixing algorithm and displays the unmixed images. The following parameters can be set:
  - **Autoscale**: Balances the brightness of the unmixed images.

- **Advanced Linear Unmixing:** The algorithm used for Linear Unmixing calculates the minimal difference between the unknown spectra and the reference spectra for all possible mixtures of the reference spectra (lowest square fit of acquired spectrum). This can produce negative values for the contribution of individual spectra. Advanced Linear Unmixing ignores the negative values and starts a new calculation based on the remaining spectra. (Without performing Advanced Linear Unmixing, these values will be set to zero).
- **Ask for Background ROI:** Subtracts a spectrum defined by a ROI in the acquired Lambda Stack before performing the Linear Unmixing. This parameter is suitable to remove homogenous background signals.
- **Ignore overexposed and underexposed pixels:** Pixels displaying above the highest or lowest intensity level will not be included in calculations.

- **Generate Channel with Residuals:** Generates an additional „Channel with Residuals“ which is a pixel-by-pixel display of the difference between fit and original data (for the channel of the Lambda Stack that shows the greatest deviation) Perfect fit for a given pixel is indicated by a pixel with intensity 0 in the corresponding channel displaying the residuals.

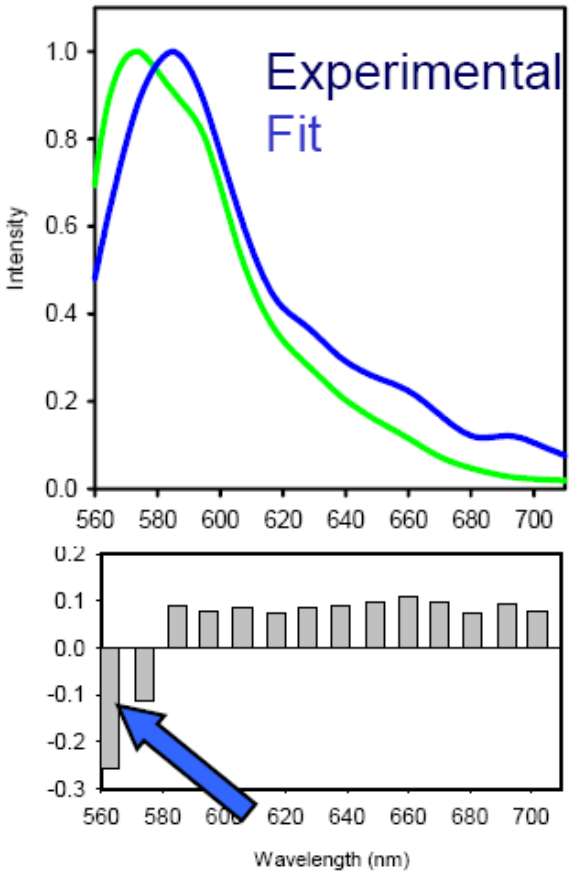


Fig. 4-183 Channel with Residuals

4.8.4.6 Concatenate Images

System requirements:

– LSM 5 system, software release 3.5 or higher

This program combines open image files (single images, z-stacks, time series) to a single file series.

Macro Panel:

Add (Click into image window): Click **Add** button followed by a click in the open image window. An image link will appear in the row of icons below (time line). The palette and the channel settings will be transferred. Overlays will only appear in concatenated images in RGB- mode.

Remove: Removes the active image link out of the concatenate image row.

Remove All: Clears the concatenate image row.

Repeat: Adjusts the number of image copies to be used in the concatenated time series. Repeated image copies can e.g. be used for adjustment of playback frame rate when creating a movie. (If a movie plays a frame rate of 15 images/sec and if an image is supposed to be seen for 2 seconds the repeat slider needs to be set at 30 image copies.)

Mode: Selects the options of data storage. Two options are possible: RGB e.g. can be used for exporting data and making movies. Raw image data e.g. can be used for further image processing and concatenating time data in image series.

– *Concatenating images in RGB mode:* All Images from time series, stacks and single images are concatenated in one series regardless of data depth (Channels, Z-depth, slice number).

– *Concatenating images in Raw image data mode:* Intersection mode: The number of channels in the concatenated image series depends on the image with the fewest channels. The color of the channel is adjusted depending on data of the first concatenated image in the row. XYZC data is treated similar.

Size selection: Selects the image size adjustment by concatenation of image data.

– *Intersection:* Adjusts the image size to a common form (In RGB mode e.g. concatenate frame size 256x 256 with 512x 512, frame size 256x 256 will be adopted to 512x 512 pixel by stretching)

– *Union:* Adopts the established image sizes (in this case 256x 256 frame size being concatenated with a frame size of 512x 512, both image sizes will be shown in the series).

– *Fixed size:* Width and height of the frame size can be set. Square images will be modified according to the smallest side of the frame (e.g. width is set to 300pixel and height is set to 250pixel, a square image will be shown in a frame size of 250x 250pixel).

Apply button: Linked images in the icon row will be concatenated to a time series image stack.

Close button: Closes the Concatenate Images macro window.

Examples:

(1) Concatenate two images:

- Open Concatenate Images macro
- Click Add button followed by a click in the open image window. An image link will appear in the row of icons below (time line).
- Click Add button followed by a click in the next open image window. A second image link will appear in the time line.
- Click Apply button, images or series will be concatenated in a new image window.

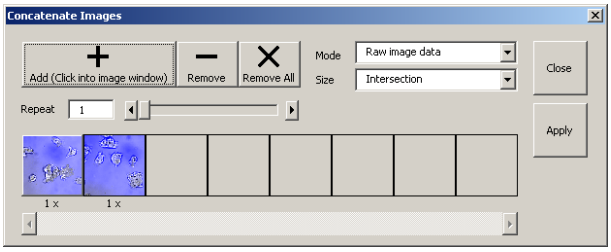


Fig. 4-184 Concatenate images window

(2) Creating a time series:

Concatenate time series images, single images or z-stacks using raw image data mode. Icons in the time line need to be in an ascending time order (check time stamp). Negative time succession results in a false depictive representation. Time data is shown in the galley view. It is calculated regarding the time stamp connected to each image data.

(3) Creating a time series by concatenating z-stacks:

The slice distance (z-depth) is preserved from the first stack in the concatenated row. The final number of slices is determined by the smallest number of slices in the concatenated z- stack row (when. Concatenating a z-stack of 20 images with a z-stack of 15 images, the concatenated time series will have two series of 15 images). Time and z-stack data can be shown in the gallery view.

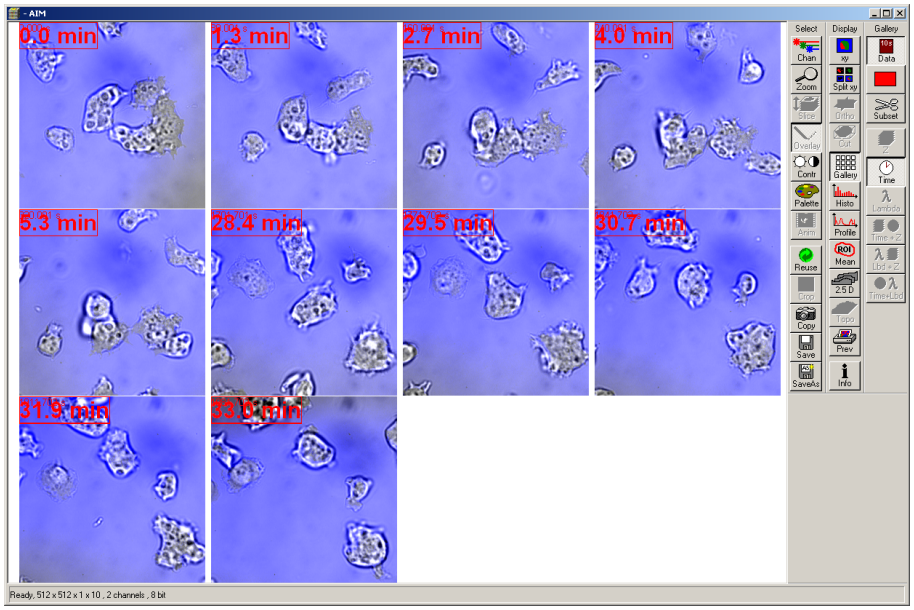


Fig. 4-185 Concatenated time series example (Gallery mode)

☞ The time stamp is automatically updated according to the recorded time information of each series. (e.g. first 5,3 min time lapse is recorded at 1.00 pm, a second autonomous 4,6 min (4 min 36 sec) time series was recorded at 1.28.24am. The concatenated time stack results in 33 minutes because of the temporal shift.).

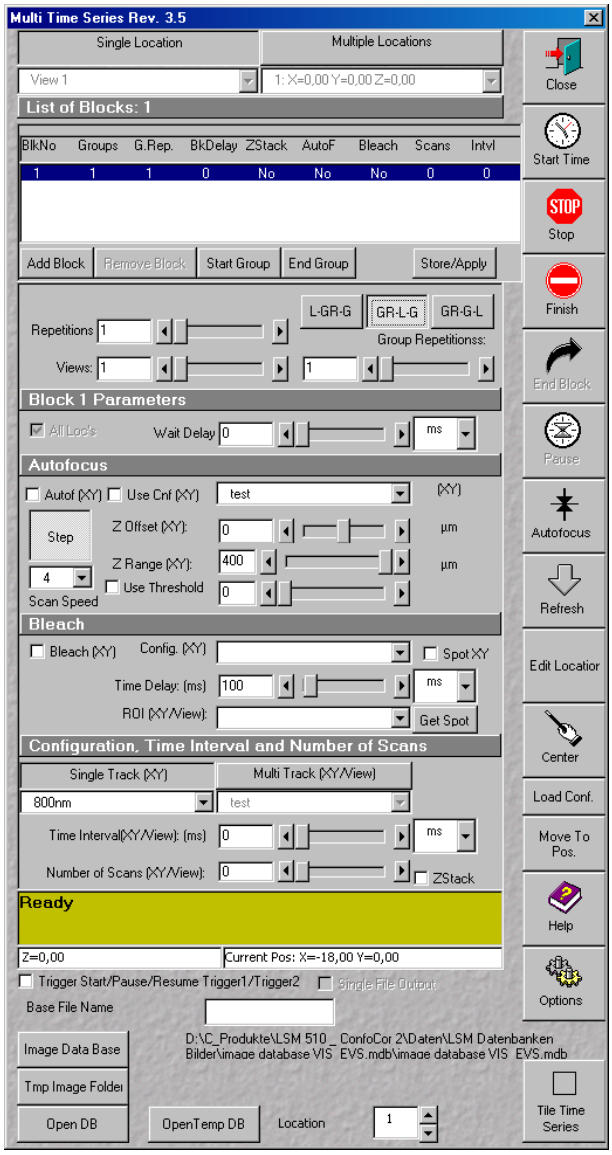


Fig. 4-186 Multiple Time Series main dialog box

4.8.4.7 Multiple Time Series Macro

The Multi Time Series program is designed to provide flexible programming of automated time dependent experiments.

- A pre-program time delay
- A pre-bleaching functionality
- An autofocus functionality
- A number of different, single time series (called a block) either at the same position of a sample or at different positions (if a motorized stage is available)
- A time series of a tile scan
- A time series of a tiled Z stack
- A Z-Stack with triggered acquisition of each single image in the stack

The basic programming unit is a single Time Series Block in line, frame or Z-stack mode. In each block the user can define a configuration for the data acquisition (single or multi-track), the number of images and the time interval/delay between images.

The user can activate an optional autofocus function before each block, pre-program a time interval before each block (from the beginning of the previous block to the beginning of the current block), and/or execute a bleach track with arbitrarily specified bleach ROI's, laser line(s) and power.

The autofocus function can be executed using a specified single-track configuration, or with the configuration used for imaging (using the first channel of the first track).

One can define the z-offset, the distance in the z direction from the position where the autofocus finds the reference feature in focus (position of maximum intensity - e.g. position of the cover slip reflection in the reflected light configuration) to the position, which is moved into focus plane when the image acquisition begins (e.g. into the tissue).

The image acquisition is done with the configuration specified for the given block.

Autofocus search parameters: Z-offset as well as the Z-range (the distance in the z direction over which the autofocus function searches for the plane of maximum intensity) can be set independently for different stage locations (on the systems with the motorized stage).

4.8.4.8 StitchArt plus Macro

Purpose of the StitchArt plus Macro

The **StitchArt plus** Macro is useful for the acquisition of larger profiles and larger fields of view than a single scan field could cover. It is applicable in reflection mode confocal imaging only.

With the StitchArt plus software profile lengths, which are required in standard tactile roughness evaluation, could be performed (for instance: 12.5 mm profile length with 10x objective, 4.0 mm profile length with 20x objective, 1.25 mm with 50x and 100x objective).

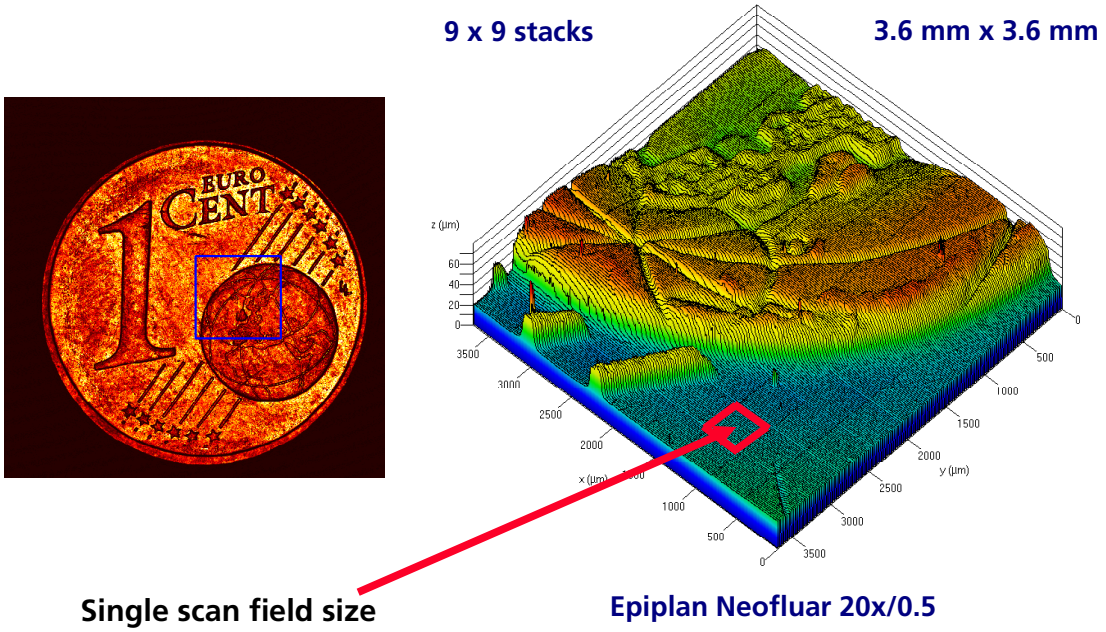


Fig. 4-187 StitchArt plus Macro

General requirements

- The system and especially the lasers have to be in operation for at least one hour.
- The motorized XY scanning stage has to be oriented as accurate as possible perpendicular to the direction of the optical axes.
- The parfocality adjustment of all objectives has to be performed and saved before running the macro.
- The pinhole has to be adjusted properly on a plane mirror or on the sample itself.
- The calibration of the objective magnification in X and Y has to be performed (An image of an XY calibration standard or another known length scale has to be taken and measured with the system in X and Y direction. The ratio between the measured and the real length value has to be calculated. The objective magnification has to be corrected with this factor and put in the objective list as a new objective).
- Pinhole diameter and amplifier offset have to be optimized by a prescan. They are automatically taken from the **Channels** tab of the **Scan Control** window.

(1) Loading the StitchArt plus Macro.

- Click on the Macro button 1.
- Click on the macro button 2.
  - The **Macro Control** window opens.
- Click on the **Load** button in the **Macro Control** window.
- Load the macro from **AIM/Macros/StitchArt-plus.lvb**.
- Click on the **Run** button in the **Macro Control** window.

(2) Starting the StitchArt plus Macro

- Click on the **Macro** Button.
- Activate the **StitchArt plus** Button.
  - The **Profile & Stack Stitching** window opens.

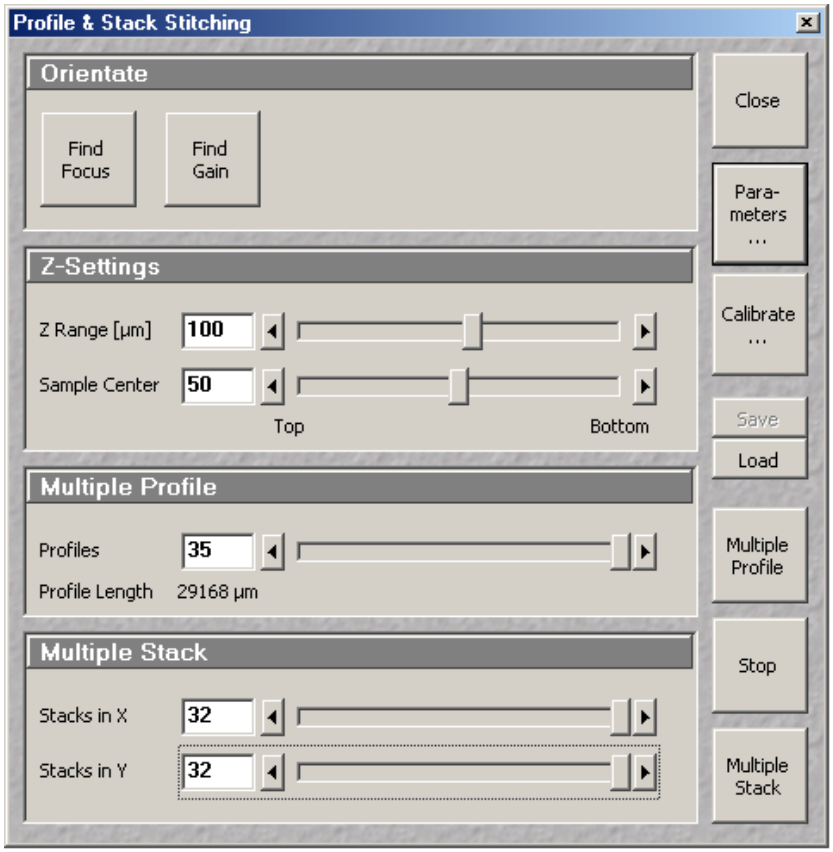


Fig. 4-188 Profile & Stack Stitching window

Orientate panel

Find Focus button — The objective chosen in the **Multiple Profiles Parameters...** window is performed and a fast line scan over Z is acquired. The **Z range** of this line scan is 0.5 mm, the single Z step 300 nm. The stage Z Position is set to the height of the major maximum intensity position.

Find Gain button — When clicking on the **Find Gain** button a **Find** procedure as described on page 4-72 is performed on the current **Z slice**.

Z-Settings panel

Z Range [µm] — This parameter defines the total **Z height** a **Multiple Profile** and/or a **Multiple Stack** is performed. **Z range** values could be changed between 20 microns and 4 mm (if working distance of the objective will permit this, otherwise an error message appears on the screen.

☞ Because in **Multiple Profile Scan** the single Z step could only be varied from 100 … 600 nm and the maximum number of Z slices is limited to 2000, the overall Z travel distance of the stage is also limited to 200 … 1200 µm, respectively.

Sample Center [%] — This button defines whether the actual Z position of the stage is rather placed on top or on the button of the materials surface.

Multiple Profile panel

Profiles — The number of single profiles which has to be acquired, can be varied between 1 and 35. (Depending on the overlap total maximum size is restricted to less than 32k).

Total Profile Length — The calculated total profile length is displayed

Multiple Stack panel

Stacks in X — The number of stacks in X dimension can be varied between 1 and 32.

Stacks in Y — The number of stacks in Y dimension can be varied between 1 and 32.

Multiple Profile button

If you click on the **Multiple Profile** button, the stage moves out of the center position of the sample and is moved to the left-hand side to perform a first single profile. After that an interim image is performed before and after the next step of the motorized XY stage. A cross correlation of the two profiles (Z) and the two (not displayed) images (lateral) is performed to assure an accurate profile match online during the experiment.

☞ If you acquire the **Multiple Profile Scan** in **Range Indicator** palette the results of the correlation could directly be observed as "blue boxes".

Select subprofiles from MULTIPLE PROFILE data

After acquisition of a **Multiple Profile** it is displayed as an XZ projection.

By variation of the **Z:XY ratio** slider on the right hand side of the image window the Z:X ratio could be changed.

- Click on the **Overlay** button in the main display window and define a region with **Rectangular Frame** button in the **Overlay** menu.
- Click on the **Extract Region** button in the **Overlay** menu. (For control the masked region is displayed in blue colour in the range indicator mode.)

Mask out MULTIPLE PROFILE data

After acquisition of a **Multiple Profile** it is displayed as an XZ projection.

- Click on the **Overlay** button and define a region with the rectangular frame.
- Click on the **Cut** button. (For control the masked region is displayed in blue color in the range indicator mode.)

Multiple Stack Button

If you click on the **Multiple Stack** button, the stage moves out of the center position of the mould (lens) and is moved to the upper left corner to perform a first single stack. After that all other stacks are acquired successive "neighbors" is performed.

☞ If a **Multiple Stack** scan is stopped before the regular end of the experiment, no correlation will be performed.

Stop Button

If you click on the **Stop** button the acquisition is stopped immediately and the stage position returns to the initial XY center position.

Close Button

If you click on the **Close** button the **Profile & Stack Stitching** window is closed.

(3) Setting Parameters for Profile & Stack Stitching

- Click on the **Parameters ...** Button in the **Profile & Stack Stitching** window.
  - The **Parameters** window opens.

☞ Pinhole diameter and receiver offset are applied as defined in the **Channels** tab of the **Scan Control** window of the LSM 5 software.

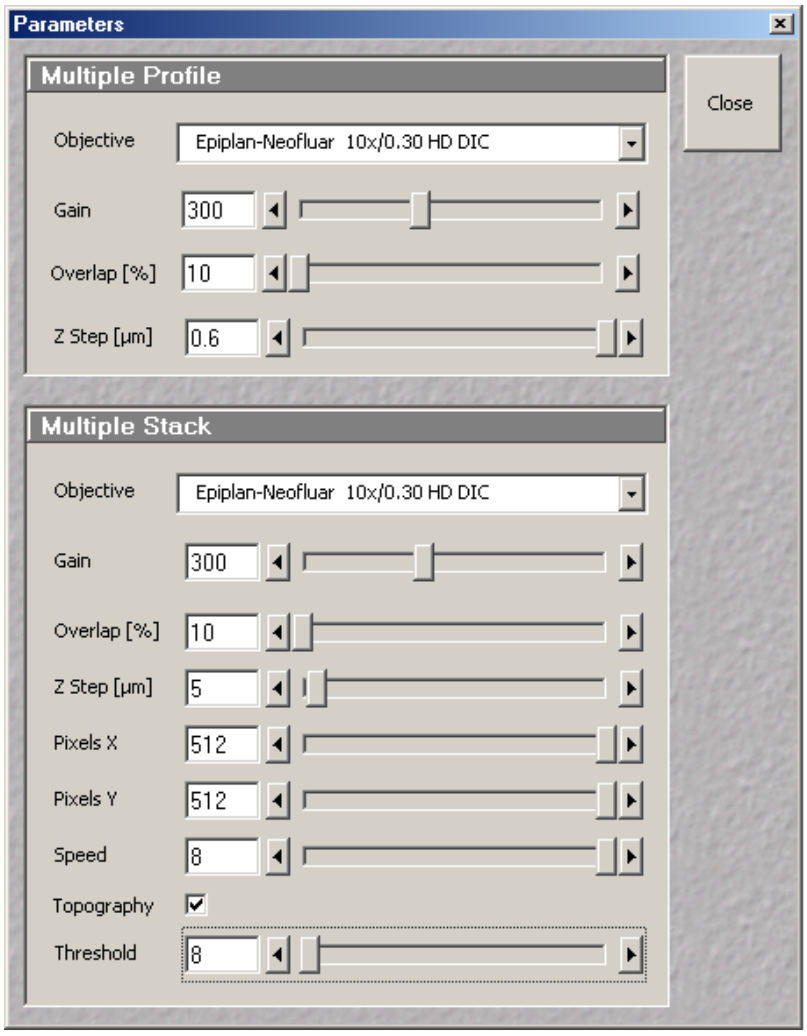


Fig. 4-189 Parameters window

Multiple Profile panel

In the Multiple Profile panel the parameters necessary for the **Multiple Profile** procedure can be defined.

Objective An objective has to be chosen from the currently used objectives of the stand.

Gain The detector gain can be modified. If the **Find Gain** procedure has been applied the optimized gain value is displayed. This value can be changed by use of the slider or by clicking on the FORWARD / BACKWARD buttons.

Overlap [%] The relative overlap between single profiles can be adjusted between 10 % and 50 % of a single profile length. For more pronounced surface features a smaller overlap is sufficient, for rather smooth surfaces larger overlap values are recommended.

Z step [µm] The single Z step in the **Multiple Profile** Mode can be varied between 0.1 µm and 0.6 µm in 0.1 µm steps.

Profile data:

Maximum format:	16384 pixels x 2048 slices
Variable overlap:	10 ... 50 % of single profile length
Scan Speed:	8 (fixed)
Scan time:	20 ... 210 seconds (depending on Z)
Height difference DZ:	0.1 ... 0.6 µm [in steps of 0.1]
Total height Z:	50 µm ... 1 mm
Auto alignment:	Cross-correlation in X, Y & Z

Multiple Stack panel

In the **Multiple Stack** panel the parameters necessary for the **Multiple Stack** procedure can be defined.

Objective An objective has to be chosen from the currently used objectives on the stand.

Gain The detector gain can be modified. If the **Find Gain** procedure has been applied the optimized gain value is displayed. This value can be changed by use of the slider or by clicking on the FORWARD / BACKWARD buttons.

Overlap [%] The relative overlap between single stacks can be adjusted between 10 % and 50 % of the single image length. For more pronounced surface features a smaller overlap is sufficient, for rather smooth surfaces larger overlap values are recommended.

Z Step [µm] The Z step between the single slices of a stack can be defined between 0.1 and 100 microns.

Pixels X The X pixel resolution of a slice of a single stack can be varied between 4 and 512 Pixels.

Pixels Y The Y pixel resolution of a slice of a single stack can be varied between 4 and 512 Pixels.

Speed The scan speed of the **Multiple Stack Scan** mode can be varied between scan speed 5 and 8 (see **Scan Speed**, page 4-75).

Topography This checkbox determines whether the result of the **Multiple Stack Scan** is treated as Multiple intensity stack array or in an reduced XYZ topography format. If this tickbox is disabled all treatments like for a single confocal image stack can be applied. If the tickbox is enabled, only topography based operations could be performed. This **Multiple Stack** experiment needs much less storage when performing the topography based experiment. But operations like orthogonal projections and intensity profiles are not any more available.

Threshold This intensity threshold has to be chosen before performing a **Multiple Stack** experiment in a topography based format and could not be changed therafter.

Adjustment functionality:

Find Focus:	Autofocus by fast Z line
Find Gain:	Auto Brightness & Contrast
Adjust scan mirrors:	To XY direction of MOT stage
Adjust spherical objective error:	On a plane mirror

☞ Macro VBA programming is described in CHAPTER VBA PROGRAMMING FOR LSM of the printed manual.

(4) Calibrating Routine

- When clicking on the **Calibrate** button in the **Profile & Stack Stitching** window the **Calibrate...** window opens.

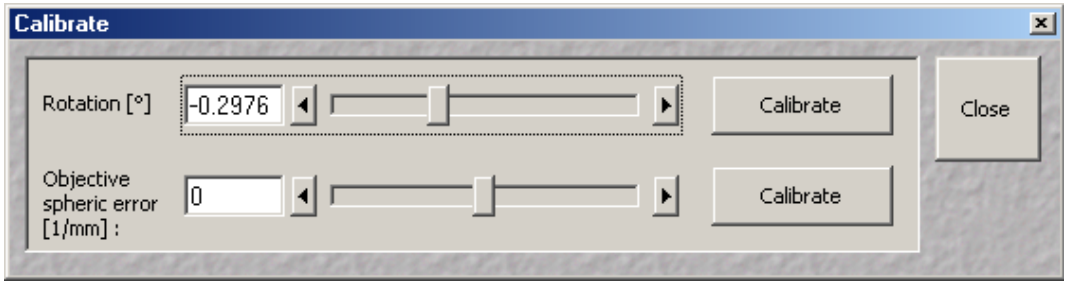


Fig. 4-190 Profile & Stack Stitching window

Calibrate Rotation Routine

With this calibration routine the direction of the scanning mirrors are adjusted to the axes of the motorized XY scanning stage.

For the **Calibrate Rotation** routine a sample with a pronounced structure in the center of the scan field has to be used. This should be an non-periodic structure, not a periodic pattern with a high spatial frequency.

The stage is moved a single step in X direction, an image before and after the step is taken. By correlation of the 2 images the scan field orientation is matched to the stage coordinate system (XY). The rotation angle between both systems is displayed.

☞ This function is also available independently from the **StitchArt plus** option.

- Load the macro from AIM/Macros/TileScanRotation.lvb

Calibrate Objective Routine

With this calibration routine the objective error of first order (spherical component) is determined and corrected. This routine has to be performed when accurately measuring radii.

For the **Calibrate Objective** routine a sample with a pronounced structure in the center of the scan field has to be used. We recommend the mirror sample provided with your system or a flatness standard. Try to bring the surface of the flatness sample in focus.

- Press the **Calibrate** button.
  - An image stack of 50 slices in 100 nm steps is acquired (centered around current position). After the topography reconstruction a 3D sphere fit is applied. The correction factor 1/R is displayed in 1/mm. This calibration is only valid for the current objective position. With a change of the objective position this calibration has to be applied for the new objective, first.

☞ This function is also available independently from the **StitchArt plus** option.

- Load the macro from AIM/Macros/SphericObjectiveCorrection.lvb

(5) Saving the images

The acquired **Multiple Profiles** and intensity based **Multiple Stacks** can either be saved as image stack based data or as a topography based data.

☞ If the **Multiple Stack** data are saved in an intensity stack based format, correlation is performed only in X- and Y- direction, **NOT** in Z-direction.

If the **Multiple Stack** data should be saved in the topography based format, the **Topography** checkbox in the **Parameters** window has to be activated and an intensity threshold has to be chosen before the experiment will be started.

☞ Try to perform a single stack first to find the optimal acquisition settings and the appropriate lower intensity threshold for topographic reconstruction.

☞ In the topography based experiment the data are correlated in X-, Y- **AND** Z-direction.

- The **Save** button in the **Profile & Stack Stitching** window saves topography based **Multiple Stacks** experiments in two file formats.

(a) XYZ-data file without correlation results (*.inf.dat)

The topography data file (.dat) contains the height data in 32-bit IEEE floating-point format. The file has no additional header. All data of the first tile are stored in the file first then followed by the data of the second tile and so on. The tile resolution and size information is stored in the .inf file.

The height values (32-bit IEEE floating-point format) are stored in the range 0…1 where the value 0 indicates that no height value has been determined for the pixel due to low intensity. All other values can be multiplied with the **RangeZ** entry from the *.inf file to obtain the metric height value.

(b) Information file with size of Multiple Stack Scan and automated correlation results (*.inf)

The information file (.inf) is formatted like a MS-Windows ini-file with sections and entries.

Section [Image information]:

Entries:

ImageSizeX ImageSizeY	The horizontal and vertical number of pixels for the tiles.
TilesX TilesY	The horizontal and vertical number of tiles that have been acquired
VoxelSizeX VoxelSizeY	The horizontal and vertical pixel to pixel distance in meter.
RangeZ	The factor that has to be applied to the floating-point height data in the data file to obtain metric height data.
Overlap	The relative fraction of the overlap region for adjacent tiles that has been used to acquire the data in the range 0..1. The value 0.5 indicates that half of the tiles overlap.

The other sections contain offsets for the individual tiles.

The section name has the format [Tile(xxx,yyy)] where xxx is the zero based horizontal index of the tile and yyy is the zero based vertical index of the tile.

Entries:

TileOffsetX TileOffsetY	The horizontal and vertical offset of the left-top corner of the tile relative to the left-top corner of the result topography in pixels. These offset values have been calculated with cross correlation from the original acquired stacks.
TileOffsetZ	The height offset for the data in the tile in meters. Note that the tile data already contain height corrected data and the macro writes always the value 0. By a multiple manual correction already manually corrected data could be more easily distinguished from uncorrected data. The value can be modified in the file and the **Load** command will use this value to shift the tiles in z-direction.

(6) Loading Multiple Stack files

Multiple Stack scan *.inf files can be loaded in the **StitchArt plus** software.

4.8.5 Visual Macro Editor

The Visual Macro Editor is an optional software module which allows the user to program a variety of scanning procedures and image calculations as well as the combination of both.

4.8.5.1 Open / Close the Visual Macro Editor Window

- Click on the **Visual** button in the **Macro** subordinate toolbar of the **Main** menu.
  - This opens the **Visual Macro Editor** window.
- Click on the ☒ button in the upper right side of the window to quit.

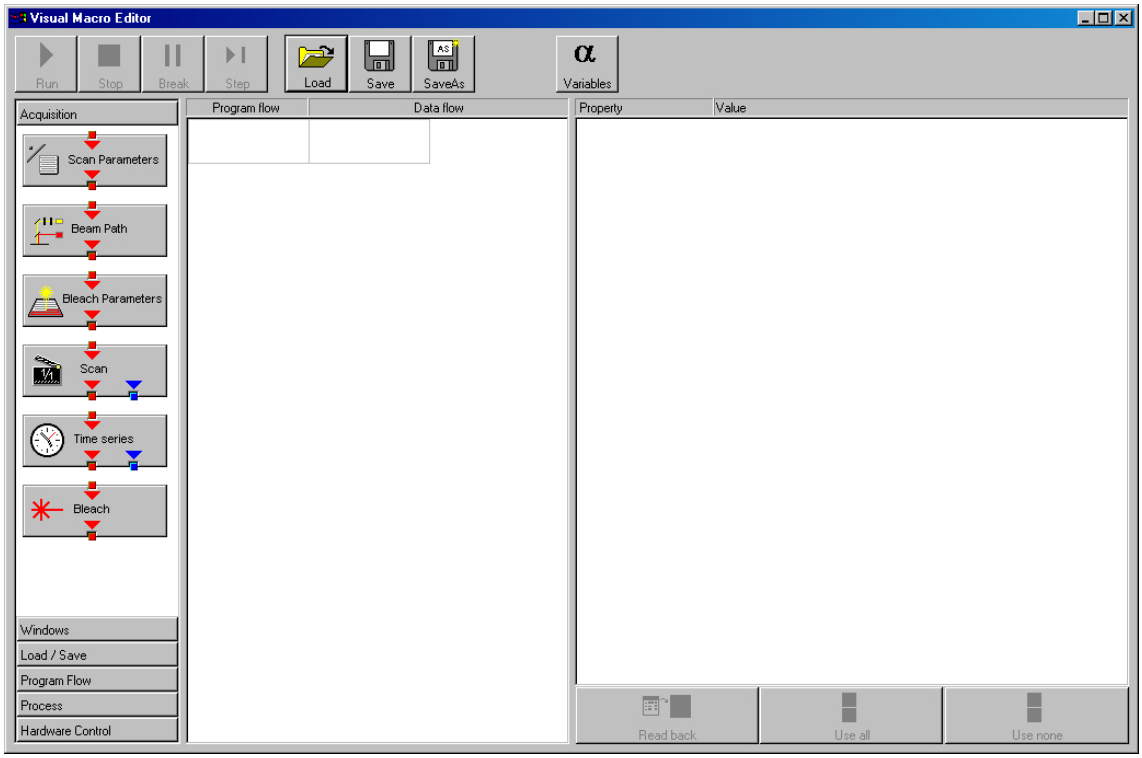


Fig. 4-191 Visual Macro Editor window

4.8.5.2 Function Description

The Editor uses predefined action blocks. The action blocks for setting up the work flow of the Macro are categorized according to their properties. The following categories are displayed on the left-hand side of the **Visual Macro Editor** control window.

- Acquisition
- Windows
- Load/Save
- Program Flow
- Process
- Hardware Control

- Click on one of the Category selection buttons will display the available action blocks.

  The action blocks can be positioned according to their function either in the **Program Flow** column or the **Data Flow** column. The color of the connection arrows of the action block defines the possibility to position the block in the program flow column or the data flow column(s).

- Click at the action block with the left mouse button (hold down the mouse button) and drag the mouse cursor into the flow column. Release the mouse button.
  - Action blocks for the program flow show red connection arrows ▼ as well as blue output arrows ▼ to connect to the data flow.
  - Action blocks for the data flow show blue connection arrows ▼ only.

☞ It is not possible to drag an action block for program flow into the data flow or an action button for data flow into the program flow.
Action blocks can be removed using the **Delete** key of the keyboard.

☞ Without using the **Image Display** action block in the data flow column, the result of the scan will not be displayed (see also description of **Scan** and **Time Series** action blocks).

The work flow is generated by connecting the action blocks using the mouse cursor.

- Click onto an output arrow with the left mouse button (hold down the mouse button) and drag the mouse cursor away.
  - A connecting line appears.
- Drag the line to the input arrow of another action block. Only arrows of the same color can be connected.

According to the action blocks a set of properties is listed which can be assigned with free or predefined values. A set of defined properties is assigned to one block only. If this block is used more than once the properties can be set differently.

Clicking the **Read Back** button will take over the current settings from the LSM main program for the displayed properties list.

- Mark the check boxes next to the parameters individually or, click **Use all** or **Use none** to check or uncheck all parameters with one click.
- For changing a values click into the Value column.

It is possible to define variables. A variable has a (property) name and a mathematical value. The **Assignment** action block can be used to assign a new value to a variable when inserted into the program flow using mathematical algorithms. The new value is then used for any subsequent calculation in the program flow where the variable is part of the calculation.

- Click the **Variable** button on the toolbar to display the variable list. Click once more to display the parameter list again.
- New variables can be added by typing in name and value in the next free line.

Toolbar buttons

Run Starts the Macro starting with the firs block in the program flow column.

Stop Stops the Macro at any time:

Break Interrupts the Macro. Clicking the button again will resume the actual program flow of the Macro:

Step Allows to go through the program flow of the Macro stepwise starting with the first block in the program flow column. Each click will perform the action of the actually highlighted block, then jump to the next block which will then be highlighted.

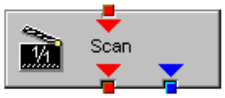
Load Use to open a specific Macro and **Save** to store the Macro**. SaveAs** allows to store the same Macro under a different name.

Variables By clicking the button the list of defined variables is displayed. New ones can be added by typing in name and value in the next free line. Variables are assigned to Macros. For each new Macro a new list of Variables has to be generated.

Acquisition Action Blocks

Loads a Beam Path Configuration form the Configuration list. Use the pull down menu in the **Property/Value** list to select the Configuration that should be used for imaging. Changing between Single Track and Multi Track allows to chose between the different Configuration lists. Multi Track also lists tracks that are used for Emission Fingerprinting or Online Fingerprinting. Depending on the type of track different settings can be loaded optionally. If they are not loaded and the parameters list of the Scan Parameter block does not offer them as being editable, for example detector gain and offset, default values will be used.

The list of properties lists all parameters that can be adjusted for the scan procedure. The value for each parameter can be typed in individually. Clicking the **Read Back** button will take over all current settings from the main software. The check boxes next to the parameters can be marked individually or, clicking **Use all** or **Use none** they can be all checked or unchecked with one click. Only the parameters with a mark in the check box will be taken into account and adjusted when the Scan Parameter block is set in the program flow column. This allows to change only some of the parameters, for example the zoom, at a certain position within the program flow as for each block the check boxes can be set.



This block performs the scanning of the sample according to the settings chosen with Beam Path and Scan Parameters. It can be linked to the Data flow column. If no connection to the data flow is made (for example: Display Image) the result of the scan will not be displayed.

The parameters for bleaching the sample can be loaded from the list of bleach settings. Bleach settings are assembled and stored in the Bleach control window of the main software.

This block performs the bleaching of the sample according to the bleach settings that have been defined and stored in the Bleach control of the main software. Use the pull down menu to select the bleach setting from the list.

The list of properties lists all parameters that can be adjusted for the acquisition of the time series.The value for each parameter can be typed in individually. Clicking the **Read Back** button will take over all current settings from the main software. The check boxes next to the parameters can be marked individually or, clicking **Use all** or **Use none** they can be all checked or unchecked with one click. Only the parameters with a mark in the check box will be taken into account and adjusted when the Time series block is set in the program flow column. This allows to change only some of the parameters, for example the used trigger, at a certain position within the program flow as for each block the check boxes can be set. This block can be connected to the data flow column.

Windows Action Blocks

This block has to be used if the actually scanned image(s) should be displayed. For each Image Display block a new image window is opened. If not saved, the image will be overwritten when the program flow is passing by again.

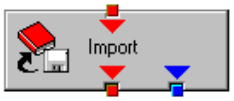
Save / Load Action Blocks

The image(s) are stored to a image database. The database taken is either the last one, that was open or the current open one. Clicking Read back updates the database to be used for storing the image(s). In addition some other parameters can be set according to the list of properties displayed.

Within the program flow already existing images can be loaded.

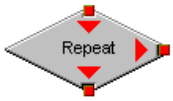
The database taken is either the last one, that was open or the current open one. Clicking Read back updates the database to be used to load the image(s) from. The images can be loaded by Index or by the name of the image.

It is possible to export images choosing a certain file format from the pull down list that appears when highlighting the line **File Format** in the properties list. Specific parameters are assigned to each file format which can be set for the image export.



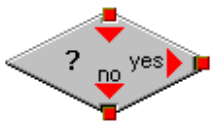
Images can be imported within the program flow. The image to be loaded can be selected by typing in the file path. The import of single images or a image series is possible. The images to be imported as series must have the same name and must be numbered in ascending order.

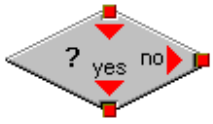
Program Flow Action Blocks



If any action(s) should be repeated a defined number of times, the Repeat block should be set into the loop of the program flow. The number of repeats is set in the properties list. The repeat is only performed using the connection of the right arrow. After finishing the repeats, the program flow connected to the arrow pointing down is performed.

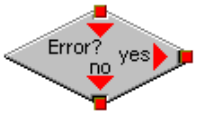
Using this block in the program flow allows to assign new values to a variable using mathematical calculations. By passing this block the variable will be transiently changed and the new value can for example be used to assign different numbers to the image name or to increase the time delay during a time series acquisition.





The two blocks are used as decision makers. Depending on the value of a predefined variable, the program flow can be directed to one of the two possible directions. The expression of the variable's value which should be used to decide on the program flow is set for each block in the properties list. It takes the actual transient value of the variable into account.

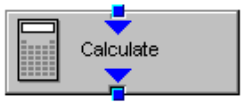
This block introduces a delay in the program flow. The delay time is started when the block is reached within the program flow.



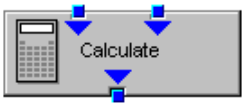
The block allows to assign a potential error message with a textbox and to decide on the Error where to direct the program flow. If no textbox is assigned the program flow will just continue according to the presence of an error message. Choosing a textbox requires the input of the user to continue with the program flow.

☞ With the Release 4.0 this works only for loading images from a database when the images cannot be found because the Index number is not present. In all other cases of an error, the error message will be displayed and the program stopped.

Process Action Blocks

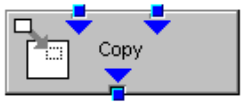


This block allows to do image calculation using the pixel intensities of the image. The operators for the formulas that can be used are described in the Ratio Dialogue within the Process Menu. The formulas set up in the Image calculation window can be copied and pasted into the properties list of the Visual Macro Editor.



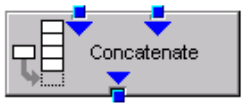
For calculations using two different sources the Calculate block with two possible inputs is used. The calculation starts when both inputs have arrived. Within program loops the next calculation will start when the inputs have arrived again in the same sequence as the first time. The operators for the formulas that can be used are described in the Ratio Dialogue within the Process Menu. The formulas set up in the Image calculation window can be copied and pasted into the properties list of the Visual Macro Editor.

The input image will be copied to a new image format that can be defined by changing the values of the listed properties.



Using this block enables to copy one image into another with the coordinates and settings put into the properties list. The destination image is connected to the left input, the source image is connected to the right input.

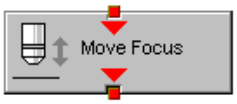
The subsequently arriving images will be concatenated according to the coordinate setting and treated as one image. A subsequent image display will be updated each time a new image arrives.



The second concatenate action block allows to concatenate images from two different acquisition blocks.

Hardware Control Action Blocks

If a motorized stage is available the movement of the stage can be set up as part of the program flow. The stage can be moved to an absolute position which can also be taken from the actual stage position using the Read back function or typed in individually. Using a relative position will use the current stage position as relating position. The values have to be typed in.



The movement of the focus can be set up as part of the program flow. The focus can be moved to an absolute position which can also be taken from the actual focus position using the Read back function or typed in individually. Using a relative position will use the current focus position as relating position. The values have to be typed in. For large travel ranges which exceed half of the objectives working distance a warning can be displayed.

4.8.5.3 Editing a Macro

Following Example shows a macro which acquires five subsequent images and moves the stage 10 microns in X and Y following each image. The images are stored and numbered in ascending order.

- Arrange and connect the action blocks (Fig. 4-192).

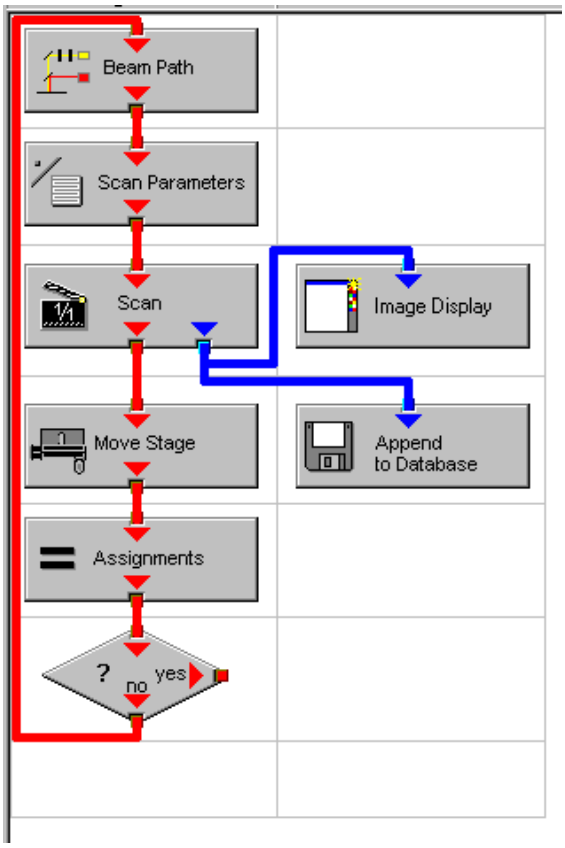


Fig. 4-192 Arrangement of action blocks

- Assign the value **1** to a variable named **Index** (Fig. 4-201).

Property	Value
Index	1

Fig. 4-193 Assignment of the variable

- Click the single action blocks and assign the values to the listed properties (beam path and scan parameters can be chosen according to the sample).

Use	Property	Value
☑	LSM Pixels X	512
☑	LSM Pixels Y	512
☑	LSM Step Y	1
☑	LSM Zoom	1.600000
☑	LSM Rotation [°]	45.000000
☑	LSM Offset X [µm]	0.000000
☑	LSM Offset Y [µm]	0.000000
☑	LSM Pixel Period [µs]	2.560000
☑	Camera Mode	Mono 1300 x 1030
☑	Camera Pixels X	1300
☑	Camera Pixels Y	1030
☑	Camera Offset X [Pixels]	0.000000
☑	Camera Offset Y [Pixels]	0.000000
☑	Pixels Z	1
☑	Scaling Z [µm]	1.000000
☑	Offset Z [µm]	0.000000
☑	Bits per Sample	8 bit

Fig. 4-195 Property list of the Scan Parameter action block

Property	Value
Configuration Type	Single Track
Configuration	488 FITC
Load Speed	No
Load Bits per Sample	No
Load Pinhole Diameter	No
Load Gain and Offset	No

Fig. 4-194 Property list of the Beam Path action block

Property	Value
Database	D:\Projekte\Release 3.5 RT\SW\Scripting\test 2.mdb
Name	"Cell culture" + Index
Description	
Notes	
Compress	Yes

Fig. 4-196 Property list of the Append to Database action block

- The database the images will be appended to is automatically displayed when highlighting the **Append to Database** action block. The last one used will be taken. To update this, open the desired database and use the **Read back** function. Type in a name for the images with or without quotation marks. All images will get the same name then. For numbering the images use quotation marks for the name and add the variable Index, which has been assigned with the value 1. This value or the transiently changed value of the variable will be added to the name of the image.

Property	Value
Mode	Relative Position
Distance X [µm]	10
Distance Y [µm]	10

Fig. 4-197 Property list of the Move stage action block.

- The movement of the stage is defined highlighting the **Move stage** action block, chosing the Mode Relative Position in the Property list and typing in the value 10 for X and Y which then will move the stage 10 microns into X and Y direction starting with the current stage position on passing that action block.

- Now the first complete scan has been performed and the image is stored. For the next cycle the variable **Index** has to get another value to make sure the numbering of the images works. Using the **Assignments** action block, the Variable Index is chosen and the value assigned is **Index + 1**. Such the variable will get the transient value 2 for the next cycle, the value 3 for the over next cycle and so on.
- The number of repeats can be defined using a **Decision** block and using the value of the variable as base for the decision. By defining that if the Expression of the variable is larger than 5 and not connecting the output arrow marked with 'yes' to any other block, the macro will stop after the acquisition of five images. Alternatively one can use at this part of the program flow the Repeat block and define the number of repeats to be performed, which would be 5 in this case (The first scan is already counted!)

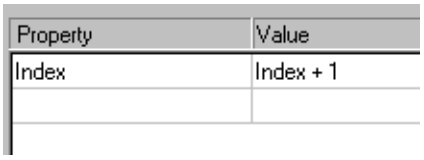


Fig. 4-198 Property list of the Assignments action block

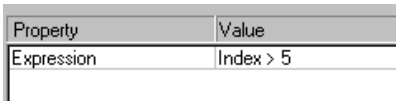


Fig. 4-199 Property list of the Decision action block

4.9 Options Menu

The **Options** menu permits performance of the following functions:

- User-Defined Examination Methods of the Expert Mode are made available to the Routine Mode.
- Deletion of Routine Mode examination methods.
- Display of a current list of dyes with preferred wavelengths for the scanning procedure.
- Display / modification of the user-accessible program **Settings** of the LSM 5 software.

- In the **Main** menu toolbar, click on **Options**.
  - This opens another, subordinate toolbar in the **Main** menu.

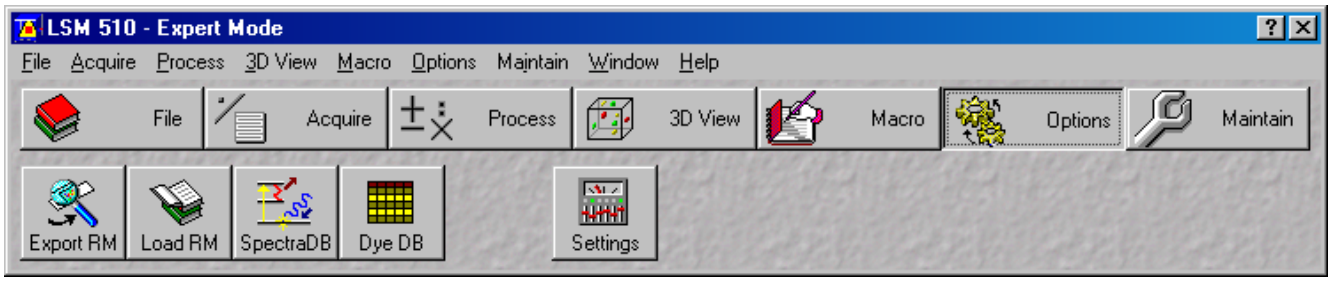


Fig. 4-200 Options menu

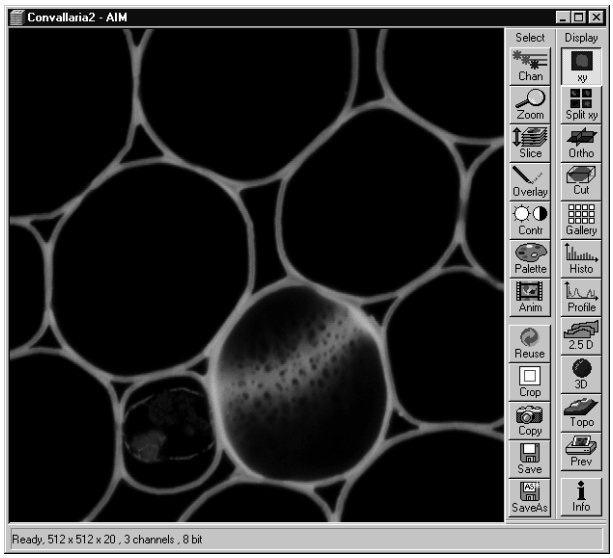


Fig. 4-201 Image Display window

4.9.1 Export RM Function

This function permits **User-defined Examination Methods** created in the **Expert Mode** to be made available to the **Routine Mode**.

- Load a Frame, Stack or Time Series image which was scanned using optimized parameters.

☞ It is also possible to export the examination method of a currently displayed image which has just been scanned.

- Click on the **Options** button of the **Main** menu and then on **Export RM** (see Fig. 4-202).
  - The **Export to Routine Mode** window appears.
- Enter any name for the method to be taken over.
- If required, enter a brief description and additional comments under **Description** and **Notes**.
- Click on **Ok**. (**Cancel** allows you to cancel the procedure).
  - The method used for image acquisition is taken over in the Routine Mode.

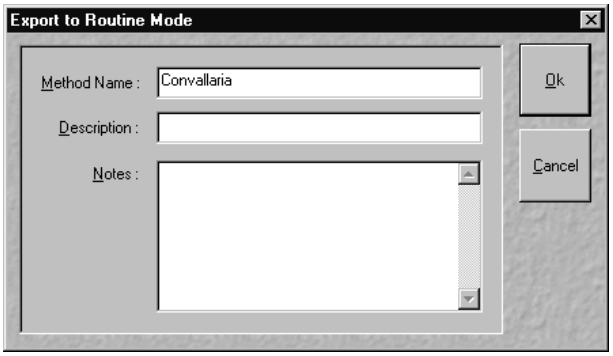


Fig. 4-202 Export to Routine Mode window

☞ The exported **User-Defined Examination Method** must be activated before it can be used in the Routine Mode (see printed manual CHAPTER ROUTINE MODE AND TOOLS).
If you want to change from the Expert Mode to the Routine Mode and vice versa, close all the windows first.
It's not possible to export ratio channels and bleach parameters. All methods will be loaded in the Routine Mode with **Zoom 1**, **Rotation 0**, **Offset 0** and without defined ROIs.

4.9.2 Load RM Function

The **Load RM** (Routine Mode) function permits the deletion of **Standard Examination Methods** and **User-defined Examination Methods** from the database of the **Routine Mode**.

- Click on the **Options** button of the **Main** menu and then on **Load RM** (see Fig. 4-203).
  - The **Routine Mode Database** is opened.

The Routine Mode database is identical to the database of the Expert Mode, though not all the functions are available. The listed methods cannot be loaded as an image.

- To delete an **Examination Method** of the Routine Mode, select the method from the **Form**, **Gallery** or **Table** display of the **Routine Mode Database**.
- Then click on the **Delete** button.
  - The selected method is deleted from the database and is no longer available in the Routine Mode.

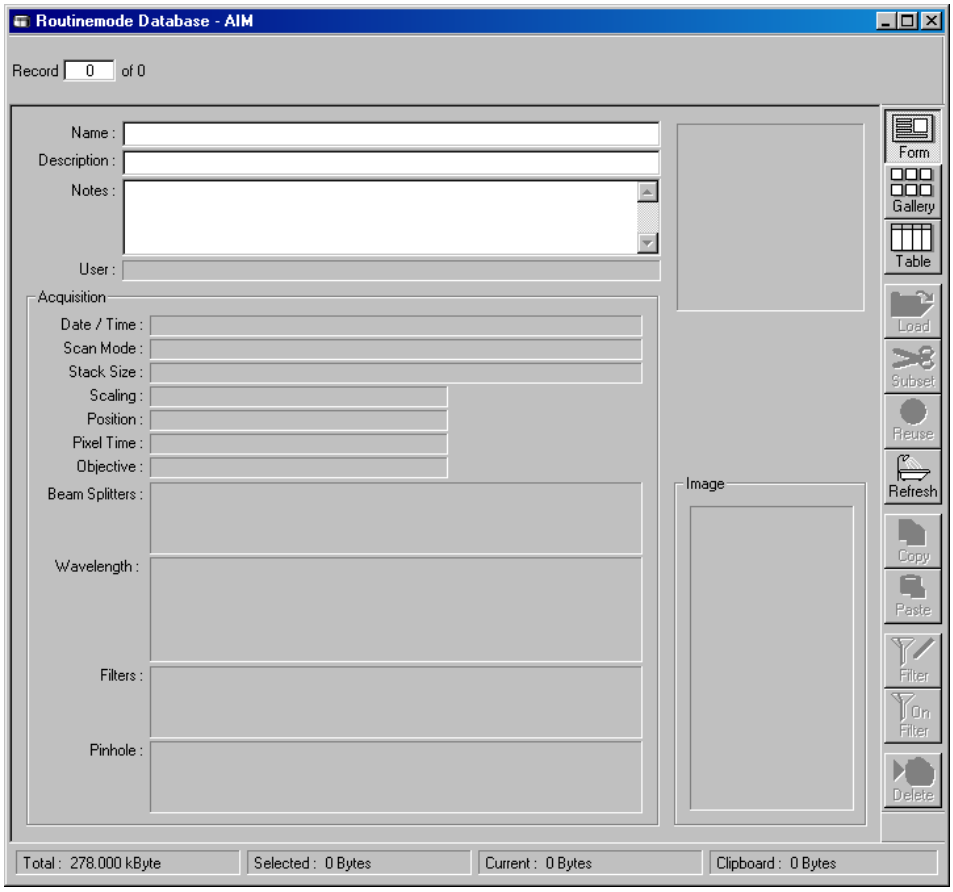


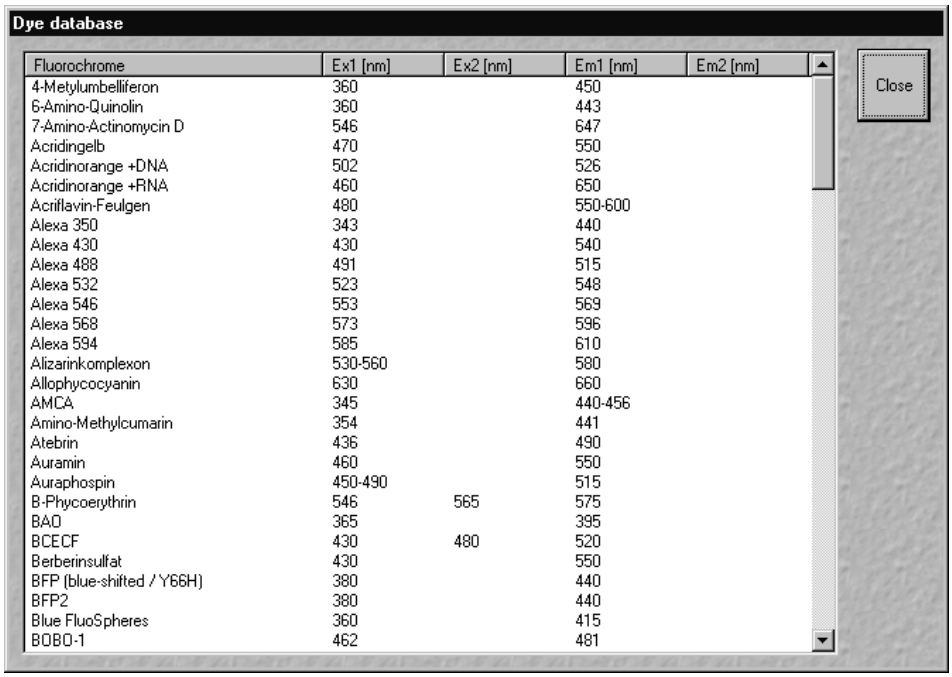
Fig. 4-203 Routine Mode Database window

4.9.3 Dye DB Function

The **Dye DB** function is for information only and permits access to the database contained in the system, including a list of suitable dyes for fluorescence microscopy.

The database contains a comparison of tables of dyes, optimum excitation wavelengths and maxima of emission wavelengths.

- Click on the **Dye DB** button in the **Options** subordinate toolbar.
  - The **Dye database** will be opened and displayed on the screen.
- Click on the **Close** button to exit the **Dye database**.



Fluorochrome	Ex1 [nm]	Ex2 [nm]	Em1 [nm]	Em2 [nm]
4-Metylumbelliferon	360		450	
6-Amino-Quinolin	360		443	
7-Amino-Actinomycin D	546		647	
Acridingelb	470		550	
Acridinorange +DNA	502		526	
Acridinorange +RNA	460		650	
Acriflavin-Feulgen	480		550-600	
Alexa 350	343		440	
Alexa 430	430		540	
Alexa 488	491		515	
Alexa 532	523		548	
Alexa 546	553		569	
Alexa 568	573		596	
Alexa 594	585		610	
Alizarinkomplexon	530-560		580	
Allophycocyanin	630		660	
AMCA	345		440-456	
Amino-Methylcumarin	354		441	
Atebrin	436		490	
Auramin	460		550	
Auraphospin	450-490		515	
B-Phycoerythrin	546	565	575	
BAO	365		395	
BCECF	430	480	520	
Berberinsulfat	430		550	
BFP (blue-shifted / Y66H)	380		440	
BFP2	380		440	
Blue FluoSpheres	360		415	
BOBO-1	462		481	

Fig. 4-204 Dye database window

4.9.4 Spectra DB Function

Spectrums created with the Lambda Mode can be stored in the Spectra Database.

- Click on the **Spectra DB** button in the **Options** subordinate toolbar.
  - The **Spectra Database** will be opened and displayed on the screen.
- Click on the **Close** button to exit the **Spectra Database**.

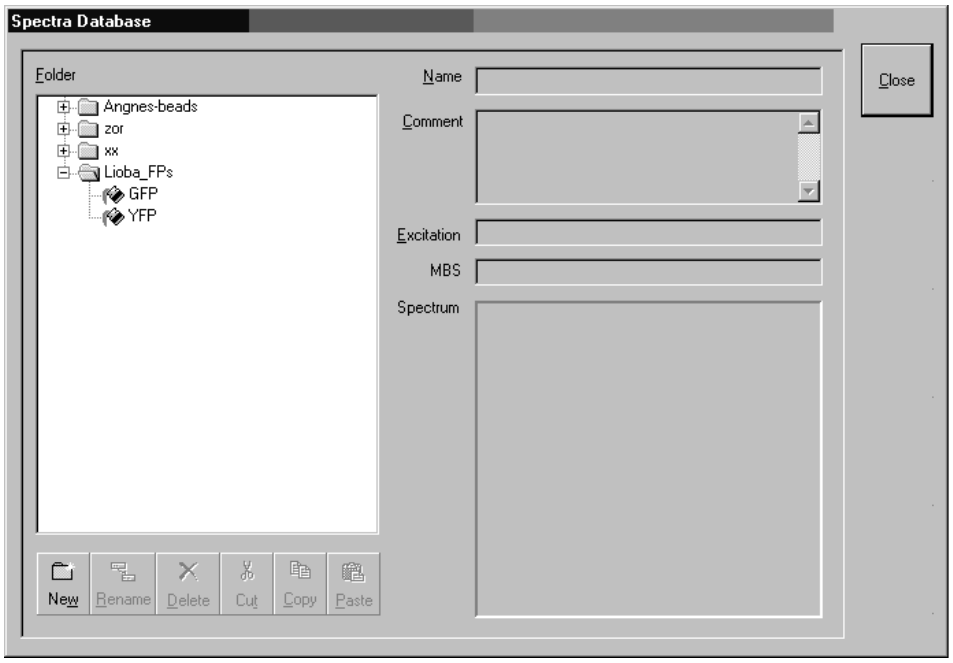


Fig. 4-205 Spectra Database window

4.9.5 Settings Function

The **Settings** function permits the individual setting and matching of software settings with regard to the following points:

Autosave
Database General
Database Table Viewer
Database Gallery Viewer
Import / Export
Scan Information
Image Status Display
Print Status Display
Recording / Reuse
Timeseries
Scan Mean of ROIs
Temporary Files
Program Start
Shutdown
Image Display Toolbars
Save

4.9.5.1 Open / Close the Settings for user : ... Window

- Click on the **Settings** button in the **Options** subordinate toolbar of the **Main** menu.
  - This opens the **Settings for user : ...** window.
- Click on the **OK** button to quit the window. The last settings will be taken over. **Cancel** enables you to cancel the procedure, with any changes you made **not** being taken over.

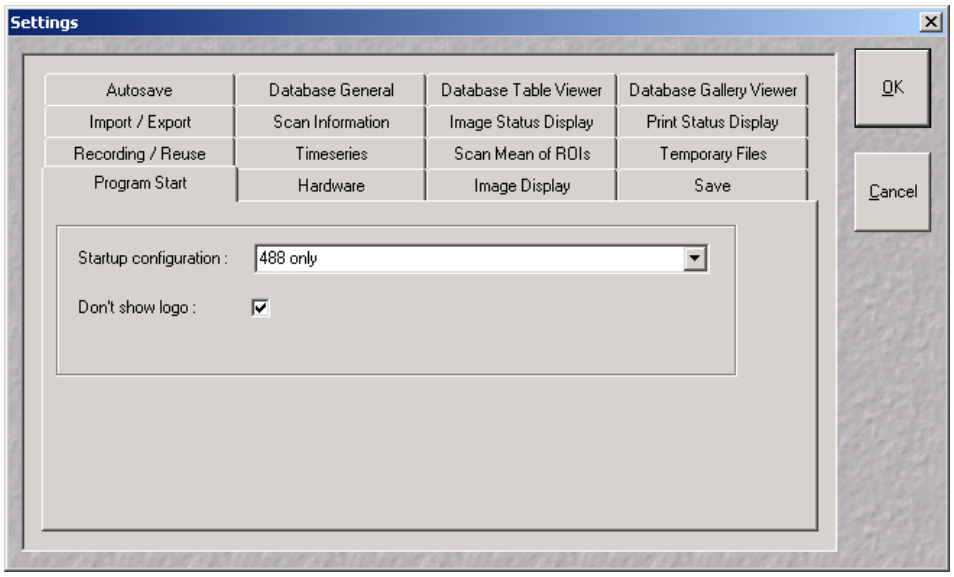


Fig. 4-206 Settings for user : ... window

4.9.5.2 Autosave

This tab permits activation or deactivation of automatic data storage. Only one option can be selected at a time.

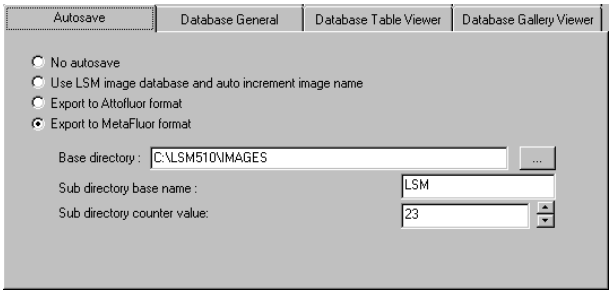


Fig. 4-207 Autosave tab

(1) No Autosave

On activation of this option, the **Autosave** function is switched off. **Save** and **Save As** give the same dialogues.

(2) Use LSM image database and auto increment image name

On activation of this option, newly recorded or modified images are stored by **Save** automatically and assigned to the name or defined in this function. The image name is automatically created using a base name and a serial number. For this, a base name must be entered in the **Base image name** input box, and a starting value for the serial number in the **Counter value** input box. The **Database** selection box permits selection of the directory in which the data will be stored.

(3) Export to Attofluor format

On activation of this option, newly recorded or modified images are stored by **Save** in the Attofluor format. The displayed **Experimental directory** selection box permits selection of the directory in which the data will be stored.

(4) Export to Metafluor format

On activation of this option, newly recorded or modified images are stored by **Save** in a subdirectory in the MetaFluor format. An existing higher layer of folders must be selected for the subdirectory from the **Base directory** selection box. Furthermore, a name for the subdirectory must be entered in the **Subdirectory base name** input box. The starting value for the images then created, to which a continuous number is automatically assigned, is set in the **Subdirectory counter** input box.

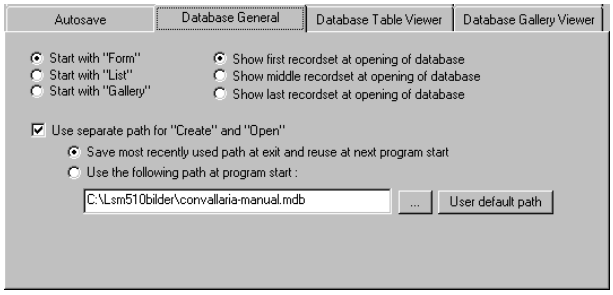


Fig. 4-208 Database General tab

4.9.5.3 Database General

This tab permits the basic starting settings for the use of databases.

(1) Start with "Form"

On opening of a database, the **Form** option is displayed first.

(2) Start with "List"

On opening of a database, the **List** option is displayed first.

(3) Start with "Gallery"

On opening of a database, the **Gallery** option is displayed first.

(4) Show first record set at opening of database

On opening of a database, the first record set is displayed.

(5) Show first record set at opening of database

On opening of a database, the middle record set is displayed.

(6) Show first record set at opening of database

On opening of a database, the last record set is displayed.

(7) Use separate path for "Create" and "Open"

This option permits the path to be changed when the **Open** or **New** database function is used.

(a) Save most recently used path at exit and reuse at next program start

On activation of this option, the path setting last used is automatically selected again in the **Open Database** or **Create New Database** window.

(b) Use the following path at program start

On activation of this option, the path for the **Open Database** or **Create New Database** window can be entered directly in the relevant selection box, or selected by clicking on the **...** button in the **Choose Directory** window. This path will then always be set when a database is opened or created.

Clicking on the **User default path** button firmly sets the **C:\users\default** path.

4.9.5.4 Database Table Viewer

The **Database Table Viewer** tab permits the definition of the columns for the table display of a database. This only requires the relevant check box to be activated with a click of the mouse.

On activation of the **Automatic column width calculation** option, the column width is calculated automatically.

On activation of **Save and use interactive column width setting,** the column width in the database can be matched as required. The individual setting will be retained when the database is closed.

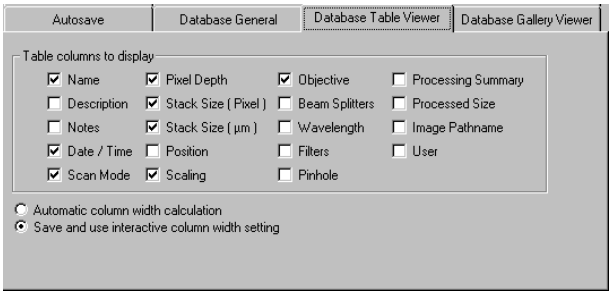


Fig. 4-209 Database Table Viewer tab

4.9.5.5 Database Gallery Viewer

The **Database Gallery Viewer** tab permits the image information to be displayed in the Gallery mode of the database to be activated by clicking on the relevant check box.

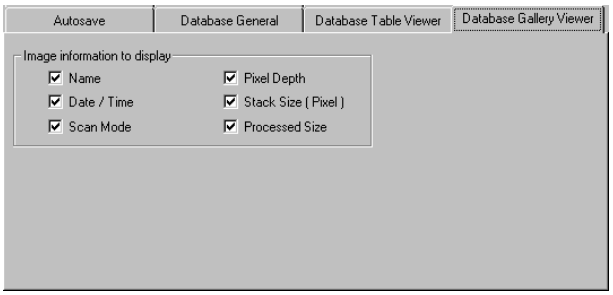


Fig. 4-210 Database Gallery Viewer tab

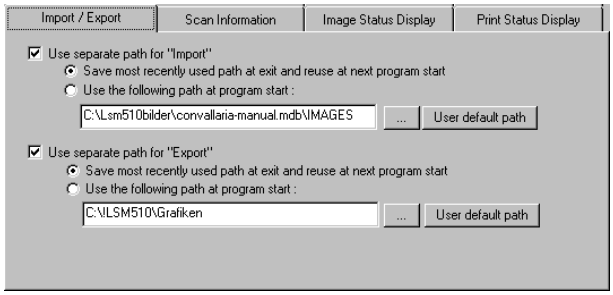


Fig. 4-211 Import / Export tab

4.9.5.6 Import / Export

Use separate path for "Import" or "Export"

This option permits the change of the path setting for use of the **Import** or **Export** function (**File** menu).

(1) Save most recently used path at exit and reuse at next program start

On activation of this option, the path used last is automatically selected again in the **Import Images** or **Export Images and Data** window.

(2) Use the following path at program start

On activation of this option, the path for the **Import Images** or **Export Images and Data** window can be entered directly in the relevant selection box, or selected by clicking on the **...** button in the **Choose Directory** window. This path will then always be set when the **Import / Export** function is used.

Clicking on the **User default path** button firmly sets the **C:\users\default** path.

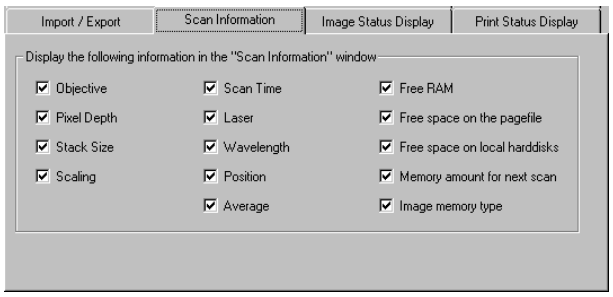


Fig. 4-212 Scan Information tab

4.9.5.7 Scan Information

This tab permits the setting of which scan information shall be displayed in the **Scan Information** window (see **Window** pulldown menu of the **Main** menu, page 4-245f).

Activation / deactivation of the information to be displayed is performed with a click of the mouse.

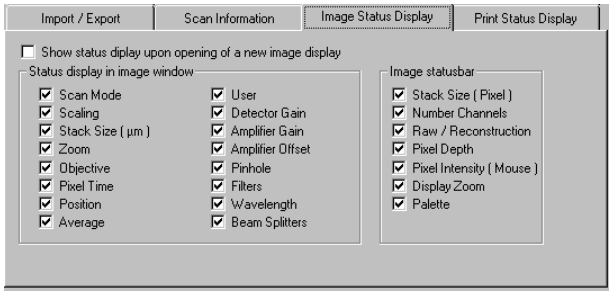


Fig. 4-213 Image Status Display tab

4.9.5.8 Image Status Display

This tab permits selection of which image information is displayed on opening of an image or on activation of the **Info** button of the **Image Display** window. Furthermore, you can determine which information will be displayed in the **Image status bar**.

On activation of the **Show status display upon opening of a new image display** check box, the image information is automatically displayed immediately after opening of the **Image Display** window (**Info** button is activated).

4.9.5.9 Print Status Display

This tab permits selection of which information is displayed in print preview.

On activation of the **Print Status Information** check box, the status information will be printed.

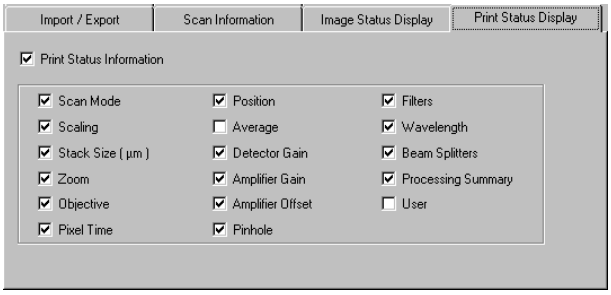


Fig. 4-214 Print Status Display tab

4.9.5.10 Recording / Reuse

The parameters to be taken into consideration for the use or load of a recording configuration are set in this tab.

As an option, you can also determine whether the objective setting shall be taken over when the **Reuse** function is used.

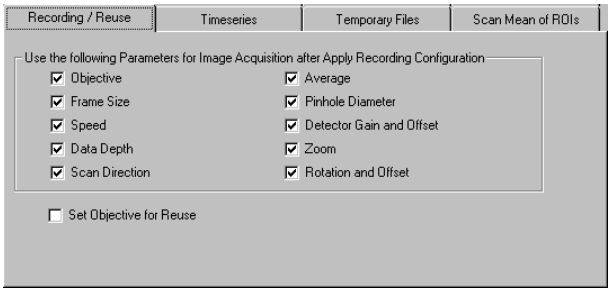


Fig. 4-215 Recording / Reuse tab

4.9.5.11 Time Series

In the **Time series** tab, you can determine whether the time for the recording of a time series is set as **Cycle Delay** or as **Time Interval**.

Cyle Delay is the interval between the end of one scan process and the beginning of the next.

Time Interval is the interval between the beginning of one scan process and the beginning of the next.

You can select the unit for **Mean of ROIs** diagrams.

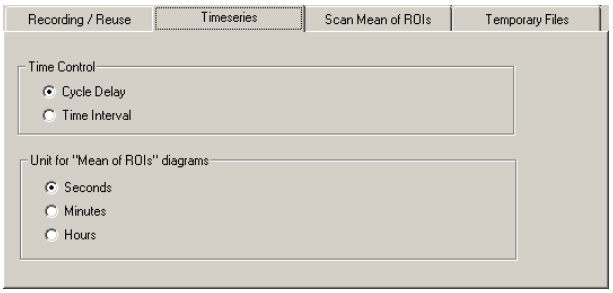


Fig. 4-216 Timeseries tab

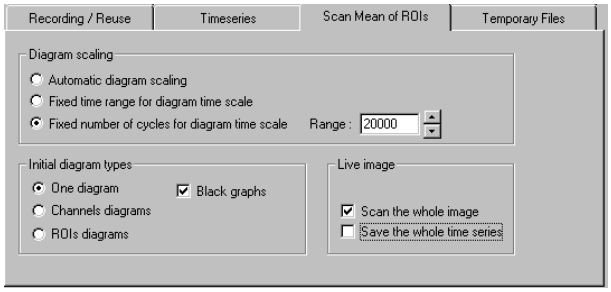


Fig. 4-217 Scan Mean of ROIs tab

4.9.5.12 Scan Mean of ROIs

The **Mean of ROIs** tab permits the presetting of the **Image Display** window for the optional **MeanROI** function (time series) to be changed with regard to scaling and display mode of the intensity time diagrams.

(1) Diagram Scaling

The following settings are possible by activating one of the option buttons:

- Automatic diagram scaling
- **Fixed time range for diagram time scale**; input of the time range in seconds via input box
- **Fixed number of cycles for diagram time scale**; input of the time range in number of cycles via input box

(2) Initial diagram types

The following settings are possible by activating the relevant option button:

- One diagram
- Channels diagram
- ROIs diagram

On activation of the **Black graphs** ☑ check box, the intensity profiles in the diagram are displayed in black (monochrome).

(3) Live image

(a) Scan the whole image

If you activate this check box, the complete live image will be scanned; only the defined ROIs will be scanned if the check box is deactivated.

(b) Save the whole time series

If you activate this check box, the complete Time Series will be scanned; only the Mean of ROI series will be scanned if the check box is deactivated.

4.9.5.13 Temporary Files

The **Temporary Files** tab permits determination of the directory in which temporary files are stored.

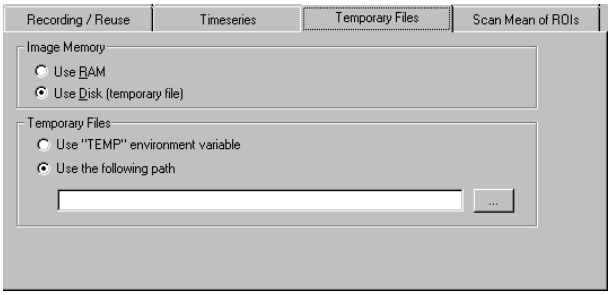


Fig. 4-218 Temporary Files tab

(1) Use "TEMP" environment variable

Temporary files are stored in the **TEMP** standard directory of the computer's hard disk.

(2) Use the following path

The directory for temporary files can be selected by clicking on the **...** button in the **Choose Directory** window.

4.9.5.14 Program Start

The **Program Start** tab permits selection of a track configuration via the **Startup configuration** selection box, which will be loaded automatically when the Expert Mode is started.

On activation of the **Don't show logo** ☑ check box, the initial screen with the Zeiss logo will not be displayed after the start of the LSM 5 software.

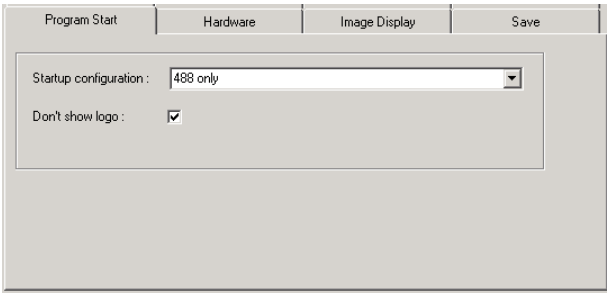


Fig. 4-219 Program Start tab

4.9.5.15 Hardware

The **Hardware** tab allows you to determine, by activation of the **Lasers off on Exit** ☑ check box, that the lasers are automatically switched off when the LSM 5 software is exited.

Allow lasers to cool for five minutes before switching of the system.

For systems with Axio Imager.Z1 with optional **Light Manager** functionality, this functionality can be switched on or off when using LSM by activating or deactivating the check box.

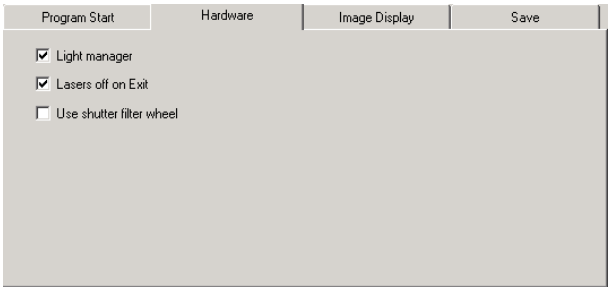


Fig. 4-220 Hardware tab

For systems equipped with an optional line selection filter in front of the Argon Multiline Laser the use of the filter wheel can be activated or deactivated by ticking the check box **Use shutter filter wheel**.

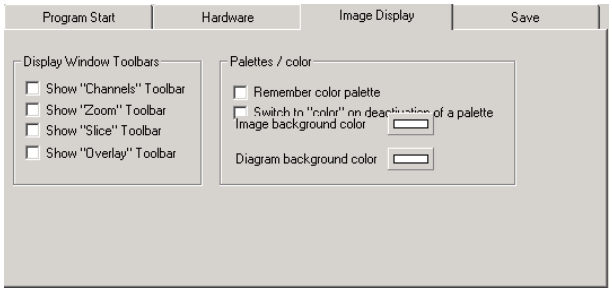


Fig. 4-221 Image Display Toolbars tab

4.9.5.16 Image Display

The **Image Display** tab enables you to determine the window toolbars which shall be automatically displayed when an **Image Display** window is opened.

Furthermore, the color mode (color / mono), to which the image display will switch when the **Color Palette** function is opened / closed, can be determined.

(1) Display Windows Toolbars

On activation of the relevant check box, the following window toolbars are automatically displayed when an **Image Display** window is opened: **Channels**, **Zoom**, **Slice**, **Overlay**.

(2) Palettes / Color

(a) Switch to "mono" on activation of a palette

If this check box ☑ is activated, the **Mono(chrome)** image display mode is switched automatically when a palette is selected in the **Color Palette** window.

(b) Switch to "color" on deactivation of a palette

If this check box ☑ is activated, the **Color** image display mode is switched automatically when a palette is deactivated in the **Color Palette** window.

(c) Remember color Palette

If this check box ☑ is activated, the consecutive image will be displayed using the same palette as the previous image.

(d) The **background color of images and diagrams** can be chosen when clicking on the respective color bar .

4.9.5.17 Save

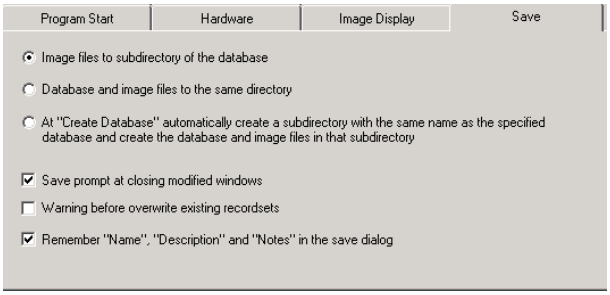


Fig. 4-222 Save tab

The **Save** tab permits the presetting for the storage of scanned or processed images to be changed.

Activation of one of the three option buttons enables you to determine the database directories to which stored images are assigned:

- Image files to subdirectory of the database
- Database and image files to the same directory
- At "Create Database" automatically create a subdirectory with the same name as the specified database and create the database and image files in that subdirectory

If the **Save prompt at closing modified windows** check box is activated, you are automatically asked on closing a changed **Image Display** window whether the image shall be stored.

If the **Warning before overwrite existing recordsets** check box is activated, this question is asked automatically on storing an image under a new name if an image file with this name already exists in the database.

If the **Remember "Name", "Description" and "Notes" in the save dialog** check box is activated, the **Name**, **Description** and **Notes** text boxes of the **Save Image and Parameter As** window show the text for the image last saved. You can edit the text boxes as required for the new image to be saved.

If the **Remember "Name", "Description" and "Notes" in the save dialog** check box is deactivated, the three text boxes are blank when the **Save Image and Parameter As** window is opened.

4.10 Maintain Menu

The **Maintain** menu contains additional modules to check and guarantee the interference-free operation of all the software and hardware components of the LSM 510. The LSM 510 and LSM 510 META can be equipped with an additional scanner, the LSM *DuoScan*. For optimal results the scan fields of the two scanners must be aligned. This procedure is described in chapter 4.8.4.1

- In the **Main** menu toolbar, click on **Maintain**. This opens another subordinate toolbar in the **Main** menu.

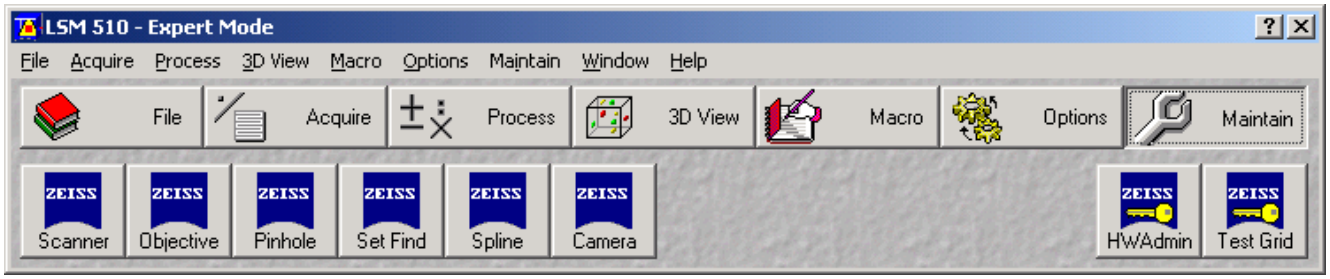


Fig. 4-223 Maintain menu

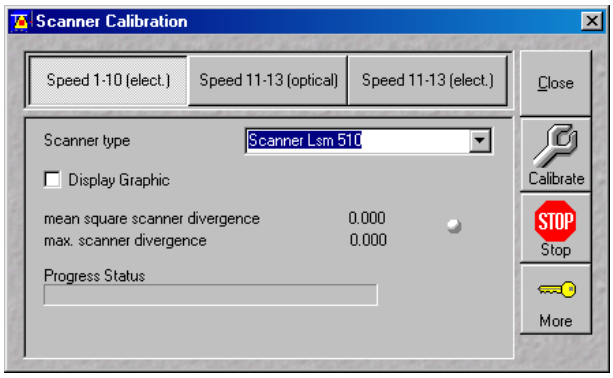


Fig. 4-224 Scanner Calibration window

4.10.1 Scanner

The **Scanner** function is used for scanner calibration.

The following type of calibrations are available:

- Electrical calibration with Speed 1-10 (unidirectional / bidirectional)
- Electrical calibration with Speed 11-13 (unidirectional)
- Optical calibration with Speed 11-13 (bidirectional)

4.10.1.1 Electrical Calibration

☞ Electrical Calibration with Speed 1-10 can be performed unidirectional / bidirectional, but with Speed 11-13 only unidirectional

(1) Preliminary notes

The electrical calibration has to be performed every 2-3 months. For electrical calibration no laser scanning is performed and for that reason no calibration sample is needed.

(2) Calibration conditions

Before the calibration process can be started, the system has to be in operation for at least one hour.

(3) Calibration procedure

- Click on the **Scanner** button in the **Maintain** subordinate toolbar of the **Main** menu.
  - This opens the **Scanner Calibration** window.
- Click on the Speed 1-10 (electr.) or Speed 11-13 (electr.) button respectively.
- For electrical calibration of speeds 11-13 the appropriate zoom factors have to be applied (11 : zoom ≥ 2.5, 12 : zoom ≥ 3.6, 13 : zoom ≥ 5.6).
- Activate the Display Graphics check box enables to check the progress of the calibration process on the Progress Status bar.
  - During successful calibration process, the status button is of green color, in case of an error it switches to red. The progress of the calibration process is indicated by the **Progress Status** bar. The calibration process is completed, when the indicator button is grayed.
- Click on the **Calibrate** button to start the automatic scanner calibration.
- Confirm warning information with **OK**.
- Click on the **Close** button to close the **Scanner calibration** window.

The **More** function is for servicing purposes only and can only be performed by authorized personnel. Its access is therefore password-protected.

4.10.1.2 Optical Calibration with Speed 11-13 (Bidirectional)

(1) Preliminary notes

The optical calibration of scan speeds 11-13 (bidirectional) can only be performed at systems with complete hardware level of Release 2.8.

The optical calibration procedure has to be repeated every two weeks in normal use and after long delay times of the system.

The minimum duration of the calibration process is 10 minutes. However, it can last up to a maximum time of 40 minutes depending on the performance of the scanner in use and the actual tuning conditions.

If the optical calibration is successfully finished, there is no need to start the electrical calibration for unidirectional scanning of speed 11-13 (in opposite, the electrical calibration would overwrite the much more accurate values of the optical calibration procedure).

With speed 11, 12 and 13, scanning is performed at scanner frequencies of 868, 1042 and 1306 Hz, respectively.

In bidirectional scanning, the total line frequencies are 1736, 2084 and 2612 Hz, the image recording times for 512 lines are 0.29, 0.25 and 0.20 seconds.

(2) Calibration conditions

- The microscope stand has to be placed totally vibration-free.
  - Note, that even power units on the granite plate or inappropriate situated cables can cause vibrations.
- Before the calibration process is started, the system has to be in operation for at least one hour (better: two hours).
  - Otherwise, the tuning results will be incorrect and the forward / backward image contents do not match with each other.
- The longest available laser wavelength of the system has to be applied.
- A 80/20 neutral beam splitter and a **None** position in the emission filter wheel of either Channel 1 or 2 has to be used in the **Scan Configuration** window.
- A Plan-Neofluar 10x/0.3 or Epiplan Neofluar 10x/0.3 objective lens has to be used.
- A special sample (see Fig. 4-225) with two identical but 90 degrees rotated gratings (one for each scan direction) has to be used as a calibration standard.
- The pinhole has to be completely opened.
- A dynamic range of 8 bit has to be used.

(3) Calibration procedure

- It is advantageous to perform the electrical calibration before starting the optical calibration process. For a first scan of the calibration standard start with scan speed 12 and zoom 3.6 (unidirectional).
- Focus on the calibration standard and adapt the dynamic range of the detector on the sample.
  - Optimize **Detector Gain** and **Ampl. Offset** values in the **Channels** sheet of the **Scan Control** window by means of the **Range Indicator** mode.

Fig. 4-225 Calibration standard

- The calibration standard has to be positioned as indicated in Fig. 4-226.
- Before starting the calibration procedure, change to the bidirectional scan mode in the **Scan Control** window.
- **Scan Corr. X** and **Scan Corr. Y** in the **Mode** sheet of the **Scan Control** window has to be set to zero.
- Click on the **Scanner** button in the **Maintain** subordinate toolbar of the **Main** menu.
  - This opens the **Scanner Calibration** window.
- Click on the **Speed 11-13** (optical) button.
- Activate the **Display Graphics** check box enables the graphical display of the calibration process.
- Click on the **More** (Less) button to display the Speed Selection menu.

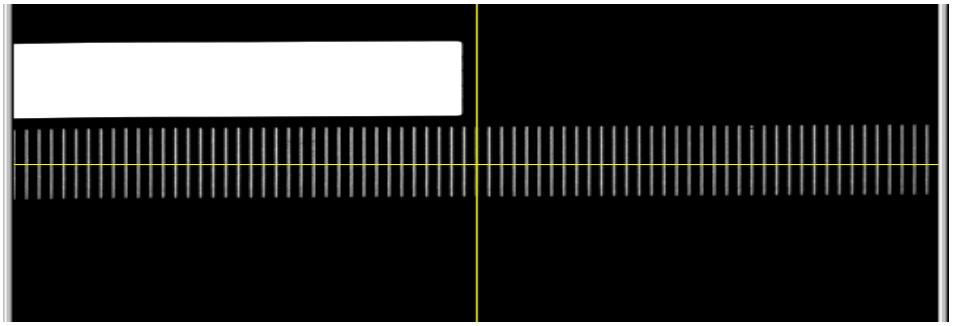


Fig. 4-226 Correct orientation of calibration sample for X scanner calibration

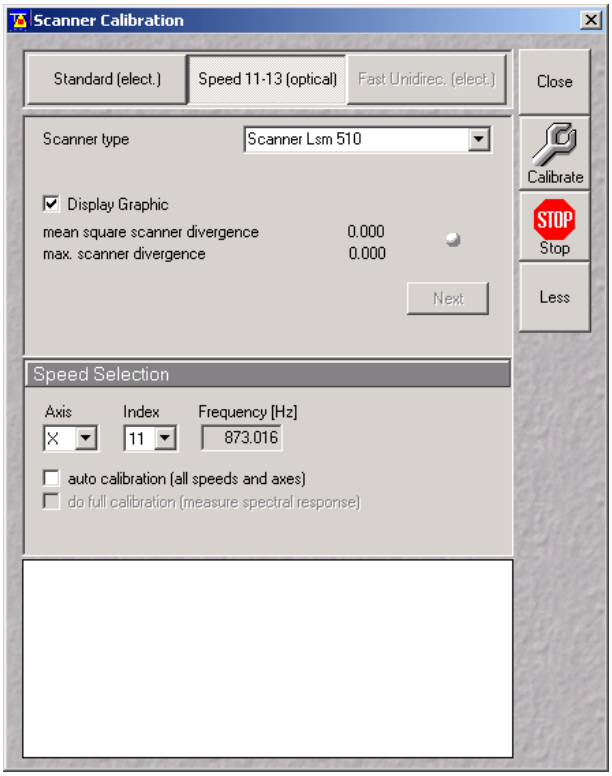


Fig. 4-227 Scanner Calibration window

- With activated **Auto Calibration** box, all speeds (index) and axis are calibrated one after another automatically.

Before the optical calibration is done a full calibration must have been performed on the system. Therefore the checkbox **do full calibration** should be ticked **the first time the optical scanner calibration is done**. This calibration takes some time and should afterwards only by repeated if the standard optical calibration (automatic or not) does no longer reveal satisfying results after several attempts. It should only be necessary to perform the full calibration again after 2-3 years of using of the system.

  – Goal of the calibration procedure is the achievement of a minimum of the **forward-backward-difference** (blue line in the graphical display) and the best possible **linearity** (black line in graphical display monitors the linearity deviation). Both lines (blue and black) should be as straight as possible and as close as possible to the **Zero level** (red line in graphical display). Green line represents the driver voltage.

- Click on the **Calibrate** button to start the scanner calibration procedure.
- Confirm warning information with **OK**.
  – The procedure starts with Speed 11 and the X-scanner. When the Auto calibration for Speed 11 is finished successfully the procedure continues with Speed 12 (higher acoustic frequency) and larger zoom.

- If necessary slightly reposition the sample and click on the **Next** button.
- Do not focus or change scan parameters during calibration procedure!
- After calibration of X-axis the orientation of the calibration grid in the calibration window changes from horizontal to vertical orientation. The second grid of the calibration sample has to be selected and again positioned as indicated in Fig. 4-228. Click on the **Next** button and continue with Y-scanner calibration.
- If the calibration for all speeds and scanners has finished successfully, quit the scanner calibration window by pressing the **Close** button.
- If the **Auto Calibration** procedure can not be performed successfully after several tries, calibrate the scanner manually.
- If the **Auto Calibration** box is disabled, the speed index and the scanner (**X-axis** or **Y-axis**) has to be chosen manually. The calibration cycle only contains one speed index and one scanner. For calibration of all speeds (11-13) and both scanners, the manual calibration procedure has to be repeated several times with changing axis and index parameters (Start with scanner X and speed 11, continue with scanner X and speed 12, then X and speed 13, ...). The position of the calibration standard has to be controlled at each new speed index. A repositioning of the calibration sample is required after scanner change (for X-scanner calibration horizontal grid, for Y-scanner vertical grid).

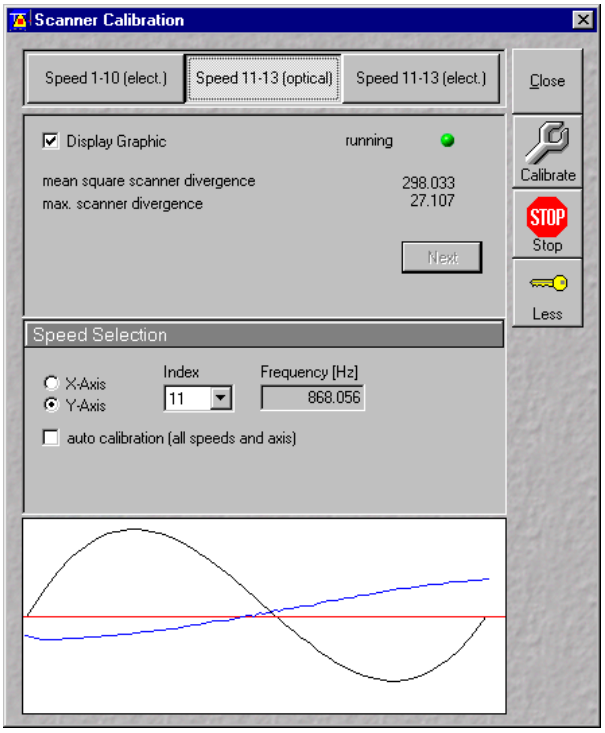


Fig. 4-228 Scanner Calibration window

4.10.1.3 Important Notes and Hints

The tuning procedure runs automatically to a large extent without any problems. However, several errors can occur. That's why it is strongly recommended to observe the complete calibration process.

If a status error message occurs or the calibration procedure is not finished properly, this can have the following reasons:

No optimal positioning of the calibration standard.

☞ One end of blue and black line jumps a bit forward and backward because software recognizes sometimes the outer line of the grating and sometimes not.

- Stop the calibration procedure.
- Check the focus of the calibration sample.
- Check if the yellow horizontal line crosses the scale pattern of the calibration standard properly.
- (If necessary) Shift the calibration standard by half a scale unit (No scale tick but a gap has to be situated directly on the edge of the image).
- Restart the calibration procedure.

No optimal setting of Detector gain and Amplifier Offset.

☞ The forward-backward-difference shows a lot of peaks and changes significantly from image to image.

- Stop the calibration procedure.
- Calibrate speed 12 (unidirectional) electrically.
- Optimize gain & offset: Ticks of the grid have to have intensity values of 250 ... 255 (just before red color in **Range Indicator** palette). Minimum intensity values have to be between 1 ... 5 (no blue parts occur in the image by applying **Range Indicator** palette).
- Restart the optical calibration procedure.

Sliders of Scan Corr. X and Scan Corr. Y in the Mode sheet of the Scan Control window were not set to zero.

☞ Calibration process does not converge.

- Stop the calibration procedure.
- Set both values to zero.
- Restart the calibration procedure.

Non-regularities of the scanner feedback.

☞ The ticks on the outer sides of the grid vary about more than 1 tick width between consecutive images (in the middle of the calibration process, the linearity is optimized and the problem starts to occur).

- Stop the calibration procedure.
- Call the LSM service hotline.

If optical calibration comes not to a successful end, please contact your service hotline.

4.10.1.4 Scanner Calibration in LSM 5 Software

In LSM 5 Software the **do full calibration (Measure spectral response)** checkbox must be set at the first calibration procedure **Speed 11-13 (optical)**.

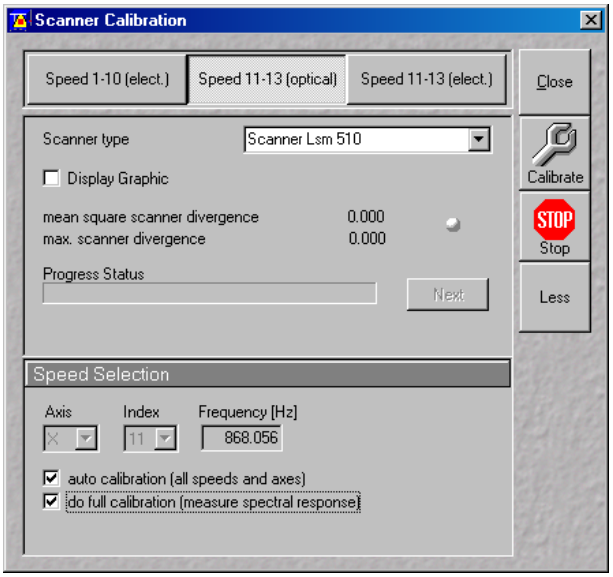


Fig. 4-229 Scanner Calibration window

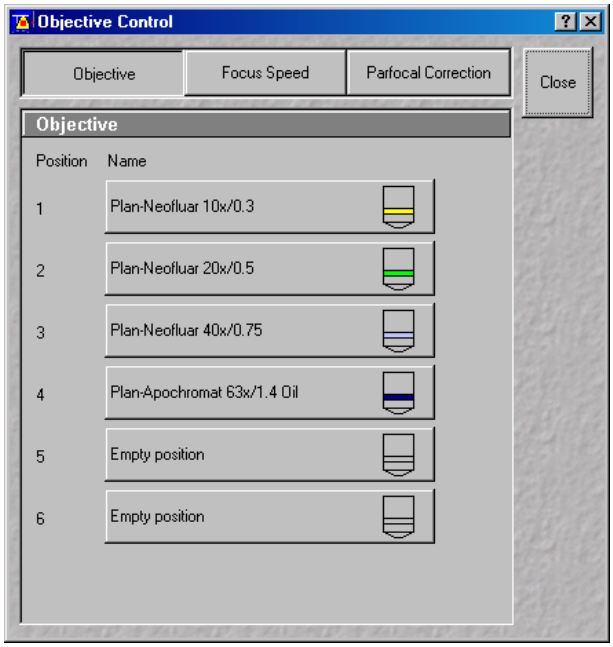


Fig. 4-230 Objective Control window

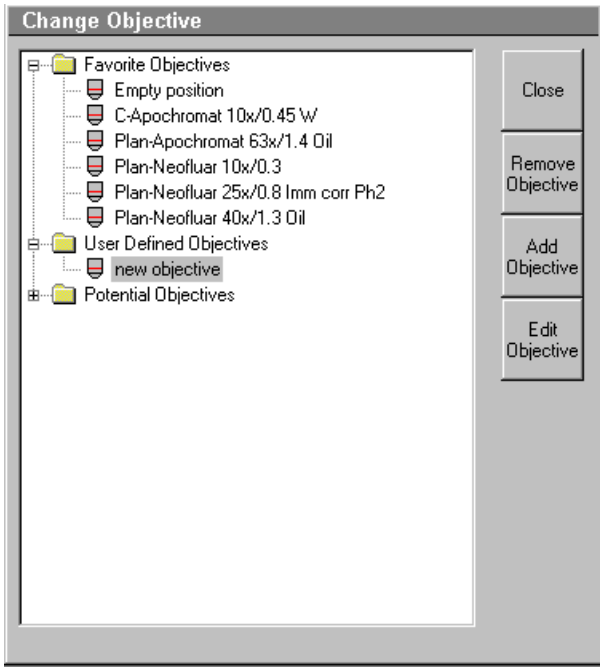


Fig. 4-231 Change Objective window

4.10.2 Objective

This function permits changed objectives to be activated and the parfocality to be set without having to exit the software.

4.10.2.1 Objective Change

- Change the required objective in the nosepiece.
- Click on the **Objective** button in the **Maintain** subordinate toolbar of the **Main** menu.
  - The **Objective Control** window appears on the screen. The **Objective** button is activated in accordance with the presetting, and the **Objective** panel is displayed in the **Objective Control** window.
- Click on the graphical button of the relevant nosepiece mount (**Position**).
  - The **Change Objective** window appears.

All available objectives are listed in the **Potential Objectives** directory of the **Change Objective** window.

- Select the new objective by double clicking from the list in the **Potential Objectives** directory.
- Click on **Close** to exit the **Change Objective** window.

(1) Add Objective

This function permits new objectives to be added to the database.

For this, proceed as follows:

- Click on the **Add Objective** button on the **Change Objective** window.
  - The **Create new Objective** window is opened.

- Enter the data of the new objective in the **Create new Objective** window, then click on the **Apply** button.

The new objective is stored in the database and included in the **Change Objectives** window. You can now activate the new objective as a favorite objective using the procedure described above.

☞ If you have activated the **Non Zeiss** check box, objectives from other manufacturers can also be included in the database.

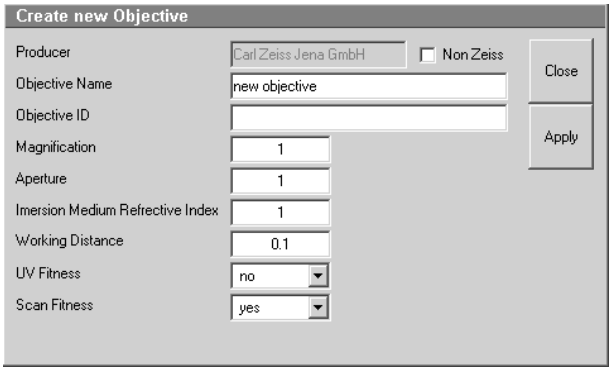


Fig. 4-232 Create new Objective window

(2) Remove Objective

You can only remove objectives in the **User Defined Objectives** directory.

- To remove an objective from the database, select it with a click of the mouse in the **Change Objective** panel and then click on **Remove Objective**. The objective is removed from the list.
- Click on **Close** to close the **Remove Objective** window.

(3) Edit Objective

You can only edit objectives in the **User Defined Objectives** directories.

- To edit an objective from the database, select it with a click of the mouse in the **Change Objective** panel and then click on **Edit Objective**. The **Edit user defined Objective** panel will open and the parameters of the Objective can be edited.
- Click on **Close** to close the **Edit user defined Objective** window.

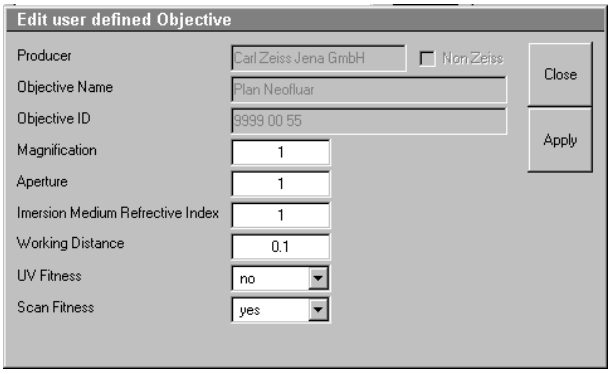


Fig. 4-233 Edit user defined Objective window

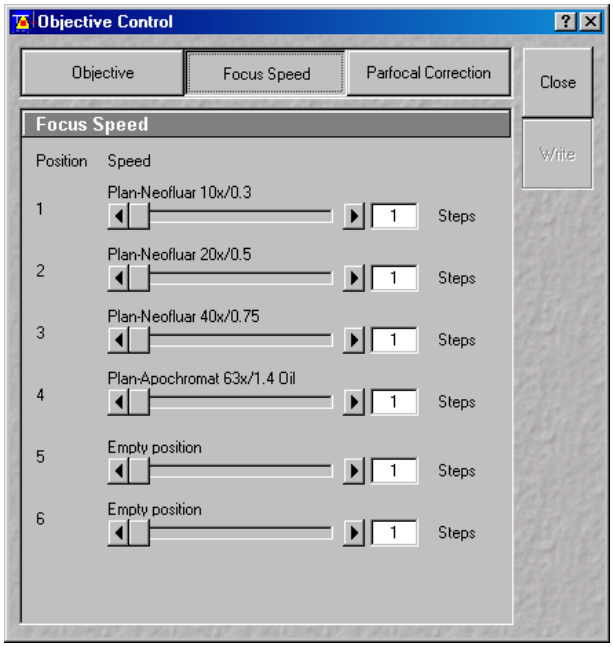


Fig. 4-234 Focus Speed window

4.10.2.2 Focus Speed Change

- Change the required objective in the nosepiece.
- Click on the **Objective** button in the **Maintain** subordinate toolbar of the main menu.
  - The **Objective Control** window appears on the screen. The **Focus Speed** has to be activated in the **Objective Control** window.
  - The focusing speed of the relevant objective can be selected by using either the slider or the input box in 40 steps.

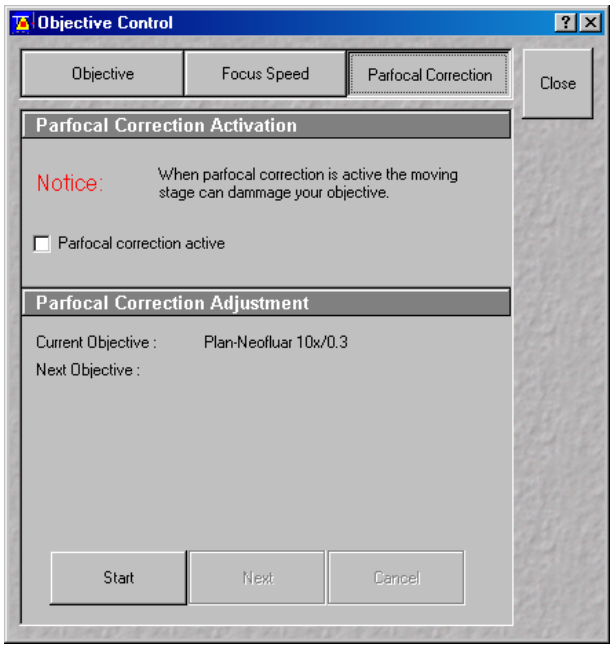


Fig. 4-235 Objective Control window

4.10.2.3 Parfocality Correction

The parfocal setting is performed via screen dialogs in successive panels.

- Click on the **Parfocal Correction** button.
  - The **Parfocal Adjustment** panel appears.
- Start the setting with the objective of the highest magnification factor (reference objective). Proceed in accordance with the displayed instructions.
- Click on **Start**.
  - The next dialog is displayed in the **Parfocal Adjustment** panel.
- Focus on your slide object.
- Click on the **Next step** button.
- Perform these steps for each objective.

- Click on the **Close** button to exit the **Objective Control** window and accept the settings.

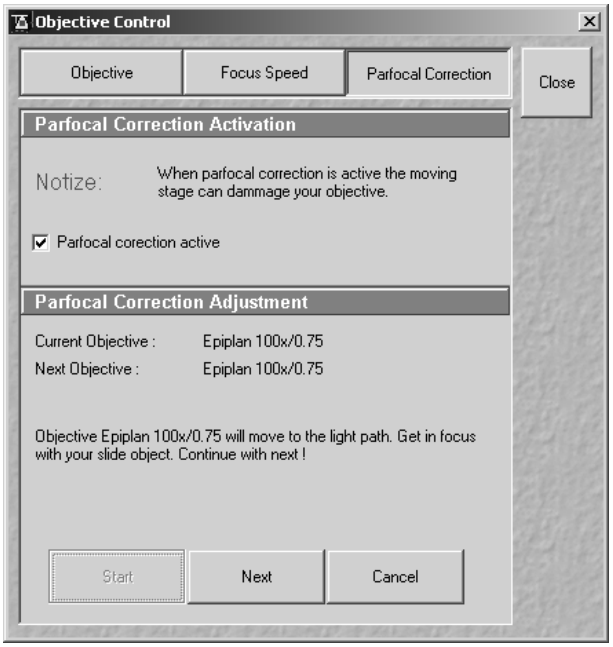


Fig. 4-236 Objective Control window

4.10.3 Pinhole Adjustment

In the **Pinhole and Collimator** window, the pinholes and collimators are optimally aligned and adjusted to the used beam path (configuration).

The position of the pinhole (X-Y-Z-coordinates) in relation to the detector makes a major contribution to image optimization.

In all existing standard configurations, the pinholes have already been adjusted at the factory. These settings are taken over for active operation when a standard configuration is loaded.

If you want to create a setting that differs from the standard configurations, adjust the pinhole as follows.

4.10.3.1 Open / Close the Pinhole & Collimator Control Window

- Click on the **Pinhole** button in the **Maintain** subordinate toolbar of the **Main** menu.
  - This opens the Pinhole & Collimator Control window.
- Click on the **Close** button to quit the window.

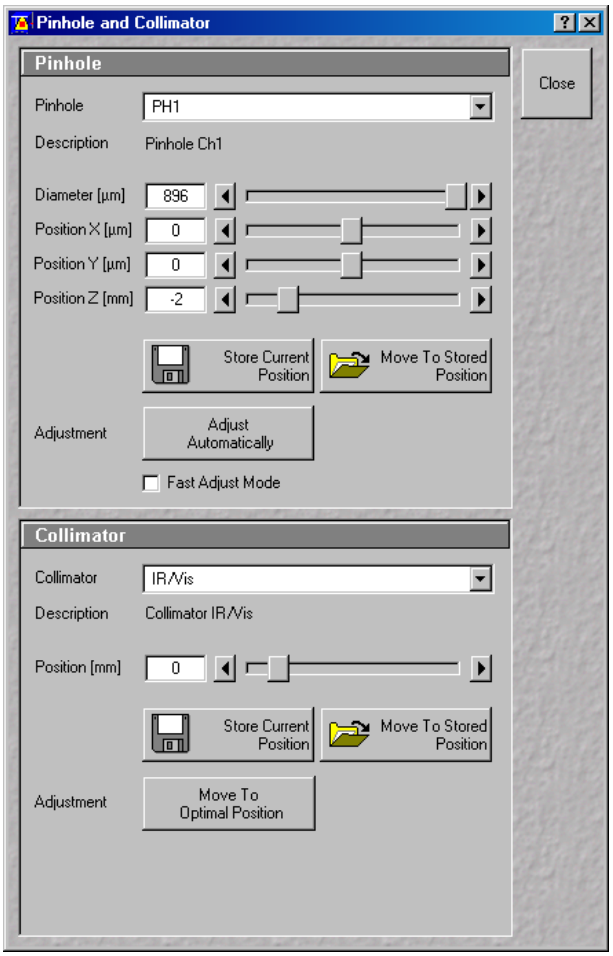


Fig. 4-237 Pinhole & Collimator Control window

4.10.3.2 Pinhole Panel

No further software function can be activated and executed during pinhole adjustment.

Pinhole / Description field: Selection of pinholes (PH1 to PH4) to be adjusted via the **Pinhole** selection box, display of the relevant channel in the **Description** field.

Diameter; Pos. X; Y; Z slider: Setting of diameter, X-, Y- and Z-position of the pinhole in relation to the beam path (Z-position can be set only for PH1) using the slider or arrow buttons, status display for setting procedure: green for ready and red for busy.

Store current Position button: Storage of the current pinhole setting.

Move to stored Position button: Pinhole setting is reset to the position last stored.

Adjust Automatically button: Automatic pinhole adjustment.

Fast Adjust mode check box: If this check box is activated, the pinhole adjustment is only performed in a limited area. Used for readjustment.

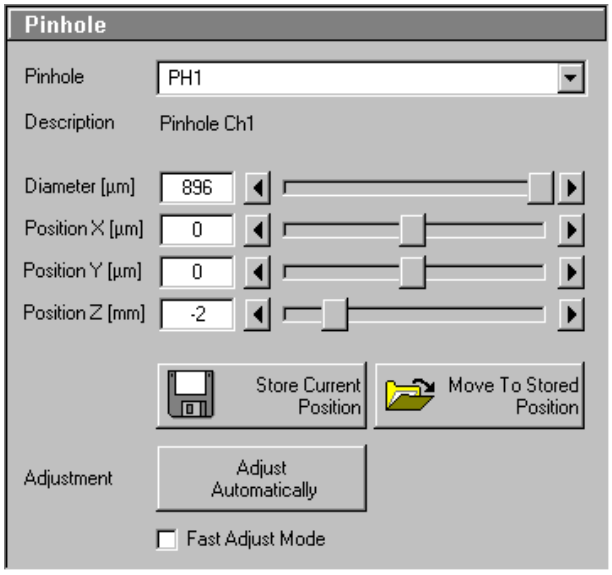


Fig. 4-238 Pinhole panel

4.10.3.3 Collimator Panel

Collimator Description field: Selection of the collimator (IR / VIS or UV / VIS) to be adjusted via **Collimator** selection box, display of selected collimator in the **Description** field.

Positions field: Setting of collimator position using the slider or arrow buttons; the display to the right of the slider indicates the current position, status display for setting procedure: green for ready and red for busy.

Store Current Position button: Stores the current collimator position.

Move To Stored Position button: Sets the collimator to the stored value.

Move to Optimal Position button: Starts the automatic collimator adjustment. Available for most common objectives.

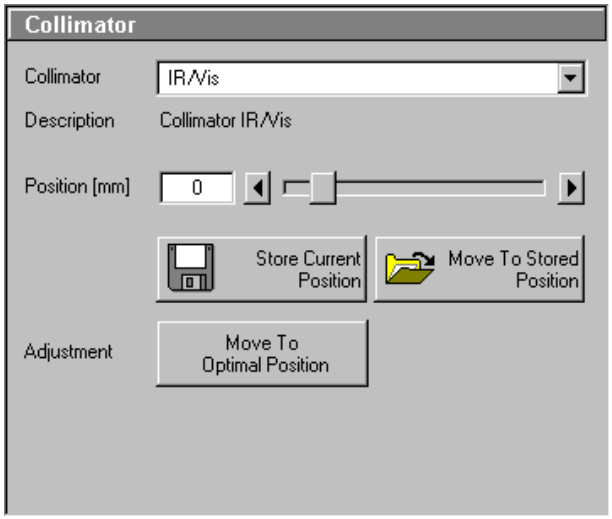


Fig. 4-239 Collimator panel

4.10.3.4 Pinhole and Collimator Adjustment

Adjustment of the LSM 510 pinholes can be performed manually or automatically.

If several channels are used to produce the image, all the used pinholes must be adjusted separately.

(1) Manual pinhole and collimator adjustment

The position of the pinhole relative to the detector in terms of X-Y-Z coordinates contributes substantially to image optimization.

Requirements to make pinhole position changes visible immediately:

- The image must be scanned by the continuous scan method.
- Select a fast scanning speed.
- Measurement with Average Number 1 only (no averaging of several measurements).
- On the **Channel Settings** panel (click on **Channels** button in the **Scan Control** window), select the pinhole diameter so as to have the best possible image contrast.

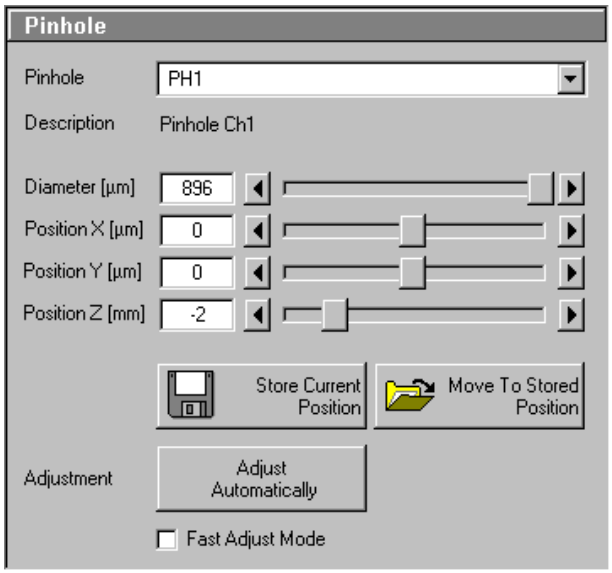


Fig. 4-240 Pinhole panel

- Click on the **Pinhole** button in the **Maintain** subordinate toolbar.
- Select the pinhole to be adjusted from the **Description** list box.
- Use the **Diameter** slider to set the smallest possible size which produces a good, high-contrast image.
  - This setting changes the pinhole diameter.
  - The **Z Slice** display box simultaneously displays the depth resolution corresponding to the pinhole diameter.

☞ Image optimization can be effected with the **Range Indicator** or in the **Line-Scan** mode.

- Optimize the pinhole position in X, Y and Z (Z only for PH1) relative to the PMT using the X, Y and Z sliders to maximum image brightness.
- Click on the **Save Current Position** button to save the pinhole adjustment.
- Removing the **Current Positions** slider in the **Collimator** panel allows the collimator to be adjusted to maximum image brightness. Optimum collimator adjustment obtained in this way can be stored by clicking on the **Save Current Position** button.
- Click on the **Stop** button to stop the continuous scan.

☞ Please do not make any program manipulations while the automatic pinhole adjustment is running (status display is red - busy).

(2) Automatic pinhole and collimator adjustment

The automatic adjustment allows the LSM 510 pinholes to be used with any combination of beam splitters.

- Click on the **Adjust Automatically** button.
  - The **Requirements for Adjustment** window will then appear.
- Meet the requirements listed in the **Requirements for Adjustment** window and press the **OK** button.
  - Pinhole adjustment will then run automatically. The adjusting procedure takes approx. 3 min.
  - The determined data are stored automatically and will be available for all further examinations using the same configuration.
- Click on the **Move to Preadjust** button in the **Collimator** panel. Optimum positioning of the collimator will be performed. The **Default** button enables the collimator to be set back to the factory-adjustment.
- Activate the **Fast Adjust Mode** check box for a faster readjustment.

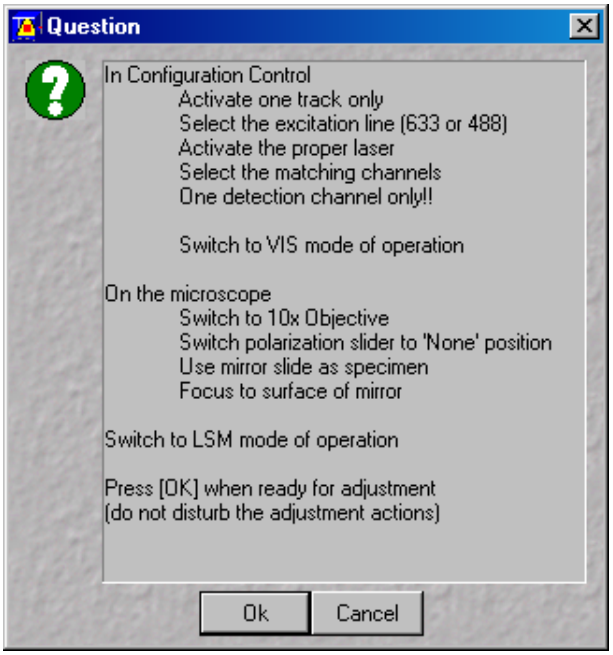


Fig. 4-241 Requirements for Adjustment window

☞ A change of the pinhole diameter made manually in the **Pinhole** panel will not be activated in the **Scan Control** window. Therefore, changes must always be made in the **Channel Settings** panel of the **Scan Control** window.

A filter change in **Autoadjust** is not displayed in the **Config. Control** window.

Configuration 1 is equipped in such a way that pinhole adjustment for channel 1 can only be made with λ = 488 nm, NFT 545, NFT 610 or NFT 570.

Please remember that the Z-coordinate for channel 1 is not optimized during the automatic pinhole adjustment. Subsequent optimization can be performed via the **Move to Preadjust** button in the **Collimator** panel of the **Pinhole & Collimator Control** window.

Please do not make any program manipulations while the automatic pinhole adjustment is running (status display is red - busy).

The optimum setting of the collimator must be performed separately for each track via the **Move to Preadjust** button in the **Pinhole & Collimator Control** window. If several tracks are activated (Recording), an average value of the positions valid for the various tracks will be set on pressing the **Preadjust** button. When all the tracks have been defined and are active (only the ticked tracks will be included in the calculation), press the **Move to Preadjust** button.

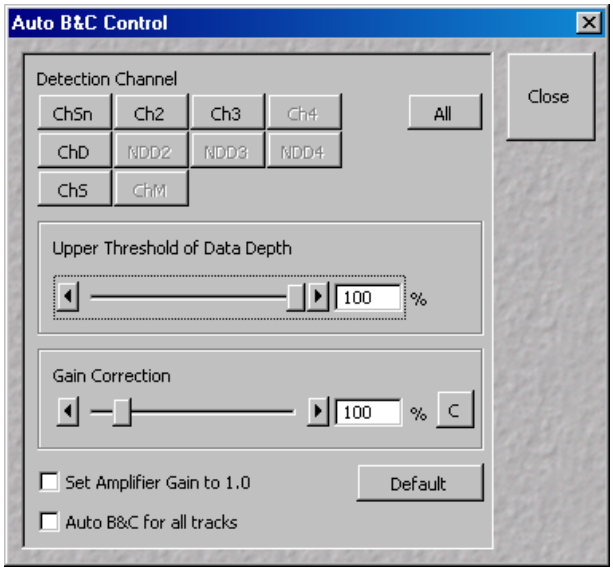


Fig. 4-242 Auto B&C Control window

4.10.4 Set Find

This function permits the preset parameters of the **Find** function (see **Scan Control**, page 4-71) to be matched individually.

- Click on the **Set Find** button in the **Maintain** subordinate toolbar of the **Main** menu.
  - The **Auto B&C Control** window appears on the screen.
- Change the settings for the **Upper Threshold of Data Depth** and **Gain Correction** using the relevant sliders.

The settings can be made individually for each detection channel or for all channels together.

For experiments with increasing of fluorescence over the time it's necessary to reduce the **Upper Threshold of Data Depth** for the **Find** function.

☞ Each slider should be used separately.

- Click on the **C** button to set the value for the **Gain Correction** to 100 %.
- If required, activate the **Set Amplifier Gain to 1.0** check box.
- Click on the **Find** button to start sample scanning with the current settings.
- Clicking on **Default** enables you to activate the default settings again.
- If required, activate **Auto B&C for all tracks** checkbox for **Find** function.

4.10.5 Spline

This function permits calibration of the Scanner position signals. This is required for the use of spline curves in the **Line** scanning mode (see **Line**, page 4-92f).

4.10.6 Camera

In the **Camera Color Adjustment** window the user can adjust the white balance and color balance of a connected camera.

Clicking the **Pic** button allows to set the white balance using the mouse cursor in the camera image.

Using the arrow buttons to adjust the color balance of the camera.

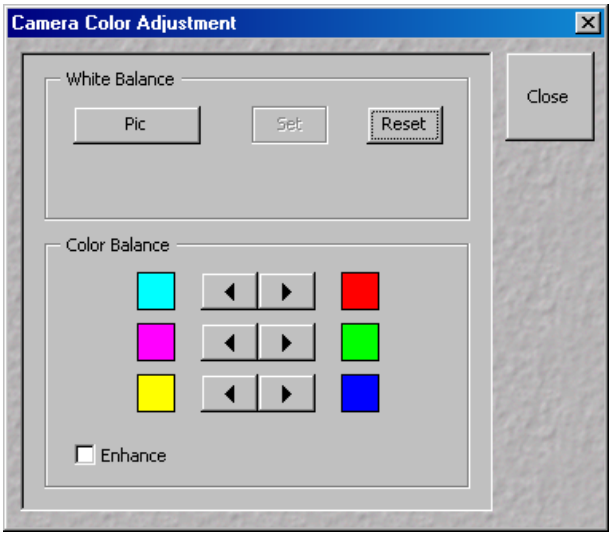


Fig. 4-243 Camera Color Adjustment window

4.10.7 HWAdmin

The **HW/Admin** function is for servicing purposes and may only be used by authorized service personnel. Its access is therefore password-protected.

4.10.8 Test Grid

The **TestGrid** function is for servicing purposes only and may only be performed by authorized personnel. Its access is therefore password-protected.

4.11 Window Menu

The **Window** menu includes the additional functions **Full Screen**, **Close All Image Windows**, **Toolbar** and **Scan Information** which are not available from a toolbar.

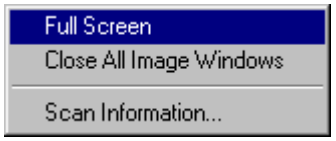


Fig. 4-244 Window menu

4.11.1 Full Screen

This function shows the active **Image Display** window in full screen size.

- Activate the image to be shown in full size by clicking on the image content.
- Click on the term **Window** in the menu bar of the **Main** menu.
  - The **Window** menu (pull-down) will be opened.
- Click on the **Full Screen** line.
  - The image will be displayed in full screen size.
- Click in the image to show it again as an **Image Display** window in normal size.

4.11.2 Close All Image Display Windows

This function closes all the opened **Image Display** windows.

- Open the **Window** menu.
- Click on the **Close All Image Windows** line.
  - All the opened **Image Display** windows will be closed.

In the **Options** menu in the function **Settings** in tab **Save** at position **Save prompt at closing modified windows** it can be determined whether a prompt is shown on **Closing of All Image Display Windows** or not.

4.11.3 Scan and System Information

This function opens the **Scan Information** window, in which the current scan data are displayed.

The extent of the data displayed in the **Scan and System Information** window depends on the settings made in the **Options** menu under **Settings** (see page 4-216).

- Open the **Window** menu.
- Click on the **Scan Information** line.
  - The **Scan and System Information** window will be displayed.
- Click on the ☒ button to close the **Scan Information** window.

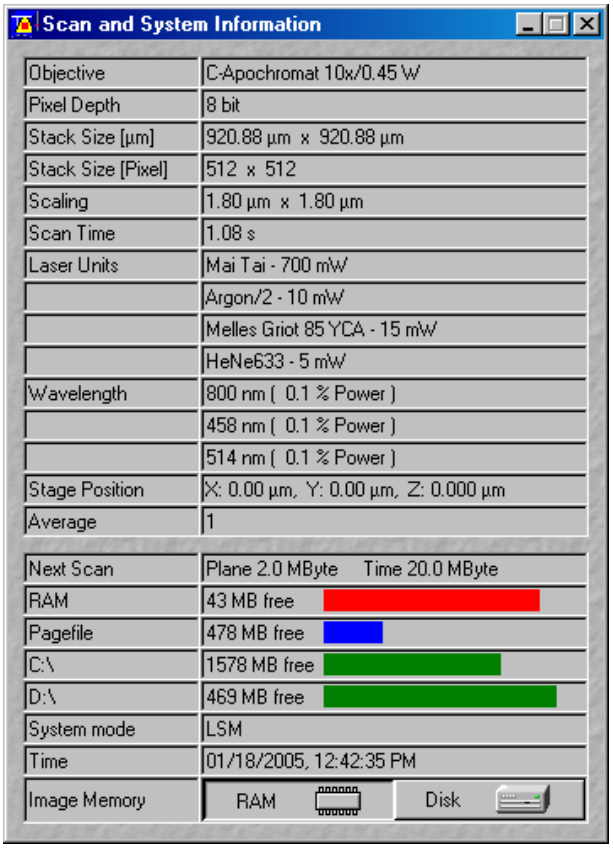


Fig. 4-245 Scan Information window

4.12 Help Menu

The **Help** menu permits activation of the Help function and of a window containing information on the installed software version.

4.12.1 Help

- Open the **Help** menu.
- Click on the **Help** line to open the online help.

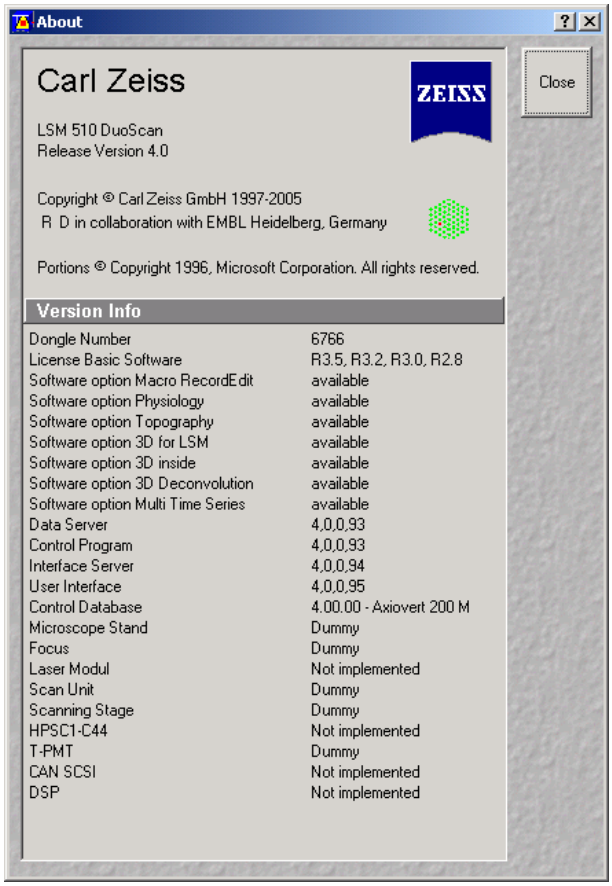


Fig. 4-246 About LSM 510 window

4.12.2 About

- Open the **Help** menu.
- Click on the **About** line to open the **About** window.

The **About** window includes important information about the software, such as the software version number, copyright, version numbers of the various program components and firmware, and the Dongle number.

- Click on the **Close** button to close the **About** window.

4.12.3 FRAP Guide

FRAP Guide opens up a SW integrated Guide for FRAP experiments. Use the buttons in the Guide to set up your experiment. Follow the steps and the descriptions. You are also referred to relevant literature and the website of the EAMNET (see also **Kinetic Analysis Tool** (optional), on page 4-153).

4.13 Display and Analysis of Images

4.13.1 Structure of the Image Display Window

The **Image Display** window shows the image or images when they are

- scanned by any scanning function (see **Scan Control** and **Time Series Control**) or
- loaded from the image database (see **Open database-Load**) or
- imported by the import function (see **Import**).

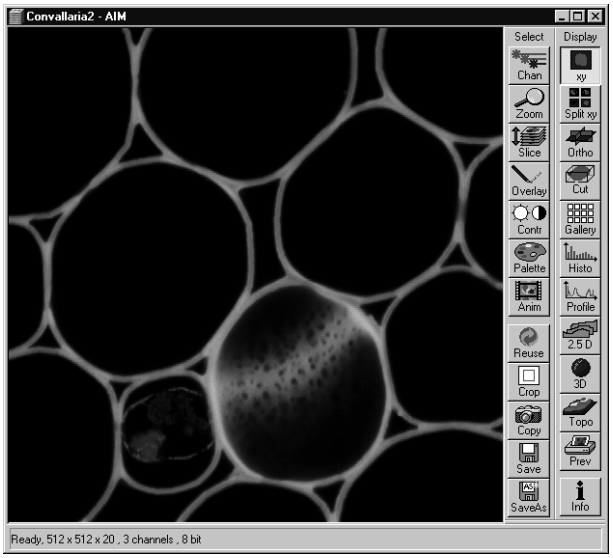


Fig. 4-247 Image Display window showing a single frame image

In addition to show images the **Image Display** window offers two toolbars for

- changing the display parameters and save an image or images (see **Select** toolbar below)
- generating new ways of displaying the data as well as analysis tools (see **Display** toolbar below).

The **Image Display** window of the LSM 5 software corresponds to the basic structure of other Microsoft ® WINDOWS applications. The **Image Display** window can be moved as required within the screen, and its vertical, horizontal and diagonal size can be matched to the current requirements (identical to Microsoft ® WINDOWS).

The caption at the top of the **Image Display** window contains the control menu for the **Image Display** window (identical to Microsoft ® WINDOWS), the name of the displayed image, and the **Minimize**, **Maximize** and **Close** buttons.

In the status line at the bottom of the **Image Display** window, the progress bar of a current scanning procedure and the parameters used for image display are shown and updated when changed.

On the left-hand side of the **Image Display** window, an overview of the scan parameter is displayed, provided that the **Info** button of the **Display** toolbar is activated.

The **Settings** function of the **Options** subordinate toolbar with the **Image Display** tab some of the functions of the **Image Display** window toolbars can be activated at the opening of a new **Image Display** window.

It is possible to display the **Chan**, **Zoom**, **Slice** and **Overlay** image display toolbars immediately on opening an **Image Display** window. The relevant check boxes to be activated in the **Image Display Toolbars** tab under **Settings** (see **Options** menu).

It is also possible to display the scan parameter of an image (**Info** button) immediately when an **Image Display** window is opened. The data to be displayed can be defined (see **Image Status Display** tab under **Settings** in the **Options** menu.

The set of functions available at the **Image Display** window toolbars depends on the type of image shown. The LSM 5 software handles the following formats:

- frame (single image and Z Stack of images)
- frame time series (time series of images and time series with Z Stack of images)

– line time series (time series of lines and time series with Z Stack of lines)
– point time series (time series of points)
– Lambda series (Lambda Stack of images and time series with Lambda Stack of images)

4.13.2 Display Modes

The following display modes are available for the different acquisition modes:

Image type		**Frame**	**Frame**	**Frame**	**Frame**	**Line**	**Line**	**Line**	**Point**
Series type			**Z Stack**	**Time**	**Lambda**		**Time**	**Lambda**	**Time**
Display functions									
xy		●	●	●	●				
xt						●	●		●
tx						●**	●		●
Split xy		●*	●*	●*	●	●	●		●
Split tx						●**	●		●
Coded								●	
Ortho			●						
Max					●			●	
Cut			●						
Gallery			●	●	●				
Histo		●	●	●	●	●	●	●	
Profile		●	●	●	●				
Diagr						●	●	●	●
Mean				● t***	● Lambda	●**	●	● Lambda	
Select						●	●	●	
2.5 D		●	●	●		●	●	●	
3D		●**	●***	●**					
Topo		●**	●***	●**					
Prev		●	●	●	●	●	●	●	●
Info		●	●	●	●	●	●	●	●

* only active in case of multi channel images

** inactive

*** optional

All display functions are exclusive functions. Only one can be active at a given time. To generate different views of the same image set use the **Duplicate** function in the **Process** menu.

☞ During image acquisition all active display functions can be used.

4.13.3 Select - Chan

This function permits to

- change the color assignment of channels of images
- switch individual channels of a multi channel image on/off
- switch to monochrome display of the image instead of color display

- Click on the **Chan** button in the **Select** toolbar.
  - The **Channels** toolbar will be displayed on the right-hand side of the **Image Display** window.

☞ Any changes done with this toolbar are effective immediately.

- Click on the **Chan** button again to remove the **Channels** toolbar.

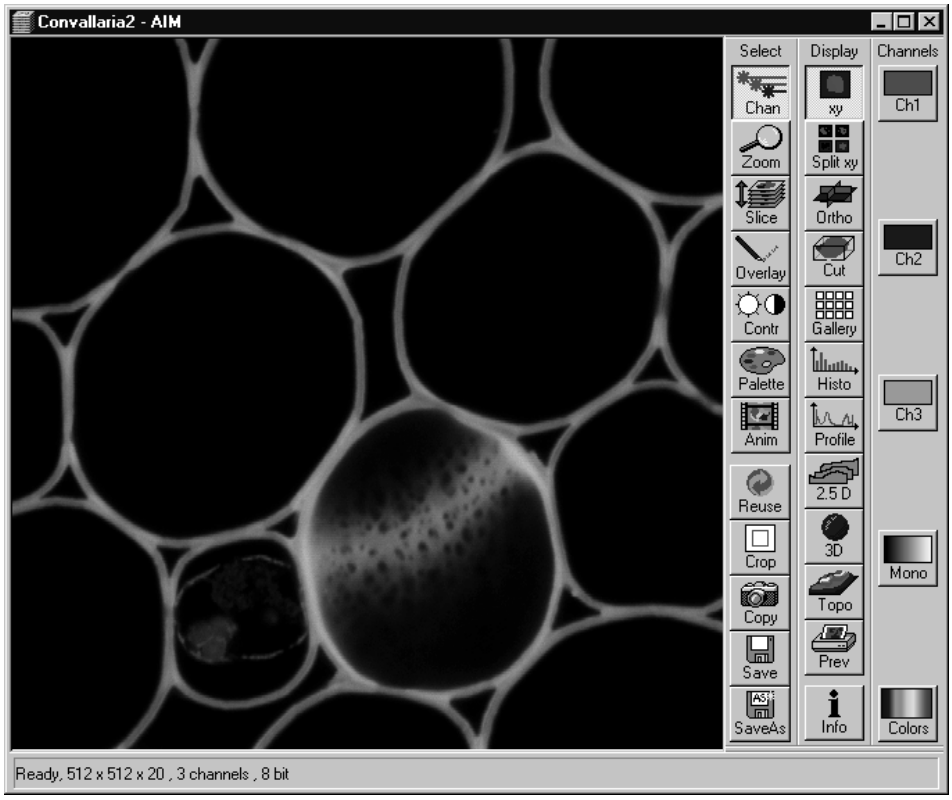


Fig. 4-248 Image Display window; Select - Chan

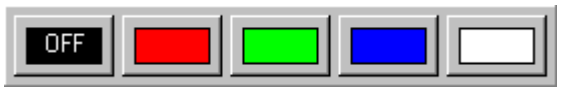


Fig. 4-249 Color selection box

4.13.3.1 Assigning Another Color to a Channel

- Click on one of the channels button in the **Channels** toolbar (e.g.: **Ch1**).
  - The color selection box with all the currently defined colors will appear.
- Click on the required color.
  - The selected color will be assigned to the current channel, the color selection box is closed and the displayed image is updated. The control box of the channel button (e.g.: **Ch1**) also shows the selected color.

4.13.3.2 Switching a Channel of a Multi Channel Image off or on

- Click on one of the channel buttons in the **Channels** toolbar (e.g.:**Ch1**).
  - The color selection box will appear.
- Click on **OFF** to deactivate the display of the relevant channel.

☞ A newly assigned color or a channel switched off is not taken into consideration during the following scanning procedure, since the setting in the **Configuration Control** window always applies here.

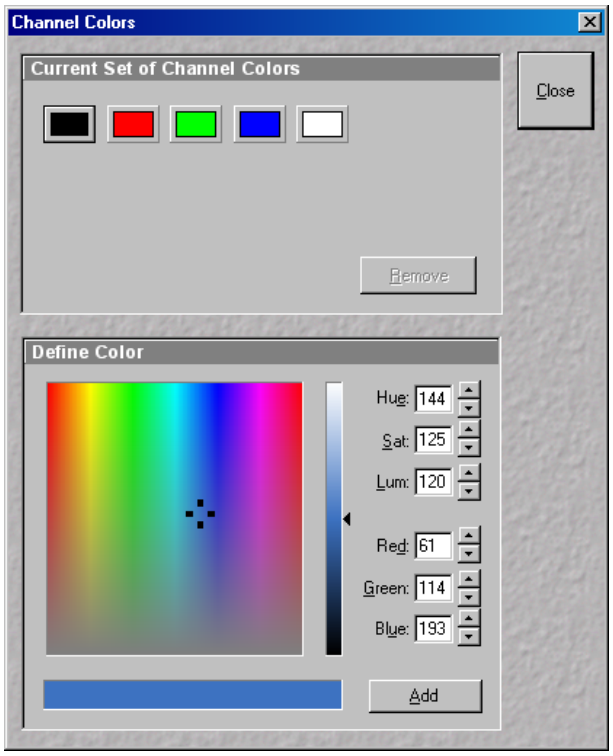


Fig. 4-250 Channel Colors window

4.13.3.3 Switching to Monochrome Image Display

- Click on the **Mono** button in the **Channels** toolbar.
  - The image will then be displayed in shades of gray exclusively. If you click on the button again, the channels will be displayed in color again.

☞ If you want to view the channels individually, select the split display by clicking on **Split xy** button in the **Display** toolbar.

4.13.3.4 Defining a New Color

- Click on the **Colors** button to open the **Channel Colors** window.
- Define a new color in the same way as in the **Configuration Control** window (see page 4-52).

4.13.4 Select - Zoom

This function allows to change the zoom factor of an image displayed.

Click on **Zoom** will display the **Zoom** toolbar. Any changes done with this toolbar are effective immediately.

The image can be zoomed by various methods. The zoom function can be performed online.

- Click on the **Zoom** button in the **Select** toolbar.
  - The **Zoom** toolbar will be displayed on the right-hand side of the **Image Display** window.
- Clicking on the **Zoom** button again will remove the **Zoom** toolbar.

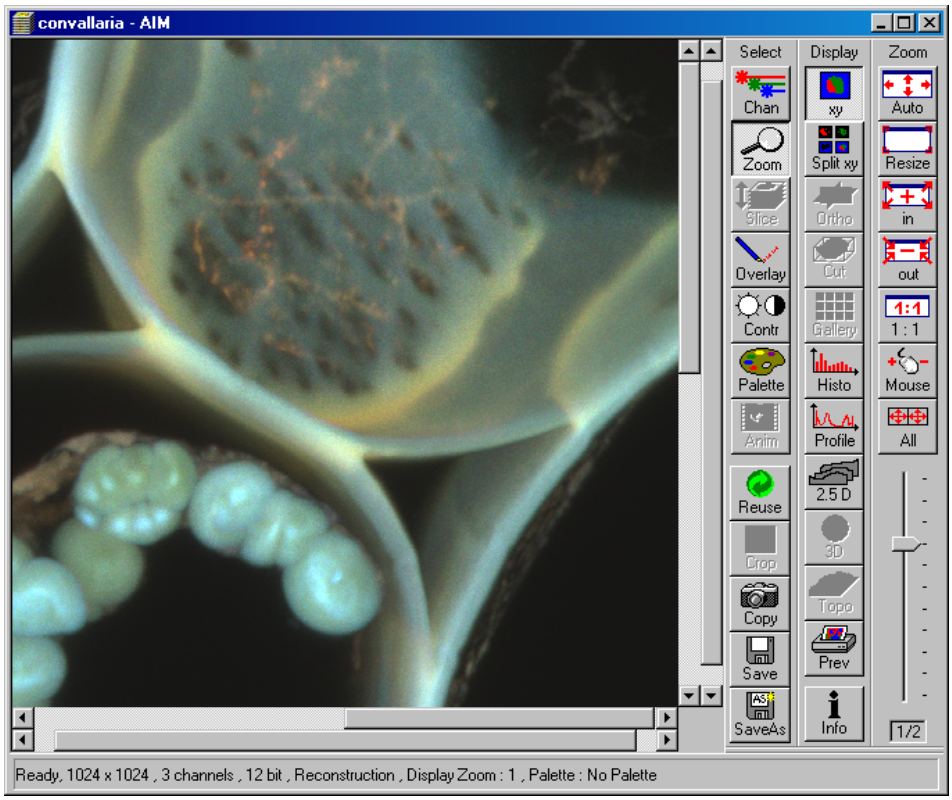


Fig. 4-251 Image Display window; Select - Zoom

Zoom-Auto The image is fitted automatically to size of the **Image Display** window.

Zoom-Resize Restores the image to its initial size.

Zoom + Enlarges the image by factor 2.

Zoom – Reduces the image by factor 2.

Zoom 1:1 Restores an image zoomed in any way to its original size.

Zoom-Mouse Allows you to enlarge / reduce the zoom factor of an image using the left / right mouse button, provided that the cursor is inside the image.

All When active it allows you to zoom all images of a gallery to the same extent without changing the display image format.

☞ **Zoom-+, Zoom-–, Zoom 1:1**, **Zoom-Mouse and All** can only be defined when the **Zoom-Auto** function is deactivated.

Slider with display box The zoom factor can be set by moving the slider. The display box below displays the current zoom factor. Factor 1 corresponds to the original size.

4.13.5 Select - Slice

This function allows to select and view individual slices from a Z Stack or a time series, when images where acquired in frame mode.

☞ The button is grayed, when these conditions are not true.

Click on **Slice** will display the **Slice** toolbar. Any changes done with this toolbar are effective immediately.

- Click on the **Slice** button in the **Select** toolbar.
  - The **Slice** toolbar is displayed on the right-hand side of the **Image Display** window.
- If you click on the **Slice** button again, the **Slice** toolbar is removed.

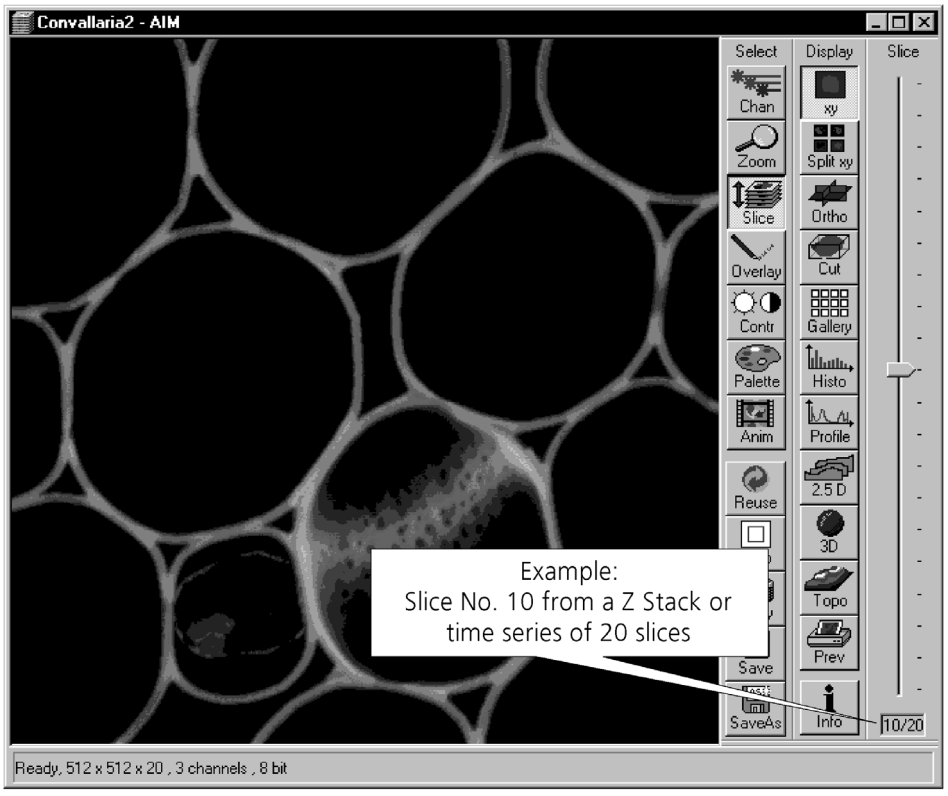


Fig. 4-252 Image Display window; Select - Slice

4.13.6 Select - Overlay

This function allows to

- select from a set of drawing functions such as rectangles and arrows
- add a scale bar to the image
- use a set of interactive measurement functions for length, angle and size

4.13.6.1 Functional Description

The overlay function uses a plane separate from the image plane (the graphics plane) and does therefore not change the content of the image(s).

The button is only available if the XY or Split XY **Display** functions are selected. Otherwise it is grayed. Some of the **Display** functions such as **Ortho** or **Cut** turn the overlay graphics off temporarily.

Any changes done with this function are effective immediately.

The overlay graphics can be stored together with images and can be retrieved from the LSM 5 image database.

- Click on the **Overlay** button in the **Select** toolbar.
  - The **Overlay** toolbar will be displayed on the right-hand side of the **Image Display** window.
- If you click on the **Overlay** button again, the **Overlay** toolbar will be removed.

Provided that the display of the overlay elements has not been deactivated by clicking on the **Off** button, the created elements will still be displayed in the **Image Display** window even after closing of the **Overlay** toolbar.

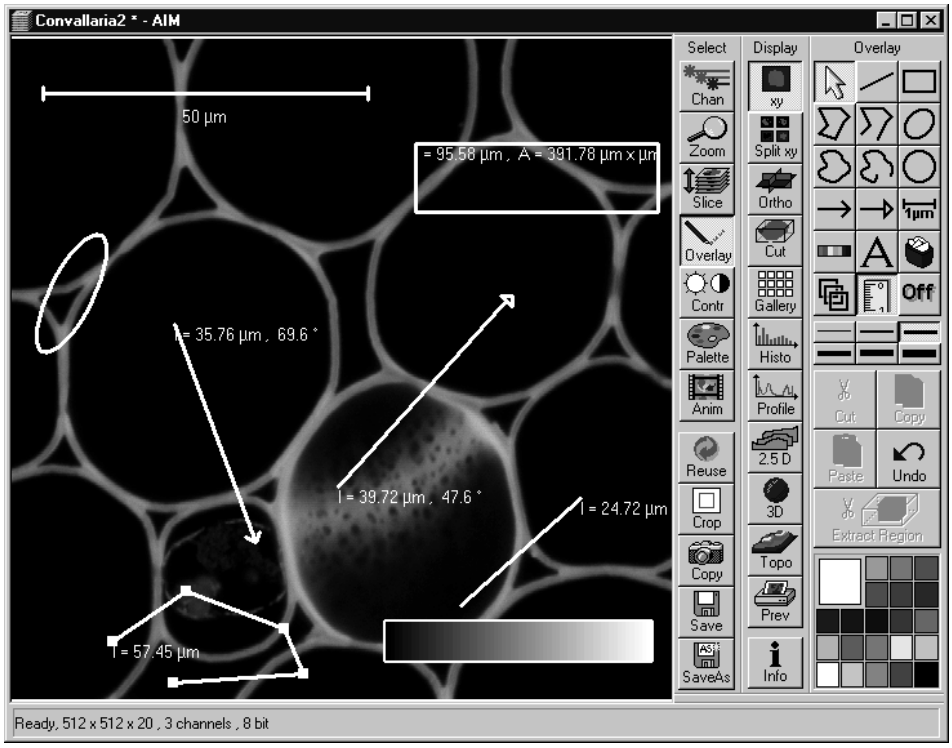


Fig. 4-253 Image Display window; Select - Overlay

4.13.6.2 Buttons in the Overlay Toolbar

The following functions can be used on activation of the buttons in the **Overlay** toolbar:

Arrow (selection) button: Activation of the mouse button for selection, resizing or movement of an overlay element in the **Image Display** window.

Resizing: Click on the handle and hold down the mouse button, drag the handle, release the mouse button.

Movement: Click on the line and hold down the mouse button, move the entire element, release the mouse button.

Line button: Creation of a straight line in the **Image Display** window.
Click and hold down the mouse button, draw a line in any required direction, release the mouse button to end the procedure.

Rectangle button: Creation of a rectangle in the **Image Display** window.

Click and hold down the mouse button, draw a rectangle in any required direction, release the mouse button to end the procedure.

Closed polyline button: Creation of a closed polyline figure in the **Image Display** window.

The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Open polyline button: Creation of an open polyline figure in the **Image Display** window.

The first click sets the starting point, each additional click adds a further line, a click with the right mouse button ends the procedure.

Ellipse button: Creation of an ellipse in the **Image Display** window.

The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and rotation direction can then be determined, the third click sets the second dimension and direction and ends the procedure.

Closed free-shape curve button: Creation of a closed Bezier figure in the **Image Display** window.

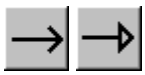
The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Open free-shape curve button: Creation of an open Bezier figure in the **Image Display** window.

The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Circle button: Creation of a circle in the **Image Display** window.

Clicking and holding down the mouse button sets the center point, drag the diameter and release the mouse button to end the procedure.



Line with arrow button: Creation of a line with arrow in the **Image Display** window.

Click and hold down the mouse button, drag the line in any required direction, release the mouse button to end the procedure.

Scale button: Creation of a horizontal or vertical scale with default increments in the **Image Display** window. Click and hold the mouse button for the starting point, drag horizontal or vertical scale, release the mouse button to end the procedure.

Gray tones / color shades button: Generates a rectangle with a display of gray tones or color shades in the image. Color shades are displayed if a palette has been loaded, with different colors being assigned to the gray tones.

A (Text) button: Creation of a text box in the **Image Display** window.

After clicking on **A**, the **Text** window will be displayed, and text can be entered via the keyboard. The **Font ...** button enables you to select the font style and size in the **Font** window. The entered text will be displayed in the left upper corner of the **Image Display** window after clicking on **OK** and can be moved to the required position using the mouse.

The **Text** window can also be activated with a double-click on a created text box, and the entered text can be edited subsequently.

Insert opens up a further window which allows you to annotate coordinates, time and Z-position with either automatic or user definable units and precision. This annotation is updated during image acquisition and can be exported with the image. The annotation can be stamped into already existing images.

Recycle bin button: All the overlay elements and dimensions dragged to the scanned image are deleted. If one overlay element was marked before, this element is now deleted from the scanned image.

Multiple button: On activation of this button, the overlay function subsequently selected is performed several times in succession, without the need to activate the function button again. This function remains selected until the **Multiple** button is deactivated again.

Measure button: Measurement of the overlay element in the **Image Display** window. On activation of the **Measure** button, the selected overlay element and all the elements created afterwards are measured and assigned with a measuring value. The measuring value can be shifted without regard to the overlay element. If of importance, the length and perimeter of a line figure, the area of a closed figure and the inclination angle of a single line will be displayed. On deactivation of the **Measure** button, the measuring value of the selected element is no longer displayed, and all the elements created afterwards will not be assigned with a measuring value.

Off button: Deactivation of the display of overlay elements in the **Image Display** window (hide overlay). Deactivation of the overlay functions.

Line button:

This button allows you to determine the line thickness of the area outline.

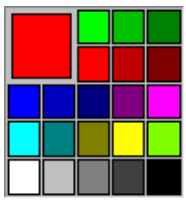
Cut button: The image contents of an overlay element are cut out, and the area will then appear in black.

Copy button: The image contents of a closed overlay element are copied to the clipboard.

Paste button: The image contents of an overlay element copied to the clipboard are inserted in the current **Image Display** window and can be positioned anywhere in the image using the mouse.

Undo button: The last **Cut** or **Paste** action can be undone by clicking on the **Undo** button.

Extract Region button: The region of a Z Stack or 4D-image surrounded by an **Overlay** element is extracted and can be displayed and stored separately in a new **Image Display** window. This function is only active if an **Overlay** element is used, that generates a closed contour.



Color selection box: The colors displayed in the **Color** selection box can be assigned to the overlay elements with a click of the mouse. The currently selected color is displayed in the larger rectangle (left top) of the selection box. A selected color is automatically assigned to the currently selected overlay element and then to all the elements created afterwards.

4.13.7 Select - Contr

This function allows to

- change the contrast and brightness of an image
- change the contrast and brightness of a channel of an image
- define interactively a new relationship between the intensities of pixels in the image memory and the displayed values of this pixel intensities on the computer screen

Click on **Contr** will display the **Contrast** toolbar. Any changes done with this toolbar are effective immediately.

Modification done by this function are for display purposes only. To permanently change the contrast and brightness of an image use the function **Contrast** in the **Process** menu.

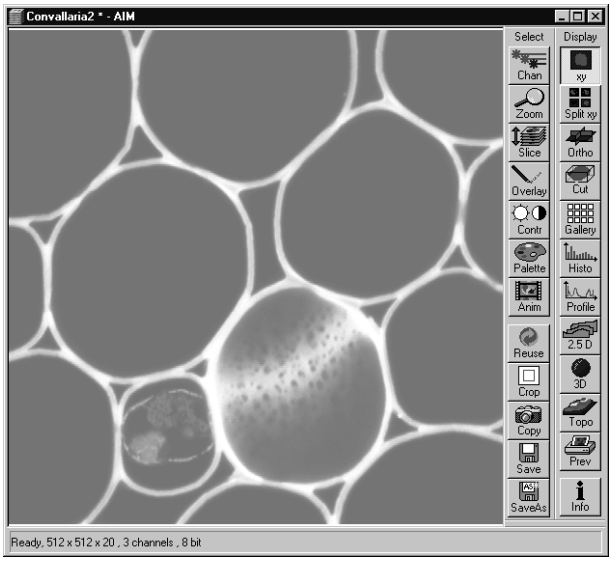


Fig. 4-254 Image Display window; Select - Contr

- Click on the **Contr** button in the **Select** toolbar.
  - The **Brightness and Contrast** window will be displayed.
- Change brightness and contrast via the sliders in the **Brightness and Contrast** window. You can adjust each channel individually by activating the channel button (e.g.: **Ch1**), or influence all channels simultaneously by clicking on **All**.
- Clicking on the **Reset** button will reset the original setting of brightness and contrast.
- Clicking on the **Close** button will close the **Brightness and Contrast** window.

Further contrast and brightness parameters can be activated or deactivated alternately using the **More** and **Less** buttons.

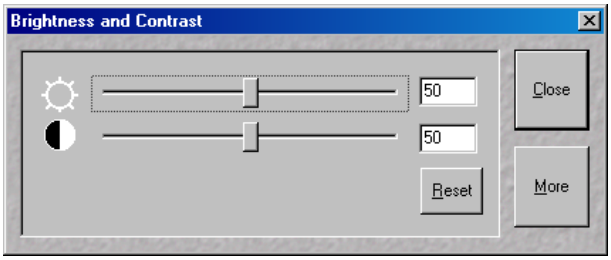


Fig. 4-255 Brightness and Contrast window

- Click on the **More** button to display the additional functions.
  - The **Brightness and Contrast** window will be enlarged, the labeling of the button changes from **More** to **Less**. If you click on **Less**, the additional functions are no longer displayed.

Simultaneously with the setting of brightness and contrast, the intensity values of the image can be set directly in the **Intensity Screen** via the **Ramp**, **PolyLine**, **Spline** and **Gamma** functions.

The intensity values can also be set either for all channels together or individually.

If the image has already been changed using the **Contrast** and **Brightness** sliders, this setting difference is displayed in the **Intensity Screen** by means of the **Shape** and **Result** lines.

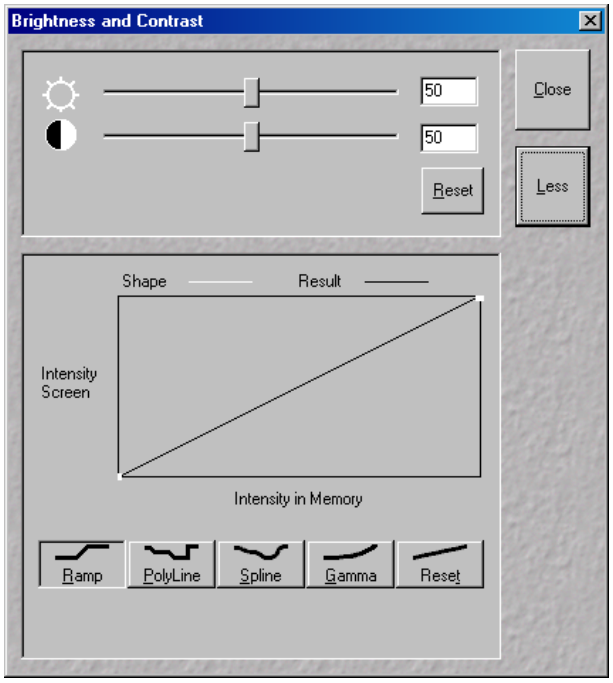


Fig. 4-256 **Brightness and Contrast window with activated Ramp function**

4.13.7.1 Ramp

The intensity is set via two knots in the **Intensity Screen**, which allows an intensity line to be created in the form of a ramp.

☞ The original line form is reset via **Reset**.

The line form will be retained even when the additional functions are no longer displayed, and on closing the **Brightness and Control** window.

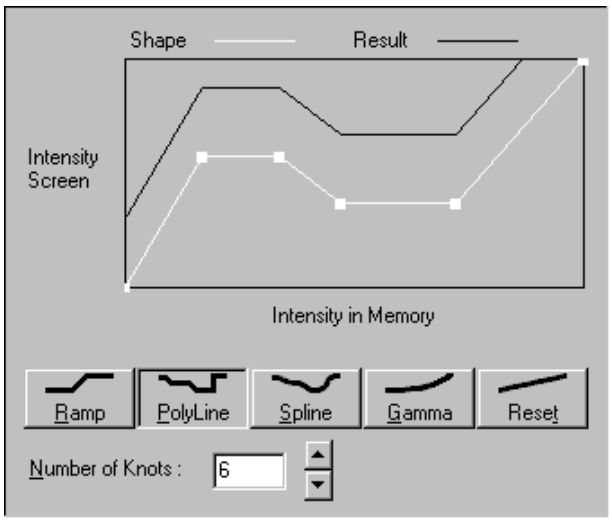


Fig. 4-257 **Brightness and Contrast window with activated PolyLine function**

4.13.7.2 PolyLine

The intensity is set in the **Intensity Screen** via a freely selectable number of knots, which permits the creation of an intensity line in the form of a polyline. The number of knots can be selected from the **Number of Knots** selection box.

☞ The original line form is reset via **Reset**.

The line form will be retained even when the additional functions are no longer displayed or when the **Brightness and Control** window is closed.

4.13.7.3 Spline

The intensity is set in the **Intensity Screen** via a freely selectable number of knots, which permits the creation of an intensity line in the form of a spline. The number of knots can be selected from the **Number of Knots** selection box.

☞ The original line form is reset via **Reset**.

The line form will be retained even when the additional functions are no longer displayed or when the **Brightness and Control** window is closed.

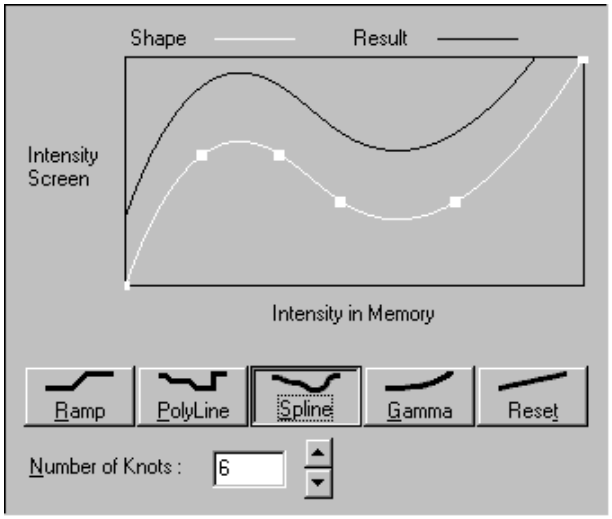


Fig. 4-258 Brightness and Contrast window with activated Spline function

4.13.7.4 Gamma

The intensity is set in the **Intensity Screen** by varying the gamma curve (clicking and dragging with the mouse) or by moving the **Gamma** slider. It is possible to set gamma values between 0.1 and 2.0.

☞ The original line form is reset via **Reset**.

The line form will be retained even when the additional functions are no longer displayed or when the **Brightness and Control** window is closed.

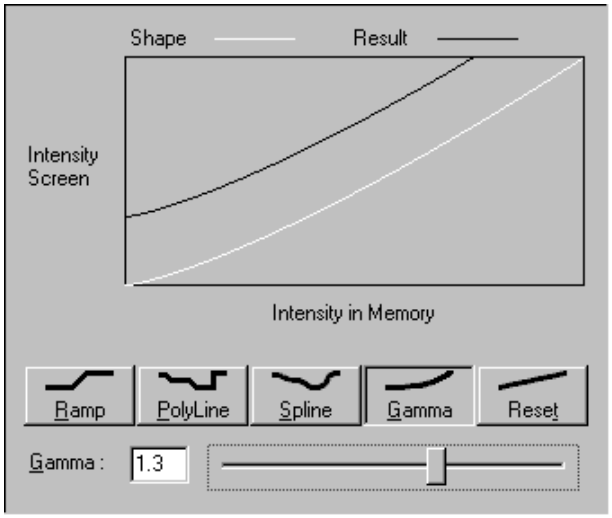


Fig. 4-259 Brightness and Contrast window with activated Gamma function

4.13.8 Select - Palette

This function allows to

- change the palette used for displaying the image(s)
- define and save new palettes
- delete palettes by removing them

Click on **Palette** will display the **Palette** toolbar. Any changes done with this toolbar are effective immediately.

The standard palettes **No palette**, **Range indicator, Glow Scale** and Rainbow are system palettes and can not be deleted.

The **Range indicator** palette is useful to optimize the gain and offset setting of images in the **Scan control** window before scanning.

Palettes are stored and retrieved together with the images when archived in the **Image Database**.

- Click on the **Palette** button in the **Select** toolbar.
  - The **Color Palette** window will be displayed.
- Select the required palette from the **Color Palette List** panel by clicking on the relevant name.
- If you want to deactivate a palette selected before, click on **No Palette** in the **Color Palette List** panel.
- Click on the **Close** button to close the **Color Palette** window.
- A changed image can be stored via the **Save As** function.

In the **Options** menu in the function **Settings** it is possible to switch to **Mono** automatically when a palette is activated and to **Colour** on deactivation of a palette.

In addition it is possible to activate / deactivate **Mono** in the **Channel** toolbar.

Some of the handling functions of the **Image Display** window toolbars can be activated at the opening of a new **Image Display** window.

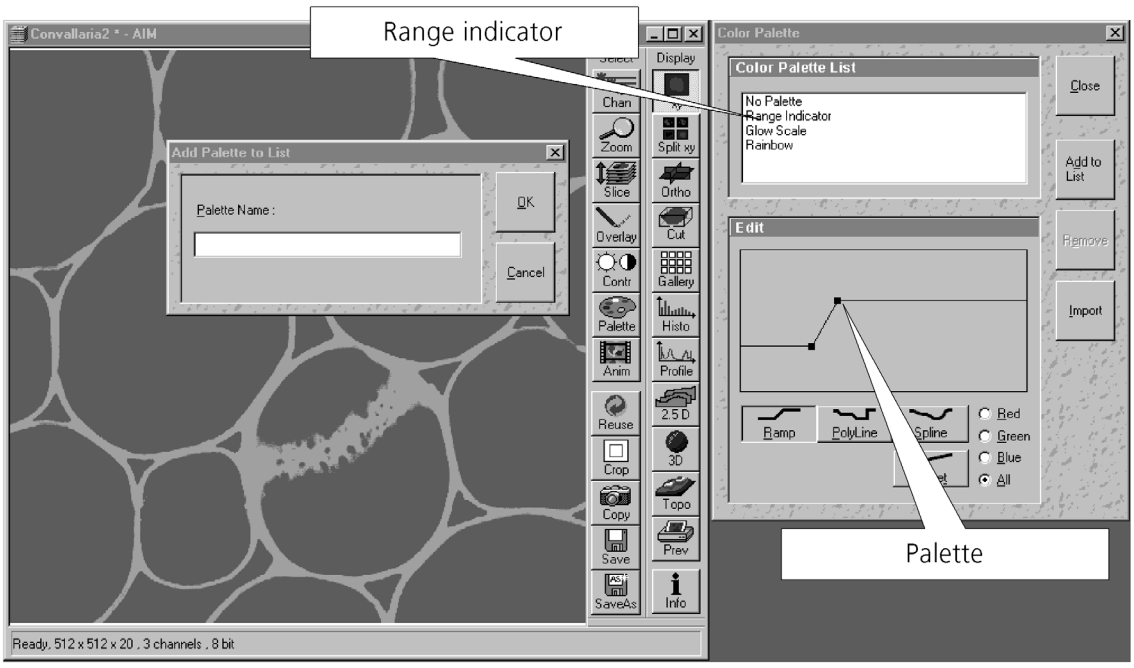


Fig. 4-260 Image Display window, Select - Palette; Color Palette and Add Palette to List window

4.13.8.1 Editing and Storing a Palette

A palette is edited by moving the knots in the **Ramp**, **Polyline** and **Spline** functions (identical to the setting in the **Contrast and Brightness** window, see page 4-257f).

The palette can be set for all colors together or separately for each color.

- Activate the relevant button: **Red**, **Green**, **Blue** or **All**.

Proceed as follows to store an edited palette under a new name:

- Click on the **Add To List** button: the **Add Palette To List** window will be displayed.
- Enter a name for the palette and click on **Ok**.
  - The palette will be stored and the name included in the **Color Palette List** panel.

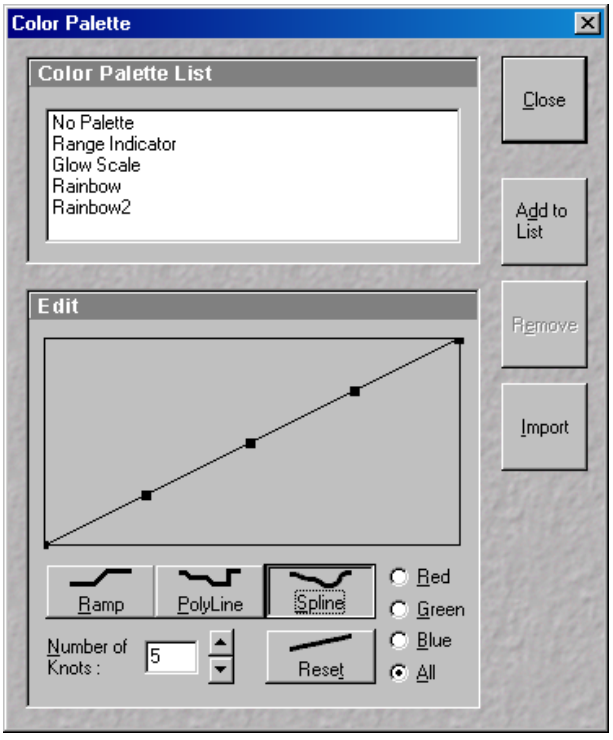


Fig. 4-261 Color Palette window

4.13.8.2 Delete a Palette

Proceed as follows to delete a palette:

- Click on the name of the palette to be deleted in the **Color Palette List** panel and then on the **Remove** button.
  - The palette will be removed from the list.

☞ The standard settings (**No Palette**, **Range Indicator**, **Glow Scale** and **Rainbow**) cannot be deleted.

4.13.8.3 Import a Palette

Proceed as follows to import a palette:

- Click on the **Import** button. The **Import Palette** window will be opened.
- Select the required palette (file extension: ***.lut**) from the relevant directory and click on **Open**.
  - The palette will be imported and displayed in the **Color Palette List** panel.

File with the extension ***.lut** are LSM 310 / 410 palette files.

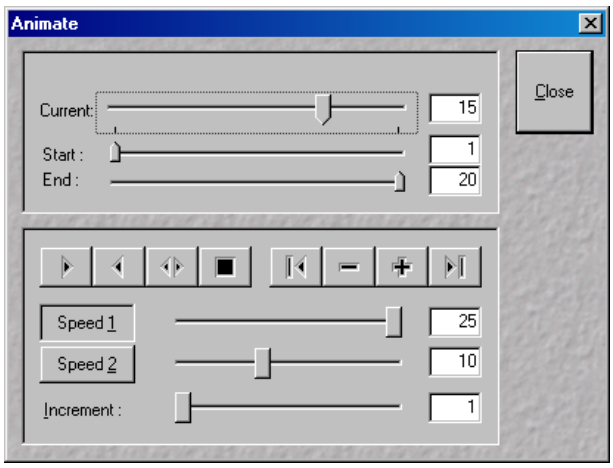


Fig. 4-262 Animate window

4.13.9 Select - Anim

This function allows to

- animate frames of a Z Stack or a time series
- specify animation parameters such as range and animation speed

Click on **Anim** will display the **Animate** toolbar. Any changes done with this toolbar are effective immediately.

When the image(s) displayed in the **Image Display** window is neither a Z Stack nor a time series this button is grayed and not accessible.

- Click on the **Anim** button in the **Select** toolbar of the **Image Display** window of a stack.
  - The **Animate** window will be displayed and the animation started immediately.
- Click on the **Close** button to close the **Animate** window and to stop the animation.

The animation is controlled via the following function elements:

Current Slice slider: Manual movement through the individual slices of a stack by moving the slider, or by entering the slice number in the input box. Slider can be accessed only, when the automatic animation is off.

Start slider: The setting of the **Start** sliders limits the number of slices to be used for the animation. Previous slices are not taken into consideration for the animation. Can be changed during automatic animation.

End slider: The setting of the **End** slider limits the number of slices to be used for the animation. Subsequent slices are not taken into consideration for the animation. Can be changed during automatic animation.

Starts the forward motion of the automatic animation. After the last slice has been passed, restart is made at the first slice.

Starts backward motion of the automatic animation. After the first slice has been passed, restart is made at the last slice.

Starts the combined forward / backward motion of the automatic animation, i.e. when the last slice has been reached, the backward motion is activated, and the forward motion is activated again on reaching the first slice.

Stops the automatic animation.

Move to the first slice.

After each click on this button, backward motion is made by the number of slices set under **Increment**.

After each click on this button, forward motion is made by the number of slices set under **Increment**.

Move to the last slice.

Speed1 /Speed2 buttons **/** sliders: Selection between two speeds, change of the relevant speed via slider or input box.

Increment slider: Reduction of the slices to be displayed by selecting an increment n (step width) of slices to be taken into consideration for the animation. If n = 3, for example, only every third slice of the stack will be displayed during the animation.

4.13.10 Select - Reuse

This function allows to

– transfer acquisition parameters of an image from the image data base to the **Microscope control**, **Configuration Control**, **Scan Control**, **Time Series Control** and **Bleach Control** windows and applies those parameters directly on the system.

The acquisition parameters of an image are displayed in the **Image Display** window and can be viewed by using the **Info** function. In the tab **Image Status Display** in the **Settings** function of the **Options** subordinate toolbar it can be determined what parameters to view with the **Info** function.

The parameters include the following:

Frame Size, Speed, Data Depth, Scan Direction, Average, Zoom Rotation, Offset, Pinhole diameter, Collimator, Detector Gain, Amplifier Offset, Amplifier Gain, Excitation, Beam Path and Scan Mode (Line, Frame, Stack, Time Series).

However, the required objective must be selected by the user.

- Click on the **Reuse** button. The acquisition parameters of the active image (stack) are applied immediately to the system.

In the **Options** menu in the function **Settings** with the **Recording/Reuse** tab, it can be determined whether the objective should also be transferred and set. Setting the microscope objective only works in microscopes with motorized objective revolvers.

4.13.11 Select - Crop

This function allows to

- interactively define the size and orientation of a rectangular scan area on the image displayed in the **Image Display** window.
- The defined area is displayed together with the **Zoom, Offset and Rotation** parameters in the **Scan Control** window in the **Mode** submenu.

Click on **Crop** will display the **Crop Rectangle** in the **Image Display** window. Any changes done with the **Crop Rectangle** are setting the parameters immediately. On the next execution of a scan (**Find**, **Fast xy**, **Single**, **Contineous** in **Scan Control** or **Start T** or **Start B** in **Time Series Control**) these new scan parameters will be used.

To reset the crop function and use default values set **Zoom=1, Offset=0** and **Rotation=0** in the **Scan Control** window in the **Mode** submenu.

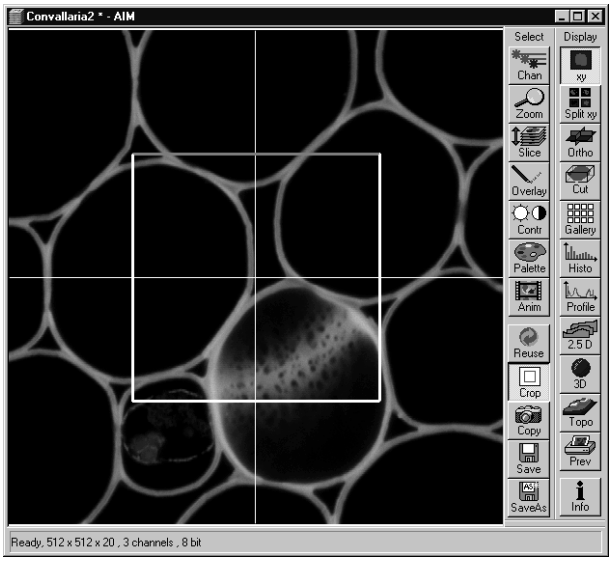


Fig. 4-263 Image Display window; Select - Crop

- Click on the **Crop** button.
  - The **Crop Rectangle** will appear on the **Image Display** window.

The **Crop Rectangle** is controlled via the following functional elements:

Offset
- Click into the crop rectangle, keep the left mouse button pressed and drag the crop rectangle to the required position. Release the mouse button.

Zoom
- Click on a corner of the crop rectangle, keep the left mouse button pressed and set the required size. Release the mouse button.

Rotation
- Click on one end of the crosslines, keep the left mouse button pressed and set the required rotation angle. Release the mouse button. The first line scanned is highlighted in blue.

Side ratio
- Click on any of the intersection points between crossline and crop rectangle, keep the left mouse button pressed and change the side ratio as required. Release the mouse button.

4.13.12 Select - Copy

This function allows to

– copy the current displayed image into the clipboard.

Click on **Copy** will be immediately effective.

From the clipboard images can be incorporated into other programs such as MS Excel, MS Powerpoint or MS Word.

To export image series, use the **Export** function in the **File** menu.

- Click on the **Copy** button.
  - The content of the **Image Display** window is copied to the clipboard.
- Start the **clipboard** application of WINDOWS.
- Select **Paste** in the **Edit** menu of the **Clipboard** application.

4.13.13 Select - Save

This function allows to

– save the image(s) of the **Image Display** window into an **Image Database**

– by not showing a dialogue and using the automatic assigned and incremented image name and a predefined existing **Image Database**

– Prerequisite: **Autosave** is checked in the **Settings** function with the **Autosave** tab

Click on **Save** will be immediately effective.

When the prerequisite is not met, the **Save As** dialogue is displayed.

In the **Options** menu in the function **Settings** with the **Autosave** tab parameters such as an automatically incremented filename can be determined and the **Autosave** activated/deactivated.

4.13.14 Select - Save As

This function allows to

- save the image(s) of the **Image Display** window into an **Image Database**
- by showing a dialogue
- use the defaults as defined in the **Settings** function with the **Save** tab

Click on the **Save As** button displays the **Save Image and Parameter As** window. Changes will be effective on closing this dialogue.

In the **Options** menu in the function **Settings** with the tab **Save** default parameters such as **Name, Description** and **Notes** can be set.

- Click on the **Save** button.
  - The Save Image and Parameter As window appears
- Enter text for the image name, description, notes or change the user name.
- Select the **Image Database** from the list of databases (MDB) or
- Open other **Image Databases** by selecting **Open MDB** or
- Create new **Image Databases** by selecting **New MDB.**

4.13.15 Display - xy

This function allows to

- display a single image in frame mode
- display multi channel images in superimposed mode

The settings of **Chan**, **Zoom**, **Slice**, **Overlay**, **Contr** and **Palette** are applied.

Click on **xy** will be immediately effective.

4.13.16 Display - Split xy

This function allows to

– display the individual channels of a multi channel image as well as the superimposed image

The settings of **Chan**, **Zoom**, **Slice**, **Overlay**, **Contr** and **Palette** apply.

Click on **Split xy** will be immediately effective.

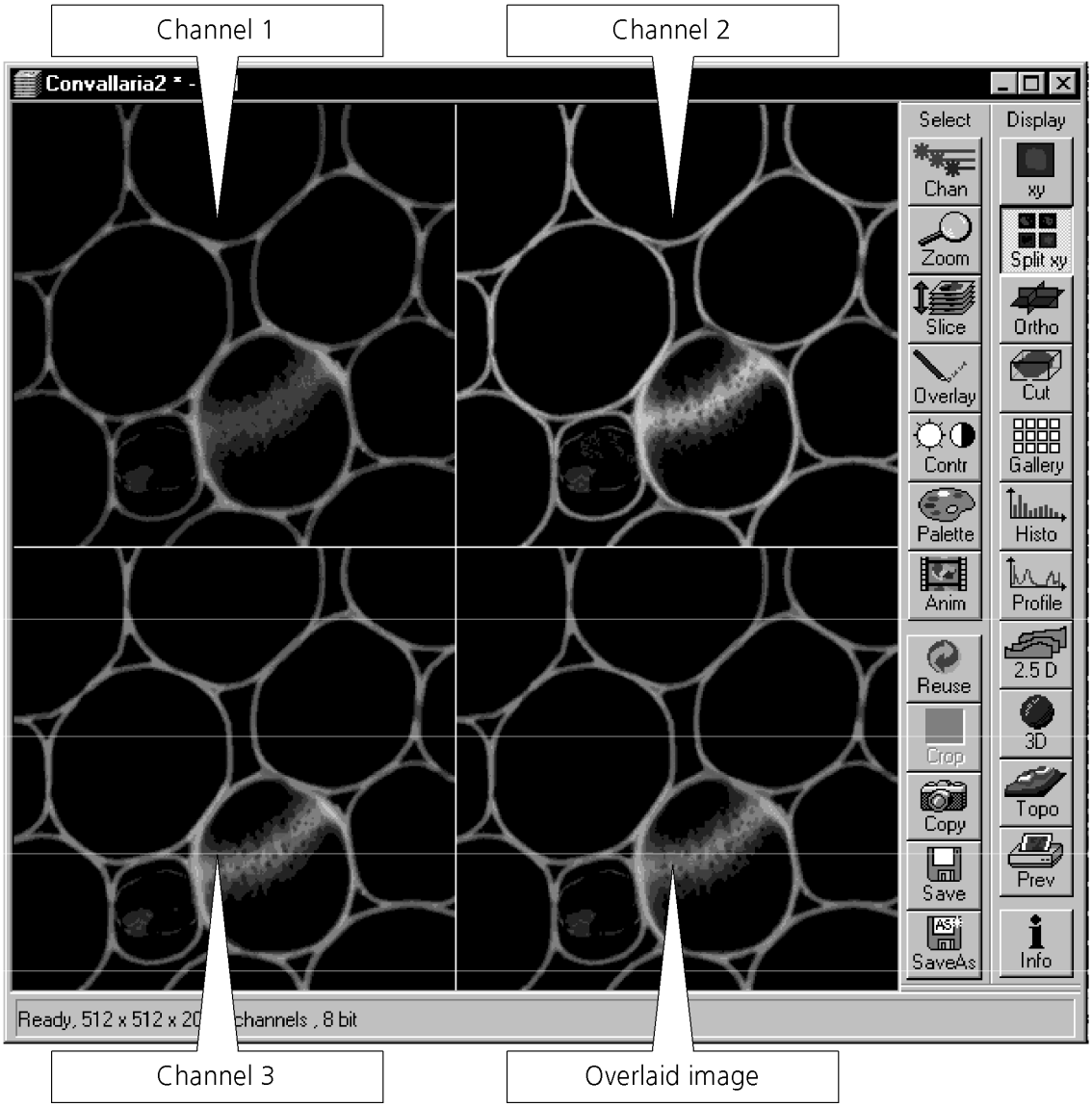


Fig. 4-264 Image Display window, Split xy display

☞ This function is useful to optimize the individual channels in a multi channel image acquisition together with the **Range Indicator** palette .

4.13.17 Display - Ortho

This function allows to

- display a Z Stack of images in an orthogonal view
- measure distances in three dimensions
- generate 2D deconvolution views of the yz and xz plane

The settings of **Chan**, **Zoom**, **Slice**, **Overlay**, **Contr** and **Palette** apply.

Click on **Ortho** will be immediately effective.

- By clicking on the **Ortho** button section lines appear in the **Display** toolbar together with orthogonal projections in the image. On the right-hand side, the **Orthogonal Sections** toolbar is shown.

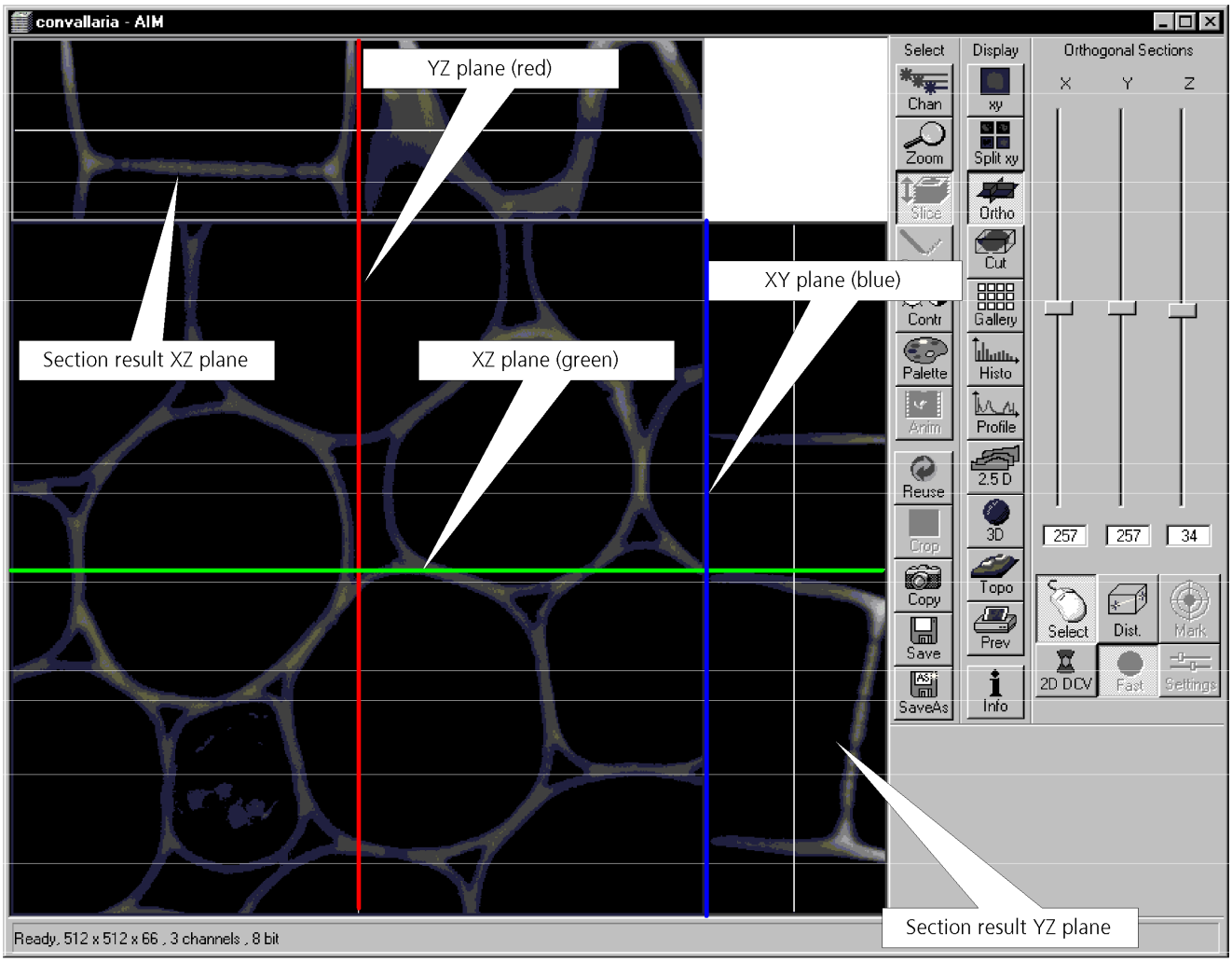


Fig. 4-265 Image Display window, Ortho display

4.13.17.1 Ortho - Select Function

- By changing the parameters X, Y and Z in the **Orthogonal Sections** toolbar, the section plane can be positioned at any XYZ coordinate of the Z Stack.

The position of section planes can be changed in various ways:

- By moving the sliders on the **Orthogonal Sections** toolbar.
  - X and Y settings may range from 1 up to the maximum number of pixels scanned (in the example shown: 512).
  - Z settings may range from 1 to a maximum of n, with n standing for the number of slices produced in the stack.
- By directly entering the relevant number value in the X-, Y- or Z-input box and pressing the **Enter** key.
- If you move the cursor into the **Image Display** window, it changes into a crosslines symbol . By dragging this symbol with the mouse you can position the XZ and YZ section planes to any point of intersection with the XY plane. A click with the left mouse button places the intersection to the desired position.
- If you move the crosslines symbol onto the intersection of the red and green section planes, it changes into the: symbol. If you now press the left mouse button and keep it pressed you can reposition both section planes **simultaneously**.
- If you move the crosslines symbol onto the green section plane, it changes into the symbol. If you now press the left mouse button and keep it pressed, you can reposition the (green) XZ section plane.
- You can reposition the (red) YZ plane in the same way using the symbol.

The result of an orthogonal section is visible at the image margin.

- Section of the XZ plane (green line) through the stack: above the XY image.
- Section of the YZ plane (red line) through the stack: right of the XY image.
- Section of the XY plane (blue, slice plane of the stack): center image.

4.13.17.2 Ortho - Distance Function

- Activating the **Dist.** button permits length measurements in 3D space.
- Click on the **Mark** button to set the first XYZ-point for the measurement of the spatial distance.
- Set the second XYZ-point for measurement by moving the X-, Y-, Z-sliders or by moving the green, red and blue lines in the image.
  - The projections of the spatial distance are shown in the image planes by yellow lines. The actual spatial distance is calculated and shown in µm below the **Select**, **Dist.** and **Mark** buttons, e.g. 3D Distance: 55.60 µm.

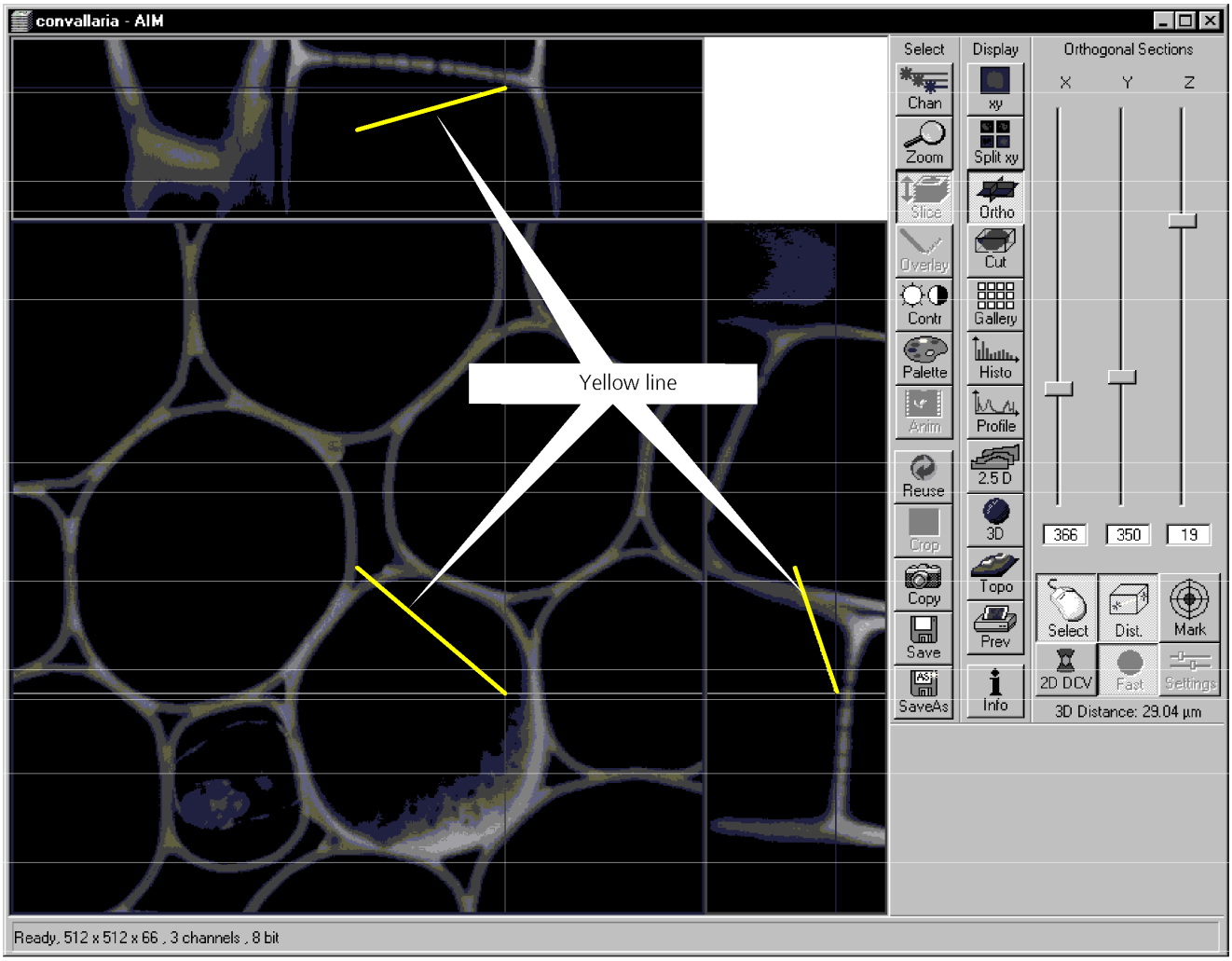


Fig. 4-266 Image Display window, Ortho Distance display

4.13.17.3 Ortho - 2D DeConVolution Function

The 2D deconvolution function causes orthogonal projection enhancement through the computed correction of the resolution in the Z-coordinate.

Image enhancement is only effective for the two projections of a fluorescence stack in the Ortho display or for an XZ-scan in fluorescence, and is also only computed for this.

- Activate the **2D DCV** button in the **Orthogonal Sections** toolbar.

If the **Fast** button is activated, calculation of the 2D-deconvolution (inverse DCV mode) is performed immediately.

The 2D DCV settings button can only be activated if a licence for the 3D DCV option has been purchased. Otherwise this button is grayed.

- Click on the **Settings** button. The **2D Deconvolution** window is opened.

The **2D Deconvolution** window contains the **Deconvolution** panel with the two tabs **Method** and **PSF**.

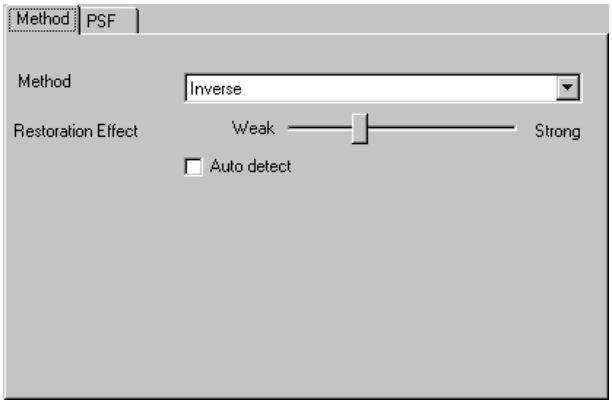


Fig. 4-267 Method tab

(1) Method tab

The **Method** tab enables you to choose between the calculation methods **Inverse** and **Iterative**.

For more details of explanation of deconvolution and the calculation methods see **3D DeConVolution** (page 4-170).

In the **Inverse** method, the **Restoration Effect** slider can be used to set the signal-to-noise ratio between **Weak** (low noise) and **Strong** (pronounced noise).

Activation of the **Auto detect** check box starts a routine for the automatic determination of the noise level in the entire image part of the Z Stack. If **Auto detect** is enabled, the **Restoration Effect** slider is disabled.

The **Iterative** method permits (in addition to the parameters of the **Inverse** method) the maximum number of iterations to be entered between 1 and 200 under **Maximum Iterations** and the **Auto Stop** function to be activated / deactivated. The **Auto Stop** function interrupts the calculation depending on the set image improvement (delta between last but one and last cycle in %), no matter whether the value under **Maximum Iterations** has been achieved or not.

(2) PSF tab (optional with 3D DCV)

The objective data are displayed in the **PSF** tab. In the case of wavelengths above 700 nm, the **NLO** button is automatically enabled.

The displayed values are always taken over by the system data, but can be edited subsequently for simulation purposes.

- Select the required method and determine the relevant parameters.

The deconvolution calculation is performed immediately after the **2D Deconvolution** window has been closed, and the image display is updated (on-line).

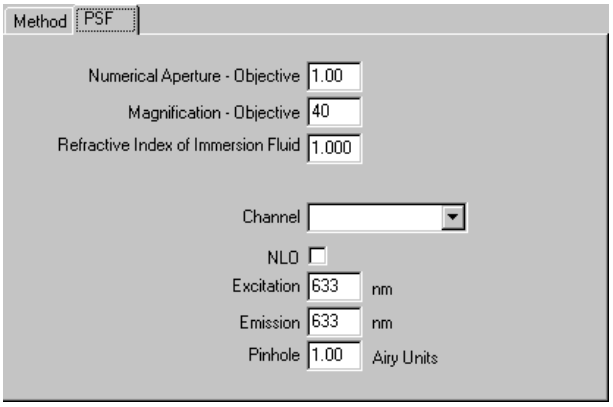


Fig. 4-268 PSF tab

4.13.18 Display - Cut

This function allows to

- display a Z Stack of images at a user defined section plane (= cut plane)
- improve the image of the section plane by trilinear interpolation

The settings of **Chan**, **Zoom**, **Slice**, **Contr** and **Palette** are applied.

- Clicking on the **Cut** button in the **Display** toolbar opens the **Cut** to the right of the **Image Display** window.

  Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.
- By varying the parameters **X**, **Y**, **Z**, **Pitch** and **Yaw**, you can position a section plane of any orientation within the stack volume.
- The resulting position of the section plane is shown as a red area below the **Trilinear Interpolation** button. At the same time, the result is shown in the **Image Display** window.
- A click on the **Reset All** button restores the original position.
- A click on the **Trilinear Interpolation** button will improve the quality of the image by performing a 3D interpolation of the image.

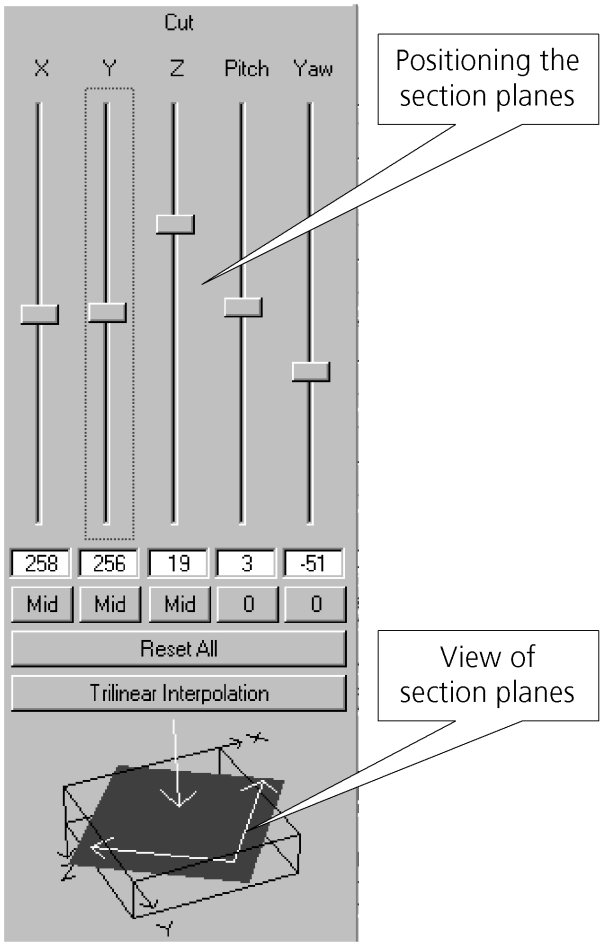


Fig. 4-269 Image Display window, Cut display

4.13.19 Display - Gallery

This function allows to

- display images (Z Stack, time series, combination of both) side by side in tiled fashion
- add data relevant to the images displayed (Z Stack slice distance, time of acquisition or wavelength)
- extract a subset of images from the original stack and store the result as a new image

The settings of **Chan**, **Zoom**, **Slice**, **Contr** and **Palette** apply.

Click on **Gallery** will display the **Gallery** toolbar. Any changes done with this toolbar are effective immediately.

- A click on the **Gallery** button in the **Display** toolbar not only produces the gallery itself but also the **Gallery** toolbar with two buttons: **Data** button and **Subset** button.
- Clicking on the **Data** button shows the Z Slice distance, the acquisition time or the wavelength or combinations.
- Clicking on the color selection button (below the **Data** button) will open a color selection window allowing you to choose - at a click of the mouse - in which color the data will be shown in the gallery display.

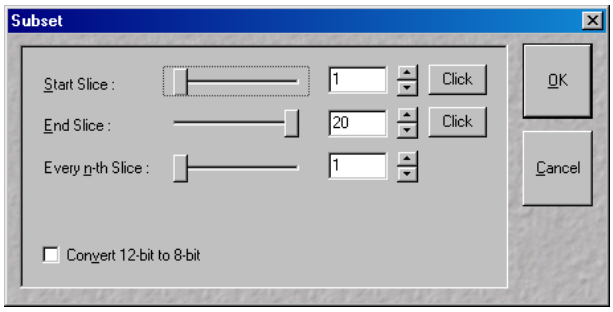


Fig. 4-270 Subset window

- Clicking on the **Subset** button opens another window entitled **Subset**, in which you can select images of the set of images displayed.
  - A stack consisting of the selected images only is generated and displayed.
- Select **Start Slice** and **End Slice** via the sliders, the input box or the **Click** (into window) button.
- Enter a value for **n** in the **Every n-th Slice** panel.
- If required, activate the **Convert 12 bit to 8 bit** check box ☑.
- Clicking on the **Ok** button will generate a new subset of images.
- **Cancel** will stop the procedure.

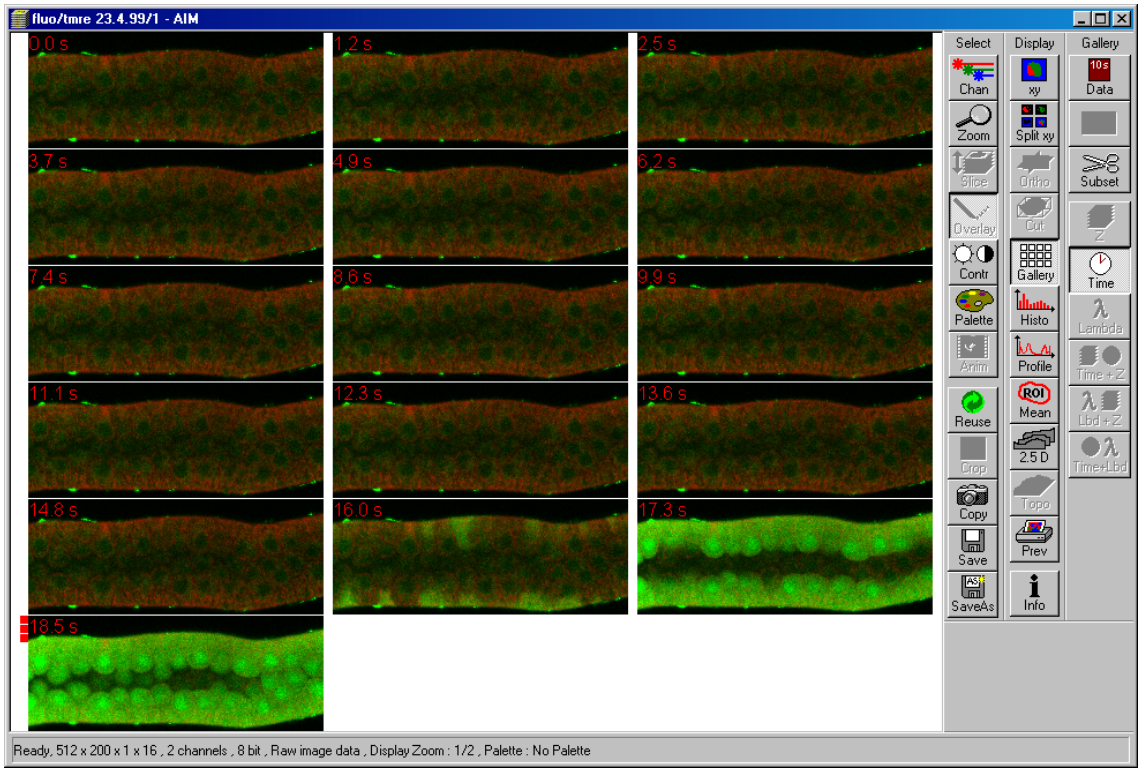


Fig. 4-271 Image Display window, Gallery display

4.13.20 Display - Histo

4.13.20.1 Display - Histo - Overview

This function allows to

- display a histogram (distribution of pixel intensities) of an image
- show the histogram values in table form
- copy table to clipboard or save as text file
- analyze the colocalization between two channels
- measure area and mean gray value and standard distribution in an area
- show separate histograms for each channel in a multi channel image

☞ Colocalization is only available in case of a two or multi channel image.

The settings of **Chan, Zoom, Slice, Contr** and **Palette** are applied.

Click on **Histo** will display the **Histogram** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

- Click on the **Histo** button. The **Histogram** toolbar will be displayed on the right.

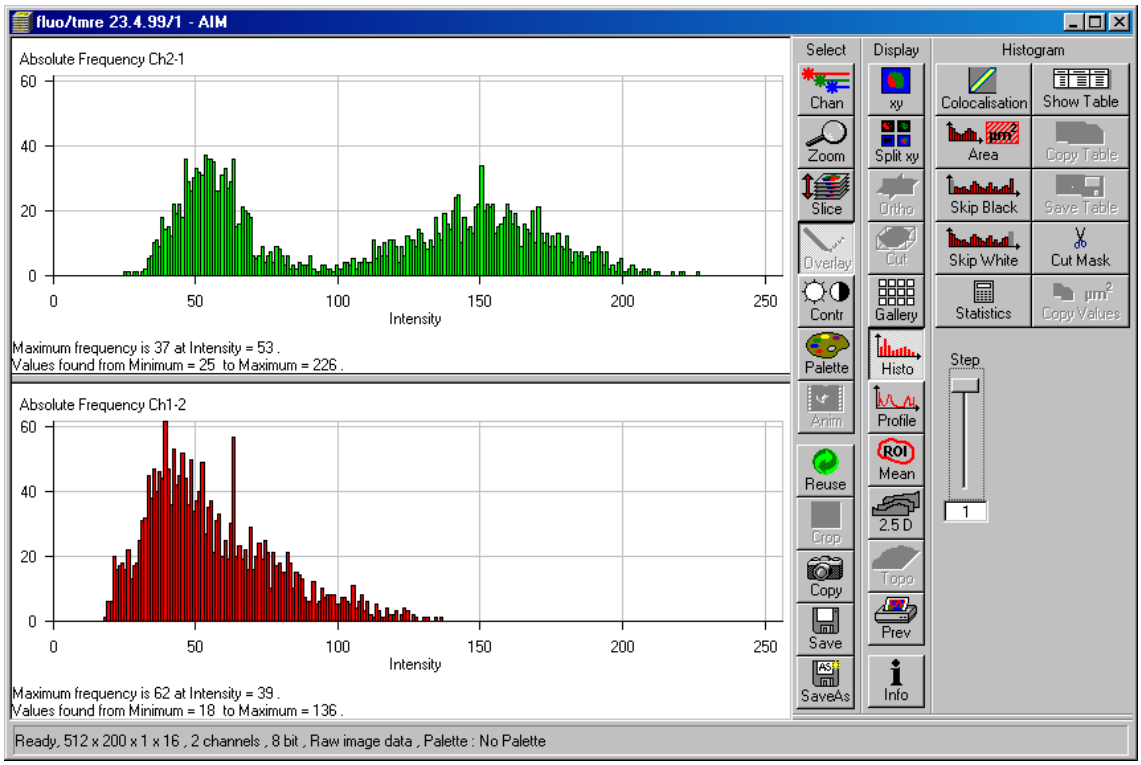


Fig. 4-272 Image Display window, Histogram display

☞ The **Histo** button can also be used online during scanning.

Histogram functions

Skip Black button	Ignore black pixels (gray value 0) in the histogram.
Skip White button	Ignore white pixels (gray value 255 or 4096) in the histogram.
Statistics	Displays statistical parameters (Mean Intensity, Standard Deviation, Number of Pixels and Size of Area) in a additional table. Area measurements of very small areas (< 10 pixels) give only approximate values
Step input box	Set the number of intensity steps which shall be displayed in the diagram. Step 1 corresponds to 256 intensity steps, Step 64 to 4 intensity steps in case of 8 bit images. Reduction is made by averaging.
Show Image button	Shows the image in the **Image Display** window beside the histogram.
Show Table button	The histogram is shown as a table at the bottom left of the **Image Display** window.
Copy Table button	The histogram table is copied to the clipboard.
Save Table button	The histogram table can be stored as a text file (extension *.txt).

Area functions

Area button	Interactive definition of area for size and intensity measurement.
Save Values button	Copies area values to the clipboard (only available if the **Area** button is activated).

4.13.20.2 Area Function

- Click on the **Area** button in the **Histogram** toolbar.
  - The function elements for **Area** measurement are displayed at the bottom right of the **Histogram** toolbar.

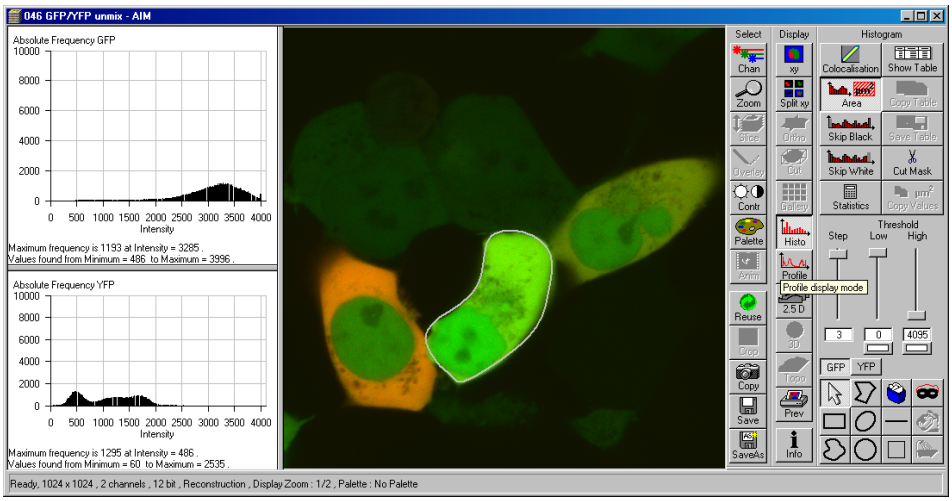


Fig. 4-273 Image Display window, Area Measure display

The following function elements are available:

Step — Set the number of intensity steps which shall be displayed in the diagram. Step 1 corresponds to 256 intensity steps, Step 64 to 4 intensity steps in case of 8 bit images. Reduction is made by averaging.

Low — **Threshold low** slider with **Color** selection button: The intensity values below threshold are not displayed. The removed areas are masked in the color selected in the **Color** selection button.

High — **Threshold high** slider with **Color** selection button: The intensity values above threshold are not displayed. The removed areas are masked in the color selected in the **Color** selection button.

Ch1-T1 — **Ch1, Ch3 ...** buttons: Selection of the channel for which the area measurement is to be performed.

Arrow (selection) button: Activation of the mouse button for selection, resizing, or movement of a mask element in the **Image Display** window.

Resize: Click on handle

Movement: Click on line and hold the mouse button, move the entire figure, release mouse button. and hold down the mouse button, drag the handle, release mouse button.

Closed polyline button: Creation of a polyline figure in the image. The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Recycle bin button: All the mask elements are deleted. If one element was marked before, this element is now deleted from the image.

Mask button: Enables the **Mask** Mode, where the region can be defined with ink.

Rectangle button: Creation of a rectangle in the image. Click and hold down the mouse button, drag a rectangle in any direction, release the mouse button to end the procedure.

Ellipse button: Creation of an ellipse in the image. The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and the rotation direction can then be determined; the third click sets the second dimension and the direction and ends the procedure.

Line button: Determines the line thickness of the area outline.

Flood fill button: Fills the overlay element or the scatter diagram with the color selected under **Mask**.

Closed free-shape curve button: Creation of a closed Bezier figure in the scatter diagram. The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Circle button: Creation of a circle in the scatter diagram. Clicking and holding down the mouse button sets the center point; drag the diameter and release the mouse button to end the procedure.

Color selection button: The colors displayed in the selection box can be assigned to the mask elements with a click of the mouse. The currently selected color is always displayed in the larger rectangle (top left) of the selection box.

Clear Mask button: Removes the color filling from an overlay element or from the scatter diagram.

- The function can be activated by clicking on one of the geometry buttons, e.g. (polyline).
- The figure of interest can be marked in the image by cursor control in conjunction with a mouse click.
- If more than one Region is drawn either the mean histogram values for all regions is displayed or only the values of the activated region if it is marked by clicking on the drawing line with the mouse.
- Areas can also be excluded from the analysis. By clicking on the **Flood fill** button (paint jar) and moving the cursor to the area to be excluded causes the remaining area to be computed and the result indicated under **Area Measure**.
- If you specify a top and bottom intensity threshold, the area lying within this intensity interval can be computed.

- Specify the thresholds either with the **Threshold low** and **Threshold high** sliders, or with the ▲▼ buttons.

4.13.20.3 Colocalisation Function

The **Colocalisation** function permits interactive analysis of two channels of an image by computing a scatter diagram (colocalisation).

- Click on the **Colocalisation** button. The scatter diagram is created and displayed beside the image.
- Click on the **xy** button of the **Display** toolbar if you want to return to the original image.

How a scatter diagram is generated

All pixels having the same positions in both images are considered a pair. Of every pair of pixels (P1, P2) from the two source images, the intensity level of pixel P1 is interpreted as X coordinate, and that of pixel P2 as Y coordinate of the scatter diagram. The value of the pixel thus addressed is increased by one every time, up to the maximum number of pixels used. This way, each pixel of the scatter diagram is a value that shows how often a particular pair of pixels has occurred.

☞ Differences between the images cause irregular spots in the scatter diagram.

Identical images produce a clean diagonal line running from bottom left to top right, because only pixel pairs (0,0), (1,1), (2,2) with the same intensity can occur. Differences between the images cause an irregular distribution in the scatter diagram.

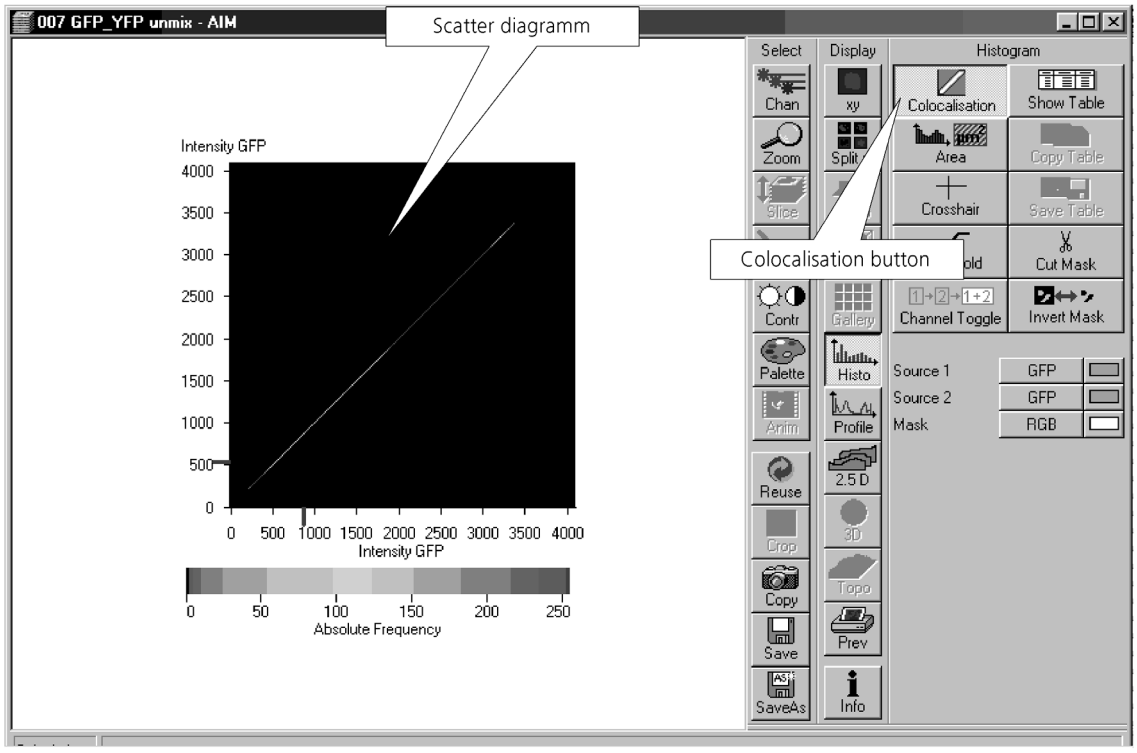


Fig. 4-274 Image Display window, Colocalization display

Function elements

The following function elements are available:

Displays the scatter Histogram of the two image channels

Adds display of the according data table

Adds display of the image

Displays a movable crosshair for different areas in the scatter histogram

Opens the **Intensity Threshold** window to sets threshold for colocalisation in the scatter histogram.

Set from Image ROIs button: Sets background threshold from ROI (**Threshold** button)

Cut Mask button.

Channel Toggle button.

Invert Mask button: Inverts the mask or the scatter diagram.

Source 1 selection box with **Color** selection box: Selection of the first channel to be selected via the selection box, assignment of a defined color via the **Color** selection box.

Source 2 selection box with **Color** selection box: Selection of the second channel to be selected via the selection box, assignment of a defined color via the **Color** selection box.

Mask selection box with **Color** selection box: Selection of **RGB** or **Overlay** display of the mask, assignment of a defined color via the **Color** selection box.

Drawing tools — Allows the selection of ROIs in the Histogram and the Image.

Save at drawing tools — Stores ROIs and threshold settings.

Enhanced colocalisation

Scatter diagram image display and data table are interactively linked.

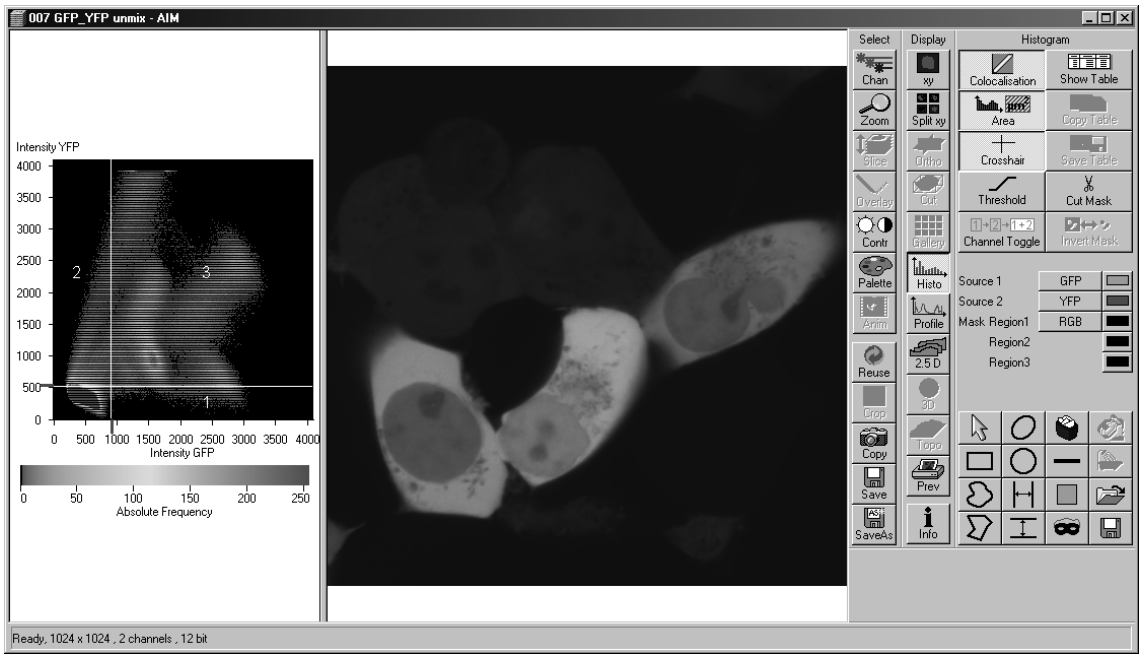


Fig. 4-275 Image Display window, Colocalization display

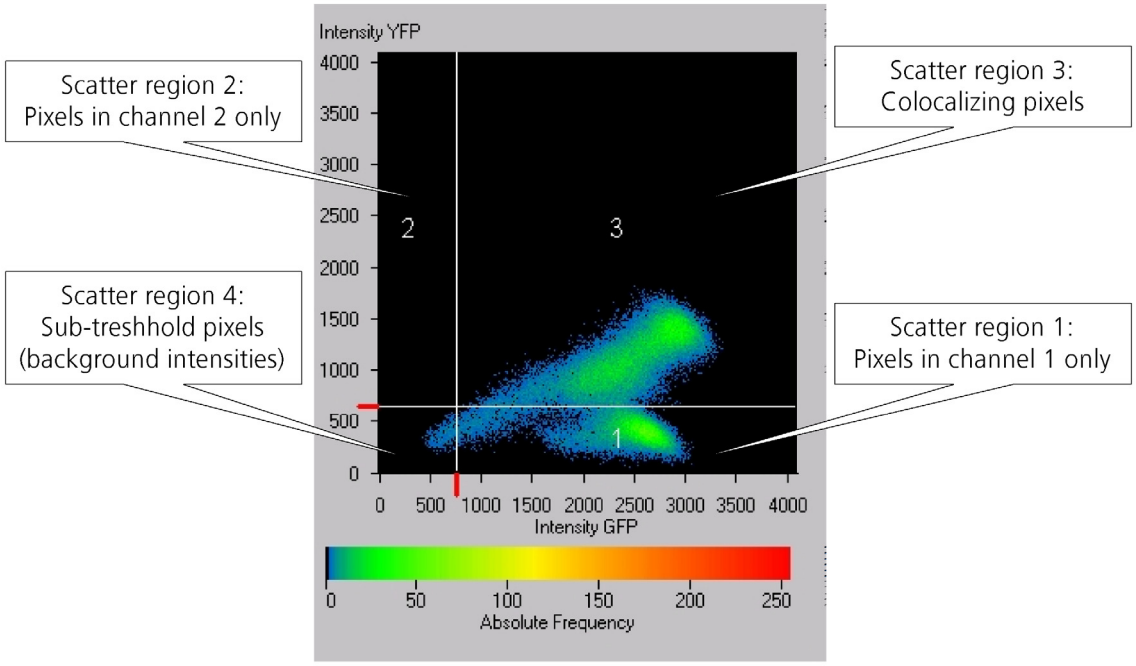


Fig. 4-276 Scatter diagram and threshold with crosshair

Quantitative Parameters

- **No. of pixels** in image ROI or scatter region
- **Area / relative area** of image ROI or scatter region
- **Mean intensities / SD** within image ROI or scatter region
- **Colocalization coefficients**
- **Weighted colocalization coefficients**
- **Overlap coefficient** after Manders
- **Correlation coefficients** (R and R^2)

Colocalization coefficients

$$c_1 = \frac{pixels_{Ch1,coloc}}{pixels_{Ch1,total}} \qquad c_2 = \frac{pixels_{Ch2,coloc}}{pixels_{Ch2,total}}$$

- Relative number of colocalizing pixels in channel 1 or 2, respectively, as compared to the total number of pixels above threshold.
- Value range 0 – 1 (0: no colocalization, 1: all pixels colocalize)
- All pixels above background count irrespective of their intensity.

Weighted colocalization coefficients

$$M_1 = \frac{\sum_i Ch1_{i,coloc}}{\sum_i Ch1_{i,total}} \qquad M_2 = \frac{\sum_i Ch2_{i,coloc}}{\sum_i Ch2_{i,total}}$$

- Sum of intensities of colocalizing pixels in channel 1 or 2, respectively, as compared to the overall sum of pixel intensities above threshold and in this channel.
- Value range 0 – 1 (0: no colocalization, 1: all pixels colocalize)
- Bright pixels contribute more than faint pixels

Correlation coefficient, Pearson´s correlation coefficient

$$R_p = \frac{\sum_i (Ch1_i - Ch1_{aver}) * (Ch2_i - Ch2_{aver})}{\sqrt{\sum_i (Ch1_i - Ch1_{aver})^2 * \sum_i (Ch2_i - Ch2_{aver})^2}}$$

- Provides information on the intensity distribution within the colocalizing region
- Value range -1 to +1
  -1,+1: all pixels are found on straight line in the scatter diagram
  0: pixels in scattergram distribute in a cloud with no preferential direction

Overlap coefficient, overlap coefficient after Manders

(Manders, Verbeek and Aten, J. Microscopy 169:375-382, 1993)

$$r = \frac{\sum_i Ch1_i * Ch2_i}{\sqrt{\sum_i (Ch1_i)^2 * \sum_i (Ch2_i)^2}}$$

- Another parameter used to quantify colocalization in image pairs
- Insensitive to differences in signal intensities between the two channels, photo-bleaching or amplifier settings
- Value range 0 – 1 (0: no colocalization, 1:all pixels colocalize)

4.13.21 Display - Profile

4.13.21.1 Function

This function allows to

- display the intensity distribution of an image along a straight or curved line
- show the intensity values in table form and copy table to clipboard or save as text file
- show separate profiles for each channel in a multi channel image

The settings of **Chan**, **Zoom**, **Slice**, **Contr** and **Palette** are applied.

Click on **Profile** will display the **Profile** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

- Click on the **Profile** button. The **Profile** toolbar will be displayed.
  - The intensity curves are shown in a graph below the image(s).
- On the **Profile** toolbar you can select the width and color of the profile line.
- You can place two markers on the profile line to measure differences in intensities and distances in the XY plane.

☞ The **Profile** button can also be used online during scanning.

- Click on the **Diagr. in Image** button to see an intensity graph superimposed on the image.

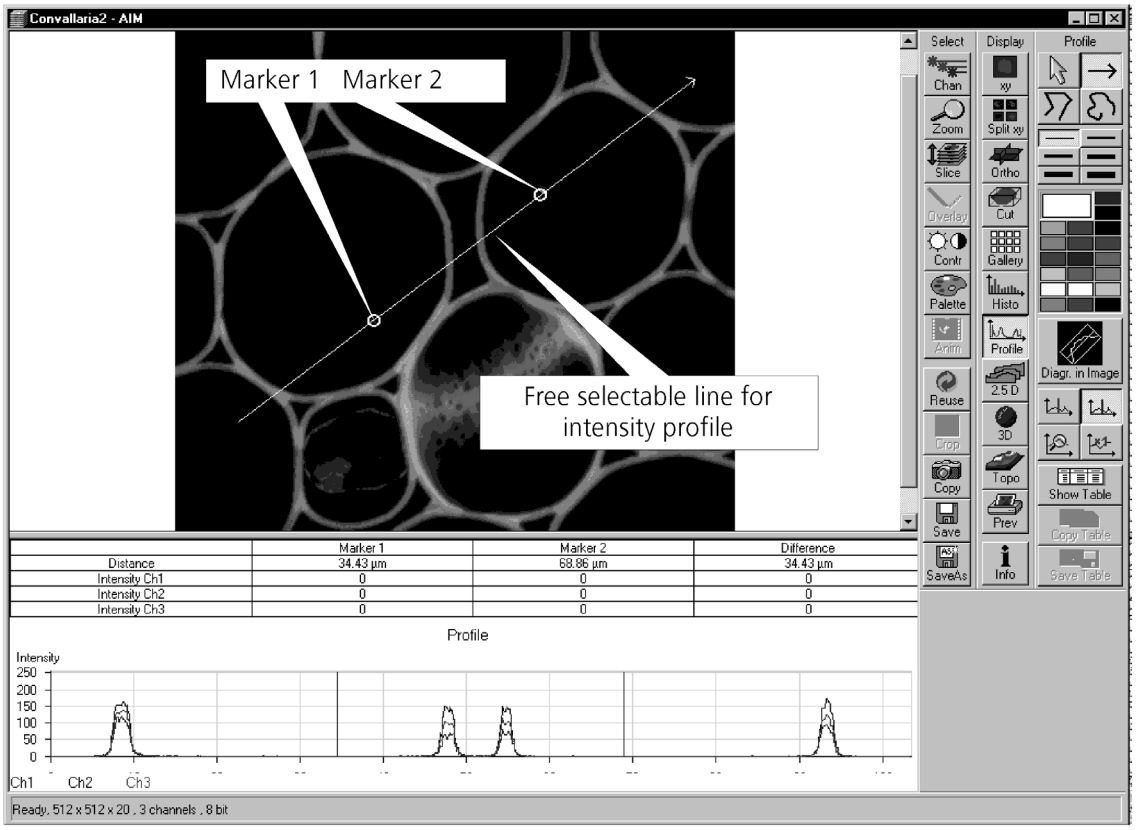


Fig. 4-277 Image Display window, Profile display

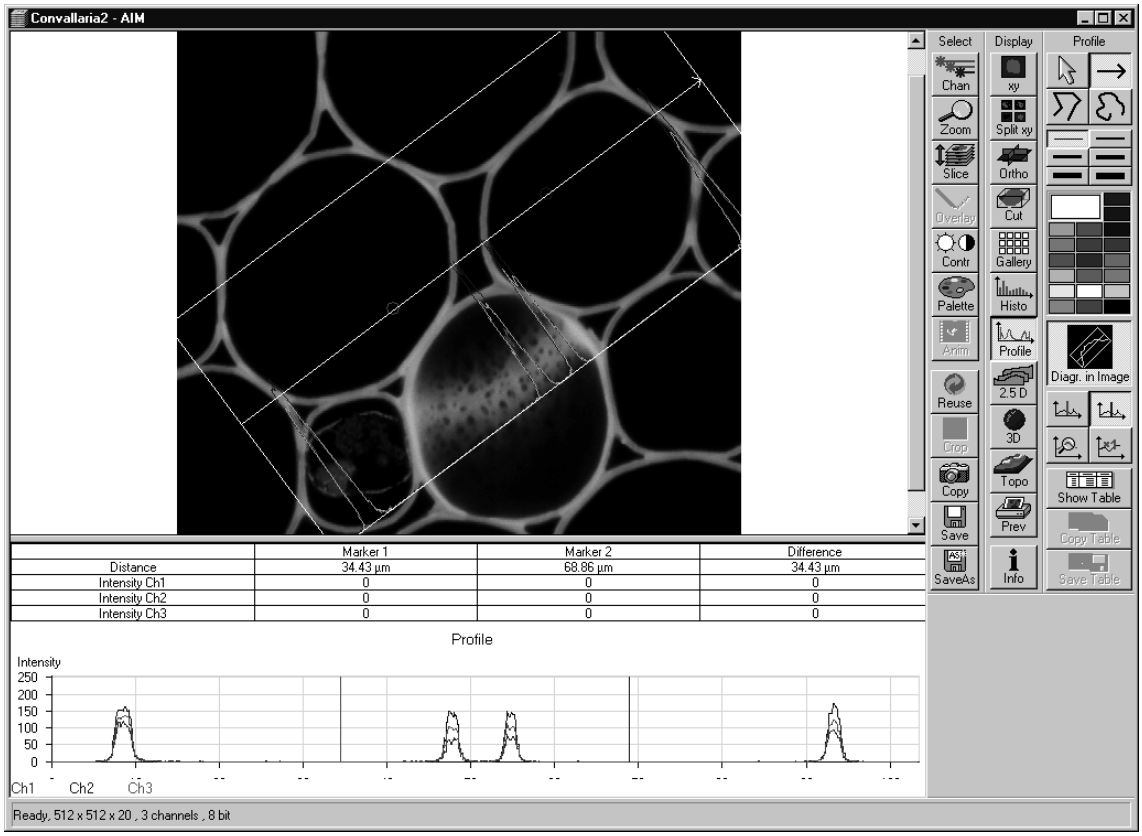


Fig. 4-278 Image Display window, Profile display

4.13.21.2 Function Elements

The **Profile** toolbar contains the following function elements:

Arrow (selection) button: Activates the mouse button for selection, resizing or movement of the profile line in the **Image Display** window.

Resize: Click on handle and hold down the mouse button, move the handle, release mouse button.

Movement: Click on line and hold down the mouse button, move the entire line, release mouse button.

Line with arrow button :(open arrow): Activates the straight profile drawing mode.

Click into the image and hold the mouse button, drag a line in any direction and release the mouse button to end the procedure.

Open polyline button: Activates the open polyline drawing mode.

The first click into the image sets the starting point, each additional click adds a further line, right mouse click ends the procedure.

Open free-shape curve button: Activates the Bezier figure drawing mode.

The first click into the image sets the starting point, each additional click adds a point, right mouse click ends the procedure.

Line button: This button allows you to determine the line thickness of the profile line. It has no influence on the way the intensity profile is generated.

Color selection box: The colors displayed in the **Color** selection box can be assigned to the overlay profile line with a click of the mouse. The currently selected color is displayed in the larger rectangle (top left) of the selection box.

Diagr. In Image button: The profile diagram is displayed in the image along the drawn profile line.

Marker 1 button (red): Activates the red marker for movement in the profile diagram; the marker shown as a red line in the diagram can now be moved to the right or left of the diagram using the mouse. The marker in the image display (red circle) follows accordingly.

Marker 2 button (blue): Activates the blue marker for movement in the profile diagram; the marker shown as a blue line in the diagram can now be moved to the right or left of the diagram using the mouse. The marker in the image display (blue circle) follows accordingly.

Zoom button: Zoom function for profile diagram. Click and drag a rectangle over the area to be enlarged in the profile diagram; the selected area is enlarged on release of the mouse button. The zoom function can be performed several times. A click with the right mouse button will reset the original size.

Reset Zoom button: Resets the zoom factor of the profile diagram to the original size.

Show Table button: The profile diagram is displayed as a table at the bottom of the **Image Display** window.

Copy Table button: The profile table is copied to the clipboard.

Save Table button: The profile table can be stored as a text file (extension *.txt).

4.13.22 Display - 2.5 D

This function allows to

- display the two-dimensional intensity distribution of an image in an pseudo 3D mode
- show the intensity values in profile, grid or filled mode
- show separate distribution for each channel in a multi channel image

☞ The **2.5 D** button can also be used online during scanning.

- Click on the **2.5 D** button.
  - The **Pseudo 3D** toolbar is displayed.

The settings of **Slice** apply. The settings of **Chan, Zoom, Contr** and **Palette** are not applied.

Click on **2.5 D** will display the **Pseudo 3D** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

The viewing plane of the **Image Display** window can be rotated, tilted either directly with the mouse or by the scroll bars on the right-hand side and the bottom of the **Image Display** window.

4.13.22.1 Direct Setting in the Image

- Click in the image and hold down the mouse button. The perspective is changed by moving the mouse button in horizontal or vertical direction.

4.13.22.2 Setting via Scrollbars

- Move the horizontal scrollbar to rotate the image around the vertical axis. The rotation angle is displayed in the yellow display box.
- Move the left vertical scrollbar to rotate the image around the horizontal axis. The rotation angle is displayed in the yellow display box.

The intensity scale can be varied by the scroll bar on the right-hand side of the **Image Display** window:

- Moving the right vertical scrollbar enables you to expand or to compress the intensity scale of the image, while the expansion of this intensity axis ranges between 10 % and 100 % of the X-scale size.

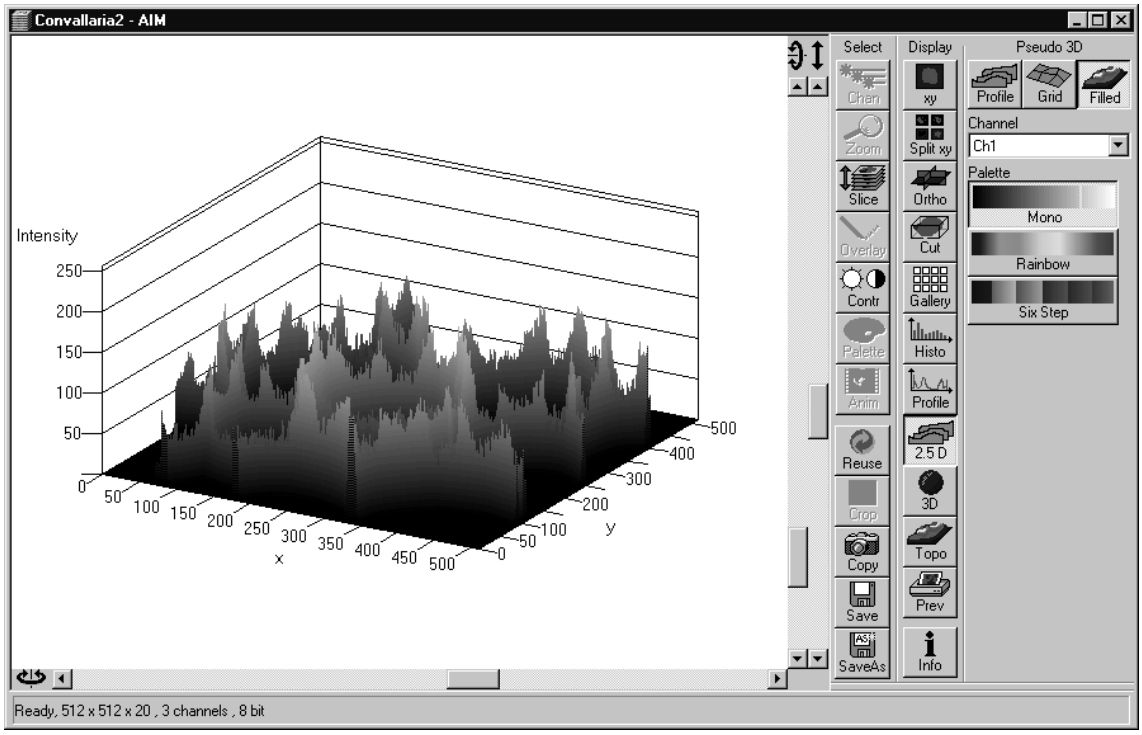


Fig. 4-279 Image Display window, 2.5 D display

The **Pseudo 3D** toolbar contains the following function elements:

Profile button	Profile display (vertical polygon display). Setting of the **Profile Distance** between 1 and 20 using the slider.
Grid button	Grid display (horizontal grid display). Setting of the **Grid Distance** between 1 and 20 using the slider.
Filled button	Color diagram (filled 3D diagram). Selection between the **Mono**, **Rainbow** and **Six Step** color palettes.
Channel list box	Permits the selection of a **Channel** in a multi channel image.

4.13.23 Display - 3D (Image VisArt)

This optional function allows to

- reconstruct and display 3 D fluorescence image stacks or time series of frames and stacks from the **Image Display** window
- select from a variety of reconstruction modes

The settings of **Chan** are applied. The settings of **Zoom, Slice, Contr** and **Palette** are not applied.

Click on **3D** will display the **3D** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

☞ The **3D** button can also be used online during scanning.

- Click on the **3D** button. The **3D** toolbar will be displayed.

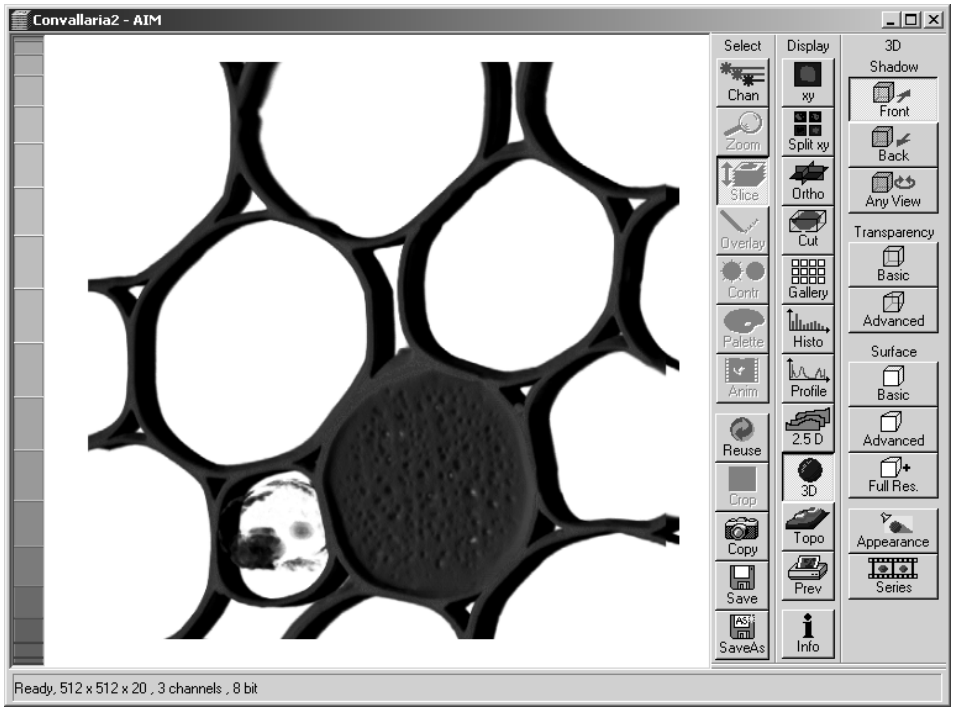


Fig. 4-280 Image Display window, 3D display

The **3D** toolbar contains the following function elements:

Shadow projection

Front button	Shadow rendering front view
Back button	Shadow rendering back view
Any View button	Shadow rendering with user defined view

Transparency projection

Basic button	Transparency rendering (voxel based)
Advanced button	Transparency rendering (voxel based) with textures

Surface projection

Basic button	Surface rendering (voxel based)
Advanced button	Surface rendering (triangle based)
Full Resolution button	High accurate surface rendering (triangle based)
Appearance button	Opens the render properties dialog
Series button	Renders a series of 3D image stack or 3D / 4D time series, opens the **Series render** dialog

4.13.23.1 Shadow Projection

With a click on **Front**, the 3D reconstructed image is displayed in a shadow projection where it is illuminated at a 45° angle from the front left.

A click on the **Back** button creates the same projection with illumination from back left.

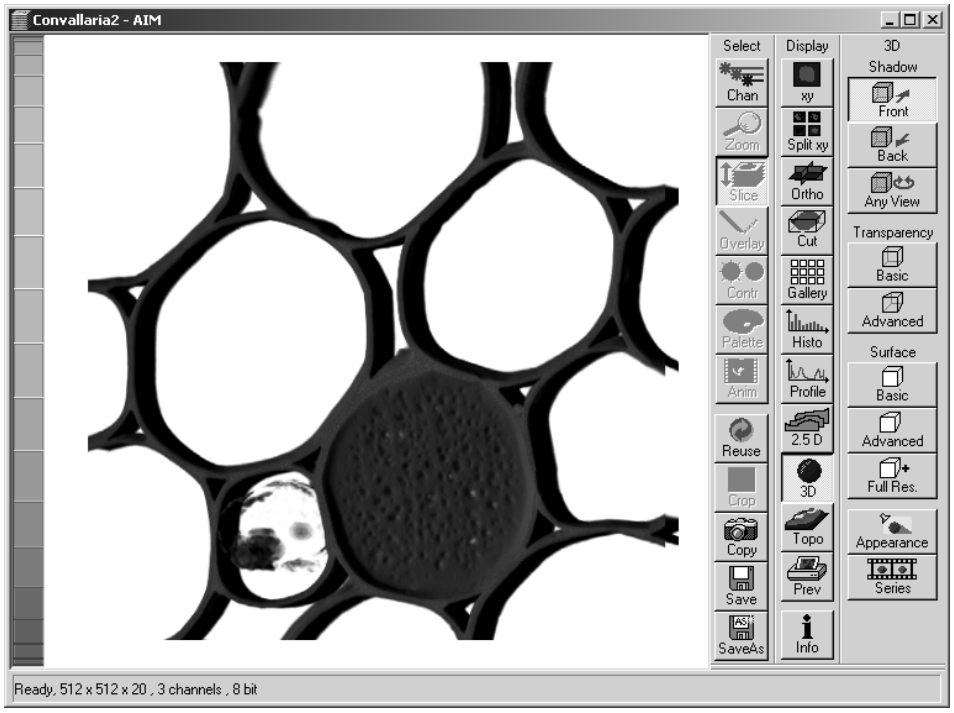


Fig. 4-281 Image Display window, 3D display, Shadow projection, Front view

The zoom wheel to the left of the **Image Display** window allows continuous zooming of the 3D reconstructed image.

A click on the **Any View** button displays the 3D reconstruction image in a shadow projection where the viewing point can be defined. In addition to the zoom setting, the image can be rotated around the three orthogonal axes via the relevant setting wheels.

However, the 3D orientation can also be set directly in the **Image Display** window by clicking, holding and dragging the 3D reconstructed image with the mouse.

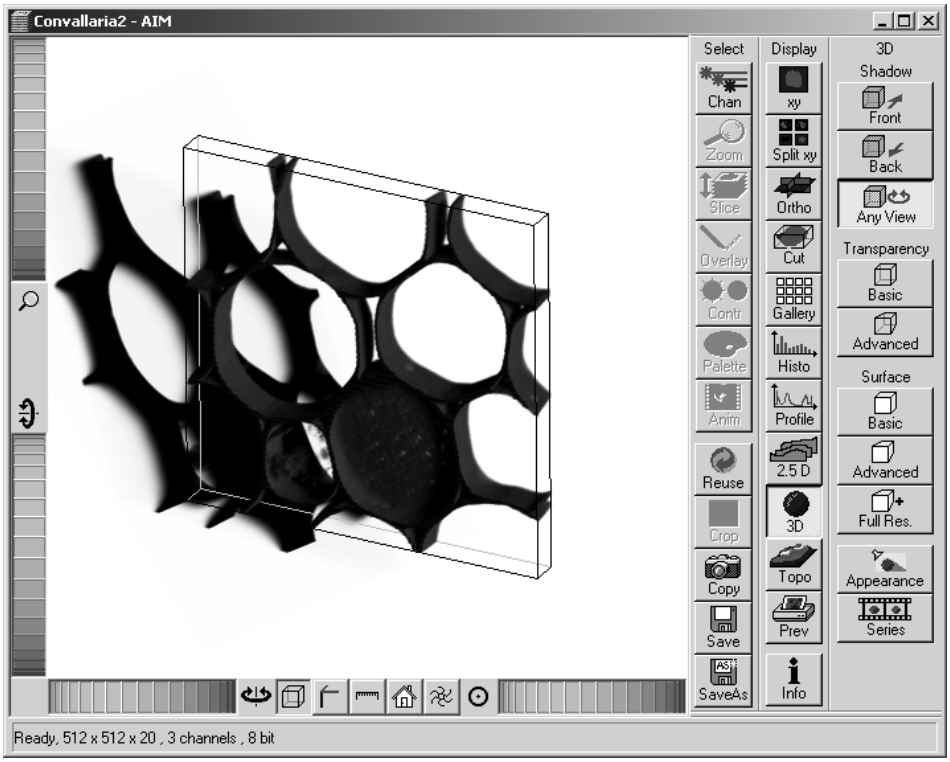


Fig. 4-282 Image Display window, 3D display, Shadow projection, Any View

The following additional buttons are available in the **Any View** shadow projection mode:

- After activation of the **Frame** button (below the image), a bounding box is drawn around the 3D reconstructed image.

☞ Depending on the used mode and hardware configuration, it can take several seconds until the 3D reconstruction is refreshed on the monitor after reorientation.

- A click on the **Coordinate System** button displays a colored coordinate system in the **Image Display** window, where the X axis is displayed in red color, the Y axis in blue and the Z axis in green.
- A click on the **Scale** button display an X-,Y- and Z-scale in the **Image Display** window.
- A click on the **Home** button resets the display parameters to the default values.

A click on the **Any View** button displays the 3D reconstruction image in a shadow projection where the viewing point can be defined. In addition to the zoom setting, the image can be rotated around the three orthogonal axes via the relevant setting wheels.

However, the 3D orientation can also be set directly in the **Image Display** window by clicking, holding and dragging the 3D reconstructed image with the mouse.

The following additional buttons are available in the **Any View** shadow projection mode:

- After activation of the **Frame** button (below the image), a bounding box is drawn around the 3D reconstructed image.

☞ Depending on the used mode and hardware configuration, it can take several seconds until the 3D reconstruction is refreshed on the monitor after reorientation.

- A click on the **Coordinate System** button displays a colored coordinate system in the **Image Display** window, where the X axis is displayed in red color, the Y axis in blue and the Z axis in green.
- A click on the **Scale** button display an X-,Y- and Z-scale in the **Image Display** window.
- A click on the **Home** button resets the display parameters to the default values.
- A click on the **Animation** button activates the animation mode. The object can be pushed by dragging in the **Image Display** window and rotates continuously. Any new push with pressed left mouse button changes the rotation direction and speed of the animation.

☞ The fastest animation results can be achieved with the advanced surface rendering mode (even without additional graphics cards).

4.13.23.2 Transparency Projection

The transparency projection generates a transparent 3D reconstructed image.

The elements for image display (zoom, 3D rotation, home, coordinate system, scale, frame and animation function) are identical to those of the **Any View** function of the shadow projection and are operated in the same way.

The transparency projections **Basic** and **Advanced** are perspective-type 3D reconstructions, with the **Advanced** projection permitting the perspective impression being varied between parallel and centric projection by changing the **View angle**. The **Advanced** projection also offers the possibility of selecting between fast and precise calculation via the **Precise / Fast** slider (at the bottom right in the 3D toolbar). Of course, the precise calculation method is more time consuming.

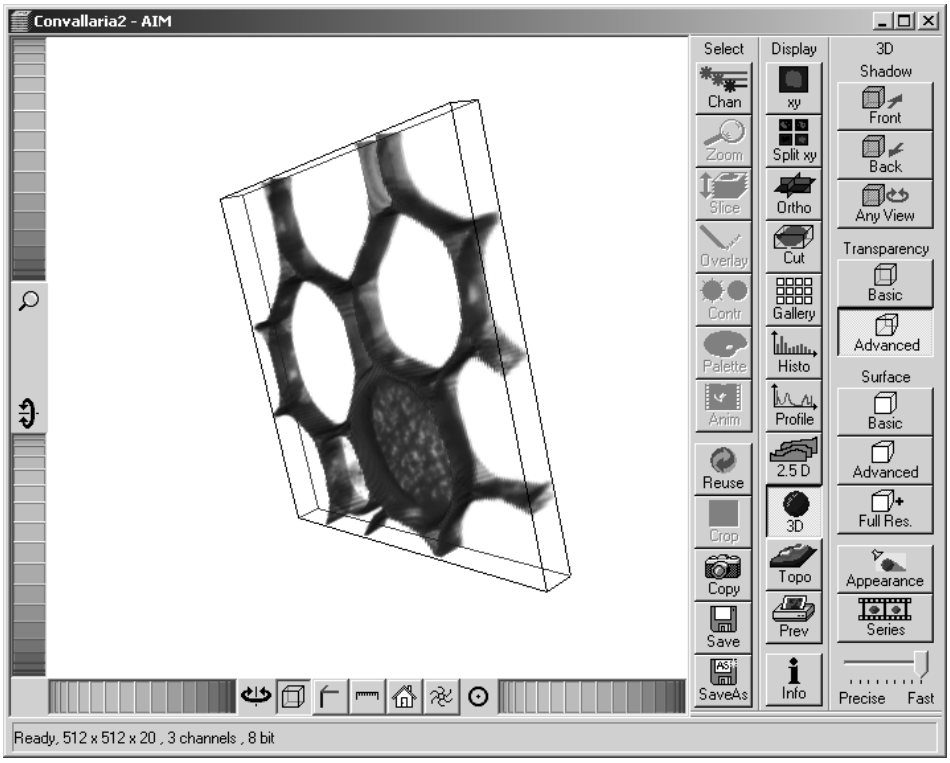


Fig. 4-283 Image Display window, 3D display, Transparency projection, Advanced

4.13.23.3 Surface Rendering

The surface rendering generates surface rendered 3D reconstructed images.

The elements for image display (zoom, 3D rotation, home, coordinate system, scale, frame and animation function) are identical to those of the transparency projection and are operated in the same way.

The surface projections **Basic** and **Advanced** are perspective-type reconstructions of the surface and differ in the fact that the calculation of the 3D information is based on voxels or triangles.

The **Advanced** projection permits the **View angle** to be varied in order to enhance the perspective impression. It is also possible to select between fast and precise calculation via the **Precise / Fast** slider (at the bottom right in the 3D toolbar). Of course, the precise calculation method is more time consuming.

The **Full resolution** projection is based on a high precision calculation method for 3D information on the basis of triangles with maximum resolution.

☞ Depending on the hardware configuration, it can take several seconds until the surface projection is refreshed on the screen.

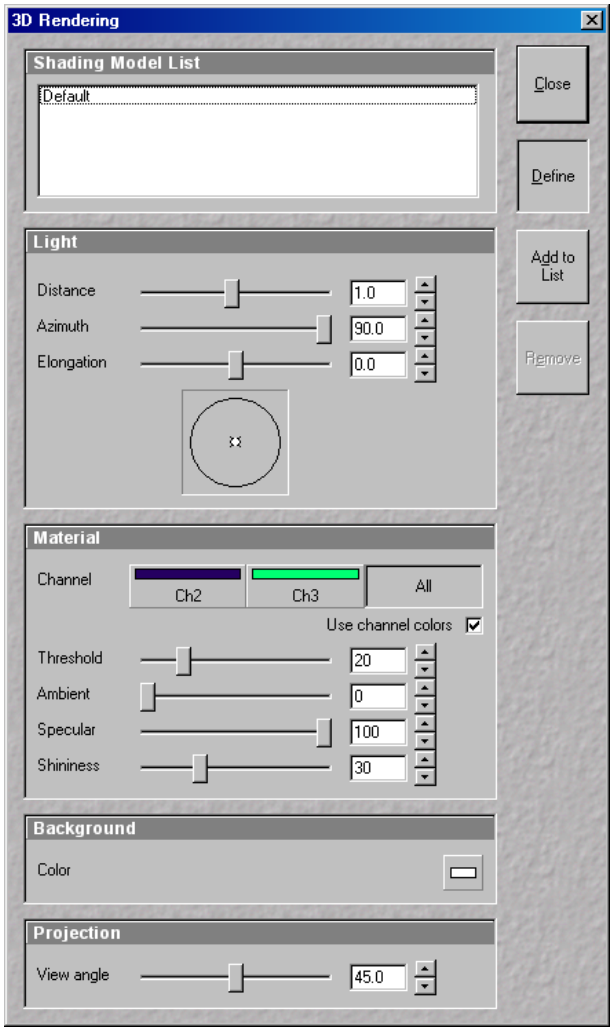


Fig. 4-284 **3D Rendering window (e.g. Surface Advanced rendering mode)**

4.13.23.4 Appearance (Settings)

The **Appearance** button opens the **3D Rendering** window.

This window allows settings for **Light**, **Material**, **Background** and **Projection** properties to be defined for **all** 3D projection modes.

Depending on the selected 3D projection mode, different setting parameters are displayed.

In the **Shadow** projection, the parameter **Roughness** is also available and can be set between **0** and **1**.

A default setting is permanently available for all modes.

If individual settings for 3D rendering are to be used again, they can be stored and loaded when required.

☞ See also **Show Processing Parameters**, page 4-310.

Proceed as follows to save individual settings:

- Click on the **Add to List** button.
- Enter a name in the opening **Add Shading Model to list** window.
- Click on **OK**.
  - The settings are saved and the entered name appears in the **Shading Model List** selection box.
- To activate the settings, double-click on the relevant name in the **Shading Model List** selection box.

Settings which are no longer required can be removed.

- Select the name of the setting to be deleted with a click of the mouse in the selection box and then click on the **Remove** button.
  - The setting is deleted.

4.13.23.5 Series

The **Series** button opens the **Render Series** window. This window allows settings for the axis to be used for rotation of the 3D reconstructed images.

- Click on the **Series** button to open the **Render Series** window.
- Select the requested projection mode by clicking on the relevant option button under **Mode**.
- Depending on the activated mode, directly set the parameters for animation in the **Render Series** window and the position of the 3D image in the **Image Display** window (zoom, rotation axes, rendering parameters).
- Click on **Apply** to start the animation

The animation is performed in a separate **Image Display** window, which permits the animation to be saved afterwards.

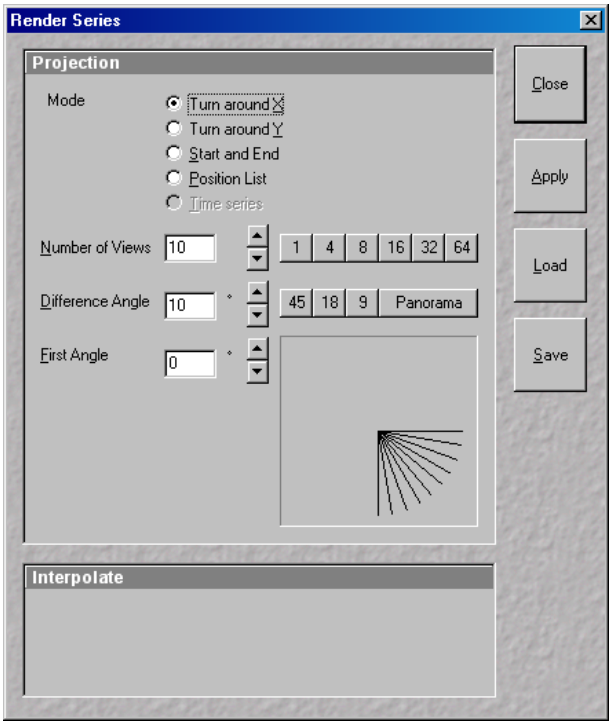


Fig. 4-285 **Render Series window (e.g. Turn around X mode)**

(1) Turn around X and Turn around Y mode

In this mode, the image is turned around the X-axis or the Y-axis exclusively.

The values for **Number of Views**, **Difference Angle** and **First Angle** can be selected accordingly (see **Projection**, page 4-162).

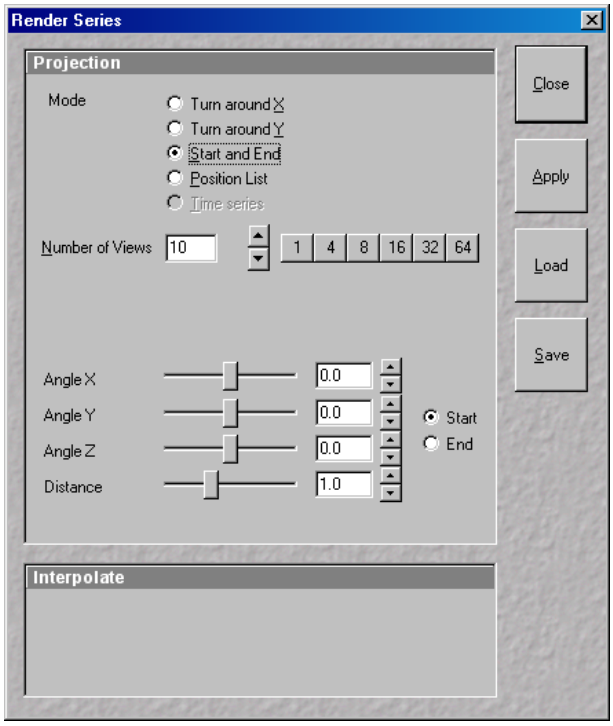


Fig. 4-286 **Render Series window (e.g. Start and End mode)**

(2) Start and End mode

In the **Start and End** mode, the image is reconstructed between a start position and an end position.

The rotation angles for X, Y and Z and the distance (zoom) can be determined using the sliders.

The value for **Number of View** can be varied.

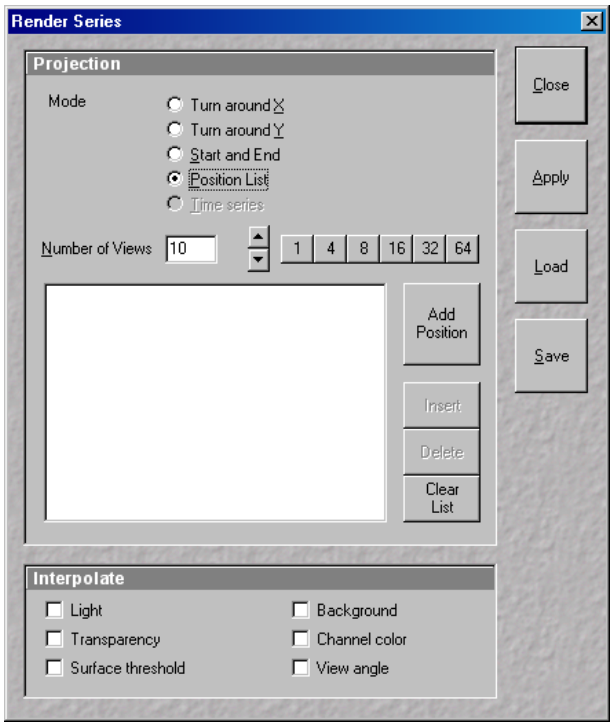


Fig. 4-287 **Render Series window (e.g. Position List mode)**

(3) Position List mode

In the **Position List** mode, the image is reconstructed between any required number of interim positions to be determined individually.

The rotation angles for X, Y and Z and the zoom can be determined directly in the image.

Every required interim position is included in the list of the **Render Series** window with a click on the **Add Position** button.

Clear List permits the contents of the list to be deleted.

The value for **Number of View** can be varied.

- Click on the **Apply** button calculates a spline along all the defined positions from the list and starts an animation along this spline track in space.

(4) Time series

When the input images is a Z Stack time series, the reconstructed images are generated for each time point.

4.13.24 Display - Topography for LSM

This optional function allows to

- process, display and measure topographic information.
- use frame Z Stacks
- and frame Z Stacks over time

The **Topo** function is mainly used for applications in material research and industry.

The settings of **Chan** and **Zoom** are applied. The settings of **Slice and Contr** are not applied. The **Palette** settings are applied in some 3D display modes.

Click on **Topo** will display the **Topography** toolbar. All changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

- Click on the **Topo** button. The **Topography** toolbar is displayed.
  - The topography of a Z Stack is displayed in the **Image Display** box of the **Image Display** window. The parameter used at the last exit of the **Topo** function are applied.

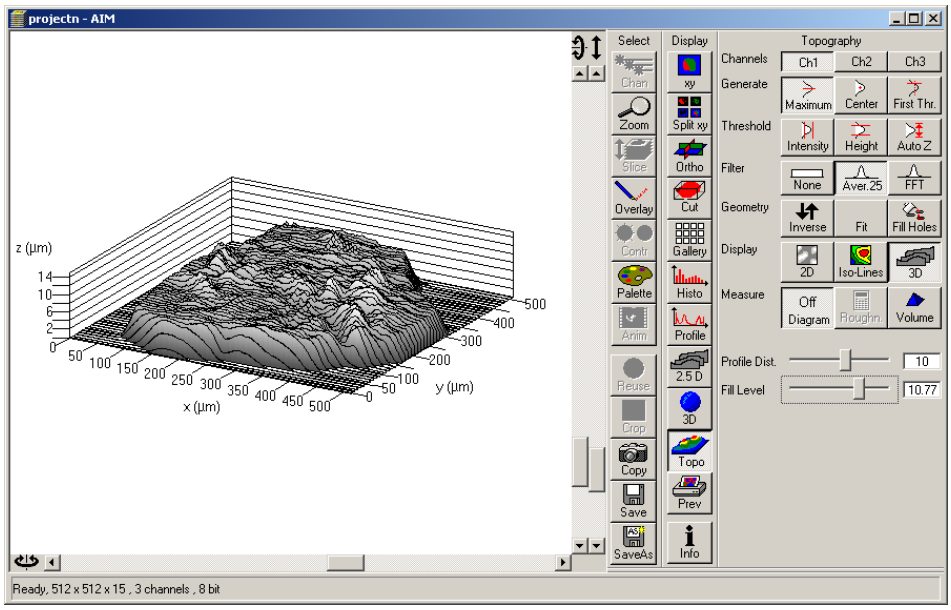


Fig. 4-288 Image Display window, Topography display

The **Topography** toolbar contains the following function elements:

Channels buttons	The selection of a channel to be used.
Generate buttons	The selection of the mode of calculation of the topography image (maximum, center of gravity, First /Last)
Threshold buttons	The selection of thresholds (intensity, height, Auto Z) to be used.
Filter buttons	The definition of filter procedures (geometrical, frequency cut-off filters) for smoothing, separation of roughness or waviness.
Geometry buttons	Inversion, various fit procedures, hole filling.
Display buttons	2D (Intensity, Height, Gradient); Iso-Lines (z Map, Intensity, Black) 3D (Profiles, Grid, Filled, Shaded).
Measure buttons	Diagrams (Profile, z Distribution, Bearing area ratio plot, Slope distribution); Roughness parameters ; Volume parameters.

4.13.24.1 Channel Selection

- Select the channel to be viewed using the relevant button (e. g.: **Ch1**).

4.13.24.2 Generate Topography

The three buttons provided in the **Generate** button bar allow you to generate the topography in different ways:

Maximum

- Click on the **Maximum** button to calculate the topography surface by finding the maximum intensity value. If the optical section with the highest intensity value is found, the intensity values of both neighboring slices are also taken into account, so that a 3 point maximum fit is calculated.
- In case it happens, that the maximum possible intensity value is present in more than one optical slice for a given pixel (saturation), the mid section of all saturated intensity slices is chosen as a reasonable approach.

Center

- Click on the **Center** button to calculate the topography surface by using the center of gravity of all summed up intensities of the stack for a given xy print.

☞ This mode provides better result for smooth surfaces of low intensity or nearly transparent surfaces. The receiver gain and offset has to be properly tuned and MarkFirst- MarkLast-positions of the stack should be located approximately in the same distance from the real surface.

First / Last

- Click on the **First** button to calculate the topography surface by using the first slice coming from the top, where the intensity reaches the value defined by the lower intensity threshold.

☞ This mode provides better result for surfaces of semitransparent materials with inclusions of higher reflectivity or transparent multilayers with subsurface layers of higher signal intensity.

Extended First / Last Mode

1. Definition of an intensity (I) threshold.
2. Starting from top / bottom of a stack to find I = 400.
3. Search of a local maximum one FWHM of actual Z PSF forwards / backwards.
4. Search of the next local maximum one FWHM forwards / backwards from the last max until you have not found any new local maximum.
5. Last local maximum is taken as surface point.

Fill Holes procedure

- Intensity of a missing pixel of a hole has to be interpolated by the distance-weighted intensity of all surrounding pixels.
- Fill hole algorithm is optimized for short calculation times.

4.13.24.3 Topography Thresholds

Intensity threshold

Click on the **Intensity** button to calculate the topography surface by using the lower and the upper intensity thresholds for image display. Use of this function is recommended to find the real surface in the case of images with pronounced noise. All image pixels with intensity less or higher than the thresholds set are ignored for the surface calculation.

- Click on the **Intensity** button to select the intensity thresholds for the surface generation. The **Intensity Threshold** window appears.
- Set the lower and upper intensity thresholds using the appropriate sliders.
- Click on **Close** to close the **Intensity Threshold** window.

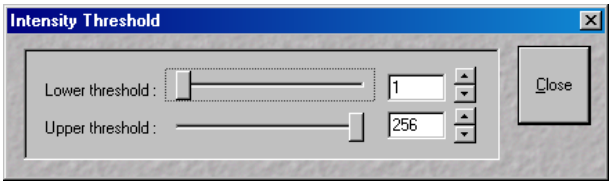


Fig. 4-289 Intensity Threshold window

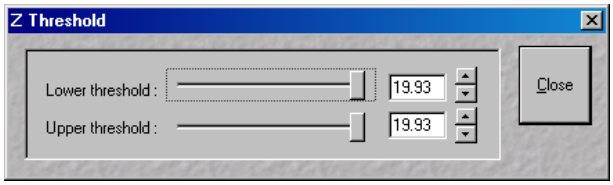


Fig. 4-290 Z Threshold window

Height threshold

Click on the **Height** button to calculate the topography surface by using the lower and the upper height thresholds for image display. Use of this function is recommended to get rid of unwanted peaks and valleys taken into account for parameter calculation. All topographic data with height values less or higher than the thresholds set are ignored for the display and parameter calculation. This threshold applies both for 2D as well as for 3D topography display modes.

- Click on the **Height** button to select the intensity thresholds for the surface generation. The **Z Threshold** window appears.
- Set the lower and upper intensity thresholds using the appropriate sliders.
- Click on **Close** to close the **Z Threshold** window.

Auto Z

By clicking on the **Auto Z** button the surface topography is displayed in the **Image Display** window in that way that it is automatically normalized to the lowest and highest Z value of the 3D topography.

- Click on the **Auto Z** button. The topography is automatically normalized with respect to the highest and lowest Z value.

4.13.24.4 Processing by Filtering

(1) Topography smoothing

The three buttons in the **Filter** button bar allow activation / deactivation of the filter functions for surface smoothing.

None button	No filter for input data.
Median / Gauss / Aver. button	Smoothing of z data using a low-pass Median, Gauss or average filter. Clicking on this button opens a selection box, where the number of neighboring pixels to be used for filtering can be specified: 1st row: small smoothing via Median/Gauss filter (Median; 3 x 3; 5 x 5; 7 x 7) 2nd row: medium smoothing via Average (9 x 9; 11 x 11; 15 x 15) 3rd row: pronounced smoothing via Average (25 x 25; 35 x 35; 45 x 45)

- To investigate the effects of various filter modes, select one of the 3D display modes (**Profiles**, **Grid**, **Filled** or **Shaded**) from the **Display** button bar.
- Click on the **Median** sub button to set the smoothing of the integrated Median filter.

  Or

- Click on a **Gauss** or **Average** sub button and select the required degree of smoothing from the selection box with a click of the mouse.

FFT button: This function performs a Fast Fourier Transformation (FFT) in the frequency range, applies highpass or lowpass filtering in the frequency range and performs the inverse FFT.

- Click on the **FFT** button, the **FFT Filter** window opens.
- Click on the arrow in the filter **Type** select box to choose an adequate filter function:
  - Gauss Lowpass
  - Gauss Highpass
  - Butterworth Lowpass
  - Butterworth Highpass
- Select a position of the **Cut off** slider to display either the lower frequencies (waviness) with the lowpass filters or the higher frequencies (roughness) with the highpass filters.
- The **Cut off** frequencies ranges from 1/1000 of the X dimension of the stack to four times of the X dimension of the stack. The dimensions of the filtering is given in units of µm.
- Select a position of the **Degree** slider. The filter functions can be calculated from 1st order to 5th order accuracy.
- Click on the **Close** button closes the **FFT Filter** window.

(2) Changing the topography geometry

The three buttons in the **Geometry** button bar allow the surface geometry to be changed.

Inverse button — Inverse surface. Depths change to heights, and vice versa.

Fit button — The following fit modes can be set:

1) No Fit

2) Plane

The topography is tilted in such a way that the mean deviation value plane is calculated.

3) Manu Tilt

4) 3Point Tilt

5) Sphere fit correction
A spherical form is eliminated, determination of micro roughness on spherical surfaces can be performed.

6) Cylinder fit correction
A cylinder form is eliminated, determination of micro roughness on cylindrical surfaces can be performed.

☞ You can display the exact values of the **Cylinder / Sphere** fit by opening a context menu in the **Image Display** box with a click of the right mouse button and selecting the **Show processing parameter** function.

Manu Tilt button — Clicking on the **Manu Tilt** button opens the **Manual Tilt** window (Fig. 4-291) for manual tilt correction.

In the manual tilt window three types of arrow buttons are present:

- One arrow changes pitch and yaw in 0.001° steps
- Two arrow changes pitch and yaw in 0.01° steps
- Three arrow changes pitch and yaw in 0.1° steps

- Click on the **Inverse** button for the inverse display of the topography. Clicking again will reset the normal display.
- Correct the tilt via the **Fit** or **Manu Tilt** functions.

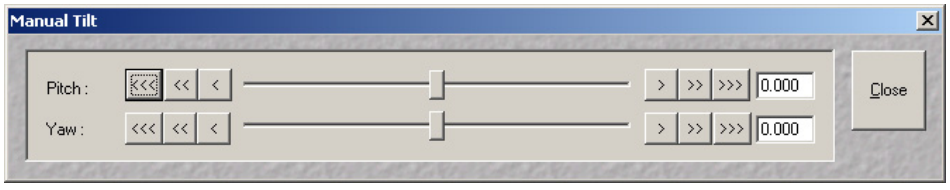


Fig. 4-291 Manual Tilt window

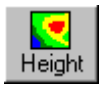
4.13.24.5 Display Modes

The three buttons in the **Display** button bar allow stacks to be displayed in the 2D, Iso-Lines or 3D display mode.

(1) 2D modes

The following 2D modes can be set:

Intensity button: Display of projection of all intensities of the stack (black-and-white display).



Height button: Height coded color map with color bar in a separate parameter window on the right hand side.

Gradient button: Display of height gradient (slope), averaged pixelwise over all neighbors (black-and-white display).

- Click on the **2D** button in the **Display** button bar.
  - The 2D display mode selected last is activated. At the same time, an additional button bar is displayed beside the **2D** button permitting selection of the required 2D display mode.
- Select the required 2D display mode with a click of the mouse.

(2) 2D Iso-Lines display mode

Iso-Lines are lines which connect points of equal height on the topography.

The following 2D Iso-Lines display modes can be set:

Intensity button: **Intensity** projection superimposed with colored iso-lines (lines of equal height).

Height button: Height function with black iso-lines.

Black button: White iso-lines on a black background.

- Click on the **Iso-Lines** button in the **Display** button bar.
  - The Iso-Lines display mode selected last is activated. At the same time, an additional button bar is displayed beside the **Iso-Lines** button permitting selection of the required Iso-Lines display mode.
  - Below the **Measure** button bar, the **Line Dist.** and **Line Offset** sliders / input boxes are displayed.
- Select the required Iso-Line display mode by clicking the left mouse button.

The additional function elements of the **Iso-Lines** display mode have the following meaning:

Line Dist. slider: Changes the distance of the iso-lines.

Line Offset slider: Setting of the height level where the Iso-Lines display starts.

☞ To apply the topography functions to a small portion of the Z Stack image use the **Overlay** function (**Overlay** button) and cut out and store as new topographic evaluation via the **Extract Region** function.

(3) 3D display

Topo animations are possible. The following 3D modes can be set:

Profiles button: Profile display.

Grid button: Grid display.

Filled button: Display of color shades.

Shaded button: Surface rendering. Can be combined with LUT. Topo animations are possible.

- Click on the **3D** button in the **Display** button bar.
  - The 3D display mode selected last is activated. At the same time, an additional button bar appears beside the **3D** button to permit selection of the required 3D display mode.
  - Below the **Measure** button bar, the **Scaling** button bar and the **Profile Dist**. and **Fill Level** sliders / input boxes are displayed.
  - The **Image Display** box contains one horizontal and two vertical scrollbars for the setting of the image viewing angle.
- Select the required 3D display mode with a click of the mouse.

The additional function elements of the **3D** display mode have the following meaning:

Profile Dist. slider: Setting of the distance of profiles and the mesh value of the grid.

Fill Level slider: Used to push through a color LUT Look Up Table (e.g.: if the **Rainbow** palette is used) in the **Profiles** / **Filled** display mode. In combination with the **Volume** button, the filling of the flood function level of the topography can be varied for volume measurements (see the **Measurement functions** paragraph).

The image viewing angle is set as follows:

Setting directly in the image

- Click in the image and hold down the mouse button. The perspective is changed by moving the mouse button in horizontal or vertical direction.

Setting via scrollbars

- Move the horizontal scrollbar to rotate the image around the vertical axis. The rotation angle is displayed in the yellow display box.
- Move the left vertical scrollbar to rotate the image around the horizontal axis. The rotation angle is displayed in the yellow display box.
- Moving the right vertical scrollbar enables you to expand the image in height or to compress it, while the Z-range between 10 % and 100 % of the X-range is scaled.

☞ You can set the x, y and Z scales to an identical ratio by opening a context menu in the **Image Display** box with a click of the right mouse button and selecting the **Metric equal ratio** function.

The displayed boxes for rotation angle and relative scaling percentage value z : x ratio permit the setting of identical perspectives for different images (e.g.: the plot of several topographies).

☞ The **Profiles** and **Filled 3D** display modes permit a color palette (e.g.: **Glowscale**, **Rainbow** or **User defined**) to be loaded or redefined by pressing the **Palette** button (see page 4-260).

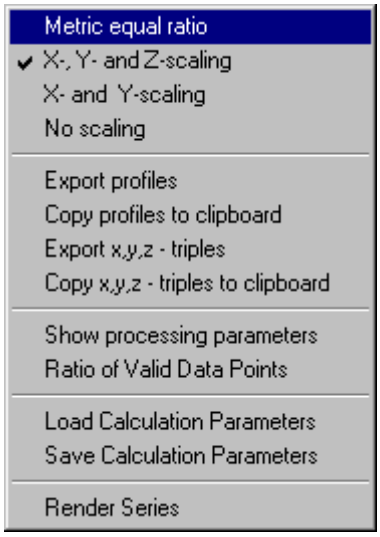


Fig. 4-292 Context menu of the 3D display mode (Profiles)

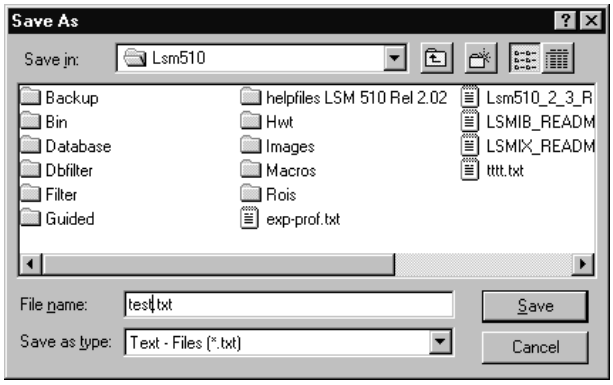


Fig. 4-293 Save As window

	X: 0.00	X: 10.55	X: 21.10	X: 31.65	X: 42.19
Y: 2.32	0.00	0.00	0.00	0.00	0.00
Y: 12.87	0.00	5.71	7.25	7.02	5.99
Y: 23.42	0.00	6.62	6.96	7.25	5.77
Y: 33.97	0.00	5.02	7.82	7.99	6.11
Y: 44.51	0.00	5.94	7.76	7.31	5.65

Fig. 4-294 Topography matrix

4.13.24.6 Context Menu of the 3D Display Mode

- Click in the **Image Display** box with the right mouse button to open the context menu.

The context menu for the 3D mode currently selected is displayed.

- Click on the required option with the left mouse button to execute the function.

(1) Metric equal ratio item

This option is available in all of the 3D display modes.

After activation of the function, the x, y and z scales are set to an identical ratio.

(2) Export profiles item

This option is available in the **Profiles** and **Grid** 3D display modes.

Use the function to save the **Profiles** or **Grid** data as a text file.

- Open the context menu with a click of the right mouse button, then click on the option **Export ...** with the left mouse button.
  - The **Save As** window is opened.
- Select the directory where you want the text file to be stored, enter a file name and click on **Save**.

A text file containing the topography in the form of an XYZ matrix is generated.

(3) Copy profiles to clipboard item

All 3D parameters shown right from the main 3D topo display window are copied in the clipboard.

(4) Export x,y,z- triples item

This option is available in the **Profiles** and **Grid** 3D display modes.

Use the function to save the **Profiles** or **Grid** data as a text file.

- Open the context menu with a click of the right mouse button, then click on the option **Export ...** with the left mouse button.
  - The **Save As** window is opened.
- Select the directory where you want the text file to be stored, enter a file name and click on **Save**.

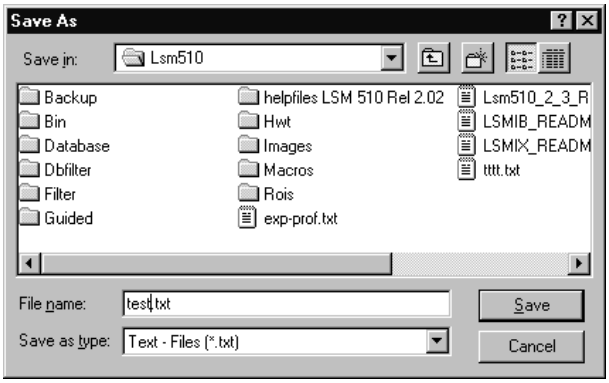


Fig. 4-295 Save As window

A text file containing the topography in the form of an XYZ table is generated.

X	Y	Z
0.00	12.87	0.00
10.55	12.87	5.71
21.10	12.87	7.25
31.65	12.87	7.02
42.19	12.87	5.99
52.74	12.87	5.19

Fig. 4-296 Topography table

(5) Copy x,y,z- triples to clipboard item

This option is available in the **Profiles** and **Grid** 3D display modes.

After selection of this option, the **Profiles** or **Grid** data are copied as an XYZ table to the clipboard and can be inserted in other programs using the **Paste** command.

☞ Please make sure that the amount of exportable data is adequate to the maximum importing size of the following software package. To lower the amount of data points, use the profile distance slider.

(6) Show processing parameters

After selection of the **Show processing parameters** function, a reporting of the following applied topo processing functionality is displayed on the right-hand side of the **Image Display** window:

- Mode (calculation mode: Max, Center, First)
- Threshold (applied intensity threshold)
- Filter
- Fit (plane, cylinder / sphere parameters)

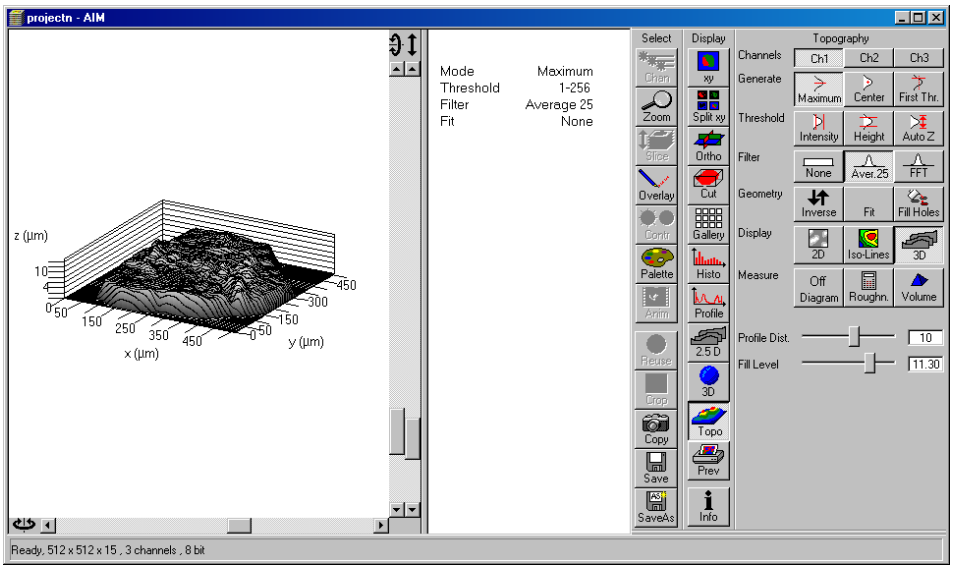


Fig. 4-297 Show processing parameters

(7) Ratio of valid data points

The ratio of valid data points (means signal intensities within a given intensity threshold) is displayed.

(8) Load Calculation Parameters

Topo routines can be loaded as tgp-files (TopoGraphic Parameters).

(9) Save Calculation Parameters

Topo routines can be saved and reloaded as tgp-files.

These files include settings for:

- reconstruction mode
- intensity threshold
- filters (including FFT)
- tilt angles (manual, 3 point fit)
- fit procedures (plane, cylinder, sphere)
- inverse
- fill holes

(10) Render properties item

This option is only available in the **Shaded** 3D display mode.

Use this function to vary the illumination conditions, reflection properties and projection settings of the topography. You can either select preset Shading Models or use parameters specifically defined as required.

The specifically defined parameters can subsequently be stored as a Shading Model and are then available at any time for further use. Shading Models can also be deleted if no longer needed.

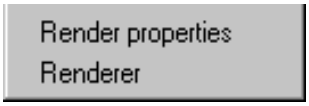


Fig. 4-298 Context menu of the 3D display mode (Shaded)

Load a Shading Model

- Open the context menu with a click of the right mouse button, then click on the option **Render properties** with the left mouse button.
  - The **3D Rendering** window is opened.
- Click on the name of the required model in the Shading Model List. The parameters are immediately set for the current topography.
- Click on **Close** to close the **3D Rendering** window again.

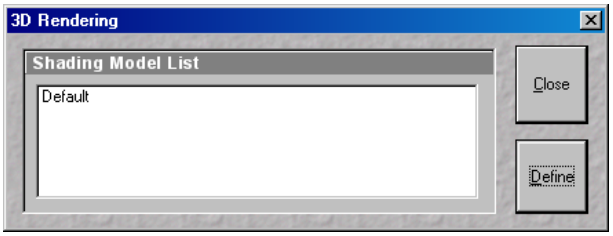


Fig. 4-299 3D Rendering window

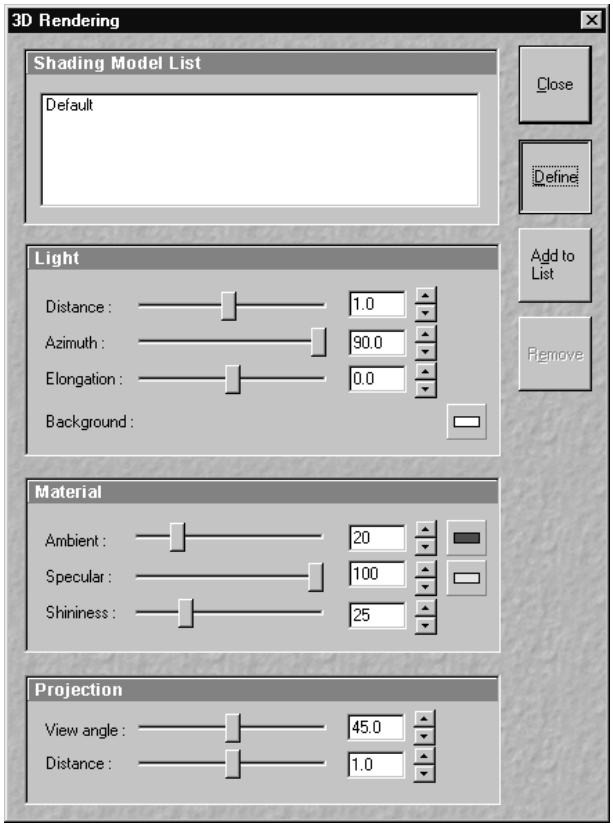


Fig. 4-300 3D Rendering window

Defining a specific Shading Model

- Open the **3D Rendering** window.
- Click on the **Define** button.
- Change the parameters of the topography using the appropriate sliders.
- Save the settings by clicking on the **Add to List** button. The **Add Shading Model to List** window is displayed.
- Enter a name for the model and click on **OK**. The model is included in the **Shading Model List**.

☞ See also **Appearance (Settings)**, page 4-296.

Light panel

Determines the properties of illumination on a sample.

Distance Goes for diffuse and **specular**, see visualization.

Azimuth See visualization. Rise angle of the "sun".

Elongation See visualization. North-south / east-west direction of the "sun".

Background Choose background color.

Material panel

Determines the reflective properties of a sample.

Ambient Specular Material properties; how many % of the light component are projected by the material into which spectral ranges.

Shininess Goes together with specular light. Shininess equal to 25 % determines diffuse light.

Projection panel

Determines the reflective properties of a sample.

View angle Determines the perspective, 0.0 parallel projection, central projection

Distance Zoom function, zoom in, zoom out

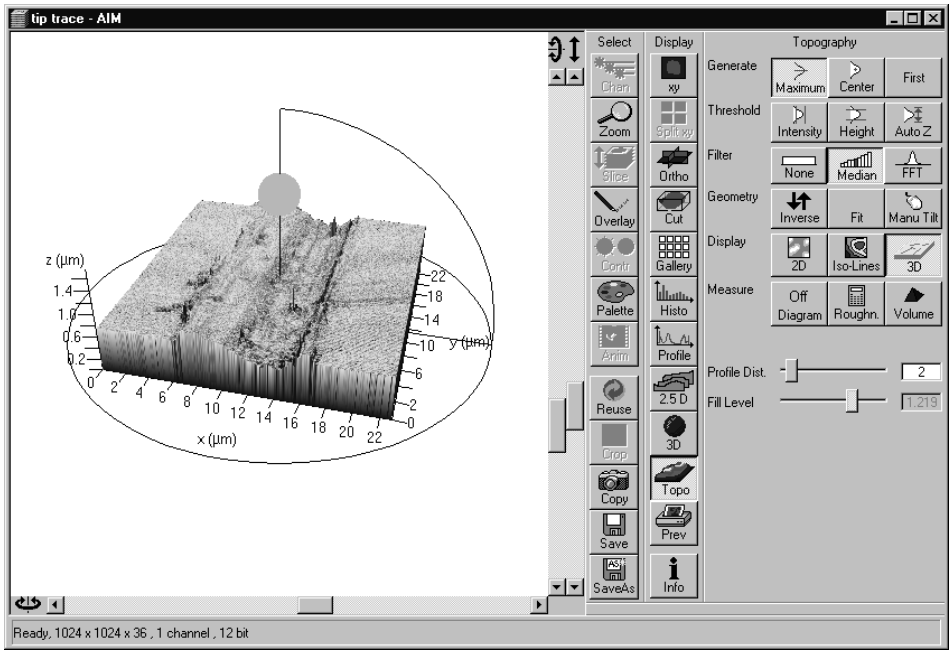


Fig. 4-301 Render function visualization aid

A zoomed rendering setting permits the zoomed section to be moved via the cursor keys after a click on the 3D window.

If a change of the 3D image angle follows, centration is made on the center again.

Deleting a Shading Model

- Select the model to be deleted in the **Shading Model List**, then click on the **Remove** button. The model is deleted.

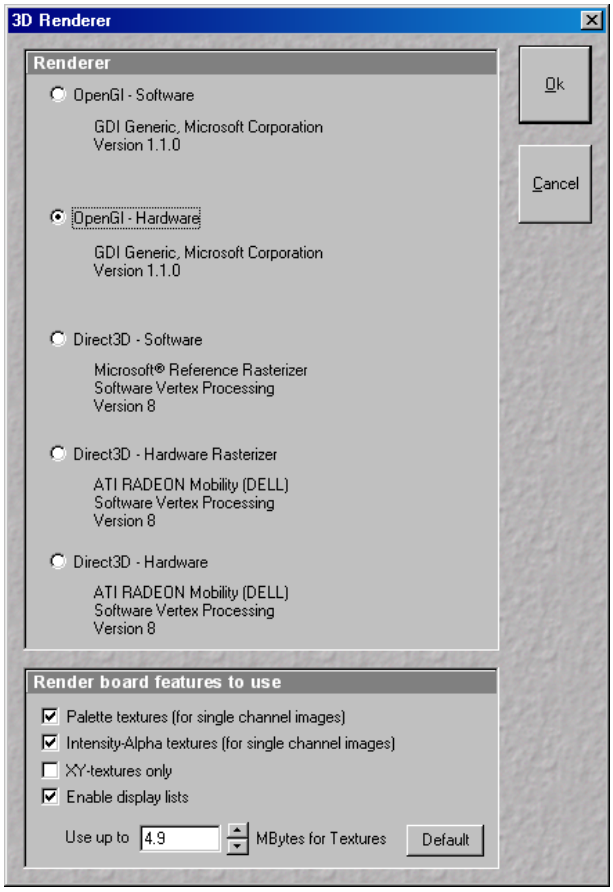


Fig. 4-302 3D Renderer window

(11) Renderer item

This option is only available in the **Shaded** 3D display mode.

After selection of the **Renderer** item, the **3D Renderer** window appears. It allows the selection of the hardware and software option which shall be used for the 3D graphics calculation.

OpenGl - Software

The graphics calculation is performed using the installed software.

OpenGl - Hardware

The graphics calculation is accelerated by using the installed graphics processor.

Direct3D – Software / Hardware Rasterizer / Hardware

These options can be used for offline versions of the LSM 5 software for PC's with the WINDOWS ® 2000 or XP operating system (not for WINDOWS ® NT).

4.13.24.7 Measurement Functions

The topography measurement functions are activated via the **Measure** button bar. The measurement functions can be performed in the 2D or 3D display mode.

Automated convention in height statistics analysis:

Topo Filters	None, median, <9x9	FFT High	FFT Low	>9x9
Data formats	**P**rimary profile	**R**oughness	**W**aviness	
2D profile	Pxx	Rxx	Wxx	n.a.
3D topography	PSxx	RSxx	SWxx	n.a.

The following measurement functions are available:

Diagram button: Diagram display. The **Profile**, **z Histo**, **Curve of tp** and **Grad. Histo** diagram display modes can be activated via the **Diagram** button and deactivated via the **Off Diagram** button. By activation of the **Diagram** button, an additional button bar is displayed for the selection of the required diagram or for deactivation. The labeling of the **Diagram** button changes depending on which diagram display mode has been activated.

Roughness button: Calculation of the roughness parameters.

Volume button: Calculation of the volume parameters.

(1) Profile measurement mode in 2D display

- Select the required 2D display of the stack via the **2D** button.
- Click on the **Diagram** button in the **Measure** button bar. Click on the **Profile** button in the button bar displayed afterwards.
  - The **Table** and **Profile** button bars are displayed below the **Measure** button bar.
  - A colored arrow (intersection line of the profile) is displayed in the image and the profile diagram appears below the image.
- If required, match the size of the **Image Display** window in order to obtain the complete display of the profile diagram.

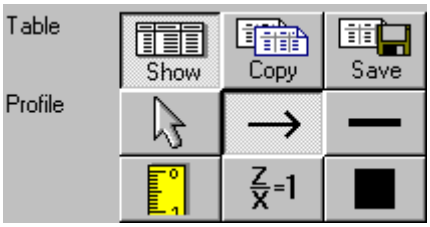


Fig. 4-303 Topography display: 2D - Profile

Additional Table / Profile buttons

The additional **Table** / **Profile** buttons have the following functions:

Show button: The profile is displayed in the form of a table at the bottom below of the **Image Display** window.

Copy button: The profile table is copied to the clipboard and can be transferred to other programs (MS Word or MS Excel) via the **Paste** function.

Save button: The profile table can be stored as a text file (ASCII).

Arrow (selection) button: Activation of the mouse button for selection, resizing or movement of the intersection line in the image.

Resizing: Click on the handle and hold down the mouse button, drag the handle, release the mouse button.

Movement: Click on the line and hold down the mouse button, move the entire intersection line, release the mouse button.

Line with arrow button (open arrow): Creation of the intersection line to define the position of the profile to be produced in the image. Click and hold the mouse button, drag the line in any required direction, release the mouse button to end the procedure. The profile diagram changes online.

Line button: This button allows you to determine the line thickness of the intersection line.

Measure button: Activates the **Profile measurement mode** in the profile diagram. The required tools are displayed to the right of the profile diagram (see **Profile measurement mode**, page 4-315).

z/x=1 button: Sets the **z/x** ratio in the profile diagram to the value **1**. Check: the following creation of a circle using the relevant tool really results in a circle in the profile display. Measured angle values correspond to the actual slope of the line displayed.

Color button: Clicking on the **Color** button opens a color selection box in which the color for the intersection line can be selected with a click of the mouse.

Activating the Profile measurement mode

If you click on the button, the **Profile** window with the tools of the profile measurement mode appears.

This window can be moved as required over the entire screen.

Fig. 4-304 Tools of the Profile measurement mode

Tools of the Profile measurement mode

The tools of the **Profile measurement mode** have the following functions:

Zoom button: Zooming of a section of the profile diagram. Click and drag a rectangle over the area to be enlarged in the profile diagram, release the mouse button to enlarge the selected area. The zoom function can be performed several times. A click with the right mouse button resizes the profile.

Marker button: Activation of the marker functions for the intersection line. The red and blue marker lines in the profile diagram can now be moved using the mouse. After movement of a marker line in the profile diagram, the relevant marker (red or blue circle) follows along the intersection line in the **2D** and **Iso-Lines** mode.

Arrow (selection) button: Activation of the mouse button for selection, resizing or movement of one of the following drawing elements in the profile diagram.

Resizing: Click on the handle and hold down the mouse button, move the handle, release the mouse button.

Movement: Click on the line and hold down the mouse button, move the entire drawing element, release the mouse button.

Inclined Line button: Creation of a straight line in the profile diagram. Display of distance, inclination angle, dxdy and dz. Click and hold down the mouse button, drag the line in any required direction, release the mouse button to end the procedure.

Free angle button: Creation of a free angle in the profile diagram. Display of the enclosed angle (max. 180 °). The first click sets the starting point, the second and third clicks define the angle and the end point.

Rectangle button: Creation of a rectangle in the profile diagram. Display of distance, area, height and width. Click and hold down the mouse button, drag the rectangle in any required direction, release the mouse button to end the procedure.

Open Polyline button: Creation of an open polyline figure in the profile diagram. Display of the length of the line figure. First click sets the starting point, any further click adds another line, click with the right mouse button ends the procedure.

Closed Polyline button: Creation of a closed polyline figure in the profile diagram. Display of the perimeter of the figure. First click sets the starting point, each further click adds another line, a click with the right mouse button closes the figure and ends the procedure.

Open free-hand curve button: Creation of an open Bezier figure in the profile diagram. Display of the length of the line figure. First click sets the starting point, each further click adds another line, a click with the right mouse button ends the procedure.

Closed free-hand curve button: Creation of a closed Bezier figure in the profile diagram. Display of the length of the line figure. First click sets the starting point, each further click adds another line, a click with the right mouse button closes the figure and ends the procedure.

Ellipse button: Creation of an ellipse in the profile diagram. Display of the area. First click sets the center point, the displayed line permits the determination of the first dimension, second click sets the first dimension, the second dimension and rotation direction can now be determined, third click sets the second dimension and direction and ends the procedure.

Circle button: Creation of a circle in the profile diagram. Display of radius and area. Clicking three times to define 3 points on the profile. A circle fit is automatically applied on the profile.

Recycle bin button: Deletes all drawing elements or the one just selected.

Line width button: Change of the line width of the drawing elements.

Color button: Clicking on the **Color** button opens a color selection box where the color of the drawing element can be selected with a click of the mouse.

x1- button: Resets the zoom factor of the profile diagram to its original size.

(2) z Histo measurement mode in 2D display

- Click on the **Diagram** button in the **Measure** button bar. Click on the **z Histo** button in the additional button bar now displayed.

The lower part of the **Image Display** box shows the 3D height distribution of the topography.

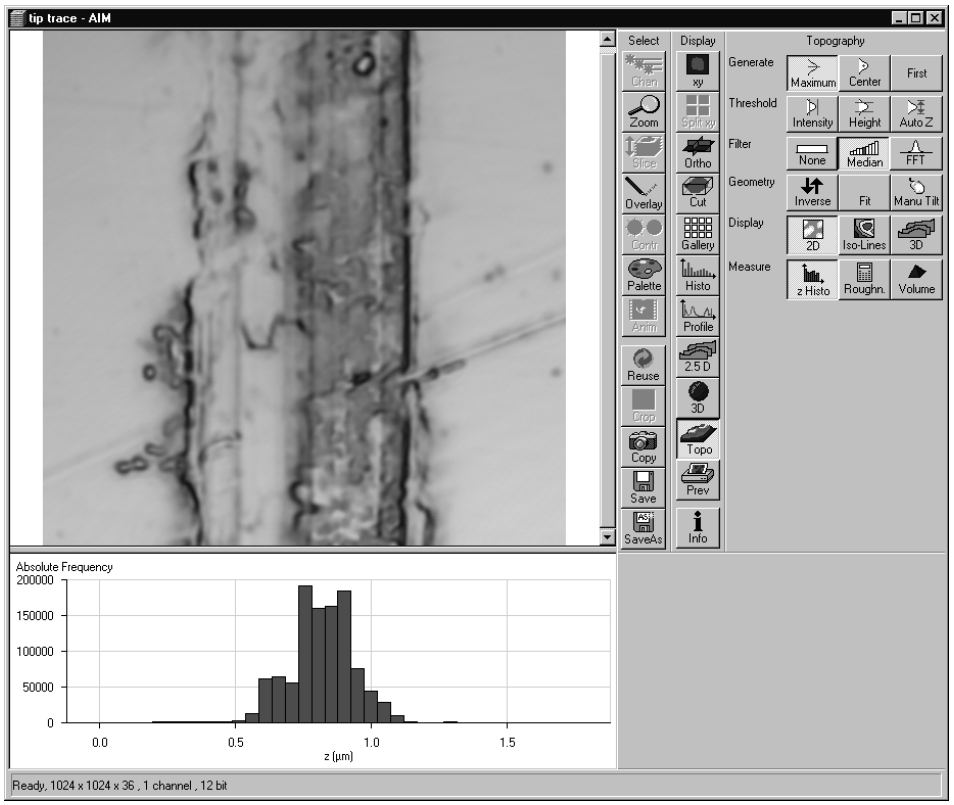


Fig. 4-305 Image Display window, Topography display: 2D - Histo

(3) Curve of tp measurement mode in 2D display

- Click on the **Diagram** button in the **Measure** button bar. Click on the **Curve of tp** button in the additional button bar now displayed.
  - The curve of the bearing area ratio as a function of the height is displayed below the image (also see **3D measurement functions**, page 4-325).

(4) Grad. Histo measurement mode in 2D display

- Click on the **Diagram** button in the **Measure** button bar. Click on the **Grad. Histo** button in the additional button bar now displayed.

The lower part of the **Image Display** box shows the gradient distribution of the topography. Before creation of the slope diagram, the image should be filtered at least once using a low-pass filter, since otherwise the rough height gradation of the image will result in a comb-shaped histogram. The Root-Mean-Square Slope (RMS Slope) parameter is calculated and displayed below the chart. The following formula is used for calculation:

$$R_{DQ} = \sqrt{\frac{1}{(N_x - 1)\cdot(N_y - 1)} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot \left\{ \left[\frac{z(x_i, y_j) - z(x_{i-1}, y_j)}{\Delta x} \right]^2 + \left[\frac{z(x_i, y_j) - (x_i, y_{j-1})}{\Delta y} \right]^2 \right\}}$$

(5) Roughness measurement mode in 2D display (Profile display)

- Click on the **Profile** button in the **Measure** button bar.
- Click on the **Roughn.** button in the **Measure** button bar.
  - The roughness parameters are calculated and displayed on the left below the image. All roughness parameters calculated from a 2D profile are named with **R**.
  - The **Copy** button is displayed below the right-hand side of the image. This button permits the roughness parameters to be copied to the clipboard and imported to another program (e.g.: MS Word or MS Excel) via the **Paste** function.

2D Amplitude parameters (Profile Roughness):

	Mean height z	Rc	Pc	Wc
Dispersion	Arithmetic mean deviation	Ra	Pa	Wa
	Root mean square deviation	Rq	Pq	Wq
Asymmetry	Skewness	Rsk	Psk	Wsk
Sharpness	Kurtosis	Rku	Pku	Wku
Extremes	Highest peak	Rp	Pp	Wp
	Lowest valley	Rv	Pv	Wv
	Absolute peak to valley	Rt	Pt	Wt
	Averaged peak to valley	Rz	Pz	Wz
	Maximum peak to valley	Rmax	Pmax	Wmax
If chosen filters are		FFT High	No, M	FFT L

Calculation of roughness parameters

The following roughness parameters are calculated (e.g. for a Y-section)

– Mean height of all profile height values R_c

$$- R_c = \frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot z(x, y_j)$$

N_x, N_y ... number of pixels in X- or Y-direction

Arithmetic mean deviation of all profile height values R_a

$$- R_a = \frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot \left[z(x, y_j) - R_c \right]$$

– Quadratic mean deviation of all profile height values R_q

$$- R_q = \sqrt{\frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot \left[z(x, y_j) - R_c \right]^2}$$

– Skewness of the distribution of all profile height values R_{SK}

$$R_{SK} = \frac{1}{N_y \cdot R_q^3} \cdot \sum_{j=1}^{N_y} \cdot z^3(x, y_j)$$

– Kurtosis of the distribution of all profile height values R_{KU}

$$R_{KU} = \frac{1}{N_y \cdot R_q^4} \cdot \sum_{j=1}^{N_y} \cdot z^4(x, y_j)$$

– Maximum peak height R_P

$$R_P = z_{\max} - R_c$$

– Maximum valley depth R_V

$$R_V = R_c - z_{\min}$$

– Maximum roughness depth R_t (= Peak to Valley / PV)

$$- S_t = z_{\max} - z_{\min}$$

maximum height difference of the overall topography along a profile.

Classification of topography in 5 equal area elements (rectangles in the 2D mode)

– average roughness depth R_z:

$$- R_z = \frac{z_{\max 1} - z_{\min 1} + z_{\max 2} - z_{\min 2} + z_{\max 3} - z_{\min 3} + z_{\max 4} - z_{\min 4} + z_{\max 5} - z_{\min 5}}{5}$$

Averaging of R_t-values of all the 5 single area elements. When combined, both parameters provide information about the homogeneity of the surface. Big differences are indicative of pronounced inclination of the overall area or of spikes.

Developed Surface Area Ratio: Σ (surface area$_{ij}$) / Σ (projected area$_{ij}$)

The percentage of the 3D surface area (sum off all triangles formed by adjacent data points) to the 2D surface area produced by projecting the 3D surface onto the threshold plane.

– maximum roughness depth R_{max}:

$$- R_{\max} = Max\ (z_{\max 1} - z_{\min 1},\ z_{\max 2} - z_{\min 2},\ z_{\max 3} - z_{\min 3},\ z_{\max 4} - z_{\min 4},\ z_{\max 5} - z_{\min 5})$$

maximum of R_t-values of all the 25 single area elements.

☞ Both the roughness parameters and the z-histogram can be changed by using filters!

(6) Roughness measurement mode in 3D display

- Click on the **Roughn.** button in the **Measure** button bar.
  - The roughness parameters are calculated and displayed on the left below the image. All roughness parameters calculated from a 3D topography are named with **S**.
  - The **Copy** button is displayed below the right-hand side of the image. This button permits the roughness parameters to be copied to the clipboard and imported to another program (e.g.: MS Word or MS Excel) via the **Paste** function.

3D Amplitude parameters (Topography Roughness):

	Mean height z	RSc	PSc	WSc
Dispersion	Arithmetic mean deviation	RSa	PSa	WSa
	Root mean square deviation	RSq	PSq	WSq
Asymmetry	Skewness	RSsk	PSsk	WSsk
Sharpness	Kurtosis	RSku	PSku	WSku
Extremes	Highest peak	RSp	PSp	WSp
	Lowest valley	RSv	PSv	WSv
	Absolute peak to valley	RSt	PSt	WSt
	Averaged peak to valley	RSz	PSz	WSz
	Maximum peak to valley	RSmax	PSmax	WSmax
If chosen filters are:		FFT High	No, M	FFT L

Calculation of roughness parameters

The following roughness parameters are calculated:

– Mean height of all surface height values S_c

$$-RS_c = \frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot z(x_i, y_j)$$

N_x, N_y ... number of pixels in X- or Y-direction

– Arithmetic mean deviation of all surface height values RS_a

$$-RS_a = \frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot \left[z(x_i, y_j) - RS_c \right]$$

– Quadratic mean deviation of all surface height values RS_q

$$-RS_q = \sqrt{\frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot \left[z(x_i, y_j) - RS_c \right]^2}$$

– Skewness of the distribution of all surface height values RS_{SK}

$$RS_{SK} = \frac{1}{N_x \cdot N_y \cdot RS_q^3} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot z^3(x_i, y_j)$$

– Kurtosis of the distribution of all surface height values S_{KU}

$$RS_{KU} = \frac{1}{N_x \cdot N_y \cdot RS_q^4} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot z^4(x_i, y_j)$$

– Maximum peak height RS_p

$$RS_p = z_{\max} - RS_c$$

– Maximum valley depth S_V

$$RS_V = RS_c - z_{\min}$$

– Maximum roughness depth RS_t (= Peak to Valley / PV)

$$-RS_t = z_{\max} - z_{\min}$$

maximum height difference of the overall topography.

Classification of topography in 25 equal area elements (rectangles in the 2D mode)

– average roughness depth S_z:

$$-RS_z = \frac{z_{max1} - z_{min1} + z_{max2} - z_{min2} + \cdots + z_{max25} - z_{min25}}{25}$$

Averaging of R_t-values of all the 25 single area elements. When combined, both parameters provide information about the homogeneity of the surface. Big differences are indicative of pronounced inclination of the overall area or of spikes.

– maximum roughness depth RS_{max}:

$$-RS_{max} = Max\ (z_{max1} - z_{min1},\ z_{max2} - z_{min2},\ \cdots,\ z_{max25} - z_{min25})$$

maximum of R_t-values of all the 25 single area elements.

☞ Both the roughness parameters and the z-histogram will be influenced by the use of filters!

4.13.24.8 3D Measurement Functions

(1) Volume measurement mode (Flood function)

- Use the 3D button to select the required 3D display of the stack.
- Click on the **Volume** button in the **Measure** button bar.
  - The volume parameters are calculated and displayed below the image.
  - The **Copy** button is displayed below the right-hand side of the image. This button permits the volume values to be copied to the clipboard and imported to other programs (e.g.: MS Word or MS Excel) via the **Paste** function.
- Setting the **Fill Level** slider enables you to change the height level of the topography. The portion of the topography lying below the set height level is filled with "water" (blue color) and the volume parameters are calculated online only for the projecting part of the topography.

☞ To use the **Fill Level** function, load the **Profiles** 3D display mode containing the **Glowscale** palette, or activate **No Palette** to obtain optimum display.

- If the **Diagram** function **Curve of tp** is also activated, a red marker line shows the position of the height level in the percentage of contact area curve.

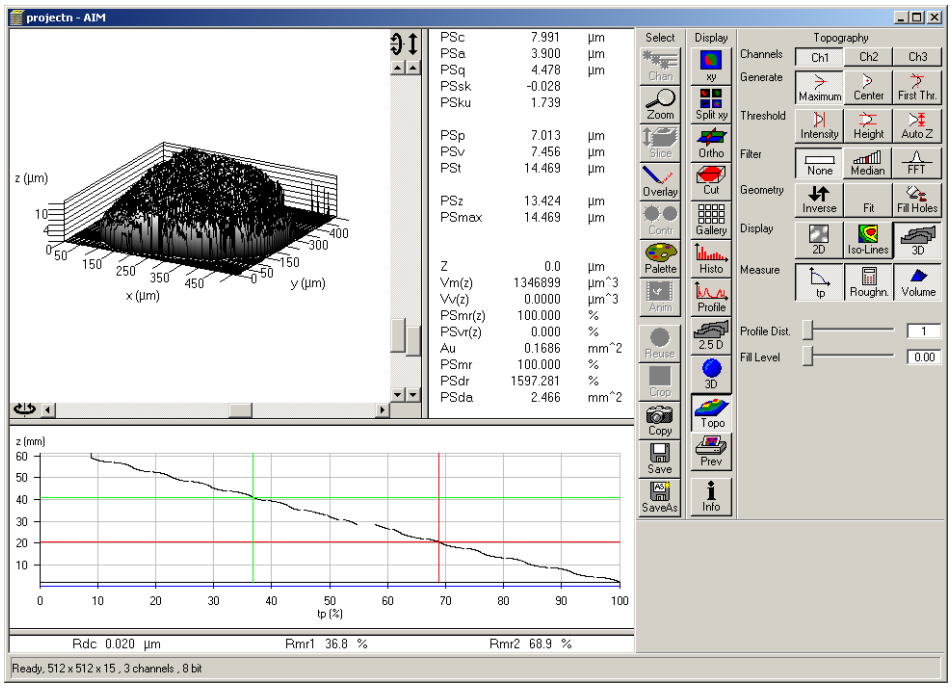


Fig. 4-306 Image Display window, Topography display: 3D - Volume

Parameters

The following parameters are calculated:

Z: height level (selectable with the **Z-Threshold** and **Fill Level** sliders). The setting of this value influences the following parameters.

Vm (z): material volume above chosen height level

Vv (z): void volume below chosen height level

Smr (z): material volume ratio

$$S_{mr}(z) = \frac{V_m(z)}{V_m(z_{\min})}$$

Svr (z): void volume ratio

$$S_{vr}(z) = \frac{V_v(z)}{V_v(z_{\max})}$$

Au: surface bearing area of the topography at Z (= projection area of those parts which are situated above chosen height level)

Smr: surface bearing area ratio of the topography at Z

percentage of contact area (= Au / (x * y) * 100 %)

Sda: true surface = sum of all triangles formed by adjacent data points of the surface reconstruction

Sdr: developed surface area ratio:

Σ (surface areaij) - Σ (projected areaij) / Σ (projected areaij) * 100 %

projected area = x * y

The percentage of the 3D surface area (sum of all triangles formed by adjacent data points of the surface reconstruction) to the 2D surface area produced by projecting the 3D surface onto the threshold plane.

absolute flat surface ⇒ is equal to base plane (Sdr = 0 %)

The increase by which the 3D surface is larger than the basic plane (e. g. 625 % is a 3D surface which is about 6.25 times larger than the projected basic plane)

(2) Profile measurement mode in 3D display

This function is performed in the same way as in the 2D display mode, with the following exceptions:

The buttons and are replaced with the buttons and . Furthermore, the **Position** slider and the input box (information of the position of the intersection line in pixels) are displayed below the **Table** and **Profile** button bar. Changing the Z-Threshold also results in a change in the profile. In the 3D image, a red marker line shows the y- and x-position of the displayed profile diagram.

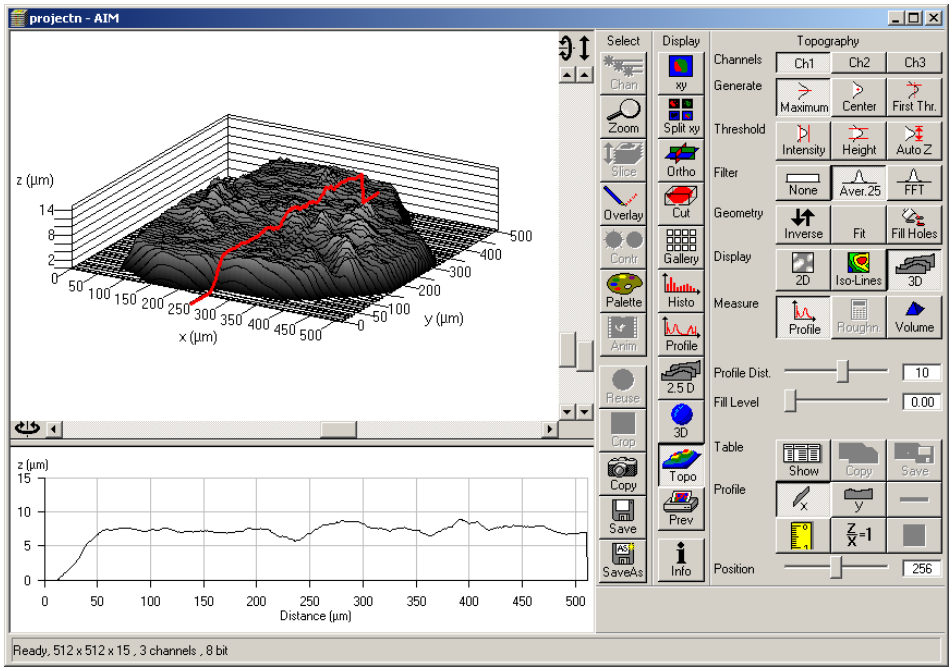


Fig. 4-307 Image Display window, Topography display: 3D - Profile

- The position of the marker line (profile intersection line) can be changed by moving the **Position** slider in x or y.
- Press the **x**- or **y**-button to select the required intersection plane.

(3) z Histo measurement mode in 3D display

This function is performed in the same way as in the 2D display mode.

(4) Curve of tp measurement mode in 3D display

This function is performed in the same way as in the 2D display mode.

Before determination of the tp bearing portion, individual peaks (noise, steep slopes) must be eliminated. The **Median** filter or a **3x3** longpass filter can be used for this purpose.

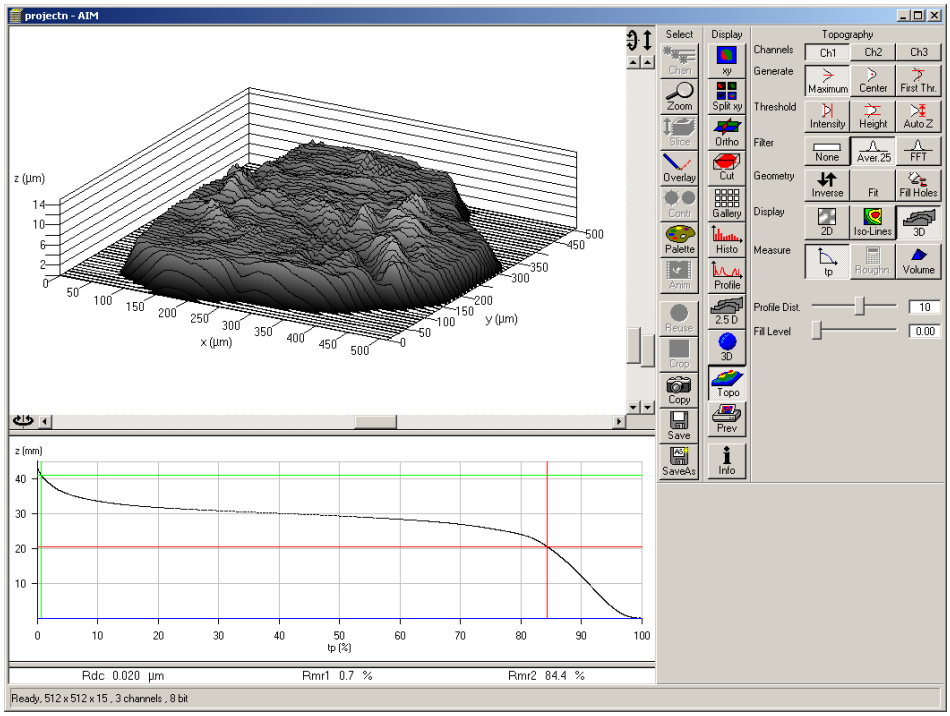


Fig. 4-308 Image Display window, Topography display: 3D – Curve of tp

Shifting the two cursor crosses permits two bearing portions to be given in percent (e.g. Smr1 = 10 %; Smr2 = 90 %) as default values for which the height difference Rdc is determined automatically.

(5) Grad. Histo measurement mode in 3D display

This function is performed in the same way as in the 2D display mode.

(6) Roughness measurement mode in 3D display

This function is performed in the same way as in the 2D display mode.

4.13.24.9 Export Data

- multiple profiles (Rel. 3.2)
- single profile
- parameters
- topography as matrix
- topography as triples

4.13.24.10 Topo ReUse

Topo routines can be saved and reloaded as tgp-files (TopoGraphic Parameters).

These files include settings for:

- reconstruction mode,
- intensity threshold,
- filters (including FFT),
- tilt angles (manual, 3 point fit),
- fit procedures (plane, cylinder, sphere),
- inverse and
- fill holes.

4.13.25 Display - Prev.

This function allows to

- compose images, graphs and text for printing
- use any image format
- change fonts and line width in graphs via context sensitive menus

The settings of Chan, Zoom, Slice, Contr and Palette apply.

In the **Options** menu in the function **Settings** with the tab **Print Status Display** parameters are determined and the **Print Status Information** is activated/deactivated.

Click on **Prev** will display the **Preview** window and the **Print** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

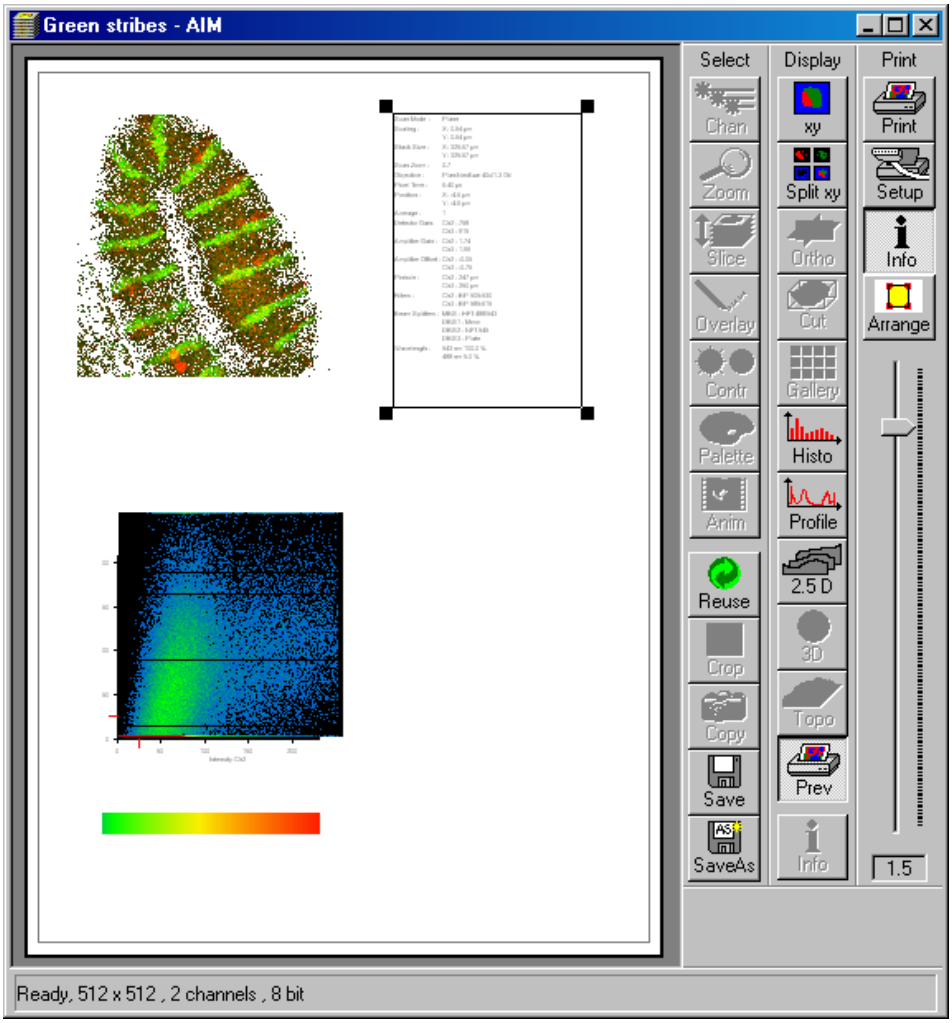


Fig. 4-309 Image Display window, Prev display with Assembly of image, intensity profile and scan info

4.13.25.1 Context Menu for Scan Information Text

The context menu (right mouse button) allows to vary the output of the **scan info**.

- Click with the right mouse button. A context menu with the options **Color** and **Font** is displayed.
- In the **Color** menu, you can select a different type color for the **scan info**, in the **Font** menu a different type font and type style.

4.13.25.2 Context Menu for Topography Images

When transferring a topography to the print preview, you can change the size and shape of type and scale lines for the 3D graphics and profile measurement results.

(1) Context menu for 3D graphics

- Click on the right mouse button. A context menu with the options **Font enlargement** and **Line width enlargement** is displayed.
- You can change the type size in the **Font enlargement** menu and the line width of the scales in the **Line width enlargement** menu.

(2) Context menu for Profile measurement function

- Click on the right mouse button. A context menu with the options **Scaling font enlargement**, **Marker font enlargement** and **Overlay font enlargement** is displayed.
- You can change the type font in the **Scaling font enlargement** menu, the size of the marker table in the **Marker font enlargement** menu and the type size of the red measurement results in the **Overlay font enlargement**.

4.13.25.3 Arranging and Printing the Print Preview

- Click on the **Arrange** button for optimum layout of image size and position relative to the textual information.
- A layout generated with **Prev.** (Preview) can be printed by clicking on the **Print** button in the **Print** toolbar.
- Clicking on the **Setup** button opens the **Print Setup** window, in which you can specify print parameters.
- Click on the slider to change the zoom value of the selected items.

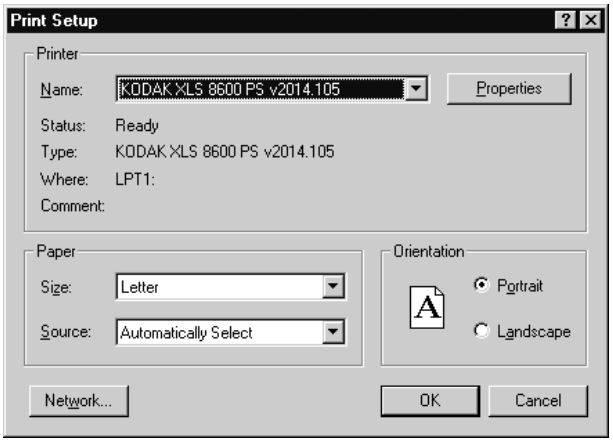


Fig. 4-310 Print Setup window

4.13.26 Display - Info

This function allows to

- display the parameters used during image acquisition of the image(s) displayed in the **Image Display** window
- use any image format
- remove the info display

The settings of **Chan, Zoom, Slice, Contr** and **Palette** are not relevant for this function.

In the **Options** menu in the function **Settings** with the tab **Image Status Display** parameters to shown are determined.

Click on **Info** will show the parameters. Click again to hide the info display.

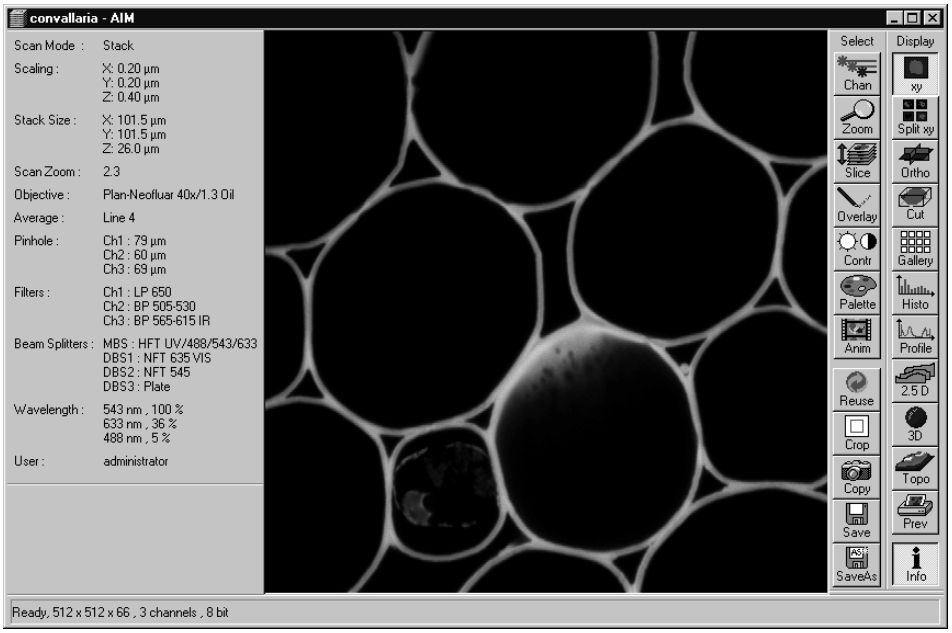


Fig. 4-311 Image Display window, Info display

4.13.27 Additional Display Mode in Time Series

4.13.27.1 Display - Mean

This function allows to

- display the intensity time diagram (mean intensity in user defined ROIs over time)
- display the intensity time diagram for volumes (3D ROIs) within a Z-Stack over time
- use frame time series and frame Z Stack time series as input
- show the intensity values in table form and copy table to clipboard or save as text file
- show separate diagrams for each channel in a multi channel image

The settings of **Chan**, **Zoom**, **Slice**, **Contr** and **Palette** apply.

Click on **Mean** will display the **Mean of ROIs** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

To use a similar functionality while scanning use the optional Mean of ROI function with the Time series control.

- Click on the **Mean ROI** button.
  - The **Mean of ROIs** image display toolbar will be displayed on the right. The used ROIs become visible in the image, and the Intensity-Time diagram is shown on the left of the **Image Display** window.

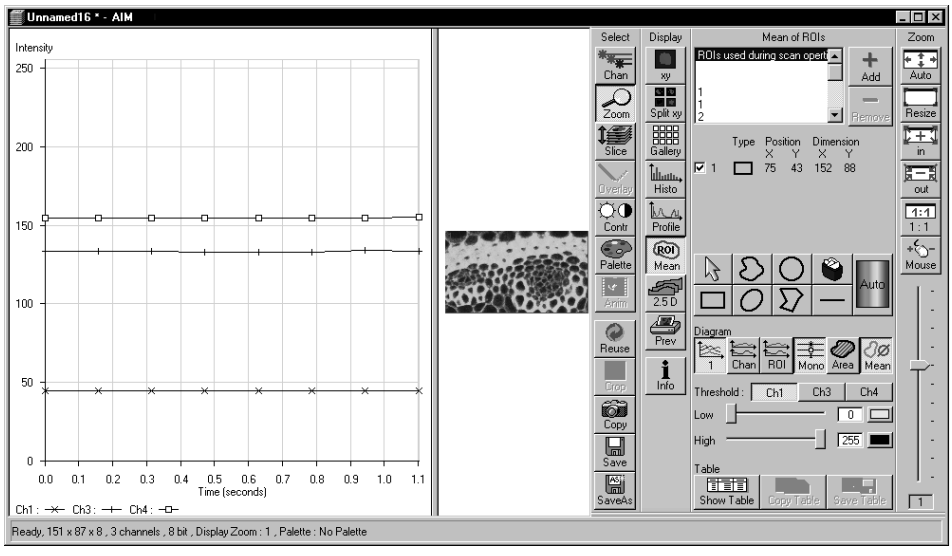


Fig. 4-312 Image Display window, Mean ROI display for time series in single plane

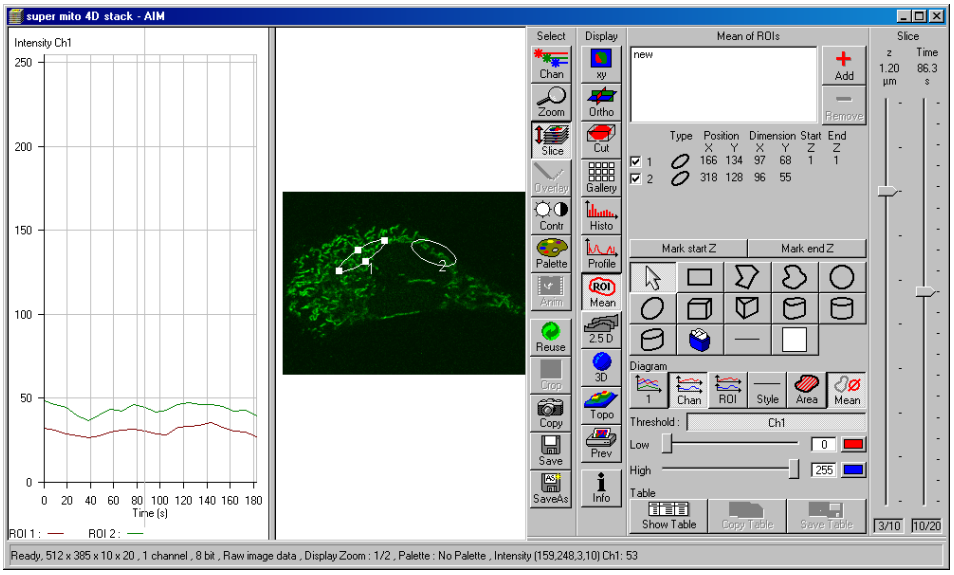
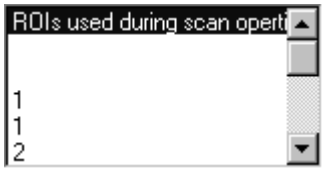


Fig. 4-313 Image Display window, Mean ROI display for time series of Z Stack

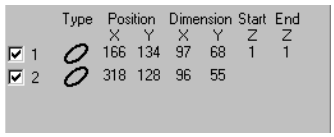
The **Mean of ROIs** toolbar contains the following function elements:



ROIs selection box: Display of the ROIs used during scanning of the time series and of the other ROIs available in the system.

Add button: Opens the **Add ROI List** window for the storage of changed or newly defined ROIs under a new name.

Remove button: Deletes the selected ROI from the ROIs selection box.



ROI data: Display of the data of the ROI selected from the ROIs selection box. On deactivation of the check box of a ROI, its intensity values from the Intensity-Time diagram are not displayed.

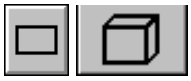
The **Mark start Z** button and **Mark end Z** button are only available for Time series with Z- Stacks when a three dimensional (for example 3D Bezier) drawing tool has been used to draw a ROI and this is still activated in the image. When clicked the actually displayed image will be set as the upper or lower border of the ROI within the stack in Z. The displayed image of the Z-Stack can be changed using the **Slice** toolbar in the Select toolbar of the image window.

Arrow button: Activation of the mouse button for resizing or movement of the ROI in the **Image Display** window.

Bezier and **3D Bezier** button: Activates the Bezier figure drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.

Circle and 3D Circle button: Activates the circle drawing mode. Clicking and holding down the mouse button sets the center point; drag the diameter and release the mouse button to end the procedure.

Recycle bin button: All the ROIs to the image are deleted.



Rectangle and 3D Rectangle button: Activates the rectangle drawing mode. Click and hold down the mouse button, drag the rectangle in any direction, release the mouse button to end the procedure.

Ellipse and **3D Elipse** button: Activates the ellipse drawing mode. The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and the rotation direction can then be determined; the third click sets the second dimension and the direction and ends the procedure.

Polyline and **3D Polyline** button: Activates polyline drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.

Line button: This button allows you to determine the line thickness of the ROI outline.

Color / Auto button: One color from the list of colors can be assigned to all ROIs. When **Auto** is pressed, the outlines of all ROIs are automatically colored differently.

Buttons for diagram display:

- **1** button: Intensity values for ROIs and channels are shown in one diagram.
- **Chan** button: Intensity values are shown separately for each channel.
- **ROI** button: Intensity values are shown separately for each ROI.
- **Mono** button: Change between color and monochrome display of the intensity time diagrams.
- **Area** button: Display of the area of the ROI in the intensity time diagram, depending on the set threshold values. Area measurements of very small areas (< 10 pixels) give only approximate values.
- **Mean** button: Display of the mean values of the relevant ROI in the intensity time diagram.

Ch1 / Ch3 / Ch4 button: Selection of the channel to be used.

Threshold low slider: The intensity values below threshold are not displayed for the **Area** function.

Threshold high slider: The intensity values above threshold are not displayed for the **Area** function.

Buttons for Table functions:

- **Copy Table** button: The table of intensity values is copied to the clipboard.
- **Show Table** button: The table of intensity values is displayed on the bottom left of the Image Display window.
- **Save Table** button: The table of intensity values can be stored as a text file.

4.13.28 Additional Display Modes in Lambda Mode

The following display modes of a Lambda Stack are available:

- Display - Coded
- Display - Max
- Display - Mean

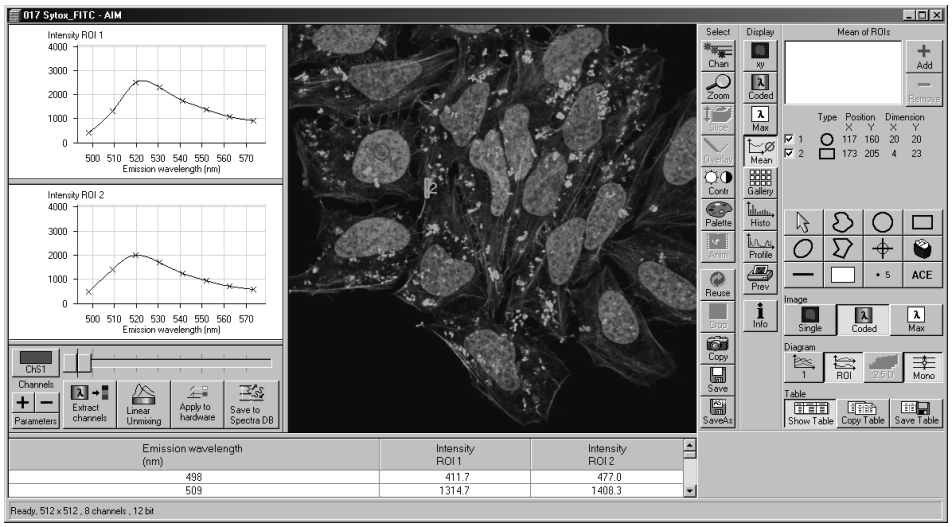


Fig. 4-314 Image Display window of a Lambda Stack, Display - Mean activated

4.13.28.1 Display - Coded

This function allows to

- display a Lambda Stack of images in a coded color view. A color palette is automatically assigned to the individual images which displays the spectral color of the most intense signal of a Lambda Stack

The settings of **Chan, Zoom, Slice** and **Contr** apply. The settings of **Palette** do not apply.

Click on **Coded** will be immediately effective.

4.13.28.2 Display - Max

This function allows to

- display a Lambda Stack of images in a maximum intensity projection.

The settings of **Chan**, **Zoom**, **Slice**, **Contr** and **Palette** apply.

Click on **Max** will be immediately effective.

4.13.28.3 Display - Mean

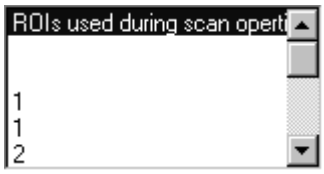
This function allows to

- display the intensity Lambda diagram (mean intensity in user defined ROIs over Lambda)
- use Lambda Stack, Lambda Stack time series, Lambda Stack Z series and Lambda Stack (Z+time) series as input
- show the intensity values in table form and copy table to clipboard or save as text file
- show separate diagrams for each ROI or all in one diagram
- generate unmixed multi channel images

The settings of **Chan**, **Zoom**, **Slice**, **Contr** and **Palette** apply. The settings of **Overlay** do not apply.

Click on **Mean** will display the **Mean of ROIs** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

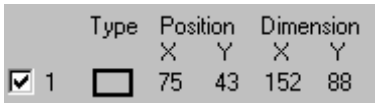
The **Mean of ROIs** toolbar contains the following function elements:



ROIs selection box: Display of the ROIs used during scanning of the time series and of the other ROIs available in the system.

Add button: Opens the **Add ROI List** window for the storage of changed or newly defined ROIs under a new name.

Remove button: Deletes the selected ROI from the ROIs selection box.



ROI data: Display of the data of the ROI selected from the ROIs selection box. On deactivation of the check box of a ROI, its intensity values from the Intensity-Time diagram are not displayed.

Arrow button: Activation of the mouse button for resizing or movement of the ROI in the **Image Display** window.

Bezier button: Activates the Bezier figure drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.

Circle button: Activates the circle drawing mode. Clicking and holding down the mouse button sets the center point; drag the diameter and release the mouse button to end the procedure.

Recycle bin button: All the ROIs to the image are deleted.

Rectangle button: Activates the rectangle drawing mode. Click and hold down the mouse button, drag the rectangle in any direction, release the mouse button to end the procedure.

Ellipse button: Activates the ellipse drawing mode. The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and the rotation direction can then be determined; the third click sets the second dimension and the direction and ends the procedure.

Polyline button: Activates polyline drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.

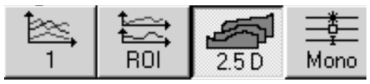
Crosshair button with user-defined spot size for Lambda Stacks.

Line button: This button allows you to determine the line thickness of the ROI outline.

Color / Auto button: One color from the list of colors can be assigned to all ROIs. When **Auto** is pressed, the outlines of all ROIs are automatically colored differently.

Buttons for Image display:

- **Single** button: Displays only a single slice out of a Lambda Stack.
- **Coded** button: Displays the Lambda Coded mode.
- **Max** button: Displays the Lambda **Max** mode.



Buttons for diagram display:

- **1** button: Intensity values for ROIs and channels are shown in one diagram over Lambda.
- **ROI** button: Intensity values are shown separately for each ROI over Lambda.
- **2.5 D** button: 3D graph of intensity, wavelength and time of ROIs in Lambda Stacks.
- **Mono** button: Change between color and monochrome display of the intensity time diagrams over Lambda.

Buttons for Table functions:

Copy Table button: The table of intensity values over Lambda is copied to the clipboard.

Show Table button: The table of intensity values over Lambda is displayed on the bottom left of the Image Display window.

Save Table button: The table of intensity values over Lambda can be stored as a text file.

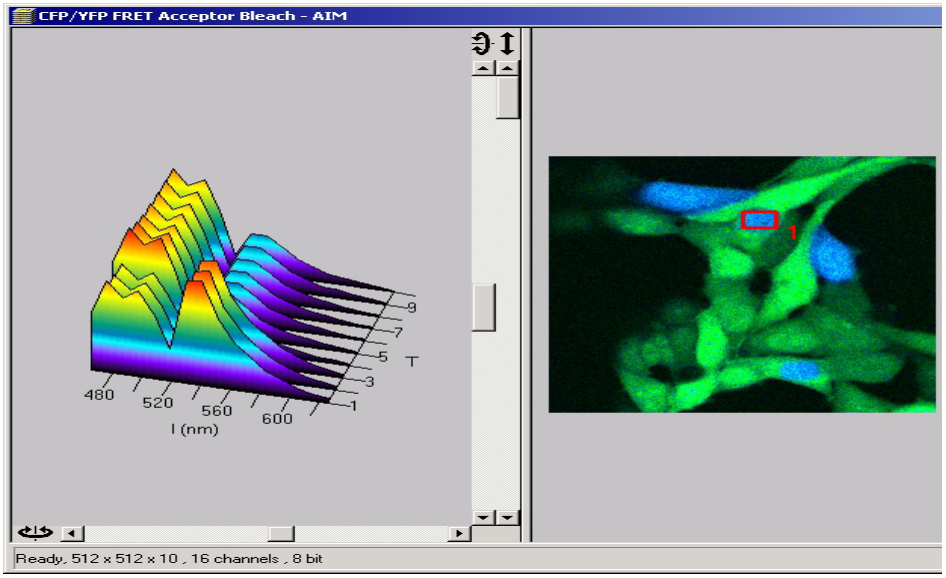


Fig. 4-315 3D graph

The toolbar below the intensity Lambda diagram has the following functions:

ChS1 button / slider — Selection of a wavelength range (use the sliders) assigned with the spectral channel ChS1. This assignment can be used for scanning (when **Apply to hardware** is pressed, see **Config Control**) and /or for extracting a single or multichannel image from the Lambda Stack (when **Extract channels** is pressed). Clicking on the color box in the ChS buttons opens the color selection box and allows a new color assignment to be made for this channel.

Channel + / – button — Add (+) or reduce (-) the number of channels displayed and available for assignment. Up to 8 channels labeled ChS1, ChS2, ChS3 ... ChS8 are available.

Extract channels button — Generates a new image or multicolor images based on the settings made in the ChS1, ChS2, ChS3 ... ChS8 buttons. Two or more Lambda channels are binned to form the channels ChS1 ... 8. The generated image is displayed in a new **Image Display** window and is no longer a Lambda Stack of images.

Linear Unmixing button — Performs the unmixing with the selected spectra.

Apply to hardware button — Sets the parameters for the META detector used in binning mode as specified in the settings ChS1, ChS2, ChS3 ... ChS8. See **Single Track** and **Multi Track** in **Config Control** for the effect of the action. The **Lambda Stack** setting is not affected by this action.

Save to spectra DB button — The values of the intensity Lambda diagram are saved in a file (extension: *.rls) in the aim/macro/roi directory. Make sure to have displayed only data of single dyes in the intensity Lambda diagram when executing this function.

4.13.28.4 Functionality for Emission Fingerprinting

Extract Channels panel

- Sum signals from selected detector elements
- Mean signals from selected detector elements
- Autoscaling of output channels (individually)

Linear Unmixing panel

- Autoscaling of output channels
- Generate an additional "Channel with Residuals"-> pixel-by-pixel display of the difference between fit and original data (for the channel of the Lambda Stack that shows the greatest deviation)
- Advanced Linear Unmixing ignored negative results and repeats the calculation.
- Ask for ROI characterizing background spectra before Linear Unmixing
- Ignore Overexposed and Underexposed Pixels. Those wont be considered for the calculation.

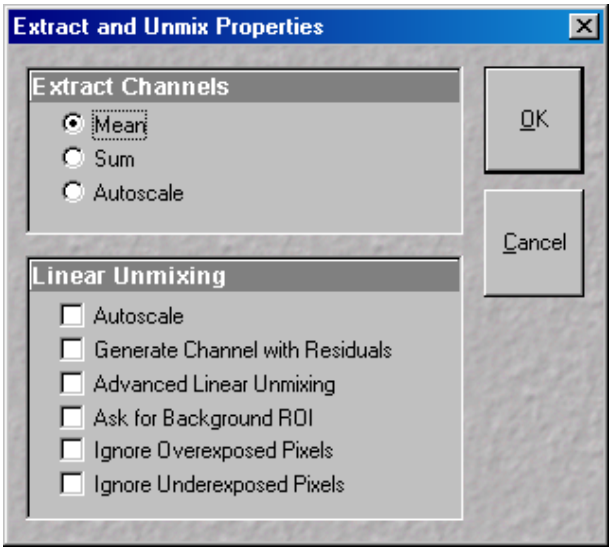


Fig. 4-316 **Extract and Unmix Properties window**

Context menu

- **Spectra Display** with option **Normalize** (right mouse click into spectra graph)

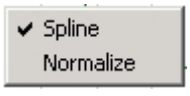


Fig. 4-317 **Context menu with Normalize function**

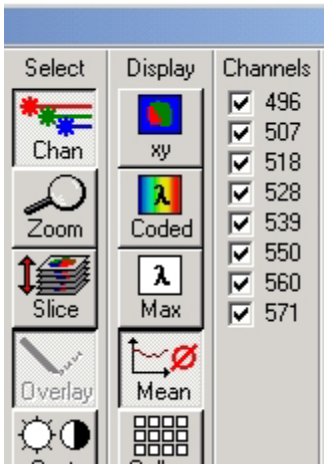


Fig. 4-318 Channels toolbar

- Deselect of individual channels in Lambda Stack affects:
  - Display in **Lambda Coded** and **Lambda max** modes, if **Chan** button in **Select** toolbar activated
  - Linear unmixing; deselected channels are not included

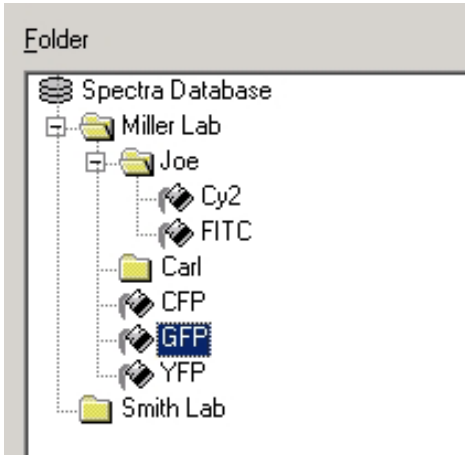


Fig. 4-319 Subfolder of Spectra Database

- Spectra Database with subfolders

4.13.28.5 Automatic Component Extraction

The use of this function permits the automatic search for the individual reference spectra in a lambda stack.

(1) Open / Close the Mean of ROI window

- Click on the **Mean of ROI** button in the image display window to open the **Mean of ROI** window.
- Click on another button to select a different function and to close the **Mean of ROI** window.

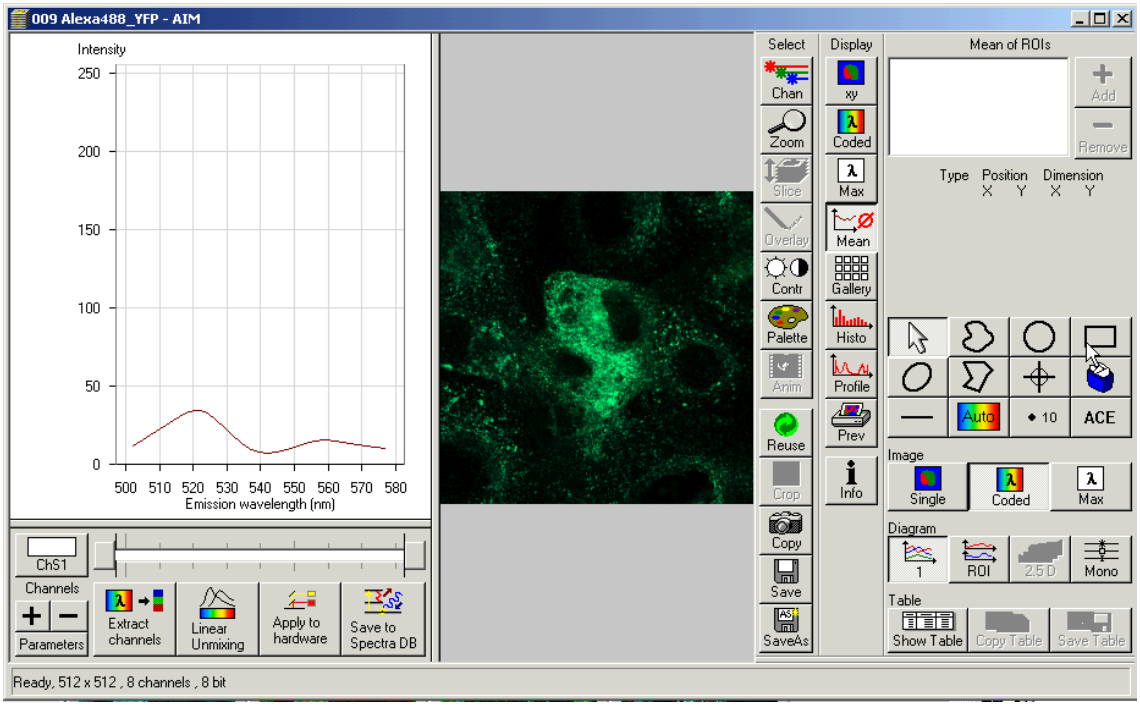


Fig. 4-320 Image Display window of a Lambda Stack, Display - Mean activated

(2) Function description

ACE button	Opens the component number window; after selecting an appropriate number of labels in your sample, press OK.
Spectra checkboxes	Select required spectra. ACE generated spectra and interactively selected spectra can be mixed here.
Drawing Tools	For interactive search and selection of spectra in the image display window.
Linear Unmixing button	Performs the unmixing with the selected spectra. The resulting images are colored according to the color of the drawing tool.

Restriction: Little to moderate spatial overlap of emission signals signals is required to obtain good results.

Benefit: Solution for Emission Fingerprinting in cases where reference spectra are not accessible via single-labeled controls.

- Select number of dyes present.
- Click **OK**.
- View results in graph.

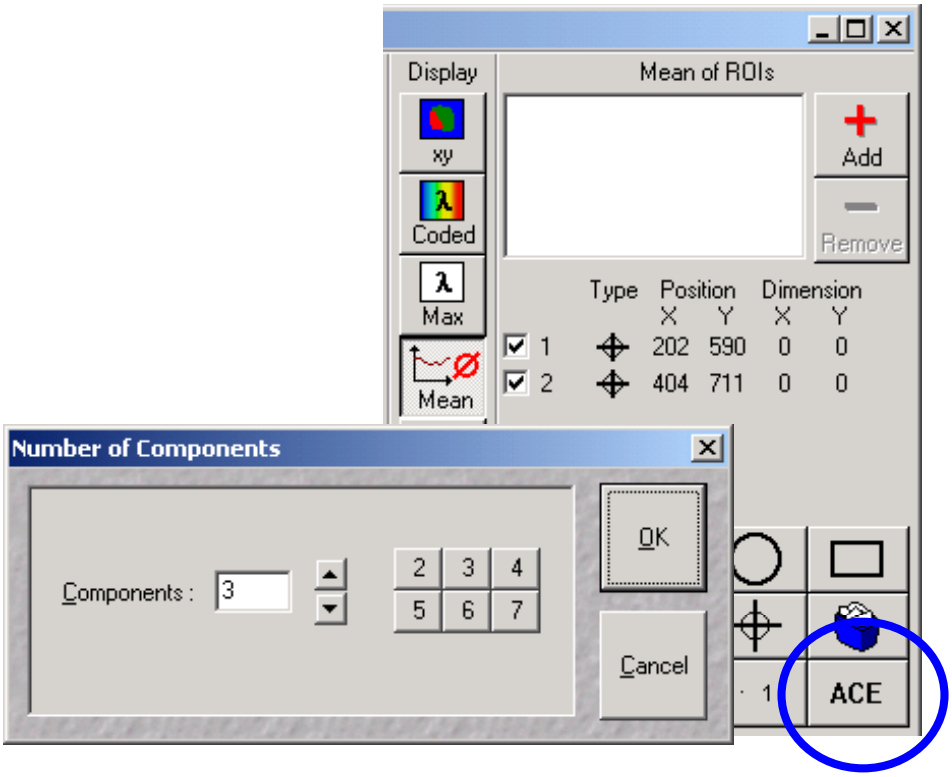


Fig. 4-321 Automatic Component Extraction function

4.14 Image Optimization

4.14.1 Single Channel

Described below is the example of the acquisition of an image, using an excitation wavelength of 543 nm and a fluorescence emission range above 560 nm. The HFT 488/543 is used as the main dichroic beam splitter.

Let the specimen be a thin section through a stem of Convallaria majalis (Lily-of-the-Valley). The description applies to the use of the Axio Imager.Z1 microscope, and analogously also to the Axiovert 200 M.

4.14.1.1 Requirements

The suitable laser is switched on.

The specimen has been positioned and focused for scanning.

- Click on the **LSM** button in the **Acquire** subordinate toolbar of the **Main** menu. The **LSM** button is activated.
- Click on the **Config** button in the **Acquire** subordinate toolbar of the **Main** menu.
  - This opens the **Configuration Control** window.
- Click on the **Single Track** button.
- Click on the

Ch3 icon and assign a color to Channel 3 in the **Channel Color Selection** window. Activate channel 3 via check box.
- Click on the icon of emission filter 3 (before Ch3) and select the **LP 560** filter.
- If required, deactivate all the other channels (ChS, Ch2, 4, monitor diode, transmission, R1-2) via check box.
- Click on the icon of the main dichroic beam splitter and select **HFT 488/543**.

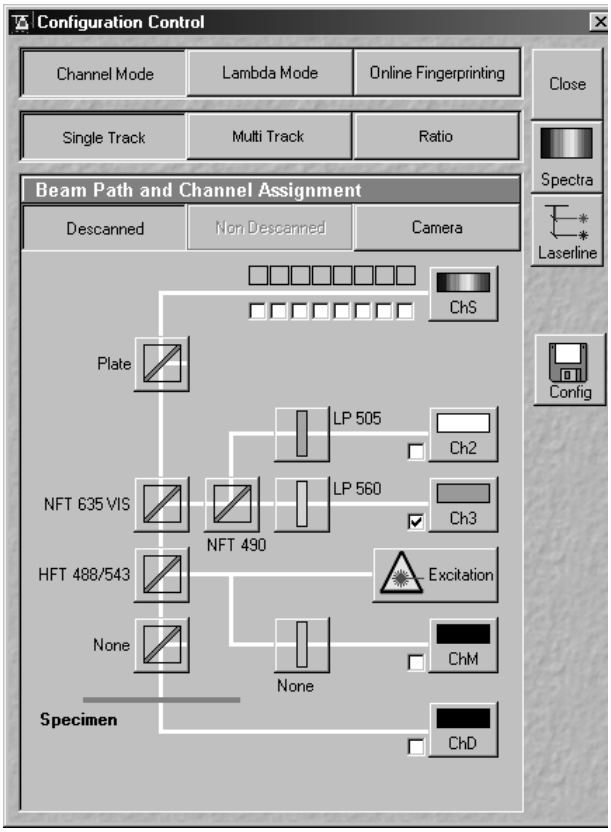


Fig. 4-322 Configuration Control window

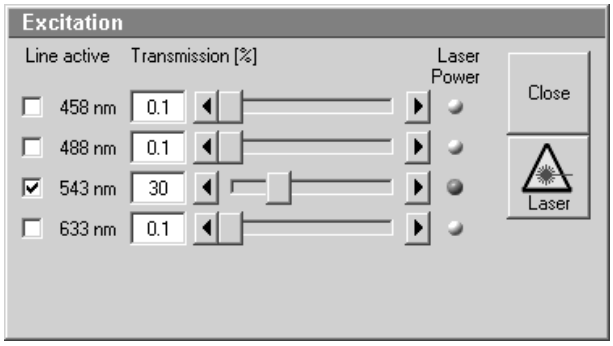


Fig. 4-323 Excitation panel

- Click on the Excitation icon, activate the **543 nm** laser line and click on **Line Active** ☑. If required, deactivate other laser lines which are not needed.
- Use the **Transmission** slider to set the laser intensity to approx. 30 % at first.

The **Beam Path and Channel Assignment** panel displays the current configuration loaded.

☞ The set laser intensity must be subsequently optimized for the current situation via the **Transmission** slider.

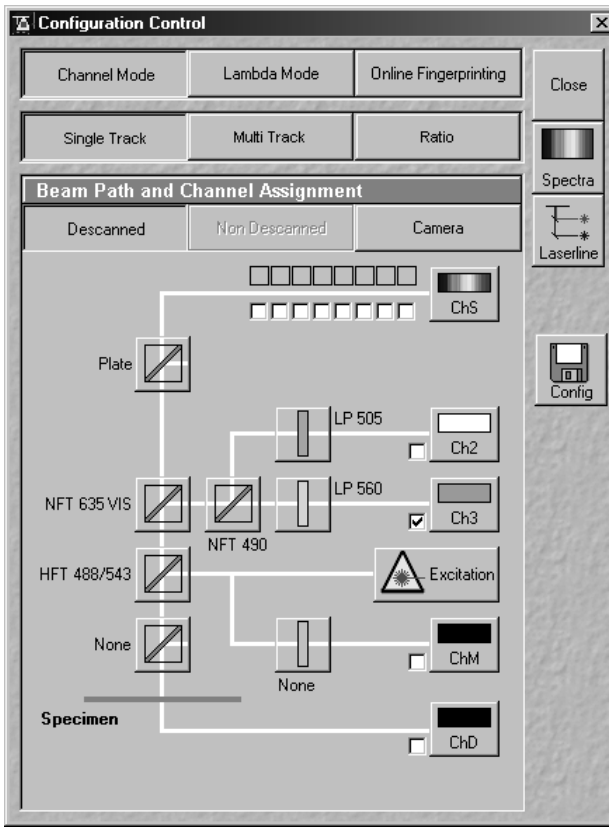


Fig. 4-324 Configuration Control window

For overlaying fluorescence and transmitted-light images, click on the **ChD** (Transmission) button in the **Beam Path and Channel Assignment** panel.

The transmitted light PMT photomultiplier will be activated.

Of course, all other transmitted light applications like

- phase contrast
- differential interference contrast (DIC)
- polarization contrast (Pol)
- darkfield

can also be performed.

☞ For the generation of images in reflection, the main dichroic beam splitter must be a neutral-density filter.
Standard equipment contains a neutral-density filter with a division ratio of 80 to 20 % (at 543 nm).

- In the **Main** menu click on the **Scan** button in the **Acquire** subordinate toolbar.
  - This opens the **Scan Control** window.
- Click on the **Mode** button.
- For a frame scan, click on the **Frame** button.
- On the **Objective Lens & Image Size** panel, select Objective and Frame size for the scan (e.g. X 512 / Y 512 scan). The **Optimal** button automatically adjusts the frame size to match the magnification and resolution of the objective. This ensures that images are acquired without collecting redundant image information.
- On the **Speed** panel, enter a scanning speed of 7, for example, to start with.
- Start with the following settings on the **Pixel Depth, Scan Direction & Scan Average** panel:

  Data depth: 8 bits

  Scan direction: → unidirectional

  Average: Number: 1
- On the **Zoom, Rotation & Offset** panel, set a zoom of 1 and a rotation of 0.

☞ Using the **Fast XY** button is a convenient way of creating an overview scan.

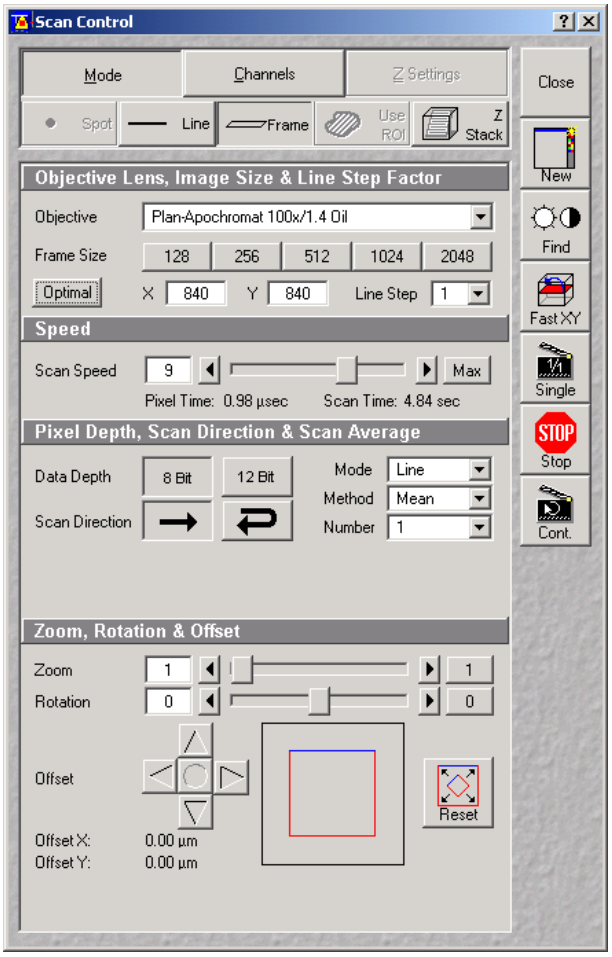


Fig. 4-325 Scan Control window (Mode)

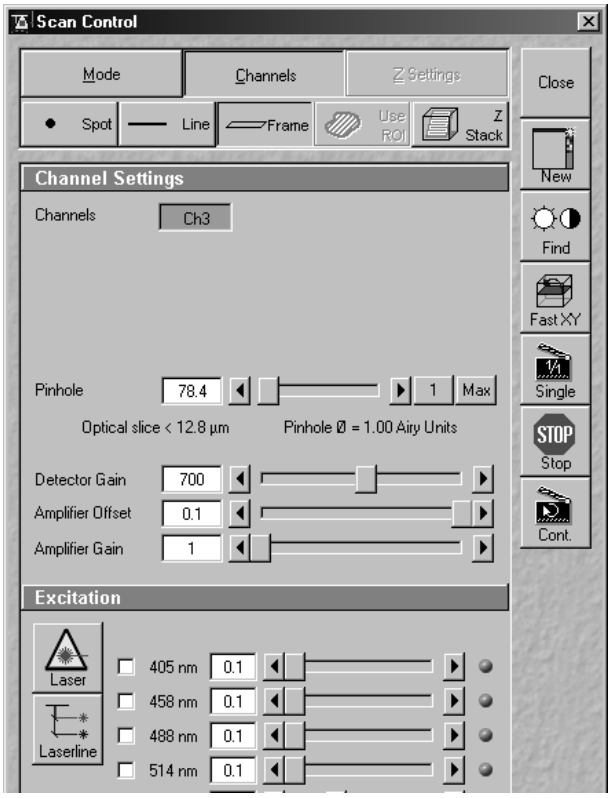


Fig. 4-326 Scan Control window (Channels)

- Click on the **Channels** button.
  - This displays the preset parameters of the configuration loaded.
- Click on the **Find** button. Make sure to position the slider correctly. Then scan while the slider is in the LSM position.
  - This starts the scanning process.
  - The image is seen to build up gradually in a new window.

☞ Function **Find** produces images of different brightness for different scan speeds.

As a rule, the first scanned image (Pre-Scan) is not ideal, since the photomultiplier is not matched to the light output. More often than not, the screen image is dull and needs subsequent optimization.

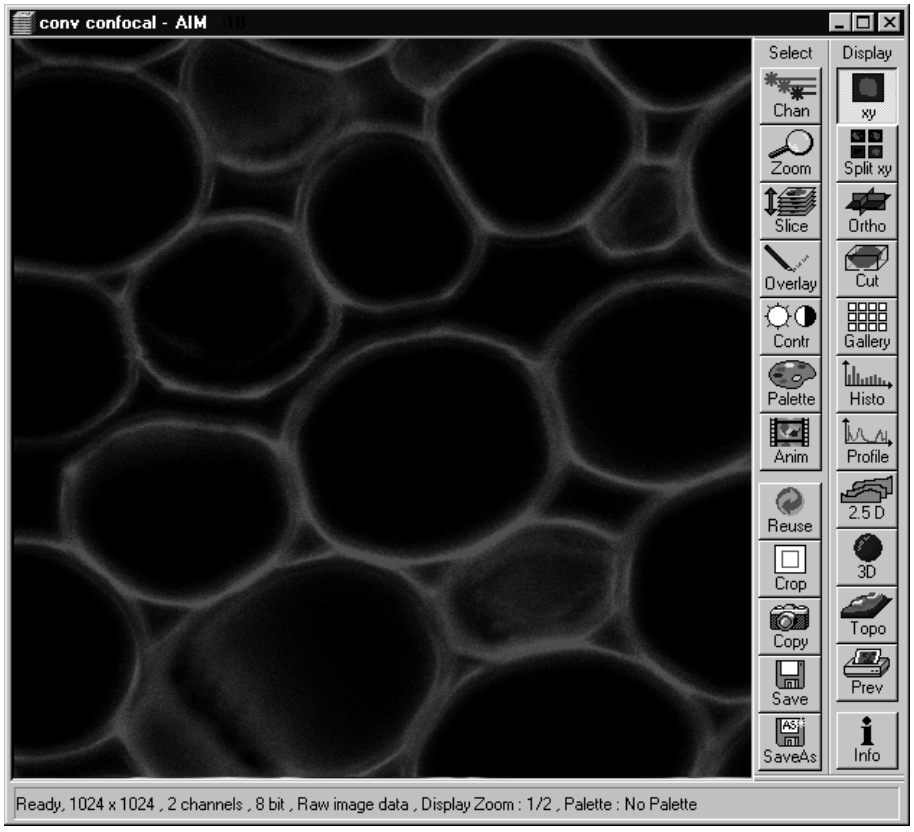


Fig. 4-327 Image Display window

4.14.1.2 Pinhole / Detector Gain / Ampl. Offset / Ampl. Gain

- In the **Scan Control** window, click on the **Cont.** button (see Fig. 4-326).
  - This starts a continuous scan.
- Use the **Pinhole** slider to set the pinhole diameter in the **Scan Control** window under **Channels**.
  - The pinhole diameter should be so small that there is still enough variation for the setting of the detector gain and that sufficient image information is still available. 1 Airy is a good value to enable a confocal fluorescence XY-image to be obtained.
  - A small pinhole diameter will increase the depth of focus, but reduce the light intensity received by the PMT photomultiplier (for reflection mode confocal images start with a pinhole value of 0.5 Airy Units).
  - The influence of the pinhole diameter on image creation is shown by the example in Fig. 4-328. The entire image was first scanned with too large a pinhole diameter. The pinhole diameter was then optimized for a defined ROI. This considerably improved the display of the specimen structures.

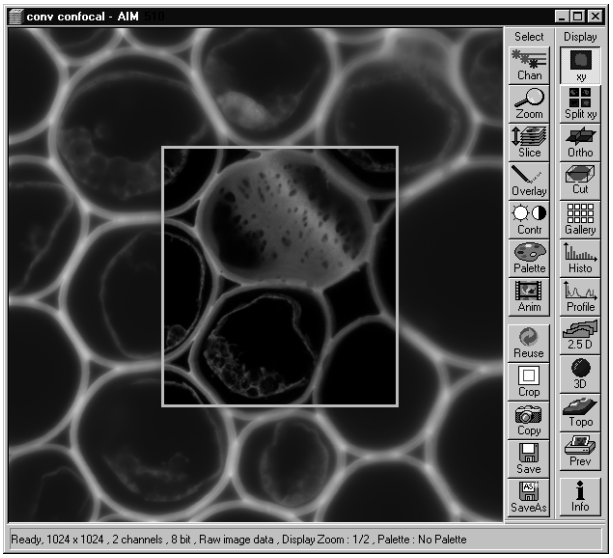


Fig. 4-328 **Image Display window with confocal ROI**

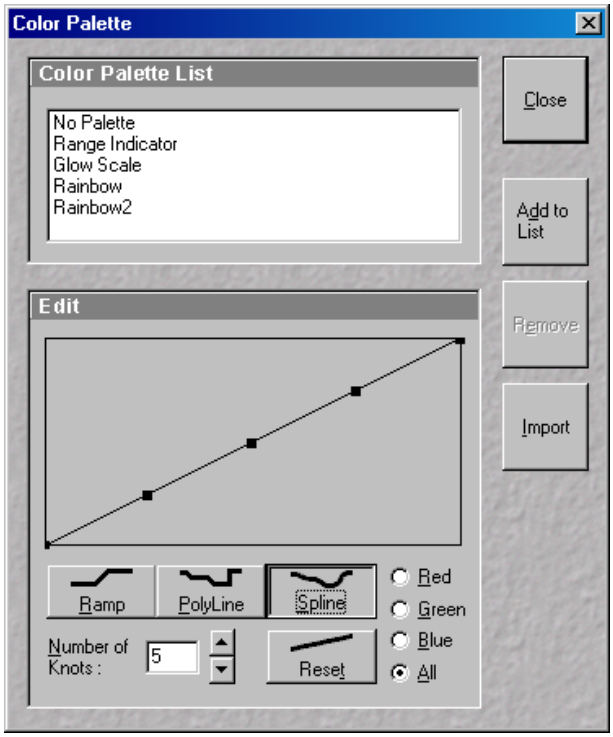


Fig. 4-329 Color Palette window

- Click on the **Palette** button in the **Select** image processing toolbar.
  - This opens the **Color Palette** window.
- In the **Color Palette List** panel, click on the Range Indicator item.
  - The scanned image appears in a false-color presentation.

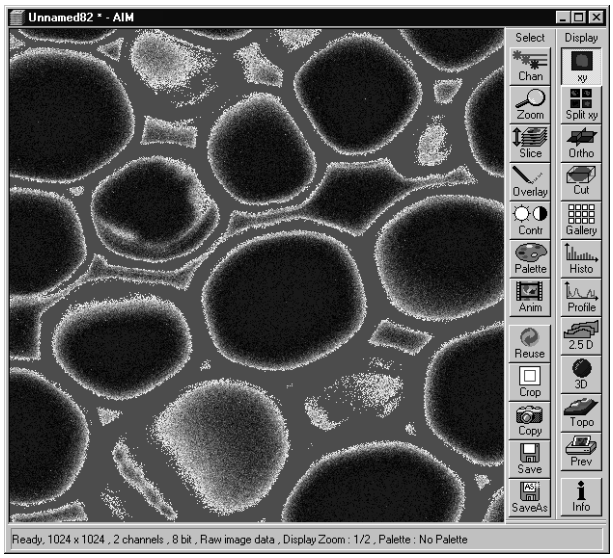


Fig. 4-330 Image Display window

If the image is too bright, it appears red on the screen.

If the image is not bright enough, it appears blue on the screen.

- On the **Channel Settings** panel of the **Scan Control** window, set the PMT (photomultiplier) gain with the **Detector Gain** slider.
  - The image should not have more than a trace of red.
  - This adjustment is very sensitive. Try using the left and right arrows to make the adjustment instead of dragging the slider bar.

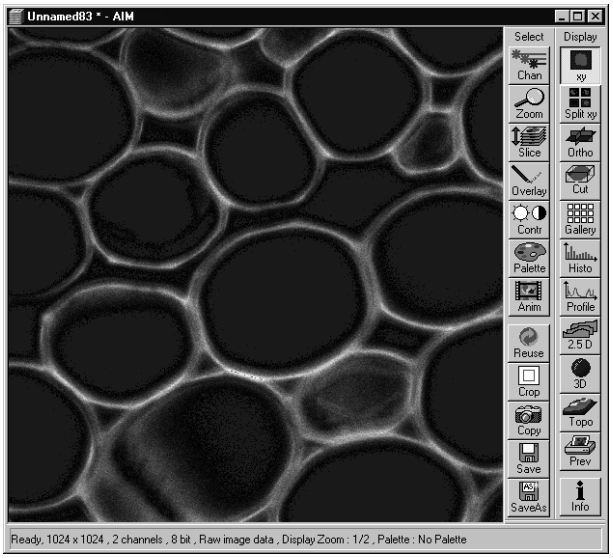


Fig. 4-331 Image Display window

- To adjust the black level (background), use the **Ampl. Offset** slider so that areas without picture content just show a trace of blue.
- If necessary, re-amplify brightness with the **Ampl. Gain** slider.

☞ Do not change the **Ampl. Gain** setting unless the settings made so far are insufficient for image optimization.

- In the Color Palette List panel of the **Color Palette** window, click on **No Palette**.
  - This deselects the **Range Indicator** and activates the new presentation.
- In the **Scan Control** window, click on the **Stop** button.
  - This stops the continuous scan.

☞ If you use the **Range Indicator** for image optimization, it may happen that the ranges marked in the **Range Indicator** will vary when the channel color is changed.

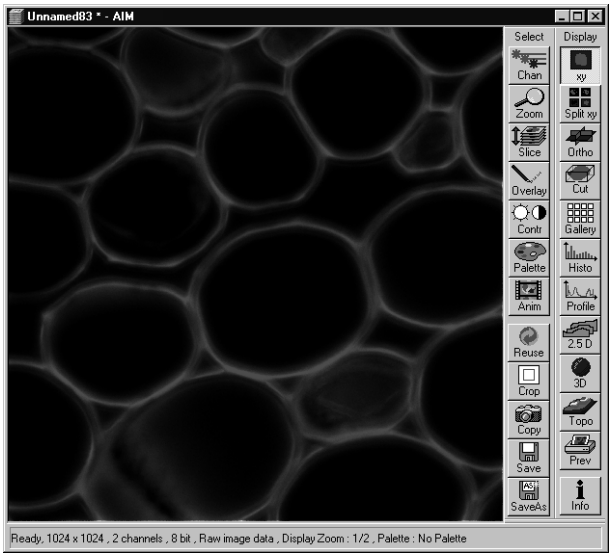


Fig. 4-332 Image Display window

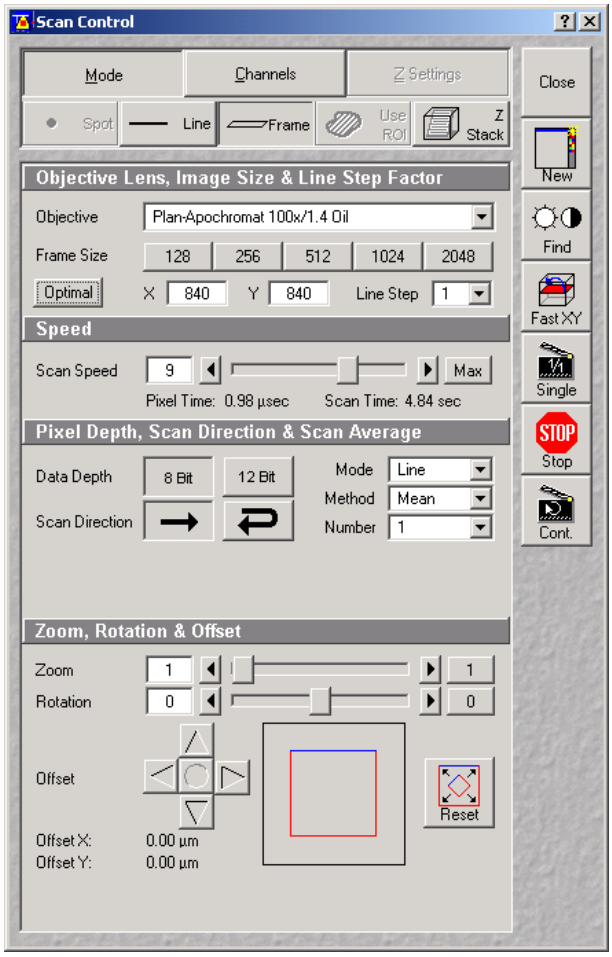


Fig. 4-333 Scan Control window

4.14.1.3 Scan Speed, Scan Average and Pixel Depth

The signal-to-noise ratio can be substantially improved by reducing the scanning speed to an acceptable level and averaging over several scans (i.e. with an average **Number** greater than 1 for the **Mean** average **Method** in the **Scan Control** window).

- Use the **Scan Speed** slider in the **Speed** panel to set the slowest acceptable scanning speed.
  - The corresponding pixel scanning time (Pixel Time) and the total scanning time (Scan Time) are shown in the dialog box.
- In the **Number** text box of the **Pixel Depth, Scan Direction & Scan Average** panel enter the number of measurements to be averaged.

☞ Image optimization can be effected much faster if you select a smaller frame, since less data have to be processed.
The greater the number of averages selected for **Mean** average **Method**, the better the image quality will be; the scanning time will be prolonged accordingly.

4.14.2 Multiple-channel

4.14.2.1 Requirements

- The suitable lasers are on.
- The specimen has been positioned and focused for scanning.
- Click on the **LSM** button in the **Acquire** subordinate toolbar of the **Main** menu. The tube slider on the microscope tube is in the **LSM** position and the **LSM** button is activated.

In the following example, 2 Channels shall be activated for the scanning procedure: one for 488 nm using emission filter BP 505-530 and one for 543 nm with LP 560. HFT 488/543 is used as the main dichroic beam splitter, and NFT 635 VIS and NFT 545 as the secondary dichroic beam splitter.

- In the **Acquire** subordinate toolbar, click on the **Config** button.
  - This opens the **Configuration Control** window.

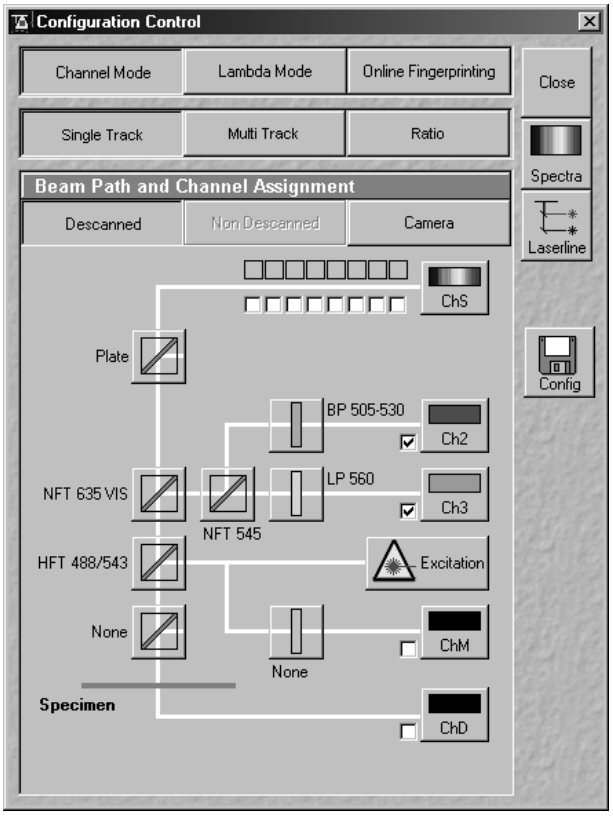


Fig. 4-334 Configuration Control window

- Click on the **Single Track** button.
- Activate (in the same way as for the single channel, see page 4-345) channel 2 and channel 3 (Ch2, Ch3), the indicated emission filters and the main and secondary dichroic beam splitter for the scanning procedure.
  - The configuration loaded is displayed in the **Beam Path and Channel Assignment** panel.
- Click on the **Scan** button in the **Acquire** subordinate toolbar of the **Main** menu.
  - This opens the **Scan Control** window.
- In the **Scan Control** window, set the parameters in the same way as described for single-channel presentation.
- Click on the **Find** button in the **Scan Control** window.
  - This starts the scanning process. The scanned image appears in a separate window.

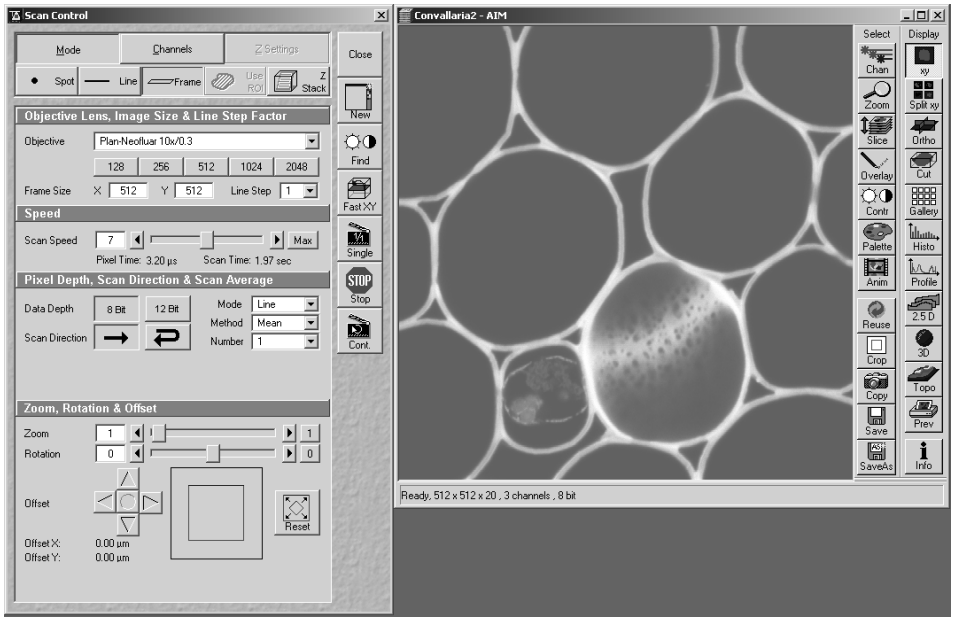


Fig. 4-335 Scan control and Image Display windows

As a rule, the first scanned image (Pre-Scan) is not ideal, since the photomultiplier is not matched to the light output. More often than not, the screen image is dull and needs subsequent optimization.

- Click on the **Channels** button in the **Scan Control** window.
  - This opens the Channel Settings and Excitation of Track panels.
  - The channels used are color-highlighted.

4.14.2.2 Image Optimization

The image optimization processes

- setting of pinhole diameter
- Detector Gain / Ampl. Offset / Ampl. Gain
- Scanning speed and Average

must be carried out separately for each channel used (see **Single Channel**, page 4-345).

For the optimum setting of the single channels, **Split xy**-display must be selected in the **Image Display** window to enable the direct viewing of the separate images of the relevant channels.

- Click on the **Cont.** button in the **Scan Control** window.
  - This starts a continuous scan.
- Click on the **Split xy** button in the **Image Display** window toolbar.
  - This displays the separate images scanned in the channels and the composite (overlay) image.

- Now click on the **Ch2** button in the **Channel Settings** panel to optimize Channel 1. Optimization is performed in the same way as for the single channel and can be monitored online in the relevant separate image of the channel.
- Then optimize the second channel by clicking on the relevant button (**Ch3**) in the **Channel Settings** panel of the **Scan Control** window.

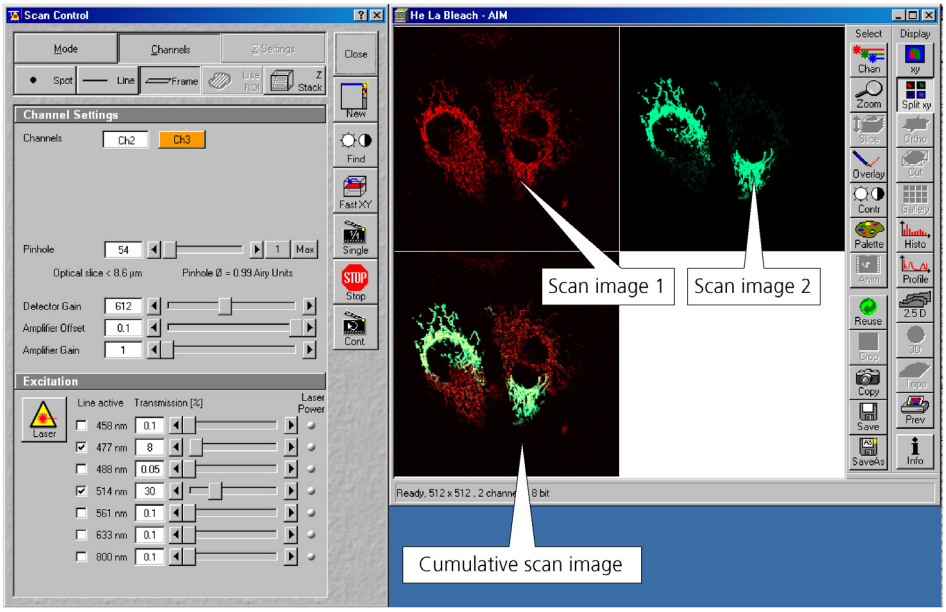


Fig. 4-336 Scan Control and Image Display windows

Now effect image optimization as explained for the single-channel mode, but separately for each channel.

- Now click on the **xy** button of the **Display** toolbar.
  - The composite scan image of two channels is presented in a common window.

☞ Image optimization can be effected much faster if you select a smaller frame, since less data have to be processed.

4.15 Extension of the LSM 510 or LSM 510 META with LSM DuoScan

The LSM 510 and LSM 510 META can be equipped with a second scanner, the LSM *DuoScan*. For optimal results the scan fields of the two scanners must be aligned. This procedure is described in chapter 4.8.4.1 The additional equipment results in different screen contents of the **Bleach control** menu (see chapter 4.5.7).

4.16 Shut-Down Procedure

Never shut down the computer by its main switch while your LSM program is still active, or else you will lose the currently set operating parameters and the images just scanned.

In the **Settings for user** dialog window, which can be activated with the **Options / Settings** buttons, activate **Laser off** or **Exit** in the **Shutdown** tab. The lasers will then automatically be switched off when you exit the LSM program.

4.16.1 Exiting the LSM Program

- Close all open windows of the LSM program by clicking on the closing icon ☒ in the top right corner of each window.
  - This closes the respective window and removes the respective icons from the taskbar.
  - After all dialog windows have been closed, the **LSM 510 Switchboard** window appears.

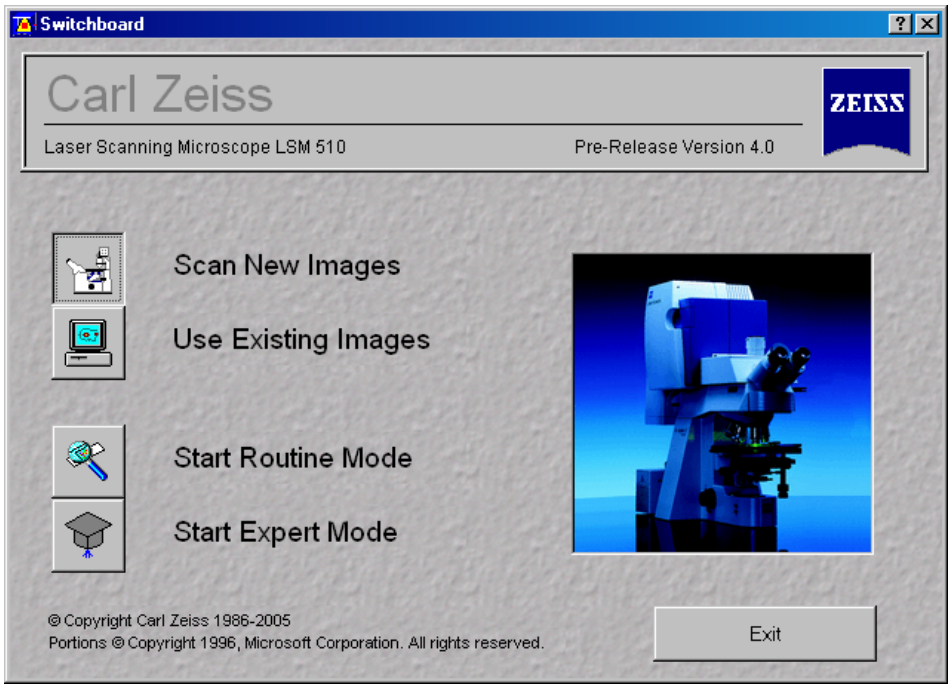


Fig. 4-337 LSM 510 Switchboard menu

- Click on the **Exit** button.
  - This terminates the LSM program.
  - The monitor screen shows the desktop of the WINDOWS XP operating system.

4.16.2 Shut Down the WINDOWS Operating System

- Move the cursor to the bottom margin of the screen.
  - This opens the taskbar containing the **Start** button.
- Click on the **Start** button of the taskbar.
  - This opens a pop-up menu.
- Click on the **Shut Down** item.
  - This opens the **Shut Down Windows** window, in which you can select between **Shut down, Restart** and **Login**.
- Unless already set by default, click on **Shut down the computer?**
- Click on the **Yes** button.

About 20 seconds after WINDOWS XP has been run down, and your computer turns off.

4.16.3 Turning Power Off

☞ Please bear in mind that a cooling phase of at least 5 minutes is required between switching off of the laser via the software and switching off of the entire system via the REMOTE CONTROL main switch or the Power Supply switch of the Enterprise UV laser.

⚠ Set the REMOTE CONTROL switches "System/PC" and "Components" or the Main switch on the System Electronic Rack and the power supply switch of the Ar Laser to position "**OFF**" after 5 minutes.
This puts your LSM 510 microscope system, including the computer, off power.

4.17 Software and Hardware Options

This section describes optional software and hardware configurations. Depending on your configuration, the content of dialogue and function may differ.

4.17.1 Software

The following software packages for Release 4.0 are available:

- Software "Physiology "
- Software "Topography "
- Software "Macro Recorder and Editor"
- Software "3D for LSM"
- Software "Multiple Time Series"
- Software "Image VisArt"
- Software "Deconvolution"
- Software "StitchArt"
- Software “FRAP”
- Software “FRET”
- Software “Visual Macro Editor”

If your configuration does not include any of the SW packages "Physiology", “FRAP” or FRET, the following functions are not available:

- Mean of ROI button in the Image Display window

If your configuration does not include the "Physiology " software package, the following functions are not available:

- Mean of ROI scan button in Time Series Control
- Ion Concentration button in the Process Menu

If your configuration does not include the “FRAP” software package, the following functions are not available:

- Kinetics button in Process Menu

If your configuration does not include the “FRET” software package, the following functions are not available:

- **FRET** Macro button in the **Macro** Menu

If your configuration does not include the “Visual Macro Editor” software package, the following functions are not available:

- **VME** button in the **Macro** Menu

If your configuration does not include the "Topography " software package, the following functions are not available:

– **Topo** button in the **Image Display** window after acquisition of image stacks

If your configuration does not include the "Macro Recorder and Editor" software package, the following functions are not available:

– **New**, **Save** and **Save as** buttons in the **Macro Control** window

– **Edit**, **Step**, **Delete**, **Editor** buttons in the **Macro Control** window

If your configuration does not include the "3D for LSM" software package, the following separate application is not available:

– **3D for LSM**

If your configuration does not include the "Multiple Time Series" software package, the following function is not available:

– Macro: "Advanced Time Series"

If your configuration does not include the "Image VisArt" software package, the following function is not available:

– **3D** button in the **Image Display** window

If your configuration does not include the "Deconvolution" software package, the following functions are not available

– **DCV Settings** button in the **Ortho** function of the **Image Display** window

– **DCV** button in the **Process** menu

If your configuration does not include the "StitchArt plus" software package, the following function is not available:

– Macro: "**StitchArt plus**"

4.17.2 Hardware

Depending on whether the following hardware components are available or not, the content of the screens may differ:

- Piezo objective focusing device
- X-Y scanning stage DC 4 × 4 or DC 100 × 90, each with MCU 28
- Stands: Axio Imager.Z1, Axio Imager. M1, Axioskop 2 FS MOT, Axiovert 200 M
- Depending on the configuration the scan head equipment may differ in filters, beam splitters and the number of photomultiplier
- Transmitted-light PMT
- Monitor diode
- LSM *DuoSan*

If your configuration does not include the Piezo objective focusing device, the following functions are not available:

- **Hyperfine Z Sectioning** in the **Z Stack** function in the **Scan Control** window
- **HRZ** parameters in the **Stage and Focus Control** window

If your configuration does not include the X-Y scanning stages DC 4 × 4 or DC 100 × 90, each with MCU 28, the following functions are not available:

- **Stage Position** and **Tile Scan** functions in the **Stage and Focus Control** window

Depending on the used microscope stand: Axio Imager.Z1, Axio Imager.M1, Axioskop 2 FS MOT or Axiovert 200 M, the following dialogue and available functions may differ:

- Context and accessibility of the **Microscope Control** window

If your configuration does not include scan head META, monitor diode and/or transmitted light PMT, the following functions may differ:

- Context and accessibility of the **Config Control** window

If your configuration does not include an AxioCam, the following functions are not available:

- **Camera** in the **Config Control** window, **Scan Control** window

If your configuration does include the LSM DuoScan the contents of the Bleach Control menu do vary (see also chapter 4.5.7)

4.18 Courses on "How to Operate the System in an Optimized Way"

Carl Zeiss is offering training courses on how to operate the system in an optimized way.

- Courses are held in our application center in Jena, Germany.
- Courses are held in English or German language, respectively.

Check out:

www.zeiss.de/lsm

for the latest dates and ask your Zeiss representative for a quotation on courses.

CHAPTER 5 VBA PROGRAMMING FOR LSM

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5 VBA PROGRAMMING FOR LSM

5.1 VBA Programming

5.1.1 General Syntax

Object oriented programming

Class Modules

5.1.2 Working with Files

I/O operations, file operations are implemented in VBA

Use of the implemented functions

Example:

```
Type CALIBRATION_PARAM
    lambda As Long
    angle As Double
    Translation As Double
End Type

Function DataFromCalibFile(strGetFile As String, calibArray() As CALIBRATION_PARAM) As Integer
    Dim hFile As Long
    Dim strLine As String
    Dim varData(1 To 3) As Variant
    Dim paramCnt As Integer

    paramCnt = -1
    'get the next free file handle
    hFile = FreeFile
    'check if file exist
    If (Len(Dir(strGetFile)) > 0) Then
        Open strGetFile For Input Access Read Shared As hFile
            If (Not EOF(hFile)) Then
                Line Input #hFile, strLine

                paramCnt = 0
                Do Until EOF(hFile)
                    Input #hFile, varData(1), varData(2), varData(3)
                    calibArray(paramCnt).lambda = varData(1)
                    calibArray(paramCnt).angle = varData(2)
                    calibArray(paramCnt).Translation = varData(3)
                    paramCnt = paramCnt + 1
                Loop
            End If
        Close #hFile
    End If

    DataFromCalibFile = paramCnt
End Function Objektstruktur
```

Use of the API function for additional functionality

5.1.3 Access to Windows API Function and external DLLs

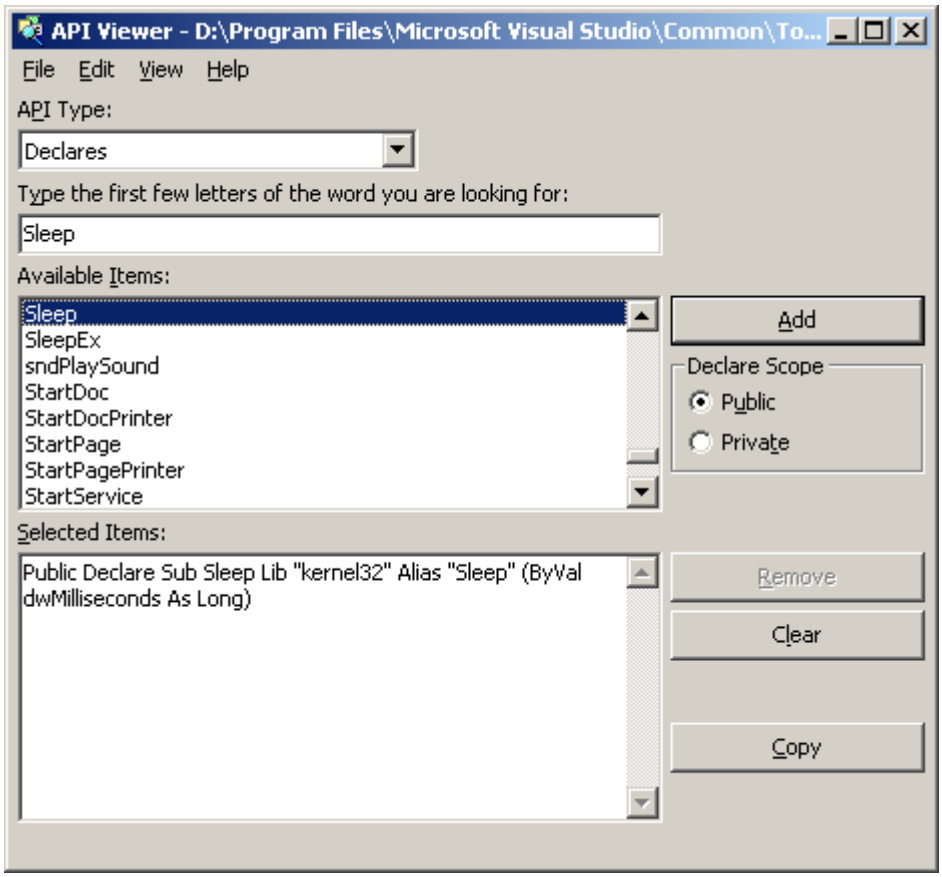
- with Type Libraries

  OLE

- with Declare Statements

  API Text Viewer

  searches WIN32API.TXT



Example:

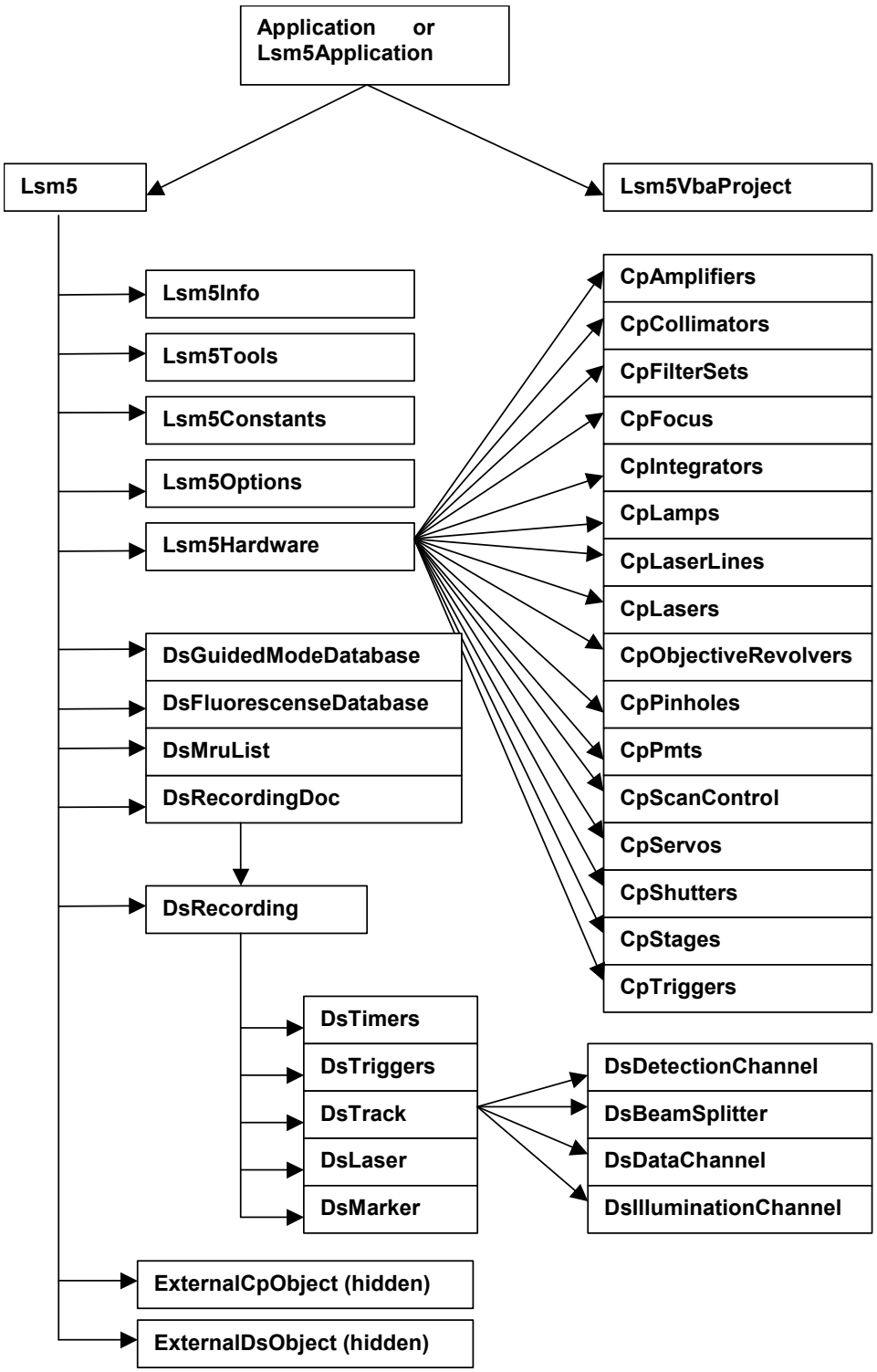
```
Declare Sub Sleep Lib "kernel32" (ByVal dwMilliseconds As Long)

Declare Function RegOpenKeyEx _
    Lib "advapi32.dll" Alias "RegOpenKeyExA" _
    (ByVal hKey As Long, ByVal lpSubKey As String, _
    ByVal ulOptions As Long, ByVal samDesired As Long, _
    phkResult As Long) As Long


Public Declare Function RegCloseKey _
    Lib "advapi32.dll" (ByVal hKey As Long) As Long


Public Declare Function RegQueryValueEx _
   Lib "advapi32.dll" Alias "RegQueryValueExA" _
   (ByVal hKey As Long, ByVal lpValueName As String, _
   ByVal lpReserved As Long, lpType As Long, _
   lpData As Any, lpcbData As Long) As Long        ' Note that if you declare the lpData  parameter
   as String, you must pass it By Value.

Public Const HKEY_CLASSES_ROOT = &H80000000

Public Const SYNCHRONIZE = &H100000
Public Const READ_CONTROL = &H20000
Public Const STANDARD_RIGHTS_READ = (READ_CONTROL)
Public Const KEY_QUERY_VALUE = &H1
Public Const KEY_ENUMERATE_SUB_KEYS = &H8
Public Const KEY_NOTIFY = &H10
Public Const KEY_READ = ((STANDARD_RIGHTS_READ Or KEY_QUERY_VALUE Or
KEY_ENUMERATE_SUB_KEYS Or KEY_NOTIFY) And (Not SYNCHRONIZE))

Public Const REG_SZ = 1                         ' Unicode null terminated string
Public Const ERROR_SUCCESS = 0&
```

```
Function FServerFromDescription(strName As String, strPath As String) As Boolean
    Dim lngResult As Long
    Dim strTmp As String
    Dim hKeyServer As Long
    Dim strBuffer As String
    Dim cb As Long
    Dim i As Integer

    FServerFromDescription = False

    strTmp = VBA.Space(255)
    strTmp = strName + "\CLSID"
    lngResult = RegOpenKeyEx(HKEY_CLASSES_ROOT, strTmp, 0&, KEY_READ, hKeyServer)

    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit
    strBuffer = VBA.Space(255)
    cb = Len(strBuffer)

    lngResult = RegQueryValueEx(hKeyServer, "", 0&, REG_SZ, ByVal strBuffer, cb)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit

    lngResult = RegCloseKey(hKeyServer)
    strTmp = VBA.Space(255)
    strTmp = "CLSID\" + Left(strBuffer, cb - 1) + "\LocalServer32"
    strBuffer = VBA.Space(255)
    cb = Len(strBuffer)
    lngResult = RegOpenKeyEx(HKEY_CLASSES_ROOT, strTmp, 0&, KEY_READ, hKeyServer)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit

    lngResult = RegQueryValueEx(hKeyServer, "", 0&, REG_SZ, ByVal strBuffer, cb)
    If (Not lngResult = ERROR_SUCCESS) Then GoTo error_exit
    strPath = Left(strBuffer, cb - 1)
```

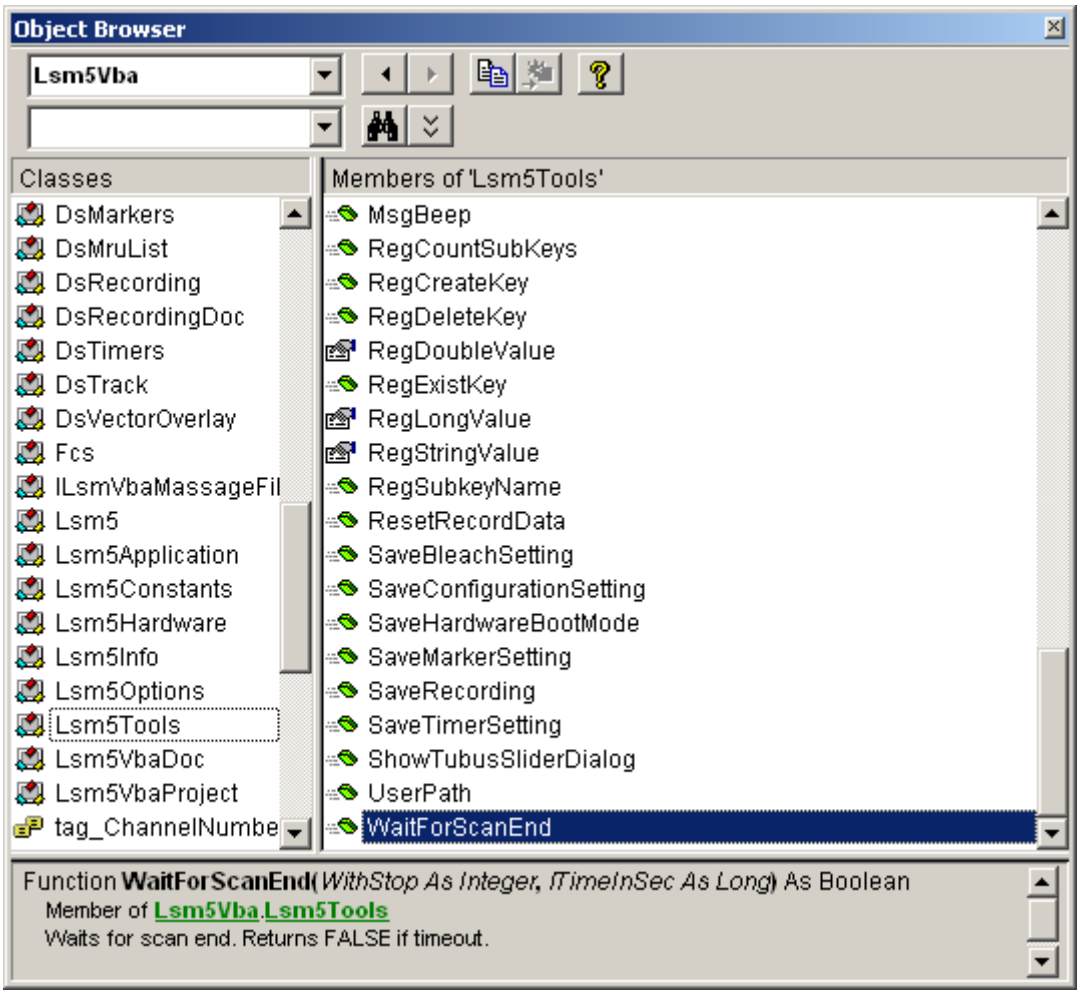
```
    lngResult = RegCloseKey(hKeyServer)
    hKeyServer = 0

    i = Len(strPath)

    Do Until (i = 0)
        If (VBA.Mid(strPath, i, 1) = "\") Then
            strPath = Left(strPath, i - 1)
            FServerFromDescription = True
            Exit Do
        End If
        i = i - 1
    Loop

error_exit:
    If (Not hKeyServer = 0) Then lngResult = RegCloseKey(hKeyServer)
    hKeyServer = 0

End Function
```

5.2 Programming for LSM

5.2.1 Object structure



To search the object structure use the object browser.



5.2.2 Differences in access to LSM Hardware with Lsm5Hardware Object / DsRecording Object

Object Browser Project / Library LsmVba auswählen und Objekt Struktur untersuchen

- Access with DsRecording Object

  Status maintains at Scan Start

```
Public Sub CopyRecording(Destination As DsRecording, Source As DsRecording)
    Dim TS As DsTrack
    Dim TD As DsTrack
    Dim DataS As DsDataChannel
    Dim DataD As DsDataChannel
    Dim DetS As DsDetectionChannel
    Dim DetD As DsDetectionChannel
    Dim IlS As DsIlluminationChannel
    Dim IlD As DsIlluminationChannel
    Dim BS As DsBeamSplitter
    Dim BD As DsBeamSplitter
    Dim lT As Long
    Dim ll As Long
    Dim success As Integer

    Destination.Copy Source
    Destination.Objective = Source.Objective
    For lT = 0 To Destination.TrackCount - 1

        Set TS = Source.TrackObjectByIndex(lT, success)
        Set TD = Destination.TrackObjectByIndex(lT, success)

        TD.Collimator1Position = TS.Collimator1Position
        TD.Collimator2Position = TS.Collimator2Position

        For ll = 0 To TD.DataChannelCount - 1
            Set DataS = TS.DataChannelObjectByIndex(ll, success)
            Set DataD = TD.DataChannelObjectByIndex(ll, success)
            DataD.ColorRef = DataS.ColorRef
        Next ll
```

```
        For ll = 0 To TD.DetectionChannelCount - 1
            Set DetS = TS.DetectionChannelObjectByIndex(ll, success)
            Set DetD = TD.DetectionChannelObjectByIndex(ll, success)
            DetD.Filter1 = DetS.Filter1
            DetD.Filter2 = DetS.Filter2
            DetD.DetectorGain = DetS.DetectorGain
            DetD.AmplifierGain = DetS.AmplifierGain
            DetD.AmplifierOffset = DetS.AmplifierOffset
            DetD.PinholeDiameter = DetS.PinholeDiameter
        Next ll

        For ll = 0 To TD.IlluminationChannelCount - 1
            Set IlS = TS.IlluminationObjectByIndex(ll, success)
            Set IlD = TD.IlluminationObjectByIndex(ll, success)
            IlD.Acquire = IlS.Acquire
            lD.Power = IlS.Power
            IlD.DetectionChannelName = IlS.DetectionChannelName
        Next ll

        For ll = 0 To TD.BeamSplitterCount - 1
            Set BS = TS.BeamSplitterObjectByIndex(ll, success)
            Set BD = TD.BeamSplitterObjectByIndex(ll, success)
            BD.Filter = BS.Filter
        Next ll

    Next lT
End Sub
```

- Access with Lsm5Hardware Object

Status will be updated at Scan Start to current recording parameters

```
Private Sub FillPinholeList()
    Dim count As Long
    Dim i As Long
    Dim ObjPinholes As CpPinholes
    Dim Servos As Object
    Dim ServoX As Object
    Dim ServoY As Object
    Dim success As Boolean

    CmbPinhole.Clear

    Set Servos = Lsm5.ExternalCpObject.pHardwareObjects.pServos
    Set ObjPinholes = Lsm5.Hardware.CpPinholes
    count = ObjPinholes.count
    For i = 0 To count - 1
        success = ObjPinholes.Select(i)
        If (success) Then
            Set ServoX = Servos.pItem(ObjPinholes.Name + "X")
            Set ServoY = Servos.pItem(ObjPinholes.Name + "Y")
            If ((Not ServoX Is Nothing) And (Not ServoY Is Nothing)) Then
                CmbPinhole.AddItem ObjPinholes.Name
            End If
        End If
    Next i
    If (count) Then CmbPinhole.ListIndex = 0        ' select first pinhole

    Set ObjPinholes = Nothing
    Set Servos = Nothing
    Set ServoX = Nothing
    Set ServoY = Nothing
End Sub
```

- Access with external Lsm5Hardware Object

5.2.3 Access to hidden Interface

- When do I need to use the hidden Interface?
- How do I find the desired Object / Method?
  Open Tool / References
  Browse CP.dll search, open
  Choose Object Browser Project / Library DS or CP
  Examine Object Structure

Example:

```
Function GetLaserKind(WaveLength As String, kind As Integer)
    Dim count As Long
    Dim CpObject As Object
    Dim Lines As Object
    Dim i As Long
    Dim Success As Integer
    Dim WaveLenghtOfIndex As Long
    Dim Attenuation As Double
    Dim Enable As Integer
    Dim Name As String

    GetLaserValueMax = False

    Set CpObject = Lsm5.ExternalCpObject()
    Set Lines = CpObject.pHardwareObjects.pLaserLines

    count = Lines.lCount
    For i = 0 To (count - 1)
        Success = Lines.bLineInfo(i, WaveLenghtOfIndex, Attenuation, Enable, Name)
        If (Success And (WaveLenghtOfIndex = WaveLenght)) Then
            kind = Lines.AttenuatorType(WaveLenght)

            Set Lines = Nothing
            Set CpObject = Nothing
            Exit Function
        End If
    Next i

    Set Lines = Nothing
    Set CpObject = Nothing

End Function
```

5.2.4 Access to scanned pictures

Example:

```
Function ChannelIndexFromChannelName(ChannelName As String, ChannelIndex As Long)
    Dim num As Long, num1 As Long, num2 As Long
    Dim i As Long
    Dim Success As Integer
    Dim DetectionChannel As DsDetectionChannel
    Dim LsmInfo As Lsm5Info
    Dim Recording As DsRecording

    ChannelIndexFromChannelName = False

    Set Recording = Lsm5.DsRecording
    Set LsmInfo = Lsm5.Info
    If (LsmInfo.NumberOfPmtsInSystem(num, num1, num2) = True) Then
        For i = 0 To (num + num1 - 1)
            Set DetectionChannel = Recording.DetectionChannelOfActiveOrder(i, Success)
            If Success Then
                If (StrComp(DetectionChannel.Name, ChannelName) = 0) Then
                    ChannelIndex = i
                    ChannelIndexFromChannelName = True
                    GoTo exit_function
                End If
            End If
        Next i
    End If

exit_function:
    Set LsmInfo = Nothing
    Set Recording = Nothing
End Function
```

```
Function GetAveragePixel(ScanDoc As DsRecordingDoc, ChannelName As String, mean As Double)
    Dim line As Variant
    Dim x As Long, xmax As Long
    Dim y As Long, ymax As Long
    Dim spl As Long
    Dim bpp As Long
    Dim Sum As Double
    Dim Channel As Long

    GetAverageScanLineParams = False

    If (ChannelIndexFromChannelName(ChannelName, Channel) = False) Then
        Exit Function
    End If

    'check for valid doc
    If (ScanDoc Is Nothing) Then Exit Function
    Sum = 0
    xmax = ScanDoc.Recording.SamplesPerLine
    ymax = ScanDoc.Recording.LinesPerFrame
    For y = 0 To ymax - 1
        line = ScanDoc.ScanLine(Channel, 0, 0, 0, spl, bpp)

        For x = 0 To spl - 1
            Sum = Sum + line(x)
        Next x
    Next y
    mean = Sum / (xmax*ymax)

    GetAverageScanLineParams = True
End Function
```

5.2.5 Backup Recording

```
.
.
.
Dim Recording As DsRecording
Dim BackupRecording As DsRecording
Dim ScanDoc As DsRecordingDoc

Set Recording = Lsm5.DsRecording
'create a backup recording
Set BackupRecording = Lsm5.CreateBackupRecording
Success = BackupRecording.Copy(Recording)

Recording.ScanMode = "Line"
Recording.FramesPerStack = 1
Recording.StacksPerRecord = 1
Recording.ScanDirection = eSingleForeward
Recording.SpecialScanMode = "NoSpecialScanMode"
Recording.TimeSeries = False
Recording.ZoomX = ZOOM_X
Recording.ROTATION = ROTATION
Recording.Sample0X = OFFSET_X
Recording.Sample0Y = OFFSET_Y
Recording.SamplesPerLine = IMAGE_SIZE
Recording.StartScanTime = 8

Success = SetChannelDetection(ChannelName, 1, AmpOffset, 100)
Set ScanDoc = Lsm5.StartContinuousScan

.
.
.

Lsm5.StopScan
'restore the backup recording
Recording.Copy BackupRecording
Set Recording = Nothing
Set BackupRecording = Nothing
```

5.2.6 Events

- What are Events good for?

- Mouse Events
- Scan state events
- Hardware Events

```
Private Sub Lsm5VbaDoc_SystemEvent(ByVal EventNr As Long, ByVal Param As Variant)
    Dim x As Long
    Dim y As Long
    Dim z As Long
    Dim t As Long
    Dim c As Long
    Dim tmp As Long
    Dim pt As POINT
    Dim dsDoc As DsRecordingDoc

    'get the active Recording Document
    Set dsDoc = Lsm5.DsRecordingActiveDocObject
    If (dsDoc Is Nothing) Then Exit Sub
    If (Not dsDoc.IsValid) Then Exit Sub

    If (EventNr = eImageWindowLeftButtonDownEvent) Then
        If dsDoc.GetCurrentMousePosition(c, t, z, y, x) <> 0 Then
            'do something
                    .
                    .
                    .


        End If
    End If

End Sub
```

CHAPTER 6 ROUTINE MODE AND TOOLS

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6 ROUTINE MODE AND TOOLS

6.1 Routine Mode

6.1.1 General

The Routine Mode of the LSM 510 software permits the fast and easy acquisition of scanning images by using time-tested **Standard Examination Methods** or by **User Defined Examination Methods**.

Standard Examination Methods are included in the LSM 510 software package and must only be activated once during the first application of the routine mode.

User Defined Examination Methods are methods which were already created and optimized in the Expert Mode. If **User Defined Examination Methods** are also to be used in the Routine Mode, they must be exported in the Routine Mode first. Apart from **User Defined Examination Methods**, stacks and time series can also be exported to the routine mode in addition to simple frames.

Images scanned in the Routine Mode are displayed in the same image windows as in the Expert mode. The tools for image processing and the image display functions are identical.

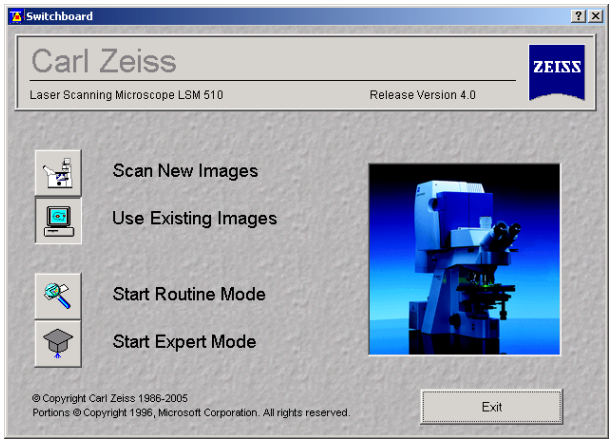

Fig. 6-1 LSM 510 Switchboard menu

6.1.2 Starting the Routine Mode

- Start the LSM program (see chapter 4).

☞ If you want to change from the Expert Mode to the Routine Mode and vice versa, close all the windows first.

- Activate the **Scan New Images** button in the LSM 510 Switchboard menu.
- Click on the **Start Routine Mode** button.
  - The **Routine Mode** window will appear.

In addition to the three standard examination methods contained in the LSM 510 software package, any required number of further examination methods can be exported from the Expert Mode to the Routine Mode.

☞ The three standard examination modes are only available if channel 1 is a Non-META PMT channel.

The maximum of five, freely selectable methods can be assigned directly to the **Standard Examination Methods** buttons.

- Click on the **Add method** button.
  - The **Select Method To Add To Standard** window appears on the screen.
- Use the checkboxes in the **Methods** frame to select / deselect a table.
- Click on the **Select All** button to select all current methods.
- Click on the **Clear** button to deselect all current methods.
- Exit the **Select Method To Add To Standard** by clicking on the **Close** button.

All the other methods required must be activated in the **User-Defined Examination Methods** selection box.

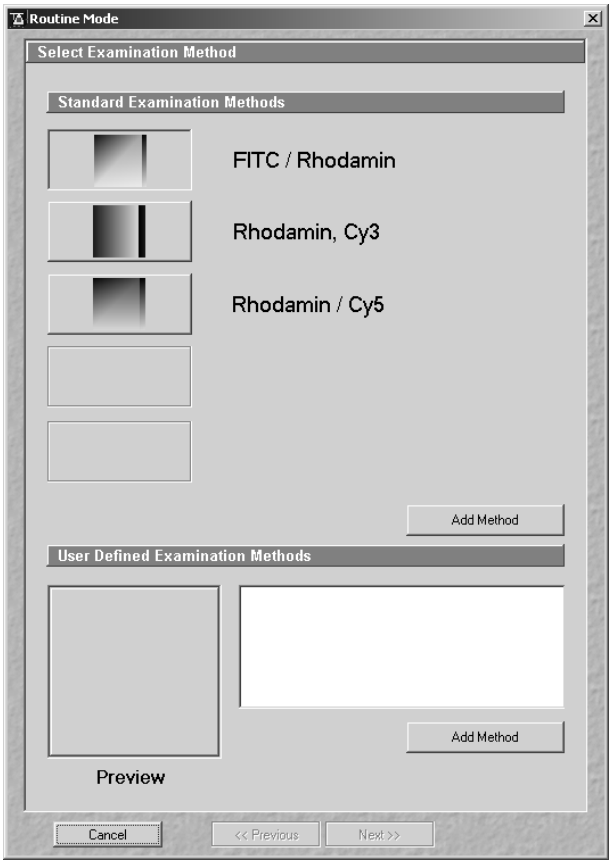

Fig. 6-2 Routine Mode - Select Examination Method window (first application)

Activate User-Defined Examination Methods in the Routine Mode

☞ The examination method to be imported has been exported from the Expert Mode to the Routine Mode using the **Export RM** function in the **Options** menu (see chapter 4).

- Start the Routine Mode.
- Click on the **Add method** button.
  - The **Select Method To Add To List** window appears on the screen.

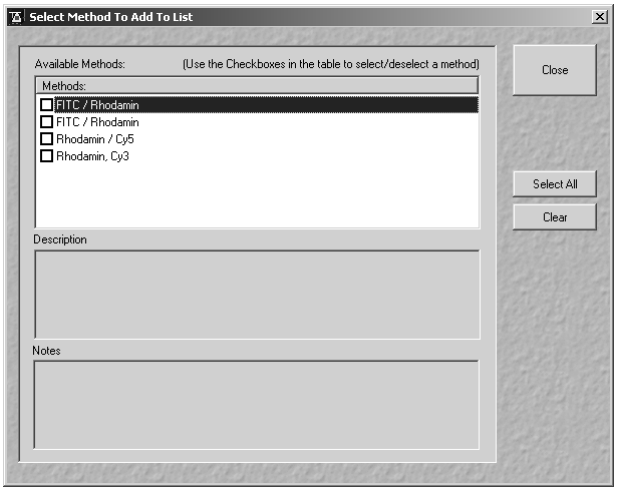

Fig. 6-3 Select Method To Add window

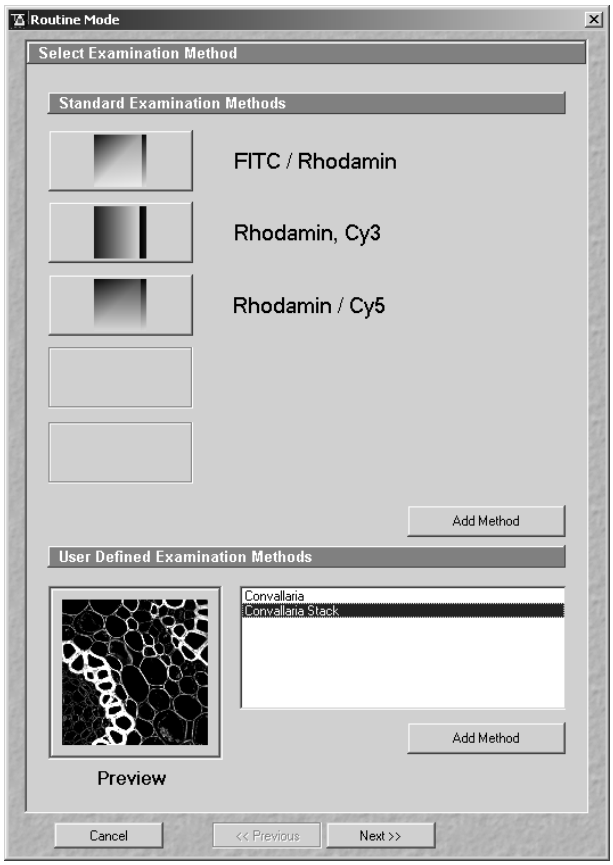

Fig. 6-4 Routine Mode - Select Examination Method window (user defined method activated)

The method taken over from the Expert Mode appears, with the entered name, in the **Methods** panel of the **Select Method To Add To List** window, though without an icon. The method can now be activated as **Standard** or **User-Defined Examination Method**.

- Click on the **Add Method** button.
  - The **Select Method To Add To List** window appears on the screen.
- Use the checkboxes in the **Methods** frame to select / deselect a table.
- Click on the **Select All** button to select all current methods.
- Click on the **Clear** button to deselect all current methods.
- Exit the **Select Method To Add To List** window by clicking on **Close**.
  - The **Routine** window appears on the screen again. The method appears in the list of **User Defined Examination Methods**.
  - An overview image (Preview) for the selected method and the name of the scan mode used are displayed in the **User Defined Examination Methods** panel.

6.1.3 Application of Examination Methods in the Routine Mode

The application of the Routine Mode is menu-controlled. The windows required for the creation of an image are limited to a minimum, and are opened one after the other using the button bar at the bottom of each window.

Lines, frames, stacks, time series and a combination of stacks and time series (4D: XYZT) can be recorded.

Additional windows will be displayed for the creation of stacks and time series. Lambda Stacks cannot be created in the Routine Mode.

The selected scan mode is displayed in all windows (except the Microscope Setup window) for checking purposes. It is possible to change to the next or previous window.

The buttons at the bottom of the relevant windows have the following functions:

Cancel button	Cancels the procedure, the Routine mode is finished.
<< Previous button	Activation of the previous window.
Next >> button	Activation of the next window.
Finish button	Ends the Routine mode; this button is active only in the **Image Acquisition** window.

6.1.3.1 Acquisition of a Frame

(1) Selection of the examination method

(a) Standard Examination Methods

- Click on the button of the required standard examination method, e.g. **FITC / Rhodamine**.

In case the current system has a META detector in channel 1 one has to define a method first for the routine mode.

– The **Routine Mode - Microscope Setup** window will appear.

(b) User Defined Examination Methods

- Click on the name of the examination method in the selection box of the **User-Defined Examination Methods** panel, e.g.: **Convallaria**.
- Then click on the **Next** button.

– The **Routine Mode - Microscope Setup** window will appear.

(2) Routine Mode - Microscope Setup window

- Prepare your specimen for examination in the same way as in the Expert mode, (see chapter 4). As in the Expert mode, different windows are available for the Axio Imager.Z1, Axioskop 2 FS MOT and the Axiovert 200 M.
- Set the microscope parameters for transmitted light or reflected light by clicking on the appropriate graph (see chapter 4).
- Clicking on the **Stage** button enables you to open the **Stage and Focus Control** window and to perform motorized focusing and specimen positioning.

The **Stage and Focus Control** window is identical to that of the Expert Mode and is handled in the same way (see chapter 4).

The **Stage** button is also available in the **Image Setup, Image Stack Setup** and **Time Setup** windows.

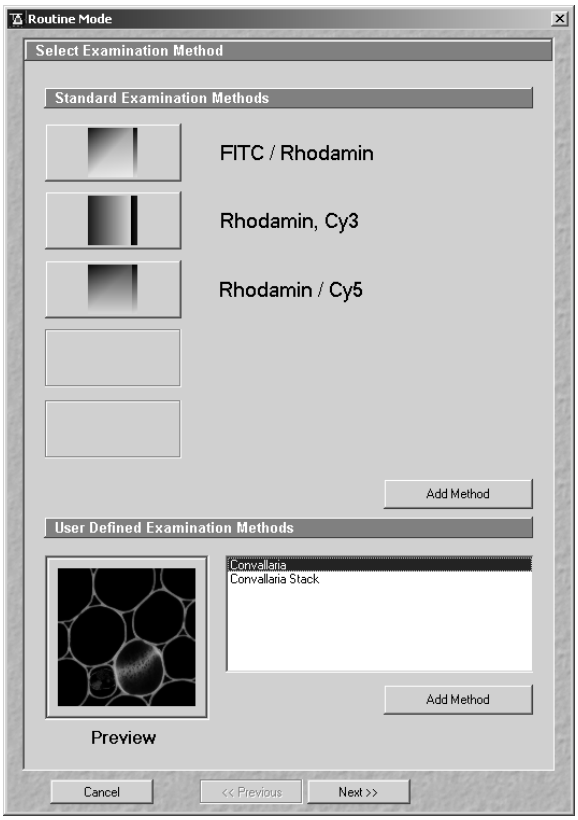

Fig. 6-5 Select Examination Method window

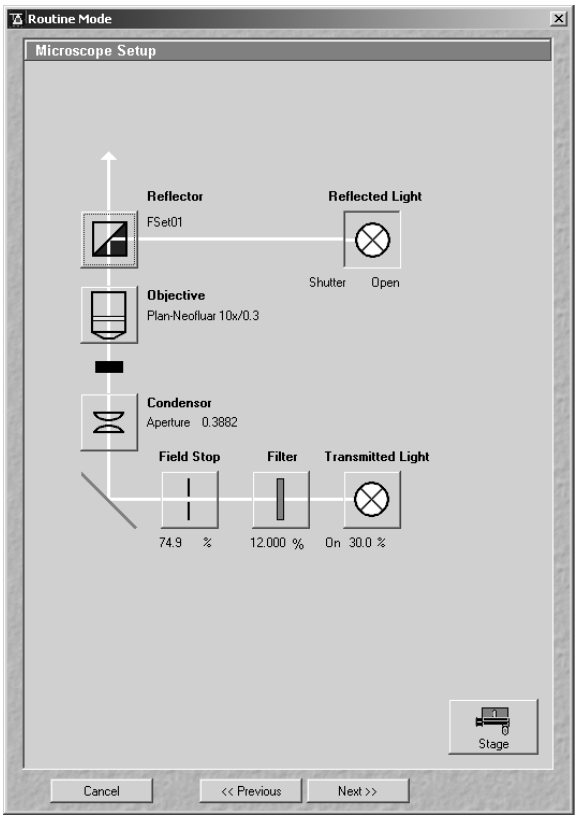

Fig. 6-6 Microscope Setup window

- When you have completed your microscope settings, click on the **Close** button to close the **Stage and Focus Control** window.
- Click on the **Next** button.
  - The **Routine Mode - Image Setup** window will appear.

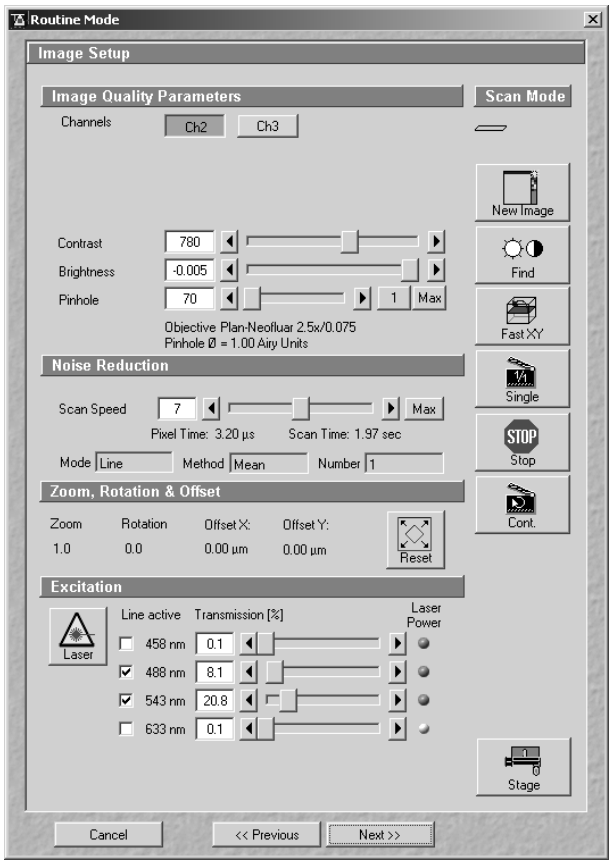

Fig. 6-7 Image Setup window

(3) Routine Mode - Image Setup window

When the **Image Setup** window is opened, all the parameters of the selected examination method are set automatically:

- **Image Size**
- **Noise Reduction** corresponds to **Speed**
- **Integration Time** corresponds to **Pixel Time**
- **Data Depth**; **Scan Direction** (bi-directional setting is taken over, but is not useful and will not be displayed)
- **Zoom**, **Rotation** and **Offset** are exported with the values **1**, **0** and **0**
- **Beam Path** configuration (also Multitracks)
- **Contrast** corresponds to **Detector Gain**
- **Brightness** corresponds to **Ampl. Offset**
- **Optical Slice Thickness**
- **Excitation**
- **Average**

The parameters for Ratio and Bleaching cannot be exported to the Routine Mode.

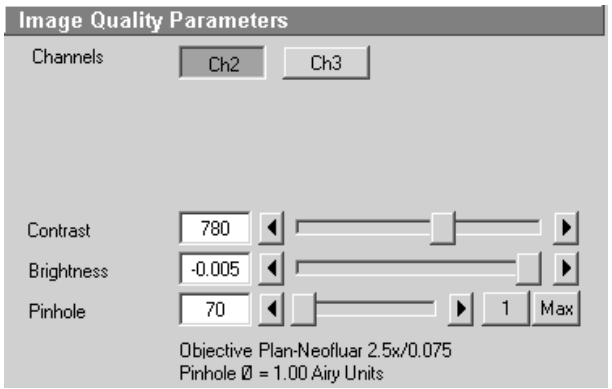

Fig. 6-8 Image Quality Parameters panel

(a) Image Quality Parameters panel

The setting possibilities of the **Image Quality Parameters** panel permit the image quality to be optimized online (continuous scan procedure).

Optimization is performed track by track (if several tracks are used) and separately for each detector (channel), as in the Expert mode.

(b) Noise Reduction panel

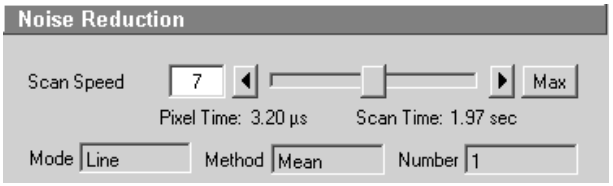

Fig. 6-9 Noise Reduction panel

In the **Noise Reduction** panel, the scan speed and, depending on it, the scan time can be influenced.

The longer the scan time, the better the noise suppression.

- Use the **Scan Speed** slider to set the slowest acceptable **Scan Time**.

Average Mode, Method and Number are taken over from the Expert Mode and displayed, but they cannot be edited.

(c) Zoom, Orientation & Offset panel

Fig. 6-10 Zoom & Orientation panel

The set zoom, rotation and offset values are displayed in the **Zoom & Rotation** panel.

If the Crop function is used in the Image window, it can be deactivated again using the **Reset** button.

- Click on the **Reset** button to deactivate the **Crop** function.

(d) Excitation panel

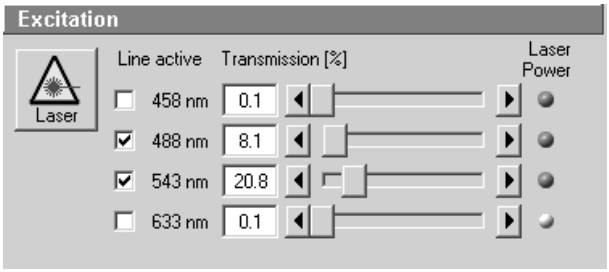

Fig. 6-11 Excitation panel

In the **Excitation** panel, the wavelength used (**Line Active** check box), the relevant transmission value (**Transmission** slider with input box) and the on / off laser status are displayed.

The laser status display indicates whether the laser has been switched on for the activated wavelength (green - on; gray - off).

If required, further wavelengths can be activated via the **Line Active** ☑ check box.

The laser setting is performed in the same way as in the Expert Mode (see chapter 4).

If the laser required for the activated wavelength is not switched on, the relevant message will be provided on the screen in a special window.

- In such a case, open the **Laser Control** window by clicking on the **Laser** button and set the relevant laser to **On**.

(e) Function buttons in the Routine Mode - Image Setup window

The selected scan mode is displayed at the top right of the **Image Setup** window for checking purposes.

The buttons arranged below have the following functions:

New Image button	Opens a new image window
Find button	Triggers single scan with automatic optimization of image brightness and contrast.
Fast XY button	Triggers continuous scan with maximum speed. This function should be used to a limited extent and only for a short period of time.
Single Scan button	Triggers single scan.
Stop Scan button	Stops the current scan procedure.
Contin. Scan button	Triggers continuous scan.
Stage button	Opens the **Stage and Focus Control** window.

(f) Start scanning

- Click on **Single Scan** or **Contin. Scan** to trigger the scanning procedure.
- If required, optimize the default parameters in the **Excitation**, **Zoom, Orientation & Offset**, **Image Quality Parameters** and **Noise Reduction** panels (using continuous scan).
- Click on **Stop Scan**.
- Click on **Next**.
  - The **Routine Mode - Image Save, Print & Export** window appears.

(4) Routine Mode - Image Save, Print & Export window

The **Routine Mode** - **Image Save, Print & Export** window is used for data backup:

The set parameters are displayed in the **Information For Method: ...** panel.

The buttons **Save**, **Save As**, **Multi Print** and **Export** provided in the **Save** panel permit you to store, print or export scanned images. The procedure is identical to that in the Expert Mode.

- Click on **Save** or **Save As** to store the acquired image.
- Click on **Finish** to exit the Routine Mode and return to the LSM 510 Switchboard menu.

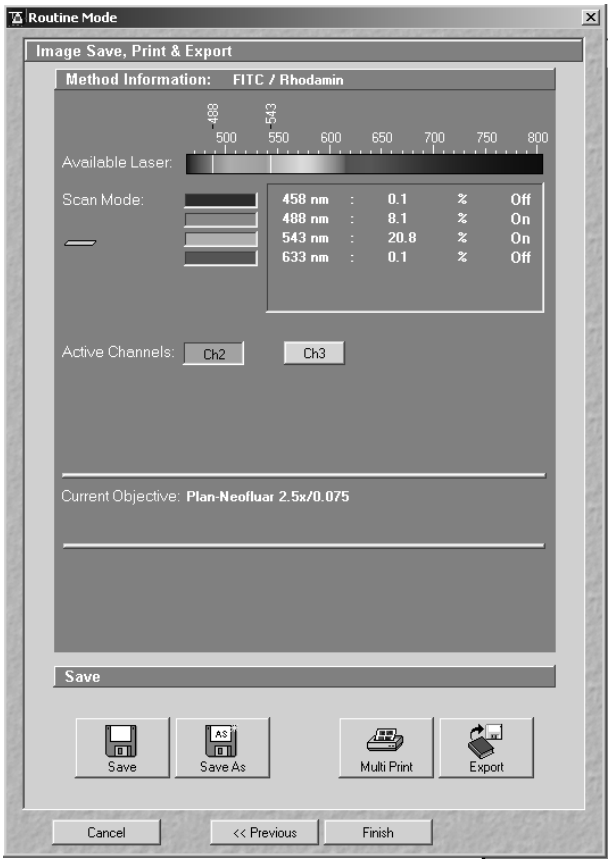

Fig. 6-12 Image Save, Print & Export window

6.1.3.2 Acquisition of a Z Stack

For the acquisition of a Z Stack, the **Routine Mode - Image Stack Setup** window showing the Z parameters is additionally displayed. The parameters correspond to those of the imported Examination Method and, if necessary, have to be matched to the specimen under examination.

- Load the required stack examination method from the **Routine Mode - Select Examination Method** window.
- Open the **Routine Mode - Microscope Setup** and **Routine Mode - Image Setup** windows one after the other by clicking on **Next**. Check the settings and change them, if required (see section 6.1.3.1, page 6-5).
- Click on the **Next** button.
  - The **Routine Mode - Image Stack Setup** window becomes visible.
- You can take over the offered parameters by clicking on the **Start** button.
  - The image stack will be recorded.
- The scan in progress can be stopped at any time by clicking on the **Stop** button.

If Z-settings must be changed, proceed as described in the following.

(1) Routine Mode - Image Stack Setup window

The buttons arranged on the right in the **Image Stack Setup** window have the following functions:

New Image button	Opens a new image window.
Start button	Triggers scanning of a stack.
Stop Scan button	Stops the current scan procedure.
XYscan button	Triggers single XY-scan.
XYcont button	Triggers continuous XY-scan.
Stage button	Opens the Stage and Focus Control window.

The definition of the stack parameters in the **Image Stack Setup** window is made in accordance with the exported Examination Method. If the exported method was created using the PIEZO 200 fine focusing stage, the Z-parameters are defined via the **Line Sel** / **Range** function of the **Hyperfine Z Sectioning** panel.

Otherwise, use the **Mark First/Last** function for the definition of the Z-parameters.

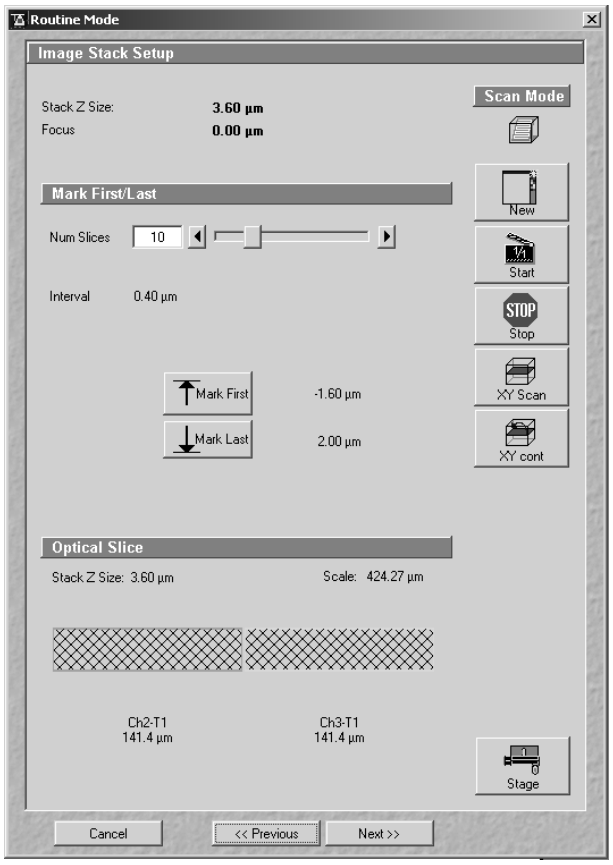

Fig. 6-13 Image Stack Setup window (Mark First/Last)

Mark First/Last function

The parameters of the Z Stack to be created are also displayed in the window and can be changed online.

Stack Z Size: Optical indication, i.e. the optical beam moves over this (displayed) Z-thickness (online). The stage (nosepiece) is moved in such a way that the stack size, dependent on the refractive index, is achieved optically.

Focus Position: Mechanical indication, i.e. if the refractive index changes, the value of the focus position also changes in relation to the "0" value (online).

Z Sectioning: Setting of **Number of Slices**, **Interval** and **Current Slice** via sliders / arrow buttons.

Mark First/Last: Determination of the Z-value for the first and last XY-image of the stack, combined with manual focusing or **Stage** control.

Optical Slice: The **Optical Slice** window displays the following information:
Black:
Stack Z Size (µm) = intervals x (number of slices - 1)
Optimal Interval = depending on the objective used and the pinhole diameter setting
Red and other colors:
Presentation of the actual data set by the operator helps to optimize stack creation.

For redefining the parameters of a Z Stack, use the setting functions of the **Mark First/Last** panel.

The optimum stack size (definition of the upper and lower limits of the stack) is determined by focus adjustment during a continuous scan.

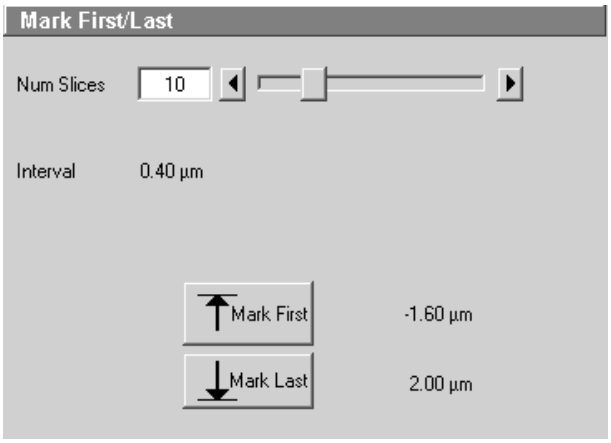

Fig. 6-14 Mark First/Last panel

- Click on the **XY cont** button.
  - A continuous XY-scan of the set focus position will be performed.
- Use the manual focusing drive or the **Stage and Focus Control** window to focus on the upper position of the specimen area where the Z Stack is to start.
- Click on the **Mark First** button to set the upper position of the Z Stack.
- Then focus on the lower specimen area where the recording of the Z Stack is to end.
- Click on the **Mark Last** button to set this lower position.
- The **Num Slices** slider enables you to set the number of slices. The limits of the Z Stack remain constant; the interval is matched accordingly.

The changes made are visualized online in the graphic display of the **Optical Slice** panel.

- Click on the **Start** button to start the recording of the Z Stack.

In case the upper and lower limits of the stack have been switched round, automatic matching will be performed by the software, since the stage of the Axio Imager.Z1 always moves from bottom to top and the nosepiece of the Axiovert 200 M always moves from top to bottom.

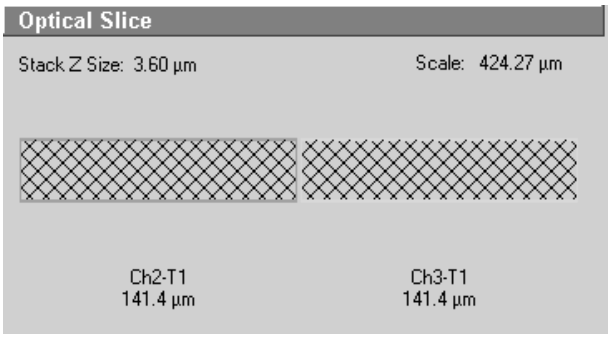

Fig. 6-15 Optical Slice panel

In case a line scan in Z is performed the following function buttons are available in the **Line** toolbar of the image created using the Line Select button **Line Sel**:

Function	**Description**
Arrow selection button	Activates the mouse pointer for the selection, positioning and size modification of the cutline in the Image window. Size modification: Click on the drag point and keep the mouse button pressed. Drag the point and release the mouse button. Shifting: Click on the line and keep the mouse button pressed. Shift the complete line and release the mouse button.
Line arrow button	Generation of a straight cutline in any direction in the Image window.
Opened free shape curve button	Generation of an open, free shape curve (spline) in the Image window. The first click sets the starting point, each further click adds a line segment. A click with the right mouse button ends the process.
Line button	Selecting the line width of the cutline.
Color button	Selecting the color of the cutline.

- Click on the **Line arrow** button or the **Opened free shape curve** button in the **Line** toolbar.
- Define a straight line or a free shape curve (spline) as the cutline for the XZ scan.

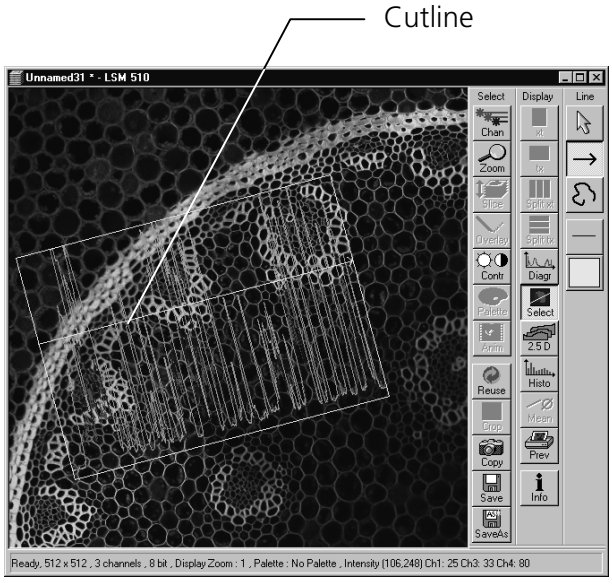

Fig. 6-16 Image window (Line Sel)

- Then click on the **Range** button.
  - The XZ-scan will be performed and displayed in the image window. At the same time, the position of the current slice is shown with a green line and the positions of the first and last slice with two red lines.

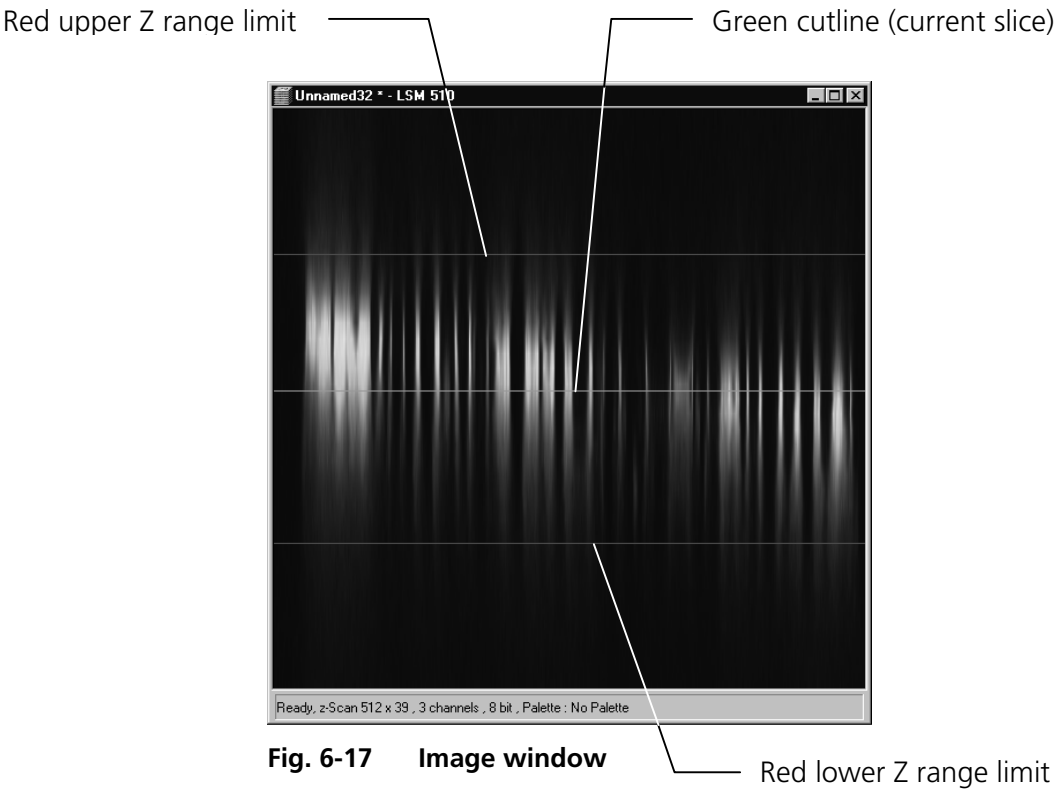

Fig. 6-17 Image window

- Moving the green line (current slice) enables you to change the current focus position (moving the stage or nosepiece in the process). If the green line (Current Slice) is shifted after the creation of **Range**, the focus position will change (the piezo fine focusing stage remains in the center position).
- Shifting one of the red lines enables you to change the stack size. The red lines (stack limits) can only be changed symmetrically to the Current-Slice position within the piezo fine focusing stage work range.

☞ Since the piezo fine focusing stage moves from bottom to top during the creation of the Z Stack, top and bottom of the Axiovert 200 M have been switched round.

- A click on the **Start** button will start the recording of the Z Stack.
- When recording of the Z Stack is completed, click on **Next**.
  - The **Routine Mode - Image Save, Print & Export** window appears.

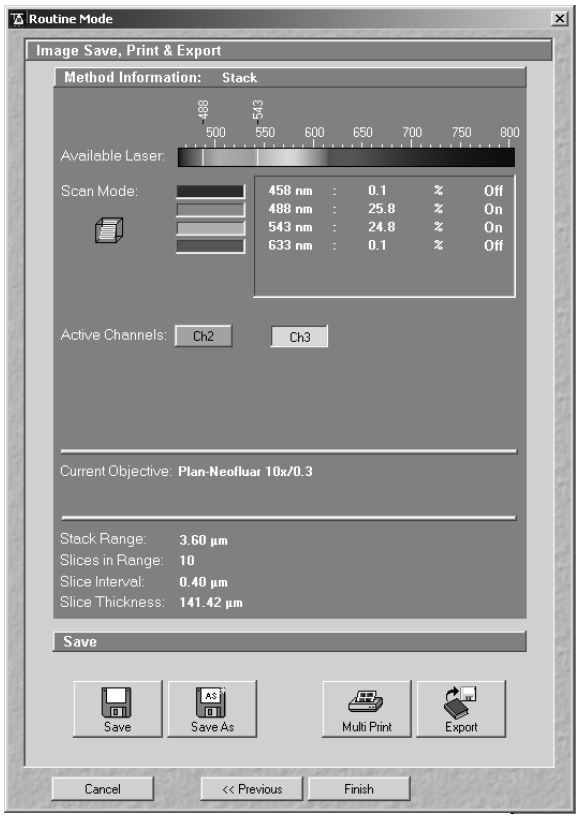

Fig. 6-18 Image Save, Print & Export window

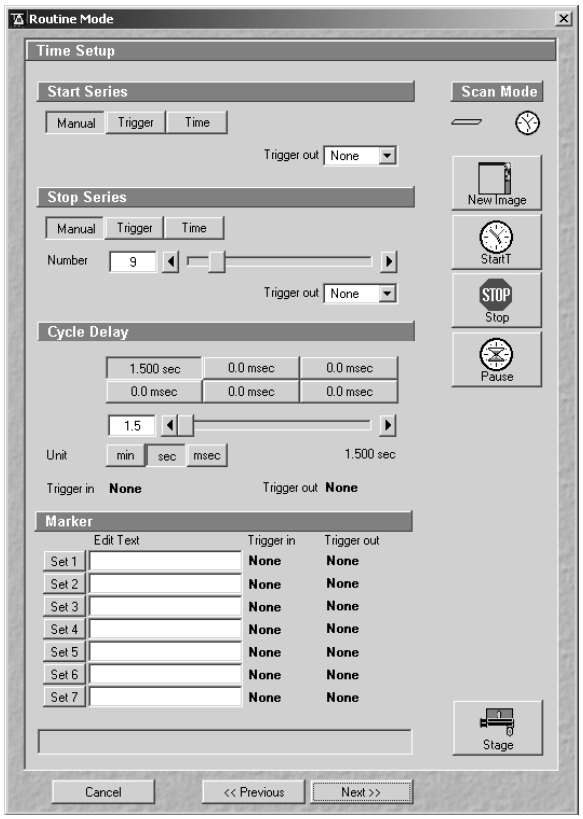

Fig. 6-19 Time Setup window

(2) Routine Mode - Image Save, Print & Export window

The set parameters are displayed in the **Information For Method: ...** panel.

The buttons **Save**, **Save As**, **Multi Print** and **Export** provided in the **Save** panel permit you to store, print or export scanned images. The procedure is identical to that in the Expert Mode.

- Click on **Save** or **Save As** to store the acquired stack image.
- Click on **Finish** to exit the Routine Mode and return to the LSM 510 Switchboard menu.

6.1.3.3 Acquisition of a Time Series

A time series is recorded in the Routine mode in the same way as a frame.

Furthermore, the Time settings are displayed in the **Routine Mode - Time Setup** window. The parameters set here correspond to those of the imported examination method and must be matched, if required.

The time series can be triggered / ended only in the way it was defined in the imported method.

- Load the required time series examination method from the **Routine Mode - Select Examination Method** window.
- Open the **Routine Mode - Microscope Setup** and **Routine Mode - Image Setup** windows one after the other by clicking on **Next**. Check the settings and change them, if required (see section 6.1.3.1, page 6-5).
- Click on the **Next** button.
  - The **Routine Mode - Time Setup** window becomes visible.

The following functions are available on the right side of the this window:

New Image button	Opens a new image window.
Start T button	Starts the time series.
Stop button	Stops the entire time series. A current scan is canceled.
Pause button	Interrupts the time series. The button labeling changes to **Resume**. A current scan is performed until its end. Pressing the **Pause** button again will resume the time series.
Stage button	Opens the **Stage and Focus Control** window.

(1) Start Series panel

Depending on the imported examination method, the time series can be started and ended manually, via trigger or by entering a time (computer time). In the **Start Series & Stop Series** panel, the relevant mode is displayed. Switching between manual, trigger and time control is not possible in the Routine mode.

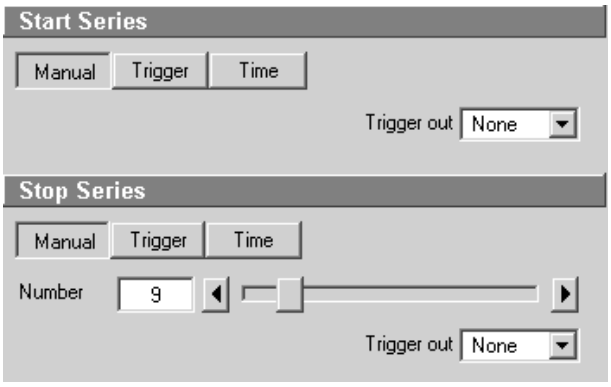

Fig. 6-20 Start & End panel

(a) Manual start of a time series

- You can take over the offered parameters by clicking on the **Start T** button.
  - The time series is recorded and displayed in the image window.
- Click on the **Stop** button to end the time series.

(b) Starting a time series via trigger

- The **Start T** button must be pressed first to start a time series via trigger.
  - **Waiting for Trigger** will be displayed in the status line.
- Then the relevant trigger key of the Trigger Control must be pressed to start the first scan procedure of the time series.
- The time series is ended by pressing the relevant trigger key.

If the number of cycles entered under **Number** has been processed without a trigger impulse having been given to end the procedure, the time series is finished.

If a trigger signal is given before the cycles have been processed, the time series will only be interrupted. **Waiting for Trigger** will be displayed in the status line. The time series can now be continued via a new trigger signal or ended via **Stop**.

(c) Starting a time series via the time

- To start a time series via the time, the **Start** button must be pressed first.
  - **Waiting for Start Time** will be displayed in the status line. The time series is started when the starting time has been reached.
  - The time series is interrupted when the end time has been reached.

If the entered **Number** of cycles has been processed, the time series is finished.

If the number of cycles has not yet been processed, the time series is only interrupted. **Waiting for Start Time** is displayed in the status line. The time series can now be continued by entering a new start time or finished via **Stop**.

The end time for the time series can be changed online.

(d) Input of the number of scan cycles

The currently set number of scan cycles is displayed in the **Number** input box of the **Start & End** panel. This number can be changed, if required.

- Set the required **Number** of scan cycles using the sliders or the arrow buttons.

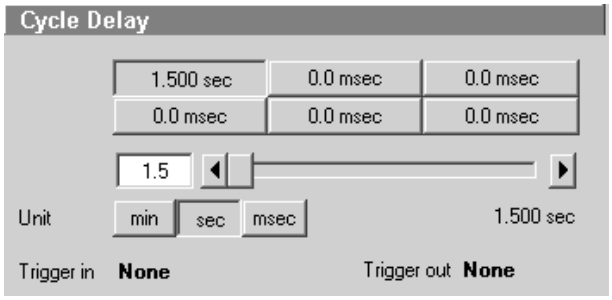

Fig. 6-21 Time Delay panel

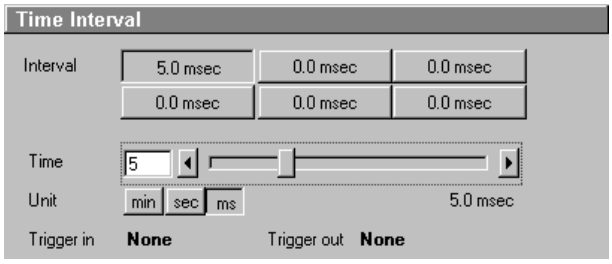

Fig. 6-22 Time Interval panel

(2) Cycle Delay / Time Interval panels

Depending on the Examination Method exported from the Expert Mode, the time series interval is defined either as a **Cycle Delay** or **Time Interval**. Accordingly, either the **Time Delay** panel or the **Time Interval** panel is displayed in the **Routine Mode - Time Settings** window.

Time Delay is the interval between the end of one scan process and the beginning of the next.

Time Interval is the interval between the beginning of one scan process and the beginning of the next.

The **Scan Interval** panel permits the intervals to be activated and changed.

The following functions are available:

Interval buttons	Activation of the interval set for the time series for the relevant button.
Time input box / arrow buttons / slider	Determination of the interval for the currently selected time button.
Unit buttons	Selection of the time unit: **min**, **sec** or **ms**.
Trigger in field	Display of the trigger keys (1-4) for the in signal.
Trigger out field	Display of the trigger keys (1-4) for the out signal.

- The interval to be used during the time series is set to a default value by activating a **Time** button.

The time displayed on the time buttons can be changed as follows, if required:

- Activate a **Time** button with a click of the mouse.
- Use the slider (arrow buttons or input box) near **Time** to set the required interval. The set time will be displayed online on the button. Select the required time unit by clicking on the relevant **Unit** button.

You can assign different times to all the six **Time** buttons.

- Changing to another interval is possible by activating another **Time** button during a current time series.

☞ If the interval change was made via trigger key in the imported examination method, the assigned **Time** button will be activated on pressing the displayed trigger key.

(3) Marker panel

The setting of a marker permits information about the moment in the current time series and any required comment to be assigned to the current scan. The time indication is set automatically, while comments must be defined before.

The markers (red squares) are visible in the Image window if the **Gallery** button (**Display** toolbar) is activated.

On storage of the image, all the markers, including the time indication and the comments, are stored along with the image contents.

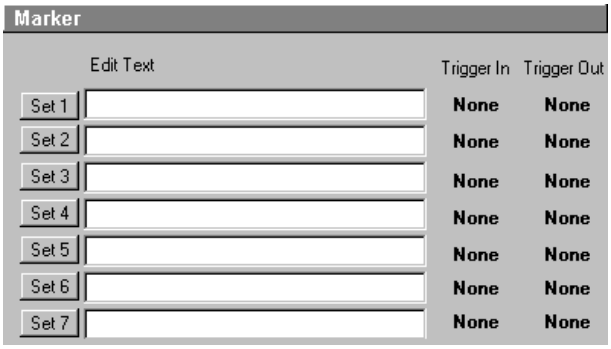

Fig. 6-23 Marker panel

The following functions are available:

Set 1-7 button	Setting of a marker during the scan procedure.
Edit Text input box (1-7)	Entry of the comments for the marker.
Trigger in field (1-7)	Display of trigger keys (1-4) used to set the marker.
Trigger out field (1-7)	Display of trigger keys (1-4) for the out signal.

- Click once in the edit text box of the required marker. Enter the comments via the keyboard. Then click outside the editing box to close this box.
- Clicking on one of the **Set 1** to **7** marker buttons will set a marker for the current scan.

(4) Recording a time series

- Set the relevant parameters for time control in the **Start Series, Stop Series**, **Time Delay** and **Marker** panels.
- Start the time series by clicking on the **Start T** button.
- If you use Trigger Control, press the relevant trigger key to start the time series with the first scan procedure.
- Use the **Set 1** to **Set 7** buttons to set markers during the scanning procedure which will allow you to evaluate interesting scanning images later.
- End the time series by clicking on the **Stop** button.
- Click on **Next**.

– The **Routine Mode** - **Image Save, Print & Export** window appears.

☞ It is also possible to record a Z Stack in the form of a time series (4D Image).

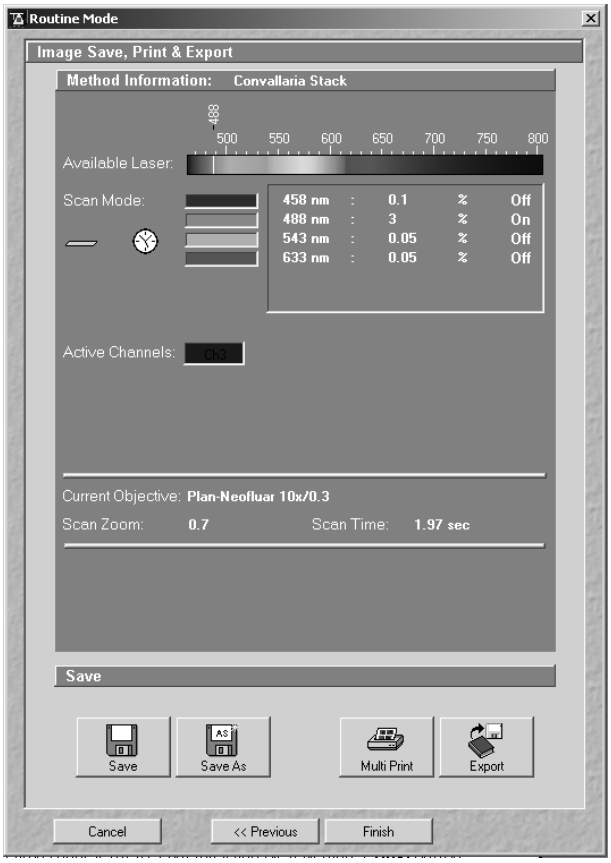

Fig. 6-24 Image Save, Print & Export window

(5) Routine Mode - Image Save, Print & Export window

The set parameters are displayed in the **Information For Method: ...** panel.

The buttons **Save**, **Save As**, **Print** and **Export** provided in the **Save** panel permit you to store, print or export scanned images. The procedure is identical to that in the Expert Mode.

The also available **Save** and **Save As** buttons permit scanned images to be stored in a database (see chapter 4).

- Click on **Finish** to exit the Routine Mode and return to the LSM 510 Switchboard menu.

6.2 Tools

6.2.1 Change Filters

The **Change Filters** tool is used to update the filter data in the software after a change of filters in the reflector turret.

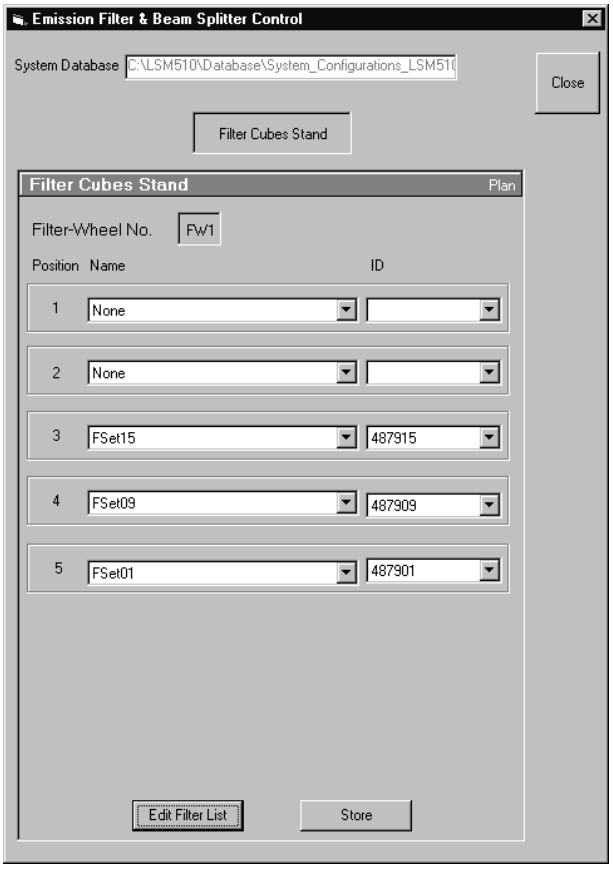

Fig. 6-25 Emission Filters & Beam Splitter Control window

- Close the LSM 510 software program.
- Insert the new filter module in the reflector turret.
- Double-click on the **Change Filters** icon on the desktop.
  - The **Emission Filter & Beam Splitter Control** window appears on the screen. The name of the currently used database is displayed in the **System Database** box, with the filter type being indicated below for checking purposes.
  - The **Filter Cubes Stand** panel shows the **Filter-Wheel No.** and the filter positions available.
  - Use the **Name** and **ID** selection boxes to enter the filters installed in the individual positions of the filter wheel.
- Open the **Name** (or **ID**) selection box of the relevant filter position and select the new filter set from the list.
- Click on the **Store** button to accept the new settings.
- Click on the **Close** button to close the **Emission Filter & Beam Splitter Control** window.

☞ All available filter sets have to registered in the filter list (see **Edit Filter List** function, next page).

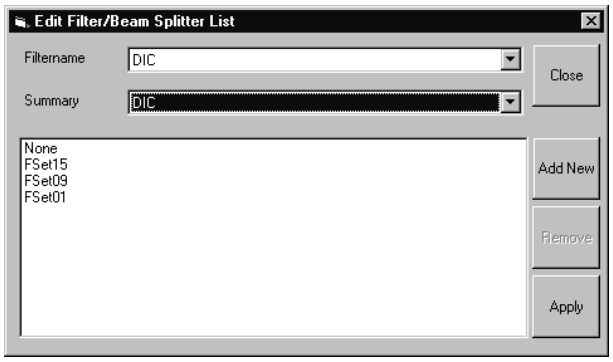

Fig. 6-26 Edit Filter/Beam Splitter List window

Edit Filter List

The **Edit Filter List** function permits updating of the filter data in the software after a change of filters on the stand.

- Close the LSM 510 software program.
- Double-click on the **Change Filters** icon on the desktop.
- Click on the **Edit Filter List** button in the **Emission Filter & Beam Splitter Control** window.
  - The **Edit Filter/Beam Splitter List** window is opened.

This window permits a list of the most frequently used filter sets to be compiled.

- Click on the arrow button in the **Filtername** list box to open it.
- Select the filter set which shall be included in the list.
- Click on the **Apply** button.

The selected filter set is included and displayed in the list (below the **Sumary** list box).

This filter set is now also available in the **Name** selection boxes of the **Filter Cubes Stand** panel and can be assigned to a filter wheel position.

To remove a filter set which is no longer needed from the list, proceed as follows:

- Click on the name of the filter set concerned in the list box of the **Edit Filter/Beam Splitter List** window.
- Click on the **Remove** button. The filter set is deleted from the list and is then no longer available in the **Filter Cubes Stand** panel of the **Emission Filter & Beam Splitter Control** window.

Add New

This function permits new filter sets to be added to the database.

For this, proceed as follows:

- Click on the **Add New** button on the **Edit Filter/Beam Splitter List** window.
  - The **Add New Filter/Beam Splitter** window is opened.
- Enter the data of the new filter set in the **Filter Cubes Stand Description** panel, then click on the **Apply** button.

The new filter set is stored in the database and included in the **New Filter Cubes Stand** panel. You can now activate the filter for a filter wheel position using the procedure described above.

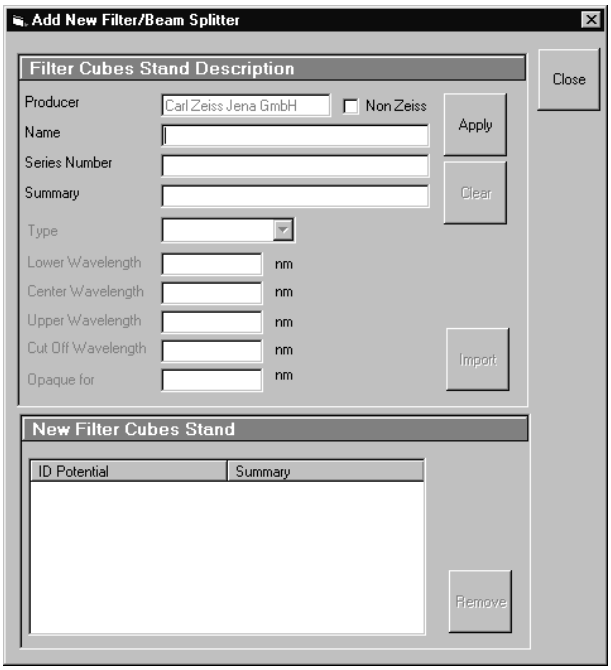

Fig. 6-27 Edit Filter/Beam Splitter List window

☞ If you have activated the **Non Zeiss** check box, filter sets from other manufacturers can also be included in the database.

- To remove an new filter set from the database, select it with a click of the mouse in the **New Filter Cubes Stand** panel and then click on **Remove**.
- Click on **Close** to close the **Add New Filter/Beam Splitter** window.

- Click on **Close** to close the **Edit Filter/Beam Splitter** List window.
- Click on the **Store** button to accept the new settings.
- Click on the **Close** button to close the **Emission Filter & Beam Splitter Control** window.

When you start the LSM 510 software, the filter data are updated.

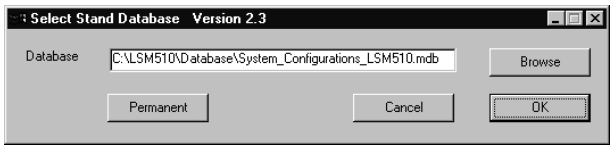

Fig. 6-28 Select Stand Database ... window

6.2.2 Stand Select

The **Stand Select** tool permits a new or updated database to be assigned to the LSM 510 software program. This function should preferably be performed by authorized service personnel.

If this is not possible, proceed as follows:

- Close the LSM 510 software program and double-click on the **Stand Select** icon on the desktop.
  - The **Select Stand Database** window appears on the screen. The currently used database is displayed in the **Database** box.

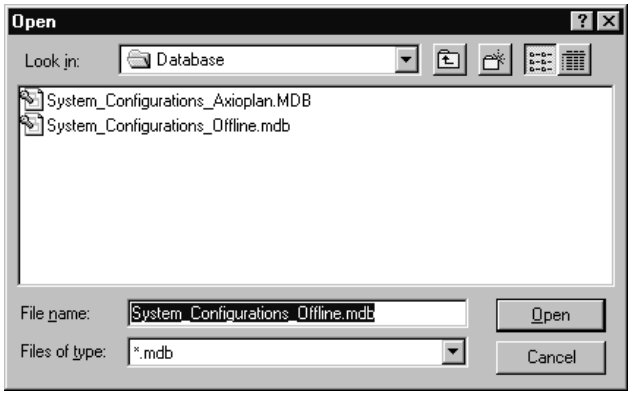

Fig. 6-29 Open window

Fig. 6-30 Select Name window

- Click on the **Browse** button to activate the new database.
  - The **Open** window appears on the screen.
- Select the directory where the new database is stored.
- Click on the name of the database (file extension: ***.mdb**) and then on the **Open** button.
  - The **Open** window is closed and the name of the new database appears in the **Database** box.
- Click on the **Permanent** button. The **Select Name** window appears.
- Select the relevant stand icon from the **Icon** list box and click on **OK**. The **Select Name** window is closed and the desktop icon is updated.
- Then click on the **OK** button in the **Select Stand Database ...** window to accept the new settings and to close the window. (Clicking on **Cancel** will cancel the procedure.)
  - After the next restart of the LSM 510 software program, the new database will be automatically read in.

6.2.3 LSM Image Browser

The **LSM Image Browser** permits images to be loaded, imported, exported and printed quickly without having to open the LSM 510 software. The **LSM Image Browser** can be used without dongle.

When images are opened, image processing functions of the LSM 510 software are available to a limited extent (**Chan**, **Zoom**, **Contr**, **Palette**, **Copy**, **Save**, **Save As**, **xy**, **Split xy**, **Prev**, **Info**).

- Click on the **LSM Image Browser** icon on the desktop of the PC. The **Zeiss LSM Image Browser** main menu is opened.

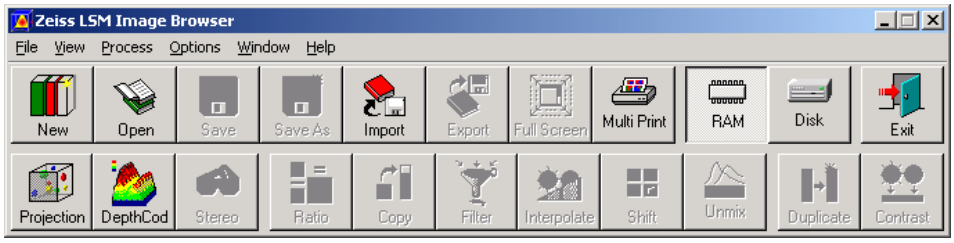

Fig. 6-31 Zeiss LSM Image Browser main menu

The following function buttons are available:

New button	Opens a new database.
Open button	Opens an existing database.
Save button	Saves the current image.
Save As button	Saves the current image under a new name.
Import button	Imports images.
Export button	Exports images.
Full Screen button	The current image is displayed on the full screen. Deactivation of the function with a click of the mouse.
Multi Print button	Several images are printed on one page.
RAM button	Use of the RAM memory for image display.
DISK button	Use of the hard disk as storage medium for image display.
Exit button	The **Zeiss LSM Image Browser** main menu is closed.

The functions **New**, **Open**, **Save**, **Save As**, **Import, Export** and **Multi Print** correspond to those of the Expert Mode of the LSM 510 software and have already been described in chapter 4.

6.2.4 LSM Image Examiner

The **LSM Image Examiner** can be used without having to open the LSM 510 software. However, this requires the installation of the relevant dongle. The **LSM Image Examiner** provides all the functions of the **LSM Image Browser**, plus the 3D functions and selected **Process** functions of the Expert Mode of the LSM 510.

When images are opened, a large scope of the image processing functions of the LSM 510 software is available (for further details see in chapter 4).

- Click on the **LSM Image Examiner** icon on the desktop of the PC. The **Zeiss LSM Image Examiner** main menu is opened.

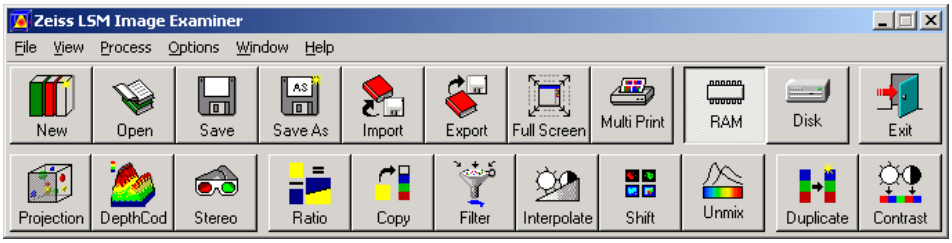

Fig. 6-32 Zeiss LSM Image Examiner main menu

In addition to the buttons of the **LSM Image Browser** mentioned above, the following function buttons are available in the lower row of the **Zeiss LSM Image Examiner** main menu:

Projection button	One single projection or a series of projections can be calculated after rotation of the data package about the X, Y or Z axis.
DepthCod button	The depth information contained in a sequence can be colored with the colors of the rainbow.
Stereo button	Stereoscopic images can be generated.
Ratio button	Permits two channels to be linked into a new channel by the creation of a ratio.
Copy button	Permits one channel each of an existing image to be copied and stored as a new image.
Filter button	Permits the subsequent processing of scanned images via the integrated filters.
Interpolate button	Permits the continuous contrast and brightness change in a stack or time series through interpolation between the starting and end values.
Shift button	Produces a congruent image with relation to the pixels of the various channels.
Unmix button	Provides the function Linear Unmixing for lamda stacks using stored reference spectra and various parameters for unmixing.
Duplicate button	Permits images (including Z Stacks and Time Series) to be duplicated completely.
Contrast button	Permits the subsequent modification of contrast and brightness of the stored image.

These functions correspond to those of the Expert Mode of the LSM 510 software and have already been described in chapter 4.

CHAPTER 7 3D FOR LSM

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7 3D FOR LSM

7.1 Overview and Explanations

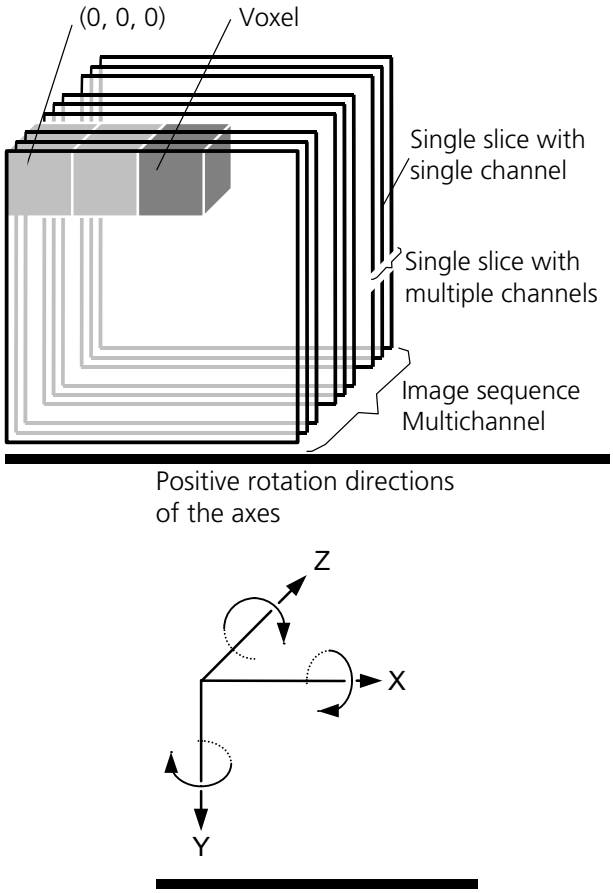


Fig. 7-1

7.1.1 The Image Sequence

The "3D for LSM" handles image sequences generated by the Zeiss LSM software. This can be three-dimensional image data or a time sequence of two-dimensional images (slices). Each slice (as well as the sequence) can consist of up to eight channels. An image sequence consists of a series of individual (2D) images and has a name that designates the entire sequence. In general an image sequence is handled as a single object in the system. Individual channels or slices can be addressed.

The following terms and definitions apply for the "3D for LSM" software.

- An image sequence is a number of individual sequential images (usually called slices in the dialog boxes), the spacing between which is equal.
- Image sequences can contain up to 12 bit of image data (per channel).
- A sequence (slice) can consist of up to eight channels.
- The maximum size of an image sequence is limited by the provided memory of the operating system.
- A voxel is the smallest element of an image sequence (the equivalent of a pixel in a 2D image). All voxels in a given image sequence are the same size.
- The coordinate system originates in the left upper front corner of the image sequence. This point has the coordinates 0, 0, 0.
- All angles are positive for rotations to the right in the direction of the positive coordinate axis (right-handed coordinate system).
- A slice is an individual image in a sequence of images. The numbering of the slices starts with "1".

Image sequences can consist of several channels. Most functions and the **Display window** are providing buttons to select all or a subset of channels stored in the selected image sequence. The **Output** image sequence will only get those channels which are selected on the input side. The button selects all channels in the image sequence to be used clicking with the left mouse button on it.

Clicking with the left mouse button on any of the number buttons toggles the state of this single channel.

Clicking with the right mouse button on any of the number buttons selects this single channel exclusively. All other channels are deselected.

7.1.2 The Image Properties

Every image sequence has its own set of properties. They contain the scaling and the scaling units. The scaling and its units are required for 3D reconstruction and measurement. If a sequence of LSM-TIFF images is read in, the image properties are loaded automatically from the file header and allocated to the image properties of the new image sequence.

7.1.3 Memory Usage

All images shown in the **Gallery** are currently loaded in the system memory of the operating system. Some functions need additional temporarily used memory during their execution.

If the memory is running low delete some images from the **Gallery**. If the images are needed afterwards they must be saved to disk first. Normally all functions produce a new result (output) image sequence. In order to save some memory, other image sequences currently presented in the **Gallery** can be selected as result position. The output image is overwritten by entry execution of a function.

7.2 User Interface

7.2.1 Introduction

This section describes the following main components of the system:

Main window — **Main window** with the **Menu**, the **Tool bar** and **Gallery**. All general system functions are located here.

Gallery — Normally several images are required in order to accomplish a particular task. These images are displayed in reduced size to provide an overview and facilitate selection. This area is located just below the **Tool bar**.

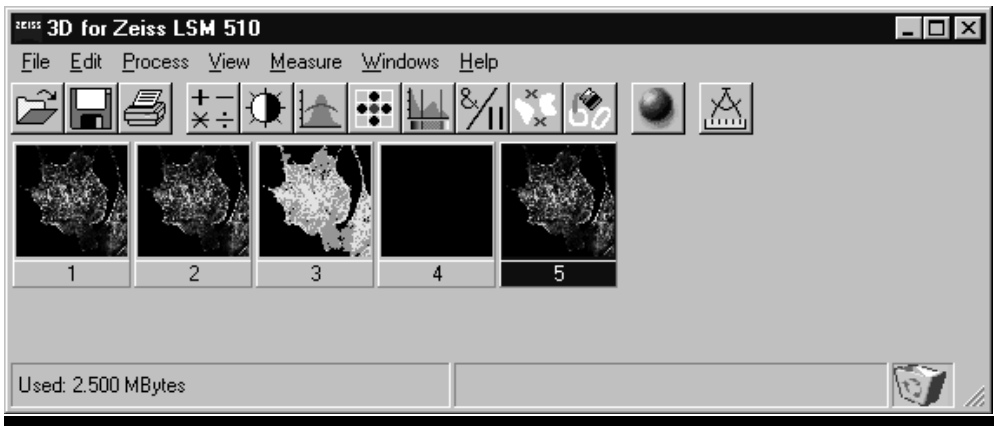


Fig. 7-2

Tool bar — This menu shows all image processing functions.

Display window This window is used to display image sequences.

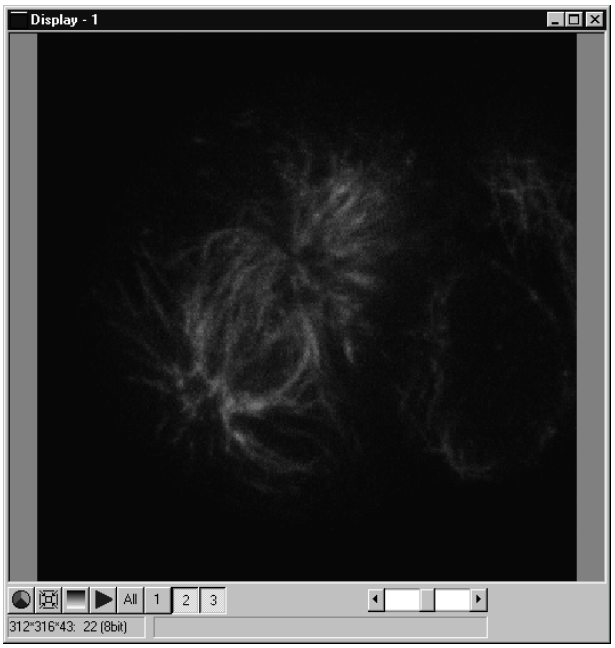


Fig. 7-3 Display window

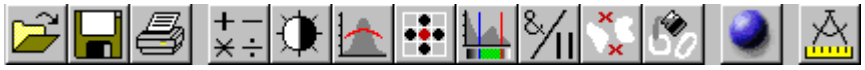
Dialog boxes All dialog boxes provide three buttons. Pressing the **OK** button executes the function with the defined parameters and closes the dialog window. Selecting the **Cancel** button does not execute the function, restores the parameters, and closes the dialog window. Pressing the **Apply** button executes the function with the defined parameters; the dialog window will stay opened.

7.2.2 Main Window

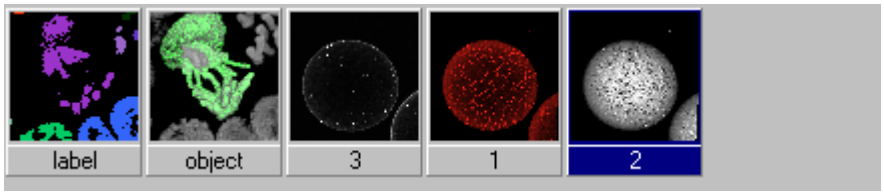
The **Main window** includes:

the **Menu**

the **Tool bar**



and the **Gallery**



File Menu

Open Image Opens a file selector dialog to load an image sequence.

Save Image As Opens a file selector to save an image or image sequence.

Save Display As Saves the currently shown contents of the Display window as a single colour image.

Print The printer parameters can be set with this tool. The standard Windows printer dialog is opened.

Exit Terminates the application.

Edit Menu

Copy Copies the contents of the **Display window** to the clipboard.

Edit Channels Allows to add or to remove channels to a single or multichannel image.

Delete All Images Deletes all images and image sequences from the memory.

Process Menu

Arithmetics Adds or subtracts the grey values of two image sequences (**Add**, **Subtract**).

Contrast Enhances the contrast and brightness of an image sequence (**Interactive**, **Automatic**, **Linearize**).

Smooth Smoothes an image sequence.

Morphology Performs morphological operations on image sequences (**Erode**, **Dilate**, **Open**, **Close**).

Segment Segmentates an image sequence to propose measurement (**Interactive**, **Automatic**).

Boolean Combines two image sequences by Boolean operations (**And**, **Or**, **Not**, **Xor**, **Mask**).

Scrap Selects or deletes objects of a defined size.

Fill Holes Fills holes in objects.

View Menu

Set Channel Colour The colour and the weight of the single channels can be defined.

Properties The properties of the image (e.g. scaling, use laser etc.) are displayed.

Render Calculates 3D reconstructions of an image sequence (Surface, Alpha).

Measurement Menu

Automatic Object Measures geometrical and densitometrical features (**General**, **Object Features**, **Volume Features**, **Condition**).

Windows Menu

Arrange All Arranges the windows automatically.

Display The current image is displayed in this window.

Help Menu

Content Opens the help for the software.

About 3D for LSM Displays status and release message of the software.

Tool Bar

This bar provides buttons with iconized images of nearly all functions. Clicking on one of the buttons will open a dialog window to define the function parameters. Selecting an entry from the menu alternatively can activate the same functions. Placing the cursor on a tool bar button will show a short description, if the window is activated.

Gallery

The **Gallery** is used as an overview of the images available in memory and their contents. It is located just below the **Tool bar**. Each small image represents a sequence. The middle slice of each image sequence is shown. The status bar of each image shows the name. The name might be a number or a string.

Every image sequence has its own channel colour assignment (see **Display window**). When an image is copied the channel colour assignment is copied too. Drag and drop techniques can be applied to copy images or define the function parameters **Input** and **Output** using the **Gallery** thumbnails.

- Position the cursor on an image in the **Gallery**.
- Press the left mouse button.
- Hold the mouse button down and move the mouse to the destination position.
- At the destination release the left mouse button, the destination image will be overwritten.

To delete an image, drag it, move it to the wastebasket, and drop it.

7.2.3 Display Window

This window is used to display an image sequence, regardless of size or type. To show multiple channel sequences each channel could have its own base colour. The user can set these colours and the weighting for each channel by pressing the corresponding button at the bottom of the window. To display a different image or image sequence, it can be dragged from the **Gallery** and dropped to the **Display window**.

The image can be displayed in full size (one pixel on the screen represents one pixel of the image) or in a zoomed size. To zoom the display view click and hold down the right mouse button on the window border and resize the window. The aspect ratio of the image will not be changed. Clicking on the button resets the **Display window** to a full size view of the image (see above).

The title bar shows the currently displayed sequence name. The status bar displays the size of the current sequence and the selected slice on the left. On the right the cursor position within the window and the corresponding intensity (grey) value of each channel is shown.

The **Display window** can be closed without any effect to the image processing functions. If no **Display window** is opened select the entry **Display** in the **Window menu**.

The scroll bar at the lower right of the window enables to show the images in a sequence. The range reaches from one to the maximum slice provided by the current sequence.

To start the automatic animation of an image sequence start the Player tool by clicking on the button . The colour selection for the channels can be activated by clicking on the button . A colour image can be displayed as a grey value image by clicking on the button .

Player

This function plays back the sequential images of an image sequence.

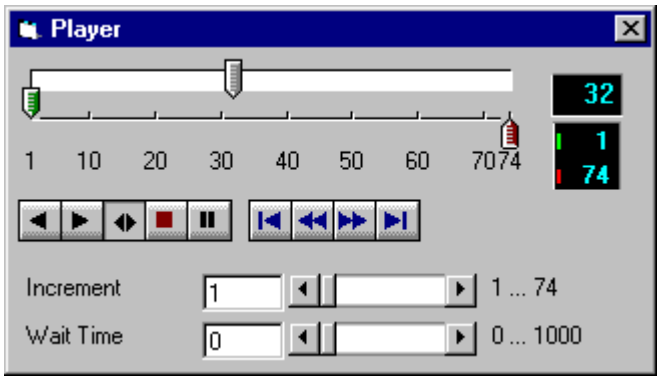


Fig. 7-4

The image sequence is displayed in the **Display window**. The display process is working as a background task; other functions can be executed while the player is running. There are several ways to stop the player:

by closing the player window

by pushing the red Stop button of the player window (the window remains open)

by closing the image window.

The **Increment** parameter specifies whether each sequential image (1) should be displayed or whether some sequential images should be skipped during display. The value 2 skips one image for every sequential image displayed, in other words, it displays only every second image.

The parameter **Wait Time** states the delay in milliseconds between two successive sequential images. The maximum display speed depends mainly on the hardware. The sequential images are always displayed in their entirety, regardless of the set delay.

Control Element of the Player

The three arrow shaped controls on the scale show the start slice and the currently displayed sequential image. The values (positions) can be changed using the mouse. Press and hold the left mouse button and move the pointer to the desired position. The set values are shown in the numerical windows at right.

Start slice

Currently displayed sequential image

End slice

The buttons in the left group start and stop playback of an image sequence.

Reverse playback

Forward playback

Play forward and then backward again (jojo)

Stop playback

Pause playback

The buttons in the middle group control the settings of the current sequential image.

Reset to start slice.

Single step backward (1 sequential image each regardless of Increment).

Single step forward (1 sequential image each regardless of Increment).

Set to end slice.

Increment Image increment.

Wait Time Displays delay between two images (in milliseconds).

Set Channel Colour

This function sets the colour and weight for the channels.

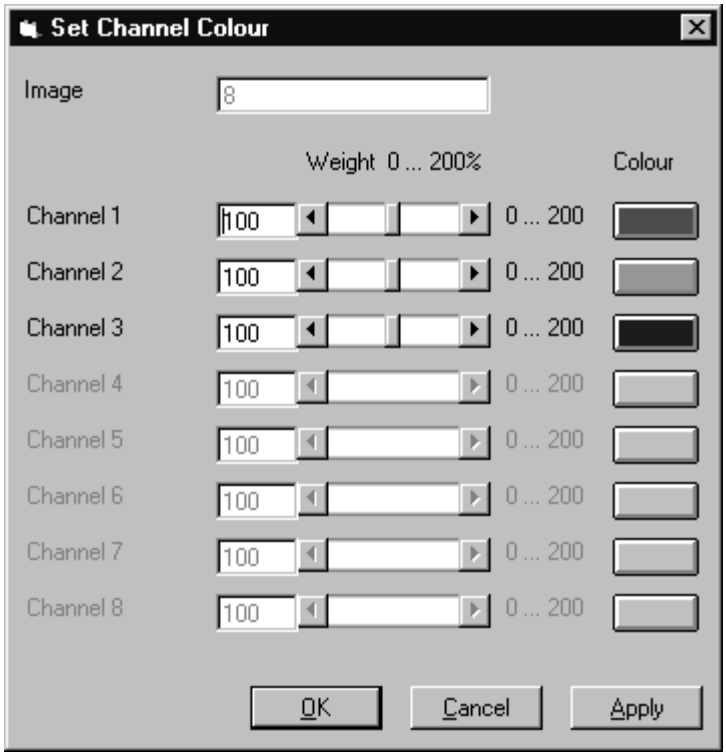


Fig. 7-5

Each image sequence can get its own colour definitions. All functions will inherit the colour definition from the **Input** sequence to the **Output** sequence. By default the colours are set to 100 % weighting and the pure base colours (red, green, blue) are defined.

The weight can be any value between 0 % and 200 %. The colour can be redefined by clicking on the coloured button on the right of the dialog. The standard Windows colour selection dialog is opened. The solution is done by clicking on one of the colours or by entering appropriate numbers in the corresponding edit boxes.

Pressing the **OK** button will close the colour selection dialog and update the **Display window** immediately.

Only those channels, which are available in the image sequence, can be defined.

Parameters:

Image	Image sequence to edit
Weight	Colour weighting for each channel
Colour	Base colour for each channel

7.3 Functions

7.3.1 Functions in the File Menu

Open Image

This function reads a Zeiss LSM (*.lsm), Zeiss LSM TIFF (*000.tif) or Carl Zeiss Vision (*0.img) image sequence from a disk or network drive.

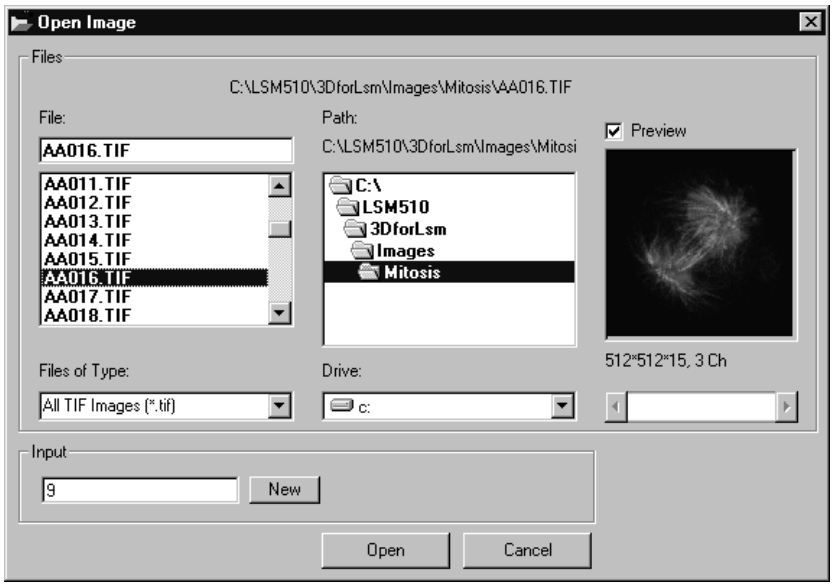


Fig. 7-6

The individual files of a Zeiss TIFF image sequence are read and saved as an image sequence in image memory. In addition, the image properties are read out of the TIFF files and allocated to the image sequence **Input**.

The directories of the current drive are listed in the **Directories** list box. Use the **Drives** list box to choose a different drive.

In case of choosing the TIFF-format in the **Files of Type** box, three number characters are always expected before the dot in the filename extension. The first number must be 000 at the end of the filename. From a complete sequence only this file is listed in the dialog, if "LSM TIF Images (*000.tif)" is selected in the **Files of Type** box. To view all TIFF files "All TIF Images (*.tif)" in the **Files of Type** box must be selected. This selection enables to start with a different file than with the very first (named *000.tif) at the end of the filenames three number digits.

Currently the Carl Zeiss Vision file format "KE Images (*0.img)" is supported. Two files per channel are saved.

Carl Zeiss Vision image sequences must have a number digit at the end of the base filename. They are used to indicate the different channels in a multichannel sequence. The numbering starts with zero (0). If a sequence is saved in the Carl Zeiss Vision format the numbers are generated automatically. To load such an image sequence "KE Images (*0.img)" in the **Files of Type** box must be selected.

The window incorporates the usual file selection controls. The bottom half displays a selection of the image properties that are stored in the image sequence.

Parameters:

BaseName Base name of the TIFF files (image sequence) to be loaded. Only the letters before the first number are stated.

Input Name of the resulting image in which the image sequence will be saved.

Save Image As

This function saves an image or image sequence to disk or network drive.

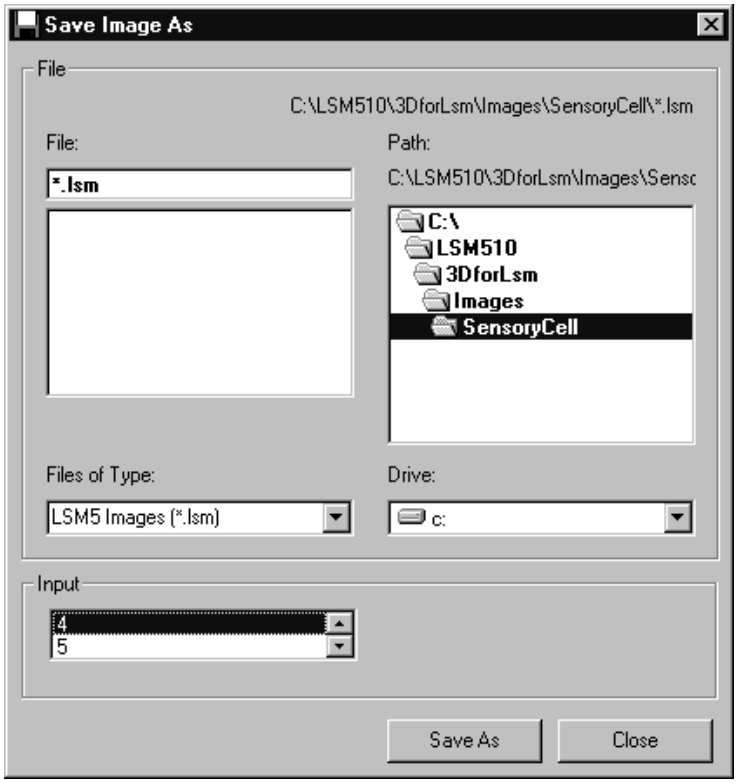


Fig. 7-7

All the files in the current directory that have the selected image format are listed in the **File Name** list box.

The directories of the current drive are listed in the **Directories** list box. Use the **Drives** list box to choose a different drive.

Use the list box **Files of Type** to select the image format. Currently the LSM image format (*.lsm) and the Carl Zeiss Vision file format "KE Images (*0.img)" is supported.

By choosing the Carl Zeiss Vision file format "KE Images (*0.img)", two files per channel are saved. On one hand the Carl Zeiss Vision type image sequence file, on the other hand the file with the image properties. One pair of files is written per channel. They are numbered automatically, starting with zero. A one number digit is added to the end of the filenames. The two files share the same filename but have different filename extensions (*.img and *.3d).

The content of the **Gallery** is shown in the **Input** section. The selection of the sequence to save is done by highlighting one of the provided names or by drag and drop from the **Gallery**.

Parameters:

Input Name of the image sequence to be saved

Filename Name of the file to be used on disk

Save Display As

This function saves the current **Display window** contents to a disk or network drive.

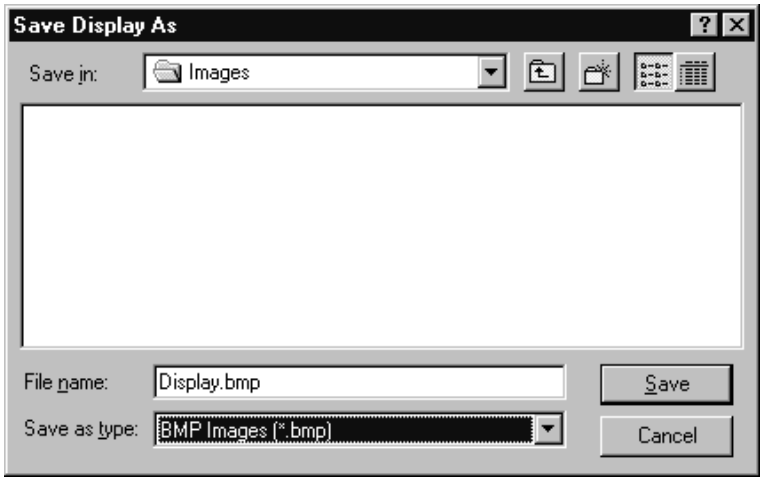


Fig. 7-8

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

The current zoom factor of the **Display window** is not taken into account, the image is saved without any zoom.

The image is saved as a true colour image with 24-bit resolution. From the **Save as Type** list box one of the provided formats can be selected.

Parameters:

None

Print

This function prints the current **Display window** contents.

The standard Windows print dialog is opened.

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

Parameters:

None

Exit

This function terminates the application completely.

All images and image sequences shown in the **Gallery** will be deleted from the memory. Save those images which might be used for any further processing.

Parameters:

None

7.3.2 Functions in the Edit Menu

Copy

This function copies the current **Display window** contents to the clipboard. No dialog is shown.

Before the execution of this function any image or image sequence can be selected to be displayed. From a multichannel sequence any channel status (on or off) combination can be defined. The colours of the shown channels can be set with the function **Set Channel Colour**.

The current zoom factor of the **Display window** is not taken into account; the image is copied without any zoom.

The image is copied as a true colour image with 24-bit resolution. Afterwards the contents can be pasted to any other Windows application.

Parameters:

None

Edit Channels

This function allows to add or to remove channels to a single or multichannel image.

On the **Add Channel** tab sheet the channels of (different) **Input** sequences can be defined to add (combine) channels to an **Output** sequence.

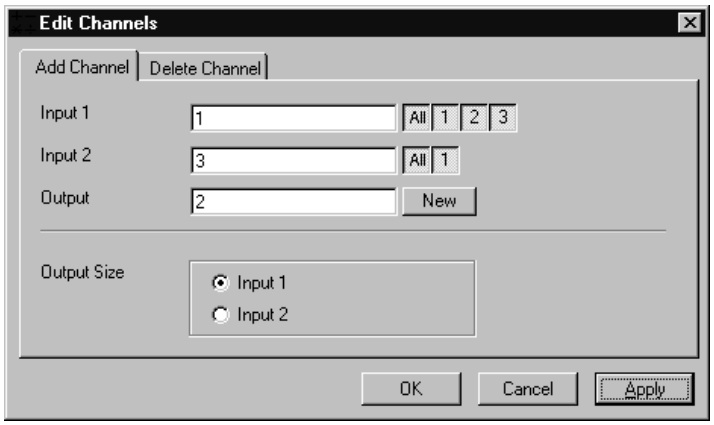


Fig. 7-9

This operation is useful to add a segmented channel (or any other result of a function) to the original image sequence. The selected channels of **Input 1** and **Input 2** are copied to **Output**. The maximum number of channels in an image sequence is eight.

If the image sequences do not have the same extents **Output Size** defines which input is taken as a reference. This selection also defines the properties for scaling and units in the output image sequences.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Output size	Defines source image sequence for size, scaling, and units

On the **Delete Channel** tab sheet channels of the **Input 1** image sequence can be selected to delete channels.

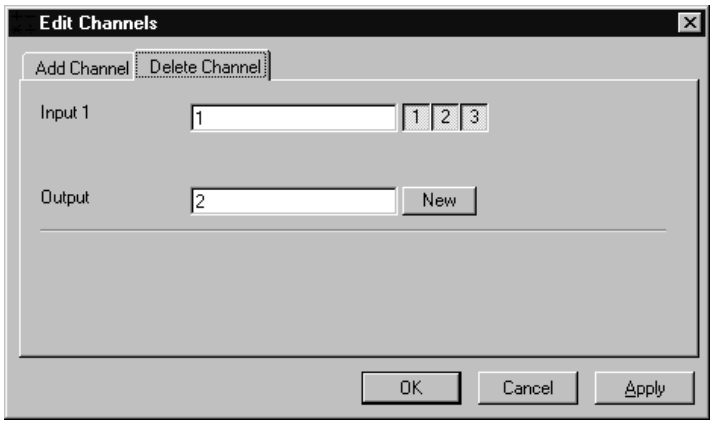


Fig. 7-10

This operation might save time and memory for further processing if not all channels are needed.

Only the selected channels of **Input 1** are copied to **Output**.

Parameters:

Input 1 Input image sequence

Output Output image sequence

Delete All Images

This function deletes all images and image sequences from the memory (**Gallery**).

The function is used whenever a completely new image sequence should be processed. In order to drop the images item by item to the wastebasket all of them can be deleted by a single function.

If any image or image sequence is needed for further use save them first.

Parameters:

None

7.3.3 Functions in the Process Menu

Arithmetics - Add

This function adds two image sequences.

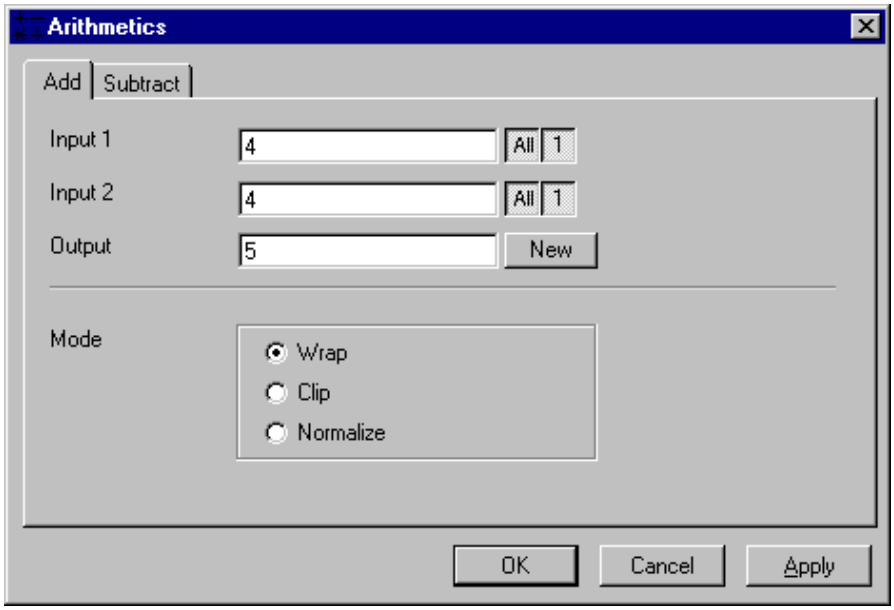


Fig. 7-11

The **Add** tab sheet of the **Arithmetics** dialog window must be selected.

If one or both input sequences are multichannel sequence, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

This function adds the two image sequences **Input 1** and **Input 2** voxel by voxel and generates the image sequence **Output**. Note that a resulting grey value may be greater than 255 (4095). The parameter **Mode** determines how a range overflow is handled:

1 - Wrap	No normalization - the grey values are displayed modulo 256 (4096). If the result is greater than 255 (4095), the value 256 (4096) is subtracted from it.
2 - Clip	Grey values which exceed 255 (4095) are replaced with 255 (4095).
3 - Normalize	The resulting grey value range is scaled to the range 0...255 (0...4095).

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Mode	1 - Wrap 2 - Clip 3 - Normalize

Arithmetics - Subtract

This function subtracts two image sequences.

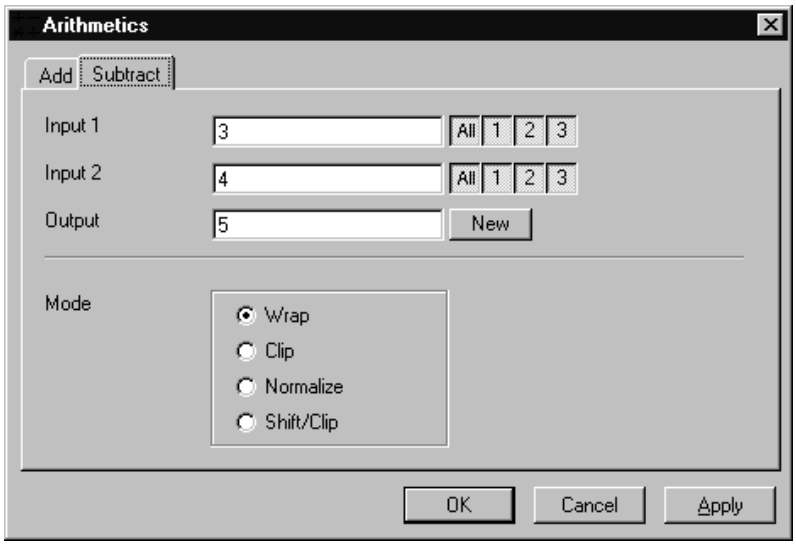


Fig. 7-12

The **Subtract** tab sheet of the **Arithmetics** dialog window must be selected.

If one or both input sequences are multichannel sequence, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

This function subtracts the two image sequences **Input 1** and **Input 2** voxel by voxel and generates the image sequence **Output**. Note that a resulting grey value may be less than 0. The parameter **Mode** determines how a range overflow (negative values) is handled.

1 - Wrap	No normalization - the grey values are displayed modulo 256 (4096). If the result is less than 0, the value 256 (4096) is added to it.
2 - Clip	Negative values are set to 0.
3 - Normalize	The resulting grey value range is scaled to the range 0...255 (0...4095).
4 - Shift/Clip	128 (2048) is added to the difference, then negative values are set to 0. Values greater than 255 (4095) are set to 255 (4095).

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Output image sequence
Mode	1 - Wrap 2 - Clip 3 - Normalize 4 - Shift/Clip

Contrast - Interactive

This function allows interactive changes of the contrast of an image sequence.

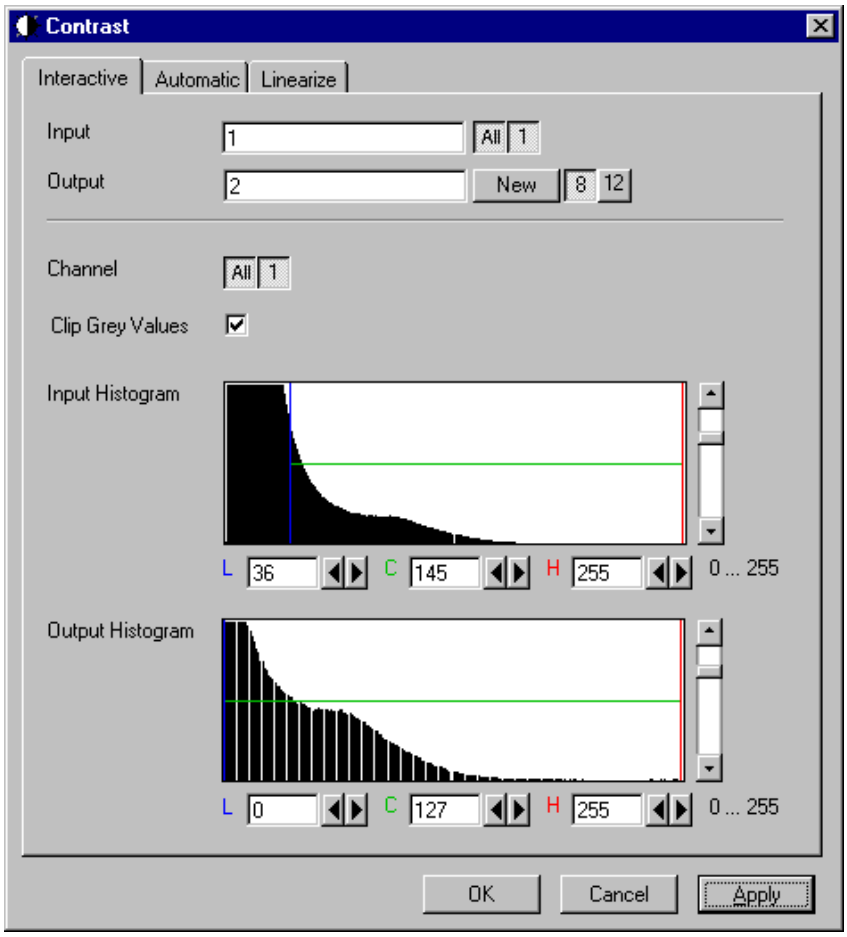


Fig. 7-13

The **Interactive** tab sheet of the **Contrast** dialog window must be selected.

A grey value range of the **Input** image sequence is scaled to another range in the **Output** image sequence. Both ranges can be edited interactively. This function is used to achieve a better view of an image sequence, or to scale a range of grey values to single value for a special coding in an image sequence. The function does not improve the result of the linear segmentation function **Segment**.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the name of the result sequence. It will get only those channels which are chosen by the **Input** parameter. The buttons labeled with 8 and 12 define the grey value (intensity) resolution in bit. Normally the result will get the same resolution as the **Input** sequence. A change will be needed if image sequences with different resolutions should be combined. Rising the grey value range to 12 bit will not enhance the display quality or measurement accuracy. The smooth and morphology functions will produce results with finer gradations.

If **Clip Grey Values** is selected, the output grey values are clipped to the **Low (L)** and **High (H)** values. If **Clip Grey Values** is not selected, output grey values beyond the **Low** and **High** value range are possible.

The **Output** histogram shows the resulting histogram. The horizontal axis represents the grey values from 0 to the maximum, which is either 255 or 4095, depending whether the input is 8 bit or 12 bit. The vertical axis represents the pixel count. The selected range is marked by the borderlines in the histogram. The blue line or **L** indicates the lower boundary, the red line or **H** the upper one, **C** indicates the center of the range.

There are three ways to change the range: clicking and dragging the borderlines with the mouse.

Entering a new value in the appropriate text boxes, clicking on the buttons or using the arrow keys from the keyboard. To alter the values within the histogram move the mouse pointer over one of the three coloured lines until the shape changes. Press and hold the left mouse button to move the line to a new position. To change the values with the arrow keys click once into the histogram. Using the left or right arrow key by its own will move the whole range. Pressing the **Shift** key additionally moves the lower boundary, the **Control** key the upper boundary.

The vertical scale of the histogram is set using the scroll bar. The units are percents of the maximum grey value distribution. This setting has no influence on the function.

Parameters:

Input	Input image sequence
Output	Output image sequence
Channel	Selection of the channel numbers for the **Output** image after contrast enhancement
Clip Grey Values	Clipping of grey values to the **Low (L)** and **High (H)** output grey values boundaries
Input L	Lower boundary of grey value range **Input**
Input C	Center of grey value range **Input**
Input H	Upper boundary of grey value range **Input**
Output L	Lower boundary of grey value range **Output**
Output C	Center of grey value range **Output**
Output H	Upper boundary of grey value range **Output**

Contrast - Automatic

This function scales the grey values of an image sequence to the maximum possible range.

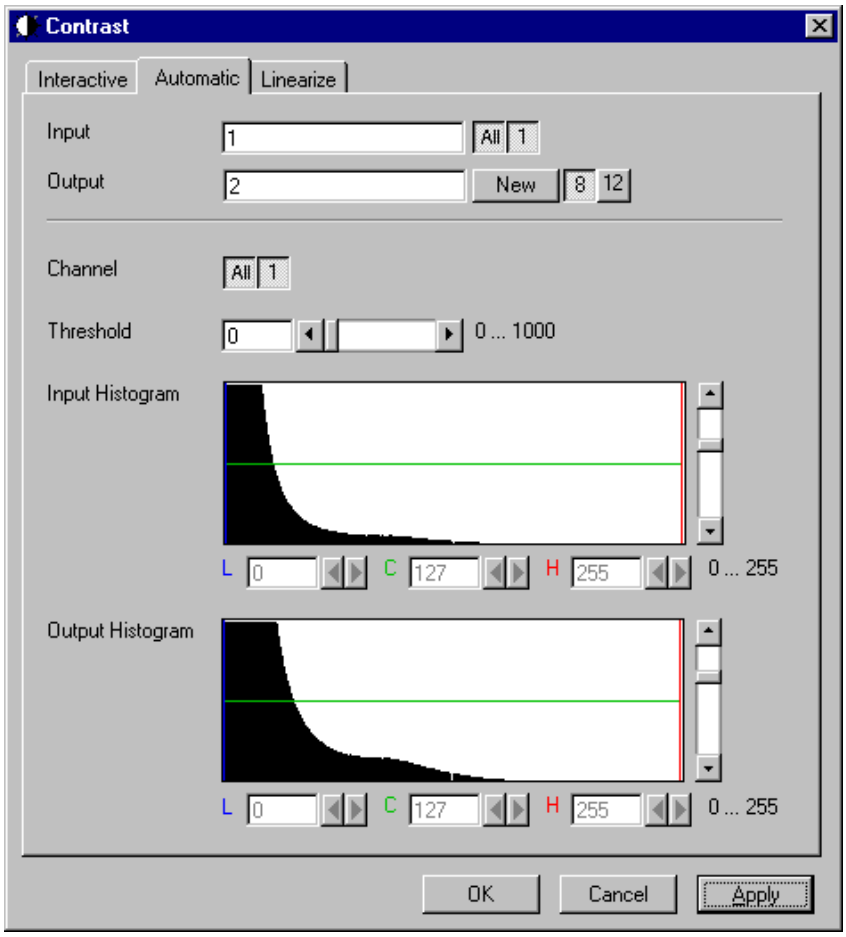


Fig. 7-14

The **Automatic** tab sheet of the **Contrast** dialog window must be selected.

This function enhances the contrast of an image sequence by spreading the grey value distribution over the maximum possible range. This function is used to achieve a better view of an image.

The light and dark grey value ranges with a low share of pixels are excluded from the operation by the parameter **Threshold**. The **Threshold** units are in thousandths of the total number of voxels. Using a value of 10 means that the scale interval is set so that 5/1000 of the total number of voxels on the light side, and 5/1000 of the total number of voxels on the dark side of the grey value distribution are excluded.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the name of the result sequence. It will get only those channels which are chosen by the **Input** parameter. The buttons labeled with 8 and 12 define the grey value (intensity) resolution in bit. Normally the result will get the same resolution as the **Input** sequence. A change will be needed if image sequences with different resolutions should be combined. Rising the grey value range to 12 bit will not enhance the display quality or measurement accuracy. The smooth and morphology functions will produce results with finer gradations.

The **Output** histogram shows the resulting histogram. They are not editable. The horizontal axis represents the grey values from 0 to the maximum, which is either 255 or 4095, depending whether the input is 8 bit or 12 bit. The vertical axis represents the pixel count. The vertical scale of the histogram is set using the scroll bar. The units are percentages of the grey value distribution maximum. This setting has no influence on the function.

Parameters:

Input	Input image sequence
Output	Output image sequence
Threshold	Exclusion value - 0...1000
Input L	Lower boundary of grey value range Input
Input C	Center of grey value range **Input**
Input H	Upper boundary of grey value range **Input**
Output L	Lower boundary of grey value range **Output**
Output C	Center of grey value range **Output**
Output H	Upper boundary of grey value range **Output**

Contrast – Linearize

This function scales a range of grey values of an image sequence to equal area fractions in the histogram.

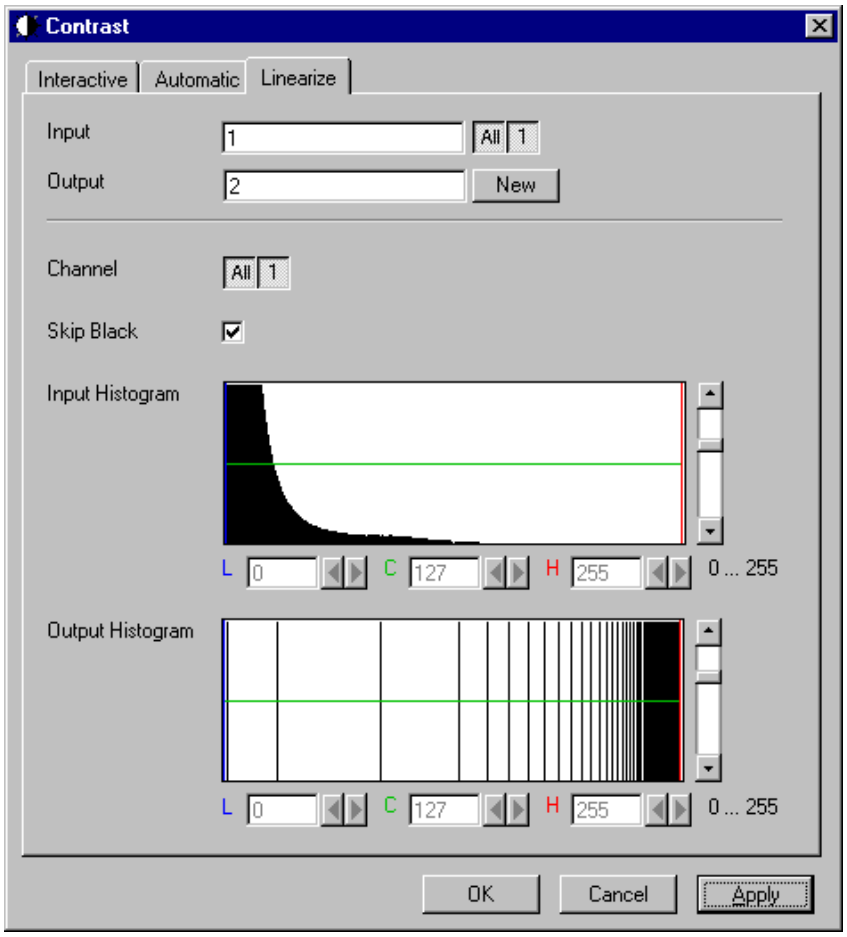


Fig. 7-15

The **Linearize** tab sheet of the **Contrast** dialog window must be selected.

This function enhances the contrast by linearizing the histogram of the image sequence to equal area fractions in the histogram. The areas (voxel count multiplied by grey value range) of all grey values in the **Output** histogram are the same. This function is used to achieve a better view of an image sequence. When **Skip Black** is checked the grey value 0 will not be taken into account for linearization.

Input indicates the sequence to enhance. If it is a multichannel sequence, a single channel, all channels, or any number can be selected. The **Input** histogram shows the grey value distribution of the selected channels of the **Input** image sequence.

Output defines the range of the result sequence. It will get only these channels which are chosen by the **Input** parameter. The grey value (intensity) resolution will be the same as the one from **Input**.

The **Output** histogram shows the resulting histogram. The horizontal axis represents the grey values from 0 to 255. The vertical axis represents the pixel count. The vertical scale of the histogram is set using the scroll bar. The units are percentages of the grey value distribution maximum. This setting has no influence to the function.

Parameters:

Image	Input image sequence
Output	Output image sequence
SkipBlack	0 - Grey value black is ignored 1 - Grey value black is taken into account
Input L	Lower boundary of grey value range Input
Input C	Center of grey value range Input
Input H	Upper boundary of grey value range Input
Output L	Lower boundary of grey value range Output
Output C	Center of grey value range Output
Output H	Upper boundary of grey value range Output

Smooth (Gauss)

This function performs a Gauss filter.

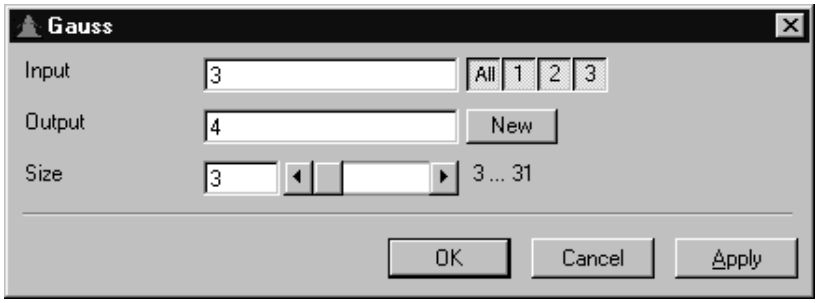


Fig. 7-16

The noise in the image sequence is reduced, the edge shape is nearly unchanged, local maxima are leveled, the dynamic range is reduced.

Image sequences should be smoothed before they are reconstructed or segmented. For most sequences a **Size** value of 3 is sufficient enough. If **Input** is a multichannel sequence, any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The grey value of every pixel is substituted by a weighted average of its surrounding neighbors. The neighbors are defined by a cube. The affected pixel is the central pixel of the filter cube. The weighted filter cube is approximated by a binomial distribution. The size of the filter cube is set using the **Size** scroll bar. Even numbers are set to the next odd value. The **Size** defines the strength of the smoothing.

Parameters:

Input	Input image sequence
Output	Output image sequence
Size	Filter size (3...31, only odd numbers)

Morphology

The following four functions perform basic operations of mathematical morphology on image sequences.

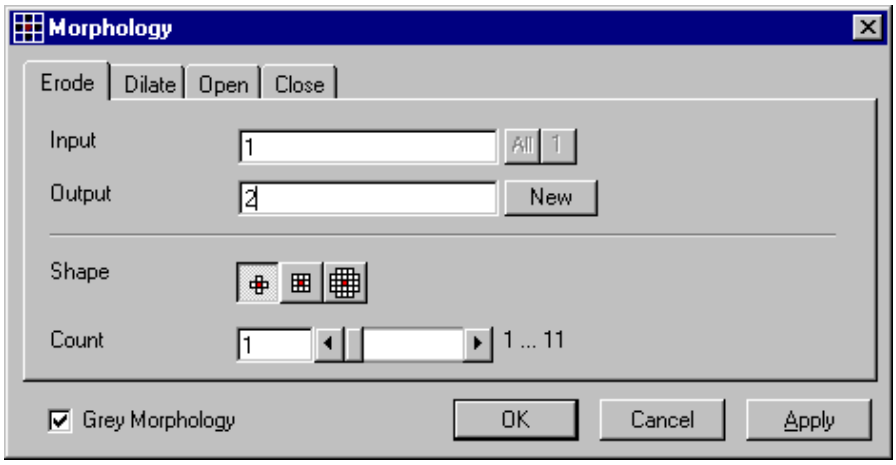


Fig. 7-17

As generalization of the morphology of two-dimensional images to three dimensions the structural elements are small volumina.

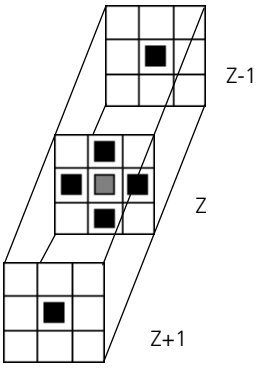
Literature

Bomans, M.; Höhne, K.-H.; Tiede, U.; Riemer, M.:
3D-Segmentation of MR Images of the Head for 3-D Display
IEEE Transactions on Medical Imaging 9, 1990, 177-183
Schiemann, T.; Bomans, M.; Tiede, U.; Höhne, K.-H.:
Interactive 3D-Segmentation of Tomographic Image Volumes
14. DAGM-Symposium Mustererkennung, Springer-Verlag 1992, 73-80

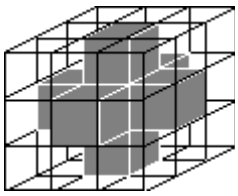
The input image sequence is analyzed voxel by voxel with a selected shape (**Shape**). The voxel to be analyzed is always the central voxel of the shape. The shape type determines which neighboring voxels are used to compute the resulting voxel.

The following structural elements are available for all morphological operations. They represent approximated spheres with an increasing radius.

Sequential image:

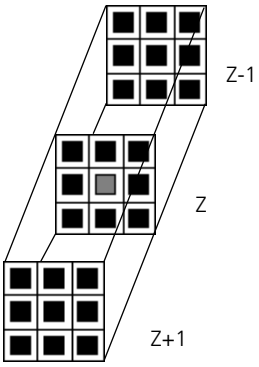


Volume view:

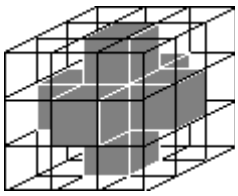


Cross shape

Sequential image:

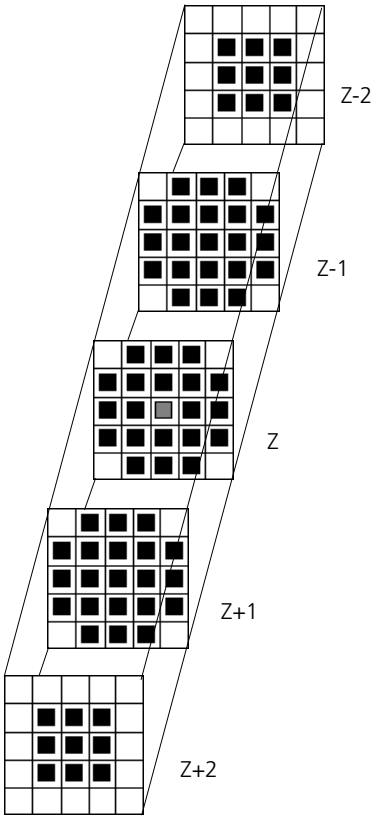


Volume view:

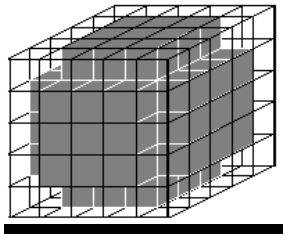


Cross shape

Sequential image:



Volume view:



Cube cross shape: created through application of "cube" and "cross" one after the other.

For regions (voxels) that are at the edge of the image sequence, it assumed for erosion that there are white voxels with a grey value of 255 (4095) outside the edge. For dilation, it is assumed that there are black voxels with the grey value 0 outside the image sequence.

If the **Grey Morphology** tickbox is activated, erosion sets the grey value of the central voxel to the minimum of all neighboring voxels affected by the structural element; dilation sets the grey value of the central voxel to the maximum.

If the **Grey Morphology** tickbox is not activated, the neighboring voxels are only distinguished by grey value 0 and non-0. For erosion the central voxel is set to 0 if any of the neighbors is 0. It is set to 255 (4095) if any neighbor is not 0. For dilation the central voxel is set to 255 (4095) if any of the neighbors is not 0. It is set to 0 if all neighbors are 0.

Erosion reduces the size of bright regions, separates thin connections between them, and makes small regions disappear. Dilation, on the other hand, makes bright regions of the image grow in size, fills gaps, and smoothes small contour details.

The result of erosion and dilation is called opening. On the one hand, this maintains to some extent the original size of the regions while not losing the smoothing effect of erosion on the image. This name stands for the operation of reducing convex bulges in the contour of the region. Thin connections between regions are eliminated, broken borders between regions are connected, and small regions disappear.

The opposite operation (first dilation, then erosion) is called closing. Concave bulges in the contours of regions are filled in; connections are formed between adjacent regions.

The following example illustrates the operations "Open" and "Close" in two dimensions:

Open = Erosion + Dilation

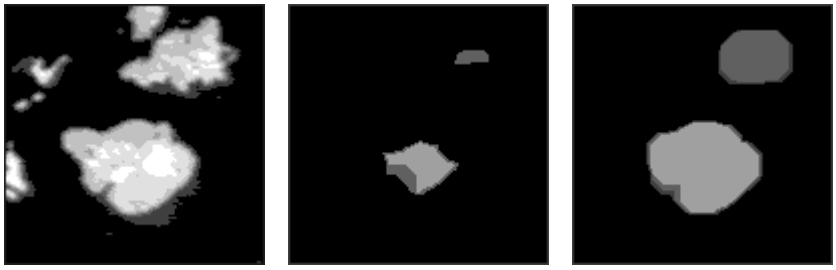


Fig. 7-18

Close = Dilation + Erosion

Fig. 7-19

The "cube cross" shape was used for the operations shown.

Morphology - Erode

This function erodes structures in an image sequence.

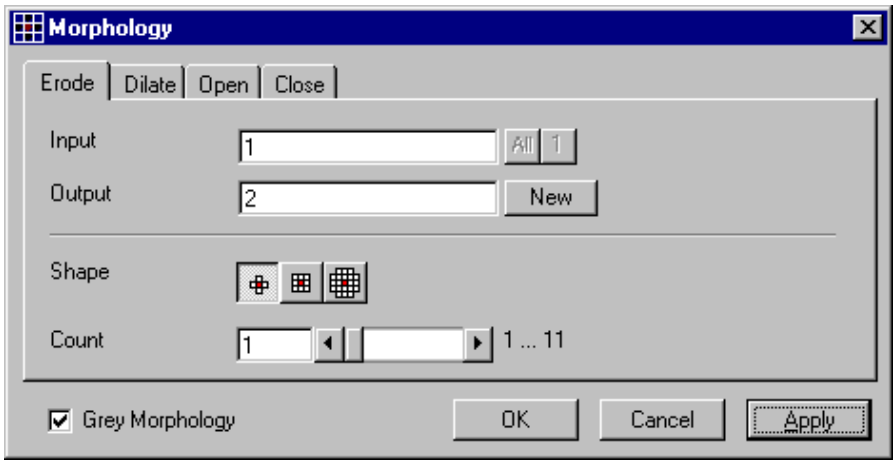


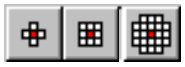
Fig. 7-20

In the **Morphology** dialog window, the tab sheet **Erode** must be selected.

Erosion makes bright regions smaller on a dark background. It also results in separation of thin connections between regions. Small regions disappear entirely.

If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results. The Input image sequence is eroded **Count** times with the shape **Shape**. The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:



If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Dilate

This function dilates structures in an image sequence.

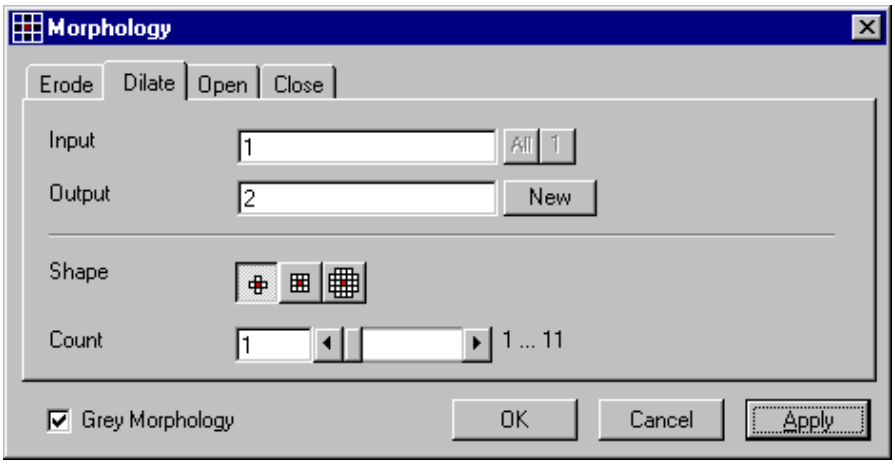


Fig. 7-21

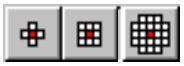
In the **Morphology** dialog window, the tab sheet **Dilate** must be selected.

Dilation makes bright regions larger on a dark background. It also results in the filling of gaps and smoothing of small contour details.

If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Input** sequential image is dilated **Count** times with the shape **Shape**. The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:



If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Open

This function carries out an opening.

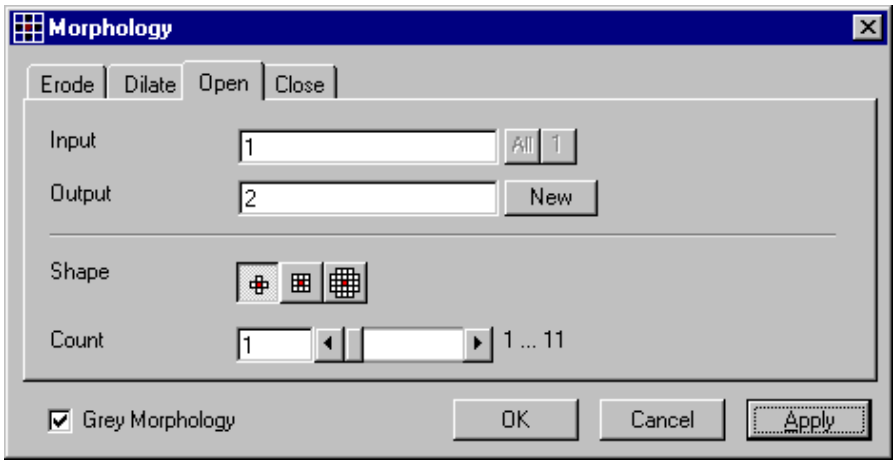


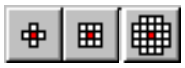
Fig. 7-22

In the **Morphology** dialog window, the tab sheet **Open** must be selected.

This function carries out an erosion followed by a dilation. For the most part, the opening maintains the original size of the regions. Thin connections between regions and small regions themselves disappear. Convex bulges in the contours of the regions are reduced. The opening is applied to the grey value image sequence **Input Count** times with the shape **Shape**. If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:



If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Morphology - Close

This function carries out a closing.

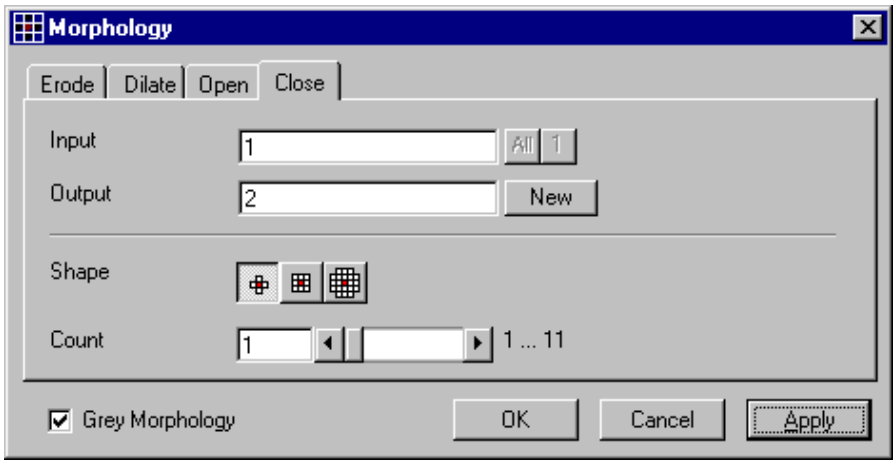


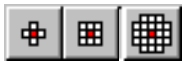
Fig. 7-23

In the **Morphology** dialog window, the tab sheet **Close** must be selected.

This function carries out a dilation followed by an erosion. For the most part, the closing maintains the original size of the regions. Connections are formed between adjacent regions; gaps and bright concave bulges in the contours of regions are filled in. The closing is applied **Count** times to the grey value image sequence Input with the shape **Shape**. If **Input** is a multichannel sequence any number and combination of channels can be selected. **Output** will only get the selected channels as results.

The **Count** scroll bar determines the number of recursive operations.

The following shapes (numbered 1 to 3 from left to right) are available:



If **Grey Morphology** is selected the function will respect all grey value shades of the sequence **Input**. If **Grey Morphology** is not selected the function will distinguish between 0 and non-0 only. The result **Output** will be a binary sequence.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Shape	Shape used 1 - cross 2 - cube 3 - cube cross
Count	Number of recursive operations
Grey Morphology	0 - Distinguish between 0 and non 0 only 1 - All grey value shades are taken into account

Segment - Interactive

This function carries out a grey value segmentation by means of thresholding.

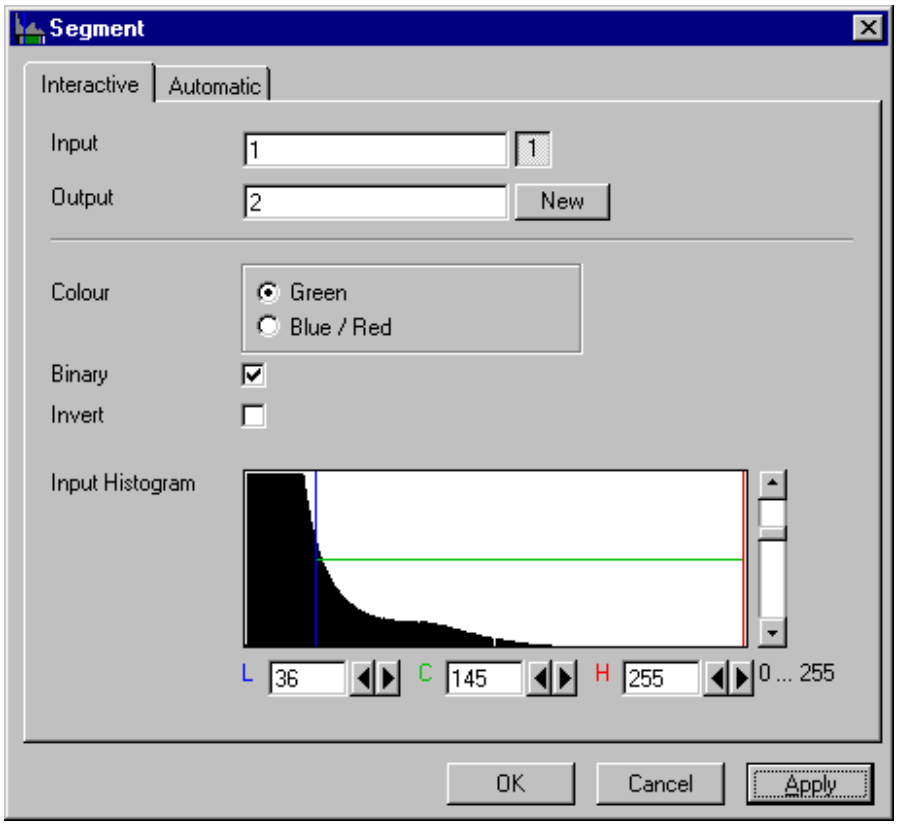


Fig. 7-24

The **Interactive** tab sheet of **Segment** dialog window must be selected.

Segmentation is especially used to generate binary regions. These are required for the measurement.

Two threshold values determine which grey value range of the **Input** image sequence is preserved and/or deleted in the **Output** image sequence. Only one channel of a multichannel sequence can be selected as **Input**. **Output** will always be a single channel sequence.

The vertical scaling of the histogram can be adjusted with the scroll bar at the right edge of the histogram. This setting has no influence on the function.

The thresholds **L**ow and **H**igh are determined either by moving the borderlines in the grey value histogram or by the scroll bars underneath. Furthermore, the values for **L**ow, **C**enter and **H**igh can be set through entry in the corresponding fields.

To move the lower (**L**) and upper (**H**) thresholds at the same time, move the vertical line in the grey value histogram or set the scroll bar (**C**).

The **Green** and **Blue/Red** option buttons of the parameter **Colour** determine whether the voxels within (**Green**) or outside (**Blue/Red**) of the grey value interval [**L, H**] are displayed with the corresponding colour.

If **Green** is selected, the voxels within the selected interval are highlighted in green. The rest of the image retains its original grey values. The voxels with the grey values **L**ow and **L**ow+1 are displayed in blue. The voxels with the grey values **H**igh and **H**igh-1 are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **L**ow, **H**igh remain unchanged. Voxels with grey values less than **L**ow are highlighted in blue; those with grey values higher than **H**igh are highlighted in red.

If the **Invert** option is selected, the grey values outside the defined interval will be segmented.

If the option **Binary** is selected, then all grey values in the range from **L**ow to **H**igh will be set to white (grey value 255) in the **Output** image sequence, while all others will be set to black (grey value 0). If the option is not selected, the grey values within the selected interval remain unchanged, while those outside the range will be set to black. The measurement function accepts both results without any difference in the results.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Colour	Green - Selected interval is displayed in green
Blue/Red	Grey values below the selected interval are displayed in blue, grey values above in red
Binary	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255, the rest to grey value 0
Invert	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
L	Low grey value threshold
C	Center of threshold interval
H	High grey value threshold

Segment - Automatic

The function carries out an automatic grey value segmentation by means of thresholding.

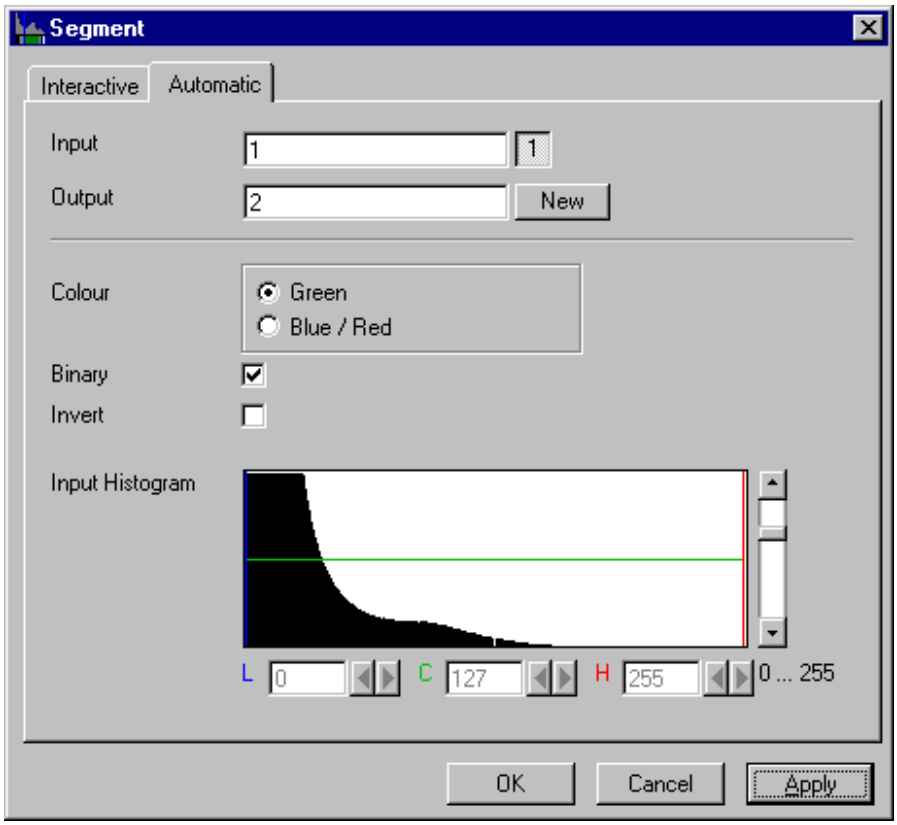


Fig. 7-25

The **Automatic** tab sheet of the **Segment** dialog window must be selected. Segmentation is especially used to generate binary regions. These are required for the measurement.

The function calculates the two strongest local minimums in the histogram of the **Input** image sequence. These values are used for the discrimination. Only one channel of a multichannel sequence can be selected as **Input**. **Output** will always be a single channel sequence. The vertical scaling of the histogram can be adjusted with the scroll bar at the right edge of the histogram. This setting has no influence on the function.

The **Green** and **Blue/Red** option buttons of the parameter **Colour** determine whether the voxels within (**Green**) or outside (**Blue/Red**) of the grey value interval [**L, H**] are displayed with the corresponding colour.

If **Green** is selected, the voxels within the selected interval are highlighted in green. The rest of the image retains its original grey values. The voxels with the grey values **L**ow and **L**ow+1 are displayed in blue. The voxels with the grey values **H**igh and **H**igh-1 are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **L**ow, **H**igh remain unchanged. Voxels with grey values less than **L**ow are highlighted in blue; those with grey values higher than **H**igh are highlighted in red.

If the **Invert** option is selected, the grey values outside the defined interval will be segmented.

If the option **Binary** is selected, then all grey values in the range from **L**ow to **H**igh will be set to white (grey value 255 (4095)) in the **Output** image sequence, while all others will be set to black (grey value 0). If the option is not selected, the grey values within the selected interval remain unchanged, while those outside the range will be set to black.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Colour	Green - Selected interval is displayed in green Blue/Red - Grey values below the selected interval are displayed in blue, grey values above in red
Binary	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255 (4095), the rest to grey value 0
Invert	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
L	Low grey value threshold
C	Center of threshold interval
H	High grey value threshold

Boolean - And

This function carries out a bit-by-bit **And** calculation for the image sequences **Input 1** and **Input 2**.

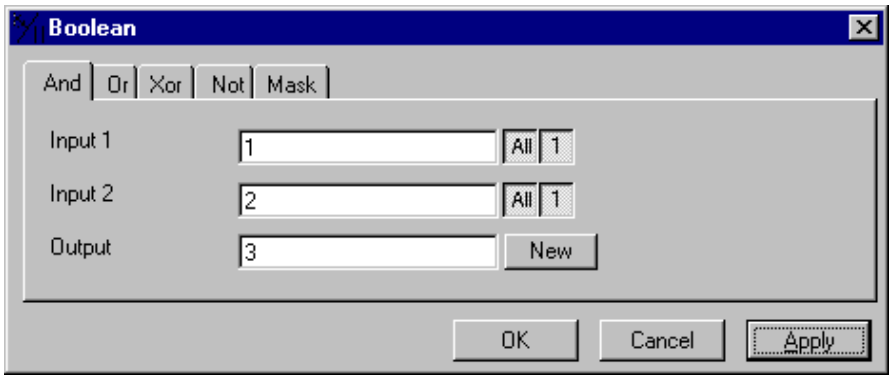


Fig. 7-26

The **And** tab sheet of the **Boolean** dialog window must be selected.

This function is especially well suited for masking images.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Or

This function carries out a bit-by-bit **Or** calculation for the images **Input 1** and **Input 2**.

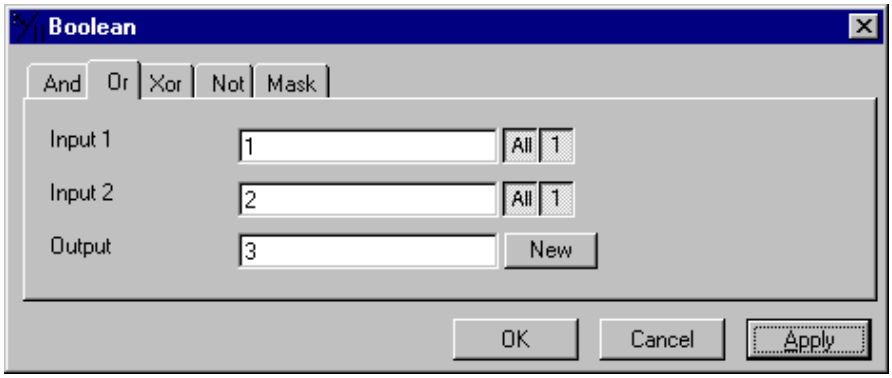


Fig. 7-27

The **Or** tab sheet of the **Boolean** dialog window must be selected.

This function can be used to combine binary masks or regions.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Xor

This function carries out a bit-by-bit **Xor** calculation for the images **Input 1** and **Input 2**.

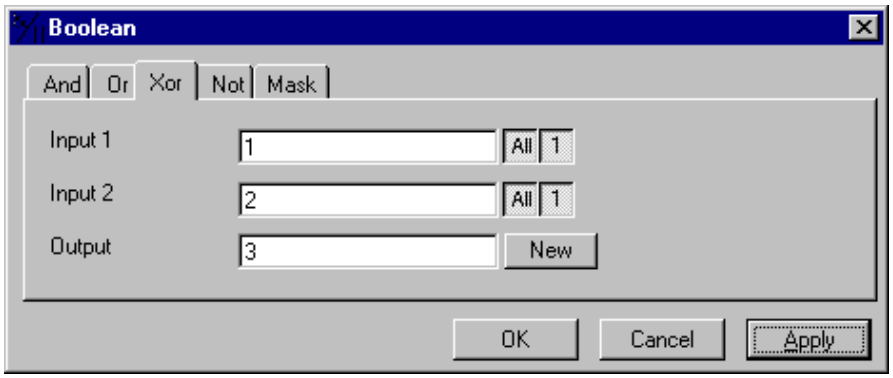


Fig. 7-28

The **Xor** option button of the **Function** option group in the **Boolean** dialog window must be selected.

This function can be used to combine binary masks or regions.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Boolean - Not

This function carries out a bit-by-bit negation of an image.

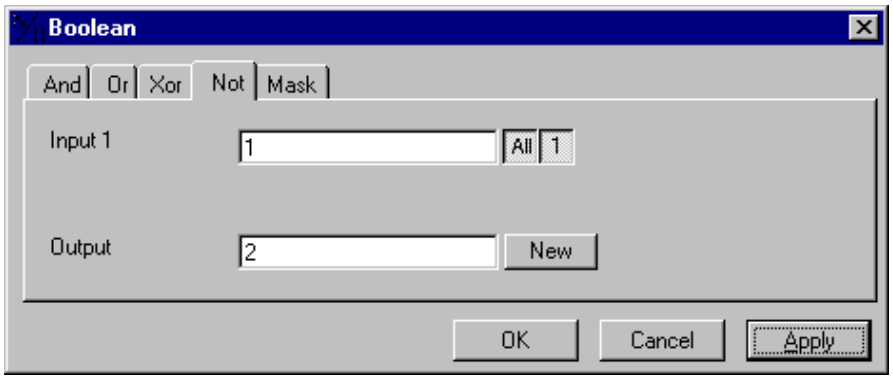


Fig. 7-29

The **Not** tab sheet of the **Boolean** dialog window must be selected.

If **Input** is a multichannel sequence any number or combination can be selected.

Parameters:

Input	Input image sequence
Output	Resulting image sequence

Boolean - Mask

This function masks a grey value image sequence.

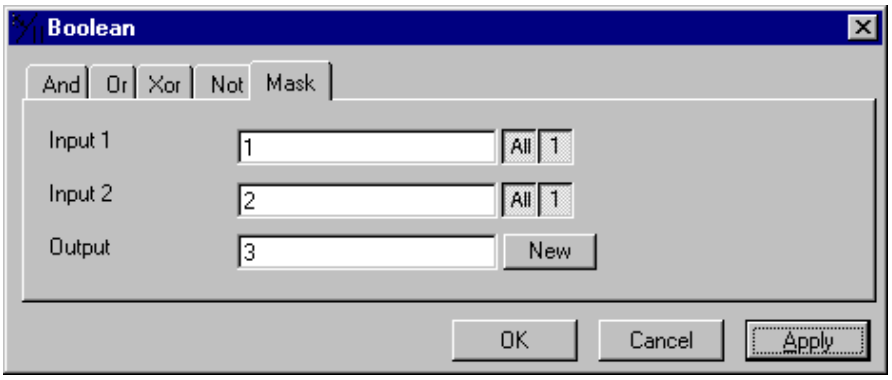


Fig. 7-30

The **Mask** tab sheet of the **Boolean** dialog window must be selected.

This function modifies the **Output** image sequence depending on the mask image sequence used.

If the grey value in **Input 2** is higher than 0, then the voxel values are copied from **Input 1** to the image sequence **Output**. If the grey value of the voxel is 0, then the voxel value of the **Output** image sequence is taken over.

If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 2** must be 1 or the same as for **Input 2**. They will be combined from left to right.

Parameters:

Input 1	First input image sequence
Input 2	Second input image sequence
Output	Resulting image sequence

Scrap

This function deletes or selects objects in a specified size range.

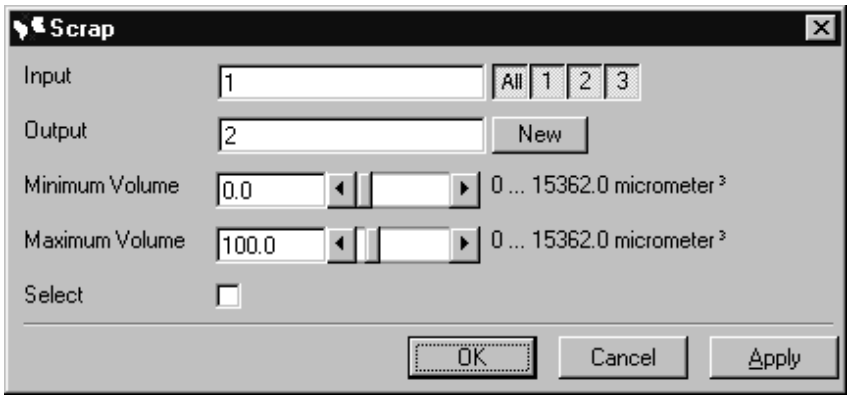


Fig. 7-31

The operation deletes or selects objects on the basis of their total volume in voxels. Objects with a volume within the range **MinVolume** to **MaxVolume** are effected.

To delete objects outside the range, the parameter **Select** must be active. If the parameter is not activated objects outside the defined volume range are deleted.

Parameters:

Input	Input image sequence
Output	Output image sequence
MinVolume	Minimum object size
MaxVolume	Maximum object size
Select	0 - Select the objects outside the size range 1 - Select the regions within the size range

Fill Holes

This function fills holes in all objects.

Fig. 7-32

All holes in objects are filled by this operation. Holes are structures, which have a grey value of 0 and are surrounded completely by voxels with a grey value not equal to 0. It is assumed that regions outside the image are black. Holes, which touch the image border, are retained.

Parameters:

Input	Input image sequence
Output	Output image sequence

7.3.4 Functions in the View Menu

Render - Surface

This function displays an image sequence according to the **gradient shading method**.

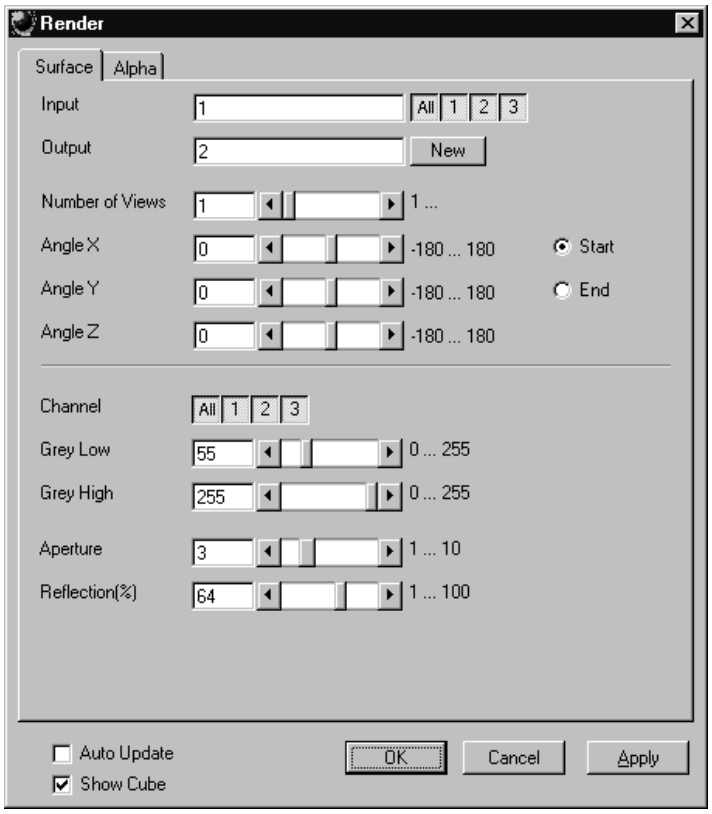


Fig. 7-33

The **Surface** tab sheet of the **Render** dialog window must be selected.

Method

The **Input** sequence defines the data to be reconstructed. If it is a multichannel sequence one or all channels can be selected for the reconstruction.

Output sets the name of the result image (sequence). If the sequence exists it is overwritten. Pressing the button **New** will generate a new name (number). The size of the sequential images in **Output** is determined by the size of the sequential images in **Input**.

Number of Views determines the number of reconstructions which should be computed. The radio buttons **Start** and **End** define which angle settings are currently shown. A definition for the angle **End** is only necessary if **Number of Views** is higher than 1. If this is true the result sequence will get views from the **Start** to the **End** angle definition. The other reconstructions are determined through the linearly interpolated intermediate angles. The direction of view is determined from the angles as follows:

The angle **Angle Z** determines the rotation of the direction of view on the Z-axis. The angle **Angle Y** determines the rotation of the direction of view on the Y-axis that has been rotated by the angle **Angle Z**. The angle **Angle X** determines the rotation of the direction of view on an X-axis that is rotated by **Angle Z** and **Angle Y**.

Channel defines if the following parameters are valid for **All** or just for one. Defining the thresholds for the channels independently is useful if the grey value boundaries of the objects differ too much in the different channels. The thresholds **Grey Low** and **Grey High** define the grey value range of the objects.

The parameter **Aperture** is a measure of the size of the highlights. Small values generate large highlights. Large values generate small highlights (similar to a spot).

Use the parameter **Reflection** to control the ratio of diffuse and reflective brightness components, i.e., the overall basic brightness compared with the highlights. When the value of **Reflection** is low, the highlights predominate; when the values are high, the region appears to be uniformly illuminated and the highlights are not so pronounced. When **Auto Update** is selected, the reconstruction is updated automatically whenever a parameter is modified (except **Input, Output**, or **Number of Views**). **Show Cube** defines whether a wire frame cube is shown in the **Display window** or not.

Application

This method can be applied, if the structures in the **Input** sequence can be segmented by grey value thresholding. Because the gradient is calculated for every pixel, the **Output** appears in very fine detail.

Noisy **Input** sequences must be smoothed (function **Smooth**) before rendering, otherwise the surface appears rough.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Number of Views	Number of reconstructions to be calculated
Angle X	Angle of rotation on the X-axis, start position
Angle Y	Angle of rotation on the Y-axis, start position
Angle Z	Angle of rotation on the Z-axis, start position
Channel	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
Grey Low	Low grey value threshold of the region to be displayed

Grey High	High grey value threshold of the region to be displayed
Aperture	Measure of the extent of the highlights
Reflection	Weight of the defuse brightness components in comparison to the highlights
Auto Update	0 - Function execution is performed on **OK** or **Apply** 1 - Function execution for the current angle is performed on any parameter change
Show Cube	0 - The wire frame cube is not shown 1 - The wire frame cube is shown in the **Display window**

Render - Surface: Method Description

This method displays the surface of structures in the **Input** sequence shaded as if a light illuminated it. The position of the light is behind the view point with parallel rays in the direction of the sequence.

The input sequence is segmented into object and background by grey value thresholding: object voxels are within the grey value range **Grey Low** to **Grey High**.

Each **Output** pixel corresponds to a point at the surface at which the ray in view direction through the **Output** pixels hits the surface. All rays are parallel.

The surface normal required for shading in this gradient renderer is the grey value gradient in the **Input** volume at the surface voxel position. It is not the geometric surface normal. The grey value gradient is determined from the grey values in a 3x3x3 cube around the surface voxel by averaging e.g. the x-gradient in y- and z-direction [4].

There is no depth cueing (far objects would appear darker).

The illumination model is a Phong model [1] (surface normal is determined for each **Output** pixel) with diffuse reflection and specular reflection. Diffuse reflection means that the surface reflects light with equal intensity in all directions. The brightness of a given surface patch depends not on the view-direction, but only on the angle between light and surface normal. Specular reflection is observed on shiny surfaces as a highlight. The light is reflected as from a mirror. The maximum intensity is observed when the view direction is the one of the mirrored light direction.

Render - Alpha

This function displays an image sequence according to the **alpha rendering method**.

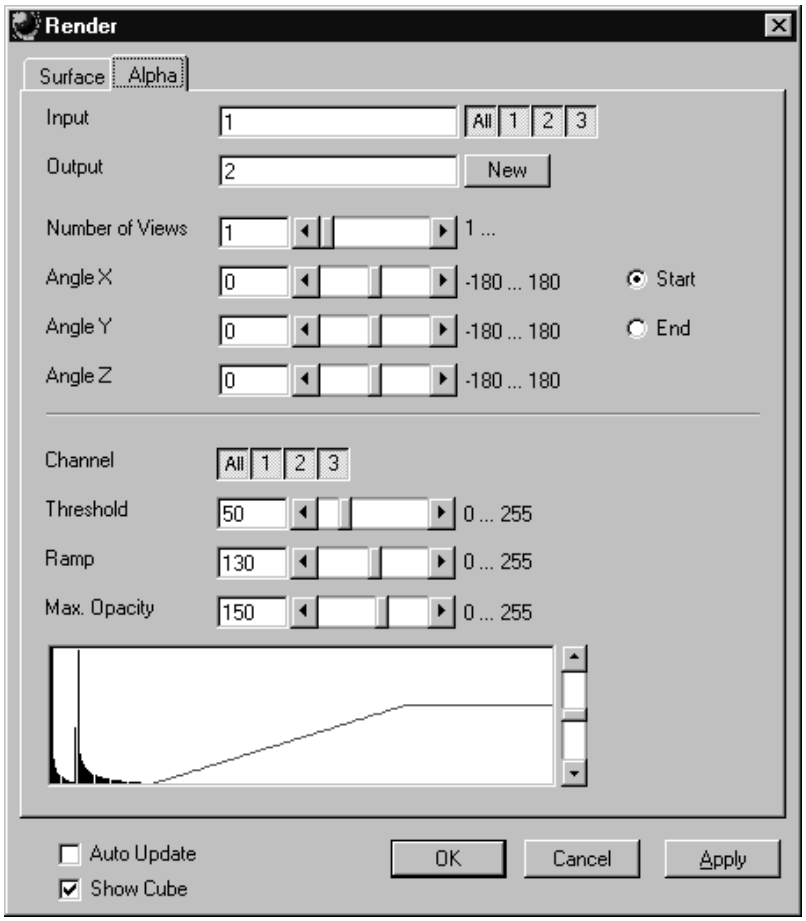


Fig. 7-34

The **Alpha** tab sheet of the **Render** dialog window must be selected.

One or more reconstructions of the input image sequence are computed according to the alpha rendering method. This type of reconstruction should be used if there is no possibility to segment the structures in the image sequence and also if the objective is to make deeply layered structures visible.

Method

The **Input** sequence defines the data to be reconstructed. If it is a multichannel sequence one or all channels can be selected for the reconstruction.

Output sets the name of the result image (sequence). If the sequence exists it is overwritten. Pressing the button **New** will generate a new name (number). The size of the sequential images in **Output** is determined by the size of the sequential images in **Input**.

Number of Views determines the number of reconstructions which should be computed. The radio buttons **Start** and **End** define which angle settings are currently shown. A definition for the angle **End** is only necessary if **Number of Views** is higher than 1. If this is true the result sequence will get views from the **Start** to the **End** angle definition. The other reconstructions are determined through the linearly interpolated intermediate angles.

The direction of view is determined from the angles as follows:

The angle **Angle Z** determines the rotation of the direction of view on the Z-axis. The angle **Angle Y** determines the rotation of the direction of view on the Y-axis that has been rotated by the angle **Angle Z**. The angle **Angle X** determines the rotation of the direction of view on an X-axis that is rotated by **Angle Z** and **Angle Y**.

Channel defines if the following parameters are valid for **All** or just for one. Defining the opacity for the channels independently is useful when the brightness and contrast of the channels differ too much. **Threshold** defines the range with no opacity. It is completely transparent. The range starts at grey value 0.

The length of slope is defined by **Ramp**. The maximum opacity value is set with the parameter **Max. Opacity**. This range ends at the maximum grey value. The **Opacity Table** shows the grey value histogram of Input with the opacity definition as a red line.

When **Auto Update** is selected, the reconstruction is updated automatically whenever a parameter is modified (except **Input, Output**, or **Number of Views**). **Show Cube** defines whether a wire frame cube is shown in the **Display window** or not.

Application

1. This method can be applied, if the structures in the Input sequence are unsharp so that objects are poorly defined by their grey value.
2. In this case, the Opacity Table is defined as a ramp. Low grey values have weight 0 to suppress the background voxels. The opacity rises with increasing grey values, depending on the parameter Ramp. The value of Max. Opacity defines the weight of the high grey values. High grey values above a threshold have weight 255 to show the "object" voxels unsuppressed. Of course a smooth step can be used.
3. The result is a display with inside structures shining through. A 3D impression can be obtained by rendering with several view directions.
4. In contrast to this, a voxel renderer like the gradient renderer would display only the surface of objects that are defined by grey value-thresholds. This surface would appear shaded as if illuminated by a light.
5. The method can also be applied to visualize pronounced structures within other enclosing structures, if the structures have different grey value ranges.
6. In this case, the Opacity Table is defined as a step. Low grey values (background) have weight 0. High grey values (inside structures) have maximum weight.

Parameters:

Input	Input image sequence
Output	Resulting image sequence
Number of Views	Number of reconstructions to be calculated
Angle X	Angle of rotation on the X-axis, start position
Angle Y	Angle of rotation on the Y-axis, start position
Angle Z	Angle of rotation on the Z-axis, start position
Channel	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
Threshold	Grey value where the opacity starts rising
Ramp	Length of the opacity slope
Max. Opacity	Maximum opacity value
Opacity Table	Maximum opacity value
Auto Update	0 - Function execution is performed on OK or Apply 1 - Function execution is performed on any parameter change
Show Cube	0 - The wire frame cube is not shown 1 - The wire frame cube is shown in the Display window

Render - Alpha: Method Description

Each **Output** pixel is a weighted sum of the **Input** voxels along a ray in view direction through the **Input** sequence. Each **Input** voxel has an opacity value, dependent only on its grey value. The opacity values are defined by the parameters **Threshold**, **Ramp**, and **Max. Opacity**.

Accumulation of pixels proceeds along the ray from back to front, i.e. from far pixels to near pixels. If a new pixel is added, it increases the result intensity by its grey value weighted by the opacity value, and attenuates the previously accumulated intensity according to the opacity value. Full intensity stops accumulation.

This calculation must be repeated for each pixel of the ray to generate one **Output** pixel. Then for each **Output** pixel to produce a 2D **Output** image for the selected view-angle. Then for each view-angle to produce an output sequence for **Number of Views** different view angles.

Render - References

[1] J.D. Foley,A.van Dam, S. K. Feiner, J.F.Hughes, Computer Graphics: Principles and Practice, Addison Wesley, Reading, MA, 1990.

[2] M. Levoy, Display of Surfaces from Volume Data, IEEE Computer Graphics & Applications, May 1988, 29-37.

[3] J. Ylä-Jääski, F.Klein, O. Kübler, Fast Direct Display of Volume Data for Medical Diagnosis, VGIP:Graphical Models and Image Processing 53,1991,7-18.

[4] K.H. Höhne, R. Bernstein, Shading 3D-Images from CT Using Gray-Level Gradients, IEEE Transactions on Medical Imaging, 5, 1986, 45-47.

[5] D.Gordon, R.A. Reynolds, Image Space Shading of 3-Dimensional Objects, CVGIP 29, 1985, 361-376.

7.3.5 Functions in the Measurement Menu

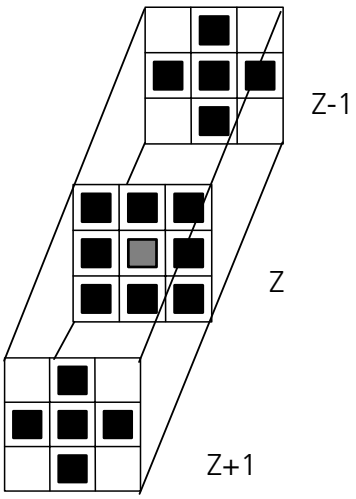
Measurement Concept

Measurement is based on regions (objects) in three-dimensional space. Segmenting an image sequence generates these. The image segmentation process produces a mask image that defines the region.

A region is a group of voxels that touch at the surfaces or at the edges, but not at the corners (18 voxel neighborhood).

This is illustrated by the following example. The voxels marked black in sequential image Z-1, Z, Z+1 all belong to the same region as the grey central voxel in sequential image Z. The volume view shows the neighborhood interrelationships as a 3D projection.

Sequential image:



Volume view:

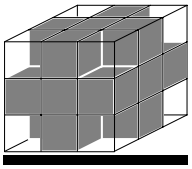


Fig. 7-35

Measurement Process

The measurement process consists of three steps: region definition, checking of the validity of the regions, and feature calculation.

Region definition:	- Automatically from the mask image
Region validation check depends on:	- Minimum volume - Measurement condition
Feature calculation depends on	- Shape of the region - Densitometric value distribution of the region - Feature parameters

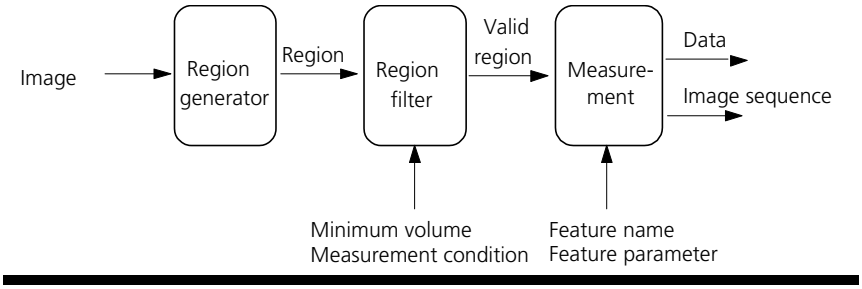


Fig. 7-36

All regions found are checked according to certain conditions. The voxel volume of each region must be equal to or greater than **MinVolume**. The measurement condition must be fulfilled. Only those regions that meet all the conditions are valid for the measurement. The region can be measured or labeled. Measurement is a process that produces data. Labeling is a process that generates an image volume.

Automatic Object Measurement – Object Features

A measurement feature describes a region characterized by a number (e.g. volume, area or a densitometrical statistic). The features can be selected on the **Object Features** and **Volume Features** tab sheets.

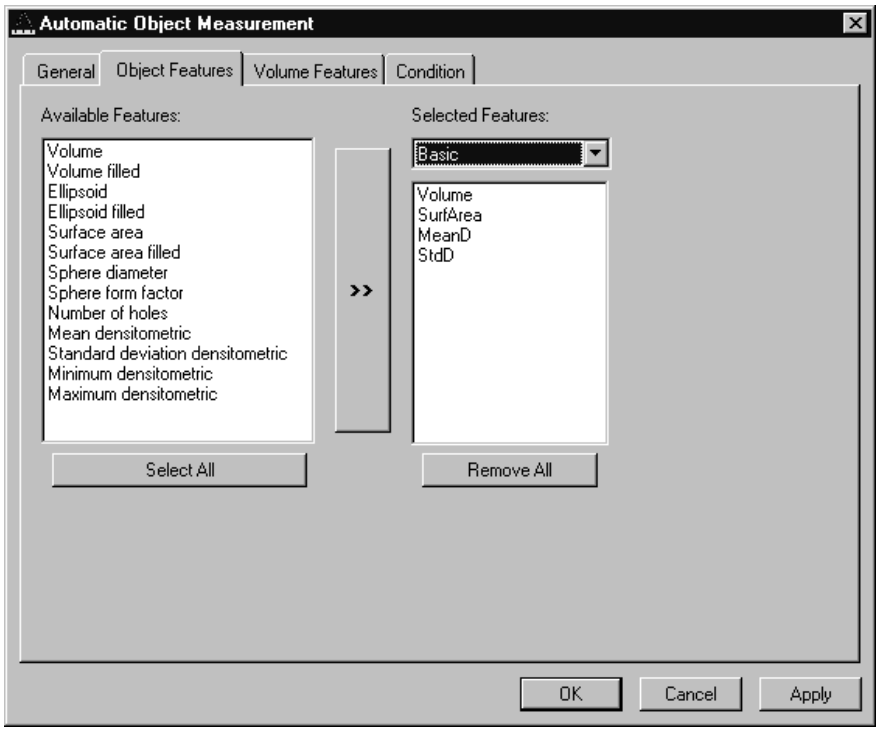


Fig. 7-37

The scalings and units are taken automatically from the assigned sequence.

The measurement features can be selected individually for each measurement. The object features generate a result value for every single object.

The dialog shows two lists. One shows the **Available Features** as groups (on the left). The other one shows the **Selected Features**. Double-clicking on items of the left list will add the **Selected Features** to the right list. Double-clicking on an item of the right list will remove this item from the list. **Selected Features** can also be transferred by clicking on the button in the middle (<< / >>) of the dialog.

The combo box above the right list represents predefined feature lists. Selecting one of the entries will fill the right list with these features; previously selected features will be overwritten.

The button **Select All** will copy all features to the list of selected features.

The button **Remove All** will clear the list of selected features.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Available Features	List of available object features
Selected Features	List of selected object features
Select All	Select all available object features for measurement
Remove All	Remove all object features from the selected features list

The following sections describe all measurement features which are defined in the system.

Object Features (geometric)

If **Object Features** are selected, one set of measurement data is calculated for each object.

Group Name	Name	Description
Volume	Volume	Volume of the object.
Volume Filled	VolumeF	Volume of the filled object.
Ellipsoid	EllipseA	Length of the main axis of the ellipsoid with the same geometrical moment of inertia as the object.
	EllipseB	Length of the middle axis of the ellipsoid with the same geometrical moment of inertia as the object.
	EllipseC	Length of the minor axis of the ellipsoid with the same geometrical moment of inertia as the object.
Ellipsoid filled	EllipseAF	Length of the main axis of the ellipse with the same geometric moment of inertia as the filled object.
	EllipseBF	Length of the middle axis of the ellipse with the same geometric moment of inertia as the filled object.
	EllipseCF	Length of the minor axis of the ellipse with the same geometric moment of inertia as the filled object.
Surface Area	SurfArea	Surface area of the object.
Surface Area Filled	SurfAreaF	Surface area of the filled object.
Sphere Diameter	Dsphere	Diameter of the sphere with the same volume. $\sqrt{6 * VOLUMEF / \pi}$
Sphere Form Factor	Fsphere	Form factor of the object. $6 \cdot \sqrt{\pi} \cdot \frac{VOLUMEF}{\sqrt{SURFAREAF^3}}$
Number of Holes	Nparts	Number of holes within an object.

Object Features (densitometric)

Group Name	Name	Description
Mean Densitometric	MeanD	Densitometric mean value of an object.
Standard Deviation Densitometric	StdD	Standard deviation of the densitometric values of an object.
Minimum Densitometric	MinD	Minimum grey value of an object.
Maximum Densitometric	MaxD	Maximum grey value of an object.

Automatic Object Measurement - Volume Features

A measurement feature describes a region characterized by a number (e.g. volume, area, or a densitometrical statistic). The features can be selected on the **Object Features** and **Volume Features** tab sheets.

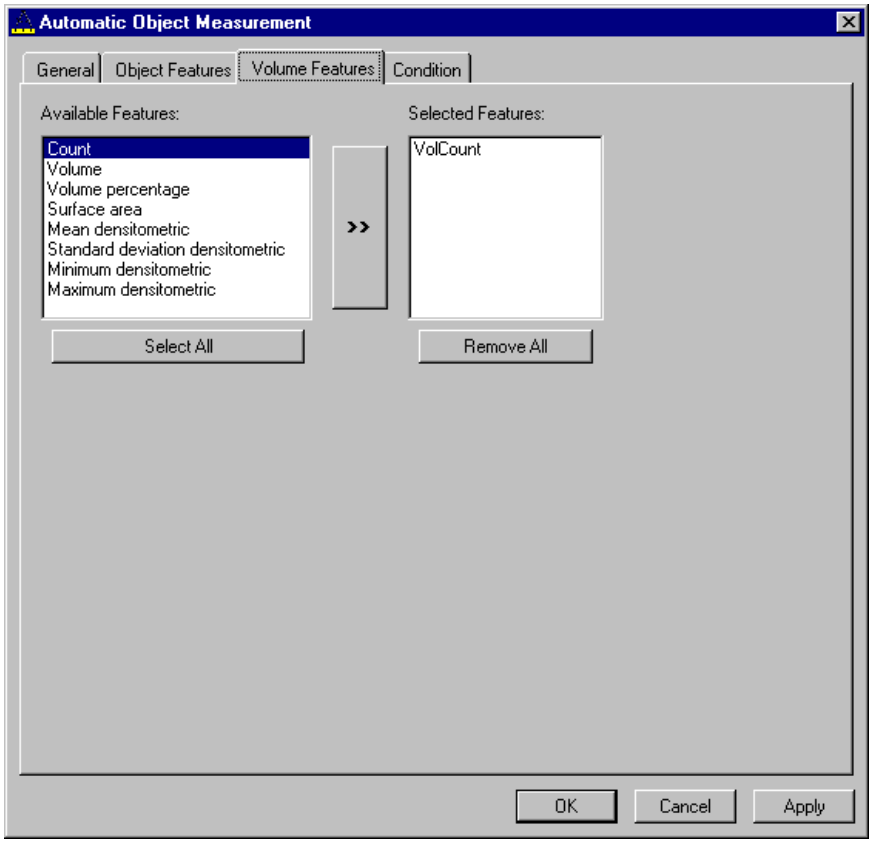


Fig. 7-38

The measurement features can be selected individually for each measurement. The object features generate a result value for every single object.

The dialog shows two lists. One shows the **Available Features** as groups (on the left). The other one shows the **Selected Features**. Double-clicking on items of the left list will add the **Selected Features** to the right list. Double-clicking on an item of the right list will remove this item from the list. **Selected Features** can also be transferred by clicking on the button in the middle (<< / >>) of the dialog.

The combo box above the right list represents predefined feature lists. Selecting one of the entries will fill the right list with these features; previously selected features will be overwritten.

The button **Select All** will copy all features to the list of selected features.

The button **Remove All** will clear the list of selected features.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Available Features	List of available object features
Selected Features	List of selected object features
Select All	Select all available object features for measurement
Remove All	Remove all object features from the selected features list

Volume Features (geometric)

The volume-related measurement generates one measured value per image sequence. The following table contains the predefined volume characteristics.

Group Name	Name	Description
Count	VolCount	Number of regions measured.
Volume	VolVolume	Total volume of all regions.
Volume Percentage	VolVolumeP	Total volume of all regions, in relation to the volume of the image sequence.

Volume Features (densitometric)

Group Name	Name	Description
Surface Area	VolSurfArea	Total surface area of all regions.
Mean Densitometric	VolMeanD	Mean grey value of all regions.
Standard Deviation Densitometric	VolStdD	Grey value standard deviation of all regions.
Minimum Densitometric	VolMinD	Minimum grey value in the image sequence.
Maximum Densitometric	VolMaxD	Maximum grey value in the image sequence.

Automatic Object Measurement - Condition

The measurement conditions are used to limit the objects to be evaluated (e.g. only objects with defined minimum value). All objects are tested against the defined conditions. If the conditions are fulfilled the feature values are written to the data table.

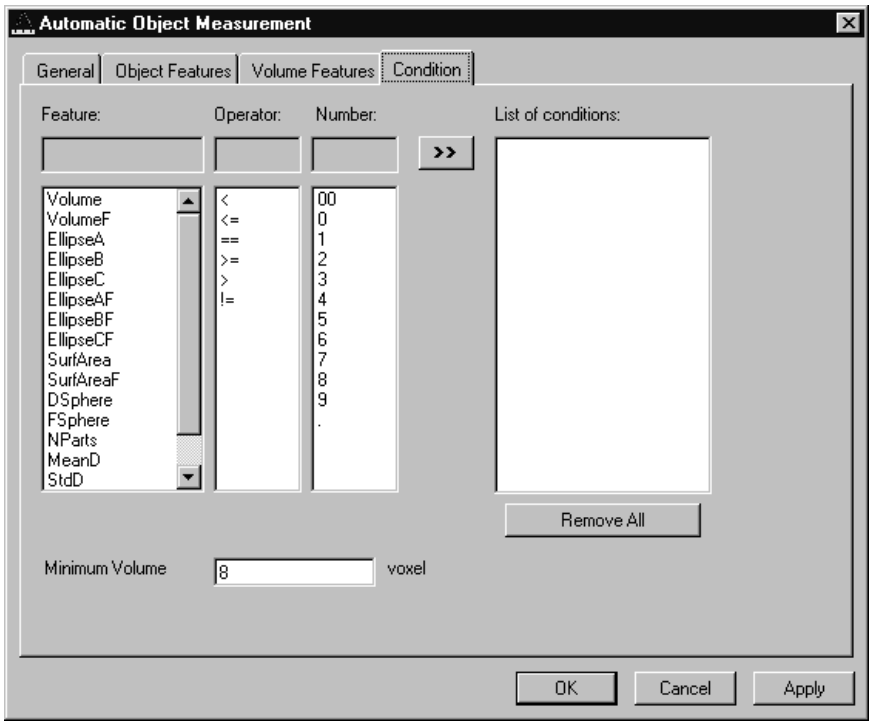


Fig. 7-39

To define the following parameter select the **Condition** tab sheet of the **Automatic Object Measurement** dialog window.

The list on the very left at the dialog shows all the measurement **Features**. The second list provides the comparison **Operators** and the next **Numbers** to define a value. This gives the possibility to compose an expression to test a feature value against a constant value. The fields above the lists will show the composed (selected) string. Clicking on the desired list entry does the selection. The button with the „>>„ characters adds this string to the **List of Conditions**. All lines of the **List of conditions** are combined with the AND expression automatically. To remove a condition line double-click on it.

The parameter **Minimum Volume** defines the minimum voxel volume for the measurement. This is an easy way to eliminate very small regions caused by noisy sequences and segmentation process.

The button **Remove All** will clear the list of defined conditions.

Clicking on the **Apply** button will execute the measurement process and switch to the **General** tab sheet of the dialog.

Parameters:

Feature	List of available object features
Operator	List of available condition operators
Number	List of numbers to compose the value
List of conditions	Defined condition list
Remove All	Remove all entries from the **List of conditions**
Minimum Volume	Minimum object volume in voxel

Automatic Object Measurement - General

This function carries out an automatic measurement and labeling.

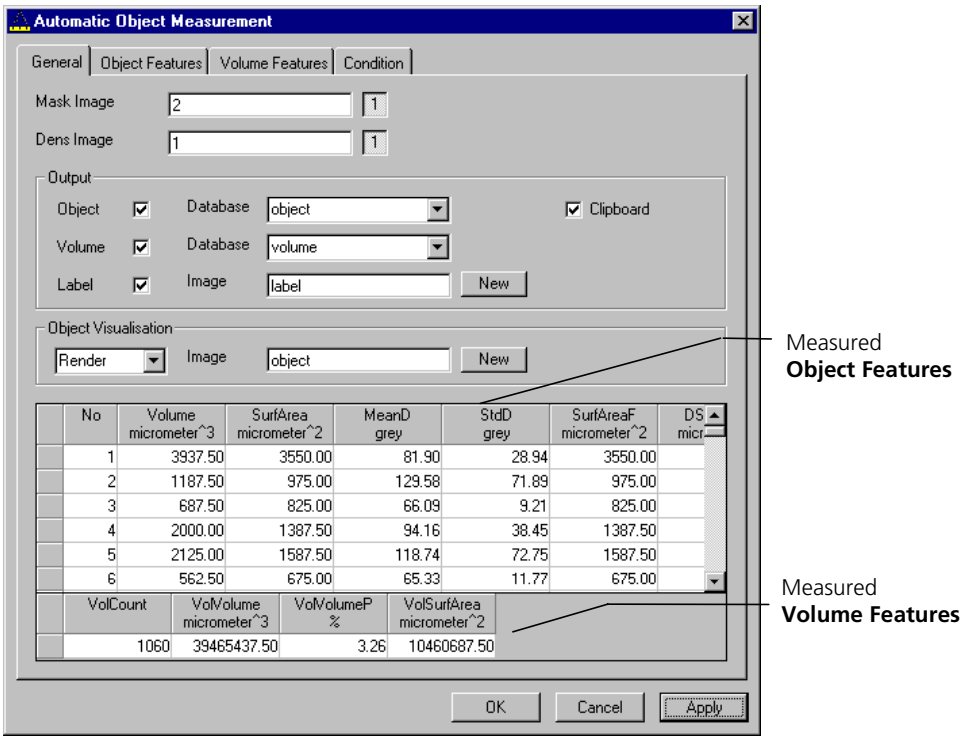


Fig. 7-40

The regions must be defined by an image sequence **Mask Image** (the objects must be separated from one another by black voxels with the grey value 0). This sequence is generated with the function **Segment**. If it is a multichannel sequence a single channel has to be chosen.

The image **Dens Image** is needed for the measurement of the densitometric features. Image sequence properties like scaling and unit are taken from **Dens Image**. A single channel of this sequence (if it is multichannel) must be selected with the buttons to the right of the parameter.

The measurement results can be stored to database files. These files are tab delimited ASCII files which can be easily imported to major Windows programs like text processing or spreat sheet application. Writing database files are independently supported for object and volume features. Activating the corresponding check boxes enables it. The name of the database is defined with the field **Database**. The files will be located in the subdirectory DATA of the main installation directory. The filename extension TXT will be added automatically.

If the check box **Label** is activated a single channel sequence will be generated. It contains all the measured objects, each object is coloured homogeneous but in different colours. To copy all measurement values to the clipboard activate the check box **Clipboard**.

A single object of interest can be visualized. Clicking on a specific row in the data grid chooses the object. By selecting a row in the data grid a new image is created with the object of interest visualized. The visualization depends on the settings in the **Object Visualisation** field. If **Render** is chosen, the object of interest is displayed with the **Surface Rendering** method. If **Mask** is chosen, the object is labelled in a pseudo colour in a new image stack.

Parameters:

Mask Image	Single channel mask image sequence that defines the objects
Dens Image	Image sequence for densitometric measurement and property source
Object	Stores measurement values of objects, including database filename
Volume	Stores volume measurement values of objects, including database filename
Label	Generates an image sequence with all objects labelled in different pseudo colours
Clipboard	Measurement values are automatically written to the clipboard

CHAPTER 8 ANNEX

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8 ANNEX

8.1 Beam Path Configuration Guide for Fluorescence Microscopy with the LSM 5

8.1.1 Optical elements in the Configuration Control window

All wavelength values given in Nanometer [nm].

8.1.1.1 Main Dichroic Beam Splitter (HFT)

- A **HFT XXX[/YYY/...]** deflects the indicated laser lines onto the specimen and allows the emitted fluorescent light to pass through.
  Example: HFT 458/514, HFT UV/488/543/633 (deflects also UV excitation light)
- A **HFT KP XXX** (KP = Short Pass) is a special type of a main dichroic used for IR multiphoton excitation. The **HFT KP 650**, deflects laser light with a wavelength longer as 650 nm onto the specimen and allows fluorescent emission light in the visible range below 650 nm to pass through.

  Example: HFT KP 650
- A **HFT KP XXX_YYY** is a combination of a HFT YYY and HFT KP XXX used for simultaneous IR multi photon and single photon excitation.
  Example: HFT KP 700_488.

8.1.1.2 Secondary Dichroic Beam Splitter (NFT)

- The **NFT XXX** is used to split the emitted light which will be guided into separate channels. Light with shorter wavelength than XXX nm is deflected, light with longer wavelength passes the NFT. A cascade of NFTs allows to distribute the emission light to more than two channels/detectors.
- The **NFT KP YYY** treats the emission light the other way round: it transmits light shorter than YYY nm and deflects above YYY nm.

8.1.1.3 Emission Filters (EF)

- A **LP XXX** (Long Pass) transmits emission light with wavelengths longer than the indicated threshold value XXX.
- A **KP XXX** (Short Pass) transmits emission light with wavelengths shorter than the indicated threshold value XXX.
- A **BP XXX-YYY** (Band Pass) transmits emission light within the indicated wavelength band.
- A **BP XXX/BB** has a transmission band for emission light with a center wavelength of XXX nm and a width of BB nm.
- The **BG 39** (Blue Green glass) blocks infrared excitation light by absorption.
- **BP ... IR** (Band Pass – Infra Red) is a band pass suitable for detection of IR excited dyes. It blocks the IR light.

8.1.1.4 Miscellaneous

- **Plates** do transmit light 100%. They are used for a correct beam guidance and will be set automatically.
- **Mirrors** do deflect 100% over the whole spectral range and can be used to guide the emission light to selected detectors.

8.1.2 Setup of Single Tracks Using Single Detectors

- Switch on the suitable lasers for excitation of the dyes in the specimen. For the UV laser and the Argon laser set the tube current of the laser to a value of app. 50% (**Excitation, Laser, Output [%]**). Example: for Alexa 488 and CY 3 switch on **Argon** (blue excitation) and **HeNe1 or DPSS 561** (green excitation).
- Activate the proper laser lines in the **Line Active** check box, set **Transmission [%]** for each active line.
  Example: Select 488 to 4% and 561 to 18%
- Select a main dichroic beamsplitter (HFT) which deflects the selected laser lines to the specimen.
  Example: HFT 488/561/633

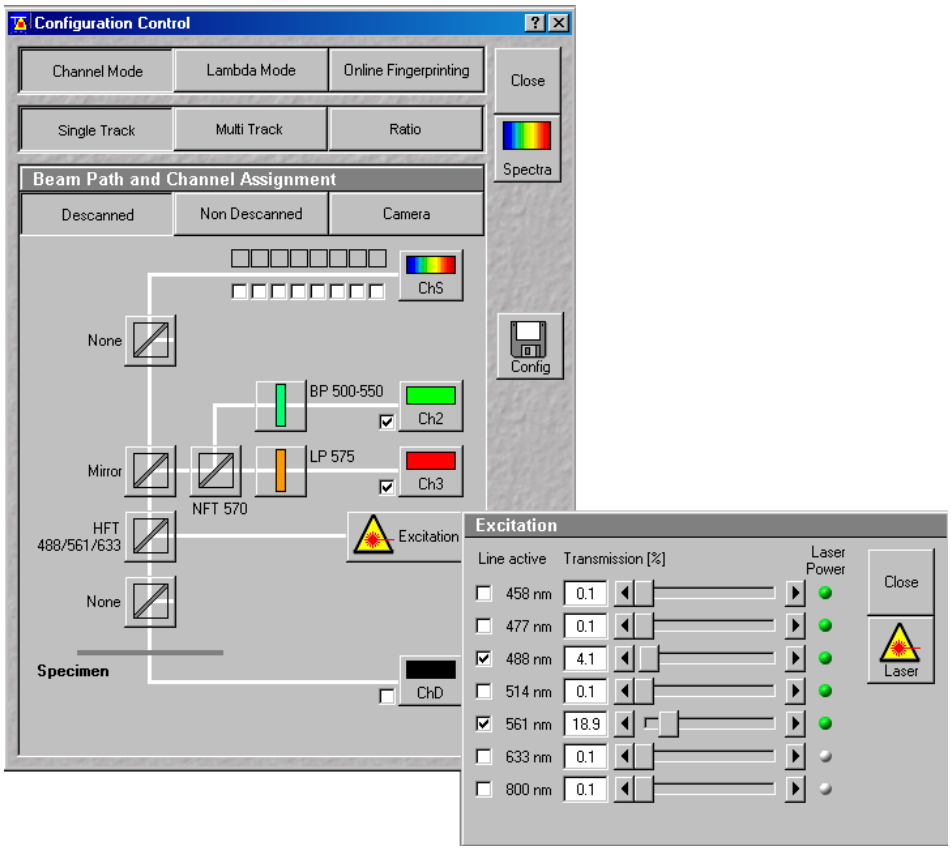

Fig. 8-1 Configuration Control window and Excitation panel

- Check the available emission filters (EM 1- 4) for transmission of fluorescent light from the specimen, in order to identify the channels for detection.
  Example: BP 500-550 in channel Ch 2 for acquisition of green emission and LP 575 in channel Ch 2 for acquisition of red emission
- Use the secondary beam splitters (NFT 1/2/3) to split and guide the emitted fluorescent light to the detectors (PMTs) of the selected channels (see above).
  Example: a mirror in NFT 1 position will deflect all emission light to pass to Ch 2 and 3. NFT 570 will allow light longer than 570 nm to pass to channel 3 and deflects light shorter then 570 towards channel Ch 2.
- Select the proper emission filters in front of the channels and activate channels. Example: select LP 575 in front of Ch 3, and BP 500-550 in front of Ch 2].
- Make sure that the active detection bands do not include any of the active laser lines Example: do not use a LP 505for detection of green emission when using the 561 nm line for green excitation

Additional hints:

- Do not forget to turn on the corresponding lasers and detectors.
- Assign appropriate colors to these activated channels. Example: Ch 2 - green (for Alexa 488 emission, Ch 3 - red (for CY3 emission)
- The Spectra dialog is a big help for checking if the configuration of the beam path was successful. It shows activated laser lines and for each channel the emission range that can be "seen" by the detector indicated by the corresponding channel color. A gray bar indicates an emission range that is guided into a channel, but the detector is not turned on.
- When simultaneously detecting more than one fluorescent dye use channel Ch 1 for detection of the emission with long wavelength, then channels Ch 4 and Ch 3 (if available) for medium wavelengths and channel Ch 2 for short wavelengths.
- Use NFT 3 for separating emission into channels Ch 1 and Ch 4 and NFT 2 for separating emission into channels Ch 2 and Ch 3

8.1.3 Multi Track Configuration

Multitracking is the method of choice for multi fluorescence imaging. It has the advantage to avoid artifacts based on emission crosstalk that occurs when using simultaneous excitation and detection. Laser lines are switched very fast and channels recorded quasi-simultaneously.

The configuration of Multiple Tracks follows the same rules described above for single track configuration. The main difference is that each track is configured to excite and detected only one fluorescent dye to prevent cross talking (or two dyes with non overlapping emission spectra).

- Create a single track for both, Alexa 488 and CY 3 detection separately (see above).
- Open the Multi Track configuration window. The system displays the Single Track setup as track one.
- Add a new track.
- Click on track one, deactivate Ch 3 (red emission detection) and switch off the green laser line (561nm) in the **Line Active** check box in the **Excitation** control window.
- Click on track two, deactivate Ch 2 (green emission detection) and switch the blue laser line (488) off.
- To extend the detection band for the green light it is now reasonable to use the LP 505 instead of BP 500-550 in track one. This is now possible since the green laser line is turned off during detection of the green fluorescence emission.
- Use the Spectra window to check the proper settings for each individual track as described above.

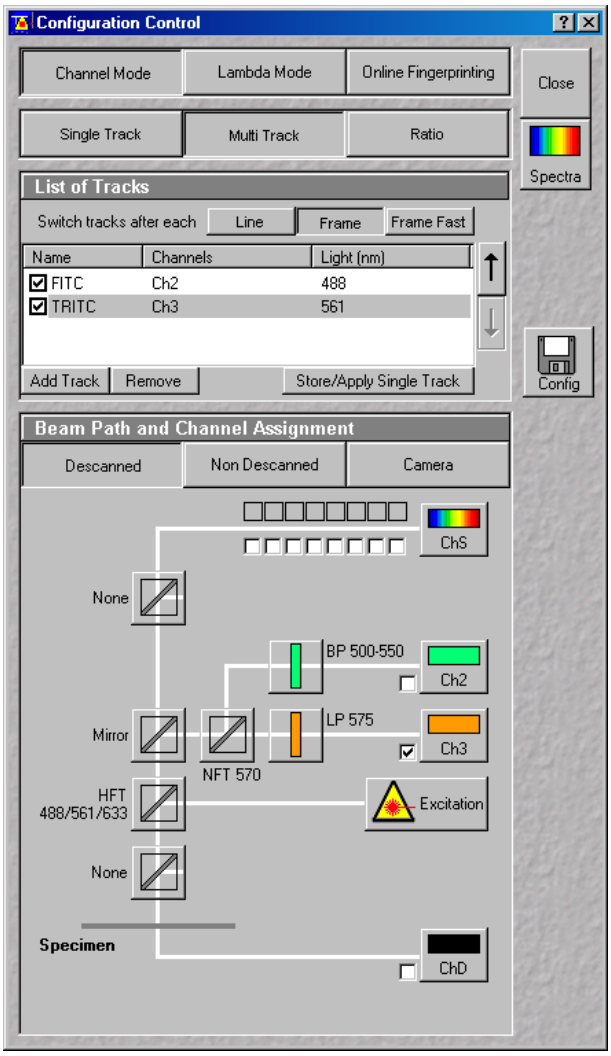

Fig. 8-2 Configuration Control window

Line and Frame Mode of Multitracking

- Settings can be used for **Line** or **Frame** wise Multitracking.
- In Line mode the lines are scanned in turns for all tracks with the corresponding laser lines turned on exclusively. Preferred for living samples with moving objects. Acquisition time can be reduced using bidirectional scan mode.
- In Frame mode whole frames are scanned in turns for all tracks with the corresponding laser lines turned on exclusively. This mode can be advantageous for dyes that tend to bleach and need time to recover.
- There are parameters that can be changed quickly in a line wise: Amplifier Gain, Amplifier Offset, Laser Line Attenuation,
- Any other changes of track settings of the selected tracks, e. g. different filters, dichroics or Detector Gain settings, need a bit more time to be changed, and require Frame mode.
- There is a fast Frame mode, that requires identical settings of these parameters.
- In our example it is now possible to use the BP 505-550 also in track two. There is no function of this BP in track two, but it guarantees equal settings in both tracks/channels, which now allows line wise Multitracking.

8.1.4 Beam path Configuration for Multi Photon Excitation

- The beam path configuration for multi photon excitation follows the same rules as described for a single and multi track configuration (see above).
- Use a KP 650 or KP 700 as main dichroic beam splitter to deflect the IR excitation light (700-1050 nm) onto the specimen.
- On detection side, set always a BG 39 in the beam path or use a IR suitable band pass filters (BP XXX-YYY IR) to prevent a bleed through of IR excitation light to the detector.

8.2 Recommendations for Excitation Laser Lines and emission filters of dyes

Dye	Laser line/HFT	Emission/EM
DAPI	364 or 405	> 385/420, max. at 461
EBFP	364 or 405	> 385/420, max. at 447
Hoechst	364 or 405	> 385/420, max. at 440
Fluoro-Gold	405 or 458	> 420/475, max. at 536
ECFP	405 or 458	> 420/475, max. at 501
Lucifer Yellow	458	> 475, max. at 536
EGFP	477 or 488	> 505, max. at 507/516
FM 1-43™	477 or 488	> 505, max. at 598
Alexa Fluor 488™	488	> 505, max. at 520
Calcium Green	488	> 505, max. at 531
Cy2™	488	> 505, max. at 508
DiO (DiOC18(3))	488	> 505, max. at 508
Fluo-3	488	> 505, max. at 520
Fluorescein (FITC)	488	> 505, max. at 520
Cy3™	514	> 530, max. at 566
EYFP	514	> 530, max. at 535
Oregon Green	514	> 530, max. at 535
SYTOX Green	514	> 530, max. at 536
FM 4-46	514 or 543	> 560, max. at 640
Alexa Fluor 546™	543	> 560, max. at 572
Calcium Orange	543	> 560, max. at 575
DiI (DiIC18(3))	543	> 560, max. at 565
DsRed	543 or 561	> 560, max. at 583
Tetramethylrhodamine (TRITC)	543 or 561	> 560, max. at 576
Rhodamine B	543	> 560/585, max. at 625
Texas Red™	561 or 594	> 570/600, max. at 620
Alexa Fluor 633™	633	> 650, max. at 654
Cy5™	633	> 650, max. at 666

Here you can note your specific combinations:

Dyes	**Laser/HFT**	**EM1**	**NFT**	**EM2**

Example:

Dyes	**Laser/HFT**	**EM1**	**NFT**	**EM2**
FITC/Cy3	488/543	BP 505-530	545	LP 560

8.3 Configurations Overview

Package Configuration	Count Channels	META Detector	Laser lines	Laser lines optional (additional)
23	**3 Channels F/R**	**No**	**ArMI 458/477/488/514, HeNe 543, HeNe 633**	**Ti:Sa 700 - 1050 nm**
23 META	**2 Channels F/R**	**Yes**	**ArMI 458/477/488/514, HeNe 543, HeNe 633**	**Ti:Sa 700 - 1050 nm**
24 META	**2 Kanäle F/R**	**Yes**	**ArMI 458/477/488/514, DPSS 561, HeNe 633**	**Ti:Sa 700 - 1050 nm**
25	**3 Channels F/R**	**Yes**	**ArMI 458/477/488/514, HeNe 543, HeNe 633**	**UV 351/364 nm**
25 META	**2 Channels F/R**	**Yes**	**ArMI 458/477/488/514, HeNe 543, HeNe 633**	**UV 351/364 nm**
26	**2 Channels F/R**	**No**	**ArMI 458/477/488/514, HeNe 543, HeNe 633**	-
26 META	**2 Channels F/R**	**Yes**	**ArMI 458/477/488/514, HeNe 543, HeNe 633**	-
27	**3 Channels F/R**	**No**	**ArMI 458/477/488/514, HeNe 543, HeNe 633**	-
28	**3 Channels F/R**	**No**	**ArMI 458/477/488/514, HeNe 543, HeNe 633**	**Laser diode 405 nm Laser line 594 nm**
28 META	**2 Channels F/R**	**Yes**	**ArMI 458/477/488/514, HeNe 543, HeNe 633**	**Laser diode 405 nm Laser line 594 nm**
29	**2 Channels F/R**	**No**	**ArMI 458/477/488/514, DPSS 561, HeNe 633**	**Laser diode 405 nm**
29 META	**2 Channels F/R**	**Yes**	**ArMI 458/477/488/514, DPSS 561, HeNe 633**	**Laser diode 405 nm**

Configuration	Main beam splitter (HFT)	Secondary beam splitter 1 (NFT 1)	Secondary beam splitter 2 (NFT 2)	Secondary beam splitter 3 (NFT 3)
23	**NT 80/20** **488/543/633** **KP 700/488** **KP 700/543** **458/514** **458** **477/543** **KP 650**	**empty** **mirror** **490** **515** **545** **635 VIS** **empty** **plate**	**mirror** **490** **545** **BG 39**	**empty** **plate** **BG 39** **mirror**
23 META	**NT 80/20** **488/543/633** **KP 700/488** **KP 700/543** **458/514** **458** **477/543** **KP 650**	**empty** **mirror** **490** **515** **545** **635 VIS** **KP 545** **Platte**	**mirror** **490** **545** **BG 39**	**empty** **plate** **BG 39** **mirror**
24 META	**NT 80/20** **488/561/633** **KP 700/488** **KP 700/561** **458/514** **458** **488/561** **KP 650**	**empty** **mirror** **490** **515** **NFT 565** **635 VIS** **KP 560** **plate**	**mirror** **490** **NFT 565** **BG 39**	**empty** **plate** **BG 39** **mirror**
25	**NT 80/20** **UV/488/543/633** **UV/488** **458/514** **477/543** **458** **UV (375)** **488**	**empty** **mirror** **490** **515** **545** **635 VIS** **empty** **plate**	**mirror** **490** **545** **plate**	**empty** **plate** **empty** **mirror**

Configuration	Main beam splitter (HFT)	Secondary beam splitter 1 (NFT 1)	Secondary beam splitter 2 (NFT 2)	Secondary beam splitter 3 (NFT 3)
25 META	**NT 80/20** **UV/488/543/633** **UV/488** **458/514** **477/543** **458** **UV (375)** **488**	**empty** **mirror** **490** **515** **545** **635 VIS** **KP 545** **plate**	**mirror** **490** **545** **plate**	**empty** **plate** **empty** **mirror**
26	**NT 80/20** **UV/488/543/633** **458/514** **477/543** **488/543** **514/633** **458** **488**	**empty** **mirror** **515** **545** **635 VIS** **empty** **empty** **plate**	**mirror**	**empty** **plate** **empty** **mirror**
26 META	**NT 80/20** **UV/488/543/633** **458/514** **477/543** **488/543** **514/633** **458** **488**	**empty** **mirror** **515** **545** **635 VIS** **empty** **KP 545** **plate**	**mirror** **515** **545** **plate**	**empty** **plate** **empty** **mirror**
27	**NT 80/20** **UV/488/543/633** **458/514** **477/543** **488/543** **514/633** **458** **488**	**empty** **mirror** **515** **545** **635 VIS** **empty** **empty** **plate**	**mirror** **514** **545** **plate**	**empty** **plate** **empty** **mirror**

Configuration	Main beam splitter (HFT)	Secondary beam splitter 1 (NFT 1)	Secondary beam splitter 2 (NFT 2)	Secondary beam splitter 3 (NFT 3)
28	**NT 80/20** **405/488/543/633** **405/488** **405/514/594** **458** **458/514** **488/594** **488/543**	**empty** **mirror** **515** **545** **595** **635 VIS** **KP 545** **plate**	**mirror** **490** **515** **545**	**empty** **plate** **empty** **mirror**
28 META	**NT 80/20** **405/488/543/633** **405/488** **405/514/594** **458** **458/514** **488/594** **488/543**	**empty** **mirror** **515** **545** **595** **635 VIS** **KP 545** **plate**	**mirror** **490** **515** **545**	**empty** **plate** **empty** **mirror**
29	**NT 80/20** **405/488/633** **405/488/561** **405/514/633** **458/514** **458/561** **488/561/633** **488**	**empty** **mirror** **490** **515** **565** **635 VIS** **KP 560** **plate**	**mirror** **490** **515** **565**	**empty** **plate** **empty** **mirror**
29 META	**NT 80/20** **405/488/633** **405/488/561** **405/514/633** **458** **458/514** **488/561** **488**	**empty** **mirror** **490** **515** **565** **635 VIS** **KP 560** **plate**	**mirror** **490** **515** **565**	**empty** **plate** **empty** **mirror**

8.4 Filter Change in the Detection Beam Path of Channels 1 and 2

For optimum investigation of specimens it is useful to employ filter wheels permitting the motor-controlled change between different filters for narrow-band or broad-band detection depending on the wavelength. The number of filters is limited by the capacity of the filter wheel. The change of the filter wheel as a whole involves complete readjustment.

The filter wheels of channels 1 (upper cover cap) and 2 (lower cover cap on the right side) of the Scanning Module have a change position in which a filter, including its mount, can be changed in a reproducible position without requiring readjustment. The filters can be rotated in their cells, and with the light path being eccentric relative to the filter center, the best transmission area of the filter for the respective wavelength or pass range can be found by rotating the filter. This is very important for the investigation of specimens of low emission.

Filter change

- By software control, move filter wheel (8-3/**5**) to the change position.
- Pull cover cap (8-3/**1**) off the Scanning Module.
- Use the filter tool (8-3/**2**) to pull the filter mount (8-3/**4**) with the filter (8-3/**3**) out of the guide well.
- Change filter to suit the application.

☞ The filter can be rotated in its mount, allowing adjustment for finding the best transmission area of the filter for the wavelength used.

- Enter the designation of this particular filter into the System Software database.

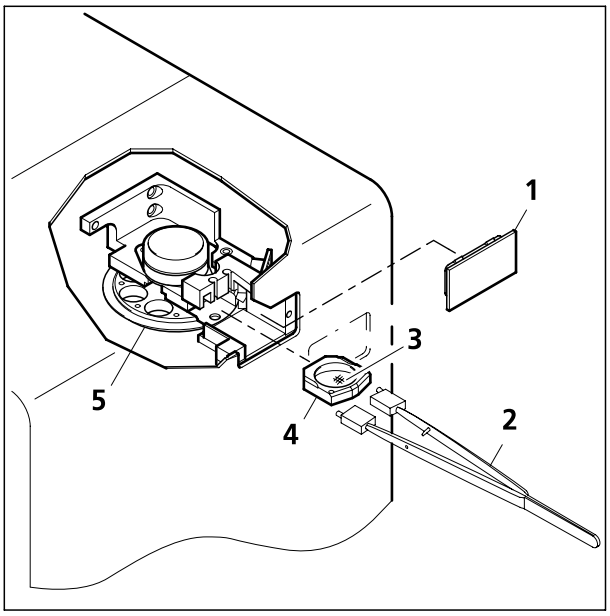

Fig. 8-3 Filter change

8.5 Piezo Objective Focussing Device

For Axio Imager.Z1, Axio Imager.M1 and Axiovert 200

Range: 250 µm

Minimum step size: 50 nm

Speed:

		Piezo objective focussing device
Slices	**Step size [µm]**	**xz-lines / s**
20	0.5	720

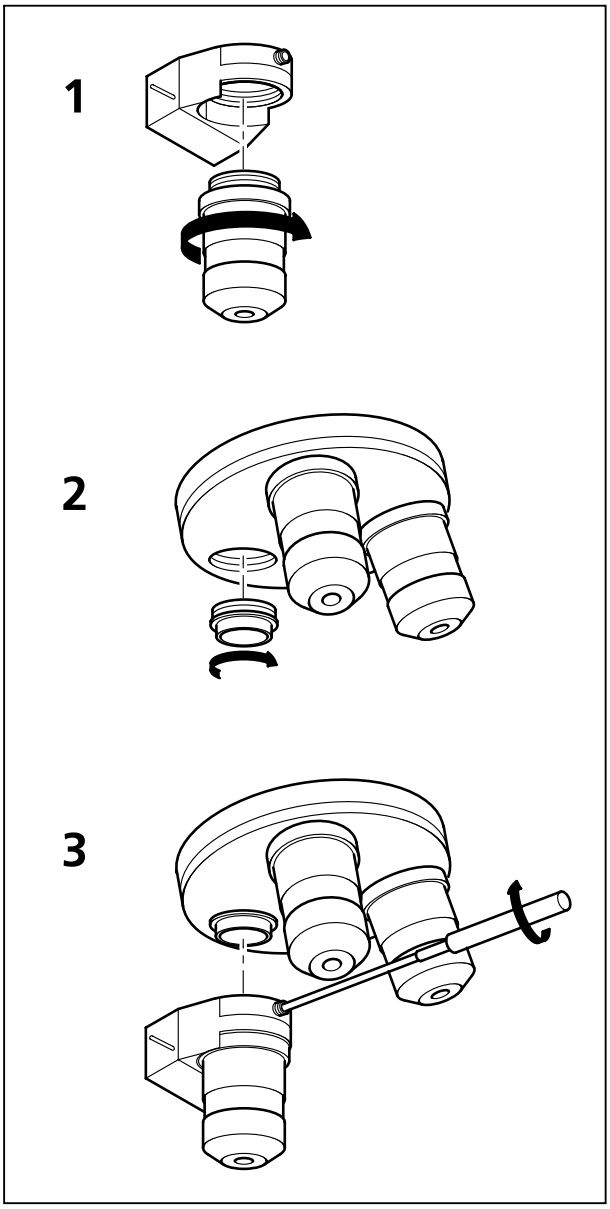

Fig. 8-4 Installation of the Piezo Objective Focusing Device

Objectives:

– W0.8/M27

– Modified Achroplan 40x / 0.8 W with reduced length to compensate for piezo height

Installation:

- Screw in your microscope objective into Piezo Objective Focusing Device (see Fig. 8-4/**1**).
- Screw the thread-ring into your microscope (see Fig. 8-4/**2**).
- Easy clamp the Piezo Objective Focusing Device on the thread-ring (see Fig. 8-4/**3**).

8.6 Specifications of Trigger-Interface LSM 510 / LSM 510 META

Application:

With the LSM 510 / LSM 510 META you can control various actions externally using Trigger-In or force external devices to work at a defined time depending on an action using Trigger-Out during time series. These actions are: Scan-Start / Stop, Bleach, Change of Scan-Interval, end of a countdown, set marker into image or even a mouse-click on a button.

Interface:

- User Port Adapter on the back of the Real Time Electronics in the System Electronic Rack
- Connector 2x 15-point high density D-Type plug, 1x coax with outer shield (Triax Lemosa Serie 00, EPL.00.650NLN)

Number:

- 8x signal IN (all able to generate an interrupt)
- 18x signal OUT (10 synchronous, 8 asynchronous relative scan position)
- 1x clock

Connector:

Coax: Triax Lemosa Serie 00, EPL.00.650NLN

Pin	Signal name	Description
Signal	PCLK_Out	PixelClock Output
Signal shield	GND	Signal ground
Outer shield	Shield	Chassis ground

15-point high density D-Type plug: Plug „A“

Pin	Signal name	description
1	SyncOUT0	Synchronous output
2	SyncOUT1	Synchronous output
3	SyncOUT2	Synchronous output
4	SyncOUT3	Synchronous output
5	GND	Signal ground
6	AsyncOUT0	Asynchronous output
7	AsyncOUT1	Asynchronous output
8	AsyncOUT2	Asynchronous output
9	AsyncOUT3	Asynchronous output
10	GND	Signal ground
11	AsyncIN0	input
12	AsyncIN1	input
13	AsyncIN2	input
14	AsyncIN3	input
15	Connector detector*	Input / Connector detector

15-point high density D-Type plug: Plug „B“

Pin	Signal name	Description
1	SyncOUT4	Synchronous output
2	SyncOUT5	Synchronous output
3	SyncOUT6 / sync outline **	Synchronous output
4	SyncOUT7 / sync outframe **	Synchronous output
5	GND	Signal ground
6	AsyncOUT4	Asynchronous output
7	AsyncOUT5	Asynchronous output
8	AsyncOUT6	Asynchronous output
9	AsyncOUT7	Asynchronous output
10	GND	Signal ground
11	AsyncIN4	input
12	AsyncIN5	input
13	AsyncIN6	input
14	AsyncIN7	input
15	Connector detector*	Input / Connector detector

*) If the pin is connected to ground, the software recognizes that at least one connector is connected to the UserPort2.
**) depending on internal switch inside real time control unit

Type/Voltage Range:

- TTL signal level 3.3 V, CMOS low power consumption
- 5.0 V tolerant input/output for interfacing with 5 V logic

Load:

Output: < 50 mA (internal serial 68 ohm resistor)

Input: 4.7 kOhm input impedance (internal 4.7 kOhm pullup to 3.3 V)

Trigger pulse description:

Signal output:

- Low level < 0.4 V, high level > 2.7 V
- Slew rate 10 ns/V

Signal input:

- Low level < 0.5 … 0.8 V, high level > 2.0 … 5.5 V
- Falling edge force interrupt
- Pulse width to detect signal > 50 ns

Caution:

- Never apply more than 5 V or negative voltages to avoid any damage.
- In and outputs are not galvanically decoupled.
- Therefore proper measures for galvanic decoupling of external devices have to be taken (opto-coupler etc.).

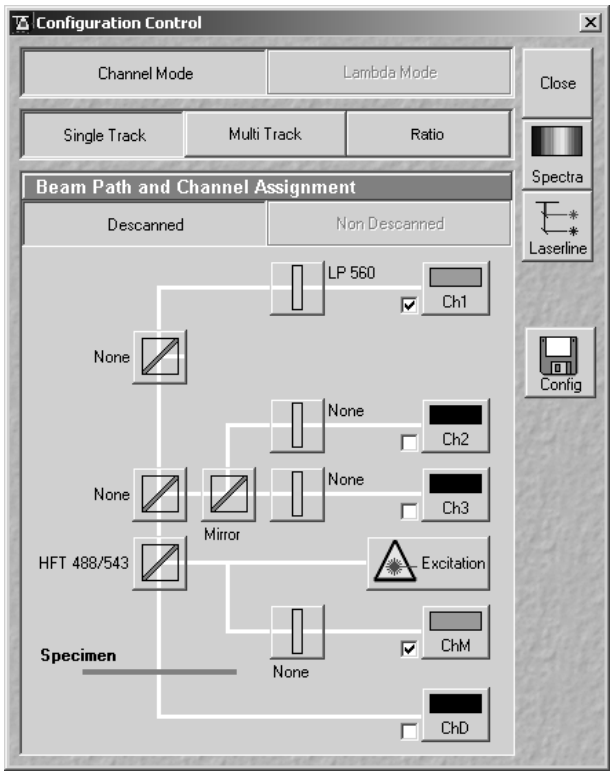

Fig. 8-5 Configuration Control window

8.7 Monitor Diode

The monitor diode is placed in the excitation ray path of the LSM 510 behind the beam splitter combining the visible and the UV ray path and in front of the main beam splitter. Therefore it allows to check the laser input in terms of power and noise. With the attenuation filter wheel in front of the diode it is possible to attenuate the laser power reaching the diode. It is not possible to select one line out of a few excitation wavelengths to be detected by the diode.

Proceed as follows to activate the diode as a detector:

- Click on the corresponding button in the **Configuration Control** window of the LSM 510 software (Expert Mode).
- Choose either **Frame** or **Line** scan.
- Change to the **Scan Control** window and press **Cont.**; the system will scan with the diode as a channel.
- Choose the right amplification of the signal obtained by using the special neutral density filters in front of the diode or / and by using the setting of the **Amplifier Gain** and **Amplifier Offset** value.
  (Scan Control - Channels ChM-1).

Application examples:

a) Checking the laser power

This function is not automated so far. To qualitatively measure the laser power, the diode can be used in such a way that the gray level obtained in the **Line Scan** mode at a certain setting of the whole system is stored as a text overlay together with the image (manually done by user). As the diode setting (Ampl. Gain, Ampl. Offset, ND filter) is stored together with the image, the setting is automatically reloaded when using the **REUSE** button. If deviations can be observed it is easy to set the laser power to the old value by means of the AOTF transmission.

b) Noise Reduction by Ratio

Contrary to the PMT signal, the signal of the monitor diode is not modulated by any specimen information. Thus it can be used to ratio the PMT signal to get rid of the laser noise (due to any laser as a physical fact) and thereby improve the signal to noise ratio of the fluorescence or reflectance image. The major condition which has to be fulfilled to use the monitor diode for this purpose is that the dominating source of noise is laser noise. The signal of the monitor diode will always be dominated by laser noise (independent of the power set at the laser, or the transmission set at the AOTF), whereas the dominating source of noise in the PMT signal can also be the shot noise of light (shot noise especially occurs in low light fluorescence application; as rule of thumb it can be noted that the shot noise is limiting the signal to noise ratio, if the PMT voltage has to be set to a value > 400 V).

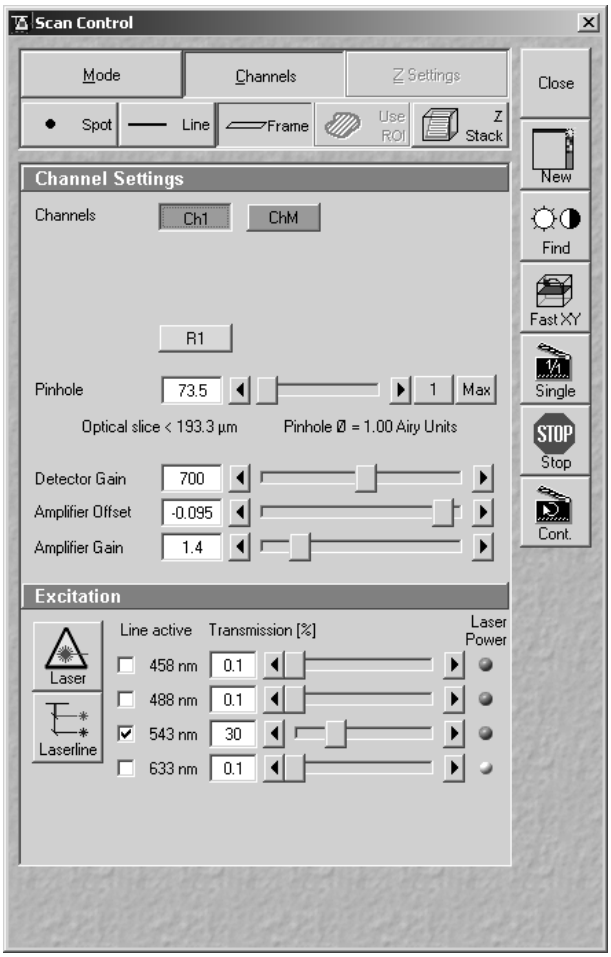

Fig. 8-6 Scan Control window

☞ Any kind of noise which can not be observed in both channels at a time will be amplified and not reduced by the ratio process. Low or high frequency laser noise is the only source of noise which is correlated in the PMT signal and the signal of the monitor diode.

Low or high frequency laser noise is mainly introduced if the Ar lasers are used at a tube current lower than 8 A (Ar-Vis) or 20 A (Ar-UV) respectively.

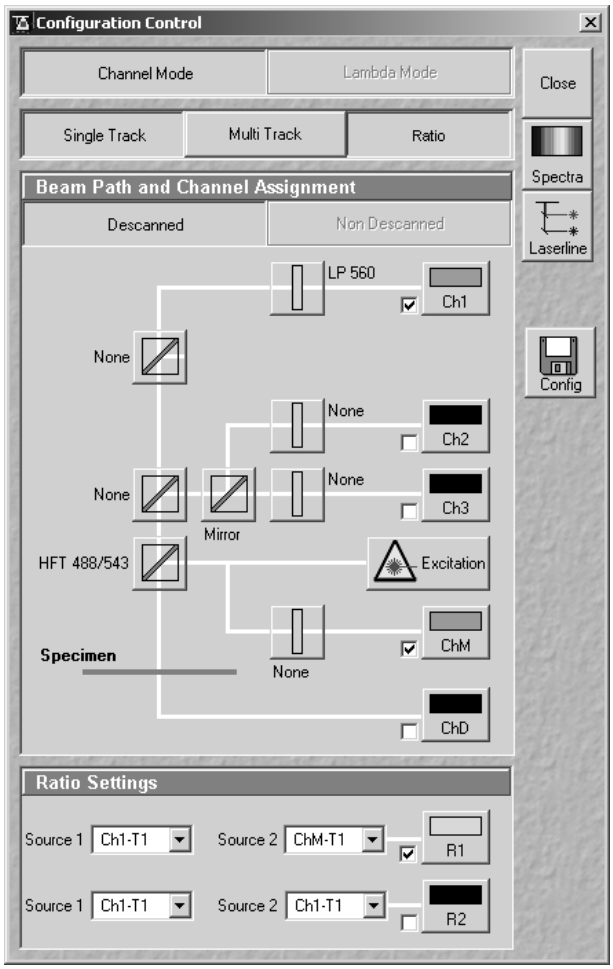

Fig. 8-7 Configuration Control window

To use the monitor diode for ratio application, proceed as follows:

- Click on the **Ratio** button.
- Activate the ratio channel R1 or R2 in the **Ratio Settings** panel in the **Configuration Control** window in addition to the monitor diode channel (ChM-1) and one PMT channel.
- Choose the appropriate PMT channel as source 1 in the **Ratio Settings** panel and ChM-1 as source 2. If this numbering is changed (inverted), the ratio image will show an inversion of gray levels if compared to the PMT image.

☞ It is not possible to do the ratio between an on-line ratio image generated with two PMT channels (as in ion-concentration sensitive ratio imaging) and the signal of the monitor diode.

The following image is an example of the reduction of correlated noise. The low frequency noise has been generated artificially.

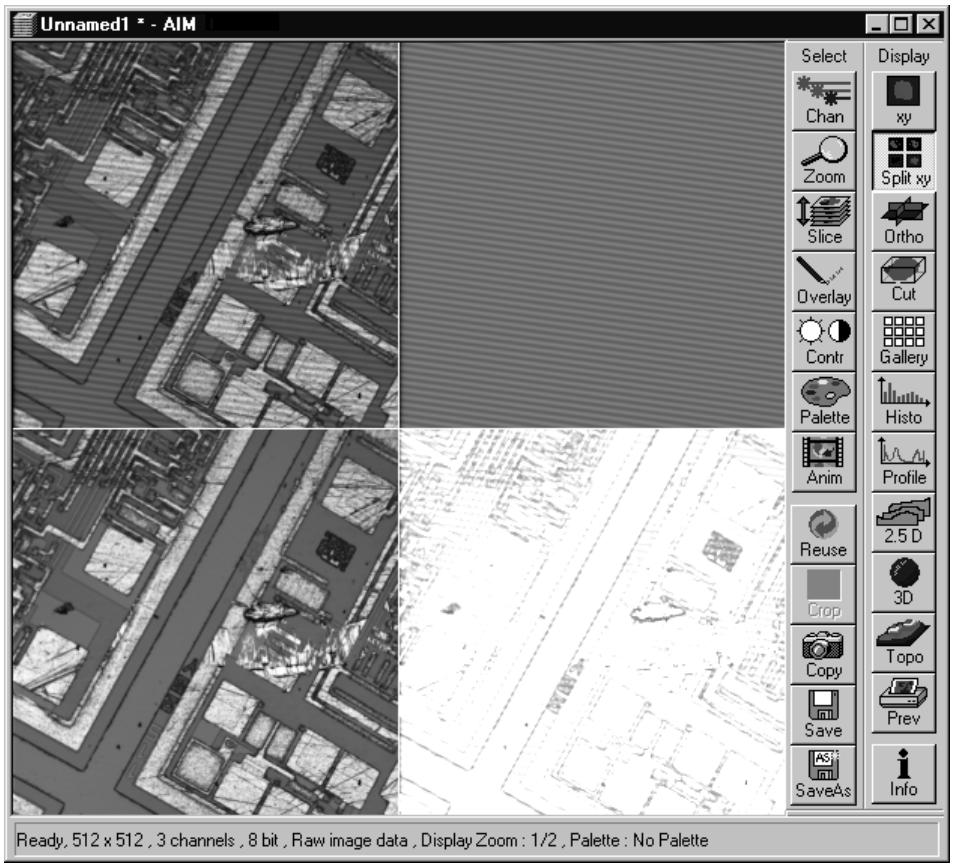

Fig. 8-8 Image Display window

The image in the upper left corner shows the PMT image plus noise, the image beneath this (upper right corner) shows the signal of the diode expanded to 512 x 512 pixels (noise without object information). The two images below show the ratio of the PMT and diode signal (left) and the sum of all signals (right). The sum-image does not contain any information and can therefore be neglected.

To get a ratio image like the one shown here, Detector Gain, Amplifier Gain, Amplifier Offset of the PMT channel, Gain and Offset of the diode channel, Gain and Offset of the ratio channel must be set in the correct way.

Each of the parameters summarized effects either the amplification of the ratio image, or the contrast of the ratio image, or the quality of the noise reduction.

The single steps to find the right setting of all the parameters to be set are listed in the following:

- Activate the Range Indicator.
- Adjustment of **Amplifier Offset**: the Offset of the PMT channel and diode channel have to fit to each other to guarantee the best noise reduction.

The best way to do the adjustment is the following:

- Choose different colors in the **Configuration Control** window for PMT and diode channel.
- Activate **Line Scan**.
- Switch off all laser lines in the **Excitation** window.
- Activate **Cont.**
- Set values for **Ampl. Gain** to 1 in each channel.
- Set the lines visible to the same level as close to the ground level as possible; the values you find for the Offset in each channel should be negative.

A final adjustment of the offset adjustment is done by visually evaluating the noise reduction in the ratio image. As the Offset value of the PMT channel influences the range setting of the ratio image much less than the Offset value of the diode channel, the fine tuning should be done via the PMT offset, if required.

☞ As mentioned before, the calculation of the ratio image is very sensitive to different signal offsets in the two channels used. As the offset is influenced by the scan speed as well as by the Amplifier Gain used, the offset calibration is not valid any more if the scan speed is changed, or the Ampl. Gain is set to a new value respectively. In most cases a new fine tuning is necessary. If this doesn't work, the complete calibration process has to be repeated.

Another possibility to calibrate the offset values is to set the values to -0.1 as default for both channels, then perform steps 3 and 4 and finally adjust the noise reduction by varying the PMT offset value.

If the ratio application is used and the offset has been set to the best reduction of noise in the ratio image, it is not allowed to change the offset of the PMT channel to change the reduction of background fluorescence, for example. This can be done only if the diode offset is corrected afterwards.

Adjustment of Detector Gain

The Gain of the PMT should be set with the help of the range indicator function. No 'red ' and no 'blue' pixels should occur in the image of the PMT.

Amplifier Gain

The diode signal is set to the right range (gray level between 50 and 200 - 8 bit image / 750 and 3500 - 12 bit image) with the help of gray filters and amplifier gain. The use of a lower filter density should be prioritized against the use of a high gain value.

The value of the amplifier gain of both channels (PMT and diode) should be set to one, if possible. Because of an increasing amplifier noise, parallel to the gain factor, a gain value of more than 2 should be avoided. The most important thing is to avoid pixels below the zero level and beyond the maximum range respectively.

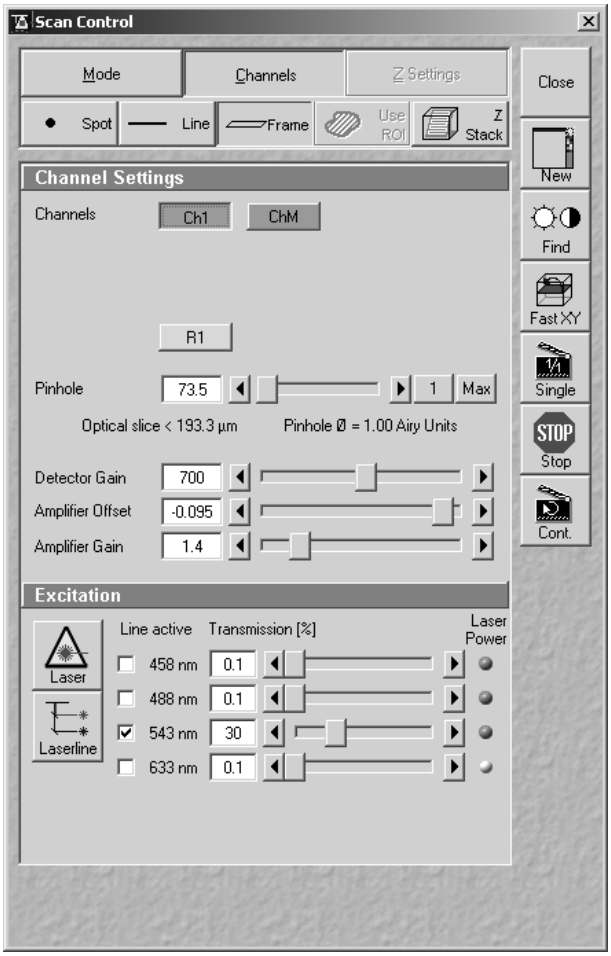

Fig. 8-9 Scan Control window

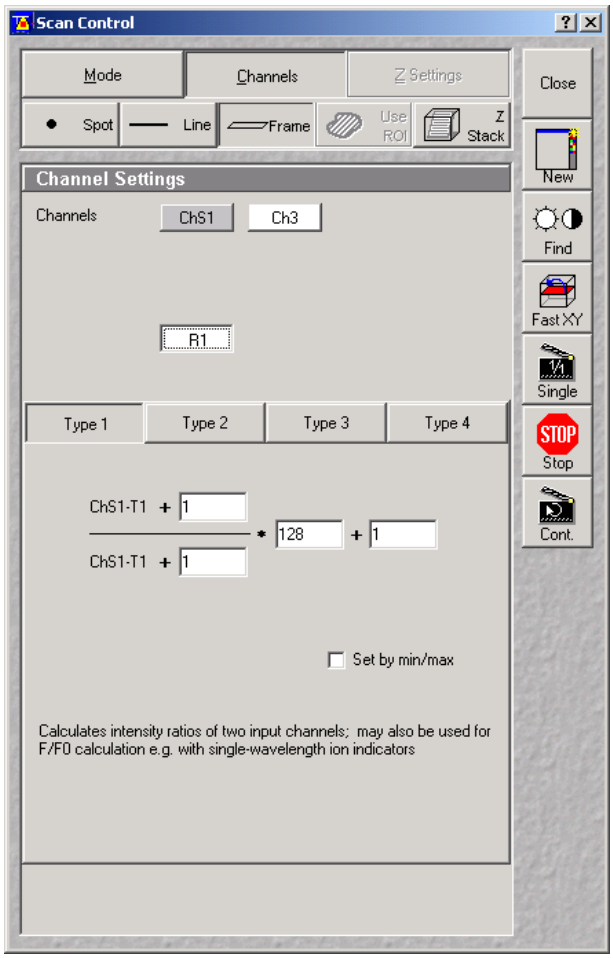

Fig. 8-10 Scan Control window

Gain and offset in Ratio channel

If the setting of the PMT channel is finished, the range of the ratio channel is adjusted by the parameters in the corresponding formula. Four types of formulas are offered when the **R1** button is pushed. The only formula needed for a ratio image with the monitor diode is type 1:

$$\frac{S1+n}{S2+m} * x+y$$

The values for n and m have to be zero, as well as the value for y. Any deviation from zero will decrease the contrast of the ratio image.

Only the value of x shall be influenced by the user. Dependent on the choice of data depth (8 or 12 bit), x is between 0 and 256 (8 bit) or between 0 and 4096 (12 bit).

Default settings are 150 and 3000 respectively. With the help of the range indicator the default value is changed until pixel overflow ('red pixels') is no more available

☞ Any new value can be set by hand-typing and pressing the **ENTER** key while the scan is running.

Any change in the setting parameters of PMT and diode signal will make a new Gain x in the ratio formula necessary.

If the adjustment of all parameters is finished, only the ratio image can be scanned or displayed by switching off the PMT channel and the diode channel in the **Configuration Control** window and leaving only the Ratio Channel turned on. As a result, only the ratio image is displayed; which can still be influenced by the settings in PMT and diode channel.

8.8 NLO Non Linear Optics Laser for LSM 510 NLO and LSM 510 META NLO

The LSM 510 can be operated with a Titanium-Sapphire-Laser to perform Multiphoton Microscopy. Depending on the type of laser the following operation procedures apply:

- The operation of software controlled lasers (Chameleon laser from Coherent or Mai Tai laser from Spectra Physics) is integrated into the LSM software. Operation of Chameleon laser (Coherent; For further details please refer to the operators manual of the laser)
  - Operation of Chameleon laser (Coherent; for further details please refer to the operators manual of the laser)

    The laser should be kept in Stand by mode for daily or weekly operation. The cooling of the laser must not be shut down during stand by. Only in case of more than two weeks without operation the laser should be shut down completely. This has to be performed according to the operation manual for the laser provided by Coherent. The shut down takes about an hour as does the start procedure afterwards. The key on the front panel of the laser power supply is used for switching between Stand by mode and On mode.

    ☞ The laser has to be in On mode to allow the LSM software to operate the laser.

  - Operation of Mai Tai laser (Spectra Physics; for further details please refer to the operators manual of the laser)

    The laser should be kept in Stand by mode for daily or weekly operation. The cooling of the laser must not be shut down during stand by. Only in case of more than two weeks without operation the laser should be shut down completely. This should be performed according to the operation manual for the laser provided by Spectra Physics. The shut down and the start procedure take up to one hour. The laser is put to On mode or Stand by mode using the LSM software. Therefore no further operation of the laser via the laser power supply unit is needed. The key on the front panel of the laser power supply is an interlock key, when removed the system cannot be operated. The power switch should be kept on for leaving the laser in Standby.

- Operation of Millenia Tsunami (Spectra Physics) or Verdi Mira lasers (Coherent)

  These lasers are not operated via the LSM software. Refer to the operators manual of the manufacturer for using these lasers in combination with the LSM 510. However it is needed to indicate the tuned wavelength in the software to ensure the appropriate setting of the AOM frequency (see below).

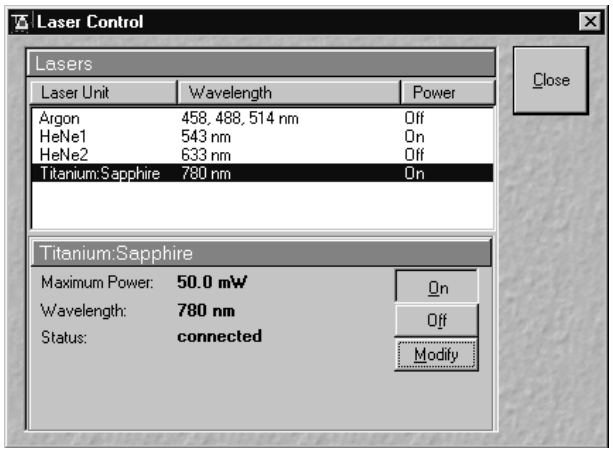

Fig. 8-11 Configuration Control window

8.8.1 Laser Control Window

The **Laser Control** window shows the NLO laser as additional laser of the system.

Operating a Chameleon laser

- Click **On** to activate the laser for the software. The system now has access to the laser and reads out the actual status of the laser.
- Click the **Modify** button. The **Laser Modify Control** window is opened (Fig. 8-12).
- Enter the desired wavelength in the **Edit Laser Wavelength** input box.
- The **Fine Tuning AO-Frequency** slider enables you to fine-tune the AO-frequency (Acousto Optical) during the continuous scanning procedure. However this should only slightly influence the intensity of the signal because the automatic presetting is calculated exactly for each wavelength.
- Click **Store** to confirm the setting. The **Laser Modify Control** window is closed, the **Laser Control** window is updated and the laser is tuned to the indicated wavelength.

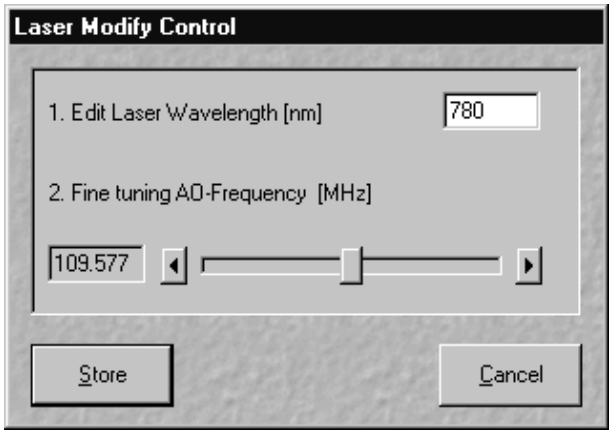

Fig. 8-12 Laser Modify Control window

Operating a Mai Tai laser

- Click **On** to activate the laser. The laser is put from Standby to On and the power ramps up. This takes a few minutes until the laser is ready to operate. The system has access to the laser and reads out the actual status of the laser.
- Click the **Modify** button. The **Laser Modify Control** window is displayed. (Fig. 8-12).
- Enter the desired wavelength in the **Edit Laser Wavelength** input box.
- The **Fine Tuning AO-Frequency** slider enables you to fine-tune the AO-frequency (Acousto Optical) during the continuous scanning procedure. However this should only slightly influence the intensity of the signal because the automatic presetting is calculated exactly for each wavelength.
- Click **Store** to confirm the setting. The **Laser Modify Control** window is closed, the **Laser Control** window is updated and the laser is tuned to the indicated wavelength.

Operating Verdi Mira or Millenia Tsunami lasers

- For other lasers: The laser itself is not switched on when clicking **On**, this has to be done directly at the laser, but the software is informed that the laser is switched on and the laser safety shutter in the AOM box can be opened.
- Click the **Modify** button. The **Laser Modify Control** window is displayed (Fig. 8-12).
- Enter the wavelength set manually on the laser in the **Edit Laser Wavelength** input box.
- The **Fine Tuning AO-Frequency** slider enables you to fine-tune the AO-frequency (Acousto Optical) during the continuous scanning procedure. However This should only slightly influence the intensity of the signal because the automatic presetting is calculated exactly for each wavelength.
- Click **Store** to confirm the setting. The **Laser Modify Control** window is closed and the **Laser Control** window is updated.

8.8.2 Configuration Control Window

Application of the NLO laser requires special main dichroic beam splitters and the relevant emission filters to be activated in the **Configuration Control** window.

NLO-excited signals can be detected in every channel by taking care on the blocking of the NIR laser excitation light.

The following filters are especially designed for detection of NLO-excited fluorescence signals:

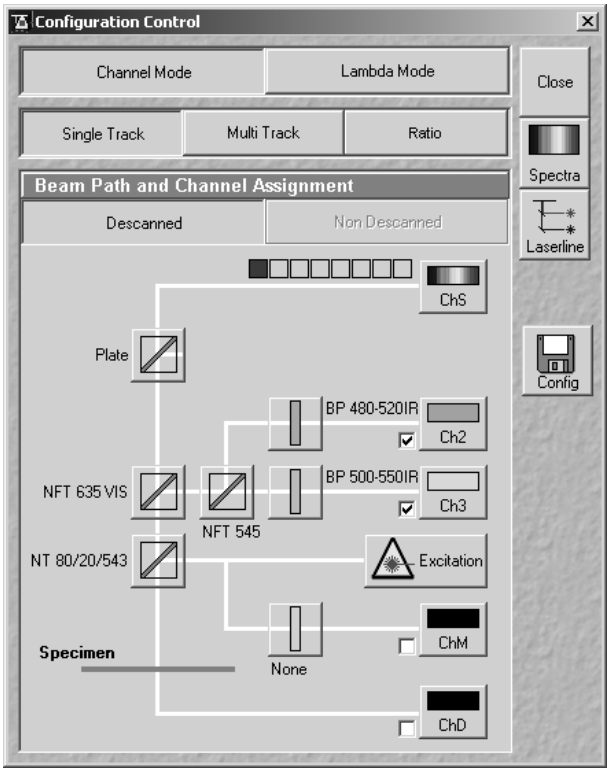

Fig. 8-13 Configuration Control window

– **HFT KP 680**
main dichroic beam splitter reflecting NIR excitation longer than 680 nm,
transmission for shorter wavelengths

– **HFT KP 700 / 488 (514, 543, 561) or combinations of the KP 700 with more than one additional laser line in the visible range.**
main dichroic beam splitter reflecting NIR excitation longer than 700 nm,
transmission for shorter wavelengths and reflecting 488 nm (or any other wavelength/s indicated) excitation for simultaneous detection of NIR and VIS excitation

– **KP 685** (short pass filter)
transmitting wavelengths shorter than 685 nm

– **BP 500-550 IR or other bandpass filters with indication of IR**
special bandpass filters for NLO blocking also in the NIR range (extension IR),
can be used without additional BG 39

– **BG 39**
highly efficient block filter for NIR, for combination with normal emission filters without IR extension

8.8.3 Pinhole and Collimator Settings

- The pinhole can be fully opened for maximum detection efficiency due to the focal excitation capabilities of the NLO effect (see **Scan Control** window, **Channel Settings**).
- In the **Pinhole & Collimator Control** window, the **NIR** collimator can be used to align the overlap of the excitation planes within the object for VIS as well as NIR excitation light wavelength in the **Collimator** panel (see **Maintain** menu, **Pinhole** button).

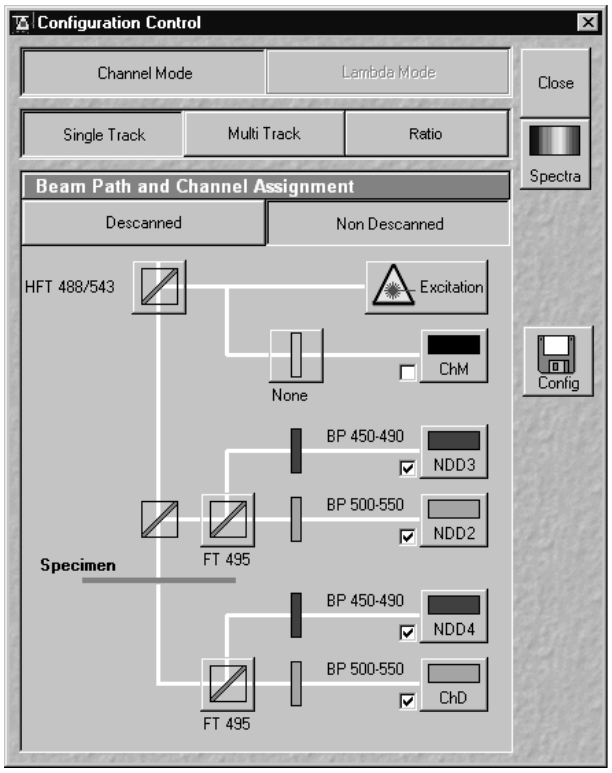

Fig. 8-14 Configuration Control window

8.9 Non Descanned Detection (NDD)

The application of Non-Descanned Detection with the LSM 510 is only useful in combination with the optional NLO laser.

The Non-Descanned Detection modules can be used on the reflected or transmitted-light beam path or simultaneously on both beam paths. This means that the maximum of four NDD channels can be configured. If two NDD channels have been assigned to the transmitted-light beam path, no transmission PMT can be implemented.

In Non-Descanned Detection, the radiation emitted by the specimen is conducted directly on the relevant detector without passing the scanner mirror again.

Non-Descanned Detection is set and configured in the **Configuration Contro**l window by activating / deactivating the buttons **Descanned Detection** and **Non-Descanned Detection** while the NDD module is being connected.

- Click on the **Non-Descanned Detection** button to change to Non-Descanned Detection.
- Configure the NDD channels analog to the Descanned Detection mode.

☞ The configuration of multitracks is also possible for NDD applications, though not in combination with the standard channels.

- Before activating image acquisition close the shutter in front of the HBO or HAL illumination. Push in the pushrod (8-15/**1**) for closing the shutter. This enhances image quality. If the hardware is still 3.0 or lower make sure that the detector gain for any used NDD is set to the minimal value before opening the manual shutter in front of the HAL / HBO illumination.
- Switching from LSM to VIS mode automatically stops scanning. Open the shutter in front of the HBO or HAL illumination for conventional florescence or transmitted light microscopy.

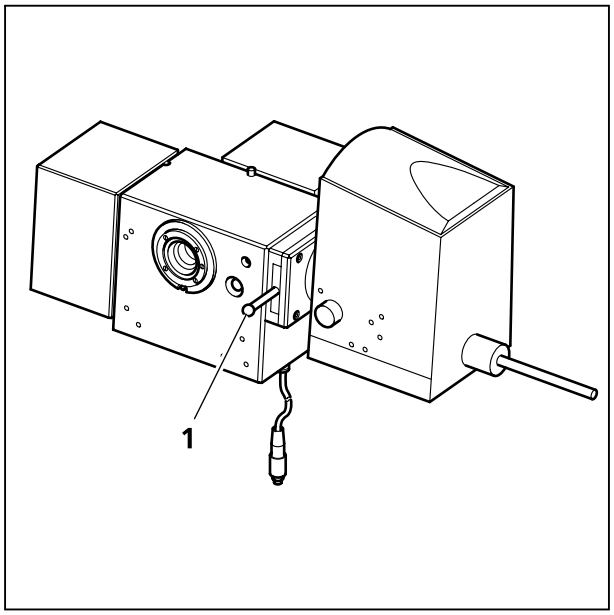

Fig. 8-15 Non descanned detection module with HBO 100 lamp

☞ Be aware that the NDD reflector cube in the reflector turret leads to a strong back reflection of HBO light into the specimen plane and the eyepiece. When observing the specimen through the ocular lens the use of the NDD reflector cube should be avoided. The light flash is not harmful but unpleasant. To avoid this an additional filter (#1261-345) can be mounted into the NDD reflector cube which greatly reduces the intensity of the back reflected light in the eyepiece. This filter reduces the intensity of the excitation light by 7 %.

8.10 AxioCam High Resolution Digital Cameras

8.10.1 High Resolution Microscopy Camera AxioCam HRm Rev.2/3.3V

Cat. No	426511-0000-000, incl. digital interface and cable
High Range Monochrome	
Number of Pixels:	1388 (H) x 1040 (V) = 1.4 Mega pixel
Chip size:	8.9 mm x 6.7 mm, equivalent to 2/3"
Spectral range:	With BK-7 protection glass up to 1000 nm, with IR barrier filter BG40 limited to about 350 nm to 700 nm
Selectable Resolution by Binning or Microscanning	

H	x	V	Acquisition Time (s) @ 20 ms exposure
1388	x	1040	0.2 (5 images / s)
2776	x	2080	0.8 (1.25 images / s)
4164	x	3120	1.7

Dynamic Range:	Better than 2000 : 1 @ 8 e readout noise
Integration Time:	1 ms to several minutes
Cooling:	Single stage Peltier cooling
Optical Interface:	C-Mount
Size:	about 11 cm x 8 cm x 6.5 cm (2.3" x 3.2" x 2.6")
Registration:	CE, cUL
Power Supply:	12 V DC, 1 A, 230 V/110 V, autodetecting

8.10.2 High Resolution Microscopy Camera AxioCam HRc Rev.2/3.3V

Cat. No	426510-0000-000, incl. digital interface and cable
High Range Color	
Number of Pixels:	1388 (H) x 1040 (V) = 1.4 Mega pixel
Chip size:	8.9 mm x 6.7 mm equivalent to 2/3"
Spectral range:	Limited by IR barrier filter BG40, about 400 nm to 700 nm
Selectable Resolution by Binning or Microscanning	

H	x	V	Acquisition Time (s) @ 20 ms exposure
1388	x	1040	0.2 (Color interpolation)
1388	x	1040	0.8 (full resolution for color channels)
2776	x	2080	2.9 (full resolution for color channels)
4164	x	3120	6.5 (full resolution for color channels)
4164	x	3120	1.6 (Fast Scan)

Dynamic Range:	Typical 2000 : 1 @ 9 e readout noise
Integration Time:	1 ms to several minutes
Cooling:	One stage Peltier cooling
Optical Interface:	C-Mount
Size:	about 11 cm x 8 cm x 6.5 cm (2.3" x 3.2" x 2.6")
Registration:	CE, cUL
Power Supply:	12 V DC, 1 A, 230 V/110 V, autodetecting

8.10.3 Microscope camera port adapters for the AxioCam

Adapter Video V200 C 2/3" 0.63x at frontport Axiovert 200 M Cat. No 000000-1071-171

This adapter is **needed for attachment** of the high-resolution AxioCam microscope cameras **on the Axiovert 200 M**

Camera Adapter 60N C 2/3" 0.63x Cat. No 426113-0000-000

This adapter is **needed for attachment** of the high-resolution AxioCam microscope cameras **on the Axio Imager.Z1, Axio Imager.M1, Axiovert 200 SP** and **Axioskop 2 FS MOT**.

☞ **No other cameras are supported by the LSM Software!**

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Multiphoton Laser Scanning Microscopy

Using the Zeiss LSM 510 META NLO

Urechis caupo
Serotonin positive nerve cells

Written by Mary Dickinson, PhD
Biological Imaging Center
California Institute of Technology
February 2002

ZEISS

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9 MULTIPHOTON LASER SCANNING MICROSCOPY

9.1 Preface

Safety Considerations when using ultrafast lasers coupled to a microscope.

Users operating ultrafast lasers have to observe all of the precautions specified in the Operating Manual for the laser and caution should be exercised when using the laser.

All users should be familiar with risks and good safety practices before access to the laser is granted.

Users must not look directly into the laser beam. Direct eye contact with the output beam from the laser will cause serious damage and possible blindness.

Every precaution should be taken to avoid exposing skin, hair or clothing to the laser, as this may cause burns.

Beware of reflected laser light and remove jewelry before working with the laser.

Avoid using organic solvents near the laser.

Protective housings should remain in place, when the laser is in use.

Safety signs have to be posted to inform people that lasers may be in use.

9.2 Introduction to Multiphoton Laser Scanning Microscopy

Multiphoton laser scanning microscopy (MPLSM) has become an important technique in vital and deep tissue fluorescence imaging. In MPLSM, fluorescent molecules are excited by the simultaneous absorption of two or more near infrared (NIR) photons. Multiphoton excitation has a quadratic dependence, producing excitation only at the focal plane; thus, out-of-focus fluorescence does not contribute to image background, and photodamage outside the plane of focus is greatly reduced. In practical terms, MPLSM makes it possible to acquire images with a high signal-to-noise ratio by using a wavelength that is less harmful to live cells. The use of NIR light makes it possible to image deeper in the specimen, due to less scatter and absorption of the incident light. However, multiphoton excitation depends on some special criteria that differ from those needed for single photon excitation events. Here we will provide a simplified explanation of the physics of multiphoton excitation.

9.2.1 Multiphoton excitation – How does it work?

In single photon excitation, a fluorescent molecule or fluorochrome (also called a chromophore) absorbs a high energy photon of light within a certain wavelength range and then, within nanoseconds, releases a photon of longer wavelength (lower energy). The absorption of a photon results in the excitation of the molecule, by displacing an electron within the molecule from the ground state to an excited state. Thus, for a single photon excitation event, excitation is directly proportional to the incident photon flux of the source, since each photon has an equal probability of exciting a molecule in the ground state. As the molecule relaxes back to the ground state, some energy is lost through non-radiative exchange (heat or vibration within the molecule), but the rest is shed as a photon of light.

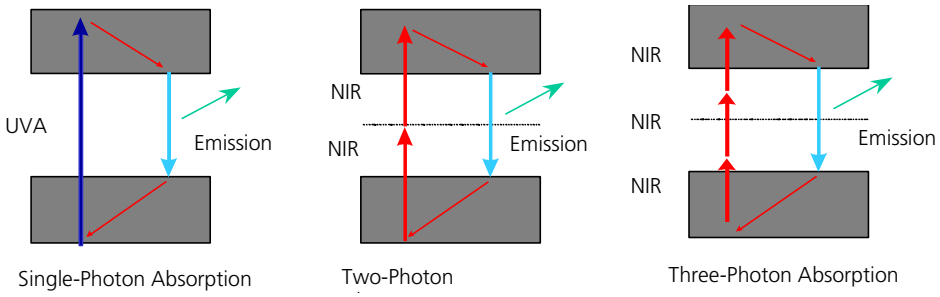

Fig. 9-1 The principle of multiphoton excitation

The energy loss accounts for the Stokes shift seen between the excitation and the emission wavelength and explains why the emission maxima is always of a lower energy, more red-shifted, from the excitation maxima. Multiphoton excitation of the fluorochrome is induced by the combined effect of two or more, lower energy, NIR photons. As a rule of thumb the energy of the two photons is roughly half the energy of the photons needed for single photon absorption, although there are clear exceptions to this rule. Multiphoton excitation can be achieved by two photons of the same or different wavelengths, but with a single laser source, two photons of the same wavelength are used.

The probability of multi-photon excitation is proportional to the incident photon flux density which is the intensity squared (I^2), because a quasi-simultaneous absorption of two photons is necessary. It follows, that for three-photon excitation, the probability of three-photon absorption is the intensity cubed (I^3). The emission characteristics of the excited fluorochrome is unaffected by the different absorption processes (Fig. 9-1).

While this appears conceptually simple, two difficulties, at the level of the fluorochrome, confound our understanding of this process. First, it is difficult to predict whether a molecule will efficiently absorb the two lower energy photons simultaneously. Drastic differences in multiphoton absorption between different molecules have been identified and it is difficult to predict by the structure of a molecule how well it will efficiently absorb simultaneous low energy photons, although some theories are emerging (see Albota et al., 1998; Rumi et al., 2000 for review). Second, the wavelengths for maximum multiphoton excitation are very difficult to predict. Deriving the multiphoton excitation wavelength maximum is clearly not as simple as doubling the single photon excitation wavelength maximum. Both of these criteria must be measured and are reflected in the multiphoton cross-section (usually referred to as δ) for a given fluorochrome (see Xu, 2000 for review).

The cross-section data indicate how well the molecule absorbs multiphoton energy at different wavelengths of NIR light. What is both interesting and perplexing about this data is that several molecules that all emit green light, i.e. excited at roughly the same wavelength via single photon absorption, can have multiphoton excitation maxima that are very different. For instance, although Fluorescein and GFP both emit green light, the multiphoton cross-section peak for Fluorescein is 770-790 nm, but is centered around 900 nm for GFP (S65T) (Xu, 2000) whereas, the single photon excitation maxima for these two molecules are both around 470-490 nm. These questions represent an intense area of investigation for physicists and chemists who specialize in multiphoton absorption (see Section 9.3.4 Choosing fluorescent probes for MPLSM).

9.2.2 Increased signal-to-noise, enhanced vitality, and deep optical sectioning in MPLSM

One of the greatest benefits of multiphoton excitation is that excitation is practically limited to the focal plane. This effect increases signal-to-noise and decreases phototoxicity. In single photon excitation, the excitation of a dye is directly proportional to the average power ($Ex \sim P_{avg}$). Thus, excitation takes place in the whole cone of focus and optical resolution is accomplished by using a confocal aperture. For multiphoton excitation, however, excitation of the dye is proportional to the squared intensity ($Ex \sim I^2$), as mentioned above. For a focused beam, intensity (I) can be described as average power (P_{avg}) divided by the cross-sectional area of the beam (A), so for multiphoton excitation, the excitation is proportional to the average power divided by the area of the beam, squared ($Ex \sim [P_{avg} /A]^2$). Thus, as the beam diameter becomes smaller (such as at the focal plane) excitation is increased and excitation out of the plane of focus becomes highly improbable and falls off with the axial distance from the focal plane with the power of 4. This explains why multiphoton excitation is mainly limited to the focal plane (Fig. 9-2). Moreover, the cross-sectional area of the beam is dependent on the NA of the objective. Objectives with a larger NA can focus light to a smaller beam waist, which is why high NA objectives are preferred for multiphoton excitation microscopy.

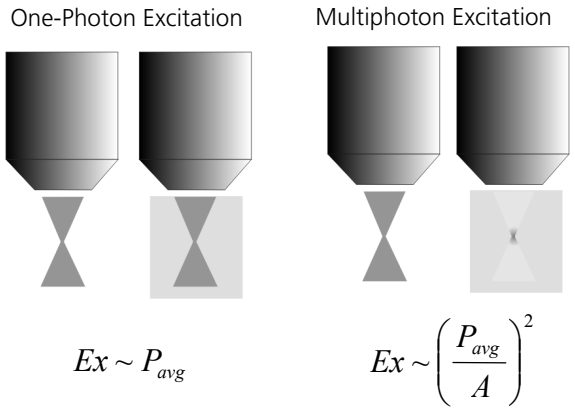

Fig. 9-2 One-photon vs. Multiphoton excitation. Two images of an objective are shown for each example. In each case, the first indicates the shape of the focused beam after passing through the objective. The second indicates the fluorescence that would be observed if the beam was focused through a cuvette containing a homogeneous solution of fluorescent dye.

Since out-of-focus fluorescence, which usually contributes to background noise in the image, is created inefficiently, MPLSM can be a better technique for imaging fine structures masked by background noise. Optical sectioning can be performed without the use of the pinhole to eliminate out-of-focus fluorescence and nearly all of the fluorescence produced at the plane of focus can be used to make the image. Although a pinhole is not normally needed using MPLSM, it is possible to use the confocal pinhole together with multiphoton excitation to prevent highly scattered photons from reaching the detector and to improve optical sectioning. This can be done on an LSM 510 NLO by carefully adjusting the collimation lens and the z-position of pinhole 1 (Refer to the alignment protocol in subsequent sections).

It is tempting to think of the decrease in background signal as an increase in resolution. This is a common misconception. In fact, due the longer excitation wavelength the optical resolution along the optical axis is worse in comparison to the resolution in a classical confocal LSM. Objects obscured by background fluorescence, may appear brighter or more defined using MPLSM, but this is not due to an increase in resolution, but rather a reduction in background noise, resulting in better contrast.

The lack of out-of-focus excitation greatly reduces bleaching, and therefore, photodamage, throughout the sample. This reduces damage caused by repeated or slow scans; however, photobleaching at the focal plane is still present. In fact, some reports indicate that bleaching may be accelerated at the plane of focus using multiphoton excitation (Patterson and Piston, 2000). Some essential, endogenous molecules within the cell can absorb UV or visible range photons (such as NAD, FADH etc.), which can destroy and deplete these molecules. Thus, it can be safer for vital imaging to use an excitation source outside of the visible range like a NIR laser. A profound example of this effect is seen in a comparative study performed by Squirrell and colleagues (Squirrell et al. 1999), where the vitality of cleavage stage hamster embryos was assessed after repeated exposure to visible range laser light and pulsed 1047 nm laser light. In these experiments, confocal imaging resulted in arrested cellular division and embryo lethality, whereas imaging using multiphoton microscopy resulted in much less embryo lethality and better data collection. In fact, at least one embryo imaged in this way was able to develop into a completely normal adult hamster named laser, illustrating the strength of this technique.

The use of NIR light has the additional benefit of being able to penetrate deeper into tissue than visible wavelength light. Compared to confocal microscopy, excitation can be achieved in deeper positions of the specimen and more data along the z-axis can be obtained. However, particularly in deep tissue imaging, care must be taken to recover as many emission photons as possible. While the incident NIR light has an advantage over visible range excitation sources, the photons that are emitted are at visible wavelengths and have the potential to be scattered or absorbed by the tissue. To improve the efficiency of collection, high numerical aperture (NA) objectives should be used, although it is often difficult to obtain lenses that have NAs above 1 with a working distance longer than 250 µm. In addition, non-descanned detectors (NDDs), which collect photons at a point closer to the specimen and do not require the emission to be focused back through the scan mechanism, can be used to improve deep tissue imaging by improving the collection efficiency of scattered photons

9.2.3 Drawbacks of using NIR light for microscopy

Although the use of NIR photons has many advantages, there is a distinct disadvantage to this mode of excitation.

Absorption of NIR light by water and some other particular molecules (such as melanocytes or condensed particles such as calcium carbonate crystals) can create dramatic local heating effects within the sample. This effect increases as the overall power from the laser is increased at the sample. It is very important to use minimized power levels to reduce the effects of local heating. For live samples, power levels above 6 mW may disturb cell replication (König et al. 1996) or even cause cells to explode, as in the case of melanocytes in zebrafish embryos. Effects of local heating can not only damage cells, but can also contribute to image artifacts (see Section 9.3.5. Samples your mother should have warned you about). As you will see in the next section, optimizing the pulse length at the specimen can improve multiphoton excitation without raising the average power.

9.2.4 Achieving Efficient Multiphoton Excitation using Ultrafast lasers

In order to achieve efficient two- or three-photon excitation, the photons must collide with the molecule simultaneously. For single-photon excitation, a continuous wave laser with a continuous photon flux can be used because the probability of excitation is directly proportional to the photon flux or average power of the source. Increasing the laser intensity (turning up the power) increases the photons delivered to the sample, increasing excitation until all of the molecules are saturated. To deliver enough photons to achieve simultaneous absorption of two NIR photons using a continuous wave laser would require enormous power. Ultrafast lasers improve the efficiency of multiphoton excitation by delivering photons in pulsed "wave packets". The high peak intensity needed for multiphoton excitation is created by concentrating photons into very brief pulses which are delivered to the sample over and over again at a rapid rate, about every 13 ns, to ensure efficient dye excitation. Instead of a steady flux of photons bombarding the fluorochrome one after another, multiple photons collide with the molecule simultaneously. This process has the advantage of delivering a high peak intensity, to satisfy the I^2 or I^3 requirement for two- or three-photon excitation, without using enormous amounts of average power.

Many lasers are now available that can produce ultrashort pulses at high repetition rates. Titanium-sapphire lasers, for instance, are capable of producing ~100 fsec pulses over a broad tunable wavelength range (690 nm-1064 nm) with a high repetition rate, ~80 MHz. Similarly, solid state, doubled neodymium doped yttrium lithium fluoride (Nd: YLF) lasers, emitting 1047 nm, 175 femtosecond pulses at 120 MHz have also be used for multiphoton excitation.

Ti: Sapphire lasers are probably the most popular lasers because of the wavelength range that is available. These lasers can operate in both a continuous wave (CW) mode or in a mode that emits pulsed light. Lasers operating in this latter mode are said to be mode-locked (ML), which refers to the fact that the laser is locking in different frequencies together to form a pulse of a particular bandwidth.

The use of ultrafast mode-locked lasers for multiphoton excitation requires to consider several additional factors which are not necessary for continuous-wave lasers used for single-photon excitation. The length of the laser pulse (referred to as the pulse length or pulse width) (τ), the peak intensity produced at the focal plane (I_{peak}), the average power of the laser at the specimen (P_{avg}), the cross-sectional area of the beam (A), and the pulse frequency (F_p) or repetition rate are all important factors for achieving and maintaining efficient multiphoton excitation in the sample.

Raising the average power of the laser, P_{avg} (controlled by the Acousto-Optic-Modulator, AOM), will raise the peak intensity. However, raising the average power will also increase the amount of heat generated in the sample, which may damage vital processes or disrupt cellular structures.

The pulse frequency, F_p, is determined by the design of the laser and is not easily manipulated. For the Coherent Chamelon Ti: Sapphire laser, Fp is equal to ~90 MHz.

The cross-sectional area of the beam, A, is an important term to consider when dealing with light focused through an objective. Simply put, reducing the cross-sectional area of the beam, for instance by focusing the beam through an objective, increases the intensity at the point of focus or in other words increases the amount of photons per area as we saw above.

The exact dimensions of A will vary greatly depending on the properties of the objective. In general, higher magnification, higher NA objectives will reduce A, similar to the way such objectives increase resolution. However, other considerations such as the transmission efficiency of the objective and the amount of dispersion produced by the objectives must also be considered as this will reduce P_{avg} or increase respectively. In addition, spherical aberration caused by the objective and sample and the diameter of the beam at the back aperture of the objective can also affect A. Thus, empirical determination of the best objective is often relied upon. (see Section 9.3.3 Objectives recommended for multiphoton excitation).

The pulse length, τ, is a measure of the duration (length) of the pulse of photons being delivered to the sample, measured at the full width, half maximum. Increasing the pulse length, decreases I_{peak}. As mentioned above, ultrafast lasers are able to supply short pulses with a high duty cycle. Unfortunately, the pulse length is very difficult to measure, especially through a microscope objective. However, it is possible to make such measurements and users should refer to Wolleschensky et al. (1998) for details. Several factors that effect the pulse length are discussed below.

9.2.5 Optimizing the Peak Intensity without Frying the Samples

Although raising the average overall power is the easiest way to increase the peak intensity, excessive heat can destroy both live and fixed samples, as well as produce unwanted imaging artifacts. Assuming that one is using a laser with a fixed pulse frequency and a high magnification, high NA objective optimized for multiphoton microscopy (see Section 9.3.3 Objectives recommended for multiphoton excitation), the pulse length becomes the most important term to minimize.

The Spectral Bandwidth refers to the spectral frequencies of the pulse. Each short pulse produced by the laser has a broad spectral band centered around the selected wavelength. Spectral bandwidth and pulse length are inversely related. The wider the bandwidth, the shorter the pulse.

The Coherent Chamelon produces pulses around 140 fsec.

GVD is a temporal broadening of the pulse length as the pulse travels through normal dispersive media such as glass (see Diels and Rudolph, 1994 for more discussion). The pulse length becomes broader in dispersive media because the red shifted frequency components, with respect to the center wavelength, travel faster than the blue shifted frequency components. Pulses with a broader spectral bandwidth are more susceptible to the effects of GVD, as there is a greater difference in wavelength, and thus, velocities within the pulse (Fig. 9-3). In addition, the amount of dispersion is related to the thickness of the dispersive media (see Wolleschensky et al. 2001 for review). When possible, thinner glass optics and lenses are used along the routing path.

Within the laser, a prism pair is used to adjust the spectral bandwidth of the laser pulses. However, outside the laser, glass elements within the microscope and scan module, within the objective, and along the routing path, can lengthen the pulse. In this case, the broader the spectral bandwidth, the more broadening of the pulses will be caused by GVD. Thus, for direct-coupled systems without the use of a prechirping unit, it is advantageous to adjust the laser so that the bandwidth of the pulse is at a minimum. For instance, if the bandwidth is less than 7 nm, the pulse length at the sample will be approximately 300 fsec. If the bandwidth is 12 nm, the pulse becomes substantially broadened by the same dispersive elements so that the pulse length is about 700 fsec at the specimen.

Fig. 9-3 Effect of GVD on short pulses with a broad bandwidth. The broader the bandwidth , the more the pulse is stretched. Longer wavelengths travel faster through the glass than shorted wavelengths in the pulse. Thus, a positively chirped pulse is broadened.

It is possible to compensate for GVD along the routing path and in the microscope to preserve the pulse length to the sample by using a prechirp unit (available from other manufactureres).

9.3 Using the LSM 510 NLO direct coupled system: Practical considerations for optimal imaging

9.3.1 Coupling an ultrafast laser to the LSM 510 NLO

Coupling an ultrafast laser to a laser scanning microscope is a fairly simple matter, if several key considerations are understood. Typically, the routing kit for coupling the LSM 510 NLO to the laser will consist of the following components: an Acousto-Optic Modulator (AOM), used to control the intensity of the beam and for beam blanking (allowing for region of interest (ROI) and bi-directional scanning and for performing Auto-Z-Brightness correction); two 45° mirrors for directing the beam to the microscope on the table; and a 1 meter lens. In the case of an upright system, the beam is brought up into the scan head via a periscope, whereas for an inverted microscope, the height of the beam will be adjusted by a smaller periscope positioned in place of the second turning mirror. These devices ensure that the beam remains parallel to the table when horizontal and enters the scan head at the proper height (see diagram). The 45° routing or turning mirrors are adjustable and will be used routinely to peak the coupling of the beam into the scan head. The 1 m lens is used to expand the beam to fill the back aperture of the objective. It is mounted in a fixed position by a trained service engineer during installation and should not be adjusted by the user (Fig. 9-4).

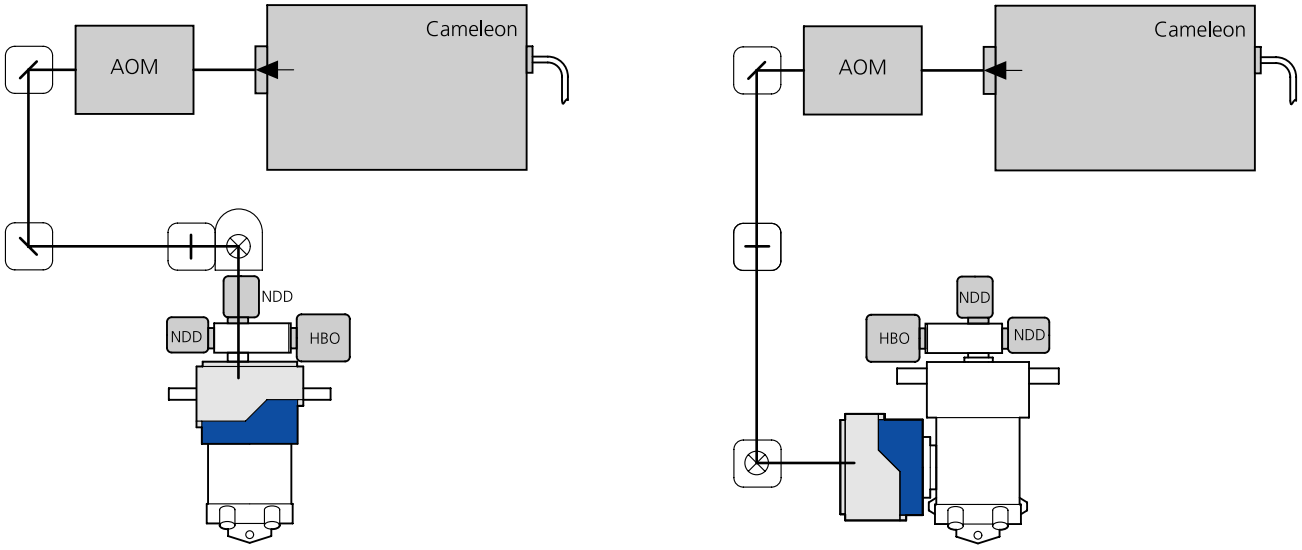

Fig. 9-4 Typical alignment scheme for the LSM 510 NLO direct-coupled system using and upright(left) or inverted(right) microscope.

9.3.2 Alignment of the ultrafast laser into the LSM 510 NLO scan head

The alignment of the laser is done by a Zeiss service engineer. However it might be needed to slightly adjust the beam coupling from time to time, depending on the room conditions. The protocols mentioned should only be performed if the user is confident with the technique, otherwise the coupling of the laser can be lost and a Zeiss service engineer is needed for realignment.

The alignment of the laser through the AOM and into the scan head is critical for producing high quality images using multiphoton excitation. Below are two protocols that can be used to optimize alignment of the beam into the scan head.

9.3.2.1 Quick alignment protocol

After the system is installed and aligned by a qualified service engineer, it is often the case that only small alignment adjustments are necessary. For instance, proper alignment should be verified after the laser is tuned to a new wavelength. Below is a quick and easy protocol that can be used to center the beam into the back aperture of the objective lens for optimum image quality.

Start by scanning a sample that is normally excited by the wavelength of the external laser. For instance, a pollen grain slide (Carolina Biological) or a Fluocells sample (Molecular Probes) can be used. (NOTE: This procedure may cause bleaching of the sample.)

Adjust the laser power and gain so that the sample is visible in the image acquisition window.

Scan the sample using the fast XY scanning mode.

Using the signal intensity as a guide, peak the alignment of the beam. Begin by using the alignment mirror closest to the laser (first turning mirror). Make improvements using both alignment pins on the mirror mount. (NOTE: a small hex wrench (Allen key) may be needed to adjust alignment). When the best signal intensity is obtained by tweaking the mirror closest to the laser, now make improvements by tweaking the mirror closest to the microscope scan head. Go back and forth between these mirrors to walk the center the beam onto the back aperture of the lens.

As a final verification of proper alignment, check the alignment of the beam onto the back aperture directly by viewing the projected beam without the objective in place. To do this, place a white card on the stage (Fig. 9-5/**A**) or lens paper on the specimen holder for an inverted microscope.

Remove one objective, select 50-100 % transmission, HFT KP 680 or 700, and Fast XY scan. When the system is scanning, a round, red spot should appear on the card (Fig. 9-5/**B**). Seeing this beam may be aided by using an IR viewer, especially at longer wavelengths.

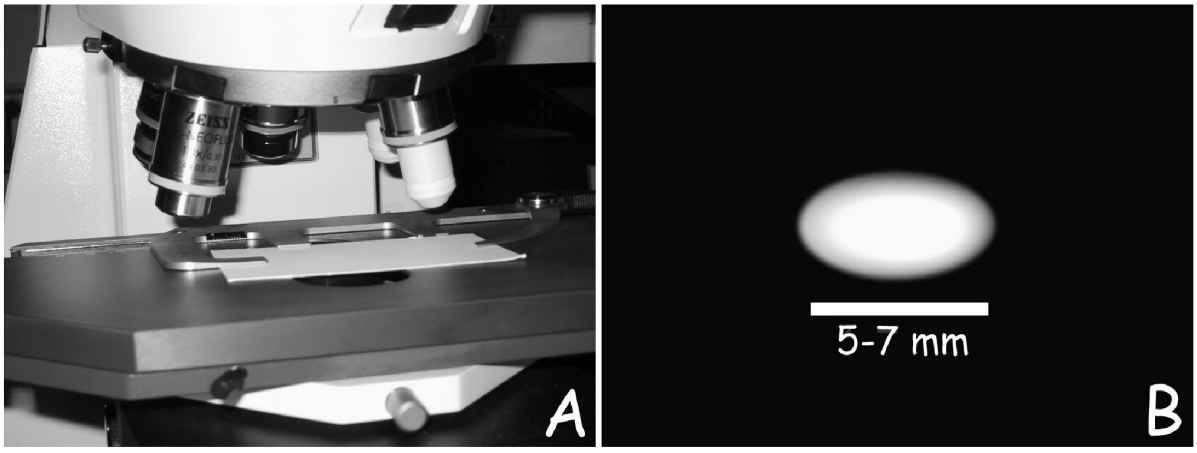

Fig. 9-5 (A) Setup for checking the beam alignment; (B) Optimal shape of the NIR laser spot

Ideally this spot should be 5-7 mm in diameter and should be completely round and uniform. If the beam appears to be clipped (flat on one side), small adjustments in the routing mirrors can improve the alignment (see above). Shadows in the illuminated spot may indicate dust or debris on the routing optics or in the scan head (check all visible elements for debris, then contact a service engineer if the problem persists).

If proper alignment cannot be established using this protocol or if the image quality appears to be poor, use the advanced alignment protocol to peak alignment.

9.3.2.2 Advanced alignment protocol

When the system is installed, the service engineer will establish alignment of the laser beam into the scan head by verifying the overlay between the NIR laser and one of the VIS lasers. Although routine use usually does not usually require a complete realignment with the scan head, tuning the laser over large wavelength increments or changing or cleaning the optics in the laser can cause gross changes in beam pointing. If this occurs, it is best to use the following protocol to re-establish proper alignment with the scan head. For this protocol, a partially reflective grid slide is needed (Part number 474028-0000-000, Test Grid Specimen for LSM). Care must be taken to use low amounts of laser power when using this slide or the grid can be easily burned.

Begin by confirming that the correct wavelength for the laser is set in the laser control panel in the software. The wavelength can be changed by selecting the modify option. Refer to the Rees analyzer or the BRF setting to determine the wavelength of the MIRA 900F.

Next, place the partially reflective grid slide on the microscope stage. Bring the grid slide in focus using the 10x Plan-Neofluar objective and transmitted light. Configure the scan head as follows (Fig. 9-6):

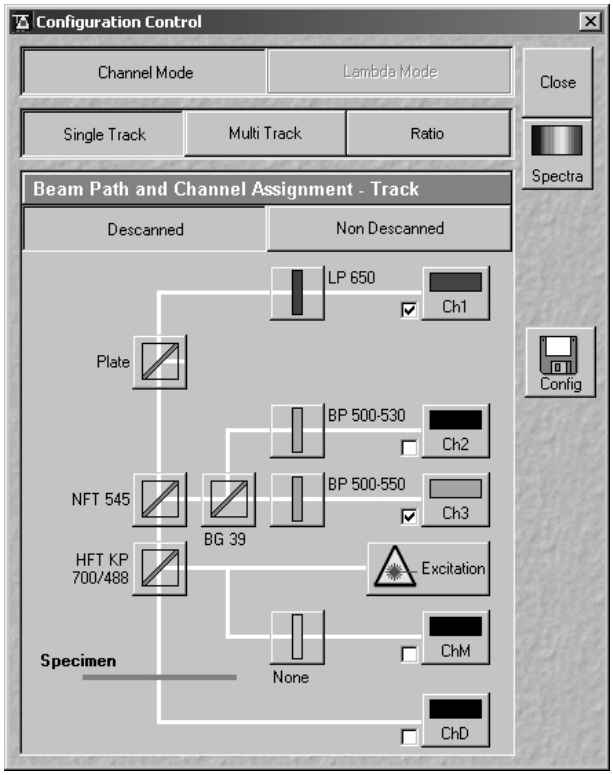

Fig. 9-6 Configuration for testing the overlay of the grid reflection images gained with the vis (543 nm) laser and the NIR laser

This configuration is designed to use the PMTs to detect the reflection of the NIR and VIS laser light off of the grid slide so an image of the grid is made on the screen. For this protocol, the 543 nm line has been chosen. The HFT KP 700/488 beam splitter is used to avoid sending too much 543 nm laser light to the reflective slide. A KP 700/543 beam splitter can be used, but care must be taken not to burn the slide. Alignment using this procedure will ensure that the NIR and VIS lasers overlay on the combining mirror when they enter the scan head.

To perform the alignment, set the laser power for both lasers at 3 % (1 % for a MIRA 900F with a 10W pump laser) and adjust only the gain to improve the intensity of the signal.

WARNING: Too much laser power can cause damage to the grid slide! Be sure attenuation is set before scanning.

Begin scanning using the FAST XY mode. An image of the grid should appear on the screen. If not, adjust the gain for each channel so that the output from both channels is visible. The two colors represent the reflection of the 543 nm line (green) and the reflection of the NIR laser (red) on the metallic grid surface. The focus may also need to be adjusted in order to get the optimal output from both channels. If the red image is out-of-focus when the green image is in focus, the collimating lens should be adjusted so that both beams are focused on the same spot. To adjust the collimator, open the Maintain panel in the LSM 510 software. While the laser is scanning, click on the pinhole button. At the bottom of this panel, there is a slider to adjust the NIR collimator.

☞ DO NOT ADJUST THE VIS COLLIMATOR.

Adjustments in the NIR collimator should improve the focus of the NIR beam. If no signal at all is detected in the red channel, turn up the laser transmission in small increments and begin peaking alignment.

☞ Note that once alignment is improved, the laser intensity will need to be attenuated to avoid burning the slide. If no red signal at all can be found, contact a service engineer.

Once both beams are in focus, begin optimizing the overlay of the grids by first adjusting the routing mirror that is closest to the laser. Make small adjustments using both alignment screws while watching the effect on the monitor screen. Adjustments of this mirror will increase the intensity of the red signal. Readjust the gain as needed. After optimizing the alignment using this mirror, begin adjusting the mirror that is closest to the scan head (NOTE: A small hex wrench [Allen key] may be needed to adjust alignment). Adjustments in this mirror will improve the alignment of the NIR overlay on the VIS overlay. Go back and forth between these mirrors to walk the center the beam onto the back aperture of the lens.

9.3.2.3 Tips on maintaining alignment with the scan head

Since the NIR beam is aligned into the scan head through mirrors and free space, it is possible that any alteration in these routing components can effect proper alignment. Below are some tips to maintaining good alignment:

- Use the simplified tuning protocol in Section **Fehler! Verweisquelle konnte nicht gefunden werden.** to tune to different wavelengths
- Do not move the position of the 1m lens. Even small adjustments of these components may cause significant changes in the alignment of the routing optics.
- If recurring alignment drifts or power fluctuations in the laser are observed, consider floating the optical table and/or regulating the room temperature more closely. Changes in the temperature of the table or optical components can often cause drift in alignment of the laser and routing optics.
- Avoid tuning the laser in increments larger than 50 nm without peaking the alignment.

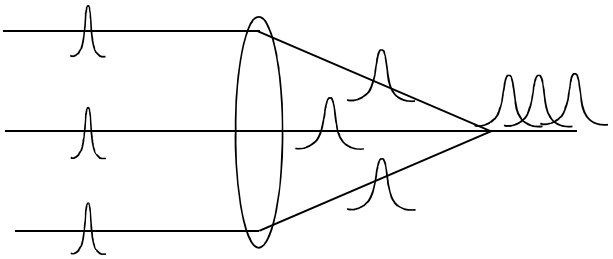

Fig. 9-7 Schematic pulse transformation by a singlet lens showing the influence of chromatic aberrations on the peak intensity due to Propagation Time Difference (PTD).

9.3.3 Objectives recommended for Multiphoton Excitation

For nonlinear microscopy, objectives should be optimized for the following parameters: high transmission in the NIR and the VIS-wavelength range, long working distance with a high numerical aperture, limited pulse broadening due to GVD, a uniform GVD across the pupil of the objective for the excitation wavelength range and a small propagation time difference (PTD). As we have already discussed, low transmission in the NIR can lower the average power at the sample, thus lowering the peak intensity.

Long working distance objectives with a high numerical aperture are clearly favored for deep imaging so that the beam can be focused deep into the tissue and the emission photons can be collected efficiently. Objectives should have limited GVD to reduce the chance that short pulses will be lengthened en route to the sample, which will again reduce the peak intensity.

In addition to GVD, chromatic aberration of objectives leads to pulse distortions. Specifically a radius-dependent group delay is introduced (Kempe et al., 1993; Netz et al., 2000). Therefore, different radial portions of the beam across the pupil of the objective arrive at different times at the focal region and cause a temporal broadening of the pulse. This results in lower peak intensity in the focal region (Fig. 9-7). This effect is also referred to as PTD.

Table 1 summarizes the important characteristic parameters for objectives recommended for nonlinear microscopy and for the optics inside the LSM 510 NLO including the microscope stand. The dispersion parameters were calculated based on the material data and the thickness of each optical element on axis. For comparison, the dispersion parameter for the optics within the LSM 510 NLO including the microscope stand and the AOM is 7500 $fsec^2$.

The variation of the dispersion parameter for different beams across the pupil of the objective is listed in the forth column. It can be seen from both parameters that the pulse broadening is nearly independent of the particular objective used and the position of the beam across the pupil of the objective. The pulse broadening due to the GVD of the different objectives and the LSM 510 NLO including the microscope stand and the AOM was calculated for 100 fs pulses at a wavelength of 800 nm and is listed in column 5.

The pulse broadening due to PTD is listed in the last column. The PTD was calculated for the whole optical setup, since it depends on the chromatic aberrations of the complete system including the LSM 510 NLO and the microscope stand for a wavelength of 800 nm. The PTD effect is negligible if the pulse length is not much shorter than 100 fsec. It can be seen that the special IR corrected objectives show a smaller PTD in comparison to the standard UV/VIS corrected objectives.

Objective	Working Distance [mm]	Dispersion Parameter [fsec]^2		Pulse broadening factor of 100 fsec pulse	Max. PTD [fsec] (*)
		On axis	Variation		
IR-Achroplan 40x/0.8 W	3.61	1714	± 20	1.14	-3
IR-Achroplan 63x/0.9 W	2.00	1494	± 15	1.11	-9
Plan Neofluar 40x/1.3 oil	0.20	2328	± 30	1.23	9
Plan Apochromat 20x/0.75	0.61	1531	± 10	1.12	10

Table 1 Summary of specific parameters for objectives recommended for 2-Photon applications. All data were measured at 800 nm. (*) Propagation time difference (PTD) is calculated for the whole optical setup including the LSM 510 NLO and the microscope stand.

Negative values indicate that pulses at the edge of the pupil are delayed with respect to pulses traveling on axis.

With regard to transmission, objectives for biomedical applications traditionally have been optimized for UV and visible range imaging. Thus, many are not corrected for transmission of NIR or IR light. The transmission of an objective depends strongly on the design of the antireflection coating. Typical transmission curves for two different objectives are shown below. The Achroplan series is coated with UV/VIS-antireflection coating giving optimal performance from 350 nm to approximately 800 nm. In contrast, the IR-Achroplan series has a VIS/IR coating that enables high transmission from 420 nm to 1400 nm. The Achroplan objectives are best for multiphoton applications, where excitation is below 850 nm and the fluorescence from the specimen is in the ultraviolet or lower visible wavelength range.

These criteria describe the imaging parameters for many ion indicators, nuclear dyes such as DAPI or Hoechst, Blue Fluorescent Protein, and Fluorescein or Alexa 488. The IR-Achroplan objectives are best for multiphoton applications requiring an excitation wavelength greater than 850 nm and where the fluorescence emission is above 420 nm (Fig. 9-8). These latter criteria apply to many dyes, such as DiI, and many of the fluorescent proteins, such as CFP, GFP, YFP, and dsRed. However, some important fluorochromes can be imaged well with either objectives.

Rhodamine, for example, has a multiphoton absorption peak at 840 nm, and like Alexa 568 it can be excited well at 800 nm. Thus, the examples are given to indicate ideal imaging criteria; however, non-optimized parameters can often be used to produce high-quality images.

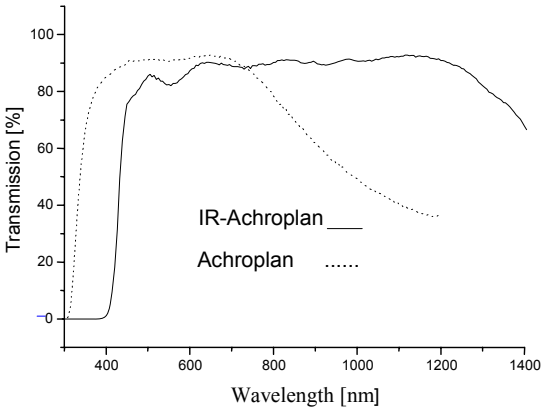

Fig. 9-8 Typical transmission curves of an IR-Achroplan and an Achroplan.

While the Achroplan objectives clearly provide the best transmission curves with the longest working distance, other objectives have proven to be useful for multiphoton imaging. For instance, the Plan-Apochromat objectives have favorable properties for multiphoton imaging as Table 1 indicates. In addition, the C-Apochromat 40x W/NA 1.2 and its 63x counterpart have proven to be very effective objectives, perhaps due to their high NA and collection efficiency. Moreover, these objectives have a 0.23 and 0.25 mm working distance, respectively, and can be very useful for collecting large 3-D data sets. Unfortunately, these objectives begin to decline in transmission at around 840 nm, so they are not optimal for dyes with more red-shifted excitation wavelength absorption maxima, but can often be used without concern for the lost photons

9.3.4 Choosing Fluorescent Probes for MPLSM

Presently, there are thousands of different fluorochrome derivatives and conjugates that can be used to study a variety of biological events. Many of these fluorochromes have been optimized for single-photon excitation and scientists are just beginning to concentrate on developing optimized fluorochromes for multiphoton imaging applications (see Albota et al, 1999). Furthermore, it is difficult to predict whether or not a particular dye will be useful for multiphoton imaging and many of us have relied on trial and error while a greater understanding of the physical principles of multiphoton excitation is evolving. That being said, there are some important parameters that one can use to help predict which dyes may be best for a particular application.

Similar to the way one would choose a fluorescent probe for conventional fluorescence microscopy, the best fluorochromes for multiphoton imaging are the ones that efficiently absorb light, reliably emit fluorescence, and are photostable. Values such as the multiphoton cross-section and the fluorescence quantum yield are important to consider when choosing a fluorochrome.

The multiphoton cross-section is a measure of how strongly a fluorochrome absorbs photons at a given wavelength and is expressed in units of 10^{-50} cm^4 sec/photon or 1 GM (Goeppert-Mayer unit, after the scientist who first predicted multiphoton absorption, Maria Goeppert-Mayer). A list of cross-sections can be found in Xu (2000) or at

www.uga.edu/caur/dyechart.pdf

The web-site shows a collection of excitation cross sections for various dyes. Currently available dyes have multiphoton cross-section values ranging from 40-200 GM (see Xu, 2000).

These values are only an indication of how well a dye will perform in biological imaging using MPLSM. A one to one correlation between the cross-section and dye performance cannot always be drawn. Measurements of multiphoton cross-section are usually performed with the dye dissolved in organic solvents or at non-physiological conditions and the local environment of a dye may influence absorption and emission characteristics. Also, these values are determined at an optimal pulse-width that cannot always be achieved at the level of the specimen. Although the cross-section is a valuable first approximation of how a dye will perform, trial and error provides the truest test for a particular application.

The multiphoton cross-section not only provides information about how well a particular molecule is excited by pulsed NIR light but also indicates the multiphoton absorption peak, a value that has been surprisingly difficult to predict. While it may seem logical that the two-photon absorption peak should be twice the one-photon peak, this has not proven to be the case. An emerging rule of thumb is that the peak is usually shorter (more blue-shifted) than twice the one-photon absorbance peak (Xu, 2000). However, one should also keep in mind that the brightest signal may not be obtained at the predicted excitation peak but at the point where the excitation peak and the power peak of the laser overlap.

Blue/Cyan dyes
Alexa 350 (780 nm-800 nm)
Hoechst (780 nm-800 nm or 900-1100 nm)
DAPI (780-800 nm or 900 nm-1100 nm)
CFP (800 nm-900 nm)
Green dyes
Oregon Green (800 nm-860 nm)
Alexa 488 (800 nm-830 nm)
GFP (840 nm-900 nm)
BODIPY (900-950 nm)
FITC (750 nm-800 nm)
DiO (780 nm-830 nm)
Yellow Dyes
YFP (890 nm-950 nm)
Orange dyes
DiA (800 nm-860 nm)
Red dyes
DiI (830-920 nm)
Rhodamine B (800 nm-860 nm)
Alexa 568 (780 nm-840 nm)

Table 2 Recommended multiphoton excitation wavelengths for common dyes.

For tunable Ti: Sapphire lasers, there is a peak output at or near 800 nm, with less average power produced at lower and higher wavelengths; as a result, it is possible to excite a given dye at a non-optimal wavelength by using more power. However, more power may also mean more heat or bleaching, so one should be careful to optimize the signal-to-power ratio.

In addition to how well a molecule absorbs light, the best molecules to choose are the ones that also efficiently release light as the molecule relaxes back to the ground state. The fluorescence quantum yield is a measure of the proportion of emission photons that are shed per excitation event.

In general, this value is the same for one- or multiphoton excitation and values for common fluorochromes can be found in Haugland (1996).

As mentioned above, photostability is an important factor when choosing a dye. Photobleaching is minimal in out-of-focus regions using MPLSM, but bleaching still occurs at the point of focus. In fact, some dyes that are relatively stable using one-photon excitation, have more rapid bleaching rates using MPLSM (Patterson and Piston, 2000). This phenomenon is not fully understood, so the bleaching rate of dyes of interest should be tested for each application. In cases where rapid bleaching occurs, we have found that ProLong (Molecular Probes, Eugene, OR) has helped stabilize the signal in fixed samples.

Table 2 is a rough guide of fluorochromes recommended by the Biological Imaging Center at Caltech. Currently, there is no comprehensive reference for all dyes excited by two- or three-photon excitation for the user to refer to for information on the peak absorbance wavelength or relative brightness of a given dye.

The information given below is based on empirical observation and does not reflect a serious, scientific investigation of all dyes, nor does it indicate the exact optimal excitation wavelength for each dye in question. Instead, this list is offered as a rough guideline of what is known to work for various imaging applications and the user is encouraged to empirically determine which dyes are most useful for their own applications. Clearly the success of any fluorescence imaging experiment relies on how well the target is labeled. The same is true for MPLSM.

9.3.5 Samples your mother should have warned you about

While multiphoton excitation has many advantages, there are also some disadvantages to using high intensity NIR or IR light for fluorochrome excitation. Probably one of the biggest disadvantages is the heat that is generated via the absorption of NIR photons by water. The local heating that is caused can affect vital cellular processes, such as cell replication (König et al., 1996) and other growth abnormalities in sensitive specimens. Thus, great care should be taken to maximize PMT settings so that minimal power levels can be used while imaging. Particular care should be taken when imaging cells or specimens that contain molecules that absorb light such as melanin.

In some cases, the absorption of NIR photons can cause dramatic local heating effects and cause cells to explode. This effect has been seen frequently in zebrafish embryos, but can be avoided by blocking melanin production with chemical agents, such as PTU or by using albino embryos.

Other molecules, such as salt crystals, can also focus light within tissues and intensify heating effects. This can also lead to imaging artifacts, perhaps due to signal produced via second harmonic generation (SHG). In Fig. 9-9 Calcium Carbonate crystals in the inner ear of the frog have produced blotchy artifacts.

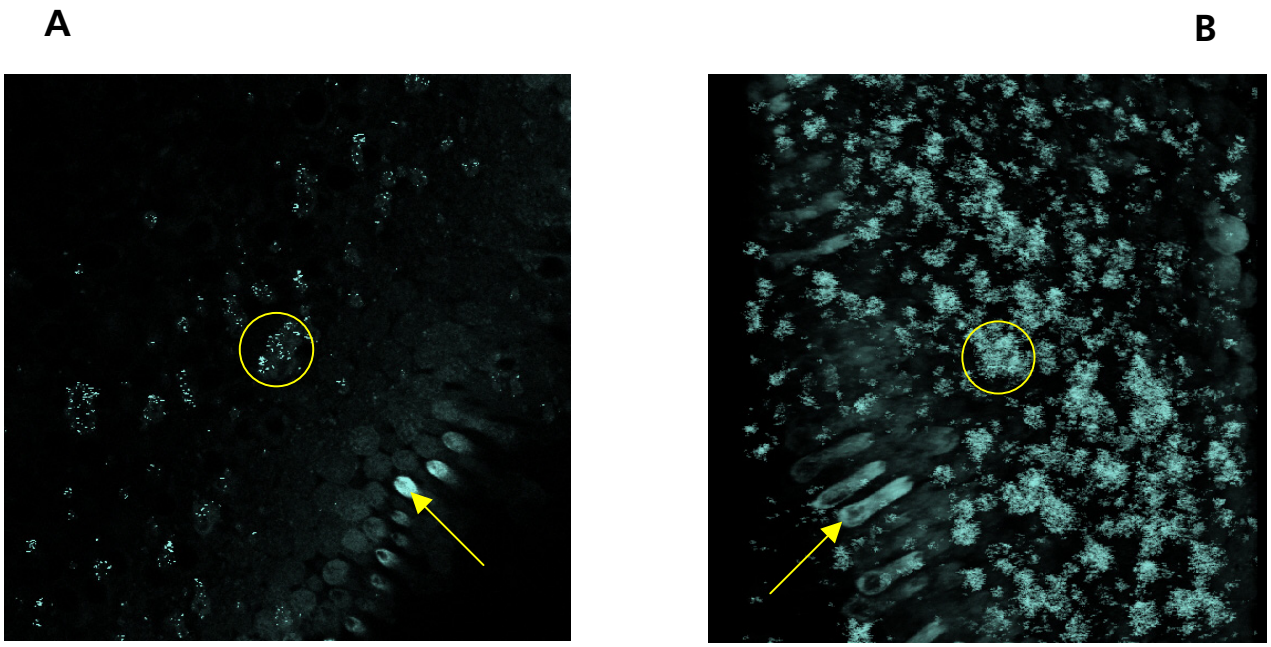

Fig. 9-9 **Multiphoton image (A) and stack of images (B) of the frog otolith. Cells have been fluorescently stained by soaking the freshly prepared specimen in FM164 (arrow). Signal produced by the artifact is shown inside the circle. Sample courtesy of Bill Roberts, University of Oregon.**

The artifacts completely obscure the three-dimensional reconstruction shown in Fig. 9-8/**B**.

This effect is not specific to Calcium Carbonate and has been seen in other samples, such as those with crystals of salt from evaporated sea water (data not shown).

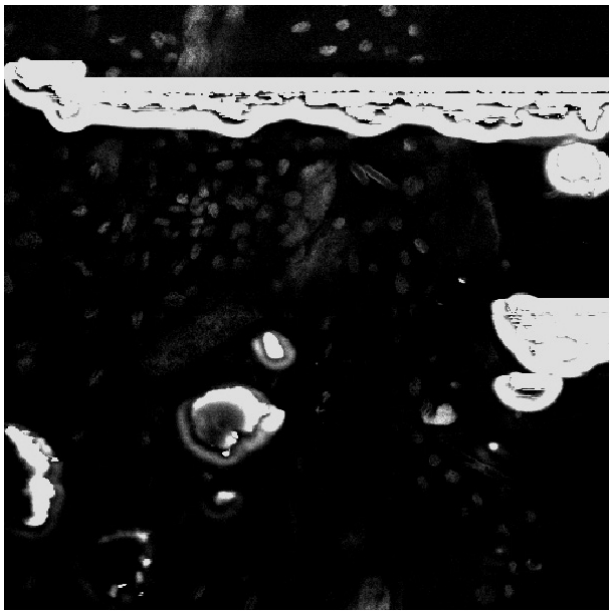

Fig. 9-10 **Bubbles have formed in the mounting media of a fixed sample of a moth embryo. The embryo was labeled with DiA, but the specific signal is obscured by the artifact produced. Sample courtesy of Patty Jansma, University of Arizona.**

Artifacts can also occur in fixed tissue. Local heating effects can cause bubbles to form in the mounting media or induce localized photochemistry in some mounting medias. It is important to test all mounting medias before use for multiphoton imaging. Fig. 9-10 shows an image where a large artifact is produced in an image using multiphoton excitation. In this example, Epon-araldite resin, generally used for EM embedding, was used. This is particularly bad for multiphoton imaging as it quickly becomes heated using focused NIR light.

Similar effects can been seen if oil and water are mixed, such as on the top of a coverslip when different immersion objectives are used on the same sample. Similar to the way water sizzles in a frying pan, steam will be produced from the focused heating of the oil and image artifacts result. Usually, these types are artifacts are dynamic as the water evaporates or the oil breaks down and the artifact may grow as you image for a longer time. It is important to clean the coverslip of your samples well with ethanol to dry off the water before mounting in oil or to clean off the oil before mounting in water.

9.4 Troubleshooting Checklist

Several key things can go wrong when imaging that will result in poor or no image being produced. Below is a checklist to help the user produce the best images possible.

9.4.1 No image being produced

Laser mode-lock:

Check to see if the laser is turned on and that it is mode-locked. For Coherent Chamelon and Spectra Physics Mai Tai lasers this is indicated in the LSM software, Laser control window.

AOM not on or too little laser power:

Check to see if the laser is turned on in the laser control panel and that the box is checked next to the percent transmission slider. If no image is seen and the laser is on, check the gain on the PMT and then increase the laser power. Check to see if increasing the percent transmission increases the power to the sample (use the monitor diode) in a linear way. If not, the AOM settings may need to be reset using the "linearize AOTF" macro.

Inefficient fluorochrome excitation (see 9.3.4):

Use a control slide to determine if the problem is in the sample. If excitation is seen with other samples, optimization of the laser wavelength may be necessary.

Laser power to the sample. Verify that the laser is focused through the objective by placing a white card on the microscope stage and removing the objective.

A bright red spot approximately 5-7 mm wide should be seen on the white card.

If no light is seen, then the beam may not be focused into the scan head or the shutter of the AOM may be closed. Check to see if there is a normal level of power going into the scan head. If no beam can be found entering the scan head, the shutter or the AOM may not be working and a service engineer should be called. If there is a beam after the 1 m lens, there may be poor alignment going into the scan head. Report this to a service engineer or attempt realignment under the guidance of a service engineer or expert user. Partial illumination of the card reflects poor alignment and the beam should be realigned into the scan head.

Non-linear power attenuation:

If changing the percent transmission leads to non-linear power attenuation, the AOM can be linearized using the AOTF fit macro. By opening this macro and following the instructions, one can verify the linearization of any of the laser lines and then run a linearization protocol to reoptimize the settings. Be sure the laser is set to the appropriate wavelength before running this macro. Choose only the NIR wavelength to optimize the AOM attenuation for the Ti: Sapphire laser.

9.4.2 Poor image quality

Lines or spaces across the image:

This can result from an unstable mode-lock. CW breakthrough appears as blank lines in the image. Q-switching will result in wavy lines in the image. A service engineer from Choherent or Spectra need to check the laser.

Poor fluorescence sensitivity:

This can result from many factors or a combination of factors. These include the following:

Correct wavelength (see 9.3.4):

Be sure that the laser is set at the best wavelength for the fluorochrome being examined. Empirical testing may be necessary to determine the best wavelength. Tuning to a different wavelength may also help to reduce autofluorescence from some tissues.

Poor Beam Alignment into the scan head (see 9.3.2.2):

Remove the objective and look at the reflection of the beam onto a white card. The beam should be round and even without any flat edges. Peak the alignment of the beam into the scan head.

Poor transmission through the objective (see 9.3.3):

Many objectives are corrected for work with UV and visible range excitation and have poor transmission of photons in the near IR.

Sliders/polarizers in the path:

DIC sliders and polarizers in the path can reduce excitation and emission sensitivity. Remove sliders or slide them to an open position.

Poor sensitivity in fixed samples:

Fixatives and mounting medias can sometimes limit the sensitivity of fluorescence detection or increase autofluorescence reducing the signal-to-noise ratio. For multiphoton excitation, the mounting media can also reduce the peak intensity of the laser deep into the sample. Empirical determination of the best fixative and mounting media for a particular application may be necessary.

9.5 References

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Handbook of Biological Confocal Microscopy (1995) ed. J. Pawley, New York: Plenum Press.

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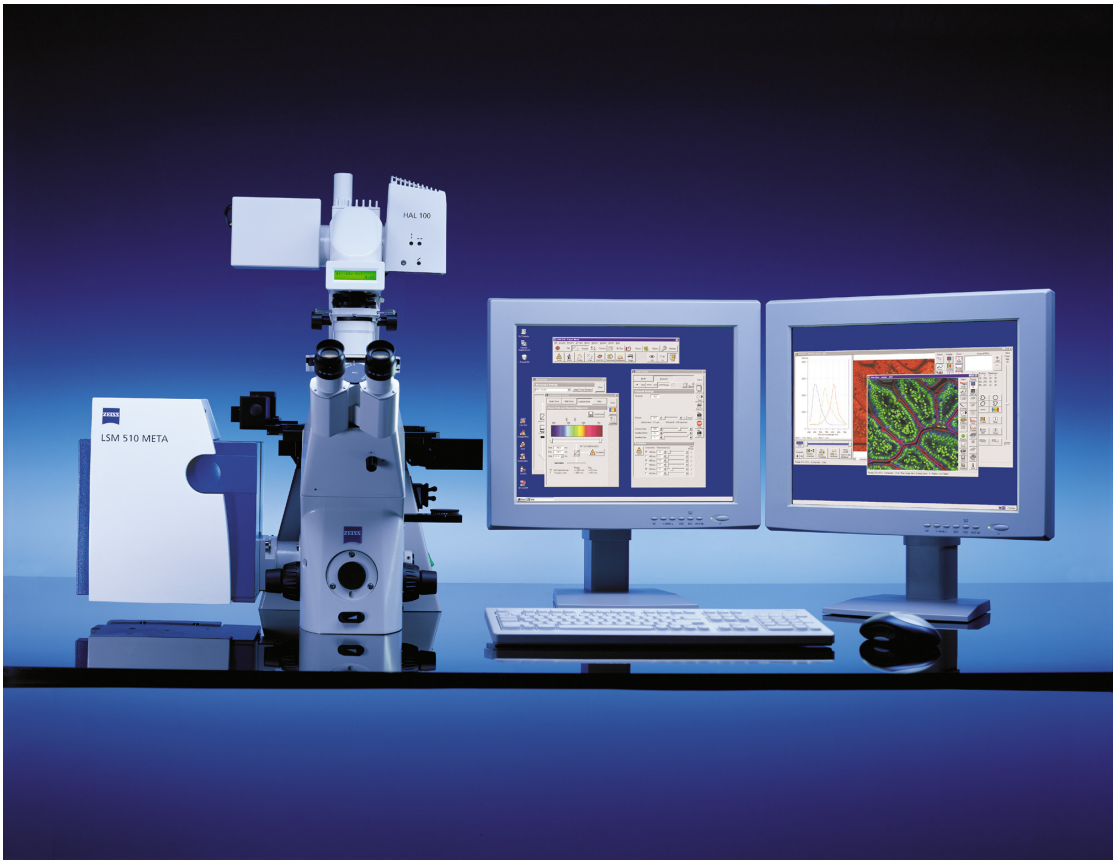
LSM 510 and LSM 510 META

Laser Scanning Microscopes



Brief Operating Manual
Release 4.0
March 2006

ZEISS

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Introduction

For your safety!
Observe the following instructions:

- The LSM 510 and LSM 510 META laser scanning microscope, including its original accessories and compatible accessories from other manufacturers, may only be used for the purposes and microscopy techniques described in this manual (intended use).
- In the Operating Manual, read the chapter *Safety Instructions* carefully before starting operation.
- Follow the safety instructions described in the operating manual of the microscope and HBO 100 mercury lamp.

Starting the System

Switching on the LSM system

- When set to **ON** the REMOTE CONTROL switch labeled **System/PC** provides power to the microscope and the computer. This allows to use the microscope and the computer without running the LSM Software (Fig. 1).
- To switch on the system completely put the **Components** switch also to **ON**. Now the complete system is ready to be initialized with the LSM Software.

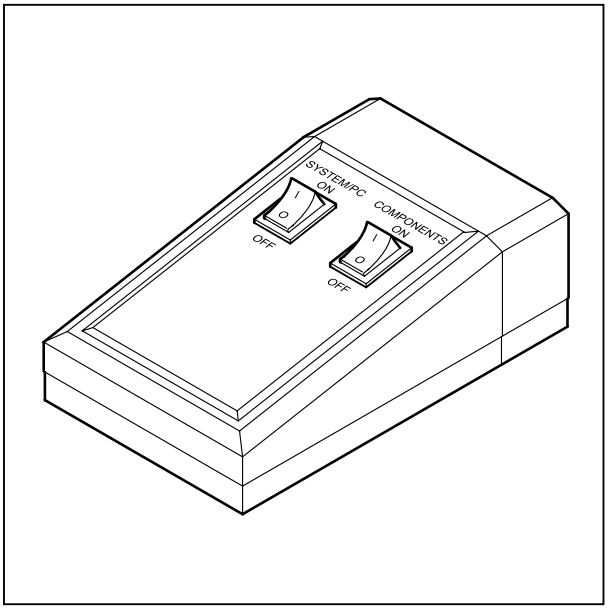


Fig. 1 **REMOTE CONTROL switch**

Switching on the HBO 100 mercury lamp

- Switch on the HBO 100 mercury lamp via the switch of the power supply, see operating manual of the mercury lamp or microscope.

Switching on the Enterprise UV-Ar Laser

- If the UV laser is required, switch it on via the toggle switch (Fig. 2/**1**) of the power supply.
  - It will be ready for operation after a few seconds.

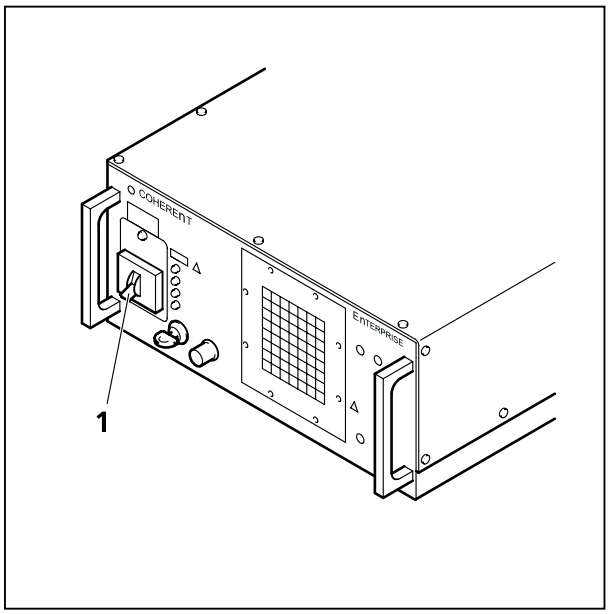


Fig. 2 **Power supply of UV-Ar laser**

Starting the LSM 5 software program

- Double click the **LSM 510** icon on the desktop of WINDOWS to start the LSM 5 software program.

The **LSM 510 Switchboard** window appears on the screen (Fig. 3).

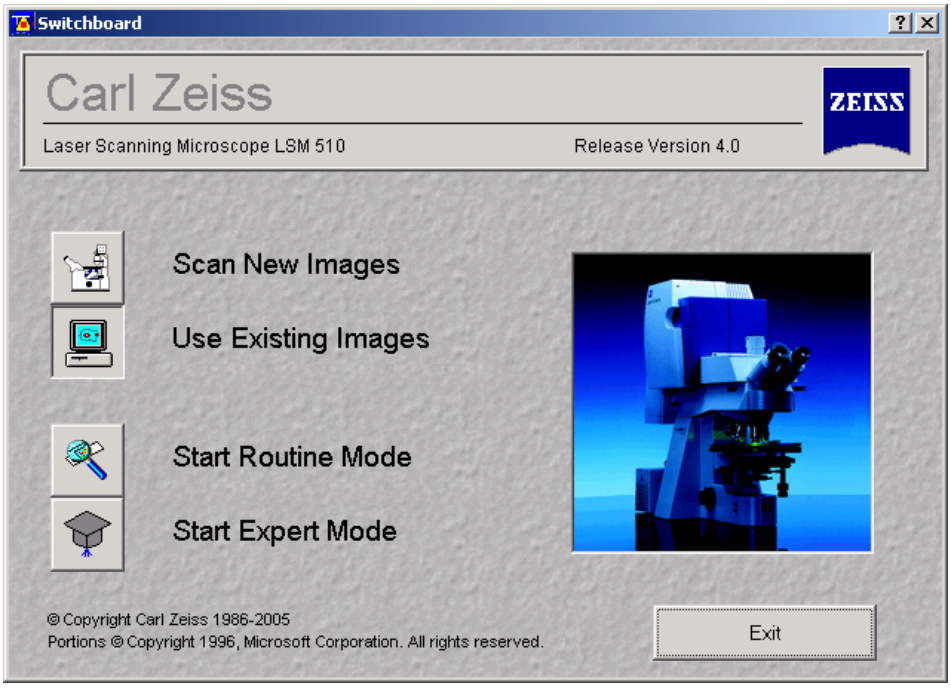


Fig. 3 **LSM 510 Switchboard menu**

- Click on the **Scan New Images** button in the **LSM 510 Switchboard** window.

Clicking on this button activates the complete LSM hardware (on-line mode).

- Click on the **Start Expert Mode** button in the **LSM 510 Switchboard** window.

The LSM 510 - Expert Mode **Main** menu appears on the screen.

Use of this mode requires to be thoroughly familiar with the exact microscope procedures and interrelations.

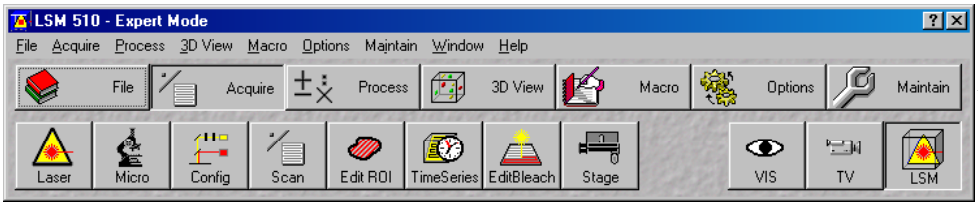


Fig. 4 **Main menu for Expert Mode**

Creating a database for acquired images

- Click on the **File** button in the **Main** menu toolbar.

The **File** subordinate toolbar appears in the **Main** menu.

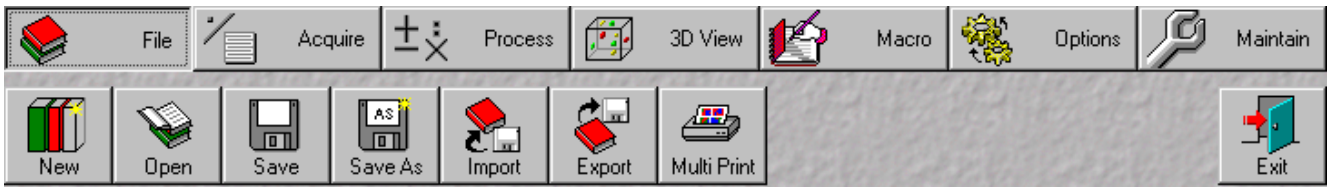


Fig. 5 **Main menu with File subordinate toolbar**

- Click on the **New** button in the **File** subordinate toolbar.

The **Create New Database** window appears.

- Select drive **C:** or **D:** from pull down menu.

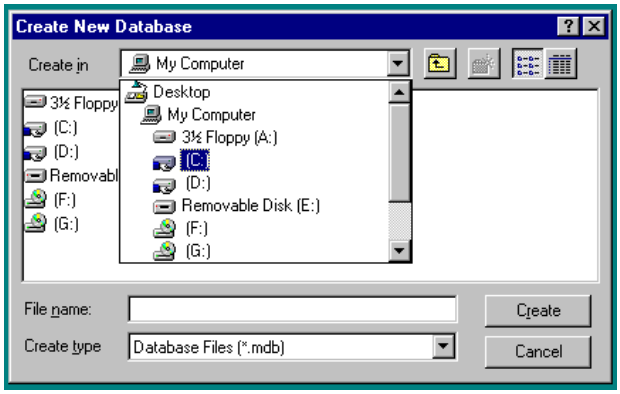


Fig. 6 **Create New Database window**

- Create a new directory if needed.

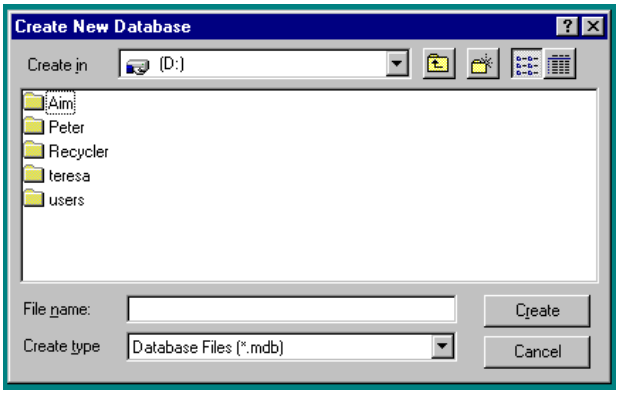


Fig. 7 **Create New Database window**

Turning on the lasers

- Click on the **Acquire** button in the **Main** menu to open the **Acquire** subordinate toolbar.

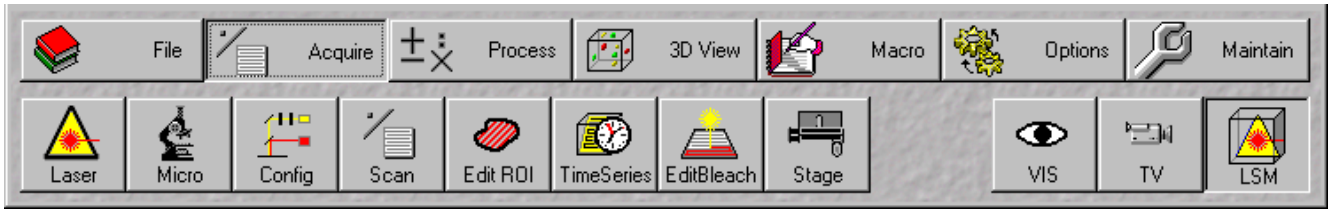
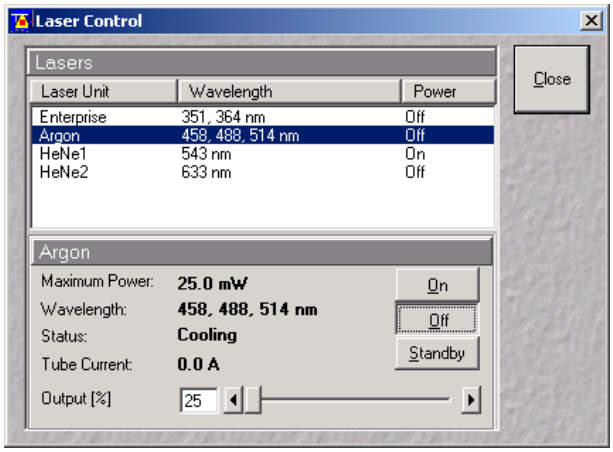


Fig. 8 **Acquire subordinate toolbar**

- Click on the **Laser** button to open the **Laser Control** window.



- Select the appropriate **Laser Unit** by clicking on the name of it.
- Click on the **Standby** button to switch required laser(s) to Standby.
- When status is **Ready** click on **On** button.
- Set **Output [%]** so that the **Tube Current** is about **4 A** (50% of the output power).

Fig. 9 **Laser Control window**

Setting the microscope

Changing between direct observation or laser scanning

The **VIS**, **TV** and **LSM** buttons switch the beam path and indicate which beam path has been set in the binocular tube of the microscope:

- Click on the **VIS** button to set the microscope for direct observation via the eyepieces of the binocular tube, lasers are off.

- Click on the **TV** button to set the microscope camera observation (if connected) via camera adapter of the binocular tube.

- Click on the **LSM** button to set the microscope screen observation via laser excitation using the LSM 510 and software evaluation.

Setting the microscope and storing the settings

- Click on the **VIS** button for direct observation.

- Click on the **Micro** button in the **Acquire** subordinate toolbar to open the **Microscope Control** window of the used microscope.

The **Microscope Control** window appears (Fig. 10).

Selecting an objective

- Open the graphical pop-up menu by clicking on the **Objective** button (Fig. 10).
- Click on the objective you want to select. The selected objective will automatically move into the beam path.

Focussing the microscope for transmitted light

- Open the graphical pop-up menu by clicking on the **Transmitted Light** button (Fig. 11).
- Click on the **On** button. Set the intensity of the Halogen illuminator using the slider.
- Click on **Close** to close the pop-up menu.
- Place specimen on microscope stage. The cover slip must be facing up.
- Use the focusing drive of the microscope to focus the required object plane.
- Select specimen detail by moving the stage in X and Y using the XY stage fine motion control.

Setting the microscope for reflected light

- Click on the **Reflected Light** button to open the shutter of the HBO 100 mercury lamp.
- Click on the **Reflector** button and select the desired filter set by clicking on it.

Storing the microscope settings

Microscope settings can be stored and up to 8 buttons assigned for fast retrieval and adjustment using the **Microscope Settings** panel.

The **Store** button permits existing microscope configurations to be stored under any name.

The **Apply** button permits existing stored microscope configurations to be loaded.

The **Delete** button permits existing microscope configurations to be deleted.

The **Assign** button permits the assignment of a microscope configuration to a button.

Note: Depending on the microscope configuration, settings must be done manually if necessary.

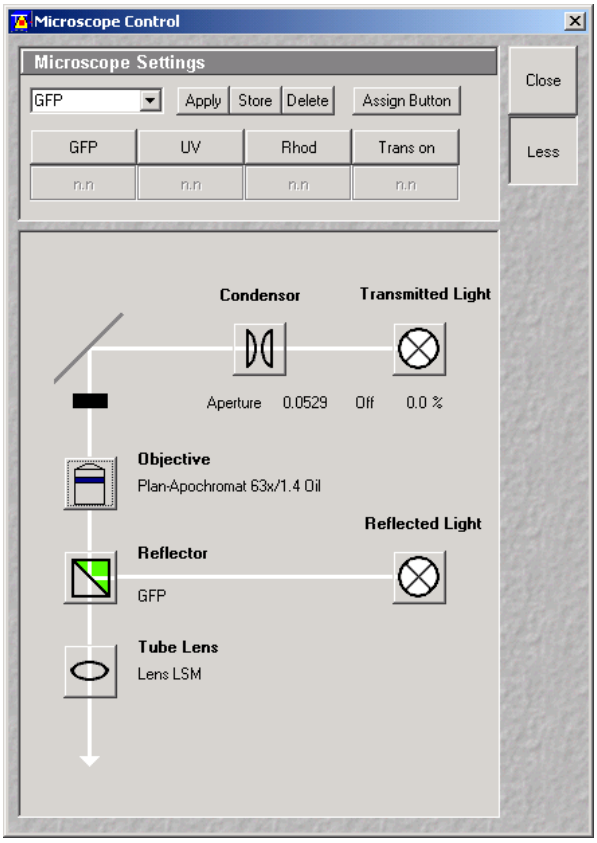


Fig. 10 **Microscope Control window, e.g.: Axiovert 200 M**

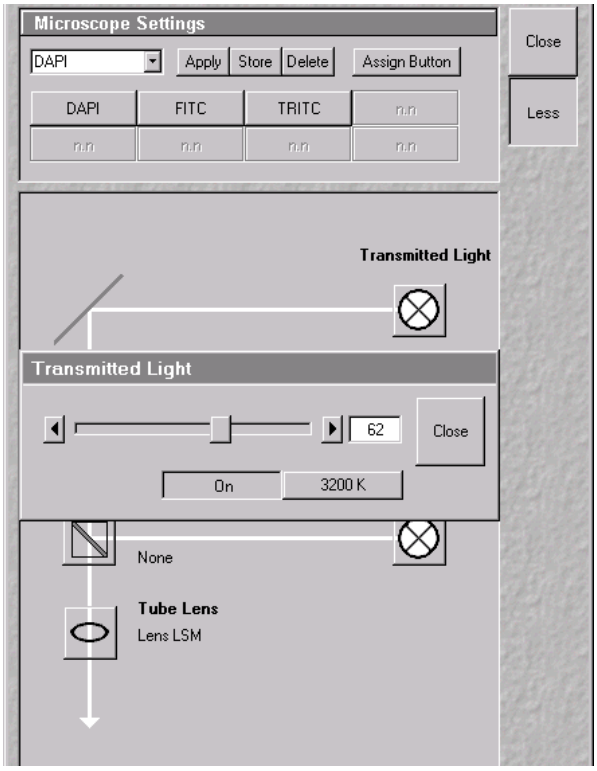


Fig. 11 **Microscope Control window with Transmitted Light pop-up menu**

Configuring the beam path and lasers

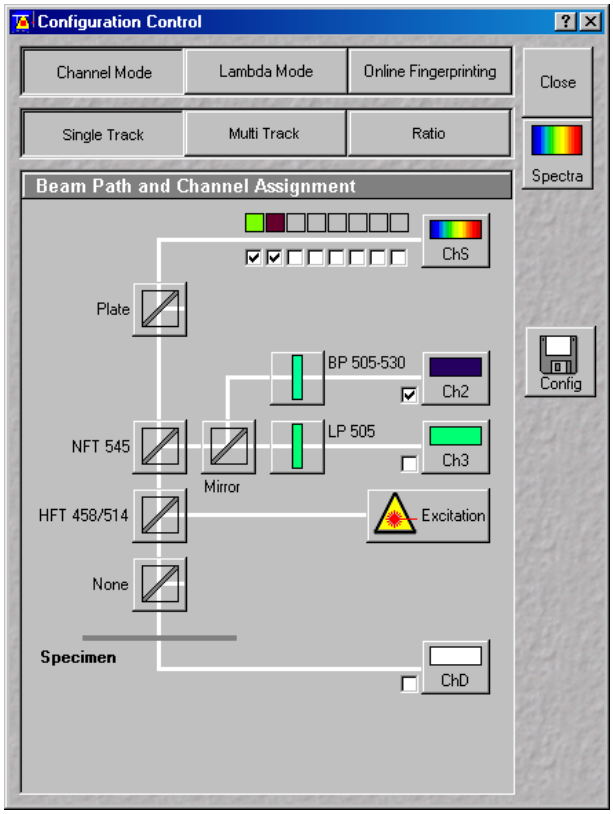


Fig. 12 **Configuration Control window for Single Track**

- Click on the **LSM** button in the **Acquire** subordinate toolbar for laser scanning.

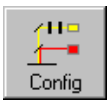
Choosing the configuration

Single Track

- Use for **single**, double and triple labeling; simultaneous scanning only
- Advantage: faster image acquisition
- Disadvantage: cross talk between channels

Multi Track

- Use for double and triple labeling; sequential scanning, line by line or frame by frame
- Advantage: when one track is active, only one detector and one laser is switched on. This reduces cross talk.
- Disadvantage: slower image acquisition



- Click on the **Config** button in the **Acquire** subordinate toolbar to open the **Configuration Control** window.

The **Configuration Control** window appears (Fig. 12).

Setting for single track configuration in Channel Mode

- Select **Channel Mode** if necessary (Fig. 12).
- Click on the **Single Track** button in the **Configuration Control** window (Fig. 12).
- Click on the **Descanned** button if necessary.

The **Beam Path and Channel Assignment** panel of the **Configuration Control** window displays the selected track configuration which is used for the scan procedure.

- You can change the settings of this panel using the following function elements:

Activation / deactivation of the excitation wavelengths (check box) and setting of excitation intensities (slider). Open the **Laser Control** window via the **Laser** button.

Selection of the main dichroic beam splitter (HFT) or secondary dichroic beam splitter (NFT) position through selection from the relevant list box.

Selection of an emission filter through selection from the relevant list box.

Activation / deactivation (via check box) of the selected channel (Ch 1-4, monitor diode ChM, META detectors ChS1-8, transmission ChD) for the scanning procedure by assigning an existing color icon or defining a new one.

- Select the appropriate filters and activate the channels.
- Click the **Excitation** button to select the laser lines and set the attenuation values (transmission in %) in the displayed window.

For the configuration of the beam path, please refer to the application-specific configurations depending on the used dyes and markers and the existing instrument configuration.

- Clicking on the **Spectr** button opens the **Detection Spectra & Laser Lines ...** window (Fig. 13) to display the activated laser lines for excitation (colored vertical lines) and channels (colored horizontal bars).

- Clicking on the **Config** button opens the **Track Configurations** window (Fig. 14) to load, store or delete track configurations.

- For storing a new track configuration enter a desired name in the first line of the **Configurations** list box and click an **Store**.
- For loading an existing configuration select it in the list box and click on **Apply**.
- For deleting an existing configuration select it in the list box and click on **Delete**.

Setting for multi track configuration in Channel Mode

The **Multi Track** function permit several tracks to be defined as one configuration (**Channel Mode Configuration**) for the scan procedure, to be stored under any name, reloaded or deleted.

The maximum of four tracks with up to 8 channels can be defined simultaneously and then scanned one after the other. Each track is a separate unit and can be configured independently of the other tracks with regard to channels, Acousto-Optical Tunable Filters (AOTF), emission filters and dichroic beam splitters.

- Select **Channel Mode** if necessary (Fig. 15).

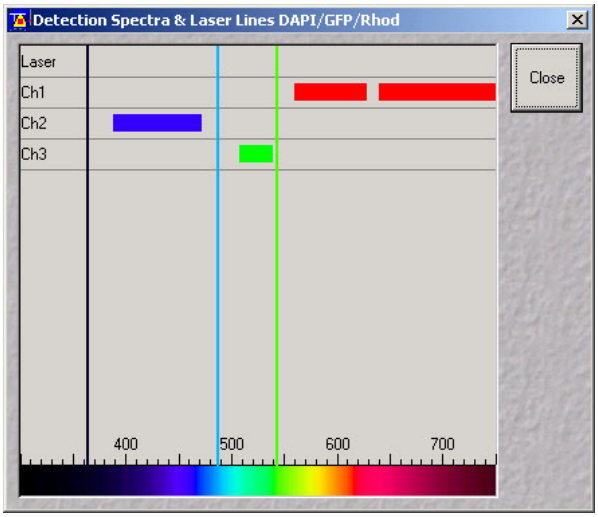


Fig. 13 **Detection Spectra & Laser Lines ... window**

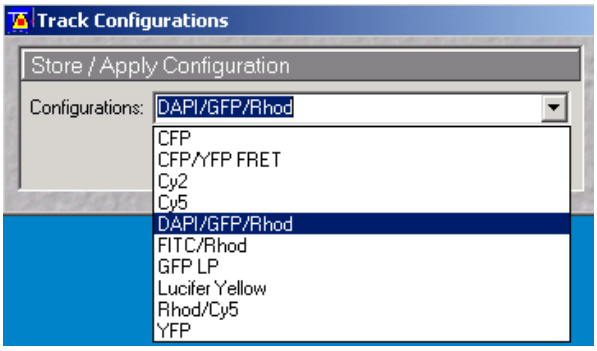


Fig. 14 **Track Configurations window**

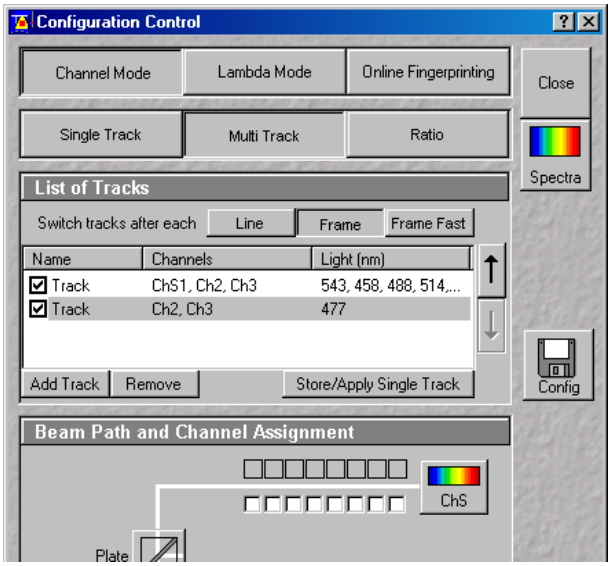


Fig. 15 **Configuration Control window for Multi Track**

- Click on the **Multi Track** button in the **Configuration Control** window (Fig. 15).
- Click on the **Descanned** button if necessary.

The following functions are available in the **List of Tracks** panel (Fig. 15).

Modes

Switch tracks after each

Line button	Tracks are switched during scanning line by line. The following settings can be changed between tracks: Laser line and intensity and channels incl. settings for gain and offset.

Switch tracks after each

Frame button	Tracks are switched during scanning frame by frame. The following settings can be changed between tracks: Laser line and intensity, all filters and beam splitters, the channels incl. settings for gain and offset and the pinhole position and diameter.
Frame Fast button	The scanning procedure can be made faster. Only and the laser line and intensity and the **Amplifier Offset** are switched, but no other hardware components. The tracks are all matched to the current track with regard to emission filter, dichroic beam splitter, setting of Detector Gain, pinhole position and diameter. When **Line** button is selected, the same rules apply as for **Frame Fast**.

Settings

Add Track button	An additional track is added to the configuration list. The maximum of four tracks can used. One track each with basic configuration is added, i.e.: one Ch 1 channel is activated, all laser lines are switched off, emission filters and dichroic beam splitters are set in accordance with the configuration last used.
Remove button	The single track previously marked in the **List of Tracks** panel in the Name column is deleted.
Store/Apply Single Track button	Opens the **Track Configurations** window. A selected track defined in a Channel Mode Configuration can also be stored as a single track for single tracking applications. Also, it's possible to load a single track in a multi tracking configuration.
	A click on this arrow button will move the selected track (highlighted in blue) one position upwards in the list box.
	A click on this arrow button will move the selected track (highlighted in blue) one position downwards in the list box.

- Configure each desired track for **Multi Track** function as described for **Single Track**.
- For storing/applying or deleting a **Channel Mode Configuration** use the **Config** button.

Scanning an image

- Click on the **Scan** button in the **Acquire** subordinate toolbar to open the **Scan Control** window.

The **Scan Control** window appears (Fig. 16).

Setting the parameters for scanning

- Select **Mode** in the **Scan Control** window.
- Select the **Frame Size** as predefined number of pixels or enter your own values (e.g. 300 x 600) in the **Objective Lens, Image Size & Line Step Factor** panel. Click on the **Optimal** button for calculation of appropriate number of pixels depending on N.A. and λ.

The number of pixels influences the image resolution!

Note: When using an Axioskop 2 FS MOT, indicate the objective that is in use in the **Scan Control** window. This ensures correct calculation of pinhole, Z stack optimization etc.

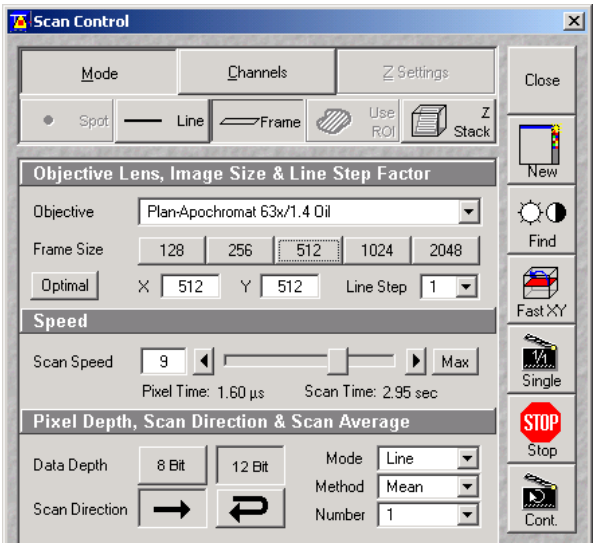


Fig. 16 **Scan Control window, Mode settings**

Adjusting the scan speed

- Use the slider in the **Speed** panel (Fig. 16) to adjust the scan speed.

A higher speed with averaging results in the best signal to noise ratio. Scan speed 8 usually produces good results. Us speed 6 or 7 for superior images.

Choosing the dynamic range

- Select the dynamic range 8 or 12 Bit (per pixel) in the **Pixel Depth, Scan Direction & Scan Average** panel (Fig. 16).

8 Bit will give 256 gray levels, 12 Bit will give 4096 levels. Publication quality images should be acquired using 12 Bit data depth. 12 Bit is also recommended when doing quantitative measurements or when imaging low fluorescence intensities.

Setting the scan averaging

Averaging improves the image by increasing the signal : noise ratio. It can be achieved line by line or frame by frame. Frame averaging helps reduce photobleaching, but does not give quite such a smooth image.

- Select the **Line** or **Frame** mode for averaging.
- Select the desired scan average method **Mean** or **Sum** in the **Method** selection box.

If you are using the **Mean** method, the image information is generated by adding up all scans pixel by pixel and then calculating the mean value.

In the **Sum** method, the pixel values of all scans are only added up, without a mean value being calculated.

- Select the Number for averaging.

Continuous averaging is possible in the **Frame** mode. In this case a **Finish** button for ending continuous averaging is displayed instead of the **Cont.** button.

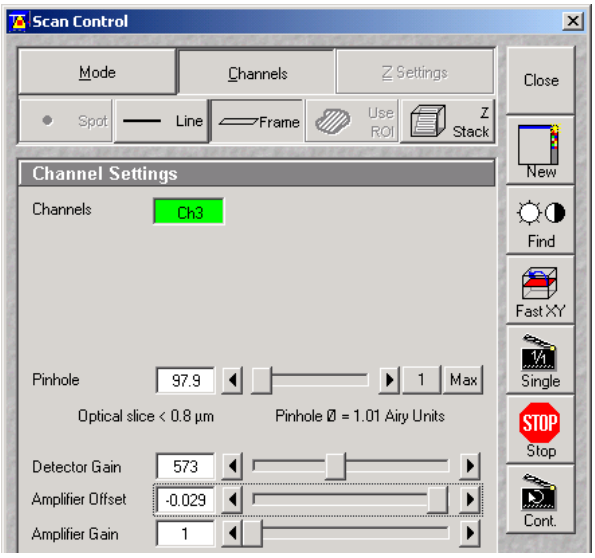


Fig. 17 **Scan Control window, Channel settings**

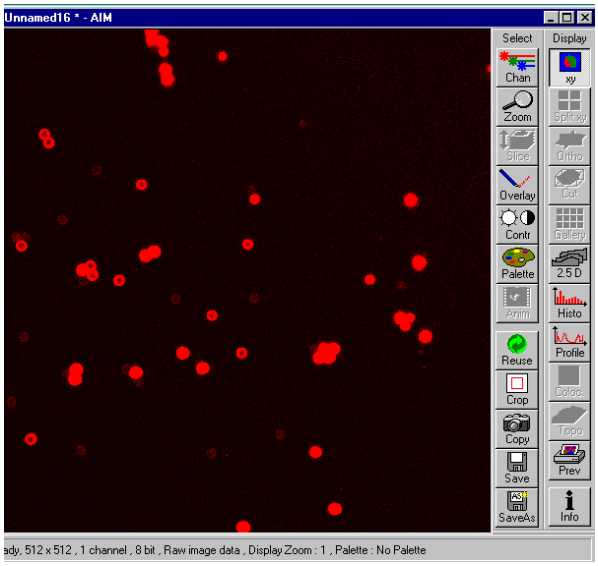


Fig. 18 **Image window**

Adjusting the pinhole

- Select **Channels** in the **Scan Control** window.
- Set the **Pinhole** size to **1** (Airy unit) for best compromise between depth discrimination and efficiency.

Pinhole adjustment changes the **Optical Slice**. When collecting multi channel images, adjust the pinholes so that each channel has the same **Optical Size**. This is important for colocalization studies.

Image acquisition

Once you have set up your parameter as defined in the above section, you can acquire a frame image of your specimen.

- Use one of the **Find**, **Fast XY**, **Single** or **Cont**. buttons to start the scanning procedure and acquire an image.

Scanned images are shown in separate windows.

- Click on the **Stop** button to stop the current scan procedure if necessary.

Select **Find** for automatically pre-adjustment of detector sensitivity.

Select **Fast** for continuous fast scanning – useful for finding and changing the focus.

Select **Single** for recording a single image.

Select **Cont.** for continuous scanning with the selected scan speed.

Select **Stop** for stopping the current scan procedure.

Image optimization

Choosing a lookup table

- Select **Palette** in the **Image** window of the scanned image (Fig. 19).

The **Color Palette** window appears.

- In the **Color Palette List** panel, click on the **Range Indicator** item (Fig. 20).

The scanned image appears in a false-color presentation (Fig. 19).
If the image is too bright, it appears red on the screen. Red = saturation (maximum).

If the image is not bright enough, it appears blue on the screen. Blue = zero (minimum).

Adjusting the laser intensity

- Set the **Pinhole** to **1** Airy Unit (Fig. 21).
- Set the **Detector Gain** high.
- When the image is saturated, reduce AOTF transmission in the **Excitation** panel (Fig. 21) using the slider to reduce the intensity of the laser light at the specimen.

Adjusting gain and offset

- Increase the **Amplifier Offset** until all blue pixels disappear, and then make it slightly positive (Fig. 21).
- Reduce the **Detector Gain** until the red pixels only just disappear.

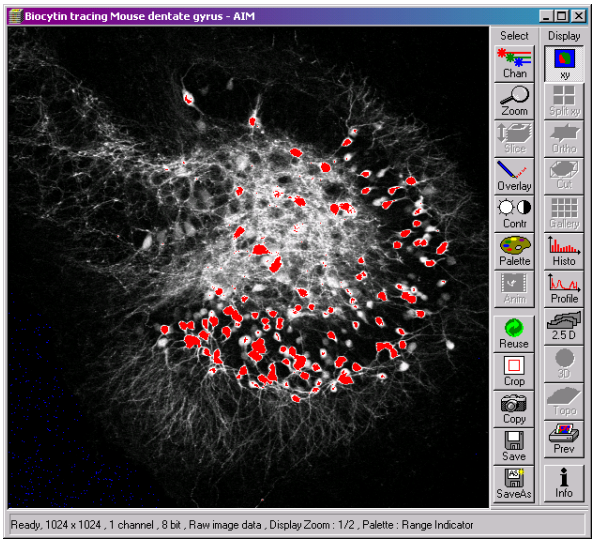


Fig. 19 **Image window**

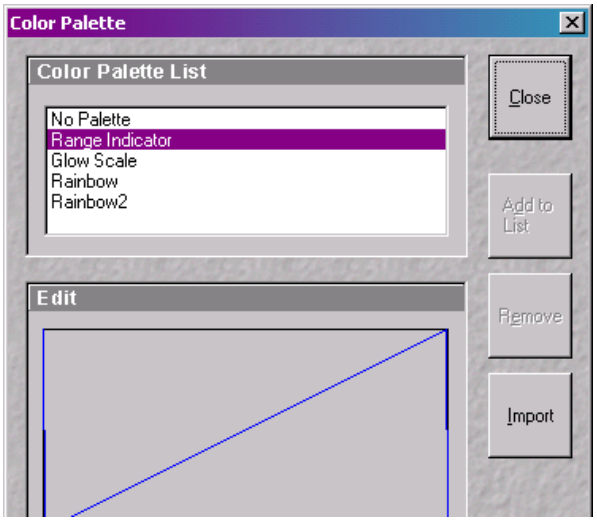


Fig. 20 **Color Palette window**

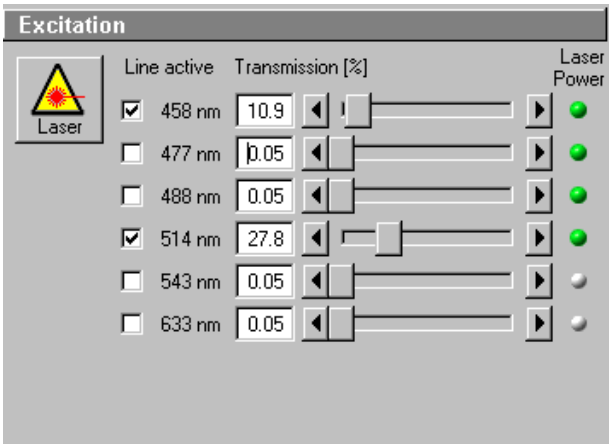


Fig. 21 **Scan Control window, Channel settings**

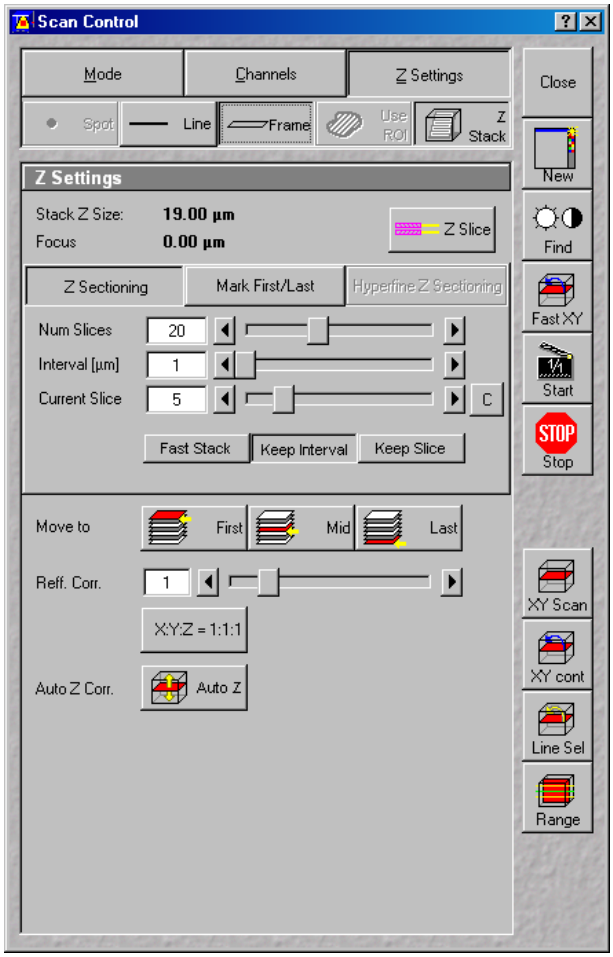


Fig. 22 **Scan Control window, Z Stack settings**

Scanning a Z stack

- Select **Z Stack** in the **Scan Control** window.
- Select **Frame** if necessary.

The **Z Settings** panel appears.

- Select **Mark First/Last** on the **Z Settings** panel.
- Click on the **XY cont** button.

A continuous XY-scan of the set focus position will be performed.

- Use the focusing drive of the microscope to focus on the upper position of the specimen area where the Z Stack is to start.
- Click on the **Mark First** button to set the upper position of the Z Stack.
- Then focus on the lower specimen area where the recording of the Z Stack is to end.
- Click on the **Mark Last** button to set this lower position.
- Click on **X:Y:Z=1:1:1** button to set the Z-interval in such a way that the voxel has identical dimensions in the X-, Y- and Z-directions (cube).
- Click on the **Start** button to start the recording of the Z Stack.

Storing an image

- Click on the **Save** or **Save As** button in the **Image** window or in the **File** subordinate toolbar of the **Main** menu.

The **Save Image and Parameter As** window appears.

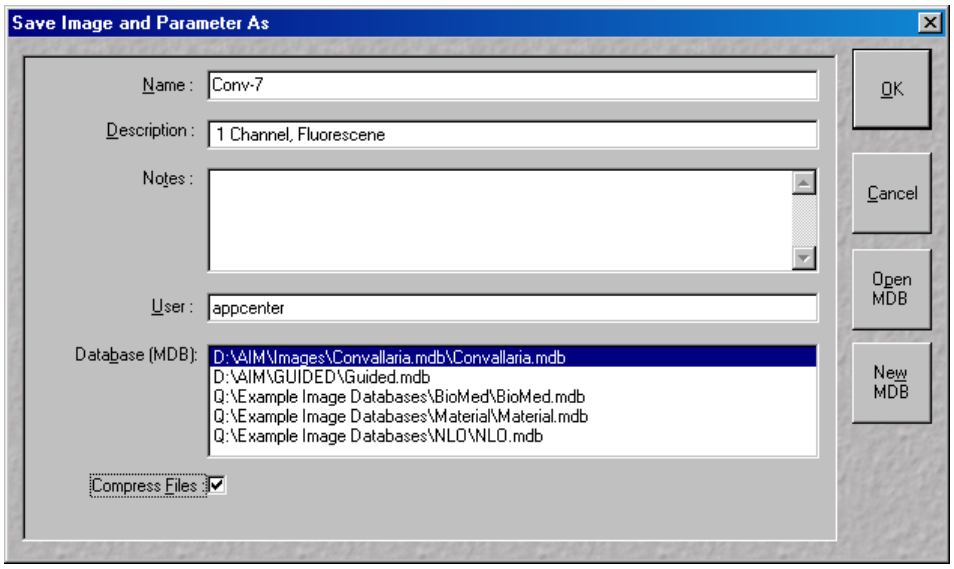


Fig. 23 **Save Image and Parameter As window**

- Enter file name, description and notes in the appropriate text boxes.
- Click on the **OK** button.

Switching off the system

- Click on the **File** button in the **Main** menu and then click on the **Exit** button to leave LSM 5 software program (Fig. 5).
- If any lasers are still running you should shut them off now in the pop up window indicating the lasers still in use.
- Shut down the computer.
- Wait until fan of Argon laser has switched off.
- On the REMOT CONTROL switch turn off the **Components** switch and the **System/PC** switch (Fig. 1).
- Switch off the HBO 100 mercury lamp.
- Switch the UV-Ar laser of via the toggle switch of the power supply (Fig. 2).