

**March 2006**

**LSM 5 LIVE / LSM 5 LIVE DuoScan**  
**Laser Scanning Microscope**  
**Release 4.0**

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 5 LIVE operating instructions

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 reminds service personnel 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.**

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## 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.

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### **2 LSM 5 *LIVE* - Setup Requirements**

The Setup Requirements section outlines the installation and supply requirements of the LSM 5 *LIVE* Microscope Systems, together with the relevant specifications.

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### **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 5 *LIVE*.

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### **4 Operation**

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.

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### **5 Tools**

This section contains a description of the use of the tools for setting the microscope.

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### **6 3D for LSM**

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.

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### **7 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.

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### **8 Certification**

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### **9 Brief Operating Manual**

This section contains the brief instructions of the LSM 5 *LIVE* Microscope Systems.

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### **10 Laser safety warning labels**

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# CHAPTER 1                      NOTES ON DEVICE SAFETY

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## 1 NOTES ON DEVICE SAFETY

### 1.1 General

The LSM 5 *LIVE* 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 5 *LIVE* 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 any thing other than the intended use of the LSM 5 *LIVE* 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 and heating inserts.

As the system is largely operated via menus on a computer, you should be acquainted 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 5 *LIVE* is a device that belongs to laser hazard class 3B. The system is equipped with safety interlocks that comply with laser hazard class 3B and 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 5 LIVE 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.



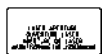
Caution: Hot surface.



Caution: UV radiation.



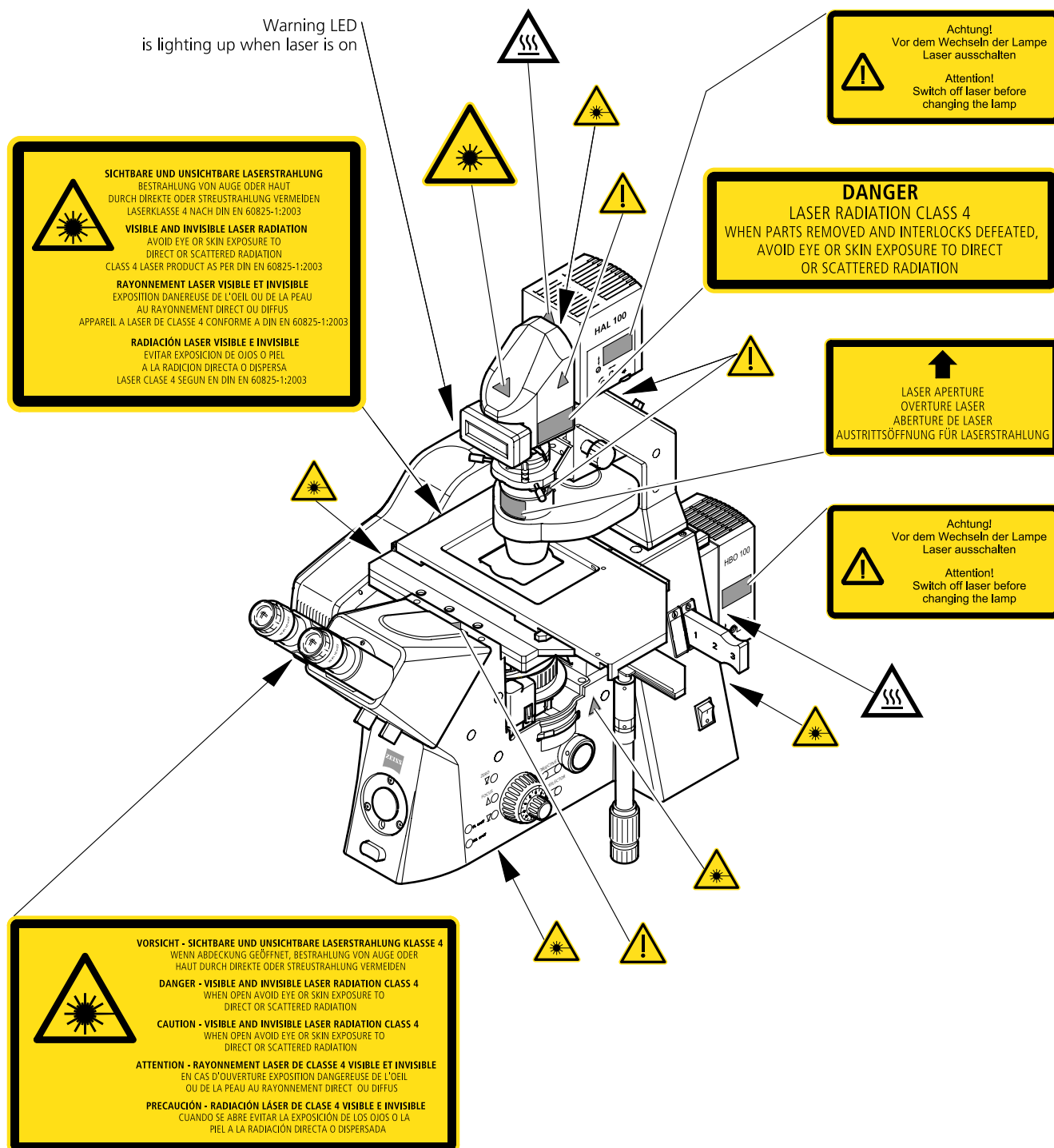
Caution: Fingers can be caught.



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 5 *LIVE* scanning module

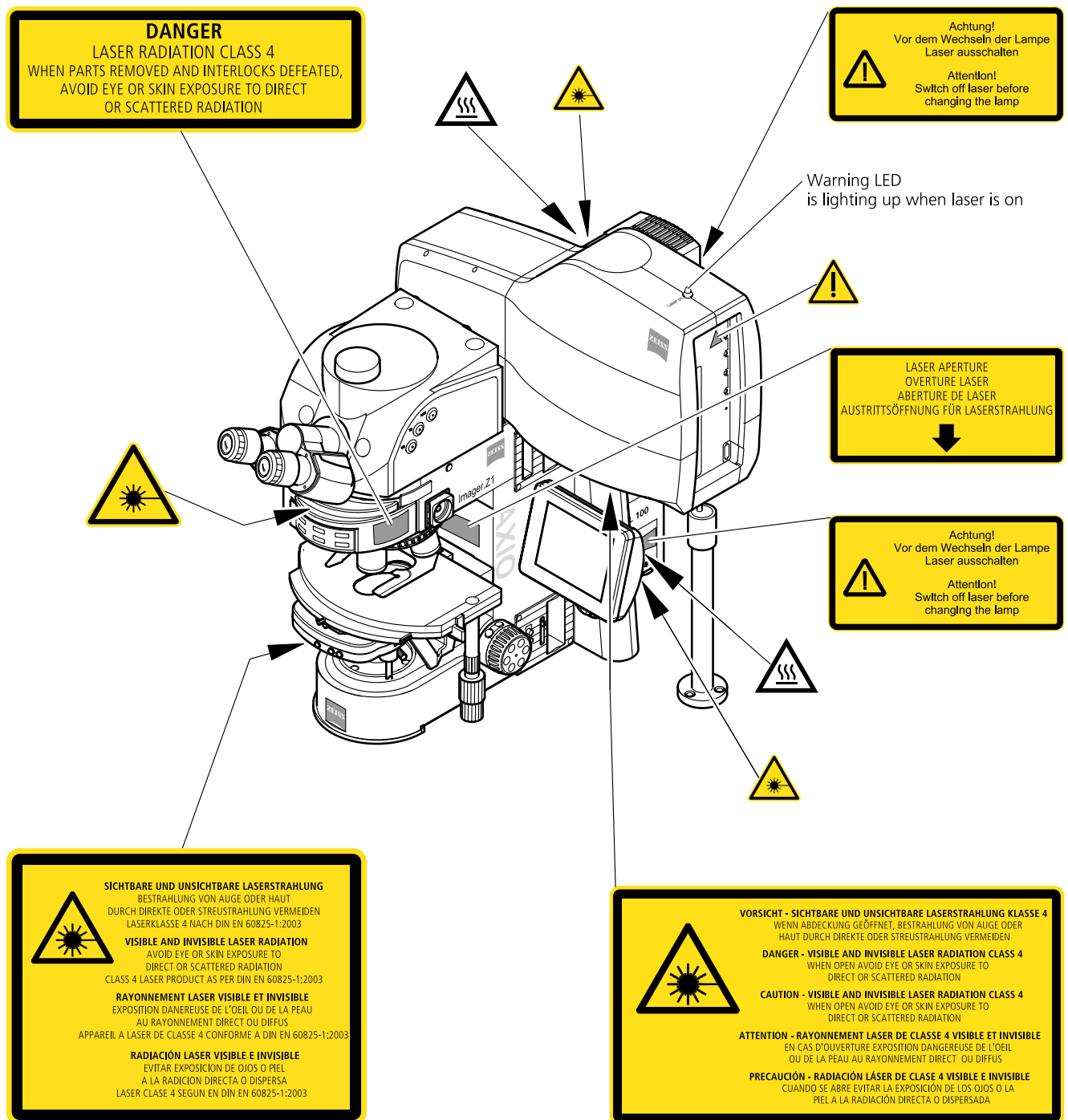


Fig. 1-2 Warning and information labels on the Axio Imager.Z1 microscope with scanning module

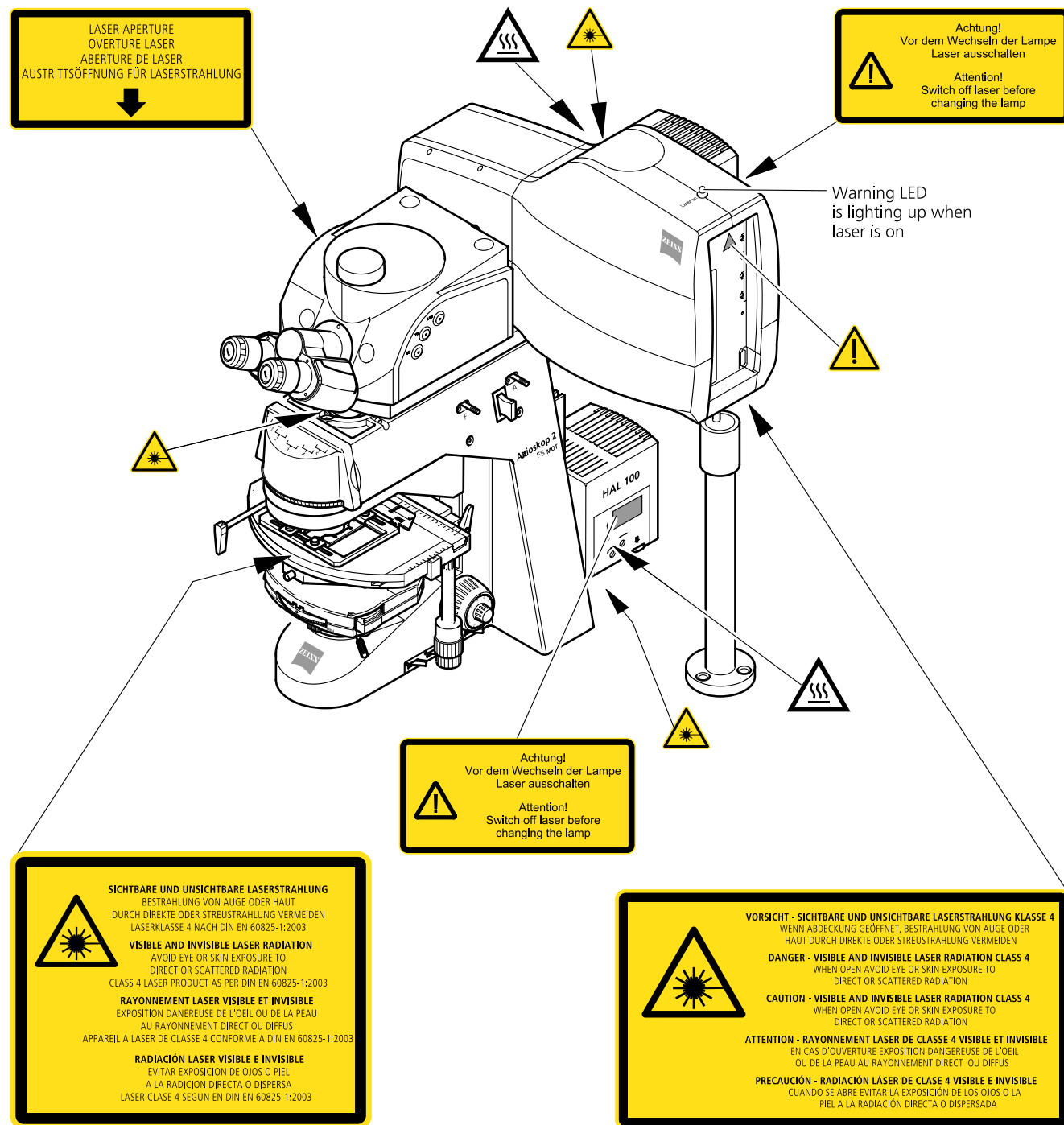
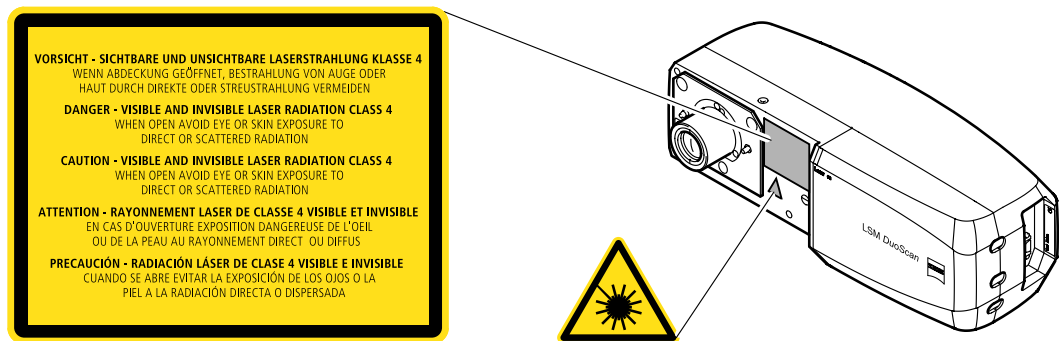


Fig. 1-3 Warning and information labels on the Axioskop 2 FS MOT microscope with scanning module



**Fig. 1-4** Warning and information labels on LSM DuoScan (systems LSM 5 LIVE DuoScan only)

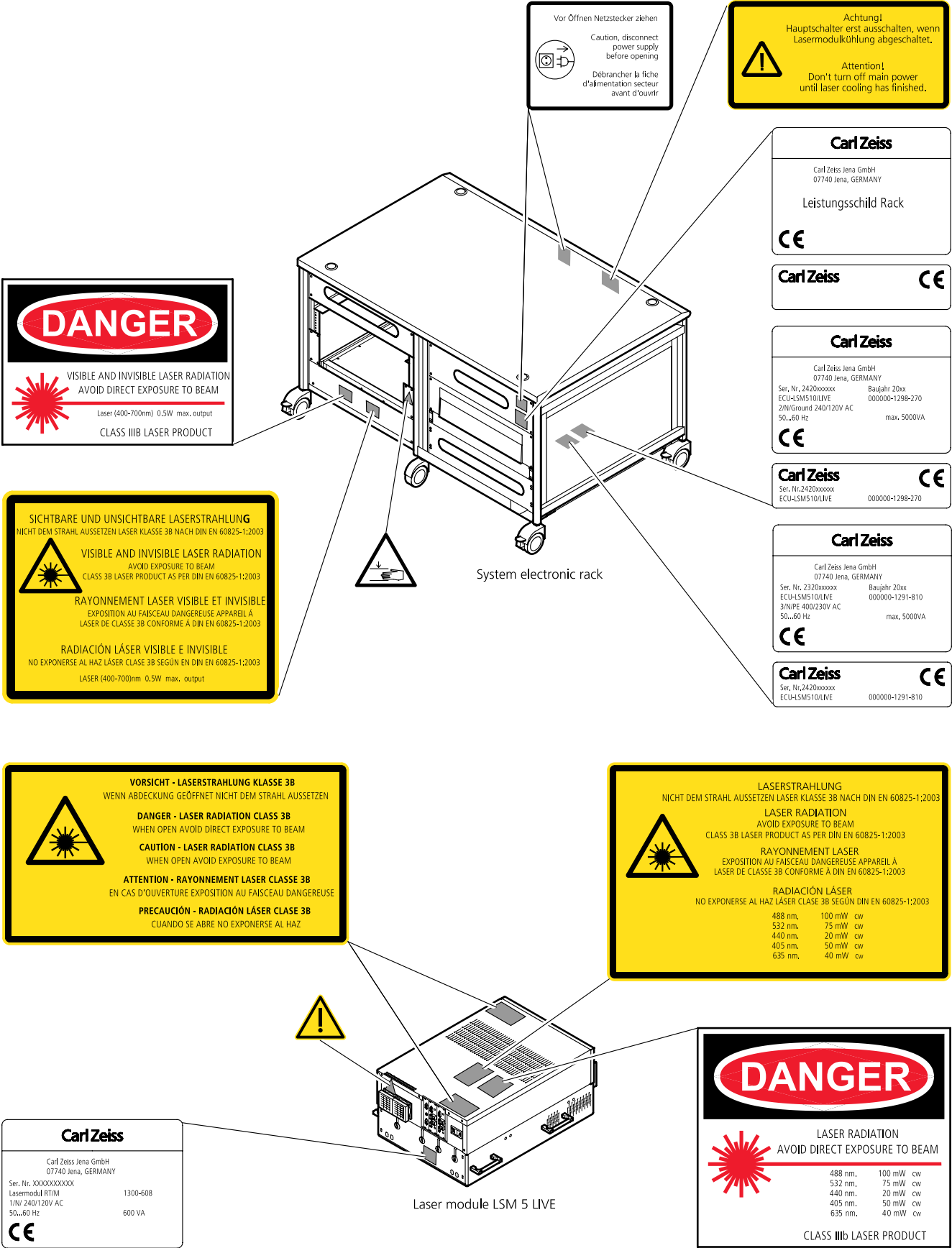
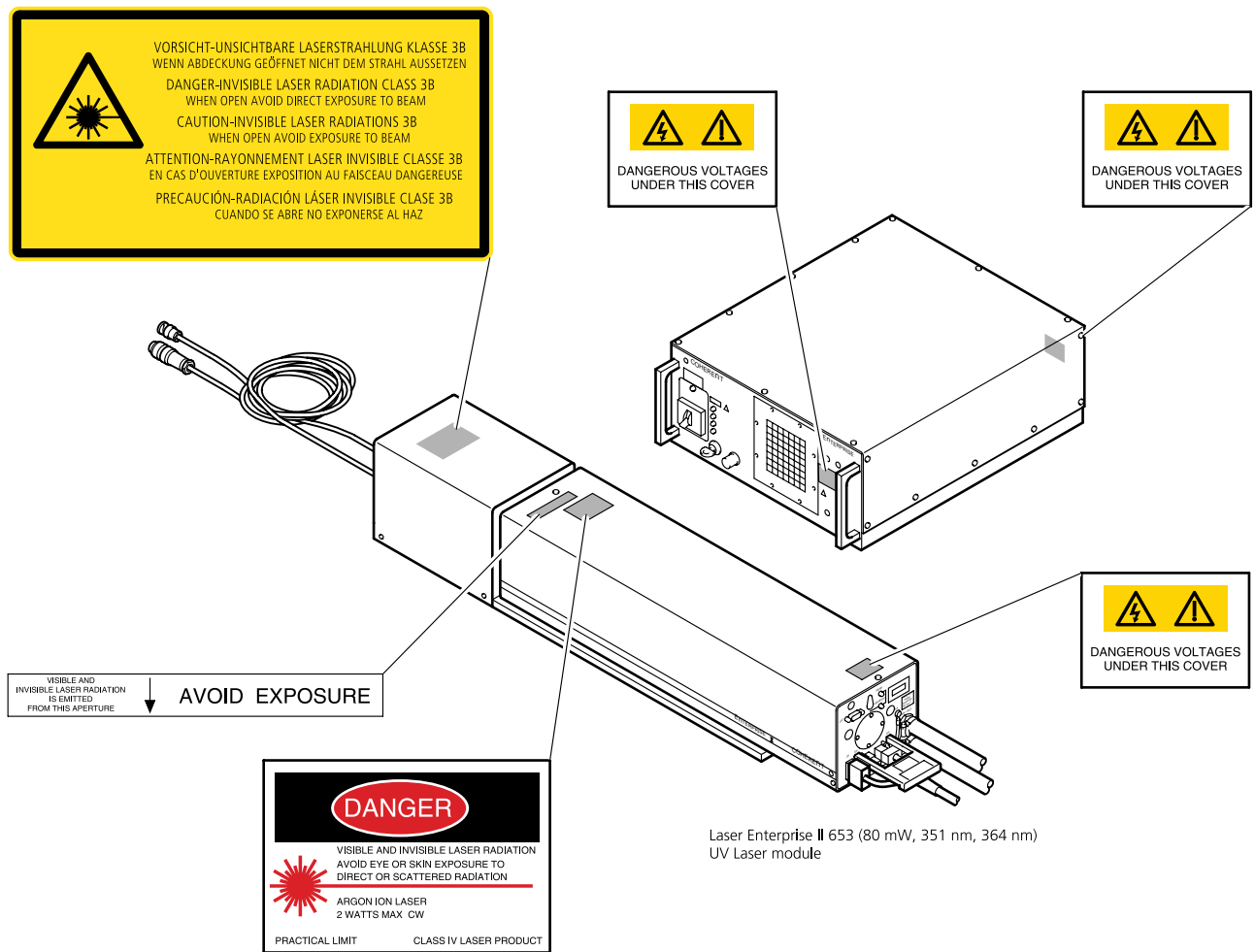


Fig. 1-4 Warning and information labels on laser components





**Fig. 1-5** Warning and information labels on UV laser components (LSM DuoScan only)

### 1.3 Regulations

Extensive knowledge of the hardware/the system is indispensable for safe operation of the LSM 5 *LIVE*.



Read these operating instructions and all device publications belonging to the system conscientiously **before** operating the LSM 5 *LIVE*! 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 5 *LIVE* has 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 Protection against diffuse laser excitation emitted around the objective and sample

Though the radiation levels around the objective and sample is very low (comparable to a common laser pointer used for e.g. talks), avoid to approach this area closer than 10 cm with your eye.

Also limit the time of skin exposure of your hands to the time needed to place the sample and to mechanically set up the acquisition.

Switch off the scanning while doing such manipulation around the objective and sample!

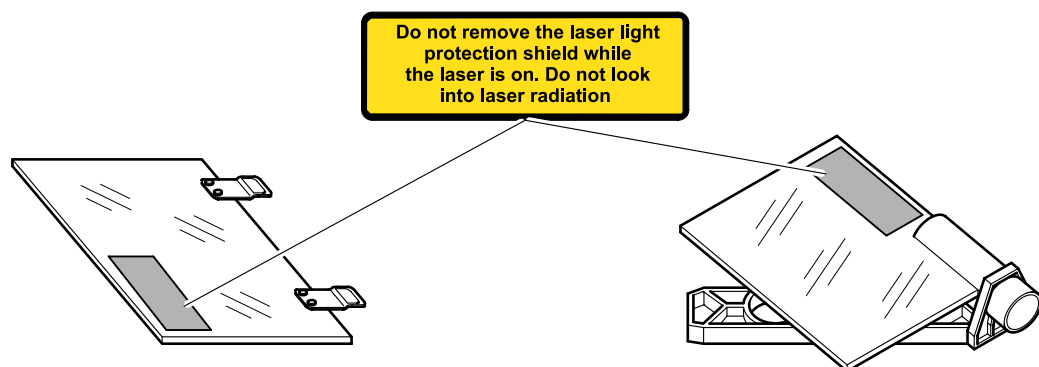
In case of scanning, don't stare into the beam!

To further reduce the radiation level of fluorescent emission and excitation light around the microscope, Carl Zeiss delivers antiglare screens for all microscopes.


Axiovert 200M: 000000-1109-741

Axio Imager.Z1/M1: 452163-0000-000

Axioskop 2 FS mot: 452163-0000-000



**Fig. 1-6 Antiglare screens for Axiovert 200M (left) and Axio Imager and Axioskop 2 FS (right)**

 Use these screens for improved working ergonomcy!

## 1.5 Protection against white light emitted around the objective and sample by an X-cite fluorescence illumination

Follow the guidelines in the X-Cite manual provided by EXFO Inc..

Don't use a neutral beamsplitter (e.g. "NT80/20" position in LSM *DuoScan* or LSM 5 *DUO* systems) in the P&C modules while looking through the eyepieces. Strong white light might project into your eyes.

In case such strong light is projected into your eye, the natural lid closure effect will protect you from any damage. Nonetheless such exposure is unpleasant and should absolutely be avoided.

## 1.6 Notes on Setting up the Microscope System



Installation and commissioning of the LSM 5 *LIVE* 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 5 *LIVE* laser scanning microscope is delivered in several crates:



The LSM 5 *LIVE* 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, addition, 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, safety interlock devices and other system components.

Please note that the LSM 5 *LIVE* 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 Laser module in and out of the System electronic rack take care not to catch your fingers.



The HBO 50 and 100 W, XBO 75 W and X-cite 120 lamps used on the light microscopes incident light path emit UV light which is harmful to the human eye and skin when observed without appropriate filters. Never remove the lamp and look direct into the emitted light.

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 5 *LIVE* should only be operated in conjunction with the system table (vibration damping).

**1.7 Power Requirements**

The LSM 5 *LIVE* comes with a mains power supply cord and plug, either CEE red (3/N/PE 400/230 V/16 A), or NEMA L 14-30P (2/N/Ground 120/240 V/30 A), 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 ( $\pm 10$ %)	100 ... 125 V AC ( $\pm 10$ %)
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 ( $\pm 10$ %) 50 / 60 Hz	208...240 V AC ( $\pm 10$ %) 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

## 1.8 Notes on Handling the Laser Components



The LSM 5 *LIVE* are laser hazard class 3B instruments. 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 5 *LIVE*. The use of any other lasers as the ones listed below is not authorized.

Laser	Class	Power
1 Diode laser 405 nm	3B	50 mW
2 Diode laser 440 nm	3B	16 mW
3 Diode laser 488 nm	3B	100 mW
4 DPSS laser 532 nm	3B	75 mW
5 Diode laser 635 nm	3B	35 mW
6 Ar laser 351/364 nm (UV, for <i>DuoScan</i> only)	4*	80 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 5 *LIVE* 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.



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.



## 1.9 Notes on Handling HBO and X-Cite Light Sources



For *LIVE* systems equipped with a rearport mounted scan head be aware that the mirror cube in the reflector turret leads to a strong back reflection of HBO light into the specimen plane and the ocular lens. When observing the specimen through the ocular lens the use of the mirror cube position should be avoided. The light flash is not harmful but unpleasant. The reflex of closing the eyelid is sufficiently protective. To avoid this situation an additional filter LP 397 (from filter cube 01, # 48 80 01) can be mounted into the mirror cube which prevents the back reflection of the HBO UV light in the ocular plane.

## 1.10 Physical Dimensions

	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
<b>Passively damped anti-vibration table</b>	130	100	75	137
<b>Scanning Module LSM 5 <i>LIVE</i></b>	55	17	30	19,5
<b>Scanning Module LSM <i>DuoScan</i></b>	39	15	13	7
<b>Microscope</b>	50	35	50	20
<b>Laser Module <i>LIVE</i></b>	66	52	22	58
<b>Laser Module, UV (for <i>DuoScan</i> only)</b>	140	20	20	60
<b>System Electronic Rack</b>	110	70	58	90
<b>Plug-in unit external laser (UV)</b>	66	52	22	9
<b>Power supply for Ar (UV)</b>	50	50	30	30
<b>Cooling unit for Ar (UV)</b>	80	45	50	30

## 1.11 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

## 1.12 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, 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.

### 1.13 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 also render all warranty claims null and void and laser safety might no longer be 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.

## 1.14 User Interface



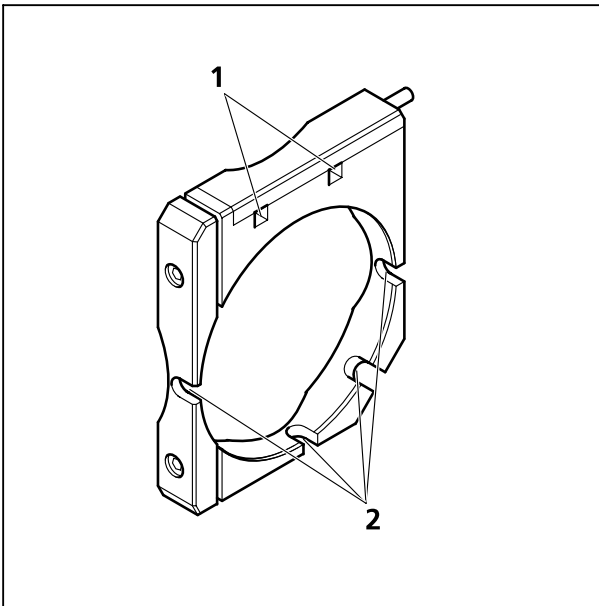
All user interface ports are equipped with a safety interlock system which warrants laser safety. These interlock devices must not be manipulated. Other interfaces which are not described here are service interfaces and are only to be operated by authorized Carl Zeiss service personnel. The following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

- Halogen and HBO lamp
- Scan head

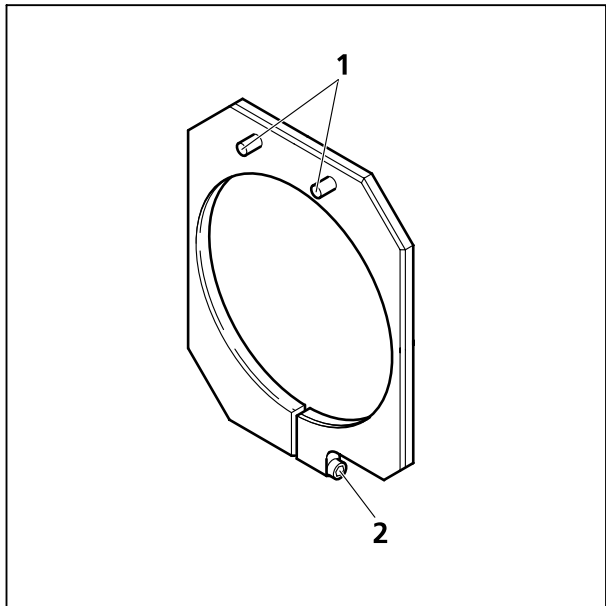
### 1.14.1 Mounting and Dismounting Lamps, TPMT and Switching Mirror

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. 1-7** Sensor ring mounted to the interface ports on the microscope side



**Fig. 1-8** Contact ring mounted to the lamp, TPMT or switching mirror

The interlock is working when the sensors of the sensor ring (Fig. 1-7/1) are depressed by the pins on the contact ring (Fig. 1-8/1). 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.

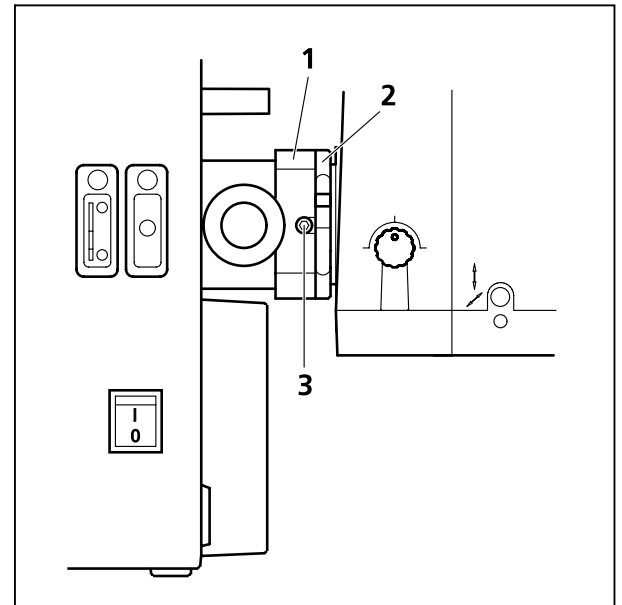


In case the system is not operating following the removal or attachment of any device on a port with safety interlock check again the connection of the Contact ring to the Sensor ring.

For dismantling the lamps, TPMT or switching mirror slightly unscrew the contact ring first (Fig. 1-8/2 or Fig. 1-9/2) so it can be moved away from the sensor ring (Fig. 1-9/1). Then unscrew the lamp, TPMT or switching mirror (main screw Fig. 1-9/3) which is in one of the recesses of the sensor ring (Fig. 1-7/2). Hold the device to be dismantled 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.



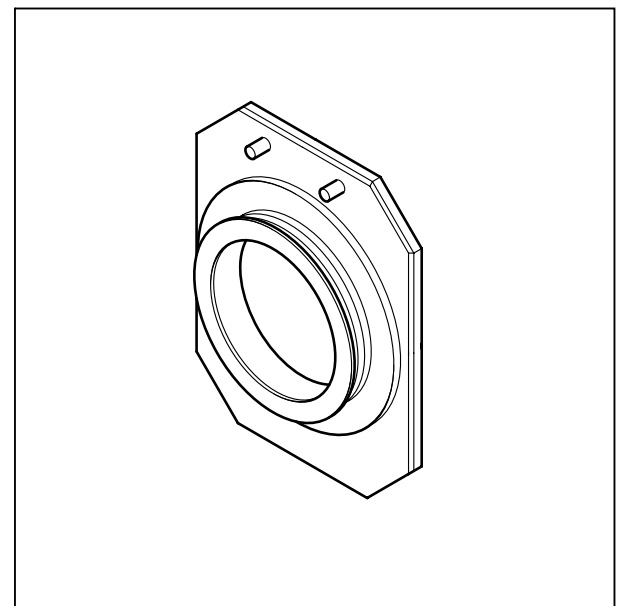
Do not remove the sensor ring from the microscope. This might result in failure of laser safety and a non operating system.



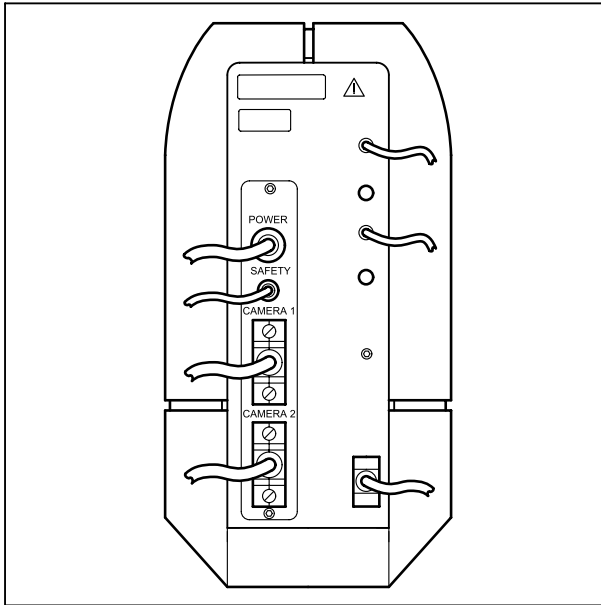
**Fig. 1-9 HBO lamp mounted to Axiovert 200 M with sensor ring and contact ring for laser safety**

For mounting any lamp, TPMT or the switching mirror back onto the microscope reverse the steps for dismantling 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. 1-10 Blind cap for closing any port equipped with an interlock device**



**Fig. 1-11** Electronic connection port of the LSM 5 *LIVE* scan head

### 1.14.2 Mounting and Dismounting the Scan Heads

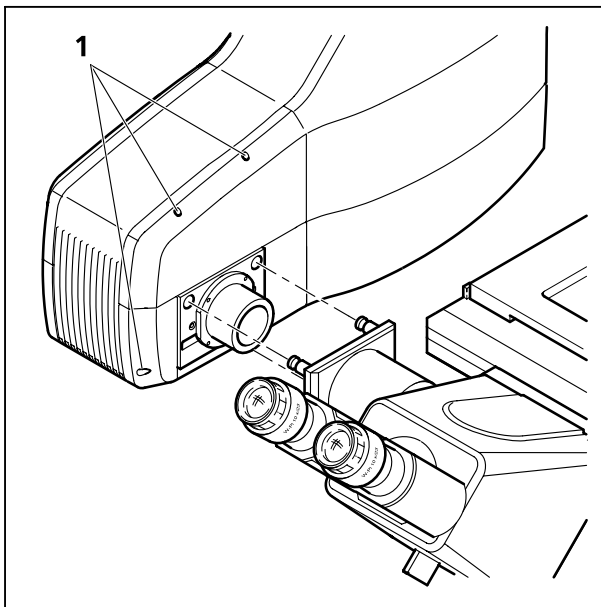
The scan heads are connected to the microscope via an integrated safety interlock. They can be moved between two microscopes. Make sure the system is shut off completely before starting the following procedure:



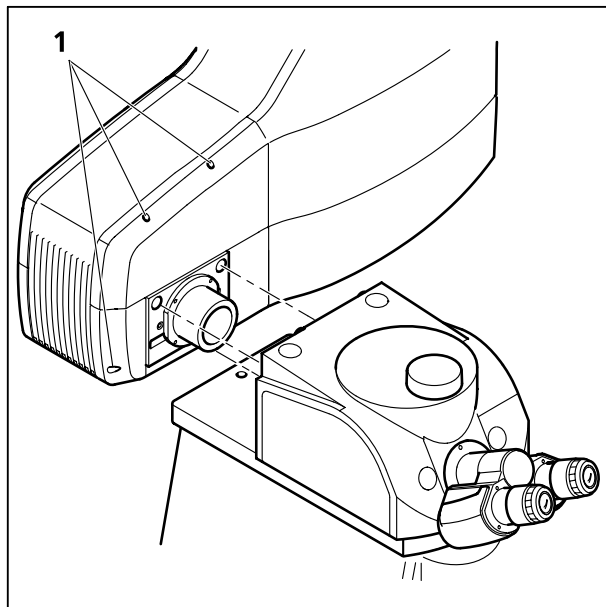
Be aware that the LSM 5 *LIVE* scan head weighs up to 19,5 kg.

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

- Loosen the three screws on LSM 5 *LIVE* or LSM *DuoScan* scan head (Fig. 1-12/1 or Fig. 1-13/1).



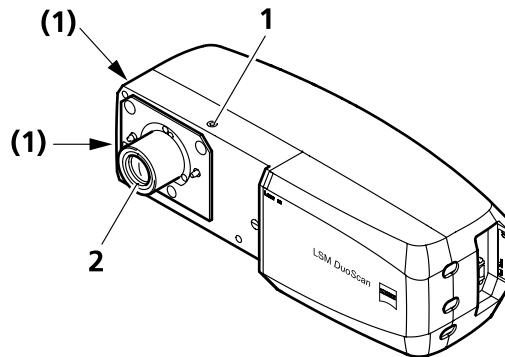
**Fig. 1-12** Port connection between LSM 5 *LIVE* and Axiovert 200 M



**Fig. 1-13** 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 three screws on the LSM 5 *LIVE* or LSM *DuoScan* scan head (Fig. 1-12/1 or Fig. 1-13/1).

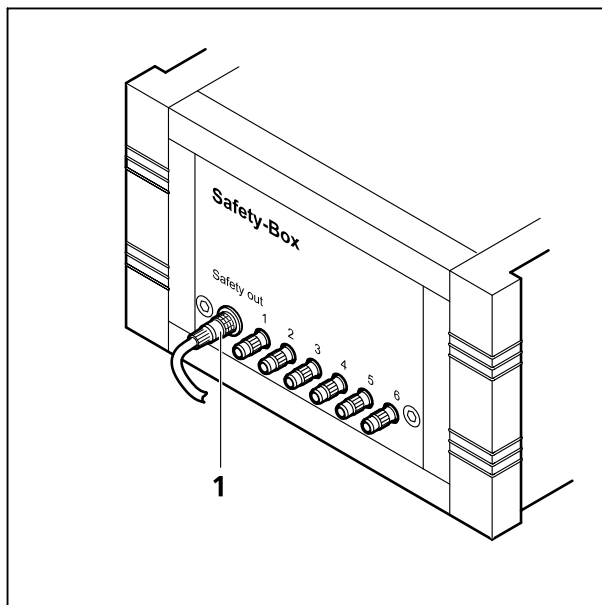
- Use the three fastening screws (Fig. 1-14/**1** and **2**) to attach or detach the LSM DuoScan to or from the microscope stand.



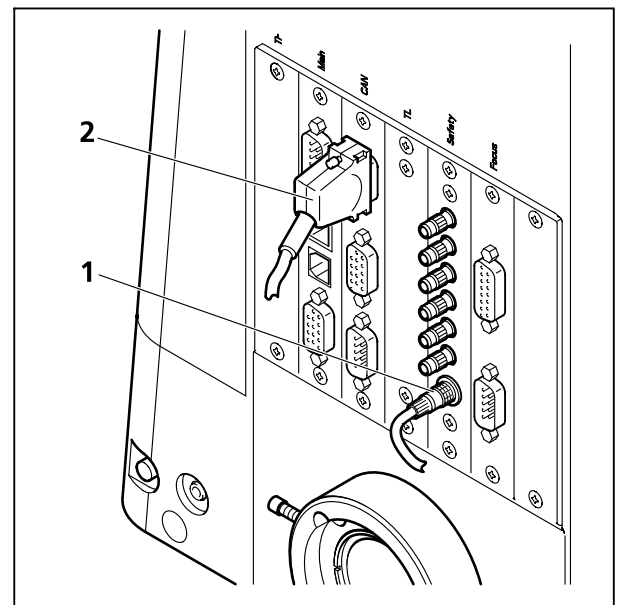
**Fig. 1-14 Fastening screws and connections on LSM DuoScan**

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

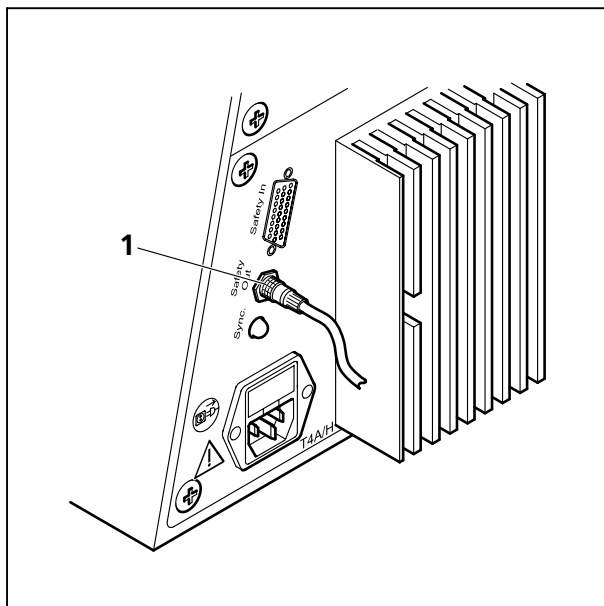
1. The connection of the microscope to the safety interface of the system is located either on the additional Safety-Box (Axioskop 2 FS MOT, Fig. 1-15/**1**) or on the rear side of the microscope (Axiovert 200 M or Axio Imager.Z1; Fig. 1-16/**1** and Fig. 1-17/**1**). This connection has to be unplugged from the microscope which is not in use and plugged into the microscope to be used following the exchange of the scan head.



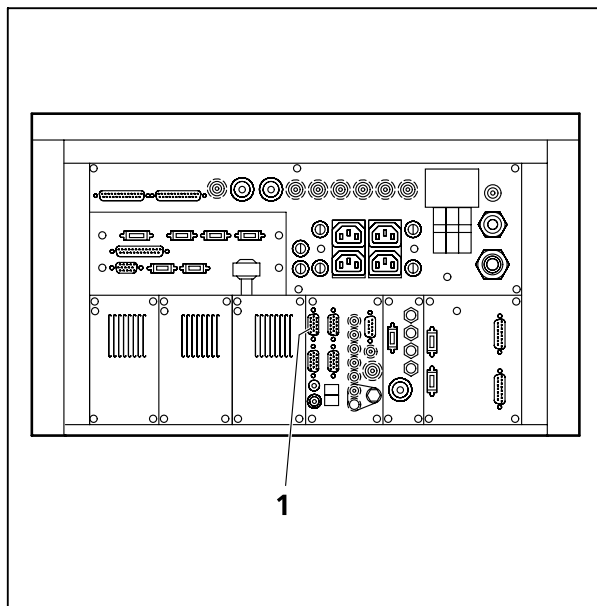
**Fig. 1-15 Safety-Box of Axioskop 2 FS MOT with main connection to safety interface (1)**



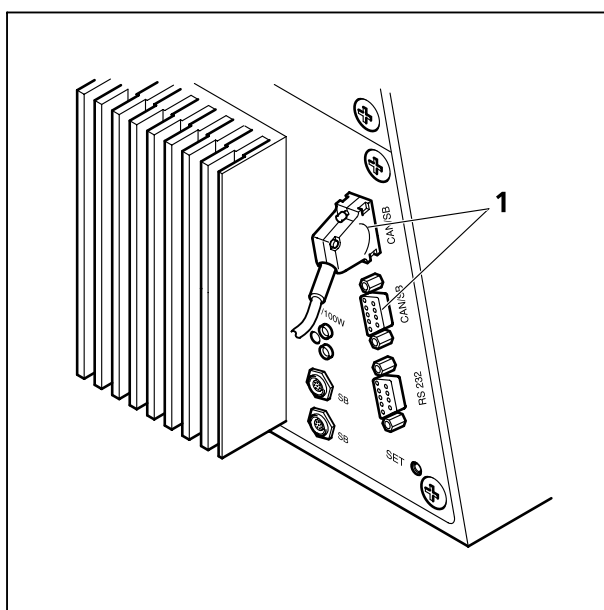
**Fig. 1-16 Connection of Axio Imager.Z1 to safety interface (1) and electronics (2)**



**Fig. 1-17** Connection of Axiovert 200 M to safety interface (1)



**Fig. 1-18** 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

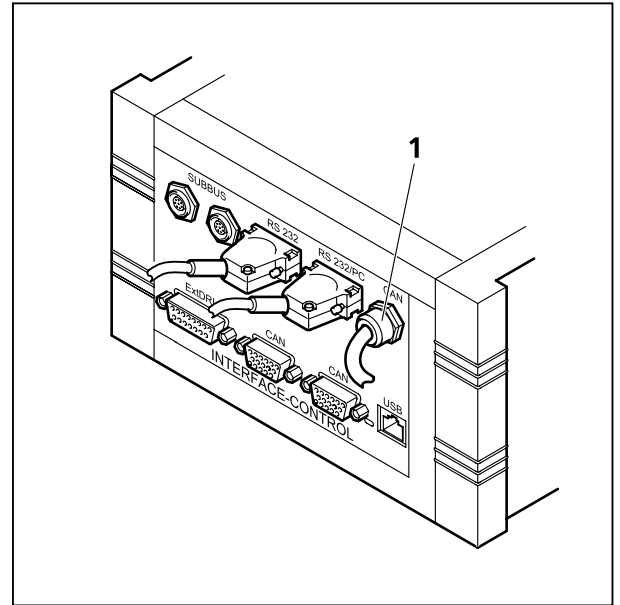


**Fig. 1-19** CAN connections on the Axiovert 200 M (1). One of the plugs is occupied with the connection to the electronics

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. 1-18. 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. 1-16/2, Fig. 1-19/1 and Fig. 1-20/1. 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.



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.



**Fig. 1-20** The Axioskop 2 FS MOT is connected to the electronics via the Interface Control. The CAN connection is fixed (1) to the control box

## CHAPTER 2    **LSM 5 *LIVE*/LSM 5 *LIVE DuoScan* - SETUP REQUIREMENTS**

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## **2 LSM 5 *LIVE* - SETUP REQUIREMENTS**

### **2.1 General Remarks**

The LSM 5 *LIVE* is a user friendly, high performance laser scanning system with unique technological performance. It is made of high precision components representing latest technological developments and a design that was guided by ergonomics, best possible performance and every day usability. The system is highly modular and flexible, but also easy to service and open for upgrades.

### **2.2 System Components and Recommended Setup**

The core of the system is a vibration isolating, air damped microscope table with a breadboard for fixation of the system and possible accessories. We strongly recommend the use of our Science Desk tables, since these have the most compact size that allows the setup of the various system configurations and future upgrades. A wide range of special accessories for physiological, molecular or cellbiological, or technical experiments (e.g. posts, shelves, grounding, faraday cages, cabinets etc.) is available worldwide by our partner Melles Griot. The science desk is a professional, open system platform for your experiments. A wide and a narrow version of the science desk table is available, matching the side- or rear port configurations of the microscope stands.

The microscope table is complemented by the laser and electronic system rack, which can be positioned at the side or at the back of the microscope table. This rack contains all electronic and laser components the LSM 5 *LIVE* needs, and is again designed with best performance, usability and future upgrades in mind. For setup and service purposes, free access is needed to the long side of the rack. Hence approx. 50 cm space should be provided to the next wall.

Use of the LSM 5 *LIVE* is mainly done by operating the software. Consequently, we provide an ergonomical computer workplace that fits the microscope table and should best be positioned on the right side of it. We recommend the use of the 45° triangle extension to get the best ergonomical setup of the complete system, but also a 0° or a 90° orientation to the microscope table is possible. The best overall system design depends on the shape of your room (see below).

### **2.3 Room Requirements**

The LSM 5 *LIVE* needs approx. an area of 7 m<sup>2</sup>. The room for the LSM 5 *LIVE* should therefore offer a minimum size of 10 m<sup>2</sup>. Due to the modular design, both square shaped rooms (approx. 3,2 m x 3 m) or rectangular shaped rooms (approx. 2.6 m x 3.9 m) match the setup requirements of the LSM 5 *LIVE*. Due to the modern diode technology of the lasers, the system will produce much less heat and noise than older confocals. Nonetheless an lab typical air condition or ventilation should be provided. A constant temperature of 23 °C would be ideal for constant and best system performance. Details should be discussed with your local Zeiss LSM specialist.

## 2.4 Minimum Space Requirements of the System

For a list of the components dimensions see below; in general, a space of 1.8 m x 3.6 m for systems with a 0° or 45° oriented computer workplace, or 2.8 m x 2.6 m for systems with a 90° oriented computer workplace is required. As mentioned, free access to the long side of the electronic and laser system rack is required for service and setup of the system. If possible, we also recommend to have a free space of 30 cm to the next wall all around the system.

## 2.5 Dimensions (Overview)

Component	Overall dimensions	Remarks
Science desk microscope table (wide)	125 cm x 100 cm	Side port systems
Science desk microscope table (narrow)	100 cm x 125 cm	Side- and Rear port systems
Electronic and laser system rack	75 cm x 115 cm	Long side must be accessible
Computer workplace (0° or 90°)	120 cm x 80 cm	
Computer workplace diagonal (45°)	130 cm x 160 cm	Recommended for ergonomics
Complete system in ideal configuration, wide table, 45° computer workplace	180 cm x 360 cm	
Complete system in ideal configuration, narrow table, 45° computer workplace	180 cm x 340 cm	Most often used setup
Complete system square setup, 90° computer workplace orientation	280 cm x 260 cm	

## 2.6 Schematic Outline of a System

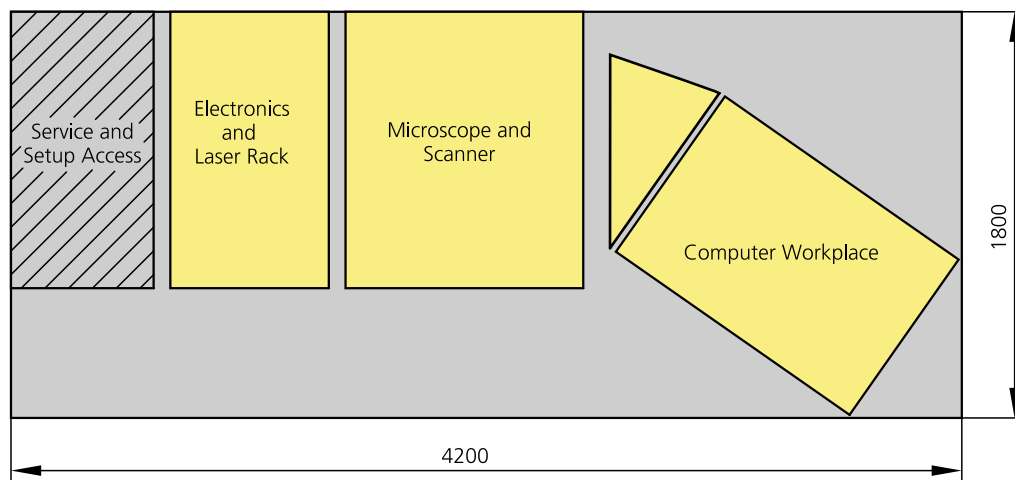


Fig. 2-1 Schematic outline of a system

## 2.7 Space Requirements

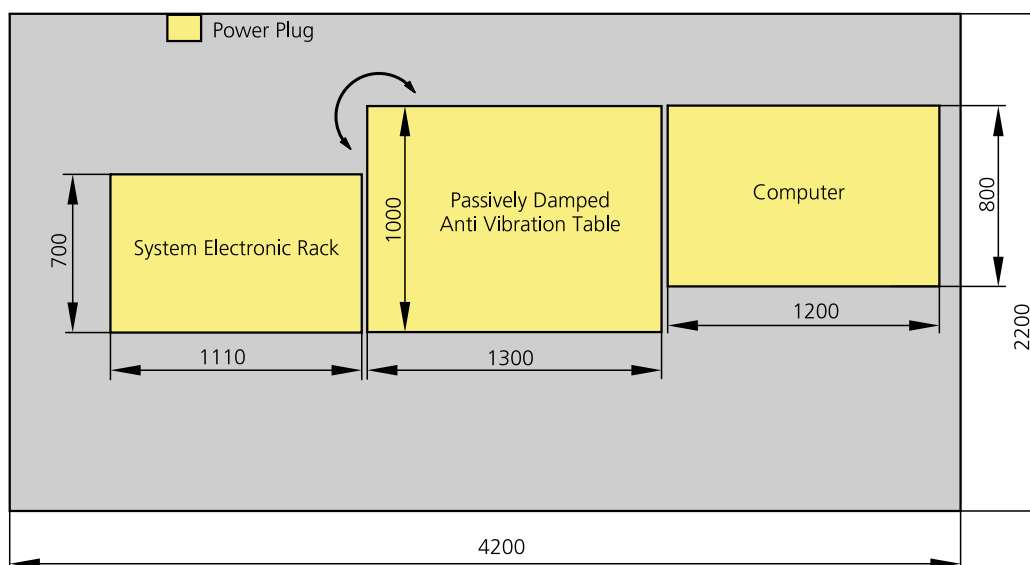
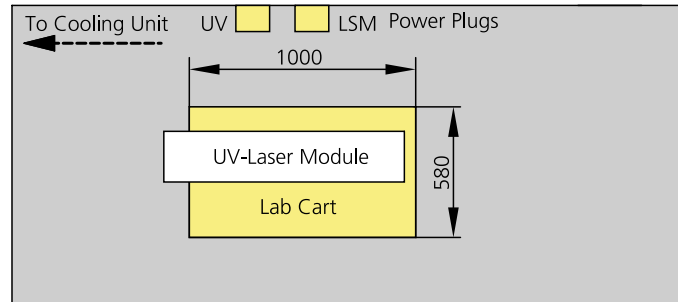


Fig. 2-2 LSM 5 *LIVE* with microscope table and PC table (dim. in mm)  
The System rack contains all electronics and laser unit.

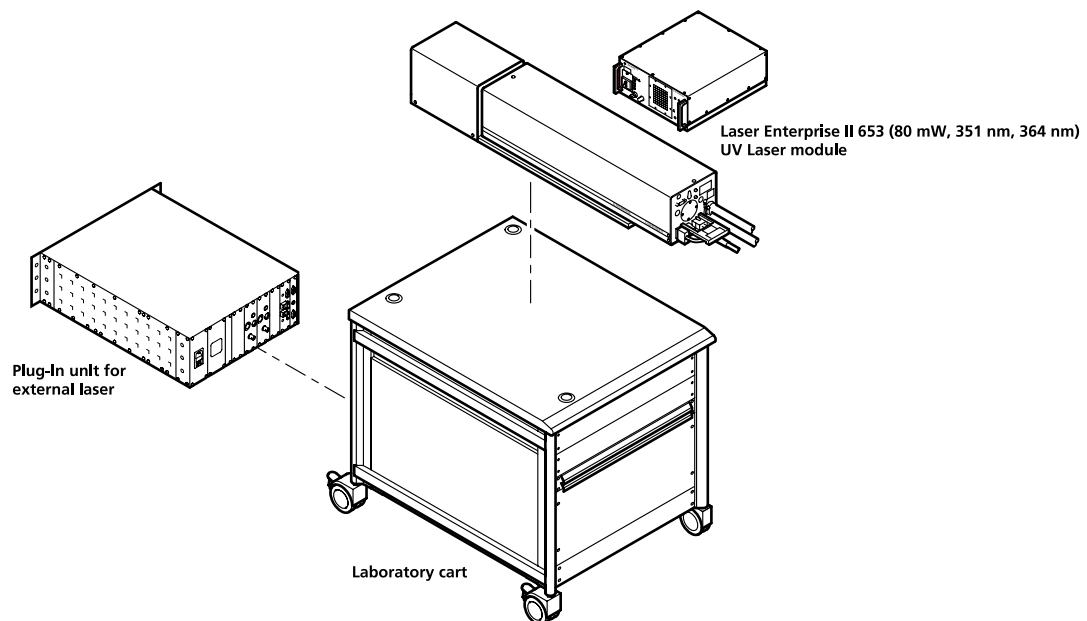
### 2.7.1 LSM DuoScan with Ar UV Laser



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



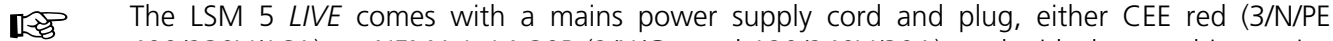
**Fig. 2-3** Space requirements for LSM DuoScan using an AR UV Laser (measurements in mm)



**Fig. 2-4** Lab container for UV laser setup

The lab container cart 000000-0465-515 is recommended to provide space for the setup of the external UV-laser, the UV laser power supply and the plug in unit for external laser.

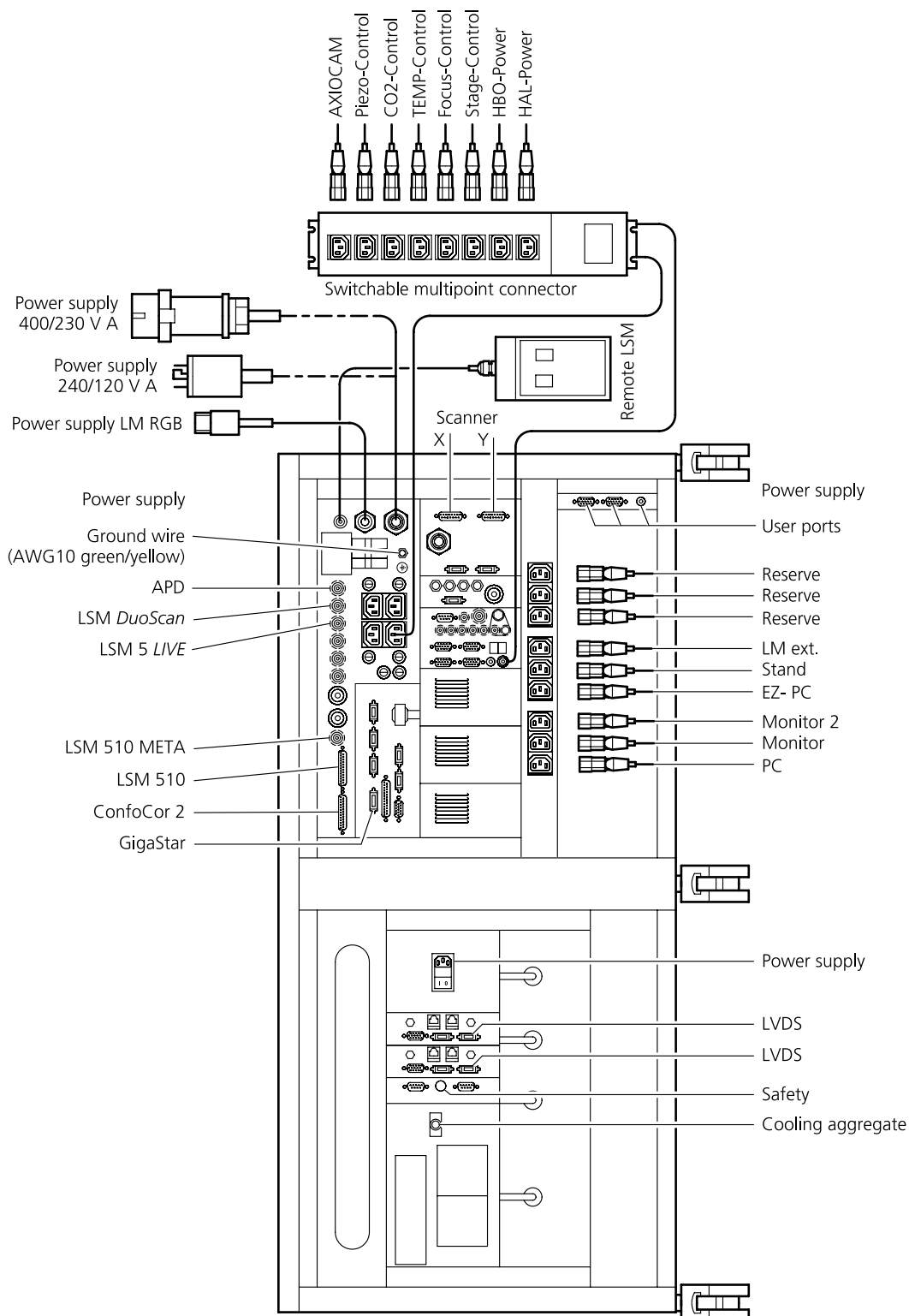
## 2.8 Power Requirements

 The LSM 5 *LIVE* 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.

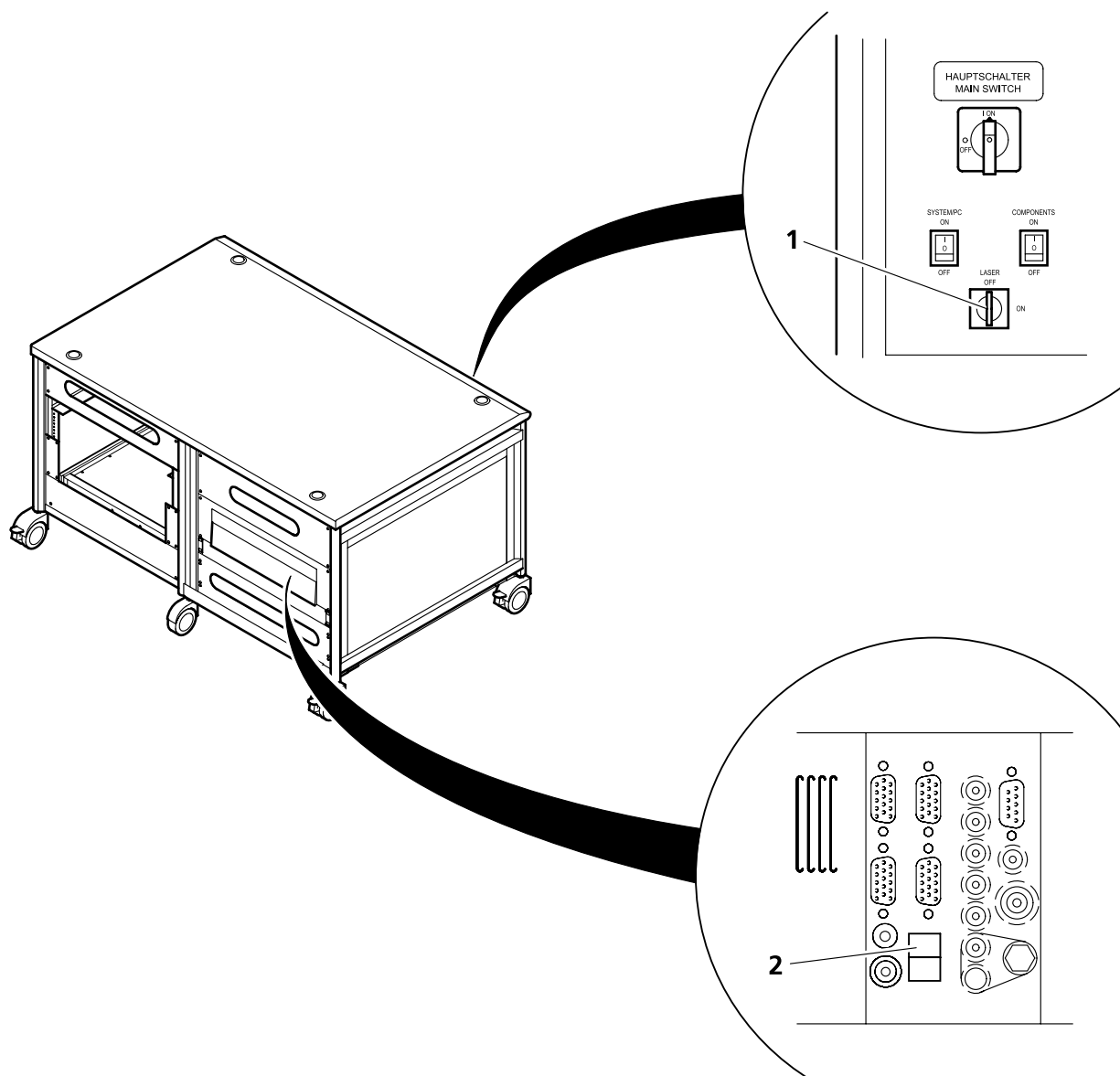
A suitable grounding point must be installed in the room.

Line voltage	220 ... 240 V AC ( $\pm 10$ %)	100 ... 125 V AC ( $\pm 10$ %)
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 (LSM <i>DuoScan</i> only)		
– Line Voltage	208...240 V AC ( $\pm 10$ %) 50 / 60 Hz	208...240 V AC ( $\pm 10$ %) 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. 2-5 Multipoint connectors**





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

**Fig. 2-6 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.

**2.9 Physical Dimensions**

	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
<b>Passively damped anti-vibration table</b>	130	100	75	137
<b>Scanning Module LSM 5 <i>LIVE</i></b>	55	17	30	19.5
<b>Scanning Module LSM <i>DuoScan</i></b>	39	15	13	7
<b>Microscope</b>	50	35	50	20
<b>Laser Module <i>LIVE</i></b>	66	52	22	58
<b>Laser Module UV *(<i>DuoScan</i> only)</b>	140	20	20	60
<b>Plug-in unit external laser (UV)*</b>	66	52	22	9
<b>System Electronic Rack</b>	110	70	58	90
<b>Power supply for Ar (UV)*</b>	50	50	30	30
<b>Cooling unit for Ar (UV)*</b>	80	45	50	30
<b>Water hose for Ar (UV)*</b>	700			
<b>Fiber optic cable, UV*</b>	300			
<b>Fiber optic cable, VIS(ible)</b>	300			
<b>Cables</b>	350			

**2.10 Dimension of Shipment Crates**

Crate containing	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
<b>Passively damped anti-vibration table</b>	145	115	115	150
<b>System Electronic Rack and Laser module</b>	135	90	100	300
<b>LSM, Microscope, Computer</b>	135	90	100	150
<b>Additional Hardware Components</b>	135	90	61	100
<b>UV laser unit</b>	125	55	50	100
<b>UV cooling unit</b>	120	60	90	50

## 2.11 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

## 2.12 Vibrations

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

---

## 2.13      **Microscopes**

Inverted Axiovert 200 M SP

Upright Axioskop 2 FS MOT

Upright Axio Imager.Z1

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 SP)  
                          100 nm (Axioskop 2 FS MOT)  
                          10 nm (Axio Imager.Z1)

Piezo Objective focus

Piezo-driven single objective drive

Max. travel 250  $\mu\text{m}$ ; resolution 5-10 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.

**2.14 Scanning Module LSM 5 LIVE**

Scanners	2 individually driven galvanometric scanners
Scanning speed	Up to 120 frames/sec (512 × 512 pixels)
Field resolution	Max. 512 × 2048 pixels (optional adjustable for each axis)
Field of view	10 × 10 mm <sup>2</sup> with a 1.25× objective
Zoom	0.5× ... 2×, continuous control
Channels	Up to 2 channels simultaneously: Optional transmitted light detection mode
Dynamic range	12-bit DAC for each detection channel
Apertures	1 or 2 individual variable aperture Computer controlled automatic alignment

**2.15 Laser Module LIVE**

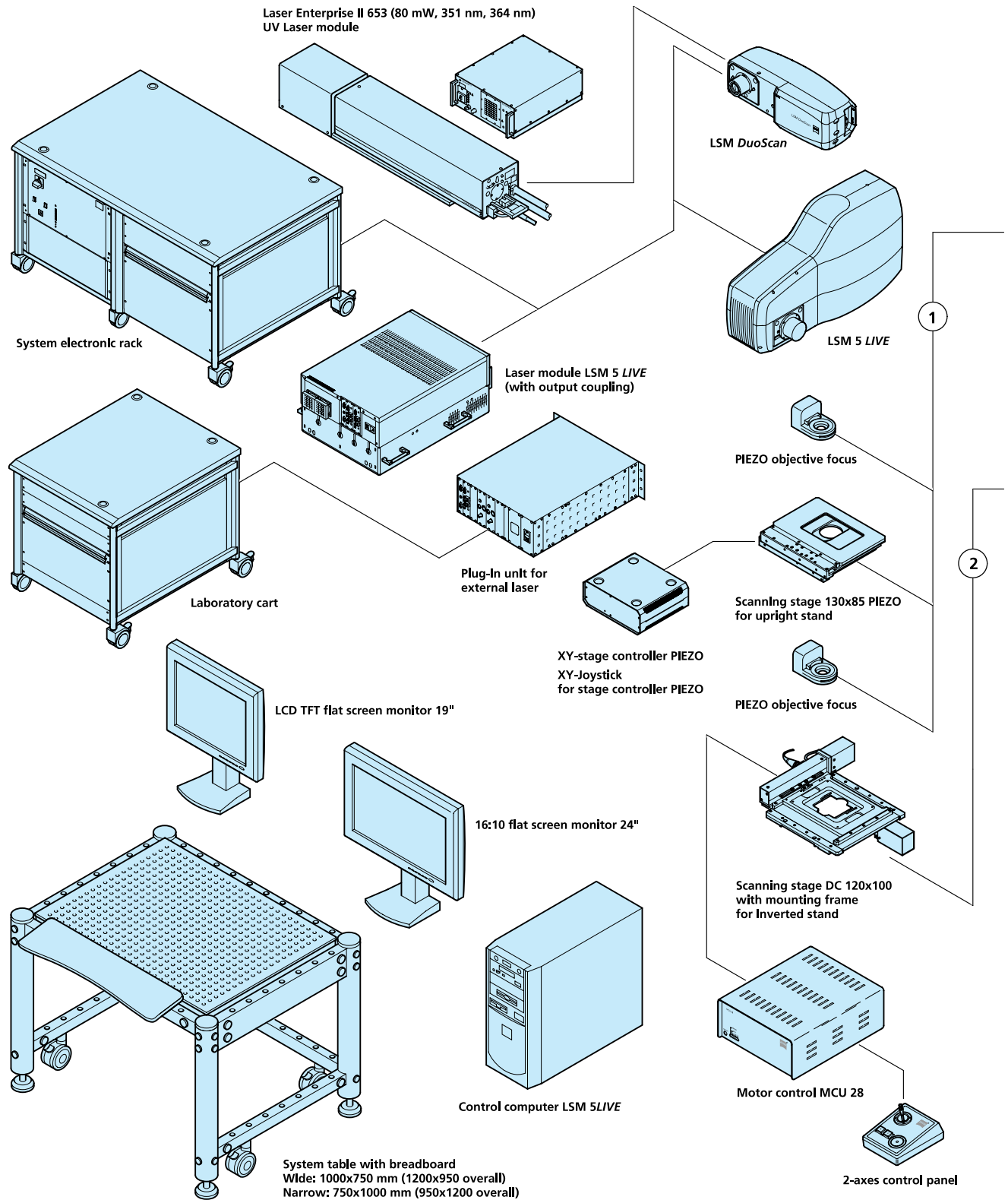
Single-mode polarization preserving fiber  
Laser beam attenuation (AOTF) for all lasers  
Diode laser (405 nm, 50 mW)  
Diode laser (440 nm, 16 mW)  
OPSS laser (488 nm, 100 mW)  
DPSS laser (532 nm, 75 mW)  
Diode laser (635 nm, 35 mW)

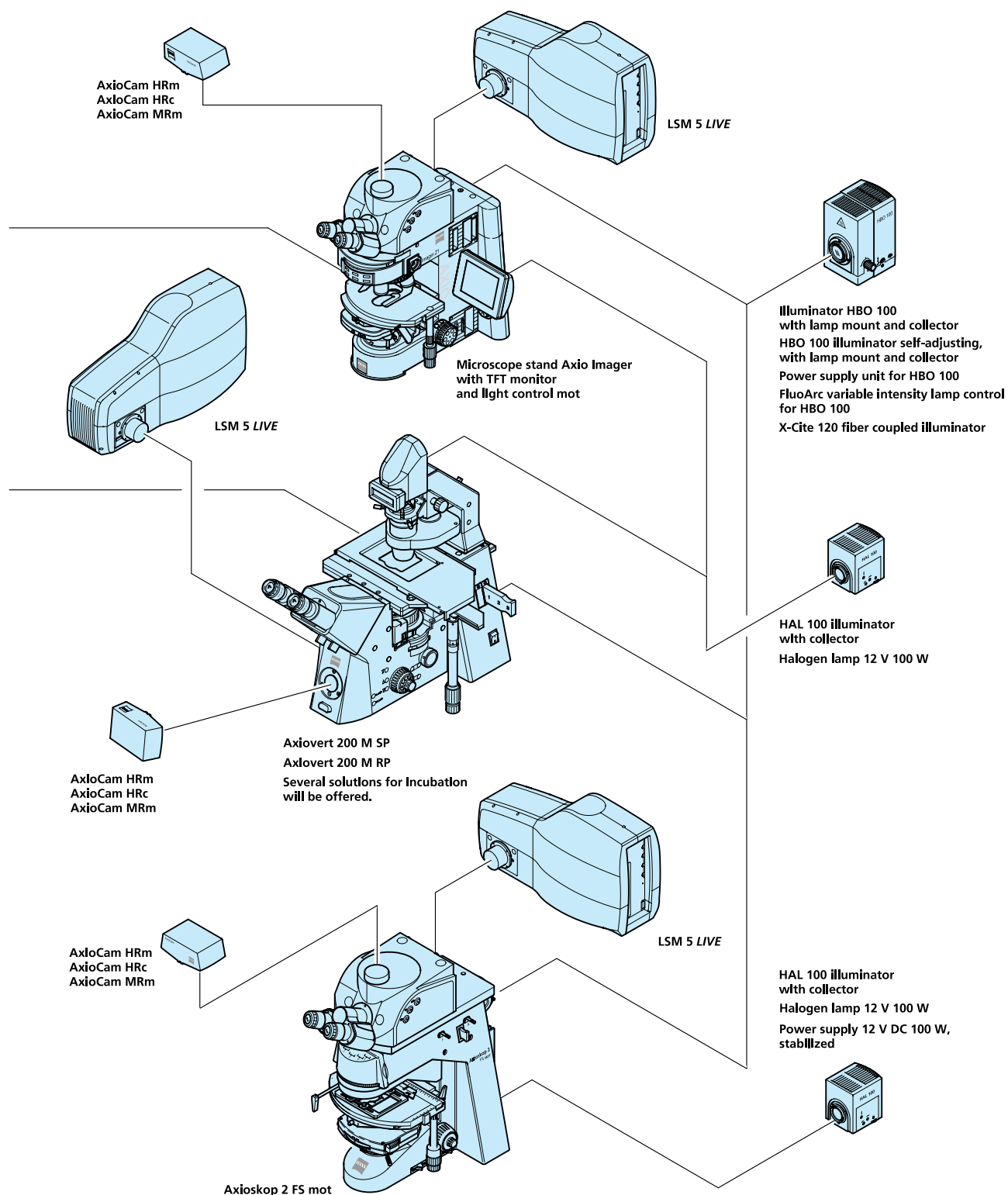
In the unlikely case of extreme fluctuations of the external power net, the laser diode will switch off or the laser power might vary. Note that this is not a defect and the laser will not be damaged.

**2.16 Laser Module UV for LSM DuoScan UV**

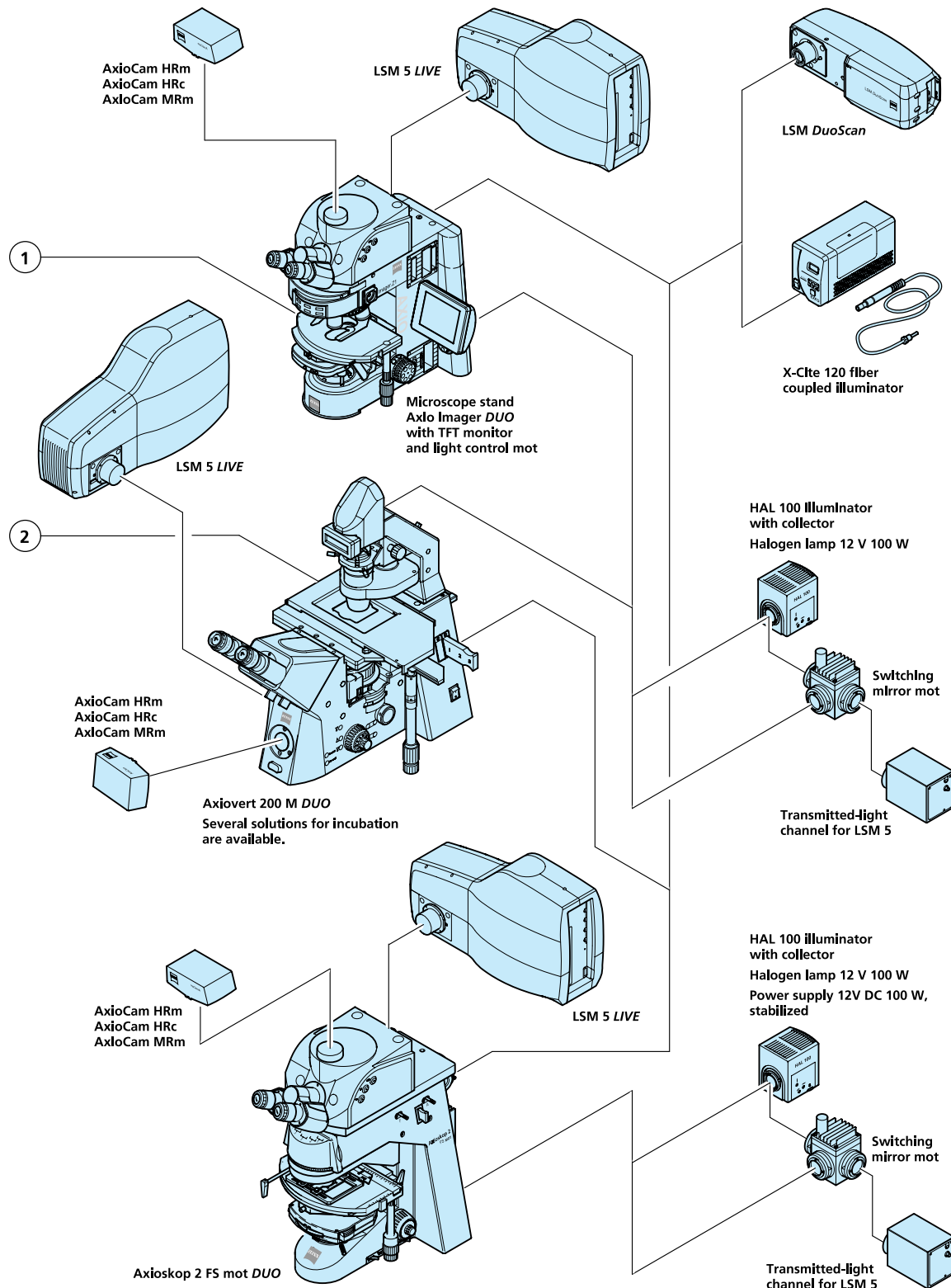
Single-mode polarization preserving fiber  
Laser beam attenuation (UV-AOTF)  
Ar UV Laser (351/364 nm, 80 mW)

## 2.17 System Overview LSM 5 LIVE





## 2.18 System Overview LSM 5 LIVE DuoScan







## **CHAPTER 3      INTRODUCTION TO LASER SCANNING MICROSCOPY**

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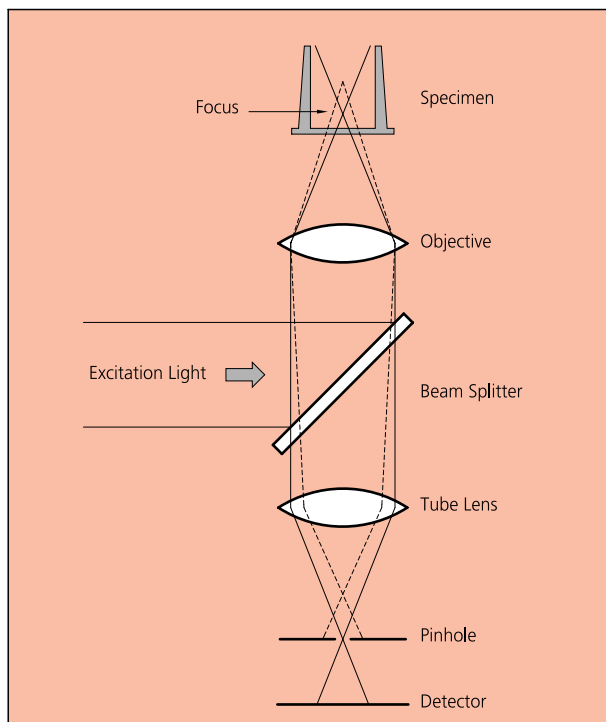
### 3 INTRODUCTION TO LASER SCANNING MICROSCOPY

#### 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 is therefore, 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**

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  $\mu\text{m}$  can be resolved, and height differences of less than 0.1  $\mu\text{m}$  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.

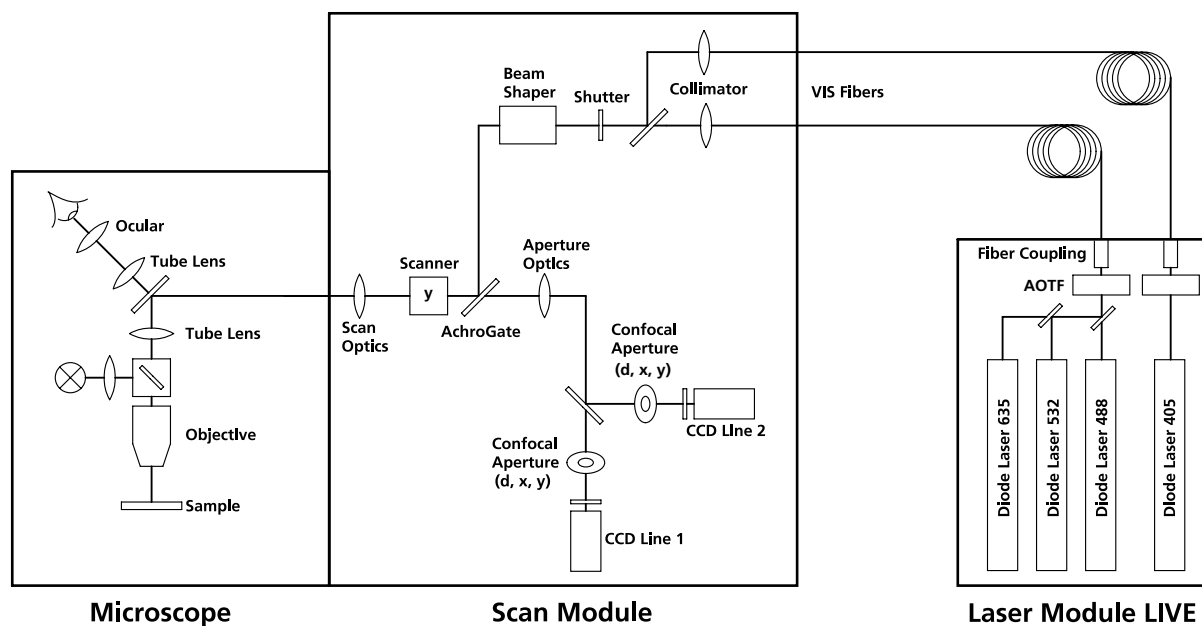
- **Physiology** (option)

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 5 *LIVE* (Schematic)**

AOTF Acousto-Optical Tunable Filter

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

The diagram above is a schematic representation of the LSM 5 *LIVE* 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 Y scanner onto the AchroGate beam splitter, which separates the emissions from the excitation light. The fluorescences are separated from each other by a Secondary Dichroic Beam Splitter and directed to individual photodetectors (CCD Line1 and CCD Line2).

---

## 3.4 Performance Features of the LSM 5 LIVE

### 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 SP) or upright (Axioskop 2 FS MOT, Axio Imager.Z1, **Axio Imager.M1**) 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.

For the VIS (visible-light) Laser Module, the user can select from up to four lasers with wavelengths of 635, 532, 488, 440, and 405 nm. Coupling of the laser light is through polarization-preserving single-mode optical fibers. One beam collimator for the visible ranges provides optimum adaptation of the respective laser wavelength to the objective used and, thus, optimum correction for Z aberrations.

The two simultaneous image acquisition channels, usable for fluorescence are ideal for the investigation of multiple fluorescence specimens. In the two channels, the diameters of the aperture and their XY positions can be optimized, and the desired emission filter placed into the beam path, by servo-motor control. In the simultaneous registration of multiple fluorescences, 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.

It is possible to superimpose a multiple fluorescence image on a brightfield, differential interference or phase image.

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...700 nm spectral range with the same high quality, producing brilliant images.

A mirror scanner system, controlled by **Realtime Electronics**, offers several advantages. The large deflection angle of the scanning mirrors allows a wide area to be scanned. With a 1.25x objective, the object area scanned is 10 x 10 mm<sup>2</sup>.

The scanning field size can be freely selected between 4 x 1 and 512 x 2048 pixels.

It is possible to carry out fastest 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 120 frames/sec (at 512 x 512 pixels); if two different lasing wavelengths are used for the scanning, two fluorochrome dyes can be viewed and documented in a quasi-simultaneous mode. This will absolutely prevent "bleeding".

### 3.4.2 Microscope Equipment of the LSM 5 *LIVE* System

The LSM 5 *LIVE* system is equipped either with the inverted Axiovert 200 M SP microscope or with the upright Axio Imager.Z1, Axioskop 2 FS MOT or Axio Imager.M1 microscopes.

Referring to the delivered operating manual "Axiovert 200 M" only differences to this manual will be explained.

#### (1) Stand

**a)** The motorized objective nosepiece 5x H DIC is firmly fixed to the stand, where no operating elements can be found for the nosepiece. Operation will be done LSM 5 software controlled. The "Restriction of revolver height to protect the objectives when changing the objectives motorized" 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.

**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 LSM-mode the analyzer must **not** be located in the beam path, analyzer rod must be pulled out.

**f)** The stands dispose of five additional ports: two side ports, front ports and base ports.

The side port or the front is equipped with the LSM 5 special interface, one of the others with the TV interface. The LSM 5 *LIVE* scanning module can be mounted to the special interface port. Different camera systems can be adapted to the TV interface using the TV adapters 452982/83/92/94/95/97/98-0000-000.

#### (2) Specimen stages and fine focus drives

**a)** Mechanical stage

The stage must be mounted with the coaxial drive 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 back. A complementary shutter built-in the stand prevents laser light from reaching the eye pieces during scanning mode.

**b)** The illuminator support is equipped with a rotatable polarizer. The Axiovert 200 M description contains the adjustment for DIC mode during conventional observation.

For scanning 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 is not allowed be located in the beam path because of the laser light already is polarized.

**c)** On the illuminator support as an option there is mounted a LSM 5 software controlled switching mirror fully motorized. Alternatively the light is directed to the LSM 5 T-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 to halogen lamp and condensers you will find in the Axiovert 200 M operating manual.

### **(4) Reflected light fluorescence**

All Axiovert 200 M fluorescence accessories exceptional the reflector slider can be used.

Further information you will find in the Axiovert 200 M operation manual.

### **(5) Imaging optics**

Optovar sliders are not usable.

The analyzer for 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 hasn't have an integrated SLR-port, but microscope cameras, as described in the Axiovert 200 M operation manual, can be used.

### **(7) TV adaptation**

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

The TV interface side port or base port can be used with TV adapters 44 or LSM adapters.



### 3.4.3 Computer Hardware and Software

The LSM 5 *LIVE* is controlled through a standard high-end Pentium PC. Linking with the electronic control system is via an ultrafast 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; this way, complete application programs can be loaded and executed by pushbutton control.

The software of the LSM 5 *LIVE* 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 a enormously wide range of image processing functions, including all standard 2D/3D (stereo, projection) functions same as 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

<b>Bleach series</b>	When doing bleach series on LSM <i>DuoScan</i> 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.
<b>Macro</b>	When using the modify images macro to convert time series data into Z stacks, an arbitrary section step of 1 $\mu\text{m}$ is applied to the stack. This will also lead to an arbitrary time stamp when reconvert the Z stack into a time series. The order of the images however is preserved under all conditions.
<b>Fast Z-stack and line scan acquisition</b>	When acquiring fast Z-stacks with line scan mode, the starting point of the stacks and the vertical dimensions can differ depending on uni- or bidirectional scanning mode. This is a mechanical effect which increases with rising line scanning speed. Avoid extremely fast speeds in the hundred(s) of frames per second range when acquiring Z-stacks.
<b>Piezo objective focus units</b>	The piezo objective focus units 1325-210, 1325-212 and 1325-213 with an increased travel range of 250 $\mu\text{m}$ 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

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## 4 OPERATION

### 4.1 Purpose

This chapter describes the operation of the LSM 5 *LIVE* Laser Scanning Microscope 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, Axiovert 200 M and Axioskop 2 FS MOT 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 5 *LIVE* 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.

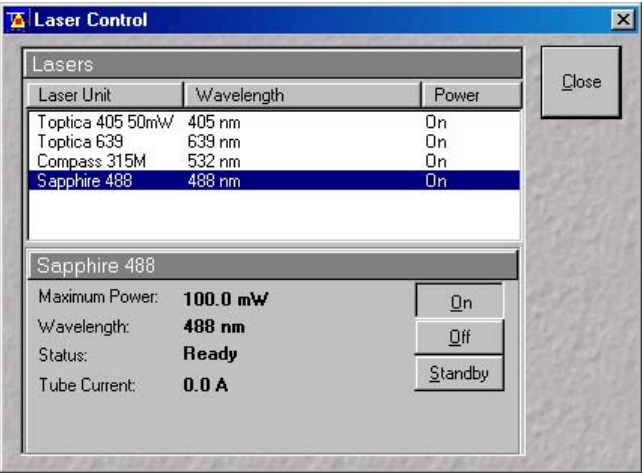
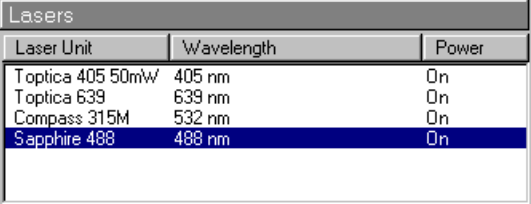
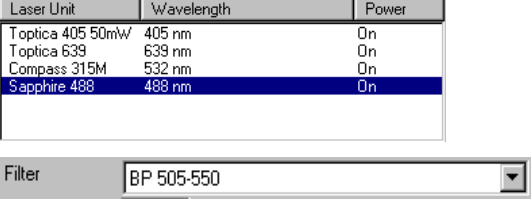




 The screen layout used in this manual is based on the former Windows ® standard layout and may vary from yours.

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
	<p>Window (e.g.: <b>Laser Control</b> window)</p> <ul style="list-style-type: none"> <li>Window displayed after activation of a function button (e.g.: <b>Laser</b> button in the toolbar of the <b>Main</b> menu).</li> </ul>
	<p>Panel (e.g.: <b>Lasers</b> panel)</p> <ul style="list-style-type: none"> <li>Limited function range within a window</li> </ul>
	<p>List box or selection box</p> <ul style="list-style-type: none"> <li>Selection of one of the displayed options at a click of the mouse.</li> <li>Open the box by clicking on the arrow button.</li> </ul>
	<p>Input box</p> <ul style="list-style-type: none"> <li>Input of text or numeric values via the keyboard.</li> </ul>
	<p>Scrollbar with slider</p> <ul style="list-style-type: none"> <li>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 <b>Shift</b> or <b>Ctrl</b> key while clicking on the arrow button to change the numeric values in coarse or fine steps.</li> </ul>
	<p>Check box</p> <ul style="list-style-type: none"> <li>Activates / deactivates setting options.</li> </ul>
	<p>Button</p> <ul style="list-style-type: none"> <li>Selection / performance of a function via mouse click.</li> </ul>

### 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 \ \*.krm
- Pibhole (aperture 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 (aperture) setting: AIM \ \*.set
- Parameter file after pinhole (aperture) 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 5 *LIVE* 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 lasers 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).

 Note that the full performance and correct adjustment of all internal elements is achieved after a warm-up time of 40 min.!

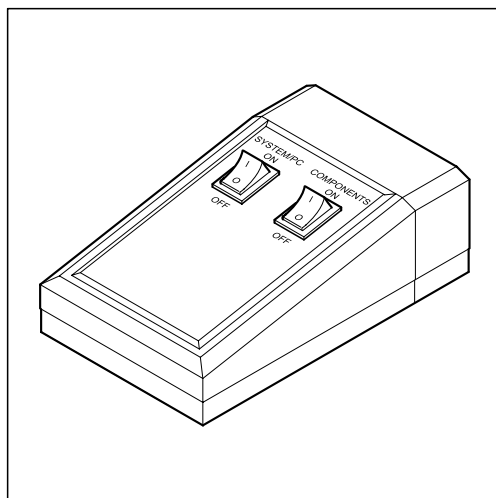



Fig. 4-1 REMOTE CONTROL switch

- 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 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 TOOLS.



LSM 5 Live

### LSM 5 LIVE icon

Start the LSM 5 software program for the LSM 5 *LIVE* laser scanning microscope.

#### 4.2.1 Switching on the HBO 100

- If the HBO 100 is required, switch it on via the toggle switch of the HBO 100 power supply.

#### 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 5 LIVE** icon on the desktop of WINDOWS to start the LSM 5 software program (Fig. 4-2).
    - The **LSM 5 LIVE Switchboard** window appears on the screen (Fig. 4-3).



Fig. 4-2 Starting the LSM 5 software

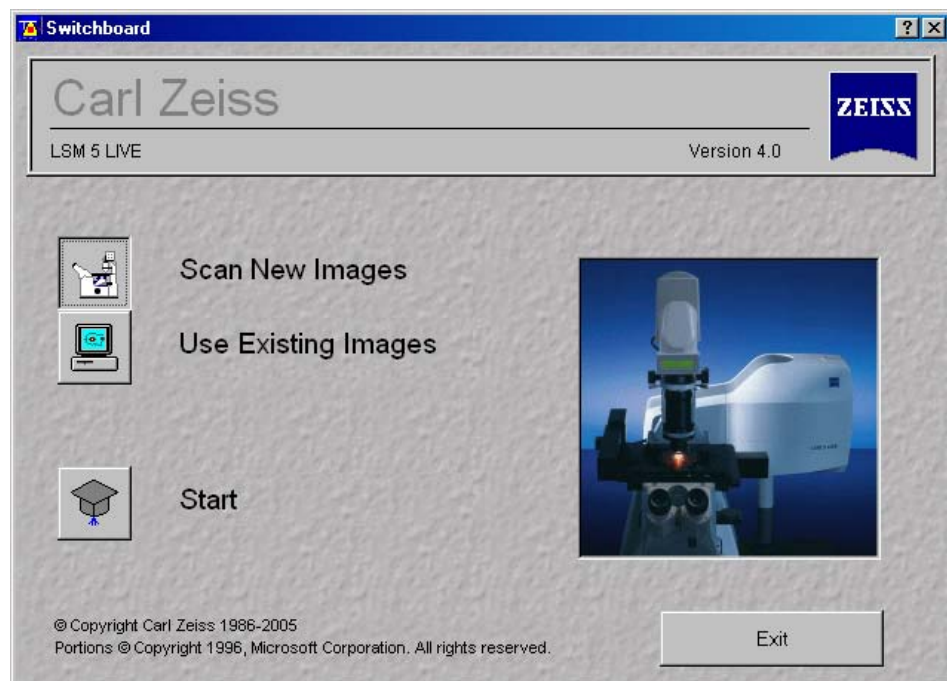


Fig. 4-3 LSM 5 LIVE Switchboard menu



**Online Mode**

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



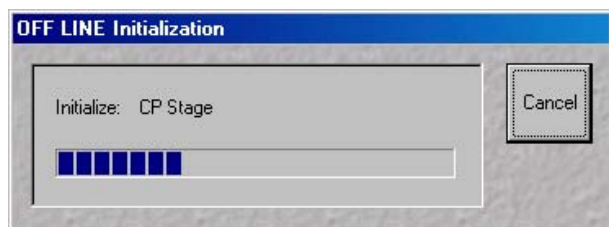
Please note that the **Online Mode** button must be activated before clicking the **Start** button. Otherwise, the hardware can not be controlled by the LSM 5 software.

**Offline Mode**

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**

Use of this button starts the software system.



**Fig. 4-4** OFF LINE Initialization window

After the start, 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 (**Online Mode Images** or **Offline Mode**), initialization is performed in the offline or online mode.



Some printers (for example KODAK Thermo Printer) will produce an error message "hard key not found" in case the printer is not switched on.

Remedy: turn on the printer before starting the LSM 5 software.


Don't switch off the KODAK printer during the scanning process.

## 4.3 Main Menu

The major functions can be selected in the **Main** menu 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 clicking **Start**.

 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** button in the **LSM 5 LIVE Switchboard** window.
  - The LSM 5 LIVE **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.

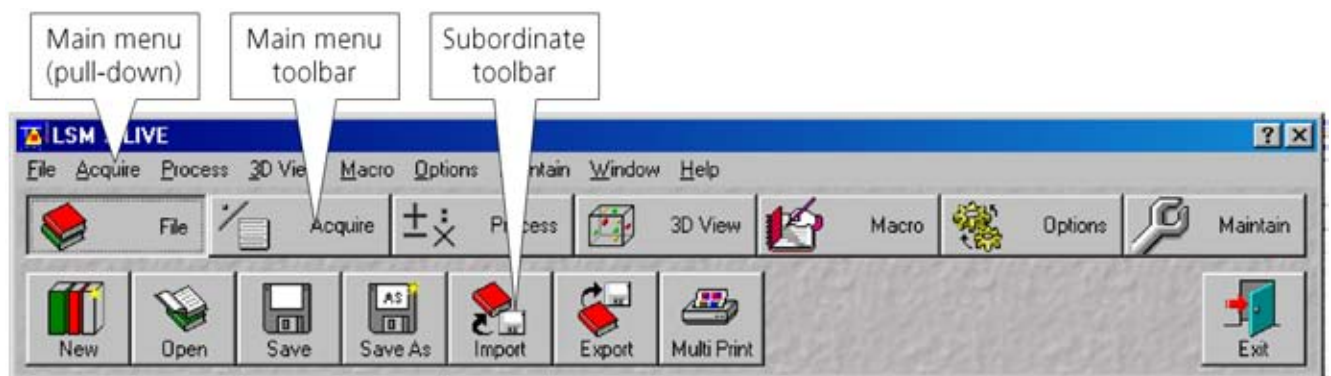
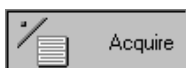


Fig. 4-5 LSM 5 LIVE Main menu



**File** button

Open, save, import and export of image data. Printing individual or several images on one page. Ending (Exit) the **Main** menu.



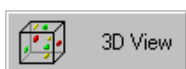
**Acquire** button

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

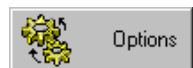
Used for three-dimensional reconstruction.



Macro

**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

**Options** button

For custom-configuration of software and hardware options.

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

**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 **Main** menu 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.

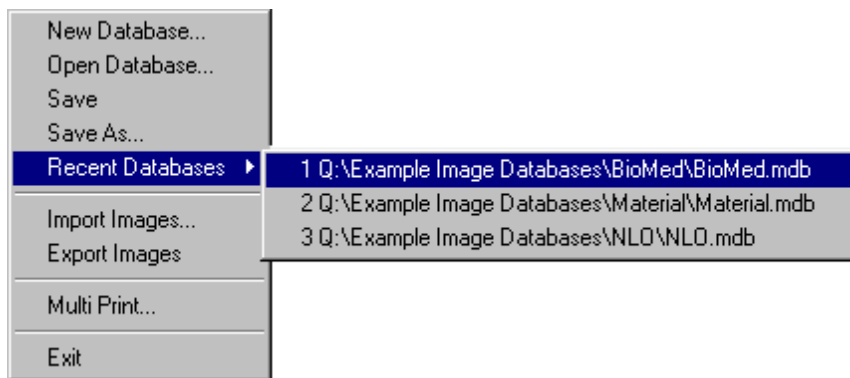


Fig. 4-6 File menu

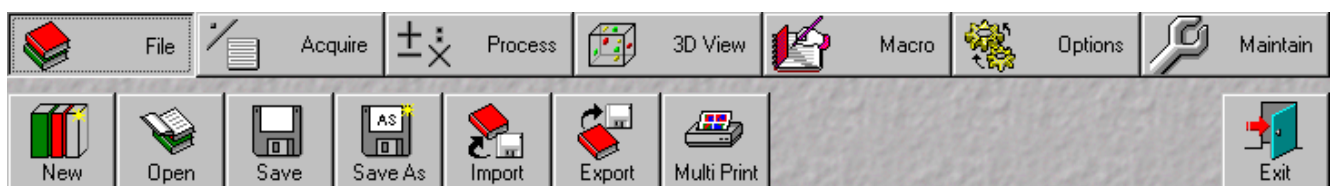
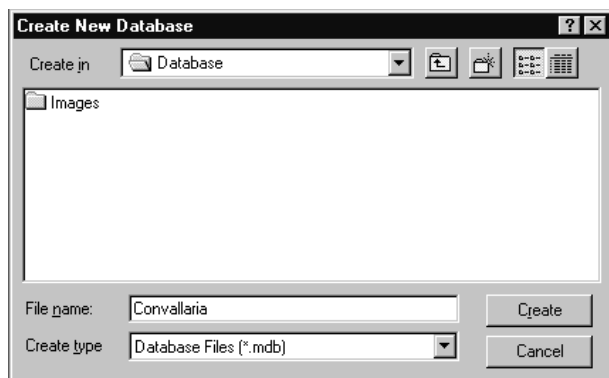


Fig. 4-7 Subordinate File menu toolbar




**Fig. 4-8 Create New Database window**

#### 4.4.1 Create New Image Database

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 .
- 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 4-19).

The new image database can be used to store an acquired or processed image (see **Saving an Image**, page 4-29).

 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 4-200).

#### 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 4-224).

- 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.

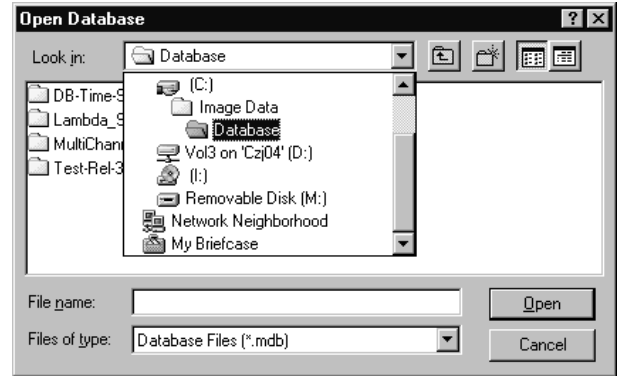



Fig. 4-9 Open Database window

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.

---

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

<b>Total: ...</b>	Display of storage volume of the entire image database
<b>Selected: ...</b>	Display of storage volume of the selected image(s)
<b>Current: ...</b>	Display of storage volume of the current image
<b>Clipboard: ...</b>	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.

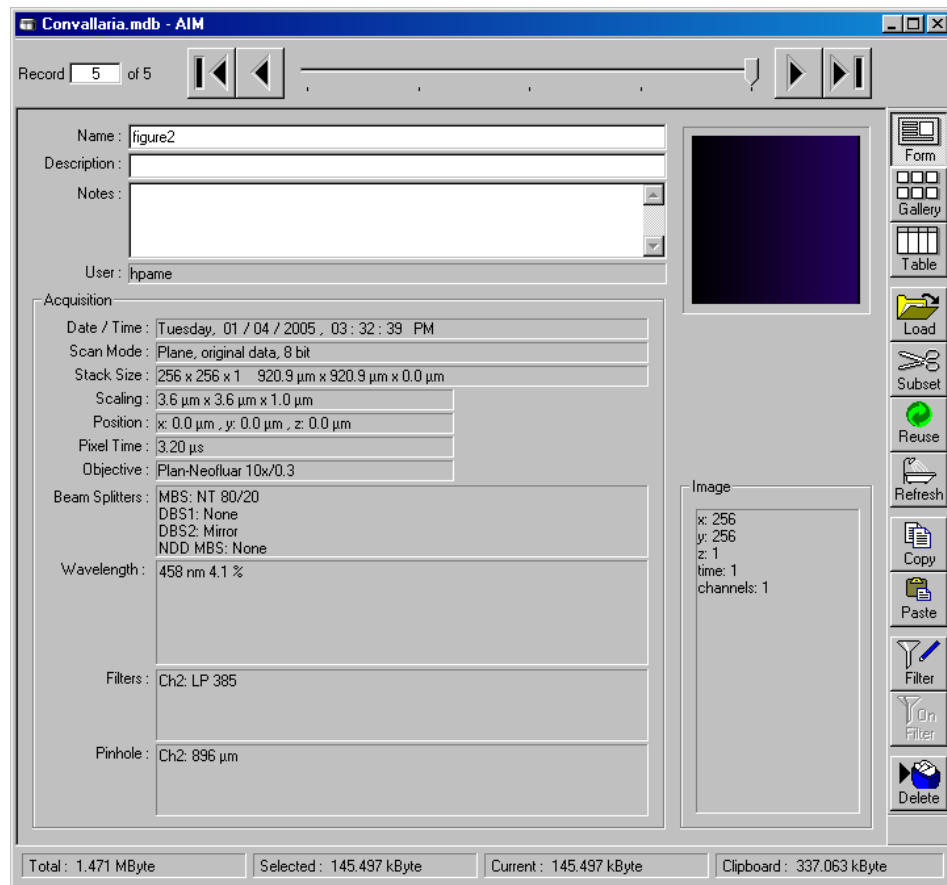


Fig. 4-10 Image Database window (Form display mode)

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 4-224.

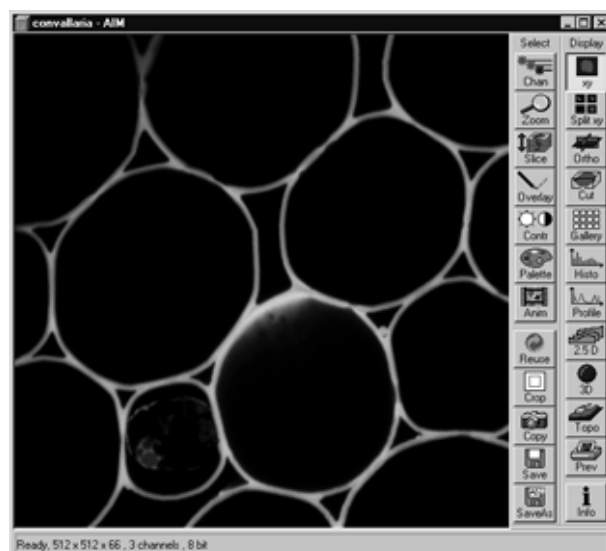
The name of the image is displayed in the **Name** input box of the **Image Database** window.

- 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.



**Fig. 4-11** Opened image displayed in the Image Display window

#### 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.

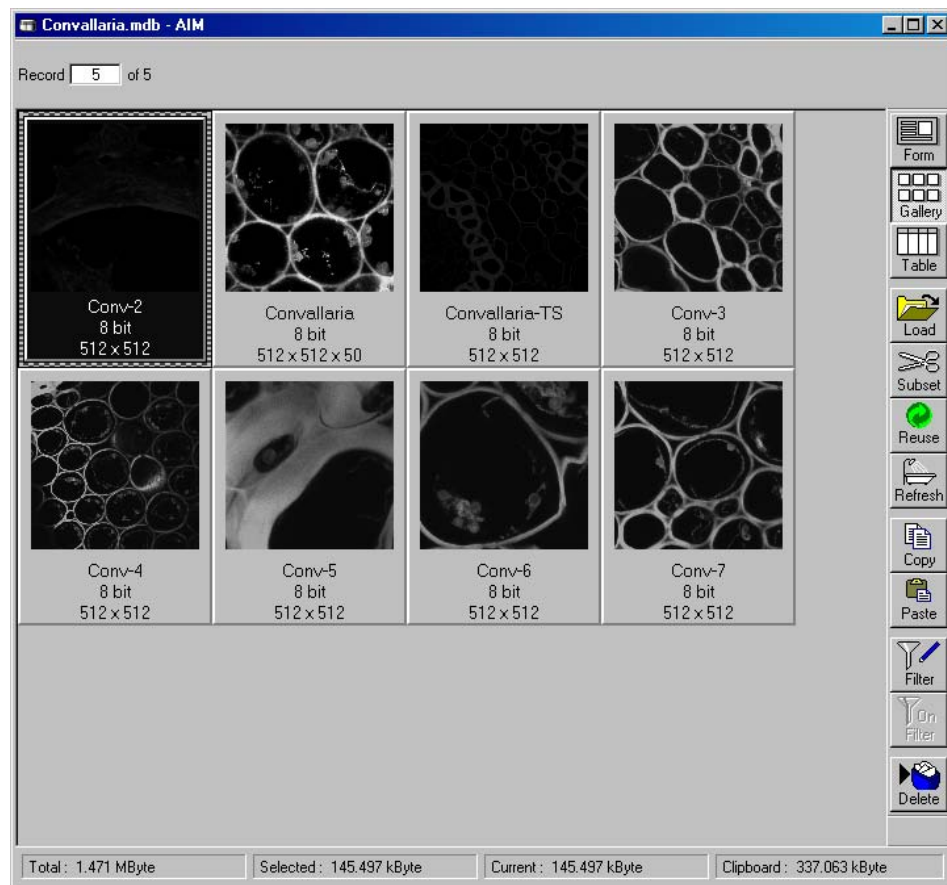


Fig. 4-12 Database window (Gallery display mode)

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.


Name	Date / Time	Scan Mode	Pixel Depth	Stack Size (Pixel)	Stack Size (µm)
figure1	01/04/2005, 02:52:48 PM	Plane, original data	8 bit	512 x 512 x 1	920.9 µm x 920.9 µm
figure2	01/04/2005, 03:29:36 PM	Plane, original data	8 bit	256 x 256 x 1	920.9 µm x 920.9 µm
figure2	01/04/2005, 03:31:56 PM	Plane, original data	8 bit	256 x 256 x 1	920.9 µm x 920.9 µm
figure2	01/04/2005, 03:32:39 PM	Plane, original data	8 bit	256 x 256 x 1	920.9 µm x 920.9 µm

Total : 1.471 MByte    Selected : 145.497 kByte    Current : 145.497 kByte    Clipboard : 337.063 kByte

Fig. 4-13 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.

#### 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.

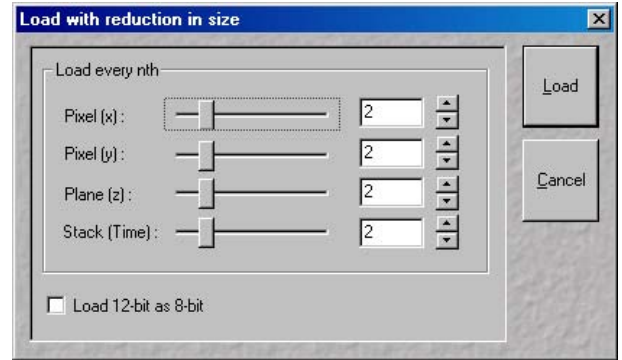


Fig. 4-14 Load with reduction in size window

#### 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.

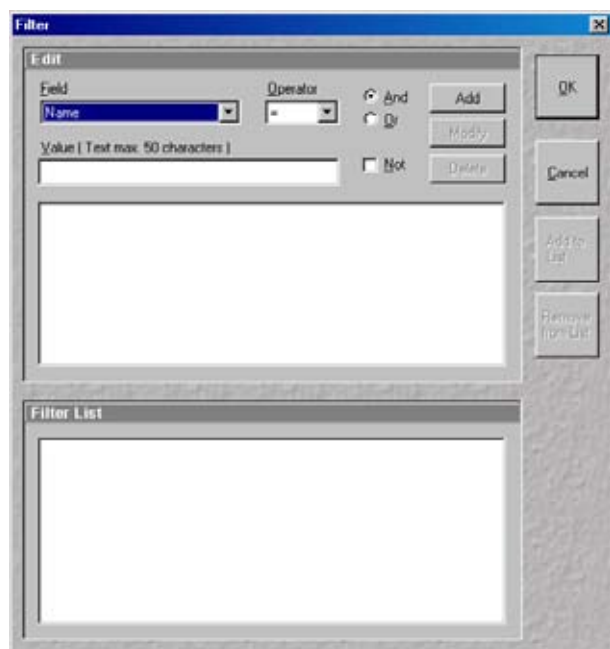


Fig. 4-15 Filter window

#### 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.

#### 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 $\mu\text{m}$
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**.

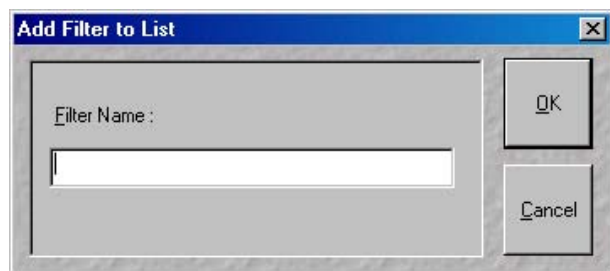


Fig. 4-16 Add Filter to List window

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.

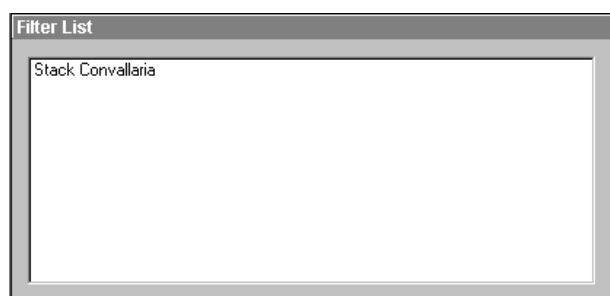


Fig. 4-17 Filter List panel

### 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.

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.

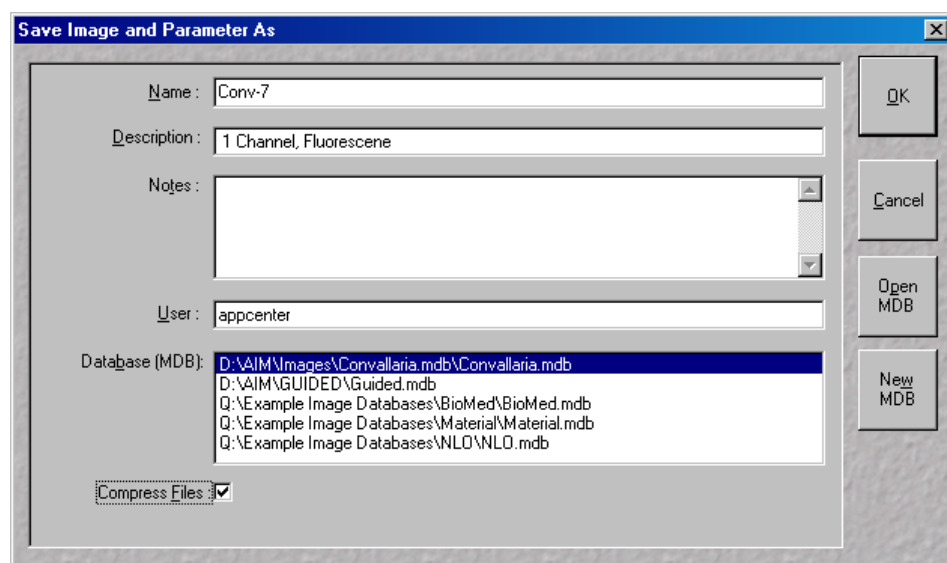
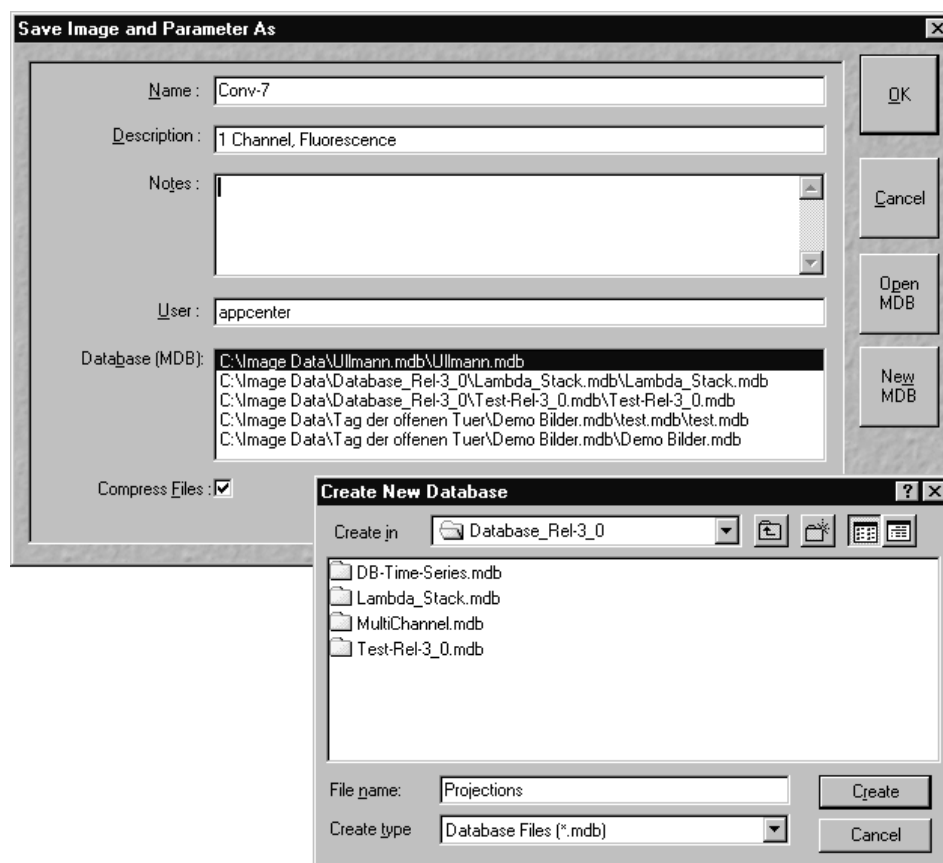


Fig. 4-18 Save Image and Parameter As window

- 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 4-18).
  - After creating the new database **Projections.mdb - AIM** window appears.



**Fig. 4-19** Save Image and Parameter As window and Create New Database window

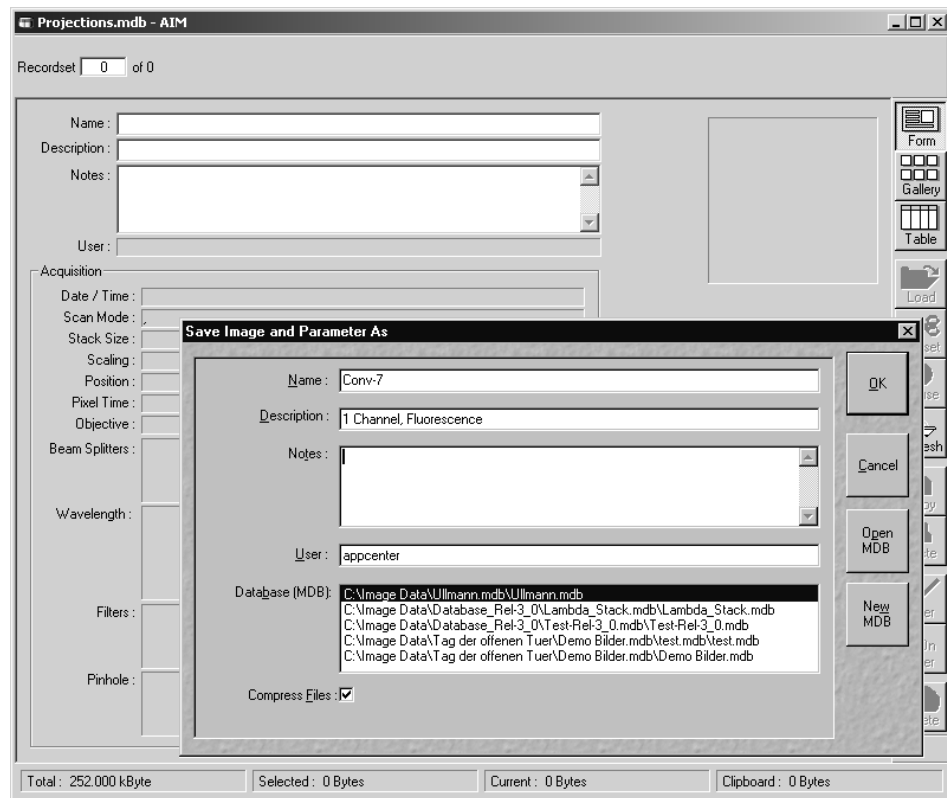


Fig. 4-20 Database window

- 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.

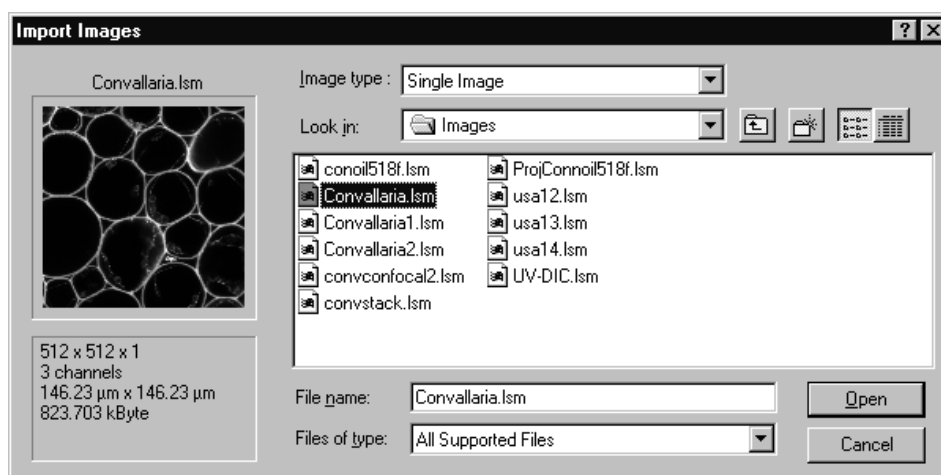



Fig. 4-21 Import Images window

- 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.

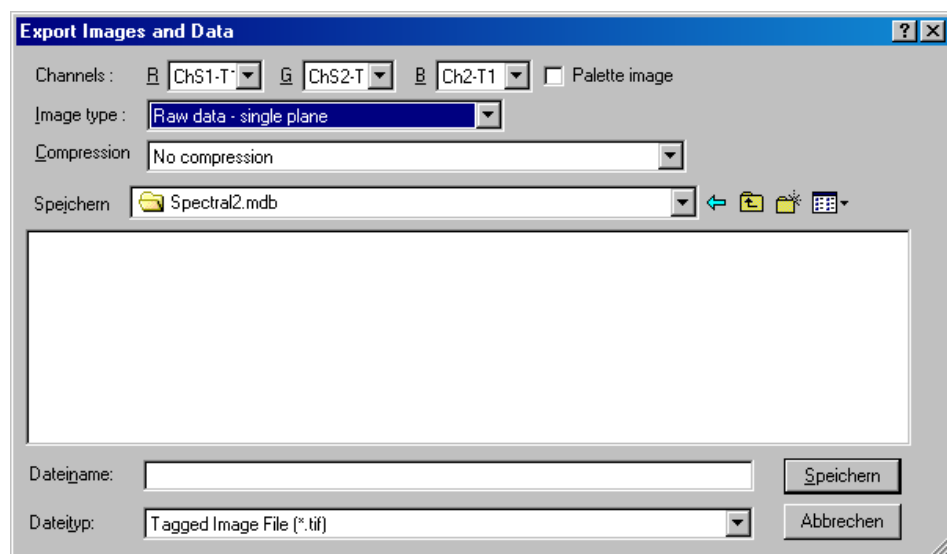
#### 4.4.6 Export of Images

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. 4-22** Export Images and Data window

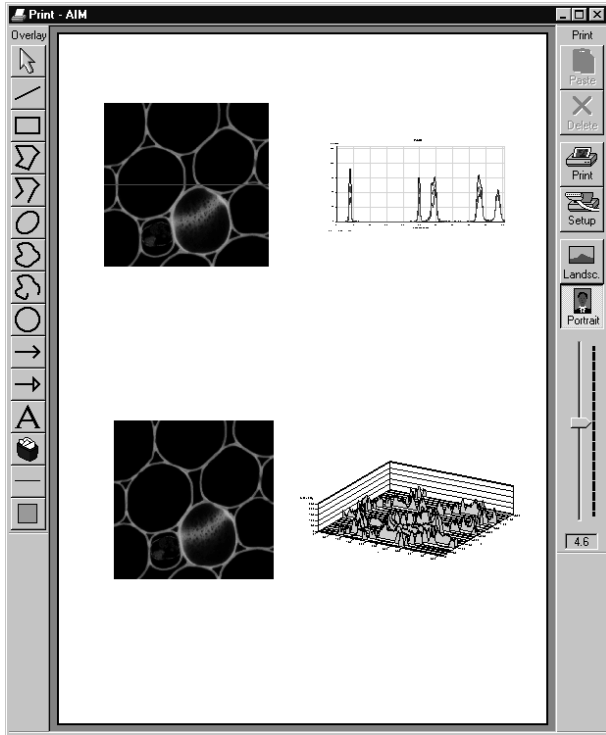


Fig. 4-23 Print - AIM window

#### 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:

<b>Paste</b>	Paste from clipboard to sheet.
<b>Delete</b>	Delete marked image.
<b>Print</b>	Start printing.
<b>Setup</b>	Printer setup.
<b>Landscape.</b>	Landscape paper orientation.
<b>Portrait</b>	Portrait paper orientation.
<b>Zoom slider</b>	Zoom function for page preview.

##### 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.



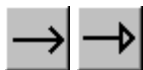
**Open free-shape curve** button: Creation of an open Bezier figure in the **Image Display** window.

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

- 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 5 LIVE **Main** menu will be closed and the **LSM 5 LIVE Switchboard** menu appears on the screen.

## 4.5 Acquire Menu

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

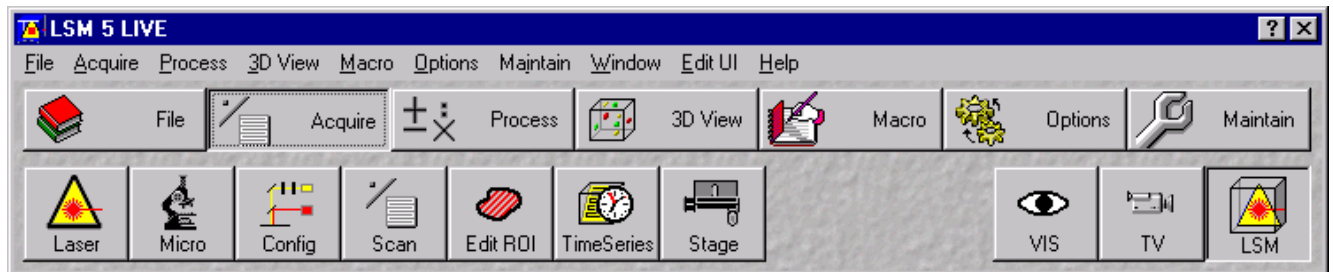



Fig. 4-24 Acquire menu

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 analyzed (option).
- **TimeSeries** permits user-specific time series to be selected for the scan procedure.
- 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.



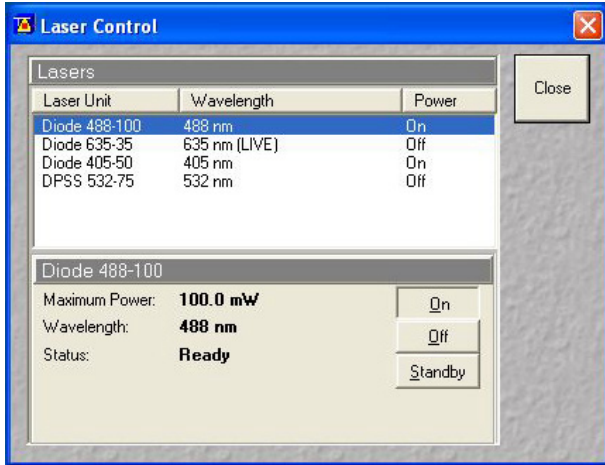


Fig. 4-25 Laser Control window

#### 4.5.1 Laser Control

The **Laser Control** panel shows the types, excitation wavelengths and operating status of all lasers available.

The subordinate laser settings panel shows the relevant and currently set **Maximum Power**, **Wavelength**, **Status** and **Tube Current** (only Sapphire 488) of the current laser.

The buttons **On**, **Off** and **Standby** permit the current laser to be set in the required status.

The name of the selected laser (Toptica ..., etc.) is displayed in the headline of this setting panel for checking.

##### 4.5.1.1 Switching on the Enterprise UV Laser

- If the UV laser is required, switch it on via the toggle switch (4-26/1) of the power supply.
  - It will be ready for operation after a few seconds.

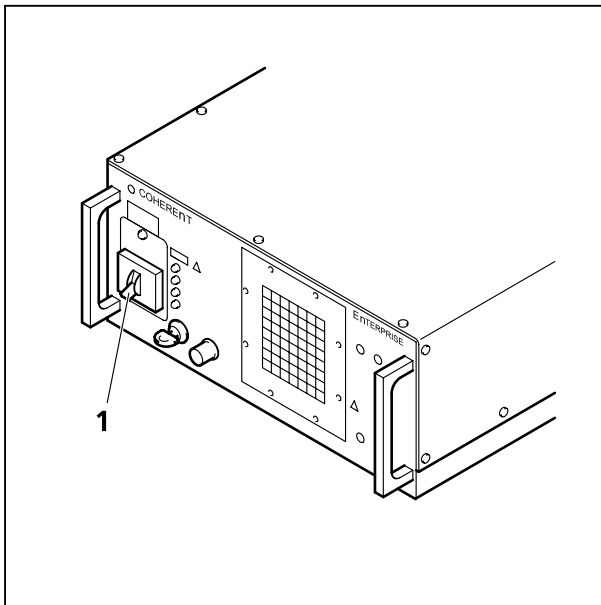


Fig. 4-26 Power supply of UV-laser

##### 4.5.1.2 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.

#### 4.5.1.3 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 and Status of the relevant laser.

#### 4.5.1.4 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 405 Diode laser (405 nm):**

- Click on the **On** button directly (**not** on **Standby**)

##### **This applies to the Toptica and the Sapphire lasers (635 / 488 nm):**

- 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.

##### **This applies to Compass 315M laser (532 nm):**

- 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 40 minutes.

##### **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.

## 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.

### 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 frame.
- 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.

<b>Reflected Light</b> button	The shutter is switched on and off. When using the X-Cite 120 additional controls are available. See below for description.
<b>Reflector</b> button	Push and click reflector cube can be selected via graphical pop-up menu.
<b>Objective</b> button	Objective can be selected via graphical pop-up menu.
<b>Condensor</b> button	Numerical aperture of the condensor is set via input box or slider. Turret position selected from graphical pop-up menu (only for motorized condensers). By clicking on the <b>Close</b> button the value is stored and the <b>Condensor</b> frame is closed.
<b>Field Stop</b> button	Opening of luminous-field diaphragm (transmitted and reflected light) can be set via input box or slider. By clicking on the <b>Close</b> button the value is stored and the <b>Field Stop</b> frame is closed.
<b>Filter</b> 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 <b>Close</b> button the value is stored and the <b>Filter</b> frame is closed.
<b>Aperture Diaphragm</b> button	Diaphragm opening is set via input box or slider. By clicking on the <b>Close</b> button the value is stored and the <b>Aperture Diaphragm</b> frame is closed.
<b>Transmitted Light</b> button	Transmitted light is switched on / off via <b>ON</b> button in the <b>Transmitted Light</b> 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 <b>3200 K</b> button in the Transmitted Light frame. By clicking on the <b>Close</b> button the <b>Transmitted Light</b> frame is closed.

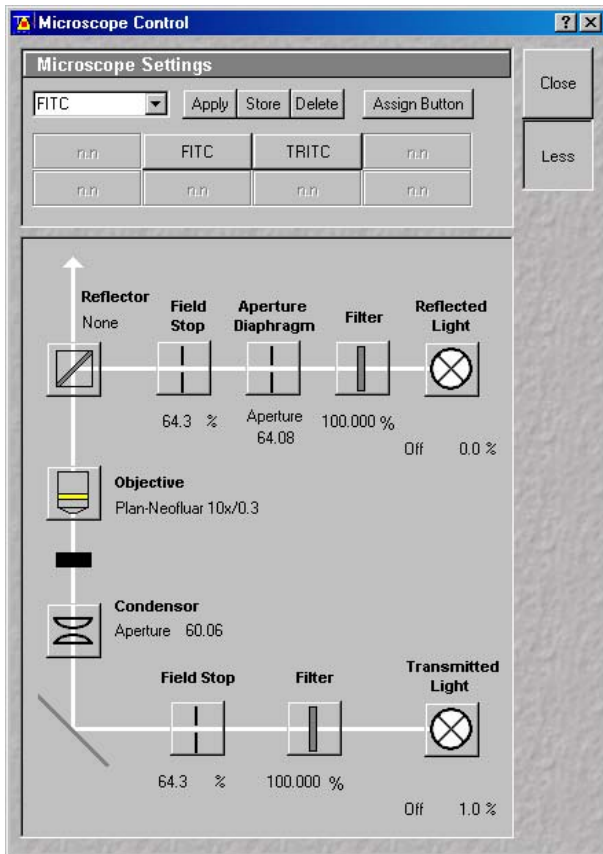


Fig. 4-27 Axio Imager.Z1 Control window

### Recording of microscope settings

The upper part of the **Axio Imager 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.

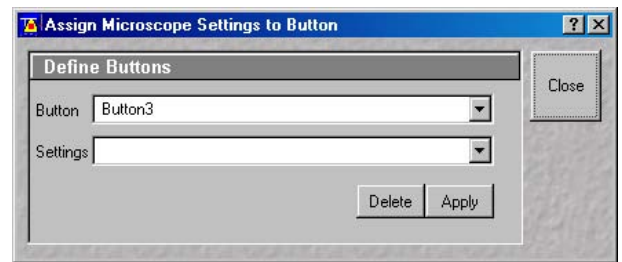



Fig. 4-28 Assign-Microscope-Settings-To-Button window

 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

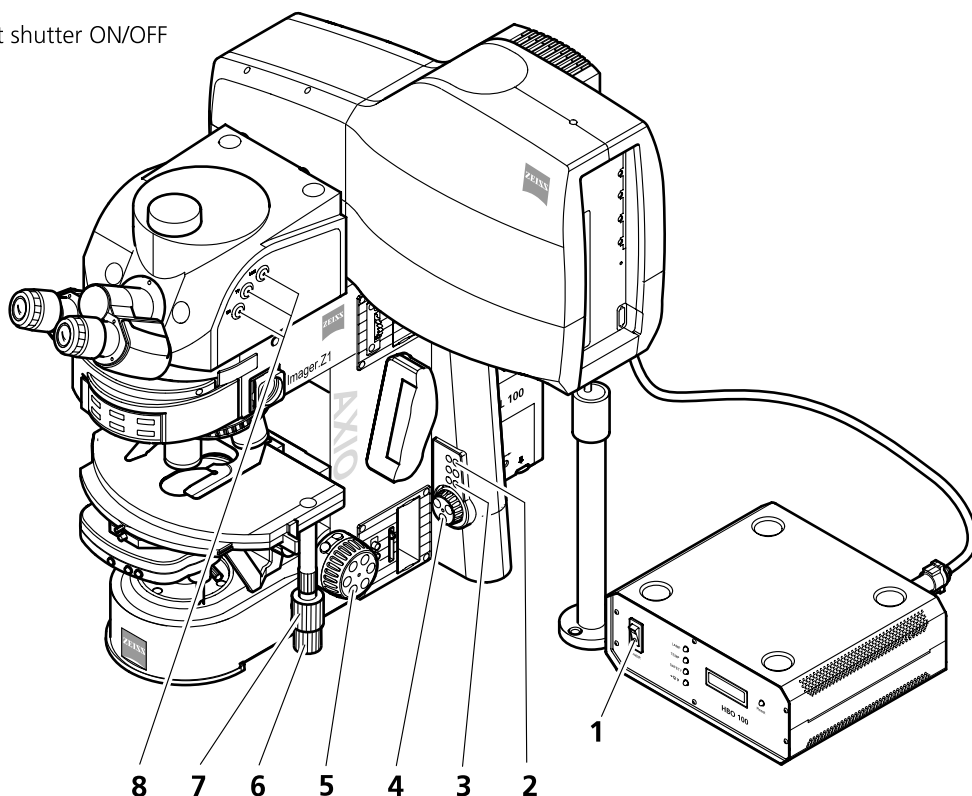


Fig. 4-29 LSM 5 LIVE with Axio Imager.Z1


### 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.

 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.

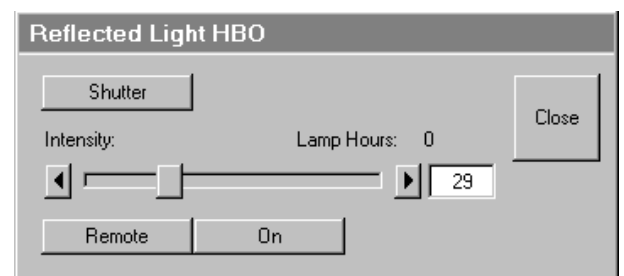



Fig. 4-30 X-Cite 120 control panel



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<b>Remote</b>	Enables the attenuation control via LSM Software. This is also enabled when clicking the <b>ON</b> button.
<b>ON</b>	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.
<b>Shutter</b>	Opens and closes the shutter in the reflected light path independent of the status of the internal lamp shutter and the attenuation.
<b>Intensity</b>	Sets the light via input box or slider.
<b>Lamp Hours</b>	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

<b>Transmitted Light</b> button	Transmitted light is switched on / off via <b>ON</b> button in the <b>Transmitted Light</b> 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 <b>3200 K</b> button in the Transmitted Light frame. The transmission light control potentiometer on the stand is disabled via the <b>Remote</b> button. By clicking on the <b>Close</b> button the <b>Transmitted Light</b> frame is closed.
<b>Condensor</b> button	Numerical aperture of the condensor is set via input box or slider. Turret position selected from graphical pop-up menu (only for motorized condensers). By clicking on the <b>Close</b> button the <b>Condensor</b> frame is closed.
<b>Objective</b> button	Objective can be selected via graphical pop-up menu.
<b>Reflector</b> button	Push and click, reflector cube can be selected via graphical pop-up menu.
<b>Tube Lens</b> button	Push and click, tube lens can be selected via graphical pop-up menu.
<b>Reflected Light</b> button	The shutter is switched on and off. When using the X-Cite 120 additional controls are available. See below for description.

---

## Recording of microscope settings

The upper part of the **Axiovert 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.

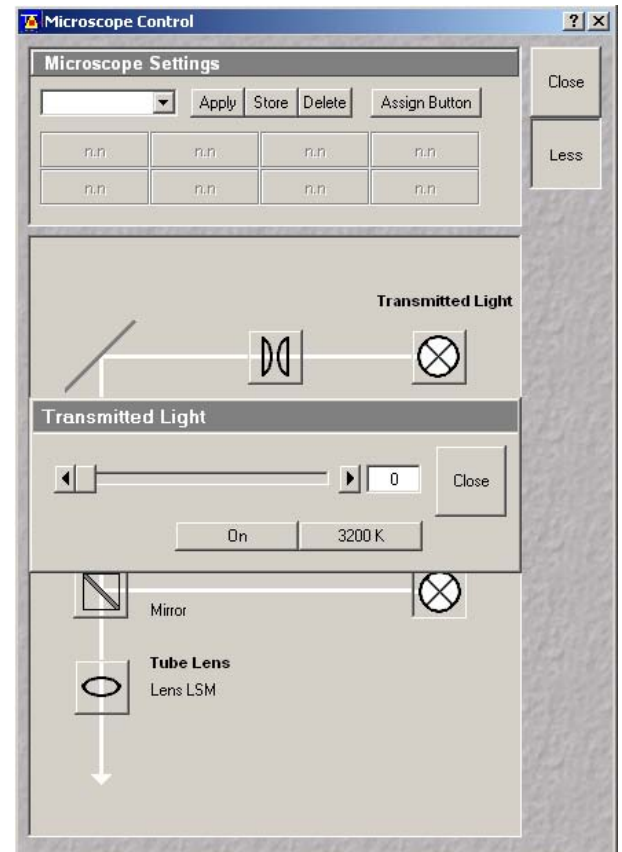


Fig. 4-31 Axiovert 200 Control window

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### 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.

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).

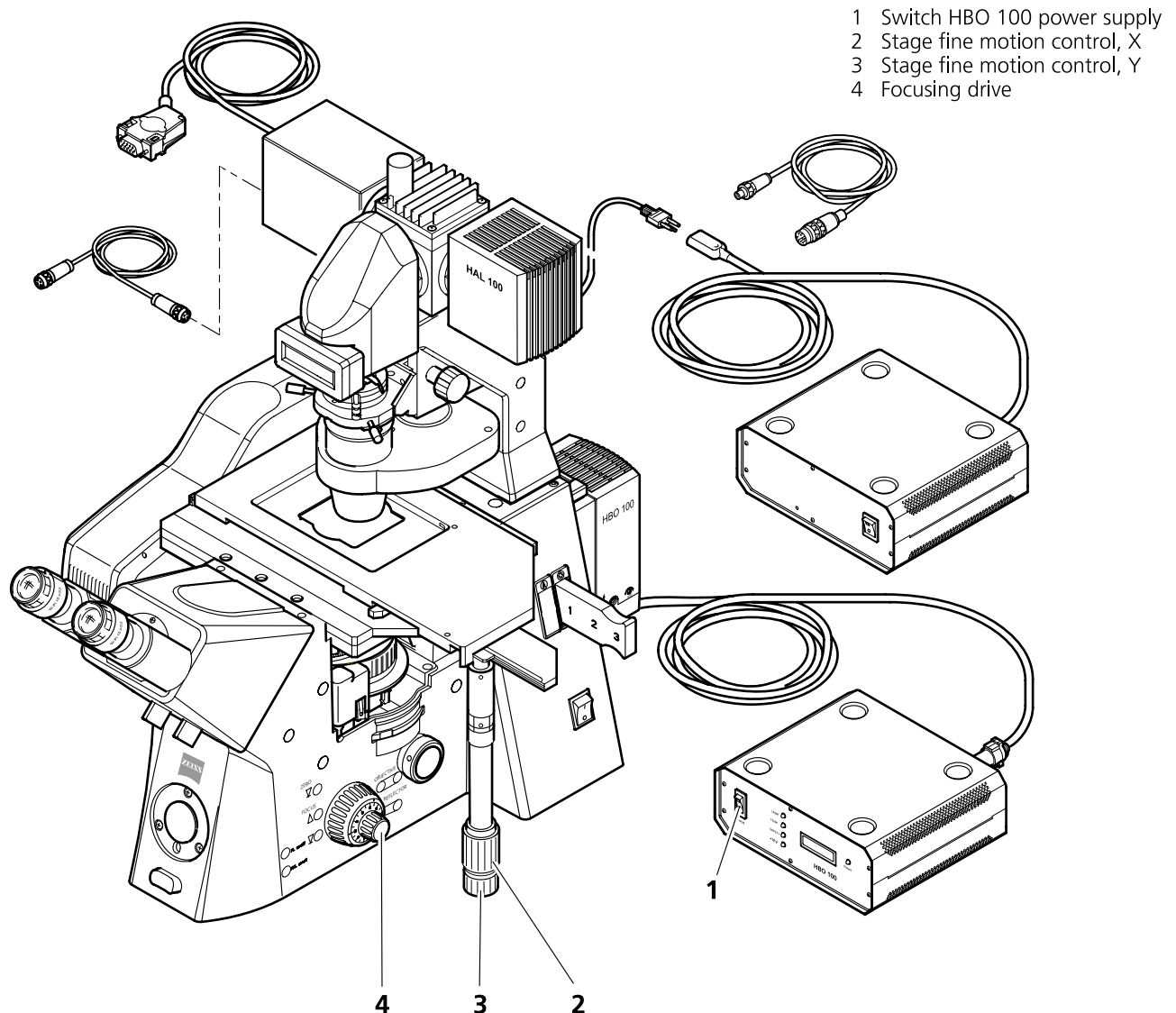


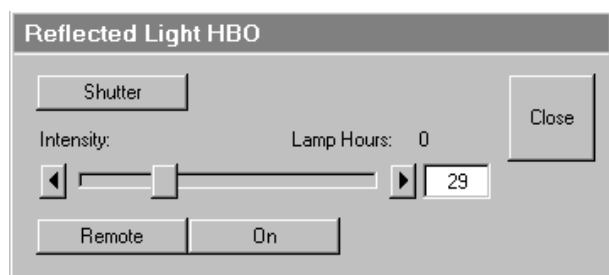
Fig. 4-32 LSM 5 LIVE with Axiovert 200 M BP

### 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

<b>Remote</b>	Enables the attenuation control via LSM Software. This is also enabled when clicking the <b>ON</b> button.
<b>ON</b>	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.
<b>Shutter</b>	Opens and closes the shutter in the reflected light path independent of the status of the internal lamp shutter and the attenuation.
<b>Intensity</b>	Sets the light via input box or slider.
<b>Lamp Hours</b>	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 (PMT photomultiplier) and tracks and the setting of the attenuators 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 (**Single Track** and **Multi Track**). Use the **Single Track** and **Multi Track** buttons to toggle between the two image acquisition modes **single tracking** and **multitracking**.

Performed settings can be stored as **Track Configurations** for **single tracking**.

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** (option). A recording configuration may contain the maximum of 4 tracks. Regardless of the number of included tracks, the maximum of 8 channels (incl. transmission) 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.

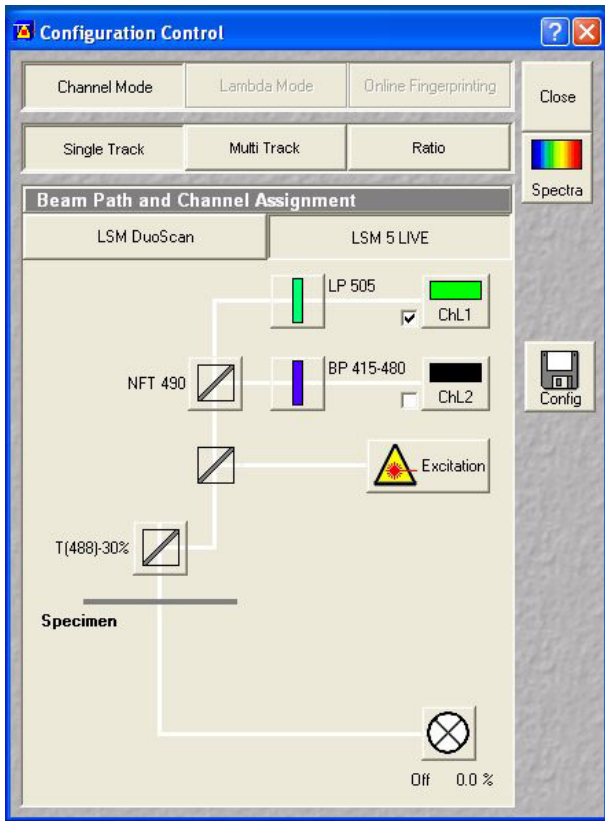


Fig. 4-34 Configuration Control window,  
Single Track activated

#### 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.

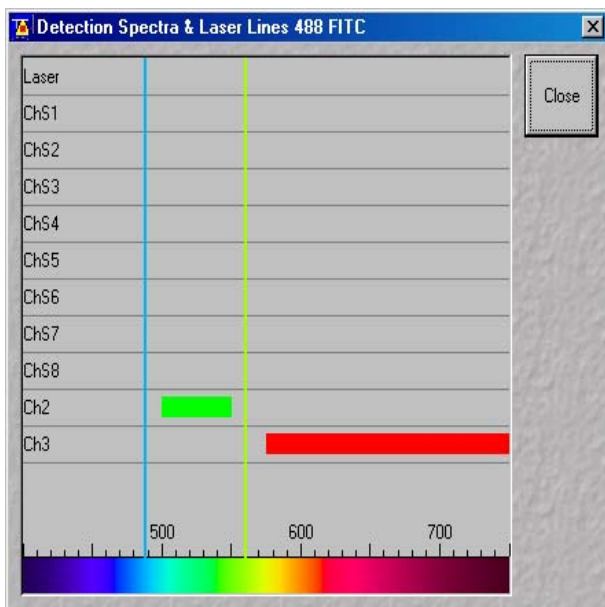


Fig. 4-35 Detection Spectra & Laser Lines window

#### 4.5.3.2 Spectra Button

The **Spectra** button opens the **Detection Spectra & Laser Lines**<sup>[Help74]</sup> 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**<sup>[Help75]</sup> 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.

#### 4.5.3.3 Config Button


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



Fig. 4-36 Track panel

#### (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.

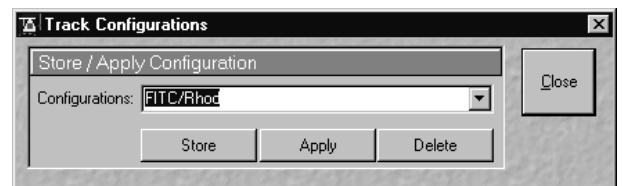


Fig. 4-37 Track Configurations window



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**.

#### 4.5.3.4 Settings for Single Track in the Channel Mode

The settings of the beam path for the scanning procedure with regard to the main beam splitter (Achrogate), 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.

##### (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.



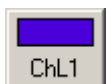
Activation / deactivation of the excitation wavelengths (check box) and setting of excitation intensities (slider). Open the **Laser Control** window via the **Laser** button.



Selection of the main beam splitter (Achrogate) 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 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.

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 5 LIVE 1 channel biomedical configurations – VarioOne GB, 488, 532 (635) nm, 1 channel FL) listed in the ANNEX of the printed manual.

The assignment of the numbered emission filters (1-2), NFT secondary dichroic beam splitters and Achrogate main beam splitters in the **Beam Path and Channel Assignment** panel is shown in the **Configuration Control** window (Fig. 4-38). The numbers of the emission filters correspond to those of the channels lying behind (PMT photomultipliers).

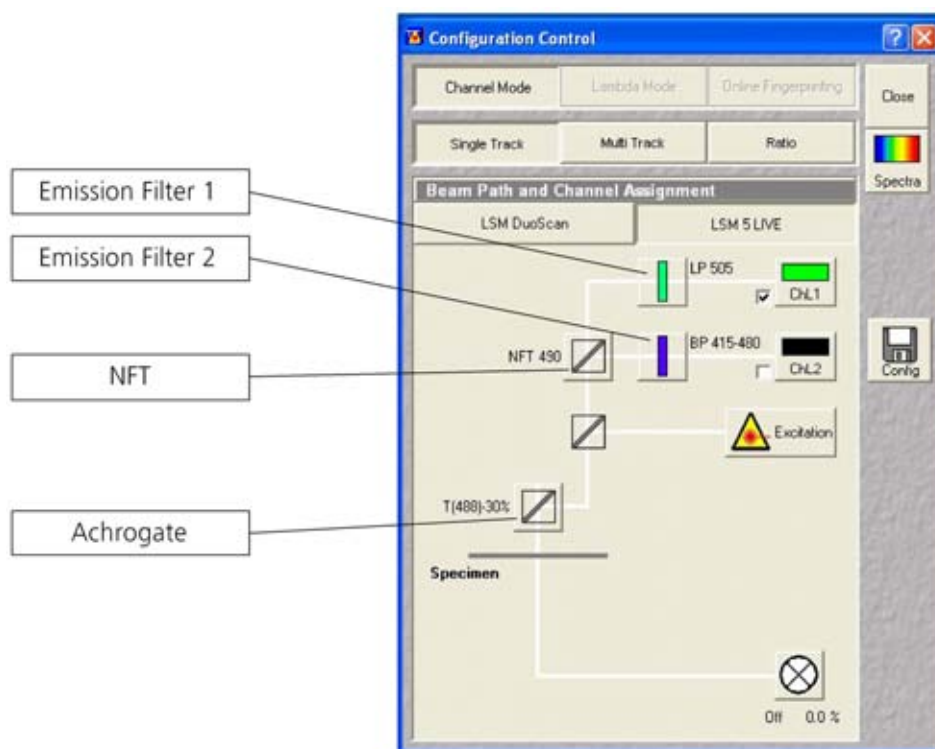







Fig. 4-38 Configuration Control window

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

- On the **Beam Path and Channel Assignment** panel, click on the Achrogate main beam splitters  (see Fig. 4-38).
  - 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 .

### (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 Achrogate main 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 5 LIVE scan module (see CHAPTER ANNEX: Filter Change in the Detection Beam Path of Channels 1 and 2 of the printed manual).

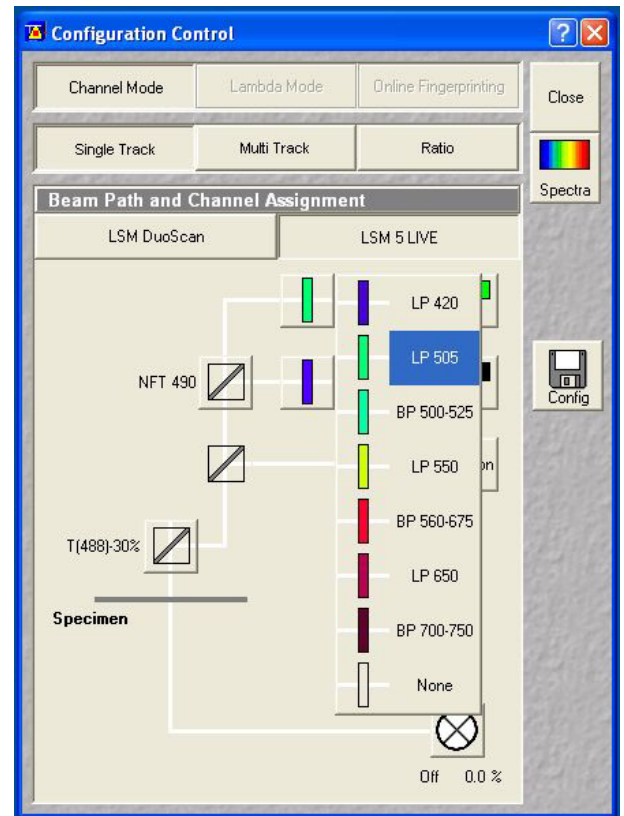



Fig. 4-39 Configuration Control window

### (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.

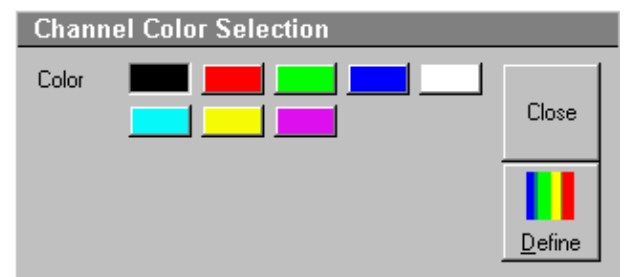


Fig. 4-40 Channel Color Selection window

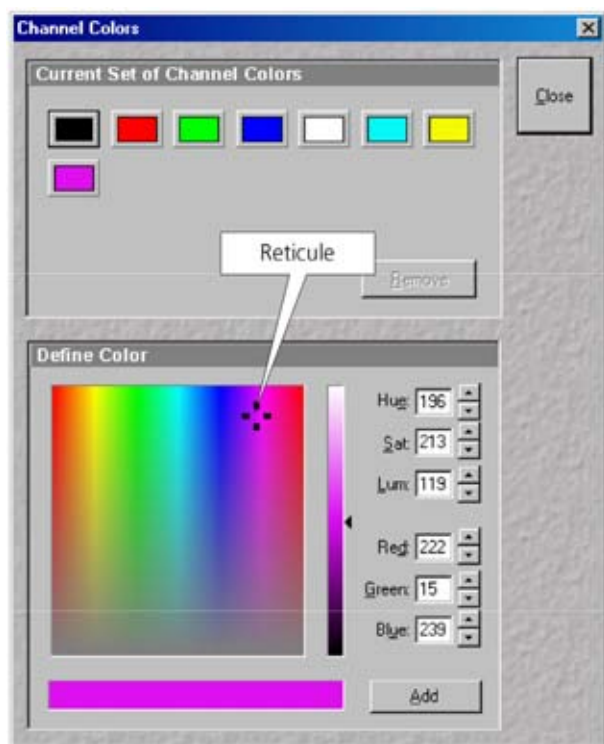



Fig. 4-41 Channel Colors window

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.

 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 photomultiplier.

## (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 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 attenuator changes accordingly.
- This allows you to adapt the laser intensity very sensitively to the job. Activate the check box for **Line Active**.

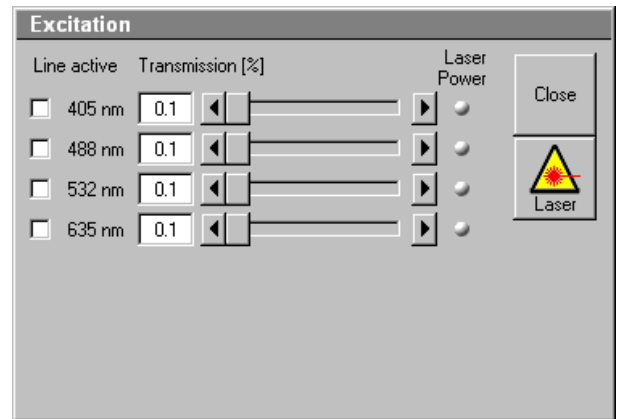


Fig. 4-42 Configuration Control window



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 for the lasers are deselected by means of the attenuator, i.e. these lasers change into standby status.

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

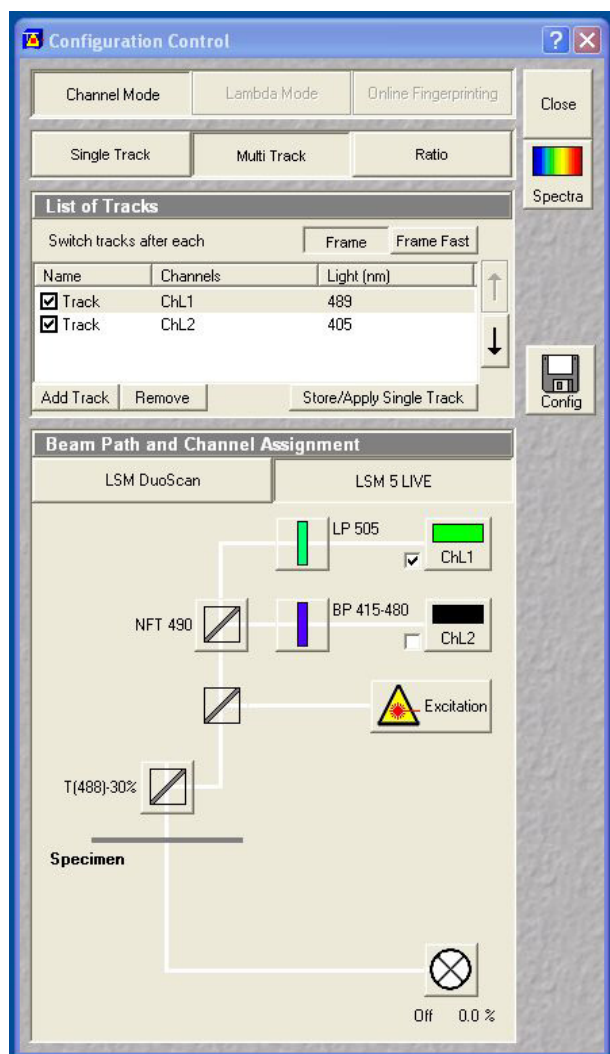


Fig. 4-43 Configuration Control window,  
Multi Track activated

#### 4.5.3.5 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, 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.

#### (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 sequence of tracks to be processed can be changed for the scan procedure.

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

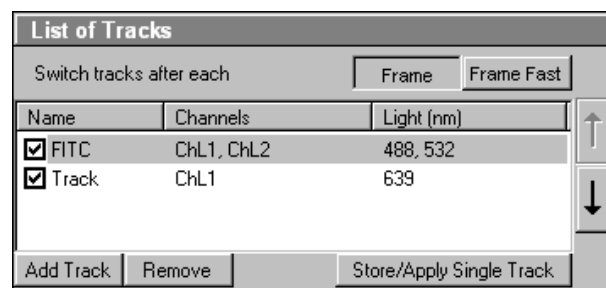


Fig. 4-44 List of Tracks panel

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.

### **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 ChL 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 ChL1-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:

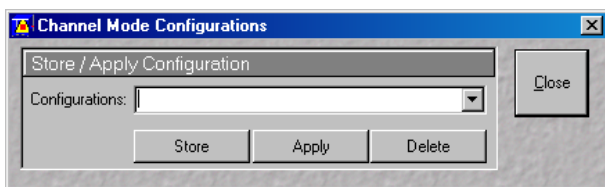




Fig. 4-45 Channel Mode Configurations window

- 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 4-200). 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**.

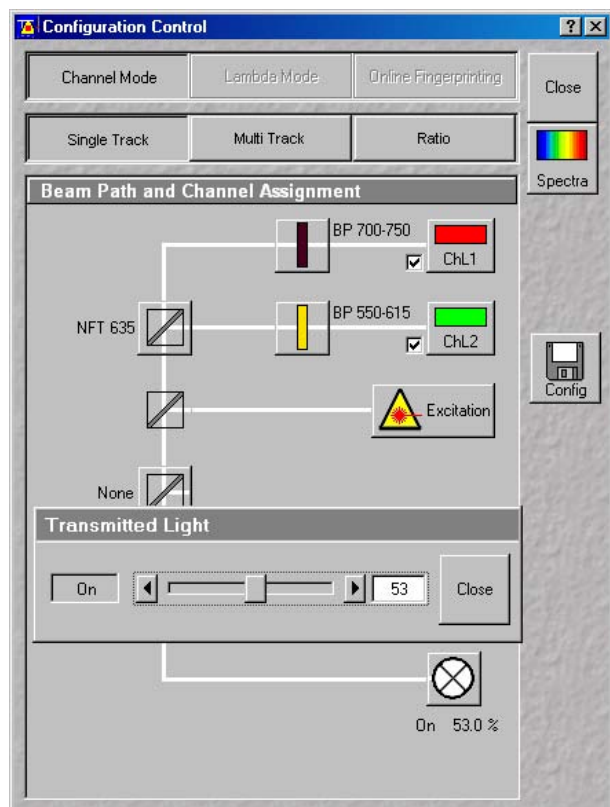
During storage via the **Config** button, all the data of **Beam Path and Channel Assignment** and the Detector Gain, Ampl. Offset, Ampl. Gain and Data Depth (8 / 12 Bit) scan parameters of all the defined tracks (multitracking) are stored. Furthermore, the used objective, the **Frame Size, Zoom, Rotation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

#### 4.5.3.6 Transmitted light preset



To add a transmitted light image, the light level of the Halogen lamp can be preselected in the configuration control window. The illumination is activated when the actual scan is started. To separate the transmitted light signal from the emitted fluorescence, choose e.g. the BP 700-750 in Ch1.

Fig. 4-46 Transmitted light preset

#### 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

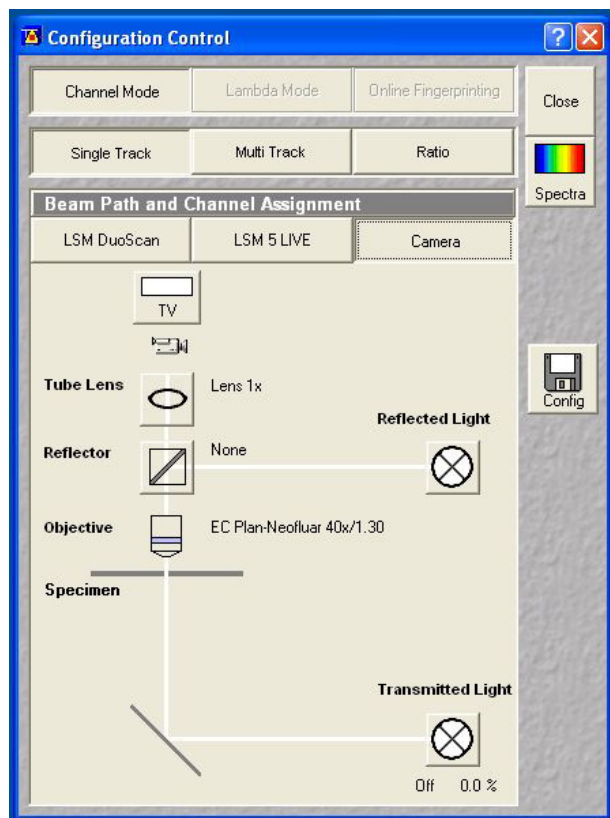


Fig. 4-47 Configuration Control window;  
camera detecting activated

#### 4.5.3.8 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.

#### 4.5.3.9 LSM 5 LIVE / DuoScan Panel

The use of this function permits the use of a LSM 5 LIVE or a LSM DuoScan in combination with a T-PMT as an alternative transmitted light detector.

- Click on the **Config** button in the **Acquire** subordinate toolbar of the main menu.
  - The **Configuration Control**<sup>[Help92]</sup> window opens.
- Activate one of the **Single Track** or **Multi Track** buttons and click on the **LSM 5 LIVE** or **LSM DuoScan** button.
  - The **Beam Path and Channel Assignment** panel for camera detection is opened.



If LSM 5 LIVE and LSM DuoScan tracks are mixed, the active detection port of the microscope has to be set according to the first track.

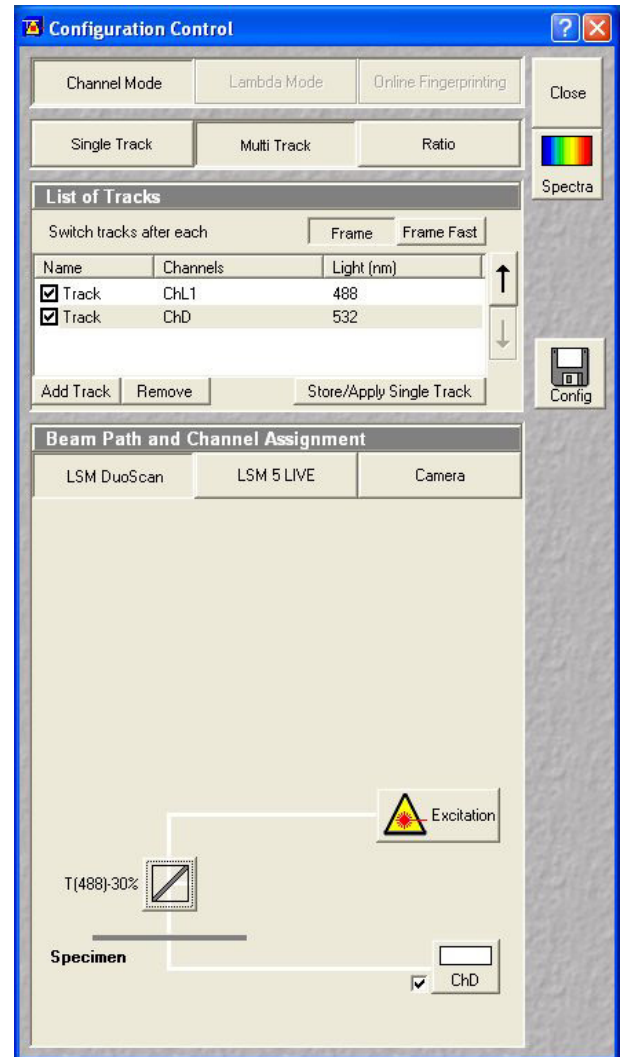


Fig. 4-48 Configuration Control window; LSM 5 LIVE / Duo Scan activated

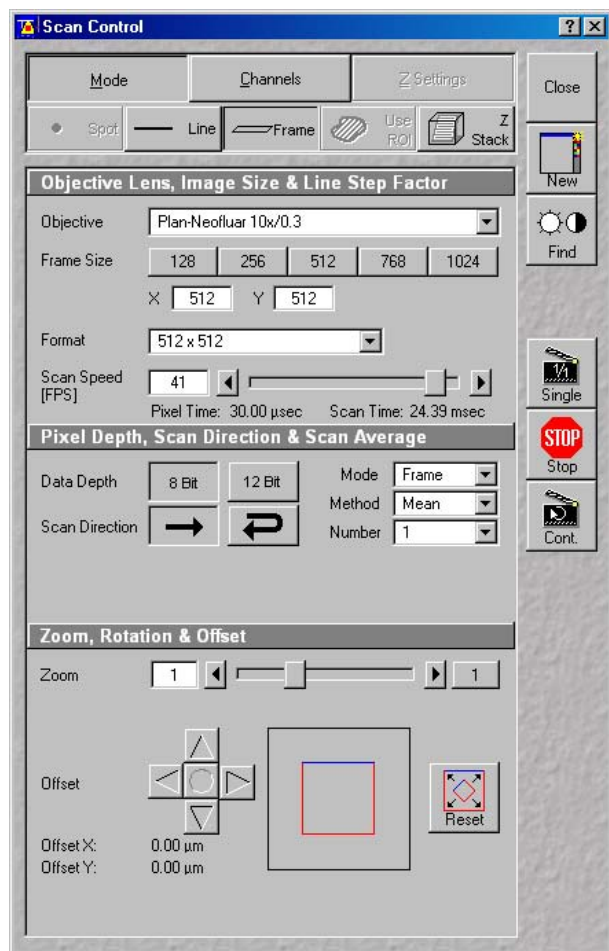


Fig. 4-49 Scan Control window

#### 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.

The following scanning modes can be performed:

##### 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)

#### 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

<b>Mode</b> button	When the button is activated, the following panels are available for the setting of the scanning parameters for the line and frame modes: <b>Objective Lens, Image Size &amp; Line Step Factor, Speed, Pixel Depth, Scan Direction &amp; Scan Average</b> and <b>Zoom, Rotation &amp; Offset</b> .
<b>Channels</b> button	When the button is activated, the <b>Channel Settings</b> and <b>Excitation of Track ...</b> panels are available for the setting of the channels and the laser excitation.
<b>Line</b> button	Activates the <b>Line</b> scan mode.
<b>Frame</b> button	Activates the <b>Frame</b> scan mode.
<b>Z Stack</b> button	Activates the <b>Z Stack</b> scan mode, display of additional buttons on the right-hand side of the <b>Scan Control</b> window.
<b>Z Settings</b> button	When the button is activated, the <b>Z Settings</b> panel is available for the Z-scan parameter definition. The <b>Z Stack</b> scan mode must be active.
<b>Close</b> button	Closes the <b>Scan Control</b> window.
<b>New</b> button	Opens a new <b>Image Display</b> window.
<b>Find</b> button	Automatic optimization of image brightness and contrast. The settings for the <b>Find</b> function can be varied as required using the <b>Maintain</b> menu.
<b>Fast XY</b> 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.
<b>Single</b> button	Single scan (named <b>Start</b> in the <b>Z Stack</b> mode).
<b>Stop</b> button	Stops the current scan procedure, no matter in which window the button is pressed (also see the <b>Time Series Control</b> window).
<b>Cont.</b> button / <b>Finish</b> button	Continuous scan (not available in <b>Spot Scan</b> mode and <b>Z Stack</b> mode). If you select the option <b>Frame</b> for <b>Mode</b> and the option <b>Continuous</b> for <b>Number</b> in the <b>Pixel Depth, Scan Direction &amp; Scan Average</b> panel, the <b>Finish</b> button is displayed instead of the <b>Cont.</b> button. In this case, continuous averaging is performed when you have started the scan. If you click on the <b>Finish</b> button, the scan/averaging process is stopped after the scan of the current image has been completed.



**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.

#### 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) 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**, **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**, **768**, **1024** 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 1024 pixels (integers). The value for **X** depends on the chosen image format.
  - The most common image formats can be set directly with the **Frame size** buttons. Note that more formats are available in the format pull down list.
- Use the **Scan Speed** slider to adjust the exposure time.
- Previous the slider is shown the number **Frames per Second**. Start with 2 to 4 frames per second.

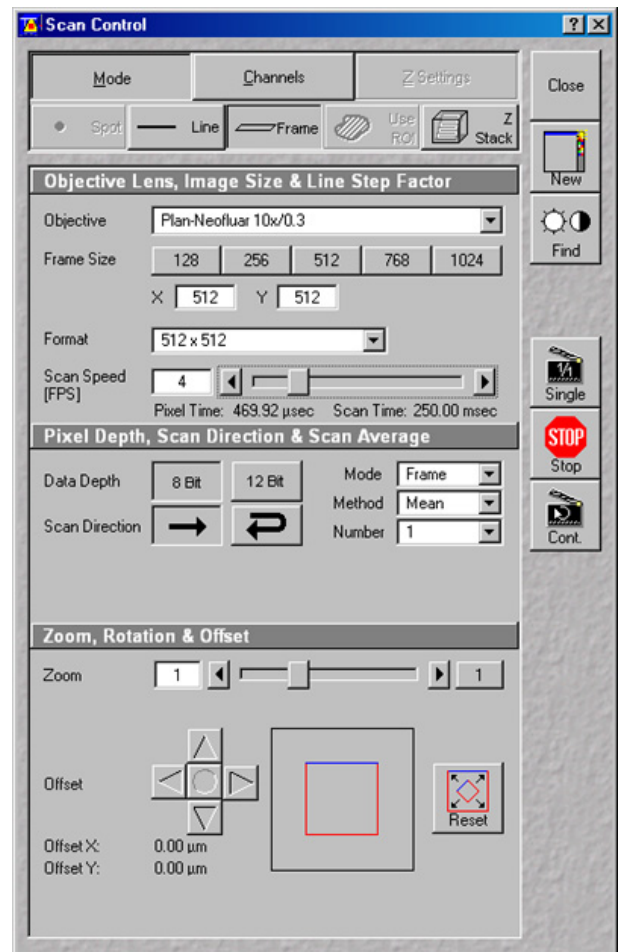


Fig. 4-50 Scan Control window - Mode/Frame

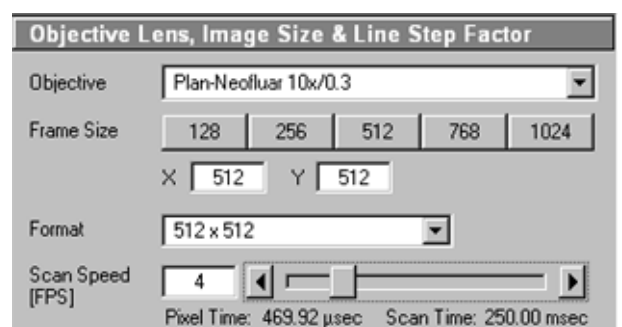


Fig. 4-51 Objective Lens, Image Size & Line Step Factor panel

The time slider displays the following information:

- Frames per second (it is possible to type in the required FPS directly, or to move the slider accordingly),
- Pixel time and
- Frame (scan) time.

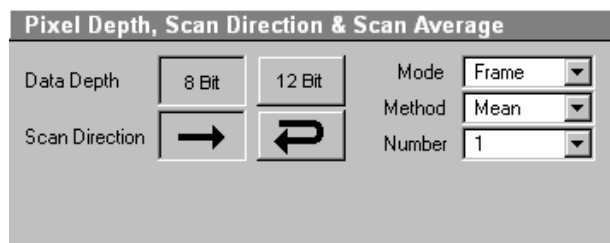




Fig. 4-52 Pixel Depth, Scan Direction & Scan Average panel

#### 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.

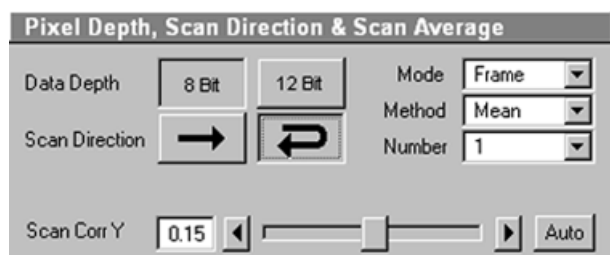
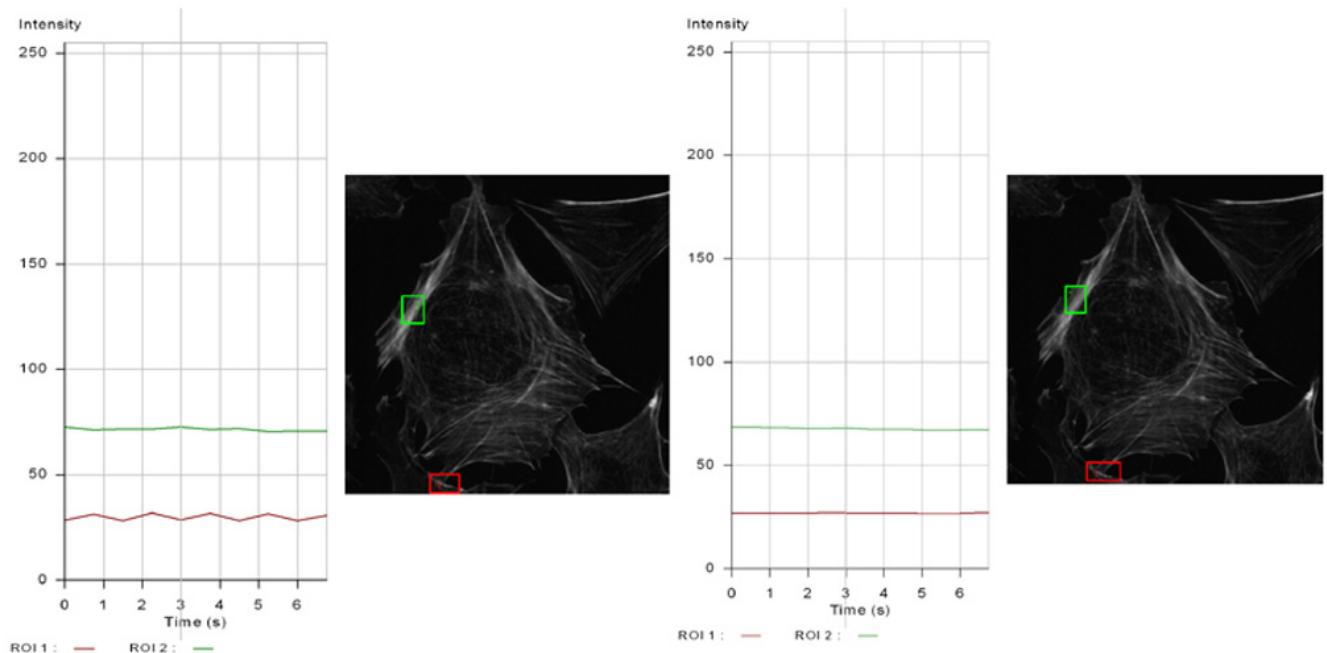


Fig. 4-53 Bidirectional Scan & Scan Correction

Unidirectional scan is always optimal regarding image quality. For possible artefacts caused by bidirectional scan (partially caused by fluoro-chromes behaviour), (jitter or flicker) the **Scan Corr Y** correction slider is available. Move slider to either side in continuous scan mode till images are stable. The **Auto** button sets the right correction automatically.

## Example




**Fig. 4-54** Example for Scan Correction

Intensity fluctuations at the end of the frame (left image) are fully compensated by the Auto button (right image).

## Average

- 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.

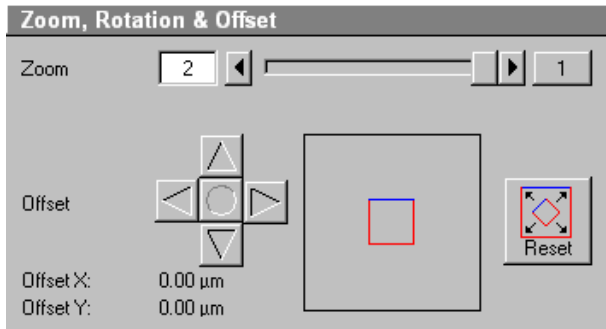
To create the image information using the **Line** average mode, each line (depending on the setting) is scanned 2, 4, 8 or 16 times during Scan Average, and then the average value per pixel is calculated. This minimizes noise interference during the scanning procedure.

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 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 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 0.5 to the maximum of 2.0, and is displayed in the relevant input box. The value 0.5 corresponds to factor 1, and value 2.0 to factor 4, related to the field of view of 18 mm in the intermediate plane. Clicking on button **1** enables immediate resetting to the zoom factor 1.
  - Recommended setting to start with: Zoom 1.
- 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.

## (2) Channels

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

### Channel Settings panel

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

Depending on the selected **Channels** button (e.g. **ChL1-T1**), the currently used settings of Pinhole (confocal aperture), Detector Gain, Amplifier Offset and Amplifier Gain are displayed.

- The slider near **Pinhole** (Confocal Aperture) enables you to change the aperture size of the relevant channel.
  - The aperture size 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 aperture size will increase the depth of focus, but reduce the light intensity received by the PMT photomultiplier.
  - When you vary the aperture size, 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 aperture size to 1 Airy unit. A click on the **Max** button sets the aperture size to the maximum.
- The sliders (and the relevant arrow buttons) near **Detector Gain**, **Ampl. Offset** and **Exposure Time** enable you to set the photomultiplier of the selected channel during continuous scanning.
  - Detector Gain: Setting of the sensitivity of the detector - setting of image contrast and brightness (values available between 0 and 50)
  - Amplifier Offset: Setting of the electronic offset - background of the image can be set (values available between -2 and 0.1)
  - Exposure Time: Setting of exposure time with display field for frame rate (Frames per Second)

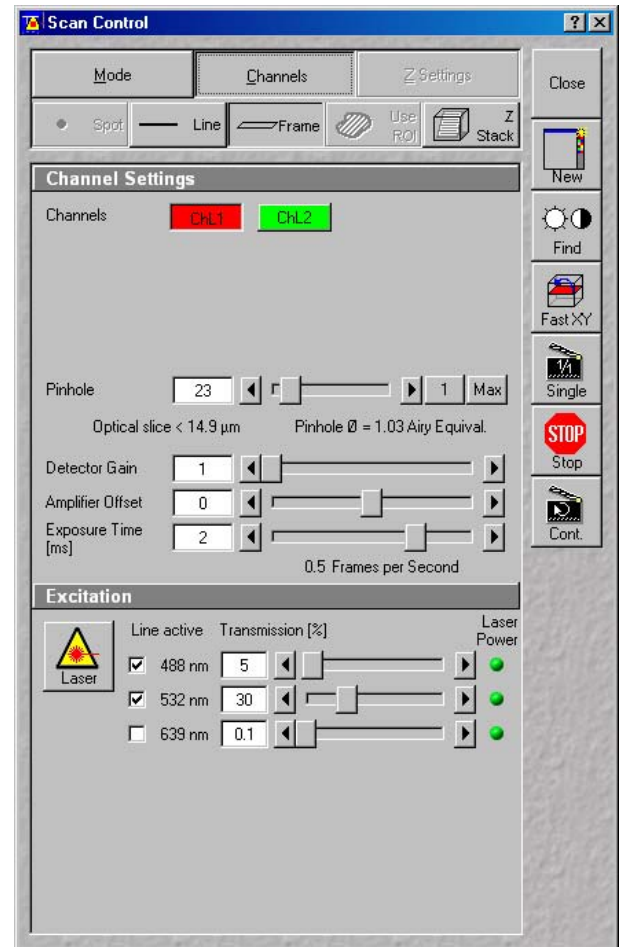


Fig. 4-56 Scan Control window – Channels

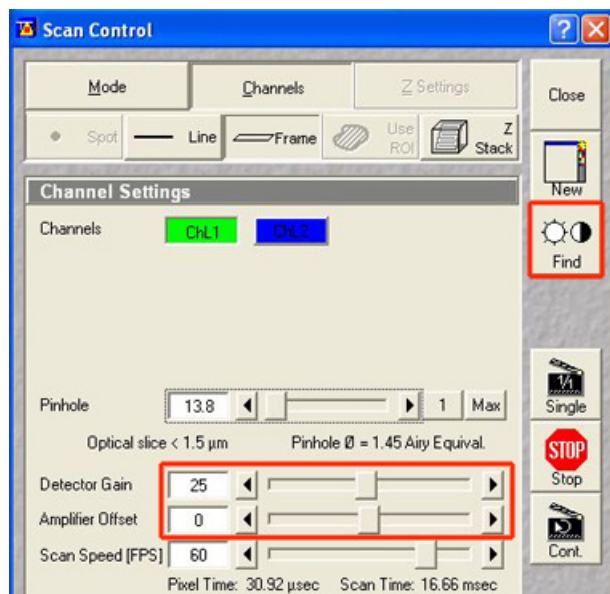


Fig. 4-57 Gain and Find


### Gain and Find

The **Find** button always sets an ideal gain for a clear background, best sensitivity and full grey resolution. This is maximally a gain value of 25.

One can manually increase this gain by moving the gain slider beyond 25 and use the digital post amplification by doing this. Be aware that from value of 37,5 on, background artefacts can be visible, since those are amplified too.

When using 12 bit grey resolution, gaps can occur in the histogram of the acquired image, in case the **Gain** slider is moved beyond a gain level of 25.

Use the **Amplifier Offset** slider to remove underexposed pixels in the image background (no blue pixels visible with "range" palette).

 The parameters **Detector Gain**, **Ampl. Offset** and **Ampl. Gain** are described in section **Confocal Aperture / Detector Gain / Ampl. Offset** (see page 4-319) in the context of image optimization.



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. 4-61).

- 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).

All parameters under Channels can be varied online.

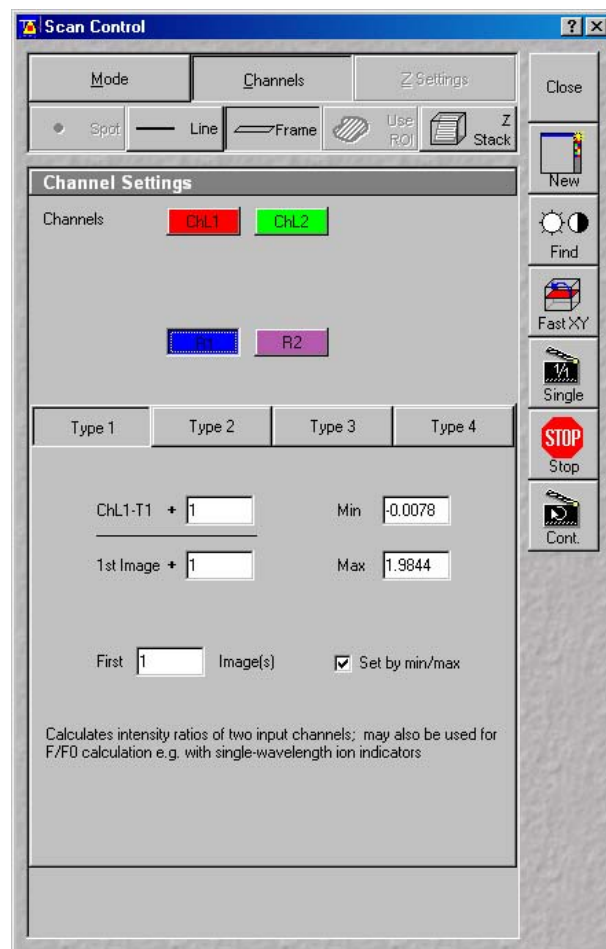


Fig. 4-58 Channel Settings panel of a Ratio Channel

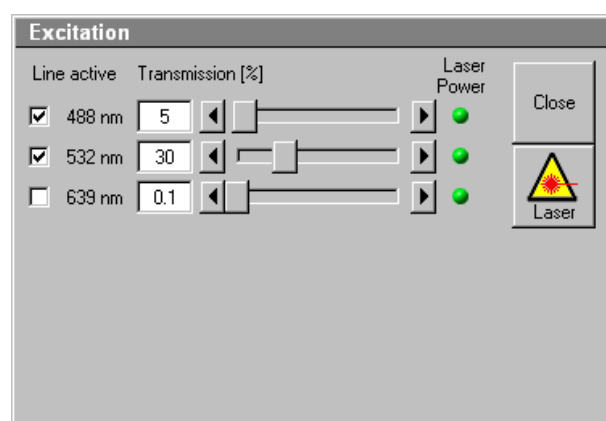
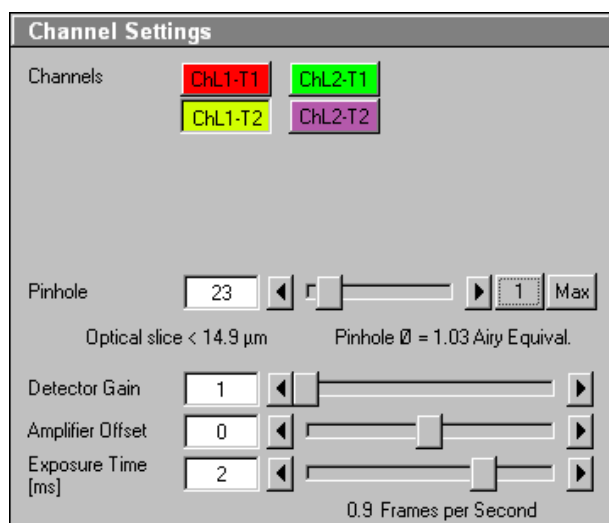


Fig. 4-59 Excitation of Track ... panel



**Acquisition of a frame**

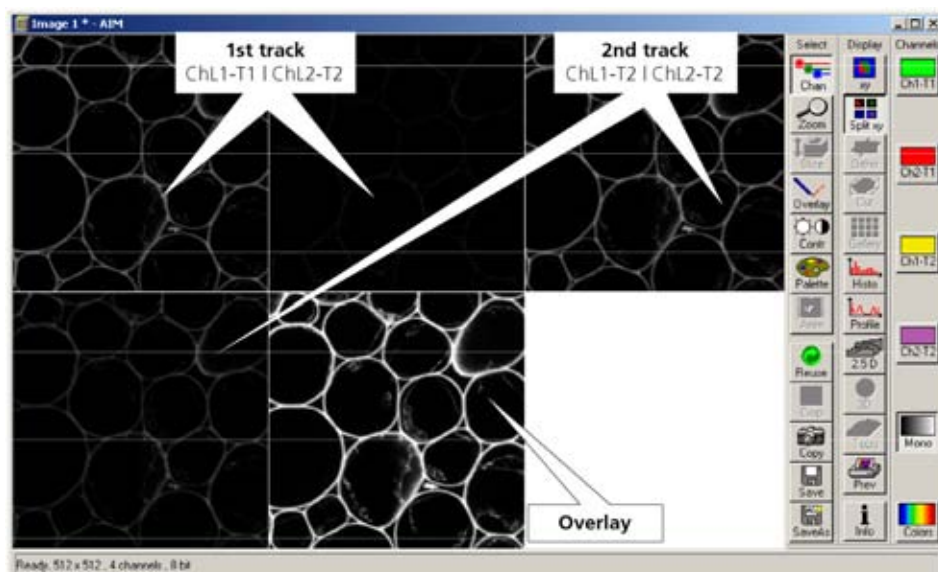
Once you have set up your parameter as defined in the above section, you can acquire a frame image of your specimen.



**Fig. 4-60** Channel Settings panel for two defined tracks plus Ratio channel

- 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 and the overlay (see Fig. 4-61). See the appropriate **Channel Settings** panel in the **Scan Control** window (Fig. 4-60).



**Fig. 4-61** Image Display window with two tracks plus ratio track (Split xy mode)

### (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.

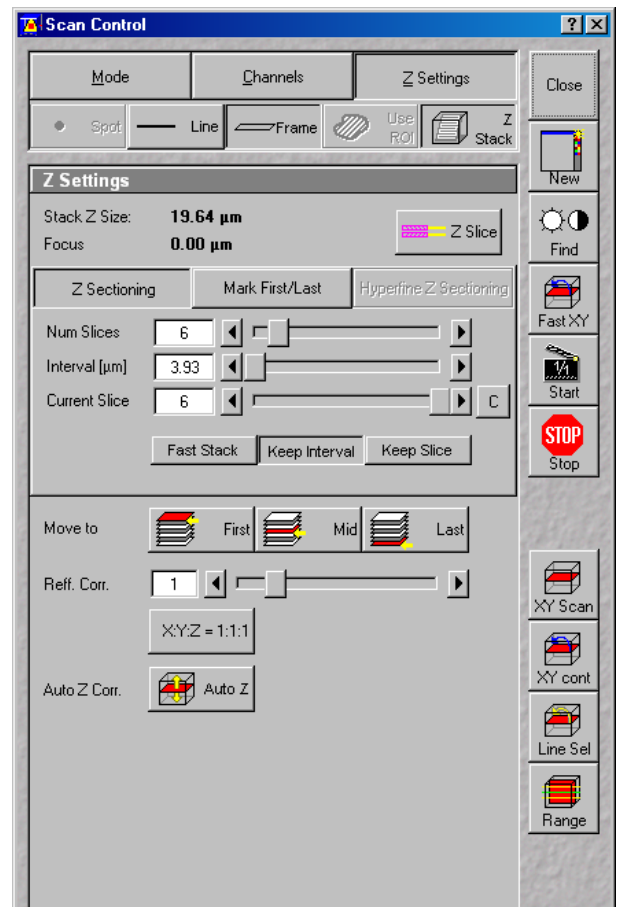


Fig. 4-62 Scan Control window – Z Settings

**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** (confocal aperture) and **Undo**) to allow the setting of the optimal interval and the optimal aperture size of fluorescence stacks. Both values influence each other and depend on the objective used.

In the case of a fixed aperture size, half the value of the smallest aperture size used is taken to determine the optimum interval. Accordingly, the aperture size 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 aperture size setting

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

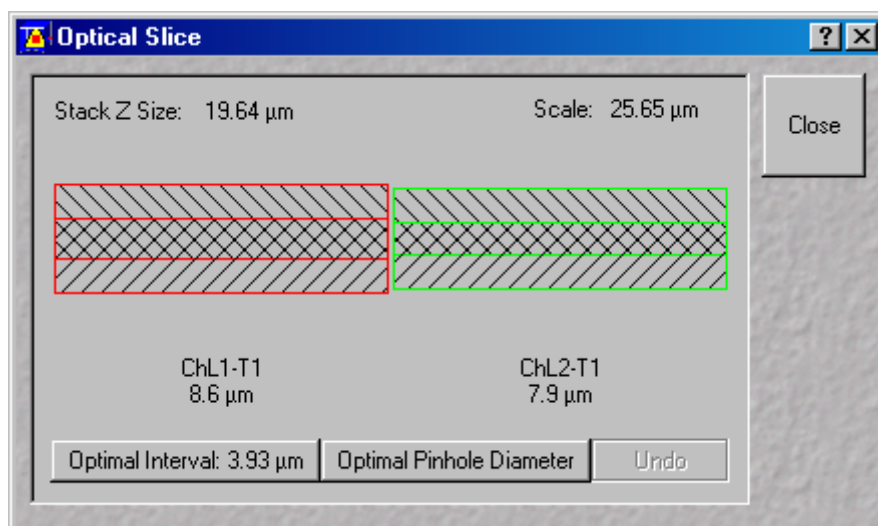


Fig. 4-63 Optical Slice window

## 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**, **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 4-84).

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**.

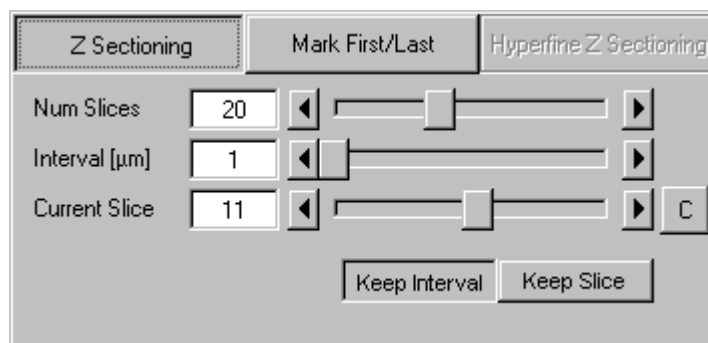
**Current Slice:** Display of the current position of the slice within the stack. Change of position via slider / arrow keys. Reset of the current slice position in the center of the stack by clicking on the **C** button. Of course, the borders of the stack are also changed if the current slice position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



**Fig. 4-64** Scan Control window - Z Sectioning tab activated

- 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.

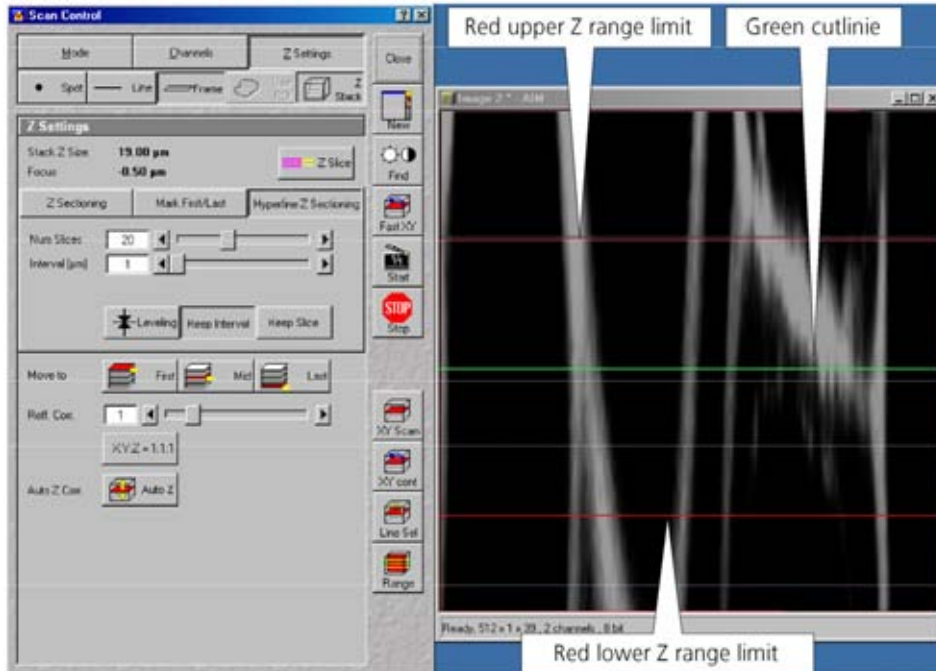


Fig. 4-65 Scan Control window and Image Display window

- 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.

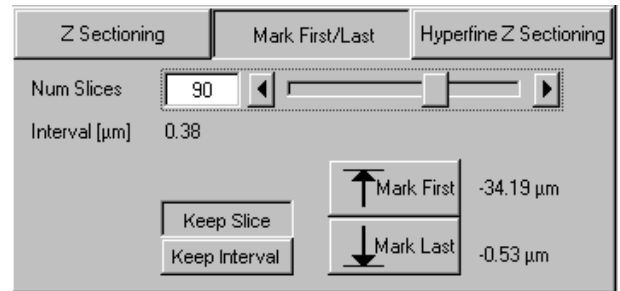
### **Mark First/Last tab**

The determination of the optimum stack size is performed here via focusing during a continuous scan.

- 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 4-114) 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. 4-66 Mark First/Last tab**

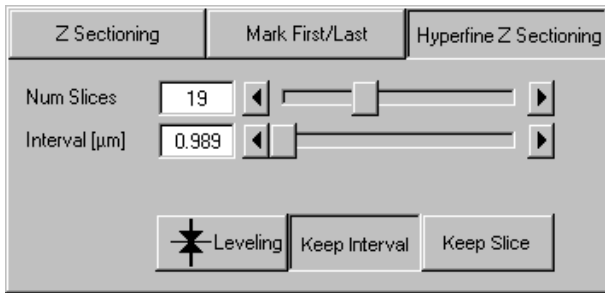


Fig. 4-67 Hyperfine Z Sectioning tab

**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 4-114).

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  $\pm 250 \mu\text{m}$ . 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.

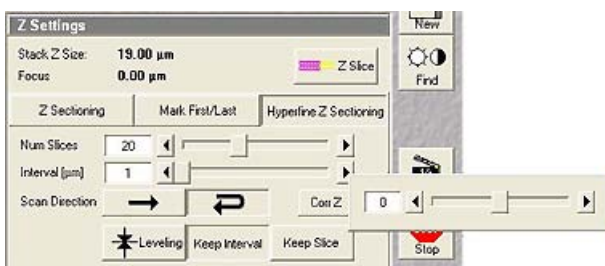



Fig. 4-68 Corr Z Slider

**Bidirectional Z-Scan in time series (4D)**

Similar effects can occur when using the fast piezo Z-drive in **Bidirectional** mode with multiple stacks. Like for X/Y-scanning, there is a uni- and a bidirectional mode here too.

- To compensate possible image deviations in the Z-direction in bidirectional focus mode, use the **Corr Z** slider by typing in the deviation occurring in the adjacent image stacks of a time series.

 If only one time point (stack) is acquired,

the **Corr Z** slider has no effect.

### **Auto Z Corr.**

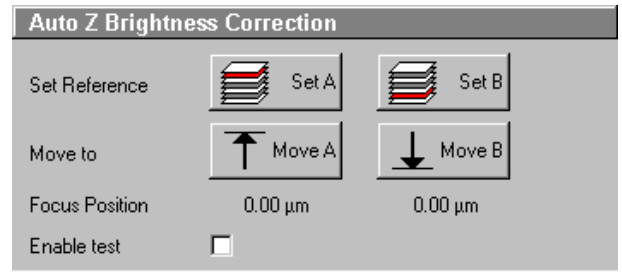
The function **Auto Z Correction** allows a linear variation of Detector Gain, Ampl. Offset, and Ampl. Gain values between the different slices of a stack.

- 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 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.



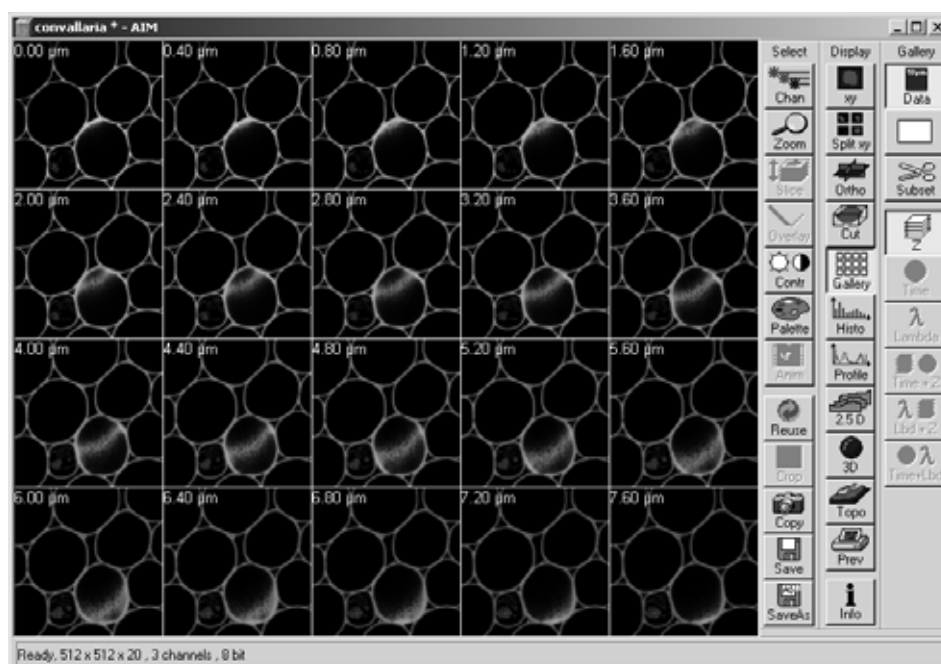
### **Acquisition of a Z Stack**

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 5 LIVE 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

#### 4.5.4.3 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.

- 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.

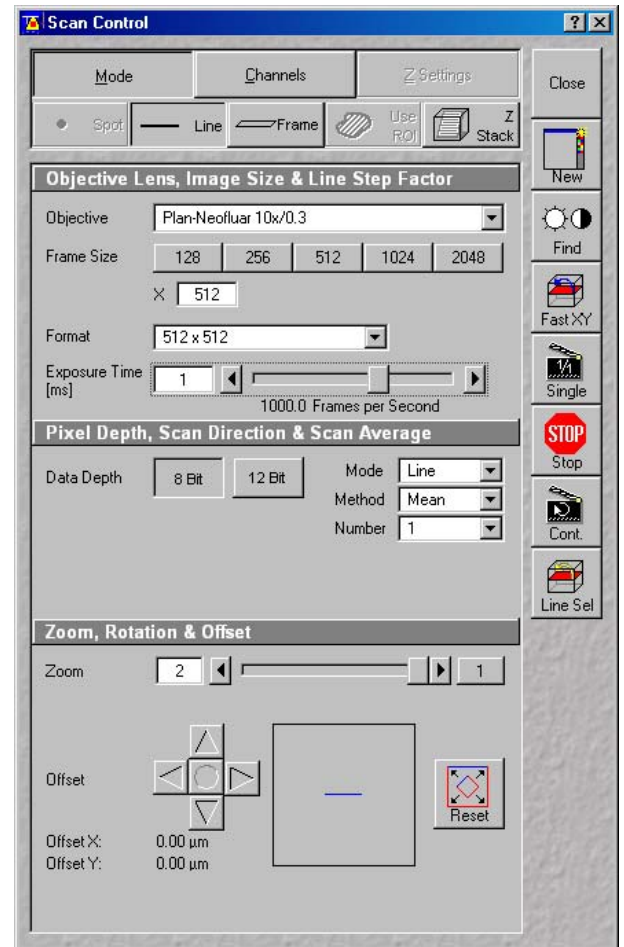


Fig. 4-71 Scan Control window - Mode/Line

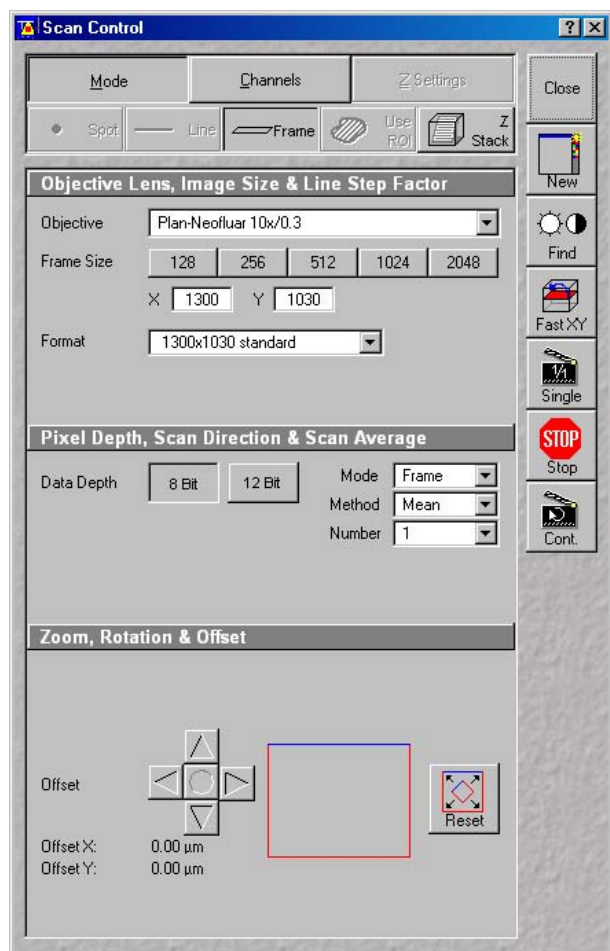


Fig. 4-72 Scan Control window - Mode, settings for camera control

#### 4.5.4.4 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**.

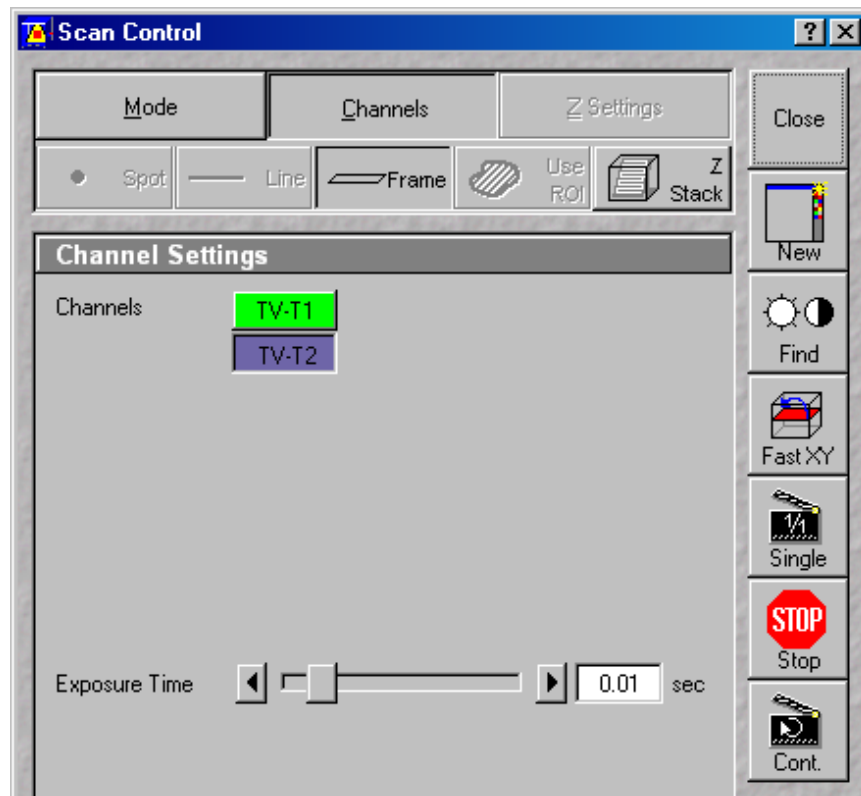


Fig. 4-73 Scan Control window - Channels, settings for camera control

<b>Channels</b> button	Displays the activated channels and possible settings.
<b>Exposure time</b>	Sets the exposure time of the camera.
<b>Find</b>	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.
<b>Fast X/Y</b>	Starts a fast online scan mode, e.g. for focusing. Also, the 5x5 binning mode can be used (to be set in <b>Mode / Format</b> ).
<b>Single</b>	Starts a single image acquisition (The <b>Image Display</b> window appears.).
<b>Cont.</b>	Starts acquisition of a series of images (The <b>Image Display</b> window appears.).
<b>Crop</b>	Defines a ROI for camera acquisition in the <b>Image Display</b> window. Note that this is just a <b>Crop</b> function, while the whole sample is illuminated. Rotation of the ROI is not possible.
<b>Info</b> button	Shows the acquisition parameters in the <b>Image Display</b> window.
<b>Close</b>	Close the <b>Scan Control</b> window.

### 4.5.5 Edit ROI (Region Of Interest)

A scan image allows certain areas (ROIs) to be defined. Definition and activation of the ROIs for the analysis 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.

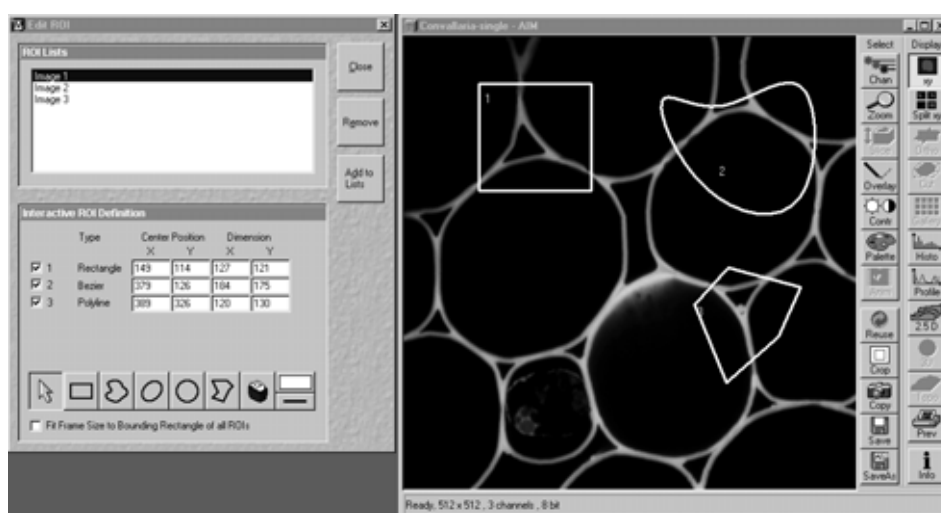


Fig. 4-74 Edit ROI window and Image Display window with ROIs

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.

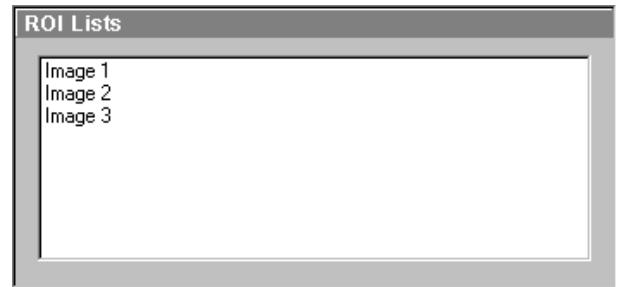


Fig. 4-75 ROI Lists panel

## (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.

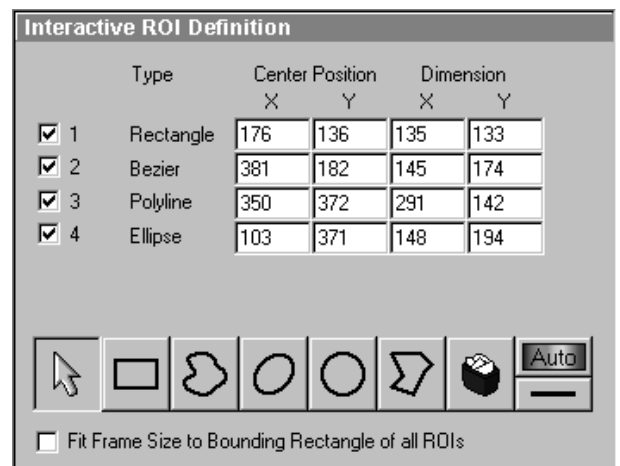


Fig. 4-76 Interactive ROI Definition panel



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.

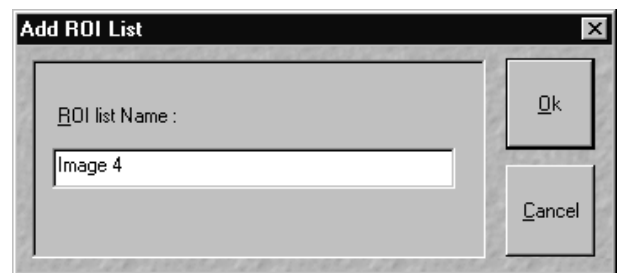


**Fit Frame Size to bounding Rectangle of all ROIs** check box: If this check box is ticked, the scan procedure is displayed only within a rectangle which is defined by the greatest extension in X and Y of all the individual figures together, i.e. the pixel number and the data quantity of the **Image 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. 4-77**      **Add ROI List window**



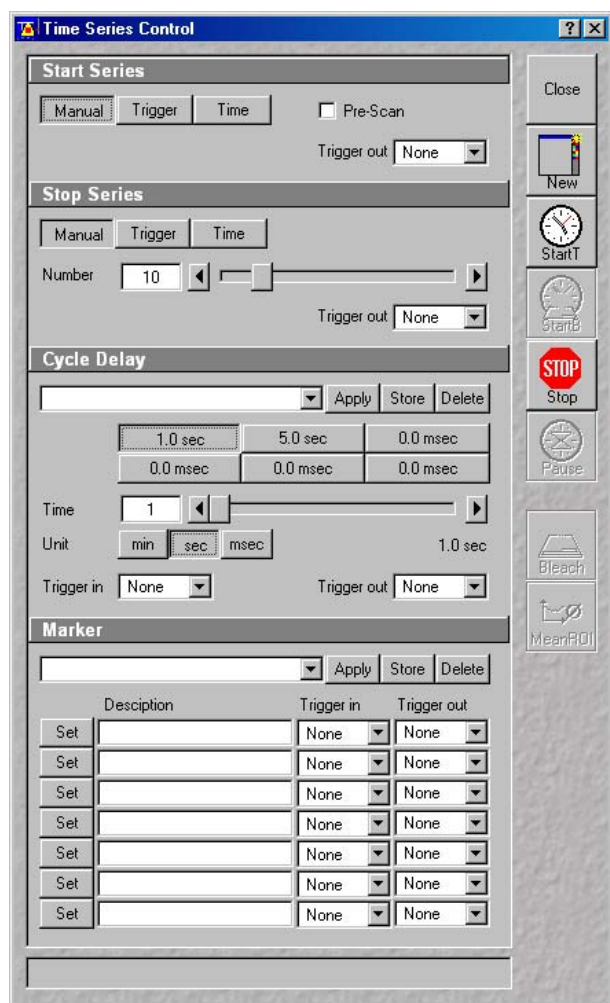


Fig. 4-78 Time Series Control window

## 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:

<b>Close</b> button	Closes the <b>Time Series Control</b> window.
<b>New</b> button	Opens a new <b>Image Display</b> window.
<b>Start T</b> button	Starts the Time Series.
<b>Stop</b> button	Stops the entire Time Series. A current scan is interrupted.
<b>Pause</b> button	Interrupts the Time Series. Button labeling is changed to <b>Resume</b> . 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.

**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. 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.

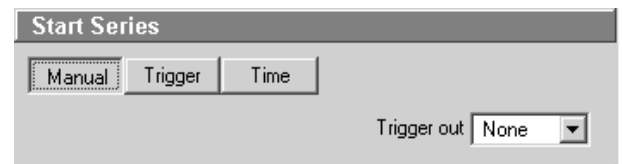



Fig. 4-79 Start Series panel

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.

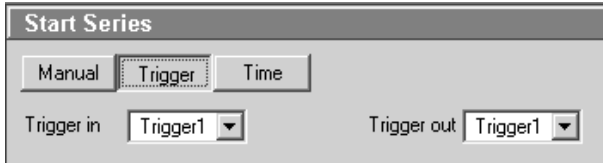


Fig. 4-80 Start Series panel

**(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.

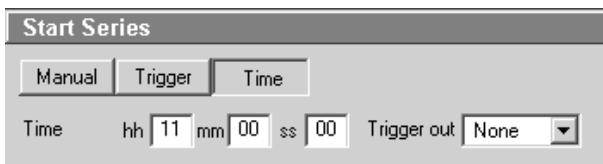



Fig. 4-81 Start Series panel

**(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.

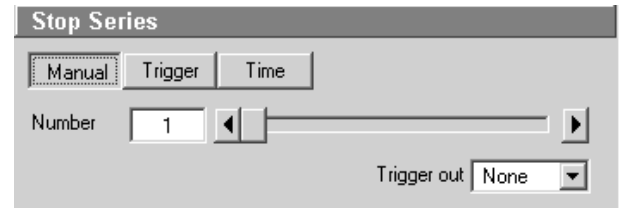


Fig. 4-82 Stop Series panel

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.

- 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.

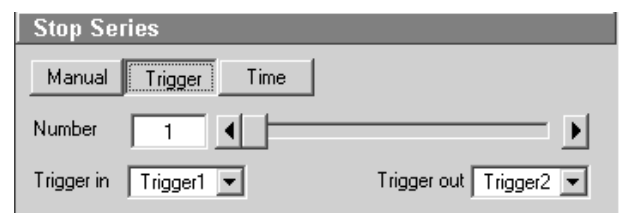



Fig. 4-83 Stop Series panel

 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**.

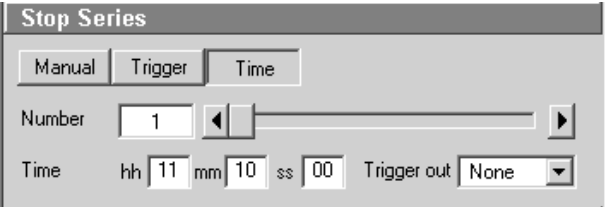



Fig. 4-84 Stop Series panel

 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.

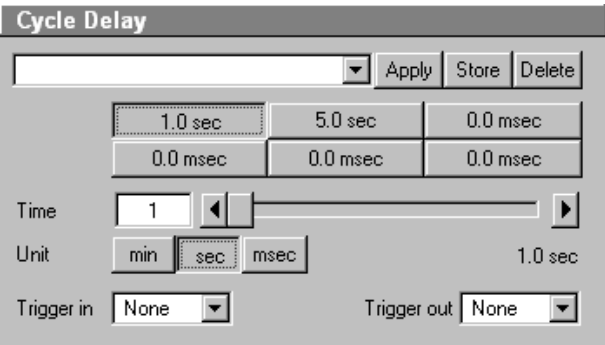


Fig. 4-85 Cycle Delay panel

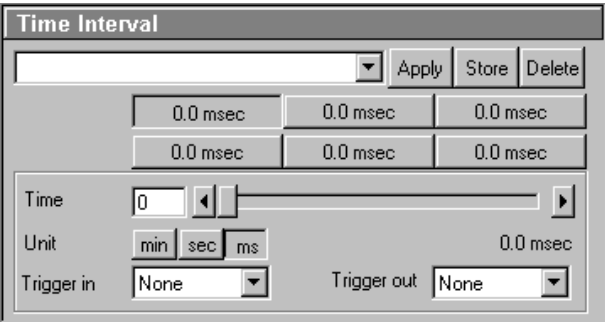


Fig. 4-86 Time Interval panel

(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.

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 **Cycle Delay** (or **Time Interval**) panel permits the intervals to be activated and changed.

## Function description

<b>Cycle delay</b> or <b>Time Interval</b> list box	List of the stored sets of Cycle Delays or Time Intervals for time series.
<b>Apply</b> button	Application of the sets of delays for time series selected in the list box.
<b>Store</b> button	Storage of sets of delays for time series.
<b>Delete</b> button	Deletion of sets of delays for time series from the list box.
<b>Time</b> buttons	Activation of the time for the time series set for the relevant button.
<b>Time</b> input box / arrow buttons / slider	Determination of the cycle time for the currently activated <b>Time</b> button.
<b>Unit</b> buttons	Selection of time units: <b>min</b> , <b>sec</b> or <b>ms</b> .
<b>Trigger in</b> list box	Selection of the trigger key (1-4) to be used to activate the <b>Time</b> button for the delay time.
<b>Trigger out</b> 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 **Cycle 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.

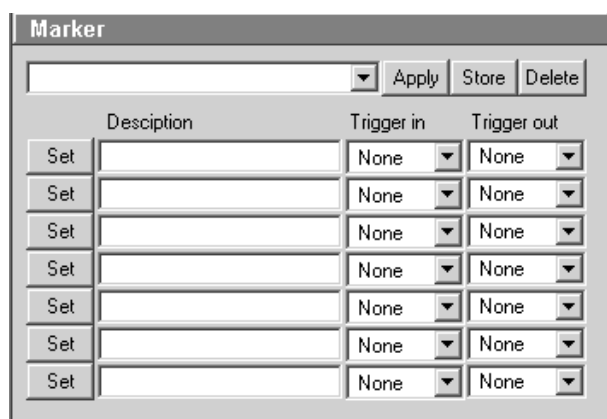
- Click on the required **Time** button.
- Open the **Trigger out** list box with a click on the arrow button.
- Choose one of the trigger keys 1 to 4 (e.g. **Trigger3**).

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. 4-87**      **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

<b>Marker</b> list box	List of the stored combinations of markers.
<b>Apply</b> button	Application of the marker combinations selected from the list box.
<b>Store</b> button	Storage of a combination of markers.
<b>Delete</b> button	Deletion of a combination of markers from the Marker list box.

---

<b>Set 1-7</b> button	Setting of a marker during the scan procedure.
<b>Description</b> input box (1-7)	Entry of the comments for the marker.
<b>Trigger in</b> list box (1-7)	Selection of the trigger key (1-4) with which the marker is to be set.
<b>Trigger out</b> 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.



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#### 4.5.6.6 Time Series of a Frame

- Set the relevant parameters for time control in the **Start Series**, **End Series** and **Cycle 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.



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. 4-88** 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

#### 4.5.6.7 Time Series of a Frame over Z Stack (option)

- 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).

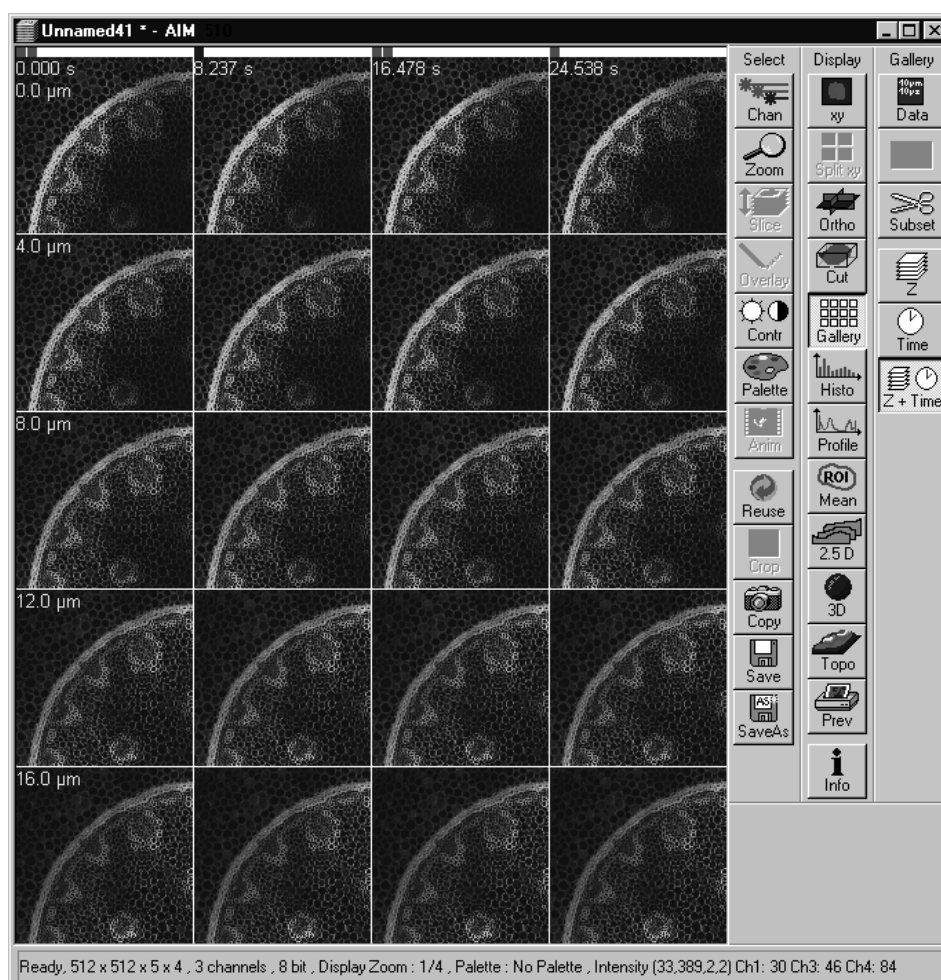


Fig. 4-89 Image Display window of a Z Stack and a Time Series Scan

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.

#### 4.5.6.8 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 4-90) is created as a function of time.

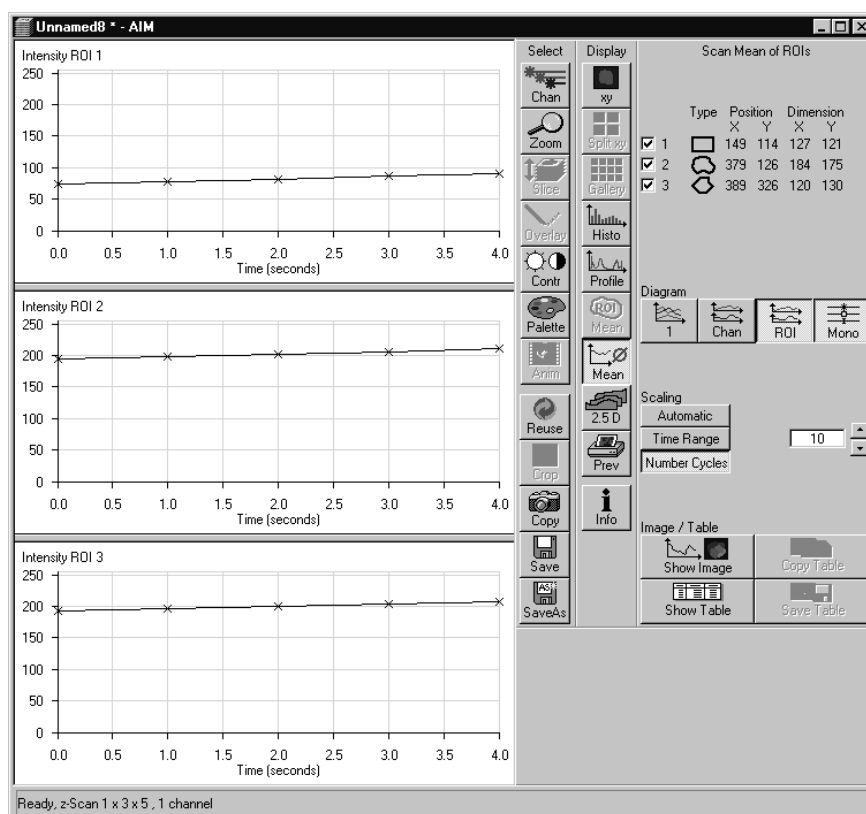


Fig. 4-90 Image Display window of a Time Series with Mean ROI

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.

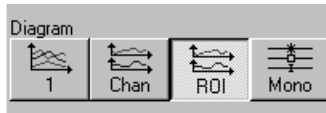
By selecting the appropriate options (see **Options** menu, **Settings – Scan Mean of ROIs**) you can activate the following additional functions:

- 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:

	Type	Position		Dimension	
		X	Y	X	Y
<input checked="" type="checkbox"/> 1		176	136	135	133
<input checked="" type="checkbox"/> 2		362	407	102	102
<input checked="" type="checkbox"/> 3		381	182	145	174

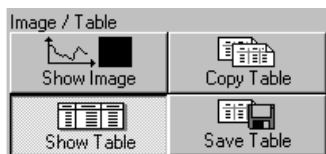
Display of the data of the ROIs used for the creation of the **MeanROI** (identical to the **Edit ROI** window). If the check box of a ROI is deactivated, the ROI's intensity values are no longer displayed in the Intensity-Time diagram.



**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 monochromatic 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.

#### 4.5.7 Stripe bleach function (LSM 5 LIVE without LSM DuoScan)

##### **Bleaching of selectable rectangular image regions with the LSM 5 LIVE**

Stripe Bleach is activated by the **EditBleach** button in the main menu. The **Bleach Control** window opens and can be used in the same way as in any other Zeiss LSM 5, e.g. the LSM 510. The only difference is that the bleach region has to be rectangular.

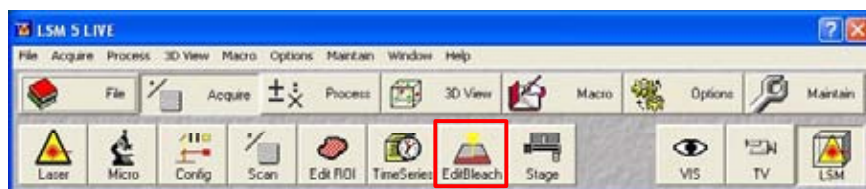


Fig. 4-91 EditBleach Button

Hence the **Define Region** button only allows to select the upper and lower border of a bleach region. It is possible to select more than one bleach region.

Bleach Regions are drawn directly in the image window. The bleach wavelength is chosen in the **Excitation** (laser) wavelength menu on the bottom of the bleach control dialogue. Check the required line and set the power with the sliders. A single bleach event can be started interactively by pressing the **Bleach** button.

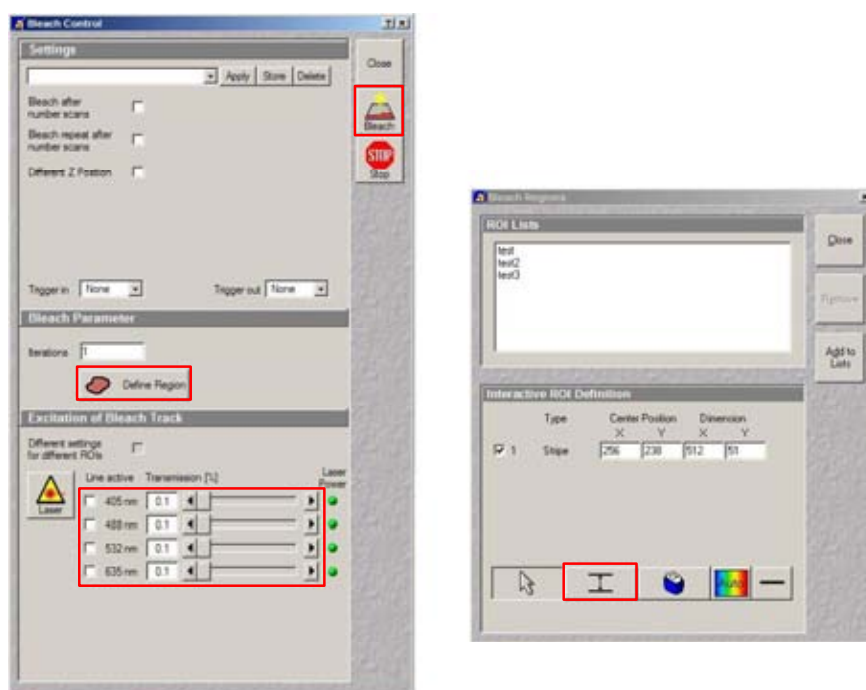


Fig. 4-92 Stripe Bleach Function

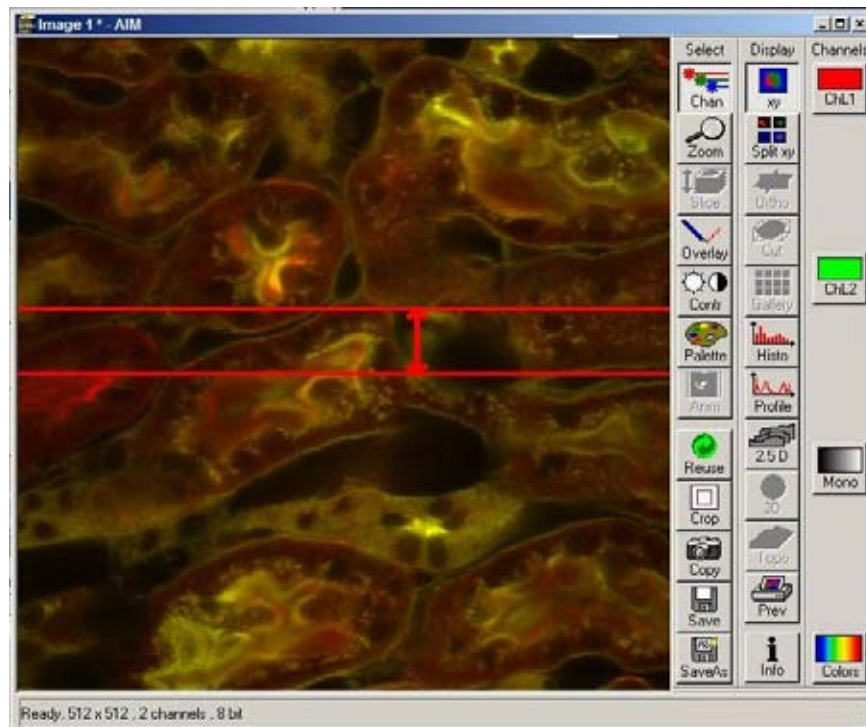


Fig. 4-93 Image Window

### Bleach series

To set up a time series with a bleach event, the bleach region is selected as described above. In the **Time Series** window, a time series is set up.

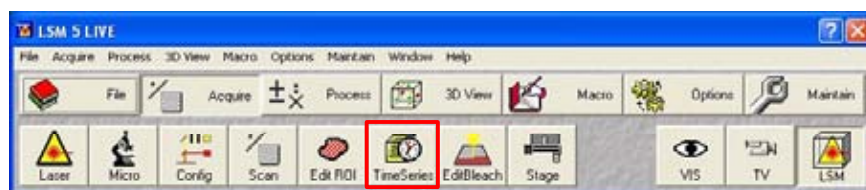


Fig. 4-94 Time Series Button



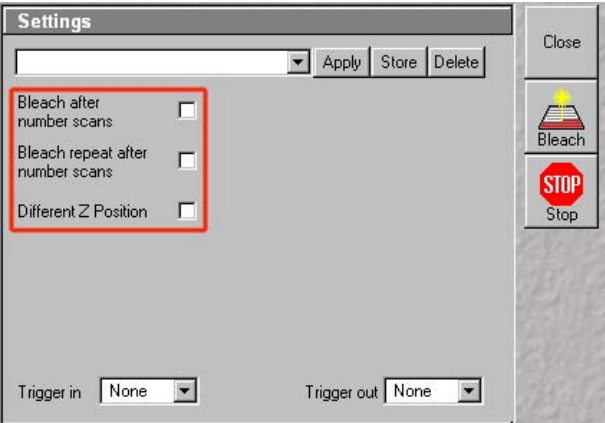


Fig. 4-95 Settings for Bleach Series

In the upper part of the **Bleach Control** window, the time parameters can be set.

Check the required boxes and type in the number of the images where the bleach event is required. The number has to be lower than the overall number of images chosen in the time series dialogue. Start the bleach series with **StartB** (!) in the **Time Series** window.

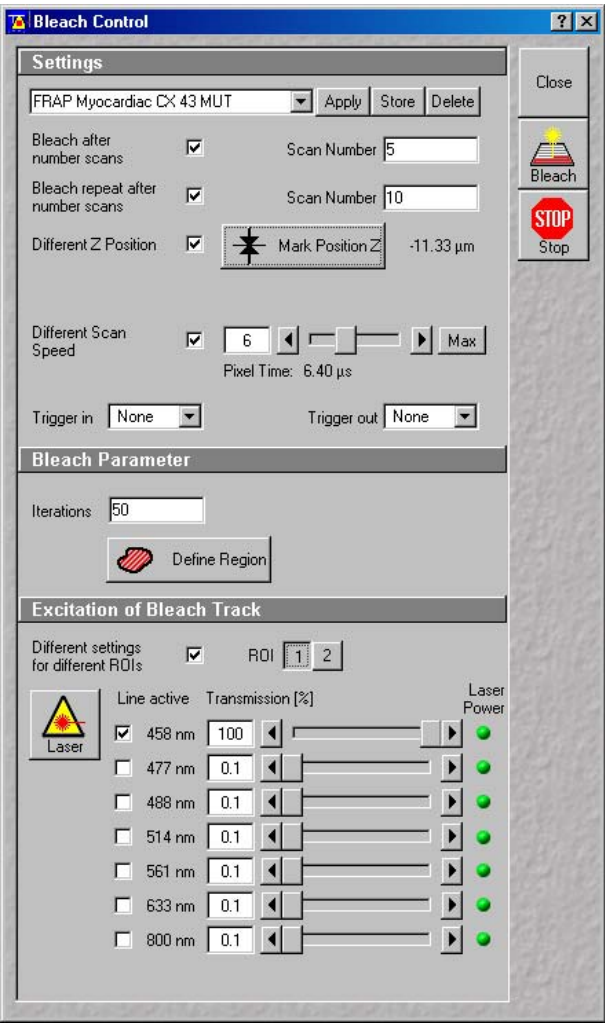


Fig. 4-96 Bleach Control Window

#### 4.5.8 Edit Bleach (LSM 5 LIVE DuoScan)

The use of this function permits the setting of bleaching parameters for spot, line or frame bleaching.

##### 4.5.8.1 Open / Close the Edit Bleach Window

- Click on the **Edit Bleach** button in the **Acquire** subordinate toolbar of the Main menu. The **Bleach Control**[Help190] 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.
- Stop** button      Ends the bleaching procedure.

#### 4.5.8.2 Settings Panel

The **Settings**<sup>[Help191]</sup> 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.

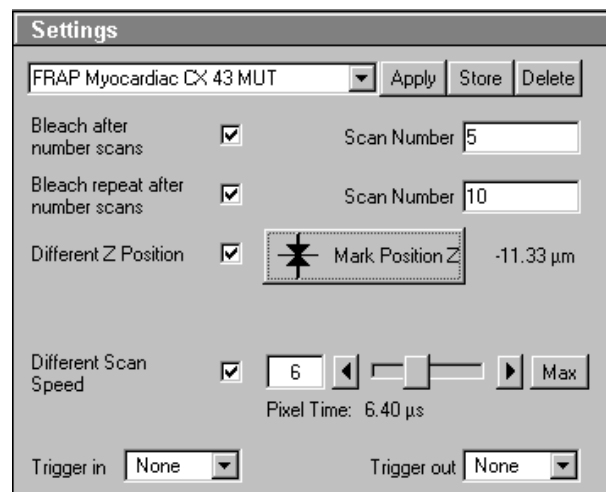


Fig. 4-97 Settings panel

- |   |  |
|---|--|
| <b>Bleach after number scans:</b>         | If this check box is ticked <input checked="" type="checkbox"/> , the bleaching procedure is automatically performed in combination with a time series. Under <b>Scan Number</b> , you must enter after how many scanning procedures bleaching is to be performed.   |
| <b>Scan Number:</b>                       | Number of Scans in a time series, after performance of which the bleaching procedure shall be started.   |
| <b>Bleach repeat after number scans:</b>  | If this check box is ticked <input checked="" type="checkbox"/> , the bleaching procedure is automatically performed in combination with a time series. Under <b>Scan Number</b> , you must enter after how many scanning procedures bleaching is to be repeated.  |
| <b>Scan Number:</b>                       | Number of Scans in a time series, after performance of which the bleaching procedure shall be repeated.  |
| <b>Different Z Position:</b>              | If this check box is ticked <input checked="" type="checkbox"/> , you can set the current stage position as the one in which the bleaching will be done by clicking the <b>Mark Position Z</b> button. This function is only available using the <b>Line</b> or <b>Frame</b> scanning mode.  |
| <b>Different Scan Speed</b>               | If this check box is ticked <input checked="" type="checkbox"/> , 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.  |
| <b>Different XY Spot Bleach Position:</b> | If this check box is ticked <input checked="" type="checkbox"/> , you can set a different XY position for spot bleaching. This function is only available using the <b>Spot</b> scanning mode. Click on the <b>Spot Select</b> button in the <b>Scan Control</b> 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**

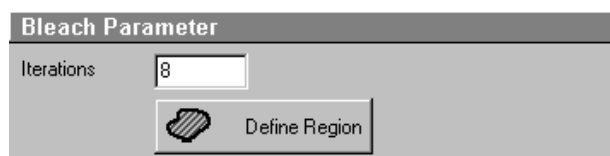
Settings which are no longer required can be deleted.

- 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**).

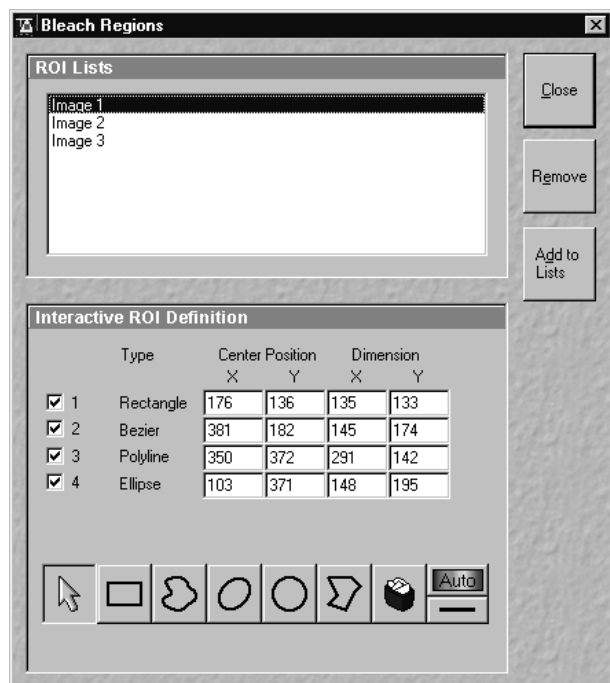
It is also possible to trigger an out signal via trigger control.



**Fig. 4-98** Bleach Parameter panel

### **4.5.8.3 Bleach Parameter Panel**

The **Bleach Parameter**<sup>[Help193]</sup> 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.




**Fig. 4-99** Bleach Regions window

- Enter the number of iterations of the bleaching procedure in the **Iterations** input box.
- Click on the **Define Region** button.
  - The **Bleach Regions**<sup>[Help194]</sup> window appears.

The definition of bleach regions corresponds to the **Edit ROI** function and is performed in the same way (see **Edit ROI**, page 4-90).

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.

#### 4.5.8.4 Excitation of Bleach Track Panel

In the **Excitation of Bleach Track** panel you can select the lasers and laser intensities for bleaching.

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**).

- 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.

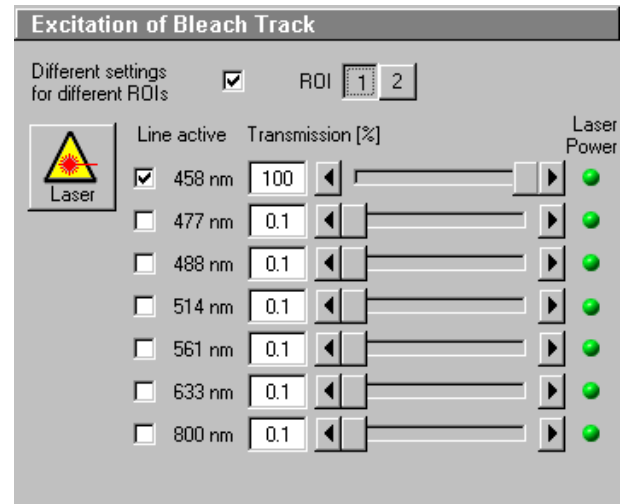


Fig. 4-100 Excitation of Bleach Track panel

#### 4.5.8.5 Start / End a Bleaching Procedure

- The bleaching process will be started via the **Bleach** button. However, it is also possible to start the bleaching process via the **Bleach** button in the **Time Series Control**<sup>[Help201]</sup> window or to combine it with a time series.

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**<sup>[Help202]</sup> in the **Bleach Control** window.

 **Stop** does not only stop the bleaching process, but the entire scanning process.

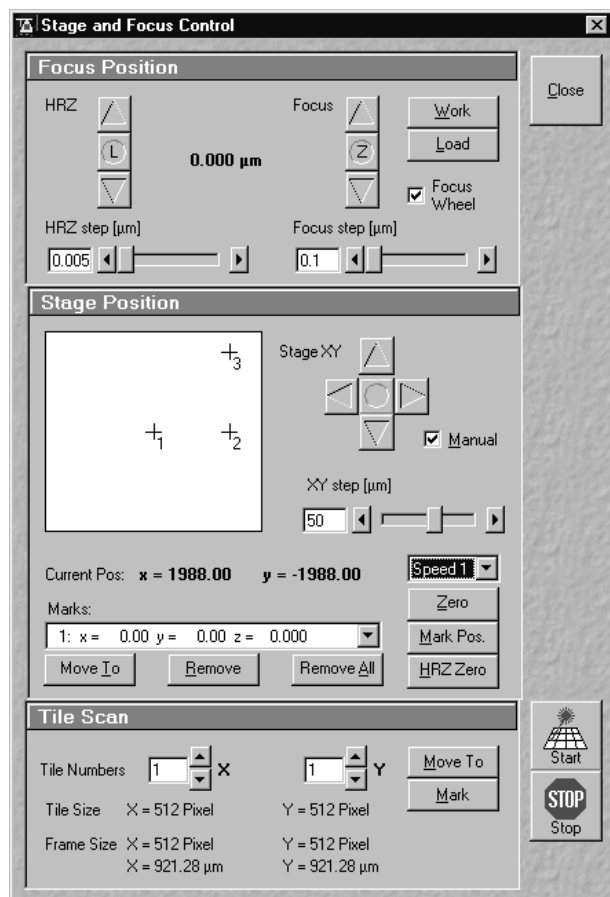


Fig. 4-101 Stage and Focus Control window

### 4.5.9 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 5 LIVE scanhead on an inverted 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.

#### 4.5.9.1 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.

#### 4.5.9.2 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.

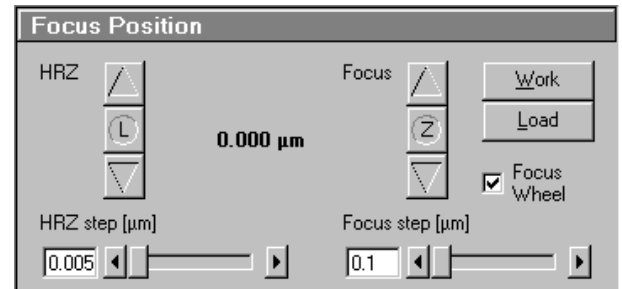


Fig. 4-102 Focus Position panel

##### 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.

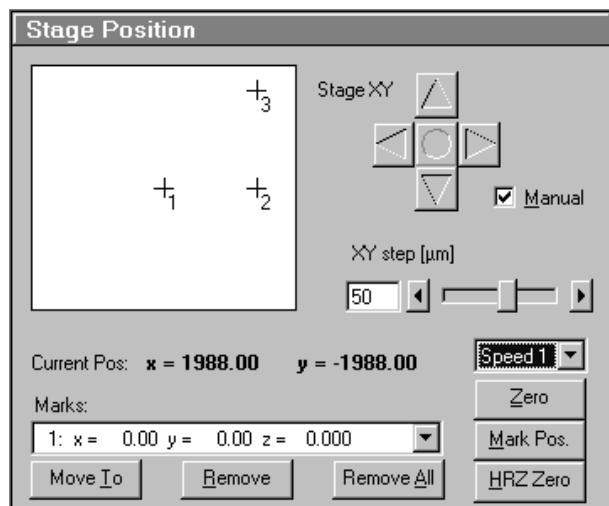


Fig. 4-103 Stage Position panel

#### 4.5.9.3 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.



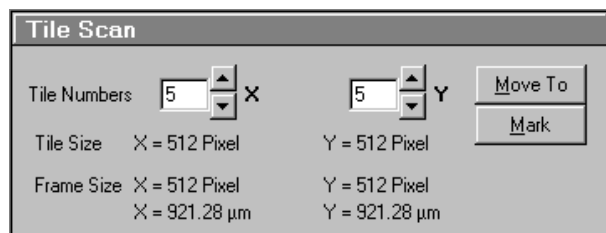


Fig. 4-104 Tile Scan window

#### 4.5.9.4 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  $\mu\text{m}$  (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  $\mu\text{m}$ .

#### 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.

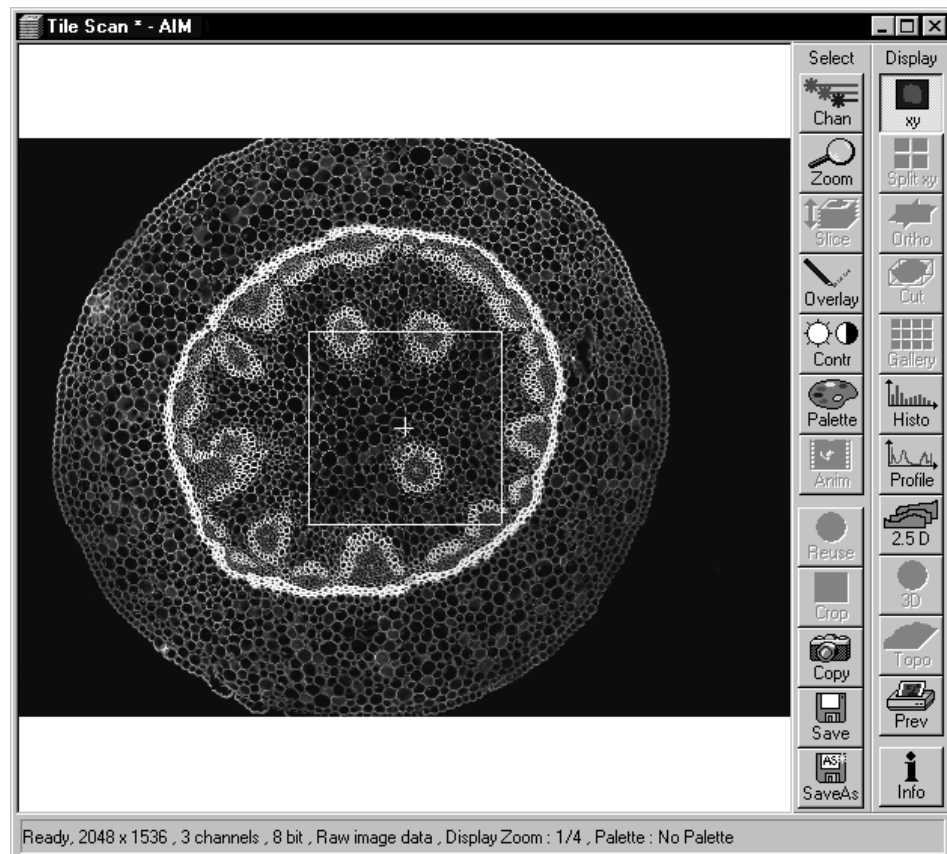


Fig. 4-105 Image Display window of a Tile Scan

- 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.

#### 4.5.10 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 5 *LIVE* 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, the beam path can be "switched" via activation of the appropriate button in the software.

## 4.6 Process Menu

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

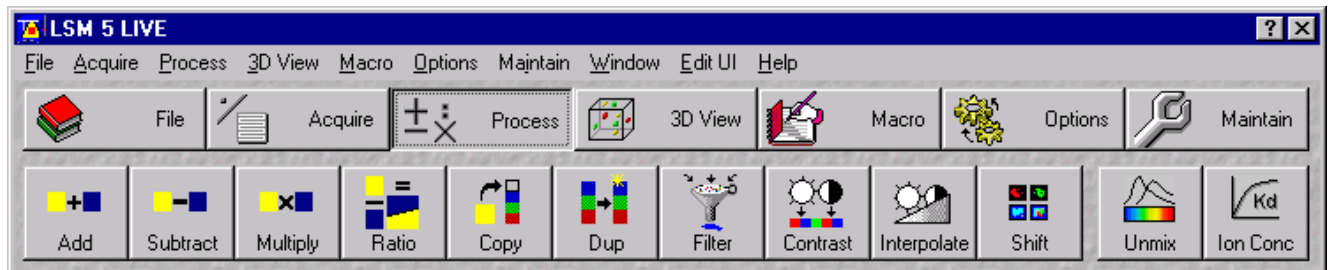


Fig. 4-106 Process menu

The functions of the **Process** menu permit already stored scan images to be subsequently linked and processed using mathematical functions and algorithms.

### 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.

#### 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.

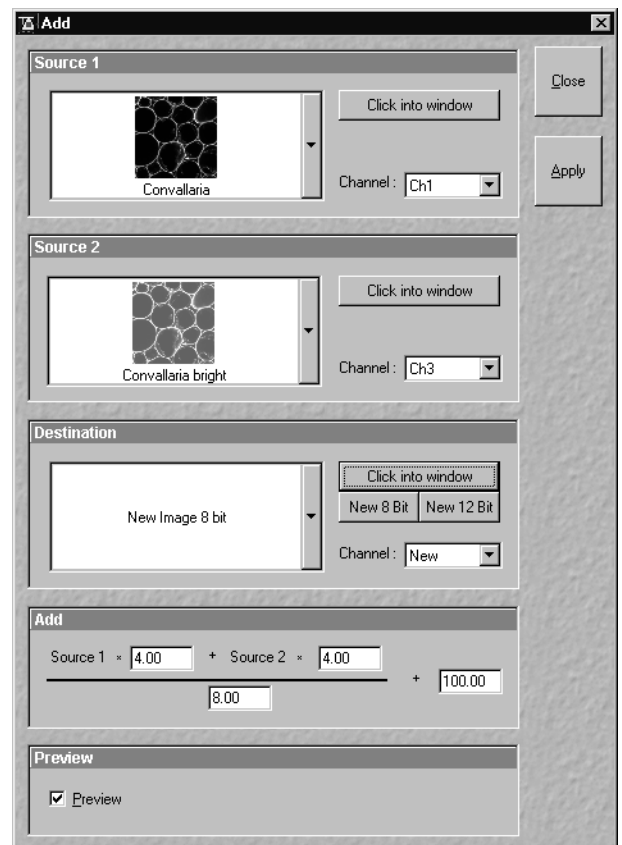


Fig. 4-107 Add window

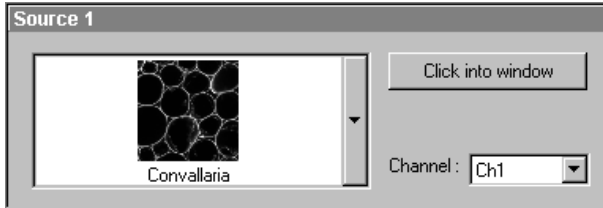


Fig. 4-108 Source 1 panel

- 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.

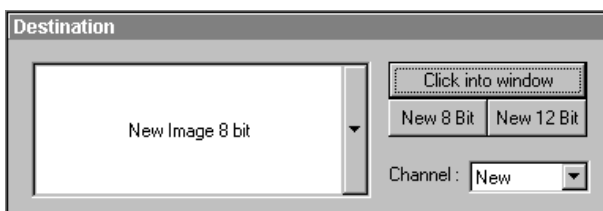


Fig. 4-109 Destination panel

- 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.

#### 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:

#### 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.

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.

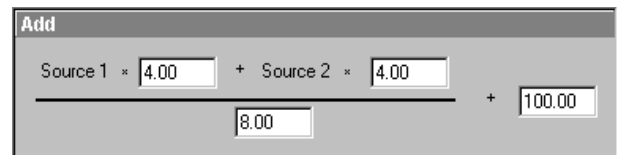


Fig. 4-110 Add panel

- 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. 4-111 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.

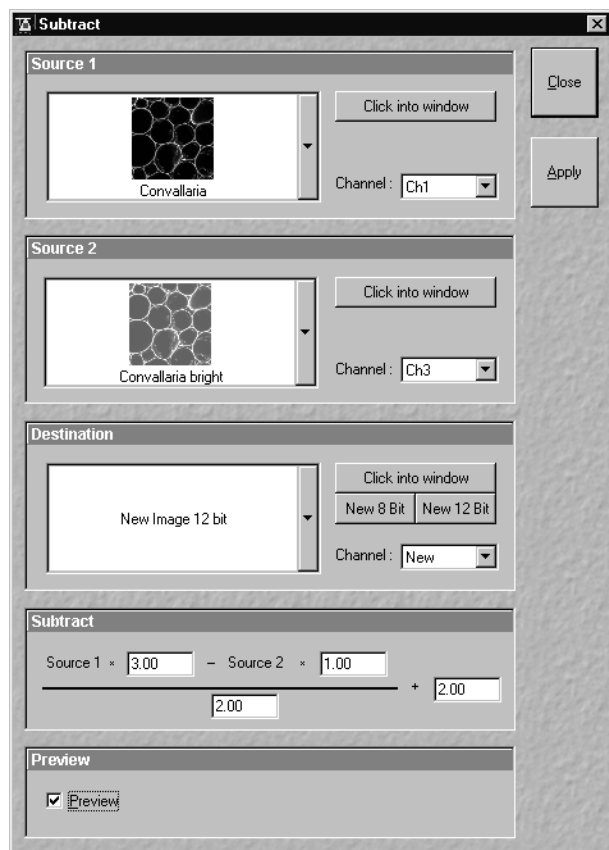


Fig. 4-112 Subtract window

## 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 4-121). 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.

#### 4.6.3.1 Open / Close the Multiply Window

- Click on the **Multiply** button in the **Process** subordinate toolbar 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 4-121). The only difference is that the mathematical formula is based on multiplication.

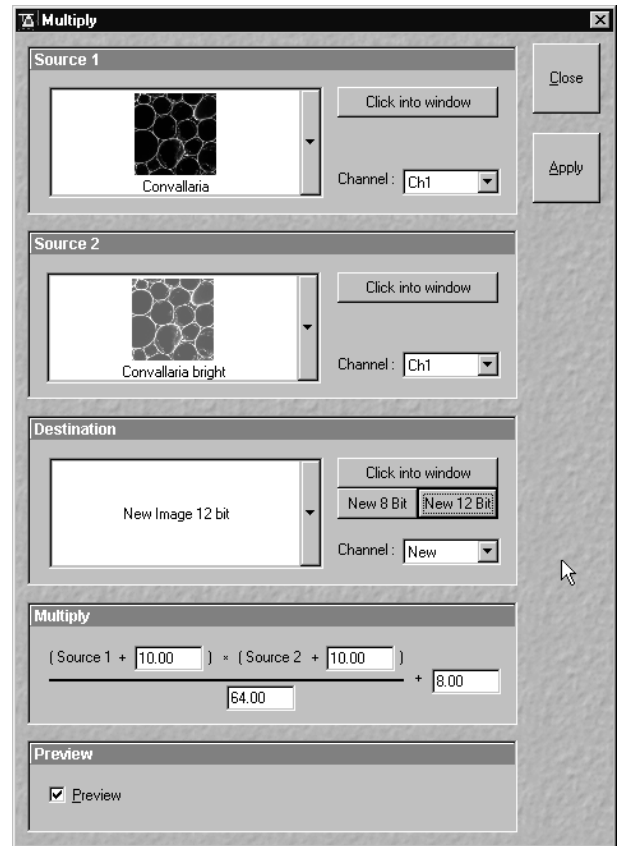


Fig. 4-113 Multiply window



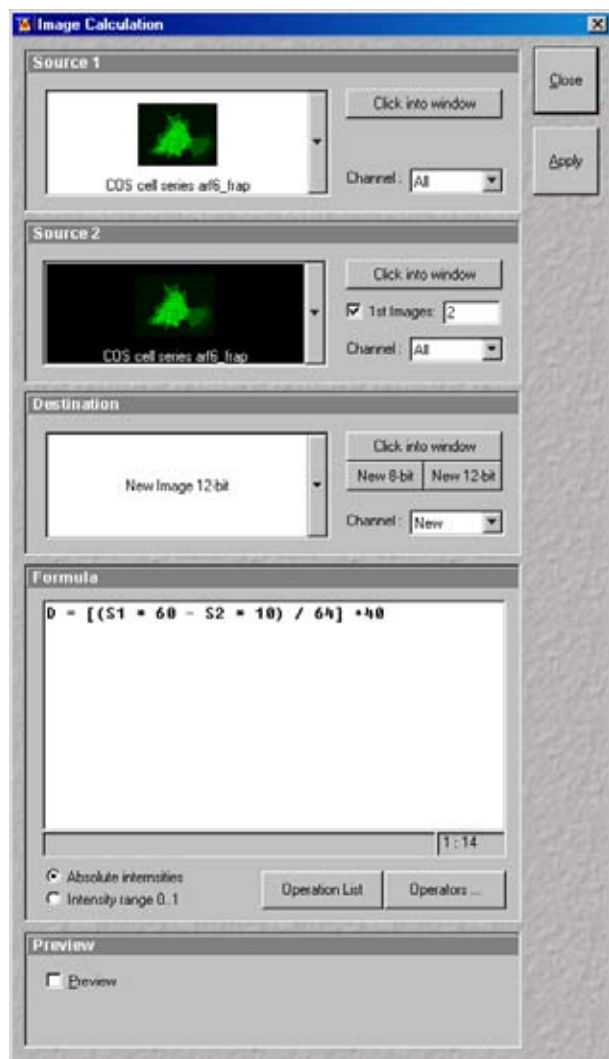


Fig. 4-114 Image Calculation window

#### 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 4-121).

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. 4-115) 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.

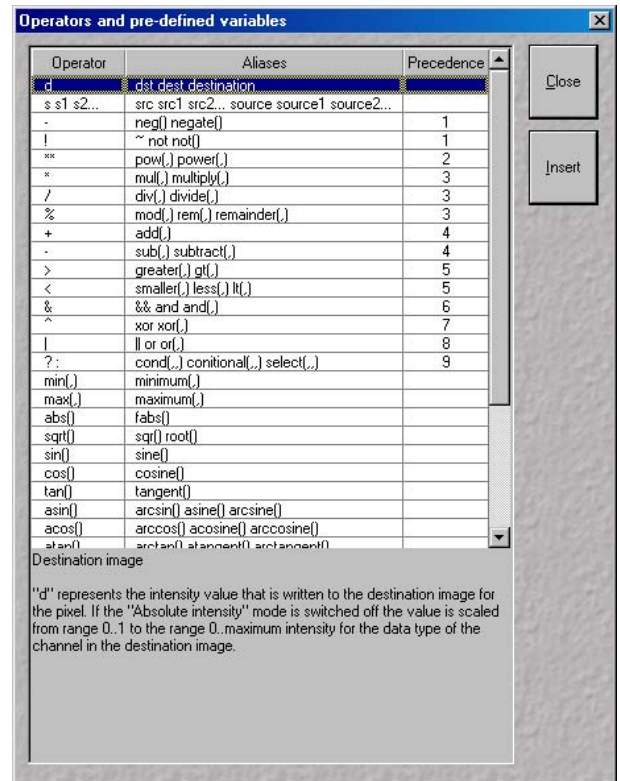


Fig. 4-115 Operators window

#### 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 4-121).

##### 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.

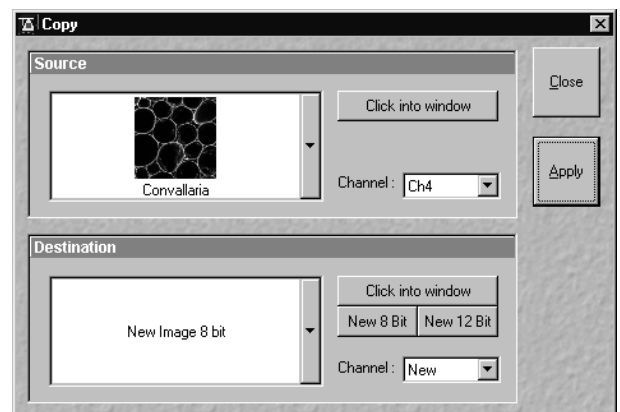


Fig. 4-116 Copy window

#### 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.

#### 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.

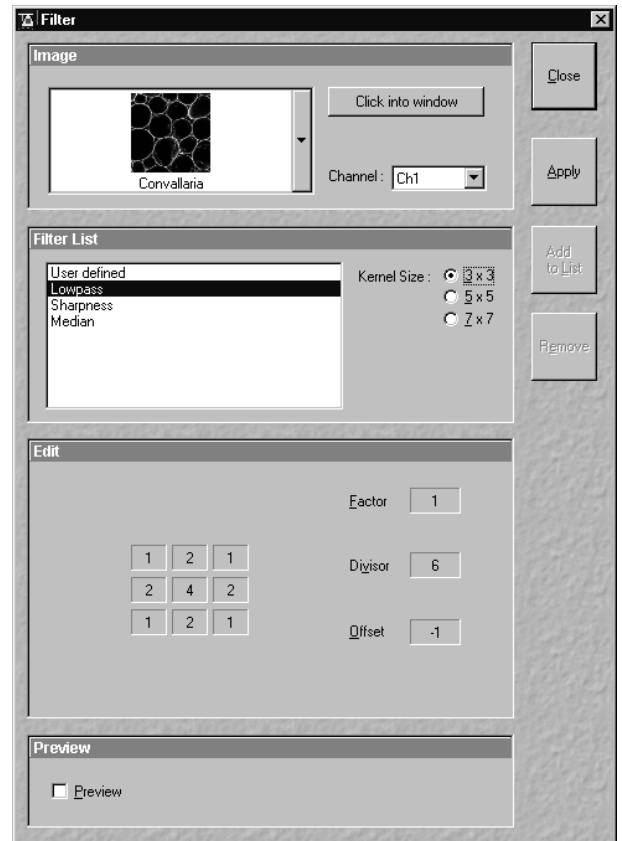


Fig. 4-117 Filter window

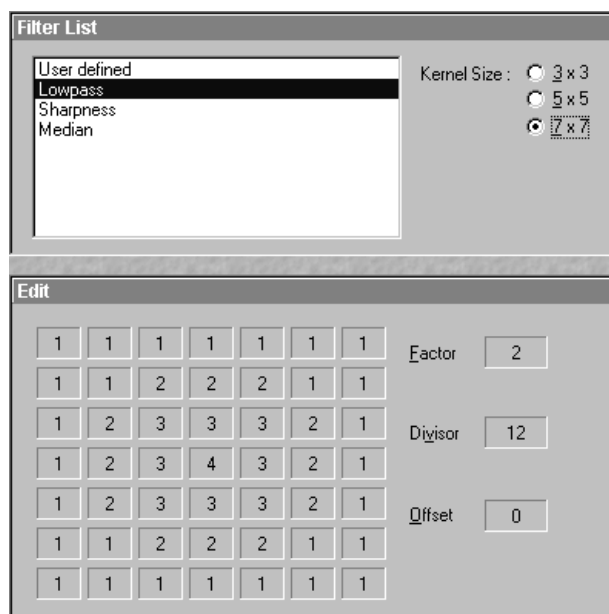


Fig. 4-118 Filter List and Edit panel (Lowpass)

**(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.

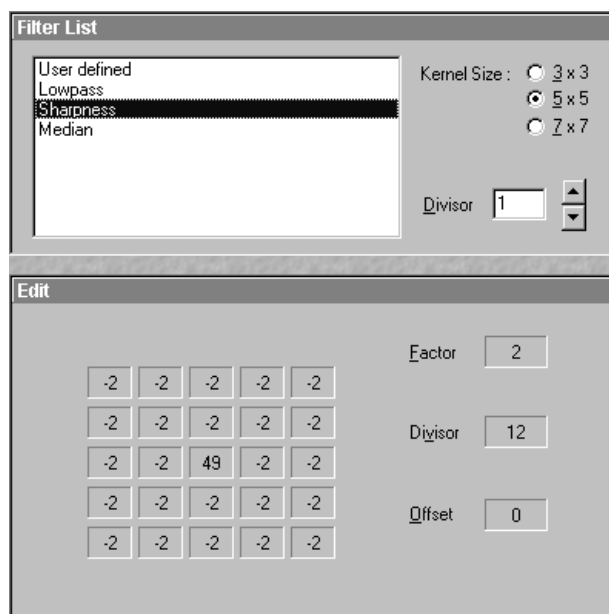


Fig. 4-119 Filter List and Edit panel (Sharpness)

**(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.

#### (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.

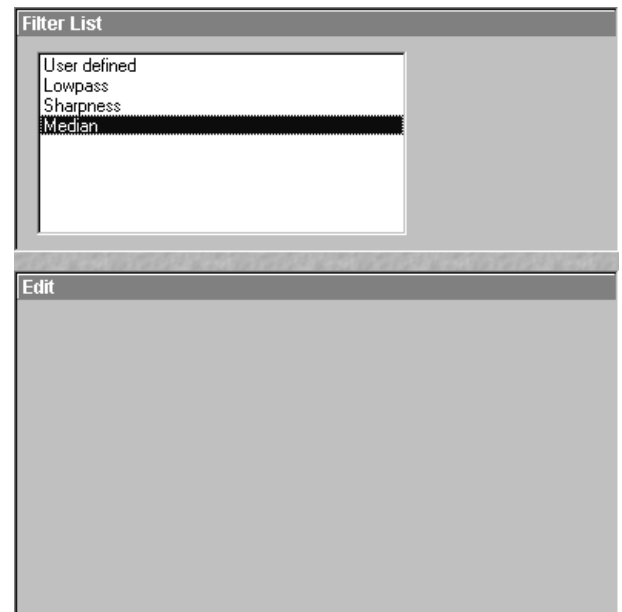


Fig. 4-120 Filter List and Edit panel (Median)

#### (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:

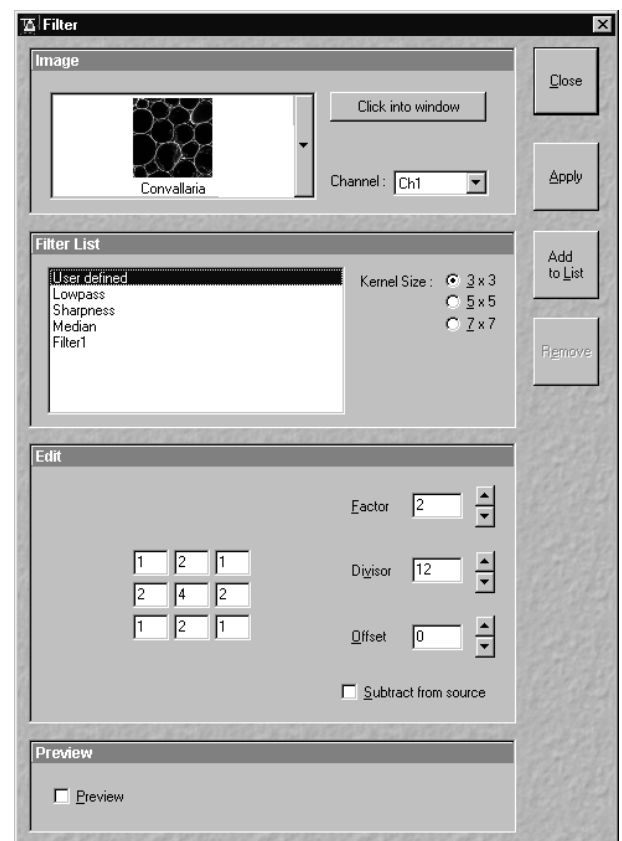


Fig. 4-121 Filter window (User-defined filter)

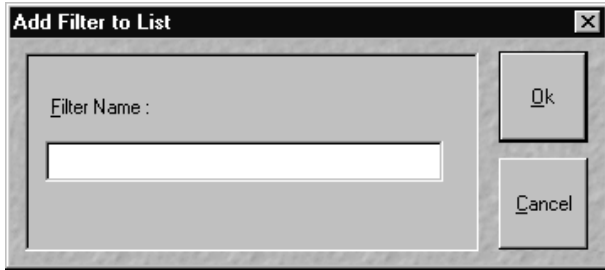


Fig. 4-122 Add Filter to List panel

- 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 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.

 The Modify Series macro can be used to convert time series data for use of the **Interpolate** function.

##### 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:

- 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.

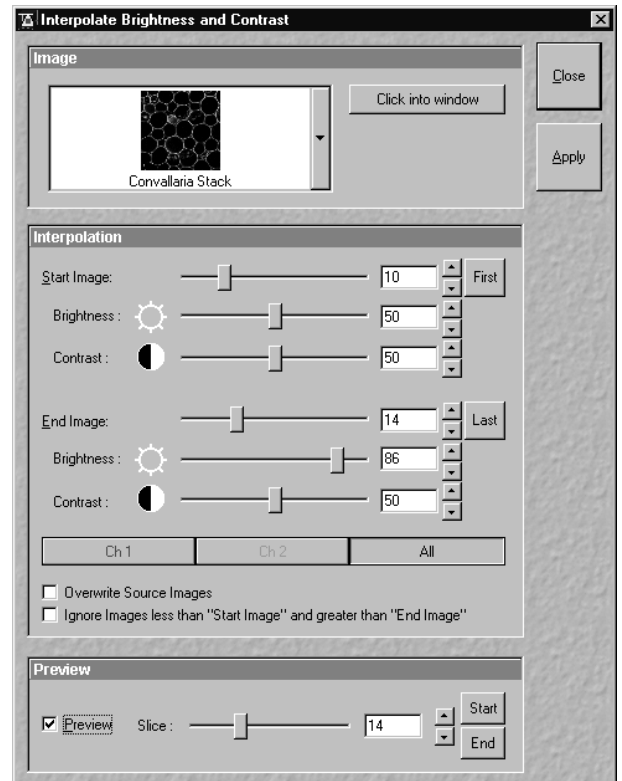


Fig. 4-124 Interpolate Brightness and Contrast window

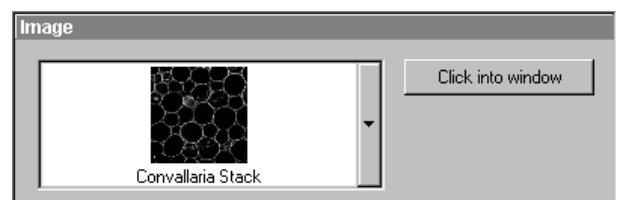


Fig. 4-125 Image panel



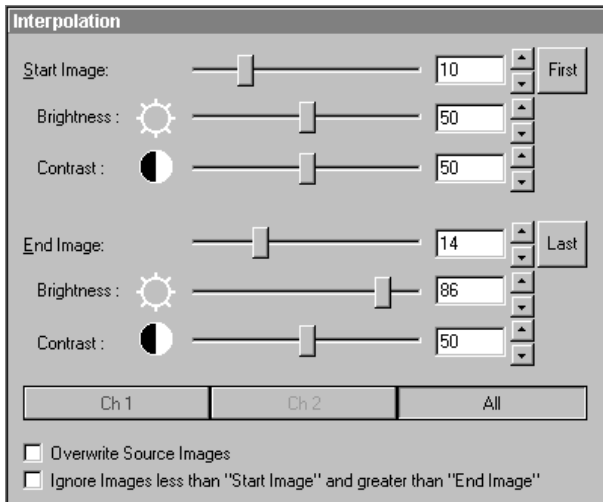


Fig. 4-126 Interpolation panel

#### 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. 4-127 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.

##### 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.

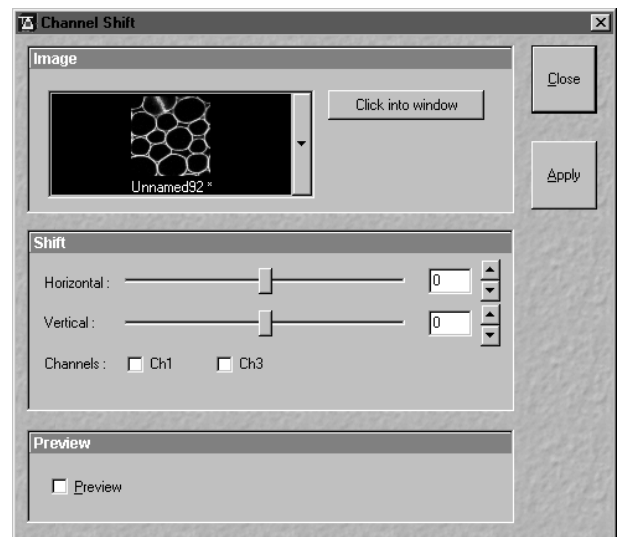
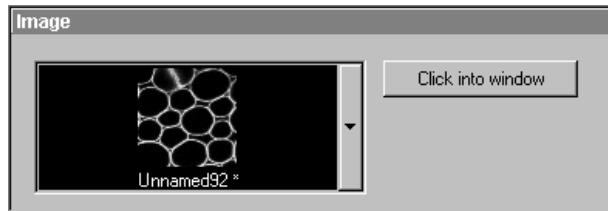



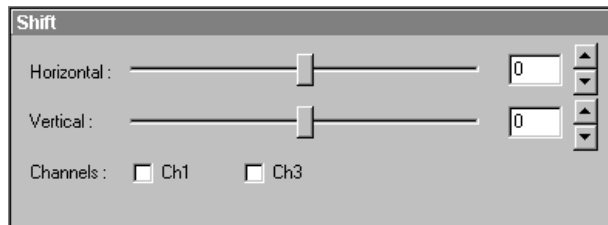
Fig. 4-128 Channel Shift window

**Fig. 4-129** 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. 4-130** 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. 4-131 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.

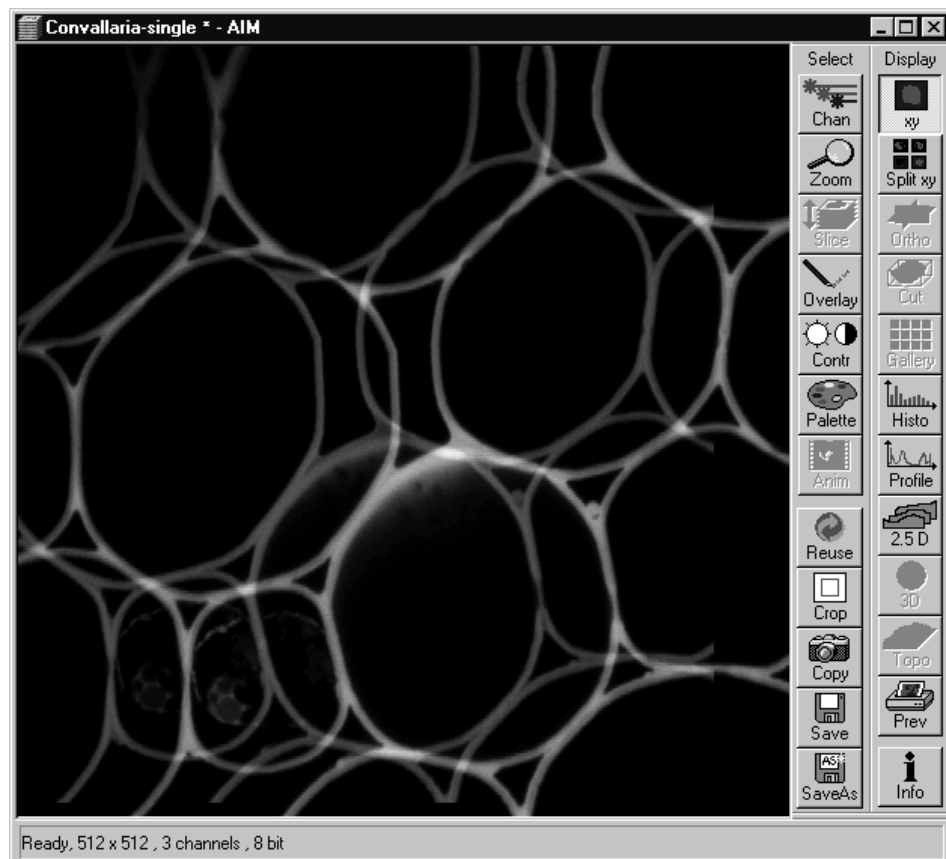


Fig. 4-132 Image Display window with channel shift

#### 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 Stack, a Stack Z series or a 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.
- 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.

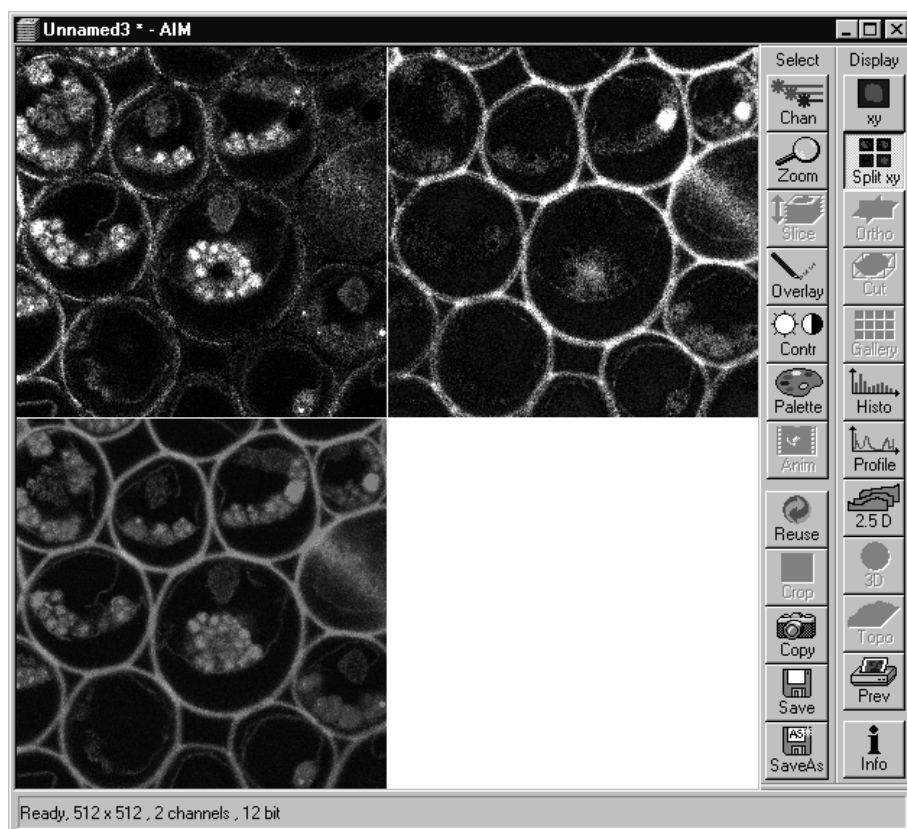
**Ignore Overexposed Pixels** and **Ignore Underexposed Pixels** will exclude this pixels for calculation of the unmixed images.

**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.



Fig. 4-133 Multichannel unmixing



**Fig. 4-134** 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.

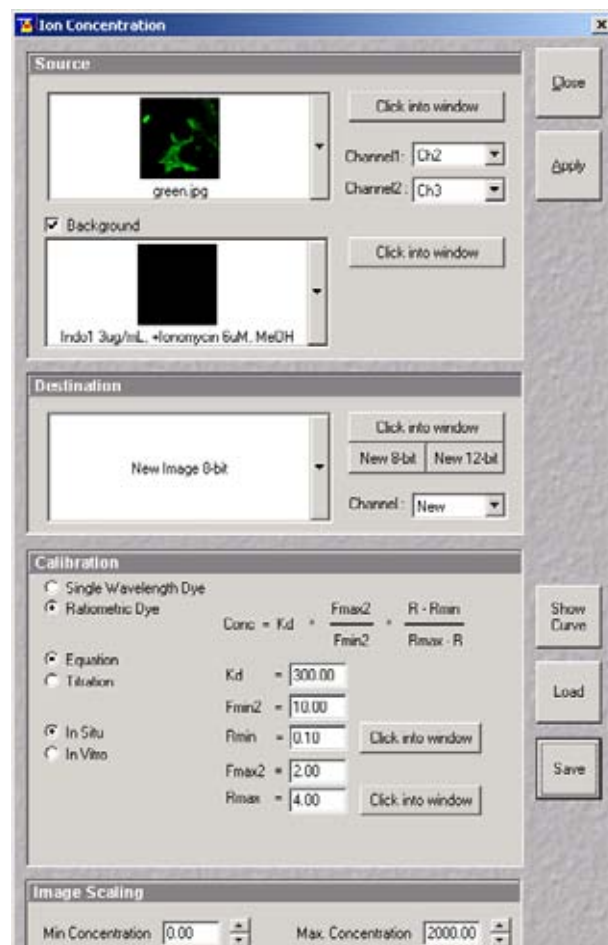


Fig. 4-135 Ion Concentration window

### 4.6.12.2 Function Description

<b>Ion Conc</b> button	Activates the <b>Ion Concentration</b> window.
<b>Source</b> window	Selects input of images to be processed.
<b>Destination</b> window	Select output and pixel depth of processed image.
<b>Calibration</b> window	Sets the six different calibration options, according to the dyes used (single wavelength, ratiometric) and required method.
<b>Show Curve</b> button	Shows resulting calibration curve.
<b>Image scaling</b> window	Sets min. and max. concentration.
<b>Preview</b> window	Activates <b>Preview</b> 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).

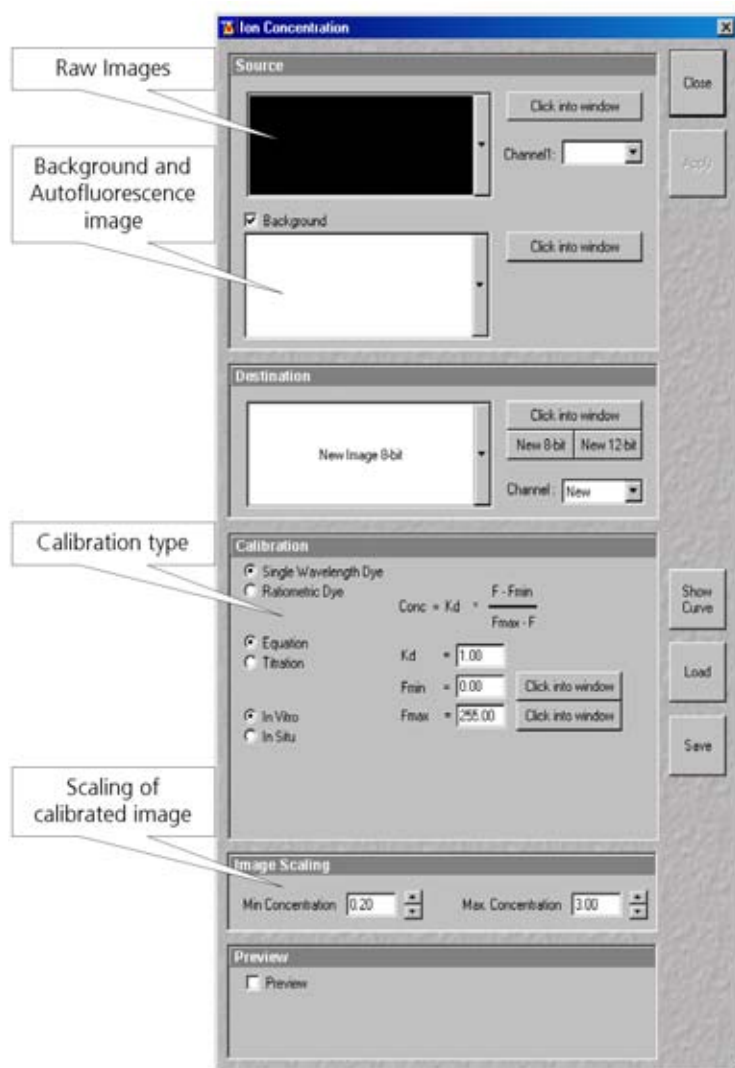


Fig. 4-136 Ion Concentration window

#### 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

$$R(t_1) = F_1(t_1) / F_2(t_1), R(t_2) = F_1(t_2) / F_2(t_2) \dots$$

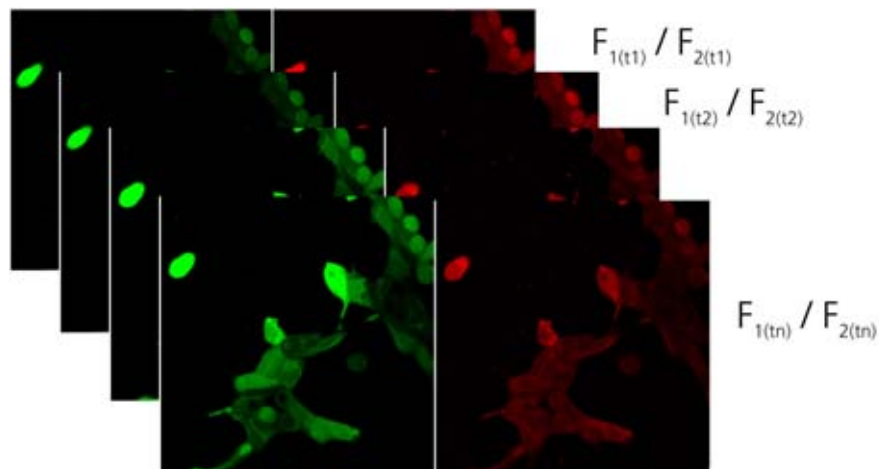


Fig. 4-137 Ratiometric Dyes – Online ratio

#### 4.6.12.6 Ratiometric Dyes - Calibration

- Subtract background/autofluorescence images from raw images to obtain  
 $R_{\text{corr}} [(F_1 - F_{1\text{Background}}) / (F_2 - F_{2\text{Background}})]$   
 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)

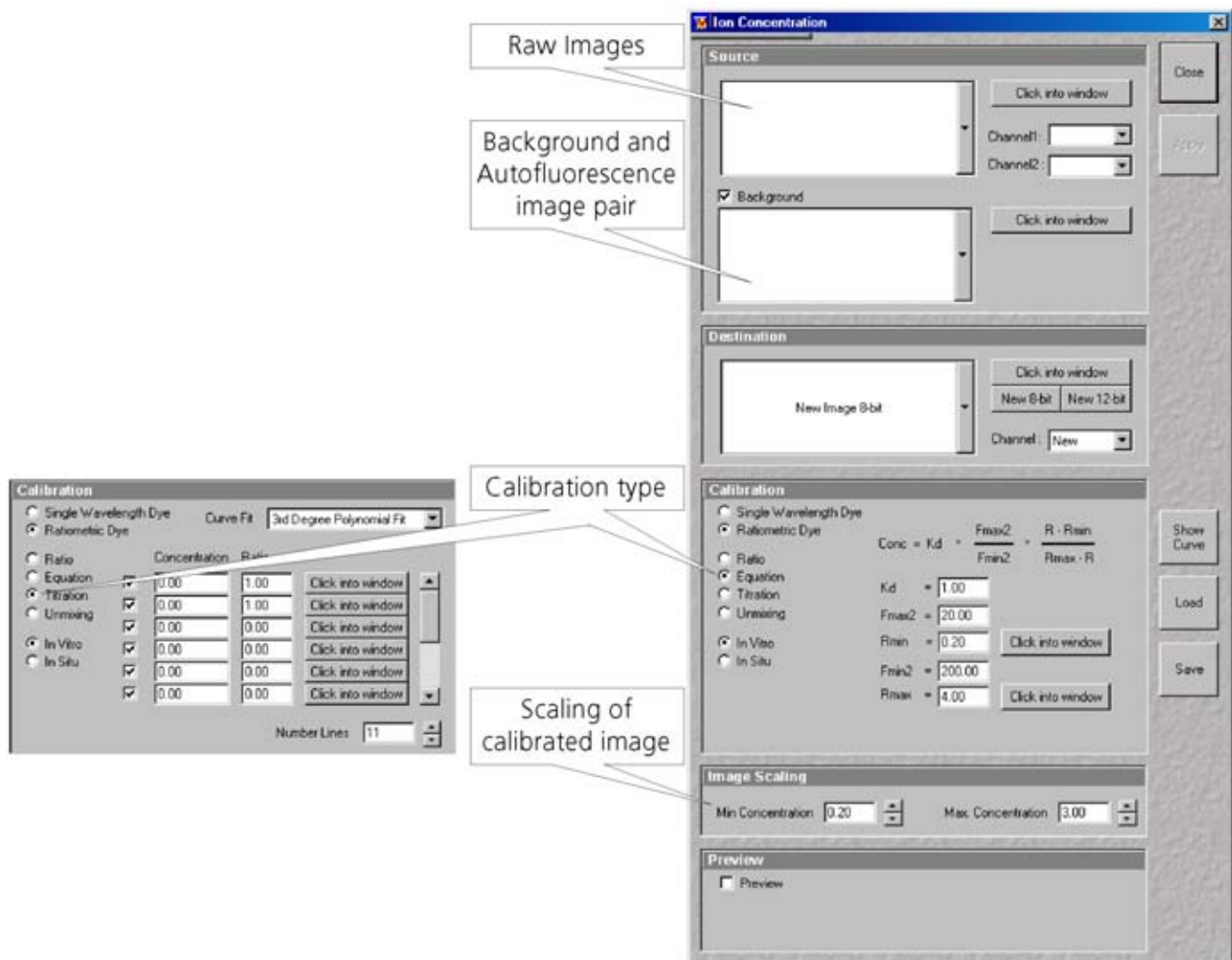


Fig. 4-138 Ratiometric Dyes - Calibration

#### 4.6.12.7 Ratiometric Dyes - Equation Calibration (Grynkiewicz)

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)

**Calibration**

☐ Single Wavelength Dye  
☒ Ratiometric Dye

Conc =  $K_d \times \frac{F_{max2}}{F_{min2}} \times \frac{R - R_{min}}{R_{max} - R}$

☐ Ratio  
☒ Equation  
☐ Titration  
☐ Unmixing

☒ In Vitro  
☐ In Situ

$K_d$  = 1.00  
 $F_{max2}$  = 20.00  
 $R_{min}$  = 0.20 Click into window  
 $F_{min2}$  = 200.00  
 $R_{max}$  = 4.00 Click into window

Fig. 4-139 Ion Concentration button

#### 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 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).

#### 4.6.14 Copy Window Contents

The use of this function permits to duplicate the displayed image.

#### 4.6.15 Copy Full Resolution

The use of this function permits to display the current image with full resolution in a new Image Display window.

#### 4.6.16 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.

#### 4.6.17 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.

##### 4.6.17.1 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.

---

#### 4.6.17.2 Function Description

<b>Click into window</b>	Allows to select the image series of the FRAP experiment to be analyzed by clicking into the appropriate image window.
<b>Channel</b> pull down menu	Allows to select single channels or all channels for analysis.
<b>Kinetic Model</b> pull down menu	Allows to select the mathematical model (mono or double exponential model) for fitting the data.
<b>Background Region</b> checkbox	Displays a button <b>Select (Click)</b> 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.
<b>Reference Region</b> checkbox	Displays a button <b>Select (Click)</b> 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.
<b>Combine Regions</b> checkbox	Opens a further dialogue with the following options: <b>Add Region, New Group, Remove Group</b> and <b>Remove All</b> . 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 <b>Add Region</b> , 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.
<b>Apply</b>	Performs the calculations and the fitting of the data.

#### 4.6.17.3 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 choose 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 4-312 for further details.

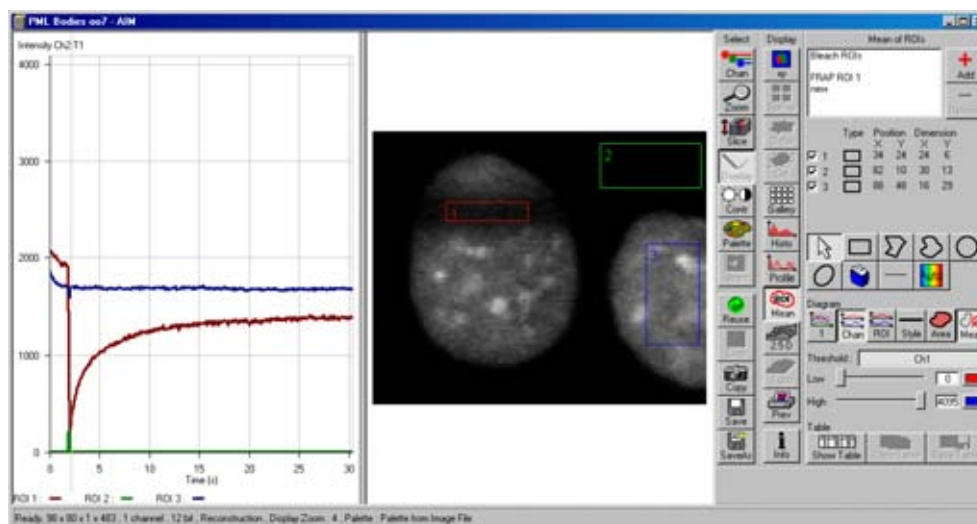


Fig. 4-140 Image window displaying the first bleached image of a images series


- 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.

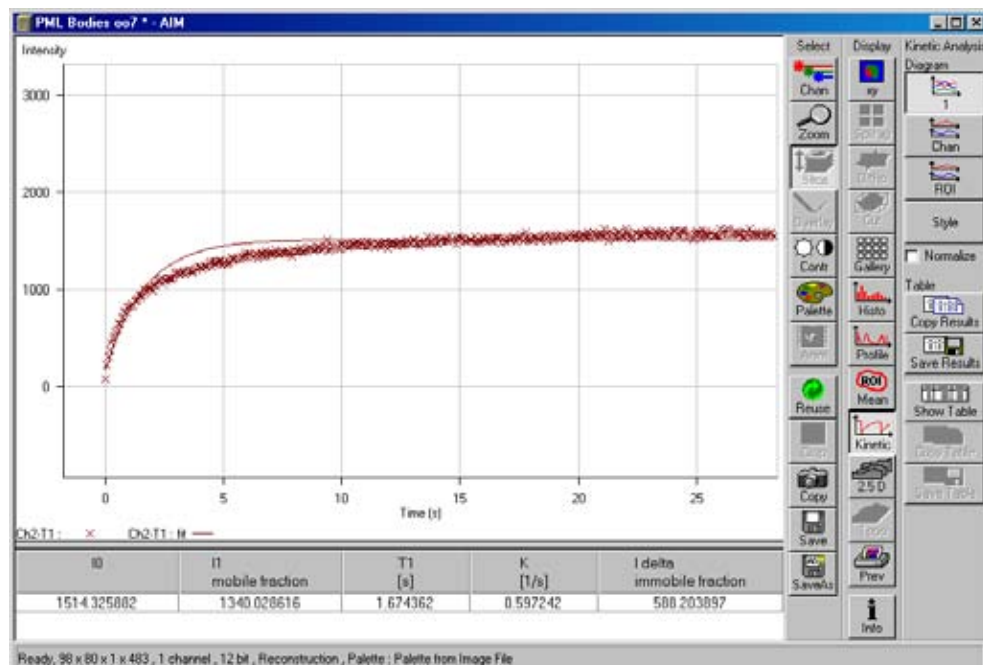
The values can be saved as a text file (**Save Table**) or copied into Excel via clipboard (**Copy Table**).

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:

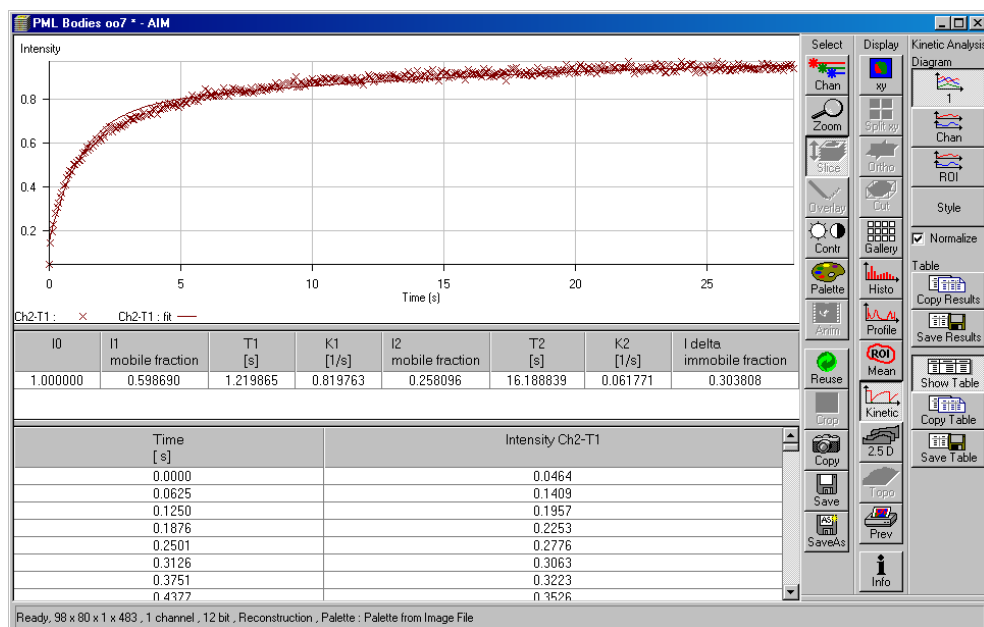
$$t_{\text{half}} = \frac{\ln 0.5}{-T1}$$


**Fig. 4-141** 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.

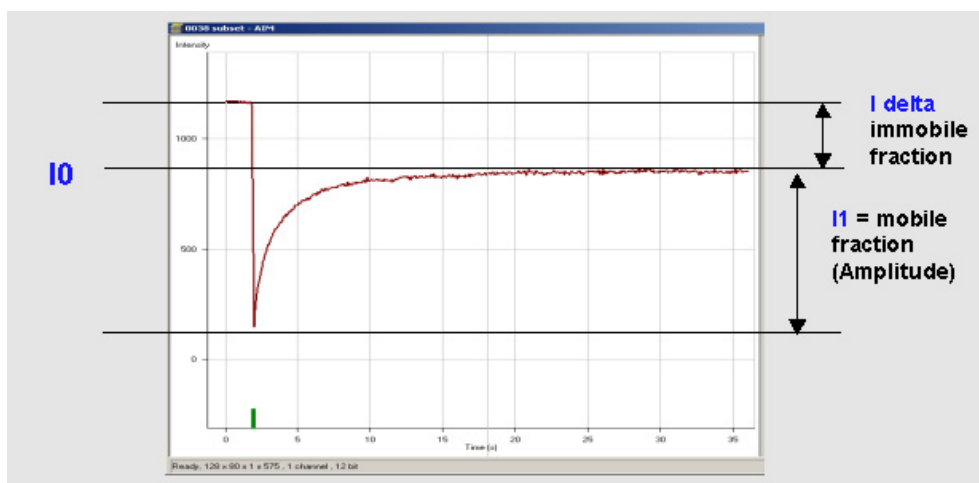


**Fig. 4-142 Image window displaying the analysis of FRAP data using a double exponential fit**

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. 4-143. Please note, that the naming of the data points is not consistent with the information on the website.



**Fig. 4-143** Schematic FRAP curve with marks at the relevant data points. I0, I1 and I delta which are calculated performing the Kinetic Analysis

## 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.

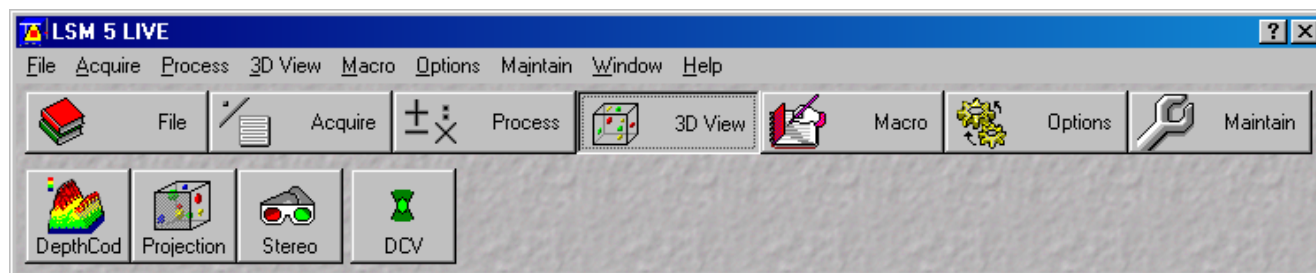


Fig. 4-144 3D View menu

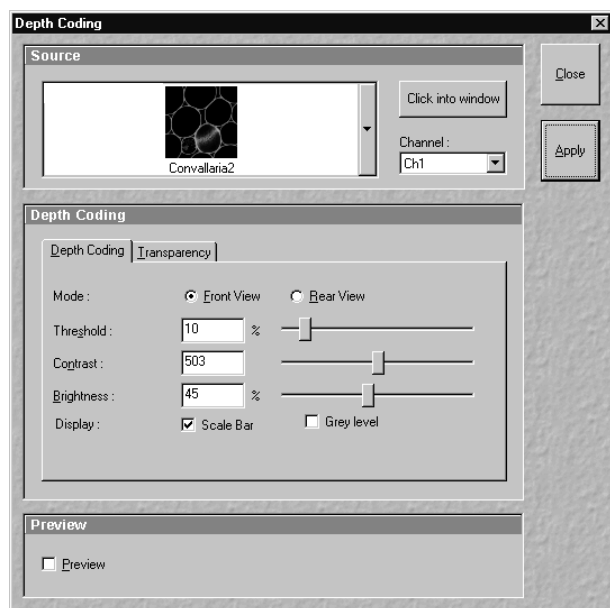


Fig. 4-145 Depth Coding window

### 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.

#### 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.

#### 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.

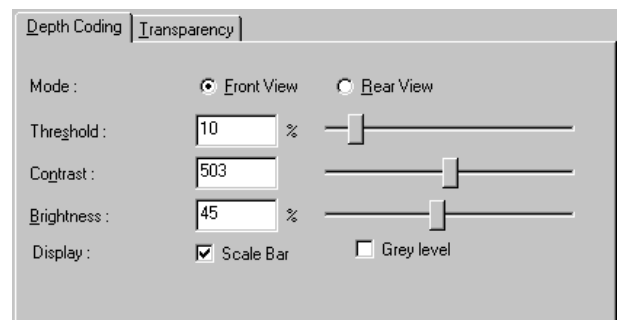


Fig. 4-146 Depth Coding tab

#### (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).

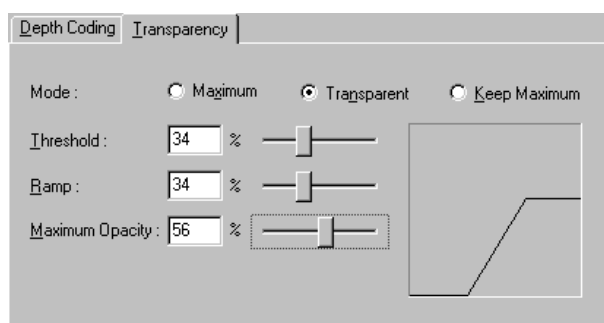


Fig. 4-147 Transparency tab

#### 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.

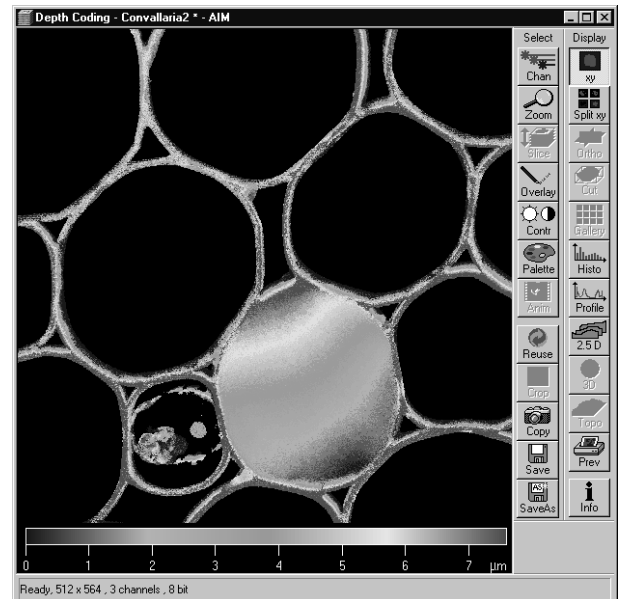


Fig. 4-148 Depth Coding image

#### 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.

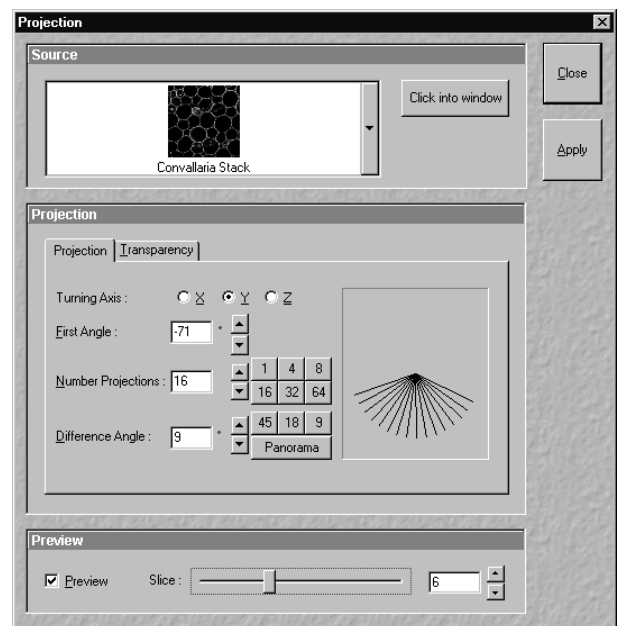


Fig. 4-149 Projection window

##### 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.

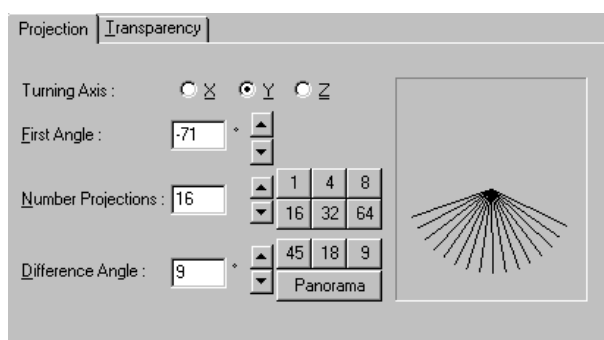


Fig. 4-150 Projection tab

## (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).

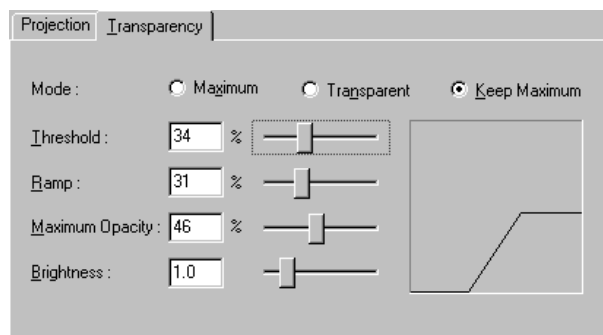


Fig. 4-151 Transparency tab

### 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.

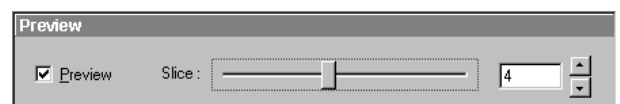


Fig. 4-152 Preview panel



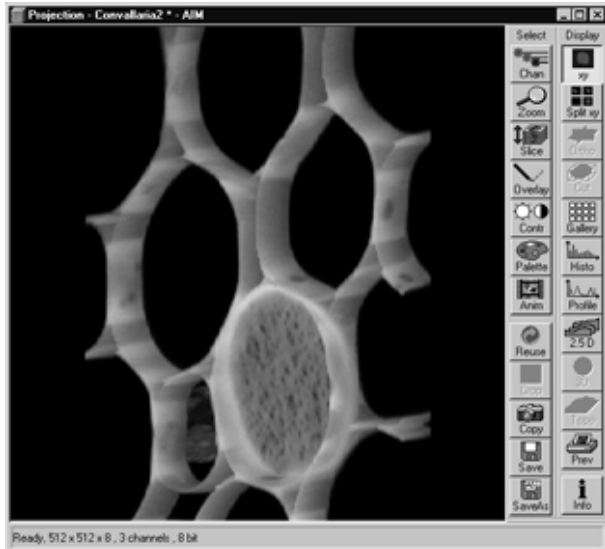


Fig. 4-153 Projection image

- 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.

 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 4-239).

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.

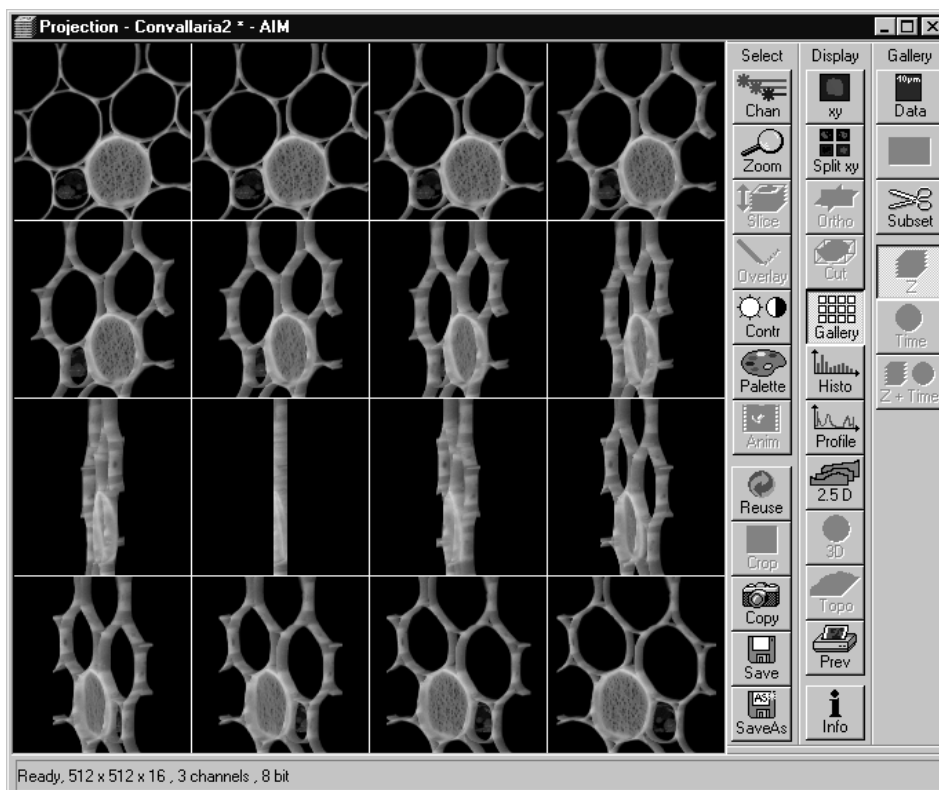


Fig. 4-154 Projection image (Gallery)

### 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.

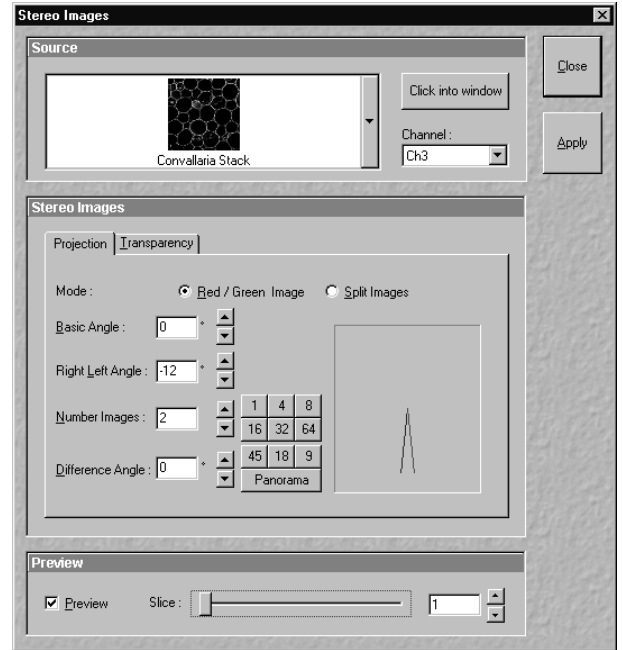


Fig. 4-155 Stereo Images window

#### 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.

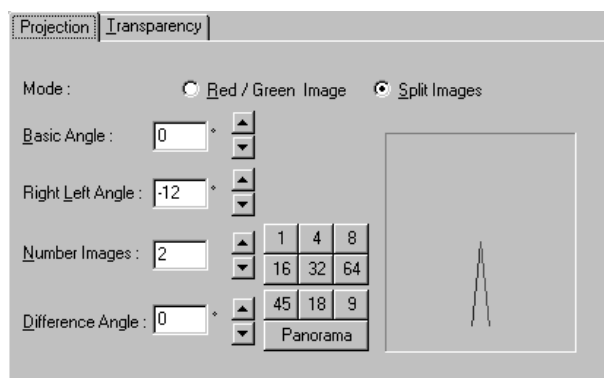



Fig. 4-156 Projection tab

 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).

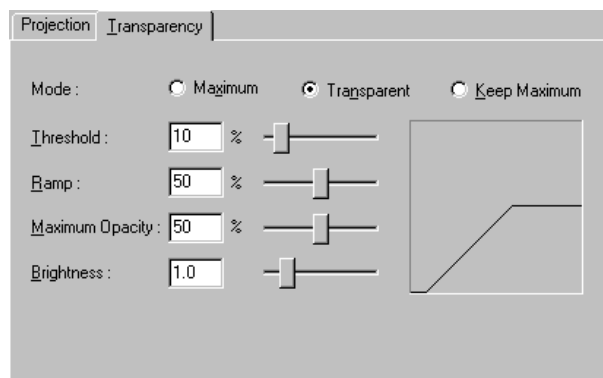


Fig. 4-157 Transparency tab



Fig. 4-158 Preview panel

#### 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.

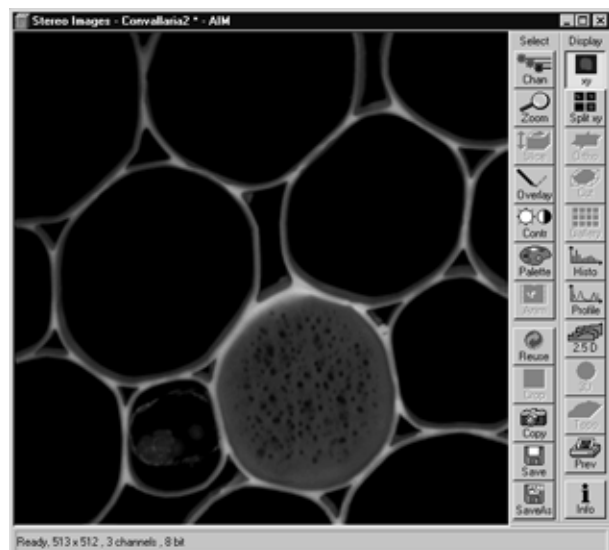



Fig. 4-159 Stereoscopic image (red / green)

- 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.

- By clicking on the **Apply** button, the two stereo pairs are presented side by side and can be viewed without red / green 3D goggles.

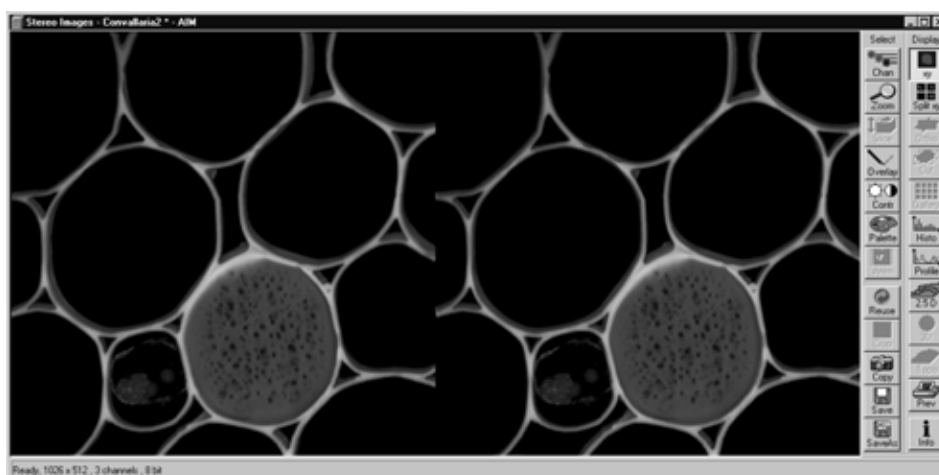


Fig. 4-160 Stereoscopic image (split)

#### 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)**.

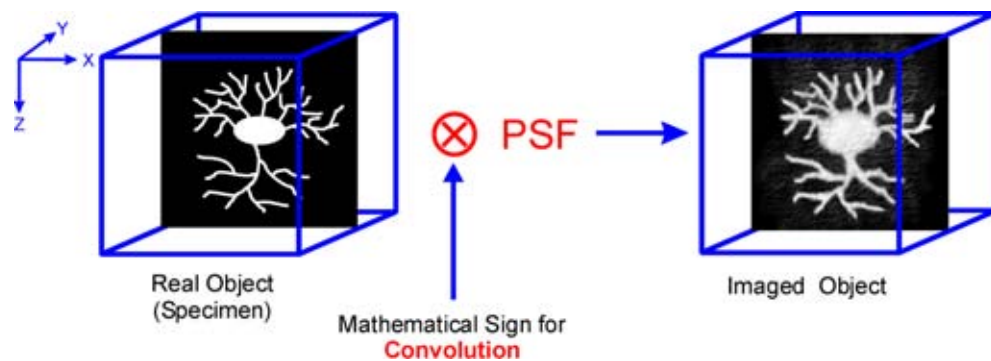


Fig. 4-161 Point Spread Function (PSF)

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:

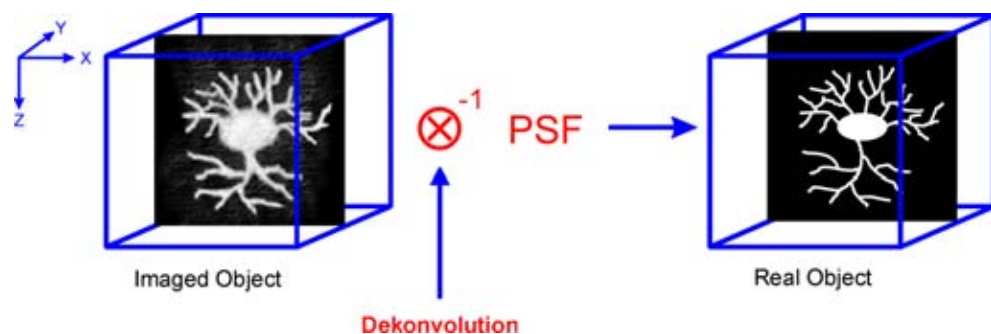
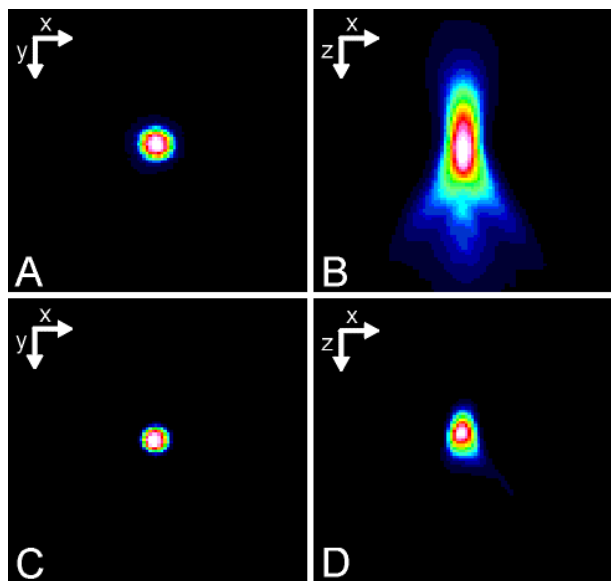


Fig. 4-162 Point Spread Function (PSF)

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  $\mu\text{m}$ .



**Fig. 4-163** Image of a fluorescent bead with a diameter of  $1\mu\text{m}$  before deconvolution (A,B) and after deconvolution (C,D)

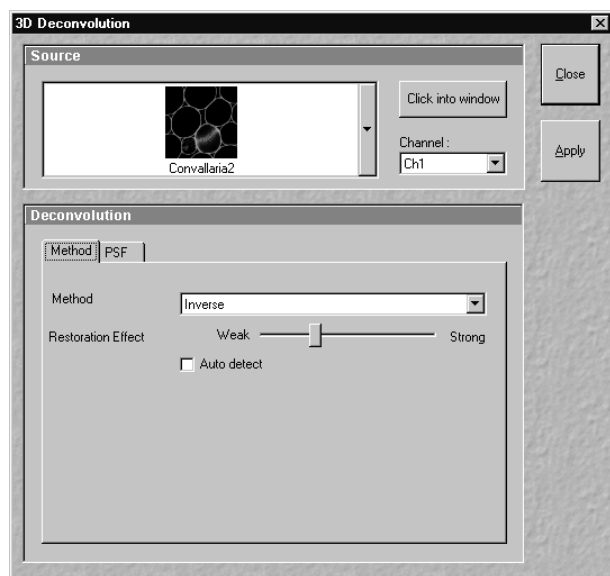
As the resolution of an optical system is significantly lower in the axial direction than in the lateral (X/Y-)direction, the greatest improvement in resolution can be achieved in the Z-direction.

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. 4-164** 3D Deconvolution window

#### 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.

#### 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:

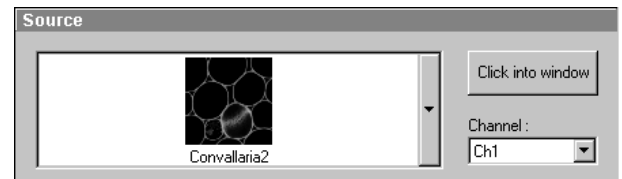


Fig. 4-165 Source panel

- 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.

#### 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**.

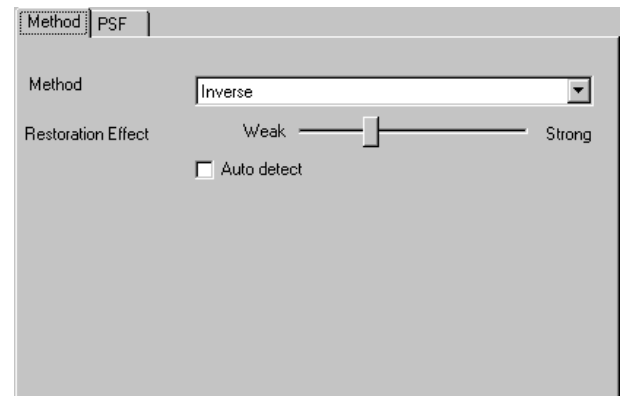


Fig. 4-166 Method tab

##### (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**.

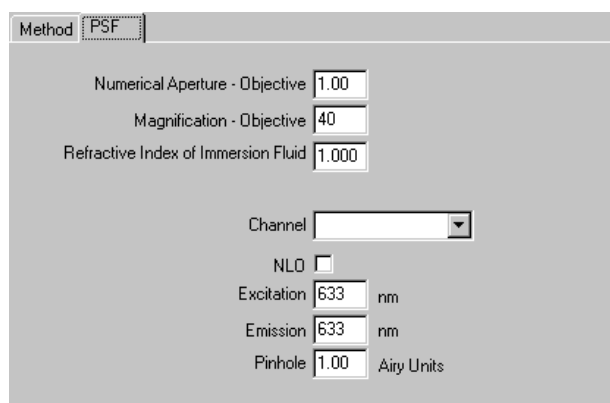


Fig. 4-167 PSF tab

## (2) PSF tab

In the 3D Deconvolution option a theoretical point spread function (PSF) is calculated from the systems settings (objective data, wavelengths, aperture size).

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.

## 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.

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 4-189 for further details.

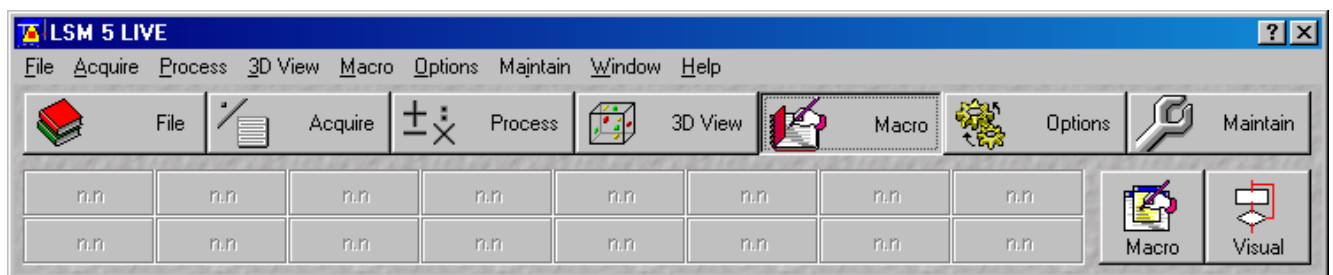


Fig. 4-168 Macro menu

### 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 5 LIVE.

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.

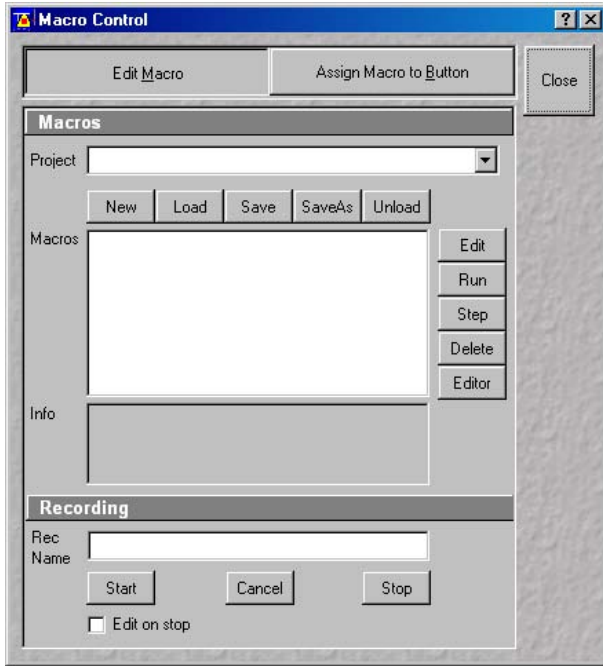


Fig. 4-169 Macro Control window

## 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.

## (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.

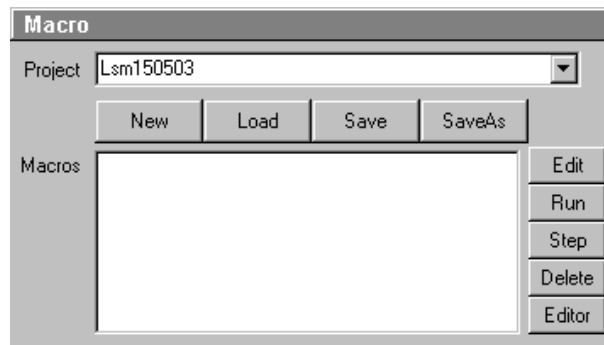


Fig. 4-170 Macro panel

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.

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.



Fig. 4-171 Open 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.

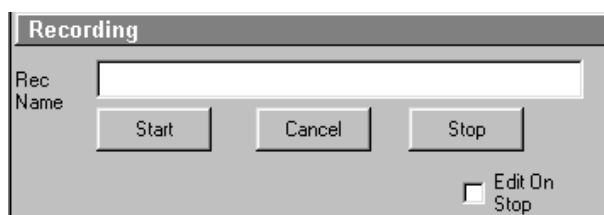


Fig. 4-172 Recording panel

### 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 4-172).

#### 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.

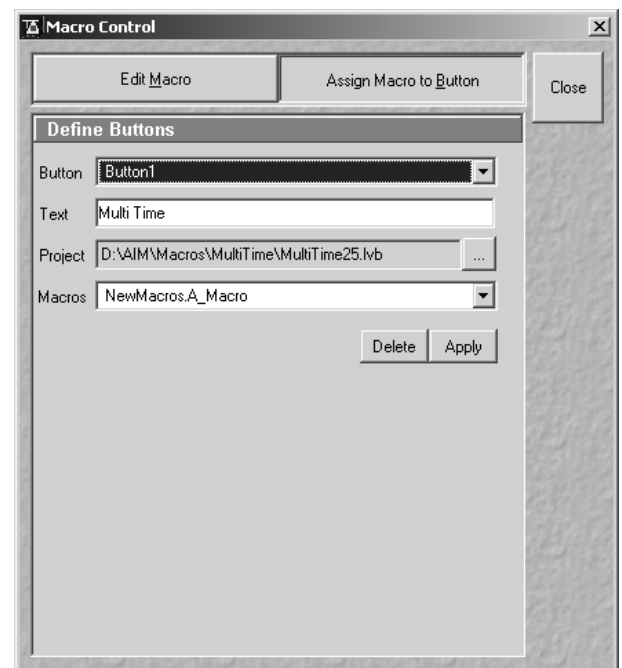


Fig. 4-173 Macro Control window

### 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
CameraColor.lvb (also Button in Maintain)	Color balance of AxioCam HRC
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.
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	<ul style="list-style-type: none"> <li>- divide complete time series through a single image/part of the series</li> <li>- duplicate a single image or part of a time series to this series.</li> </ul>
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 as guide tour on the installation CD-ROM or DVD.
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 ASCII 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
Kollimatic.lvb	Automatically generates the calibration matrix of a LSM 5 <i>LIVE</i> system for optimal confocal aperture and collimator settings
Lambdatrans.lvb	Time series alternating between lambda and transmission mode



Name	Description
LsmHWAdmin.lvb (also Button in Maintain)	Direct service hardware access (password protected)
LsmHWAdminEx.lvb	Calibration service macro (password protected)
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 ( <b>Option</b> )
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 ( <b>Software option</b> )
OptimizeGDC.lvb OptimizeGDCV3_2.lvb	Optimize the max. peak power of fiber coupled Ti:Sa lasers (Release 3.0/3.2)
Optimize_Live.lvb	Temporarily adjusts the confocal aperture position of a LSM 5 <i>LIVE</i> 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.
Reset_live_servos.lvb	Reinitializes all servo motors of a LSM 5 <i>LIVE</i> 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.
ScanfieldTransform.lvb	Adjusts LSM 510 or LSM 5 <i>LIVE</i> image match relative to each other or to LSM <i>DuoScan</i> ROI's
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 a LSM 5 <i>LIVE</i>
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**, **Multiple Time Series** and **StitchArt plus** 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 Distance Macro

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  $\mu\text{m}$ ).
  - 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.

##### 4.8.4.2 Profile Macro

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  $\mu\text{m}$  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.

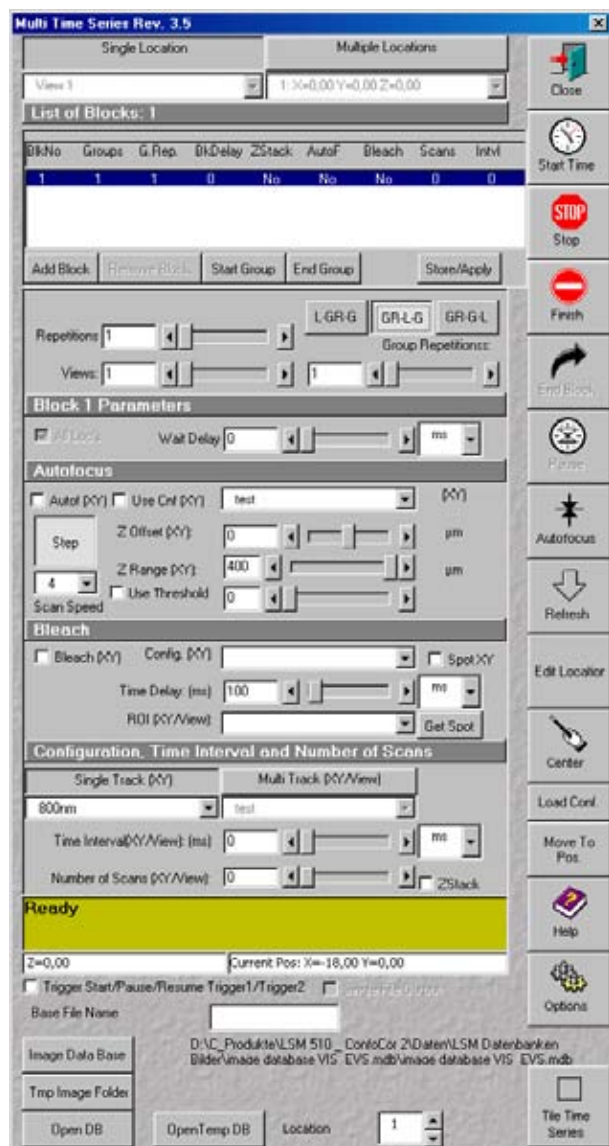


Fig. 4-174 Multiple Time Series main dialog box

#### 4.8.4.3 Multiple Time Series Macro

The Multi Time Series program is designed to provide flexible programming of automated time dependent experiments.

- A pre-program Cycle Delay
- A pre-bleaching functionality (not available)
- 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).

---

#### 4.8.5 Kollimatic

VBA Macro to calibrate the LSM 5 *LIVE* depending on the current system configuration (e.g. objectives, filters,...)

##### **Prerequisites:**

- System Warm-Up (at least 2 hours; for procedure please read the User's Manual) and environmental conditions according to setup requirements.
- "OptimizeLIVE.lvb" Macro: ensure both channel sliders set to 0
- Proper adjustment of collimators and Confocal Slit Aperture (CSA) 1 + 2 (this has to be done by the local Service Representative)
- AOTF RGB + V linearized properly with Macro AOTFFitlin.lvb
- All used objectives have been stored in the database according their exact order in the nose piece.
- Necessary calibration slides are available  
1x Bead Slide 1µm for dry objectives, # **000000-1371-460**,  
1x Bead Slide 200nm for immersion objectives, # **000000-1371-461**;  
to be ordered from Carl Zeiss Jena.

##### **Basic Functions of the Macro**

The KolliMatic macro features two main parts, Basic Calibration and Calibration Matrix:

##### **A - Basic Calibration**

- Needs to be done only once with one of the recommended objectives (should be part of the shipment in order to be able to repeat the Basic Calibration without calibrating all objectives again (see objective list down below))
- Is for calibrating both collimators RGB + V
- Is for calibrating the Y-position of the Beam Shift Compensator (BSC) for all present NFT positions




On currently delivered LSM 5 *LIVE* Systems, the Basic Calibration is done in the factory (This can be confirmed by checking that "BasicCalibrationLog.txt" is present in C:\AIM\Bin).

Objective list (objectives are listed in order of most to least recommended)

- Plan Apochromat 20x / 0.75
- Plan Neofluar 40x / 1.3 Oil
- C-Apochromat 10x / 0.45W
- C-Apochromat 40x / 1.2W corr
- Plan Apochromat 5x / 0.16
- Plan Neofluar 10x / 0.3
- Plan Apochromat 63x / 1.4 Oil
- C-Apochromat 63x / 1.2W corr
- Plan Neofluar 20x / 0.5

**B - Calibration Matrix**

- Is based on the calibration results of the Basic Calibration
- Needs to be performed for each objective separately
- Is for calibrating the collimator position of both collimators RGB + V only for the selected Objective depending on:  
Laser Line, Zoom + EF Filter Position
- Is for calibrating the BSC Y-position depending on:  
Laser Line, Zoom + EF Filter Position

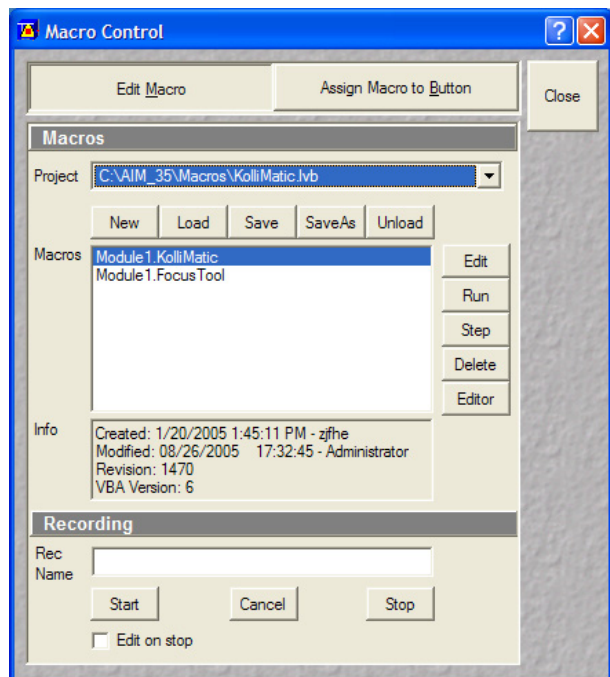
 On currently delivered LSM 5 LIVE Systems, the Calibration Matrix is set up in the factory for every objective belonging to the system (This is done together with the Basic Calibration).

Without running Calibration Matrix, the positions of the collimators and BSC Y should be OK. This is due to the Basic Calibration, but the results are not fully optimized.

**Installation of the Macro:**

If not already done, copy the required files to the following destinations:

File Name	Destination
KolliMatic.lvb	C:\AIM\Macros
PotentialKollimaticConfig.ini	C:\AIM\Bin
KolliMatic_Instructions.pdf	C:\AIM\Macros



**Fig. 4-175 Macro Control Window of the main LSM Software**

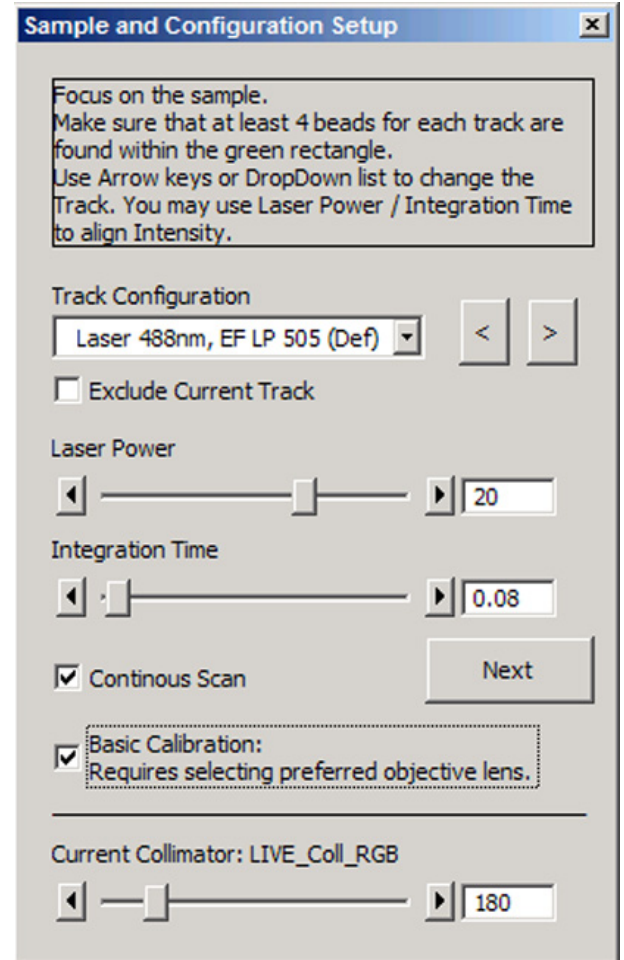
**Starting the Macro:**

- Start the LSM software and check first, if all prerequisites have been fulfilled (see Paragraph 1. Prerequisites)
- Put the appropriate calibration slide (depending on current objective: immersion or non immersion) onto the microscope stage,
- Activate all lasers, and using the and focus with the 488 nm line start scanning and focus onto the beads
- Put the appropriate calibration slide (depending on current objective: immersion or non immersion) on the microscope stage, start single scan
- Use the Macro Menu of the main LSM software to **Load** and **Run** the macro **KolliMatic** (See Fig.4-175)

Please wait a few seconds, the macro will set up beam path and all necessary settings automatically.

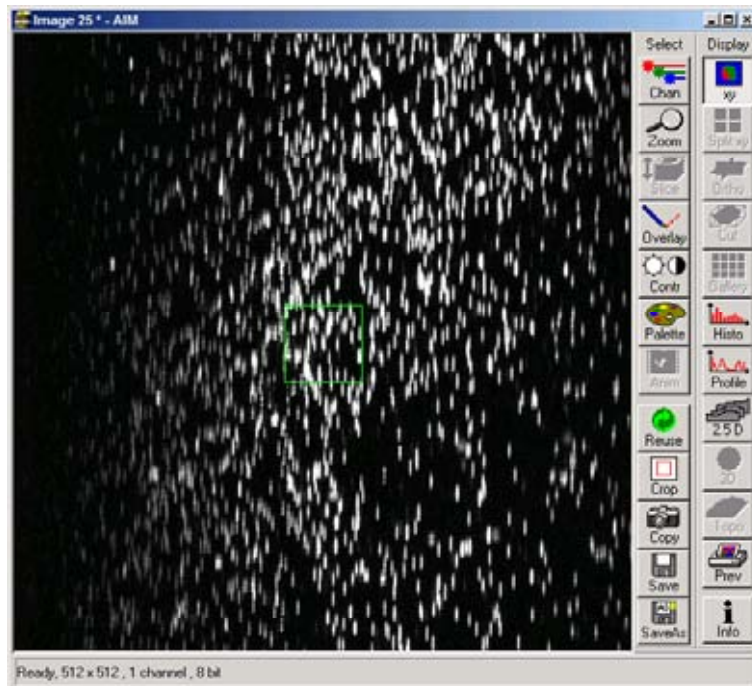
### **Preparations for using the Calibration Slides:**

- Follow the steps shown in the macro **Sample and Configuration Setup** window
- Use the arrows or the drop down menu in order to select one track for each available laser line (no need to select several tracks for the same laser line), start with a track for the lowest wavelength laser (i.e. typically the 405 nm laser line.)
- Select an area of the bead sample that provides as many beads as possible, at least 4 (fit into the green rectangle)
- In Fig. 4-176, the **Basic Calibration** has been selected (Check box **Basic Calibration: Requires selecting preferred objective lens**), therefore some more tracks are available
- Use the **Laser Power** as a first option in order to set the intensity, second option is **Integration Time**
- In order to start / stop the scan, just select / deselect the check box **Continuous Scan**
- Focus on the beads
- Defocus with the **Collimator** slider until the beads become line shaped
- Focus with the microscope focus drive to get the vertical lines as thin as possible (Fig. 4-177)
- Now use the **Collimator** slider to get a round spot out of the line (Fig. 4-178)
- Repeat these steps for a track with the 488 nm laser line
- After one track for each laser line has been adjusted, click **Next**.

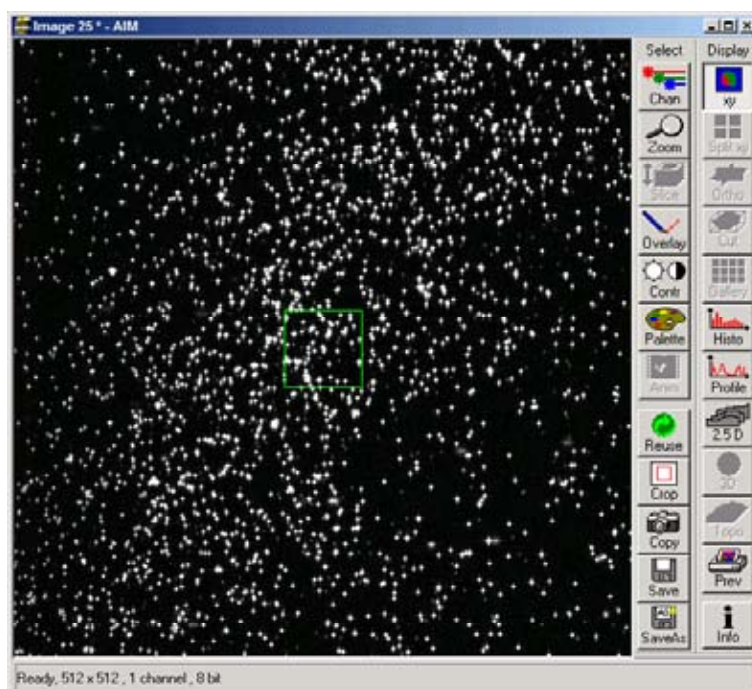


**Fig. 4-176** Figure Sample and Configuration Setup Window of the KolliMatic Macro






**Fig. 4-177** Beads focused for maximal vertical thinness

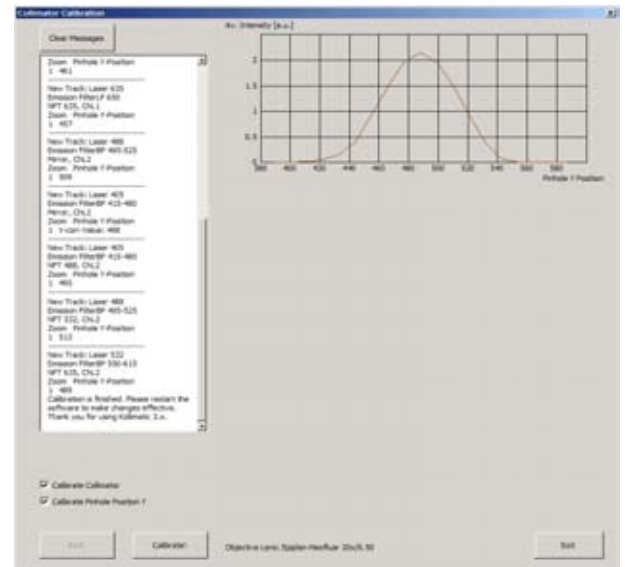


**Fig. 4-178** Beads optimally focused

## **Running the Basic Calibration**

 If you have selected the check box for **Basic Calibration**, please follow these instructions. In order to run the **Calibration Matrix** only, move on to paragraph 7.


- Make sure that both check boxes have been selected (Fig. 4-179)
- Double check if the objective settings (shown at the bottom of the macro window) match the currently used objective
- Click the button **Calibrate**
- In order to follow the calibration process, just click into the **Message** window; a scroll bar will appear
- The Basic Calibration takes about 15 min
- The displayed curve should resemble a Gaussian profile
- After the macro has finished, a message will pop up with the request to restart the LSM software to make the changes effective.
- Press **Exit** and restart the LSM software



**Fig. 4-179 Collimator Calibration Window**



## Running the Calibration Matrix

 If you haven't selected the check box for Basic Calibration, the "Calibration Matrix" will be selected automatically. In this case, when the Calibration Matrix is running, the Basic Calibration is disabled.

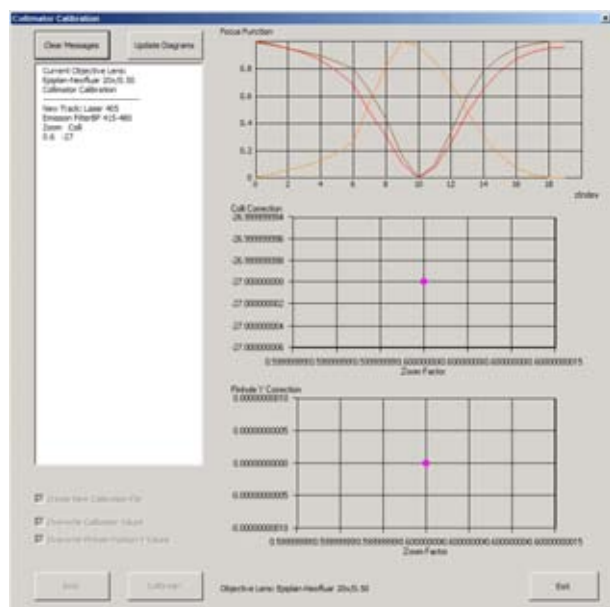


Fig. 4-180 Running the Calibration Matrix

- This window provides three check boxes (Fig. 4-180)
- The **recommended setting** is to select **Create new calibration file**. All previous files will be deleted.

### Exceptions:

#### No check box checked

- Especially after an unexpected interruption of the program, it is possible to continue from the position where the process stopped (saving time).

#### Overwrite Collimator values

- The values for the chosen objective will be added or updated.
- After selecting the **Collimator** option, the **Pinhole Y** option will be selected automatically.

- Double check if the objective settings (shown at the bottom of the **Macro** window) match the currently used objective
- Click the button **Calibrate**

### Meaning of the Graph (Figure):

Brown: Focal plane of the CCD Line Detector  
 Red: Focal plane of the collimator  
 Orange: Estimated focal plane

The brown and red graphs should be close to this curve.

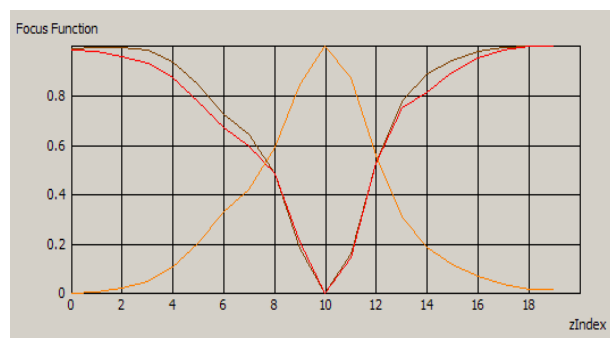


Fig. 4-181 Graph of Focus Function

- In order to follow the calibration process, just simply click into the **Message** window and; a scroll bar will appear (Fig. 4-182)
- The Calibration Matrix takes about 25 min for each objective
- After the macro has finished, a message will pop up with a the request to switch the objective lens in order to make the changes effective
- If necessary, repeat the procedure for the other objectives
- Use the **Update Diagrams** button in order to check, if an objective has already been calibrated
  - Select an objective, press the **Update Diagrams** button
  - If no graphs appear, this particular objective has yet needs to be calibrated
- Click **Exit** to finish

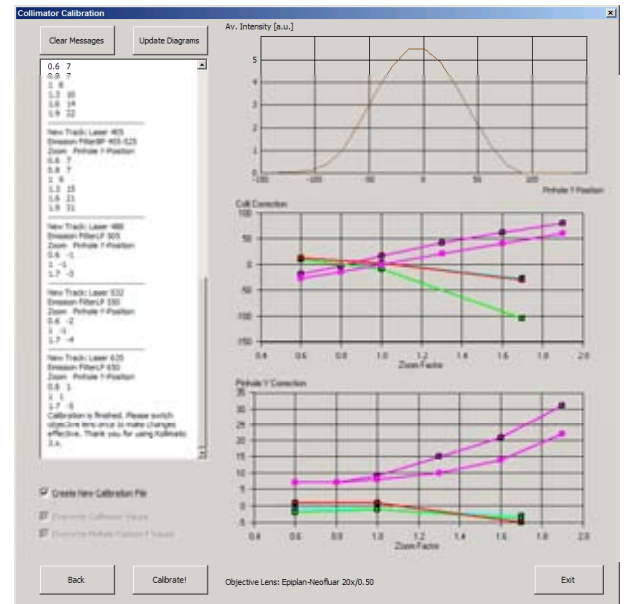


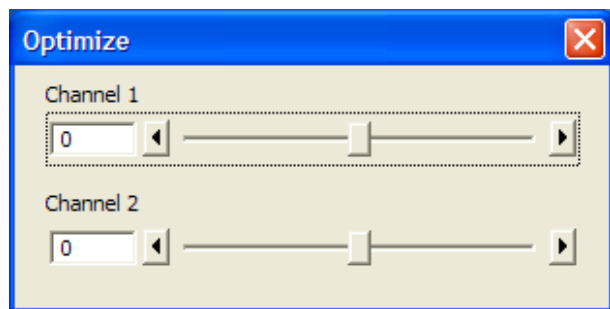
Fig. 4-182 Collimator Calibration window after calibration

#### Meaning of the Graph (Figure):

- Each coloured line represents an individual laser line.
- Each coloured calibration point represents an emission filter.
- Standard graphs are displayed with black points.

#### Meaning of the Colours:

- <420 nm: → Pink
- 421...475 nm: → Blue
- 476...495 nm: → Turquoise
- 496...550 nm: → Green
- 551...590 nm: → Yellow
- >590 nm: → Red

**Important Information!****Fig. 4-183** Optimize Macro

If the calibration has been completed successfully with the Calibration Matrix,

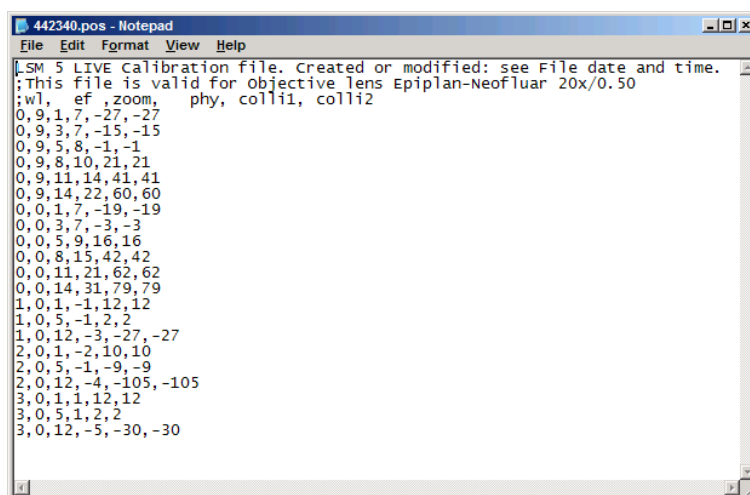
**DO NO LONGER USE THE PINHOLE AND COLLIMATOR FUNCTIONALITY OF THE LSM SOFTWARE IN THE MAINTAIN MENU.**

The use of the pinhole sliders to raise the quality of the images is well known from the other LSM Systems.


On the LSM 5 LIVE, only the **Optimize** sliders of the "OptimizeLIVE.lvb" macro have to be used (Fig. 4-183) to compensate for misalignments (e.g. temperature influences).

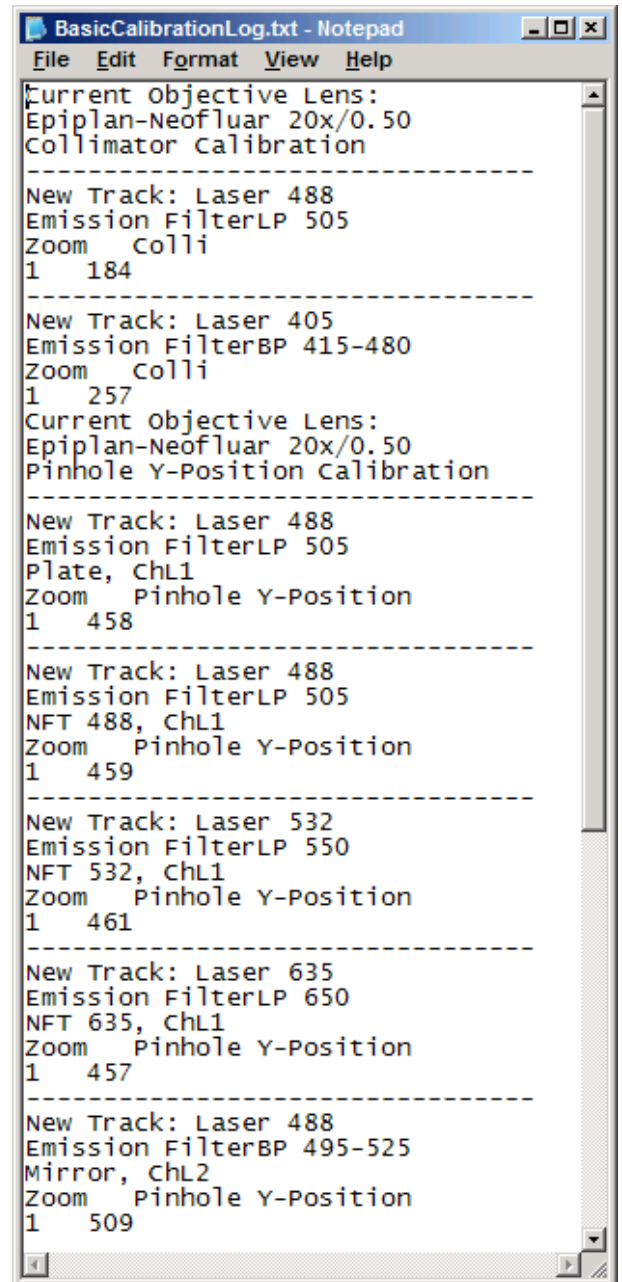
After the Calibration Matrix has optimized a particular objective, the results will be stored automatically in a file as shown below. These files can be found in **C:\AIM\BIN**.

**DO NOT CHANGE ANY OF TH VALUES!**

**Fig. 4-184** Calibration file

The calibration results of the Basic Calibration will be saved in a file named **"BasicCalibrationLog.txt"** (to be found in the BIN-Folder as well).

 Changes won't effect the stored values.



```

BasicCalibrationLog.txt - Notepad
File Edit Format View Help
Current Objective Lens:
Epiplan-Neofluar 20x/0.50
Collimator Calibration
-----
New Track: Laser 488
Emission FilterLP 505
Zoom Colli
1 184
-----
New Track: Laser 405
Emission FilterBP 415-480
Zoom Colli
1 257
Current Objective Lens:
Epiplan-Neofluar 20x/0.50
Pinhole Y-Position Calibration
-----
New Track: Laser 488
Emission FilterLP 505
Plate, ChL1
Zoom Pinhole Y-Position
1 458
-----
New Track: Laser 488
Emission FilterLP 505
NFT 488, ChL1
Zoom Pinhole Y-Position
1 459
-----
New Track: Laser 532
Emission FilterLP 550
NFT 532, ChL1
Zoom Pinhole Y-Position
1 461
-----
New Track: Laser 635
Emission FilterLP 650
NFT 635, ChL1
Zoom Pinhole Y-Position
1 457
-----
New Track: Laser 488
Emission FilterBP 495-525
Mirror, ChL2
Zoom Pinhole Y-Position
1 509

```

Fig. 4-185 BasicCalibrationLog file

#### 4.8.6 Optimize Macro

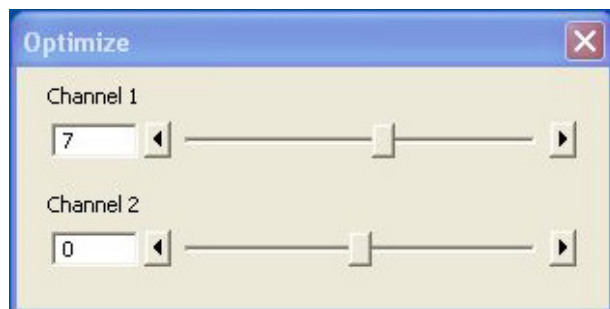


Fig. 4-186 Optimize window

but can enhance image brightness efficiently.

For temporary optimization of the LSM 5 *LIVE* beampath, try to avoid a modification of the general adjustment in the pinhole dialogue. Use the **Optimize** slider instead. The general setting of the confocal aperture might still be correct for other scan parameters.

The **Optimize** slider can be set independently for each channel. The slider is moved to the one or the other side while scanning continuous. The brightest image result is the optimal setting. This setting doesn't affect the general adjustment of the LSM 5 *LIVE* collimator and confocal aperture,

#### 4.8.7 Relnit Macro



Fig. 4-187 Reset window

To improve the internal adjustment of default positions of motor driven parts, the **Reset Servo** macro (Relnit) is available.

After one hour, we recommend to start the **Relnit** macro to reset all default positions for the actual warm up state of the system. Repeat this whenever you experience changes in the system performance. After running the macro, close the window.

#### 4.8.8 Image Match (Scanfield Transformation) Macro

Adjusts the Image match between two scanning imaging systems or a manipulation and a scanning imaging system.

To adjust the image match, set up a multitracking that creates one channel for each scanning and/or manipulation system. In case of the LSM DuoScan, use a T-PMT to create an image with the DuoScan system.

- Start the **Image Match (Scanfield Transformation)** macro.
- Use the Image match calibration sample delivered with the system. This sample has a fluorescent layer and a reflective grid, useful to create images in fluorescent, reflective or transmission light mode.
- Start continuous scan, and set laser power and gain to appropriate levels. The point scanning system will be the speed limiting system, so choose a fast scanning speed to get a faster image refresh.
- Use **unidirectional** scan to avoid adjustment errors due to bidirectional image jitters.
- Set the matching zoom factors with one of the **Match DuoScan zoom/offset** or **Match LIVE zoom/offset** buttons relative to each other. It is recommended to adjust the LSM zoom.

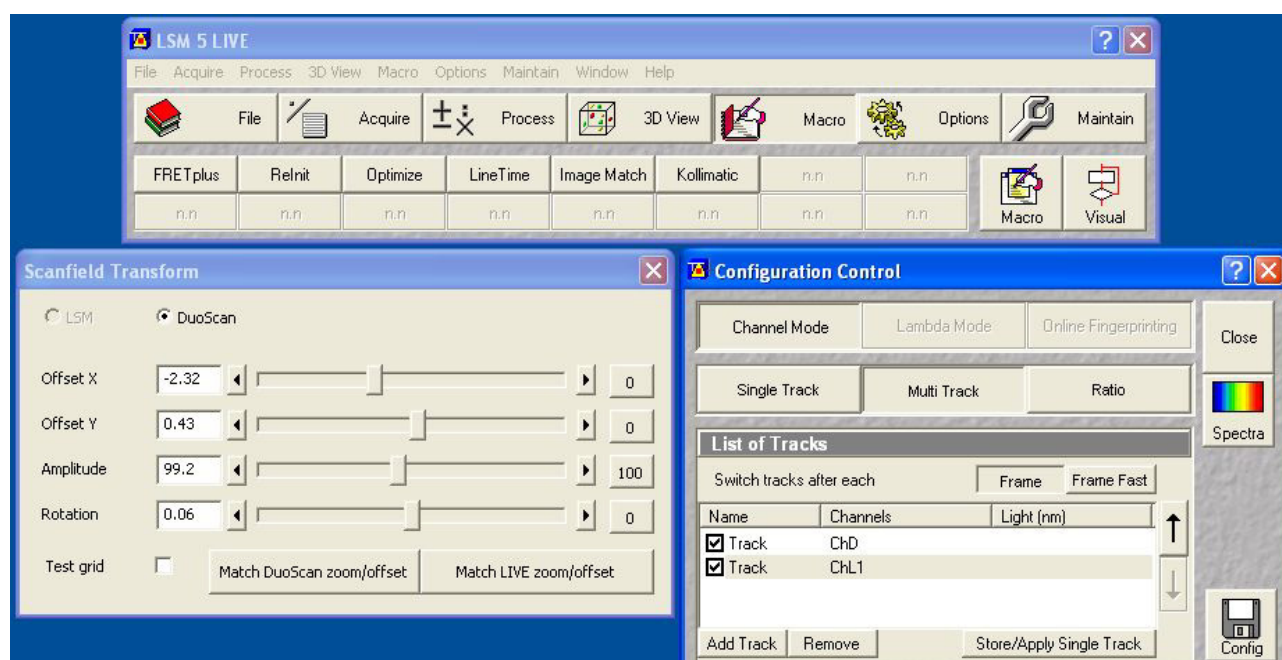


Fig. 4-188 Scanfield Transform window

These buttons set the two scanheads at a matching scanfield diameter, according to the following table (LSM 5 LIVE = Zoom 1.0):

Microscope stand	LSM 5 LIVE DuoScan	LSM 5 DUO
Inverted (port config)	(SP/RP) DuoScan = Zoom 1.4142	(RP/SP) LSM 510 = Zoom 1.33978
Upright (port config)	(Tube/RP) DuoScan = Zoom 1.4142	(RP/Tube) LSM 510 = Zoom 1.25295

- Choose **Split image** for the image window display. You will see the scanned areas of both scanning systems and an overlay.

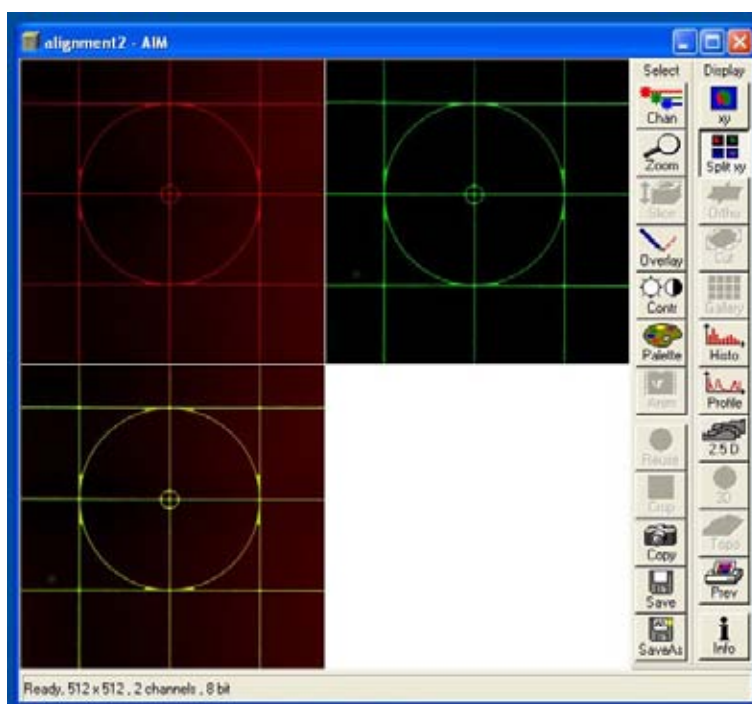



Fig. 4-189 Split image


- Choose **LSM (510)** or **DuoScan** to adjust the image match.
- While scanning, move the sliders of the Image match macro to get a perfect overlay of the scanning and/or manipulation system relative to each other.
- Offset X/Y** adjusts the horizontal and vertical image shift
- Amplitude** adjusts a size difference between the images
- Rotation** compensates for a tilt in one of the images.

 Store the final image, and use the **ReUse** function for future image matching. The **Multitrack** setup and the zoom factors will then be set automatically.

## 4.8.9 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.

### 4.8.9.1 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[amy362].

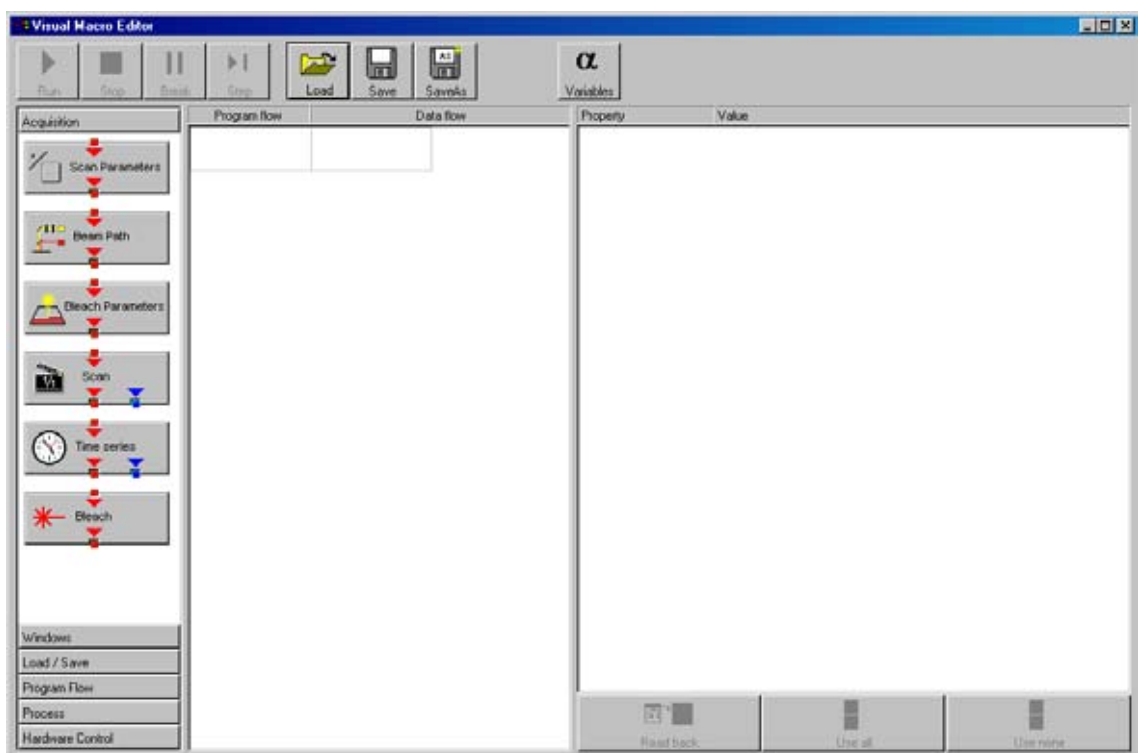


Fig. 4-190 Visual Macro Editor window



#### 4.8.9.2 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

<b>Run</b>	Starts the Macro starting with the first block in the program flow column.
<b>Stop</b>	Stops the Macro at any time:
<b>Break</b>	Interrupts the Macro. Clicking the button again will resume the actual program flow of the Macro:
<b>Step</b>	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.
<b>Load</b>	Use to open a specific Macro and <b>Save</b> to store the Macro. <b>SaveAs</b> allows to store the same Macro under a different name.
<b>Variables</b>	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 from 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 choose 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.

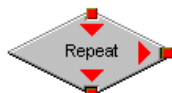


It is possible to export images choosing a certain file format from the pull down list that appears when highlighting the line **File Format** in the properties list. Specific parameters are assigned to each file format which can be set for the image export.

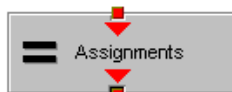


Images can be imported within the program flow. The image to be loaded can be selected by typing in the file path. The import of single images or a image series is possible. The images to be imported as series must have the same name and must be numbered in ascending order.

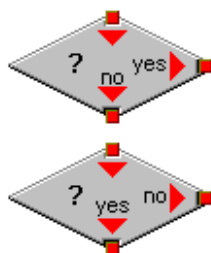
## Program Flow Action Blocks



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.



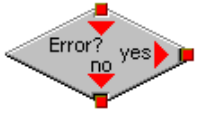
Using this block in the program flow allows to assign new values to a variable using mathematical calculations. By passing this block the variable will be transiently changed and the new value can for example be used to assign different numbers to the image name or to increase the time delay during a 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.

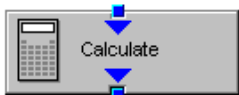


The block allows to assign a potential error message with a textbox and to decide on the Error where to direct the program flow. If no textbox is assigned the program flow will just continue according to the presence of an error message. Choosing a textbox requires the input of the user to continue with the program flow.



With the Release 3.5 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.

### 4.8.9.3 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. 4-191).

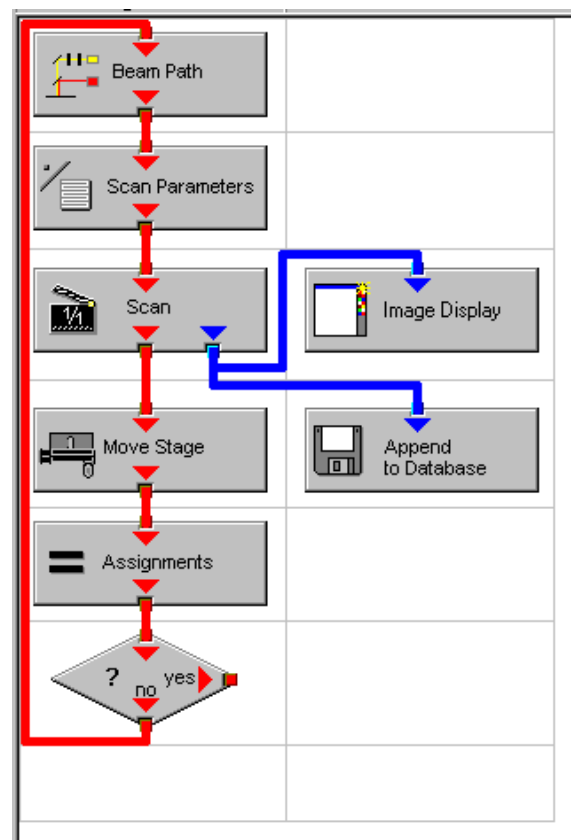


Fig. 4-191 Arrangement of action blocks

- Assign the value **1** to a variable named **Index** (Fig. 4-192).

Property	Value
Index	1

Fig. 4-192 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
<input checked="" type="checkbox"/>	LSM Pixels X	512
<input checked="" type="checkbox"/>	LSM Pixels Y	512
<input checked="" type="checkbox"/>	LSM Step Y	1
<input checked="" type="checkbox"/>	LSM Zoom	1.600000
<input checked="" type="checkbox"/>	LSM Rotation [°]	45.000000
<input checked="" type="checkbox"/>	LSM Offset X [µm]	0.000000
<input checked="" type="checkbox"/>	LSM Offset Y [µm]	0.000000
<input checked="" type="checkbox"/>	LSM Pixel Period [µs]	2.560000
<input checked="" type="checkbox"/>	Camera Mode	Mono 1300 x 1030
<input checked="" type="checkbox"/>	Camera Pixels X	1300
<input checked="" type="checkbox"/>	Camera Pixels Y	1030
<input checked="" type="checkbox"/>	Camera Offset X [Pixels]	0.000000
<input checked="" type="checkbox"/>	Camera Offset Y [Pixels]	0.000000
<input checked="" type="checkbox"/>	Pixels Z	1
<input checked="" type="checkbox"/>	Scaling Z [µm]	1.000000
<input checked="" type="checkbox"/>	Offset Z [µm]	0.000000
<input checked="" type="checkbox"/>	Bits per Sample	8 bit

**Fig. 4-194** Property list of the Scan Parameter 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. 4-195** Property list of the Append to Database action block

Property	Value
Mode	Relative Position
Distance X [µm]	10
Distance Y [µm]	10

**Fig. 4-196** Property list of the Move stage 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. 4-193** Property list of the Beam Path 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.
- The movement of the stage is defined highlighting the **Move stage** action block, choosing 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 use 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!)

Property	Value
Index	Index + 1

**Fig. 4-197** Property list of the Assignments action block

Property	Value
Expression	Index > 5

**Fig. 4-198** Property list of the Decision action block



## 4.9 Options Menu

The **Options** menu permits performance of the following functions:

- 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.

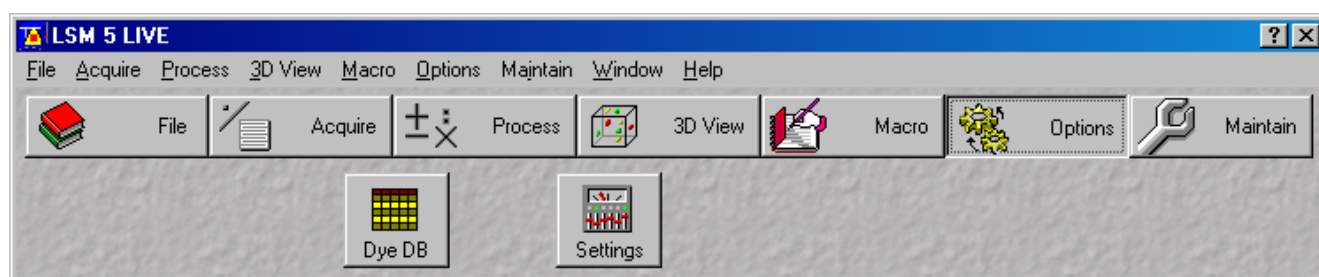


Fig. 4-199 Options menu

#### 4.9.1 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**.

Dye database					
Fluorochrome	Ex1 [nm]	Ex2 [nm]	Em1 [nm]	Em2 [nm]	
4-Methylumbelliferon	360		450		
6-Amino-Quinolin	360		443		
7-Amino-Actinomycin D	546		647		
Acridingelb	470		550		
Acridinorange +DNA	502		526		
Acridinorange +RNA	460		650		
Acridavir-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		
Auraphosfin	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. 4-200 Dye database window

## 4.9.2 Settings Function

The **Settings** function permits the individual setting and matching of software settings with regard to the following points:

Autosave	Recording / Reuse
Database General	Timeseries
Database Table Viewer	Scan Mean of ROIs
Database Gallery Viewer	Temporary Files
Import / Export	Program Start
Scan Information	Hardware
Image Status Display	Image Display Toolbars
Print Status Display	Save

### 4.9.2.1 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.

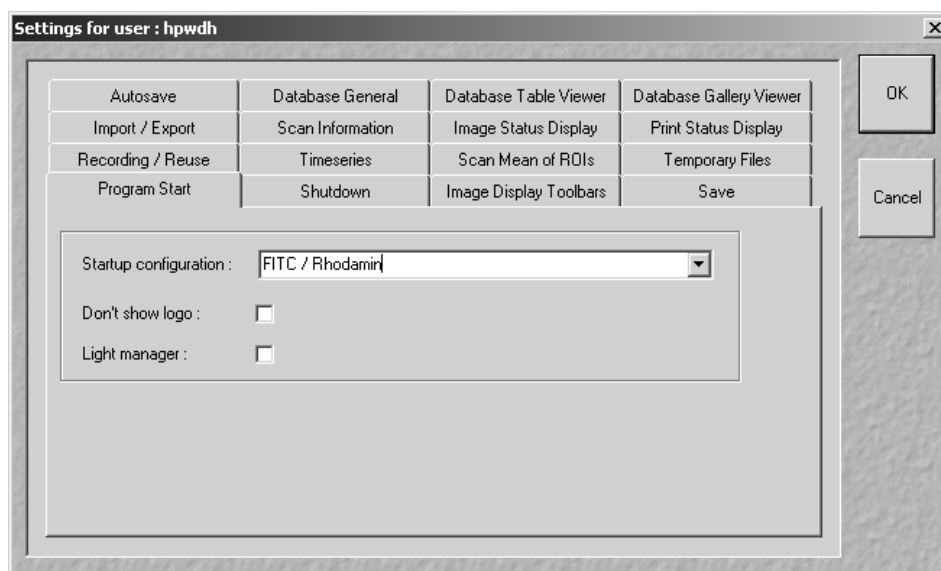


Fig. 4-201 Settings for user : ... window

#### 4.9.2.2 Autosave

This tab permits activation or deactivation of automatic data storage. Only one option can be selected at a time.

##### (1) No Autosave

On activation of this option, the **Autosave** function is switched off. **Save** and **Save As** give the same dialogues.

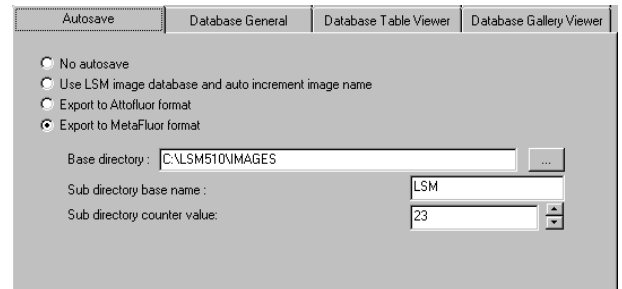


Fig. 4-202 Autosave tab

##### (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.

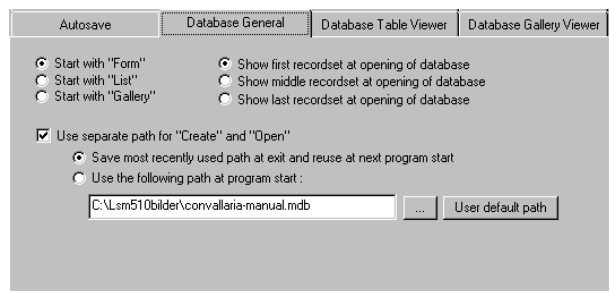


Fig. 4-203 Database General tab

### 4.9.2.3 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.

#### 4.9.2.4 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.

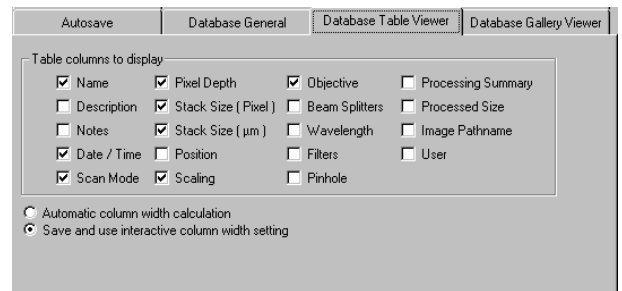


Fig. 4-204 Database Table Viewer tab

#### 4.9.2.5 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.

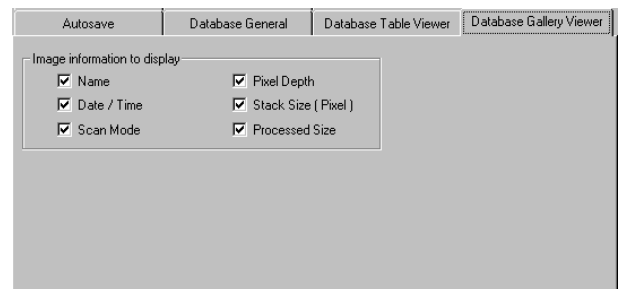


Fig. 4-205 Database Gallery Viewer tab

#### 4.9.2.6 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.

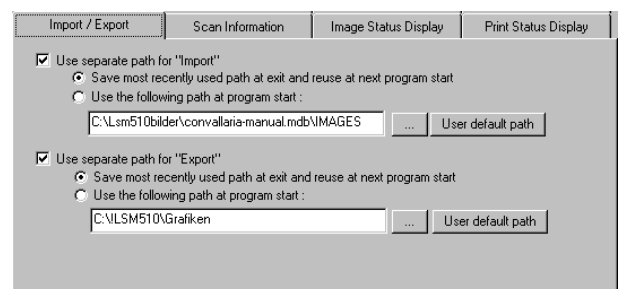


Fig. 4-206 Import / Export tab

Clicking on the **User default path** button firmly sets the **C:\users\default\** path.

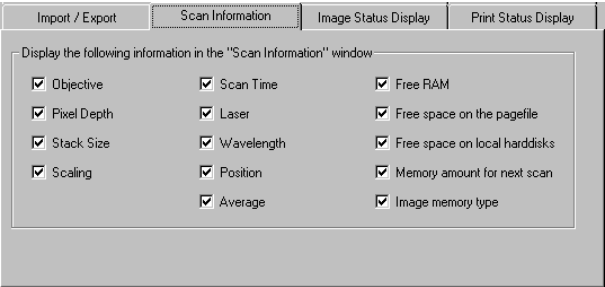


Fig. 4-207 Scan Information tab

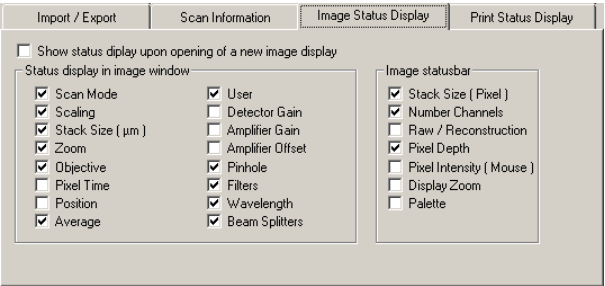


Fig. 4-208 Image Status Display tab

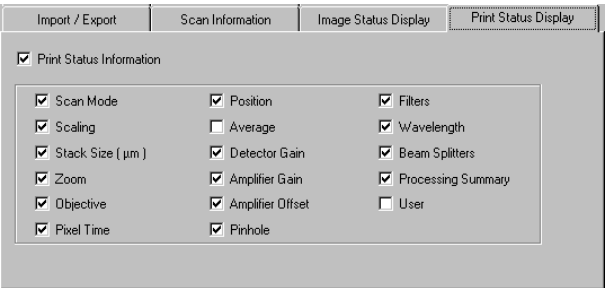


Fig. 4-209 Print Status Display tab

#### 4.9.2.7 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 4-222f).

Activation / deactivation of the information to be displayed is performed with a click of the mouse.

#### 4.9.2.8 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).

#### 4.9.2.9 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.

#### 4.9.2.10 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.

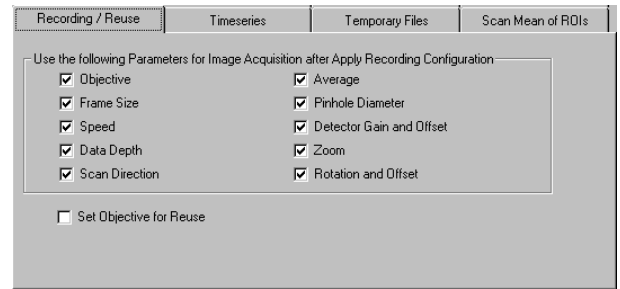


Fig. 4-210 Recording / Reuse tab

#### 4.9.2.11 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**.

**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.

You can select the unit for **Mean of ROIs** diagrams.

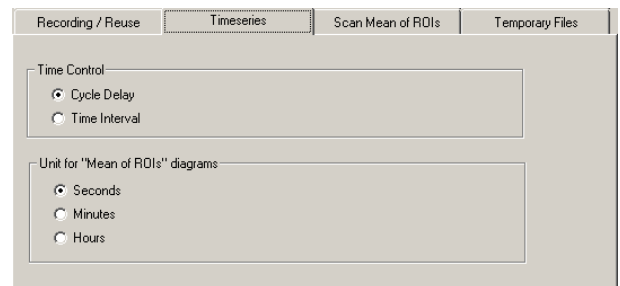


Fig. 4-211 Timeseries tab

#### 4.9.2.12 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

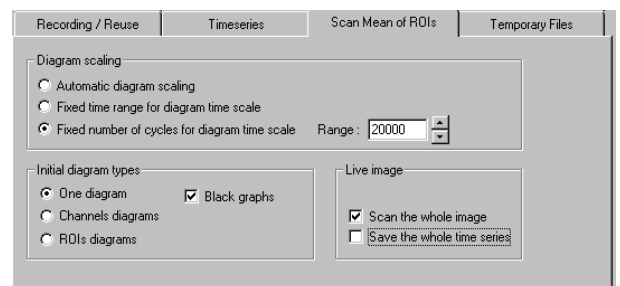


Fig. 4-212 Scan Mean of ROIs tab



## (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.

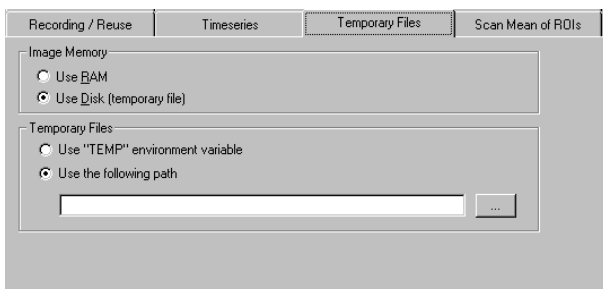


Fig. 4-213 Temporary Files tab

### 4.9.2.13 Temporary Files

The **Temporary Files** tab permits determination of the directory in which temporary files are stored.

#### (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.

#### 4.9.2.14 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 software program 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.

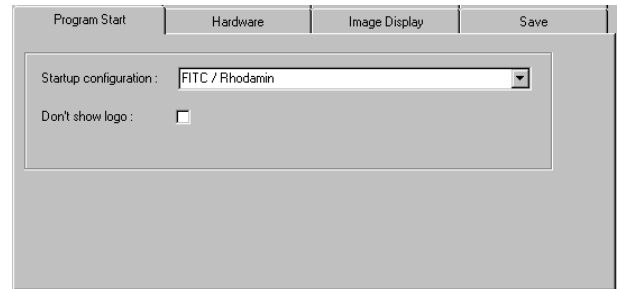


Fig. 4-214 Program Start tab

#### 4.9.2.15 Hardware

The **Hardware** tab allows you to set several hardware defaults at the shutdown of the system.

The **Lasers off on Exit** 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 off the system.

**Light manager** activates or deactivates the light microscope stands light manager.

**Shutter filter wheel** activates or deactivates additional laser attenuation by grey filters (LSM 510 only).

**Simultaneous grab and bleach** activates or deactivates the simultaneous grab and bleach option in the bleach menu. This function can create fluorescence artifacts depending on sample properties and confocal aperture setting (it is recommended to close the confocal aperture to 1 airy unit).



Fig. 4-215 Hardware tab

#### 4.9.2.16 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.

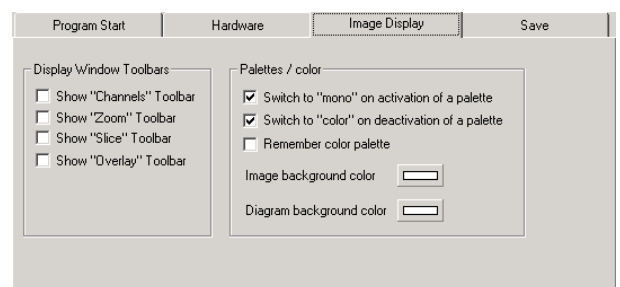


Fig. 4-216 Image Display tab

#### (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.

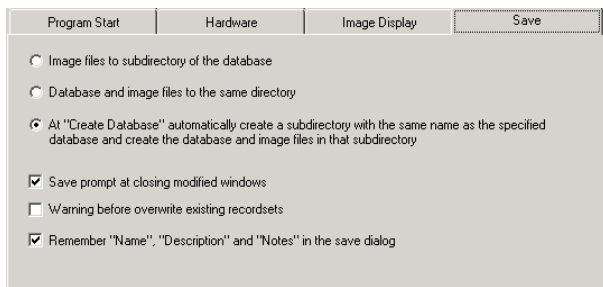
- (b) Switch to "color" on deactivation of a palette

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 .

**4.9.2.17 Save**

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 5 LIVE.

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

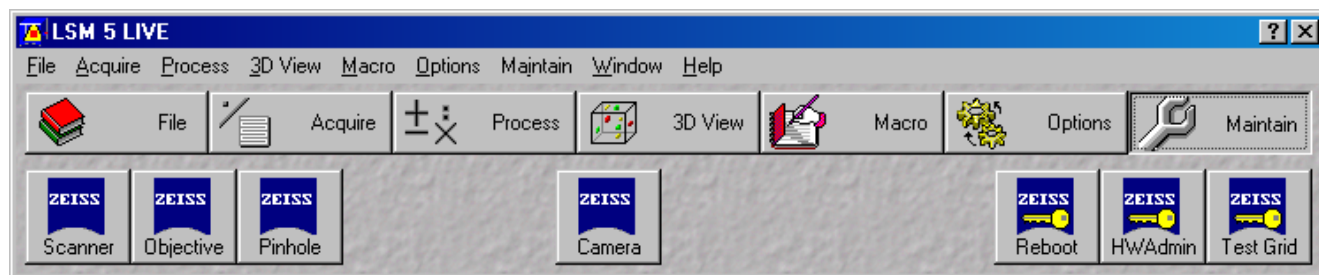


Fig. 4-218 Maintain menu

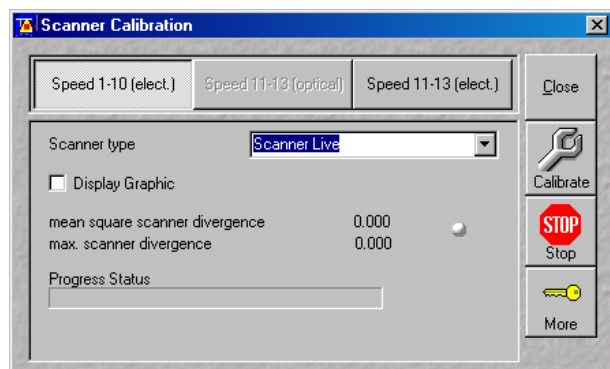


Fig. 4-219 Scanner Calibration window

### 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 10-11 (unidirectional)

#### 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.
- 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 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:

#### 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.

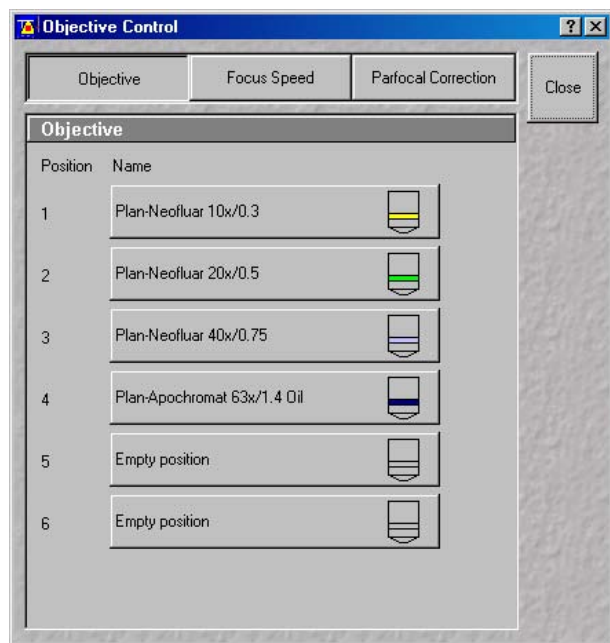


Fig. 4-220 Objective Control window

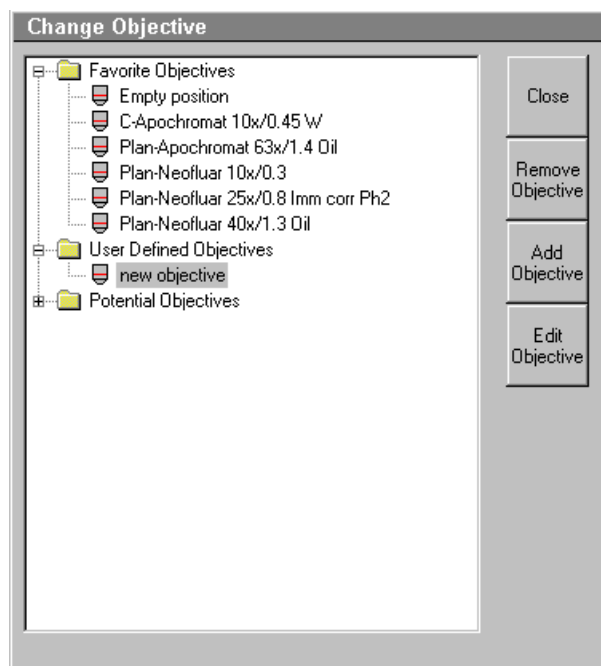


Fig. 4-221 Change Objective window

## 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.

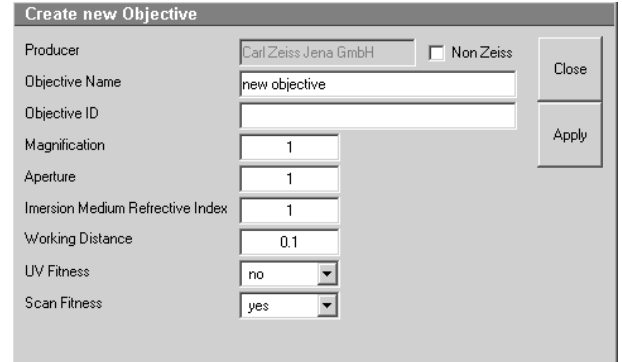


Fig. 4-222 Create new Objective window

## (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.

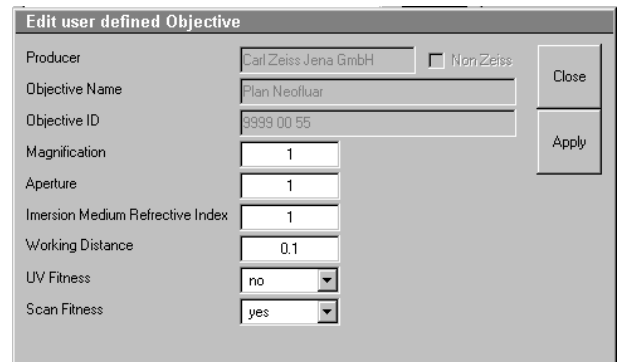


Fig. 4-223 Edit user defined Objective window



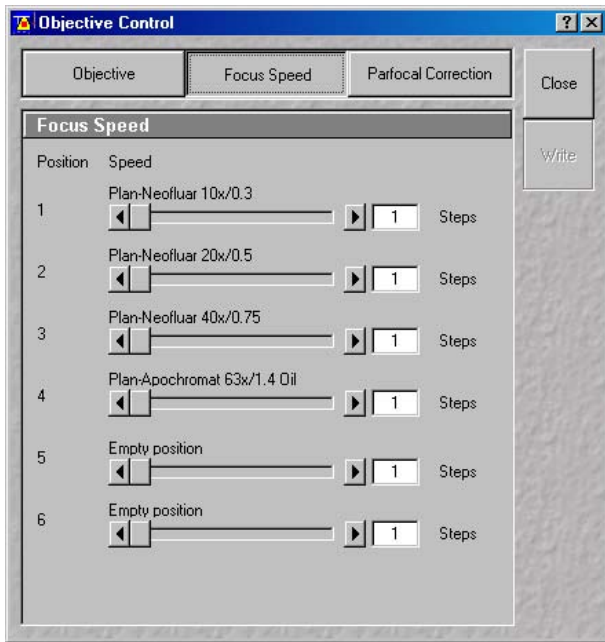


Fig. 4-224 Focus Speed window

#### 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.

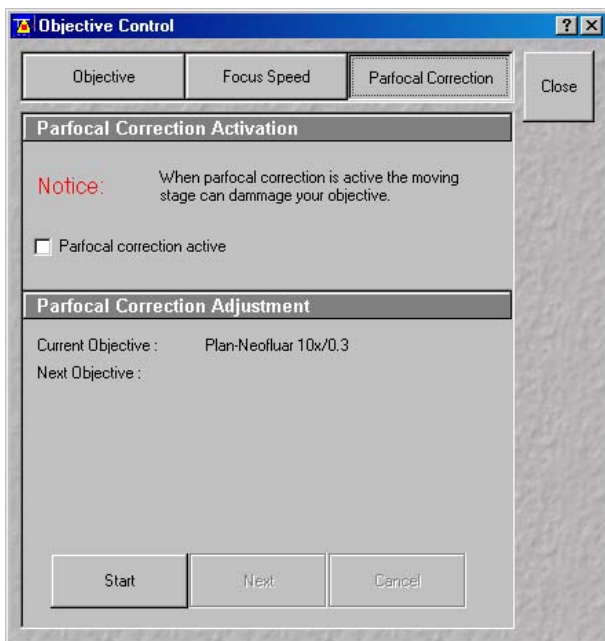


Fig. 4-225 Objective Control window

#### 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.

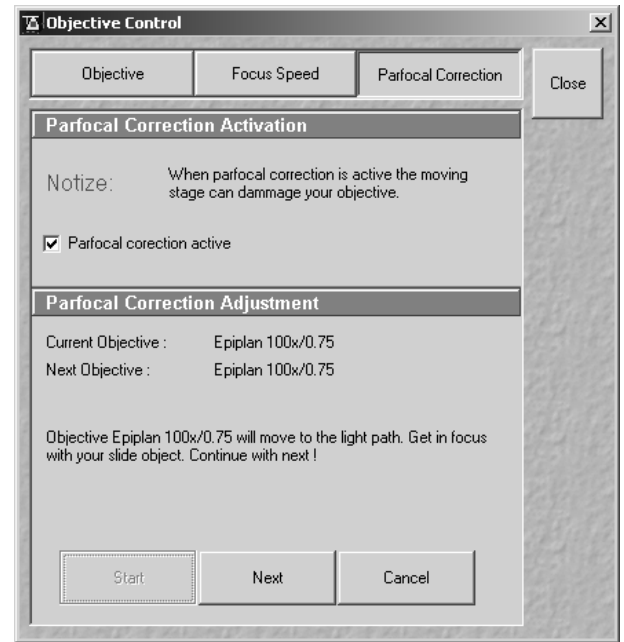


Fig. 4-226 Objective Control window


### 4.10.3 Pinhole Adjustment


In the **Pinhole and Collimator** window, the aperture are optimally aligned and adjusted to the used beam path (configuration).

The position of the aperture (pixel shift and Y-coordinate) in relation to the detector makes a major contribution to image optimization.

In all existing standard configurations, the apertures 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 aperture as follows.

 The X-position does not influence brightness, but shifts the whole image position in X-direction.

 Use the Optimize and Kollimatic functions (see chapter **Macro Menu**) first, the Pinhole Adjustment is not recommended on the LSM 5 LIVE as a general tool.

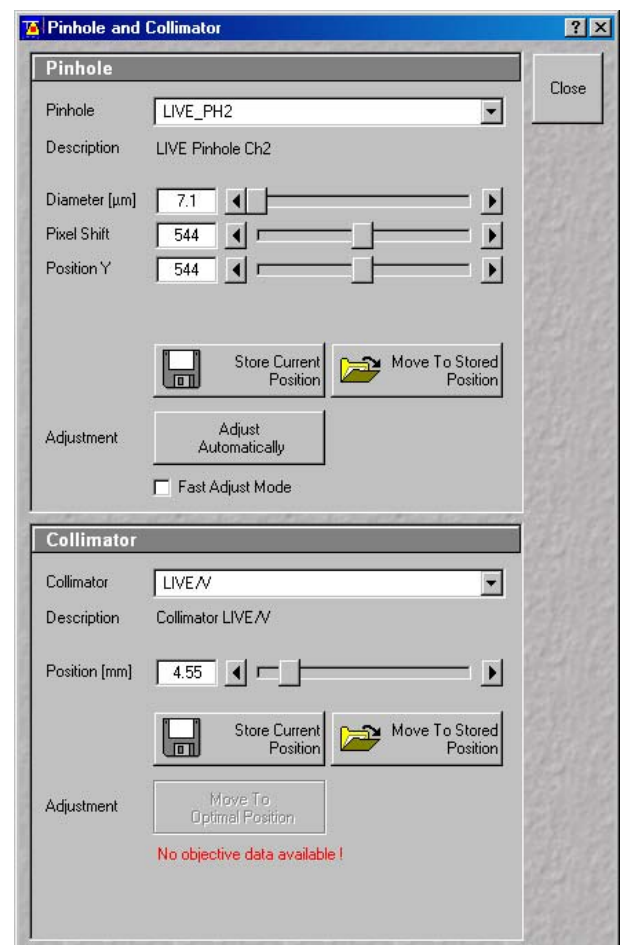


Fig. 4-227 Pinhole and Collimator window

#### 4.10.3.1 Open / Close the Pinhole and Collimator Window

- Click on the **Pinhole** button in the **Maintain** subordinate toolbar of the **Main** menu.
  - This opens the **Pinhole and Collimator** window.
- Click on the **Close** button to quit the window.

#### 4.10.3.2 Pinhole Panel

No further software function can be activated and executed during aperture adjustment.

**Pinhole / Description** field: Selection of apertures (PH1 to PH2) to be adjusted via the **Pinhole** selection box, display of the relevant channel in the **Description** field.

**Diameter; Pixel Shift; Position Y** slider: Setting of size, pixel shift and Y-position of the aperture in relation to the beam path 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 aperture setting.

**Move to stored Position** button: Aperture setting is reset to the position last stored.

**Adjust Automatically** button: Automatic aperture adjustment.

**Fast Adjust mode** check box: If this check box is activated, the aperture adjustment is only performed in a limited area. Used for readjustment.

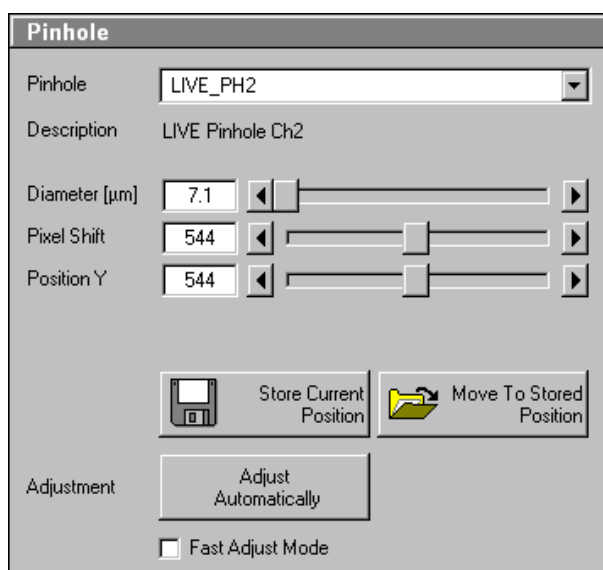


Fig. 4-228 Pinhole panel

#### 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.

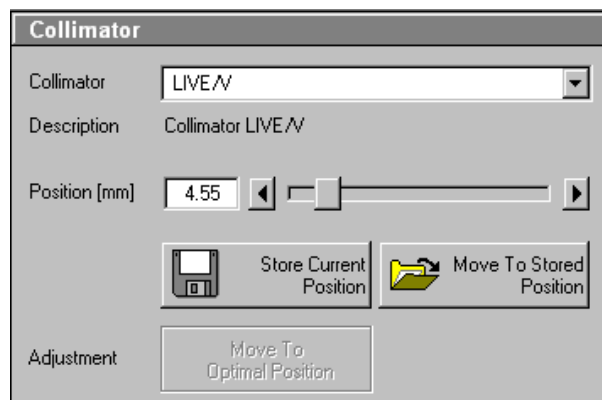


Fig. 4-229 Collimator panel

#### 4.10.3.4 Aperture and collimator Adjustment

Adjustment of the LSM 5 *LIVE* apertures can be performed manually or automatically.

If several channels are used to produce the image, all the used apertures must be adjusted separately.

##### (1) Manual aperture and collimator adjustment

The position of the aperture relative to the detector in terms of Y coordinate contributes substantially to image optimization.

Requirements to make aperture 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 aperture size so as to have the best possible image contrast.

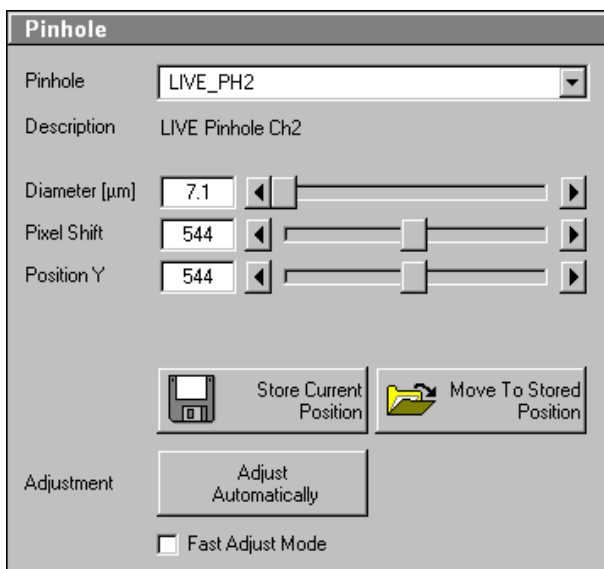




Fig. 4-230 Pinhole panel

- Click on the **Pinhole** button in the **Maintain** subordinate toolbar.
- Select the aperture (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 aperture size.
  - The **Z Slice** display box simultaneously displays the depth resolution corresponding to the aperture size.

 Image optimization can be effected with the **Range Indicator** or in the **Line-Scan** mode.

- Optimize the pixel shift and the aperture position in Y relative to the PMT using the **Pixel Shift** and **Position Y** sliders to maximum image brightness.
- Click on the **Save Current Position** button to save the aperture adjustment.
- Removing the **Position** 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 aperture adjustment is running (status display is red - busy).

## (2) Automatic aperture and collimator adjustment

The automatic adjustment allows the LSM 5 LIVE apertures 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.
  - Aperture 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.
- The **Move To Stored Position** button enables the collimator to be set back to the factory-adjustment.
- Activate the **Fast Adjust Mode** check box for a faster readjustment.

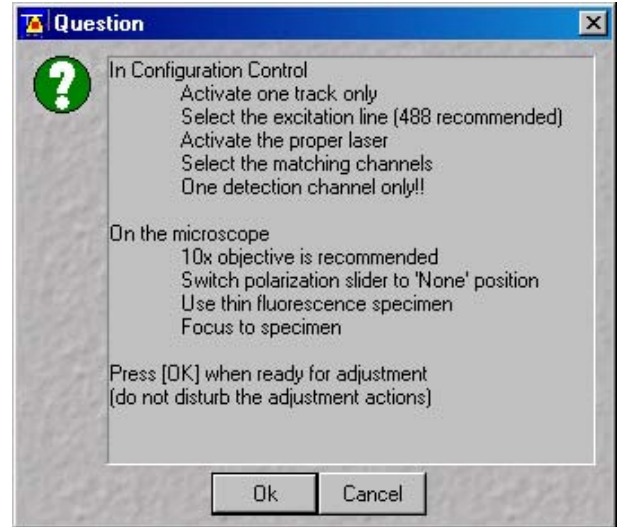



Fig. 4-231 Requirements for Adjustment window

 A change of the aperture size 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.

Please do not make any program manipulations while the automatic aperture adjustment is running (status display is red - busy).

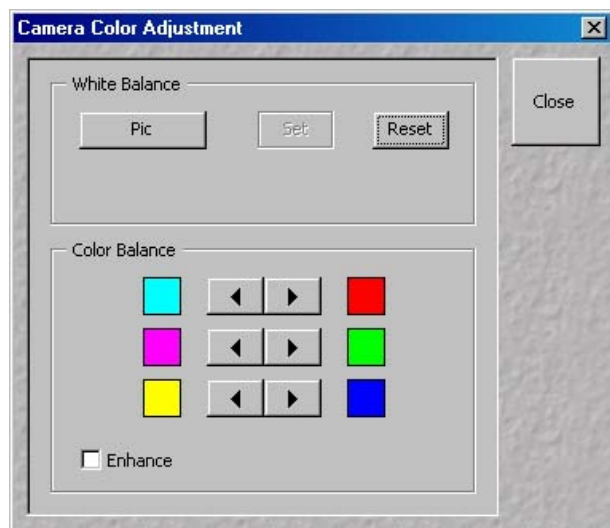


Fig. 4-232 Camera Color Adjustment window

#### 4.10.4 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.

#### 4.10.5 Reboot

The **Reboot** function is for servicing purposes only and may only be performed by authorized personnel. Its access is therefore password-protected.

#### 4.10.6 HW/Admin

The **HW/Admin** function is for servicing purposes and may only be used by authorized service personnel. Its access is therefore password-protected.

#### 4.10.7 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.

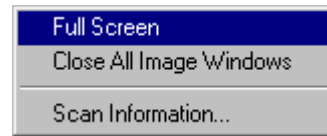


Fig. 4-233 Window menu

### 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.



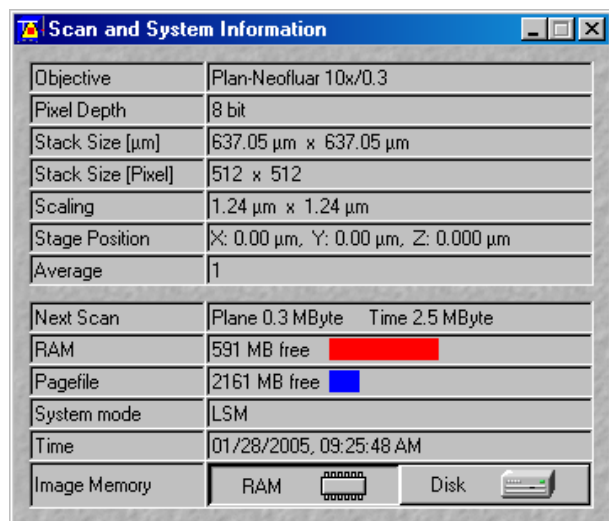



Fig. 4-234 Scan Information window

#### 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 4-200).

- 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.

## 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.

### 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.

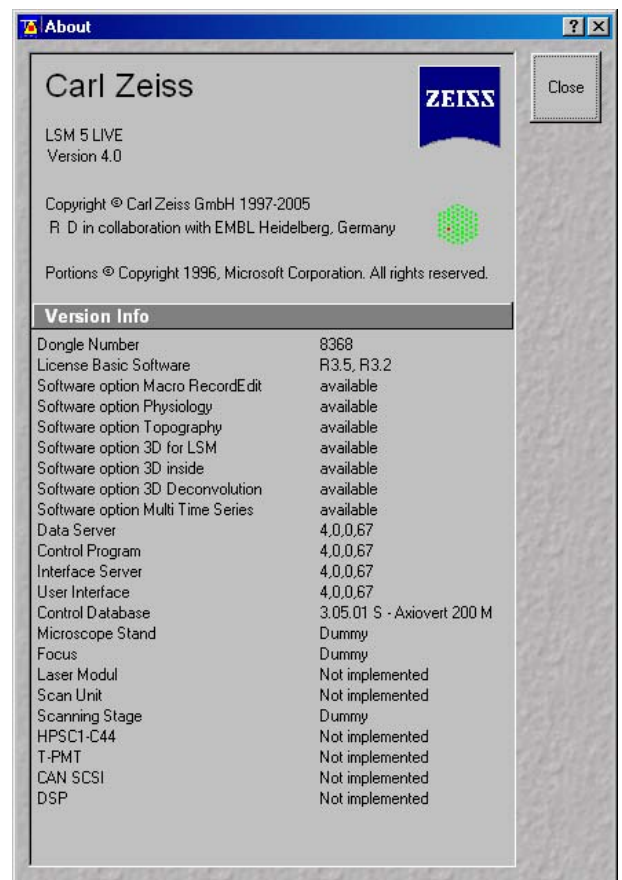


Fig. 4-235 About LSM 5 LIVE window

### 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 4-146).

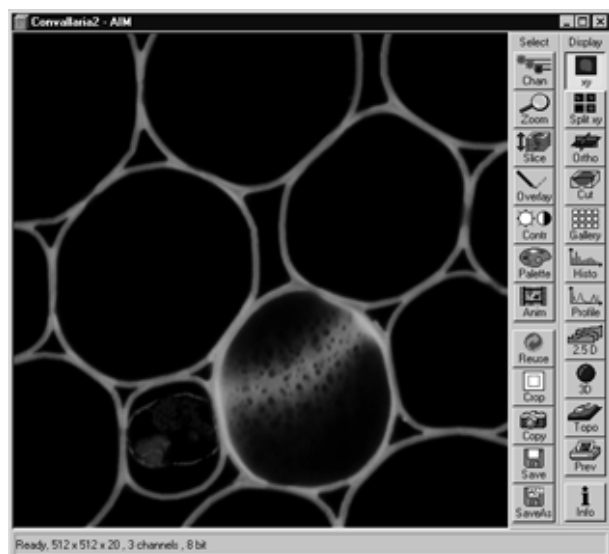


Fig. 4-236 Image Display window showing a single frame image

## 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**).

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)

#### 4.13.2 Display Modes

The following display modes are available for the different acquisition modes:

Image type		Frame	Frame	Frame	Line	Line
Series type			Z Stack	Time		Time
Display functions						
xy		•	•	•		
xt					•	•
tx					•**	•
Split xy		•*	•*	•*	•	•
Split tx					•**	•
Coded						
Ortho			•			
Max						
Cut			•			
Gallery			•	•		
Histo		•	•	•	•	•
Profile		•	•	•		
Diagr					•	•
Mean				• t***	•**	•
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.

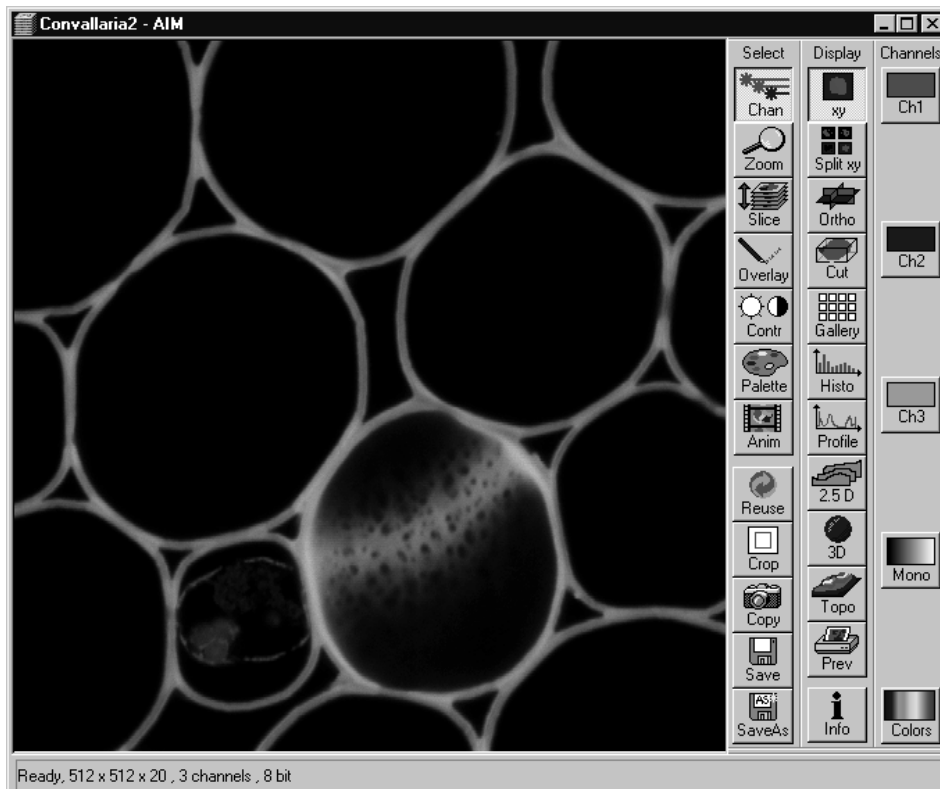


Fig. 4-237 Image Display window; Select - Chan

#### 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.



Fig. 4-238 Color selection box


#### 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.

#### 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 4-51).

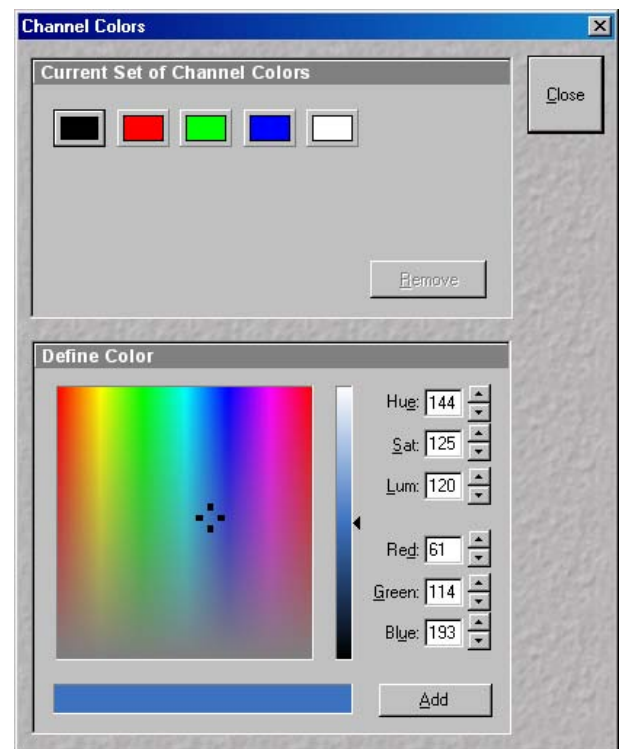


Fig. 4-239 Channel Colors window

#### 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.

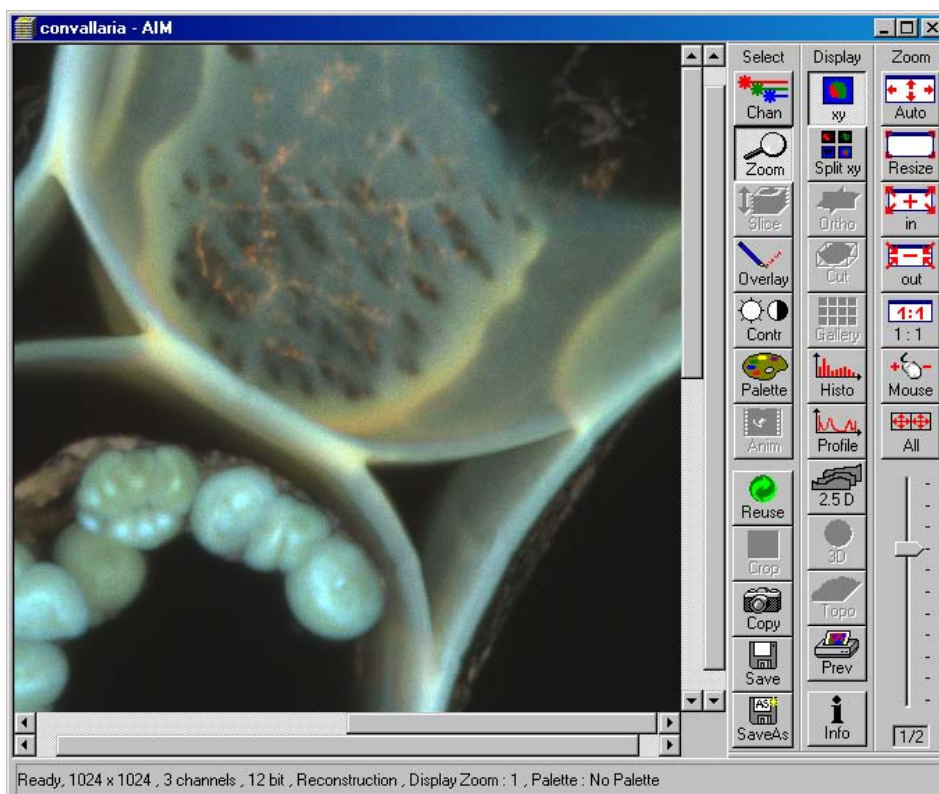


Fig. 4-240 Image Display window; Select - Zoom

<b>Zoom-Auto</b>	The image is fitted automatically to size of the <b>Image Display</b> window.
<b>Zoom-Resize</b>	Restores the image to its initial size.
<b>Zoom +</b>	Enlarges the image by factor 2.
<b>Zoom –</b>	Reduces the image by factor 2.
<b>Zoom 1:1</b>	Restores an image zoomed in any way to its original size.
<b>Zoom-Mouse</b>	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 were 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.

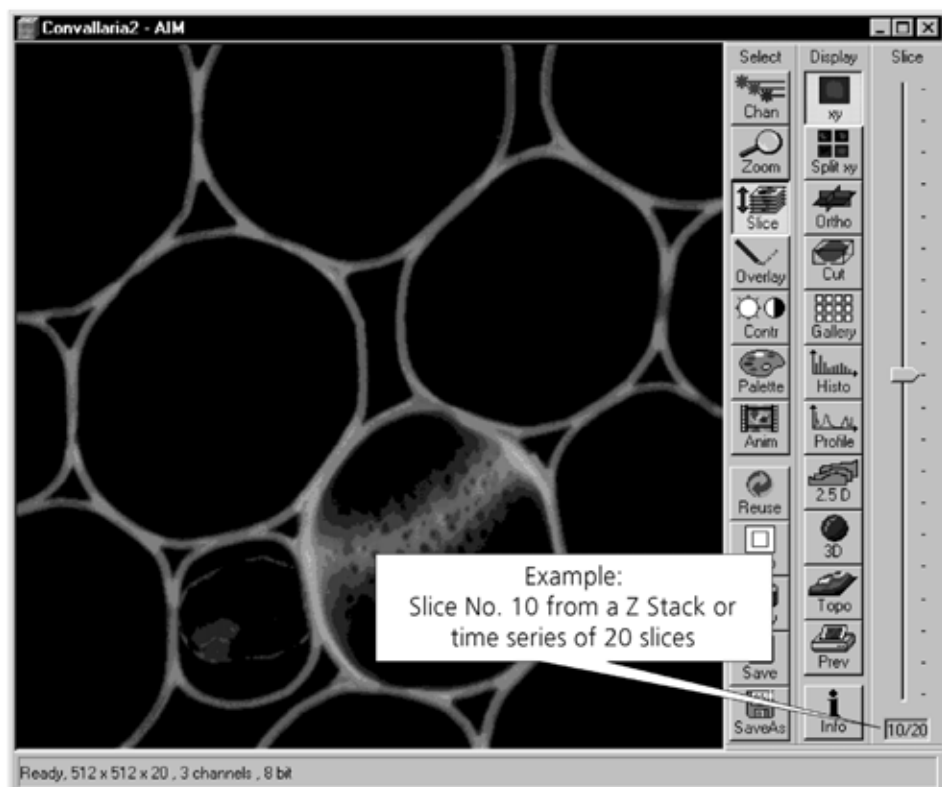


Fig. 4-241 Image Display window; Select - Slice



#### 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.

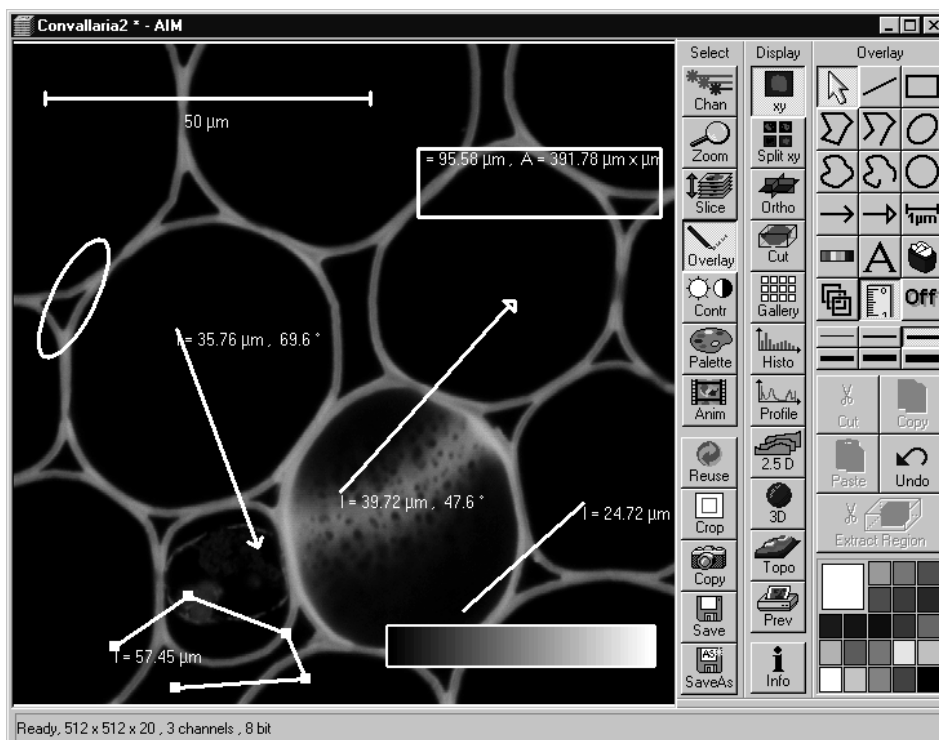


Fig. 4-242 Image Display window; Select - Overlay

#### 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.



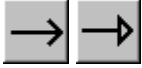
**Open free-shape curve** button: Creation of an open Bezier figure in the **Image Display** window.

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.



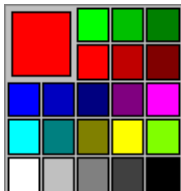
**Paste** button: The image contents of an overlay element copied to the clipboard are inserted in the current **Image Display** window and can be positioned anywhere in the image using the mouse.



**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.

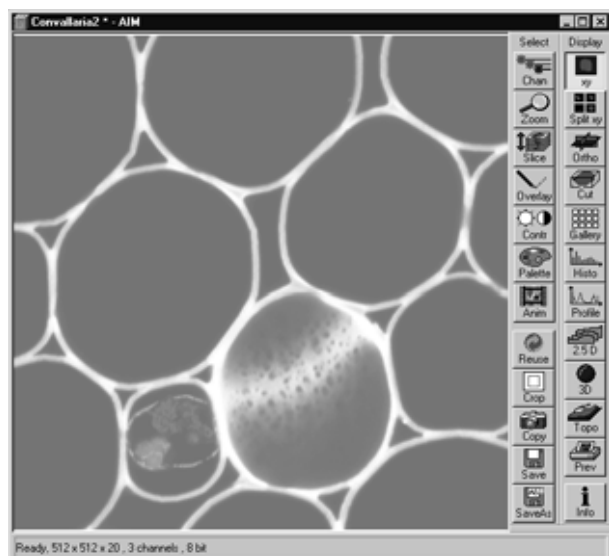


Fig. 4-243 Image Display window; Select - Contr

#### 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.

- 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**.

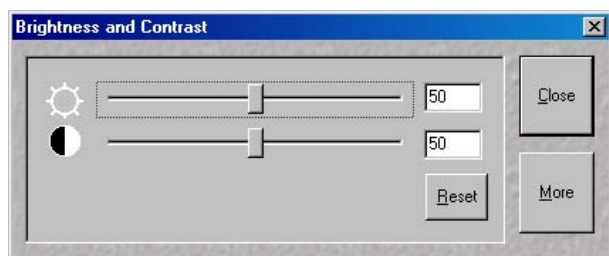


Fig. 4-244 Brightness and Contrast window

- 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.

- 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.

#### 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.

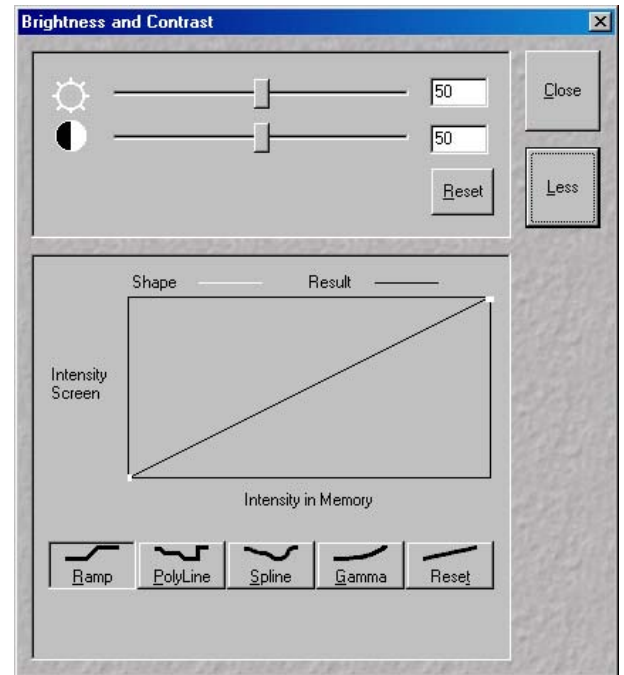



Fig. 4-245 Brightness and Contrast window with activated Ramp function

#### 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.

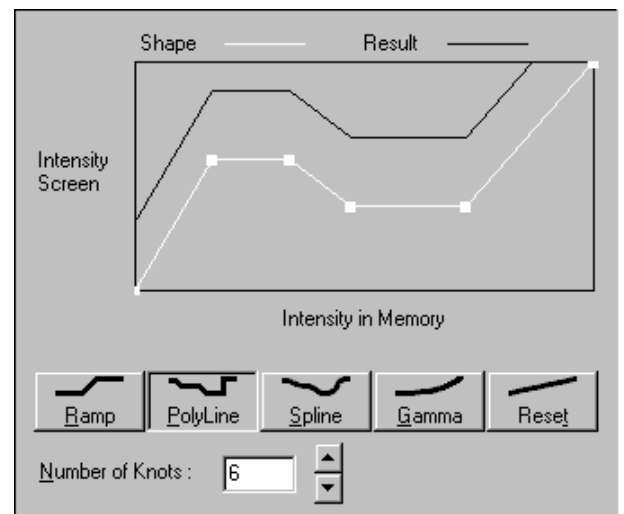


Fig. 4-246 Brightness and Contrast window with activated PolyLine function

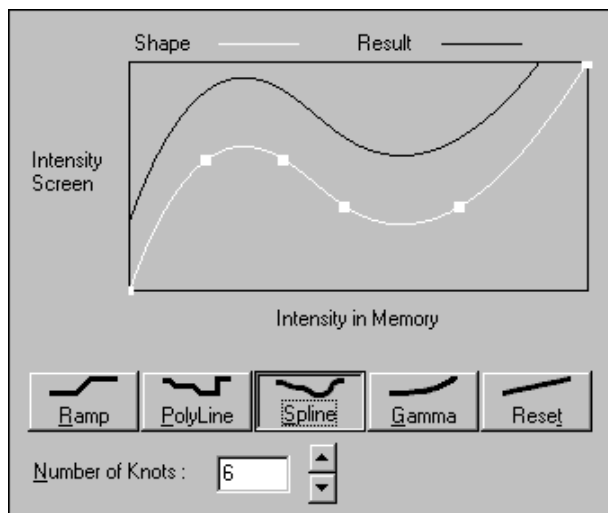


Fig. 4-247 Brightness and Contrast window with activated Spline function

#### 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.

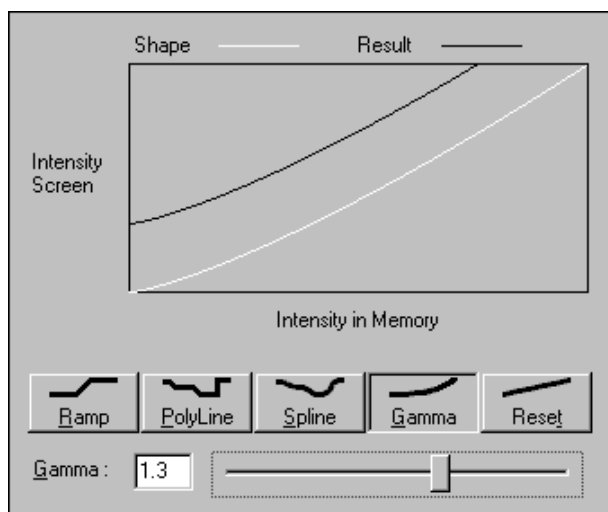


Fig. 4-248 Brightness and Contrast window with activated Gamma function

#### 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.

#### 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.



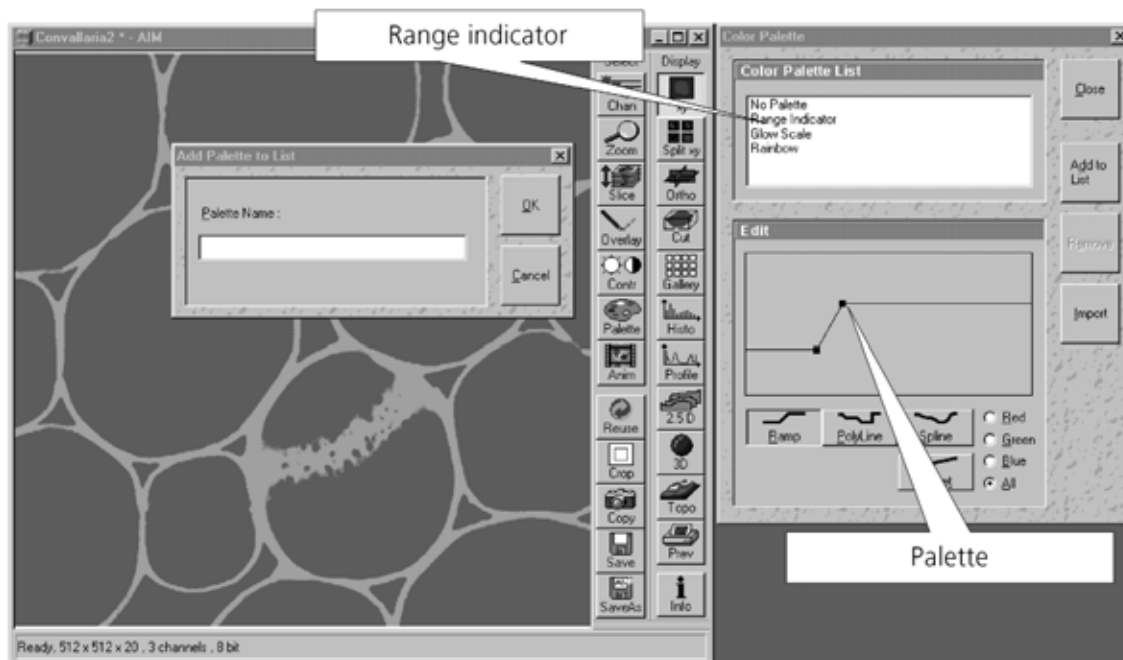


Fig. 4-249 Image Display window, Select - Palette; Color Palette and Add Palette to List window

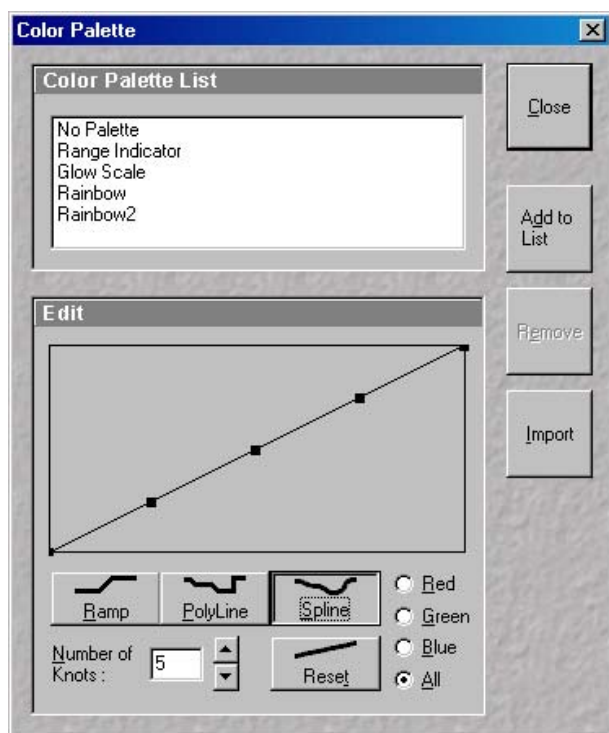


Fig. 4-250 Color Palette window

#### 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 4-234f).

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.

#### 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.

#### 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.

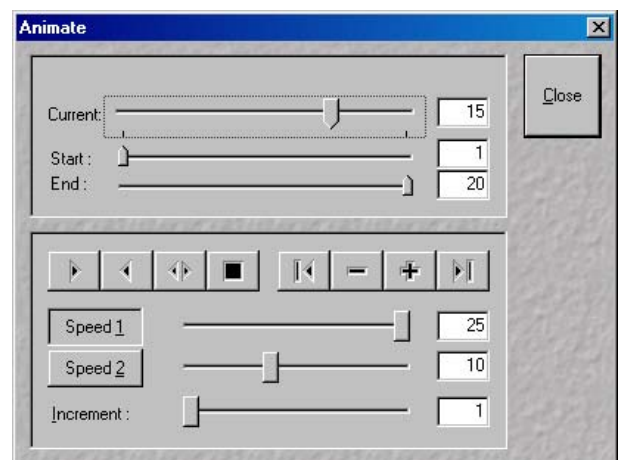


Fig. 4-251 Animate window

- 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 :

**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 :

**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 :

**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.

Speed 1  
Speed 2

**Speed1 /Speed2** buttons / sliders: Selection between two speeds, change of the relevant speed via slider or input box.

Increment :

**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** and **Time Series 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, Aperture Size (confocal aperture), 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.

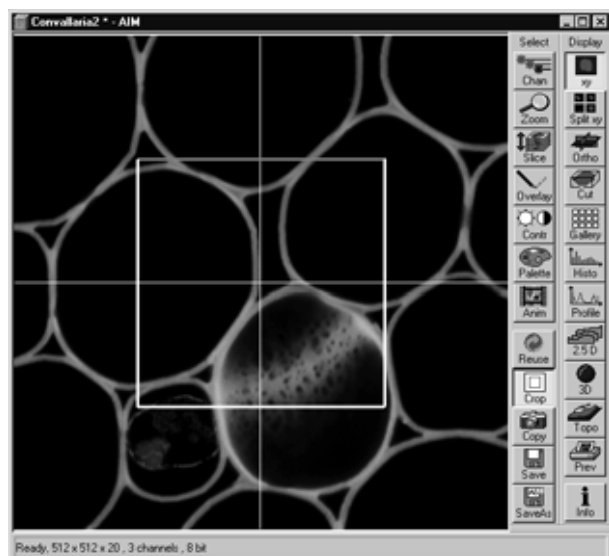


Fig. 4-252 Image Display window; Select - Crop

#### 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 and Offset** 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**, **Continuous** 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** and **Offset=0** in the **Scan Control** window in the **Mode** submenu.

- 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:

- |                   |  |   |
|-------------------|--|---|
| <b>Offset</b>     |  | • Click into the crop rectangle, keep the left mouse button pressed and drag the crop rectangle to the required position. Release the mouse button.                                 |
| <b>Zoom</b>       |  | • Click on a corner of the crop rectangle, keep the left mouse button pressed and set the required size. Release the mouse button.  |
| <b>Side ratio</b> |  | • 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.

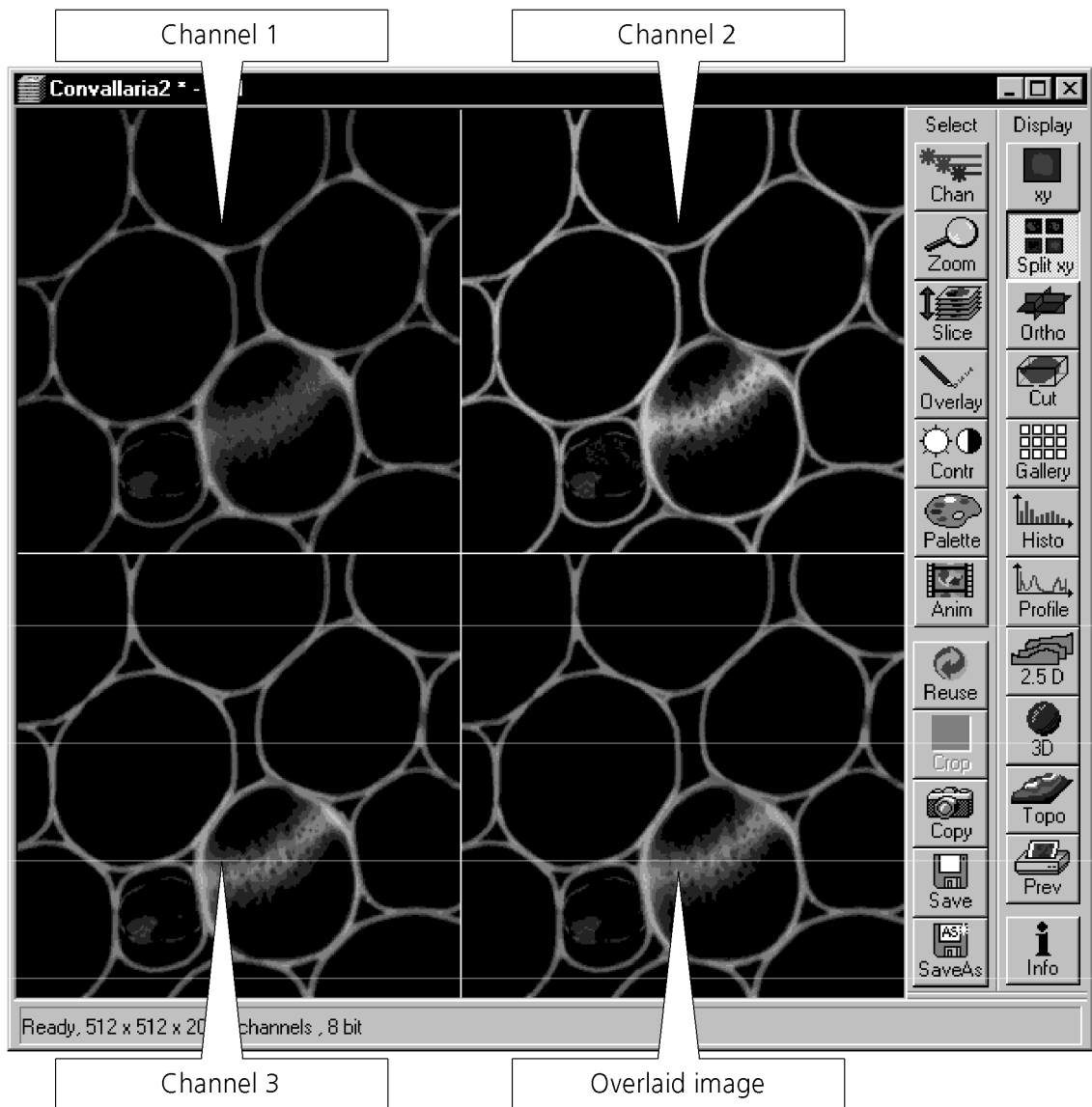


Fig. 4-253 Image Display window, Split xy display



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.

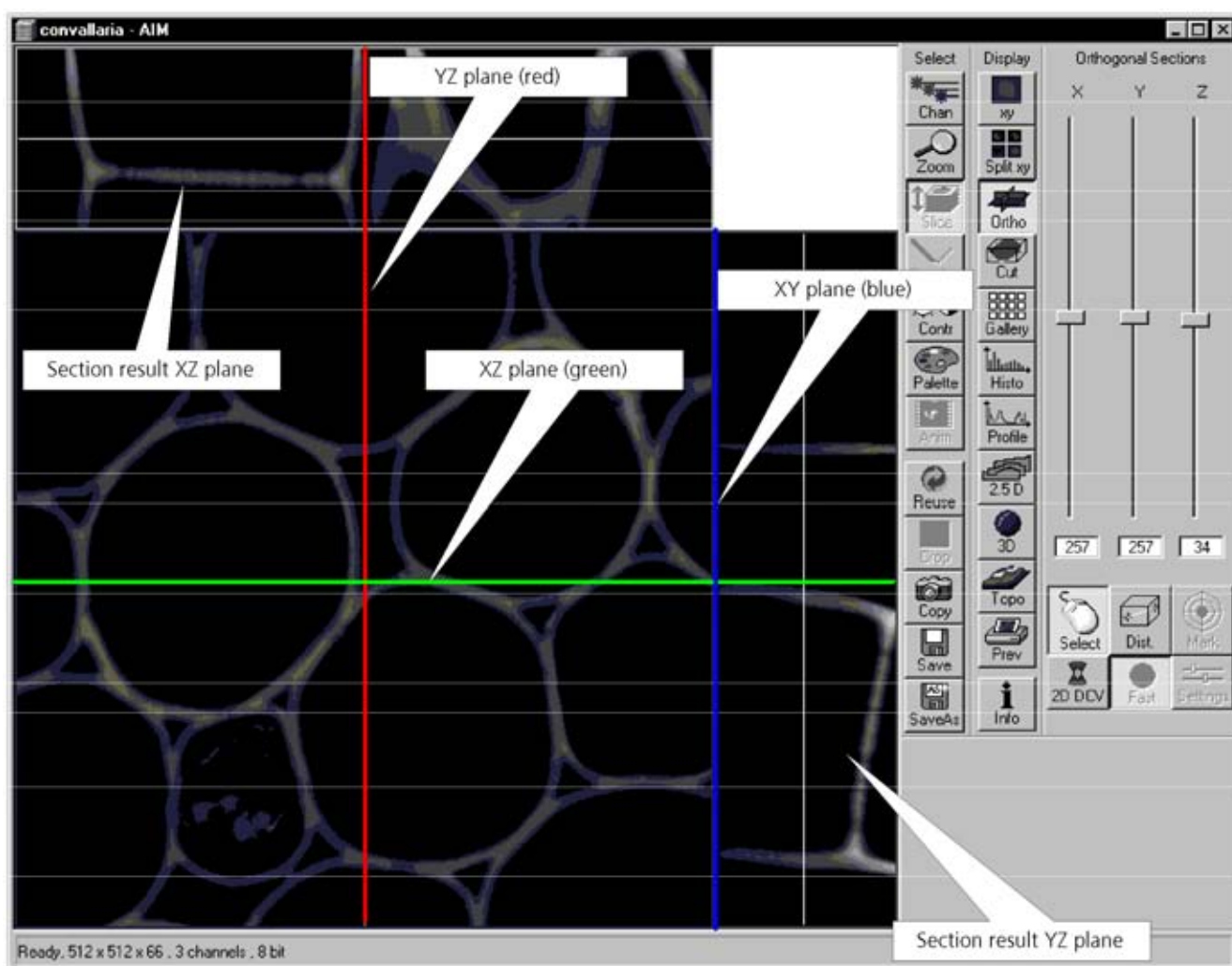








Fig. 4-254 Image Display window, Ortho display

#### 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  $\mu\text{m}$  below the **Select**, **Dist.** and **Mark** buttons, e.g. 3D Distance: 55.60  $\mu\text{m}$ .

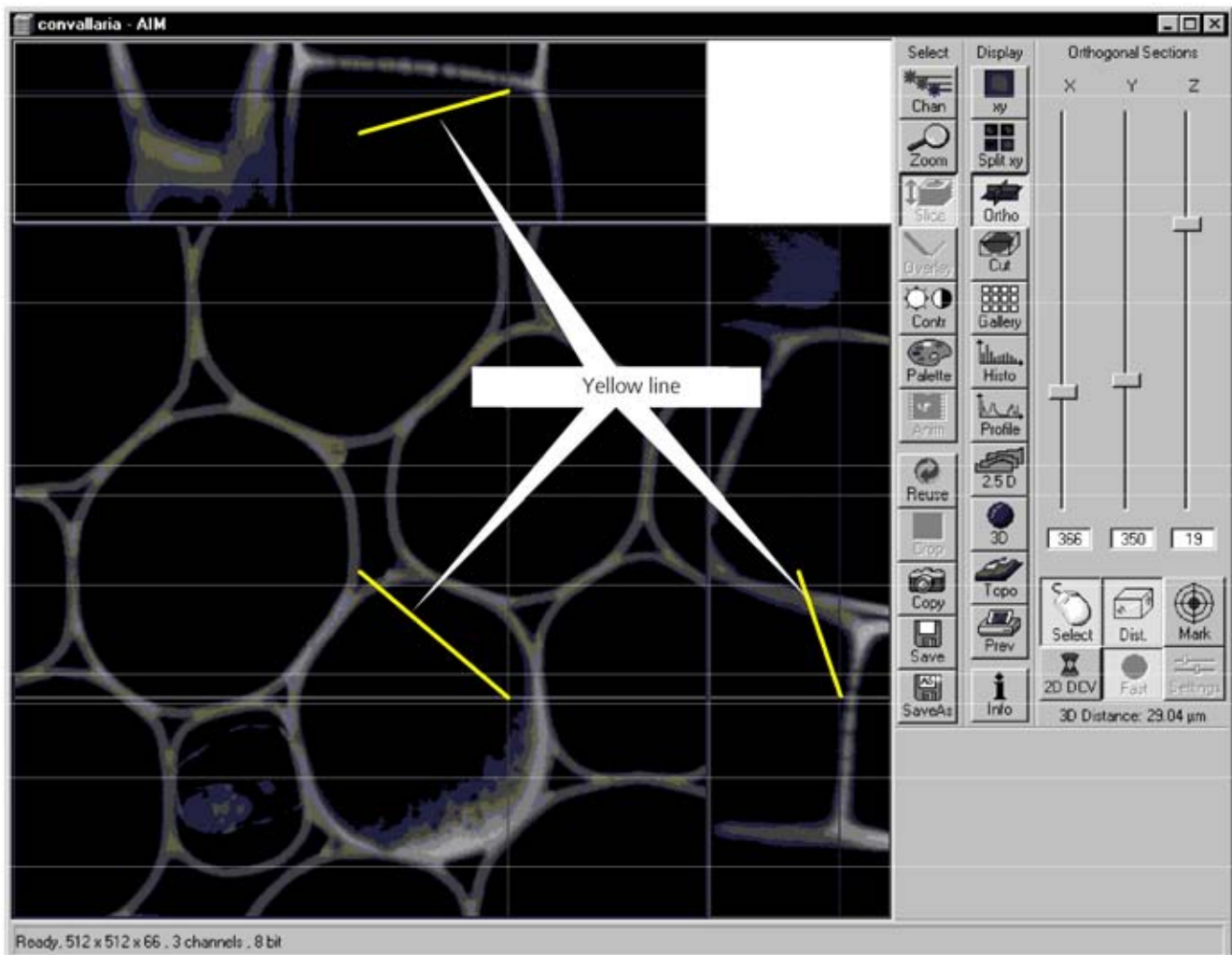


Fig. 4-255 Image Display window, Ortho Distance display

#### 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**.

##### (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 4-163).

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.

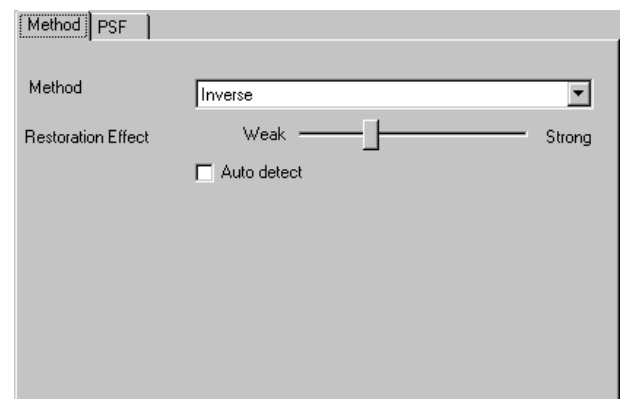


Fig. 4-256 Method tab

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.

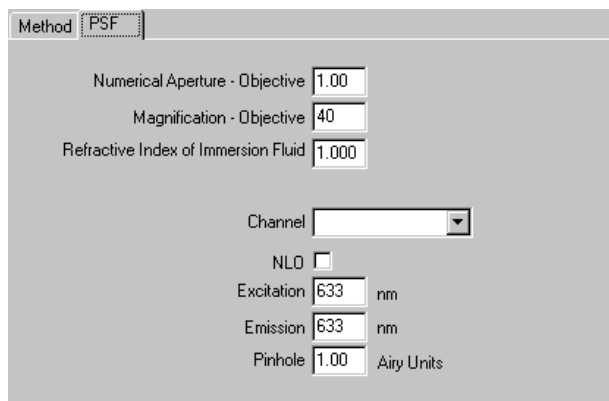


Fig. 4-257 PSF tab

## (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).

### 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.

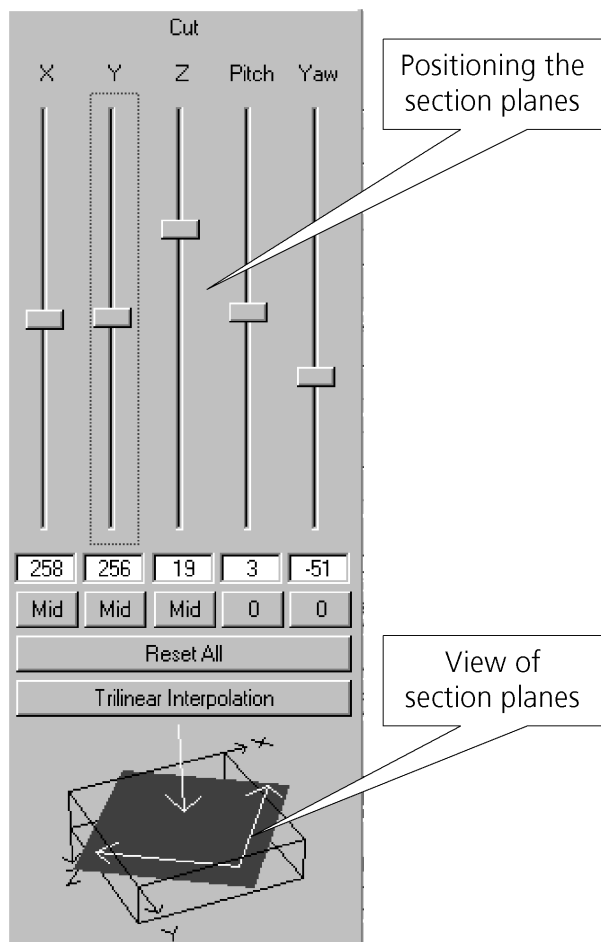


Fig. 4-258 Image Display window, Cut display

#### 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.
- 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.

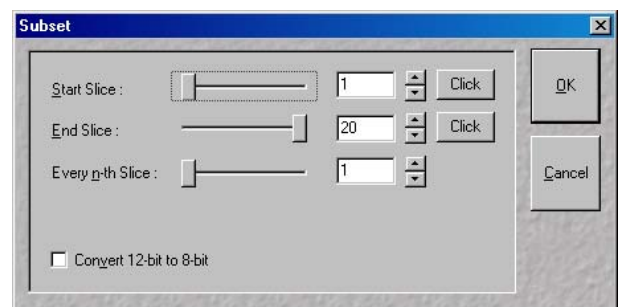


Fig. 4-259 Subset window

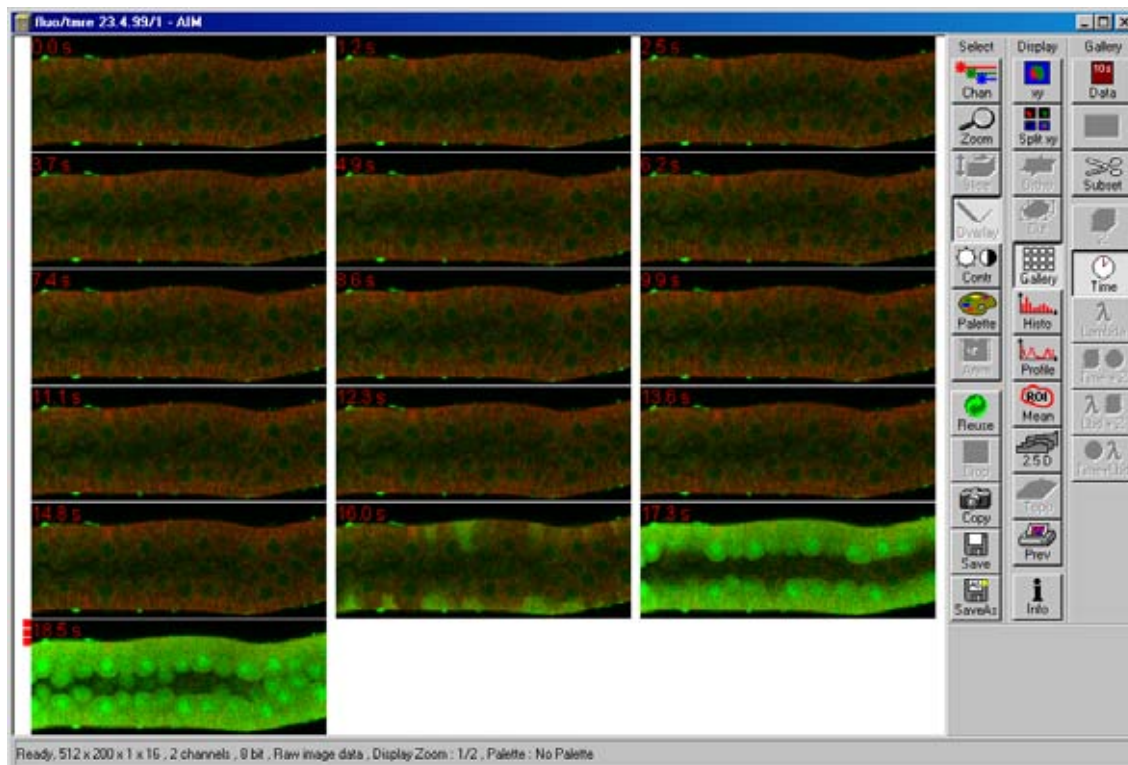



Fig. 4-260 Image Display window, Gallery display

## 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.

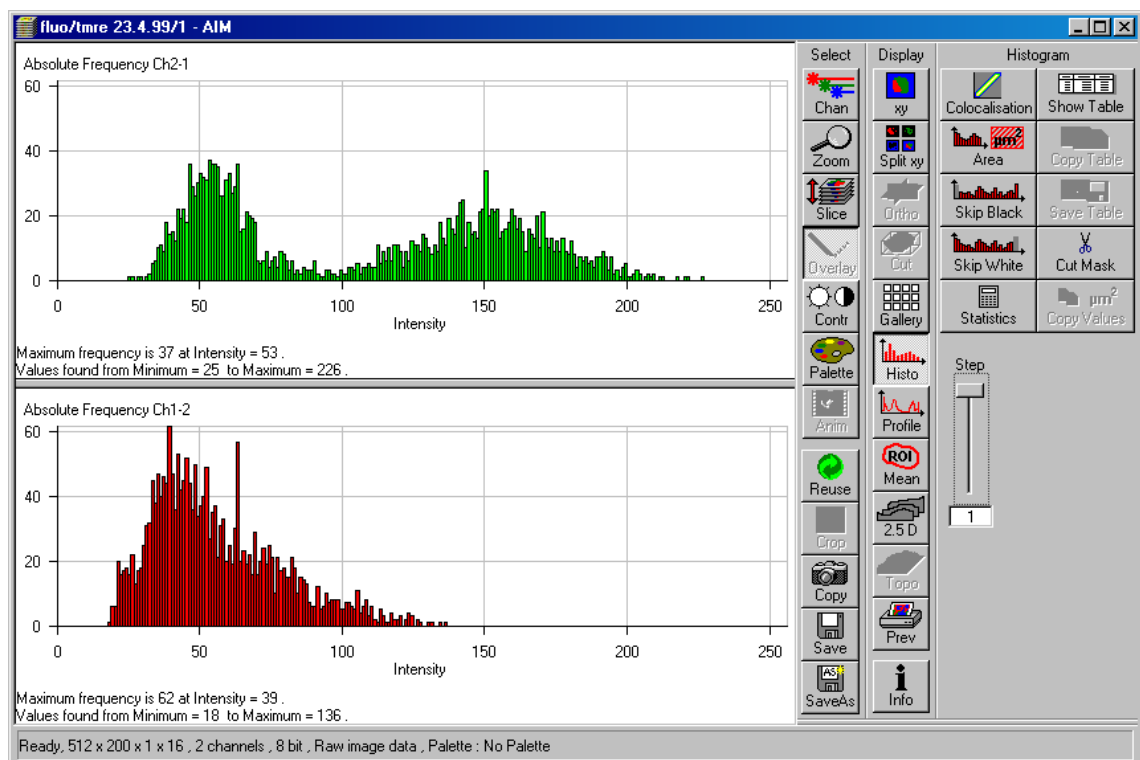


Fig. 4-261 Image Display window, Histogram display

 The **Histo** button can also be used online during scanning.



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**Histogram functions**

<b>Skip Black</b> button	Ignore black pixels (gray value 0) in the histogram.
<b>Skip White</b> button	Ignore white pixels (gray value 255 or 4096) in the histogram.
<b>Statistics</b>	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
<b>Step</b> 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.
<b>Show Image</b> button	Shows the image in the <b>Image Display</b> window beside the histogram.
<b>Show Table</b> button	The histogram is shown as a table at the bottom left of the <b>Image Display</b> window.
<b>Copy Table</b> button	The histogram table is copied to the clipboard.
<b>Save Table</b> button	The histogram table can be stored as a text file (extension *.txt).
<b>Area functions</b>	
<b>Area</b> button	Interactive definition of area for size and intensity measurement.
<b>Save Values</b> button	Copies area values to the clipboard (only available if the <b>Area</b> 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.

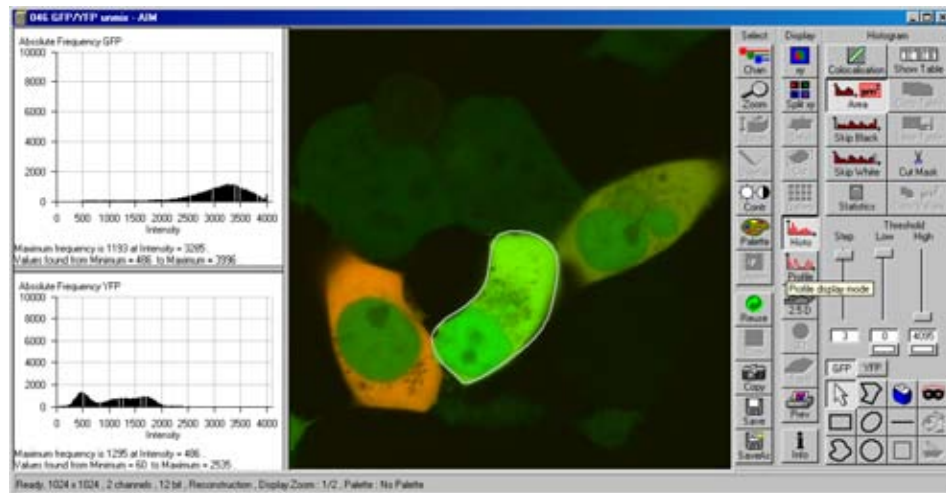


Fig. 4-262 Image Display window, Area Measure display

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.

ChS1-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.

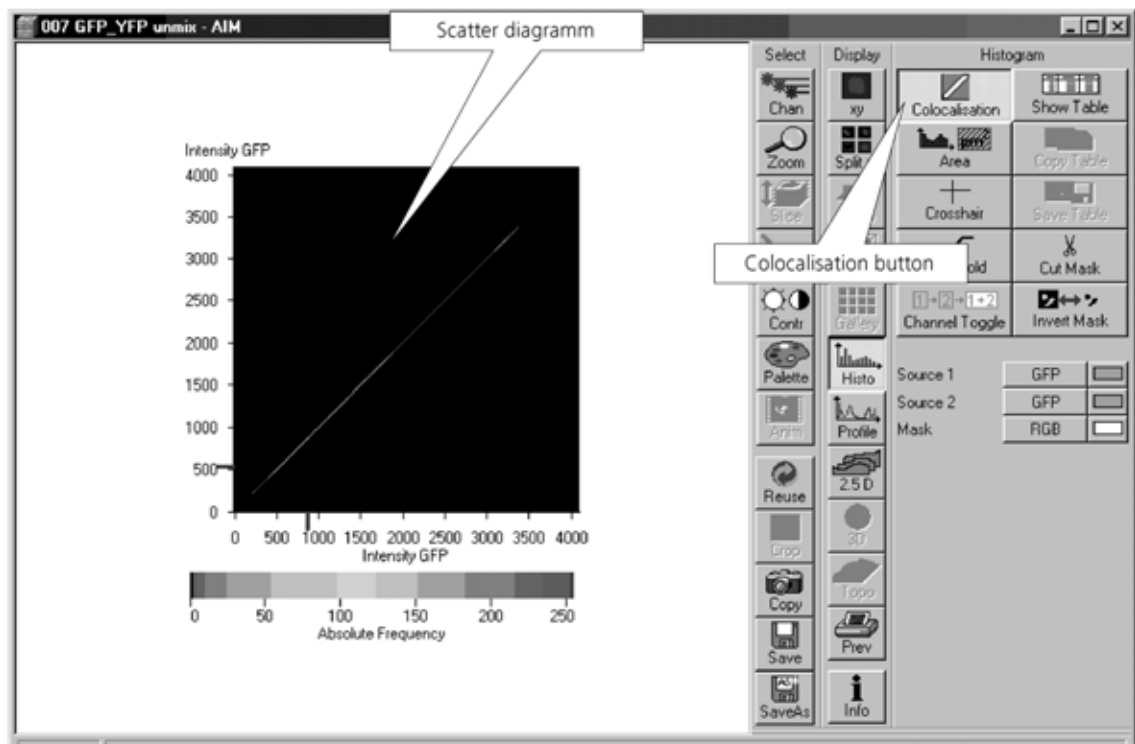


Fig. 4-263 Image Display window, Colocalization display

## 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)



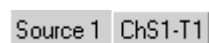
**Cut Mask** button.



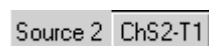
**Channel Toggle** button.



**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.

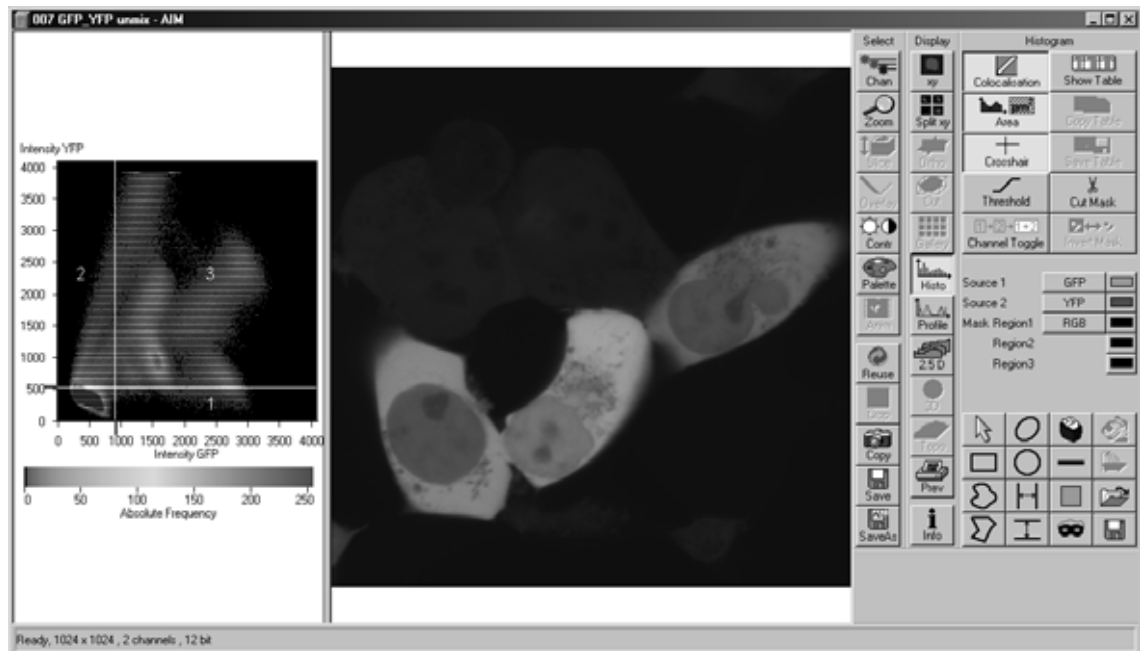


Fig. 4-264 Image Display window, Colocalization display

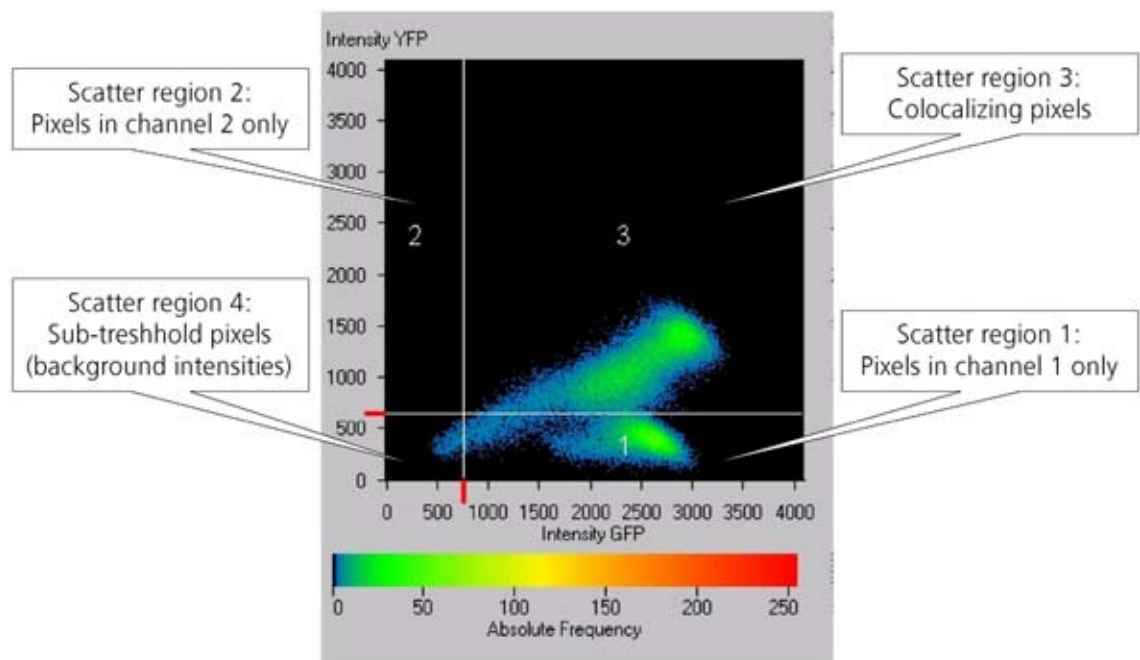


Fig. 4-265 Scatter diagram and threshold with crosshair

**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<sup>2</sup>)

**Colocalization coefficients**

$$c_1 = \frac{\text{pixels}_{Ch1,coloc}}{\text{pixels}_{Ch1,total}} \quad c_2 = \frac{\text{pixels}_{Ch2,coloc}}{\text{pixels}_{Ch2,total}}$$

- 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**

$$M_1 = \frac{\sum_i Ch1_{i,coloc}}{\sum_i Ch1_{i,total}} \quad M_2 = \frac{\sum_i Ch2_{i,coloc}}{\sum_i Ch2_{i,total}}$$

- 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.



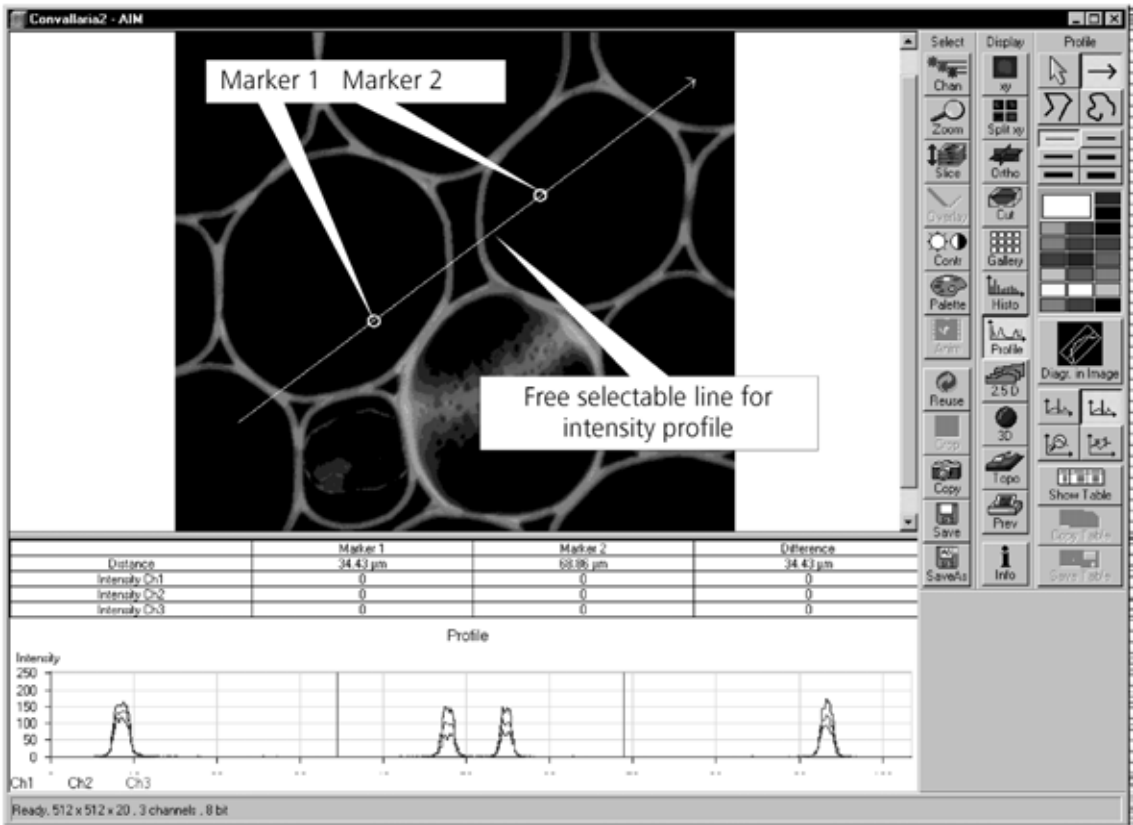


Fig. 4-266 Image Display window, Profile display

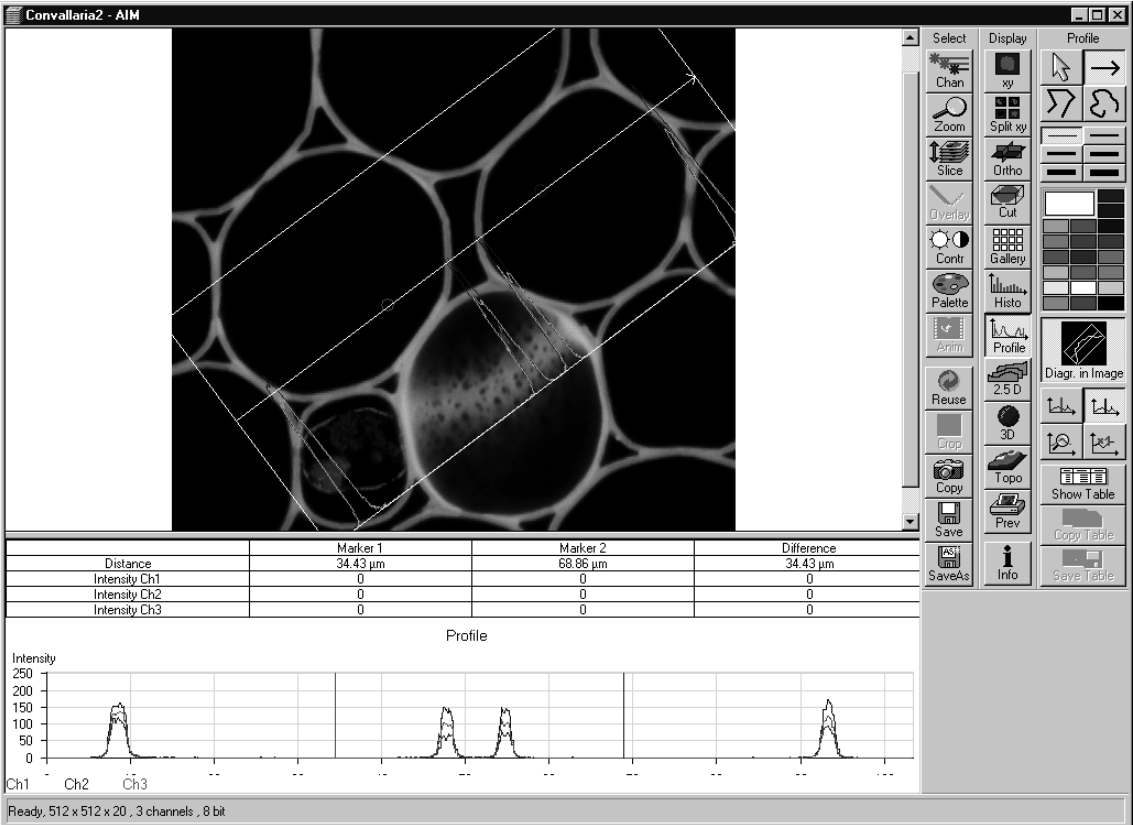


Fig. 4-267 Image Display window, Profile display

#### 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.



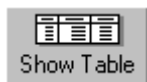
**Marker 2** button (blue): Activates the blue marker for movement in the profile diagram; the marker shown as a blue line in the diagram can now be moved to the right or left of the diagram using the mouse. The marker in the image display (blue circle) follows accordingly.



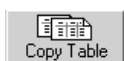
**Zoom** button: Zoom function for profile diagram. Click and drag a rectangle over the area to be enlarged in the profile diagram; the selected area is enlarged on release of the mouse button. The zoom function can be performed several times. A click with the right mouse button will reset the original 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).

#### 4.13.21.3 Display - Mean

This function allows to

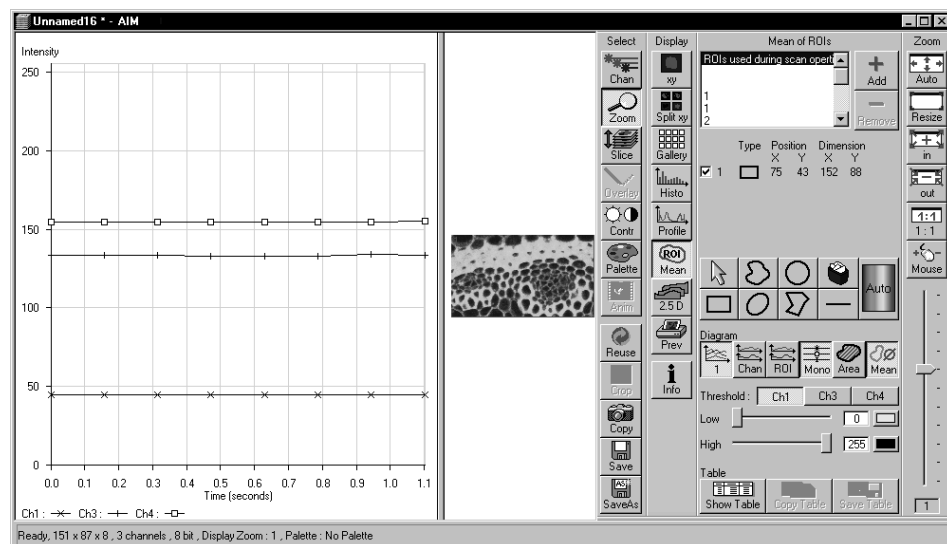
- display the intensity time diagram (mean intensity in user defined ROIs over time)
- display the intensity time diagram for volumes (3D ROIs) within a Z-Stack over time
- use frame time series and frame Z Stack time series as input
- show the intensity values in table form and copy table to clipboard 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. 4-268** Image Display window, Mean ROI display for time series in single plane

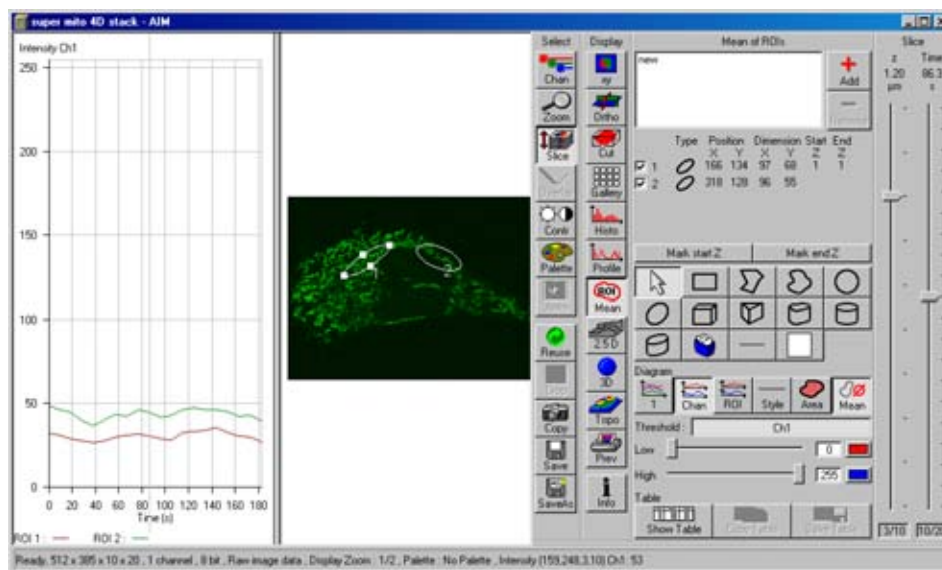


Fig. 4-269 Image Display window, Mean ROI display for time series of Z Stack

#### 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.

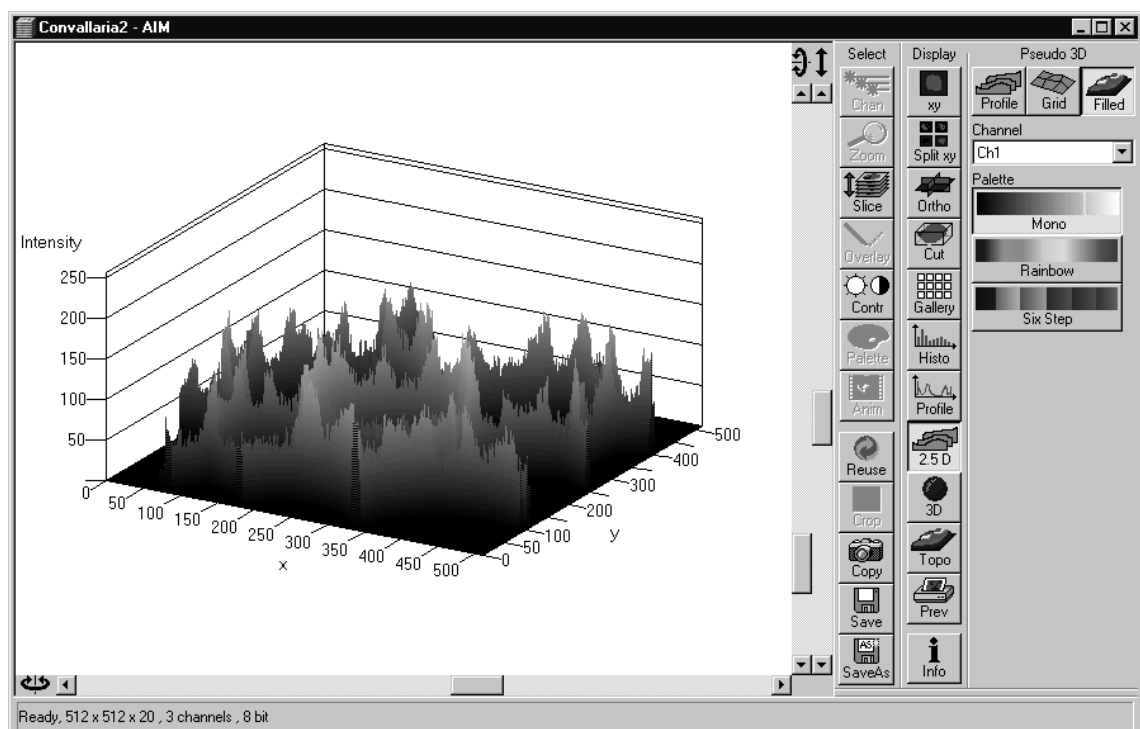


Fig. 4-270 Image Display window, 2.5 D display

The **Pseudo 3D** toolbar contains the following function elements:

- |                         |   |
|-------------------------|---|
| <b>Profile</b> button   | Profile display (vertical polygon display). Setting of the <b>Profile Distance</b> between 1 and 20 using the slider.     |
| <b>Grid</b> button      | Grid display (horizontal grid display). Setting of the <b>Grid Distance</b> between 1 and 20 using the slider.            |
| <b>Filled</b> button    | Color diagram (filled 3D diagram). Selection between the <b>Mono</b> , <b>Rainbow</b> and <b>Six Step</b> color palettes. |
| <b>Channel</b> list box | Permits the selection of a <b>Channel</b> 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.

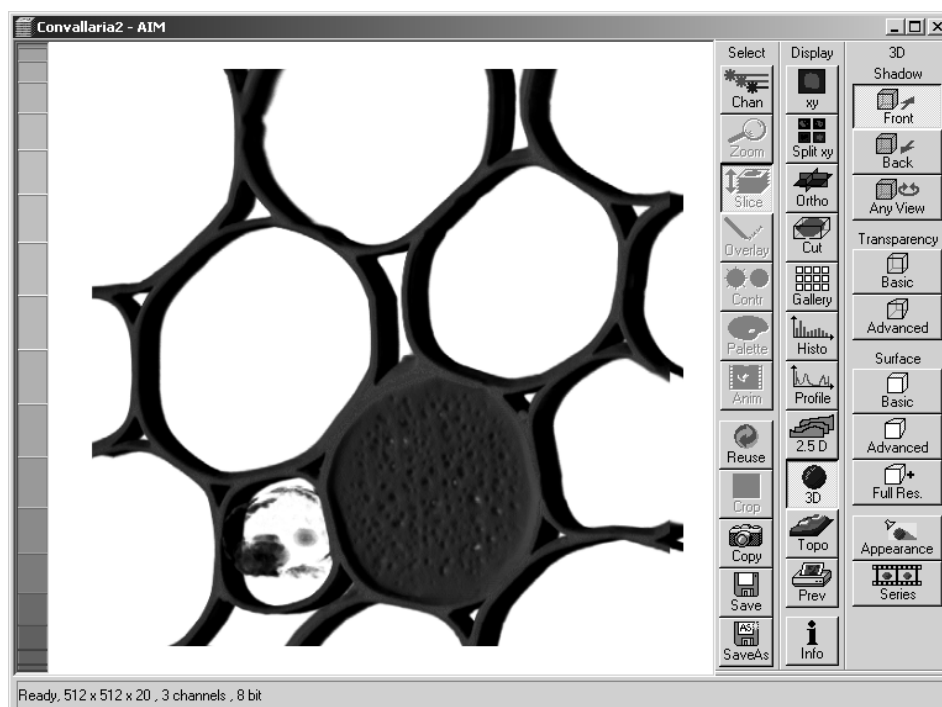


Fig. 4-271 Image Display window, 3D display

The **3D** toolbar contains the following function elements:

#### Shadow projection

<b>Front</b> button	Shadow rendering front view
<b>Back</b> button	Shadow rendering back view
<b>Any View</b> button	Shadow rendering with user defined view

## Transparency projection

<b>Basic</b> button	Transparency rendering (voxel based)
<b>Advanced</b> button	Transparency rendering (voxel based) with textures

## Surface projection

<b>Basic</b> button	Surface rendering (voxel based)
<b>Advanced</b> button	Surface rendering (triangle based)
<b>Full Resolution</b> button	High accurate surface rendering (triangle based)
<b>Appearance</b> button	Opens the render properties dialog
<b>Series</b> button	Renders a series of 3D image stack or 3D / 4D time series, opens the <b>Series render</b> 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.

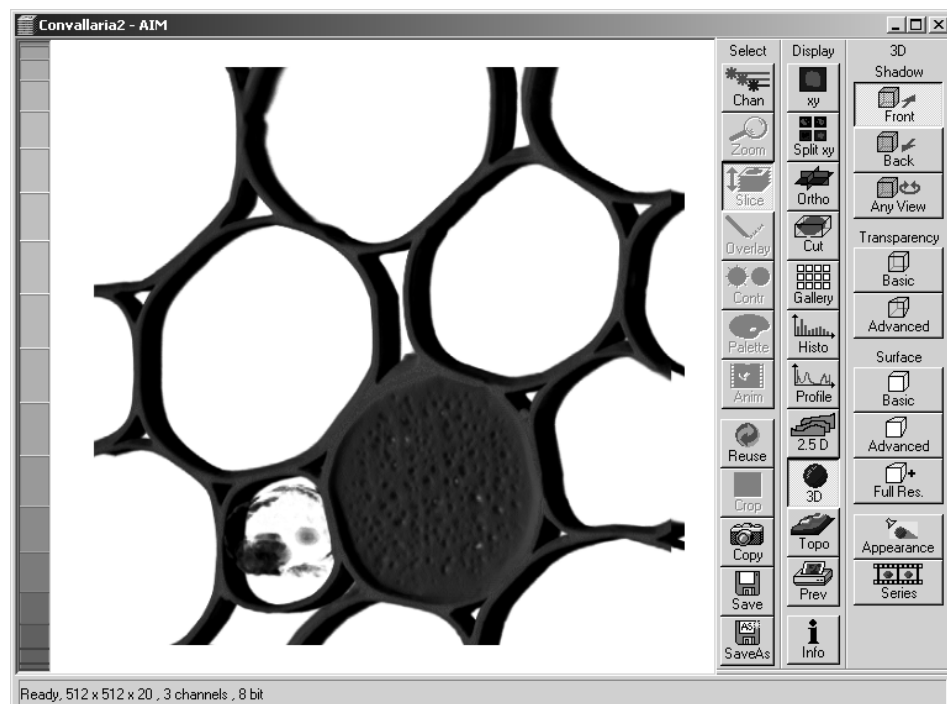


Fig. 4-272 Image Display window, 3D display, Shadow projection, Front view



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.

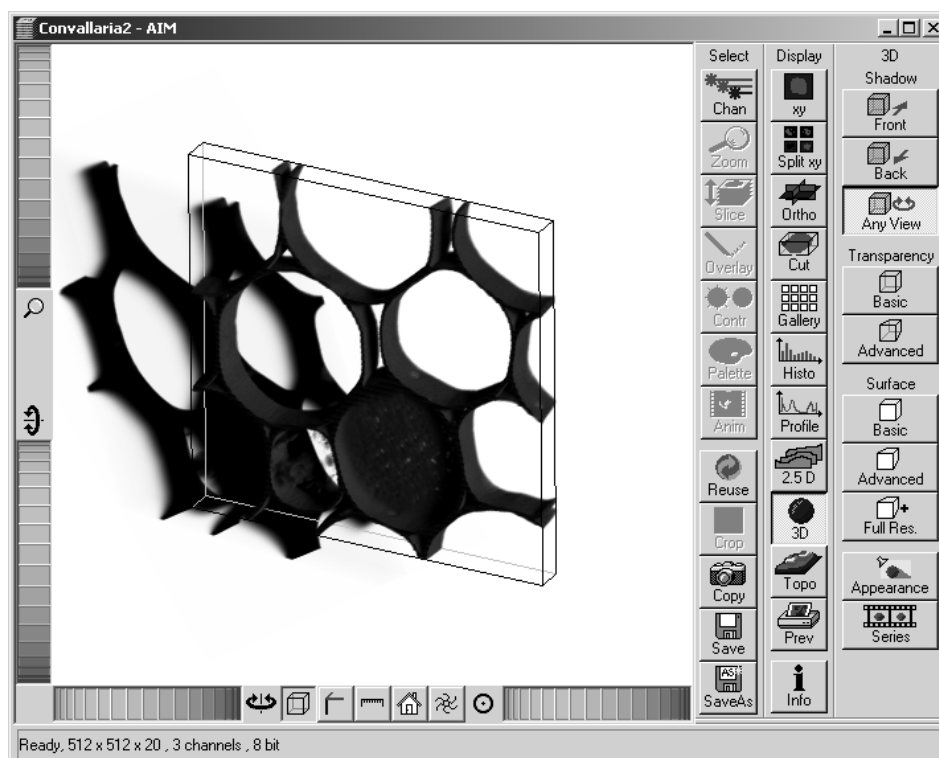


Fig. 4-273 Image Display window, 3D display, Shadow projection, Any View

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.

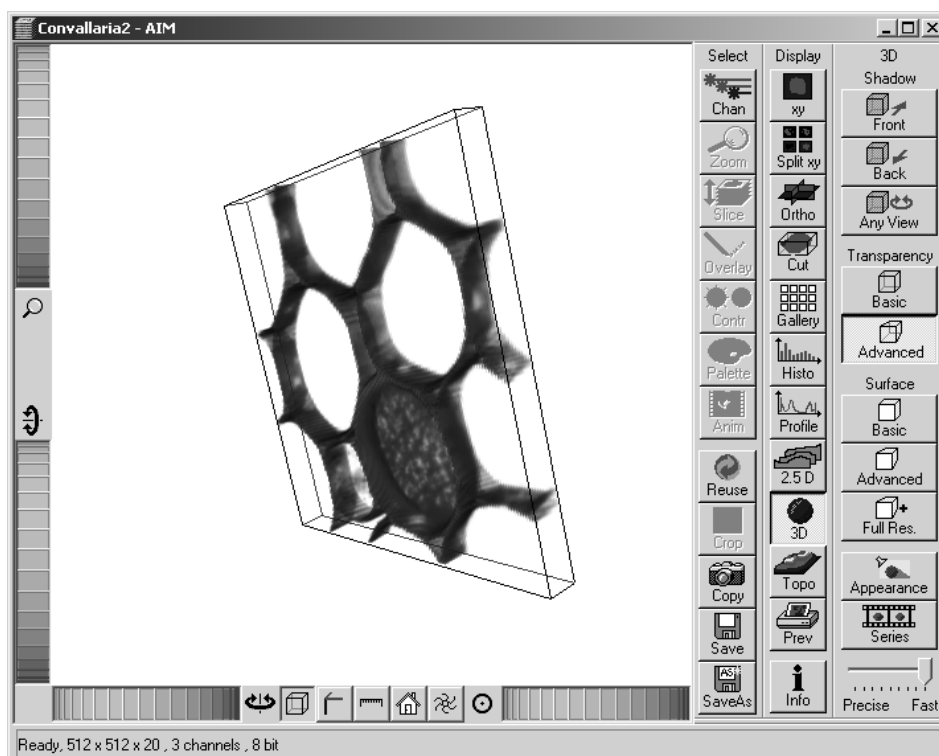


Fig. 4-274 Image Display window, 3D display, Transparency projection, Advanced

#### 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.

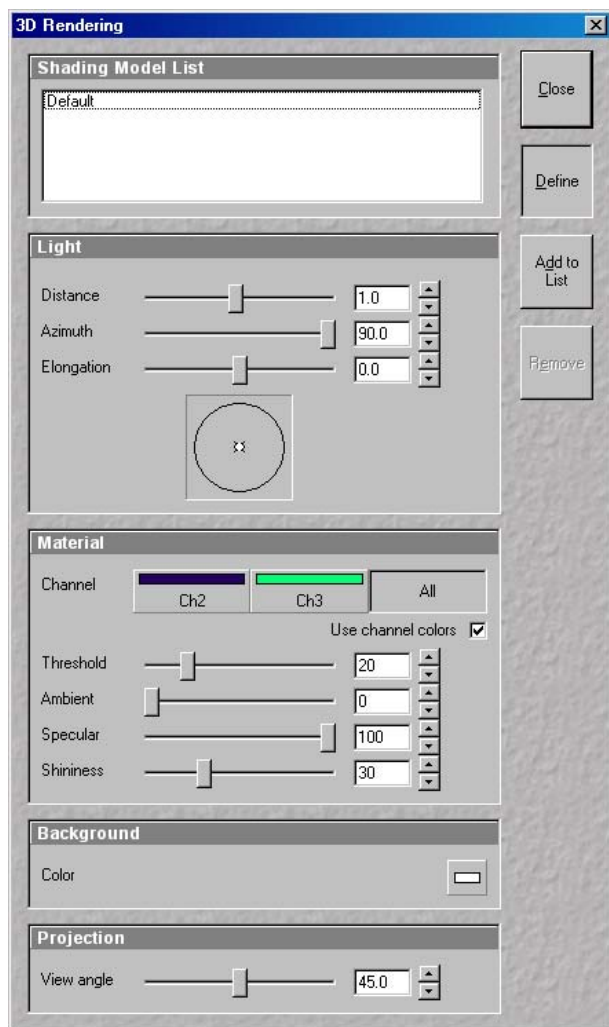
The surface projections **Basic** and **Advanced** are perspective-type reconstructions of the surface and differ in the fact that the calculation of the 3D information is based on voxels or triangles.

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. 4-275** 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 4-289.

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.

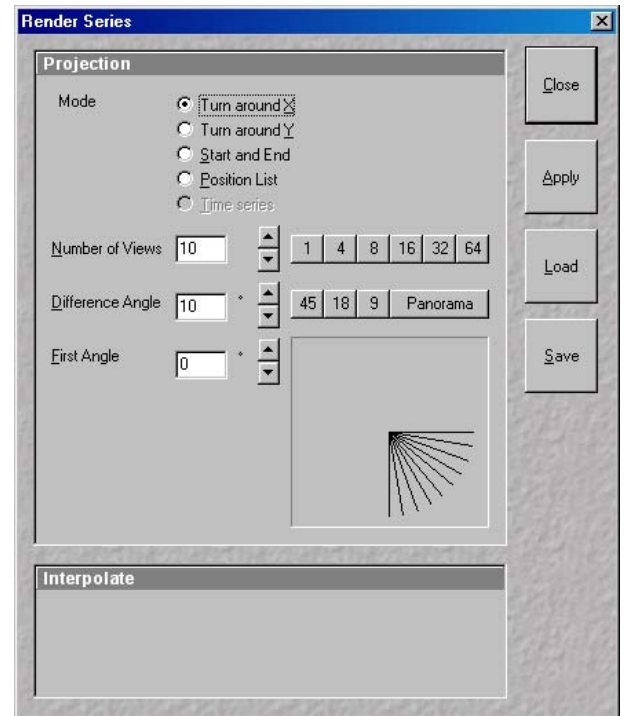


Fig. 4-276 **Render Series window**  
(e.g. Turn around X mode)

#### (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 4-155).

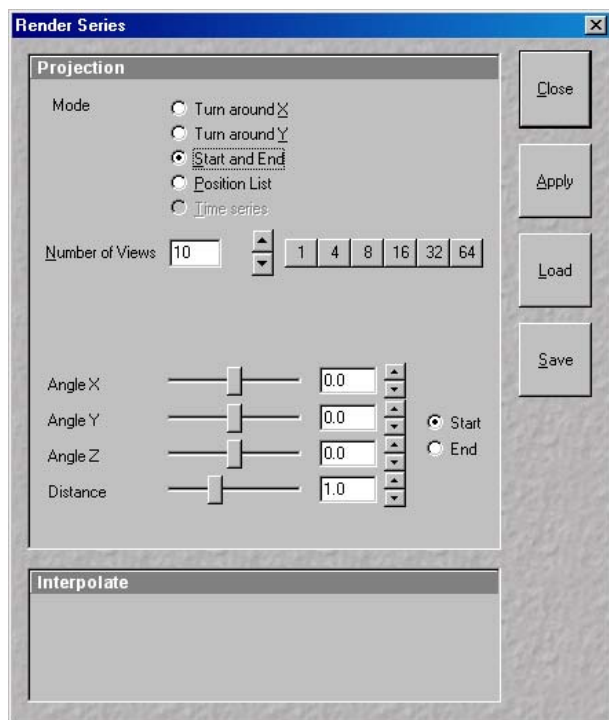


Fig. 4-277 Render Series window  
(e.g. Start and End mode)

## (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.

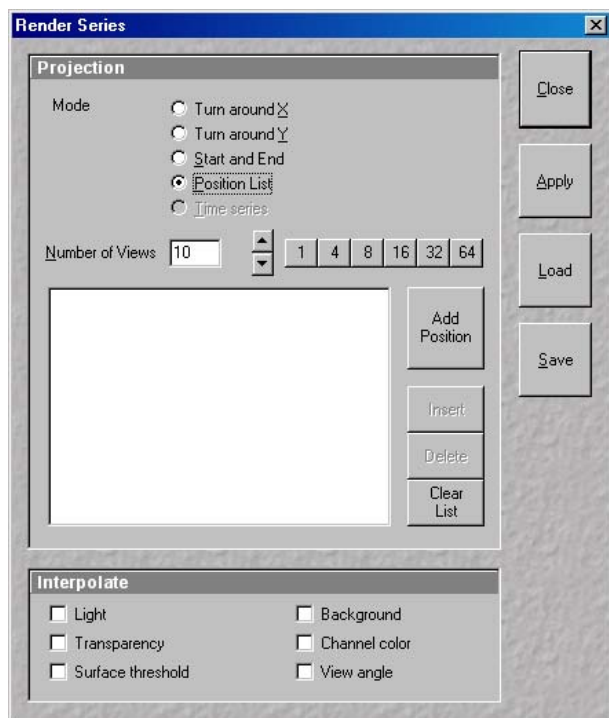


Fig. 4-278 Render Series window  
(e.g. Position List mode)

## (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.

- If you click on the right mouse button in the main display window of Image VisArt two options appear:
  - Render properties
  - Renderer

This functionality helps to find the optimal performance when working with different hardware renderers and to speed up some advanced rendering modes.}

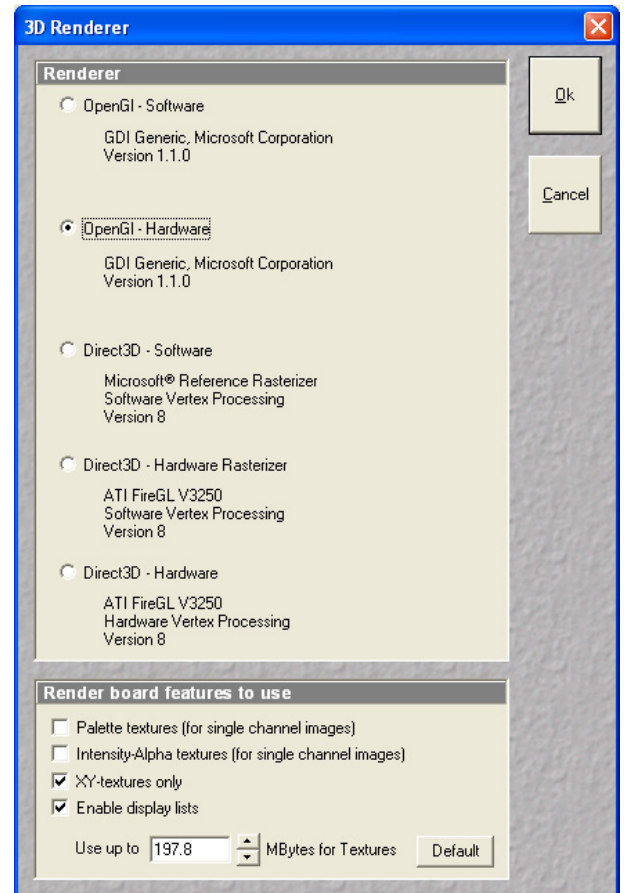


Fig. 4-279 3D Renderer window



#### 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.

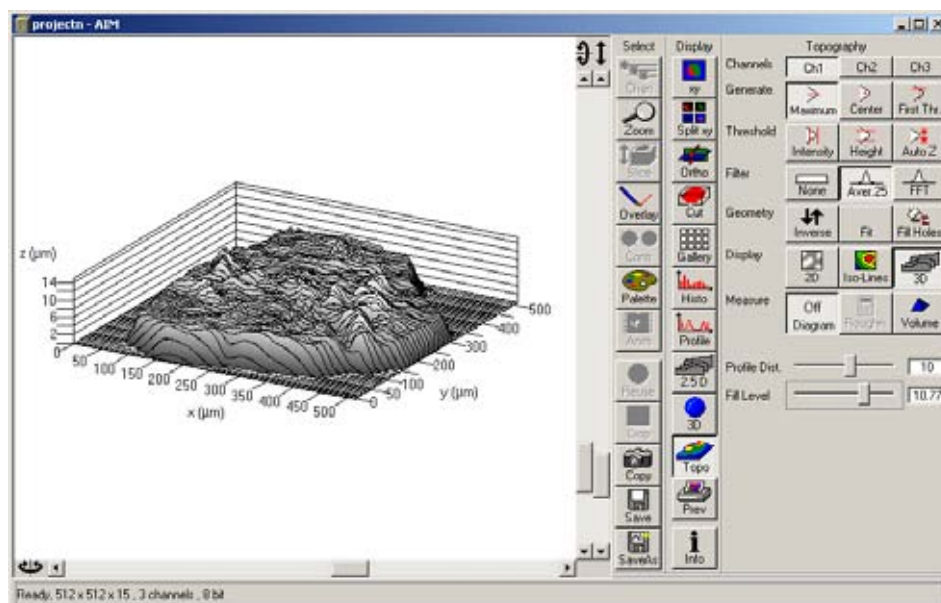


Fig. 4-280 Image Display window, Topography display

The **Topography** toolbar contains the following function elements:

<b>Channels</b> buttons	The selection of a channel to be used.
<b>Generate</b> buttons	The selection of the mode of calculation of the topography image (maximum, center of gravity, First /Last)
<b>Threshold</b> buttons	The selection of thresholds (intensity, height, Auto Z) to be used.
<b>Filter</b> buttons	The definition of filter procedures (geometrical, frequency cut-off filters) for smoothing, separation of roughness or waviness.
<b>Geometry</b> buttons	Inversion, various fit procedures, hole filling.
<b>Display</b> buttons	2D (Intensity, Height, Gradient); Iso-Lines (z Map, Intensity, Black) 3D (Profiles, Grid, Filled, Shaded).
<b>Measure</b> 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

- Definition of an intensity (I) threshold.
- Starting from top / bottom of a stack to find  $I = 400$ .
- Search of a local maximum one FWHM of actual Z PSF forwards / backwards.
- Search of the next local maximum one FWHM forwards / backwards from the last max until you have not found any new local maximum.
- 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.

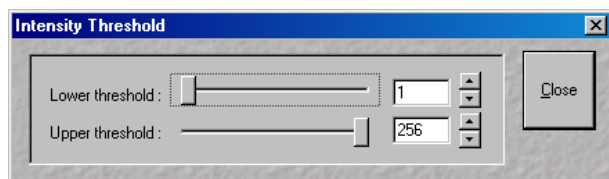


Fig. 4-281 Intensity Threshold window

- 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.

## 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.

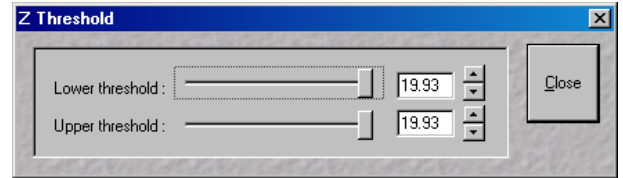


Fig. 4-282 Z Threshold window

- 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:

1<sup>st</sup> row: small smoothing via Median/Gauss filter (Median; 3 x 3; 5 x 5; 7 x 7)

2<sup>nd</sup> row: medium smoothing via Average (9 x 9; 11 x 11; 15 x 15)

3<sup>rd</sup> 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  $\mu\text{m}$ .
- Select a position of the **Degree** slider. The filter functions can be calculated from 1<sup>st</sup> order to 5<sup>th</sup> 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. 4-283) 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.

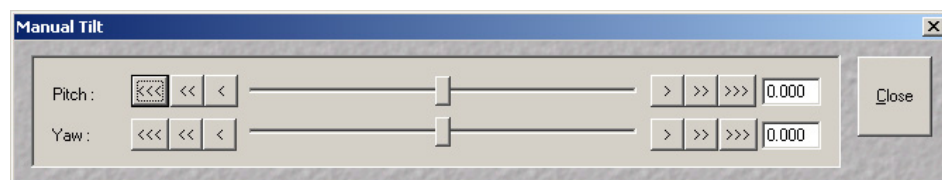


Fig. 4-283      Manual Tilt window

#### 4.13.24.5 Display Modes

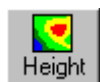
The three buttons in the **Display** button bar allow stacks to be displayed in the 2D, Iso-Lines or 3D display mode.

##### (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.

**Line Dist.** slider: Changes the distance of the iso-lines.

Line Offset

**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.

**Profile Dist.** slider: Setting of the distance of profiles and the mesh value of the grid.

Fill Level

**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 4-237).

#### 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.

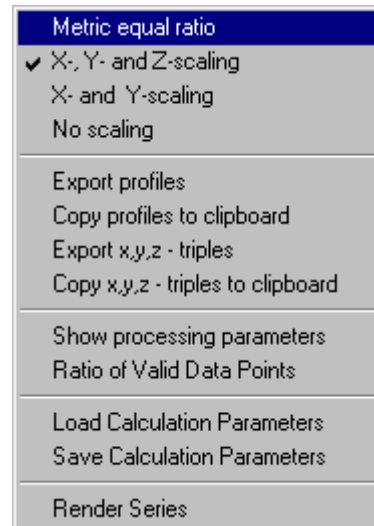


Fig. 4-284 Context menu of the 3D display mode (Profiles)

##### (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.

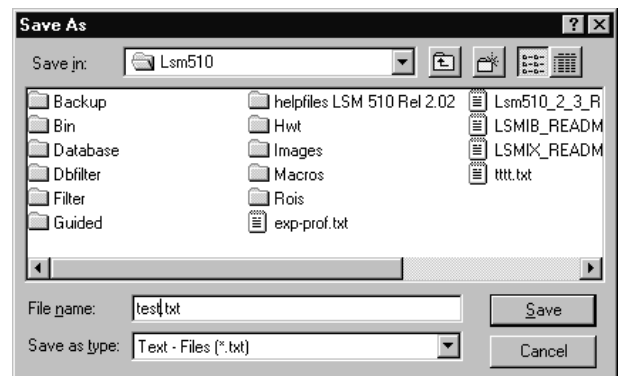


Fig. 4-285 Save As window

	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. 4-286 Topography matrix

##### (3) Copy profiles to clipboard item

All 3D parameters shown right from the main 3D topo display window are copied in the clipboard.

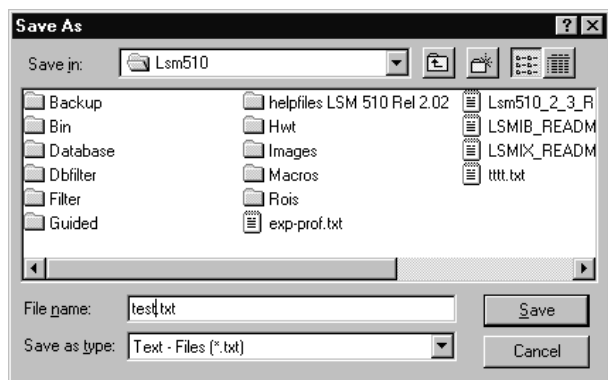


Fig. 4-287 Save As window

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. 4-288 Topography table

#### (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**.

A text file containing the topography in the form of an XYZ table is generated.

#### (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)

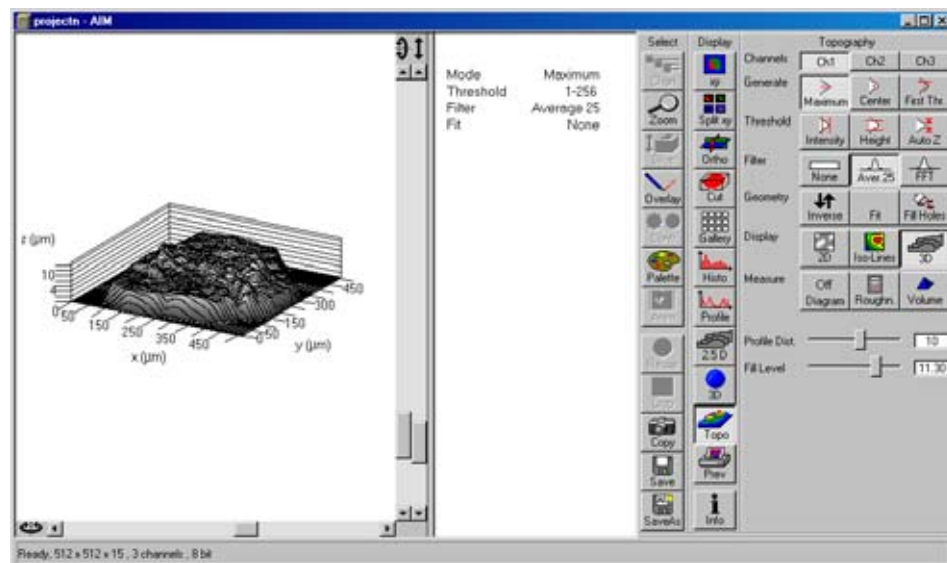


Fig. 4-289 Show processing parameters

## (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

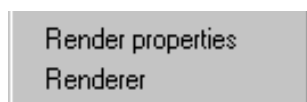


Fig. 4-290 Context menu of the 3D display mode (Shaded)

### (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.

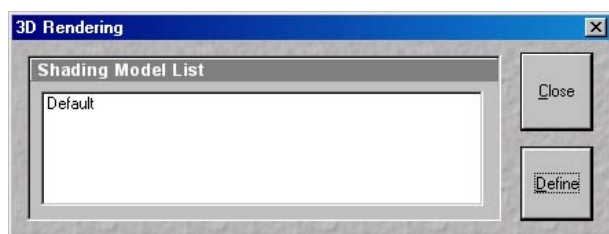


Fig. 4-291 3D Rendering window

### 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.

## 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 4-274.

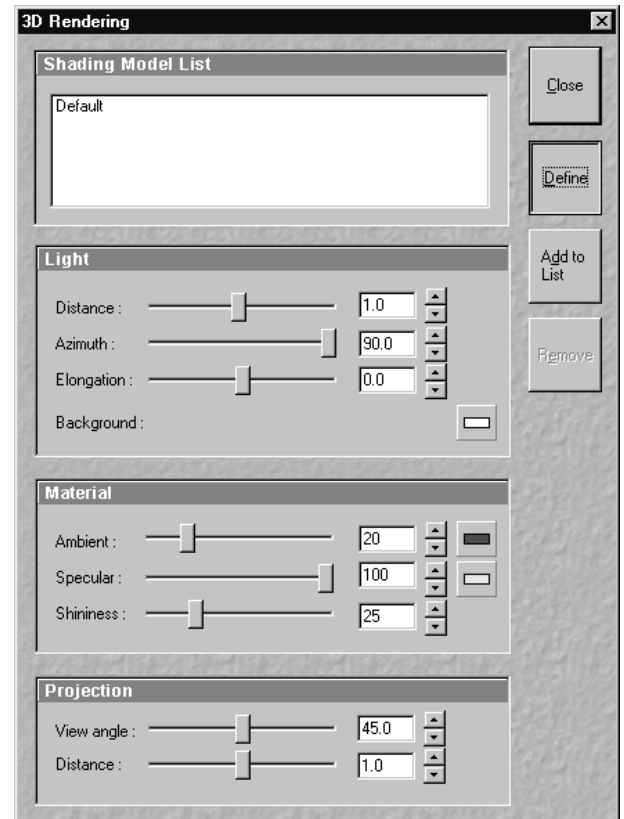


Fig. 4-292 3D Rendering window

## 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**

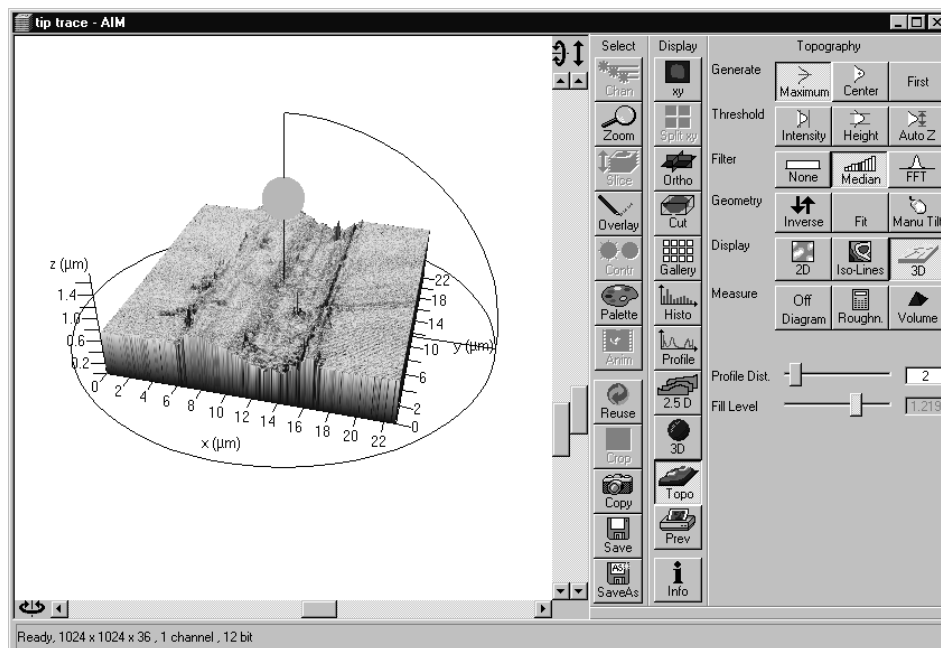
Determines the reflective properties of a sample.

**View angle**

Determines the perspective, 0.0 parallel projection, central projection

**Distance**

Zoom function, zoom in, zoom out



**Fig. 4-293** 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.

### (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).

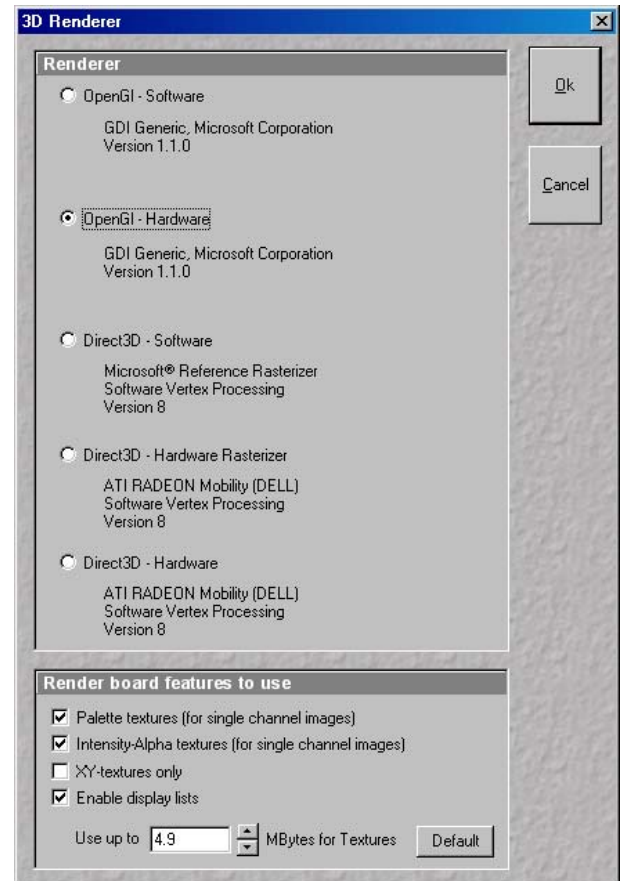


Fig. 4-294 3D Renderer window



#### 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:

<b>Topo Filters</b>	None, median, <9x9	FFT High	FFT Low	>9x9
Data formats	<b>P</b> Primary profile	<b>R</b> oughness	<b>W</b> 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. 4-295 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 4-294).



**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.

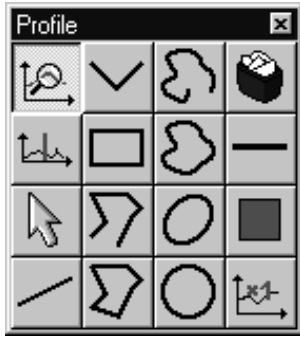



Fig. 4-296 Tools of the Profile measurement mode

### 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.

### 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, dx dy 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.

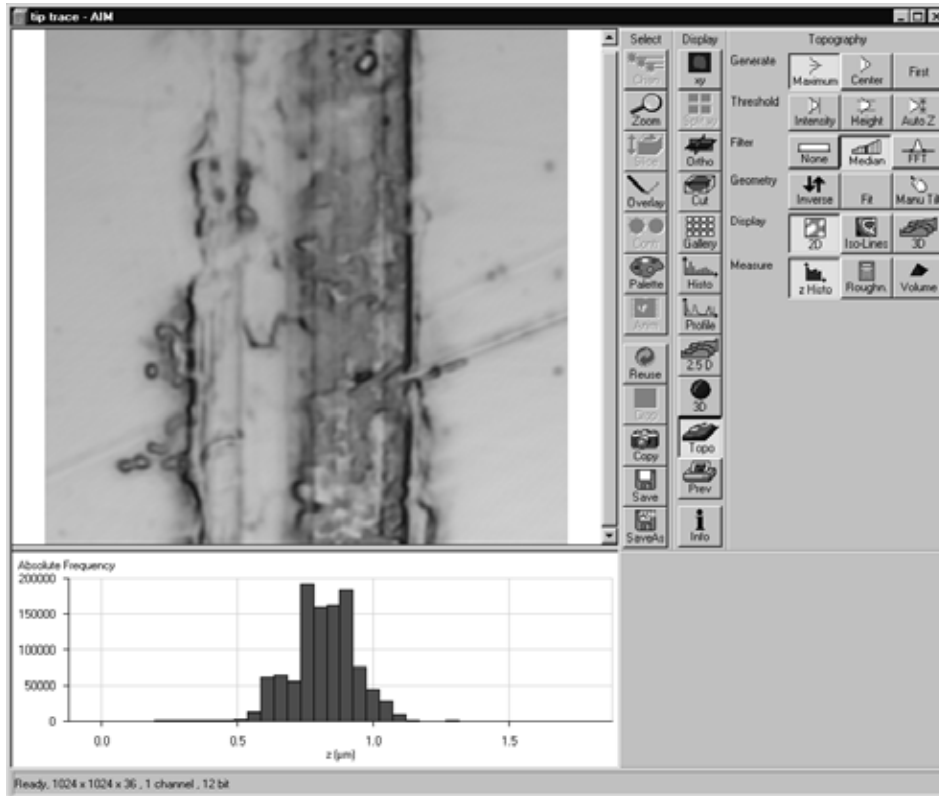


Fig. 4-297 Image Display window, Topography display: 2D - Histo

## (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 4-304).

#### (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:

$$R_{DQ} = \sqrt{\frac{1}{(N_x - 1) \cdot (N_y - 1)} \cdot \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} \left\{ \left[ \frac{z(x_i, y_j) - z(x_{i-1}, y_j)}{\Delta x} \right]^2 + \left[ \frac{z(x_i, y_j) - z(x_i, y_{j-1})}{\Delta y} \right]^2 \right\}}$$

#### (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	Ps	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  $R_c$

$$-R_c = \frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot z(x, y_j)$$

$N_x, N_y \dots$  number of pixels in X- or Y-direction

Arithmetic mean deviation of all profile height values  $R_a$

$$-R_a = \frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot [z(x, y_j) - R_c]$$

- Quadratic mean deviation of all profile height values  $R_q$

$$-R_q = \sqrt{\frac{1}{N_y} \cdot \sum_{j=1}^{N_y} \cdot [z(x, y_j) - R_c]^2}$$

- 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:  $\Sigma$  (surface area<sub>ij</sub>) /  $\Sigma$  (projected area<sub>ij</sub>)

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  $R_{\max}$ :

$$- R_{\max} = \text{Max} (z_{\max 1} - z_{\min 1}, z_{\max 2} - z_{\min 2}, z_{\max 3} - z_{\min 3}, z_{\max 4} - z_{\min 4}, z_{\max 5} - z_{\min 5})$$

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  $S_c$

$$-RS_c = \frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot z(x_i, y_j) \quad N_x, N_y \dots \text{number of pixels in X- or Y-direction}$$

- Arithmetic mean deviation of all surface height values  $RS_a$

$$-RS_a = \frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot [z(x_i, y_j) - RS_c]$$

- Quadratic mean deviation of all surface height values  $RS_q$

$$-RS_q = \sqrt{\frac{1}{N_x \cdot N_y} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot [z(x_i, y_j) - RS_c]^2}$$

- 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  $RS_{KU}$

$$RS_{KU} = \frac{1}{N_x \cdot N_y \cdot RS_q^4} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot z^4(x_i, y_j)$$

- 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  $S_z$ :

$$-RS_z = \frac{Z_{\max 1} - Z_{\min 1} + Z_{\max 2} - Z_{\min 2} + \dots + Z_{\max 25} - Z_{\min 25}}{25}$$

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  $RS_{\max}$ :

$$-RS_{\max} = \text{Max} (Z_{\max 1} - Z_{\min 1}, Z_{\max 2} - Z_{\min 2}, \dots, Z_{\max 25} - Z_{\min 25})$$


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.

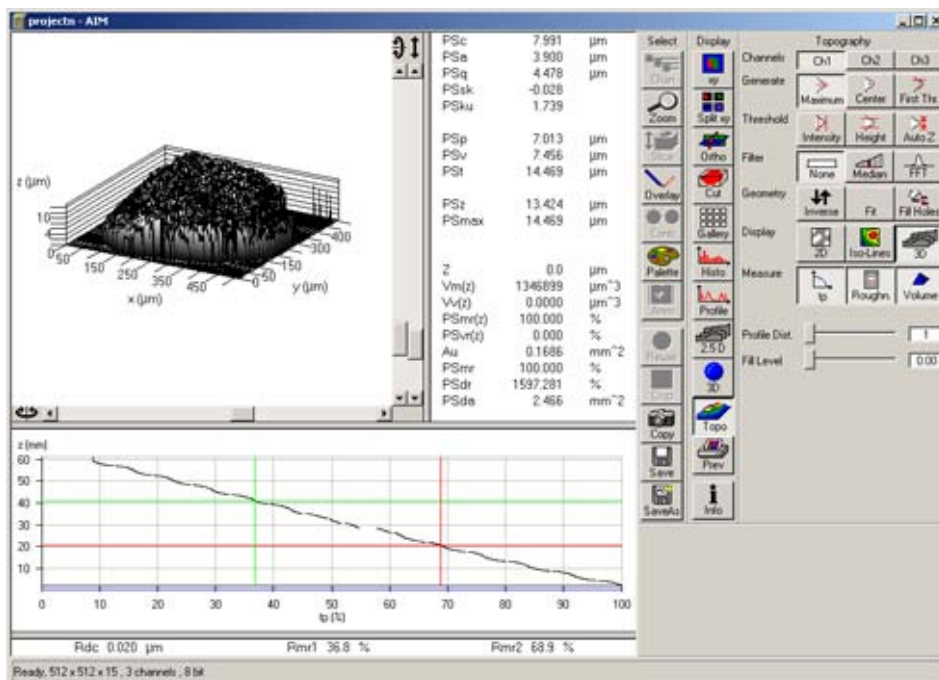


Fig. 4-298 Image Display window, Topography display: 3D - Volume

## 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 (=  $A_u / (x * y) * 100 \%$ )

**Sda:** true surface = sum of all triangles formed by adjacent data points of the surface reconstruction

**Sdr:** developed surface area ratio:

$$\Sigma (\text{surface area}_{ij}) - \Sigma (\text{projected area}_{ij}) / \Sigma (\text{projected area}_{ij}) * 100 \%$$

$$\text{projected area} = x * y$$




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  $\Rightarrow$  is equal to base plane ( $S_{dr} = 0 \%$ )

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.

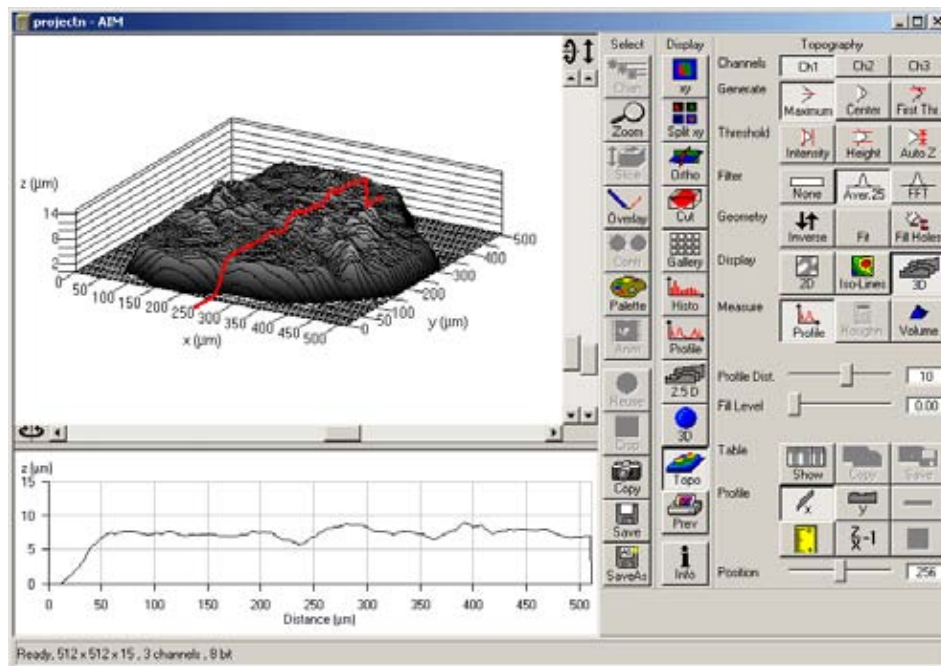


Fig. 4-299 Image Display window, Topography display: 3D - Profile

- 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.

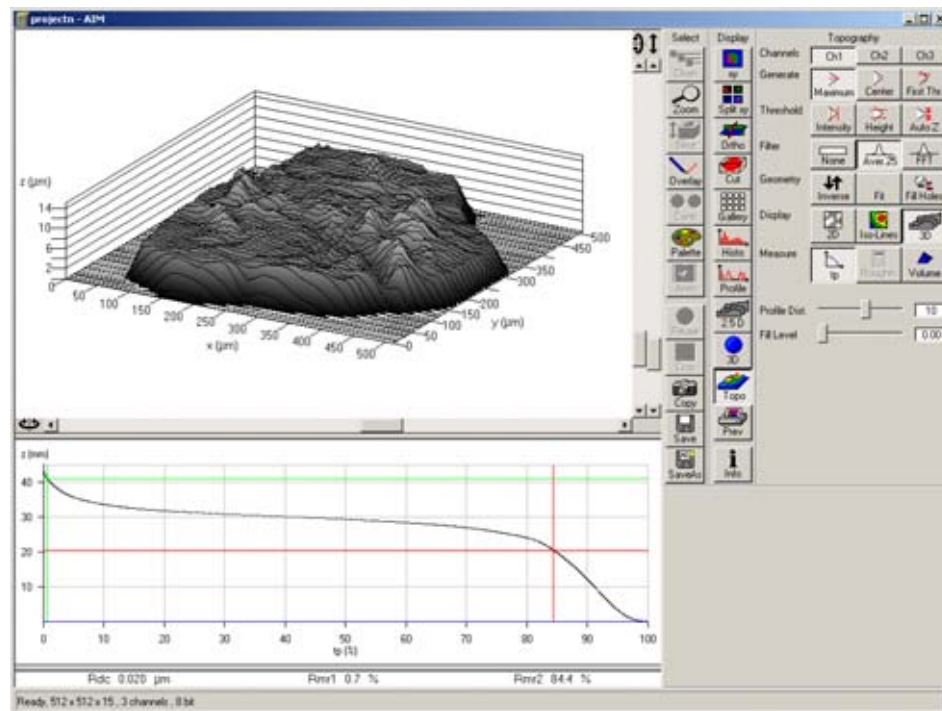


Fig. 4-300 Image Display window, Topography display: 3D – Curve of tp

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.

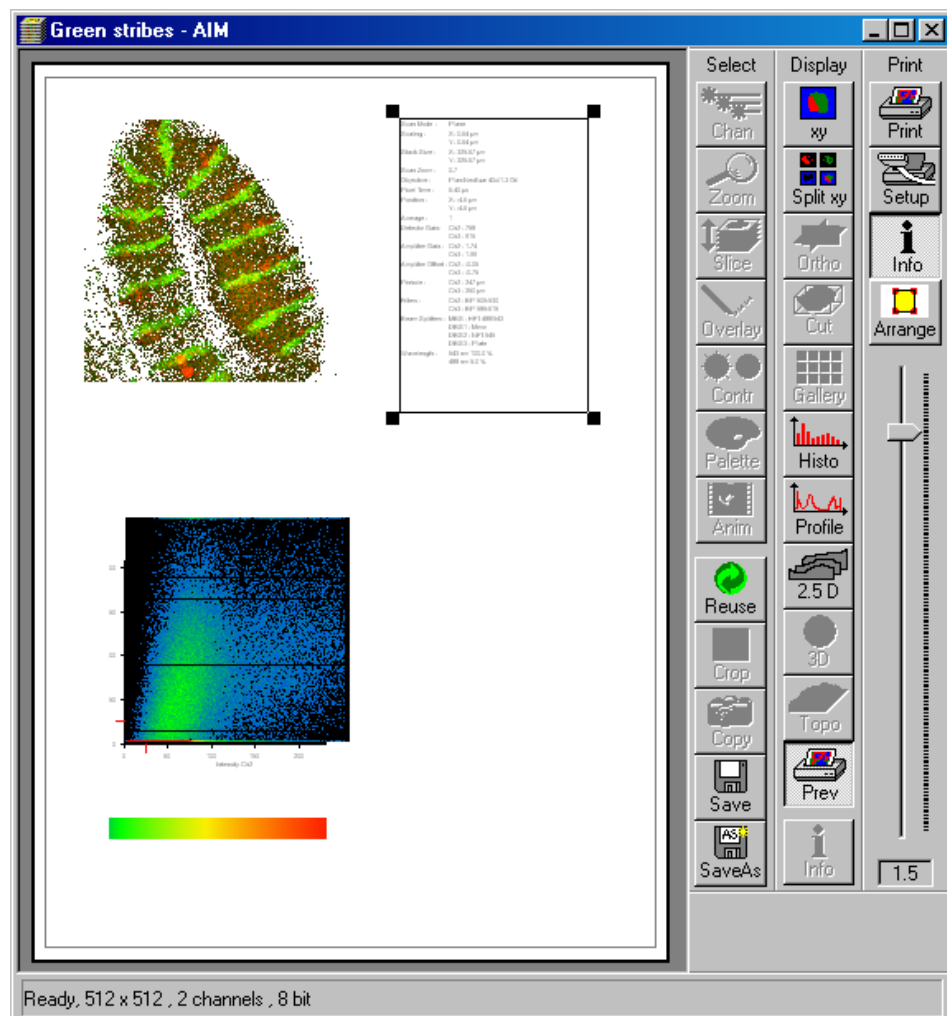
This function allows to

- 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. 4-301** 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**.

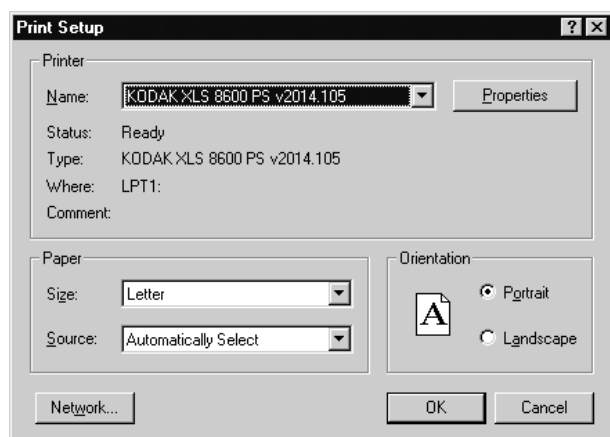


Fig. 4-302 Print Setup window

#### 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.

#### 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.

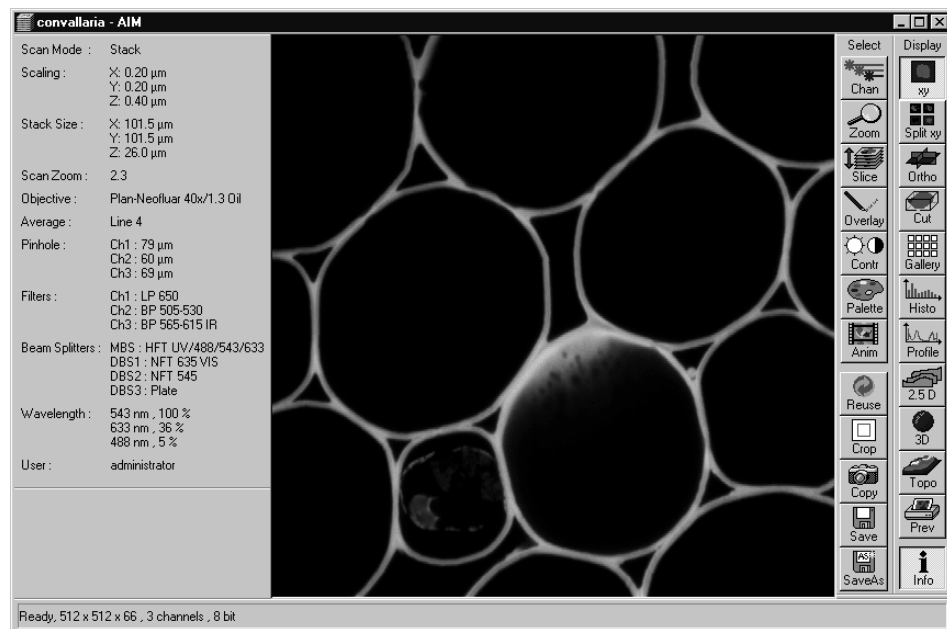


Fig. 4-303 Image Display window, Info display

## 4.13.27 Additional Display Mode in Time Series

### 4.13.27.1 Display - Mean

This function allows to

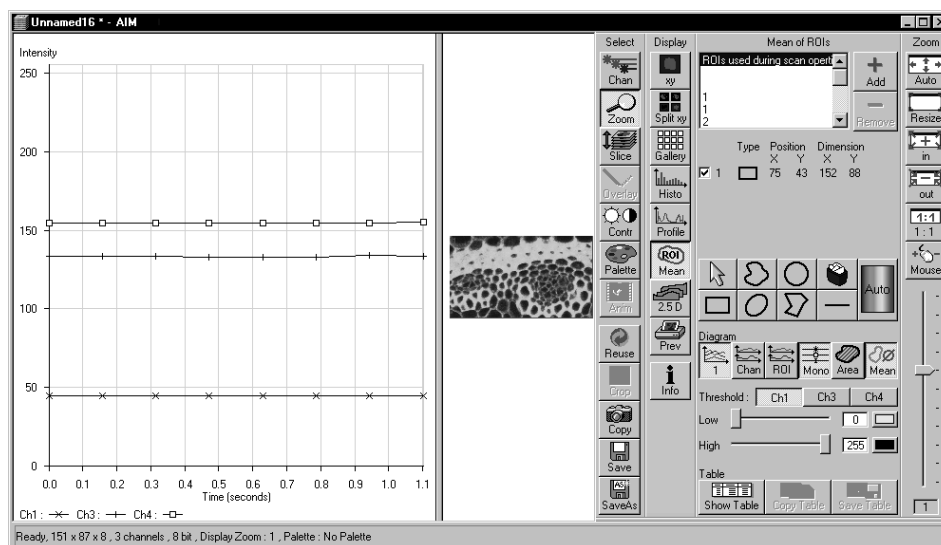
- display the intensity time diagram (mean intensity in user defined ROIs over time)
- display the intensity time diagram for volumes (3D ROIs) within a Z-Stack over time
- use frame time series and frame Z Stack time series as input
- show the intensity values in table form and copy table to clipboard 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.

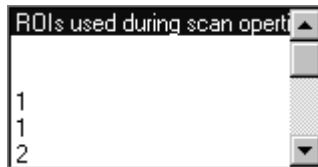
To use a similar functionality while scanning use the optional Mean of ROI function with the Time series control.

- 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. 4-304** Image Display window, Mean ROI display for time series in single plane

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.

	Type	Position		Dimension	
		X	Y	X	Y
<input checked="" type="checkbox"/> 1		75	43	152	88

**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.



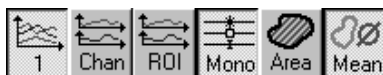
**Polyline** button: Activates polyline drawing mode. The first click sets the starting point, each additional click adds a further line, a double-click on the starting point closes the figure and ends the procedure.



**Line** button: This button allows you to determine the line thickness of the ROI outline.

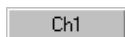


**Color / Auto** button: One color from the list of colors can be assigned to all ROIs. When **Auto** is pressed, the outlines of all ROIs are automatically colored differently.



#### 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 / Ch2 / 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.

## 4.14 Image Optimization

### 4.14.1 Single Channel



Described below is the example of the acquisition of an image, using an excitation wavelength of 532 nm and a fluorescence emission range above 550 nm.

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.

- The **LSM** button has to be activated either in the LSM software or on the LSM button on the LSM tube itself.
- 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  **ChL1** icon and assign a color to Channel 1 in the **Channel Color Selection** window. Activate channel 1 via check box.
- Click on the  icon of emission filter 1 (before ChL1) and select the **LP 550** filter.
- If required, deactivate all the other channels (ChL2) via check box.

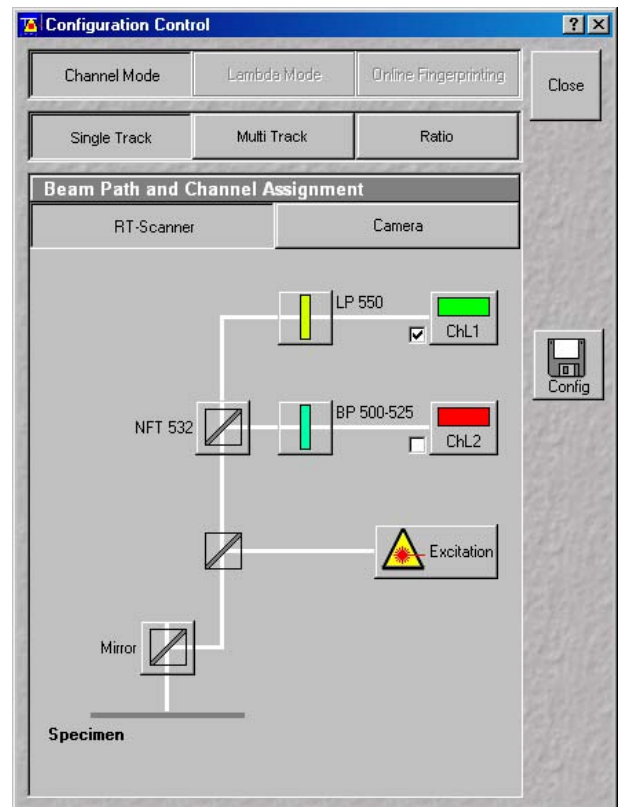


Fig. 4-305 Configuration Control window

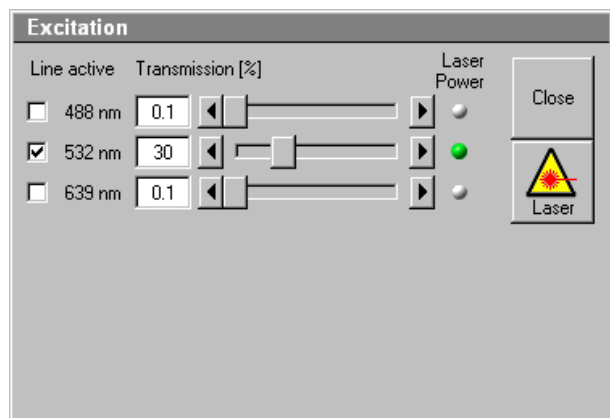



Fig. 4-306 Excitation panel

- Click on the  icon, activate the **532 nm** laser line by clicking 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.

For overlaying fluorescence and transmitted-light images, activate a second channel without a laser line but the Halogen illumination of the light microscope as the transmitted image light source.



Choose the BP 675-725 in the emission filter menu and swing in the according red filter at the condensor


Of course, all other transmitted light applications like

- phase contrast
- differential interference contrast (DIC)
- polarization contrast (Pol)
- darkfield

can also be performed.

- 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 & Line Step Factor** panel, select Objective and Frame size for the scan (e.g. X 512 / Y 512 scan).
- Enter a **Exposure Time** of 5 to 10 frames per second, 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.

 Using the **Fast XY** button is a convenient way of creating an overview scan.

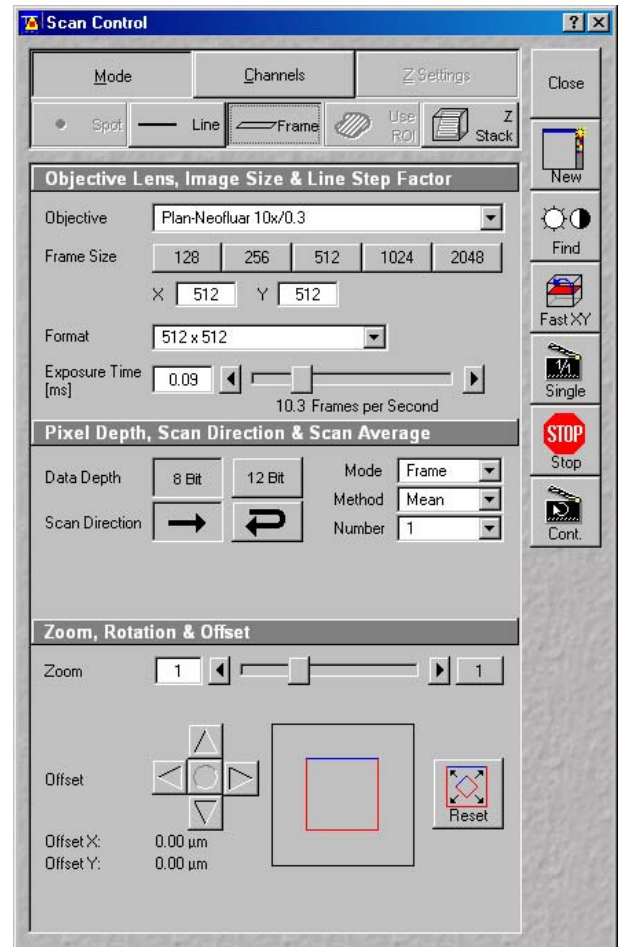


Fig. 4-307 Scan Control window (Mode)



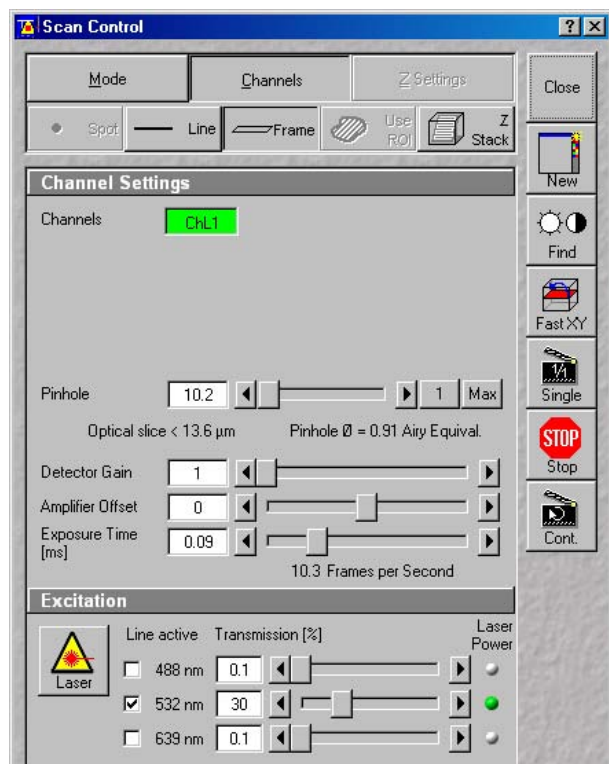


Fig. 4-308 Scan Control window (Channels)

- 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.

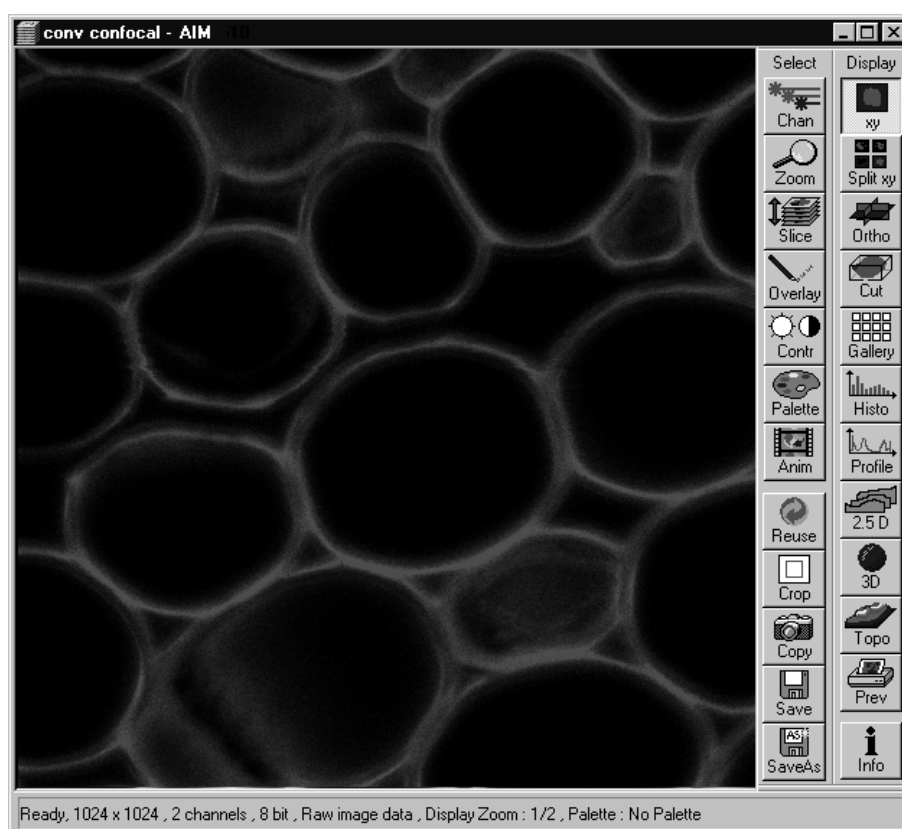


Fig. 4-309 Image Display window

#### 4.14.1.2 Confocal Aperture / Detector Gain / Ampl. Offset

- In the **Scan Control** window, click on the **Cont.** button (see Fig. 4-308).
  - This starts a continuous scan.
- Use the **Pinhole** slider (confocal aperture) to set the aperture size in the **Scan Control** window under **Channels**.
  - The aperture size 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 aperture size will increase the depth of focus, but reduce the light intensity received by the CCD detector.
  - The influence of the aperture size on image creation is shown by the example in Fig. 4-310. The entire image was first scanned with too large a aperture size. The aperture size was then optimized for a defined ROI. This considerably improved the display of the specimen structures.
- 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.

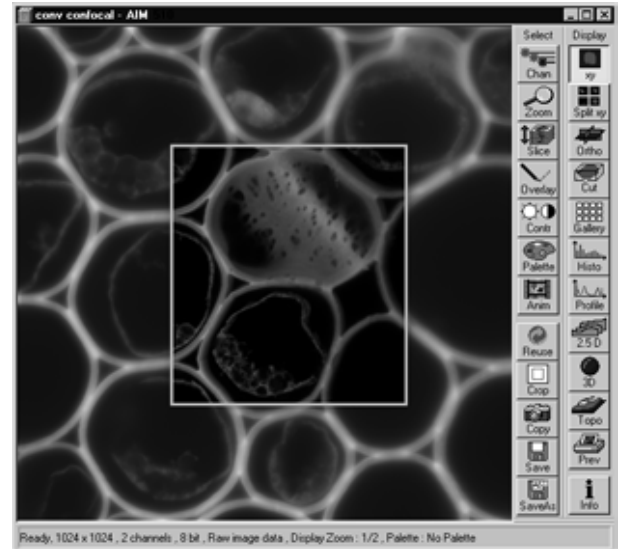


Fig. 4-310 Image Display window with confocal ROI

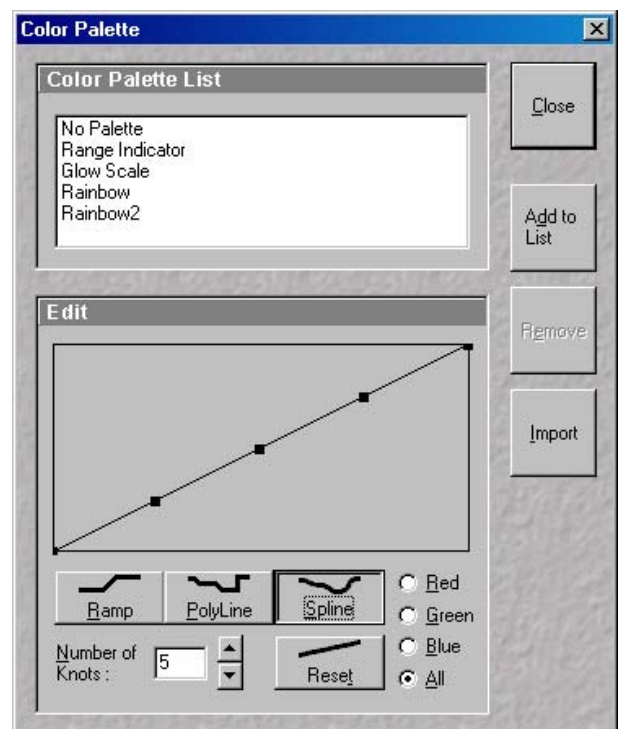


Fig. 4-311 Color Palette window

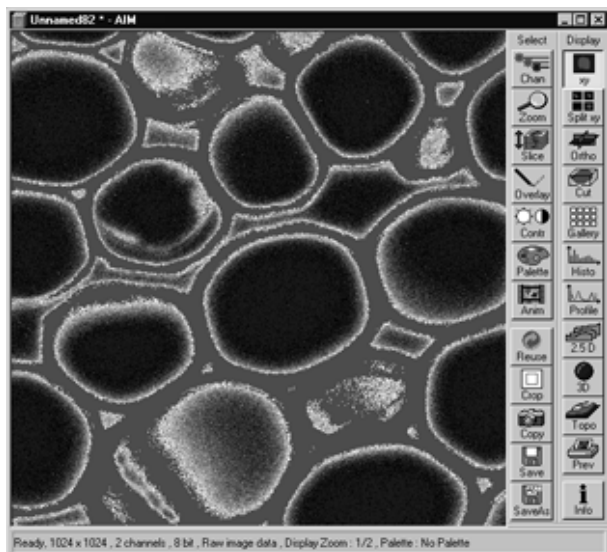


Fig. 4-312 Image Display window

If the image is too bright, it appears red on the screen.

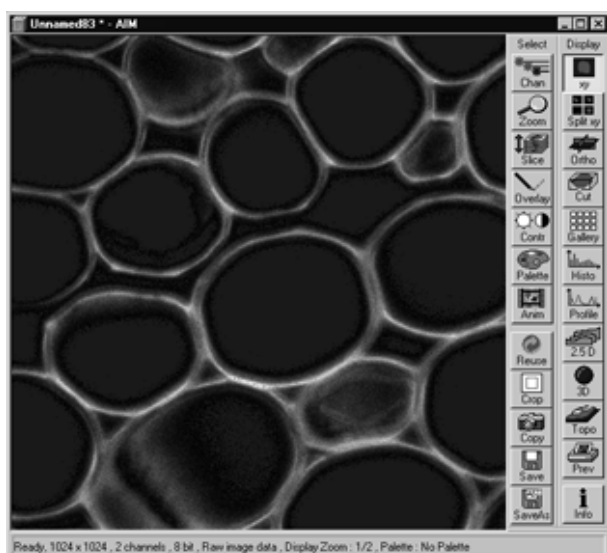



Fig. 4-313 Image Display window

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 CCD detector 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.

- To adjust the black level (background), use the **Ampl. Offset** slider so that areas without picture content just show a trace of blue.
- 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.

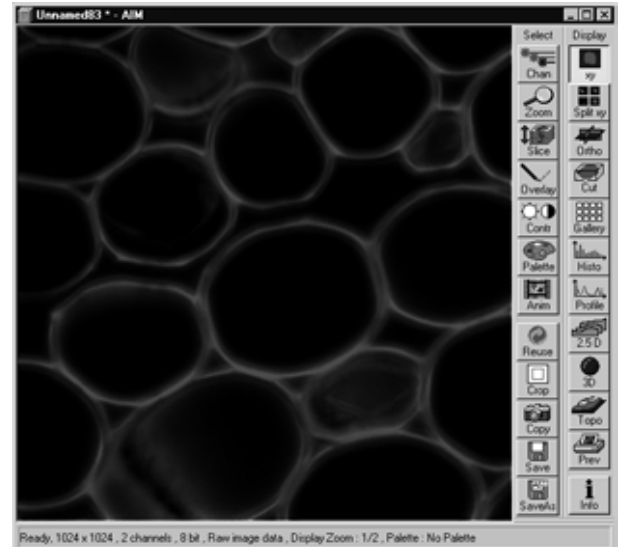



Fig. 4-314 Image Display window

#### 4.14.1.3 Exposure Time, 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 **Exposure Time** slider in the **Objective Lens, Image Size and Line & Step Factor** panel to set the slowest acceptable exposure time speed.
  - The corresponding value for **Frames per Second** is shown below the slider.
- 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.

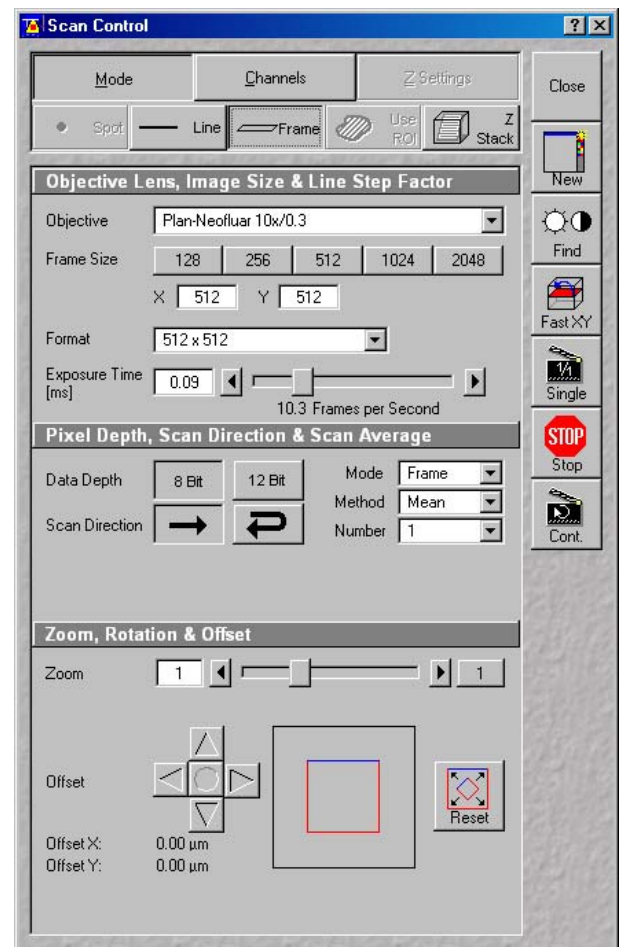


Fig. 4-315 Scan Control window

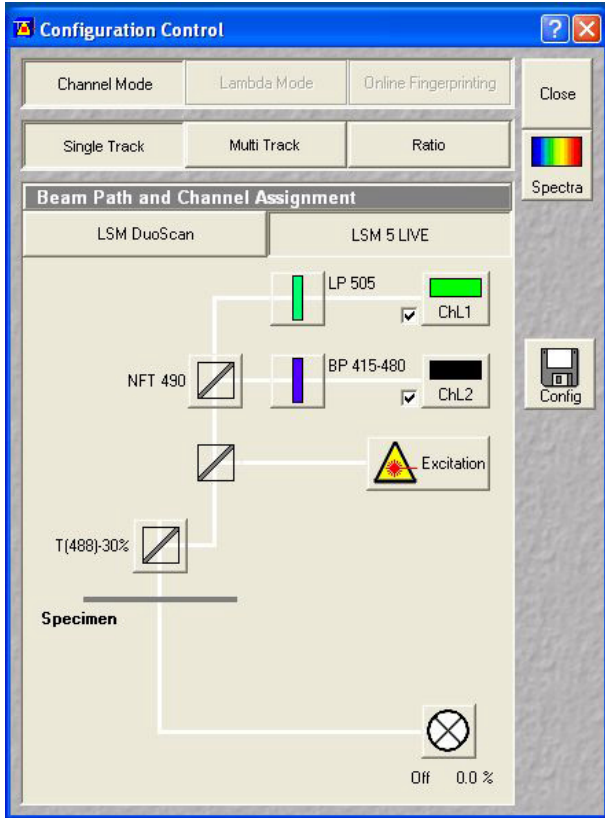


Fig. 4-316 Configuration Control window

#### 4.14.2 Multiple-channel

##### 4.14.2.1 Requirements

- The suitable lasers are on.
- The specimen has been positioned and focused for scanning.
- The **LSM** button has to be activated either in the LSM software or on the LSM button on the LSM tube itself.

In the following example, 2 Channels shall be activated for the scanning procedure: one for 488 nm using emission filter BP 500-525 and one for 532 nm with LP 550. NFT 532 is used as the secondary dichroic beam splitter.

- In the **Acquire** subordinate toolbar, click on the **Config** button.
  - This opens the **Configuration Control** window.

- Click on the **Single Track** button.
- Activate (in the same way as for the single channel, see page 4-315) channel 2 (ChL2), 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.

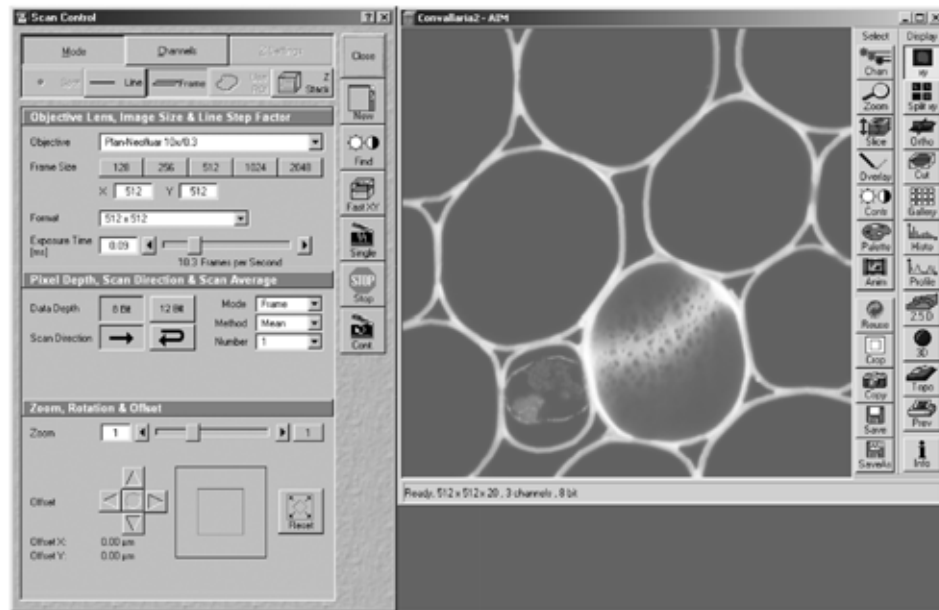


Fig. 4-317 Scan control and Image Display windows

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 aperture size
- Detector Gain / Ampl. Offset
- Scanning speed and Average

must be carried out separately for each channel used (see **Single Channel**, page 4-315).

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 **ChL1** 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 (**ChL2**) in the **Channel Settings** panel of the **Scan Control** window.

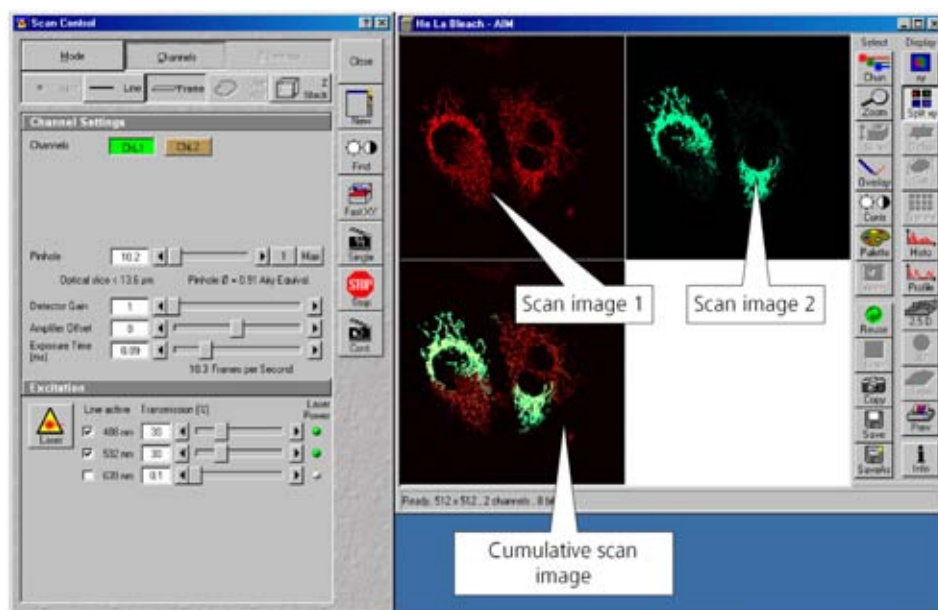



Fig. 4-318 Scan Control and Image Display windows

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 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.

### 4.15.1 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 5 LIVE Switchboard** window appears.



Fig. 4-319 LSM 5 LIVE Switchboard menu

- Click on the **Exit** button.
  - This terminates the LSM program.
  - The monitor screen shows the desktop of the WINDOWS XP operating system.



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#### 4.15.2 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.

#### 4.15.3 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.

#### 4.15.4 Turning off the HBO 100

- Switch off the HBO 103 via the toggle switch of the HBO 100 power supply.

## 4.16 Software and Hardware Options

This section describes optional software and hardware configurations. Depending on your configuration, the content of dialogue and function may differ.

### 4.16.1 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 plus"
- Software "Visual Macro Editor"
- Software "FRAP"

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 "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**"

#### 4.16.2 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, 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

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 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 an AxioCam, the following functions are not available:

- **Camera** in the **Config Control** window, **Scan Control** window

#### **4.17 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](http://www.zeiss.de/lsm)**

for the latest dates and ask your Zeiss representative for a quotation on courses.

# CHAPTER 5 TOOLS

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## 5 TOOLS

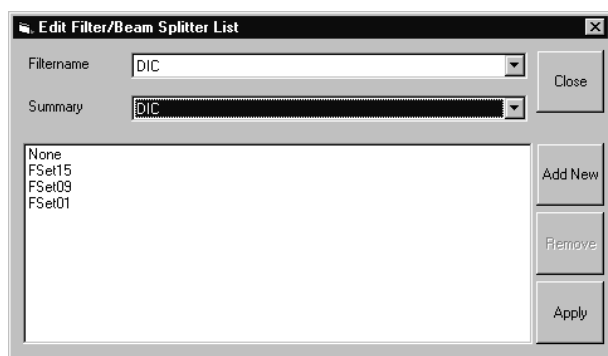
### 5.1 Change Filters

The **Change Filters** tool is used to update the filter data in the software after a change of filters in the reflector turret of the microscope, the emission filters and the beam splitters of the LSM 5 LIVE.

All filter data are updated in the **Emission Filters & Beam Splitter Control** window. After activation of the appropriate button (**Emission Filters**, **Filter Cubes Stand** or **Beam Splitters LSM**), the special input mask for the selected filter type is displayed. Since the procedures of updating or entering a new filter type are identical for all types, only the updating of filters in the reflector turret (Filter Cubes Stand) will be described in the following.

- Close the LSM 5 LIVE 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.
- Click on the **Filter Cubes Stand** button. The **Filter Cubes Stand** panel is displayed.
- 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 be registered in the filter list (see **Edit Filter List** function, next page).



**Fig. 5-1** 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 5 LIVE 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 **Summary** 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

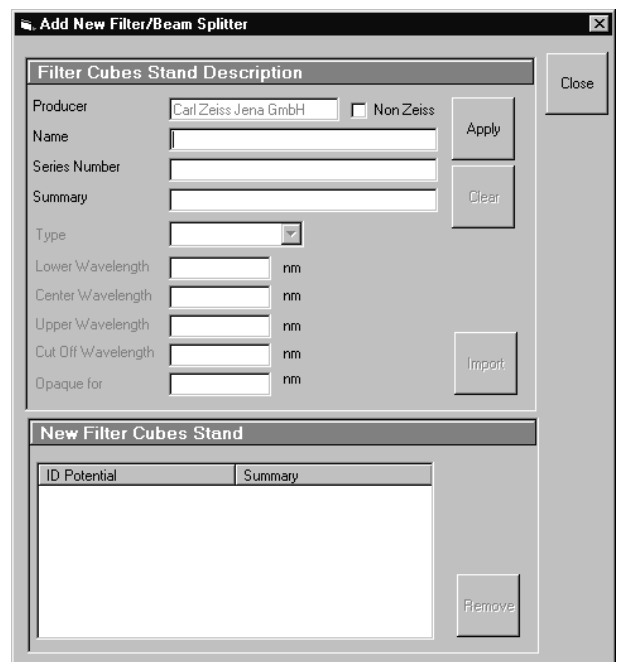
This function permits new filter sets to be added to the database.

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.

 If you have activated the **Non Zeiss** check box, filter sets from other manufacturers can also be included in the database.



**Fig. 5-2** **Edit Filter/Beam Splitter List window**

- 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 5 LIVE software, the filter data are updated.



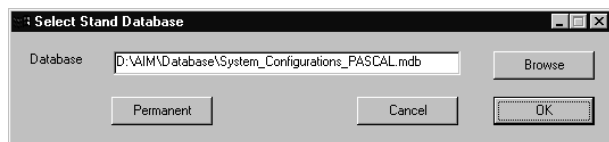


Fig. 5-3 Select Stand Database ... window

## 5.2 Stand Select

The **Stand Select** tool permits a new or updated database to be assigned to the LSM 5 *LIVE* software program. This function should preferably be performed by authorized service personnel.

If this is not possible, proceed as follows:

- Close the LSM 5 *LIVE* 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.

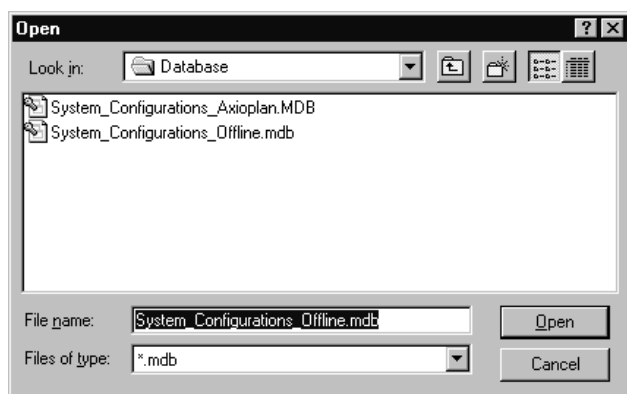


Fig. 5-4 Open window

- 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 5 *LIVE* software program, the new database will be automatically read in.

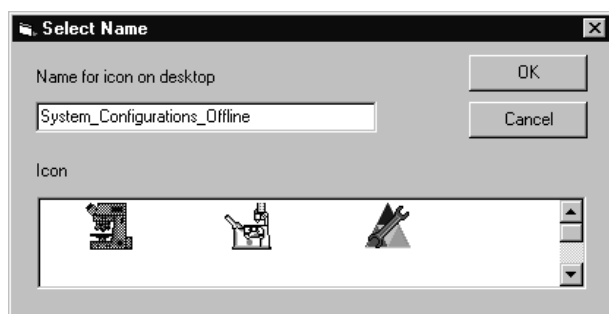


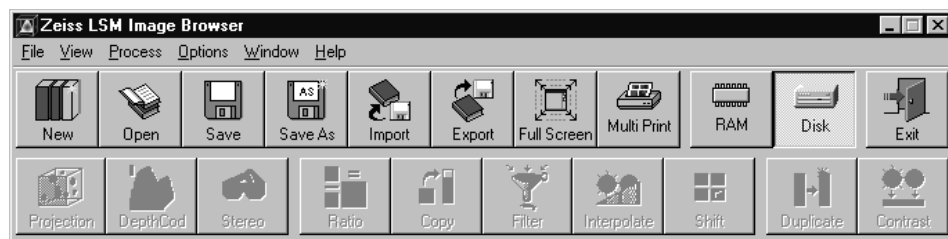
Fig. 5-5 Select Name window

### 5.3 LSM Image Browser

The **LSM Image Browser** permits images to be loaded, imported, exported and printed quickly without having to open the LSM 5 LIVE software. The **LSM Image Browser** can be used without dongle.

When images are opened, image processing functions of the LSM 5 LIVE 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. 5-6 Zeiss LSM Image Browser main menu**

The following function buttons are available:

<b>New</b> button	Opens a new database.
<b>Open</b> button	Opens an existing database.
<b>Save</b> button	Saves the current image.
<b>Save As</b> button	Saves the current image under a new name.
<b>Import</b> button	Imports images.
<b>Export</b> button	Exports images.
<b>Full Screen</b> button	The current image is displayed on the full screen. Deactivation of the function with a click of the mouse.
<b>Multi Print</b> button	Several images are printed on one page.
<b>RAM</b> button	Use of the RAM memory for image display.
<b>DISK</b> button	Use of the hard disk as storage medium for image display.
<b>Exit</b> button	The <b>Zeiss LSM Image Browser</b> 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 5 LIVE software and have already been described in chapter 4.

## 5.4 LSM Image Examiner

The **LSM Image Examiner** can be used without having to open the LSM 5 LIVE 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 5 LIVE.

When images are opened, a large scope of the image processing functions of the LSM 5 LIVE software is available (for further details see chapter 4).

- Click on the **LSM Image Examiner** icon on the desktop of the PC. The **Zeiss LSM Image Examiner** main menu is opened.

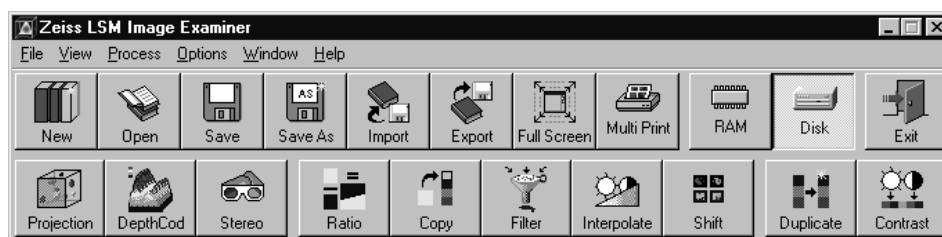


Fig. 5-7 Zeiss LSM Image Examiner main menu

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:

<b>Projection</b> 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.
<b>DepthCod</b> button	The depth information contained in a sequence can be colored with the colors of the rainbow.
<b>Stereo</b> button	Stereoscopic images can be generated.
<b>Ratio</b> button	Permits two channels to be linked into a new channel by the creation of a ratio.
<b>Copy</b> button	Permits one channel each of an existing image to be copied and stored as a new image.
<b>Filter</b> button	Permits the subsequent processing of scanned images via the integrated filters.
<b>Interpolate</b> button	Permits the continuous contrast and brightness change in a stack or time series through interpolation between the starting and end values.
<b>Shift</b> button	Produces a congruent image with relation to the pixels of the various channels.
<b>Duplicate</b> button	Permits images (including Z Stacks and Time Series) to be duplicated completely.
<b>Contrast</b> 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 5 LIVE software and have already been described in chapter 4.

## CHAPTER 6                      3D FOR LSM

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## 6 3D FOR LSM

### 6.1 Overview and Explanations

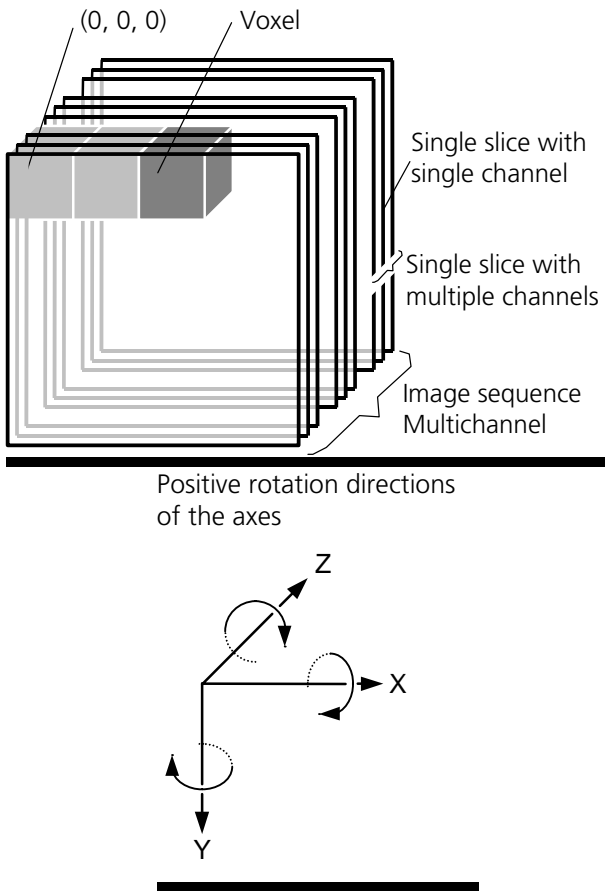


Fig. 6-1

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.

#### 6.1.1 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

## 6.1.2 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.

## 6.1.3 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.

## 6.2 User Interface

### 6.2.1 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**.

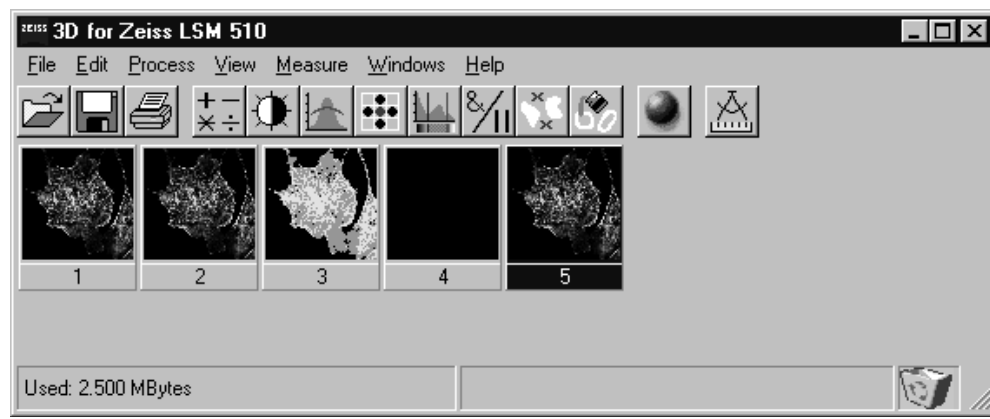
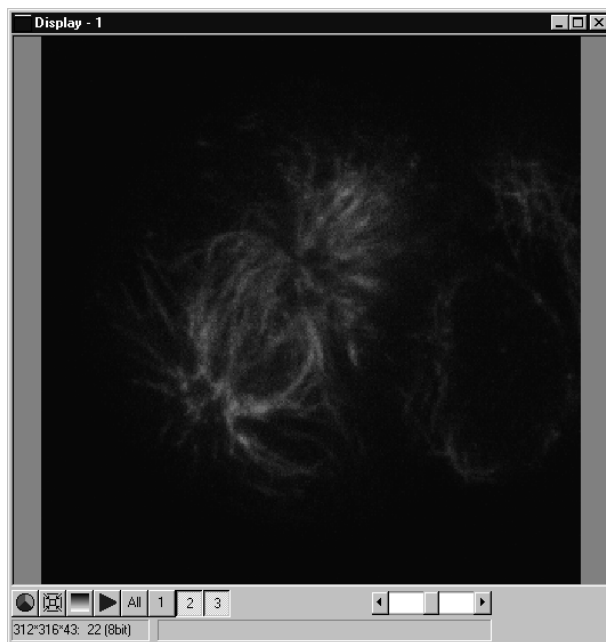


Fig. 6-2

**Tool bar**      This menu shows all image processing functions.

**Display window** This window is used to display image sequences.



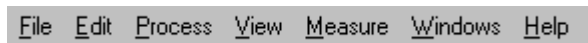
**Fig. 6-3 Display window**

**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.

## 6.2.2 Main Window

The **Main window** includes:

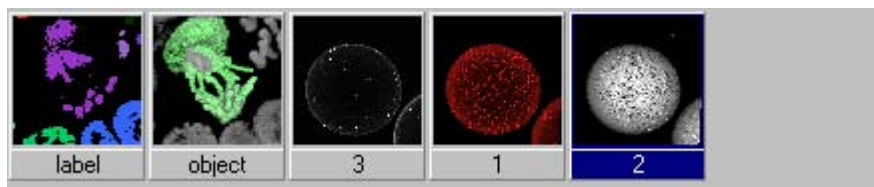
the **Menu**



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

Smooths 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.

### 6.2.3 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.

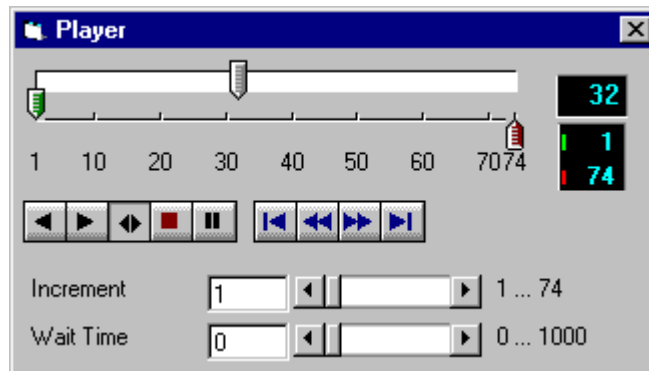


Fig. 6-4

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.

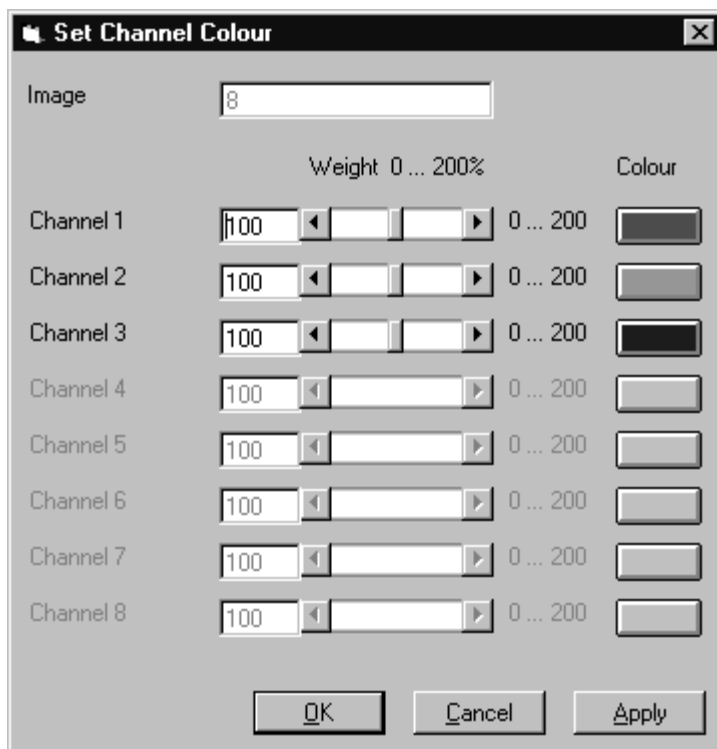


Fig. 6-5

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:

<b>Image</b>	Image sequence to edit
<b>Weight</b>	Colour weighting for each channel
<b>Colour</b>	Base colour for each channel

## 6.3 Functions

### 6.3.1 Functions in the File Menu

#### Open Image

This function reads a Zeiss LSM (\*.lsm), Zeiss LSM TIFF (\*.000.tif) or Carl Zeiss Vision (\*.img) image sequence from a disk or network drive.

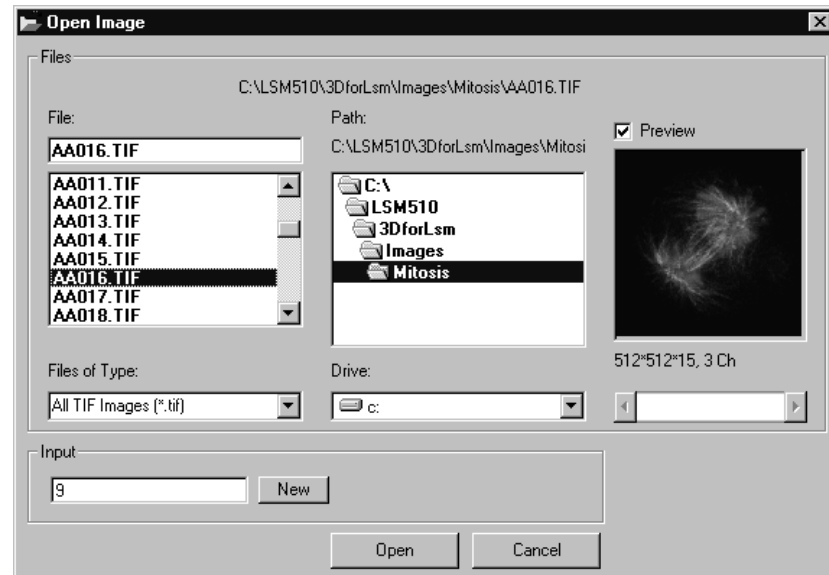


Fig. 6-6

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 (\*.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:

<b>BaseName</b>	Base name of the TIFF files (image sequence) to be loaded. Only the letters before the first number are stated.
<b>Input</b>	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.

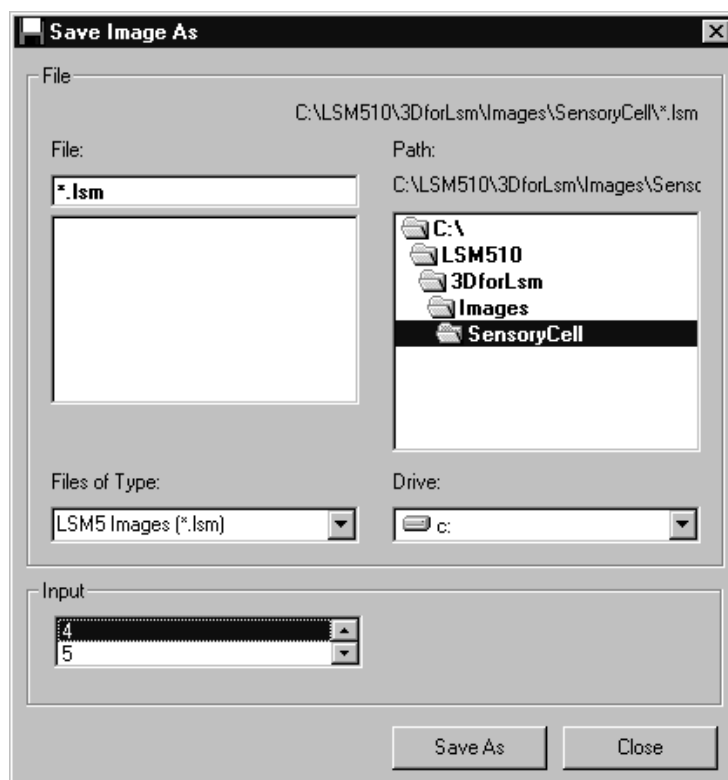


Fig. 6-7

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 (\*.ism) and the Carl Zeiss Vision file format "KE Images (\*0.img)" is supported.

By choosing the Carl Zeiss Vision file format "KE Images (\*.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. 6-8**

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**

### 6.3.2 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.

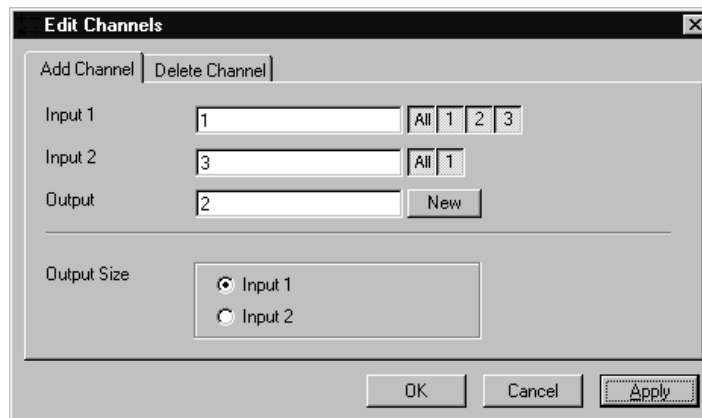


Fig. 6-9

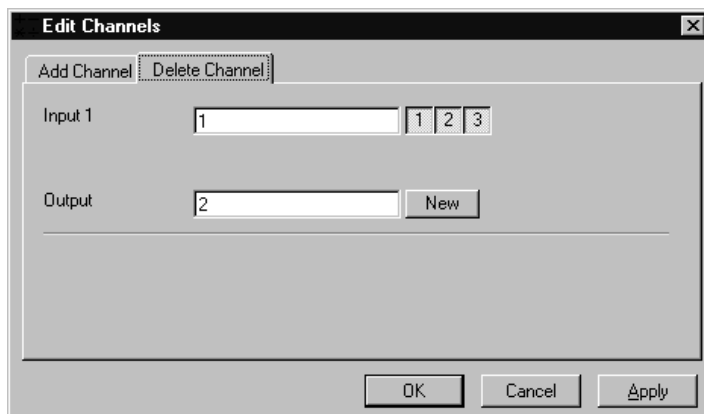
This operation is useful to add a segmented channel (or any other result of a function) to the original image sequence. The selected channels of **Input 1** and **Input 2** are copied to **Output**. The maximum number of channels in an image sequence is eight.

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:

<b>Input 1</b>	First input image sequence
<b>Input 2</b>	Second input image sequence
<b>Output</b>	Output image sequence
<b>Output size</b>	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. 6-10**

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:*

<b>Input 1</b>	Input image sequence
<b>Output</b>	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**

### 6.3.3 Functions in the Process Menu

#### Arithmetics - Add

This function adds two image sequences.

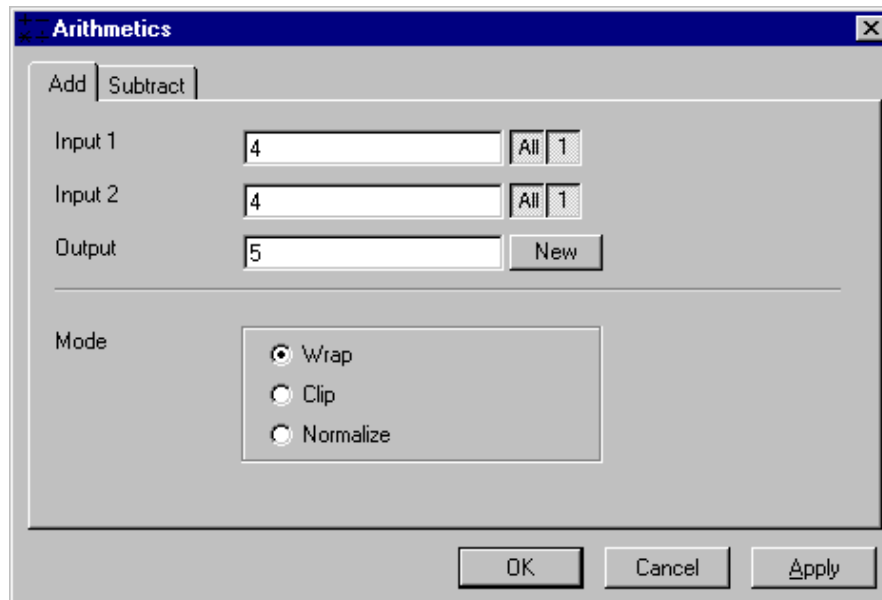


Fig. 6-11

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:

- |                |                                       |
|----------------|---------------------------------------|
| <b>Input 1</b> | First input image sequence            |
| <b>Input 2</b> | Second input image sequence           |
| <b>Output</b>  | Output image sequence                 |
| <b>Mode</b>    | 1 - Wrap<br>2 - Clip<br>3 - Normalize |

## Arithmetics - Subtract

This function subtracts two image sequences.

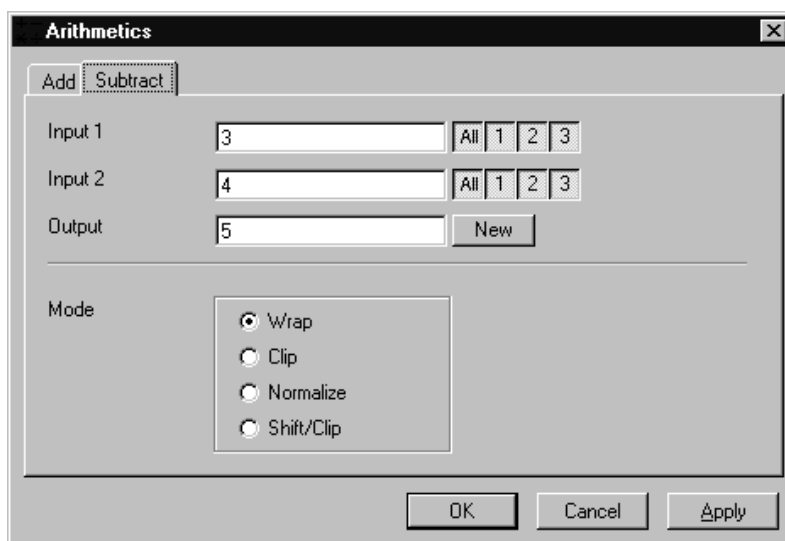


Fig. 6-12

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:

- |                |   |
|----------------|---|
| <b>Input 1</b> | First input image sequence                              |
| <b>Input 2</b> | Second input image sequence                             |
| <b>Output</b>  | Output image sequence                                   |
| <b>Mode</b>    | 1 - Wrap<br>2 - Clip<br>3 - Normalize<br>4 - Shift/Clip |

## Contrast - Interactive

This function allows interactive changes of the contrast of an image sequence.

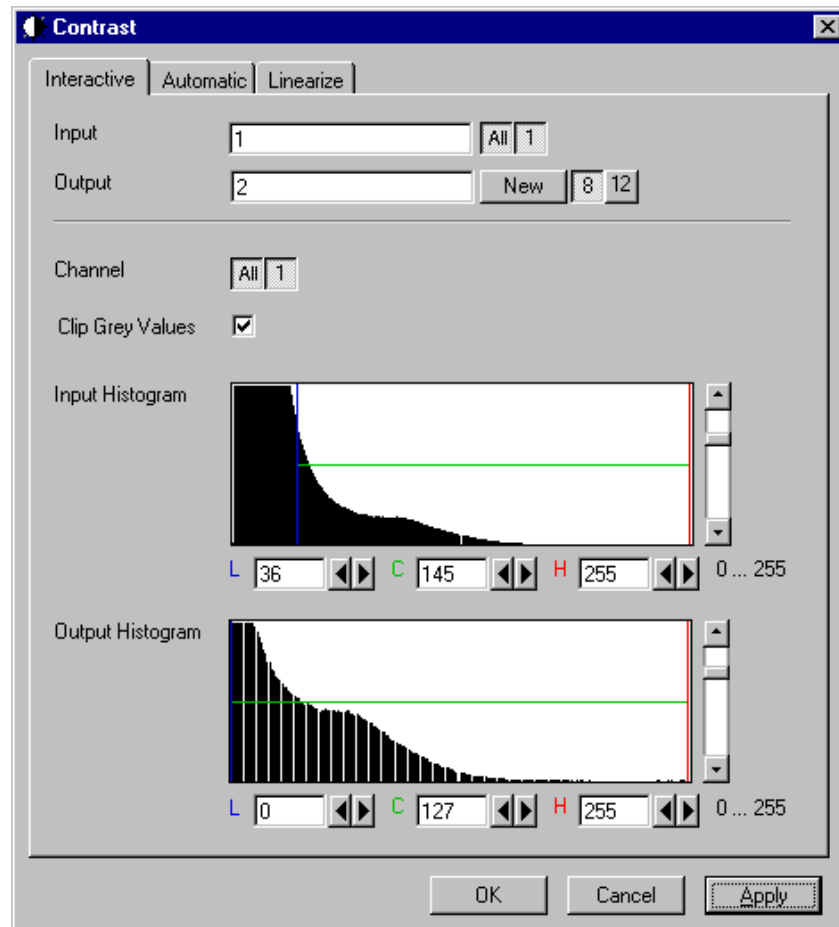


Fig. 6-13

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. Raising 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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Output image sequence
<b>Channel</b>	Selection of the channel numbers for the <b>Output</b> image after contrast enhancement
<b>Clip Grey Values</b>	Clipping of grey values to the <b>Low (L)</b> and <b>High (H)</b> output grey values boundaries
<b>Input L</b>	Lower boundary of grey value range <b>Input</b>
<b>Input C</b>	Center of grey value range <b>Input</b>
<b>Input H</b>	Upper boundary of grey value range <b>Input</b>
<b>Output L</b>	Lower boundary of grey value range <b>Output</b>
<b>Output C</b>	Center of grey value range <b>Output</b>
<b>Output H</b>	Upper boundary of grey value range <b>Output</b>

## Contrast - Automatic

This function scales the grey values of an image sequence to the maximum possible range.

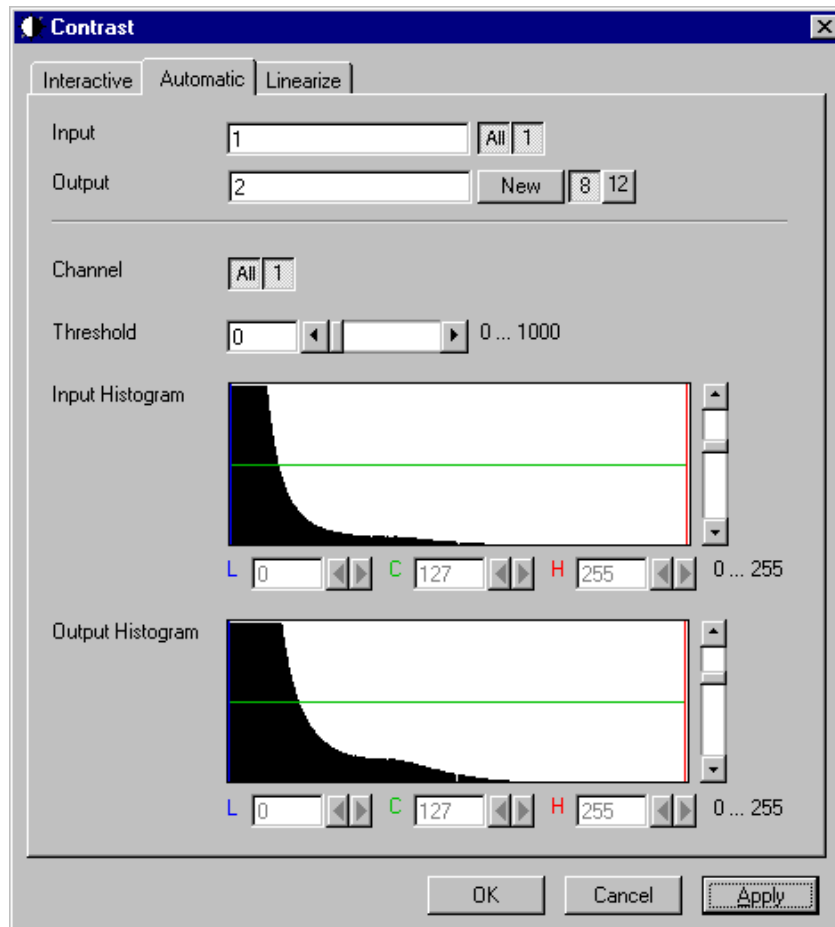


Fig. 6-14

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. Raising 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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Output image sequence
<b>Threshold</b>	Exclusion value - 0...1000
<b>Input L</b>	Lower boundary of grey value range <b>Input</b>
<b>Input C</b>	Center of grey value range <b>Input</b>
<b>Input H</b>	Upper boundary of grey value range <b>Input</b>
<b>Output L</b>	Lower boundary of grey value range <b>Output</b>
<b>Output C</b>	Center of grey value range <b>Output</b>
<b>Output H</b>	Upper boundary of grey value range <b>Output</b>

## Contrast – Linearize

This function scales a range of grey values of an image sequence to equal area fractions in the histogram.

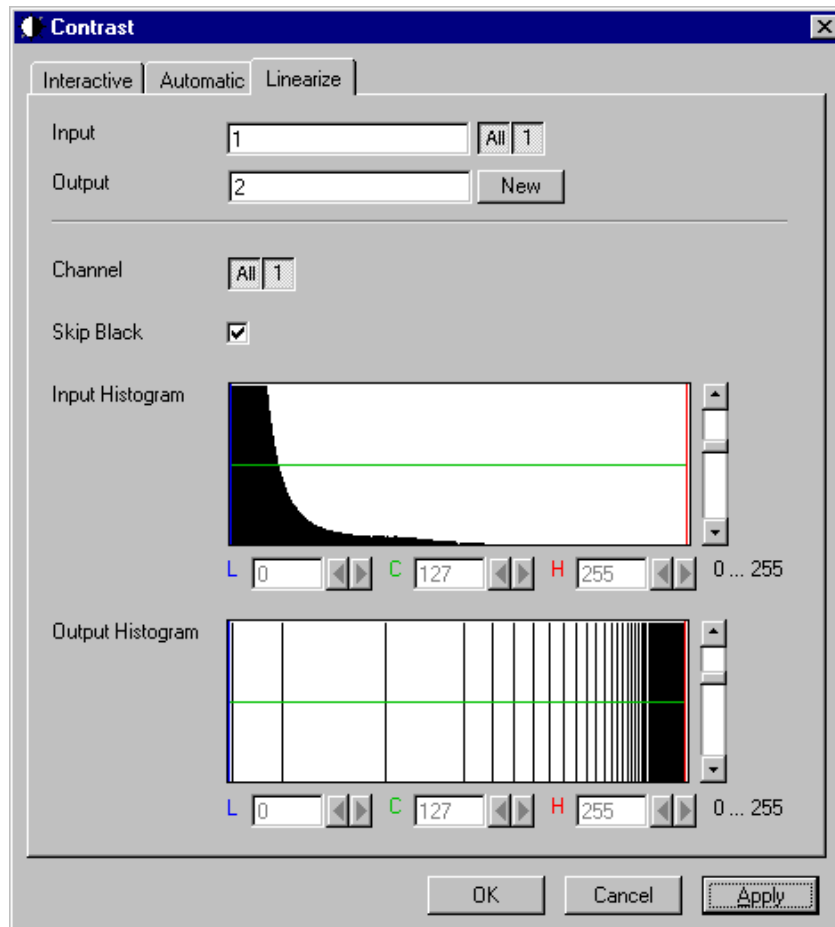


Fig. 6-15

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**.

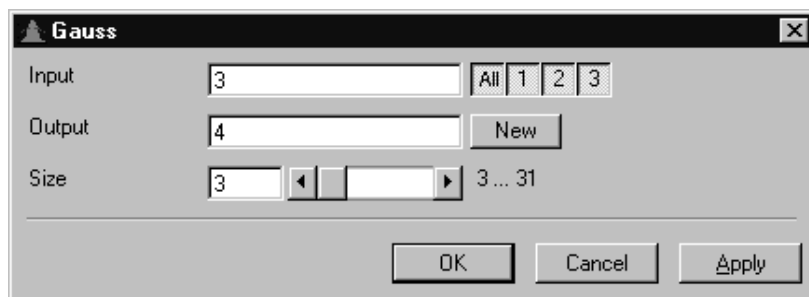
The **Output** histogram shows the resulting histogram. The horizontal axis represents the grey values from 0 to 255. The vertical axis represents the pixel count. The vertical scale of the histogram is set using the scroll bar. The units are percentages of the grey value distribution maximum. This setting has no influence to the function.

*Parameters:*

<b>Image</b>	Input image sequence
<b>Output</b>	Output image sequence
<b>SkipBlack</b>	0 - Grey value black is ignored 1 - Grey value black is taken into account
<b>Input L</b>	Lower boundary of grey value range Input
<b>Input C</b>	Center of grey value range Input
<b>Input H</b>	Upper boundary of grey value range Input
<b>Output L</b>	Lower boundary of grey value range Output
<b>Output C</b>	Center of grey value range Output
<b>Output H</b>	Upper boundary of grey value range Output

**Smooth (Gauss)**

This function performs a Gauss filter.



**Fig. 6-16**

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.

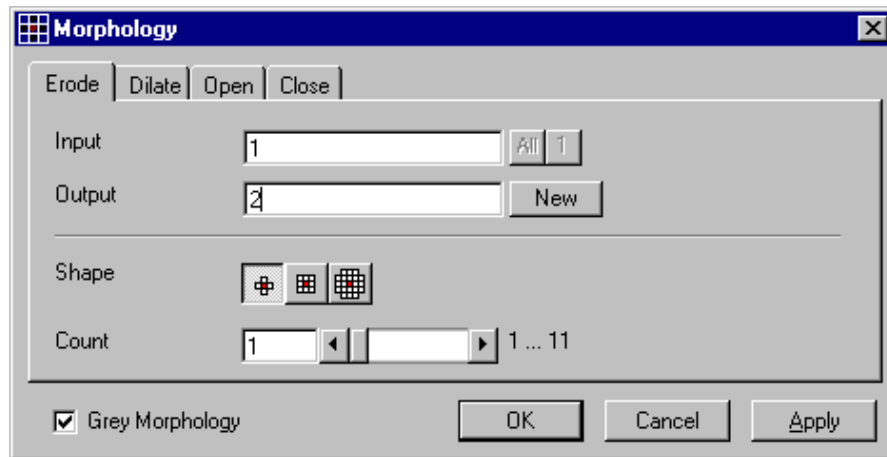
The grey value of every pixel is substituted by a weighted average of its surrounding neighbors. The neighbors are defined by a cube. The affected pixel is the central pixel of the filter cube. The weighted filter cube is approximated by a binomial distribution. The size of the filter cube is set using the **Size** scroll bar. Even numbers are set to the next odd value. The **Size** defines the strength of the smoothing.

*Parameters:*

<b>Input</b>	Input image sequence
<b>Output</b>	Output image sequence
<b>Size</b>	Filter size (3...31, only odd numbers)

## Morphology

The following four functions perform basic operations of mathematical morphology on image sequences.



**Fig. 6-17**

As generalization of the morphology of two-dimensional images to three dimensions the structural elements are small volumina.

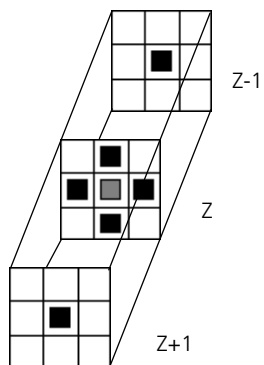
### Literature

- 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

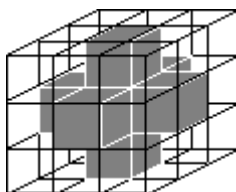
The input image sequence is analyzed voxel by voxel with a selected shape (**Shape**). The voxel to be analyzed is always the central voxel of the shape. The shape type determines which neighboring voxels are used to compute the resulting voxel.

The following structural elements are available for all morphological operations. They represent approximated spheres with an increasing radius.

Sequential image:

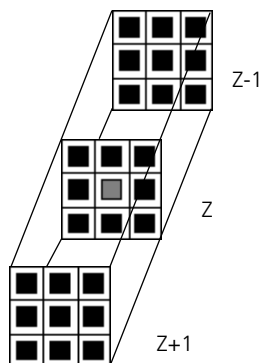


Volume view:

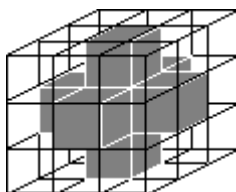


Cross shape

Sequential image:

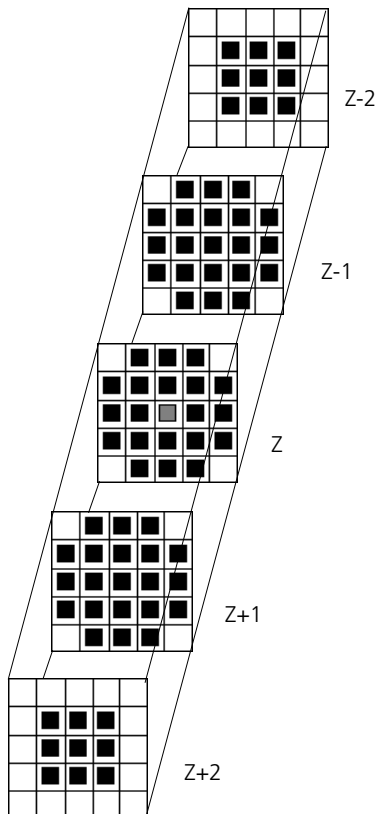


Volume view:

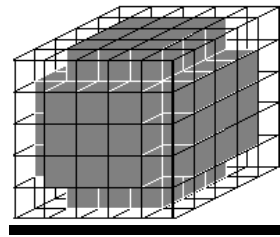


Cross shape

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 is 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.

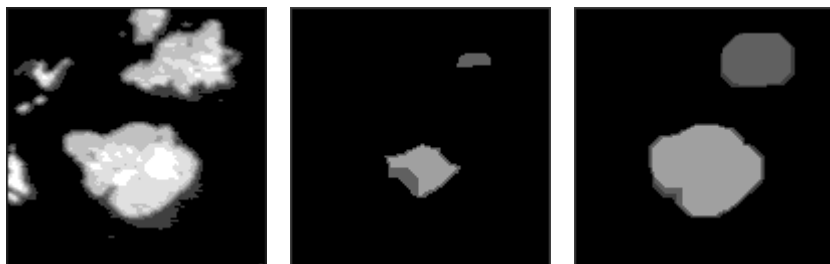
Erosion reduces the size of bright regions, separates thin connections between them, and makes small regions disappear. Dilation, on the other hand, makes bright regions of the image grow in size, fills gaps, and smoothes small contour details.

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. 6-18**

Close = Dilation + Erosion



**Fig. 6-19**

The "cube cross" shape was used for the operations shown.

## Morphology - Erode

This function erodes structures in an image sequence.

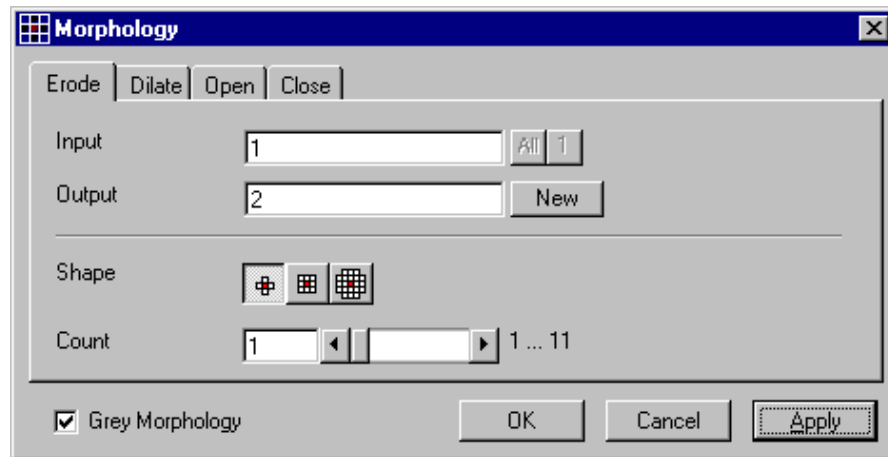


Fig. 6-20

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Shape</b>	Shape used 1 - cross 2 - cube 3 - cube cross
<b>Count</b>	Number of recursive operations
<b>Grey Morphology</b>	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.

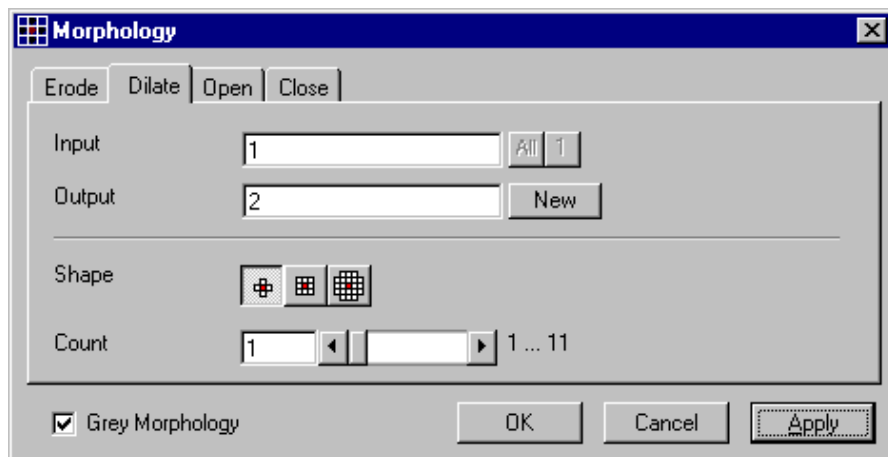


Fig. 6-21

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Shape</b>	Shape used 1 - cross 2 - cube 3 - cube cross
<b>Count</b>	Number of recursive operations
<b>Grey Morphology</b>	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.

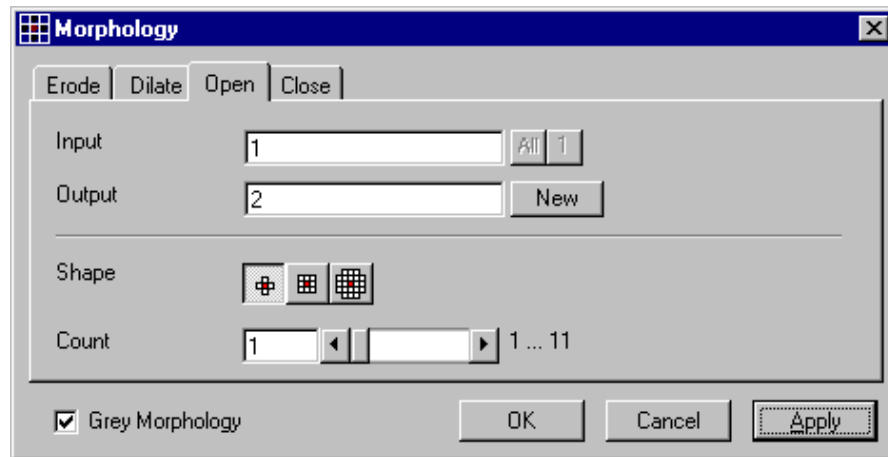


Fig. 6-22

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Shape</b>	Shape used 1 - cross 2 - cube 3 - cube cross
<b>Count</b>	Number of recursive operations
<b>Grey Morphology</b>	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.

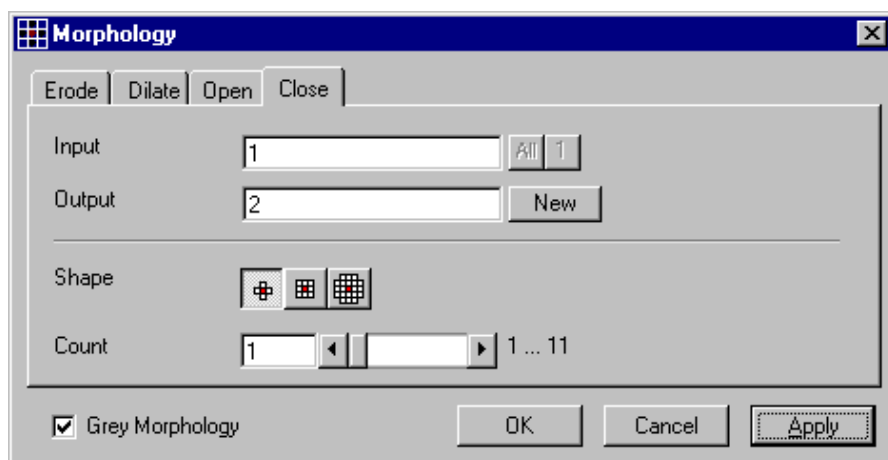


Fig. 6-23

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Shape</b>	Shape used 1 - cross 2 - cube 3 - cube cross
<b>Count</b>	Number of recursive operations
<b>Grey Morphology</b>	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.

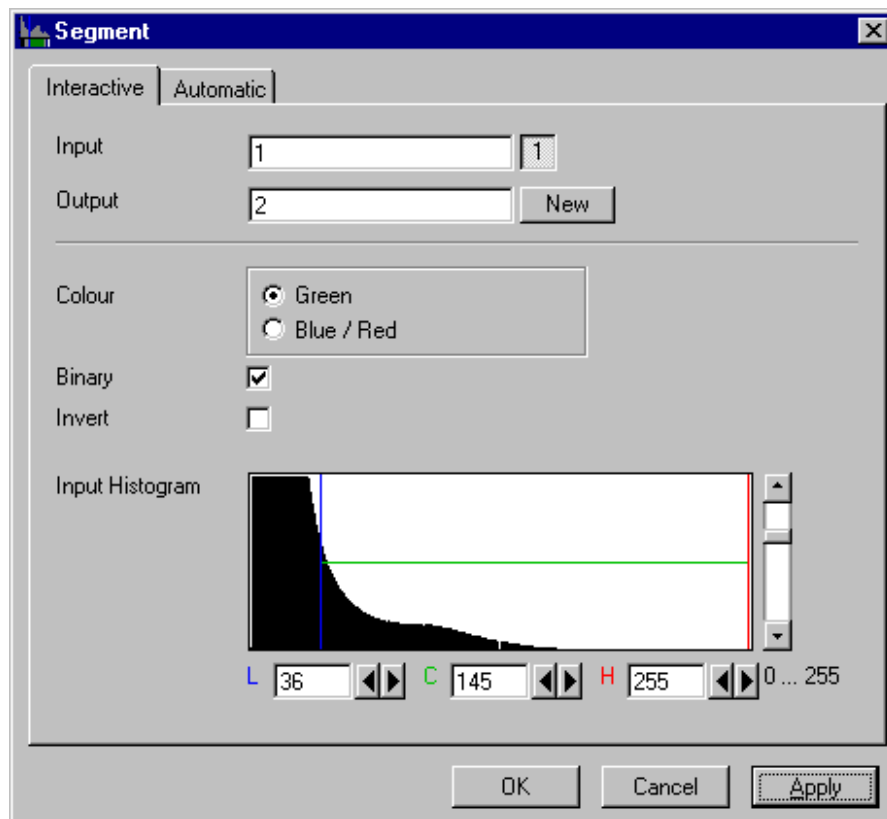


Fig. 6-24

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 **Low** and **High** are determined either by moving the borderlines in the grey value histogram or by the scroll bars underneath. Furthermore, the values for **Low**, **Center** and **High** 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 **Low** and **Low+1** are displayed in blue. The voxels with the grey values **High** and **High-1** are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **Low**, **High** remain unchanged. Voxels with grey values less than **Low** are highlighted in blue; those with grey values higher than **High** 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 **Low** to **High** 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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Colour</b>	Green - Selected interval is displayed in green
<b>Blue/Red</b>	Grey values below the selected interval are displayed in blue, grey values above in red
<b>Binary</b>	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255, the rest to grey value 0
<b>Invert</b>	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
<b>L</b>	Low grey value threshold
<b>C</b>	Center of threshold interval
<b>H</b>	High grey value threshold

## Segment - Automatic

The function carries out an automatic grey value segmentation by means of thresholding.

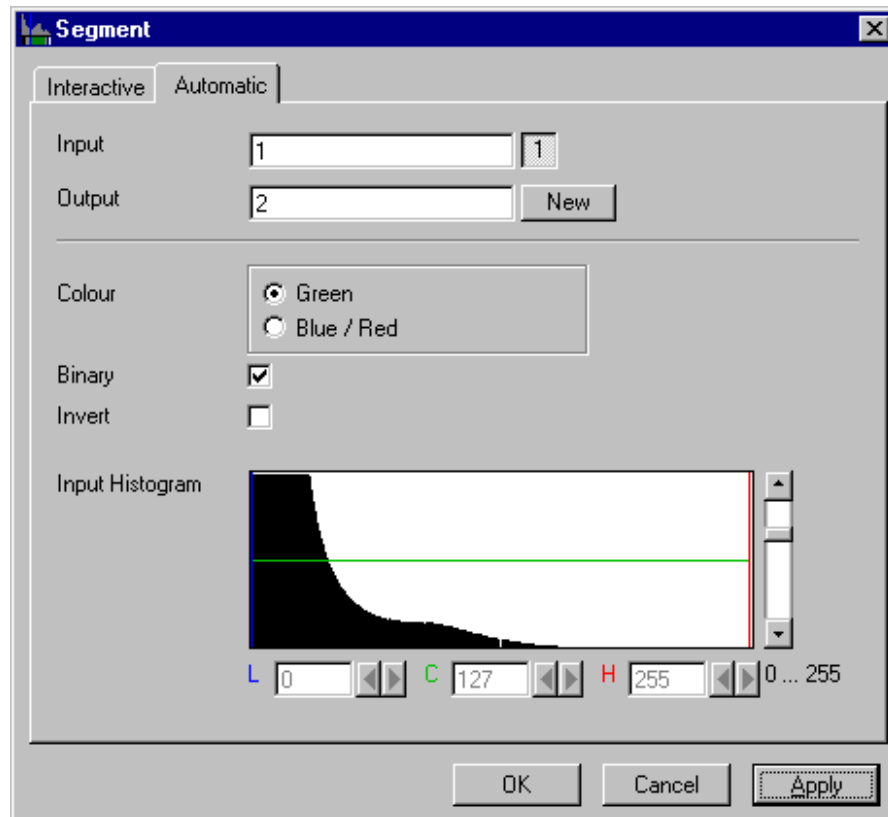


Fig. 6-25

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 **Low** and **Low+1** are displayed in blue. The voxels with the grey values **High** and **High-1** are displayed in red.

If **Blue/Red** is selected, the voxels with grey values within the interval **Low**, **High** remain unchanged. Voxels with grey values less than **Low** are highlighted in blue; those with grey values higher than **High** 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 **Low** to **High** 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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Colour</b>	Green - Selected interval is displayed in green Blue/Red - Grey values below the selected interval are displayed in blue, grey values above in red
<b>Binary</b>	0 - Selected voxels retain the original grey value 1 - Selected voxels are set to grey value 255 (4095), the rest to grey value 0
<b>Invert</b>	0 - Grey values inside the selected interval are segmented 1 - Grey values outside the selected interval are segmented
<b>L</b>	Low grey value threshold
<b>C</b>	Center of threshold interval
<b>H</b>	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**.

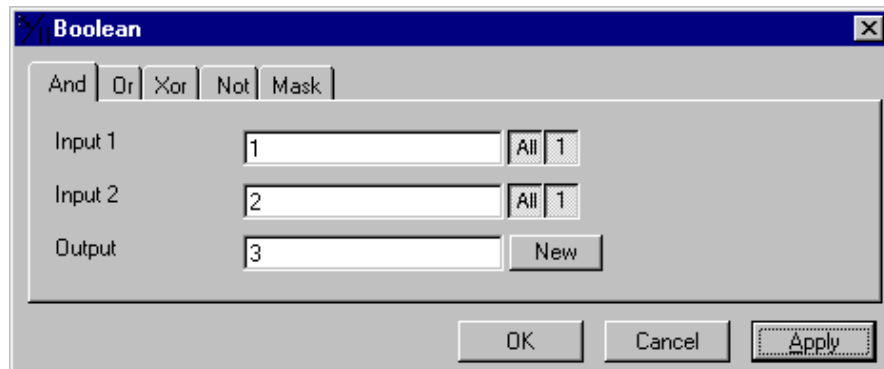


Fig. 6-26

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:

<b>Input 1</b>	First input image sequence
<b>Input 2</b>	Second input image sequence
<b>Output</b>	Resulting image sequence

## Boolean - Or

This function carries out a bit-by-bit **Or** calculation for the images **Input 1** and **Input 2**.

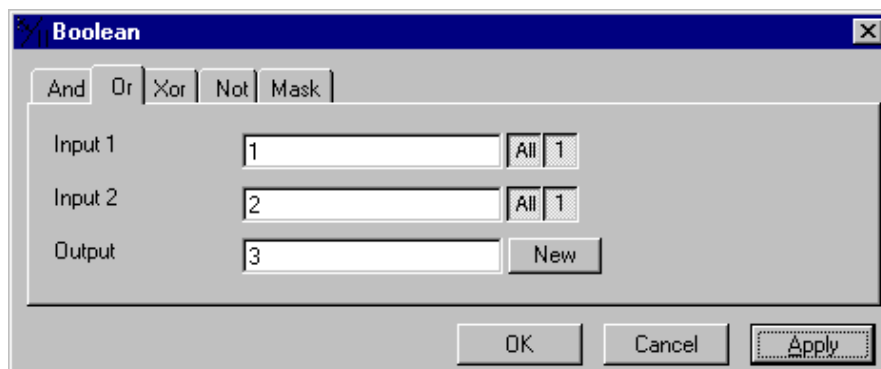


Fig. 6-27

The **Or** tab sheet of the **Boolean** dialog window must be selected.

This function can be used to combine binary masks or regions.



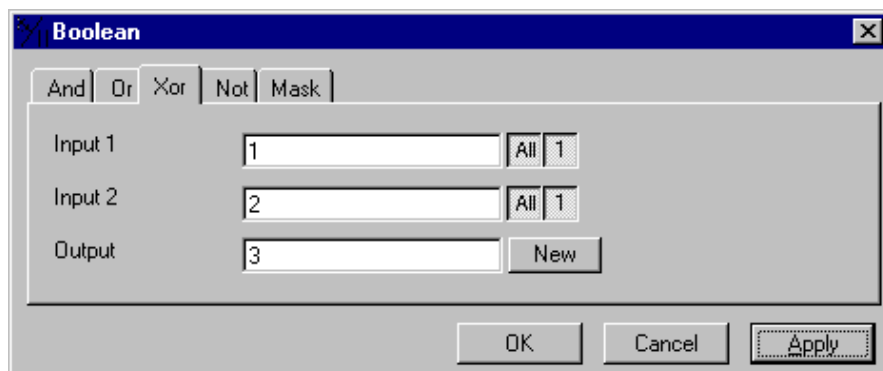
If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

*Parameters:*

<b>Input 1</b>	First input image sequence
<b>Input 2</b>	Second input image sequence
<b>Output</b>	Resulting image sequence

### Boolean - Xor

This function carries out a bit-by-bit **Xor** calculation for the images **Input 1** and **Input 2**.



**Fig. 6-28**

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.

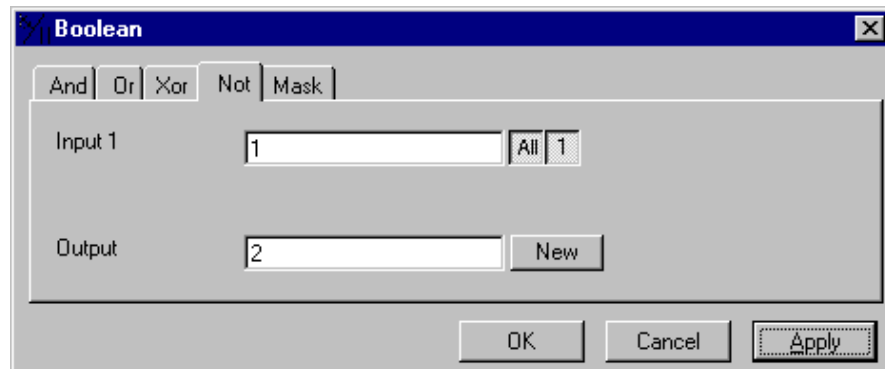
If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 1** and **Input 2** must be the same. They will be combined from left to right.

*Parameters:*

<b>Input 1</b>	First input image sequence
<b>Input 2</b>	Second input image sequence
<b>Output</b>	Resulting image sequence

## Boolean - Not

This function carries out a bit-by-bit negation of an image.



**Fig. 6-29**

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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence

## Boolean - Mask

This function masks a grey value image sequence.

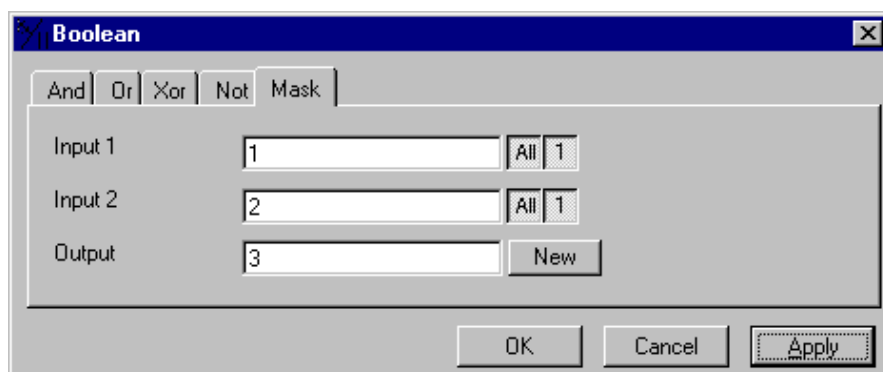


Fig. 6-30

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.

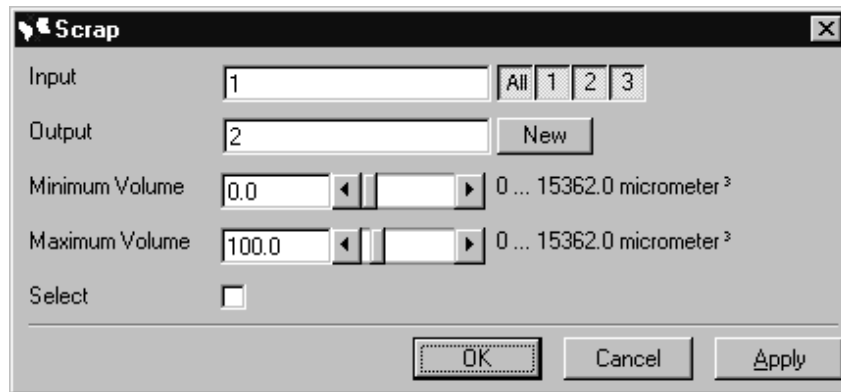
If one or both input sequences are multichannel sequences, any number or combination can be selected. The number of selected channels for **Input 2** must be 1 or the same as for **Input 1**. They will be combined from left to right.

### Parameters:

<b>Input 1</b>	First input image sequence
<b>Input 2</b>	Second input image sequence
<b>Output</b>	Resulting image sequence

## Scrap

This function deletes or selects objects in a specified size range.



**Fig. 6-31**

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Output image sequence
<b>MinVolume</b>	Minimum object size
<b>MaxVolume</b>	Maximum object size
<b>Select</b>	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. 6-32**

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Output image sequence

### 6.3.4 Functions in the View Menu

#### Render - Surface

This function displays an image sequence according to the **gradient shading method**.

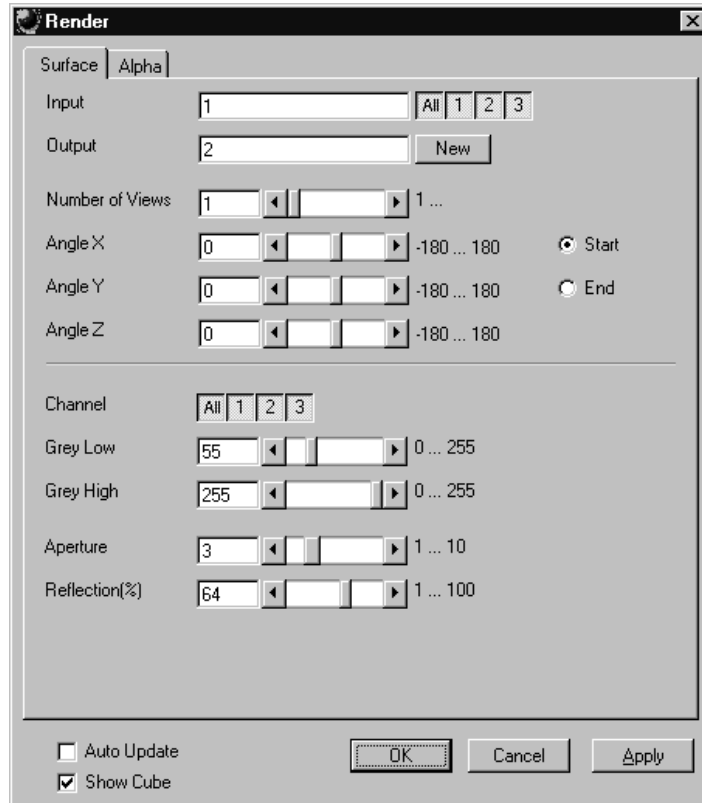


Fig. 6-33

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:

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Number of Views</b>	Number of reconstructions to be calculated
<b>Angle X</b>	Angle of rotation on the X-axis, start position
<b>Angle Y</b>	Angle of rotation on the Y-axis, start position
<b>Angle Z</b>	Angle of rotation on the Z-axis, start position
<b>Channel</b>	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
<b>Grey Low</b>	Low grey value threshold of the region to be displayed

---

<b>Grey High</b>	High grey value threshold of the region to be displayed
<b>Aperture</b>	Measure of the extent of the highlights
<b>Reflection</b>	Weight of the diffuse brightness components in comparison to the highlights
<b>Auto Update</b>	0 - Function execution is performed on <b>OK</b> or <b>Apply</b> 1 - Function execution for the current angle is performed on any parameter change
<b>Show Cube</b>	0 - The wire frame cube is not shown 1 - The wire frame cube is shown in the <b>Display window</b>

### 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**.

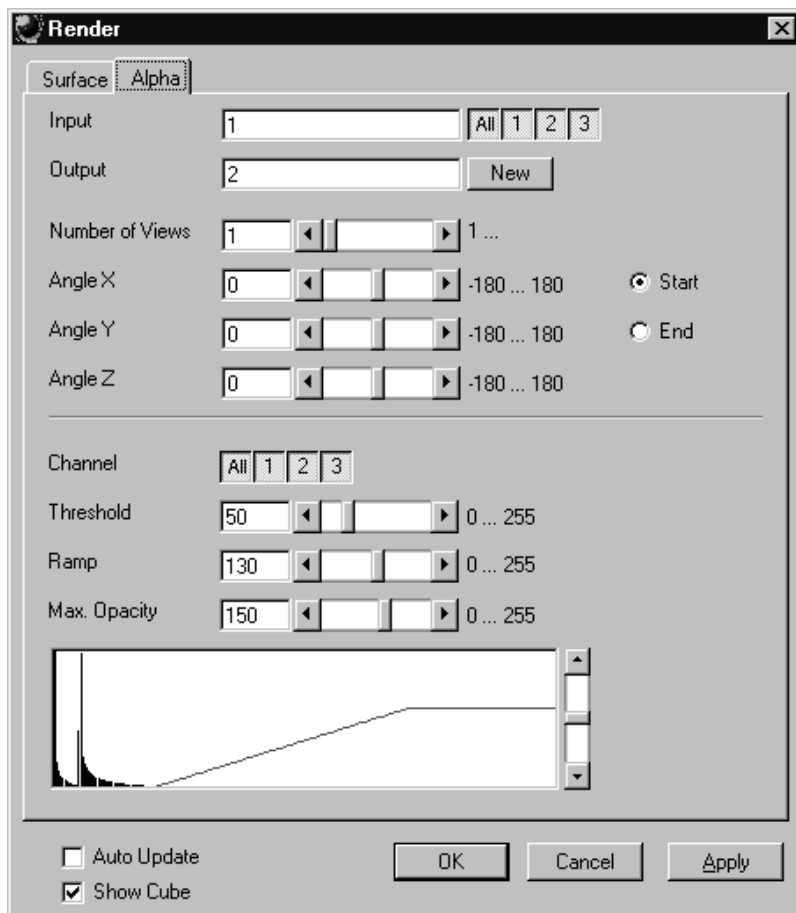


Fig. 6-34

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:*

<b>Input</b>	Input image sequence
<b>Output</b>	Resulting image sequence
<b>Number of Views</b>	Number of reconstructions to be calculated
<b>Angle X</b>	Angle of rotation on the X-axis, start position
<b>Angle Y</b>	Angle of rotation on the Y-axis, start position
<b>Angle Z</b>	Angle of rotation on the Z-axis, start position
<b>Channel</b>	All - The following parameters are valid for all channels X - The following parameters are valid for the selected channel only
<b>Threshold</b>	Grey value where the opacity starts rising
<b>Ramp</b>	Length of the opacity slope
<b>Max. Opacity</b>	Maximum opacity value
<b>Opacity Table</b>	Maximum opacity value
<b>Auto Update</b>	0 - Function execution is performed on OK or Apply 1 - Function execution is performed on any parameter change
<b>Show Cube</b>	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.

### 6.3.5 Functions in the Measurement Menu

#### Measurement Concept

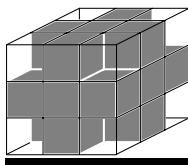
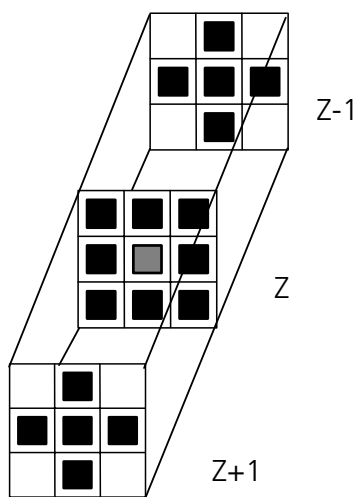
Measurement is based on regions (objects) in three-dimensional space. Segmenting an image sequence generates these. The image segmentation process produces a mask image that defines the region.

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. 6-35**

## Measurement Process

The measurement process consists of three steps: region definition, checking of the validity of the regions, and feature calculation.

Region definition:

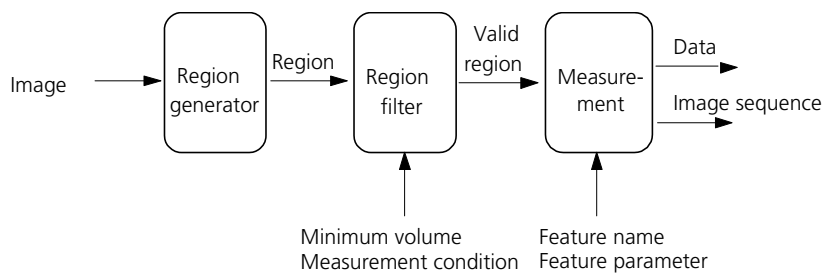
- Automatically from the mask image

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. 6-36**

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.

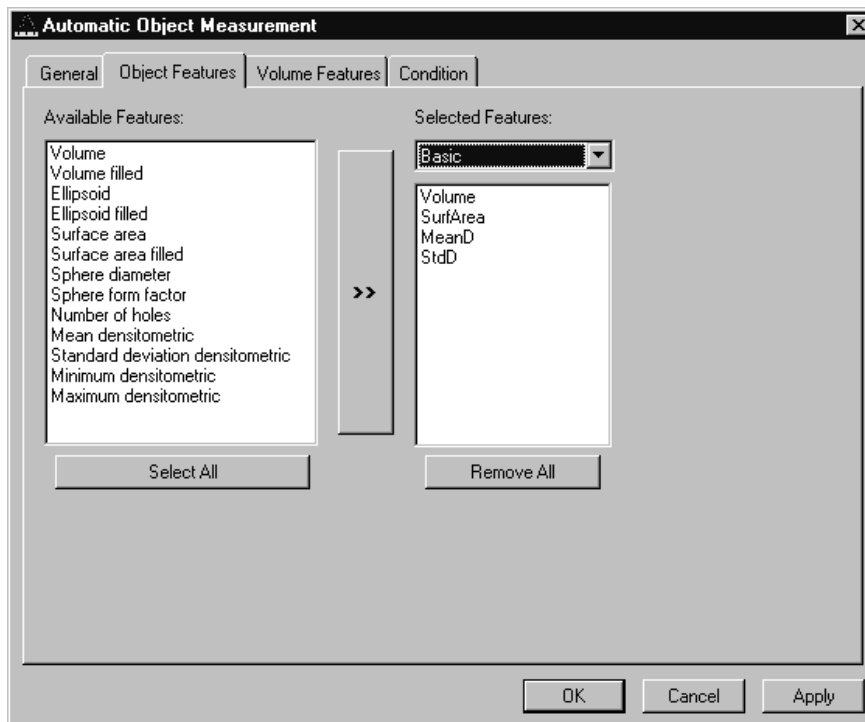


Fig. 6-37

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:*

<b>Available Features</b>	List of available object features
<b>Selected Features</b>	List of selected object features
<b>Select All</b>	Select all available object features for measurement
<b>Remove All</b>	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. $\sqrt{6 * \text{VOLUMEF} / \pi}$
Sphere Form Factor	Fsphere	Form factor of the object. $6 \cdot \sqrt{\pi} \cdot \frac{\text{VOLUMEF}}{\sqrt{\text{SURFAREA}^3}}$
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.

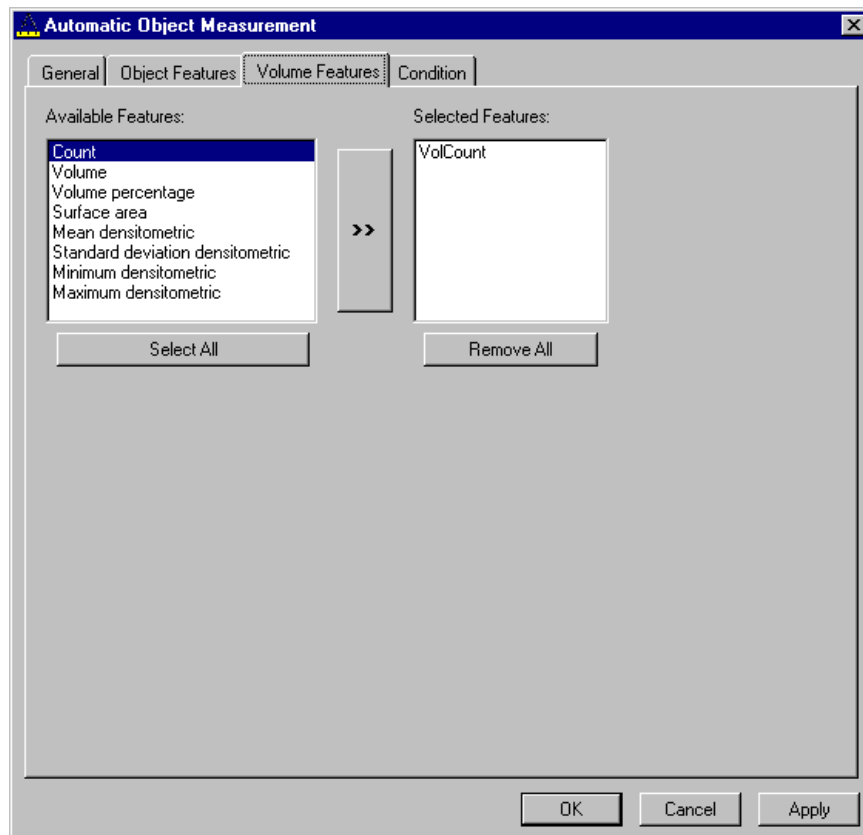


Fig. 6-38

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:*

<b>Available Features</b>	List of available object features
<b>Selected Features</b>	List of selected object features
<b>Select All</b>	Select all available object features for measurement
<b>Remove All</b>	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.

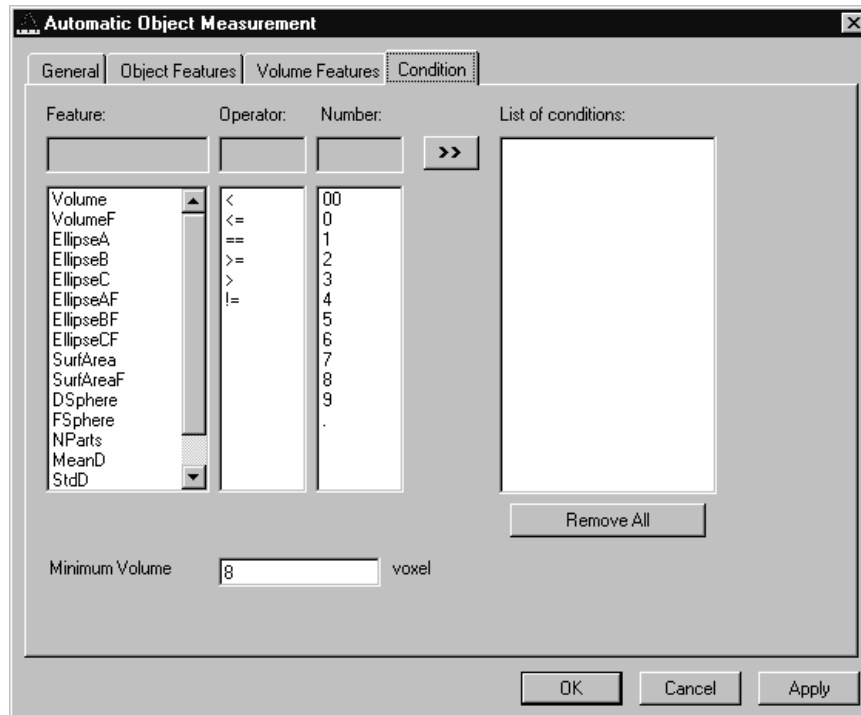


Fig. 6-39

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:*

<b>Feature</b>	List of available object features
<b>Operator</b>	List of available condition operators
<b>Number</b>	List of numbers to compose the value
<b>List of conditions</b>	Defined condition list
<b>Remove All</b>	Remove all entries from the <b>List of conditions</b>
<b>Minimum Volume</b>	Minimum object volume in voxel

## Automatic Object Measurement - General

This function carries out an automatic measurement and labeling.

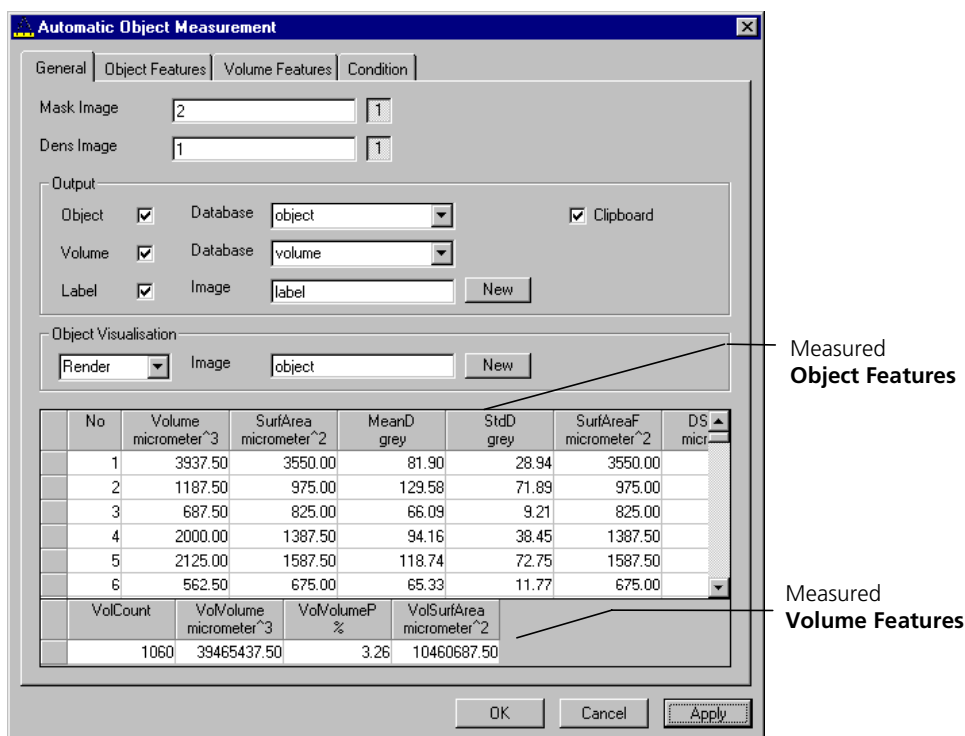


Fig. 6-40

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 spread 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:*

<b>Mask Image</b>	Single channel mask image sequence that defines the objects
<b>Dens Image</b>	Image sequence for densitometric measurement and property source
<b>Object</b>	Stores measurement values of objects, including database filename
<b>Volume</b>	Stores volume measurement values of objects, including database filename
<b>Label</b>	Generates an image sequence with all objects labelled in different pseudo colours
<b>Clipboard</b>	Measurement values are automatically written to the clipboard



## CHAPTER 7                      ANNEX

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**7 ANNEX****7.1 Recommendations for Excitation Laser Lines and Emission Filters of Dyes**

<b>Dye</b>	<b>Laser line/HFT</b>	<b>Emission/EM</b>
DAPI	405	> 420, max. at 461
EBFP	405	> 420, max. at 447
Hoechst	405	> 420, max. at 440
Fluoro-Gold	405 or 440	> 420/475, max. at 536
ECFP	405 or 440	> 420/475, max. at 501
Lucifer Yellow	440 or 488	> 475, max. at 536
EGFP	488	> 505, max. at 507/516
FM 1-43™	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™	532	> 530, max. at 566
EYFP	532	> 530, max. at 535
Oregon Green	532	> 530, max. at 535
SYTOX Green	532	> 530, max. at 536
FM 4-46	532	> 560, max. at 640
Alexa Fluor 546™	532	> 560, max. at 572
Calcium Orange	532	> 560, max. at 575
Dil (DiIC18(3))	532	> 560, max. at 565
DsRed	532	> 560, max. at 583
Tetramethylrhodamine (TRITC)	532	> 560, max. at 576
Rhodamine B	532	> 560/585, max. at 625
Texas Red™	532	> 560/585, 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	488/532	BP 500-525	532	LP 550

## 7.2 Configurations Overview

### 7.2.1 LSM 5 *LIVE* 1 Channel Biomedical Configurations

1 channel, FL	VarioOne GB
Laser lines	488, 532 (635) nm
Emission-filters channel 1	LP 505 BP 495-525 SK LP 550 BP 550-615 LP 650 SK BP 700-750

**7.2.2 LSM 5 *LIVE* 2 Channel Biomedical Configurations**

<b>2 channel, FL</b>	<b>VarioTwo VRGB</b>
<b>Laser lines</b>	<b>(405/440), 488, 532 (635) nm</b>
<b>Secondary beam splitter</b>	Plate mirror NFT 488 NFT 532 NFT 635
<b>Emission-filters channel 1</b>	LP 420 LP 505 BP 495-525 SK LP 550 BP 560-675 SK LP 650 SK BP 700-750
<b>Emission-filters channel 2</b>	BP 415-480 SK BP 455-525 BP 495-525 SK BP 550-615

### 7.3 Changing Filters in the Scanning Module

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.

Please ask your LSM service engineer to do this exchange in order to maintain your warranty.

### 7.4 Detaching / Attaching the Scanning Module from / to Microscope Stands

Tool needed: 3 mm Allen key



The user can remove the Scanning Module from one microscope and attach it to another within a few minutes. Described below is the change-over from an Axioskop 2 FS MOT to an Axiovert 200 M in sideport configuration.



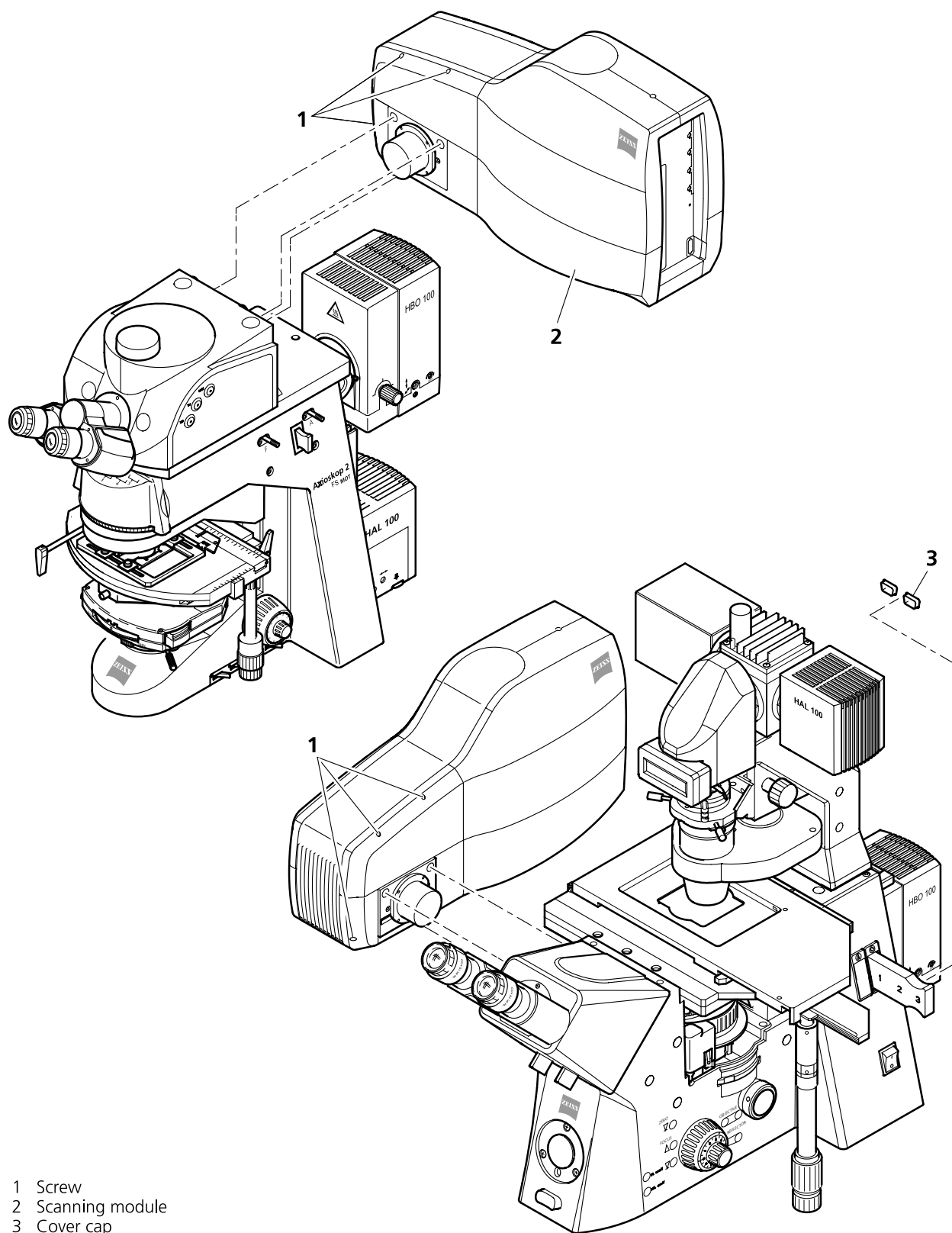
**Before** the change-over, **shut down** the system as described in chapter 4 in order to avoid damage to the system and loss of data.

- Loosen the three screws (7-1/1) at the Scanning Module (7-1/2) fitted to the Axioskop 2 FS MOT.
- Cautiously pull Scanning Module off the Axioskop 2 FS MOT stand.
- Attach Scanning Module to the left sideport of the Axiovert 200 M, minding the guide pins (7-1/5), and secure it with the three screws (7-1/1).



As the Scanning Module is heavy, weighing about 19.5 kg, it is easier if the changeover is carried out by two persons.

- Pull off covering caps (7-1/3) from the CAN-BUS and RS232 interface ports at the rear of the Axiovert 200 M, remove the two cables 457411-9011 (CAN-BUS) and 457411-9012 (RS232) from the Axioskop 2 FS MOT, plug them into the Axiovert 200 M and secure them there.
- Switch the LSM 5 LIVE on.
- Click on the **Stand select** icon to update the system database with the new database of the Axiovert 200 M microscope.
- Restart the LSM 5 LIVE program.

**Fig. 7-1** Change-over of the Scanning Module

---

## **7.5 Hints on the Use of the Piezo Fine Focusing Stage**

### **7.5.1 General Description**

The Piezo fine focusing stage is a compact attachment for the Axio Imager.Z1 and Axiovert 200 M microscope stages, which allows the particularly fast and high-precision fine focusing of the object. The Piezo stage permits fine focusing over a range of 250 µm, with the smallest step width being less than 10 nm, reproducibility better than 40 nm, and the maximum speed amounting to 60 Hz. The stage allows the use of specimens with a weight of less than 100 g.

The piezo stage is not used if manual coarse focusing is performed. To position the objective in relation to the optical Z-axis, the standard XY-microscope stage is used.

The piezo stage features a mount for standard object carriers of 76 mm x 26 mm x 1 mm and a milled-out receptacle for Ø 36 mm x 1 mm Petri dishes.

### **7.5.2 Application Fields**

- High-precision fine focusing and translation of the object along the optical axis.
- Fast and high-precision mounting of one-dimensional Z-line sections.
- Fast and high-precision mounting of two-dimensional R-Z-longitudinal sections.
- Fast and high-precision mounting of XY-Z-Stacks for the three-dimensional reconstruction of the object.
- Exact measurement of Point-Spread-Functions for deconvolution.

### **7.5.3 Additional Information on the Operation**

The piezo fine-focusing stage is a high-precision, sensitive accessory for the LSM 5 *LIVE* from Carl Zeiss and must therefore be treated carefully.

High mechanical stress, such as the use of specimens weighing more than 100 g or the application of pressure or knocks on the movable stage tongue, can result in damage and therefore in failure of the stage function.

To be able to fully utilize the outstanding precision attainable with the fine focusing stage, anything which could interfere with its operation, especially mechanical knocks and impact of the LSM 5 *LIVE* components, should be avoided. We would recommend you to always use the actively vibration-damped table.

The specifications of the stage are obtained only after a heating phase of approx. 30 minutes. Furthermore, the installation conditions for the LSM 5 *LIVE* system must be observed.

The maximum reproducibility (better than 40 nm) for moving to an absolute position in Z is achieved by always moving to the required position from below.

Fine focusing is performed mechanically via an inclined position of the stage tongue. Therefore, the lifting range Z at the location of the image field depends on the position of the piezo stage in relation to the optical axis. This means: if the user shifts the object on the microscope stage to the right via the piezo stage, the lift will be different from the one in the zero position of the stage (max. 250 µm) and also from the one after a shift of the stage to the left.

If the LSM 5 *LIVE* system is equipped with a motorized scanning stage, this shift is read back to •x and the lift is calibrated automatically if the zero position of the piezo stage has been matched to the zero position of the scanning stage via an initialization run. For this, activate the **Stage** button of the **Acquire** toolbar. Then position the scanning stage in such a way that the optical axis of the microscope corresponds to the zero position of the piezo stage, i.e. to the center of the specimen holder in the stage tongue. Then perform initialization by pressing the **piezo Null** button. This step must be repeated after every new start of the system. Also see the notes on the operation of the motorized scanning stages.

If the system is equipped with a manual microscope stage, the user has the option of performing the calibration by entering the •x shift in mm via the **Calibration** slider.

The shift is read off from the microscope stages. In the case of the manual AxioImager stage, •x can be read directly from the scale adhered to the front of the stage. In the case of the manual Axiovert 200 M stage, a scale is located on the right of the knob, where the 45 mm •x shift relative to the zero position of the microscope stage can be read off. The •x value is positive for both stages if shift from the zero position is made to the right and negative if the shift is made to the left.

On account of the inclined position of the stage tongue, the object is also shifted laterally during the fine focusing motion. This lateral shift is negligibly small if, as recommended by us, specimen carriers with thickness 1.0 mm are used exclusively. Otherwise, the marked lateral shift of the object during fine focusing can result in image distortion. For the same reason, Petri dishes without fixation ring must be used exclusively.

The nosepiece of the Axiovert 200 M stand is moved to the load position prior to switching off the LSM 5 *LIVE* system and the piezo stage is then moved to the lowest position to avoid damage of the objective or object by a possible collision. The user must refocus after start-up of the system. Before an objective change in the Axiovert 200 M or the Axio Imager.Z1 the nosepiece and the microscope stage must be moved to the load position by the user, and then back to the work position to prevent the objectives from hitting the piezo components. This is performed automatically if the objectives are changed menu-controlled via the relevant buttons of the LSM 5 *LIVE* program.

## 7.6 Piezo Objective Focussing Device

For upright stands Axio Imager.Z1, Axio Imager.M1, Axioskop 2 FS MOT

Range: 250  $\mu\text{m}$

Minimum step size: 5 nm

Speed:

		Piezo objective focussing device	Piezo focussing stage
Slices	Step size [ $\mu\text{m}$ ]	xz-lines / s	xz-lines / s
20	0.5	60	60

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. 7-2/1).
- Screw the thread-ring into your microscope (see Fig. 7-2/2).
- Easy clamp the Piezo Objective Focusing Device on the thread-ring (see Fig. 7-2/3).

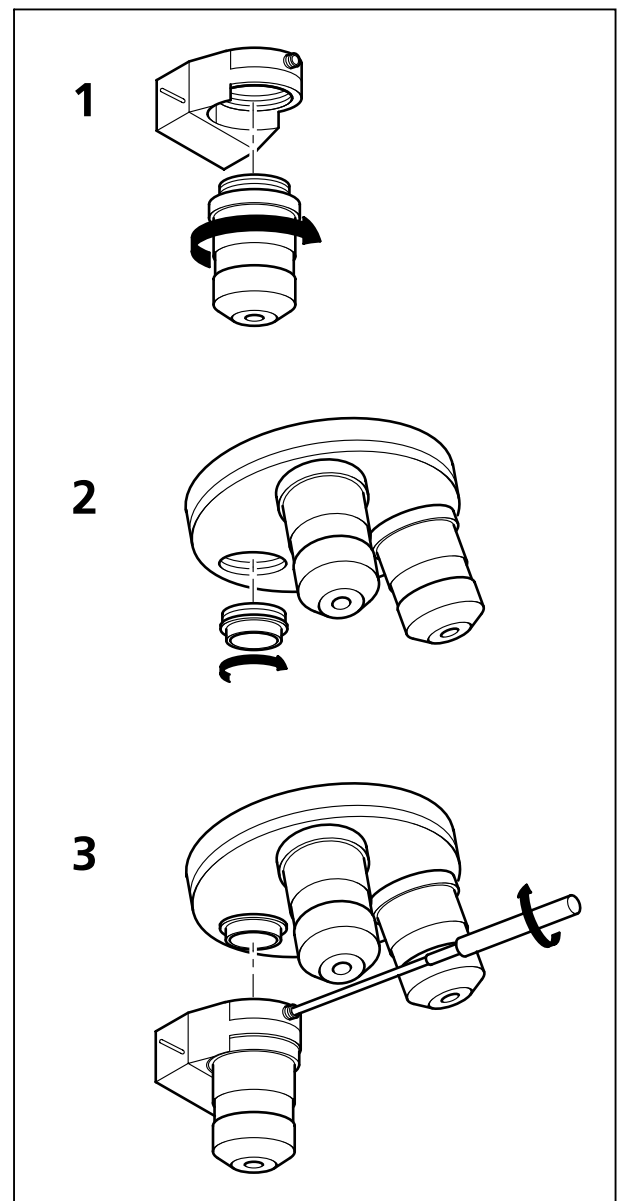


Fig. 7-2 Installation of the Piezo Objective Focusing Device



## 7.7 Specifications of Trigger-Interface LSM 5 *LIVE*

### Application:

With the LSM 5 *LIVE* 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 Control Unit in the ECU of LSM 5 *LIVE*:
- Connector 2x high density D-Type plug, 1x coax with outer shield

### 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

### High density D-Type 15pol.: 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

### High density D-Type 15pol.: 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

\*) pull to signal ground

\*\*) 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 > 2.8 V
- Slew rate 10 ns/V

Signal input:

- Low level < 0.8 V, high level > 2.0 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.).

**Hint:**

Use the Zeiss "Trigger Box" (1315-620) for immediate use of the User-Port:

- 4 momentary-on switches, 2 BNC connectors including optional signal inversion for input
- 8 leds and 2 BNC connectors for output

## 7.8 AxioCam High Resolution Digital Cameras

### 7.8.1 Microscopy Camera AxioCam MRm Rev.2

High resolution microscopy camera AxioCam MRm Rev. 2 (D)

Cat. No 000000-0445-554

Mid Range Monochrome

incl. AxioVision AC, digital interface, cable and IR barrier filter BG40 (enclosed)

Number of Pixels: 1388 (H) x 1040 (V) = 1.4 Mega pixel

Pixel size: 6.45 µm x 6.45 µm

Chip size: 8.9 mm x 6.7 mm, equivalent to 2/3"

Spectral range: With protection glass limited appr. 350 nm to 1000 nm,  
With IR barrier filter BG40 appr. 350 nm to 700 nm

NIR-Mode: Increase of IR sensitivity

Max. Full Well Capacity: Approx. 17.000 e

Selectable Resolution:

H	x	V
276	x	208 (Binning 5x5)
346	x	260 (Binning 4x4)
462	x	346 (Binning 3x3)
694	x	520 (Binning 2x2)
1388	x	1040 (single shot)

Live frame rates (depending on hardware and software configuration):

H	x	V	Binning Factor	Frame Rate @20ms
1388	x	1040	1	7
694	x	520	2	13
462	x	346	3	17

Readout of Sensor

Sub Regions("ROI"): Adjustable

Digitalization: 12 bits / 18 Mhz pixel clock

Dynamic Range: Typical 1700 : 1 at 10 e readout noise

Integration time: 1 ms to 20 s

Cooling: One stage Peltier cooling

Control signals: TTL output for controlling of external shutters

Interface: PCI interface card (32 bit / 5 V) with one cable (5m)  
for data, control and power supply

Optical Interface: C-Mount

Thread depth for objectives: max. 5 mm

---

Max. file size per image:	About 2.8 MB at 1388 x 1040 @ 1 x 12 bit
Operating Systems:	Win 2000, Win XP
Size / Weight:	About 11 cm x 8 cm x 4.5 cm (2.3" x 3.2" x 1.7") / 370 g
Housing:	Blue anodized aluminum, with cooling fins, 1/4" photo thread for tripod mount
Registration:	CE, cUL
Power Supply:	Via PC
Environment conditions:	0° ... +35° Celsius, 10% .... 80% relative air humidity, no condensing, free air circulation required

### 7.8.2 High Resolution Microscopy Camera AxioCam HRm Rev.2

Cat. No	000000-0445-553, 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
694 x 520	0.07 (13 images / s)
1388 x 1040	0.2 (5 images / s)
2776 x 2080	
4164 x 3120	
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:	GS, CE, cUL
Power Supply:	12 V DC, 1 A, 230 V/110 V, autodetecting

### 7.8.3 High Resolution Microscopy Camera AxioCam HRc

Cat. No	000000-0412-312, incl. digital interface and cable		
High Range Color			
Number of Pixels:	1300 (H) x 1030 (V) = 1.3 Mega pixel		
Chip size:	8.7 mm x 6.9 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
432	x	342	0.07 (Color interpolation)
1300	x	1030	0.2 (Color interpolation)
1300	x	1030	0.7 (full resolution for color channels)
2600	x	2060	
3900	x	3090	
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:	GS, CE, cUL		
Power Supply:	12 V DC, 1 A, 230 V/110 V, autodetecting		

#### 7.8.4 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**

**Adapter Video 60 C 2/3" 0.63x**

Cat. No 000000-1069-414

This adapter is **needed for attachment** of the high-resolution AxioCam microscope cameras **on the Axio Imager.Z1** and **Axioskop 2 FS MOT**.

For an additional documentation port to be connected to the camera port of the tubes with interface 60:

**Double video adapter**

Cat. No 000000-1058-640

For connection to interface 60, 2 switching positions for switching to 100% mirror or to interface for P&C modules.

**Adapter Video 44 C 2/3" 0.63x**

Cat. No 452997-0000-000

This adapter is **needed for attachment** of the high-resolution AxioCam microscope cameras **on the Axiovert 200 M SP**.



**No other cameras are supported by the LSM Software!**

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# **LSM 5 *LIVE***

## **LSM 5 *LIVE DuoScan***

### **Laser Scanning Microscope**



Brief Operating Manual  
Release 4.0  
March 2006



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## Introduction



### **For your safety!**

#### **Observe the following instructions:**

- The LSM 5 *LIVE* / LSM 5 *LIVE DuoScan* 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

- Set the **MAIN SWITCH** on the system rack to **ON** position (Fig. 1).
- When set to **ON** the **System/PC** switch 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 to **ON**. Insert the key into the key-operated **LASER** switch and switch also to **ON**.

Now the complete system is ready to be initialized with the LSM Software.

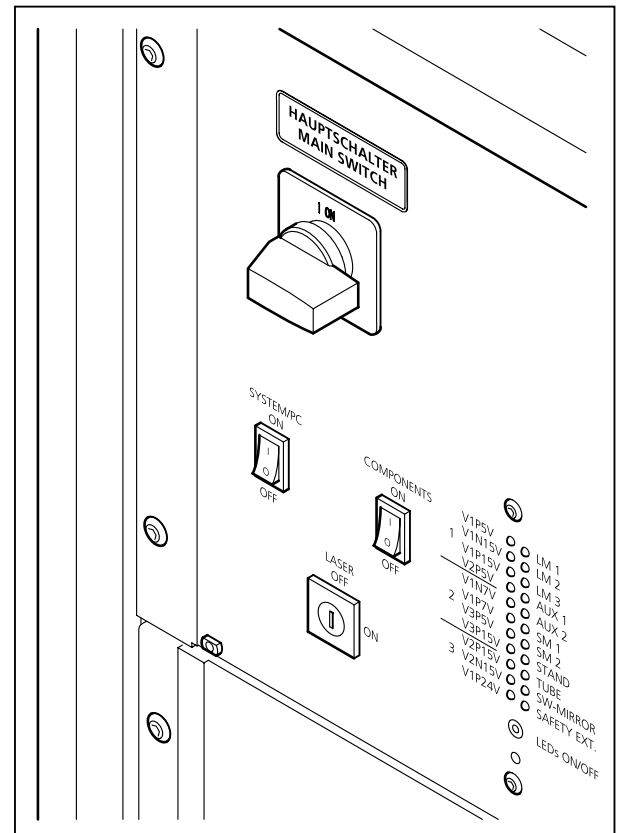


Fig. 1 System rack

### 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.

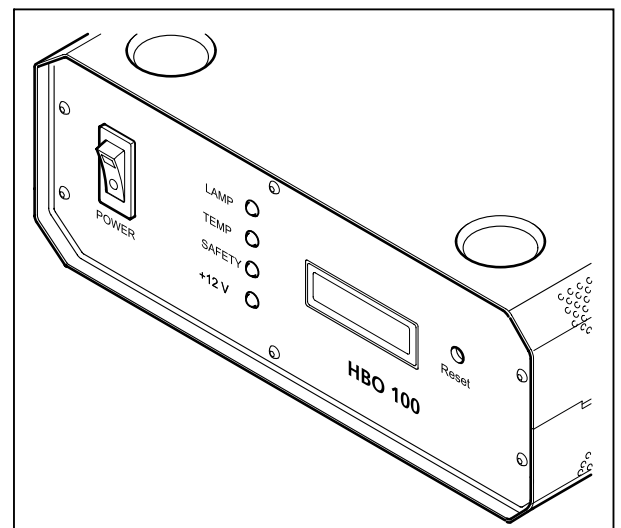


Fig. 2 HBO 100 power supply

## Starting the LSM 5 software program



- Double click the **LSM 5 LIVE** icon on the desktop of WINDOWS to start the LSM 5 software program.

The **LSM 5 LIVE Switchboard** window appears on the screen (Fig. 3).



Fig. 3 LSM 5 LIVE Switchboard menu



- Click on the **Scan New Images** button in the **LSM 5 LIVE Switchboard** window. Clicking on this button activates the complete LSM hardware (on-line mode).



- Click on the **Start** button in the **LSM 5 LIVE Switchboard** window.

The LSM 5 LIVE - **Main** menu appears on the screen.

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



← **Main** menu (pull-down)

← **Main** menu toolbar

← Subordinate toolbar

Fig. 4 Main menu



## 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.

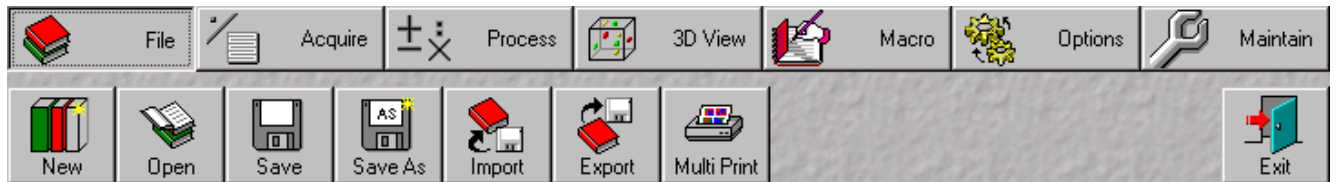
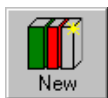


Fig. 5 Main menu with File subordinate toolbar



- 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.

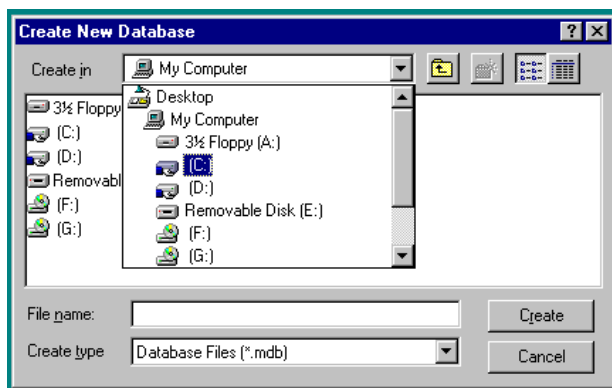


Fig. 6 Create New Database window

- Create a new directory if needed.

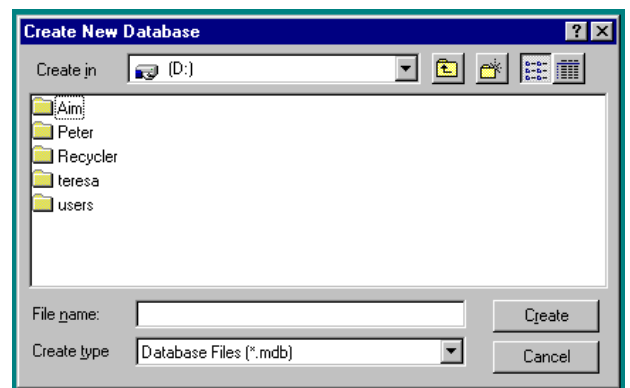
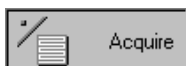


Fig. 7 Create New Database window

## Turning on the lasers



- Click on the **Acquire** button in the **Main** menu to open the **Acquire** subordinate toolbar.

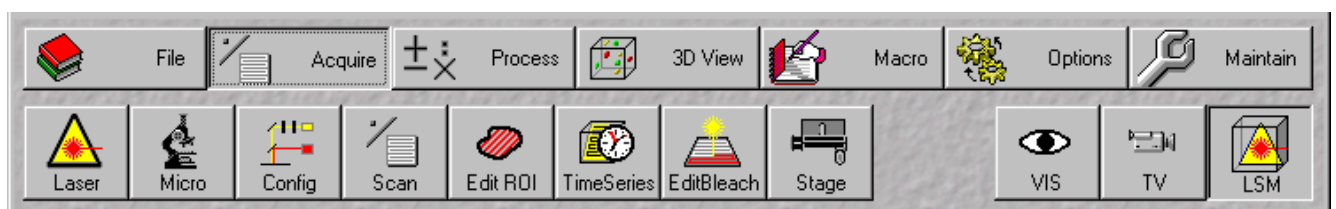
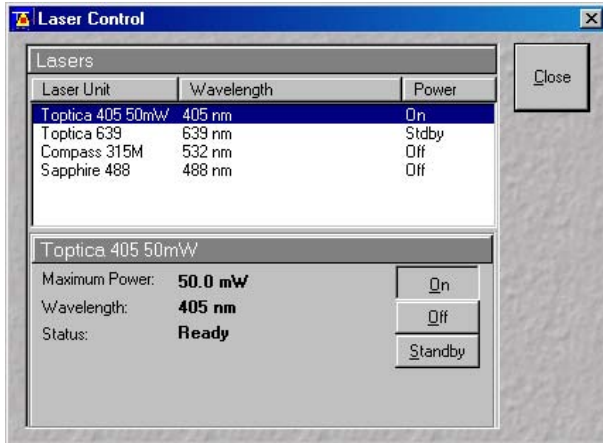


Fig. 8 Acquire subordinate toolbar



- 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 **On** button to switch required laser(s) to on (or standby if required).

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 5 *LIVE* 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).

- 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.

- 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.

- 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.

Microscope settings can be stored and up to 8 buttons assigned for fast retrieval and adjustment using the **Microscope Settings** panel.

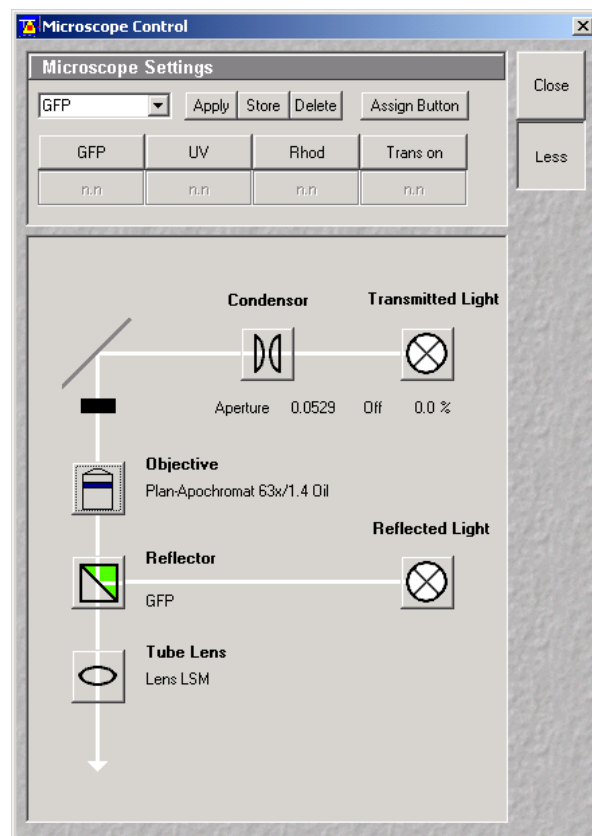
The **Store** button permits existing microscope configurations to be stored under any name.

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

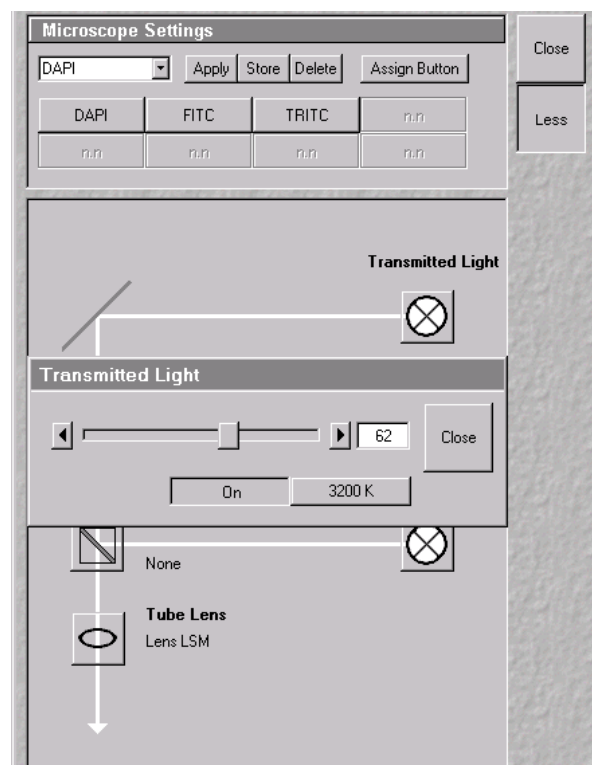
The **Delete** button permits existing microscope configurations to be deleted.

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

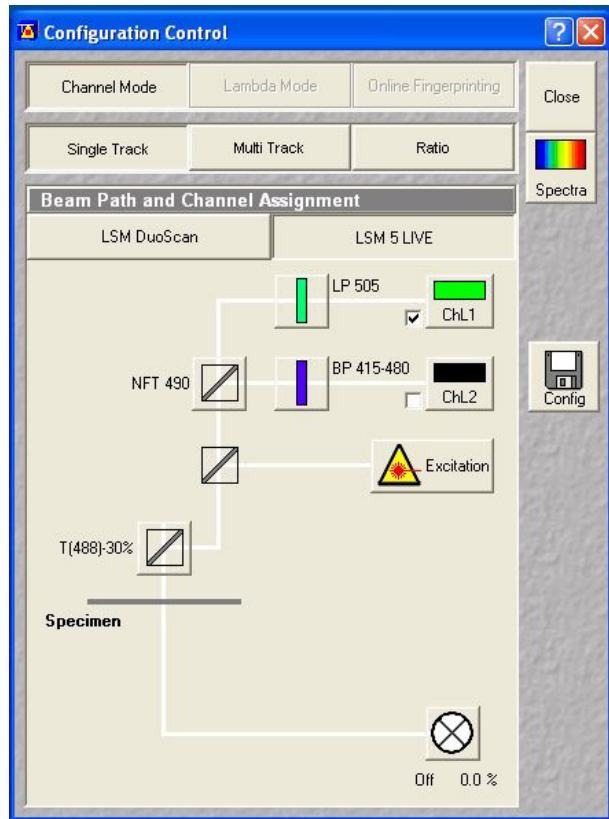


Fig. 12 Configuration Control window for Single Track

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

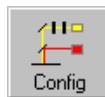
### Choosing the configuration

#### 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).

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 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 (ChL 1, ChL 2) 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 **Config** button opens the **Track Configurations** window (Fig. 13) 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**.

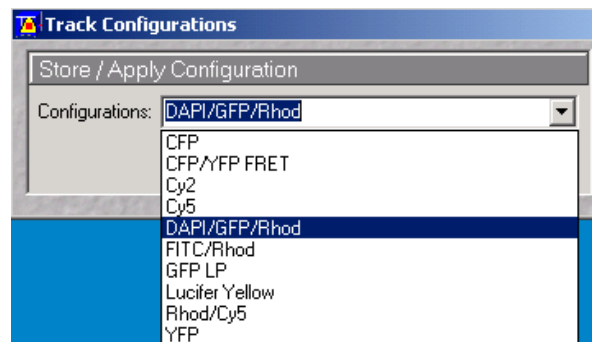


Fig. 13 Track Configurations window

## 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. 14).

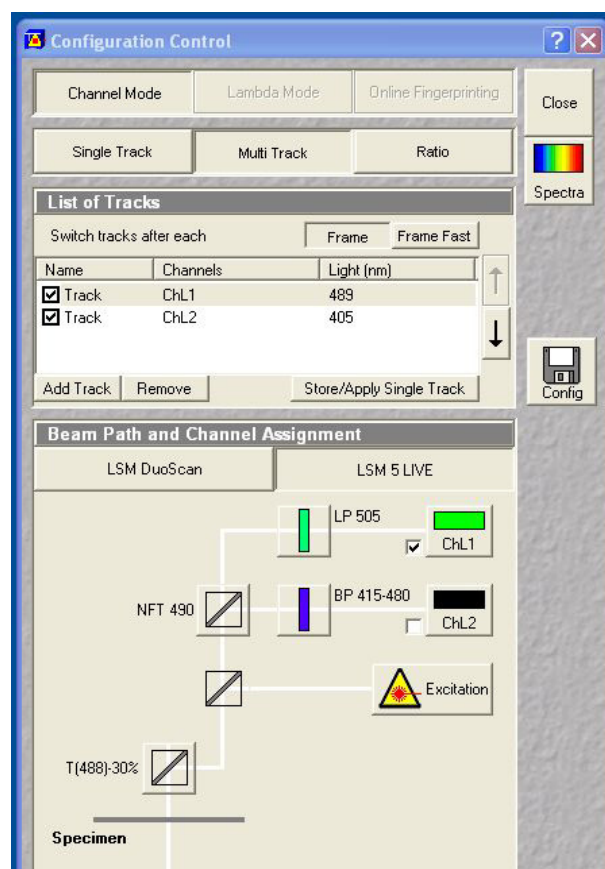


Fig. 14 Configuration Control window for Multi Track

- 
- Click on the **Multi Track** button in the **Configuration Control** window (Fig. 14).

The following functions are available in the **List of Tracks** panel (Fig. 14).

**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 ChL 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 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 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



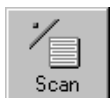
A click on this arrow button will move the selected track (highlighted in blue) one position upwards in the list box.



A click on this arrow button will move the selected track (highlighted in blue) one position downwards in the list box.

- 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. 15).

### 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.

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.

### Adjusting the exposure time

- Use the **Exposure Time** slider in the **Objective Lens, Image Size & Line Step Factor** panel (Fig. 15) to adjust the exposure time.

Below the slider is shown the number **Frames per Second**. Start with 2 to 4 frames per second.

### Choosing the dynamic range

- Select the dynamic range 8 or 12 Bit (per pixel) in the **Pixel Depth, Scan Direction & Scan Average** panel (Fig. 15).

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.

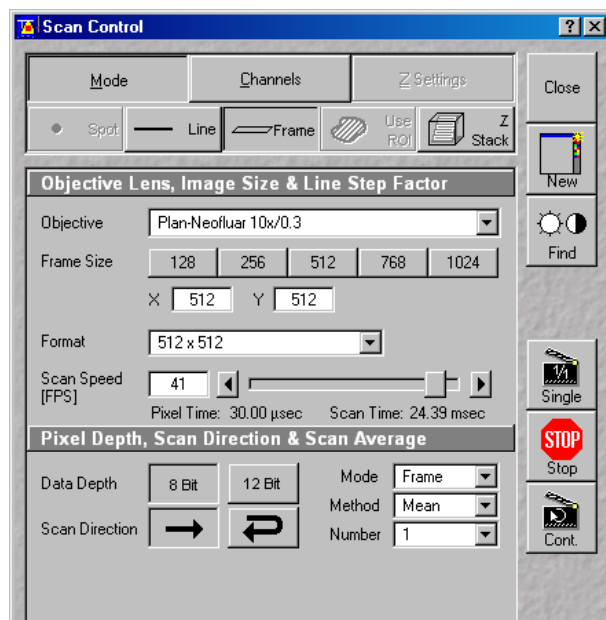


Fig. 15 Scan Control window, Mode settings



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.

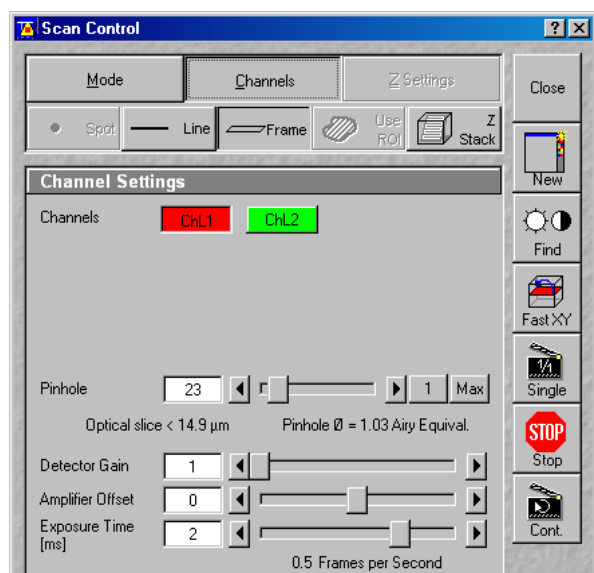


Fig. 16 Scan Control window, Channel settings

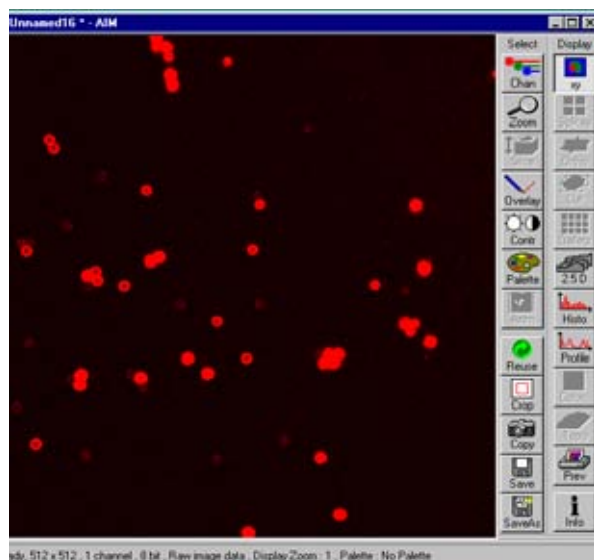


Fig. 17 Image window

## 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. 18).

The **Color Palette** window appears.

- In the **Color Palette List** panel, click on the **Range Indicator** item (Fig. 19).

The scanned image appears in a false-color presentation (Fig. 18).

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. 20).
- Set the **Detector Gain** slider to a middle position.
- When the image is saturated, reduce AOTF transmission in the **Excitation** panel (Fig. 20) 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. 20).
- Reduce or increase the **Detector Gain** until the red pixels only just disappear.

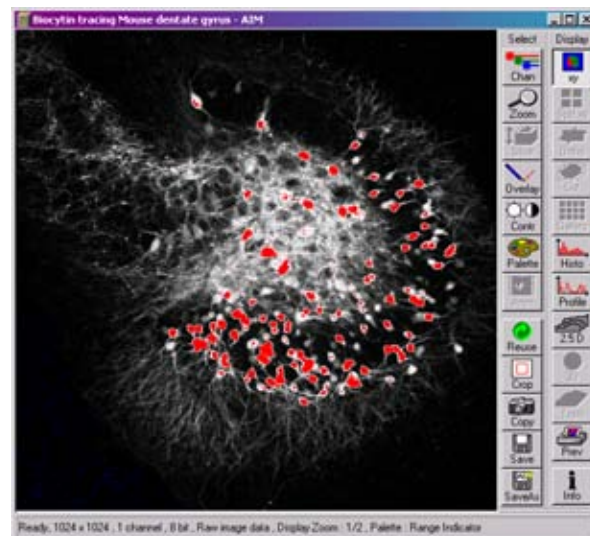


Fig. 18 Image window

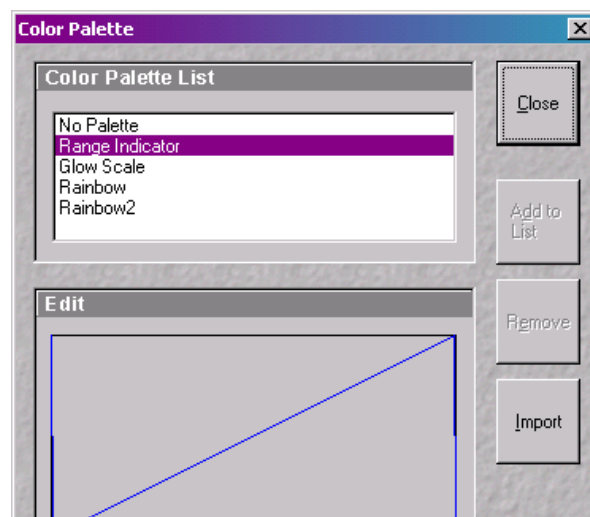


Fig. 19 Color Palette window

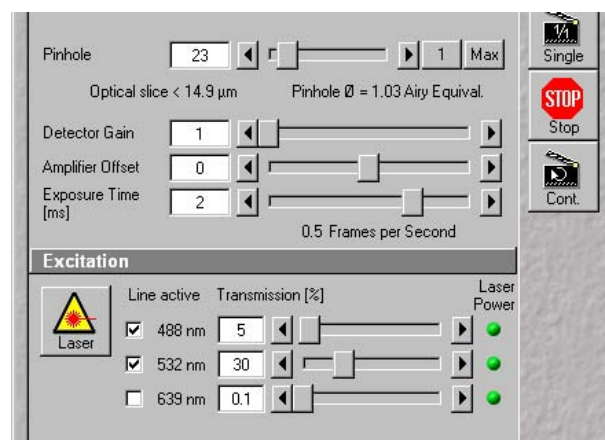


Fig. 20 Scan Control window, Channel settings

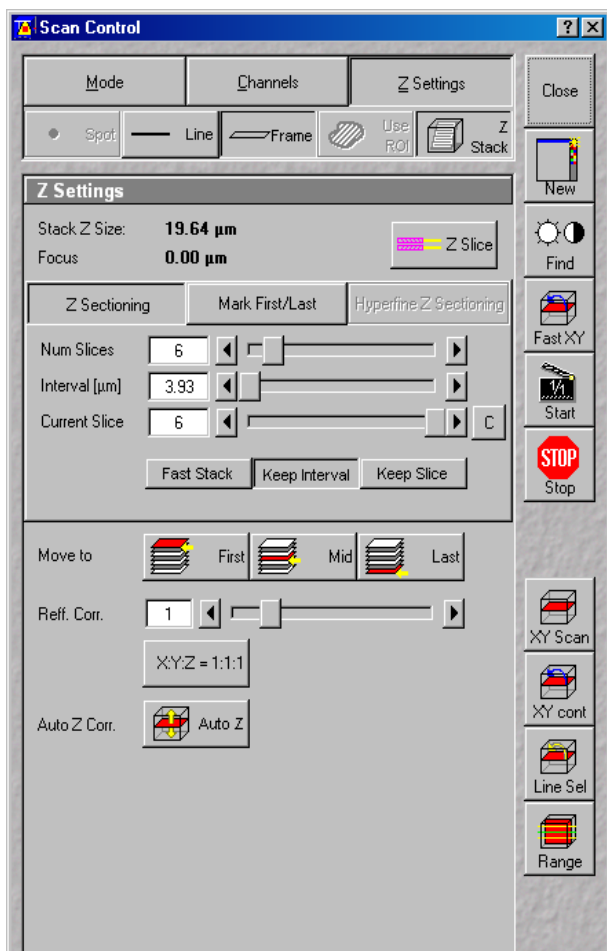


Fig. 21 Scan Control window, Z Stack settings

## 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.

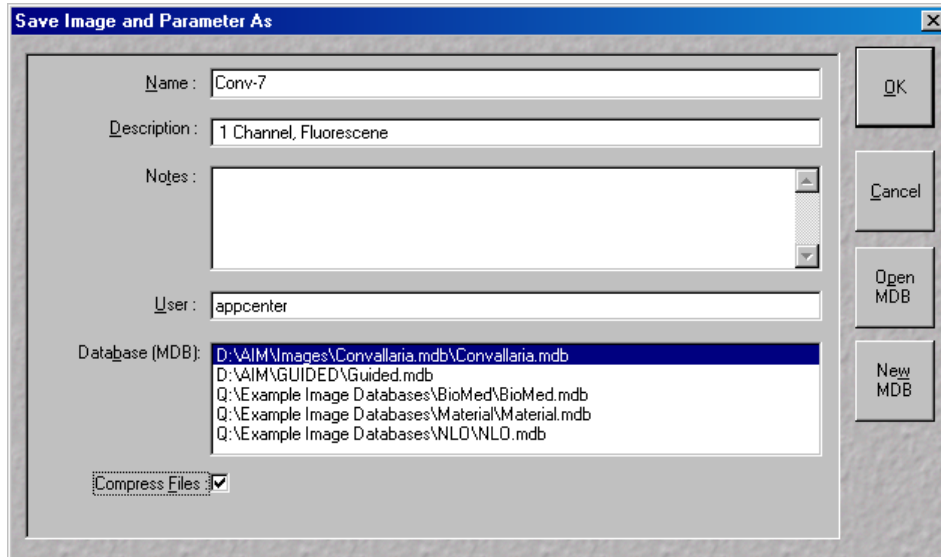
- Use the focusing drive of the microscope to focus on the upper position of the specimen area where the Z Stack is to start.
- Click on the **Mark First** button to set the upper position of the Z Stack.
- Then focus on the lower specimen area where the recording of the Z Stack is to end.
- Click on 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. 22** 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).
- Shut down the computer.
- Put the **LASER** key switch to **OFF** position. Remove the key from the system rack.
- Put the **COMPONENTS** switch and the **System/PC** switch to **OFF** position (Fig. 1).
- Put the **MAIN SWITCH** on the system rack to **OFF** position (Fig. 1).
- Switch off the HBO 100 mercury lamp (Fig. 2).

