Operating Manual
ConfoCor 3
Release 4.0

March 2006

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

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

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CHAPTER 1 NOTES ON DEVICE SAFETY

CONTENTS

1 NOTES ON DEVICE SAFETY

Information on safety issues for the ConfoCor 3 that deviate from those for the LSM 510 and LSM 510 META replace the latter. Apart from that all other information on the safety for the LSM 510 and LSM 510 META remain unrestricted valid.

1.1 General

The LSM 510 and LSM 510 META laser scanning microscopes as well as the ConfoCor 3, including their original accessories and compatible accessories from other manufacturers, may only be used for the purpose of microscopic and spectroscopic 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.

The ConfoCor 3 spectroscope is used for fluorescence correlation spectroscopy, whereas the beam is parked in a sample, which might consist of a solution or a cell, fluorescence fluctuations recorded and analyzed by the so-called correlation function.

☞ Installation and commissioning of the LSM 510, LSM 510 META and the ConfoCor 3 systems must be performed by authorized Carl Zeiss service staff. The system should not be used prior to instruction by a Carl Zeiss representative.

The manufacturer will not assume liability for any malfunction or damage caused by anything other than the intended use of the LSM 510, LSM 510 META or ConfoCor 3 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 LSM 510, the LSM 510 META, the microscope, the HBO, the HAL and additional optional devices, if ordered, as the UV Laser, the piezo focusing device, the heating inserts and the Ti:Sa Laser.

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

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

1.2 Warning and Information Labels

The warning and information labels attached on the LSM 510, LSM 510 META and ConfoCor 3 must be observed. Check whether all of the labels shown below are provided on your instrument, and contact Carl Zeiss Germany or one of the service agencies if you should discover that any of the labels should be missing. You will receive a free replacement.

Description of labels

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

Attention: Laser irradiation hazards possible when operating the system.

Attention: High voltage.

Pull the mains plug before opening the device housing.

Caution: Hot surface.

Caution: UV radiation.

Caution: Fingers can be caught.

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

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

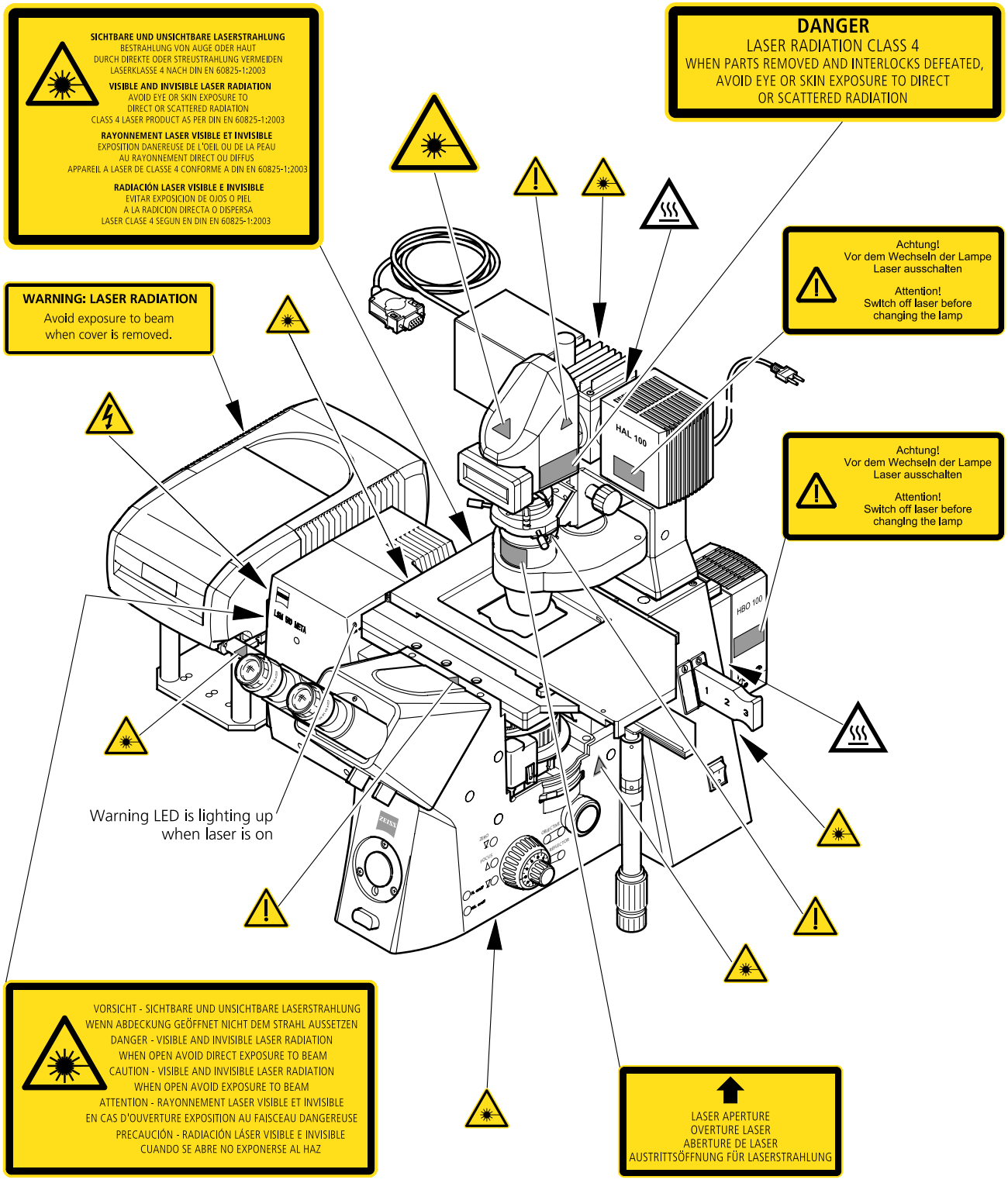


Fig. 1-1 Warning and information labels on the Axiovert 200 M microscope with the LSM 510 META scanning module and the ConfoCor 3 detection module

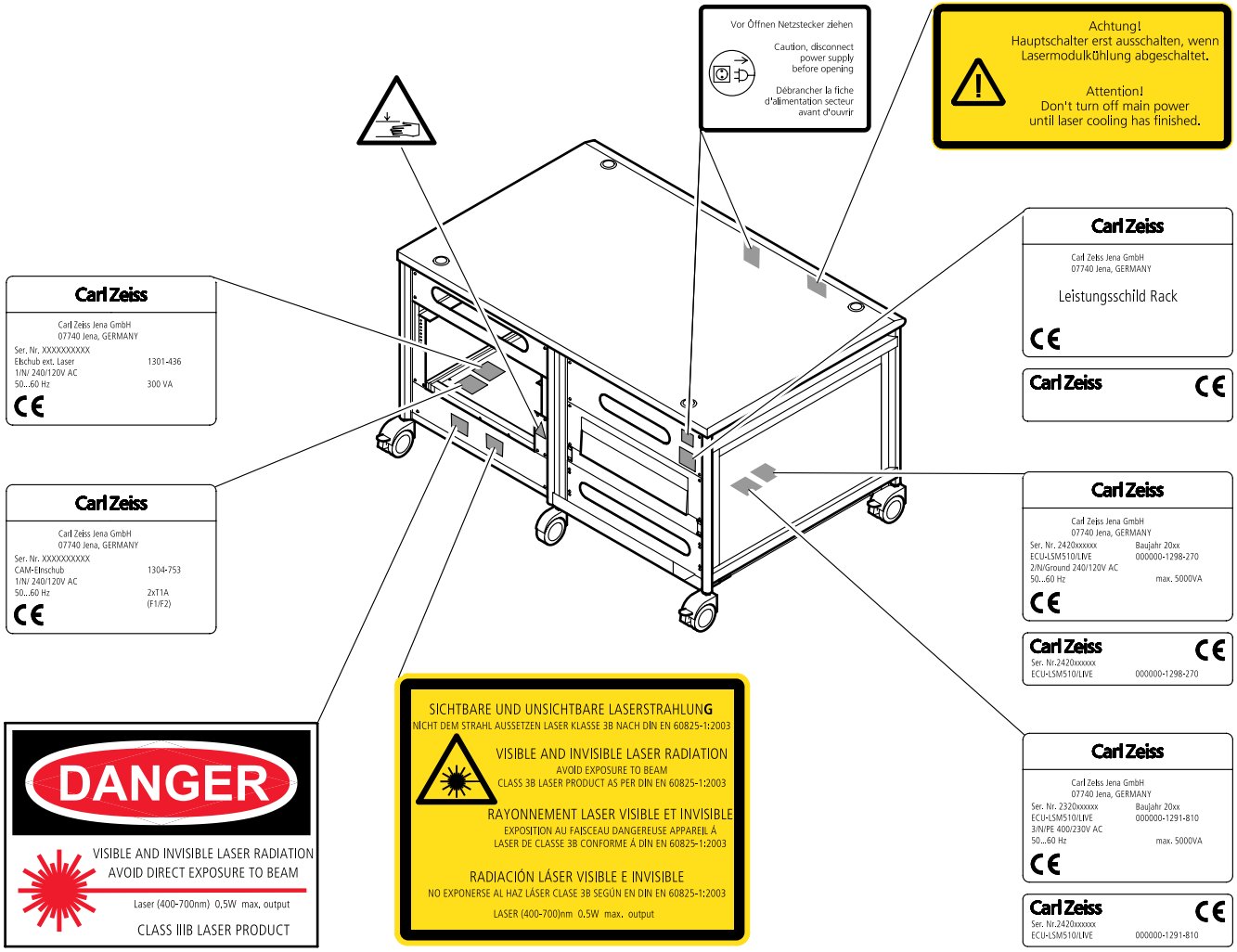
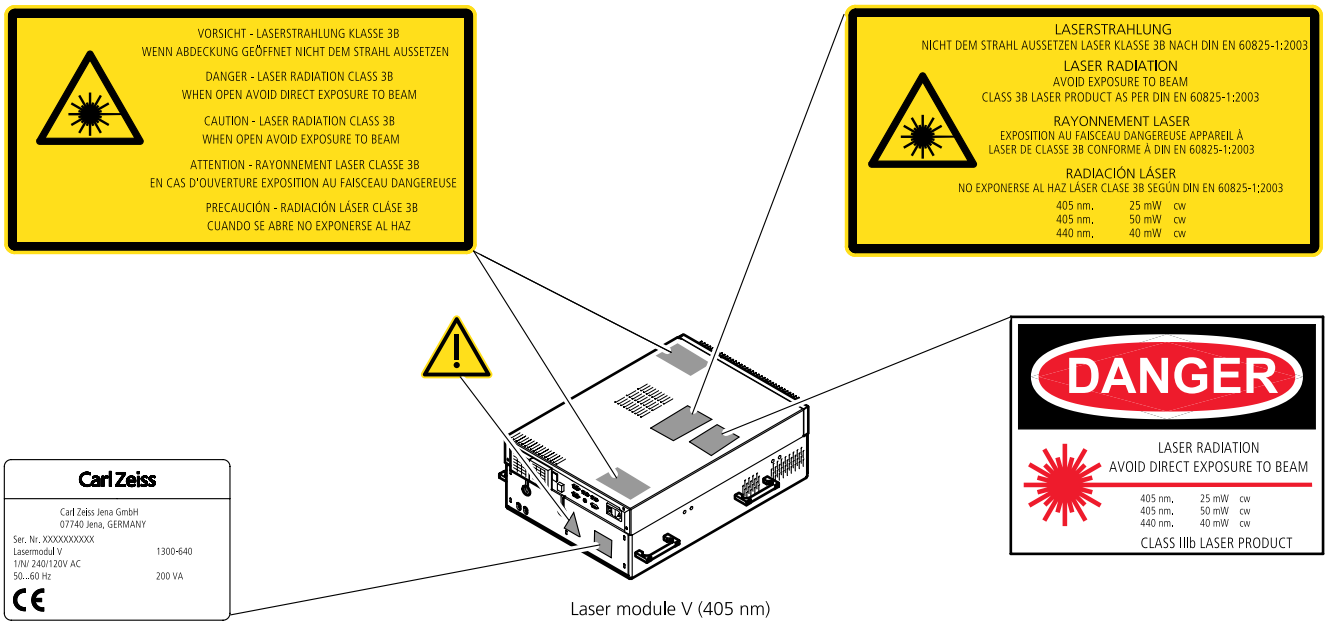
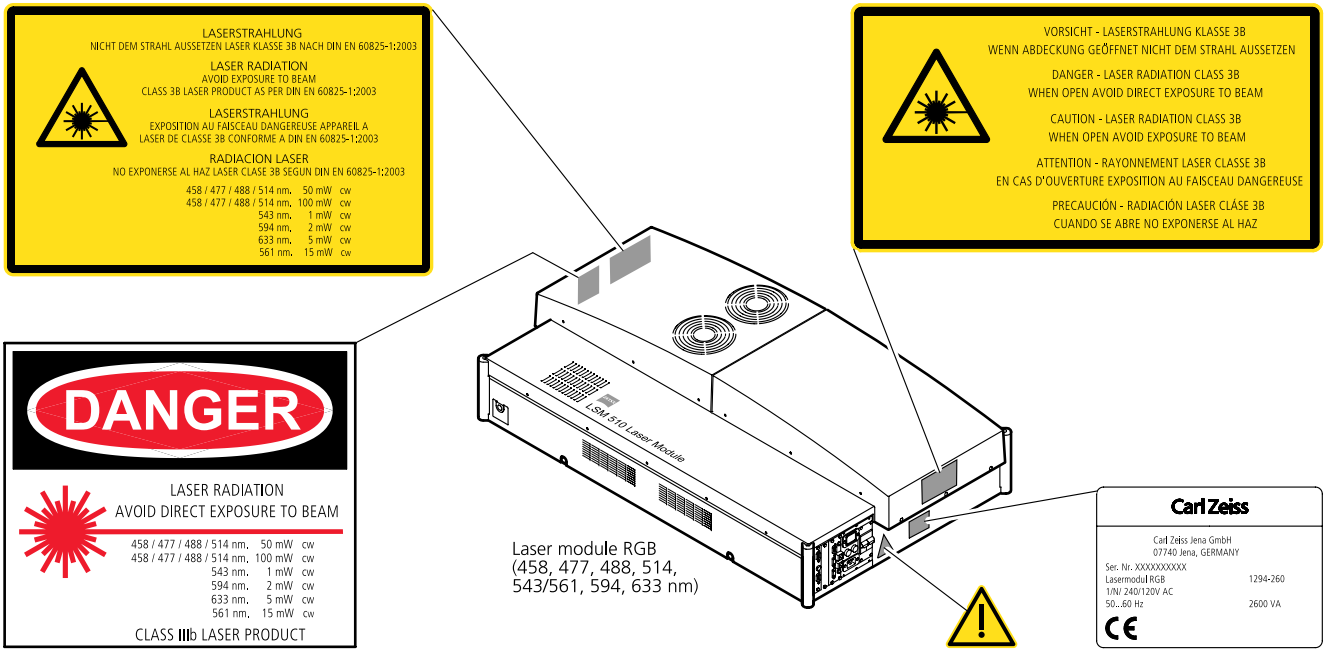


Fig. 1-2 Warning and information labels on the system electronic rack



Laser module V (405 nm)



Laser module RGB
(458, 477, 488, 514,
543/561, 594, 633 nm)

Fig. 1-3 Warning and information labels on laser components

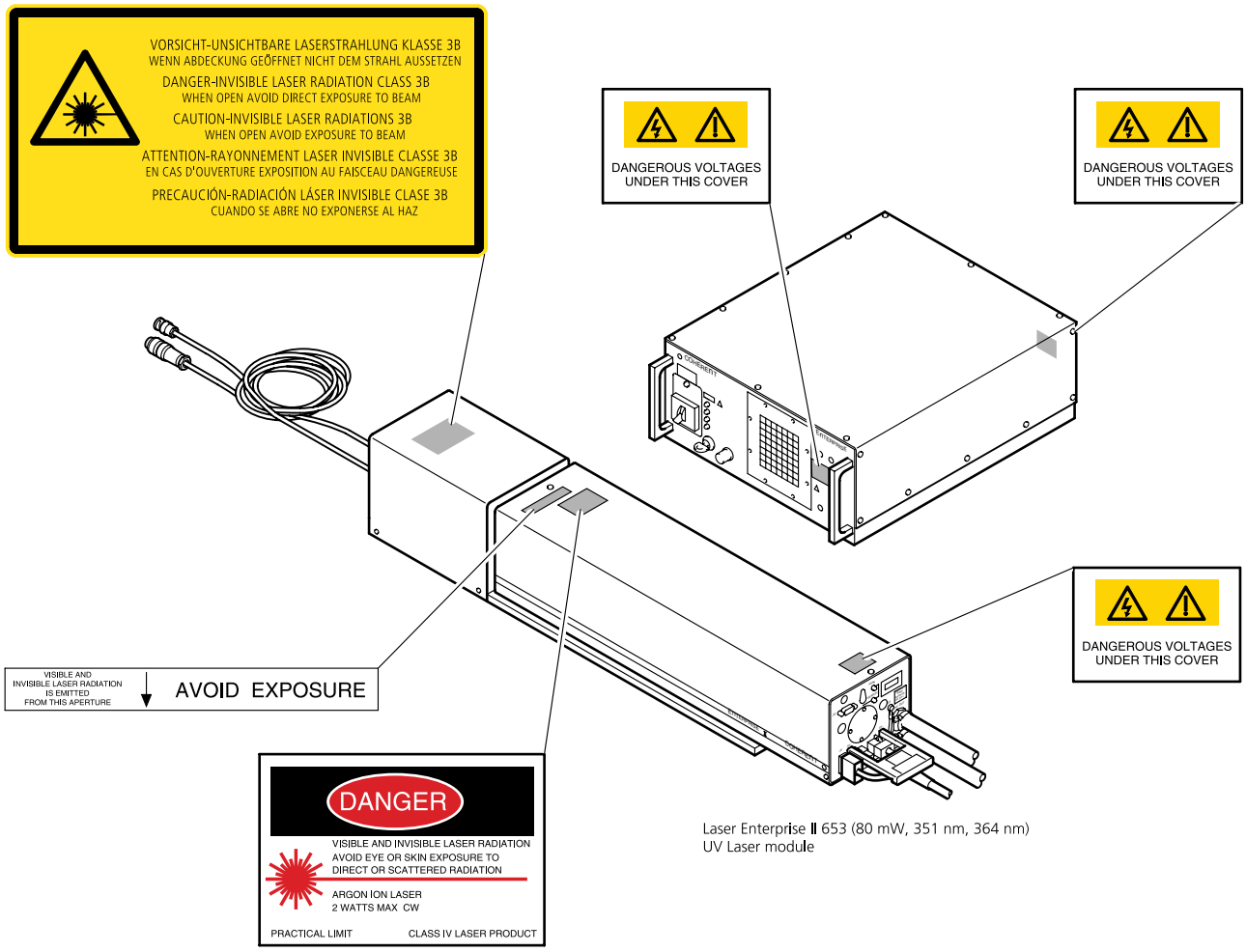


Fig. 1-4 Warning and information labels on laser components

1.3 Regulations

Extensive knowledge of the hardware/the system is indispensable for safe operation of the LSM 510, LSM 510 META and the ConfoCor 3.

☞ Read these operating instructions and all device publications belonging to the system conscientiously **before** operating the LSM 510, LSM 510 META or the ConfoCor 3! You can obtain additional information on the hardware configuration delivered and on optional system extensions from the manufacturer or via the service hotline.

⇒ The LSM 510, LSM 510 META and ConfoCor 3 have been designed, built and tested in conformity with the following regulations and guidelines:

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

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

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

Low voltage directive: 73/23/EWG

EMC directive: 89/336/EWG

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

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

The products and their 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 products.

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

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

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

1.4 Notes on Setting up the Microscope and Spectroscope Systems

☞ Installation and commissioning of the LSM 510, LSM 510 META and the ConfoCor 3 systems must be performed by authorized Carl Zeiss service staff. The system should not be used prior to instruction by a Carl Zeiss representative.

The LSM 510, LSM 510 META laser scanning microscope and the ConfoCor 3 spectroscope are delivered in several crates.

⚠ The LSM 510, LSM 510 META and ConfoCor 3 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.

☞ Please note the following for the ConfoCor 3:

- The ConfoCor3 requires an Axiovert 200 microscope and is attached to channel 4 of the LSM 510 (META). Note that only system tables with sufficient anti-vibration functionality should be used.
- A scanning stage is recommended for automatic positioning for solution measurements. For cell measurements the scanning mirrors are recommended.
- For FCS measurements with VIS and UV light the C-Apochromat 40 x water objective NA 1.2 is recommended. For measurements with NLO light the C-Apochromat 63 x water objective NA 1.2 is recommended.

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

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

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

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

Please note that the LSM 510, LSM 510 META and ConfoCor 3 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 V in and out of the System electronic rack take care not to catch your fingers.

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

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

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

To avoid vibrations during operation, the LSM 510, LSM 510 META and ConfoCor 3 should only be operated in conjunction with the system table (vibration damping).

1.5 Power Requirements

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

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

1.6 Notes on Handling the Laser Components

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

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

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

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

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

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

If used properly, the LSM 510, LSM 510 META and ConfoCor 3 will not pose any laser radiation risks for operating staff. Nevertheless, you should observe the following warnings:

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

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

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

1.7 Physical Dimensions

	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Large system table	150	80	78	100
Small system table	80	65	78	60
Passively damped anti-vibration table	130	100	75	137
Active anti-vibration table (NLO) for Mai Tai Laser or Chameleon	120	140	75	200
Active anti-vibration table (NLO) for Verdi Mira or Millenia Tsunami Laser	180	140	75	400
Scanning Module LSM 510	25	20	25	15
Scanning Module LSM 510 META	28	27	30.5	13
Module ConfoCor 3	49	27	18	25
Microscope	50	35	50	20
Laser Module RGB	110	70	28	95
Laser Module, UV	140	20	20	60
Laser Module V (405 nm)	66	52	22	30
Plug-in unit external laser	66	52	22	9
System Electronic Rack	110	70	58	90
Power supply for Ar (UV)	50	50	30	30
Cooling unit for Ar (UV)	80	45	50	30

1.8 Environmental Requirements

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

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

1.9 Notes on Handling the Computer and Data Media

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

☞ Do make sure, though, that you receive your LSM-ConfoCor 3 system with the operating system installed, with initialization and start files set up and with the LSM-ConfoCor program also installed.

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

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

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

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

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

Backup your data on a regular basis.

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

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

1.10 Notes on Care, Maintenance and Service

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

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

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

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

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

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

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

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

1.11 User Interface

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

- ConfoCor 3 filter wheels
- ConfoCor 3 external connectors

The ConfoCor 3 module must not be detached from the LSM 510 or LSM 510 META scan modules!

1.11.1 Changing Filter Wheels in the ConfoCor 3

By opening the lid (see Fig. 1-5 and Fig. 1-6) you have access to all the filter wheels in the ConfoCor 3.

You can remove and replace filter wheels by push and click.

Be careful not to disrupt any connectors in doing this.

Please note that the data base has to be exchanged if filter wheels are replaced with another filter set.

When opening the lid, the safety regulations will switch off the laser light.

Do not remove the ConfoCor 3 detection head from its attachment to the LSM 510 or LSM 510 META.

Laser light can escape the system through the external port used as the attachment site for the ConfoCor 3 that can lead to bodily damage.

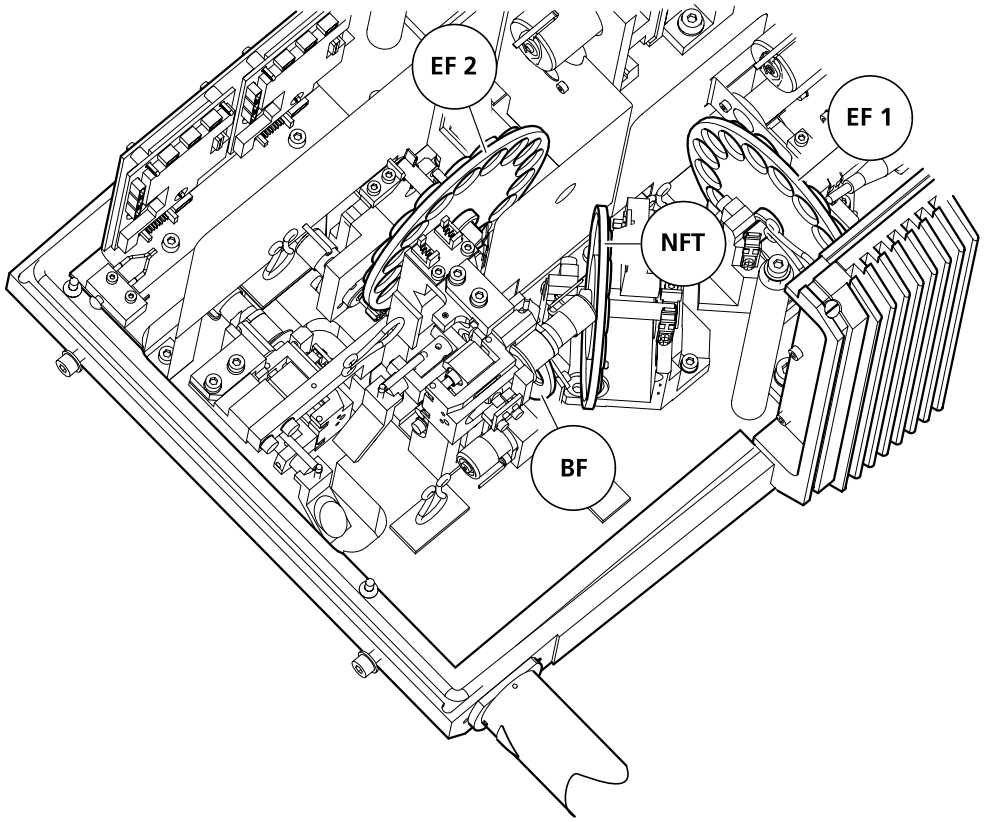


Fig. 1-5 **Top view APD module. The location of exchangeable filter wheels are indicated (BF = block filter; NFT = secondary beam splitter; EF 1 = emission filter 1; EF-2 = emission filter 2)**

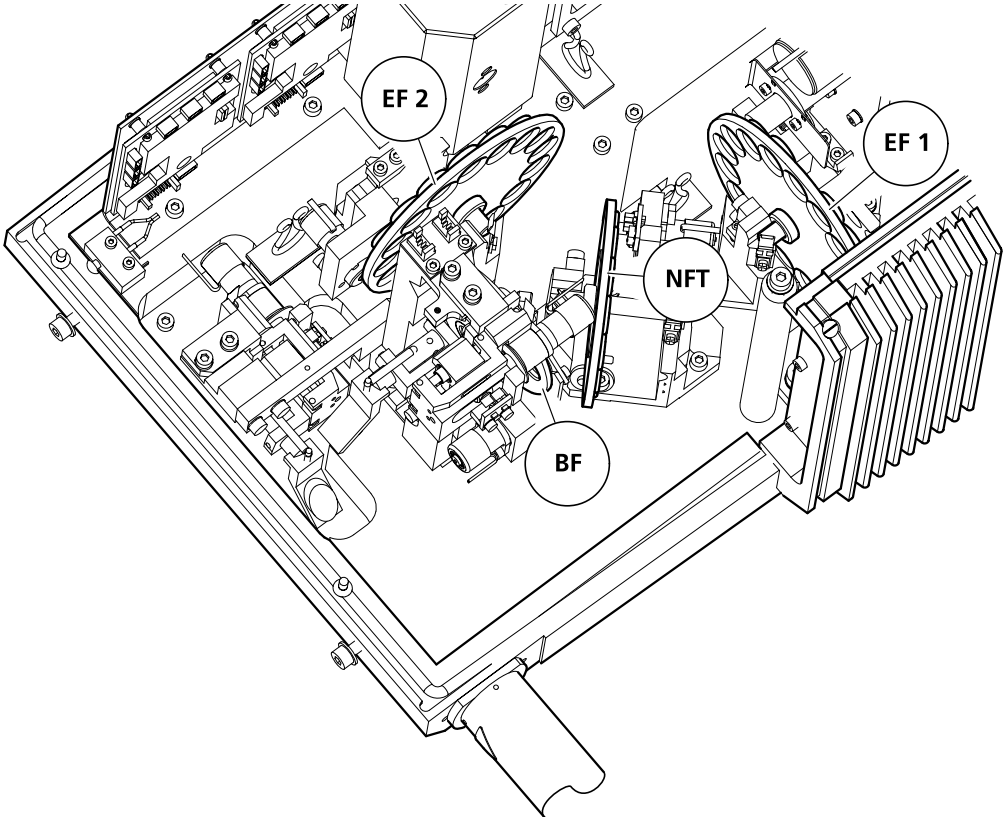


Fig. 1-6 **Top view GaAsP module. The location of exchangeable filter wheels are indicated (BF = block filter; NFT = secondary beam splitter; EF 1 = emission filter 1; EF-2 = emission filter 2)**

1.11.2 Using External Connectors

In the rear of the GaAsP and APD modules, you have external access to the TTL pulses of both channels delivered at the APD1 and APD2 plugs for the APD module (see Fig. 1-7) or at the FCS1 and FCS2 plugs of the GaAsP module (see Fig. 1-8).

In the GaAsP module you have also access to the NIM pulse at the FLIM1and FLIM2 plugs.

☞ Please note that either plug is of BNT type and BNT triax cables should used. If using BNC connectors and coax cables the signal quality can suffer dramatically.

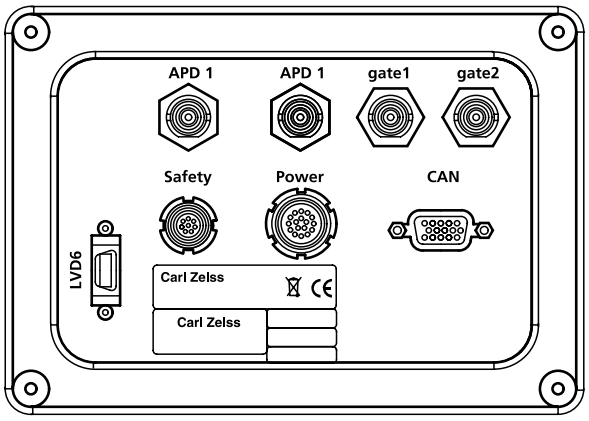


Fig. 1-7 Rear view APD module

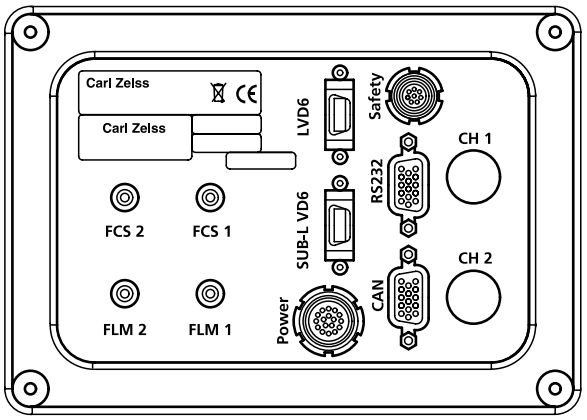


Fig. 1-8 Rear view GaAsP module

The assignment of the connectors is as follows:

Name	**APD**	**GaAsP**	**Meaning**
LVDS	14pin MDR	14pin MDR	Main communication link
Sub-LVDS		14pin MDR	Communication to optional module
Safety	8pin LEMO EXJ-1B	8pin LEMO EXJ-1B	Laser safety
Power	16pin LEMO ECJ-2B	16pin LEMO ECJ-2B	Power consumption: less than 20W
CAN	15pin HD Sub-D female	15pin HD Sub-D female	Optional CAN Port / Service Port
RS232		9pin Sub-D male	Service Port
APD 1 / 2	Triax BNT		Photon pulse- level: 2,5V@50Ω, 30 nsek long
FCS 1 / 2		Triax LEMO ERN-00	Photon pulse- level: 2,3V@50Ω, 25 nsek long
Gate 1 / 2	Triax BNT		Gate APD- TTL level
FLIM 1 / 2		Triax LEMO ERN-00	Double amplified PMT signal (up to –2V)
CH 1 / 2		4pin Binder (not implemented)	No connector

To connect the ConfoCor 3 to an optional correlator card, use the connectors APD 1 /2 or FCS 1 / 2, respectively. As long as the maximum count rate is not reached, each TTL Pulse corresponds to one photon. Take care to use 50Ω termination to avoid reflections and use double shielded 'triax' cable to avoid heavy distortions of the signal. To connect to BNC equipment, connect inner shield to ground and leave outer shield open.

For gating the APD use TTL signals.

To connect the ConfoCor 3 to an optional FLIM card, use the connectors FLIM 1 / 2 or APD 1 / 2 respectively. APD 1 / 2 delivers directly the APD signal (jitter 350psec), whereas FLIM 1 / 2 delivers an unshaped and amplified negative pulse of the PMT signal ranging from -0,9V of up to -2V (jitter 280psec).

CHAPTER 2 SETUP REQUIREMENTS

CONTENTS

2 SETUP REQUIREMENTS

2.1 Space Requirements

2.1.1 ConfoCor 3 and LSM on large system table: 2 m × 3,50 m

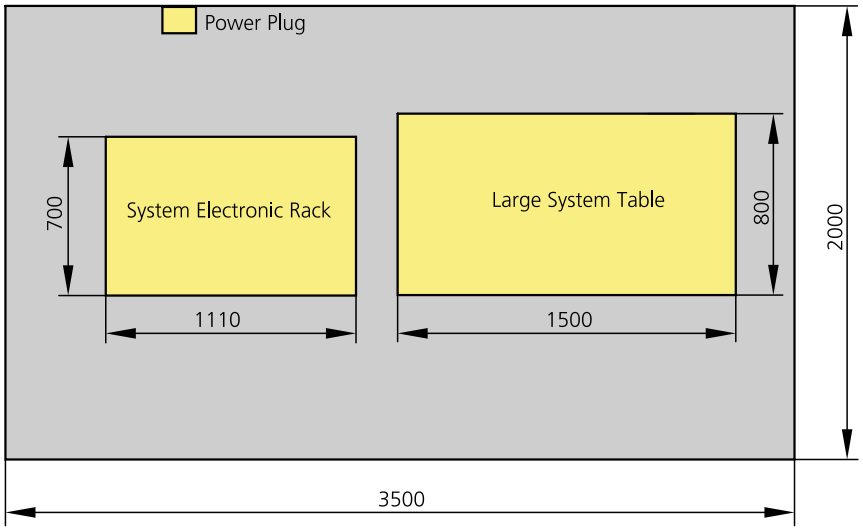


Fig. 2-1 Space requirements for ConfoCor 3 and LSM on large system table (measurements in mm)

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

2.1.2 ConfoCor 3 and LSM on small system table: 2 m x 3,5 m

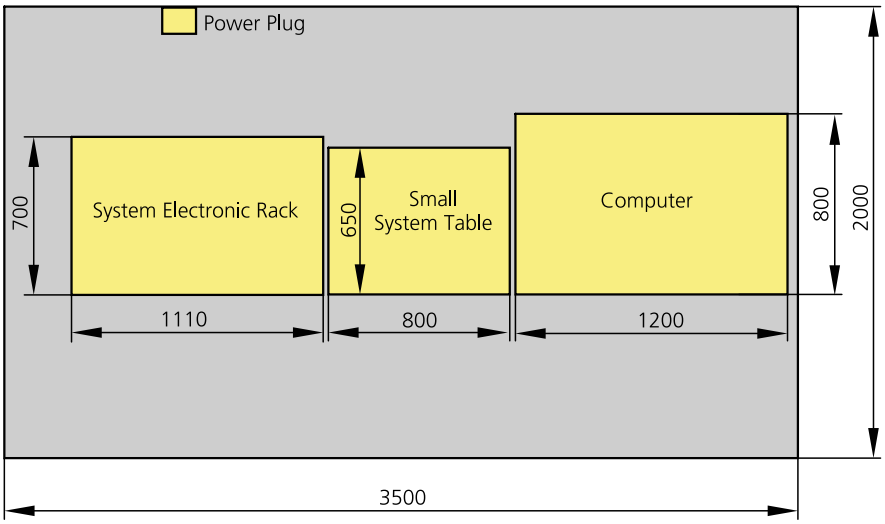


Fig. 2-2 Space requirements for ConfoCor 3 and LSM on small system table (measurements in mm)

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

2.1.3 ConfoCor 3 and LSM on passive anti vibration table: 2,2 m x 4,2 m

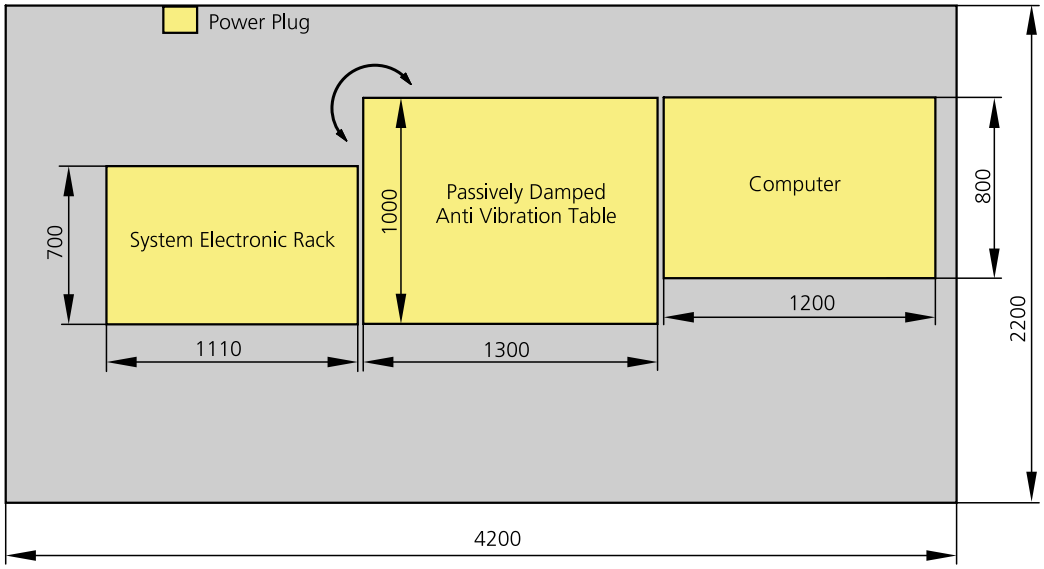


Fig. 2-3 Space requirements for ConfoCor 3 and LSM on Passively Damped Anti Vibration Table (system table with breadboard).
Depending on the ordered table it might be turned by 90°.
(measurements in mm)

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

2.1.4 ConfoCor 3, LSM and 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

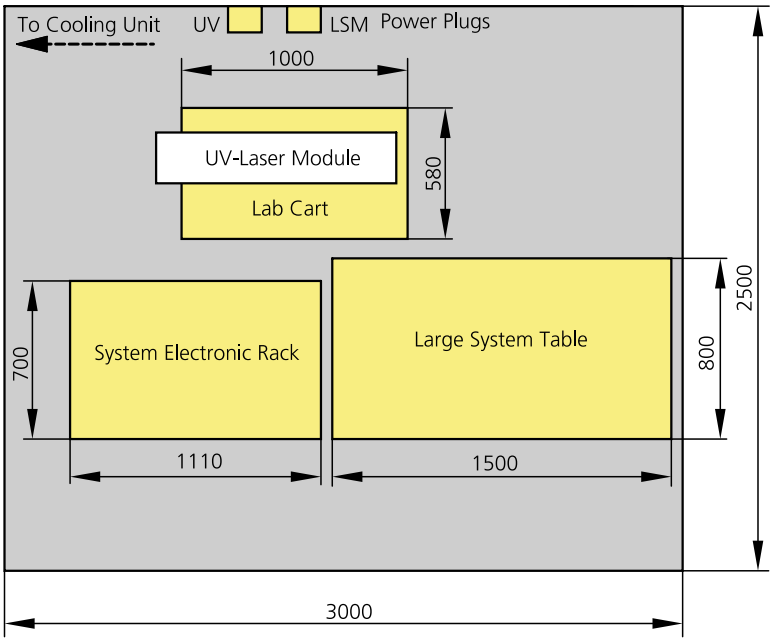


Fig. 2-4 Space requirements for ConfoCor 3, LSM and AR UV Laser (measurements in mm)

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

2.1.5 ConfoCor 3 and LSM prepared for Two Photon Laser (NLO)

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

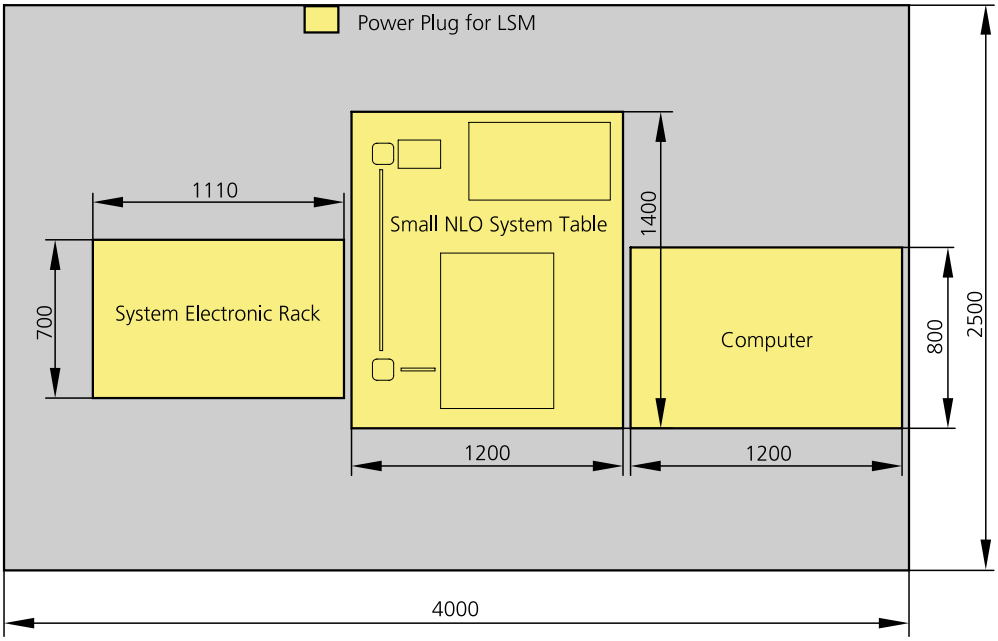


Fig. 2-5 Space requirements for ConfoCor 3, LSM and Two Photon Laser and Small NLO System Table (measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the system, the power supply for the microscope, the scanning unit and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack. Power supply and cooling unit of the NLO Laser can be stored under the system table. The electronics for the NLO laser have to be set aside the System Electronic Rack. An additional lab cart (000000-0465-515) is recommended. The NLO laser incoupling unit will be placed will be conducted below the ConfoCor 3 unit.

2.1.5.2 Coherent “Verdi Mira” or Spectra Physics “Millenia Tsunami”. Directly-coupled to inverted Microscope: 3,5 m x 3,5 m

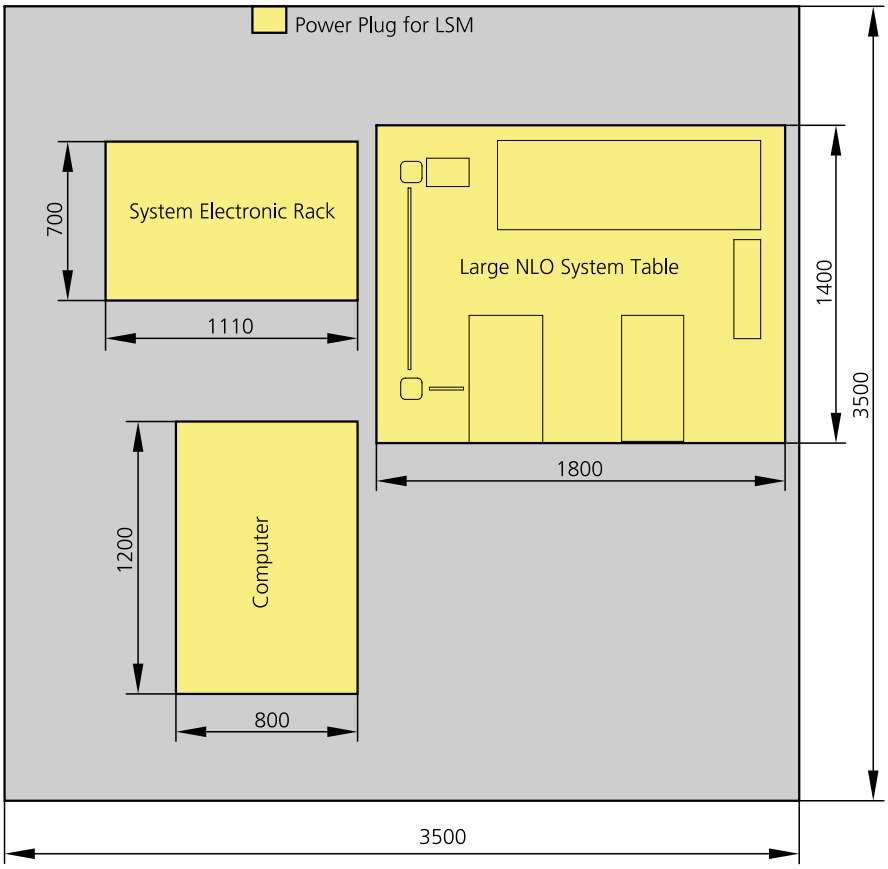


Fig. 2-6 Space requirements for Two Photon Laser and Large NLO System Table. Set up of ConfoCor 3, LSM and inverted microscope (measurements in mm)

The System Electronic Rack contains all electronics for control of the hardware components of the system, the power supply for the microscope, the scanning unit and the Laser Module RGB equipped with the choice of lasers. The Laser Module RGB is set on top of the System Electronic Rack. Power supply and cooling unit of the NLO Laser can be stored under the system table. Coherent “Chameleon” or Spectra Physics “Mai Tai” lasers can also be used. The electronics for the NLO laser have to be set aside the System Electronic Rack. An additional lab cart (000000-0465-515) is recommended.

2.2 Power Requirements

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

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

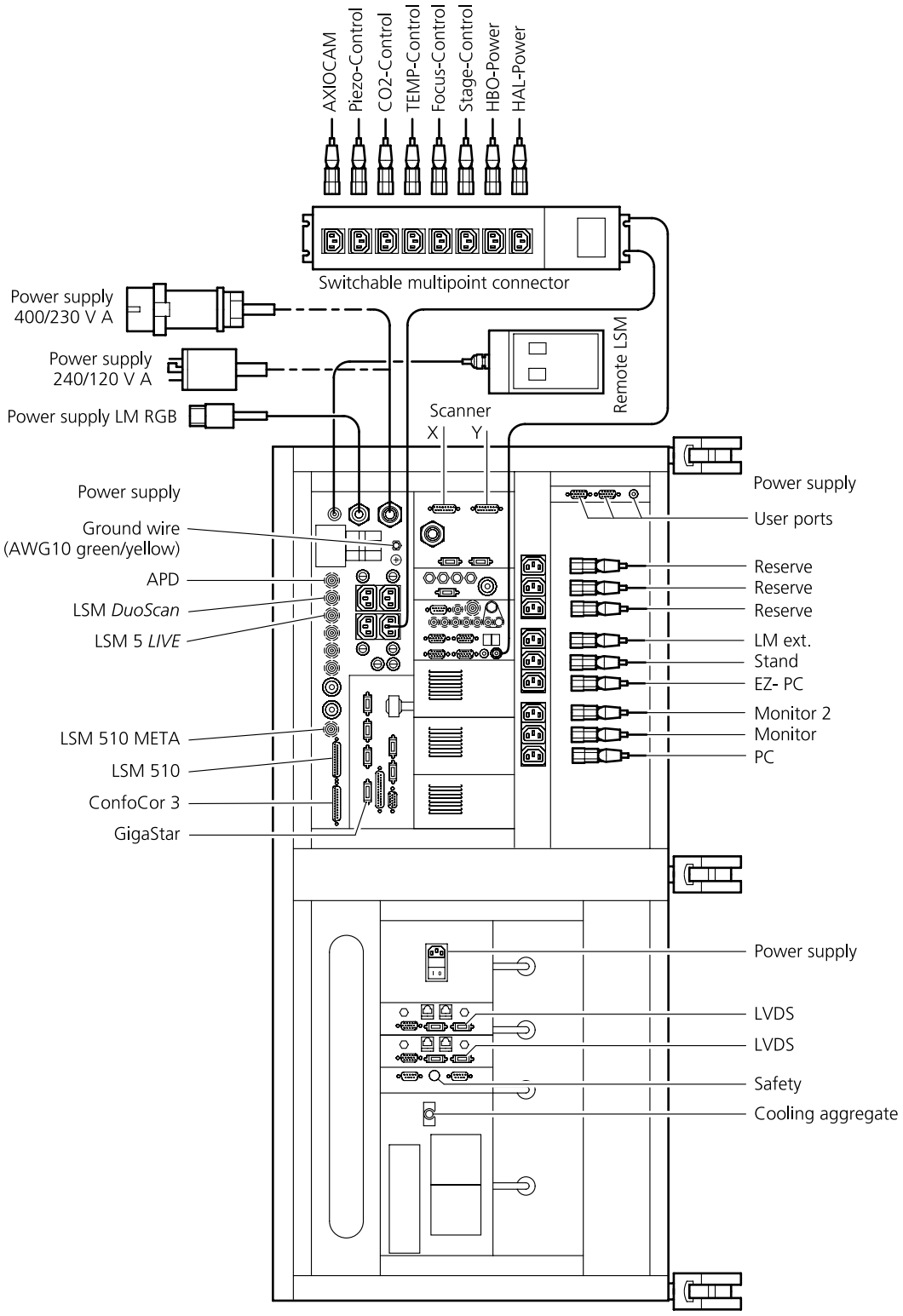


Fig. 2-7 **Power connector for LSM 510 / LSM 510 META and Laser Module V. Free/reserve outlets may be used to supply power to additional equipment. No more than 1 A can be provided by each outlet. (Scheme is turned 90° to real system.)**

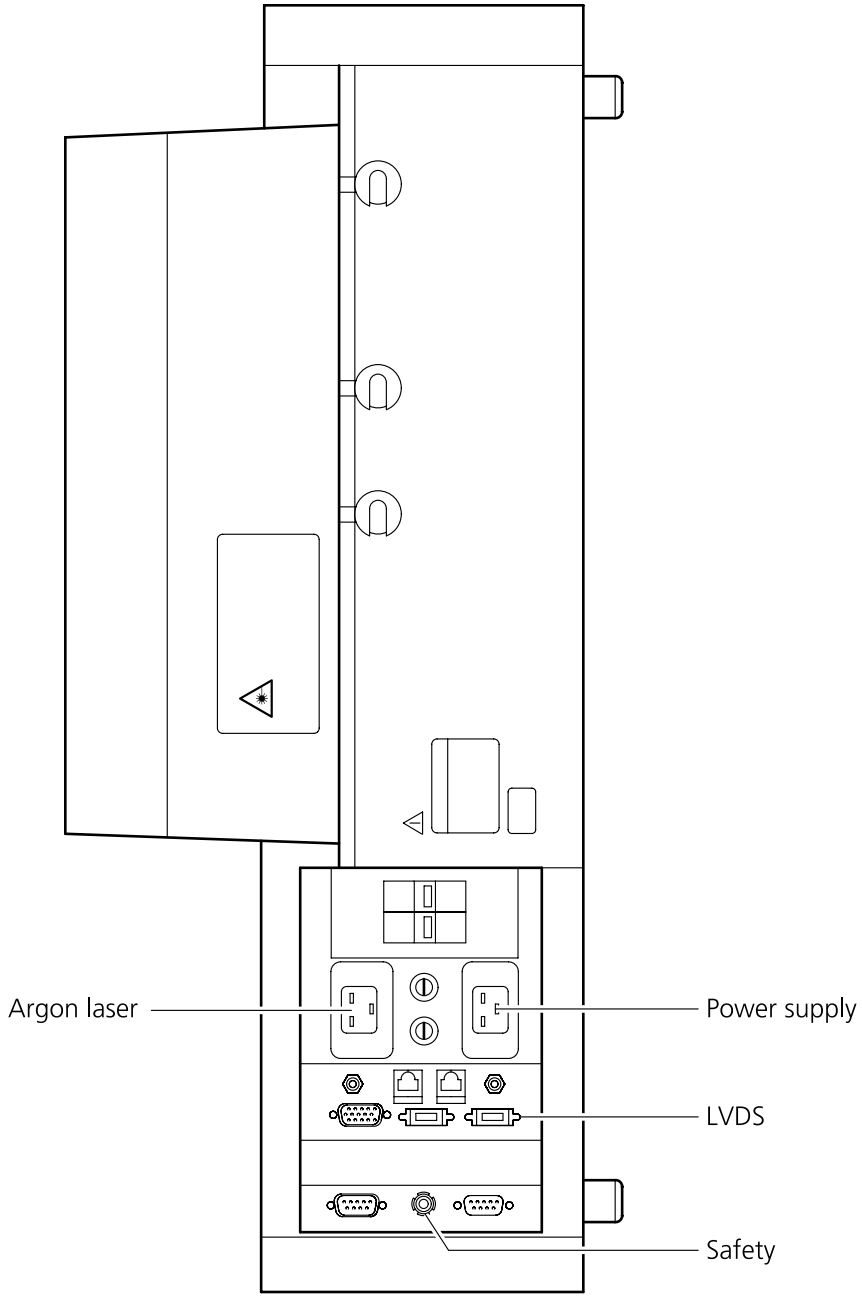
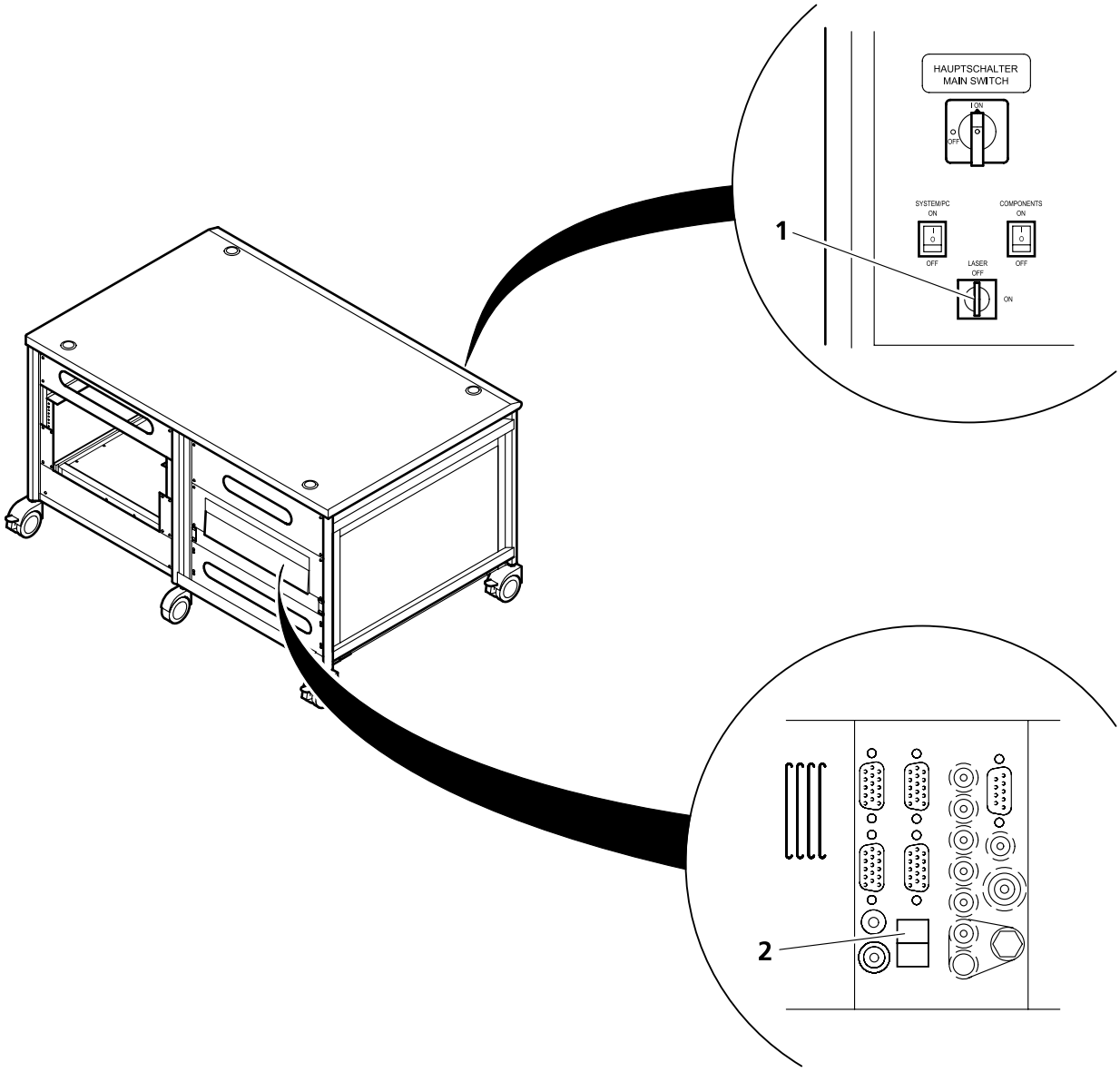


Fig. 2-8 Power connector for Laser Module RGB (Scheme is turned 90° to real system.)



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

Fig. 2-9 Key-interlock Laser ON/OFF and interface for connection of door interlock

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

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

Two door interlocks can be connected.

2.3 Physical Dimensions

	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Large system table	150	80	78	100
Small system table	80	65	78	60
Passively damped anti-vibration table	130	100	75	137
Active anti-vibration table (NLO) for Mai Tai Laser or Chameleon	120	140	75	200
Active anti-vibration table (NLO) for Verdi Mira or Millenia Tsunami Laser	180	140	75	400
Scanning Module LSM 510	25	20	25	15
Scanning Module LSM 510 META	28	27	30.5	13
Module ConfoCor 3	49	27	18	25
Microscope	50	35	50	20
Laser Module RGB	110	70	28	95
Laser Module, UV	140	20	20	60
Laser Module V (405 nm)	66	52	22	30
Plug-in unit external laser	66	52	22	9
System Electronic Rack	110	70	58	90
Power supply for Ar (UV)	50	50	30	30
Cooling unit for Ar (UV)	80	45	50	30
Water hose for Ar (UV)	700			
Fiber optic cable, VIS(ible)	300			
Fiber optic cable, UV	300			
Fiber optic cables at NLO systems	400			
Cables	350			
SCSI cable	350			

2.4 Dimension of Shipment Crates

Crate containing	Length (cm)	Width (cm)	Height (cm)	Weight (kg)
Large system table	160	85	95	120
Small system table	90	75	80	80
Passively damped anti-vibration table	145	115	115	150
Active anti-vibration table (NLO) for Mai Tai Laser or Chameleon	145	160	110	330
Active anti-vibration table (NLO) for Mira or Tsunami Laser	200	160	110	460
System Electronic Rack and Laser module	135	90	100	300
LSM, Microscope, Computer	135	90	100	150
Module ConfoCor 3	67	42	31	27
Support ConfoCor 3	67	42	31	10
Upgrade package containing module and support ConfoCor 3	80	58	83	40
Additional Hardware Components	135	90	61	100
UV laser unit	125	55	50	100
UV cooling unit	120	60	90	50

2.5 Environmental Requirements

1. Operation, specified performance	T = 22 °C ± 3 °C without interruption (24 h a day independently whether system is operated or switched-off)
2. Operation, reduced performance	T = 10 °C to 30 °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 measure-ments ≥ 2 h
7. Relative humidity	< 65 % at 30 °C
8. Operation altitude	max. 2000 m
9. Loss of heat	4 kW

2.6 Vibrations

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

2.7 Microscopes

Inverted Axiovert 200 M BP or SP

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

Z motor

DC servomotor, opto-electronically coded

Least Z interval: 50 nm (Axiovert 200 M BP or SP)

Piezo Objective focus

Piezo-driven single objective drive

Max. travel 250 µm; resolution 10 nm

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

2.8 Scanning Module LSM 510 and LSM 510 META

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

2.9 Detection Module ConfoCor 3

Channels	a) 2 channels (APD) or b) 2 channels (GaAsP PMT)
Dynamic range	12-bit DAC for each detection channel
Pinholes	1 individual pinhole (channel 4 of LSM 510 or LSM 510 META) Computer controlled automatic alignment

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

Single-mode polarization preserving fiber

Laser beam attenuation for all lasers by VIS-AOTF

HeNe laser (543 nm, 1 mW)

HeNe laser (594 nm, 2 mW)

HeNe laser (633 nm, 5 mW)

DPSS laser (561 nm, 10 mW)

Ar laser (458, 477, 488, 514 nm, 30 mW)

2.11 Laser Module V (405 nm)

Single-mode polarization preserving fiber

Laser beam attenuation by UV-AOTF

Diode laser (405 nm, 30 mW)

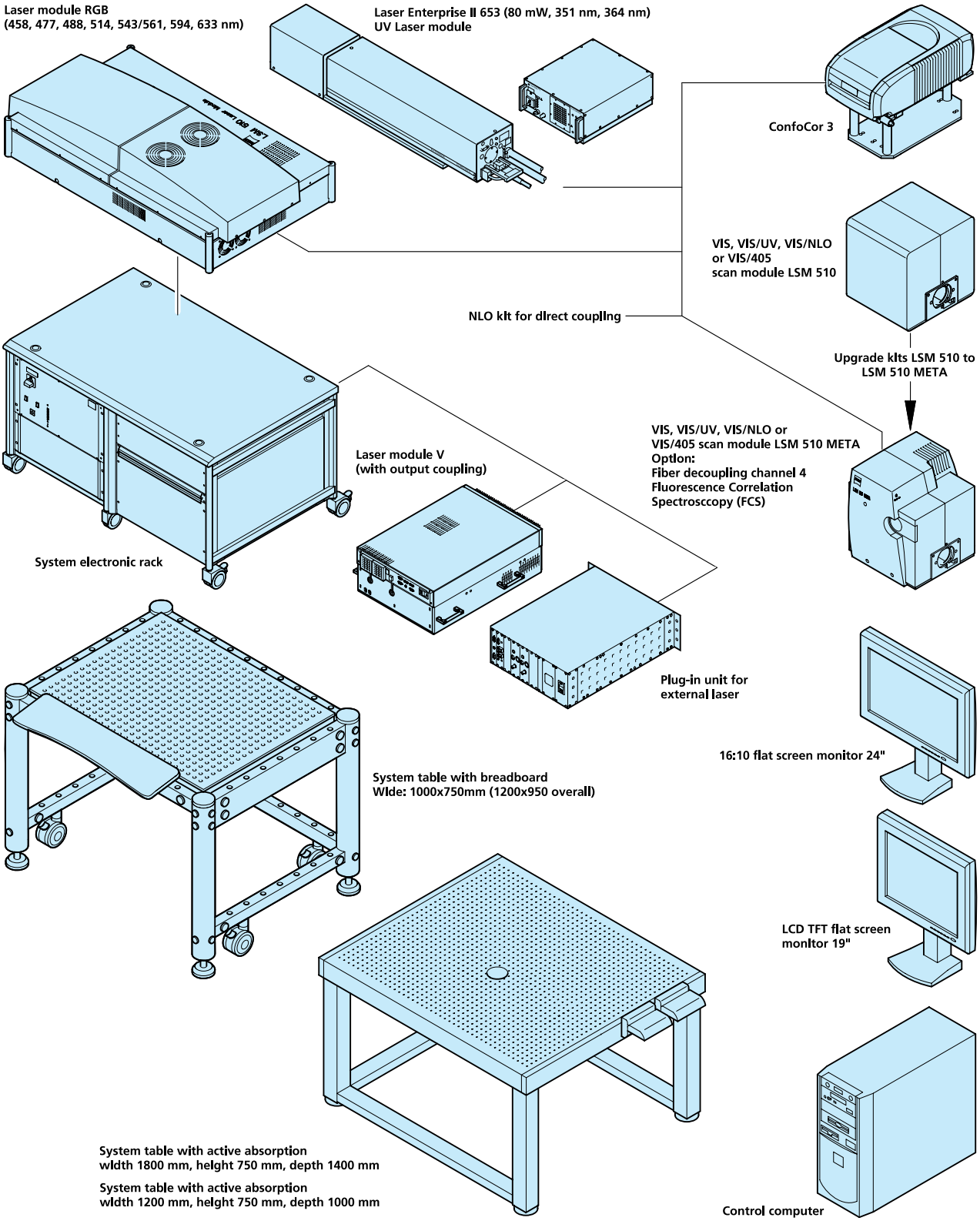
2.12 Laser Module UV (351, 364 nm)

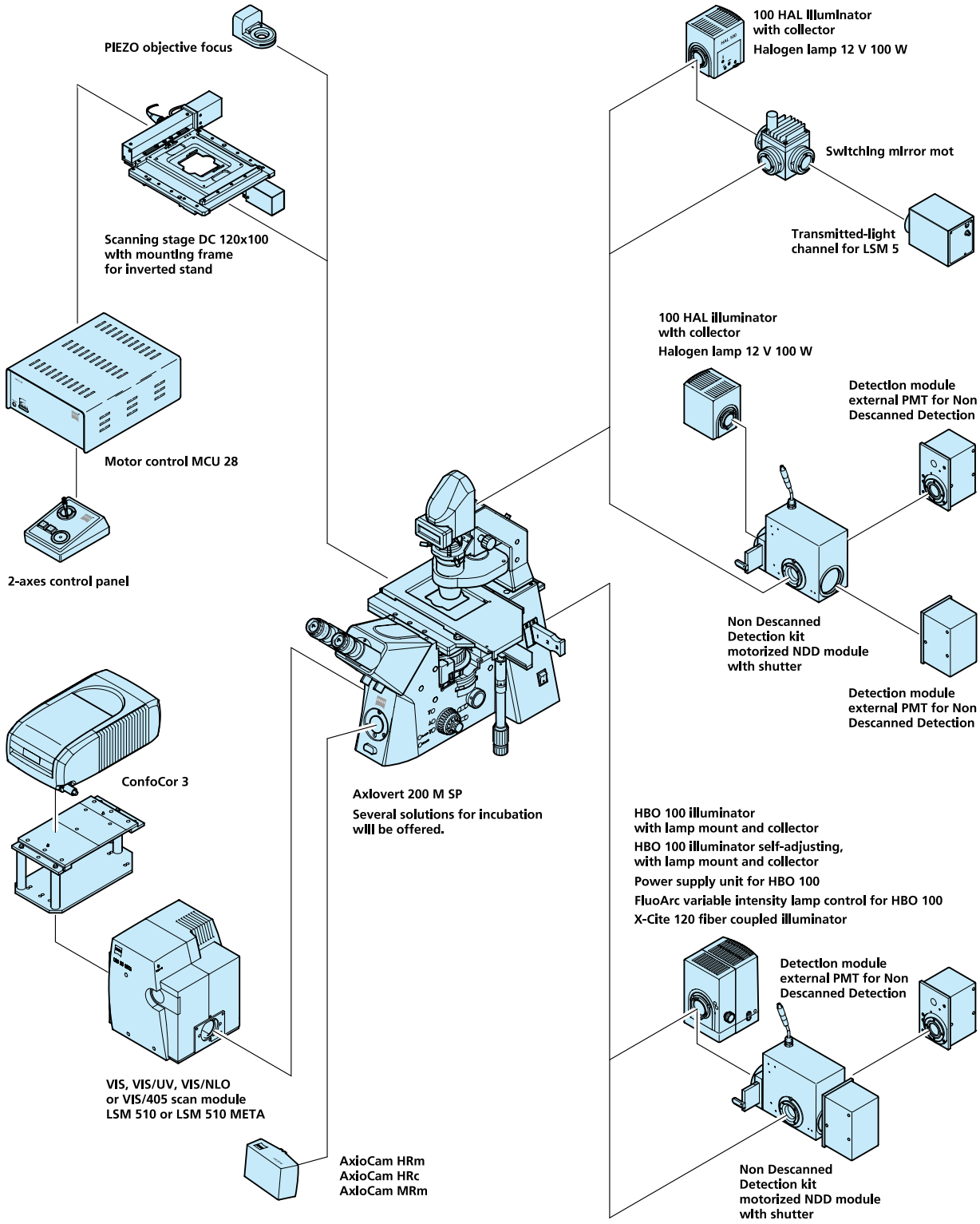
Single-mode polarization preserving fiber

Laser beam attenuation for all lasers by UV-AOTF

Ar laser (351, 364 nm, 80 mW)

2.13 System Overview ConfoCor 3 and LSM 510 / LSM 510 META




PIEZO objective focus
Scanning stage DC 120x100
with mounting frame
for inverted stand
Motor control MCU 28
2-axes control panel
ConfoCor 3
VIS, VIS/UV, VIS/NLO
or VIS/405 scan module
LSM 510 or LSM 510 META
AxioCam HRm
AxioCam HRc
AxioCam MRm
Axiovert 200 M SP
Several solutions for incubation
will be offered.
100 HAL Illuminator
with collector
Halogen lamp 12 V 100 W
Switching mirror mot
Transmitted-light
channel for LSM 5
100 HAL illuminator
with collector
Halogen lamp 12 V 100 W
Detection module
external PMT for Non
Descanned Detection
Non Descanned
Detection kit
motorized NDD module
with shutter
Detection module
external PMT for Non
Descanned Detection
HBO 100 illuminator
with lamp mount and collector
HBO 100 illuminator self-adjusting,
with lamp mount and collector
Power supply unit for HBO 100
FluoArc variable intensity lamp control for HBO 100
X-Cite 120 fiber coupled illuminator
Detection module
external PMT for Non
Descanned Detection
Non Descanned
Detection kit
motorized NDD module
with shutter

CHAPTER 3 INTRODUCTION TO LASER SCANNING MICROSCOPY AND FLUORESCENCE CORRELATION SPECTROSCOPY

CONTENTS

3 INTRODUCTION TO LASER SCANNING MICROSCOPY AND FLUORESCENCE CORRELATION SPECTROSCOPY

3.1 Principle of Laser Scanning Microscopy

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

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

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

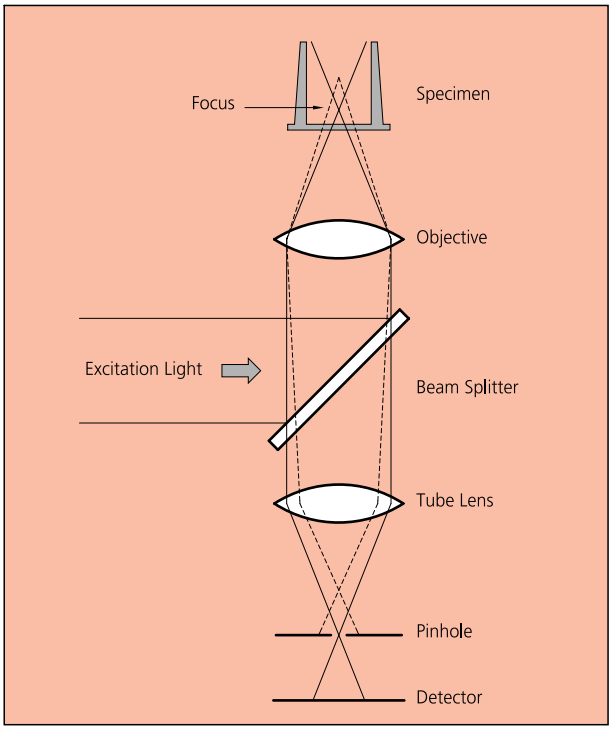


Fig 3-1 Principle of confocal imaging

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

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

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

3.2 Principle of Fluorescence Correlation Spectroscopy

Fluorescence Correlation Spectroscopy (FCS) analyzes the diffusion time of molecules and their differences if they have bound together. This is done by fluctuation analysis of fluorescence-labeled molecules within a well defined volume element. In most experiments, Brownian motion drives the fluctuation. The volume element is the confocal volume defined by the excitation spot of a well focused laser beam and the selected emission region defined by the properly aligned pinhole of the detection optics (see Fig 3-1).

As such, the setup is the same as for a Laser-Scanning Microscope (LSM), but in the latter we are not interested in the fluctuations but in the average intensity. As a matter of fact, what is the signal in FCS is noise in the LSM. Since the fluctuations are more pronounced, if less molecules are in the volume, FCS requires little molecule numbers (1-10). Whereas LSM is a scanning technique, FCS uses the beam is parked in one spot.

The fluctuations are analyzed by treating the measured photon counts with mathematical methods called correlation functions (see Fig. 3-2). The amplitude of the function is inverse proportional to the molecule number and the decay time gives the residence time of the molecule in the confocal volume and hence its diffusion time. If the two interacting molecules are of different size, only the smaller one has to be labeled using fluorescent dyes. This method is called auto-correlation. In this case the total auto-correlation is the sum of the two different species. If the diffusion constants of both partners are similar, they are both labeled with different dyes and cross-correlation is used. Often, photophysical processes like triplet states impinge on the correlation function, but can be accommodated in the model. Then the total correlation is the product of the single processes.

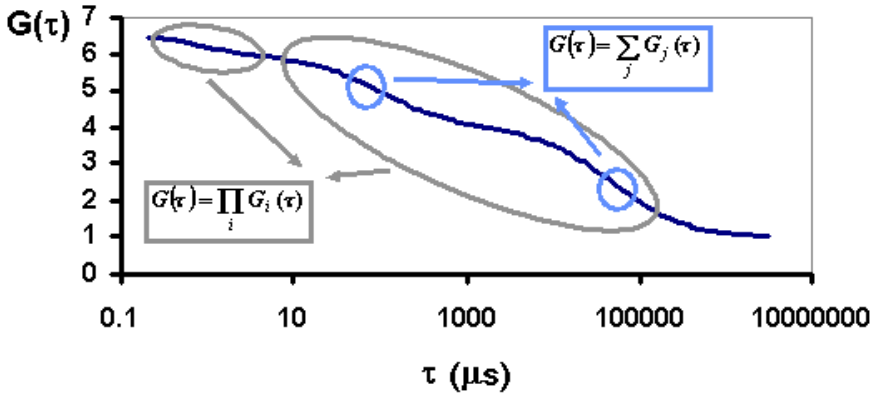


Fig. 3-2 Correlation functions

Figure 3-2 shows a correlation curve for a two component translational diffusion with triplet. The two components can be separated on behalf of their diffusion time (circles). Note that the contribution of the two components add up, whereas the contribution of the total diffusion process and the triplet multiply to obtain the total correlation.

Because of the tiny size of the confocal volume and its nature, the measurement can be carried out, in principle, in every area that is reachable by light and that is not smaller than an *Escherichia coli* bacterium (approximately 0.2 fl). In particular, measurements can be done inside living cells or on cell membranes. In order to be able to place the measurement volume at its proper place, it is advantageous to combine FCS with powerful light microscopy, particularly a confocal LSM.

3.3 Optical Diagram of the ConfoCor 3 and the LSM 510 and LSM 510 META (Schematic)

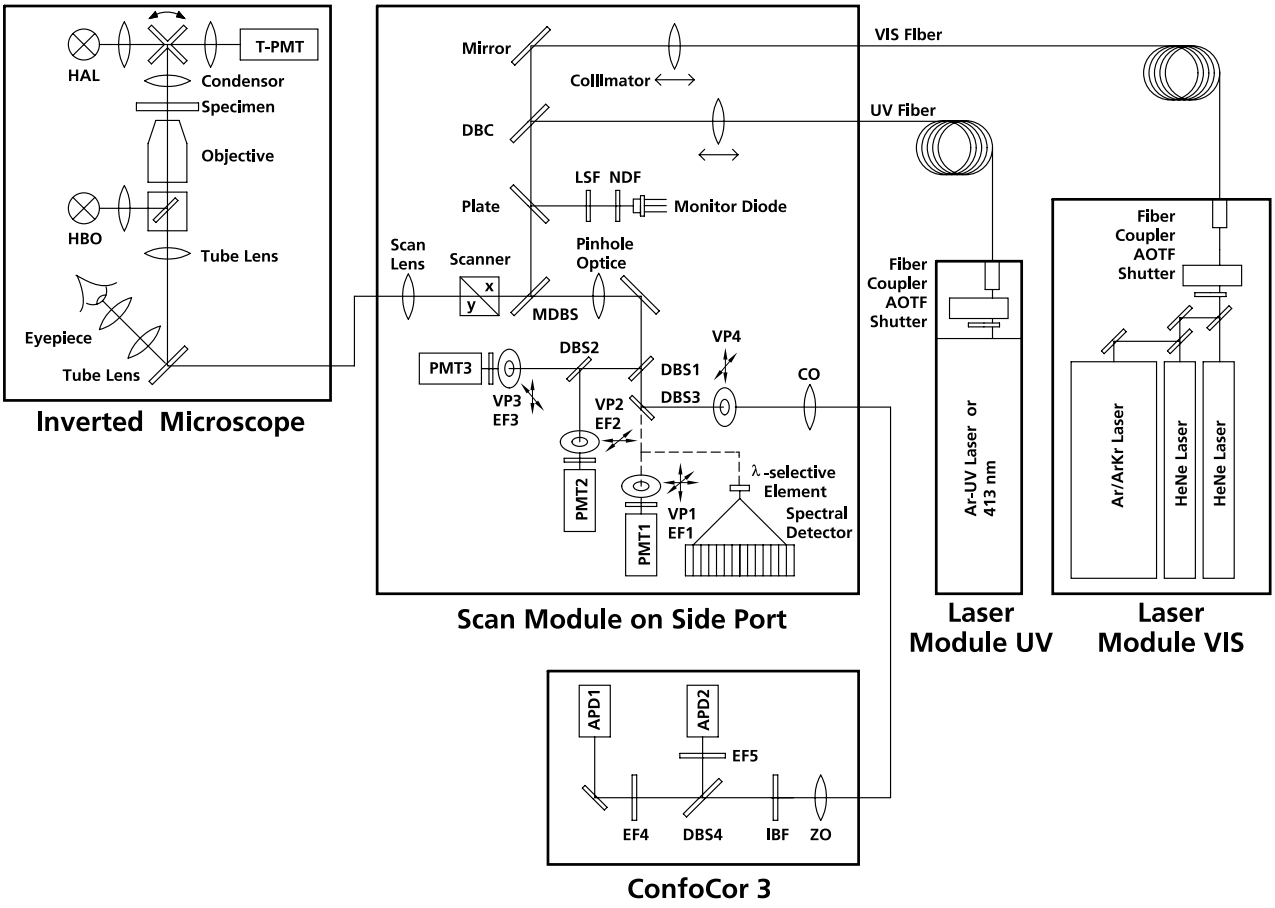


Fig. 3-3 Optical path, schematic (3-channel configuration)

AOTF	Acousto Optical Tunable Filter
APD	Avalanche photodiode
CO	Collimation Optics
DBC	Dichroic Beam Combiner
DBS	Dichroic Beam Splitter
EF	Emission Filter
HAL	Halogen Lamp
HBO	Mercury Vapor Short-Arc Lamp
IBF	Infrared Blocking Filter
LSF	Line Selection Filter
MDBS	Main Dichroic Beam Splitter
NDF	Neutral Density Filter
VP	Variable Pinhole
PMT	Photomultiplier
T-PMT	Transmission-Photomultiplier
ZO	Zoom Optics

The diagram above is a schematic representation of the LSM 510-ConfoCor 3 system.

Laser light is focused onto the specimen through an objective in a diffraction-limited mode. Light emitted at the focal plane and at planes below and above it is directed via an XY scanner onto a main dichroic beam splitter (MDBS), which separates the emissions from the excitation light. The fluorescences are separated from each other by a series of dichroic beam splitters (DBS1 ... maximally DBS4) and directed to individual photomultipliers (PMT1 ... maximally PMT3) or via Channel 4 into the ConfoCor 3 module. Here light passes though block filters for NLO light (if applicable), and is directed via a dichroic beam splitter (DBS 5) to avalanche photodiodes (APD1 ... maximally APD2) or GaAsP (GaAsP1 … maximally GaAsP2) detectors. The pinholes (VP) block all out of focus light, whereas emission filters (EF) are used to select a specific spectrum of the emitted light and to suppress any remaining excitation light.

3.4 Performance Features of the ConfoCor 3 and the LSM 510 or LSM 510 META

3.4.1 Optical and Mechanical Aspects

The highly integrated system design makes for the shortest possible optical paths, top-grade optical precision and high stability. The compact scanning module can be fitted to an inverted (Axiovert 200 M BP or SP) or upright (Axio Imager.Z1, Axio Imager.M1 or Axioskop 2 FS MOT) microscope in less than three minutes. On the Axiovert 200 M, the scanning module may be mounted either to the base port directly below the microscope or to the side port. Please note that the Axiovert is required for attachment of the ConfoCor 3.

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

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

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

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

The three internal image acquisition channels, usable for reflection or fluorescence, and an additional transmitted-light channel are ideal for the investigation of multiple fluorescence specimens. The ConfoCor 3 is attached to the fourth channel. The ConfoCor 3 comes with two detector types: Gallenium Arsenide Phosphide detectors (GaAsP) or avalanche photodiodes (APD). The APDs will deliver a TTL pulse, the GaAsP detectors deliver TTL pulse, NIM pulse and an analogue signal. Separately in each of the four channels, the diameters of the pinholes and their XY positions can be optimized, and the desired emission filter placed into the beam path, by servo-motor control. In the case of pinhole VP1, this adjustment also includes positioning along Z. In the simultaneous registration of multiple fluorescence, identical optical sections can be obtained in each confocal channel. This is of importance, e.g., with the FISH method (fluorescence in-situ hybridization) used for genome analysis in cytogenetic studies.

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

A fiber-optic cable connection to external special detectors, such as cooled PMTs or spectrometers, is not available if the ConfoCor 3 is attached.

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. In the ConfoCor 3 all filter wheels can be exchanged by push and click.

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

A two-mirror scanner system, controlled by a real time electronics, offers several advantages. The large deflection angle of the scanning mirrors allows a wide area to be scanned. With a 1.25× objective, the object area scanned is 10 × 10 mm². The scanning field size can be freely selected between 4 × 1 and 2048 × 2048 pixels.

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

For ConfoCor 3 operation, the mirrors will be parked.

Selection of the specimen detail of interest for zooming is fast and convenient, and the zoomed image is automatically centered. This saves the job of specimen centration with the microscope stage.
Using a bi-directional scanning facility will double the scanning rate to approx. 5 frames/sec (at 512 × 512 pixels); if two different laser wavelengths are used for the two scanning directions (wavelength 1 for left-to-right, and wavelength 2 for right-to-left scanning), two fluorochrome dyes can be viewed and documented in a quasi-simultaneous mode. This will prevent cross talk between detection channels.

3.4.2 Microscope Equipment of the ConfoCor 3 and the LSM 510 or LSM 510 META System

The LSM 510 or LSM 510 META system is equipped either with the inverted Axiovert 200 M BP or SP microscope required in combination with the ConfoCor 3.

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

(1) Stand

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

b) The reflector mount is motorized and provided with the Axiovert 200 M reflector turret. The reflector turret has 5 positions: One transmitting light position, which is identical to the LSM position, and four further positions for fluorescence filter sets (reflector modules). If you want to use more than five conventional fluorescence filter sets, it is advisable to use a further reflector turret. When changing the reflector turret position you must make sure that the turret will click into position, since otherwise the image area will be cut. **c)** The stand has a motorized focusing drive (fine coarse). Sensitivity of the focusing drive is adjusted to the delivered objectives by the manufacturer. If you want to use other objectives, sensitivity and parfocality can be adjusted via the Axioset program.

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

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

(2) Specimen stages and fine focus drives

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

b) Scanning stage

c) Piezo objective focus drive

(3) Transmitted-light illumination

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

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

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

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

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

(4) Reflected light fluorescence

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

(5) Imaging optics

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

(6) Photo equipment

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

(7) TV adaptation

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

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

3.4.3 Computer Hardware and Software

The LSM 510- and LSM 510 META-ConfoCor 3 are controlled via a standard high-end Pentium PC. Linking to the electronic control system is made via Gigabit Ethernet interface. The PC comes with the WINDOWS XP operating system.

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

The software of the LSM 510, LSM 510 META and ConfoCor 3 have two levels. On the simple operator interface level, a result will be achieved after a few prompts; graphical prompting of the user in conjunction with automatic setting of many parameters is an ideal tool for daily routine jobs. The expert level offers perfect facilities for individual settings of functions and parameters.

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

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

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

CHAPTER 4 CONFOCOR 3 IN COMBINATION WITH LSM 510 AND LSM 510 META

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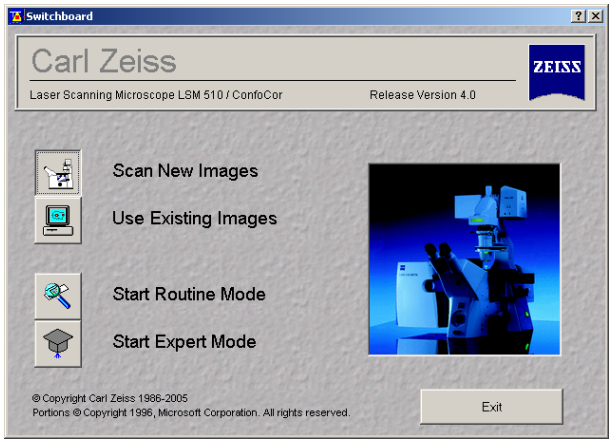


Fig. 4-1 **LSM 510-ConfoCor 3 Switchboard window**

4 OPERATION OF CONFOCOR 3 IN EXPERT MODE

4.1 Main Menu for ConfoCor 3

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

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

In the standard setting of the LSM 510-ConfoCor 3 software, the toolbars are automatically displayed after the start of the Expert Mode.

☞ However, since the LSM 510-ConfoCor 3 software is operated more conveniently with the help of the toolbars, only this method of function activation will be described in the following.

- Click on the Start Expert Mode button in the **LSM 510-ConfoCor 3 Switchboard** window.
  - The LSM FCS - Expert Mode **Main** menu appears on the screen.

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

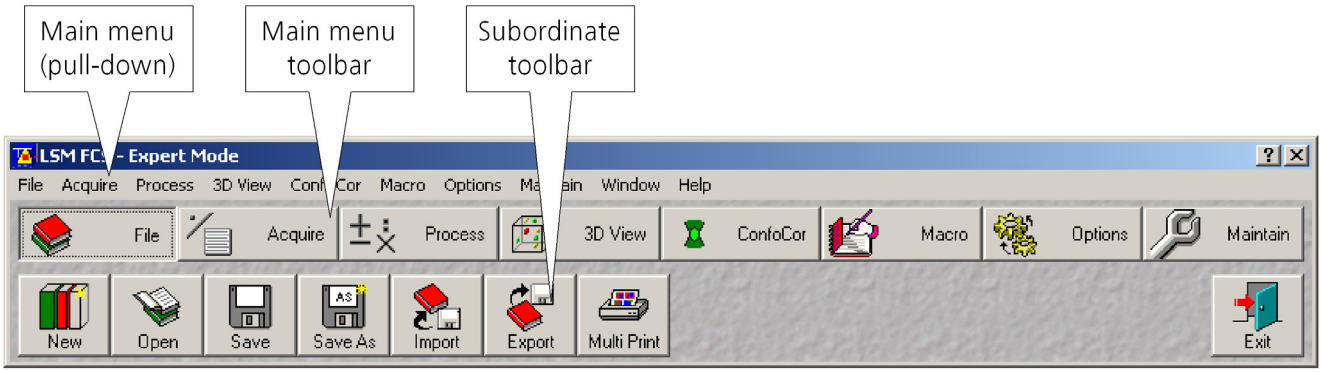


Fig. 4-2 **LSM FCS- Expert Mode Main menu**

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

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

	**Process** button	Used for processing of acquired images.
	**3D View** button	Used for three-dimensional reconstruction.
	**Macro** button	Makes it possible for the user to store frequently used processes (Macro recorder) and to run them automatically (Macro play). It is possible to write new macros or to edit existing ones.
	**ConfoCor** button	Loading FCS data files and methods; defining and selecting models; define settings; executing measurement and data analysis
	**Options** button	For custom-configuration of software and hardware options, and for exporting system operating sequences to the Routine Mode. This menu item enables access to the coloring table. In the **Settings** window you can specify essential operating modes and informative help, organized by tabs, which have an effect on the user interface.
	**Maintain** button	Service mode for adjustment and setting of other parameters (e.g. objectives).

4.2 File Menu for ConfoCor 3

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 510-ConfoCor 3 software, or the export of images to other software packages. The **Print** function allows individual or several images to be arranged on a print page for printout. The **Expert Mode** can be ended via the **Exit** function.

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

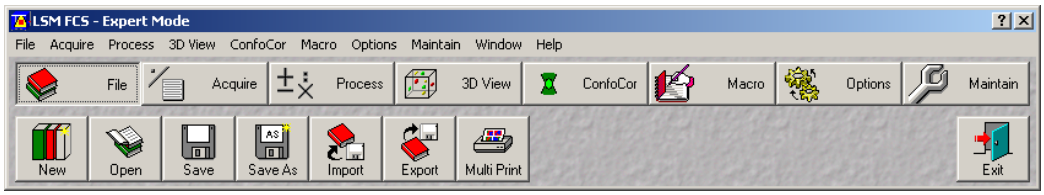


Fig. 4-3 LSM-FCS - File menu

The **New** button allows you to create a new database

The **Open** button allows you to open an existing database

The **Save** button allows you to save an existing database with the name and file location unchanged

The **Save As** button allows you to save an existing database under another name and different file location.

The **Import** button allows you to import an existing image. You have the option of different file formats.

The **Export** button allows you to exort the highlighted image in different file formats.

The **Multi Print** button allows you to print a highlighted image.

4.3 Acquire Menu for ConfoCor 3

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

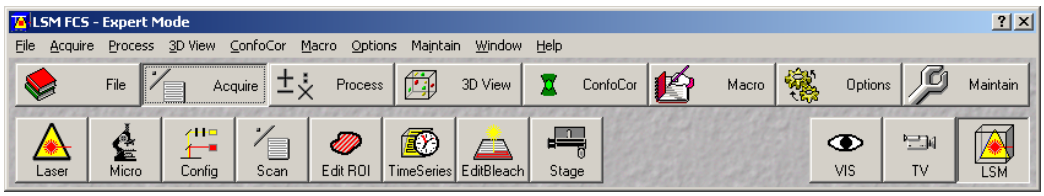


Fig. 4-4 LSM-FCS - Acquire menu

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

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

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

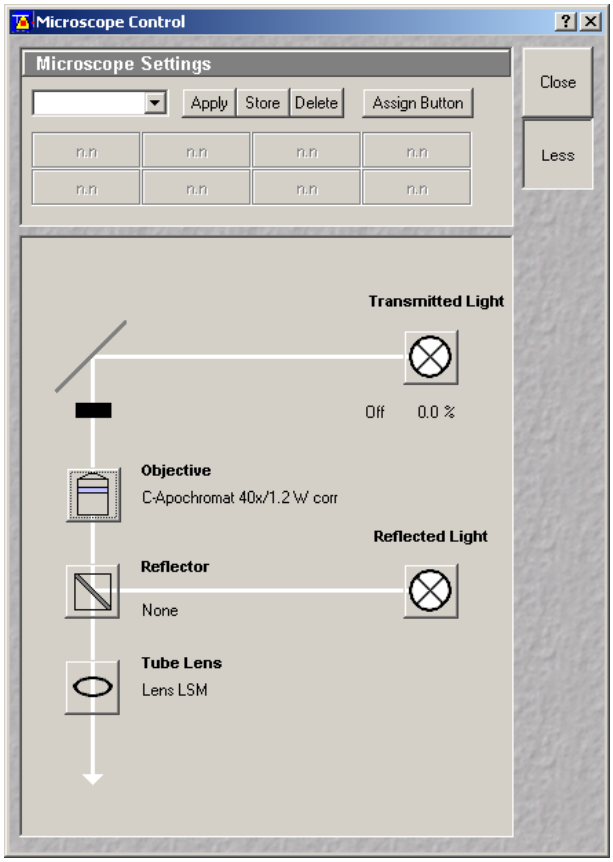


Fig. 4-5 **Microscope Control window**

4.3.1 Micro Button

The **Microscope Control** (**Micro** button) window permits motorized functions (objective and reflector change settings) and the illumination mode (transmitted light) of the connected microscope to be controlled via the software (see Fig. 4-5).

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.

- 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.

The **Microscope Control** window contains the following functions:

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

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

Objective button — Objective can be selected via graphical pop-up menu. Please note, that for FCS only the C-Apoochromat 40x/60x W N1.2 are specified

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

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

Reflected Light button The shutter is switched on and off.

4.3.1.1 Reflected-light Observation (Epi-fluorescence)

- Turn on the HBO 50 power supply switch.
- Click on the check box for **Reflected Light ☑ On**.
- In the **Reflector Turret** list box, select the desired filter set by clicking on it.
  - The filter is automatically moved into the beam path to enable observation in epi-fluorescence.
- In the **Tubelens** list box, select the desired tube lens by clicking on it.
- Swing the required objective for FCS measurements into the working position. This is performed by selecting the objective in the **Objective** selection box in the **Axiovert Control** window.

We recommend to use the C-Apochromat 40x/1.2 W corr on account of its optimized optics.

4.3.1.2 Transmitted-light Observation

- Click on the check box for **Transmitted Light ☑ On**.
- Activate the condensor function in the **Condensor** panel.
- Swing the required objective for FCS measurements into the working position. This is performed by selecting the objective in the **Objective** selection box in the **Axiovert Control** window.
- Select **Light Remote** or **3200 K** or set the transmitted light intensity via slider.

4.3.2 VIS, TV, LSM Buttons

The **VIS**, **TV** and **LSM** buttons switch the beam path of the microscope (VIS for viewing, TV for camera, LSM for LSM laser operation with monitor observation).

For the measuring process, the **LSM** button in the toolbar subordinate to the **ConfoCor** item must be activated.

☞ Please note, in the LSM 510 – ConfoCor 3 the major beam splitter is shared between the systems. A change in its setting will only affect the system, where the change was made.

4.4 ConfoCor Menu for ConfoCor 3

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

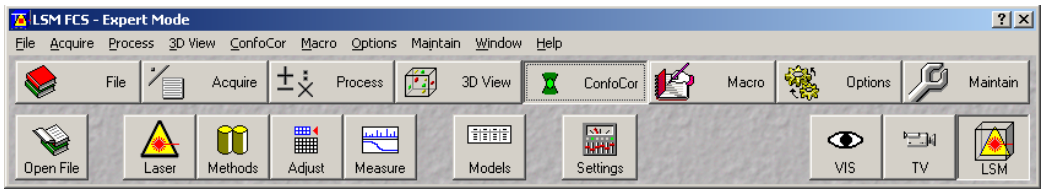


Fig. 4-6 ConfoCor menu

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

- Open File
- Laser
- Methods
- Adjust
- Measure
- Models
- Settings

4.4.1 Open File

This function is intended for use of already measured data in the FCS mode. It also allows you to import data stored with the ConfoCor 1, ConfoCor 2 or the ConfoCor 3. In addition, raw data stored with the ConfoCor 2 and ConfoCor 3 can be loaded.

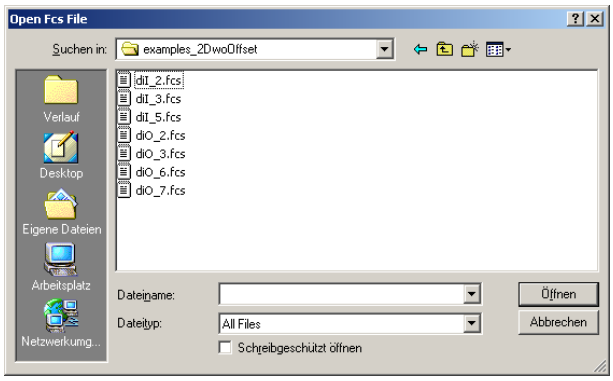


Fig. 4-7 Open FCS file window

- To open previously stored data, click on the **Open File** button in the **ConfoCor** subordinate toolbar of the **Main** menu.
  - This opens the **Open FCS file** window for the selection of drives, directories and subdirectories in which data files have been stored.
- If you want to load a data file in 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.
- Select the appropriate data file(s) via mouse click and click on the **Open** button.
  - This opens the FCS data file in a separate window from saved data or opens FCS data files in one window of exported data.

4.4.2 Laser Control for ConfoCor 3

The **Laser** function allows you to switch on and off the lasers and set the tube currents if applicable.

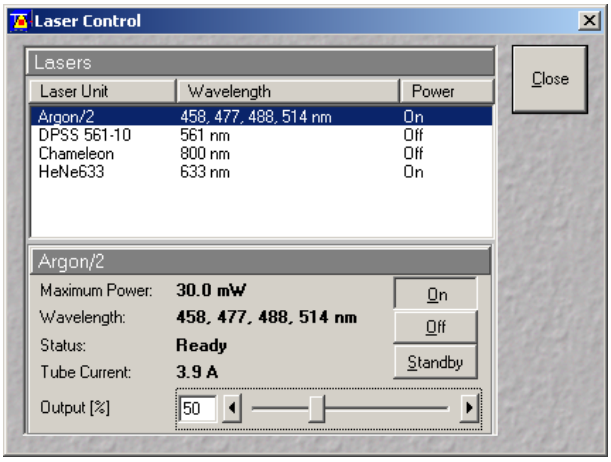


Fig. 4-8 Laser Control window

- Click on the **Laser** button in the **ConfoCor** subordinate toolbar of the **Main** menu.
  - This opens the **Laser Control** window (see Fig. 4-8), which consist of two panels. The upper **Lasers** panel lists all the available lasers, that can be selected within the display box. The selected laser can than be controlled then in the lower panel that bears the name of the selected channel.
  - The Argon laser is first switched to Standby, until it is warmed up. Then the laser can be switched on by pressing the **On** button and the tube current set by the **Output (%)** slider. A good value is setting the tube current to 50% output. The laser can be switched off by pressing the **Off** button or brought to standby by pressing the **Standby** button. Whenever the laser is required, he will automatically go from **Standby** to **On** and does not need to be switched on in this menu. Other lasers with a constant tube current can be either switched **On** or **Off**.

4.4.3 Methods

The **Methods** function shows all existing measure methods for selection or deletion.

- Click on the **Methods** button in the **ConfoCor** subordinate toolbar of the **Main** menu.
  - This opens the **Select Method** window (see Fig. 4-9). The name of the currently selected method will appear in the status bar of the window.

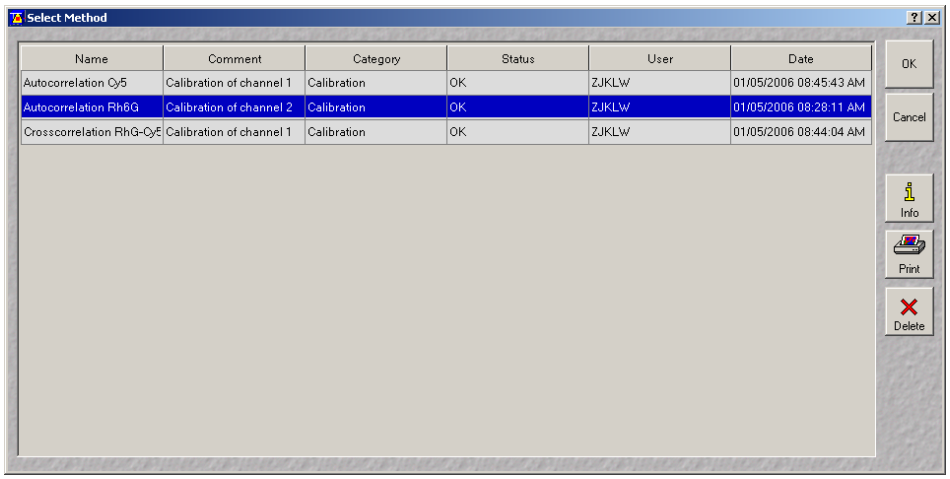


Fig. 4-9 Select Method window

- You can select the analysis method you want to use by double-clicking on the corresponding list entry. After the double click the analysis method is selected and the **Select Method...** window will be closed automatically.
- Alternatively you can select the analysis method by a single click – which will highlight the selected method – and a subsequent click on the **OK** button. The window will then be closed automatically.
- The **Cancel** button of the **Select Method...** window allows you to leave the dialog without any action.
- The **Print** button opens the **Print Setup** window. Set the print parameters and start the print function by clicking **OK**. The **Print Setup** window is closed automatically.
- The **Delete** button allows you to delete a method from the database. To do so, click on the method entry in the list and then click the **Delete** button. A window will pop up requesting confirmation of this action. If you confirm, the method will really be deleted from the database.

☞ Use this function with extreme care! You might lose valuable data!

- After a click on the **Info** button the **Method Information** window will pop up which shows a detailed description of the analysis method (see Fig. 4-10).

☞ All parameters will load with this method.

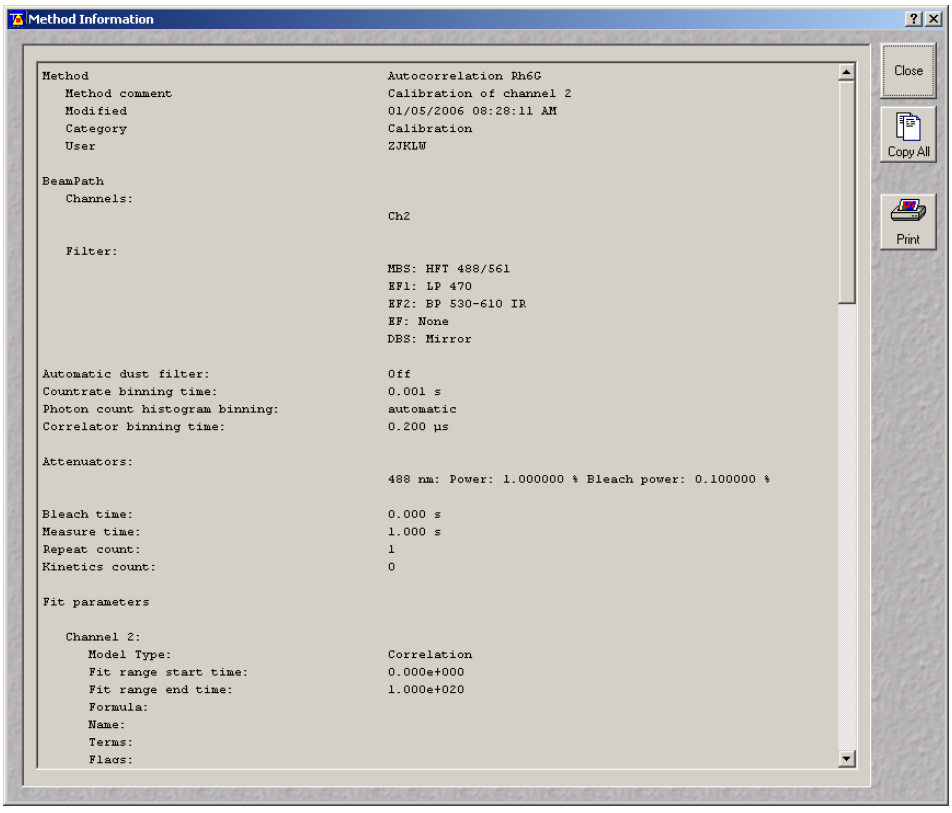


Fig. 4-10 Method Information window

- Click on the **Copy All** button to copy the details to the clipboard. Afterwards you can insert the details in a WINDOWS application (e.g. Winword) and save it.

- Click the **Print** button to print the details table. This opens the **Print Setup** window. Set the print parameters and start the print function by clicking **OK**. The **Print Setup** window is closed automatically.
- Click the **Close** button to close the **Method Details** window.

4.4.4 Adjust

The **Adjust** button opens the **Carrier Position** window (see Fig. 4-11), which is used to define and orient the sample carrier.

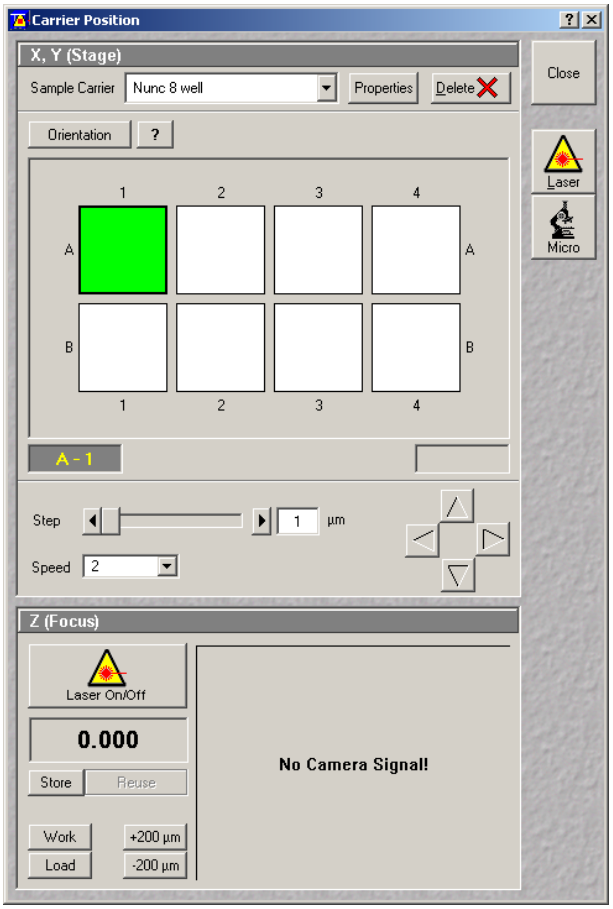


Fig. 4-11 Carrier Position window

4.4.4.1 Define a New Sample Carrier

- In the **Carrier Position** window, use the select dropdown menu box, if you want to edit a pre-existing carrier than press the **Properties** button. If you want to create a new carrier, just press the **Properties** button. The selected Carrier configuration will be displayed. Enter the number of chambers and the distance (mm) between the chambers in the appropriate **Column** and **Row** input boxes.

4.4.4.2 Save a New Sample Carrier

- Click on the **Save** button to save the new sample carrier.
  - The **Save Carrier** window appears on the screen.
- Choose a memorable name for the new sample carrier which you are likely to remember.
- Click on **OK** to save the new sample carrier.
- Click on **Cancel** if you don't want to save.

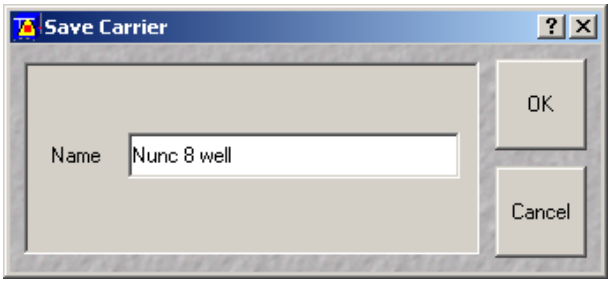


Fig. 4-12 Save Sample Carrier window

4.4.4.3 Delete a Carrier Configuration

A no longer required sample carrier can be deleted as follows:

- Select the sample carrier to be deleted from the **Sample Carrier** selection box.
- Click on the **Delete** button. Confirm the deleting in the following window by clicking **OK**.
- When you are finished, close the **Carrier Definition** window by clicking on **Close**.

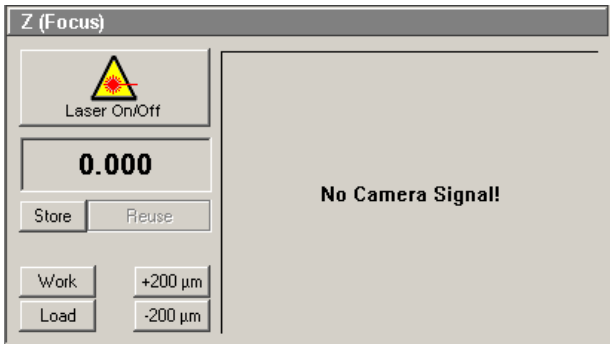


Fig. 4-13 Z (Focus) panel

4.4.4.4 Z (Focus) Panel

The functions of the **Z (Focus)** panel allows you to position the focus in Z direction (see Fig. 4-13). Following function elements are available:

Laser On/Off button

Switches selected laser lines on/off

Z-position display field

Displays the current Z position.

Store button

Stores the current Z position.

Reuse button

Moves the z drive to the stored Z position.

Work button

Moves the stage / nosepiece back to the Work position. This is the position last set before the **Load** button was pressed.

Load button

Lowers the stage / nosepiece to make it easier for you to change the sample carrier (or objective).

+200 µm button

Moves the Z drive for +200 µm per mouse click (upwards).

-200 µm button

Moves the Z drive for –200 µm per mouse click (downwards).

☞ The Z-position stored by activation of the **Store** button is a relative parameter and is only valid during the current session. Accordingly, this position can be approached in a defined way only during this session via **Reuse**.

Since the sample is just a solution in many cases and has no structure, we cannot "focus" the instrument by looking at sample features. Cover slip reflection has to be used instead to find the glass- / solution interface. The position of the glass surfaces is detected most conveniently via line scanning.

Proceed as follows:

- Make sure that the front lens of the water immersion objective is wetted by a drop of water. Use fluorescence-free double-distilled water, since otherwise the immersion water will cause background fluorescence and deteriorate the correlation signal.
- Choose a chamber by clicking on one in the sample carrier drawing in the **X, Y (Stage)** panel of the **Carrier Position** window.
- Set up a beam path in **Configuration Control** (see Fig. 4-14). Convenient settings are 488 line at 2 % AOTF, 80/20 splitter in the major beam splitter and an appropriate channel and emission filter, for example channel 3 with a KP680 id available.

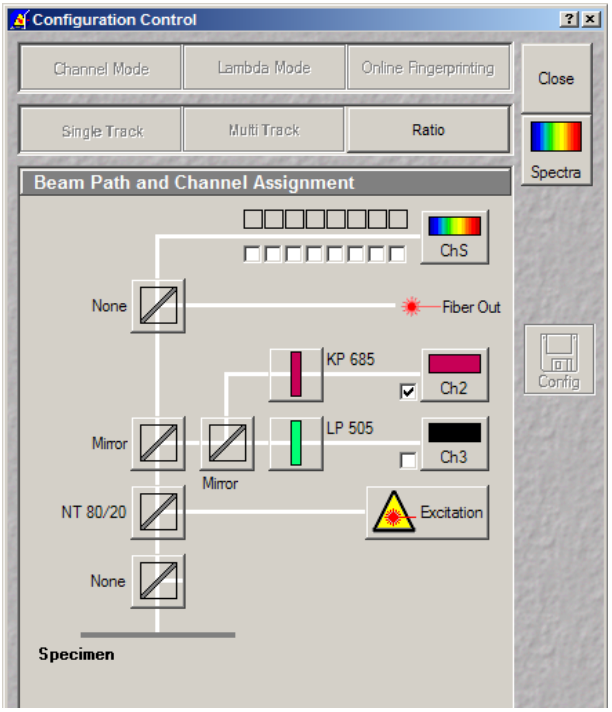


Fig. 4-14 **Beam path configuration for finding glass reflection**

- Set up scan mode (see Fig. 4-15). Choose **Line** scan, highest Scan speed, 512 x 512 and **Zoom 1** for convenience.

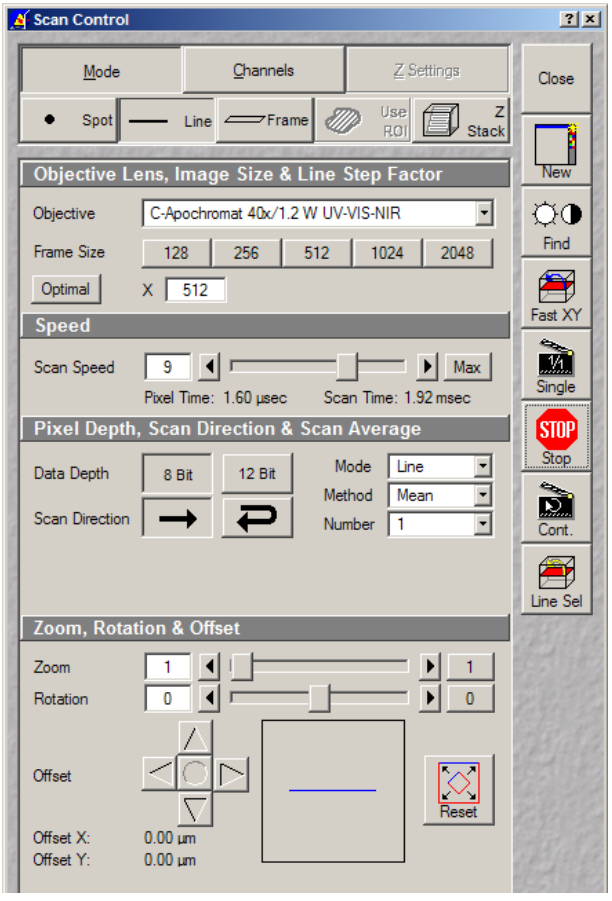


Fig. 4-15 **Image settings for line scan mode**

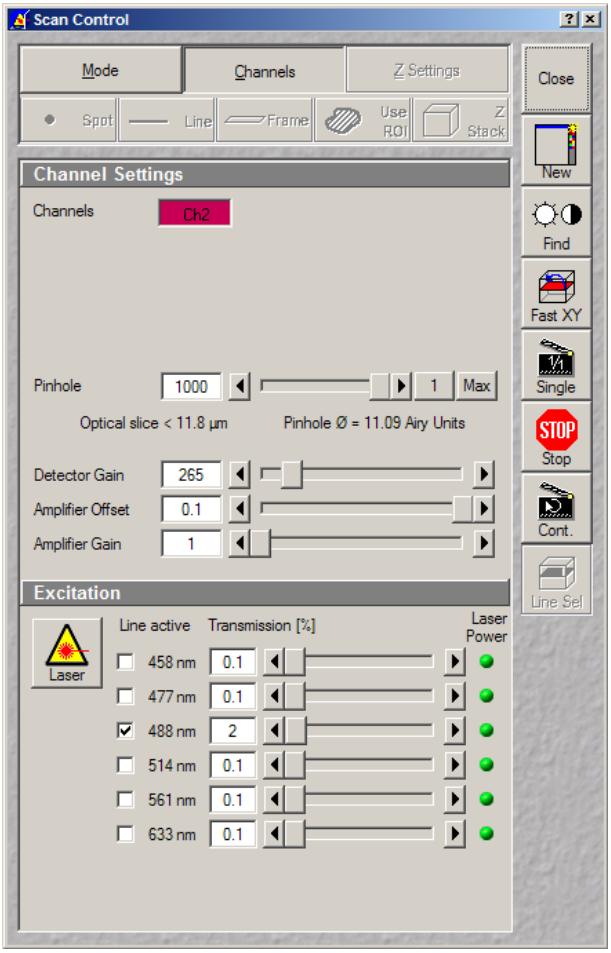


Fig. 4-16 **Channel settings for finding glass reflection**

- Set up scan control (see Fig. 4-16). Open Pinhole to maximum. Set detector gain between 250 and 300, and laser power to 2 %.

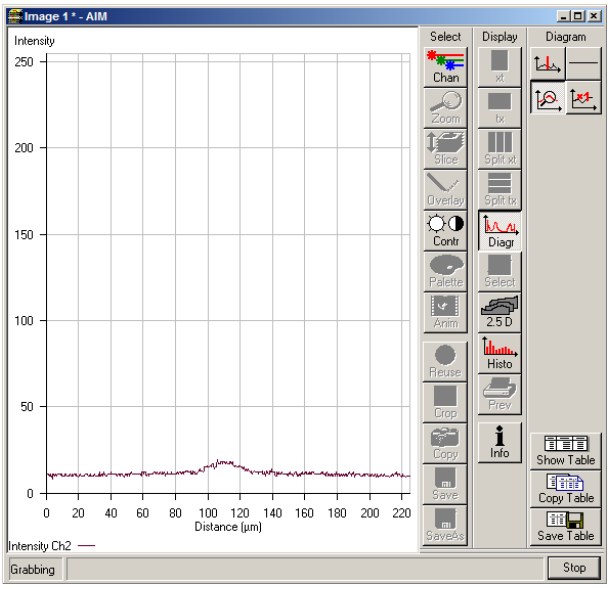


Fig. 4-17 **Line Scan diagram far away from the lower cover slip surface**

- Start scan and press **Diagr** button (see Fig. 4-17).
- The diagram shows a line with a hill, which is the result of reflected light not focused yet on the lower glass surface. You might adjust the line to lower or higher values by altering the gain.

- Move the objective cautiously upward by turning the Z focus knob of the microscope stand. A lens moving upward will be indicated by increasing numbers in the position field to the left of the camera window.
- If the focus position approaches the lower cover slip surface, the position of the line will shift to higher values (see Fig. 4-18).

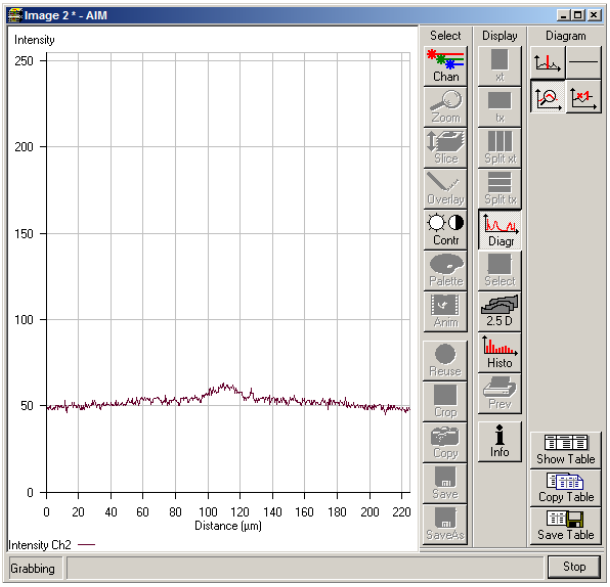


Fig. 4-18 Line diagram near the lower cover slip surface

- Continue cautiously moving upwards. The line position will reach its maximum (see Fig. 4-19).

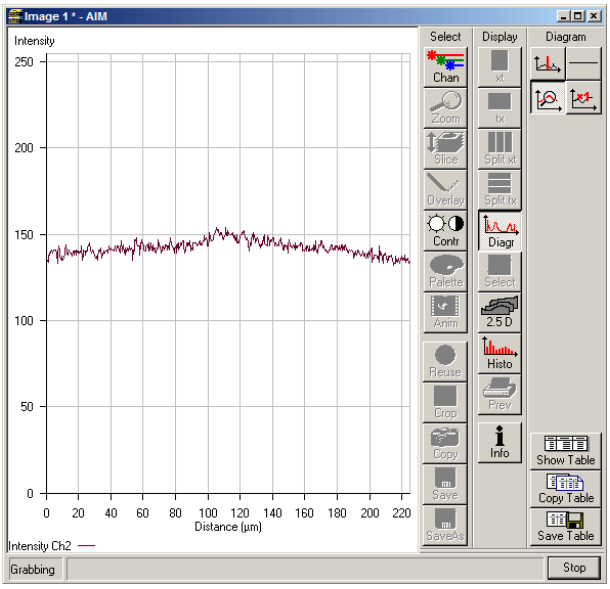


Fig. 4-19 Line diagram at lower cover slip surface

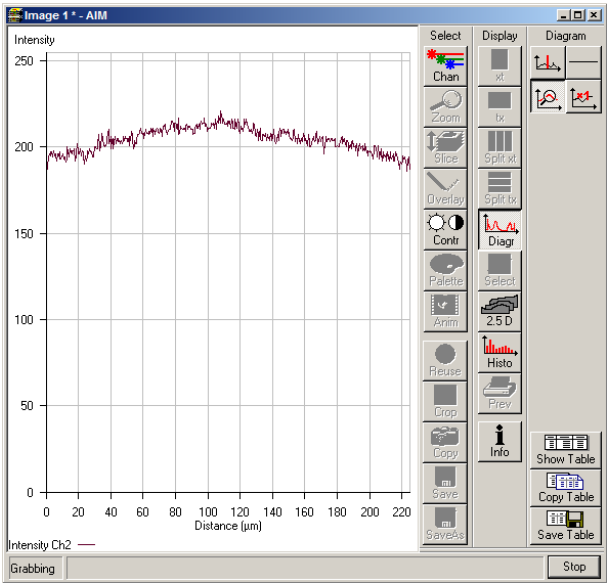


Fig. 4-20 Line diagram at upper cover slip surface

- Having cautiously moved over a short distance the line position will decline again to a minimum and than rise again to a second maximum (see Fig. 4-20), which corresponds to the upper cover slip surface has been found.
- Store the reached position using the **Store** button.

Now you can position the detection volume into the sample in a well-defined way: if you use one of the C-Apochromat water immersion objectives, position the detection volume 150-200 µm deep into the sample to get rid of disturbing interface effects. The easiest way of doing this is by clicking the **+200 µm** button.

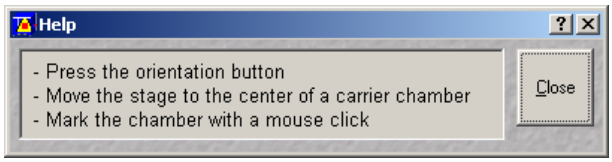


Fig. 4-21 Help window

4.4.5 Orienting the Carrier in X and Y

- Click on the **Orientation** button in the **X, Y (Stage)** panel
- Select one of the chambers of the sample for orientation in x and y.
- Position the center of one chamber over the center of the objective by clicking the arrow keys with the mouse or by using the control panel's joystick (if available).
- When you have finished the **Orientation** automatically deactivates. The stage is now oriented.
- Now you can move any chamber into the measurement position by clicking the appropriate field on the sample carrier.

The selected chamber is then automatically approached via the motorized microscope stage. When the defined position has been reached, the position of the chamber is displayed in the lower cleft corner in the **Positions** panel and is indicated in green.

☞ By pressing the **?** button, a **Help** window appears (see Fig. 4-21), explaining the work flow for orienting the Carrier.

4.4.6 Measure

The **Measure** function opens the **Measurement** window. It is used for optimizing an existing method or for creating a new one.

- Click on the **Methods** button in the **ConfoCor** subordinate toolbar of the **Main** menu (see Fig. 4-6).
  - The **Measurement** window will appear (see Fig. 4-22).

A measurement has three major sections:

- **System Configuration.** It is used for defining beam paths and methods and adjusting the pinholes and collimators.
- **Acquisition.** It is used for setting the measurement parameters and positioning the laser beam in respect to the sample via the stage or the scanners.
- **Processing.** It is used for analyzing the data and how data are handled.
- These major sections are available in separate subwindows by clicking the appropriate button in the **Method Optimization** window.

The following functions are available on the right-hand side of the **Method Measurement** window:

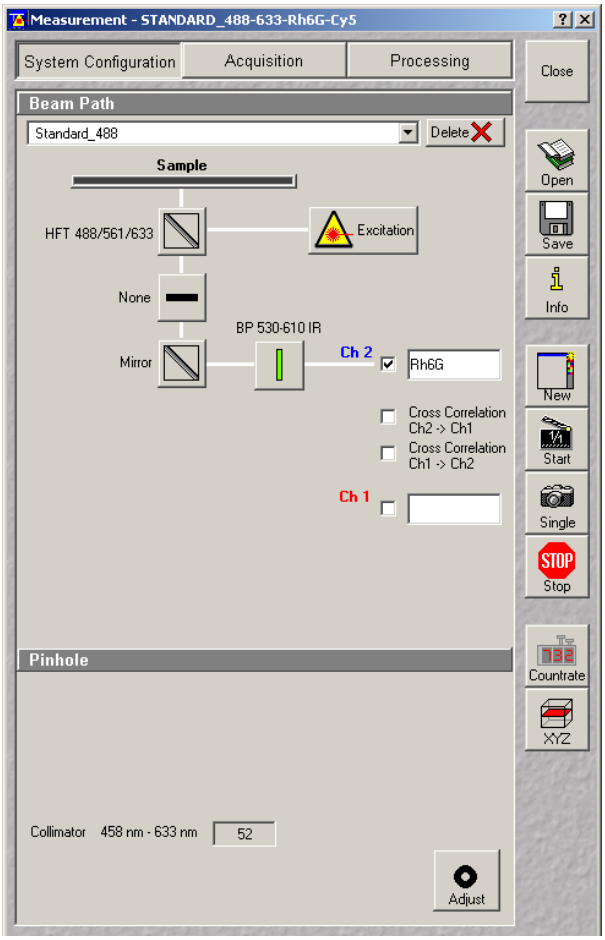


Fig. 4-22 Measurement window

(1) Close button

The **Measurement** window is closed.

(2) Open button

The **Select Method** window (see Fig. 4-23) will open that allows you to select a predefined method.

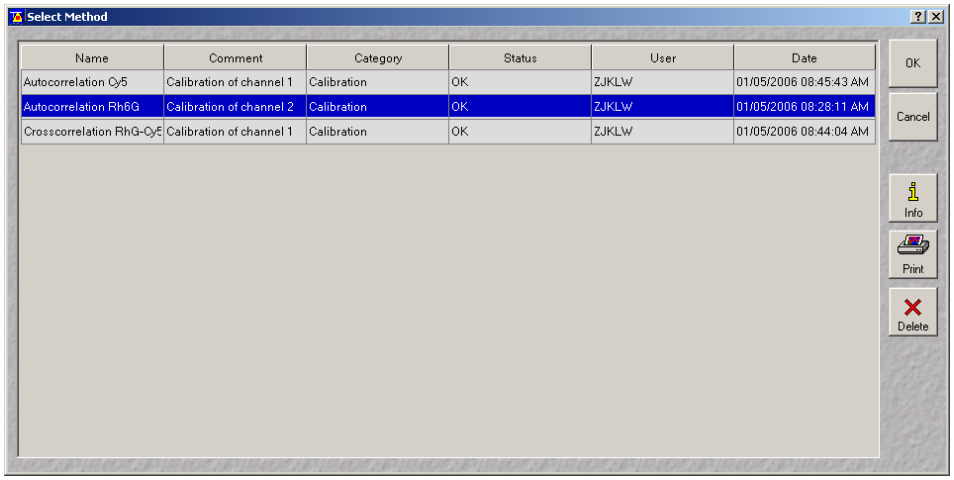


Fig. 4-23 Select Method window

(3) Save button

The **Save Method** window will appear. Saves the optimized or new created measurement method.

(4) Info button

The current **Method Information** window will appear that inform you on the actual settings of the method (see Fig. 4-24).

Display - Info

- The **Info** button is used to open the current **Method Information** window in which all the relevant parameters of the method are displayed.

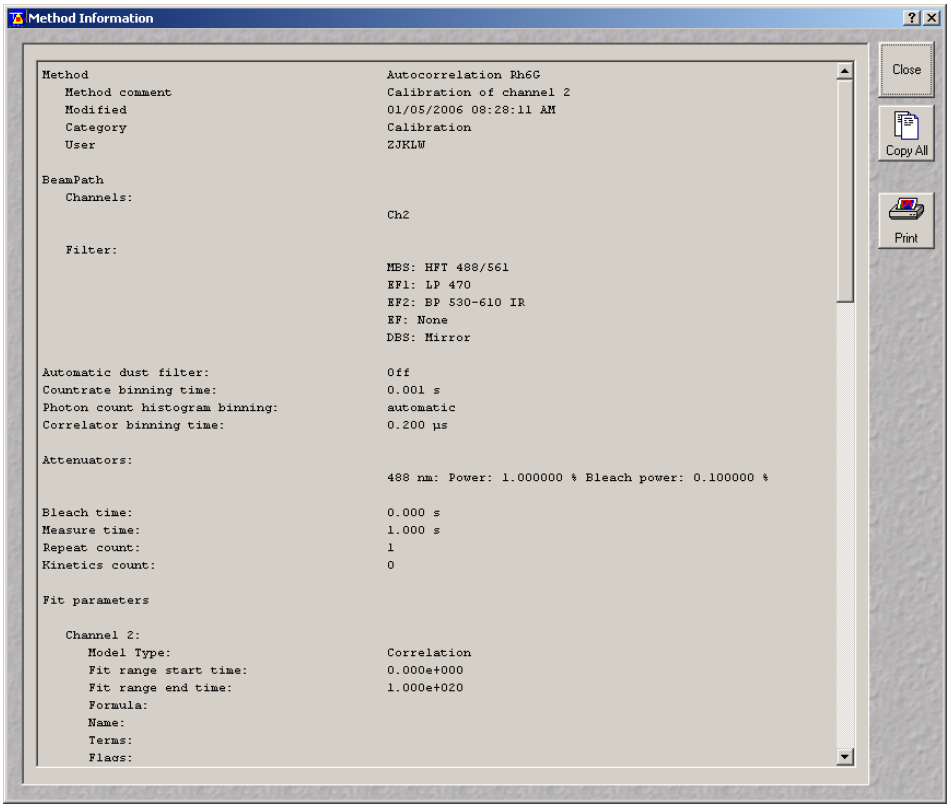


Fig. 4-24 Method Information window

- With **Copy All**, the complete parameter set will be copied to the clipboard.

A click on the **Print** button opens the **Print Setup** window and allows the parameters of the method to be printed in the form of a table.

(5) New button

Opens a new **Data Display Window**.

(6) Start button

Starts the measuring procedure.

Measurement procedure

After setting the parameters the measurement can be run.

- Click on the **Start** button on the right-hand side of the **Measurement** window to start the measurement and data analysis process.
  - The FCS data evaluation window will appear.

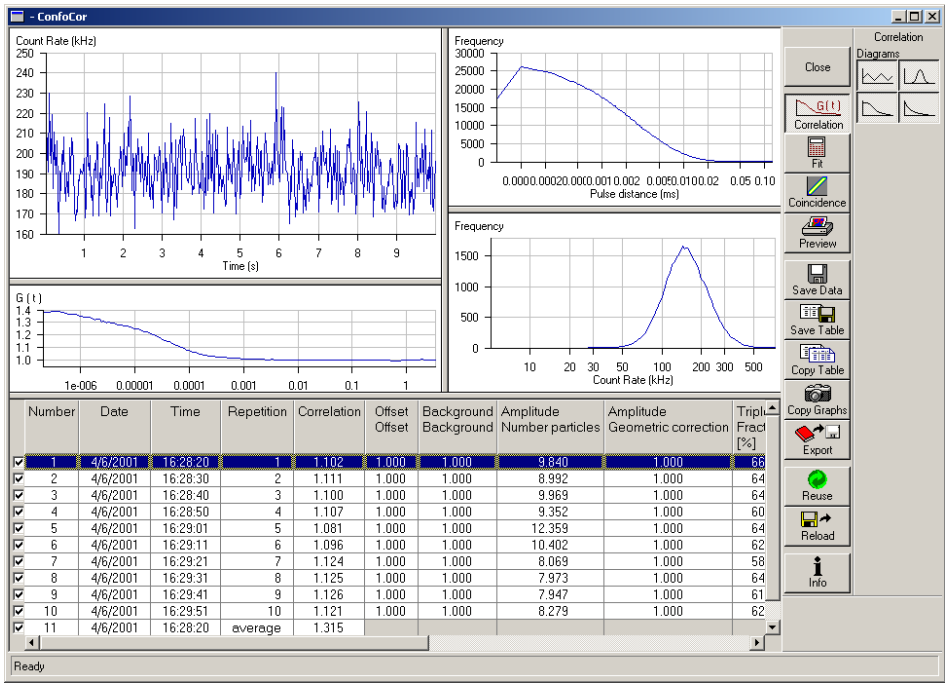


Fig. 4-25 FCS data evaluation window

☞ The further procedure will be explained in section 4.5, Data Evaluation and Result Presentation for FCS Measurements.

(7) Single button

Starts a single measurement with the current settings.

(8) Stop button

Ends the measuring procedure.

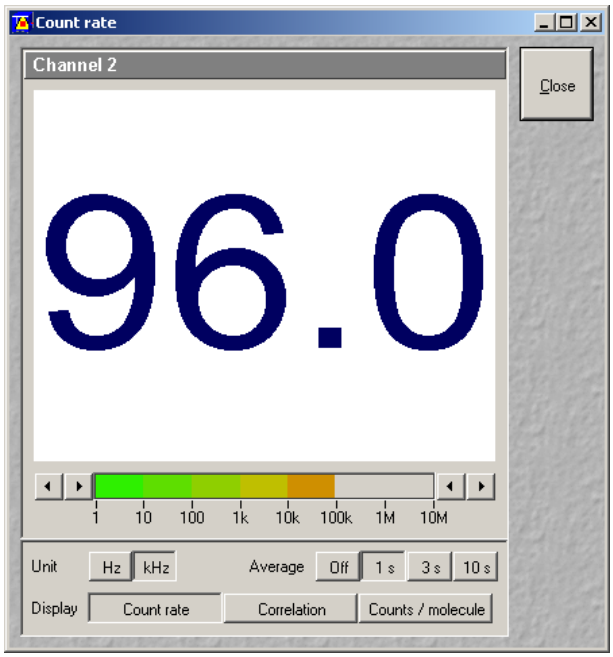


Fig. 4-26 Count rate window

(9) Count rate button

Opens the **Count rate** window (see Fig. 4-26).

(a) Count rate window

- Click on the **Count rate** button on the right-hand side to open the **Count rate** window.

In two **Channel** and one **Correlation** panels, the **Count rate** window shows the values for Count rate, Correlation or Counts / Molecule depending on the activated **Display** button.

The **Channel** panels and the **Correlation** panel feature a big display field for the count rate and, at the underside, a scale allowing the measuring range for the displayed count rate to be read from a colored bar. A click on one of the four arrow buttons permits the measuring range display to be narrowed or widened.

The buttons in the lower range of the **Count rate** window have the following functions:

(b) Unit

Selecting the unit **Hz** or **kHz**.

(c) Average

Averaging after **1 s**, **10 s** or **Off**.

(d) Display

Selecting one of the display modes **Count rate**, **Correlation** or **Counts / Molecule**. The arrow buttons allow to adjust the scale bar range.

(10) X,Y,Z buttons

Opens the **X,Y,Z-Scan** window (see Fig. 4-27) for performing a X,Y,Z-scan.

(a) X,Y,Z-Scan for positioning the focus within structured samples

To make the positioning of the detection volume easier, the ConfoCor 3 is provided with a X,Y,Z-Scan possibility.

This means, that the focus is moved in either the X, Y, or Z direction over a preselected distance while the count rate in one or both of the detection channels as defined by the channel check boxes is recorded. A maximum (or minimum) in the resulting curve (Fig. 4-28) usually indicates a region of interest. The focus can be positioned to this region by a simple mouse click.

- Click on the **X,Y,Z-Scan** button on the right-hand side of the **Method Optimization** window.
  - The **X,Y,Z-Scan** operating window appears on the screen (Fig. 4-27).

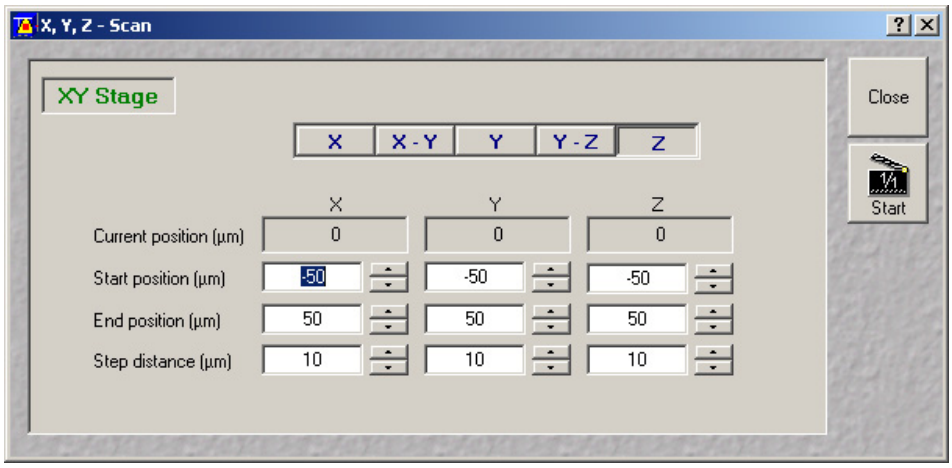


Fig. 4-27 X,Y,Z-Scan operating window

(b) For X,Y,Z-Scanning proceed as follows:

- Press the **VIS** button.
- Position the object in X and Y.
- Find the upper cover slip surface (interface between the glass and the cell) by carefully focusing the objective.
- Switch to the **FCS** mode by clicking on the **FCS** button in the **Analysis** subordinate toolbar.

The next steps depend on whether you already have a functional measurement method for your particular problem or not.

- If you already have a functional method: Select the method first using the **Select Method** button in the **Analyse FCS** subordinate toolbar of the **Main** menu.
- If you do not have a functional method: Create a new method.
- Select the **Channel**, **Start** and **End** position and the **Number** of **positions**.

- Clicking on **Start** opens a new window showing a count rate graph (Fig. 4-28).

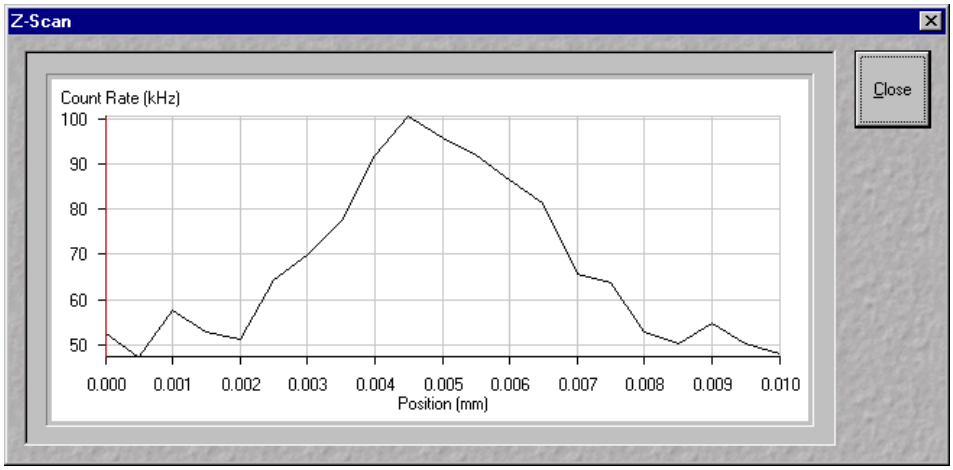


Fig. 4-28 Z-Scan result window

When the scan has been finished, a vertical red line appears at the current position. This line can be dragged with the mouse when the left button is held down.

- Position the vertical red line where the focus should be placed by using the mouse in case of a z-Scan, or where the sample should be positioned in x or y.
- Then close the window with **Close**. The **Refresh Positions** button will actualize the current position if pressed.

☞ Please note, that X, Y scans are performed by the stage regardless if the Scanner button is pressed or not.

Fig. 4-29 Context menu of the X,Y,Z-Scan result window

The data of the **X,Y,Z-Scan** result window can be copied to the clipboard via the context menu or saved directly as an ASCII file.

- Click in the **X,Y,Z-Scan** result window with the right mouse button to open the context menu.
- Select the line **Copy data to clipboard** with a click of the mouse if you want to insert the data into other WINDOWS programs directly via the clipboard. The Paste function enables you to insert these data directly into the required program.
- Select **Export data to file** to save the data in an external ASCII file (.txt).

4.4.7 System Configuration

- Click on the **System Configuration** button on top of the **Measurement** window (Fig. 4-22).
  - The **System Configuration** subwindow appears on the screen.

4.4.7.1 Beam Path Panel

The **Beam Path** panel allows new / existing beam path configurations to be created / edited and saved. The **Delete** function enables you to delete existing beam paths.

- Click on the **System Configuration** button in the **Measurement** window
  - The **Beam Path** window will be displayed as the upper submenu.
- When the beam path definition is finished, you can use the buttons on the right to start a task.

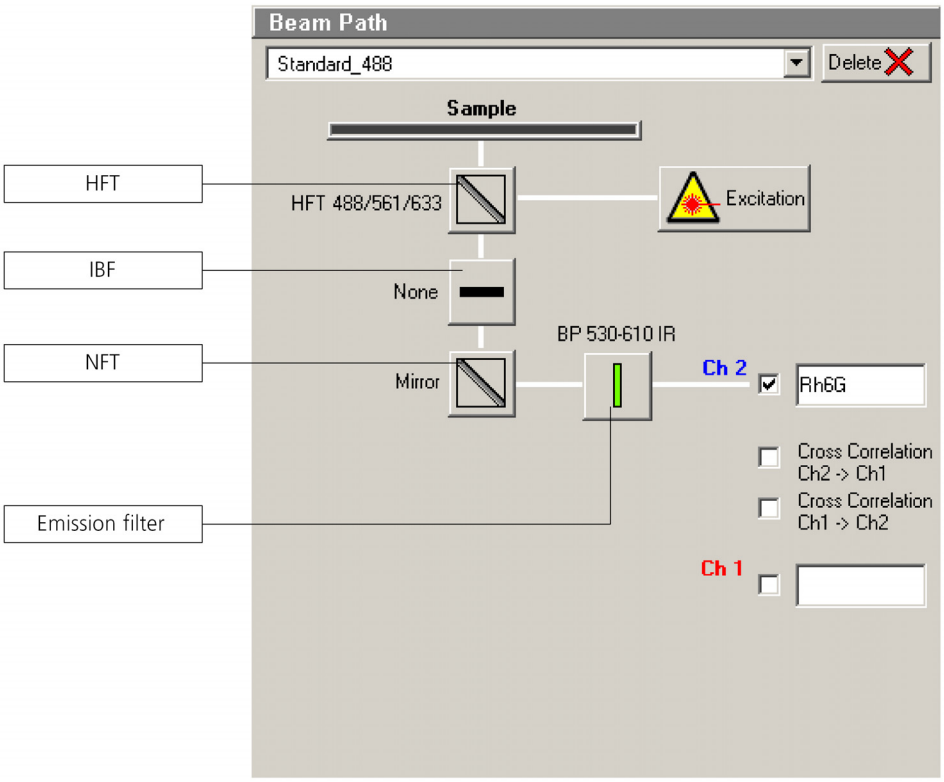


Fig. 4-30 ConfoCor - Beam Path panel

(1) Loading a predefined beam path

In the **Beam Path** panel you can select a saved beam path configuration by the drop down menu. The selected beam path will be shown as a drawing. The delete button will delete the selected beam path.

If a two-channel beam path is selected, you can activate / deactivate both the channels to be used and the calculation of the cross correlation. You can obtain cross-correlation curves by either shifting channel 1 versus channel 2 (Ch1 ->2) or channel 2 versus channel 1 (Ch2->1).

- Select a corresponding beam path from the **Beam Path** list box.
- Activate / deactivate the channels and the cross correlation function via the appropriate check boxes.

☞ It is possible to define a new beam path or edit a loaded beam path in this menu. If a new settings are required, please select the correct filters and beam splitters, press **Save**, select **Only beam path**, make the appropriate entries for **Name** and press **Ok**. Once a new beam path is ready, it can be immediately used in the **Measurement** window.

(2) Define a beam path

(a) Light path definition

The following filter and dichroics can be set.

 button

Activation / deactivation of a main dichroic beam splitter (HFT) through selection from the relevant list box (see Fig. 4-30). The HFT reflects the specified laser lines and allows the resulting fluorescence spectrum to pass through. Note that the HFT is from the LSM module.

 button

Activation / deactivation of secondary dichroic beam splitter (NFT) through selection from the relevant list box (see Fig. 4-30). The NFT splits the fluorescence spectrum onto the various detection channels.

 button

Activation / deactivation of an infrared blocking filter (IBF) through selection from the relevant list box (see Fig. 4-30). The IBF is used to block any IR excitation light in NLO applications.

 button

Activation / deactivation of an emission filter (EF) or infrared blocking filter (IBF) through selection from the relevant list box (Fig. 4-30). The EF is used to narrow the fluorescence spectrum. The bandpass (BP) allows the range within the specified border wavelengths to pass through. The longpass (LP) allows the range above the specified border wavelength to pass through. TH short pass (KP) allows the range below the specified border wavelength to pass through.

Channel check boxes

Selection of the channels to be used:

- Ch1 and Ch2 are physical channels for auto-correlation
- Ch2 → Ch1 and Ch1 → Ch2 are software channels for cross-correlation

Ch1 / Ch2 input boxes

For entering of a special comment (e.g. used dye).

Save button

For saving the new or edited configuration.

Delete button

Deletion of an existing configuration.

(b) Laser definition

Press the **Excitation** button and the laser control window will appear. It consists of two panels, one for the laser settings that will be used for the measurement and pinhole adjustment and the second for those used for the pre-bleach (see Fig. 4-31).

☞ Note, that lasers for measurements and pre-bleach can be selected independently from each other.

The **Excitation** panel allows to activate / deactivate the wavelengths via check boxes and to set the requested laser attenuation (%) using the sliders and by choosing the power attenuation in the selection box.

In the **AOTF Dampening Factor (%)** box the AOTF can be set to suppress the laser light over all by a factor of 1, 10, 100 and 1000 fold by moving the slider to the respective dampening factor of a 100, 10, 1 an 0.1, respectively.

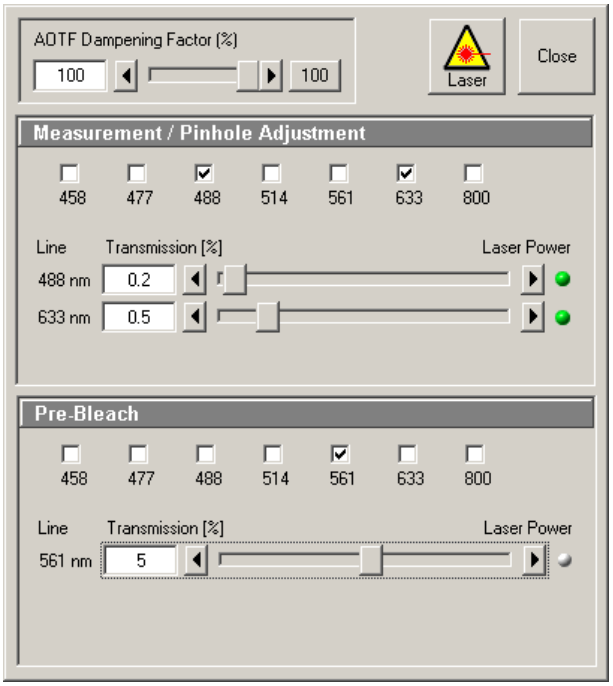


Fig. 4-31 Laser attenuation window

☞ If **Automatic laser dampening** is activated under FCS **Settings**, the range of available attenuation is increased to lower power settings. In this case the **AOTF Dampening Factor (%)** box is not displayed. If **Automatic laser dampening** is not activated under FCS **Settings**, the **AOTF Dampening Factor (%)** box is displayed. Any change in one of the dampening boxes is updated in the other.

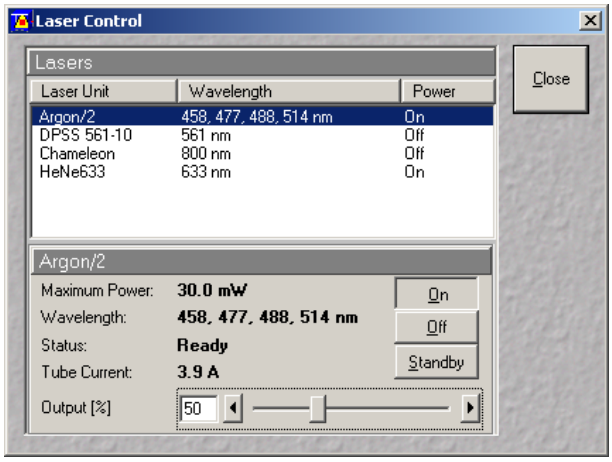


Fig. 4-32 Laser control window

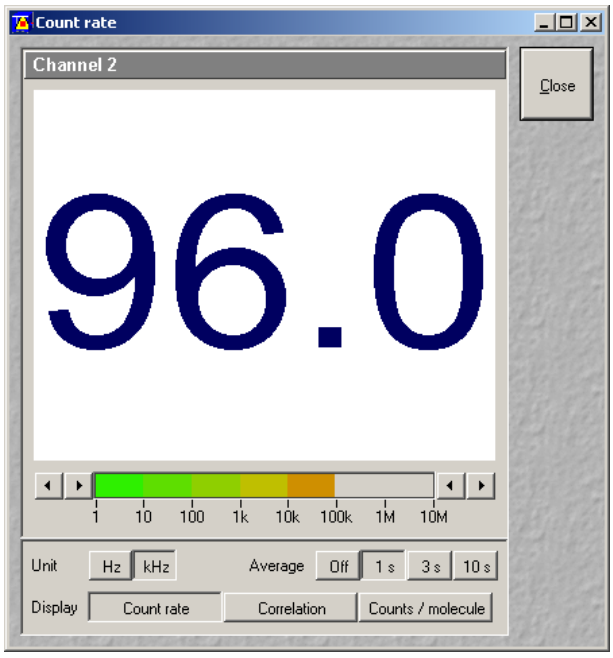


Fig. 4-33 Count rate window

Furthermore you can open the **Laser Control** window via **Laser** button to switch on / off the necessary lasers.

- Activate / deactivate the laser line via mouse click.
- If necessary switch on / off the lasers using the **Laser** button. When finished, close the **Laser Control** window.
- Set the laser attenuation using the sliders. For this purpose, open the **Count rate** window by clicking the **Count rate** button on the right-hand side of the **Measurement** window.

A good starting point is to set the intensity in such a way that a count rate between 50 kHz and 200 kHz is obtained.

- For most dyes, the Counts/Molecule setting should be optimized in a second step to a value just under its maximum by changing the laser power.

If carriers of different slide thickness are employed the Counts / Molecule setting should be optimized by using the correction ring on the lens.

The correction ring is turned counterclockwise or clockwise until a maximum value is obtained. The correction ring should also be used for adjusting the Counts / Molecule setting whenever the immersion media is changed. This is especially important in cases where the refractive index of the immersion media is different from that of the sample.

- When finished, close the **Count Rate** window using the **Close** button.

The **Close** button will close the **Laser attenuation** window and returns to the **Beam Path** panel.

The **Laser** button will open the **Laser control** window (see Fig. 4-32). The **Lasers** panel shows the types, excitation wavelengths and operating statuses of the lasers available.

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

- Click on the **Laser** button in the **ConfoCor 3** 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.

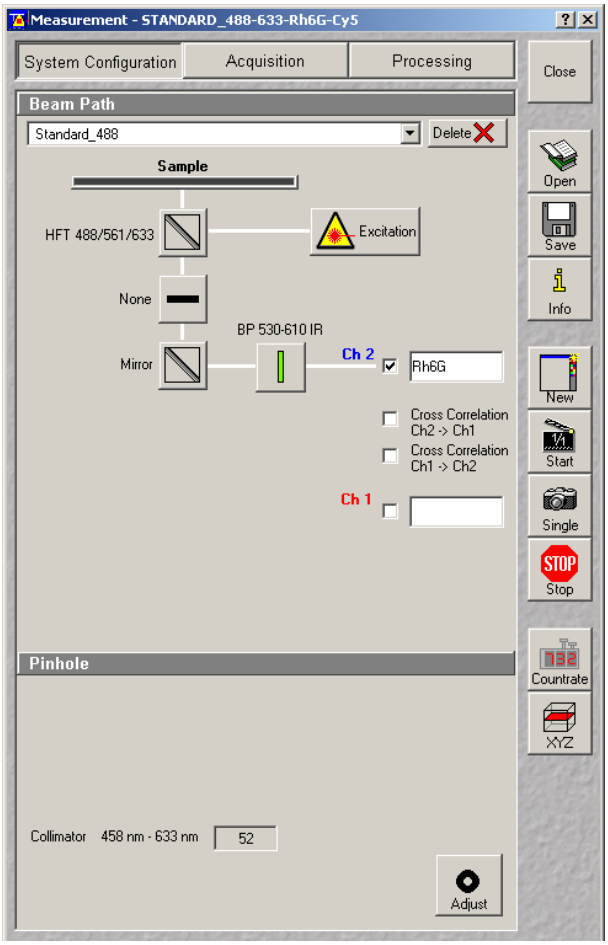


Fig. 4-34 **Measurement window with System Configuration panel activated**

Lasers panel (upper)

List of available lasers, including the display of relevant wavelengths and switching status. Selection of the laser to be switched on / off and setting of the laser output is performed in the subordinate setting panel.

Laser settings panel (lower)

Switch on / off the required laser or set Standby operation. Display Maximum Power, Wavelength, Status and Tube Current (only Enterprise and Argon) of the relevant laser. Set the laser output for Argon.

(c) 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 Ar-multiline laser:

- Click on the **Standby** button.
  - Wait for the laser to heat up, until the **Status ready - Standby** message appears.
- 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.

- 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.

This applies to HeNe lasers:

- After selecting the laser, click on the **On** button.
  - The required laser for image acquisition is now available.
- You can switch on an off lasers and set the tube current if applicable.

☞ Note that the Argon laser must be first set into standby and after warming can be switched on. The **Close** button returns to the **Laser attenuation** window.

(d) Procedure for defining a new beam path configuration

- First tick the laser or lasers (in case of cross -correlation) you need for excitation of your sample.
- Then choose a (main) dichroic beam splitter which should correspond to the chosen laser(s). Click on the **Beam Splitter** symbol  and a list box will appear. Then click on the beam splitter you need.
- Select an infrared block filter in case you need one with NLO applications by clicking on the corresponding  button.

☞ Please note that you can use IR lasers for FCS, there is, however, no specifications for the performance of the instrument for these excitation.

- Select the second beam splitter in a similar way to the first one.
  - For cross correlation, choose an appropriate filter like the NFT 488/633 beam splitter.
  - For auto correlation measurements in detection channel 1select **Plate** in the second beam splitter position.
  - All auto correlation measurements in detection channel 2select **Mirror** in the second beam splitter position.

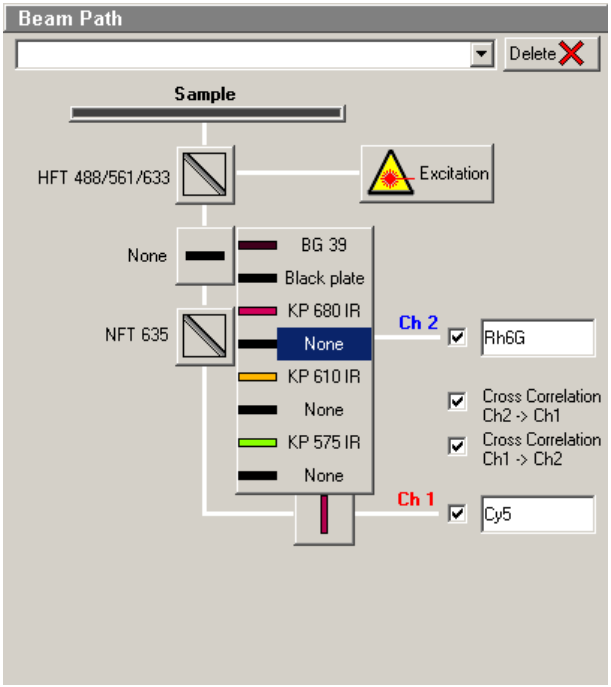


Fig. 4-35 IBF Infrared Filter list box open

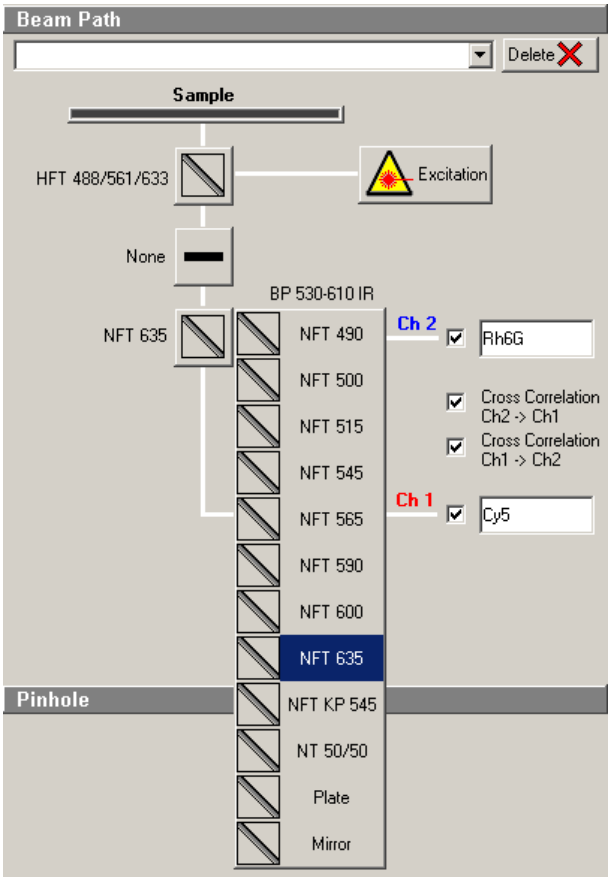


Fig. 4-36 NFT Secondary Beam Splitter list box open

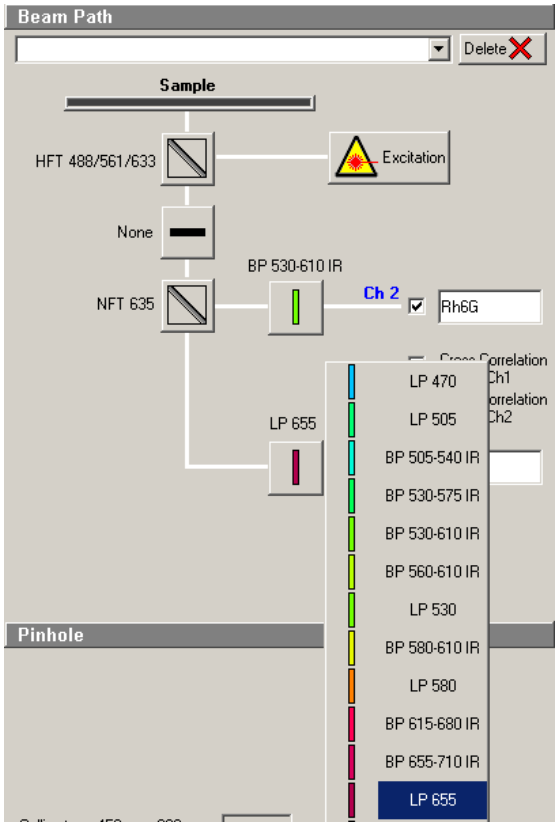


Fig. 4-37 Emission Filter 1 list box open

- Click on the **Emission Filter** symbol(s)  to get the list box allowing you to select the emission filters you need.

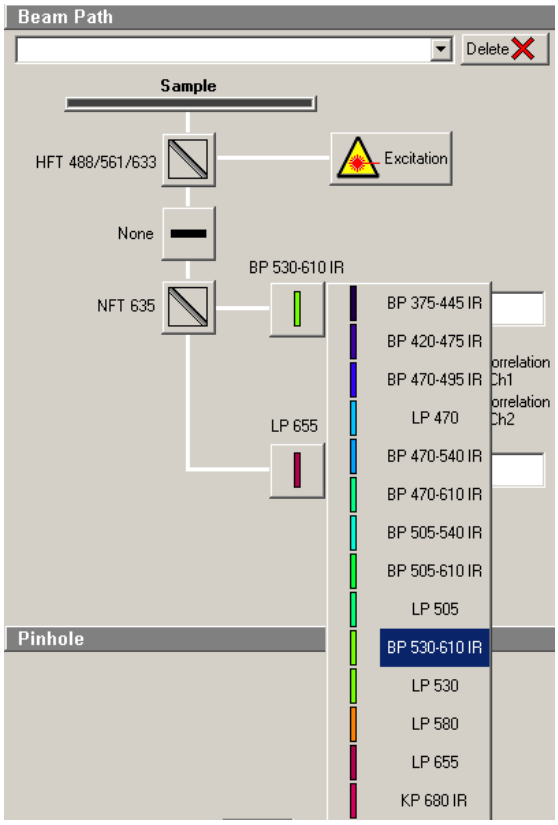


Fig. 4-38 Emission Filter 2 list box open

- It is recommended to give the channel a name which is easy to remember.

(e) Save a new beam path configuration

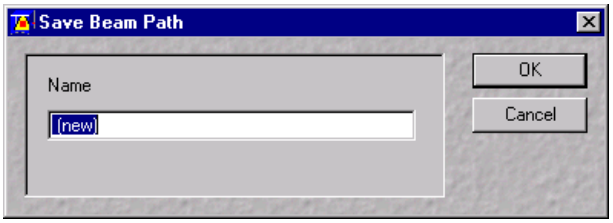


Fig. 4-39 Save Beam Path window

- Click the **Save** button to save the new beam path.
  - The **Save Beam Path** window appears.
  - Activate **Only Beam Path**
- Choose a memorable name for the new beam path which you are likely to remember.
- Click on **OK** to save the new beam path.

☞ Before you can use a newly defined beam path, you have to adjust the pinhole(s) for this particular beam path.

(f) Delete a beam path configuration

A no longer required beam path can be deleted as follows:

- Select the configuration to be deleted from the **Beam Path** selection box.
- Click on the **Delete** button. Confirm the deleting in the following window by clicking **OK**.
- A beam path can only be deleted if it is not used by any method. If you intend to delete such a beam path, you will have to delete the corresponding method first.

(g) Pinhole panel

The **Pinhole** panel (Fig. 4-40) allows you to set the pinhole diameter. It also displays the current Collimator position. The wavelength window for the respective **Collimator** is indicated to the left of the position window.

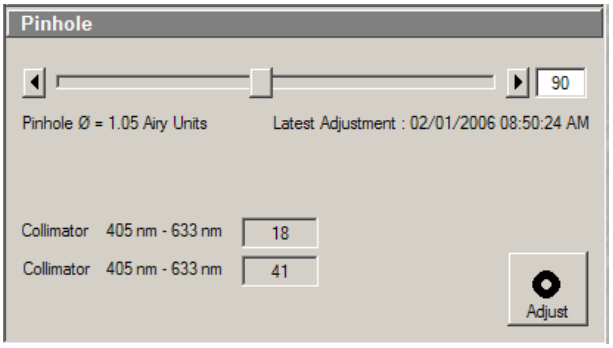


Fig. 4-40 Pinhole panel for ConfoCor 3

(h) Pinhole Diameter

In the **Pinhole** panel you can set the pinhole diameters for channel 4 of the LSM using the slider or the input box (see Fig. 4-40).

- It is recommended to set the pinhole diameter to a size corresponding to the used excitation wavelength that corresponds to 1 AIRY unit:
  458 nm 66 µm
  488 nm 70 µm
  514 nm 74 µm
  543 nm 78 µm
  633 nm 90 µm

☞ Pinhole sizes are given in µm and AIRY units.

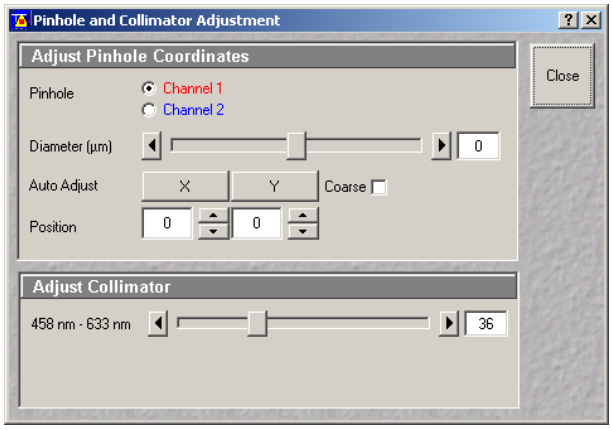


Fig. 4-41 **Pinhole and Collimator Adjustment window**

(i) Pinhole Adjustment and Collimator Settings

Pressing the **Adjust** button will open the **Pinhole and Collimator Adjustment** window. The window has two panels, one for pinhole adjustment (upper) and one for the collimator settings (lower) (see Fig. 4-41).

4.4.8 Adjust Pinhole Coordinate Panel

The pinhole is adjusted using a dye solution. The general approach is to move the pinhole cyclically in x and y until the intensity maximum is found. For each excitation wavelength a suitable dye must be used. We recommend:

- Rhodamine 6G for 458, 488 and 514 nm
- Tetra-Methyl-Rhodamine (TMR) for 543 nm and
- Cy 5 for 633 nm.

It is also recommended to work with a relatively concentrated solution (10^{-5} mol) and low laser power to achieve smooth intensity curves.

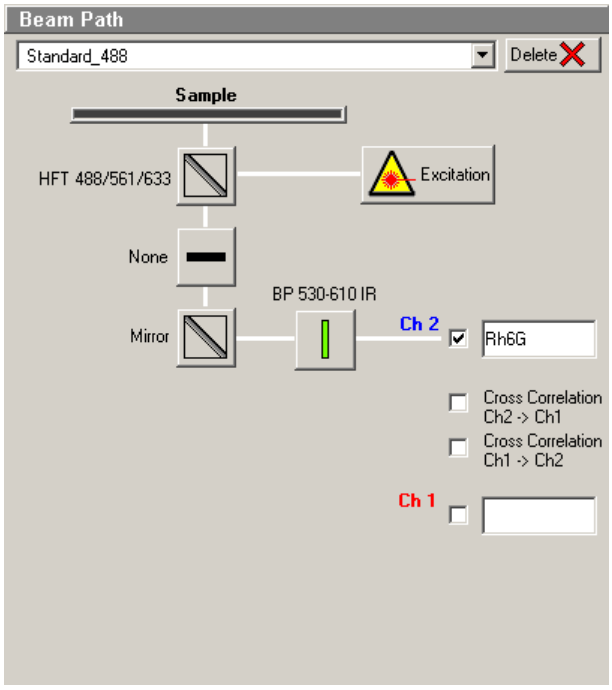


Fig. 4-42 **Beam Path panel**

- Click on the **Adjust** button in the **Pinhole** panel of the **Measurement** window.
  - This opens the **Pinholes and Collimator Adjustment** window.
- Click on the **Close** button to quit the window.

4.4.8.1 Beam Path Panel

Here you can select the beam path for pinhole adjustment. The date of the last pinhole adjustment is shown in the **Last Adjustment** display box.

- Select the beam path in the selection box.
  - The selected beam path appears in a drawing.
  - For the definition of a new beam path, see section 4.4.7 System Configuration.

4.4.8.2 Adjust Coordinates Panel

The **Adjust Coordinates** panel is used to adjust the pinhole of channel 4 of the LSM. Adjustment can be performed manually or automatically. Manual adjustment is not recommended, since the relevant procedure is very complex.

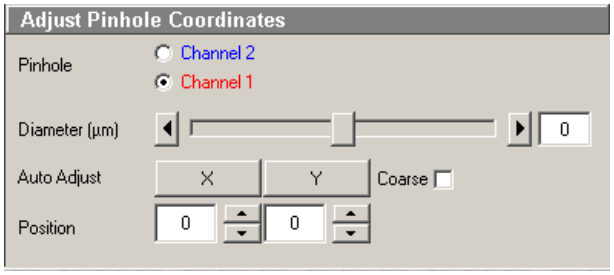


Fig. 4-43 Adjust Coordinates panel

☞ Please note that there is only 1 pinhole for both channels. You can adjust the pinhole to either channel. The values for the pinhole alignment done with the last channel will be stored and used.

Following functions are available:

Pinhole

Selection of the channel for adjustment.

Diameter

Setting of pinhole diameter to the used wavelength (see Fig. 4-43).

Auto Adjust buttons:

- **X fine / X coarse**
  Starts the automatic coarse or fine adjustment for X direction depending on the activation / deactivation of the **Coarse** check box.
- **Y fine / Y coarse**
  Starts the automatic coarse or fine adjustment for Y direction depending on the activation / deactivation of the **Coarse** check box.

☞ Z-positions can be changed by altering the Collimator position

Coarse check box

If ticked, the coarse adjustment for X or Y is performed, if not ticked, the fine adjustment for X or Y is performed.

Position input boxes

Display / input of the pinhole position in X and Y.

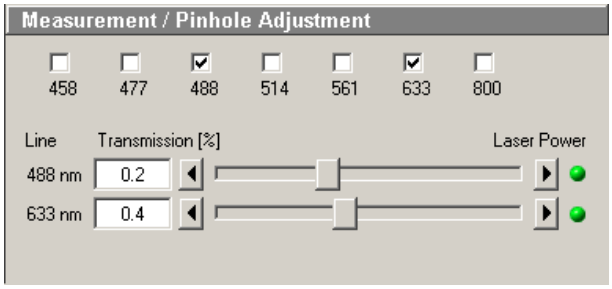


Fig. 4-44 **Laser control window for measurements and pinhole adjustment**

4.4.8.3 Excitation Panel

The **Excitation** panel allows you to activate / deactivate the wavelengths via check boxes and to set the laser attenuation (%) using the sliders. Furthermore you can open the **Laser Control** window via **Excitation** button to switch on / off the necessary lasers (see Fig. 4-42).

The **AOTF Dampening Factor (%)** display box allows you to reduce the overall power by factors of 1, 10, 100 and 1000.

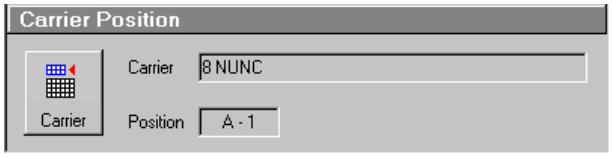


Fig. 4-45 **Carrier Position panel**

4.4.8.4 Carrier Position Panel

In the **Carrier** and the **Position** display boxes, the carrier and the currently selected position are shown.

If the carrier or the position have to be changed for adjustment use, the **Carrier** button to open the **Carrier Position** panel (see Fig. 4-6).

To adjust the pinhole proceed as follows:

- Mount a carrier with a suitable dye solution and orient the sample stage as described in section 4.4.1 Open File.
- Click the **Adjust** button in the **Pinhole** panel of the **Measurement** window. The **Measurement / Pinhole Adjustment** window will appear (Fig. 4-44).

In the **Beam Path** panel of the **Measurement** window you will see a drawing of the current beam path. Select the beam path you want to adjust in the **Beam Path** selection box in the **Beam Path** panel of the **Measurement** window.

- Select the test sample. Click on the **Sample Carrier** button in the **Positions** panel of the **Acquire** window. Then select the sample by mouse click.
- Use the slider in the **Laser Control** window to set the laser intensity to minimum.
- Click on the **Count Rate** button at the right-hand side. This will open the **Count Rate** window.
- Then cautiously increase the laser intensity until a count rate of about 100 kHz is indicated.
- Close the **Count Rate** window.
- Set the pinhole diameter to a size corresponding to the used excitation wavelength:
  458 nm 66 µm
  488 nm 70 µm
  514 nm 74 µm
  543 nm 78 µm
  633 nm 90 µm

- Select the pinhole you want to adjust by clicking on the corresponding **Ch1** or **Ch2** radio button in the **Adjust Pinholes Coordinates** panel of the window.

☞ In the case of a cross correlation beam path, adjust the **one** pinhole to either of the **two** channels.

Each of the pinhole axes must be adjusted separately. If the **Coarse** check box is activated, the corresponding axis buttons will be labeled **X Coarse** and **Y Coarse** respectively and the pinhole will travel over the maximum range for each axis.

If the **Coarse** check box is deactivated, the buttons will be labeled **X Fine** and **Y Fine** respectively. In this case the pinhole travel will be limited to speed up adjustment.

☞ If the pinholes are adjusted for the first time, the coarse adjustment must be performed first and then the fine adjustment, each time for X, Y and Z (only coarse). For subsequent readjustments, the fine adjustment is normally sufficient.

- Perform the pinhole adjustment in the following sequence:
  X fine; Y fine

If one of the above buttons, e.g. **X Coarse** or **X Fine** etc., is clicked, the instrument will behave as follows:

– The **Automatic Pinhole Adjustment** window will appear (Fig. 4-46). It is possible to exit the process using the **Cancel** button. When the process is finished, quit with **OK**.

– In the Count Rate/Position diagram, the black line corresponds to the measured intensity (count rate). The red line corresponds to the intensity curve fitted from it to find the optimum pinhole position.

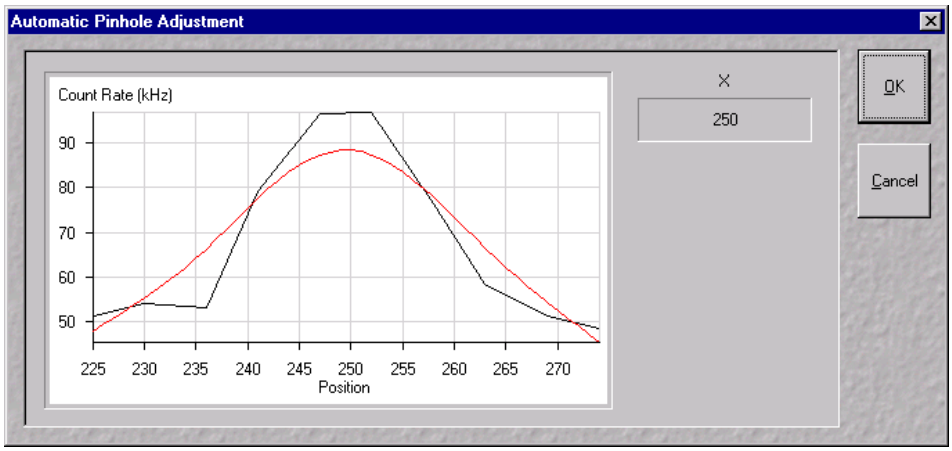


Fig. 4-46 Automatic Pinhole Adjustment

- Repeat the procedure for all axes.
- You will find peaks for x-Coarse, and y-coarse, in any case.
- A click with the right mouse button on the diagram opens the context menu. Selection of the option **Copy to clipboard** or **Export data to file** permits the data to be copied to the clipboard or saved directly in an ASCII file.

When the whole pinhole adjustment is completed, close the dialog with **Close**.

☞ You might be able to increase count rate by adjusting the collimator in the **Adjust Collimator** The position of the collimator influence the z-settings.

Fig. 4-47 Adjust Collimator panel

4.4.9 Adjust Collimator Panel

The **Adjust Collimator** panel permits the setting of the collimator for FCS measurements.

Under normal conditions, the collimator settings should not be changed.

In some cases when the pinhole is close to the limit of the pinhole range for a certain wavelength it might be useful to alter the collimator settings carefully.

☞ This function should be used with extreme care. An unsuitable collimator setting can render the pinhole adjustment impossible.

- To change the collimator setting, click the **Adjust** button in the **Pinhole** panel of the **Measurement** window (see Fig. 4-49).
- The **Collimator** tab will appear in the **Adjust Collimator** panel.

Now the new collimator settings for the **Collimator VIS** or **Collimator IR** (if applicable) can be entered either by drawing the slider, clicking on the arrows or directly entering a new number.

☞ If the collimator setting is changed, an information is given indicating the previous position as long as the x and y coordinates of the pinholes are not changed.

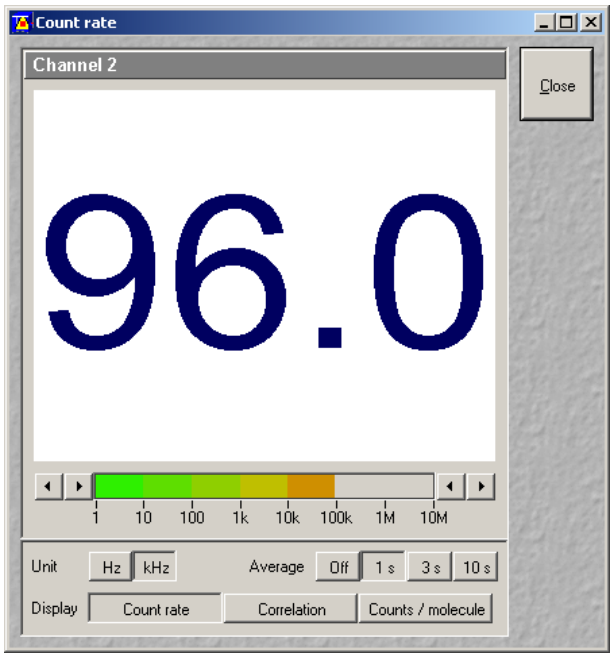


Fig. 4-48 Count rate window

- Set the laser attenuation using the sliders. For this purpose, open the **Count rate** window by clicking the **Count rate** button on the right-hand side of the **Count rate** window.

A good starting point is to set the intensity in such a way that a count rate between 50 kHz and 200 kHz is obtained.

- For most dyes, the Counts/Molecule setting should be optimized in a second step to a value just under its maximum by changing the laser power.

If carriers of different slide thickness are employed the Counts / Molecule setting should be optimized by using the correction ring on the lens.

The correction ring is turned counterclockwise or clockwise until a maximum value is obtained. The correction ring should also be used for adjusting the Counts / Molecule setting whenever the immersion media is changed. This is especially important in cases where the refractive index of the immersion media is different from that of the sample.

- When finished, close the **Count Rate** window using the **Close** button.

4.4.10 Acquisition

- Click on the **Acquisition** button on top of the **Measurement** window.
  - The **Acquisition** subwindow appears on the screen.

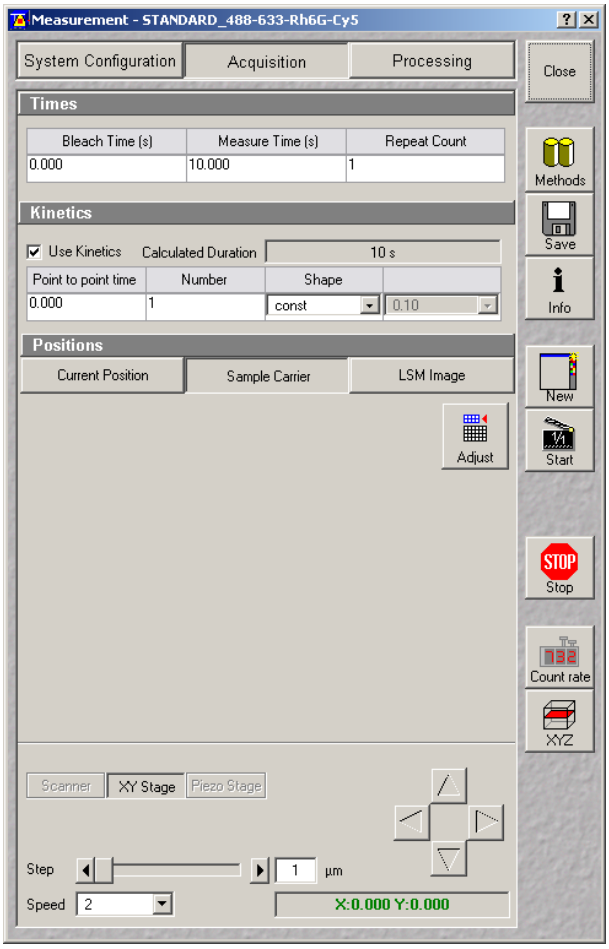


Fig. 4-49 Measurement window, Application

4.4.10.1 Times Panel

In the **Times** panel you can set the values for the **Bleach Time**, **Measure Time** and **Repeat Count** (see Fig. 4-50).

Bleach Time:

Bleach Time is the time prior to the measuring procedure during which the laser already has an effect on the sample. The **Bleach Time** is taken into consideration only **once** (at the beginning of a measuring cycle).

Measure Time:

Measure Time is the period of **one** measurement.

Repeat Count:

The **Repeat Count** value determines the number of measurements, i. e. how often **Measure Time** is to be performed in a row.

- Set the **Bleach Time**.
  Start with the **Bleach Time** of 0 s. If the signal decreases during the subsequent measurement, this might be caused by bleaching, as a remedy, you can prebleach the sample before measurement by setting the **Bleach Time** to a non-zero value. If no **laser** check box was activated, no bleaching occurs, but the measurement will be delayed for the bleach time. Bleaching occurs with the lasers checked and the power defined by the AOTF setting under **System configuration** in the **Measurement**. If the box is checked, the laser power defined by the slider is used for each selected laser.

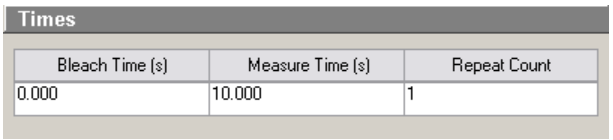


Fig. 4-50 Times panel

- Set the **Measure Time**.
  If you do not have any idea about the behavior of your sample, start with a measurement time of about 20 seconds. Depending on the signal-to-noise ratio of your correlation curve, decrease or increase the measurement time. Apart from that, set the measurement time to the correct value right from the beginning. As a rule of thumb, the measurement time should be a 1000 fold the diffusion time of your molecule under investigation.
- Set the **Repeat Count**.
  Start with **Repeat Count** set to 1. If you want to obtain information about the variation of your fitted values later on, increase the repeat count to produce a measurement series. 10 times is a good value to start with

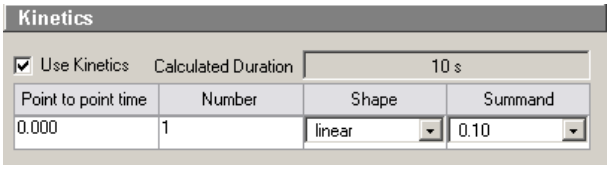


Fig. 4-51 Kinetics panel

4.4.10.2 Kinetics Panel

In the **Kinetics** panel you can set kinetics parameters.

- If a kinetics measurement is required, tick the **Kinetics** check box.

The input boxes **Point to Point time, Number, Shape** and **Void** (const. Shape) / **Summand** (linear shape) **/ Factor** (exponential shape) permit entry of the time spacing between consecutive and the number of measurements.

☞ Please note that the hierarchy in which measurements are performed is channel – repetition – position – kinetics. For example all the selected chambers or positions are measured once in one cycle in the determined order. We will refer to a cycle as the period between the start and end of a measurement including all positions.

Depending on these four parameters, The total measurement time, including the time the system needs to change position, will be displayed in the **Calculated Duration** display box.

☞ Note, if the **Point to Point time** is chosen too short compared to the needed measurement time, for example only 1 s if the measurement time is 10 s with 1 repetition selected, than the system will automatically calculate the minimal needed **Point to Point time**, in the example 10 s.

The **Number** box allows you to select, how many cycles you want to perform. One cycle consists of the measurement protocol defined in the times window and the repeats for all positions.

The **Shape** and **Void / Summand / Factor** selection boxes allow you to choose from three possibilities of distributing the time spacing between single consecutive cycles.

const:

The period between two successive measuring cycles is constant and will be exactly the time selected in the **Point to Point time** selection box. For example, if 20 is selected, the start of each measurement cycle is delayed by 20 s from the end of the previous one. In this case the **Void / Summand / Factor** box is void and inactive.

Definition of variables:

Point to Point time of the measurement = p_i (given start value=p_0)

Start time of the measurement: $p_{t,i}$

Number of cycles: n

The cycle count = i (note: i starts with 0; so for first cycle i = 0; or general i = n-1)

Summand/factor = f

With the definition of variables for **const**: $p_i = p_0$; $p_{t,I} = i \times p_0$; $i > 0$

lin:

The period between two successive measuring cycles as specified in the **Point to Point time** selection box is increased linearly by a summand of which the initial value is defined in the **Summand** selection box. With the definitions of variables: $p_i=p_0+(i-1) \times f$; $p_{t,i}= i \times p_0 + (i-1) \times f$; $i>0$

exp:

The period between two successive measuring cycles as specified in the **Point to Point time** selection box is increased by an exponential factor of which the initial base is defined in the **Factor** selection box.

☞ Note, that the first measurement is therefore assigned a 0 for the exponent.

With the definitions of variables: $p_i = p_0 \times f^{i-1}$; $p_{t,i} = p_0 \times (\Sigma f^{i-1})$; $i > 0$

- Enter the time spacing you want to start with in the **Point to Point time** input box of your kinetics measurement.
- Enter the number of repeated cycles in the **Number** box
- In the **Shape** selection box, set one of the shape options **const**, **lin** or **exp**.
- Enter a number in the **Summand/Factor** selection box.

☞ Note, if **const** was selected, the **Summand/Factor** selection box is inactivated (void).

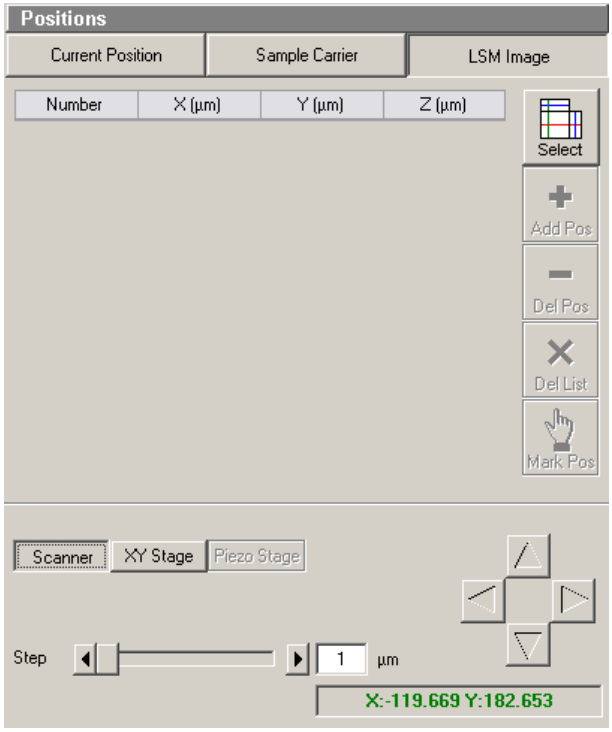


Fig. 4-52 **Positions panel with LSM image mode activated**

4.4.10.3 Positions Panel

In the **Positions** panel you can select the carrier and the sample position by the stage or by the scanners.

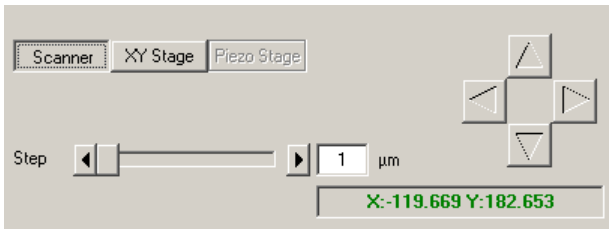


Fig. 4-53 **Positions panel, Scanner**

4.4.10.4 Moving the Scan Mirrors

To activate scanner positioning press the **Scanner** button at the bottom of the panel. The Scanner Control options will appear (see Fig. 4-53).

- Move the scanning mirrors in the appropriate position by clicking on the arrow keys.

Each mouse click moves the scanners in the appropriate direction by one step.

- Set the required step width via the **Step** slider or the relevant input box.

The position of the scanner is indicated in the lower X,Y display box.

☞ If the **Scanner** button is activated, all positioning will be done via the scanning mirrors.

4.4.10.5 Moving the Microscope Stage

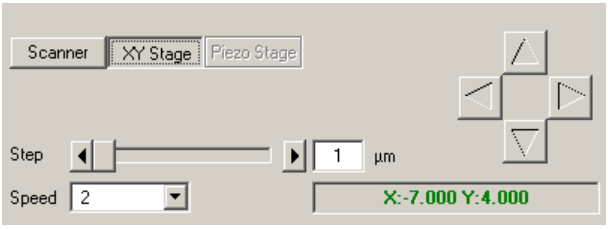


Fig. 4-54 Positions panel, XY Stage

To activate stage positioning click on the **XY Stage** button. In this case the stage control options will be available (see Fig. 4-54).

- Move the stage in the appropriate position by clicking on the arrow keys.

Each mouse click moves the microscope stage in the appropriate direction by one step.

- Set the required step width via the **Step** slider or the relevant input box.

The travel speed of the microscope stage can be set to steps **1**, **2** and **3**.

Step 2 is preferably used for standard positioning.

Step 1 (slow speed) should only be used for very precise positioning, since positioning for longer paths requires more time.

Step 3 is suitable if large vessels are used, since precise center positioning only plays a minor role in such cases.

- Select the required travel speed of the microscope stage via the **Speed** selection box.

☞ When the required chamber is approached by activation of the arrow keys, the relevant chamber is displayed in white with a black frame, since normally the defined position cannot be set in this way.

☞ If the Piezo Stage Option has been chosen the Piezo Stage instead of the normal XY stage will be used.

☞ If the **XY Stage** button is activated, all positioning will be done via the scanning stage.

There are three ways to position the laser beam with respect to the sample by pressing either of the following buttons:

- **Current Position:** no automated positioning possible. Sample has to be manually positioned in respect to the laser beam
- **Sample Carrier:** automated positioning used for maneuvering between wells of a carrier
- **LSM image:** automated positioning using an LSM image

4.4.10.6 Current Position

– If the **Current Position** is pressed, The **Positions** panel changes to allow to activate the **Crosshair** function. If you press the **Crosshair** button, a crosshair will be displayed in a scanned image. You can position the crosshair by clicking into the image with the left mouse button. You can inactivate the crosshair if you press the **Crosshair** button again. You can lock that position by pressing the **Lock** button. Pressing the **Lock** button again, will unlock the crosshair.

– When using this method, FCS measurements are performed on a fixed position with parked scanners and without automatic table movement. In this case the structure of interest must be moved to the position manually under LSM control prior to FCS measurements.

☞ The crosshair serves only the function to mark a site of interest. But the laser will stay with the scanning mirrors parked. If you want to measure with stage movement, the crosshair can be used to indicate the position of the laser, and the site of interest can be moved beneath it.

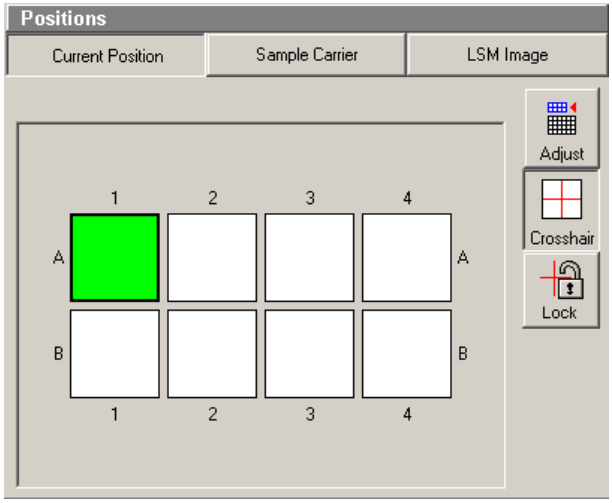


Fig. 4-55 **Current position panel with activated crosshair**

4.4.10.7 Using the Crosshair Function

In this mode you have to position your sample manually.

- Activate the **current pos** button via mouse click and click into the image.
  – The crosshair appears in the LSM scan image. Place the cross at the position, where the Laser beam is positioned as determined in section 4.4.7 System Configuration. We recommend that having set the crosshair you do not close that **Scan** window. Then the crosshair will be preserved within the new scan. The crosshair will be positioned on the site were you click with the left mouse button. If you have activated the **Lock** button, than the position will be fixed.
- Scan the image continuously and position the site of interest under the crosshair.

4.4.10.8 Using the Carrier

If you press the **Adjust** button, the Carrier Position window will open. If you adjust the carrier (see section 4.4.12 Loading and Configuring Sample Carriers), than the carrier will be displayed in the current position window. You can now approach any well by clicking on it.

☞ The wells will be approached by the scanning stage. Even if the **Scanner** button is activated, selecting a well will automatically deactivate the **Scanner** and activates **the X,Y-Stage** mode. If you take an image of the well, than the position can be changed in **Scanner** mode within the well.

☞ This mode correspond to the **Sample Carrier** mode, if **single position** is selected. Regardless which well is selected, in the result table of the **FCS results** window the position is indicated as "1".

4.4.11 Sample Carrier

The **Sample Carrier** function opens the **Carrier Position** options. It is used for selecting the carrier and the sample, for orienting the sample in x- and y-direction and for setting in Z position.

- Click on the **Sample Carrier** button in the **Positions** panel.
  - The sample select options pop up (Fig. 4-56).

☞ Before the sample can be positioned make sure that the microscope stand is set up for FCS measurements and that the appropriate laser(s) is (are) switched on.

- Close the **Sample Carrier** window by clicking one of the other positioning options.

☞ Note, the carrier is only visible, if it was adjusted in the **Carrier Position** window that opens by pressing the **Adjust** button.

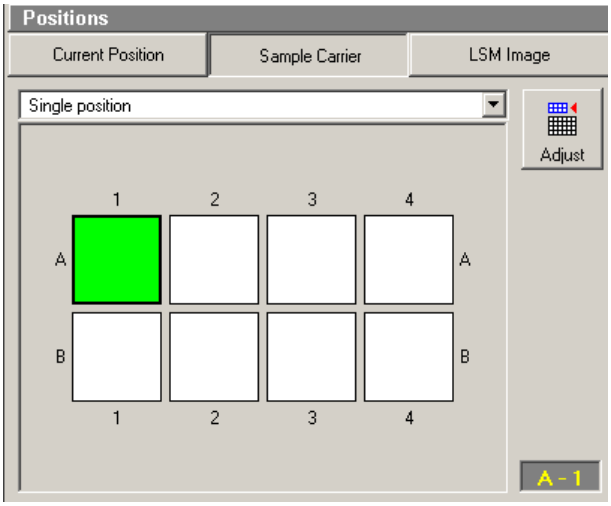


Fig. 4-56 Positions panel, sample carrier adjusted

4.4.12 Loading and Configuring Sample Carriers

Press the **Adjust** button. This opens the **Carrier Position** window (see Fig. 4-57).

Window contains two panels:

- The **(X,Y) Stage** panel
- The **Z (Focus)** panel

On the right hand side you have the following buttons:

Close Pressing the **Close** button will close the **Carrier Position** window.

Laser Pressing the **Laser** button will open the **Laser Control** window.

Micro Pressing the **Micro** button will open the **Microscope Control** window.

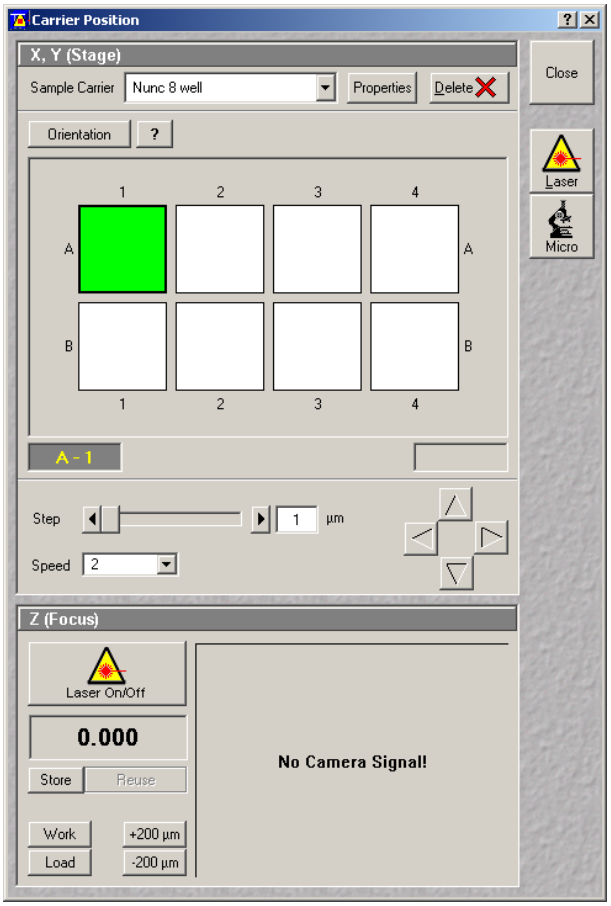


Fig. 4-57 Carrier Position window

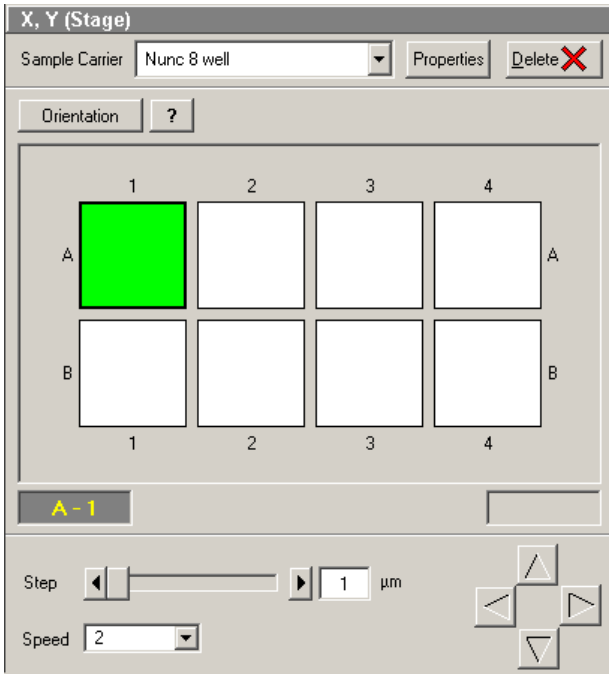


Fig. 4-58 X, Y (Stage) panel

(1) X, Y (Stage) panel

In the **X, Y (Stage)** panel you can select the sample carrier and orient the sample carrier in x- and y-direction.

(a) Selecting the sample carrier

- Select the sample carrier from already stored ones in the **Sample Carrier** selection drop down menu. This will load in the defined configuration.
- You can delete stored carrier configuration by pressing the **Delete** button. This will delete the currently selected carrier configuration.

(b) Defining a new Carrier

- Click the Properties button. The Carrier Properties window will open.

The **Properties** windows allows new / existing sample carriers to be created / edited and saved.

- Click on the **Properties** button in the **Carrier Position** window.
  - The **Carrier Definition** window is opened.

Columns - Number input box

Setting of the number of columns for the sample carrier.

Columns - Distance input box

Setting of the distance between the chambers of the column.

Rows - Number input box

Setting of the number of rows for the sample carrier.

Rows - Distance input box

Setting of the distance between the chambers of the row.

Close button

The **Carrier Definition** window is closed.

Save button

For saving the new or edited sample carrier.

Delete button

Deletion of an existing sample carrier.

4.4.13 Selecting a Chamber at the Sample Carrier

A scheme of the selected sample carrier is shown in the center part of the panel. The lines of the chambers are marked with letters, and the columns with numbers.

You have different options to select chambers in a loaded carrier by choosing an option from the positions drop down menu (see Fig. 4-59).

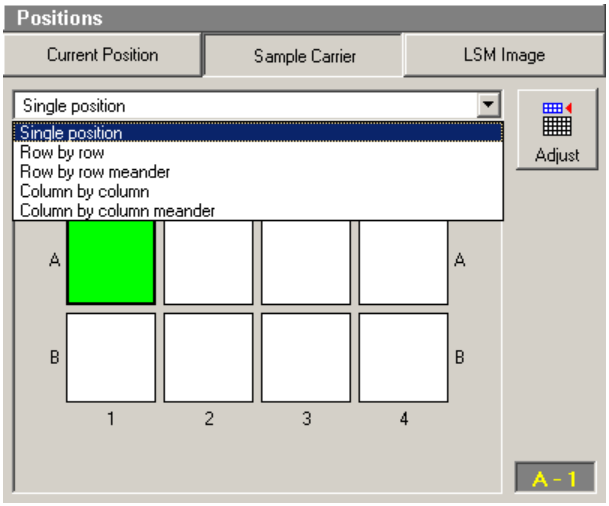


Fig. 4-59 Positions drop down menu

4.4.13.1 Single Position

Select from the drop down menu **Single Position**.

In the **Positions** panel (see Fig. 4-60) you can select the chamber you want to take the measurement in (only one is selectable).

- Click on the chamber you want to use. The motorized stage moves the selected chamber to the defined position under the objective.
- If you want to move to other positions within a chamber, click onto the respective chamber.

The chamber to be used for the X and Y alignment of the stage can be selected at a click of the mouse.

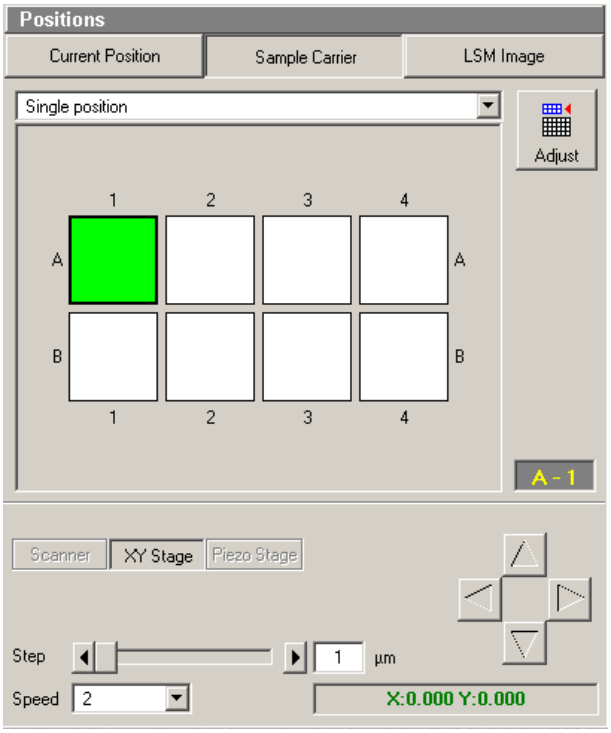


Fig. 4-60 Current position panel

☞ Note, that before clicking on a chamber you have to orient the carrier. For a non-oriented carrier, all chambers are displayed in white. Please orient first before you select a position. After orientation, the selected well will appear green. The position of the current well is displayed in the **left lower corner** (left field, e.g. **A - 2**). The current position of the mouse pointer is shown in the **right lower corner**.

- Select the chamber by clicking on it. The stage moves to that chamber of which the dimensions must be specified earlier. This well becomes green when the stage has finished movement.

☞ You can also use the arrow buttons or the joy stick to move the microscope stage, but in those cases, the orientation gets lost. The selected chamber will not any more be highlighted in green in this case. You can set the size of the steps to be performed via the **Step** slider (or the input box).

☞ Regardless which well is selected, in the result table of the **FCS results** window the position is indicated as "1".

The travel speed of the microscope can be set to steps **1**, **2** and **3**.

Step **1** (slow speed) should only be used for very precise positioning, since positioning for longer paths requires more time.

Step **2** is preferably used for standard positioning.

Step **3** is suitable if large vessels are used, since precious center positioning only plays a minor role in such cases.

- Select one of the three speeds from the **Speed** selection box.
- Select the step size from the Step selection box with the slider or numerically.
- The current coordinate position of the stage is indicated in the lower right corner in X and Y.

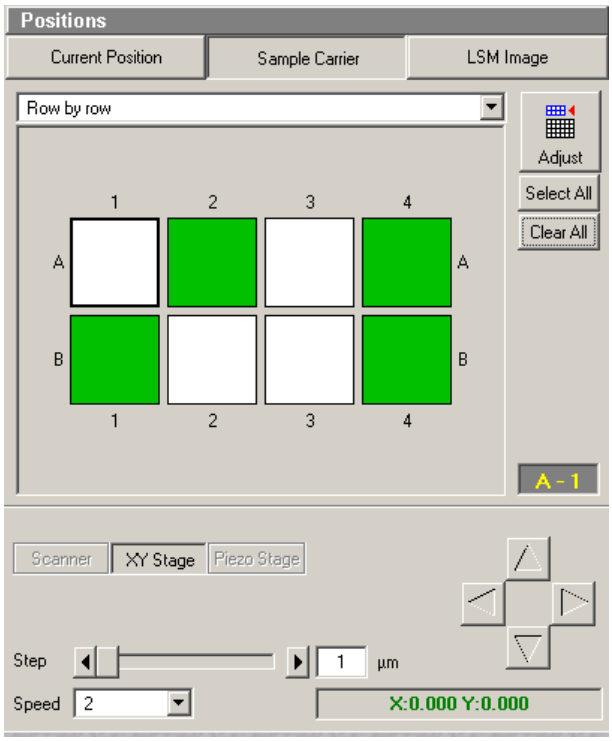


Fig. 4-61 Sample Carrier panel

4.4.13.2 Multiple Positions

Select from the drop down menu other than **Single Position**.

In the **Positions** panel you can select the chambers you want to take the measurement in (all are selectable, see Fig. 4-61).

The samples to be measured can be selected in the schematic drawing of the sample carrier. Activated chambers will be highlighted in the drawing in green. In the **Sample Carrier** display box, the selected sample carrier is shown for information.

- Select the chambers and the work direction for the measuring procedure by loading the respective routine from the drop down menu.

Individual chambers can be selected / deselected by clicking on them with the mouse.

Alternatively, a whole block of chambers can be selected / deselected by drawing a frame which contains the centers of the selected or deselected chambers or by pressing the **Select All** / **Deselect All** buttons

If multiple samples are selected, the work direction can be chosen as well.

Following routines can be loaded:

Row by row. Measurement is performed row by row. Each row starts from the left site.

Row by row meander. Measurement is performed row by row. Rows start alternatively from the left and right. First row starts from the left.

Column by column. Measurement is performed column by column. Each column starts from the top.

Column by column meander. Measurement is performed column by column. Columns start alternatively from the top and bottom. First column starts from the top.

Following functions are available:

Select All button

Selects all chambers of the sample carrier.

Deselect All button

Deselects all chambers of the sample carrier.

Adjust button

Opens the **Carrier Position** window.

4.4.14 LSM Image

If **LSM Image** is activated, you can define positions for FCS measurements in a just scanned LSM image (Fig. 4-62).

The **Positions** list shows the numbers and coordinates of the selected positions in the LSM scan image.

Select button

Activates the cursor (crossline) to allow the definition of positions in the scan image. Shows or hides just selected positions if activated or not activated.

☞ Please note, if the **Scanner** button is pressed, the scanner will be positioned to the high-lighted position. If the **XY Stage** button is pressed, the stage will not be positioned to the high-lighted position. Only after triggering a measurement the stage will move to the indicated position and will go back to the initial position after the end of the measurement. If you want to obtain the count rate by pressing the **Count Rate** button at the indicated position, you have to start a measurement beforehand.

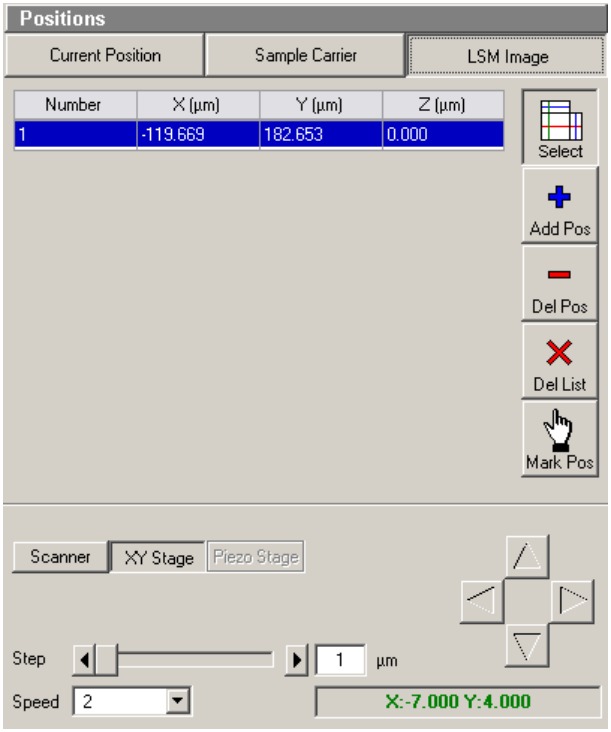


Fig. 4-62 LSM image panel

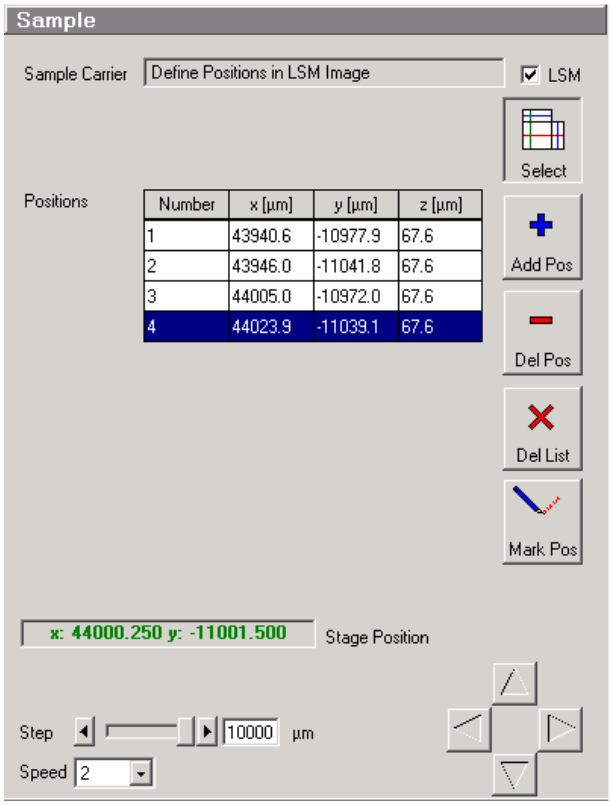


Fig. 4-63 **Sample panel for defining positions**

Add Pos button

Adds the position of the cursor to the **Positions** list. A crossline is set at the selected cursor position in the image. To select further positions click at the appropriate position in the image and then on **Add Pos**. If more than one position is selected the crosslines are getting current numbers.

Del Pos button

Deletes the selected (highlighted) position in the **Positions** list and the corresponding crossline in the scan image.

Del List button

Deletes all positions in the **Positions** list and all crosslines in the scan image.

Mark Pos button

Marks the selected positions as overlay elements in the scan image. The crosslines are also visible in the scan image if the **Select** button is deactivated or the **Method Measurement** window is closed.

☞ Please note, positioning is by scanning mirrors if the **Scanner** button or by stage, if the **XY Stage** button is pressed. If more than one position was selected, measurement is only taken at the highlighted position. Using the crosshair the mirrors do not move and positioning has to be done manually with the stage.

4.4.15 Processing

- Click on the **Processing** button on top of the **Measurement** window.
  - The **Processing** subwindow appears on the screen (see Fig. 4-64).

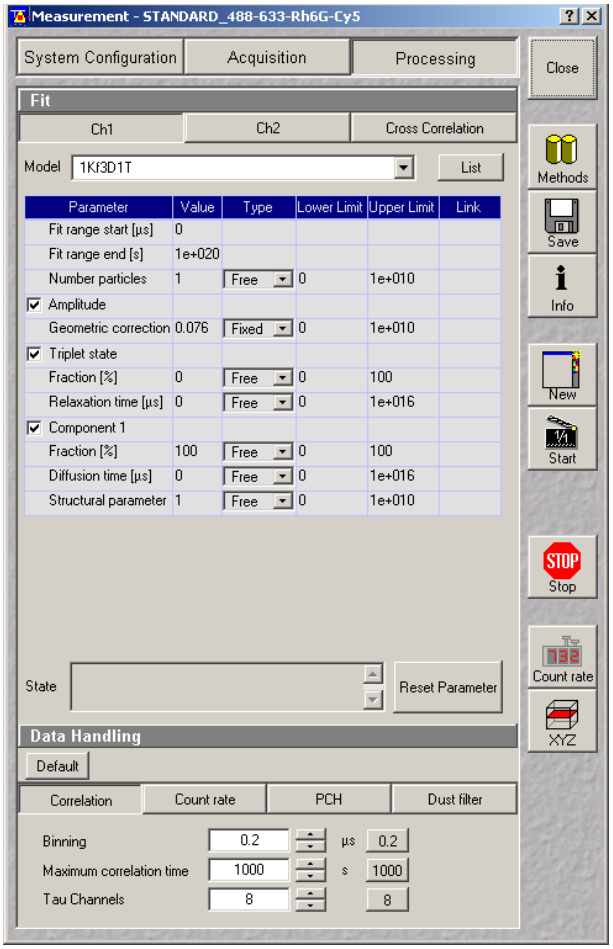


Fig. 4-64 Processing window

4.4.15.1 Fit Panel

The **Fit** panel of the menu allows you to load in a fit model and define, how the parameters are to be fitted.

The channel is selected by pressing either the **Ch1**, **Ch2** or **Cross Correlation** button, if applicable. To each channel a different Fit model can be assigned.

By pressing the **List** button, the Model list window will appear. You can close the window by pressing the **Close** button. You can delete the highlighted model by pressing the **Delete** button. You can modify the existing highlighted model by pressing the **Modify** button or you can define a new model by pressing the **New** button.

Load a predefined model by selecting the appropriate one from the **Model** drop down menu.

You can select and deselect parameters by checking / de-checking the check boxes of the **parameter** column.

You can type in values for the **Fit range start** and **Fit range end** values.

You can type in values for the parameters under the **value** column.

You can define the values of the various model parameters as **Start** values, or you can leave them **free** or **fix** them by selecting the appropriate routine from the pull down menu under the **type** column.

You can set **upper** and **lower limits** by typing the limits into the boxes under the **upper limit** and **lower limit** columns.

You can **globally link** parameters for different setting by selecting the appropriate routine from the pull down menu under the **global** column.

It is generally accepted that non-linear fitting procedures yield more reliable results when the number of free parameters is low. It is recommended to fix parameters which are known from independent measurements. Good candidates for fixing are diffusion times of the free dye and the free (i. e. not bound) partner, which had been determined in previous measurements and the structural parameter, that is an instrumental parameter.

☞ If you press the **Save** button and select All settings, the next time the method is called up data will be fitted to the settings defined in the **Fit** panel.

You can reset all manipulations by pressing the **Reset** button. The current status is displayed in the **Status** display window.

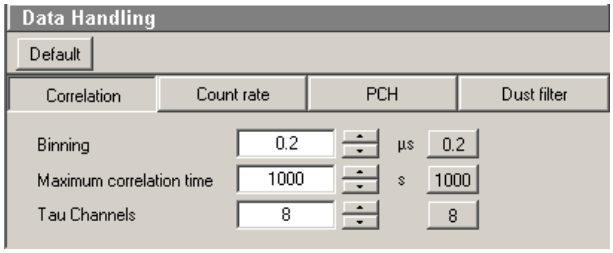


Fig. 4-65 Data Handling panel, Correlation

4.4.15.2 Data Handling Panel

The **Data Handling** panel allows you to select how certain measurements should be recorded. All changed settings can be reset to the default settings by pressing the **Default** button (see Fig. 4-65). You have different options.

(1) Correlation

Pressing the **Correlation** button will activate the correlation settings (see Fig. 4-65). Here you can set the initial binning time, the initial tau channels and the maximum tau time by adjusting the number in the **Binning**, **Maximum correlation time** and **Tau Channels** display boxes. You can either directly enter values or use the arrows. The units are indicated at the right site of the display boxes. Default values can be entered by pressing the **0.2**, **1000** and **8** buttons. Please note that with the default settings, the algorithm works the fastest.

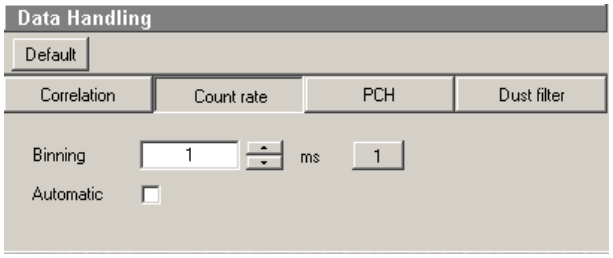


Fig. 4-66 Data Handling panel, Count rate

(2) Count-Rate

Pressing the **Count-Rate** button will activate the count-rate settings (see Fig. 4-66). You can either check the **Automatic** check-box, in which case dynamic binning will be applied, or you can de-activate the check box and enter a number of a constant binning time in the **Binning** display window. You can either type in values or use the arrows. Units are displayed at the right of the display box. The default value of 1 ms can be selected by pressing the **1** button.

(3) PCH

Pressing the **PCH** button will activate the photon counting histogram settings (see Fig. 4-67). You can either check the **Automatic** check-box, in which case 32 different binning times will be used, or you can de-activate the check box and enter a number of a constant binning time in the **Binning** display window. You can either type in values or use the arrows. Units are displayed at the right of the display box. The default value of 10 µs can be selected by pressing the **10** button.

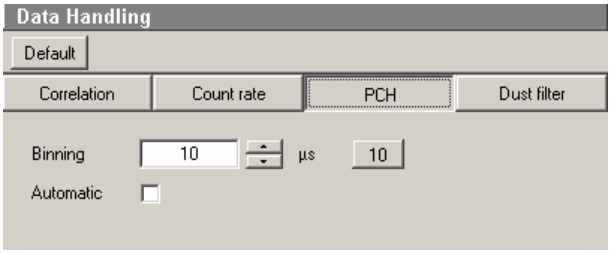


Fig. 4-67 Data Handling panel, PCH

☞ In automatic binning mode, binning starts with a value of 50 ns, which is doubled 32 times. So binning times are 50 x 2^n, with n=1 to 32. The histogram with the best dynamic range will be selected and displayed.

(4) Dust-filter

Pressing the **Dust-filter** button will activate the dust-filter settings (see Fig. 4-68). The **Dust-Filter** selection box allows you to activate an electronic dust filter that will be active in operation during the measurement. The threshold in % is set by typing or by using the arrows. All measurement points within a binned count rate time window having a deviation of more than the specified value from the average count rate will be cut out and not used for the correlation analysis. The default value of 10% can be activated by pressing the **10** button.

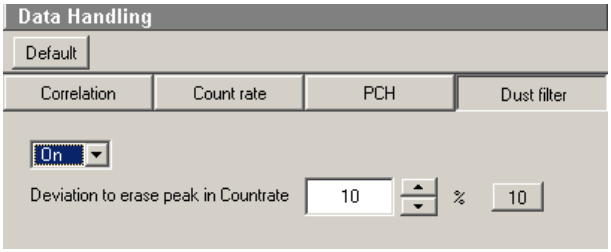


Fig. 4-68 Data Handling panel, Dust-Filter

☞ Please note that the cut off count rate is defined as the exceeding of the average count rate during a certain measurement period. Thus, the consecutive fast succession of low peaks might accumulate the same count rate as one high peak within a certain period of time and hence, the cut off is not defined by the peak height but rather by the counts / binning time.

4.4.16 Fitting the Correlated Data to Model Equations

In the following, the mathematical equations of the correlation functions and the fit equations will be more detailed. The acquired correlation functions must be fitted to models in order to retrieve meaningful parameters. It depends on the process, which model is the most appropriate. If the underlying process is known, the model can be chosen a priori. For example, if one studies diffusion in a membrane, a 2-D diffusion model should be applied. In other cases, the process is not known, for example, if one deals with free or anomalous diffusion. In this case, one can screen different potential models and look for the best fit taken into account the X^2 value. Often two models work nearly the same, for example, a two component free diffusion model can give you as satisfactorily a fit as a one component anomalous diffusion model and without prior knowledge on the system it will be impossible to decide, which is the better one. In principle,. models can be ruled out, if the fit does not work, however, a working model is only a potential candidate but does not signify it to be the correct one. Care should be taken to minimize the free parameters as much a possible to improve on the fit quality. It does not make too much sense to fit to three components without fixing parameters of at least one. If , for example, the diffusion time of a free ligand can be determined in a pre-experiment, that value should be fixed to reduce the number of floating parameters for the evaluation of the binding experiment to its receptor.

The software of the ConfoCor was designed to be flexible. That means that the user can define or assemble equations that do not make sense. Care should therefore be exerted and used formulas matched with the ones from literature to obtain meaningful results. Also, the presence of a model does not automatically mean, that the recorded data are of a quality that allows it usage. For example, anti-bunching requires a lot of care in data acquisition like long measurement times and cross-correlation to reduce dead times of the detectors and elimination of after-pulsing artefacts. It is in the responsibility of the user to set up his experiments accordingly.

4.4.16.1 The Correlation Function

The auto-correlation function is defined as follows:

$$G^{\delta I}(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t+\tau) \rangle}{\langle \delta I(t) \rangle^2} = \frac{\frac{1}{T} \cdot \int_0^T (\delta I(t) \cdot \delta I(t+\tau)) dt}{\frac{1}{T^2} \cdot \int_0^T (\delta I(t))^2 dt} = \frac{T \cdot \int_0^T (\delta I(t) \cdot \delta I(t+\tau)) dt}{\int_0^T (\delta I(t))^2 dt} \quad \textbf{(1a)}$$

or

$$G^{I}(\tau) = \frac{\langle I(t) \cdot I(t+\tau) \rangle}{\langle I(t) \rangle^2} = \frac{\frac{1}{T} \cdot \int_0^T (I(t) \cdot I(t+\tau)) dt}{\frac{1}{T^2} \cdot \int_0^T (I(t))^2 dt} = \frac{T \cdot \int_0^T (I(t) \cdot I(t+\tau)) dt}{\int_0^T (I(t))^2 dt} \quad \textbf{(1b)}$$

where $\langle\ \rangle$ denotes the time average and $\delta I(t) = I(t) - \langle I(t) \rangle$ describes the fluctuations around the mean intensity.

For long time average of I (no bleaching) the following relation exists:

$$G^{I}(\tau) = 1 + G^{\delta I}(\tau) \quad \textbf{(1c)}$$

Definition of the cross-correlation function

The formalism for the cross-correlation function is identical to the auto-correlation function, with the exception that the signal in one channel is not compared to itself, but to a signal in a second channel. Lets assign the indices "r" and "b" for the red and blue channel, respectively, than the cross-correlation function would read as follows:

$$G_X^{\delta I}(\tau) = \frac{\langle \delta I_b(t) \cdot \delta I_r(t+\tau) \rangle}{\langle I_b(t) \rangle \cdot \langle I_r(t) \rangle} = \frac{\langle \delta I_r(t) \cdot \delta I_b(t+\tau) \rangle}{\langle I_b(t) \rangle \cdot \langle I_r(t) \rangle} \quad \textbf{(1d)}$$

$$G_X^{I}(\tau) = \frac{\langle I_b(t) \cdot I_r(t+\tau) \rangle}{\langle I_b(t) \rangle \cdot \langle I_r(t) \rangle} = \frac{\langle I_r(t) \cdot I_b(t+\tau) \rangle}{\langle I_b(t) \rangle \cdot \langle I_r(t) \rangle} \quad \textbf{(1e)}$$

Note that the ConfoCor 3 calculated G^{I} functions. The acquired correlation functions are than compared to model equations.

4.4.16.2 Model Equations

In the following available equations used for the fits are given that define the accessible parameters. For some equations useful conversions to other parameters are listed as well. The total correlation is given by equation 2:

$$G_{tot}^{I}(\tau) = 1 + d + B \quad + A \cdot \prod_k \sum_l G_{k,l}(\tau) \quad \textbf{(2)}$$

where d is the offset, B the background correction, A the amplitude and $G_{k,l}(\tau)$ the correlation for a single process. The suffixes k and l signify correlation terms for dependent and independent processes, respectively, that are multiplied with or added to each other.

The total correlation is therefore an amplitude corrected for background, which is multiplied to the product of the single correlation terms that are dependent and hence convolute each other. Any offset can beaded to the correlation values. In cases, when the processes are independent from each other, the single correlations terms add up, for example in cases where there are more than one component all bearing the same label or of bunching terms that are independent from each other. If independent and dependent processes are present, all independent terms will add up and are multiplied with the dependent terms.

One can distinguish between different classes of processes: anti-bunching, bunching and diffusion. In addition, the corrected amplitude has to be added.

4.4.16.3 Amplitudes

The Amplitude of the correlation function is influenced by the offset, background and the number of particles in dependence of the geometric factor. The amplitude is also influenced by the process of correlation.

1) The "1"

In a normal correlation, the curve converges to 1, in case intensities I are correlated as is the case with the ConfoCor 3 software. Note that in other cases, if fluctuations δI are correlated, the correlation function converges to 0. If no bleaching occurs

$$G^{\partial I}(\tau) = 1 + G^{I}(\tau) \quad \textbf{(2)}$$

You can therefore easily convert $G^{I}(\tau)$ to $G^{\delta I}(t)$ values by adding a fixed offset of –1.

2) Offset d

$$d = c \quad \textbf{(3)}$$

c is any rational number that can be negative or positive.

In the ConfoCor software the offset can be a fit parameter or a fixed value.

In some cases, especially if very slow or immobile components are present, there can be a positive offset from 1. This offset can be taken into account by fitting to d. On the other hand, if the offset is known, it can be fixed. The offset will be added to each correlation value.

3) Background B

$$B = (1 - \frac{I_b}{I_t})^2 \quad \textbf{(4)}$$

where I_b is the background intensity and I_t is the total intensity.

The background in the ConfoCor Software is always a fixed value and never a fit parameter. This means that the background must be user defined.

Note that the background in this case refers to a non-correlating background. If there is no background intensity, B = 1, otherwise B < 1. The background can be determined by measuring an unlabeled solution or cell at the same settings than the real experiment and recording the count rate I_b. The real experiment with the labelled species will give I_t. A non-correlating background will result in a lower amplitude and hence overestimation of molecule numbers, if not corrected for. Note the squared correction term.

4) Amplitude A

$$A = \frac{\gamma}{N} = G(0) - 1 \qquad \textbf{(5a)}$$

where γ is the geometric factor accounting for the point spread function (PSF) and N the number of particles.

In the ConfoCor software γ can be a fit or a predefined fixed value. In case γ is a fit value N must be fixed in the fit procedure. N is normally a fit parameter.

Please note that γ takes different values for different fitting models depending on the assumed intensity distribution of the points spread function (PSF):

γ_C=1.000 (cylindrical),

γ_{2DG}=0.500 (2-D Gaussian),

γ_{3DG}=0.350 (3-D Gaussian),

γ_{GL}=0.076 (Gaussian-Lorentzian).

γ can also be calibrated, if a known concentration c of a dye is measured. In this case N can be fixed and γ fitted. The obtained number can be entered as the calibrated fixed number. N can be calculated from equation

$$c = \frac{N}{V \cdot L_A} \qquad \textbf{(5c)}$$

with V being the confocal volume and L_A = 6.023 x 10^{23} mol^{-1} the Avogadro number)

The volume V is calculated from equation

$$V = \pi^{3/2} \cdot \omega_r^2 \cdot \omega_z \qquad \textbf{(5d)}$$

with ω_z axial focus radius and ω_r the lateral focus radius. The radii themselves have to be determined by a calibration measurement using a dye with a high quantum yield and a known diffusion coefficient D from the fitted diffusion time τ_d and the structural parameter S employing a free diffusion model with triplet state.

The following relations exists:

$$\tau_d = \frac{\omega_r^2}{4 \cdot D} \text{ for 1 photon excitation} \quad \textbf{(5e)}$$

$$\tau_d = \frac{\omega_r^2}{8 \cdot D} \text{ for 2 photon excitation} \quad \textbf{(5f)}$$

$$S = \frac{\omega_z}{\omega_r} \quad \textbf{(5g)}$$

Equation 5e or 5f, dependent on the excitation source, can be used to retrieve ω_r; with its knowledge ω_z can be calculated from equation 5g.

Please note, that N can have different meanings in different fit models. For biology, normally the number of diffusing particles is of interest. In this case, if photo-physical processes (triplet, blinking, stretched exponentials) are involved, it is recommended to use their normalized forms, since then, the number of molecules correspond directly to the number of diffusing particles. If photo-physical terms are not normalized, the number measured is the total number of diffusing particles an those undergoing photo-physical processes.

4.4.16.4 Anti-bunching Terms

Anti-bunching is the phenomenon that a molecule cannot produce emitted photons as long as it stays in the excited state. Hence during the transition time required to drop back to the ground state, which corresponds in most of the cases to the lifetime if no other photo-physical processes are involved, no photon can be expected, which results in auto-correlation and hence a drop of the correlation function below 1.

1) Dependent to other terms

$$G_a(\tau) = (1 - C - C \cdot e^{-\tau/\tau_a}) \text{ not normalized} \quad \textbf{(6a)}$$

$$G_a(\tau) = (1 - \frac{C \cdot e^{-\tau/\tau_a}}{1 - C}) \text{ normalized} \quad \textbf{(6b)}$$

where C is the amplitude and τ_a the transition time, also referred to as the lifetime.

2) Independent in combination with other terms

$$G_a(\tau) = (-C \cdot e^{-\tau/\tau_a}) \quad \textbf{(6c)}$$

C in this case is either a fit parameter or a fixed value and often takes the value 9/5.

There are two cases to be distinguished: First, if the anti-bunching is dependent with other processes, than equations 6a and 6b in the non-normalized or normalized form must be used and the terms are multiplied with other correlation terms. In case the anti-bunching is treated independent to other processes, than equation 6c is the correct one to use and the term is added to other correlation terms.

3) Stretched exponential – anti-bunching

This is a more general term adding frequency and stretched factors to the exponent.

$$G_k(t) = 1 - K_1 - K_1 \cdot e^{(-k_1 \cdot \tau / \tau_{k1})^{\kappa 1}}$$ not normalized **(6d)**

$$G_k(t) = 1 - \frac{K_1 \cdot e^{(-k_1 \cdot \tau / \tau_{k1})^{\kappa 1}}}{1 - K_1}$$ normalized **(6e)**

where K_1 is the fraction of molecule, and τ_{k1} the exponential decay time, k_1 the frequency factor and κ_1 the stretch factor.

K_1 and τ_{k1} are fit parameters; k_1 is a fixed parameter and must be user defined; κ_1 is either a fit parameter or can be fixed.

Note, fixing k_1 and κ_1 to "1" result s in a simple anti-bunching term.

4) Double stretched exponential – anti-bunching

This is a double exponential function, where the exponentials are substracted.

$$G_k(t) = 1 - K_1 - K_1 \cdot e^{(-k_1 \cdot \tau / \tau_{k1})^{\kappa 1}} - K_2 - K_2 \cdot e^{(-k_2 \cdot \tau / \tau_{k2})^{\kappa 2}}$$ not-normalized **(6f)**

$$G_k(t) = 1 - \frac{K_1 \cdot e^{(-k_1 \cdot \tau / \tau_{k1})^{\kappa 1}} + K_2 \cdot e^{(-k_2 \cdot \tau / \tau_{k2})^{\kappa 2}}}{1 - K_1 - K_2}$$ normalized **(6g)**

where K_1 and K_2 are the fractions of molecules, and τ_{k1} and τ_{k2} the exponential decay times, k_1 and k_2 the frequency factors and κ_1 and κ_2 the stretch factors.

K_1, K_2, τ_{k1} and τ_{k2} are fit parameters; k_1 and k_2 are fixed parameters and must be user defined; κ_1 and κ_2 are fit parameters or can be fixed.

4.4.16.5 Bunching Terms

Bunching is the phenomenon of a burst of photons during a certain time interval, the duration of which is determined by photo-physical processes including triplet, blinking, flickering and protonation. This terms are exponential decay functions. Formally, they look the same, only the exponential decay might be different.

1) Triplet

$$G_t(\tau) = (1 - T_t + T_t \cdot e^{-\tau/\tau_t}) \text{ not normalized} \quad \textbf{(7a)}$$

$$G_t(\tau) = (1 + \frac{T_t \cdot e^{-\tau/\tau_t}}{1 - T_t}) \text{ normalized} \quad \textbf{(7b)}$$

where T_t is the triplet fraction, that is the number of molecules undergoing triplet states and τ_t the triplet decay time.

T_t and τ_t are fitted parameters.

Triplet is based on an un-allowed intersystem crossing from the excited to the so-called triplet state. This state lasts for 1 – 5 µs. If the electron drops back to the ground state, no photon is emitted and hence during the triplet state the molecule is in a dark state. Triplet is indicated as a rise in the correlation amplitude, which is indicated as a deviation from the flattening curve at shorter correlation times. If not normalized, the triplet fraction contributes to the total number of molecules.

2) Blinking

$$G_b(\tau) = (1 - T_b + T_b e^{-\tau/\tau_b}) \text{ non-normalized} \quad \textbf{(7c)}$$

$$G_b(\tau) = (1 + \frac{T_b \cdot e^{-\tau/\tau_b}}{1 - T_b}) \text{ normalized} \quad \textbf{(7d)}$$

where T_b is the blinking fraction, that is the number of molecules in the dimmer state and τ_b the blinking decay time of the dimmer state. Note, if the blinking term is not normalized, the number of blinking molecules will influence the total number of molecules.

T_b and τ_b are fitted parameters.

Blinking is based on the phenomenon that the electron distribution over conjugated systems can change in dependence on the local environment, for example changes in the pH, which will lead to molecules in a bright and dim or dark states. It is therefore a kinetic process that can be described in the following way with the following relations:

$$B \xleftrightarrow{k_F;k_R} D \qquad \textbf{(7f)}$$

$$\tau_b = \frac{1}{k_F + k_R} \qquad \textbf{(7g)}$$

$$T_b = \frac{k_F \cdot k_R \cdot (\eta_B - \eta_D)^2}{(k_F + k_R) \cdot \left(k_F \cdot \eta_B^2 + k_R \cdot \eta_D^2\right)} \quad \text{with the constraint } \eta_B > \eta_D \qquad \textbf{(7h)}$$

with B and D representing the brighter and darker states, k_F and k_R the forward and backward reaction rates and η_B and η_D the emission yields or molecular brightness of molecule species (D or B) in Hz or the relative dimensionless brightness. In case, where darker state is completely dark (η_D=0), equation 7hn simplifies to

$$T_b = \frac{k_R}{k_F + k_R} \qquad \textbf{(7i)}$$

Note that Blinking is referred to a process that does not lead to a covalent modification in the chemical bonds. If covalent changes occur the process is referred to as Flickering, which is formally treated in the same way.

3) Independent triplet and blinking

In this case the terms are just representatives for two independent bunching terms that are linked by addition (double exponential term). Note that the triplet fraction, if present, could be potentially fitted to either of the terms.

$$G_t(\tau) = (1 - T_1 + T_1 \cdot e^{-\tau/\tau_{t1}} - T_2 + T_2 \cdot e^{-\tau/\tau_{t2}}) \text{ not-normalized} \qquad \textbf{(7j)}$$

$$G_t(\tau) = (1 + \frac{T_1 \cdot e^{-\tau/\tau_{t1}} + T_2 \cdot e^{-\tau/\tau_{t2}}}{1 - T_1 - T_2}) \text{ normalized} \qquad \textbf{(7k)}$$

where T_1 and T_2 are the fractions of molecules in the triplet state, and τ_{t1} and τ_{t2} the triplet exponential decay times.

T_1, T_2, τ_{t1} and τ_{t2} are all fitted parameters.

4) Dependent triplet and blinking

In this case the terms are just representatives for two dependent bunching terms that are linked by multiplication. Note that the triplet fraction, if present, could be fitted to either of the terms.

$$G_t(\tau) = (1 - T_1 + T_1 \cdot e^{-\tau/\tau_{t1}})(1 - T_2 + T_2 \cdot e^{-\tau/\tau_{t2}}) \text{ not normalized} \quad \textbf{(7l)}$$

$$G_t(\tau) = (1 + \frac{T_1 \cdot e^{-\tau/\tau_{t1}}}{1 - T_1})(\frac{1 + T_2 \cdot e^{-\tau/\tau_{t2}}}{1 - T_2}) \text{ normalized} \quad \textbf{(7m)}$$

where T_1 and T_2 are the fractions of molecules in the triplet state, and τ_{t1} and τ_{t2} the triplet exponential decay times.

T_1, T_2, τ_{t1} and τ_{t2} are all fitted parameters.

5) Stretched exponential - bunching

In some reactions like, the kinetics cannot be fitted to simple exponential functions but require stretched exponentials.

$$G_k(t) = 1 - K_1 + K_1 \cdot e^{(-k_1 \cdot \tau/\tau_{k1})^{\kappa 1}} \text{ not normalized} \quad \textbf{(7n)}$$

$$G_k(t) = 1 + \frac{K_1 \cdot e^{(-k_1 \cdot \tau/\tau_{k1})^{\kappa 1}}}{1 - K_1} \text{ normalized} \quad \textbf{(7o)}$$

where K_1 is the fraction of molecule, and τ_{k1} the exponential decay time, k_1 the frequency factor and κ_1 the stretch factor.

K_1 and τ_{k1} are fit parameters; k_1 is a fixed parameters and must be user defined; κ_1 is either a fit parameter or can be fixed.

Note, fixing k_1 and κ_1 to "1" result s in a simple bunching term.

6) Double stretched exponential - bunching

This is a double exponential function, where the exponentials are added.

$$G_k(t) = 1 - K_1 + K_1 \cdot e^{(-k_1 \cdot \tau / \tau_{k1})^{\kappa 1}} - K_2 + K_2 \cdot e^{(-k_2 \cdot \tau / \tau_{k2})^{\kappa 2}} \text{ not-normalized} \quad \textbf{(7p)}$$

$$G_k(t) = 1 + \frac{K_1 \cdot e^{(-k_1 \cdot \tau / \tau_{k1})^{\kappa 1}} + K_2 \cdot e^{(-k_2 \cdot \tau / \tau_{k2})^{\kappa 2}}}{1 - K_1 - K_2} \text{ normalized} \quad \textbf{(7q)}$$

where K_1 and K_2 are the fractions of molecules, and τ_{k1} and τ_{k2} the exponential decay times, k_1 and k_2 the frequency factors and κ_1 and κ_2 the stretch factors.

K_1, K_2, τ_{k1} and τ_{k2} are fit parameters; k_1 and k_2 are fixed parameters and must be user defined; κ_1 and κ_2 are fit parameters or can be fixed.

This term is often required to fit protonation, with the second stretch factor and the frequency factors are set to "1".

4.4.16.6 Diffusion Terms

Diffusion is driven by Brownian motion. We can distinguish translational, rotational and flow.

1) Rotational diffusion

In the most general form, rotation can be described as the sum of 5 exponential terms

$$g_r(\tau) = 1 - R_a + R_a \cdot \sum_{m=1}^{5} c_m \cdot e^{-r_m \cdot \tau / \tau_{r,m}} \quad \textbf{(8a)}$$

with R_a being the amplitude, c_m the relative amplitude, r_m the frequency factor and $\tau_{r,m}$ the rotational diffusion time.

However, there are special cases that are of more use.

In symmetric rotation, the general formula reduces to:

$$G_r(\tau) = 1 - R_a + R_a \cdot e^{-\tau/\tau_r} \text{ not normalized} \quad \textbf{(8b)}$$

$$G_r(\tau) = 1 + \frac{R_a \cdot e^{-\tau/\tau_r}}{1 - R_a} \text{ normalized} \quad \textbf{(8c)}$$

with R_a being the rotational amplitude and τ_r the rotational diffusion time.

R_a and τ_r are both fit parameters.

If rotation occurs independent from other processes, the formula used as an additive term is defined as:

$$G_r(\tau) = R_a \cdot e^{-\tau/\tau_r} \quad \textbf{(8d)}$$

R_a in this case is either a fit parameter or a fixed value and often takes the value 4/5.

In case of asymmetric rotation, the term is as follows:

$$G_r(\tau) = 1 - R_a + R_a \cdot (c_1 \cdot e^{-r_1 \cdot \tau/\tau_{r,1}} + c_2 \cdot e^{-r_2 \cdot \tau/\tau_{r,2}}) \text{ not normalized} \quad \textbf{(8e)}$$

$$G_r(\tau) = 1 + \frac{R_a \cdot (c_1 \cdot e^{-r_1\tau/\tau_{r,1}} + c_2 \cdot e^{-r_2\tau/\tau_{r,2}})}{1 - R_a}$$ normalized **(8f)**

with R_a being the amplitude, c_1 and c_2 relative amplitudes, r_1 and r_2 frequency factors and $\tau_{r,1}$ and $\tau_{r,2}$ the rotational diffusion times.

R_a, $\tau_{r,1}$ and $\tau_{r,2}$ are fitted parameters; c_1 and c_2 as well as r_1 and r_2 are fixed values and must be user defined.

Rotational frequencies take often the following values:

r_1	1
r_2	10/3

The relative amplitudes dependent on the polarisation of the excitation light and the analyser in the emission beam path and are as follows:

Ex	lin. pol.	lin. pol.	lin. pol.	unpol.	unpol.	unpol.
Em	parallel	perpendicular	all	parallel	perpendicular	all
c_1	80	20/9	860/9	5/9	215/9	20
c_2	64/9	4	4	16/9	1	1

2) Translational diffusion

In its general form, translational diffusion is defined as:

$$G_d(\tau) = \left(\sum_{i=1}^{3} \frac{\Phi_i}{\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i}\right)^{e_{d1}} \cdot \left(\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i} \cdot 1/S^2\right)^{1/2}\right)^{e_{d2}}} \right) \text{ with the constraint } \sum_i \Phi_i = 1 \qquad \textbf{(8g)}$$

with $\tau_{d,i}$ representing the diffusional correlation time of molecule species i , S the structural parameter that is the ratio of axial to lateral focus radii, α_i the anomaly parameter or temporal component of molecule species I, e_{d1}, e_{d2} fixed exponentials to define dimensionality of diffusion (1-D: e_{d1}=1/2; e_{d2}=0; 2-D: e_{d1}=1; e_{d2}=0; 3-D: e_{d1}=1; e_{d2}=1)

e_{d1} and e_{d2} are fixed values and have to be user defined. The following values define 1-, 2-and 3-D diffusion:

e_{d1}	e_{d2}	dimensionality
1/2	0	1-D
1	0	2-D
1	1	3-D

Note that in the ConfoCor 3 software this values are automatically selected with the choice of dimensionality.

S is either a fit parameter or a fixed value. It is an instrumental parameter and can be determined by a calibration experiment using a dye solution with a known diffusion as a fit result.

α_i is either a fitted value for anomalous diffusion or a fixed value (set to "1) for free diffusion. The following relation exists

α	Diffusion process
=1	Free diffusion
<1	Anomalous sub-diffusion
<1	Anomalous super-diffusion

Note that α_i is set automatically to "1", if free diffusion is selected. If anaomalous diffusion is selected, the parameter will float.

$\tau_{d,i}$ are fitted parameters. They can be converted to diffusion coefficients D_i using formulas 5e or 5f. The ConfoCor software allows you to directly fit to D_i values, but in this case the lateral radius ω_r has to be specified as a fixed value.

Please note that in the case of anomalous diffusion The following relations exist:

$$\tau^{\alpha}{}_{d,i} = \frac{\omega_r^2}{\Gamma_{2,i}} \quad \text{1 photon excitation} \qquad \textbf{(8h)}$$

$$\tau^{\alpha}{}_{d,i} = \frac{\omega_r^2}{2 \cdot \Gamma_{2,i}} \quad \text{2 photon excitation} \qquad \textbf{(8i)}$$

with Γ representing the transport coefficient of the fractional time dimension.

Please note the following relation between D and G :

$$D(t) = \Gamma \cdot t^{\alpha - 1} \qquad \textbf{(8j)}$$

If activating the fitting to the diffusion coefficient in the ConfoCor 3 the Γ values have to be calculated from the D values by the following conversion:

$$\Gamma = 4 \cdot D \qquad \textbf{(8k)}$$

The Φi values are fit parameters. They account for different brightness of different components. In principle, if molecules of different brightness are present, the apparent molecular brightness is defined as

$$\eta = \frac{\sum_{i=1}^{3} f_i \cdot \eta_i^2}{\sum_{i=1}^{3} f_i \cdot \eta_i} \qquad \textbf{(8l)}$$

with η_i being the brightness of the molecules in kHz or the dimensionless relative brightness values. The brightness of the species have to be determined beforehand in control experiments.

Note that the brightness contributes as the square to the correlation function, in other words a double as bright molecule will contribute 4 fold more. Therefore, the fitted number of molecules must be corrected for to obtain the real number N_{diff} of diffusing particles; please note that to obtain the diffusing particle number directly, other terms should be used in their normalized form :

$$N_{diff} = N \cdot \frac{(\sum_{i=1}^{3} f_i \cdot \eta_i)^2}{\sum_{i=1}^{3} f_i \cdot \eta_i^2} \qquad \textbf{(8m)}$$

If one wants to know the true fraction f_i of each species, with the known brightness values those can be retrieved from the relation

$$\Phi_i = \frac{f_i \cdot \eta_i^2}{\sum_{i=1}^{3} f_i \cdot \eta_i^2} \text{ with the constraints } \sum_i \Phi_i = 1 \text{ and } \sum_i f_i = 1 \qquad \textbf{(8n)}$$

If there is no brightness difference between the components, Φ_i will become to f_i.

In the ConfoCor software you can fit directly to the fractions even in case of different brightness values. In this case, the brightness values have to be fixed parameters and defined by the user. The fit formula converts from equation 5a in combination with 8g taking into account the corrected amplitude to equation 8o:

$$G_{tot}(\tau) = \frac{\gamma}{N} \cdot \frac{\sum_{i=1}^{3} f_i \cdot \eta_i^2}{(\sum_{i=1}^{3} f_i \cdot \eta_i)^2} \cdot \left(\sum_{i=1}^{3} \frac{\frac{f_1 \cdot \eta_1^2}{\sum_{i=1}^{3} f_i \cdot \eta_i^2}}{\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i}\right)^{e_{d1}} \cdot \left(\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i} \cdot 1/S^2\right)^{1/2}\right)^{e_{d2}}} \right) = \frac{\gamma}{N} \cdot \left(\sum_{i=1}^{3} \frac{\frac{f_1 \cdot \eta_1^2}{(\sum_{i=1}^{3} f_i \cdot \eta_i)^2}}{\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i}\right)^{e_{d1}} \cdot \left(\left(1+\left(\tau/\tau_{d,i}\right)^{\alpha_i} \cdot 1/S^2\right)^{1/2}\right)^{e_{d2}}} \right) \qquad \textbf{(8o)}$$

with fixed brightness values ηi.

3) Flow

Flow signifies active transport either via cytoplasmic movement or directed transport.

If flow occurs in the absence of translational diffusion, the term is defined as follows:

$$G_f(\tau) = e^{-\left(\tau/\tau_f\right)^2} \quad \textbf{(8m)}$$

In the presence of translational diffusion, the term alters to:

$$G_f(\tau) = e^{-\frac{(\tau/\tau_f)^2}{1+(\tau/\tau_d)}} \quad \textbf{(8n)}$$

with τ_f representing the average residence time for flow and τ_d the diffusion correlation time.

Note, in the ConfoCor 3 software, the correct term is automatically loaded in dependence on the absence or presence of a translational term.

With the knowledge of the lateral radius ω_r given as a fixed value, the software allows to fit directly to the velocity v instead of the average residence time. The following relation exists:

$$v = \frac{\omega_r}{\tau_f} \quad \textbf{(8o)}.$$

4.4.16.7 Photon Counting Histogram (PCH)

In the photon counting histogram (PCH) no closed formulas exist, to which the data can be fitted. Hence in this case, fitting is done numerically.

4.4.17 Models

If you press the **Models** button in the ConfoCor submenu toolbar, the **Model List** window will open (see Fig. 4-69).

The window lists all the stored models with the **Model name** and the **Type**. You can select a model by highlighting it.

The type is of either **Assemble**, **PCH** or **User defined** depending where the model was created, in **Correlation**, **PCH** or **Formula**, respectively.

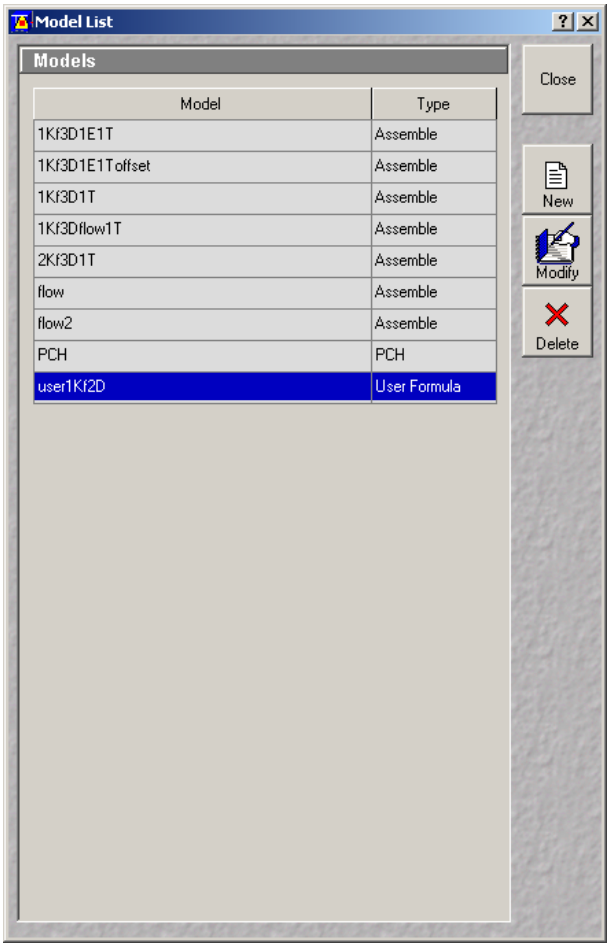


Fig. 4-69 Model List window

You have the following options:

4.4.17.1 Close

Pressing the **Close** button will close the Model List window.

4.4.17.2 New

Pressing the **New** button will open the **Model** window, where you can define a model by predefined terms or user defined.

4.4.17.3 Modify

Pressing the **Modify** button will open the **Model** window, where you can alter the highlighted pre-existing method.

4.4.17.4 Delete

Pressing the **Delete** button will delete the highlighted method. You will be prompted if you want to delete the model. Press yes or no I you want to delete or keep the model, respectively.

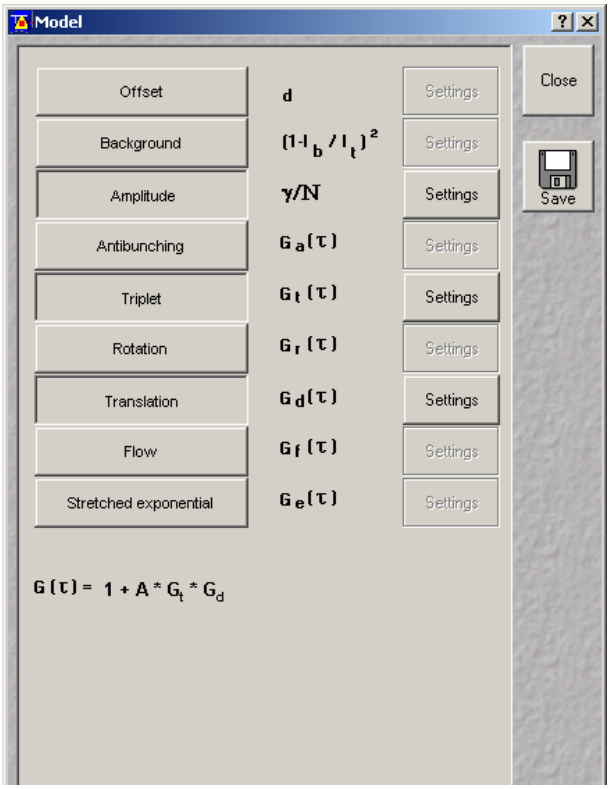


Fig. 4-70 **Model window, new model**

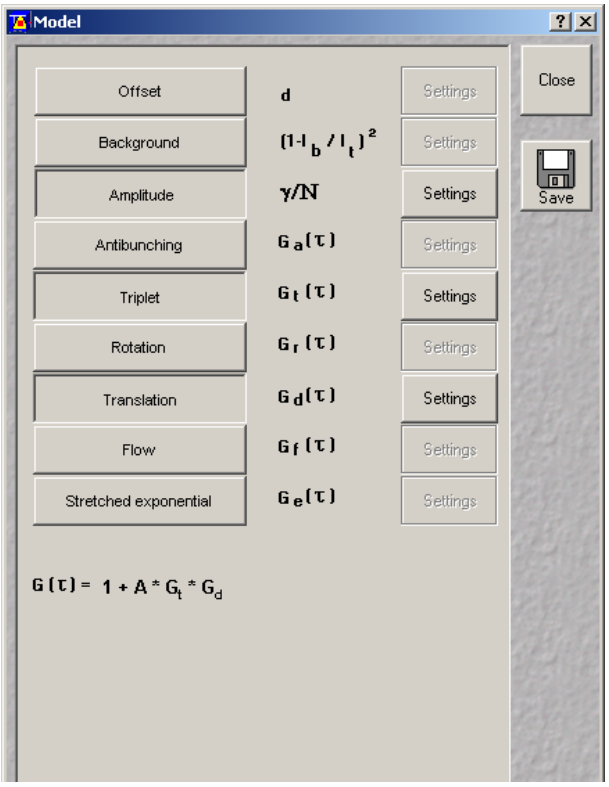


Fig. 4-71 **Model window, predefined**

4.4.17.5 Defining a Model

Press the **New** or **Modify** button and the **Model** window will appear (see Fig. 4-69).

☞ Note, the **Modify** button will load the highlighted configuration and either the **Correlation**, **PCH,** or **Formula** is available (see Fig. 4-83); in **New** all options are available and can be selected by pressing the **Correlation**, **PCH** or **Formula** button (see Fig. 4-70).

You have three options to define a model:

- **Correlation**: assemble a fluorescence correlation spectroscopy (FCS) model
- **PCH**: assemble a photon counting histogram model
- **Formula**: program a user defined model

(1) Correlation

You can assemble a with predefined terms.

- Press the **Correlation** button to activate the options for assembling a model.

You have the following options (see Fig. 4-71):

- Press the **Close** button to exit the **Correlation** window.
- Press the **Save** button to save a defined model. The **Save Model** window will appear. You can type in a name. Pressing **Ok** will save the model in a database. Pressing **Cancel** will close the **Save Model** window without saving the model.

- To define a model, just activate the respective terms that are available.
- You can choose from:
  - **Offset**: Deviation from 1
  - **Background**: Unspecific background correction

- **Amplitude**: Number of molecules and geometric factor
- **Antibunching**: Antibunching term
- **Triplet**: Exponential term for triplet state, blinking, flickering or other bunching terms
- **Rotation**: Rotational diffusion term
- **Translation**: Translational diffusion term
- **Flow**: Flow term
- **Stretched exponential**: Stretched exponential term for protonation and other kinetics

All highlighted terms are assembled in a way that is displayed in **G(τ)=** display box.

For all terms specific settings can be applied. These settings are accessible if the respective **Settings** button is pressed. All **Settings** windows can be closed by pressing the **Close** button. The settings windows are bipartite: Above you can select parameters and define values, below is a description box that display the formula in its most general form, explains the parameters and gives useful conversions of the fitted parameter to other interesting parameters. Press the close button to exit the **Setting** window.

The following settings are available:

Offset

- You can either set the offset (see Fig. 4-72) to 0 by activating the **Normalized** option.
- Or you set an offset by activating the **Calibrated** option. This enables you to type in a value in the **Calibrated** selection box, or by setting a value using the arrows.

☞ Note, the offset can have positive or negative numbers and the value specified will be added to all correlation values.

The description box gives you information on the offset.

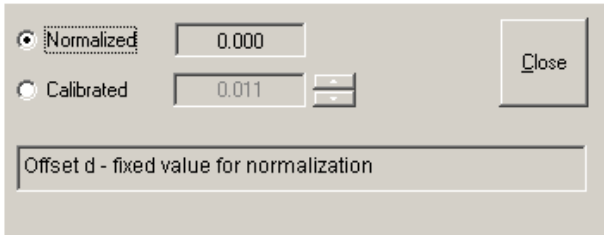


Fig. 4-72 Settings Offset panel

Background

- You can set an offset by setting a value in the **Background** selection box (see Fig. 4-73). Either type in a value or use the arrows.

The description box gives you information on the background.

☞ If there is no background, the background correction factor will be set to 1.

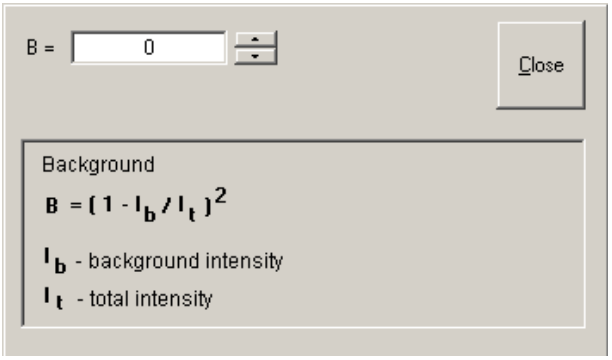


Fig. 4-73 Settings Background panel

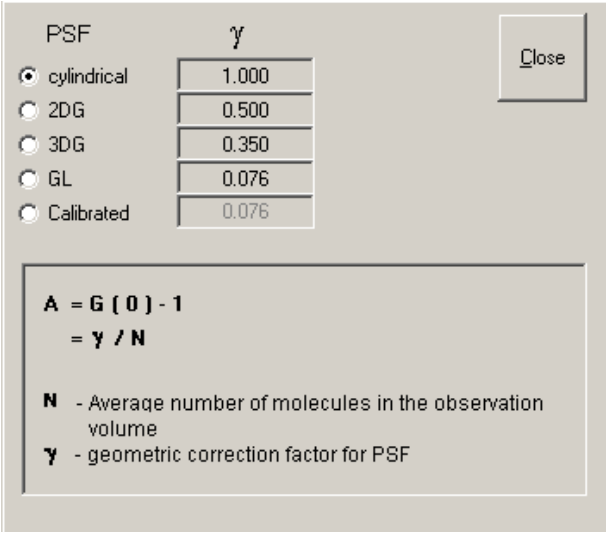


Fig. 4-74 Settings Amplitude panel

Amplitude

- You can set a value for the geometric factor γ, which describes the point-spread function (see Fig. 4-74).
- By activating the corresponding option you can select between a cylindrical, 2 dimensional Gaussian (2DG), 3 dimensional Gaussian (3DG) and Gaussian-Lorentzian (GL) PSFs. If you activate the Calibrate selection box, you can type in a user defined number or use the arrows to set a value.

The description box gives you information on the amplitude

☞ At least one the γ factor or the number of molecules N have to be fixed in the fit procedure.

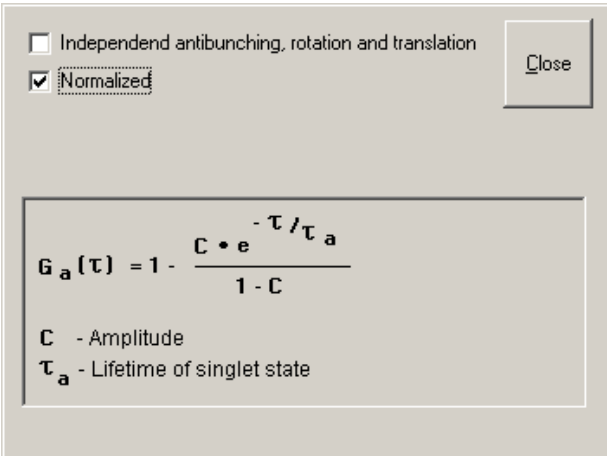


Fig. 4-75 Settings Antibunching panel

Antibunching

You can select the anti-bunching term in its normalized form by checking the **Normalized** check box, or in its non-normalized form by de-checking the box (see Fig. 4-75).

You can select the **Independend antibunching, rotation and translation** form by checking the respective box. This equation will be used as an additiv term to rotational and translational diffusion terms.
You have the option to leave the amplitude value to **Free** or to **9/5** by activating the corresponding option.

The description box gives you information on the anti-bunching.

Triplet

The triplet represents bunching terms, that are exponential decay functions.

You can select the triplet term(s) in its/their normalized form by checking the **Normalized** check box, or in its/their non-normalized form by de-checking the box (see Fig. 4-76).

You have several options for the bunching terms which you can select by the **Components** drop down menu (see Fig. 4-77):

Triplet: 1 exponential function

Blinking: 1 exponential function

Independent Triplet and Blinking: Sum of 2 exponential functions

Dependent Triplet and Blinking: Product of 2 exponential functions

The description box gives information on the bunching terms.

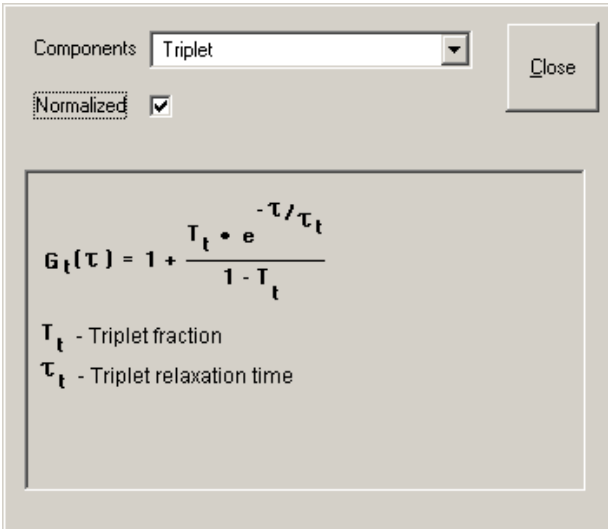


Fig. 4-76 Settings Triplet panel

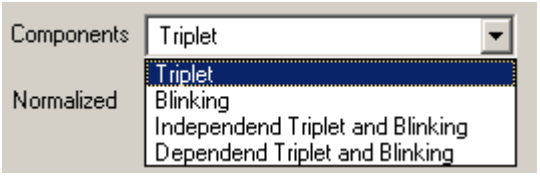


Fig. 4-77 Components drop down menu

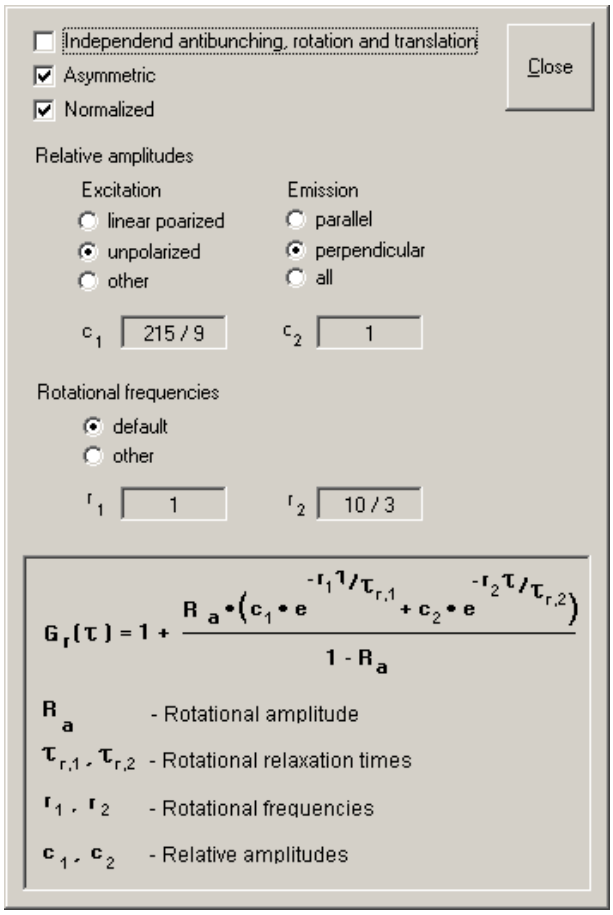


Fig. 4-78 Settings Rotation panel

Rotation

You can select between symmetric rotation (1 exponential term) and asymmetric rotation (double exponential term) by selecting / deselecting the **Asymmetric** check box (see Fig. 4-78).

You can select between normalized and non-normalized rotation functions by selecting / deselecting the **Normalized** check box.

If the **Asymmetric** box is checked, you can define relative amplitudes and rotational frequencies (note, these parameters are no fit values and have to be defined by the user).

Relative amplitudes are defined by selecting the excitation polarization (**linear polarized** or **unpolarized**) and the emission detection (**parallel**, **perpendicular** or **all**), which will result in the corresponding values displayed in the **c1** and **c2** display boxes. Alternatively, if **others** is activated, you can type in user defined values.

Rotational frequencies can be selected by activating the Default settings with the corresponding values will be shown in the **r1** and **r2** display boxes, or by user defined values that can be entered when **others** is activated.

You can select the **Independend antibunching, rotation and translation** by checking the respective box. This equation will be used as an additiv term to anti-bunching and translational diffusion terms. You have the option to leave the amplitude value to **Free** or to **4/5** by activating the corresponding option.

The description box gives information on the rotational diffusion terms.

Translation

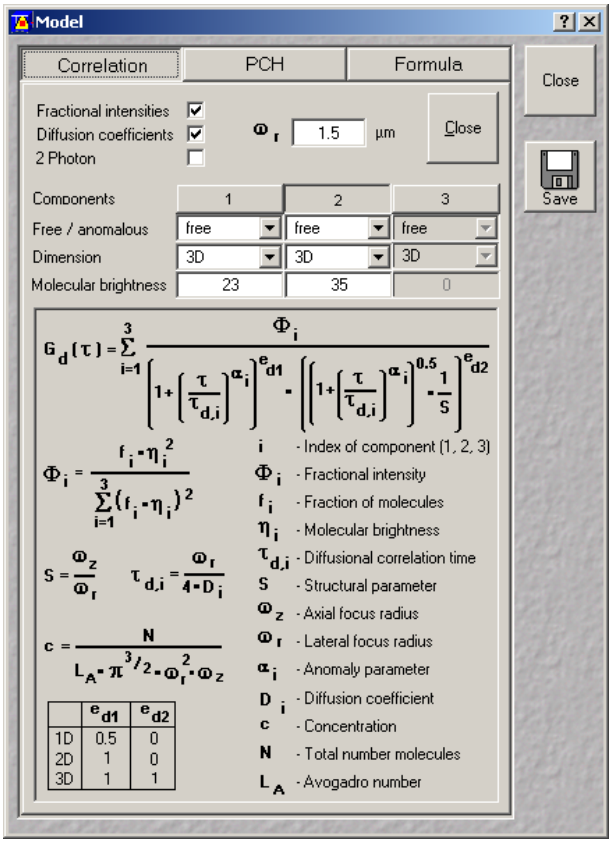


Fig. 4-79 Settings Translation panel

- You have the following possibilities for setting parameters (see Fig. 4-79):
- You can either fit to fractions (used normally when no brightness differences are observed between different components) or to fractional intensities, when the **Fractional Intensities** option is selected. In this case, you have to provide the absolute or relative brightness values of the components into the **Molecular brightness** selection boxes.
- You can either fit to the diffusion time, or you can directly fit to the Diffusion coefficient by activating the **Diffusion coefficients** option. In the latter case you have to provide the dimension of the radius of the confocal volume in the ω_r selection box. Either type in a value or use the arrow keys.
- You must also specify if you use two photon excitation by checking the **2 Photon** check box, since that will influence the fit formula in the case the **Diffusion coefficients** option was chosen.
- You can select the numbers of components (1, 2 and 3) by pressing the **Components 1**, **2** or **3** buttons.
- For each component you can select free or anomalous diffusion in the pull down menus of the **Free/anomalous** selection box.
- For each component you can set the dimensionality of diffusion in the pull down menus of the **Dimension** selection boxes. You can toggle between 1-D, 2-D and 3-D.
- For each component you can enter brightness values in the **Brightness** select boxes. This values are only displayed, if the **Fractional Intensities** option is selected. You can type in absolute values (that must have the same units) or relative values.

The description box gives information on the translational diffusion terms.

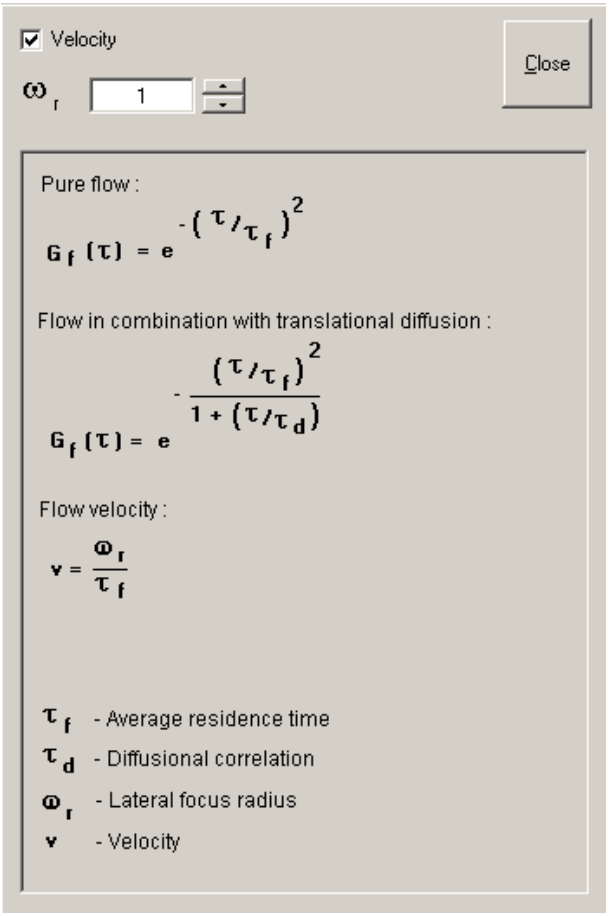


Fig. 4-80 Settings Flow panel

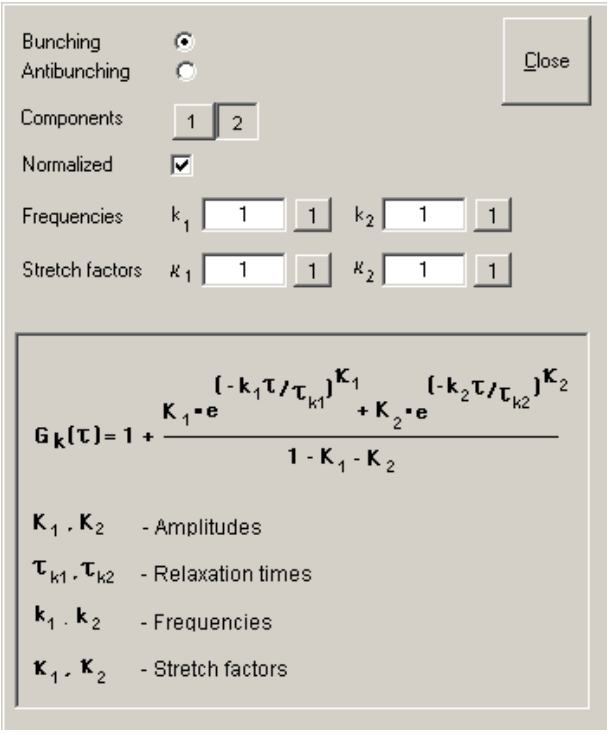


Fig. 4-81 Stretched exponential panel

Flow

You can determine, if you want to fit to the velocity directly instead of the diffusion time by checking the **Velocity** check box (see Fig. 4-80). In this case you have to provide the radial dimension of the confocal volume in the ω_r selection box. Type in the number or use the arrow keys.

The description box gives you information on the flow terms. Note, that the system automatically toggles between the pure flow and the one that is used when the **Translation** term is activated.

Stretched exponential

You can select between bunching and anti-bunching terms by activating the **Bunching** or **Antibunching** option (see Fig. 4-81).

You can select between 1 (mono exponential) or 2 (double exponential) stretched exponential terms by pressing the **Components 1** or **2** button.

You can select between normalized and non-normalized terms by selecting / deselecting the **Normalized** check box.

☞ Note, the frequencies and stretch factors are no fit parameters and must be defined in the **Frequencies** $\mathbf{k_1}$ and $\mathbf{k_2}$ as well as the **Stretch factors** κ_1 and κ_2 display boxes. Either enter a value or press the **1** button for the default setting.

The description box will give information on the stretched exponential terms.

(2) PCH

PCH = Photon Counting Histogram allows you to determine concentrations and the molecular brightness of molecules.

You can choose between 1, 2 or 3 components by activating the **Components 1**, **2** and **3** buttons, respectively (see Fig. 4-82). For each activated component you can enter a brightness value in the **Specific brightness** enter boxes.

You can also enter **Instrumental parameters** values in the respective **square (a1)**, **cubic (a2)** and **power (a3)** enter boxes. These values have to be determined in independent calibration experiments using a defined dye solution and correct for the deviation of the confocal volume from a true Gaussian distribution.

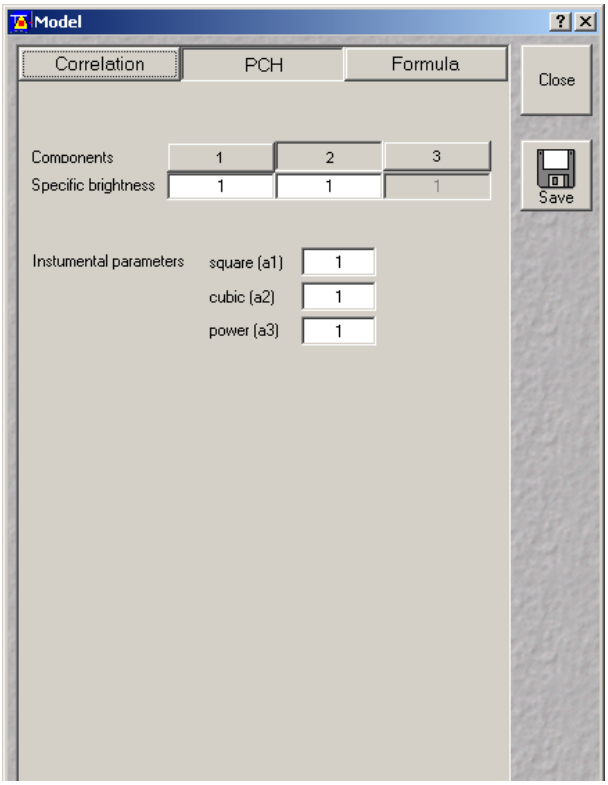


Fig. 4-82 Model window, PCH

(3) Formula

Defining a model with user defined terms

- Press the **Formula** button for the user defined model options (see Fig. 4-83).

This opens up a calculator, that you can use to edit your formula. You can also type in directly in the Selection box.

All variables that are defined at the beginning, are taken as fixed variables, all others as fit parameters.

The formula is continuously parsed and any expected operation indicated in the description box at the bottom.

- Press the **Save** button to open the **Save Model** window. The formula will be automatically parsed or compiled and any syntax errors will be displayed. Give the model a name and press **Ok** to save it. Press **Cancel** to close the window without saving.
- The description box at the bottom will inform you on the parameters and operations.
- Press the **Close** button to leave the **User defined** Window.

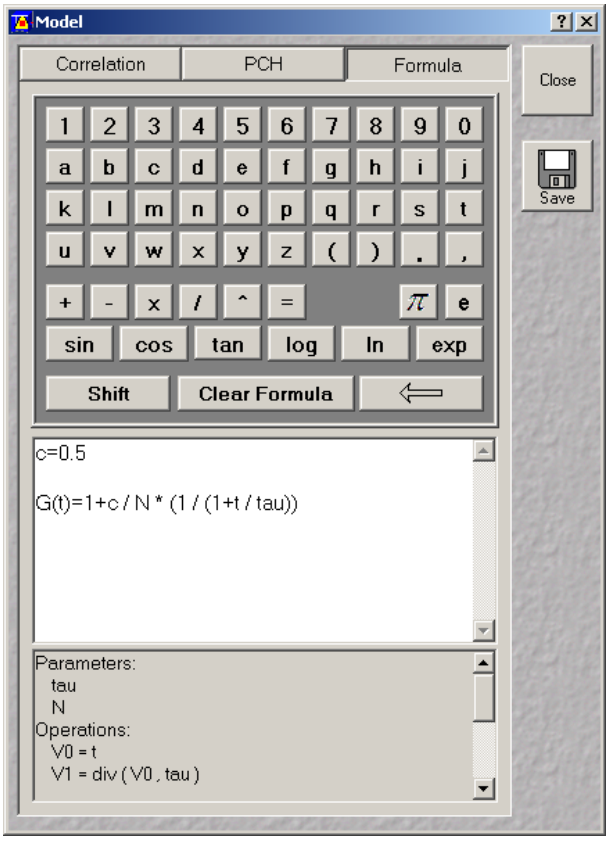


Fig. 4-83 Model window, Formula

4.4.18 Settings

Pressing the **Settings** button of the **ConfoCor** submenu bar opens the **Options for ConfoCor** window (see Fig. 4-84). You can close the window by pressing the **Close** button. The menu has three submenus:

- Measurement
- Auto Save
- LSM+ConfoCor

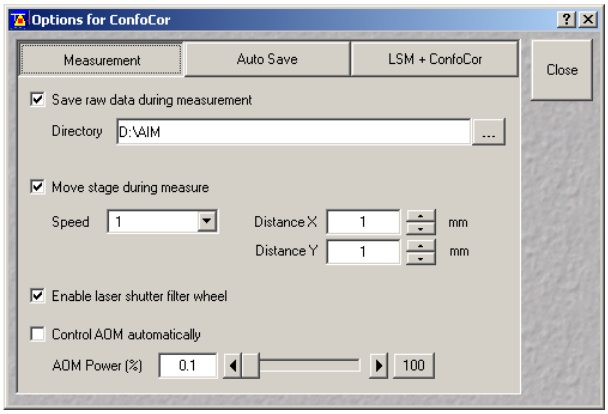


Fig. 4-84 **Options for ConfoCor window, Measurement**

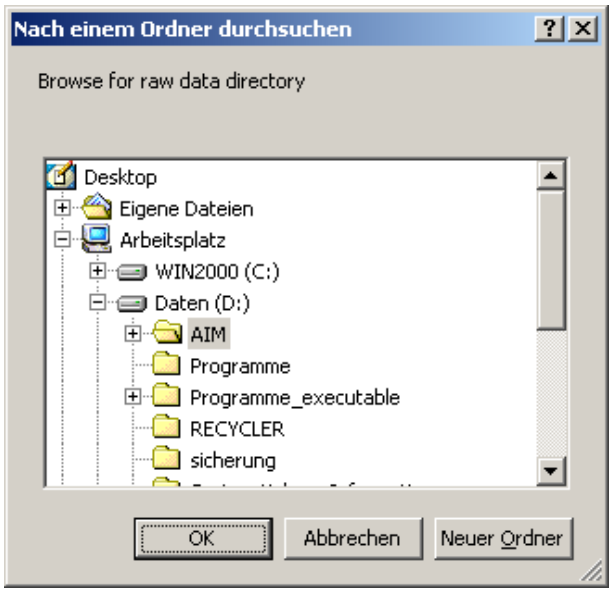


Fig. 4-85 **Save raw data**

4.4.18.1 Measurement

(1) Raw data

If the **Save raw data during measurement** check box is activated (see Fig. 4-84), raw data will be saved in a specified directory.

☞ If the check-box is de-activated, raw data will be saved in the Temp file. If the **FCS results** window is closed, the raw data will be lost in this case.

When you tick the **Save raw data during measurement** check box, the raw data (photon trace) will be stored on disk. This option is used when access to the raw data is required to analyze the data in a different way than by calculating the correlation functions.

The raw data file structure is described in section 4.5.3 Description of the Raw Data Format.

- In the **Directory** field, the directory will be set where the data should be saved.
- When the ... button is clicked, a WINDOWS directory selection dialog will open (see Fig. 4-85).
- The maximum number of files can be set (up to 100).

☞ Each single measurement is stored in one raw data file. For two channel experiments, each channel will be stored in a separate file.

(2) Stage movement

If the **Move stage during measure** check box is activated, the stage will move during an measurement

This option allows you to move the stage during measurement. In this case the stage will be moved forth and back during the entire measurement time. This option is useful during rare event detection when large, slowly moving objects in extremely small concentrations have to be registered. Since these aggregates are diffusing very slowly without such movement the measurement times would be prohibitively long. It should be taken into account that, in this case, the data stream is analyzed with the coincidence analysis rather than by correlation analysis.

If the **Use Piezo Stage if available** check box is checked, the Piezo Stage will be used whenever the **Piezo** button is pressed in the measurement window.

(3) Activating the Piezo Stage

You have the option to mount a Piezo Stage onto your normal stage. Be sure to select on the control unit of the Piezo Stage **the closed loop** operation. If closed loop is selected the corresponding LCD will light up. To work with the Piezo Stage, it should be activated under FCS Options in **Measure** tab by pressing the **Piezo Stage** button.

If the **Move stage during measure** check box is checked, the following settings are possible in the dialog:

- Travel distance of the stage in millimeters: Only integer numbers of millimeters are possible.
- Speed of the stage: Only the three preset stage speeds 0.48 mm/s, 4.81 mm/s and 24.01 mm/s are possible.
- Travel direction: The stage can be moved along the x- or y-axis of the stage.

☞ If you haven't chosen the closed loop operation mode, the Piezo Stage will not work properly in the LSM 510-ConfoCor 3 Software.

The Piezo Stage allows for 2D translational motion in the x and y direction with a maximum motion of 100 μm in each direction. The resolution is 1 nm. Positioning accuracy using the Piezo Stage is 10 nm and less.

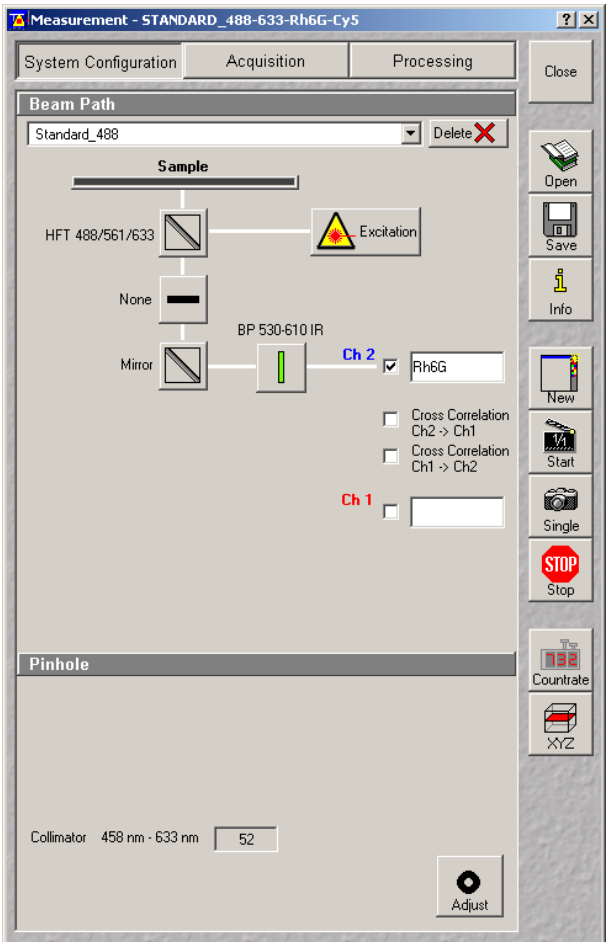


Fig. 4-86 Measurement window

(4) Working with the Piezo Stage

Open the **Measurement** window (see Fig. 4-86) and press the **Piezo Stage** button.

Any stage movement done with the stage control will address the Piezo Stage.

Movement by the joystick will overrule the Piezo Stage and the normal stage will be addressed.

The position of the Piezo Stage will be centered in respect to the normal stage whenever the **Piezo Stage to 0** button was pressed.

The position in x and y are displayed in the **Stage Position** display box.

If the Piezo Stage is activated the x, and y-Scans in the X,Y,Z-Menu will be nevertheless done by the scanning mirrors.

☞ Note, the numbers displayed will not correspond to the positions in the LSM mode, if the Piezo Stage is activated. Position numbers correspond to the numbers displayed on the Piezo Stage unit. Zeroing will display 40.000 in x and y. If the standard stage is used, the numbers will be the same.

(5) Shutters

If additional shutters (before the HeNe lasers and DSSP lasers and a line suppression filter wheel for the Argon laser) are available in the laser module, they can be activated by checking the **Enable laser shutter filter wheel** check box.

In this case, if the HeNe and DSSP filters are not used, a shutter will be placed in front of them; a line selection filter will be used for the laser line activated for the Argon laser.

☞ Note, that activating two lines of the Argon, only in the case of 458/514 a filter be in place, otherwise a non-position.

☞ This function is recommended when measuring close (within 0.2 mm) close to the cover slip glass to suppress any residual excitation light.

(6) AOTF dampening

If the **AOTF control automatically** check box is activated, the laser AOTF setting will be extended for the dynamic range in the lower range. Please note that in case two activated laser are set at different ranges, the one in the lower range will be set to the lowest available AOTF of the higher range.

If the box is unchecked, the AOTF can be suppressed overall by 1, 10, 100 or a 1000 fold. By moving the slider of the **AOTF Power (%)** selection box, the respective dampening factor can be set (100%, 10%, 1%, 0.1%).

4.4.18.2 Auto Save

If the **Auto Save** check box is activated (see Fig. 4-87), all curves will be saved automatically in the directory, which is specified **Directory** selection box (see figure settings-auto-save).

The **Auto Save** panel of the window enables you to instruct the program to save the results automatically during the measurement process without human intervention.

- Activate the **Auto Save** check box via mouse click.
- The directory for the automatically saved files can be chosen as well in the **Directory** selection box.

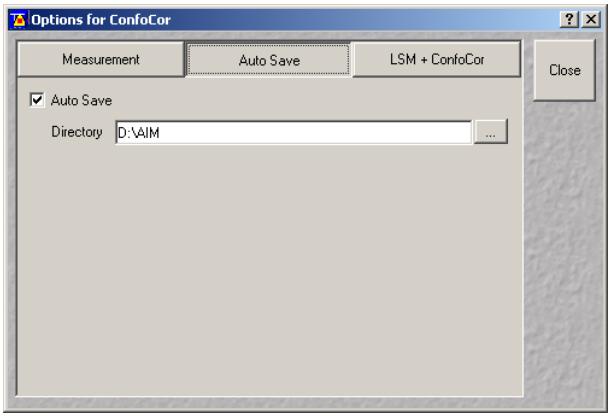


Fig. 4-87 Option for ConfoCor window, Auto Save

The **Task Name** specified in the **Task Description** panel is normally used as file name. For checking purposes, it is then displayed in the **Filename** display box.

4.4.18.3 LSM + ConfoCor

The **LSM + ConfoCor** function (see Fig. 4-88) can be used to type in offset values between LSM scanners and the stage, if this is used for FCS measurements. The offset can be determined by bleaching holes into a dye layer and determining the offset between the crosses and actual bleach spot. Type in the corresponding numbers.

☞ If the scanners are uses, offsets has to be corrected with the **Bidirectional** sliders. The numbers typed inhere are then meaningless.

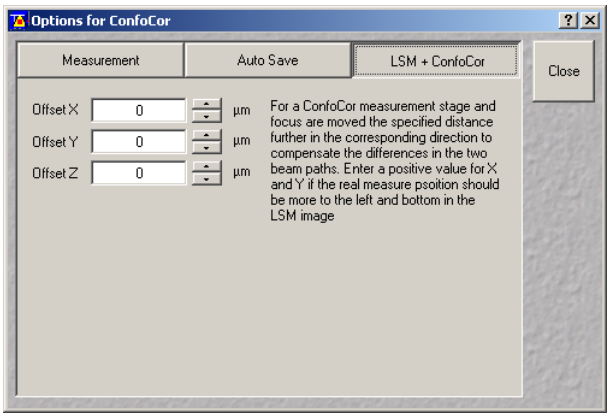


Fig. 4-88 Option for ConfoCor window, LSM+ConfoCor

4.4.19 Compensating Offsets between FCS and LSM

4.4.19.1 Determination of the Offset between LSM Scanner and FCS Stage Positioning

(1) Alignment in X, Y

You can compensate manually or by using a Macro.

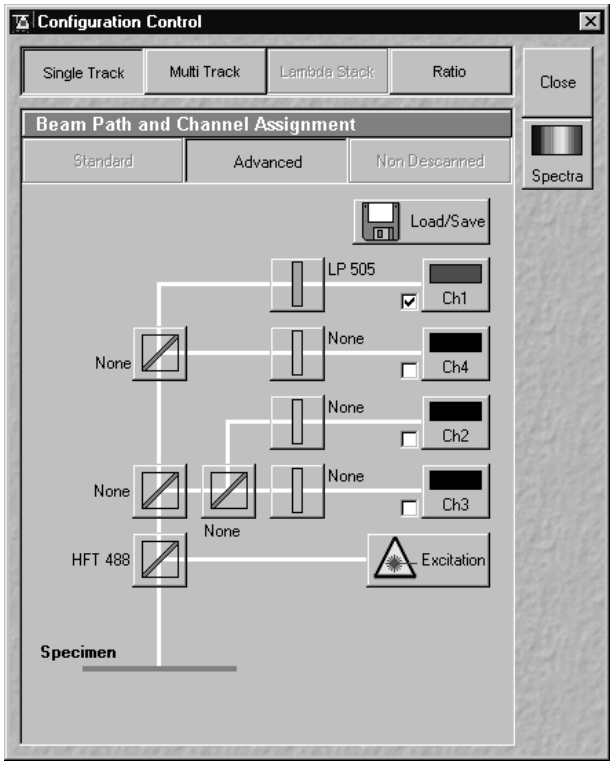


Fig. 4-89 LSM beam path for imaging the test layer of Rh6G

(a) Manual Adjustment

Do the manual adjustment according to the following procedure:

- Obtain an LSM image of the Rh6G layer.
- Set the following LSM parameters in the **Configuration Control** (see Fig. 4-89):
  - Channel 1: select
  - Excitation: 488 nm at 2.1%, tube current 6.1 Amps
  - Main beam splitter: HFT 488
  - Emission filter of channel 1: LP505

- Set the following LSM parameters in the **Scan Control** (see Fig. 4-90):
  - Frame Size: X=512, Y=512
  - Scan Speed: 8
  - Data Depth: 8 Bit
  - Scan direction: ->
  - Mode: Line
  - Method: Mean
  - Number: 4
  - Zoom: 5
  - Rotation: 0

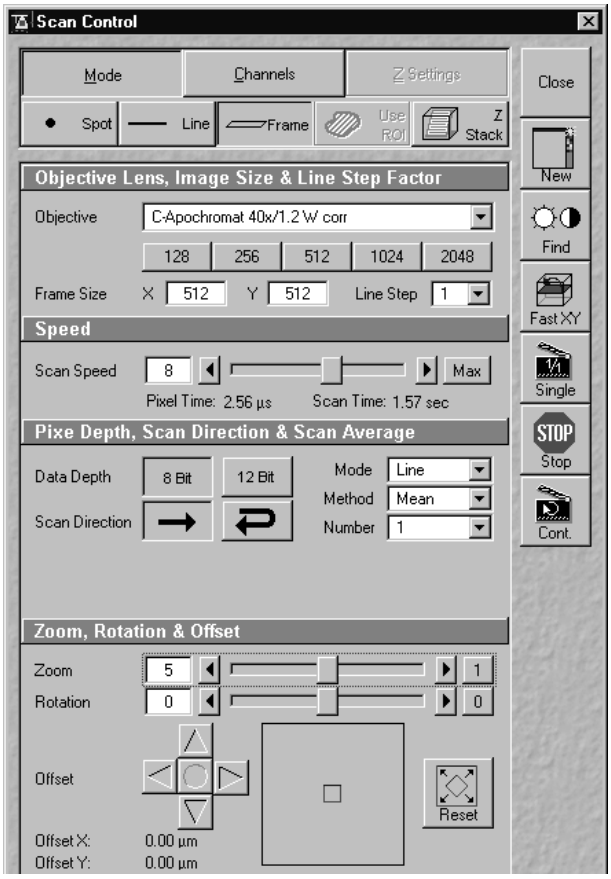


Fig. 4-90 Settings at the LSM control

- Optimize focus position and **Detector Gain** value for a good LSM image (see Fig. 4-91).
- Leave the LSM image window and LSM control window on the computer screen.

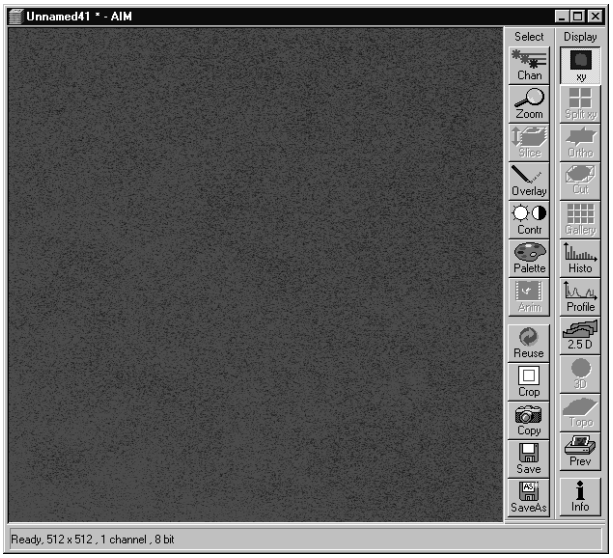


Fig. 4-91 LSM image of the Rh6G layer before bleaching. Usually some texture is observed.

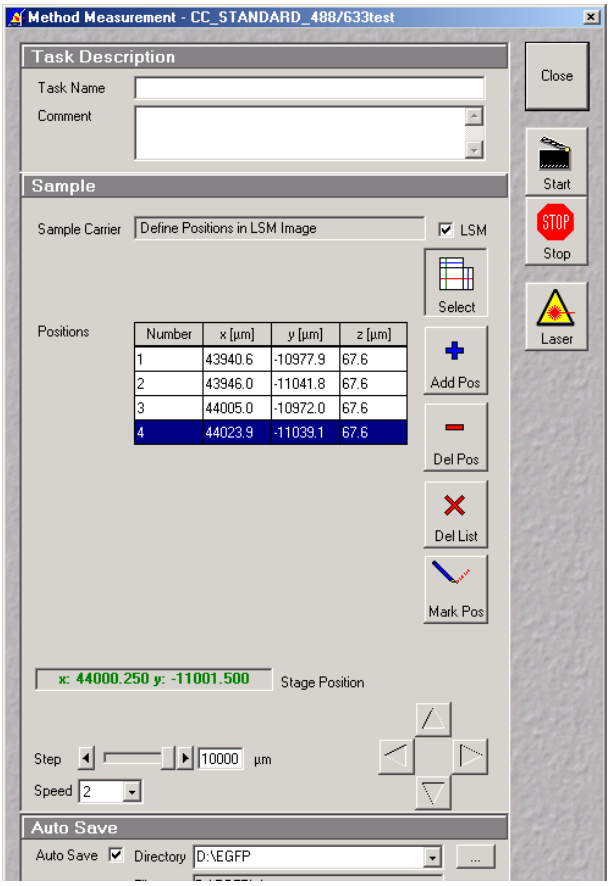


Fig. 4-92 **The Method Measurement window in the LSM mode**

- Switch to FCS.
  - In the main toolbar press the **FCS** button.
  - Click **Measurement** button.
  - Select a suitable beam path.
  - Press **LSM Image** button.
  - Mark the points in the LSM image that you want to bleach by positioning the cross and pressing the **Add** button. The marked points will be listed. Click **Mark Pos** to overlay the markers in the LSM image (see Fig. 4-92 and Fig. 4-93).

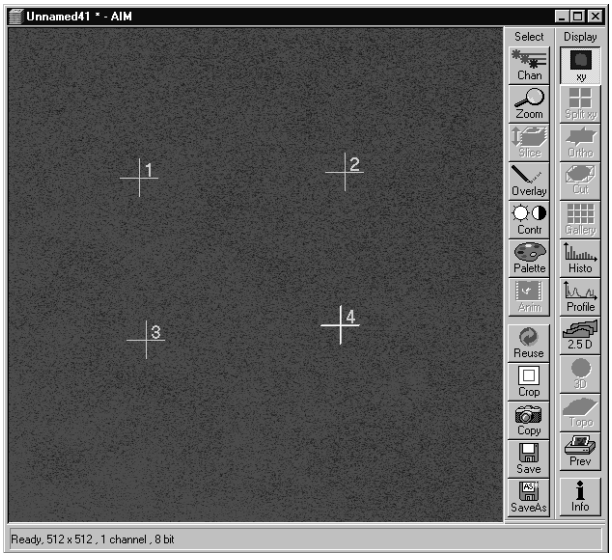


Fig. 4-93 **LSM image of the Rh6G layer with markers for FCS-LSM adjustment**

- Bleach the marked sites.
  - Click **Start**. The usual FCS measurement window appears.
  - Once finished, close the FCS measurement window without saving.

- Rescan the Rh6G layer.
  - Switch to LSM mode.
  - Click **Single** in the **Scan Control**.
  - Once the scan is finished you see the bleached spots (see Fig. 4-94).

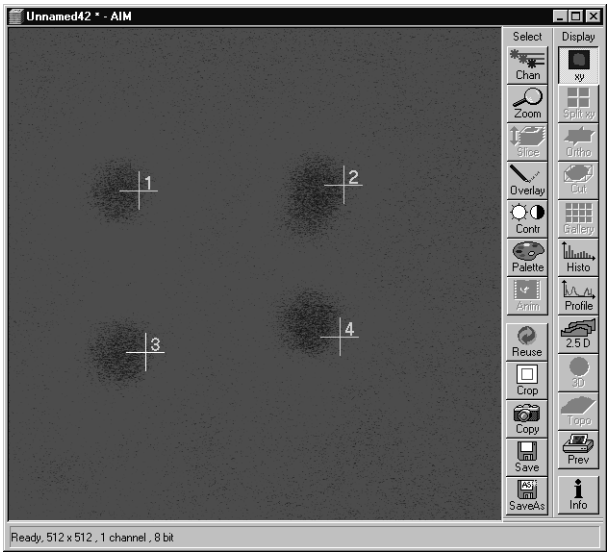


Fig. 4-94 **LSM image of the Rh6G layer after bleaching. Deviations between the markers and the bleached spots are noticeable.**

- Measure the distance between the burned spots and the markers (see Fig. 4-95).
  - Click on **Overlay**.
  - Click on the **1 µm** button.
  - Draw a line between the center of the bleached spot to the vertical and horizontal lines of the marker cross.

- Determine the average X and Y deviation values between the markers and bleached spots. Depending on the direction of these deviations increase or decrease the X and Y values by the measured mean deviation. Type these X and Y values into **Settings** / **LSM+ConfoCor**.

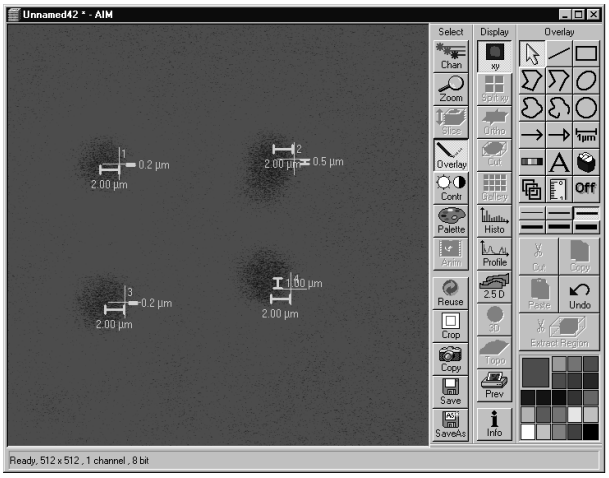


Fig. 4-95 **LSM image after bleaching with overlaid distance markers**

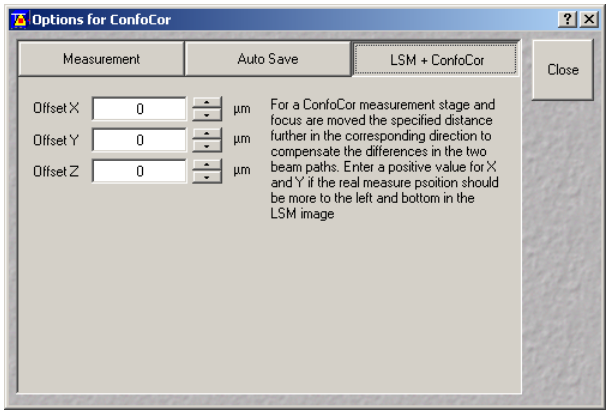


Fig. 4-96 **The Settings for user window with the LSM+Confocor activated**

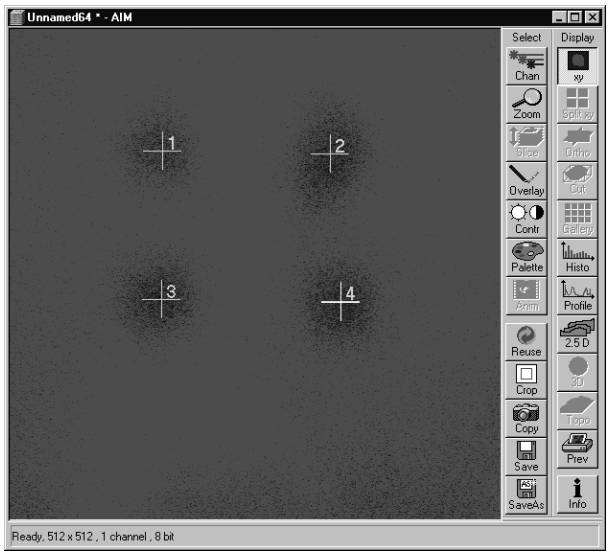


Fig. 4-97 **LSM image after bleaching and correction of offset values**

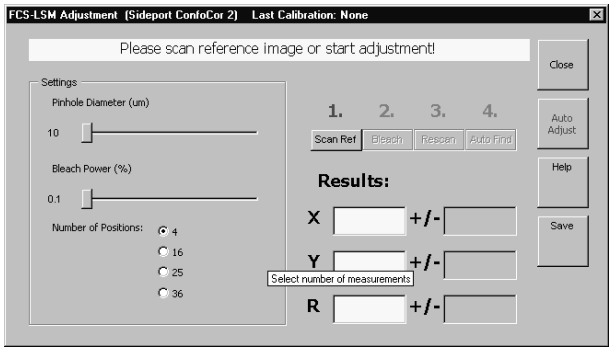


Fig. 4-98 **The Macro "Fcs-Lsm" display before offset compensation**

- Type the measured distance correction values under **Settings** in the **LSM + ConfoCor** button (see Fig. 4-96).
  - If the bleached point is left to the marker: type distance in X as positive value
  - If the bleached point is right to the marker: type distance in X as negative value
  - If the bleached point is below the marker: type distance in Y as positive value
  - If the bleached point is right to the marker: type distance in Y as negative value
  - Offset Z: no entry
- Check the correctness of your settings by repeating the adjustment procedure (see Fig. 4-97).

If the deviations between bleached areas and markers is marginal, the LSM/FCS superposition alignment is sufficient to guarantee a precision of 1 µm.

☞ Please note, that precision is higher if you decide to make a measurement in one section of the image rather than all over it. In this case only deviations in that sector should be taken into account for calculating the offset values.

(b) Adjustment Using the LSM/FCS Macro

- Before running the Macro choose a suitable method in the **Method file** menu in FCS and set the beam path for the LSM. Please, focus on the Rhodamine 6 Green layer.
- Activate the Macro toolbar by clicking the **Macro** button.
- Choose the **Lsm-Fcs** macro by clicking the corresponding button.
- The **Requirements for Adjustment** window will be displayed.
- Click **Ok** to run the Macro, **Cancel** to abort.
- Once **Ok** was chosen, the **Lsm-Fcs** macro window will be displayed (see Fig. 4-98).

- Set the **Pinhole diameter** to 10 and the **Bleach power** to 1%. If holes are too weak or getting to big you should increase or decrease the **Bleach power,** respectively.
- Choose the numbers of Bleach spots by checking the corresponding number.
- You have now two possibilities to continue: Stepwise or automatic.
- Stepwise Procedure:
  - Press the **Scan Ref** button which is highlighted and labeled by a red **1**. A scan is performed. Once finished the **Bleach button** will be highlighted and labeled with a red **2**. The **1** will turn to black.
  - Press the **Bleach button**. The bleach is performed. The **FCS evaluation** window will appear. Once ready, the **Rescan** button will be highlighted and labeled with a red **3**. The **2** will turn to black The **FCS evaluation** window will disappear.
  - Press the **Rescan button**. The image is rescanned. You will see a number of bleached spots according to your settings which corresponds to the positions defined by the rigid FCS beam path. You will also see yellow crosses which are the positions of the laser beam for the LSM. Because there might be an offset between both, crosses do not necessarily correspond with the bleached spots. Once ready, the **Autoscan** button is highlighted and labeled with a red **4**. The **3** will turn to black.
  - Press the **Autoscan** button. Once finished, the **4** will turn black. The program will find the burned spots which will turn red and label them by green circles. The distance between the green circles and yellow crosses is indicated by the green line and the value displayed as the **R** value (see Fig. 4-99). The program also calculates the deviation in X and Y, the values which are also displayed under **X** and **Y** offsets (see Fig. 4-100). The program also calculates the scan drift during the procedure and gives a warning if the scan drift was to great.
  - Press the **Save** button to store the offset values. Deviations between LSM and FCS will than be automatically compensated with the corresponding offsets.

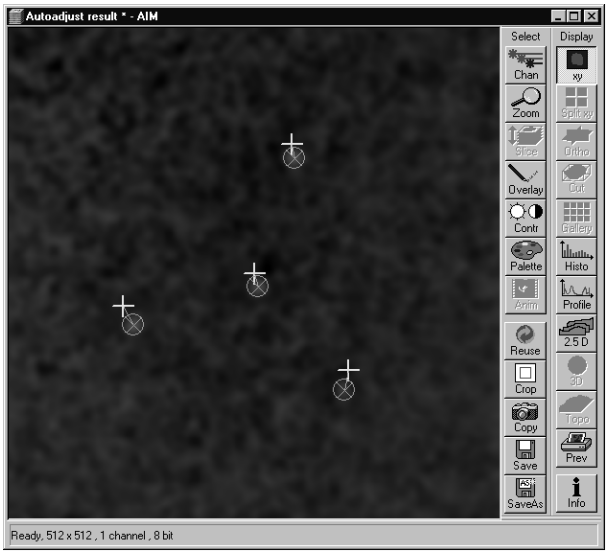


Fig. 4-99 **LSM image after performance of the "Fcs-Lsm" Macro**

☞ Please note that you can do a readjustment without leaving the **Lsm-Fcs Macro**. However, all buttons will be left highlighted and numbers will stay black. That means that you have to watch for yourself when one step is finished and you have to remember, which is the next step you have to activate.

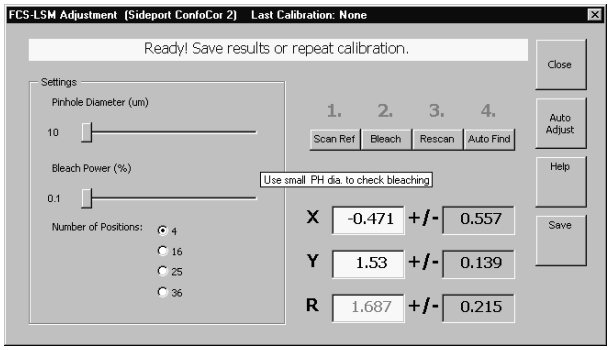


Fig. 4-100 The "Fcs-Lsm" Macro after offset compensation

- Press the **Help** button if you wish to view the **Requirements for adjustment** display. Press **Close** to leave the Macro.

☞ By activating the **Macro** a **Stop Macro** window will appear. You can leave the **Macro** at any time by pressing the **Stop Macro** button.

Automatic Procedure:

– Press the **Auto Adjust** button. The Macro will automatically perform Steps 1. to 4. The corresponding submenu button will be highlighted.

– Once finished, offsets are displayed. Press **Save** to store the offsets, **Help** to view the **Requirements for adjustment** display or **Close** to leave the Macro.

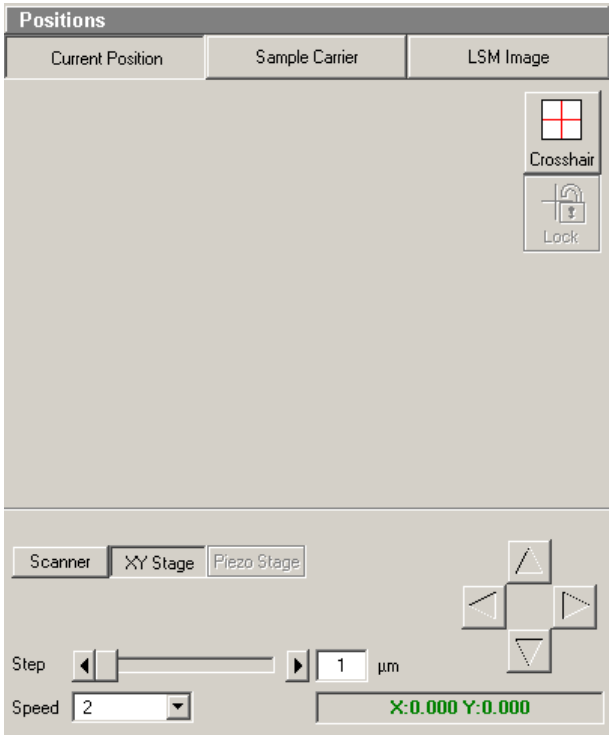


Fig. 4-101 Positions panel, Current Position

(c) Determination of the Fixed X,Y Position of the Laser Beam in the Measurement... Window in the Current Position mode

- Get a LSM image of a Rh6G layer.
  – Go to **Current Position** mode
  – Click the **Crosshair** button (see Fig. 4-101).

- Perform a bleach at the position of the fixed laser beam.
  – Click **Start**. The usual FCS measurement window appears.
  – Once finished, close the FCS measurement window without saving.

- Rescan the Rh6G layer.
  – Click **Single** in the **Scan Control**.
  – Once the scan is finished you see a bleached spot corresponding to the position of the rigid laser beam (see Fig. 4-102).

☞ Since the scanners are parked, the spot should lie in the center of the image, if no crop function and offsets were used.

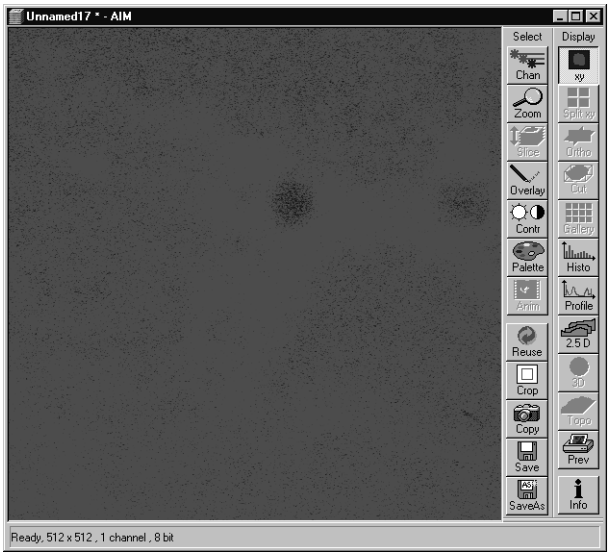


Fig. 4-102 LSM image after bleaching in the Method Optimization

- Record the position of the spot.
  - With the crosshair. Place crosshair above the bleached spot (see Fig. 4-103). We recommend to use the crosshair for positioning your cell.
  - With the cursor. Make sure that under **Options** / **LSM Settings** in the **Image Status Display** tab the **Pixel Intensity** box is checked to activate the display of the coordinates. The coordinates will than be displayed at the lower bar of the image.
  - With an overlay arrow. Click **Overlay** and activate the **Arrow** button. Place the arrowhead in the middle of the spot.

☞ If the **Position** box is checked, the **Crosshair** function is inactivated.

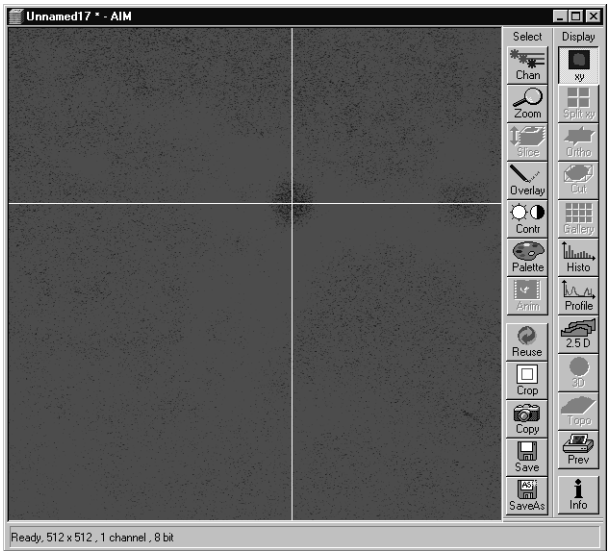


Fig. 4-103 LSM image after bleaching with crosshair

(2) Alignment in Z

No compensation in z is necessary

4.4.19.2 Determination of the Offset between LSM Scanner and FCS Scanner Positioning

There is no need to compensate between defined positions and the actual measurement positions because of scanning mirror positioning. However, the precise arrest position of the mirrors depend on the used scan zoom and speed. To adjust for differences, the compensation sliders of the bidirectional Scan can be adjusted. For the **Method Optimization** in the **Crosshair** mode you locate the position of the beam and place the structure of interest at the defined site. For Z adjustment you have to use the **Method Optimization** window. In all cases you need a thin layer of a fluorescent dye, preferentially Rhodamine 6 Green, dried on a glass bottom of for example a Labtec 8-well chamber.

Alignment in X, Y in the Positions Mode

No compensation is necessary. A defined site is positioned by the scanning mirrors with pixel precision (see Fig. 4-104).

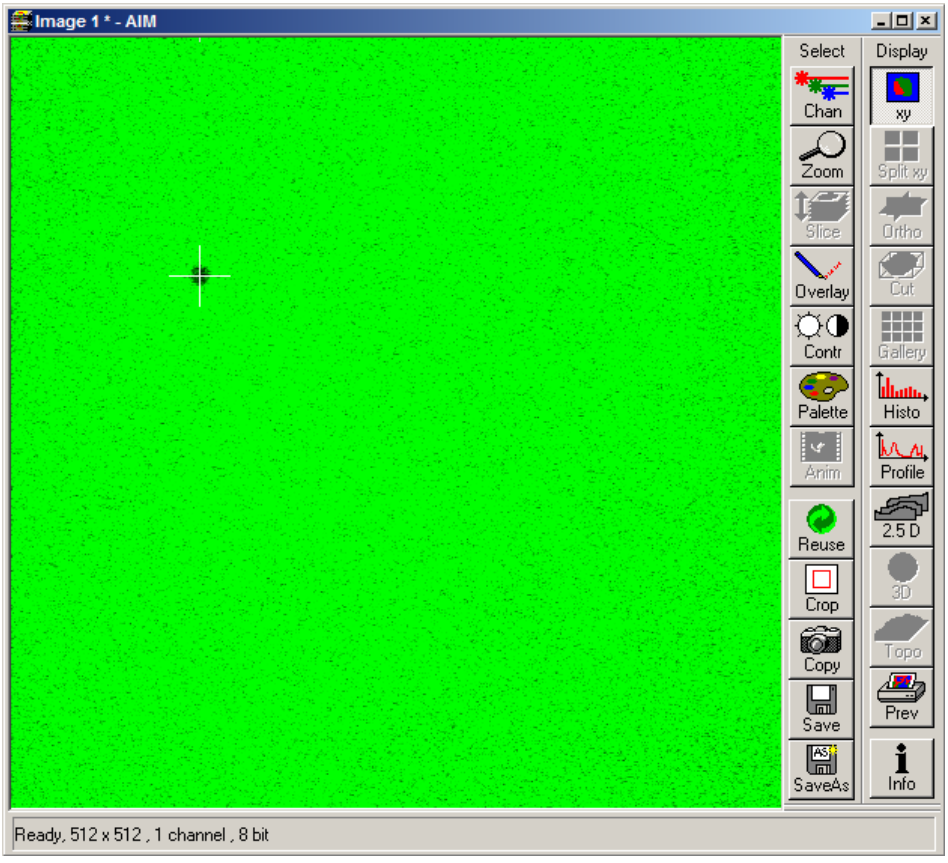


Fig. 4-104 **Bleaching of a spot in a dye layer in Method Optimize via Position list. Note the correspondence of the defined (cross) and actual (bleached spot) positions.**

To compensate for Scan Zoom and Scan Speed go to **Scan Control** under **Mode**. Use a sample with high contrast, like "Convolaria", and focus. Activate **Bidirectional Scan** (bended arrow) under **Scan direction** (see Fig. 4-105). During scanning press the **Auto** button. The system will adjust automatically the **Scan Corr X** and **Y** values. Alternatively you can do the compensation manually.

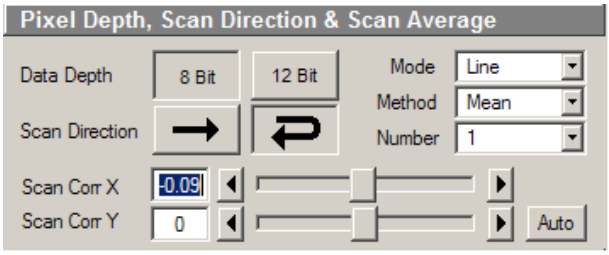


Fig. 4-105 **The Pixel Depth, Scan Direction & Scan Average Window**

To this end use an edge of a structure or a line. If the **Scan Corr** is not adjusted, you will see a pixel shift (see Fig. 4-106).

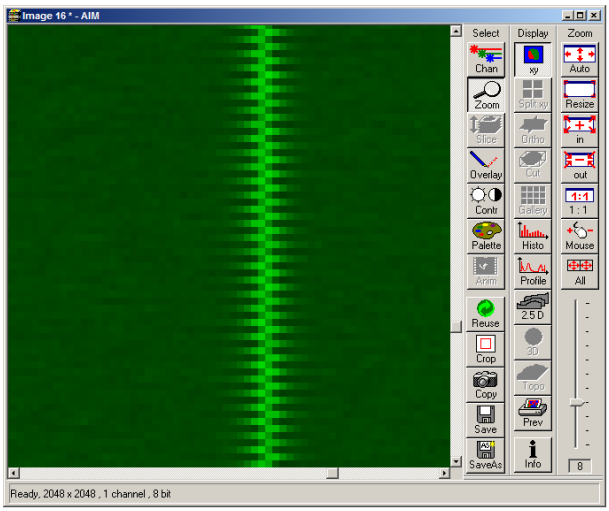


Fig. 4-106 **Image of a line before scan correction with pixel shift in**

Move the **Scan Corr X** slider until the pixel shift is at the minimum (see Fig. 4-107). Rotate the image by 90 degrees and redo the same adjustment for **Scan Corr Y**.

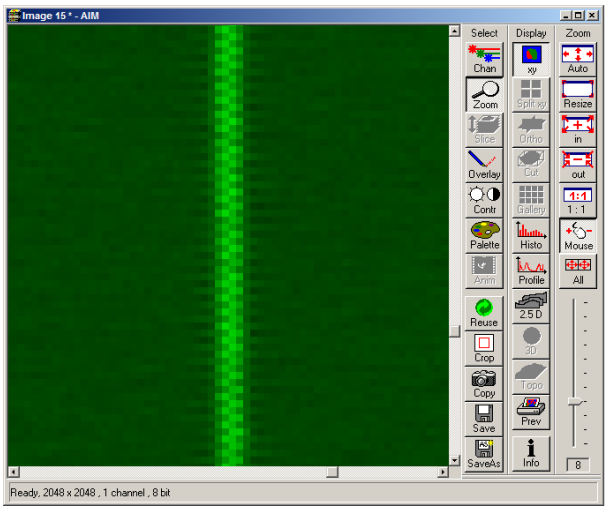


Fig. 4-107 **Image of a line after pixel shift compensation in X**

4.5 Data Evaluation and Result Presentation for FCS Measurements

4.5.1 Structure of the Data Evaluation Window

The data evaluation window (Fig. 4-108) of the FCS software part corresponds to the basic structure of other Microsoft ® WINDOWS applications.

The control line at the top of the data evaluation window contains the control menu for the data evaluation window (identical to Microsoft ® WINDOWS), the name of the displayed data file, and the **Minimize**, **Maximize** and **Close** buttons (identical to Microsoft ® WINDOWS).

In the status line at the bottom of the data evaluation window, the progress of a current measuring procedure are shown online. During the measurement you can stop the procedure by clicking the **Stop** button.

The major part of the window contents the measuring results (graph and table) corresponding to the selected display modes.

To its right, the **Display** toolbar is always shown in the standard setting. Depending on whether some buttons in the **Display** toolbar are activated / deactivated, further toolbars (e.g.: **Correlation**, **Print, Fit**) are displayed / not displayed on the right-hand side of the data evaluation window.

The data evaluation 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).

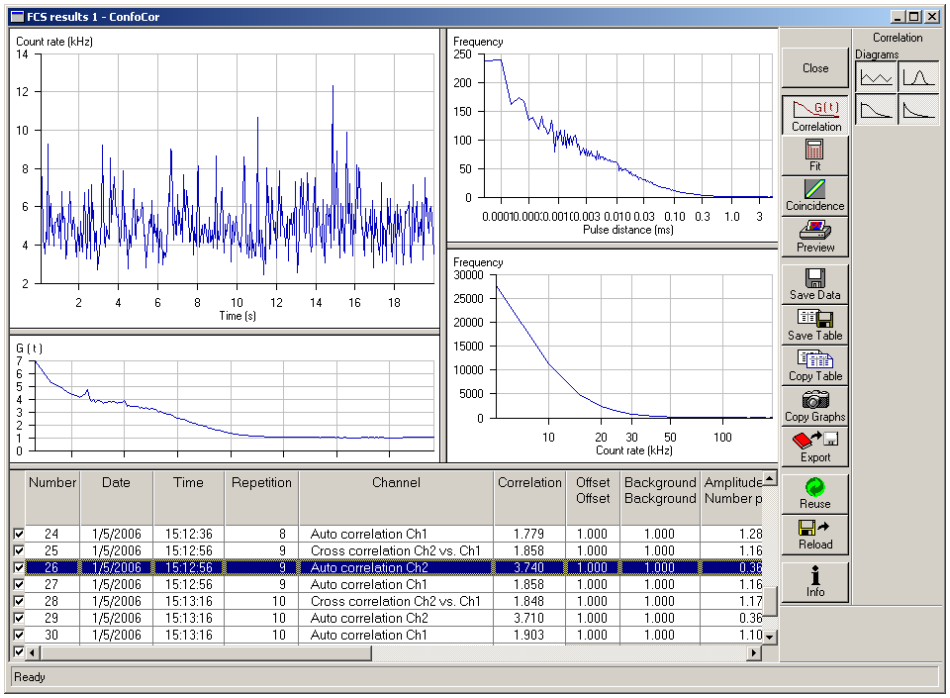


Fig. 4-108 Data evaluation window

4.5.2 Open / Close the Data Evaluation Window

- Click **Start** either from the **Measure** window to trigger a new measurement, or open an existing data file from the database by pressing the **Open FCS** button.
  - The **Data Evaluation** window will appear (Fig. 4-108).
- Click the **Close button** to leave the Data evaluation window.

Formats that will be recognized by pressing the **Open FCS** button include:

- ConfoCor1 data files. If more than 1 file is selected they will be opened in the same **Data Evaluation** window. According to the setting at the **Average** selection box in the **Table Properties** window, which is obtained by clicking the right mouse button within the table and selecting **Properties**, the average of the correlation or the average of the fit results are shown in the last row.
- ConfoCor 2 and 3 data files (*.fcs). Each data file will be opened in a separate **Data evaluation** window.

☞ Note, that exported data files will be opened within the same window. ConfoCor 2 raw data files: Each file will be opened in a different **Data evaluation** window.

4.5.3 Description of the Raw Data Format

4.5.3.1 ConfoCor 1

For the ConfoCor 1 no raw data format was available

4.5.3.2 ConfoCor 2

This raw data format is the one for ConfoCor 2 (and ConfoCor 3) data files of Rel 3.5 and lower.

The data format is an exact representation of the dual channel photon trace within the limits given by the digitalization. This means that the data are recorded without losses within these limits.

The basic idea is to record the time between subsequent pulses from the detector in units of elapsed clock cycles (run length encoding). However, the format is modified to conserve space at high count rates and to be capable to handle dual channel data.

Data will be recorded in 16 bit words. Whenever a pulse is detected or the counter counting the clock cycles overruns 255 (FF hex), a word will be recorded. The word will contain information how many clock cycles elapsed (*1 ... 255*) since the last word had been recorded and additionally what happened in the four cycles of data generation *bt1 ... bt4*.

The recorded word has the following structure:

Bit	Meaning
0 (LSB) ... 7	clock counter value (starting at 1) during the triggering event trigger events are pulse recordings or counter overruns zero is reserved and only transmitted at the end of the measurement
8	1, if pulse recorded in channel 1 during cycle bt1; else 0
9	1, if pulse recorded in channel 2 during cycle bt1; else 0
10	1, if pulse recorded in channel 1 during cycle bt2; else 0
11	1, if pulse recorded in channel 2 during cycle bt2; else 0
12	1, if pulse recorded in channel 1 during cycle bt3; else 0
13	1, if pulse recorded in channel 2 during cycle bt3; else 0
14	1, if pulse recorded in channel 1 during cycle bt4; else 0
15 (MSB)	1, if pulse recorded in channel 2 during cycle bt4; else 0

Table 1 Structure of the recorded word

We hope the two examples will make these statements clearer. The tables show part of the running pulse train (from left to right) divided into clock cycles. "1" in the corresponding box indicates that a pulse arrived in this cycle. The "counter" row shows the counter readings. The lowest row indicates when the word has been recorded.

Example 1

The following words will be recorded:

- at W1:
  high byte: 00011001(bin) = 19 (hex); low byte: 123 (dec) = 7B (hex); resulting word = 7B19 (hex)
- at W2:
  high byte: 00000000(bin) = 00 (hex); low byte: 255 (dec) = FF (hex); resulting word = FF00 (hex)

				bt1	bt2	bt3	bt4							bt1	bt2	bt3	bt4		
CH 1				1		1						…							
CH 2					1							…							
counter	120	121	122	123	0	0	0	1	2	3	4	…	254	255	0	0	0	1	2
							⇑ W1										⇑ W2		

Table 2 Example 1

Example 2

The following words will be recorded:

- at W3:
  high byte: 00010001(bin) = 11 (hex); low byte: 123 (dec) = 7B (hex); resulting word = 7B11 (hex)
- at W4:
  high byte: 00100100(bin) = 24 (hex); low byte: 255 (dec) = FF (hex); resulting word = FF24 (hex)

				bt1	bt2	bt3	bt4							bt1	bt2	bt3	bt4		
CH 1				1		1						…			1				
CH 2												…				1			
counter	120	121	122	123	0	0	0	1	2	3	4	…	254	255	0	0	0	1	2
							⇑ W3										⇑ W4		

Table 3 Example 2

The clock runs at a clock rate of 20 MHz. This means a maximum data rate of 10 Mbyte/s. If no pulses are recorded, the clock rate drops to approx. 155 Kbyte/s according to counter overflows.

The first 30 bytes of the raw data file contain the comment "ConfoCor_2_-_Raw_data_file_1.0" and have to be ignored.

4.5.3.3 ConfoCor 3

This raw data format is for ConfoCor 3 (and ConfoCor 2) Rel 4.0 and higher.

☞ In the ConfoCor 3 the format is already processed into one that describes the time distance between photons.

Raw data files can be opened directly. They are also opened automatically if linked to an .fcs measurement. In this case all raw data files associated with the .fcs file will be opened in the same window automatically.

The format is explained in a figure (Fig. 4-109). The figure displays the raw data in an editor that shows the raw data in file offset, hexadecimal and as ASCII.

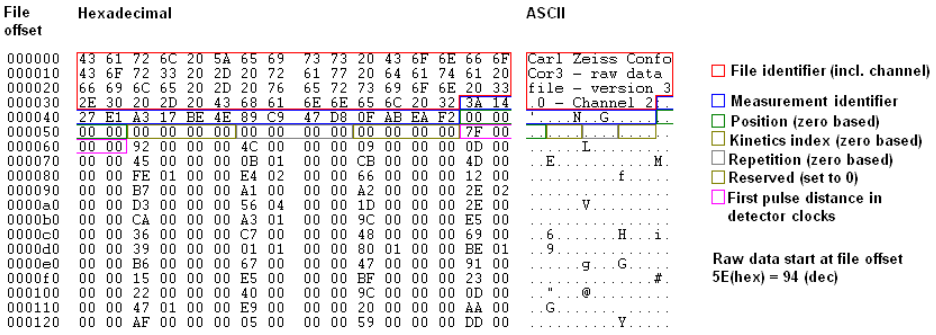


Fig. 4-109 ConfoCor 3 format

The first 52 bytes (bytes 1-52) represent the File identifier with the channel number. The identifier is a randomly created number that will be assigned to all repetitions of the same measurement.

The next 16 bytes (bytes 53-68) is the measurement identifier. This will be assigned to all channels of the same repetition. Hence, in a cross-correlation experiment, the two auto-correlation pairs that belong together can be identified.

The next 4 bytes (bytes 69-72) encode the position of the measurement and are zero based.

The next 4 bytes (bytes 73-76) encode the kinetic index and are zero based.

The next 4 bytes (bytes 77-80) encode the repetition number and are zero based.

The next 4 bytes (bytes 81-84) are reserved for comments an set to 0.

The next 4 bytes (bytes 85 -corresponding to a file offset of 5E (hex)=94 (dec)-to 88) code for the first pulse distance in detector clocks.

The next bytes (starting from byte 89) code for follow up pulse distances.

4.5.4 Display - Correlation

- If not selected, click on the **Correlation** button to activate the **Correlation** display mode.
  - The **Correlation** display toolbar will appear to the right of the **Data Evaluation** window (Fig. 4-108).

Up to four windows with different graphs become visible in the upper left part of the window depending on the activated **Diagrams** buttons. By clicking on the appropriate button, the diagram can be toggled between **ON** and **OFF**. The size of the diagrams can be matched as required by moving the border lines. Click on the border line, hold down the mouse button, move the line in the required direction; and release the mouse button.

The measuring results are displayed in a table below the diagrams. The width of the columns can also be changed by moving the border lines. The order of the columns can also be changed. For this purpose, click on the head line of the relevant column, hold down the mouse button and move the column to the required position. When the mouse button is released, the column is inserted in the new position.

If more than one line of the table is selected, an appropriate legend can be added to the diagrams by activating the required **Diagram Legend** check boxes.

To select a line in the table, click on it with the mouse (multiple choice is possible by additionally pressing the **Shift** or **Ctrl** key). Selected lines are highlighted in color.

After completion of the measurement, the correlation curves are fitted to the model curve using the parameters provided by the measurement method.

The fitting results are written into the table in the lower part of the window.

Any line of the table can be made the current one by clicking on it. This also means that the corresponding graphs are shown in the displays.

Pressing the right mouse button, when the cursor is within the table, will open a menu (see Fig. 4-110) offering different options (see next page):

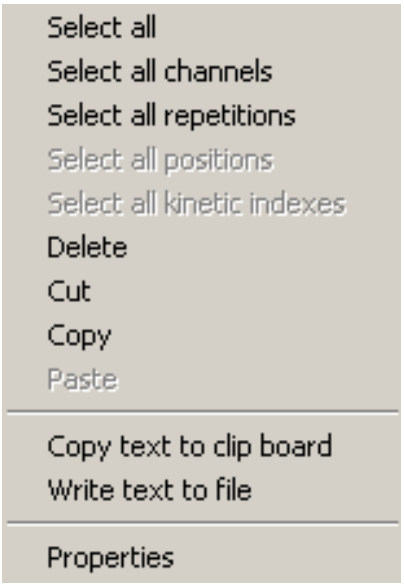


Fig. 4-110 Table menu

Select all All the channels from the different measurement and the average channels will be selected.

Select all channels All channels belonging to the currently selected one and to one measurement of repetitive measurements will be selected and highlighted.

Select all repetitions All channels belonging to the currently highlighted one of repetitive measurements will be selected and highlighted.

Select all positions All channels belonging to the same measurement position than the highlighted channel will be selected.

Select all kinetic indices All channels belonging to the same kinetic time point than the highlighted channel will be selected.

Delete The highlighted columns will be deleted.

Cut The highlighted columns will be stored in the clipboard. Only if the data are pasted in a new window, the data are deleted from the old one.

Paste Will paste columns currently stored in the clipboard into the table.

Copy text to clipboard The table will be copied into the clipboard and can be opened in other Text programs.

Write text to file Will store the table in a .txt file. You will be prompted to choose a name and a folder.

Properties Choosing **Properties** will open the **Table Properties** window (see Fig. 4-111).

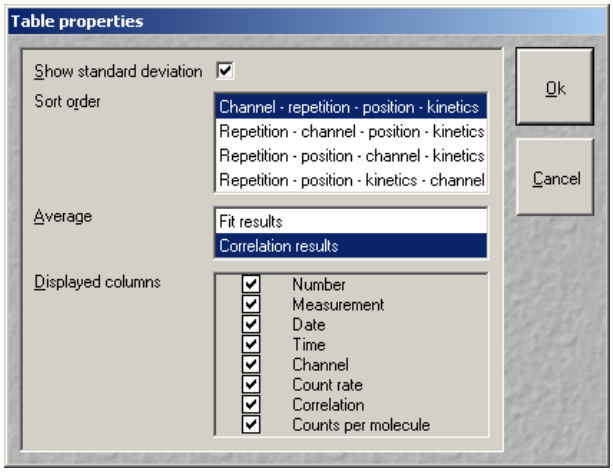


Fig. 4-111 Table properties window

If the **Show standard deviation** box is checked, all parameters will be displayed with their values and the calculated standard deviation from different measurements.

In the **Sort order** display window you can choose, how different measurements are grouped together. The hierarchy of grouping of categories is determined by their listed sequence.

In the **Average** display window you can select, how the data are averaged. You can either select **Fit Results**, in which case the single values of the parameters are averaged, or **Correlation results**, where the single data points of each correlation functions are averaged and an average correlation function is calculated.

☞ Please note, only in the latter case the displayed average correlation function can be meaningfully fitted.

The **Displayed columns** selection window allows you to change the appearance of the table with regard to columns to be displayed. All parameters with their boxes checked will be displayed.

The table of measuring rows in the lower part of the **Data Evaluation** window contains a check box on the left-hand side of each row. Deactivation of these check boxes will exclude the relevant rows from all subsequent evaluation procedures. These settings will be stored by saving the data.

However, immediate reactivation is possible via the check box, if required.

The order of the displayed columns can be adjusted using drag and drop.

The **Correlation** toolbar contains the following function elements for different displays:

Count Rate Trace button

Shows the **Count Rate** diagram: Here the count rate(s) vs. running time is displayed. If a cross correlation set-up is used, the count rate trace for each channel is displayed. If you have set an electronic dust filter in **Processing** or loaded Raw data files with an electronic dust filter activated the cut off regions will be displayed as grayed out areas. Correlation analysis is than performed separately with all sections of the correlation curves that are disrupted by cut off regions and the calculated curves are averaged.

You can zoom in the image by pressing the left mouse button and drawing a rectangle of the area you want to zoom in. If the button is disengaged, the zoom image is displayed.

By clicking the right mouse button you obtain the count rate context menu (see Fig. 4-112).

If you have zoomed in, you can go to the original image size by choosing the **Reset diagram zoom** option.

If you have a raw data file available, you can define cut regions by choosing the **New cut region** option. The cut region size can be adapted by two sliders. The cut out region is thereby displayed as a matted box (see Fig. 4-113). You can select independent cut regions for different channels of a cross correlation experiments. In cross-correlation calculations, a cut region in one channel will automatically define the same cut region in the other channel. You can select more cut regions by repeatedly choosing this option.

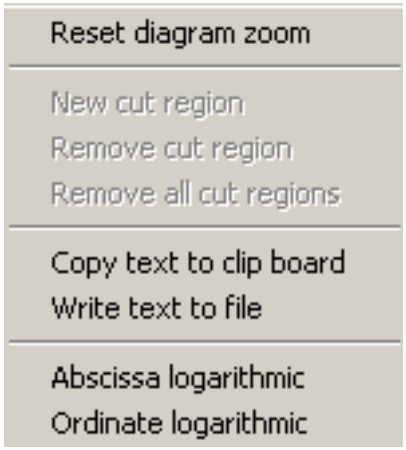


Fig. 4-112 Count Rate menu

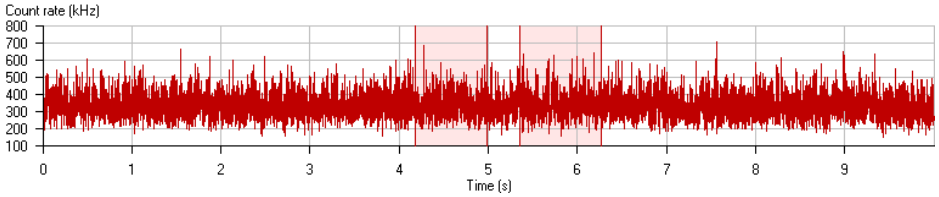


Fig. 4-113 Cut Out Regions

These regions can overlap. **Remove cut region** will remove the last cut region. By repeatedly choosing this option, the cut regions in the reverse order of their creation will be removed. **Remove all cut regions** will remove all cut regions simultaneously.

☞ Note, that the remaining parts of the count rate will be separately correlated and the average will be calculated and displayed. **Copy text to clipboard** will copy the diagram coordinates into the clipboard, from which they can be pasted into other programs like Excel. **Write text to file** will store the diagram coordinates in a .txt file. You will be prompted to choose a name and a folder before saving.

- Choosing **Abscissa logarithmic** or **Ordinate logarithmic** will display the abscissa or ordinate in a logarithmic scaling.

 **Correlation Curve** button

Shows the **Correlation Curve** graph: In case of auto correlation measurement, the developing correlation curve is displayed. In case of cross correlation measurement, three curves will be shown: the two auto correlation curves for each of the channels and the cross correlation curve.

The zoom function can be used to display certain diagram areas of interest in an enlarged form: Use the left mouse button to click on the margin of the area of interest in the diagram, keep the mouse button pressed and draw a rectangular above the area of interest in any required direction. On release of the mouse button, the selected area is displayed in an enlarged form in the diagram. The scaling of axes is matched automatically.

By clicking the right mouse button you can choose between different options in the context menu (see correlation-menu).

The context menu of the relevant diagram can be used to reset the zoom to its original value.

- Click on the diagram with the **right** mouse button.
  - The context menu is opened.
- Click on the line **Reset Diagram Zoom** with the left mouse button.
  - The zoom value is reset.

The data (measuring values) of each diagram can be copied to the clipboard or stored directly as an ASCII file via the context menu.

- Click on the diagram with the right mouse button to open the context menu.
- Select the line **Copy data to clipboard** with a click of the mouse if you want to insert the data directly into other WINDOWS programs via the clipboard. The Paste function permits direct insertion of these data into the required program.
- Select **Write text to file** to store the data in an external ASCII file (.txt). Normalized values will also be exported in this way.

☞ Note that the **Export** button in the **Display Correlation** window will only export the original values.

The diagram display can be manipulated with the context menu as follows:

- Click on the diagram with the right mouse button to open the context menu.
  - Selection of **Abscissa logarithmic** will scale the x-axis logarithmically.
  - Selection of **Ordinate logarithmic** will scale the y-axis logarithmically.
  - Selection of **Normalize** will normalize the curves to 2 in the following way (see Fig. 4-115):

The G(0) value corresponding to the number of diffusing particles will be normalized to 2, not the total correlation to which also other processes like triplet state can contribute. All other values (subtracted by 1) will than be multiplied by the correction factor defined by the ratio of 1 to [$G(\tau_{n2})$-1] and increased by1, hence $G(\tau)_{normalized}$=[1/ (G(0)-1) * (G(τ)-1)]+1. Deactivating **Normalize** will display the normal correlation curve (see Fig. 4-114).

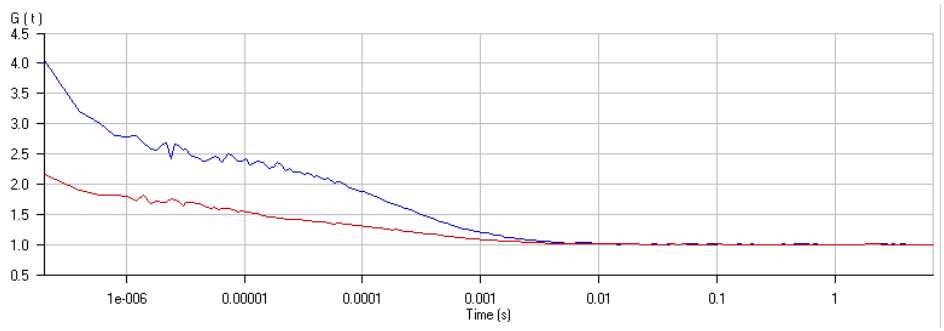


Fig. 4-114 The Correlation Curve window in normal display

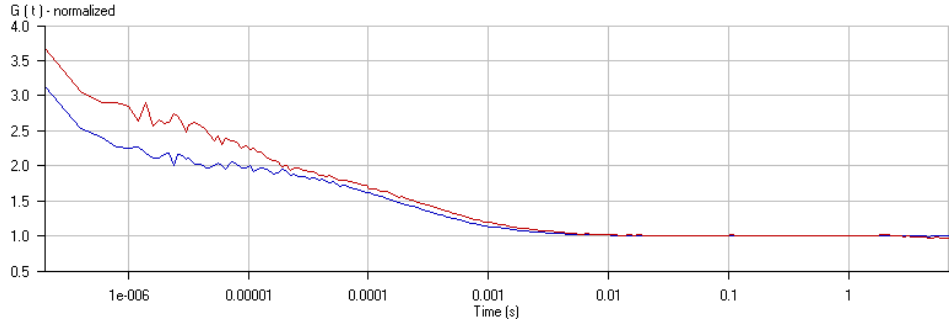


Fig. 4-115 The Correlation Curve window in normalized display

 **Count Rate Histogram** button

Shows the **Count Rate** histogram: To obtain this histogram, the number of pulses (or: photons recorded from the detector) in a moving time window are recorded and included in a histogram. You can determine the binning time in **Processing** under PCH or iIf you load a raw data file with the **Reload** button.

You can zoom in the image by pressing the left mouse button and drawing a rectangle of the area you want to zoom in. If the button is disengaged, the zoom image is displayed.

By clicking the right mouse button you can choose between different options in the context menu.

If you have zoomed in, you can go to the original image size by choosing the **Reset diagram zoom** option.

Copy text to clipboard will copy the diagram coordinates into the clipboard, from which they can be pasted into other programs like Excel. **Write text to file** will store the diagram coordinates in a .txt file. You will be prompted to choose a name and a folder before saving.

Choosing **Abscissa logarithmic** or **Ordinate logarithmic** will display the abscissa or ordinate in a logarithmic scaling.

 **Pulse Density** button

Shows the **Pulse Density** histogram: Here the times elapsed between two subsequent pulses (or: photons recorded from the detector) are measured and the times are included in a histogram.

You can zoom in the image by pressing the left mouse button and drawing a rectangle of the area you want to zoom in. If the button is disengaged, the zoom image is displayed.

By clicking the right mouse button you can choose between different options in the context menu (see pdh-menu).

Copy text to clipboard will copy the diagram coordinates into the clipboard, from which they can be pasted into other programs like Excel. **Write text to file** will store the diagram coordinates in a .txt file. You will be prompted to choose a name and a folder before saving.

Choosing **Abscissa logarithmic** or **Ordinate logarithmic** will display the abscissa or ordinate in a logarithmic scaling.

☞ Note that, normalization only works for a curve, for which a fit has been conducted in the **Fit window**.

4.5.5 Display - Fit

- Click on the **Fit** button to activate the **Fit** display mode.
  - A **Fit** menu subordinate will appear (Fig. 4-116).

In general this menu allows you to work with already measured and probably also fitted correlation curves. The intention is to have the possibility of performing another fit using different fitting parameters if the automatic fit did not yield optimal results.

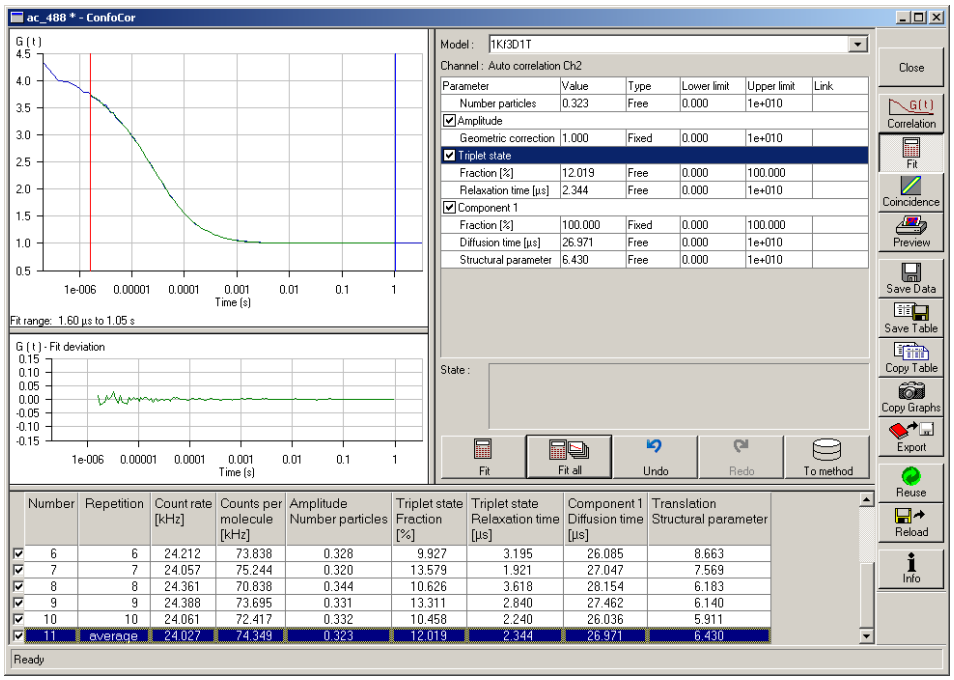


Fig. 4-116 Display fit

The upper graph shows the correlation curve with the overlaid fit graph. It also displays the fit range defined by the red (start value) and the blue (end value) bars. The lower graph depicts the fit residuals.

- Select one or more channels in the lower table. The last activated channel will be displayed in the **Channel** display.
- Use the **Model** pull down menu to select a predefined model. All the terms and components of the model will be displayed activated. All parameters to one term are listed as a block in the **Parameter** column. The parameters' values are displayed in the **Values** column.
- You can deselect and select terms by de-checking / checking the boxes on the left of each term.
- Set the range of the curves to be fitted by setting the red and blue bars in the curve fit window. Press the **Fit** button to fit the loaded curves to the model. If you press **Fit all** the other channels in the window corresponding to the highlighted ones will be fitted as well.

☞ Note, all other channels will not be fitted and the model has to be selected anew, if such a channel is selected.

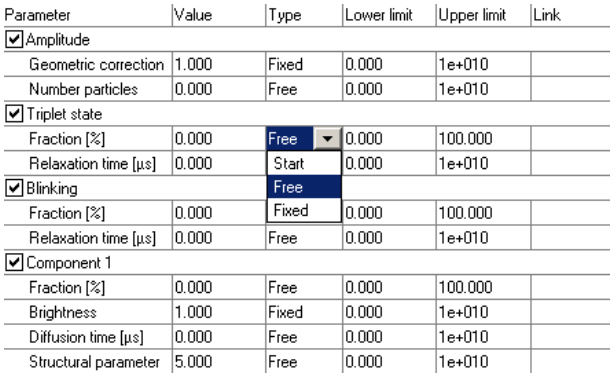


Fig. 4-117 Fit Type selection

- An additional button bar (**Free**, **Fix** or **Start**) will appear if you click at the right-hand side of the parameter fields in the **Type** column (see Fig. 4-117).
  - If you select **Free**, the parameter will be defined as free. After the fit, the field will contain the fit result for the parameter. The start values of the parameter (initial guesses) will be calculated by an algorithm.
  - **Fix** allows you to fix the parameter. This is useful if you know its value from other measurements.
  - **Start** will assign a start value to the parameter and leaves the parameter free to fit. In this case, no initial guesses will be made.

- Upper and lower values for the fit parameters can be entered in the fields under the **Upper limit** and **Lower limit** columns. Defaults are the extreme possible values. If fit limits are exceeded, the fit will try to find another solution or will fail.
- Parameters can be linked globally by an additional button bar (**link measurement M**, **link kinetic indexes K**, **link positions P**, **link repetitions R, link channels C**). You can select the type of linkage by checking the corresponding boxes. After closing the button bar, the linkage type is displayed as a one letter abbreviations. If a linkage is activated that does not apply for a measurement, it will be just disregarded. Global fitting will fit all linked parameters of different measurements to the same value. On-off measurements will interfere with global fitting and should be deactivated. The following linkages of a parameter is possible:
  Link measurement: links a parameter from different measurements
  Link kinetic indexes: links a parameter from the same time points
  Link positions: links a parameter obtained from measurements at the same site
  Link repetitions: links a parameter for all repetitions of one measurement
  Link channels: link a parameter for the same channels
- **Start Channel** and **End Channel** enable you to determine which part of the correlation curve should be fitted to the model. Moving the red or blue line with the mouse permits the parameters **Start Channel** and **End Channel** to be determined directly in the Correlation-Time diagram. The start and end position of the channels will be displayed as the absolute position (1 – 255) or correlation times (in µsec) and are represented by the red and blue bar, respectively.

☞ **Caution:** The numbers indicate which channels are used for correlation. If you want to know the number of channels omitted from the respective end you have to subtract the **Start channel** number by 1 and subtract from 255 the **End channel** number, respectively.

The **State** display box informs you about the fitting conditions. The quality of the fit is displayed in the **chi²** display box of the result table. The X^2 (chi^2) value should approach zero for highest quality.

- When you are finished, click the **Fit** button and the fit procedure will b e run for the fit settings field (on the right-hand side of the diagrams).
  - When the fit is completed, the free parameters will be replaced with the new fitting results and the fit result graph in the correlation curve diagram will be redrawn.

– The result table will also be updated for the highlighted channels.

- With a click on the **Fit all** button, the fit procedure is performed for all activated measurements (via the check box of the row) in the result table of those channels that were of the same type as the highlighted ones.
- With the **Undo** button, you can cancel a previous command. Repeatedly pressing will cancel commands in the reverse of their execution. The **Redo** button will re-execute the previous undone command. Repeatedly pressing will re-execute previous undone commands in the order of their previous execution.

The **ToMethod** function permits the fitted parameters to be transferred directly to an existing or a new Measuring Method.

- Open the **Measure** window by clicking the appropriate button in the **ConfoCor** subordinate toolbar of the **Main** menu and press the **Processing** button. Choose the same model as used in the fit.
- Then click on the **Write to Method** button.

The parameters of the selected fitting model are assigned to the method selected in the **Processing** window and stored.

- If the parameters shall be assigned to a new method, click on the **Save** button and enter a new name for the method in the appearing **Save Method** window. Confirm your entry by clicking on **OK**.

☞ All the result table functions in the **Fit** mode are identical to those in the **Correlation** mode.

The zoom function can be used to display certain diagram areas of interest in an enlarged form:

- Use the left mouse button to click on the margin of an area of interest in the diagram, keep the mouse button pressed and draw a rectangular above the area of interest in any required direction. On release of the mouse button, the selected area is displayed in an enlarged form in the diagram. The scaling of axis is matched automatically.

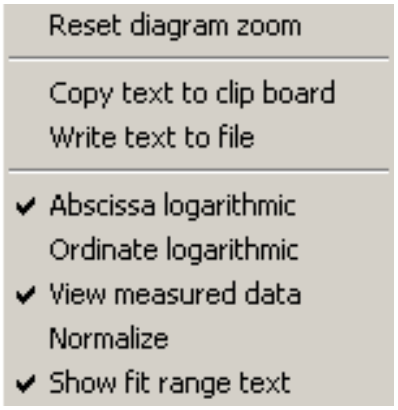


Fig. 4-118 Context menu of the Fit diagram

The context menu can be used to reset the zoom to its original value.

- Click on the diagram with the **right** mouse button to open the context menu (see Fig. 4-118).
- A click on the **Reset Diagram Zoom** with the **left** mouse button will reset the zoom value.

The data of the diagram can be copied to the clipboard or stored directly as an ASCII file via the Context menu.

- Click on the diagram with the **right** mouse button to open the context menu.

- Select the line **Copy data to clipboard** with a click of the mouse if you want to insert the data directly into other WINDOWS programs via the clipboard. The **Paste** function permits direct insertion of these data into the required program.
- Select **Write to file** to store the data in an external ASCII file (.txt). Only the coordinates of displayed curves are exported.

The display of the diagram can be adapted by use of the context menu.

- Click on the diagram with the right mouse button to open the context menu.
- Select **Abscissa logarithmic** or **Ordinate logarithmic** to scale the x- or y-axis, respectively, logarithmically.

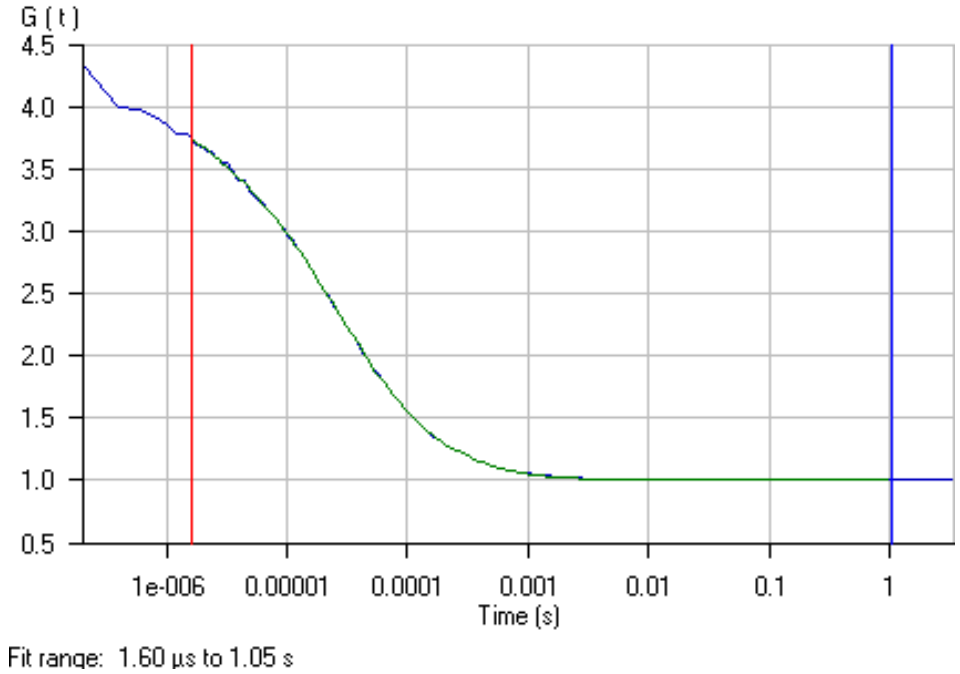


Fig. 4-119 Correlation Curve window in normal display

- Select **View measured data** will display the measured Correlation curve in addition to the Fit curve (see Fig. 4-119). If this option is not selected, only the Fit curve is displayed (see Fig. 4-121).
- Select **Normalize** to display the correlation curves in a normalized form (see Fig. 4-120). The amplitude of the diffusion contribution will be normalized to 2, not the total amplitude.
- Select **Show fit range text** to display the start and end values of the fitted data defined by the red and blue bars.

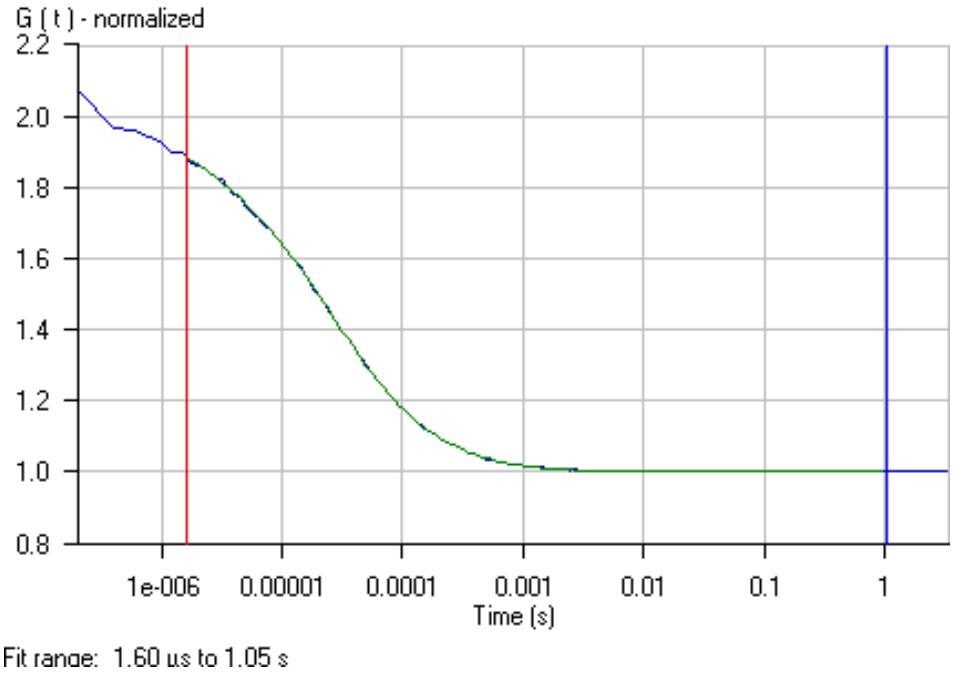


Fig. 4-120 Correlation Curve window in normalized display

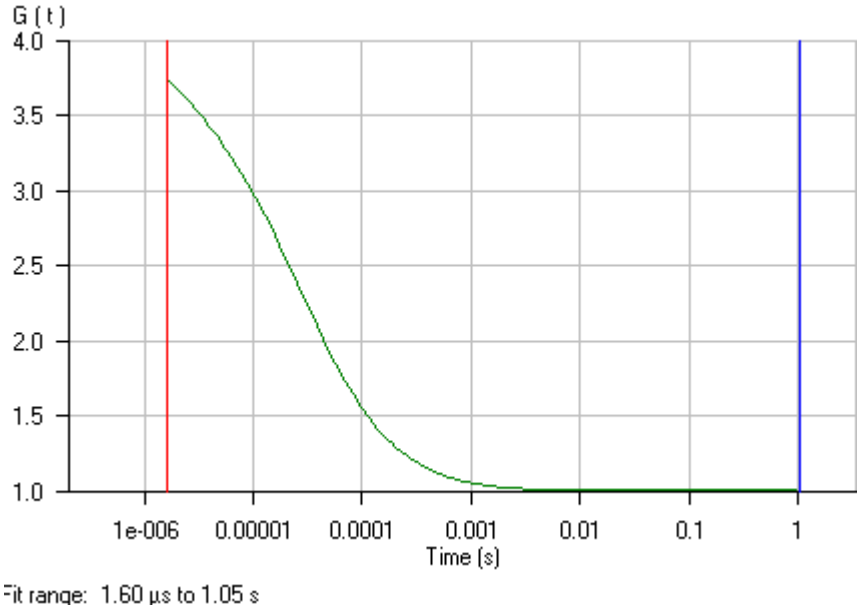


Fig. 4-121 Correlation Curve window in fit curve display ("View measured data" unchecked)

The zoom function can be used to display certain areas of interest in the **Fit deviation** diagram see fit-deviation-window) in an enlarged form:

- Use the left mouse button to click on the margin of the area of interest in the **Fit deviation** diagram, keep the mouse button pressed and draw a rectangular above the area of interest in any required direction. On release of the mouse button, the selected area is displayed in the diagram in an enlarged form. The scaling of axes is matched automatically.

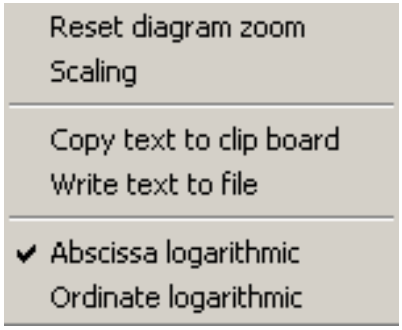


Fig. 4-122 **Context menu of the Fit deviation diagram**

The context menu of the **Fit deviation** diagram (can be used to reset the zoom to its original value (see fit-deviation-context).

- Click on the diagram with the **right** mouse button.
  - The context menu (see Fig. 4-122) is opened.
- Click on the line **Reset Diagram Zoom** with the left mouse button.
  - The zoom value is reset.

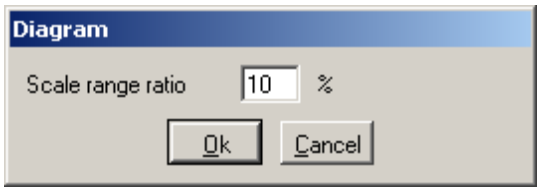


Fig. 4-123 **Diagram scaling window**

The scaling of the G(t) axis of the **Fit deviation** diagram can be varied in percentage values of the fit scaling via the context menu.

- Click on the diagram with the right mouse button to open the context menu.
- Select the line **Scaling** with a click of the mouse.
  - The **Diagram** scaling window (see Fig. 4-123) is opened.
- Enter the required percentage value (from 1 to 100) for the scaling and click on **OK**.
  - The scaling is changed accordingly.
  - Enter **Cancel** if you want too close the window without changes

The data of the diagram can be copied to the clipboard or stored directly as an ASCII file via the context menu.

- Click on the diagram with the right mouse button to open the context menu.
- Select the line **Copy data to clipboard** with a click of the mouse if you want to insert the data directly into other WINDOWS programs via the clipboard. The Paste function permits direct insertion of these data into the required program.
- Select **Write to file** to store the data in en external ASCII file (.txt).

The table of measuring rows in the lower part of the **Data Evaluation** window contains a check box on the left-hand side of each row. Deactivation of these check boxes will exclude the relevant rows from all subsequent evaluation procedures.

However, immediate reactivation is possible via the check box, if required.

4.5.6 Display - Coincidence

- Click on the **Coincidence** button to activate the **Coincidence** display mode.
  - The coincidence analysis generates a two-dimensional color-coded count rate histogram (Fig. 4-124).

This is an alternative to the standard correlation analysis during rare event detection. If the FCS is used to look for small amounts of double-labeled objects (molecules, aggregates etc.) in liquids, the measurement time to generate a correlation curve which can be evaluated by the standard model may be prohibitively long. As a rule of thumb, approximately 1000 transitions of labeled aggregates are required to generate a correlation curve of sufficient quality.

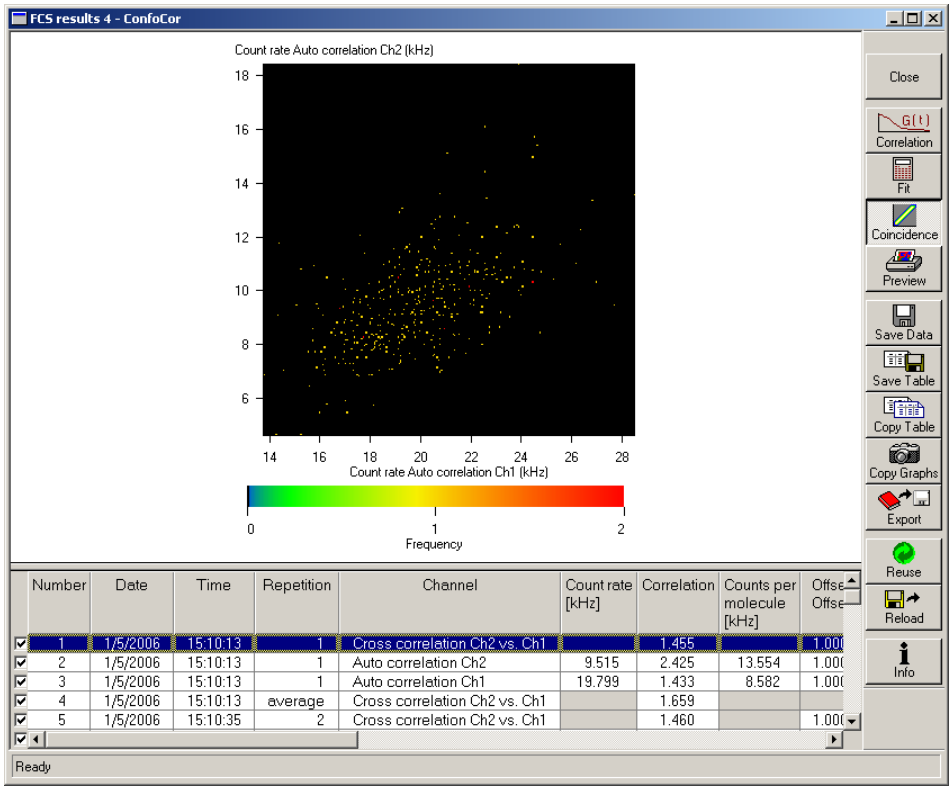


Fig. 4-124 Coincidence diagram window

During the measurement in small time windows the count rates in channel 1 (red) and channel 2 (blue) are registered and the respective frequencies are calculated. The coordinates of the entry respective to the x- and y-axes of the diagram are given. Once the coordinates of the cell are found, the value will be incremented.

If double-labeled aggregates are present, the diagonal of the diagram will be populated.

☞ All the table functions in the **Coincidence** mode are identical to those in the **Correlation** mode

4.5.7 Display - Preview

- Click on the **Preview** button to activate the **Preview** display mode.
  - A print preview and the additional **Print** subordinate toolbar will appear on the screen (Fig. 4-125).

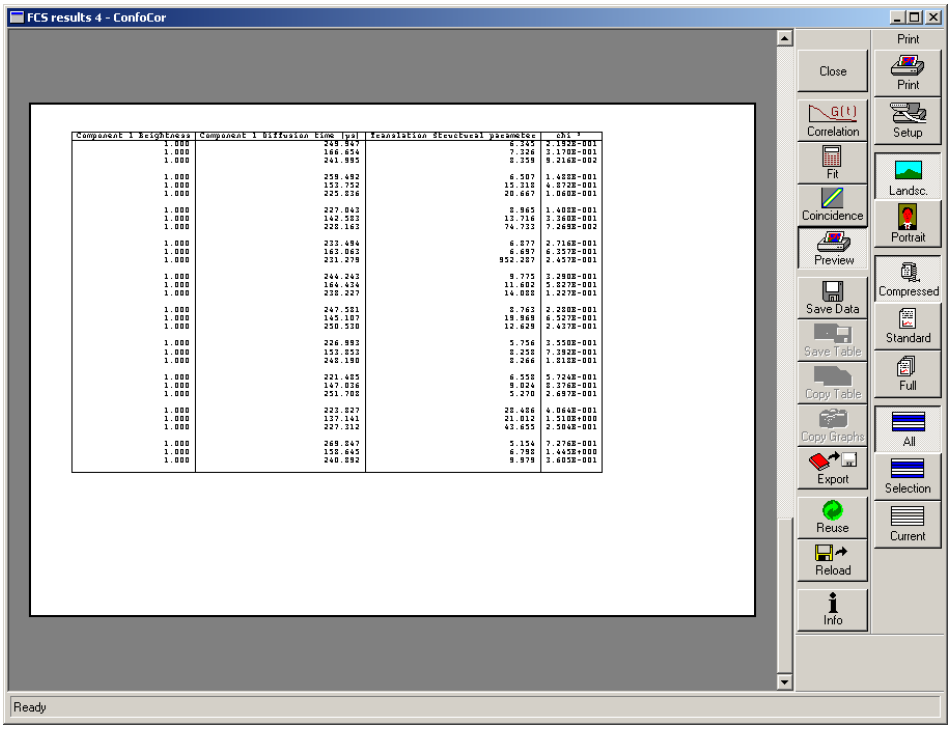


Fig. 4-125 Preview / Print window

The **Preview / Print** window shows an overview, as a table and/or a graph, of the results of a measurements and the relevant measuring parameters of the used measuring method. This result overview can be printed directly on a connected printer.

For this purpose, the following function buttons are available in the **Print** subordinate toolbar (at the right-hand side of the **Preview / Print** window):

Print button

Starts the print function.

Setup button

Opens the printer setup window to set the print parameters.

Landsc. button

Selects **Landscape** paper orientation.

Portrait button

Selects **Portrait** paper orientation.

Compressed button

Selects the **Compressed Report** preview without diagrams.

Standard button

Selects the **Standard Report** preview with correlation and fit curves.

Full button

Selects the **Full Report** preview with all diagrams.

All button

Prints all measurements.

Selection button

Prints selected measurements.

Current button

Prints currently selected measurements only.

To print a report, proceed as follows:

- Select the **Compressed**, **Standard** or **Full** button.
- Select the **All**, **Selection** or **Current** button.
- Click on **Landsc.** or **Portrait** for paper orientation.
- If required, change the printer settings using the printer **Setup** button.
- Click on **Print** to start the print procedure.

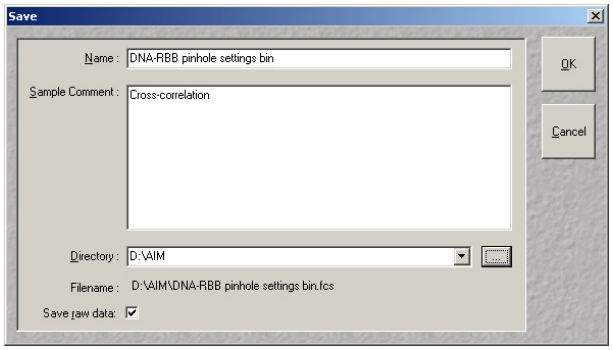


Fig. 4-126 Save window

4.5.8 Display - Save Data

- **Save** allows you to save the whole data set (curves, fitting results, fit parameters, raw data) to disk. If **Save** is selected, the **Save** window appears (see Fig. 4-126).

The **Save** menu offers the possibility of entering file name in the **Name** field.

It also offers the possibility of entering a comment in the **Comment** field.

The directory can be chosen in the **Directory** field. A directory can be selected if the button to the right is pressed.

It is possible to store the data as ANSI text (space-consuming, but readable) or as a binary file.

If the **Save raw data** box is checked, the raw data will be stored in a file bearing the same name than the .fcs file, which contains all channels as separate files.

- **OK** will confirm the action, **Cancel** will exit the menu without further action.

4.5.9 Display - Copy Table

- With **Copy Table**, the whole table will be copied to the WINDOWS NT clipboard. The contents of the clipboard can be used by most WINDOWS NT applications via the **Paste** function.

4.5.10 Display - Save Table

- With **Save Table**, the result table can be written to a white space in a separate text file. If the button is clicked, a file menu will appear offering the possibility to select a directory and to enter a file name.

4.5.11 Display - Copy Graphs

- Click on the **Copy Graphs** button to copy the displayed graphs to the clipboard.
  - Using the Paste function of Microsoft WINDOWS, you can paste the graphs into other WINDOWS applications.

4.5.12 Display - Export

- With **Export**, the measured data will be exported in a ConfoCor 1-compatible format and can be handled using the FCS Access Fit program. In the **Export** window (see Fig. 4-127) you can enter a name in the **File name** field and a type in the **Data type** field. In the **Save** pull down menu you can select a directory. You can specify if only the Selected rows (highlighted), the Current row (the current selected one) or all rows are exported. Exported files can be opened again with the **Open FCS** button. All selected files will be loaded in one **Correlation display** window.

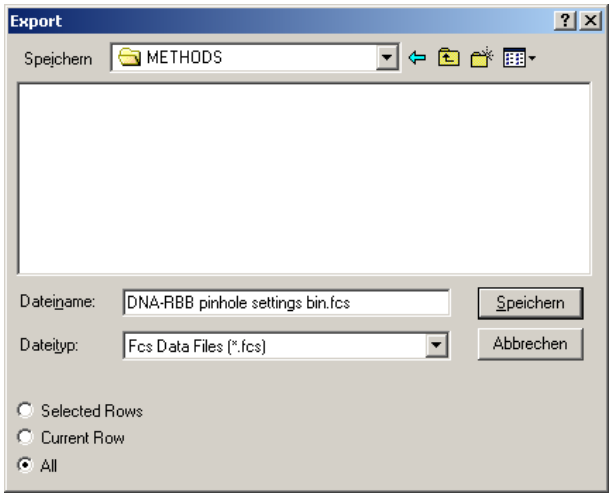


Fig. 4-127 Export window

4.5.13 Reuse

- With a click on the **Reuse** button, the configuration used to record the of the present fit curve will be loaded.

4.5.14 Reload

The **Reload** can be used for already opened raw data files. The **Correlate** window will appear (see Fig. 4-128).

You have the following options:

- Automatic dust filter
- Correlator settings
- Count rate settings
- Photon Counting Histogram settings

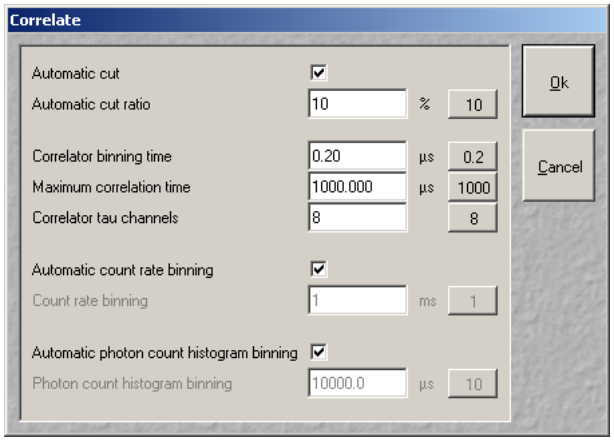


Fig. 4-128 Correlate window

4.5.14.1 Automatic Dust Filter

If the **Automatic cut** box is checked, you can type in a threshold in % in the **Automatic cut ratio** display box. This sets a threshold intensity for an electronic dust filter. The value (0 … 100) will define the threshold in percentage. A default value of **10%** can be selected by pressing the button on the right.

If the integrated count rate over a certain count interval will exceed the average count rate by that threshold, this special interval is discarded for correlation analysis. For example, if the system detects a count rate in a certain time interval that exceeds the average count rate by over 30 % and the threshold was set at 30, this interval is discarded for correlation analysis. The time intervals before and after the discarded region are separately correlated and the results averaged. These holds also true, if more than one region is discarded.

The single regions taken into account and disrupted by discarded regions are separately correlated and the resulting average will be displayed.

☞ Note, that due to built up of an average, this kind of dust filter does not work for count rates exceeding the average that will come at the beginning of the measurement time before the system had the possibility to calculate the average count rate. Also, due to the necessity to average signals over a certain integration time, more than only the peak area will be discarded. Another outcome of the necessity to average the count rate signal is that several small peaks following close to each other will be treated as a huge peak and might be cut out. This means, in the **Automatic cut** mode accumulated count rates rather than peaks are cut out. For cross-correlation experiments, any of the regions discarded in either autocorrelation function will not be used. Cut off regions are framed by stippled boxes and appear matted in the Count Rate window.

4.5.14.2 Correlator Settings

You can determine the start value of the **Correlator binning time** in µs, the **Maximum correlator time** in s and the start value for the **Correlator tau channels** by typing the values in the respective selection boxes. Default values (**0.2** µs, **1000** s and **8**) can be loaded by pressing the corresponding default buttons on the right.

4.5.14.3 Count Rate Settings

You can either check the **Automatic count rate binning** box, in which case a dynamic binning is chosen, or deactivate the box and type in a value in ms in the **Count rate binning** selection box for a constant binning with the defined value. The default value of **1** ms can be loaded by pressing the default button to the right.

4.5.14.4 Photon Counting Histogram Settings

You can either check the **Automatic photon counting histogram binning** box, in which case 32 histograms with different binning are calculated and the one with the best dynamic range (3 standard deviations) is chosen, or deactivate the box and type in a value in µs in the **Photon count histogram binning** selection box for a constant binning with the defined value. The default value of **10** µs can be loaded by pressing the default button to the right.

4.5.15 Info

Pressing the **Info** button will open the **Method Information** window (see method-information). With **Copy All**, the complete parameter set will be copied to the clipboard.

A click on the **Print** button opens the **Print Setup** window and allows the parameters of the method to be printed in the form of a table.

4.6 Combined Application of LSM Scanning and FCS Measurement Procedure

4.6.1 Taking FCS Measurements within a Cell

The simultaneous installation of the ConfoCor 3 onto the channel 4 of the LSM 510 permits examination of points selected within a single LSM image with the ConfoCor 3.

For combined application, proceed as follows:

- Click on the **ConfoCor** button in the of the **Main** menu to select the FCS mode.
- Click on the **Measure** button in the **ConfoCor** subordinate toolbar to open the **Measurement** window. Select the FCS measurement method by clicking the methods button and select a method. Close the window.
- Open the **Adjust** window. Initialize stage and focus. Switch on the lasers using the **Laser** button. Close the **Laser Control** and the **Adjust** windows.
- Click on the **VIS** button in the **ConfoCor** subordinate toolbar
- Select the specimen area to be examined by moving the stage and focus.
- Click on the **LSM** button in the **ConfoCor** subordinate toolbar
- Select the appropriate beam path configuration in the **Configuration Control** window.
- Open the **Scan Control** window by clicking the **Scan** button in the **Acquire** subordinate toolbar. Set the scan parameters and click on **Single** to scan an image of the sample.
- When finished, select the **Acquisition** mode in the **Measurement** window.

There are two ways of defining the positions, where FCS measurements should be performed in an image: **Current Position** and **LSM Image**.

4.6.1.1 Using the Current Position

When using this method, FCS measurements are performed on a fixed position without scanner or stage. In this case the structure of interest must be moved to the position manually under LSM control prior to FCS measurements. In either case, f If **Scanner** or **XY Stage** are used and no rotation was defined, than the laser position with the parked mirrors is the centre of the image. In case of rotation the position hof the laser beam with this rotation within the image has to be determined by a bleach experiment. You can use the Crosshair in any case to mark the position of the laser beam.

- Open the **Measurement** window by clicking on the **Measurement** button in the **ConfoCor** subordinate toolbar. Press the **Current Position** button in the **Position** panel via mouse click. The **Position** panel changes to allow the activation of the **Crosshair** function.
- Activate the **Crosshair** button via mouse click and click into the image.
  - The crosshair appears in the LSM scan image. Place the cross at the position, where the Laser beam is positioned. If you have activated the **Lock** button, than the position will be fixed.
- Scan the image continuously and position the site of interest under the crosshair.

4.6.1.2 Using the LSM Image Window

With this method you can conveniently choose several points of interest at which FCS measurements will be performed. You can approach this positions either with the scanning stage is the **XY stage** button is activated, or by the mirrors, if the **Scanning** button is activated.

- Open the **Measurement** window by clicking the **Measure** button in the **ConfoCor** subordinate toolbar. Press **LSM Image** and select between **Scanner**, **XY stage** or **Piezo stage**.
  - The **Positions** panel changes to allow the definition of positions in the LSM scan image.
- Activate the **Select** button via mouse click.
  - The cursor (crossline) appears in the LSM scan image.
- Move the cursor to the desired position in the scan image (drag and drop). Click on the **Add Pos** button to select this position. The coordinates of the position appears in the **Positions** list of the **Sample** panel.
- In a Z Stack, you can also vary the Z-plane in which measurement is to be performed. For this purpose, select the required Z slice in the stack or set the Z value via the focusing drive of the microscope.
- Select further positions to be measured in the same way.
- Click on the **Mark Pos** button to fix the selected positions as overlay elements in the image.

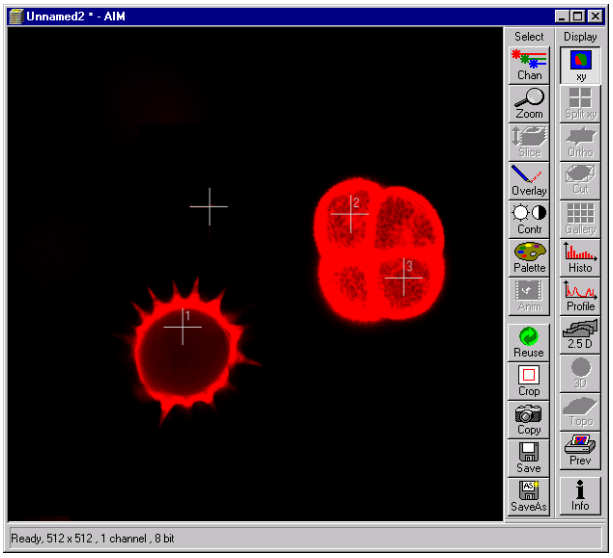


Fig. 4-129 LSM scan image with cursor (crossline) and 3 selected positions

☞ The X-, Y- and Z- coordinates of the selected positions are only stored in the **Positions** table and not in the scanned image (stack). Accordingly, all the marked positions in all the Z-planes are displayed in the scanned image (Z Stack), no matter in which Z-plane they lie.

- When finished, click on the **Start** button in the **Measurement** window to start the FCS measurements of the selected positions.
  - The measurement procedure starts and the data and evaluation window appears on the screen.

☞ Please note that the positions selected for FCS measurements in the **Measurement** window using the ConfoCor 3 are approached by the scanning stage, Piezo stage or scanning mirrors, whichever was chosen. In case of scanning mirrors, the stage does not move. All positions will be approached sequentially.

☞ In case of x and y scan, it is the scanning mirrors that are used. Hence there is no offset between defined positions and actual positions. However, a scan correction in x and y can be required, which is done by changing the **Scan Corr X** and **Scan Corr Y** positions in the Scan window, if the bidirectional scan was chosen. If stages are used, the offsets have to be entered in the **Settings** menu under **LSM + ConfoCor**.

4.6.1.3 Performing FCS Measurements on the Cell Membrane

Due to the shape of the confocal volume, the membrane should not be approached from the side but instead rather from the top of the cell. Please note that it is better to use the upper membrane for measurement, since the lower membrane might be too close to the glass bottom surface resulting in disturbing reflections. You can home in on the membrane either manually or using the **Z Scan** feature in the **Method Optimization ...** window.

(1) Manual Focusing on the Membrane

- Acquire an image of the cell.
- Place the focus above the cell and scan continuously.
- Focus slowly down until you see a fluorescence signal.
- Stop focusing.

Due to its high sensitivity ConfoCor 3 can still detect signals that cannot be imaged by the LSM. If the signal is too low for imaging, the membrane can still be detected using the **Z Scan** feature in the **Measurement** window.

(2) Focusing on the Membrane by a Z Scan

The **Z Scan** measures the count rate at previously defined Z positions. It is performed without table movement, that is at the fixed position of the laser beam.

- Position the region of interest at the site of the fixed laser beam path as described in chapter 14.6.1.
- Optional: Perform a **Z Stack** that includes the membrane of the cell with the LSM.
- Open the **Measurement** window by clicking on the **Measurement** button in the **ConfoCor** subordinate toolbar. Press the **Current Position** check box or the **LSM Image** button in the **Position** panel via mouse click.
  - The **Position** panel changes and shows the **Adjust** and the **Crosshair** buttons for **Current Position** or the Position list for **LSM Image**.
- Activate the **Crosshair** button via mouse click or define a position.
  - The crosshair or cross appears in the LSM scan image.
- Move the cursor to the appropriate position in case of **Current Position** or select by pressing the **Add** button in **LSM Image** followed by **Mark Pos** to fix the position as an overlay element
- Press the **Z Scan** button. The **Z Scan** display shows the current Z position of the laser beam. Check the corresponding channel if not yet activated.
- Select the start and end position of the Z Stack. If you have acquired a Z Stack in the LSM mode, use the values defined by the **Mark First/Last** button. Note that the values defined by the Z Stack in the LSM mode are not automatically used in the **Z Scan** of the FCS mode.
- Enter the number of positions. If you have acquired a Z Stack, use the value defined in the **Z Sectioning** tab.

- Press the **Start** button to perform a Z scan. Press the **Cancel** button if you want to leave the Z Scan settings.
- After pressing the **Start** button a Z scan is performed and the intensity displayed in dependence of the Z position (see Fig. 4-130). The red line in the diagram shows the actual Z position. Peaks show Z levels of high signal intensity and may correlate to labeled membranes. Note, that the glass surface will also give a peak. To determine its position, just perform a Z Scan at a position where there is no cell. Place the red line at the peak that corresponds to the membrane to select this Z position.

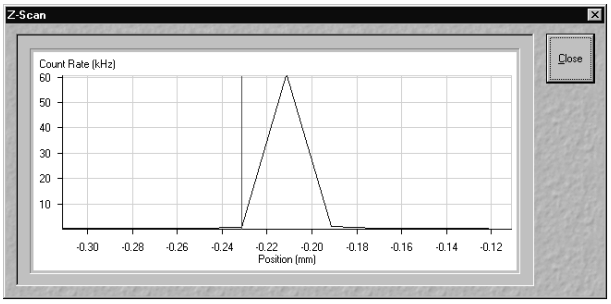


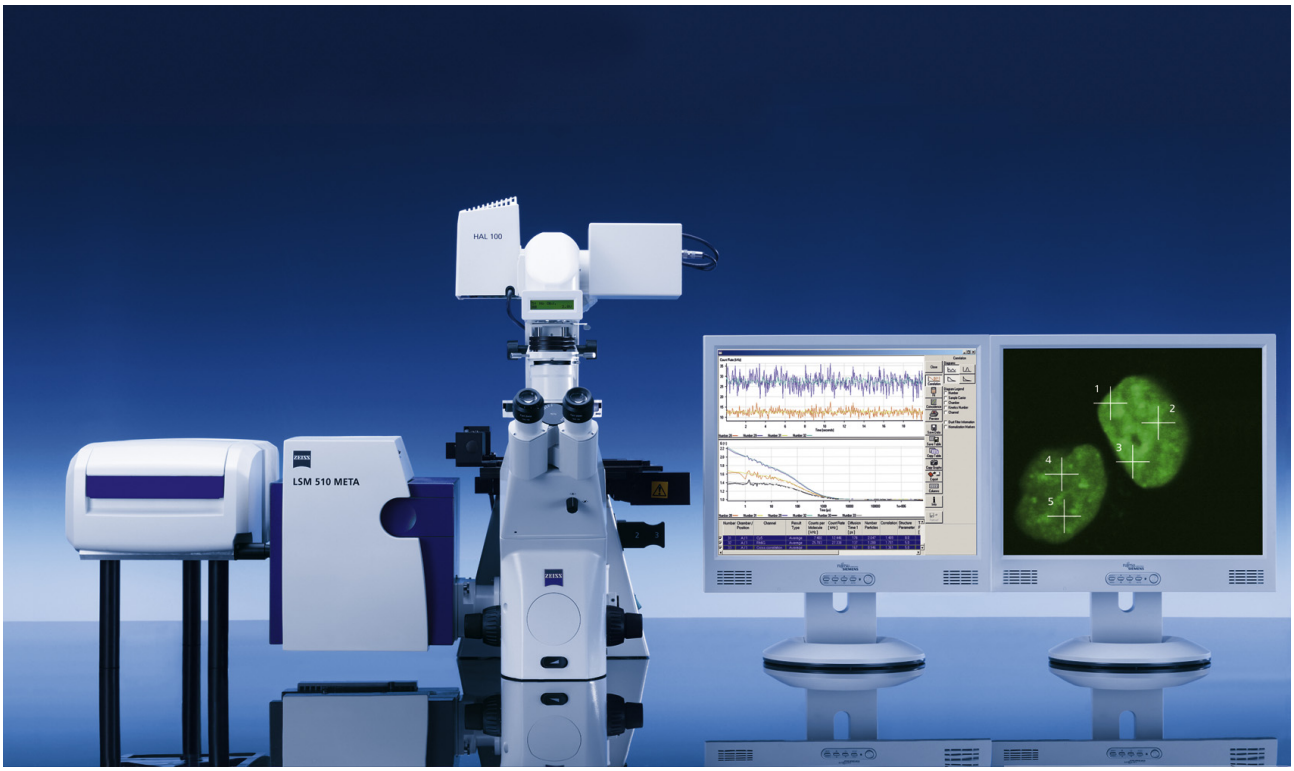
Fig. 4-130 Z-Scan image

- If no clear signal can be detected, or the peak of interest lies too close to the border, close the **Scan** window and perform a new Z scan. Choose a different Z range by modifying start and end positions to values that lie closer to the position at which the signal should be expected.
- If the Z position has been selected, close the **Image Display** window by clicking on the **Close** button.
- Press the **Start** button in the **Measurement** window to perform an FCS measurement.
  - The FCS measurement is performed at the same X/Y position as the Z scan.

4.7 Index

LSM 510 / LSM 510 META and ConfoCor 3

Laser Scanning Microscopes



Brief Operating Manual
Release 4.0
March 2006

ZEISS

Contents

Introduction

For your safety!
Observe the following instructions:

- The LSM 510- and LSM 510 META-ConfoCor 3 laser scanning microscope, including its original accessories and compatible accessories from other manufacturers, may only be used for the purposes and microscopy techniques described in this manual (intended use).
- In the Operating Manual, read the chapter *Safety Instructions* carefully before starting operation.
- Follow the safety instructions described in the operating manual of the microscope and HBO 100 mercury lamp.

Starting the System

Switching on the LSM system

- When set to **ON** the REMOTE CONTROL switch labeled **System/PC** provides power to the microscope and the computer. This allows to use the microscope and the computer without running the LSM Software (Fig. 1).
- To switch on the system completely put the **Components** switch also to **ON**. Now the complete system is ready to be initialized with the LSM Software.

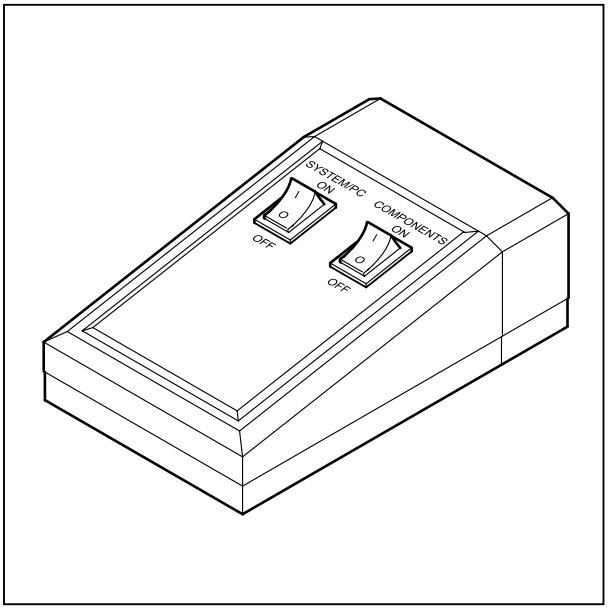


Fig. 1 **REMOTE CONTROL switch**

Switching on the HBO 100 mercury lamp

- Switch on the HBO 100 mercury lamp via the switch of the power supply, see operating manual of the mercury lamp or microscope.

Switching on the Enterprise UV-Ar Laser

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

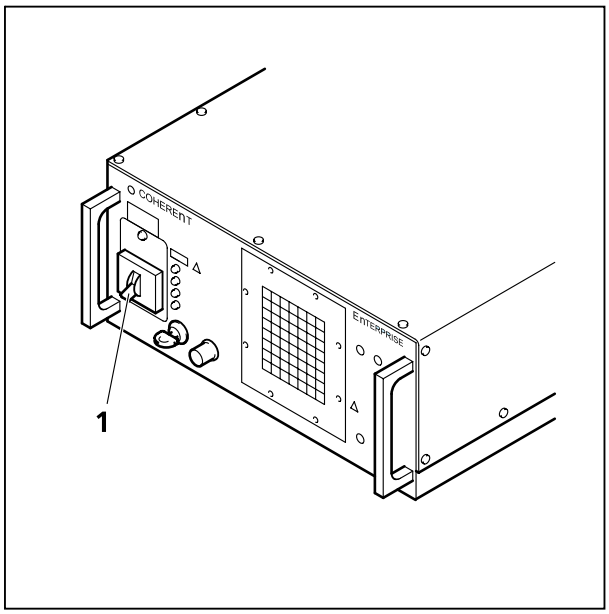


Fig. 2 **Power supply of UV-Ar laser**

Starting the LSM 5 software program

- Double click the **LSM 510-FCS** icon on the desktop of WINDOWS to start the LSM 5 software program.

The **LSM 510 / ConfoCor Switchboard** window appears on the screen (Fig. 3).

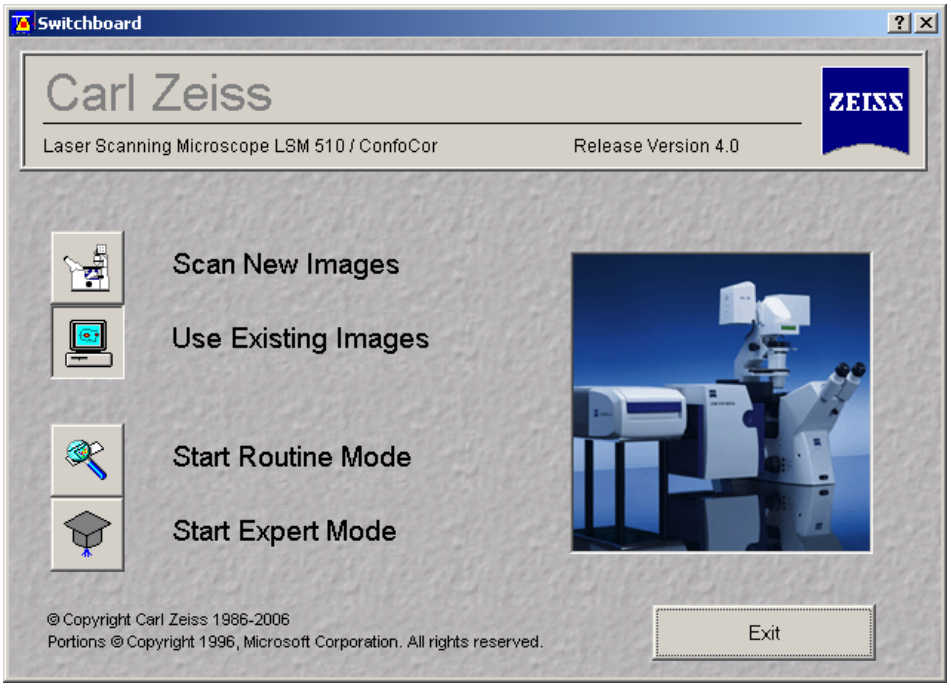
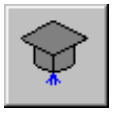


Fig. 3 **LSM 510-ConfoCor Switchboard menu**

- Click on the **Scan New Images** button in the **LSM 510-ConfoCor 3 Switchboard** window.

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



- Click on the **Start Expert Mode** button in the **LSM 510-ConfoCor 3 Switchboard** window.

The LSM 510 - Expert Mode **Main** menu appears on the screen.

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

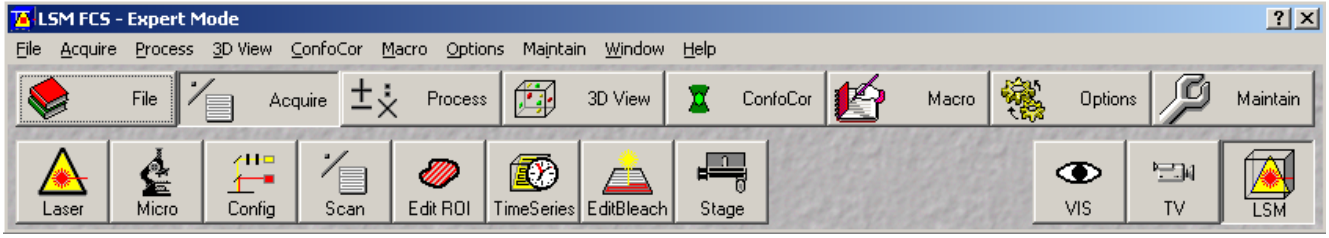


Fig. 4 **Main menu for Expert Mode**

Creating a database for acquired images

- Click on the **File** button in the **Main** menu toolbar.

The **File** subordinate toolbar appears in the **Main** menu.

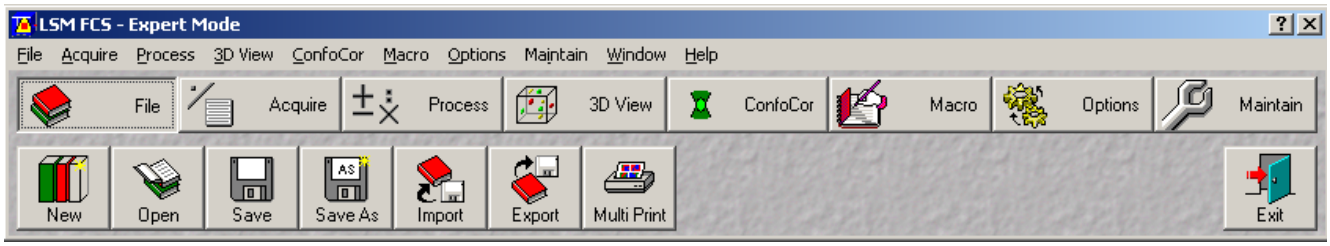


Fig. 5 **Main menu with File subordinate toolbar**

- Click on the **New** button in the **File** subordinate toolbar.

The **Create New Database** window appears.

- Select drive **C:** or **D:** from pull down menu.

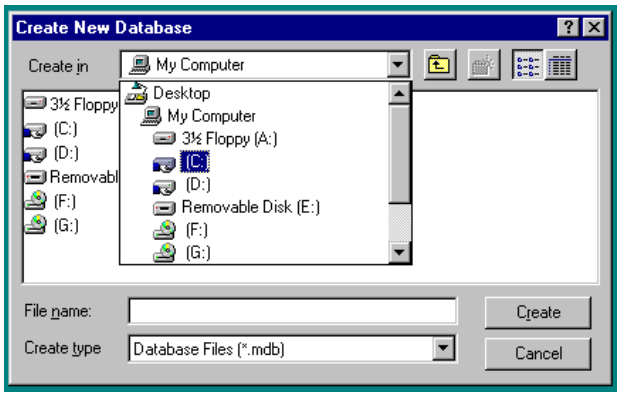


Fig. 6 **Create New Database window**

- Create a new directory if needed.

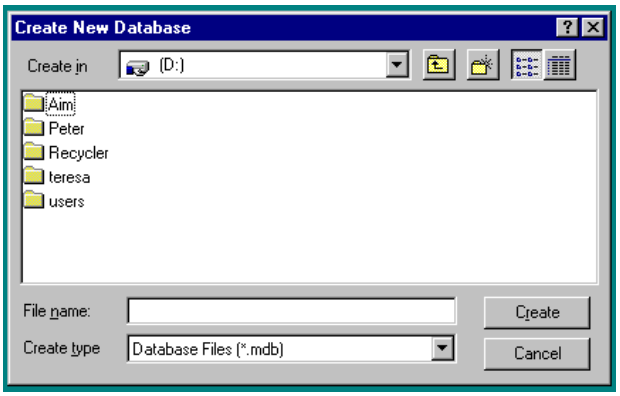
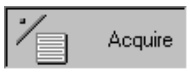


Fig. 7 **Create New Database window**

Turning on the lasers



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

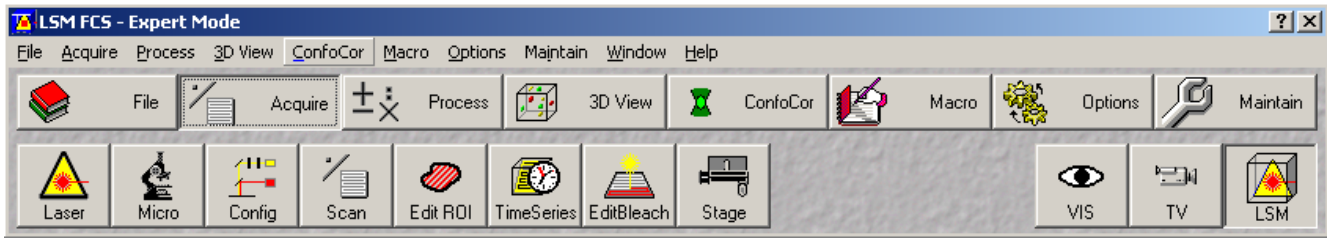
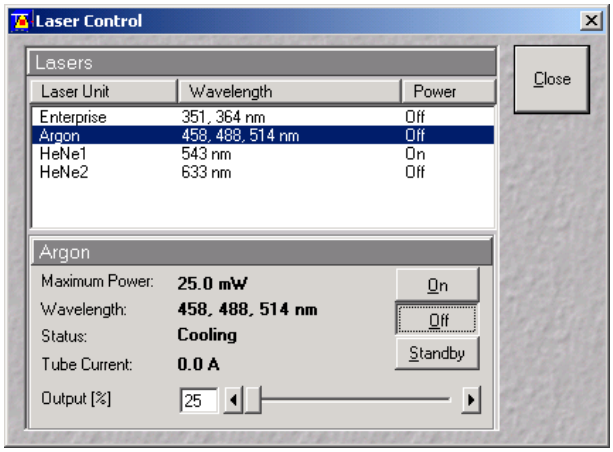


Fig. 8 **Acquire subordinate toolbar**

- Click on the **Laser** button to open the **Laser Control** window.



- Select the appropriate **Laser Unit** by clicking on the name of it.
- Click on the **Standby** button to switch required laser(s) to Standby.
- When status is **Ready** click on **On** button.
- Set **Output [%]** so that the **Tube Current** is about **4 A** (50% of the output power).

Fig. 9 **Laser Control window**

Setting the microscope

Changing between direct observation or laser scanning

The **VIS**, **TV** and **LSM** buttons switch the beam path and indicate which beam path has been set in the binocular tube of the microscope:

- Click on the **VIS** button to set the microscope for direct observation via the eyepieces of the binocular tube, lasers are off.

- Click on the **TV** button to set the microscope camera observation (if connected) via camera adapter of the binocular tube.

- Click on the **LSM** button to set the microscope screen observation via laser excitation using the LSM 510 and software evaluation.

Setting the microscope and storing the settings

- Click on the **VIS** button for direct observation.

- Click on the **Micro** button in the **Acquire** subordinate toolbar to open the **Microscope Control** window of the used microscope.

The **Microscope Control** window appears (Fig. 10).

Selecting an objective

- Open the graphical pop-up menu by clicking on the **Objective** button (Fig. 10).
- Click on the objective you want to select. The selected objective will automatically move into the beam path.

Focussing the microscope for transmitted light

- Open the graphical pop-up menu by clicking on the **Transmitted Light** button (Fig. 11).
- Click on the **On** button. Set the intensity of the Halogen illuminator using the slider.
- Click on **Close** to close the pop-up menu.
- Place specimen on microscope stage. The cover slip must be facing up.
- Use the focusing drive of the microscope to focus the required object plane.
- Select specimen detail by moving the stage in X and Y using the XY stage fine motion control.

Setting the microscope for reflected light

- Click on the **Reflected Light** button to open the shutter of the HBO 100 mercury lamp.
- Click on the **Reflector** button and select the desired filter set by clicking on it.

Storing the microscope settings

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

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

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

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

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

Note: Depending on the microscope configuration, settings must be done manually if necessary.

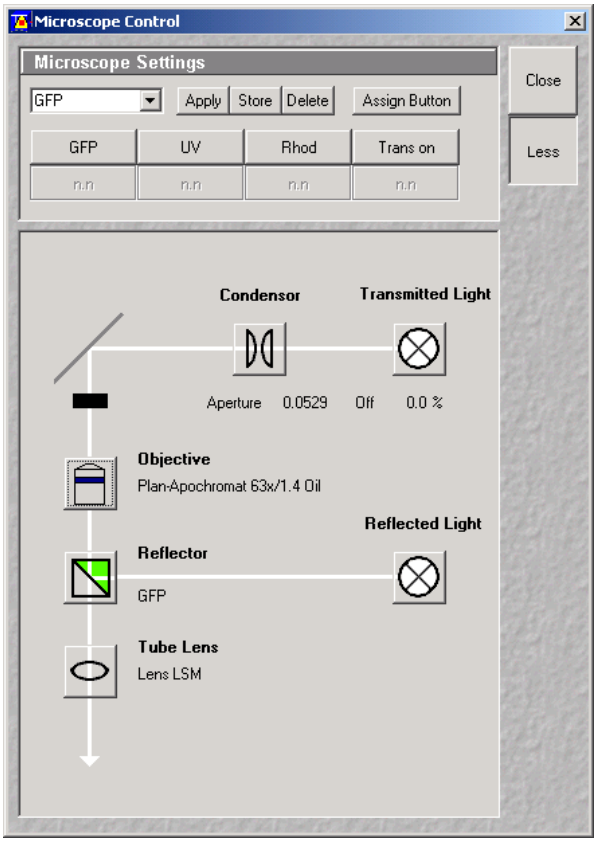


Fig. 10 **Microscope Control window, e.g.: Axiovert 200 M**

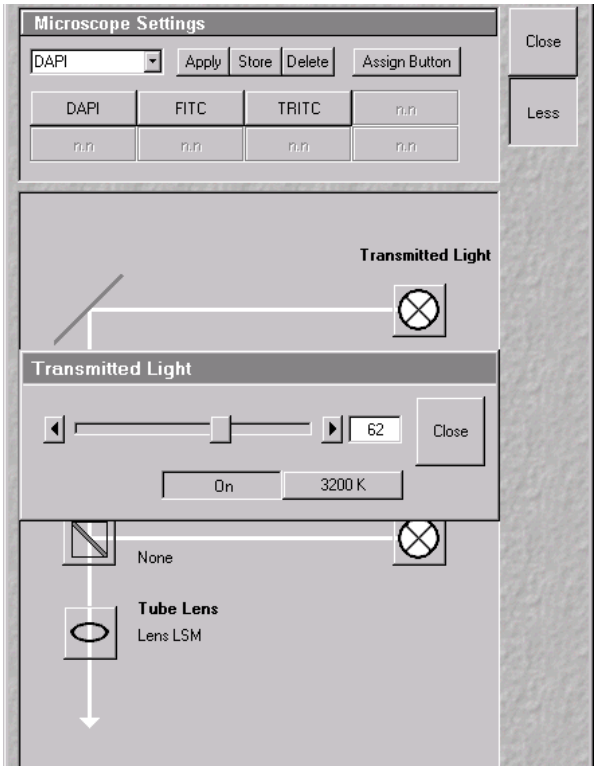


Fig. 11 **Microscope Control window with Transmitted Light pop-up menu**

Configuring the beam path and lasers

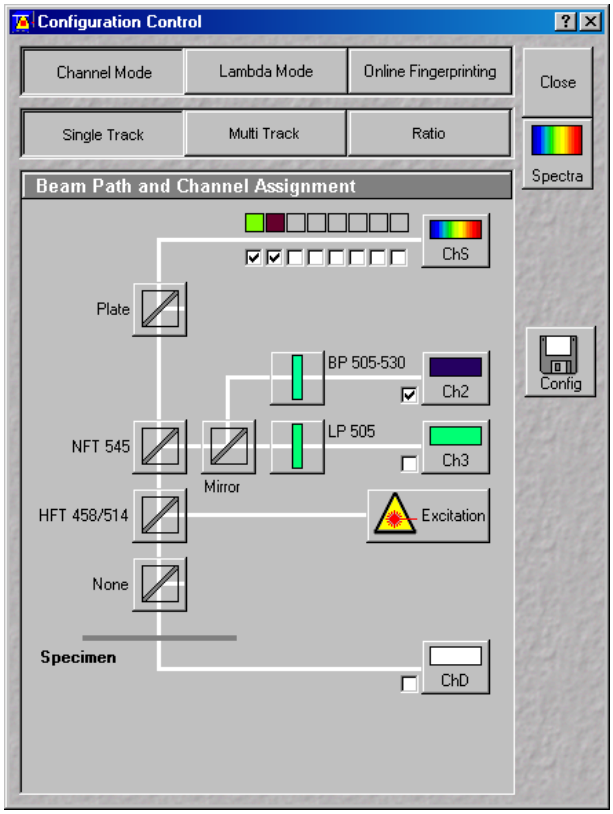


Fig. 12 **Configuration Control window for Single Track**

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

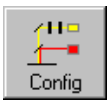
Choosing the configuration

Single Track

- Use for **single**, double and triple labeling; simultaneous scanning only
- Advantage: faster image acquisition
- Disadvantage: cross talk between channels

Multi Track

- Use for double and triple labeling; sequential scanning, line by line or frame by frame
- Advantage: when one track is active, only one detector and one laser is switched on. This reduces cross talk.
- Disadvantage: slower image acquisition



- Click on the **Config** button in the **Acquire** subordinate toolbar to open the **Configuration Control** window.

The **Configuration Control** window appears (Fig. 12).

Setting for single track configuration in Channel Mode

- Select **Channel Mode** if necessary (Fig. 12).
- Click on the **Single Track** button in the **Configuration Control** window (Fig. 12).
- Click on the **Descanned** button if necessary.

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

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

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

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

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

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

- Select the appropriate filters and activate the channels.
- Click the **Excitation** button to select the laser lines and set the attenuation values (transmission in %) in the displayed window.

For the configuration of the beam path, please refer to the application-specific configurations depending on the used dyes and markers and the existing instrument configuration.

- Clicking on the **Spectr** button opens the **Detection Spectra & Laser Lines ...** window (Fig. 13) to display the activated laser lines for excitation (colored vertical lines) and channels (colored horizontal bars).

- Clicking on the **Config** button opens the **Track Configurations** window (Fig. 14) to load, store or delete track configurations.

- For storing a new track configuration enter a desired name in the first line of the **Configurations** list box and click an **Store**.
- For loading an existing configuration select it in the list box and click on **Apply**.
- For deleting an existing configuration select it in the list box and click on **Delete**.

Setting for multi track configuration in Channel Mode

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

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

- Select **Channel Mode** if necessary (Fig. 15).

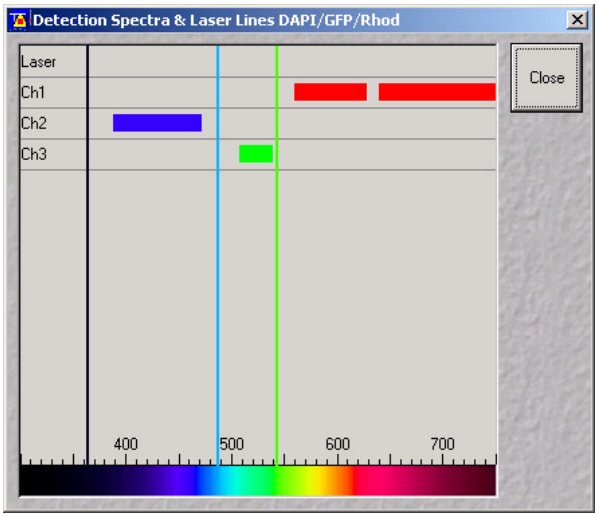


Fig. 13 **Detection Spectra & Laser Lines ... window**

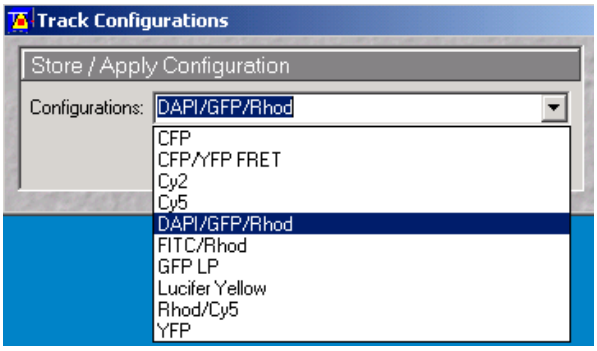


Fig. 14 **Track Configurations window**

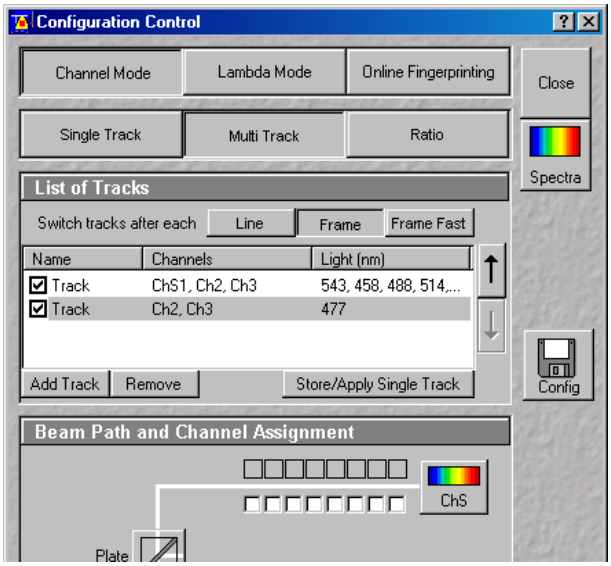


Fig. 15 **Configuration Control window for Multi Track**

- Click on the **Multi Track** button in the **Configuration Control** window (Fig. 15).
- Click on the **Descanned** button if necessary.

The following functions are available in the **List of Tracks** panel (Fig. 15).

Modes

Switch tracks after each

Line button	Tracks are switched during scanning line by line. The following settings can be changed between tracks: Laser line and intensity and channels incl. settings for gain and offset.

Switch tracks after each

Frame button	Tracks are switched during scanning frame by frame. The following settings can be changed between tracks: Laser line and intensity, all filters and beam splitters, the channels incl. settings for gain and offset and the pinhole position and diameter.
Frame Fast button	The scanning procedure can be made faster. Only and the laser line and intensity and the **Amplifier Offset** are switched, but no other hardware components. The tracks are all matched to the current track with regard to emission filter, dichroic beam splitter, setting of Detector Gain, pinhole position and diameter. When **Line** button is selected, the same rules apply as for **Frame Fast**.

Settings

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

- Configure each desired track for **Multi Track** function as described for **Single Track**.
- For storing/applying or deleting a **Channel Mode Configuration** use the **Config** button.

Scanning an image

- Click on the **Scan** button in the **Acquire** subordinate toolbar to open the **Scan Control** window.

The **Scan Control** window appears (Fig. 16).

Setting the parameters for scanning

- Select **Mode** in the **Scan Control** window.
- Select the **Frame Size** as predefined number of pixels or enter your own values (e.g. 300 x 600) in the **Objective Lens, Image Size & Line Step Factor** panel. Click on the **Optimal** button for calculation of appropriate number of pixels depending on N.A. and λ.

The number of pixels influences the image resolution!

Note: When using an Axioskop 2 FS MOT, indicate the objective that is in use in the **Scan Control** window. This ensures correct calculation of pinhole, Z stack optimization etc.

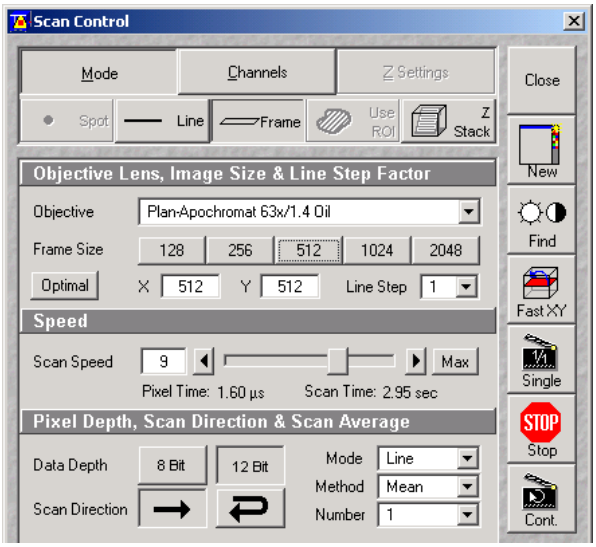


Fig. 16 **Scan Control window, Mode settings**

Adjusting the scan speed

- Use the slider in the **Speed** panel (Fig. 16) to adjust the scan speed.

A higher speed with averaging results in the best signal to noise ratio. Scan speed 8 usually produces good results. Us speed 6 or 7 for superior images.

Choosing the dynamic range

- Select the dynamic range 8 or 12 Bit (per pixel) in the **Pixel Depth, Scan Direction & Scan Average** panel (Fig. 16).

8 Bit will give 256 gray levels, 12 Bit will give 4096 levels. Publication quality images should be acquired using 12 Bit data depth. 12 Bit is also recommended when doing quantitative measurements or when imaging low fluorescence intensities.

Setting the scan averaging

Averaging improves the image by increasing the signal : noise ratio. It can be achieved line by line or frame by frame. Frame averaging helps reduce photobleaching, but does not give quite such a smooth image.

- Select the **Line** or **Frame** mode for averaging.
- Select the desired scan average method **Mean** or **Sum** in the **Method** selection box.

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

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

- Select the Number for averaging.

Continuous averaging is possible in the **Frame** mode. In this case a **Finish** button for ending continuous averaging is displayed instead of the **Cont.** button.

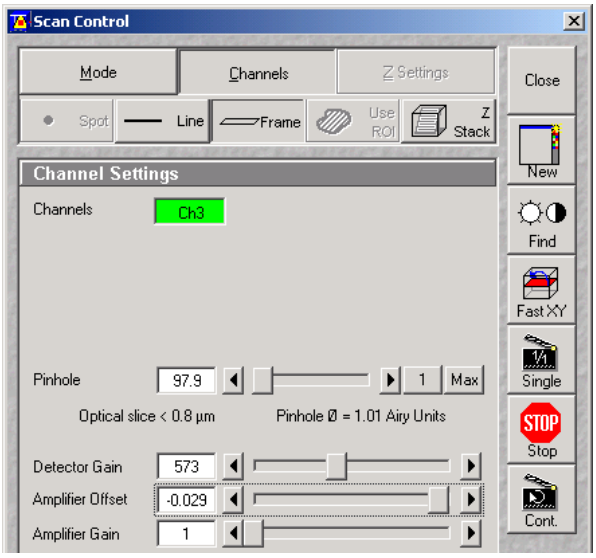


Fig. 17 **Scan Control window, Channel settings**

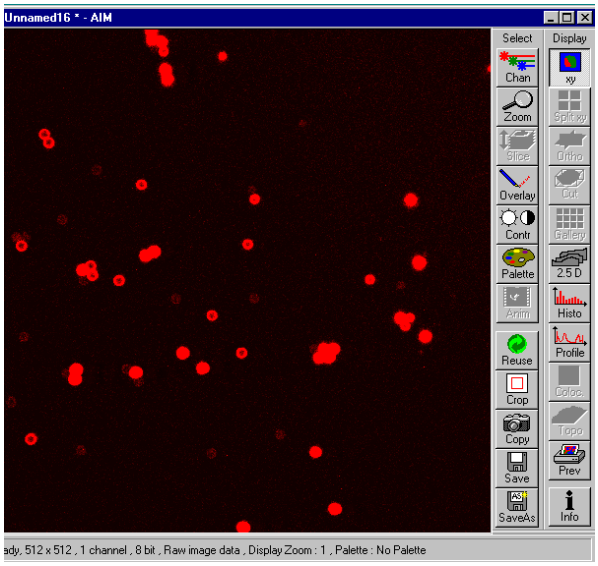


Fig. 18 **Image window**

Adjusting the pinhole

- Select **Channels** in the **Scan Control** window.
- Set the **Pinhole** size to **1** (Airy unit) for best compromise between depth discrimination and efficiency.

Pinhole adjustment changes the **Optical Slice**. When collecting multi channel images, adjust the pinholes so that each channel has the same **Optical Size**. This is important for colocalization studies.

Image acquisition

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

- Use one of the **Find**, **Fast XY**, **Single** or **Cont**. buttons to start the scanning procedure and acquire an image.

Scanned images are shown in separate windows.

- Click on the **Stop** button to stop the current scan procedure if necessary.

Select **Find** for automatically pre-adjustment of detector sensitivity.

Select **Fast** for continuous fast scanning – useful for finding and changing the focus.

Select **Single** for recording a single image.

Select **Cont.** for continuous scanning with the selected scan speed.

Select **Stop** for stopping the current scan procedure.

Image optimization

Choosing a lookup table

- Select **Palette** in the **Image** window of the scanned image (Fig. 19).

The **Color Palette** window appears.

- In the **Color Palette List** panel, click on the **Range Indicator** item (Fig. 20).

The scanned image appears in a false-color presentation (Fig. 19).
If the image is too bright, it appears red on the screen. Red = saturation (maximum).

If the image is not bright enough, it appears blue on the screen. Blue = zero (minimum).

Adjusting the laser intensity

- Set the **Pinhole** to **1** Airy Unit (Fig. 21).
- Set the **Detector Gain** high.
- When the image is saturated, reduce AOTF transmission in the **Excitation** panel (Fig. 21) using the slider to reduce the intensity of the laser light at the specimen.

Adjusting gain and offset

- Increase the **Amplifier Offset** until all blue pixels disappear, and then make it slightly positive (Fig. 21).
- Reduce the **Detector Gain** until the red pixels only just disappear.

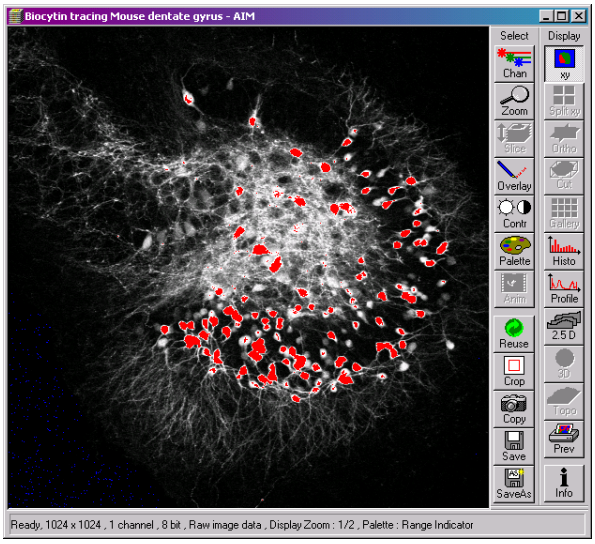


Fig. 19 **Image window**

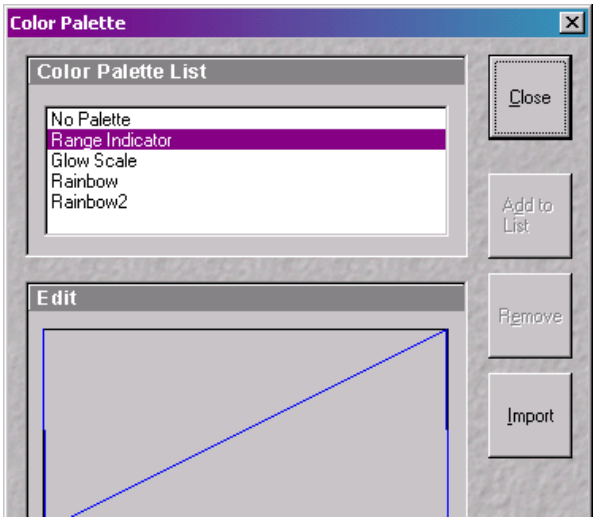


Fig. 20 **Color Palette window**

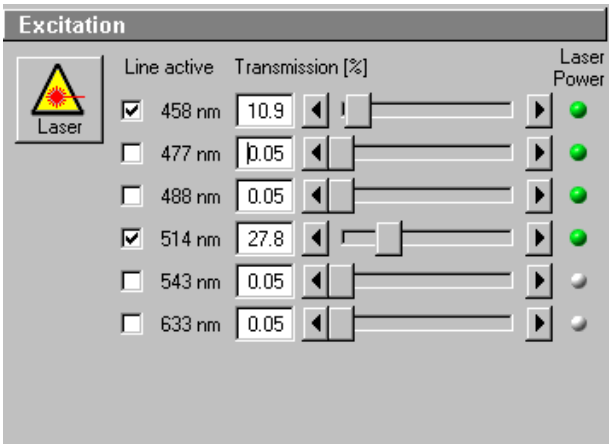


Fig. 21 **Scan Control window, Channel settings**

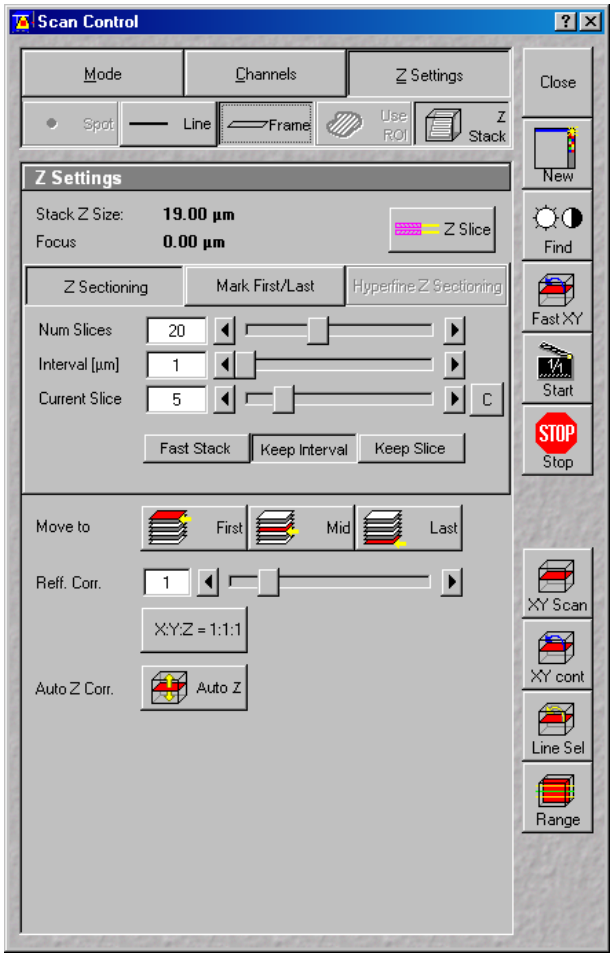


Fig. 22 **Scan Control window, Z Stack settings**

Scanning a Z stack

- Select **Z Stack** in the **Scan Control** window.
- Select **Frame** if necessary.

The **Z Settings** panel appears.

- Select **Mark First/Last** on the **Z Settings** panel.
- Click on the **XY cont** button.

A continuous XY-scan of the set focus position will be performed.

- Use the focusing drive of the microscope to focus on the upper position of the specimen area where the Z Stack is to start.
- Click on the **Mark First** button to set the upper position of the Z Stack.
- Then focus on the lower specimen area where the recording of the Z Stack is to end.
- Click on the **Mark Last** button to set this lower position.
- Click on **X:Y:Z=1:1:1** button to set the Z-interval in such a way that the voxel has identical dimensions in the X-, Y- and Z-directions (cube).
- Click on the **Start** button to start the recording of the Z Stack.

Storing an image

- Click on the **Save** or **Save As** button in the **Image** window or in the **File** subordinate toolbar of the **Main** menu.

The **Save Image and Parameter As** window appears.

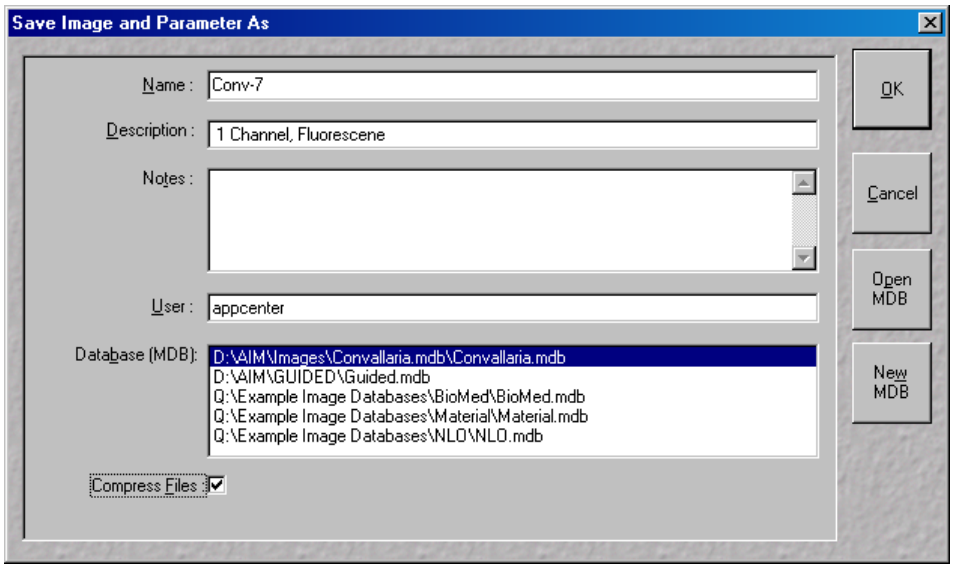


Fig. 23 **Save Image and Parameter As window**

- Enter file name, description and notes in the appropriate text boxes.
- Click on the **OK** button.

Using the ConfoCor

- Click on the **ConfoCor** button in the **Main** menu toolbar.

The **ConfoCor** subordinate toolbar appears in the **Main** menu.

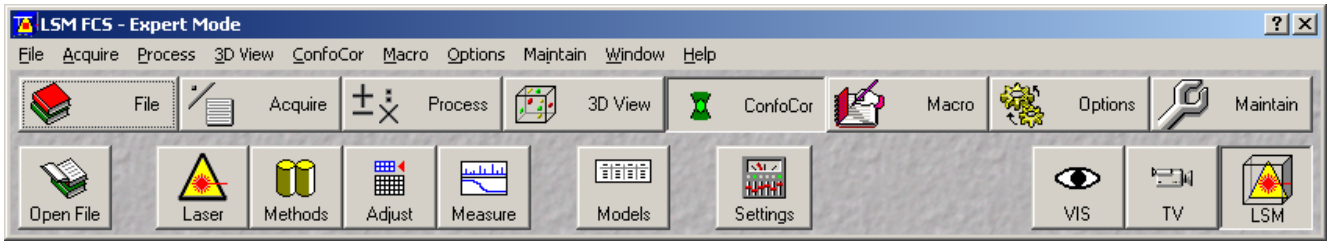
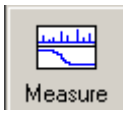


Fig. 24 **ConfoCor subordinate toolbar**

Setting up the configuration



- Click on the **Measure** button to open the **Measurement** window.

- Press the **System Configuration** button to activate the **System Configuration** panel.

The **Beam Path and Pinhole** panels of the **measurement** window display the selected track configuration which is used for the FCS procedure and the pinhole size.

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

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

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

Selection of a block filter.

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

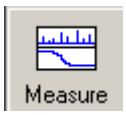
Activation / deactivation (via check box) of the selected channel (Ch 1-2 for the FCS procedure.

Pinhole slider — Setting of the Pinhole diameter.

Press the **Count rate** button to open the **Count rate** window. In **Excitation** set the laser power to obtain a satisfactorily count rate.

Pinhole Alignment for a new defined beam path. After adjusting the sample carrier, align the pinhole in x and y by first conducting a coarse and then a fine alignment.

Taking a measurement



- Click on the **Measure** button to open the **Measurement** window.

- Press the **Acquisition** button to activate the Acquisition panels.

The **Times, Kinetics and Position** panels of the **Measurement** window display the selected measurement configuration and the positions which is used for the FCS procedure.

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

Times panel enter boxes	Enter the bleach time, measurement time and repletion number.
Kinetics check box	Activate / deactivate if you want to conduct kinetics or a 1 time point measurement.
Position panels	Select a carrier or position in an LSM image.

Press the **Methods** button to open the **Select Method** window. You can switch the method to the highlighted one by just clicking the **Ok** button. If your current method is not stored, all settings will be lost.

Press the **Save** button to open the **Save Method** window. You can save either only the beam path configuration or the whole method (including (including configuration and processing settings).

Press the **Info** button **Current Method Information** window, which displays information on the system set up and the measurement procedure.

Press the **New** button to open the **FCS results ... - ConfoCor** window. If a measurement is triggered, all data are displayed in that window if highlighted.

Press the **Start** button to trigger a measurement. If no **FCS results ... - ConfoCor** window a new one will be opened. If **FCS results ... - ConfoCor** windows are open, data will be written to the highlighted one. All defined positions will be approached consecutively.

Press the **Single** button to trigger one measurement at the highlighted position. Note, this button is only available in the **LSM Image** panel.

Press the **Stop** button to end a measurement. All data accumulated so far will be available and can be stored.

Press the **Count rate** button to open the **Count rate** window. This allows you to optimize your experiment by changing the laser power and the pinhole size.

Press the **XYZ** button to open **X,Y,Z-Scan** window. The current coordinates will be displayed. You can define boundaries where a scan is performed with simultaneous acquisition of the count rate. This allows you, for example, to identify labeled molecules accumulated in the membrane.

Press **XY Stage** if positioning should be accomplished by the motorized stage.

Press **Scanner** if the positioning should be accomplished by the scanning mirrors

After the end of a measurement, the data are displayed **FCS results ... - ConfoCor** window (see Fig. 25).

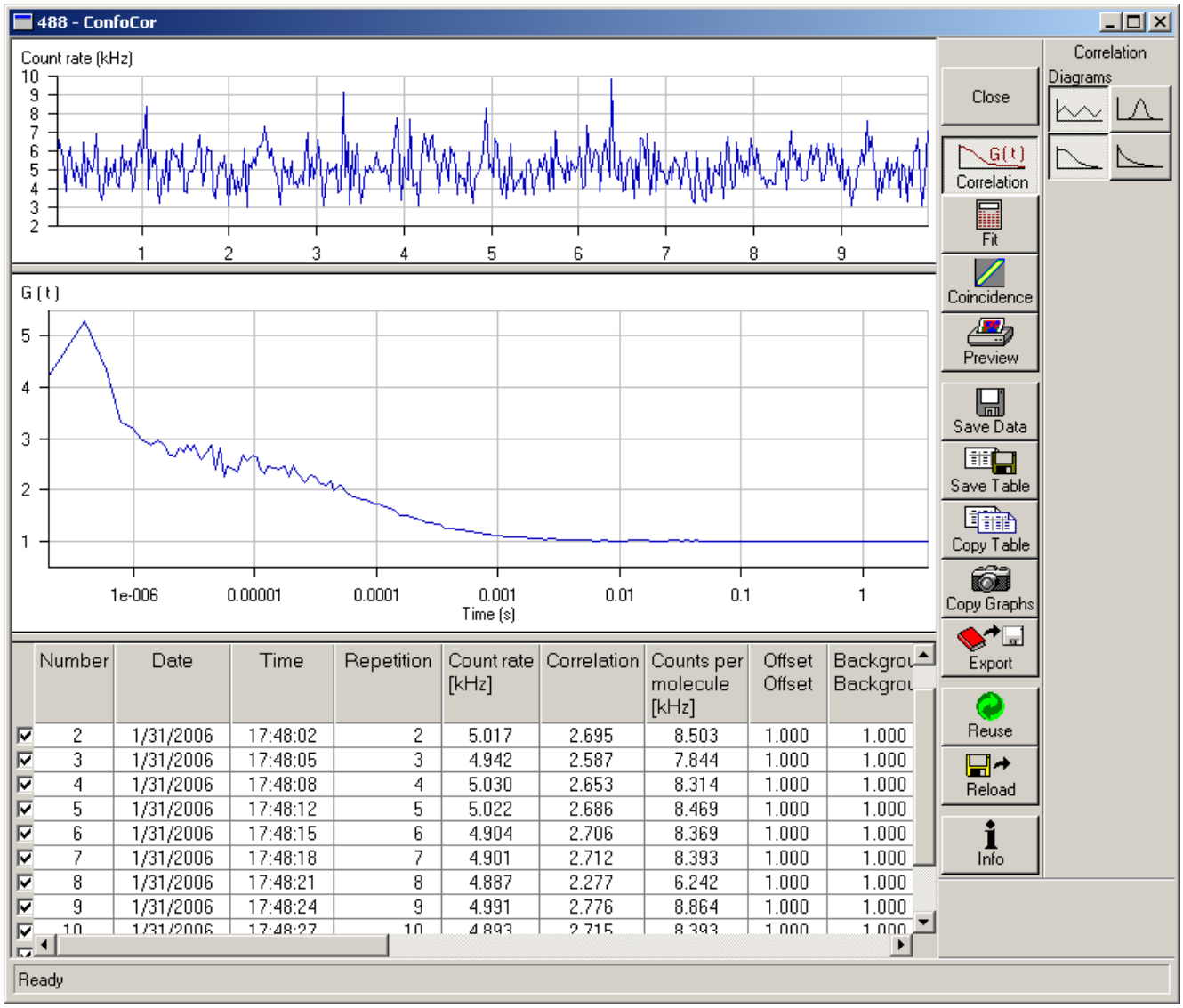


Fig. 25 **FCS results ... - ConfoCor window with Correlation activated**

You have the following function elements:

Press the **Save Data** button to open the **Save** window. You can save the whole data set in an ANSI text format. Optionally you can save the raw data trace.

Pressing the **Reuse** button will set the system configuration to exactly the same values, as used in the experiment.

Pressing the Reload button will open the current measurement, if stored raw data are available

Press the Fit button to open the Fit panel, which allows you to analyze your data.

Analyzing the data

Data can be analyzed in the **Fit** display of the **FCS results ... - ConfoCor** window (see Fig. 26).

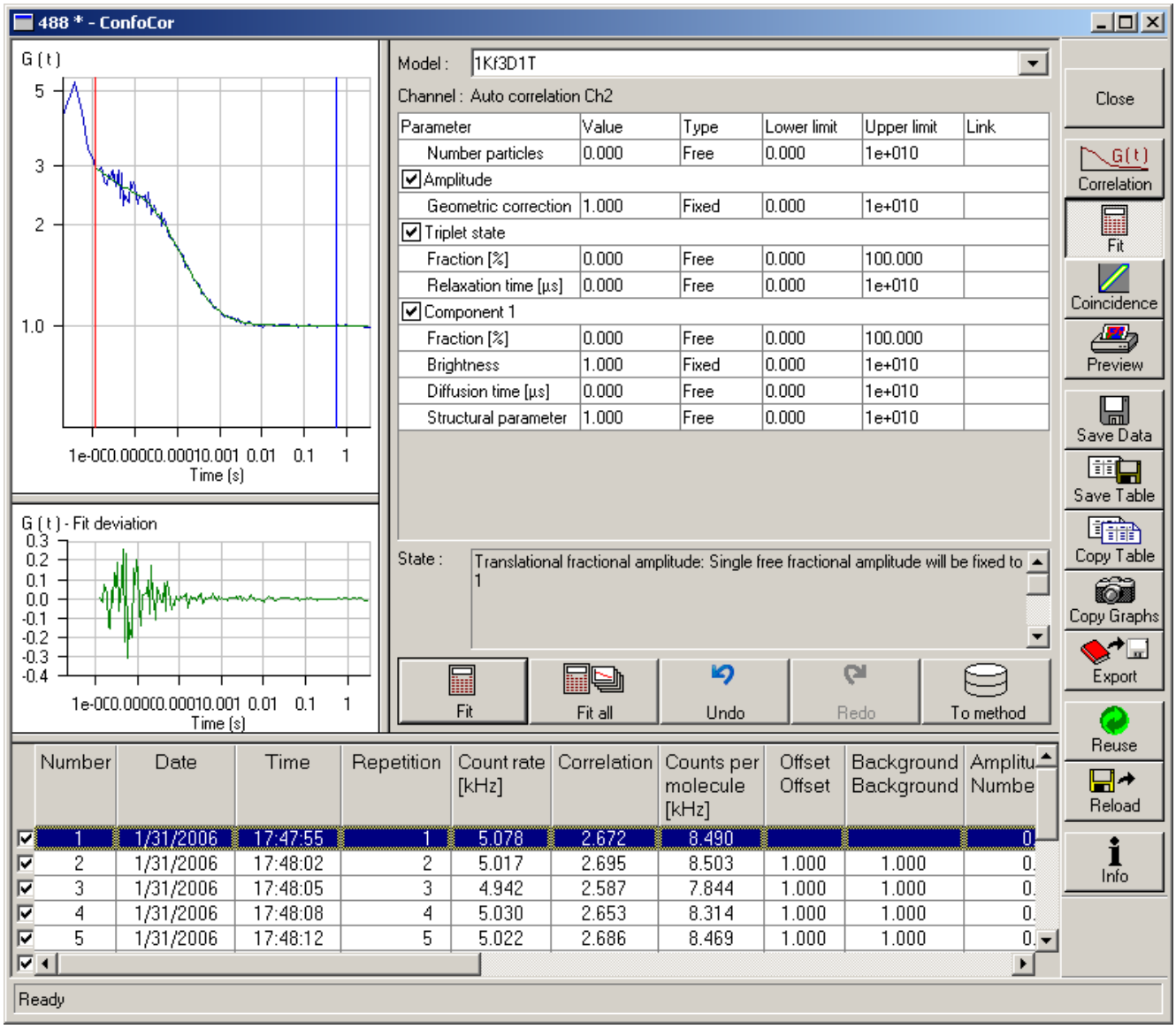


Fig. 26 **FCS results ... - ConfoCor window with Fit activated**

You have the following options:

Curve window — Set the red and blue bars to define the start and end points of the fit.

Model scroll bar — Load a predefined model. You can assemble a model by pressing the **Model** button in the ConfoCor submenu.

Table — You can define the conditions of the fit by activating / deactivating terms, setting the type of a parameter (fixed, free or start value), defining limits and globally link parameters.

Pressing the **Fit** button will fit the current loaded correlation functions to the defined model. The fitted data will be displayed.

Pressing the **Fit all** button will fit to all measurements of the same channels than the selected ones.

Pressing the **Undo** button will cancel the last operation, or previous ones as well, if the button is pressed repeatedly.

Pressing **Redo** will redo the last cancelled operation, or previous ones, if the button is pressed repeatedly.

Pressing the **Write to Method** button will write back the settings to the method. If the method is stored, the settings will be active when the method is selected the next time.

Switching off the system

- Click on the **File** button in the **Main** menu and then click on the **Exit** button to leave LSM 5 software program (Fig. 5).
- If any lasers are still running you should shut them off now in the pop up window indicating the lasers still in use.
- Shut down the computer.
- Wait until fan of Argon laser has switched off.
- On the REMOT CONTROL switch turn off the **Components** switch and the **System/PC** switch (Fig. 1).
- Switch off the HBO 100 mercury lamp.
- Switch the UV-Ar laser of via the toggle switch of the power supply (Fig. 2).