IMPORTANT NOTES ON CHAPTER 5

These operation instructions describe standard and optional hardware and software configurations. Depending on your order and therefore on the configuration, the content of the screens may differ.

Software

Eight different configuration packages of the LSM Software Release 3.2 are available:

- Software "LSM control" and an additional license
- Software "Physiology evaluation"
- Software "Topography evaluation"
- Software "Macro Recorder and Editor"
- Software "3D for LSM"
- Software "Multiple Time Series"
- Software "Image VisArt"
- Software "Deconvolution"
- Software "StitchArt"

If your configuration does not include the "Physiology evaluation" software package, the following functions are inactive:

- **Mean of ROI** scan button in **Time Series** control
- **Mean of ROI** button in the image display after frame scan

If your configuration does not include the "Topography evaluation" software package, the following functions are inactive:

- **Topo** button in the image display after acquisition of image stacks

If your configuration does not include the "Macro Recorder and Editor" software package, the following functions are inactive:

- **New**, **Save** and **Save as** buttons in the **Macro Control**
- **Edit**, **Step**, **Delete**, **Editor** buttons in the **Macro Control**

If your configuration does not include the "3D for LSM" software package, the following functions are inactive:
Separate software "3D for LSM"

If your configuration does not include the "Multiple Time Series" software package, the following functions are not available:
– Macro: "Advanced Time Series"

If your configuration does not include the "Image VisArt" software package, the following functions are inactive:
– **3D** button in the image window

If your configuration does not include the "Deconvolution" software package, the following functions are inactive:
– **DCV** button in the image window and in the process main menu

If your configuration does not include the "StitchArt" software package, the following function is not available:
– Macro: "StitchArt"

Hardware

Depending on whether the following hardware components are available or not, the content of the screens may differ:

– HRZ 200 fine focusing stage
– Piezo objective focusing device
– X-Y scanning stage DC 4 × 4 or DC 100 × 90, each with MCU 28
– Depending on the configuration the Scan head equipment may differ in filters, beamsplitters and the number of photomultiplier
– Transmitted-light PMT
– Stands:

  Axiovert 100 M, Axiovert 200 mot
– Monitordiode
– Programmable AOTF
– Non Descanned Detectors
– META detection module

If your configuration does not include an AxioCam, the following functions are not available:
– **Camera** in the **Config Control** window, **Scan Control** window

Limitations notes for the Release 3.2 (date of issue: 11/2002)

No.	Function	Description	Fix
Hardware			
1.	Hardware	Starting the system new after a longer time, some errors messages are possible in the error log	Starting the system once again
2.	Hardware	For precise measurements over long time ranges constant environmental conditions are necessary (temperature, humidity)	
3.	Computer	The computer (generation Pentium 3) has problems to initialize the operating system sometimes (blue screen)	Restart of the computer is necessary
Software			
Main menu			
4.	Scan & system information	The display of state of Ar-laser is off in the Scan & system information if laser is in standby mode	
File			
5.	Import	For the import of more than one FCS image the view of chambers is not correct	
6.	Export/Import	Export of 4D series as LSM 4 Tif is handled as z stack	
7.	Export/Import	*.eps – format will exported not correctly for more than one channel	
Acquire – Laser Control			
Acquire – Microscope Control			
8.	Microscope Control	You select another objective for an **Axioplan 2 Imaging** if the LSM software is not active: If you start the LSM software now – the last registered objective in the LSM software moves back in the beam path	Make sure, that you use **not** 2 objectives for different immersion media
Acquire – Configuration Control			
9.	Configuration	The movement of the NDD switching unit (to HBO/HAL mirror position) during changes from LSM to VIS position will not updated in the configuration control	
10.	Configuration	During visual adjustment with help of HBO/HAL lamp the detector gain of NDD detectors must be minimized to avoid decrease of sensitivity of photomultipler	
11.	Configuration-META-Offline	There is a bug in the demo database: the slider of lambda stacks shows a wrong spectral range and a higher number of pmt elements	Only in offline mode! (ignore it)
12.	NDD-Configuration	The switching between NDD and descanned mode does not work properly, if the laserline attenuation is not adjusted	Use the AOTFfit-macro to adjust the laserlines

Acquire – Scan Control			
13.	Scan Control	Auto Correction for bidirectional scanning does not work for Lambda stacks	You have to adjust the scanner with a single track before you start your Lambda stack
14.	Scan Control	Spline scan with more than 4 channels does not work	
15.	Scan Control	The Gain Slider for monitordiode works upside down	In this case inform your service
16.	Scan Control	Offset and Amplifier Gain Adjustment does not work for Spot Scan	Adjust the detector before you start the spot series
17.	Z Settings	The movement of focus for Axioskop 2 FS is to slow for Move **First / Mid / Last** – therefore it´s possible, that the focus movement is not finished during the image acquisition	Generate a second image if the focus is finished
18.	Z Settings	Z Values in the LSM software and at the display of Axiovert 200 are different	Ignore it
Acquire – Edit ROI			
19.	Edit ROI	Creation of ROIs with 1 Pixel width is not possible	Minimum is a 2 pixel width
20.	Edit ROI	ROIs for xz planes are possible to define but not to scan	
Acquire – Time Series Control			
21.	Time series	xz-t-series: Roi/mean in the image menu shows Roi-values in xy-coordinates and calculated the area in xy-units and not in xz-units	
Acquire – Bleach Control			
Macros			
22.	Multi Time Series – macro	There is no information about bleaching during the measurement	
23.	AOTF Fit	Sometimes it occurs that after the linearisation the maximum transmission value is only 99 %	(rounding error) Ignore it
24.	AOTF Fit	Sometimes the macro creates wrong calibration files – in this case you get messages in the error log about the laserlines	Adjust it again
Analyse FCS			
25.	Carrier	The carrier window needs a bit time to open because the little camera in the ConfoCor head has to initialize	
26.	Carrier	The laser beam is on every time if the Carrier window is open (also for the LSM-FCS combination system when the slider are in the LSM position)	
27.	Measure	The coordinates for LSM and FCS are not the same for the combination system	
Options			
28.	FCM Settings	The coordinates for X and Y are exchanged for the case that the LSM scan head is situated on the sideport and the FCS head is situated on the baseport (for LSM-FCM-Combi)	

Maintain			
29.	Objective	In the LSM software you get another information about the number of focus speed for the objectives than in the external program AxioSet (for microscope settings)	Ignore it
Image			
30.	Image	At an image with 2k × 2k the µm-scale is to small in **Print Preview** and in printed Picture.	
31.	Pinhole	The FCS pinholes are displayed in the LSM Pinhole Control, but it is not possible to adjust FCS pinholes in this dialog (for LSM-FCS-Combi)	
Tools			
32.	CLM	**CLM** for Axioskop 2 FS don´t exist	
33.	Change Filters	**Change Filter** for Axioskop 2 FS does not exist	
Help			
34.	Help	The heading of help text for Combi-system still contains Release 2.8	

CHAPTER 5 OPERATION IN EXPERT MODE

CONTENTS

5 OPERATION IN EXPERT MODE

5.1 Purpose of this Section and other Operating Manuals

This section describes the operation of the LSM-FCS Combination System exemplified by typical applications in conjunction with the LSM-FCS software and its graphic user environment.

When starting up and operating the microscope system, mind the operating instruction manual for the Axiovert 200 M microscope:

– B 40-080 e Axiovert 200 M, Operating Manual

5.1.1 Software

The LSM-FCS software, Version 3.2, controls the microscope, the scanning and laser modules, tools (filters, stand, CLM) and the image acquisition process, and displays and analyzes the images. It is based on the network-capable graphic 32-bit Microsoft ® WINDOWS NT 4.0 operating system and WINDOWS 2000, respectively.

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

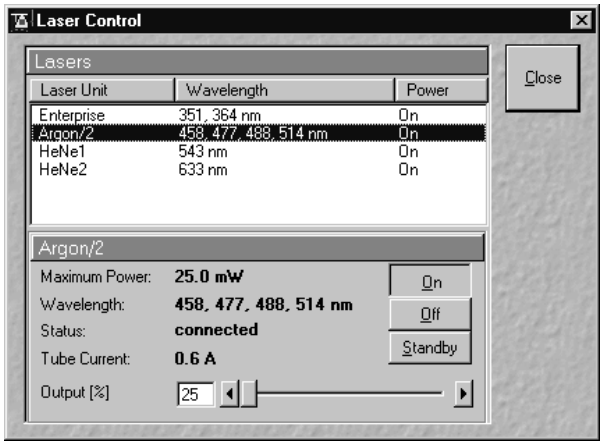
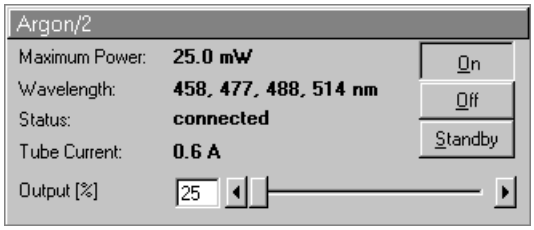
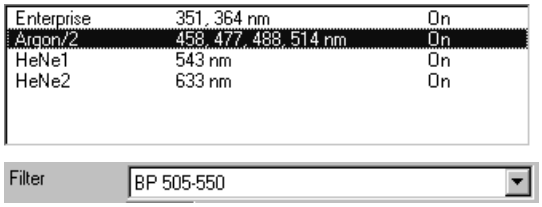
The LSM-FCS software is menu-controlled and normally uses its own windows for the activation of the various functions; within these windows, further submenus (panels) can be displayed and removed.

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

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

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

5.1.2 Windows and Window Elements

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

Window element	Description / Explanation
	Check box – Activates / deactivates setting options.
	Button – Selection / performance of a function via mouse click.

5.1.3 Convention for the Text in this Manual

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

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

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

5.1.4 Backup

System backup

- A complete backup is contained on the enclosed optical disk.

User files backup

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

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

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

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

5.1.5 Software Operation

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

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

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

5.2 Switching on the System

The LSM system is turned on with the **REMOTE CONTROL** switch. This switches all the system components on except for the "Enterprise" UV laser.

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

If **REMOTE CONTROL** switch is not used, turn the system on with the "**I**" button on the laser module; in addition, the jumper plug supplied must be connected at the **POWER REMOTE CONTROL** terminal.

- Turn the **REMOTE CONTROL** switch to "**ON**" position (see Fig. 5-1).
  - This switches the entire system on.
  - Microscope and laser will be ready for operation after a short time.
  - Computer boots up.
  - Computer hardware system test runs.

☞ Drive "A" of the computer must not contain a diskette.

The monitor shows a dialog box for selection of the operating system version.

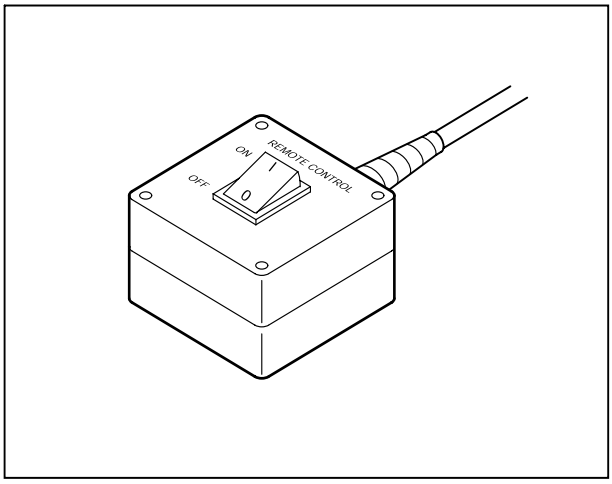


Fig. 5-1 REMOTE CONTROL switch

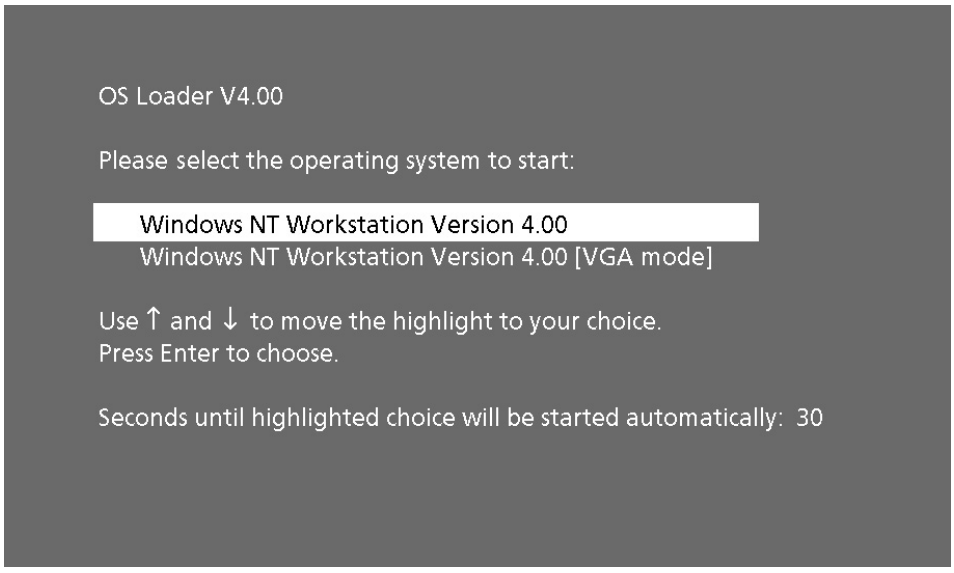


Fig. 5-2 Selecting the operating system version

Fig. 5-3 Begin Logon window

- Confirm the default setting of the "Windows NT Workstation Version 4.00" by pressing the **Enter** key.
  - WINDOWS NT operating system is being loaded.
  - The **Begin Logon** window appears on the screen.

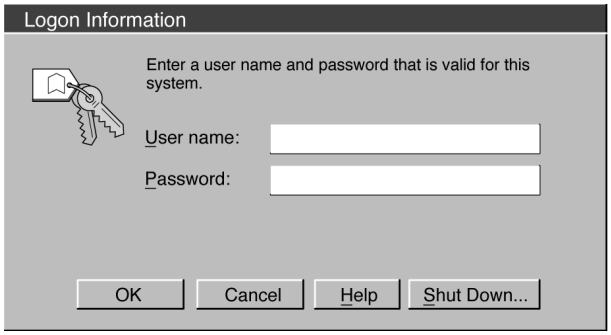


Fig. 5-4 Logon Information window

5.2.1 Log on to WINDOWS NT

- Press the three keys **Ctrl**, **Alt** and **Del** at the same time.
  - The **Logon Information** window appears on the screen, permitting you to log on to the WINDOWS NT 4.0 operating system.
- Enter the valid user name into the **User name** text box.
- Enter your password into the **Password** text box.

- After entries, confirm by clicking the **OK** button or **Enter**.
  - The WINDOWS NT operating system desktop appears on the screen, showing a number of icons.

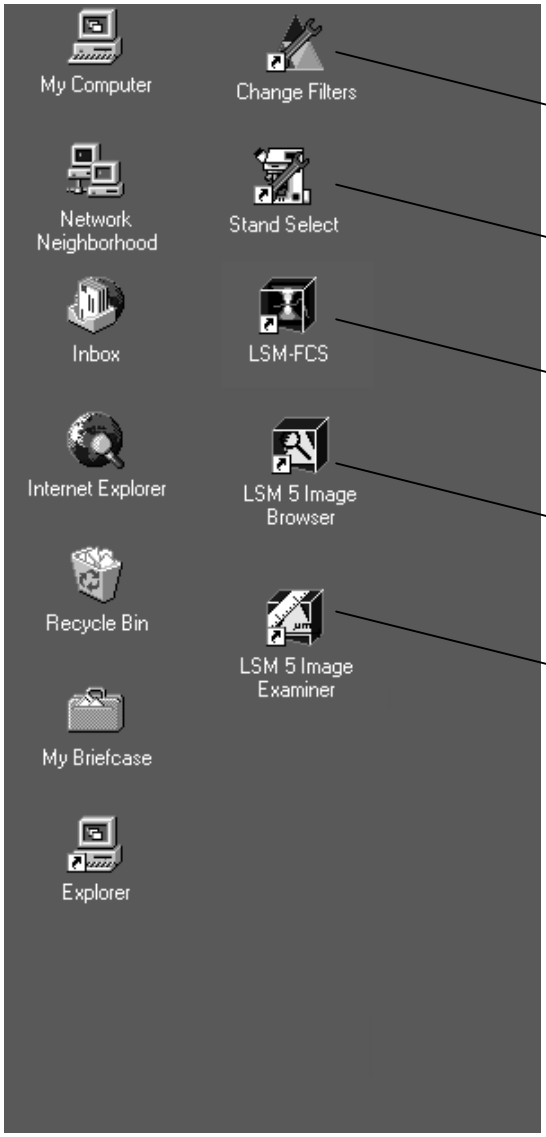


Fig. 5-5 WINDOWS NT operating system desktop

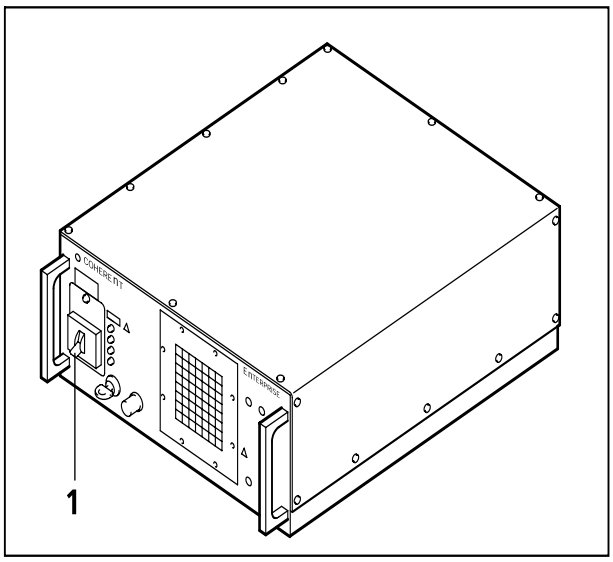


Fig. 5-6 Power supply of UV-Ar laser

5.2.2 Switching on the Enterprise UV Laser

- If the UV laser is required, switch it on via the toggle switch (5-6/**1**) of the power supply.
  - It will be ready for operation after a few seconds.

☞ Only for LSM.

Fig. 5-7 Starting the LSM-FCS program

5.2.3 Starting the LSM-FCS Program

The LSM-FCS software program can be operated in two different modes (with or without connected instrument system). In the on-line mode, the entire program package (image recording and analysis) is available, while only a part of the software functions (image analysis only of already stored images) and no hardware functions are available in the off-line mode. Of course, the off-line mode can also be started when the instrument system is connected. In that case, it is not necessary that the lasers and the microscope are switched on.

- Double-click on the **LSM-FCS** icon on the desktop of WINDOWS to start the LSM-FCS software program (see Fig. 5-5).
  - The **LSM-FCS Switchboard** menu appears on the screen (see Fig. 5-8).

Fig. 5-8 LSM-FCS Switchboard menu

The **LSM-FCS Switchboard** menu presents the following items for selection:

- **Scan New Images**

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

- **Use Existing Images**

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

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

- **Start Routine Mode**

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

☞ In the Routine mode, the FCS measurement functions are not available.

– **Start Expert Mode**

Use of this mode requires to be thoroughly familiar with the exact microscope procedures and interrelations.
You need to set all parameters and functions upon your own decision; this mode therefore provides you with the greatest flexibility of operation.
It is also possible, however, to call up stored configurations and to modify the parameters / settings if necessary.

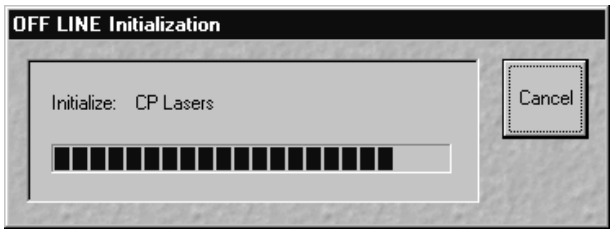


Fig. 5-9 OFF LINE Initialization window

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

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

☞ Existing images can only be loaded and processed in the **Expert Mode**.

If you want to change from the **Expert Mode** to the **Routine Mode** and vice versa, close all the windows first.

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

Remedy: turn on the printer before starting the LSM-FCS software.

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

5.3 Main Menu

- Click on the **Start Expert Mode** button.
  - The LSM-FCS - Expert Mode **Main** menu appears on the screen.

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

Fig. 5-10 LSM-FCS Switchboard menu

Fig. 5-11 LSM-FCS - Expert Mode Main menu

The major functions can be selected in the **Main** menu of the Expert Mode either via the pull-down menus in the menu bar or via a 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-FCS software, the toolbars are automatically displayed after the start of the Expert Mode. However, display of the toolbars can be deactivated via the **Window** pull-down menu (see **Toolbar**, page 5-273).

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

The buttons of the **Main** menu (upper toolbar) have the following meanings:

File button	Open, save, import and export of image data or FCS measurement results. 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.
Analyse FCS button	Selecting measurement method and carrier for FCS measurements. Executing measurement and data analysis. Optimizing measurement method.
Config FCS button	Calling up and setting the necessary operating parameters for FCS measurements. During the preparation for and execution of measurements via laser excitation. This menu item is used as the working dialog between the computer and the microscope.
Macro button	Makes it possible for the user to store frequently used processes (Macro recorder) and to run them automatically (Macro play). It is possible to write new macros or to edit existing ones.
Options button	For custom-configuration of software and hardware options, and for exporting system operating sequences to the Routine Mode. This menu item enables access to the coloring table. In the **Settings for User** window you can specify essential operating modes and informative help, organized by tabs, which have an effect on the user interface.
Maintain button	Service mode for adjustment and setting of other parameters (e.g. objectives).

5.4 File Menu

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

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

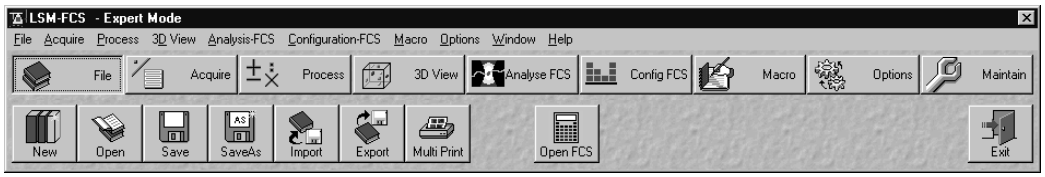


Fig. 5-12 File menu

5.4.1 Create New Image Database

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

- Click on the **New** button in the **File** subordinate toolbar of the **Main** menu.
  - This opens the **Create New Database** window for the selection of drives, directories and subdirectories.
- Enter the name of the image database you want to create in the **File name** text box, e. g. **Convallaria**.

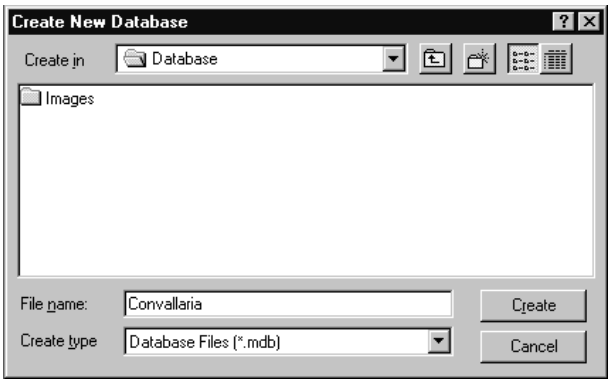


Fig. 5-13 Create New Database window

- If you want to create the image database in a certain folder (drive / directory), click on the arrow button next to the **Create in** box.
  - This opens a drop-down list box showing all folders available for selection.

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

- If you want to create a new folder, click on the button.

- Click on the **Create** button.
  - This creates the new image database in the selected drive and directory.
  - The database files of the LSM-FCS software have the filename extension ***.mdb**.

The **Convallaria.mdb** window appears, presenting the opened image database with 0 of 0 image entries.

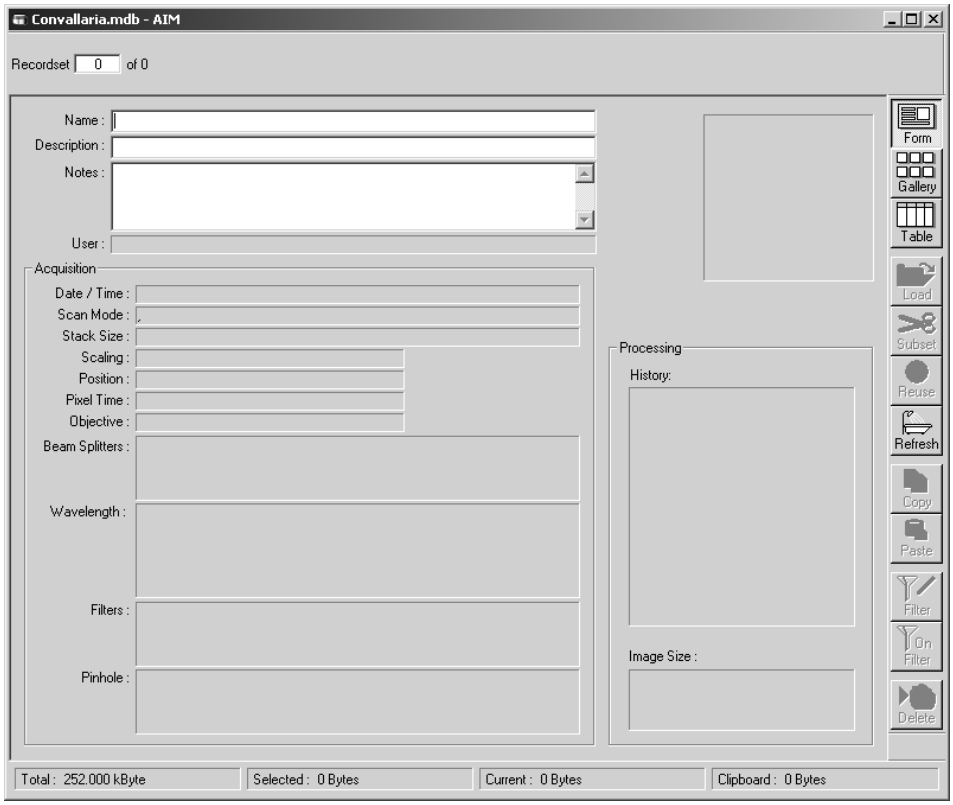


Fig. 5-14 New Database window

The new image database can be used to store an acquired or processed image (see **Saving an Image**, page 5-36).

☞ The start settings and the extent of the parameters displayed in the image database window can be changed as required via the **Settings** function in the **Options** subordinate toolbar (see **Settings Function**, page 5-241).

5.4.2 Open Existing Image Database

The **Open** function allows one or several databases to be opened. The images stored in the database(s) are displayed in thumbnail form; they can be selected and loaded into the **Image Display** window (see page 5-275).

Fig. 5-15 Open Database window

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

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

- Open the image database by a double click on the respective key icon (e.g. **Test-Rel-3_0.mdb**), or click on the name of the image database for selection and open it by clicking on the **Open** button.
  - This opens the image database window, e.g. **Test-Rel-3_0.mdb - AIM**, in which you can select from a variety of options.
- Click on the ☒ button in the title bar of the **Database** window (see Fig. 5-16) to close this window.

5.4.3 Image Database window

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

- Form
- Gallery
- Table

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

The buttons on the right have the following functions:

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

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

Total: ...	Display of storage volume of the entire image database
Selected: ...	Display of storage volume of the selected image(s)
Current: ...	Display of storage volume of the current image
Clipboard: ...	Display of storage volume of the image(s) contained in the clipboard

5.4.3.1 Form display mode

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

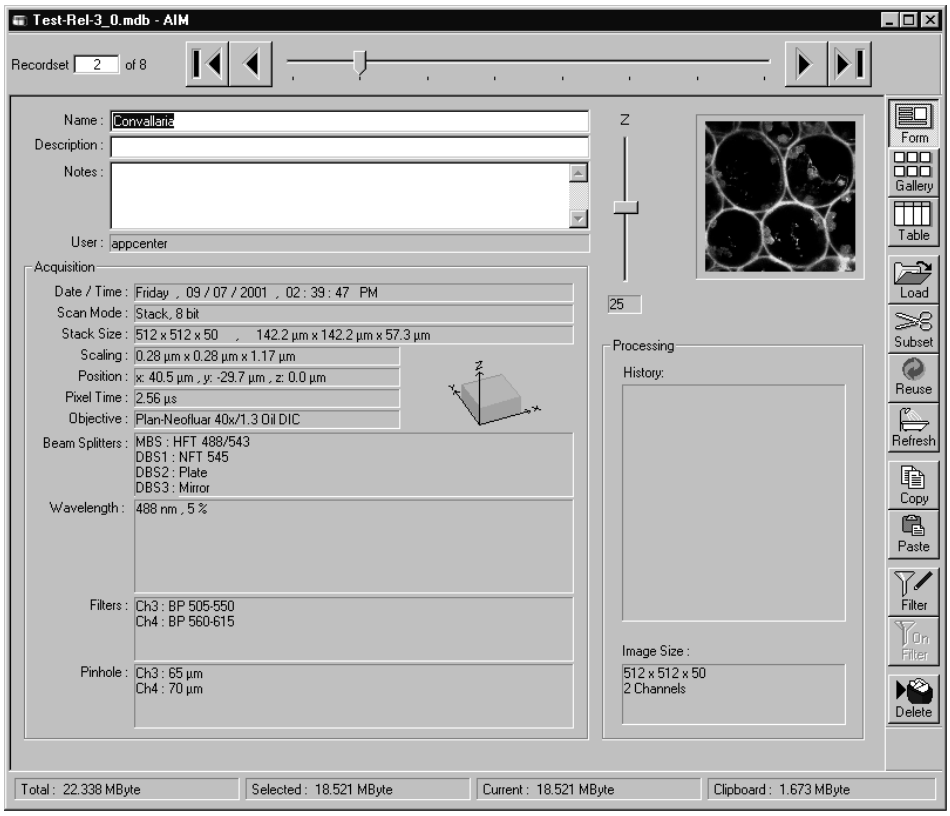


Fig. 5-16 Image Database window (Form display mode)

In the **Options** menu in the function **Settings** it is possible to define
- the start mode of the image database (Form, Gallery, Table)
- the **Recordset** displayed (first, last, middle) and
- the parameters shown.

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

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

 show the next image

 show the previous image

 show the last image of the image database

 show the first image of the image database

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

- You can browse through the series by dragging the **Slice** slider using the mouse.

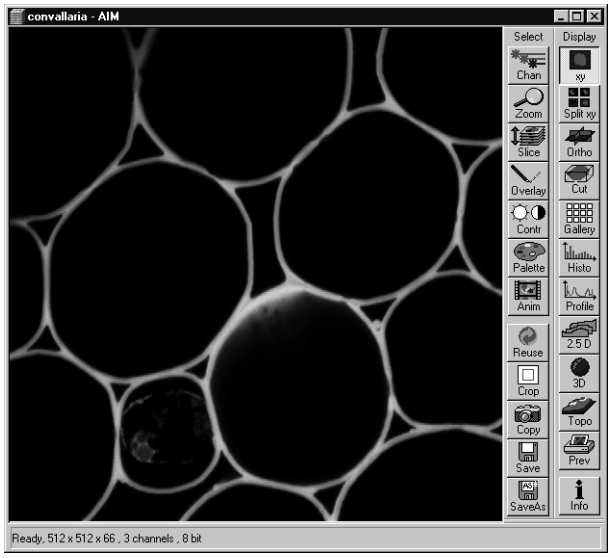


Fig. 5-17 Opened image

- Click on the **Load** button to open the selected image.

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

For a description of the toolbars **Select** and **Display** see **Display and Analysis of Images**, page 5-275).

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

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

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

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

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

5.4.3.2 Gallery display mode

- Click on the **Gallery** button. All images of the image database, e.g. **Test-Rel3_0.mdb**, (image series) are shown in a tiled arrangement of thumbnails on the screen.

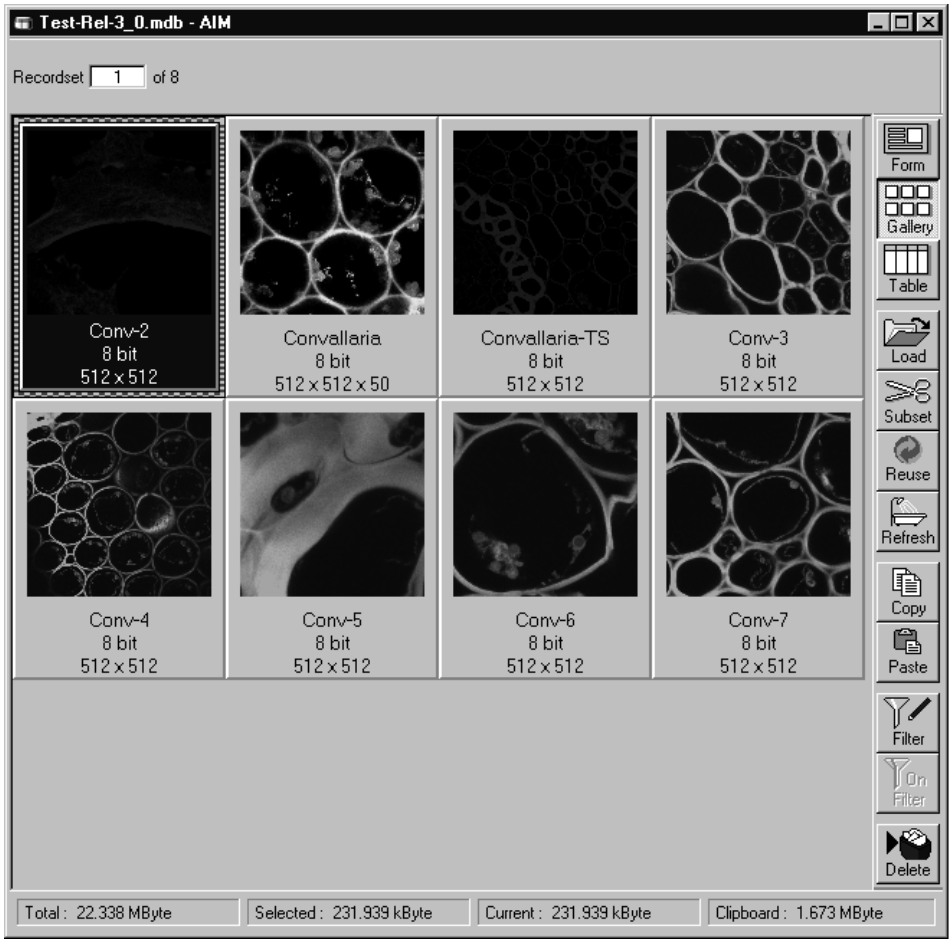


Fig. 5-18 Database window (Gallery display mode)

In the **Options** menu in the function **Settings** it is possible to define
- the start mode of the image database (Form, Gallery, Table)
- the recordset displayed (first, last, middle) and
- the parameters shown.

- To select **one** of the images of the database for normal-size presentation, double-click on the desired image. The same can be achieved by clicking on the desired image in the gallery and then clicking on the **Load** button.

- To select several single images press & hold down the **Ctrl**-key and select each desired image by a click of the mouse. If several images have been selected, they will all be opened and displayed one after the other.

- To select a number of consecutive images press & hold down the **Shift**-key, click on the first and the last image to be selected. All the images between these two will also be included in the selection. If several images have been selected, they will all be opened and displayed one after the other.

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

5.4.3.3 Table display mode

- Click on the **Table** button.
  - All images of the image database, e.g. **Test-Rel3_0.mdb**, (image series) are shown in **Table** display mode on the screen.

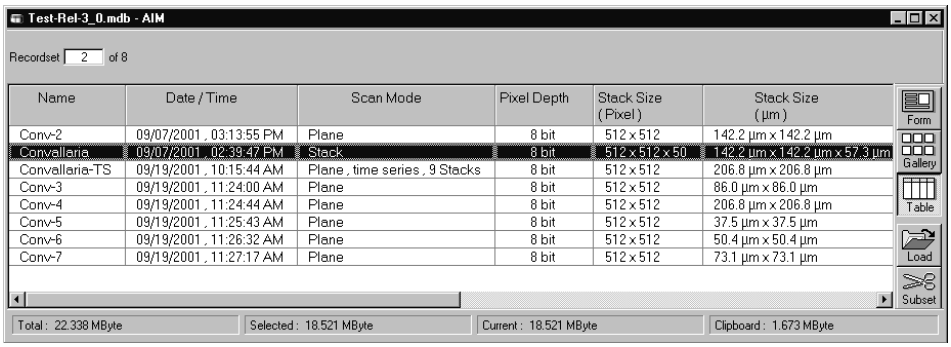


Fig. 5-19 Database window (Table display mode)

In the **Options** menu in the function **Settings** it is possible to define
- the start mode of the image database (Form, Gallery, Table)
- the recordset displayed (first, last, middle) and
- the parameters shown.

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

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

5.4.3.4 Load function

- Click on the **Load** button to load the selected images into the **Image Display** window.

5.4.3.5 Subset function

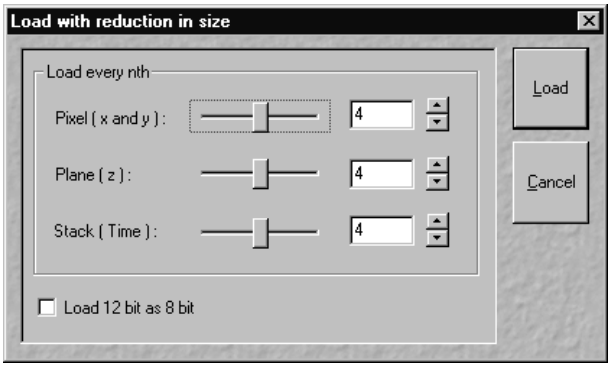


Fig. 5-20 Load with reduction in size window

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

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

5.4.3.6 Refresh function

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

5.4.3.7 Copy / Paste function

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

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

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

- Open the relevant databases and position both windows side to side.
- Select the required images (multiple selection by keeping the **Shift** key pressed) from one database.

- Click on a selected image and keep the mouse button pressed, move the mouse button to the window of the other database (a small rectangular appears near the mouse button) and release the mouse button again (Drag & Drop).

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

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

The images will then be available in both image databases.

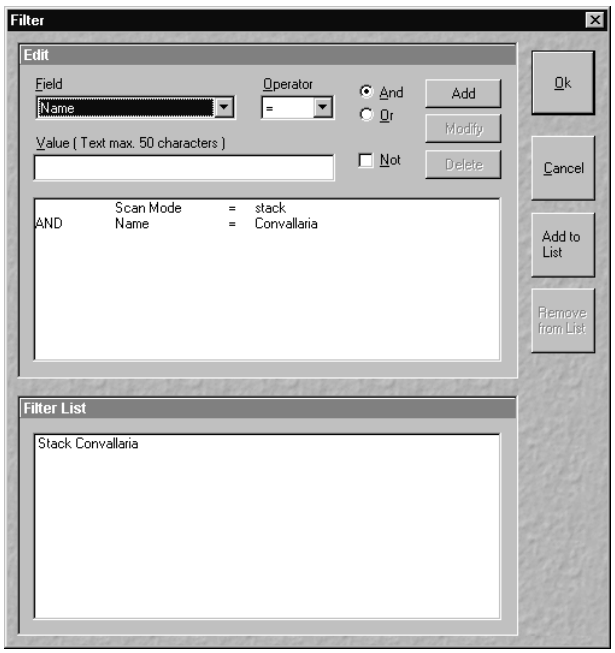


Fig. 5-21 Filter window

5.4.3.8 Filter function

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

Edit panel

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

- Activate the required feature in the work box.
- Click on **Delete**. The filter feature will be deleted from the work box.

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

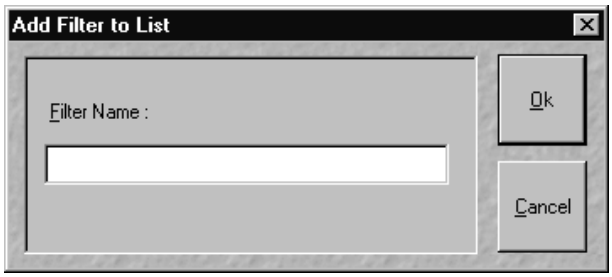


Fig. 5-22 Add Filter to List window

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

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

Filter List panel

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

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

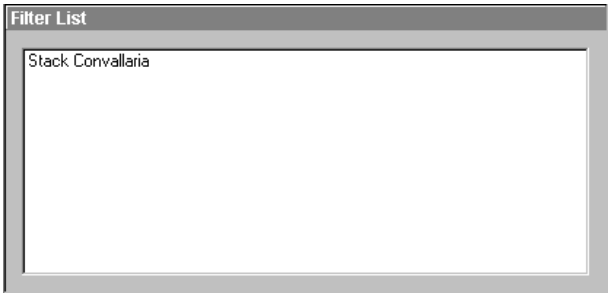


Fig. 5-23 Filter List panel

Filters which are no longer needed can be deleted.

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

5.4.3.9 On Filter function

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

5.4.3.10 Delete function

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

5.4.4 Save an Image to the Image Database

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

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

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

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

☞ **Save**

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

Save As

Stores a previously stored and called up image under a different name. If images are called up and stored again, the original data and time display will be retained.

Clicking on either of these buttons opens the **Save As** window to create and open an image database.

When the **Compress Files** ☑ check box is activated, the images are stored in a compressed form.

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

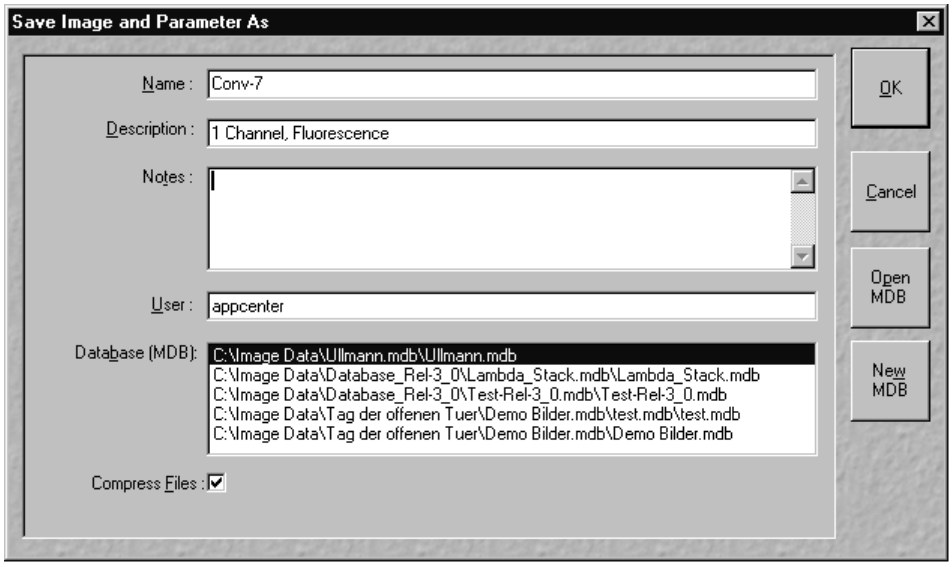


Fig. 5-24 Save Image and Parameter As window

- Enter the name of the image in the **Name** text box, e.g. **Conv-7**.
- Click on the **New MDB** button.
  - This opens the **Create New Database** window in which you can create a new image database.
- Enter the name of the database you want to create in the **File name** text box, e.g. **Projections**.
- If you want to create the image database in a certain folder (drive / directory), click on the arrow button next to the **Create in** box.
  - This opens a drop-down list box showing all folders available for selection.
- After selection, click on the **Create** button.
  - This creates the image database in the selected drive and directory.

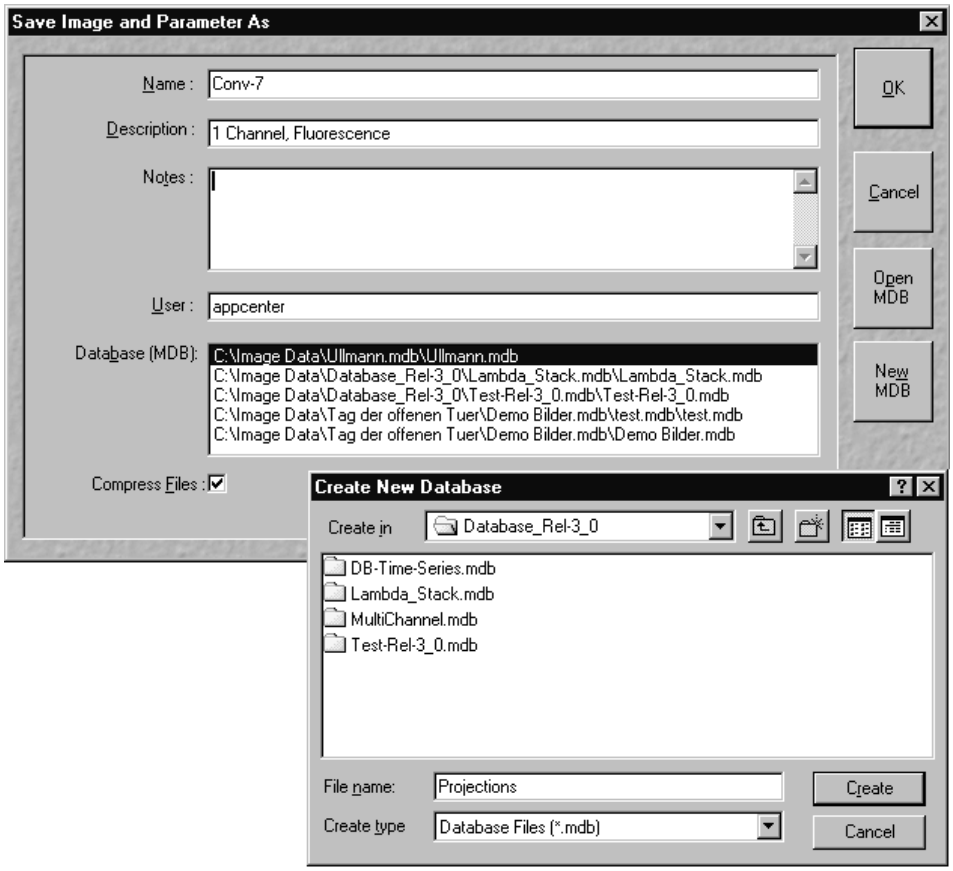


Fig. 5-25 Save Image and Parameter As window and Create New Database window

- The **Projections.mdb - AIM** window appears.

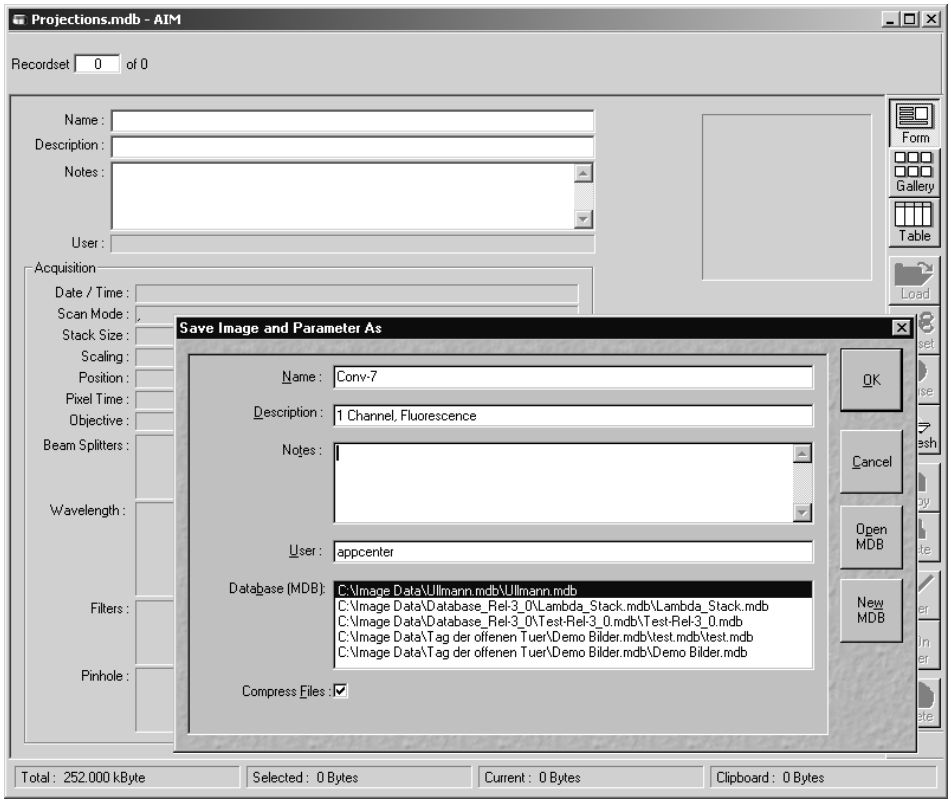


Fig. 5-26 Database window

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

5.4.5 Import of Images

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

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

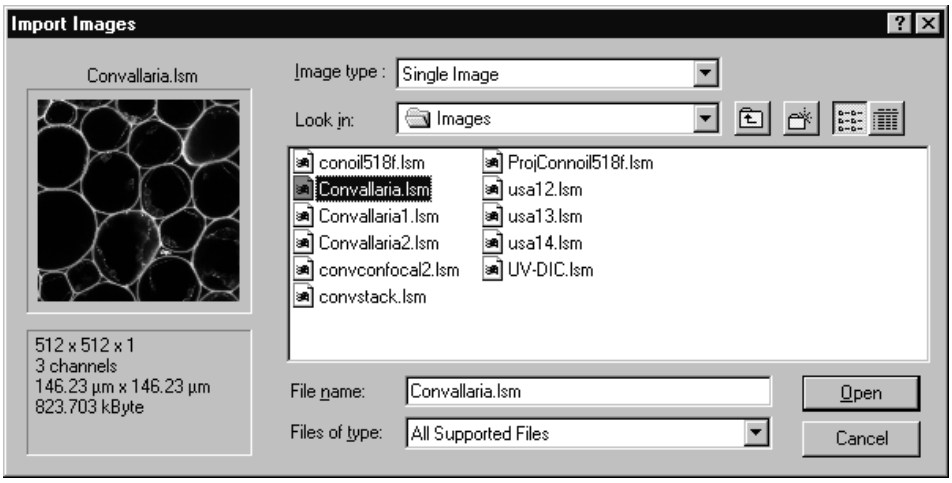


Fig. 5-27 Import Images window

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

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

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

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

5.4.6 Export of Images

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

- Select the image to be exported.
- Click on the **Export** button in the **File** subordinate toolbar of the **Main** menu.
  - This opens the **Export Images and Data** window.
- Under **Save in**, select the data medium and the directory to which the image is to be exported.
- Enter a name for the image under **File name**.
- Select the image format into which the image is to be exported under **Image type** (**Single Image with raw data**, **Contents of the Image Display window**, **Full resolution**).
- Click on the **Save** button.
  - The image is stored on the relevant data medium / directory.

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

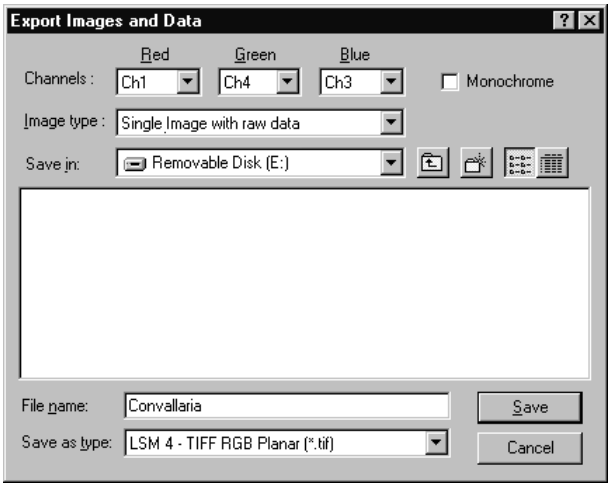


Fig. 5-28 Export Images and Data window

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

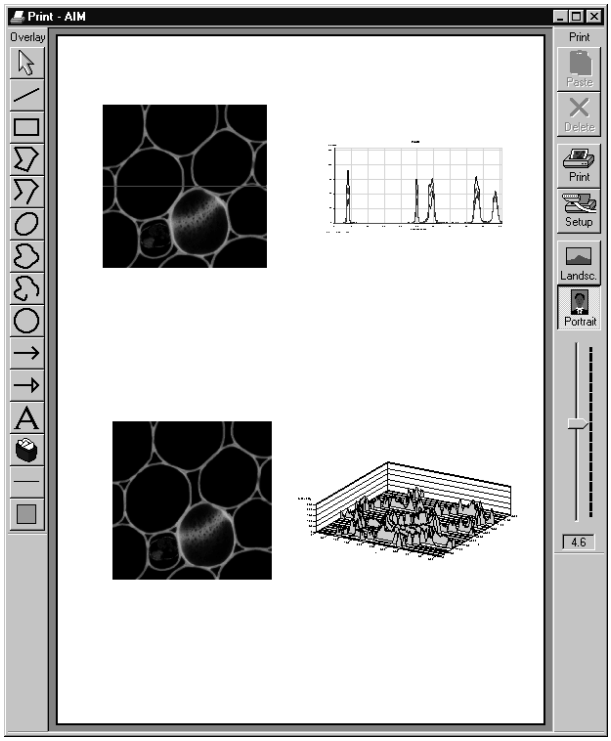


Fig. 5-29 Print - AIM window

5.4.7 Multi Print

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

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

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

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

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

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

Arrow (selection) button: Activation of the mouse button for selection, resizing or movement of an overlay element in the **Image Display** window.
Resizing: Click on the handle and hold down the mouse button, drag the handle, release the mouse button.
Movement: Click on the line and hold down the mouse button, move the entire element, release the mouse button.

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

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

Closed polyline button: Creation of a closed polyline figure in the **Image Display** window.
The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

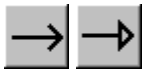
Open polyline button: Creation of an open polyline figure in the **Image Display** window.
The first click sets the starting point, each additional click adds a further line, a click with the right mouse button ends the procedure.

Ellipse button: Creation of an ellipse in the **Image Display** window.
The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and rotation direction can then be determined, the third click sets the second dimension and direction and ends the procedure.

Closed free-shape curve button: Creation of a closed Bezier figure in the **Image Display** window.
The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Open free-shape curve button: Creation of an open Bezier figure in the **Image Display** window.
The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Circle button: Creation of a circle in the **Image Display** window.
Clicking and holding down the mouse button sets the center point, drag the diameter and release the mouse button to end the procedure.



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

A (Text) button: Creation of a text box in the **Image Display** window.
After clicking on **A**, the **Text** window will be displayed, and text can be entered via the keyboard. The **Font ...** button enables you to select the font style and size in the **Font** window. The entered text will be displayed in the left upper corner of the **Image Display** window after clicking on **OK** and can be moved to the required position using the mouse.
The **Text** window can also be activated with a double-click on a created text box, and the entered text can be edited subsequently.

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

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

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

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

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

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

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

5.4.8 Open FCS - Load a FCS data 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 or the ConfoCor 2.

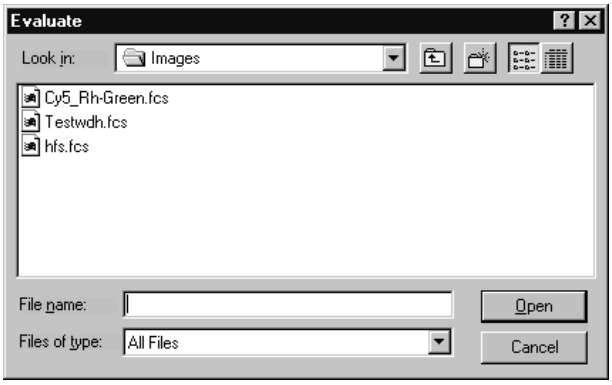


Fig. 5-30 Evaluate window

- To open previously stored data, click on the **Open FCS** button in the **File** subordinate toolbar of the **Main** menu.
  - This opens the **Evaluate** 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 (see Fig. 5-31).

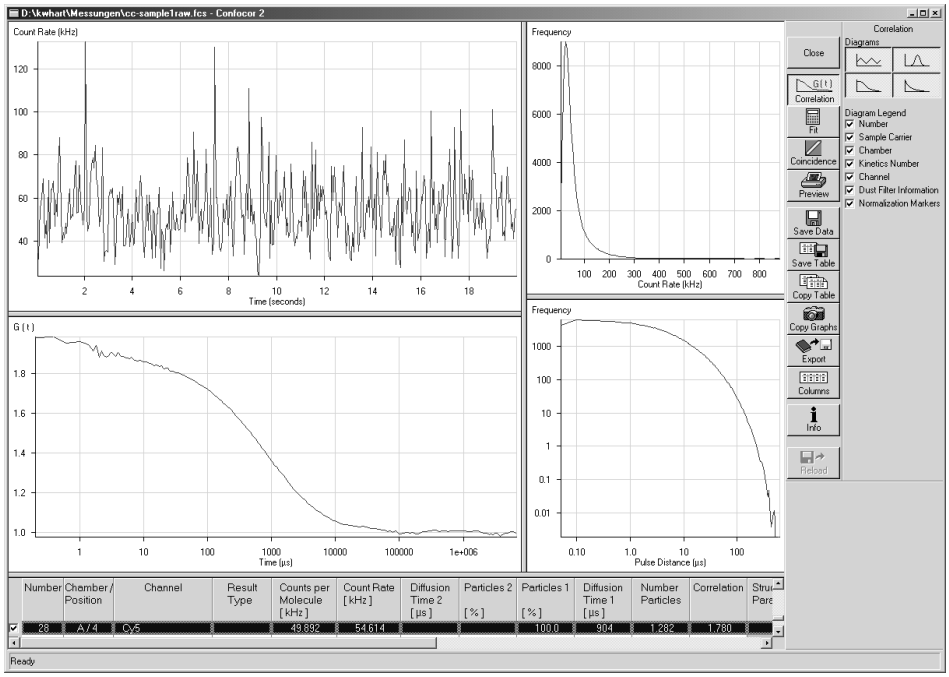


Fig. 5-31 FCS data file window

When the files have been opened, the data display window as described in the beginning of chapter 5.17, **Data Evaluation and Result Presentation for FCS Measurements**, will be shown. It offers the same possibilities as described in chapter 5.17, page 5-399.

5.4.9 Exit the Expert Mode

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

5.5 Acquire Menu

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

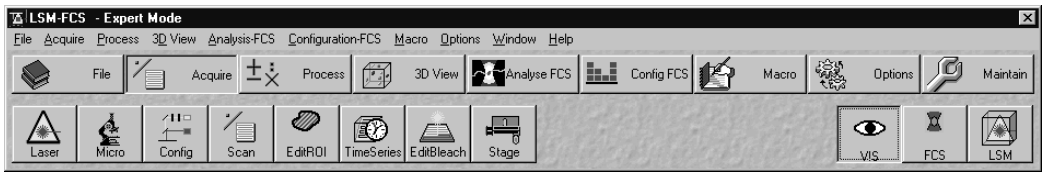


Fig. 5-32 Acquire menu

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.

5.5.1 Laser Control

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

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

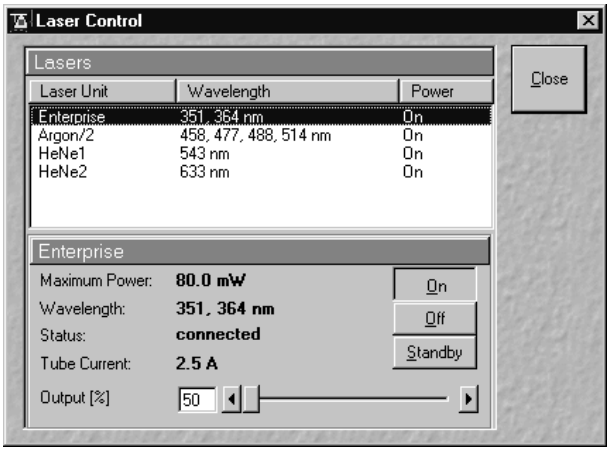


Fig. 5-33 Laser Control window

5.5.1.1 Opening / Closing the Laser Control window

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

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

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

5.5.1.2 Function description

Lasers panel (upper) — List of available lasers, including the display of relevant wavelengths and switching status.

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

Laser settings panel (lower) — Switch on / off the required laser or set Standby operation.

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

Set the laser output for Enterprise and Argon.

5.5.1.3 Settings

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

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

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

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

Thus, the laser needed for image acquisition is available.

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

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

To switch on the Enterprise laser, proceed as follows:

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

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

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

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

This applies to HeNe Diode lasers:

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

☞ The Argon, HeNe1 and HeNe2 lasers are available for FCS measurements.

5.5.2 Microscope Control

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

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

5.5.2.1 Open the Microscope Control window

- Click on the **Micro** button.
  - This opens the **Axiovert Control** window on the screen (Fig. 5-34).

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

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

5.5.2.2 Microscope Control window for Axiovert 200 M

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

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

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

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

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

Reflected Light button — The shutter is switched on and off.

Recording of microscope settings

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

Complete microscope configurations can be created and applied.

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

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

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

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

Load a microscope configuration

An existing microscope configuration can be loaded as follows:

- Click on the arrow button.
  - This opens a list box of all stored microscope configurations.

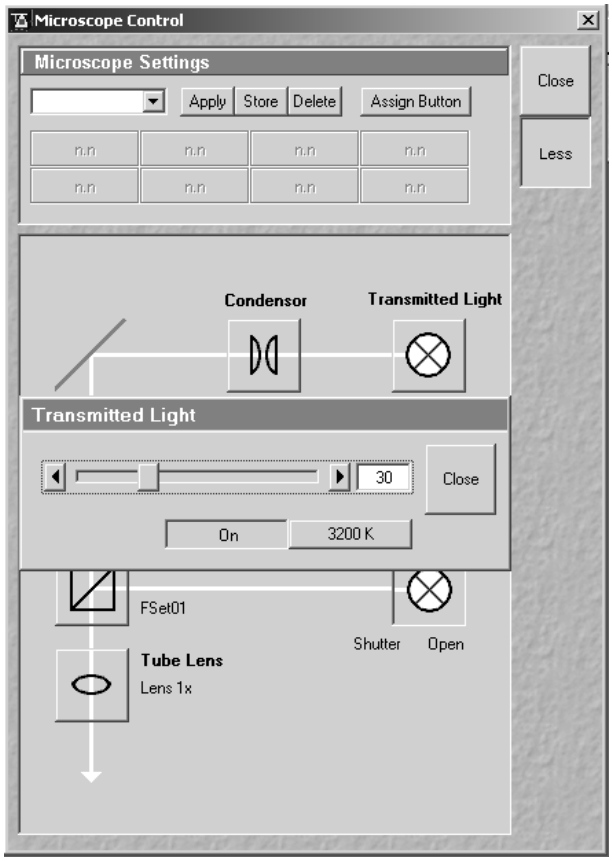


Fig. 5-34 Axiovert Control window

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

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

Store a microscope configuration

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

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

Delete a microscope configuration

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

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

Assignment of a microscope configuration to a button

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

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

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

- Click on the arrow in the **Settings** list box and select a microscope configuration.

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

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

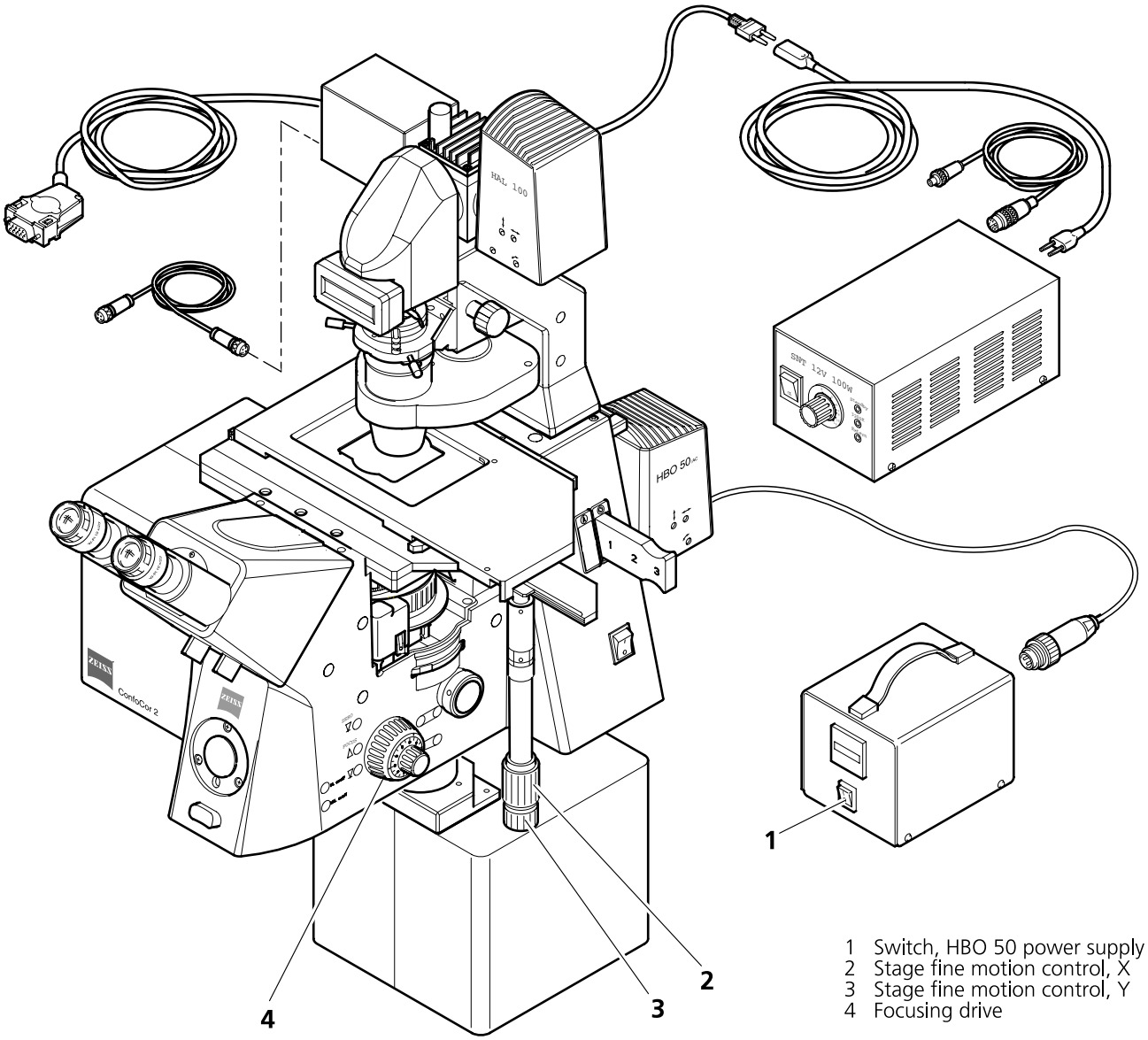
- Click on the **VIS** button in the **Acquire** subordinate toolbar.
- Place specimen on microscope stage.
  - The cover slip must be facing down.
- In the **Objective** list box, select the required objective.
- Use the focusing drive (5-35/**4**) to focus the required specimen plane.
- Select specimen detail by moving the stage in X and Y via the XY stage fine motion control (5-35/**3** and **2**).

(1) Transmitted-light observation

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

(2) Reflected-light observation (Epi-fluorescence)

- Turn on the HBO 50 power supply switch (5-35/**1**).
- Click on the **Reflected Light** button and set the shutter in the **Open** position.
- Click on the **Reflector** button and select the desired filter set by clicking on it.
  - The filter is automatically moved into the beam path to enable observation in epi-fluorescence.
- Click on the **Tube Lens** button and select the tube lens.
- Click on the **Objective** button and select the objective.



1 Switch, HBO 50 power supply
2 Stage fine motion control, X
3 Stage fine motion control, Y
4 Focusing drive

Fig. 5-35 LSM-FCS with Axiovert 200 M BP

5.5.2.3 Select the LSM mode

Switchover to the scanning mode is then required.

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

5.5.3 Configuration Control

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

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

The **Configuration Control** window has a different appearance, depending on which selection button has been activated (**Channel** mode or **Lambda Mode**). The **Lambda Mode** is only available if the system contains the META detector. Only in the **Channel Mode** a subordinate toolbar exist which contains a **Single Track**, **Multi Track** or **Ratio** button. In the **Channel** mode use the **Single Track** and **Multi Track** buttons to toggle between the two image acquisition modes **single tracking** and **multitracking**. In addition, you can activate the **Ratio** button for the activation of up to two **Ratio** channels.

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

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

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

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

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

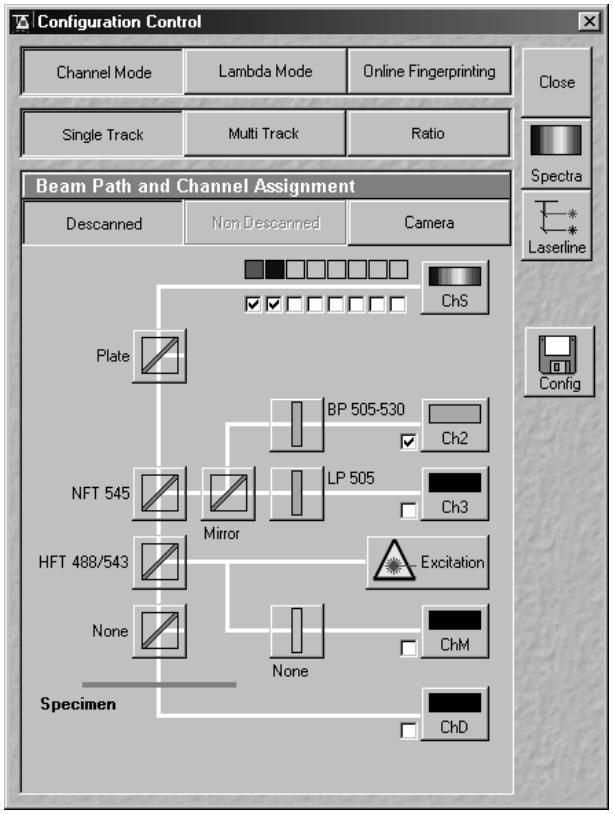


Fig. 5-36 Configuration Control window, Single Track activated

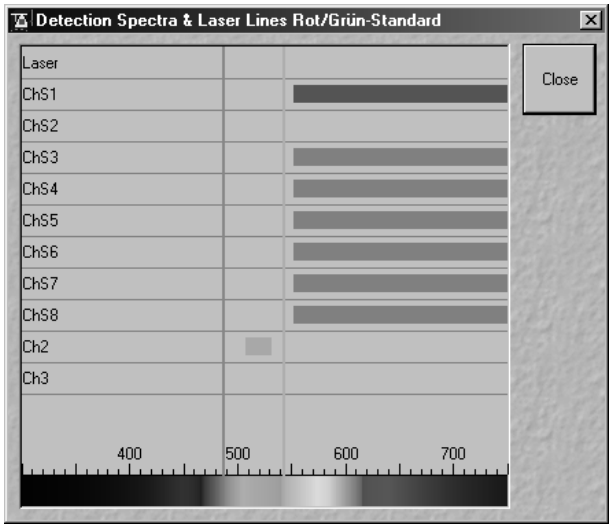


Fig. 5-37 Detection Spectra & Laser Lines window

5.5.3.1 Open / Close the Configuration Control window

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

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

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

5.5.3.2 Spectra button

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

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

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

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

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

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

5.5.3.3 Laserline button

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

If more than 6 excitation laser lines (wavelengths) are available from the connected lasers, these can no longer be completely displayed in the **Line Active** column of the **Excitation** window. In such a case, the required laser lines (if not displayed) must be allocated.

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

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

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

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

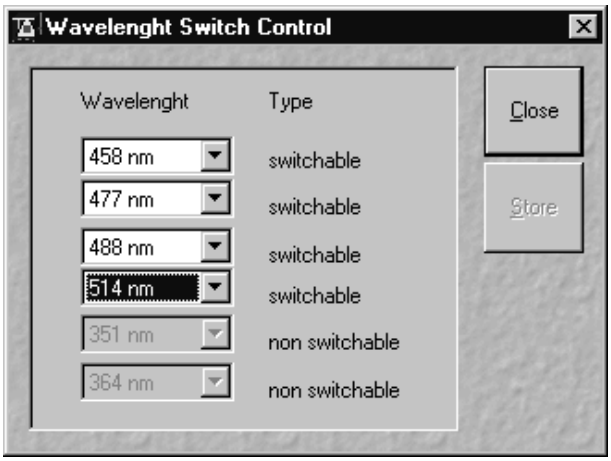


Fig. 5-38 Wavelength & Switch Control window

Fig. 5-39 Track panel

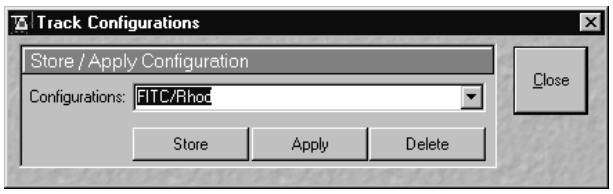


Fig. 5-40 Track Configurations window

5.5.3.4 Config button

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

(1) Load a track configuration

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

- Click on the **Config** button, the **Track Configurations** window appears on the screen.

On the **Store / Apply Configuration** panel, click on the arrow button .

- This opens a list box of all stored track configurations.

- Browse through the configurations by clicking, or use the scroll bar at the side of the list box.
- Click on the desired configuration.
  - The selected configuration is shown in the first line of the **Configurations** list box (e.g.: FITC/Rhod).
- Click on the **Apply** button.
  - This results in the stored instrument parameters being taken over for active use. The track configuration selected before is overwritten.

☞ The optical diagram of the configuration selected appears on the **Beam Path and Channel Assignment** panel. The newly loaded track has been automatically activated for the scanning procedure. The **Track Configurations** window is closed automatically.

In the **Options** menu in the function **Settings** it is possible to define the parameters to be used when applying a track configuration.

(2) Store a track configuration

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

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

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

(3) Delete a track configuration

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

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

5.5.3.5 Settings for Single Track in the Channel Mode

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

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

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

(1) Beam Path and Channel Assignment panel

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

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

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

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

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

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

The **Fiber Out** port of the LSM 510 permits connection of a MCS or another detector. The connected MCS is operated via a macro which can be started via the **Macro** function (see page 5-223ff).

For the configuration of the beam path, please refer to the application-specific configurations depending on the used dyes and markers and the existing instrument configuration (e.g.: module LSM - config. 16) listed in the annex.

The assignment of the numbered emission filters (1-4), NFT secondary dichroic beam splitters (1-3) and HFT main dichroic beam splitters in the **Beam Path and Channel Assignment** panel is shown in the **Configuration Control** window (Fig. 5-41). The numbers of the emission filters correspond to those of the channels lying behind (PMT photomultipliers).

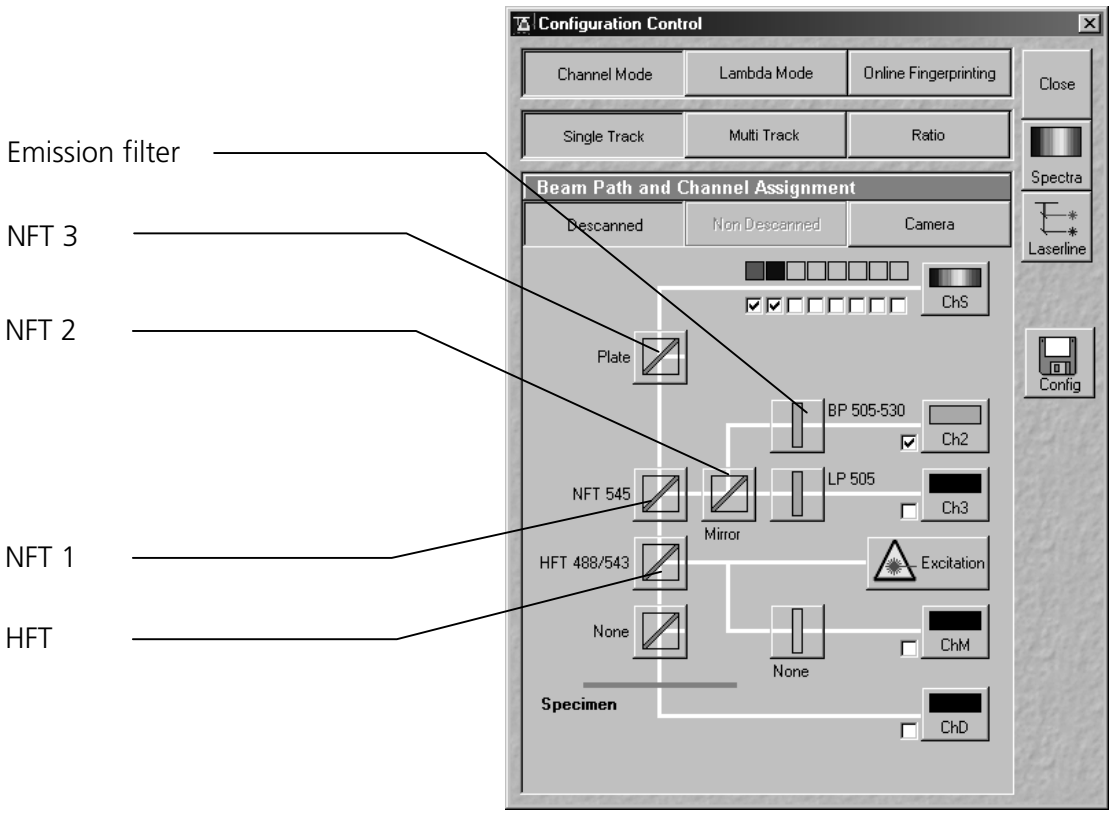


Fig. 5-41 Configuration Control window

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

- On the **Beam Path and Channel Assignment** panel, click on the HFT main dichroic beam splitters (see Fig. 5-41).
  - This opens a graphical pop-up window of all beam splitters available.
- To select a beam splitter, click on the respective line of the list.
  - The selected beam splitter moves into the beam path.
- Proceed accordingly to configure the NFT secondary dichroic beam splitters.

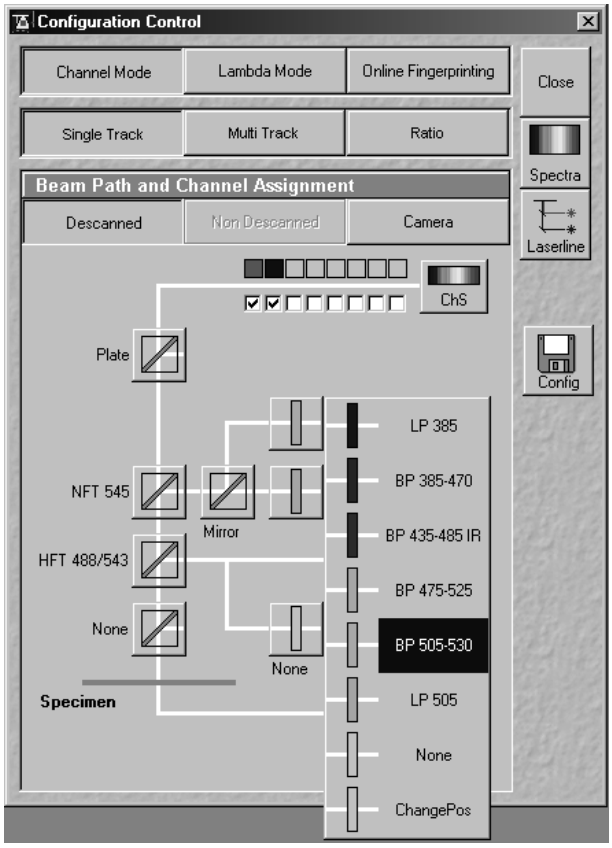


Fig. 5-42 Configuration Control window

(3) Beam path - Emission filter

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

☞ For channels 1 and 2, it is possible to change the filters directly on the LSM scan module (see Annex: Filter change in the beam path of channels 1 and 2).

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

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

This changes the color of the channel symbol.

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

Fig. 5-43 Channel Color Selection window

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

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

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

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

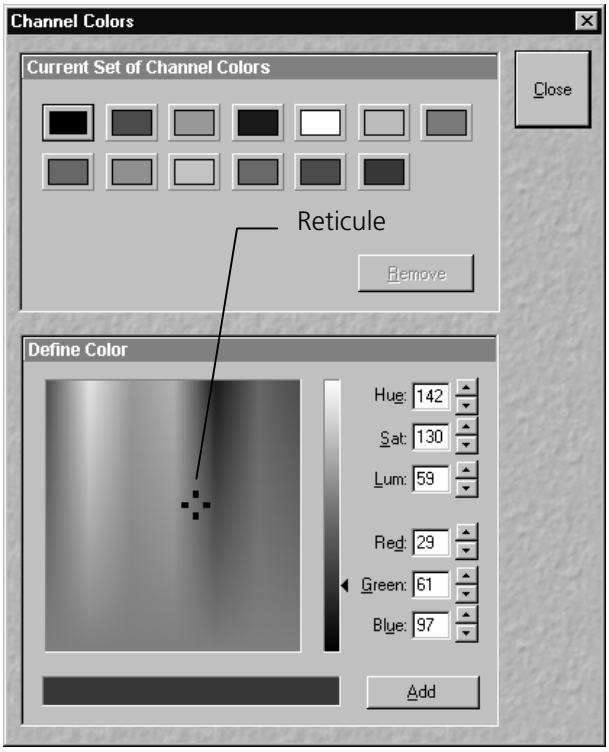


Fig. 5-44 Channel Colors window

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

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

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

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

☞ The  symbols for the transmitted-light PMT photomultiplier (ChD Transmission) and monitor diode (ChM) can be activated in the same way as the photomultipliers of channels 1 to 4. The use of the monitor diode function is described in detail in the annex of this manual.

When changing from the NFT 1secondary dichroic beam splitter, the appropriate setting of the NFT 3 secondary dichroic beam splitter is performed automatically.

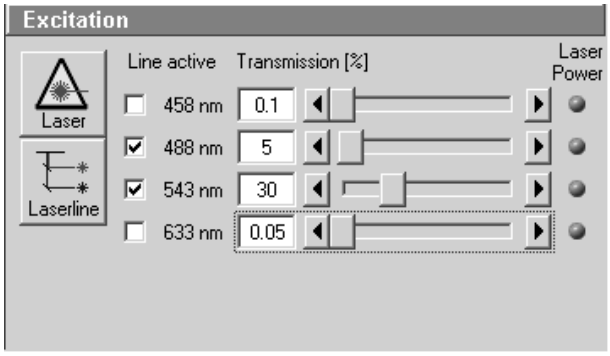


Fig. 5-45 Configuration Control window

(5) Beam path - Laser attenuation

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

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

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

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

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

5.5.3.6 Settings for Multi Track in the Channel Mode

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

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

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

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

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

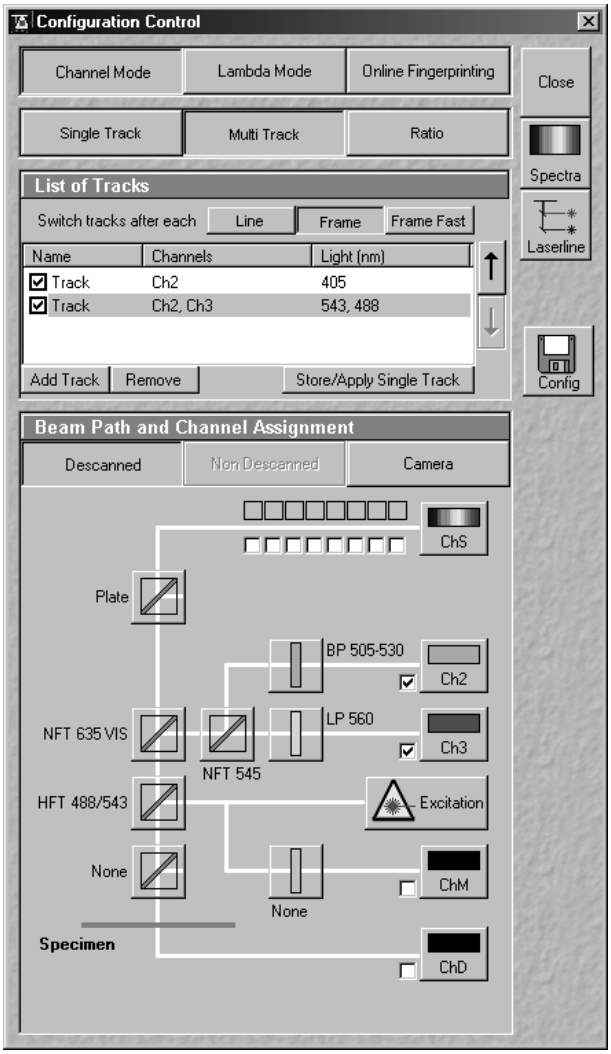


Fig. 5-46 Configuration Control window, Multi Track activated

(1) Beam Path and Channel Assignment panel

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

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

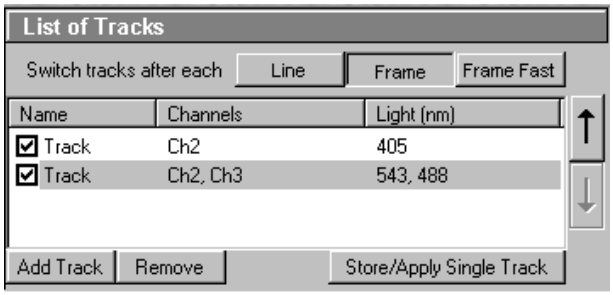


Fig. 5-47 List of Tracks panel

(2) List of Tracks panel

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

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

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

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

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

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

The configuration of the selected track is displayed in the **Beam Path and Channel Assignment ...** panel.

- To select a track for the display of the beam path configuration, click on its name.
  - The selected track is highlighted in gray or blue.

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

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

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

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

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

Settings

Add Track button — An additional track is added to the configuration list. The maximum of four tracks can be added. One track each with basic configuration is added, i.e.: one Ch 1 channel is activated, all laser lines are switched off, emission filters and dichroic beam splitters are set in accordance with the configuration last used.

Remove button — The single track previously marked in the **List of Tracks** panel in the Name column is deleted.

Store/Apply button — Opens the **Track Configurations** window. A selected track defined in a Recording Configuration can also be stored as a single track for single tracking applications. Also, it's possible to load a single track in a multitracking configuration.

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

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

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

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

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

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

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

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

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

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

(3) Config button in Multi Track mode

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

Load a recording configuration

An existing recording configuration can be loaded as follows:

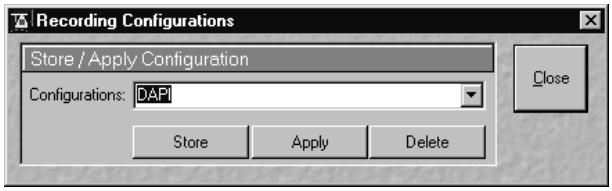


Fig. 5-48 Recording Configurations window

- Click on the **Config** button, the **Recording Configurations** window appears on the screen.
- On the **Store / Apply Configuration** panel, click on the arrow button ▼.
  - This opens a list box of all stored recording configurations.

- Browse through the configurations by clicking, or use the scroll bar at the side of the list box.
- Click on the desired configuration.
  - The selected configuration is shown in the first line of the **Configurations** list box (e.g.: DAPI).
- Click on the **Apply** button.
  - The program loads those parameters of the selected **Recording Configuration** which have been activated in the **Options** menu under **Settings / Recording Configuration** (see section 5.11.5, page 5-241). The **Recording Configurations** window is automatically closed.

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

Store a recording configuration

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

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

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

Delete a recording configuration

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

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

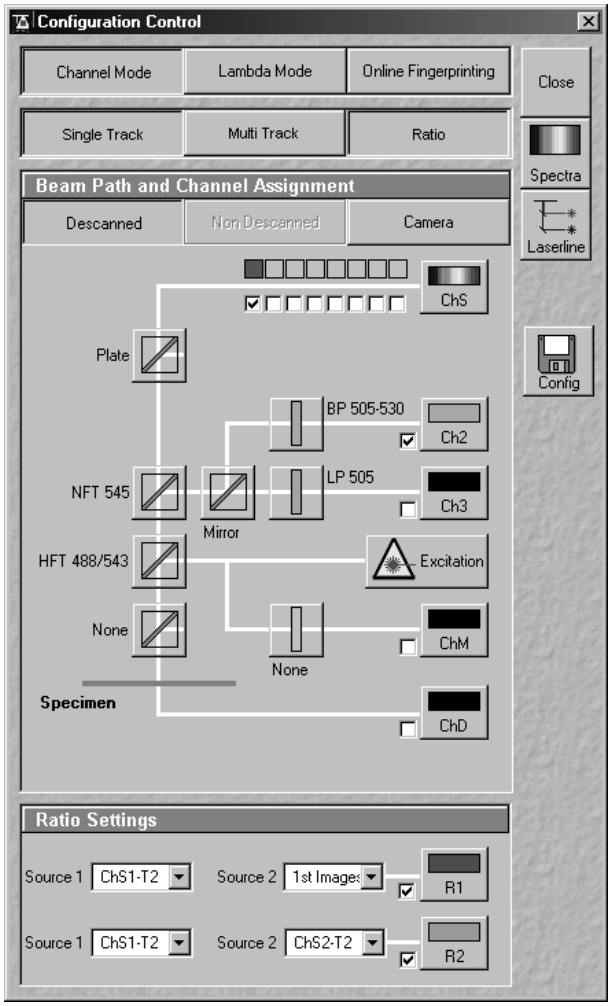


Fig. 5-49 Configuration Control window; Ratio activated

5.5.3.7 Ratio Settings panel

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

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

The **Ratio Settings** panel is only available in the **Single Track** and **Multi Track** mode.

Source 1 in ratio settings

Selects source 1 data channel in **Configuration Control**.

Source 2 in ratio settings

Selects source 2 data channel in **Configuration Control**, including the option to select "1st Image" for R1 and/or R2 (e.g. to calculate F/F_0 for single wavelength dyes).

R1/R2 in Scan Control

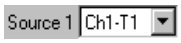
R1/R2 can be selected as channels in the **Scan Control** window. Five preset formulas can be chosen for online display of radiometric or single wavelength dyes.

Set by min/max (in Scan Control window - Channels mode)

Allows the definition of the display scaling according to the expected minimal and maximal values.

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

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



Selection of the channels of which the ratio is to be formed from the relevant list box.

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

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

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

☞ The ratio to be formed between the selected channels can be defined more precisely using three formulas in the **Scan Control** window after activation of the **Channels** button and a click on the relevant ratio button (e.g.: **R1**).

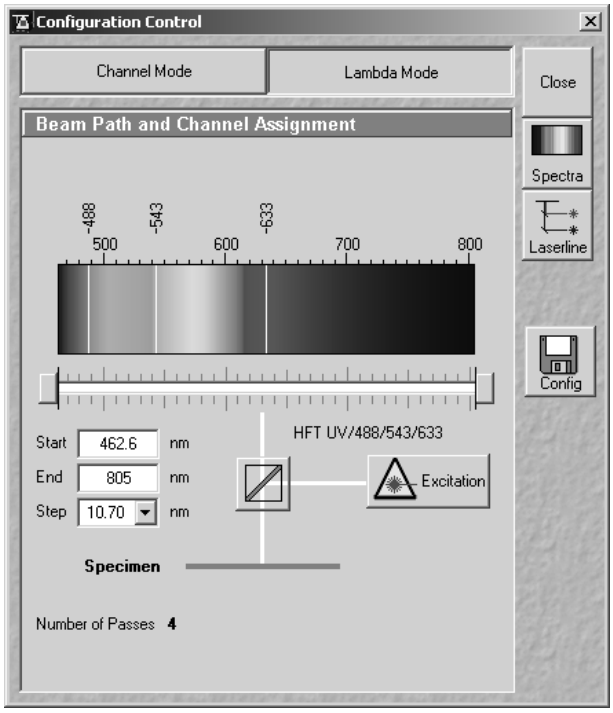


Fig. 5-50 Configuration Control window; Lambda Mode activated

5.5.3.8 Settings in the Lambda Mode

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

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

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

(1) Beam Path and Channel Assignment panel

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

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

Beam Path configuration

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

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

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

Control buttons

Close — Closes the **Configuration Control** window.

Spectra — The **Spectra** button opens the **Detection Spectra & Laser Lines** window (see page 5-56).

Laserline — The **Laserline** button opens the **Wavelength Switch Control** window (see page 5-57).

Config — The **Config** button permits existing track configurations to be loaded, stored under any name, or deleted (see page 5-58).

(2) Beam path - Excitation

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

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

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

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

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

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

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

(4) Beam path - META detector settings

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

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

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

Step check box: Step size can be increased to 21.4 nm by binning of a pair of adjacent channels.

5.5.3.9 Settings in the Online Fingerprinting Mode

The use of this function permits the selection of reference spectra together with the excitation settings, to allow an immediate display of the unmixing results during the scanning.

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

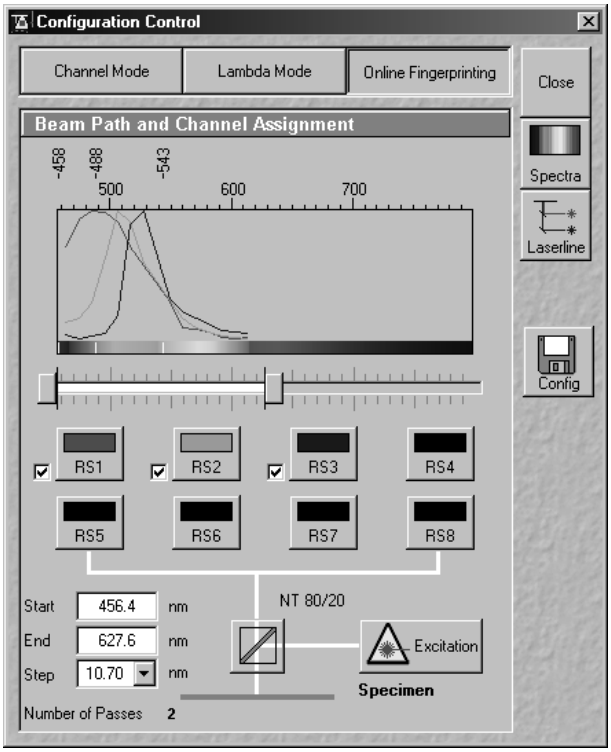


Fig. 5-51 Configuration Control window; Online Fingerprinting Mode activated

Beam Path configuration

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

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

Control buttons for reference spectra

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

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

Start box	Start value of required spectral range.
End box	End value of required spectral range.
Step box	Step width in spectral range (10.70 recommended).

5.5.3.10 Non Descanned panel

The functions of the **Non Descanned** panel are described in **chapter 9** of this manual (section 9.11, page 9-35).

5.5.3.11 Camera Detection panel

The use of this function permits the use of a Zeiss AxioCam HR camera as an alternative external detector.

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

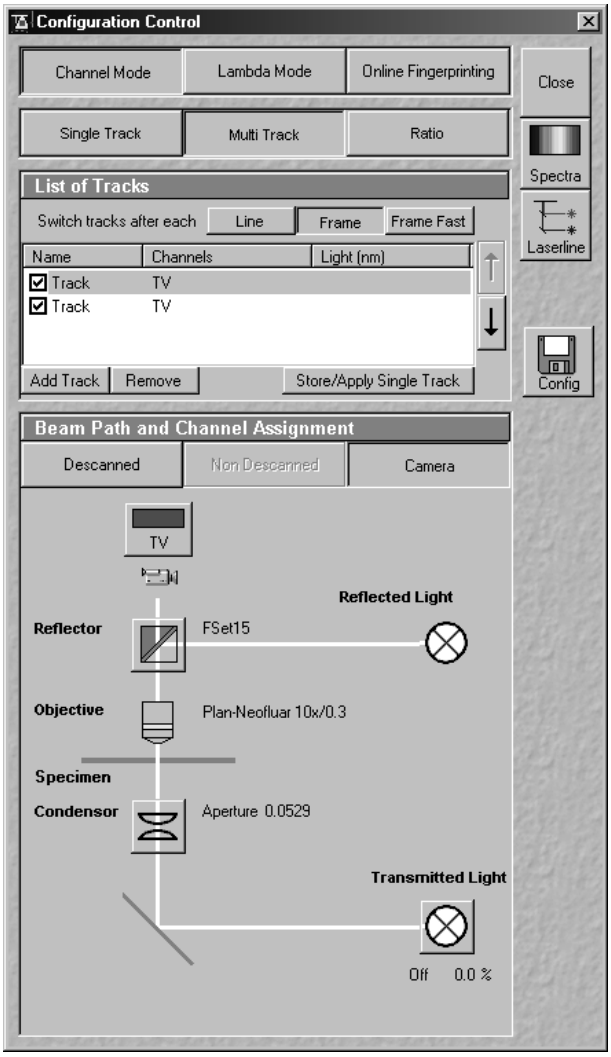


Fig. 5-52 Configuration Control window; camera detecting activated

Control buttons

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

Reflector Selects a beamsplitter for the excitation/emission.

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

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

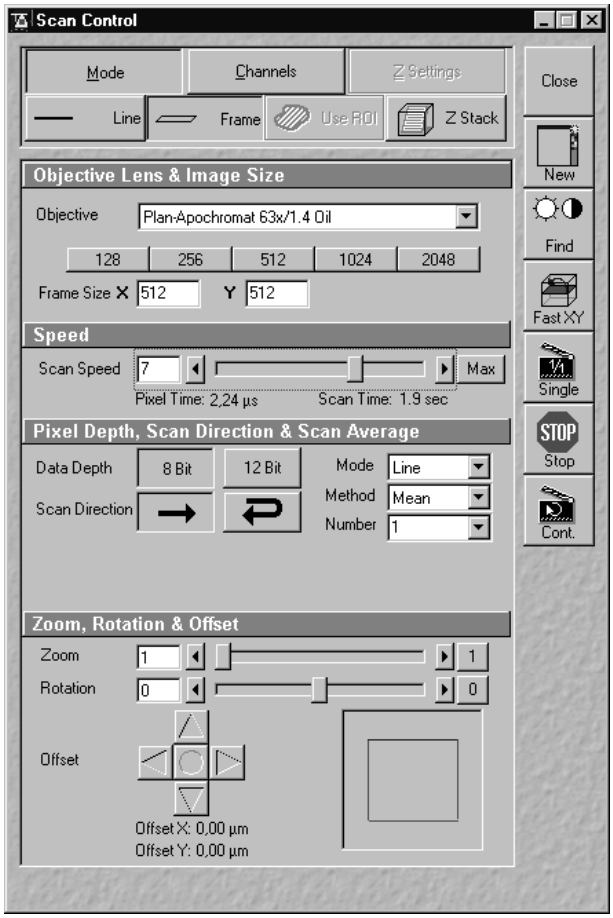


Fig. 5-53 Scan Control window

5.5.4 Scan Control

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

The microscope must be in the LSM mode, i.e. the relevant sliders on the relevant microscope stand must be in the **LSM** position. The **LSM** button in the **Acquire** subordinate toolbar is activated when the LSM mode has been set.

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

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

The following scanning modes can be performed:

Spot

- scanning of a spot (Spot + Time Series)

Line

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

Frame

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

5.5.4.1 Open / Close the Scan Control window

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

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

Generally available buttons

Mode button	When the button is activated, the following panels are available for the setting of the scanning parameters for the line and frame modes: **Objective Lens, Image Size & Line Step Factor**, **Speed**, **Pixel Depth, Scan Direction & Scan Average** and **Zoom, Rotation & Offset**.
Channels button	When the button is activated, the **Channel Settings** and **Excitation of Track ...** panels are available for the setting of the channels and the laser excitation.
Spot button	Activate the **Spot** scan mode
Line button	Activates the **Line** scan mode.
Frame button	Activates the **Frame** scan mode.
Use ROI button	Activates the scanning procedure only within a **ROI** (region of interest) to be defined first.
Z Stack button	Activates the **Z Stack** scan mode, display of additional buttons on the right-hand side of the **Scan Control** window.
Z Settings button	When the button is activated, the **Z Settings** panel is available for the Z-scan parameter definition. The **Z Stack** scan mode must be active.
Close button	Closes the **Scan Control** window.
New button	Opens a new **Image Display** window.
Find button	Automatic optimization of image brightness and contrast. The settings for the **Find** function can be varied as required using the **Maintain** menu, **Set Find** (see page 5-270).
Fast XY button	Continuous scan with high speed. This function should be used to a limited extent and only for a short period of time. Fast XY switches temporarily to 512 x 512 frame size.
Single button	Single scan (named **Start** in the **Z Stack** mode).
Stop button	Stops the current scan procedure, no matter in which window the button is pressed (also see the **Time Series Control** and **Bleach Control** windows).

Cont. button / **Finish** button — Continuous scan (not available in the **Z Stack** mode). If you select the option **Frame** for **Mode** and the option **Continuous** for **Number** in the **Pixel Depth, Scan Direction & Scan Average** panel, the **Finish** button is displayed instead of the **Cont.** button. In this case, continuous averaging is performed when you have started the scan. If you click on the **Finish** button, the scan/averaging process is stopped after the scan of the current image has been completed.

Additional button in the Spot mode

Spot Sel button — Automatically defines spot on the **Image Display** window by positioning of two perpendicular lines

Additional button in the Line mode

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

Additional buttons in the Z Stack mode

Start button — Triggers the scan of a stack.

XYscan button — Triggers a single XY-scan

XYcont button — Triggers continuous XY-scan.

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

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

5.5.4.2 Frame

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

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

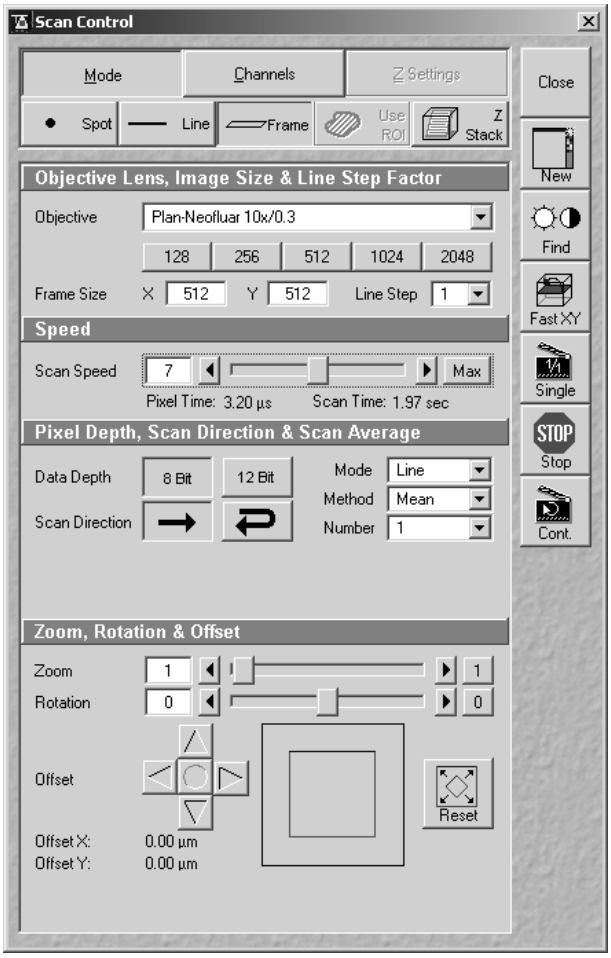


Fig. 5-54 Scan Control window - Mode/Frame

(1) Mode

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

Objective Lens, Image Size & Line Step Factor panel

- Open the **Objective** list box and select the objective to be used via a click of the mouse (identical to Microscope Control). When using immersion oil objectives, make sure to perform immersion as required.
- Select the **Frame Size** from the default sizes via the buttons **128**, **256**, **512**, **1024**, **2048,** or enter the required values via the keyboard. Recommended setting to start with: 512 x 512 pixels.
  - It is also possible to enter different values for X and Y. The value for **Y** is freely selectable between 1 and 2048 pixels (integers). The value for **X** must always be an integral multiple of 4. The maximum value for **X** is also 2048 pixels.

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

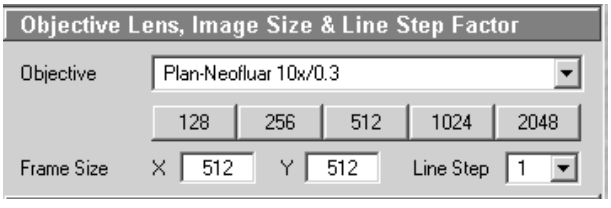


Fig. 5-55 Objective Lens, Image Size & Line Step Factor panel

Fig. 5-56 Speed panel

Speed panel

- Select the **Scan Speed** from the 13 preset steps via slider or input box. Recommended: 7 for the first scan. A click on the **Max** button sets the maximum speed for the current zoom.

- The Scan Speed determines on the Pixel Time. In the case of different image formats, the Pixel Time is constant for the same Scan Speed, but the Scan Time is different.
- Pixel Time: dwell time of the laser beam on the pixel
- Scan Time: duration of the acquisition for the entire frame
- The minimum Pixel Time of 0.64 µs is only achieved at resolutions 512 x n and above, the maximum Pixel Time of 204.8 µs only with frame sizes larger than 1024 x n.
- A longer Pixel Time for even smaller frame sizes is possible; maximum: 6553.6 µs.

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

Speed:	1 ... 8	9	10	11	12	13
Zoom:	0.7	1.0	1.4	2.4	3.2	5.6

☞ Note that **Lambda Scan** mode can only be performed with specifications from speed 1 ... 10 at 512 x 512 pixels.

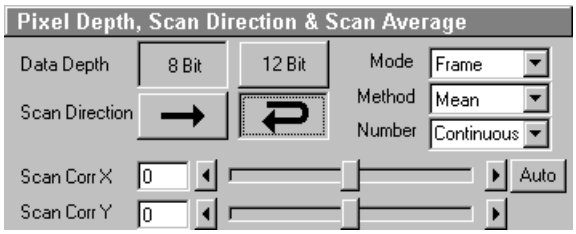


Fig. 5-57 Pixel Depth, Scan Direction & Scan Average panel

Pixel Depth, Scan Direction & Scan Average panel

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

- Select the **Line** or **Frame** mode for averaging.
- Select the desired scan average method **Mean** or **Sum** in the **Method** selection box.
- Select the desired scan average from the available values **2**, **4**, **8** and **16** in the **Number** selection box or **Continues** (only for **Frame** average mode).

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

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

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

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

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

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

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

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

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

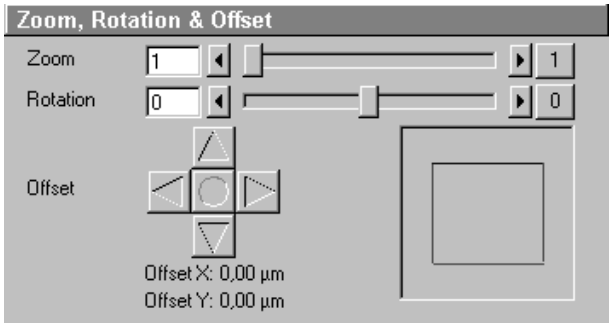


Fig. 5-58 Zoom, Rotation & Offset panel

Zoom, Rotation & Offset panel

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

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

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

- Set the desired zoom factor via the slider (**Zoom**) or by clicking on the arrow buttons.
  - The zoom factor can be set continuously in the range from 0.7 to the maximum of 8, and is displayed in the relevant input box. The value 0.7 corresponds to factor 1, and value 8 to factor 11, related to the field of view. From zoom factor 5.6, the magnification will be empty, and the zoom factors will be displayed in red in that case. Clicking on button **1** enables immediate resetting to the zoom factor 1.
  - Recommended setting to start with: Zoom 1.
- To rotate the scan area, use the slider (**Rotation**) or click on the arrow buttons.
  - Clicking on button **0** enables immediate resetting to 0°.
  - Recommended setting to start with: Rotation 0°.
- Move the scan area by clicking on the 4 arrow buttons (**Offset**).
  - The offset of the scan area from the center of the field of view is displayed online in µm for X and Y.
  - A click on the center button will recenter the scan area to the field of view.
  - Clicking, holding and drawing the rectangle with the mouse permits the scan area to be moved directly within the field of view.
  - Recommended setting to start with: Offset X = 0, Y = 0

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

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

(2) Channels

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

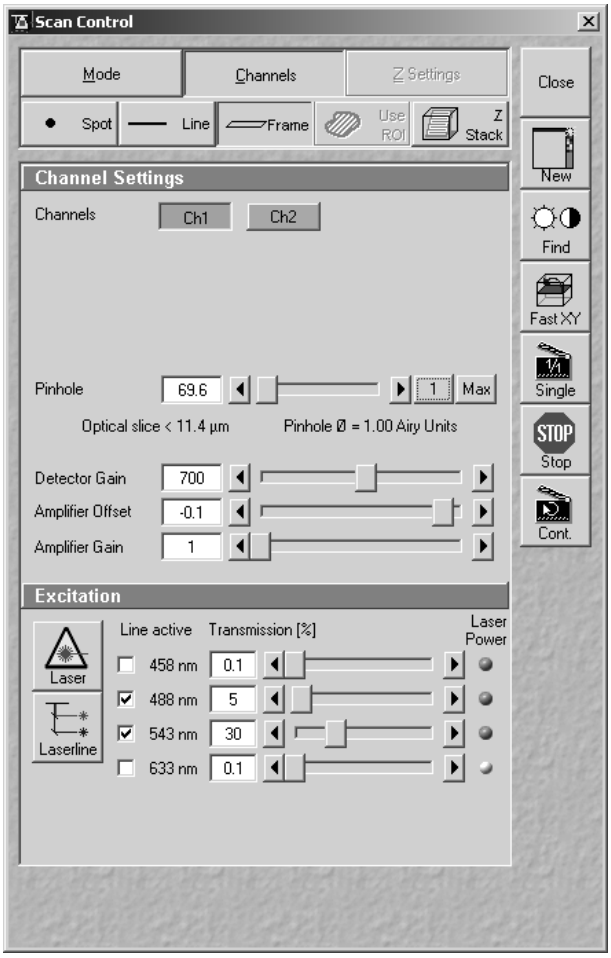


Fig. 5-59 Scan Control window – Channels

Channel Settings panel

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

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

- The slider near **Pinhole** enables you to change the pinhole diameter of the relevant channel.
  - The pinhole diameter is indicated in **µm**, **Optical Slice** and **Airy Units**. The Airy value depends on the aperture of the objective, excitations and the emission wavelength.
  - A small pinhole diameter will increase the depth of focus, but reduce the light intensity received by the PMT photomultiplier.
  - When you vary the pinhole diameter, an Optical Slice value is displayed. For optimum depth resolution, Airy values should be small, but in fluorescence applications not below 1.0 to keep the intensity loss within a reasonable limit.
  - A click on the **1** button sets the pinhole to a diameter of 1 Airy unit. A click on the **Max** button sets the pinhole diameter to the maximum.
- The sliders (and the relevant arrow buttons) near **Detector Gain**, **Ampl. Offset** and **Ampl. Gain** enable you to set the photomultiplier of the selected channel during continuous scanning.
  - Detector Gain: Setting of the high voltage of the PMT photomultiplier - setting of image contrast and brightness (values available between 80 and 1250)
  - Amplifier Offset: Setting of the electronic offset - background of the image can be set (values available between -2 and 0.1)
  - Amplifier Gain: Amplification factor (values available between 1 and 3)

☞ The parameters **Detector Gain**, **Ampl. Offset** and **Ampl. Gain** are described in section **Pinhole / Detector Gain / Ampl. Offset / Ampl. Gain** (page 5-392) in the context of image optimization. In case the **Lambda Mode** has been chosen in the **Configuration Control** window only the META channel **ChS** is displayed in the **Channel** settings of the **Scan Control** window.

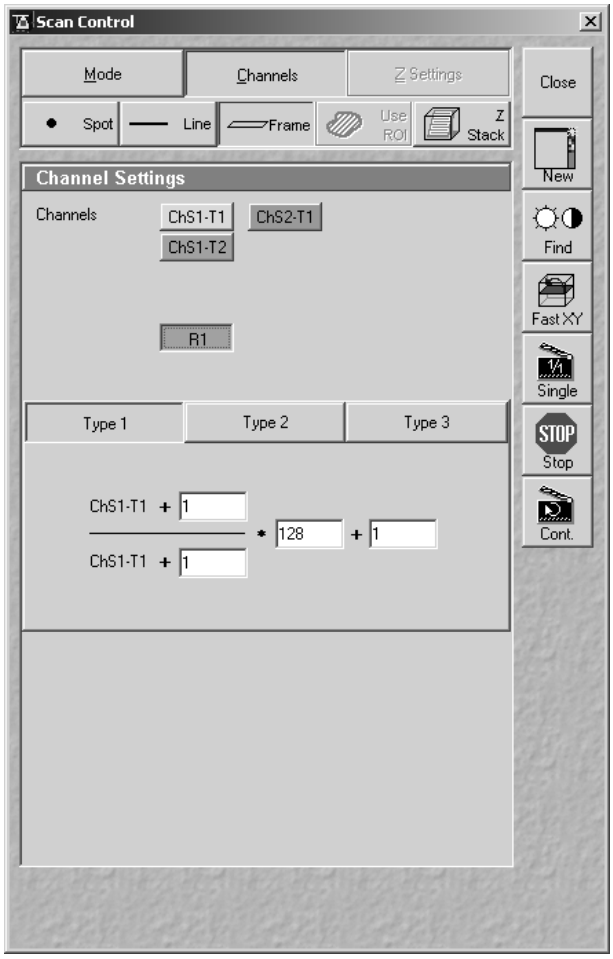


Fig. 5-60 Channel Settings panel of a Ratio Channel

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

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

Clicking on the required tabs enables you to choose from five formulas (**Type 1** to **5**) for ratio calculation. The relevant decimal values can be entered in the input boxes via the keyboard. The entered values remain unchanged even after switchover to another formula and can be reactivated any time.

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

The ratio channels are displayed in the **Image Display** window (see Fig. 5-63).

- Select the required formula and enter the relevant values.

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

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

Excitation panel

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

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

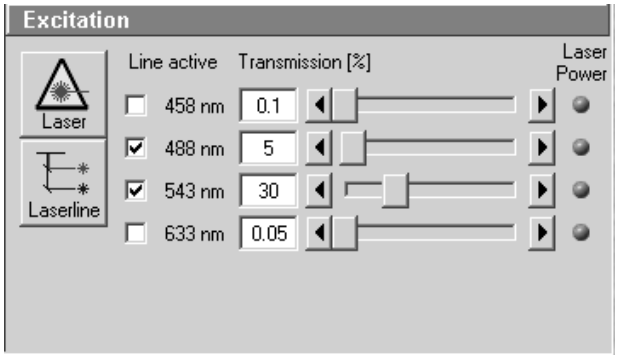


Fig. 5-61 Excitation of Track ... panel

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

☞ If bi-directional scanning with12-bit technology, several channels and scan speeds of 9 or 10 are used at the same time, a data jam can occur and difficulties can therefore arise if 233 MHz PC's (or lower) are used. All parameters under Channels can be varied online.

Acquisition of a frame

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

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

The following scan image shows the result with two defined tracks plus the **Ratio** channel and the overlay (see Fig. 5-63). The appropriate **Channel Settings** panel in the **Scan Control** window is shown in Fig. 5-62.

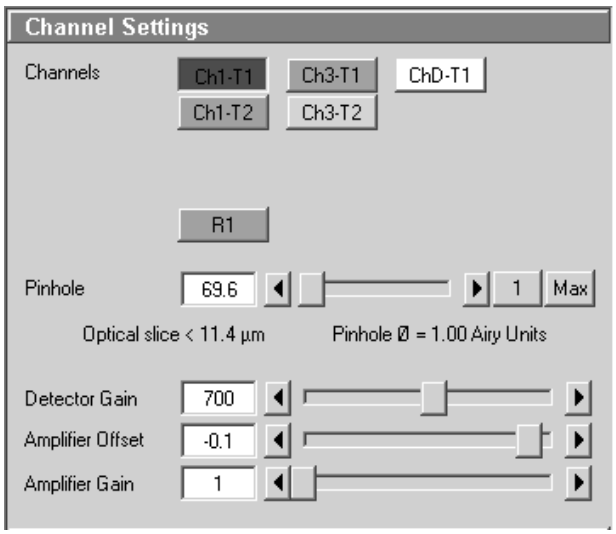


Fig. 5-62 Channel Settings panel for two defined tracks plus Ratio channel

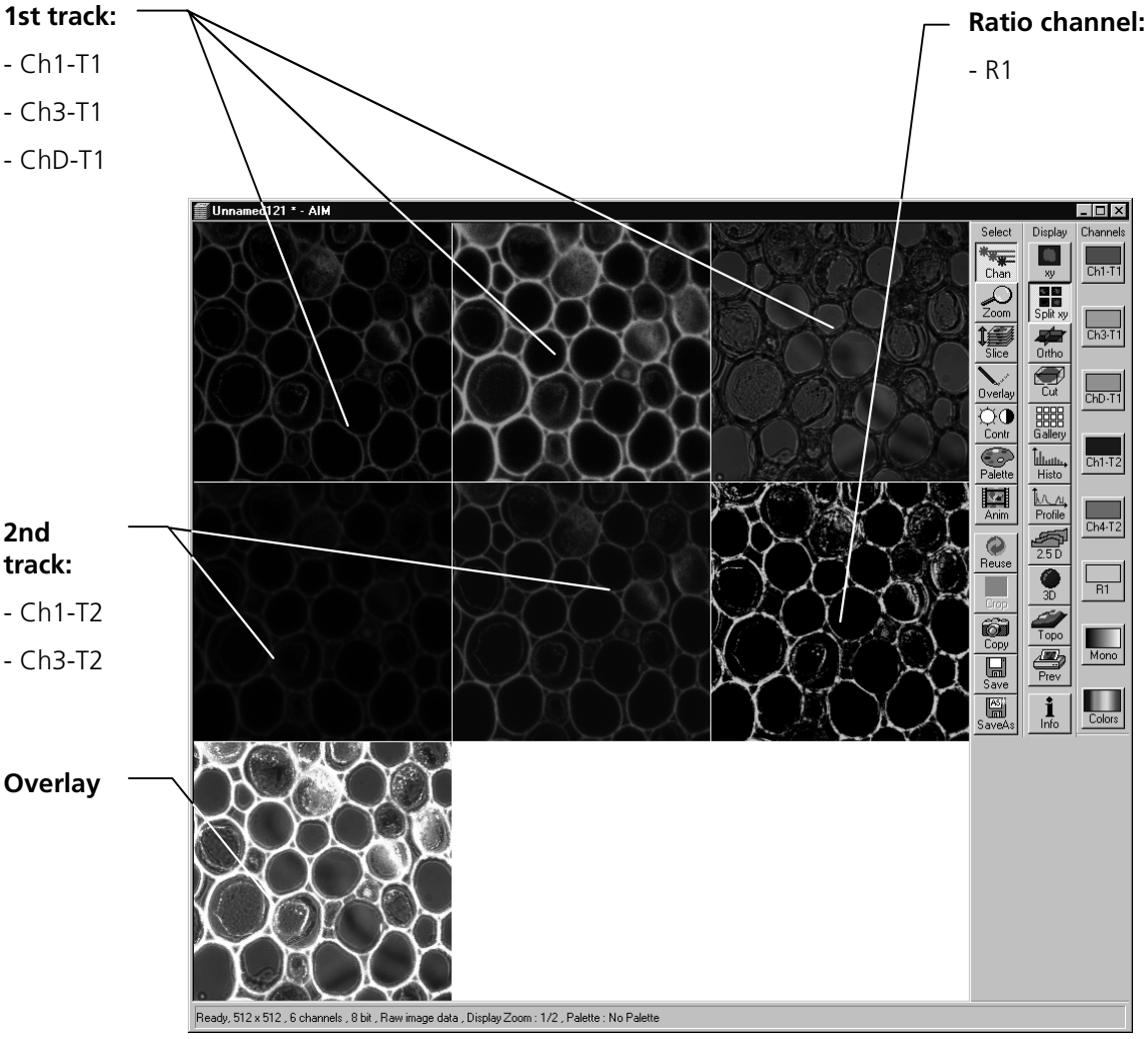


Fig. 5-63 Image Display window with two tracks plus ratio track (Split xy mode)

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

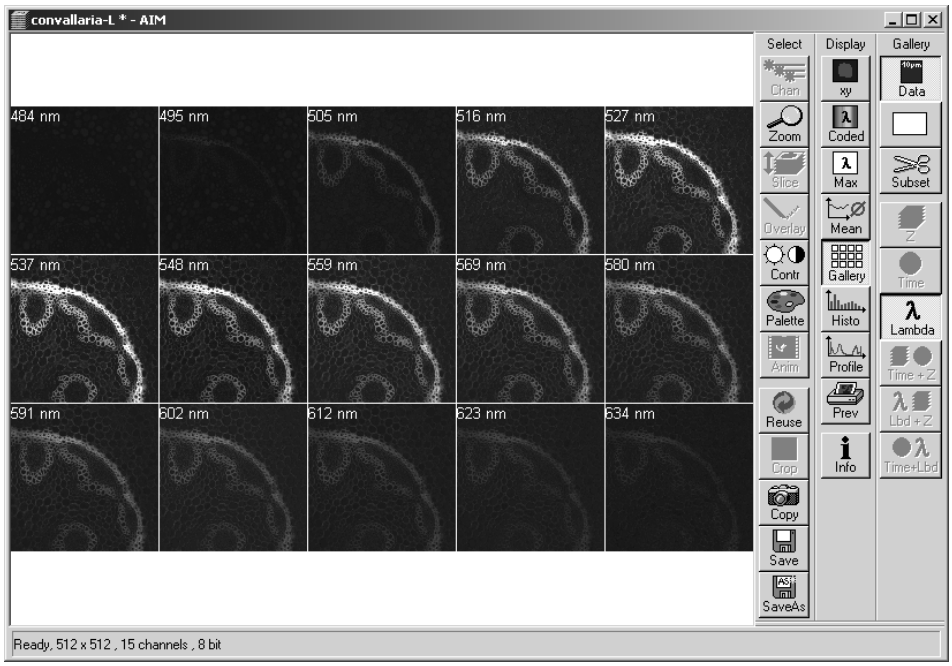


Fig. 5-64 Image Display window with a Lambda Stack

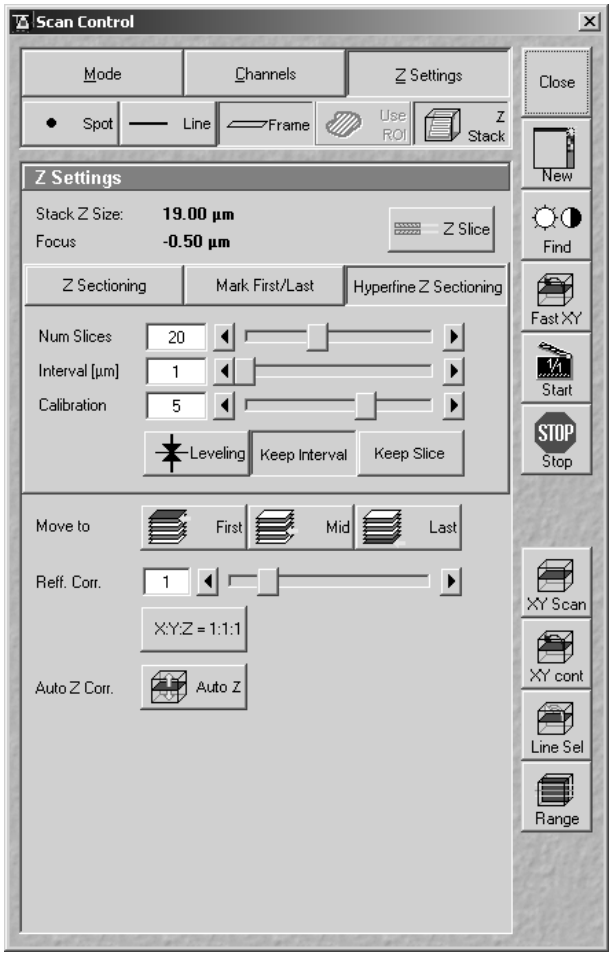


Fig. 5-65 Scan Control window - Z Settings

(3) Z Stack

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

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

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

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

Z Settings panel - overview

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

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

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

Z Slice: Opens the **Optical Slice** window.
The **Optical Slice** window contains two buttons (**Optimal Interval: ... µm** and **Optimal Pinhole Diameter**) to allow the setting of the optimum interval and the optimum pinhole diameter of fluorescence stacks. Both values influence each other and depend on the objective used.

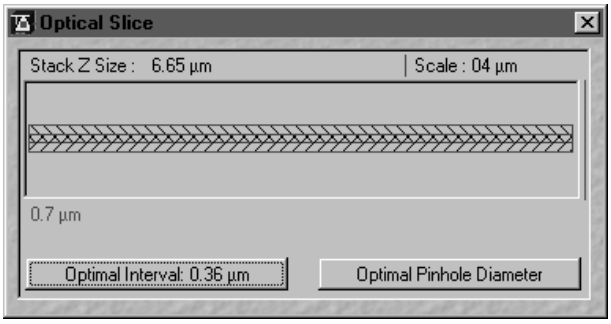


Fig. 5-66 Optical Slice window

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

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

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

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

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

Tabs

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

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

Hyperfine Z Sectioning: Tab for production of a Z Stack using the optional HRZ 200 fine focusing stage.

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

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

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

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

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

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

Auto Z Corr. This function permits the set values of the scan parameters **Detector Gain**, **AOTF**, **Ampl. Offset** and **Ampl. Gain** (as measure for the brightness level) to be varied between two freely selectable slices of a stack to be recorded. During the scan procedure, the interim values of these three parameters are automatically linearly interpolated between the initial and end values (see page 5-98).

The parameters of a Z Stack can be defined using the **Z Sectioning** tab, the **Mark First/Last** tab or - if the optional HRZ 200 fine focusing stage is connected - the **Hyperfine Z Sectioning** tab:

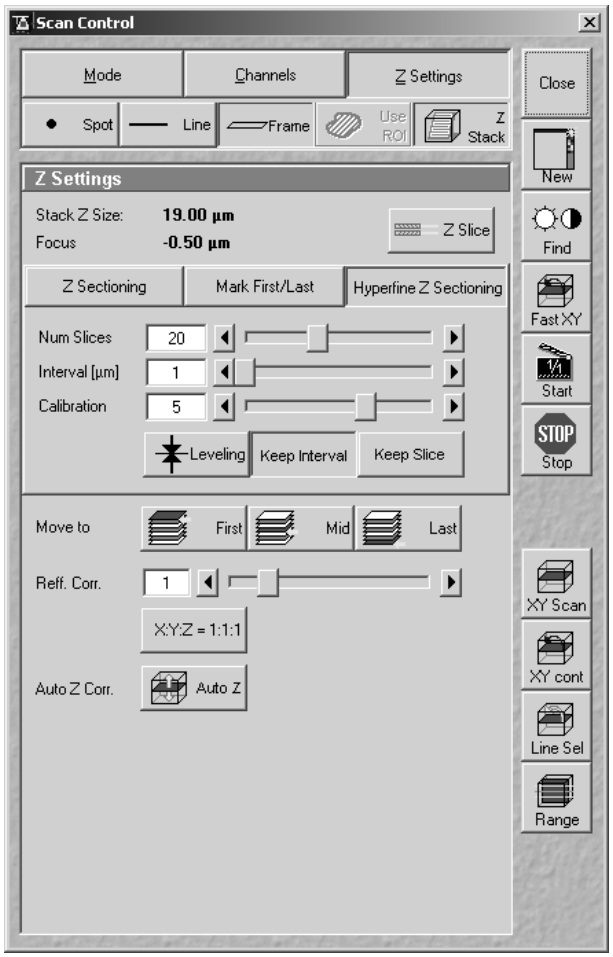


Fig. 5-67 Scan Control window - Z Sectioning tab activated

Z Sectioning tab

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

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

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

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

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

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

The optimum stack size is determined with the help of the **Line Sel** and **Range** functions:

- Click on the **Line Sel** button.
  - An XY-scan of the current slice is performed. The cutline is displayed in the image center. The **Line** toolbar is displayed on the right-hand side of the **Image Display** window.

The **Line** toolbar permits you to define the position, shape, width and color of the cutline in the **Image Display** window.

The following function buttons are available:

Arrow selection button: Activates the mouse pointer for the selection and positioning of the cutline in the **Image Display** window and for changing its length.
Length change: Click on the drag point and keep the mouse button pressed. Drag the point and release the mouse button.
Shifting: Click on the line and keep the mouse button pressed. Shift the complete line and release the mouse button.

Line arrow button: Generation of a straight cutline in any direction in the **Image Display** window.

Opened free shape curve button: Generation of an open, free shape curve (spline) in the **Image Display** window. The first click sets the starting point, each further click adds a line segment. A click with the right mouse button ends the process.

Line button: Selecting the line width of the cutline.

Color button: Selecting the color of the cutline.

Click on the **Line arrow** button or the **Opened free shape curve** button in the **Line** toolbar.

- Define a straight line or a free shape curve (spline) as the cutline for the XZ scan.

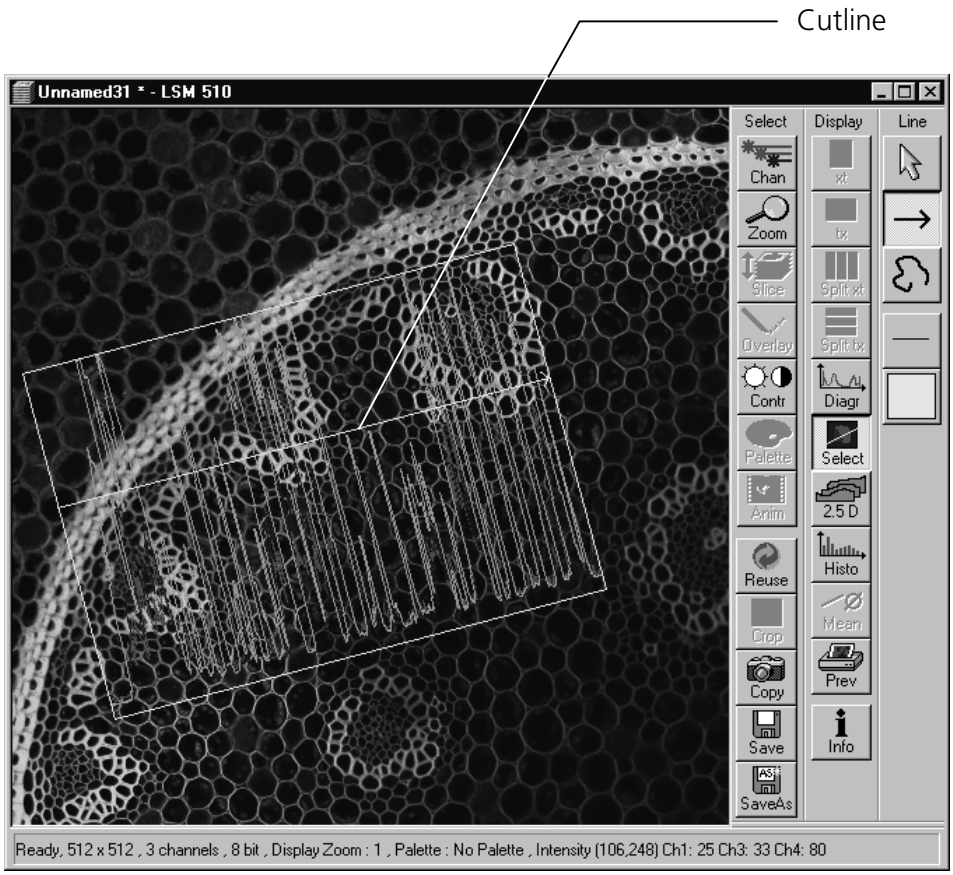


Fig. 5-68 Image Display window with cutline displayed

- Then click on the **Range** button.
  - The XZ-scan will be performed and displayed in the **Image Display** window. At the same time, the position of the current slice is shown with a green line and the positions of the first and last slice with two red lines.

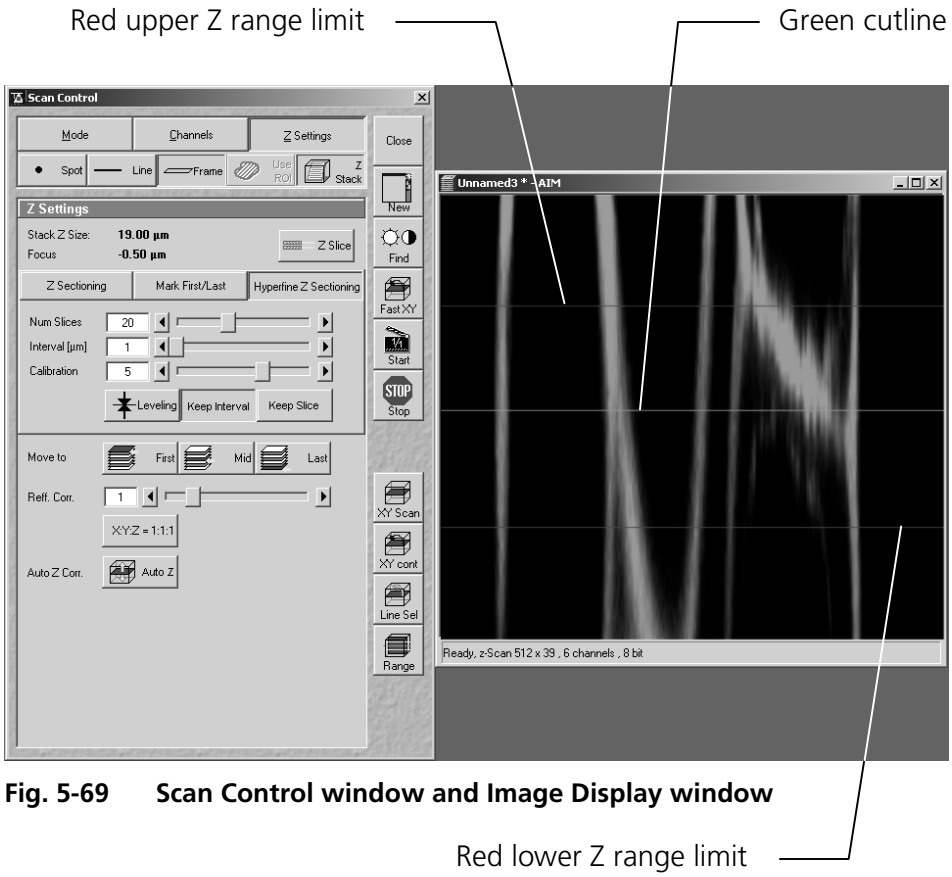


Fig. 5-69 Scan Control window and Image Display window

- Moving the green line (current slice) enables you to change the current focus position (moving the stage or nosepiece in the process). The stack limits are also changed, while interval and Num Slice remain unchanged.
- Shifting one of the red lines enables you to change the stack size; in that case, the interval size is matched, and the Num Slice remains constant.
  - Changing the values of **Num Slice**, **Interval** and **Current Slice** in the **Z Sectioning** tab will, of course, also change the positions of the red and green lines in the **Image Display** window.
- A click on the **Start** button will start the recording of the Z Stack.
  - The settings of the entire **Scan Control** window (**Mode**, **Channels, Z settings**) will be used when the stack is produced.

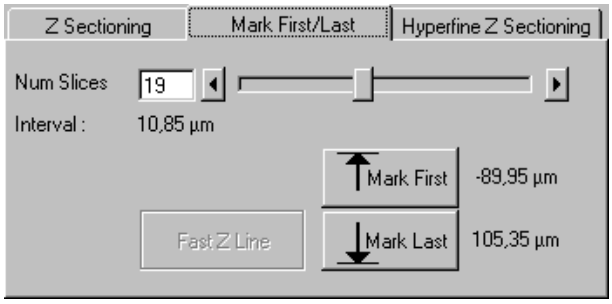


Fig. 5-70 Mark First/Last tab

Mark First/Last tab

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

- Click on the **XYcont** button.
  - A continuous XY-scan of the set focus position will be performed.
  - If you have reduced the scan speed or have set image averaging, you should use the fast scanning mode to find the lowest and highest points of focus. These settings are made under **Mode** in the **Scan Control** menu, or directly via the **FAST XY** button.
- Use the manual focusing drive or the **Stage and Focus Control** window (see **Stage**, page 5-134) to focus on the upper position of the specimen area where the Z Stack is to start.
- Click on the **Mark First** button to set the upper position of the Z Stack.
- Then focus on the lower specimen area where the recording of the Z Stack is to end.
- Click on the **Mark Last** button to set this lower position.
- The **Num Slices** slider enables you to set the number of slices. The limits of the Z Stack remain constant, the interval is matched accordingly.
- Click on the **Start** button to start the recording of the Z Stack.

In case the upper and lower limits of the stack have been switched round, automatic matching will be performed by the software, since the nosepiece of the Axiovert 200 M always moves from top to bottom.

☞ Setting via **Range** is not possible via the **Mark First/Last** function, i.e. the lines cannot be shifted.

The **Fast Z Line** functions is not available in frame mode.

When you change from **Mark First/Last** to **Z Sectioning** or vice versa, the values are updated in the **Z Sectioning** tab.

Hyperfine Z Sectioning tab

Activation of this tab is only possible if the HRZ 200 fine focusing stage or piezo objective focusing device has been connected.

The HRZ 200 or piezo objective focusing device can be controlled via software (see **Stage**, page 5-134).

The accuracy of the HRZ 200 or piezo objective focusing device regarding the step width in the Z-direction lies in the range of 10 nm.

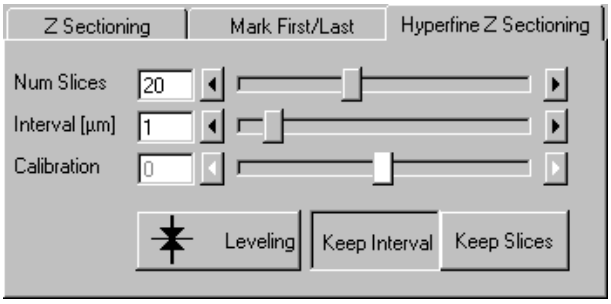


Fig. 5-71 Hyperfine Z Sectioning tab

The HRZ 200 or piezo objective focusing device allows stacks to be produced considerably quicker than via the focus of the microscope stand.

The focus position remains unchanged.

- Clicking on the additional **Leveling** button moves the HRZ 200 to the zero position, while the motor focus moves into the opposite direction at the same time, i.e. the position of the object in relation to the objective remains unchanged. This function is used to set defined initial conditions.
- The **Calibration** slider must normally be left in the default position 0. Calibration is required only if the examined image field is located clearly outside the center of the specimen carrier on the HRZ 200.

☞ Calibration is not required for the motorized stage. In that case, the **Calibration** function cannot even be activated (see Annex: Hints on the use of the HRZ 200 or piezo objective focusing device).

- Use the slider or the arrow keys to set the number of slices for the Z Stack.
- Use the slider or the arrow keys to set the size of the interval.

Num Slices and **Interval** can be varied independently of each other within the HRZ 200 work range of ±100 µm. When change is made to **Z Sectioning,** or vice versa, values are also taken over, provided they are within the HRZ 200 or piezo objective focusing device work range.

If a larger range is set for the Z Stack under **Z Sectioning** or **Mark First/Last**, the **Interval** is matched accordingly when changing to **Hyperfine Z Sectioning**, while **Num Slice** remains constant.

- Use **XYcont**, **Line Sel** and **Range** to determine the parameters of the Z Stack (identical to Z Sectioning).

If the green line (Current Slice) is shifted after the creation of **Range**, the focus position will change (the HRZ 200 or piezo objective focusing device remains in the center position). The red lines (stack limits) can only be changed symmetrically to the Current-Slice position within the HRZ 200 or piezo objective focusing device work range.

☞ Since the HRZ 200 moves from bottom to top during the creation of the Z Stack, top and bottom of the Axiovert 200 M have been switched round.

Auto Z Corr.

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

- Click on the **Auto Z** button, the **Auto Z Brightness Correction** window opens.

The buttons **Set A** and **Set B** permit definition of two distinct gain / offset / AOTF settings at two different Z positions A and B.

Pressing the **Move A** and Move **B** buttons permits the defined Z-position to be directly approached.

The **Enable test** check box permits simulation of the value changes for **Detector Gain**, **Ampl. Offset**, **Ampl. Gain** and **Attenuation** in the **Scan Control** window without the scanners being in operation.

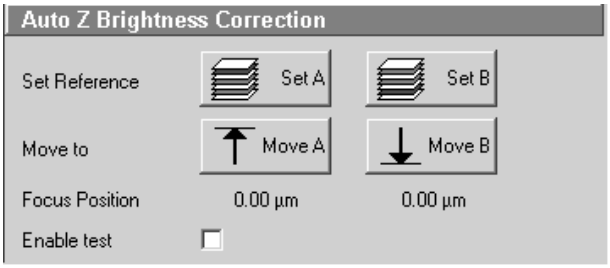


Fig. 5-72 Z Brightness level control window

☞ If a Z Stack is performed and the **Auto Z Brightness Correction** window is opened this correction is automatically performed equal whether the **Enable test** box is enabled or disabled.

- Use the focusing drive to set the Z-position where the brightness level correction is to be started.
- In the **Scan Control** window, set the initial values for **Detector Gain**, **Ampl. Offset** and **Ampl. Gain.** If required, start the continuous scan procedure for this purpose. Click on the **Set A** button.
- Use the focusing drive to set the Z-position where the brightness level correction is to be ended.
- Set the end value for **Detector Gain**, **Ampl. Offset** and **Ampl. Gain** in the **Scan Control** window**.** Click on the **Set B** button.
- If required, check the change of the set values by activating **Enable test.**

After the start of the scan procedure, the brightness level values are linearly interpolated between the defined references A and B.

☞ Note that the total Z range where the interpolation takes place can exceed the Z reference A and B.

Acquisition of a Z Stack

Once you have set up your image as defined in the above section, you can collect a series of confocal images through the different focal planes of your specimen.

- Click on the **Start** button on the **Scan Control** window. The system will automatically start the creation of a Z Stack. Be careful not to bump the air table or the microscope until Z sectioning is completed. Each successive Z Slice can be viewed by changing to the **Gallery** Mode. This button is located on the right-hand side of the image.

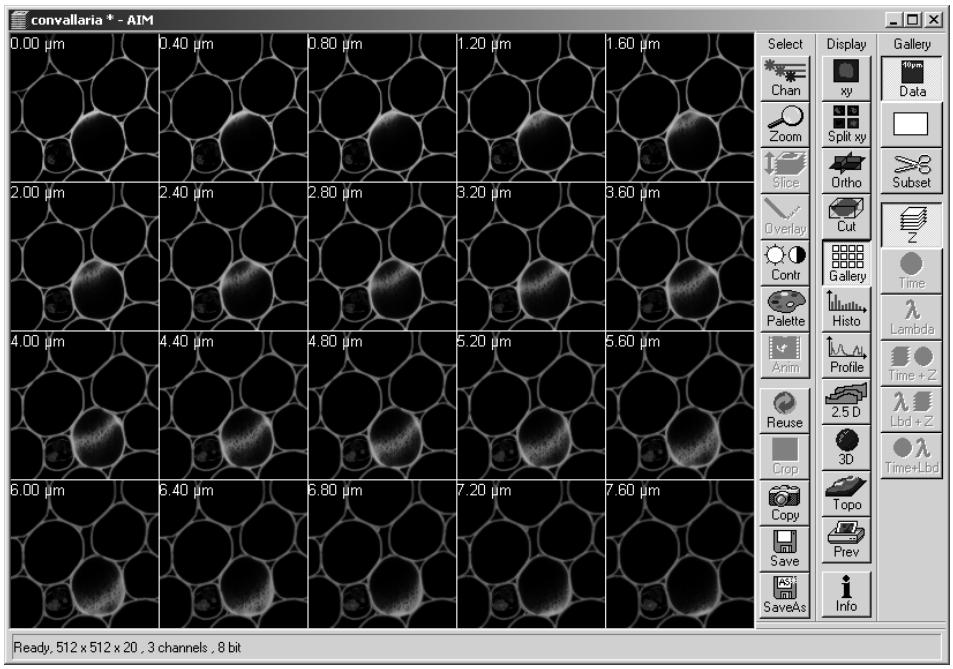


Fig. 5-73 Image Display window of a Z Stack

A black bar will be shown under the image and will move from left to right, showing that the LSM-FCS is in the process. The laser will automatically stop scanning when the Z Stack is completed.

The entire stack of images can be saved using the **Save** or **Save As** buttons on the right-hand side of the image.

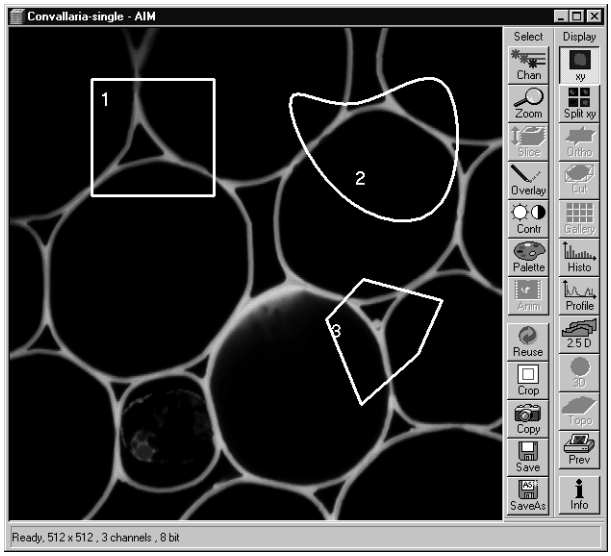


Fig. 5-74 Image Display window created via the Use ROI function

(4) Use ROI (Region Of Interest)

Performance of the **Frame** and **Z Stack** scan modes can be limited to one or several freely definable sections within the the **Image Display** window using the **Use ROI** function.

The laser scans the entire line length, but is limited in the Y-direction by the ROIs. The Scan Time is therefore reduced.

Definition and activation of the ROIs to be used is performed via the **Edit ROI** function (**Acquire** subordinate toolbar).

If no ROI has been activated, the **Use ROI** button is not available.

- Click on the **Edit ROI** button in the **Acquire** subordinate toolbar to open the **Edit ROI** window.
- Define one or several ROIs as required or select an existing ROI from the **ROI Lists** panel (see **Edit ROI**, page 5-108).
- The selected ROI is automatically activated when the **Edit ROI** window is closed with a click on **Close**.
- Click on the **Use ROI** button in the **Scan Control** window to perform the scan procedure in the defined ROI exclusively.

Only the regions of interest defined before are visible in the new scanning image, the other areas remain dark.

☞ The Scan Time is updated when ROIs are used.

- Clicking on the **Use ROI** button again will deactivate the function.

5.5.4.3 Line

In the **Line** mode, fluorescent or reflected light along a freely definable line is displayed in the form of an intensity profile.

All the possibilities of creating an image (Frame, Z Stack) are also available in the **Line** mode.

The **Line** and **Frame** buttons are activated alternately and exclude each other.

If the **Line** button has been selected, the **Line Sel** (selection) button also appears on the right-hand side of the **Scan Control** window. It permits positioning of the line to be scanned as required within the **Image Display** window (Frame in XY-plane).

- Set all the parameters for the Scan procedure (**Mode** and **Channels** or **Z Settings**) in the same way as for the scanning of a frame or a Z Stack.
- Then click on the **Line Sel** button.
  - A frame will be scanned and the currently selected scan line and its intensity profile will be displayed. The **Line** toolbar is displayed on the right-hand side of the **Image Display** window.

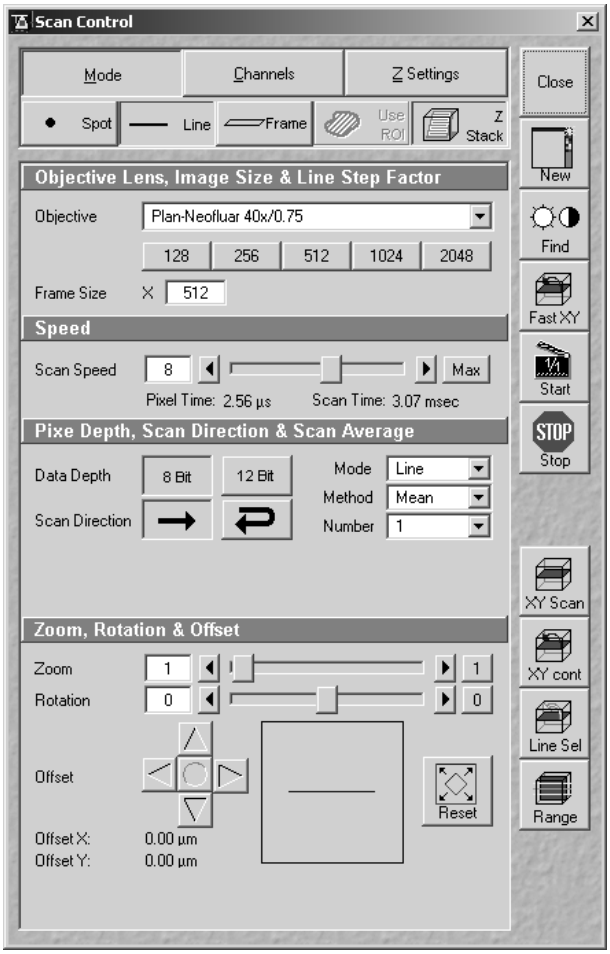


Fig. 5-75 Scan Control window - Mode/Line

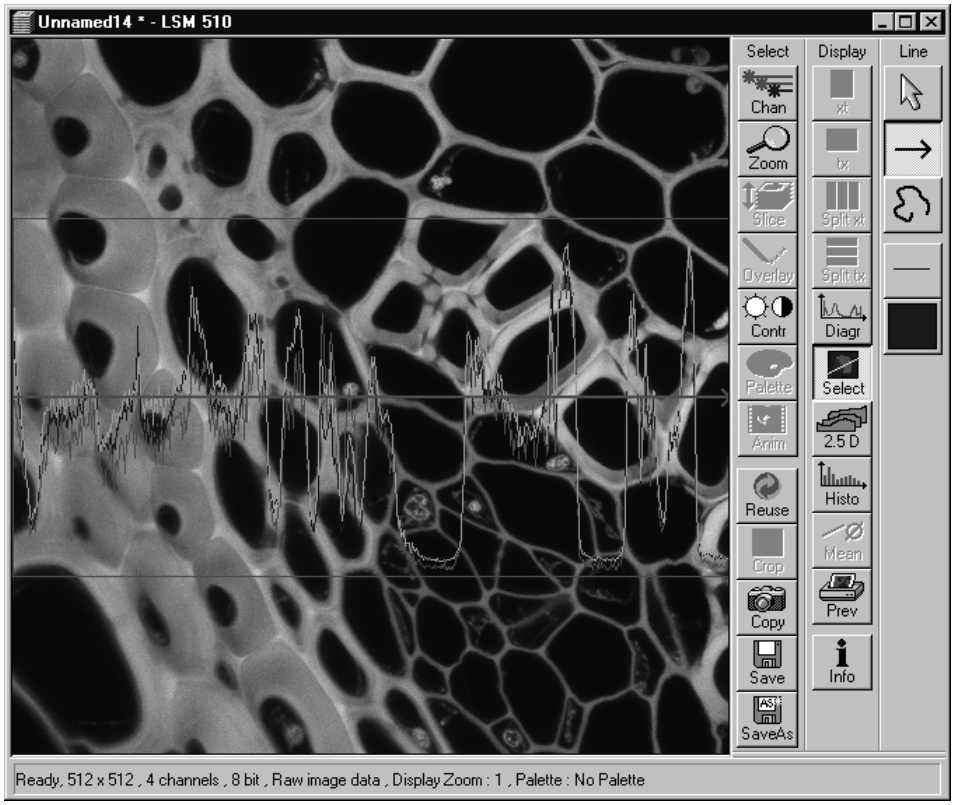


Fig. 5-76 Image Display window after activation of the Line Sel button

The **Line** toolbar permits you to define the position, shape, width and color of the scan line in the **Image Display** window.

The scan line can be defined either as a straight line or a free shape curve (spline).

The following function buttons are available in the **Line** toolbar:

Arrow selection button: Activates the mouse pointer for the selection and positioning of the scan line in the **Image Display** window and for changing its length.
Length change: Click on the drag point and keep the mouse button pressed. Drag the point and release the mouse button.
Shifting: Click on the line and keep the mouse button pressed. Shift the complete line and release the mouse button.

Line arrow button: Generation of a straight scan line in any direction in the **Image Display** window.

Opened free shape curve button: Generation of an open, free shape curve (spline) in the **Image Display** window. The first click sets the starting point, each further click adds a line segment. A click with the right mouse button ends the process.

Line button: Selecting the line width of the scan line.

Color button: Selecting the color of the scan line.

(1) Defining a straight line as the scan line

- Activate the **Line arrow** button of the **Line** toolbar. Click on the spot in the frame at which the line is to start and keep the mouse button pressed.
- Then drag the line to its desired end position and let go off the mouse button again.

The position of the line in the image can be changed as follows:

- Activate the **Arrow selection** button. To change the position in the X/Y-direction, click on the line and keep the mouse button pressed.
- Then move the lines to the desired position and let go off the mouse button again.
- To change the rotation direction or the length of the line, click on the start or end point of the line and keep the mouse button pressed.
- Change the rotation direction and / or the length of the line as required and let go off the mouse button again.

The intensity profile for the defined line is displayed on-line.

After release of the mouse button, the relevant intensity profile along the drawn line will be displayed. In the **Zoom, Rotation & Offset** panel, the current, changed angle and the offset in X and Y are displayed.

- When the **Line Sel** button is pressed again, a frame will be scanned in such a way that the selected line lies exactly in the center of the Y-axis again and is parallel to the X-axis.

☞ The position of the Line (rotation and offset) can also be changed directly in the **Zoom, Rotation & Offset** panel of the **Scan Control** window.

In the **Line** mode, Line Stacks can also be recorded over a defined period of time (see **Time Series**, page 5-114).

Line Scan is only possible in the unidirectional mode.

(2) Defining a free shape curve (spline) as the scan line

- Activate the **Free shape curve** button of the **Line** toolbar.
- Draw the your shape curve (spline) in the **Image Display** window using the mouse. The first click sets the starting point, each further click adds a line segment. A click with the right mouse button ends the line definition.

The scanner represented by a white line immediately begins with the on-line tracing of the defined free shape curve. The laser excitation remains inactive in this process.

If the defined free shape curve becomes too complicated or the selected **Scan Speed** is too high, the following message appears in the status bar of the **Image Display** windows:

Maximum scanner speed exceeded!

- In this case, reduce the **Scan Speed** set in the **Scan Control** window.
- If the generated contour and the line traced by the scanner are not in coincidence, reduce the **Scan Speed** by a further amount.

If no sufficient coincidence of the two lines can be achieved by the reduction of the scan speed, you have to calibrate the scanner position signal.

- Click on the **Maintain** button in the **Main** menu and then on the **Spline** button.
  - The **Calibrate Spline Scan** window is opened.
- Bring the generated contour and the scanner line to coincidence by varying the amplitude or offset values for X and Y.
- If necessary, match the free shape curve to the scanner line.
- Then click on the **Single** or **Cont.** button to execute the scan process, with the laser activated.

A **Line** scan is performed along the defined freehand shape curve, and the intensity profile is displayed at the bottom of the **Image Display** window.

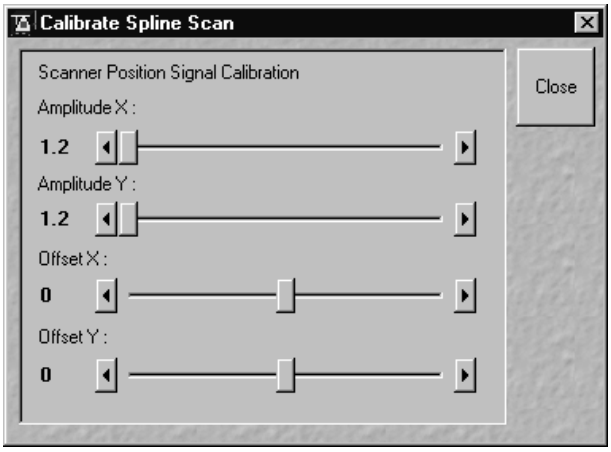


Fig. 5-77 Calibrate Spline Scan

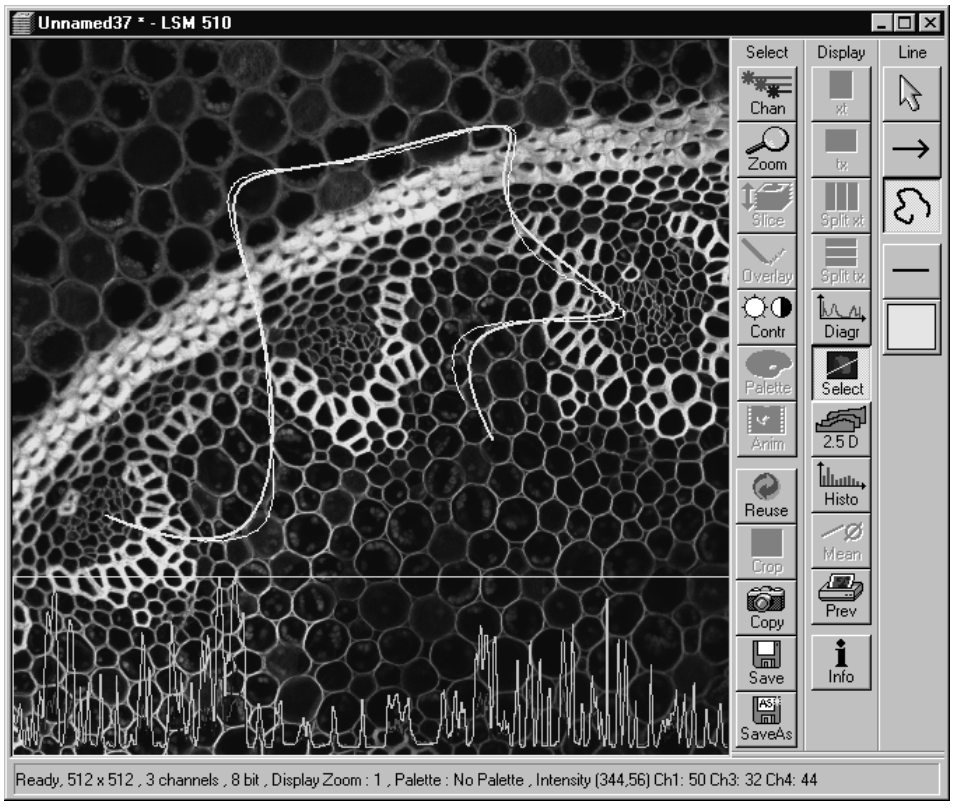


Fig. 5-78 Image Display window after definition of a freehand shape curve for the line scan process

Irrespective of the length of the defined freehand shape curve, the intensity profile is always calculated (by interpolation) and displayed in accordance with the pixel value set under **Line Length** (2048 pixels maximum).

- Click on the **Stop** button to terminate the scan procedure.

☞ As soon as the free shape curve is modified, the laser excitation is deactivated and the scanner again starts to trace the newly generated free shape curve.

(3) Selecting the width and color of the scan line

- Line color and width can be set via the **Line** and **Color** buttons of the **Line** toolbar.

(4) Line Stack

The intensity profile of a defined straight line or free shape curve can also be recorded as a Z Stack. To do this, proceed in the same way as for the Frame Stack.

5.5.4.4 Spot

In the **Spot** mode fluorescent or reflected light occurring from a single voxel xyz is detected. In this mode a spot can be defined by two perpendicular lines in the **Image Display** window.

In the spot mode the Z Stack button is not available. After definition of the spot position the only possible scan mode is a time series of a spot.

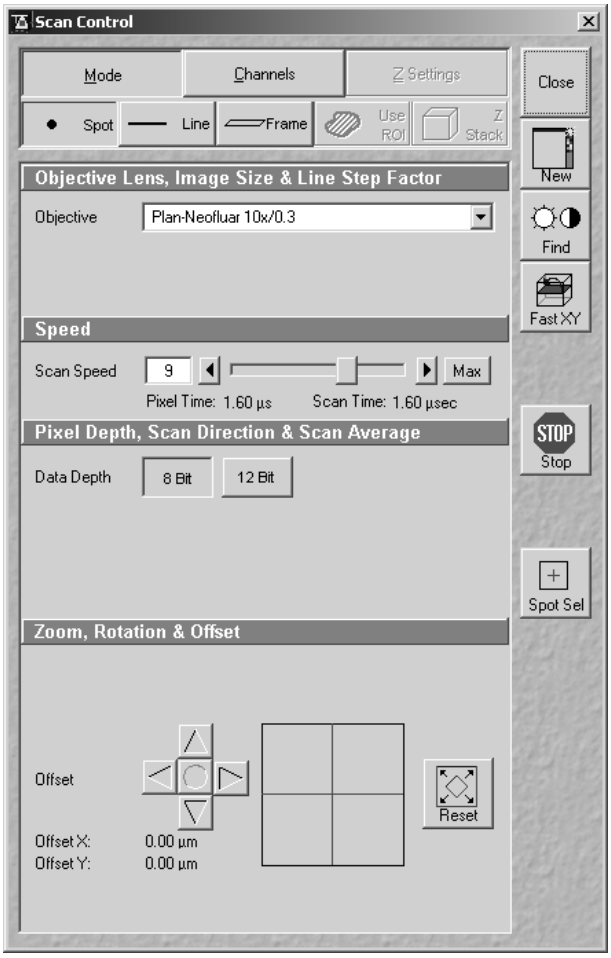


Fig. 5-79 Scan Control window - Mode/Spot

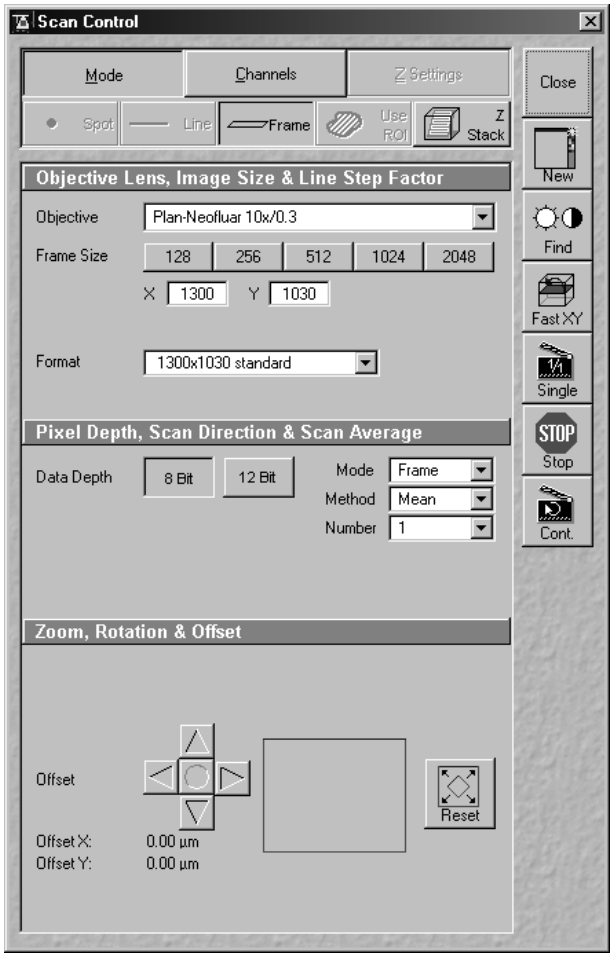


Fig. 5-80 Scan Control window - Mode, settings for camera control

5.5.4.5 Camera control

The use of this function permits the control of the external CCD-camera settings.

(1) Open / Close the Scan control window for camera control

- In the **Configuration Control** window, activate the **Camera** button.
- Click on the **Scan** button in the **Acquire** subordinate toolbar of the main menu.
- Click on the **Close** button

(2) Function description

Mode button	Displays the selected objective, frame size and pixel depth.
Frame Size	Selects between square formats or free defined frame sizes.
Format	Selects between a range of default camera resolutions. The 5x5 binning mode can be used for focusing in realtime.
Data Depth	Sets the pixel depth.
Zoom/Offset	Shifts a subregion in the frame.
Reset	Resets the frame/subregion to default value selected in **Format**.

Channels button — Displays the activated channels and possible settings.

Exposure time — Sets the exposure time of the camera.

Find — Starts a prescan and sets the exposure time automatically.
In case of a camera multitracking, only one channel should be selected in Configuration Control in order to speed up the find function.

Fast X/Y — Starts a fast online scan mode, e.g. for focusing. Also, the 5x5 binning mode can be used (to be set in **Mode** / **Format**).

Single — Starts a single image acquisition (The **Image Display** window appears.).

Continuous — Starts acquisition of a series of images (The **Image Display** window appears.).

Crop — Defines a ROI for camera acquisition in the **Image Display** window. Note that this is just a **Crop** function, while the whole sample is illuminated. Rotation of the ROI is not possible.

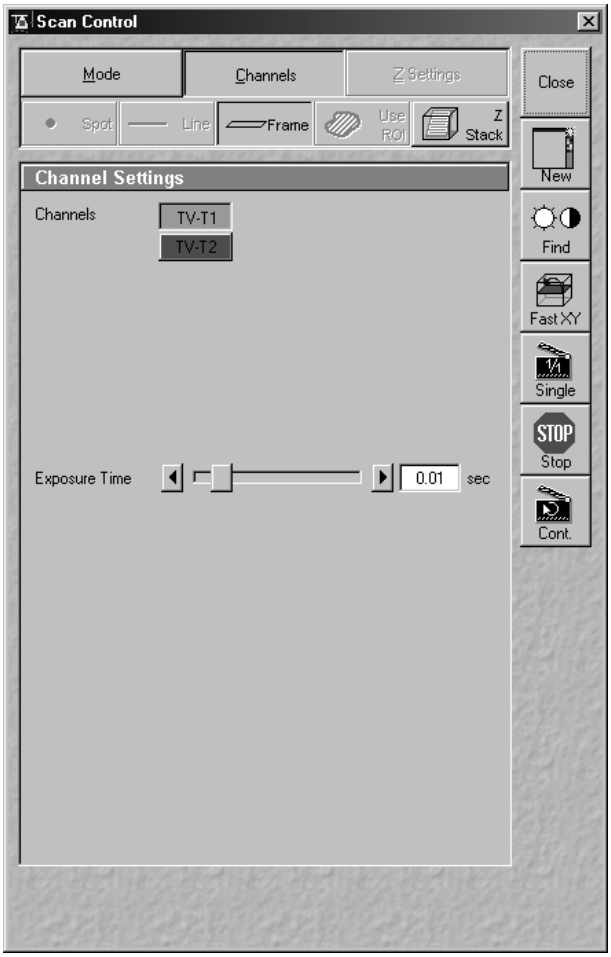


Fig. 5-81 **Scan Control window - Channels, settings for camera control**

Info button — Shows the acquisition parameters in the **Image Display** window.

Close — Close the **Scan Control** window.

5.5.5 Edit ROI (Region Of Interest)

A scan image allows certain areas (ROIs) to be defined. Only these areas of interest will be scanned. The laser beam will be switched on only in these areas via Acousto-Optical Tunable Filters (AOTF). Definition and activation of the ROIs for the scan procedure is performed in the **Edit ROI** window.

5.5.5.1 Open / Close the Edit ROI window

- Click on the **Edit ROI** button in the **Acquire** subordinate toolbar of the **Main** menu. The **Edit ROI** window appears on the screen and the ROIs defined last are visible in the **Image Display** window.
- Click on the **Close** button in the **Edit ROI** window. The **Edit ROI** window is closed and the ROIs disappear from the **Image Display** window.

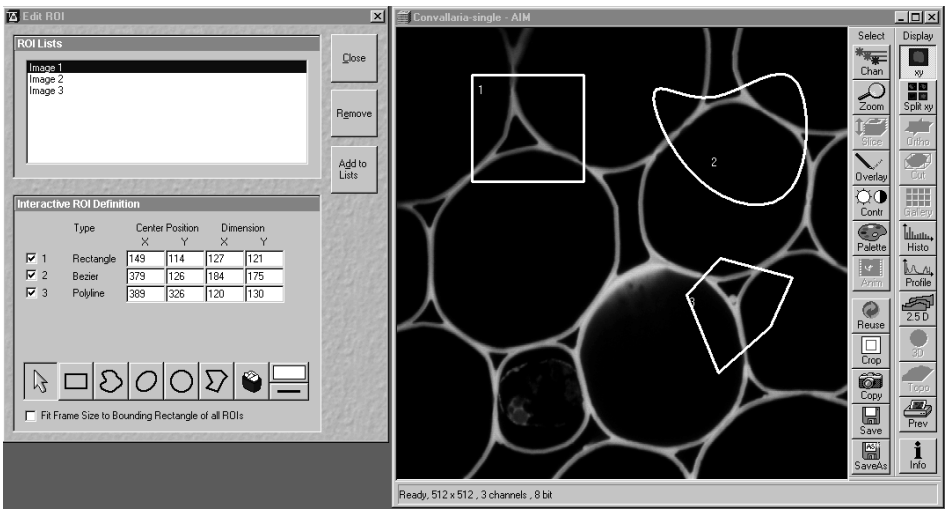


Fig. 5-82 Edit ROI window and Image Display window with ROIs

The **Use ROI** status display (button) in the **Scan Control** window shows whether the ROI mode is activated or not. If ROIs shall not be taken in consideration during scanning, the **Use ROI** button must be deactivated prior to the scanning procedure.

When **Edit ROI** is activated and the first ROI is drawn in the **Image Display** window, the **Use ROI** is activated automatically.

5.5.5.2 Function description

The following functions are available on the right side of the **Edit ROI** window:

Close button	The **Edit ROI** window is closed.
Remove button	An entry marked in **ROI Lists** (stored ROI configuration) is deleted.
Add to Lists button	The **Add ROI List** window is opened.

(1) ROI Lists panel

In the ROI Lists panel, all the currently defined and stored ROI configurations are shown.

- Click on the ROI configuration which you want to use for the scan procedure.
  - The selected ROI configuration is highlighted in blue and displayed in the opened **Image Display** window.
- To produce a new ROI configuration, an already stored configuration can be activated, changed and stored under a new name using the **Add to List** button.
- To delete a stored ROI configuration from the list, click on its name first (highlighted in blue) and then on the **Remove** button.

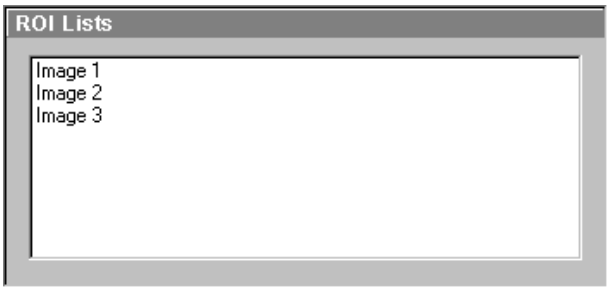


Fig. 5-83 ROI Lists panel

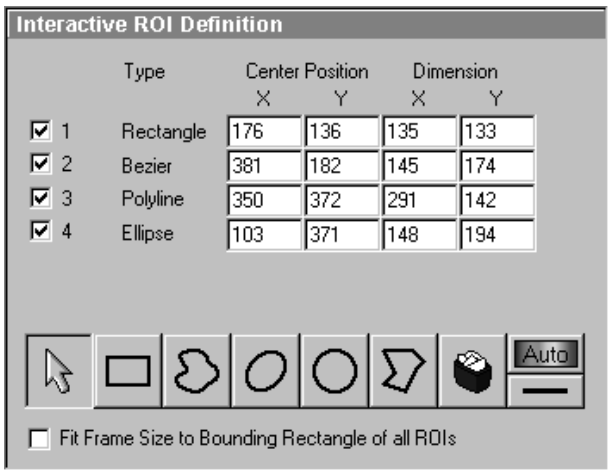


Fig. 5-84 Interactive ROI Definition panel

(2) Interactive ROI Definition panel

In the **Interactive ROI Definition** panel, the parameters of the ROI configuration just selected from the **ROI Lists** panel are displayed. Furthermore, it contains all the functions required for the creation of ROIs.

The X and Y values for **Center Position** and **Dimension** can be edited.

- Activate the relevant text box with a mouse click and enter the new value via the keyboard.
- If you click outside the edited text box, the new value will be taken over and the ROI figure be shifted to the new position.

The upper part of the panel gives an overview of all the individual figures stored under the selected name according to type, position within the **Image Display** window (in pixels) and greatest dimension in X and Y (in pixels). The origin of the position indication lies in the left top corner of the **Image Display** window.

☑ Check box (e.g.: 1 - 4): Clicking on this check box allows a ROI to be deactivated. The tick disappears from the check box, as does the relevant marked area from the scanning image. Clicking on the check box again will reactivate the ROI.

Arrow button: Activation of the mouse button to change the size or move the ROIs in the **Image Display** window.

Rectangle button: Draw of a rectangle in the **Image Display** window; click and keep mouse button pressed, drag the rectangle in any direction, let go off the mouse button to end the procedure.

Bezier button: Draw of a bezier figure in the **Image Display** window; first click sets the starting point, each additional click adds a line, double-click on the starting point closes the figure and ends the procedure.

Ellipse button: Draw of an ellipse in the **Image Display** window; first click sets the center point, displayed line permits determination of the extension, second click sets the first dimension, then the second dimension and the rotation direction can be determined, third click sets the second dimension and direction and ends the procedure.

Circle button: Draw of a circle in the **Image Display** window; click and keep the mouse button pressed to set the center point, drag the diameter, let go off mouse button again to end the procedure.

Polyline button: Draw of a polyline figure in the **Image Display** window; first click sets the starting point, each further click adds a line, double-click on the starting point closes the figure and ends the procedure.

Recycle bin button: All the ROIs dragged to the scanning image are deleted. If an area outline was marked before, this area is now deleted in the scanning image.

Auto / Color button: A defined color from the list of colors can be assigned to the ROIs. In that case, the same color is assigned to all the individual figures. In the **Auto** position, the outlines of the dragged ROIs are automatically colored differently.

Line button: This button allows you to determine the line thickness of the area outline. This is for display purposes only. The scanned line is not effected.

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

- In the toolbar of the **Interactive ROI Definition** panel, click on the symbol of the area you want to use to mark the region of interest in the scanning image. Five different area symbols are available in the form of buttons.
- Click on the marking area and keep the mouse button pressed to drag the area into the region of interest in the scanning image. The marking area will be numbered automatically and entered in the **Interactive ROI Definition** panel with its position and dimension parameters and the appropriate number.
- The dragged marking area is marked by clicking on its outline; its size can be changed by clicking on the marking points. Clicking on the area edge beside the marking points allows repositioning of the area on the scanning image.

☞ The digits of the ROIs can be shifted independently of the contours of the figure.

- If you have framed all the required ROIs in accordance with steps 2 to 4, you can store these ROIs under any required name via the **Add to Lists** button.
- The **Add ROI List** window will appear. Enter any required name to store the ROIs and click on the **OK** button.
- This stored ROI configuration appears in the **ROI Lists** panel of the **Edit ROI** window.

Fig. 5-85 Add ROI List window

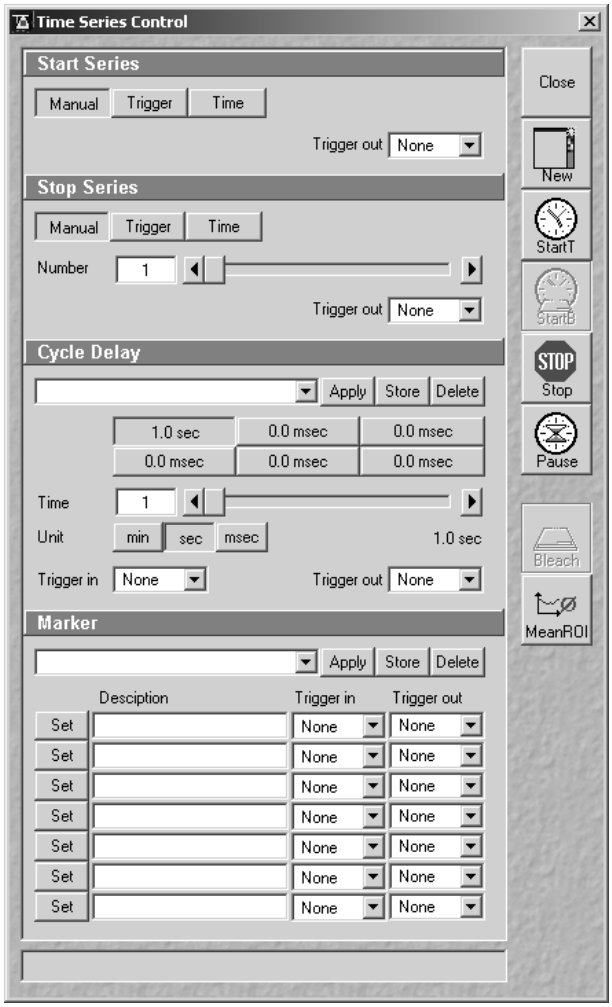


Fig. 5-86 Time Series Control window

5.5.6 Time Series

The **Time Series Control** window allows the definition of parameters for time series.

The **Time Series** function offers the following options for the creation of image series:

- Definition of break times between 0.1 ms and 10 hours.
- Determination of the number of steps from 1 to 10,000 for one scanning procedure.
- Setting of markers.
- Interruption of time control via pause function, and resume of the time series function.
- Triggering of time series via:
  - numeric input
  - external trigger pulses
  - time (of the PC)

5.5.6.1 Open / Close the Time Series Control window

- Click on the **Time Series** button in the **Acquire** subordinate toolbar of the **Main** menu.

The **Time Series Control** window appears on the screen.

- Click on the **Close** button to close the **Time Series Control** window.

5.5.6.2 Function description

The following functions are available on the right-hand side of the **Time Series Control** window:

Close button	Closes the **Time Series Control** window.
New button	Opens a new **Image Display** window.
Start T button	Starts the Time Series.
Start B button	Starts the Time Series in combination with a bleach procedure. Bleach procedure must be defined first in the **Bleach Control** window.
Stop button	Stops the entire Time Series. A current scan is interrupted.
Pause button	Interrupts the Time Series. Button labeling is changed to **Resume**. A current scan is performed until the end. When the button is pressed again, the Time Series is immediately continued with the next scan procedure.
Bleach button	Starts a Bleach procedure without a Time Series. Bleach procedure must be defined first in the **Bleach Control** window.
Mean ROI button	Creates a Time Series with the intensity values of the Frame or the default ROIs. An average value is formed of the intensity values of the Frame or the ROIs determined in the relevant scan procedure. These average values are displayed in an extended **Image Display** window as a function of the time which has passed.

The status line, in which the phases of the current Time Series or notes for the user are displayed, is in the lower part of the **Time Series Control** window.

(1) Start Series panel

In this panel, the parameters for the start of the time series are set.

Fig. 5-87 Start Series panel

The following functions are available:

Manual button	The time series is started manually with a click on the **Start T** or **Start B** button.
Trigger button	The time series is started via a trigger signal from Trigger Control.
Time button	The time series is started when the set time is reached. The internal computer time applies.

Time input box — Input of the time for the start of the time series (**Time** button activated).

Trigger in list box — Selection of the trigger key (1-4) with which the start is to be triggered (**Trigger** button activated).

Trigger out list box — Selection of the trigger keys (1-4) for the out signal.

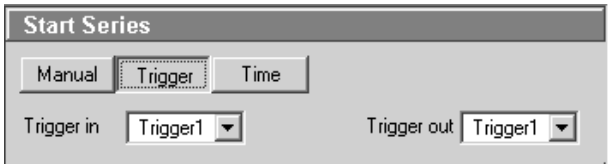


Fig. 5-88 Start Series panel

(a) Start via Trigger

For the start via trigger control (**Trigger** button activated), first determine the trigger key which is to trigger the start of the Time Series.

- Open the **Trigger in** list box with a click on the arrow button.
- Choose one of the trigger keys 1 to 4 (e.g. **Trigger1**).

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

- Open the **Trigger out** list box with a click on the arrow button.
- Choose one of the trigger keys 1 to 4 (e.g. **Trigger1**).

In this example, the scan procedure is triggered on pressing key **1** of the trigger control, and an out signal is given at the same time.

☞ When starting a Time Series via Trigger, the **Start T** or **Start B** button must be pressed first. **Waiting for Trigger** will then be displayed in the status line.

Then the relevant trigger key on the Trigger Control must be pressed to start the first scan procedure of the Time Series.

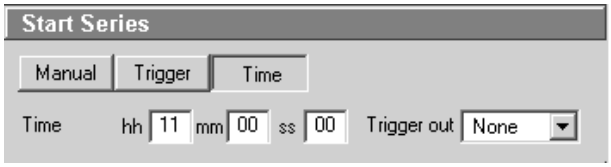


Fig. 5-89 Start Series panel

(b) Start via Time

For the start via the time set on the PC (**Time** button activated), the start time must be entered first in the **Time** input box.

- Click in the **Time** input box to open it.
- Enter a start time via the keyboard. Then click outside the input box once to close it again.

☞ When starting a Time Series via the time, the **Start T** or **Start B** button must also be pressed in this case. **Waiting for Start Time** will be displayed in the status line.

The Time Series is started when the starting time has been reached.

The starting time for the Time Series can be changed online.

(2) Stop Series panel

In this panel, the parameters for the end of the Time Series are set and the number of cycles is determined.

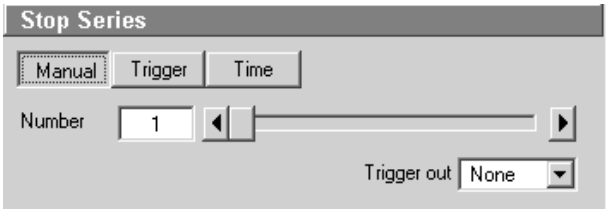


Fig. 5-90 Stop Series panel

The following functions are available:

Manual button	The time series is finished manually with a click on the **Stop** button.
Trigger button	The time series is finished via a trigger signal.
Time button	The time series is finished when the set time has been reached. The internal computer time applies as the set time.
Number input box / arrow keys / slider	Determination of the number of images acquired or image stacks for the time series.
Time input box	Input of the time for the end of the time series (**Time** button activated).
Trigger in list box	Selection of the trigger keys (1-4) with which the end is to be triggered (**Trigger** button activated).
Trigger out list box	Selection of the trigger keys (1-4) for the out signal.

- Use the slider near **Number** to select the images or image stacks for the time series.

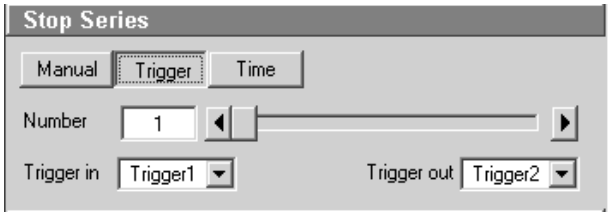


Fig. 5-91 Stop Series panel

(a) Stop via Trigger

To end the Time Series via Trigger Control (**Trigger** button activated), first determine the trigger key which is to end the Time Series.

- Open the **Trigger in** list box with a click on the arrow button.
- Choose one of the trigger keys 1 to 4 (e.g. **Trigger2**).

It is also possible to trigger an out signal via Trigger Control.

- Open the **Trigger out** list box with a click on the arrow button.
- Choose one of the trigger keys 1 to 4 (e.g. **Trigger2**).

In this example, the Time Series is ended on pressing key **2** of the Trigger Control, and an out signal is given at the same time.

☞ If the entered number of cycles has been processed without a trigger impulse having been given to end the procedure, the Time Series is finished.

If a trigger signal is given before the cycles have been processed, the Time Series will only be interrupted. **Waiting for Trigger** will be displayed in the status line. The Time Series can now be continued via a new trigger signal or ended via **Stop**.

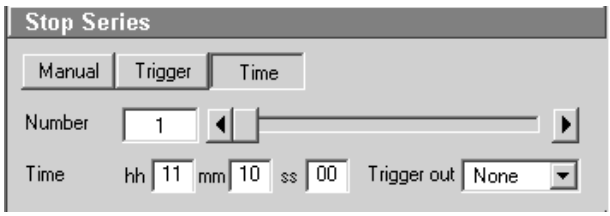


Fig. 5-92 Stop Series panel

(b) Stop via Time

To end the Time Series via the time set on the PC (**Time** button activated), the end time must first be entered in the **Time** input box.

- Click on the **Time** input box to open it.
- Enter the end time via the keyboard. Then click outside the input box once to close the box.

☞ The Time Series is interrupted when the end time has been reached.

If the entered **Number** of cycles has been processed, the Time Series is finished.

If the number of cycles has not yet been processed, the Time Series is only interrupted. **Waiting for Start Time** is displayed in the status line. The Time Series can now be continued by entering a new start time, or finished via **Stop**.

The end time for the Time Series can be changed online.

(3) Time Delay / Time Interval panels

Depending on the settings in the **Time Series** tab (see **Options** menu, **Settings**), the time series interval is defined either as a **Time Delay** or **Time Interval**. Accordingly, either the **Time Delay** panel or the **Time Interval** panel is displayed in the **Time Series Control** window.

Time Delay is the interval between the end of one scan process and the beginning of the next.

Time Interval is the interval between the beginning of one scan process and the beginning of the next.

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

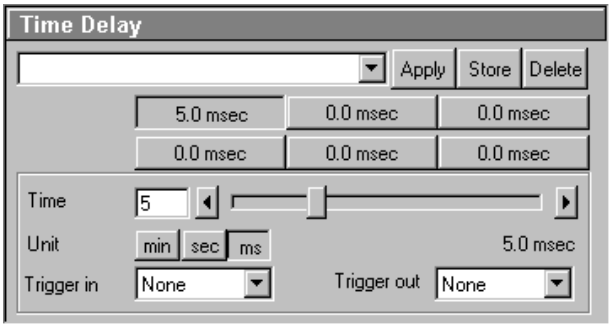


Fig. 5-93 Time Delay panel

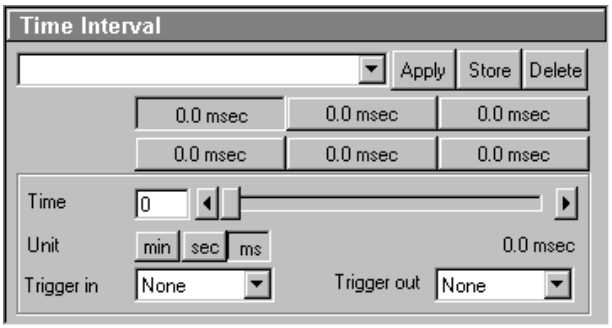


Fig. 5-94 Time Interval panel

The following functions are available:

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

- The delay time or time interval to be used during the Time Series is set to a default value by activating a **Time** button.

For this purpose, the relevant time must be assigned to the **Time** button first.

- Activate a **Time** button with a click of the mouse.
- Set the required delay time or time interval via the slider (arrow keys or input box) near **Time**. The set time is displayed online on the button. Select the time unit by clicking on the relevant button near **Unit**.

You can assign different times to all the six **Time** buttons and store this assignment either as a set of delays or of time intervals.

- Enter a name in the **Time Delay** list box or **Time Interval** list box and click on **Store** to store the set of delays.

If required, a set of delays or time intervals can be activated again quickly.

- Open the list box with a click on the arrow button and select the required set with a click of the mouse.
- Then click on the **Apply** button to activate the set. The stored delays are assigned to the **Time** buttons.

Sets of delays or Sets of time intervals which are no longer required can be deleted.

- Open the list box and select the required set.
- Click on the **Delete** button. The set will be removed.

The **Time** buttons can also be activated via keys 1 to 4 of the Trigger Control.

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

It is also possible to trigger an out signal via Trigger Control.

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

In this example, the relevant **Time** button is activated on pressing key **3** of the Trigger Control, and an out signal is given at the same time.

☞ The delays or time intervals can be changed online with a click on another **Time** button. The new delay will be applied immediately.

A change of the delay during a Time Series is displayed in the **Image Display** window if the **Gallery** button (**Display** toolbar) is activated.

(4) Marker panel

The setting of a marker permits information about the moment in the current time series and any required comment to be assigned to the current scan. The time indication is set automatically, while comments must be defined before.

The markers (red squares) are visible in the **Image Display** window if the **Gallery** button (**Display** toolbar) is activated.

On storage of the image, all the markers, including the time indication and the comments, are stored along with the image contents.

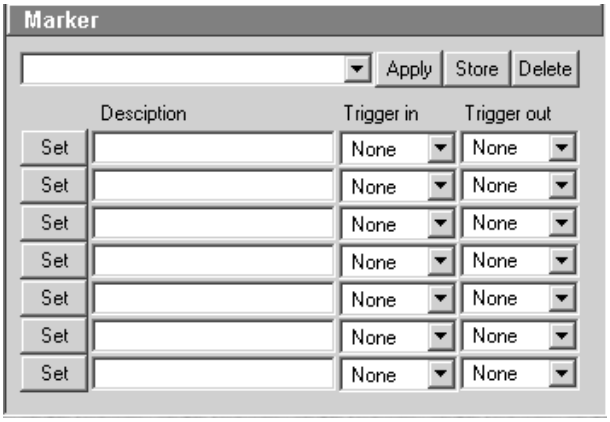


Fig. 5-95 Marker panel

The following functions are available:

Marker list box	List of the stored combinations of markers.
Apply button	Application of the marker combinations selected from the list box.
Store button	Storage of a combination of markers.
Delete button	Deletion of a combination of markers from the Marker list box.
Set 1-7 button	Setting of a marker during the scan procedure.
Edit Text input box (1-7)	Entry of the comments for the marker.
Trigger in list box (1-7)	Selection of the trigger key (1-4) with which the marker is to be set.
Trigger out list box (1-7)	Selection of the trigger key (1-4) for the out signal.

- A marker for the current scan is set by clicking on one of the **Set 1** to **7** marker buttons.

The assignment of any required comment for the marker must be performed as follows:

- Click in the **Edit Text** box of the required marker key (e.g.: **Set 1**) to open the editing box.
- Enter the comments via the keyboard. Then click outside the editing box to close this box again.

You can assign comments of any required length to all the seven **Set** buttons and store this assignment as a combination of marker keys.

- Enter a name in the Marker list box and click on **Store** to store the combination.

If required, a combination of markers can be activated again quickly.

- Open the Marker list box with a click on the arrow button and select the required combination with a click of the mouse.
- Then click on the **Apply** button to activate the combination. The relevant comments are displayed in the **Edit Text** boxes of the **Set** buttons.

Combinations which are no longer required can be deleted.

- Open the Marker list box and select the required combination.
- Click on the **Delete** button. The combination will be removed.

The marker buttons can also be activated via keys 1 to 4 of the Trigger Control.

- Click on the required **Set** button.
- Open the **Trigger in** list box with a click on the arrow button.
- Select one of the trigger keys 1 to 4 (e.g. **Trigger4**).

It is also possible to trigger an out signal via Trigger Control.

- Click on the required **Set** button.
- Open the **Trigger out** list box with a click on the arrow button.
- Select one of the trigger keys 1 to 4 (e.g. **Trigger4**).

In this example, the relevant **Set** button is activated on pressing key **4** of the Trigger Control, a marker is set in the Scan and an out signal given at the same time.

5.5.6.3 Time Series of a Frame

- Set the relevant parameters for time control in the **Start Series**, **End Series** and **Time Delay** panels.
- Start the Time Series with a click on the **Start T** or **Start B** button.
- If you use Trigger Control, confirm the relevant Trigger key to start the Time Series with the first scan procedure.
- Use the **Set 1** to **Set 7** buttons to set markers during the scanning procedure which will allow you to evaluate interesting scanning images later.

☞ Time end will finish time series even if you have created a program which would exceed the time end.

Bleach times will be added.

No break is possible during bleaching.

If you want to integrate a bleaching procedure in a time series, start must be triggered via **Start B**. The bleaching procedure must be defined first in the **Bleach Control** window (see page 5-129).

If a time series is interrupted before its programmed end, the programmed number of images will be taken over in the database. However, only those images are stored which were created before interruption of the time series. This is due to the fact that the original image parameters are to be taken over via the **Reuse** function.

If a stop time for time series is entered via the **Trigger** button or the **Time** button, the recording of the series will not be definitely finished. It is possible to either continue the series via new settings of **Trigger** and **Time** or to definitely finish the time series via the **Stop** key.

The following example of a scanning image was taken using the **Time Series** function. Both the time and the markers set during the scanning procedure are projected in the image series in different colors.

If the cursor is moved to a marker position in the scanning image, the relevant information on the image detail is automatically provided in an additional window.

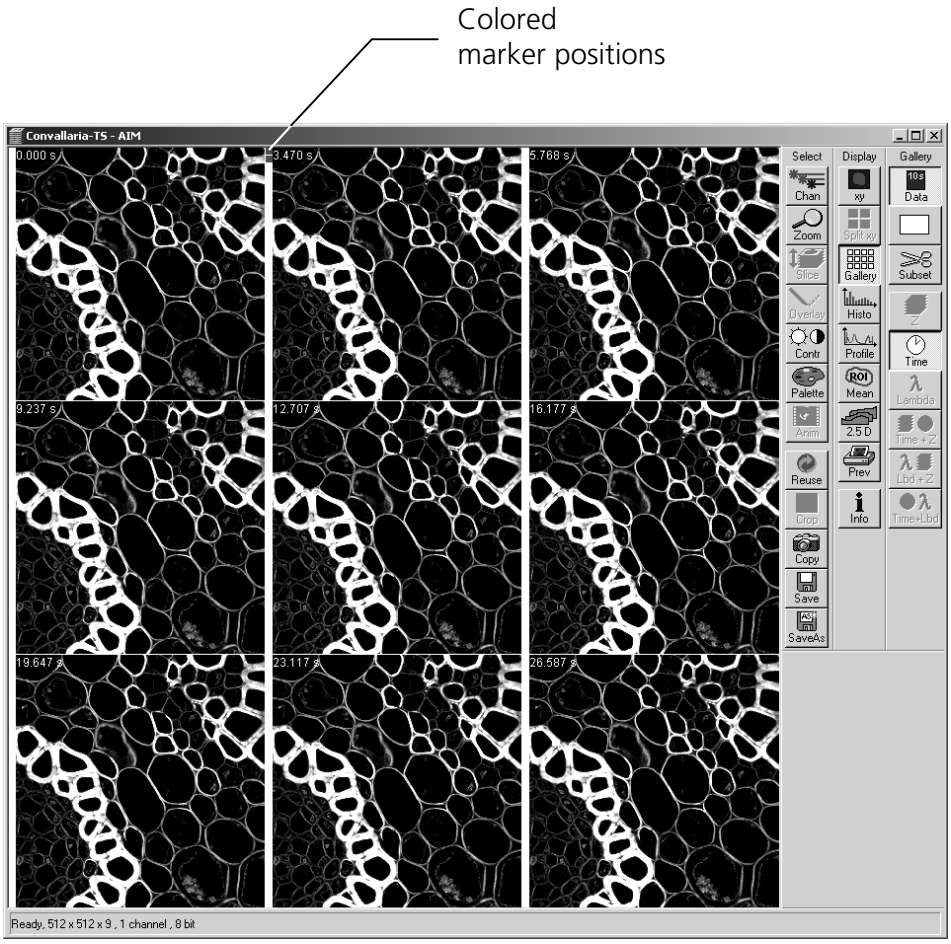


Fig. 5-96 Image Display window of a Time Series Scan

The image markers have different colors with the following meaning:

- red: manually set marker with time indication and comments
- blue: automatically set marker with change of delay
- green: automatically set marker at the beginning and at the end of a bleaching procedure

5.5.6.4 Time Series of a frame over Z Stack

- First, set all parameters required for recording a Z Stack in the **Scan Control** window.
- Then set the parameters required for recording the time series in the **Time Series Control** window (identical procedure as for the time series of a frame).
- Start the time series by clicking on **Start T**.
  - Complete stacks are now recorded at the defined time intervals. The result is displayed in the form of the combined **Image Display** window of the stack and time series (4D).

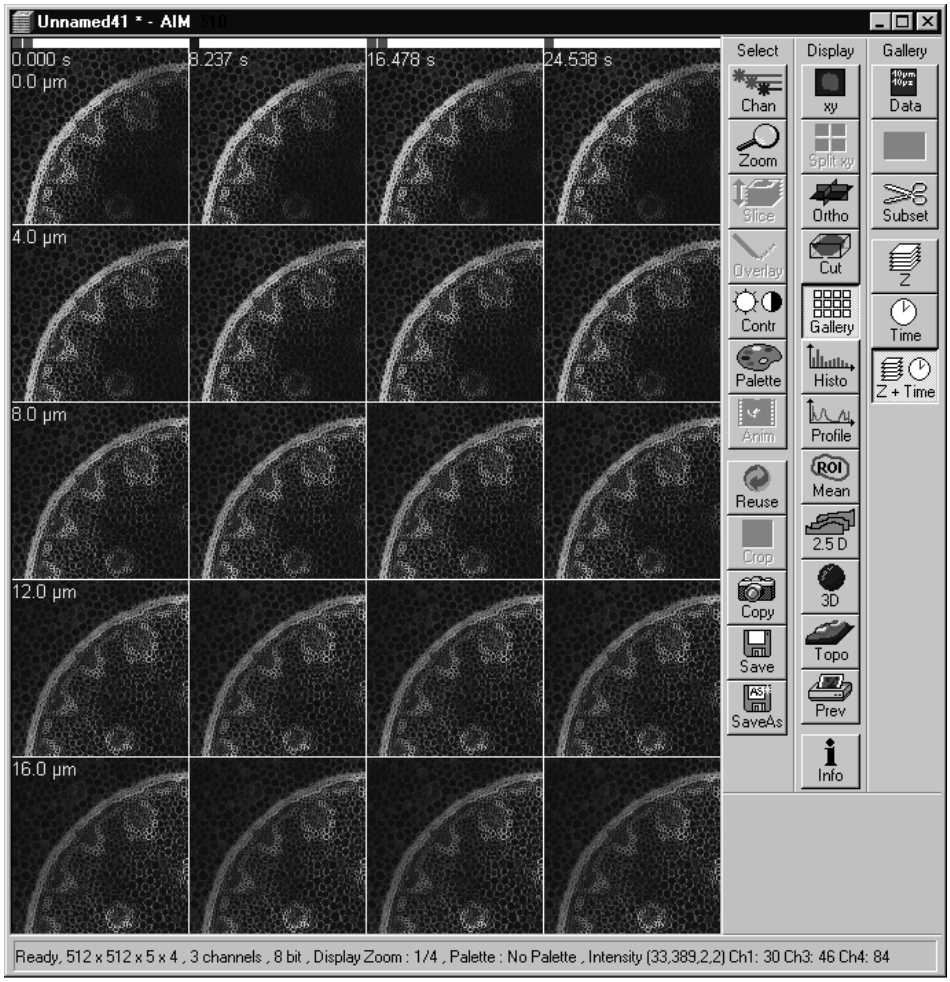


Fig. 5-97 Image Display window of a Z Stack and a Time Series Scan

The additional **Z**, **Time** and **Z + Time** buttons are available in the **Gallery** toolbar of the **Image Display** window.

When you click on the **Z** button, the individual frames of the Z Stack are displayed for the selected Time Slice. When you click on the **Time** button, the individual frames of the time series are displayed for the selected Z Slice.

For Z Stacks over the time (4D) following offline functions will be enlarged:

- Slice (**Z** slider and **Time** slider)
- Gallery (**Z**, **Time** and **Z + Time** buttons)
- 3D (slider for single time index)

To select the Z or Time Slices, use the appropriate sliders which are displayed if the **Slice** button in the **Image Display** window has been activated.

When you click on the **Z + Time** button, all individual frames will be displayed.

5.5.6.5 Time Series of a frame over a Z Stack over Lambda

- Activate the **META** mode sheet in the configuration control and set the relevant parameters.
- Set the relevant parameters for the **Start Series**, **End Series** and **Time Delay** panels.
- Start the time series with a click on the **Start T** or **Start B** button.

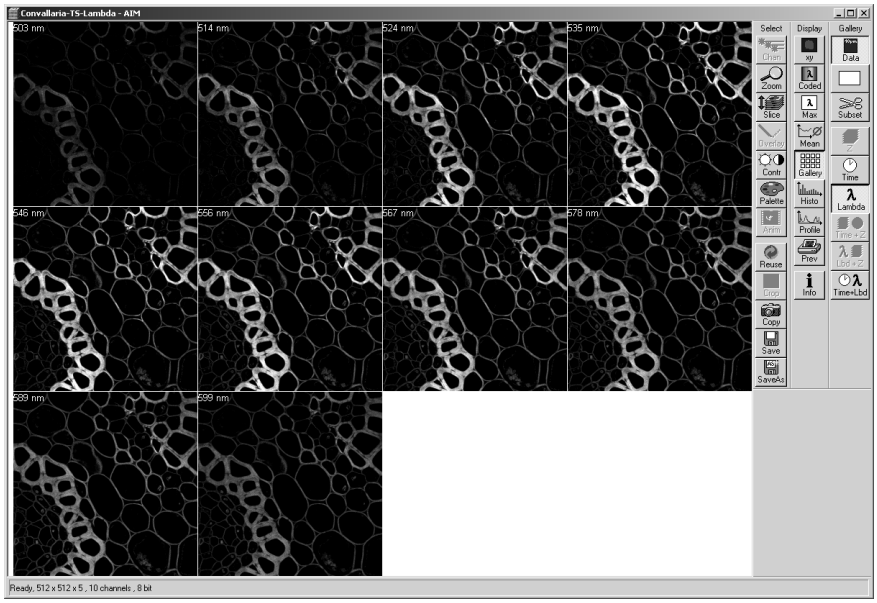


Fig. 5-98 Image Display window of a Time Series over Lambda

5.5.6.6 Time Series with Mean ROI

- Set all the parameters in the same way as for Time Series of a frame.
- Then click on the **Mean ROI** button in the time series frame.

A mean intensity profile of the defined ROIs is created as a function of time.

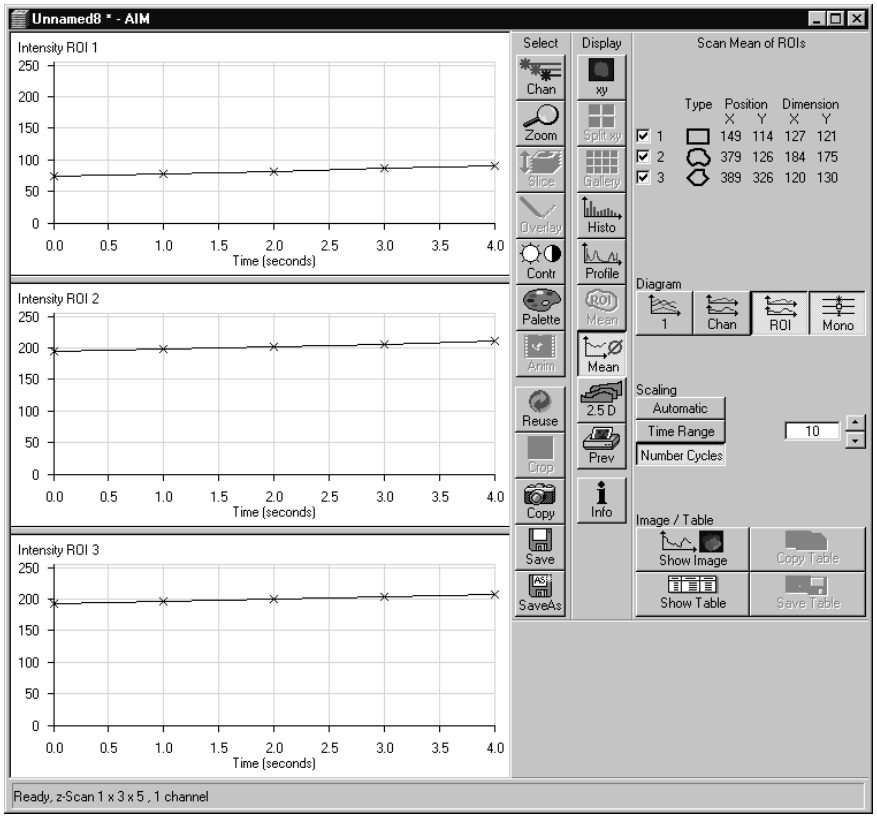


Fig. 5-99 Image Display window of a Time Series with Mean ROI

The **Image Display** window of the **Mean ROI** function is structured differently than that of a frame.

On the left-hand side of the **Image Display** window, the intensity time profiles per ROI are displayed graphically.

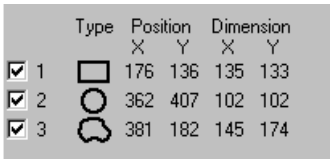
The **Select** and **Display** toolbars, which are also available in the standard **Image Display** window, are positioned in the center.

The **Scan Mean of ROIs** toolbar with further function elements is additionally displayed on the right-hand side. The major purpose of these function elements is to vary the display of the recorded Mean ROI.

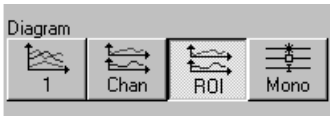
By selecting the appropriate options (see **Options** menu, **Settings** – **Scan Mean of ROIs)** you can activate the following additional functions:

- Display of the live image in the **Image Display** window of the **Mean ROI** function (used ROIs only)
- Scan of the complete image (if Live Image has been activated)
- Saving of the complete time series (if Live Image has been activated)

The following functions are available:

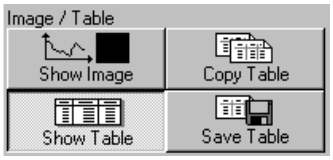


Display of the data of the ROIs used for the creation of the **MeanROI** (identical to the **Edit ROI** window). If the check box of a ROI is deactivated, the ROI's intensity values are no longer displayed in the Intensity-Time diagram.



1 button: Intensity values for ROI and Channels are displayed in a diagram. **Chan** button: Intensity values are displayed separately for each channel used. **ROI** button: Intensity values are displayed separately for each ROI used. **Mono** button: Switches between color and monochromic display of intensity profiles.

Automatic button: Automatic scaling of the display of Intensity-Time diagrams. **Time Range** button: Display of Intensity-Time diagrams is scaled depending on the Time Range set in the input box shown on the left. **Number Times** button: Display of Intensity-Time diagrams is scaled depending on the Number Cycle set in the input box shown on the left.



Show Image button: Shows the scan image in the **Image Display** window to the side of the intensity diagram. This button is active only if the **Live Image** option is activated. **Copy Table** button: The table of intensity values is copied to the clipboard. **Show Table** button: The table of intensity values is displayed at the bottom left of the **Image Display** window. **Save Table** button: The table of intensity values can be stored as a text file.

5.5.7 Edit Bleach

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

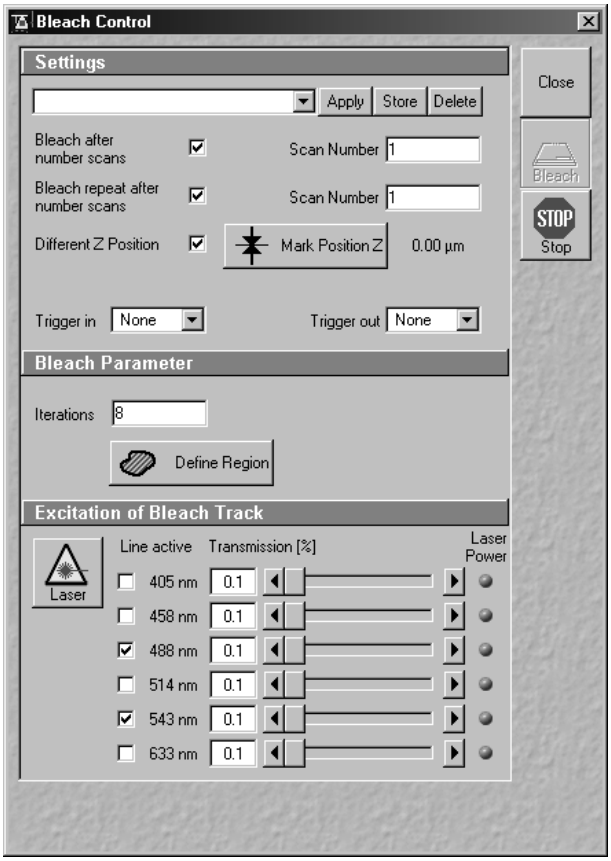


Fig. 5-100 Bleach Control window

5.5.7.1 Open / Close the Edit Bleach window

- Click on the **Edit Bleach** button in the **Acquire** subordinate toolbar of the **Main** menu. The **Bleach Control** window appears on the screen.
- Click on the **Close** button to close the **Bleach Control** window.

5.5.7.2 Function description

The following functions are available on the right-hand side of the **Bleach Control** window:

Close button	The **Bleach Control** window is closed.
Bleach button	Starts the bleaching procedure.
Stop button	Ends the bleaching procedure.

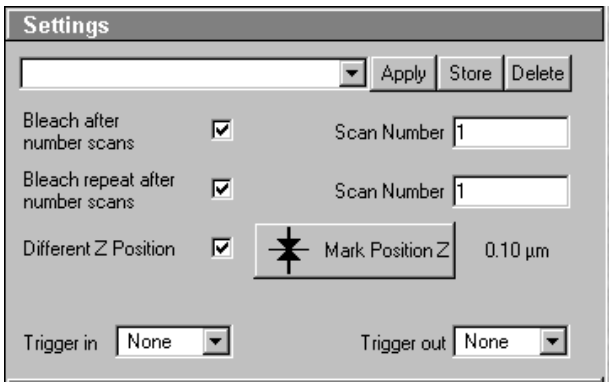


Fig. 5-101 Settings panel

(1) Settings panel

The **Settings** panel allows you to determine when and how the bleaching process shall be done (only works in connection with time series).

Furthermore, all the settings of the **Bleach Control** window can be stored, reactivated or deleted in this panel.

Bleach after number scans: If this check box is ticked ☑, the bleaching procedure is automatically performed in combination with a time series. Under **Scan Number,** you must enter after how many scanning procedures bleaching is to be performed.

Scan Number: Number of Scans in a time series, after performance of which the bleaching procedure shall be started.

Bleach repeat after number scans: If this check box is ticked ☑, the bleaching procedure is automatically performed in combination with a time series. Under **Scan Number,** you must enter after how many scanning procedures bleaching is to be repeated.

Scan Number: Number of Scans in a time series, after performance of which the bleaching procedure shall be repeated.

Different Z Position: If this check box is ticked ☑, you can set the current stage position as the one in which the bleaching will be done by clicking the **Mark Position Z** button. This function is only available using the **Line** or **Frame** scanning mode.

Different XY Spot Bleach Position: If this check box is ticked ☑, you can set a different XY position for spot bleaching. This function is only available using the **Spot** scanning mode. Click on the **Spot Select** button in the **Scan Control** window. A new image is produced and two crosshairs appear in the image. The red crosshair marks the spot that will be imaged. The green crosshair marks the spot that will be bleached. Move the center of the crosshairs to the desired positions and perform bleaching.

Proceed as follows to store the entire settings of the **Bleach Control** window:

- Enter a name in the Settings list box and click on **Store** to store the settings.

If required, stored settings for the bleaching procedure can be reactivated quickly.

- Open the Settings list box with a click on the arrow button and select the required name with a click of the mouse.
- Then click on the **Apply** button to activate these settings. The **Bleach Control** window will be updated accordingly.

Settings which are no longer required can be deleted.

- Open the Settings list box and select the required name.
- Click on the **Delete** button. This stored setting will be removed.

The bleaching procedure can also be activated via keys 1 to 4 of the Trigger Control.

- Open the **Trigger in** list box with a click on the arrow button.
- Select one of the trigger keys 1 to 4 (e.g. **Trigger4**).

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

Fig. 5-102 Bleach Parameter panel

(2) Bleach Parameter panel

The **Bleach Parameter** panel allows you to determine how often the bleaching process shall be performed, and to select the area for bleaching in the scan image via the **Define Region** button.

- Enter the number of iterations of the bleaching procedure in the **Iterations** input box.

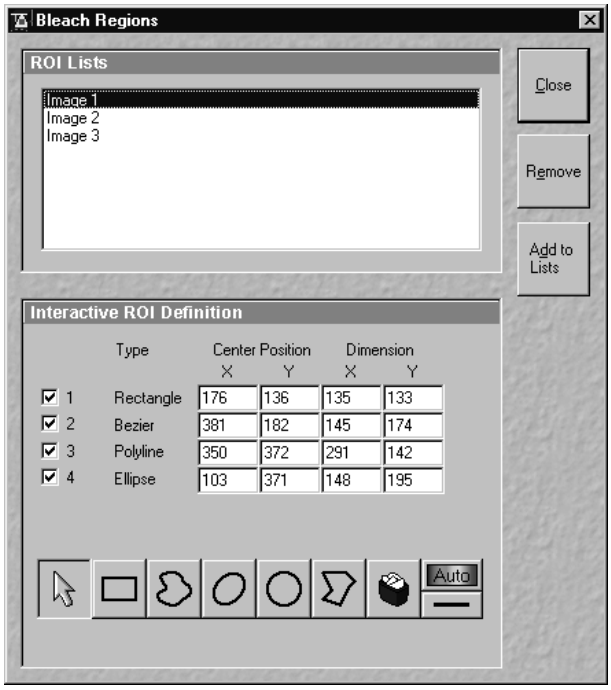


Fig. 5-103 Bleach Regions window

- Click on the **Define Region** button.
  - The **Bleach Regions** window appears.

The definition of bleach regions corresponds to the **Edit ROI** function and is performed in the same way (see section **Edit ROI**, page 5-108).

ROIs already defined with **Edit ROI** are also available in the **Bleach Regions** window. They can be activated directly, modified - if required - and stored under a new name.

☞ ROIs newly defined in **the Bleach Regions** window will then also be available in the **Edit ROI** window.

- Define the required bleach regions in the scan image or use an existing ROI.

(3) Excitation of Bleach Track panel

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

The setting of the lasers for the bleaching procedure corresponds to that for the scanning procedure and must be performed accordingly (see **Laser Control**, **Configuration Control** and **Scan Control**).

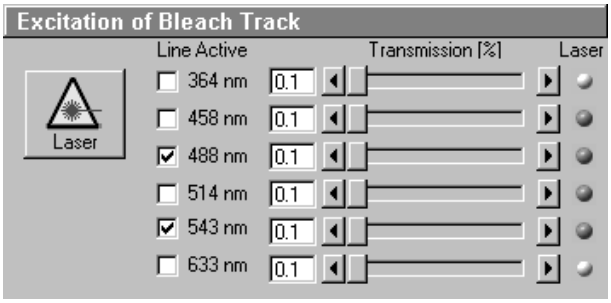


Fig. 5-104 Excitation of Bleach Track panel

- Select the required laser wavelength and its intensity under **Excitation**.
- If required, switch the relevant laser to **On** (**Laser** button).

(4) Start / End a bleaching procedure

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

When a trigger key is activated to start the bleaching procedure, the **Waiting for Trigger** message first appears in the status line of the **Bleach Control** window. In that case, the bleaching procedure is started after activation of the relevant trigger key.

- The bleaching process can be finished via **Stop** in the **Bleach Control** window.

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

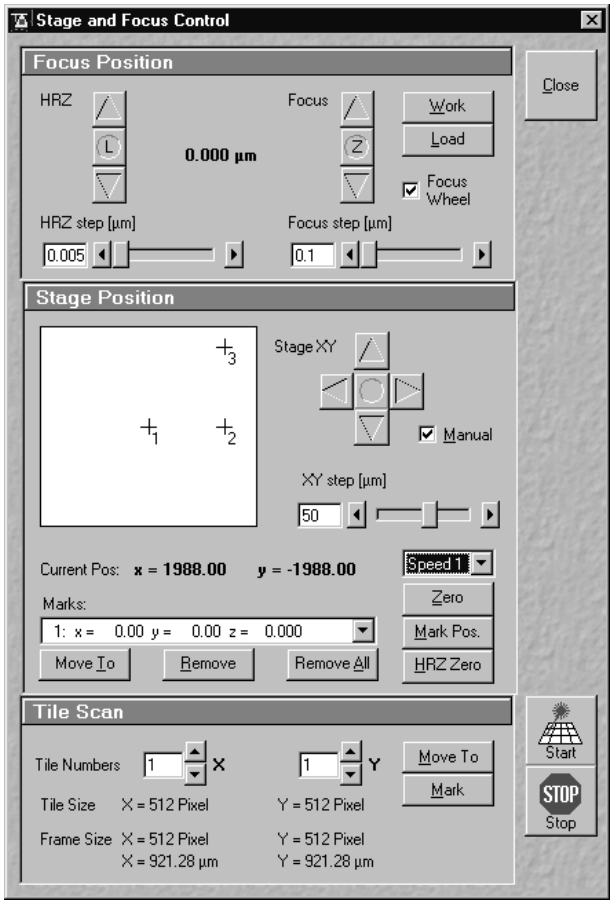


Fig. 5-105 Stage and Focus Control window

5.5.8 Stage

The following software description applies to systems which are equipped with a motorized stage.

This window enables you to activate both the motor focus and the scanning stage.

The **Focus Position** and **Stage Position** panels include the function keys for the performance of defined moves and the display of the current Z and X, Y positions.

By use of an LSM 510 META scanhead on an Axiovert 200 MOT sideport system care should be taken when moving the motorized XY scanning stage to the maximum positions, so that fingers are not bruised between scan head and stage.

5.5.8.1 Open / Close the Stage and Focus Control window

- Click on the **Stage** button in the **Acquire** subordinate toolbar of the **Main** menu. The **Stage and Focus Control** window appears on the screen.
- Click on the **Close** button in the **Stage and Focus Control** window to close this window.

5.5.8.2 Function description

The following functions are available on the right-hand side of the **Stage and Focus Control** window:

Close button	The **Stage and Focus Control** window is closed.
Start button	Starts the tile scanning procedure.
Stop button	Ends the scanning procedure.

(1) Focus Position panel

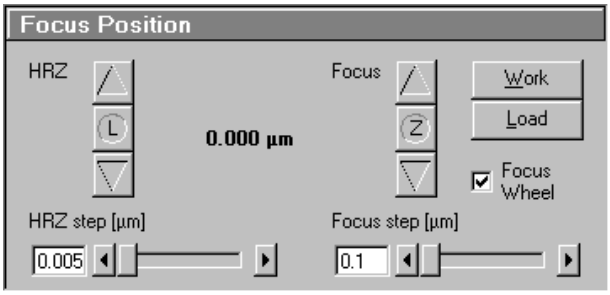


Fig. 5-106 Focus Position panel

Focus buttons (Z Moves)

Clicking on the **Up** arrow button moves the specimen stage / nosepiece upwards.

Clicking on the **Z** button sets the current Z-position to zero.

Clicking on the **Down** arrow button moves the specimen stage / nosepiece downwards.

Focus Step slider

0.1 µm is the smallest value which can be set, and 100 µm the highest.
Clicking on the arrow keys changes the step size by 1 µm.
Pressing the **CTRL** key and clicking changes the step size by 0.05 µm.
Pressing the **Shift** key and clicking changes the step size by 10 µm.

Work button

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

Load button

Clicking on the **Load** button lowers the specimen stage / nosepiece to make it easier for you to change the specimen (or objective).

Focus Wheel check box

Clicking on this check box activates / deactivates the focus wheel of the microscope.

Use of the optional HRZ 200 fine focusing stage or piezo objective focusing device

The **HRZ Step** slider is used to set the step width of the fine focusing stage.

Use the arrows of **HRZ** to move the fine focusing stage upwards or downwards in steps.

As soon as the focus position is changed (via handwheel or software), the HRZ 200 stage is automatically leveled.

A click on the **L** button moves the HRZ 200 fine-focusing stage in the center position of its travel range and the focus position is reset accordingly. Therefore, the same Z-level remains visible (the current position is not set to zero).

The motor focus of the stand is operated in the same way via the relevant buttons. Moving into the **Work** or **Load** position is always performed via the motor focus and not via the HRZ stage.

☞ Please see the annex for further information on the HRZ 200 fine focusing stage: Hints on the use of the HRZ 200 fine focusing stage.

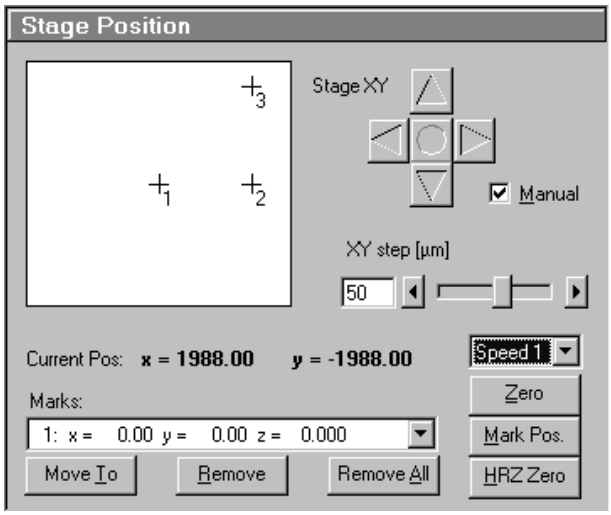


Fig. 5-107 Stage Position panel

(2) Stage Position panel

The **Stage Position** panel shows a symbolic specimen carrier in the left upper.

The buttons for moving to a position and mark it are below or on its right.

The **Current Position** display for X and Y is below.

Below that, you will find the **Marks** selection box of marked positions and the possibility to activate and delete them.

Moving the scanning stage

The scanning stage can be moved using the joystick, or software-controlled using the **Stage XY** buttons, or manually.

Stage XY buttons

Clicking on the arrow buttons moves the stage in X or Y direction.

Clicking on the **Center** button moves the stage in the XY = 0 position.

XY Step slider

1 µm is the smallest value which can be set for XY movement, and 100 µm the highest.

Manual check box

This check box activates / deactivates the motor control of the stage and the joystick, if available.

If **Manual** is active, the scanning stage can be moved manually via the knurled screws. The **Move To** and **Center** function buttons in **Stage Position** are without a function. The **Current Position** is updated. You can zero the display via **ZERO** and mark manually set positions (**Mark pos.**).

The scanning stage cannot be moved via the software or the joystick.

If **Manual** is deactivated, the scanning stage can be moved via the software or the joystick. All the functions of the **Stage Position** window are available.

Current Pos(ition) field

Current Pos displays the currently set stage position in relation to the zero position.

Marks selection box

Clicking on the arrow button displays the table of the session-related marked specimen areas. The table includes the ordinal number, the X-position and the Y-position. Click on the appropriate mark to select it for operation.

Move To button

Clicking on the **Move To** button moves the stage to the position selected before from the **Marks** selection box.

Remove

The **Remove** command enables a selected position to be deleted from the table. The position then also disappears from the specimen carrier display.

☞ The selected position is deleted, the position with the next number in sequence moves up one number.

Remove All

The **Remove All** command deletes all the entries marked in the current session.

Speed selection box

Clicking on the arrow key displays the table of the available speeds for stage movement. Click on the appropriate speed to select it for operation.

Zero button

Zeros the **Current Position** display and thus sets the currently set stage position to 0 in relation to X and Y. The already marked object areas thus receive new X and Y-coordinates.

Mark Pos. button

Mark Pos. allows the **Current Position** to be marked. This marked position is then stored in the **Marks** selection box in sequence. The marked position is shown on the specimen carrier with a cross and its ordinal number.

HRZ Zero button

Zeros the **Current Position** display and thus sets the currently set stage position to 0 in relation to X and Y. The already marked object areas thus receive new X and Y-coordinates.

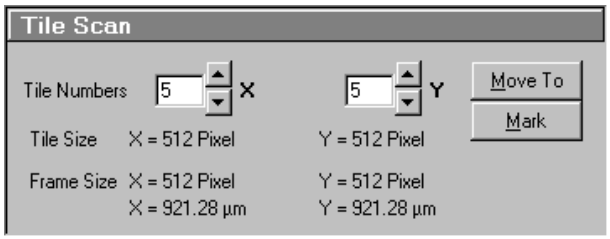


Fig. 5-108 Tile Scan window

(3) Tile Scan panel

This function permits a frame to be created as an overview image of the specimen with a maximum size of 4096 x 4096 pixels. According to settings, such a frame is divided in XY-tiles of 1 x 1 to the maximum of 15 x 15. A tile of special interest (target) can then be selected for scanning.

The application of the **Tile Scan** function requires an objective with a minimum magnification factor of 2.5x.

Tiles Numbers X / Y input box

Input of the number of tiles for **X** or **Y** from which the frame is to be composed.

Tile Size X / Y display

Display of the size of a single tile in µm (corresponds to the value selected in the **Scan Control** window).

Frame Size X / Y display

Display of the frame size of the tile scan for **X** or **Y**. Specification in pixels and µm.

Move To button

If the **Move To** button is activated, a rectangle with a target allowing the selection of the region of interest is positioned in the center of the scanned frame. Click and hold down the left mouse button to drag the rectangle to the required specimen area. When you release the mouse button, the stage moves to the selected position.

Mark button

If the **Mark** button is activated, marks previously set in the Tile Scan image are displayed, and further marks can be added at spots of special interest by a mouse click in the Tile Scan image. By activating the **Move To** button, the stage can be moved to the individual marks set in Tile Scan in the same way as it is moved to the marks set in the **Stage Position** panel.

- Set the number of tiles for the frame in the **Tiles Numbers X / Y** input boxes of the **Tile Scan** window.
  - The resulting frame size is displayed on-line.
- Click on **Start**.
  - The overview frame is scanned and displayed on the screen in a new **Image Display** window.

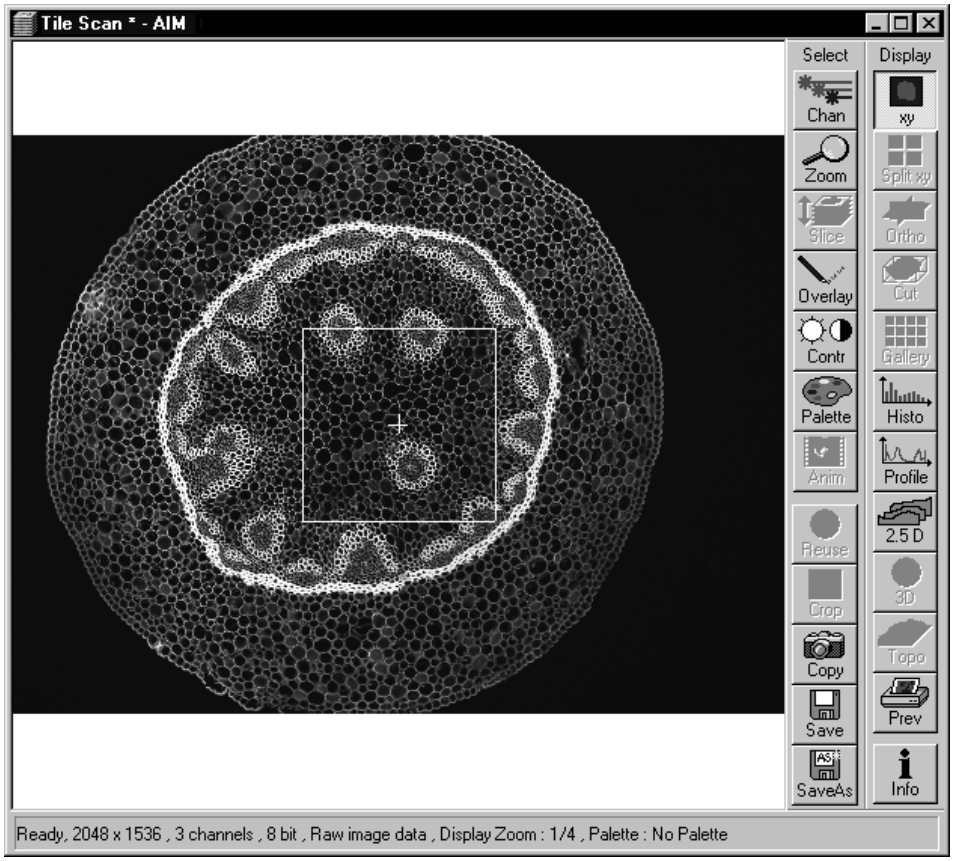


Fig. 5-109 Image Display window of a Tile Scan

- Activate the **Move To** button.
- In the Tile Scan image, move the target to the required spot of the frame (dragging with the mouse).
  - The microscope stage then travels to the selected position.

Or:

- Activate the **Mark** button.
- Set a mark at the spot of interest by clicking with the mouse in the Tile Scan image. A cross with the consecutive number of the mark is displayed in the Tile Scan image. The new mark is also displayed in the specimen carrier (**Stage Position** panel) and included in the **Marks** selection box.
- Select the mark in the **Marks** selection box and click on the **Move To** button in the **Stage Position** panel. The stage moves to the selected position.

- Then click on the **Single** button in the **Scan Control** window to scan the selected area as a single image.
  - The single image is scanned and displayed in a new **Image Display** window.

☞ **Overlay** functions cannot be activated in the **Tile Scan Image Display** window.

The created overview frame can then be stored like any other scan image. If a stored overview frame is opened again, the rectangle with target will appear again. However, it can be deleted using the **Overlay** function.

5.5.9 VIS, FCS and LSM Buttons

The **VIS**, **FCS** and **LSM** buttons are included in the **Acquire** subordinate toolbar of the **Main** menu.

They switch the beam path and indicate which beam path has been set in the binocular tube of the microscope:

- **VIS**: observation via the eyepieces of the binocular tube, lasers are off
- **FCS**: screen observation via laser excitation using the FCS measurements and software evaluation
- **LSM**: screen observation via laser excitation using the LSM image acquisition and software evaluation

5.6 Process Menu

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

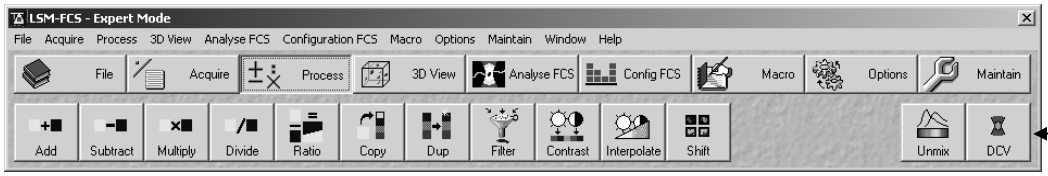


Fig. 5-110 Process menu

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

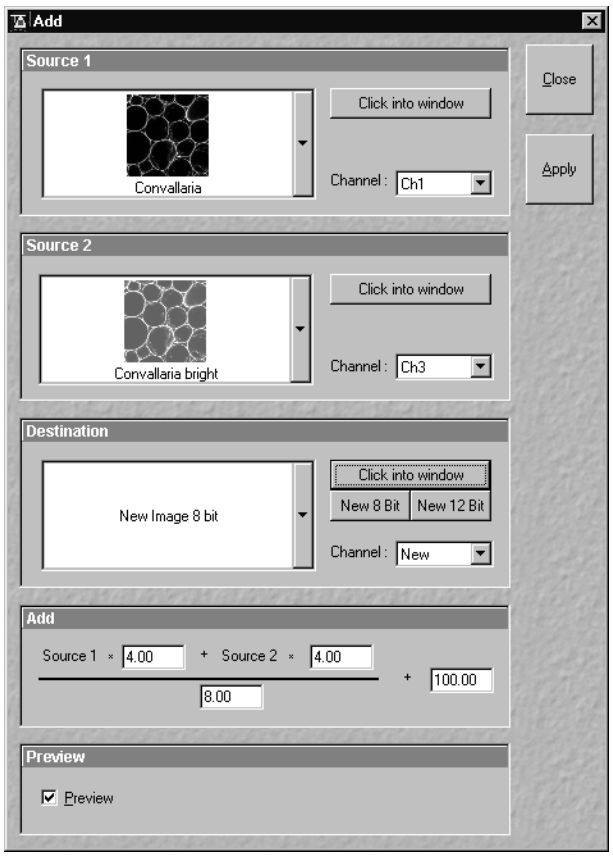


Fig. 5-111 Add window

5.6.1 Add

The **Add** function links two channels each of one or two images into a new channel through addition. The channel created in this way can be stored via the **Save As** function. (This also works with extracted META channels.)

5.6.1.1 Open / Close the Add window

- Click on the **Add** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Add** window.
- Click on the **Close** button to quit the **Add** window.

5.6.1.2 Source panel

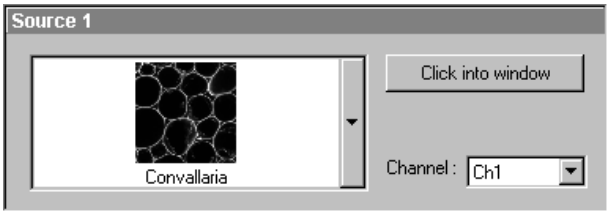


Fig. 5-112 Source 1 panel

In the **Source 1** panel, the first image source for the addition process is determined. The current image is displayed in the display box of the image selection box.

Proceed as follows to select an image via the image selection box:

- Click on the arrow button. The image selection box is opened and all the currently loaded images are displayed in a minimized form.
- Click on the required image. This image will then appear in the display box of the image selection box and has been selected as Source 1.

Use the **Click into window** button to directly select the opened image:

- Click on the **Click into window** button first and then double-click on the relevant **Image Display** window. The selected image will then be displayed in the display box of the image selection box and has been activated as Source 1.

The channel which is to be used for the **Add** operation is selected via the **Channel** selection box:

- Click on the arrow button. The **Channel** selection box is opened and shows all the recorded channels of the relevant image.
- Click on the required channel to activate it.

In the **Source 2** panel, the second image source for the addition process is determined. The procedure is identical to that for Source 1.

- Select the image for Source 2 and the relevant channel.

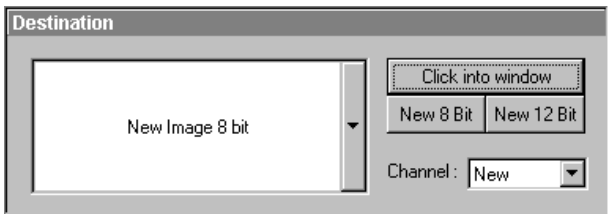


Fig. 5-113 Destination panel

5.6.1.3 Destination panel

In the **Destination** panel, it is determined in which **Image Display** window the **Add** operation is performed, and the data format which the newly created image shall have.

The **Add** operation can be performed in an already opened window or in a new **Image Display** window.

- Click on the arrow button of the image selection box to open this box.
- Click on the relevant image if the **Add** operation shall be performed in an existing **Image Display** window.

  or
- Click on **New Image 8 bit** or **New Image 12 bit** to use a new **Image Display** window.

☞ You can also use the **Click into window** button for image selection.

Clicking on the **New 8 bit** or **New 12 Bit** button enables you to determine directly and quickly whether the new image is to be created in the 8-bit or 12-bit format.

If an existing **Image Display** window is used to perform the Add function, you must determine whether an existing channel shall be overwritten with the Add operation or whether a new channel shall be added.

- In the **Channel** selection box, click on the channel which shall be overwritten, or click on **New** for a new channel.

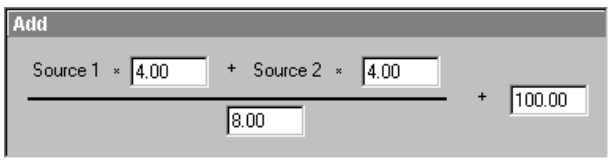


Fig. 5-114 Add panel

5.6.1.4 Add panel

In the **Add** panel, the currently set formula for the **Add** operation is displayed. The editable input boxes permit the formula to be changed with any numeric values.

- Click in the required input box and enter the relevant value.
- Click on the **Apply** button to perform the operation in the activated window or a new **Image Display** window.
- The new image can then be stored via the **Save As** function.

5.6.1.5 Preview panel

Fig. 5-115 Preview panel

The Preview function enables you to preview the result of the defined **Add** operation in a preview window.

- Activate the ☑ **Preview** check box with a click of the mouse. The **Add - Preview Image Display** window is displayed with the operation result.
- Deactivate the **Preview** check box to close the **Add - Preview Image Display** window.

☞ After a change of the formula in the **Add** panel, click in the **Add - Preview Image Display** window for an update.

5.6.2 Subtract

The **Subtract** function links two channels each of one or two images into a new channel by subtraction. The channel created in this way can be stored via the **Save As** function.

5.6.2.1 Open / Close the Subtract window

- Click on the **Subtract** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Subtract** window.
- Click on the **Close** button to quit the **Subtract** window.

5.6.2.2 Performance of the Subtract function

This function is performed in the same way as the **Add** function (see **Add**, page 5-142). The only difference is that the mathematical formula is based on subtraction.

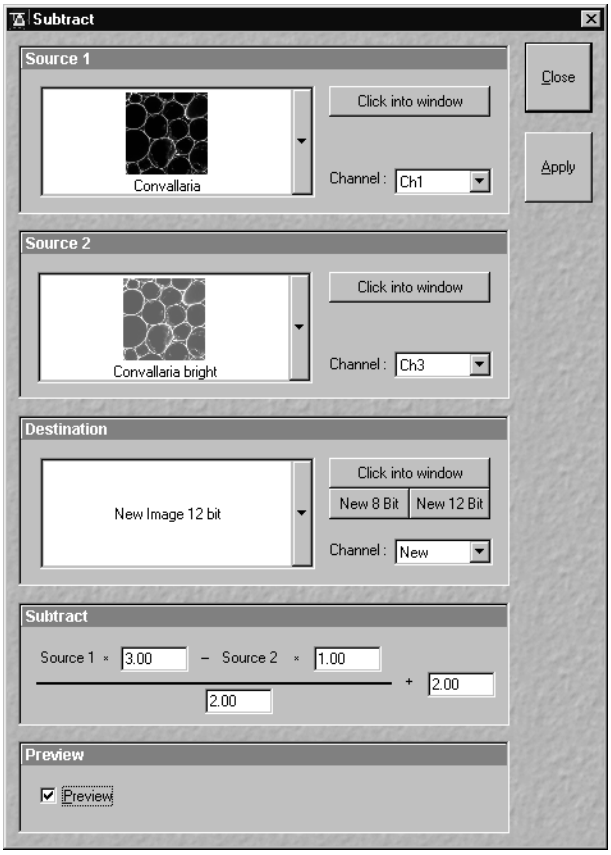


Fig. 5-116 Subtract window

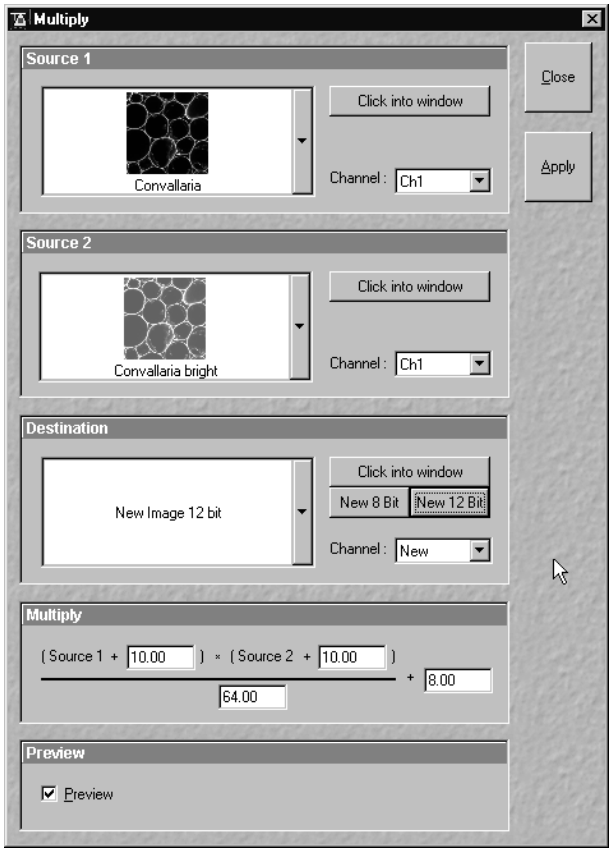


Fig. 5-117 Multiply window

5.6.3 Multiply

The **Multiply** function permits two channels each to be linked into a new channel by multiplication. The channel created in this way can be stored via the **Save As** function.

5.6.3.1 Open / Close the Multiply window

- Click on the **Multiply** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Multiply** window.
- Click on the **Close** button to quit the **Multiply** window.

5.6.3.2 Performance of the Multiply function

This function is performed in the same way as the **Add** function (see **Add**, page 5-142). The only difference is that the mathematical formula is based on multiplication.

5.6.4 Ratio

The **Ratio** function permits two channels to be linked into a new channel by the creation of a ratio. The channel created in this way can be stored via the **Save As** function.

5.6.4.1 Open / Close the Ratio window

- Click on the **Ratio** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Ratio** window.
- Click on the **Close** button to quit the **Ratio** window.

5.6.4.2 Performance of the Ratio function

This function is performed in the same way as the **Add** function (see **Add**, page 5-142).

However, three different formulas can be used for ratio creation, each of which can be activated by clicking on the ⊙ button.

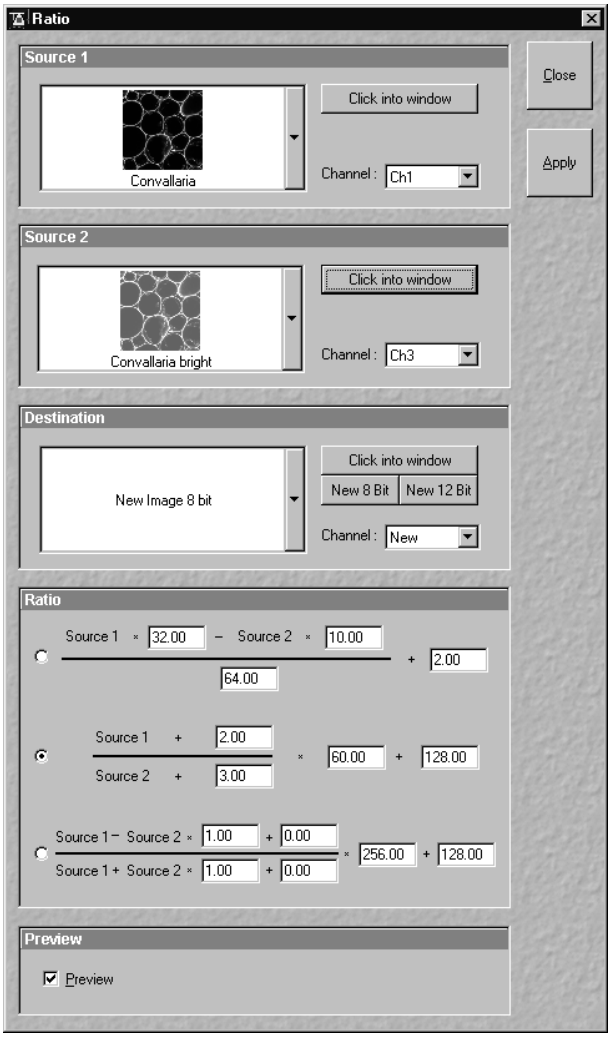


Fig. 5-118 Ratio window

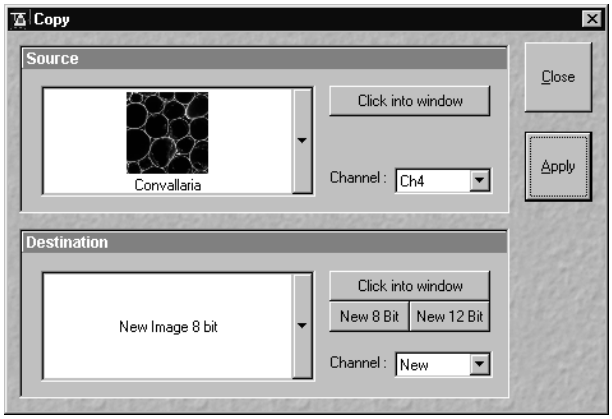


Fig. 5-119 Copy window

5.6.5 Copy (Channel)

The **Copy** function permits one channel each of an existing image to be copied and stored as a new image.

The selection of Source, Channel and Destination is made in the same way as in the **Add** function (see **Add**, page 5-142).

5.6.5.1 Open / Close the Copy window

- Click on the **Copy** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Copy** window.
- Click on the **Close** button to quit the **Copy** window.

5.6.5.2 Performance of the Copy function

- Select Source, Channel and Destination and then click on the **Apply** button.
  - The image of the copied channel is then displayed in a new window or in the **Image Display** window activated for it.
- The new image can be stored via the **Save As** function.

☞ For Z Stacks or Time Series, the entire series of the selected channel is copied.

5.6.6 Duplication (Image)

This function permits images (including Z Stacks and Time Series) to be duplicated completely.

- If several images have been opened, select the image to be duplicated.
- Click on the **Dup** button in the **Process** subordinate toolbar of the **Main** menu.
  - The selected image is duplicated and displayed in a new **Image Display** window.
- Use the **Save As** function to store the image under a new name.

5.6.7 Filter

The filter function permits the subsequent processing of scanned images via the integrated **Lowpass**, **Sharpness** and **Median** filters. Furthermore, **User-defined** filters can be installed by the user. User-defined filters can be stored, reloaded and removed.

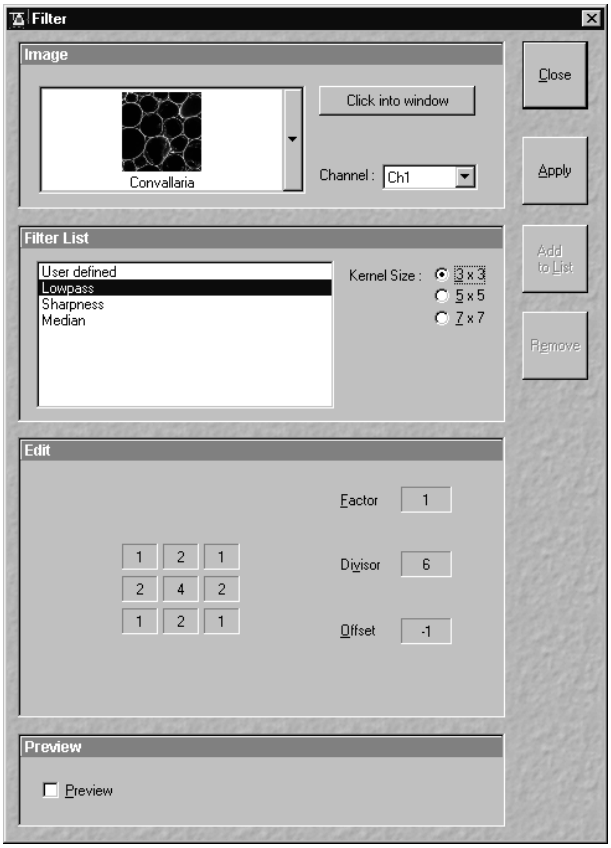


Fig. 5-120 Filter window

5.6.7.1 Open / Close the Filter window

- Click on the **Filter** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Filter** window.
- Click on the **Close** button to quit the **Filter** window.

5.6.7.2 Image panel

In the **Image** panel, the image or channel to be processed is selected.

The currently selected image is displayed in the image selection box.

Proceed as follows to select an image via the image selection box:

- Click on the arrow button. The image selection box is opened and all the currently loaded images are displayed in a minimized form.
- Click on the required image, which will then appear in the display box of the image selection box and will be available for filtering.

☞ You can also use the **Click into window** button to select the image.

- Open the **Channel** selection box with a click on the arrow button and select the channel to be processed.

5.6.7.3 Filter List and Edit panel

In the **Filter List** panel, the filters and the matrix size (**Kernel Size**) are selected.

The matrix of the selected filter and the set filter parameters **Factor**, **Divisor** and **Offset** are displayed in the **Edit** panel.

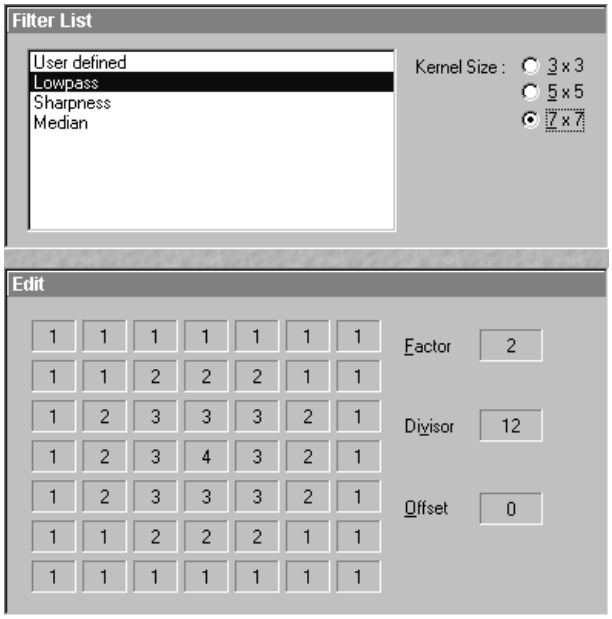


Fig. 5-121 Filter List and Edit panel (Lowpass)

(1) Kernel Size

The size of the filter matrix can be modified here. The effect of a filter increases along with the matrix size. However, this also increases the time required for filtering.

- Select the required matrix size by clicking on one of the selection buttons **3 x 3**, **5 x 5** or **7 x 7**.

(2) Lowpass filter

With the lowpass filter, the gray value of each center pixel is replaced with the average value of the surrounding neighbor pixels. The viewed neighbor pixels are defined by a square. The modified pixel now is the center pixel of the filter matrix.

Image noise will be reduced by the application of the lowpass filter. The cutoff of regions will blur. Local maxima will be flattened. The dynamic range will be reduced considerably.

This filter permits the matrix size to be modified only in the 3 preset steps.

(3) Sharpness filter

With the sharpness filter, the original image is filtered with a lowpass filter first. The result of this filtering is then subtracted from the original image.

This will improve image sharpness.

The matrix size can be modified in the 3 preset steps.

Furthermore, divisor values ranging from **1** to **78** can be entered. The higher the divisor value, the lower the image sharpness.

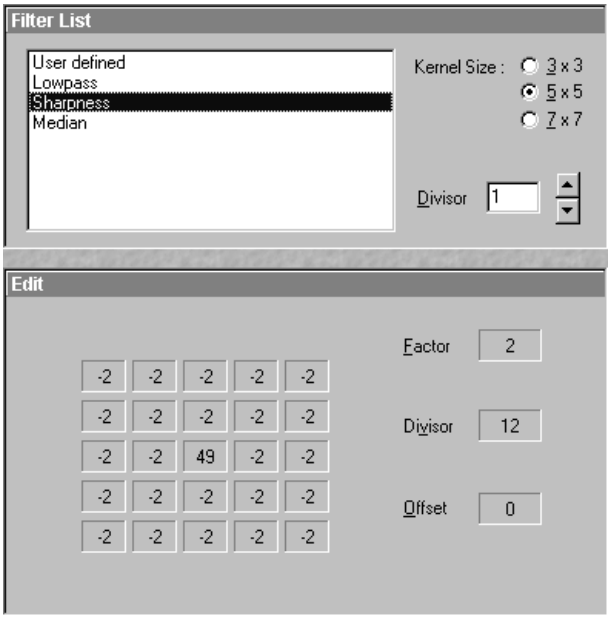


Fig. 5-122 Filter List and Edit panel (Sharpness)

(4) Median filter

With the median filter, the gray value of each center pixel is replaced with the median value of the surrounding neighbor pixels. The viewed neighbor pixels are defined by a square. The modified pixel now is the center pixel of the filter matrix.

The median value is defined as the middle value (not average) of all the gray values sorted in ascending order within a matrix.

Image noise will be reduced by the application of the median filter. The cutoff of regions will slightly blur. Local maxima will be flattened. The dynamic range will be reduced considerably.

The settings of this filter can not be modified.

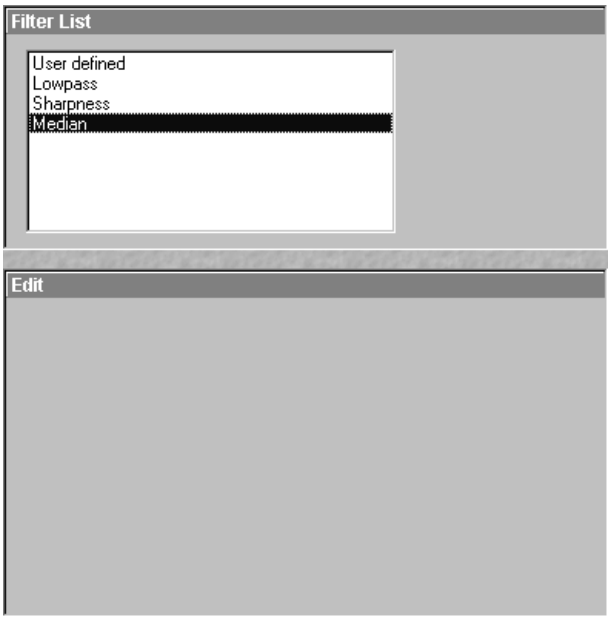


Fig. 5-123 Filter List and Edit panel (Median)

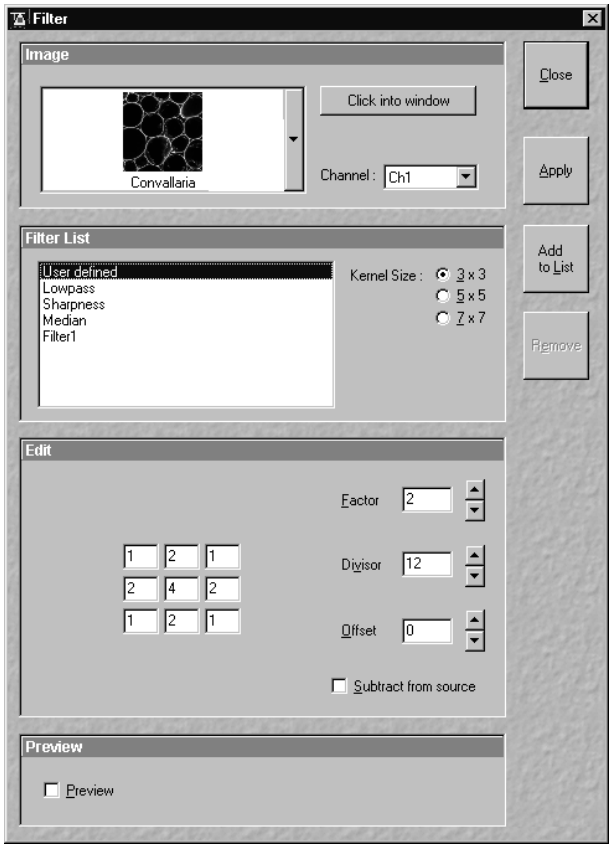


Fig. 5-124 Filter window (User-defined filter)

Fig. 5-125 Add Filter to List panel

(5) User-defined filter

The **User-defined** function permits you to create your own filters. In addition to the **Kernel Size**, the parameters **Factor**, **Divisor** and **Offset** can be modified here.

The filter result can be subtracted from the original image via the **Subtract from Source** check box.

Proceed as follows to store **User-defined** filters:

- Click on the **Add To List** button and enter a name in the **Add Filter To List** window. The name will be included in the **Filter List**.

Proceed as follows to activate stored, **User-defined** filters:

- Click on the name of the filter in the **Filter List**. The filter will then be activated immediately.

Proceed as follows to delete **User-defined** filters:

- Click on the name of the filter in the **Filter List** and then on the **Remove** button. The filter will be deleted.

- After selection of the required filter, click on the **Apply** button to start the filter procedure.
  - Filtering will be performed and displayed in the current **Image Display** window.
- In the case of images with several channels, activate the **xy** button in the **Display** image toolbar to display all the channels. Each channel must be filtered separately.
- Use the **Save As** function to store the newly created image.

5.6.7.4 Preview panel

The Preview function allows you to have the result of the **Filter** operation displayed as a preview image.

Fig. 5-126 Preview panel

- Activate the ☑ **Preview** check box with a click of the mouse. The **Filter - Preview Image Display** window with the filter result will be displayed.
- Deactivate the **Preview** check box to close the **Filter - Preview Image Display** window.

☞ After a change of the filter settings, click in the **Filter - Preview Image Display** window once to update it.

5.6.8 Contrast

The **Contrast** function permits the subsequent modification of contrast and brightness of the stored image.

- Open the image to be processed and click on the **Contrast** button.
  - The function is performed with firmly set parameters and the result is displayed in a new **Image Display** window. The procedure can be repeated as often as required.
- The newly created image can be stored using the **Save As** function.

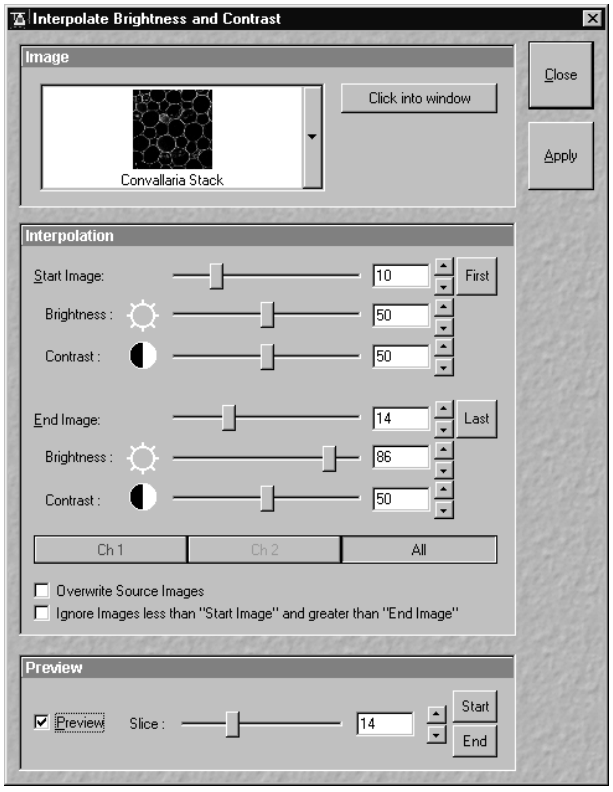


Fig. 5-127 Interpolate Brightness and Contrast window

5.6.9 Interpolate

This function permits the continuous contrast and brightness change in a stack or Time Series through interpolation between the starting and end values. This permits the subsequent compensation of specimen bleaching which occurred during image recording. Interpolation can be defined for the entire image or only for individual channels.

5.6.9.1 Open / Close the Interpolate Brightness and Contrast window

- Click on the **Interpolate** button in the **Process** subordinate toolbar of the **Main** menu (also see page 5-142).
  - This opens the **Interpolate Brightness and Contrast** window.
- Click on the **Close** button to quit the window.

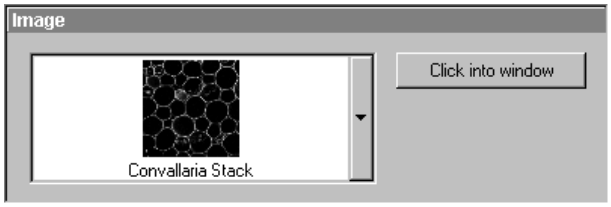


Fig. 5-128 Image panel

5.6.9.2 Image panel

The image to be processed is selected in the **Image** panel.

The currently selected image is shown in the display box of the image selection box.

Proceed as follows to select a series via the image selection box:

- Click on the arrow button. The image selection box will be opened and all the currently loaded images will be displayed in a minimized form.
- Click on the required image, which will then appear in the display box of the image selection box and has been selected for the interpolation procedure.

☞ You can also use the **Click into window** button for image selection.

5.6.9.3 Interpolation panel

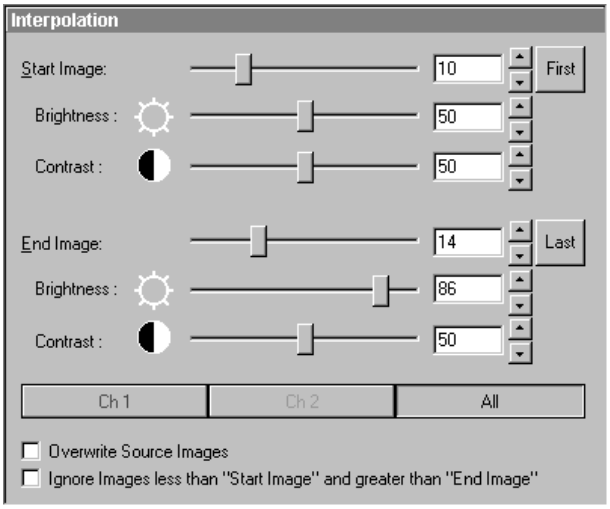


Fig. 5-129 Interpolation panel

In the **Interpolation** panel, the parameters for the interpolation procedure are set.

- Use the **Start Image** slider to select the slice at which the interpolation procedure shall start. Clicking on the **First** button permits the fast selection of the first slice in the series.
- Use the **Brightness** and **Contrast** sliders to set the image brightness and contrast for the first slice (**Start Image**).
- Use the **End Image** slider to select the slice at which the interpolation procedure shall end. Clicking on the **Last** button permits the fast selection of the last slice in a series.
- Use the **Brightness** and **Contrast** sliders to set the image brightness and contrast for the last slice (**End Image**).
- Use the available Channel buttons (e.g.: **Ch1**) to select the channel for interpolation or click on the **All** button if the entire image is to be interpolated.
- Having set the parameters, click on the **Apply** button. Interpolation will be performed in a new **Image Display** window.
- The newly created image (series) can be stored using the **Save As** function.

☞ If you activate the **Overwrite Source Images** check box, interpolation will be performed in the current **Image Display** window.

If you activate the **Ignore Images less than "Start Image" and greater than "End Image"** check box, only the slices lying between Start Image and End Image will be taken into consideration for interpolation. Otherwise, brightness and contrast will also be changed for the other slices.

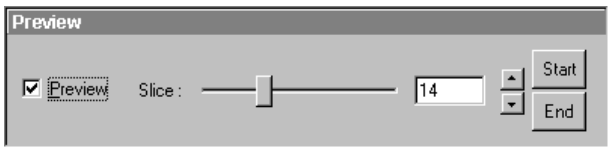


Fig. 5-130 Preview panel

5.6.9.4 Preview panel

The **Preview** function enables you to see the result of interpolation for one slice each in a preview window.

- Activate the ☑ **Preview** check box with a click of the mouse.
  - The **Interpolate C&B - Preview Image Display** window will be displayed. At the same time, the **Slice** slider with the relevant input box and arrow keys and the two buttons **Start** and **End** are displayed in the **Preview** panel.
- Use the slider or input box / arrow keys to set the slice which shall be displayed in the preview window.
- Clicking on the **Start** or **End** button permits the fast activation of the **Start Image** or **End Image** for previewing.
- Deactivate the **Preview** check box to close the **Interpolate C&B - Preview Image Display** window.

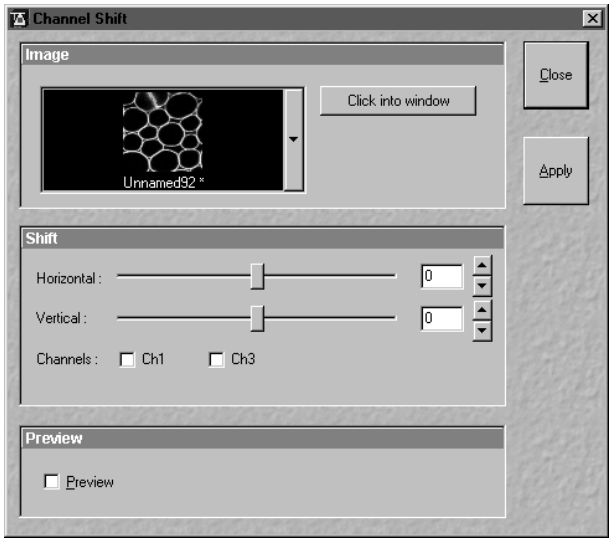


Fig. 5-131 Channel Shift window

5.6.10 Channel Shift

The **Channel Shift** function is used to produce a congruent image with relation to the pixels of the various channels.

This pixel correction function is particularly important in UV applications.

5.6.10.1 Open / Close the Channel Shift window

- Click on the **Shift** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Channel Shift** window.
- Click on the **Close** button to quit the window.

5.6.10.2 Image panel

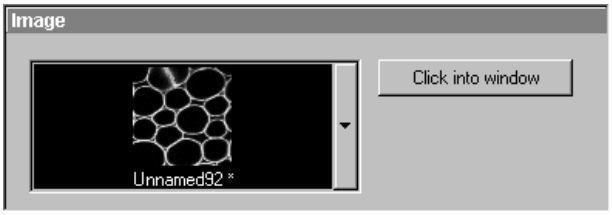


Fig. 5-132 Image panel

- Click on the arrow button. The image selection box will be opened and all the currently loaded images are displayed in a minimized form.
- Click on the required image, which will then appear in the display box of the image selection box and has been selected for the **Shift** function.

☞ You can also use the **Click into window** button for image selection.

5.6.10.3 Shift panel

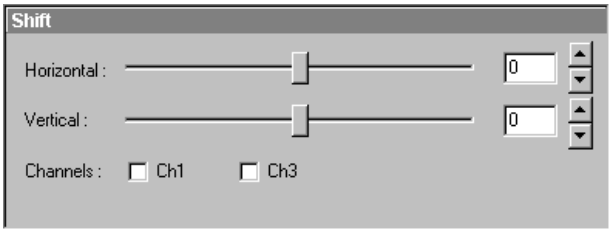


Fig. 5-133 Shift panel

- Select the channels required for processing in the **Shift** box by clicking on the **Ch1** or **Ch3** buttons. A tick ☑ will appear in the button when the channels are activated.
- Use the scrollbar or the ▲ and ▼ buttons to select the pixel shift in the horizontal and vertical direction.
- Click on the **Apply** button to activate the setting.

5.6.10.4 Preview panel

Fig. 5-134 Preview panel

- If ☑ **Preview** is activated, a preview of the shift is shown in a separate **Image Display** window.

The following image shows the result of a pixel shift via the **Shift** function. This image change can be stored in the image database via the **Save** or **Save As** buttons.

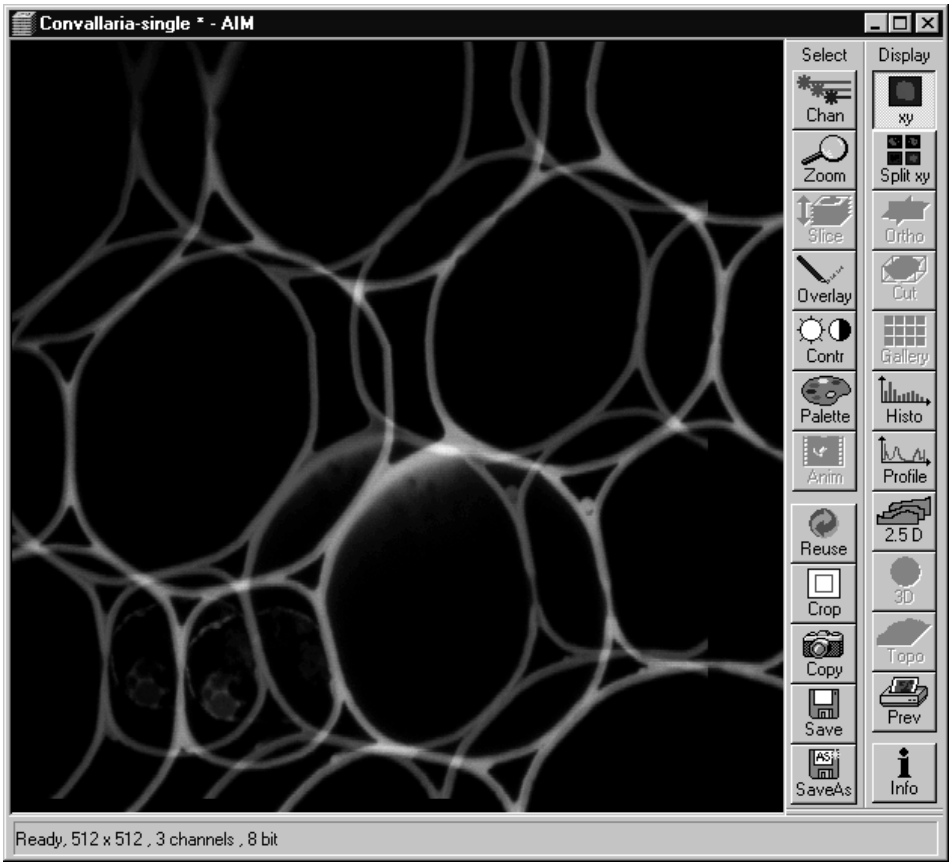


Fig. 5-135 Image Display window with channel shift

For applications requiring 3- or 4-channel scanning, proceed in the same way as described for the 1- or 2-channel mode.

5.6.11 Unmix

The **Unmix** functionality permits to extract the emission of single fluorescence dyes (e.g. GFP only, YFP only etc.) from the overall emission band of strongly overlapping multifluorescence signal intensities by a pixelwise linear unmixing procedure .

Mathematically, experimental fluorescence spectra of monolabelled samples are taken as an external reference. Up to 8 different reference signals can be varied in this least-square-fit based algorithm to produce an 8 channel multifluorescence stack without any partial overlap between the channels.

5.6.11.1 Open / Close the Unmix window

- Click on the **Unmix** button in the **Process** subordinate toolbar of the **Main** menu.
  - This opens the **Linear Unmixing** window.
- Click on the **Close** button to quit the **Unmix** window.

5.6.11.2 Source panel

In the **Source** panel the image source for the linear unmixing process has to be defined.

This has to be a Lambda Stack, a Lambda Stack Z series or a Lambda Stack T series.

Proceed as follows to select an image via the image selection box:

- Click on the arrow button. The image selection box is opened and all the currently loaded images, stacks, time series with a Lambda dimension are displayed in a minimized form.
- Click on the required image. This image will then appear in the display box of the image selection box and has been selected.

5.6.11.3 Definition of Channels panel

In the **Channels** panel the number of reference spectra (number of fluorescence channels) can be selected from the channel selection boxes.

- Select the references fluorescence dye spectra which are present in the sample with the check boxes.
- If necessary change the colors of the relevant fluorescence channel.
- If no predefined reference spectra exist, please define reference signals via the **Save to Spectra DB** button in the **Display** - **Mean** functionality (see page 5-379).
- After definition of the required reference spectra set click on the **Apply** button.
- A new window with the resulting channels of the unmixing procedure opens immediately.

☞ Try to avoid saturation of fluorescence signals in the stack to be unmixed.

To get the highest quality unmixing results, please define an extra background channel, if possible.

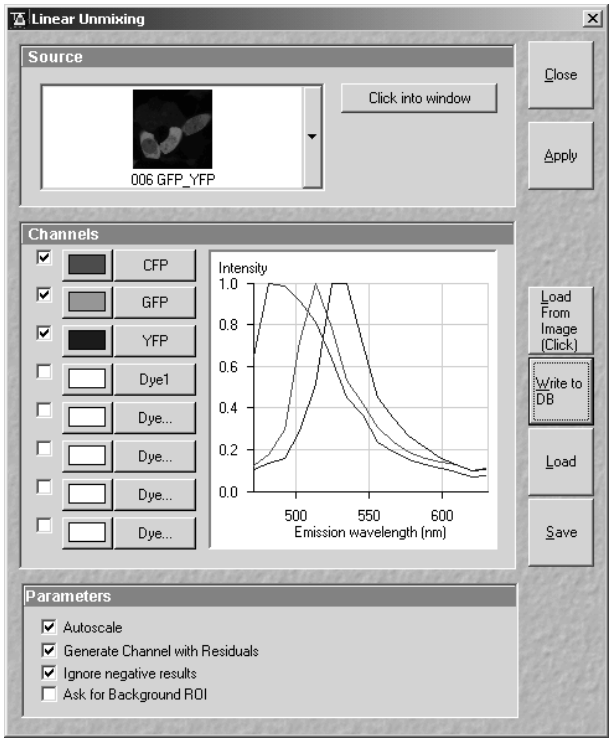


Fig. 5-136 Handling of Reference Spectra

Handling of Reference Spectra

- **Reference Spectra** used for **Linear Unmixing** are stored with result images
- ReUsable and storable via **Load from Image** and **Write to DB** buttons
- New file type *.umx for **Save** and **Load** of combinations (configurations) of **Reference Spectra**

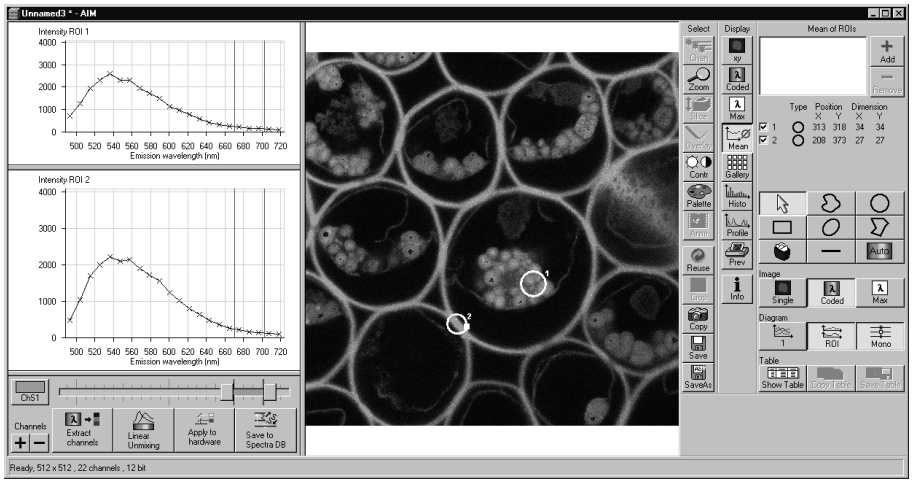


Fig. 5-137 Image Display window before unmixing

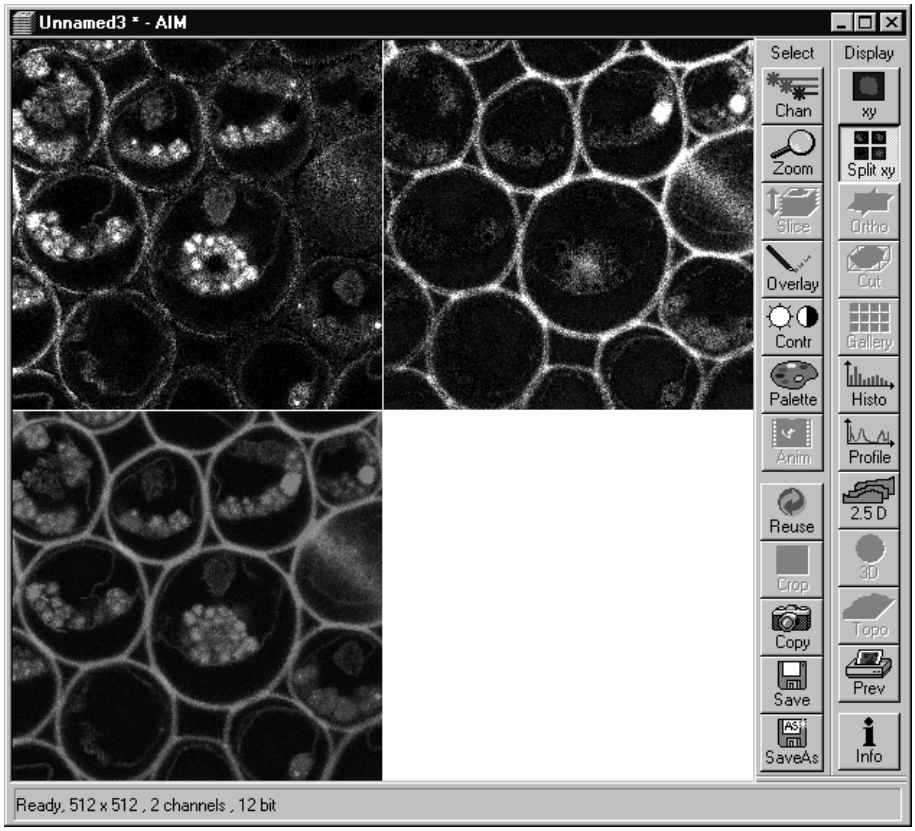


Fig. 5-138 Image Display window after unmixing

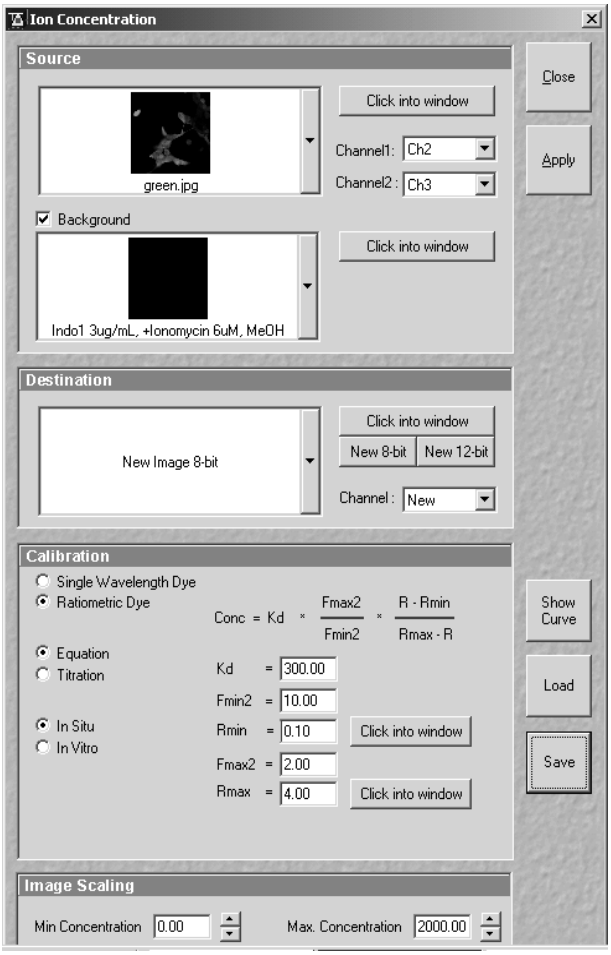


Fig. 5-139 Ion Concentration window

5.6.12 Ion Concentration

The use of this function (option) permits the calibration of ion concentrations in physiological experiments.

(1) Open / Close the Ion Concentration window

Click on the **Ion Conc** button in the **Process** subordinate toolbar of the main menu.

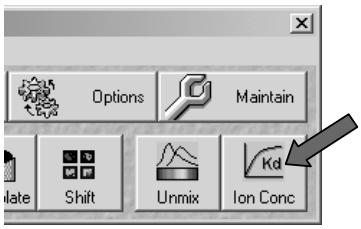
Click on the **Close** button.

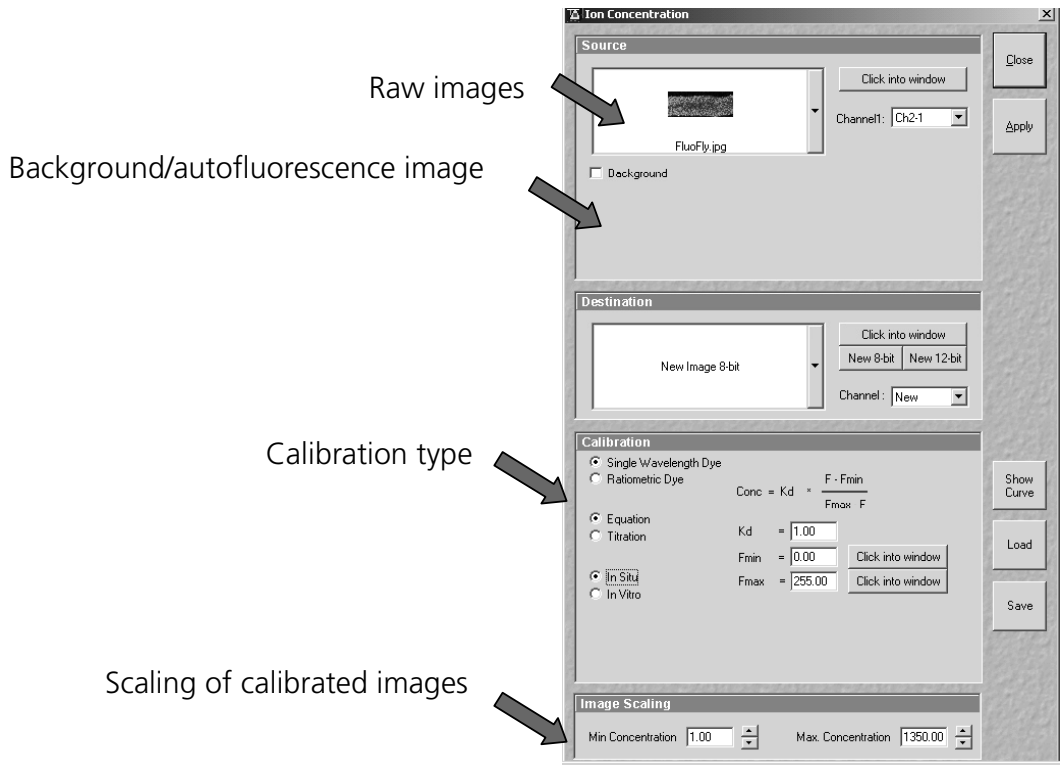
(2) Function description

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

(3) Single wavelength dyes – offline Calibration

- Subtract background/autofluorescence image from raw images to obtain
- Perform equation- or titration calibration (compare F with a calibration curve -> titration calibration or put F values in calibration formula)





(4) Ratiometric Dyes

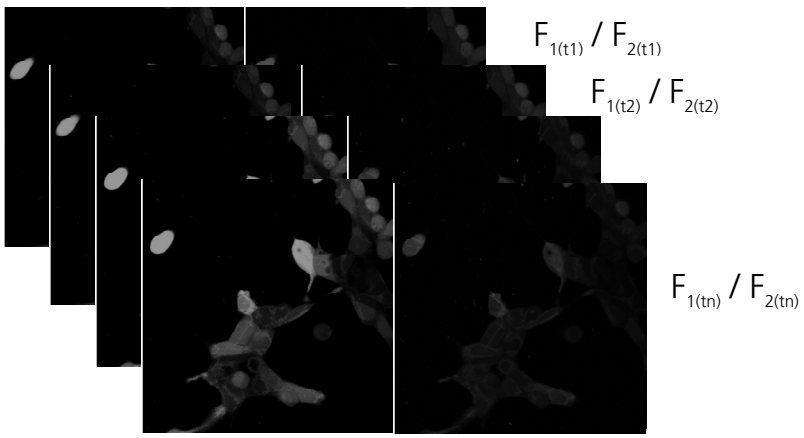
- Fura-2, Indo-, SNARF, Cameleon, Ratiometric Pericam, Phluorin,...

- Display fluorescence ratio R over time
- Display fluorescence ratio R corrected for background/autofluorescence over time
- Calculate absolute ion concentrations (pixel by pixel) via titration calibration (known ion concentrations applied to the cells – in situ – or in solutions – in vitro or equation calibration where possible [Fura-2, Indo-, SNARF]

- Calculation of R eliminates artifacts and uncertainties caused by
  - inhomogenous dye distribution
  - photobleaching
  - may be applied with moving cells

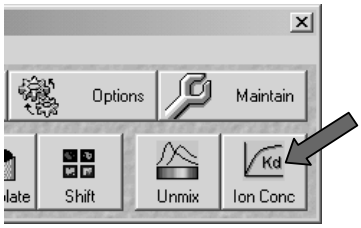
(5) Ratiometric Dyes - Online ratio

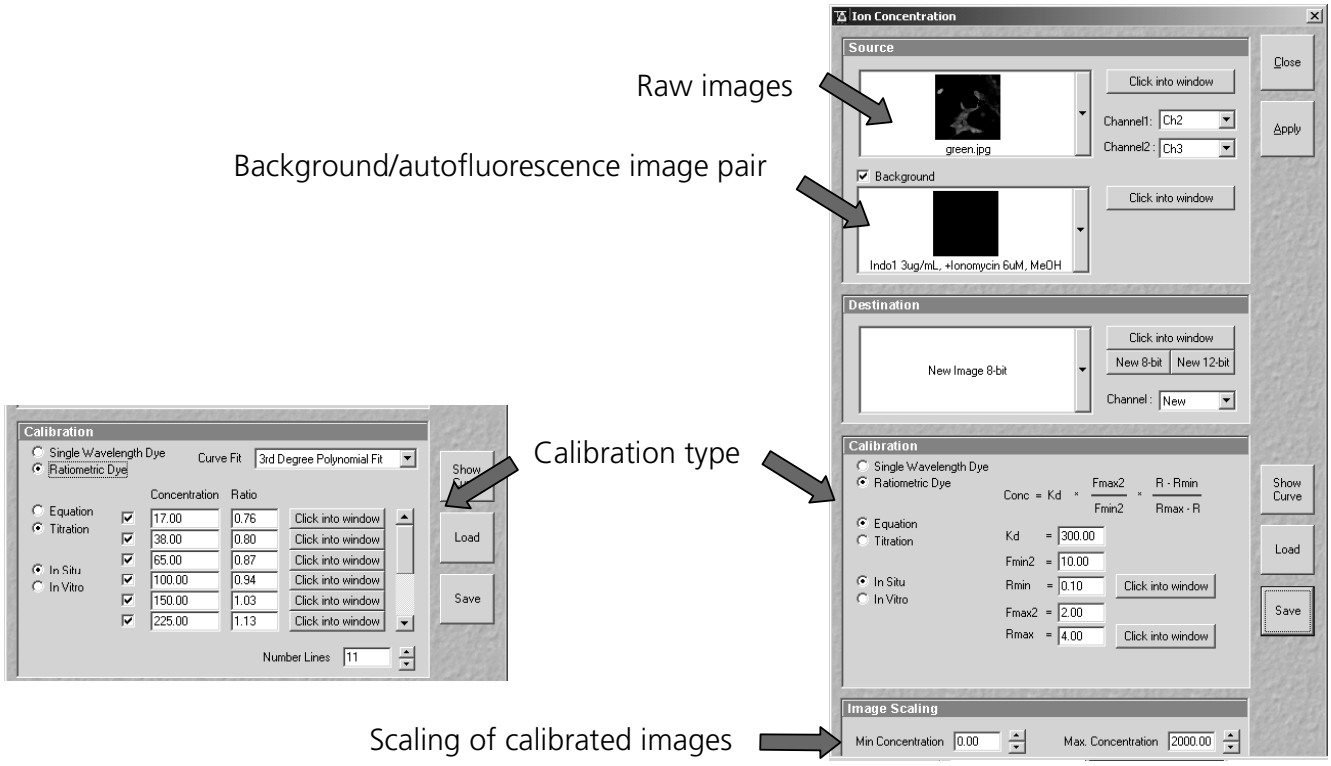
$R_{(t1)} = F_{1(t1)} / F_{2(t1)}$, $R_{(t2)} = F_{1(t2)} / F_{2(t2)}$ ...



(6) Ratiometric Dyes - Calibration

- Subtract background/autofluorescence images from raw images to obtain R_{korr} [=F_1-$F_{1Background}$)/(F_2-$F_{2Background}$)] when calibration reference is not obtained with the experimental sample (in situ)
- Calculate ratio R
- Perform equation- or titration calibration (compare R with a calibration curve -> titration calibration or put R values in calibration formula)





(7) Ratiometric Dyes - Equation Calibration (Grynkiewicz)

Fura-2, Indo-1,..

K_D (dissociation constant) taken from literature

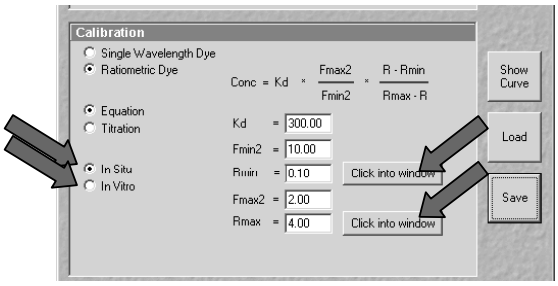
R_{min}: derived from ion-free state of the dye (e.g. 0 Ca^{2+})

R_{max}: derived from ion-bound state of the dye (e.g. saturated with Ca^{2+})

F_{min2} and F_{max2} are the minimum and maximum fluorescence intensities at wavelength 2

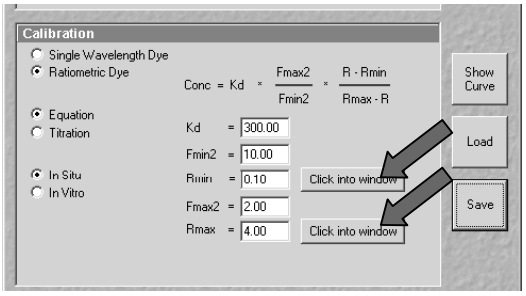
R_{min}, R_{max}, F_{min2} and F_{max2} may be determined in the cells under investigation (in situ) or in solutions (in vitro)

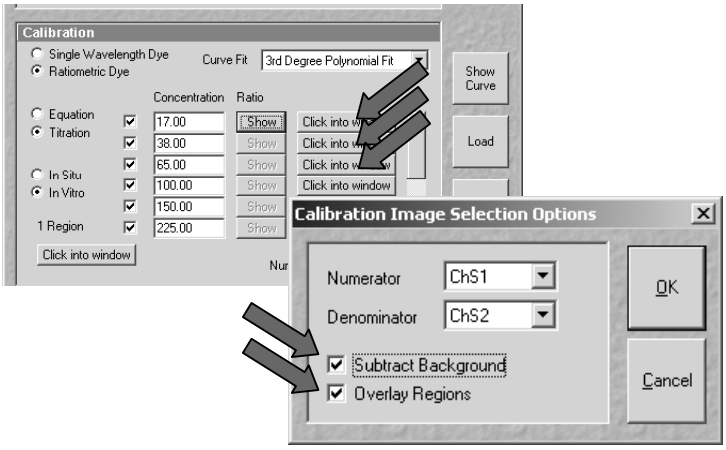
Calibration parameters may be saved and reloaded (*.cal)



(8) Options for Calibration Image Selection (equation- or titration calibration)

- Click into image window.
- Select source channel(s).
- Optional background subtraction
- Optional calculation of parameters from overlay region(s)



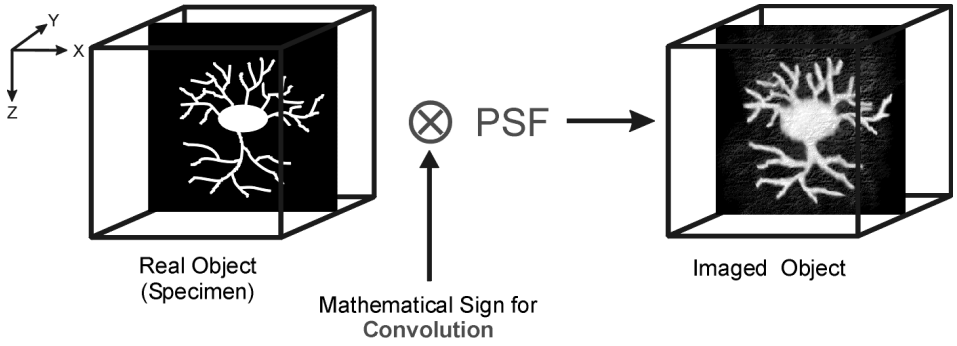


5.6.13 3D DeConVolution (DCV)

The 3D Deconvolution option is used for the resolution enhancement of fluorescence image stacks.

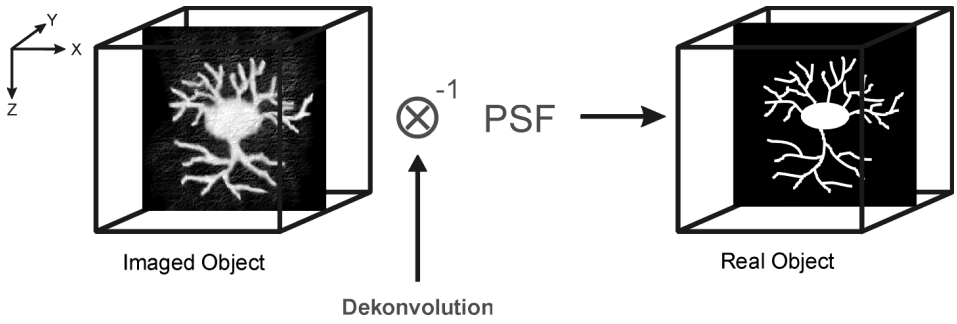
5.6.13.1 Background

When a three-dimensional object is reproduced by an optical system the resulting image of the object does not correspond exactly to the object's actual form. The image of the object is "distorted" as it passes through the optical system. In physical terms the actual object is convolved by the optical system's **Point Spread Function** (PSF).



The **Point Spread Function** describes how the light of a point object is distorted by the optical system. This "convolution" makes the image appear grainy and structures in the image seem blurred. This effect is most prominent in the axial (Z-)direction as each lens is optimized for the two-dimensional image of the object.

If the PSF is known it is possible to use mathematical algorithms to undo this distortion. The image of the object is deconvolved using the PSF and the actual form is reconstructed:



The effect of 3D deconvolution can be demonstrated impressively on objects with a known form. As a rule fluorescent beads are used for this purpose. The following figure shows the 3D deconvolution of an image stack with a fluorescent bead with a diameter of 1 µm.

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

The Z Stack must meet the following requirements:

- At least two-fold oversampling in xyz (z: half of optimal interval button)
- High signal-to-noise ratio
- Detector gain < 500 V

Calculation is either made for one channel of the opened image which must first be selected accordingly, or for all channels of a stack.

Calculation is started via **Apply** and can be stopped using the **ESC** key, if required.

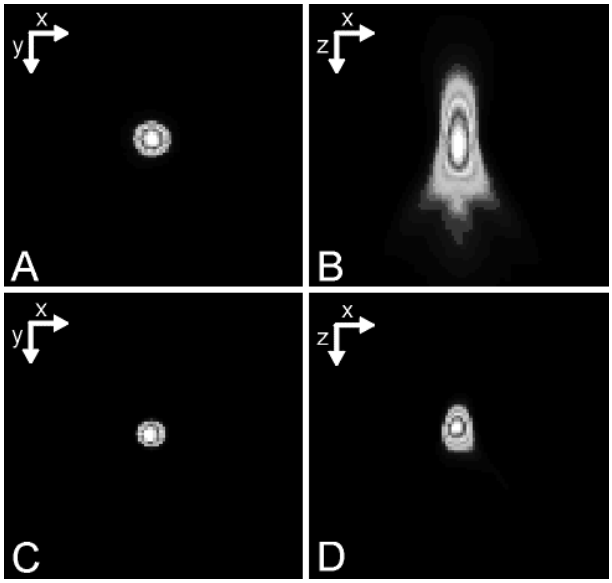


Fig. 5-140 Image of a fluorescent bead with a diameter of 1µm before deconvolution (A,B) and after deconvolution (C,D)

5.6.13.2 Open / Close the 3D Deconvolution window

- Click on the **DCV** button in the **Process** subordinate toolbar of the **Main** menu (also see page 5-142).
  - This opens the **3D Deconvolution** window.
- Click on the **Close** button to quit the window.

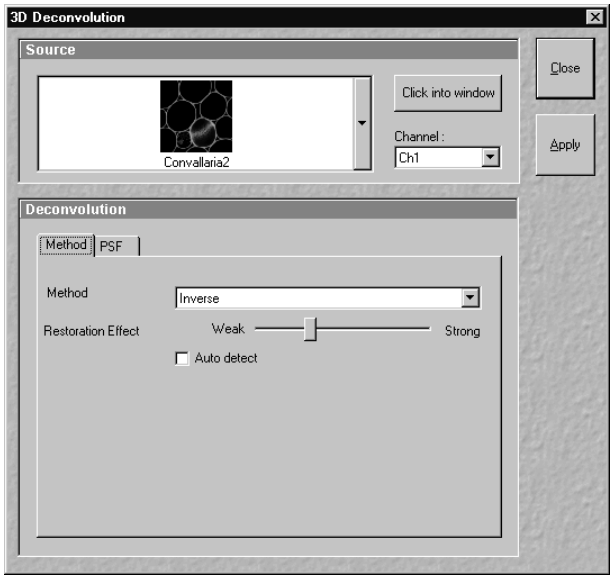


Fig. 5-141 3D Deconvolution window

Fig. 5-142 Source panel

5.6.13.3 Source panel

The image to be processed is selected in the **Source** panel. The currently selected image is shown in the display box of the image selection box. Proceed as follows to select a image via the image selection box:

- Click on the arrow button. The image selection box will be opened and all the currently loaded images will be displayed in a minimized form.
- Click on the required image, which will then appear in the display box of the image selection box and has been selected for the interpolation procedure.

☞ You can also use the **Click into window** button for image selection.

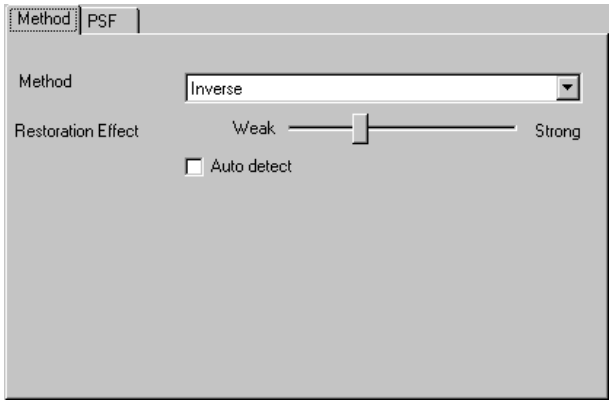


Fig. 5-143 Method tab

5.6.13.4 Deconvolution panel

The **Deconvolution** panel contains the two tabs **Method** and **PSF**.

(1) Method tab

The **Method** tab permits selection between the calculation methods **Nearest Neighbour**, **Inverse** and **Iterative**.

(a) Nearest Neighbor

The Nearest Neighbor method is the simplest and fastest algorithm which in principle corresponds to a 3D sharpness filter.

(b) Inverse Filter

The regularized inverse filter generally achieves better results than the Nearest Neighbor algorithm. It is well suited to process several image stacks for a preselection of images for the use of the iterative high-end methods.

© Constrained Iterative

The best image quality is achieved using the Constrained Iterative Maximum Likelihood Algorithm. Increasing the resolution in the image, especially in the Z-direction, is only possible with this method. Due to the complex mathematical method, depending on the image size and the PC being used the calculation can take up to several hours.

In the **Inverse** method, the **Restoration Effect** slider permits the noise-to-signal ratio to be selected between the settings **Weak** (low noise) and **Strong** (pronounced noise).

Activation of the **Auto detect** check box will start a routine for the automatic determination of the noise level in the entire image part of the Z Stack (not available in the **Nearest Neighbour** method). If **Auto detect** is enabled, the **Restoration Effect** slider is disabled.

The **Iterative** method permits (in addition to the parameters of the **Inverse** method) the maximum number of iterations to be entered between 1 and 200 under **Maximum Iterations** and the **Auto Stop** function to be activated / deactivated. The **Auto Stop** function interrupts the calculation depending on the set image improvement (delta between last but one and last cycle in %), no matter whether the value under **Maximum Iterations** has been achieved or not.

The **Nearest Neighbour** method permits entry of the **Number of Neighbours** and the **Sharpness in Focus** value in addition to the **Restoration Effect**.

(2) PSF tab

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

The PSF data are displayed in the **Method** tab. In the case of wavelengths above 700 nm, the **NLO** button is automatically enabled.

The displayed values are always taken over by the system data, but can be edited subsequently for simulation purposes.

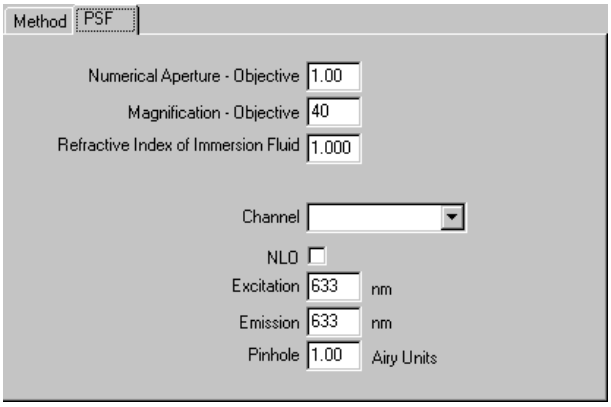


Fig. 5-144 PSF tab

5.7 3D View Menu

The 3D View functions serve to record and play back series of images for 3D display of microscopic structures.

- In the **Main** menu toolbar, click on **3D View**.
  - This opens another, subordinate toolbar in the **Main** menu.

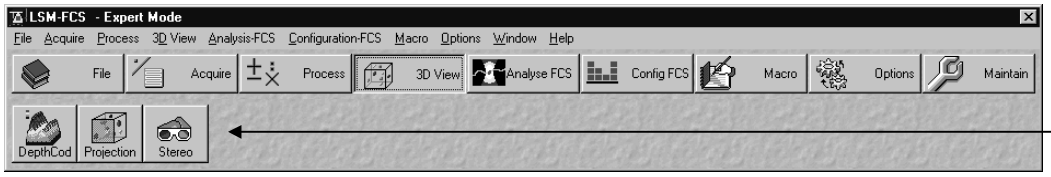


Fig. 5-145 3D View menu

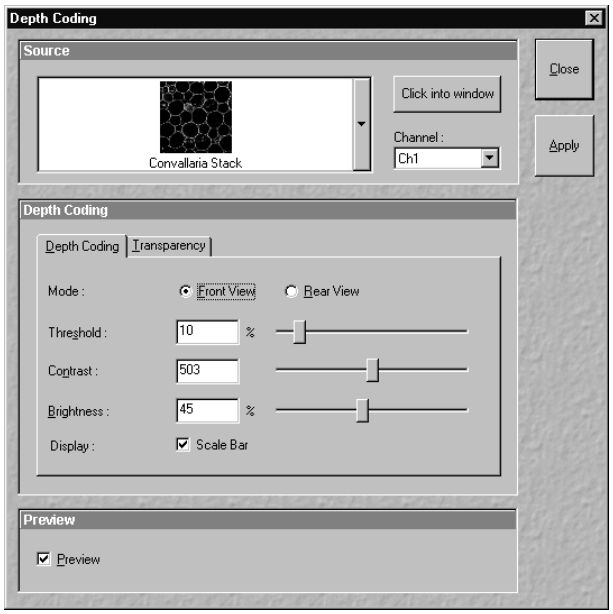


Fig. 5-146 Depth Coding window

5.7.1 3D DepthCod (Color Coded Depth Map)

By means of the **Depth Coding** function, the depth information contained in a sequence can be colored with the colors of the rainbow, in which case "blue " stands for front and "red" stands for rear.

A stack of images must be available.

5.7.1.1 Open / Close the Depth Coding window

- Click on the **DepthCod** button in the **3D View** subordinate toolbar of the **Main** menu.
  - This opens the **Depth Coding** window.
- Click on the **Close** button to quit the window.

5.7.1.2 Source panel

In the **Source** panel, the image source is selected. The currently selected image is displayed in the display box of the image selection box. Proceed as follows to select an image via the image selection box:

- Click on the arrow button. The image selection box will be opened and all the currently loaded images are displayed in a minimized form.
- Click on the required image, which will then be shown in the display field of the image selection box and be available for the following operation.

The **Click into window** button enables you to select the opened image directly:

- Click on the **Click into window** button first and then double-click on the relevant **Image Display** window. The selected image will then be shown in the display box of the image selection box.

Select the channels to be processed via the **Channel** selection box:

- Click on the arrow button to open the selection box. Click on the required channel to activate it.

5.7.1.3 Depth Coding panel

- On the **Depth Coding** panel you can set the desired parameters. Activate the **Scale Bar** check box ☑ if you want a color scale to be shown.

(1) Depth Coding tab

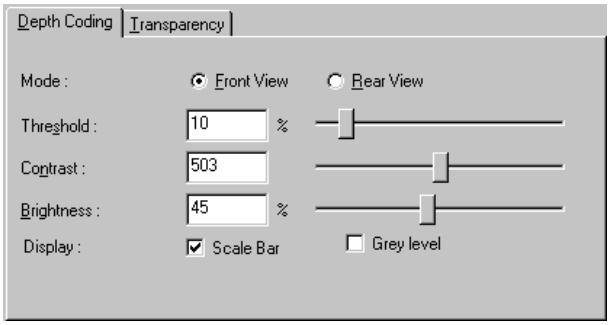


Fig. 5-147 Depth Coding tab

Mode **Front View**: The image is viewed from the front / above when this option is activated.

Mode **Rear View**: The image is viewed from the rear / below when this option is activated.

Threshold: All brightness values below the Threshold (range: 0 to 255) are ignored or treated like 0 when determining the depth and the display.

Contrast: Defines the factor with which the contrast of the overlaid series affects the contrast of the depth-coded color.

Brightness: Defines the factor with which the brightness of the overlaid series affects the brightness of the depth-coded color.

Display Scale Bar: Displays a colored scale in the image.

Display Grey level: The depth information is displayed in gray levels.

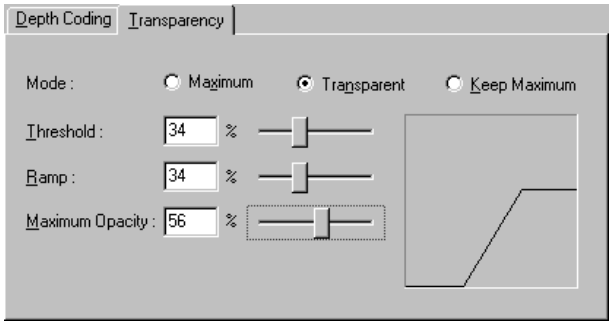


Fig. 5-148 Transparency tab

(2) Transparency tab

Mode **Maximum**: The color is defined by the Z position of the brightness value.

Mode **Transparent**: The transparent projection is built up from the rear to the front. The color is defined by the Z position at which the original was last higher than or equal to Threshold.

Mode

Keep Maximum: Activating this option modifies the specification governing calculation of the projection.

Threshold: Pixel value at which the ramp rises (variable from 0 to 100 %).

Ramp: Slope of the ramp (variable from 0 to 100 %; 0 % corresponds to a vertical rise).

Maximum Opacity: Degree of visibility at the top corner of the ramp (variable from 0 to 100 %; 0 corresponds to the bottom edge in the diagram).

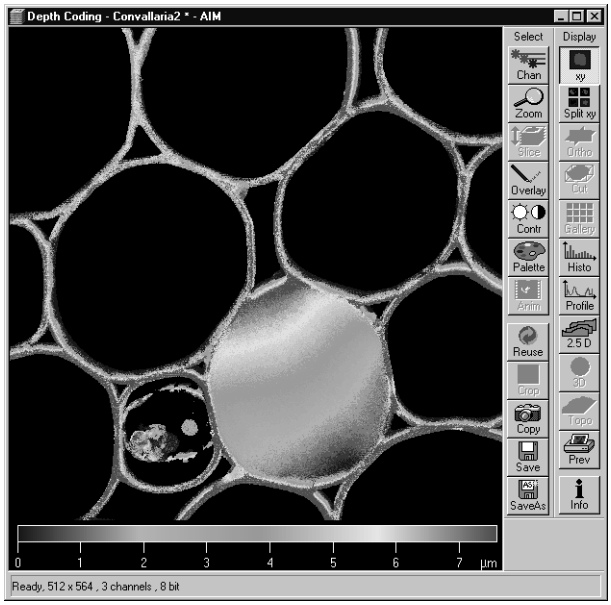


Fig. 5-149 Depth Coding image

5.7.1.4 Preview panel

The **Preview** function permits you to regard the influence of parameter changes in an **Image Display** window.

- After finding the optimum adjustment using the **Preview** function, you have to generate the final version of the image using the **Apply** button.
  - The system then generates a color-coded depth map for the selected channel.

5.7.2 Projection

By means of the **Projection** function, one single projection or a series of projections can be calculated after rotation of the data package about the X, Y or Z axis.

A stack of images must be available.

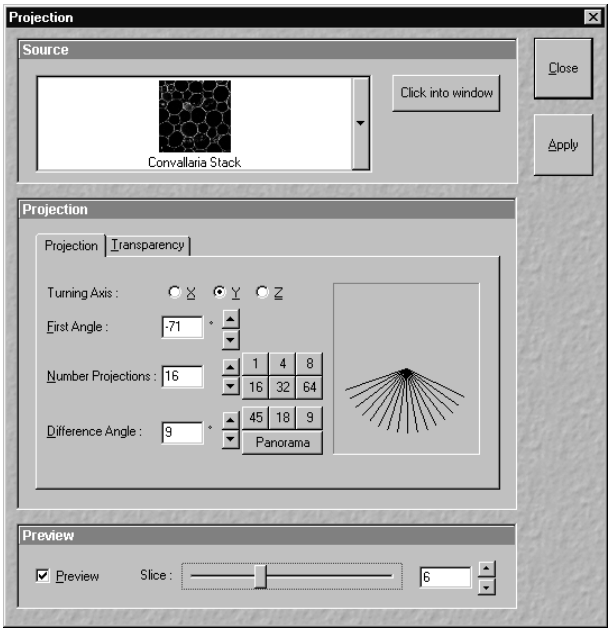


Fig. 5-150 Projection window

5.7.2.1 Open / Close the Projection window

- Click on the **Projection** button in the **3D View** subordinate toolbar of the **Main** menu.
  - This opens the **Projection** window.
- Click on the **Close** button to quit the window.

5.7.2.2 Source panel

- Select the image for the projection operation from the image selection box.

5.7.2.3 Projection panel

- On the **Projection** panel, set the parameters needed for the animation: **Turning Axis**, **First Angle**, **Number Projections** and **Difference Angle** in the **Projection** tab and the **Mode** parameters in the **Transparency** tab.

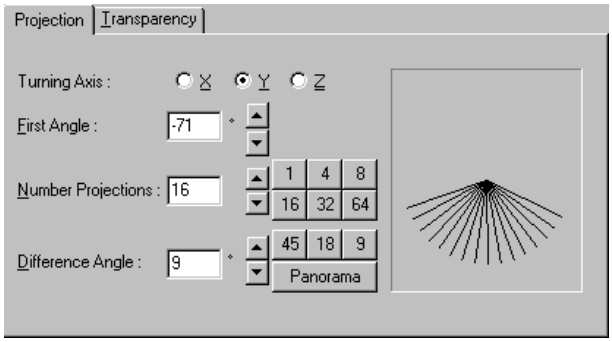


Fig. 5-151 Projection tab

(1) Projection tab

Turning Axis X/Y/Z: Selects the axis about which the data package is to be rotated.

First Angle: Rotation angle in degrees.

Number Projections: Number of projections for a sequence (variable from 0 to 100).

Difference Angle: Angle increment of a sequence.

☞ The number keys permit the direct selection of preset values for **Number Images** and **Difference Angle**. If the **Panorama** button is pressed, a panorama sequence of the image series is computed.

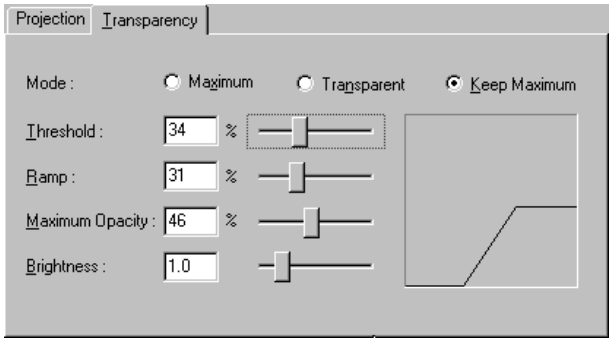


Fig. 5-152 Transparency tab

(2) Transparency tab

Mode **Maximum**: The color is defined by the Z position of the brightness value.

Mode **Transparent**: The transparent projection is built up from the rear to the front. The color is defined by the Z position at which the original was last higher than or equal to Threshold.

Mode

Keep Maximum: Activating this option modifies the specification governing calculation of the projection.

Threshold: Pixel value at which the ramp rises (variable from 0 to 100 %).

Ramp: Slope of the ramp (variable from 0 to 100 %; 0 % corresponds to a vertical rise).

Maximum Opacity: Degree of visibility at the top corner of the ramp (variable from 0 to 100 %; 0 % corresponds to the bottom edge in the diagram).

Brightness: The image can be brightened again by modifying the value (from 0.2 to 5).

5.7.2.4 Preview panel

Fig. 5-153 Preview panel

The **Preview** function permits you to regard the influence of parameter changes in an **Image Display** window.

The **Slice** slider enables you to select the slice which shall be displayed in the **Preview Image Display** window.

- After finding the optimum adjustment using the **Preview** function, you have to generate the final version of the image using the **Apply** button.
  - The projection appears. The computation can be followed in the image or by the progress bar.

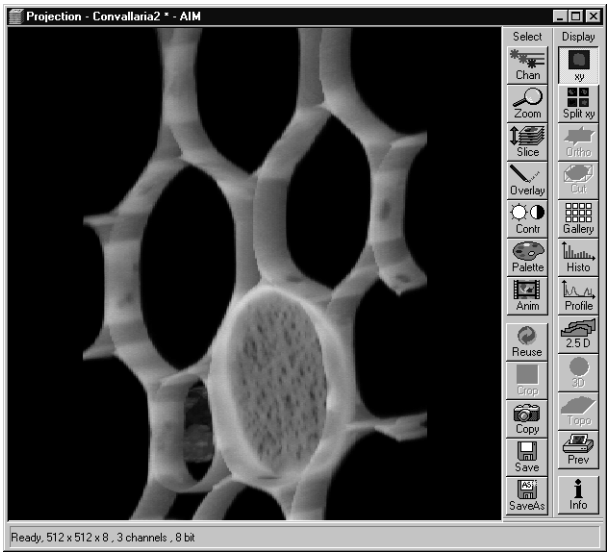


Fig. 5-154 Projection image

☞ The computed 3D sequence can be animated with the **Anim** button in the **Select** toolbar.

In addition, the **Animate** window appears, in which you can influence the direction and speed of 3D image rotation (see section 5.15.8, page 5-295).

You can browse through the rotation sequence manually with the **Slice** button in the **Select** toolbar and the **Slice** slider.

- To view the computed 3D sequence as a gallery on the screen, click on the **Gallery** button in the **Display** toolbar.

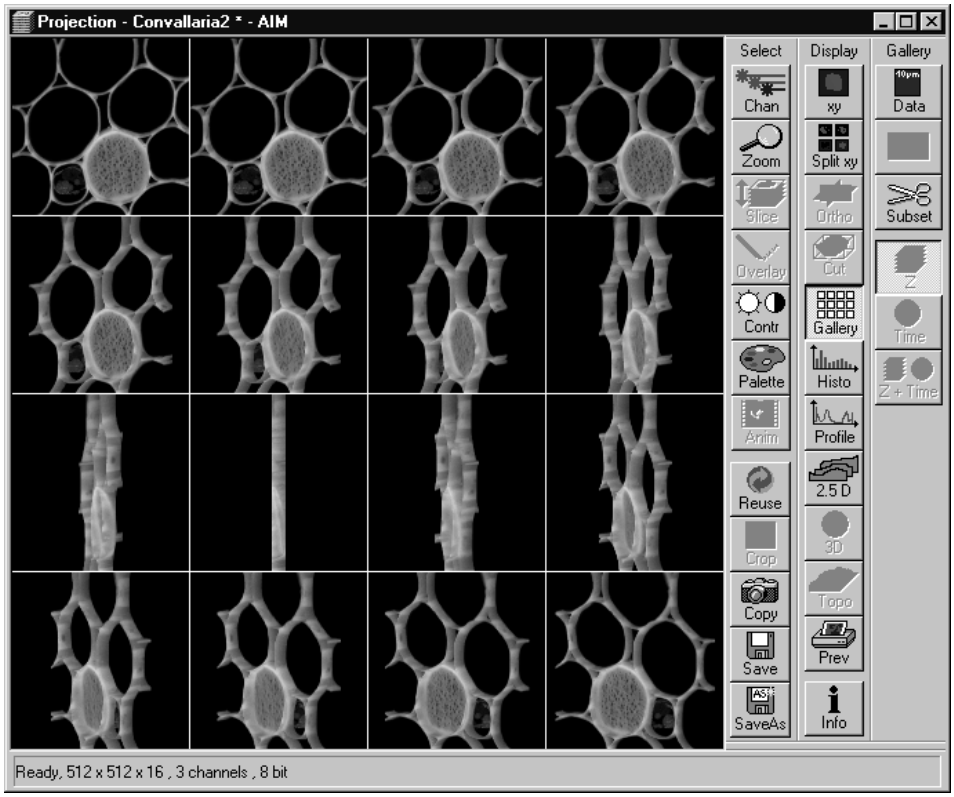


Fig. 5-155 Projection image (Gallery)

5.7.3 Stereo

Stereoscopic images can be generated in a variety of ways by means of the **Stereo** function.

A stack of images must be available.

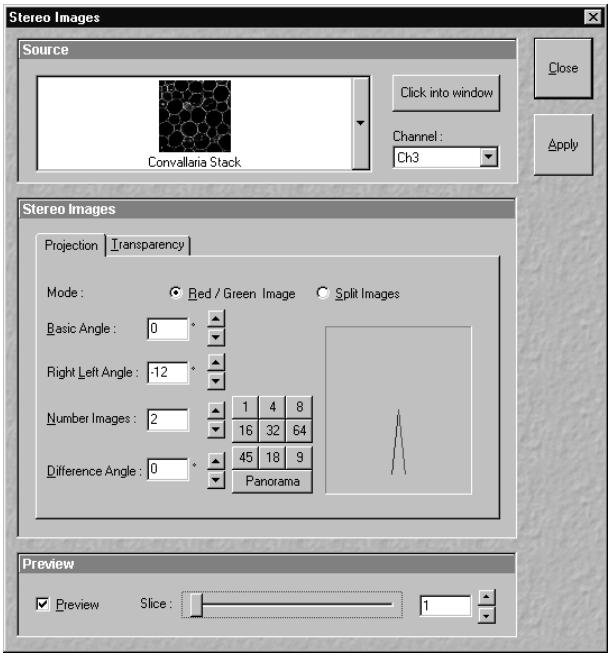


Fig. 5-156 Stereo Images window

5.7.3.1 Open / Close the Stereo Images window

- Click on the **Stereo** button in the **3D View** subordinate toolbar of the **Main** menu.
  - This opens the **Stereo Images** window.
- Click on the **Close** button to quit the window.

5.7.3.2 Source panel

- Select the image for the projection operation from the image selection box.
- Select the channel to be used from the **Channel** selection box.

5.7.3.3 Stereo Images panel

- In the **Stereo Images** panel, set the parameters needed for stereoscopic viewing: **Mode**, **Basic Angle**, **Right Left Angle**, **Number Images** and **Difference Angle** in the **Projection** tab and the **Mode** parameters in the **Transparency** tab.

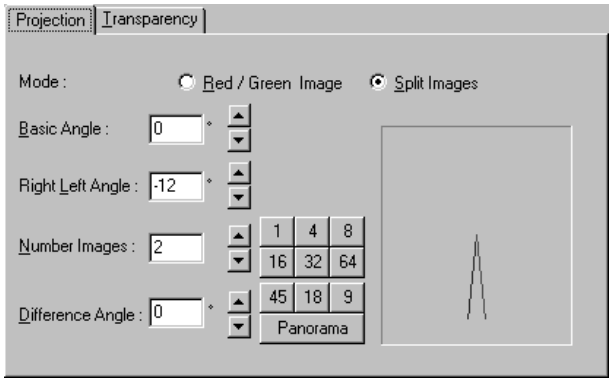


Fig. 5-157 Projection tab

(1) Projection tab

Mode

Red / Green Image: This displays a stereo image for red / green anaglyph observation using red / green spectacles.

Mode

Split Images: This displays a pair of stereo images for observation through a stereoscope. Colored stereo images are also possible.

Basic Angle: Direction angle at which the specimen is viewed; 0° from the front, 180° from the rear.

Right Left Angle: Angle between right and left (red and green) image.

Number Images: Number of 3D images (slices).

Difference Angle: Angle increment of a sequence.

☞ The number keys permit the direct selection of preset values for **Number Images** and **Difference Angle**. If the **Panorama** button is pressed, a panorama sequence of the image series is computed.

(2) Transparency tab

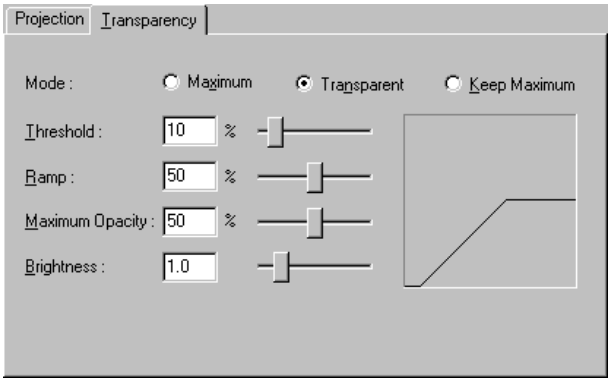


Fig. 5-158 Transparency tab

Mode **Maximum**: The color is defined by the Z position of the brightness value.

Mode **Transparent**: The transparent projection is built up from the rear to the front. The color is defined by the Z position at which the original was last higher than or equal to Threshold.

Mode **Keep Maximum**: Activating this option modifies the specification governing calculation of the projection.

Threshold: Pixel value at which the ramp rises (variable from 0 to 100 %).

Ramp: Slope of the ramp (variable from 0 to 100 %; 0 % corresponds to a vertical rise).

Maximum Opacity: Degree of visibility at the top corner of the ramp (variable from 0 to 100 %; 0 corresponds to the bottom edge in the diagram).

Brightness: The image can be brightened again by modifying the value (from 0.2 to 5).

5.7.3.4 Preview panel

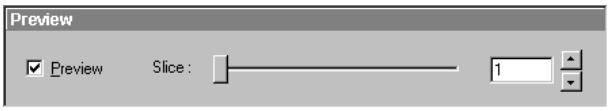


Fig. 5-159 Preview panel

The **Preview** function permits you to regard the influence of parameter changes in an **Image Display** window.

The **Slice** slider enables you to select the slice which shall be displayed in the **Preview Image Display** window.

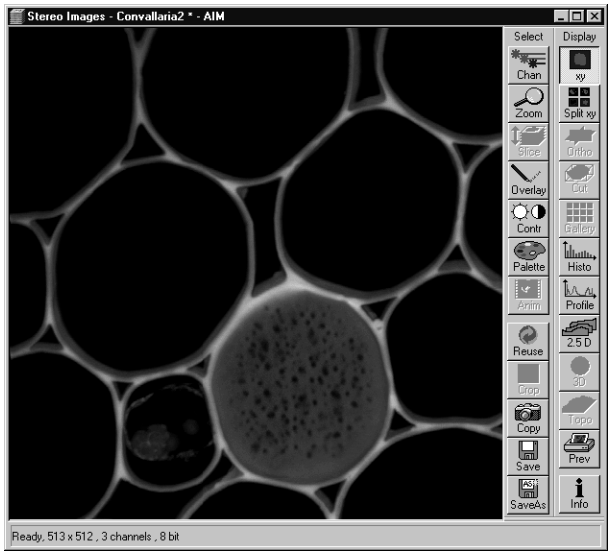


Fig. 5-160 Stereoscopic image (red / green)

- To start computation of the stereoscopic image, click on the **Apply** button.
  - The image is built up twice (once each for the red and green colors), resulting in a stereoscopic image.

☞ The stereoscopic effect can only be seen with the aid of red / green 3D goggles. The red lens is to be used for the right eye and the green lens for the left eye.

The presentation can be modified by selecting the **Split Images** (Mode) option in the **Projection** tab of the **Stereo Images** panel.

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

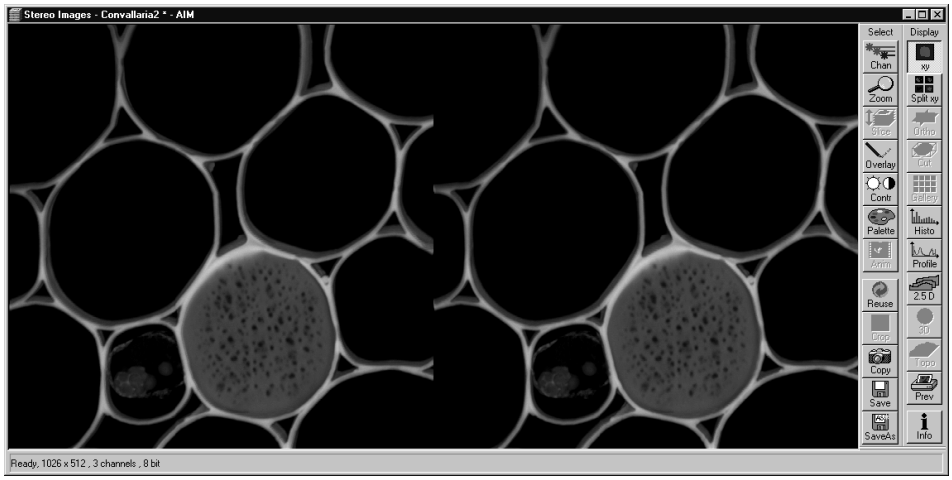
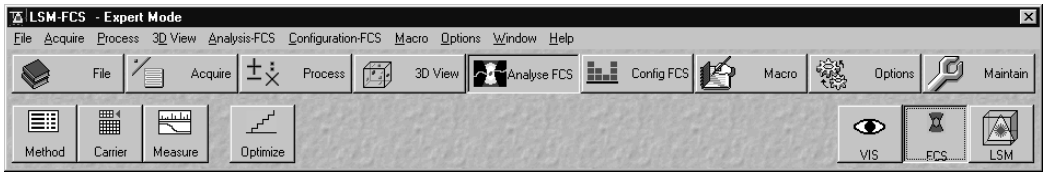


Fig. 5-161 Stereoscopic image (split)

5.8 Analyse FCS Menu

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



← **Analyse FCS** subordinate toolbar

Fig. 5-162 Analyse FCS menu

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

- Method.
- Carrier.
- Measure.
- Optimize (if necessary).

The **VIS**, **FCS** and **LSM** buttons switch the beam path of the microscope (VIS for viewing, FCS for FCS measurements via laser excitation with monitor observation, LSM for LSM laser operation with monitor observation).

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

5.8.1 Select Method

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

- Click on the **Method** button in the **Analyse FCS** subordinate toolbar of the **Main** menu.
  - This opens the **Method Selection ...** window (Fig. 5-163). The name of the currently selected method will appear in the status bar of the window.

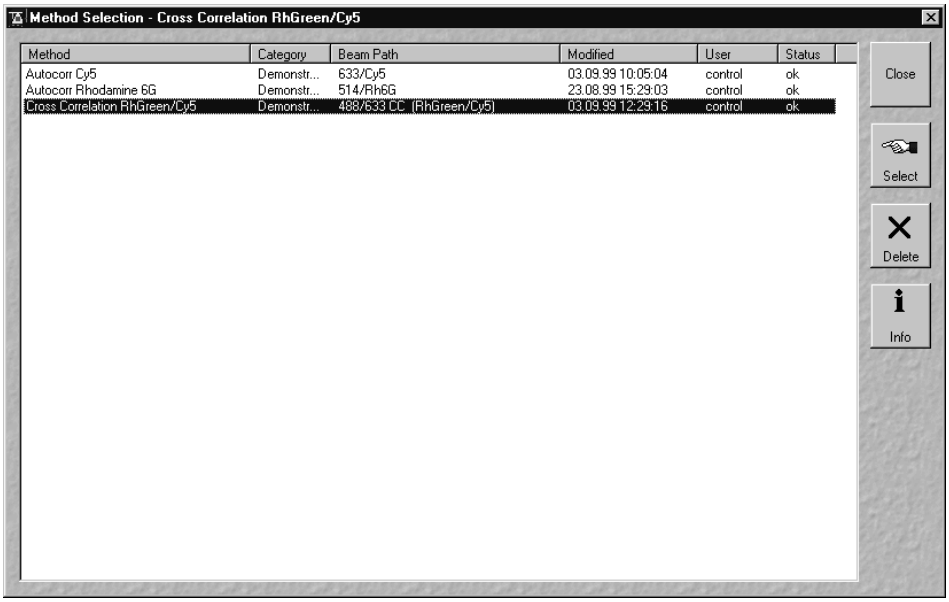


Fig. 5-163 Method Selection ... window

- 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 **Method Selection ...** 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 **Select** button. The window will then be 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 a window will pop up which shows a detailed description of the analysis method (Fig. 5-164).

☞ All parameters will load with this method.

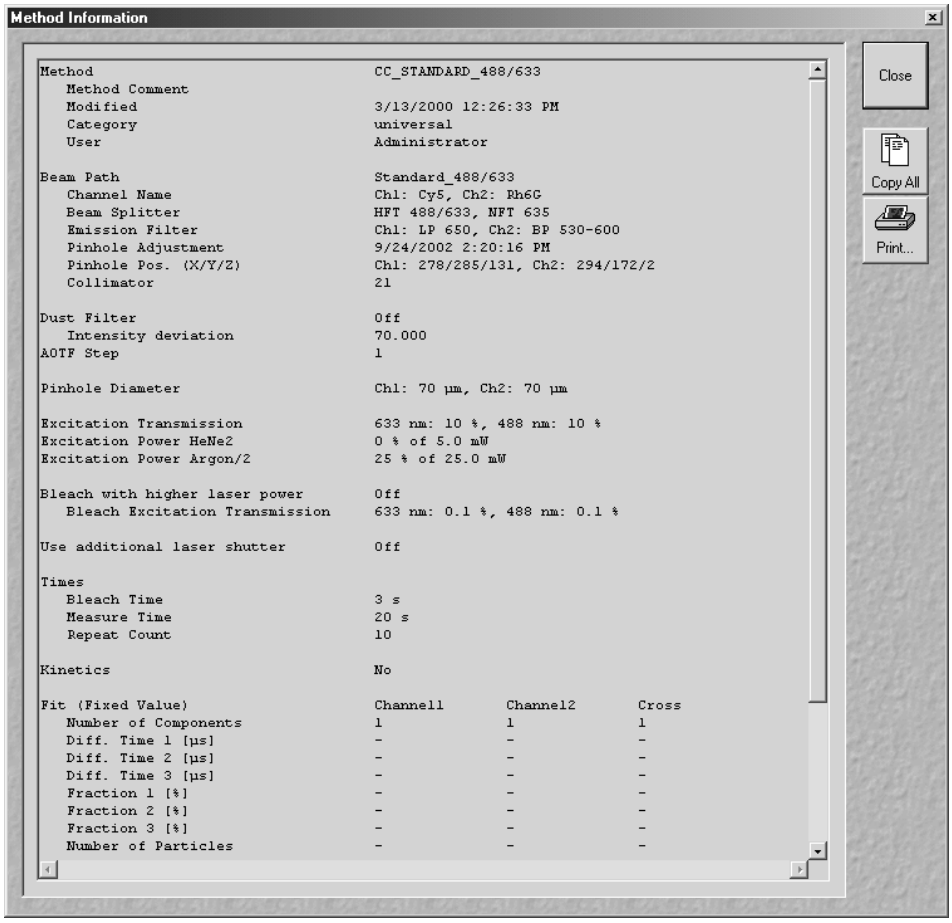


Fig. 5-164 Detailed description of the analysis method open

- 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.

- The **Close** button of the **Method Selection ...** window allows you to leave the dialog without any action.

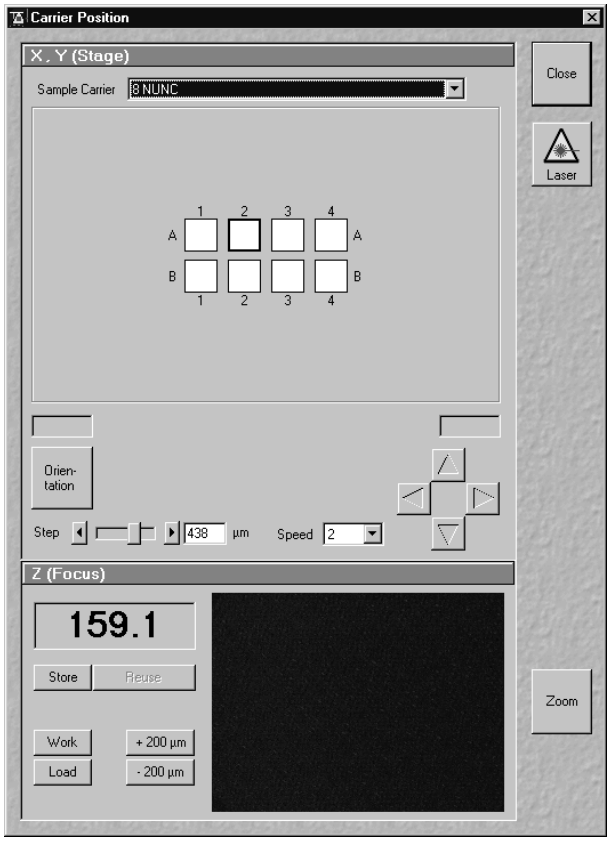


Fig. 5-165 Carrier Position window

5.8.2 Select Carrier

The **Carrier** function opens the **Carrier Position** window. 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.

5.8.2.1 Opening / Closing the Carrier Position window

- Click on the **Carrier** button in the **Analyse FCS** subordinate toolbar of the **Main** menu (Fig. 5-162).
  - The sample select window (**Carrier Position** window) pops up (Fig. 5-165).

☞ 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.

- Click on the Laser button to open the **Laser Control** window (for settings see **Config FCS** menu, page 5-208).

- Close the **Carrier Position** window by clicking the **Close** button.

5.8.2.2 Function description

(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.

Selecting the sample carrier

- Select the sample carrier you are currently using in the **Sample Carrier** selection box of the **X, Y (Stage)** panel.

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.

The chamber to be used for the X and Y alignment of the stage can be selected at a click of the mouse. It then appears with a black frame and is displayed in the **Current Stage Position** display field (left field, e.g. **A - 2**). The current position of the mouse pointer is shown in the right field.

- Select the chamber for X and Y alignment of the stage.

Orienting the Sample in x and y

- Now click on the **Orientation** button in the **X, Y (Stage)** panel (Fig. 5-167).
- Select one of the chambers of the sample for orientation in x and y.
  - The selected chamber is displayed in yellow with a black frame.
- Position the center of the selected 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, click the **Orientation** button once more. The stage is now oriented.

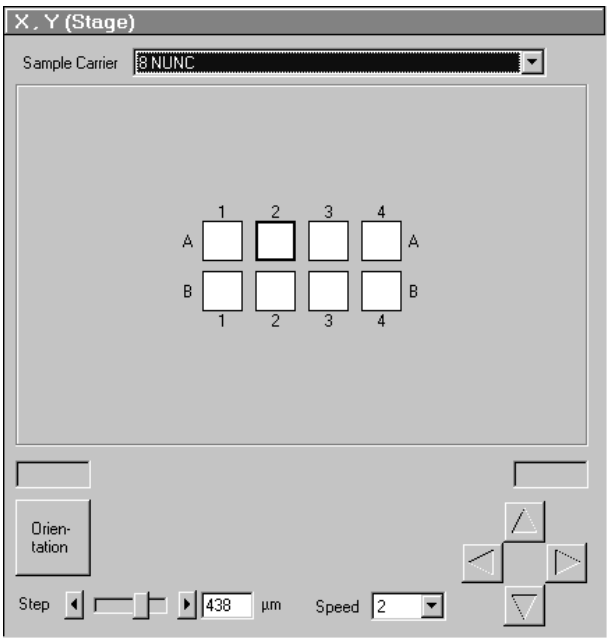


Fig. 5-166 X, Y (Stage) panel

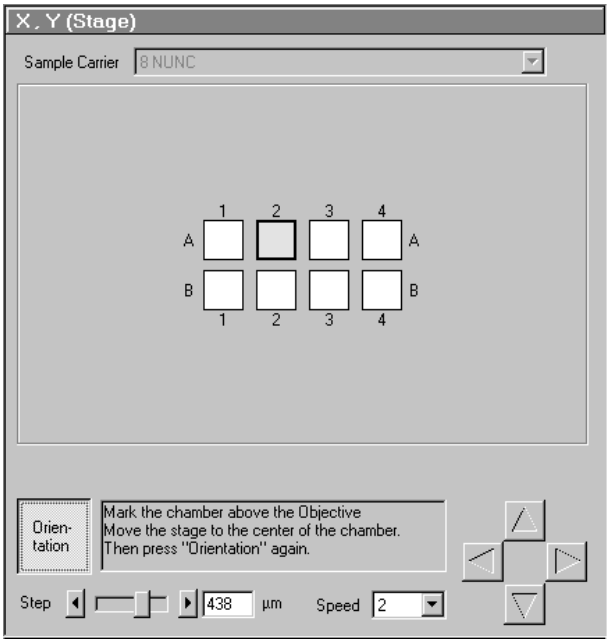


Fig. 5-167 X, Y (Stage) panel (Sample drives unlocked)

- 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 chamber is displayed in the **Carrier Position** window in yellow with a black frame.

Moving the microscope stage

- 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.

(2) Z (Focus) panel

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

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).
Zoom button (on the right-hand side of the **Carrier Position** window)	Opens **Camera** window for zoom function.

☞ 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.

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.
- The camera window shows interference fringes caused by the laser light (Fig. 5-168).

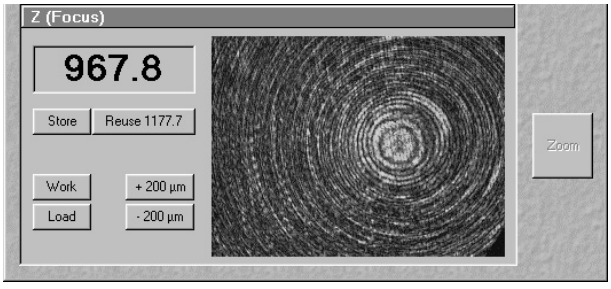


Fig. 5-168 Camera image far away from the lower cover slip surface

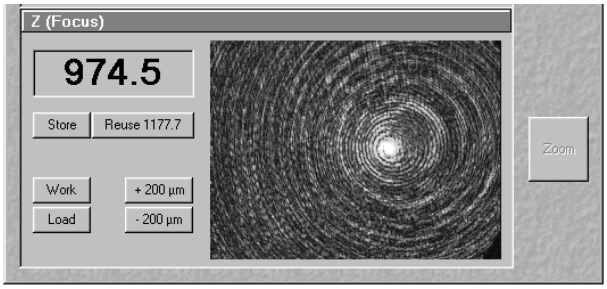


Fig. 5-169 Camera image near the lower cover slip surface

- 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 camera window looks similar to (Fig. 5-169).

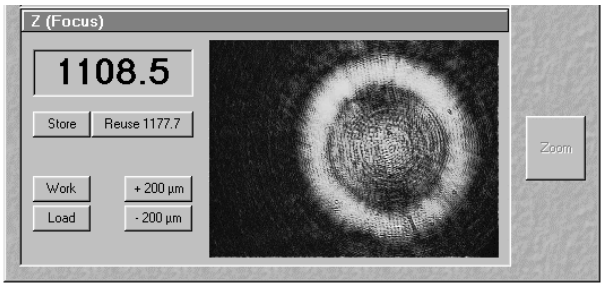


Fig. 5-170 Lower cover slip surface

- Continue cautiously moving upwards. If the camera image looks like (Fig. 5-170), you are close to the lower cover slip surface.
- Having cautiously moved over a short distance the cover slip reflection should look as shown in (Fig. 5-171). The upper cover slip surface has been found.
- Store the reached position using the **Store** button.

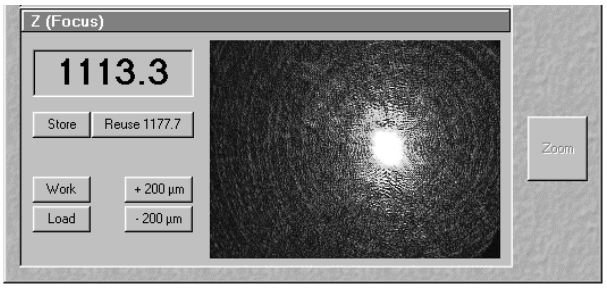


Fig. 5-171 Upper cover slip surface

- 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.

5.8.3 Measure Method

The **Measure** function opens the **Method Measurement ...** window. It is used for setting the measurement parameters and for starting the measurement.

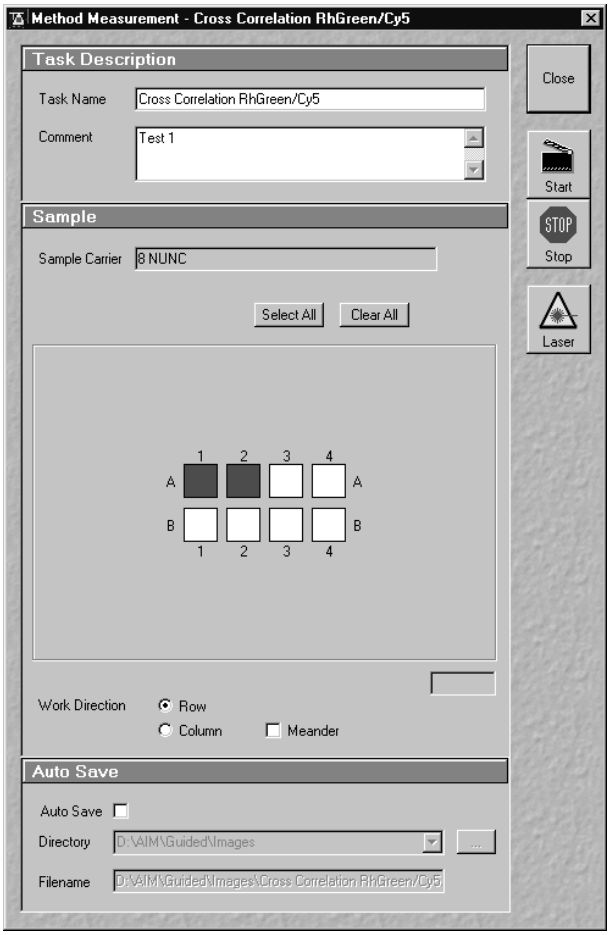


Fig. 5-172 Method Measurement ... window

5.8.3.1 Opening / Closing the Method Measurement ... window

- Click on the **Measure** button in the **Analyse FCS** subordinate toolbar of the **Main** menu (Fig. 5-162).
  - The **Method Measurement ...** window will appear on the screen (Fig. 5-172). The name of the currently used method will appear in the status bar of the window.

- Close the **Method Measurement ...** window by clicking the **Close** button.

5.8.3.2 Function description

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

Close button	The **Method Measurement ...** window is closed.
Start button	Starts the measuring procedure.
Stop button	Ends the measuring procedure.
Laser button	Opens the **Laser Control** window.

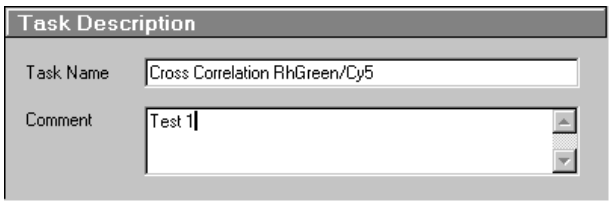


Fig. 5-173 Task Description panel

(1) Task Description panel

In the **Task Description** panel you can enter the task name for the measuring procedure.

The **Comment** field allows you to store a comment on the measurement you are performing.

- Enter a task name and a comment (if necessary) for operation.

The task name will be used as file name when saving the measured data. It must therefore meet the WINDOWS conventions for file names.

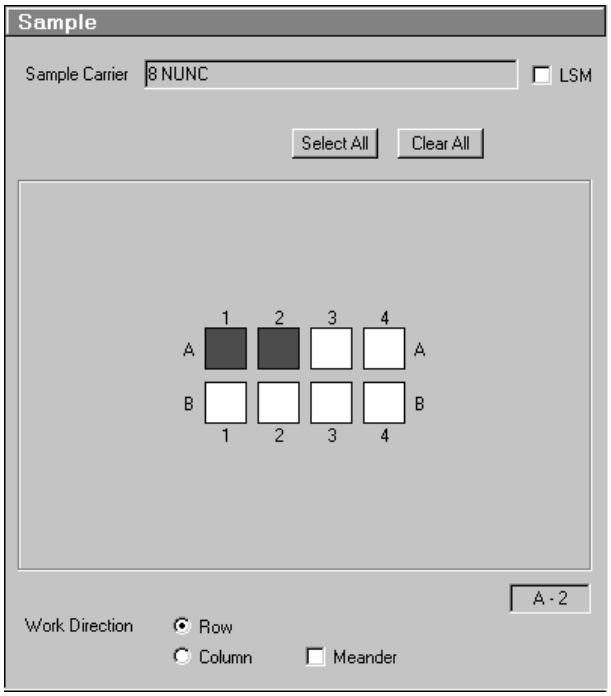


Fig. 5-174 Sample panel

(2) Sample panel

The samples to be measured can be selected by mouse in the schematic drawing of the sample carrier. Activated chambers will be highlighted in the drawing. 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 using the mouse.

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.

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

Following functions are available:

LSM check box	If **LSM** is activated, the **Sample** panel changes to define positions in an opened LSM scan image.
Select All button	Selects all chambers of the sample carrier.
Clear All button	Deselects all chambers of the sample carrier.
Row radio button	If **Row** is chosen, measurement is performed row by row.
Column radio button	If **Column** is chosen, measurement is performed column by column.
Meander check box	If **Meander** is chosen, measurement of the row or column is made in meanders.

(3) Sample panel with activated LSM check box

If **LSM** is activated, you can define positions for FCS measurements in a just scanned LSM image (see also section 5.18).

You can either press the **Crosshair** button or check the **LSM** box (Fig. 5-175). If the Crosshair button is pressed, a Crosshair will appear in the Scan Image. If the **LSM** box is checked, the **Sample Carrier** display box is activated (Fig. 5-175).

In the **Sample Carrier** display box it is shown that the **Define Positions in LSM Image** mode is activated.

The **Positions** list shows the numbers and coordinates of the selected positions in the LSM scan image (Fig. 5-176).

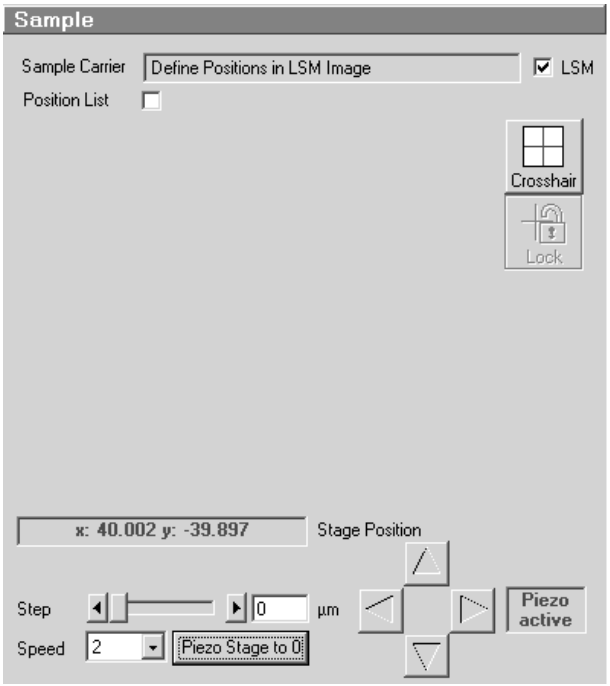


Fig. 5-175 Sample panel

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.

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.

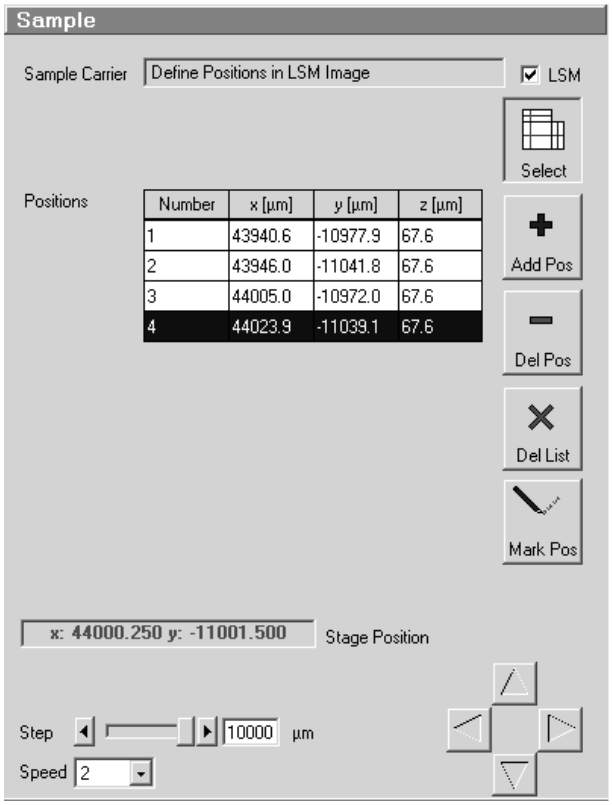


Fig. 5-176 Sample panel for defining positions

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.

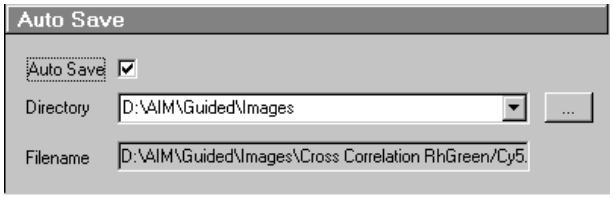


Fig. 5-177 Auto Save panel

(4) Auto Save panel

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.

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.

5.8.3.3 Measurement procedure

After setting the parameters the measurement can be run.

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

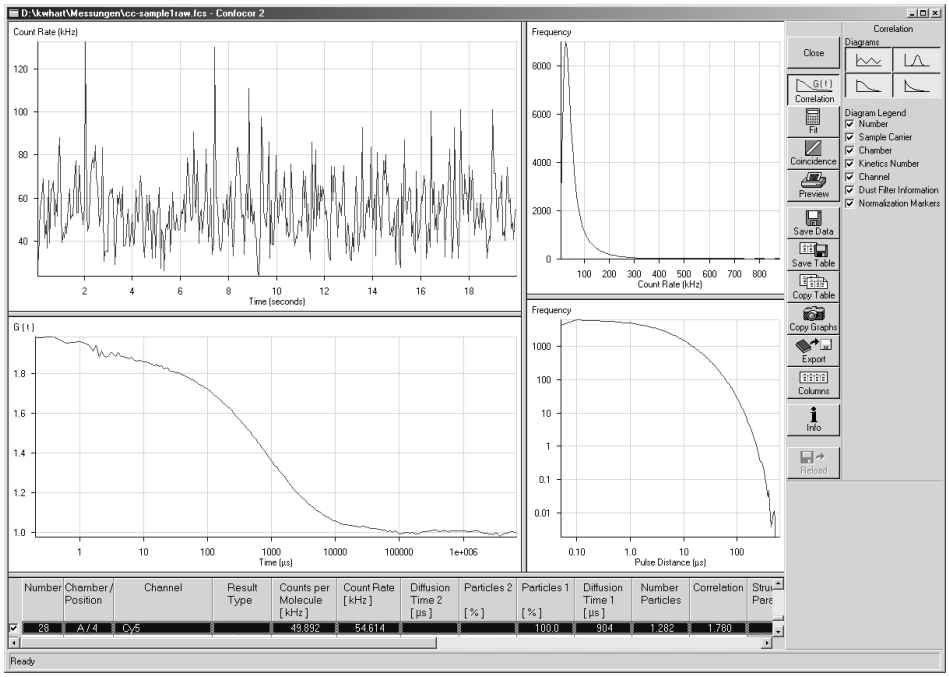


Fig. 5-178 FCS data evaluation window

☞ The further procedure will be explained in section 5.17, *Data Evaluation and Result Presentation for FCS Measurements*.

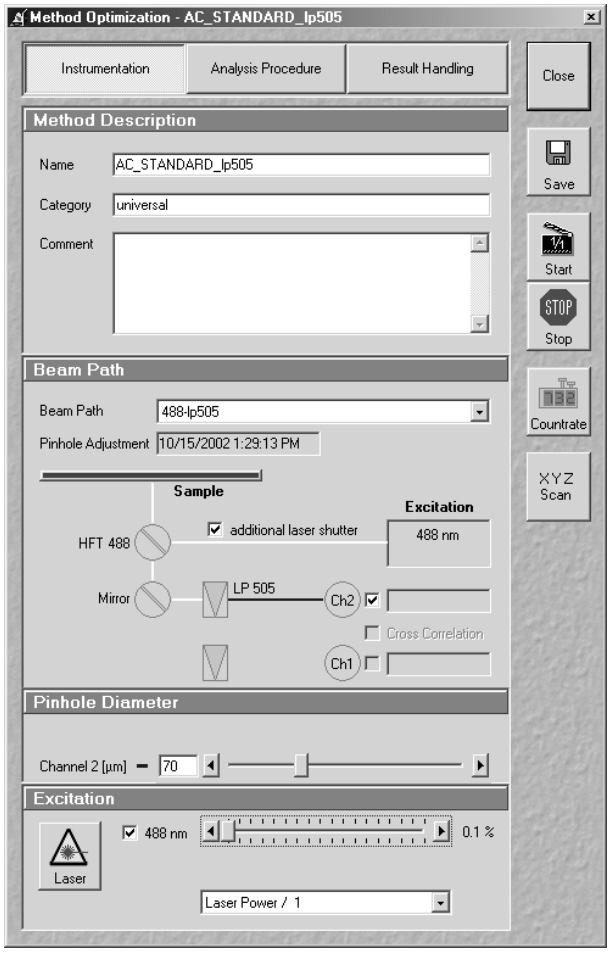


Fig. 5-179 Method Optimization ... window (Instrumentation subwindow)

5.8.4 Optimize Method

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

5.8.4.1 Opening / Closing the Method Optimization ... window

- Click on the **Optimize** button in the **Analyse FCS** subordinate toolbar of the **Main** menu (Fig. 5-162).
  - The **Method Optimization ...** window will appear.

- Close the **Method Optimization ...** window by clicking the **Close** button.

5.8.4.2 Function description

An analysis method has three major sections:

- **Instrument** settings
- **Analysis Procedure** settings
- **Result Handling** parameters.

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 Optimization ...** window:

Close button	The **Method Optimization ...** window is closed.
Save button	Saves the optimized or new created measurement method.
Start button	Starts the measuring procedure for optimizing.
Stop button	Ends the measuring procedure.
Count rate button	Opens the **Count rate** window.
X,Y,Z-Scan button	Opens the **X,Y,Z-Scan** window for performing a X,Y,Z-scan.

5.8.4.3 Instrumentation

- Click on the **Instrumentation** button on top of the **Method Optimization ...** window (Fig. 5-179).
  - The **Instrumentation** subwindow appears on the screen.

(1) Method Description panel

In the **Method Description** panel, an existing method name for the measuring procedure can be edited, or a new one entered. In the **Category** filed, a category name for the method can be entered.

The **Comment** field allows you to store a comment on the method you are creating.

- Enter a name, a category and a comment for the method.

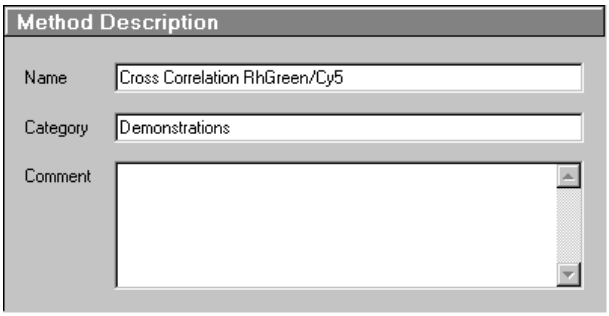


Fig. 5-180 Method Description panel

(2) Beam Path panel

In the **Beam Path** panel you can select a saved beam path configuration. The selected beam path will be shown as a drawing.

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.

In the **Pinhole Adjustment** display box, the date of the last pinhole adjustment is shown.

- 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.

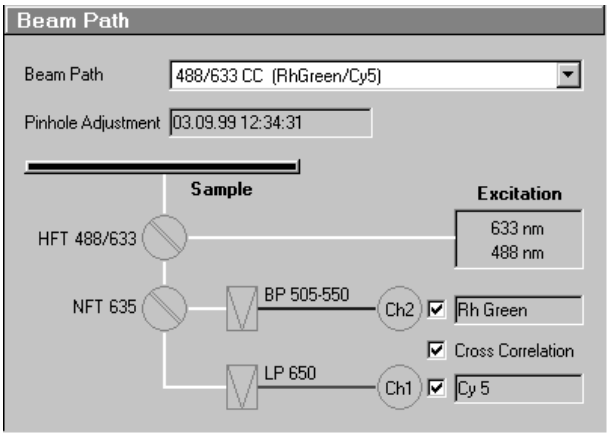


Fig. 5-181 Beam Path panel

☞ It is not possible to define a new beam path in this menu. If a new beam path is required, open the **Beam Path Definition** menu via the **Define Beam Path** button (section 5.9.4, page 5-218) in the **Config FCS** subordinate toolbar of the **Main** menu.

Once a new beam path is ready, it can be immediately used in the **Method Optimization ...** window.

Fig. 5-182 Pinhole Diameter panel

(3) Pinhole Diameter panel

In the **Pinhole Diameter** panel you can set the pinhole diameters for channel 1 and 2 using the sliders or the input boxes.

- It is recommended to 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

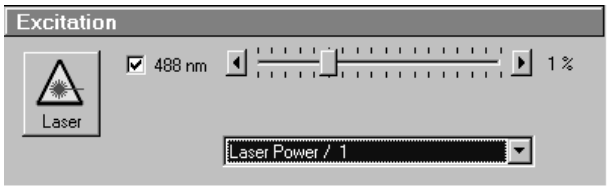


Fig. 5-183 Excitation panel

(4) Excitation panel

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.

The selection box allows you to choose from four possibilities of setting the laser power:

Laser power / 1: power defined by AOTF slider.

Laser power / 10: 10 fold reduced laser power.

Laser power / 100: 100 fold reduced laser power.

Laser power / 1000: 1000 fold reduced laser power.

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 **Method Optimization ...** 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.

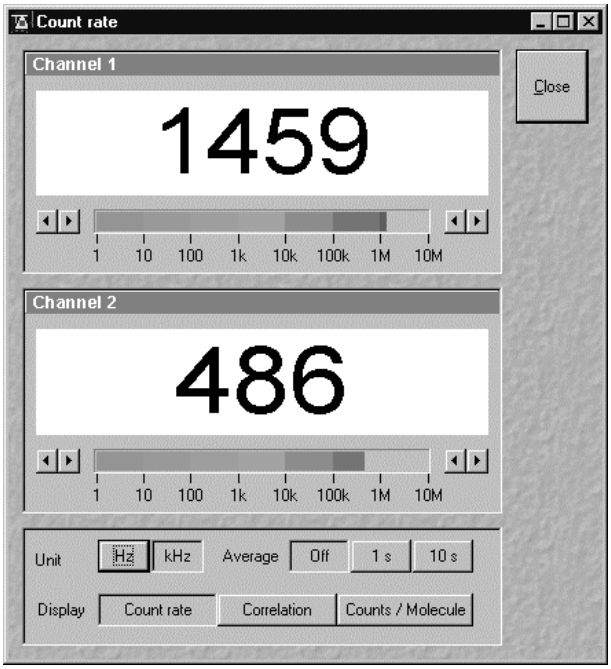


Fig. 5-184 Count rate window

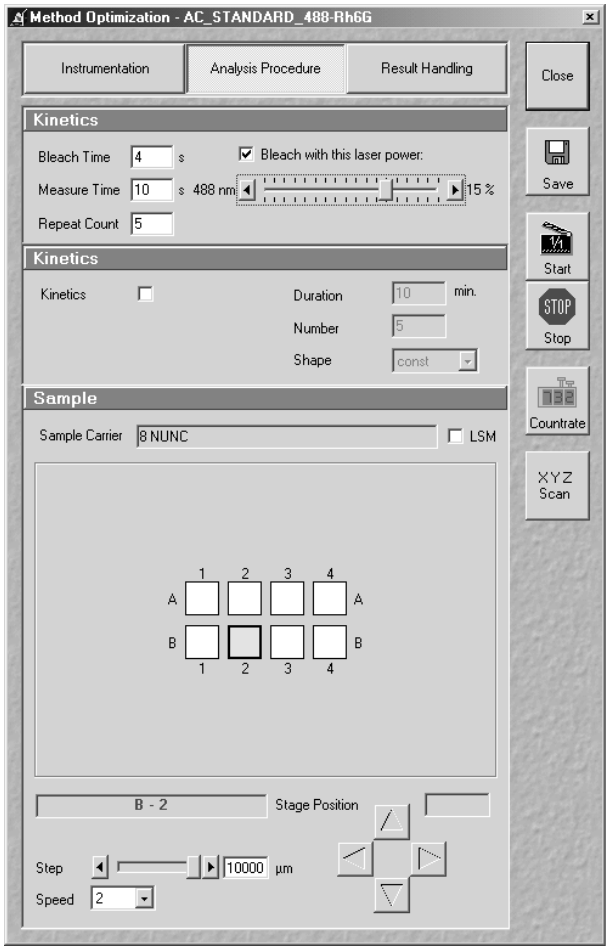


Fig. 5-185 Method Optimization ... window (Analysis Procedure subwindow)

5.8.4.4 Analysis Procedure

- Click on the **Analysis Procedure** button on top of the **Method Optimization ...** window.
  - The **Analysis Procedure** subwindow appears on the screen.

(1) Times panel

In the **Times** panel you can set the values for the **Bleach Time**, **Measure Time** and **Repeat Count**.

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 the **Bleach with this laser power** check box was not activated, bleaching occurs with the laser power defined by the AOTF setting under **Instrumentation** in the **Method Optimisation**. If the box is checked, the laser power defined by the slider is used.
- 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.
- 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.

(2) Kinetics panel

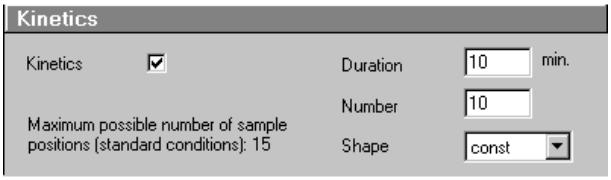


Fig. 5-186 Kinetics panel

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

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

The input boxes **Duration** and **Number** permit entry of the overall duration of the measurement and the measuring cycles to be performed during this period. Measuring cycle means that all the selected chambers are measured once in the determined order.

Depending on these two parameters, the maximum number of chambers to be measured is calculated automatically and displayed under **Maximum possible number of sample positions (standard conditions): ...**. If **0** is displayed, the measurement cannot be performed, and a relevant message is displayed on the screen if you try to start the measurement nevertheless.

The **Shape** selection box allows you to choose from three possibilities of distributing the measuring cycles (**Number)** to the overall measuring time (**Duration**):

const: The period between two successive measuring cycles is constant.

lin: The period between two successive measuring cycles is increased linearly, i.e. it is doubled each time.

log: The period between two successive measuring cycles is increased logarithmically.

- Enter the duration (**Duration** input box) of your kinetics measurement and the number (**Number** input box) of individual correlation measurements you want to perform.
- In the **Shape** selection box, set one of the shape options **const**, **lin** or **log**.

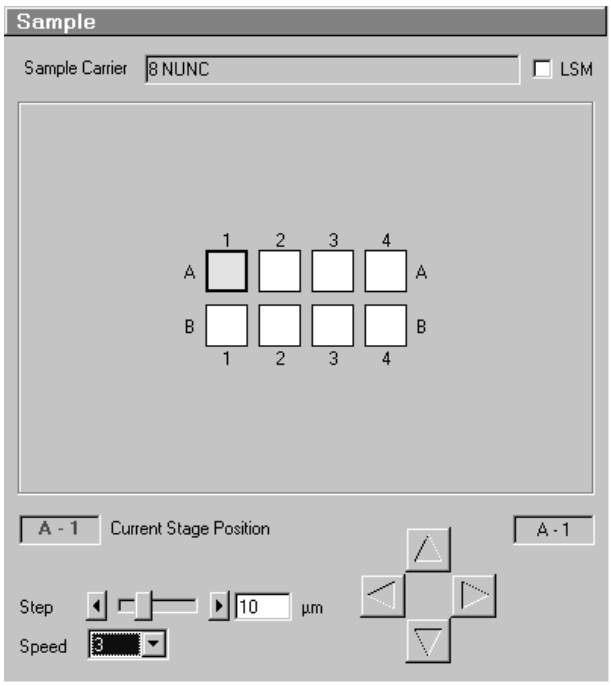


Fig. 5-187 Sample panel

(3) Sample panel

In the **Sample** panel you can select the chamber for the optimization procedure (only one is selectable).

- Click on the chamber you want to use for sample development.
  - The motorized microscope stage moves the selected chamber to the defined position under the objective.
- If you want to move to other positions within a chamber, use the arrow buttons to move the microscope stage.
- Set the size of the steps to be performed via the **Step** slider (or the 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 one of the three travel speeds from the **Speed** selection box.

- By activating the **LSM** check box, you can define the positions in a LSM image in the same way as described in section 5.8.3, page 5-193.

5.8.4.5 Result Handling

- Click on the **Result Handling** button on top of the **Method Optimization ...** window.
  - The **Result Handling** subwindow appears on the screen.

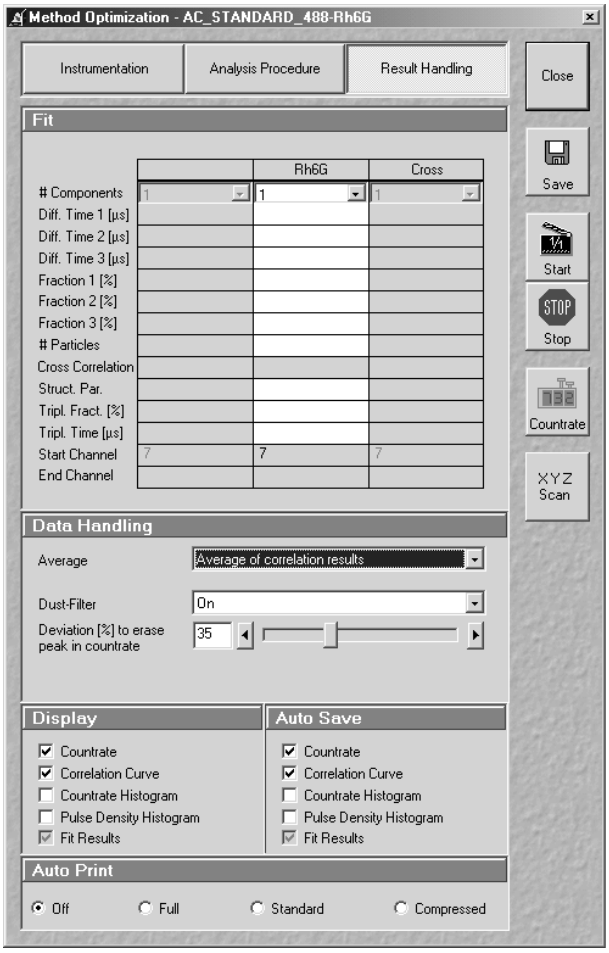


Fig. 5-188 Method Optimization ... window (Result Handling subwindow)

(1) Fit panel

The **Fit** panel of the menu allows you to fix various model parameters, which otherwise will be a fit result. The fixation of the number of **Components** in this model is *mandatory*, while the fixation of all other parameters is optional.

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.

- Enter the model parameters to be fixed in the input boxes.

(2) Data Handling panel

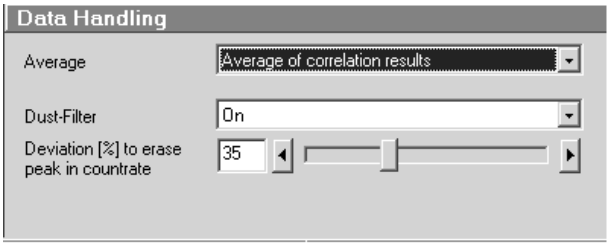


Fig. 5-189 Data Handling panel

The **Data Handling** panel allows you to select if you want as an average the mean values of the correlation curves or the mean of the fit results by choosing the appropriate fit procedure in the **Average** selection box. In the first case, the single values of the different correlation curves are averaged and the new curve fitted. In the second case, the fitted parameters of each correlation curve are averaged.

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 the slider. 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.

☞ Please note that the cut off count rate is defined as the exceeding of the average count rate during a certain measurement period. Thu, 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.

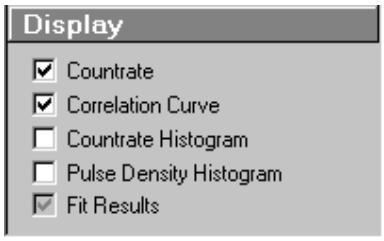


Fig. 5-190 Display panel

(3) Display panel

In the **Display** panel you can tick which result curves (**Count Rate**, **Correlation Curve**, **Count Rate Histogram** and **Pulse Density Histogram**) will be displayed when the measurement runs. The meaning of these curves is explained in the **Measurement** window.

- Select the appropriate options via mouse click.

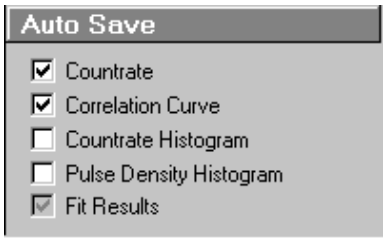


Fig. 5-191 Auto Save panel

(4) Auto Save panel

The **Auto Save** panel allows you to determine which of the resulting curves will be saved automatically.

- Select the appropriate options via mouse click.

Fig. 5-192 Auto Print panel

(5) Auto Print panel

In the **Auto Print** panel you can determine whether or not you want to have an automatic protocol printout (with varying degree of detail).

- Select the appropriate radio buttons via mouse click.

5.8.4.6 X,Y,Z-Scan for positioning the focus within structured samples

To make the positioning of the detection volume easier, the ConfoCor 2 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. 5-194) 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. 5-193).

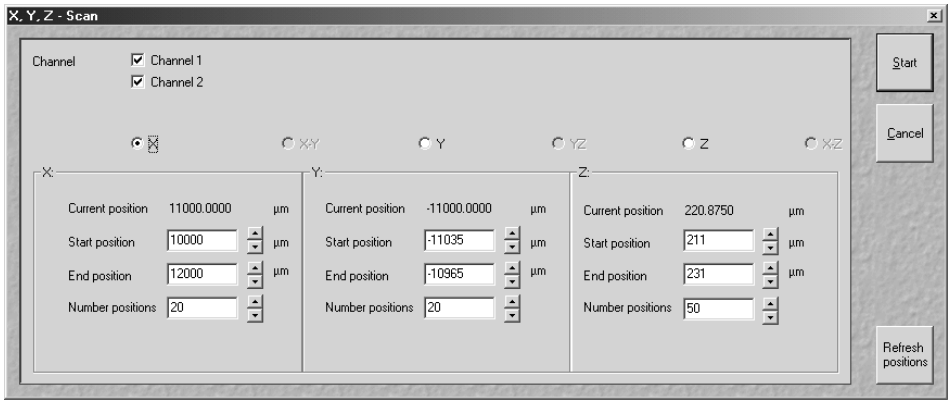


Fig. 5-193 X,Y,Z-Scan operating window

For X,Y,Z-Scanning proceed as follows:

- If you use the Axiovert 100, first switch to the visual or TV mode using the tube sliders on the microscope stand so that you are able to view the object (the label near the tube sliders will show the correct tube slider position) or press the **VIS** button (Axiovert 200).
- 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. 5-194).

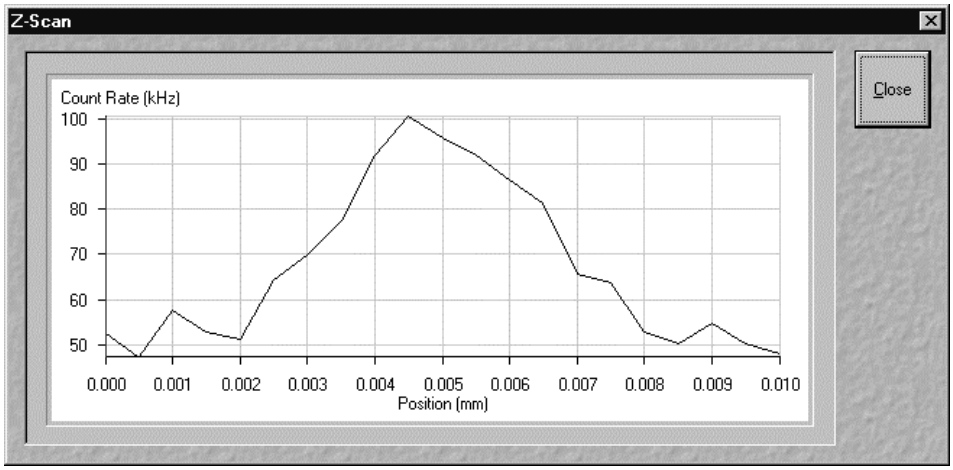


Fig. 5-194 Z-Scan result window

When the scan has been finished, a vertical red line appears at the y-axis at the left-hand side. 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.
- Then close the window with **Close**. The **Refresh Positions** button will actualise the current position if pressed.

Fig. 5-195 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).

5.8.5 VIS, FCS and LSM Buttons

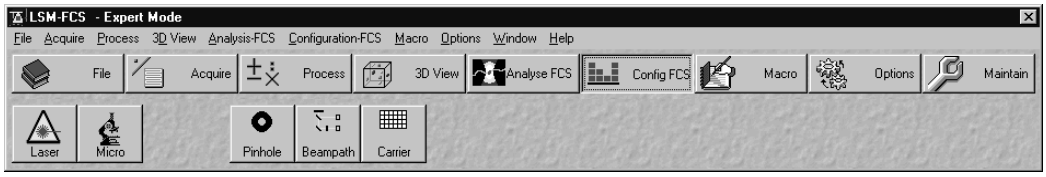
The **VIS**, **FCS** and **LSM** buttons are included in the **Analyse FCS** subordinate toolbar of the **Main** menu.

They switch the setting of the beam path of the microscope:

- **VIS**: observation via the eyepieces of the binocular tube
- **FCS**: screen observation via laser excitation using the FCS measurements and software evaluation
- **LSM**: screen observation via laser excitation using the LSM image acquisition and software evaluation

5.9 Config FCS Menu

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



← **Config FCS** subordinate toolbar

Fig. 5-196 Config FCS menu

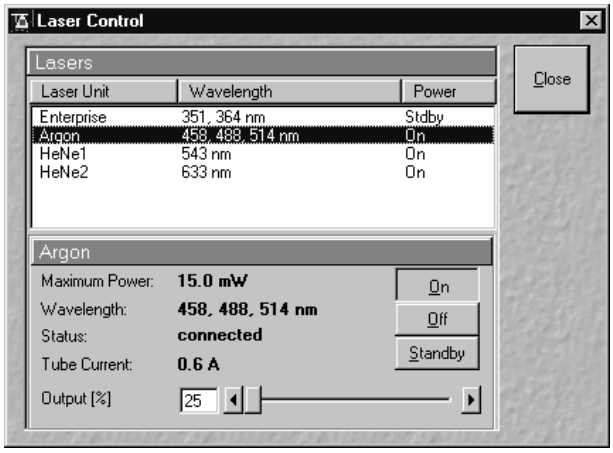


Fig. 5-197 Laser Control window

5.9.1 Laser Control

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.

5.9.1.1 Opening / Closing the Laser Control window

- Click on the **Laser** button in the **Config FCS** 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.

5.9.1.2 Function description

Lasers panel (upper)	List of available lasers, including the display of relevant wavelengths and switching status. Selection of the laser to be switched on / off and setting of the laser output is performed in the subordinate setting panel.
Laser settings panel (lower)	Switch on / off the required laser or set Standby operation. Display Maximum Power, Wavelength, Status and Tube Current (only Enterprise and Argon) of the relevant laser. Set the laser output for Argon.

5.9.1.3 Settings

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

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

This applies to the 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.

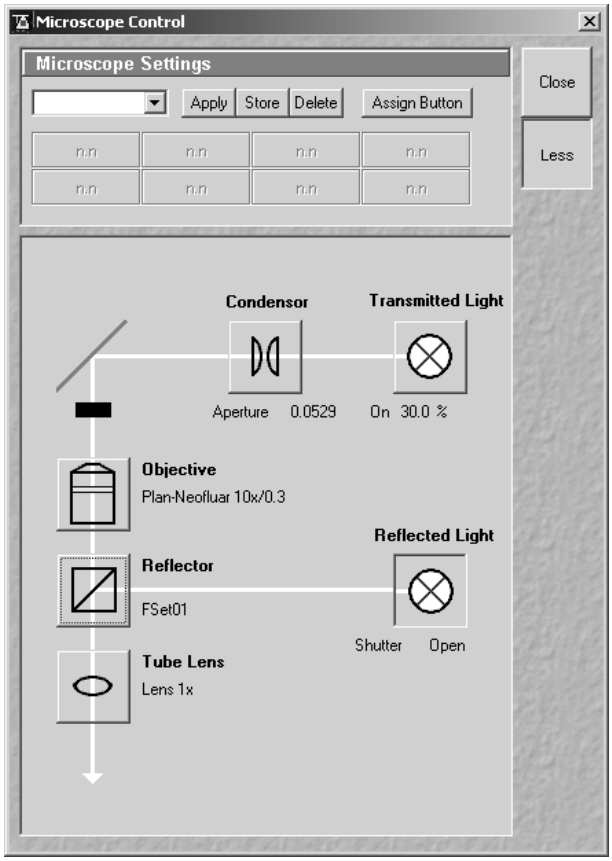


Fig. 5-198 Axiovert Control window

5.9.2 Micro

The **Axiovert 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.

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.

5.9.2.1 Open the Axiovert Control window

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

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

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

5.9.2.2 Function description

Transmitted Light button	Transmitted light is switched on / off via **ON** button in the **Transmitted Light** frame, setting of light intensity can be varied via input box or slider. 3200 K color temperature for photo documentation can be switched on via **3200 K** button in the Transmitted Light frame. The transmission light control potentiometer on the stand is disabled via the **Remote** button. By clicking on the **Close** button the **Transmitted Light** frame is closed.
Condensor button	Numerical aperture of the condensor is set via input box or slider. Turret position selected from graphical pop-up menu (only for motorized condensors). By clicking on the **Close** button the **Condensor** frame is closed.
Objective button	Objective can be selected via graphical pop-up menu.
Reflector button	Push and click, reflector cube can be selected via graphical pop-up menu.
Tube Lens button	Push and click, tube lens can be selected via graphical pop-up menu.
Reflected Light button	The shutter is switched on and off.

(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.

- Click on the **FCS** button on the right-hand side of the **Analyse FCS** subordinate toolbar in the **Main** menu (Fig. 5-162), to select the FCS detection head.

(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.
- Click on the **FCS** button on the right-hand side of the **Analyse FCS** subordinate toolbar in the **Main** menu (Fig. 5-162), to select the FCS detection head.

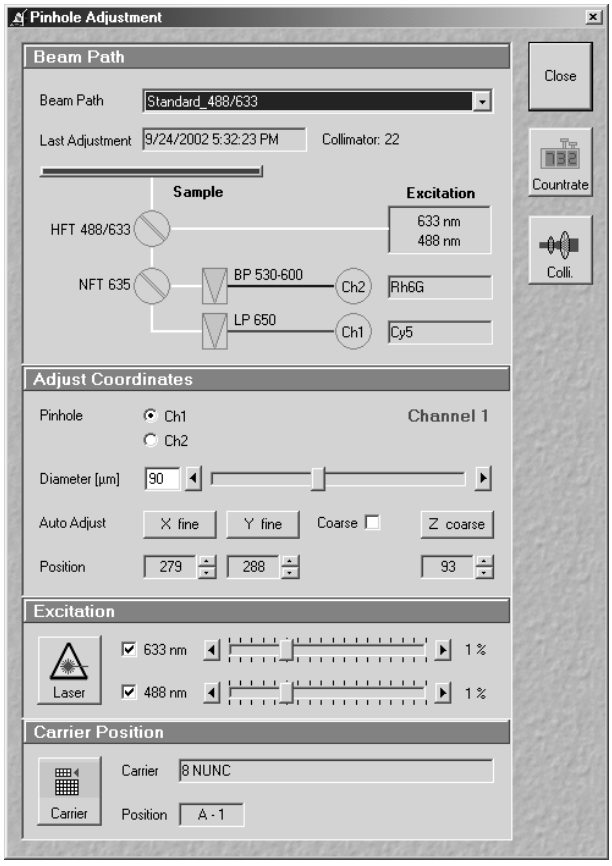


Fig. 5-199 Pinhole Adjustment window

5.9.3 Adjust Pinhole

The pinhole is adjusted using a dye solution. The general approach is to move the pinhole cyclically in x, y and z 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.

5.9.3.1 Open / Close the Pinhole Adjustment window

- Click on the **Pinhole** button in the **Config FCS** subordinate toolbar of the **Main** menu.
  - This opens the **Pinhole Adjustment** window.
- Click on the **Close** button to quit the window.

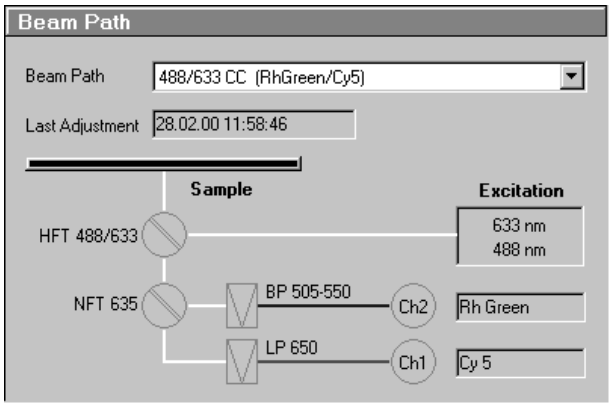


Fig. 5-200 Beam Path panel

5.9.3.2 Function description

(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 5.9.4, page 5-218.

(2) Adjust Coordinates panel

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

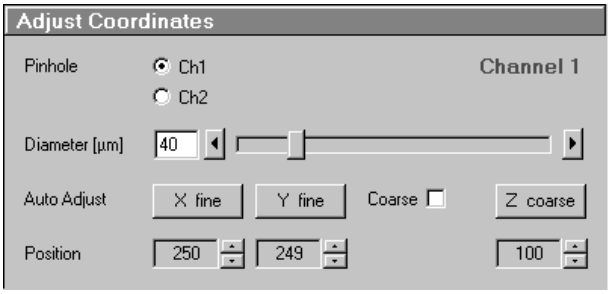


Fig. 5-201 Adjust Coordinates panel

Following function are available:

Pinhole Selection of the channel for adjustment.

Diameter Setting of pinhole diameter to the used wavelength (see page 5-216).

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 coarse** Starts the automatic coarse adjustment for Z. Since fine Z adjustments would be meaningless, they have not been included as a feature.

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, Y and Z.

(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 **Laser** button to switch on / off the necessary lasers (see section 5.9.1, page 5-208).

The **Laser Power** display box allows you to reduce the overall power by factors of 10, 100 and 1000.

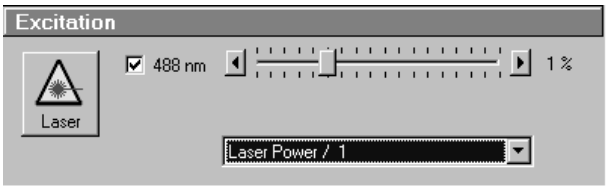


Fig. 5-202 Excitation panel

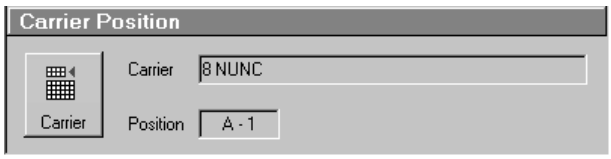


Fig. 5-203 Carrier Position panel

(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** window (see section 5.8.2, page 5-186).

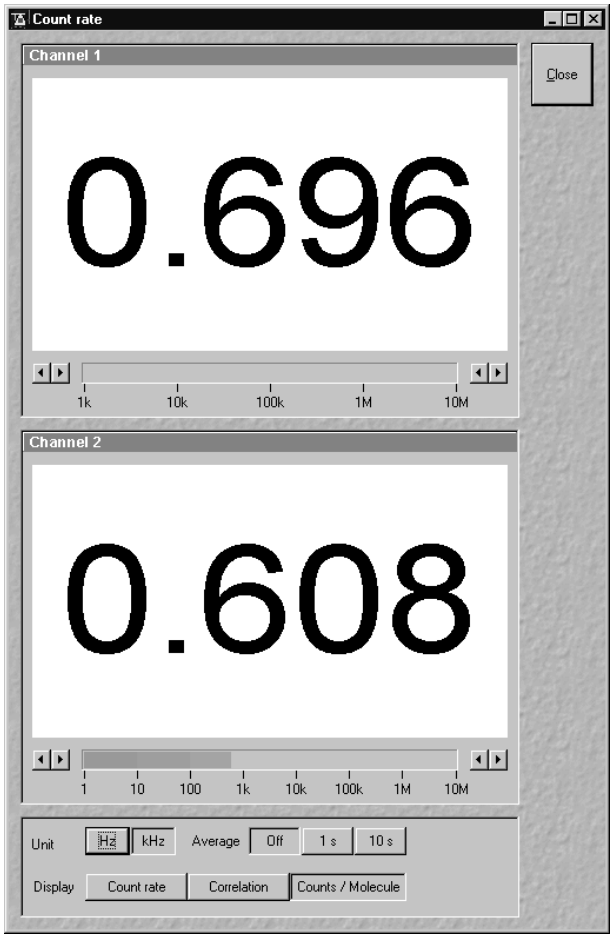


Fig. 5-204 Count rate window

(5) 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:

Unit:	Selecting the unit **Hz** or **kHz**.
Average:	Averaging after **1 s**, **10 s** or **Off**.
Display:	Selecting one of the display modes **Count rate**, **Correlation** or **Counts / Molecule**.

(6) FCS Hardware Settings window

- Click on the **Coll.** button to open the **FCM Hardware settings** window. The slider allows you to set the Collimator position.

If the Collimator setting was changed, the **Pinhole Adjustment** window will inform about the **Old** and **New** positions, as long as no Pinhole alignment was done in x, y, or z.

As soon as one Pinhole alignment was done and the **OK** button was clicked, only the current value will be displayed.

The Collimator setting will be stored with the respective method and will be automatically used when the method is called up.

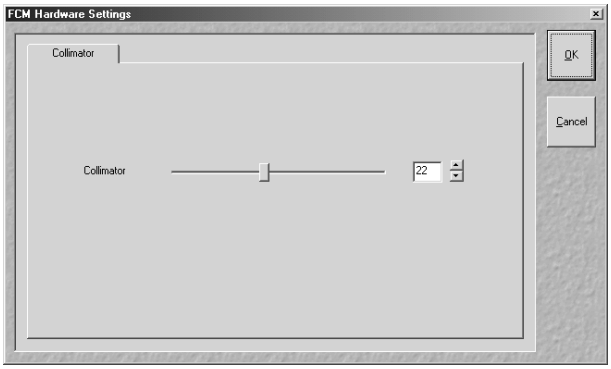


Fig. 5-205 FCM Hardware Settings

5.9.3.3 To adjust the pinhole proceed as follows

- Mount a cuvette with a suitable dye solution and orient the sample stage as described in section 5.8.1, page 5-184.
- Click the **Pinhole** button in the **Config FCS** toolbar subordinate of the **Main** menu. The **Pinhole Adjustment** window will appear (Fig. 5-199).

In the **Beam Path** panel of the **Pinhole Adjustment** window you will see a drawing of the current beam path including a line which indicates when this beam path had last been adjusted.

- Select the beam path you want to adjust in the **Beam Path** selection box in the **Beam Path** panel of the **Pinhole Adjustment** window.
- Select the test sample. Click on the **Carrier** button close to the bottom of the window. The **Carrier Position** window will appear. Then select the sample by mouse click.
- Use the slider in the **Excitation** section of the menu to set the laser intensity to minimum.
- Click on the **Count Rate** button at the right-hand side of the **Pinhole adjustment** window. 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 Coordinates** panel of the window.

☞ In the case of a cross correlation beam path, adjust the **two** pinholes subsequently in **two** runs.

Each of the pinhole axe 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
  - Z coarse
  - X fine; Y fine

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

- The **Automatic Pinhole Adjustment** window will appear (Fig. 5-206). 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.

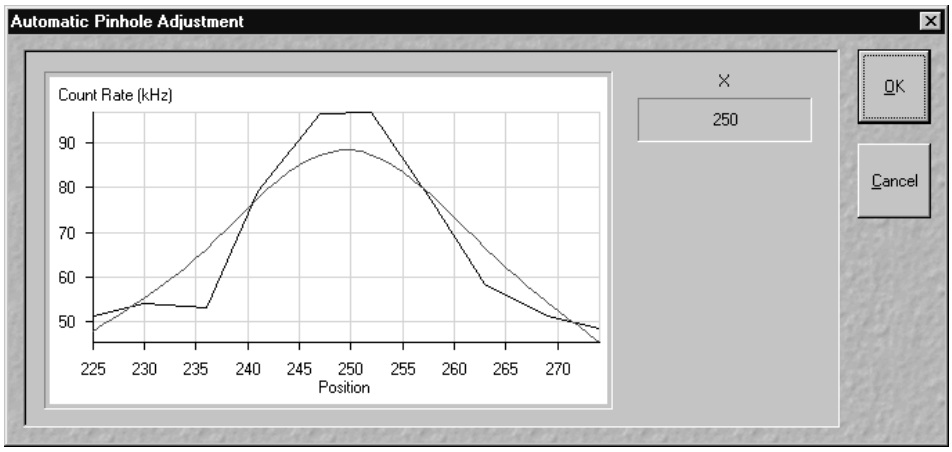


Fig. 5-206 Automatic Pinhole Adjustment

- Repeat the procedure for all axes.
- You will find peaks for x-Coarse, and y-coarse, in any case. If you fail to find the peak in z-coarse proceed as follows. Go to the **FCM Hardware settings** window by clicking the **Coll.** button. Move slider one step up, if peak is expected to the right; move slider one step down, if peak is suspected to the left. Repeat x-, y-, z-alignment and repeat changing Collimator setting, until z-peak is within the window.
- 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**.

5.9.4 Define Beam Path

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

5.9.4.1 Open / Close the Beam Path Definition window

- Click on the **Beampath** button in the **Config FCS** subordinate toolbar of the **Main** menu.
  - The **Beam Path Definition** window is opened.
- When the beam path definition is finished, click on the **Close** button to quit the **Beam Path Definition** window.

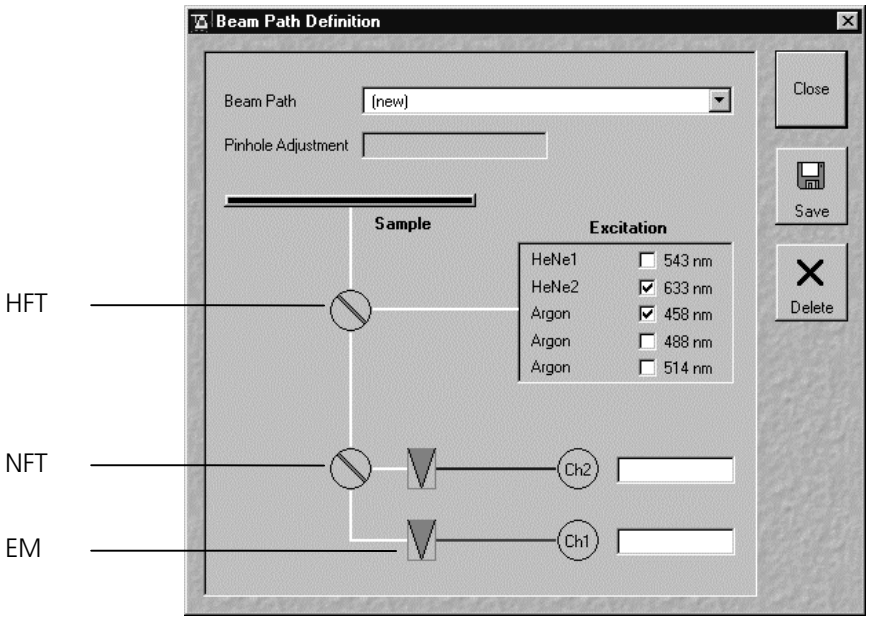


Fig. 5-207 Beam Path Definition window

HFT (main beam splitter)

The main beam splitter (HFT) reflects the specified laser lines and allows the resulting fluorescence spectrum to pass through.

NFT (secondary beam splitter)

The secondary beam splitter (NFT) splits the fluorescence spectrum onto the various detection channels.

EM (emission filter)

The emission filter (EM) 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.

5.9.4.2 Function description

Beam Path selection box	Selection of a new (scratch) or existing beam path for editing.
Pinhole Adjustment display box	Display of the last pinhole adjustment of existing configurations.
	Activation / deactivation of a main dichroic beam splitter (HFT) or secondary dichroic beam splitter (NFT) through selection from the relevant list box (Fig. 5-207).
	Activation / deactivation of an emission filter (EM) through selection from the relevant list box (Fig. 5-207).
Excitation check boxes field	Selection of the wavelength to be used.
Ch1 / Ch2 input boxes	For entering of a special comment (e.g. used dye).
Close button	The **Beam Path Definition** window is closed.
Save button	For saving the new or edited configuration.
Delete button	Deletion of an existing configuration.

5.9.4.3 Define 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 the second beam splitter in a similar way to the first one.
  - For cross correlation, choose the NFT 488/633 beam splitter.
  - Auto correlation measurements with an excitation wavelength of 633 nm should be carried out in detection channel 1. In this case, select **Plate** in the second beam splitter position.
  - All the other auto correlation measurements will be carried out in detection channel 2. To work with this channel, select a **Mirror** in the second beam splitter position.

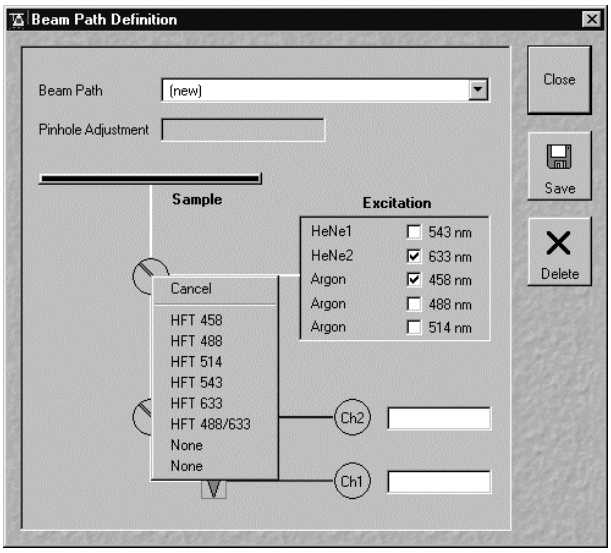


Fig. 5-208 Beam Splitter list box open

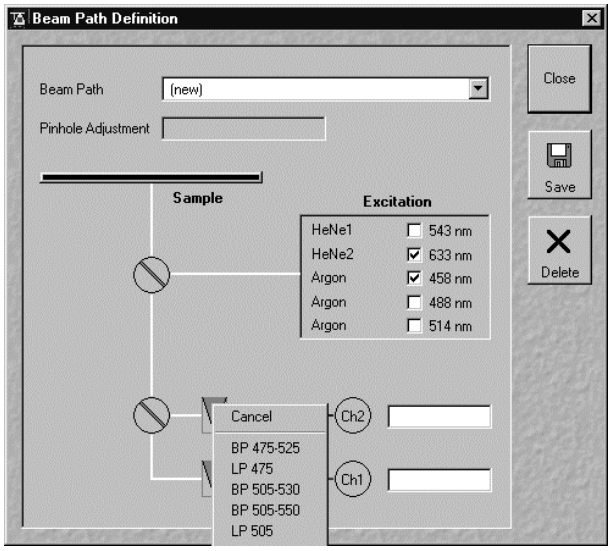


Fig. 5-209 Emission Filter list box open

- Click on the **Emission Filter** symbol(s) to get the list box allowing you to select the emission filters you need.
- It is recommended to give the channel a name which is easy to remember.

Fig. 5-210 Save Beam Path window

5.9.4.4 Save a new beam path configuration

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

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

5.9.4.5 Delete a beam path configuration

A no longer required configuration 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.
- When you are finished, close the **Beam Path Definition** window by clicking on **Close**.

5.9.5 Define Carrier

The **Carrier** function allows new / existing sample carriers to be created / edited and saved. The **Delete** function enables you to delete existing sample carriers.

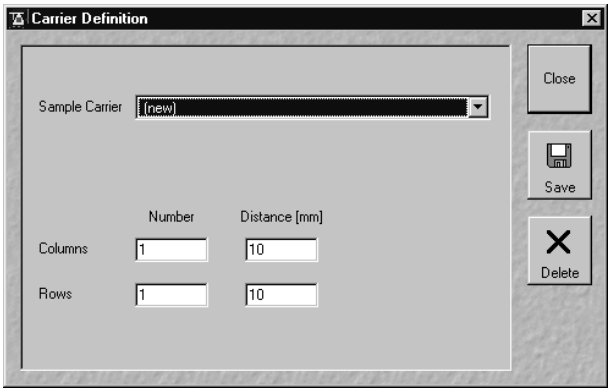


Fig. 5-211 Define Carrier window

5.9.5.1 Open / Close the Carrier Definition window

- Click on the **Carrier** button in the **Configuration** subordinate toolbar of the **Main** menu.
  - The **Carrier Definition** window is opened.
- When the beam path definition is finished, click on the **Close** button to quit the **Define Beam Path** window.

5.9.5.2 Function description

Sample Carrier selection box	Selection of a new or existing sample carrier for editing.
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.

5.9.5.3 Define a new sample carrier

- In the **Sample Carrier** selection box, use the **(new)** entry or select the sample carrier to be edited.
- Enter the number of chambers and the distance (mm) between the chambers in the appropriate **Column** and **Row** input boxes.

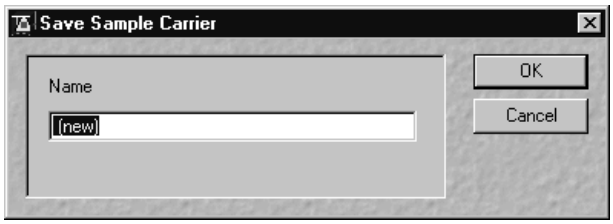


Fig. 5-212 Save Sample Carrier window

5.9.5.4 Save a new sample carrier

- Click on the **Save** button to save the new sample carrier.
  - The **Save Sample 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.

5.9.5.5 Delete a beam path 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**.

5.10 Macro Menu

The macro function permits the recording, running and editing of command sequences and their allocation to buttons in the **Macro** menu.

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

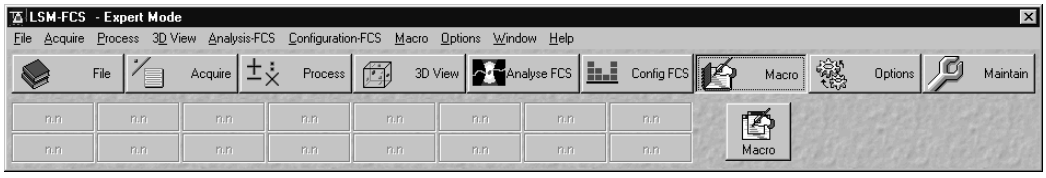


Fig. 5-213 Macro menu

5.10.1 Macro Language

"Visual Basic for Applications", called VBA in the following, is used as the Macro language. This language is well known through its widespread use as Macro language in the "Microsoft Word for Windows" and "Microsoft Excel for Windows" products. Experience with "Microsoft Visual Basic" would also be beneficial for macro-programming of the LSM-FCS.

An Integrated Development Environment, called IDE in the following, is available for the editing and debugging of macros. IDE includes an "online help program" where the VBA language is described in detail.

Macros are stored in project files. One project file can include several macros.

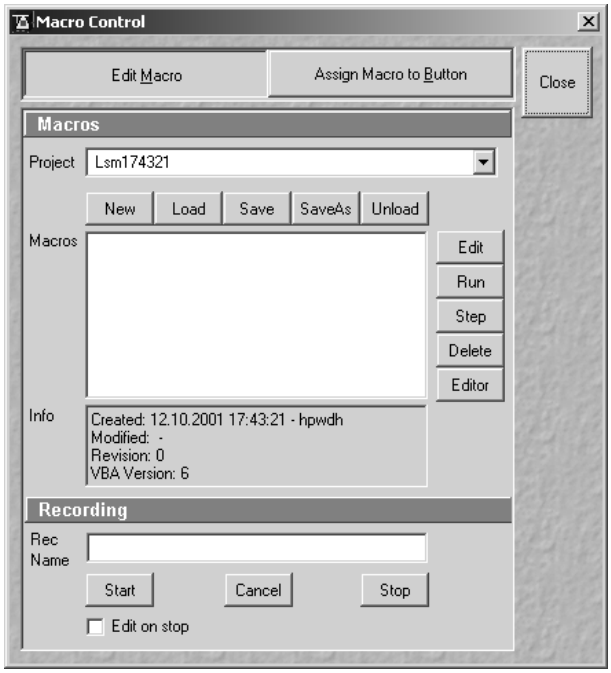


Fig. 5-214 Macro Control window

Fig. 5-215 Macro panel

5.10.2 Macro Control

5.10.2.1 Open / Close the Macro Control window

- Click on the **Macro** button in the **Macro** subordinate toolbar of the **Main** menu.
  - This opens the **Macro Control** window.
- Click on the **Close** button to quit the window.

5.10.2.2 Edit Macro function

This function allows you to manage project data. Macros can be recorded, stored, performed, edited and, if required, deleted.

- Press the **Edit Macro** button to switch to the **Macro** and **Recording** panels.

(1) Macro panel

New button: Creates a new project.

Load button: Opens an existing project.

Save button: Stores the project on the hard disk.

Save As button: Stores an existing project under a new name.

Unload button: Removes the selected macro from the **Macros** list.

Edit button: Allows macros to be edited and debugged. The editor (**Microsoft Visual Basic**) is automatically located at the beginning of the relevant macro.

Run button: Runs a macro.

Step button: Opens the editor for line-by-line editing / debugging.

Delete button: Deletes the selected macro.

Editor button: Opens the editor. Displays the processed area of the macro

edited last.

Macros are stored and managed in project files (*.lvb). Before you can record or edit a macro, you have to create a project as follows:

- Press the **New** button to create a project file.
  - A new project is created and displayed in the **Project** selection box (e.g.: **LSM 150503**). The project name is automatically default, but can be edited afterwards.

To activate an existing project, proceed as follows:

- Press the **Load** button.
  - The **Open** window will be opened.
- Select the relevant project file (data extension: ***.lvb**) from the **Macros** list box. Click on the **Open** button.
  - The project file will be opened and the macros contained in it are displayed in the **Macros** selection box of the **Macro Control** window.

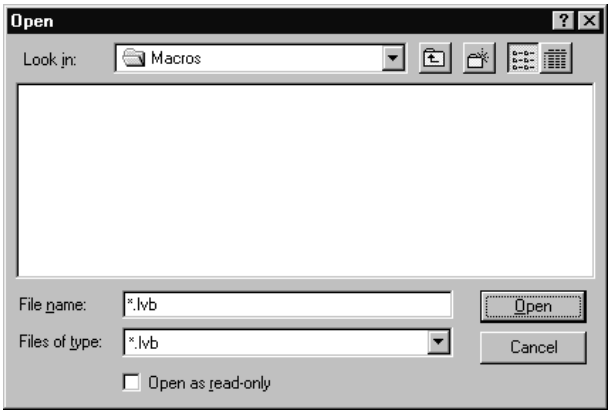


Fig. 5-216 Open window

Recorded macros are stored in main memory first. Before the macros can be assigned to the buttons in the **Macro** submenu, the project must be stored on the hard disk.

- Press the **Save** button under the project name in the **Macro Control** window and determine the file name in the **Project** selection box, if required.

(2) Recording panel

Before recording a command sequence, you can enter the name for the macro to be created in the **Rec Name** input box of the **Recording** panel.

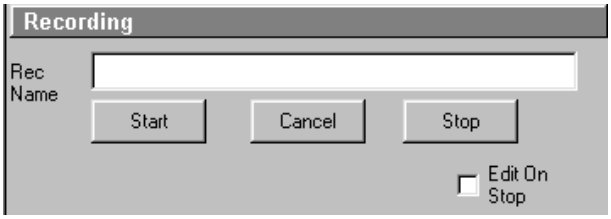


Fig. 5-217 Recording panel

Start button:	Starts recording.
Cancel button:	Cancels the recording procedure.
Stop button:	Stops recording.
☑ **Edit On Stop**:	On stopping the recording procedure, the macro editor is automatically opened at the relevant position.

Proceed as follows to record a macro:

- Enter a name for the macro to be created under **Rec Name** in the **Recording** panel.
- Click on the **Start** button to start recording the macro.
- Then perform the operations to be stored, e.g.:
  - Click on the **Find** button in the **Scan Control** window. A Find scan will be performed.
  - Click on the **New** button in the **Scan Control** window. A new **Image Display** window will be opened.
  - Click on the **Single** button in the **Scan Control** window. A **Single** scan will be performed.
- Then click on the **Stop** button to end the recording. (**Cancel** enables you to cancel recording)
  - If recording was successful, the entered **Rec Name** will then also be available in the **Macros** list box of the **Macro** panel. The new macro is automatically assigned to the current project. It is possible to assign as many macros as required to a project.
- Click on the **Save** button to store the new macro.

Proceed as follows to perform a macro:

- Select the required macro from the **Macros** list box of the **Recording** panel.
- Click on the **Run** button to start performing the macro.

☞ Provided that a macro is linked to a button in the **Macro** subordinate toolbar, you only need to click on this button to perform the macro.

Proceed as follows to delete a macro:

- Select the required macro from the **Macros** list box of the **Recording** panel.
- Click on the **Delete** button. The macro will be removed from the list.

Proceed as follows to edit a macro:

- Select the required macro from the **Macros** list box of the **Recording** panel.
- Click on the **Edit** button. The Microsoft Visual Basic editing window will be opened.
- Make the required changes (also see the notes on page 5-228).

5.10.2.3 Assign Macro to Button function

This function permits stored macros to be linked with one button each in the **Macro** subordinate toolbar.

- Press the **Assign Macro to Button** button to switch to the **Define Buttons** panel.

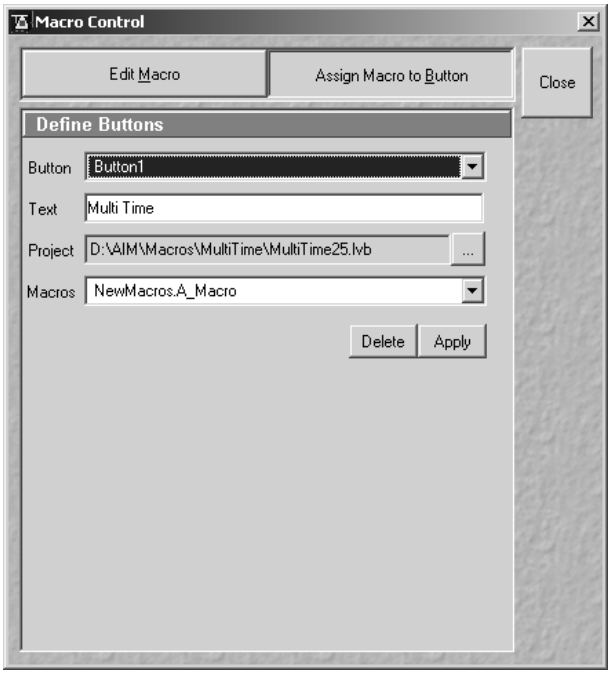


Fig. 5-218 Macro Control window

Define Buttons panel

Proceed as follows to link a macro to a button of the **Macro** subordinate toolbar:

- Select the button number from the **Button** selection box.
- Enter the button labeling in the **Text** editing box.
- Select the name of the project file from the **Project** box using the **...** button
- Select the macro name from the **Macros** box.
- Press the **Apply** button to assign the relevant macro to the specified button in the **Macro** toolbar.

Proceed as follows to delete the linking between a button in the **Macro** subordinate toolbar and a macro:

- Select the button number from the **Button** selection box.
- Press the **Delete** button to delete the linking.

5.10.2.4 Editing and debugging of macros

The **Edit** button activates IDE (Integrated Development Environment) which allows macros to be edited and debugged. Under the **Help - Contents and Index** menu item, IDE contains detailed "online" help on its operation and on the VBA macro language. Therefore, only a few hints are provided in the following:

You should activate the required toolbars. We would recommend you to activate the **Debug** toolbar via the **View - Toolbars -Debug** menu item.

The following buttons in the toolbar can help you when debugging macros:


Starts running the command lines.


Stops running the command lines.


Interrupts processing of the command lines (pause).


Sets a breakpoint in the line with the text cursor.


Processes a command line and steps into subprocedures.


Processes a command line and steps over subprocedures.


Exits the subprocedure (step out).


Displays the value of the marked expression (Watch). If nothing is marked, the value of the variable above the text cursor is displayed.


Activates the **Watch** window in which values of variables and expressions can be displayed. For this, text is marked in the code window and dragged into the **Watch** window. Variables can be modified in the **Watch** window.

In the left-hand edge of the code window you will find an arrow beside the current command line. A new current command line can be determined by moving the arrow via the mouse. This makes it possible to skip command lines or to process command lines several times.

5.10.3 Overview of available Macros (all LSM releases)

Documentation files (*.rtf, *.doc) of advanced macros will be located in the macro directory.

Name	Description
AOTFfit.lvb	Linearize laser attenuation (AOTF or mechanical)
AOTFfitlin.lvb	New method to linearize laser attenuation (AOTF or mechanical)
Autofocus.lvb	Automatic focusing according to a set configuration
Bleach.lvb	Bleaching of a rectangular area or a line; combines old macros BleachRectangle.lvb, BleachLine.lvb and Spot.lvb
CameraColor.lvb (also Button in Maintain)	Color balance of Axiocam HRc
Centerv28-30.lvb	Centers the field of view around the position marked with the cross tool;
CopyPasteOverlays28-30.lvb	Copies actual overlay drawing into the clipboard and pastes the drawing into a selected image window
CopyPasteRoi.lvb	Copies drawing element of overlay into clipboard and pastes it into other selected windows
CopyRoisToOverlay28-30.lvb	Copies ROIs to overlay drawings; both can be viewed and measured at the same time;
CpCanTrace.lvb	Checks communication of PC with CAN-Bus/net
CpDsp.lvb	Checks communication of PC with DSP
DeleteMultiTimeRecipies.lvb	Deletes all available Multiple Time Series set ups
Distance.lvb	Example macro for measurement
Distance28-30.lvb	Release 3.0: measures the distance using the mouse;
DivideThroughReferenceImage.lvb	- divide complete time series through a single image/part of the series - duplicate a single image or part of a time series to this series.
EventPollPeriod.lvb	Not documented
FastModeSwitch.lvb	Store settings from "Scan-Control" and reuse.
FileExport.lvb	Exports one or more selected images according to the set file format in one go; Exports image intensity values in ASCI format;
GDC_calib.txt	Description
HotKey.lvb	Shift focus with a button and start Single-Scan
KSPlastv25.lvb	KS software macro

Name	Description
Kundenmacro32.doc	Documentation
Lambdatrans.lvb	Time series alternating between lambda and transmission mode
LsmHWAdmin.lvb (also Button in Maintain)	Direct service hardware access (password protected)
LsmHWAdminEx.lvb	Calibration service macro (password protected)
LsmHWAdminMeta.lvb	Calibration for META channel
LsmTime.lvb	Triggered Time scan Macro
Macro_Description.pdf	Description
MCS30.lvb Lsm.mac	Control of spectrometer
MetaExport.lvb	Export of META image files including all channels as tif, bmp...
ModifySeries30.lvb ModifySeries30.rtf	Modifies Z Stacks and Time Stacks like Rotation of the stacks, being mirrored, Conversion of time stacks into z-stacks and vice versa;
MultiProfile.lvb	StitchArt macro (**Software option**)
MultiStack.lvb	Similar to StitchArt, but generates stacks only (no profiles) and uses the settings in scan control.
MultiTime28-32.lvb MultiTime28-32.rtf	Set up of time series experiments including repeated imaging, bleaching and autofocusing with defined configurations at multiple locations and for various views at each location (**Software option**)
OptimizeGDCV3_0.lvb OptimizeGDCV3_2.lvb	Optimize the max. peak power of fiber coupled Ti:Sa lasers (Release 3.0/3.2)
Parameters.lvb	Check the scan parameters
Pixel28-30.lvb	Displays and stores the mean intensity values of each line of one or more channels of one or more images; data from each line are stored as a txt file in the current folder;
Profile.lvb	Displays the pixel values along a line
Profilev28-30.lvb	Opens VBA editor for profile
Reboot.lvb (also Button in Maintain)	Service reset of scan-module (password protected)
Scalebar30.lvb Scalebar30.rtf	Indication of self defined intensity levels assigned to a ROI as scale bar in the image; also attaches tick marks and concentration values to the grayscale/color wedge.
SetFind.lvb (also Button in Maintain)	Sets properties of the Find function
Spline.lvb (also Button in Maintain)	Calibration of a spline-scan

Name	Description
TileScanRotation.lvb	Defines rotation of a tile-scan
TimeSeriesShutter.Lvb	Close laser shutter in time series on a LSM 5 PASCAL
Trigger.lvb	Trigger test
TuneNLOLaser32.lvb	Change wavelength of a tunable Ti:Sa laser and run excitation series

Remarks:

- During installation, default macros will be installed according to their type either in AIM\, AIM\HWT or AIM\Macros\. Self generated Macros will be in AIM\Macros.
- In case of a new installation, old macros will be stored in AIM\Macros\BackupMacros or AIM\Backup\, to avoid problems with identical names of existing and new macros.

5.10.4 Sample Macros

The LSM-FCS software package includes e. g. the **Distance**, **Profile**, **Spot** and **Multiple Time Series** sample macros.

They can be easily executed by clicking on the relevant button in the **Macros** subordinate toolbar.

During the execution of a macro, the **Stop Macros** window is always displayed on the screen. This enables a macro to be stopped any time by pressing the **Stop** button.

The functions of the sample macros are explained below.

5.10.4.1 Distance macro

This macro permits measurement of the distance of a line created in the scan image.

- Click on the **Distance** button in the **Macro** subordinate toolbar.
  - An XY scanning image of the specimen is recorded and displayed. At the same time, the **Mouse position test** window appears on the screen.
- Then draw a line over the distance to be measured by clicking and holding down the mouse button. The click of the mouse sets the starting point, releasing the mouse sets the end point of the line.
  - After release of the mouse button, the length of the line in the scanning image is displayed (in µm).
  - Any required number of lines can be defined in the image. The previous line is deleted.
- A click on the **Exit** button in the **Mouse position test** window will end the macro.

5.10.4.2 Profile macro

This macro permits the gray values of a line created in the scanning image to be determined pixel by pixel.

- Click on the **Profile** button in the **Macro** subordinate toolbar.
  - An XY scanning image of the specimen is recorded and displayed. The **Profile** window is shown on the screen.
- Then click and hold down the mouse button to draw a line in the scanning image for which the gray values shall be determined.
  - The current numbers of the pixels of the created line to which the relevant gray value is assigned now appear in the **Profile** window.
  - At the same time, the distance of the created line is displayed in µm for checking.
  - Any required number of lines can be defined in the image. The previous line is deleted.
- A click on **Cancel** will end the macro.

5.10.4.3 Bleach macro

This macro permits the specimen to be excited with the laser as required along a line created in the scanning image.

- Click on the **Bleach** button in the **Macro** subordinate toolbar.
  - An XY scanning image of the specimen is recorded and displayed. The **Spot Scan** window is shown on the screen.
- Click on the **Select Excitation Line** button.
- Create a free-hand line (spline) in the scanning image over the area to be excited by clicking and holding down the mouse button.
- Then determine the duration of the excitation by moving the **Exposure Time** slider.
- Click on the **Excite** button to trigger the excitation procedure.
- A click on **Exit** will end the macro.

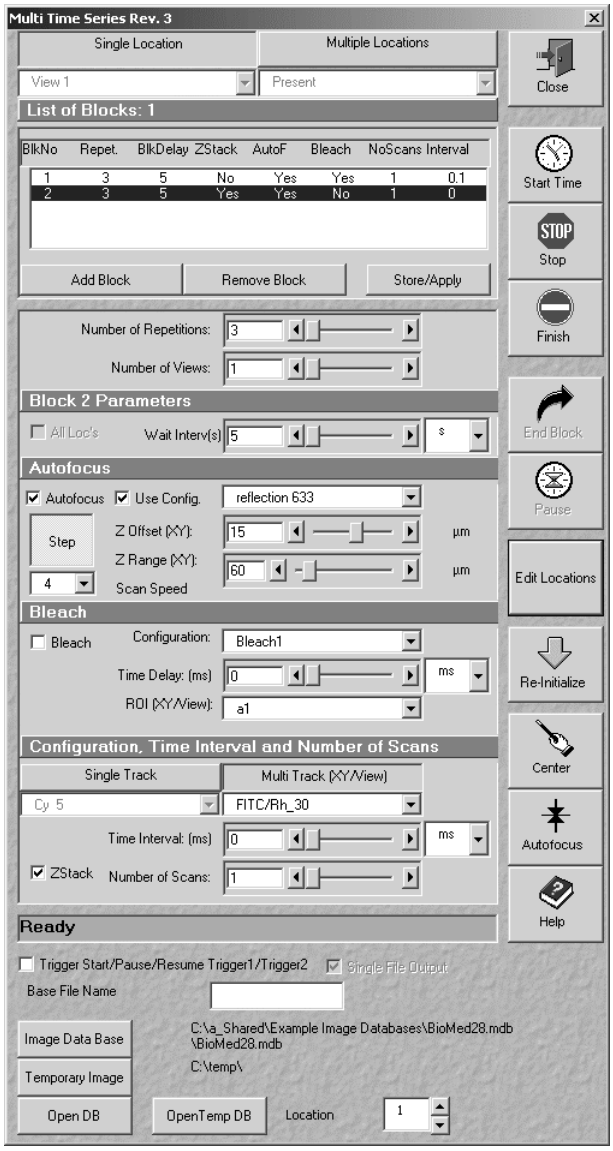


Fig. 5-219 Multiple Time Series main dialog box

5.10.4.4 Multiple Time Series macro, Rev. 3

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

- A pre-program time delay
- A pre-bleaching functionality
- An autofocus functionality
- A number of different, single time series (called a block) either at the same position of a sample or at different positions (if a motorized stage is available)
- A time series of a tile scan
- A time series of a tiled Z stack

The basic programming unit is a single Time Series Block in line, frame or Z-stack mode In each block user can define configuration for the data acquisition (single or multi-track), the number of images and the time interval/delay between images.

User can activate an optional autofocus function before each block, pre-program time interval before each block (from the beginning of the previous block to the beginning of the current block), and/or execute bleach track with arbitrarily specified bleach ROI's, laser line(s) and power.

The autofocus function can be executed using specified single-track configuration, or with the configuration used for imaging (using the first channel of the first track).

One can define the z-offset, the distance in the z direction from the position where the autofocus finds the reference feature in focus (position of maximum intensity - e.g. position of the cover slip reflection in the reflected light configuration) to the position, which is moved into focus plane when the image acquisition begins (e.g. into the tissue).

The image acquisition is done with the configuration specified for the given block.

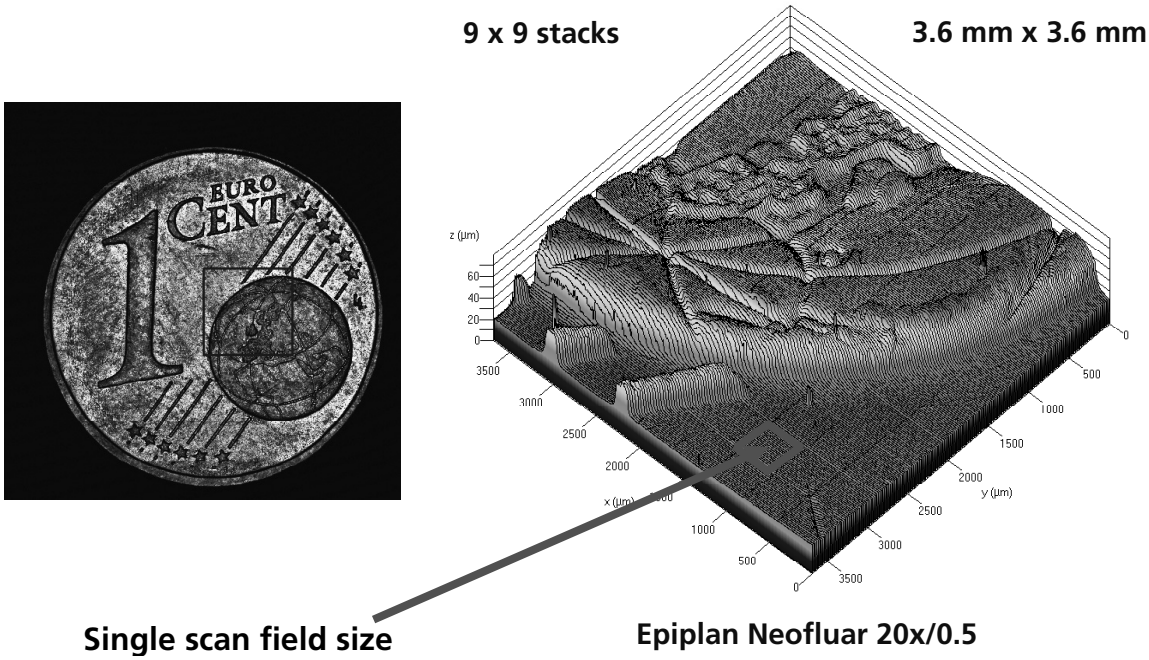
Autofocus search parameters: Z-offset as well as the Z-range (the distance in the z direction over which the autofocus function searches for the plane of maximum intensity) can be set independently for different stage locations (on the systems with the motorized stage).

5.10.4.5 StitchArt macro

The StitchArt software option for LSM 510 MAT and LSM 5 PASCAL MAT permits the automated 3D measurement of large samples:

- Highly resolved topographies of 10 times larger scan field
- Extra large profiles of more than 10 times of length of scan field

Example:



(1) Multiple Profile Mode

Maximum format: 16384 pixels x 2048 slices
Variable overlap: 10 ... 50 % of single profile length
Scan Speed: 8 (fixed)
Scan time: 20 ... 210 seconds (depending on Z)
Height difference DZ: 0.1 ... 0.6 µm [in steps of 0.1]
Total height Z: 50 µm ... 1 mm
Auto alignment: Cross-correlation in X, Y & Z

Maximum profile lengths:

Objective**:**	5x	10x	20x	50x	100x
Single X Profile (0.7):	2.6 mm	1.3 mm	650 µm	260 µm	130 µm
Multiple Profile (1.0):	30.0 mm	15.0 mm	7.5 mm	3.0 mm	1.5 mm

(2) Multiple Stack mode

Single stack format (X): 4x4 ... 512x512 pixels
Number of stacks: 1x1 ... 16x16
Variable overlap: 10 ... 50 % of single image size
Scan Speed: 5 ... 8
Scan time: 1 minute ... 10 hours
Height step DZ: 0.5 ... 100 microns
Total height Z: 4 mm
Auto alignment: Cross-correlation in X & Y

Maximum stack sizes:

Objective**:**	5x	10x	20x	50x	100x
Single XYZ Stack (0.7):	2.6 mm	1.3 mm	650 µm	260 µm	130 µm
Multiple Stack (1.0):	26.7 mm	13.3 mm	6.7 mm	2.6 mm	1.3 mm

Adjustment functionality:

Find Focus: Autofocus by fast Z line
Find Gain: Auto Brightness & Contrast
Adjust scan mirrors: To XY direction of MOT stage
Adjust spherical objective error: On a plane mirror

☞ Macro VBA programming is described in chapter 6.

5.11 Options Menu

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

- User-Defined Examination Methods of the Expert Mode are made available to the Routine Mode.
- Deletion of Routine Mode examination methods.
- Display of a current list of dyes with preferred wavelengths for the scanning procedure.
- Display / modification of the user-accessible program **Settings** of the LSM-FCS software.

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

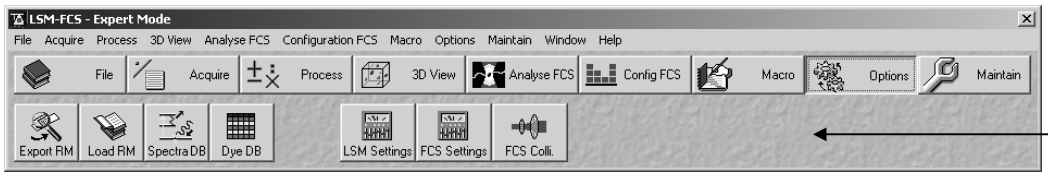


Fig. 5-220 Options menu

5.11.1 Export RM Function

This function permits **User-defined Examination Methods** created in the **Expert Mode** to be made available to the **Routine Mode**.

- Load a Frame, Stack or Time Series image which was scanned using optimized parameters.

☞ It is also possible to export the examination method of a currently displayed image which has just been scanned.

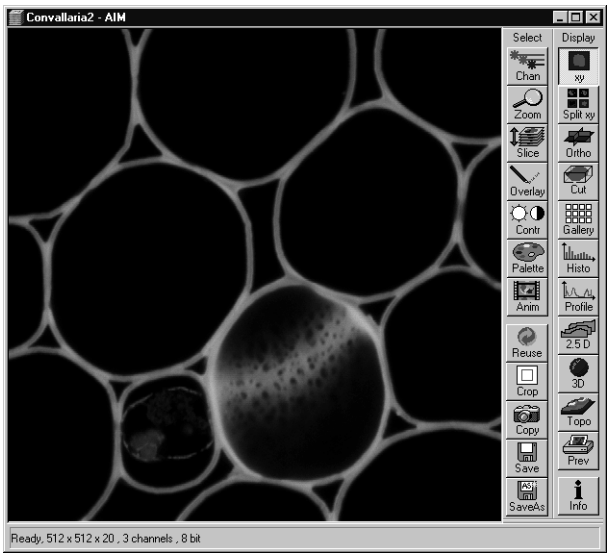


Fig. 5-221 Image Display window

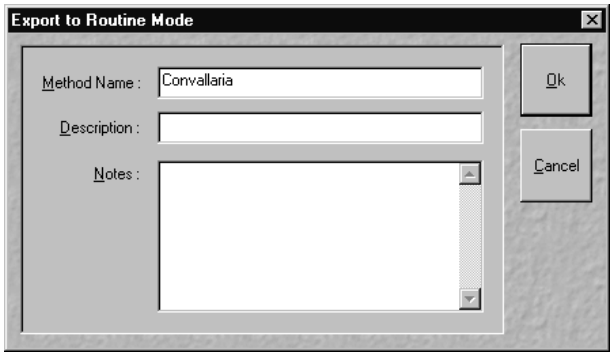


Fig. 5-222 Export to Routine Mode window

- Click on the **Options** button of the **Main** menu and then on **Export RM** (see Fig. 5-220).
  - The **Export to Routine Mode** window appears.
- Enter any name for the method to be taken over.
- If required, enter a brief description and additional comments under **Description** and **Notes**.
- Click on **Ok**. (**Cancel** allows you to cancel the procedure).
  - The method used for image acquisition is taken over in the Routine Mode.

☞ The exported **User-Defined Examination Method** must be activated before it can be used in the Routine Mode (see **Routine Mode**, chapter 5).

If you want to change from the Expert Mode to the Routine Mode and vice versa, close all the windows first.

It's not possible to export ratio channels and bleach parameters. All methods will be loaded in the Routine Mode with **Zoom 1**, **Rotation 0**, **Offset 0** and without defined ROIs.

5.11.2 Load RM Function

The **Load RM** (Routine Mode) function permits the deletion of **Standard Examination Methods** and **User-defined Examination Methods** from the database of the **Routine Mode**.

- Click on the **Options** button of the **Main** menu and then on **Load RM** (see Fig. 5-220).
  - The Routine Mode Database is opened.

The Routine Mode database is identical to the database of the Expert Mode, though not all the functions are available. The listed methods cannot be loaded as an image.

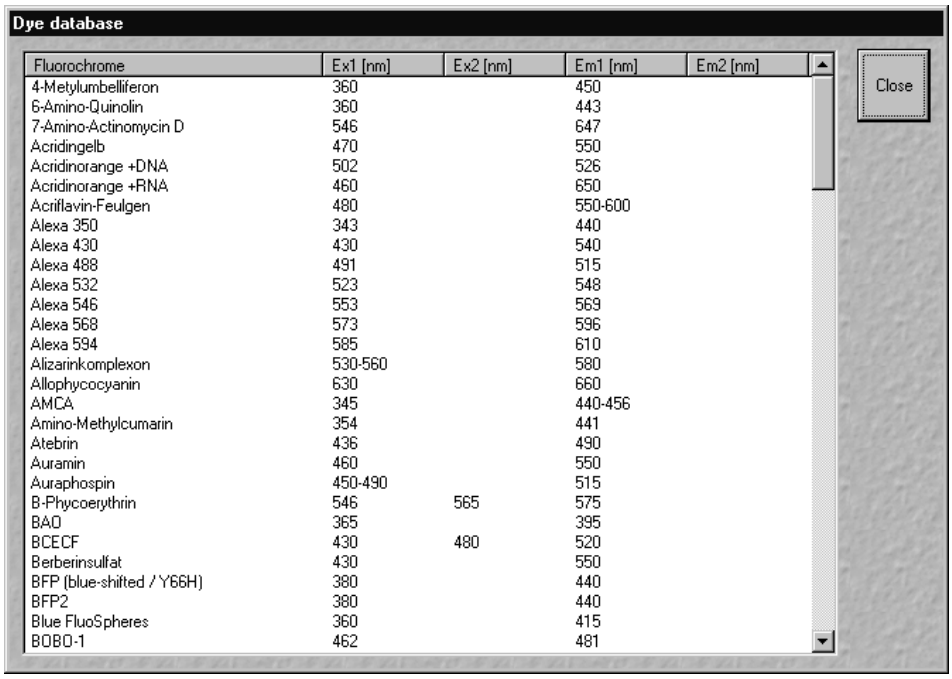
- To delete an **Examination Method** of the Routine Mode, select the method from the **Form**, **Gallery** or **Table** display of the **Routine Mode Database**.
- Then click on the **Delete** button.
  - The selected method is deleted from the database and is no longer available in the Routine Mode.

5.11.3 Dye DB Function

The **Dye DB** function is for information only and permits access to the database contained in the system, including a list of suitable dyes for fluorescence microscopy.

The database contains a comparison of tables of dyes, optimum excitation wavelengths and maxima of emission wavelengths.

- Click on the **Dye DB** button in the **Options** subordinate toolbar.
  - The **Dye database** will be opened and displayed on the screen.



Fluorochrome	Ex1 [nm]	Ex2 [nm]	Em1 [nm]	Em2 [nm]
4-Metylumbelliferon	360		450	
6-Amino-Quinolin	360		443	
7-Amino-Actinomycin D	546		647	
Acridingelb	470		550	
Acridinorange +DNA	502		526	
Acridinorange +RNA	460		650	
Acriflavin-Feulgen	480		550-600	
Alexa 350	343		440	
Alexa 430	430		540	
Alexa 488	491		515	
Alexa 532	523		548	
Alexa 546	553		569	
Alexa 568	573		596	
Alexa 594	585		610	
Alizarinkomplexon	530-560		580	
Allophycocyanin	630		660	
AMCA	345		440-456	
Amino-Methylcumarin	354		441	
Atebrin	436		490	
Auramin	460		550	
Auraphospin	450-490		515	
B-Phycoerythrin	546	565	575	
BAO	365		395	
BCECF	430	480	520	
Berberinsulfat	430		550	
BFP (blue-shifted / Y66H)	380		440	
BFP2	380		440	
Blue FluoSpheres	360		415	
BOBO-1	462		481	

Fig. 5-223 Dye database window

- Click on the **Close** button to exit the **Dye database**.

5.11.4 Spectra DB Function

Spectrums created with the Lambda Mode can be stored in the Spectra Database.

- Click on the **Spectra DB** button in the **Options** subordinate toolbar.
  - The **Spectra Database** will be opened and displayed on the screen.

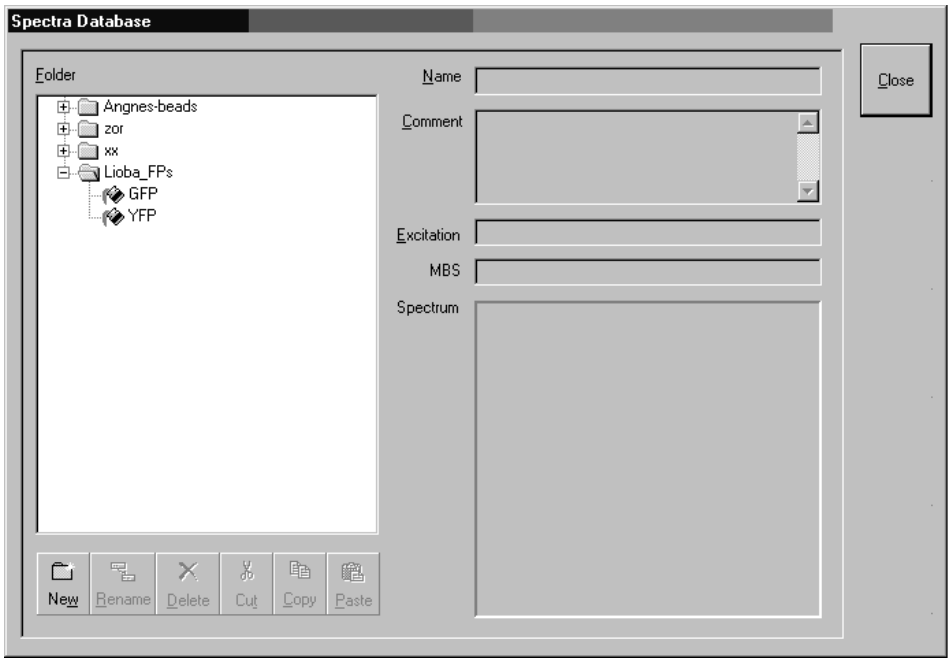


Fig. 5-224 Spectra Database window

- Click on the **Close** button to exit the **Spectra Database**.

5.11.5 LSM Settings Function

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

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

5.11.5.1 Open / Close the Settings for user : ... window

- Click on the **Settings** button in the **Options** subordinate toolbar of the **Main** menu.
  - This opens the **Settings for user : ...** window.
- Click on the **OK** button to quit the window. The last settings will be taken over. **Cancel** enables you to cancel the procedure, with any changes you made **not** being taken over.

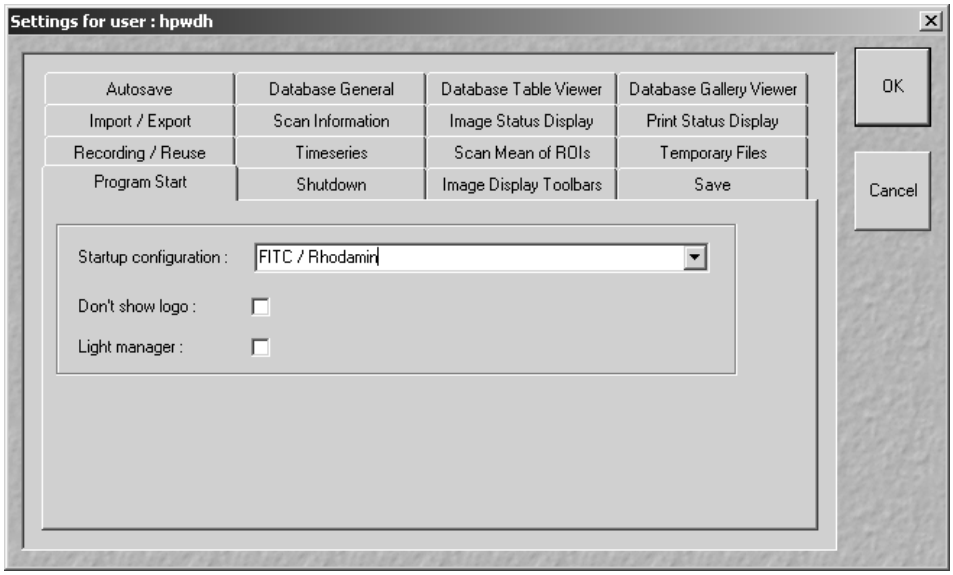


Fig. 5-225 Settings for user : ... window

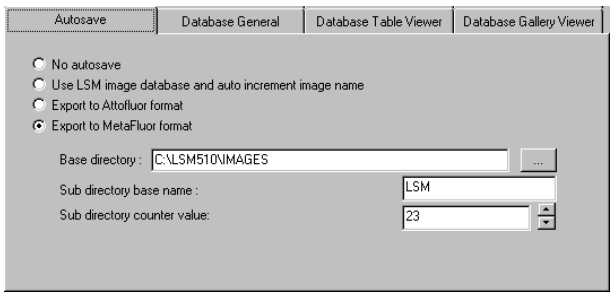


Fig. 5-226 Autosave tab

5.11.5.2 Autosave

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

(1) No Autosave

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

(2) Use LSM image database and auto increment image name

On activation of this option, newly recorded or modified images are stored by **Save** automatically and assigned to the name or defined in this function. The image name is automatically created using a base name and a serial number. For this, a base name must be entered in the **Base image name** input box, and a starting value for the serial number in the **Counter value** input box. The **Database** selection box permits selection of the directory in which the data will be stored.

(3) Export to Attofluor format

On activation of this option, newly recorded or modified images are stored by **Save** in the Attofluor format. The displayed **Experimental directory** selection box permits selection of the directory in which the data will be stored.

(4) Export to Metafluor format

On activation of this option, newly recorded or modified images are stored by **Save** in a subdirectory in the MetaFluor format. An existing higher layer of folders must be selected for the subdirectory from the **Base directory** selection box. Furthermore, a name for the subdirectory must be entered in the **Subdirectory base name** input box. The starting value for the images then created, to which a continuous number is automatically assigned, is set in the **Subdirectory counter** input box.

5.11.5.3 Database General

This tab permits the basic starting settings for the use of databases.

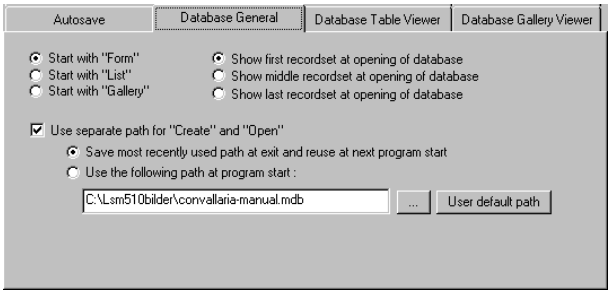


Fig. 5-227 Database General tab

(1) Start with "Form"

On opening of a database, the **Form** option is displayed first.

(2) Start with "List"

On opening of a database, the **List** option is displayed first.

(3) Start with "Gallery"

On opening of a database, the **Gallery** option is displayed first.

(4) Show first recordset at opening of database

On opening of a database, the first recordset is displayed.

(5) Show first recordset at opening of database

On opening of a database, the middle recordset is displayed.

(6) Show first recordset at opening of database

On opening of a database, the last recordset is displayed.

(7) Use separate path for "Create" and "Open"

This option permits the path to be changed when the **Open** or **New** database function is used.

(a) Save most recently used path at exit and reuse at next program start

On activation of this option, the path setting last used is automatically selected again in the **Open Database** or **Create New Database** window.

(b) Use the following path at program start

On activation of this option, the path for the **Open Database** or **Create New Database** window can be entered directly in the relevant selection box, or selected by clicking on the **...** button in the **Choose Directory** window. This path will then always be set when a database is opened or created.

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

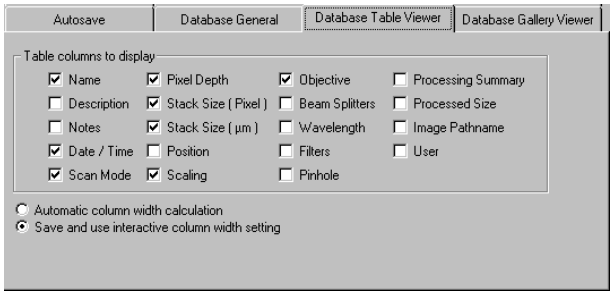


Fig. 5-228 Database Table Viewer tab

5.11.5.4 Database Table Viewer

The **Database Table Viewer** tab permits the definition of the columns for the table display of a database. This only requires the relevant check box to be activated with a click of the mouse.

On activation of the **Automatic column width calculation** option, the column width is calculated automatically.

On activation of **Save and use interactive column width setting,** the column width in the database can be matched as required. The individual setting will be retained when the database is closed.

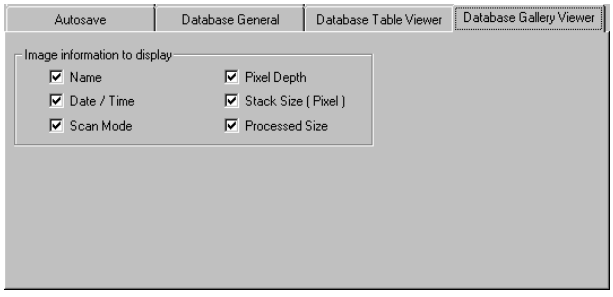


Fig. 5-229 Database Gallery Viewer tab

5.11.5.5 Database Gallery Viewer

The **Database Gallery Viewer** tab permits the image information to be displayed in the Gallery mode of the database to be activated by clicking on the relevant check box.

5.11.5.6 Import / Export

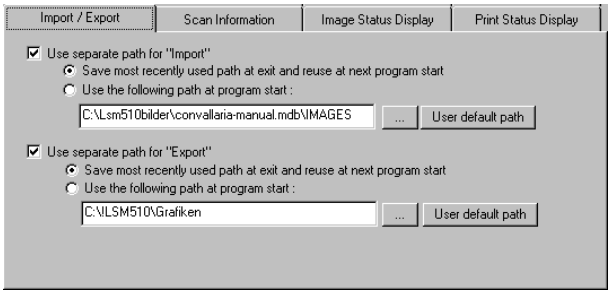


Fig. 5-230 Import / Export tab

Use separate path for "Import" or "Export"

This option permits the change of the path setting for use of the **Import** or **Export** function (**File** menu).

(1) Save most recently used path at exit and reuse at next program start

On activation of this option, the path used last is automatically selected again in the **Import Images** or **Export Images and Data** window.

(2) Use the following path at program start

On activation of this option, the path for the **Import Images** or **Export Images and Data** window can be entered directly in the relevant selection box, or selected by clicking on the **...** button in the **Choose Directory** window. This path will then always be set when the **Import / Export** function is used.

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

5.11.5.7 Scan Information

This tab permits the setting of which scan information shall be displayed in the **Scan Information** window (see **Window** pull-down menu of the **Main** menu, page 5-272f).

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

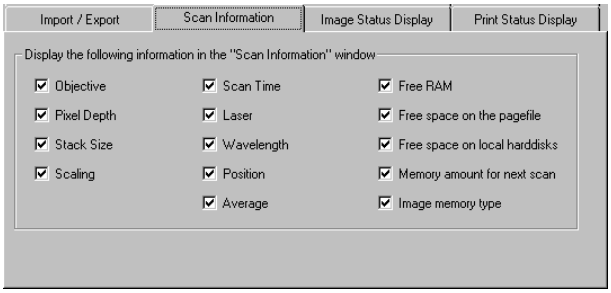


Fig. 5-231 Scan Information tab

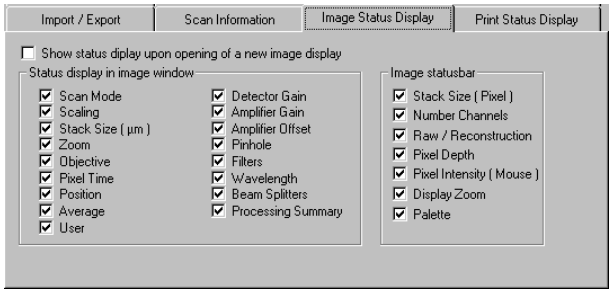


Fig. 5-232 Image Status Display tab

5.11.5.8 Image Status Display

This tab permits selection of which image information is displayed on opening of an image or on activation of the **Info** button of the **Image Display** window. Furthermore, you can determine which information will be displayed in the **Image status bar**.

On activation of the **Show status display upon opening of a new image display** check box, the image information is automatically displayed immediately after opening of the **Image Display** window (**Info** button is activated).

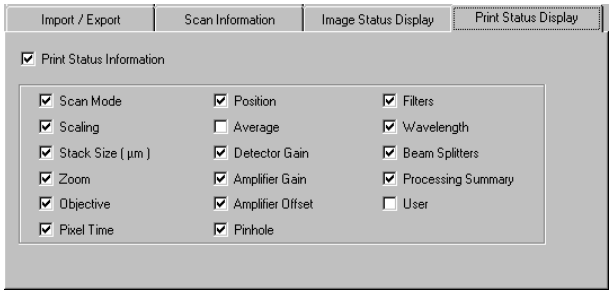


Fig. 5-233 Print Status Display tab

5.11.5.9 Print Status Display

This tab permits selection of which information is displayed in print preview.

On activation of the **Print Status Information** check box, the status information will be printed.

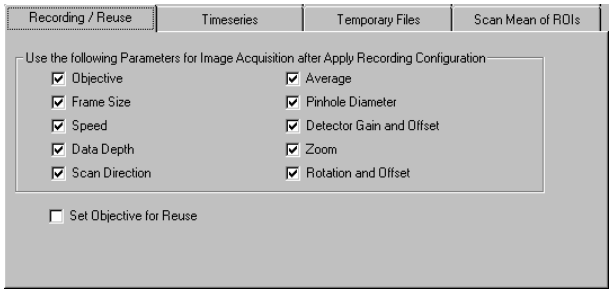


Fig. 5-234 Recording / Reuse tab

5.11.5.10 Recording / Reuse

The parameters to be taken into consideration for the use or load of a recording configuration are set in this tab.

As an option, you can also determine whether the objective setting shall be taken over when the **Reuse** function is used.

5.11.5.11 Time series

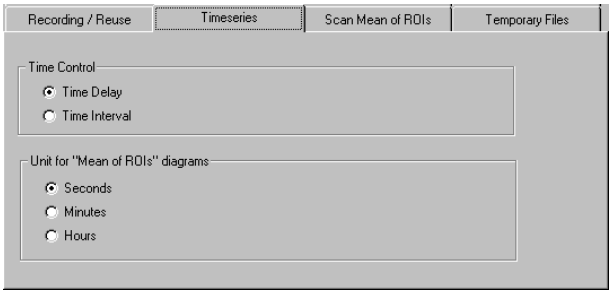


Fig. 5-235 Timeseries tab

In the **Timeseries** tab, you can determine whether the time for the recording of a time series is set as **Time Delay** or as **Time Interval**.

Time Delay is the interval between the end of one scan process and the beginning of the next.

Time Interval is the interval between the beginning of one scan process and the beginning of the next.

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

5.11.5.12 Scan Mean of ROIs

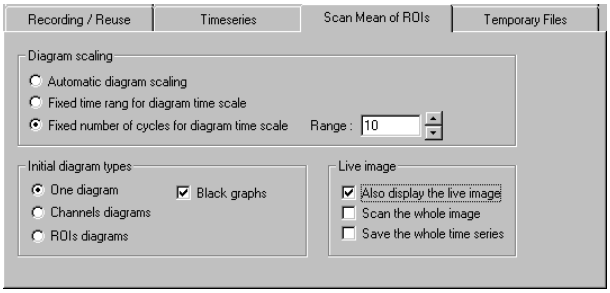


Fig. 5-236 Scan Mean of ROIs tab

The **Mean of ROIs** tab permits the presetting of the **Image Display** window for the optional **MeanROI** function (time series) to be changed with regard to scaling and display mode of the intensity time diagrams.

(1) Diagram Scaling

The following settings are possible by activating one of the option buttons:

- **Automatic diagram scaling**
- **Fixed time range for diagram time scale**; input of the time range in seconds via input box
- **Fixed number of cycles for diagram time scale**; input of the time range in number of cycles via input box

(2) Initial diagram types

The following settings are possible by activating the relevant option button:

- One diagram
- Channels diagram
- ROIs diagram

On activation of the **Black graphs** ☑ check box, the intensity profiles in the diagram are displayed in black (monochrome).

(3) Live image

If you activate the **Also display the live image** check box, the live image will be additionally displayed in the **Image Display** window of the Mean of ROI function during the Mean of ROI scan.

On activation of the **Also display the live image** check box, two further options become available in the **Scan Mean of ROIs** tab:

- Scan the whole image check box
- Save the whole time series check box

(a) Scan the whole image

If you activate this check box, the complete live image will be scanned; only the defined ROIs will be scanned if the check box is deactivated.

(b) Save the whole time series

If you activate this check box, the complete Time Series will be scanned; only the Mean of ROI series will be scanned if the check box is deactivated.

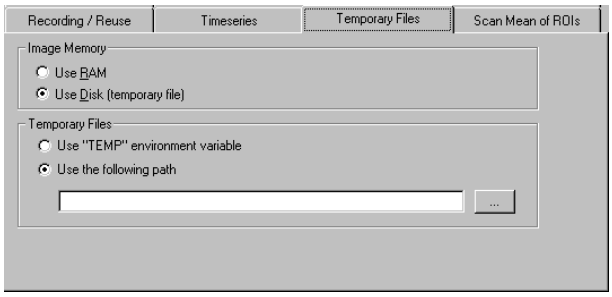


Fig. 5-237 Temporary Files tab

5.11.5.13 Temporary Files

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

(1) Use "TEMP" environment variable

Temporary files are stored in the **TEMP** standard directory of the computer's hard disk.

(2) Use the following path

The directory for temporary files can be selected by clicking on the **...** button in the **Choose Directory** window.

5.11.5.14 Program Start

The **Program Start** tab permits selection of a track configuration via the **Startup configuration** selection box, which will be loaded automatically when the Expert Mode is started.

On activation of the **Don't show logo** ☑ check box, the initial screen with the Zeiss logo will not be displayed after the start of the LSM-FCS software.

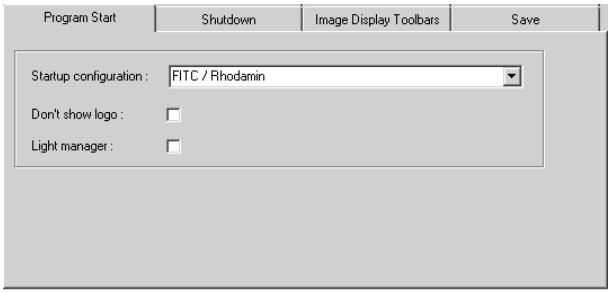


Fig. 5-238 Program Start tab

5.11.5.15 Shutdown

The **Shutdown** tab allows you to determine, by activation of the **Lasers off on Exit** ☑ check box, that the lasers are automatically switched off when the LSM-FCS software is exited.

Allow lasers to cool for five minutes before switching of the system.

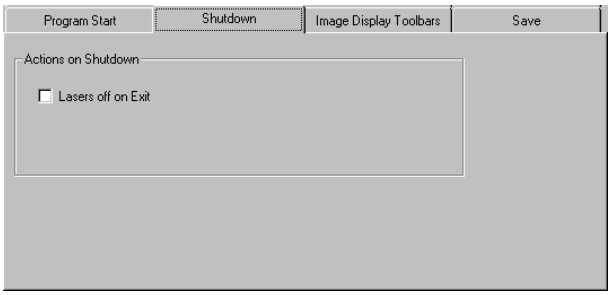


Fig. 5-239 Shutdown tab

5.11.5.16 Image Display Toolbars

The **Image Display Toolbars** tab enables you to determine the window toolbars which shall be automatically displayed when an **Image Display** window is opened.

Furthermore, the color mode (color / mono), to which the image display will switch when the **Color Palette** function is opened / closed, can be determined.

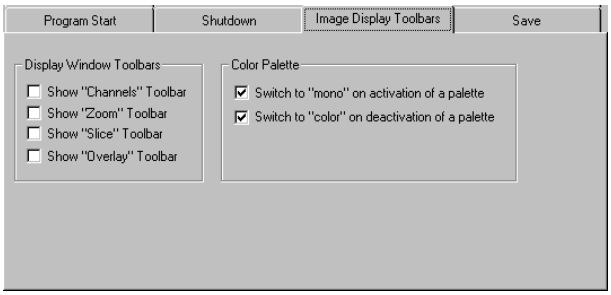


Fig. 5-240 Image Display Toolbars tab

(1) Display Windows Toolbars

On activation of the relevant check box, the following window toolbars are automatically displayed when an **Image Display** window is opened: **Channels**, **Zoom**, **Slice**, **Overlay**.

(2) Color Palette

Switch to "mono" on activation of a palette

If this check box ☑ is activated, the **Mono(chrome)** image display mode is switched automatically when a palette is selected in the **Color Palette** window.

Switch to "color" on deactivation of a palette

If this check box ☑ is activated, the **Color** image display mode is switched automatically when a palette is deactivated in the **Color Palette** window.

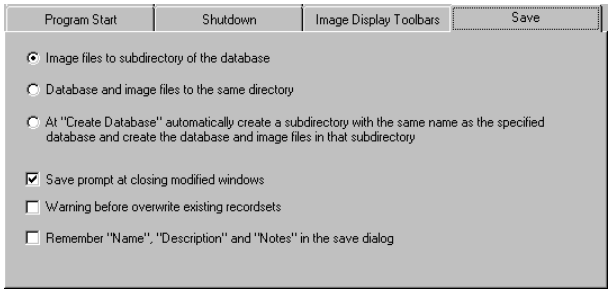


Fig. 5-241 Save tab

5.11.5.17 Save

The **Save** tab permits the presetting for the storage of scanned or processed images to be changed.

Activation of one of the three option buttons enables you to determine the database directories to which stored images are assigned:

- Image files to subdirectory of the database
- Database and image files to the same directory
- At "Create Database" automatically create a subdirectory with the same name as the specified database and create the database and image files in that subdirectory

If the **Save prompt at closing modified windows** check box is activated, you are automatically asked on closing a changed **Image Display** window whether the image shall be stored.

If the **Warning before overwrite existing recordsets** check box is activated, this question is asked automatically on storing an image under a new name if an image file with this name already exists in the database.

If the **Remember "Name", "Description" and "Notes" in the save dialog** check box is activated, the **Name**, **Description** and **Notes** text boxes of the **Save Image and Parameter As** window show the text for the image last saved. You can edit the text boxes as required for the new image to be saved.

If the **Remember "Name", "Description" and "Notes" in the save dialog** check box is deactivated, the three text boxes are blank when the **Save Image and Parameter As** window is opened.

5.11.6 FCS Settings Function

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

- **Open / Save**
- **Raw Data**
- **Measure**
- **LSM+ConfoCor**

5.11.6.1 Open / Close the Settings for user : ... window

- Click on the **Settings** button in the **Options** subordinate toolbar of the **Main** menu.
  - This opens the **Settings for user : ...** window.
- Click on the appropriate tab to change this option.
- Click on the **OK** button to quit the window. The last settings will be taken over. **Cancel** enables you to cancel the procedure, with any changes you made **not** being taken over.

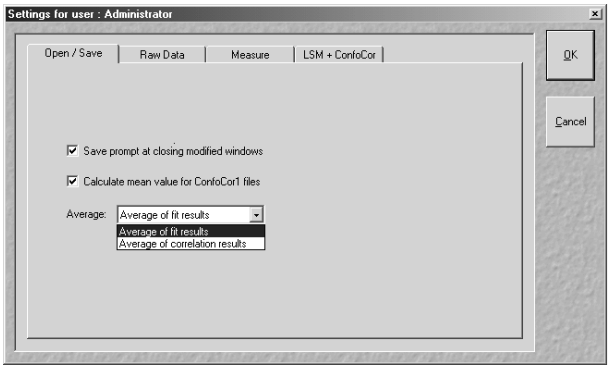


Fig. 5-242 Settings for user : ... window (Open / Save tab activated)

5.11.6.2 Open / Save tab

Ticking the **Save prompt at closing modified windows** option box will set the following procedure: if the field is ticked and you exit a window in which some settings were changed a dialog will ask you to save these changes.

Ticking the **Calculate mean value for ConfoCor 1** files option box will give you a calculation of the mean of opened ConfoCor 1 files in the last row. You can choose in the **Average** display box if you would like to **Average the correlation results** (every measurement point of the single correlation curves is averaged) or **Average the fit results** (values of parameters obtained from the single correlation curves are averaged).

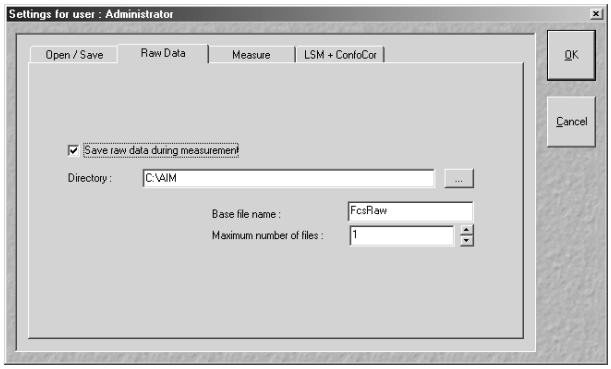


Fig. 5-243 Raw Data tab

5.11.6.3 Raw Data tab

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 Appendix A.

- In the **Directory** field, the directory will be set where the data should be saved. When the ... button is clicked, a WINDOWS NT directory selection dialog will open.
- In the **Base File Name** field, the base file name is set.
- The maximum number of files can be set (up to 100).

☞ Each single measurement is stored in one raw data file. When the set limit is reached, the oldest file will be overwritten and the data in this file are lost. It is the responsibility of the operator to save the data.

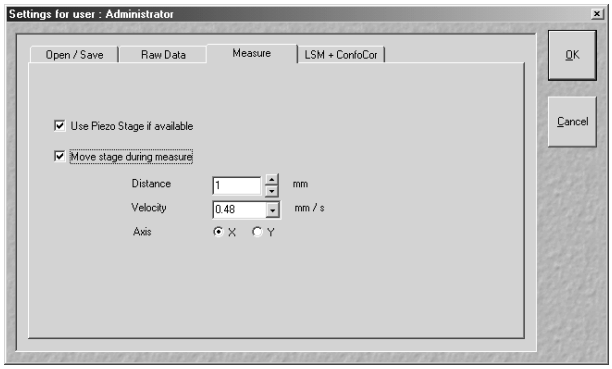


Fig. 5-244 Measure tab

5.11.6.4 Measure tab

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 **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.

5.11.6.5 LSM+ConfoCor tab

This function permits the lateral matching of the ConfoCor 2 beam path with that of the LSM through entry of the relevant offset values.

Determination of these values and their setting will be performed by service staff during start-up of the system.

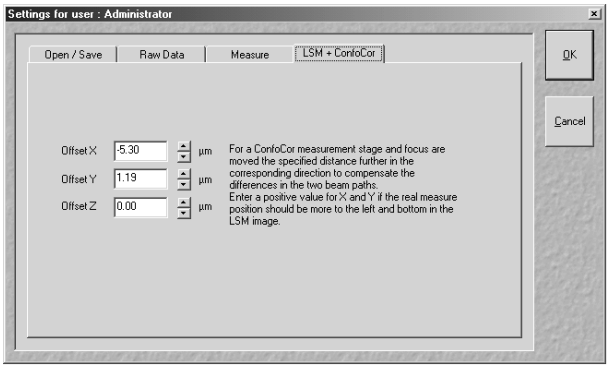


Fig. 5-245 LSM+ConfoCor tab

5.11.7 FCS Collimator Settings Function

This function 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.

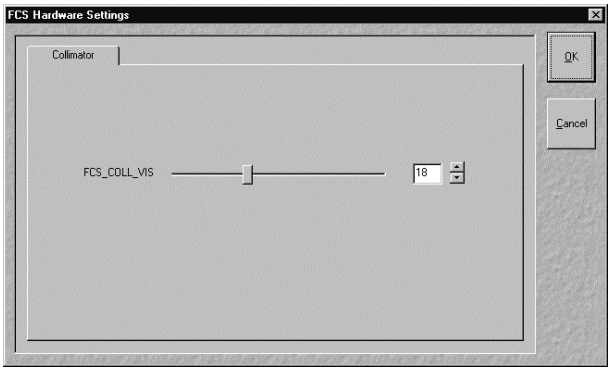


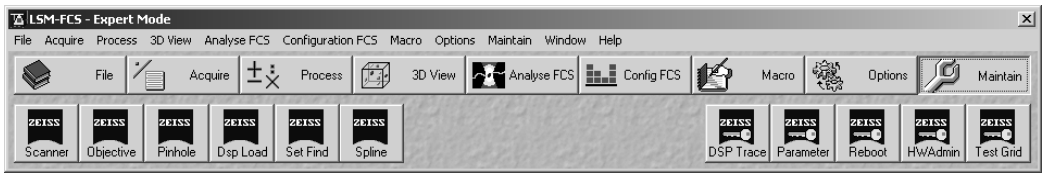
Fig. 5-246 Collimator tab

- To change the collimator setting, click the **FCS Colli.** button in the **Options** toolbar subordinate of the **Main** menu (Fig. 5-220).
- The **Collimator** tab will appear.
- Now the new collimator settings can be entered either by drawing the slider, clicking on the arrows or directly entering a new number.

5.12 Maintain Menu

The **Maintain** menu contains additional modules to check and guarantee the interference-free operation of all the software and hardware components of the LSM-FCS.

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



← **Maintain** subordinate toolbar

Fig. 5-247 Maintain menu

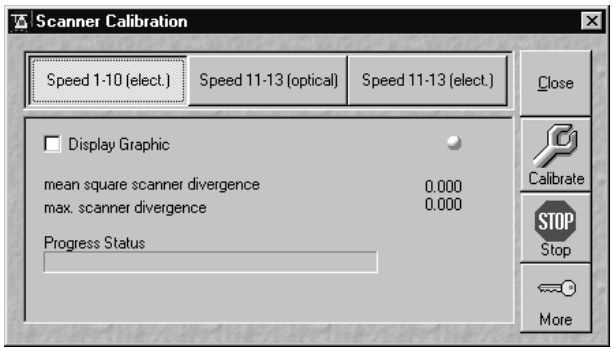


Fig. 5-248 Scanner Calibration window

5.12.1 Scanner

The **Scanner** function is used for scanner calibration and involves a manipulation of the LSM-FCS hardware.

5.12.1.1 Calibration with Speed 1-10 (electr., unidirectional / bidirectional) and Speed 11-13 (electr., only unidirectional)

(1) Preliminary notes

The electrical calibration has to be performed every 2-3 months. For electrical calibration no laser scanning is performed and for that reason no calibration sample is needed.

(2) Calibration conditions

Before the calibration process can be started, the system has to be in operation for at least one hour.

(3) Calibration procedure

- Click on the **Scanner** button in the **Maintain** subordinate toolbar of the **Main** menu.
  - This opens the **Scanner Calibration** window.
- Click on the **Speed 1-10 (electr.)** or **Speed 11-13 (electr.)** button respectively.
- For electrical calibration of speeds 11-13 the appropriate zoom factors have to be applied (11 : zoom ≥ 2.5, 12 : zoom ≥ 3.6, 13 : zoom ≥ 5.6).
- Activate the **Display Graphics** check box enables to check the progress of the calibration process on the **Progress Status** bar.
  - During successful calibration process, the status button is of green color, in case of an error it switches to red. The progress of the calibration process is indicated by the **Progress Status** bar. The calibration process is completed, when the indicator button is grayed.
- Click on the **Calibrate** button to start the automatic scanner calibration.
- Confirm warning information with **OK**.
- Click on the **Close** button to close the **Scanner calibration** window.

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

5.12.1.2 Calibration with Speed 11-13 (optical, bidirectional)

(1) Preliminary notes

The optical calibration of scan speeds 11-13 (bidirectional) can only be performed at systems with complete hardware level of Release 2.8.

The optical calibration procedure has to be repeated every two weeks in normal use and after long delay times of the system.

The minimum duration of the calibration process is 10 minutes. However, it can last up to a maximum time of 40 minutes depending on the performance of the scanner in use and the actual tuning conditions.

If the optical calibration is successfully finished, there is no need to start the electrical calibration for unidirectional scanning of speed 11-13 (in opposite, the electrical calibration would overwrite the much more accurate values of the optical calibration procedure).

With speed 11, 12 and 13, scanning is performed at scanner frequencies of 868, 1042 and 1306 Hz, respectively.

In bidirectional scanning, the total line frequencies are 1736, 2084 and 2612 Hz, the image recording times for 512 lines are 0.29, 0.25 and 0.20 seconds.

(2) Calibration conditions

- The microscope stand has to be placed totally vibration-free.
  - Note, that even power units on the granite plate or inappropriate situated cables can cause vibrations.
- Before the calibration process is started, the system has to be in operation for at least one hour (better: two hours).
  - Otherwise, the tuning results will be incorrect and the forward / backward image contents do not match with each other.
- The longest available laser wavelength of the system has to be applied.
- A 80/20 neutral beamsplitter and a **None** position in the emission filter wheel of either Channel 1 or 2 has to be used in the **Scan Configuration** window.
- A Plan-Neofluar 10x/0.3 or Epiplan Neofluar 10x/0.3 objective lens has to be used.
- A special sample (see Fig. 5-249) with two identical but 90 degrees rotated gratings (one for each scan direction) has to be used as a calibration standard.
- The pinhole has to be completely opened.
- A dynamic range of 8 bit has to be used.

(3) Calibration procedure

- It is advantageous to perform the electrical calibration before starting the optical calibration process. For a first scan of the calibration standard start with scan speed 12 and zoom 3.6 (unidirectional).
- Focus on the calibration standard and adapt the dynamic range of the detector on the sample.
  - Optimize **Detector Gain** and **Ampl. Offset** values in the **Channels** sheet of the **Scan Control** window by means of the **Range Indicator** mode.

Fig. 5-249 Calibration standard

- The calibration standard has to be positioned as indicated in Fig. 5-250.
- Before starting the calibration procedure, change to the bidirectional scan mode in the **Scan Control** window.
- **Scan Corr. X** and **Scan Corr. Y** in the **Mode** sheet of the **Scan Control** window has to be set to zero.
- Click on the **Scanner** button in the **Maintain** subordinate toolbar of the **Main** menu.
  - This opens the **Scanner Calibration** window.

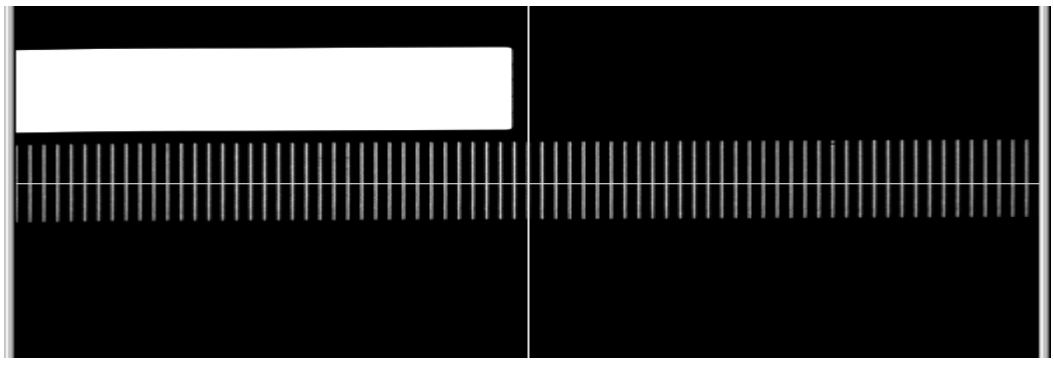


Fig. 5-250 Correct orientation of calibration sample for X scanner calibration

- Click on the **Speed 11-13 (optical)** button.
- Activate the **Display Graphics** check box enables the graphical display of the calibration process.
- Click on the **More (Less)** button to display the **Speed Selection** menu.

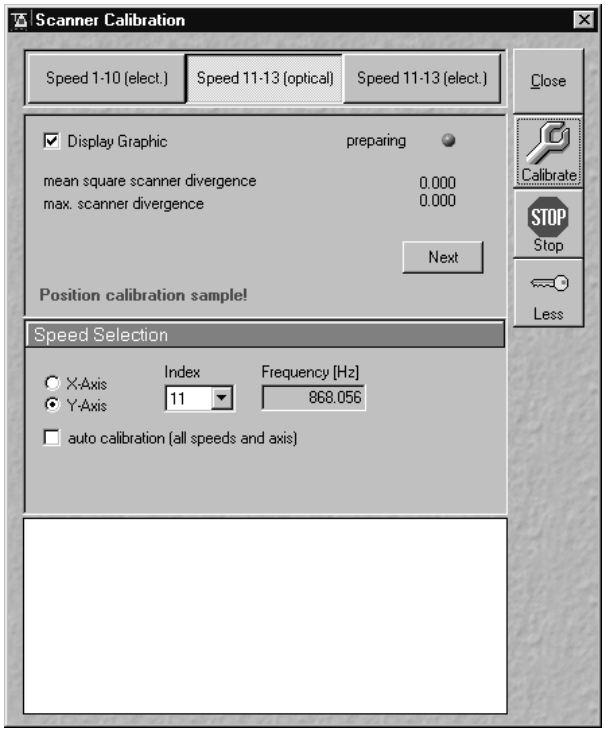


Fig. 5-251 Scanner Calibration window

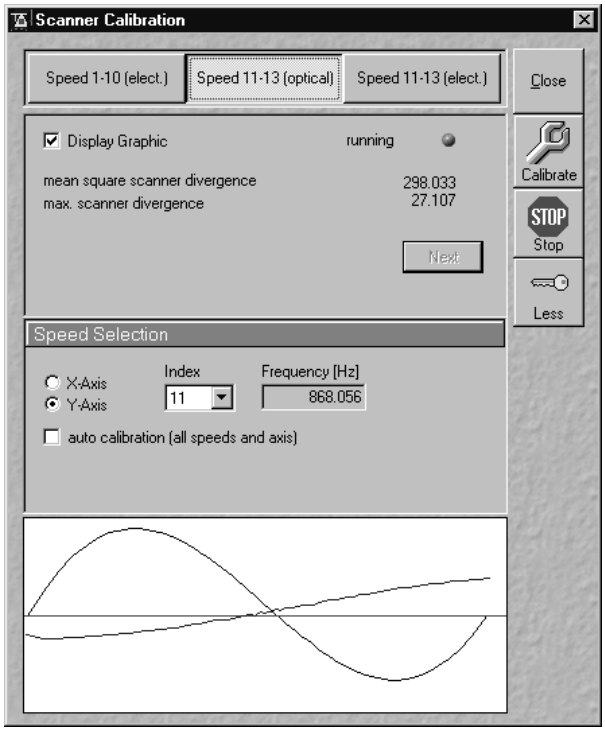


Fig. 5-252 Scanner Calibration window

- With activated **Auto Calibration** box, all speeds (index) and axis are calibrated one after another automatically.
  - Goal of the calibration procedure is the achievement of a minimum of the **forward-backward-difference** (blue line in the graphical display) and the best possible **linearity** (black line in graphical display monitors the linearity deviation). Both lines (blue and black) should be as straight as possible and as close as possible to the **Zero level** (red line in graphical display). Green line represents the driver voltage.
- Click on the **Calibrate** button to start the scanner calibration procedure.
- Confirm warning information with **OK**.
  - The procedure starts with Speed 11 and the X-scanner. When the Auto calibration for Speed 11 is finished successfully the procedure continuos with Speed 12 (higher acoustic frequency) and larger zoom.
- If necessary slightly reposition the sample and click on the **Next** button.
- Do not focus or change scan parameters during calibration procedure!
- After calibration of X-axis the orientation of the calibration grid in the calibration window changes from horizontal to vertical orientation. The second grid of the calibration sample has to be selected and again positioned as indicated in Fig. 5-250.
- Click on the **Next** button and continue with Y-scanner calibration.
- If the calibration for all speeds and scanners has finished successfully, quit the scanner calibration window by pressing the **Close** button.
- If the Auto Calibration procedure can not be performed successfully after several tries, calibrate the scanner manually.

- If the **Auto Calibration** box is disabled, the speed index and the scanner (**X-axis** or **Y-axis**) has to be chosen manually. The calibration cycle only contains one speed index and one scanner. For calibration of all speeds (11-13) and both scanners, the manual calibration procedure has to be repeated several times with changing axis and index parameters (Start with scanner X and speed 11, continue with scanner X and speed 12, then X and speed 13, ...). The position of the calibration standard has to be controlled at each new speed index. A repositioning of the calibration sample is required after scanner change (for X-scanner calibration horizontal grid, for Y-scanner vertical grid).

(4) Important notes and hints

The tuning procedure runs automatically to a large extent without any problems. However, several errors can occur. That's why it is strongly recommended to observe the complete calibration process.

If a status error message occurs or the calibration procedure is not finished properly, this can have the following reasons:

No optimal positioning of the calibration standard.

Indication: One end of blue and black line jumps a bit forward and backward because software recognizes sometimes the outer line of the grating and sometimes not.

- Stop the calibration procedure.
- Check the focus of the calibration sample.
- Check if the yellow horizontal line crosses the scale pattern of the calibration standard properly.
- (If necessary) Shift the calibration standard by half a scale unit (No scale tick but a gap has to be situated directly on the edge of the image).
- Restart the calibration procedure.

No optimal setting of Detector gain and Amplifier Offset.

Indication: The forward-backward-difference shows a lot of peaks and changes significantly from image to image.

- Stop the calibration procedure.
- Calibrate speed 12 (unidirectional) electrically.
- Optimize gain & offset: Ticks of the grid have to have intensity values of 250 ... 255 (just before red color in **Range Indicator** palette). Minimum intensity values have to be between 1 ... 5 (no blue parts occur in the image by applying **Range Indicator** palette).
- Restart the optical calibration procedure.

Sliders of Scan Corr. X and Scan Corr. Y in the Mode sheet of the Scan Control window were not set to zero.

Indication: Calibration process does not converge.

- Stop the calibration procedure.
- Set both values to zero.
- Restart the calibration procedure.

Non-regularities of the scanner feedback.

Indication: The ticks on the outer sides of the grid vary about more than 1 tick width between consecutive images (in the middle of the calibration process, the linearity is optimized and the problem starts to occur).

- Stop the calibration procedure.
- Call the LSM service hotline.

If optical calibration comes not to a successful end, please contact your service hotline.

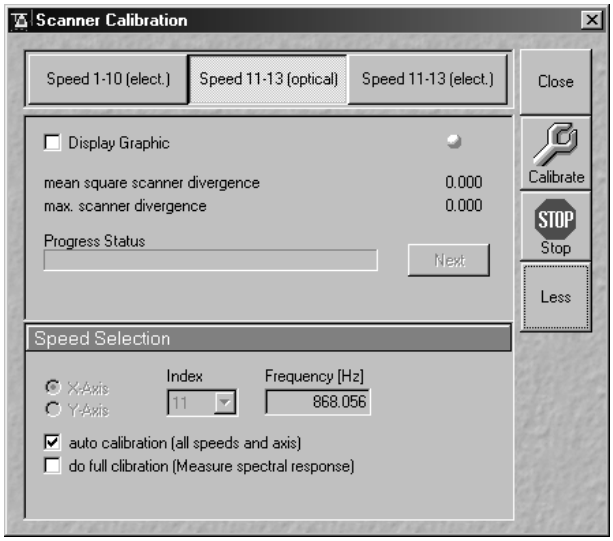


Fig. 5-253 Scanner Calibration window

Scanner calibration in LSM 5 Software, Release 3.0

In LSM 5 Software, Release 3.0, the **do full calibration (Measure spectral response)** checkbox must be set at the first calibration procedure **Speed 11-13 (optical)**.

5.12.2 Objective

This function permits changed objectives to be activated and the parfocality to be set without having to exit the software.

5.12.2.1 Objective change

- Change the required objective in the nosepiece.
- Click on the **Objective** button in the **Maintain** subordinate toolbar of the **Main** menu.
  - The **Objective Control** window appears on the screen. The **Objective** button is activated in accordance with the presetting, and the **Objective** panel is displayed in the **Objective Control** window.
- Click on the graphical button of the relevant nosepiece mount (**Position**).
  - The **Change Objective** window appears.

All available objectives are listed in the **Potential Objectives** directory of the **Change Objective** window.

- Select the new objective by double clicking from the list in the **Potential Objectives** directory.
- Click on **Close** to exit the **Change Objective** window.

(1) Add Objective

This function permits new objectives to be added to the database.

For this, proceed as follows:

- Click on the **Add Objective** button on the **Change Objective** window.
  - The **Create new Objective** window is opened.

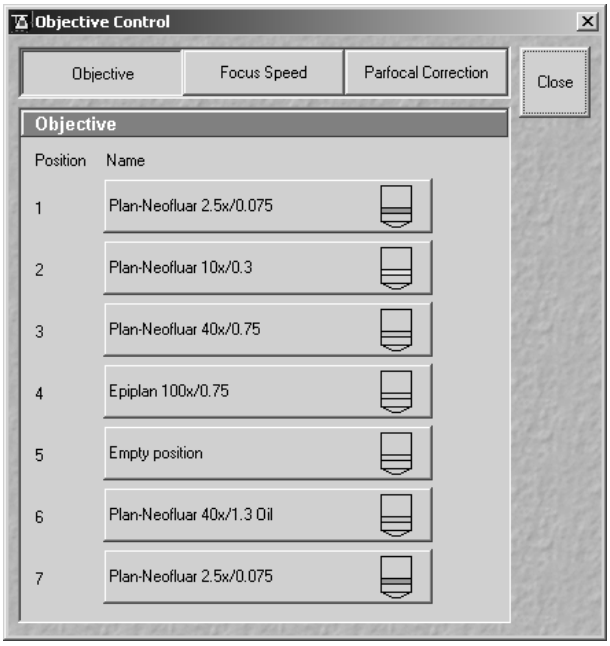


Fig. 5-254 Objective Control window

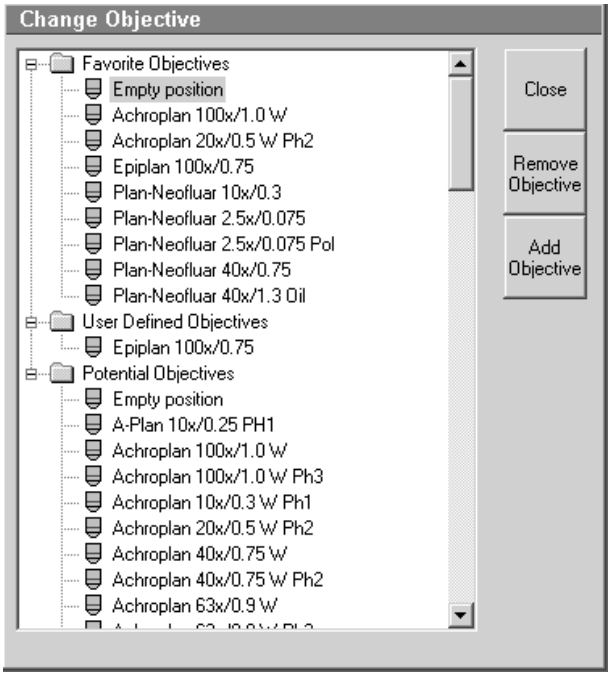


Fig. 5-255 Objective Control window

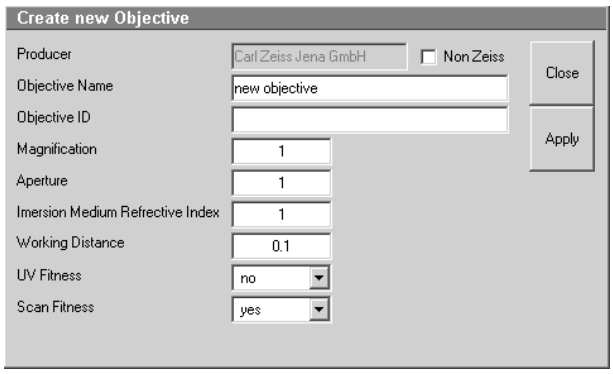


Fig. 5-256 Create new Objective window

- Enter the data of the new objective in the **Create new Objective** window, then click on the **Apply** button.

The new objective is stored in the database and included in the **Change Objectives** window. You can now activate the new objective as a favorite objective using the procedure described above.

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

(2) Remove Objective

You can only remove objectives in the **Favorite Objectives** and the **User Defined Objectives** directories.

- To remove an objective from the database, select it with a click of the mouse in the **Change Objective** panel and then click on **Remove Objective**. The new objective appears in the **User Defined Objectives** directory.
- Click on **Close** to close the **Create new Objective** window.

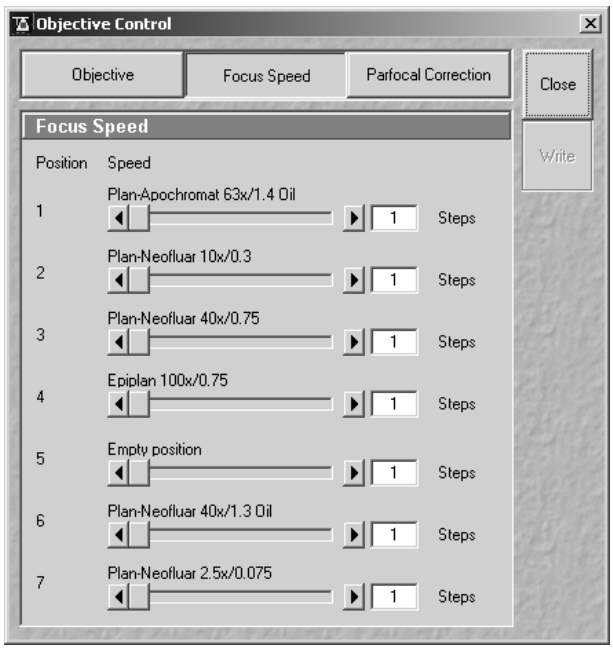


Fig. 5-257 Focus Speed window

5.12.2.2 Focus speed change

- Change the required objective in the nosepiece.
- Click on the **Objective** button in the **Maintain** subordinate toolbar of the main menu.
  - The **Objective Control** window appears on the screen. The **Focus Speed** has to be activated in the **Objective Control** window.
  - The focusing speed of the relevant objective can be selected by using either the slider or the input box in 40 steps.

5.12.2.3 Parfocality Correction

The parfocal setting is performed via screen dialogs in successive panels.

- Click on the **Parfocal Correction** button.
  - The **Parfocal Adjustment** panel appears.
- Start the setting with the objective of the highest magnification factor (reference objective). Proceed in accordance with the displayed instructions.
- Click on **Start**.
  - The next dialog is displayed in the **Parfocal Adjustment** panel.
- Focus on your slide object.
- Click on the **Next step** button.
- Perform these steps for each objective.
- Click on the **Close** button to exit the **Objective Control** window and accept the settings.

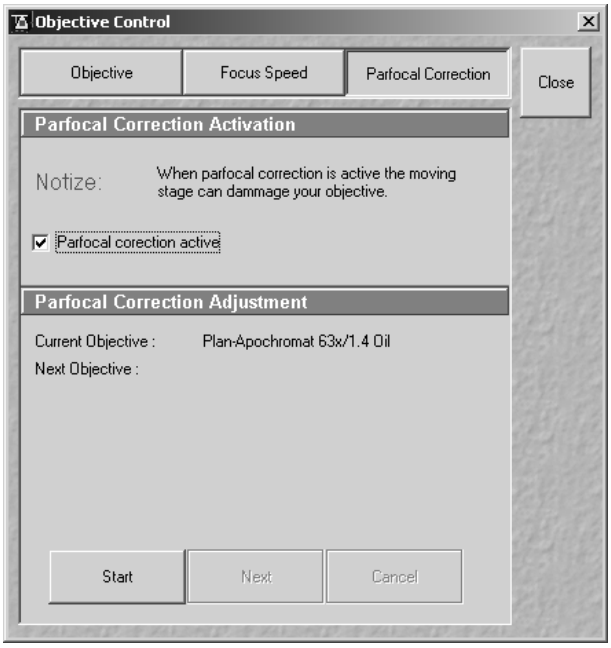


Fig. 5-258 Objective Control window

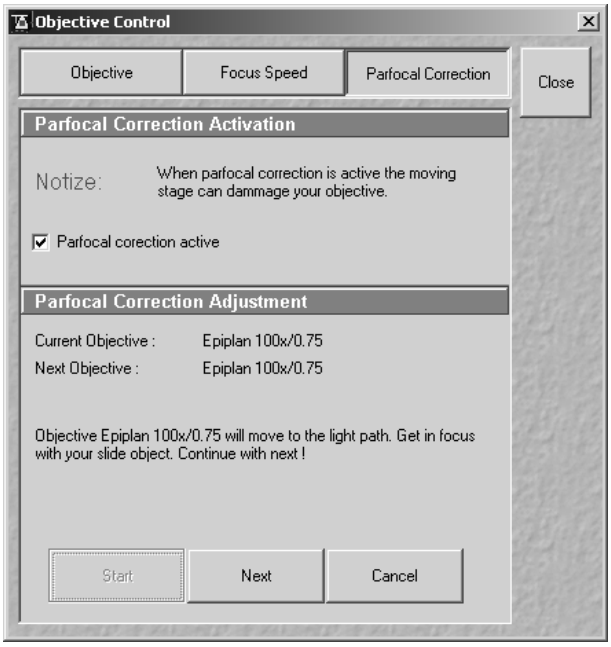


Fig. 5-259 Objective Control window

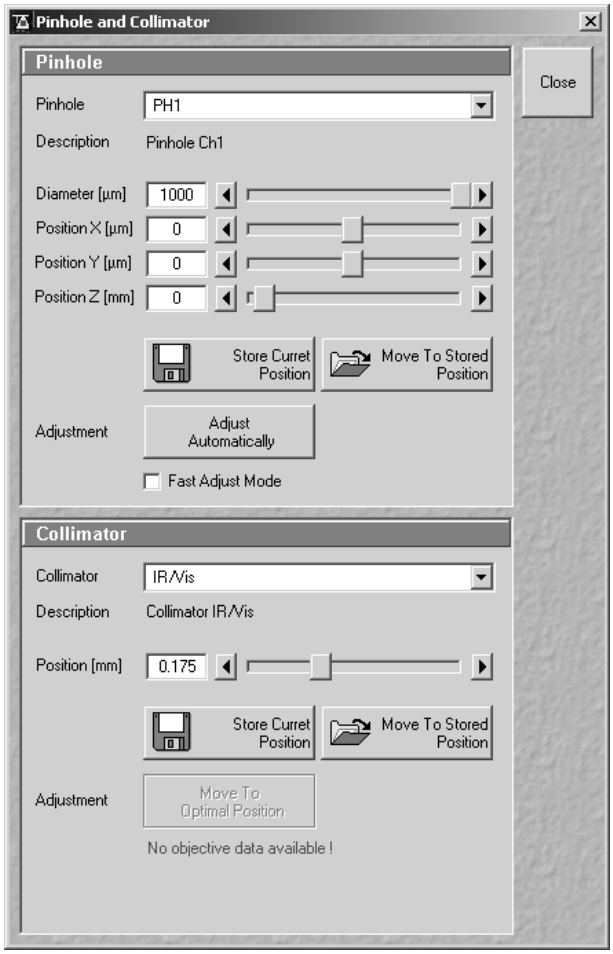


Fig. 5-260 Pinhole & Collimator Control window

5.12.3 Pinhole Adjustment

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

The position of the pinhole (X-Y-Z-coordinates) in relation to the detector makes a major contribution to image optimization.

In all existing standard configurations, the pinholes have already been adjusted at the factory. These settings are taken over for active operation when a standard configuration is loaded.

If you want to create a setting that differs from the standard configurations, adjust the pinhole as follows.

5.12.3.1 Open / Close the Pinhole & Collimator Control window

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

5.12.3.2 Function description

(1) Pinhole panel

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

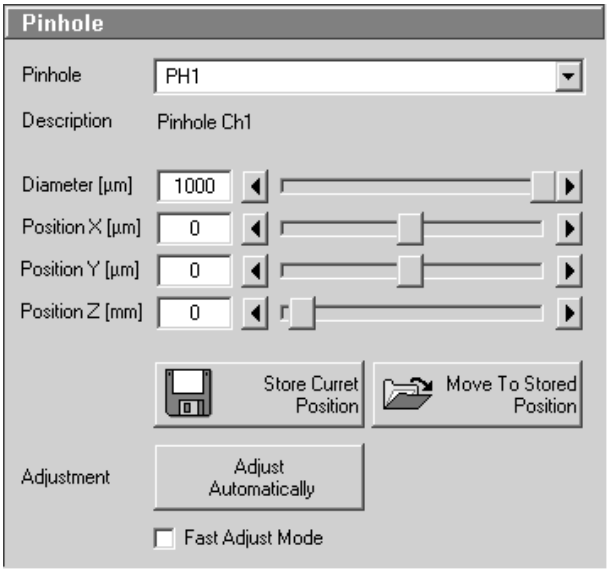


Fig. 5-261 Pinhole panel

Pinhole / Description field: Selection of pinholes (PH1 to PH4) to be adjusted via the **Pinhole** selection box, display of the relevant channel in the **Description** field.

Diameter; Pos. X; Y; Z slider: Setting of diameter, X-, Y- and Z-position of the pinhole in relation to the beam path (Z-position can be set only for PH1) using the slider or arrow buttons, status display for setting procedure: green for ready and red for busy.

Store current Position button: Storage of the current pinhole setting.

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

Adjust Automatically button: Automatic pinhole adjustment.

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

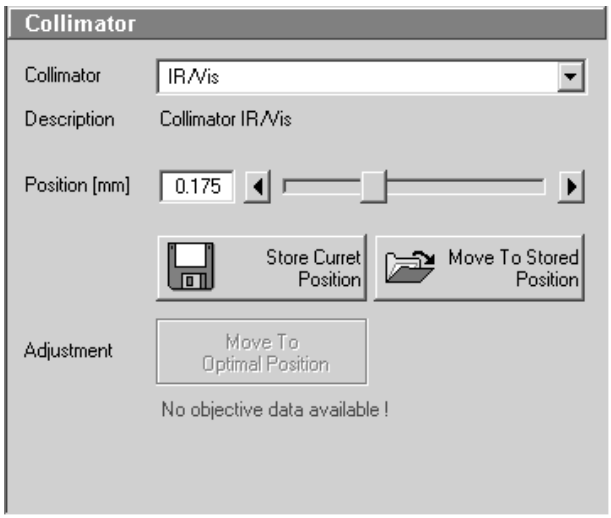


Fig. 5-262 Collimator panel

(2) Collimator panel

Collimator

Description field: Selection of the collimator (IR / VIS or UV / VIS) to be adjusted via **Collimator** selection box, display of selected collimator in the **Description** field.

Positions field: Setting of collimator position using the slider or arrow buttons; the display to the right of the slider indicates the current position, status display for setting procedure: green for ready and red for busy.

Store Current Position button: Stores the current the collimator position.

Move To Stored Position button: Sets the collimator to the stored value.

Move to Optimal Position button: Starts the automatic collimator adjustment. Available for most common objectives.

5.12.3.3 Pinhole and collimator adjustment

Adjustment of the LSM-FCS pinholes can be performed manually or automatically.

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

(1) Manual pinhole and collimator adjustment

The position of the pinhole relative to the detector in terms of X-Y-Z coordinates contributes substantially to image optimization.

Requirements to make pinhole position changes visible immediately:

- The image must be scanned by the continuous scan method.
- Select a fast scanning speed.
- Measurement with Average Number 1 only (no averaging of several measurements).
- On the **Channel Settings** panel (click on **Channels** button in the **Scan Control** window), select the pinhole diameter so as to have the best possible image contrast.

- Click on the **Pinhole** button in the **Maintain** subordinate toolbar.
- Select the pinhole to be adjusted from the **Description** list box.
- Use the **Diameter** slider to set the smallest possible size which produces a good, high-contrast image.
  - This setting changes the pinhole diameter.
  - The **Z Slice** display box simultaneously displays the depth resolution corresponding to the pinhole diameter.

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

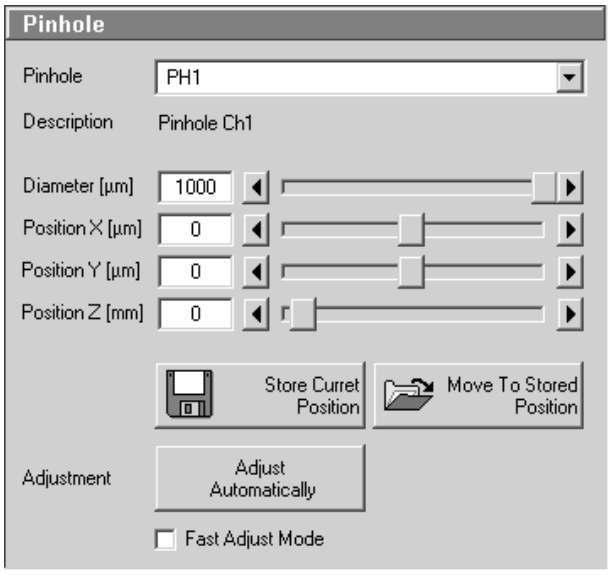


Fig. 5-263 Pinhole panel

- Optimize the pinhole position in X, Y and Z (Z only for PH1) relative to the PMT using the X, Y and Z sliders to maximum image brightness.
- Click on the **Save Current Position** button to save the pinhole adjustment.

- Removing the **Current Positions** slider in the **Collimator** panel allows the collimator to be adjusted to maximum image brightness. Optimum collimator adjustment obtained in this way can be stored by clicking on the **Save Current Position** button.
- Click on the **Stop** button to stop the continuous scan.

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

(2) Automatic pinhole and collimator adjustment

The automatic adjustment allows the LSM-FCS pinholes to be used with any combination of beam splitters.

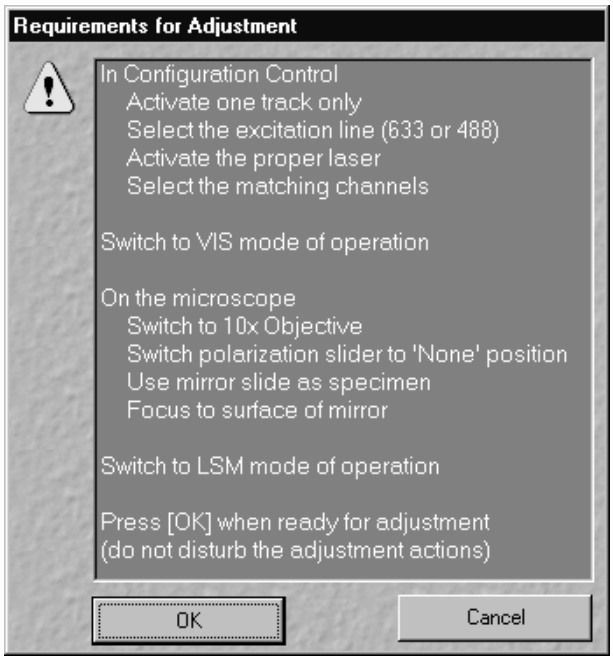


Fig. 5-264 Requirements for Adjustment window

- Click on the **Adjust Automatically** button.
  - The **Requirements for Adjustment** window will then appear.
- Meet the requirements listed in the **Requirements for Adjustment** window and press the **OK** button.
  - Pinhole adjustment will then run automatically. The adjusting procedure takes approx. 3 min.
  - The determined data are stored automatically and will be available for all further examinations using the same configuration.
- Click on the **Move to Preadjust** button in the **Collimator** panel. Optimum positioning of the collimator will be performed. The **Default** button enables the collimator to be set back to the factory-adjustment.
- Activate the **Fast Adjust Mode** check box for a faster readjustment.

☞ A change of the pinhole diameter made manually in the **Pinhole** panel will not be activated in the **Scan Control** window. Therefore, changes must always be made in the **Channel Settings** panel of the **Scan Control** window.

A filter change in **Autoadjust** is not displayed in the **Config. Control** window.

Configuration 1 is equipped in such a way that pinhole adjustment for channel 1 can only be made with λ = 488 nm, NFT 545, NFT 610 or NFT 570.

Please remember that the Z-coordinate for channel 1 is not optimized during the automatic pinhole adjustment. Subsequent optimization can be performed via the **Move to Preadjust** button in the **Collimator** panel of the **Pinhole & Collimator Control** window.

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

The optimum setting of the collimator must be performed separately for each track via the **Move to Preadjust** button in the **Pinhole & Collimator Control** window. If several tracks are activated (Recording), an average value of the positions valid for the various tracks will be set on pressing the **Preadjust** button. When all the tracks have been defined and are active (only the ticked tracks will be included in the calculation), press the **Move to Preadjust** button.

5.12.4 DSP (Digital Signal Processor)

The **DSP** function is used to display the current performance of the system processor for checking purposes.

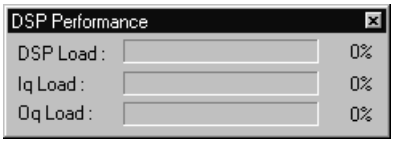


Fig. 5-265 DSP Performance window

- Click on the **DSP** button in the **Maintain** subordinate toolbar of the **Main** menu.
  - This opens the **DSP Performance** window.
- Click on the ☒ button to close the **DSP Performance** window.

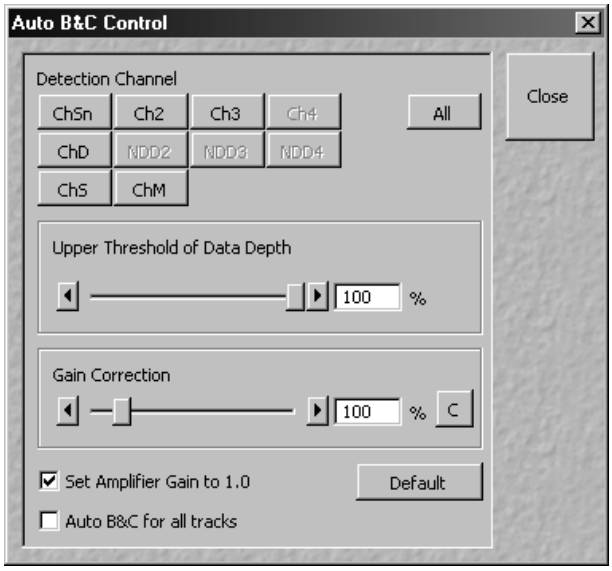


Fig. 5-266 Auto B&C Control window

5.12.5 Set Find

This function permits the preset parameters of the **Find** function (see **Scan Control**, page 5-72)) to be matched individually.

- Click on the **Set Find** button in the **Maintain** subordinate toolbar of the **Main** menu.
  - The **Auto B&C Control** window appears on the screen.
- Change the settings for the **Upper Threshold of Data Depth** and **Gain Correction** using the relevant sliders.

The settings can be made individually for each detection channel or for all channels together.

For experiments with increasing of fluorescence over the time it's necessary to reduce the **Upper Threshold of Data Depth** for the **Find** function.

☞ Each slider should be used separately.

- Click on the **C** button to set the value for the **Gain Correction** to 100 %.
- If required, activate the **Set Amplifier Gain to 1.0** check box.
- Click on the **Find** button to start sample scanning with the current settings.
- Clicking on **Default** enables you to activate the default settings again.
- If required, activate **Auto B&C for all tracks** checkbox for **Find** function.

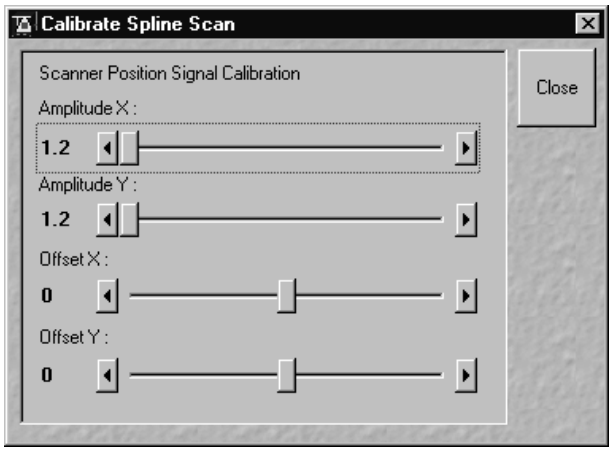


Fig. 5-267 Calibrate Spline Scan window

5.12.6 Spline

This function permits calibration of the Scanner position signals. This is required for the use of spline curves in the **Line** scanning mode (see section 5.5.4, **(3) Line**, page 5-101f).

5.12.7 DSP Trace

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

5.12.8 Parameter

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

5.12.9 Reboot

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

5.12.10 HW/Admin

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

5.12.11 Test Grid

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

5.13 Window Menu

The **Window** menu includes the additional functions **Full Screen**, **Close All Image Windows**, **Toolbar** and **Scan Information** which are not available from a toolbar.

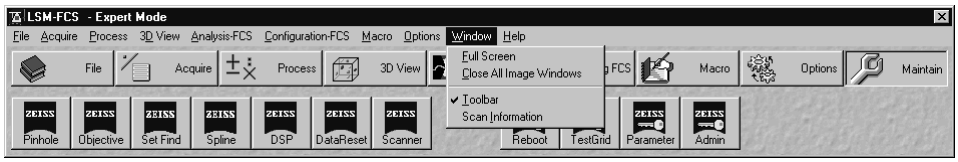


Fig. 5-268 Window pull-down-menu

5.13.1 Full Screen

This function shows the active **Image Display** window in full screen size.

- Activate the image to be shown in full size by clicking on the image content.
- Click on the term **Window** in the menu bar of the **Main** menu.
  - The **Window** menu (pull-down) will be opened.
- Click on the **Full Screen** line.
  - The image will be displayed in full screen size.
- Click in the image to show it again as an **Image Display** window in normal size.

5.13.2 Close All Image Display Windows

This function closes all the opened **Image Display** windows.

- Open the **Window** menu.
- Click on the **Close All Image Windows** line.
  - All the opened **Image Display** windows will be closed.

In the **Options** menu in the function **Settings** in **Save** tab at position **Save prompt at closing modified windows** it can be determined whether a prompt is shown on **Closing of All Image Display Windows** or not.

5.13.3 Toolbar

This function activates / deactivates (alternately) the toolbar and the subordinate toolbar of the **Main** menu.

- Open the **Window** menu.
- Click on the **Toolbar** line.
  - The toolbars of the **Main** menu are displayed / not displayed.

Fig. 5-269 Main menu without toolbars

5.13.4 Scan and System Information

This function opens the **Scan Information** window, in which the current scan data are displayed.

The extent of the data displayed in the **Scan and System Information** window depends on the settings made in the **Options** menu under **Settings** (see page 5-241).

- Open the **Window** menu.
- Click on the **Scan Information** line.
  - The **Scan and System Information** window will be displayed.
- Click on the ☒ button to close the **Scan Information** window.

In the **Options** menu in the function **Settings** in the change of parameters shown can be determined in the **Scan Information** tab.

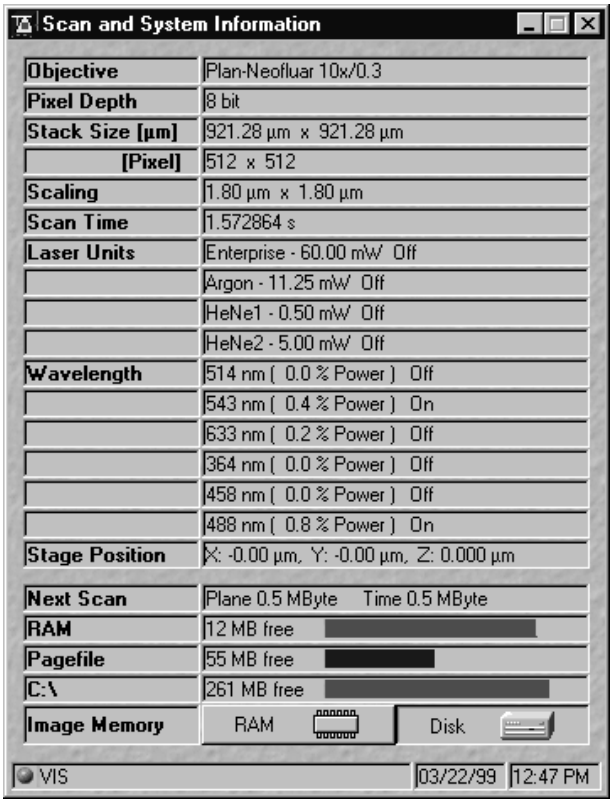


Fig. 5-270 Scan Information window

5.14 Help Menu

The **Help** menu permits activation of the Help function and of a window containing information on the installed software version.

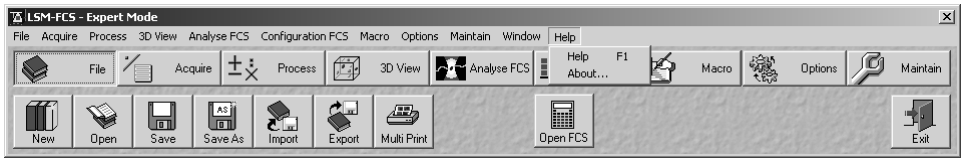


Fig. 5-271 Help pull-down menu

5.14.1 Help

- Open the **Help** menu.
- Click on the **Help** line to open the online help.

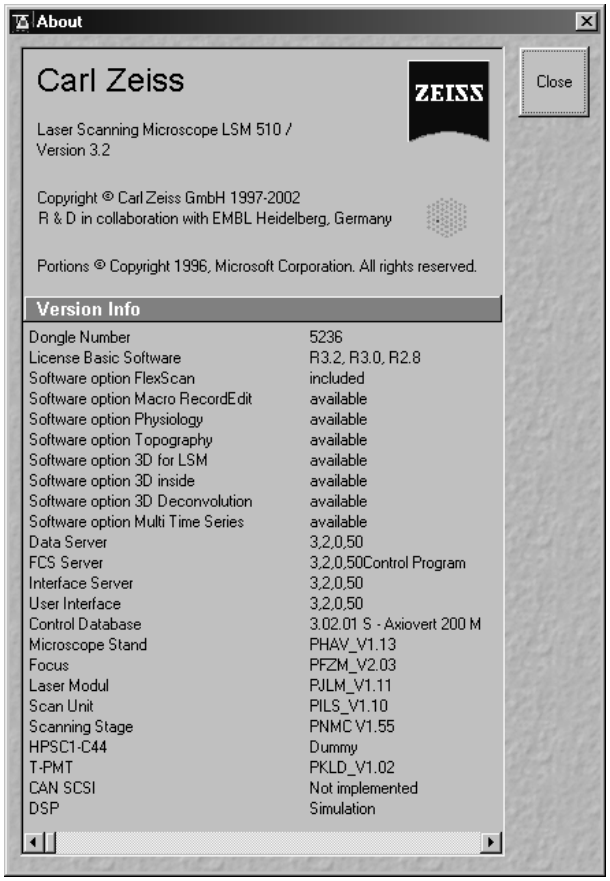


Fig. 5-272 About LSM-FCS window

5.14.2 About

- Open the **Help** menu.
- Click on the **About** line to open the **About** window.

The **About** window includes important information about the software, such as the software version number, copyright, version numbers of the various program components and firmware, and the Dongle number.

- Click on the **Close** button to close the **About** window.

5.15 Display and Analysis of Images

5.15.1 Structure of the Image Display Window

The **Image Display** window shows the image or images when they are

- scanned by any scanning function (see **Scan control** and **Time series control**) or
- loaded from the image database (see **Open database-Load**) or
- imported by the import function (see **Import**).

In addition to show images the **Image Display** window offers two toolbars for

- changing the display parameters and save an image or images (see **Select** toolbar below)
- generating new ways of displaying the data as well as analysis tools (see **Display** toolbar below).

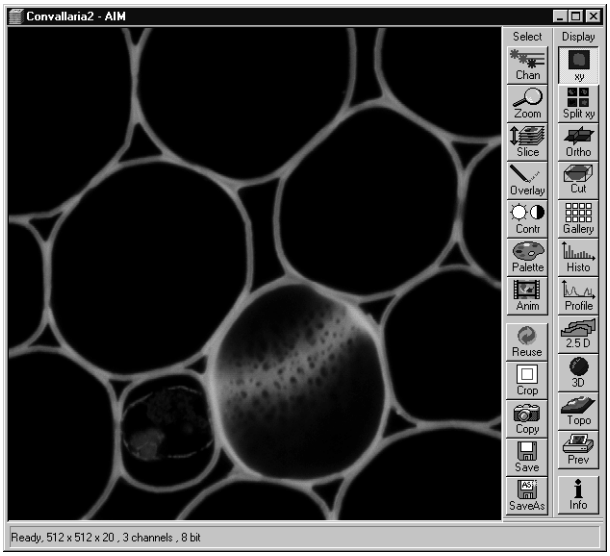


Fig. 5-273 Image Display window showing a single frame image

The **Image Display** window of the LSM-FCS software corresponds to the basic structure of other Microsoft ® WINDOWS applications. The **Image Display** window can be moved as required within the screen, and its vertical, horizontal and diagonal size can be matched to the current requirements (identical to Microsoft ® WINDOWS).

The caption at the top of the **Image Display** window contains the control menu for the **Image Display** window (identical to Microsoft ® WINDOWS), the name of the displayed image, and the **Minimize**, **Maximize** and **Close** buttons.

In the status line at the bottom of the **Image Display** window, the progress bar of a current scanning procedure and the parameters used for image display are shown and updated when changed.

On the left-hand side of the **Image Display** window, an overview of the scan parameter is displayed, provided that the **Info** button of the **Display** toolbar is activated.

The **Settings** function of the **Options** subordinate toolbar with the **Image display toolbars** tab some of the functions of the **Image Display** window toolbars can be activated at the opening of a new **Image Display** window.

It is possible to display the **Chan**, **Zoom**, **Slice** and **Overlay** image display toolbars immediately on opening an **Image Display** window. The relevant check boxes to be activated in the **Image Display Toolbars** tab under **Settings** (see **Options** menu).

It is also possible to display the scan parameter of an image (**Info** button) immediately when an **Image Display** window is opened. The data to be displayed can be defined (see **Image Status Display** tab under **Settings** in the **Options** menu.

The set of functions available at the **Image Display** window toolbars depends on the type of image shown. The LSM 5 software handles the following formats:

- frame (single image and Z Stack of images)
- frame time series (time series of images and time series with Z Stack of images)
- line time series (time series of lines and time series with Z Stack of lines)
- point time series (time series of points)
- Lambda series (Lambda Stack of images and time series with Lambda Stack of images)

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

Image type		**Frame**	**Frame**	**Frame**	**Frame**	**Line**	**Line**	**Line**	**Point**
Series type			**Z Stack**	**Time**	**Lambda**		**Time**	**Lambda**	**Time**
Display functions		xy	xy	xy	xy	xt, tx*	xt, tx		xt, tx
		Split xy	Split xy	Split xy	Coded	Split xt, Split tx*	Split xt Split tx	Coded	Split xt Split tx
		Ortho*	Ortho		Max			Max	
		Cut*	Cut						
		Gallery*	Gallery	Gallery	Gallery				
		Histo	Histo	Histo	Histo	Histo	Histo	Histo	
		Profile	Profile	Profile	Profile	Diagr	Diagr	Diagr	Diagr
				Mean t**	Mean Lambda	Mean*	Mean	Mean Lambda	
		2.5 D	2.5 D	2.5 D		Select	Select	Select	
		3D**	3D**	3D**		2.5 D	2.5 D	2.5D	
		Topo**	Topo**	Topo**					
		Prev	Prev	Prev	Prev				
		Info	Info	Info	Info	Prev	Prev	Prev	Prev
						Info	Info	Info	Info

* inactive

** optional

All display functions are exclusive functions. Only one can be active at a given time. To generate different views of the same image set use the **Duplicate** function in the **Process** menu.

During image acquisition all active display functions can be used.

5.15.2 Select - Chan

This function permits to

- change the color assignment of channels of images
- switch individual channels of a multi channel image on/off
- switch to monochrome display of the image instead of color display

Click on **Chan** will display the **Channels** toolbar. Any changes done with this toolbar are effective immediately.

- Click on the **Chan** button in the **Select** toolbar.
  - The **Channels** toolbar will be displayed on the right-hand side of the **Image Display** window.
- Click on the **Chan** button again to remove the **Channels** toolbar.

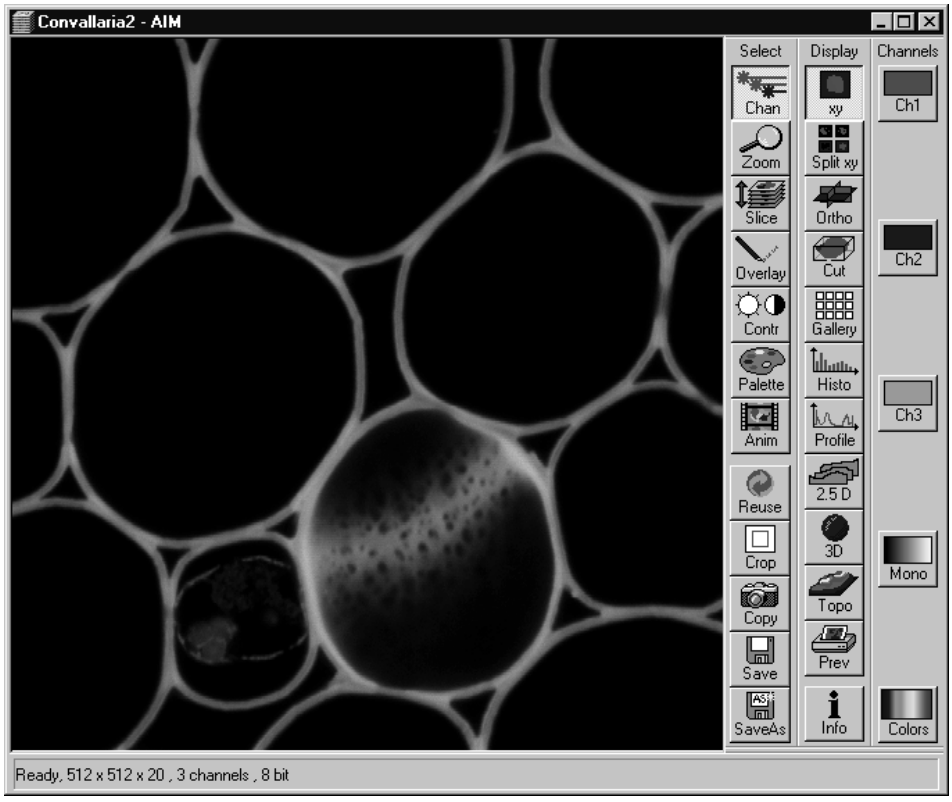


Fig. 5-274 Image Display window; Select - Chan

(1) Assigning another color to a channel

- Click on one of the channels button in the **Channels** toolbar (e.g.: **Ch1**).
  - The color selection box with all the currently defined colors will appear.

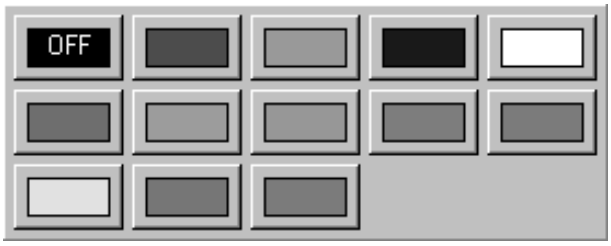


Fig. 5-275 Color selection box

- Click on the required color.
  - The selected color will be assigned to the current channel, the color selection box is closed and the displayed image is updated. The control box of the channel button (e.g.: **Ch1**) also shows the selected color.

(2) Switching a channel of a multi channel image off or on

- Click on one of the channel buttons in the **Channels** toolbar (e.g.:**Ch1**).
  - The color selection box will appear.
- Click on **OFF** to deactivate the display of the relevant channel.

☞ A newly assigned color or a channel switched off is not taken into consideration during the following scanning procedure, since the setting in the **Configuration Control** window always applies here.

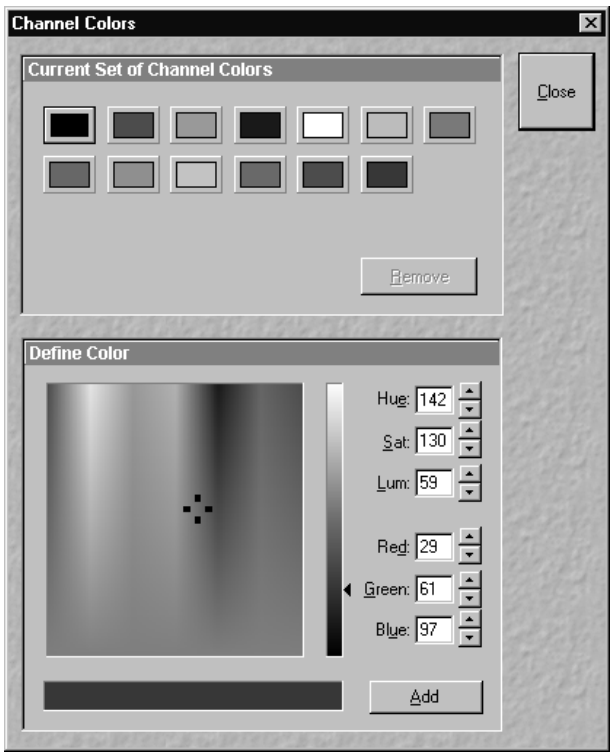


Fig. 5-276 Channel Colors window

(3) Switching to monochrome image display

- Click on the **Mono** button in the **Channels** toolbar.
  - The image will then be displayed in shades of gray exclusively. If you click on the button again, the channels will be displayed in color again.

☞ If you want to view the channels individually, select the split display by clicking on **Split xy** button in the **Display** toolbar.

(4) Defining a new color

- Click on the **Colors** button to open the **Channel Colors** window.
- Define a new color in the same way as in the **Configuration Control** window (see page 5-63).

5.15.3 Select - Zoom

This function allows to change the zoom factor of an image displayed.

Click on **Zoom** will display the **Zoom** toolbar. Any changes done with this toolbar are effective immediately.

The image can be zoomed by various methods. The zoom function can be performed online.

- Click on the **Zoom** button in the **Select** toolbar.
  - The **Zoom** toolbar will be displayed on the right-hand side of the **Image Display** window.
- Clicking on the **Zoom** button again will remove the **Zoom** toolbar.

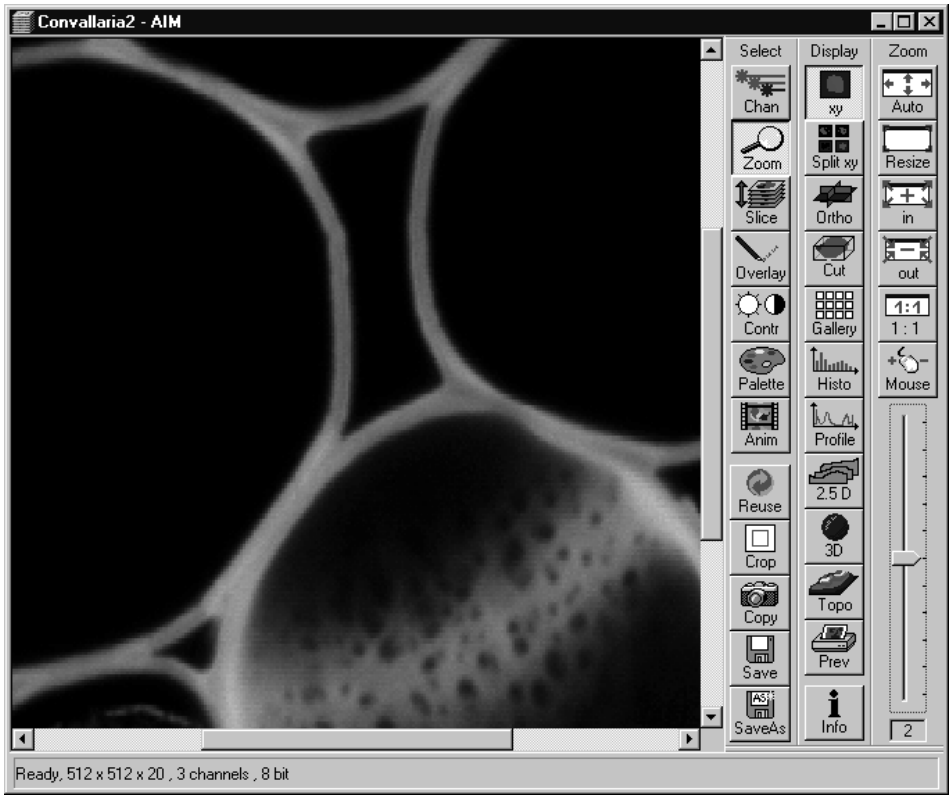


Fig. 5-277 Image Display window; Select - Zoom

Zoom-Auto The image is fitted automatically to size of the **Image Display** window.

Zoom-Resize Restores the image to its initial size.

Zoom-+ Enlarges the image by factor 2.

Zoom-– Reduces the image by factor 2.

Zoom 1:1 Restores an image zoomed in any way to its original size.

Zoom-Mouse Allows you to enlarge / reduce the zoom factor of an image using the left / right mouse button, provided that the cursor is inside the image.

☞ **Zoom-+, Zoom-–, Zoom 1:1** and **Zoom-Mouse** can only be defined when the **Zoom-Auto** function is deactivated.

Slider with display box The zoom factor can be set by moving the slider. The display box below displays the current zoom factor. Factor 1 corresponds to the original size.

5.15.4 Select - Slice

This function allows to

– select and view individual slices from a Z Stack or a time series, when images where acquired in frame mode.

The button is grayed, when these conditions are not true.

Click on **Slice** will display the **Slice** toolbar. Any changes done with this toolbar are effective immediately.

- Click on the **Slice** button in the **Select** toolbar.

– The **Slice** toolbar is displayed on the right-hand side of the **Image Display** window.

- If you click on the **Slice** button again, the **Slice** toolbar is removed.

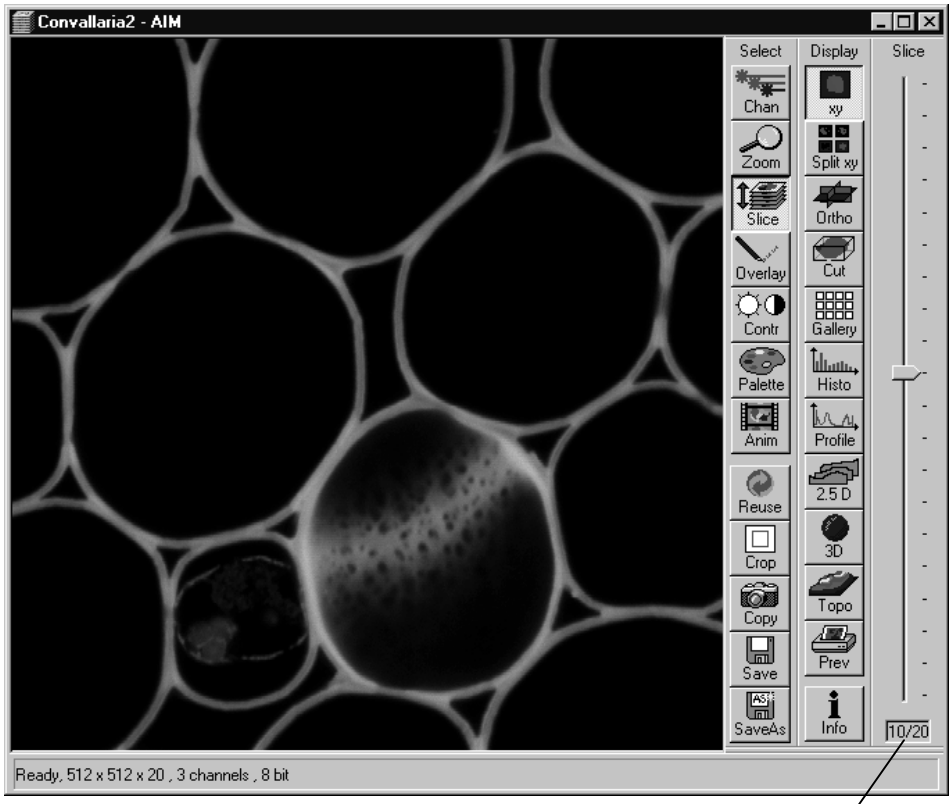


Fig. 5-278 Image Display window; Select - Slice

Example:
Slice No. 10 from a Z Stack or
time series of 20 slices

- Select the slices using the slider on the right.

5.15.5 Select - Overlay

This function allows to

- select from a set of drawing functions such as rectangles and arrows
- add a scale bar to the image
- use a set of interactive measurement functions for length, angle and size

The overlay function uses a plane separate from the image plane (the graphics plane) and does therefore not change the content of the image(s).

The button is only available if the XY or Split XY **Display** functions are selected. Otherwise it is grayed. Some of the **Display** functions such as **Ortho** or **Cut** turn the overlay graphics off temporarily.

Any changes done with this function are effective immediately.

The overlay graphics can be stored together with images and can be retrieved from the LSM 5 image database.

- Click on the **Overlay** button in the **Select** toolbar.
  - The **Overlay** toolbar will be displayed on the right-hand side of the **Image Display** window.
- If you click on the **Overlay** button again, the **Overlay** toolbar will be removed.

Provided that the display of the overlay elements has not been deactivated by clicking on the **Off** button, the created elements will still be displayed in the **Image Display** window even after closing of the **Overlay** toolbar.

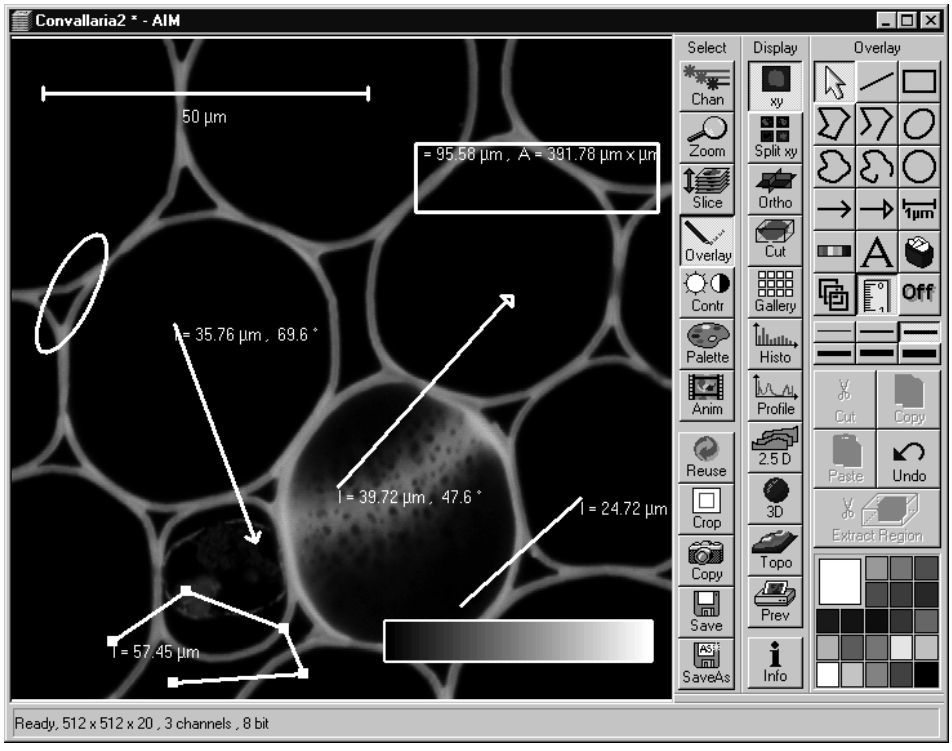


Fig. 5-279 Image Display window; Select - Overlay

The following functions can be used on activation of the buttons in the **Overlay** toolbar:

Arrow (selection) button: Activation of the mouse button for selection, resizing or movement of an overlay element in the **Image Display** window.
Resizing: Click on the handle and hold down the mouse button, drag the handle, release the mouse button.
Movement: Click on the line and hold down the mouse button, move the entire element, release the mouse button.

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

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

Closed polyline button: Creation of a closed polyline figure in the **Image Display** window. The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

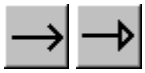
Open polyline button: Creation of an open polyline figure in the **Image Display** window. The first click sets the starting point, each additional click adds a further line, a click with the right mouse button ends the procedure.

Ellipse button: Creation of an ellipse in the **Image Display** window. The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and rotation direction can then be determined, the third click sets the second dimension and direction and ends the procedure.

Closed free-shape curve button: Creation of a closed Bezier figure in the **Image Display** window. The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Open free-shape curve button: Creation of an open Bezier figure in the **Image Display** window. The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Circle button: Creation of a circle in the **Image Display** window. Clicking and holding down the mouse button sets the center point, drag the diameter and release the mouse button to end the procedure.



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

Scale button: Creation of a horizontal or vertical scale with default increments in the **Image Display** window. Click and hold the mouse button for the starting point, drag horizontal or vertical scale, release the mouse button to end the procedure.

Gray tones / color shades button: Generates a rectangle with a display of gray tones or color shades in the image. Color shades are displayed if a palette has been loaded, with different colors being assigned to the gray tones.

A (Text) button: Creation of a text box in the **Image Display** window. After clicking on **A**, the **Text** window will be displayed, and text can be entered via the keyboard. The **Font ...** button enables you to select the font style and size in the **Font** window. The entered text will be displayed in the left upper corner of the **Image Display** window after clicking on **OK** and can be moved to the required position using the mouse. The **Text** window can also be activated with a double-click on a created text box, and the entered text can be edited subsequently.

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

Multiple button: On activation of this button, the overlay function subsequently selected is performed several times in succession, without the need to activate the function button again. This function remains selected until the **Multiple** button is deactivated again.

Measure button: Measurement of the overlay element in the **Image Display** window. On activation of the **Measure** button, the selected overlay element and all the elements created afterwards are measured and assigned with a measuring value. The measuring value can be shifted without regard to the overlay element. If of importance, the length and perimeter of a line figure, the area of a closed figure and the inclination angle of a single line will be displayed. On deactivation of the **Measure** button, the measuring value of the selected element is no longer displayed, and all the elements created afterwards will not be assigned with a measuring value.

Off button: Deactivation of the display of overlay elements in the **Image Display** window (hide overlay). Deactivation of the overlay functions.

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

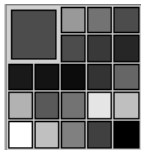
Cut button: The image contents of an overlay element are cut out, and the area will then appear in black.

Copy button: The image contents of a closed overlay element are copied to the clipboard.

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

Undo button: The last **Cut** or **Paste** action can be undone by clicking on the **Undo** button.

Extract Region button: The region of a Z Stack or 4D-image surrounded by an **Overlay** element is extracted and can be displayed and stored separately in a new **Image Display** window. This function is only active if an **Overlay** element is used, that generates a closed contour.



Color selection box: The colors displayed in the **Color** selection box can be assigned to the overlay elements with a click of the mouse. The currently selected color is displayed in the larger rectangle (left top) of the selection box. A selected color is automatically assigned to the currently selected overlay element and then to all the elements created afterwards.

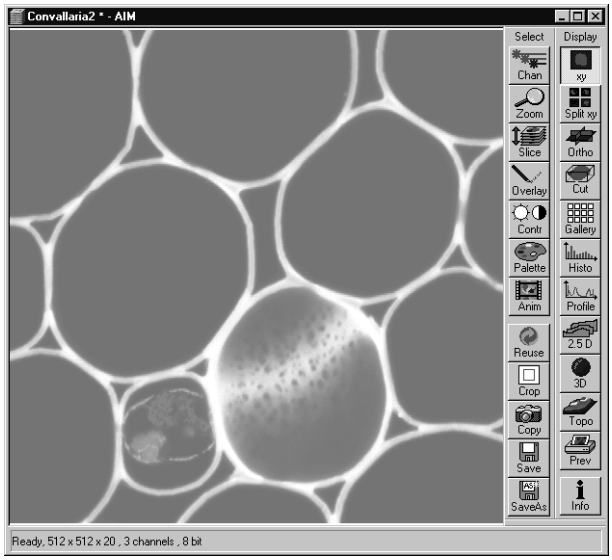


Fig. 5-280 Image Display window; Select - Contr

5.15.6 Select - Contr

This function allows to

- change the contrast and brightness of an image
- change the contrast and brightness of a channel of an image
- define interactively a new relationship between the intensities of pixels in the image memory and the displayed values of this pixel intensities on the computer screen

Click on **Contr** will display the **Contrast** toolbar. Any changes done with this toolbar are effective immediately.

Modification done by this function are for display purposes only. To permanently change the contrast and brightness of an image use the function **Contrast** in the **Process** menu.

- Click on the **Contr** button in the **Select** toolbar.
  - The **Brightness and Contrast** window will be displayed.
- Change brightness and contrast via the sliders in the **Brightness and Contrast** window. You can adjust each channel individually by activating the channel button (e.g.: **Ch1**), or influence all channels simultaneously by clicking on **All**.

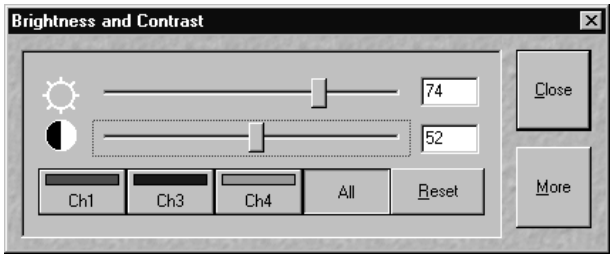


Fig. 5-281 Brightness and Contrast window

- Clicking on the **Reset** button will reset the original setting of brightness and contrast.
- Clicking on the **Close** button will close the **Brightness and Contrast** window.

Further contrast and brightness parameters can be activated or deactivated alternately using the **More** and **Less** buttons.

- Click on the **More** button to display the additional functions.
  - The **Brightness and Contrast** window will be enlarged, the labeling of the button changes from **More** to **Less**. If you click on **Less**, the additional functions are no longer displayed.

Simultaneously with the setting of brightness and contrast, the intensity values of the image can be set directly in the **Intensity Screen** via the **Ramp**, **PolyLine**, **Spline** and **Gamma** functions.

The intensity values can also be set either for all channels together or individually.

If the image has already been changed using the **Contrast** and **Brightness** sliders, this setting difference is displayed in the **Intensity Screen** by means of the **Shape** and **Result** lines.

(1) Ramp

The intensity is set via two knots in the **Intensity Screen**, which allows an intensity line to be created in the form of a ramp.

The original line form is reset via **Reset**.

The line form will be retained even when the additional functions are no longer displayed, and on closing the **Brightness and Control** window.

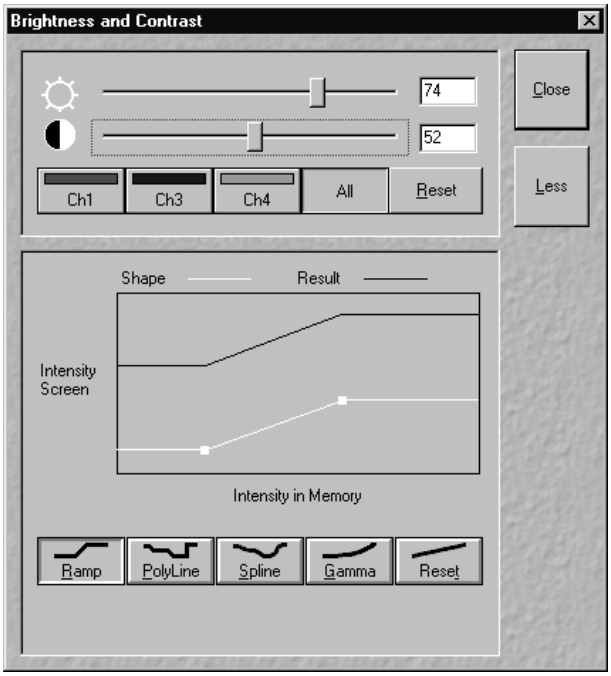


Fig. 5-282 Brightness and Contrast window with activated Ramp function

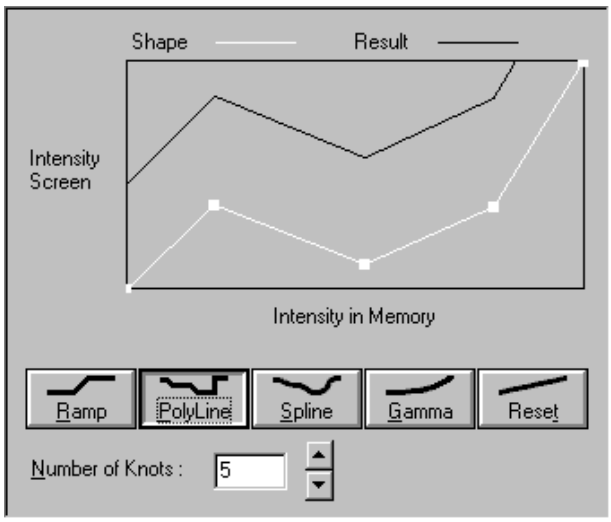


Fig. 5-283 Brightness and Contrast window with activated PolyLine function

(2) PolyLine

The intensity is set in the **Intensity Screen** via a freely selectable number of knots, which permits the creation of an intensity line in the form of a polyline. The number of knots can be selected from the **Number of Knots** selection box.

The original line form is reset via **Reset**.

The line form will be retained even when the additional functions are no longer displayed or when the **Brightness and Control** window is closed.

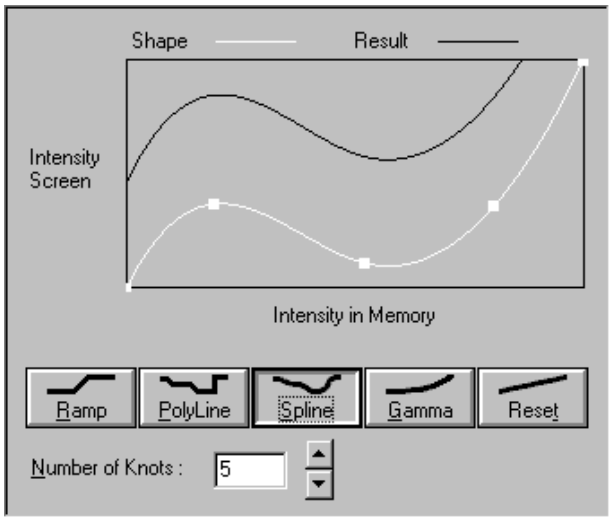


Fig. 5-284 Brightness and Contrast window with activated Spline function

(3) Spline

The intensity is set in the **Intensity Screen** via a freely selectable number of knots, which permits the creation of an intensity line in the form of a spline. The number of knots can be selected from the **Number of Knots** selection box.

The original line form is reset via **Reset**.

The line form will be retained even when the additional functions are no longer displayed or when the **Brightness and Control** window is closed.

(4) Gamma

The intensity is set in the **Intensity Screen** by varying the gamma curve (clicking and dragging with the mouse) or by moving the **Gamma** slider. It is possible to set gamma values between 0.1 and 2.0.

The original line form is reset via **Reset**.

The line form will be retained even when the additional functions are no longer displayed or when the **Brightness and Control** window is closed.

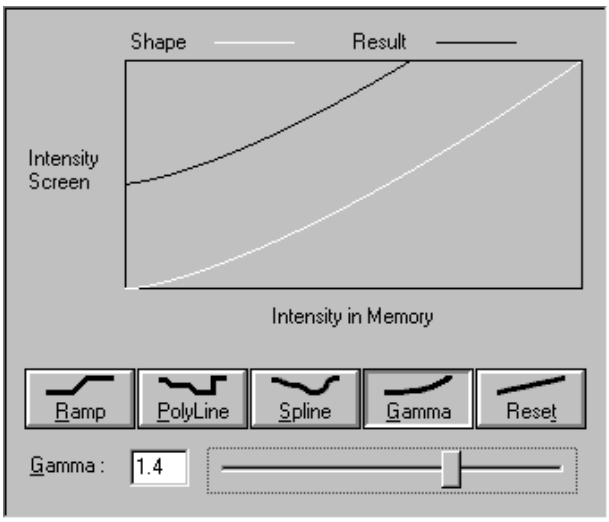


Fig. 5-285 Brightness and Contrast window with activated Gamma function

5.15.7 Select - Palette

This function allows to

- change the palette used for displaying the image(s)
- define and save new palettes
- delete palettes by removing them

Click on **Palette** will display the **Palette** toolbar. Any changes done with this toolbar are effective immediately.

The standard palettes **No palette**, **Range indicator, Glow Scale** and Rainbow are system palettes and can not be deleted.

The **Range indicator** palette is useful to optimize the gain and offset setting of images in the **Scan control** window before scanning.

Palettes are stored and retrieved together with the images when archived in the **Image Database**.

- Click on the **Palette** button in the **Select** toolbar.
  - The **Color Palette** window will be displayed.
- Select the required palette from the **Color Palette List** panel by clicking on the relevant name.
- If you want to deactivate a palette selected before, click on **No Palette** in the **Color Palette List** panel.
- Click on the **Close** button to close the **Color Palette** window.
- A changed image can be stored via the **Save As** function.

In the **Options** menu in the function **Settings** it is possible to switch to **Mono** automatically when a palette is activated and to **Colour** on deactivation of a palette.

In addition it is possible to activate / deactivate **Mono** in the **Channel** toolbar.

Some of the handling functions of the **Image Display** window toolbars can be activated at the opening of a new **Image Display** window.

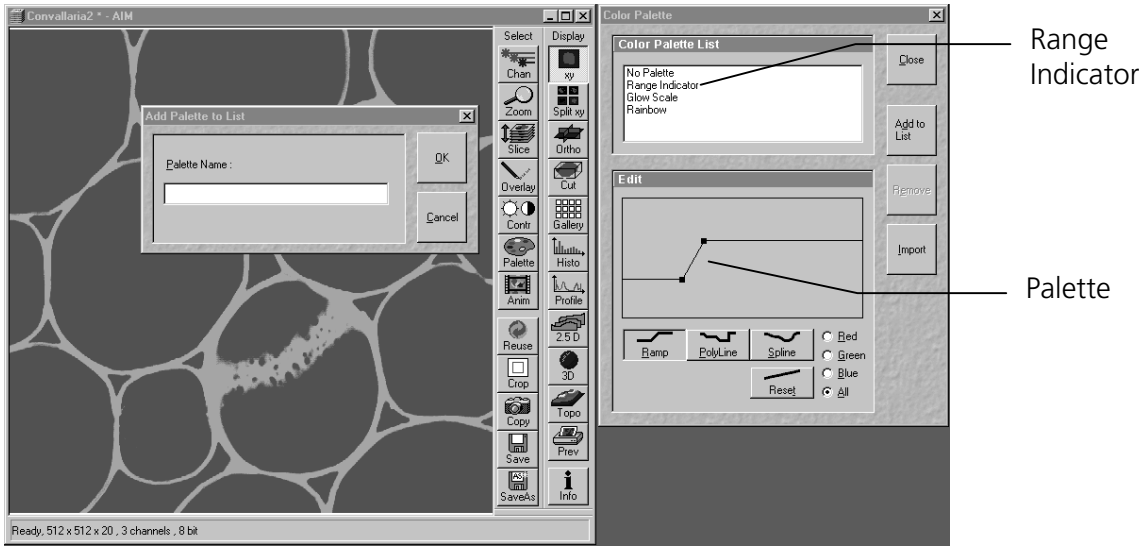


Fig. 5-286 Image Display window, Select - Palette; Color Palette window and Add Palette to List window

(1) Editing and storing a palette

A palette is edited by moving the knots in the **Ramp**, **Polyline** and **Spline** functions (identical to the setting in the **Contrast and Brightness** window, see page 5-288f).

The palette can be set for all colors together or separately for each color.

- Activate the relevant button: **Red**, **Green**, **Blue** or **All**.

Proceed as follows to store an edited palette under a new name:

- Click on the **Add To List** button: the **Add Palette To List** window will be displayed.
- Enter a name for the palette and click on **Ok**.
  - The palette will be stored and the name included in the **Color Palette List** panel.

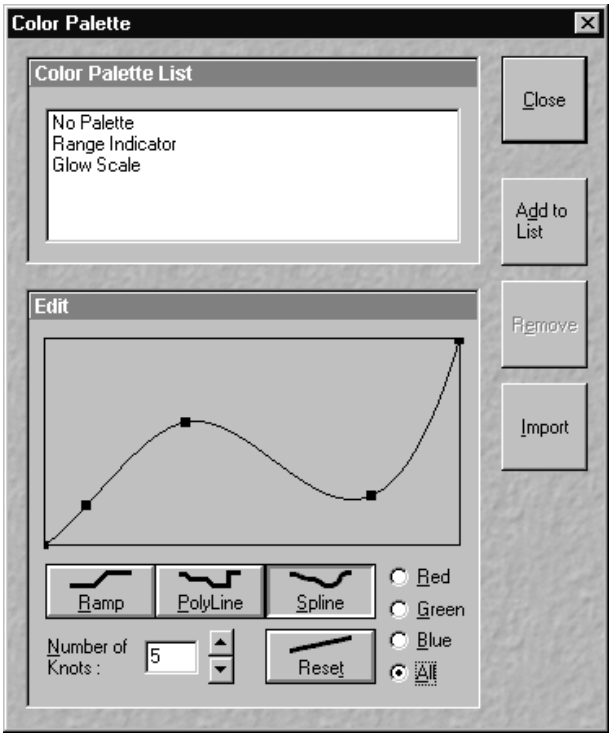


Fig. 5-287 Color Palette window

(2) Delete a palette

Proceed as follows to delete a palette:

- Click on the name of the palette to be deleted in the **Color Palette List** panel and then on the **Remove** button.
  - The palette will be removed from the list.

☞ The standard settings (**No Palette**, **Range Indicator**, **Glow Scale** and **Rainbow**) cannot be deleted.

(3) Import a palette

Proceed as follows to import a palette:

- Click on the **Import** button. The **Import Palette** window will be opened.
- Select the required palette (file extension: ***.lut**) from the relevant directory and click on **Open**.
  - The palette will be imported and displayed in the **Color Palette List** panel.

File with the extension ***.lut** are LSM 310 / 410 palette files.

5.15.8 Select - Anim

This function allows to

- animate frames of a Z Stack or a time series
- specify animation parameters such as range and animation speed

Click on **Anim** will display the **Animate** toolbar. Any changes done with this toolbar are effective immediately.

When the image(s) displayed in the **Image Display** window is neither a Z Stack nor a time series this button is grayed and not accessible.

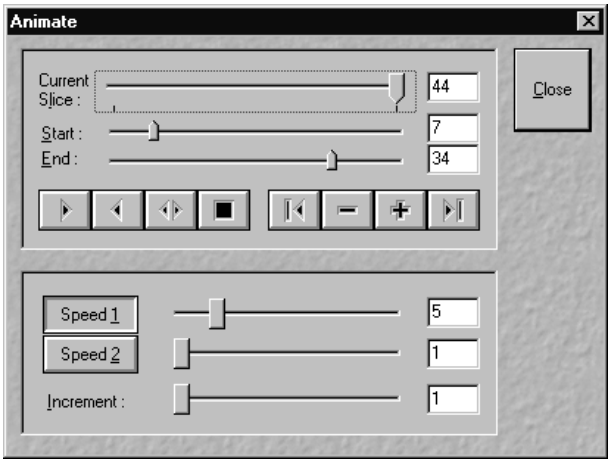


Fig. 5-288 Animate window

- Click on the **Anim** button in the **Select** toolbar of the **Image Display** window of a stack.
  - The **Animate** window will be displayed and the animation started immediately.
- Click on the **Close** button to close the **Animate** window and to stop the animation.

The animation is controlled via the following function elements:

Current Slice : **Current Slice** slider: Manual movement through the individual slices of a stack by moving the slider, or by entering the slice number in the input box. Slider can be accessed only, when the automatic animation is off.

Start : **Start** slider: The setting of the **Start** sliders limits the number of slices to be used for the animation. Previous slices are not taken into consideration for the animation. Can be changed during automatic animation.

End : **End** slider: The setting of the **End** slider limits the number of slices to be used for the animation. Subsequent slices are not taken into consideration for the animation. Can be changed during automatic animation.

Starts the forward motion of the automatic animation. After the last slice has been passed, restart is made at the first slice.

Starts backward motion of the automatic animation. After the first slice has been passed, restart is made at the last slice.

Starts the combined forward / backward motion of the automatic animation, i.e. when the last slice has been reached, the backward motion is activated, and the forward motion is activated again on reaching the first slice.

Stops the automatic animation.

Move to the first slice.

After each click on this button, backward motion is made by the number of slices set under **Increment**.

After each click on this button, forward motion is made by the number of slices set under **Increment**.

Move to the last slice.

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

Increment slider: Reduction of the slices to be displayed by selecting an increment n (step width) of slices to be taken into consideration for the animation. If n = 3, for example, only every third slice of the stack will be displayed during the animation.

5.15.9 Select - Reuse

This function allows to

- transfer acquisition parameters of an image from the image data base to the **Microscope control**, **Configuration Control**, **Scan Control**, **Time Series Control** and **Bleach Control** windows and applies those parameters directly on the system.

The acquisition parameters of an image are displayed in the **Image Display** window and can be viewed by using the **Info** function. In the **Image Status Display** tab in the **Settings** function of the **Options** subordinate toolbar it can be determined what parameters to view with the **Info** function.

The parameters include the following:

Frame Size, Speed, Data Depth, Scan Direction, Average, Zoom Rotation, Offset, Pinhole diameter, Detector Gain, Amplifier Offset, Amplifier Gain, Excitation, Beam Path and Scan Mode (Line, Frame, Stack, Time Series). However, the required objective must be selected by the user.

- Click on the **Reuse** button. The acquisition parameters of the active image (stack) are applied immediately to the system.

In the **Options** menu in the function **Settings** with the **Recording/Reuse** tab, it can be determined whether the objective should also be transferred and set. Setting the microscope objective only works in microscopes with motorized objective revolvers.

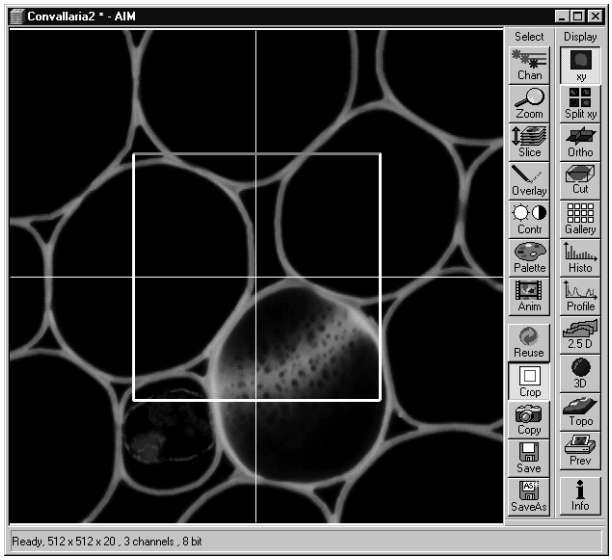


Fig. 5-289 Image Display window; Select - Crop

5.15.10 Select - Crop

This function allows to

- interactively define the size and orientation of a rectangular scan area on the image displayed in the **Image Display** window.
- The defined area is displayed together with the **Zoom, Offset and Rotation** parameters in the **Scan Control** window in the **Mode** submenu.

Click on **Crop** will display the **Crop Rectangle** in the **Image Display** window. Any changes done with the **Crop Rectangle** are setting the parameters immediately. On the next execution of a scan (**Find**, **Fast xy**, **Single**, **Contineous** in **Scan Control** or **Start T** or **Start B** in **Time Series Control**) these new scan parameters will be used.

To reset the crop function and use default values set **Zoom=1, Offset=0** and **Rotation=0** in the **Scan Control** window in the **Mode** submenu.

- Click on the **Crop** button.
  - The **Crop Rectangle** will appear on the **Image Display** window.

The **Crop Rectangle** is controlled via the following functional elements:

Offset

- Click into the crop rectangle, keep the left mouse button pressed and drag the crop rectangle to the required position. Release the mouse button.

Zoom

- Click on a corner of the crop rectangle, keep the left mouse button pressed and set the required size. Release the mouse button.

Rotation

- Click on one end of the crosslines, keep the left mouse button pressed and set the required rotation angle. Release the mouse button. The first line scanned is highlighted in blue.

Side ratio

- Click on any of the intersection points between crossline and crop rectangle, keep the left mouse button pressed and change the side ratio as required. Release the mouse button.

5.15.11 Select - Copy

This function allows to

– copy the current displayed image into the clipboard.

Click on **Copy** will be immediately effective.

From the clipboard images can be incorporated into other programs such as MS Excel, MS Powerpoint or MS Word.

To export image series, use the **Export** function in the **File** menu.

- Click on the **Copy** button.
  - The content of the **Image Display** window is copied to the clipboard.
- Start the **clipboard** application of WINDOWS.
- Select **Paste** in the **Edit** menu of the **Clipboard** application.

5.15.12 Select - Save

This function allows to

- save the image(s) of the **Image Display** window into an **Image Database**
- by not showing a dialogue and using the automatic assigned and incremented image name and a predefined existing **Image Database**
- Prerequisite: **Autosave** is checked in the **Settings** function with the **Autosave** tab

Click on **Save** will be immediately effective.

When the prerequisite is not met, the **Save As** dialogue is displayed.

In the **Options** menu in the function **Settings** with the **Autosave** tab parameters such as an automatically incremented filename can be determined and the **Autosave** activated/deactivated.

5.15.13 Select - Save As

This function allows to

- save the image(s) of the **Image Display** window into an **Image Database**
- by showing a dialogue
- use the defaults as defined in the **Settings** function with the **Save** tab

Click on the **Save As** button displays the **Save Image and Parameter As** window. Changes will be effective on closing this dialogue.

In the **Options** menu in the function **Settings** with the **Save** tab default parameters such as **Name, Description** and **Notes** can be set.

- Click on the **Save** button.
  - The **Save Image and Parameter As** window appears
- Enter text for the image name, description, notes or change the user name.
- Select the **Image Database** from the list of databases (MDB) or
- Open other **Image Databases** by selecting **Open MDB** or
- Create new **Image Databases** by selecting **New MDB.**

5.15.14 Display - xy

This function allows to

- display a single image in frame mode
- display multi channel images in superimposed mode

The settings of **Chan, Zoom, Slice, Overlay, Contr** and **Palette** are applied.

Click on **xy** will be immediately effective.

5.15.15 Display - Split xy

This function allows to

– display the individual channels of a multi channel image as well as the superimposed image

The settings of **Chan, Zoom, Slice, Overlay, Contr** and **Palette** apply.

Click on **Split xy** will be immediately effective.

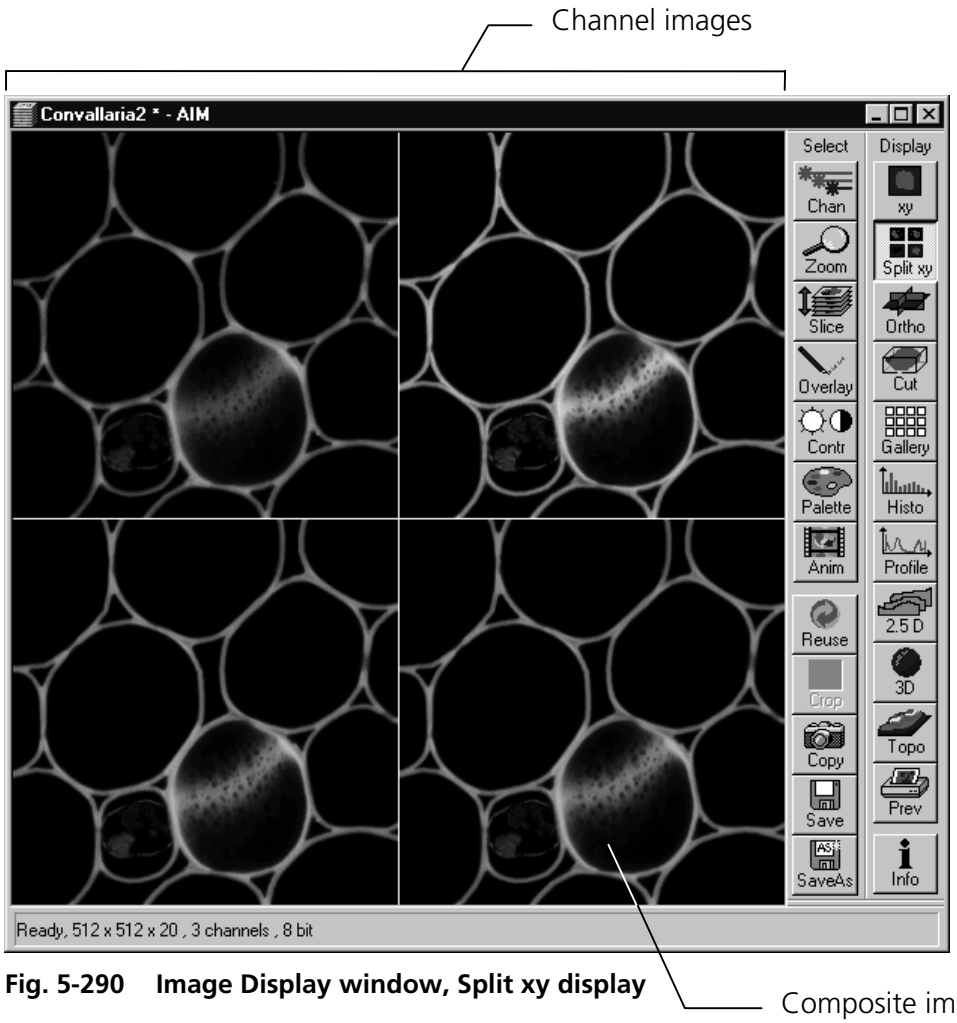


Fig. 5-290 Image Display window, Split xy display

☞ This function is useful to optimize the individual channels in a multi channel image acquisition together with the **Range Indicator** palette .

5.15.16 Display - Ortho

This function allows to

- display a Z Stack of images in an orthogonal view
- measure distances in three dimensions
- generate 2D deconvolution views of the yz and xz plane

The settings of **Chan, Zoom, Slice, Overlay, Contr** and **Palette** apply.

Click on **Ortho** will be immediately effective.

- By clicking on the **Ortho** button section lines appear in the **Display** toolbar together with orthogonal projections in the image. On the right-hand side, the **Orthogonal Sections** toolbar is shown.

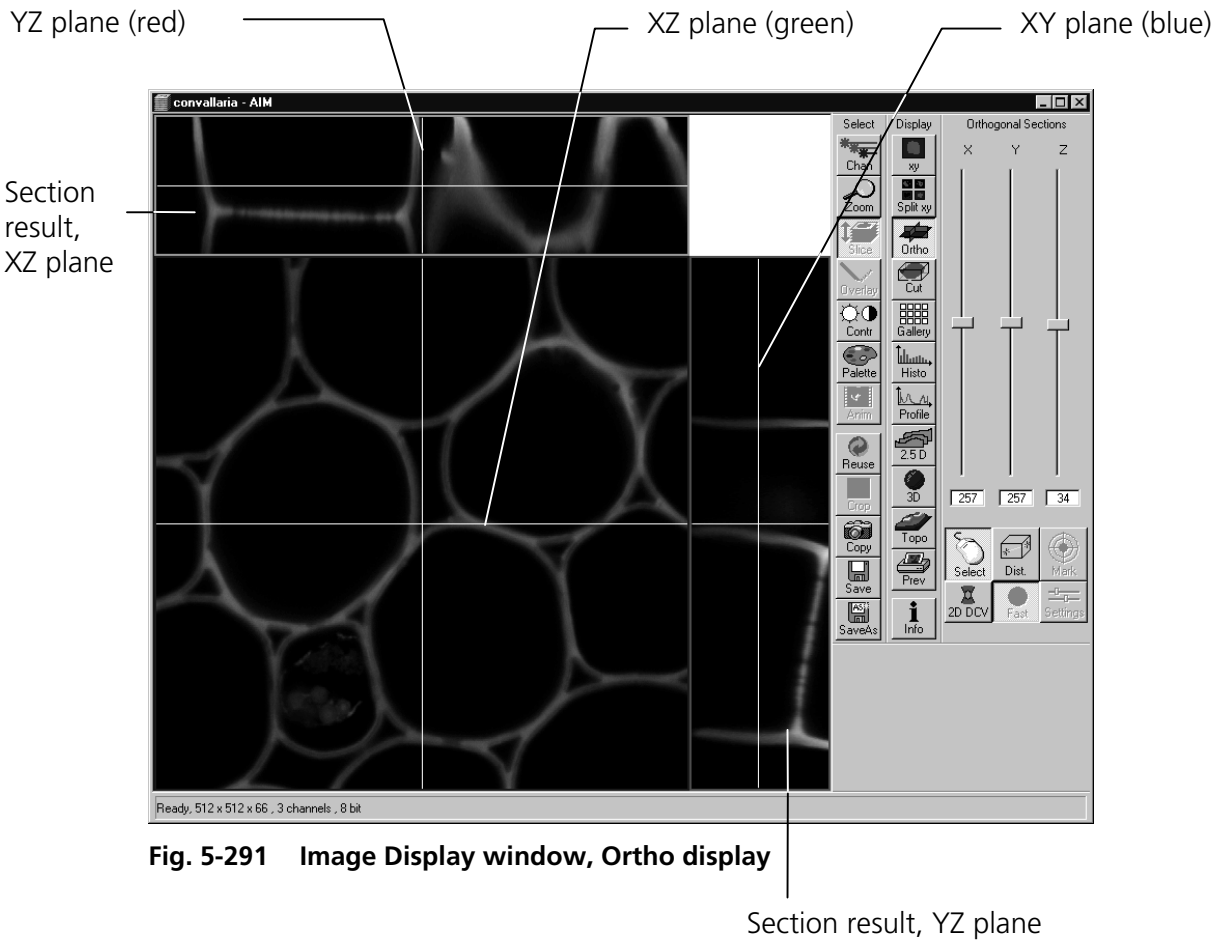


Fig. 5-291 Image Display window, Ortho display

5.15.16.1 Ortho - Select function

- By changing the parameters X, Y and Z in the **Orthogonal Sections** toolbar, the section plane can be positioned at any XYZ coordinate of the Z Stack.

The position of section planes can be changed in various ways:

- By moving the sliders on the **Orthogonal Sections** toolbar.
  - X and Y settings may range from 1 up to the maximum number of pixels scanned (in the example shown: 512).
  - Z settings may range from 1 to a maximum of n, with n standing for the number of slices produced in the stack.
- By directly entering the relevant number value in the X-, Y- or Z-input box and pressing the **Enter** key.
- If you move the cursor into the **Image Display** window, it changes into a crosslines symbol  . By dragging this symbol with the mouse you can position the XZ and YZ section planes to any point of intersection with the XY plane. A click with the left mouse button places the intersection to the desired position.
- If you move the crosslines symbol  onto the intersection of the red and green section planes, it changes into the:  symbol. If you now press the left mouse button and keep it pressed you can reposition both section planes **simultaneously**.
- If you move the crosslines symbol  onto the green section plane, it changes into the  symbol. If you now press the left mouse button and keep it pressed, you can reposition the (green) XZ section plane.
- You can reposition the (red) YZ plane in the same way using the  symbol.

The result of an orthogonal section is visible at the image margin.

- Section of the XZ plane (green line) through the stack: above the XY image.
- Section of the YZ plane (red line) through the stack: right of the XY image.
- Section of the XY plane (blue, slice plane of the stack): center image.

5.15.16.2 Ortho - Distance function

- Activating the **Dist.** button permits length measurements in 3D space.

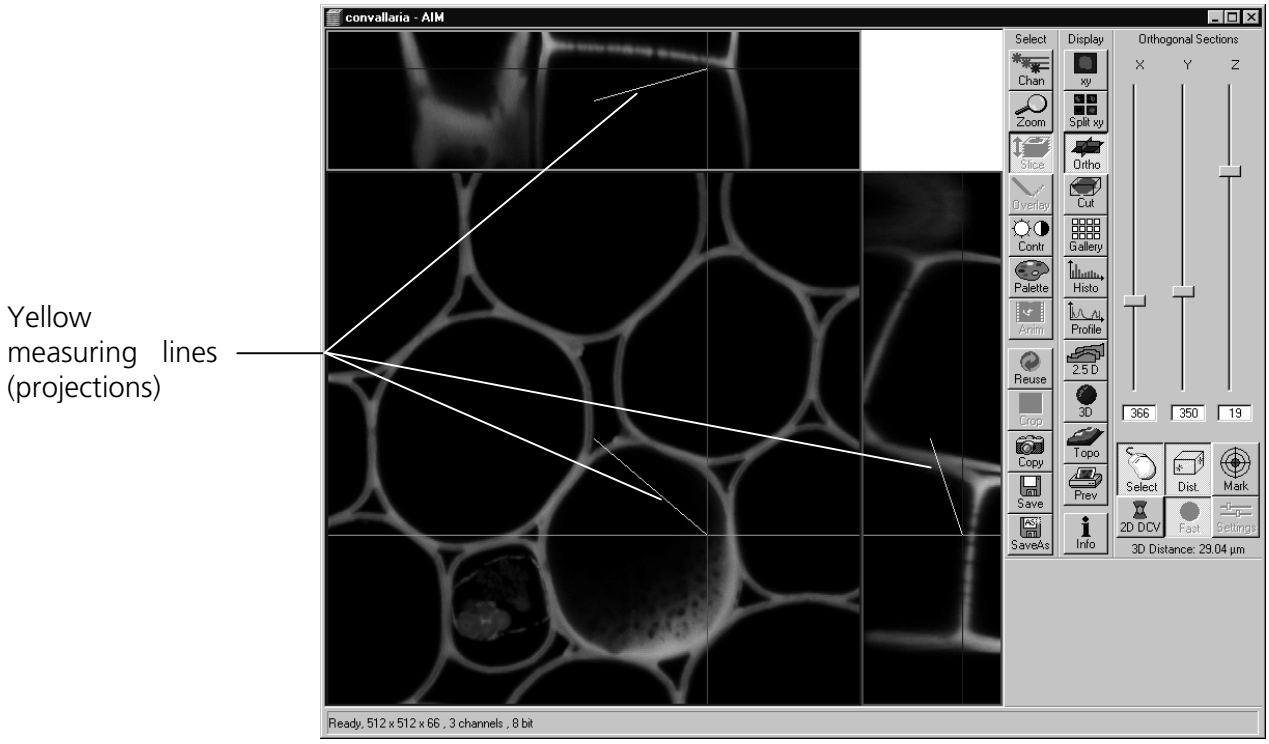


Fig. 5-292 Image Display window, Ortho display

- Click on the **Mark** button to set the first XYZ-point for the measurement of the spatial distance.
- Set the second XYZ-point for measurement by moving the X-, Y-, Z-sliders or by moving the green, red and blue lines in the image.
  - The projections of the spatial distance are shown in the image planes by yellow lines. The actual spatial distance is calculated and shown in µm below the **Select**, **Dist.** and **Mark** buttons, e.g. 3D Distance: 55.60 µm.

5.15.16.3 Ortho - 2D DeConVolution function

The 2D deconvolution function causes orthogonal projection enhancement through the computed correction of the resolution in the Z-coordinate.

Image enhancement is only effective for the two projections of a fluorescence stack in the Ortho display or for an XZ-scan in fluorescence, and is also only computed for this.

- Activate the **2D DCV** button in the **Orthogonal Sections** toolbar.

If the **Fast** button is activated, calculation of the 2D-deconvolution (inverse DCV mode) is performed immediately.

The 2D DCV settings button can only be activated if a licence for the 3D DCV option has been purchased. Otherwise this button is grayed.

- Click on the **Settings** button. The **2D Deconvolution** window is opened.

The **2D Deconvolution** window contains the **Deconvolution** panel with the two tabs **Method** and **PSF**.

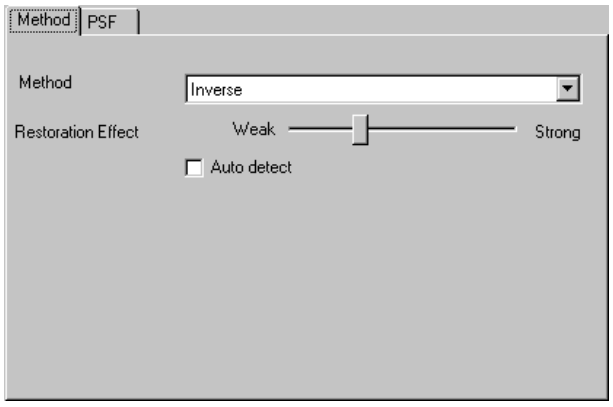


Fig. 5-293 Method tab

(1) Method tab

The **Method** tab enables you to choose between the calculation methods **Inverse** and **Iterative**.

For more details of explanation of deconvolution and the calculation methods see section **3D DeConVolution** (page 5-163).

In the **Inverse** method, the **Restoration Effect** slider can be used to set the signal-to-noise ratio between **Weak** (low noise) and **Strong** (pronounced noise).

Activation of the **Auto detect** check box starts a routine for the automatic determination of the noise level in the entire image part of the Z Stack. If **Auto detect** is enabled, the **Restoration Effect** slider is disabled.

The **Iterative** method permits (in addition to the parameters of the **Inverse** method) the maximum number of iterations to be entered between 1 and 200 under **Maximum Iterations** and the **Auto Stop** function to be activated / deactivated. The **Auto Stop** function interrupts the calculation depending on the set image improvement (delta between last but one and last cycle in %), no matter whether the value under **Maximum Iterations** has been achieved or not.

(2) PSF tab (optional with 3D DCV)

The objective data are displayed in the **Method** tab. In the case of wavelengths above 700 nm, the **NLO** button is automatically enabled.

The displayed values are always taken over by the system data, but can be edited subsequently for simulation purposes.

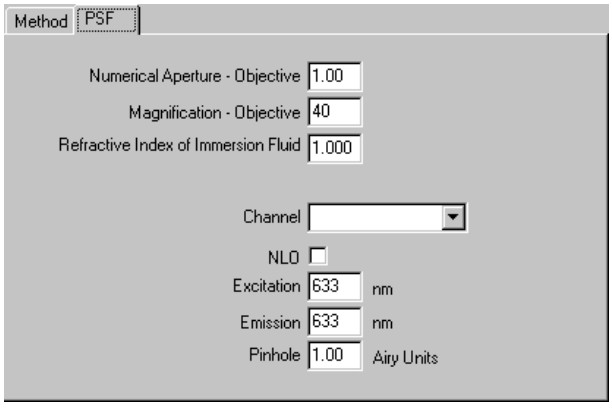


Fig. 5-294 PSF tab

- Select the required method and determine the relevant parameters.

The deconvolution calculation is performed immediately after the **2D Deconvolution** window has been closed, and the image display is updated (on-line).

5.15.17 Display - Cut

This function allows to

- display a Z Stack of images at a user defined section plane (= cut plane)
- improve the image of the section plane by trilinear interpolation

The settings of **Chan**, **Zoom**, **Slice**, **Contr** and **Palette** are applied.

Click on **Cut** will display the **Cut** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

- Clicking on the **Cut** button in the **Display** toolbar opens the **Cut** toolbar to the right of the **Image Display** window.

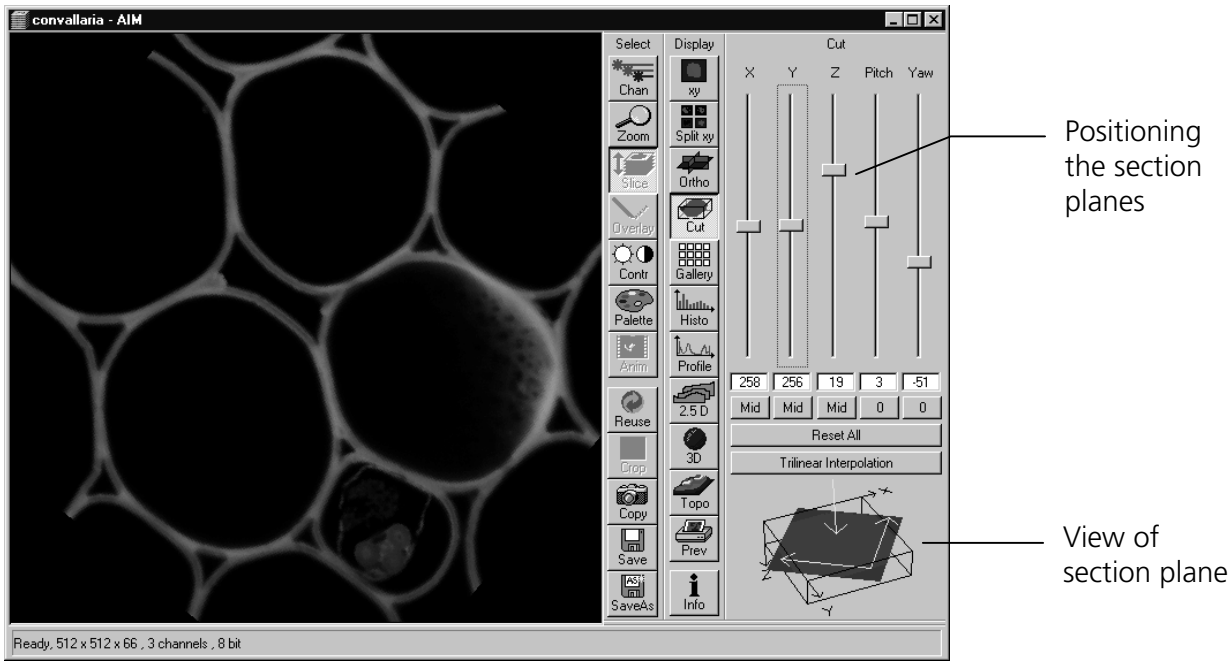


Fig. 5-295 Image Display window, Cut display

- By varying the parameters **X**, **Y**, **Z**, **Pitch** and **Yaw**, you can position a section plane of any orientation within the stack volume.
- The resulting position of the section plane is shown as a red area below the **Trilinear Interpolation** button. At the same time, the result is shown in the **Image Display** window.
- A click on the **Reset All** button restores the original position.
- A click on the **Trilinear Interpolation** button will improve the quality of the image by performing a 3D interpolation of the image.

5.15.18 Display - Gallery

This function allows to

- display images (Z Stack, time series, combination of both) side by side in tiled fashion
- add data relevant to the images displayed (Z Stack slice distance, time of acquisition or wavelength)
- extract a subset of images from the original stack and store the result as a new image

The settings of **Chan, Zoom, Slice, Contr** and **Palette** apply.

Click on **Gallery** will display the **Gallery** toolbar. Any changes done with this toolbar are effective immediately.

- A click on the **Gallery** button in the **Display** toolbar not only produces the gallery itself but also the **Gallery** toolbar with two buttons: **Data** button and **Subset** button.

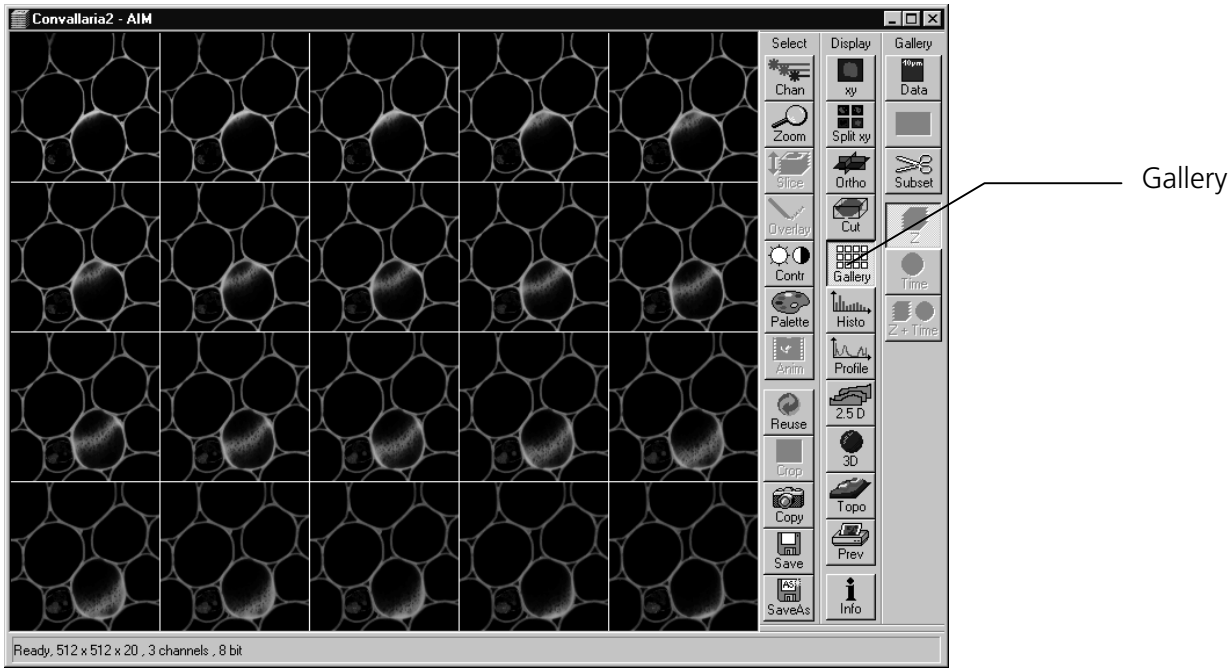


Fig. 5-296 Image Display window, Gallery display

- Clicking on the **Data** button shows the Z Slice distance, the acquisition time or the wavelength or combinations.
- Clicking on the color selection button (below the **Data** button) will open a color selection window allowing you to choose - at a click of the mouse - in which color the data will be shown in the gallery display.

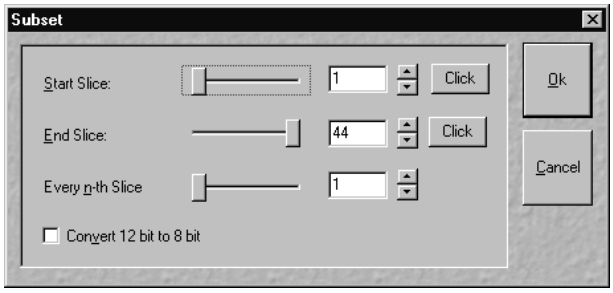


Fig. 5-297 Subset window

- Clicking on the **Subset** button opens another window entitled **Subset**, in which you can select images of the set of images displayed.
  - A stack consisting of the selected images only is generated and displayed.
- Select **Start Slice** and **End Slice** via the sliders, the input box or the **Click** (into window) button.
- Enter a value for **n** in the **Every n-th Slice** panel.
- If required, activate the **Convert 12 bit to 8 bit** check box ☑.
- Clicking on the **Ok** button will generate a new subset of images.
- **Cancel** will stop the procedure.

5.15.19 Display - Histo

(1) Display - Histo - Overview

This function allows to

- display a histogram (distribution of pixel intensities) of an image
- show the histogram values in table form
- copy table to clipboard or save as text file
- analyze the colocalization between two channels
- measure area and mean gray value and standard distribution in an area
- show separate histograms for each channel in a multi channel image

Colocalization is only available in case of a two or multi channel image.

The settings of **Chan, Zoom, Slice, Contr** and **Palette** are applied.

Click on **Histo** will display the **Histogram** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

- Click on the **Histo** button. The **Histogram** toolbar will be displayed on the right.

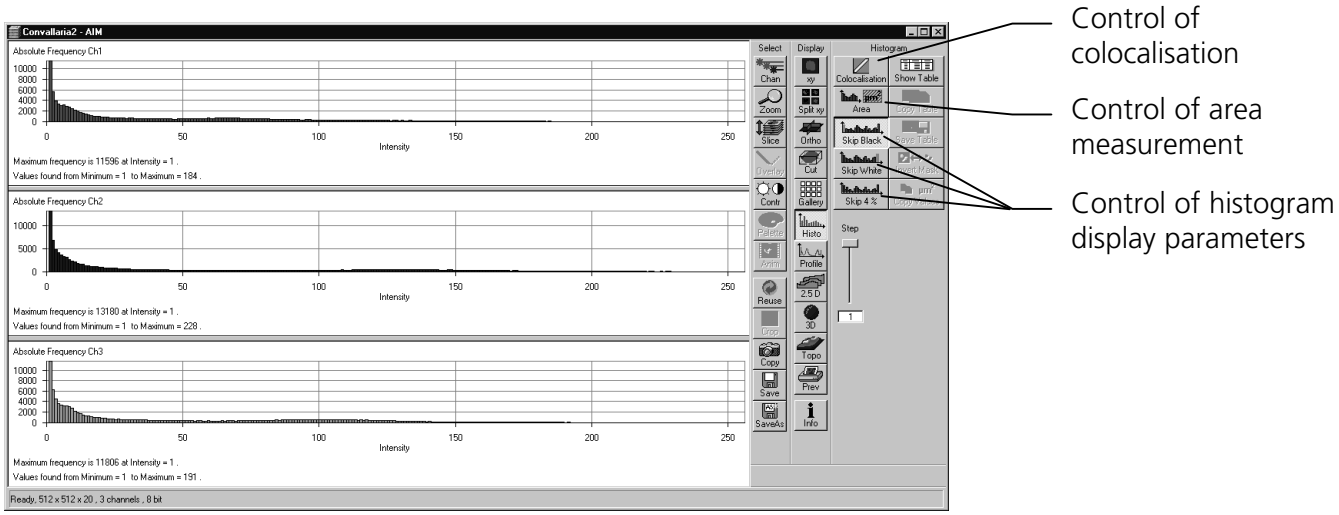


Fig. 5-298 Image Display window, Histogram display

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

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

Histogram functions

Skip Black button	Ignore black pixels (gray value 0) in the histogram.
Skip 4 % button	Ignore the lower 4% of the intensities in the histogram.
Skip White button	Ignore white pixels (gray value 255 or 4096) in the histogram.
Step input box	Set the number of intensity steps which shall be displayed in the diagram. Step 1 corresponds to 256 intensity steps, Step 64 to 4 intensity steps in case of 8 bit images. Reduction is made by averaging.
Show Image button	Shows the image in the **Image Display** window beside the histogram.
Show Table button	The histogram is shown as a table at the bottom left of the **Image Display** window.
Copy Table button	The histogram table is copied to the clipboard.
Save Table button	The histogram table can be stored as a text file (extension *.txt).

Area function

Area button	Interactive definition of area for size and intensity measurement.
Save Values button	Copies area values to the clipboard (only available if the **Area** button is activated).

(2) Area function

- Click on the **Area** button in the **Histogram** toolbar.
  - The function elements for **Area** measurement are displayed at the bottom right of the **Histogram** toolbar.

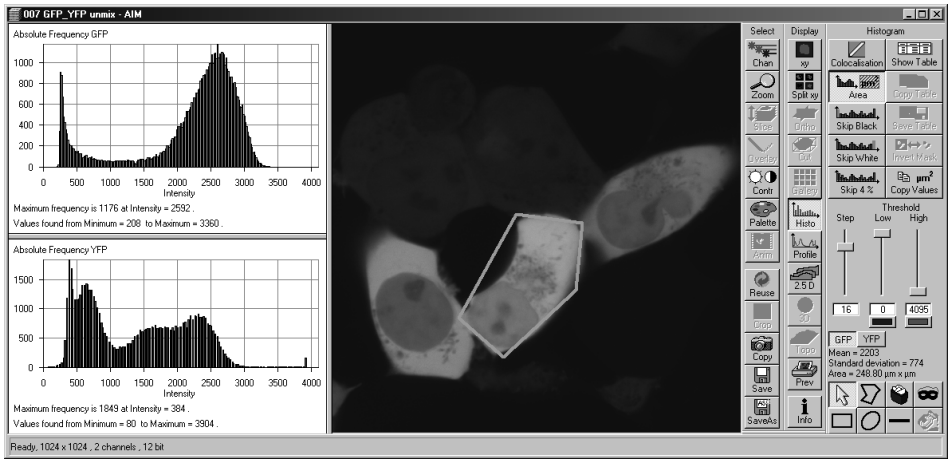


Fig. 5-299 Image Display window, Area Measure display

The following function elements are available:

Step

Set the number of intensity steps which shall be displayed in the diagram. Step 1 corresponds to 256 intensity steps, Step 64 to 4 intensity steps in case of 8 bit images. Reduction is made by averaging.

Low

Threshold low slider with **Color** selection button: The intensity values below threshold are not displayed. The removed areas are masked in the color selected in the **Color** selection button.

High

Threshold high slider with **Color** selection button: The intensity values above threshold are not displayed. The removed areas are masked in the color selected in the **Color** selection button.

Ch1-T1

Ch1, Ch3 ... buttons: Selection of the channel for which the area measurement is to be performed.

Mean = 29
Standard deviation = 57
Area = 0.0530 mm x mm

Display box: Display of the mean value and the standard deviation of the non-masked area. Area measurements of very small areas (< 10 pixels) give only approximate values.

Arrow (selection) button: Activation of the mouse button for selection, resizing, or movement of a mask element in the **Image Display** window.
Resize: Click on handle and hold down the mouse button, drag the handle, release mouse button.
Movement: Click on line and hold the mouse button, move the entire figure, release mouse button.

Closed polyline button: Creation of a polyline figure in the image. The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Recycle bin button: All the mask elements are deleted. If one element was marked before, this element is now deleted from the image.

Mask button: Enables the **Mask** Mode, where the region can be defined with ink.

Rectangle button: Creation of a rectangle in the image. Click and hold down the mouse button, drag a rectangle in any direction, release the mouse button to end the procedure.

Ellipse button: Creation of an ellipse in the image. The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and the rotation direction can then be determined; the third click sets the second dimension and the direction and ends the procedure.

Line button: Determines the line thickness of the area outline.

Flood fill button: Fills the overlay element or the scatter diagram with the color selected under **Mask**.

Closed free-shape curve button: Creation of a closed Bezier figure in the scatter diagram. The first click sets the starting point, each additional click adds a further line, a click with the right mouse button closes the figure and ends the procedure.

Circle button: Creation of a circle in the scatter diagram. Clicking and holding down the mouse button sets the center point; drag the diameter and release the mouse button to end the procedure.

Color selection button: The colors displayed in the selection box can be assigned to the mask elements with a click of the mouse. The currently selected color is always displayed in the larger rectangle (top left) of the selection box.

Clear Mask button: Removes the color filling from an overlay element or from the scatter diagram.

- The function can be activated by clicking on one of the geometry buttons, e.g.  (polyline).
- The figure of interest can be marked in the image by cursor control in conjunction with a mouse click.

- Clicking on the **Flood fill** button (paint jar) and moving the cursor to the area to be excluded causes the remaining area to be computed and the result indicated under **Area Measure**.

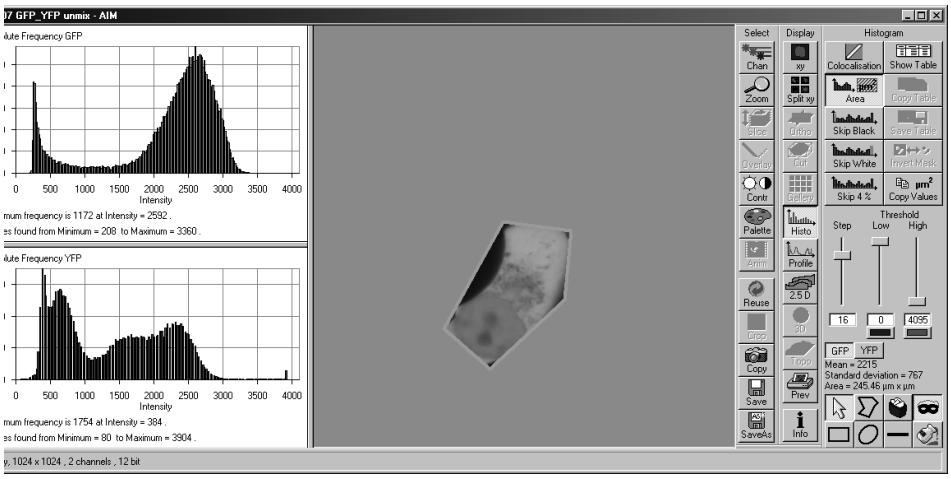


Fig. 5-300 Image Display window, Area Measure display

- If you specify a top and bottom intensity threshold, the area lying within this intensity interval can be computed.
- Specify the thresholds either with the **Threshold low** and **Threshold high** sliders, or with the ▲ and ▼ buttons.

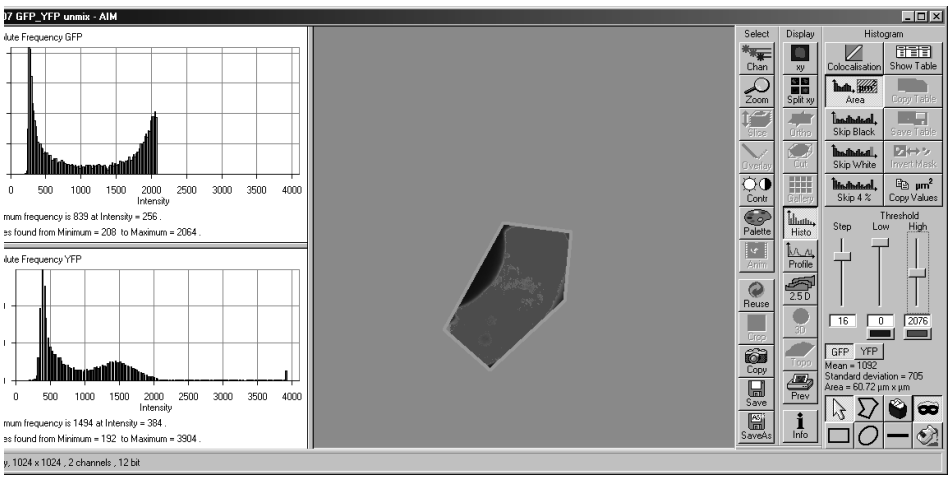


Fig. 5-301 Image Display window, Area Measure display

- Click on the **xy** button of the **Display** toolbar if you want to return to the original image.

(3) Colocalisation function

The **Colocalisation** function permits interactive analysis of two channels of an image by computing a scatter diagram (colocalisation).

- Click on the **Colocalisation** button. The scatter diagram is created and displayed beside the image.

How a scatter diagram is generated:

All pixels having the same positions in both images are considered a pair. Of every pair of pixels (P1, P2) from the two source images, the intensity level of pixel P1 is interpreted as X coordinate, and that of pixel P2 as Y coordinate of the scatter diagram. The value of the pixel thus addressed is increased by one every time, up to the maximum number of pixels used. This way, each pixel of the scatter diagram is a value that shows how often a particular pair of pixels has occurred.

Differences between the images cause irregular spots in the scatter diagram.

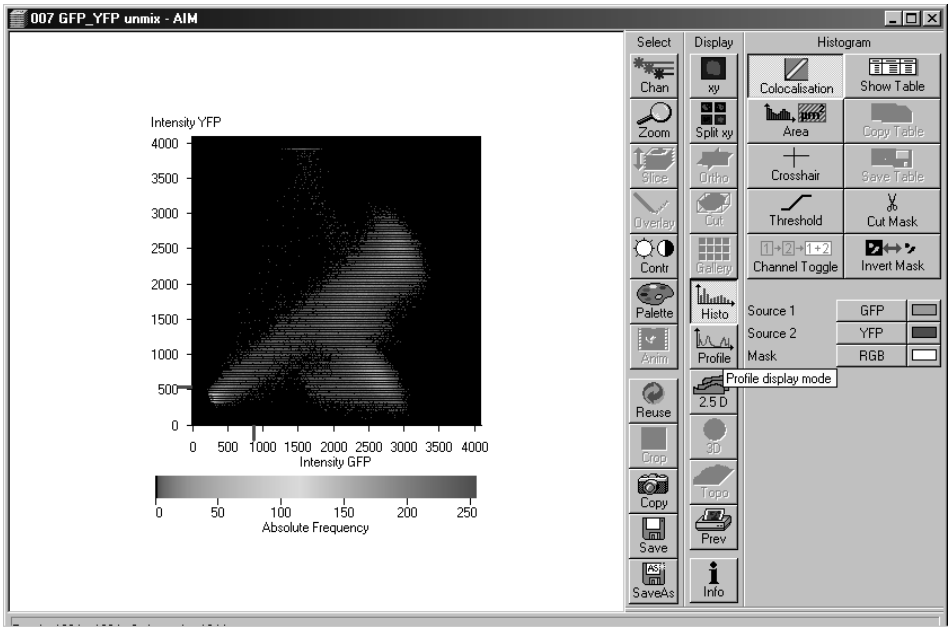


Fig. 5-302 Image Display window, Colocalization display

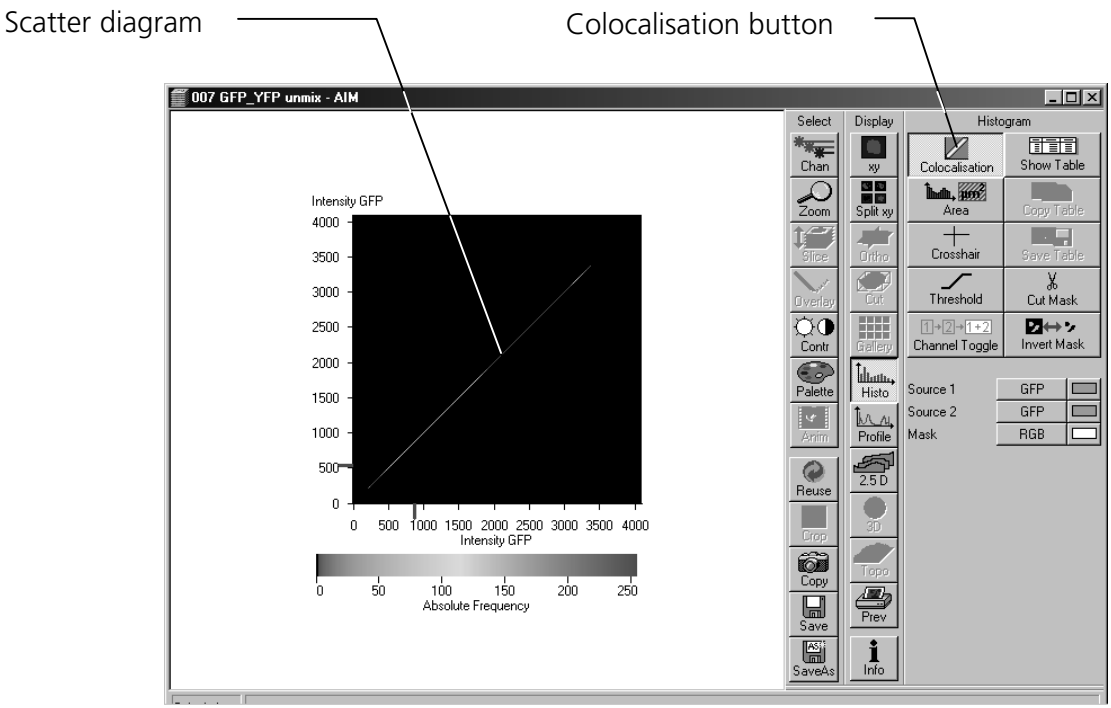


Fig. 5-303 Image Display window, Colocalization display

Identical images produce a clean diagonal line running from bottom left to top right, because only pixel pairs (0,0), (1,1), (2,2) with the same intensity can occur. Differences between the images cause an irregular distribution in the scatter diagram.

The following function elements are available:

Colocalisation button — Displays the scatter Histogram of the two image channels

Show table button — Adds display of the according data table

Area button — Adds display of the image

Crosshair button — Displays a movable crosshair for different areas in the scatter histogram

Threshold button — Opens the **Intensity Threshold** window to sets threshold for colocalisation in the scatter histogram.

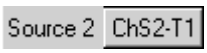
Set from Image ROIs button: Sets background threshold from ROI (**Threshold** button)

Cut Mask button:

Channel Toggle button:

Invert Mask button: Inverts the mask or the scatter diagram.

Source 1 selection box with **Color** selection box: Selection of the first channel to be selected via the selection box, assignment of a defined color via the **Color** selection box.



Source 2 selection box with **Color** selection box: Selection of the second channel to be selected via the selection box, assignment of a defined color via the **Color** selection box.

Mask selection box with **Color** selection box: Selection of **RGB** or **Overlay** display of the mask, assignment of a defined color via the **Color** selection box.

Drawing tools — Allows the selection of ROIs in the Histogram and the Image

Save at drawing tools — Stores ROIs and threshold settings

Enhanced colocalisation

Scattergram, image display and data table are interactively linked:

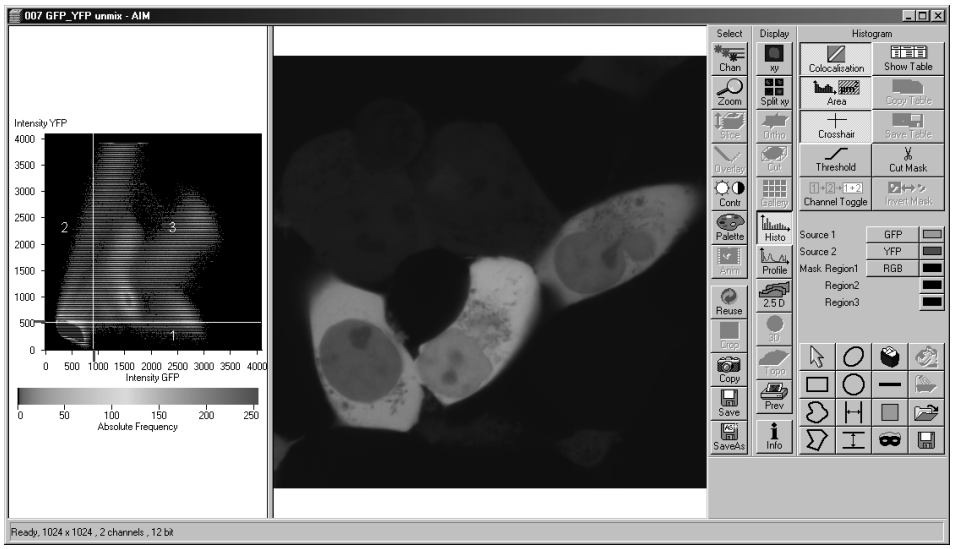


Fig. 5-304 Image Display window, Colocalization display

Scattergram and thresholding with crosshair:

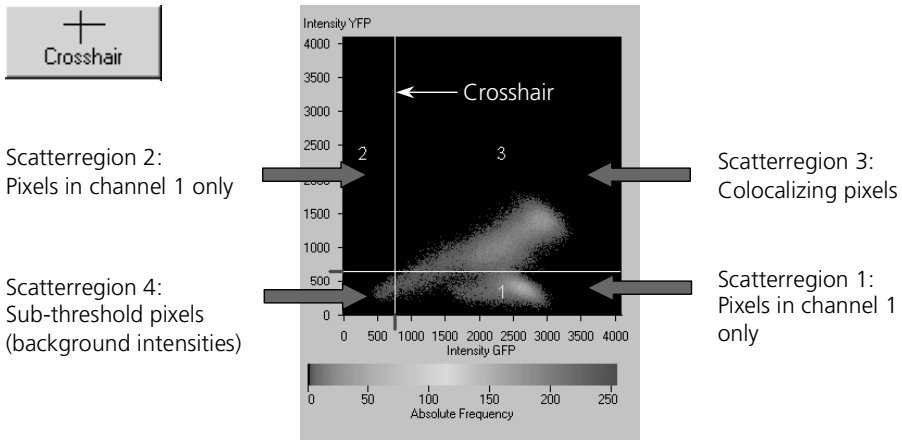
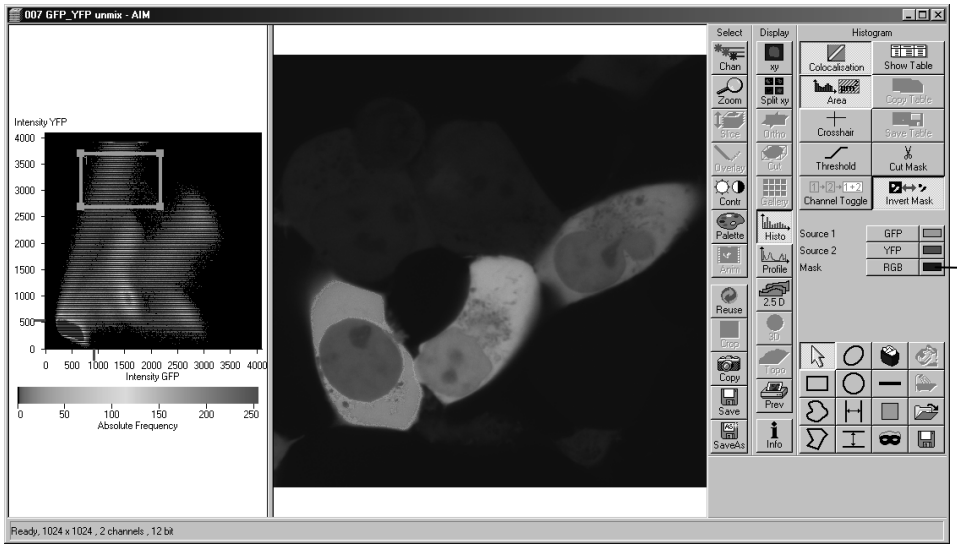


Fig. 5-305 Scattergram and thresholding with crosshair

Select ROIs in scattergram and view corresponding pixels in image display:



Color assignment for pixels in ROI (RGB or overlay)

Fig. 5-306 Image Display window, Colocalization display

Select ROIs in image display and view corresponding pixels in scattergram:

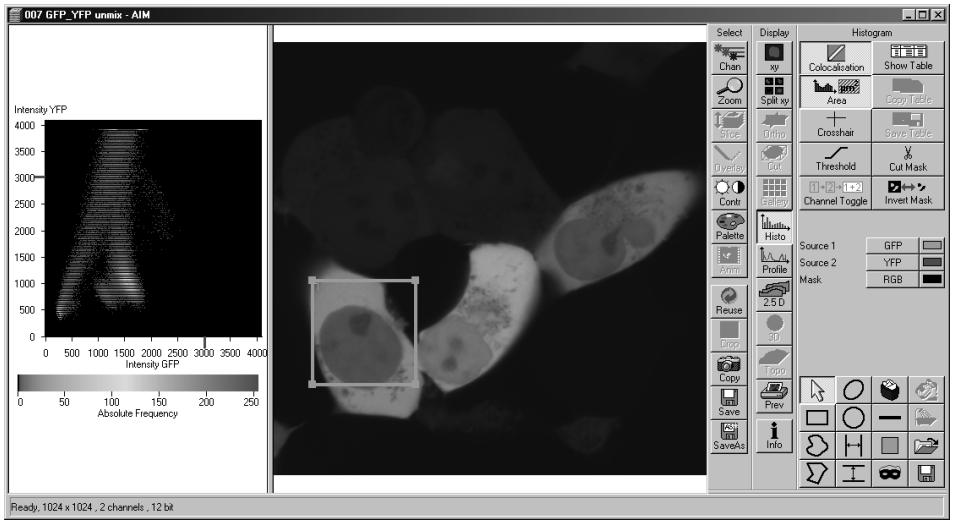


Fig. 5-307 Image Display window, Colocalization display

Quantitative Parameters:

- **No. of pixels** in image ROI or scatter region
- **Area / relative area** of image ROI or scatter region
- **Mean intensities / SD** within image ROI or scatter region
- **Colocalization coefficients**
- **Weighted colocalization coefficients**
- **Overlap coefficient** after Manders
- **Correlation coefficients** (R and R^2)

Colocalization coefficients

$$c_1 = \frac{pixels_{Ch1,coloc}}{pixels_{Ch1,total}} \qquad c_2 = \frac{pixels_{Ch2,coloc}}{pixels_{Ch2,total}}$$

- Relative number of colocalizing pixels in channel 1 or 2, respectively, as compared to the total number of pixels above threshold.
- Value range 0 – 1 (0: no colocalization, 1: all pixels colocalize)
- All pixels above background count irrespective of their intensity.

Weighted colocalization coefficients

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

- Sum of intensities of colocalizing pixels in channel 1 or 2, respectively, as compared to the overall sum of pixel intensities above threshold and in this channel.
- Value range 0 – 1 (0: no colocalization, 1: all pixels colocalize)
- Bright pixels contribute more than faint pixels

Correlation coefficient, Pearson´s correlation coefficient

$$R_p = \frac{\sum_i (Ch1_i - Ch1_{aver}) * (Ch2_i - Ch2_{aver})}{\sqrt{\sum_i (Ch1_i - Ch1_{aver})^2 * \sum_i (Ch2_i - Ch2_{aver})^2}}$$

- Provides information on the intensity distribution within the colocalizing region
- Value range -1 to +1
  - -1,+1: all pixels are found on straight line in the scattergram
  - 0: pixels in scattergram distribute in a cloud with no preferential direction

Overlap coefficient, overlap coefficient after Manders
(Manders, Verbeek and Aten, J. Microscopy 169:375-382, 1993)

$$r = \frac{\sum_i Ch1_i * Ch2_i}{\sqrt{\sum_i (Ch1_i)^2 * \sum_i (Ch2_i)^2}}$$

- Another parameter used to quantify colocalization in image pairs
- Insensitive to differences in signal intensities between the two channels, photo-bleaching or amplifier settings
- Value range 0 – 1 (0: no colocalization, 1:all pixels colocalize)

5.15.20 Display - Profile

This function allows to

- display the intensity distribution of an image along a straight or curved line
- show the intensity values in table form and copy table to clipboard or save as text file
- show separate profiles for each channel in a multi channel image

The settings of **Chan, Zoom, Slice, Contr** and **Palette** are applied.

Click on **Profile** will display the **Profile** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

- Click on the **Profile** button. The **Profile** toolbar will be displayed.
  - The intensity curves are shown in a graph below the image(s).
- On the **Profile** toolbar you can select the width and color of the profile line.

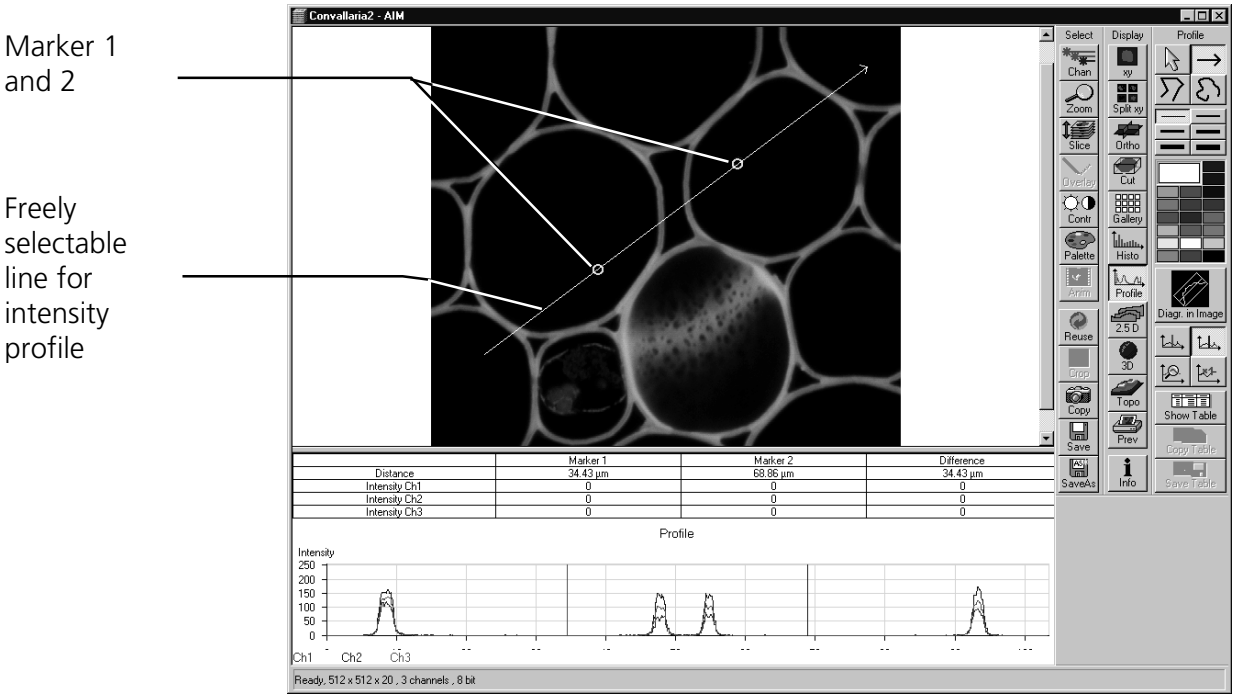


Fig. 5-308 Image Display window, Profile display

- You can place two markers on the profile line to measure differences in intensities and distances in the XY plane.

- Click on the **Diagr. in Image** button to see an intensity graph superimposed on the image.

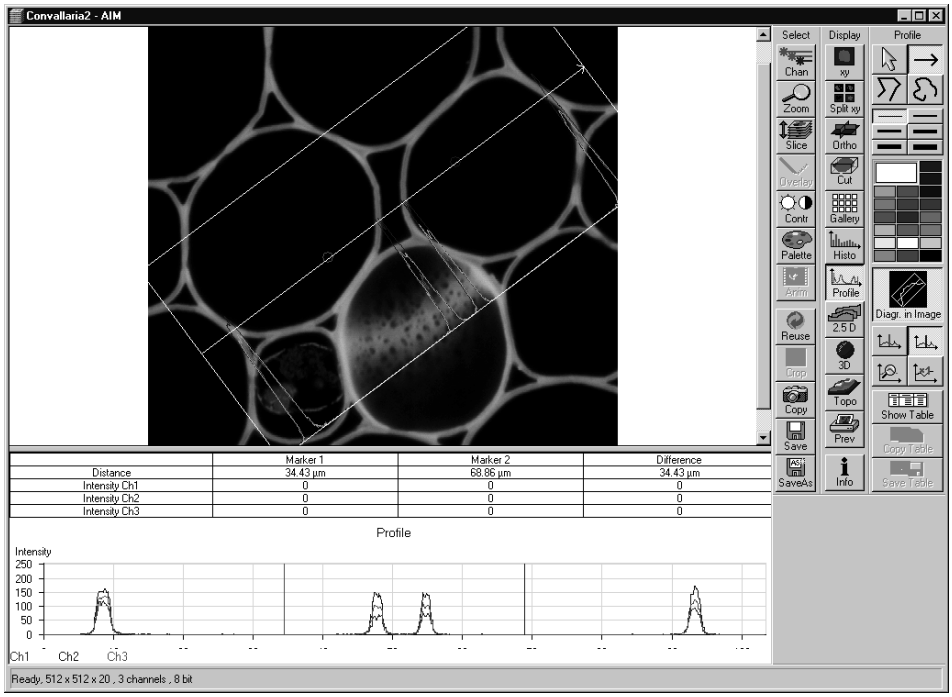


Fig. 5-309 Image Display window, Profile display

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

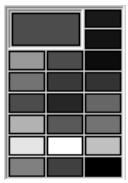
Arrow (selection) button: Activates the mouse button for selection, resizing or movement of the profile line in the **Image Display** window.
Resize: Click on handle and hold down the mouse button, move the handle, release mouse button.
Movement: Click on line and hold down the mouse button, move the entire line, release mouse button.

Line with arrow button :(open arrow): Activates the straight profile drawing mode.
Click into the image and hold the mouse button, drag a line in any direction and release the mouse button to end the procedure.

Open polyline button: Activates the open polyline drawing mode.
The first click into the image sets the starting point, each additional click adds a further line, right mouse click ends the procedure.

Open free-shape curve button: Activates the Bezier figure drawing mode.
The first click into the image sets the starting point, each additional click adds a point, right mouse click ends the procedure.

Line button: This button allows you to determine the line thickness of the profile line. It has no influence on the way the intensity profile is generated.



Color selection box: The colors displayed in the **Color** selection box can be assigned to the overlay profile line with a click of the mouse. The currently selected color is displayed in the larger rectangle (top left) of the selection box.

Diagr. In Image button: The profile diagram is displayed in the image along the drawn profile line.

Marker 1 button (red): Activates the red marker for movement in the profile diagram; the marker shown as a red line in the diagram can now be moved to the right or left of the diagram using the mouse. The marker in the image display (red circle) follows accordingly.

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

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

Reset Zoom button: Resets the zoom factor of the profile diagram to the original size.

Show Table button: The profile diagram is displayed as a table at the bottom of the **Image Display** window.

Copy Table button: The profile table is copied to the clipboard.

Save Table button: The profile table can be stored as a text file (extension *.txt).

5.15.21 Display - 2.5 D

This function allows to

- display the two-dimensional intensity distribution of an image in an pseudo 3D mode
- show the intensity values in profile, grid or filled mode
- show separate distribution for each channel in a multi channel image

- Click on the **2.5 D** button.
  - The **Pseudo 3D** toolbar is displayed.

The settings of **Slice** apply. The settings of **Chan, Zoom, Contr** and **Palette** are not applied.

Click on **2.5 D** will display the **Pseudo 3D** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

The viewing plane of the **Image Display** window can be rotated, tilted either directly with the mouse or by the scroll bars on the right-hand side and the bottom of the **Image Display** window.

(1) Direct setting in the image

- Click in the image and hold down the mouse button. The perspective is changed by moving the mouse button in horizontal or vertical direction.

(2) Setting via scrollbars

- Move the  horizontal scrollbar to rotate the image around the vertical axis. The rotation angle is displayed in the yellow display box.
- Move the  left vertical scrollbar to rotate the image around the horizontal axis. The rotation angle is displayed in the yellow display box.

The intensity scale can be varied by the scroll bar on the right-hand side of the **Image Display** window:

- Moving the  right vertical scrollbar enables you to expand or to compress the intensity scale of the image, while the expansion of this intensity axis ranges between 10 % and 100 % of the X-scale size.

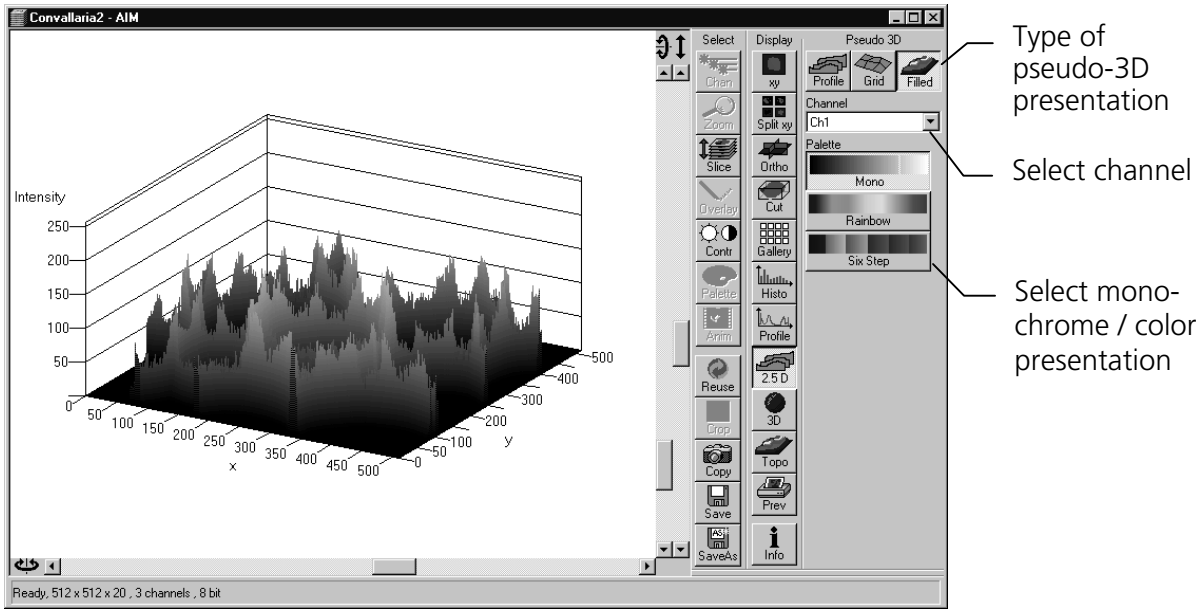


Fig. 5-310 Image Display window, 2.5 D display

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

Profile button	Profile display (vertical polygon display). Setting of the **Profile Distance** between 1 and 20 using the slider.
Grid button	Grid display (horizontal grid display). Setting of the **Grid Distance** between 1 and 20 using the slider.
Filled button	Color diagram (filled 3D diagram). Selection between the **Mono**, **Rainbow** and **Six Step** color palettes.
Channel list box	Permits the selection of a **Channel** in a multi channel image.

5.15.22 Display - 3D (Image VisArt)

This optional function allows to

- reconstruct and display 3 D fluorescence image stacks or time series of frames and stacks from the **Image Display** window
- select from a variety of reconstruction modes

The settings of **Chan are** applied. The settings of **Zoom, Slice, Contr** and **Palette** are not applied.

Click on **3D** will display the **3D** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

- Click on the **3D** button. The **3D** toolbar will be displayed.

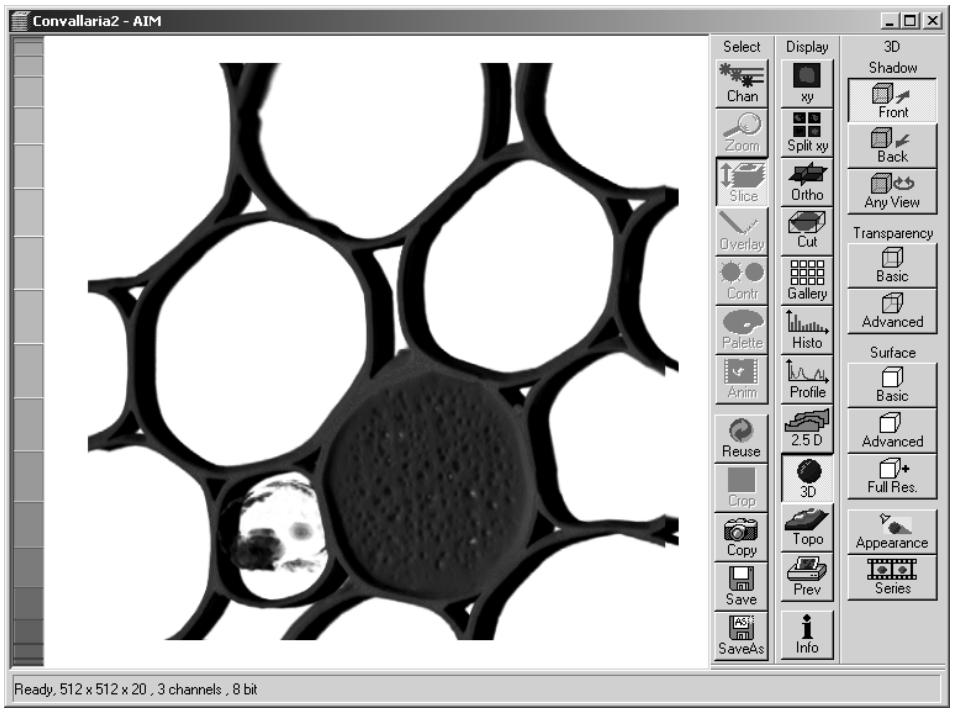


Fig. 5-311 Image Display window, Profile display

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

Shadow projection

Front button	Shadow rendering front view
Back button	Shadow rendering back view
Any View button	Shadow rendering with user defined view

Transparency projection

Basic button	Transparency rendering (voxel based)
Advanced button	Transparency rendering (voxel based) with textures

Surface projection

Basic button	Surface rendering (voxel based)
Advanced button	Surface rendering (triangle based)
Full Resolution button	High accurate surface rendering (triangle based)

Appearance button	Opens the render properties dialog
Series button	Renders a series of 3D image stack or 3D / 4D time series, opens the **Series render** dialog

5.15.22.1 Shadow Projection

With a click on **Front**, the 3D reconstructed image is displayed in a shadow projection where it is illuminated at a 45° angle from the front left.

A click on the **Back** button creates the same projection with illumination from back left.

The zoom wheel to the left of the **Image Display** window allows continuous zooming of the 3D reconstructed image.

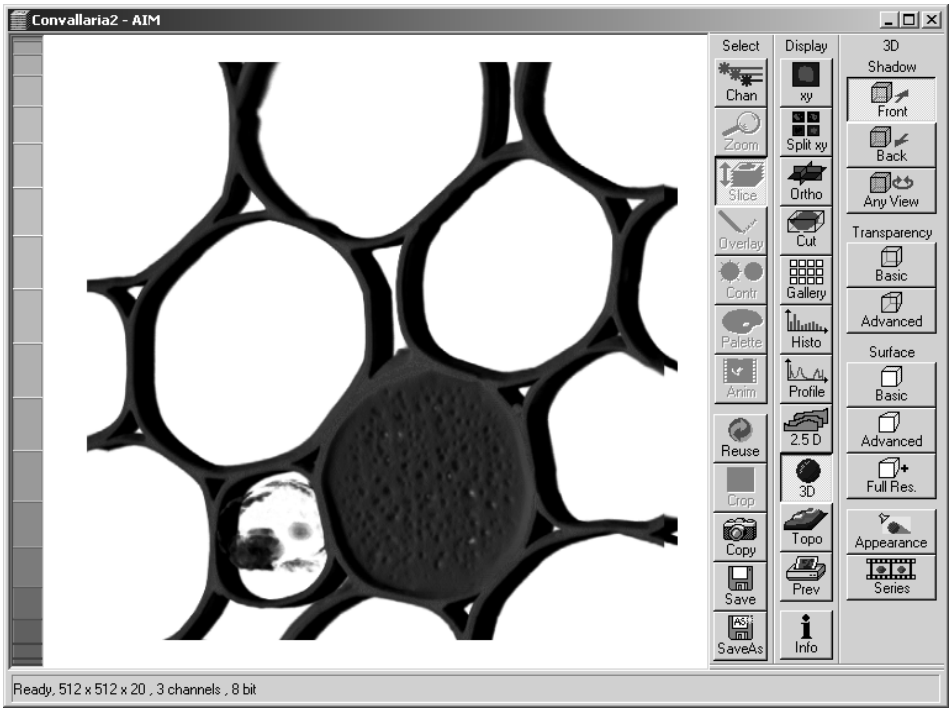


Fig. 5-312 Image Display window, 3D display, Shadow projection, Front view

A click on the **Any View** button displays the 3D reconstruction image in a shadow projection where the viewing point can be defined. In addition to the zoom setting, the image can be rotated around the three orthogonal axes via the relevant setting wheels.

However, the 3D orientation can also be set directly in the **Image Display** window by clicking, holding and dragging the 3D reconstructed image with the mouse.

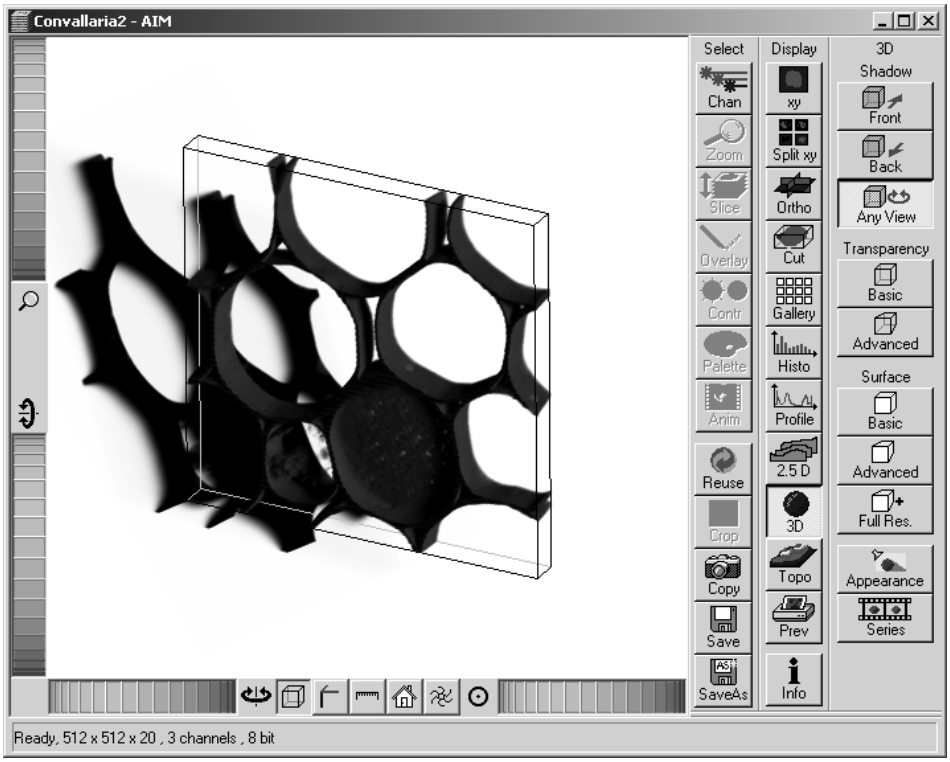


Fig. 5-313 Image Display window, 3D display, Shadow projection, Any View

The following additional buttons are available in the **Any View** shadow projection mode:

- After activation of the **Frame** button (below the image), a bounding box is drawn around the 3D reconstructed image.

☞ Depending on the used mode and hardware configuration, it can take several seconds until the 3D reconstruction is refreshed on the monitor after reorientation.

- A click on the **Coordinate System** button displays a colored coordinate system in the **Image Display** window, where the X axis is displayed in red color, the Y axis in blue and the Z axis in green.
- A click on the **Scale** button display an X-,Y- and Z-scale in the **Image Display** window.
- A click on the **Home** button resets the display parameters to the default values.

- A click on the **Animation** button activates the animation mode. The object can be pushed by dragging in the **Image Display** window and rotates continuously. Any new push with pressed left mouse button changes the rotation direction and speed of the animation.

☞ The fastest animation results can be achieved with the advanced surface rendering mode (even without additional graphics cards).

5.15.22.2 Transparency Projection

The transparency projection generates a transparent 3D reconstructed image.

The elements for image display (zoom, 3D rotation, home, coordinate system, scale, frame and animation function) are identical to those of the **Any View** function of the shadow projection and are operated in the same way.

The transparency projections **Basic** and **Advanced** are perspective-type 3D reconstructions, with the **Advanced** projection permitting the perspective impression being varied between parallel and centric projection by changing the **View angle**. The **Advanced** projection also offers the possibility of selecting between fast and precise calculation via the **Precise / Fast** slider (at the bottom right in the 3D toolbar). Of course, the precise calculation method is more time consuming.

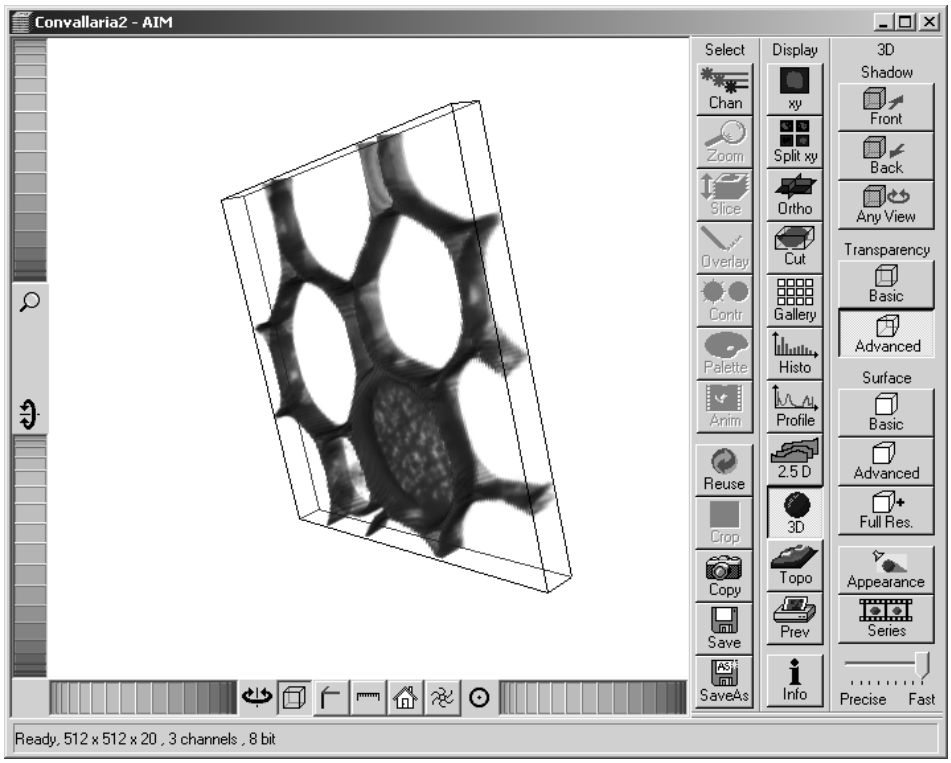


Fig. 5-314 Image Display window, 3D display, Transparency projection, Advanced

5.15.22.3 Surface Rendering

The surface rendering generates surface rendered 3D reconstructed images.

The elements for image display (zoom, 3D rotation, home, coordinate system, scale, frame and animation function) are identical to those of the transparency projection and are operated in the same way.

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

The **Advanced** projection permits the **View angle** to be varied in order to enhance the perspective impression. It is also possible to select between fast and precise calculation via the **Precise / Fast** slider (at the bottom right in the 3D toolbar). Of course, the precise calculation method is more time consuming.

The **Full resolution** projection is based on a high precision calculation method for 3D information on the basis of triangles with maximum resolution.

☞ Depending on the hardware configuration, it can take several seconds until the surface projection is refreshed on the screen.

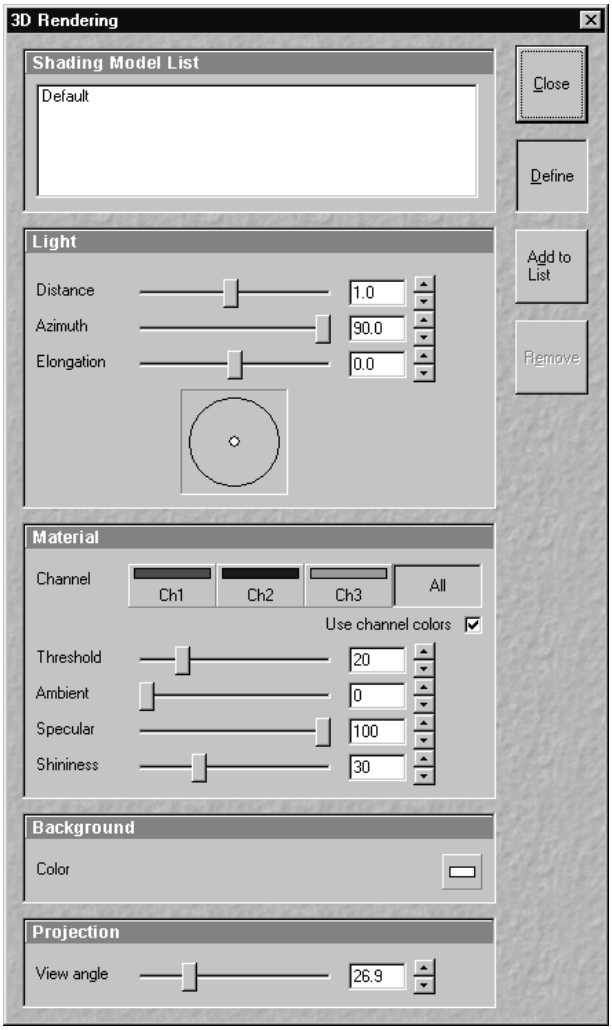


Fig. 5-315 3D Rendering window (e.g. Surface Advanced rendering mode)

5.15.22.4 Appearance (Settings)

The **Appearance** button opens the **3D Rendering** window.

This window allows settings for **Light**, **Material**, **Background** and **Projection** properties to be defined for **all** 3D projection modes.

Depending on the selected 3D projection mode, different setting parameters are displayed.

In the **Shadow** projection, the parameter **Roughness** is also available and can be set between **0** and **1**.

A default setting is permanently available for all modes.

If individual settings for 3D rendering are to be used again, they can be stored and loaded when required.

Proceed as follows to save individual settings:

- Click on the **Add to List** button.
- Enter a name in the opening **Add Shading Model to list** window.
- Click on **OK**.
  - The settings are saved and the entered name appears in the **Shading Model List** selection box.

- To activate the settings, double-click on the relevant name in the **Shading Model List** selection box.

Settings which are no longer required can be removed.

- Select the name of the setting to be deleted with a click of the mouse in the selection box and then click on the **Remove** button.
  - The setting is deleted.

5.15.22.5 Series

The **Series** button opens the **Render Series** window. This window allows settings for the axis to be used for rotation of the 3D reconstructed images.

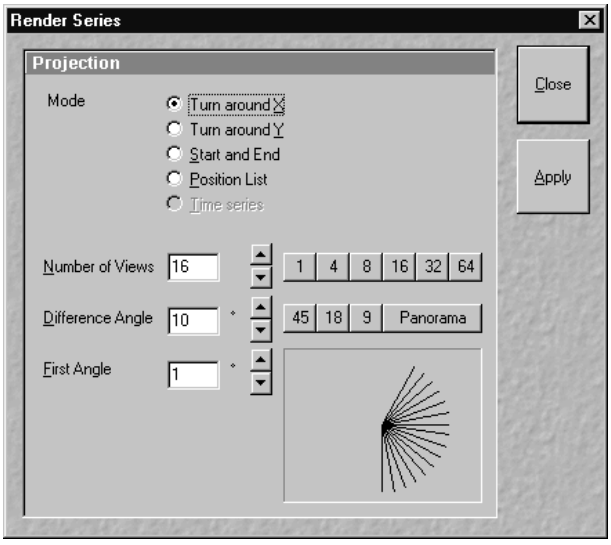


Fig. 5-316 Render Series window (e.g. Turn around X mode)

- Click on the **Series** button to open the **Render Series** window.
- Select the requested projection mode by clicking on the relevant option button under **Mode**.
- Depending on the activated mode, directly set the parameters for animation in the **Render Series** window and the position of the 3D image in the **Image Display** window (zoom, rotation axes, rendering parameters).
- Click on **Apply** to start the animation

The animation is performed in a separate **Image Display** window, which permits the animation to be saved afterwards.

(1) Turn around X and Turn around Y mode

In this mode, the image is turned around the X-axis or the Y-axis exclusively.

The values for **Number of Views**, **Difference Angle** and **First Angle** can be selected accordingly (see section 5.7.2 **Projection**, page 5-175).

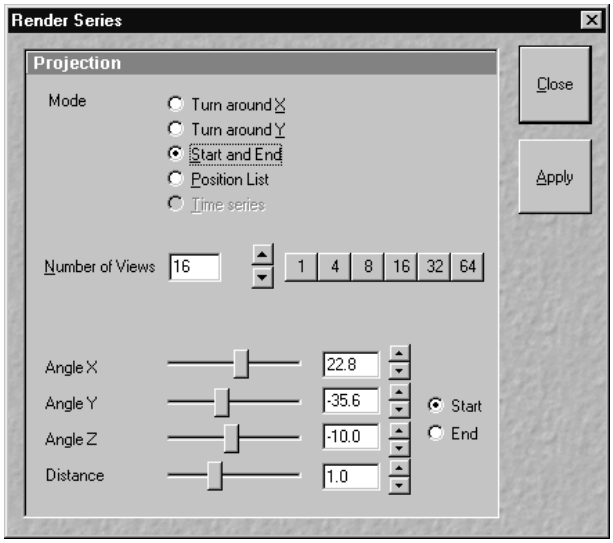


Fig. 5-317 Render Series window (e.g. Start and End mode)

(2) Start and End mode

In this mode, the image is reconstructed between a start position and an end position.

The rotation angles for X, Y and Z and the distance (zoom) can be determined using the sliders.

The value for **Number of View** can be varied.

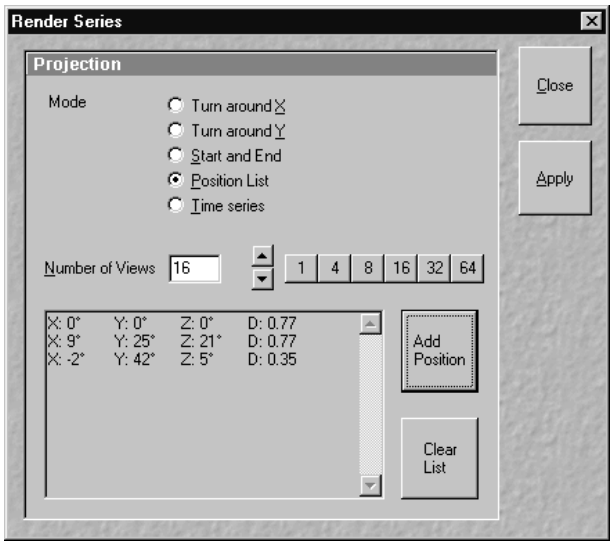


Fig. 5-318 Render Series window (e.g. Position List mode)

(3) Position List mode

In this mode, the image is reconstructed between any required number of interim positions to be determined individually.

The rotation angles for X, Y and Z and the zoom can be determined directly in the image.

Every required interim position is included in the list of the **Render Series** window with a click on the **Add Position** button.

Clear List permits the contents of the list to be deleted.

The value for **Number of View** can be varied.

- Click on the **Apply** button calculates a spline along all the defined positions from the list and starts an animation along this spline track in space.

(4) Time series

When the input images is a Z Stack time series, the reconstructed images are generated for each time point.

5.15.23 Display - Topo for LSM

This optional function allows to

- process, display and measure topographic information.
- use frame Z Stacks
- and frame Z Stacks over time

The **Topo** function is mainly used for applications in material research and industry.

The settings of **Chan** and **Zoom** are applied. The settings of **Slice and Contr** are not applied. The **Palette** settings are applied in some 3D display modes.

Click on **Topo** will display the **Topography** toolbar. All changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

- Click on the **Topo** button. The **Topography** toolbar is displayed.
  - The topography of a Z Stack is displayed in the **Image Display** box of the **Image Display** window. The parameter used at the last exit of the **Topo** function are applied.

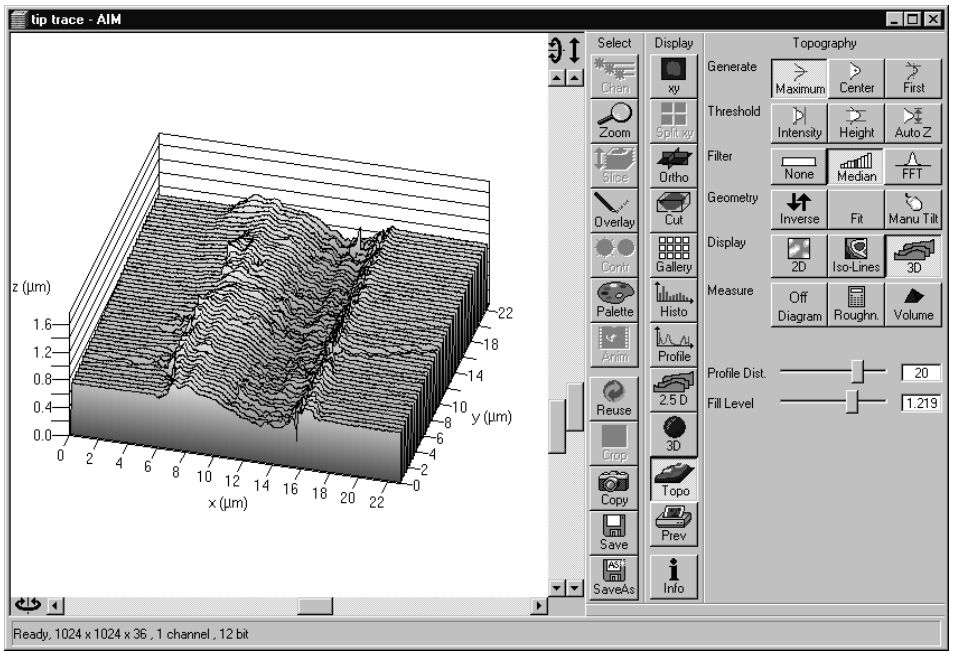


Fig. 5-319 Image Display window, Topography display

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

Channels buttons	The selection of a channel to be used.
Generate buttons	The selection of the mode of calculation of the topography image (maximum, center of gravity, first intensity).
Threshold buttons	The selection of thresholds (intensity, height, Auto Z) to be used.
Filter buttons	The definition of filter procedures (geometrical, frequency cut-off filters) for smoothing, separation of roughness or waviness.
Geometry buttons	Automated correction procedures, changes of geometry, tilting.
Display buttons	2D (Intensity, z Map, Gradient); Iso-Lines (z Map, Intensity, Black) 3D (Profiles, Grid, Filled, Shaded).
Measure buttons	Diagrams (Profile, z Distribution, Bearing area ratio plot, Slope distribution); Roughness parameters; Volume parameters.

5.15.23.1 Channel Selection

- Select the channel to be viewed using the relevant button (e. g.: **Ch1**).

5.15.23.2 Generate Topography

The three buttons provided in the **Generate** button bar allow you to generate the topography in different ways:

Maximum

- Click on the **Maximum** button to calculate the topography surface by finding the maximum intensity value. If the optical section with the highest intensity value is found, the intensity values of both neighboring slices are also taken into account, so that a 3 point maximum fit is calculated.

Center

- Click on the **Center** button to calculate the topography surface by using the center of gravity of all summed up intensities of the stack for a given xy print.

☞ This mode provides better result for smooth surfaces of low intensity or nearly transparent surfaces. The receiver gain and offset has to be properly tuned and MarkFirst- MarkLast-positions of the stack should be located approximately in the same distance from the real surface.

First

- Click on the **First** button to calculate the topography surface by using the first slice coming from the top, where the intensity reaches the value defined by the lower intensity threshold.

☞ This mode provides better result for surfaces of semitransparent materials with inclusions of higher reflectivity or transparent multilayers with subsurface layers of higher signal intensity.

Extended First / Last Mode

1. Definition of an intensity (I) threshold.
2. Starting from top / bottom of a stack to find I = 400.
3. Search of a local maximum one FWHM of actual Z PSF forwards / backwards.
4. Search of the next local maximum one FWHM forwards / backwards from the last max until you have not found any new local maximum.
5. Last local maximum is taken as surface point.

5.15.23.3 Topography Thresholds

Intensity threshold

Click on the **Intensity** button to calculate the topography surface by using the lower and the upper intensity thresholds for image display. Use of this function is recommended to find the real surface in the case of images with pronounced noise. All image pixels with intensity less or higher than the thresholds set are ignored for the surface calculation.

- Click on the **Intensity** button to select the intensity thresholds for the surface generation. The **Intensity Threshold** window appears.
- Set the lower and upper intensity thresholds using the appropriate sliders.
- Click on **Close** to close the **Intensity Threshold** window.

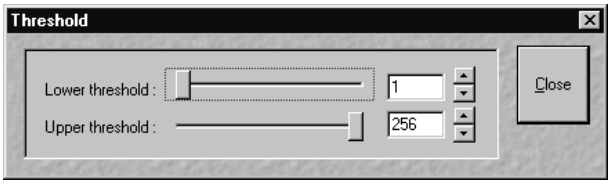


Fig. 5-320 Threshold window

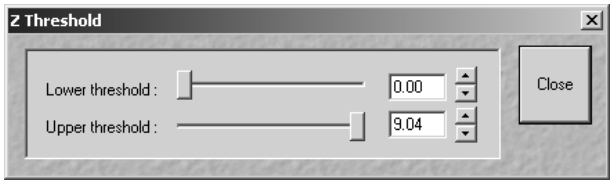


Fig. 5-321 Z Threshold window

Height threshold

Click on the **Height** button to calculate the topography surface by using the lower and the upper height thresholds for image display. Use of this function is recommended to get rid of unwanted peaks and valleys taken into account for parameter calculation. All topographic data with height values less or higher than the thresholds set are ignored for the display and parameter calculation. This threshold applies both for 2D as well as for 3D topography display modes.

- Click on the **Height** button to select the intensity thresholds for the surface generation. The **Z Threshold** window appears.
- Set the lower and upper intensity thresholds using the appropriate sliders.
- Click on **Close** to close the **Z Threshold** window.

Auto Z

By clicking on the **Auto Z** button the surface topography is displayed in the **Image Display** window in that way that it is automatically normalized to the lowest and highest Z value of the 3D topography.

- Click on the **Auto Z** button. The topography is automatically normalized with respect to the highest and lowest Z value.

5.15.23.4 Processing by Filtering

(1) Topography smoothing

The three buttons in the **Filter** button bar allow activation / deactivation of the filter functions for surface smoothing.

None button — No filter for input data.

Median / Gauss / Aver. button — Smoothing of z data using a low-pass Median, Gauss or average filter. Clicking on this button opens a selection box, where the number of neighboring pixels to be used for filtering can be specified:

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

2nd row: medium smoothing via Average (9 x 9; 11 x 11; 15 x 15)

3rd row: pronounced smoothing via Average (25 x 25; 35 x 35; 45 x 45)

- To investigate the effects of various filter modes, select one of the 3D display modes (**Profiles**, **Grid**, **Filled** or **Shaded**) from the **Display** button bar.
- Click on the **Median** sub button to set the smoothing of the integrated Median filter.

  Or
- Click on a **Gauss** or **Average** sub button and select the required degree of smoothing from the selection box with a click of the mouse.

FFT button: This function performs a Fast Fourier Transformation (FFT) in the frequency range, applies highpass or lowpass filtering in the frequency range and performs the inverse FFT.

- Click on the **FFT** button, the **FFT Filter** window opens.
- Click on the arrow in the filter **Type** select box to choose an adequate filter function:
  - Gauss Lowpass
  - Gauss Highpass
  - Butterworth Lowpass
  - Butterworth Highpass
- Select a position of the **Cut off** slider to display either the lower frequencies (waviness) with the lowpass filters or the higher frequencies (roughness) with the highpass filters.
- The **Cut off** frequencies ranges from 1/1000 of the X dimension of the stack to four times of the X dimension of the stack. The dimensions of the filtering is given in units of µm.
- Select a position of the **Degree** slider. The filter functions can be calculated from 1st order to 5th order accuracy.
- Click on the **Close** button closes the **FFT Filter** window.

(2) Changing the topography geometry

The three buttons in the **Geometry** button bar allow the surface geometry to be changed.

Inverse button — Inverse surface. Depths change to heights, and vice versa.

Fit button — The following fit modes can be set:
1) **No Fit**
2) **Plane**
The topography is tilted in such a way that the mean deviation value plane is calculated.
3) **Cylinder fit correction**
A cylinder form is eliminated, determination of micro roughness on cylindrical surfaces can be performed.
4) **Sphere fit correction**
A spherical form is eliminated, determination of micro roughness on spherical surfaces can be performed.

If **Cylinder** / **Sphere fit** is chosen, the **Manu Tilt** button is disabled.
☞ You can display the exact values of the **Cylinder / Sphere** fit by opening a context menu in the **Image Display** box with a click of the right mouse button and selecting the **Show processing parameter** function.

Manu Tilt button — Manual tilt correction.

Clicking on the **Manu Tilt** button activates the function (enabled). The sliders for manual tilt correction are displayed on the right and below the **Image Display** box. Vary the tilt by adjusting the horizontal and vertical sliders or the arrows. The tilting angle is varied in steps of 1 degree. By additional pressing of the Ctrl key, the tilting angle can be varied in steps of 0.1 degrees. A yellow box showing the tilt angle currently set is displayed next to each slider for checking purposes. A second click on the **Manu Tilt** button ends this function and saves the setting (save and disabled). The sliders for tilt correction disappear from the display.
You can also change the tilt angle directly in the **Image Display** box. Click the left mouse button in the image and hold it down. Moving the mouse pointer in horizontal or vertical direction tilts the topography by an axis parallel or vertical to the screen. On releasing the mouse button, the change of the tilt is stored and the function is deactivated (disabled). To reset the manual tilt correction, click the **Fit** button.

- Click on the **Inverse** button for the inverse display of the topography. Clicking again will reset the normal display.
- Correct the tilt via the **Fit** or **Manu Tilt** functions.

5.15.23.5 Display Modes

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

(1) 2D modes

The following 2D modes can be set:

Intensity button: Display of projection of all intensities of the stack (black-and-white display).

z Map button: Height coded color map with color bar.

Gradient button: Display of height gradient (slope), averaged pixelwise over all neighbors (black-and-white display).

- Click on the **2D** button in the **Display** button bar.
  - The 2D display mode selected last is activated. At the same time, an additional button bar is displayed beside the **2D** button permitting selection of the required 2D display mode.
- Select the required 2D display mode with a click of the mouse.

(2) 2D Iso-Lines display mode

Iso-Lines are lines which connect points of equal height on the topography.

The following 2D Iso-Lines display modes can be set:

Intensity button: **Intensity** projection superimposed with colored iso-lines (lines of equal height).

z Map button: **z Map** function with black iso-lines.

Black button: White iso-lines on a black background.

- Click on the **Iso-Lines** button in the **Display** button bar.
  - The Iso-Lines display mode selected last is activated. At the same time, an additional button bar is displayed beside the **Iso-Lines** button permitting selection of the required Iso-Lines display mode.
  - Below the **Measure** button bar, the **Line Dist.** and **Line Offset** sliders / input boxes are displayed.
- Select the required Iso-Line display mode by clicking the left mouse button.

The additional function elements of the **Iso-Lines** display mode have the following meaning:

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

Line Offset slider: Setting of the height level where the Iso-Lines display starts.

☞ To apply the topography functions to a small portion of the Z Stack image use the **Overlay** function (**Overlay** button) and cut out and store as new topographic evaluation via the **Extract Region** function.

(3) 3D display

Topo animations are possible. The following 3D modes can be set:

Profiles button: Profile display.

Grid button: Grid display.

Filled button: Display of color shades.

Shaded button: Surface rendering. Can be combined with LUT. Topo animations are possible.

- Click on the **3D** button in the **Display** button bar.
  - The 3D display mode selected last is activated. At the same time, an additional button bar appears beside the **3D** button to permit selection of the required 3D display mode.
  - Below the **Measure** button bar, the **Scaling** button bar and the **Profile Dist**. and **Fill Level** sliders / input boxes are displayed.
  - The **Image Display** box contains one horizontal and two vertical scrollbars for the setting of the image viewing angle.
- Select the required 3D display mode with a click of the mouse.

The additional function elements of the **3D** display mode have the following meaning:

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

Fill Level **Fill Level** slider: Used to push through a color LUT Look Up Table (e.g.: if the **Rainbow** palette is used) in the **Profiles / Filled** display mode. In combination with the **Volume** button, the filling of the flood function level of the topography can be varied for volume measurements (see the **Measurement functions** paragraph).

Fill Holes procedure

- Intensity of a missing pixel of a hole has to be interpolated by the distance-weighted intensity of all surrounding pixels.
- Fill hole algorithm is optimized for short calculation times.

The image viewing angle is set as follows:

Setting directly in the image

- Click in the image and hold down the mouse button. The perspective is changed by moving the mouse button in horizontal or vertical direction.

Setting via scrollbars

- Move the  horizontal scrollbar to rotate the image around the vertical axis. The rotation angle is displayed in the yellow display box.
- Move the  left vertical scrollbar to rotate the image around the horizontal axis. The rotation angle is displayed in the yellow display box.
- Moving the  right vertical scrollbar enables you to expand the image in height or to compress it, while the Z-range between 10 % and 100 % of the X-range is scaled.

☞ You can set the x, y and Z scales to an identical ratio by opening a context menu in the **Image Display** box with a click of the right mouse button and selecting the **Metric equal ratio** function.

The displayed boxes for rotation angle and relative scaling percentage value z : x ratio permit the setting of identical perspectives for different images (e.g.: the plot of several topographies).

☞ The **Profiles** and **Filled** 3D display modes permit a color palette (e.g.: **Glowscale**, **Rainbow** or **User defined**) to be loaded or redefined by pressing the **Palette** button (see page 5-292).

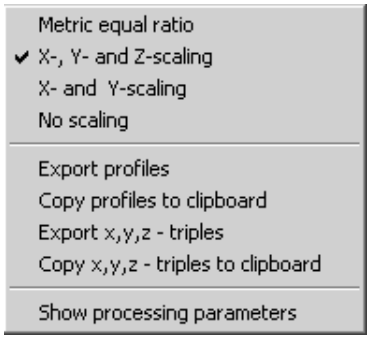


Fig. 5-322 Context menu of the 3D display mode (Profiles)

5.15.23.6 Context Menu of the 3D Display Mode

- Click in the **Image Display** box with the right mouse button to open the context menu.

The context menu for the 3D mode currently selected is displayed.

- Click on the required option with the left mouse button to execute the function.

(1) Metric equal ratio item

This option is available in all of the 3D display modes.

After activation of the function, the x, y and z scales are set to an identical ratio.

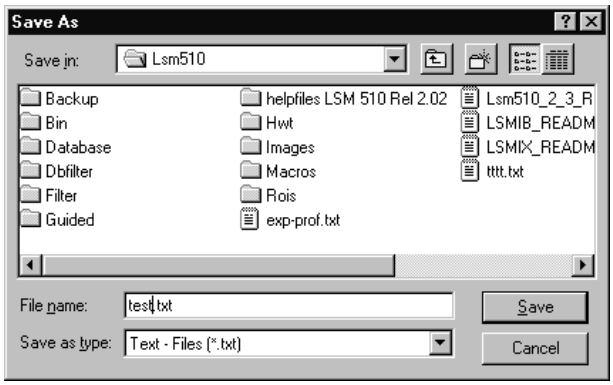


Fig. 5-323 Save As window

(2) Export ... item

This option is available in the **Profiles** and **Grid** 3D display modes.

Use the function to save the **Profiles** or **Grid** data as a text file.

- Open the context menu with a click of the right mouse button, then click on the option **Export ...** with the left mouse button.
  - The **Save As** window is opened.
- Select the directory where you want the text file to be stored, enter a file name and click on **Save**.

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

	X: 0.00	X: 10.55	X: 21.10	X: 31.65	X: 42.19
Y: 2.32	0.00	0.00	0.00	0.00	0.00
Y: 12.87	0.00	5.71	7.25	7.02	5.99
Y: 23.42	0.00	6.62	6.96	7.25	5.77
Y: 33.97	0.00	5.02	7.82	7.99	6.11
Y: 44.51	0.00	5.94	7.76	7.31	5.65

Fig. 5-324 Topography matrix

(3) Copy ... to clipboard item

This option is available in the **Profiles** and **Grid** 3D display modes.

After selection of this option, the **Profiles** or **Grid** data are copied as an XYZ matrix to the clipboard and can be inserted in other programs using the **Paste** command.

(4) Export x,y,z- triples item

This option is available in the **Profiles** and **Grid** 3D display modes.

Use the function to save the **Profiles** or **Grid** data as a text file.

- Open the context menu with a click of the right mouse button, then click on the option **Export ...** with the left mouse button.
  - The **Save As** window is opened.
- Select the directory where you want the text file to be stored, enter a file name and click on **Save**.

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

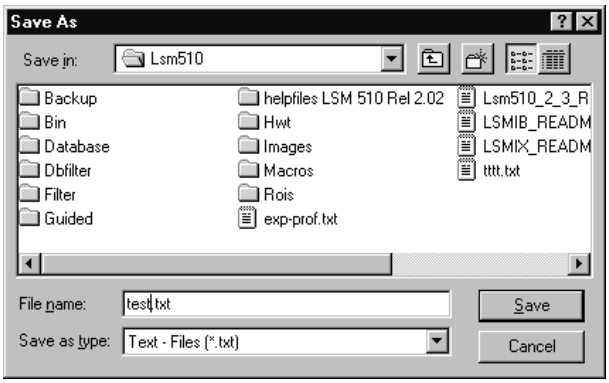


Fig. 5-325 Save As window

X	Y	Z
0.00	12.87	0.00
10.55	12.87	5.71
21.10	12.87	7.25
31.65	12.87	7.02
42.19	12.87	5.99
52.74	12.87	5.19

Fig. 5-326 Topography table

(5) Copy x,y,z- triples to clipboard item

This option is available in the **Profiles** and **Grid** 3D display modes.

After selection of this option, the **Profiles** or **Grid** data are copied as an XYZ table to the clipboard and can be inserted in other programs using the **Paste** command.

☞ Please make sure that the amount of exportable data is adequate to the maximum importing size of the following software package. To lower the amount of data points, use the profile distance slider.

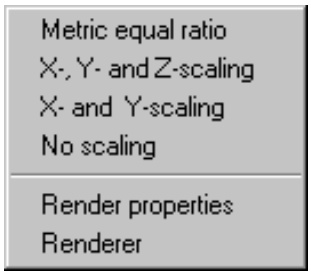


Fig. 5-327 Context menu of the 3D display mode (Shaded)

(6) Render properties item

This option is only available in the **Shaded** 3D display mode.

Use this function to vary the illumination conditions, reflection properties and projection settings of the topography. You can either select preset Shading Models or use parameters specifically defined as required.

The specifically defined parameters can subsequently be stored as a Shading Model and are then available at any time for further use. Shading Models can also be deleted if no longer needed.

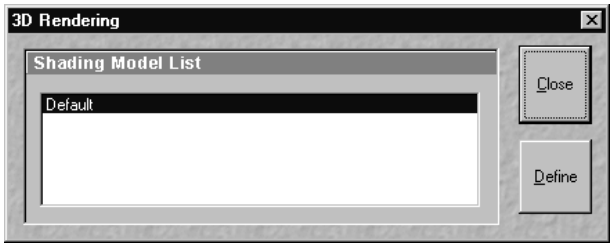


Fig. 5-328 3D Rendering window

Load a Shading Model

- Open the context menu with a click of the right mouse button, then click on the option **Render properties** with the left mouse button.
  - The **3D Rendering** window is opened.
- Click on the name of the required model in the Shading Model List. The parameters are immediately set for the current topography.
- Click on **Close** to close the **3D Rendering** window again.

Defining a specific Shading Model

- Open the **3D Rendering** window.
- Click on the **Define** button.
- Change the parameters of the topography using the appropriate sliders.
- Save the settings by clicking on the **Add to List** button. The **Add Shading Model to List** window is displayed.
- Enter a name for the model and click on **OK**. The model is included in the **Shading Model List**.

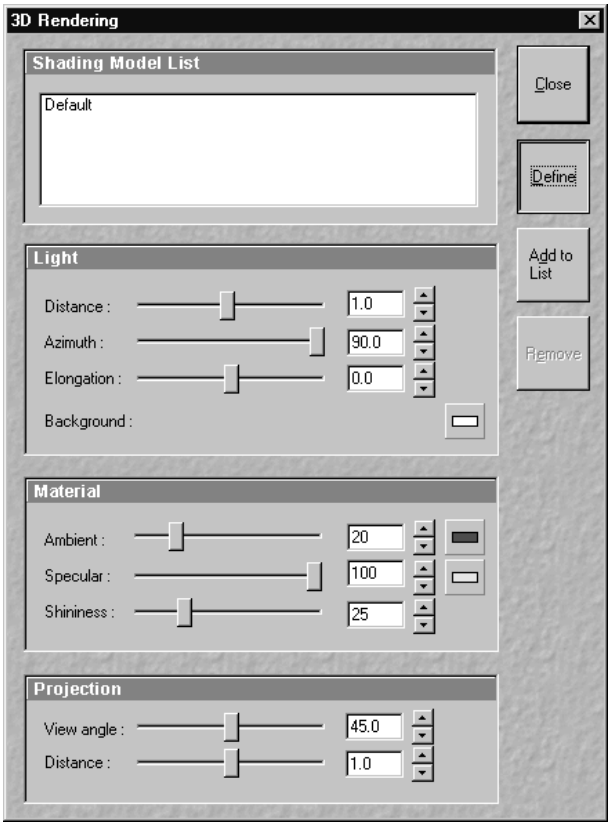


Fig. 5-329 3D Rendering window

Light panel

Determines the properties of illumination on a sample.

Distance Goes for diffuse and **specular**, see visualization.

Azimuth See visualization. Rise angle of the "sun".

Elongation See visualization. North-south / east-west direction of the "sun".

Background Choose background color.

Material panel

Determines the reflective properties of a sample.

Ambient
Specular Material properties; how many % of the light component are projected by the material into which spectral ranges.

Shininess Goes together with specular light. Shininess equal to 25 % determines diffuse light.

Projection panel

Determines the reflective properties of a sample.

View angle Determines the perspective, 0.0 parallel projection, central projection

Distance Zoom function, zoom in, zoom out

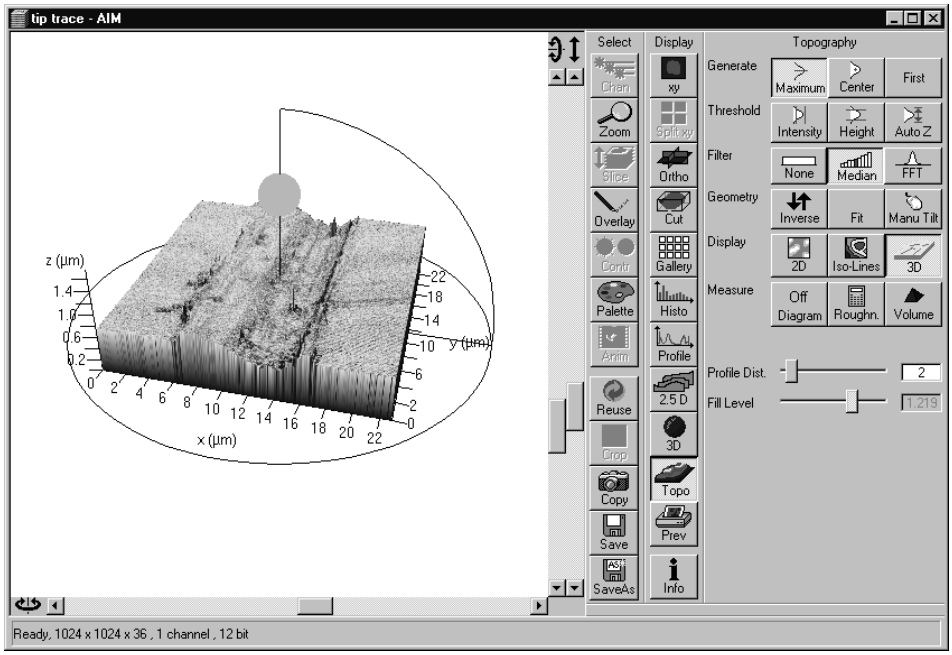


Fig. 5-330 Render function visualization aid

A zoomed rendering setting permits the zoomed section to be moved via the cursor keys after a click on the 3D window.

If a change of the 3D image angle follows, centration is made on the center again.

Deleting a Shading Model

- Select the model to be deleted in the **Shading Model List**, then click on the **Remove** button. The model is deleted.

(7) Renderer item

This option is only available in the **Shaded** 3D display mode.

After selection of the **Renderer** item, the **3D Renderer** window appears. It allows the selection of the hardware and software option which shall be used for the 3D graphics calculation.

OpenGl - Software

The graphics calculation is performed using the installed software.

OpenGl - Hardware

The graphics calculation is accelerated by using the installed graphics processor.

Direct3D – Software / Hardware Rasterizer / Hardware

These options can be used for offline versions of the LSM 5 software for PC's with the WINDOWS 98 or 2000 operating system (not for WINDOWS NT).

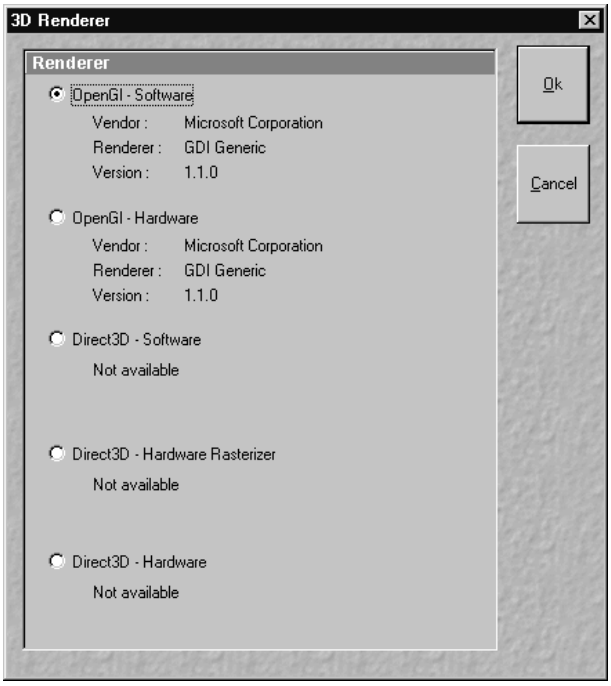


Fig. 5-331 3D Renderer window

(8) Show processing parameters

After selection of the **Show processing parameters** function, a reporting of the following applied topo processing functionality is displayed on the right-hand side of the **Image Display** window:

- Mode {calculation mode: Max, Center, First)
- Threshold (applied intensity threshold)
- Filter
- Fit (plane, cylinder / sphere parameters).

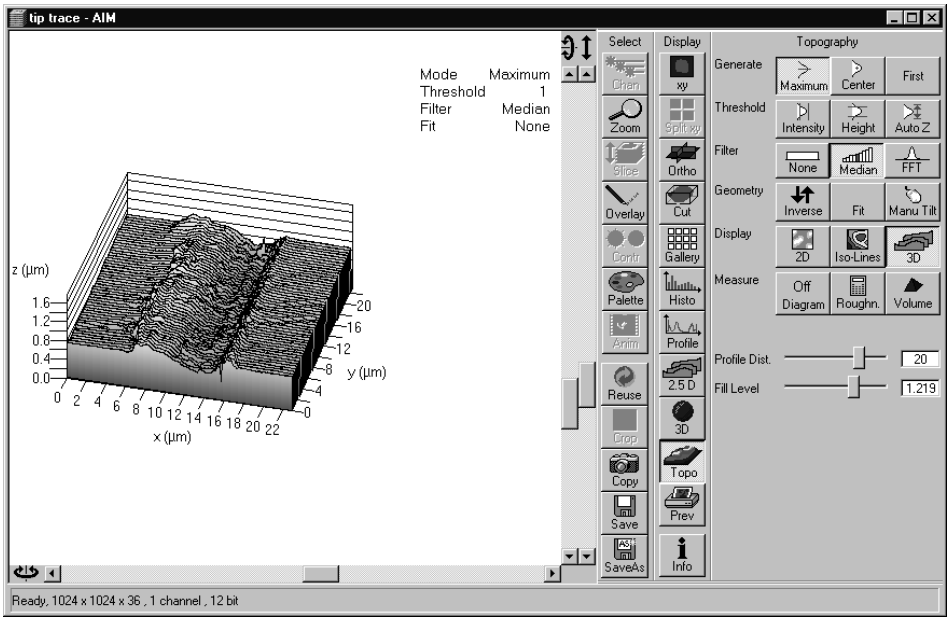


Fig. 5-332 Show processing parameters

5.15.23.7 Measurement Functions

The topography measurement functions are activated via the **Measure** button bar. The measurement functions can be performed in the 2D or 3D display mode.

Automated convention in height statistics analysis:

Topo Filters	None, median, <9x9	FFT High	FFT Low	>9x9
Data formats	**P**rimary profile	**R**oughness	**W**aviness	
2D profile	Pxx	Rxx	Wxx	n.a.
3D topography	SPxx	SRxx	SWxx	n.a.

The following measurement functions are available:

Diagram button: Diagram display. The **Profile**, **z Histo**, **Curve of tp** and **Grad. Histo** diagram display modes can be activated via the **Diagram** button and deactivated via the **Off Diagram** button. By activation of the **Diagram** button, an additional button bar is displayed for the selection of the required diagram or for deactivation. The labeling of the **Diagram** button changes depending on which diagram display mode has been activated.

Roughness button: Calculation of the roughness parameters.

Volume button: Calculation of the volume parameters.

(1) Profile measurement mode in 2D display

- Select the required 2D display of the stack via the 2D button.
- Click on the **Diagram** button in the **Measure** button bar. Click on the **Profile** button in the button bar displayed afterwards.
  - The **Table** and **Profile** button bars are displayed below the **Measure** button bar.
  - A colored arrow (intersection line of the profile) is displayed in the image and the profile diagram appears below the image.
- If required, match the size of the **Image Display** window in order to obtain the complete display of the profile diagram.

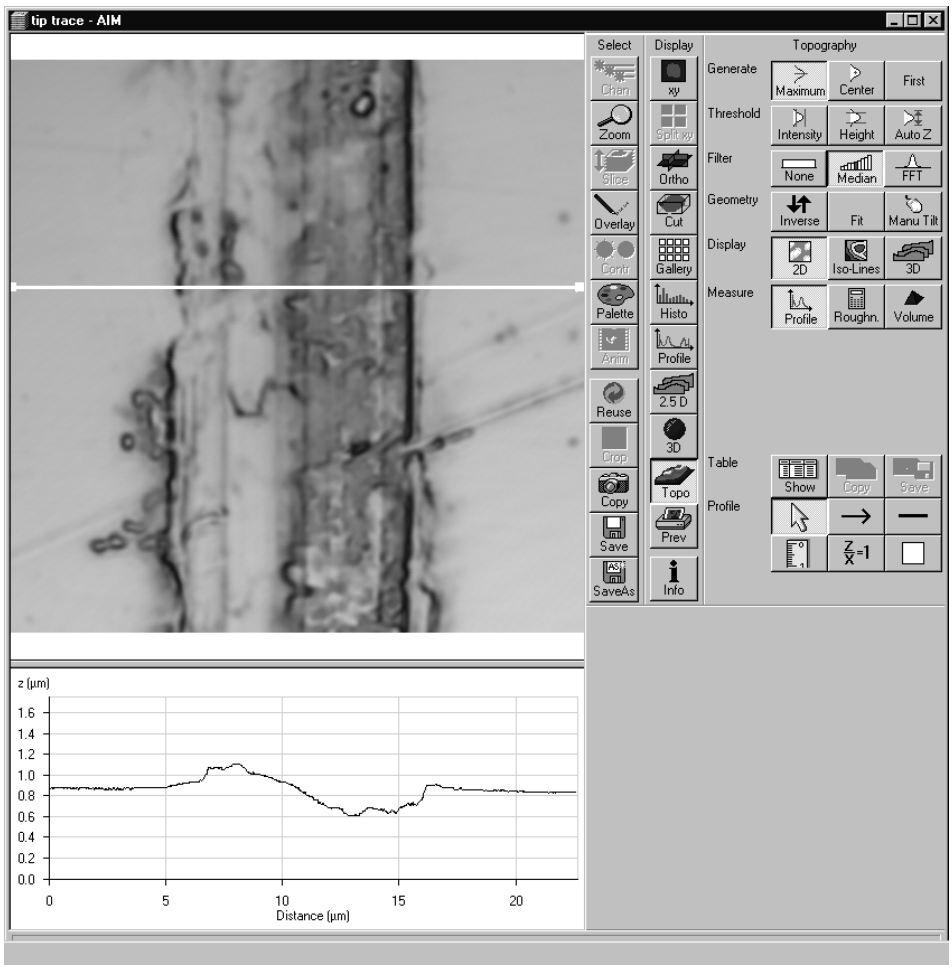


Fig. 5-333 Image Display window, Topography display: 2D - Profile

The additional **Table** / **Profile** buttons have the following functions:

Show button: The profile is displayed in the form of a table at the bottom below of the **Image Display** window.

Copy button: The profile table is copied to the clipboard and can be transferred to other programs (MS Word or MS Excel) via the **Paste** function.

Save button: The profile table can be stored as a text file (ASCII).

Arrow (selection) button: Activation of the mouse button for selection, resizing or movement of the intersection line in the image.
Resizing: Click on the handle and hold down the mouse button, drag the handle, release the mouse button.
Movement: Click on the line and hold down the mouse button, move the entire intersection line, release the mouse button.

Line with arrow button (open arrow): Creation of the intersection line to define the position of the profile to be produced in the image. Click and hold the mouse button, drag the line in any required direction, release the mouse button to end the procedure (The profile diagram changes online.

Line button: This button allows you to determine the line thickness of the intersection line.

Measure button: Activates the **Profile measurement mode** in the profile diagram. The required tools are displayed to the right of the profile diagram (see **Profile measurement mode**, page 5-356).

z/x=1 button: Sets the **z/x** ratio in the profile diagram to the value **1**. Check: the following creation of a circle using the relevant tool really results in a circle in the profile display. Measured angle values correspond to the actual slope of the line displayed.

Color button: Clicking on the **Color** button opens a color selection box in which the color for the intersection line can be selected with a click of the mouse.

Fig. 5-334 Tools of the Profile measurement mode

Profile measurement mode

If you click on the  button, the **Profile** window with the tools of the profile measurement mode appears.

This window can be moved as required over the entire screen.

The tools of the **Profile measurement mode** have the following functions:

Zoom button: Zooming of a section of the profile diagram. Click and drag a rectangle over the area to be enlarged in the profile diagram, release the mouse button to enlarge the selected area. The zoom function can be performed several times. A click with the right mouse button resizes the profile.

Marker button: Activation of the marker functions for the intersection line. The red and blue marker lines in the profile diagram can now be moved using the mouse. After movement of a marker line in the profile diagram, the relevant marker (red or blue circle) follows along the intersection line in the **2D** and **Iso-Lines** mode.

Arrow (selection) button: Activation of the mouse button for selection, resizing or movement of one of the following drawing elements in the profile diagram.
Resizing: Click on the handle and hold down the mouse button, move the handle, release the mouse button.
Movement: Click on the line and hold down the mouse button, move the entire drawing element, release the mouse button.

Inclined Line button: Creation of a straight line in the profile diagram. Display of distance, inclination angle, dxdy and dz. Click and hold down the mouse button, drag the line in any required direction, release the mouse button to end the procedure.

Free angle button: Creation of a free angle in the profile diagram. Display of the enclosed angle (max. 180 °). The first click sets the starting point, the second and third clicks define the angle and the end point.

Rectangle button: Creation of a rectangle in the profile diagram. Display of distance, area, height and width. Click and hold down the mouse button, drag the rectangle in any required direction, release the mouse button to end the procedure.

Open Polyline button: Creation of an open polyline figure in the profile diagram. Display of the length of the line figure. First click sets the starting point, any further click adds another line, click with the right mouse button ends the procedure.

Closed Polyline button: Creation of a closed polyline figure in the profile diagram. Display of the perimeter of the figure. First click sets the starting point, each further click adds another line, a click with the right mouse button closes the figure and ends the procedure.

Open free-hand curve button: Creation of an open Bezier figure in the profile diagram. Display of the length of the line figure. First click sets the starting point, each further click adds another line, a click with the right mouse button ends the procedure.

Closed free-hand curve button: Creation of a closed Bezier figure in the profile diagram. Display of the length of the line figure. First click sets the starting point, each further click adds another line, a click with the right mouse button closes the figure and ends the procedure.

Ellipse button: Creation of an ellipse in the profile diagram. Display of the area. First click sets the center point, the displayed line permits the determination of the first dimension, second click sets the first dimension, the second dimension and rotation direction can now be determined, third click sets the second dimension and direction and ends the procedure.

Circle button: Creation of a circle in the profile diagram. Display of radius and area. Clicking three times to define 3 points on the profile. A circle fit is automatically applied on the profile.

Recycle bin button: Deletes all drawing elements or the one just selected.

Line width button: Change of the line width of the drawing elements.

Color button: Clicking on the **Color** button opens a color selection box where the color of the drawing element can be selected with a click of the mouse.

x1- button: Resets the zoom factor of the profile diagram to its original size.

(2) z Histo measurement mode in 2D display

- Click on the **Diagram** button in the **Measure** button bar. Click on the **z Histo** button in the additional button bar now displayed.

The lower part of the **Image Display** box shows the 3D height distribution of the topography.

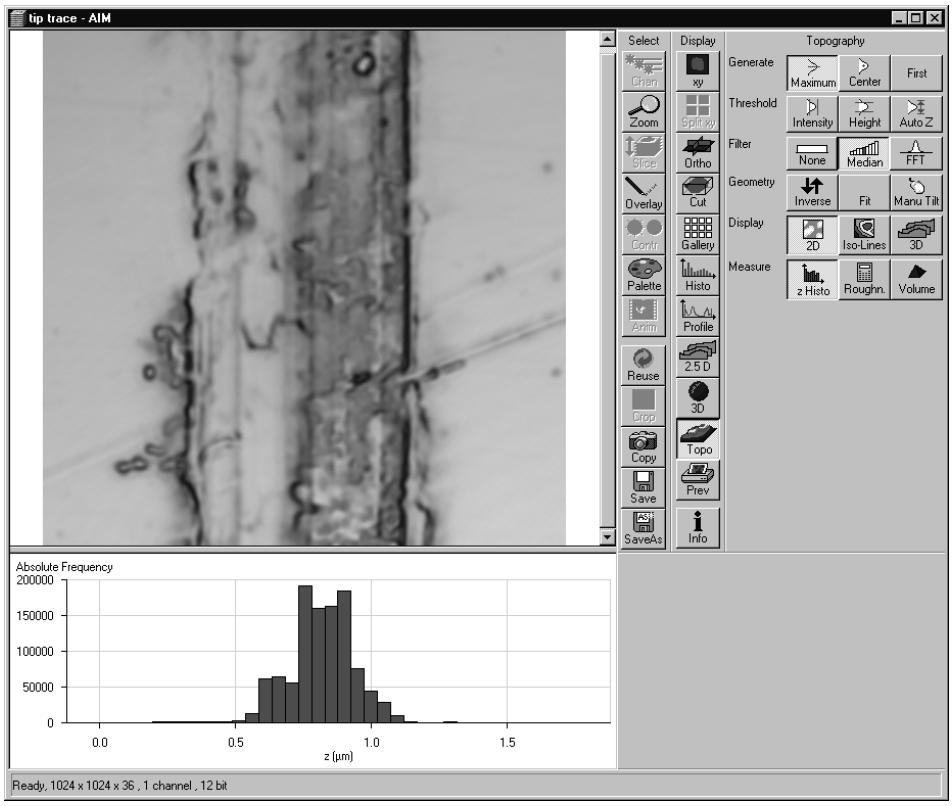


Fig. 5-335 Image Display window, Topography display: 2D - Histo

(3) Curve of tp measurement mode in 2D display

- Click on the **Diagram** button in the **Measure** button bar. Click on the **Curve of tp** button in the additional button bar now displayed.
  - The curve of the bearing area ratio as a function of the height is displayed below the image (also see **3D measurement functions**, page 5-365).

(4) Grad. Histo measurement mode in 2D display

- Click on the **Diagram** button in the **Measure** button bar. Click on the **Grad. Histo** button in the additional button bar now displayed.

The lower part of the **Image Display** box shows the gradient distribution of the topography. Before creation of the slope diagram, the image should be filtered at least once using a low-pass filter, since otherwise the rough height gradation of the image will result in a comb-shaped histogram. The Root-Mean-Square Slope (RMS Slope) parameter is calculated and displayed below the chart. The following formula is used for calculation:

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

(5) Roughness measurement mode in 2D display (Profile display)

2D Amplitude parameters (Profile Roughness):

	Mean height z	Rc	Pc	Wc
Dispersion	Arithmetic mean deviation	Ra	Pa	Wa
	Root mean square deviation	Rq	Pq	Wq
Asymmetry	Skewness	Rsk	Psk	Wsk
Sharpness	Kurtosis	Rku	Pku	Wku
Extremes	Highest peak	Rp	Pp	Wp
	Lowest valley	Rv	Pv	Wv
	Absolute peak to valley	Rt	Pt	Wt
	Averaged peak to valley	Rz	Pz	Wz
	Maximum peak to valley	Rmax	Pmax	Wmax
If choosen filters are		FFT High	No, M	FFT L

- Click on the **Profile** button in the **Measure** button bar.

- Click on the **Roughn.** button in the **Measure** button bar.
  - The roughness parameters are calculated and displayed on the left below the image. All roughness parameters calculated from a 2D profile are named with **R**.
  - The **Copy** button is displayed below the right-hand side of the image. This button permits the roughness parameters to be copied to the clipboard and imported to another program (e.g.: MS Word or MS Excel) via the **Paste** function.

The following roughness parameters are calculated (e.g. for a Y-section)

- Mean height of all profile height values R_c

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

N_x, N_y ... number of pixels in X- or Y-direction

- Arithmetic mean deviation of all profile height values R_a

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

- Quadratic mean deviation of all profile height values R_q

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

- Skewness of the distribution of all profile height values R_{SK}

$$R_{SK} = \frac{1}{N_y \cdot R_q^3} \cdot \sum_{j=1}^{N_y} \cdot z^3(x, y_j)$$

- Kurtosis of the distribution of all profile height values R_{KU}

$$R_{KU} = \frac{1}{N_y \cdot R_q^4} \cdot \sum_{j=1}^{N_y} \cdot z^4(x, y_j)$$

- Maximum peak height R_P

$$R_P = z_{\max} - R_c$$

- Maximum valley depth R_V

$$R_V = R_c - z_{\min}$$

– Maximum roughness depth R_t (= Peak to Valley / PV)

$$- R_t = z_{\max} - z_{\min}$$

maximum height difference of the overall topography along a profile.

Classification of topography in 5 equal area elements (rectangles in the 2D mode)

– average roughness depth R_z:

$$- R_z = \frac{z_{\max 1} - z_{\min 1} + z_{\max 2} - z_{\min 2} + z_{\max 3} - z_{\min 3} + z_{\max 4} - z_{\min 4} + z_{\max 5} - z_{\min 5}}{5}$$

Averaging of R_t-values of all the 5 single area elements. When combined, both parameters provide information about the homogeneity of the surface. Big differences are indicative of pronounced inclination of the overall area or of spikes.

Developed Surface Area Ratio: Σ (surface area$_{ij}$) / Σ (projected area$_{ij}$)

The percentage of the 3D surface area (sum off all triangles formed by adjacent data points) to the 2D surface area produced by projecting the 3D surface onto the threshold plane.

– maximum roughness depth R_{max}:

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

maximum of R_t-values of all the 25 single area elements.

☞ Both the roughness parameters and the z-histogram can be changed by using filters!

(6) Roughness measurement mode in 3D display

3D Amplitude parameters (Topography Roughness):

	Mean height z	SRc	SPc	Wc
Dispersion	Arithmetic mean deviation	SRa	SPa	Wa
	Root mean square deviation	SRq	SPq	Wq
Asymmetry	Skewness	SRsk	SPsk	Wsk
Sharpness	Kurtosis	SRku	SPku	Wku
Extremes	Highest peak	SRp	SPp	Wp
	Lowest valley	SRv	SPv	Wv
	Absolute peak to valley	SRt	SPt	Wt
	Averaged peak to valley	SRz	SPz	Wz
	Maximum peak to valley	SRmax	SPmax	Wmax
If choosen filters are:		FFT High	No, M	FFT L

- Click on the **Roughn.** button in the **Measure** button bar.
  - The roughness parameters are calculated and displayed on the left below the image. All roughness parameters calculated from a 3D topography are named with **S**.
  - The **Copy** button is displayed below the right-hand side of the image. This button permits the roughness parameters to be copied to the clipboard and imported to another program (e.g.: MS Word or MS Excel) via the **Paste** function.

The following roughness parameters are calculated:

- Mean height of all surface height values S_c

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

N_x, N_y ... number of pixels in X- or Y-direction

- Arithmetic mean deviation of all surface height values S_a

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

- Quadratic mean deviation of all surface height values S_q

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

- Skewness of the distribution of all surface height values S_{SK}

$$S_{SK} = \frac{1}{N_x \cdot N_y \cdot S_q^3} \cdot \sum_{i=1}^{N_x} \cdot \sum_{j=1}^{N_y} \cdot z^3(x_i, y_j)$$

- Kurtosis of the distribution of all surface height values S_{KU}

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

- Maximum peak height S_P

$$S_P = z_{\max} - S_c$$

- Maximum valley depth S_V

$$S_V = S_c - z_{\min}$$

- Maximum roughness depth S_t (= Peak to Valley / PV)

$$-S_t = z_{\max} - z_{\min}$$

maximum height difference of the overall topography.

Classification of topography in 25 equal area elements (rectangles in the 2D mode)

- average roughness depth S_z:

  $$- S_z = \frac{z_{\max 1} - z_{\min 1} + z_{\max 2} - z_{\min 2} + \cdots + z_{\max 25} - z_{\min 25}}{25}$$

  Averaging of R_t-values of all the 25 single area elements. When combined, both parameters provide information about the homogeneity of the surface. Big differences are indicative of pronounced inclination of the overall area or of spikes.

- maximum roughness depth S_{max}:

  $$- S_{\max} = Max\ (z_{\max 1} - z_{\min 1},\ z_{\max 2} - z_{\min 2},\ \cdots,\ z_{\max 25} - z_{\min 25})$$

  maximum of R_t-values of all the 25 single area elements.

☞ Both the roughness parameters and the z-histogram will be influenced by the use of filters!

5.15.23.8 3D Measurement Functions

(1) Volume measurement mode (Flood function)

- Use the 3D button to select the required 3D display of the stack.
- Click on the **Volume** button in the **Measure** button bar.
  - The volume parameters are calculated and displayed below the image.
  - The **Copy** button is displayed below the right-hand side of the image. This button permits the volume values to be copied to the clipboard and imported to other programs (e.g.: MS Word or MS Excel) via the **Paste** function.

- Setting the **Fill Level** slider enables you to change the height level of the topography. The portion of the topography lying below the set height level is filled with "water" (blue color) and the volume parameters are calculated online only for the projecting part of the topography.

☞ To use the **Fill Level** function, load the **Profiles** 3D display mode containing the **Glowscale** palette, or activate **No Palette** to obtain optimum display.

- If the **Diagram** function **Curve of tp** is also activated, a red marker line shows the position of the height level in the percentage of contact area curve.

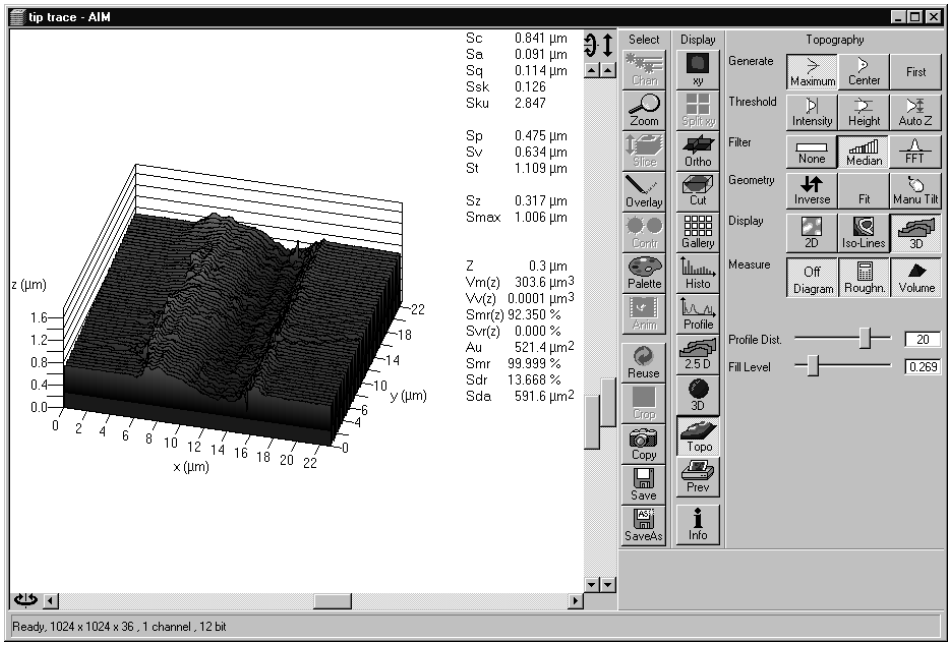


Fig. 5-336 Image Display window, Topography display: 3D - Volume

The following parameters are calculated:

Z: height level (selectable with the **Z-Threshold** and **Fill Level** sliders). The setting of this value influences the following parameters.

Vm (z): material volume above chosen height level

Vv (z): void volume below chosen height level

Smr (z): material volume ratio

$$S_{mr}(z) = \frac{V_m(z)}{V_m(z_{\min})}$$

Svr (z): void volume ratio

$$S_{vr}(z) = \frac{V_v(z)}{V_v(z_{\max})}$$

Au: surface bearing area of the topography at Z (= projection area of those parts which are situated above chosen height level)

Smr: surface bearing area ratio of the topography at Z
percentage of contact area (= Au / (x * y) * 100 %)

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

Sdr: developed surface area ratio:
Σ (surface area$_{ij}$) - Σ (projected area$_{ij}$) / Σ (projected area$_{ij}$) * 100 %
projected area = x * y
The percentage of the 3D surface area (sum of all triangles formed by adjacent data points of the surface reconstruction) to the 2D surface area produced by projecting the 3D surface onto the threshold plane.
absolute flat surface $\Rightarrow$ is equal to base plane (Sdr = 0 %)
The increase by which the 3D surface is larger than the basic plane (e. g. 625 % is a 3D surface which is about 6.25 times larger than the projected basic plane)

(2) Profile measurement mode in 3D display

This function is performed in the same way as in the 2D display mode, with the following exceptions:

The buttons and are replaced with the buttons and . Furthermore, the **Position** slider and the input box (information of the position of the intersection line in pixels) are displayed below the **Table** and **Profile** button bar. Changing the Z-Threshold also results in a change in the profile. In the 3D image, a red marker line shows the y- and x-position of the displayed profile diagram.

red
marker line
(x position)

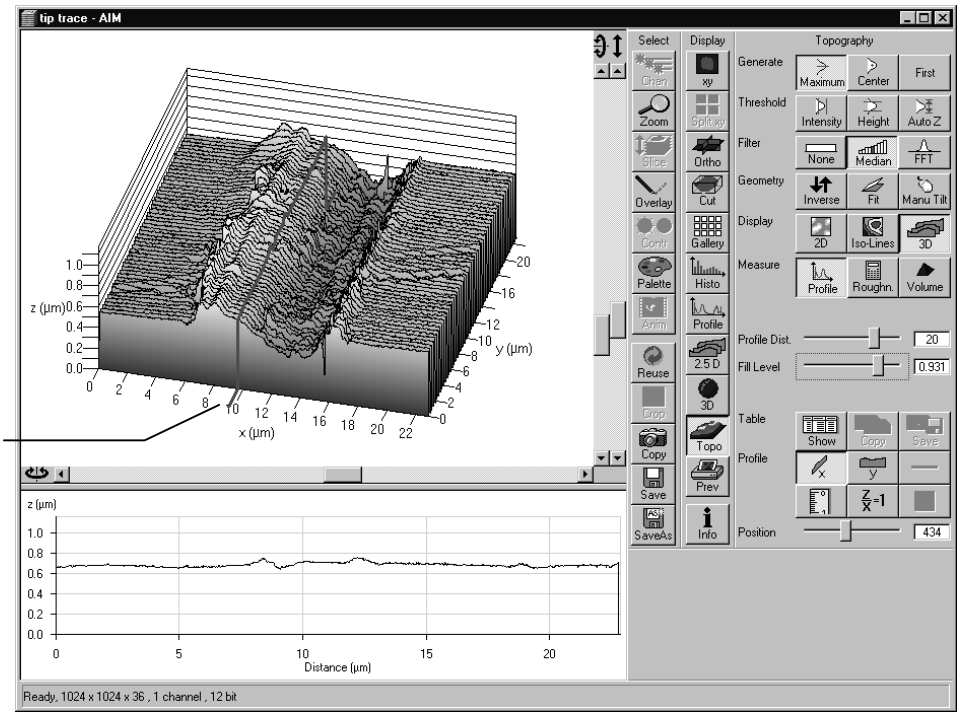


Fig. 5-337 Image Display window, Topography display: 3D - Volume

- The position of the marker line (profile intersection line) can be changed by moving the **Position** slider in x or y.
- Press the **x**- or **y**-button to select the required intersection plane.

(3) z Histo measurement mode in 3D display

This function is performed in the same way as in the 2D display mode.

(4) Curve of tp measurement mode in 3D display

This function is performed in the same way as in the 2D display mode.

Before determination of the tp bearing portion, individual peaks (noise, steep slopes) must be eliminated. The **Median** filter and perhaps a **3x3** longpass filter can be used for this purpose.

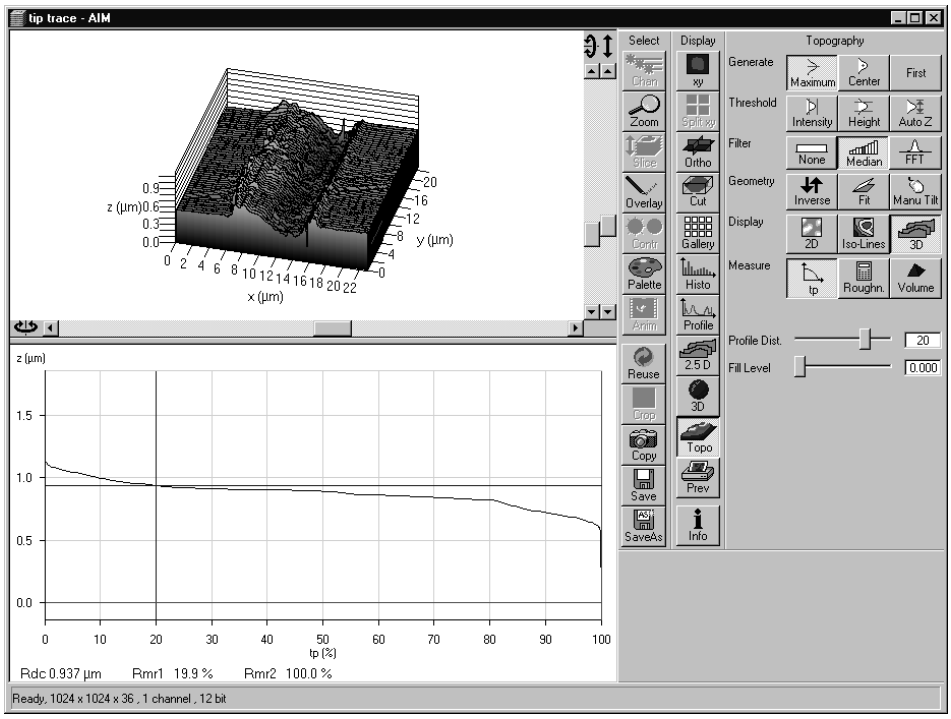


Fig. 5-338 Image Display window, Topography display: 3D – Curve of tp

Shifting the two cursor crosses permits two bearing portions to be given in percent (e.g. Smr1 = 10 %; Smr2 = 90 %) as default values for which the height difference Rdc is determined automatically.

(5) Grad. Histo measurement mode in 3D display

This function is performed in the same way as in the 2D display mode.

(6) Roughness measurement mode in 3D display

This function is performed in the same way as in the 2D display mode.

5.15.23.9 Export data

- multiple profiles (Rel. 3.2)
- single profile
- parameters
- topography as matrix
- topography as triples

5.15.23.10 Topo ReUse

Topo routines can be saved and reloaded as tgp-files (TopoGraphic Parameters).

These files include settings for:

- reconstruction mode,
- intensity threshold,
- filters (including FFT),
- tilt angles (manual, 3 point fit),
- fit procedures (plane, cylinder, sphere),
- inverse and
- fill holes.

5.15.24 Display - Prev.

This function allows to

- compose images, graphs and text for printing
- use any image format
- change fonts and line width in graphs via context sensitive menus

The settings of Chan, Zoom, Slice, Contr and Palette apply.

In the **Options** menu in the function **Settings** with the **Print Status Display** tab parameters are determined and the **Print Status Information** is activated/deactivated.

Click on **Prev** will display the **Preview** window and the **Print** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

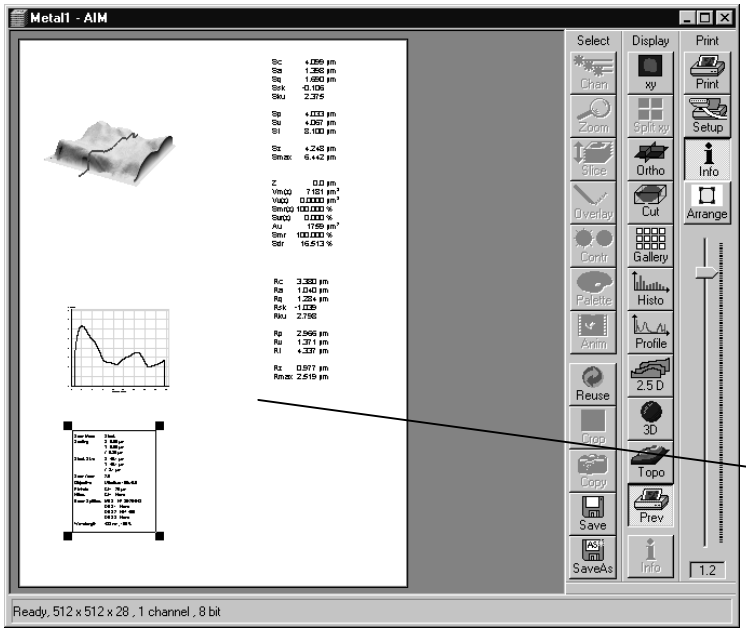


Fig. 5-339 Image Display window, Prev display

(1) Context menu for scan information text

The context menu (right mouse button) allows to vary the output of the **scan info**.

- Click with the right mouse button. A context menu with the options **Color** and **Font** is displayed.
- In the **Color** menu, you can select a different type color for the **scan info**, in the **Font** menu a different type font and type style.

(2) Context menu for Topography Images

When transferring a topography to the print preview, you can change the size and shape of type and scale lines for the 3D graphics and profile measurement results.

(a) Context menu for 3D graphics

- Click on the right mouse button. A context menu with the options **Font enlargement** and **Line width enlargement** is displayed.
- You can change the type size in the **Font enlargement** menu and the line width of the scales in the **Line width enlargement** menu.

(b) Context menu for Profile measurement function

- Click on the right mouse button. A context menu with the options **Scaling font enlargement**, **Marker font enlargement** and **Overlay font enlargement** is displayed.
- You can change the type font in the **Scaling font enlargement** menu, the size of the marker table in the **Marker font enlargement** menu and the type size of the red measurement results in the **Overlay font enlargement**.

(3) Arranging and printing the Print Preview

- Click on the **Arrange** button for optimum layout of image size and position relative to the textual information.
- A layout generated with **Prev.** (Preview) can be printed by clicking on the **Print** button in the **Print** toolbar.
- Clicking on the **Setup** button opens the **Print Setup** window, in which you can specify print parameters.
- Click on the slider to change the zoom value of the selected items.

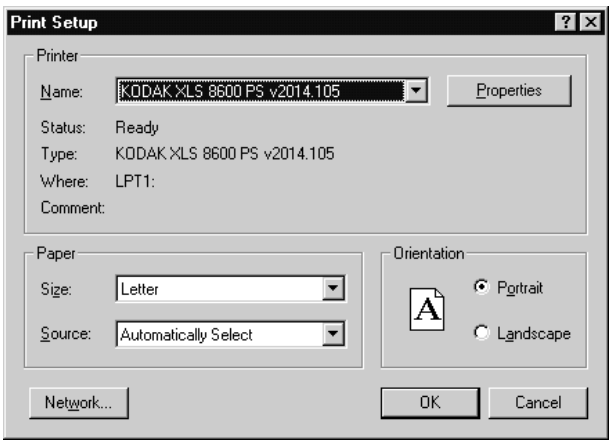


Fig. 5-340 Print Setup window

5.15.25 Display - Info

This function allows to

- display the parameters used during image acquisition of the image(s) displayed in the **Image Display** window
- use any image format
- remove the info display

The settings of **Chan, Zoom, Slice, Contr** and **Palette** are not relevant for this function.

In the **Options** menu in the function **Settings** with the **Image Status Display** tab parameters to shown are determined.

Click on **Info** will show the parameters. Click again to hide the info display.

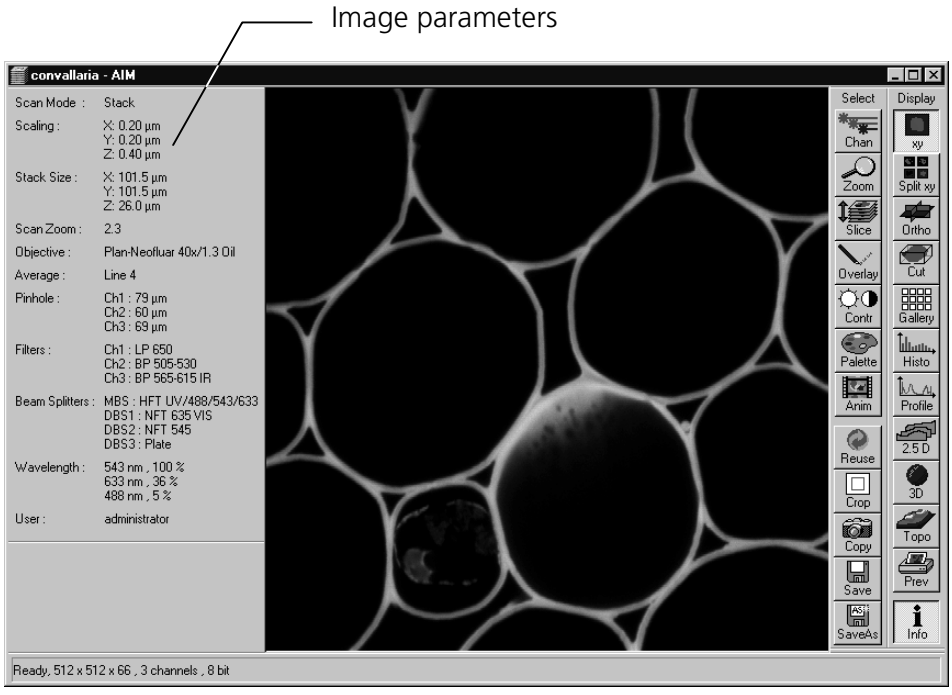


Fig. 5-341 Image Display window, Info display

5.15.26 Additional Display Mode in Time Series

5.15.26.1 Display - Mean

This function allows to

- display the intensity time diagram (mean intensity in user defined ROIs over time)
- use frame time series and frame Z Stack time series as input
- show the intensity values in table form and copy table to clipboard or save as text file
- show separate diagrams for each channel in a multi channel image

The settings of **Chan**, **Zoom**, **Slice**, **Contr** and **Palette** apply.

Click on **Mean** will display the **Mean of ROIs** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

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

- Click on the **Mean ROI** button.
  - The **Mean of ROIs** image display toolbar will be displayed on the right. The used ROIs become visible in the image, and the Intensity-Time diagram is shown on the left of the **Image Display** window.

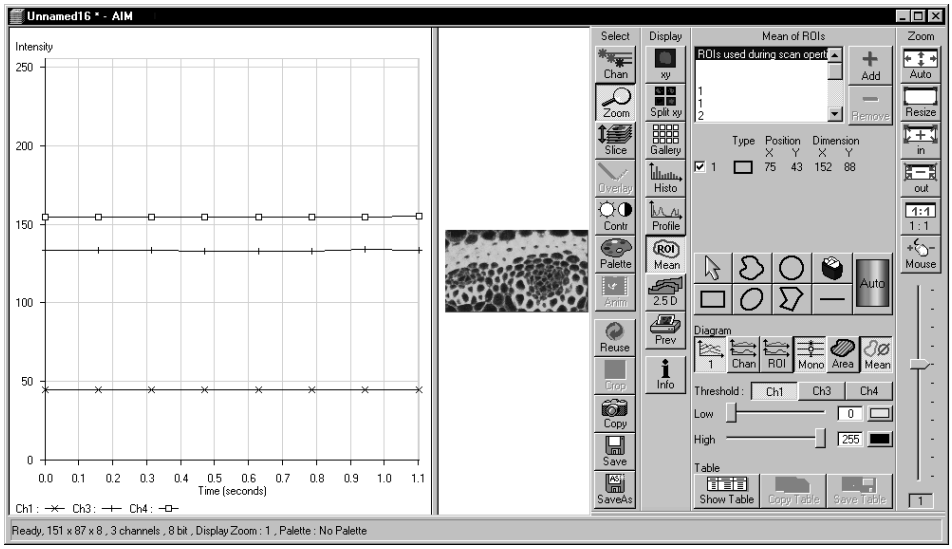
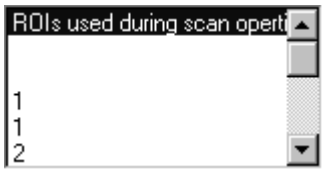


Fig. 5-342 Image Display window, Mean ROI display

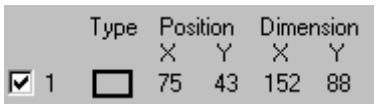
The **Mean of ROIs** toolbar contains the following function elements:



ROIs selection box: Display of the ROIs used during scanning of the time series and of the other ROIs available in the system.

Add button: Opens the **Add ROI List** window for the storage of changed or newly defined ROIs under a new name.

Remove button: Deletes the selected ROI from the ROIs selection box.



ROI data: Display of the data of the ROI selected from the ROIs selection box. On deactivation of the check box of a ROI, its intensity values from the Intensity-Time diagram are not displayed.

Arrow button: Activation of the mouse button for resizing or movement of the ROI in the **Image Display** window.

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

Circle button: Activates the circle drawing mode. Clicking and holding down the mouse button sets the center point; drag the diameter and release the mouse button to end the procedure.

Recycle bin button: All the ROIs to the image are deleted.

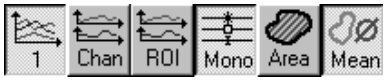
Rectangle button: Activates the rectangle drawing mode. Click and hold down the mouse button, drag the rectangle in any direction, release the mouse button to end the procedure.

Ellipse button: Activates the ellipse drawing mode. The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and the rotation direction can then be determined; the third click sets the second dimension and the direction and ends the procedure.

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

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

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



Buttons for diagram display:

1 button: Intensity values for ROIs and channels are shown in one diagram.

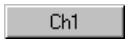
Chan button: Intensity values are shown separately for each channel.

ROI button: Intensity values are shown separately for each ROI.

Mono button: Change between color and monochrome display of the intensity time diagrams.

Area button: Display of the area of the ROI in the intensity time diagram, depending on the set threshold values. Area measurements of very small areas (< 10 pixels) give only approximate values.

Mean button: Display of the mean values of the relevant ROI in the intensity time diagram.



Ch1 / Ch3 / Ch4 button: Selection of the channel to be used.

Threshold low slider: The intensity values below threshold are not displayed for the **Area** function.

Threshold high slider: The intensity values above threshold are not displayed for the **Area** function.

Buttons for Table functions:

Copy Table button: The table of intensity values is copied to the clipboard.

Show Table button: The table of intensity values is displayed on the bottom left of the **Image Display** window.

Save Table button: The table of intensity values can be stored as a text file.

5.15.27 Additional Display Modes in Lambda Mode

The following display modes of a Lambda Stack are available:

- Display - Coded
- Display - Max
- Display - Mean

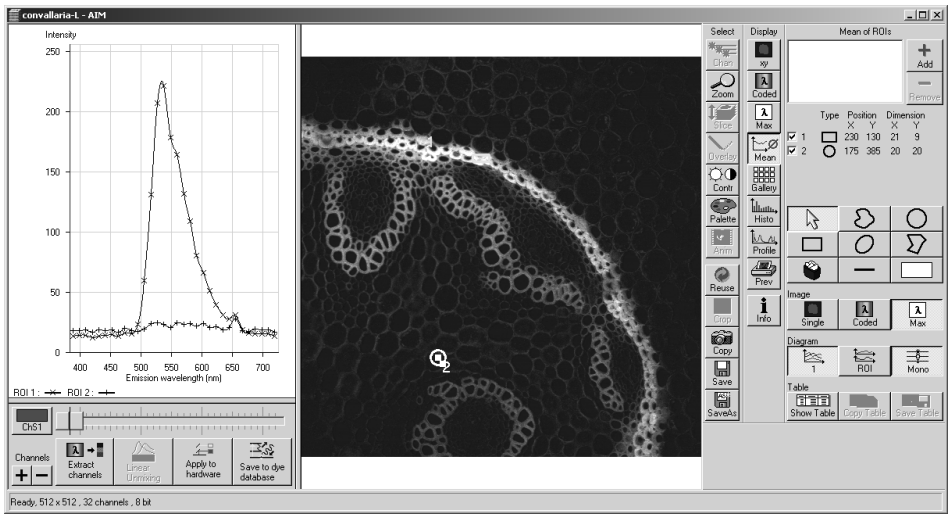


Fig. 5-343 Image Display window of a Lambda Stack, Display - Mean activated

5.15.27.1 Display - Coded

This function allows to

- display a Lambda Stack of images in a coded color view. A color palette is automatically assigned to the individual images which displays the spectral color of the most intense signal of a Lambda Stack

The settings of **Chan, Zoom, Slice** and **Contr** apply. The settings of **Palette** do not apply.

Click on **Coded** will be immediately effective.

5.15.27.2 Display - Max

This function allows to

- display a Lambda Stack of images in a maximum intensity projection.

The settings of **Chan**, **Zoom**, **Slice**, **Contr** and **Palette** apply.

Click on **Max** will be immediately effective.

5.15.27.3 Display - Mean

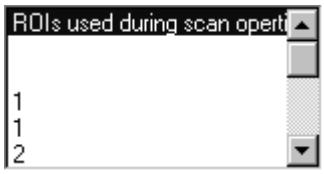
This function allows to

- display the intensity Lambda diagram (mean intensity in user defined ROIs over Lambda)
- use Lambda Stack, Lambda Stack time series, Lambda Stack Z series and Lambda Stack (Z+time) series as input
- show the intensity values in table form and copy table to clipboard or save as text file
- show separate diagrams for each ROI or all in one diagram
- generate unmixed multi channel images

The settings of **Chan**, **Zoom**, **Slice**, **Contr** and **Palette** apply. The settings of **Overlay** do not apply.

Click on **Mean** will display the **Mean of ROIs** toolbar. Any changes done with this toolbar are effective immediately. The content of the overlay plane is temporarily deleted while the toolbar is displayed.

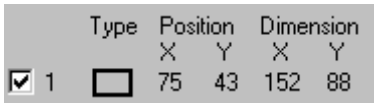
The **Mean of ROIs** toolbar contains the following function elements:



ROIs selection box: Display of the ROIs used during scanning of the time series and of the other ROIs available in the system.

Add button: Opens the **Add ROI List** window for the storage of changed or newly defined ROIs under a new name.

Remove button: Deletes the selected ROI from the ROIs selection box.



ROI data: Display of the data of the ROI selected from the ROIs selection box. On deactivation of the check box of a ROI, its intensity values from the Intensity-Time diagram are not displayed.

Arrow button: Activation of the mouse button for resizing or movement of the ROI in the **Image Display** window.

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

Circle button: Activates the circle drawing mode. Clicking and holding down the mouse button sets the center point; drag the diameter and release the mouse button to end the procedure.

Recycle bin button: All the ROIs to the image are deleted.

Rectangle button: Activates the rectangle drawing mode. Click and hold down the mouse button, drag the rectangle in any direction, release the mouse button to end the procedure.

Ellipse button: Activates the ellipse drawing mode. The first click sets the center point, the displayed line permits the determination of the first dimension, the second click sets the first dimension, the second dimension and the rotation direction can then be determined; the third click sets the second dimension and the direction and ends the procedure.

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

Crosshair button with user-defined spot size for Lambda Stacks.

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

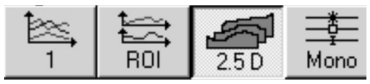
Color / Auto button: One color from the list of colors can be assigned to all ROIs. When **Auto** is pressed, the outlines of all ROIs are automatically colored differently.

Buttons for Image display:

Single button: Displays only a single slice out of a Lambda Stack.

Coded button: Displays the Lambda **Coded** mode (see chapter 0).

Max button: Displays the Lambda **Max** mode (see chapter 5.15.27.2).

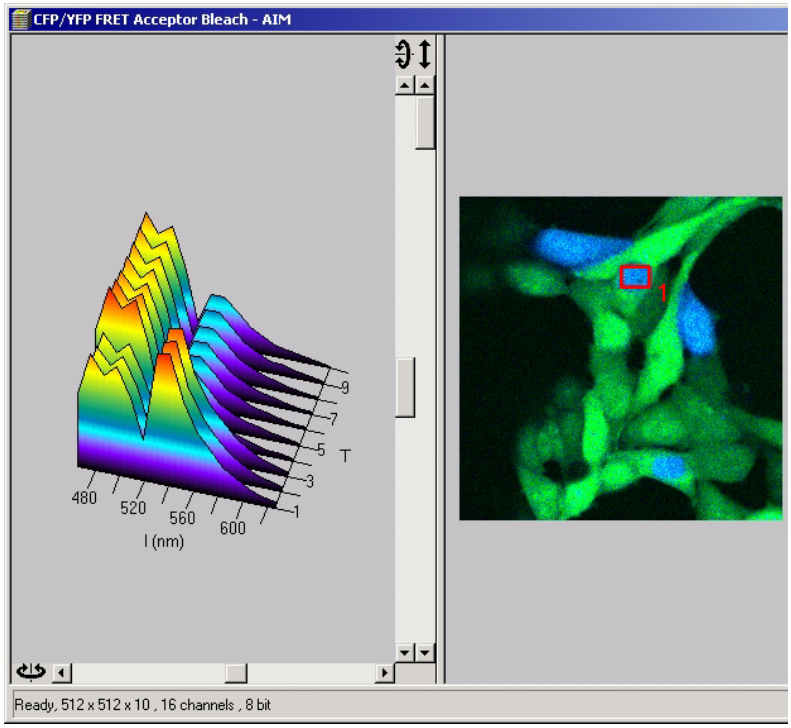


Buttons for diagram display:

1 button: Intensity values for ROIs and channels are shown in one diagram over Lambda.

ROI button: Intensity values are shown separately for each ROI over Lambda.

2.5 D button: 3D graph of intensity, wavelength and time of ROIs in Lambda Stacks.



Mono button: Change between color and monochrome display of the intensity time diagrams over Lambda.

Buttons for Table functions:

Copy Table button: The table of intensity values over Lambda is copied to the clipboard.

Show Table button: The table of intensity values over Lambda is displayed on the bottom left of the **Image Display** window.

Save Table button: The table of intensity values over Lambda can be stored as a text file.

The toolbar below the intensity Lambda diagram has the following functions:

ChS1 button / slider — Selection of a wavelength range (use the sliders) assigned with the spectral channel ChS1. This assignment can be used for scanning (when **Apply to hardware** is pressed, see **Config Control**) and /or for extracting a single or multi channel image from the Lambda Stack (when **Extract channels** is pressed). Clicking on the color box in the ChS buttons opens the color selection box and allows a new color assignment to be made for this channel.

Channel + / – button — Add (+) or reduce (-) the number of channels displayed and available for assignment. Up to 8 channels labeled ChS1, ChS2, ChS3 ... ChS8 are available.

Extract channels button — Generates a new image or multicolor images based on the settings made in the ChS1, ChS2, ChS3 ... ChS8 buttons. Two or more Lambda channels are binned to form the channels ChS1 ... 8. The generated image is displayed in a new **Image Display** window and is no longer a Lambda Stack of images.

Linear Unmixing button — Performs the unmixing with the selected spectra.

Apply to hardware button — Sets the parameters for the META detector used in binning mode as specified in the settings ChS1, ChS2, ChS3 ... ChS8. See **Single Track** and **Multi Track** in **Config Control** for the effect of the action. The **Lambda Stack** setting is not affected by this action.

Save to spectra DB button — The values of the intensity Lambda diagram are saved in a file (extension: *.rls) in the aim/macro/roi directory. Make sure to have displayed only data of single dyes in the intensity Lambda diagram when executing this function.

5.15.27.4 Functionality for Emission Fingerprinting

Extract Channels

- Sum or mean signals from selected detector elements
- Autoscaling of output channels (individually)

Linear Unmixing

- Autoscaling of output channels
- Generate an additional "Channel with Residuals"-> pixel-by-pixel display the difference between fit and original data (for the channel of the Lambda Stack that shows the greatest deviation)
- Ignore negative results
- Ask for ROI characterizing background spectra before Linear Unmixing

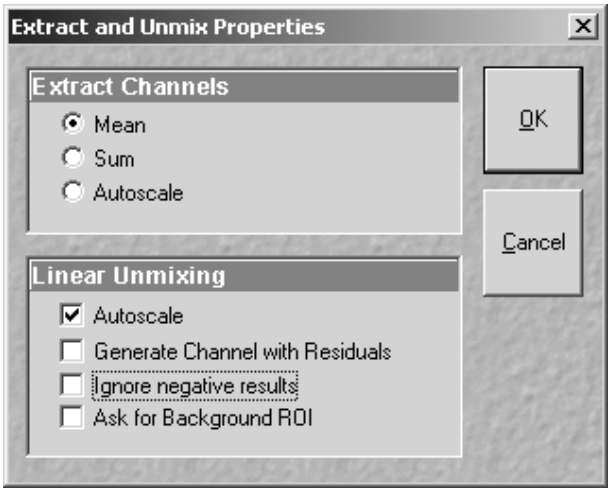


Fig. 5-344 Extract and Unmix Properties window

- **Spectra Display** with option **Normalize** (right mouse click into spectra graph)

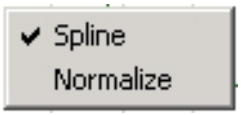


Fig. 5-345 Context menu with Normalize function

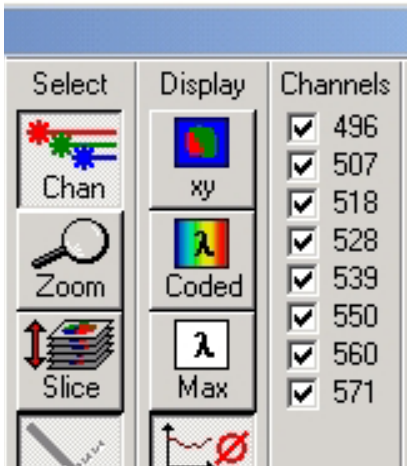


Fig. 5-346 Channels toolbar

- Deselect of individual channels in Lambda Stack affects:
  - Display in **Lambda Coded** and **Lambda max** modes, if **Chan** button in **Select** toolbar activated
  - Linear unmixing; deselected channels are not included

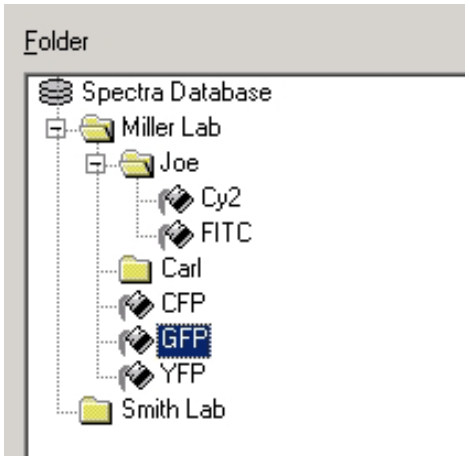


Fig. 5-347 Subfolder of Spectra Database

- Spectra Database with subfolders

5.15.27.5 Automatic Component Extraction

The use of this function permits the automatic search for the individual reference spectra in a lambda stack.

(1) Open / Close the Mean of ROI window

- Click on the **Mean of ROI** button in the image display window.
- Click on another button to select a different function.

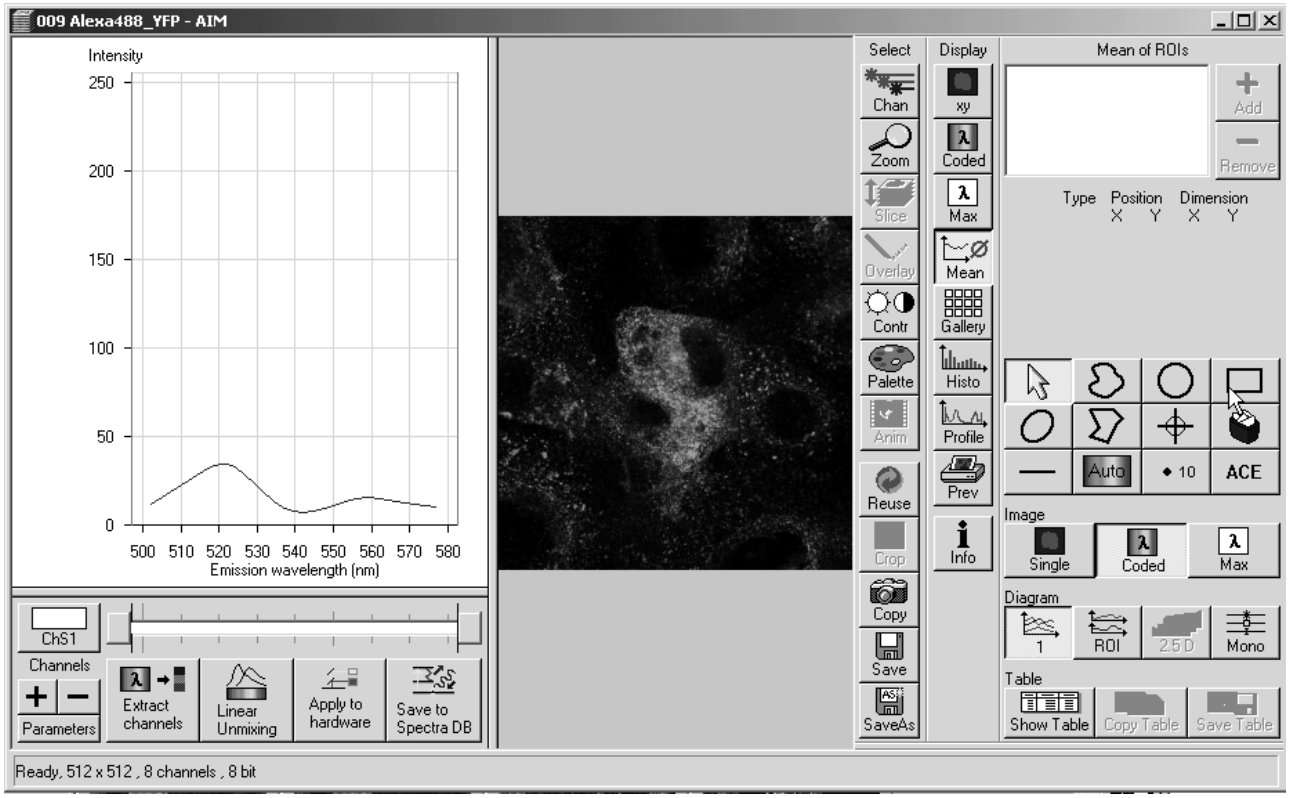


Fig. 5-348 Image Display window of a Lambda Stack, Display - Mean activated

(2) Function description

ACE button	Opens the component number window; after selecting an appropriate number of labels in your sample, press OK.
Spectra checkboxes	Select required spectra. ACE generated spectra and interactively selected spectra can be mixed here.
Drawing Tools	For interactive search and selection of spectra in the image display window.
Linear Unmixing button	Performs the unmixing with the selected spectra.

- Select number of dyes present.
- Click **OK**.
- View results in graph.

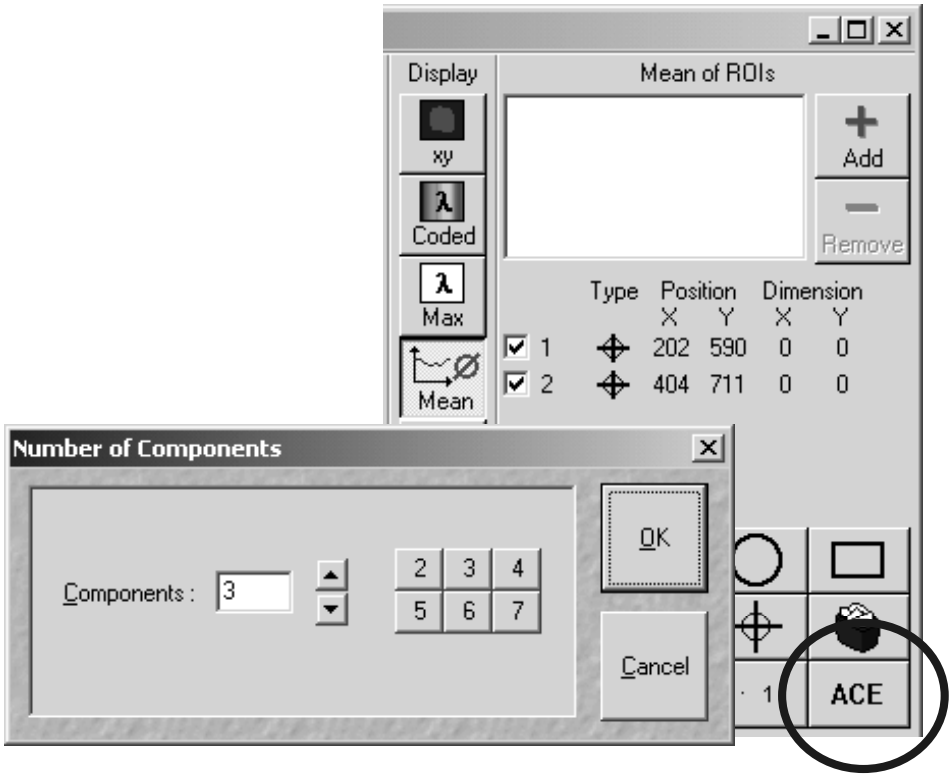


Fig. 5-349 Automatic Component Extraction function

Restriction:	Little to moderate spatial overlap of emission signals
Benefit:	Solution for Emission Fingerprinting in cases where reference spectra are not accessible via single-labeled controls

5.16 Image Optimization

5.16.1 Single Channel

Described below is the example of the acquisition of an image, using an excitation wavelength of 543 nm and a fluorescence emission range above 560 nm. The HFT 488/543 is used as the main dichroic beam splitter.

Let the specimen be a thin section through a stem of Convallaria majalis (Lily-of-the-Valley).

5.16.1.1 Requirements

- The suitable laser is switched on.
- The specimen has been positioned and focused for scanning.
- Click on the **LSM** button in the **Acquire** subordinate toolbar of the **Main** menu. The tube slider on the microscope tube is in the **LSM** position and the **LSM** button is activated.
- Click on the **Config** button in the **Acquire** subordinate toolbar of the **Main** menu.
  - This opens the **Configuration Control** window.
- Click on the **Single Track** button.
- Click on the  **Ch3** icon and assign a color to Channel 3 in the **Channel Color Selection** window. Activate channel 3 via check box.
- Click on the  icon of emission filter 3 (before Ch3) and select the **LP 560** filter.
- If required, deactivate all the other channels (ChS, Ch2, 4, monitor diode, transmission, R1-2) via check box.
- Click on the  icon of the main dichroic beam splitter and select **HFT 488/543**.

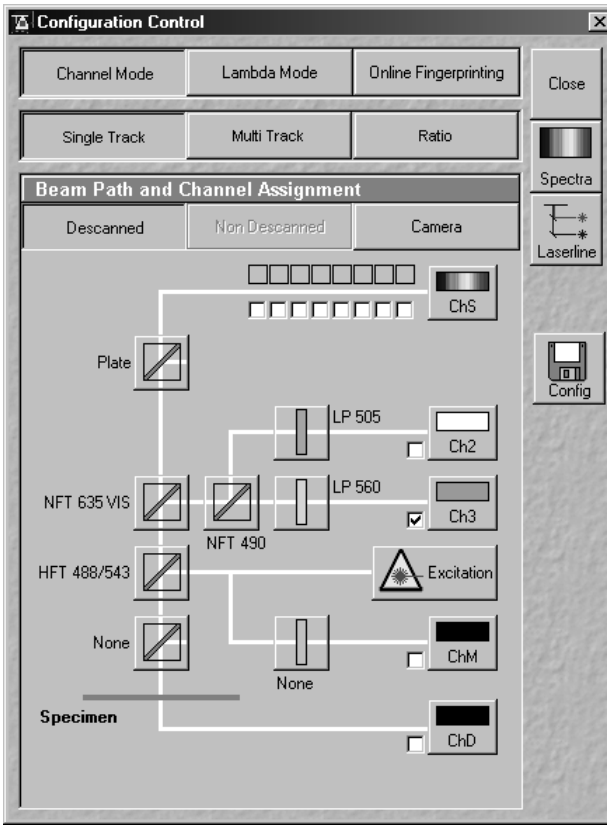


Fig. 5-350 Configuration Control window

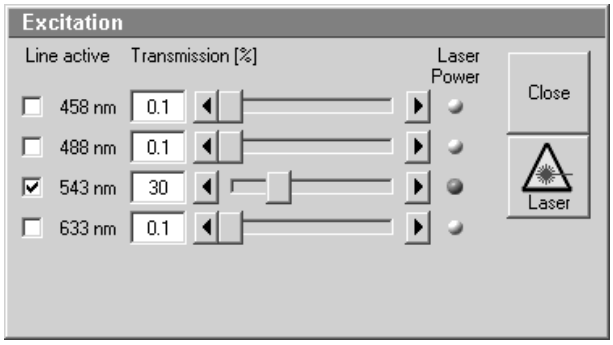


Fig. 5-351 Excitation panel

- Click on the Excitation icon, activate the **543 nm** laser line and click on **Line Active** ☑. If required, deactivate other laser lines which are not needed.
- Use the **Transmission** slider to set the laser intensity to approx. 30 % at first.

The **Beam Path and Channel Assignment** panel displays the current configuration loaded.

☞ The set laser intensity must be subsequently optimized for the current situation via the **Transmission** slider.

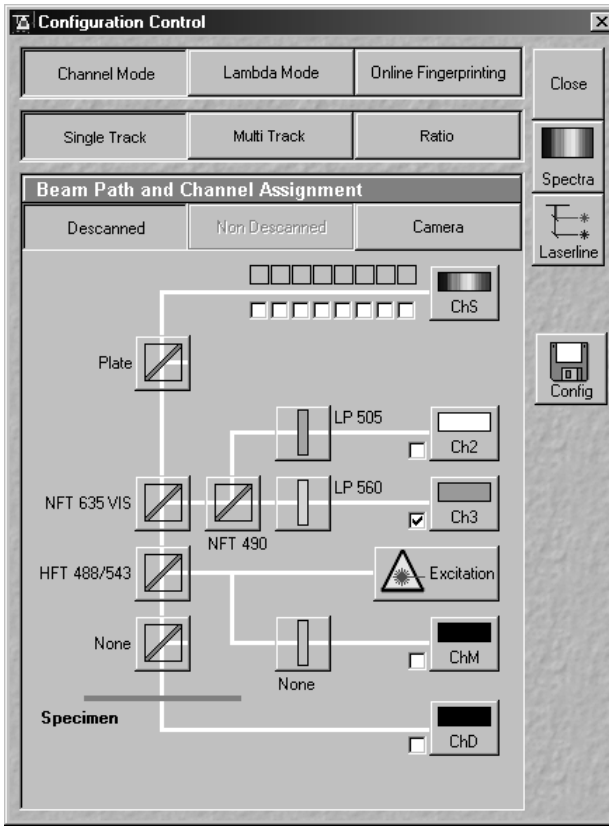


Fig. 5-352 Configuration Control window

For overlaying fluorescence and transmitted-light images, click on the **ChD** (Transmission) button in the **Beam Path and Channel Assignment** panel.

The transmitted light PMT photomultiplier will be activated.

Of course, all other transmitted light applications like

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

can also be performed.

☞ For the generation of images in reflection, the main dichroic beam splitter must be a neutral-density filter.

Standard equipment contains a neutral-density filter with a division ratio of 80 to 20 % (at 543 nm).

- In the **Main** menu click on the **Scan** button in the **Acquire** subordinate toolbar.
  - This opens the **Scan Control** window.
- Click on the **Mode** button.
- For a frame scan, click on the **Frame** button.
- On the **Objective Lens & Image Size** panel, select Objective and Frame size for the scan (e.g. X 512 / Y 512 scan).
- On the **Speed** panel, enter a scanning speed of 7, for example, to start with.
- Start with the following settings on the **Pixel Depth, Scan Direction & Scan Average** panel:

  Data depth: 8 bits
  Scan direction: → unidirectional
  Average: Number: 1
- On the **Zoom, Rotation & Offset** panel, set a zoom of 1 and a rotation of 0.

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

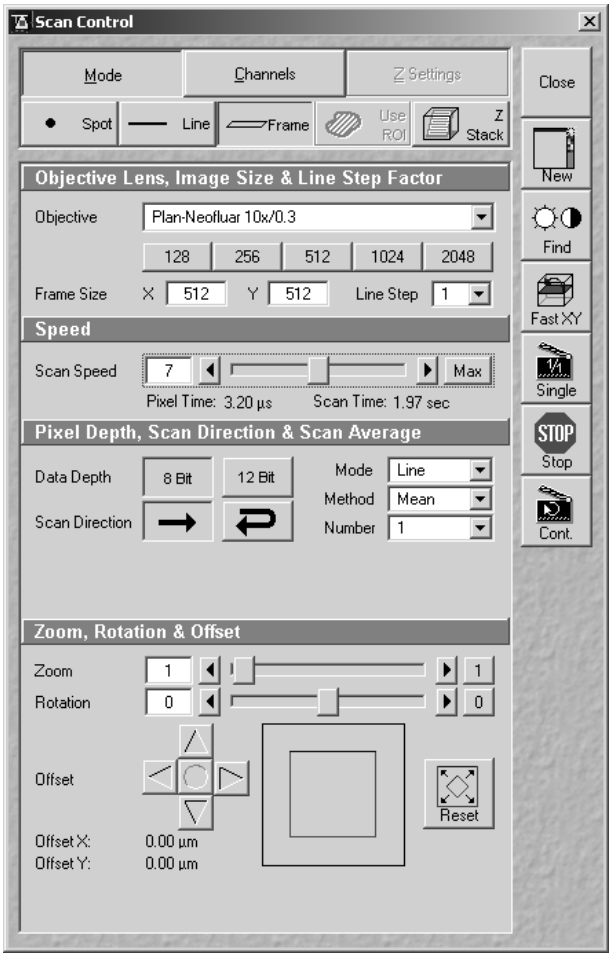


Fig. 5-353 Scan Control window (Mode)

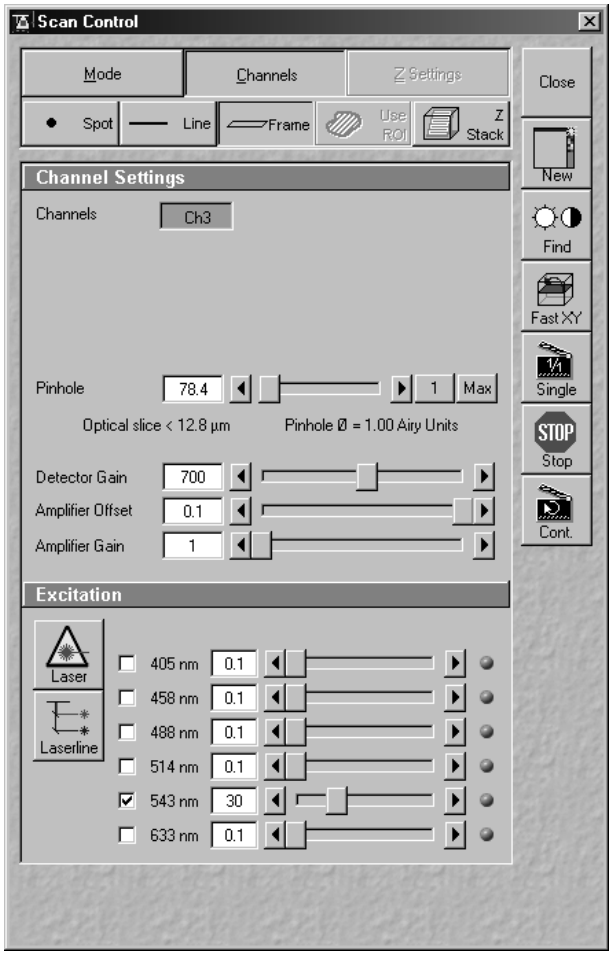


Fig. 5-354 Scan Control window (Channels)

- Click on the **Channels** button.
  - This displays the preset parameters of the configuration loaded.

- Click on the **Find** button. Make sure to position the slider correctly. Then scan while the slider is in the LSM position.
  - This starts the scanning process.
  - The image is seen to build up gradually in a new window.

☞ Function **Find** produces images of different brightness for different scan speeds.

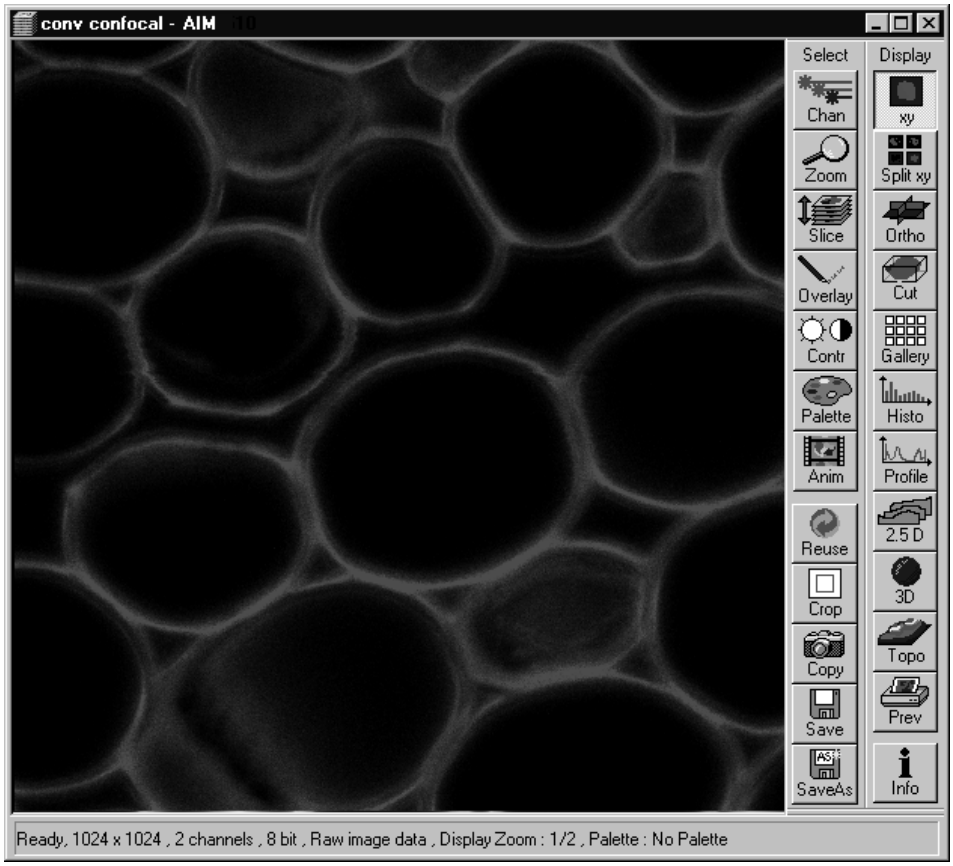


Fig. 5-355 Image Display window

As a rule, the first scanned image (Pre-Scan) is not ideal, since the photomultiplier is not matched to the light output. More often than not, the screen image is dull and needs subsequent optimization.

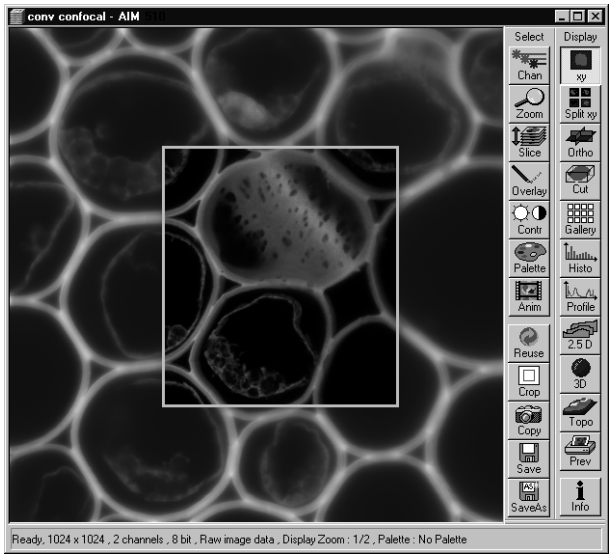


Fig. 5-356 Image Display window with confocal ROI

5.16.1.2 Pinhole / Detector Gain / Ampl. Offset / Ampl. Gain

- In the **Scan Control** window, click on the **Cont.** button (see Fig. 5-354).
  - This starts a continuous scan.
- Use the **Pinhole** slider to set the pinhole diameter in the **Scan Control** window under **Channels**.
  - The pinhole diameter should be so small that there is still enough variation for the setting of the detector gain and that sufficient image information is still available. 1 Airy is a good value to enable a confocal fluorescence XY-image to be obtained.
  - A small pinhole diameter will increase the depth of focus, but reduce the light intensity received by the PMT photomultiplier (for reflection mode confocal images start with a pinhole value of 0.5 Airy Units).
  - The influence of the pinhole diameter on image creation is shown by the example in Fig. 5-356. The entire image was first scanned with too large a pinhole diameter. The pinhole diameter was then optimized for a defined ROI. This considerably improved the display of the specimen structures.

- Click on the **Palette** button in the **Select** image processing toolbar.
  - This opens the **Color Palette** window.
- In the **Color Palette List** panel, click on the Range Indicator item.
  - The scanned image appears in a false-color presentation.

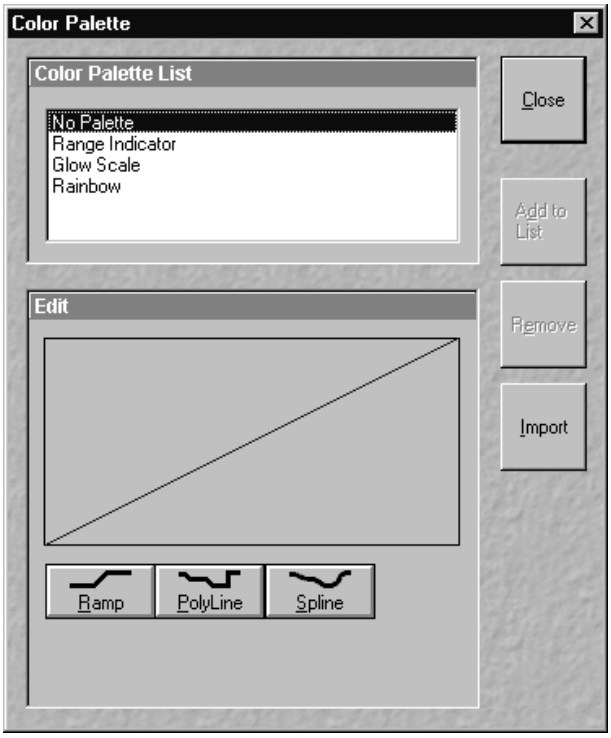


Fig. 5-357 Color Palette window

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

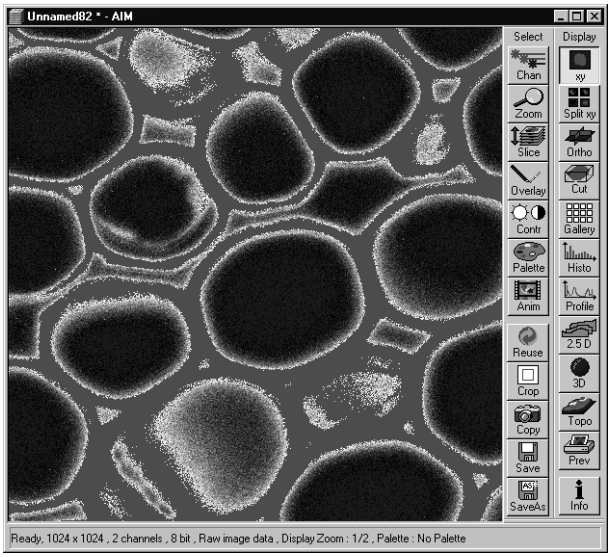


Fig. 5-358 Image Display window

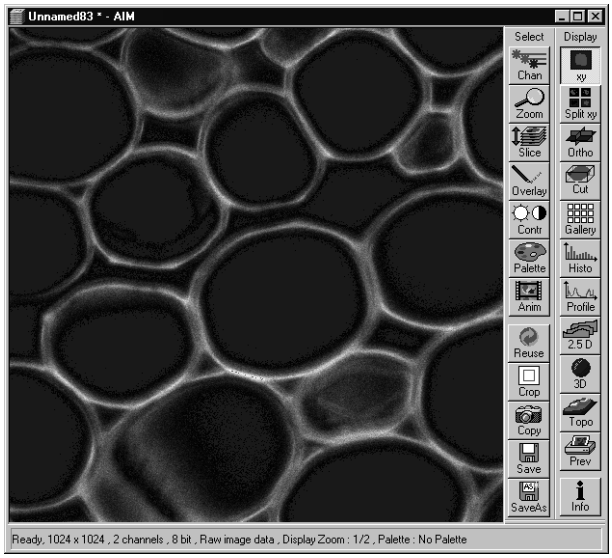


Fig. 5-359 Image Display window

If the image is not bright enough, it appears blue on the screen.

- On the **Channel Settings** panel of the **Scan Control** window, set the PMT (photomultiplier) gain with the **Detector Gain** slider.
  - The image should not have more than a trace of red.
  - This adjustment is very sensitive. Try using the left and right arrows to make the adjustment instead of dragging the slider bar.

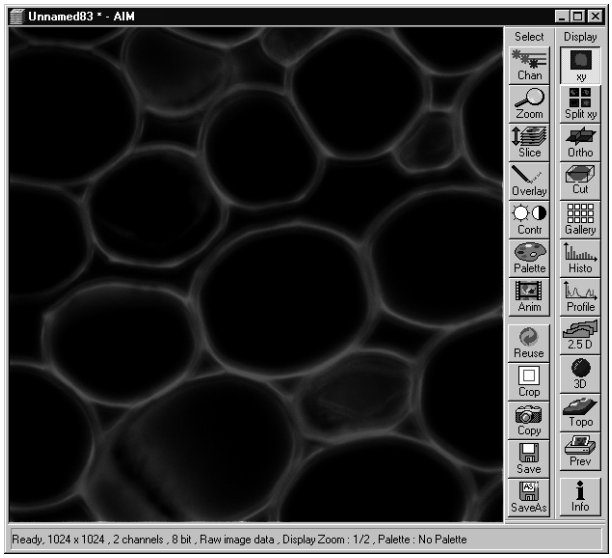


Fig. 5-360 Image Display window

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

☞ Do not change the **Ampl. Gain** setting unless the settings made so far are insufficient for image optimization.

- In the Color Palette List panel of the **Color Palette** window, click on **No Palette**.
  - This deselects the **Range Indicator** and activates the new presentation.
- In the **Scan Control** window, click on the **Stop** button.
  - This stops the continuous scan.

☞ If you use the **Range Indicator** for image optimization, it may happen that the ranges marked in the **Range Indicator** will vary when the channel color is changed.

5.16.1.3 Scan Speed, Scan Average and Pixel Depth

The signal-to-noise ratio can be substantially improved by reducing the scanning speed to an acceptable level and averaging over several scans (i.e. with an average **Number** greater than 1 for the **Mean** average **Method** in the **Scan Control** window).

- Use the **Scan Speed** slider in the **Speed** panel to set the slowest acceptable scanning speed.
  - The corresponding pixel scanning time (Pixel Time) and the total scanning time (Scan Time) are shown in the dialog box.
- In the **Number** text box of the **Pixel Depth, Scan Direction & Scan Average** panel enter the number of measurements to be averaged.

☞ Image optimization can be effected much faster if you select a smaller frame, since less data have to be processed.

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

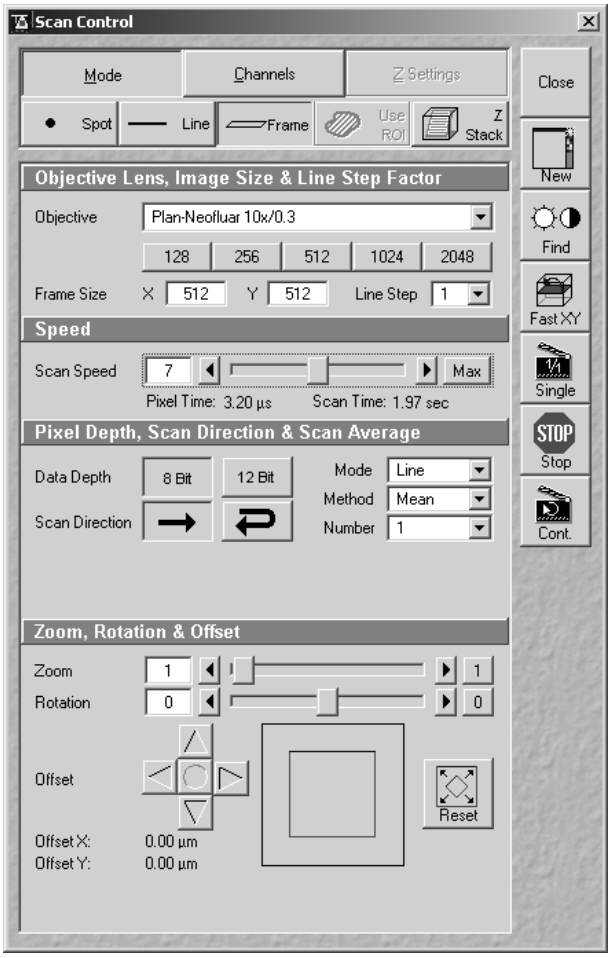


Fig. 5-361 Scan Control window

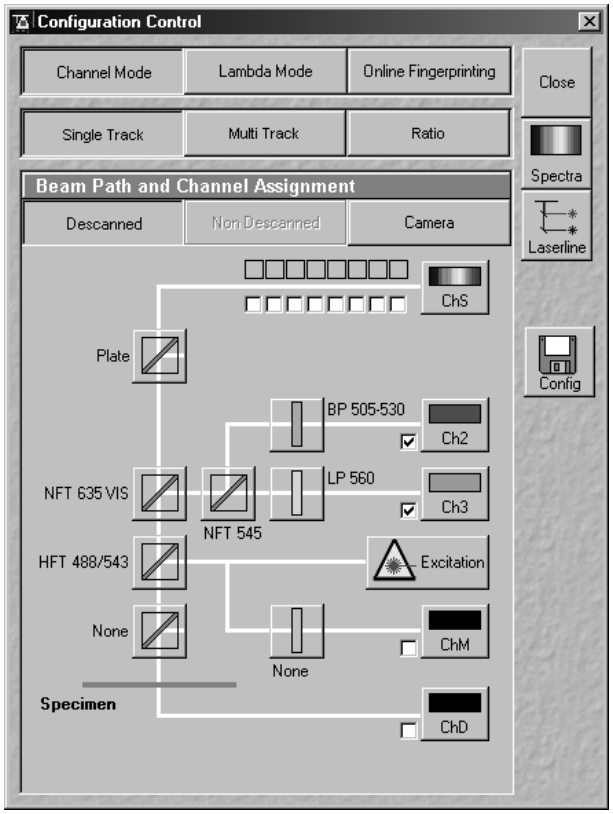


Fig. 5-362 Configuration Control window

5.16.2 Multiple-channel

5.16.2.1 Requirements

- The suitable lasers are on.
- The specimen has been positioned and focused for scanning.
- Click on the **LSM** button in the **Acquire** subordinate toolbar of the **Main** menu. The tube slider on the microscope tube is in the **LSM** position and the **LSM** button is activated.

In the following example, 2 Channels shall be activated for the scanning procedure: one for 488 nm using emission filter BP 505-530 and one for 543 nm with LP 560. HFT 488/543 is used as the main dichroic beam splitter, and NFT 635 VIS and NFT 545 as the secondary dichroic beam splitter.

- In the **Acquire** subordinate toolbar, click on the **Config** button.
  - This opens the **Configuration Control** window.
- Click on the **Single Track** button.
- Activate (in the same way as for the single channel, see page 5-387) channel 2 and channel 3 (Ch2, Ch3), the indicated emission filters and the main and secondary dichroic beam splitter for the scanning procedure.
  - The configuration loaded is displayed in the **Beam Path and Channel Assignment** panel.
- Click on the **Scan** button in the **Acquire** subordinate toolbar of the **Main** menu.
  - This opens the **Scan Control** window.
- In the **Scan Control** window, set the parameters in the same way as described for single-channel presentation.
- Click on the **Find** button in the **Scan Control** window.
  - This starts the scanning process. The scanned image appears in a separate window.

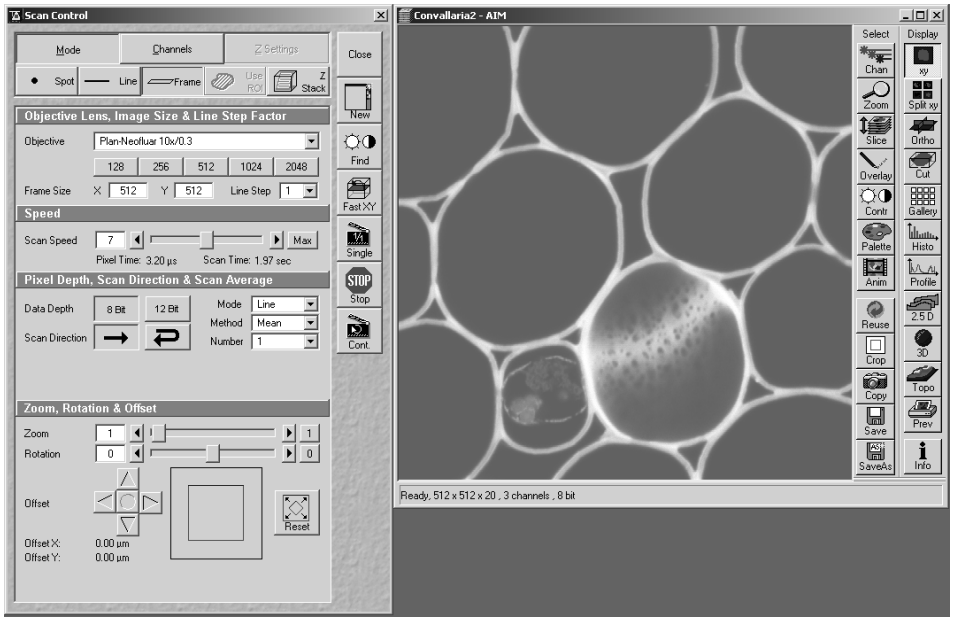


Fig. 5-363 Scan control and Image Display windows

As a rule, the first scanned image (Pre-Scan) is not ideal, since the photomultiplier is not matched to the light output. More often than not, the screen image is dull and needs subsequent optimization.

- Click on the **Channels** button in the **Scan Control** window.
  - This opens the Channel Settings and Excitation of Track panels.
  - The channels used are color-highlighted.

5.16.2.2 Image Optimization

The image optimization processes

- setting of pinhole diameter
- Detector Gain / Ampl. Offset / Ampl. Gain
- Scanning speed and Average

must be carried out separately for each channel used (see section **(1) Single channel**, page 5-387).

For the optimum setting of the single channels, **Split xy**-display must be selected in the **Image Display** window to enable the direct viewing of the separate images of the relevant channels.

- Click on the **Cont.** button in the **Scan Control** window.
  - This starts a continuous scan.

- Click on the **Split xy** button in the **Image Display** window toolbar.
  - This displays the separate images scanned in the channels and the composite (overlay) image.
- Now click on the **Ch2** button in the **Channel Settings** panel to optimize Channel 1. Optimization is performed in the same way as for the single channel and can be monitored online in the relevant separate image of the channel.
- Then optimize the second channel by clicking on the relevant button (**Ch3**) in the **Channel Settings** panel of the **Scan Control** window.

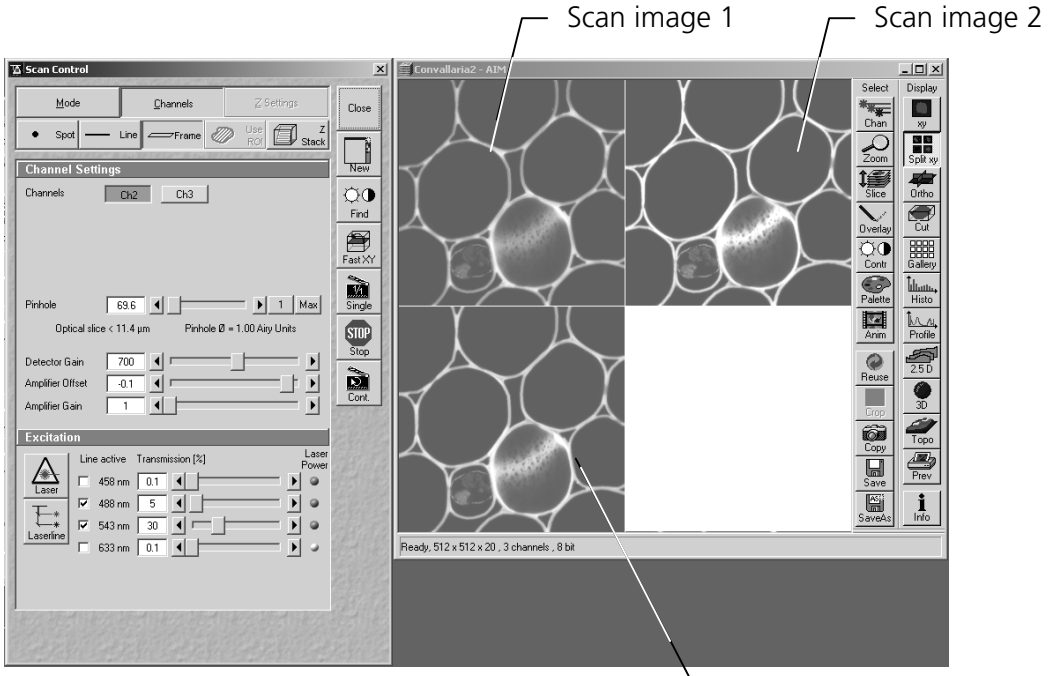


Fig. 5-364 Scan Control and Image Display windows

Now effect image optimization as explained for the single-channel mode, but separately for each channel.

- Now click on the **xy** button of the **Display** toolbar.
  - The composite scan image of two channels is presented in a common window.

☞ Image optimization can be effected much faster if you select a smaller frame, since less data have to be processed.

5.17 Data Evaluation and Result Presentation for FCS Measurements

5.17.1 Structure of the Data Evaluation Window

The data evaluation window (Fig. 5-365) 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).

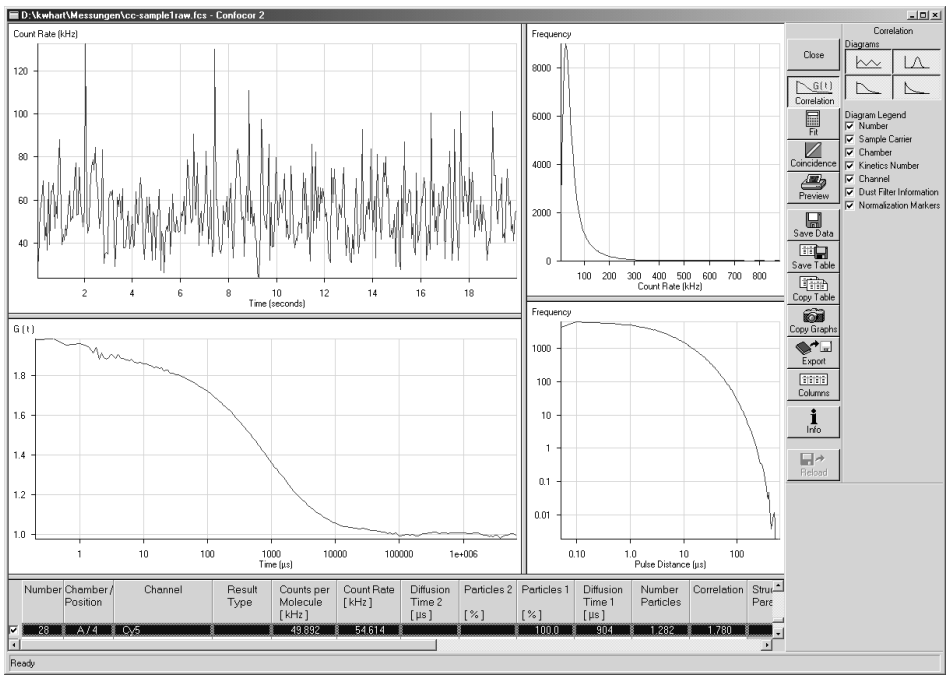


Fig. 5-365 Data evaluation window

5.17.2 Open / Close the Data Evaluation Window

- Click **Start** either from the **Optimize Method** or from the **Measure Method** window, or open an existing data file from the database.
  - The **Data Evaluation** window will appear (Fig. 5-365).

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 under **Options FCS Settings** in **Open / Save** tab the average of the correlation or the average of the fit results are shown in the last row.
- ConfoCor2 data files (*.fcs). Each data file will be opened in a separate **Data evaluation** window.
- ConfoCor2 raw data files: Each file will be opened in a different **Data evaluation** window. If you open a raw data file the **Options** menu for loading raw data files will appear (see Fig. 5-366). You have several options to open raw data files.

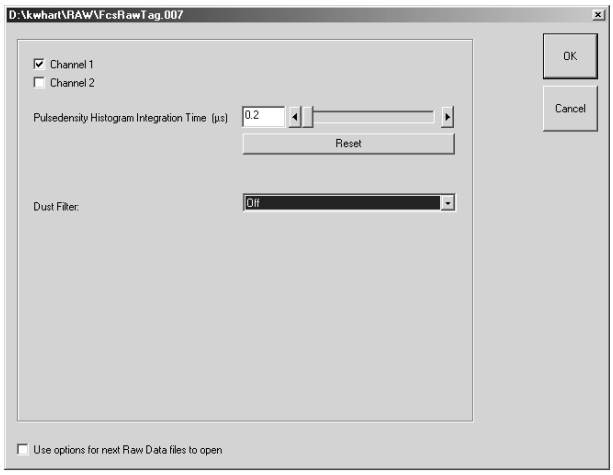


Fig. 5-366 The Options menu for loading raw data files with dust filter inactivated

- You have the possibility to set the integration time for display in the Count Rate histogram by typing in or setting with the slider the bin time in µs in the **Pulsedensity Histogram Integration Time (µs)** display box. The **Reset** button returns the setting to the default value of 0.2 µs.
- You have the possibility to select the channels you want to have displayed by checking the corresponding **Channel 1** and **Channel 2** boxes, respectively. If both channels are activated and you open up an one channel autocorrelation data file, only the respective channel will be displayed.
- You can check the **Use options for next Raw Data files to open** box to automatically select the set options the next time you load raw data.
- You can apply an electronic dust filter in the **Dust Filter** display box in the following ways:
  - Selection of **Off** (see Fig. 5-366) will inactivate the dust filter.

– Selection of **Auto** (see Fig. 5-367) will allow you to set a threshold intensity in the **Dust Filter Threshold Intensity** display box by typing in a value or selecting a specific value with the slider. The value (0 … 100) will define the threshold in percentage. If now 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.

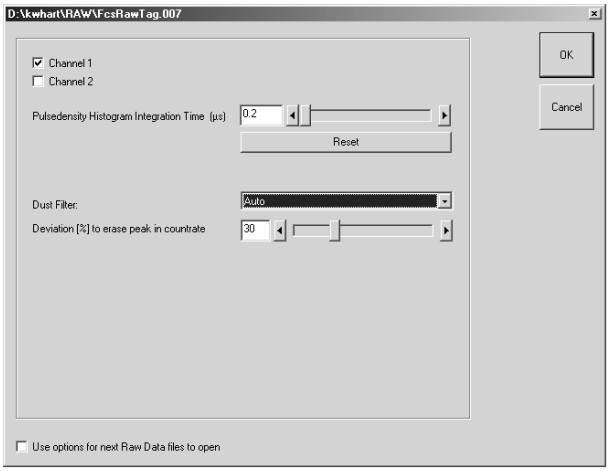


Fig. 5-367 The Options menu for loading raw data files with dust filter set to automatic

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 **Auto** 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 greyed in the Count Rate window.

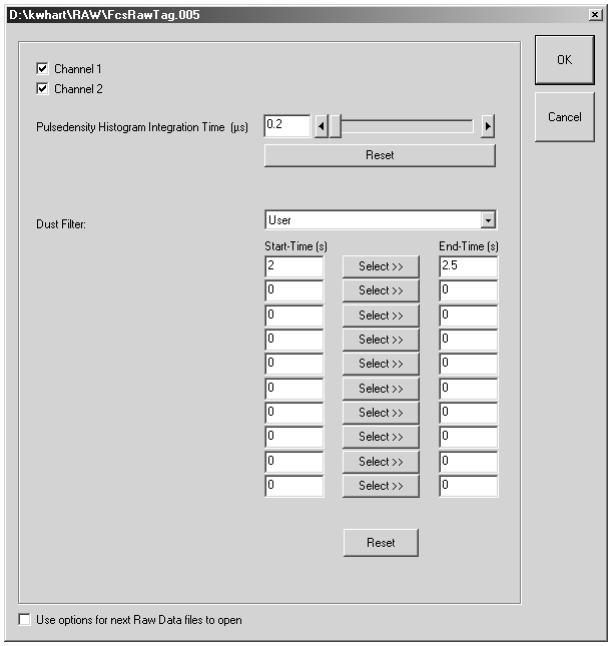


Fig. 5-368 The Options menu for loading raw data files with dust filter set to user defined

– Selection of **User** (see Fig. 5-368) will give you the possibility to select up to ten regions that should be discarded for correlation analysis (for auto and cross-correlation). Type in the respective start times and end times in s of the regions you want to discard. To this end it is recommended to open the raw data with the dust filter set to **Off**, look for the peaks you want to cut out, and reopen the same raw data file by pressing the **Reload** button. Type in the corresponding boundery values and click ok. Alternatively, press **Select**, click with the left mouse button in the old opened file and position the two black bars to frame the region that you want to discard. Press **Select** again, which will display the position of the boundaries. Click ok and the diagram will reopen. The discarded regions will appear greyed out (see Fig. 5-369, 5-370). The regions taking into account and separated by discarded regions will be separately correlated and the average will be displayed. The **Reset** button will reset all typed in values to zero.

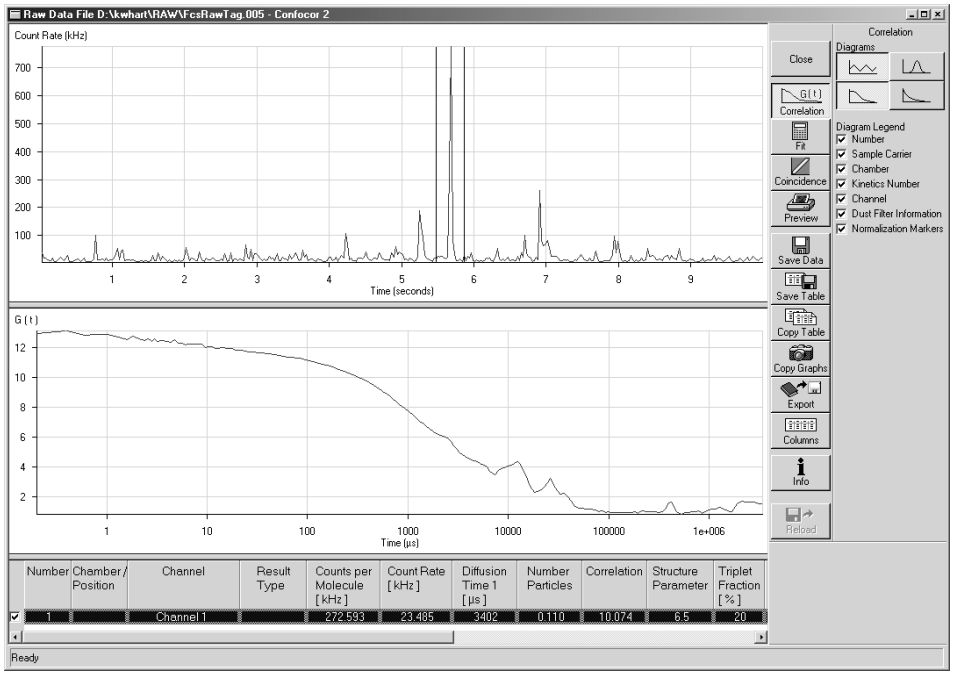


Fig. 5-369 The Count Rate Trace window with region of interest framed

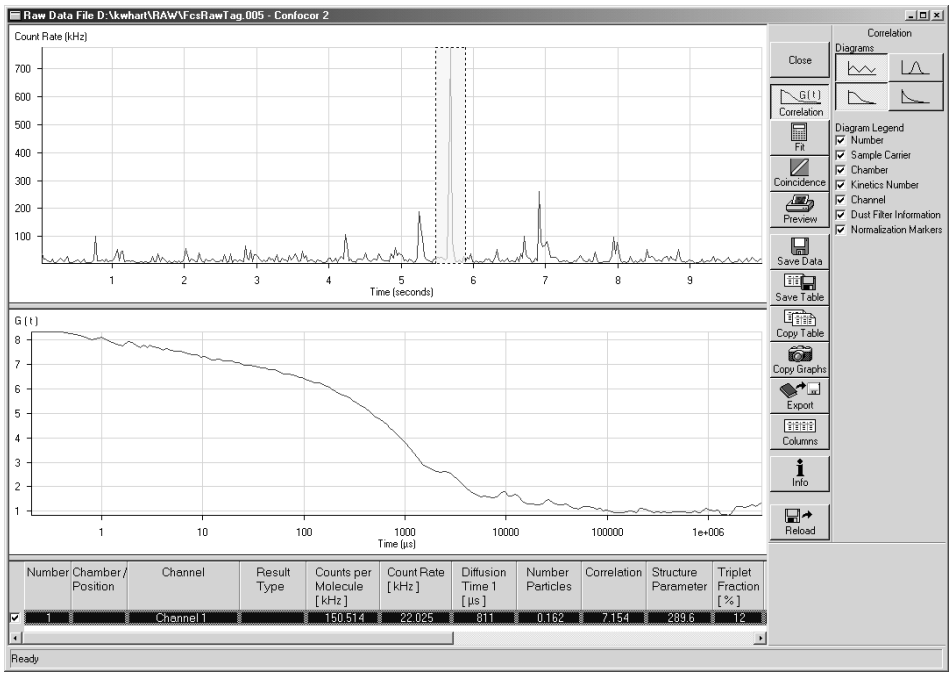


Fig. 5-370 The Count Rate Trace window with region of interest removed

The **Display** toolbar on the right allows you to select additional data post-processing actions. These will be described in detail in the following sections.

- When you are finished, close the **Data evaluation** window by clicking the **Close** button in the **Display** toolbar.

5.17.2.1 Description of the Raw Data Format

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.

				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 1

The following words will be recorded:

- at W1:
  high byte: 00011001(bin) = 19 (hex); low byte: 123 (dec) = 7B (hex); resulting word = 197B (hex)
- at W2:
  high byte: 00000000(bin) = 00 (hex); low byte: 255 (dec) = FF (hex); resulting word = 00FF (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

Example 2

The following words will be recorded:

- at W3:
  high byte: 00010001(bin) = 11 (hex); low byte: 123 (dec) = 7B (hex); resulting word = 117B (hex)
- at W4:
  high byte: 00100100(bin) = 24 (hex); low byte: 255 (dec) = FF (hex); resulting word = 24FF (hex)

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.

5.17.3 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. 5-365).

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.

The appearance of the table can be changed with regard to columns to be displayed and colors of certain areas by using the **Columns** function (see section 5.17.12, page 5-423).

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

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 under the **Method Optimisation** window in **Result Handling** or loaded Raw data files with an electronic dust filter activated the cut off regions will be displayed as greyed out areas if the **Dust Filter Information** check box in the **Display – Column** is checked. 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.

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. By clicking the right mouse button you can choose between displays. Selection of **Use fit limits to restrict view range** will display only the part of the correlation curve that is used for fitting as determined in the **Fit** window by the **Start** and **End** channels, respectively (see Fig. 5-372). Selection of this option will automatically activate this form of display for the Fit diagram as well. Selection of **Normalized View** will display all checked correlation curves with two defined values normalized to 1 and 2, respectively (see Fig. 5-373). The position of the bar more to the right defines the correlation time $\tau_{n1}$to which the corresponding correlation values will be normalized to 1. The difference of $G(\tau_{n1})$ to 1 will than be added to all the other values. Therefore $G(\tau)_{new} = G(\tau)_{old} + (1-G(\tau_{n1}))$. The position of the bar more to the left defines the correlation time for which the correlation value will be normalized to 2. All other values (substracted 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 $G(\tau)_{new}=[1/ (G(\tau_{n2})-1) * (G(\tau)_{old} -1)]+1$. The bars are only visible if the **Normalization Markers** box in the **Display – Column** is checked. Selection of none of these options will display the normal correlation curve (see Fig. 5-371)

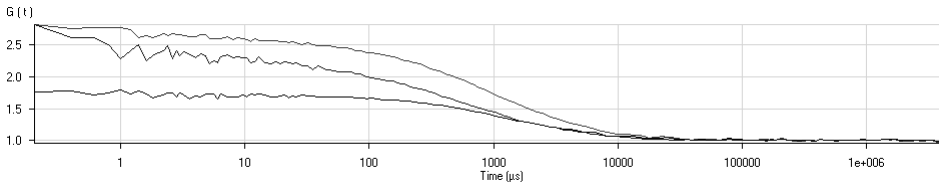


Fig. 5-371 The Correlation Curve window in normal display

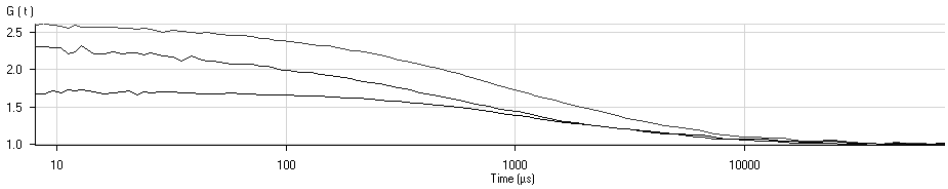


Fig. 5-372 The Correlation Curve window in restricted display

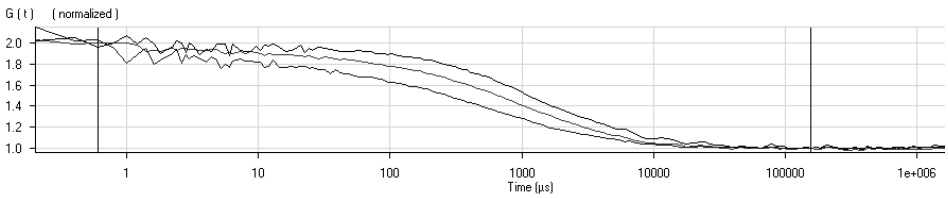


Fig. 5-373 The Correlation Curve window in normalized display

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. If you load a raw data file, you can determine the size of the time window (binning time in µs) by changing the default value of 0.2 µs in the **Pulsedensity Histogram Integration Time (µs)** display box of the **Options** menu for loading raw data files.

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.

Number check box — When this box is activated, the number of the measurement is displayed as a legend for the measurements shown in the diagram.

Sample Carrier check box — When this box is activated, the type of the sample carrier of the measurements shown in the diagram is displayed as a legend.

Chamber check box — When this box is activated, the name of the chamber is displayed as a legend for the measurements shown in the diagram.

Kinetics Number check box — When this box is activated, the kinetics number is displayed as a legend for the measurements shown in the diagram.

Channel check box — When this box is activated, the name of the channel is displayed as a legend for the measurements shown in the diagram.

Dust Filter Information check box — When this box is activated, the boxes defining the cut off regions in the intensity trace windows will be displayed.

Normalization Markers check box — When this box is activated, the normalization markers in the correlation curve if in the normalization view mode will be displayed.

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.

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.

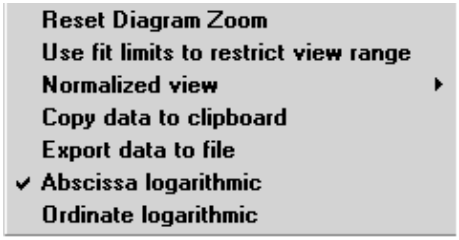


Fig. 5-374 Context menu of the Correlation diagrams

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 **Abcissa logarithmic** will scale the x-axis logarithmically.
  - Selection of **Ordinate logarithmic** will scale the y-axis logarithmically.
  - Selection of **Use fit limits to restrict view range** will display only the part of the correlation curve that is used for fitting.
  - Selection of **Normalized View** will give you a context submenu from which you can choose the following options (see Fig. 5-375):

Fig. 5-375 Subcontext menu of the Normalized view

  - ✓ Select **Off** to inactivate the normalization of the curve
  - ✓ Select **First Marker (on 2.0)** to normalize the correlation curve at the position of the black bar to 2.
  - ✓ Select **Second Marker (on 1.0)** to normalize the correlation curve at the position of the black bar to 1.0.
  - ✓ Select **Both Markers (on 2.0 and 1.0)** to normalize the correlation curve to 2.0 at the position of the left black bar and to 1.0 at the position of the right black bar. The position of the bar more to the right defines the correlation time τ_{n1} to which the corresponding correlation value will be normalized to 1. The difference of $G(\tau_{n1})$ to 1 will than be added to all the other values. Therefore $G(\tau)_{norm1} = G(\tau) + (1-G(\tau_{n1}))$. The position of the bar more to the left defines the correlation time for which the correlation values will be normalized to 2. Note that this value is defined by the arithmetic mean of the correlation values at position defined by the bar and the correlation values defined by the correlation times just before and after. All other values (substracted by 1) will than be multiplied by the correction factor defined by the ratio of 1 to $(G(\tau_{n2})-1)$ and increased by 1, hence $G(\tau)_{norm2}=[1/ (G(\tau_{n2-1})-1) * (G(\tau)_{norm1}-1)] + 1$.

  Note that selection of any normalized view will automatically normalize the display in the fit curve as well. The normalization bars will only be visible, if the **Normalization Markers** check box is checked.

☞ Not all Options are equally available for all diagrams.

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 **Export data to file** to store the data in an external ASCII file (.txt).

The table of measuring rows in the lower part of the **Data Evaluation** window contains a check box on the right-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.

Deactivated rows can be hidden in the table by activating the check box **Hide unused Rows** with a click of the mouse on the **Table** toolbar (see **Display – Columns**, page 5-423).

5.17.4 Display - Fit

- Click on the **Fit** button to activate the **Fit** display mode.
  - A **Fit** menu subordinate will appear (Fig. 5-376).

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.

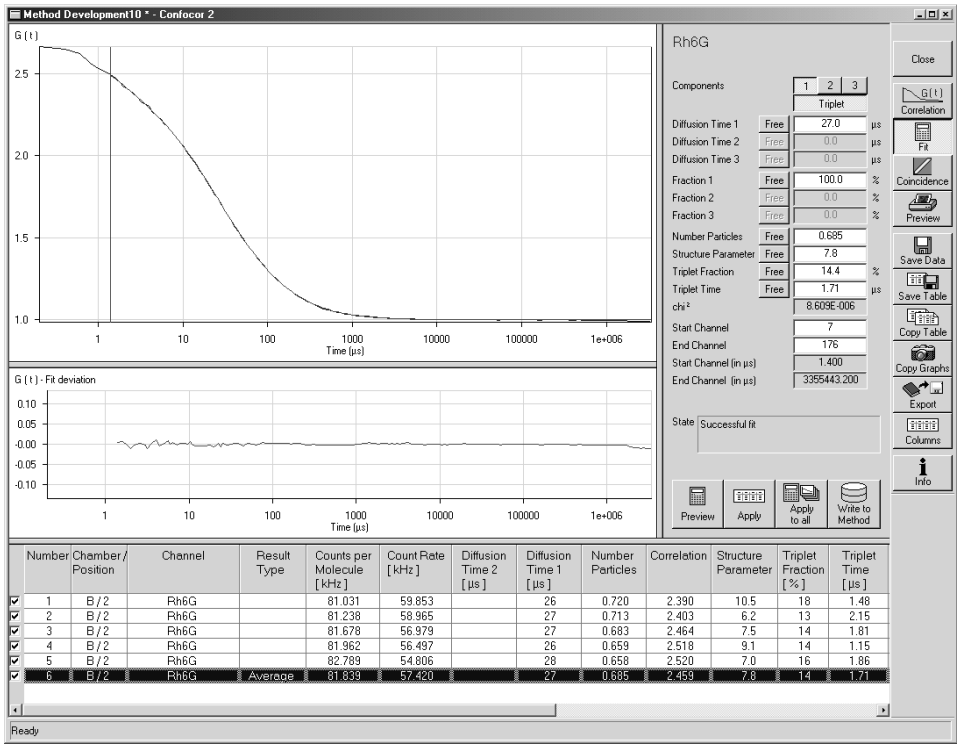


Fig. 5-376 Display fit

The upper graph shows the correlation curve with the overlaid fit graph.

The lower graph depicts the fit residuals.

- Use the **1, 2** and **3** buttons to select a one-, two- or three-component model. If a one- or two-component model is selected, the parameters not used are grayed.
- Clicking on the **Triplet** button allows you to change between taking the triplet fraction into account or not.

- An additional button bar (**Free** or **Fix**) will appear if you click on the button at the right-hand side of the parameters.
  - If you click on **Free**, the parameter will be defined as free. After the fit, the field will contain the fit result for the parameter.
  - **Fix** allows you to fix the parameter. This is useful if you know its value from other measurements.
- **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 substract the Start channel number by 1 and substract from 255 the End** channel number, respectively.

The **State** display box informs you whether the fitting operation has been successful or not. This merely indicates, if the algorithm was able to fit the results. The quality of the fit is displayed in the **chi²** display box. The X^2 (chi^2) value should approach zero for highest quality.

- When you are finished, click the **Preview** button and the fit procedure will be 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 remains unaffected by the Fitting procedure.
- Click on the **Apply** button to also apply the procedure performed in the fit settings field to the current (highlighted) measurement in the result table.
- With a click on the **Apply to all** button, the fit procedure is performed for all activated measurements (via the check box of the row) in the result table.

The **Write to Method** function permits the fitted parameters to be transferred directly to an existing or a new Measuring Method.

- Open the **Optimize Method** window by clicking the appropriate button in the **Analyse FCS** subordinate toolbar of the **Main** menu.
- Then click on the **Write to Method** button.

The parameters of the selected fitting model are assigned to the method selected in the **Method Optimization ...** 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 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.

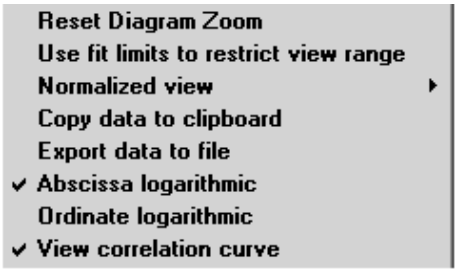


Fig. 5-377 Context menu of the Fit diagram

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. 5-377).
- 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 **Export data 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 **Abcsissa logarithmic** or **Ordinate logarithmic** to scale the x- or y-axis, respectively, logarithmically.
- Select **Use fit limits to restrict view range** to display only the part of the correlation curve that is used for fitting as determined by the **Start channel** and **End channel**, respectively (see Fig. 5-380). Selection of this option will automatically activate this form of display for the Correlation diagram as well.

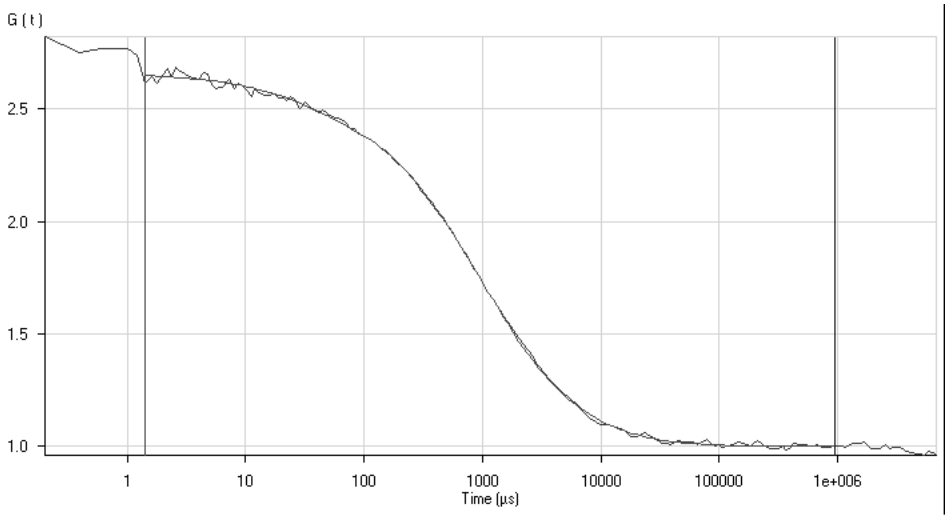


Fig. 5-378 The Correlation Curve window in normal display

- Select **Normalized view** to display the correlation curves in a normalized form. Two bars define the positions of the curve that will be normalized to 1 and 2, respectively (see Fig. 5-381). You have the option to normalize to 2 and 1 separately, or to normalize to both **Options** are given in the subcontext menu (see Fig. 5-379). In this case the left bar defines the position were the curve is normalized to 2, whereas the right bar specifies the position at which the curve is normalized to 1.

Fig. 5-379 Subcontext menu of the Normalized view

The bars are only visible if the **Normalization Markers** box in the **Display – Column** is checked. Not that activating the **Normalization view** in the fit display will automatically activate the **Normalization view** in the correlation display as well. The normalization bars are only visible if the **Normalization Markers** check box is checked.

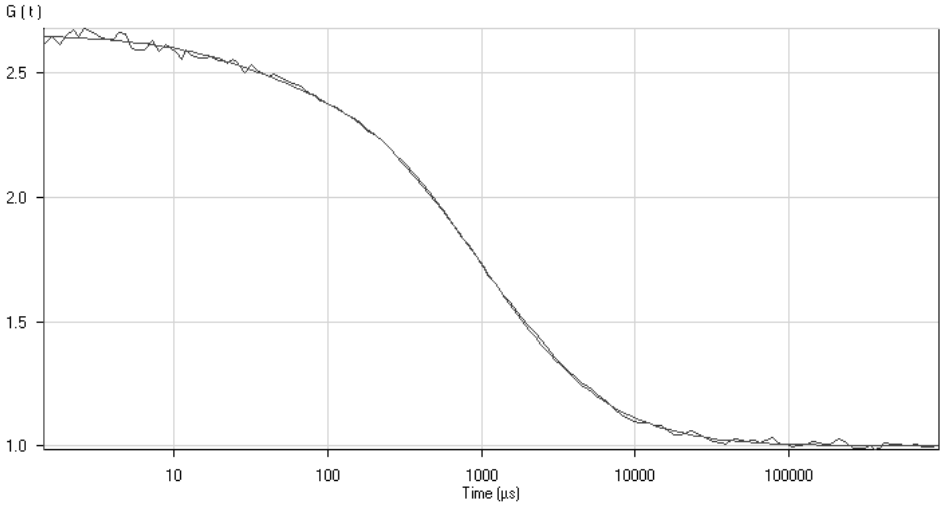


Fig. 5-380 The Correlation Curve window in restricted display

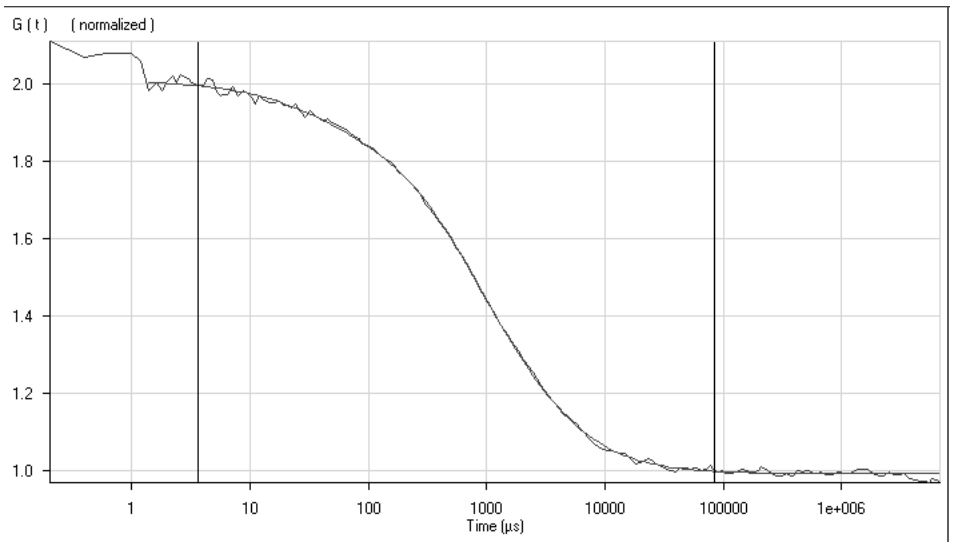


Fig. 5-381 The Correlation Curve window in normalized display

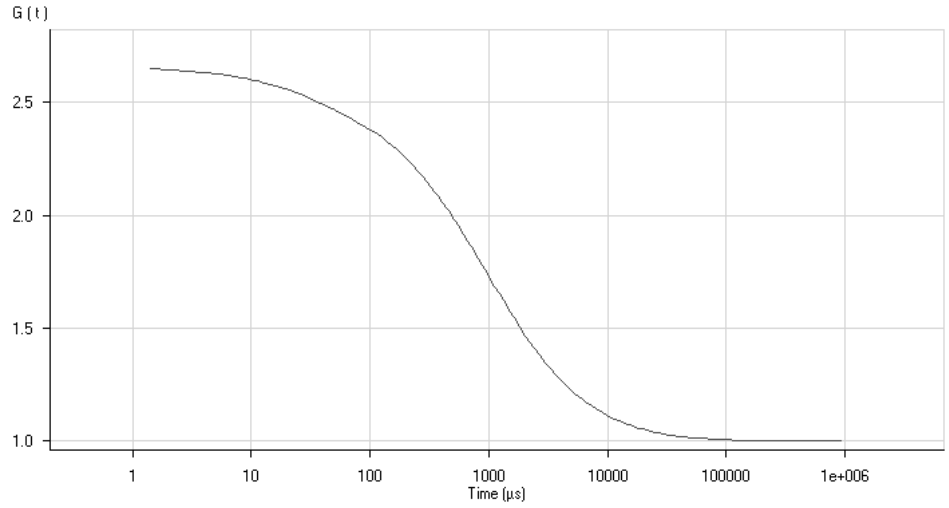


Fig. 5-382 The Correlation Curve window in fit curve display ("View correlation curve" unchecked)

- Selelect **View correlation curve** will display the Correlation curve in addition to the Fit curve (see Fig. 5-378). If this option is not selected, only the Fit curve is displayed (see Fig. 5-382).

The zoom function can be used to display certain areas of interest in the **Fit deviation** diagram 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.

The context menu of the **Fit deviation** 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.

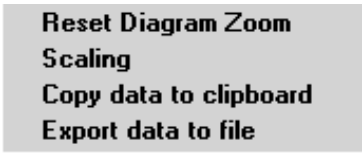


Fig. 5-383 Context menu of the Fit deviation diagram

The scaling of the G axis (t) 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 is opened.
- Enter the required percentage value (from 1 to 100) for the scaling and click on **OK**.
  - The scaling is changed accordingly.

Fig. 5-384 Diagram scaling window

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 **Export data 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 right-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.

Deactivated rows can be hidden in the table by activating the check box **Hide unused Rows** with a click of the mouse on the **Table** toolbar (see **Display - Columns**).

5.17.5 Display - Coincidence

- Click on the **Fit** button to activate the **Fit** display mode.
  - The coincidence analysis generates a two-dimensional color-coded count rate histogram (Fig. 5-385).

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.

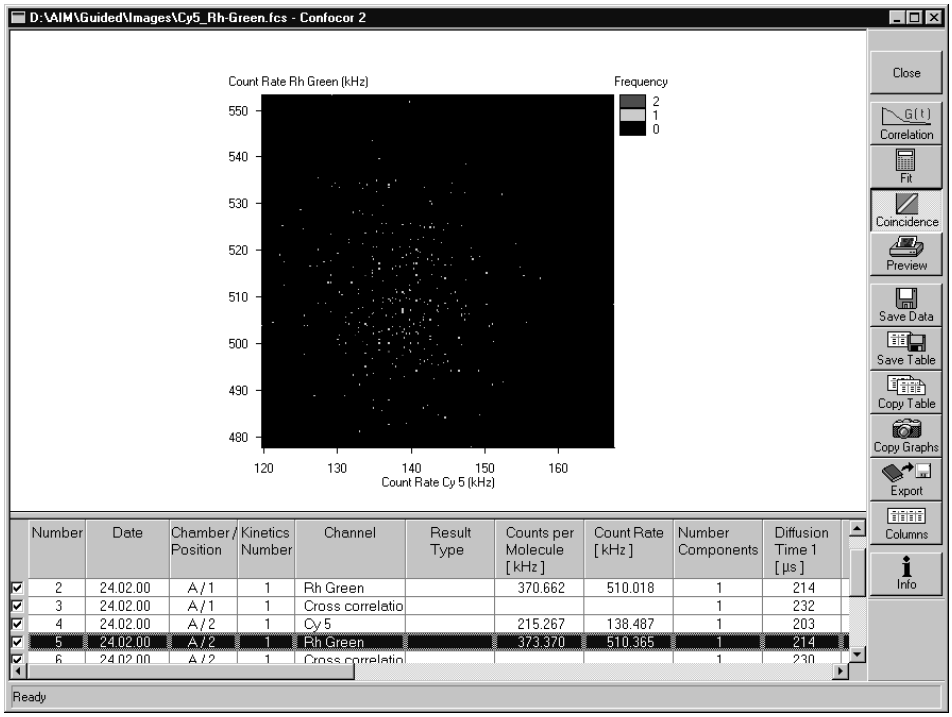


Fig. 5-385 Coincidence diagram window

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

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5.17.6 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. 5-386).

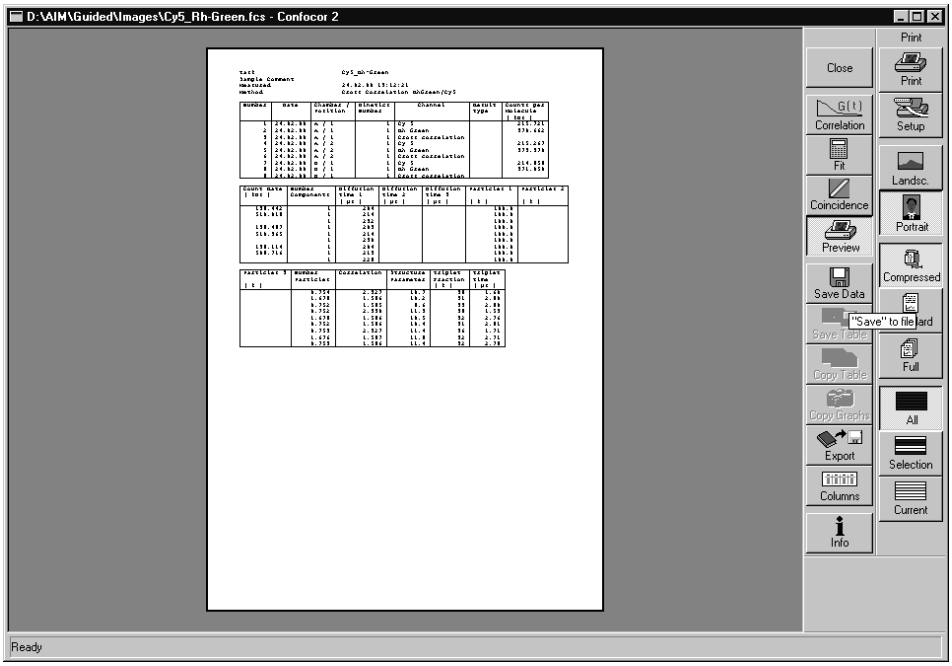

Fig. 5-386 Preview / Print window

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.

5.17.7 Display - Save Data

- **Save** allows you to save the whole data set (curves, fitting results, fit parameters) to disk. If **Save** is selected, the **Save** menu appears.

The **Save** menu offers the possibility of entering the task name from which the file name is derived.

It also offers the possibility of entering a comment in the comment field.

The directory can be chosen.

It is possible to store the data as ANSI text (space-consuming, but readable) or as a binary file.

- **OK** will confirm the action, **Cancel** will exit the menu without further action.

5.17.8 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.

5.17.9 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.

5.17.10 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.

5.17.11 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.

5.17.12 Display - Columns

- With a click on the **Columns** button, the **Table** subordinate toolbar can be toggled on and off.

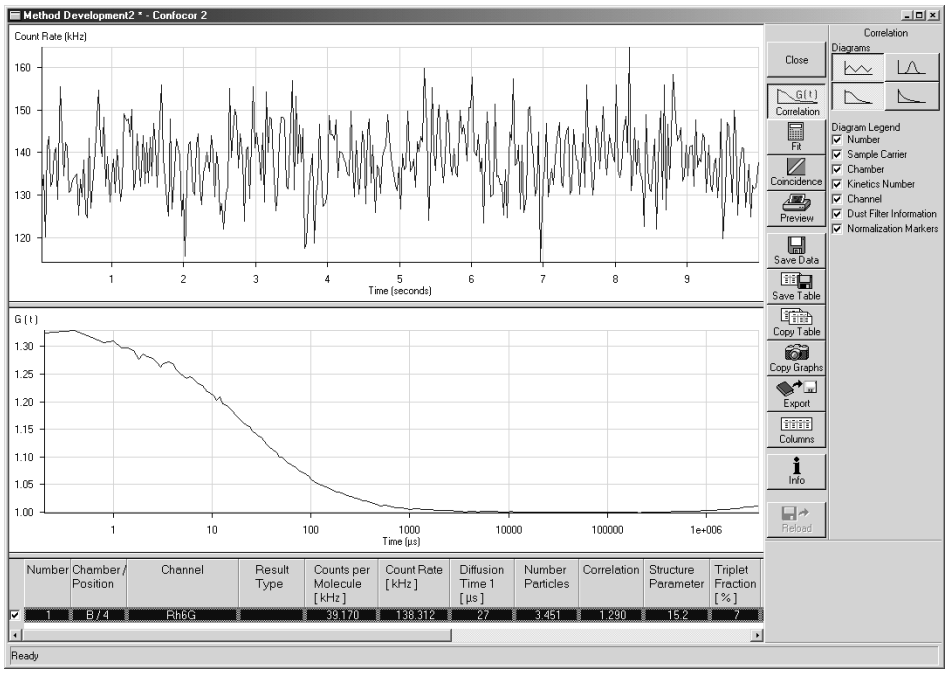

Fig. 5-387 Display Column with Table subordinate toolbar

- By ticking the appropriate **Columns** check box in the **Table** subordinate toolbar, it is possible to include any fitting parameter into the displayed table or remove it from there.
- It is possible to configure the colors for "special situation" highlighting. On clicking the buttons of the **Table** subordinate toolbar, a menu will pop up which allows you to choose the desired color.
- By ticking the **Order by Correlation** check box, the table of measurement results is arranged in the order of correlation (only useful for more than one correlation type).
- Activate the **Hide unused Rows** check box if deactivated rows shall be hidden from the table of measuring values.

5.17.13 Display - Info

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

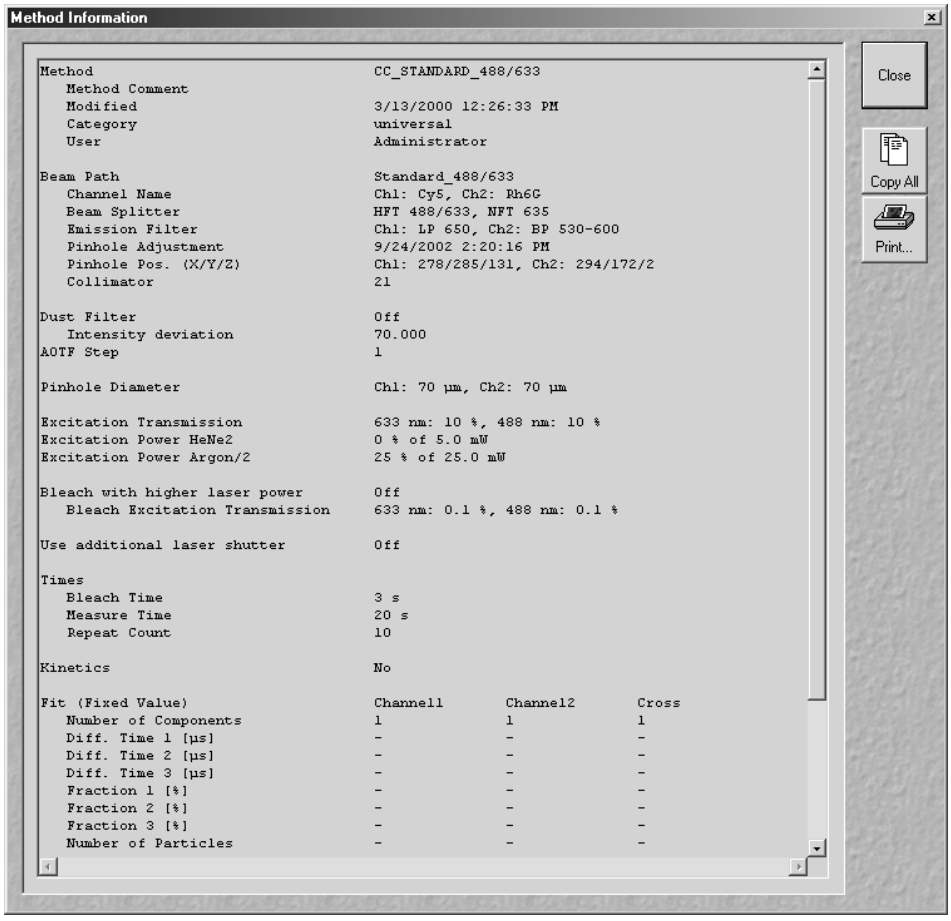

Fig. 5-388 Method Information window

- 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.

5.18 Combined Application of LSM Scanning and FCS Measurement Procedure

5.18.1 Taking FCS Measurements within a Cell

The simultaneous installation of the LSM 510 and the ConfoCor 2 to the stand of the Axiovert 200 M permits examination of points selected with the ConfoCor 2 within a single LSM image.

For combined application, proceed as follows:

- Click on the **FCS** button in the **Analyse FCS** subordinate toolbar of the **Main** menu to select the FCS mode.
- Click on the **Method** button in the **Analyse FCS** subordinate toolbar to open the **Method Selection ...** window. Select the FCS measurement method and click the **Select** button. Close the window.
- Open the **Carrier Position** window. Initialize stage and focus. Switch on the lasers using the **Laser** button. Close the **Laser Control** and the **Carrier Position** windows.
- Click on the **VIS** button in the **Analyse FCS** subordinate toolbar
- Select the specimen area to be examined by moving the stage and focus.
- Click on the **LSM** button in the **Analyse FCS** 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 **FCS** mode in the **Analyse FCS** subordinate toolbar.

There are two ways of defining the positions, where FCS measurements should be performed. One is activated in the **Method Measurement ...** window (treated in section 5.18.1.1) and the other in the **Method Optimization ...** window (see section 5.18.1.2).

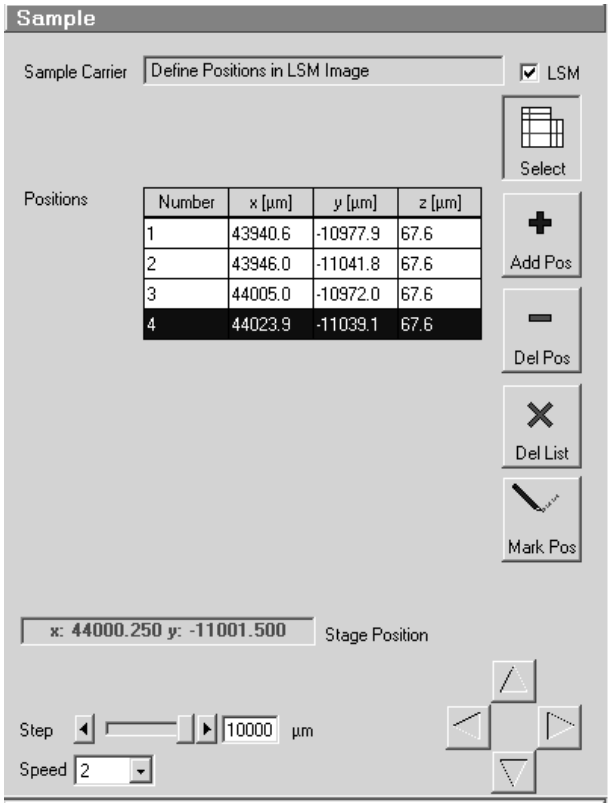

Fig. 5-389 Sample panel for defining positions in the Method Measurement

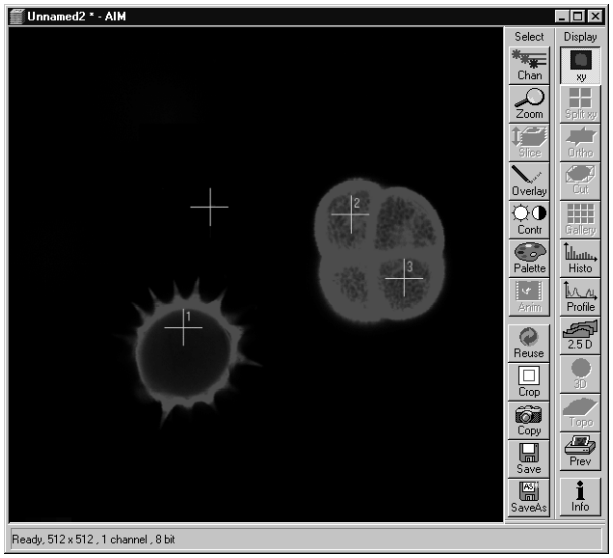

Fig. 5-390 LSM scan image with cursor (crossline) and 3 selected positions

5.18.1.1 Using the Method Measurement ... Window

With this method you can conveniently choose several points of interest at which FCS measurements will be performed.

- Open the **Method Measurement ...** window by clicking the **Measure** button in the **Analyse** subordinate toolbar. Activate the **LSM** check box in the **Sample** panel via mouse click. If the Piezo Stage Option has been choosen in **FCS Options** in **Measure** tab than checking the **LSM** box will automatically activate the usage of the Piezo Stage. A little display left to the stage control buttons will appear telling that the "Piezo stage active". Note, that it will not be possible to use the normal stage if the **LSM** box is activated in the FCS mode. If you want to use the normal stage with the **LSM** box checked, you have to deactivate the Piezo Stage in **FCS Options** before starting the LSM-FCS software. In the LSM mode, always the normal stage is active and the Piezo stage cannot be activated.
  - The **Sample** 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.

☞ 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 **Method 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 **Method Measurement ...** window using the ConfoCor 2 are approached by moving the microscope stage, since the FCS beam path is rigidly fixed in one position. When the last selected position has been approached and measured, the stage will return to the start position.

Possible offsets during scanning with the LSM 510 are compensated by the stage movement in such a way that the marked points will be hit.

An accuracy of 1 µm can be obtained. We recommend to take measurements not all over the frame but in one section only to increase the accuracy. Refer to chapter 5.18.3 for more information.

5.18.1.2 Using the Method Optimization ... Window

When using this method, FCS measurements are performed on a fixed position without table movement if the **Crosshair** function is activated or at one defined position if the **Position** function is used. In the former case the structure of interest must be moved to the position manually under LSM control prior to FCS measurements. This positioning allows for a higher accuracy of <1 µm deviation.

- Open the **Method Optimization ...** window by clicking on the **Optimization** button in the **Analyze** subordinate toolbar. Activate the **LSM** check box in the **Sample** panel via mouse click. If the Piezo Stage Option has been chosen checking the **LSM** box will automatically activate the usage of the Piezo Stage. Note, that it will not be possible to use the normal stage if the **LSM** box is activated. If you want to use the normal stage with the **LSM** box checked, you have to deactivate the Piezo Stage in the **Config. Tool** before starting the LSM-FCS software. In the LSM mode, always the normal stage is active and the Piezo stage cannot be activated
  - The **Sample** panel changes to allow the definition of positions in the LSM scan image or to activate the **Crosshair** function.

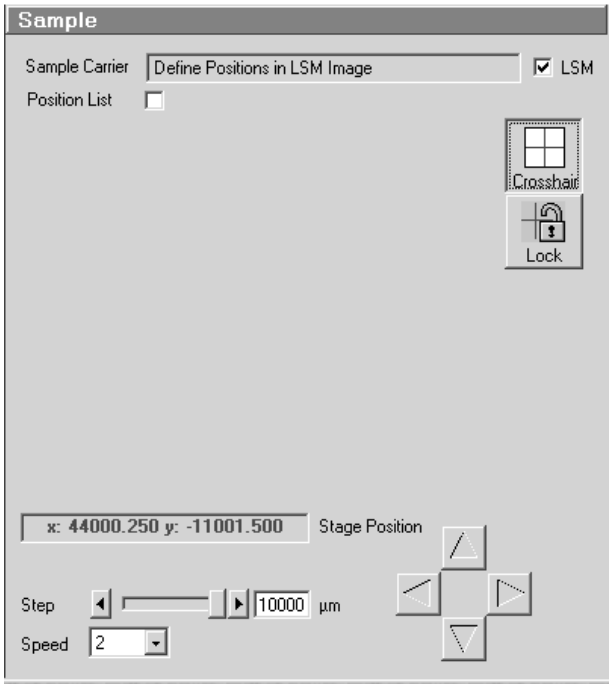

Fig. ss23 Sample panel with activated crosshair

(1) Using the Crosshair function

In this mode you have to position your sample manually.

- 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 as determined in section 5.18.3.2. We recommend that having set the crosshair with the bleaching procedure as outlined in section 5.18.3.2 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.

(2) Using the Position function

In this mode, the position will be approached by the scanning table.

- Check the Position box. The **Sample** panel changes to allow the definition of positions in the LSM scan image
- Click onto the **Select** button. Move the cursor to the desired position in the scan image (drag and drop). Select that position by clicking the **Add** button. Repeat until all positions are marked, than click the **Mark Position** button to store all positions in the overlay.

☞ In the optimization window only the currently selected position will be approached by the scanning table used for an FCS measurement, not all your defined positions. Select the position by clicking into the corresponding row of the displayed **Positions** table.

- After defining the site of interest, click on the **Start** button in the **Method Optimization ...** window to start the FCS measurement.
  - The measurement procedure starts and the data and evaluation window appears on the screen.

☞ Please note that the position selected for FCS measurements in the **Method Optimization ...** window using the ConfoCor 2 is not approached by moving the microscope stage if the **Crosshair** function is activated. To find out the position of the fixed laser beam refer to chapter 5.18.3. Since no stage movement is involved, the manual positioning of your sample will be more precise with an accuracy of at least 1 µm.

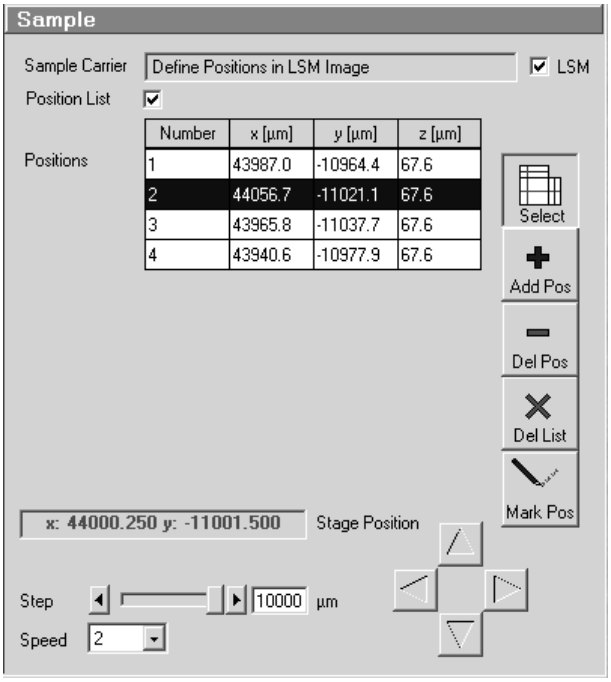

Fig. ss24 Sample panel for defining positions in the Method Measurement

5.18.2 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.

5.18.2.1 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 and proceed as described in sections 5.18.1.1 and 5.18.1.2.

Due to its high sensitivity ConfoCor2 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 **Method Optimization ...** window.

5.18.2.2 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 5.18.3.2.
- Optional: Perform a **Z Stack** that includes the membrane of the cell as outlined in chapter 5.5.4 with the LSM.
- Open the **Method Optimization ...** window by clicking on the **Optimization** button in the **Analyze** subordinate toolbar. Activate the **LSM** check box in the **Sample** panel via mouse click.
  - The **Sample** panel changes and shows the position of the Laser beam.
- Activate the **Select** button via mouse click.
  - The cursor (crossline) appears in the LSM scan image.
- Move the cursor to the appropriate position.
- Select by pressing the **Add** button.
- Press **Mark Pos** to fix the position as an overlay element.

☞ You might also activate the **Crosshair** and manually placing your sample at the position of the laser beam as defined in section 5.18.3.2.

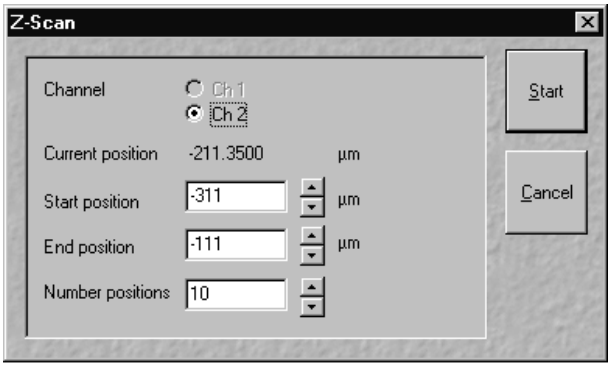

Fig. 5-391 Z-Scan display window

- Press the **Z Scan** button. The **Z Scan** display shows the current Z position of the laser beam. (see Fig. 5-391). Note, that after initialization of the LSM 510 / ConfoCor2 software the position is the same both for the FCS and LSM mode.
- 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. 5-392). 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.

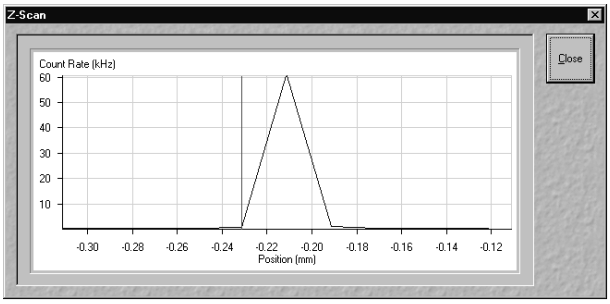

Fig. 5-392 Z-Scan image

- 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 **Method Optimization ...** window to perform an FCS measurement.
  - The FCS measurement is performed at the same X/Y position as the Z scan.

5.18.3 Compensating Offsets between FCS and LSM

As described in chapter 5.18.1.1 you can select the positions in the **Method Measurement ...** window or the Method **Optimization ...** window in the **Positions** mode, at which the FCS measurements should be performed. Before you use this function you have to adjust the offsets in X,Y between the FCS and the LSM mode following the description in section 5.18.3.1. 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. This procedure is described in section 5.18.3.2. For Z adjustment you have to use the **Method Optimization ...** window as described in section 5.18.3.3. 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.

5.18.3.1 Alignment in X, Y for the Method Measurement ... and the Method Optimization in the Positions Mode

You can compensate manually or by using a Macro.

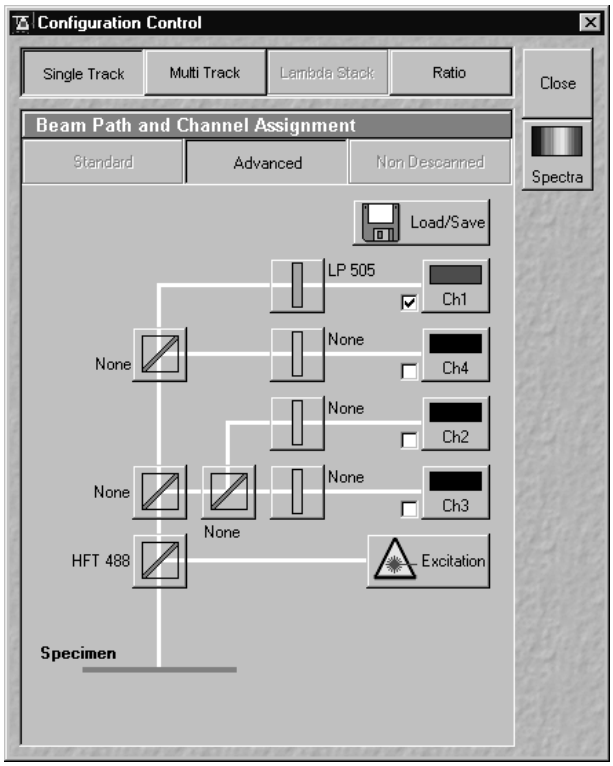

Fig. 5-393 LSM beampath for imaging the test layer of Rh6G

5.18.3.1.1 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. 5-393):
  - 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. 5-394):
  - 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

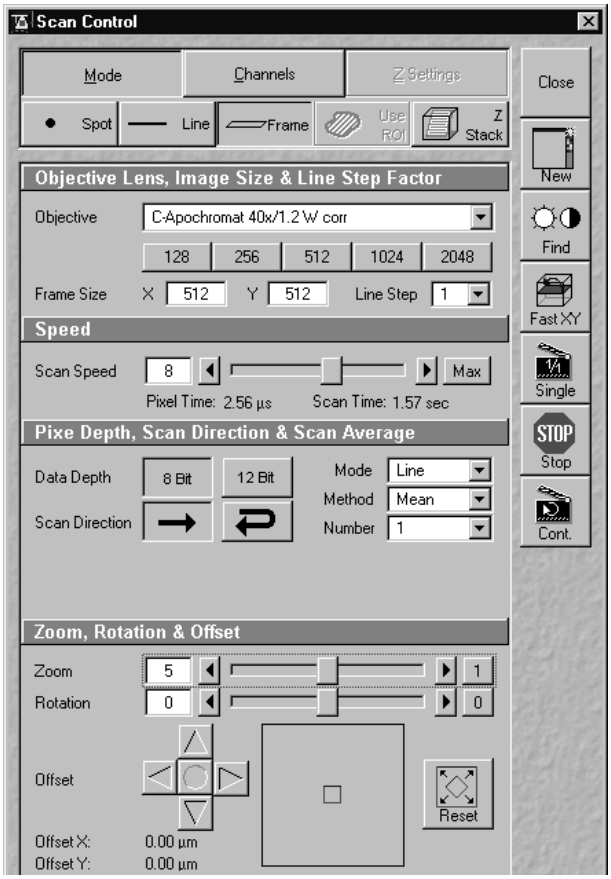

Fig. 5-394 Settings at the LSM control

- Optimize focus position and **Detector Gain** value for a good LSM image (see Fig. 5-395).
- Leave the LSM image window and LSM control window on the computer screen.

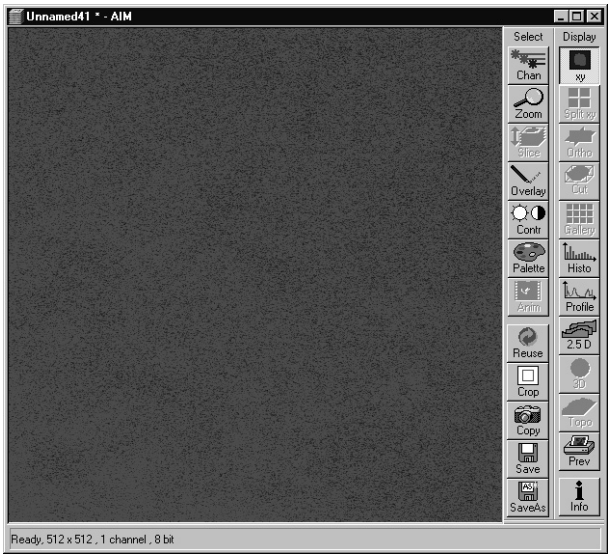

Fig. 5-395 LSM image of the Rh6G layer before bleaching. Usually some texture is observed.

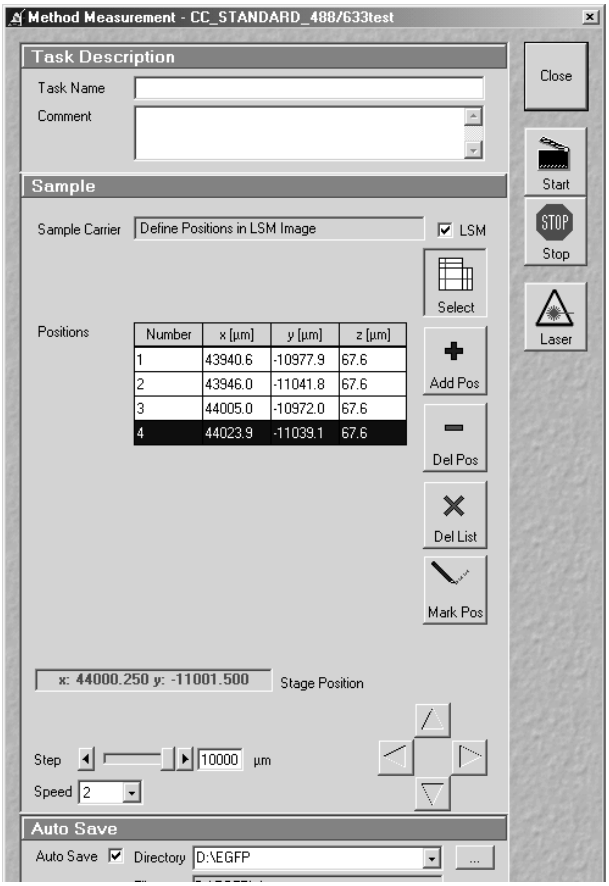

Fig. 5-396 The Method Measurement window in the LSM mode

- Switch to FCS mode.
  - In the main toolbar press the **FCS** button.
  - Click **Analyze / Select Method**: Select "**LSM-FCS_STANDARD_488-Rh6G**"
  - Click **Analyze / Measure**: activate the check box **LSM**.
  - 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. 5-396 and Fig. 5-397).

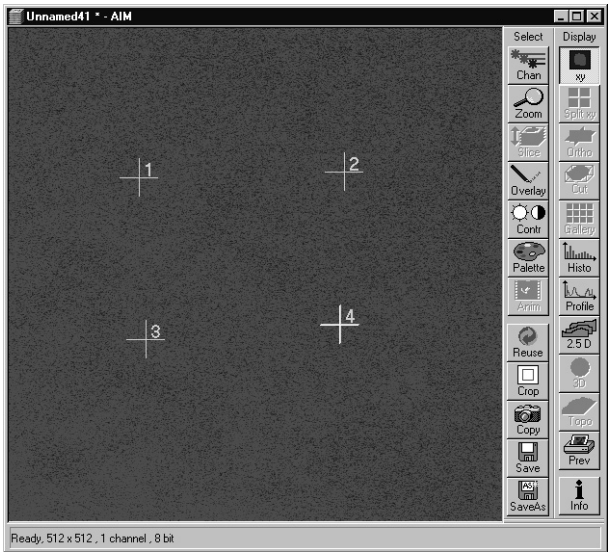

Fig. 5-397 LSM image of the Rh6G layer with markers for FCS-LSM adjustment

- 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. 5-398).

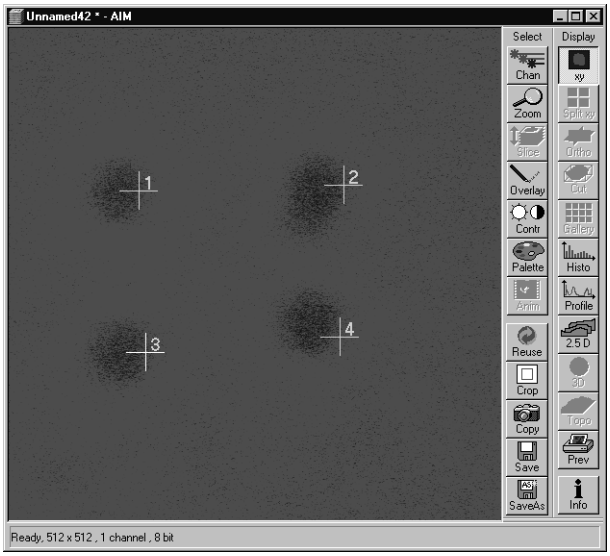

Fig. 5-398 LSM image of the Rh6G layer after bleaching. Deviations between the markers and the bleached spots are noticeable.

- Measure the distance between the burned spots and the markers (see Fig. 5-399).
  - 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 **FCS Settings** / **LSM+ConfoCor**.
  - Click **Options** / **FCM Settings** / **LSM+Confocor**.

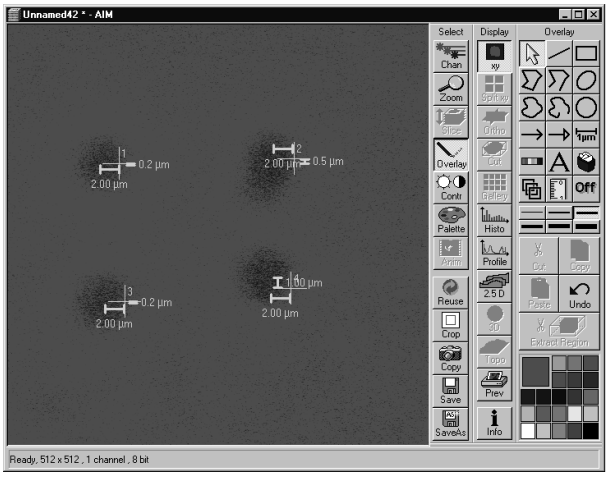

Fig. 5-399 LSM image after bleaching with overlaid distance markers

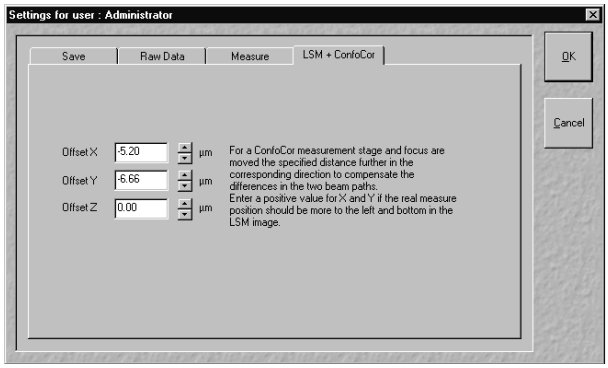

Fig. 5-400 The Settings for user window with the LSM+Confocor tab selected

- Type the measured distance correction values under **Options / FCS Settings** in the **LSM + ConfoCor** tab (see Fig. 5-400).
  - 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

☞ Note that this correction is valid only for the ConfoCor2 sitting at the sideport. ConfoCor2 attached at the baseport requires other algebraic signs.

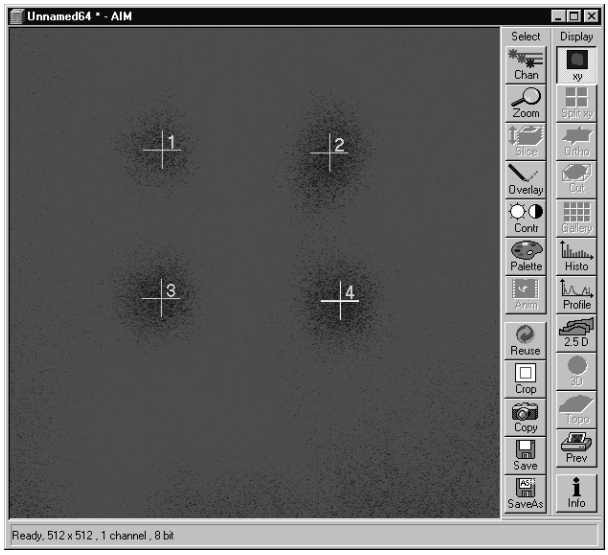

Fig. 5-401 LSM image after bleaching and correction of offset values

- Check the correctness of your "FCM Settings" by repeating the adjustment procedure (see Fig. 5-401).
  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.

5.18.3.1.2 Adjustment Using the LSM/FCS Macro

Before running the Macro choose the Method "**LSM-FCS_STANDARD_488-Rh6G**" in the Method file menue in FCS and set the beam path for the LSM according to 5.18.3.1.1. 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. 5-402).
- 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.

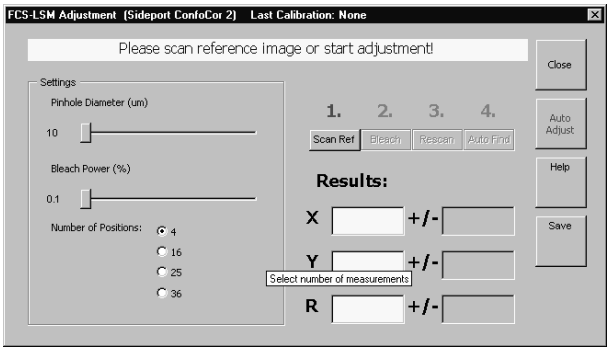

Fig. 5-402 The Macro "Fcs-Lsm" display before offset compensation

- 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.

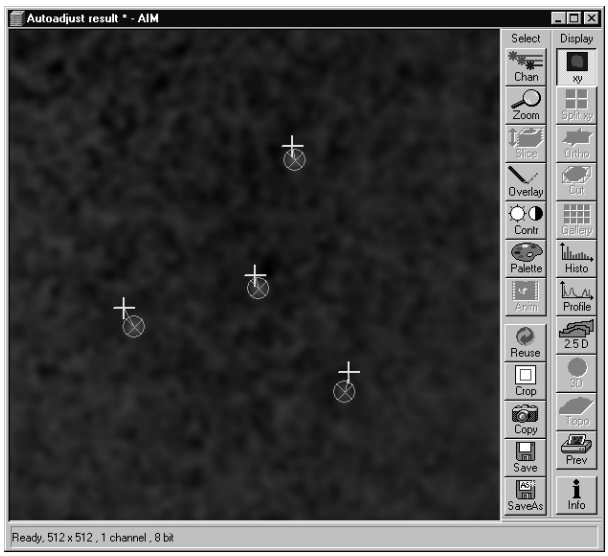

Fig. 5-403 LSM image after performance of the "Fcs-Lsm" Macro

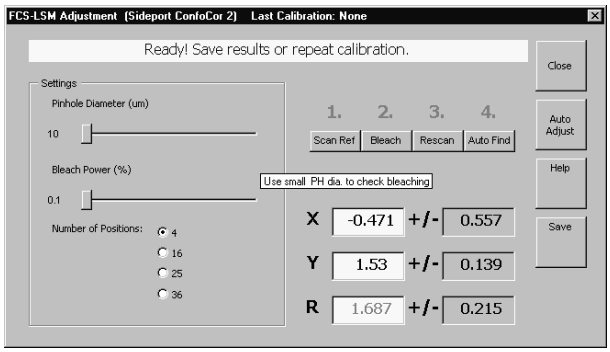

Fig. 5-404 The "Fcs-Lsm" Macro after offset compensation

- 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. 5-403). The program also calculates the deviation in X and Y, the values which are also displayed under **X** and **Y** offsets (see Fig. 5-404). 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.
- 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.
- 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.

5.18.3.2 Determination of the Fixed X,Y Position of the Laser Beam in the Method Optimization ... Window in the Crosshair Mode

- Get a LSM image of a Rh6G layer as described under 5.18.3.1.1.
- Switch to FCS mode.
  - In the main toolbar press the FCS button.
  - Click **Analyze / Select Method**: Select "**LSM-FCS_STANDARD_488-Rh6G**"
  - Click **Analyze / Optimization**: activate the check box **LSM**.
  - Click the **Crosshair** box (see Fig. 5-405).
- 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.

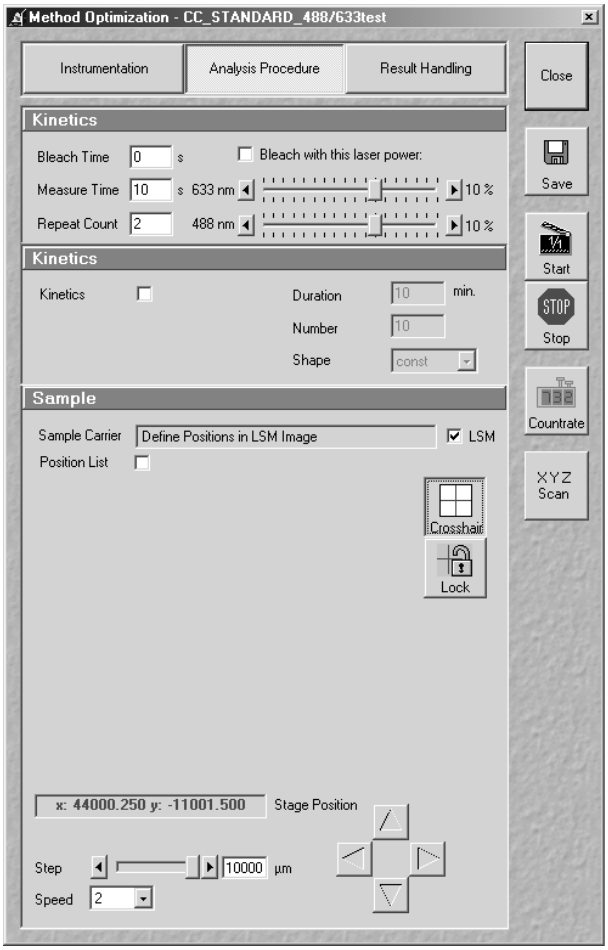

Fig. 5-405 The Method Optimization ... window in the LSM mode with the Crosshair function activated

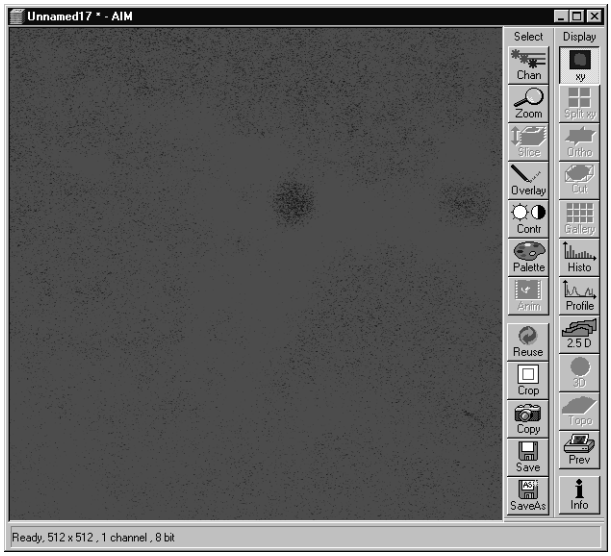

Fig. 5-406 LSM image after bleaching in the Method Optimization

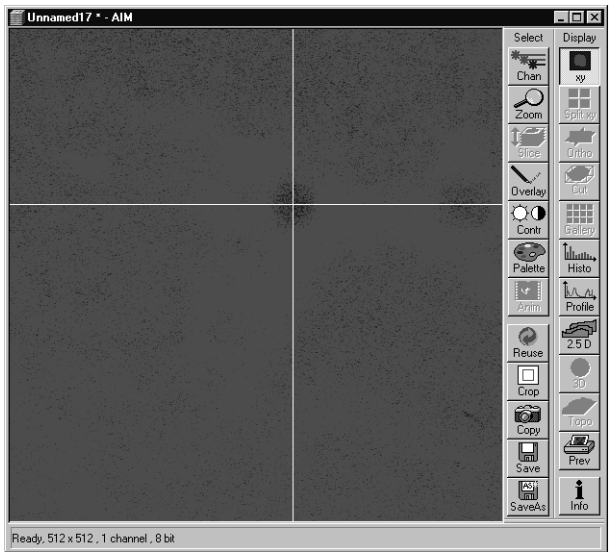

Fig. 5-407 LSM image after bleaching with crosshair

- Rescan the Rh6G layer.
  - Switch to LSM mode.
  - 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. 5-406).
- Record the position of the spot.
  - With the crosshair. Place crosshair above the bleached spot (see Fig. 5-407). 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.

5.18.3.3 Alignment in Z

- In the LSM mode take an image of the dye layer and focus at the Z level of maximum intensity. Record the corresponding Z position.
- Switch to FCS mode.
- Open the **Method Optimization ...** window by clicking the **Optimization** button in the **Analyze** subordinate toolbar. Activate the **LSM** check box in the **Sample** panel via mouse click.
  - The **Sample** panel changes to allow the definition of positions in the LSM scan image.
- Activate the **Select** button or the **Crosshair** via mouse click.
  - If you have pressed the **Select** button the cursor (crossline) appears in the LSM scan image. Choose a position by moving the cursor and pressing the **Add** button.
- Press the **Z Scan** button. The Z-Scan display shows the current Z position of the laser beam. (see Fig. 5-391). Note, that after initialization of the LSM 510 / ConfoCor software the position will be the same for both the FCS and LSM mode.
- Check the corresponding channel if not yet activated.
- Set the start and end position of the Z Stack around the current position.
- Enter the number of positions.
- Press **Start** to perform the Z scan.
- A Z scan is performed and the intensity displayed in dependence of the Z position (see Fig. 5-392). The major peak corresponds to the labeled layer. Place the red line at this peak value to select this Z position. Record the position and the direction the red bar was moved.
- Calculate the offsets between LSM and ConfoCor2 by subtracting both Z values.
- Type the calculated values under **Options / FCS Settings** in the **LSM + ConfoCor** tab (see Fig. 5-400).
  - Offset X: no entry
  - Offset Y: no entry
  - Offset Z: type in the positive or negative number, if you have moved the red line to the right or left, respectively.

5.18.4 Piezo Stage

5.18.4.1 Activating the Piezo Stage

You have the option to mount a Piezo stage onto your normal stage. If you want to have access to the Piezo stage in the LSM-FCS software, the **Piezo stage** checkbox in the AIM Configuration tool has to be checked. To this end double click on the **ConfTool** icon, select **FCS** tab and check the corresponding box (see Fig. 5-408). 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 checking the **Use Piezo stage if available** check box. The Piezo stage will be automatically used in the FCS-mode when the **LSM** box is checked in the **Method Measurement** or the **Optimization Measurement / Analysis Procedure**. If you do not want to use the Piezo Stage with the **LSM** box checked , you have to deactivate the check box.

Fig. 5-408 Activating the Piezo Stage in the Conf.Tool

☞ Note: If you haven't chosen the closed loop operation mode, the Piezo stage will not work properly in the LSM-FCS Software."

The Piezo stage allows for 2D translational motion in the x and y direction with a maximum motion of 80 µm in each direction. The resolution is 1 nm. Positioning accuracy using the Piezo Stage is 10 nm and less.

5.18.4.2 Working with the Piezo stage

Open **Method Optimization / Analysis** procedure (see Fig. 5-409) or **Method Measurement** and check the **LSM** box. In the **FCS** mode the Piezo stage is automatically used.

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 done by the Piezo stage.

☞ Note: The numbers displayed will not correspond to the positions in the LSM mode, if the Piezo stage is activated. If the standard stage is used, the numbers will be the same.

☞ Note: The Piezo stage will only be active in the **FCS** mode. In the **VIS** and **LSM** mode the normal stage will be used.

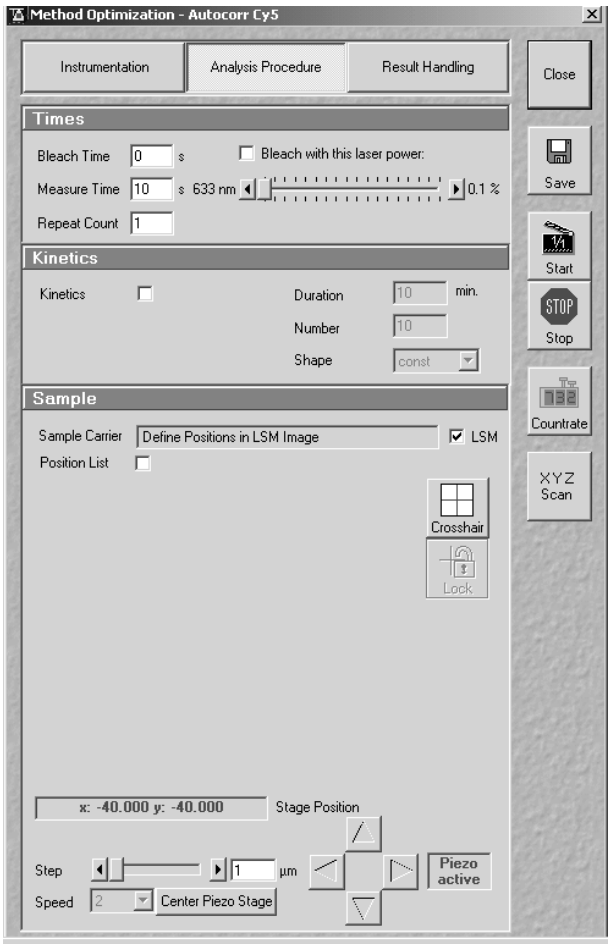

Fig. 5-409 Method optimization window (Analysis Procedure subwindow) with LSM box checked and Piezo stage active

5.19 Shut-Down Procedure

Never shut down the computer by its main switch while your LSM-FCS program is still active, or else you will lose the currently set operating parameters and the images just scanned.

☞ In the **Settings for user** dialog window, which can be activated with the **Options / Settings** buttons, activate **Laser off** or **Exit** in the **Shutdown** tab. The lasers will then automatically be switched off when you exit the LSM-FCS program.

5.19.1 Exiting the LSM-FCS Program

- Close all open windows of the LSM-FCS program by clicking on the closing icon ☒ in the top right corner of each window.
  - This closes the respective window and removes the respective icons from the taskbar.
  - After all dialog windows have been closed, the **LSM-FCS Switchboard** window appears.

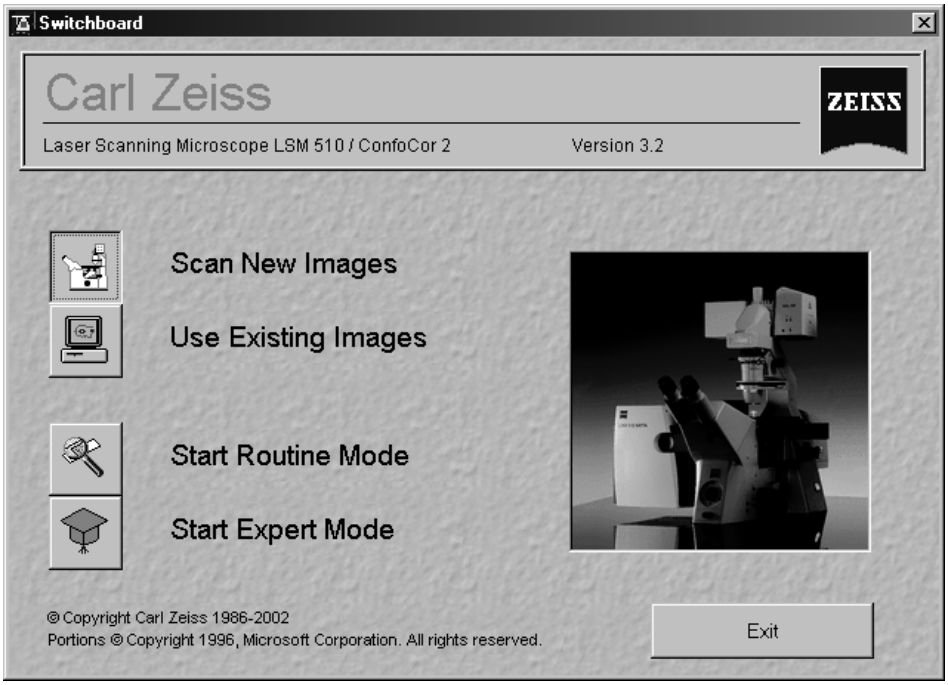

Fig. 5-410 LSM-FCS Switchboard menu

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

5.19.2 Shut Down the WINDOWS Operating System

- Move the cursor to the bottom margin of the screen.
  - This opens the taskbar containing the **Start** button.
- Click on the **Start** button of the taskbar.
  - This opens a pop-up menu.
- Click on the **Shut Down** item.

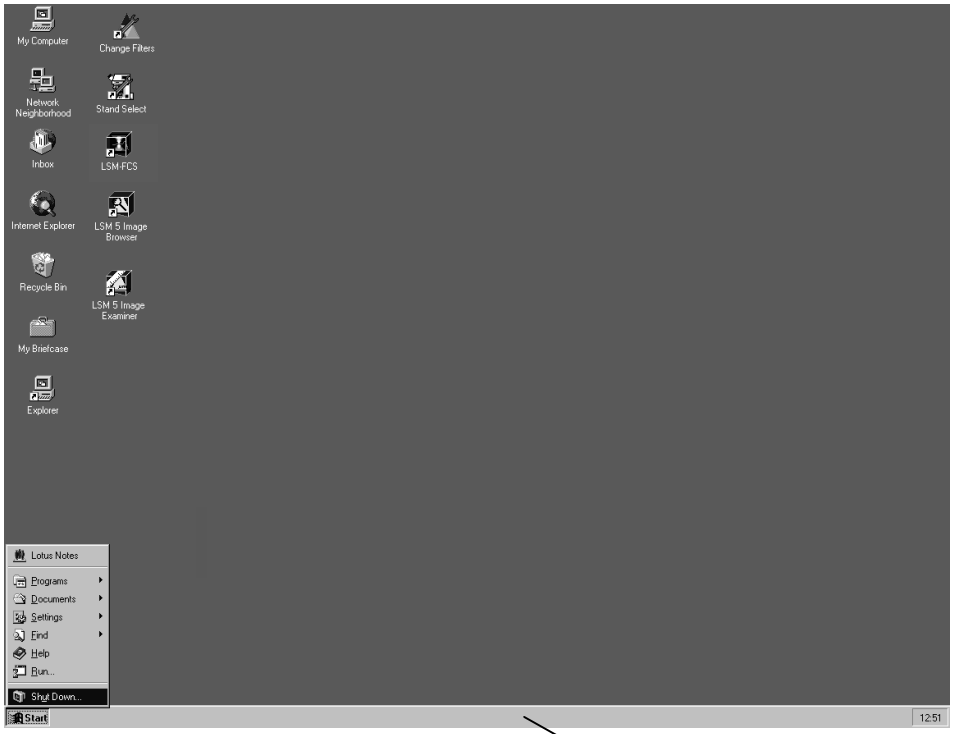

Fig. 5-411 Start menu

Fig. 5-412 Shut Down window

– This opens the **Shut Down Windows** window, in which you can select between **Shut down, Restart** and **Login**.

- Unless already set by default, click on **Shut down the computer?**
- Click on the **Yes** button.

The screen now displays the message

Shutdown in Progress - Please wait while the system writes unsaved data to the disk.

About 20 seconds after WINDOWS NT has been run down, the **Shutdown Computer** window appears which tells you that you can now turn off your computer.

5.19.3 Turning Power Off

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

⚠ Throw the REMOTE CONTROL main switch and the power supply switch of the Ar Laser to position "**OFF**" after 5 minutes.

– This puts your LSM-FCS microscope system, including the computer, off power.

5.20 Software and Hardware Options

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

5.20.1 Software

The following software packages for Release 3.0 are available:

- Software "Physiology evaluation"
- Software "Topography evaluation"
- Software "Macro Recorder and Editor"
- Software "3D for LSM"
- Software "Multiple Time Series"
- Software "Image VisArt"
- Software "Deconvolution"
- Software "StitchArt"

If your configuration does not include the "Physiology evaluation" software package, the following functions are not available:

- **Mean of ROI** scan button in **Time Series Control**
- **Mean of ROI** button in the **Image Display** window

If your configuration does not include the "Topography evaluation" software package, the following functions are not available:

- **Topo** button in the **Image Display** window after acquisition of image stacks

If your configuration does not include the "Macro Recorder and Editor" software package, the following functions are not available:

- **New**, **Save** and **Save as** buttons in the **Macro Control** window
- **Edit**, **Step**, **Delete**, **Editor** buttons in the **Macro Control** window

If your configuration does not include the "3D for LSM" software package, the following separate application is not available:

- **3D for LSM**

If your configuration does not include the "Multiple Time Series" software package, the following function is not available:

- Macro: "Advanced Time Series"

If your configuration does not include the "Image VisArt" software package, the following function is not available:

- **3D** button in the **Image Display** window

If your configuration does not include the "Deconvolution" software package, the following functions are not available

- **DCV Settings** button in the **Ortho** function of the **Image Display** window
- **DCV** button in the **Process** menu

If your configuration does not include the "StitchArt" software package, the following function is not available

- Macro: "StitchArt"

5.20.2 Hardware

Depending on whether the following hardware components are available or not, the content of the screens may differ:

- HRZ 200 fine focusing stage
- Piezo objective focusing device
- X-Y scanning stage DC 4 × 4 or DC 100 × 90, each with MCU 28
- Depending on the configuration the scan head equipment may differ in filters, beamsplitters and the number of photomultiplier
- Transmitted-light PMT
- Monitor diode
- Programmable AOTF

If your configuration does not include the HRZ 200 fine focusing stage, the following functions are not available:

- **Hyperfine Z Sectioning** in the **Z Stack** function in the **Scan Control** window
- **HRZ** parameters in the **Stage and Focus Control** window

If your configuration does not include the X-Y scanning stages DC 4 × 4 or DC 100 × 90, each with MCU 28, the following functions are not available:

- **Stage Position** and **Tile Scan** functions in the **Stage and Focus Control** window

If your configuration does not include scan head META, monitor diode and/or transmitted light PMT, the following functions may differ:

- Context and accessibility of the **Config Control** window

If your configuration does not include programmable AOTF, the following functions are not available:

- **Laserline** in the **Config Control** window and **Channels** in the **Scan Control** window

If your configuration does not include an AxioCam, the following functions are not available:

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

5.21 System Configuration Tool

Transparent configuration of system hardware

Start by double-click on the **ConfTool.exe** in directory **/AIM**

Configures:

- Scanning stages, focus accessories, AxioCam
- Microscope and microscope accessories (lamps, reflector cubes ...)
- Substitutes direct editing of system databases

Benefits:

- Fast and easy integration of new hardware
- Optional rotation/flip of scanned images according to image orientation in visual observation

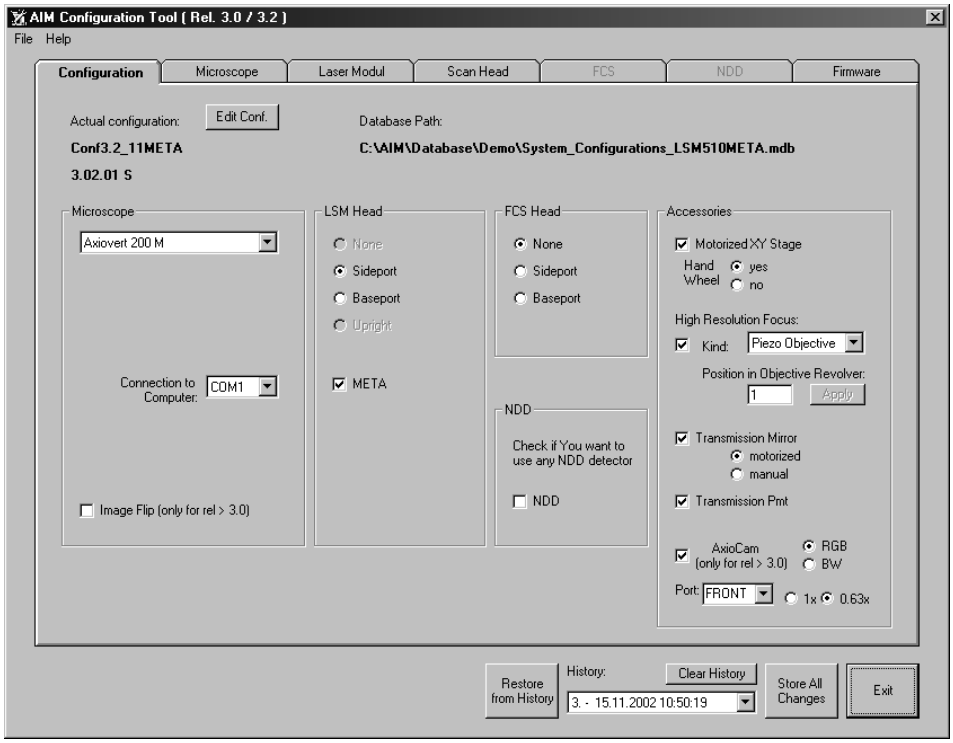

Fig. 5-413 Configuration Tool menu

5.22 Courses on How to Operate the System in an Optimized Way

Carl Zeiss is offering training courses on how to operate the system in an optimized way.

Courses are held in our application center in Jena, Germany.

Check out

www.zeiss.de/lsm

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