

ConfoCor 2

Fluorescence Correlation

Microscope

Operating Manual
Release 3.2



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

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

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How to make best use of the ConfoCor 2 operating manuals

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



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



This WARNING symbol warns against hazards from dangerously high voltages.



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



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



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



The MAINS PLUG symbol reminds service personnel to pull the mains plug before opening the device housing.

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

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

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

Scope of Equipment Supplied

Country:
Order number:
Serial number:
Delivery date:
Custom configuration:

Axiovert 200 M SP	000000-1028-779	<input type="checkbox"/>
Axiovert 200 M BP	000000-1028-780	<input type="checkbox"/>

Objectives:
.....
.....

ConfoCor 2:

ConfoCor 2 detection head	single-channel VIS 000000-1048-915	<input type="checkbox"/>
ConfoCor 2 detection head	dual-channel VIS 000000-1048-916	<input type="checkbox"/>

Control computer with 21" monitor	000000-1032-142	<input type="checkbox"/>
Control computer with two 21" monitors	000000-1032-143	<input type="checkbox"/>

Large system table	453031-0000-000	<input type="checkbox"/>
System baseplate	453030-0000-000	<input type="checkbox"/>

XY scanning stage for Axiovert 200 M BP	000000-1017-917	<input type="checkbox"/>
---	-----------------	--------------------------

The ConfoCor 2 in the configuration as checked above

was installed and handed to the customer in functional condition

on

by

Phone:

Fax:

The customer has been instructed on how to operate and
maintain the equipment.

(Place).....(date).....

.....
Carl Zeiss Jena GmbH
Microscopy Division

.....
Customer

One copy to be kept by customer
One copy to be kept by Carl Zeiss

1 **Notes on Device Safety**

This chapter provides general notes on device safety, safe operation, and possible hazards caused by disregarding the operating manuals.

2 **Setup Requirements**

The Setup Requirements chapter outlines the installation and supply requirements of the ConfoCor 2, together with the relevant specifications.

3 **Operation**

Detailed software description.

4 **Appendix**

List of key words.

CHAPTER 3 OPERATION

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3 OPERATION

3.1 General

This chapter describes the operation of the Fluorescence Correlation Spectrometer ConfoCor 2 exemplified by typical applications in conjunction with the ConfoCor 2 software and its graphic user environment. It will be much easier to understand if you are already familiar with fluorescence correlation spectroscopy in general.



Before switching on the ConfoCor 2, please carefully read chapter 1 entitled *Notes on device safety*.

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

3.1.1 Software

The ConfoCor 2 software, Version 3.2, controls the microscope, the FCS-Detection unit and laser modules, tools (filters, stand, CLM) and the data acquisition process, and displays and analyzes the data. It is based on the network-capable graphic 32-bit Microsoft ® WINDOWS NT 4.0 / WINDOWS 2000 operating system.

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The installation of the software for the ConfoCor 2 and the basic settings of the equipment components are carried out by 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.

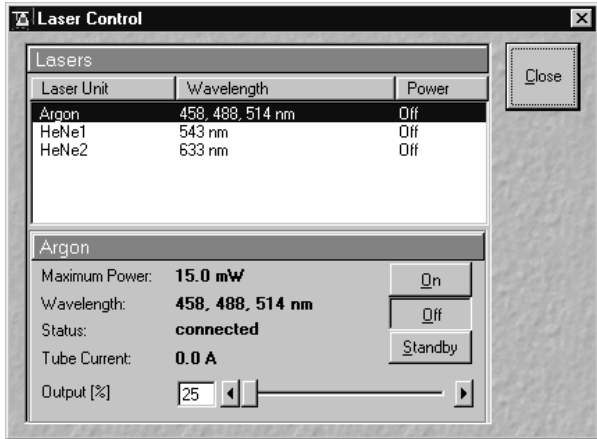
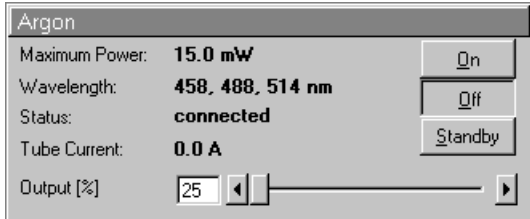
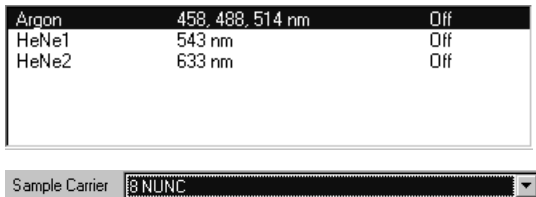


The ConfoCor 2 software is menu-controlled and normally uses its own windows for the activation of the various functions; within these windows, further submenus (panels) can be displayed and removed again.

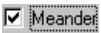

Theoretically, the number of simultaneously opened windows for software operation or image display is unlimited, but should not be too excessive so that an overview is still possible.

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.

3.1.2 User Interface and Text Formats

3.1.2.1 WINDOWS and Window Elements

Window element	Description / Explanation
	<p>Window (e.g.: Laser Control window)</p> <ul style="list-style-type: none"> Window displayed after activation of a function button (e.g.: Laser button in the Configuration subordinate toolbar).
	<p>Panel (e.g.: Argon panel)</p> <ul style="list-style-type: none"> Limited function range within a window
	<p>List box or selection box</p> <ul style="list-style-type: none"> Selection of one of the displayed options at a click of the mouse. Open the box by clicking on the arrow button.
	<p>Input box</p> <ul style="list-style-type: none"> Input of text or numeric values via the keyboard.
	<p>Scrollbar with slider</p> <ul style="list-style-type: none"> 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. <p>Press the Shift or Ctrl key while clicking on the arrow button to change the numeric values in coarse or fine steps.</p>

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

3.1.2.2 Text Formats

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

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

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

3.1.2.3 Software Operation

The ConfoCor 2 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 the online help for the relevant information.

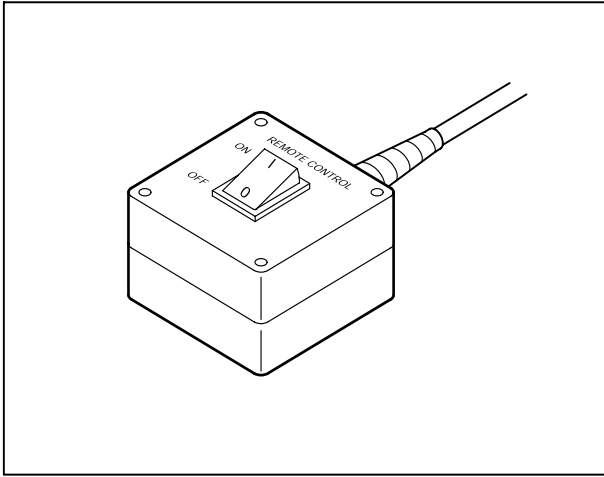


Fig. 3-1 REMOTE CONTROL switch

3.2 Switching on the System

It is recommended to switch on the instrument hardware before booting the control computer.

- The ConfoCor 2 is turned on by switching the **REMOTE CONTROL** switch (Fig. 3-1) to position "**ON**". This switches on all system components.
- If the **REMOTE CONTROL** switch is not used, turn on the system with the "**I**" on the laser module.
- In addition, the jumper plug supplied must be plugged in at the **POWER REMOTE CONTROL** terminal.

If the **REMOTE CONTROL** switch is in the position "**ON**", the following processes will run:

- The entire system is switched on
- Microscope and laser will be ready after a short while
- Computer boots up
- Computer hardware system test runs



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

When the system is controlled by the **REMOTE CONTROL** switch, the other switches on the laser module are without function.

The monitor shows a dialog box for selection of the operating system version (Fig. 3-2).

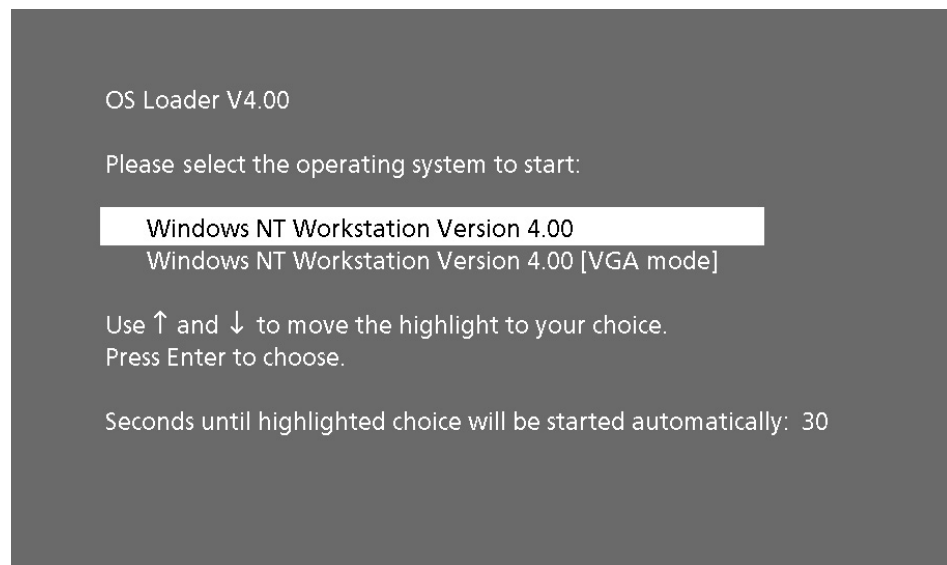


Fig. 3-2 Selecting the operating system version

- 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. 3-3).

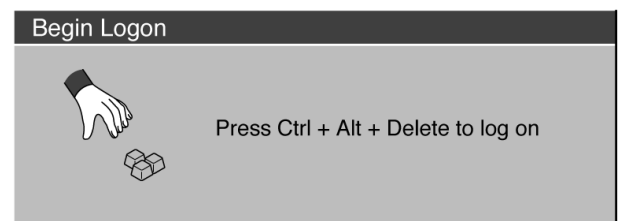


Fig. 3-3 Begin Logon window

3.2.1 Log on to WINDOWS NT

- Now 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 (Fig. 3-4).
- Enter the valid user name into the **User name** text box.
- Enter the valid password into the **Password** text box.

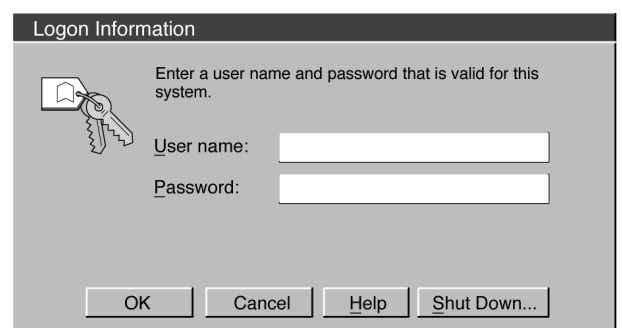


Fig. 3-4 Logon Information window

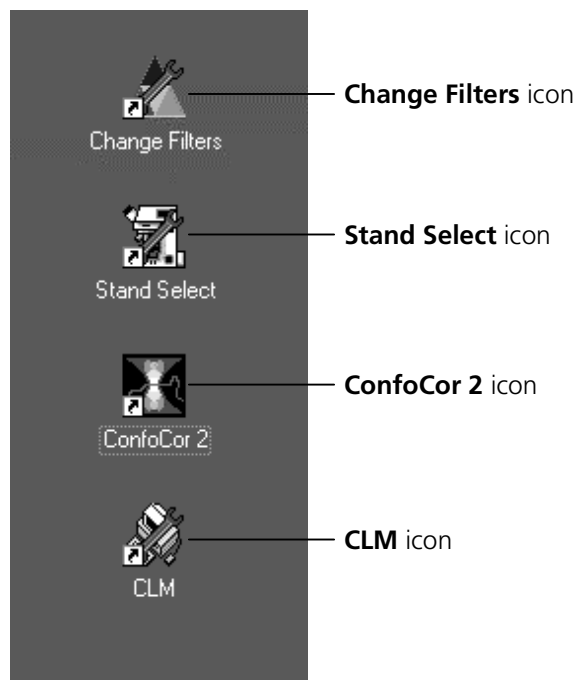


Fig. 3-5 **WINDOWS NT operating system desktop (section)**

- After you have made the two entries, confirm them by clicking the **OK** button or by hitting the **Enter** key.
 - The WINDOWS NT operating desktop appears on the screen, showing a number of icons (Fig. 3-5).

3.2.2 **Starting the ConfoCor 2 Software Program**

The ConfoCor 2 software program can be performed in two different modes (with or without connected instrument system). In the on-line mode, the entire program package (instrument control, data acquisition, data analysis and presentation) is available, while only a part of the software functions (data analysis only of already stored FCS-data files) 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, the lasers and the microscope need not be switched on.

 The use of the ConfoCor 2 software program requires installation of the relevant dongle.

- Double-click on the **ConfoCor 2** icon on the desktop of WINDOWS NT or WINDOWS 200 to start the ConfoCor 2 software program (see Fig. 3-5).
 - The **ConfoCor 2 Switchboard** menu appears on the screen (see the following page, Fig. 3-6).

- Select the mode you want to run by clicking the appropriate button: **Online Mode** or **Offline Mode**.

- **Online Mode**

Clicking on this button activates the complete ConfoCor 2 hardware.

- **Offline Mode**

This item allows you to process and analyze previously acquired data files with the ConfoCor 2 software. In this mode, control of the hardware (laser module ...) is not possible.



Fig. 3-6 ConfoCor 2 Switchboard menu (Online Mode activated)

- Click the **Start** button to run the ConfoCor 2 software.
- If you click the **Exit** button, the program will be terminated.

3.3 Quickstart

3.3.1 Analysis Method

Central paradigm of the user interface conception of the ConfoCor 2 is the **analysis method**. The analysis method is a complete description of the measurement and data evaluation method. It includes instrument settings such as choice of laser lines and their respective intensities, choice of filters and beam splitters, pinhole settings, measurement time(s), repeat counts and so on. Also part of the measurement method are preset parameters for the fitting procedure.

Once a method is worked out and filed, it can be easily reused. We also provide a convenient way to develop and optimize methods.

3.3.2 General Preparations

After clicking the **Start** button in the **ConfoCor 2 Switchboard** menu, the instrument is running and the **Main** menu appears (Fig. 3-7). From this moment the instrument can be almost entirely controlled by software.

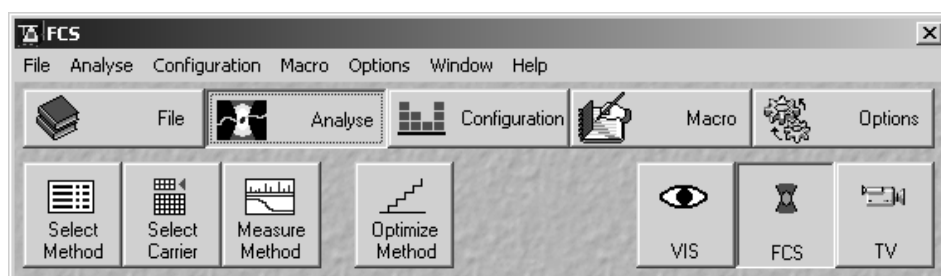


Fig. 3-7 Main menu

3.3.2.1 Setting the Axiovert 200 M Microscope

This step is used to set:

- microscope objective
 - specimen position
 - specimen focus
- The first step is to set up the microscope for FCS measurements. This is done in the **Axiovert Control** window (Fig. 3-9) or manually at the microscope stand. This window is reached by clicking the **Micro** button in the **Configuration** subordinate toolbar of the **Main** menu.

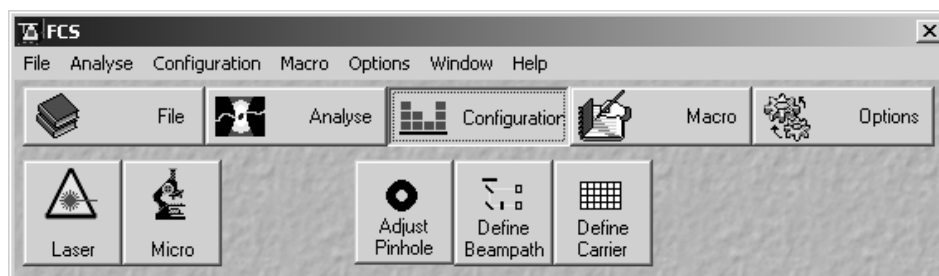


Fig. 3-8 Main menu with activated Configuration subordinate toolbar

To set-up the Microscope:

- Swing the required objective for FCS measurements into the working position. This is performed by clicking in the **Objective** panel of the **Axiovert Control** window.

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

- Set the reflector turret to the position without an epifluorescence filter-set by choosing the **None** entry.
- Activate the **Transmitted Light** panel by clicking on the **Transmitted Light** button and switch off the transmitted light attachment.
- Close the window using the **Close** button.
- Click on the **FCS** button on the right-hand side of the **Analyse** subordinate toolbar in the **Main** menu (Fig. 3-7) to select the FCS detection head.

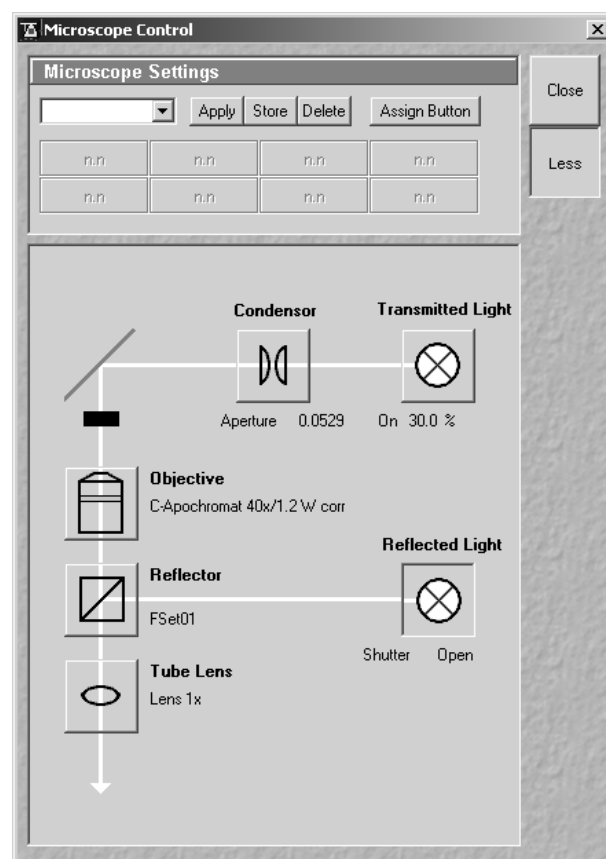


Fig. 3-9 Axiovert Control window

3.3.2.2 Turning on the Lasers

This step is used to switch the lasers and set the intensity.

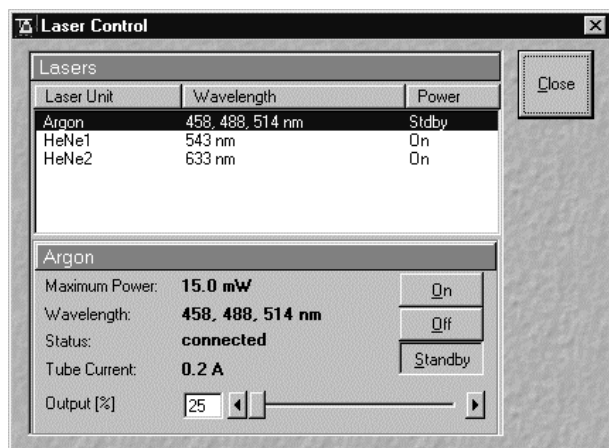


Fig. 3-10 Laser Control

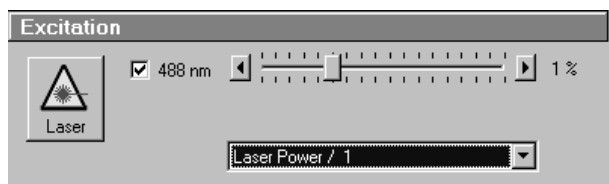


Fig. 3-11 Laser Intensity Control

- Click on the **Laser** button in the **Configuration** subordinate toolbar of the **Main** menu.
 - You will see a **Laser Control** window with a list of available lasers (Fig. 3-10). Your screen may differ from the one displayed.
- Click on the laser(s) featuring the appropriate wavelength to excite the dyes in your sample.
- In case of the argon laser, click on the **Standby** button first.
 - **Warming up** appears in the line status.
 - After 2 minutes when the warming-up phase is finished, the **Ready** message appears.
- Then click on the **On** button.
 - The laser is switched on.
- Use **Output** [%] scrollbar to set the intensity to approximately 6 to 8 ampere.
- In case of the HeNe laser, click on the **On** button directly.
- Close the window using the **Close** button.

3.3.2.3 Selection of a Method

This step is used to select a defined method with prepared parameters.

- Click the **Select Method** button in the **Analyse** toolbar subordinate of the **Main** menu. The **Method Selection ...** window will appear (Fig. 3-12).

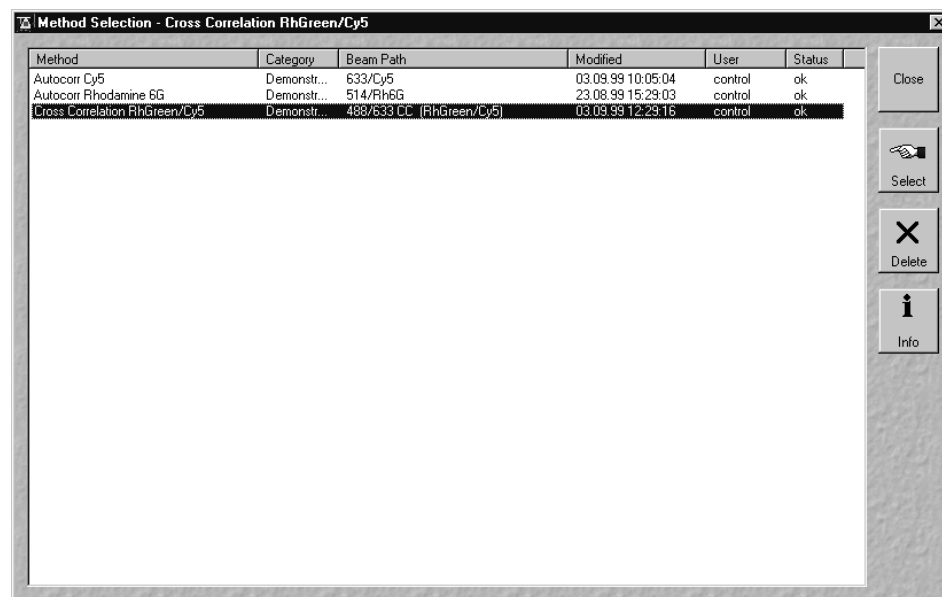


Fig. 3-12 Method Selection ... window

- 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.

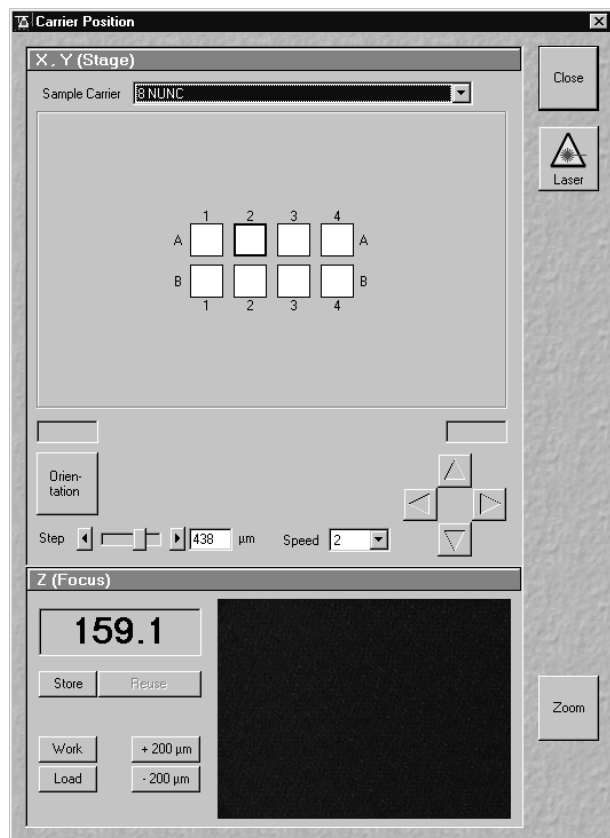


Fig. 3-13 Carrier Position window

3.3.2.4 Mounting and Orienting the Sample

This step is used to mount a sample on the motorized stage and to orient the sample with respect to the center of the used objective (or with respect to the optical axis).

- This is done with the **Select Carrier** button of the **Analyse** toolbar subordinate of the **Main** menu (Fig. 3-7).
 - The sample select window (**Carrier Position** window) pops up (Fig. 3-13).



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.

(1) Selecting Carrier

- Select the sample carrier you are currently using in the **Sample Carrier** selection box of the **Carrier Position** window (Fig. 3-13).

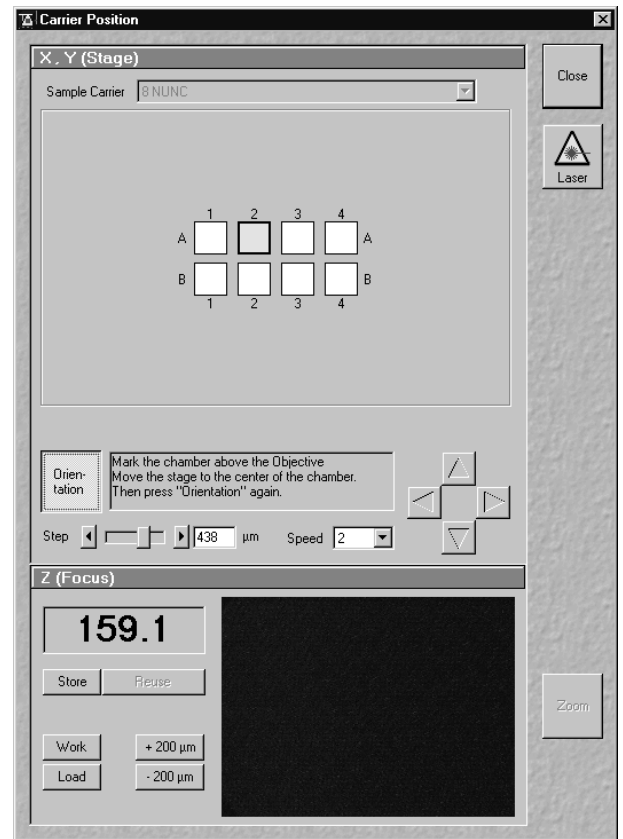
(2) Orienting the Sample in x and y

- Now click on the **Orientation** button in the **Carrier Position** window (Fig. 3-14).
 - Select one of the chambers of the sample for orientation in x and y via mouse click.
 - 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.
-
- Now you can move any chamber into the measurement position by clicking the appropriate field on the sample carrier.

The selected chamber is then approached automatically 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.



If 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.

**Fig. 3-14 Sample drives unlocked**

(3) Positioning the Focus in z for Solutions

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.

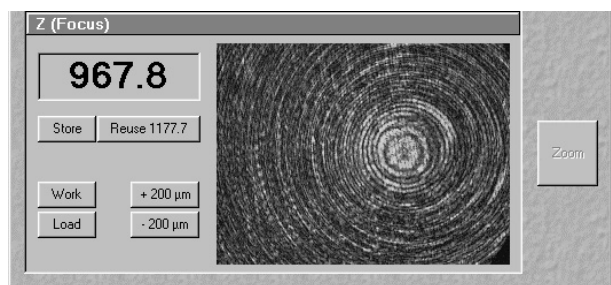


Fig. 3-15 Camera image far away from the lower cover slip surface

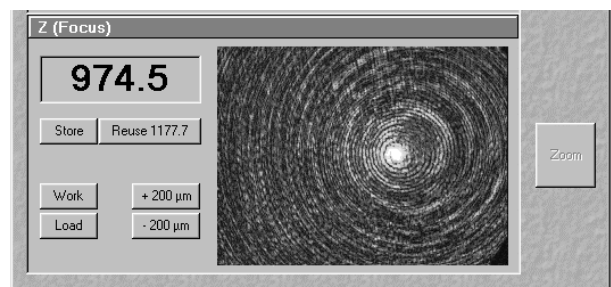


Fig. 3-16 Camera image near the lower cover slip surface

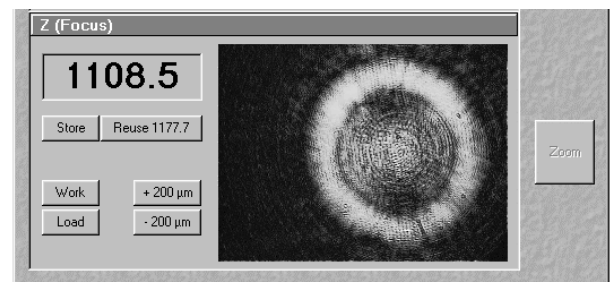


Fig. 3-17 Lower cover slip surface

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.
- The camera window shows interference fringes caused by the laser light (Fig. 3-15).
- 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. 3-16).
- Continue cautiously moving upwards. If the camera image looks like (Fig. 3-17), 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. 3-18). The upper cover slip surface has been found.
- Store the reached position using the **Store** button.



All positions are given in relative coordinates.

- 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.
- Close the **Carrier Position** window by clicking the **Close** button.

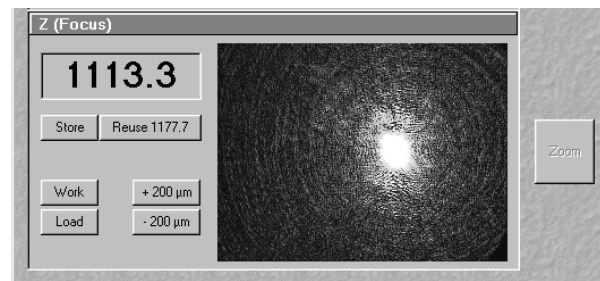


Fig. 3-18 Upper cover slip surface

3.3.2.5 Running the Measurements

Now measurements can be run.

- Click the **Measure Method** button in the **Analyse** toolbar subordinate of the **Main** menu. The **Method Measurement ...** window will appear on the screen (Fig. 3-19). The name of the currently used method will appear in the status bar of the window.
- Give your measurement a **Task Name** by entering a string in the **Task Name** input box. Under this name, all the measurement data will be stored, regardless of whether the measurement covers only a single sample or a whole series. It is recommended to choose a name which is easy to remember.

The **Comment** input box allows you to store a comment on the measurement you are performing.

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.

The buttons **Select All** and **Clear All** select or deselect all chambers.

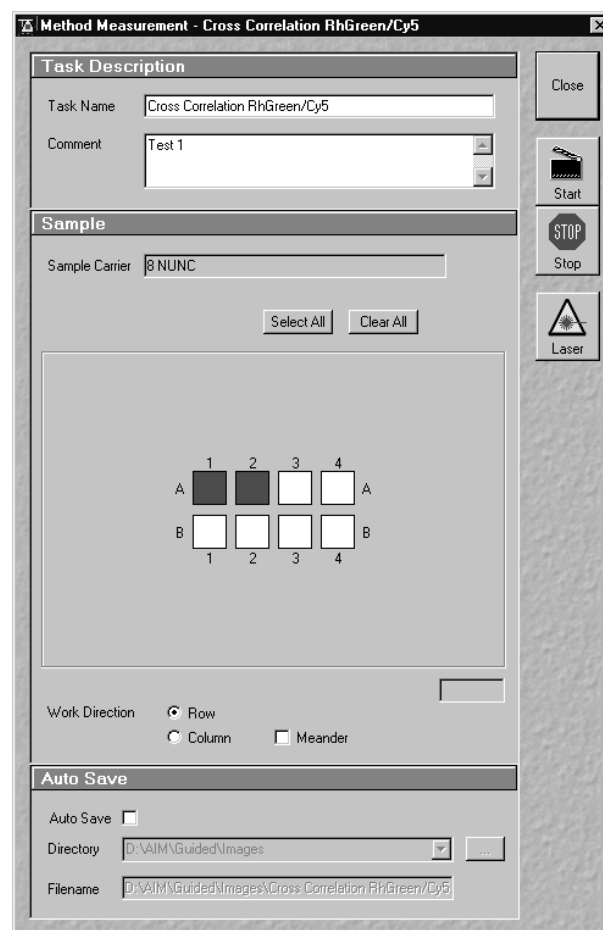


Fig. 3-19 Method Measurement ... window

- 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 (**Row, Column, Meander**) can be chosen as well.

- Click on the **Start** button on the right-hand side of the **Method Measurement ...** window.

3.3.3 Data Evaluation and Result Presentation

- When you click **Start** from the **Method Measurement ...** window, the **Data Evaluation** window will appear (Fig. 3-20).

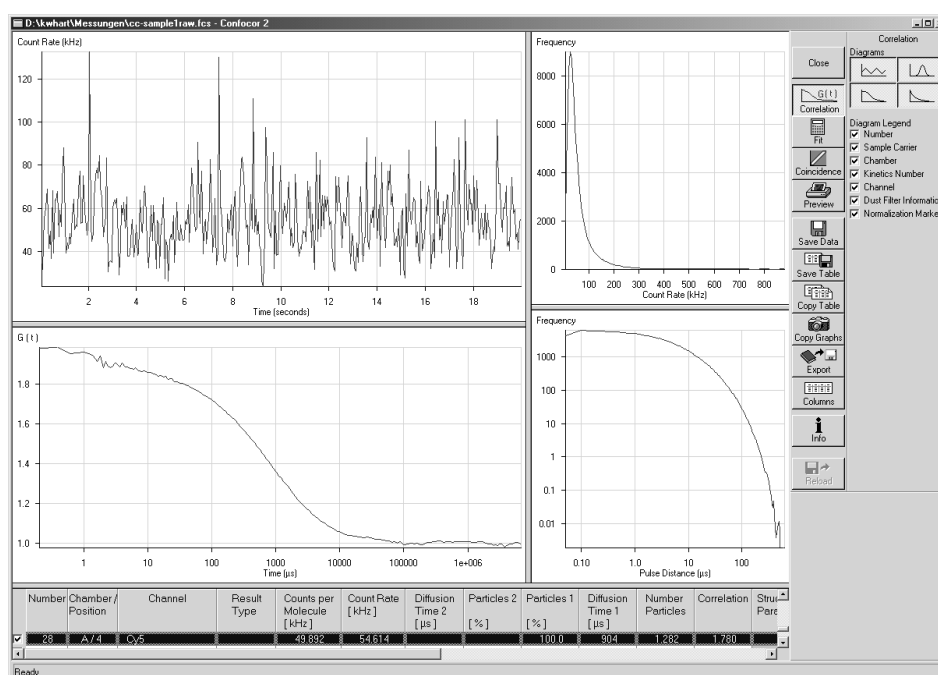


Fig. 3-20 Data Evaluation window

Up to four windows with different graphs become visible in the upper left part of the window.

The **Count Rate** diagram:

- Here the count rate(s) vs. running time is (are) displayed. If a cross correlation set-up is used, the count rate trace for each channel is displayed.

The **Correlation Curve** graph:

- In case of auto correlation measurement, the developing correlation curve is displayed.
- In case of cross correlation measurement, three curves will be shown: the two auto correlation curves for each of the channels and the cross correlation curve.

The **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.

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.

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.

- The current line in the table is marked (preset color: gray).
- Special situations which occurred during the fit are marked with special colors in the table:
 - Unsuccessful fit (preset color: red)
 - Fixed fit parameters (preset color: gray)
 - Auto cut-off (preset color: yellow)
- The used colors can be configured in the **Columns** menu.
- The displayed columns can be chosen in the **Columns** menu.
- The order of the columns in the table can be altered by standard WINDOWS means: the column caption can be dragged to the desired position.
- 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.

3.3.3.1 Display Correlation

If you click on the **Correlation** button (Fig. 3-20), the **Diagrams** subordinate at the very right-hand side of the window will appear. By clicking on the appropriate button the diagram can be toggled between **ON** and **OFF**.

3.3.3.2 Manual Fit

When you click on the **Fit** button, a **Fit** menu subordinate will appear (Fig. 3-21). 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.

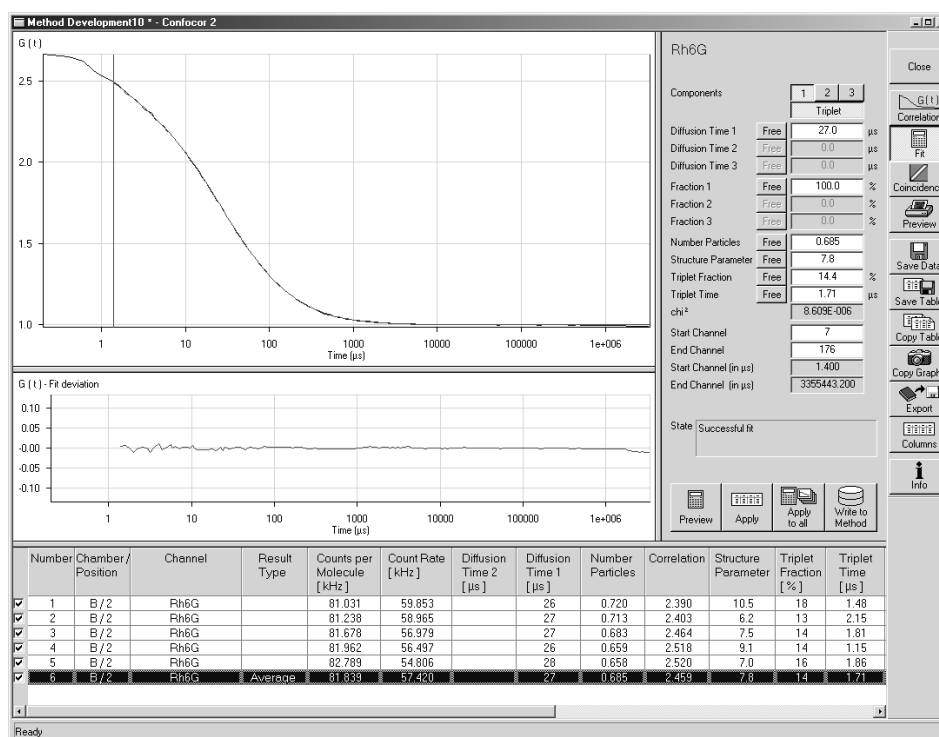


Fig. 3-21 Display fit

- 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 parameter.
 - 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.
- With **Start Channel** and **End Channel** it is possible to determine which part of the correlation curve should be fitted to the model.



Caution: The number indicates how many channels are omitted from the respective end.

- When you have finished, click the **Calculate** button and the fit procedure will be run. 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.

3.3.3.3 Data Storage and Export

- **Save Data** allows you to save the whole data set (curves, fitting results, fit parameters). 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.

3.3.3.4 Exit

- To exit the program, click the **Exit** button in the **File** toolbar subordinate of the **Main** menu.

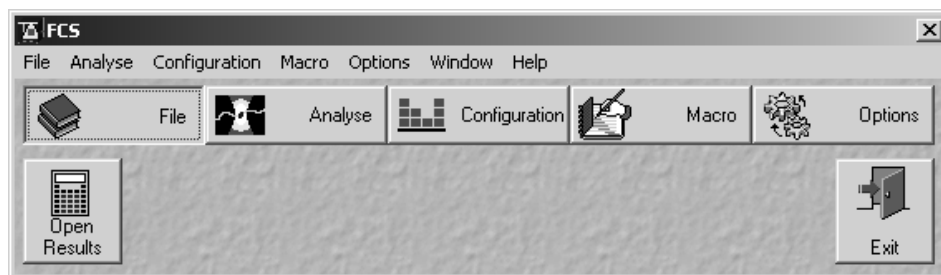


Fig. 3-22 Main menu – File subordinate toolbar

- After clicking **Exit**, the switchboard will appear.
- You can decide at this point to restart the program by clicking on **Start** again.
- If you click **Exit** on the switchboard, the program will be closed.

 If any of the lasers is still switched on, a warning will appear and ask you to switch off the laser.

3.4 Detailed Description

3.4.1 Main Menu

- Click on the **Start** button in the **ConfoCor 2 Switchboard** menu.
 - The ConfoCor 2 **Main** menu appears on the screen.

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



Fig. 3-23 ConfoCor 2 Switchboard menu

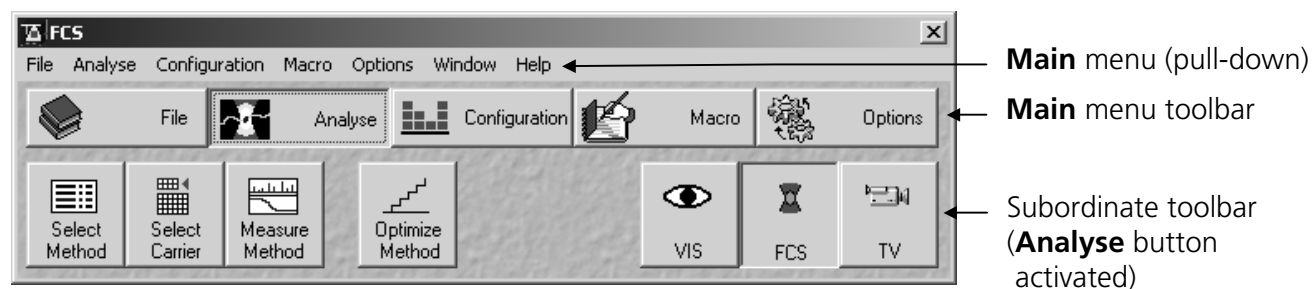


Fig. 3-24 ConfoCor 2 Main menu

The major functions can be selected in the **Main** menu either via the pull-down menus in the menu bar or via the toolbar.

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

The buttons of the **Main** menu (upper toolbar) enable following functions:

File button	Open results (FCS and Raw data files). Ending (Exit) the ConfoCor 2 program.
Analyse button	Selecting measurement method and carrier. Executing measurement and data analysis. Optimizing measurement method.
Configuration button	Calling up and setting the necessary operating parameters. During the preparation for and execution of FCS 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 load and run macros automatically (Macro play).
Options button	<p>For custom-configuration of software and hardware options.</p> <p>This menu item enables access to the collimator settings.</p> <p>In the Settings for User window you can specify essential operating modes, organized by registers, which have an effect on the user interface.</p>

3.4.2 File Menu

The **Open Results** function of the **File** menu permits stored FCS data files to be opened.

The **ConfoCor 2 software program** can be ended via the **Exit** function.

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

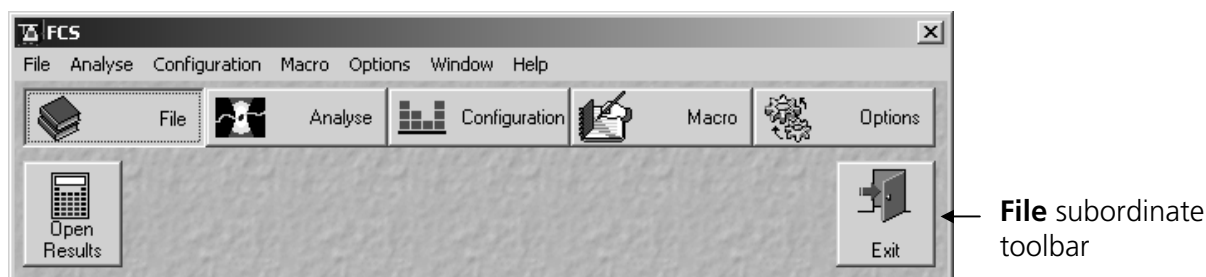


Fig. 3-25 File menu

3.4.2.1 Open Results - Load a FCS data file

This function is intended for use of already measured data. It also allows you to import data stored with the ConfoCor, the predecessor of the ConfoCor 2.

- To open previously stored data, click on the **Open Results** 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. 3-27).



Fig. 3-26 Evaluate window

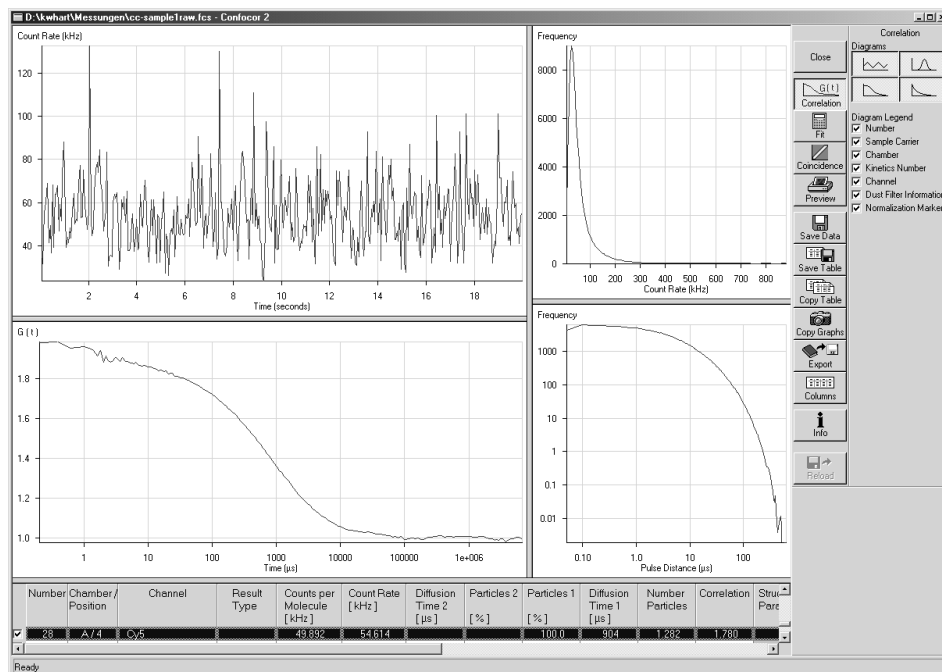


Fig. 3-27 FCS data file window

When the files have been opened, the data display window as described in the beginning of chapter 3.5, *Data Evaluation and Result Presentation*, will be shown. It offers the same possibilities as described in chapter 3.5, page 3-80.

3.4.2.2 Exit the ConfoCor 2 Software Program

- Close all open windows of the ConfoCor 2 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 ConfoCor 2 **Main** menu will be closed and the **ConfoCor 2 Switchboard** menu appears on the screen.
- You can decide at this point to restart the program by clicking on **Start** again.
- If you click **Exit** on the switchboard, the program will be closed.



If any of the lasers is still switched on, a warning will appear and ask you to switch off the laser.

3.4.3 Analyse Menu

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

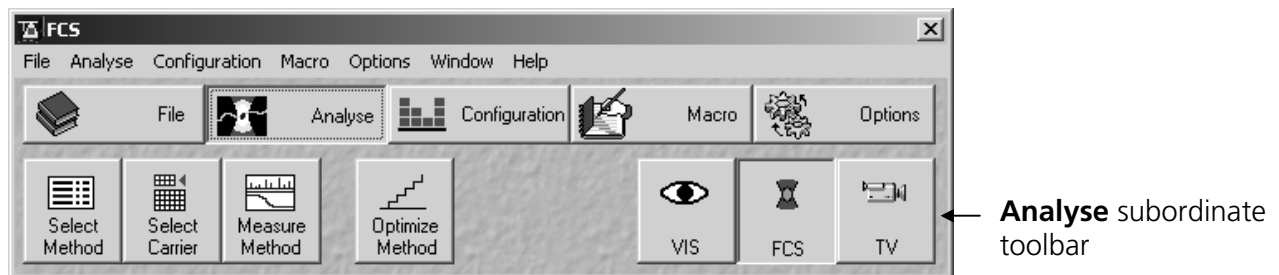


Fig. 3-28 Analyse menu

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

- Select method.
- Select carrier.
- Measure method.
- Optimize method (if necessary).

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

For the measuring process, the **FCS** button in the toolbar subordinate to the **Analyse** button must be activated.

3.4.3.1 Select Method

The **Select Method** function shows all existing measuring methods for selection or deletion.

- Click on the **Select Method** button in the **Analyse** subordinate toolbar of the **Main** menu.
 - This opens the **Method Selection ...** window (Fig. 3-29). The name of the currently selected method will appear in the status bar of the window.

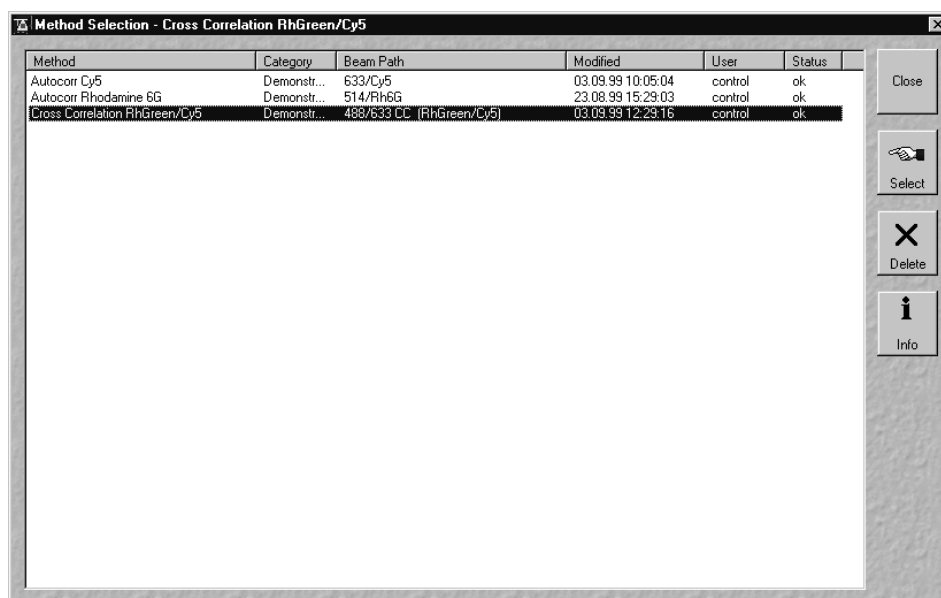


Fig. 3-29 Method Selection ... window

- 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. 3-30).

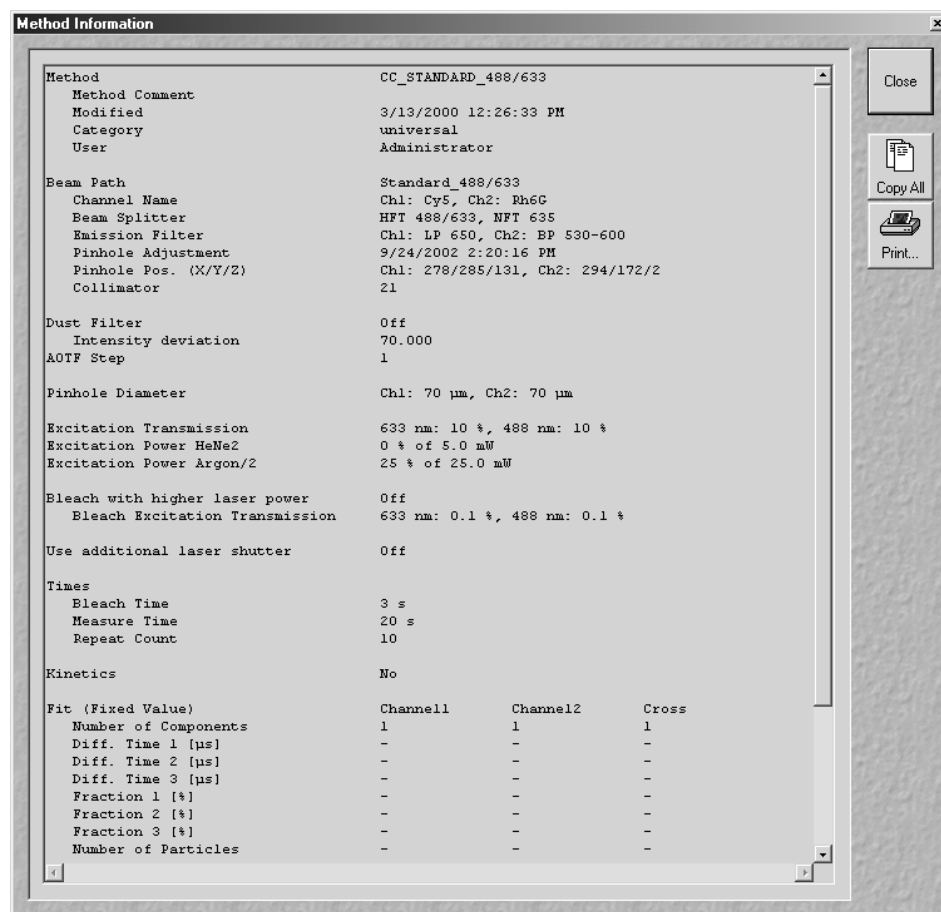


Fig. 3-30 Detailed description of the analysis method

- 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.

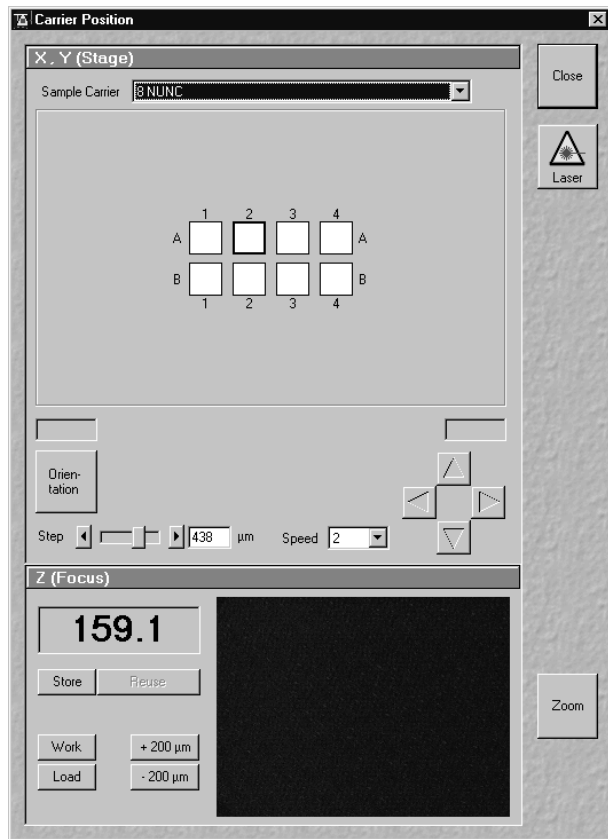


Fig. 3-31 Carrier Position window

3.4.3.2 Select Carrier

This 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.

(1) Opening / Closing the Carrier Position window

- Click on the **Select Carrier** button in the **Analyse** subordinate toolbar of the **Main** menu (Fig. 3-28).
 - The sample select window (**Carrier Position** window) pops up (Fig. 3-31).



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 **Configuration** menu, page 3-53).
- Close the **Carrier Position** window by clicking the **Close** button.

(2) Function description**(a) 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.

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.

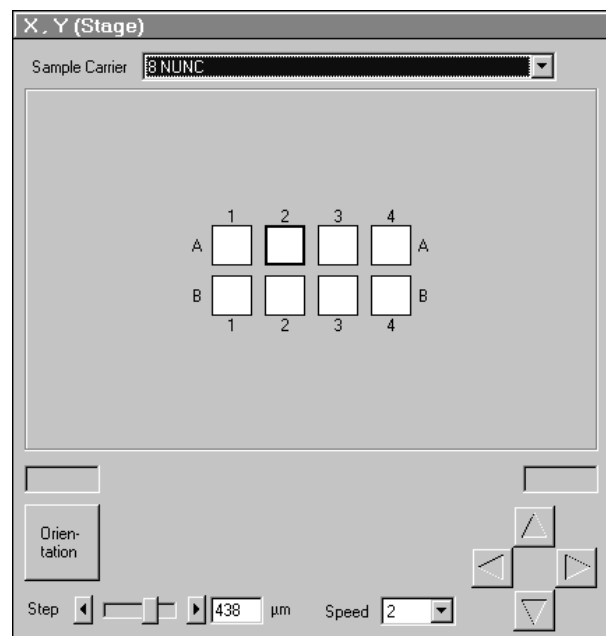
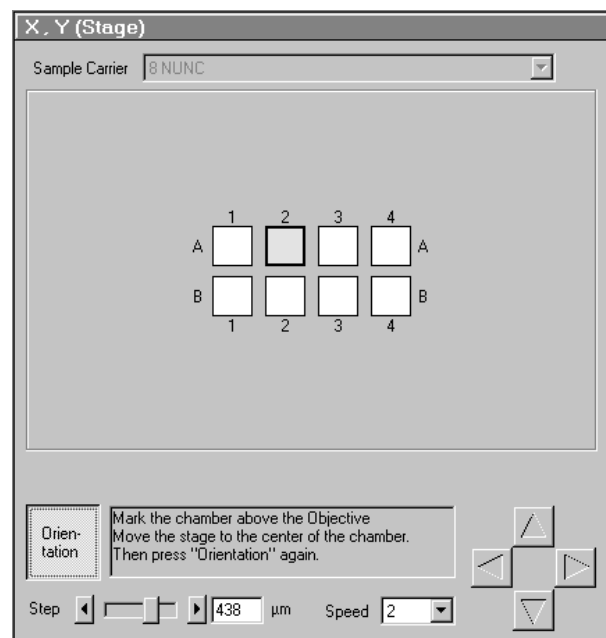


Fig. 3-32 X, Y (Stage) panel

Orienting the Sample in x and y

- Now click on the **Orientation** button in the **X, Y (Stage)** panel (Fig. 3-33).
- 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.
- 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.



**Fig. 3-33 X, Y (Stage) panel
(Sample drives unlocked)**

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.

(b) Z (Focus) panel

The functions of the **Z (Focus)** panel allows you to position the focus in z-direction. The 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	Lowars 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. 3-34).

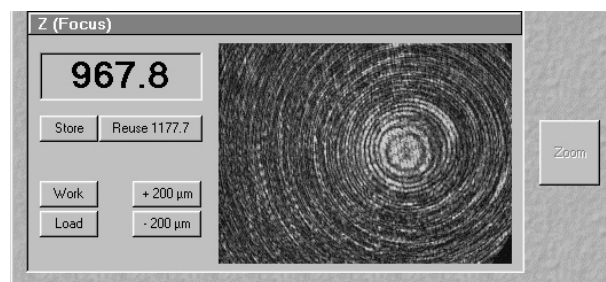


Fig. 3-34 Camera image far away from the lower cover slip surface

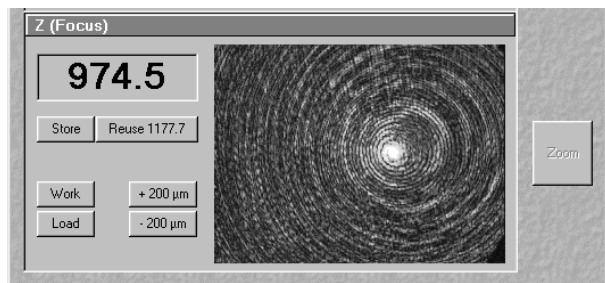


Fig. 3-35 Camera image near the lower cover slip surface

- 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. 3-35).

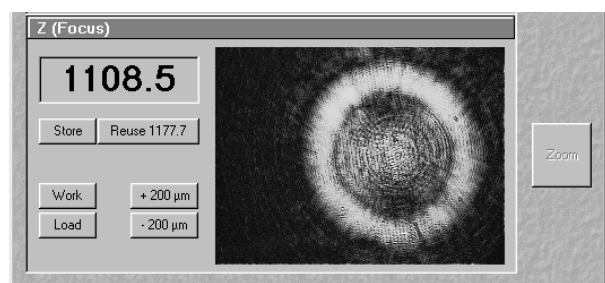


Fig. 3-36 Lower cover slip surface

- Continue cautiously moving upwards. If the camera image looks like (Fig. 3-36), 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. 3-37). The upper cover slip surface has been found.
- Store the reached position using the **Store** button.

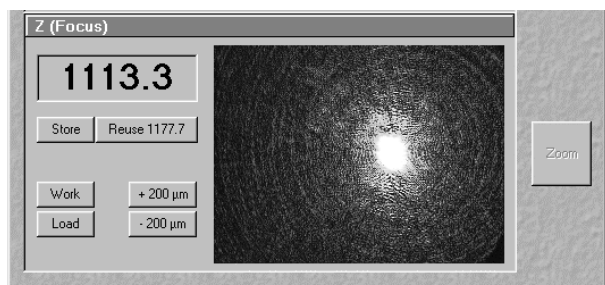


Fig. 3-37 Upper cover slip surface

- 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.

3.4.3.3 Measuring Method

This function opens the **Method Measurement ...** window. It is used for setting the measurement parameters and for starting the measurement.

(1) Opening / Closing the Method Measurement ... window

- Click on the **Measure Method** button in the **Analyse** subordinate toolbar of the **Main** menu (Fig. 3-28).
 - The **Method Measurement ...** window will appear on the screen (Fig. 3-38). 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.

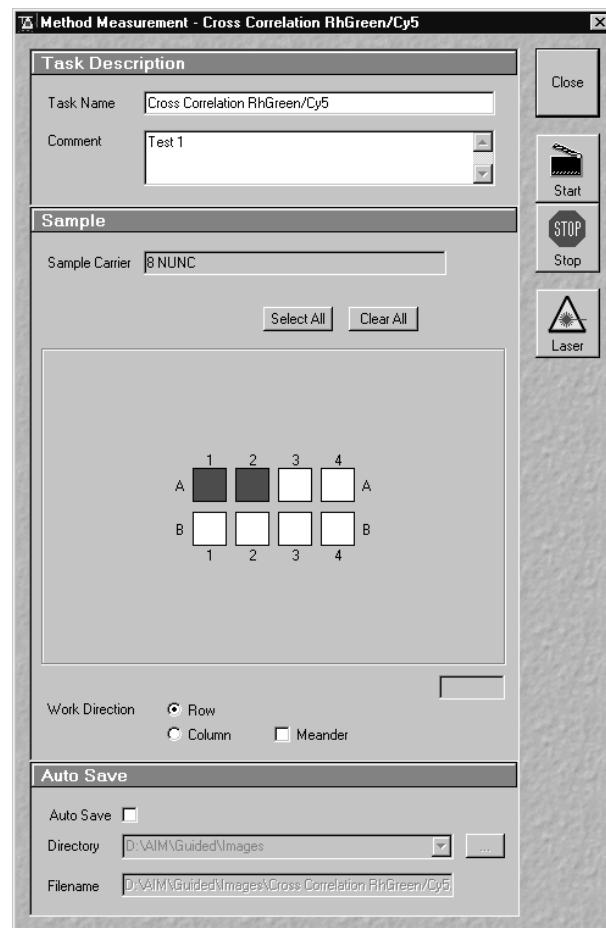
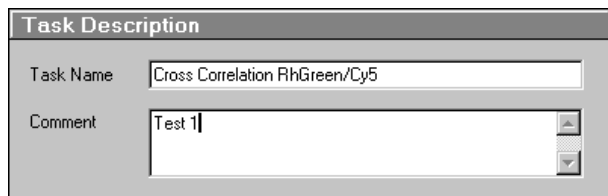


Fig. 3-38 Method Measurement ... window

(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.

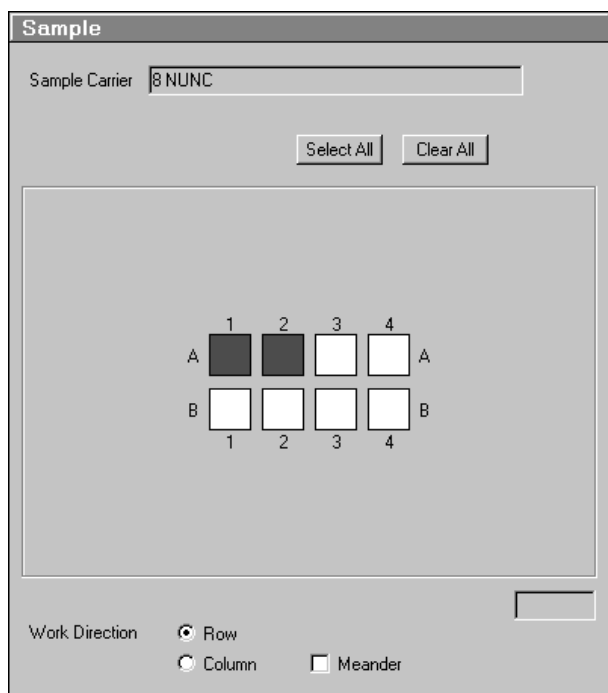
**Fig. 3-39 Task Description panel****(a) Task Description panel**

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. 3-40 Sample panel****(b) 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.

The following functions are available:

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 checkbox	If Meander is chosen, measurement of the row or column is made in meanders.

(c) 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** checkbox 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.

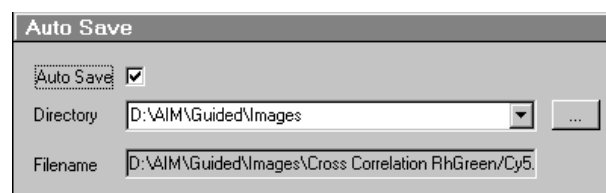


Fig. 3-41 Auto Save panel

(3) Measurement procedure

After setting of the parameters, the measurement can be performed.

- Click on the **Start** button on the right-hand side of the **Method Measurement ...** window to start the measurement and data analysis process.
 - The ConfoCor 2 data evaluation window will appear.

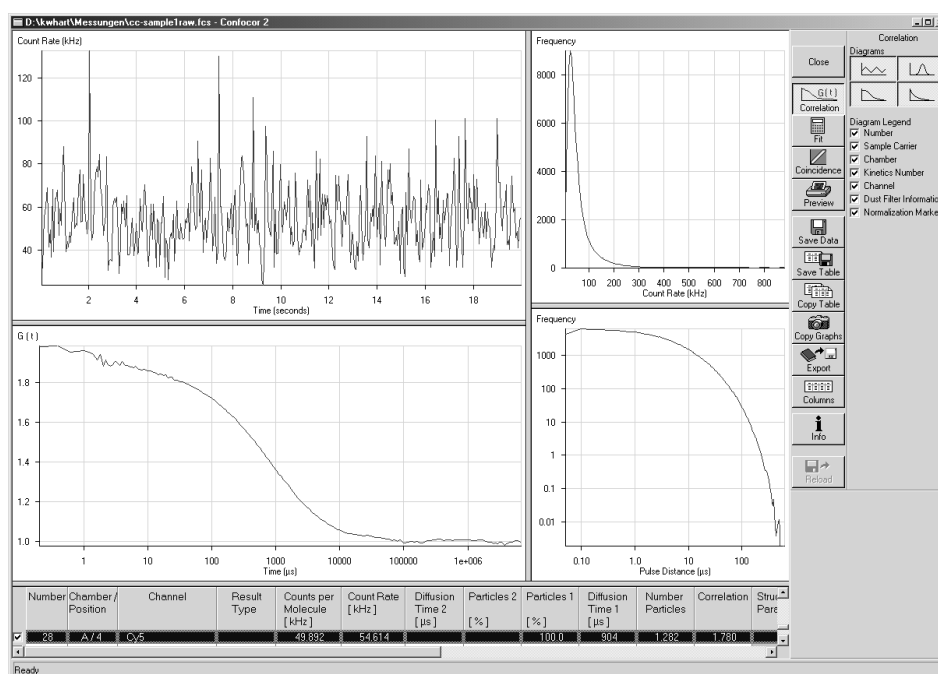



Fig. 3-42 ConfoCor 2 data evaluation window

 The further procedure will be explained in section 3.5, *Data Evaluation and Result Presentation*.

3.4.3.4 Optimize Method

This function opens the **Method Optimization ...** window. It is used for optimizing an existing method or for creating a new one.

(1) Opening / Closing the Method Optimization ... window

- Click on the **Optimize Method** button in the **Analyse** subordinate toolbar of the **Main** menu (Fig. 3-28).
 - The **Method Optimization ...** window will appear.
- Close the **Method Optimization ...** window by clicking the **Close** button.

(2) Function description

An analysis method has three major sections:

- Instrument** settings
- Analysis Procedure** settings
- Result Handling** parameters.

These major sections become available in separate subwindows by clicking the appropriate button in the **Method Optimization ...** window (description see next pages).

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 newly created measurement method.
Start button	Starts the measuring procedure for optimization.
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.

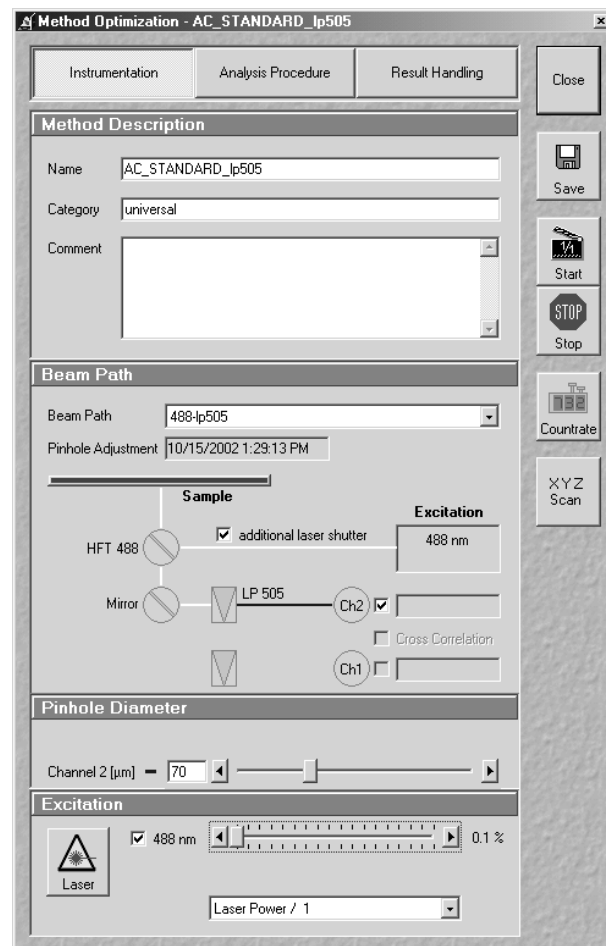


Fig. 3-43 Method Optimization ... window (Instrumentation subwindow)

(3) Instrumentation

- Click on the **Instrumentation** button on top of the **Method Optimization ...** window (Fig. 3-43).
 - The **Instrumentation** subwindow appears on the screen.

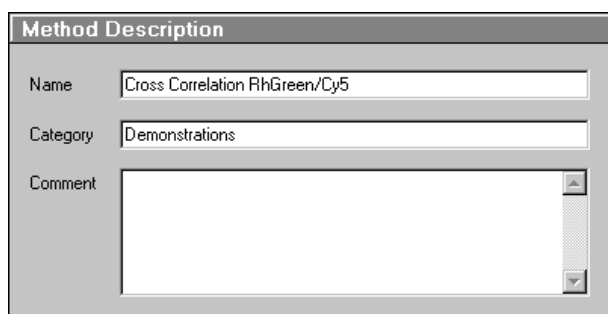


Fig. 3-44 Method Description panel

(a) 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** field, 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.

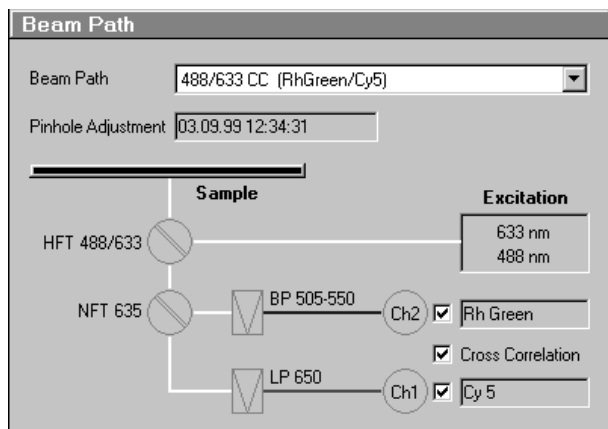


Fig. 3-45 Beam Path panel

(b) 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 checkboxes.



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 3.4.4.4, page 3-63) in the **Configuration** subordinate toolbar of the **Main** menu.

Once a new beam path is created and the pinholes calibrated, the beam path can be used in the **Method Optimization ...** window.

(c) 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. 3-46 Pinhole Diameter panel****(d) Excitation panel**

The **Excitation** panel allows you to activate / deactivate the wavelengths via checkboxes and to set the requested laser attenuation (%) using the sliders.

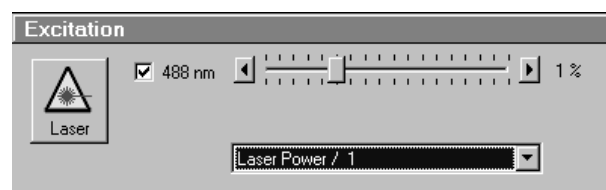
The selection box allows you to choose from four possibilities of setting the laser power:

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.

**Fig. 3-47 Excitation panel**

Furthermore, you can open the **Laser Control** window via the **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.

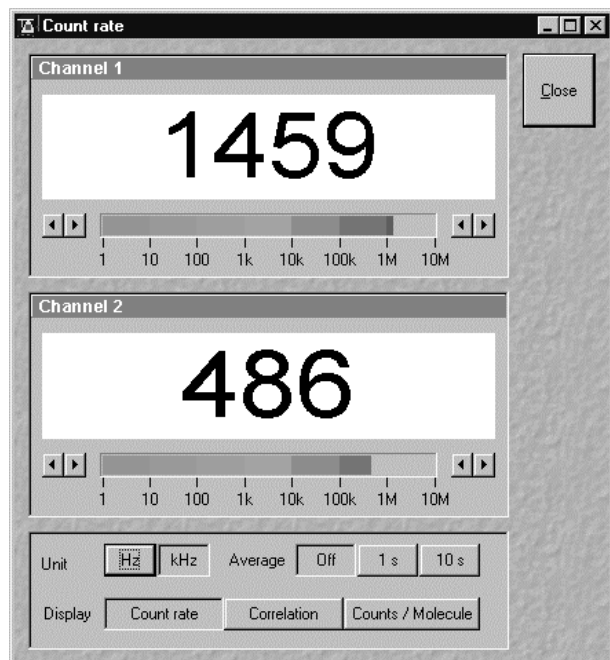


Fig. 3-48 Count rate window

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

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

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

(4) Analysis Procedure

- Click on the **Analysis Procedure** button on top of the **Method Optimization ...** window.
 - The **Analysis Procedure** subwindow appears on the screen.

(a) Times panel

In the **Times** panel you can set the values for **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.

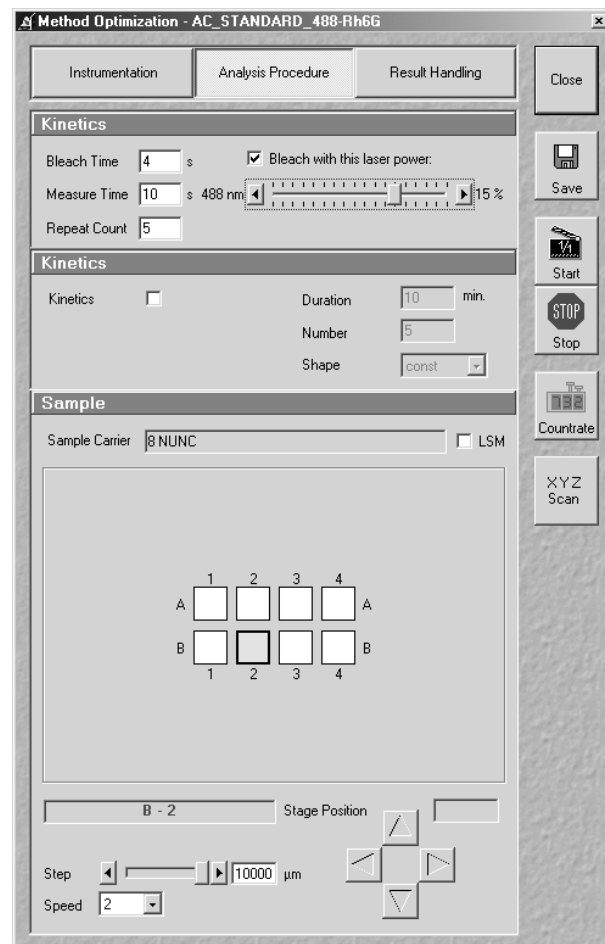


Fig. 3-49 Method Optimization ... window (Analysis Procedure subwindow)

- 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.

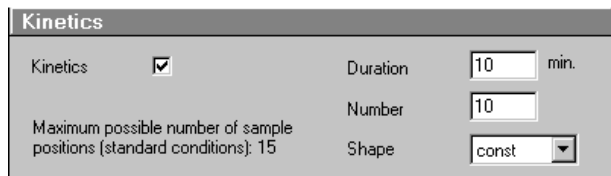


Fig. 3-50 Kinetics panel

(b) Kinetics panel

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

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

The input boxes **Duration** and **Number** permit entry of the overall duration of the measurement and the number of 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**.

(c) Sample panel

In the **Sample** panel you can select the chamber for the optimization procedure (only one chamber can be selected).

- 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).

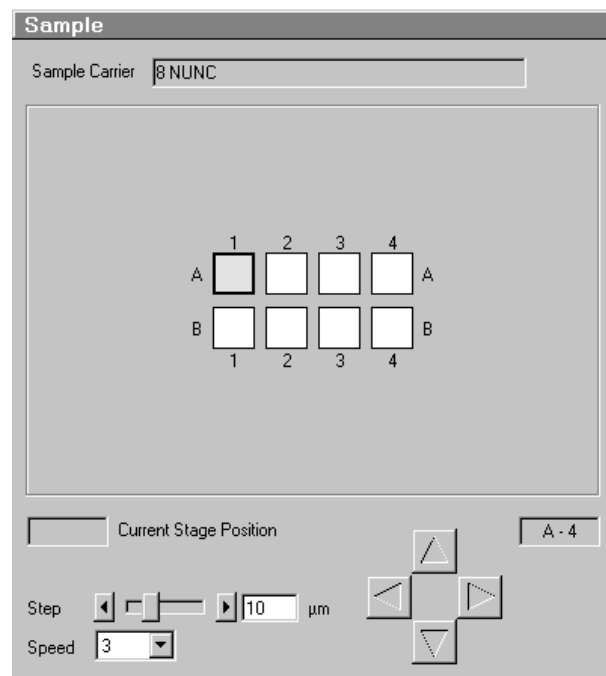


Fig. 3-51 Sample panel

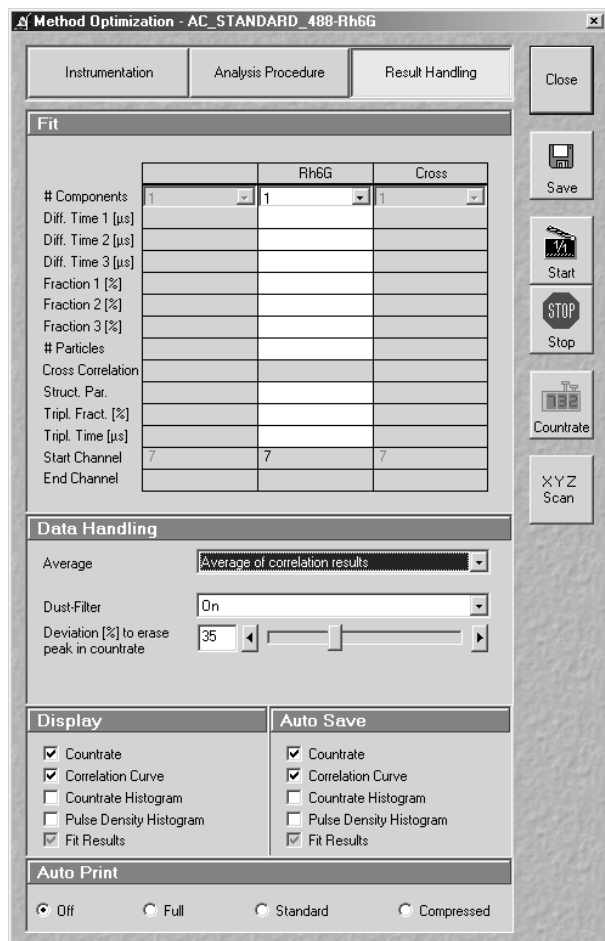
The travel speed of the microscope stage can be set to steps **1**, **2** and **3**.

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.



**Fig. 3-52 Method Optimization ... window
(Result Handling subwindow)**

(5) Result Handling

- Click on the **Result Handling** button on top of the **Method Optimization ...** window.
 - The **Result Handling** subwindow appears on the screen.

(a) 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 determined in previous measurements.

- Enter the model parameters to be fixed in the input boxes.

(b) 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.

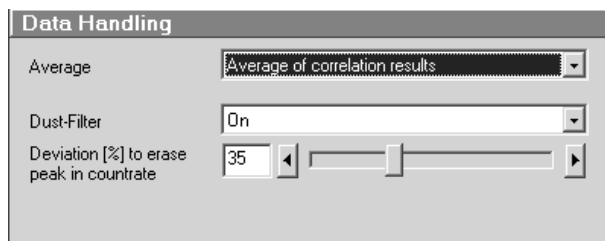



Fig. 3-53 Data Handling panel

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.

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

(c) 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.

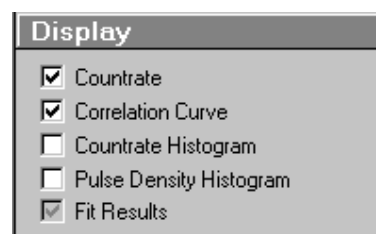


Fig. 3-54 Display panel

(d) 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.

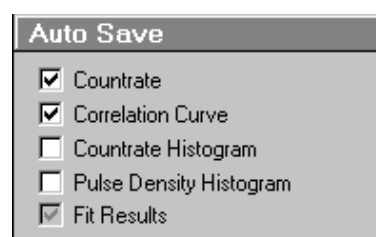


Fig. 3-55 Auto Save panel

(e) 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.

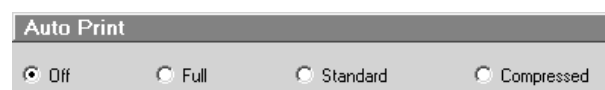


Fig. 3-56 Auto Print panel

(5) 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 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. 3-58) 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. 3-57).

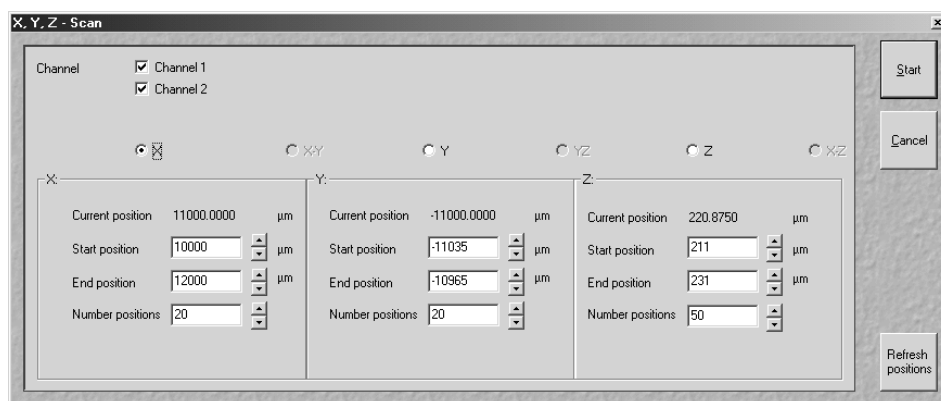


Fig. 3-57 X,Y,Z-Scan operating window

For X,Y,Z-Scanning, proceed as follows:

- If you use the Axiovert 100, first switch to the visual or TV mode using the silver sliders on the microscope stand so that you are able to view the object (the label near the silver sliders will show the correct silver 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 focussing the objective.
- Switch to the **FCS** mode by clicking on the **FCS** button in the **Analyse** 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** 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. 3-58).

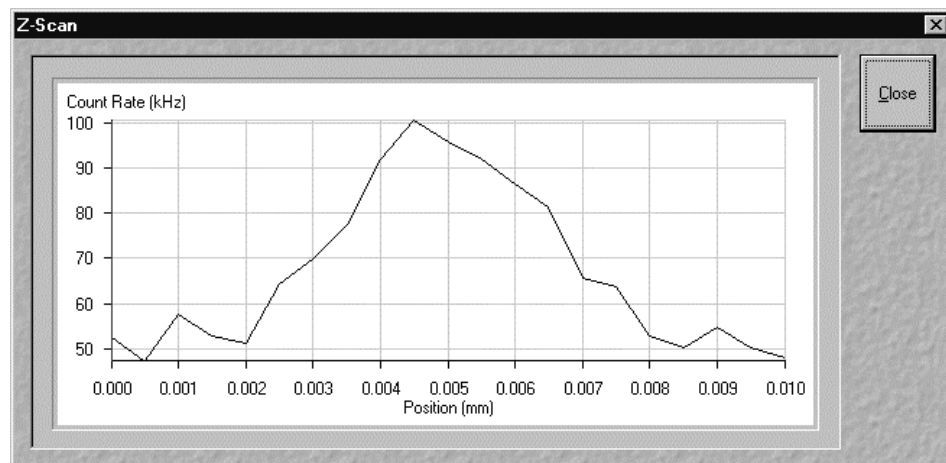


Fig. 3-58 Z-Scan result window

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

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.

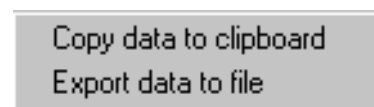


Fig. 3-59 Context menu of the X,Y,Z-Scan result window

- 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).

3.4.3.5 VIS, FCS and TV Buttons

The **VIS**, **TV** and **FCS** buttons are included in the **Analyse** 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
- **TV:** camera observation (if connected) via camera adapter of the binocular tube
- **FCS:** screen observation via laser excitation using the ConfoCor 2 and software evaluation

3.4.4 Configuration Menu

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

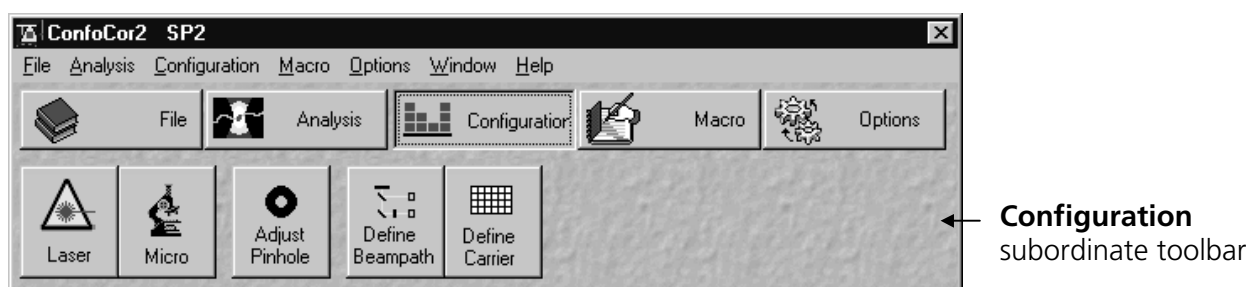


Fig. 3-60 Configuration menu

3.4.4.1 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.

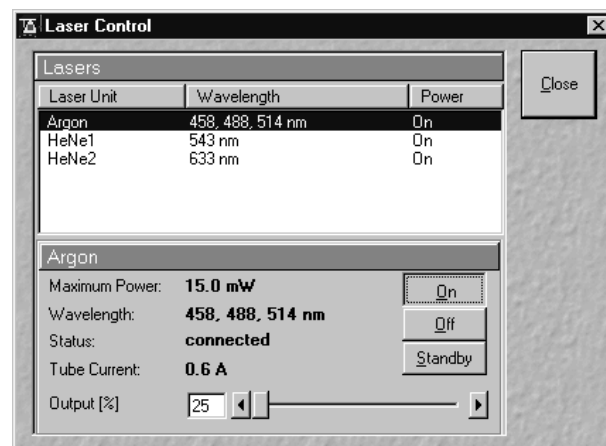


Fig. 3-61 Laser Control window

(1) Opening / Closing the Laser Control window

- Click on the **Laser** button in the **Configuration** 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.

(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 Argon) of the relevant laser. Sets the laser output for Argon.

(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:

The following 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.

The following applies to HeNe lasers:

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

3.4.4.2 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.

(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.

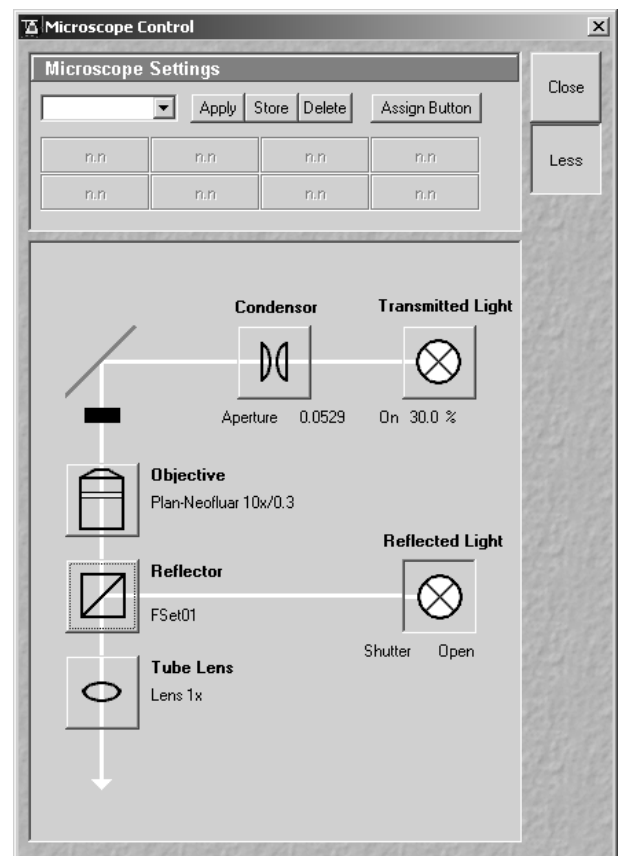


Fig. 3-62 Axiovert Control window

(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 condensers). 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.

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

- Turn on the HBO 50 power supply switch.
- Click on the checkbox 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** subordinate toolbar in the **Main** menu (Fig. 3-24) to select the FCS detection head.

(b) Transmitted-light observation

- Click on the checkbox for **Transmitted Light** ☒ **On**.
- Activate the condenser function in the **Condenser** 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** subordinate toolbar in the **Main** menu (Fig. 3-24) to select the FCS detection head.

3.4.4.3 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.

(1) Open / Close the Pinhole Adjustment window

- Click on the **Adjust Pinhole** button in the **Configuration** subordinate toolbar of the **Main** menu.
 - This opens the **Pinhole Adjustment** window.
- Click on the **Close** button to quit the window.

(2) Function description

(a) 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 3.4.4.4, page 3-63.

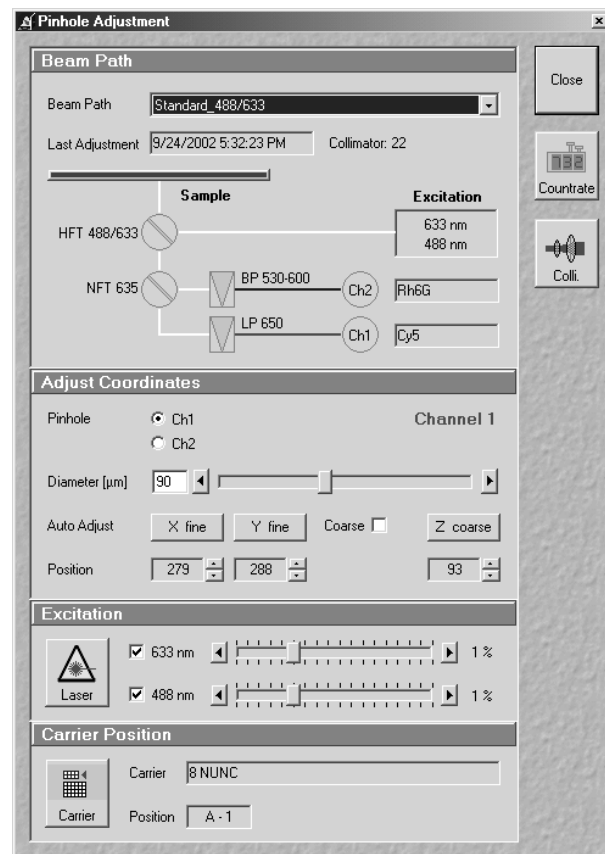


Fig. 3-63 Pinhole Adjustment window

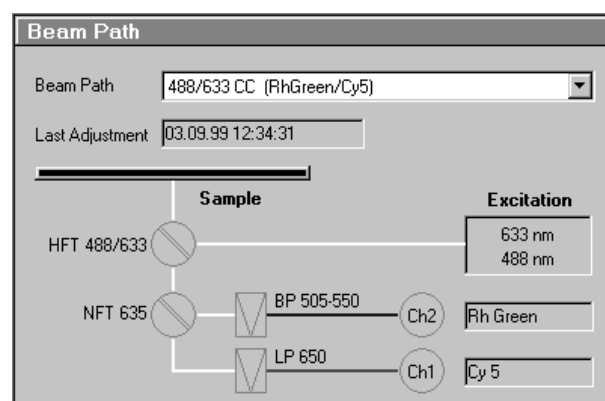


Fig. 3-64 Beam Path panel

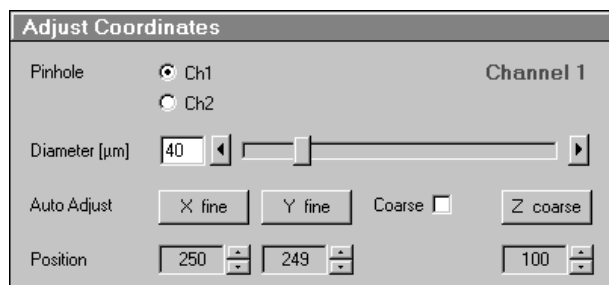


Fig. 3-65 Adjust Coordinates panel

(b) 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.

The following functions are available:

Pinhole	Selection of the channel for adjustment.
Diameter	Setting of pinhole diameter to corresponding the used wavelength (see page 3-61).
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 checkbox.
Y fine / Y coarse	Starts the automatic coarse or fine adjustment for Y direction depending on the activation / deactivation of the Coarse checkbox.
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 checkbox	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.

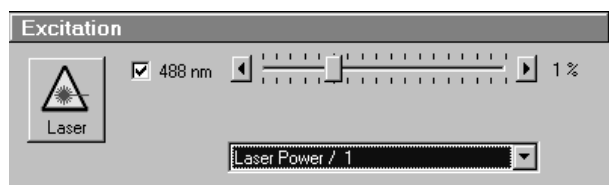


Fig. 3-66 Excitation panel

(c) Excitation panel

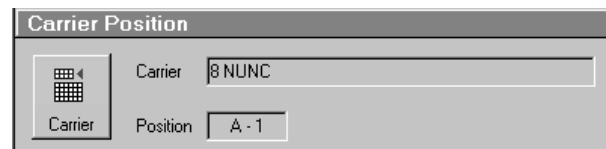
The **Excitation** panel allows you to activate / deactivate the wavelengths via checkboxes and to set the laser attenuation (%) using the sliders. Furthermore, you can open the **Laser Control** window via the **Laser** button to switch on / off the necessary lasers (see section 3.4.4.1, page 3-53).

The **Laser Power** display box allows you to reduce the overall power by factors of 10, 100 and 1000.

(d) 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 3.4.3.2, page 3-32).

**Fig. 3-67 Carrier Position panel****(e) Count rate window**

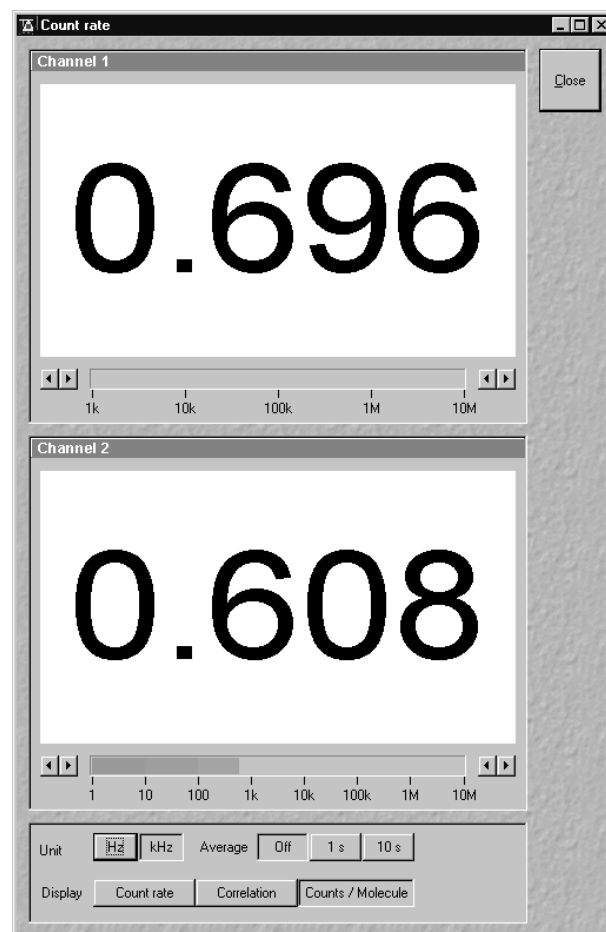
- Click on the **Count rate** button on the right-hand side to open the **Count rate** window.

In two **Channel** panels and one **Correlation** panel, 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**.

**Fig. 3-68 Count rate window**

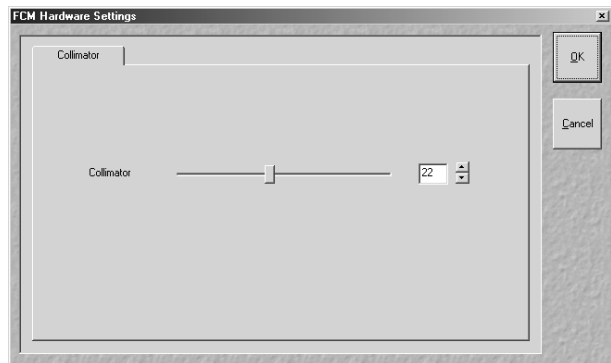


Fig. 3-69 FCM Hardware Settings window

(f) 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.

(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 3.4.3.1, page 3-30.
- Click the **Pinhole** button in the **Configuration** toolbar subordinate of the **Main** menu. The **Pinhole Adjustment** window will appear (Fig. 3-63).

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 axes must be adjusted separately. If the **Coarse** checkbox 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** checkbox 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. 3-70). 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.

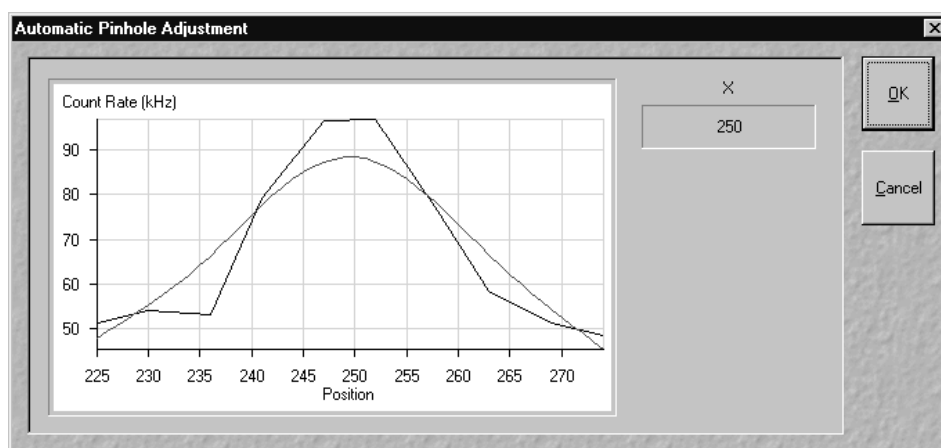


Fig. 3-70 Automatic Pinhole Adjustment

- 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**.

3.4.4.4 Define Beam Path

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

(1) Open / Close the Beam Path Definition window

- Click on the **Define Beam Path** button in the **Configuration** 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.

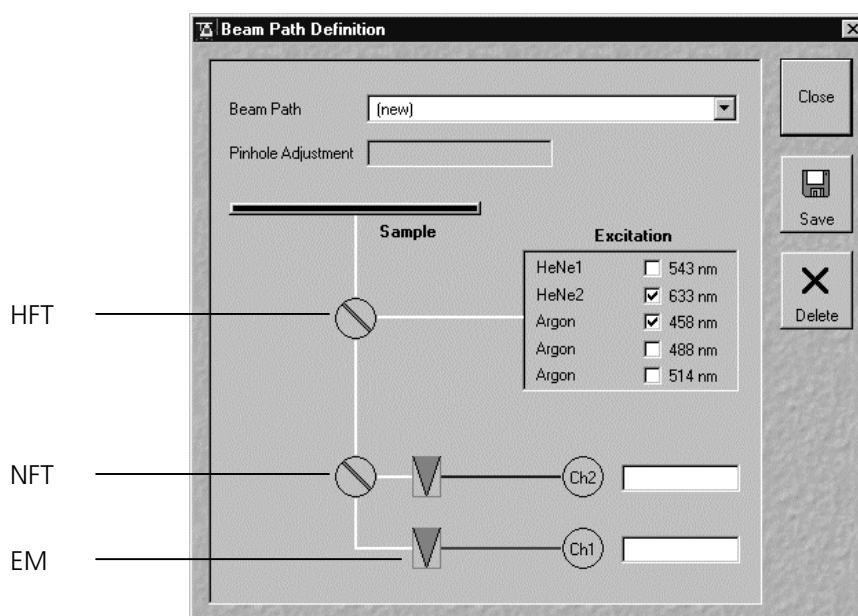


Fig. 3-71 Beam Path Definition window

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.

(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. 3-71).



Activation / deactivation of an emission filter (EM) through selection from the relevant list box (Fig. 3-71).

Excitation checkboxes 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.

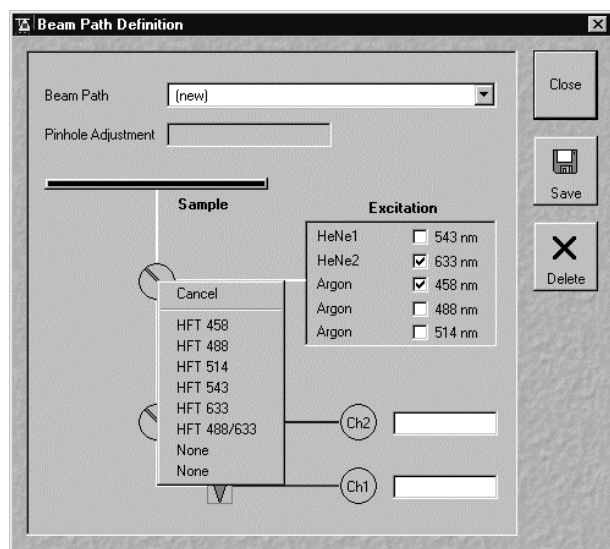



Fig. 3-72 Beam Splitter list box

(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.

- 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.

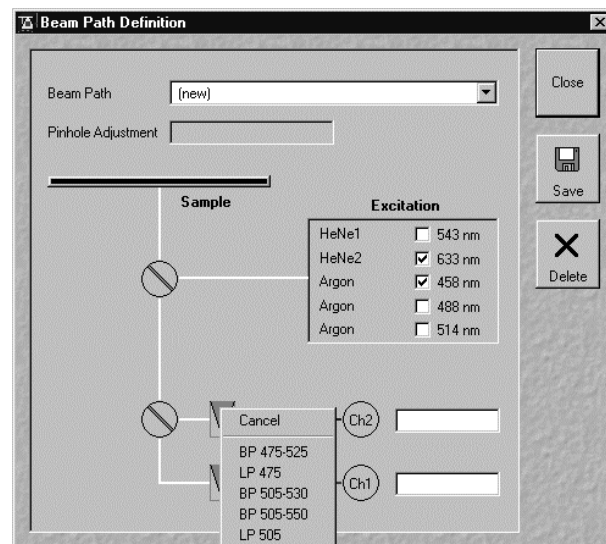


Fig. 3-73 Emission Filter list box

(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.

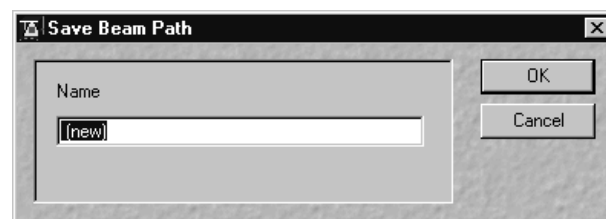



Fig. 3-74 Save Beam Path window

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

(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 deletion 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**.

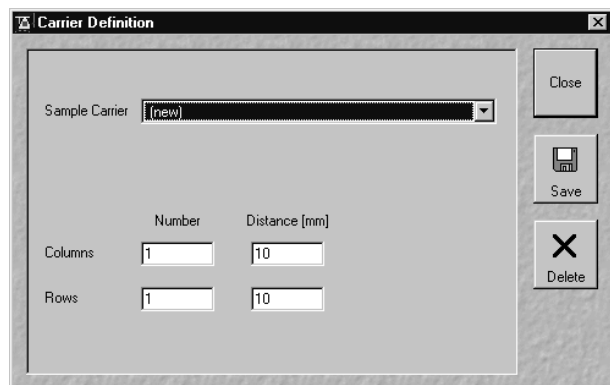


Fig. 3-75 Define Carrier window

3.4.4.5 Define Carrier

The **Define Carrier** function allows new / existing sample carriers to be created / edited and saved. The **Delete** function enables you to delete existing sample carriers.

(1) Open / Close the Carrier Definition window

- Click on the **Define 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.

(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

Saving of the new or edited sample carrier.

Delete button

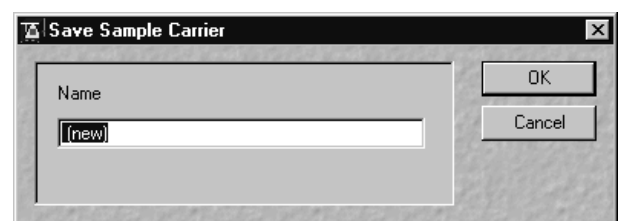
Deletion of an existing sample carrier.

(3) Define a new sample carrier

- In the **Sample Carrier** selection box, use **(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.

(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.

**Fig. 3-76 Save Sample Carrier window****(5) Delete a sample carrier**

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 deletion in the following window by clicking **OK**.
- When you are finished, close the **Carrier Definition** window by clicking on **Close**.

3.4.5 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. 3-77 Macro menu

3.4.5.1 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 ConfoCor 2.

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.

3.4.5.2 Macro Control

(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.

(2) Edit Macro function

This function allows you to load, run and unload macros.

- Press the **Edit Macro** button to switch to the **Macro** panel.

Load button: Opens an existing project.

Run button: Runs a macro.

Unload button: Removes the selected macro from the **Macros** list.

Macros are stored and managed in project files (*.lvb).

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.

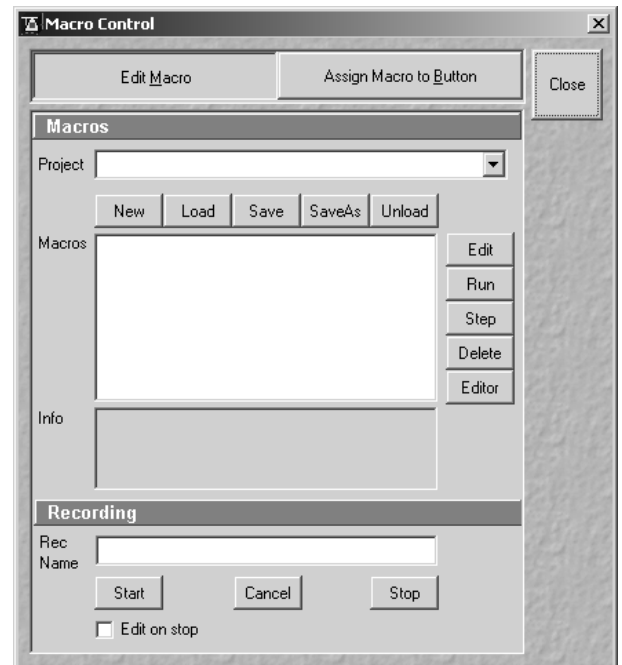


Fig. 3-78 Macro Control window

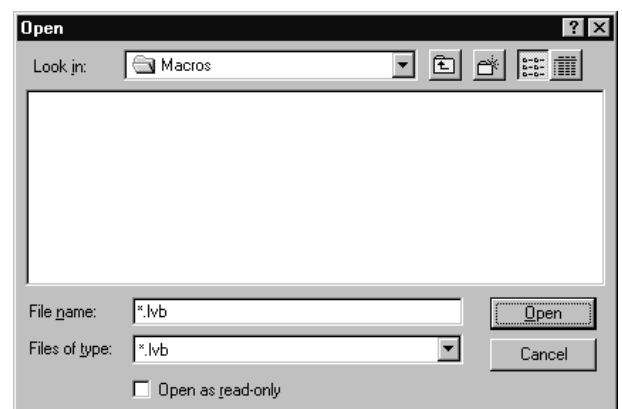



Fig. 3-79 Open window

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.

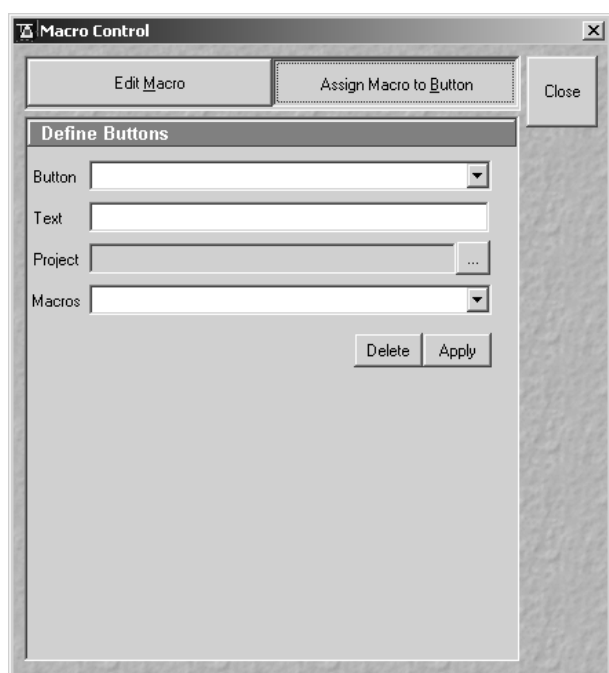


Fig. 3-80 Macro Control window

(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 button **Assign Macro to Button** to switch to the **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.

3.4.6 Options Menu

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

- Display / modification of the user-accessible program **Settings** of the ConfoCor 2 software.
 - **Collimator** setting.
 - Additional function **Objectives** (not available in the **Options** subordinate toolbar).
 - Additional function **Dye Database** (not available in the **Options** subordinate toolbar).
- In the **Main** menu toolbar, click on **Options**.
 - This opens another, subordinate toolbar in the **Main** menu.

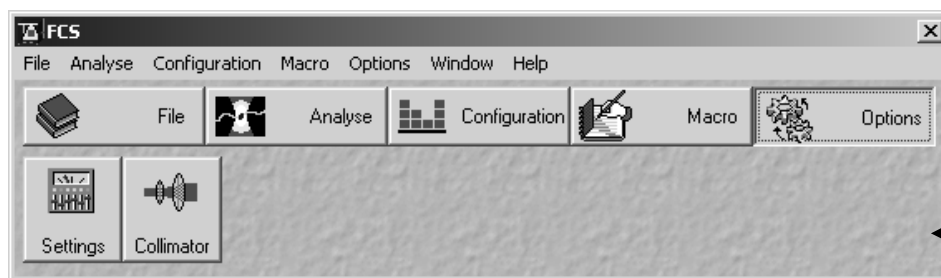


Fig. 3-81 Options menu

3.4.6.1 Settings Function

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

- **Open / Save**
- **Raw Data**
- **Measure**

(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.

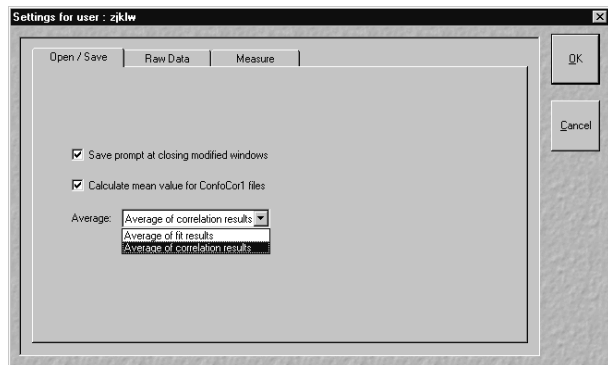


Fig. 3-82 Settings for user : ... window ("Save" register activated)

- Click on the appropriate register 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.

(2) Open / Save register

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).

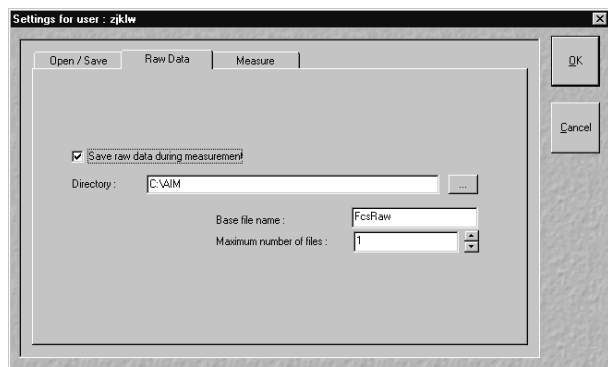


Fig. 3-83 Raw Data register

(3) Raw Data register

When you tick the **Save raw data during measurement** checkbox, 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/2000 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).

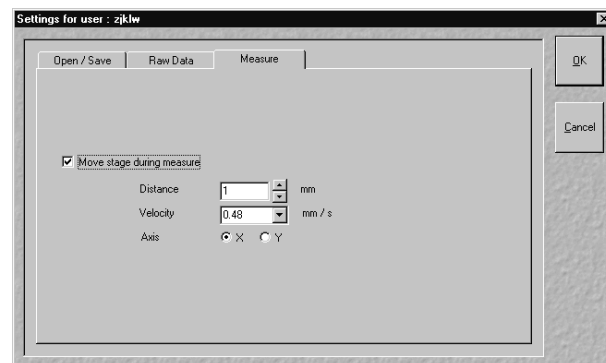
Each single measurement is stored in one raw data file. When the set limit is reached, the oldest file will be overwritten and the data in this file are lost. It is the responsibility of the operator to save the data.

(4) Measure register

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** checkbox 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.

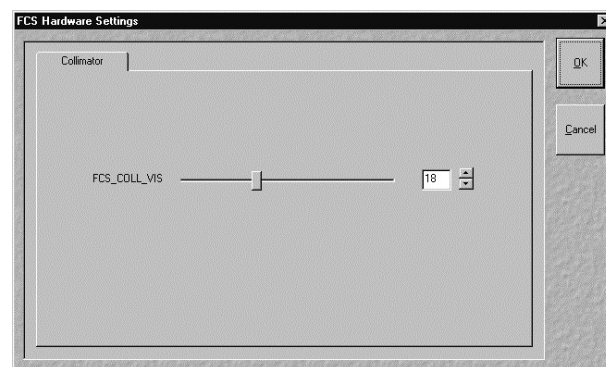
**Fig. 3-84 Measure register****3.4.6.2 Collimator Settings**

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. If collimator setting is unsuitable, pinhole adjustment can become impossible.

**Fig. 3-85 Collimator register**

- To change the collimator setting, click the **Collimator** button in the **Options** toolbar subordinate of the **Main** menu (Fig. 3-81).
- The **Collimator** register 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.

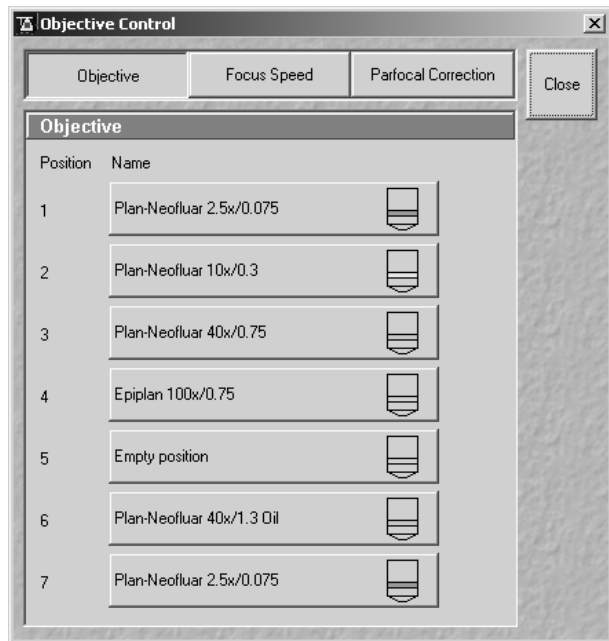


Fig. 3-86 Objective Control window

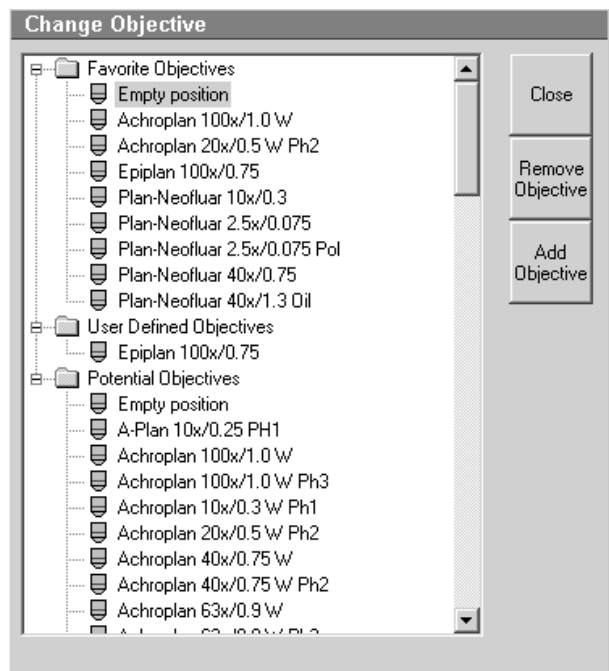


Fig. 3-87 Objective Control window

3.4.6.3 Objective

This function permits changed objectives to be activated and the parfocality to be set without having to exit the software (only available in the **Options** pull-down menu).

(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.

(a) Add Objective


This function permits new objectives to be added to the database.

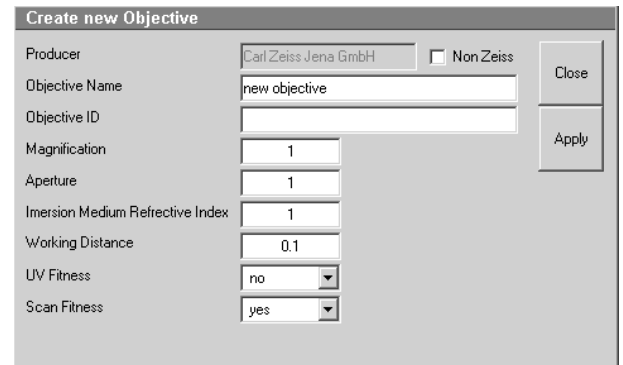
For this, proceed as follows:

- Click on the **Add Objective** button on the **Change Objective** window.
 - The **Create new Objective** window is opened.

- Enter the data of the new objective in the **Create new Objective** window, then click on the **Apply** button.

The new objective is stored in the database and included in the **Change Objectives** window. You can now activate the new objective as a favorite objective using the procedure described above.

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



The 'Create new Objective' window contains the following fields and controls:

- Producer:** A text box containing 'Carl Zeiss Jena GmbH' and a checkbox labeled 'Non Zeiss'.
- Objective Name:** A text box containing 'new objective'.
- Objective ID:** An empty text box.
- Magnification:** A numeric input box with the value '1'.
- Aperture:** A numeric input box with the value '1'.
- Imersion Medium Refractive Index:** A numeric input box with the value '1'.
- Working Distance:** A numeric input box with the value '0.1'.
- UV Fitness:** A dropdown menu with 'no' selected.
- Scan Fitness:** A dropdown menu with 'yes' selected.
- Buttons:** 'Close' and 'Apply' buttons on the right side.

Fig. 3-88 Create new Objective window

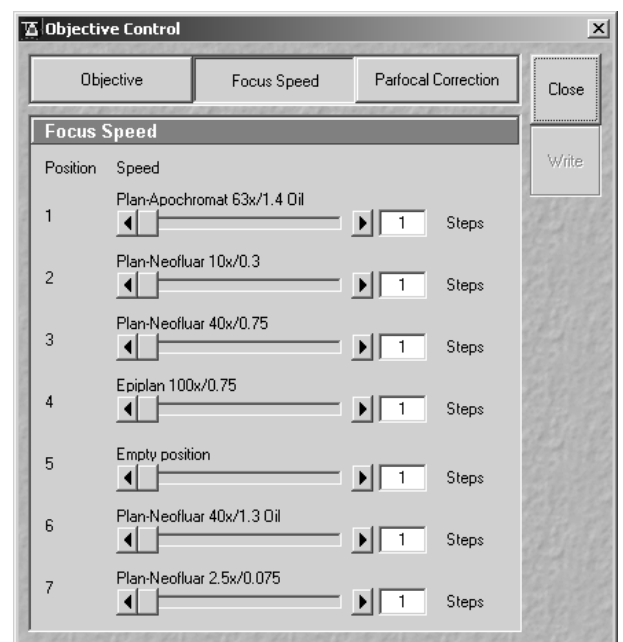
(b) Remove Objective

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.

(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.



The 'Objective Control' window has three tabs: 'Objective', 'Focus Speed', and 'Parfocal Correction'. The 'Focus Speed' tab is active, showing a table of focus speeds for different objectives.

Position	Speed	Steps
1	Plan-Apochromat 63x/1.4 Oil	1 Steps
2	Plan-Neofluar 10x/0.3	1 Steps
3	Plan-Neofluar 40x/0.75	1 Steps
4	Epiplan 100x/0.75	1 Steps
5	Empty position	1 Steps
6	Plan-Neofluar 40x/1.3 Oil	1 Steps
7	Plan-Neofluar 2.5x/0.075	1 Steps

Each row includes a slider and a numeric input box for selecting the speed in steps. The 'Write' button is located at the bottom right of the window.

Fig. 3-89 Focus Speed window



Fig. 3-90 Objective Control window

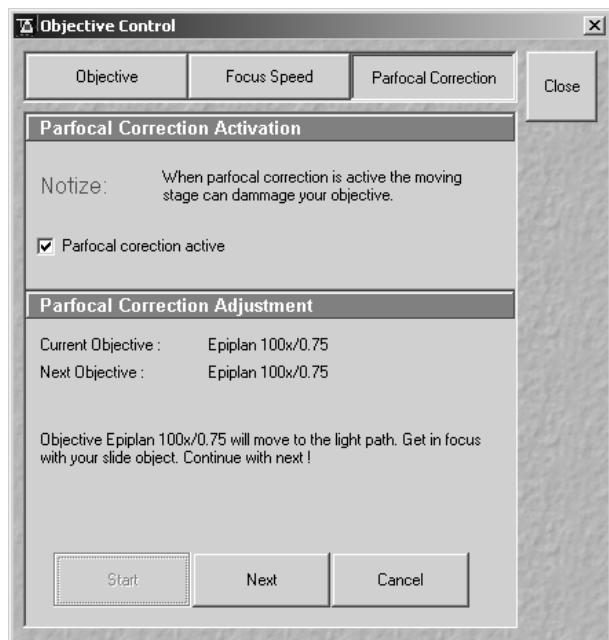


Fig. 3-91 Objective Control window

(3) Parfocality Correction

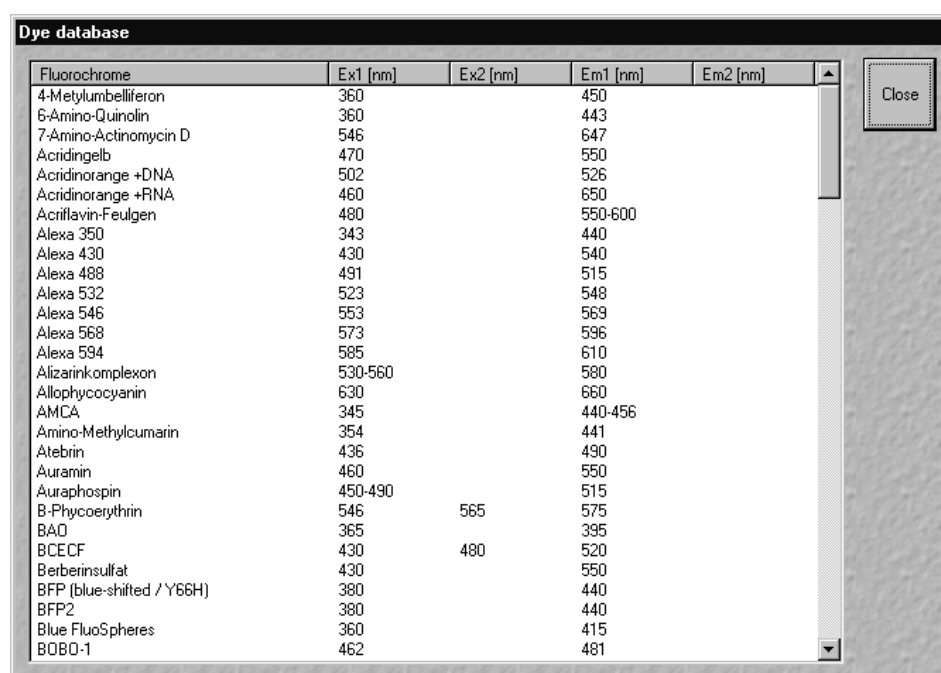
The parfocal setting is performed via screen dialogs in successive panels.

- 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.

3.4.6.4 Dye Database Function

The **Dye Database** 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 term **Options** in the menu bar of the **Main** menu.
 - The **Options** menu (pull-down) will be opened.
- Click on the **Dye Database** line.
 - The **Dye database** will be opened and displayed on the screen.



Fluorochrome	Ex1 [nm]	Ex2 [nm]	Em1 [nm]	Em2 [nm]
4-Methylumbelliferon	360		450	
6-Amino-Quinolin	360		443	
7-Amino-Actinomycin D	546		647	
Acridingelb	470		550	
Acridinorange +DNA	502		526	
Acridinorange +RNA	460		650	
Acridflavin-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. 3-92 Dye database window

- Click on the **Close** button to exit the **Dye database**.

3.4.6.5 Hardware Admin Function

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

3.4.7 Window Menu

The **Window** menu includes the additional function **Close All** which is not available from a toolbar.

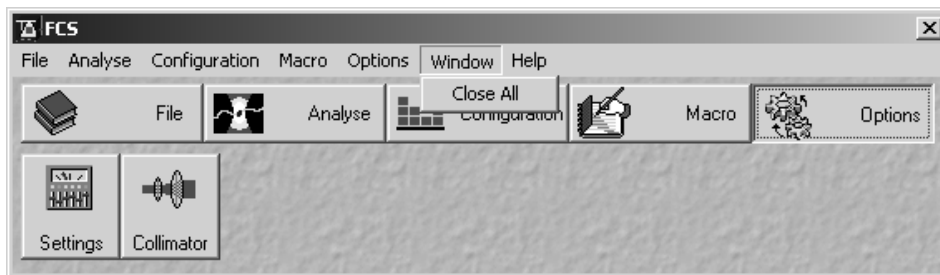


Fig. 3-93 Window pull-down menu

This function closes all the opened analysis windows.

- Open the **Window** menu.
- Click on the **Close All** line.
 - All the opened analysis windows will be closed.

3.4.8 Help Menu

The **Help** menu permits activation of the Help function and of a window containing information on the ConfoCor 2 and the installed software version.

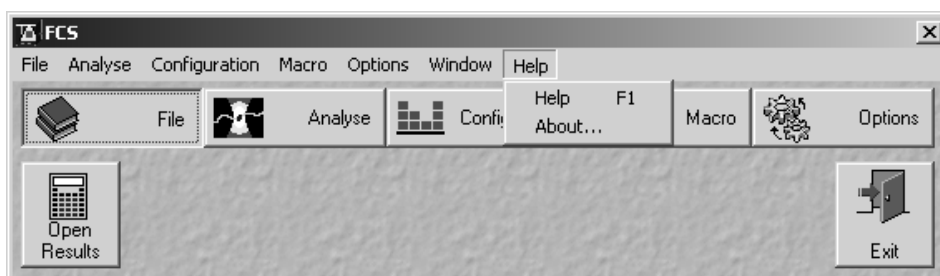


Fig. 3-94 Help pull-down menu

3.4.8.1 Help

- Open the **Help** menu.
- Click on the **Help** line to open the online help.

3.4.8.2 About

- Open the **Help** menu.
- Click on the **About** line to open the **About ConfoCor 2** window.

The **About ConfoCor 2** 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  or **Close** button to close the **About** window.

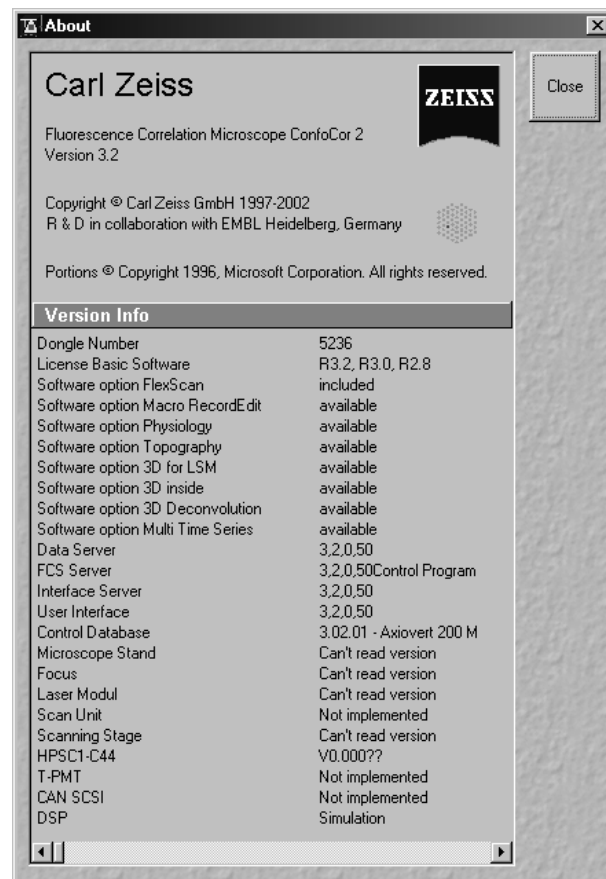


Fig. 3-95 About ConfoCor 2 window

3.5 Data Evaluation and Result Presentation

3.5.1 Structure of the Data Evaluation Window

The data evaluation window (Fig. 3-96) of the ConfoCor 2 software 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 is shown online. During the measurement you can stop the procedure by clicking the **Stop** button.

The major part of the window contains 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 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).

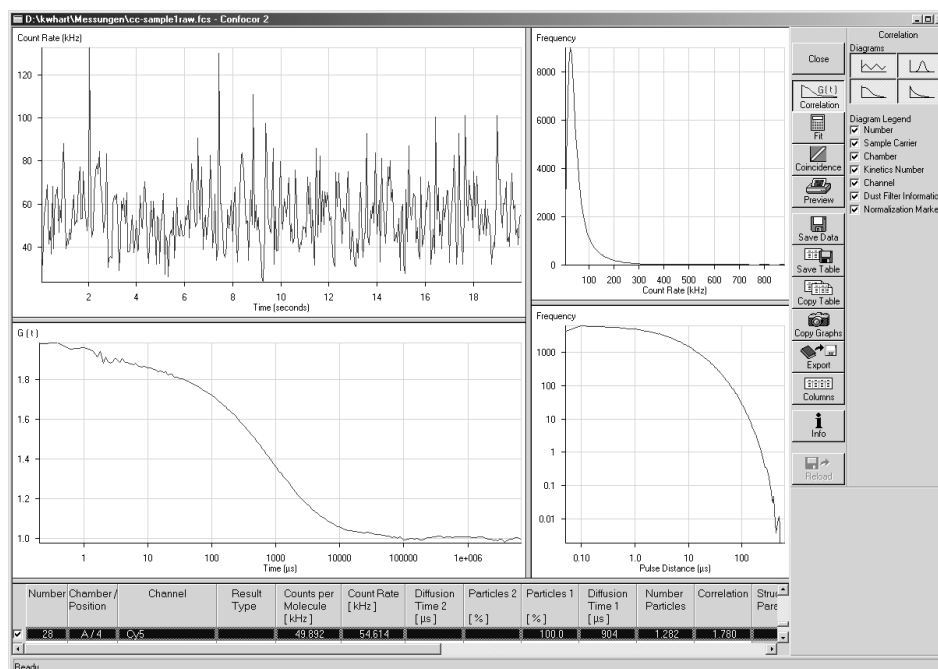


Fig. 3-96 Data evaluation window

3.5.2 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. 3-96).

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. 3-97). You have several options to open raw data files.
- 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. 3-97) will inactivate the dust filter.

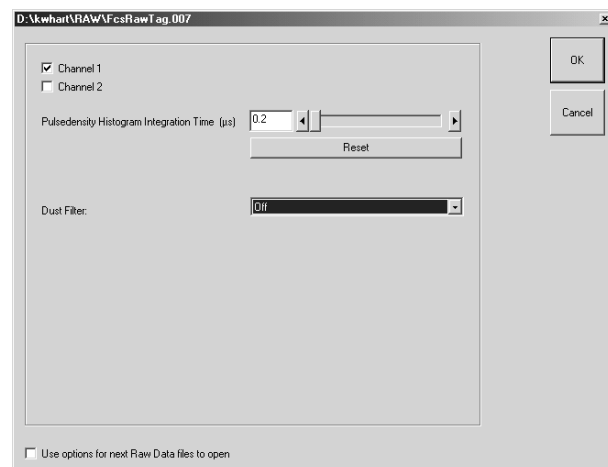


Fig. 3-97 The Options menu for loading raw data files with dust filter inactivated

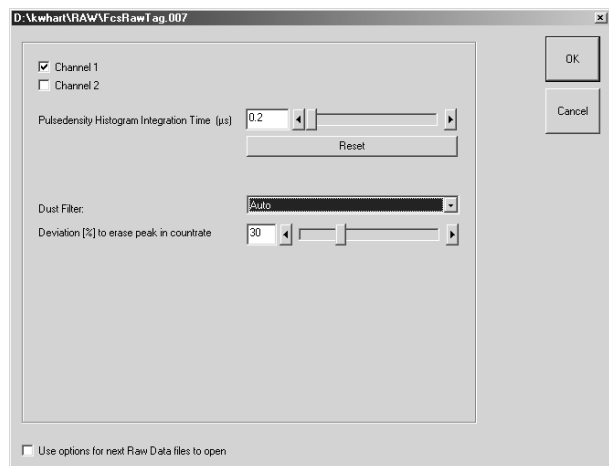


Fig. 3-98 The Options menu for loading raw data files with dust filter set to automatic

- Selection of **Auto** (see Fig. 3-98) 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.

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.

- Selection of **User** (see Fig. 3-99) 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 boundary 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. 3-100, 3-101). 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.

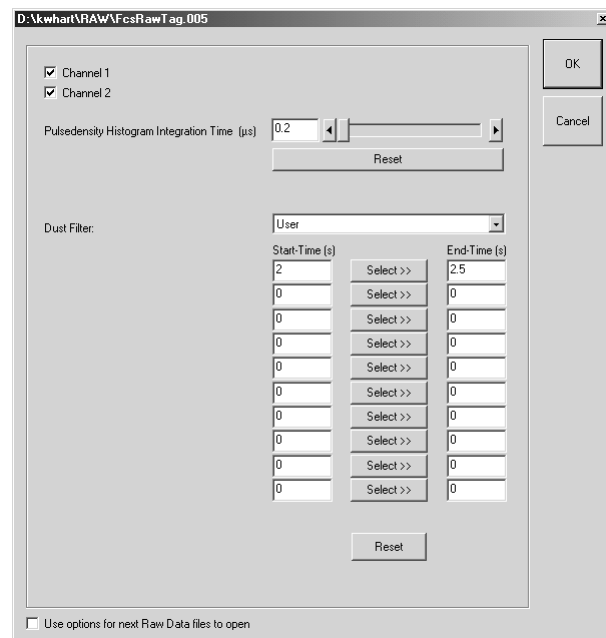


Fig. 3-99 The Options menu for loading raw data files with dust filter set to user defined

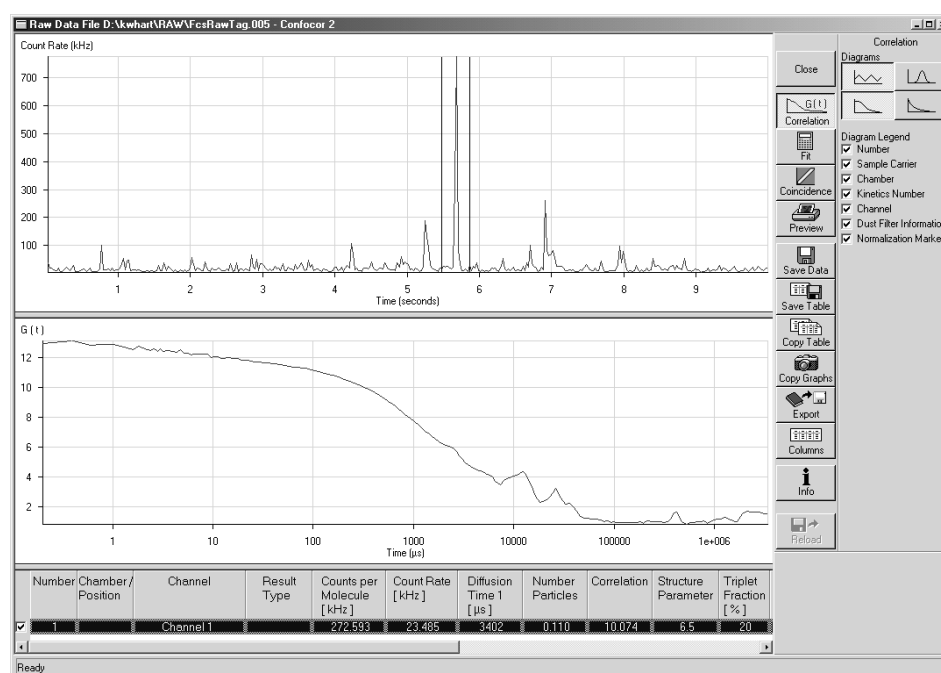


Fig. 3-100 The Count Rate Trace window with region of interest framed

OPERATION

Data Evaluation and Result Presentation Open / Close the Data Evaluation Window

Carl Zeiss

ConfoCor 2

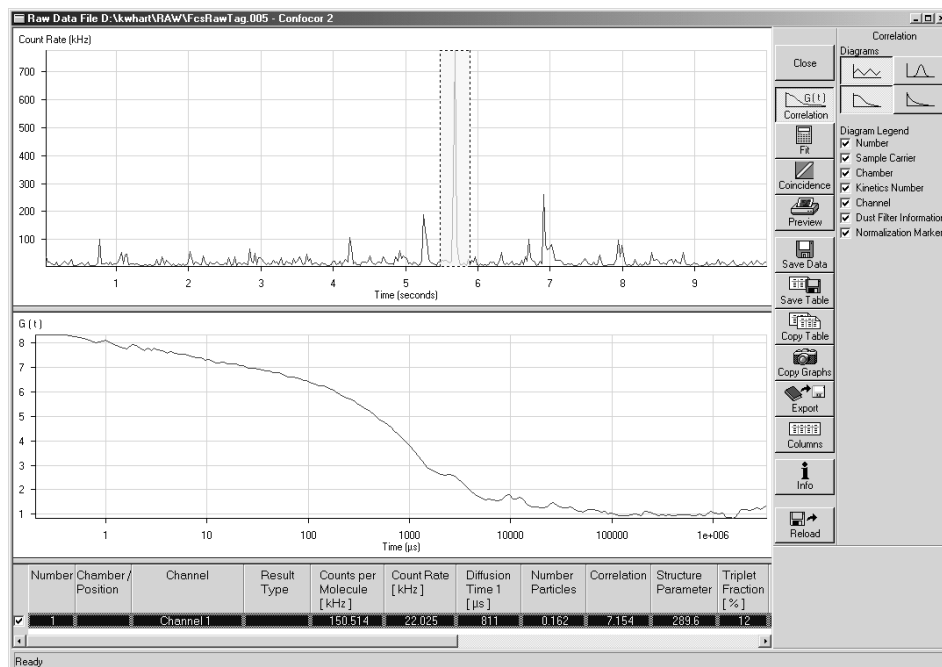


Fig. 3-101 The Count Rate Trace window with region of interest removed

The **Display** toolbar on the right allows you to select additional data via 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.

3.5.3 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. 3-96).

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** checkboxes.

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 3.5.12, page 3-102).

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 then 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. 3-103). 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. 3-104). The position of the bar more to the right defines the correlation time τ_{n1} to which the corresponding correlation values will be normalized to 1. The difference of $G(\tau_{n1})$ to 1 will then 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 (subtracted by 1) will then be multiplied by the correction factor defined by the ratio of 1 to $(G(\tau_{n2}) - 1)$ and increased by 1, 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. 3-102)

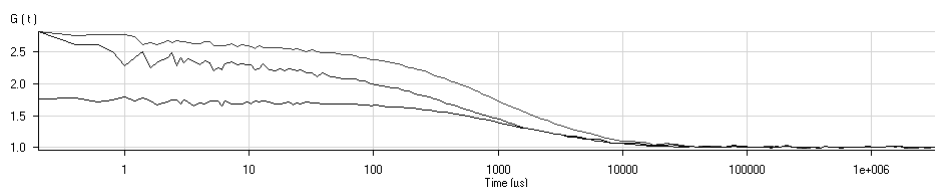


Fig. 3-102 The Correlation Curve window in normal display

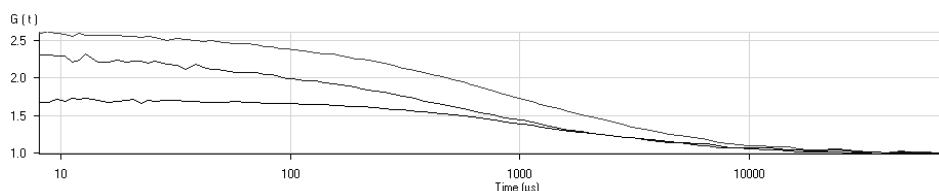


Fig. 3-103 The Correlation Curve window in restricted display

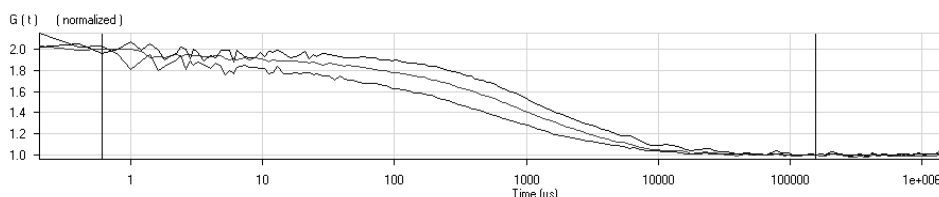


Fig. 3-104 The Correlation Curve window in normalized display



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.

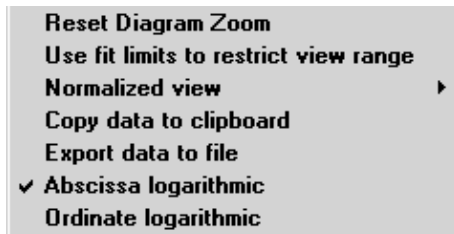


Fig. 3-105 Context menu of the Correlation diagrams

The context menu of the relevant diagram can be used to reset the zoom to its original value.

- Click on the diagram with the **right** mouse button.
 - The context menu is opened.
- Click on the line **Reset Diagram Zoom** with the left mouse button.
 - The zoom value is reset.

The 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. 3-106):

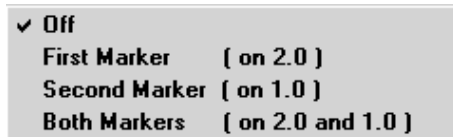


Fig. 3-106 Subcontext menu of the Normalized view

- ✓ 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 then 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 the position defined by the bar and the correlation values defined by the correlation times just before and after. All other values (subtracted by 1) will then be multiplied by the correction factor defined by the ratio of 1 to $(G(\tau_{n2}) - 1)$ and increased by 1, hence $G(\tau)_{norm2} = [1 / (G(\tau_{n2}) - 1) * (G(\tau)_{norm1} - 1)] + 1$.

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 checkbox on the right side of each row. Deactivation of these checkboxes will exclude the relevant rows from all subsequent evaluation procedures.

However, immediate reactivation is possible via the checkbox, if required.

Deactivated rows can be hidden in the table by activating the checkbox **Hide unused Rows** with a click of the mouse on the **Table** toolbar (see **Display – Columns**, page 3-102).

3.5.4 Display - Fit

- Click on the **Fit** button to activate the **Fit** display mode.
 - A **Fit** menu subordinate will appear (Fig. 3-107).

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.

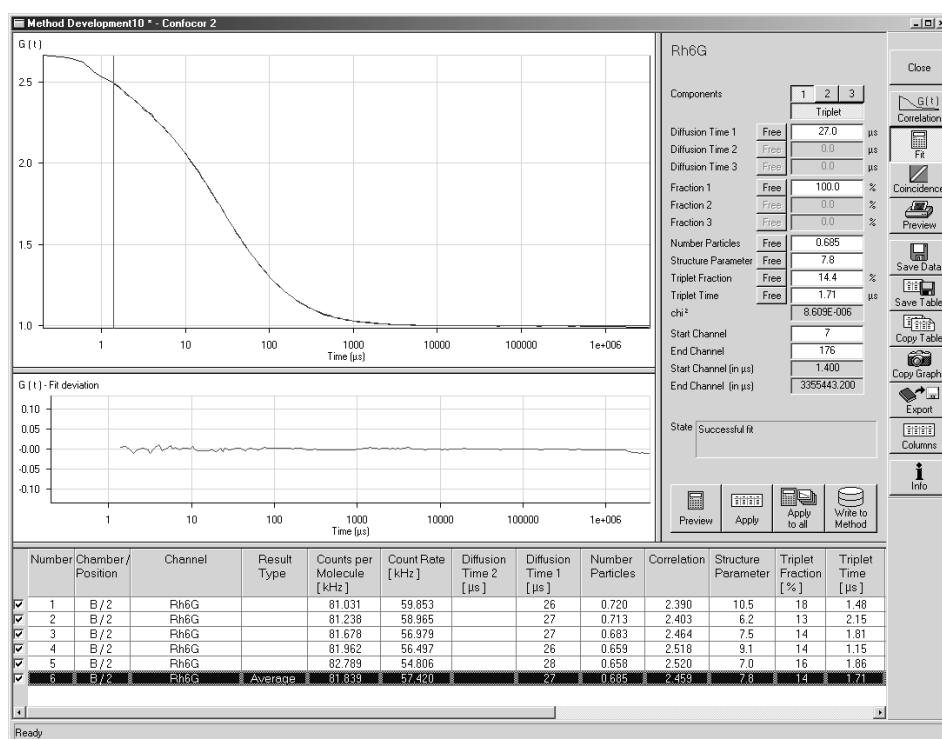


Fig. 3-107 Display fit

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 subtract the Start channel number by 1 and subtract 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 (chi²) 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 checkbox 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** 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.

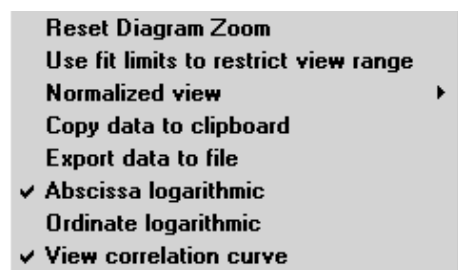


Fig. 3-108 Context menu of the Fit diagram

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.

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. 3-108).
- 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 **Abscissa 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. 3-111). Selection of this option will automatically activate this form of display for the Correlation diagram as well.

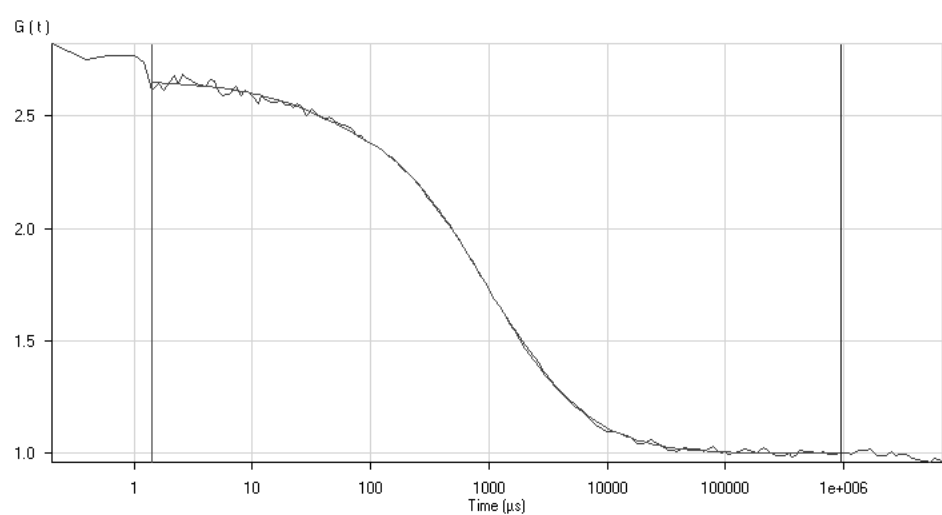


Fig. 3-109 The Correlation Curve window in normal display

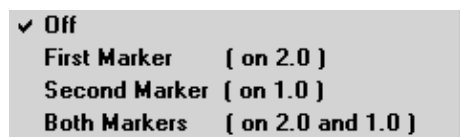


Fig. 3-110 Subcontext menu of the Normalized view

- 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. 3-112). 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. 3-110). In this case the left bar defines the position where the curve is normalized to 2, whereas the right bar specifies the position at which the curve is normalized to 1.

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.

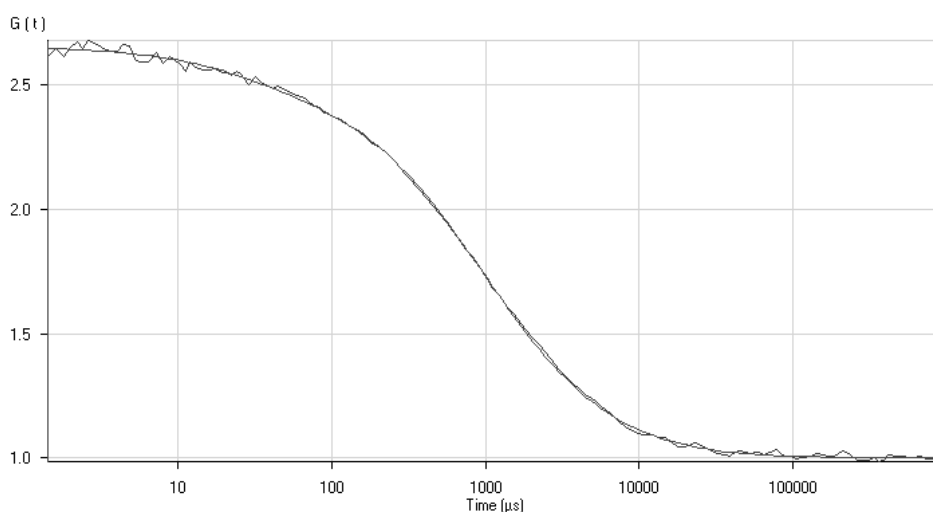


Fig. 3-111 The Correlation Curve window in restricted display

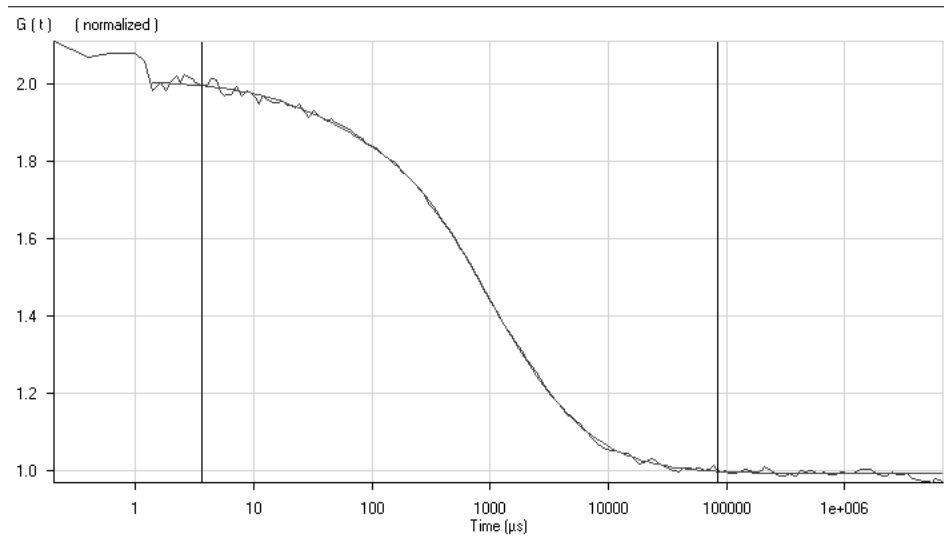


Fig. 3-112 The Correlation Curve window in normalized display

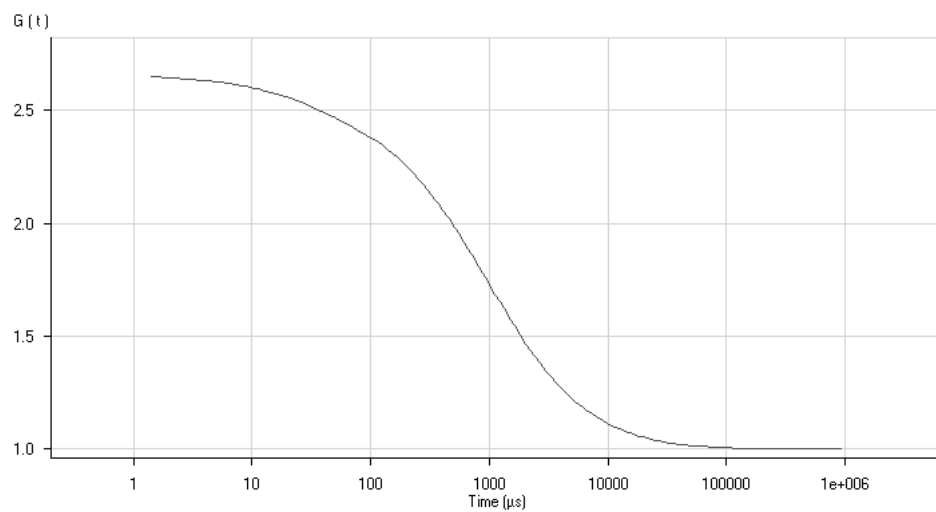


Fig. 3-113 The Correlation Curve window in fit curve display ("View correlation curve" unchecked)

- Select **View correlation curve** will display the Correlation curve in addition to the Fit curve (see Fig. 3-109). If this option is not selected, only the Fit curve is displayed (see Fig. 3-113).

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.

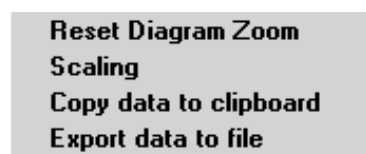


Fig. 3-114 Context menu of the **Fit deviation** diagram

- Click on the diagram with the **right** mouse button.
 - The context menu is opened.
- Click on the line **Reset Diagram Zoom** with the left mouse button.
 - The zoom value is reset.

The 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.



Fig. 3-115 Diagram scaling window

- 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.

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).

The table of measuring rows in the lower part of the **Data Evaluation** window contains a checkbox on the right side of each row. Deactivation of these checkboxes will exclude the relevant rows from all subsequent evaluation procedures.

However, immediate reactivation is possible via the checkbox, if required.

Deactivated rows can be hidden in the table by activating the checkbox **Hide unused Rows** with a click of the mouse on the **Table** toolbar (see **Display - Columns**).

3.5.5 Display - Coincidence

- Click on the **Coincidence** button to activate the **Coincidence** display mode.
 - The coincidence analysis generates a two-dimensional color-coded count rate histogram (Fig. 3-116).

This is an alternative to the standard correlation analysis during rare event detection. If the ConfoCor 2 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.

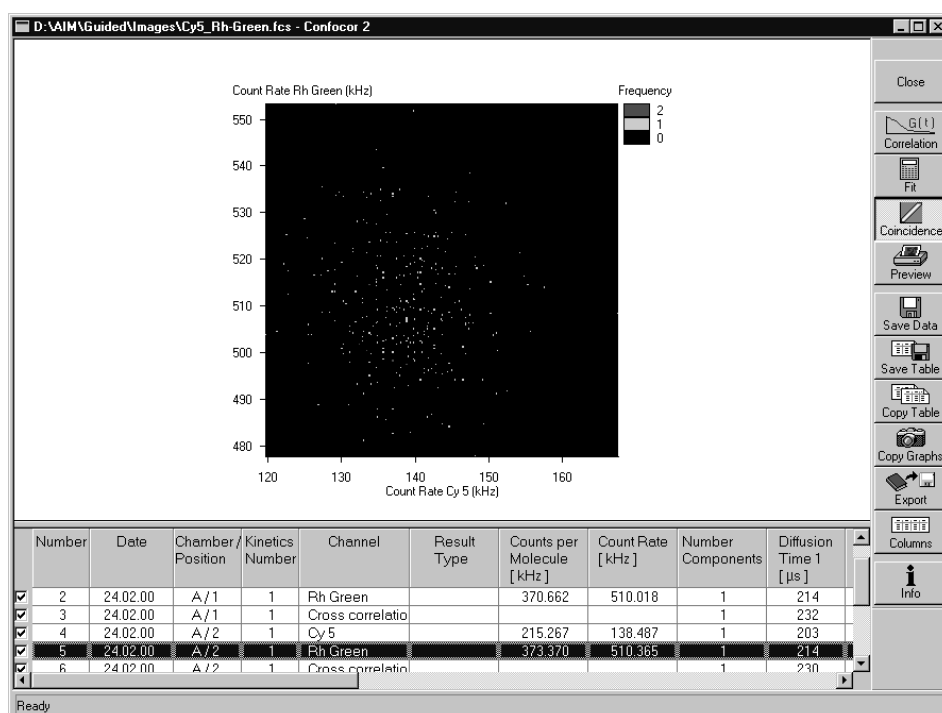


Fig. 3-116 Coincidence diagram window

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.

3.5.6 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. 3-117).

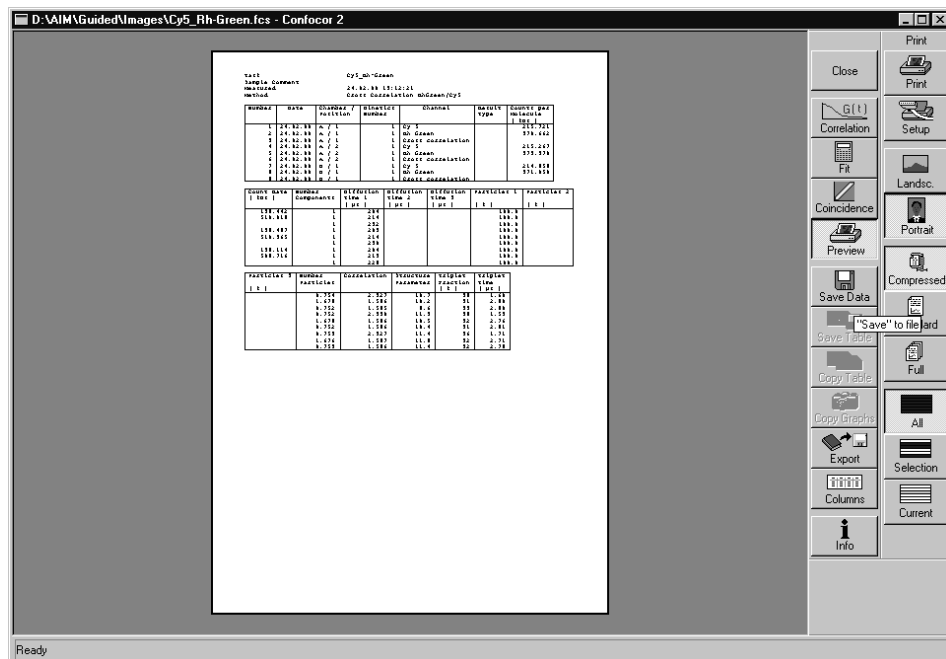


Fig. 3-117 Preview / Print window

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 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.

3.5.7 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.

3.5.8 Display - Copy Table

- With **Copy Table**, the whole table will be copied to the WINDOWS clipboard. The contents of the clipboard can be used by most WINDOWS applications via the **Paste** function.

3.5.9 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.

3.5.10 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.

3.5.11 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.

3.5.12 Display - Columns

- With a click on the **Columns** button, the **Table** subordinate toolbar can be toggled on and off.

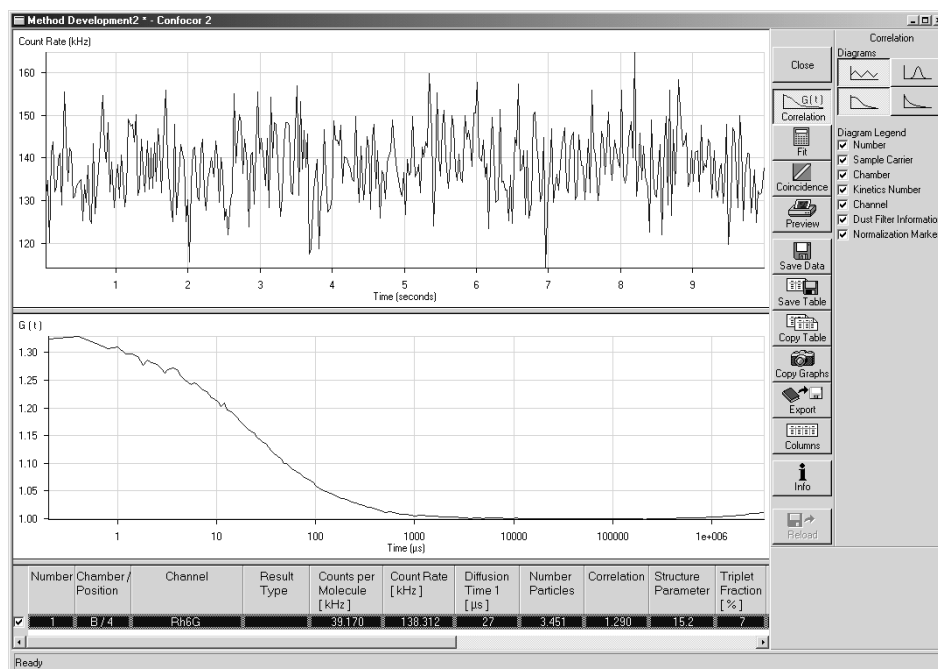


Fig. 3-118 Display Column with Table subordinate toolbar

- By ticking the appropriate **Columns** checkbox 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** checkbox, 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** checkbox if deactivated rows shall be hidden from the table of measuring values.

3.5.13 Display - Info

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

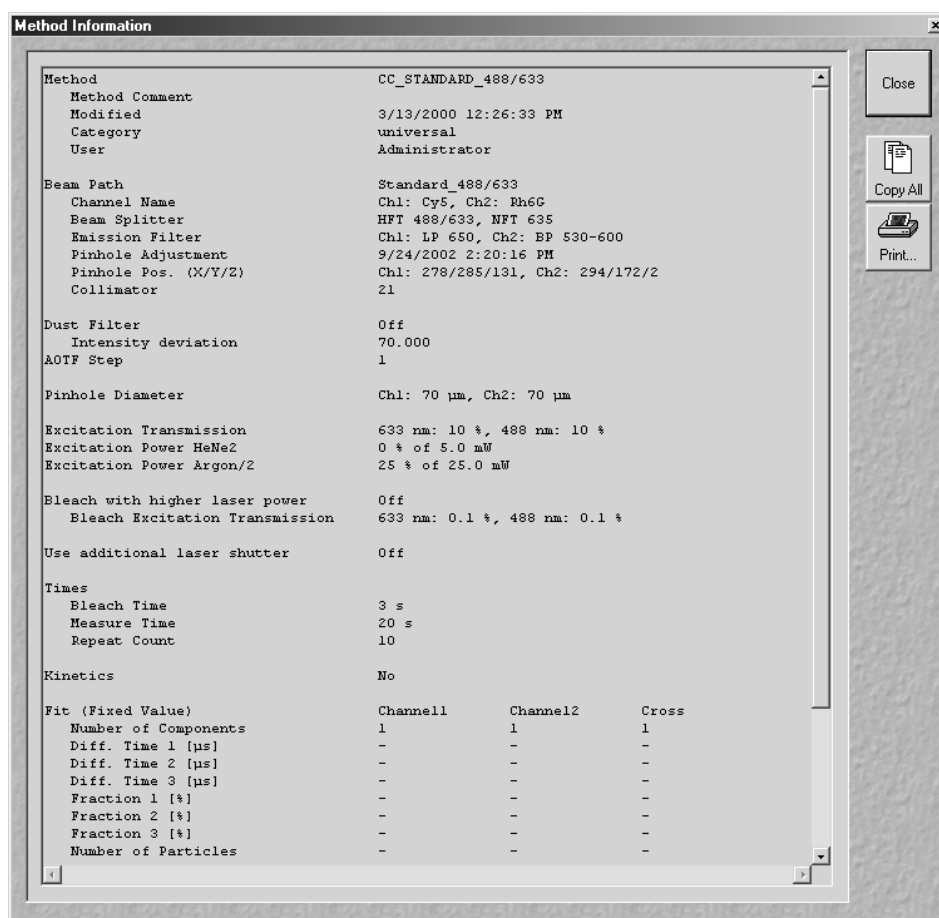


Fig. 3-119 Method Information window

- 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.

3.6 Shut-Down Procedure



Never shut down the computer by its main switch while your ConfoCor 2 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 on Exit** in the **Shutdown** register. The lasers will then automatically be switched off when you exit the ConfoCor 2 program.

3.6.1 Exiting the ConfoCor 2 Program

- Close all open windows of the ConfoCor 2 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 **ConfoCor 2 Switchboard** window appears.



Fig. 3-120 ConfoCor 2 Switchboard menu

- Click on the **Exit** button.
 - This terminates the ConfoCor 2 program.
 - The monitor screen shows the desktop of the WINDOWS NT operating system.

3.6.2 Running Down the Operating System

- Move the cursor to the bottom margin of the screen.
 - This opens the taskbar containing the **Start** button.
- Click on the **Start** button of the taskbar.
 - This opens a pop-up menu.
- Click on the **Shut Down** item.
 - This opens the **Shut Down Windows** window, in which you can select between **Shut down**, **Restart** and **Login**.

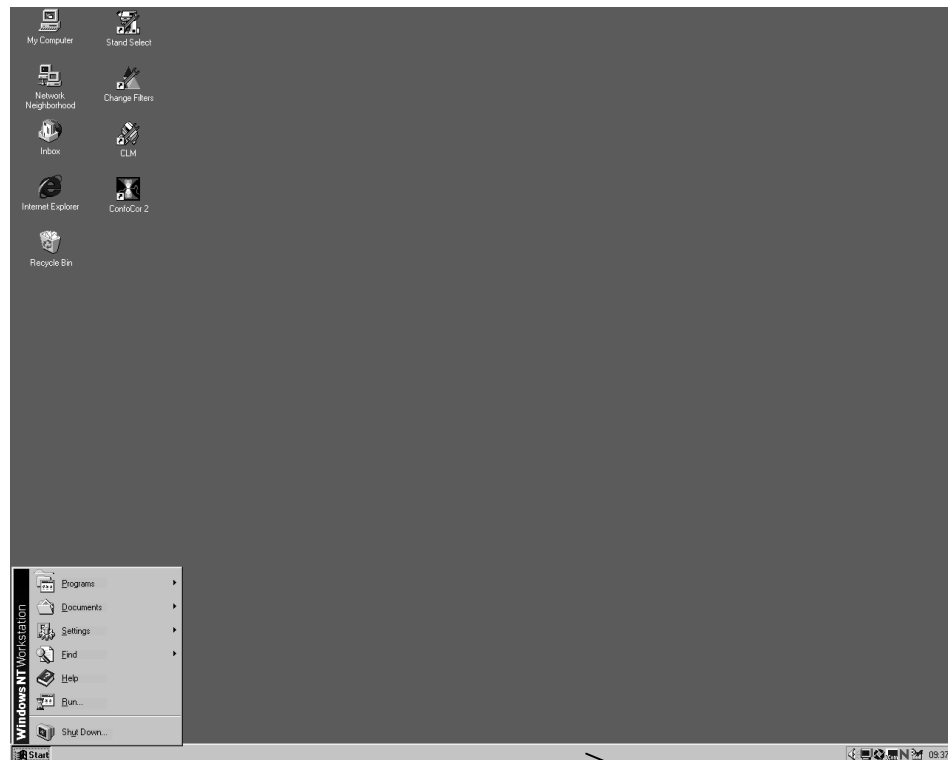


Fig. 3-121 Start menu

Taskbar



Fig. 3-122 Shut Down window

- 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.

After WINDOWS has been run down, the **Shutdown Computer** window appears which tells you that you can now turn off your computer.

3.6.3 Turning Power Off



Throw the REMOTE CONTROL main switch and the power supply switch of the Ar laser to position "**OFF**" after 5 minutes.

- This puts your ConfoCor 2 microscope system, including the computer, off power.

3.7 Tools

3.7.1 Change Filters

The **Change Filters** tool is used to update the filter data in the software after a change of filters in the reflector turret.

- Close the ConfoCor 2 software program.
- Insert the new filter module in the reflector turret.
- Double-click on the **Change Filters** icon on the desktop.
 - The **Emission Filter & Beam Splitter Control** window appears on the screen. The name of the currently used database is displayed in the **System Database** box, with the filter type being indicated below for checking purposes.
 - The **Filter Cubes Stand** panel shows the **Filter-Wheel No.** and the filter positions available.
 - Use the **Name** and **ID** selection boxes to enter the filters installed in the individual positions of the filter wheel.
- Open the **Name** (or **ID**) selection box of the relevant filter position and select the new filter set from the list.
- Click on the **Store** button to accept the new settings.
- Click on the **Close** button to close the **Emission Filter & Beam Splitter Control** window.



All the available filter sets have to be registered in the filter list (see **Edit Filter List** function, next page).

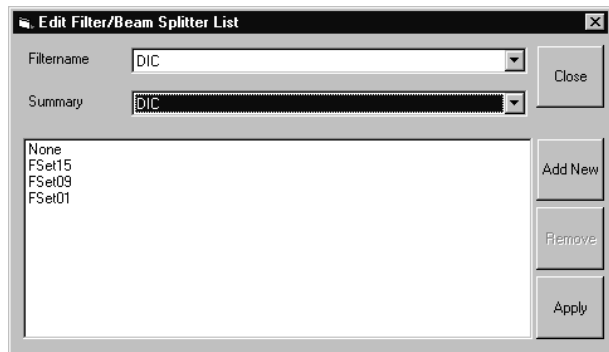


Fig. 3-123 Edit Filter/Beam Splitter List window

(1) Edit Filter List

The **Edit Filter List** function permits updating of the filter data in the software after a change of filters on the stand.

- Close the ConfoCor 2 software program.
- Double-click on the **Change Filters** icon on the desktop.
- Click on the **Edit Filter List** button in the **Emission Filter & Beam Splitter Control** window.
 - The **Edit Filter/Beam Splitter List** window is opened.

This window permits a list of the most frequently used filter sets to be compiled.

- Click on the arrow button in the **Filter name** list box to open it.
- Select the filter set which shall be included in the list.
- Click on the **Apply** button.

The selected filter set is included and displayed in the list (below the **Summary** list box).

This filter set is now also available in the **Name** selection boxes of the **Filter Cubes Stand** panel and can be assigned to a filter wheel position.

To remove a filter set which is no longer needed from the list, proceed as follows:

- Click on the name of the filter set concerned in the list box of the **Edit Filter/Beam Splitter List** window.
- Click on the **Remove** button. The filter set is deleted from the list and is then no longer available in the **Filter Cubes Stand** panel of the **Emission Filter & Beam Splitter Control** window.


(2) Add New

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

For this, proceed as follows:

- Click on the **Add New** button on the **Edit Filter/Beam Splitter List** window.
 - The **Add New Filter/Beam Splitter** window is opened.
- Enter the data of the new filter set in the **Filter Cubes Stand Description** panel, then click on the **Apply** button.

The new filter set is stored in the database and included in the **New Filter Cubes Stand** panel. You can now activate the filter for a filter wheel position using the procedure described above.

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

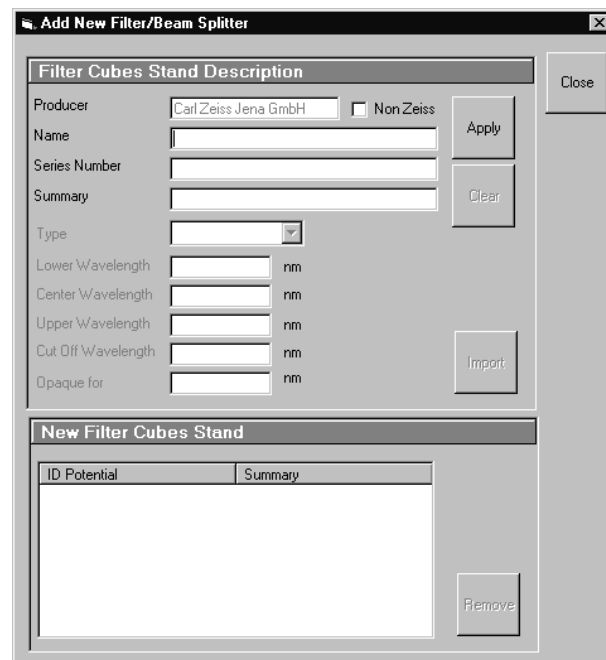


Fig. 3-124 Edit Filter/Beam Splitter List window

- To remove a new filter set from the database, select it with a click of the mouse in the **New Filter Cubes Stand** panel and then click on **Remove**.
- Click on **Close** to close the **Add New Filter/Beam Splitter** window.
- Click on **Close** to close the **Edit Filter/Beam Splitter** List window.
- Click on the **Store** button to accept the new settings.
- Click on the **Close** button to close the **Emission Filter & Beam Splitter Control** window.

When you start the ConfoCor 2 software, the filter data are updated.

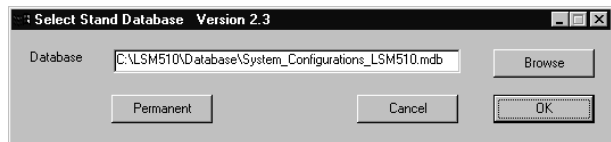


Fig. 3-125 Select Stand Database ... window

3.7.2 Stand Select

The **Stand Select** tool permits a new or updated database to be assigned to the ConfoCor 2 software program. This function should preferably be performed by authorized service personnel.

If this is not possible, proceed as follows:

- Close the ConfoCor 2 software program and double-click on the **Stand Select** icon on the desktop.
 - The **Select Stand Database** window appears on the screen. The currently used database is displayed in the **Database** box.

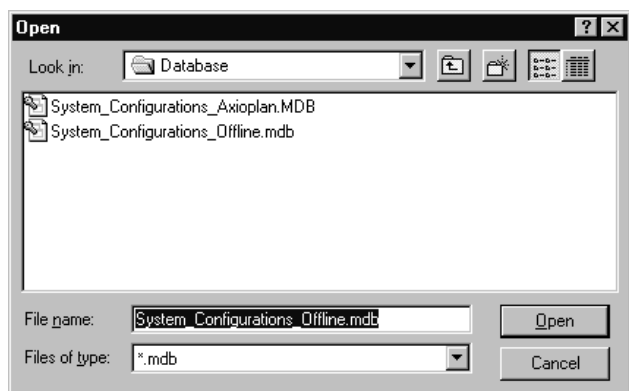


Fig. 3-126 Open window



Fig. 3-127 Select Name window

- Click on the **Browse** button to activate the new database.
 - The **Open** window appears on the screen.
- Select the directory where the new database is stored.
- Click on the name of the database (file extension: ***.mdb**) and then on the **Open** button.
 - The **Open** window is closed and the name of the new database appears in the **Database** box.
- Click on the **Permanent** button. The **Select Name** window appears.
- Select the relevant stand icon from the **Icon** list box and click on **OK**. The **Select Name** window is closed and the desktop icon is updated.
- Then click on the **OK** button in the **Select Stand Database ...** window to accept the new settings and to close the window. (Clicking on **Cancel** will cancel the procedure.)
 - After the next restart of the ConfoCor 2 software program, the new database will be read automatically.

3.7.3 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

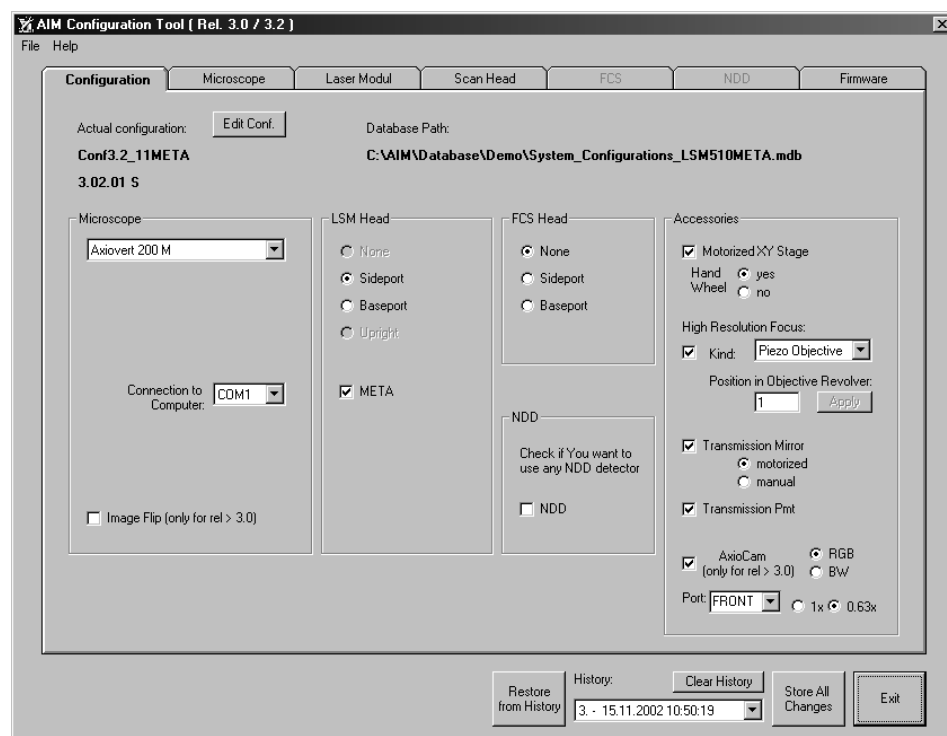


Fig. 3-128 Configuration Tool menu

3.8 Annex

3.8.1 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 <i>bt1</i> ; else 0
9	1, if pulse recorded in channel 2 during cycle <i>bt1</i> ; else 0
10	1, if pulse recorded in channel 1 during cycle <i>bt2</i> ; else 0
11	1, if pulse recorded in channel 2 during cycle <i>bt2</i> ; else 0
12	1, if pulse recorded in channel 1 during cycle <i>bt3</i> ; else 0
13	1, if pulse recorded in channel 2 during cycle <i>bt3</i> ; else 0
14	1, if pulse recorded in channel 1 during cycle <i>bt4</i> ; else 0
15 (MSB)	1, if pulse recorded in channel 2 during cycle <i>bt4</i> ; 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.

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CHAPTER 4 APPENDIX

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4 APPENDIX

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