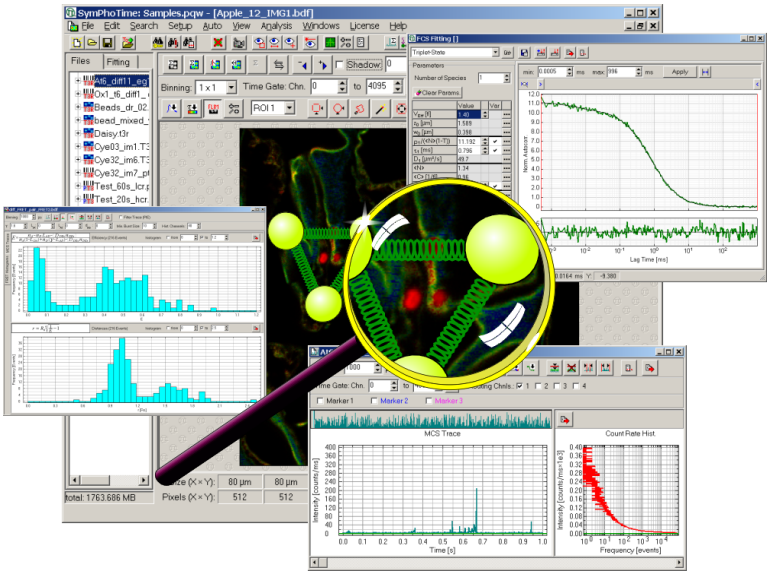
SymPhoTime

PicoQuant GmbH
Unternehmen für optoelektronische Forschung und Entwicklung

Confocal TCSPC Data Acquisition and Analysis Software



User's Manual and Technical Data

Version 5.1

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1. Introduction

1.1 SymPhoTime in a Nutshell

Originally designed for use with PicoQuant's MicroTime 200 confocal microscope, the SymPhoTime software has evolved into a versatile tool applicable from laser scanning microscopy to cuvette measurements.

Of pivotal importance are the Time–Tagged Time–Resolved (TTTR) measurement modes of the TimeHarp 200, PicoHarp 300 and HydraHarp 400 TCSPC devices, which allow the performance of vastly different measurement tasks based on one single data format, yet without any sacrifice of information available from each single photon. This allows all measurement data to be handled in a standardised yet flexible way.

The SymPhoTime software is designed with almost unlimited flexibility for integration of virtually all algorithms and methods for the analysis of fluorescence dynamics that users may require. Based on the powerful TTTR data collection, users can perform an unlimited number of analysis steps without losing track of the interdependence and origin of their measurement and analysis data. Results can be obtained through a vast set of analysis tools, such as intensity time trace, burst analysis, lifetime histogramming, fluorescence correlation spectroscopy, lifetime imaging, to name only a few.

Getting Started

1.2 Requirements

The SymPhoTime software is suitable for PCs running Windows XP or Vista. It demands a reasonable performance of the PC. For routine work, a 586–class machine with at least a 400 MHz CPU clock and a minimum of 512 MB RAM is necessary. For improved performance in data acquisition or during complex analysis tasks like FLIM or FCS, a multiple–core CPU is recommended.

The software takes approximately 20 MB, however, not including the storage space for data files. To use the software efficiently, a high screen resolution is needed. For daily work, a screen resolution of at least 1200 × 1024 pixels is recommended. Even better is a two monitor set–up.

The SymPhoTime package is protected by a Hardlock protection module (dongle) that must be connected to the appropriate port of the PC (or server, if it is a network dongle) during operation. In order to recognise its presence and to use the Hardlock, a software driver should be installed for the respective operating system and port selected. The driver is automatically installed with the SymPhoTime software package.

1.3 Feature Overview

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1.4 Installation Procedure

The software is supplied pre–installed and on CD, together with a copy protection module (Hardlock). On the installation CD you will find the following files and directories:

`setup.exe`	self–extracting installation file
`Readme.txt`	installation notes
`techdocs`	Various application notes
`other products`	Information on other PicoQuant products
`MT200`	drivers and diagnosis software for the MicroTime 200 hardware:
`Video`	Frame grabber driver and associated software installation files
`PCAN`	MT200 shutter control (CAN) drivers
`NanoCapture`	Scanner diagnosis and calibration software setup
`Hardlock`	Copy protection module network installation and drivers
`LSM_Remote_Control`	Client for remote control of the SPT via TCP/IP for usage with LSMs

If this software is purchased as part of a complete system, it will be pre–installed upon delivery. Usually there will be no need for re–installation. The most probable situation where a user may need to perform an installation, will be on a PC used for data analysis only. In this case, or if the software was purchased as a standalone product, install the software by running `setup.exe` and follow the instructions of the program.

If the controlling PC of a MicroTime 200 microscope needs to be re–installed, make sure that the drivers of all devices are correctly installed. (The drivers for all possible components are located in the appropriate subfolders of the `MT200` directory.) Then run `setup.exe` selecting the "operation" installation.

As the complete installation for a microscope system is complex and difficult, it is strongly recommended to contact the support, when the necessity of a re–installation occurs.

Network Hardlock

The software installation of the Hardlock drivers for each client terminal is identical to the standard Hardlock installation. The Hardlock network module must be connected to a computer which is network–accessible from all PCs designed to run the SymPhoTime software. For this computer (often a network server), the Hardlock driver installation is different from the usual procedure. Please refer to the documentation in the subfolder `MT200\Hardlock\` and contact your network administrator.

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1.5 License Information

The functionality of the software package depends on the purchased license. To show the license configuration choose *License* | *Info…* from the main menu.

The serial number of the Hardlock module, the location of the current license and the available software package are shown. Currently, there are four packages available within the SymPhoTime software, called "Point", "Image", "Complete" and "Point Analysis".

"Complete" includes the functions of both "Point" and "Image". "Point Analysis" limits the "Point" license to the analysis functions for evaluating TTTR files recorded by the TimeHarp, PicoHarp or HydraHarp software.

1.6 System Configuration Using Maintenance Mode

Maintenance mode is only for experienced users, who are building their own experimental setup using PicoQuant equipment together with the SymPhoTime software.

If you have purchased a MicroTime 200 or an LSM upgrade kit, the delivered system is already configured correctly. Usually, changes of the configuration will not be necessary. Please contact PicoQuant before changing settings in maintenance mode. Inadequate settings may cause serious problems.

To work properly with any experimental set–up, the SymPhoTime software needs to know its hardware configuration. The SymPhoTime stores this configuration data on two levels. The first level constitutes a kind of "known good" configuration for the system. These settings are called *Factory Defaults*. They are the same for all user accounts, therefore they are stored in a single configuration file (`MicroTime 200.cfg`). To retrieve this configuration, a user can select *Configuration* | *Restore Factory Defaults* from the main menu.

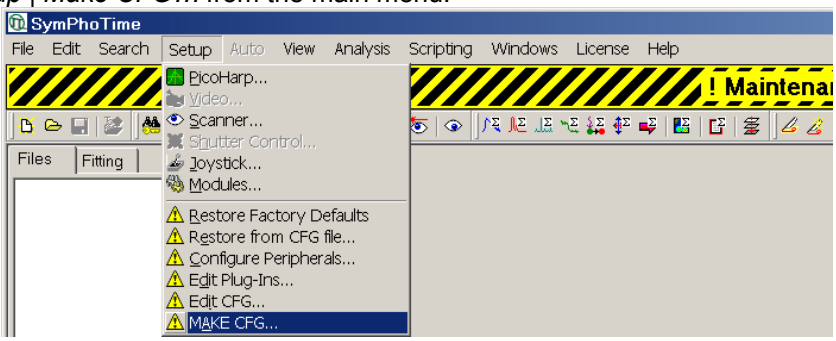
The second level of configuration data storage allows individual users to customise the system and the software. These customisations are saved in the Windows Registry for each individual user account.

Some hardware–related settings are critical for the performance of the system and must not be changed in day–to–day routine. These settings can only be edited in maintenance mode. Changes of these settings are at first stored in the Windows Registry and do not affect the *Factory Defaults*. To replace the original settings of the *Factory Defaults*, select *Setup* | *Make CFG…* from the main menu and replace the existing `MicroTime 200.cfg`. This is possible in maintenance mode only, and you need administrator privileges.

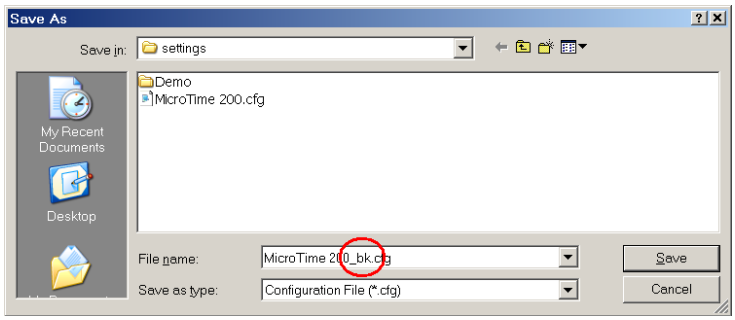
The software can be started in maintenance mode from the Windows Start menu entry of the SymPhoTime by using the link *Configuration* | *Maintenance Mode*. This link is only available if the software has been installed in "Operation" configuration.

Before changing any settings in maintenance mode, save a backup of the current configuration file (administrator privileges are required):

1. Select *Setup* | *Make CFG…* from the main menu:



2. The following dialog opens:

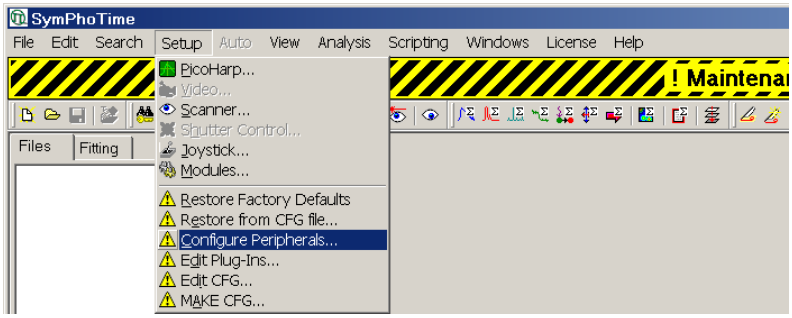


3. The existing configuration file `MicroTime 200.cfg` is visible in the file list. Select a different name for the backup file, for example `MicroTime 200_bk.cfg`.
4. Press *Save*.

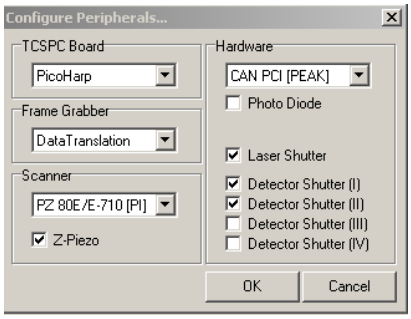
If it should become necessary to retrieve the settings from this backup file, they may be recovered by selecting *Setup* | *Restore from CFG file…* from the main menu.

1.6.1 Configuration of the Peripheral Electronic Setup

In maintenance mode, select *Setup* | *Configure Peripherals* from the main menu:



The following dialog opens:

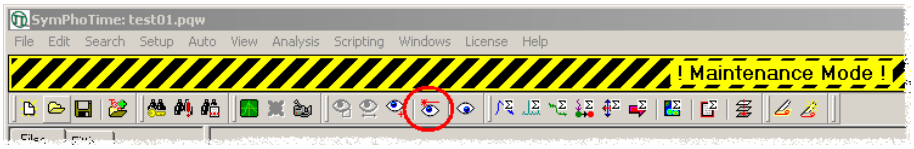


In this dialog, please select the components that are part of your system:

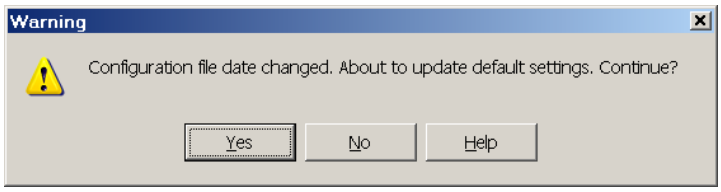
- *TCSPC Board*: Only one TCSPC device can be used at a time. Please select *HydraHarp, PicoHarp* or *TimeHarp*, according to the TCSPC electronics that is installed on your computer.
- *Frame Grabber*: This section is for users of a MicroTime 200 that is equipped with a video camera for readout by a frame grabber. In all other cases, please select *<none>*.
- *Scanner*: The following scanners are supported by the SymPhoTime: *PZ 80E/E-710[PI]* or *KDT 180-100-lm*. For LSM use please select *<none>*.
- *Hardware*: **Only for MicroTime 200.** Select *CAN PCI [PEAK]* or *CAN USB [PEAK]*, depending on the connection between the computer and the MT200 mainboard. Check the boxes for all shutters that are present in your MicroTime 200.

1.6.2 Configuration of the SymPhoTime Software for Usage with an LSM

1. Open a workspace.
2. Open the *Acquire LSM Measurements* menu pressing the following button:



3. In the *<user configurable LSM>* window, type in the configuration data for your specific LSM as shown below. In case your LSM is not listed, please contact PicoQuant.
4. Press *Set Defaults*. The software saves the new LSM configuration data to the Windows Registry. **Please note:** At this point, the changed settings will only affect the current user account. Furthermore, they can still be overwritten by selecting *Configuration* | *Restore Factory Defaults* from the main menu.
5. To transfer the settings to the *Factory Defaults*, select *Setup* | *Make CFG…* from the main menu and replace the existing `MicroTime 200.cfg`.
   Please note: Should it become necessary to install the software on a new PC, this settings file needs to be transferred to the new installation directory.
6. Restart the program in normal mode. For each user account the software recongises the change in the configuration file the first time it is started. The following message appears:

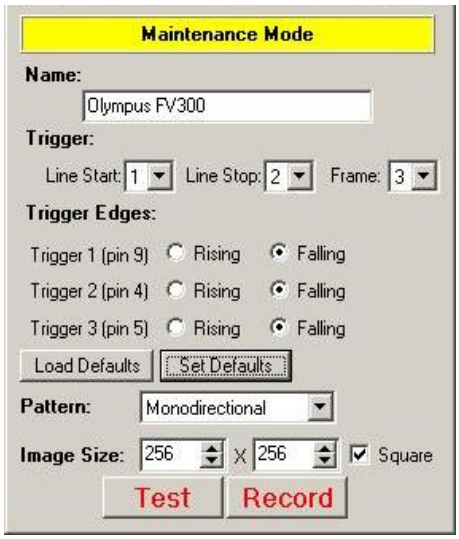


   Press *Yes* to transfer the changed settings to the default settings of the corresponding user account.

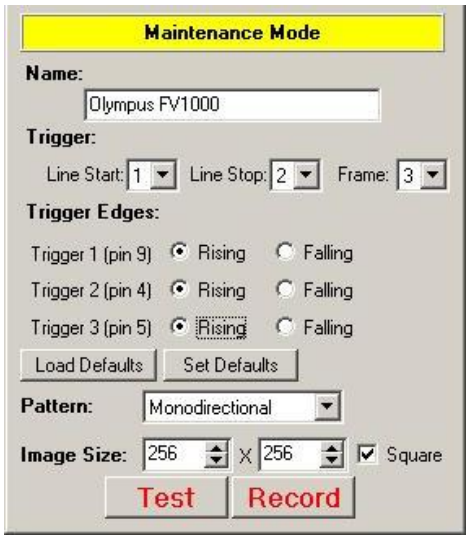
Configuration data for selected LSM companies and LSM types:

Please note that the settings that are displayed in this section are for the PicoHarp300. If you use the TimeHarp200 the “Trigger” settings for “Line Start” and “Line Stop” must be exchanged, e.g. “Line Start 2 and Line Stop 1” must be changed to “Line Start 1 and Line Stop 2”.

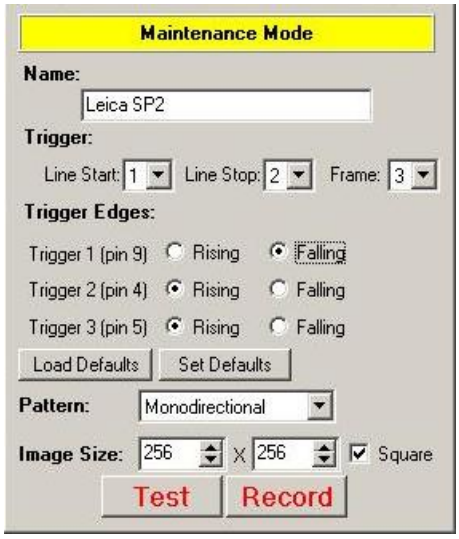
Olympus FV 300:



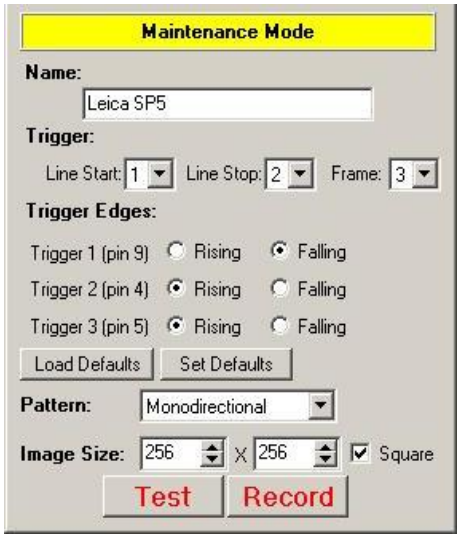
Olympus FV 1000:



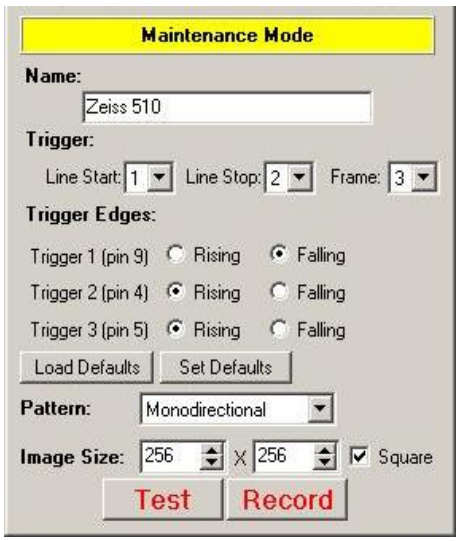
Leica SP2:



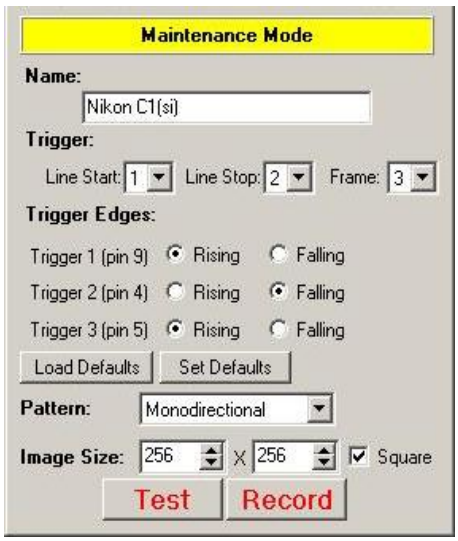
Leica SP5:



Zeiss LSM 510:



Nikon C1:



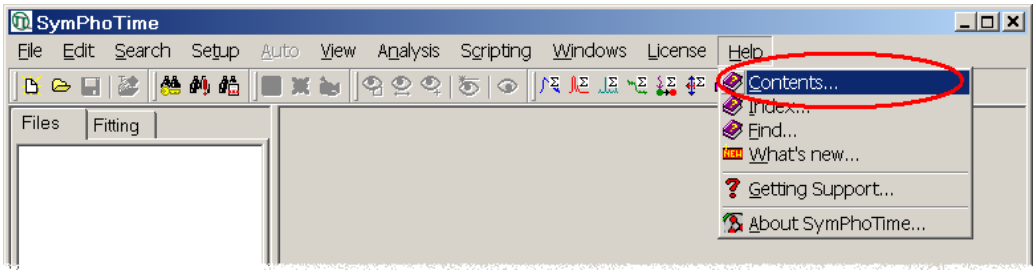
2. SymPhoTime in General

This section introduces the basic concepts and the user interface of the SymPhoTime software. Its purpose is to guide through the first steps in using this package. A complete description of all features cannot be provided in this manual. Please refer to the help file, which is installed with the software.

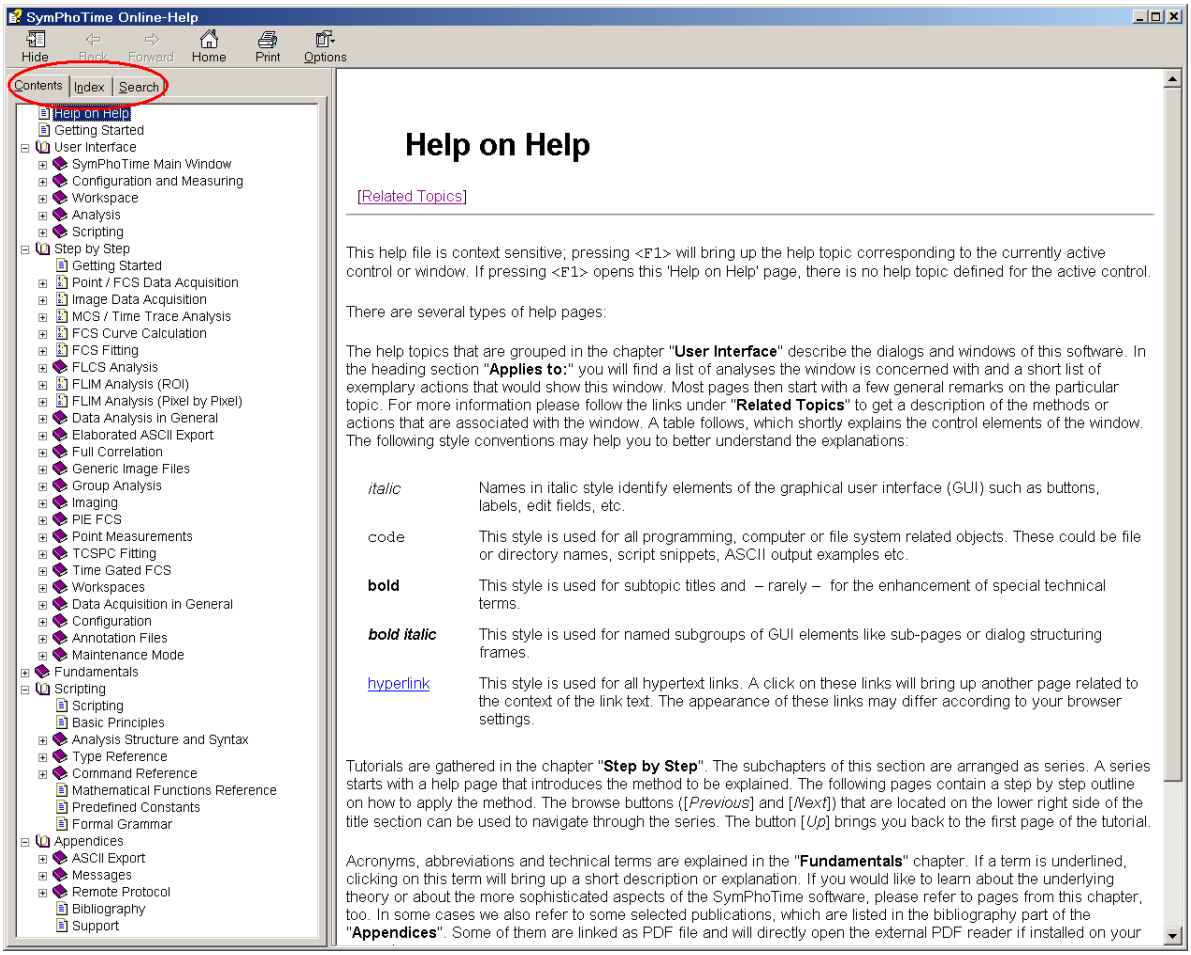
2.1 How to Get Help

Reference information, an extensive description of the user interface and additional step by step tutorials can be found in the help file that is installed with the package.

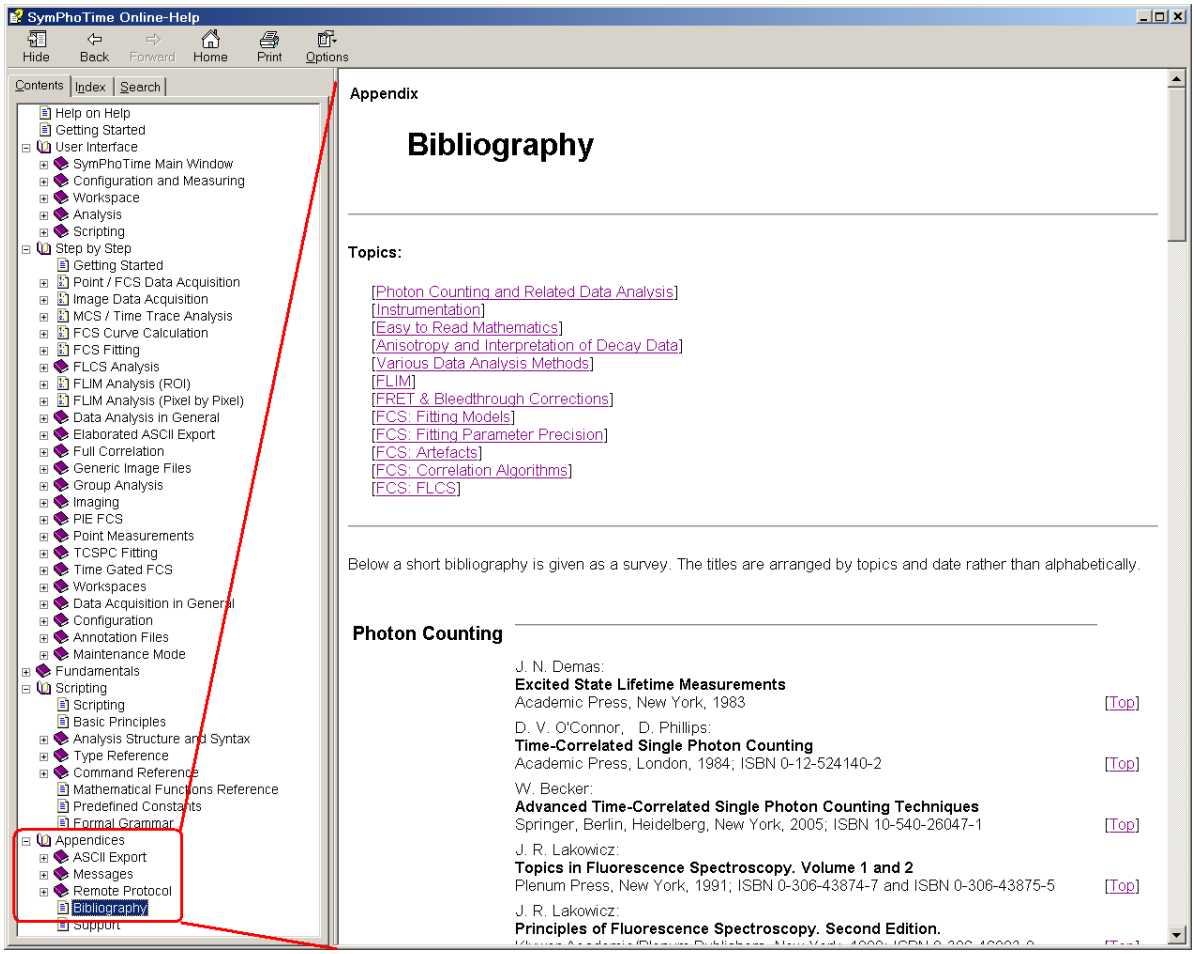
The help file can be directly accessed by *Help* | *Contents…* from the main menu.



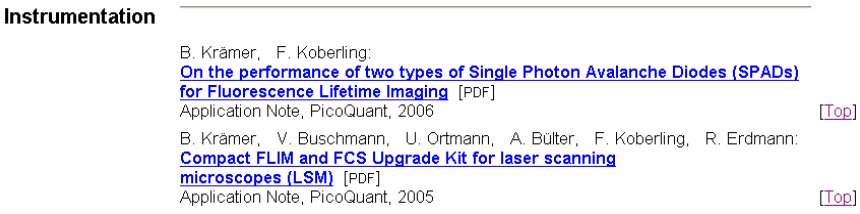
Either use the *Contents* of the help file to find information about the user interface, or to select a step–by–step tutorial. An extensive *Index* allows you to locate information by a keyword search.



For further reading on the "science behind", please refer to *Bibliography* in the *Appendices* section:

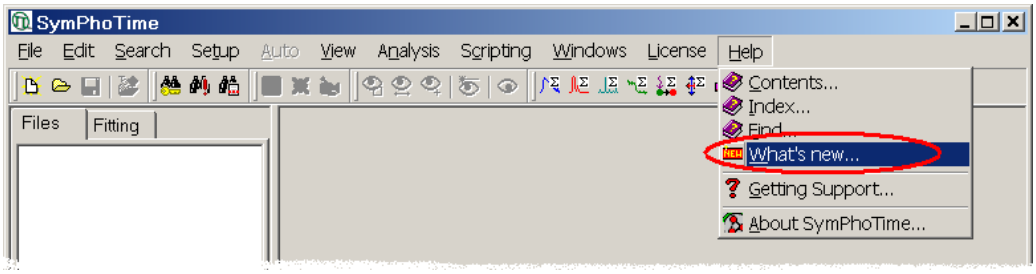


Some of the referenced articles are included the help file. These references are displayed as links. Just click on any of these entries to view the corresponding article in `pdf` format:



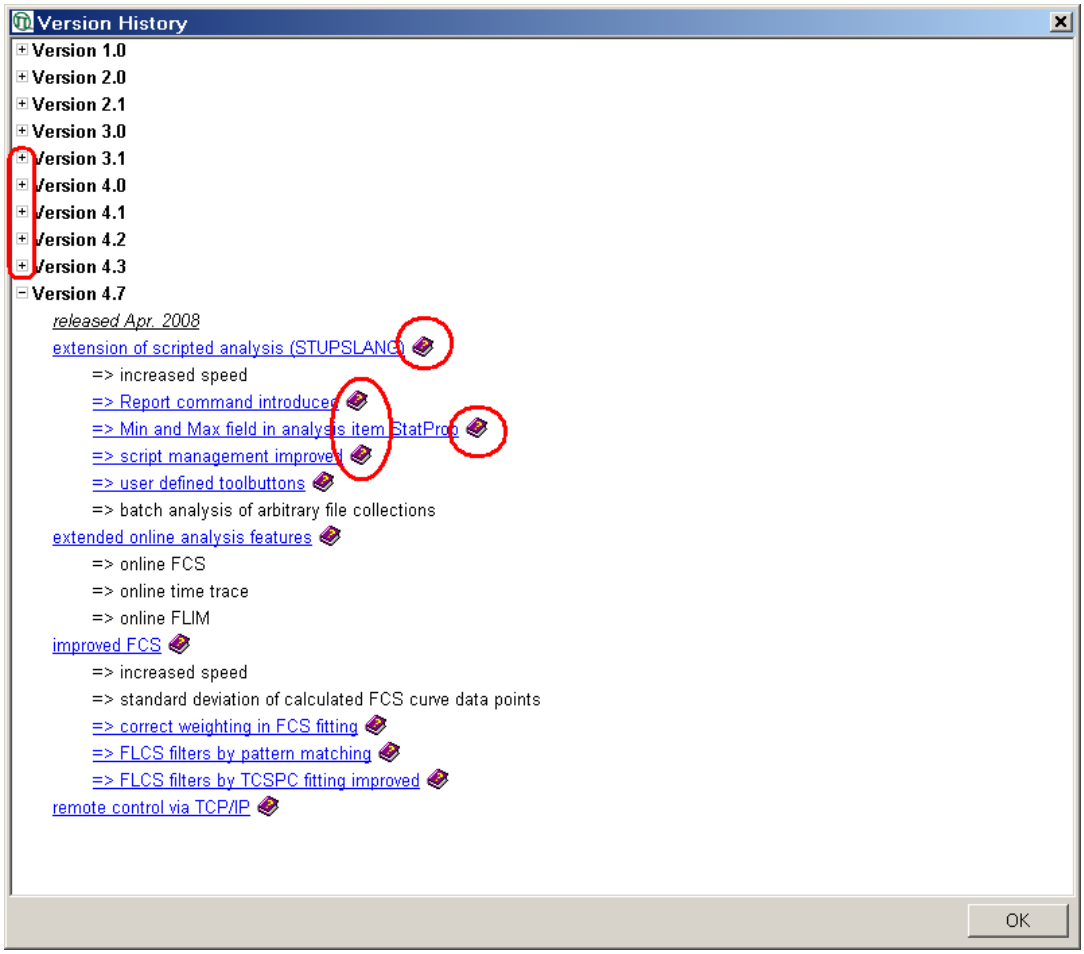
What's new...

A version history of the symphotime package can be viewed by *Help* | *What's new…* from the main menu.



The software presents an overview of all changes and implementations since the first released version. Click on the + buttons to expand the view for a version entry.

Furthermore, every entry that is displayed with a help icon serves as a direct link into the help file. Just click on the blue, underlined text to bring up the corresponding help page:



Getting Help for GUI Elements

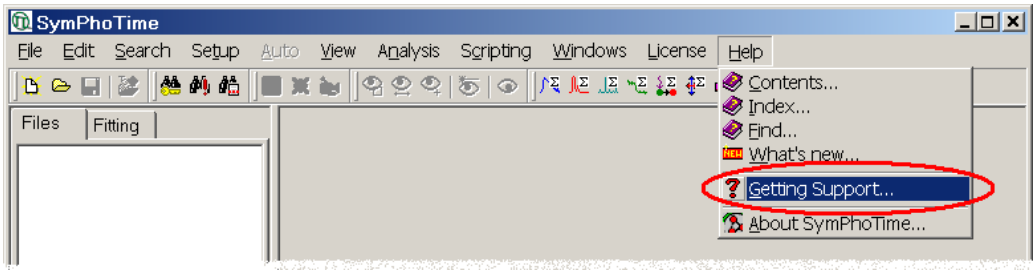
The help system is context sensitive. By pressing <F1>, a help page will be displayed that describes the active control or dialog.

Nearly every message (error, warning, etc.) of this software provides a *Help* button. Press this help button to bring up a corresponding help page. It explains the situation leading to the message and presents possible reasons or solutions.

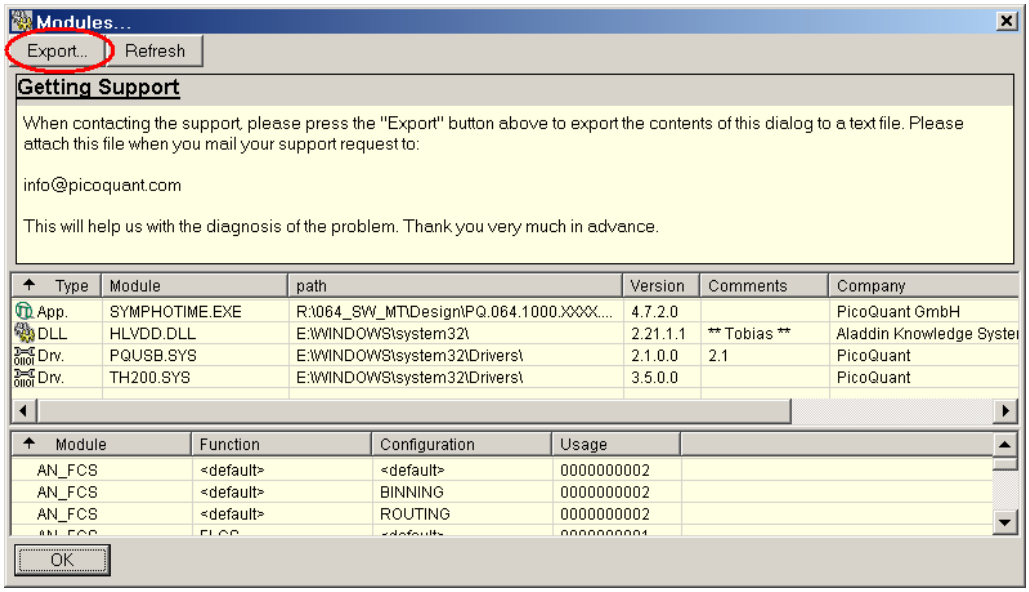
Getting Support

If you experience any problems with this software package, please do not hesitate to contact the support. Just e–mail us a description of the problem and of any relevant circumstances, e.g. the data in question.

When you contact the support, please bring up the *Modules* dialog:



Press the *Export* button of this dialog to export its contents to a text file:



This is an example, how this text file may look like:

```
"MODULES"
"App.","SYMPHOTIME.EXE","E:\Program Files\PicoQuant\SymPhoTime\","5.0.0.0","Feb. 2009","PicoQuant GmbH",
"2003 - 2009 PicoQuant"
"DLL","HLVDD.DLL","E:\WINDOWS\system32\","2.21.1.1","** Tobias **","Aladdin Knowledge Systems Ltd.",
"Copyright (c) 1985-2005 Aladdin Knowledge Systems Ltd."
"DLL","OLCLIB.DLL","E:\Program Files\PicoQuant\SymPhoTime\","0.10.0.0","","PicoQuant GmbH",
"Copyright © PicoQuant 1997-2007"
"DLL","TIMESCAN.DLL","E:\Program Files\PicoQuant\SymPhoTime\","3.5.0.0","Feb. 2007","PicoQuant GmbH",
"PicoQuant 2002 - 2007"
"DLL","ZSCAN.DLL","E:\Program Files\PicoQuant\SymPhoTime\","3.5.0.0","Feb. 2007","PicoQuant GmbH",
"PicoQuant 2003 - 2007"
"DLL","PHMTLIB.DLL","E:\Program Files\PicoQuant\SymPhoTime\","2.0.0.0",
"Programming library for PicoHarp 300 TCSPC system on Windows ME/2K/XP.","PicoQuant",
"Copyright © PicoQuant 1997-2006"
"Drv.","PQUSB.SYS","E:\WINDOWS\system32\Drivers\","2.1.0.0","2.1","PicoQuant","Copyright © 2004-2006"
"Drv.","TH200.SYS","E:\WINDOWS\system32\Drivers\","3.5.0.0","","PicoQuant",
"Sheldon Instruments 1999, PicoQuant 2006"
"Functions"
"AN_FCS","<default>","<default>","0000000002"
"AN_FCS","<default>","BINNING","0000000002"
"AN_FCS","<default>","ROUTING","0000000002"
"AN_FCS","FLCS","<default>","0000000001"
"AN_FCS_FIT","FITTING","PUREDIFF","0000000010"
"AN_IMG","SHOW_ROI","<default>","0000000056"
"AN_IMG","SHOW_ROI","MASK","0000000056"
"AN_IMG","RAINBOW","AV_LT","0000000013"
"AN_IMG","RAINBOW","MOD_INT","0000000018"
"AN_IMG","TOGGLE_FLIM","<default>","0000000006"
"AN_IMG","ADD_IMG_CALC","<default>","0000000003"
"AN_IMG","CALC_FAST","<default>","0000000002"
"AN_IMG","DISP_LTHISTO","<default>","0000000004"
"AN_IMG","RAINBOW","INT_IMG","0000000022"
"AN_IMG","<default>","<default>","0000000002"
"AN_IMG","REDRAW","<default>","0000000006"
"AN_IMG","RAINBOW","INT_FRAC","0000000001"
"AN_IMG","RAINBOW","LT_RAT","0000000001"
"AN_IMG","DISP_TCSPC","<default>","0000000003"
"AN_IMG","ROI","FREEHAND","0000000001"
"AN_IMG","RGB_SCHEME","<default>","0000000004"
```

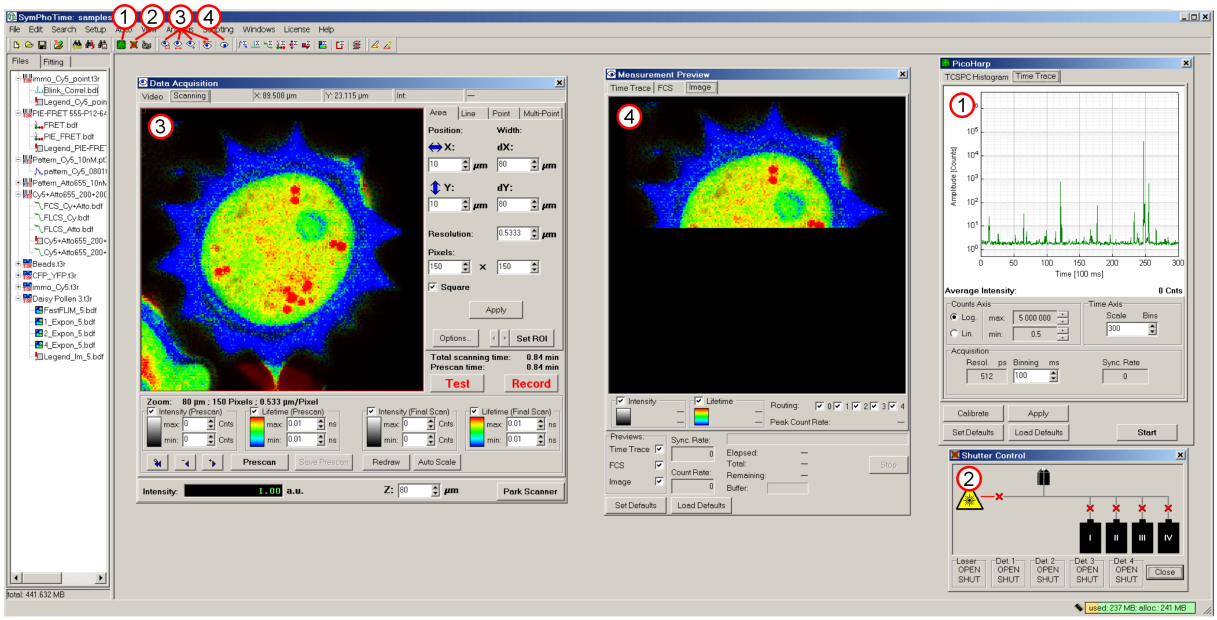
Depending on your system configuration the actual contents of this file may vary.

Please attach this file when you mail your support request to info@picoquant.com. This will help us with the diagnosis of the problem. Thank you very much in advance. Your feedback will help us to improve the product and its documentation.

2.2 User Interface

This chapter contains an overview of the SymPhoTime user interface. It is intended to provide the necessary background information for the step–by–step tutorials starting on page 17.

Data Acquisition



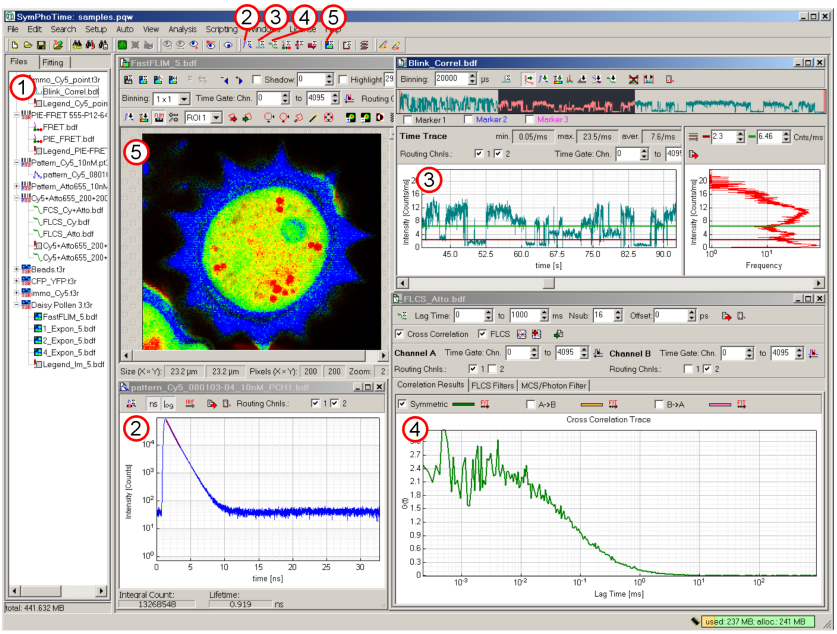
1. **Oscilloscope**
   The oscilloscope can be used in two different modes: *TCSPC Histogram* or *Time Trace*. The display can be toggled between these two modes by selecting the corresponding page of the dialog. To see a TCSPC histogram, select the appropriate page and press the *Start* button.

2. **Shutter Control**
   This dialog shows a schematic drawing of the MicroTime 200's optical parts. Shutters are displayed as ✕, when closed, or ●, when open. To open or close a shutter, click on its symbol, or click on the *OPEN* or *SHUT* label in the corresponding group box in the lower part of the dialog.

3. **Data Acquisition Panel**
   This dialog is not relevant for LSM users. It allows MicroTime 200 users to configure and run image scans, line scans and time trace measurements.

4. **Online Measurement Preview**
   The *Measurement Preview* allows users of both the MT200 and LSMs to monitor the progress of an image scan or a time trace measurement. It provides online image analysis (FLIM and fluorescence intensity) and an online fluorescence time trace or FCS curve display.

Analysis



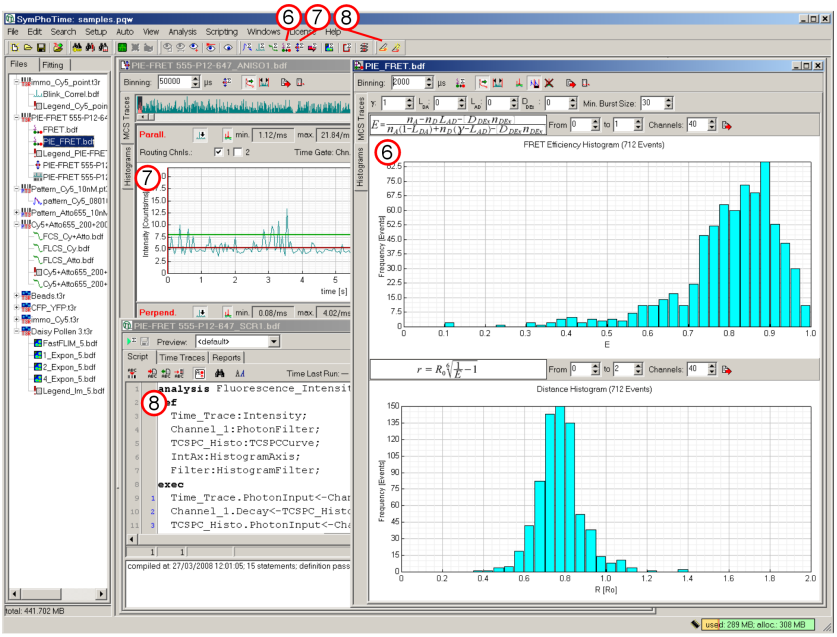
1. **Workspace Tree View**
   A workspace is the structure used for data management with the SymPhoTime software: Measurements produce TTTR files (`*.t3r`, `*.pt2` or `*.pt3`) in this folder. Likewise, any analysis performed on the TTTR files results in binary result files (`*.bdf`). The workspace documents the dependency between TTTR files and their derived data files.

2. **TCSPC Histogram**
   Time **C**orrelated **S**ingle **P**hoton **C**ounting (TCSPC) histogram (or decay curve) for a TTTR file.

3. **Time Trace / MCS Trace**
   Fluorescence time trace and fluorescence lifetime trace calculation, including time gated TCSPC histogramming, **B**urst **I**ntegrated **F**luorescence **L**ifetime (BIFL), lifetime histogramming, on / off histogramming, burst size histogramming and **P**hoton **C**ounting **H**istogram / **C**ount **R**ate **H**istogram (PCH / CRH).

4. **FCS**
   Fluorescence **C**orrelation **S**pectroscopy, **F**luorescence **C**ross **C**orrelation **S**pectroscopy (FCCS) and **F**luorescence **L**iftime **C**orrelation **S**pectroscopy (FLCS).

5. **Imaging**
   Time gated imaging, FRET imaging, **F**luorescence **L**ifetime **IM**aging (FLIM), including lifetime histogramming.



6. **FRET, PIE FRET**
   Förster **R**esonance **E**nergy **T**ransfer, with or without **P**ulsed **I**nterleaved **E**xcitation (PIE), burst integrated or binwise analysis, including bleedtrough corrections.

7. **Anisotropy**
   Steady state anisotropy histogramming, burst integrated or binwise analysis.

8. **Scripting (STUPSLANG)**
   Sym**P**ho**T**ime **U**ser **P**rogramming **S**cript **LANG**uage: User programmable multistage analysis including user defined equations, free histogramming, filtering etc., both for imaging and time trace analysis.

2.3 Basic Concepts

This chapter contains an introduction to the principles behind the SymPhoTime software package. It is intended to provide the necessary background information for the step–by–step tutorials starting on page 17.

Workspaces

A workspace is a structure used for data management with the SymPhoTime software. It is implemented as a standard file folder (i.e. directory). The name of the folder corresponds to the workspace name, which is also displayed in the title bar of the main window.

Measurements produce TTTR files (`*.t3r`, `*.pt2` or `*.pt3`) in this folder. Likewise, any analysis performed on the TTTR files will result in binary result files (`*.bdf`) saved in this folder. The organisational structure of the workspace is stored in a workspace file (`*.pqw`), which is an index to the TTTR files and to the derived data files.

To denote the relationship of the result files with their parent TTTR file, the workspace is displayed as a tree-like structure on the left side of the main window, which is used to handle and view the files of a workspace.

A workspace may contain TTTR files, derived data files and fitting results files. Because fitting results files are not necessarily associated with a TTTR file, they are displayed on a separate tab of the tree view control.

To show a `*.bdf` derived file, double click on the corresponding tree entry.

To rename a file, highlight it by a single mouse click. After a second click the file name can be edited. The program will not accept the new name if there already exists a file with this name.

Press <DEL> to delete highlighted file(s).

Comments, Annotations and TTTR Header Information

The simplest way to attach a brief description (or any other textual information) to a data file is to edit its comment. This is applicable to any workspace file, except bitmaps (`*.bmp`). Highlight the selected file to be commented, and select *Edit* | *Comment…* from the main menu to bring up a dialog allowing to edit the file comment. For TTTR files the *Headers* tab of this dialog displays its file header information.

File comments are limited to 256 characters. For more complex documentation tasks, a more advanced data documentation feature is available, called annotation.

Choose *Analysis* | *Annotation Textfile* from the main menu to bring up a window which allows to enter more extensive comments. Here, a descriptive text of arbitrary length can be typed in.

Automated Measurements

Automated measurements perform repeated point, line or area measurements, sometimes varying some parameters between the single measurements, like the Z–position of the PIFOC. The main menu entries under *Auto* give access to the automated measurement modes which are available with the current installation (greyed, if no workspace is loaded or if measurements are impossible for whatever reasons).

An automated measurement generates a TTTR group in the workspace. The TTTR files that are recorded for the series are added to this group. They can be treated like any other TTTR file, additionally the files of a group can be analysed automatically: It is possible to apply an analysis of a `*.bdf` file of a single group member to all other members of the same group: Highlight the `*.bdf` file in the workspace tree view, and select *Analysis* | *Analyse Group* from the main menu.

Analysis in General

Analysis of TTTR files always follows the same pattern. First select the TTTR file in the workspace tree view, then start the analysis choosing *Analysis* from the main menu or click on the appropriate tool button.

For the sake of simplicity, we can divide the measurements and their corresponding analyses into two groups. Analyses of measurements from a single "fixed" spot (i.e. detection volume), like MCS, FCS, FRET, and anisotropy will be referred to as point type analyses. Analyses of any measurement involving scanning (line and area scans) can be treated as imaging. Due to the very universal TTTR format, MCS, TCSPC histogramming, and even FCS trace calculations are possible with any `*.t3r` file, including those obtained by scanning. Please keep in mind that not every possible operation leads to a useful or meaningful result.

Whenever a results file is produced, it will be shown in a child window and will be inserted in the workspace tree under its parent TTTR file. Once a results window is closed, double clicking on its workspace entry will bring it up again.

3. Step–by–Step Tutorials

The SymPhoTime software can be used both as a complete data acquisition and analysis tool as well as a mere (offline) analysis tool without the presence of the hardware. To start the complete package, execute `SymPhoTime (Operation)`. If your software is configured to support the hardware and it is not available (for example it is not turned on), you might get error messages. To start the program in analysis mode, execute `SymPhoTime (Analysis)`. The appropriate shortcuts and icons are created by the installer and can be found in the *PicoQuant – SymPhoTime* group of the Windows Start menu.

3.1 Data Acquisition

The data acquisition tutorials of this section refer to the MicroTime 200. Users of Laser Scanning Microscopes (LSM) are referred to the LSM Upgrade Kit manual.

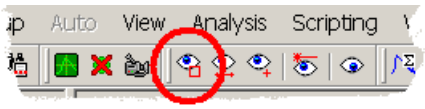
3.1.1 Imaging

Fluorescence Intensity / FLIM

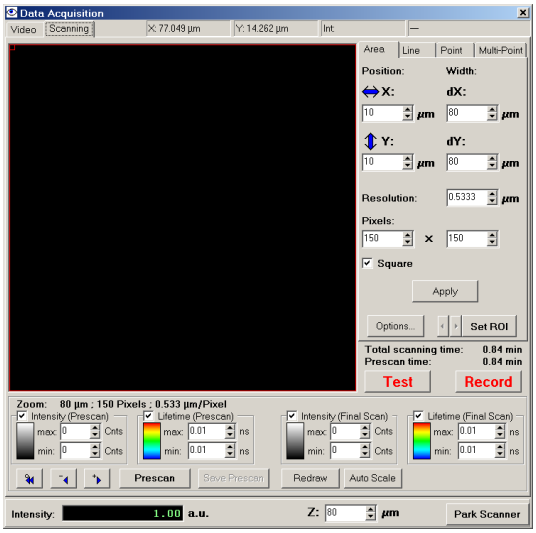
Description of image data acquisition, including prescanning (for generating a preview), final recording, setting of scanner parameters and definition of a region of interest (ROI) for scanning.

The FLIM tutorial refers to the MicroTime 200. Users of Laser Scanning Microscopes (LSM) are referred to the LSM Upgrade Kit manual. Please read chapter "FLIM Data Acquisition" of the hardware manual first to prepare the instrument.

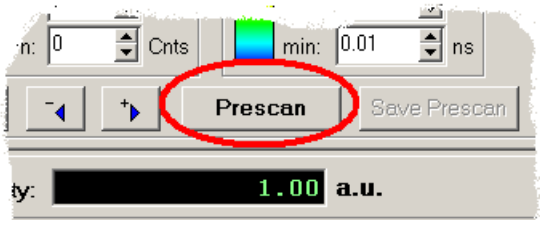
1. Create a new workspace by selecting *File* | *New Workspace…* from the main menu.
   Response: The software opens a dialog for specifying name and location of the new workspace.
2. Select a name and a location for the new workspace. To optimise data acquisition efficiency it is advisable to locate the workspace on the local hard drive. Please make sure that you have read and write permission for the folder you choose. Press *OK* to create the workspace.
   Response: The name of the new workspace is displayed in the title bar of the main window.
3. Press the *Acquire Images* toolbutton.



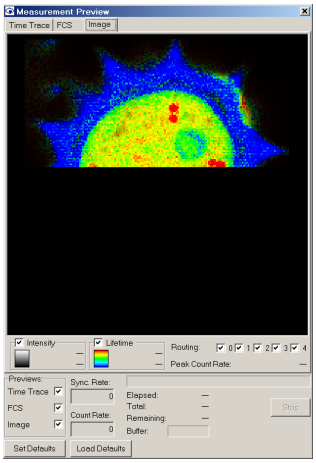
Response: The software opens the *Data Acquisition* dialog:



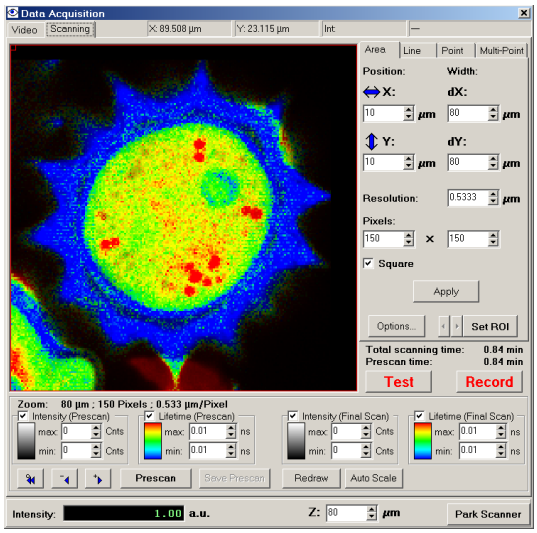
4. Press the "Prescan" button:



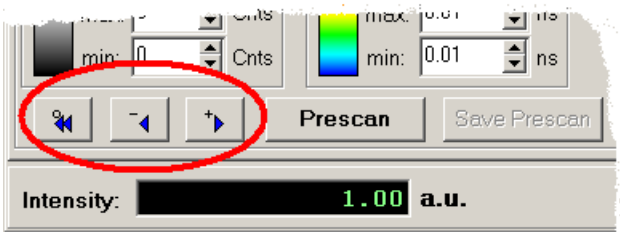
Response: The software starts the measurement and opens the *Measurement Preview* dialog on the *Image* page. The scanned image is displayed according to the progress of the scanning.



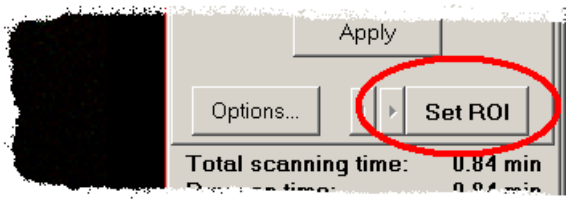
Liftime information is displayed by a false colour scheme, whereas the intensity information determines the brightness of each displayed pixel. After the scanning completes, the scanned image is transferred to the *Scanning* page of the *Data Acquisition* dialog.



5. Zoom to a suitable region by clicking and dragging on the displayed image. The toolbuttons beneath the image display can be used to navigate through the zoom history.

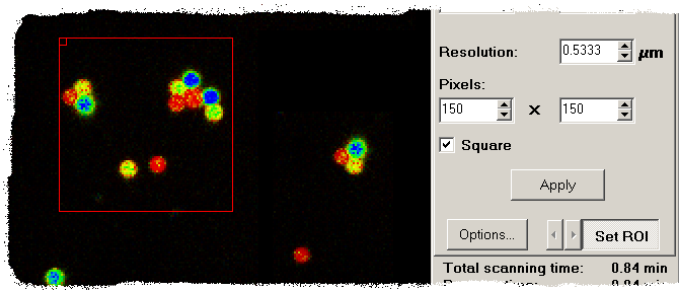


6. To improve the resolution of the zoomed image, the prescan can be repeated.
   Response: The newly scanned image replaces the previous prescan.
7. To define an area for the final scan, press the *Set ROI* button:

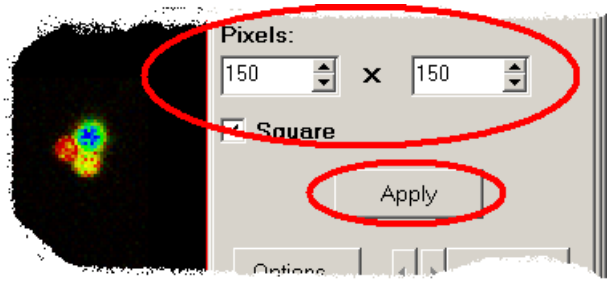


Response: The mouse cursor of the image display changes to the following shape:

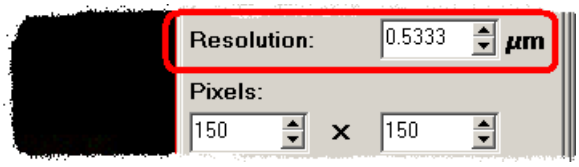
8. Select a suitable region of interest for the final scan by clicking and dragging on the displayed image.
   Response: The selected ROI is displayed as a red rectangle in the displayed image.



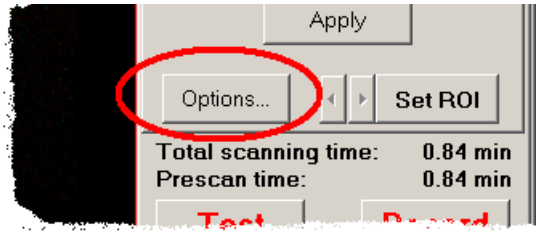
9. To set the number of pixels of the final scan, edit the values of the *Pixels* edit boxes and press the *Apply* button.



Response: The image resolution is updated in accordance with the new *Pixels* values.

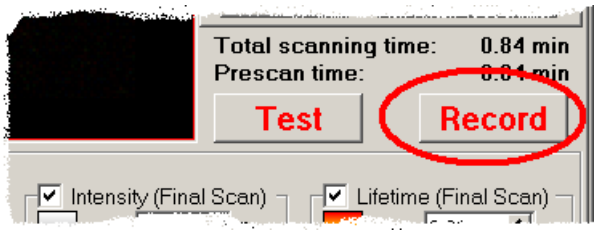


10. To set the scanning parameters, for example the scanning velocity, press the *Options* button:

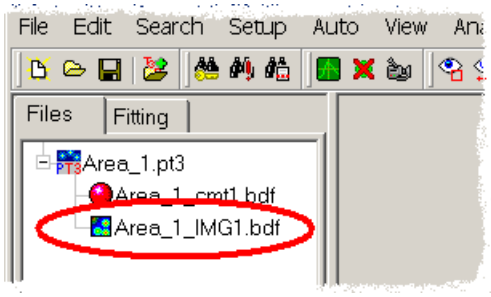


Response: The software opens a dialog for scanner configuration. Press *OK* to close this dialog.

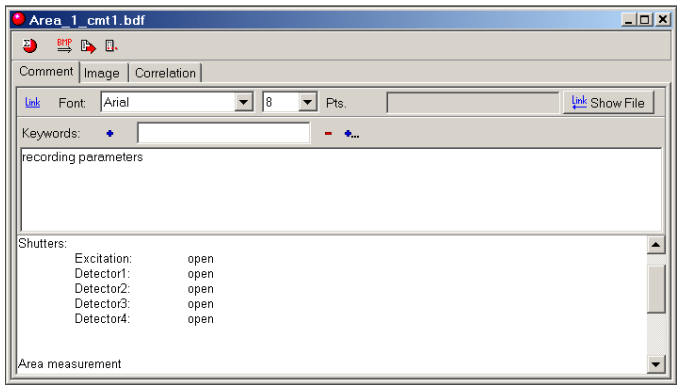
11. Press *Record*.



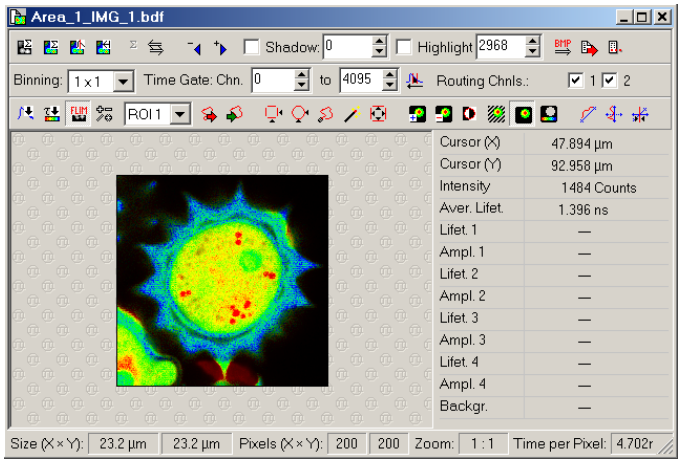
Response: The software starts the measurement and opens the *Measurement Preview* on the *Image* page. The scanned image is displayed according to the progress of the scanning. After the scanning completes, the scanned image is transferred to the *Scanning* page of the *Data Acquisition* dialog, where it is fitted into the ROI. The scanned image file is added to the workspace. It is displayed in the *Files* view on the left side of the main window.



A *Measurement Comment* window is created, which displays measurement parameters. You can add additional information.



12. To visualise the recorded image double click on its entry in the *Files* view.
Response: The image file is displayed in an *Imaging* window.



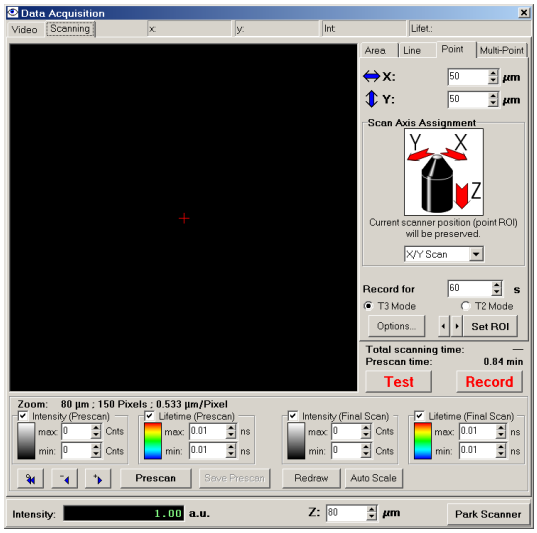
3.1.2 Time Trace

Point / FCS

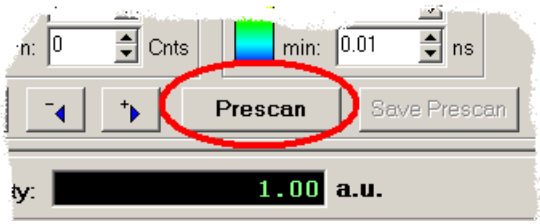
Description of point data acquisition: Scanning a preview image, defining the location (ROI) for the point measurement, defining the measurement duration for the captured FCS curve or fluorescence time trace.

1. Create a new workspace by selecting *File* | *New Workspace…* from the main menu.
   Response: The software opens a dialog for specifying name and location of the new workspace.
2. Select a name and a location for the new workspace. To optimise data acquisition efficiency it is advisable to locate the workspace on the local hard drive. Please make sure that you have read and write permission for the folder you choose. Press *OK* to create the workspace.
   Response: The name of the new workspace is displayed in the title bar of the main window.
3. Press the *Acquire Point Measurements* toolbutton.

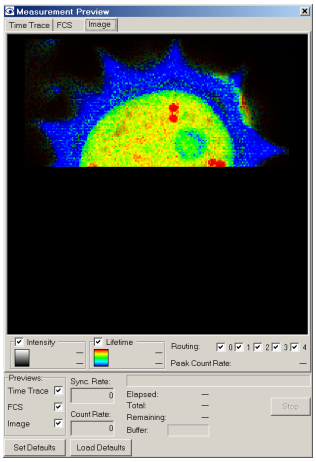
Response: The *Data Acquisition* dialog will be opened.



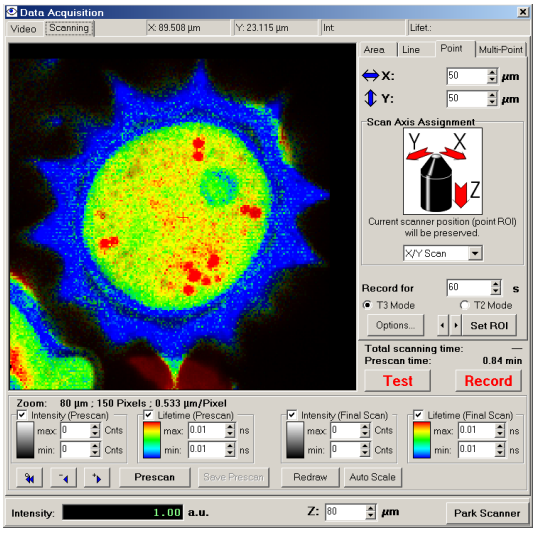
4. Press the *Prescan* button:



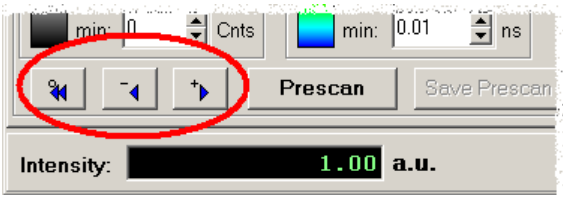
Response: The software starts the measurement and opens the *Measurement Preview* dialog on the *Image* page. The scanned image is displayed according to the progress of the scanning.



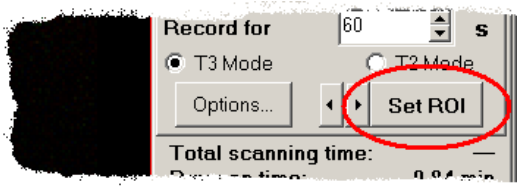
Liftime information is displayed by a false colour scheme, whereas the intensity information determines the brightness of each displayed pixel. After the scanning completes, the scanned image is transferred to the *Scanning* page of the *Data Acquisition* dialog.



5. Zoom to a suitable region by clicking and dragging on the displayed image. The toolbuttons beneath the image display can be used to navigate through the zoom history.

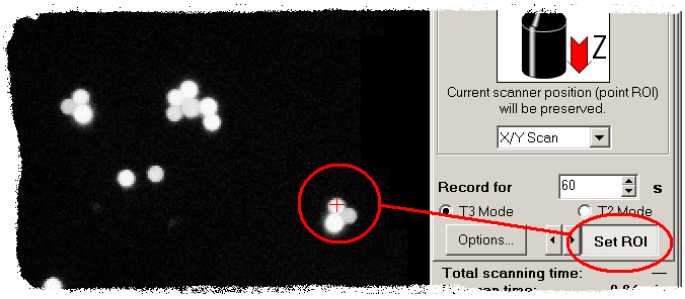


6. To improve the resolution of the zoomed image, the prescan can be repeated.
   Response: The newly scanned image replaces the previous prescan.
7. To select a point for the measurement, press the *Set ROI* button:

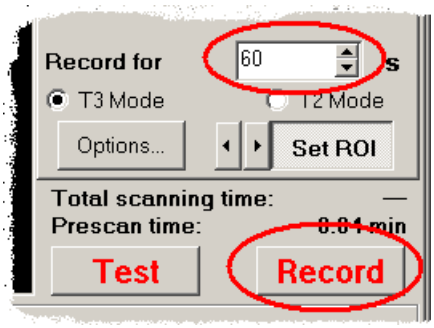


Response: The mouse cursor of the image display changes to the following shape:

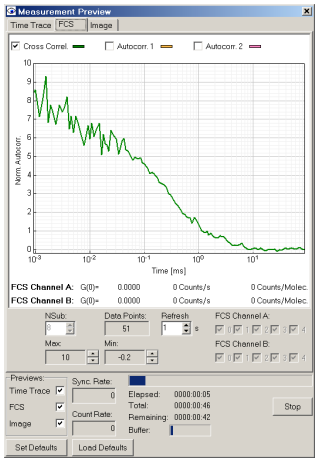
8. Click on the desired location in the image.
   Response: The scanning stage is immediately repositioned.

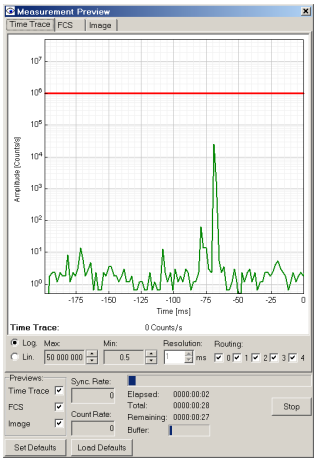


9. Set the measurement time in the *Record for* edit box and press the *Record* button.

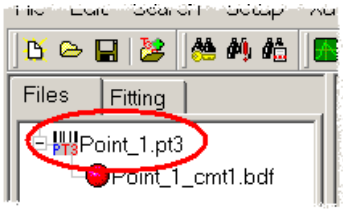


Response: The software starts the measurement and opens the *Measurement Preview*. Two of the three pages of this dialog can be selected for point measurements: On the *FCS* page an online FCS preview is displayed, whereas on the *Time Trace* page the count rate of the fluorescence photons is plotted as a function of time.

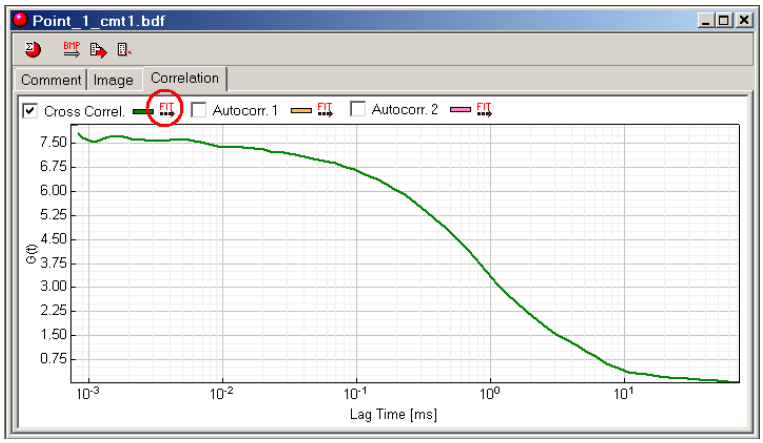




After the measurement completes, the recorded file is added to the workspace. It is displayed in the *Files* view on the left side of the main window:



A *Measurement Comment* window is created, which displays measurement parameters. You can add additional information. The online FCS curve is saved to the *Correlation* page of the *Measurement Comment* window. It can directly be fitted by pressing one of the *Fit* buttons.



3.1.3 Remote Control of the SymPhoTime for LSMs

The remote control is a small program which is designed to be executed on the LSM PC. It allows to control the data acquisition for FLIM and FCS measurements from the LSM PC.The program doesn't need to be installed but may simply be copied from the SymPhoTime install CD to the LSM PC. On the CD, it can be found as: `LSM_Remote_Control\Client_SymPhoTime_RC.exe`

The PC running the LSM software and the PC running the SymPhoTime software have to be connected via a Local Area Network (LAN) using the TCP / IP protocol. By default, the LSM Remote Control assumes the IP–address of the SymPhoTime PC to be `192.168.43.3` .

The IP address of the SymPhoTime can be set by

1. *Start* | *Control Panel*.
2. Open the "*Network Connections*" icon.
3. Click with the right mouse button on the desired connection and chose "*Properties*"
4. Click on "*Internet Protocoll (TCP / IP)*"
5. Click on "*Properties*"
6. Click on "*Use the following IP address*" and enter the desired IP address
7. Close all opened windows by clicking "*OK*"

If another IP–address has to be used for the SymPhoTime PC, please call the program with the following command line option: `Client_SymPhoTime_RC.exe -ip=xxx.xxx.xxx.xxx`
(where `xxx.xxx.xxx.xxx` represents the IP–address of the SymPhoTime PC). The IP–address currently used is shown in the foot–region of the RC program window.

Please make sure that the connection is not rejected by a firewall on either PC. The connection uses port 6000 on the side of the SymPhoTime PC. More detailed information about the connection syntax can be found in the SymPhoTime online help.

Remote Controlled Data Acquisition for FLIM

1. Start the program on the LSM PC. Be sure that SymPhoTime is already up and operational for data acquisition. This requires at least a workspace to be opened and the acquisition hardware to be on–line and initialised.

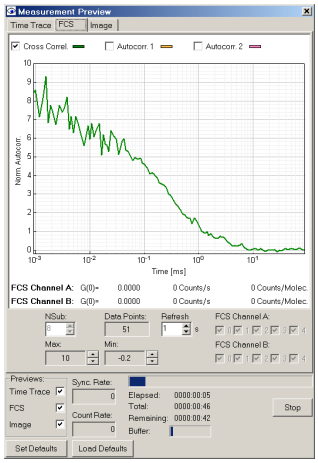
2. Choose the *FLIM* page of the RC–window.
   Response: The dialog offers to enter FLIM image parameters:

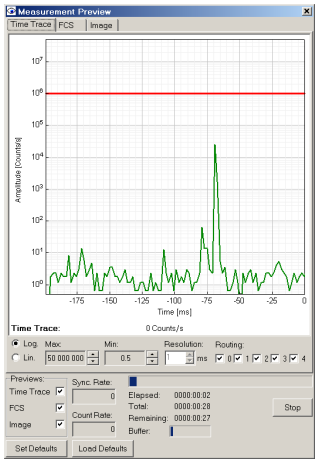
3. Set the scanning *Pattern* according to the mode of your LSM scanner.
4. Set the *Image Size* according to the scanning resolution of your LSM.
   You may enter the dimensions by spin control or the edit fields as well.
   Any format from 16 x 16 pxels up to 512 x 512 pixels is allowed.
5. Press *Test* to perform a test scan.
   The data will be shown as a preview but not be stored.
6. If all preliminary settings are done, press *Record* to start the final measurement.
   Response: The software starts the measurement and opens a *Measurement Preview*. The scanned image is displayed according to the progress of the scanning.
7. After the scanning completes, press *Stop* to end the data acquisition. The preview window closes and the scanned image file named `SRV_nnn` is added to the workspace, where `nnn` is the consecutive number. It is displayed in the *Files* view on the left side of the main window.

Remote Controlled Data Acquisition for Point / FCS Measurements

1. Start the program on the LSM PC. Be sure that SymPhoTime is already up and operational for data acquisition. This requires at least a workspace to be opened and the acquisition hardware to be on–line and initialised.
2. Choose the *FCS* page of the RC–window.
   Response: The dialog offers to enter the measurement time:

3. Set the measurement time in the *Record for* edit box and press the *Record* button.
   Response: The software starts the measurement and opens the *Measurement Preview*. Two of the three pages of this dialog can be selected for point measurements: On the *FCS* page an online FCS preview is displayed, whereas on the *Time Trace* page the count rate of the fluorescence photons is plotted as a function of time.





4. After the measurement completes by either pressing the *Stop* button or waiting for the measurement time to expire, the recorded file named `SRV_nnn` is added to the workspace, where `nnn` is the consecutive number. It is displayed in the *Files* view on the left side of the main window.

Note: The remote control program is designed to always stay on top of the programs on your desktop. This may or may not collide with other programs using the same feature. If the window of the remote control does not stay on top of the LSM application program, please reduce the LSM program window size so that the remote control program can be placed outside the LSM program window.

3.2 Data Analysis

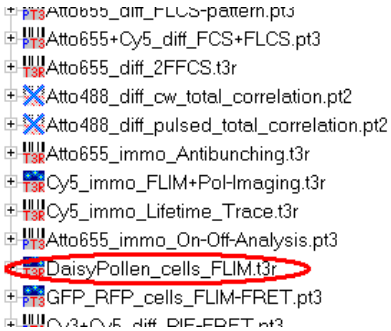
In the following sections a representative selection of analyses is demonstrated. For a description of (PIE–) FRET, Anisotropy, On / Off histogramming, etc. please refer to the help file.

3.2.1 FLIM

ROI

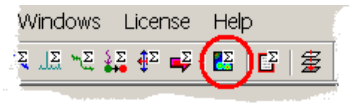
FLIM analysis by selection of a Region Of Interest (ROI) in the image and calculation of the TCSPC histogram (fluorescence lifetime decay) for the fluorescence photons of the ROI. Fit of the lifetime decay.

1. Open the sample workspace:
   `<Documents and Settings>\All Users\Documents\SymPhoTime\samples\samples.pqw`
   Alternatively, if you click on the link `samples` in the group *PicoQuant – SymPhoTime* of the Windows Start menu, the software is opened with the sample workspace.
   Response: The sample workspace contains several examples of measurements and analyses. The files of the sample workspace are displayed in the *Files* view on the left side of the main window.



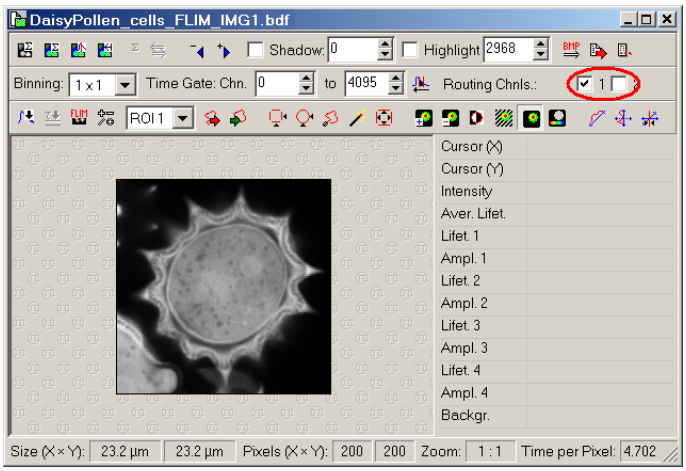
2. Click on the file `DaisyPollen_cells_FLIM.t3r`.
   Response: The file is highlighted in the workspace tree view.

3. Press the *Image* toolbutton.

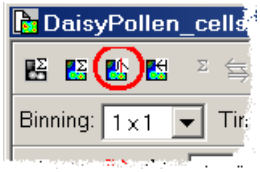


Response: The software calculates a simple intensity image from the raw data file.

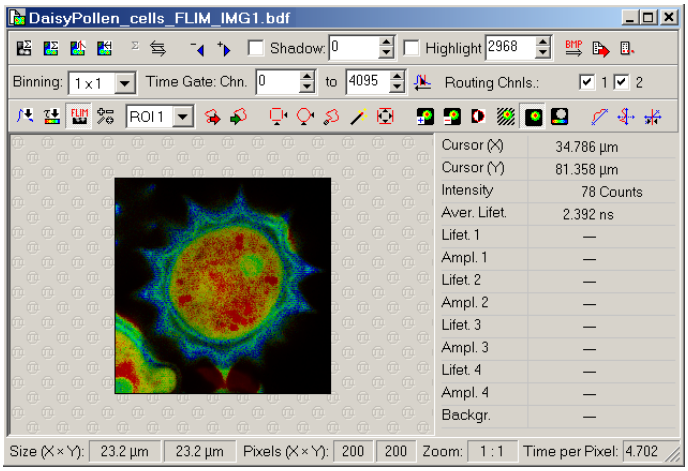
4. Select *Routing Channel 1* by checking the corresponding box and unchecking all other boxes.



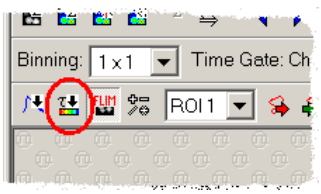
5. Press the *Fast FLIM* toolbutton.



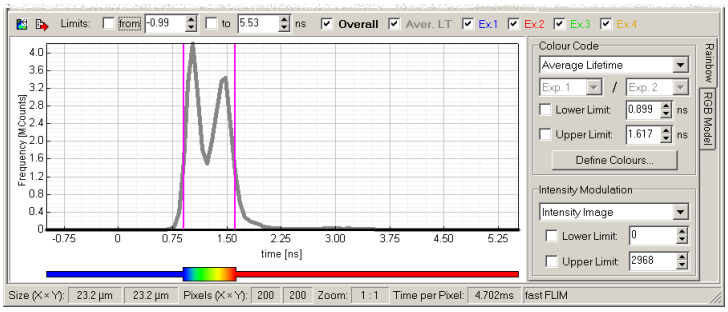
Response: The software applies the routing selection and calculates the so–called fast FLIM image. The fast FLIM image uses the average arrival time of fluorescence photons after the excitation pulse as a measure for the average lifetime.



6. Press the *colours and lifetime histogram…* toolbutton.

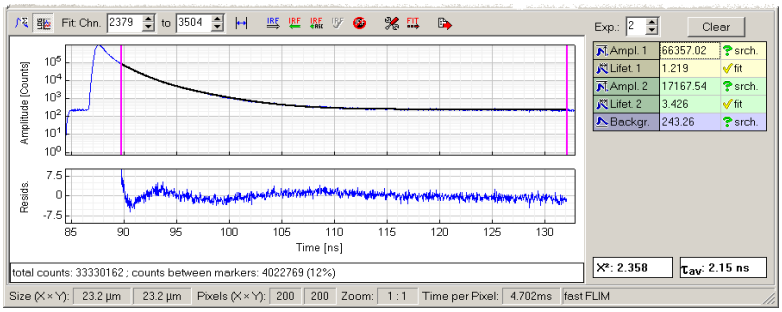


Response: The *Imaging* window is extended by a plot of the lifetime histogram. This plot shows the frequency, with which each lifetime occurs in the image. The colour coding of the FLIM image is visualised by the bar below the lifetime histogram.

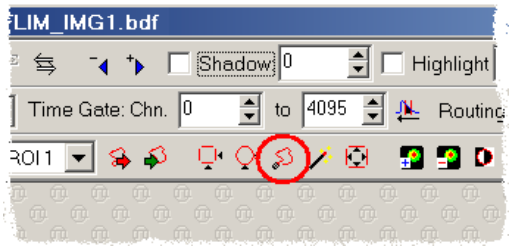


7. The colour contrast can be adjusted by dragging the pink marks in the lifetime histogram.
   Response: The software updates the color code of the image.
8. Press the *fitting and TCSPC histogram…* toolbutton.

Response: The *Imaging* window is extended by a plot of the TCSPC curve from all image pixels.

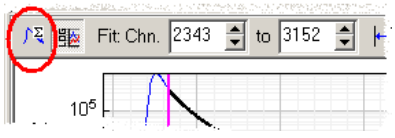


9. Press the *select free ROI* toolbutton.



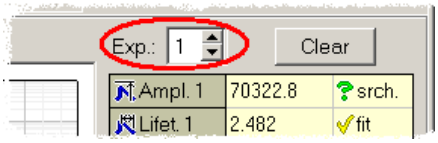
Response: ROI selection is activated for the click and drag action.

10. Hold down the left mouse button and encircle a suitable ROI with the mouse on the image. Release the left mouse button.
    Response: The software hides all image pixels that are not selected.
11. Press the *recalculate TCSPC histogram* toolbutton.

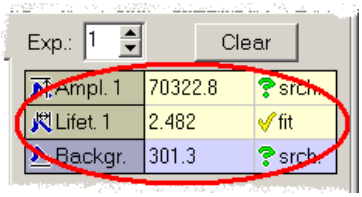


Response: The software calculates the TCSPC curve for the selected ROI.

12. Select a mono–exponential fitting model by entering `1` into the *Exp.* edit box.



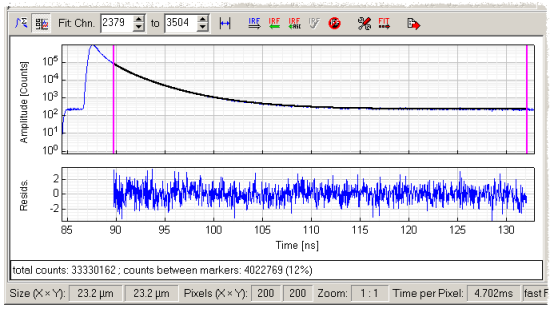
Response: The parameter table updates the model parameter list:



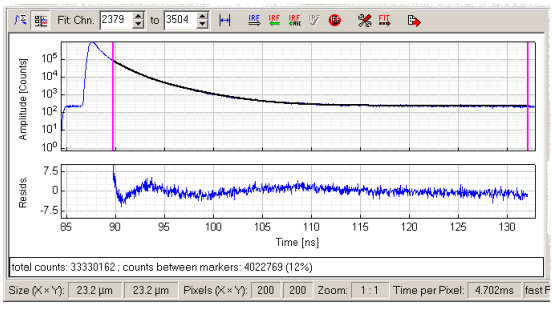
13. Press the *Clear* button.
Response: The software resets the fitting parameters.
14. Press the *Fit* toolbutton.

Response: The software performs a tailfit to optimise the fitting parameters for the TCSPC curve of the ROI.

15. Check the residuals trace. For an acceptable fit the residuals trace should not show any trends:



If there are trends, like in the following example, the mono–exponential model does not describe the TCSPC curve of the ROI sufficiently:

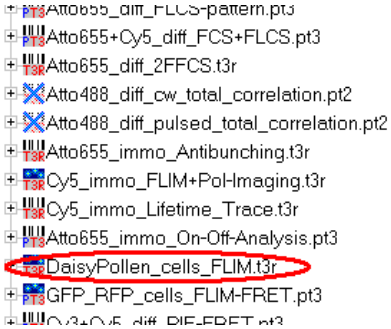


In this case increase the number of exponentials in the *Exp.* edit box and continue with step 13.

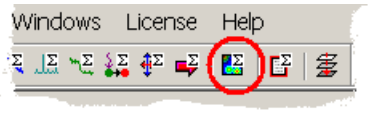
Pixel by Pixel

FLIM analysis by fitting a FLIM image pixel by pixel with a double–exponential and three–exponential tail fit. Display of the calculated data using an RGB false colour model. The calculated amplitudes for the first, second and third exponential are depicted in three different colours.

1. Open the sample workspace:
   `<Documents and Settings>\All Users\Documents\SymPhoTime\samples\samples.pqw`
   Alternatively, if you click on the link `samples` in the group *PicoQuant – SymPhoTime* of the Windows Start menu, the software is opened with the sample workspace.
   Response: The sample workspace contains several examples of measurements and analyses. The files of the sample workspace are displayed in the *Files* view on the left side of the main window.

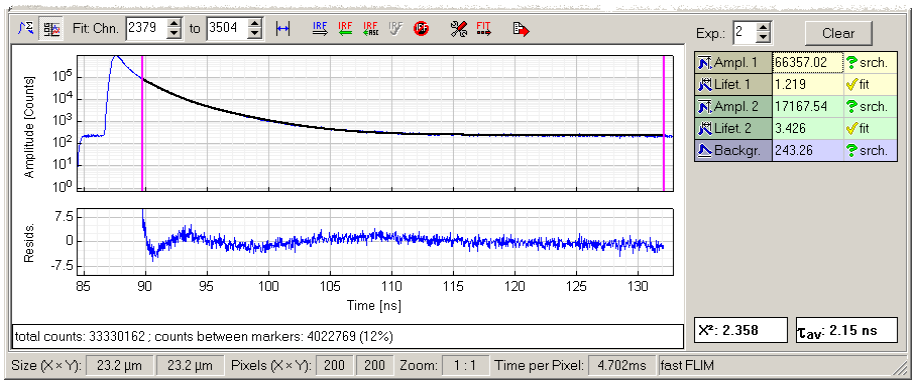


2. Click on the file `DaisyPollen_cells_FLIM.t3r`.
   Response: The file is highlighted in the workspace tree view.
3. Press the *Image* toolbutton.

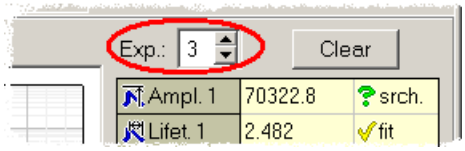


   Response: The software calculates a simple intensity image from the raw data file.
4. Press the *fitting and TCSPC histogram…* toolbutton.

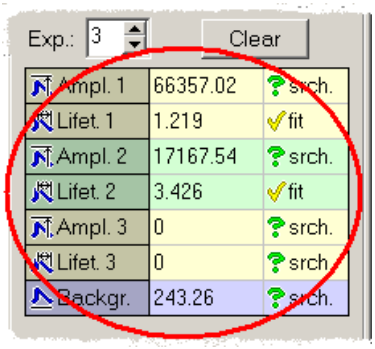
   Response: The *Imaging* window is extended by a plot of the TCSPC curve from all image pixels.



5. Select a three–exponential fitting model by entering 3 into the *Exp.* edit box.



Response: The parameter table updates the model parameter list:

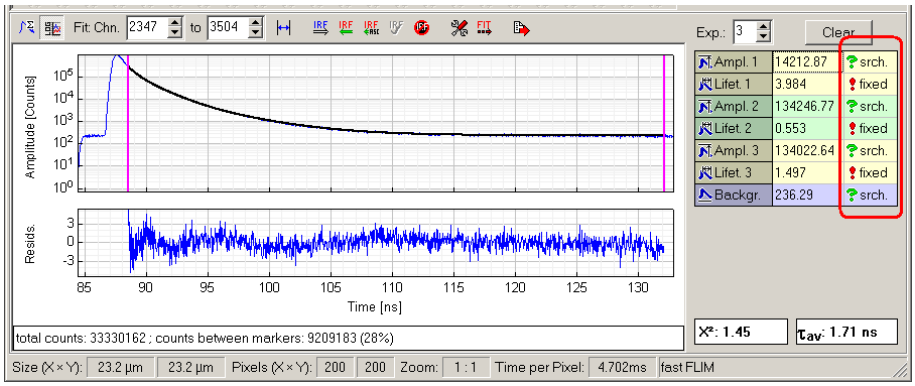


6. Press the *Clear* button.
Response: The software resets the fitting parameters.

7. Press the *Fit* toolbutton.

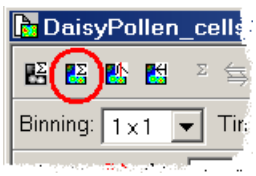
Response: The software performs a tailfit to optimise the fitting parameters for the TCSPC curve of the ROI.

8. Mark all lifetime parameters as "*fixed*" and all other parameters as search by (repeated) klicking on the last column of the parameter table for each row.

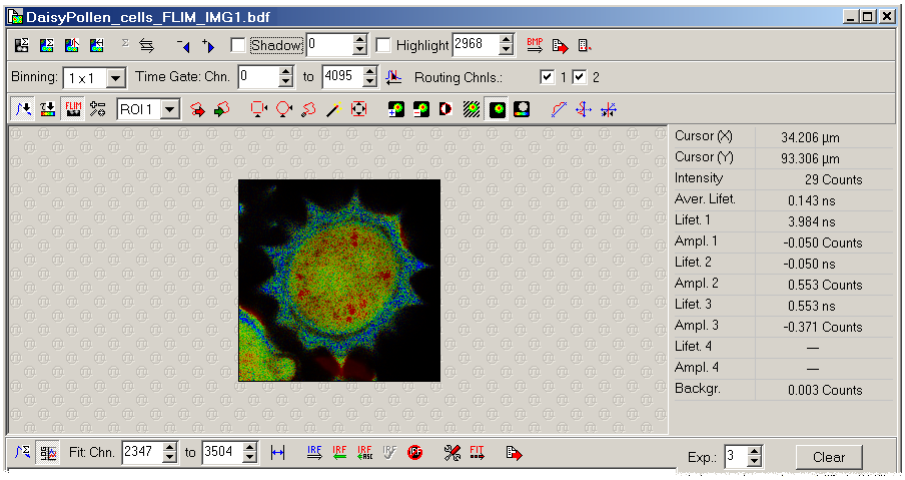


Response: The software initialises the optimisation behaviour of the fitting parameters for the following image fit. The parameters marked as "*fixed*" will be the same for all image pixels, whereas the parameters marked as "*search*" will be subject to optimisation.

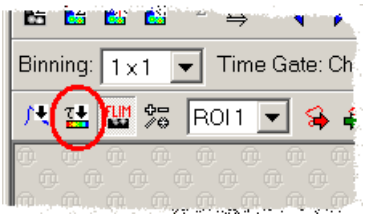
9. Press the *Recalculate* button.



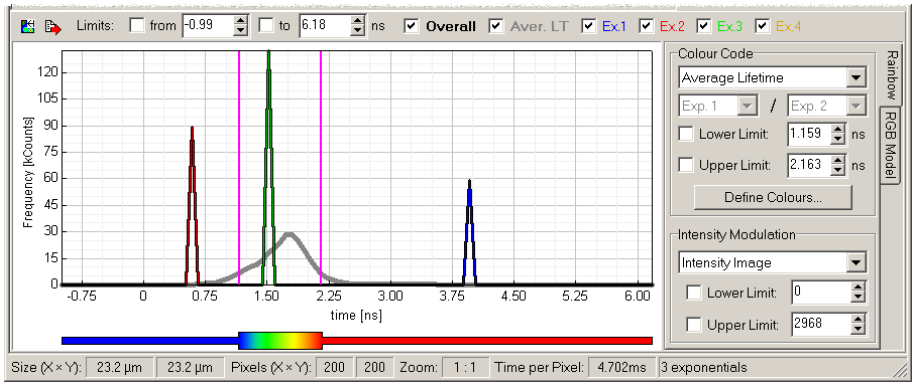
Response: The software starts the pixel by pixel fitting. This may take several minutes. The fitting can also be confined to a ROI. After the calculation the image is displayed as a false colour plot.



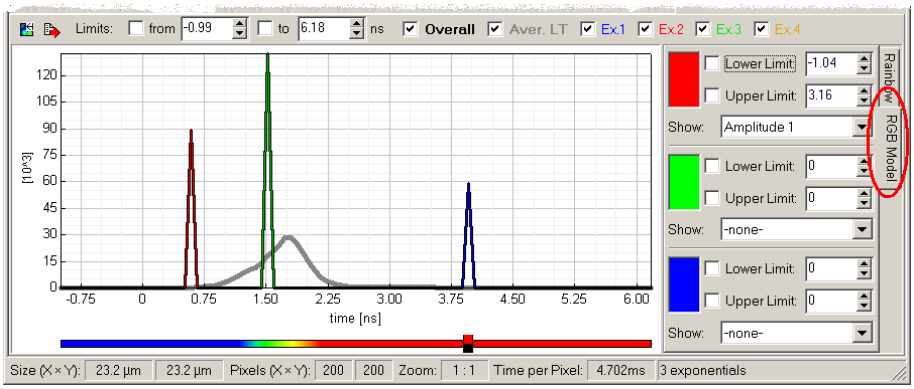
10. Press the *colours and lifetime histogram…* toolbutton.



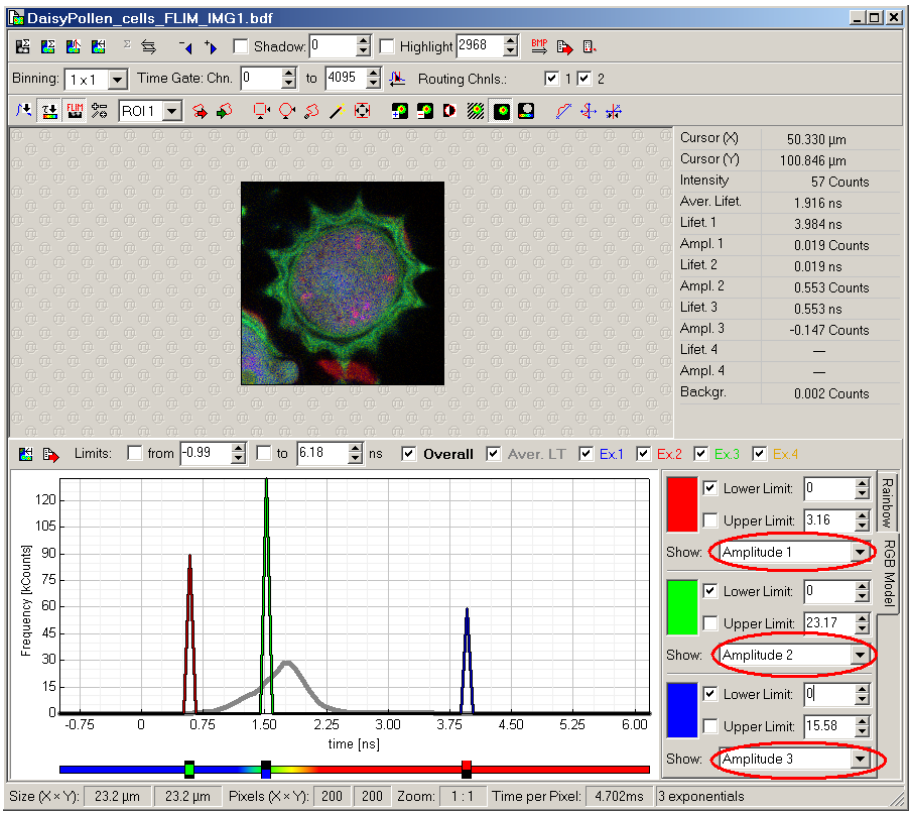
Response: The *Imaging* window is extended by a plot of the lifetime histogram. This plot shows the frequency, with which each lifetime occurs in the image. The colour coding of the FLIM image is visualised by the bar below the lifetime histogram.



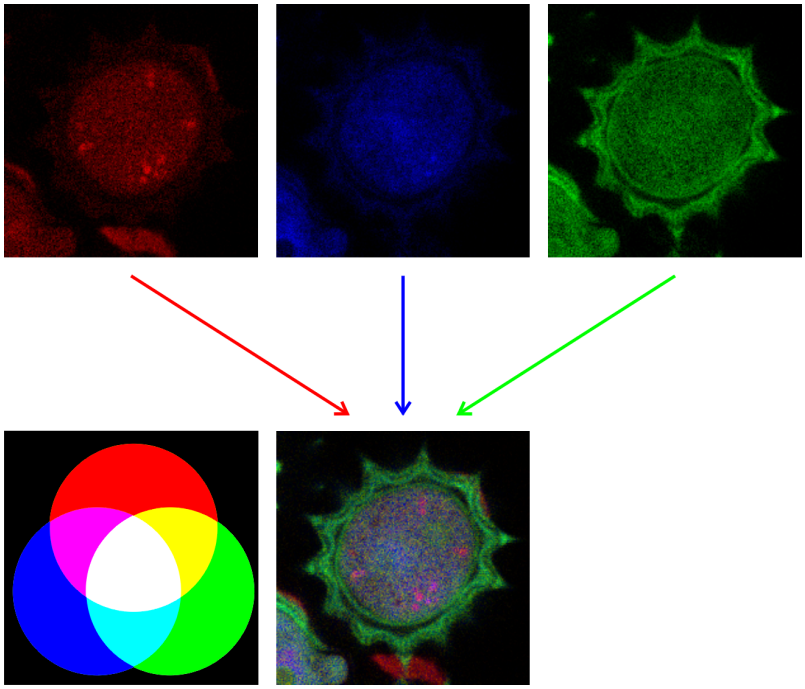
11. Select the *RGB Model* page.
Response: The software selects a different false colour scheme:



12. Select *Amplitude 1* for the red channel, *Amplitude 2* for the green channel and *Amplitude 3* for the blue channel.



Response: The fitted amplitudes are displayed in different colours. The brightness of each colour corresponds to the amplitude of the exponential.

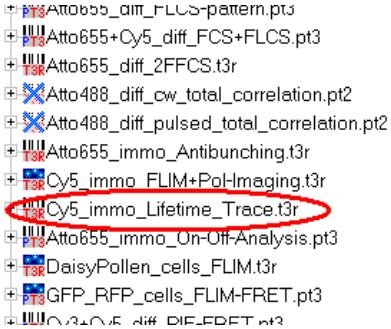


3.2.2 MCS / Time Trace Analysis

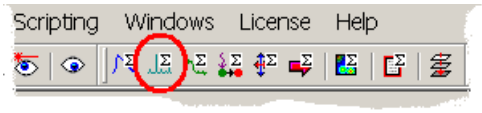
Fluorescence Lifetime Trace Calculation

Calculation of the intensity time trace, selection of routing (detection) channels and binning width. Display of the TCSPC histogram, double–exponential tail fit. Fit of each time bin of the time trace. Display of the lifetimes of the first and second exponential in a lifetime histogram.

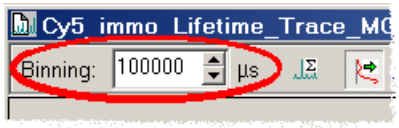
1. Open the sample workspace:
   `<Documents and Settings>\All Users\Documents\SymPhoTime\samples\samples.pqw`
   Alternatively, if you click on the link `samples` in the group *PicoQuant – SymPhoTime* of the Windows Start menu, the software is opened with the sample workspace.
   Response: The sample workspace contains several examples of measurements and analyses. The files of the sample workspace are displayed in the *Files* view on the left side of the main window.



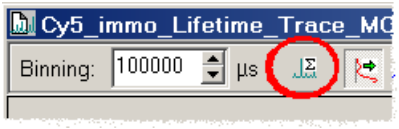
2. Click on the file `Cy5_immo_Lifetime_Trace.t3r`.
   Response: The file is highlighted in the workspace tree view.
3. Press the *MCS trace* toolbutton.



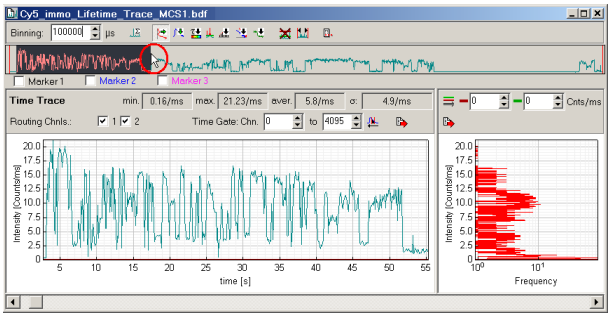
   Response: The software opens the raw data file in an *MCS* window.
4. Set a *Binning* of 100000 µs.



5. Press the *recalculate MCS* toolbutton.

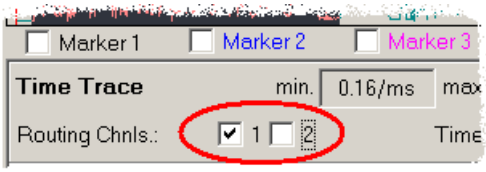


   Response: The software calculates an intensity time trace from the raw data file.
6. Select a suitable time window for display by click and drag on the overall plot.

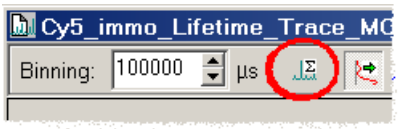


Response: The selected time window is highlighted in the overall plot. This highlighted region is displayed in the detail plot below.

7. Select *Routing Channel 1* by checking the corresponding box and unchecking all other boxes.

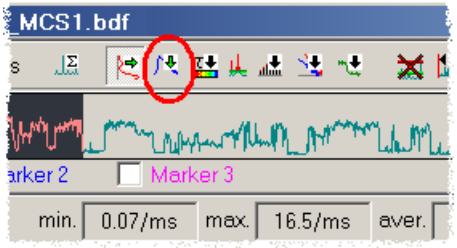


8. Press the *recalculate MCS* toolbutton.



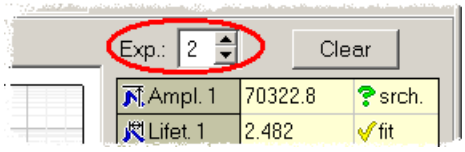
Response: The software calculates an intensity time trace for routing channel 1.

9. Press the *TCSPC histogram* toolbutton.



Response: The *MCS* window is extended by a plot of the TCSPC curve from the complete time trace.

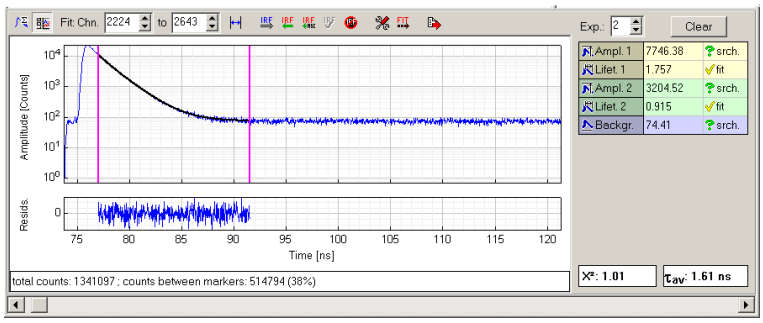
10. For this example, select a double–exponential fitting model by entering `2` into the *Exp.* edit box.



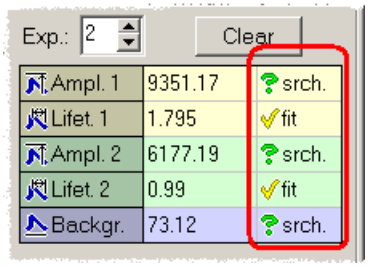
11. Press the *Clear* button.
Response: The software resets the fitting parameters.

12. Press the *Fit* toolbutton.

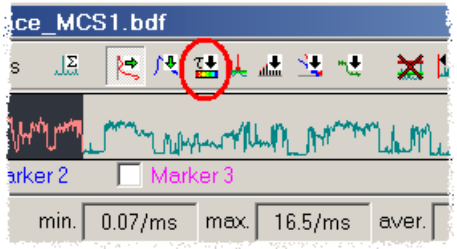
Response: The software performs a tailfit to optimise the fitting parameters for the TCSPC curve.



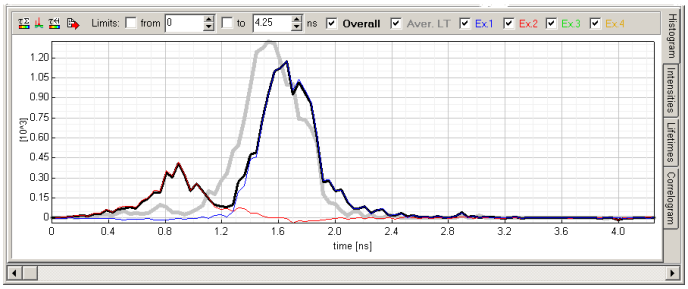
13. Set the lifetime parameters to "*fit*", set all other parameters to "*search*".



14. Press the *lifetime histogram* toolbutton.



Response: The software calculates the fluorescence lifetime trace, i.e. it applies the fitting model to each time bin. After the calculation the *MCS* window is extended by a plot of the lifetime histogram.



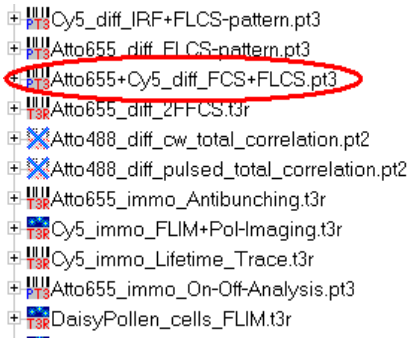
This plot shows the frequency, with which each lifetime occurs in the image. The intensity time traces corresponding to each exponential can be viewed on the *Intensities* page. The intensity is calculated as $Amp_i \cdot \tau_i$ for each exponential *i* and each time bin. The lifetime traces can be viewed on the *Lifetimes* page. Interdependencies between calculated parameters can be displayed on the *Correlogram* page.

3.2.3 FCS Analysis

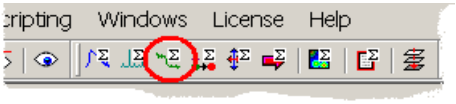
FCS Curve Calculation

Selection of router (detection) channels, calculation of a cross–correlation curve.

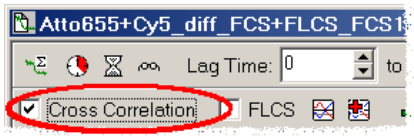
1. Open the sample workspace:
   `<Documents and Settings>\All Users\Documents\SymPhoTime\samples\samples.pqw`
   Alternatively, if you click on the link `samples` in the group *PicoQuant – SymPhoTime* of the Windows Start menu, the software is opened with the sample workspace.
   Response: The sample workspace contains several examples of measurements and analyses. The files of the sample workspace are displayed in the *Files* view on the left side of the main window.



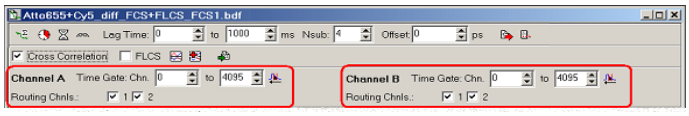
2. Click on the file `Atto655+Cy5_diff_FCS+FLCS.pt3`.
   Response: The file is highlighted in the workspace tree view.
3. Press the *FCS trace* toolbutton.



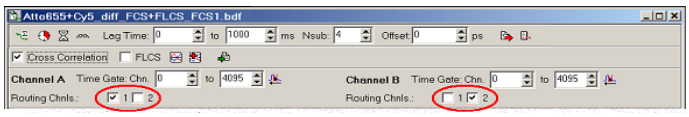
   Response: The software opens the raw data file in an *FCS* window.
4. Check the *Cross Correlation* box.



   Response: The software toggles the user interface to cross–correlation by replacing the *Autocorrelation* control group by two control groups *Channel A* and *Channel B*:

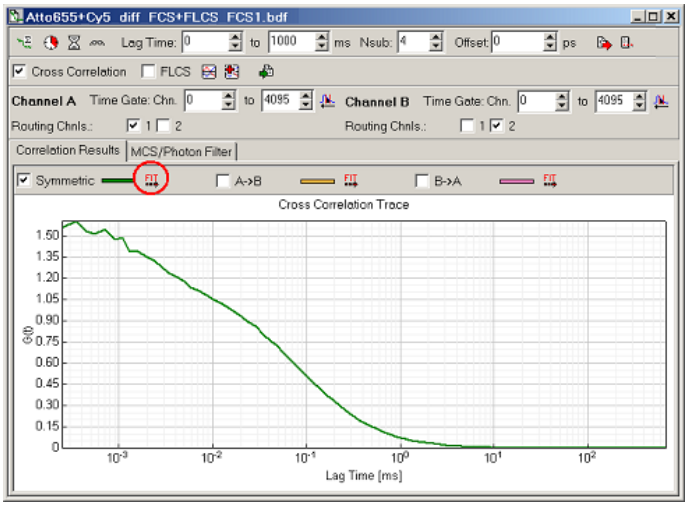


5. For *Channel A* check *Routing Channel 1*, uncheck *Routing Channel 2*, for *Channel B* check *Routing Channel 2*, uncheck *Routing Channel 1*:



6. Press the *Calculate FCS Trace* toolbutton.

Response: The software starts to calculate the cross–correlation curve of *Routing chnl. 1* with *Routing chnl. 2*. This may take up to one minute. When the calculation completes, the software displays the cross–correlation curve. The curve can be fitted by pressing the *Fit* toolbutton (see p. 51).



3.2.4 FLCS Curve Calculation

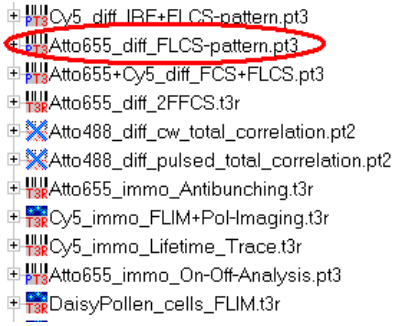
FLCS can be regarded as a fusion of Time–Correlated Single Photon Counting and FCS. With FLCS it is possible to separate the autocorrelation function of various signal components.

Because each signal component is identified by its temporal behaviour on the picosecond to nanosecond time scale (TCSPC), the FLCS experiment must be performed using pulsed laser excitation instead of a conventional CW illumination.

Background and Afterpulsing Removal

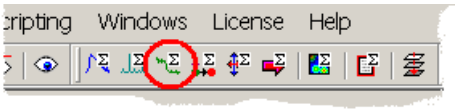
Selection of router (detection) channels, calculation of an autocorrelation curve: The afterpulsing artefacts of the SPAD detectors are visible. Application of FLCS for afterpulsing removal.

1. Open the sample workspace:
   `<Documents and Settings>\All Users\Documents\SymPhoTime\samples\samples.pqw`
   Alternatively, if you click on the link `samples` in the group *PicoQuant – SymPhoTime* of the Windows Start menu, the software is opened with the sample workspace.
   Response: The sample workspace contains several examples of measurements and analyses. The files of the sample workspace are displayed in the *Files* view on the left side of the main window.



2. Click on the file `Atto655_diff_FLCS-pattern.pt3`.
   Response: The file is highlighted in the workspace tree view.

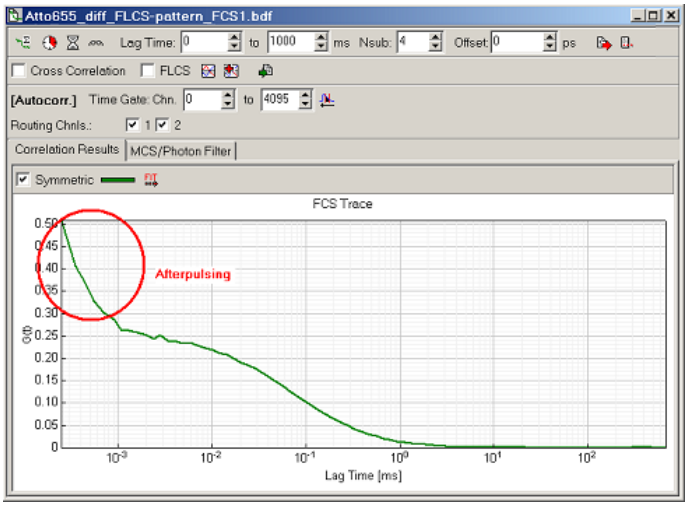
3. Press the *FCS trace* toolbutton.



Response: The software opens the raw data file in an *FCS* window.

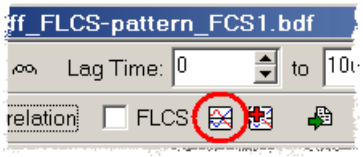
4. Start the correlation by pressing *calculate FCS trace* (i.e. do not check the *FLCS* box, nor the *Cross Correlation* box).

Response: The software calculates the autocorrelation function:

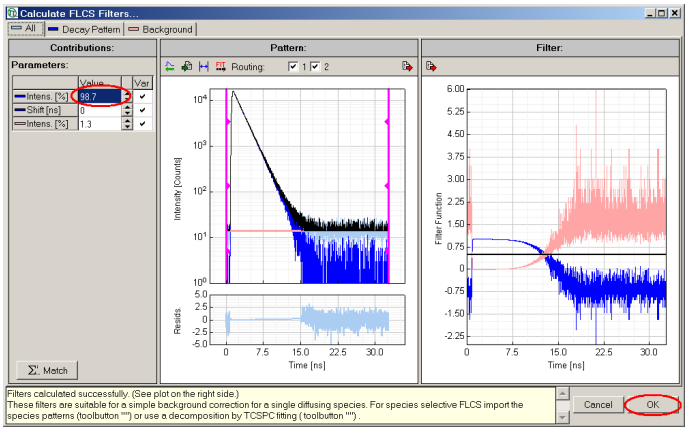


Please note: By selecting all available routing channels for autocorrelation analysis, the routing information is neglected, i.e. all photons are treated as originating from a single (logical) detector. This autocorrelation function is equivalent to a standard FCS result. The afterpulsing of the detector causes a fast initial decay of the calculated autocorrelation function and hampers the analysis when trying to resolve these contributions by FCS fitting (see p. 51).

5. Now, FLCS is applied to the same data. First, we identify the decay patterns. Press *calculate FLCS filters (REPLACE)*.

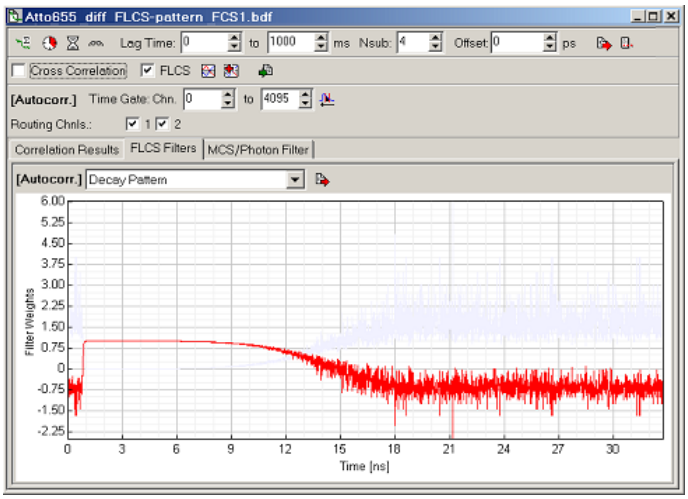


Response: The software activates the page *FLCS Filters* and opens a dialog for FLCS filter calculation:



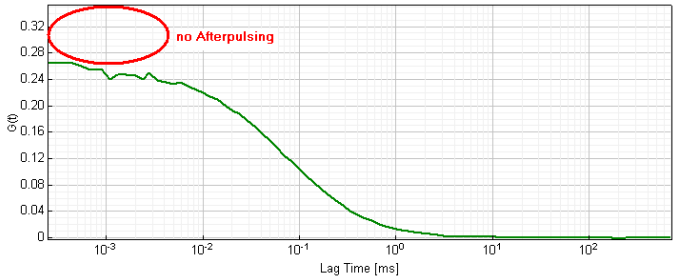
The software automatically determines the average background value and subtracts it from the total histogram. The resulting dark blue histogram is the pure Atto655 pattern. ~99% of all photons have been emitted with this decay pattern. Two corresponding filter functions are displayed at the right hand side.

6. Press OK.
   Response: The software returns to the FCS window and transfers the filter functions.



7. Back in the main FCS form, make sure that the filter function *Decay Pattern* is selected and press *calculate FCS trace*.

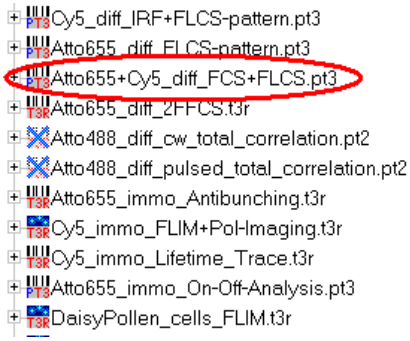
Response: The software calculates the correlation curve. The result should look like this:



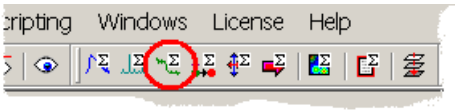
Separating the Contribution of two Fluorophores (Lifetime Fitting Decomposition)

FLCS analysis using decay patterns that are obtained by decay curve fitting. Exponential decomposition, FLCS filter definition and component separation.

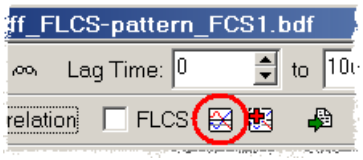
1. Open the sample workspace:
   `<Documents and Settings>\All Users\Documents\SymPhoTime\samples\samples.pqw`
   Alternatively, if you click on the link `samples` in the group *PicoQuant – SymPhoTime* of the Windows Start menu, the software is opened with the sample workspace.
   Response: The sample workspace contains several examples of measurements and analyses. The files of the sample workspace are displayed in the *Files* view on the left side of the main window.



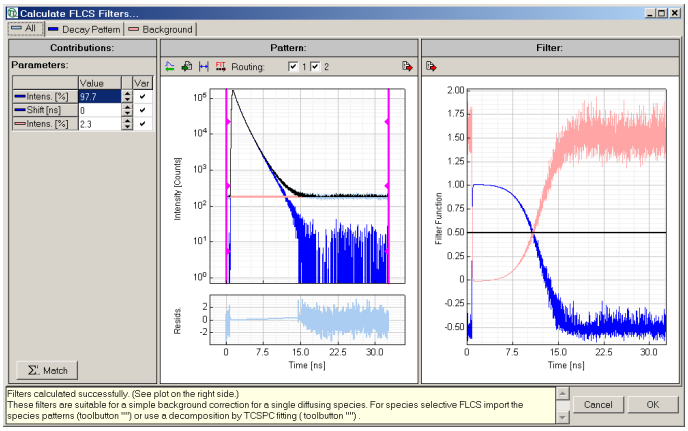
2. Click on the file `Atto655+Cy5_diff_FCS+FLCS.pt3`.
   Response: The file is highlighted in the workspace tree view. It contains intensity contributions from two diffusing fluorophores. The goal is to generate separated FCS curves for both fluorophores.
3. Press the *FCS trace* toolbutton.



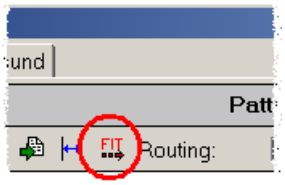
   Response: The software opens the raw data file in an *FCS* window.
4. Make sure that the *Cross Correlation* tick box is empty. Press *calculate FLCS filters (REPLACE)*.



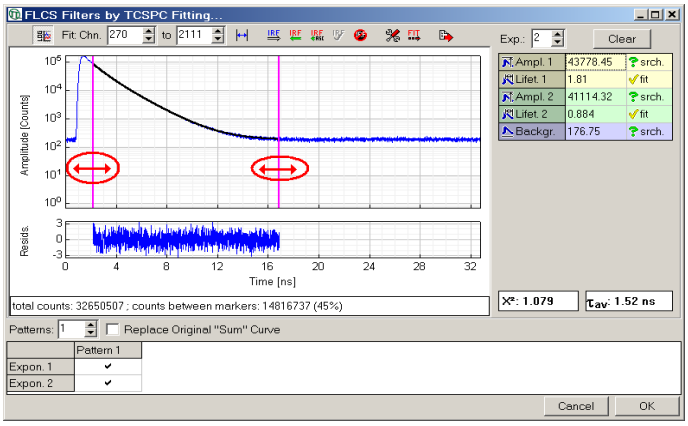
   Response: The software opens a dialog for FLCS filter calculation:



5. Press the *FIT* (*decompose*) button.

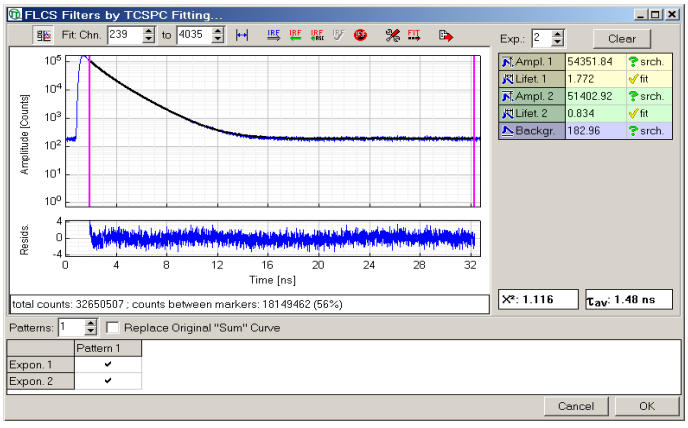


Response: The software opens a dialog for decomposition into exponential components.

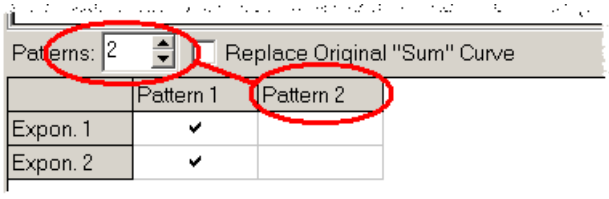


6. On this dialog, press the *Fit* button to perform a simple double–exponential tail fit.

Response: A possible result is shown on the screen shot below:

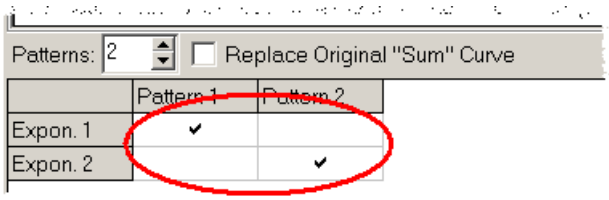


7. Set the number of patterns to `2`:

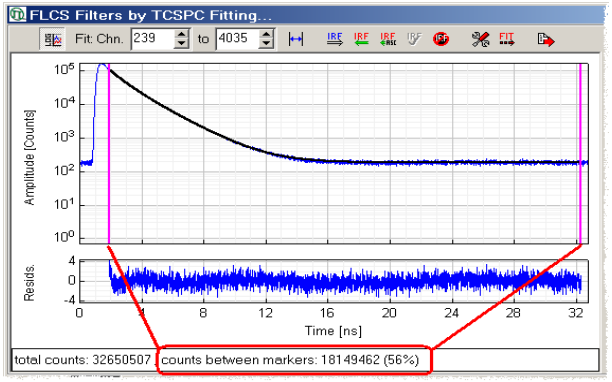


Response: The patterns / exponentials association table is extended to two columns.

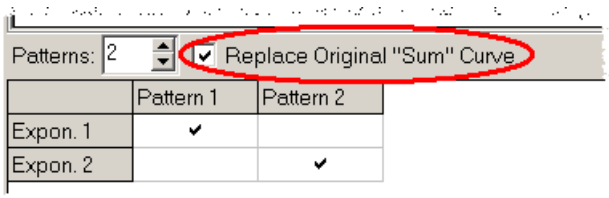
8. Assign the two exponentials to the patterns: check (Pattern 1 | Expon. 1) and (Pattern 2 | Expon. 2):



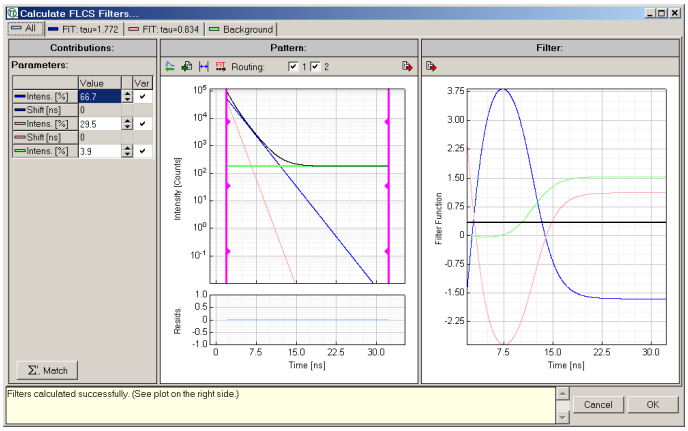
Please note that the selected data range for fitting is equivalent to time–gating. Filters can be calculated only for the fitted data range. Counts outside these limits will be neglected, which is equivalent to time–gating. Observe that only a fraction of the counts is between the markers.



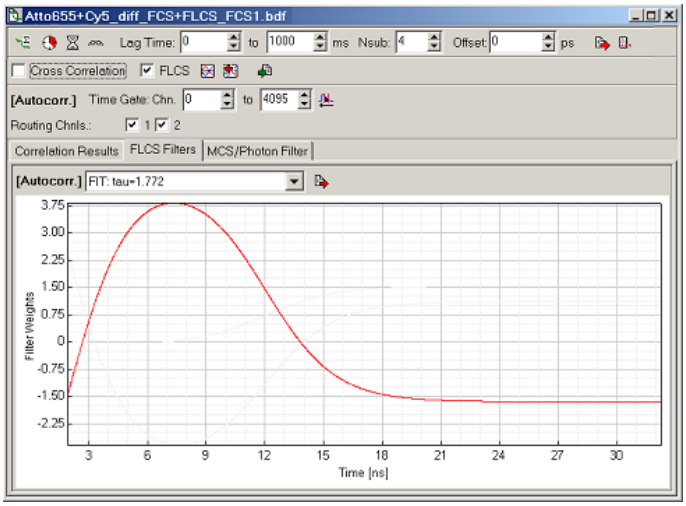
9. Check *Replace Original "Sum" Curve* and press *OK* to accept the fitting results.



Response: The software returns to the *Calculate FLCS Filters…* window and derives the FLCS filters automatically based on the exponential decomposition. The original TCSPC histogram is replaced by the sum of all exponential contributions to reduce the noise level.

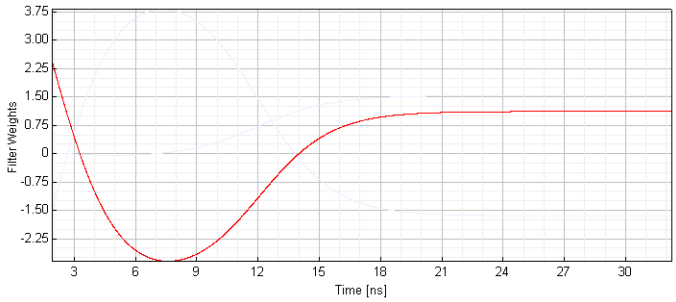


10. Back in the *Calculate FLCS Filters…* window, press *OK*.
    Response: The software returns to the *FCS* window and transfers the filter functions.



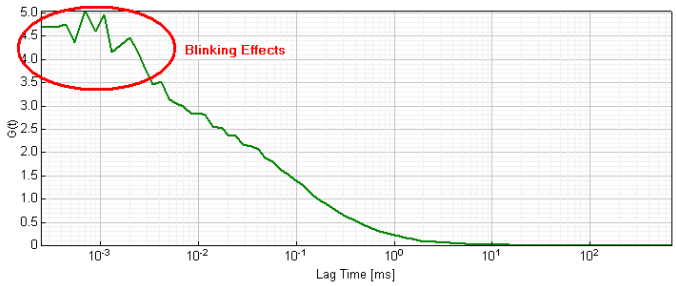
11. Back in the *FCS* window, select the filter function that is associated with the shorter of the lifetimes (which corresponds to Cy5).

Response: The software highlights the selected filter in red colour in the filter plots:



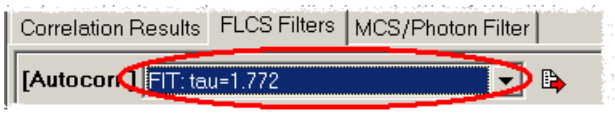
12. Press *calculate FCS trace*.

Response: The result is the separated correlation curve for Cy5:

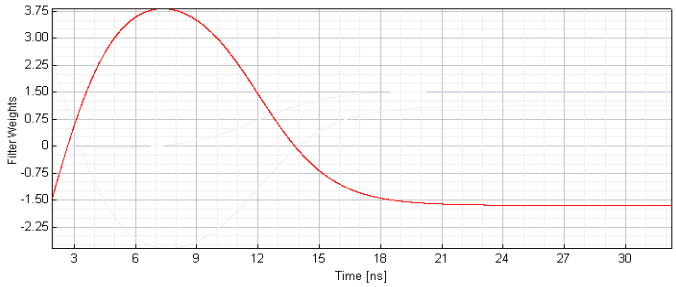


Please note the presence of contributions that indicate blinking.

13. On the *FLCS Filters* page, select the filter function that is associated with the longer of the lifetimes (which corresponds to Atto655).

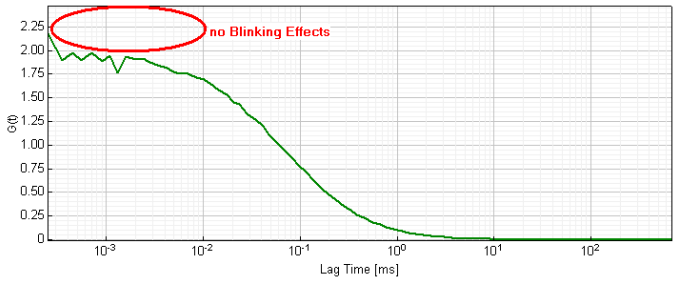


Response: The software highlights the selected filter in red colour in the filter plots:



14. Press *calculate FCS trace*.

Response: The result is the separated correlation curve for Atto655:

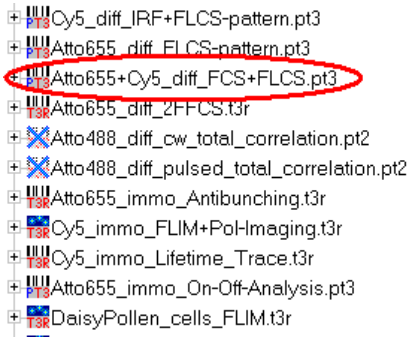


The absence of blinking effects in this curve indicates that all contributions of Cy5 are indeed removed by the FLCS filtering.

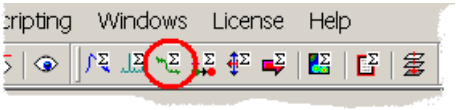
Separating the Contribution of two Fluorophores (Pattern Matching)

FLCS analysis using experimentally obtained decay patterns. Decomposition into patterns, FLCS filter definition and component separation.

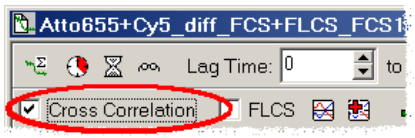
1. Open the sample workspace:
   `<Documents and Settings>\All Users\Documents\SymPhoTime\samples\samples.pqw`
   Alternatively, if you click on the link `samples` in the group *PicoQuant – SymPhoTime* of the Windows Start menu, the software is opened with the sample workspace.
   Response: The sample workspace contains several examples of measurements and analyses. The files of the sample workspace are displayed in the *Files* view on the left side of the main window.



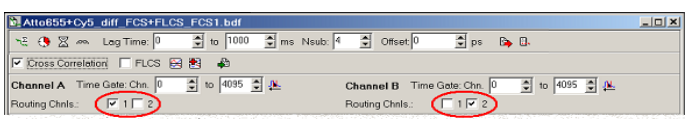
2. Click on the file `Atto655+Cy5_diff_FCS+FLCS.pt3`.
   Response: The file is highlighted in the workspace tree view. This sample file contains intensity contributions from two different diffusing fluorescent species. The goal is to generate separated FCS curves for each species.
3. Press the *FCS trace* toolbutton.

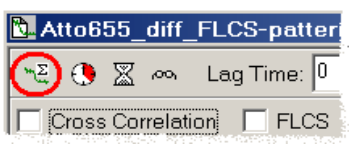


   Response: The software opens the raw data file in an *FCS* window.
4. Check the *Cross Correlation* box.

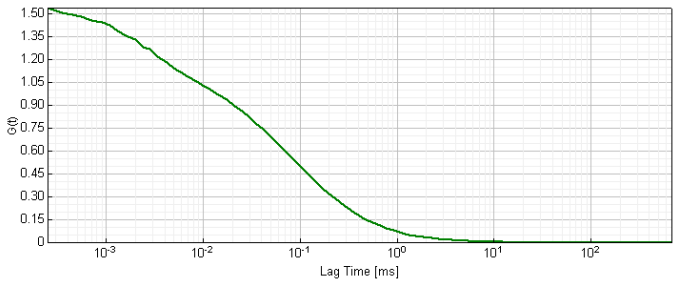


   Response: The software activates cross–correlation and splits the *Time Gate* and *Routing Chnls* controls into two independent data channels *Channel A* and *Channel B.*
5. Assign *Routing Chnl. 1* as data *Channel A* and *Routing Chnl. 2* as data *Channel B* and press *calculate FCS trace*.

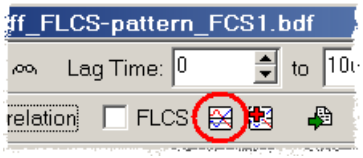




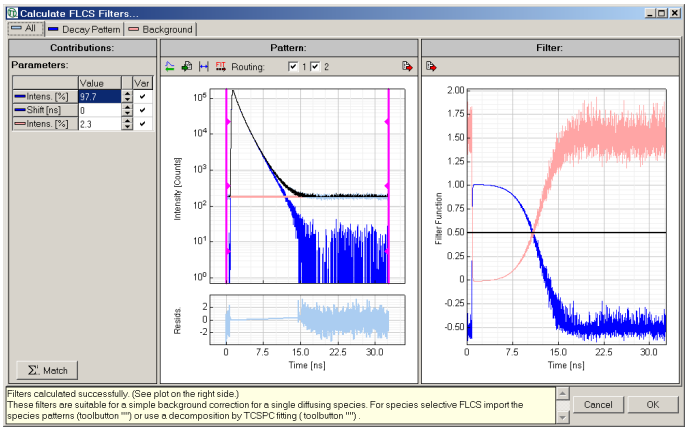
Response: The resulting cross–correlation function (corresponding to the mixture) is really complicated due to the photophysics of Cy5 (blinking) and the presence of two independently diffusing components:



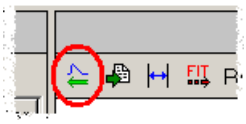
6. Leave this window open for comparison and highlight the `Atto655+Cy5_diff_FCS+FLCS.pt3` data file again. Press *FCS trace*. Press *calculate FLCS filters (REPLACE)*.



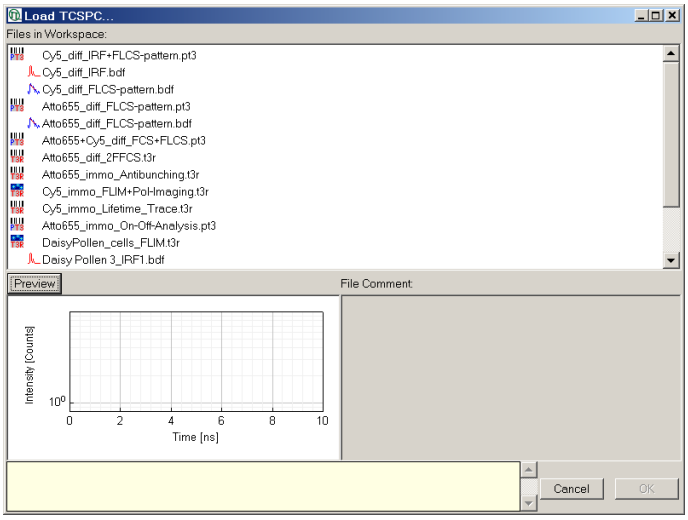
Response: The software opens a dialog for decomposition of the original decay into exponential components.



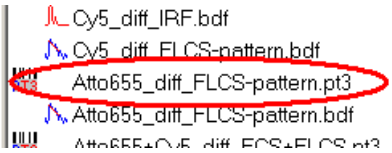
7. Press *load pattern*.



Response: The software opens a dialog for loading a measured TCSPC curve.

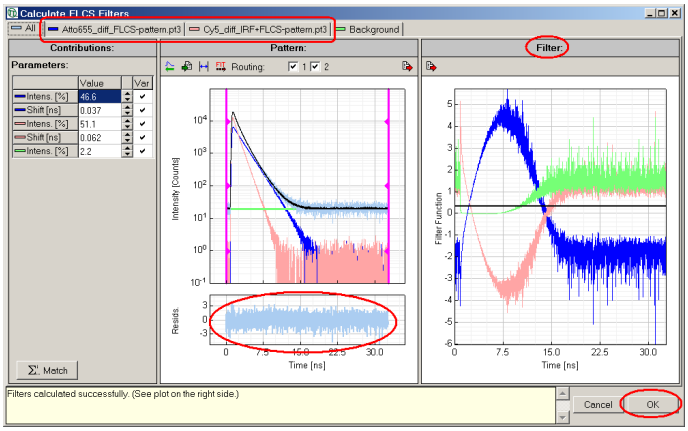


8. Select `Atto655_diff_FLCS-pattern.pt3`. This file contains a TCSPC curve for pure Atto655 fluorescence. Press *OK*.

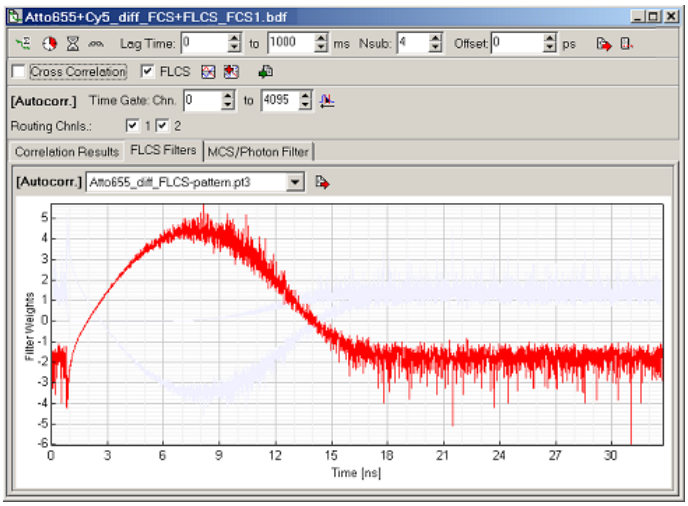


Response: The software imports the selected file as a pattern.

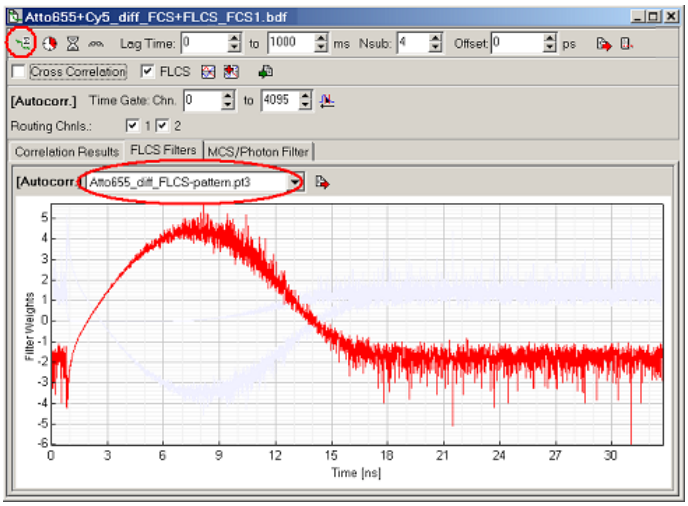
9. Repeat steps 7. and 8. for the file `Cy5_diff_IRF+FLCS-pattern.pt3`, which contains a TCSPC curve for pure Cy5 fluorescence.
Response: The software decomposes the overall TCSPC curve of the `Cy5+Atto655_200+200.pt3` data file and calculates the corresponding set of FLCS filters. The decomposition is nearly perfect (check the residuals trace):



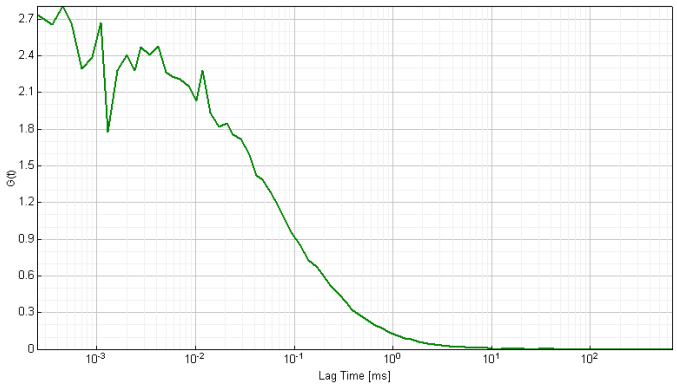
10. Press *OK* to accept the filters.
    Response: The software returns to the *FCS* window and transfers the filter functions.



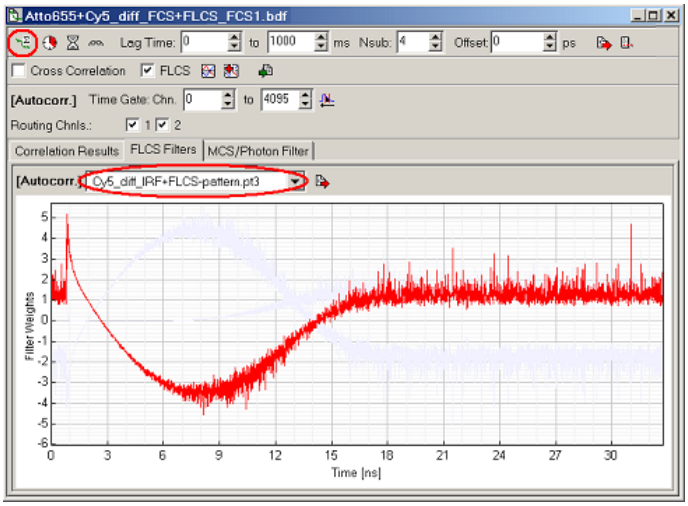
11. In the main *FCS* window, select the filter function for Atto655 and press *calculate FCS trace*.



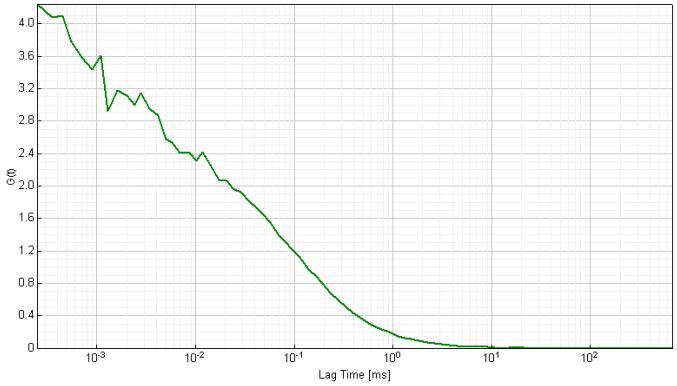
Response: The software calculates the dedicated autocorrelation function for Atto655:



12. Repeat step 11. for the Cy5 fluorophore.



Response: The result should be this:

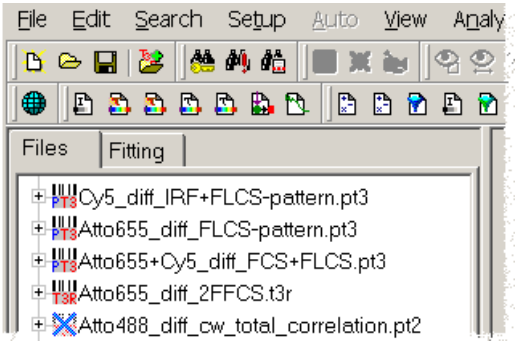


13. The reader is encouraged to compare the correlation curves for Atto655 and Cy5 in the context of an FCS fit (which is introduced in the following section), as well as to try to resolve these contributions by the conventional approach, i.e. by fitting a multi–component FCS model to the autocorrelation function of the mixture, which was calculated in step 5.

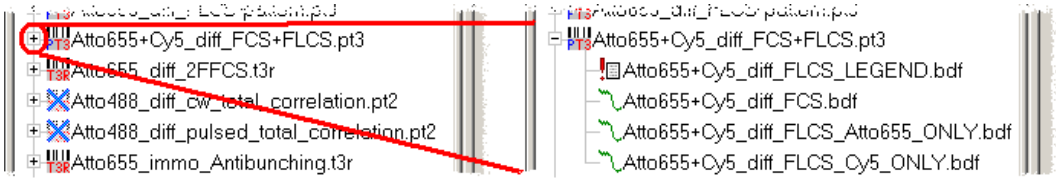
3.2.5 FCS Fitting

Selection of a FCS fitting model, estimation of the model parameters, assessment of the goodness of fit.

1. Open the sample workspace:
   `<Documents and Settings>\All Users\Documents\SymPhoTime\samples\samples.pqw`
   Alternatively, if you click on the link `samples` in the group *PicoQuant – SymPhoTime* of the Windows Start menu, the software is opened with the sample workspace.
   Response: The sample workspace contains several examples of measurements and analyses. The files of the sample workspace are displayed in the *Files* view on the left side of the main window.

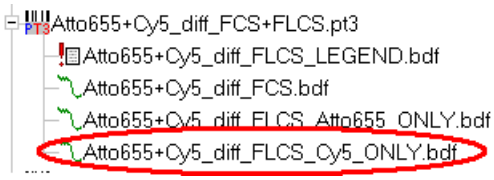


2. Expand the *File* treeview entry for the file `Atto655+Cy5_diff_FCS+FLCS.pt3` by clicking on its [+] button.

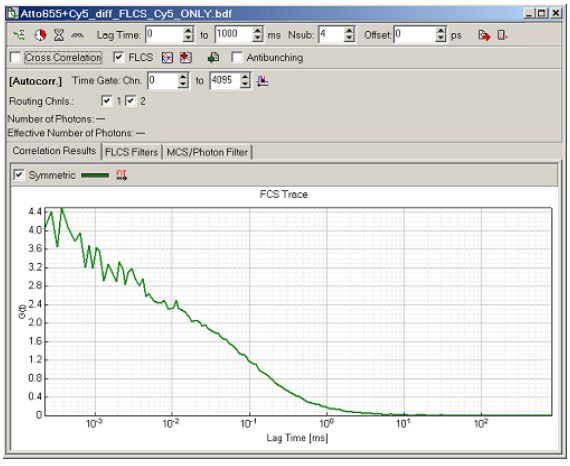


   Response: The software shows the analysed data files for this raw data file.

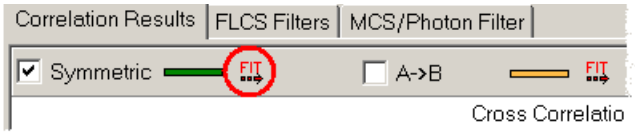
3. Double click on the file `Atto655+Cy5_diff_FLCS_Cy5_ONLY.bdf`.



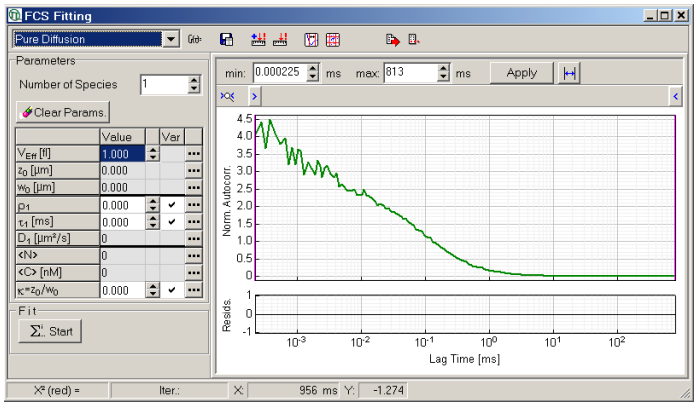
   Response: The software opens the analysed data file in an *FCS* window.



4. Press the leftmost *Fit* toolbutton.

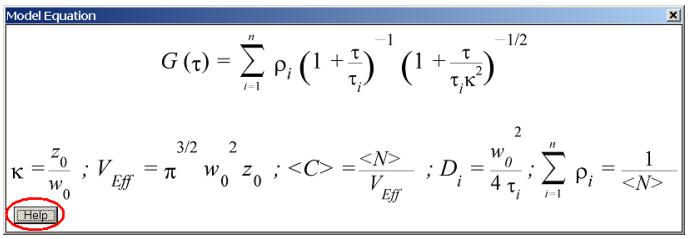


Response: The software opens the FCS curve in an *FCS Fitting* window.



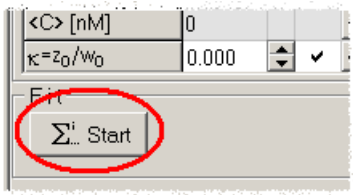
5. Press the *show model equation* toolbutton.

Response: The software displays the equations of the currently selected model.



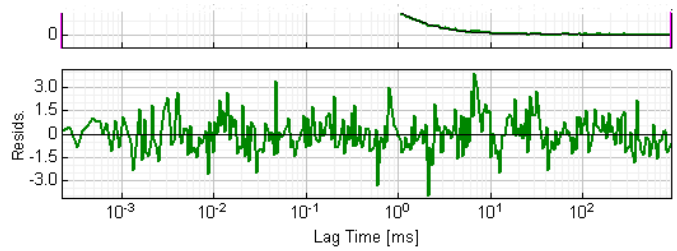
To display an explanation of the parameters press the *Help* button.

6. In the *FCS Fitting* window, press *Start*.

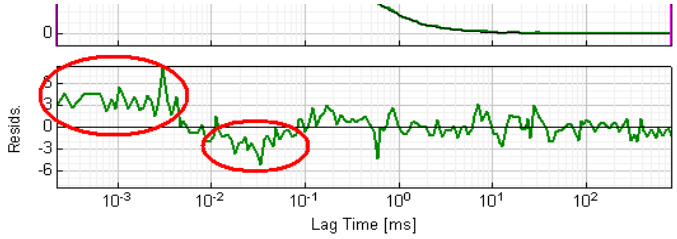


Response: The software estimates and optimises the model parameters.

7. Check the residuals trace. For an acceptable fit the residuals trace should not show any trends:

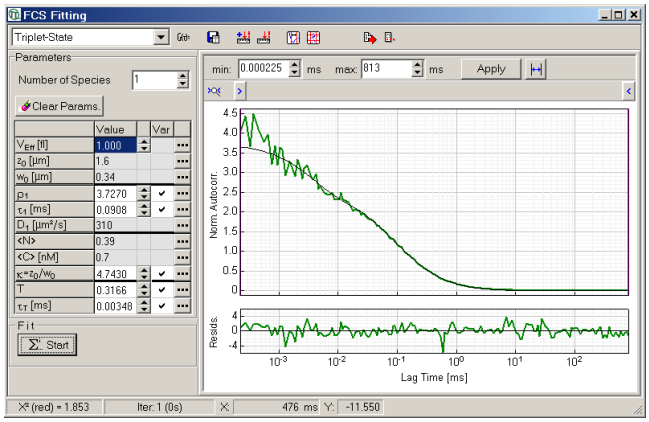


If there are trends, like in the following example, the *Pure Diffusion* model does not describe the correlation curve sufficiently:



In this case a different model has to be used. For this example, select the model *Triplet State* in the model selection box.

Press *Start* to repeat the fit.
Response: The software estimates and optimises the parameters of the *Triplet State* model:



4. User Scripts (STUPSLANG)

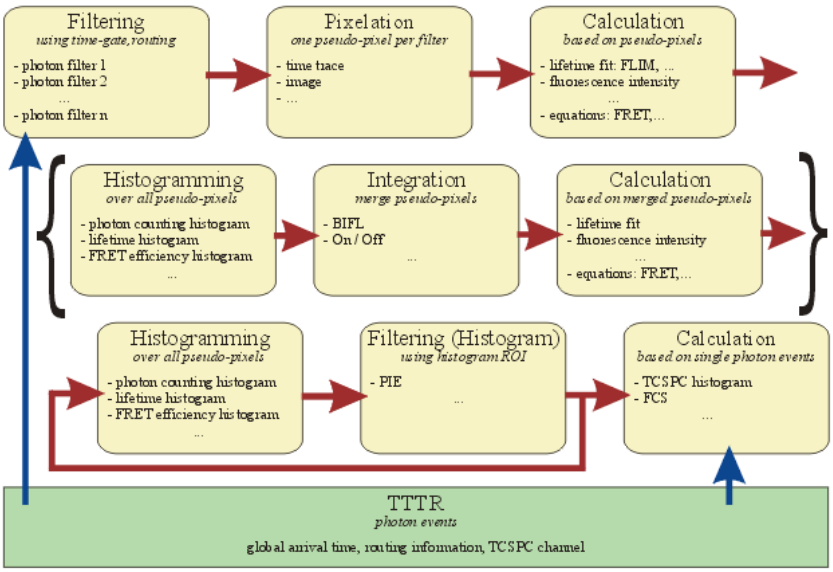
4.1 Scope and Purpose

SymPho**T**ime **U**ser **P**rogramming **S**cript **LANG**uage (STUPSLANG) is intended to allow users to implement analysis techniques which are not part of the standard SymPhoTime environment. Its approach is to supply a limited amount of simple analysis steps, which may be freely combined to allow more complex analyses. Although STUPSLANG is based on a formal grammar, it is **not** intended to be a programming language. The motivation for these limitations was to keep the script code a simple, linear list of analysis steps.

A complete documentation of the scripting language is beyond the scope of this short introduction. Please refer to the help file for further reading.

4.2 Basic Principles

The following figure summarises the typical course of a scripted analysis. Please refer to the sections below for an explanation of the involved concepts.



pseudo–pixels

The central concept of STUPSLANG is the so–called "pseudo–pixel". It is best illustrated on a fluorescence image: In this case a pseudo–pixel simply is an image pixel. pseudo–pixels have the following properties:

- fluorescence intensity (i.e. the number of photons within this pixel)
- pixel size (e.g. the area corresponding to a pixel)
- TCSPC histogram (i.e. a fluorescence decay)

Closer examination of the concept of a pixel reveals that the time bins of an MCS trace behave exactly like an image pixel, except for the pixel size, which is, of course, not an area, but the time span associated with a pixel. This means a "pseudo–pixel" is an abstract term for all objects which may be seen as an analogon of an image pixel.

As a benefit of this approach, the analysis steps for imaging and MCS trace analysis are exactly the same. Once the sorting of photon events into pseudo–pixels is done, the subsequent analyses are reduced to using the properties of the pseudo–pixel to extract useful information, for example by performing a lifetime fit on the TCSPC histogram.

In the case of a simple fluorescence intensity analysis, each image pixel corresponds to a pseudo–pixel. Now let an experiment be performed with two detectors: When calculating the fluorescence decays that are associated with each detector, one image pixel would be associated with two pseudo–pixels, one for each detector.

This example illustrates that a pseudo–pixel is always associated with a kind of abstract photon filter, which has two main properties: a set of routing channels and a time gate, which define which photon events may "pass" the filter.

Histogramming: Integrating and Filtering

Another central concept of the scripting is the histogramming over the pseudo–pixels. The frequency of a given parameter value throughout all pseudo–pixels of an image or time trace is calculated. An example would be the lifetime histogram of a FLIM image or a BIFL analysis. Correlations between pairs of parameters may also be investigated. This would result in a 2D–histogram, in which the frequency of pairs of parameter values is estimated.

While these histograms are of interest per se, they also offer the possibility of excluding certain pseudo–pixels from the analysis. For example, a simple histogram of the fluorescence intensity can be used to separate different intensity levels of a blinking molecule. The blinking level of interest may be singled out by defining a region of interest in the photon counting histogram. When such a filter is applied, other parameters, e.g. the lifetime, can be histogrammed only for the selected pseudo–pixels. In the above example this would result in a selective lifetime histogram for the blinking state.

This method becomes even more powerful in the context of a 2D–histogram, i.e. a correlogram between pairs of parameters. For a blinking molecule, the blinking states might even be separated with a better precision if the lifetime of each blinking state is taken into account, in addition to the fluorescence intensity.

There is yet another concept connected with histogramming. In a BIFL analysis, groups of pseudo–pixels that constitute a "burst" have to be formed. The decays etc. of the pseudo–pixels would have to be calculated for this group. Now, a burst would be defined as a contiguous region of pseudo–pixels for which the fluorescence intensity is above a given threshold. Again, the histogram may be used to define a suitable threshold. Another example would be on / off histogramming. The separation between "on" and "off" states makes use of the photon counting histogram.

While integration of pseudo–pixels is similar to filtering, it introduces the new concept of bracketing several pseudo–pixels to form a "burst". It should be mentioned, that because imaging and time trace analysis are considered to be essentially the same, "burst" analysis is also possible for imaging: It is useful, e.g. for single molecule experiments: A single molecule would show in the image as a peak; "burst" analysis would treat these peaks in analogy to the bursts in a time trace.

Running and Editing Scripts

There are several predefined scripts, which may also serve as demo code. All scripting functions are accessible via the *Script Manager…* entry of the *Scripting* menu. Select a TTTR file to be analysed. Then, either select one of the scripts that are listed there or select *New*, to load a template for a new user defined script. User defined scripts are accessible under *Script Manager…* as well.

Once a script is selected, the selected TTTR file is opened, and the development environment for scripting is started. To execute a script, press the *Run Analysis Script* toolbutton.

5. Data Formats

5.1 Workspace

The workspace is implemented as a standard file folder. The name of the folder corresponds to the workspace name. The workspace file (`*.pqw`) is an ASCII text file. Raw data are stored as so–called TTTR files. There are three types of TTTR files, containing images (*Area*), *Line* scans or *Point* measurements:

TimeHarp / PicoHarp / HydraHarp Image
TimeHarp / PicoHarp / HydraHarp Line scan
TimeHarp / PicoHarp / HydraHarp Point measurement

TTTR files may be grouped. Groups originate from automated measurements and are marked by a special icon  . TTTR group members are visualised as branches of the group.

From TTTR files, lifetime histograms, intensity information, correlation data etc. can be extracted. This will produce derived data files with the extension `.bdf`. Possible derived data files are, e.g.:


TCSPC histogram

MCS trace

FCS trace

Image


FRET analysis

Anisotropy analysis

ASCII export filter

Annotation

Fitting

Derived FCS files can be analysed by fitting model equations to the data. These fitting sessions may be, but not necessarily are, associated with a single raw data file. For this reason they are visualised in a separate page of the workspace tree view called *Fitting*. There are two groups of fitting results, *FCS Fit* and *FCS Calibration*. Possible fitting results files are:


FCS fitting file

FCS calibration table

5.2 ASCII Export Filter

The files that are exported by the *ASCII Export Filter* include a header, which contains information on the workspace, the raw data TTTR file and the *Export Filter* file (`*.bdf`). Furthermore, the filter properties of the exported traces are shown. If a trace is set as a burst detection trace, the data will be exported burstwise. If none is set, the data will be exported binwise.

Following the file header, the bin (burst) data is listed for each trace. Binwise exported data has a time column denoting the bin time; for burstwise exported data, burst start and burst stop times are listed in the first two columns.

The example below shows a burstwise exported file:

```
Workspace:
E:\Samples\samples.pqw

TTTR File:
diff_FRET_pair.t3r

BDF File:
E:\Samples\diff_FRET_pair_PPR1.bdf
```

```
Traces:
  Trace  1 [burst detection trace]
    Routing Channels: Ch.1;

    Time Gate: [0 - 4095]
    Threshold : 7.722
    Background: 1.146
  Trace  2
    Routing Channels: Ch.2;
    Time Gate: [0 - 4095]
    Threshold : 13.152
    Background: 4.633

Burst Data:

    start[s]     stop[s]          Trace  1              Trace  2
                              intensity   aver.chn.   intensity   aver.chn.
    1.119000    1.120000         8.85     2997.50        2.37     2849.71
    1.334000    1.336000        15.71     3019.94        9.73     2864.63

    1.979000    1.980000         9.85     3012.45       -0.63     2866.50
    2.553000    2.554000        10.85     2989.33        0.37     2882.00
    2.734000    2.735000         6.85     3000.13        3.37     2859.75
    3.105000    3.106000        10.85     3014.25        4.37     2895.00
    3.438000    3.440000        26.71     2989.90       -1.27     2855.13
    4.365000    4.366000         7.85     2943.78        3.37     2875.63
```

5.3 TTTR File

The SymPhoTime software records TTTR files in TimeHarp 200 version 5.0, PicoHarp 300 version 2.0 or HydraHarp 400 version1.0 format respectively. The software can import and handle (checked) and write (highlighted in green) the following format versions:

	TimeHarp 100	TimeHarp 200	PicoHarp 300	HydraHarp 400
1.0			☑	☑
1.1			☑	
2.0		☑	☑	
2.1				
2.2	☑			
2.3	☑			
3.0	☑	☑		
3.1				
3.2				
4.0		☑		
5.0		☑		
5.1		☑		
5.2		☑		
5.3		☑		
6.0		☑		

TTTR mode data files (`*.t3r`) are binary files with a structure described below. The file contains a header with selected setup parameters and multiple time–tagged events, called TTTR records.

TimeHarp TTTR 5.0 format:

Data Item	Type	Description
Ident	char [16]	the string ‘TimeHarp 200’
SoftwareVersion	char [6]	'5.0'

HardwareVersion	char [6]	currently ‘2.4’ or '2.5'
FileTime	char [18]	the file creation date and time
CR/LF	char [2]	0x0D,0x0A carriage return, line feed
Comment	char [256]	any ASCII string
NumberOfChannels**	int32	number of time channels (normally 4096)
NumberOfCurves**	int32	meaningless in TTTR file
BitsPerChannel**	int32	meaningless in TTTR file
RoutingChannels	int32	1 or 4
NumberOfBoards**	int32	reserved, now 1
ActiveCurve	int32	meaningless in TTTR file
MeasurementMode**	int32	2=TTTR
SubMode	int32	0=Standard (Timed), 1,2=Reserved, 3=Image
RangeNo*	int32	0=base resol., 1=x2, 2=x4, 3=x8, 4=x16,5=x32
Offset*	int32	offset (approx.) in ns
AcquisitionTime*	int32	acquisition time in ms
StopAt	int32	meaningless in TTTR file
StopOnOvfl	int32	meaningless in TTTR file
Restart	int32	meaningless in TTTR file
DisplayLinLog*	int32	lin=0, log=1
DisplayTimeAxisFrom*	int32	lower time axis bound for display in ns
DisplayTimeAxisTo*	int32	upper time axis bound for display in ns
DisplayCountAxisFrom*	int32	lower count axis bound for display
DisplayCountAxisTo*	int32	upper count axis bound for display
The following block will appear in the file for each i = 1 to 8		
DisplayCurve[i].MapTo	int32	meaningless in TTTR file
DisplayCurve[i].Show	int32	meaningless in TTTR file
end of block.		
The following block will appear in the file for each i = 1 to 3		
Param[i].Start	float	reserved for automated measurements
Param[i].Step	float	reserved for automated measurements
Param[i].End	float	reserved for automated measurements
end of block		
RepeatMode	int32	reserved for automated measurements
RepeatsPerCurve	int32	reserved for automated measurements
RepeatTime	int32	reserved for automated measurements
RepeatWaitTime	int32	reserved for automated measurements
ScriptName	char [20]	reserved for automated measurements
The following block will appear in the file for each i = 1 to NumberOfBoards [currently this must be 1]		
BoardSerial	int32	Board serial number
CFDZeroCross*	int32	CFD zero cross level in mV
CFDDiscriminatorMin*	int32	CFD min discriminator level in mV
SYNCLevel*	int32	SYNC trigger level in mV
CurveOffset*	int32	reserved, now 0
Resolution	float	Resolution in ns
end of block		
TTTRGlobclock	int32	clock in ns, normally 100
ExtDevices	int32	in version 5.0 and 5.1: Reserved (not used) in version 5.2 and 5.3: 1 = PRT 400, 2 = NRT 400
Reserved	int32	
Reserved	int32	
Reserved	int32	
Reserved	int32	
Reserved	int32	
SyncRate	int32	SYNC rate as displayed in meter
AverageCFDRate	int32	Average CFD rate as displayed in meter
StopAfter	int32	Stopped after this many ms

StopReason	int32	0=TimeOver, 1=Manual, 2= Overflow, 3=Error
NumberOfRecords**	int32	Number of TTTR records
SpecHeaderLength**	int32	Length of special header to follow (in 4 byte portions)
Block of SpecHeaderLength		
Reserved	int32 []***	Special header for imaging
end of block		
The following block will appear in the file for each i = 1 to NumberOfRecords		
TTTRRecord	uint32	TTTR record, structured as follows (MSBit at the left): Reserved[1], Valid[1], Route[2], Data[12], TimeTag[16] if Valid==1 then Data = Channel else Data = Overflow[1], Reserved[8], Marker[3]
end of block		

* These items are stored as they were set/displayed upon entering TTTR mode.
** Items may vary and thereby change the file structure dynamically. A program must interpret these before reading further data.
*** Type formally denoted as Int32 (4 bytes) for sizing purposes, but actual type may vary according to header definition.

A TTTR record is 32 bit long and always contains a 16–bit time–tag. The first 16 bits contain information either about a photon event (routing and start–stop time, i.e. TCSPC channel number) or about another event (marker or overflow). The *Valid* flag must be monitored in order to identify true photon events.

If this flag is 0, the record marks an overflow or the arrival of an external trigger pulse (marker) and carries no information about a photon arrival. The *Data* field then contains a "special" event and is structured differently. For reconstructing the true absolute time tag, the *Overfl bit* must be monitored. If this bit is 1, an overflow of the *TimeTag* has occurred and you need to add 2^{16} to obtain the true time tag. The three least significant bits of the *Data* field denote arrival (bit=1) of any of the three possible external markers.

If the *Valid* flag is 1, then the record describes a real photon event. In this case, the *Data* field contains the channel number of the photon. The *Route* bits identify the detector channel that the photon came from.

PicoHarp T2 / T3 mode 2.0 format:

Data Item	Type	Description
Ident	char [16]	the string ‘PicoHarp 300’
FormatVersion	char [6]	currently '2.0'
CreatorName	char [18]	for this Software 'SymPhoTime'
CreatorVersion	char [12]	currently '5.0'
FileTime	char [18]	the file creation date and time
CR/LF	char [2]	0x0D,0x0A carriage return, line feed
Comment	char [256]	any ASCII string
NumberOfCurves**	int32	meaningless in TTTR file
BitsPerRecord**	int32	32
RoutingChannels	int32	1 or 4
NumberOfBoards**	int32	reserved, now 1
ActiveCurve	int32	meaningless in TTTR file
MeasurementMode**	int32	2=T2 or 3=T3
SubMode	int32	0=Standard (Timed), 1,2=Reserved, 3=Image (not for T2–mode)
RangeNo*	int32	0=base resol., 1=x2, 2=x4, 3=x8, 4=x16, … 7=x128
Offset*	int32	offset in ns
AcquisitionTime*	int32	acquisition time in ms
StopAt	int32	meaningless in TTTR file
StopOnOvfl	int32	meaningless in TTTR file
Restart	int32	meaningless in TTTR file
DisplayLinLog*	int32	lin=0, log=1
DisplayTimeAxisFrom*	int32	lower time axis bound for display in ns
DisplayTimeAxisTo*	int32	upper time axis bound for display in ns
DisplayCountAxisFrom*	int32	lower count axis bound for display

DisplayCountAxisTo*	int32	upper count axis bound for display
The following block will appear in the file for each i = 1 to 8		
DisplayCurve[i].MapTo	int32	meaningless in TTTR file
DisplayCurve[i].Show	int32	meaningless in TTTR file
end of block.		
The following block will appear in the file for each i = 1 to 3		
Param[i].Start	float	reserved for automated measurements
Param[i].Step	float	reserved for automated measurements
Param[i].End	float	reserved for automated measurements
end of block		
RepeatMode	int32	reserved for automated measurements
RepeatsPerCurve	int32	reserved for automated measurements
RepeatTime	int32	reserved for automated measurements
RepeatWaitTime	int32	reserved for automated measurements
ScriptName	char [20]	reserved for automated measurements
The following block will appear in the file for each i = 1 to NumberOfBoards [currently this must be 1]		
HardwareIdent	char [16]	currently 'PicoHarp 300'
HardwareVersion	char [8]	currently '1.0'
HardwareSerial	int32	hardware serial number
SyncDivider	int32	input channel 0 (Sync) input divider
CFDZeroCross0*	int32	input channel 0 CFD zero cross level in mV
CFDLevel0*	int32	input channel 0 CFD discriminator level in mV
CFDZeroCross1*	int32	input channel 1 CFD zero cross level in mV
CFDLevel1*	int32	input channel 1 CFD discriminator level in mV
Resolution	float	Resolution in ns
RouterModelCode	int32	0=none, 1=PHR_402 , 2=PHR_403, 3=PHR_800
RouterEnabled	int32	1=enabled, 0=disabled (routing off)
RtChan1_InputType	int32	0=custom, 1=NIM, 2=TTL
RtChan1_InputLevel	int32	in mV
RtChan1_InputEdge	int32	1=rising, 0=falling
RtChan1_CFDPresent	int32	0=not present, 1=present
RtChan1_CFDLevel	int32	in mV
RtChan1_CFDZeroCross	int32	in mV
RtChan2_InputType	int32	0=custom, 1=NIM, 2=TTL
RtChan2_InputLevel	int32	in mV
RtChan2_InputEdge	int32	1=rising, 0=falling
RtChan2_CFDPresent	int32	0=not present, 1=present
RtChan2_CFDLevel	int32	in mV
RtChan2_CFDZeroCross	int32	in mV
RtChan3_InputType	int32	0=custom, 1=NIM, 2=TTL
RtChan3_InputLevel	int32	in mV
RtChan3_InputEdge	int32	1=rising, 0=falling
RtChan3_CFDPresent	int32	0=not present, 1=present
RtChan3_CFDLevel	int32	in mV
RtChan3_CFDZeroCross	int32	in mV
RtChan4_InputType	int32	0=custom, 1=NIM, 2=TTL
RtChan4_InputLevel	int32	in mV
RtChan4_InputEdge	int32	1=rising, 0=falling
RtChan4_CFDPresent	int32	0=not present, 1=present
RtChan4_CFDLevel	int32	in mV
RtChan4_CFDZeroCross	int32	in mV
end of block		
ExtDevices	int32	external devices, bitwise coded
Reserved	int32	
Reserved	int32	
InpRate0	int32	input 0 count rate

InpRate1	int32	input 1 count rate
StopAfter	int32	stopped after this many ms
StopReason	int32	0=TimeOver, 1=Manual, 2= Overflow, 3=Error
NumberOfRecords**	int32	number of TTTR records in either T2 or T3 format
SpecHeaderLength**	int32	length of special header to follow (in 4 byte portions)
Block of SpecHeaderLength		
Reserved	int32 []***	Special header for imaging
end of block		
The following block will appear in T2–mode file for each i = 1 to NumberOfRecords		
TTTR T2–mode Record	uint32	The bit allocation in the record is, starting from the MSB: channel : 4 time : 28 The channel code 15 (all bits ones) marks a special record. Special records can be overflows or external markers. To differentiate this, the lower 4 bits of time must be checked. ● If they are all zero, the record marks an overflow. ● If they are >=1 they are external markers.
end of block		
The following block will appear in T3–mode file for each i = 1 to NumberOfRecords		
TTTR T3–mode Record	uint32	The bit allocation in the record is, starting from the MSB: channel : 4 dtime : 12 nsync : 16 The channel code 15 (all bits ones) marks a special record. Special records can be overflows or external markers. To differentiate this, dtime must be checked. ● If it is zero, the record marks an overflow. ● If it is >=1 the individual bits are external markers.
end of block		

* These items are stored as they were set/displayed upon entering TTTR mode.
** Items may vary and thereby change the file structure dynamically. A program must interpret these before reading further data.
*** Type formally denoted as Int32 (4 bytes) for sizing purposes, but actual type may vary according to header definition.

HydraHarp T2 / T3 mode 1.0 format:

Data Item	Type	Description
Ident	char [16]	the string 'HydraHarp'
FormatVersion	char [6]	currently '1.0'
CreatorName	char [18]	for this Software 'SymPhoTime'
CreatorVersion	char [12]	currently '5.0'
FileTime	char [18]	the file creation date and time
CR/LF	char [2]	0x0D,0x0A carriage return, line feed
Comment	char [256]	any ASCII string
NumberOfCurves**	int32	meaningless in TTTR file
BitsPerRecord**	int32	32
ActiveCurve	int32	meaningless in TTTR file
MeasurementMode**	int32	2=T2 or 3=T3
SubMode	int32	0=Standard (Timed), 1,2=Reserved, 3=Image (not for T2–mode)
Binning*	int32	0=base resol., 1=x2, 2=x4, 3=x8, 4=x16, … 10=x1024
Resolution	double	in ps (as resulting from base resolution and binning)
Offset*	int32	offset in ns
AcquisitionTime*	int32	acquisition time in ms
StopAt	int32	meaningless in TTTR file
StopOnOvfl	int32	meaningless in TTTR file
Restart	int32	meaningless in TTTR file
DisplayLinLog*	int32	lin=0, log=1

DisplayTimeAxisFrom*	int32	lower time axis bound for display in ns
DisplayTimeAxisTo*	int32	upper time axis bound for display in ns
DisplayCountAxisFrom*	int32	lower count axis bound for display
DisplayCountAxisTo*	int32	upper count axis bound for display
The following block will appear in the file for each i = 1 to 8		
DisplayCurve[i].MapTo	int32	meaningless in TTTR file
DisplayCurve[i].Show	int32	meaningless in TTTR file
end of block.		
The following block will appear in the file for each i = 1 to 3		
Param[i].Start	float	reserved for automated measurements
Param[i].Step	float	reserved for automated measurements
Param[i].End	float	reserved for automated measurements
end of block		
RepeatMode	int32	reserved for automated measurements
RepeatsPerCurve	int32	reserved for automated measurements
RepeatTime	int32	reserved for automated measurements
RepeatWaitTime	int32	reserved for automated measurements
ScriptName	char [20]	reserved for automated measurements
HardwareIdent	char [16]	currently 'HydraHarp 400'
HardwarePartNo	char[8]	PicoQuant part number, currently '930010'
HardwareSerial	int32	hardware serial number
NumberOfModules	int32	current number of modules present (max. 10)
The following block will appear in the file for each i = 1 to 10		
ModuleInfo[i].ModelCode	int32	hardware serial number
ModuleInfo[i].Version	int32	hardware version code
end of block.		
BaseResolution	double	base resolution in ps
InputsEnabled	int64	bitfield to identify enabled inputs
InputChannelsPresent**	int32	number of input channels present in hardware
RefClockSource	int32	0=internal, 1=external
ExtDevices	int32	external devices, bitwise coded
MarkerSettings	int32	bitfield to identify marker ebabling and edges
SyncDivider	int32	divider for Sync input
SyncCFDLevel	int32	Sync input CFD discriminator level in mV
SyncCFDZeroCross	int32	Sync input CFD zero cross level in mV
SyncOffset	int32	Sync input timing offset in ps
The following block will appear in the file for each i = 1 to InputChannelsPresent**		
InpChan[i].ModuleIdx	int32	input module index
InpChan[i].CFDLevel	int32	input module CFD discriminator level in mV
InpChan[i].CFDZeroCross	int32	input module CFD zero cross level in mV
InpChan[i].Offset	int32	input module timing offset in ps
end of block.		
The following block will appear in the file for each i = 1 to InputChannelsPresent**		
InputRate[i]	int32	input count rate as displayed by rate meter
end of block.		
The following is a T2 / T3 mode specific header		
SyncRate	int32	sync count rate as displayed by rate meter
StopAfter	int32	stopped after this many ms
StopReason	int32	0=timeover, 1=manual, 2=overflow, 3=error
ImgHdrSize**	int32	size of special header to follow before the records start
NumRecords**	int64	number of T2 / T3 event records
end of block.		

The following is a special header that may not be present at all: for each i = 0 to ImgHdrSize**		
Reserved	int32 []***	Special header for imaging
end of block		
The following block will appear in T2–mode file for each i = 1 to NumberOfRecords		
TTTR T2–mode Record	uint32	The bit allocation in the record is, starting from the MSB: special : 1 channel : 6 time : 25 If the special bit is clear, it's a regular event record. Add its time field and the overflow time accumulator to get the correct time information. If the special bit is set, the following interpretation of the channel code is given: ● code 63 (all bits ones) identifies a time overflow, increment the overflow time accumulator. ● code 0 (all bits zeroes) identifies a sync event, ● codes from 1 to 15 identify external markers.
end of block		
The following block will appear in T3–mode file for each i = 1 to NumberOfRecords**		
TTTR T3–mode Record	uint32	The bit allocation in the record is, starting from the MSB: special : 1 channel : 6 time : 15 nsync : 10 If the special bit is clear, it's a regular event record. Add its nsync field and the sync overflow accumulator to get the correct sync event count. If the special bit is set, the following interpretation of the channel code is given: ● code 63 (all bits ones) identifies a sync count overflow, increment the sync coint overflow accumulator. ● codes from 1 to 15 identify external markers.
end of block		

* These items are stored as they were set/displayed upon entering TTTR mode.
** Items may vary and thereby change the file structure dynamically. A program must interpret these before reading further data.
*** Type formally denoted as Int32 (4 bytes) for sizing purposes, but actual type may vary according to header definition.

Special Header

The special header is used for imaging measurements. Any program interpreting a TTTR file has at least to read in its length, which is denoted in the field *SpecHeaderLength* in multiples of four bytes. For point measurement TTTR files *SpecHeaderLength* is 0. For images the *SubMode* field is set to 3. For the PI E710 piezo–controller, it has the following format:

SymPhoTime / PI E710 Imaging Header:			
	Dimensions	int32	0,1=reserved, 2=line, 3=area
	Ident	int32	1=PI E710
	TimePerPixel	int32	Time per pixel in units of 0.2 ms
	Acceleration	int32	Acceleration interval length in % of total width
	Pattern	int32	0=monodirectional scan; else bidirectional scan
	Reserved	int32	
	X0	float	upper left corner of scanned area in µm (X)
	Y0	float	upper left corner of scanned area in µm (Y)
	PixX	int32	number of pixels (X)
	PixY	int32	number of pixels (Y)
	PixResol	float	µm per pixel
	TStartTo	float	Time from trigger to first point of the following scanned line
	TStopTo	float	Time from trigger to last point of the following scanned line
	TStartFro	float	Bidirectional scan: analogue TStartTo for “fro“ direction
	TStopFro	float	Bidirectional scan: analogue TStopTo for “fro“ direction
End Header			

When utilising the KDT180–100–lm scanning stage, the imaging header has the following format:

SymPhoTime / KDT180–100–lm Imaging Header:			
	Dimensions	int32	0,1=reserved, 2=line, 3=area
	Ident	int32	4= KDT180–100–lm
	Velocity	int32	units of ticks/s
	Acceleration	int32	units of ticks/s²
	Pattern	int32	0=monodirectional scan; 1=bidirectional scan
	Reserved	int32	
	X0	float	upper left corner of scanned area in µm (X)
	Y0	float	upper left corner of scanned area in µm (Y)
	PixX	int32	number of pixels (X)
	PixY	int32	number of pixels (Y)
	PixResol	float	µm per pixel
End Header			

For an LSM image file, the imaging header has the following format:

SymPhoTime / LSM Imaging Header:			
	Dimensions	int32	0, 1=reserved, 2=line, 3=area
	Ident	int32	3= LSM
	Frame	int32	frame trigger index
	LineStart	int32	line start trigger index
	LineStop	int32	line stop trigger index
	Pattern	int32	0=monodirectional scan; 1=bidirectional scan
	PixX	int32	number of pixels (X)
	PixY	int32	number of pixels (Y)
End Header			

In *Dimensions,* the spatial dimensionality of the image is denoted, in which 2 is reserved for repeated line scans and an image (two dimensional) is denoted by 3. The *Ident* number identifies the actual header format.

To reconstruct the images the marker signals embedded in the photon data stream have to be retrieved. The PI E710 and KDT180-100-lm formats assume that a line trigger is provided in the TTTR file. This trigger pulse is fed into the TCSPC device during the recording of the image TTTR file, where it is stored in the most significant bit of the *Marker* field. A line neither needs to start at the trigger pulse nor to stop at the trigger pulse of the next line. *TStart* and *TStop* denote (if present) the relative positions of the line between two subsequent pulses, *TStart*=0 means coincidence of the line start with the trigger pulse, 0.5 means a line start halfway to the next pulse, etc. Similarly, a *TStop*=1 marks coincidence of the line stop with the following line trigger pulse. *TStartFro* and *TStopFro* are used to position the "fro" line in a bidirectional scan. They are not meaningful when the field *Pattern* is set to 0.

Please refer to the TimeHarp manual for the specification of the SCX 200 type special header.

Data types

The data types used in the files correspond to standard C/C++ data types as follows:

char	byte, characters in ASCII
int32	32 bit signed integer
int64	64 bit signed integer
uint16	16 bit unsigned integer
uint32	32 bit unsigned integer
float	32 bit floating point number
double	64 bit floating point number
time_t	32 bit time value as defined in all C libraries

Note that the format for date and decimal point may depend on your Windows settings. Note also that, on platforms other than x86 machines, byte swapping may occur when the TTTR data files are read for further processing. We recommend using the native x86 environment consistently.

5.4 ASCII–Exported Image Files

Exporting an image *.bdf file in ASCII format will generate comparably large files. Images consist of several layers, containing (if available) the amplitudes and lifetimes of the exponential components (up to four) of the current FLIM analysis, the fluorescence intensity, the average lifetime and the background level of each pixel. The ASCII exported files start with a header displaying general image information e.g.:

```
Pixels (x/y):  5 / 5
Spatial Resolution:     1.77049160003662E-0001 µm
Layers:         9
TimeGate:       Chn. 0 to 4095
Width:          0.708 µm Height: 0.708 µm
Pixel Resolution: 0.177 µm
```

If a pixel binning has been applied, the spatial resolution of the original image is different from the actual pixel resolution. “Pixels” denotes the number of exported image pixels in x and y direction.

The layer data follows the header. Each layer starts with its name. The following lines correspond to the horizontal image lines. The software exports only the smallest rectangular region containing the current ROI. If an exported pixel is not part of the ROI, a minus ('–') sign replaces the displayed value.

6. References

PicoQuant maintains a database of publications mentioning PicoQuant devices. It can be found at our website at http://www.picoquant.com/_scientific.htm. It is a valuable source if you would like to know which laboratories are using PicoQuant products or how broad the field of various applications is. Furthermore, numerous measurement examples are published on the PicoQuant website. Please visit the MicroTime 200 section of http://www.picoquant.com/_systems.htm.

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Optics Express, 2003, 11, (26), 3583-3591

FCS: FLCS

[45] M. Böhmer, M. Wahl, H.–J. Rahn, R. Erdmann, J. Enderlein:
Time–resolved fluorescence correlation spectroscopy
Chemical Physics Letters, 2002, 353, 439-445

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FLCS — Fluorescence Lifetime Correlation Spectroscopy
Application Note, PicoQuant, 2006

7. Appendix

7.1 Technical Reference Data

Data Acquisition

TCSPC device.....TimeHarp 200, PicoHarp 300 or HydraHarp 400

Supported Configurations…100 × 100 (× 100) µm Piezo Scanning Stage or 10 × 10 cm Wide Range Scanner Laser Scanning Microscopes (LSM), Stand–Alone TCSPC

Routing.....1 to 4 detectors

Measurement Modes.....TTTR: t3r, t2, t3

Analysis

Supported Methods.....FLIM, FRET, Anisotropy, PIE, FCS, FCCS, FLCS, SMD, PCH, Lifetime Histogram, Fluorescence Time/Lifetime Traces, BIFL, On / Off Histogram, Burst Size Histogram, TCSPC Lifetime Fitting, FCS Fitting, User Scripting (STUPSLANG).

TCSPC Fitting

Methods.....FLIM, fluorescence time traces, BIFL, lifetime histogramming

Models.....1 to 4 exponentials, iterative reconvolution

Optimisation.....least squares, MLE, Marquardt–Levenberg, Monte Carlo

Error test/assessment.....χ^2, distribution weighted residuals

Error analysis.....asymptotic standard errors

FCS Fitting

Models.....pure diffusion, triplet–state, conformational, protonation; 2D/3D Gauss. PSF

Optimisation.....least squares, Marquardt–Levenberg, Monte Carlo

Error test / assessment.....χ^2, distribution weighted residuals

Error analysis.....asymptotic standard errors, Bootstrap and support plane analysis

User Interface

Graphical user interface.....Windows® GUI, menu or mouse driven

Preferences.....saved in Windows registry

Data Formats

Supported formats.....TimeHarp t3r, PicoHarp pt3, pt2, HydraHarp ht3, ht2

Operating Environment

Required PC….minimum 400 MHz CPU clock, 4096 MB memory; recommended: dual–core; RAM >=512 MB

Display.....1200 × 1024 or better

Disk space.....20 MB (except data storage)

Operating system.....Windows XP / Vista

Protection module port.....USB (parallel on request)

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7.2 Abbreviations

BIFL	Burst Integrated Fluorescence Lifetime
BNC	British Naval Connector or Bayonet Nut Connector or Bayonet Neill Concelman
CAN	Controller Area Network
CCD	Charge–Coupled Device
CFD	Constant Fraction Discriminator
cps	Counts per Second
FCS	Fluorescence Correlation Spectroscopy
FIFO	First In, First Out (buffer type)
FLIM	Fluorescence Lifetime Imaging
FRET	Förster Resonance Energy Transfer
FWHM	Full–Width at Half–Maximum
IO	Input / Output
IRF	Instrument Response Function
LED	Light Emitting Diode
LSM	Laser Scanning Microscope
MCS	Multichannel Scaling
OD	Optical Density
PC	Personal Computer
PCI	Peripheral Component Interconnect
PIE	Pulsed Interleaved Excitation
PMT	Photomultiplier Tube
RGB	Red–Green–Blue (colour scheme)
ROI	Region of Interest
SMA	Sub–Miniature version A (connector type)
SMD	Single Molecule Detection
SPAD	Single Photon Avalanche Diode
SYNC	Synchronisation (signal)
TCSPC	Time–Correlated Single Photon Counting
TTL	Transistor–Transistor Logic
TTTR	Time–Tagged Time–Resolved

7.3 Support

If you observe any errors or bugs, please try to find a reproducible error situation. Please e–mail us a detailed description of the problem and of any relevant circumstances, together with the data in question. When you contact the support, please bring up the *Modules* dialog (for example *Help* | *Getting Support…* from the main menu) and press the *Export* button of this dialog to export its contents to a text file. Please attach this file when you mail your support request to info@picoquant.com. This will help us with the diagnosis of the problem. Thank you very much in advance. Your feedback will help us to improve the product and its documentation.

In any case, we would like to offer you our complete support. Please do not hesitate to contact PicoQuant if you would like assistance with your system.

Of course, we also appreciate good news: If you have obtained exciting results with this software or published scientific papers, we would also like to know! Please send an e–mail to info@picoquant.com containing the appropriate citation. Gain additional publicity! PicoQuant maintains a database of publications mentioning PicoQuant devices and / or written by us. It can be found at our website at http://www.picoquant.com/_scientific.htm. It is a valuable source if you want to know which laboratories are using PicoQuant products or how broad the field of various applications is.

All information given here is reliable to the best of our knowledge. However, no responsibility is assumed for possible inaccuracies or omissions. Specifications and external appearance are subject to change without notice.

Retraction of old devices

Waste electrical products must not be disposed of with household waste. This equipment should be taken to your local recycling centre for safe treatment.

WEEE–Reg.–Nr. DE 96457402

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