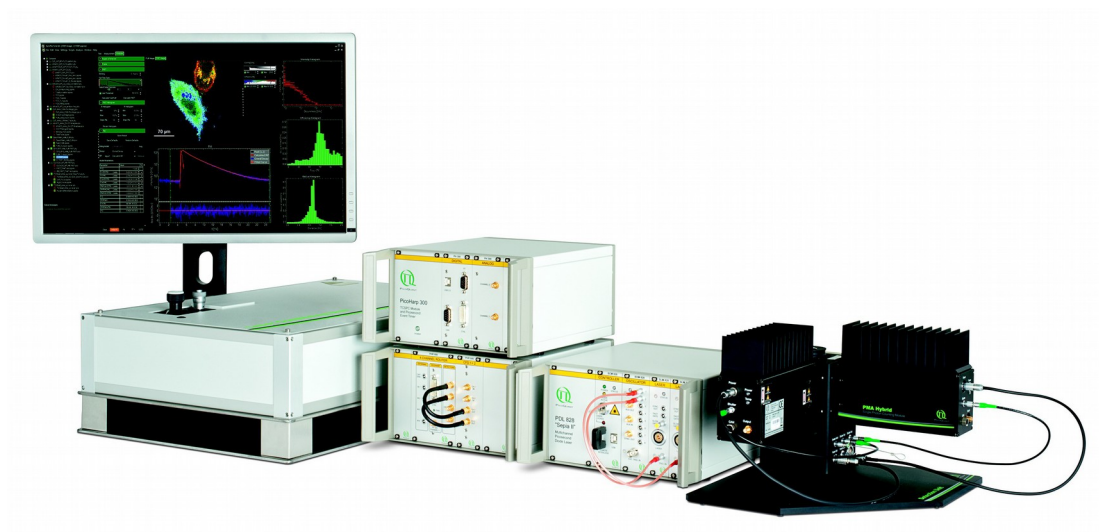


LSM Upgrade Kit

Compact FLIM and FCS
Upgrade Kit for
Zeiss LSM
Laser Scanning Microscopes



User's Hardware Manual and Technical Data

Version 5.1

with PicoQuant Software Integration

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1. Introduction

This manual describes the basic procedures to acquire FLIM - images and point measurements for FCS using the LSM-upgrade Kit in combination with your LSM.

- In regard to the LSM upgrade kit manual the Zeiss LSM microscopes have no differences. However, we refer only to the Zeiss LSM microscope in order to facilitate the reading.

More detailed explanation about the technical details of the setup as well as software analysis can be found in your additional documentation:

- **Laser Combining Unit - Manual (LCU - Manual):** This manual should be consulted for information about the Laser Combining Unit, which contains the lasers of your LSM. It explains setting the correct intensities and also contains a detailed description for realignment. It is only delivered if pulsed lasers from PicoQuant are delivered with the system.

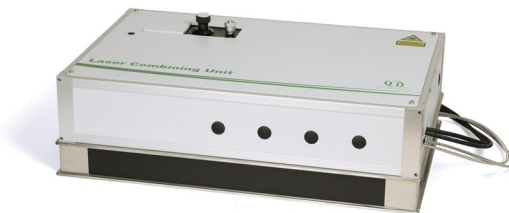


Fig. 1.1: Laser Combining Unit (LCU)

- **Detection Unit - Manual:** This manual varies depending on your detection system. Here you find basic information about alignment of the detection path and how to change filters.

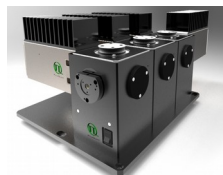


Fig. 1.2: Detection Unit for LSM FLIM / FCS Upgrades:
Multi channel PMA and SPAD detection unit

- **PicoHarp 300 and TimeHarp260 Manual:** Here you find all information about your Time Correlated Single Photon Counting (TCSPC) device. This manual also includes an introduction about single photon counting.



Fig. 1.3: PicoHarp 300 and TimeHarp260

-
- **Light Sources:** In the green folder you'll also find detailed information about the properties of your pulsed diodes lasers.

- **Router (PHR800):** The router is needed in systems equipped with a PicoHarp 300 and more than one detector.



Fig. 1.4: PHR 800 router. The configuration for a Multi-PMT setup is displayed.

- **Laser Driver (PDL):** The laser driver manual varies depending on your laser driver and explains how to set different intensities, repetition rates, and – if applicable – different pulse patterns.



Fig. 1.5: Laser driver for pulsed diode lasers: PDL 828 "Sepia" (left) and PDL 800-D (right)

- **Light Sources:** In the green folder you'll also find detailed information about the properties of your pulsed diodes lasers like pulse widths and emission wavelength.
- **Software Manual (SymPhoTime 64 - Manual):** Here you find all information about the software installation. The SymPhoTime 64 software contains a detailed, context sensitive online help function (press F1 for accessing the online help).
- **Pre-Installation Requirements:** This is an important document that already should have been considered before installation. If you want to change the configuration of your LSM, you'll find detailed information, if the desired change can be realized and additional parts are necessary. In case of doubt, contact a PicoQuant representative.
- **Appendix:** The appendix contains specific information about your individual LSM Upgrade Kit, including a description of the included parts, filter handling and a cable plan, which helps restoring the configuration after disassembly.

2. Operation

2.1. Safety Instructions



The delivered instruments are pre-set by PicoQuant to operate on the power outlet line voltage for the country of delivery. Nevertheless, please check that the actual line voltage corresponds to the value set on these instruments!

If pulsed lasers from PicoQuant are present at the system, please check that the actual line voltage corresponds to the value set on the PDL-800-D laser driver!

Never connect or disconnect any cable while the data acquisition and control electronics are ON. Charged signal cables can destroy the devices!

Protect the photon detectors (SPAD or PMT) as much as possible, particularly from excessive light intensities, e.g. the microscope illumination lamp, unattenuated backscattered excitation, etc.

In case of operation with the DSN 102, the SPAD detector(s) must be switched off using the DSN 102 power supply unit BEFORE switching off the system by the main power button.



In case of **NDD-detection**, the PMT-detector is mounted via a special adapter (part 75076) to fit onto the Zeiss LSM NDD-port. **This connection must not be disassembled** due to laser safety regulations.

2.2. Starting Hardware and Software Equipment

To **start** the LSM itself, its detectors and cw lasers, please refer to your Zeiss LSM manual. In the following we **assume a running Laser Scanning Microscope** with the sample already in place and in focus. The target region of interest for FLIM is already identified. The LSM upgrade kit can be started completely separate from the Zeiss LSM itself.

The LSM upgrade kit hard- and software should be started in the following order:

1. Start both the Zeiss and PicoQuant systems.
2. Start the **laser driver** and enable laser emission. Prior to data acquisition, the laser head should be operated for a few minutes to stabilize.
3. Start both **PCs**.
4. Start the **SymPhoTime 64 software**.
5. **Open** an already existing **workspace** or create a new one.
6. Start the **ZEN** software
7. Start the **ZEN PicoQuant Application** on your LSM-PC. The PicoQuant FLIM + FCS software integration tool will open in an additional ZEN-Software window. This window stays always on top and will allow a seamless operation for FLIM and FCS measurements on the system.
8. If pulsed lasers from PicoQuant are mounted to the system, start the **laser driver** and enable laser emission. Prior to data acquisition, the laser head should be operated for a few minutes to stabilize. If you use a PDL 828 "Sepia II", you can set the laser settings in the SymPhoTime 64 software.
9. If available, place correct **fluorescence filters** in the corresponding filter holder position of the external PicoQuant detector unit. Switch the detector unit on.
10. If you want to work with user specific settings, load your **User Configuration**.

The Upgrade Kit is now ready to use.

2.3. FLIM Data Acquisition

Scope:

Acquire a FLIM image using a pulsed laser, the Zeiss LSM scanhead with appropriate scanning and dichroic settings and external PicoQuant TCSPC detectors.

2.3.1. Starting Point

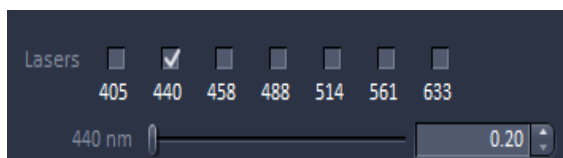
1. **Zeiss LSM:** Choose and pre-scan with the Zeiss LSM (internal detectors) the correct region where you want to perform a FLIM measurement. Finally stop the scanning process.
2. All components (laser, detectors, software) of the PicoQuant FLIM / FCS Upgrade are already running. For details about powering on see chapter 2.2.
3. **SymPhoTime 64 software:** The desired **User Configuration** of the SymPhoTime 64 has been loaded. The SymPhoTime 64 allows configuring the view of the acquisition window. Thus the user can preconfigure the online analysis he wants to see during acquisition, e.g. Images (also of the different spectral channels if more than one detector is present), a Time Trace, a Decay (TCSPC) Histogram. Several online analyses can be displayed simultaneously.
4. **SymPhoTime 64 software:** A workspace has been loaded or created. The workspace defines where the data should be stored to.

2.3.2. Changing from continuous wave to pulsed excitation

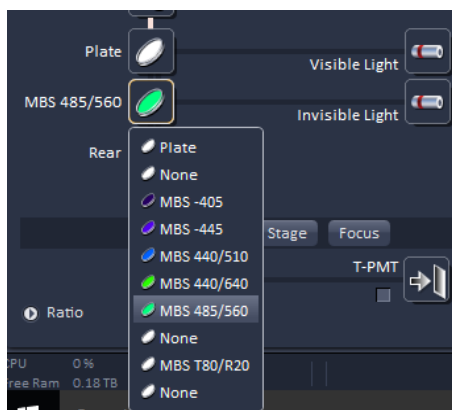
Pulsed Laser driver:

If a pulsed laser from **PicoQuant** is present, make sure that the **laser repetition rate is correctly set**. In general, 40 MHz is the best frequency to start with. Only if you observe that your decay does not fit in the TCSPC histogram, lower the frequency to 20 MHz. For details of adapting the repetition rate, see chapter 2.3.7.

Zeiss LSM: Select the correct pulsed laser line. If pulsed lasers provided by PicoQuant are present, note that they are fed through the InVIS - port of the LSM880. In order to activate them (independent from the line you use), activate the 440 – laser line in the Zen software. The chosen intensity in the Zen software is irrelevant, as this is adjusted on the LCU.



Zeiss LSM: If pulsed lasers provided by PicoQuant are present, it is also necessary to choose a suited dichroic at the InVis port, not at the VIS-port.



2.3.3. Changing from internal Zeiss LSM PMTs to external TCSPC detectors

1. **Zeiss LSM - software:** Configure the detection beam path inside the LSM scan head. If the system is equipped with external PicoQuant detectors, guide the beam to the fiber exit port by selecting "Plate". In the "Mirror"-position as shown in Fig. 2.1, the light is guided onto the internal detectors. Setting the Beam splitter wheel before the Fiberout to "Plate" enables the fluorescence to reach the external TCSPC detectors via the attached Multi-mode fiber. If you have the PicoQuant Upgrade using Zeiss BiG.2 detectors, configure the beam path selecting "Plate". If you are working with two-photon excitation and are using a PMT from PicoQuant connected to the NDD port, guide the light to the NDD detector position.

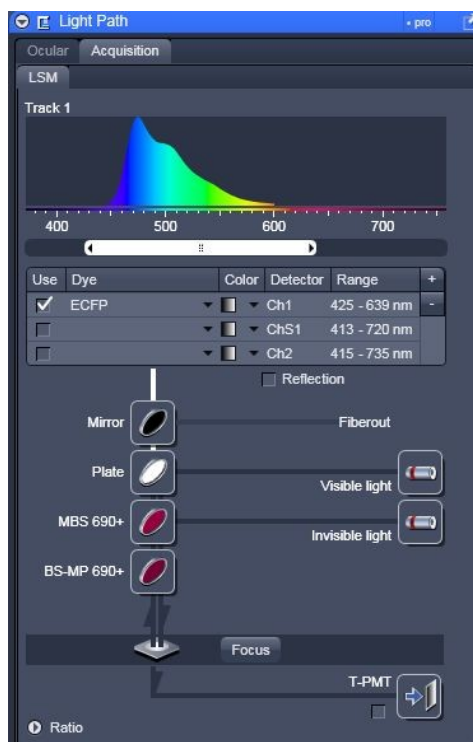


Fig. 2.1: Beam Path and Channel Control window of the LSM software: For FLIM data acquisition take care of the correct excitation HFT (major dichroic) and guide the light to the fiber out by switching the "mirror" set before the "Fiberout" to a "Plate"

2. **PicoQuant Detection Unit** (if applicable): Make sure you have **suitable fluorescence detection filters** (bandpass / longpass) in the corresponding filter holders of the detection unit. When using a Multi Channel Detection Unit, make sure that the beam splitters in the beam splitting tower is set to distribute the emission light to the detectors correctly.

2.3.4. Set suitable scan parameters

1. In the **Zeiss ZEN - software**, set the correct scan pattern:
 - Use a **monodirectional scan** as indicated in Fig. 2.2.

- **Averaging** can only be used in conjunction with the „**Frame**“ option. The „**Line**“ option will scan every line several times which cannot be correctly interpreted by the SymPhoTime 64 software. In order to access the “Frame” option, click on “show all”.

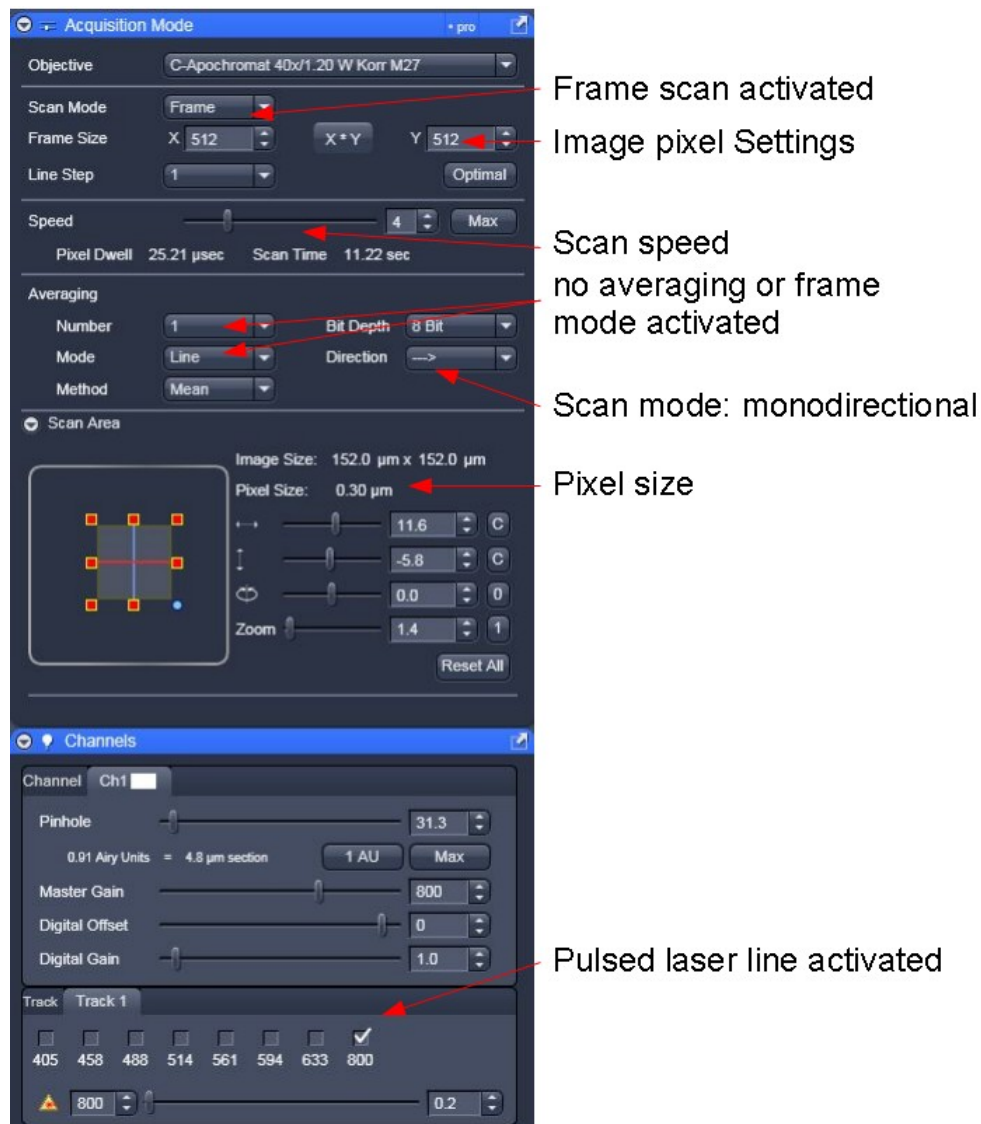


Fig. 2.2: LSM scanning settings in the LSM software

2.3.5. PicoQuant FLIM + FCS software integration tool

With the PicoQuant FLIM+FCS Software integration tool (ZEN PicoQuant Application) it is possible to run FLIM and FCS Measurements, see Figure 2.3.

For the files to be saved in the SymPhoTime64 workspace, File Name, Group Name and an additional comment can be given.

All settings and scan parameters made in the ZEN software are automatically transferred to the SymPhoTime 64 software and saved in the raw data file (.ptu) as a comment.

After starting the tool, select “Maintain / Info” and open the “Connection” window. The “Connection State” should be on “idle”. If this is not the case, click on “connect”. Make sure that the SymPhotime 64 software is up and running since the tool is connecting to this software.

Go back to the “Application” tab.

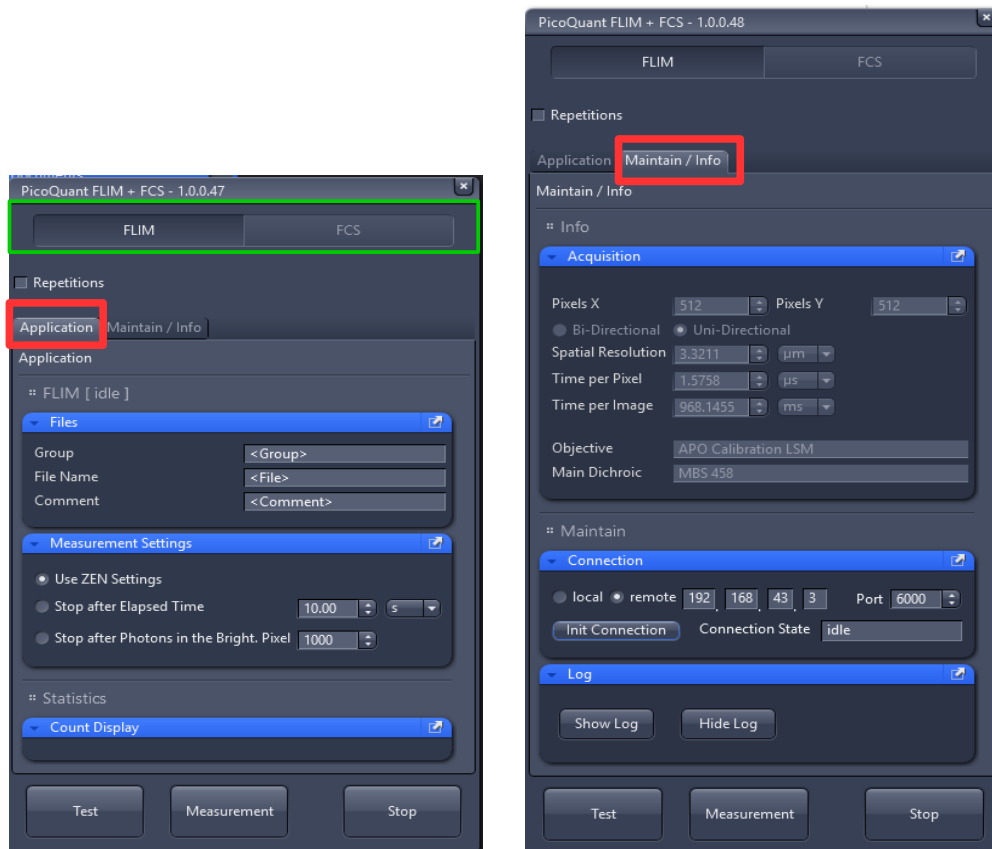


Fig2.3: PicoQuant FLIM+FCS software integration tool. Left Application tab, right Maintain/Info tab, the selection between FLIM and FCS Measurements is highlighted in green

Regarding the measurement settings of the PicoQuant FLIM+FCS software tool it is possible to select between:

- **Caution:** As long as you use the PicoQuant FLIM+FCS software tool **do not start or stop** a measurement in the SymPhoTime 64 software by clicking on the “Start” or “Stop” button.
- **FLIM or FCS:** For FLIM measurements the FLIM button of the PicoQuant FLIM+FCS software tool needs to be clicked.
- **“Use ZEN Settings”:** this setting should always be selected for all automated measurements which are controlled by the ZEN software like z-stacks.
- **“Stop after Elapsed Time” / “Stop after Photons in the Bright. Pixel”:** These two settings can be used when it is necessary to measure for a certain time or to collect a specific number of photons. Make sure that the Zen settings like “Time Series” are unchecked.

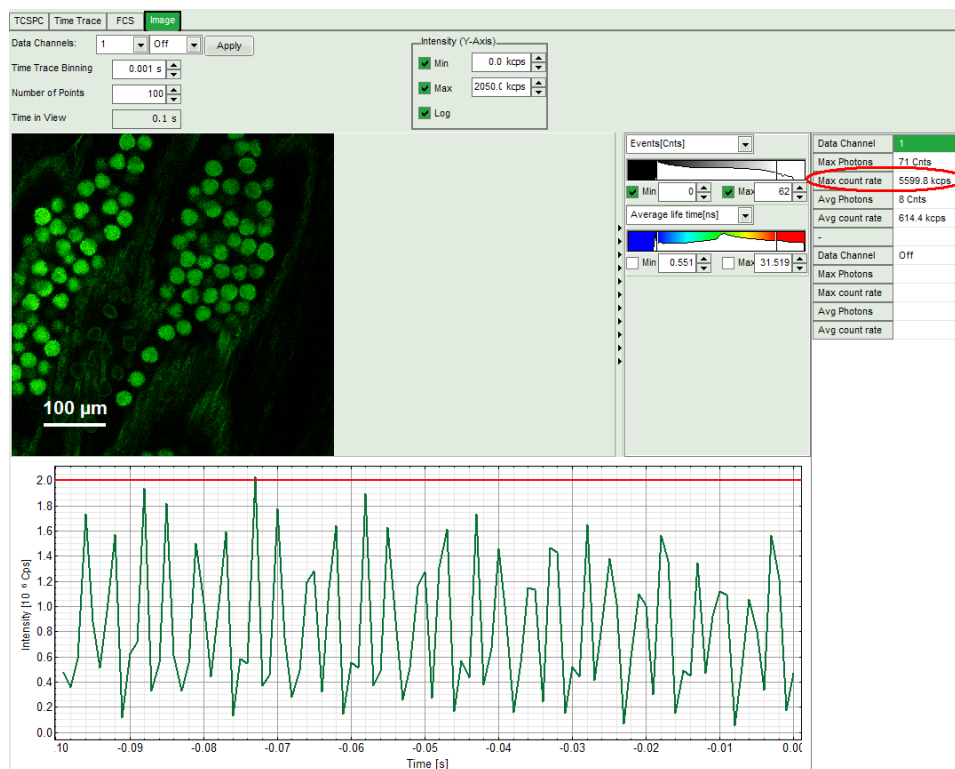
2.3.6. Optimize the detected photon count rate

1. **PicoQuant FLIM + FCS tool:** To set up the right parameters for data acquisition you can start a test measurement by clicking on "Test" at the PicoQuant FLIM + FCS software tool. The measurement is running continuously while only one frame at a time is displayed in the SymPhoTime 64 software.



Fig. 2.4: Start a test measurement with the ZEN PicoQuant Application

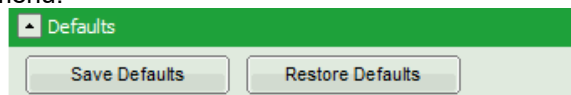
4. **SymPhoTime 64 software:** During the LSM data recording the test measurement preview is displayed, together with a time trace (Fig. 2.5). Make sure that the Peak Count Rate (marked in red) is below the values given in the table at Fig. 2.5. The Peak Count Rate is determined from the maximum in the time trace. The "Time trace binning" should be set to 0.001 s. Here you can watch the changes of the count rate over time. A red line indicates a threshold of 10% of the repetition rate. The intensity should always remain below this value. If only very tiny lifetime-changes are expected, stay even well below this number. In case you have a rapidFLIM setup, this so called pile-up limit has not to be regarded and count rates up to 30 Mcps can be used.



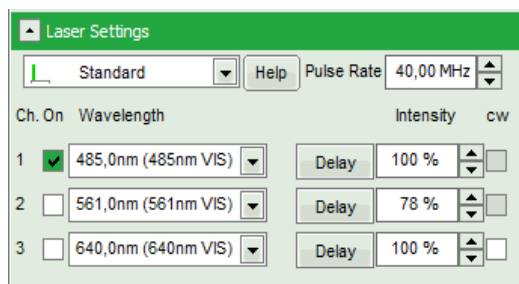
PDL setting (Laser repetition rate)	Upper Count rate limit for different detectors, in [KCounts / s]		
	PMA PMT, PMA Hybrid or PDM SPAD	BiG.2	Excelitas AQR, Confocor
40 MHz	4000	4000	1000
20 MHz	2000	2000	1000
10 MHz	1000	1000	1000
5,0 MHz	500	500	500
2,5 MHz	250	250	250

Fig. 2.5: Top: Online monitoring of the of the photon count rate in the SymPhoTime 64 preview window. On the left, a FLIM image is displayed. The intensity time trace is shown below. An intensity of 10% of the repetition frequency is indicated by the red line in the graph. Adjust the laser intensity so that the intensity graph is below the red line. Bottom: Count rate limit for different combinations of excitation repetition rates and detectors.

- **SymPhoTime 64 software:** The Image **preview settings** for a measurement can be pre-set, when no measurement is running. Defaults can be saved using the “Save Defaults” button in the Default drop down menu.



- To finally save the settings in a User profile, select “Settings” => “Save as User Profile” in the main menu bar. This way, all settings can be saved in a user specific profile and loaded with the next start of the software.



- **Adjust the photon count rate** by changing the intensity of the pulsed TPE or PicoQuant lasers that are attached to the system. The intensity of the PicoQuant lasers can either be adjusted at the laser combining unit (LCU) or – if you don't have a LCU, but a directly fiber coupled laser – in an analogous way at the laser scaffold with the intensity regulation knob. For attenuation via changing the **laser current** see the laser driver manual. If the pulsed lasers are controlled by a **SEPIA II**, the intensity can be regulated in the SymPhoTime 64 software. Therefore, check the laser head you want to use in the “laser Settings”-drop-down menu and set the intensity. Keep in mind that changing the laser intensity via the laser current does affect the pulse shape of the laser. Setting the intensity close to the lower laser threshold will ensure the narrowest pulse width, but the intensity will also be lowest. Note that the % values in the menu refer to the current that drives the laser and do not belong to a linear intensity scale.
- To ensure the shortest possible FLIM data acquisition time we recommend to **increase the photon count rate** if possible up to the pile-up limit. Of course, in case of weak fluorescing samples this limit cannot be reached even when using maximal laser power.
- Using the “Image” tab of the “Test” preview window in the SymPhoTime 64 you will see a **preview** of the **FLIM** image. The lifetime displayed in the image by the color code is calculated by the mean arrival time of the fluorescence photons after the laser pulse. It allows the optimization of the field of view and focus setting.

Supporting information:

Unnecessary small photon count rates extend the data acquisition time, because a certain amount of photons is needed for the FLIM analysis. On the other hand, too high photon count rates can lead to artefacts and falsify the FLIM analysis. The upper limit for the count rate is given by:

- TCSPC principle: To avoid **pile-up** artefacts (overestimation of early photons) the detected photon count rate should be in general less than 10 % of the laser repetition rate. When using a laser repetition rate of 20 MHz this results in a photon count rate of 2 Mcps (2.000 cts / ms).
- TCSPC detector: Some detectors introduce **artefacts** when being operated at **high count rates**. The AQR type SPAD from Excelitas shows above 1 Mcps photon count rate significant IRF broadening and a count rate dependent IRF shift.

2.3.7. Choose the correct laser repetition rate

For **PicoQuant pulsed diode lasers** the laser repetition rate can be set at the PDL laser driver or – if the lasers are controlled with a SEPIA II – in the “Laser Settings” drop-down – menu of the SymPhoTime 64 software. In general the repetition rate should be as high as possible to achieve the highest possible photon count rate. However, the time window after a laser pulse should be still large enough to allow the population of excited dye molecules to deplete completely before the next laser pulse.

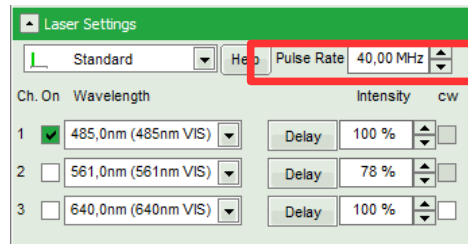


Fig. 2.6: SymPhoTime 64 repetition rate

For example, the time between 2 laser pulses at 80 MHz repetition rate is 12.5 ns. If a fluorophore with 6 ns lifetime is measured, due to the statistical process more than 12 % of the photons are emitted after 12.5 ns. In this case the repetition rate has to be lowered.

Start a continuous “Test” measurement via the PicoQuant FLIM+FCS software tool. Check the decay behavior of your sample using the TCSPC tab of the Test - window of the SymPhoTime 64 software. Activate the TCSPC Histogram-tab, and monitor the fluorescence decay as shown in Fig. 2.7. At 40 MHz the **time window is optimally adapted** (right panel in Fig. 2.7), while at 20 MHz the fluorescence has already decayed at less than half of the detection window (left in Fig. 2.7). At 80 MHz laser repetition rate, the fluorescence would not decay completely before the end of the time window is reached. Due to a “wrap around” the decay tail would be observed even before the actual pulse. Therefore 40 MHz would be the optimal repetition rate in this case.

Stop the test measurement.

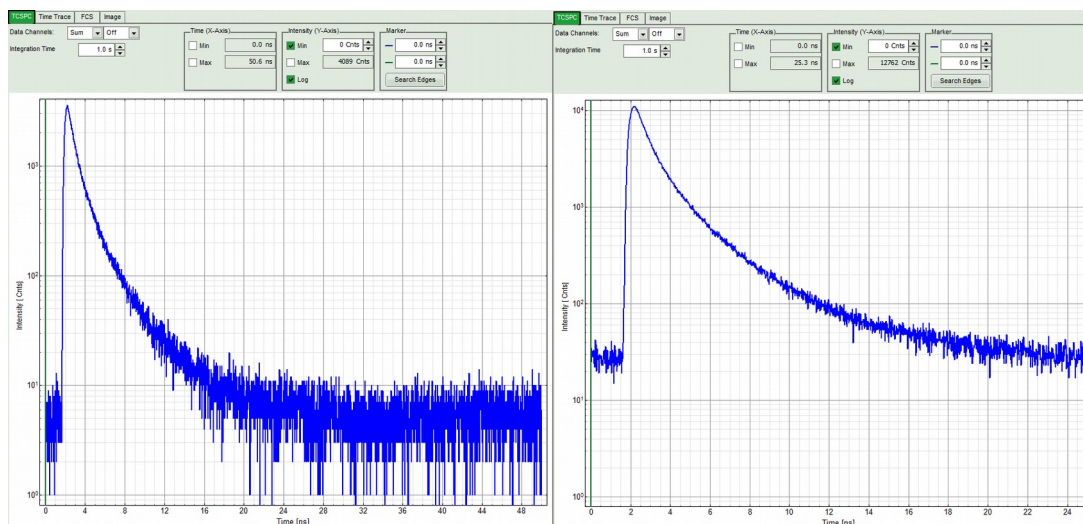


Fig. 2.7: Fluorescence decay at unnecessary low (left, 20 MHz) and optimal (right, 40 MHz) laser repetition rate. With an even higher repetition rate, a part of the fluorescence would appear at the beginning of the TCSPC-window.

2.3.8. Start FLIM data acquisition

1. **PicoQuant FLIM + FCS tool:** By using the PicoQuant FLIM+FCS software tool it is possible to start the measurement simultaneously within ZEN and SymPhoTime 64 by clicking on “Measurement” in the FLIM tab.



The measurement parameters of the ZEN software (image size, scanning pattern, bi- or monodirectional scanning, image dimensions e.g.) are used, when “Use ZEN Settings” is selected. This is necessary for automated measurements like z-stacks. The measurement parameters are entered in the comment field of the SymPhoTime 64 software.

In case the same image should be scanned several times it is possible to select a certain number of repetition cycles by ticking the “Repetitions” box. In the Repetitions window the number of repetition cycles can be selected, see Fig. 2.10.

2. **SymPhoTime 64 software:** During **LSM data recording** the chosen **online analysis** with FLIM images, TCSPC-Histograms, countrate-display etc. are displayed, see Fig. 2.8. The layout of the online display can be arranged prior to the measurement. When scanning several frames, all frames are integrated over time. This display allows you to evaluate the quality of the FLIM data captured. Please note that the FLIM preview image is just integrated for the display, the stored raw data still allows you to analyze the data separately frame by frame.

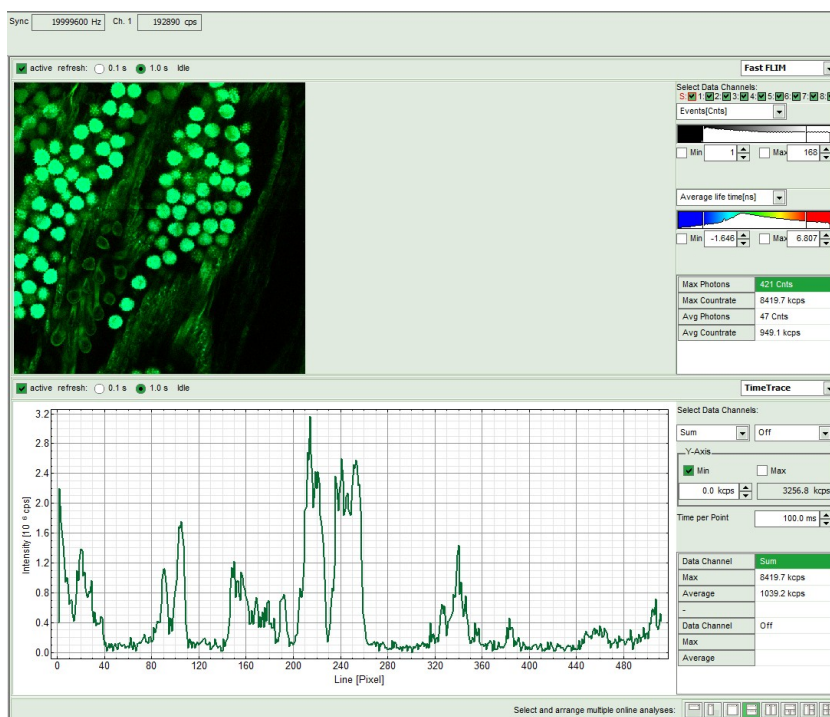


Fig. 2.8: SymPhoTime Measurement Preview being displayed during LSM data acquisition. The FLIM image is displayed on the upper left. The display parameters are shown on the right. Below, a timetrace of the intensity variations over time is plotted.

3. **Z-stack:** when performing a z-stack measurement the online preview of the SymPhoTime 64 is summing up all frames over time. So the displayed image does not really make sense. Anyway it is possible to evaluate every single frame (= z-slice) separately within the "Multi Frame FLIM"-analysis.

Please note: For a z-stack it is necessary to tick the box "Use Zen Settings" inside the PicoQuant FLIM + FCS software tool.

Please note: When using the **averaging** option of the ZEN software it is necessary to set the averaging mode to **Frame**, which was already described in Chapter 2.3.4.

An averaging of "x" is used in order to increase the collected number of photons in each z-slice by scanning each slice "x" times. This means that the number of frames in the measurement is "x" times larger than the selected number of z-slices.

(Example: a z-stack of 20 slices with an averaging of 2 means that every z-slice is scanned twice. The resulting number of frames in the recorded image is 40. This can be analyzed using a frame binning of 2 in the FLIM-analysis window of the "Multi Frame FLIM"-analysis, see Fig. 2.9)

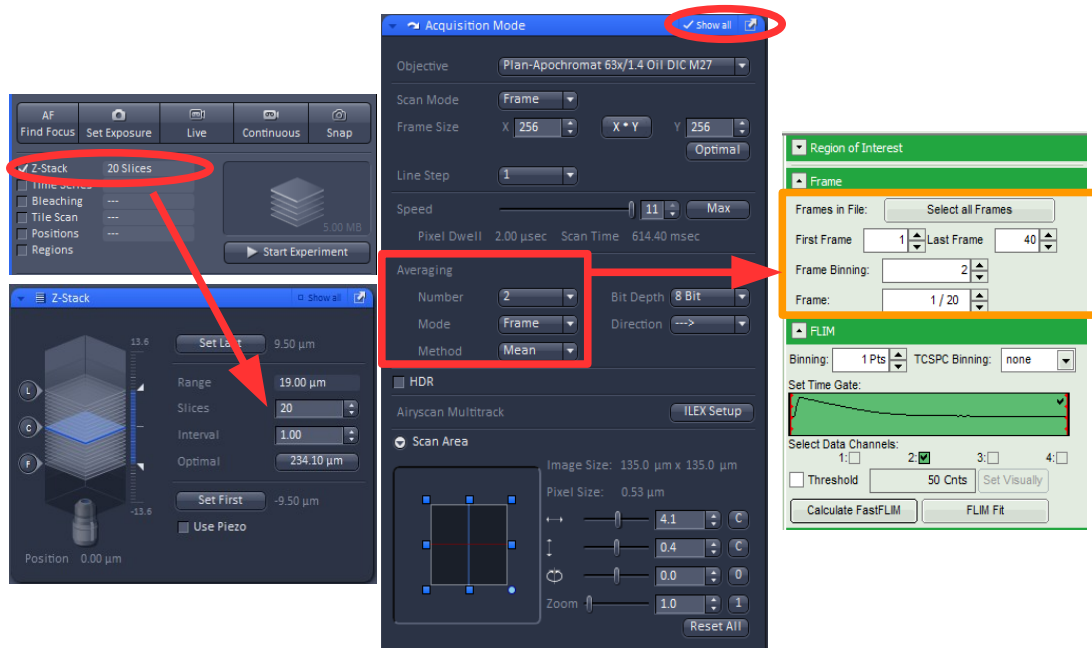


Fig. 2.9: Settings of a z-stack measurement with an averaging of 2 in the ZEN software (Averaging Mode has to be set to Frame, otherwise each line is scanned several times, which cannot be processed with the SymphoTime 64 software) and the correct selection of a frame binning when analyzing the raw data frame by frame with "Multi Frame FLIM"-Analysis in the SymphoTime 64 software

- The **LSM scanning process stops** either after having scanned the pre - set number of frames, if the system is set for averaging a specific number of frames, or by just manually interrupting the measurement by clicking on the "Stop" - button of the PicoQuant FLIM+FCS software tool.



- SymPhoTime 64 software:** The recorded raw – data - file (*.ptu) is displayed in the workspace menu on the left and can now be opened for analysis.

2.3.9. Resulting raw data file and documentation

- SymPhoTime 64 software:** The result of the measurement is a .ptu-raw data file which contains the maximum available information for every detected photon (temporal, spatial and detection channel information). Furthermore the online analysis files for each measurement are saved as .pqres-files.

In case an online Fast - FLIM-image shall be processed, highlight the raw data (.ptu) file and open this file with e.g. the FLIM - Analysis option in the Analysis-tab. If you double-click on the "OnlineFastFLIM_... .pqres-file", the FLIM analysis is directly opened.

- Every raw data file is stored together with the actual settings and it contains all measurement parameters known by the SymPhoTime 64 software. To access, double click on the raw (" .ptu")-file. A Comment field with all informations is opened, see Fig. 2.10.
- The file name and all additional information (measurement type, sample, etc.) can be entered **before** the measurement in the "File-Name" and "Comment"-fields of the **PicoQuant FLIM+FCS software** tool of the ZEN software. Furthermore it is possible to repeat the measurement several times by ticking the "repetition" box and inserting a specific number of cycles. The information about the current repetition cycle is transferred for each measurement in the file name of the .ptu file as a number (T_1), see Fig. 2.10. Additionally also the number of the measurement position used for FCS measurements is saved in the file name (P_1).

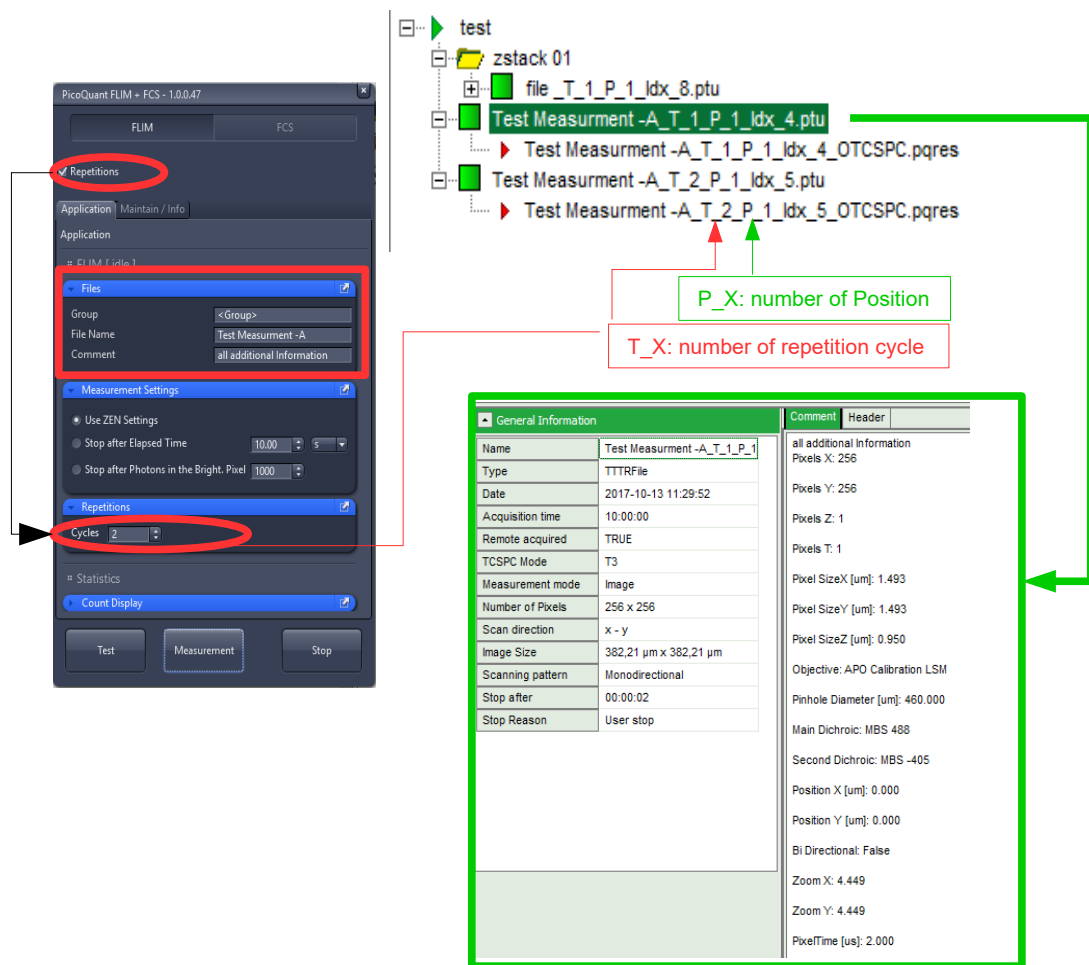
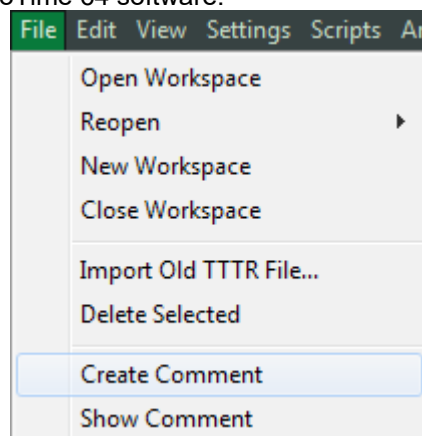


Fig. 2.10: Left: Insert File Name and Comment into PicoQuant FLIM + FCS software tool. When ticking the "Repetition" box it is possible to repeat the FLIM measurement several times. The number of repetition cycles and the position numbers are saved in the file name within the SymPhoTime 64 workspace. Double-clicking on the *.ptu file opens the comment with all informations about the measurement (marked in green)

- Note** that the raw data file **cannot be changed after** the measurement; if you need to add information after the measurement, you can add a comment file via "Create Comment" the main menu bar in the SymPhoTime 64 software.



- The **image dimensions** that are used for image size calculations are found in the ZEN “Acquisition Mode” Drop-down menu as “Pixel size” and are automatically transferred to the **SymPho-Time 64** -software (Fig. 2.11)

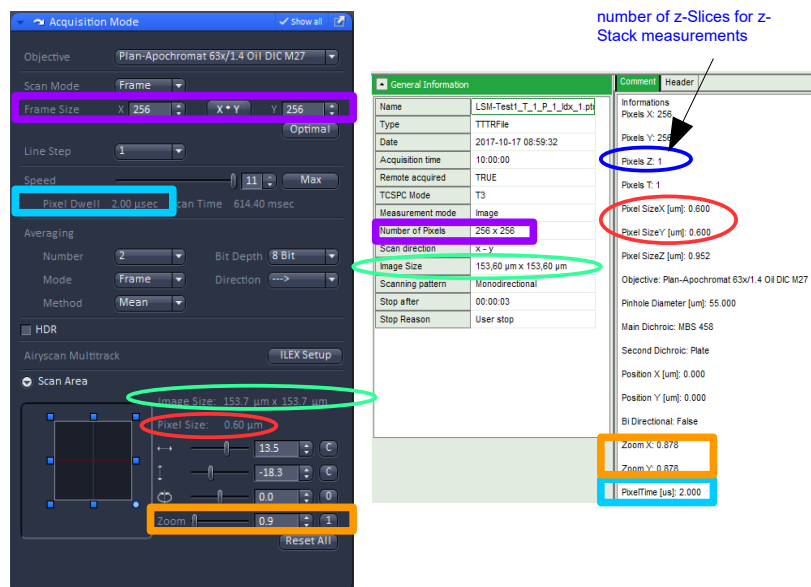


Fig. 2.11: The Image size and pixel dwell time information are shown in the ZEN software display of the “Acquisition Mode” drop-down – menu (left). This information is also visible in the comment of the raw data file in the SymPhoTime 64 software, accessible by double-clicking on the .ptu file.

- SymPhoTime 64 software:** Specific recorded frames can be selected for evaluation in the FLIM-analysis. Highlight the raw data file, go to the main “Analysis” tab, and select the “FLIM”-analysis from the “Imaging” drop-down menu. The FLIM analysis window pops up. The frames chosen for analysis can be entered in the field “Frame” (from “First Frame” up to “Last Frame”).

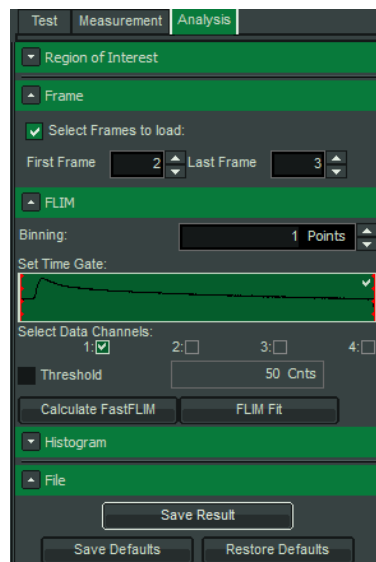


Fig. 2.12: Changing the selection of frame numbers for analysis in the SymPhoTime Software.

2.3.10. Measure an instrument response function (IRF)

For accurate measurements of lifetimes near to the timing resolution, the instrument response function (IRF) should be taken into account. The width of the IRF displays the timing resolution of the instrument. An IRF can be obtained by performing the following steps:

1. Place a sample with an **negligible short lifetime** (e.g. Erythrosin B or Fluorescein, dissolved in a saturated KI-solution. For fluorescein, a slightly basic pH value is necessary to dissolve a sufficient amount of dye) in the laser focus on the microscope stage. The dye chosen should have a similar spectral emission range as the sample that should be measured. Potassium iodide is a strong quencher and reduces the fluorescence lifetime to a few picoseconds. See e.g. [Applied Spectroscopy, Vol.63, p.0363-0368 \(2009\)](#). For 2 photon-excitation, also second harmonic generation may be used to measure the IRF.
2. Place a suitable **emission filter** in the filter holder in front of the PicoQuant detector, ideally the same as used later for the measurements.
3. **Zeiss LSM – software:** Set the LSM settings in the same way as for a **FLIM measurement**. This way the IRF can be monitored by the PicoQuant detectors. Start a test measurement using the PicoQuant FLIM+FCS tool.
4. **SymPhoTime 64 software:** You can now check the decay behavior of your sample using the TCSPC preview of the “Test” tab. Activate the TCSPC Histogram-tab and monitor the fluorescence decay as shown in Fig. 2.13. In the “TCSPC-settings” drop-down menu, also the time width per channel can be set. Adjust the count rate to approximately 100 kCounts/s. It is important that the laser power output is not changed at the laser driver or in the SymPhoTime – software, if a SEPIA II laser driver is present at the system. Only adjust the laser power at the Laser Combining Unit (LCU) or attenuator at the laser head. Changing the laser driver or software intensity settings changes the shape and temporal position of the laser pulse. You can use the driver settings to optimize the pulse shape of the laser for shortest IRF width.

This does not apply to for lasers used for multiphoton excitation, as for those lasers, the pulse width is always negligibly short.

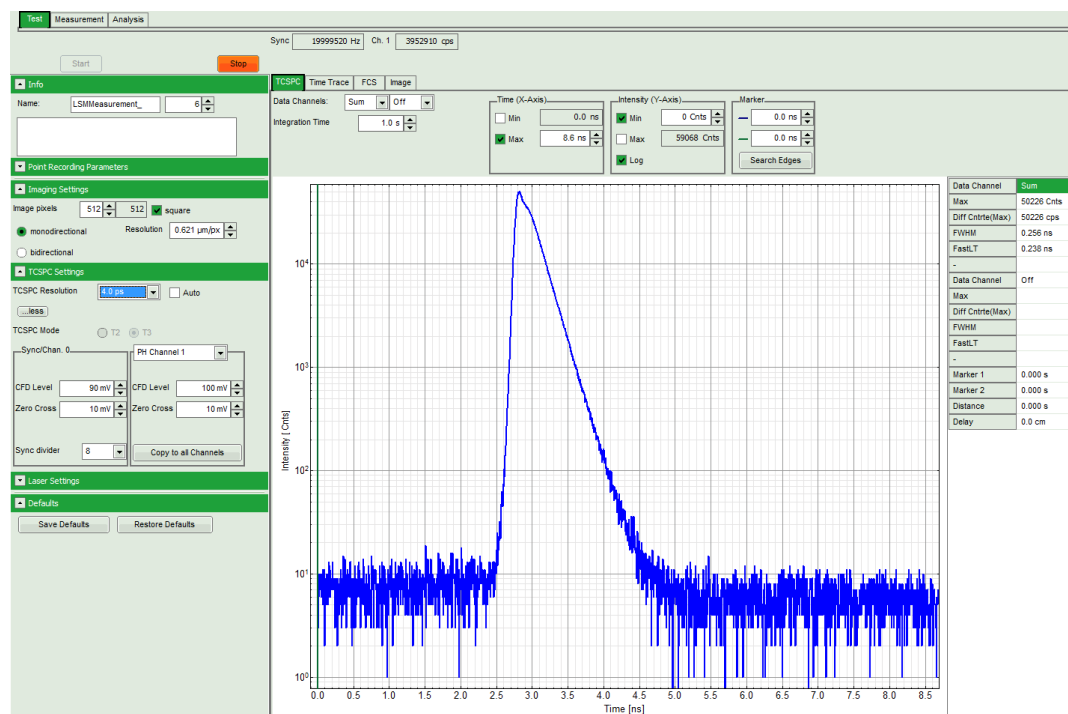


Fig. 2.13: TCSPC-preview window, recorded with a PH300, MPD SPAD and PicoQuant laser source. The time width per channel can be set with the “TCSPC Settings” drop-down menu. The count rate is displayed over the preview graphs for each channel separately.

5. **PicoQuant FLIM + FCS tool:** Stop the test measurement by pressing “Stop” in the Zeiss PicoQuant FLIM+FCS tool.
Step 4 are only for checking the IRF shape and settings, they do not need to be performed routinely.
6. **Zeiss LSM – software:** For measuring a IRF curve it is advisable to stop the measurement after a certain number of photons is collected. In order to do that within a FLIM Measurement it is necessary to tick “Time Series” in the Acquisition Tab of the ZEN software and select the maximum number of cycles, as already described in 2.3.5.
7. **PicoQuant FLIM + FCS tool:** Select Stop after 10000 Photons in the Measurement Settings tab within the the PicoQuant FLIM+FCS tool and start a measurement by clicking on “Measurement”.
8. When the decay histogram in the TCSPC preview has reached 10000 counts in the peak channel, the image acquisition in the SymPhoTime and LSM – software is stopped automatically. You can open the TCSPC histogram of the captured image, using the “TCSPC-Histogram” - analysis of the SymPhoTime 64 software. It contains the IRF.
9. Replace the IRF solution with your sample and start the sample measurement.

2.3.11. Remarks

- Your LSM allows **storing** defined **configurations**. Acquiring FLIM images is easier, when one configuration for FLIM and one configuration for FCS measurements is stored. Storing and loading configurations is possible in the “Configuration Control” window of the ZEN software. A configuration allows to pre-set the correct beam path and major dichroic.

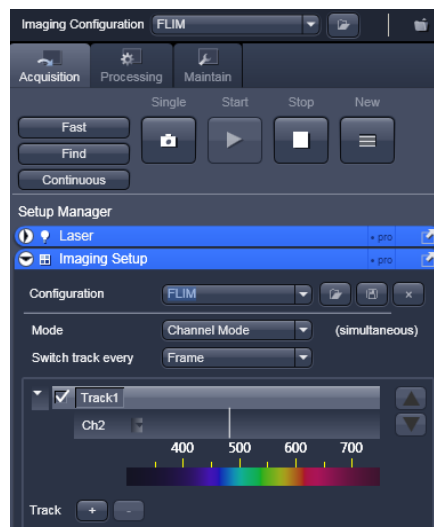


Fig. 2.14: Storing and applying stored configurations is possible via the “Imaging setup” tab. Clicking the “open” icon in the “Configuration” line opens a stored configuration. Entering a new configuration name and clicking on the “save” icon stores the actual configuration.

- The SymPhoTime software is **unable to compensate** for **hysteresis** effects that may be induced by bidirectional scanning, thus if such effects are noticed, it is preferable to perform monodirectional scans.
- The **laser intensity** of the diode lasers from **PicoQuant** can be **adjusted** in two different ways: On the one hand, the light intensity can be reduced at the LCU using the filter wheel and the scaffold, on the other hand, the intensity can be controlled at the PDL800-D or the SEPIA laser driver. If the intensity is changed at the PDL800-D or the SEPIA II, this changes the pulse shape and therefore the instrument response function (IRF). Take care to record the IRF and the fluorescence decay with the

same laser settings at the PDL800 or the SEPIA II if you want to perform reconvolution fit. The pulse width of the laser and therefore the time response of the instrument (the width of the IRF) can be optimized with the power settings at the laser driver.

- The **fluorescence detection sensitivity** of the optical system can also be increased by opening the pinhole in the LSM. On the other hand this may reduce the signal to background ratio as well as the optical resolution of the system.
- In order to obtain the **optimal lifetime information** it is in general preferable to analyze FLIM images with less pixels than standard intensity “only” images, as the accuracy of the lifetime information is directly dependent on the number of photons per pixel. That may become crucial, if several lifetimes are present. The SymPhoTime 64 software allows to perform post-acquisition pixel binning for optimization of the best compromise between lifetime accuracy and image resolution.

2.4. Data acquisition for FCS measurements

Scope:

Perform a point measurement at a specific position. With sensitive detectors like BiG.2 or PMA-Hybrid, Fluorescence Correlation Spectroscopy (FCS) measurements can be performed. In all point measurements, the fluorescence is recorded at a certain position in the sample and analyzed in the SymPhoTime 64 software.

If lifetime information is not needed, also steady state lasers from the Zeiss LSM might be used for the fluorescence excitation.

For performing and analyzing point measurements, your SymPhoTime 64 license must cover these measurements.

2.4.1. Starting Point

The following description assumes that

- Your **sample** is already placed onto the microscope and brought into **focus**. If you are measuring in solution, it is assumed that the laser is focussed into the solution. If you are measuring in live cells, take into account that due to the analysis method in FCS, cells appearing relatively dark in the manual epifluorescence view should be selected.
- The **correct dichroic mirror** for your wavelength has been selected in the ZEN software, and the **correct emission filter(s)** has/have been placed into your detection unit. The light is directed correctly to the fiber exit port as for a FLIM – image acquisition.

Measuring with pulsed excitation	Measuring with cw excitation
Select the pulsed laser and select the optimal repetition rate	If measuring with cw lasers, the SYNC signal of the laser driver unit must still be present . This ensures that a trigger signal is provided, which is needed by the TCSPC unit.

2.4.2. PicoQuant FLIM + FCS software tool for FCS

PicoQuant FLIM + FCS tool: It is possible to define several measurement parameters within the PicoQuant FLIM + FCS software tool. By clicking on FCS several options for point measurements can be adjusted, see Fig. 2.15:

- By ticking the “Time series” box, it is possible to repeat the measurement several times. The number of repetition cycles can be set in the “Time Series / Position Settings” tab. The “Point Time” refers to the measurement time per point. The “Delay Time” corresponds to the time gap between two measurements (different position or different cycles).

Note: when using “Time Series” options for FCS measurements only the settings in PicoQuant FLIM + FCS software tool should be used. Time Series option within the ZEN-Software should be disabled.

- Various measurement positions in an image can be selected directly in the ZEN software and loaded into the PicoQuant FLIM + FCS software tool by clicking “From Overlay..”. This is explained in more detail in Chapter 2.4.3

Note: The box “Positions” should only be checked, when at least one position is loaded. Otherwise the measurement does not start.

- “Use ZEN Settings” in the “Measurement Settings” window.
- Only if “Positions” and “Time Series” are not checked, enter the measurement time into the “Measurement Settings” tab by selecting “Stop after Elapsed Time”. This time refers to the overall duration of the measurement.

Select FCS Measurement

"stop after Elapsed Time" refers to the overall measurement time

Measurements can be repeated several times by selecting "x" cycles
Point Time = Measurement time per Point
Delay Time = Time between two measurements (different point or cycle)

Load several measurement positions, which are selected within the ZEN software by clicking "From Overlay"
Note: When no positions are loaded the upper box named "Positions" should not be ticked!!

Position No	x (μm)	y (μm)	z (μm)
1	-1000.6	-1619.9	0.0
2	-297.6	-308.1	0.0
3	1030.8	84.9	0.0

Fig. 2.15: Measurement Parameters which can be adjusted within the PicoQuant FLIM+FCS software tool. Blue: selection between FLIM and FCS, purple: overall measurement time, orange: time series options, green: Positions for automated multi-point measurements. If “Time Series” is selected, “Measurement Settings” should be set to “Use ZEN Settings”.

2.4.3. Choose location and time series for the point measurement

There are two different ways of selecting the positions

- **Point measurement at one position only:**

1. **Zeiss LSM software:** Change the light-path settings within the ZEN Software by opening the Image Settings tab. The light should be guided to the internal Zeiss detectors by selecting “**Mirror**” (see Fig. 2.16 marked in red). Furthermore it is necessary to adjust the wavelength prior to any measurement, so that the laser light is not detected. This can be done by shifting the bar highlighted with a yellow arrow in Fig. 2.16.

For selecting one point open the “Acquisition Mode” tab in the ZEN software and click on “Spot Select”. A new image of your sample is recorded and afterwards the crosshair inside the image can be shifted to the correct position where the point measurement should be performed, see Fig. 2.16.

Once the spot is selected the light-path has to be set back to “**Plate**” in order to use the PicoQuant or BiG.2 detectors for the FCS measurement.

2. **PicoQuant FLIM + FCS tool:** Click on the FCS tab within the PicoQuant FLIM + FCS software tool, select a certain overall measurement time at “stop after Elapsed Time” or choose “Time series” and define the number of repetition cycles, the measurement time per point and the delay time in-between two measurements.
3. **PicoQuant FLIM + FCS tool:** Insert the file name and additional informations into the “File Name” and “Comment” fields and start a measurement by clicking on “Test” or “Measurement”.
Note: When performing a “Test” - measurement the recorded data is **not** saved in the SymPhoTime 64 software as a .ptu File. Therefore it is advisable to use the “Test” measurement option when laser-power and other parameters still need to be optimized. The optimization process is described in Chapter 2.4.4

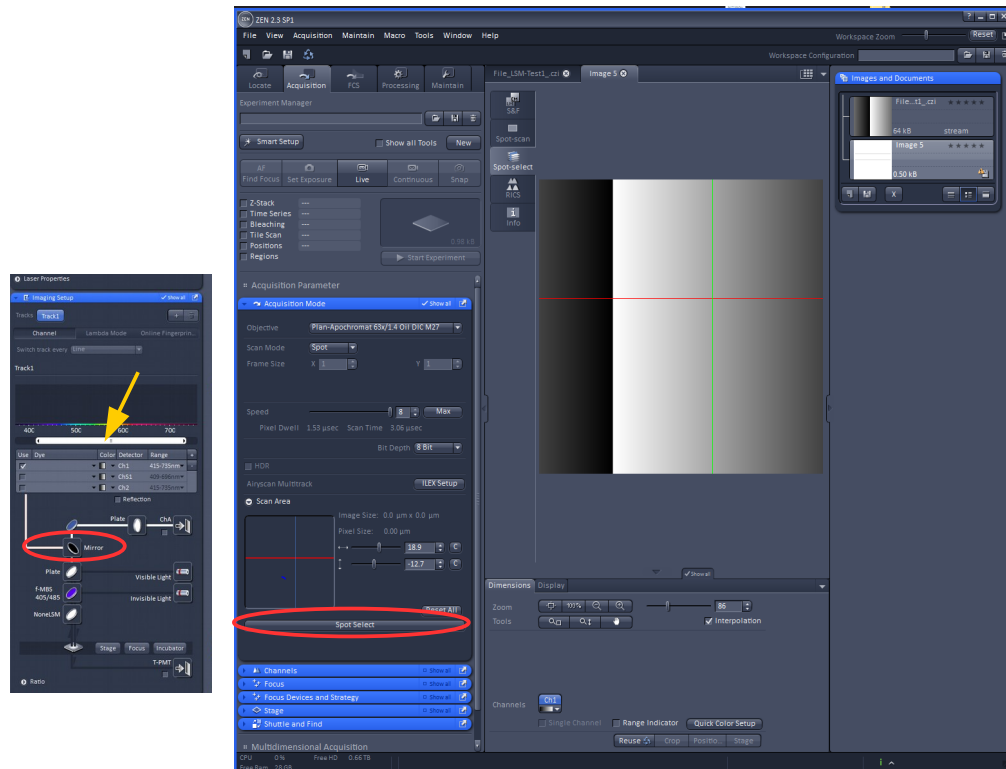
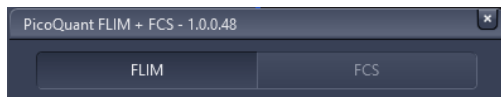


Fig. 2.16: Switch from PicoQuant to Zeiss detectors by changing the Light-path from "Mirror" to "Plate". Set the correct detection wavelength for Zeiss detectors by shifting the bar highlighted with a yellow arrow. Selecting the position of the point measurement in the Image window. Clicking "Spot Select", indicated in red, results in scanning one image. Clicking at the desired position inside the recorded image marks the spot to be measured. The scanner moves to that position when the Measurement is started.

- **Automated point measurements at several positions:**

1. **PicoQuant FLIM + FCS tool:** First go to the FLIM Measurement tab in the PicoQuant FLIM + FCS software tool of the ZEN software. This allows you to take an image of your sample, for selecting interesting locations for the later on point measurements.



2. **Zeiss LSM software:** In order to define one or several specific positions for FCS measurements the following procedure has to be done, see Fig. 2.17:
 - Set the correct light-path within the "Image Settings" tab. The light is guided to the internal Zeiss Detectors by selecting "**Mirror**" (see Fig. 2.16 marked in red). Furthermore it is necessary to adjust the wavelength prior to any measurement, so that the laser light is not detected. This can be done by shifting the bar highlighted with a yellow arrow in Fig. 2.16.
 - Perform a "Snap" measurement of your sample (1) once all measurement conditions for image data acquisition are set correctly (image size, laser power, repetition rate, scan parameters, ect.). See Chapter 2.3 for more detailed information on FLIM data acquisition.

The "Snap" measurement results in scanning one frame. Once the "snap" image is displayed it is possible to select either one or several point positions for the FCS measurements.
 - Beneath the displayed image tick "show all" (2), open the "Graphics" tab (3). Tick the boxes "Keep Tool" and "Auto Color" (4).
 - Select "Cross" in the drop down menu (5) and click on all positions in the image, where a FCS measurement should be performed (6). The selected positions are also displayed in the List within the "Graphics" tab. By clicking on "ID" (7) the positions in the image are numbered, so it is possible to identify them afterwards.
 - Save the image including all defined measurement positions by clicking on the "save" button at the "Images and Documents" window (8). This Image can be loaded into the ZEN software afterwards at any time displaying the points for the FCS measurement.
 - The numbers of the points will be displayed in the file name of the .ptu file in the SymPhoTime 64 software once a measurement is performed. The exact measurement position will be saved in the comment of the .put-raw data file.
 - Change the light-path settings back to "**Plate**", that the PicoQuant detectors are used for the following measurements.

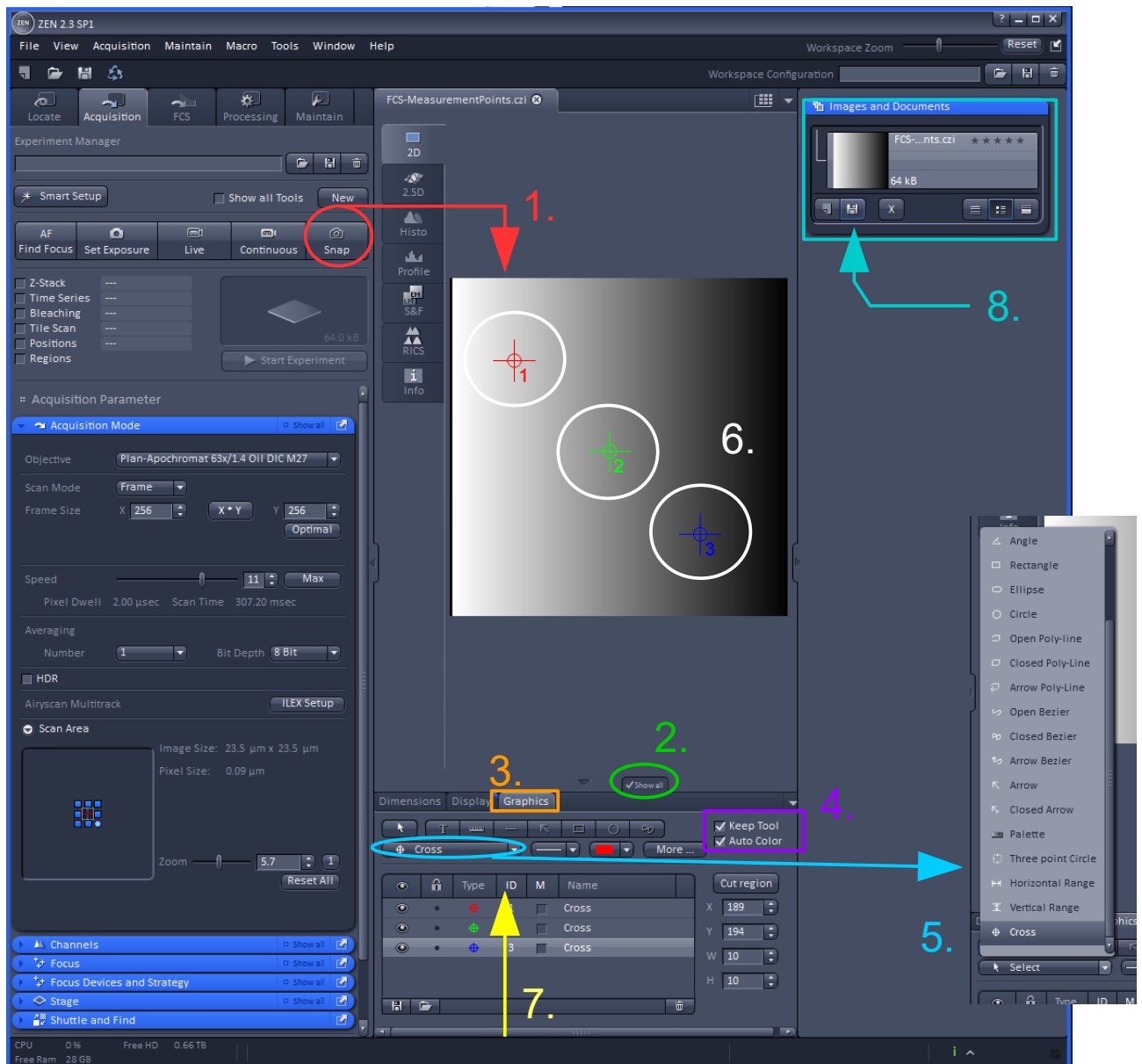
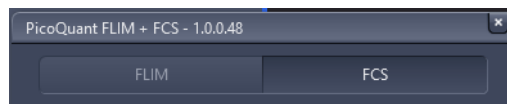


Fig. 2.17: Detailed description on how to define different measurement positions for FCS measurements at a specific sample

3. **PicoQuant FLIM + FCS tool:** Switch to the FCS measurement tab in the PicQuant FLIM + FCS software tool of the ZEN software. When ticking the “Positions” box it is possible to load all previously defined measurement positions via “From Overlay” in the “Positions” tab, see green box of Fig. 2.18. It is possible to delete some of the positions individually.



4. **PicQuant FLIM + FCS tool:** select a certain measurement time at “stop after Elapsed Time” or choose “Time series” and define the number of repetition cycles, the measurement time per point and the delay time in-between two measurements (between two positions or two cycles)

Insert the file name and all additional informations into the “File Name” and “Comment” field and start a measurement by clicking on “Measurement”.

Note: When performing a “Test” - measurement the recorded data is **not** saved in the SymPhoTime 64 software as a .ptu file. Therefore it is advisable to use the “Test” measurement option when laser-power and other parameters still need to be optimized. The optimization process is described in Chapter 2.4.4

One Example of a Multi-Point-Time-Series measurement is given in Fig. 2.18.

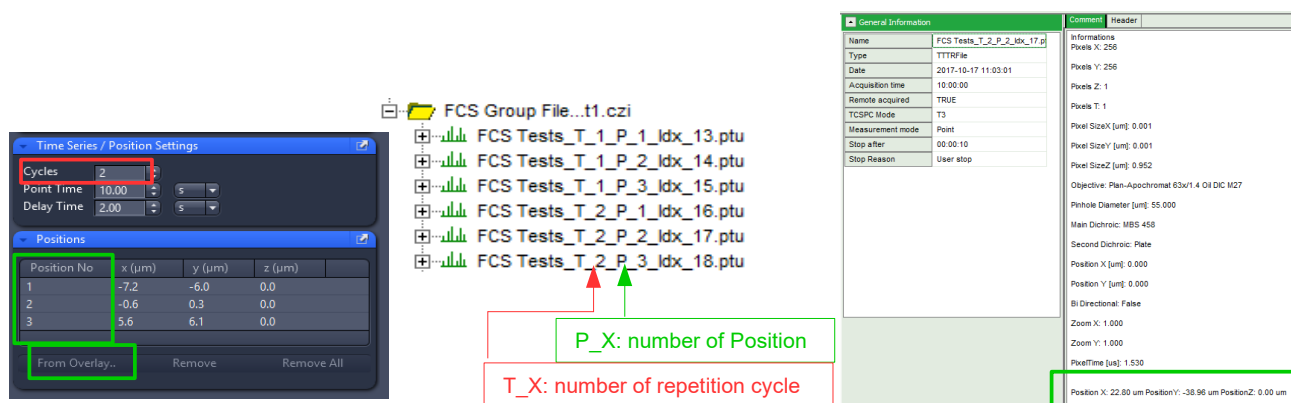


Fig. 2.18: Example of the resulting name in the SymPhoTime 64 software when performing a Time Series of 2 Repetition Cycles with 3 different measurement points. The correct coordinates of the point is saved in the comment of the .ptu file.

2.4.4. FCS data acquisition

- PicoQuant FLIM + FCS tool:** In order to optimize the photon count rate and measurement settings for a FCS measurement start a test-measurement first by clicking on “Test”.
- SymPhoTime 64:** The FCS-curve is calculated during the test-measurement and displayed in the FCS tab, see Fig. 2.19. The display of cross- and the two autocorrelation traces can be selected using the “data Channels” menu above the trace. The update time of the FCS trace can be adjusted with the “Integration Time”. On the right panel, together with the average count rate also the $G(0)$ value and the molecular brightness (“cpm”) are displayed for each channel. The molecular brightness is calculated by $G(0)$ times the average count rate and displays the mean fluorescence count rate per dye molecule. The system is optimized (for example by adjusting the objective collar) when the molecular brightness is maximal. If two detection channels are selected in the preview, a cross-correlation curve is also automatically created. The Time Trace can be observed by switching to the “Time Trace” tab. The changes of the count rate over time can be observed. The binning should be set to 1000 ms in order to observe the photoinduced bleaching of the probe. An optimum between low bleaching rate and good FCS data quality can be found by adjusting the laser power.

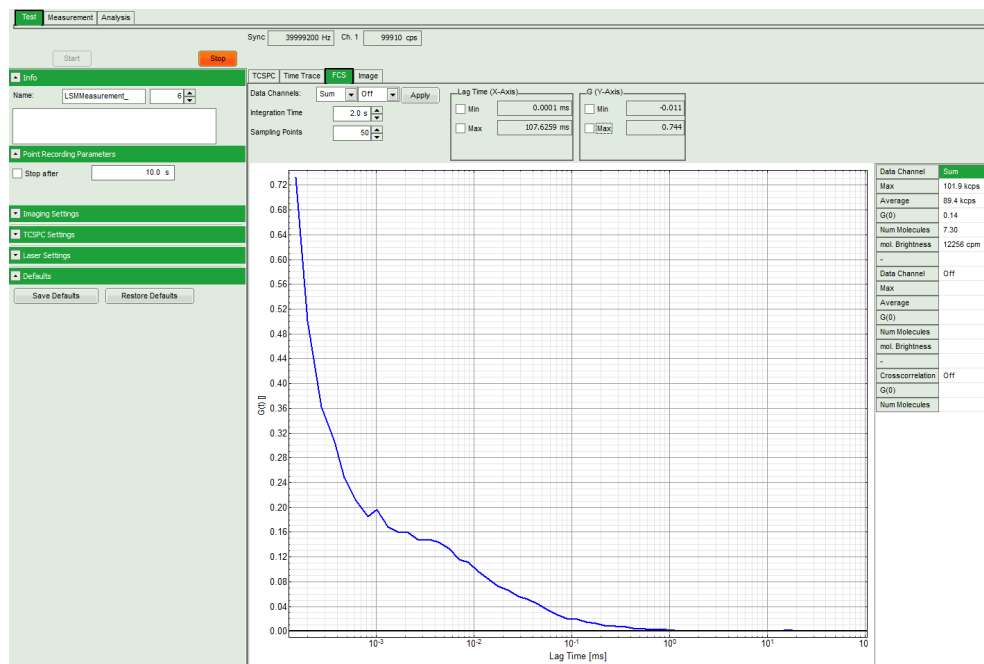


Fig. 2.19: Online monitoring of the of the FCS trace and photon count rate in the SymPhoTime 64 preview window. On the right, the average count rate and the molecular brightness are displayed.

3. **SymPhoTime 64:** At **very high count rates**, depending on the used computer, the online FCS calculation can become very slow. In this case use a low refresh rate for the online display, less number of points to be calculated or decrease the concentration.
4. **Optimize the detected photon count rate:**
 - Similar guidelines as described for FLIM-measurements apply. Additionally for certain analysis modes some special requirements are of interest:
 - **Too high laser powers** can lead to artefacts and falsify the fluorescence lifetime analysis. One problem is the fluorescence **saturation** which gives rise to an increase of the confocal detection volume and diffusion time. The second problem is **photo-bleaching** which leads to a shortening of the measured diffusion time. The laser power should thus be adjusted that the signal to noise in the FCS curve is maximal and the measured diffusion time is still not affected. Best is to measure a series of FCS curves with different laser powers to find the optimal setting.
 - Crucial for FCS analysis is a **high countrate per molecule** (molecular brightness). In order to achieve it, a balance between efficient excitation and photo-bleaching has to be found. A lower concentration with higher laser power is preferable, ideal concentrations for FCS span 0.1 - 500 nM.
 - For **analysis of individual molecule transits**, the concentrations need to be sufficiently low, so that individual intensity bursts can be recognized.
 - **Zeiss ZEN - software:** If the sample is sensitive to photo-bleaching, select a different area with similar brightness for optimization of the settings.
 - **Zeiss ZEN - software:** Adjust the countrate by de- or increasing the laser power in the ZEN software.
 - If the measurement is performed with **PicoQuant lasers**, adjust the intensity at the Laser combiner Unit (LCU) or at the scaffold at the single directly coupled laser, respectively, or – if the lasers are attached to a SEPIA II – regulate the intensity in the SymPhoTime 64 - software, see Fig. 2.20

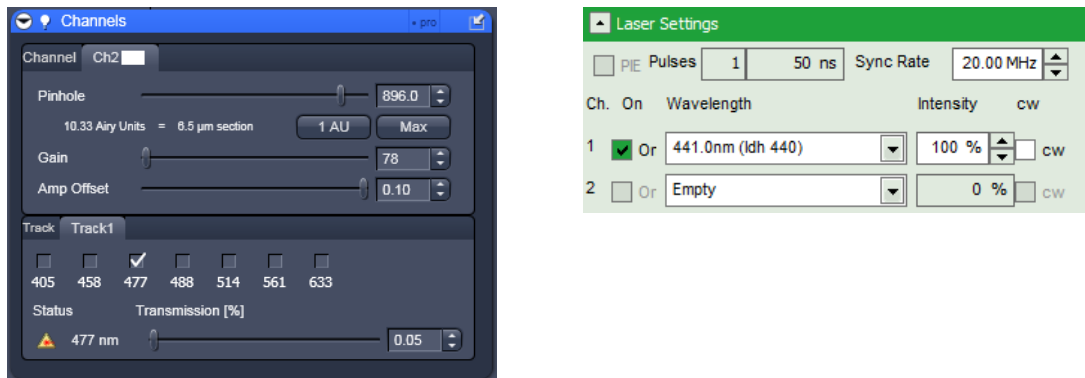


Fig. 2.20: Intensity regulation in the LSM software (left) for pulsed lasers from Zeiss, and in the SymPhoTime 64 software for pulsed diodes from PicoQuant (right). Note that it is preferable to adjust the laser intensity for lasers provided by PicoQuant at the LCU, as changing the intensity in the SymPhoTime software will also change the shape of the laser pulse, while adjusting the intensity at the LCU does not influence this.

5. **PicoQuant FLIM + FCS tool:** after all settings are optimized a measurement can be started by clicking on “Measurement” in the PicoQuant FLIM + FCS software tool. Make sure that all Time Series and Position Settings are set correctly as previously described in Chapter 2.4.3
6. **SymPhoTime 64 software:** The measurement preview is displayed according to the chosen preview settings within the SymPhoTime 64 software e.g. as set in Fig. 2.21. The FCS curve is now integrated over time.
7. **SymPhoTime 64 software:** The data recording stops when the set measurement time is over or by just manually interrupting the data acquisition. Do **not stop** the measurement in the SymPhoTime 64 software but use the PicoQuant FLIM + FCS software tool.

The recorded FCS - file is displayed in the workspace menu on the left and can now be opened for analysis. Therefore select in the “FCS “ drop-down-menu of the “Analysis” – tab the FCS or FLCS analysis. The FCS and FLCS analysis scripts are described in detail in the SymPhoTime 64 online help.

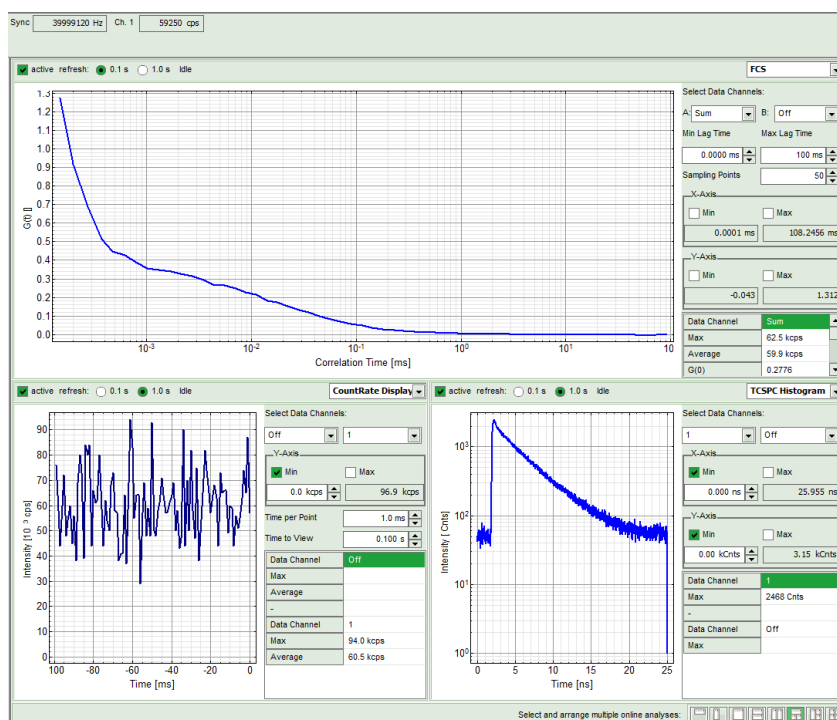


Fig. 2.21: Online Preview of the FCS measurement. In this configuration, the FCS curve is displayed in the upper panel, while the Count Rate is displayed simultaneously on the lower left and the TCSPC histogram on the lower right.

2.4.5. Remarks

- For measuring in biological cells filled primarily with water, a **water immersion objective** with a high NA (1.2) should be used for FCS measurements. Optimize the molecular brightness by adjusting the objective collar while measuring in a dye water mixture of around 20 nM concentration (number of molecules in the confocal volume should be between 1 and 10).
- **FCS measurements** can **only** be performed using **BiG.2**, **SPADs** or **PMA-Hybrid detectors**, as only these detectors are sufficiently sensitive. Using cross - correlation between two detectors allows complete suppression of detector afterpulsing effects on the correlation curve . If just one detector is present and pulsed excitation is used, FLCS can be used in order to suppress detector afterpulsing. This is not necessary for PMA-Hybrids, since these detectors are nearly free from this artefact. Afterpulsing leads to a strong increase of the amplitude of the correlation curve in the μs – time range.
- For a solid FCS-analysis, the **count-rate** has to be **stable**. Signal decrease due to photo-bleaching will result in an offset of the FCS curve. The offset can be fitted in the “Triplet extended” fitting model.
- The **calculation time** of a FCS measurement depends on the **measurement time** and the detector **count rate**. A longer measurement time leads to less noise in the FCS curve.

3. Troubleshooting

In general, make sure that all components of the Zeiss system as well as of the FLIM / FCS upgrade kit are switched on.

3.1. Hardware configuration gets lost or software needs to be installed again

The actual **hardware configuration** of your LSM FLIM / FCS Upgrade (LSM trigger signal configuration, name of the TCSPC device and its settings, number of detection channels, predefined view settings....) is saved in a **settings.pfs** file. Restoring the configuration from the settings.pfs file is also useful, if the software has to be installed again.

- Use “restore factory defaults” in the “settings” main menu bar. Apart from the designed folder location of the SymPhoTime, the original settings of the LSM-FLIM / FCS Upgrade are stored on the external USB memory storage delivered with the instrument.

In case the **hardware equipment is changed**, the settings.pfs file needs to be changed in order to adapt the software to the respective hardware changes. Please contact PicoQuant in this case. As the system is delivered already configured, it is not recommended to change settings without PicoQuant's supervision.

For storing individual user settings (e.g. incl. previews), the software can be started in the user mode and saved via “Settings” / “Save User Settings as”. This creates a *.pus file, which contains the user specific settings. When changing from one user setting to another, the software needs to restart.

3.2. Instrument is loosing sensitivity

Note:

- It is recommended to purchase a **power meter** in the lab in order to measure the laser intensities for selected wavelengths. This is especially for FCS measurements of value since the laser power is a crucial parameter for FCS.

- If you have purchased a LSM FLIM / FCS Upgrade Kit with 1 or 2 SPAD or PMA Hybrid detectors attached, a good way to check the performance of the instrument is the acquisition of **FCS-traces** of a **dye solution** with a dye that can be effectively excited. At a given laser intensity, filters and objective, and distance to the cover slide-surface, the **molecular brightness** is a stable, characteristic value which can be used to monitor the instrument's performance. It is displayed in the FCS-measurement – preview of the test-mode. Suited dyes for different wavelengths are: ATTO655 for 635 nm, Rhodamine 6G for 532 nm and ATTO488 for 485 nm excitation. The molecular brightness is the **count rate** of the **detectors divided** by the **number of molecules** present in the detection volume. It is displayed in the FCS preview. In general, a 10 nM solution is used. For a day to day comparison, use the same laser power, best controlled with a power meter. A more detailed description for performance of FCS measurements is given in the point-measurement section.

If a performance drop is noticed, first the origin of the performance drop needs to be found. A drop in system performance may be caused by:

1) Misaligned Zeiss LSM

- If the in-coupling within the standard LSM is misaligned or the laser performance is dropping, refer to the LSM manual or contact your local LSM representative.

2) Misaligned Detection Unit

- Refer to your detector manual or contact PicoQuant.

3) Misaligned Pinhole

- One crucial parameter of the Zeiss LSM system is the pinhole alignment. It can be monitored by optimizing the countrate of a high concentrated dye solution (e.g. ATTO 488) at a concentration of around 10 μM . Optimize the pinhole for the highest countrate. For the pinhole setting read your Zeiss LSM manual. Refer also to chapter 3.4.

4) Decreasing Laser Performance of lasers delivered by PicoQuant

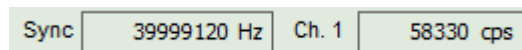
- Check the **laser intensity** with a laser power meter. If the laser output from PicoQuant lasers is too low, either the LCU has to be readjusted or the laser needs to be repaired or replaced. Please read the manual of the laser(s) and LCU or contact PicoQuant.

3.3. No image is displayed during measurement

Can laser light be seen over the objective during measurement?

If no:

- Is the pulsed laser activated and switched on?
- Is the **pulse repetition rate** correct? The Pulse frequency is displayed in a control window over the preview windows.



When starting a TCSPC measurement in the test mode, dark counts should be displayed (see Fig. 2.13). A peak must not necessarily appear, but at least background noise should be displayed. If the repetition rate is 0, check whether at the PDL800B the SYNC is set to internal or, if you use a SEPIA II, the pulse settings in the “Laser Settings” drop-down menu are correct.

Note that **pulsed diode lasers** from **Zeiss** only generate a Trigger signal, when they are selected and the measurement is running!

If yes:

- Do you **see dark counts** when activating the **Time trace Tab** in the “Preview” window? If not, check whether the detectors are still on (the DSN102 (if applicable) shuts down a detector automatically if the count rate exceeds a limiting count rate).
- Is a pulsed laser activated (for Zeiss diode lasers, this is necessary for providing a SYNC-signal)?
- Check, whether **correct emission filters** are placed. If you have a 2 - Detector unit, make sure that the beam splitter is set to the correct position. Check if the filter is placed firmly in the filter holder.
- When all detectors are on and background counts are displayed in the TCSPC window, place a **fluorescent sample** on the Zeiss microscope, set all settings in the Zeiss software for FLIM and start scanning, with the “Time Trace” measurement in the “Test”-tab window running. An **increase in the count rate** should be observed, especially when the laser intensity is increased during the scan. If the count rate does not increase, make sure that the **shutters** in front of the detectors are not closed (i.e. the top of the 2 channel detector box should be placed correctly, all filters should sit in the filter holder correctly). Shutters should click softly when opening and closing.
- Please make sure that the optical fiber to the PicoQuant detector(s) is connected to the scan head.
- If the shutter is not the problem, take a FLIM image as described in the acquire a FLIM image section. Although no counts are displayed, a .ptu file is generated. **Calculate a time trace** from your FLIM - *.ptu - file by choosing the “*Intensity Time Trace*” analysis in the time-trace analysis drop-down menu (only available if you have a full software licence including point analysis). The displayed time trace should contain the photon counts. Select “*marker 1 - 3*” to show **line start, line stop and frame markers** that are passed from the instrument. You may have to select a suited display range in order to display the marker signals. If no marker signals are present, check cable connection between the Zeiss LSM and the LSM Upgrade Kit. Also try to start the LSM and the PicoQuant system new. If no marker signals are displayed in spite of a present connection, contact PicoQuant. If you don't have the Point Measurement Mode included in your license, check cable connections and send the file to PicoQuant.

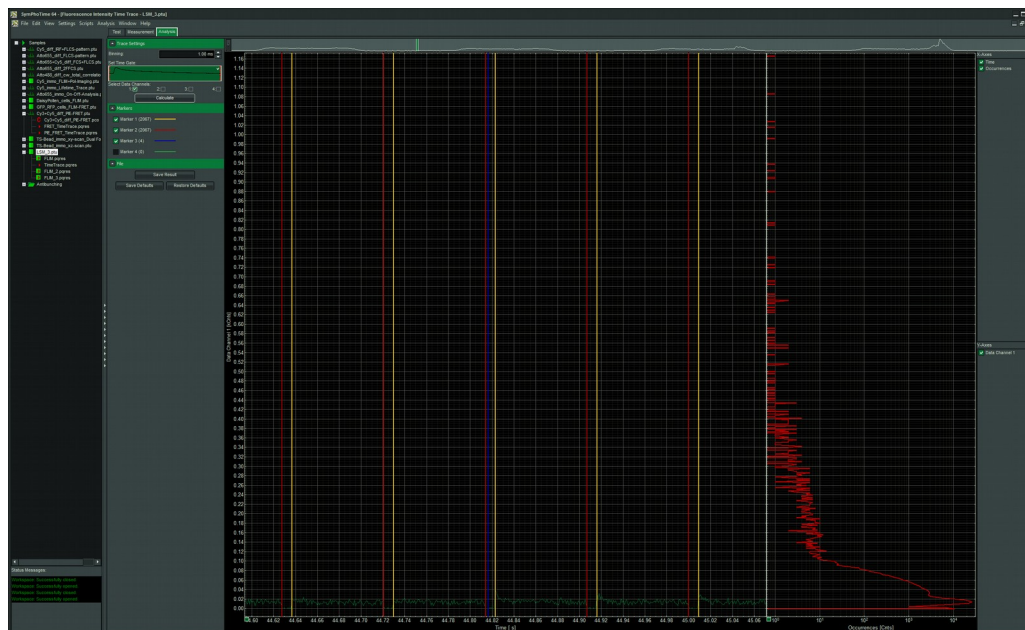


Fig. 3.1: The Time Trace analysis, where the line and frame - markers can be displayed.

3.4. Image looks blurry, or sensitivity is low – how to align the Pinhole

- The pinhole settings are crucial for image quality and results of FCS measurements
- If the Pinhole is:
 1. Completely open:
 - => the image resolution is not optimal, but the sensitivity is highest.
 - => In FCS the confocal volume is maximal, but the shape of the confocal volume will deviate from the assumed model and fits may be poor. Also, the non-correlated background will be highest.
 2. Small but at wrong position:
 - => sensitivity will be poor in FCS and in imaging applications.
- Optimal Pinhole settings depend on the wavelength used.
- To align the Pinhole, put a bright fluorescent sample on the microscope (e.g. a dye solution of around 10 μM of concentration) and focus into the solution.
- Adjust the Pinhole via the “Maintenance” menu of the Zeiss ZEN Software (for Details, see the Zeiss ZEN LSM manual):
 1. Go to Pinhole adjustment settings. If the sensitivity of the instrument is too low, open the pinhole completely. Does the intensity increase significantly? Set the desired pinhole diameter to 1 Airy unit. Note that cw lasers controlled by Zeiss are adjusted using the pinhole settings for lasers from the “VIS”-port. For pulsed diode lasers from Zeiss or PicoQuant, TiSa-lasers or the InTune-laser, adjust the settings for lasers from the “InVIS”-port.
 2. Start a FCS measurement by clicking “Test” and open a time trace analysis in the SymPhoTime 64 software. Set the scale to linear display (check-box “Log” should be empty).
 3. Move the “Position X”, until the signal has maximally increased and repeat the same procedure for “Position Y”. Now, the Pinhole is optimally aligned.
 4. Save the settings.

4. Recommended literature

Publications related to the LSM FLIM / FCS upgrade hardware, software, and underlying key technologies:

Wahl M., Koberling F., Patting M., Rahn H., Erdmann R.: *Time-resolved confocal fluorescence imaging and spectroscopy system with single molecule sensitivity and sub-micrometer resolution*. Current Pharmaceutical Biotechnology, Vol.05, p.299-308 (2004)

Koberling F., Wahl M., Patting M., Rahn H.-J., Kapusta P., Erdmann R.: *Two-channel fluorescence lifetime microscope with two colour laser excitation, single-molecule sensitivity, and submicrometer resolution*. Proceedings of SPIE, Vol.5143, p.181-192 (2003)

Ortmann U., Dertinger T., Wahl M., Rahn H., Patting M., Erdmann R.: *Compact TCSPC upgrade package for laser scanning microscopes based on 375 to 470 nm picosecond diode lasers* Proceedings of SPIE, Vol.5325, p.179 (2004)

Benda A., Hof. M., Wahl M., Patting M., Erdmann R., Kapusta P.: *TCSPC upgrade of a confocal FCS microscope*. Review of Scientific Instruments, Vol.76, 033106 (2005)

Further literature can be found in the help menu of the SymPhoTime software.

The following application and technical notes are available from PicoQuant upon request:

Buschmann V., Bleckmann A., Bülter A., Krämer B., Nikolaus J., Schwarzer R., Korte T., Ziomkowska J., Herrmann A., Koberling F.: *Polarization Extention Unit for LSM Upgrade Kits*

Koberling F., Schuler B.: *FRET analysis of freely diffusing molecules using the MicroTime 200*

Krämer B., Koberling F.: *Lifetime based hydrophobicity analysis of hepatocytes using the MicroTime 200*

Krämer B., Koberling F., Tannert A., Korte T., Hermann A.: *Lifetime based analysis of lipid organization in hepatocytes using the MicroTime 200*

Orthaus S., Buschmann V., Bülter A., Fore S., König M., Erdmann R.: *Quantitative in vivo imaging of molecular distances using FLIM-FRET*

Wahl M.: *Time-correlated single photon counting in fluorescence lifetime analysis*

Wahl M.: *Time tagged time resolved fluorescence data collection*

Numerous measurement examples are published on the PicoQuant website. Please visit the LSM FLIM / FCS Upgrade section of http://www.picoquant.com/_systems.htm.

5. Abbreviations

BNC	British Naval Connector or Bayonet Nut Connector or Bayonet Neill Concelman
CCD	Charge-Coupled Device
CFD	Constant Fraction Discriminator
cps	Counts per Second
cw	Continuous wave (not pulsed)
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
LCU	Laser Combining Unit
LED	Light Emitting Diode
LSM	Laser Scanning Microscope
MCS	Multichannel Scaling
OD	Optical Density
PC	Personal Computer
PCI	Peripheral Component Interface
PIE	Pulsed Interleaved Excitation
PMT	Photomultiplier Tube
RGB	Red-Green-Blue (colour scheme)
ROI	Region of Interest
SMA	SubMiniature version A (connector type)
SMD	Single Molecule Detection
SPAD	Single Photon Avalanche Diode
SYNC	Synchronization (signal)
TCSPC	Time-Correlated Single Photon Counting
TTL	Transistor-Transistor Logic
TTTR	Time-Tagged Time-Resolved
TCSPC	Time-correlated Single Photon Counting

6. Support and Warranty

If you observe any errors or bugs, please try to find a reproducible error situation. E-mail a detailed description of the problem and relevant circumstances to **info@picoquant.com**. In case of software problems, attach the log file of the measurement. The log - file to any measurement can be generated under *Help -> About → Get Support* in the SymPhoTime 64 Software. Store the generated text as a textfile and mail it to PicoQuant. Your feedback will help us to improve the product and documentation.


Questions about the software can also be pasted to the SymPhoTime user forum (see <http://forum.picoquant.com/>).

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

Of course, we also appreciate good news: If you have obtained exciting results with the LSM FLIM / FCS Upgrade or published scientific papers, we would also like to know! Please send us 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>. 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.

Thank you very much in advance for your kind cooperation!

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<p>Retraction of old devices</p> <p>Waste electrical products must not be disposed of with household waste. This equipment should be taken to your local recycling centre for safe treatment.</p> <p>WEEE-Reg.-Nr. DE 96457402</p>	
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