LSM Upgrade Kit

Compact FLIM Upgrade Kit for
Zeiss LSM 980
Laser Scanning Microscopes

PicoQuant

User's Hardware Manual and Technical Data

Version 1.5

with PicoQuant Software Integration

Table of Contents

1. Introduction

This manual describes the basic procedures to acquire FLIM - images 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.
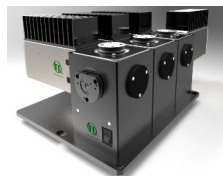

Fig. 1.2: Detection Unit for LSM FLIM / FCS Upgrades:
Multi channel PMA and SPAD detection unit

Fig. 1.3: MultiHarp150 and TimeHarp260

- **MultiHarp150 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.

- **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. 1.4: 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 - Manual):** Here you find all information about the software installation. The SymPhoTime software contains a detailed, context sensitive online help function. Press F1 for accessing the online help. When placing the cursor into a number field and pressing F1, the help in the context of the field is opened.

- **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.

- **System Specifications:** The System Specifications 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. General safety information

 CAUTION! Before using this device, make sure that you have read and understood the content of this user manual. Store this documentation in a safe and easily accessible place for future reference.

Incorrect handling of this product may result in personal injury or physical damage. The manufacturer assumes no responsibility and cannot be held liable for any injury / damages resulting from operating the device outside of the normal usage defined in this manual.

2.1. Warning Symbols and Conventions

The following symbols and conventions will be used throughout this manual. Please take time to familiarize yourself with their meaning before proceeding.

	The **general safety alert symbol** is used to alert you to hazards that may lead to personal injury or physical damage. Follow all associated safety instructions to avoid possible injury or death.
	A **high voltage warning symbol** is used to indicate the presence of un-insulated, dangerous voltage inside the enclosure. Note that this voltage may be sufficient to constitute a risk of shock.
	The **laser radiation warning symbol** alerts you that the device can generate laser radiation. Follow all applicable laser safety instructions to avoid injury or damages.
	The device's susceptibility to electrostatic discharge (ESD) is indicated by the **ESD warning symbol**. Ensure that you follow proper ESD protection rules to avoid damaging the device.
CAUTION!	Make sure to follow any instructions prefaced with "**CAUTION!**" to avoid personal injury or damaging the device.
WARNING!	The "**WARNING!**" label prefaces any instructions that have to be followed to avoid sever injury or death.
NOTICE	Important tips and information for device operation that do not include a risk of injury or damage are prefaced with the "*NOTICE*" label.
	This symbol indicates that an earth terminal needs to be connected to the ground (to avoid risks of electrical shock).

2.2. Safety Instructions

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.

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

2.3. Laser Safety Instructions

WARNING! **Visible and invisible laser radiation**

The LSM FLIM Upgrade may be equipped with one or more pulsed diode lasers. To avoid hazardous radia-tion exposure you should carefully obey the safety instructions that are provided with your Laser Coupling Unit (LCU) and Multi Detection Unit (MDU) operation manual.

If your instrument uses another excitation system, follow the safety instructions of the relevant manual. **Pulsed diode lasers can emit laser light of up to class 3B / IIIB.** Please refer to the labels affixed to the LCU and MDU.

Laser class 3B / IIIb require that special laser safety glasses are worn. The installation room of the LSM Upgrade Kit must be labeled as laser area.

Lasers can be hazardous and have unique safety requirements. Permanent eye injury and blindness are pos-sible if lasers are used incorrectly. Pay close attention to each safety related CAUTION and WARNING state-ment in the user manual. Read all instructions carefully BEFORE operating this device.

Required Laser Safety Measures

Please observe the laser safety measures for class 3b / IIIb lasers in accordance with applicable national and federal regulations. The owner / operator is responsible for observing the laser safety regulations.

What does the owner / operator have to observe?

- The owner / operator of this product is responsible for proper and safe operation and for following all applicable safety regulations.

- The owner / operator is fully liable for all consequences resulting from the use of the laser for any purposes other than those listed in the operating manual. The laser may be operated only by persons who have been instructed in the use of this laser and the potential hazards of laser radiation.

- The owner / operator is responsible for performing and monitoring suitable safety measures (accord-ing to IEC/EN 60825-1 and the corresponding national regulations).

- The owner / operator is also responsible for naming a laser safety officer or a laser protection adviser (according to the standard IEC/EN 60825-1: "Safety of laser products, Part 1: Classification of sys-tems, requirements and user guidelines" and the respective national regulations).

- When using lasers of **class 3B / IIIB**, it is required to wear special eye protection (laser safety gog-gles).
- The room in which the LSM Upgrade Kit is installed must be labeled as a laser area.

The following security instructions must be followed at all times.

General Safety Instructions for Operation

- Never look directly into a laser beam or a reflection of the laser beam. Avoid all contact with the laser beam.

- Do not introduce any reflective objects into the laser beam path.

- Every person involved with the installation and operation of this device has to:

    ○ Be qualified

    ○ Follow the instructions of this manual

- As it is impossible to anticipate every potential hazard, please be careful and apply common sense when operating the laser diode heads and associated driver unit. Observe all safety precautions relevant to Class 3B / IIIB lasers

- Use of controls or adjustments or performance of procedures other than those specified herein may result in hazardous radiation exposure.

- The laser power levels accessible if the unit is opened can cause instant blindness, skin burns and fires. Class 3B / IIIb lasers can present a major hazard through exposure to the direct (intra-beam) or reflected (specular or diffuse) laser beams when the laser is inadvertently "on" and there is a direct line-of-sight path to the laser beam or its reflection. If your instrument uses another excitation system, follow the safety instructions of the relevant manual.

- Never remove the optical fiber or light guide from the system when the lasers are powered.

- Do not disable the interlocks on the Multi Detection Unit.

3. Operation

3.1. 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 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 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 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 measurements on the system.

8. If you use a PDL 828 "Sepia II" laser driver, you can set the laser settings in the ZENPicoQuant Application 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 (SymPhoTime)** and **experiment settings (ZEN)**

    The Upgrade Kit is now ready to use.

3.2. 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.

3.2.1. Starting Point

1. **Zeiss LSM:** Choose and 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 Upgrade are already running. For details about powering on see chapter 3.1.

3. **SymPhoTime software:** A workspace has been loaded or created. The workspace defines where the data should be stored to.

3.2.2. 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. It can be set in the Zeiss PicoQuant application under the section "Laser Settings" or by using a manual laser driver at the driver itself. 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 3.2.8.

Zeiss LSM: Select the correct pulsed laser line. If pulsed lasers provided by PicoQuant are present, note that they are guided through the invisible laser port of the Zeiss LSM. In order to activate them (independent from the line you use), the 440 – laser line in the Zen software must be checked. This is automatically done by the Zeiss PicoQuant application at each start of a measurement.
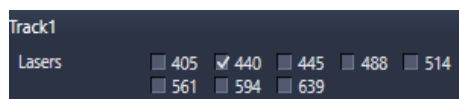
Zeiss LSM: If pulsed lasers provided by PicoQuant are present, it is also necessary to choose a suited MBS (mean beam splitter) at the invisible laser port. The MBS at the visible laser port should be set to "Plate".

3.2.3. 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 equiped with external PicoQuant detectors, guide the beam to the fiber exit port by selecting "Plate" (see red arrow in Fig. 3.1). Also if you have the PicoQuant Upgrade using Zeiss BiG.2 detectors, configure the beam path selecting "Plate". In the "Mirror"-position the light is guided onto the internal detectors.
   If you are working with two-photon excitation (NLO) and are using a PMT from PicoQuant connected to the NDD port, guide the light to the NDD detector position.
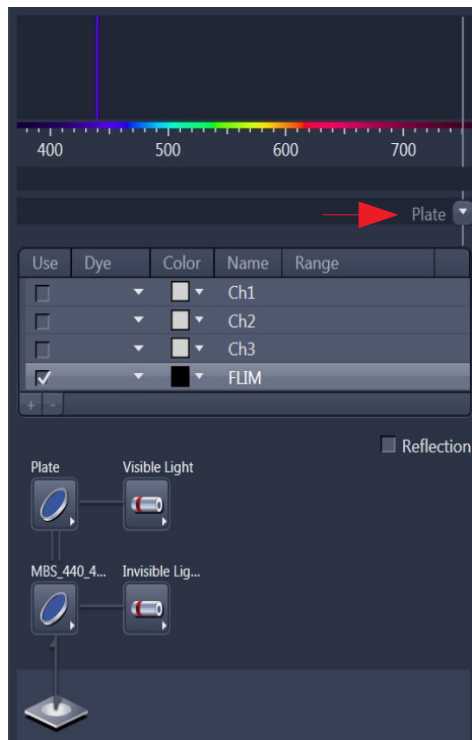
Fig. 3.1: Light path and channel control window of the LSM software: For FLIM data acquisition take care of the correct excitation MBS in the Invisible Light Path and guide the light to the external detectors by switching to "Plate" (see red arrow).

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, please check that the beam splitters and filters distribute the emission light to the detectors correctly.

3.2.4. PicoQuant laser control in ZEN PicoQuant application (optional)

It is possible to control the PicoQuant Laser driver settings (PDL 828) via ZEN software using the laser control.

1. Open the "Laser Settings" window in ZEN PicoQuant application. This is only available if the system is equipped with a Sepia laser controller (PDL828).
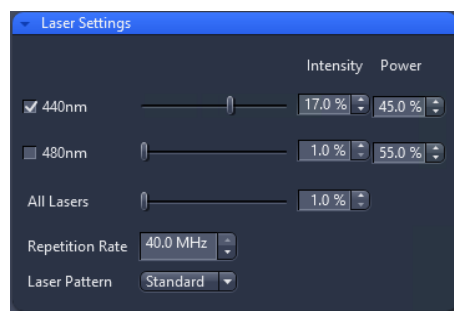
Fig. 3.2 Settings for PicoQuant pulsed lasers: Lasers can be switched on/off with the checkbox and the intensity can be changed via the sliders over two orders of magnitue. The electrical power can be set, it alters the pulse width as well as power. Standard setting is smallest pulse width. In addition, the repetition rate and the laser pattern can be selected. **Standard:** all lasers are pulsing at the same time. **PIE:** Pulsed Interleaved Excitation, the lasers of different wavelengths pulse one after another.

2. the appearance and features of the **Laser Settings** window depend on the LCU-Type that is used.

A) **LCU without motorization (manual attenuation of lasers)**:

The sliders are greyed out, change of intensity of the lasers is only possible with the electrical "Power" setting or manually at the laser or laser combining unit LCU.

B) **LCU with motorized attenuators (AMOT LCU)**:
In case that you use the system with motorized attenuators in the LCU it is possible to adjust the intensity of the lasers during a "Test" measurement using the sliders shown in Fig. 3.2 on the right side. The electrical laser power can be set to a certain value in the field named "Power". Factory preset are settings which correspond to the laser power that leads to the smallest laser pulse.

The laser intensity scale of the sliders for controlling the attenuation of the lasers within the software only displays approximate intensity values.

3. It is possible to select or deselect the lasers by clicking on the checkbox next to the displayed Laser wavelength. Furthermore the **Pulse Mode** as well as the **Pulse Rate** can be set. All the settings are transferred to SymPhoTime as soon as a measurement is started or during a "Test" measurement. (see Fig. 3.2)

Laser Pattern **PIE** refers to Pulsed Interleaved Excitation. In this mode the lasers of different wavelengths are pulsing one after another. In analysis, the excitation wavelength is chosen by the selection of the emission time window.
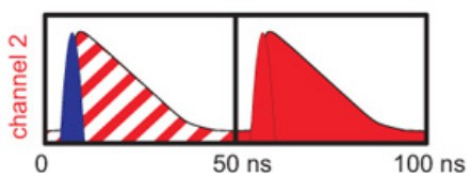

Fig. 3.3: Pulsed Interleaved Excitation (PIE). The excitation wavelengths (blue or red) is set by selecting the corresponding emission window at the TCSPC histogram.

Please note that there is a special feature of the PicoQuant PDL regarding the Pulse Mode **"PIE-Pattern"**:

A) The given **Repetition Rate** of the **laser setting** describes the pulse rate of all laser pulses of different wavelengths together, while the resulting Sync Rate displayed in the SymPhoTime shows the Sync-Rate of the generated Pulse Pattern. For example when using 3 Lasers with a Pulse Rate of 10MHz the distance between each Laser Pulse is 100ns. The Resulting Sync is one third, i.e. 3.33 MHz.

B) Once for example 3 Lasers are selected in PIE-Pattern Mode with a Pulse Rate of 10MHz and a measurement is started, the Pulse Pattern (Sync 3,33MHz) will remain constant even in case one or two lasers are deselected. This feature is useful in order to set the correct laser power for every single laser pulse within the Pulse Pattern. If the Pattern should be changed to for example 2 Laser wavelengths only (Pulse Rate 10MHz, Sync 5MHz) it is necessary to deselect all Lasers, start and stop a measurement and after that select the two laser pulses that should be used.

3.2.5. Set suitable scan parameters

1. In the **Zeiss ZEN - software**, set the correct scan pattern:

    - Use a **mono-directional scan** as indicated in Fig. 3.4.

    - **Recommended settings:** "Frame" scan, Mono-directional scan (→), Line Step equal to 1, Averaging "None". Scan Speed setting of "7" is a good start, however FLIM imaging works also at higher or lower speeds.
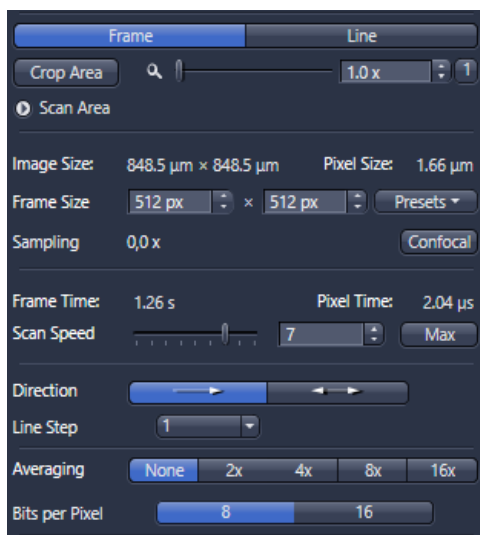
Fig. 3.4: LSM scanning settings in the LSM software: For FLIM use "Frame" scan, Mono-directional scan (→), Line Step equal to 1, Averaging "None" and Scan Speed setting of 7.

3.2.6. Zeiss PicoQuant application

With the ZEN PicoQuant Application it is possible to run FLIM measurements, see Figure 3.5.

For the files to be saved in the SymPhoTime 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 software and saved in the raw data file (.ptu) as a comment.

Fig3.5: Zeiss PicoQuant application.
Setttings for FLIM measurements can be set in this application. FLIM test measurements and measurements can be started and stopped.
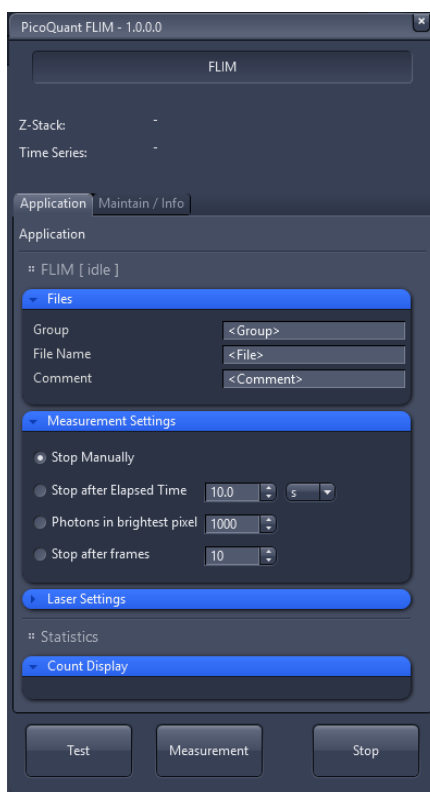
Using the measurement settings of the Zeiss PicoQuant application it is possible to select between:

- **"Files"**

  ○ **Group:** after typing a group name, a windows folder with that name will be opened at the SymPhoTime workspace.

  ○ **File:** all files will be named in SymPhoTime with the name entered in this field

  ○ **Comment:** additional experiment information can be entered here which will be stored with the *.ptu file. The content can be displayed in SymPhoTime by a double-click at the *.ptu file or by using "File → show comment".

- **"Measurement Settings"**

  ○ **Stop Manually:** The measurement is running as long it is not stopped by clicking on "Stop".

  ○ **Stop after Elapsed Time / Photons in Brightest Pixel / Stop after Frames:**
  These settings can be used when it is necessary to measure for a certain time or to collect a specific number of photons or frames. The FLIM image, which reaches first the specified number of photons in the brightest pixel will stop the measurement if "Photons in Brightest Pixel" is selected.

3.2.7. Optimize the detected photon count rate

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

Fig. 3.6: To start a test measurement with the
ZEN PicoQuant application click on "Test"

4. **SymPhoTime software:** The test measurement preview is displayed, together with a time trace (Fig. 3.7). Make sure that the Peak Count Rate (marked in red) is below the values given in the table at Fig. 3.7 The Peak Count Rate is determined from the maximum in the time trace. The "Time trace binning" should be set to 0.01 ms. 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 classical pile-up limit. The intensity should always remain below this value for as standard TCSPC setup. In case you have a rapidFLIM setup with a low dead time TCSPC unit (MultiHarp 150) and PMA Hybrid detector, count rates as high as 65 Mcps can be measured. In this case the red line can be ignored.
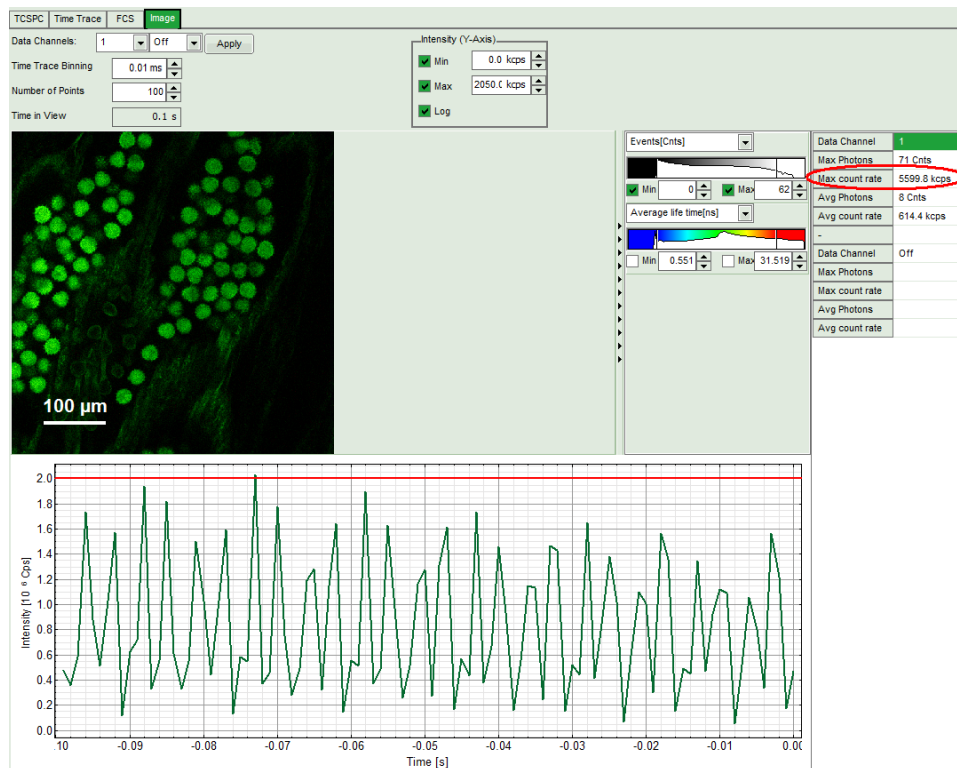
PDL setting (Laser repetition rate)	Upper Count rate limit for different detectors, in [MCounts / s]			
	PMA Hybrid rapidFLIM	PMA PMT, PMA Hybrid	BiG.2	Excelitas SPAD
40 MHz	65	4	4	1
20 MHz	32	2	2	1
10 MHz	16	1	1	1
5,0 MHz	8	0.5	0.5	0.5
2,5 MHz	4	0.25	0.25	0.25

Fig. 3.7: Top: Online monitoring of the of the photon count rate in the SymPhoTime FLIM 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.
Bottom: Count rate limit for different combinations of excitation repetition rates and detectors. rapidFLIM systems can acquire FLIM images at much higher photon count rates.

•   *SymPhoTime software:* The Image **preview settings** for a measurement can be preset, 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. If the pulsed lasers are controlled by a **Sepia laser driver**, the intensity can be set in the Zeiss PicoQuant application (see figure 3.8). Keep in mind that changing the laser intensity via the laser electrical power (Power setting in Zeiss PicoQuant application) does affect the pulse shape of the laser. Setting the power close to the lower laser threshold will ensure the narrowest pulse width, but the intensity will also be lowest. This is the default setting. Note that the % values in the menu refer to the current that drives the laser and do not belong to a linear intensity scale.
  In case laser settings are available at Zeiss PicoQuant application, the "Laser Settings" of the SymPhoTime should not be used.
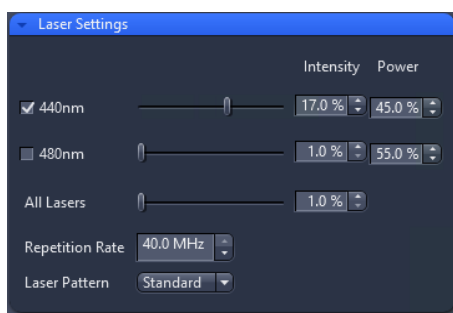

Fig. 3.8 Settings for PicoQuant pulsed lasers: Lasers can be switched on/off with the checkbox and the intensity can be changed via the sliders over two orders of magnitue. The electrical power can be set, it alters the pulse width as well as power. Standard setting is smallest pulse width. In addition, the repetition rate and the laser pattern can be selected. **Standard:** all lasers are pulsing at the same time. **PIE:** Pulsed Interleaved Excitation, the lasers of different wavelengths pulse one after another.

- 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 (classical FLIM setup) or beyond (rapidFLIM setup). 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 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.

3.2.8. 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 laser driver – in the "Laser Settings" drop-down – menu of the Zeiss PicoQuant application. 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. 3.9: Repetition rate can be adjusted in the "Laser Settings" of the Zeiss PicoQuant application

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 "Test" measurement via the Zeiss PicoQuant application. Check the decay behavior of your sample using the TCSPC tab of the Test - window of the SymPhoTime software. Activate the TCSPC Histogram-tab, and monitor the fluorescence decay as shown in Fig. 3.10. At 40 MHz the **time window is optimally adapted** (right panel in Fig. 3.10), while at 20 MHz the fluorescence has already decayed at less than half of the detection window (left in Fig. 3.10). 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.
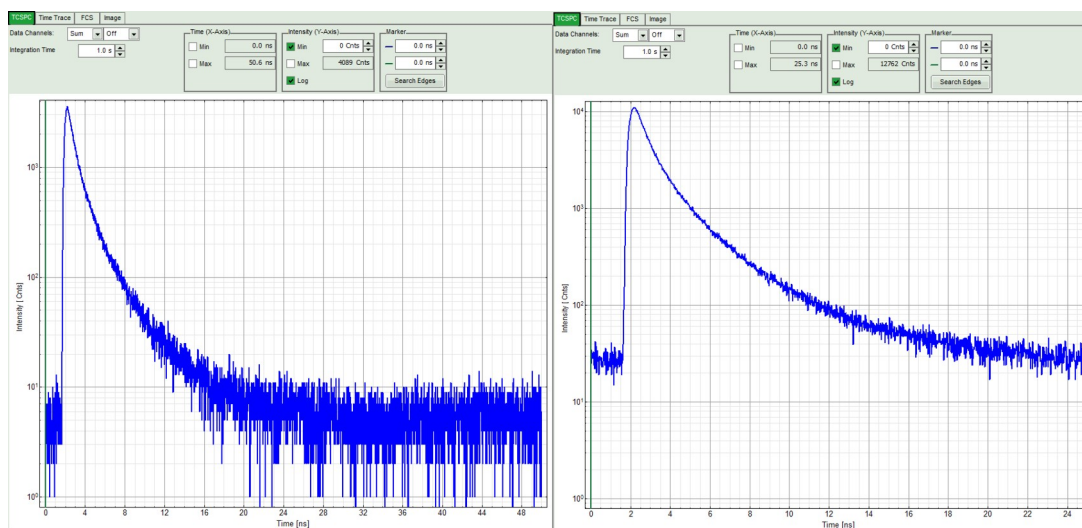

Fig. 3.10: Fluorescence decay at unnecessary low (left, 20 MHz) and optimal (right, 40 MHz) laser repetition rate. With an even higher repetition rate, a part of the fluorescence would appear at the beginning of the TCSPC-window.

3.2.9. Start FLIM data acquisition

1. **Zeiss PicoQuant application:** By using the Zeiss PicoQuant application it is possible to start the FLIM measurement by clicking on "Measurement".

The measurement parameters of the ZEN software (image size, scanning pattern, monodirectional scanning, image dimensions e.g.) are used as in the ZEN software selected. Time series and z-stacks can be selected as well. The measurement parameters are entered automatically in the comment field of the SymPhoTime *.ptu files.
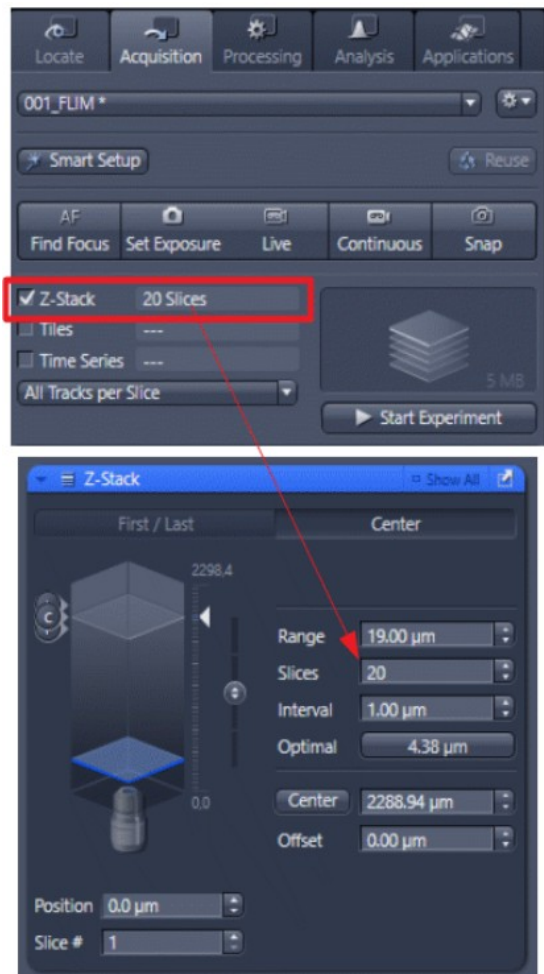

Fig. 3.11: Settings of a z-stack measurement for e.g. 20 slices with an axial distance of 1 µm

2. **SymPhoTime software: During LSM data recording** the chosen **online analysis** with FLIM images, TCSPC-Histograms, countrate-display etc. are displayed, see Fig. 3.12. 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 frame by frame.
Please note that for z-stacks and time series the correct file naming is only activated, if a group and file name is entered under "Files" in the Zeiss PicoQuant application.
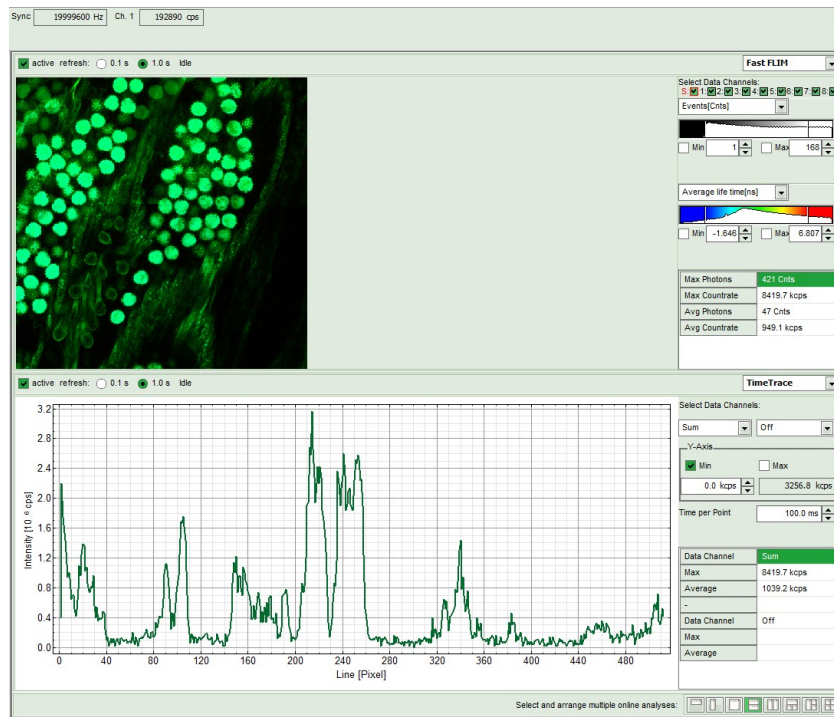
Fig. 3.12: SymPhoTime measurement preview being displayed during LSM data acquisition. The FLIM image is displayed on the upper left. The display parameters are shown on the right. Below, a timetrace of the intensity variations over time is plotted. Due to user set configurations of the SymPhotime software, the screen can look differently.

3. The **LSM scanning process stops** either after reaching the set stop condition, or by just manually interrupting the measurement by clicking on the "Stop" - button of the Zeiss PicoQuant application.

4. **SymPhoTime software:** The recorded raw – data - file (*.ptu) is displayed in the workspace menu together with the result files *.pqres and can now be opened for analysis by double click.

3.2.10. Resulting raw data file and documentation

1. **SymPhoTime 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 date file is stored together with the actual settings and it contains all measurement parameters known by the SymPhoTime software. To access, double click on the raw (".ptu")-file. A Comment field with all informations is opened, see Fig. 3.13.

    •   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 **Zeiss PicoQuant application** of the ZEN software. Furthermore it is possible to repeat the measurement several times by using the "Time Series" option in the ZEN software. 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. 3.13. Additionally also the number of the measurement position is saved in the file name (P_1).
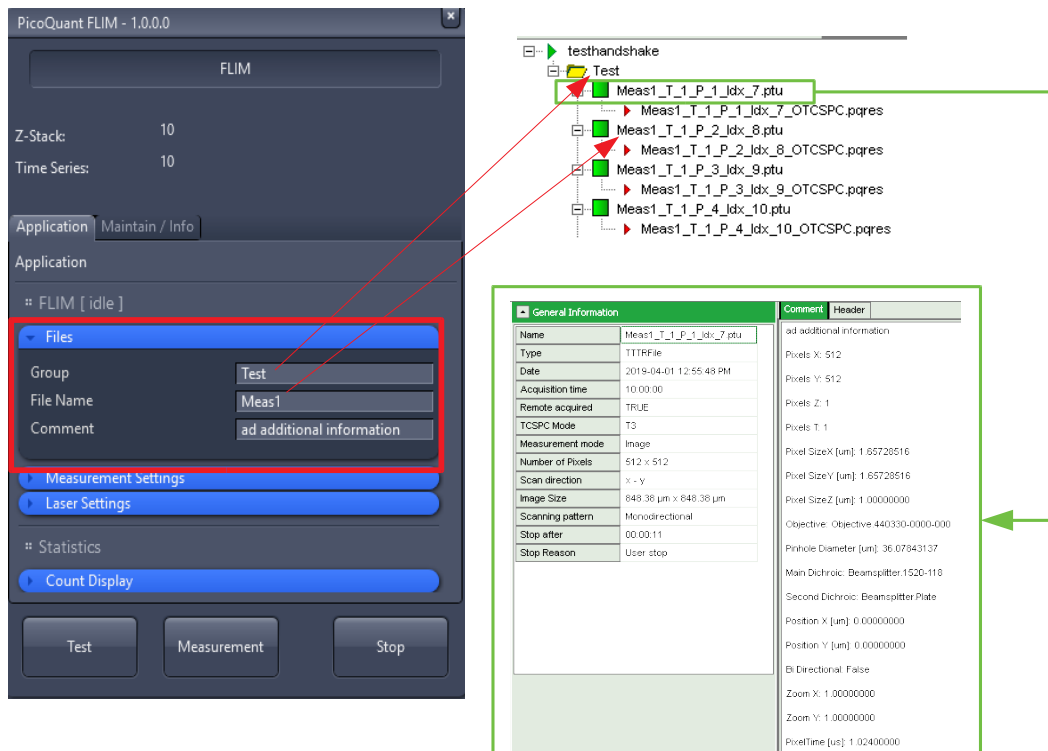
Fig. 3.13: The number of repetition cycles and the position numbers are saved in the file name within the SymPhoTime workspace. Double-clicking on the *.ptu file opens the comment with all informations about the measurement (marked in green)

- **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 software.
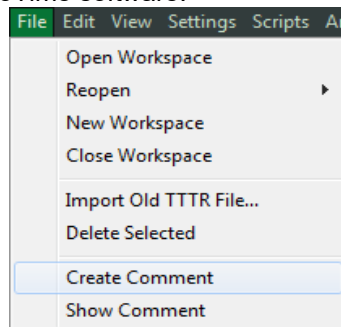
- The **image dimensions** that are used for image size calculations are found in the ZEN "Acqusition Mode" Drop-down menu as "Pixel size" and are automatically transferred to the **SymPho-Time** -software (Fehler: Verweis nicht gefunden)

- **SymPhoTime 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").
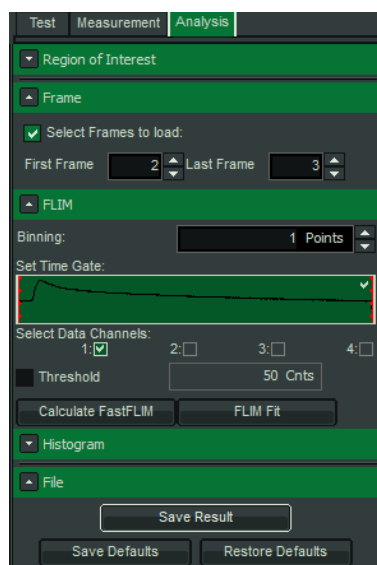
Fig. 3.14: Changing the selection of frame numbers for analysis in the SymPhoTime Software.

3.2.11. 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 by using sample with Urea crystals.

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 Zeiss PicoQuant application.

4. **SymPhoTime 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. 3.15. 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 "Power" setting of the Zeiss PicoQuant application, if a Sepia laser driver is present at the system. Changing the electrical power of the laser changes the shape and temporal position of the laser pulse. However, you can use the driver settings to optimize the pulse shape of the laser for shortest IRF width. The settings should then kept constant for the following FLIM measurements.

   This does not apply to for lasers used for multiphoton excitation, as for those lasers, the pulse width is always negligibly short.
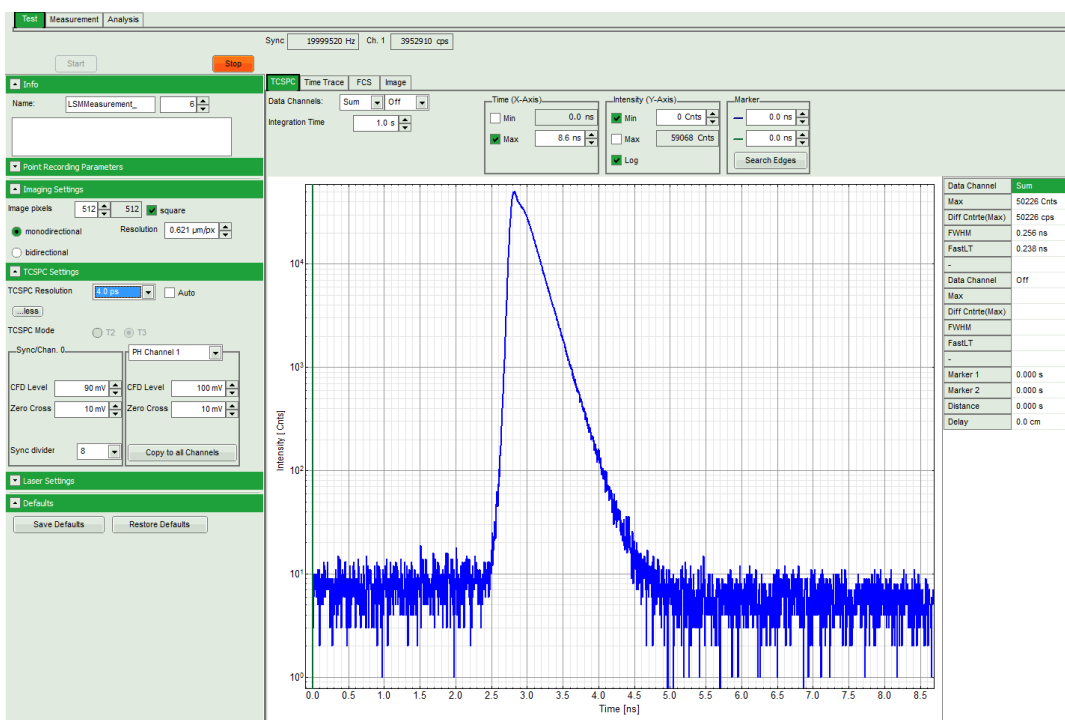
Fig. 3.15: TCSPC-preview window, recorded with a PH300, MPD SPAD and PicoQuant laser source. The time width per channel can be set with the "TCSPC Settings" drop-down menu. The count rate is displayed over the preview graphs for each channel separately.

5. **Zeiss PicoQuant application:** Stop the test measurement by pressing "Stop".
   Step 4 are only for checking the IRF shape and settings, they do not need to be performed routinely.

6. **Zeiss PicoQuant application:** Select Stop after 1000 Photons in the brightest pixel and start a measurement by clicking on "Measurement".

7. When the decay histogram in the TCSPC preview has reached 1000 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 software. It contains the IRF.

8. Replace the IRF solution with your sample and start the sample measurement.

3.2.12. Remarks

- Your LSM allows **storing** defined experiment **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 preset the correct beam path and MBS.
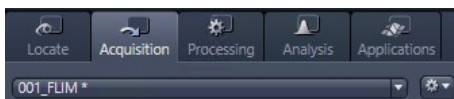

Fig. 3.16: Storing and applying stored configurations is possible in the ZEN software. Clicking on the settings icon in the opens a stored configuration. An actual configuration can be saved.

- 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.

- 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 software allows to perform post-acquisition pixel binning for optimization of the best compromise between lifetime accuracy and image resolution.

4. FCS Measurements

4.1. Choose location and time series for the point measurement

Point measurement at one position:

1. **Zeiss PicoQuant Application:** Select the appropriate laser wavelength and intensity of the PicoQuant laser.

2. **Zeiss LSM software:** Change the light-path settings within the ZEN Software by opening the Image Settings tab. Configure the light path for the PicoQuant laser. The fluorescence light should first be guided to the internal Zeiss detectors by selecting **"Mirror"**. Furthermore it is necessary to adjust the detection wavelength range for the spectral detector.

   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. 4.1.

   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.
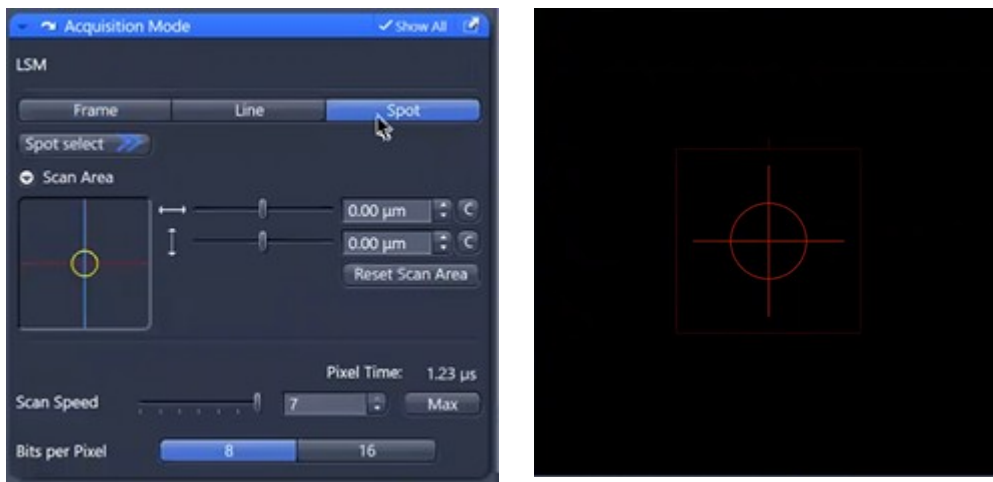

Fig. 4.1:
Switch to "Spot" measurement in Acquisition Mode window.
Selecting the position of the point measurement in the Image window. Clicking "Spot Select" results in scanning one image. Clicking at the desired position inside the recorded image marks the spot to be measured. The scanner moves to that position when the Measurement is started.

3. **ZEN software:** Activate Time Series (compare Fig. 4.2).

Fig. 4.2:
Activate "Time Series" (left image). The measurement duration is set in the "Time Series" window by entering an appropriate number of cycles. Enter the desired number of measurement cycles (right image) to define the measurement duration.

4. **Zeiss LSM software:** Click on "Continous". In this way the laser spot is guided to the selected location inside the sample and the laser light is switched on.

5. **SymPhoTime software:** Select in the Test Mode "TCSPC" and start a test measurement by clicking on "Start". Check that a TCSPC decay is visible and the count rate is appropriate for the FCS measurement (between 5 kcps and 1 Mcps)

6. **SymPhoTime software:** Select in the Test Mode "FCS". An FCS curve should be visible, compare Fig. 4.3.

Fig. 4.3:
FCS curve displayed in the "Test Mode". Count rate and molecular brightness are shown in the small windows which can be activated by double clicking on the respective values at the right side of the FCS curve.

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.

7. 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.

In case the measurement takes place inside a FCS solution, adjust the correction collar of the objective to optimize the molecular brightness. Optimize the laser intensity to obtain a good compromize between high molecular brightness and low photobleaching. Photobleaching can be observed using the "Time Trace" display and can occur if the number of flourophores is limited e.g. inside biological cells.

8. **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.

9. **Optimize** the detected **photon count rate** when measuring inside the final probe:

    1. **Too high laser power** can lead to artefacts and falsify the fluorescence correlation 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.

    2. 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.

    3. For **analysis of individual molecule transits**, the concentrations need to be sufficiently low, so that individual intensity bursts can be recognized.

    4. **Zeiss ZEN - software:** If the sample is sensitive to photo-bleaching, select a different area with similar brightness for optimization of the settings.

5. **Zeiss PicoQuant Application:** Adjust the countrate by de- or increasing the laser power (PicoQuant lasers) or using the **Zeiss ZEN – software** adjust the countrate by de- or increasing the laser power (Zeiss lasers).

4.2. FCS data acquisition

1. **SymPhoTime 64:** after all settings are optimized a measurement can be started by selecting "Measurement", adjusting the measurement time with "Stop after" and clicking on "Start" (Fig. 4.2).

Fig. 4.4: Measurement of the FCS trace and photon count rate in the SymPhoTime 64.

2. **SymPhoTime 64 software:** The measurement preview is displayed according to the chosen preview settings within the SymPhoTime 64 software (compare Fig. 4.5). The FCS curve is now integrated over time.
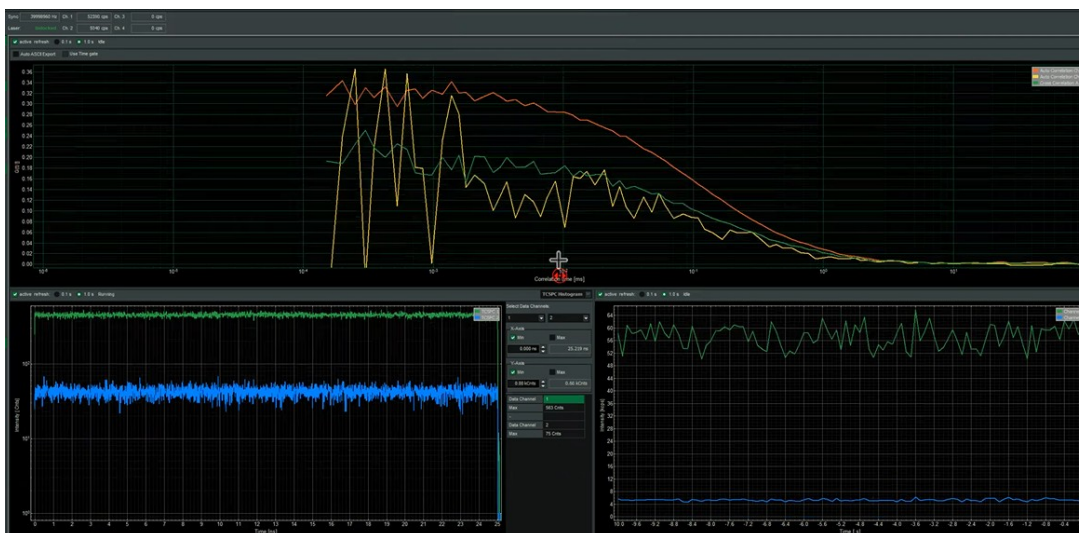

Fig. 4.5: Measurement of the FCS trace and photon count rate in the SymPhoTime 64.

3. **SymPhoTime 64 software:** The data recording stops when the set measurement time is over or by just manually interrupting the data acquisition.

4. 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.

5. Troubleshooting

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

5.1. 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.

Laser settings in SymPhoTime: The entries of the different laser wavelengths should appear in the "Laser Settings" window starting in the upper line with the shortest wavelength up to the longest longest wavelength at the last row.
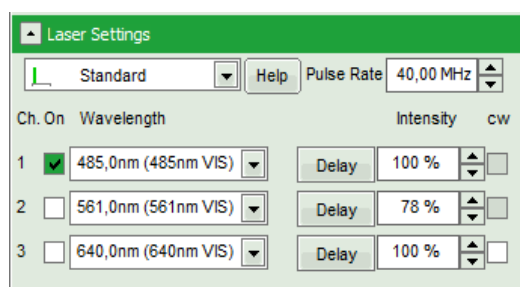
5.2. Instrument is loosing sensitivity

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 so 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 5.4.

**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.

5.3. 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.

Sync	39999120 Hz	Ch. 1	58330 cps
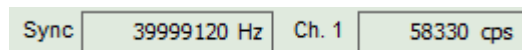
When starting a TCSPC measurement in the test mode, dark counts should be displayed (see Fig. 3.15). A peak must not necessarily appear, but at least background noise should be displayed. If the repetition rate is 0, check whether at the PDL800B the SYNC is set to internal or, if you use a SEPIA II, the pulse settings in the "Laser Settings" drop-down menu are correct.

If yes:

- Do you **see dark counts** when activating the **Time trace Tab** in the "Preview" window? If not, check whether the detectors are switched on.

- Is a pulsed laser activated?

- 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.
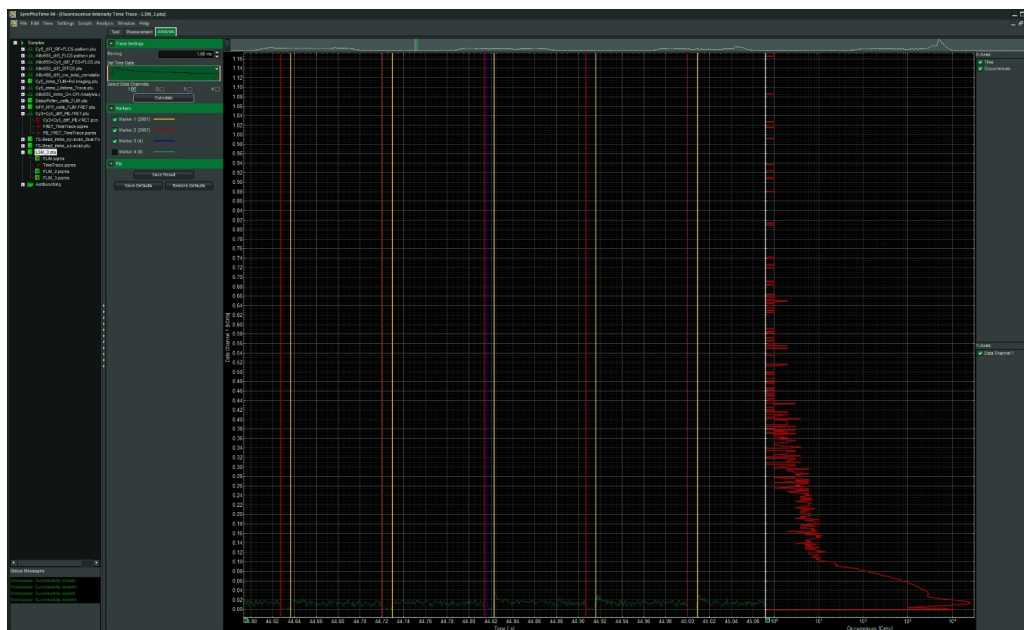
Fig. 5.1: The Time Trace analysis, where the line and frame - markers can be displayed.

5.4. 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.

    2. 1 AU (Airy Unit) but at wrong position:

        => sensitivity will be poor

- Optimal Pinhole settings depend on the MBS used.

- To align the Pinhole, put a bright fluorescent sample on the microscope (e.g. a dye solution of around 100 µM of concentration) and focus into the solution.

- Adjust the Pinhole via the "System Maintenance and Calibration" menu in the "Tools" section of the Zeiss ZEN Software (for Details, see the Zeiss ZEN LSM manual):

    1. Go to Pinhole adjustment settings. 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, PicoQuant or NLO lasers, adjust the settings for lasers from the "Invisible"-port.

    2. Start a FLIM measurement and use the maximum zoom setting. Click "Test" in the "Zeiss PicoQuant application" and open a time trace analysis in the SymPhoTime software. Set the scale to linear display (check-box "Log" should be empty).

    3. Adjust the laser intensity to reach a count rate of 2 Mcps (2 million counts per second).

    4. Go to Tools → "System Maintenance and Calibration". Move the "Position X", until the signal has maximally increased and repeat the same procedure for "Position Y". Now, the Pinhole is optimally aligned.

    5. Save the settings.

6. 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.

Application and technical notes:

Numerous measurement examples, technical and application notes are published on the PicoQuant website. Please visit the LSM FLIM / FCS Upgrade section of http://www.picoquant.com.

Many informations regarding the techniques described in this manual can be found on www.tcspc.com.

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*

7. 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	Continous 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

8. 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 **suport@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 Software. Store the generated text as a textfile and mail it to PicoQuant. Your feedback will help us to improve the product and documentation.

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 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!

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	