LSM Upgrade Kit

Compact FLIM and FCS
Upgrade Kit for **Nikon AX**
Laser Scanning Microscopes
with complete integration

User's Hardware Manual and Technical Data

Version 1.4

Table of Contents

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.

FLIM measurements are fully integrated into the NIS Software. All necessary settings and configurations are made in the NIS software. However, for the data analysis the SymPhoTime can be used. For FCS measurements both softwares, NIS and SymPhoTime are used to aquire the data.

For data analysis please refer to www.tcspc.com. In this website, supported by PicoQuant, useful information in the filed of time-resolved microscopy and spectroscopy is continuosly loaded. This includes step-by-sep tutorials for data analysis with the SymPhoTime software, demonstration videos and technical articles.

For technical or software inquires send an e-mail to support@picoquant.com.
Please refer to the serial number of your system (for help locating the serial number please see here: http://www.picoquant.com/contact/serialnumber)

More detailed explanation about the technical details of the setup 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.

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.

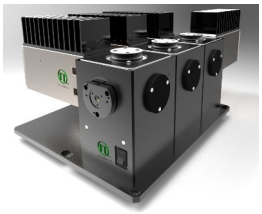


Fig. 1.2: Detection Unit for LSM FLIM / FCS Upgrades: Multi channel PMA / SPAD detection unit

- **MultiHarp 150, HydraHarp and TimeHarp 260 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: MultiHarp150 and TimeHarp 260

- **Laser Driver (Sepia and 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 diode lasers.
- **Software Manual (SymPhoTime 64 - 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 whether additional parts are necessary. In case of doubt, contact a PicoQuant representative.
- **System Specifications:** The System Specifications contains specific information about your individual LSM FLIM / FCS Upgrade, 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 severe 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 country 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 radiation 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 possible if lasers are used incorrectly. Pay close attention to each safety related CAUTION and WARNING statement 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 (according 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 systems, 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 goggles).
- 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 Nikon AX 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 hardware and software should be started in the following order:

1. Start both the PicoQuant LSM Upgrade and enable laser emission by turning the key switch on the laser driver clockwise. Prior to data acquisition, the laser head should be operated for a few minutes to stabilize. Switch the detector unit on.
2. Start the Nikon AX.
3. Start the **NIS** software and click on "AX with PicoQuant" in the selection box.
4. Place correct **fluorescence filters** in the corresponding filter holder position of the external PicoQuant detector unit.
5. In NIS: Select "**FLIM**".
6. If you want to work with user specific settings, load your **optical configuration (NIS)**.
7. If you want to analyze your data in SymPhoTime start **SymPhoTime in Analysis Mode**

   The LSM FLIM & FCS Upgrade is now ready to use.

3.2. Preparing NIS for FLIM

Position the probe under the microscope and perform all steps that you see a nice confocal image. Now change the lightpath to a prestored FLIM position or click on „AX – FLIM&FCS".

Fig. 3.1: Activate FLIM & FCS functionality

Now FLIM can be selected (Fig. 3.2). FLIM is only possible with Galvano monodirectional scanning. Select the scanning velocity (1 - 2 µs is recommended). Averaging should be 1. You can deactivate / activate FLIM detectors by clicking on the respective detector button. “AutoSignal” is not working for FLIM.
Now click on “Experiment Setup…”.

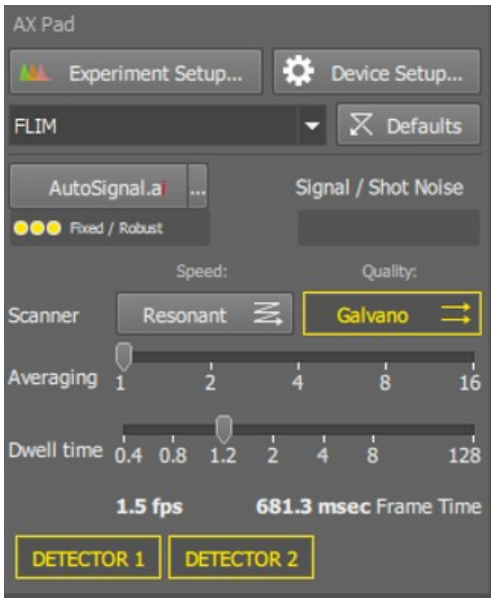


Fig. 3.2 FLIM / FCS selector, Experimental setup and Device Setup can be opened. Scanning speed of 1.2 µs per pixel is recommended for FLIM imaging.

In the **Experimental Setup** several settings can be set (Fig. 3.3):

- TCSPC Device
  - In case the system is equipped with two TCSPC units, they can be selected. Standard setting is the rapidFLIM MultiHarp 150 TCSPC unit.
- Acquisition Settings
  - During “Live” (not Capture), a number of 1 to 3 frames can be averaged to have a noise free FLIM image.
  - The maximum of displayed detection channels can be set.
  - The main dichroic can be selected

PicoQuant Diode Laser	LDH 405	LDH 440	LDH 485	LDH 510	LDH 530	LDH 640
Recommended Nikon AX excitation dichoics	405/488 405/488/561 405/488/561/640 405/488/543/640	457/514	405/488 405/488/ 561/640	20/80	20/80	405/488/561/640 405/488/543/640

Tab. 3.1: Suited dichroic mirrors for using with PicoQuant diode lasers

- Laser port and detector port can be selected. Standard is port 2 for both settings.
  - In case Nikon contineous wave (cw) lasers should be used for FCS, the laser port is number 1.

- Detector Settings
  - The detector channels can be named, e.g. “EGPF”.
  - The main emission wavelength of the dye can be entered.
  - The excitation laser line for the emission channel can be selected. Due to Pulsed Interleaved Excitation (PIE), a crosstalk of another excitation laser line into this emission channel is avoided.
  - Channel range can be checked in case the detection channel should be restricted to a selected range of the TCSPC histogram. The maximum number of time channels of the TCSPC histogram is given by the “Real Channels” indicator, see Fig. 3.4.

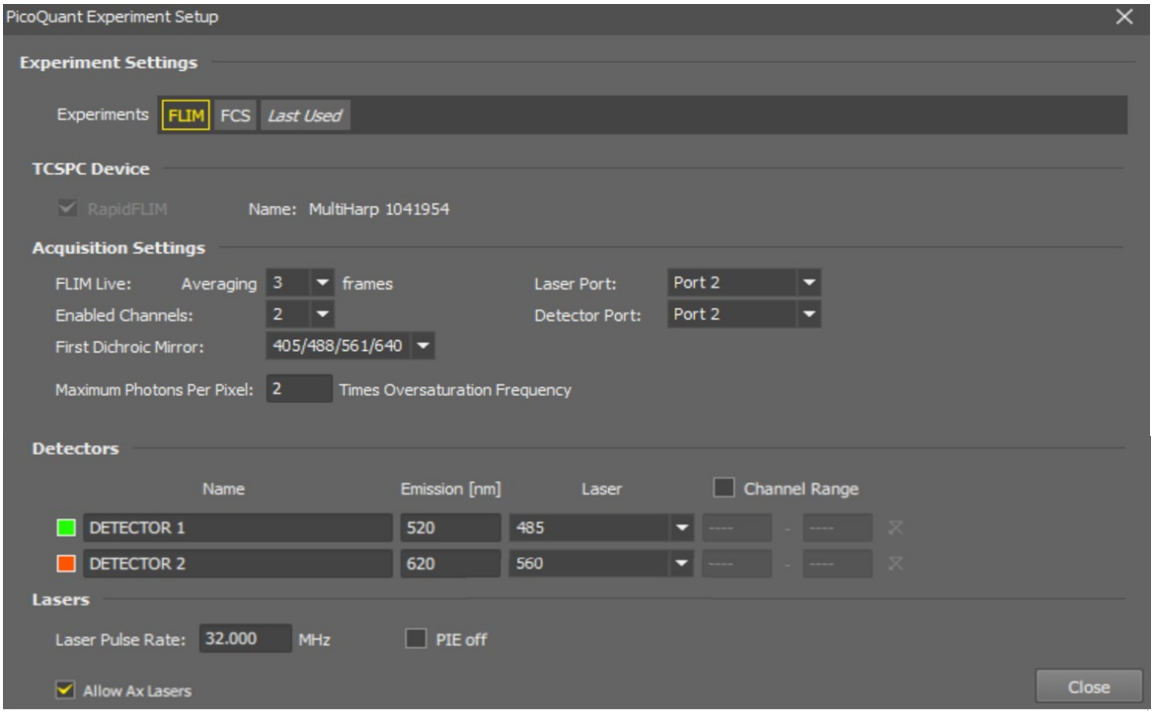


Fig. 3.3 Experiment Setup window

- Laser settings:
  - Laser pulse rate setting. Control in the TCSPC decay window (right click in the captured FLIM image → PicoQuant → TCSPC decay) if the decay curve is completely decaying to the level which is achieved while lasers are switched off. If this is not the case a lowering of the laser pulse rate can help. For standard dyes including fluorescing proteins like GFP a laser pulse rate between 20 and 40 MHz is best suited.

- “Maximum Photons per Pixel” Setting: In order to reduce the file size of the captured FLIM image, the maximum number of photons which can be displayed and stored per pixel is limited. The standard value of this limit is two times above the pile-up limit. E.g. if the pile-up limit is reached with 4 photons per pixel, 8 photons per pixel are still displayed correctly. In case measurements should be done at higher photon count rates this value can be set higher on the cost of bigger FLIM image file sizes. Eventually more PC RAM is needed since the FLIM image files are stored temporaily in the computer RAM.
- PIE Setting: PIE (pulsed interleaved excitation) is activated as standard. With PIE, every detection channel will only be illuminated by the laser selected in the detector section.
- FCS (Fluorescence Correlation Spectroscopy) can also be performed with the Nikon cw (contineous wave) lasers. This can be beneficial since for some dyes beaching is reduced by applying cw illumination.

Measurement Control

Fig. 3.4 shows the control for the measurement duration (Capture). It can be set either in timing units (sec, min) or as a number of frames to acquire. In addition, a number of photons in the brightest pixel of the FLIM image can be set.

- Time Channels:
  - as standard, the highest number of time channels should be selected (e.g. 4096). The system then selects the best bin width (e.g. 40 ps) and calculates the highest possible number of time channels.
- IRF
  - automatically, after a “Live” measurement, the IRFs are calculated for all active detector channels. IRF is necessary to calculate correctly the lifetimes displayed in colors in the FLIM images. This is indicated by “IRF: OK”. In case “IRF not available” is displayed, perform a “Live” measurement.
  - Next to the detectors count rate indicator, which shows the light intensity measured, a pile-up indicator flashes with red light, as soon the fluorescence intensity goes beyond the pile-up limit. Detector Pile-up, which is created by the finite time width of the detectors’ electric pulses, can cause errors of the lifetime readings in FLIM images. In case the absolute lifetime is crucial keep care that the measurement does not reach the pile-up limit by lowering the laser intensity accordingly.
    Pile-up limit indication can also be seen in the FLIM image displayed as red pixels if switched on by the oversaturation indicator which is located above the FLIM image  .

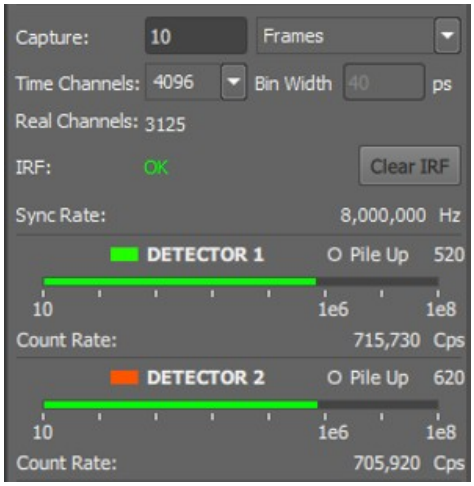


Fig. 3.4 Capture time and time channels selector. Indicator for real (applied) time channels and bin width of each time channel, IRF determination as well as count rate. “Pile Up” indicator illumines red if pile up limit is reached. Pile up can lead to errors in the lifetime determination.

Device Setup

FLIM and FCS AX Device Setup displays the current TCSPC unit and sync (pulse) rate of the pulsed lasers.

With PTU Export Settings, for each measurement a ptu FLIM or FCS file can be automatically created. When Live PTU Export is checked, also during live measurements a *.ptu file is created.

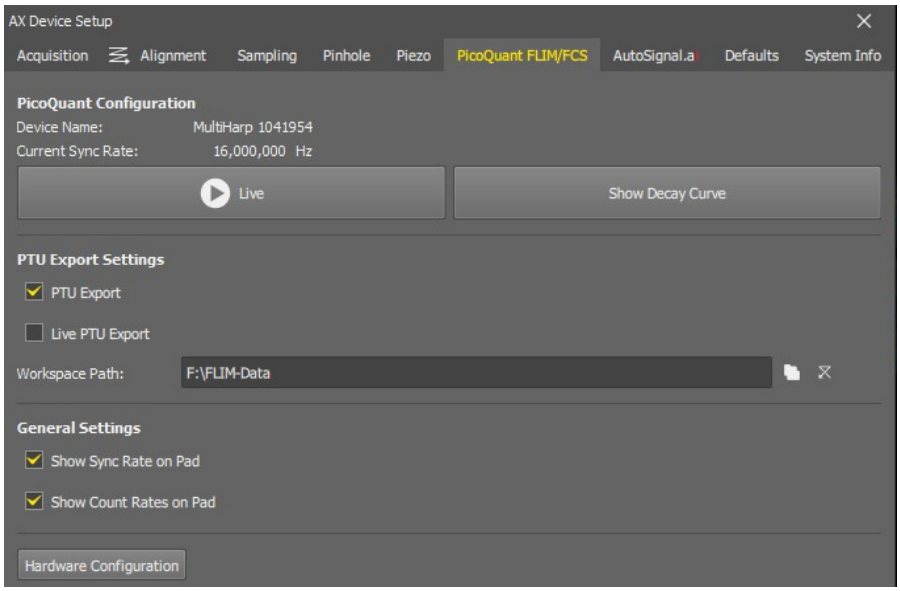


Fig. 3.5 FLIM and FCS AX Device Setup.

PTU files are the raw data files which contain all lifetime information. They can be read by SymPhoTime64. **The Workspace Path sets the location for all FLIM and FCS ptu files.**

The easiest way to save the settings for FLIM or FCS measurements is by presetting defined **optical configurations** in the NIS software. It is useful to create at least one standard configuration for FLIM and FCS. Buttons can be named e.g. “FLIM”.

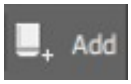


Fig. 3.6: Add “optical configuration” for FLIM or FCS measurements, compare also Fig. 3.1.

3.2.1. PicoQuant laser control in NIS

In case your system is equipped with a computer controlled laser unit from PicoQuant, it is possible to control the pulsed lasers via NIS software.

The intensity of the lasers is set using the sliders shown in Fig. 3.7. Pulse form and width are not changed. There is a slider “All L.” which controls the laser intensity of all pulsed lasers together.

The electrical laser power is automatically set to an optimized value that corresponds to the shortest laser pulse. This allows the best timing resolution of the system.

However, if more laser power is needed, “H.P.” (H.P. - High Pulse) can be ativated. The electrical laser power goes to its maximum. This leads to a broadening of the laser pulse and to a reduction in timing precision.

Attention: the laser intensity scale of the sliders for controlling the attenuation of the lasers within the software only displays approximate intensity values. The sliders adjust the laser intensity on a logarithmic scale of about two orders of magnitude.

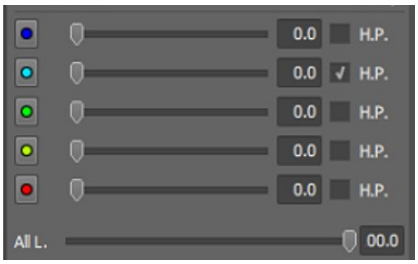


Fig. 3.7 Settings of PicoQuant lasers

It is possible to select or deselect the lasers by clicking on one of the Laser wavelength buttons. To set the **Pulse Mode (PIE on/off)** and change the **Pulse Rate** open the experimental setup window (see Fig. 3.3).

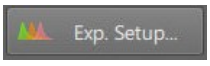
The selected **Pulse Rate** describes the pulse rate of all laser pulses of different wavelengths together, while the resulting Sync Rate shows the Sync-Rate of the generated Pulse Pattern. For example when using 3 Lasers with a pulse rate of 10MHz the distance between each laser pulse of equal wavelength is 100ns. The resulting sync rate is one third, i.e. 3.33 MHz, since the three lasers are pulsing in a pulsed interleaved pattern.

In standard setting PIE is on. In this case, lasers of different wavelengths are pulsing not at the same time but one after another. By selecting the desired laser wavelength for each detector (e.g. 485 nm for DETECTOR 1, compare Fig. 3.3), cross talk between channels can be minimized. Only the fluorescence detected in a specific detector channel is displayed which is generated by the selected laser excitation wavelength.

For Multi Photon Excitation (MPE) lasers, the lightpath 

must be used. Emission is stable, if the field in the MPE control panel changes from flashing yellow to green. The emission must be switched on and the Shutter should be open (compare Fig. 3.8).

Fig. 3.8 Settings of Multi Photon (MPE) lasers

The wavelengths setting of the MPE laser is reachable under “Experiment Setup”. 

In the setup window the MPE laser wavelength can be set by clicking on “Adjust Wavelength…”.

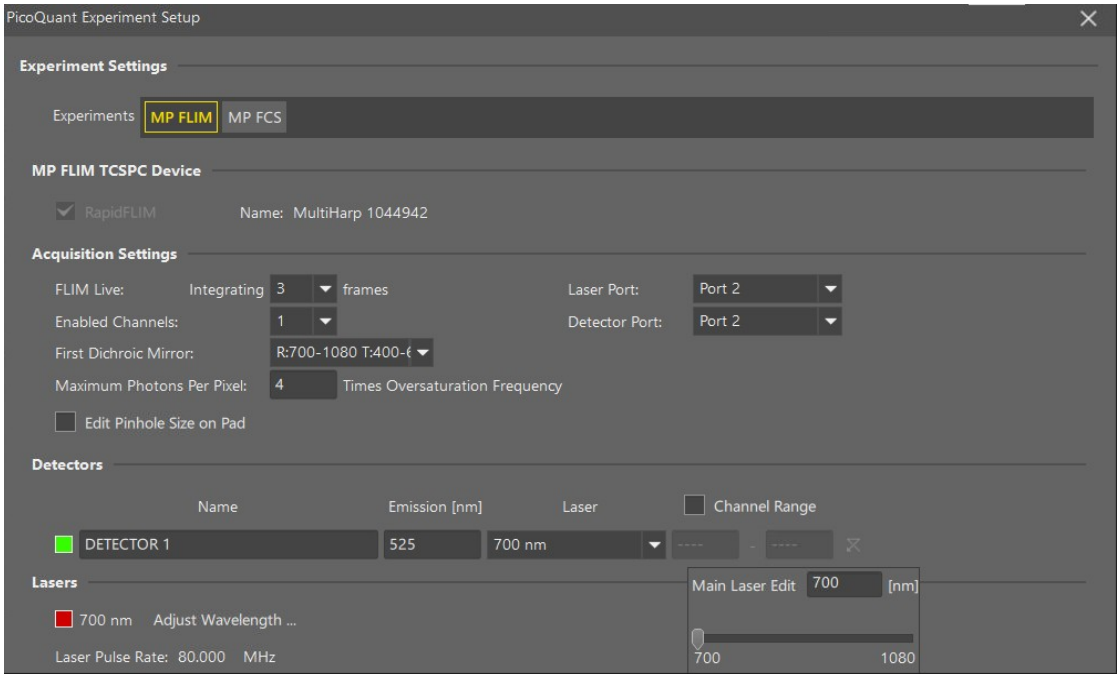


Fig. 3.9 Experiment Setup window for Multi Photon Excitation FLIM. Please choose a correct “First Dichroic Mirror” for the selected MPE wavelength, e.g. “R:700-1080”.

3.3. Settings for FLIM Acquisition

3.3.1. Starting Point

Before taking a FLIM image, select the region which you want to image and adjust the axial position accordingly. There are two convenient ways in which the user can switch between normal confocal and FLIM acquisition:

- Create two NIS user configurations, one for normal confocal usage and one optimized for FLIM.
- At the Pad switch between the detection ports FLIM and DU4. Attention: **To Change from "FLIM"-mode to "DU4"-mode the FLIM button must be deactivated by clicking once more on the FLIM button.**

From this point the manual assumes that the image region is selected and in focus.

3.3.2. Determining the best conditions for a FLIM measurement

The goal during the optimization is to determine the **best excitation and detection rates** with a "**Scan**" measurement.

1. Turn on PicoQuant equipment (laser and laser drivers, electronics and detection unit) and place the desired emission filter in the detection unit.
2. Activate **FLIM** and click 

. A test measurement will be automatically started.
3. After the scan has been activated, the image appears. Adjust the focus and open the decay curve by right clicking in the FLIM image area, selecting "PicoQuant Plugin" and "Show Decay Curve" (see Fig. 3.10) and observe the decay behavior (see Fig. 3.11).

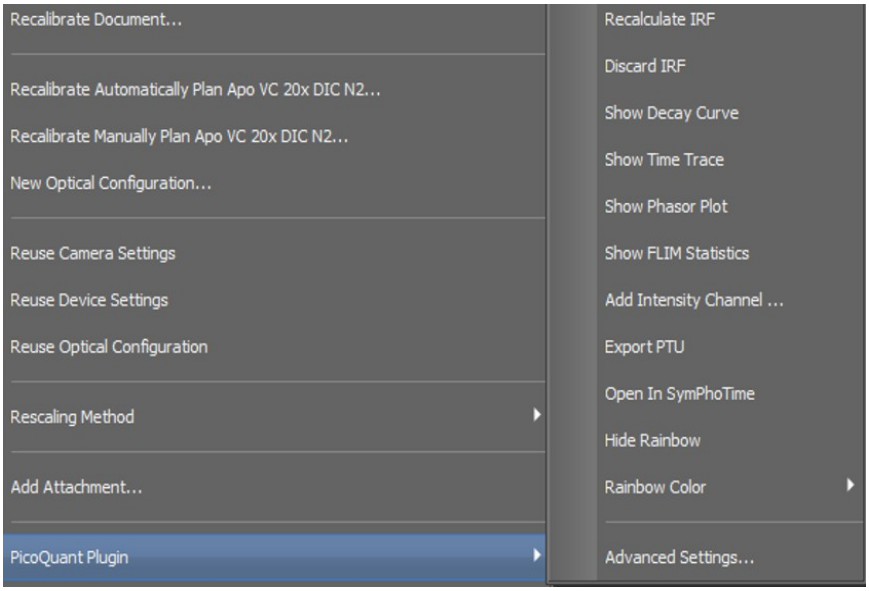


Fig. 3.10: Open the PicoQuant Plugin by right click in the image.

The decay window appears and shows the decay as well as the automatically calculated instrument response function (IRF, displyed in red). Decay and IRF can be displayed individually for each channel. The channel is selected in the lower area of the FLIM image display window.

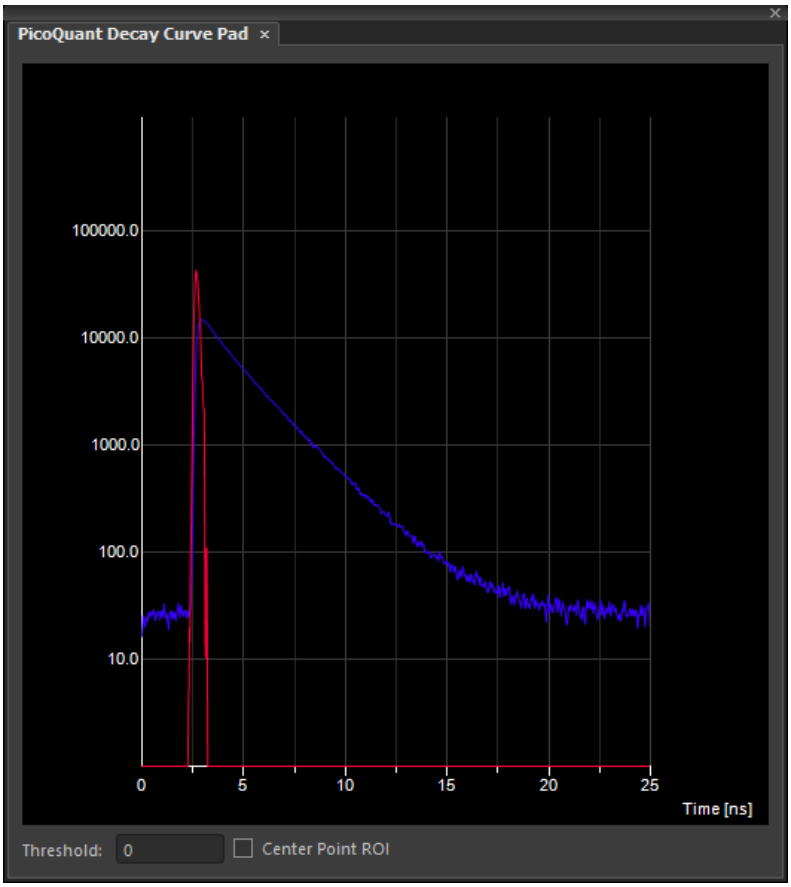


Fig. 3.11: Picoquant Decay Curve Pad showing fluorescent decay (blue) and calculated IRF (red)

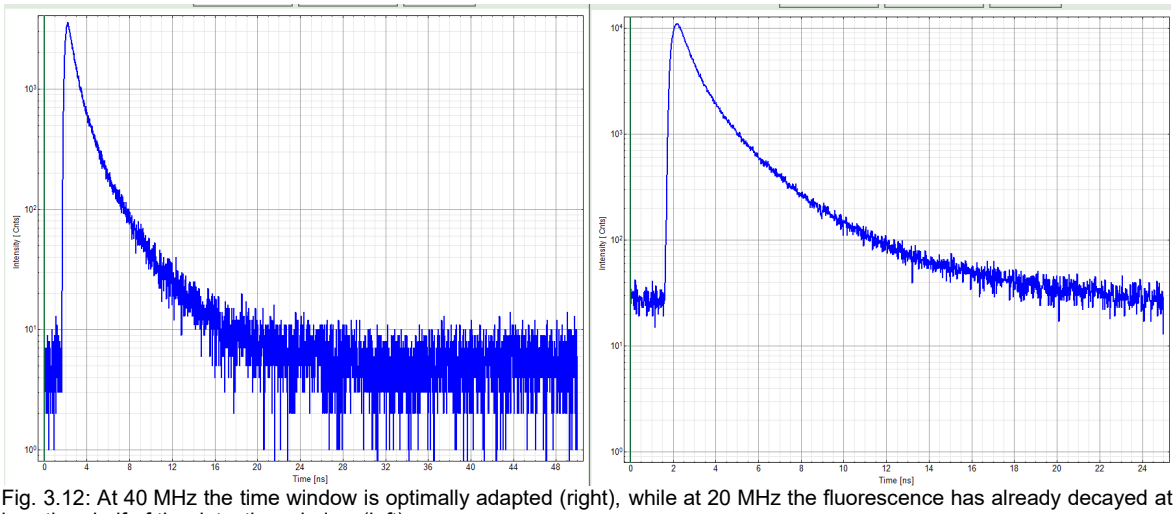


Fig. 3.12: At 40 MHz the time window is optimally adapted (right), while at 20 MHz the fluorescence has already decayed at less than half of the detection window (left).
At 80 MHz laser repetition rate (not shown), the fluorescence would not decay completely before the end of the time window is reached. Due to a “wrap around” the decay tail would be observed even before the actual pulse. In this case the “wrap around” correction of SymPhoTime should be used to analyze the FLIM image. It is included in the “rapidReconvolution” fit option for FLIM.

4. **The best repetition rate is the highest at which the fluorescence decay is complete**. In other words, the pulse sequence has to be as high as possible, in order to increase the overall count rate, but slow enough to allow the population of excited state to be completely depleted within two laser pulses[1]. The best way to quickly visualize this is by checking that at the beginning and end of the decay there is flat background, and that the background after the decay does not occupy more than 20-30% of the window.

5. For PicoQuant pulsed diode lasers operated with the Sepia II laser driver the **laser repetition rate** (pulse rate) can be set under **experimental setup** (see Fig. 3.3). In case a manual laser driver is used (e.g. PDL-800D), please set the repetition rate at the laser driver manually.

1 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, more than 12 % of the photons are emitted due to the statistical process after 12.5 ns. In this case the laser pulse repetition rate has to be lowered.

For most fluorescence measurements a **repetition rate of 20 - 40 MHz** is a good choice.

6. Using this rapidFLIM system, **a count rate in the brightest pixel of up to 40% with respect to the pulse rate** can be set. Count rates above can lead to distorted decays due to the pulse pile-up effect[1]. It is recommended to adjust the laser intensity in a way, that a high bleaching rate of the image is avoided and a detection count rate below the pile up limit is achieved. For detector count rates above the pile up limit, a red pile up indicator flashes (see Fig. 3.13). If it flashes red, the laser intensity should be reduced using the laser intensity settings. Count rates above the pile up limit lead to an error for the calculated lifetimes in the FLIM analysis.
7. Pixels in the FLIM image which are affected by pulse pile-up, can be marked in a selected color by activating the over-saturation indicator  .

Fig. 3.13: Indication of Pile Up limit

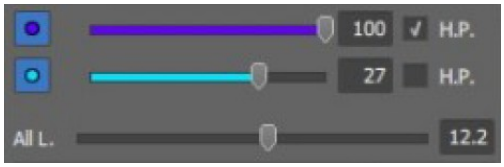


Fig. 3.14: Selection of the correct laser intensity

1 Pulse pile-up is arising from the finite electrical pulse width of the detectors after photon detection. Classical pile-up is not occuring in a rapidFLIM system since the dead-time of the TCSPC electronic MultiHarp 150 is sufficiently small (650 ps).
For more information about the pile effect and electronics dead-time please refer to the TCSPC card manual.

3.3.3. FLIM measurement

1. Activate the PTU Export in **"Device Setup".** Please specify a workspace Path, where all FLIM image data are saved for further analysis in SymPhoTime software. The "PTU Export" feature allows to generate and save automatically with each FLIM capture a PTU file which contains the complete information of the recorded FLIM image.

   PTU file export is especially useful for rapidFLIM applications since it can be used in conjunction with the rapidFLIM data fitting in SymPhoTime. This analysis method corrects for artifacts which come from the finite pulse width of the detectors leading to a so called "pulse pile-up". By applying the pile up correction, acquisition at very high count rates in the range of the laser repetition rate and higher can be performed. Selection of excitation laser or detection channel must be performed in SymPhoTime by using the PTU export feature.

   Please define also a workspace path for FLIM data storage (see Fig. 3.15).

Fig. 3.15: When the PTU Export is activated, all scan data and each measurement will be saved in a workspace.

2. Select the "**Capture"** criteria in FLIM mode**.**

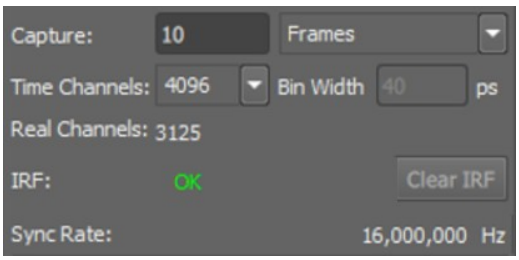


Fig. 3.16: Duration of a FLIM measurement. Choose between stop after frames, stop after photon counts or stop after a certain time.

3. Press "Scan" to start a test measurement. Display parameters including color coded lifetime range are available by clicking the blue arrow a the top of the window (see Fig. 3.17).
4. After the "Scan" is stopped, the software automatically determines the IRF functions which are needed to calculate the lifetimes in the FLIM image correctly. The lifetimes displayed in the FLIM image are calculated by the mean arrival times of fluorescence photons in the corresponding pixel.

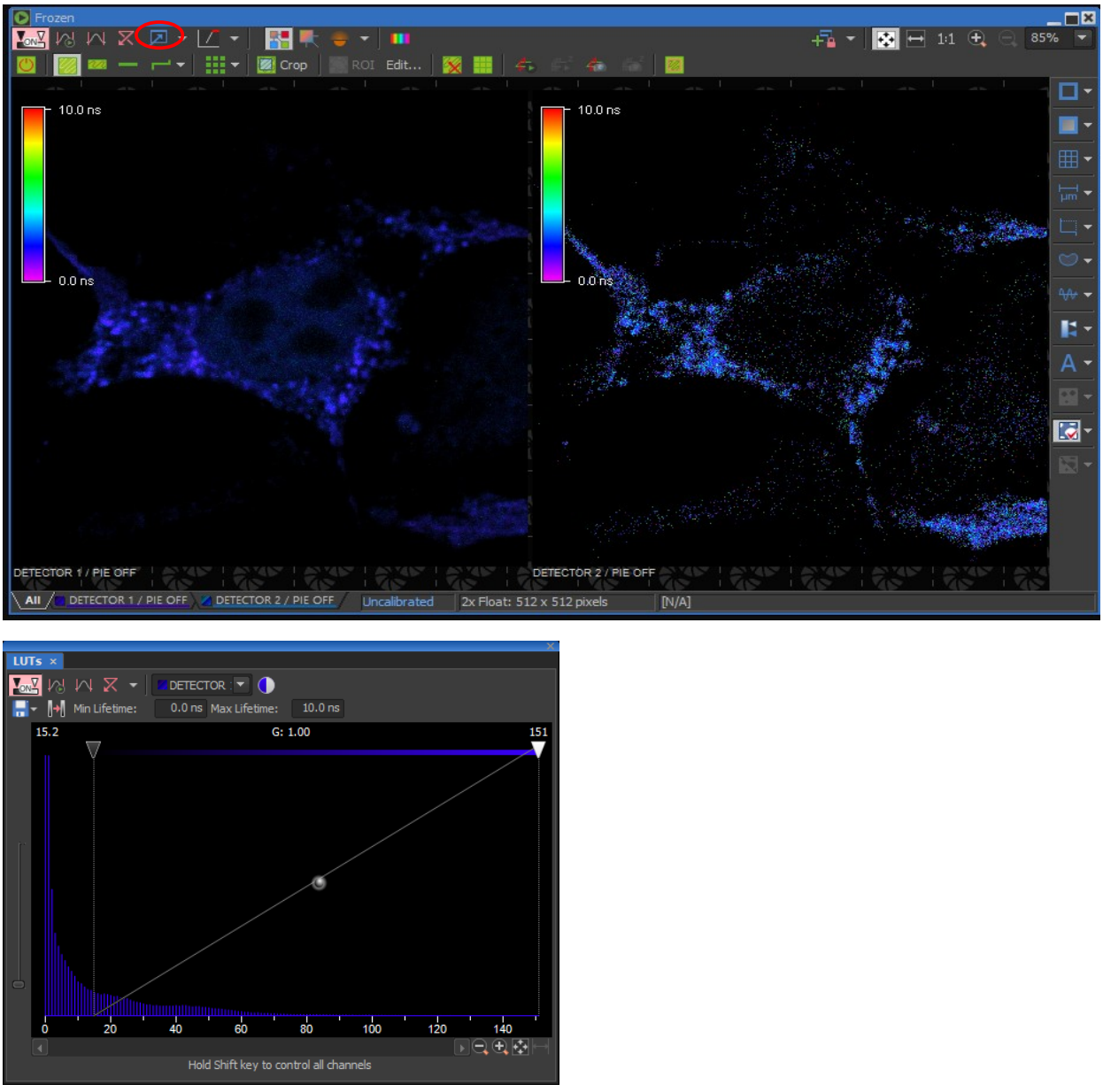


Fig. 3.17: Measurement preview displayed during LSM acquisition. In the LUTs window the intensity scale as well as the lifetime display can be set (e.g. here the false color display shows 0 ns in magenta while lifetimes of 10 ns are depicted in red).

5. Press 

to start the final FLIM measurement.
5. The measurement comes to its end when the finishing criterion is reached. Alternatively it can be interrupted at any time by pressing again the capture button.
6. As a rule of thumb for homogeneous samples, a FLIM measurement can be interrupted when the brightest pixel has acquired 1000 photons for an average lifetime calculation.

It is possible to use ND Acquisition as well as Jobs in the same way as usual, piezo z-stack is also supported.

3.3.4. PhLIM measurement

For phosphorescence image recording, the PhLIM mode (Phosphorescence Lifetime Image) can be used. By using this mode, the repetition rate of the laser is automatically reduced to capture lifetimes in the microsecond range.
The laser excitation changes automatically to the burst mode. A burst of laser pulses excites the phosphorescence after which there is a time where no laser pulses occur. This laser pulse (burst) repetition rate can be set in “Experiment Settings”.
Burst ratio defines the number of laser pulses per burst. Burst base rate determines frequency of the laser pulses inside a burst.
PIE mode is not available when PhLIM is selected.

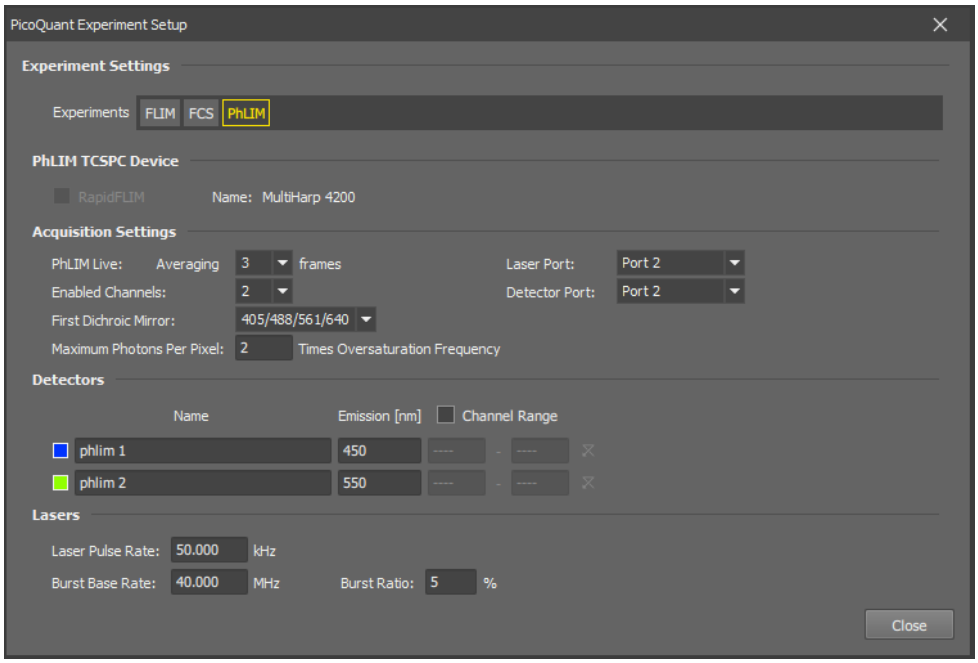


Fig. 3.18: Experiment Settings for PhLIM. In addition to “laser pulse rate” also “burst base rate” and “burst ratio” can be set.

For a laser pulse rate of f = 50 kHz the time between two bursts is 1 / f = 20 µs. As a rule of thumb this time should be 10 times longer than the expected decay time of the chromophore. In addition the pixel dwell time should be ten times longer than the time between two bursts to avoid image artifacts.

Pile-up limit indication is switched off for PhLIM since pile-up does not occur for PhLIM measurements by using the MultiHarp TCSPC devices.

The fitting of the PhLIM images can be performed with a Tail-fit algorithm in SymPhoTime. Therefore the IRF is not needed and not calculated in NIS.

3.3.5. Data Analysis using SymPhoTime

After completing the FLIM measurements the data can be analyzed with the SymPhoTime software. SymPhoTime contains all the necessary analysis methods that are suitable for time correlated data. There are two ways to open the FLIM data in SymPhoTime. You can start the SymPhoTime software from the NIS software.

1. Open the “PicoQuant Plugin” by right click in the FLIM display window and select “Open in SymPhoTime”. The image, that has been selected will open in SymPhoTime. In this way it is possible to obtain the data from a desired detection channel and a desired laser wavelength, which contains a single frame.

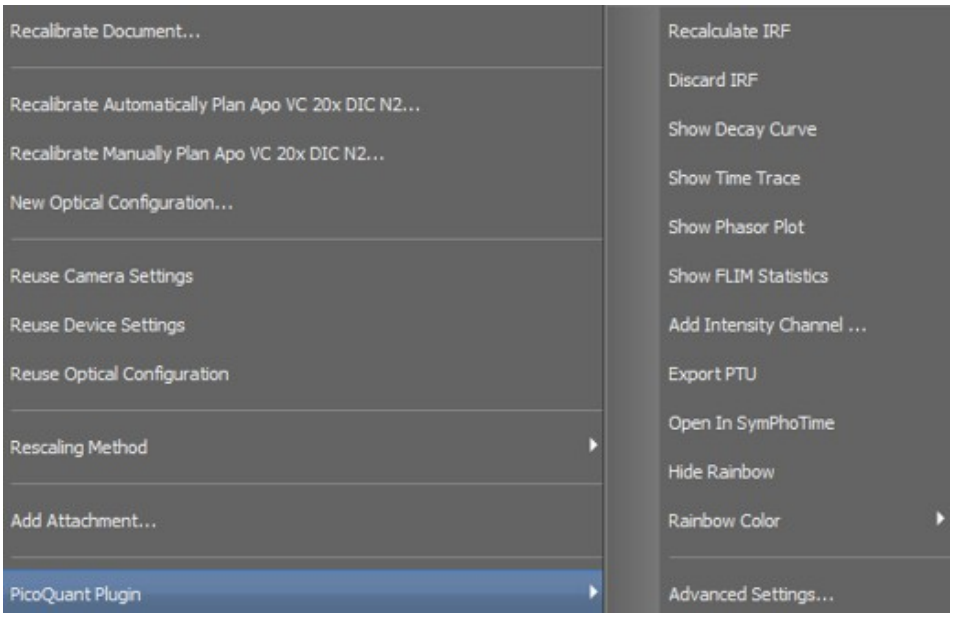


Fig. 3.19 „Open In SymPhoTime“ to analyze the FLIM image in SymPhoTime

In SymPhoTime a workspace (windows folder) is created at the path specified in “Device Setup” (compare Fig. 3.15). The user can select each measurement for analysis. New measurement data from NIS of the same day are loaded into this workspace automatically.

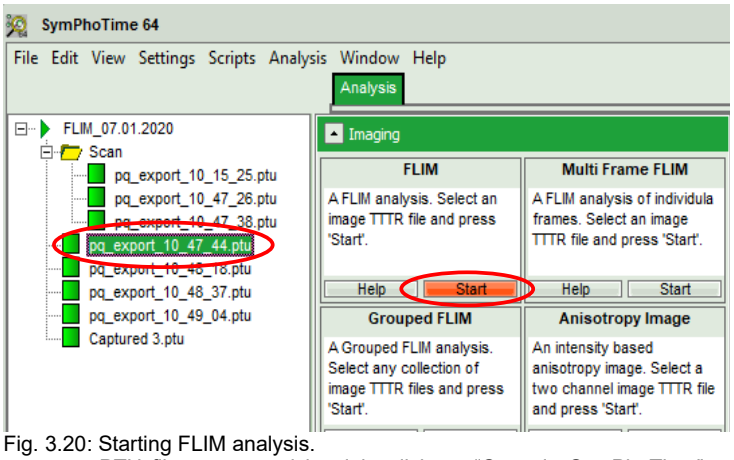


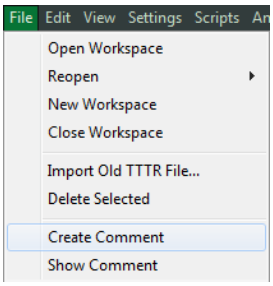
Fig. 3.20: Starting FLIM analysis.

- PTU files generated by right click on “Open in SymPhoTime” are listed as “Captured...ptu” files. No rapidFLIM correction is possible with these files. They contain the selected detector channel excited by the selected laser line. The FLIM images consist of one frame (all frames during measurement are summed up).
- PTU file generated by the “PTU export” feature (see Fig. 3.15) are displayed in the workspace as “pq_export_hour_min_sec.ptu”. All ptu files generated by “Scan” imaging are listed in the “Scan” folder. RapidFLIM correction is possible, the PTU file contains all data channels. The correct laser line must be chosen by applying time gating in the TCSPC window in case of PIE excitation. Frame selection is possible.

In the next step “Analysis” is selected to select a suitable analysis method (e.g. start FLIM or start FLIM FRET analysis).

3.3.6. Resulting raw data file and documentation in SymPhoTime

- **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 ("Create Comment") via the main menu bar.



- **SymPhoTime 64 software (only available for "pq-export" files):** Specific recorded frames can be selected for analysis 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").

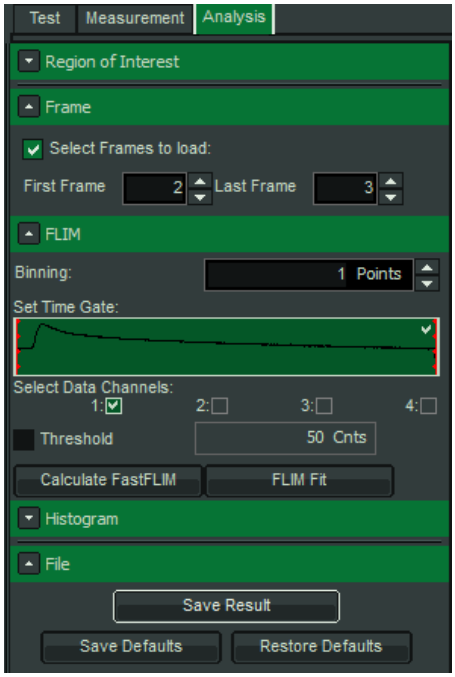


Fig. 3.21: Changing the selection of frame numbers for analysis in the SymPhoTime 64 Software (only for "PTU-export" files).

3.3.7. Measure an Instrument Response Function (IRF)

The width of the IRF displays the timing resolution of the instrument. An IRF can be obtained following these steps:

1. Estimation of IRF in Nikon NIS software

   The NIS software estimates the IRF automatically from the decay measured during a FLIM "Scan" measurement. To check the IRF and observe its behavior perform a right click at the image display window and select **"PicoQuant Plugin → Show Decay Curve".**

If the settings are changed (e.g. repetion rate or the laser power is changed from optimal power to “H.P. - High Power”) the IRF should be recalculated. This is done automatically with the next “Scan” measurement. The IRF is recalculated everytime the configuration is changed.

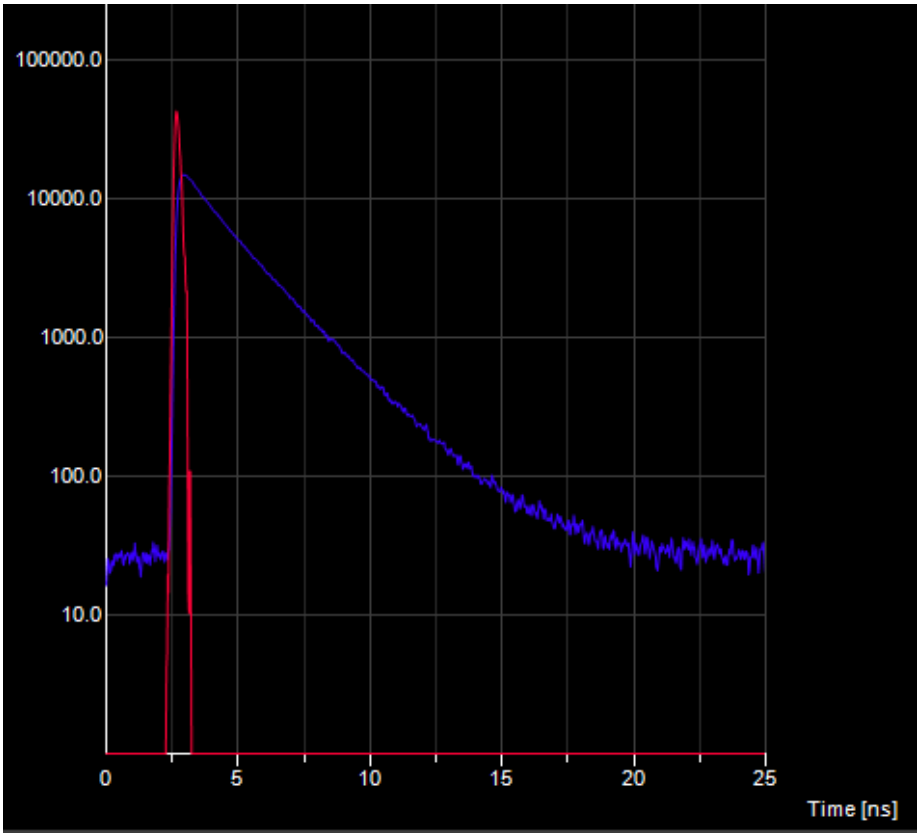


Fig. 3.22 PicoQuant Decay Curve Pad showing a calculated instrument response function (in red)

2. Measurement of IRF and import in SymPhoTime Software

For accurate measurements of lifetimes close to the timing resolution of the instrument, the instrument response function (IRF) should be measured instead of estimated:

a) Place a sample with a **very short lifetime** in the laser focus on the microscope stage (e.g. Erythrosin B or Fluorescein, dissolved in a saturated Potassium iodide solution in concentration near to the saturation limit. For Fluorescein, a slight basic pH value is necessary to dissolve a sufficient amount of dye). The dye chosen should have a similar emission range as the sample that should be measured afterwards. 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 two photon excitation, also second harmonic generation may be used to measure the IRF (e.g. by use of urea crystals).

b) Place a suited **emission filter** in the filter holder in front of the PicoQuant detector, ideally the same as used later for the measurements.

c) Start the scanning process by pressing 

.

d) **PicoQuant Decay Curve Pad:** You can now check the decay behavior of your sample using the PicoQuant Decay Curve Pad, as shown in Fig. 3.22. Adjust the count rate to approximately 50 kCounts/s. The laser power can be changed optically by attenuating or electronically by changing the electrical power of the laser diodes. Optical attenuation does not change the IRF while electrical power changes will affect the IRF which has in most cases a faster response at lower electrical laser powers. You can use the electrial laser power settings to optimize the pulse shape of the laser for shortest IRF width.

e) **NIS software:** Stop the test measurement by pressing 

.

f) **NIS software:** Set the measurement time to 60 seconds and take a measurement by pressing 

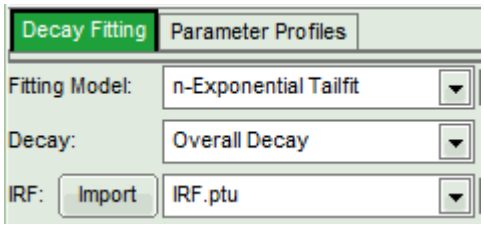
.

g) When the decay histogram in the TCSPC preview has reached 10 000 counts in the peak channel, **stop** the image acquisition. Do not let it increase above 100 000 counts.

h) Now the acquired image can be transferred to SymPhoTime (right mouse click on the FLIM image → PicoQuant plugin → Open in SymPhoTime). Rename the FLIM image as IRF record.

i) Replace the IRF solution with your sample again.

j) Import the IRF in your final FLIM image by clicking the “Import” button. Please note, that every detector / laser has a slightly different IRF. The IRF must therefore be recorded for every detector / laser combination separately. For best results it should be measured on the same day the final FLIM measurements take place.



3.4. Settings for FCS Acquisition

3.4.1. FCS measurements

FCS measurements are recorded at a single point in the image. Prior to performing a FCS measurement, a FLIM or standard image has to be taken in order to find the location where the FCS measurement should be performed. The configuration with respect to FLIM can be the same or may differ, since in FCS it is also possible to excite with CW lasers from Nikon, or simultaneously with Nikon and PicoQuant lasers (**Port 1** for Nikon lasers, or **Port 2** for pulsed PicoQuant lasers). Port selection is done in the “Device Setup” window.

After recording the image please select AX – FLIM & FCS.

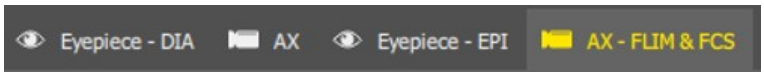


Fig. 3.23: Selection of FLIM and FCS functionality.

In the drop down menu at the AX Pad select FCS (Fig. 3.24).

Fig. 3.24: Selection of FCS measurement.

Lets imagine we would like to excite with a PicoQuant laser at 485 nm.

1. In this case activate the 485 nm laser and we choose a suitable dichroic in “Device Setup” window.
2. In “Device Setup” select **Port 2** for PicoQuant lasers and **Port 2** for PicoQuant detectors.
3. Select FCS capture time in seconds.

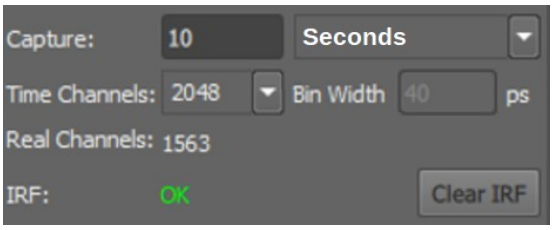


Fig. 3.25: Selection of FCS measurement duration.

4. Set the pinhole size to 1 AU (Airy Unit) at the excitation wavelength (in our example 485 nm).

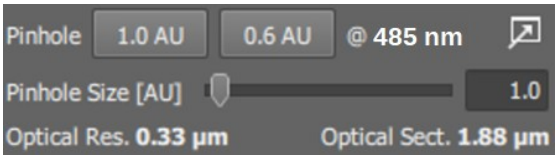


Fig. 3.26: Selection of confocal pinhole size in A.U..

5. In the captured image, go with the mouse to the spot where you want perform the FCS measurement. By default, FCS measurement point is created in the center of scan area. User can move existing points in scan area by dragging and add new points with double clicks. If user wants to reset scan area to the default (= only one point in the center), user has to rightclick and select “Reset Points” from menu. (Fig. 3.27).

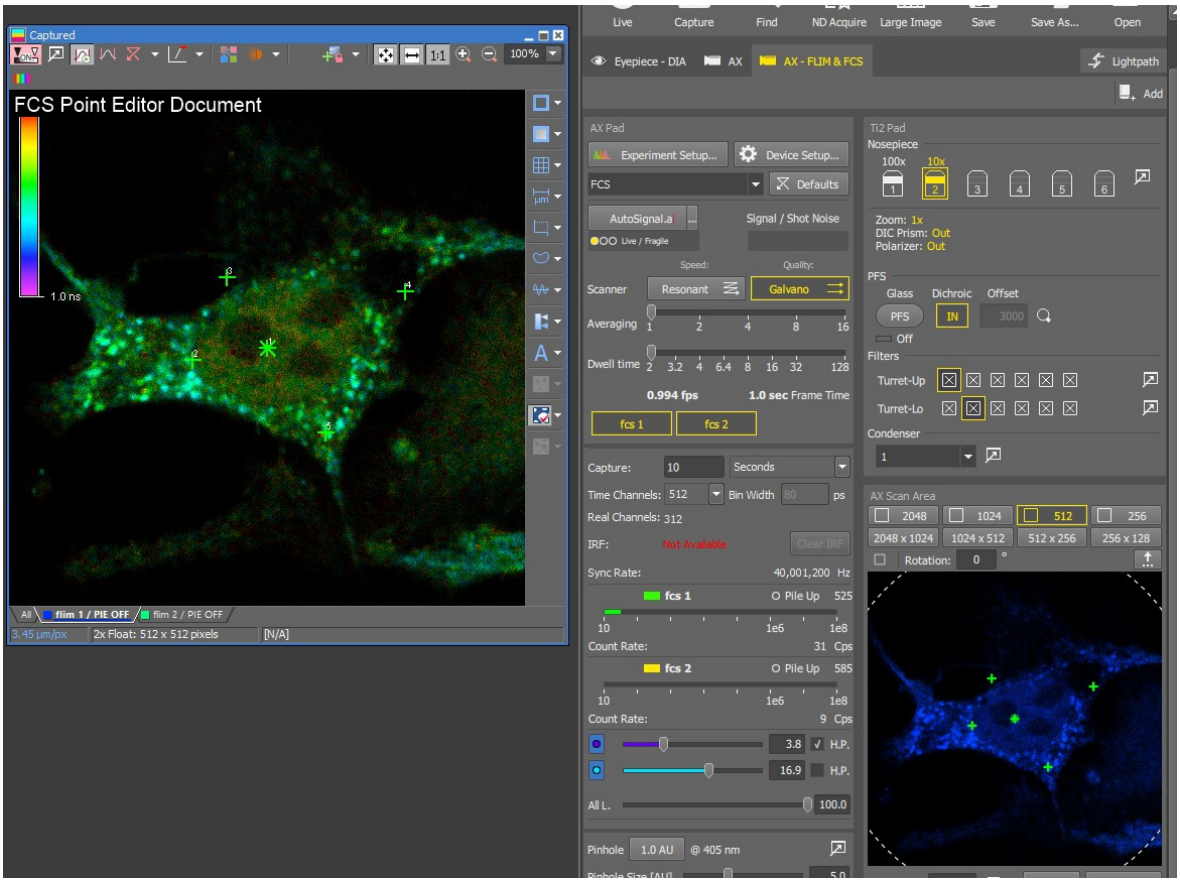


Fig. 3.27: Selection of FCS measurement location.

When multiple points are defined, “capture” runs measurement on each point sequentially. When “live” runs, measurement runs on current active point only. The FCS measurement point appears in the AX Scan Area window as a small green dot (Fig. 3.28).
User can define up to 64 points which will be measured. These points can be defined in Scan Area panel as well as in “FCS Point editor document”.

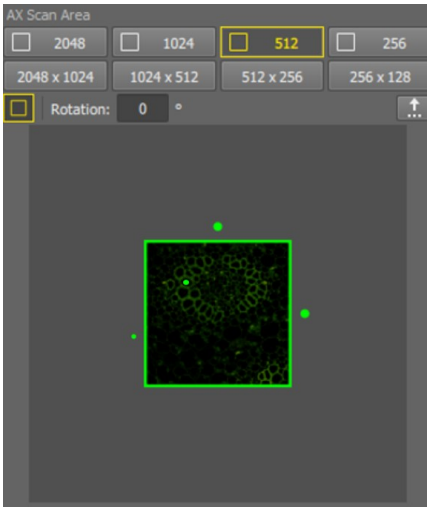


Fig. 3.28: FCS measurement point is visible as a small green dot in field of view.

6. Aside from scan area, the user can also define FCS measurement points in FCS Point Editor Document. This makes defining points easier, because the scan area may be too small to precisely set each point. When user selects FCS experiment, the last document which was acquired in either AX or FLIM / PhLIM mode is marked as FCS Point Editor Document. On this document, the user can move measurement points by dragging and access FCS point defining menu by right-click:

1. Reset Points – only one point in the center is defined and all other points removed

2. Add FCS Point [x,y] – adds measurement point on the right-clicked location. If user right-clicks above already defined point, these options are available too:

3. Set FCS Point [x,y] as Active

4. Remove FCS Point [x,y]

5. Keep Only FCS Point [x,y]

When user runs FCS capture, a measurement is run sequentially on each defined FCS point. For the FCS document, content of current scan area is used as the document image and each measurement point is marked in the document with white cross. The active point is marked with white star. The user can select the active point by left-click.

FCS measurement curves show data from the active measurement point, if export to PTU / open in SPT are selected, the data from this point are used for export / SPT.

7. At the captured image perform a right click and select “PicoQuant Plugin” → “FCS Autocorrelation”. The FCS correlation plot opens (Fig. 3.29). If “Calculate Cross-correlation” is checked, also the cross-correlation will be displayed (green curve in Fig. 3.29).

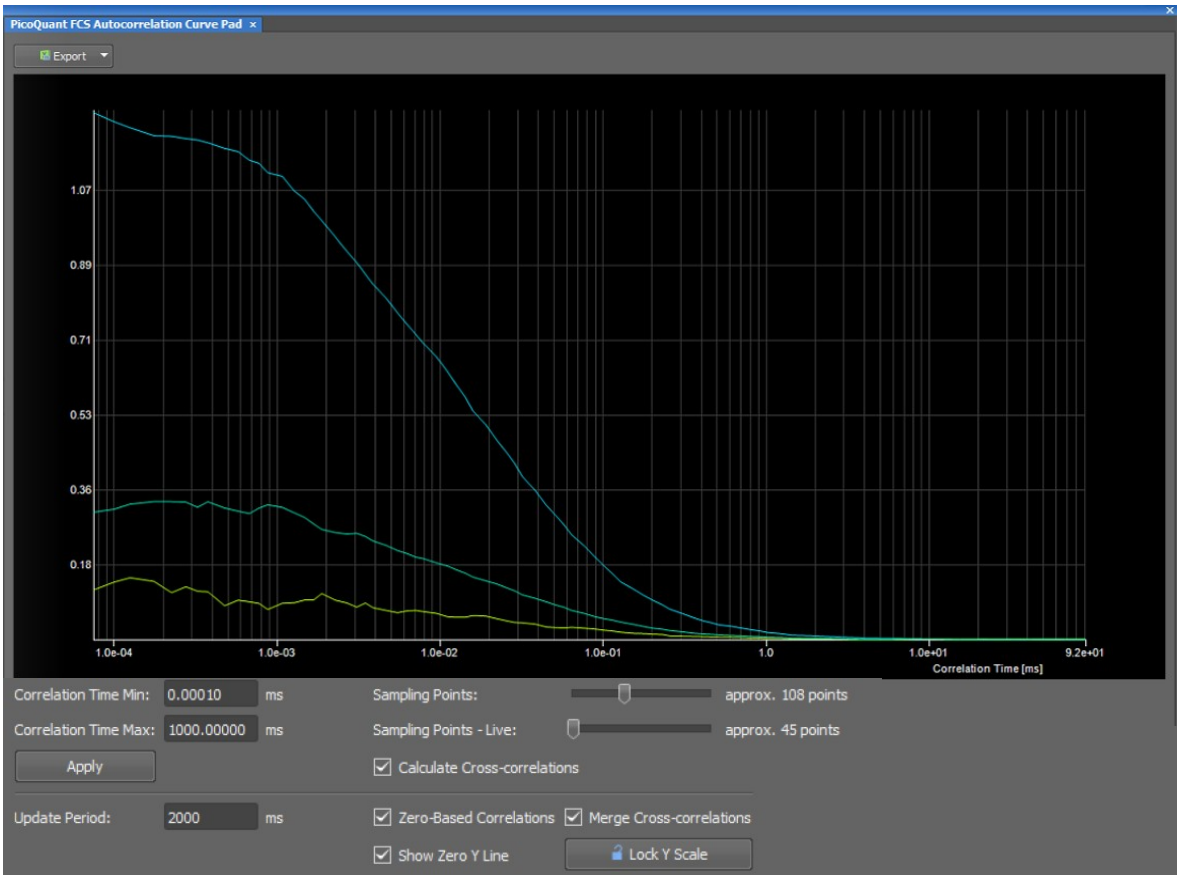


Fig. 3.29: FCS correlation display. This panel shows autocorrelations and crosscorrelations for FCS document components. User can adjust number of curve sampling points and correlation time range. After changing the settings, user has to click “Apply” to apply the settings. Update Period field sets how often the curves are recalculated during acquisition.

8. In order to optimize the laser intensity and objective collar, start a the measurement with 

.

9. After all settings are optimized, the final FCS measurement is started with 

. The measurement settings can be stored as optical configuration.

3.4.2. Requirements for FCS

FCS is a technique which requires single molecule sensitivity and hence is much more demanding than FLIM. In the following table the general requirements to perform FCS with a PicoQuant Upgrade Kit are listed.

Nikon AX	LSM Upgrade Kit	Sample
• Water immersion objective with NA 1.2 or higher with correction collar. • Clean optics and perfect alignment (pinhole alignment is crucial). • Cover sample from room light.	• SPADs or PMA Hybrid PMTs. • SPT64 version with license for point measurements. • Clean filter optics and perfect alignment.	• Concentration in the range of 0.1 - 500 nM. • High Absorption Coefficient and Emission Quantum yield. • Photostability.

Unlike FLIM measurements, FCS can also be performed with CW excitation from Nikon lasers. If pulsed lasers from PicoQuant are selected instead, the user will have access to the timing information and the technique is renamed as FLCS (Fluorescence Lifetime Correlation Spectroscopy).

The steps to perform FCS measurements are the following:

- Optimise AX pinhole setting with a strongly fluorescent solution.
- Perform a calibration measurement to determine the confocal volume with a ~20 nM concentrated dye solution.
- A region of interest is identified with a Nikon confocal measurement.
- The best conditions for the FCS are determined with a “Live” measurement.
- The FCS measurement is performed (“Capture”).

3.4.3. Optics optimization

FCS is a single molecule sensitive technique and hence it demands a perfect alignment of the system. Furthermore its analysis is based on the assumption of a perfect 3D Gaussian confocal volume. Therefore good confocality must be ensured and distortions from a Gaussian volume are to be minimized. In this regard, there are two important things that the user should check, **the pinhole alignment**, and that the thickness of the cover slip is properly set with the objective **correction collar**. Both things can be monitored by maximization of the fluorescent signal. The objective should be a water immersion objective with a numerical aperture N.A. of around 1.2.

1. Place a strongly fluorescent solution (>100µM concentration) in focus. The color of such a dye solution is clearly visible. The solution has to have similar spectral properties to the target molecule to be investigated with FCS (for example, if you want to measure FCS with GFP, you can use ATTO488 or Fluorescein to check the optics). Likewise use a cover slip or microscope dish analogue to that in which the FCS sample will be measured. Important is an equal thickness of the glass between objective and sample.
2. **NIS software:** Select the optical configuration for FCS.
3. **System:** Introduce in the PicoQuant detection unit a filter adequate for the sample.
4. **NIS software:** Focus into the volume, being at least 20 µm away from the cover slide.
5. **NIS software:** In the **FLIM image** select a point in the center of the image and start at FCS test by clicking 

.
6. **NIS software:** With a right click on the FLIM image, select “PicoQuant Plugin” → “Show Time Trace” and monitor the photon count rate over time.
7. Adjust the laser intensity to get a reading of around 2 Mcps. **Only if this count rate is reached** proceed to the next step.

8. **NIS software:** Open “Experimental Setup” → “Pinhole” and adjust the pinhole to reach the highest count rate possible. Move the pinhole position with the arrows and observe the variation of the time trace signal.

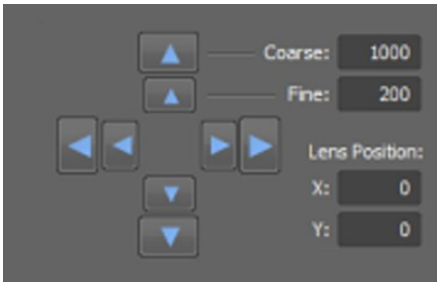


Fig. 3.30: Pinhole adjustment

9. **Microscope:** Move the correction collar at the water immersion objective and observe the variation of the time trace. Leave it at the maximum.
10. The system is now ready for a the FCS calibration measurement.

3.4.4. Perform a calibration measurement to determine the confocal Volume

Prior to a FCS measurement, the size and shape of the confocal volume needs to be determined. The size of the confocal volume depends on the optics selected (objective, excitation and detection channels, thickness of the cover slide) and will be introduced as fixed parameter during the FCS analysis.

1. There are several dyes that can be used as standard for calibration. Choose one which matches the spectral properties of your probe[1]. For example, for GFP, ATTO488 can be used as standard.
2. Select the same FCS configuration used during the Optics Optimization in the previous section and make sure the X-Y values from the Lens Position (pinhole) are set to their optimized positions.
3. Put the standard dye and focus into the volume, at least 20µm away from the cover slip.
4. In the **FLIM image** select a point in the center of the image and start at FCS “test” by clicking 

.
5. Go to the Autocorrelation (FCS) window and note the value of the **molecular brightness (MB)**. Increase the laser power till achieving a maximum in the MB. Further increase of the laser power will typically decreases the MB.
6. Determine the minimum laser intensity at which the MB reaches its maximum value and then reduce the laser power until achieving a value of 20% of that maximum[2].
7. Stop the test measurement and take a calibration measurement for at least 60 s by pressing 

.
8. Right click in the FLIM image and “PicoQuant Plugin” → “Open in SymPhoTime”. The FCS calibration measurement is now stored in the selected workspace.

3.4.5. FCS measurement

Before performing a FCS measurement, the target ROI has to be identified. This is performed with a FLIM measurement or standard Nikon AX measurement. Once the ROI is identified and in focus, the user can change to FCS acquisition.

1. In the **recorded image** select the point in which you want to perform the measurement. If you are measuring in solution, it is assumed that the laser is focused into the solution. If you are measuring in live cells, take into account that FCS works best with very dim cells. Therefore cells barely visible in the epi-fluorescence view should be selected.

1 See application note “Absolute Diffusion Coefficients: Compilation of Reference Data for FCS Calibration” on PicoQuant Website

2 The idea is to measure in the region where the MB dependence with the excitation intensity is linear. Best is to create a plot of power vs Count rate. Since this is time consuming, 20% from the maximum can be selected to do it quickly on the safe side.

2. Start a test measurement by clicking 

.
3. **NIS and SPT 64 software:** The diffusion time of molecules under investigation are dependent on the size of the molecules observed. If the diffusion time through the confocal volume gets longer less laser power should be applied. One has to make sure that the molecules are not bleached while diffusing through the confocal volume. This can be investigated by performing measurements at different laser powers. An indication for bleaching is a FCS curve which does not decay to zero at long lag times. For optimal setting the laser power is set as high as possible by not leading to a reduction in the diffusion time or to an offset of the FCS curve.
4. Select the desired acquisition time and start the measurement by clicking 

.
5. The FCS curve is calculated during the measurement and displayed in the FCS autocorrelation curve pad (Fig. 3.29). The display of the cross-correlation trace can be selected by checking “Calculate Cross-correlations”. The number of points calculated can be adjusted with “Sampling Points”. More points lead to a higher quality but need longer to be computed.
6. The Time Trace can be observed by opening the “Time Trace Pad”. The changes of the count rate over time can be observed (Fig. 3.31).

Fig. 3.31: Online Preview of the time trace.

3.4.6. FCS analysis

For FCS analysis please refer to the online help installed with the SPT64 software (acces by pressing F1 in the SPT64 software. Alternatively consult the step-by-step tutorials at www.tcsp.com

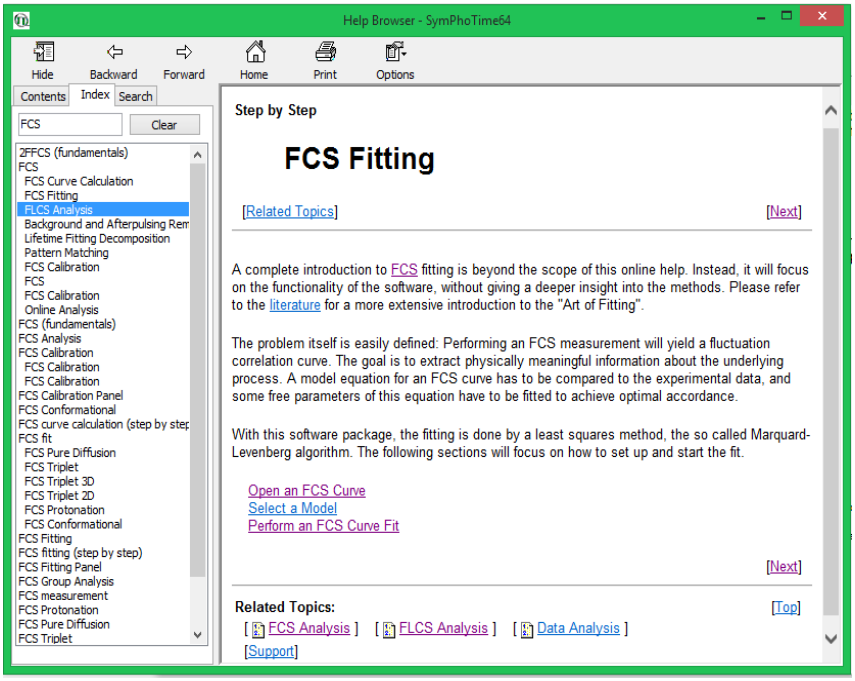


Fig. 3.32: Online Help with information regarding analysis routines

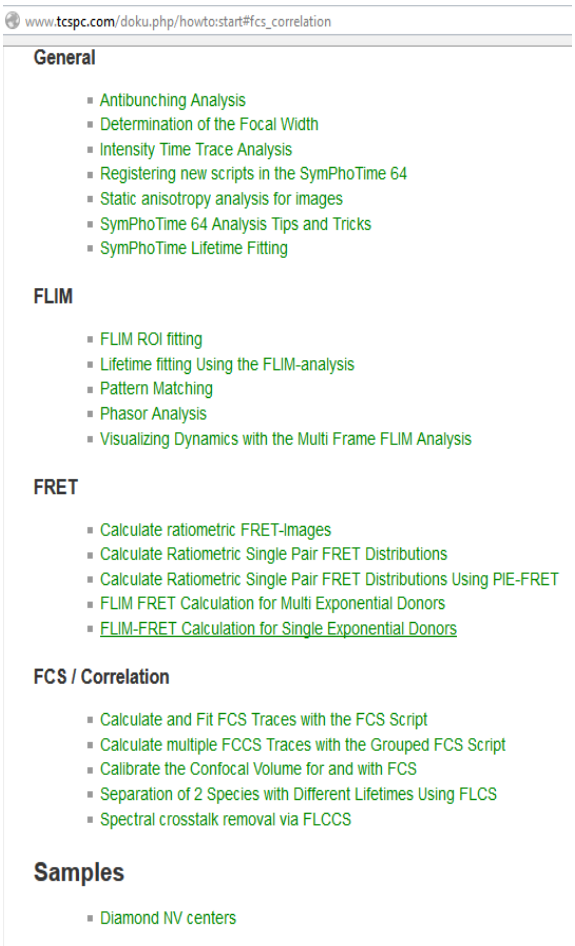


Fig. 3.33: "How to" section from the TCSPC wiki https//:TCSPC.com.

3.4.7. Remarks

- Usually, a **water immersion objective** with a high NA (1.2) is used for FCS measurements. Optimize the fluorescence count-rate by adjusting the objective collar.
- **FCS measurements** can **only** be performed using **avalanche photodiodes** 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 the afterpulsing is very low for these detector types.
- For a solid FCS-analysis, the **count rate** has to be **stable**. Signal decrease due to photobleaching may lead to an increased FCS correlation amplitude at long lag times.
- The **calculation time** of an FCS curve depends on the **measurement time** and the detector **count rate**.

4. Right Click Menu

When a right click is performed in a FLIM image, a menu appears, containing the "PicoQuant Plugin" specific options. Contents of the menu will be described in the following.

Recalculate IRF
Click this option, if you want to recalculate IRF for the current document. This will not change IRFs stored in the plugin settings. This option is only available with PQ HW connected.

Discard IRF
Click this option, if you want to discard IRF for the current document. Again, this has no effect on the IRFs stored in the plugin settings. This option does not require PQ HW to be connected.

Show Decay Curve
This menu option shows decays of currently selected detector channels or all channels if "All" component is selected. It shows IRFs as well, which are shown with same color but slightly transparent. If ROI is used, decay is shown only for the area in ROI. If "center point ROI" option is checked, only decay from the center point of the ROI is visible. If the decay curve threshold value is set, only pixels with intensity above the threshold are included in the decay curve. It is also possible to inspect the data in the decay by leftclicking into the graph.

Show Time Trace
This menu option shows Time Trace Pad, which is active during live mode and plots photon count per detector channel to a 10 second graph. Maximum values per detector channel are remembered and shown as dotted line. By clicking "Reset Maximum Value", the current maximum is discarded. Since this pad is only used during live mode, it is available only with PQ HW connected. This pad is also useful for hardware alignment, because changes of the photon rate are immediately visible.

Show FLIM statistics
This panel shows current mouse coordinates in the document and statistics for each FLIM channel in the document.

Fig. 4.1: FLIM statistics

Add Intensity Channel
It is possible to generate the intensity channel from a FLIM / PhLIM component, which can be used e.g. for thresholding in NIS-Elements. In the image below, intensity channel was generated from the Detector A / 485nm channel.

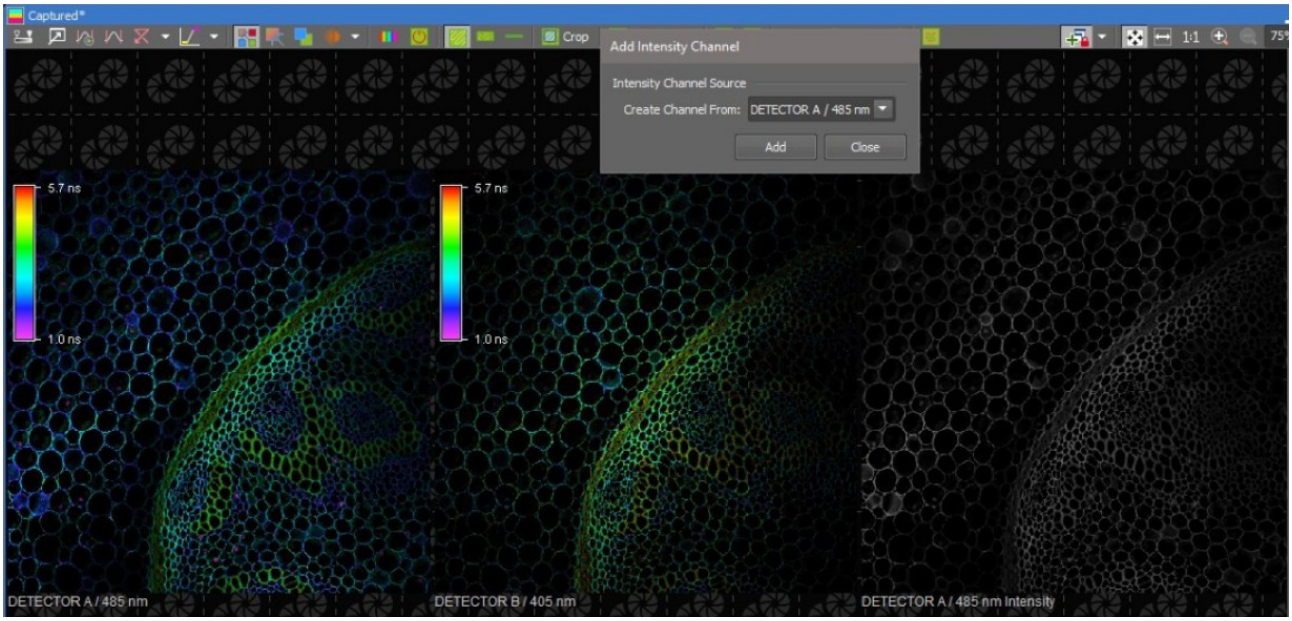


Fig. 4.2: Intensity channel is added. In this way a standard intensity image is generated from a FLIM measurement which does not contain lifetime information.

Export PTU
This menu option generates a ptu file from the document, which can then be opened in SymPhoTime or some 3rd party application such as FLIMFit. Please note, that this is different from the "Export PTU" option in “Device Setup”. PTU file created from the export menu option is generated from the already acquired image, while the PTU export option in “Device Setup” results in the ptu file being created during acquisition.
For FCS documents, all FCS components are exported to one ptu, enabling the SymPhoTime to calculate cross-correlations. If there are multiple points in the FCS document, only current point is exported.

Export All to PTU – ND documents only
This menu option generates ptu files for all ND frames and puts them in a common directory.

Open in SymPhoTime
It is possible to open the document directly in the SymPhoTime application by using this menu option. If there are multiple points in the FCS document, only current point is opened.

Open All in SymPhoTime – ND documents only
This menu option opens all frames of ND document in SymPhoTime.

Rainbow Color
This menu option lets the user change the FLIM / PhLIM color scale. The user can either use one of the predefined color scales or define his own scale. By default, "Rainbow Contrast - Magenta Below" is used.

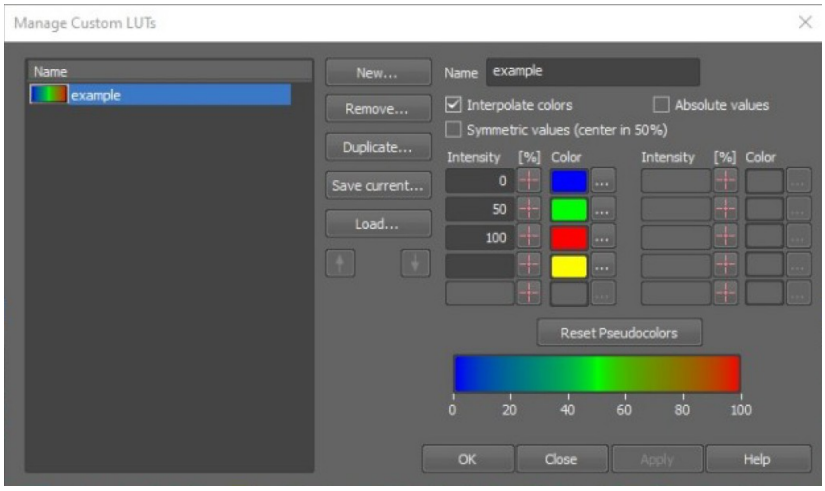


Fig. 4.3: Manage LUTs. With this menu, your own color scale can be created.

Advanced Settings
It is possible to switch between the intensity modulated display and display without intensity modulation.

User can also enable lifetime binning, 3x3 and 5x5 options are available.

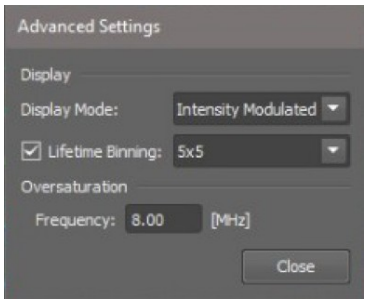


Fig. 4.4: Advanced settings. With this menu, display mode and lifetime binning can be set. The countrate for which oversaturation is displayed can be changed. Oversaturation is enabled in the same way as in the non-FLIM images. The oversaturation frequency is based on the pile-up limit. If the laser pulse rate is 20 MHz and the pile-up limit is 40% of the pulse rate (standard for MultiHarp TCSPC), pixels with the frequency above 8 MHz (8 million photons per second) are marked as oversaturated.

Additional Information:

Image Transformations
On FLIM / PhLIM images, usual image transformations as flip, rotate, crop are supported, as well as extracting and reordering of image components.

Large Image
It is possible to create a FLIM / PhLIM Large Image from XY multipoint in the following way: Image -> ND Processing -> Stitch Multipoint to Large Image.

Volume View, Slices View, Tiled View
Volume view, slices view and tiled view work in the same way as for non-FLIM images.

5. Phasor Plot

In the right click menu, “Show Phasor Plot” displays 2D plot of the FLIM / PhLIM data which can be used to separate pixels with different lifetime properties. Data for currently selected component are shown. It is important to have IRF calculated for the image to have meaningful plot display. Color of the pixel in the 2D plot is determined by number of pixels with same plot properties. There are two modes of the 2D plot selectable by the "Mode" combo box:

Phasor Plot: pixels are mapped to XY coordinates based on their lifetimes. Pixels with monoexponential decay are shown on the semicircle. Intensity / Lifetime Plot: X axis value is determined by the pixel lifetime, while Y axis value shows intensity of the pixel.

Min Intensity: If set, only pixels with at least this intensity are used for plot graph calculation.

Amplitude Correction, Phasor Plot mode: pixels with monoexponential decay should be visible on the semicircle. Due to backround fluorescence, they will appear inside the semi cicle. To calibrate the Phasor Plot, use sample which is known to be monoexponential and set amplitude correction for each detector channel so that the center of the drawn data area sits on the semicircle.

It is possible to use ellipse selection to color pixels with similar 2D plot properties in the image. Red, green and blue ellipsis are available, each highlighting the pixels with red/green/blue color. The "Intensity Modulated Highlighting" can be checked, resulting in highlighted pixels keeping their intensity. This selection is available for both modes of the plot.

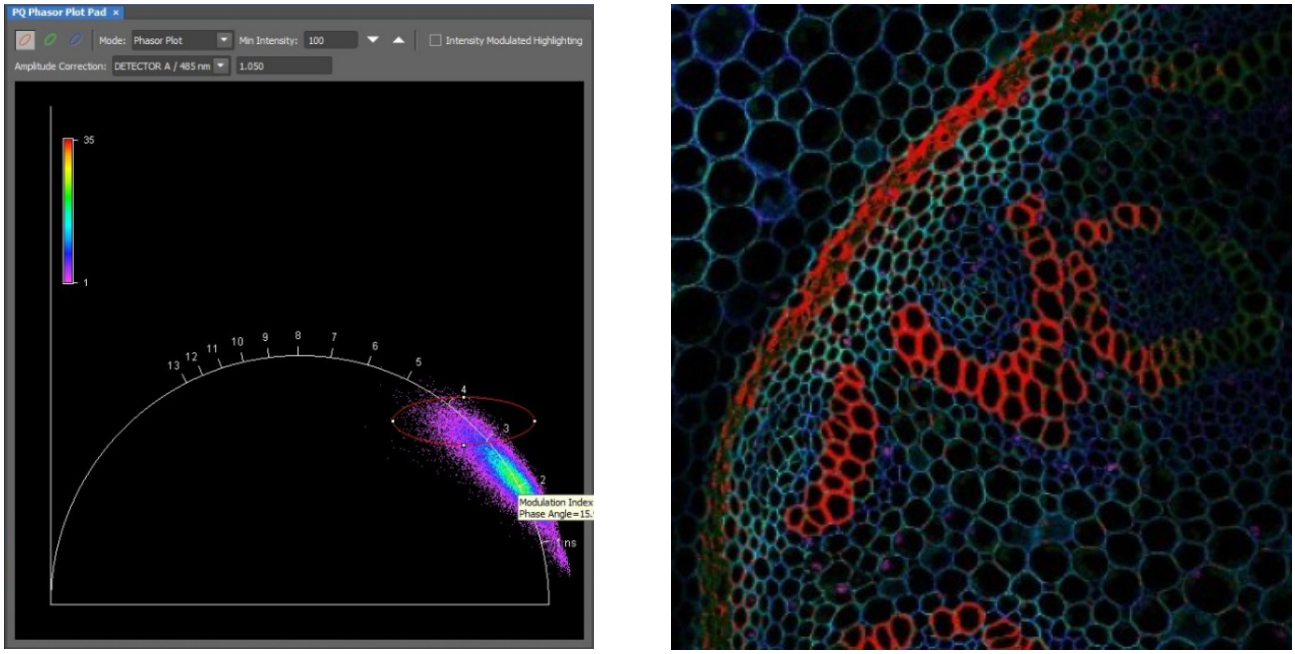


Fig. 5.1: Phasor Plot. Pixels marked by the red ellipse appear red in the FLIM image. By right-clicking the plot, it is also possible to select menu option to export the pixels selected to binary.

6. LUTs, Histogram and ROI Statistics

Standard NIS tools, LUTs, Histogram and ROI Statistics work in a similar way as with standard images. The graph in LUTs shows intensities as usual and by default the LUT autoscale function is enabled, but there are two fields added "Min Lifetime" and "Max Lifetime", where the range of FLIM / PhLIM color scale can be adjusted. By default, the range is set from 0 to 1/5th of the maximum lifetime detectable by the HW.

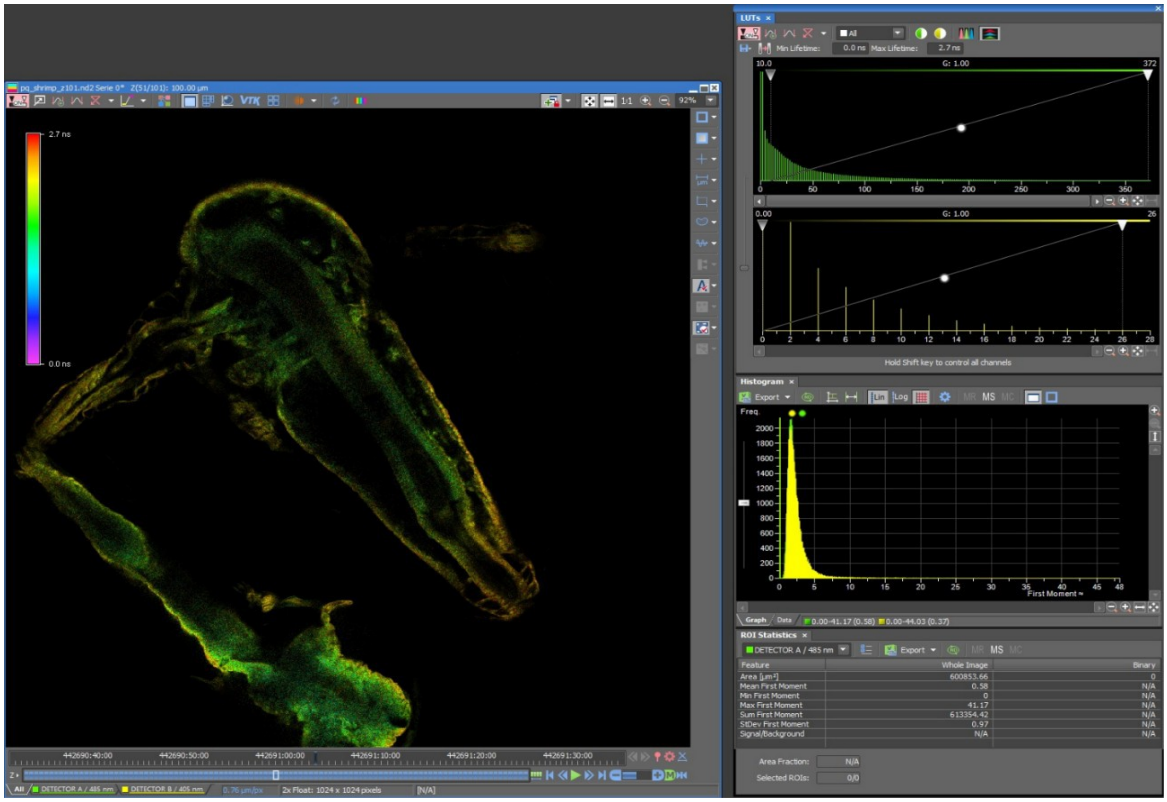


Fig. 6.1: Display of intensity and lifetime histograms. Lifestime range and intensity range (minimal and maximal number of photons displayed) can be set.

7. Troubleshooting

In general, make sure that all components of the Nikon AX system as well as of the Upgrade Kit are switched on.

7.1. Hardware configuration gets lost or SPT64 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 folder named C:\ProgramData\Laboratory Imaging\Platform.

In case the **hardware equipment is changed**, please contact PicoQuant. As the system is delivered already configured, it is not recommended to change settings without PicoQuant's supervision.

SymPhoTime: For storing individual user settings, they can be 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 will restart.

FLIM & FCS hardware settings in NIS software are accessible under “Device Setup” → “PicoQuant FLIM/FCS” → “Hardware Configuration”. A password is required to access these settings. Please ask PicoQuant if you need access to these settings.

7.2. Instrument is loosing sensitivity

Note:

- It is recommended to purchase a **laser power meter** in the lab in order to measure the laser intensities for selected wavelengths. This is especially important for FCS measurements since the laser power is a crucial parameter for FCS.
- If you have purchased a LSM FLIM / FCS Upgrade with 1 or 2 SPAD or Hybrid-PMT 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 (MB)** is a stable, characteristic value which can be used to monitor the instrument's performance. Suited dyes for different wavelengths are: ATTO655 for 635 nm, Rhodamine 6G for 532 nm or 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. In general, a 10 to 20 nM solution is used. For a day to day comparison, use the same laser power, best controlled with a power meter.
- If a performance drop is noticed, first the origin of the performance drop needs so be found. Please make first sure that the objective lens is clean. A drop in system performance may be further caused by:

1) Misaligned Pinhole

- Optimize the Pinhole Settings as described above. Use a high concentrated dye sample (approx. 100 µM) and optimize the detected fluorescence count rate.
- Set the laser intensity to observe approx. 2 Mcps. This will result in a smooth time trace curve and even a small misalignment can be corrected. Please note down the setting. In this way you can refer to it for later measurements.
- Note down the pinhole settings in X and Y.
- Optimize the count rate by changing the pinhole alignment.
    - In **“Device Setup”** → **“Pinhole”** the pinhole alignment can be performed

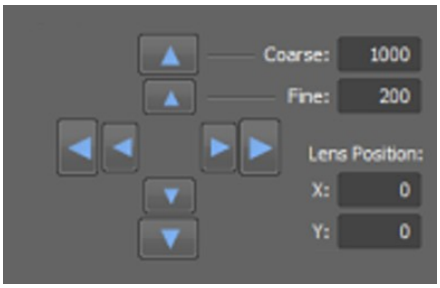


Fig. 7.1: Pinhole alignment.

2) Correction collar setting

- For FCS measurements in water rich sample, an apochromatic corrected water immersion objective with an NA of 1.2 or higher has to be employed. This objective leads to only minimal optical aberrations inside the sample. Optimize the correction collar in order to achieve the highest fluorescence count rate. Use a high concentrated dye sample (approx. 100 µM). Set the laser intensity to observe approx. 2 Mcps. This will result in a smooth time trace curve and even a small misalignment can be corrected.
- For FCS measurements, alignments 1) and 2) should be repeated in a regular fashion.

3) Decreasing Laser Performance

- 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. In this case please contact PicoQuant.

4) Decreasing Detection Sensitivity

- Misalignment of the LSM itself: The first important step is to align the pinhole (see above). If this procedure does not help, contact your Nikon service representative.
- Dirty objective. Please make sure the objective lens is clean.
- Misalignment of the detector(s): Refer to your detector manual.

7.3. No image is displayed during measurement

Can laser light be seen over the objective during measurement?

If no:

- Is the "**Sync Rate"** correct? The sync rate is displayed in the AX Pad.

When starting a TCSPC measurement in the "Live" mode, dark counts should be displayed. A peak must not necessarily appear, but at least background noise should be displayed. If the repetition rate is 0, check whether at the PDL800-D the SYNC is set to internal or, if you use a Multi Photon Excitation (MPE) laser, if the laser is switched on.

If yes:

- Do you **see dark counts** at the detector count rate meter (compare Fig. 3.4). If not, check whether the detectors are still on. In case of SPAD detectors, the DSN 102 - if applicable - shuts down a detector automatically if the count rate exceeds a limiting count rate. Please make sure that the SPAD detectors are switched on at the DSN 102.
- Check, whether **correct emission filters** are placed. If you have a 2 - Detector unit, make sure that the beam splitter is selected accordingly.

- When all detectors are on and background counts are displayed in the TCSPC window, place a **fluorescent sample** on the Nikon microscope, set all settings in the Nikon software for FLIM and start scanning, and open the “Time Trace Pad”. 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 (the filter holder should be placed correctly in the detector unit). Shutters should click softly when opening and closing the filter lid.
- 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.
  Open the measurement in SymPhoTime. **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 Nikon AX and the LSM FLIM / FCS Upgrade (TCSPC Unit). Also try to start the Nikon AX 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 recorded FLIM file to PicoQuant.

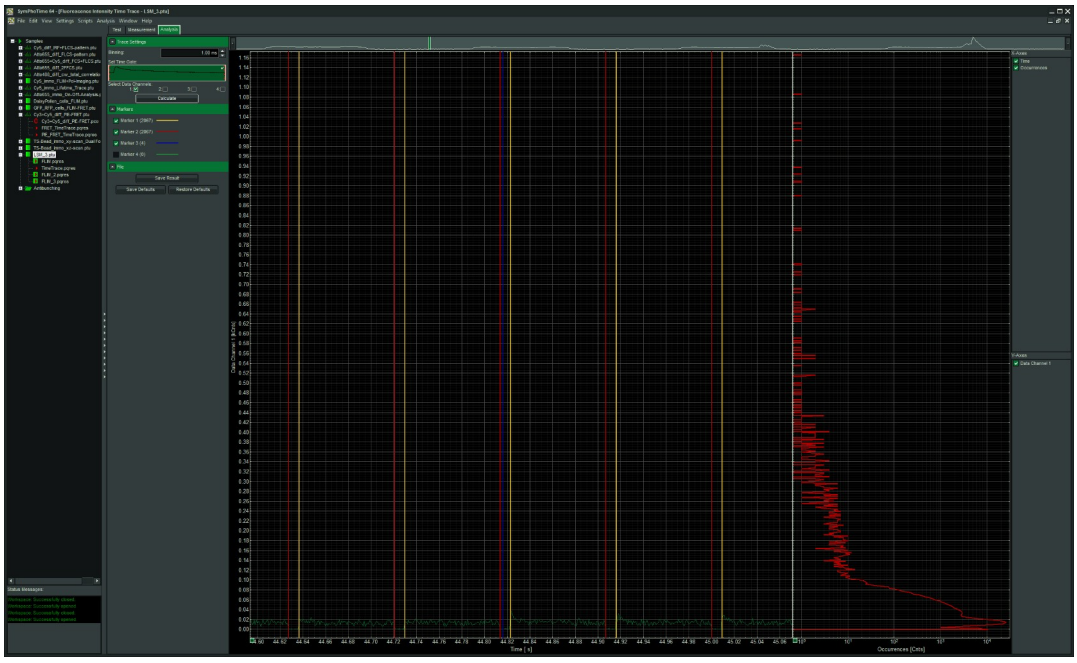


Fig. 7.2: The Time Trace analysis, where the line and frame - markers can be displayed in yellow and red.

8. Recommended literature

Publications related to the LSM FLIM / FCS upgrade hardware, software, and underlying key technologies can be found on the Web. Please visit the LSM FLIM / FCS Upgrade section of
http://www.picoquant.com/scientific

and also the TCSPC – Wiki:
http://tcspc.com/

9. Abbreviations

BNC	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

10. 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 https://forum.picoquant.com).

In any case, we would like to offer you our complete support. Please do not hesitate to contact Nikon 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 https://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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PicoQuant GmbH

Rudower Chaussee 29 (IGZ), 12489 Berlin, Germany

Telephone: +49 / (0)30 / 1208820-0
Fax: +49 / (0)30 / 1208820-90
e-mail: info@picoquant.com
WWW: https://picoquant.com