

LSM Upgrade Kit

Compact FLIM and FCS
Upgrade Kit for **Nikon A1**
Laser Scanning Microscopes
with Software Integration



User's Hardware Manual and Technical Data

Version 3.9

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1. Introduction

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

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

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.

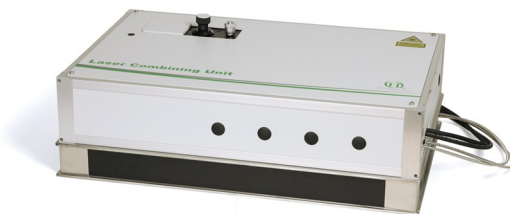


Fig. 1.1: Laser Combining Unit (LCU)

- **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.

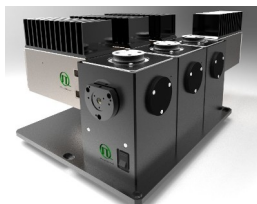


Fig. 1.2: Detection Unit for LSM FLIM / FCS Upgrades:
Multi channel PMA / SPAD detection unit

- **MultiHarp 150 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 (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.
- **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. Operation

2.1. Safety Instructions



Laser Warning

The LSM FLIM / FCS Upgrade is 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 PDL diode laser operation manual. If your instrument uses another excitation system, follow the safety instructions of the relevant manual.

Please check that the actual line voltage corresponds to the value set on the PDL-800-D laser driver!

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

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

2.2. Starting Hardware and Software Equipment

To **start** the LSM itself, its detectors and cw lasers please refer to your Nikon A1 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 Nikon 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 the **PC**.
4. Start the **SymPhoTime 64 software**.
5. **Open** an already existing **workspace** or create a new one.
6. Start **NIS** software
7. Place correct **fluorescence filters** in the corresponding filter holder position of the external PicoQuant detector unit and the confocal microscope. Switch the detector unit on.
8. If you want to work with user specific settings, load your **user configuration (SymPhoTime)** and **optical configuration (NIS)**. The

Upgrade is now ready to use.

2.3. Preparing NIS for FLIM and FCS

The easiest way to acquire FLIM or FCS measurements is by presetting defined **layout and optical configurations** in the NIS software.

- **Layout:** arrangement of the main window which displays all the necessary controls to perform the measurements. It can be common for FLIM and FCS measurements. It is selected at the bottom bar of the NIS software.
- **Optical configurations:** arrangement of the optical and electronic settings to perform the measurement. It is useful to create at least one standard configuration for FLIM and another for FCS. It is selected at the top bar of the NIS software and buttons can be configured named e.g. “FLIM” and “FCS”.

The Layout and optical configurations are normally prepared during the system installation. Shall this be the case continue in section 2.4 or 2.5

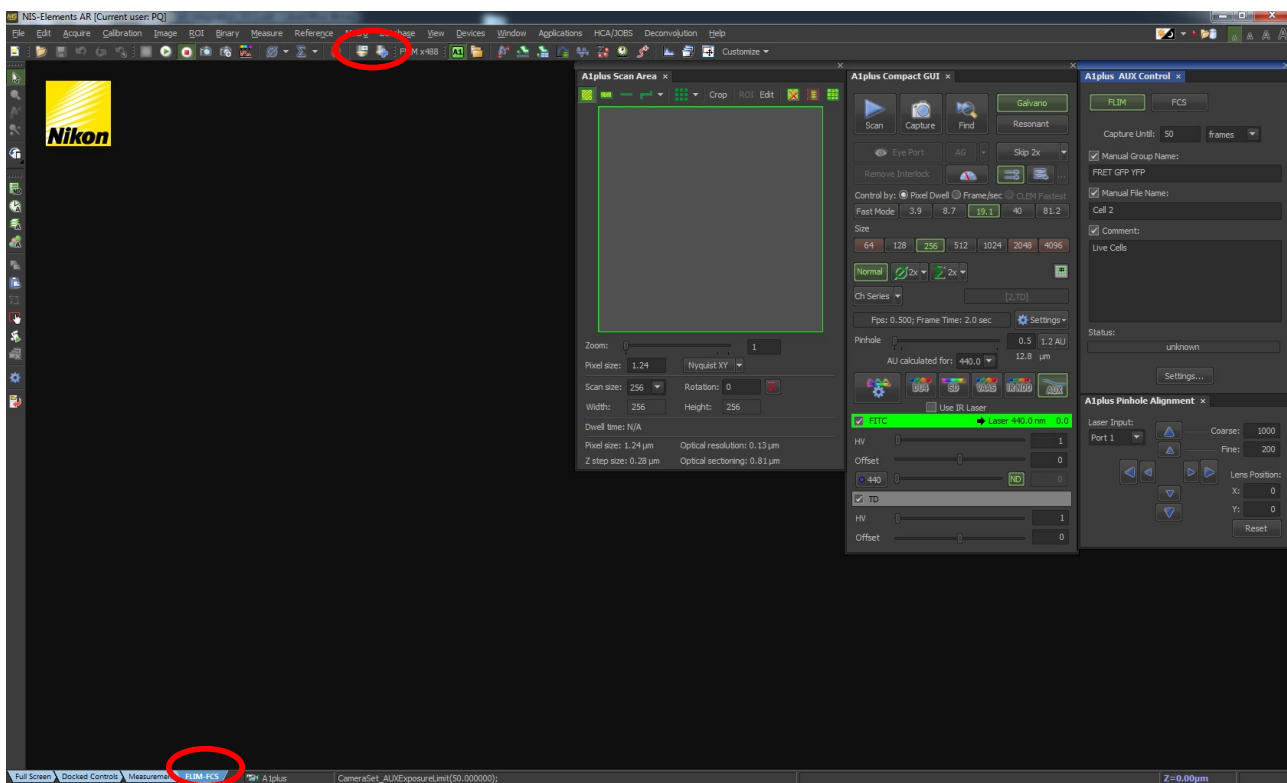


Fig. 2.1: Layout for FLIM and FCS measurements

2.3.1. Creation of a Layout for FLIM/FCS measurements

In order to perform FLIM or FCS measurements the **AUX port** must be displayed and enabled. Furthermore, the **A1plus AUX control**, **A1plus Compact GUI**, **A1plus Pinhole alignment** and **A1plus Scan Area** should be open.

1. In the main NIS window do right click, go to **Acquisition Controls** and then Click in **A1plus AUX control**, **A1plus Compact GUI**, **A1plus Pinhole alignment** and **A1plus Scan Area**. If applicable, open also the **A1 PDL Pad** for the laser settings of pulsed lasers.

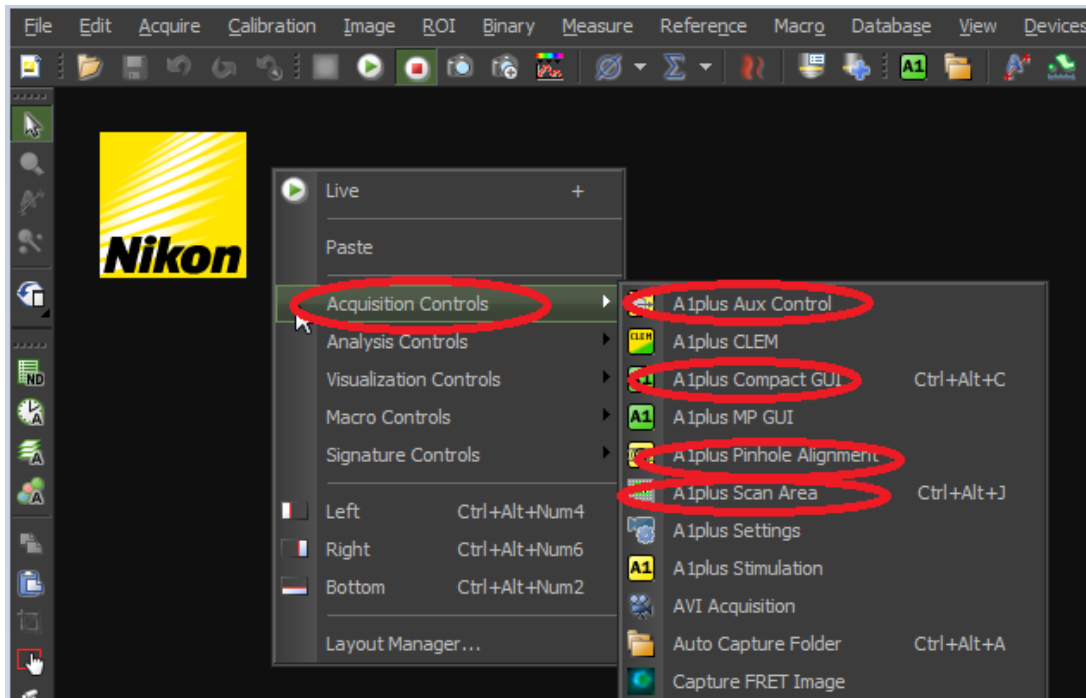


Fig. 2.2: Activation of the necessary windows to perform FLIM and FCS measurements.

Upon clicking on each of them its corresponding window will be opened.

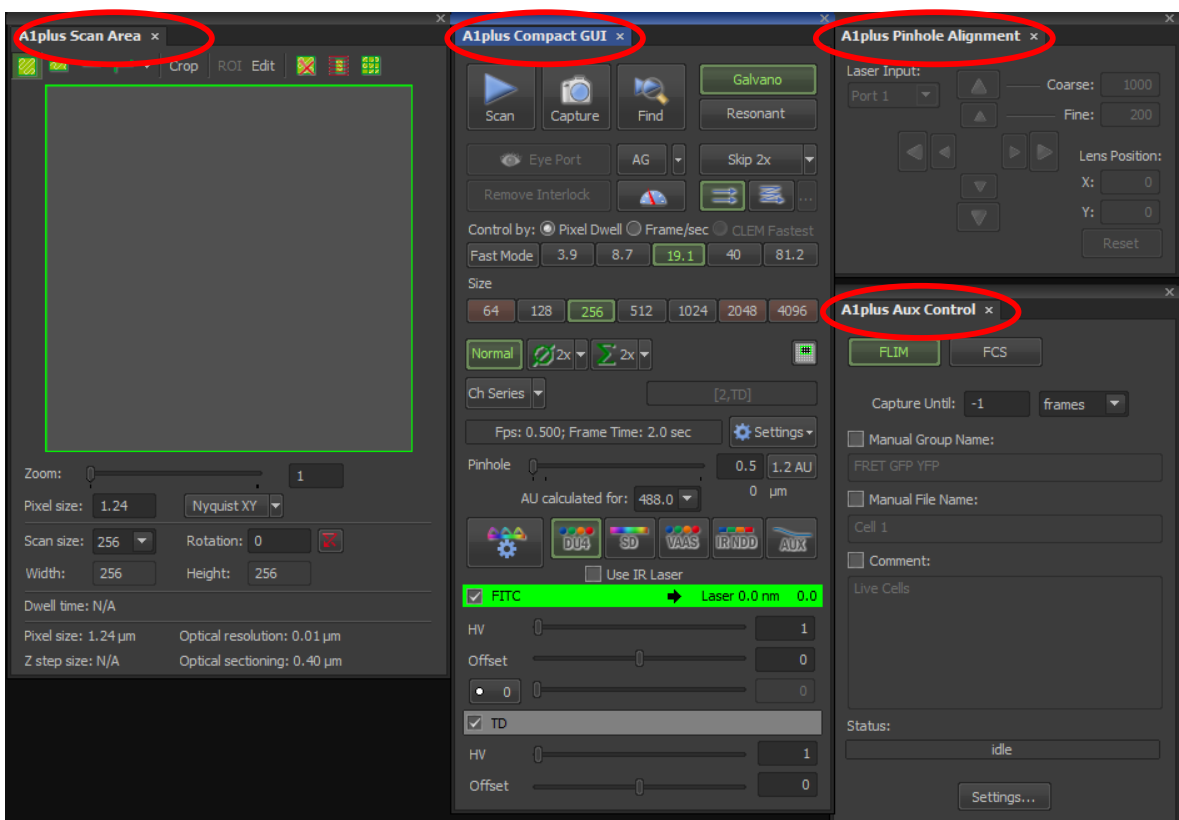


Fig. 2.3: Windows to perform FLIM and FCS measurements

2. In the case that the AUX port was not visible in the **A1plusCompact GUI**, the **A1plus Aux Control** will not be displayed from the drop-down menu of the **Acquisition Controls**. In this case go to the **A1plus Compact GUI** and click in the **Settings** button. Then click in **Show AUX Detector**.

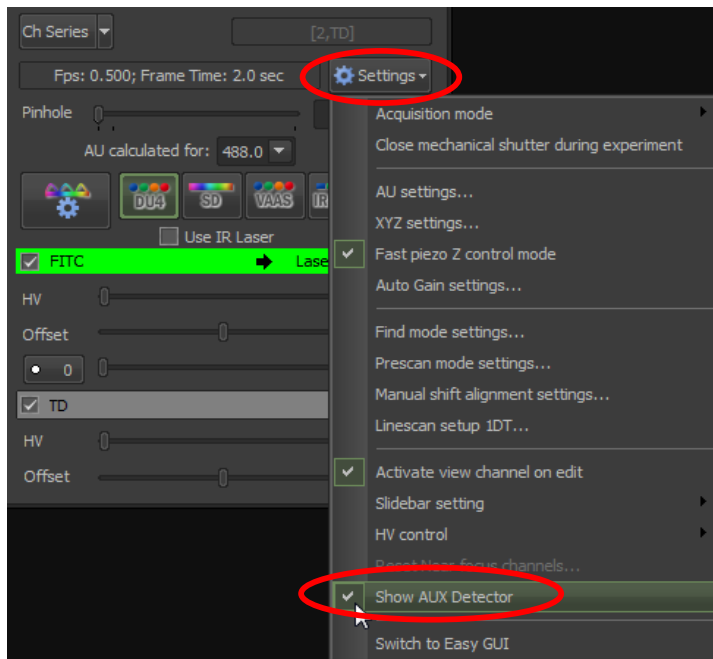


Fig. 2.4: Activation of the AUX display, in the A1plus Compact GUI.

3. To activate the AUX port click on its icon. Upon activation the icon will be highlighted and the NIS acquisition will follow the FLIM/FCS sequences.

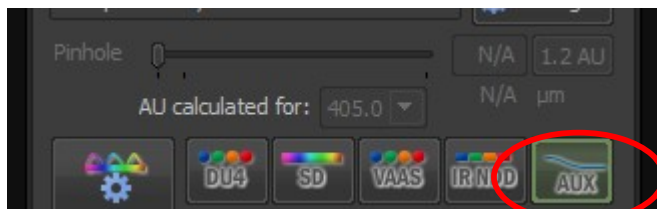


Fig. 2.5: When the AUX port is activated is becomes highlighted.

4. Now the Layout is ready for FLIM/FCS acquisitions. At the bottom of the NIS window do right click in one of the existing Layouts and then save as FLIM-FCS or create a new one.

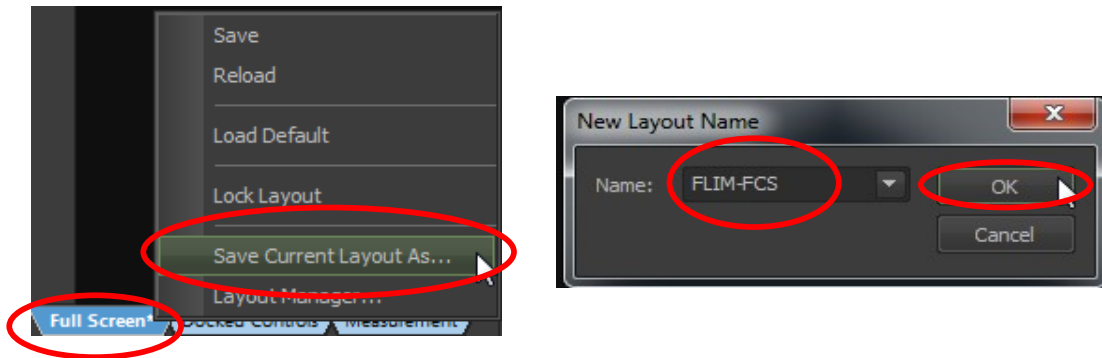


Fig. 2.6: Saving of the newly created FLIM-FCS layout.

The layout for FLIM and FCS measurements is now saved.

2.3.2. PicoQuant laser control in NIS using PQ PDL Pad (optional)

It is possible to control the PicoQuant Laser driver settings (PDL 828) via NIS software using the **A1plus PQ PDL**.

1. In the main NIS window do a right click, go to **Acquisition Controls** and then select **A1plus PQ PDL**. Upon clicking on **A1plus PQ PDL** its corresponding window will be opened.

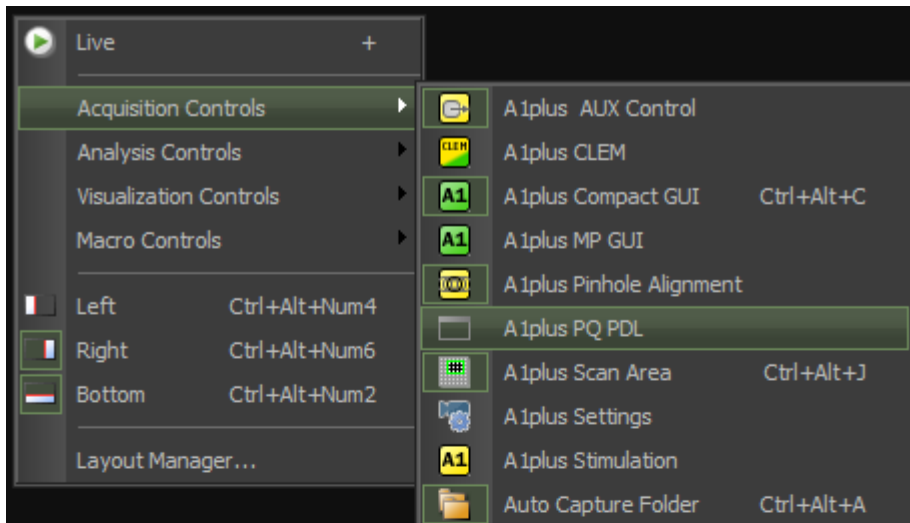


Fig. 2.7 Activation of the A1plus PQ PDL window to control the PicoQuant Laser Driver in NIS Software

2. the appearance and features of the **A1plus PQ PDL** window depend on the LCU-Type that is used.

A) **LCU without motorization** (see Fig. 2.8 left):

In case that you use the system without motorized attenuators in the LCU it is only possible to change the electrical laser power via the sliders.

B) **LCU with motorized attenuators** ((see Fig. 2.8 right):

In case that you use the system with motorized attenuators in the LCU it is possible to set the intensity of the lasers during a measurement using the sliders shown in Fig. 2.8 on the right side. The electrical laser power can be set to a certain value in the field named "Head". Here it is useful to select a value which corresponds to the laser power that leads to the smallest laser pulse possible. By clicking on "**Configure**" a **PicoQuant PDL Configuration** window will pop up. The correct attenuators used for the respective wavelengths are set during the installation of

the system. Attention: to access this configuration, the windows display settings scaling factor needs to be set to 100%. The setting is preset during installation of the system.

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

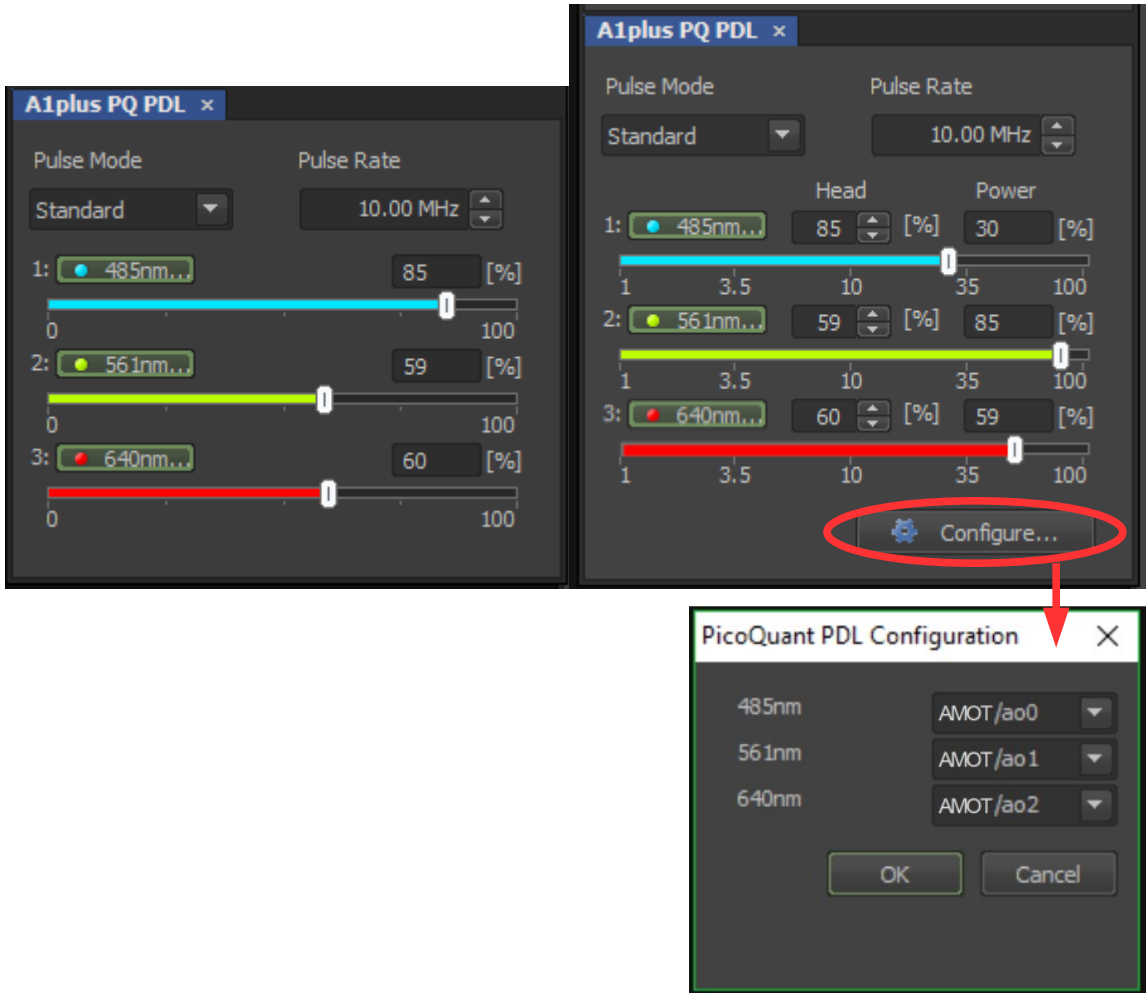


Fig. 2.8 A1plus PQ PDL window, **left:** LCU without motorization The sliders can be used to change the electrical laser power. **right:** LCU with motorization. The electrical laser power can be set in the field named “Head”, while the laser attenuation can be configured using the sliders or the field named “Power”. In the PicoQuant PDL Configuration window the analogue output lines of the NI board can be selected. They are preset during installation of the system.

3. It is possible to select or deselect the lasers by clicking on 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. (see Fig. 2.9)

Please note that there is a special feature of the PDL pad 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.

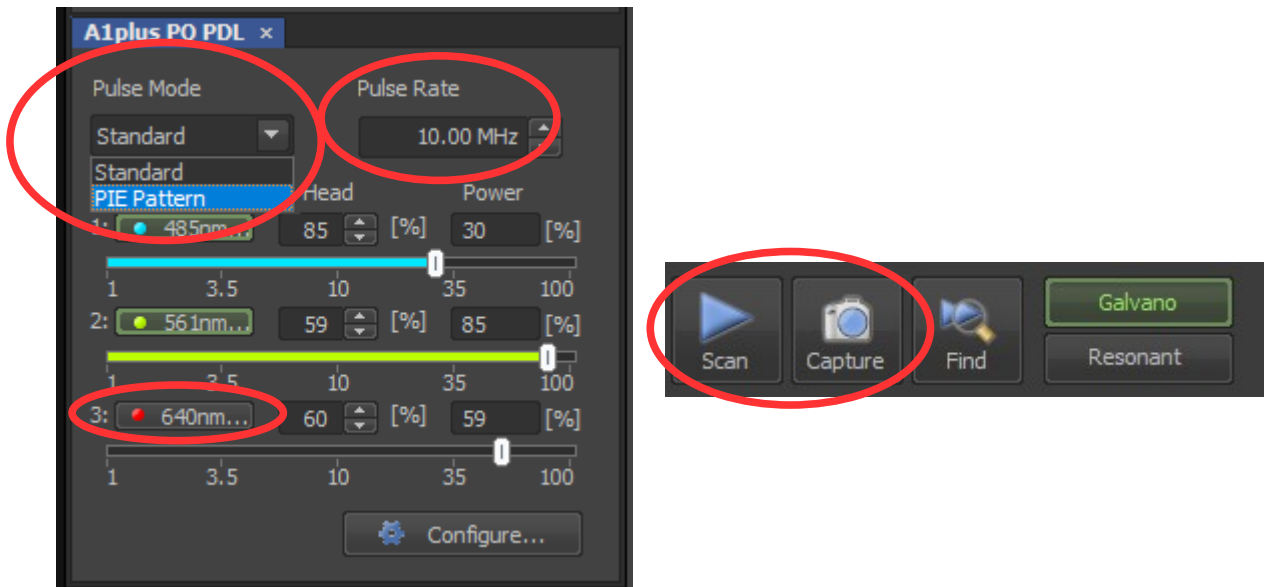


Fig. 2.9 Selection of Pulse Mode, Pulse Rate and Lasers and transfer the settings by starting a measurement.

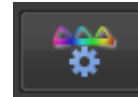
2.3.3. Creation a Configuration for FLIM measurements

The configurations pre-sets the optical and electronic settings in the Nikon A1 to perform the measurements:

- Excitation laser port (1, 2, 1+2)
- Excitation laser
- Main dichroic
- Scanning mode (Scanning mirrors, Scanning speed, mono- / bidirectional, averaging...)
- Pixel size
- Pinhole size

It should be configured depending on the specific optics and lasers in your LSM, as well as depending on the type of experiments.

As an example lets imagine that the A1 confocal microscope is equipped with two pulsed PicoQuant lasers at 440nm and 488nm, as well as with several CW lasers from Nikon. Lets imagine we want to perform a FLIM image exciting with the 488 pulsed laser from PicoQuant and that we want image with a PicoQuant detector containing a filter in the green region.



1. In the **A1plus Compact GUI** click in the **Optical Path Settings**

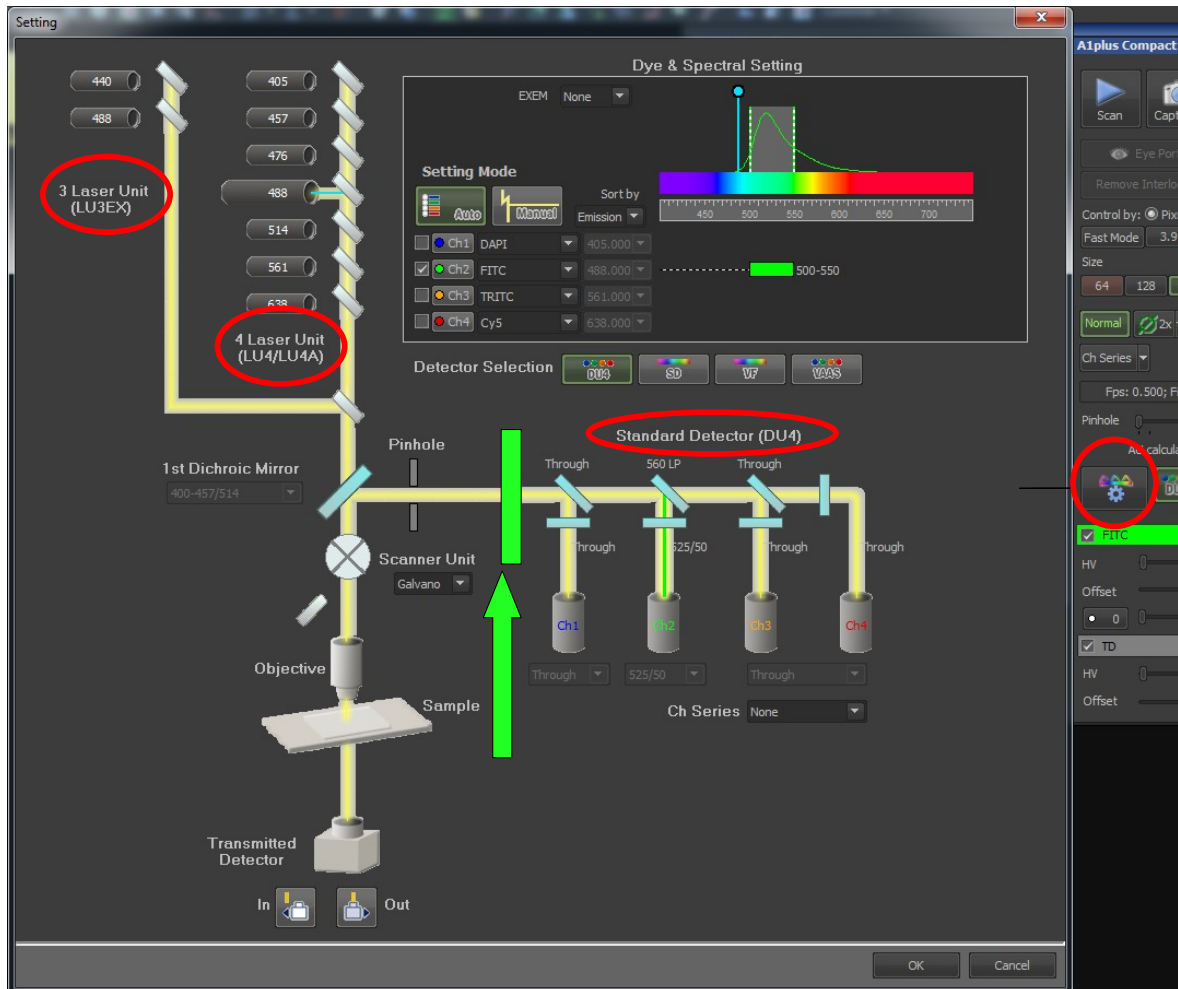


Fig. 2.10: Optical Path Settings. Please be aware when using the Nikon laser combiner LUN-V, the PicoQuant pulsed laser can not be displayed in the Settings window. PicoQuant pulsed lasers are selected with the “port” selector, PicoQuant lasers are at port 2, Nikon lasers at port 1.

The window shows a diagram of the Optical Path. Pulsed lasers from **PicoQuant (3Laser Unit)** and cw lasers from **Nikon (4 Laser Unit)** are displayed in the upper left part of the window (PicoQuant lasers are not displayed in case a Nikon laser combiner LUN-V is used). In case of LUN-V laser combiner, PicoQuant lasers are selected by the port switch. PicoQuant lasers are at port 2, while the Nikon lasers are at port 1. Detection channels from **Nikon (DU4)** are shown in the central part of the Window. The PicoQuant detectors are not displayed in this optical diagram. There is an optical element placed between the pinhole and the Nikon Detection Channels that deviates the light towards the PicoQuant detection. This element is not present in the Optical Path Settings window, however in the image above its position is marked with a green arrow.

2. To be able to select any excitation laser, the **Setting Mode** has to be set to **Manual**. Then, the list of all available lasers can be opened from each Nikon detection Channel. Lasers are ordered according to their wavelength, but separated in two groups. First appear the lasers from the Nikon laser combiner and after the lasers from the PicoQuant laser combiner (not applicable for Nikon laser combiner LU-N).

Despite that for FLIM the Nikon detectors (DU4) will not be used, it is useful to select a channel in the NIS software which resembles the PicoQuant detector. This will enable image comparison between normal confocal and FLIM, both with the same settings, by switching between the DU4 and AUX ports, respectively.

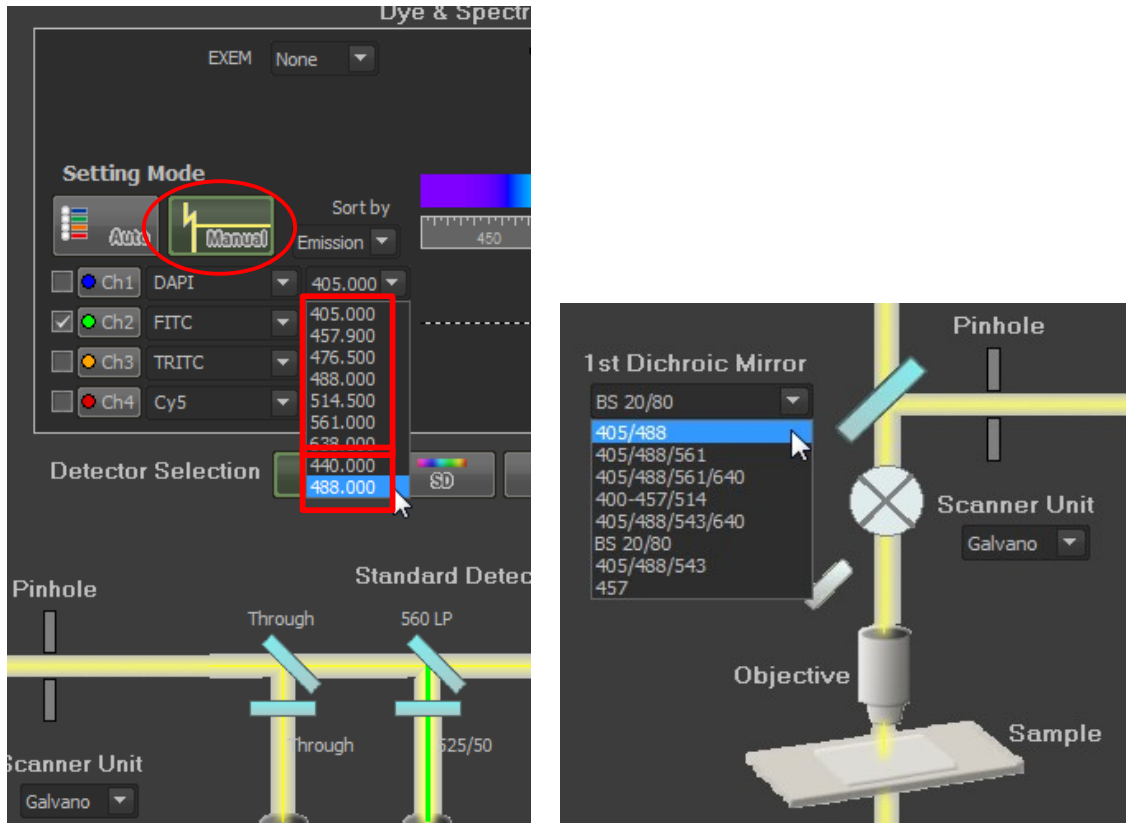


Fig. 2.11: Change the Setting Mode to Manual in order to be able to select any of the lasers. Pick a suitable excitation dichroic for the combination of excitation and detection selected.

3. Choose a **suitable excitation dichroic** (see Fig. 2.11) for the pulsed excitation wavelength. Recommended dichroics for PicoQuant pulsed diode laser wavelength can be found in table Tab. 2.1:

PicoQuant Laser	Diode	LDH 405	LDH 440	LDH 485	LDH 510	LDH 530	LDH 640
Recommended Nikon A1 excitation dichroics		405/488	457/514	405/488	20/80	20/80	405/488/561/640
		405/488/561		405/488/561/640			405/488/543/640
		405/488/561/640					
		405/488/543/640					

Tab. 2.1: Suited dichroic mirrors for using with PicoQuant diode lasers

4. In the **A1plus CompactGUI** window some settings also have to be selected. For standard FLIM measurements:
 - Scan with the Galvano mirrors (Resonant can not be used for FLIM).

- Mono-directional scan (bidirectional sometimes leads to artefacts).
- Scanning speed with a pixel dwell time of 2 μ s or higher (higher scanning speeds will lead to incorrect FLIM data).
- Size of 512 x 512 pixels.
- No averaging (averaging can not be interpreted by the SPT64) → “Normal” must be selected.
- Pinhole size of 1.2 AU (you may increase it if the signal is too low, at expenses of confocal contrast).
- Select the proper excitation laser for the calculation of the pinhole AU.
- Click in the **AUX** to open the light-path towards the PicoQuant detectors and to enable FLIM acquisition.
- **Attention:** Channel series (CH Series) as well as averaging (→ should be on “Normal”) must be switched off in order to activate the AUX button.

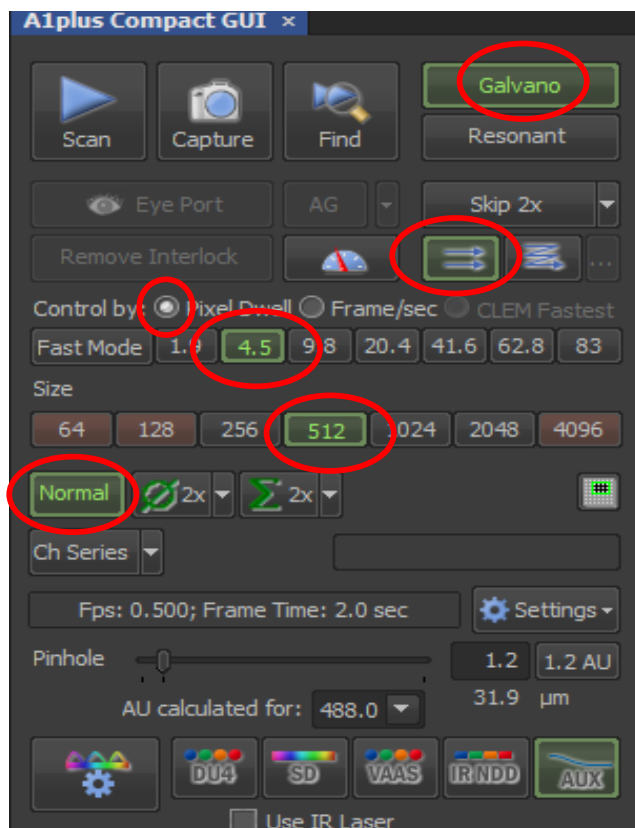


Fig. 2.12: Standard FLIM settings for the A1plus Compact GUI.

- In the **A1plus Pinhole Alignment** window the excitation port can be selected:
 - **Port 1** corresponds to the Nikon laser combiner.
 - **Port 2** corresponds to the PicoQuant Laser combiner.
 - **Port 1+2** corresponds to simultaneous excitation with the Nikon and PicoQuant laser combiners.

For FLIM measurements select **Port 2**. Note that the **A1plus Pinhole Alignment** also enables to optimize the pinhole position (this will be important for FCS).

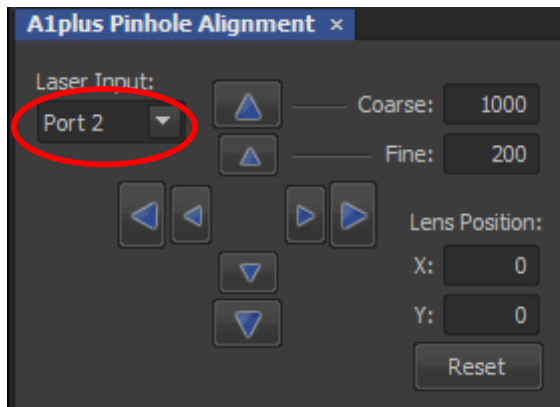


Fig. 2.13: Selection of Port 2 for pulsed excitation with PicoQuant lasers.

- In the **A1plus AUX control** window select FLIM as well as the criterion to select the duration of your FLIM measurement (number of frames, time or accumulated counts).

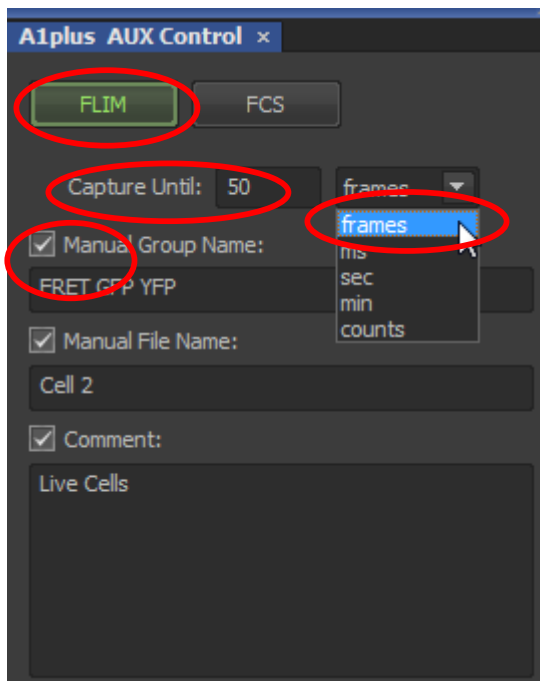


Fig. 2.14: Selection of FLIM or FCS as well as the duration of the measurement. In addition the names for the measurements in the SPT can be set. The group name opens a folder in the SPT with the given name. Comments can be written in the section “comment”. They will be displayed in the SPT software when double-clicking on a recorded *.ptu file.

- The configuration for FLIM measurements is now ready and can be saved. In the top bar of the NIS software click in the icon of *New Optical Configuration*, name it for example as FLIM x488 (i.e. FLIM with excitation at 488) and click **Finish**. The new configuration will be now permanently displayed in the top bar of the NIS software. You can also create further configurations for other excitation lasers or measurement conditions.

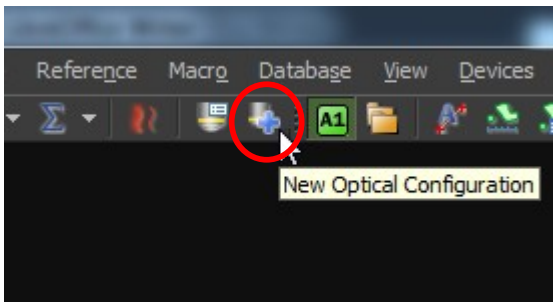


Fig. 2.15: Creation a new Optical Configuration

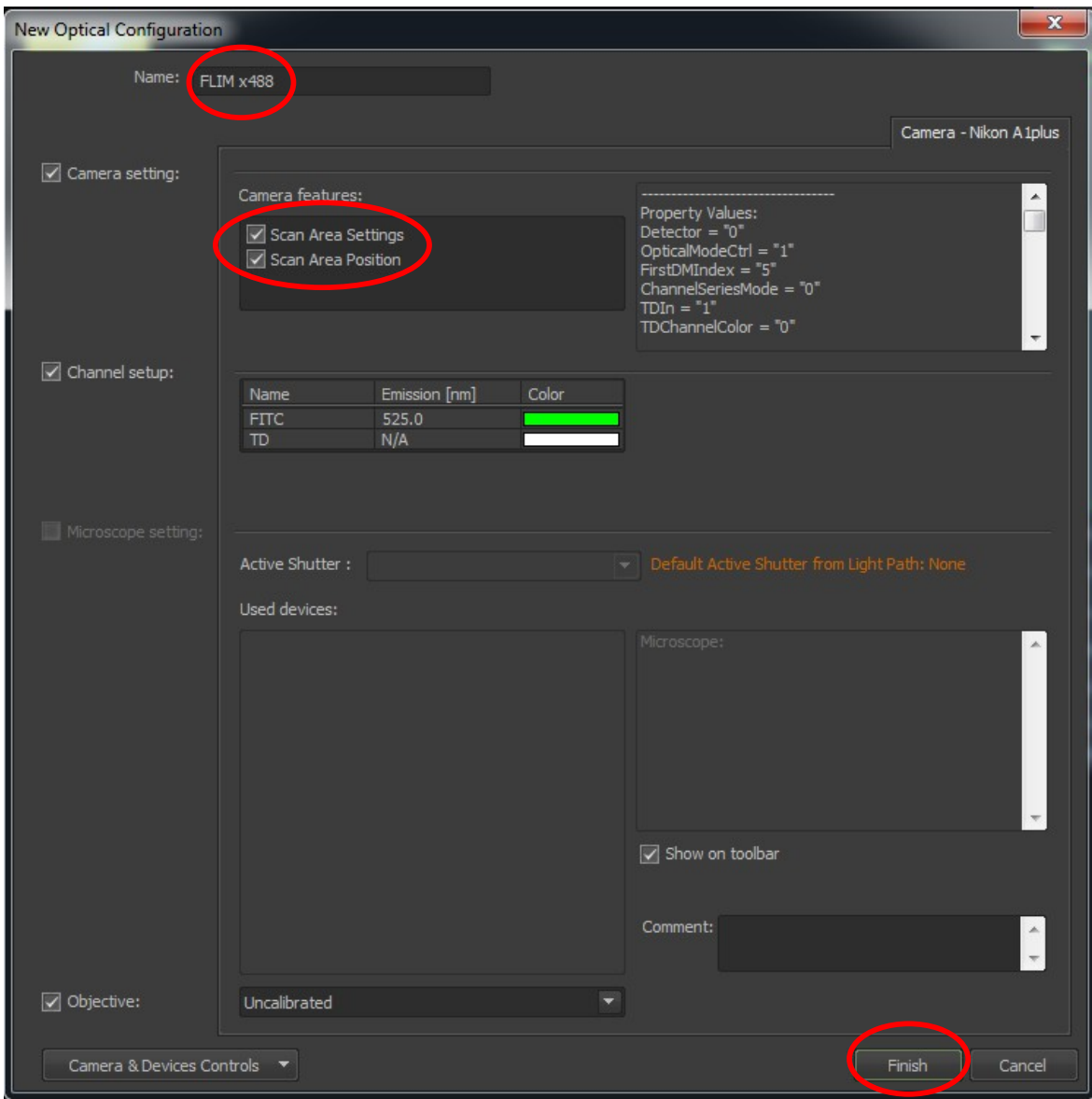


Fig. 2.16: Saving of an Optical Configuration. If "Scan Area Settings" and "Scan Area Position" are not checked, the "Optical Configuration" will not store these settings.

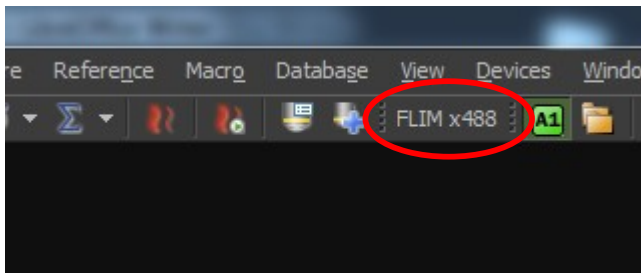


Fig. 2.17: User configuration “FLIM x488” in the top bar of the NIS main window.

2.3.4. Creating a Configuration for FCS measurements

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** and **Port 1+2** in the **A1plus Pinhole Alignment**, respectively).

Lets imagine we would like to excite simultaneously with a PicoQuant laser at 488nm and with continuous CW laser from Nikon at 560nm.

1. In this case we select both lasers from the Optical Path Settings Menu and we choose a dichroic suitable for both excitations.

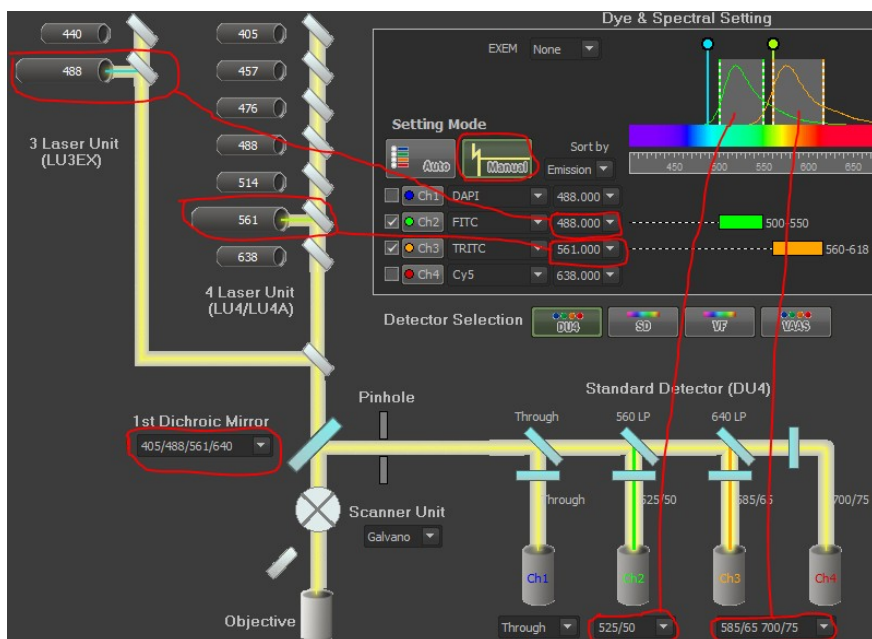


Fig. 2.18: Optical configuration with simultaneous Cw excitation from Nikon at 560nm and pulsed from PicoQuant at 485nm. Please note that with Nikon laser combiner LUN-V the PicoQuant pulsed lasers are not displayed. They can only be selected by using the Port – selector for Port 1 (Nikon lasers) and Port 2 (PicoQuant lasers).

2. In the **A1plus Pinhole Alignment** we select **Port 1 + 2** and In the **A1plus Scan Area**, select the point scan area. This can be set to a free point or to arrays with several points (2 x 2, 4 x 4, etc...). Please note that in older Nikon A1 instruments the “Port 1+2” selection will lead to very low laser power or bad results due to non optimal alignment of this configuration.

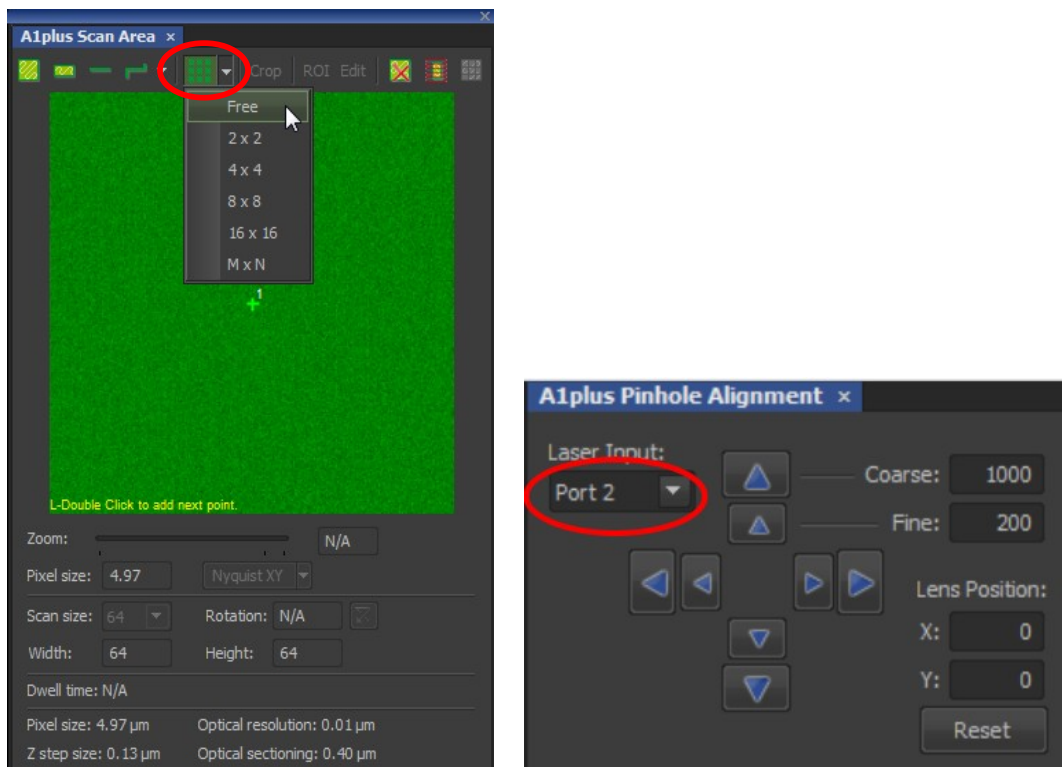


Fig. 2.19: Selection of the point location and excitation port.

3. In the **A1plus Compact GUI** select the AUX port and the pinhole size. Select the laser for which the pinhole AU is calculated (the laser with longer wavelength, in this case 560nm). In the **A1plus AUX Control** select FCS and to capture to a given number of seconds. Set the pinhole size to 1 AU.

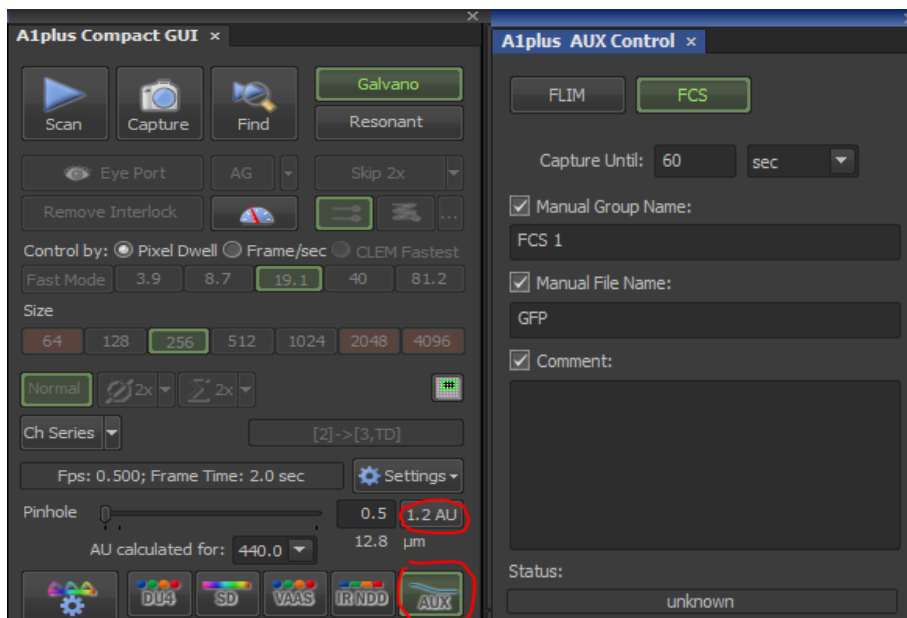


Fig. 2.20: Activation of the AUX port. Selection of FCS as well as the duration of the measurement. In addition the names for the measurements in the SPT can be set. The group name opens a folder in the SPT with the given name. Comments can be written in the section “comment”. They will be displayed in the SPT software when double-clicking on a recorded *.ptu file.

4. The configuration is now ready and can be saved. For saving follow the same steps described in the previous sections.

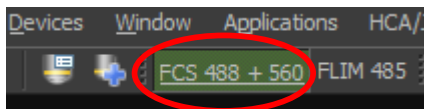


Fig. 2.21: Icons for the two newly created configurations for FLIM and FCS.

2.4. Acquisition for FLIM

1. **Nikon Software NIS:** A region of interest is identified
2. **NIS and SPT64:** The best conditions for the FLIM are determined with a “Test” measurement. These are the excitation and detection rates.
3. **NIS and SPT64:** The FLIM measurement is performed.

2.4.1. Starting Point in NIS

Before taking a FLIM image, the target ROI has to be identified. This is normally performed with the Nikon A1 (cw lasers and Nikon detectors). Once the ROI is identified and in focus, the user can change to FLIM acquisition. There are two convenient ways in which the user can switch between normal confocal from Nikon and FLIM:


- Create an additional NIS user configuration for normal confocal with the same settings as for FLIM, but with the exception of the excitation port and excitation laser.
- Within the FLIM configuration switch between the detection ports AUX and DU4 in the **A1plus Compact GUI**.

The first option is preferred when the wavelength of the PicoQuant lasers is also available in the Nikon laser combiner. In this way the user can fully tune in the NIS software the excitation and detection to obtain the best confocal image. The second option is preferred when the wavelength of the PicoQuant laser is not available in the Nikon laser combiner. In this case the user can tune with the NIS the detection gain. However the excitation power will have to be tuned manually at the PicoQuant laser combiner.

From this point the manual assumes that the target ROI is identified and in focus.

2.4.2. Determining the best conditions for a FLIM measurement

The goal during the optimization is to determine the **best excitation and detection rates** with a “Test” measurement. Note that “Test” measurements are not saved.

1. Turn on SPT64 software and PicoQuant equipment (laser and laser drivers, electronics and detection unit) and place the desired emission filter in the detection unit.
2. In the NIS software Activate the **FLIM configuration** and click . A test measurement will be automatically initiated in the SPT64 software.

- In the SPT64 software go to **TCSPC** and observe the decay behavior. Tune the laser intensity with the attenuation knob at the laser combiner (or directly at the laser attenuation knob in the case you only have one pulsed laser) till the observed decay curve has a peak between 10^3 and 10^5 counts.

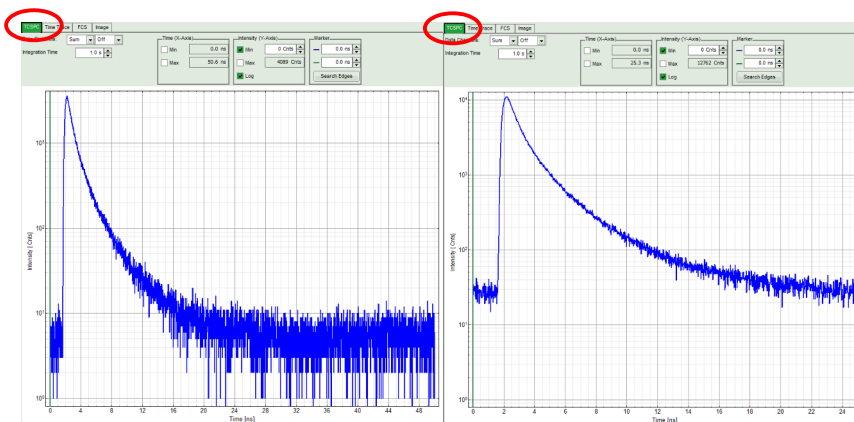


Fig. 2.22: Fluorescence decay at unnecessary low (left, 20 MHz) and optimal (right, 40 MHz) laser repetition rate. With an even higher repetition rate, a part of the fluorescence would appear at the beginning of the TCSPC-window.

- 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 pulses¹. 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 window².

For PicoQuant pulsed diode lasers the **laser repetition rate** (called Sync rate in the SPT64) can be set at the PDL laser driver or – if the lasers are controlled with a SEPIA II – in the “Laser Settings” drop-down – menu of the SymPhoTime 64 - software.

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

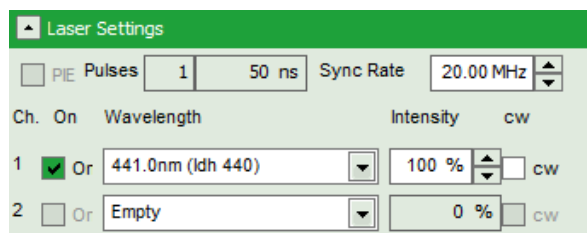


Fig. 2.23: Laser settings in SPT64.

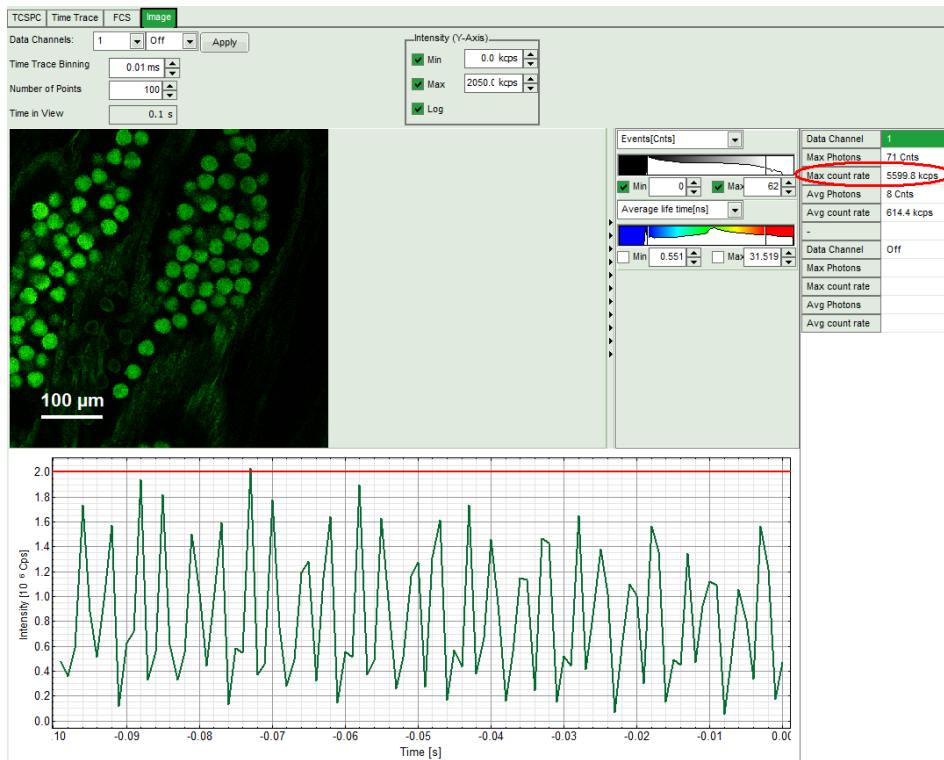
- For example, the time between 2 laser pulses at 80 MHz repetition rate is 12.5 ns. If a fluorophore with 6 ns lifetime is measured, more than 12 % of the photons are emitted due to the statistical process after 12.5 ns. In this case the repetition rate has to be lowered.
- In the example above at 40 MHz the **time window is optimally adapted**, while at 20 MHz the fluorescence has already decayed at less than half of the detection window. 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. This would modify the lifetime results.

5. Change to the **Image** tab in the SPT64. The goal now is to **set the count rate in the brightest pixel at ca 10% with respect to the excitation rate**. Furthermore, for Excelitas and τ -SPAD detectors, the maximum count rate should never exceed 1MHz¹. High count rates can lead to distorted decays due to the pile up effect². The count rates can be monitored in the Time Trace below the FLIM image (Fig. 2.24).

For the setting of the correct laser intensity the user can look at the Time Trace, displayed below the FLIM image in the Test mode. The red line indicates a threshold of 10% of the repetition rate. The intensity spikes should always remain below this line in order to avoid pile up. For a proper monitoring of this time trace set the "Time trace binning" to 0.01 ms. That means that each point in the time trace corresponds to 10 μ s recording time. If the pixel dwell time is set to \sim 5 μ s then a point in the time trace is the average count rate of two adjacent pixels.

Systems with the TimeHarp 250 N using the rapidFLIM technology are not restricted to these count rate limits.

1 Some detectors introduce artefacts when being operated at high count rates. The AQR type SPAD from Excelitas above 1 MHz photon count rate show significant IRF broadening and a count rate dependent IRF shift.
2 For more information about the pile effect and electronics dead-time please refer to the TCSPC card manual.



PDL setting (Laser repetition rate)	Upper count rate limit for different detectors [kcounts / s]		
	PMA PMT, PMA Hybrid or MPD SPAD	Excelitas SPAD	τ-SPAD
1 (40 MHz)	4000	1000	1000
2 (20 MHz)	2000	1000	1000
4 (10 MHz)	1000	1000	1000
8 (5,0 MHz)	500	500	500
16 (2,5 MHz)	250	250	250

Fig. 2.24: Top: Online monitoring of the of the photon count rate in the SymPhoTime 64 preview window. On the left, a FLIM image is displayed. The intensity time trace is shown below. An intensity of 10% of the repetition frequency is indicated by a red line in the graph (laser repetition frequency was set to 20 MHz). Please select a “time binning” of 0.01 ms (10μs) for correct display of the peak countrates.

The laser power should be adjusted that the count rate does not exceed the red line. In the table the count rate limits are displayed for different combinations of excitation repetition rates and detectors.

The “Max count rate” displayed on the right side can be inaccurate since it is calculated on a single pixel basis which might not have enough photons for an accurate measurement of the maximal count rate. P

2.4.3. FLIM measurement

1. **SPT64 software.** Create a Workspace or open an already existing one.
2. **NIS software.** Go to the **A1plus AUX control** select **FLIM**, **File Name** and if relevant also a Group Name and a Comment. These names will be transferred to the SPT64. The advantage of saving files under the same Group Name is that all the individual measurements can be easily analysed together with the same analysis settings. Select also the **“Capture until” criterion**.




3. Press  to start the measurement. Sample names along with the measurement settings are automatically transferred to the SPT64.



Fig. 2.25: Selection of a FLIM measurement up to 50 frames, and controls to start the measurement. “Scan” makes a test measurement, which is not saved, and “Capture” makes a final measurement which is saved.

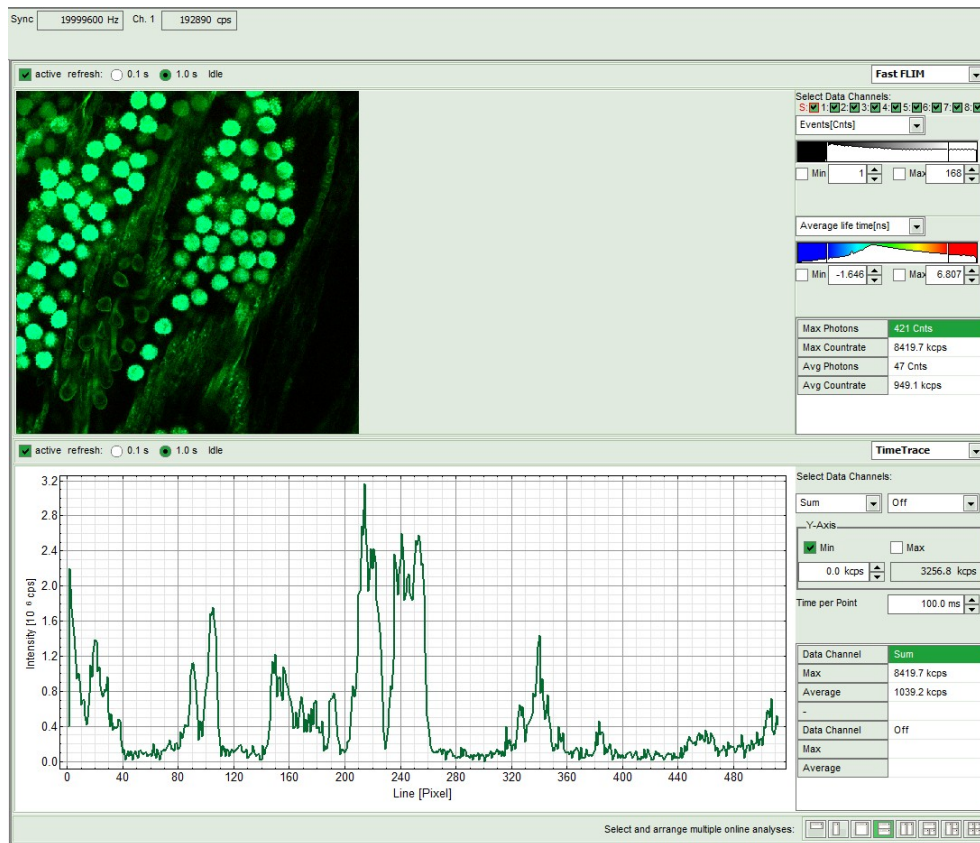
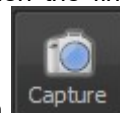
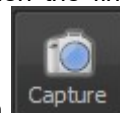


Fig. 2.26: SymPhoTime Measurement Preview being displayed during LSM data acquisition. In this preview, the FLIM image is displayed on the upper left. The display parameters are shown on the right. Below, a timetrace is shown, which represents a profile through lines of the image.

4. **SPT64 software:** During LSM data recording the chosen online analyses are displayed, e.g. one or – in case of several detectors - several FLIM -images. When scanning several frames, all frames are summed up. 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 analyse the data separately frame by frame.
5. **Nikon A1 software:** The measurement comes to its end when the finishing criterion is reached.



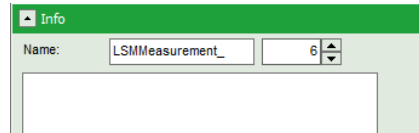
Alternatively it can be interrupted at any time by pressing again . Data will be automatically saved in the SPT64. As a rule of thumb for homogeneous samples, a FLIM measurement can be interrupted when the brightest pixel has acquired 1000 photons.

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

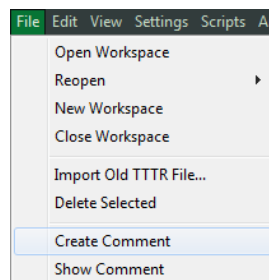
2.4.4. Resulting raw data file and documentation

- **SymPhoTime 64 software:** The measurement result is a .ptu-raw data file, containing for every detected photon the maximum available information (temporal, spatial, detection channel information), together with the online analysis files “.pqres”. If e.g. a online Fast-FLIM-image is to 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. It contains all actual measurement parameters known by the SymPhoTime software. To access, double click on the raw (“.ptu”)-file. The

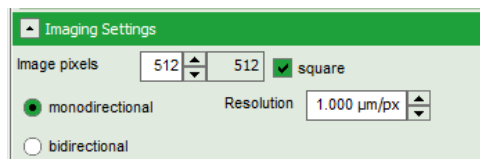
raw data file contains all actual measurement parameters known by the SymPhoTime software; individual information (measurement type, sample, etc) can be entered **before** the measurement via the “Info” drop-down-menu or in the comment field of the A1Plus AUX Command (see Fig. 2.25).



- **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.



- The **image dimensions** and “Resolution” are automatically transferred from the NIS software to **SymPhoTime 64 - software** when the measurement is started.



- **SymPhoTime 64 software:** 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”).

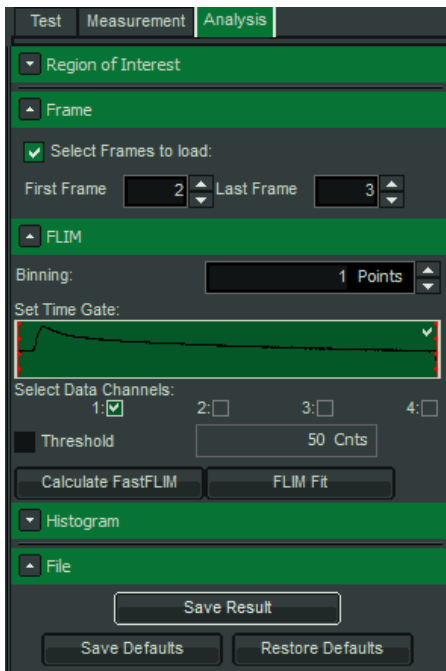
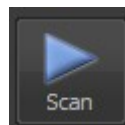


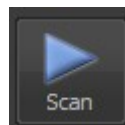
Fig. 2.27: Changing the selection of frame numbers for analysis in the SymPhoTime 64 Software.

2.4.5. Measure an instrument response function (IRF)

For accurate measurements of lifetimes close to the timing resolution of the instrument, 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 following these steps:

1. Place a sample with an **negligible short lifetime** in the laser focus on the microscope stage (e.g. Erythrosin B or Fluorescein, either dissolved in a saturated KI-solution. 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 2 photon-excitation, also second harmonic generation may be used to measure the IRF.
2. Place a suited **emission filter** in the filter holder in front of the PicoQuant detector, ideally the same as used later for the measurements.



3. **Nikon A1 software:** Start the scanning process by pressing .
4. **SymPhoTime 64 software:** You can now check the decay behavior of your sample using the TCSPC preview of the “Test” tab, as shown in Fig. 2.28. In the “TCSPC-settings” drop-down-menu, also the time width per channel can be set. Adjust the count rate to approximately 50 kCounts/s. It is important that the laser power output is not changed at the laser driver itself or in the SymPhoTime – software, if a SEPIA II is present at the system, but only at the Laser Combining Unit (LCU) or attenuator at the laser head. Changing the laser driver or software intensity settings changes the shape and temporal position of the laser pulse. However you can use the driver settings to optimize the pulse shape of the laser for shortest IRF width.

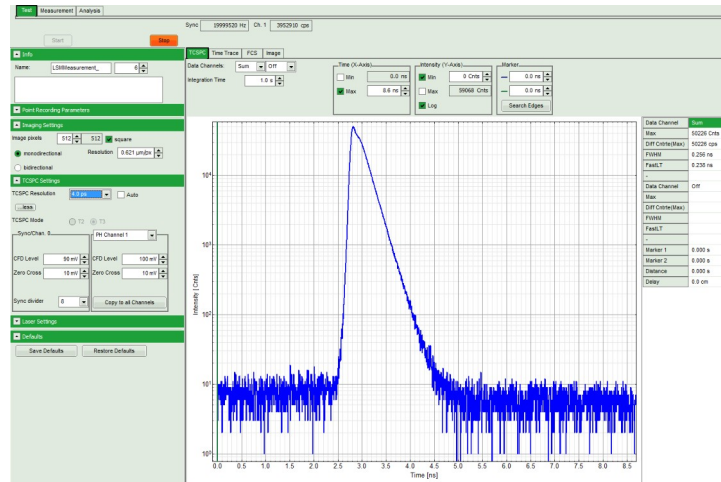
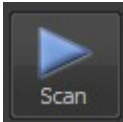



Fig. 2.28: TCSPC-preview window, recorded with a PH300, MPD SPAD and PicoQuant laser source. The time width per channel can be set with the “TCSPC Settings” drop-down menu. The count rate is displayed over the preview graphs for each channel separately.

5. **NIS software:** Stop the test measurement by pressing . Steps 4 and 5 are only for checking of the IRF shape and settings, they do not need to be performed routinely.

6. **NIS software:** Now take a measurement by pressing .
7. 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 till 100 000 counts. You can open the TCSPC histogram of the captured image, using the “TCSPC-Histogram” - analysis of the SymPhoTime 64 software. It contains the IRF.
8. Replace the IRF solution with your sample again.

2.5. Acquisition for FCS

2.5.1. Requirements for FCS

FCS is a techniques 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 A1	LSM Upgrade Kit	Sample
<ul style="list-style-type: none"> Water immersion objective with NA 1.2 or higher with correction collar. Clean optics and perfect alignment. Cover sample from room light. Point measurement mode. 	<ul style="list-style-type: none"> SPADs or PMA Hybrid PMTs. SPT64 version with license for point measurements. Clean optics and perfect alignment. 	<ul style="list-style-type: none"> 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). If the user decides to excite simultaneously with two colors, CW from Nikon and pulsed from PicoQuant, the user will have access to FLCCS.


The steps to perform FCS measurements are the following:

- NIS and SPT64** - Optimise A1 pinhole setting with a strongly fluorescent solution.
- NIS and SPT64** - Perform a calibration measurement to determine the confocal volume with a 20 nM concentrated dye solution.
- NIS** - A region of interest is identified with a Nikon confocal measurement.
- NIS and SPT64** - The best conditions for the FCS are determined with a "Test" measurement.
- NIS and SPT64** - The FCS measurement is performed.

2.5.2. Optics optimization

FCS is a single molecule sensitive technique and hence it demands a perfect alignment of the system. Furthermore its analysis relies in 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 coverslide is properly set with the objective **correction ring**. Both things can be monitored by maximization of the fluorescent signal.

- Place a strongly fluorescent solution (>100µM concentration) in focus. 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.
- NIS software:** Select the optical configuration for FCS. This can be performed by calling NIS in the standard FCS configuration and modify it accordingly. For example, if you want to measure GFP and excite with PicoQuant laser take the configuration created before and modify the Laser Port to be Port 2, and select only the green channel with 488 PicoQuant excitation.

3. Introduce in the PicoQuant detection unit a filter that matches the NIS configuration. Note that if you want to excite with a Nikon laser, the PicoQuant laser driver has also to be switched on¹.
4. **NIS software:** Focus into the volume, being at least 20 μ m away from the cover slide.
5. **NIS software:** In the **A1plus scan Area** select a point in the centre of the image and start at FCS test by clicking .
6. **SPT64 software:** Select the "Time Trace" Tab and monitor the photon count rate over time.
7. Adjust the laser intensity to get a reading of around 2 Mcps.
8. **NIS software:** Go to the **A1plus pinhole alignment** window and note the X Y position of the lens.

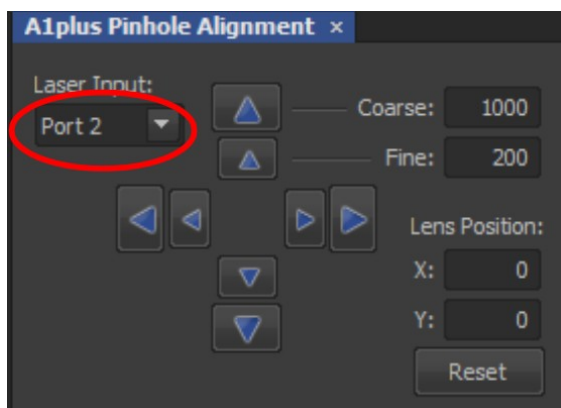


Fig. 2.29: Selection of Port 2 for pulsed excitation with PicoQuant lasers.

9. **NIS software:** Move the lens position with the arrows and observe the variation of the time trace signal. Move X Y in all directions and note the X Y values at which the maximum intensity is achieved.
10. **Microscope:** Move the correction collar at the objective and observe the variation of the time trace. Leave it at the maximum.
11. If desired, the PicoQuant Laser combiner can also be aligned by looking at the fluorescence intensity time trace. Otherwise the system is now ready for a the calibration measurement.



2.5.3. 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 probe². For example, for GFP, ATTO488 can be used as standard.
2. **NIS software:** 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. **NIS software:** Put the standard dye and focus into the volume, at least 20 μ m away from the coverslide.


¹ The PDL laser driver provides the sync signal to the PicoQuant electronics. The sync signal is necessary in order to count photons. If the system lacks lasers from PicoQuant, then a Sync generator device is connected to the electric to provide the sync signal.

² See application Note "Absolute Diffusion Coefficients: Compilation of Reference Data for FCS Calibration" on PicoQuant Website

4. **NIS software:** In the **A1plus scan Area** select a point in the centre of the image and start at FCS “test” by clicking .
5. **SPT-64 software:** Go to FCS 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 decrease the MB.
6. **NIS software/PicoQuant laser combiner:** 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 maximum¹.
7. **NIS software:** Stop the test measurement and take a calibration measurement for at least 60 s by pressing .
8. **SPT-64 software:** The calibration measurement is automatically stored when the measurement is over.

2.5.4. FCS measurement

Before performing a FCS measurement, the target ROI has to be identified. This is normally performed with the Nikon A1 (cw lasers and Nikon detectors). Once the ROI is identified and in focus, the user can change to FCS acquisition.

1. **NIS software:** In the **A1plus scan Area** select the point in which you want to perform the measurement. If you are measuring in solution, it is assumed that the laser is focussed into the solution. If you are measuring in live cells, take into account that due to the high sensitivity of the SPAD or Hybrid-PMT detectors, cells appearing relatively dark in the manual epi-fluorescence view should be selected.
2. **NIS software:** Start a test measurement by clicking .
3. **NIS and SPT64 software/PicoQuant laser combiner:** For measurements inside living cells it has to be noted that the diffusion time of the molecules under investigation are dependent on the size of the molecules observed. A longer diffusion time through the confocal volume needs less laser power since the dye molecules are excited by the laser radiation for a longer time. 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. For optimal setting the laser power is set as high as possible by not leading to a reduction in the diffusion time.

¹ 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 in the safe side.

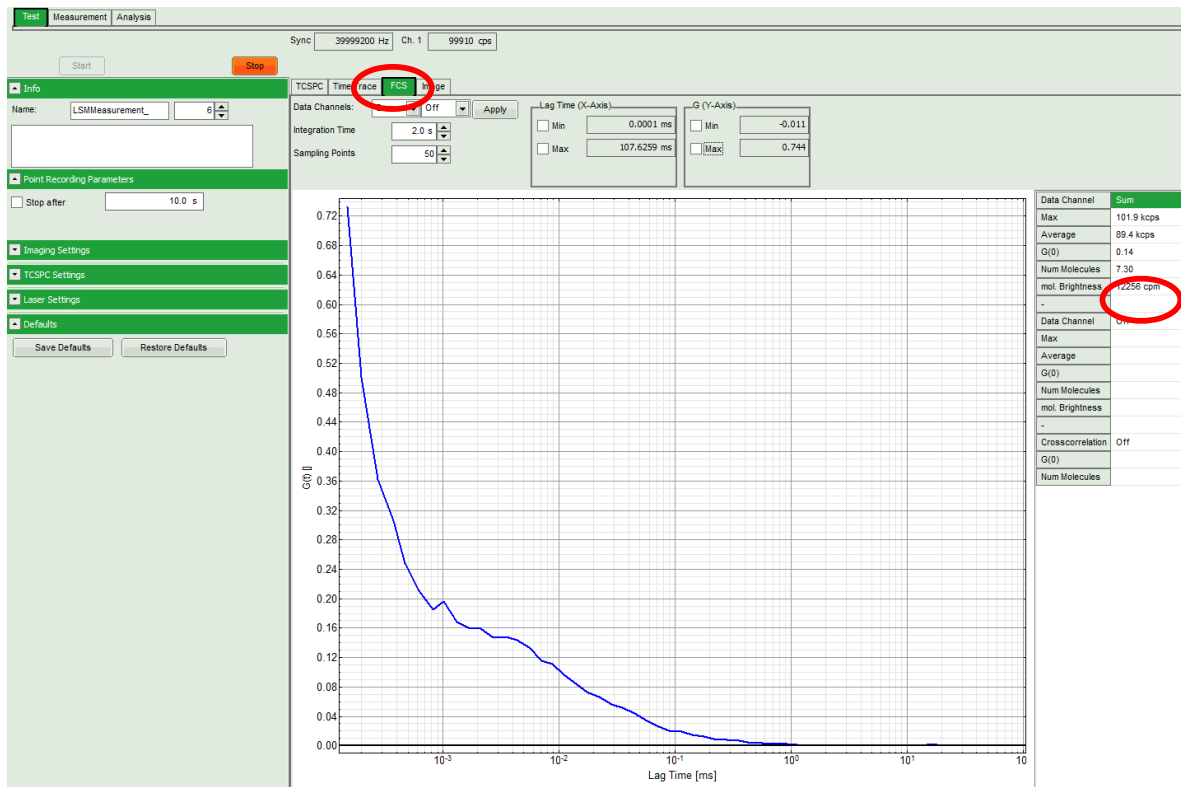
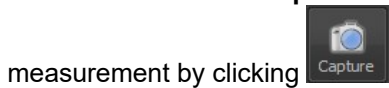


Fig. 2.30: Online monitoring of the of the FCS trace and photon count rate in the SymPhoTime preview window. On the right, the average count rate and the molecular brightness are displayed.

- NIS software:** In the **A1puls AUX control** select the desired acquisition time and start the



measurement by clicking

- SPT-64 software:** The FCS-curve is calculated during the measurement and displayed in the FCS tab (Fig. 2.30). 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 “mol. 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. If two curves are selected in the preview, a cross-correlation curve is also automatically calculated. 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 for the assessment of bleaching and to 1 ms for the observation of the intensity fluctuations.

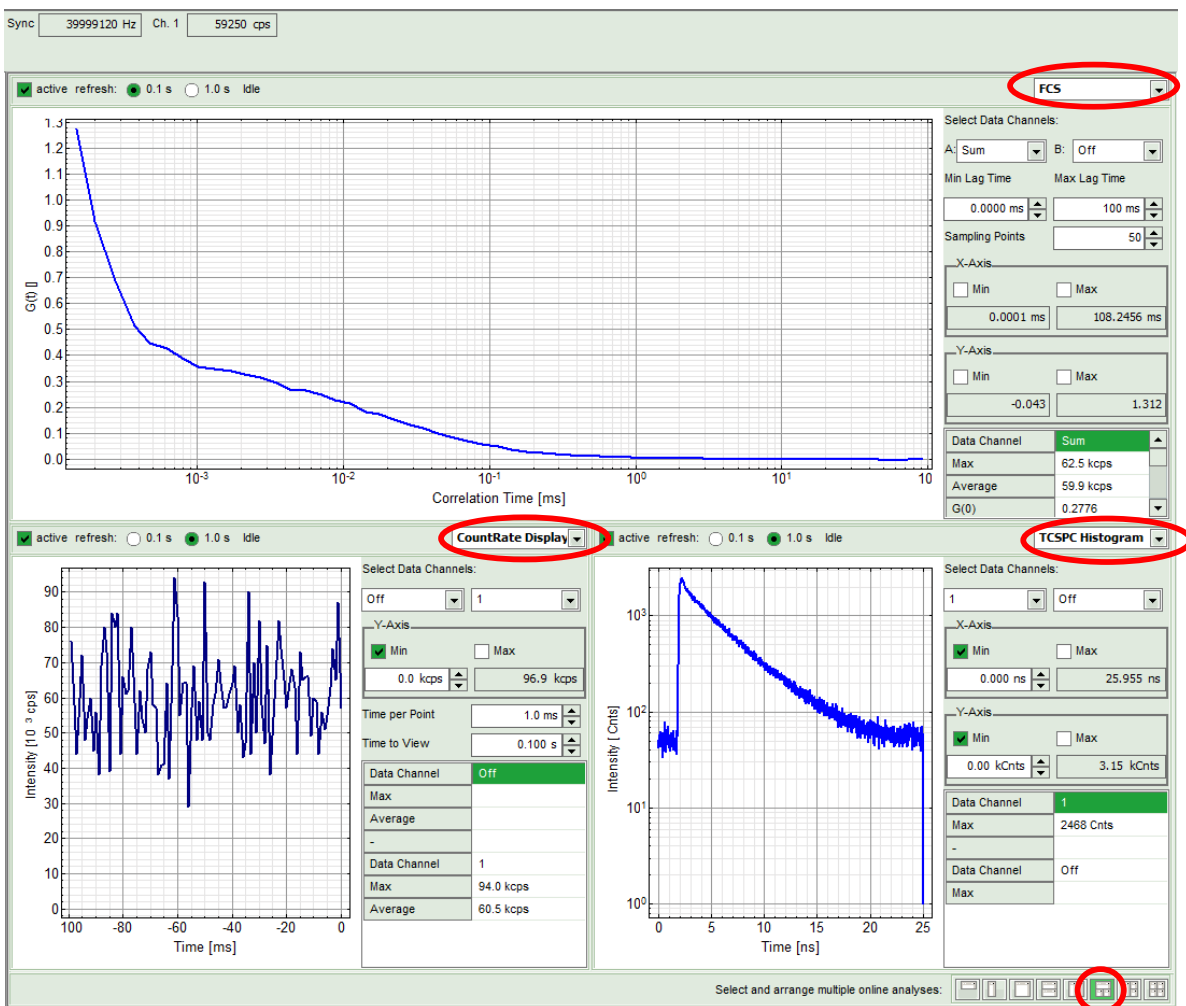


Fig. 2.31: Online Preview of the FCS measurement. In this configuration, the FCS curve is displayed in the upper panel, while the Time Trace is displayed simultaneously on the lower left and the TCSPC histogram on the lower right.

2.5.5. FCS analysis

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

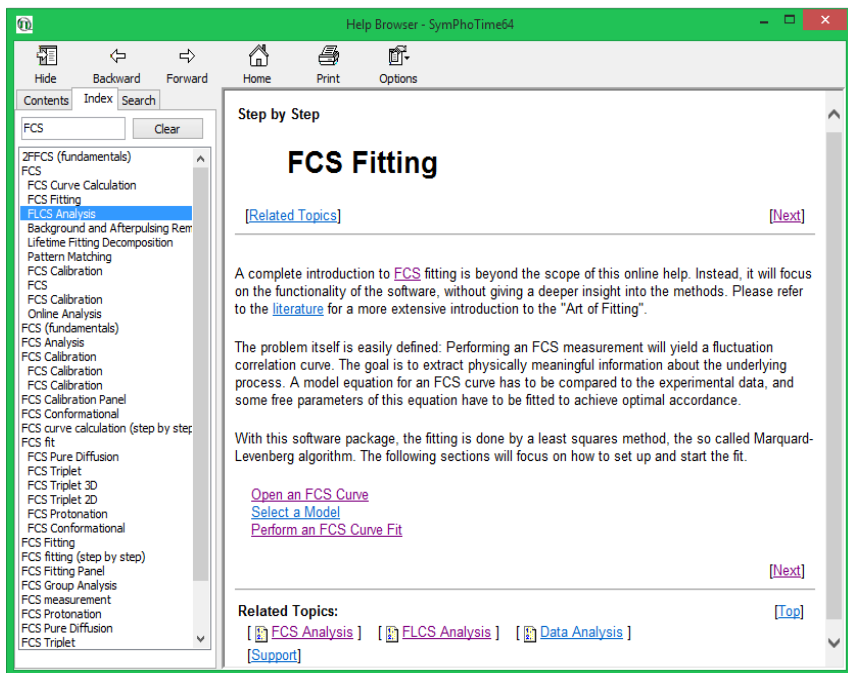


Fig. 2.32: Online Help with plenty of theory and technical information regarding analysis routines

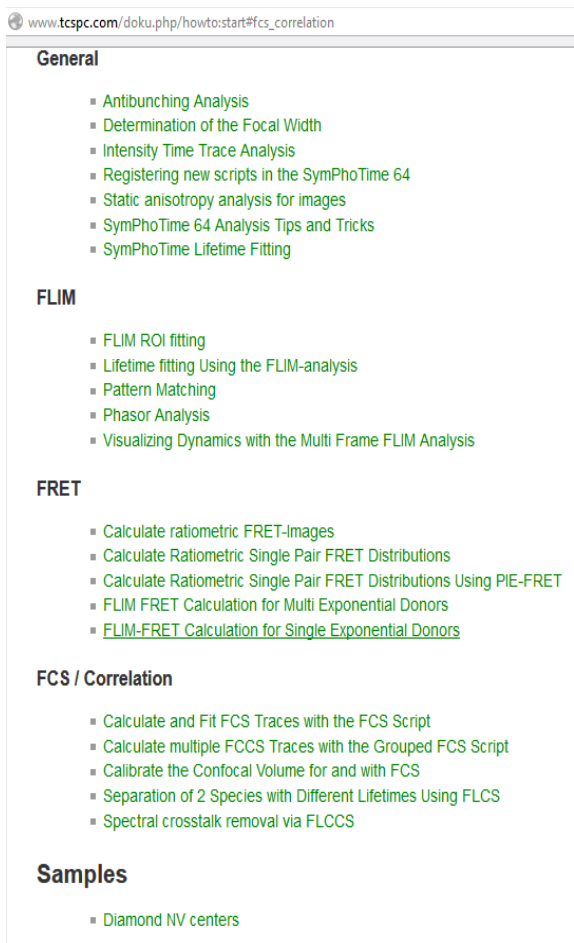


Fig. 2.33: "How to" section from the TCSPC wiki.

2.5.6. Remarks

- After changing of the dichroic of the scan head the **pinhole must be optimized**. The pinhole can be adjusted with an Allen key optimizing the fluorescence countrate of fluorescence sample (see Fig. 2.1, position 2)
- Usually, a **water immersion objective** with a high NA (1.2) is used for FCS measurements. Optimize the fluorescence countrate by adjusting the objective collar.
- **FCS measurements** can **only** be performed using **avalanche photodiodes** or **Hybrid PMTs** as 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 Hybrid PMTs.
- For a solid FCS-analysis, the **count rate** has to be **stable**. Signal decrease due to photobleaching may lead to an increasing FCS correlation amplitude at long lag times.
- The **calculation time** of an FCS measurement depends on the **measurement time** and the detector **count rate**.

3. Troubleshooting

In general, make sure that all components of the Nikon A1 system as well as of the upgrade kit are switched on.

3.1.1. Measurements can not be started from the NIS software

To be able to start the FLIM acquisition with the NIS software, the NIS, SPT64 and both computers have to be properly connected and configured. This is performed by PicoQuant during the system installation. Shall the communication be lost, it is necessary to be re-establish it. Connection between both PCs can be checked if both PCs can be controlled with the same mouse and keyboard. If this is not the case then contact PicoQuant. If the both PCs are connected but it is not possible to start FLIM or FCS measurements with the NIS software, then check the following protocol.

Configuration of the SymPhotime64 Software

The "Remote Interface" must be activated. In the main Window go to **Settings**, and then to **Hardware Setup**. Click in the "active" box "Remote Interface" and then press "Save as default".

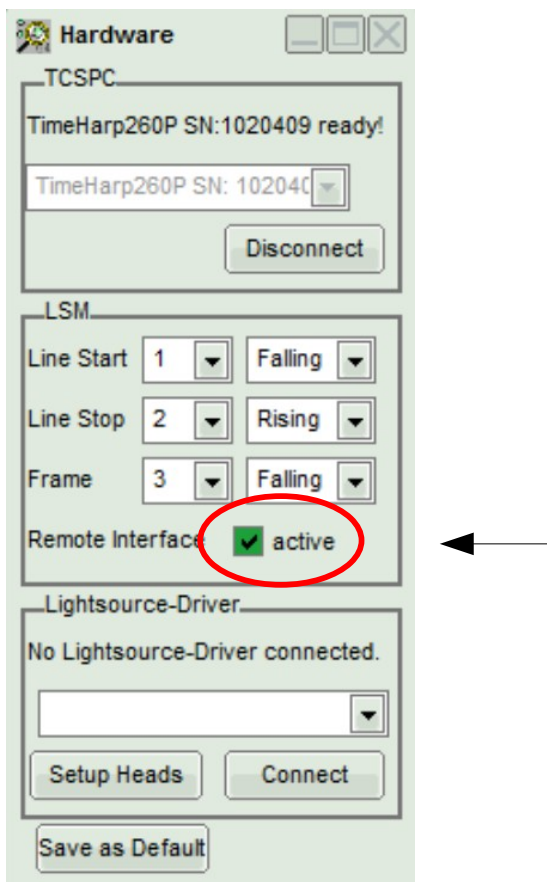


Fig. : Activation of the remote interface to allow the measurements to be initiated from the NIS software.

Configuration of the NIS Software

Go to the **A1plus AUX** control window and check the "status" of the communication between both computers. "Unknown" indicates that the communication is lost, whereas "idle" means that the communication is running.

If the "status" is "unknown" click in Settings. A new window is opened. Check that the IP and port addresses coincide with those in the figure below and click "connect". The connection should be re-established and the Status updated to "idle".

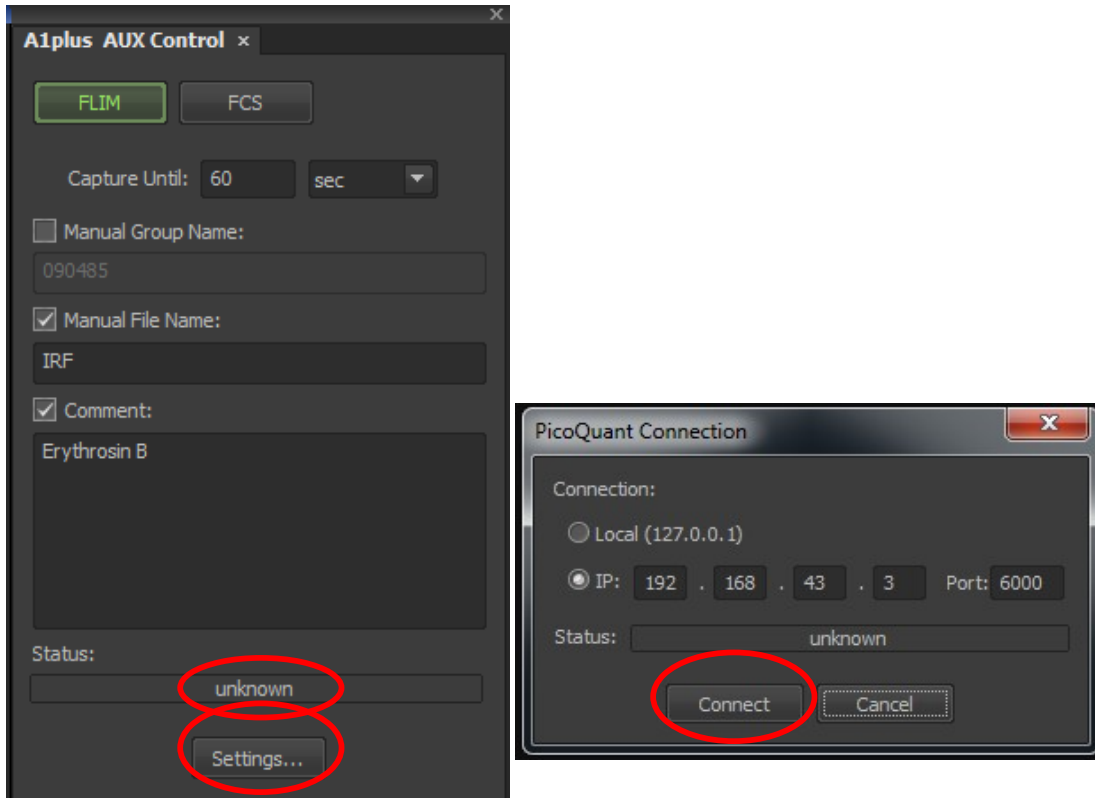


Fig. : Status and connection settings for Nikon and PicoQuant computer communication.

3.2. 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 **settings file**. Restoring the configuration from the .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 *.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, 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.

3.3. Instrument is losing 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 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** 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 or Fluorescein (in a solution with high pH) 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 to 20 nM solution is used. For a day to day comparison, use the same laser power, best controlled with a power meter. A more detailed description for performance of FCS measurements is given in the point-measurement section.
- If a performance drop is noticed, first the origin of the performance drop needs to be found. A drop in system performance may be caused by:

1) Misaligned Pinhole

- Optimize the Pinhole Settings as described above. Use a high concentrated dye sample (approx. 100 μ M) 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 the setting that you can refer to it for later measurements.

2) Correction collar setting

- For FCS measurements in biological water rich sample, an apochromatic corrected water immersion objective with an NA of 1.2 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 μ M). 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. Please read the manual of the laser(s) and LCU

4) Decreasing Detection Sensitivity

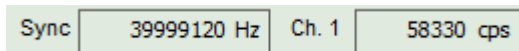
- Misalignment of the LSM itself: The first important step is to align the pinhole (see above). If this procedure does not help, contact your Nikon service representative.
- Misalignment of the detector(s): Refer to your detector manual.

3.4. No image is displayed during measurement

Can laser light be seen over the objective during measurement?

If no:

- Is the **pulse repetition rate** correct? The pulse frequency is displayed in a control window over the preview windows.



When starting a TCSPC measurement in the test mode, dark counts should be displayed (see Fig. 2.28). 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 SEPIA II, whether 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 still on (the DSN102 - if applicable - shuts down a detector automatically if the count rate exceeds a limiting count rate).
- 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.
- 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, 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 (the filter should sit in the filter holder correctly). Shutters should click softly when opening and closing.
- 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 Nikon A1 and the LSM FLIM / FCS Upgrade. Also try to start the Nikon A1 and the PicoQuant system new. If no marker signals are displayed in spite of a present connection, contact PicoQuant. If you don't have the Point Measurement Mode included in your license, check cable connections and send the file to PicoQuant.

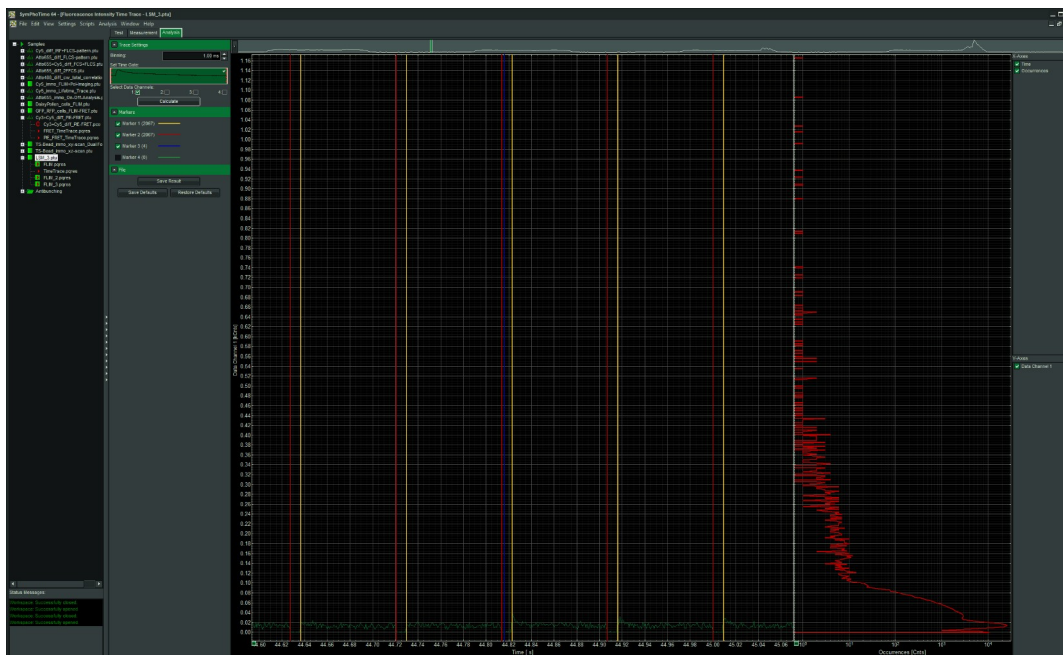


Fig. 3.1: The Time Trace analysis, where the line and frame - markers can be displayed.

3.5. 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 at wrong position:
 - => sensitivity will be poor in FCS and in imaging applications.
- To align the pinhole, place a bright fluorescent sample on the microscope (e.g. a dye solution) and focus into the solution. The pinhole position can be moved in the AUX – menu of the Nikon software(Fig. 3.1)

4. 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/>

5. Abbreviations

BNC	British Naval Connector or Bayonet Nut Connector or Bayonet Neill Concelman
CCD	Charge-Coupled Device
CFD	Constant Fraction Discriminator
cps	Counts per Second
cw	Continuous wave (not pulsed)
FCS	Fluorescence Correlation Spectroscopy
FIFO	First In, First Out (buffer type)
FLIM	Fluorescence Lifetime Imaging
FRET	Förster Resonance Energy Transfer
FWHM	Full-Width at Half-Maximum
IO	Input-Output
IRF	Instrument Response Function
LCU	Laser Combining Unit
LED	Light Emitting Diode
LSM	Laser Scanning Microscope
MCS	Multichannel Scaling
OD	Optical Density
PC	Personal Computer
PCI	Peripheral Component Interface
PIE	Pulsed Interleaved Excitation
PMT	Photomultiplier Tube
RGB	Red-Green-Blue (colour scheme)
ROI	Region of Interest
SMA	SubMiniature version A (connector type)
SMD	Single Molecule Detection
SPAD	Single Photon Avalanche Diode
SYNC	Synchronization (signal)
TCSPC	Time-Correlated Single Photon Counting
TTL	Transistor-Transistor Logic
TTTR	Time-Tagged Time-Resolved

6. Support and Warranty

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

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

In any case, we would like to offer you our complete support. Please do not hesitate to contact 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 http://www.picoquant.com/_scientific.htm. It is a valuable source if you want to know which laboratories are using PicoQuant products or how broad the field of various applications is.

Thank you very much in advance for your kind cooperation!

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