

LSM FLIM / FCS Upgrade



PICOQUANT
Unternehmen für optoelektronische
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Compact Lifetime and FCS
Upgrade for **Nikon C1si/C2**
Laser Scanning Microscopes
Full Integration



User's Hardware Manual and Technical Data

Version 2.3

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1. Introduction

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

- In regard to the LSM upgrade kit manual the **Nikon C1(si)** and **C2** microscopes have no differences.
- The **Nikon C1si/C2** has two fiber exit ports. The spectral exit port is used for FLIM and FCS. In case the user has the spectral detection box, the detection fiber must be swapped between spectral and FLIM / FCS operation. The spectral detector (si) is not necessary for the FLIM / FCS operation, only the scan head must be of the C2si type.

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

- **Laser Combining Unit - Manual (LCU - Manual):** This manual should be consulted for information about the Laser Combining Unit, which contains the lasers of your LSM. It explains setting the correct intensities and also contains a detailed description for realignment.



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 Units for LSM FLIM / FCS Upgrades from left to right: Multi channel PMA / SPAD detection unit, Single channel SPAD detection unit

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



Fig. 1.3: PicoHarp 300 and TimeHarp 260

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



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

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



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

- **Light Sources:** In the green folder you'll also find detailed information about the properties of your pulsed diodes lasers.
- **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.
- **Pre-Installation Requirements:** This is an important document that already should have been considered before installation. If you want to change the configuration of your LSM, you'll find detailed information, if the desired change can be realized and additional parts are necessary. In case of doubt, contact a PicoQuant representative.
- **Appendix:** The appendix contains specific information about your individual LSM 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.

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

2.2. Starting Hardware and Software Equipment

To **start** the LSM itself, its detectors and cw lasers, please refer to your Nikon C1si/C2 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 FLIM / FCS Upgrade can be started completely separate from the Nikon C1si/C2 itself.

The LSM FLIM / FCS Upgrade hard- and software should be started in the following order:

1. Start the **laser driver** and enable laser emission. Prior to data acquisition, the laser head should be operated for a few minutes to stabilize. If you use a PDL 828 "Sepia", you can set the laser settings in the SymPhoTime 64 software.
2. Start the external **PicoHarp 300** TCSPC device, if applicable also the **PHR 800** router and the **DSN 102** SPAD power supply
3. Start the **PC**.
4. Start the **SymPhoTime 64 software**.
5. **Open** an already existing **workspace** or create a new one.
6. Place correct **fluorescence filters** in the corresponding filter holder position of the external PicoQuant detector unit and the confocal microscope. If you have a Dual Channel SPAD Detector Unit, set the variable beam splitter unit to the correct position (see Dual - Channel Detection Unit manual for details).
7. **Make sure that no light is focussed onto the detector(s) and power** them on. Either power on the detector on its unit or, if using a DSN 102, start the respective detector by pressing the corresponding channel button on the DSN 102.
8. If you want to work with user specific settings, load your **User Configuration**.

The FLIM / FCS Upgrade is now ready to use.

2.3. FLIM Data Acquisition

Scope:

Acquire a FLIM image using a PicoQuant pulsed laser, the Nikon C1si/C2 scanhead with appropriate scanning and dichroic settings and external PicoQuant TCSPC detectors.

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.

2.3.1. Starting Point

1. **Nikon C1si/C2:** Choose and pre-scan with the Nikon C1si/C2 (its cw lasers and internal detectors) the correct region where you want to perform a FLIM measurement. For the ad hoc inspection make sure that you are using a suitable cw excitation wavelength, the right excitation dichroic, a useful microscope objective and suitable detection wavelengths range for the internal Nikon C1si/C2 detectors. Finally stop the scanning process.
2. All components (laser, detectors, software) of the PicoQuant FLIM / FCS Upgrade are already running. For details about powering on see chapter 2.2.
3. **SymPhoTime 64 software:** The SymPhoTime 64 allows configuring the view of the acquisition window. Thus the user can preconfigure the online analysis he wants to see during acquisition, e.g. Images (also of the different spectral channels if more than one detector is present), a Time Trace, a Decay (TCSPC) Histogram. Several online analyses can be displayed simultaneously. If already available, load the desired **User Settings** in the SymPhoTime 64.
4. **SymPhoTime 64 software:** A workspace has been loaded or created. The workspace defines where the data should be stored to.

2.3.2. Changing from continuous wave to pulsed excitation

1. **PQ Laser driver:** Make sure that the **laser repetition rate is correctly set**. In general, 40 MHz is the best frequency to start with. Only if you observe that your decay does not fit in the TCSPC histogram, lower the frequency to 20 MHz. This applies only to pulsed diode lasers, the repetition rate of multiphoton lasers cannot be adjusted (unless with a pulse picker).
2. **Nikon C1si/C2 scan head:** Choose a **suitable excitation dichroic** for the pulsed excitation wavelength.

2.3.3. Changing from internal Nikon C1si/C2 detection to external TCSPC detectors

1. **Nikon C1si/C2 scan head:** If you want to do your time resolved experiments with the external PicoQuant detector, connect the optical fiber of the PQ detector with the fiber exit port of the Nikon C1si/C2 scan head. Normally, the Nikon detector box is connected to this port. It must be unplugged.
2. **Nikon C1si/C2 scan head:** If you want to do your time resolved experiments with the external PicoQuant detectors, switch the exit port lever at the scan head to “spectrum” detection. Make sure that at the exit port for “Spectrum” detection the fiber to the PQ detectors is plugged in.



Fig. 2.1: The Nikon C1si/C2 scan head. 1: dichroic, 2: pinhole adjustment, 3: switch for “standard” and “spectrum” detection. FLIM and FCS is performed with the “spectrum” setting.

3. **PQ Detection Unit:** Make sure you have **suitable fluorescence detection filters** (bandpass / longpass) in the corresponding filter holders of the detection unit(s). When using a Dual Channel SPAD Detection Unit, make sure that the beam splitter in the beam splitting tower is set to distribute the emission light to the detectors correctly (see your detection unit manual for details).

2.3.4. Set suitable scan parameters

In the **Nikon C1si/C2 - software**, set the correct scan pattern:

- Use a **monodirectional scan**.
- Please use no **averaging mode** of the Nikon C1si/C2 for the scanning process.

2.3.5. Starting Point in NIS

Before taking a FLIM image, the target ROI has to be identified. This is normally performed with the Nikon C1si/C2 (cw lasers and Nikon detectors). Once the ROI is identified and in focus, the user can change to FLIM acquisition.

2.3.6. 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 PQ 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.
3. 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 10e3 and 10e5 counts.

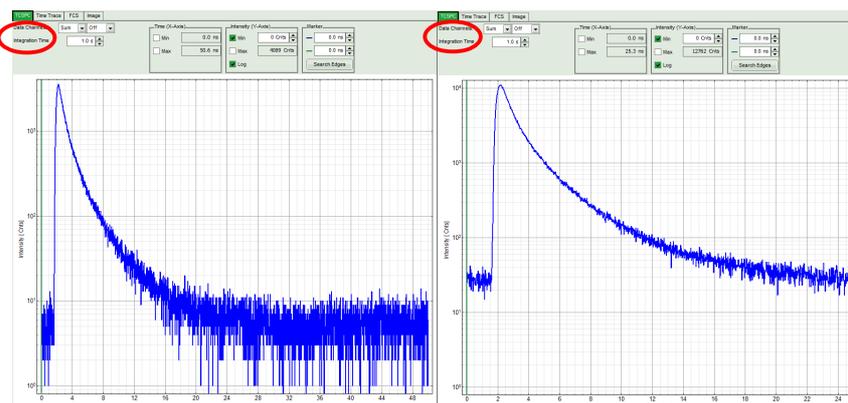


Fig. 2.2: Fluorescence decay at unnecessary low (left, 20 MHz) and optimal (right, 40 MHz) laser repetition rate. With an even higher repetition rate, a part of the fluorescence would appear at the beginning of the TCSPC-window.

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 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.

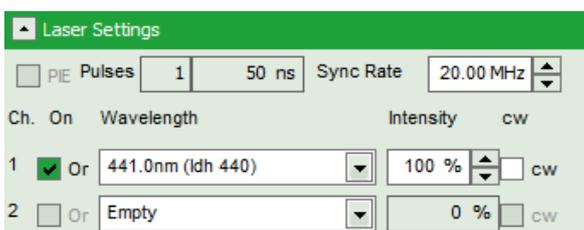


Fig. 2.3: Laser settings in SPT64.

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 1MHz³. High count rates can lead to distorted decays due to the pile up effect⁴. The count rates can be monitored in the Time Trace below the FLIM image (Fig. 2.4).

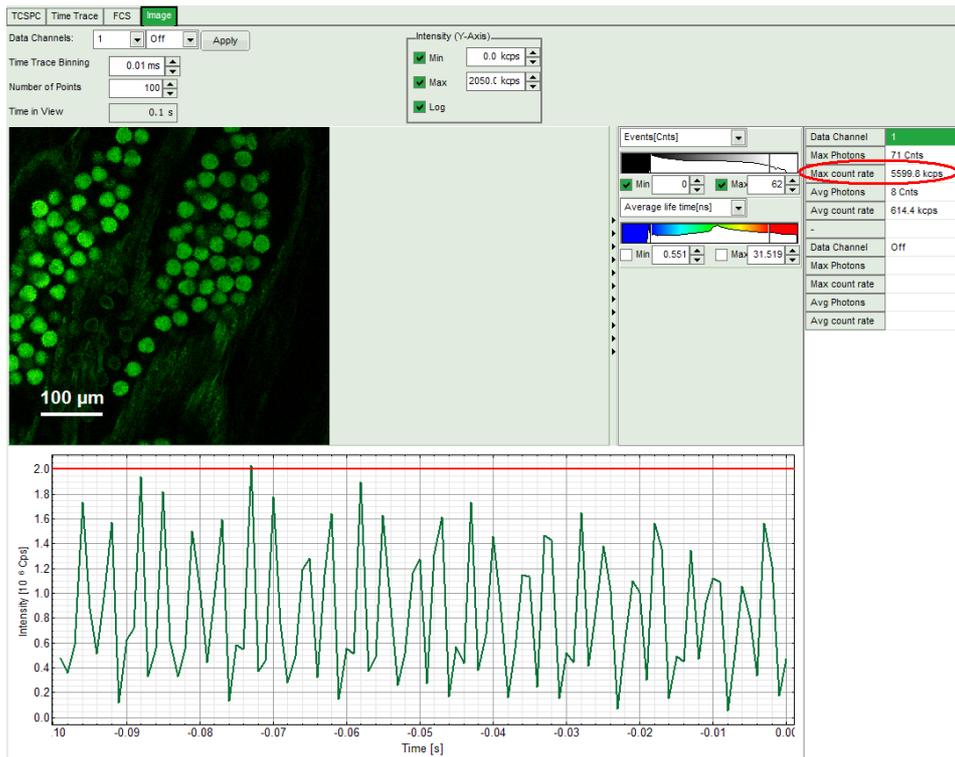
For the setting of the correct laser intensity the user can look at the Time Trace, displayed below the FLIM image in the Test mode. The red line indicates a threshold of 10% of the repetition rate. The intensity spikes should always remain below this line in order to avoid pile up. For a proper monitoring 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.

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 repetition rate has to be lowered.

² 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.

³ 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.

⁴ 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. 2.4: 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

2.3.7. FLIM measurement

1. **SPT64 software.** Create a Workspace or open an already existing one.
2. **NIS software.** Go to the **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. 2.5: 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.

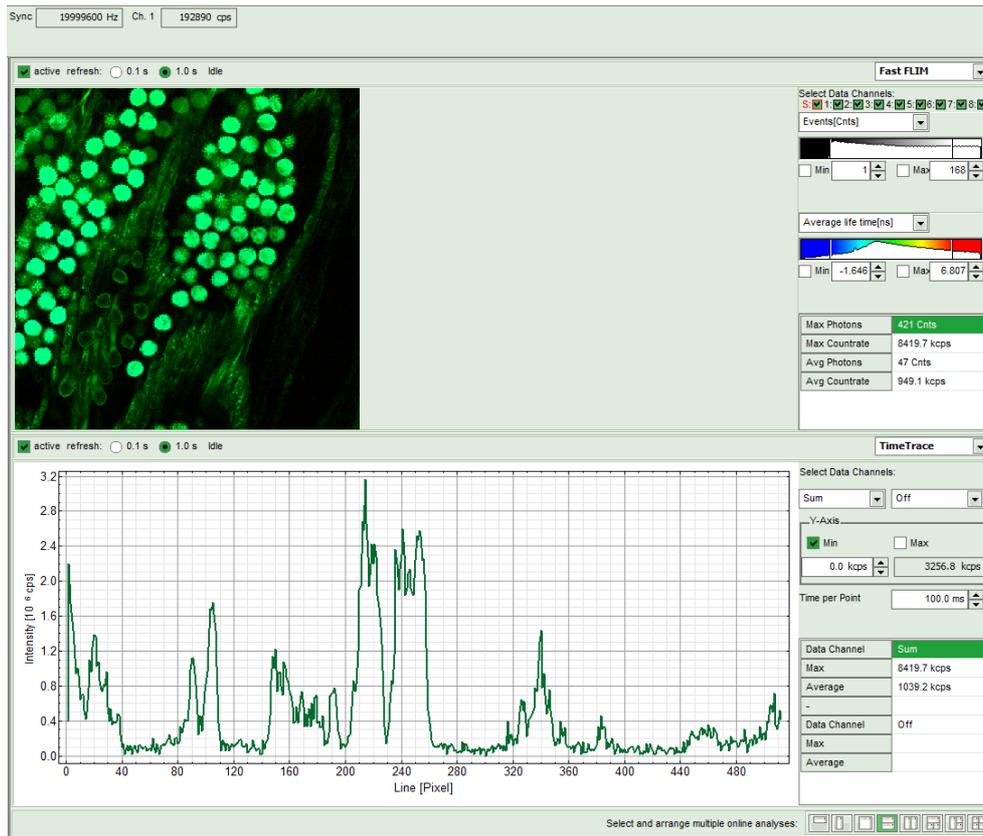
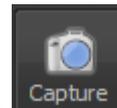


Fig. 2.6: SymPhoTime Measurement Preview being displayed during LSM data acquisition. In this preview, the FLIM image is displayed on the upper left. The display parameters are shown on the right. Below, a timetrace is shown, which represents a profile through lines of the image.

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 C1si/C2 software:** The measurement comes to its end when the finishing criterion is reached.



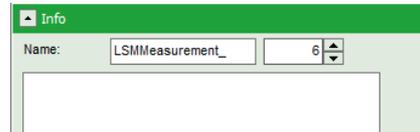
Alternatively it can be interrupted at any time by pressing again . Data will be automatically saved in the SPT64. As a rule of thumb for homogeneous samples, a FLIM measurement can be interrupted when the brightest pixel has acquired 1000 photons.

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.

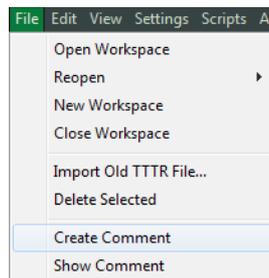
2.3.8. Resulting raw data file and documentation

- **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 data file is stored together with the actual settings. It contains all actual measurement

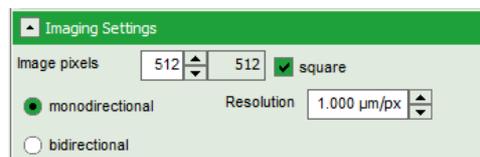
parameters known by the SymPhoTime software. To access, double click on the raw (".ptu")-file. The raw data file contains all actual measurement parameters known by the SymPhoTime software; individual information (measurement type, sample, etc) can be entered **before** the measurement via the "Info" drop-down-menu or in the comment field of the C1si/C2Plus AUX Command (see Fig. 2.5).



- **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").

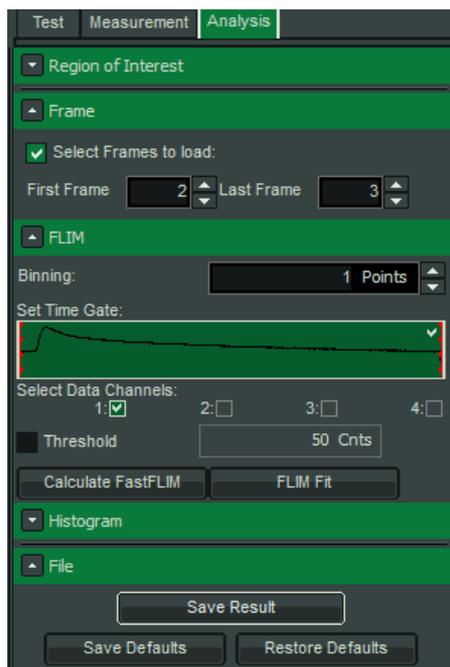
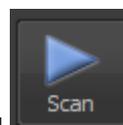


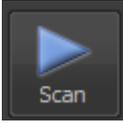
Fig. 2.7: Changing the selection of frame numbers for analysis in the SymPhoTime 64 Software.

2.3.9. 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 PQ detector, ideally the same as used later for the measurements.



3. **Nikon C1si/C2 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. 2.8. 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.

2.4. Acquisition for FCS

2.4.1. 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 PQ Upgrade Kit are listed.

Nikon C1si/C2	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 PQ 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:

- **NIS (Nikon Software) and SPT64** - Optimise C1si/C2 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.

2.4.2. 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.

1. 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.
2. **NIS software**: Select the optical configuration for FCS.

3. **Nikon C1si/C2 scan head:** If you want to do your time resolved experiments with the external PicoQuant detectors, switch the exit port lever at the scan head to “spectrum” detection. Make sure that at the exit port for “Spectrum” detection the fiber to the PQ detectors is plugged in.



Fig. 2.9: The Nikon C1si/C2 scan head. 1: dichroic, 2: pinhole adjustment, 3: switch for “standard” and “spectrum” detection. FLIM and FCS is performed with the “spectrum” setting.

4. Introduce in the PQ detection unit an optimized filter for your measurements. Note that if you want to excite with a Nikon laser, the PQ laser driver has also to be switched on¹.
5. **NIS software:** Focus into the volume, being at least 20µm away from the cover slide.
6. **NIS software:** In the **C1si/C2plus scan Area** select a point in the centre of the image and start at FCS test by clicking .
7. **SPT64 software:** Select the “Time Trace” Tab and monitor the photon count rate over time.
8. Adjust the laser intensity to get a reading of around 2 Mcps.
9. **Nikon scanhead:** Use an Allen key to optimize the pinhole setting in X and Y (the holes for the Allen key are marked by a number 2 in Fig. 2.9).
10. **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.
11. **Microscope:** Move the correction collar at the objective and observe the variation of the time trace. Leave it at the maximum.
12. If desired, the PQ 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.

2.4.3. Perform a calibration measurement to determine the Confocal Volume

Prior to a final 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 as used before.

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

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

3. **NIS software:** Put the standard dye and focus into the volume, at least 20µm away from the coverslide.
4. **NIS software:** In the **C1si/C2plus 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 decreases the MB.
6. **NIS software/PQ 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.

2.4.4. FCS measurement

Before performing a FCS measurement, the target ROI has to be identified. This is normally performed with the Nikon C1si/C2 (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 **C1si/C2plus 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/PQ 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.

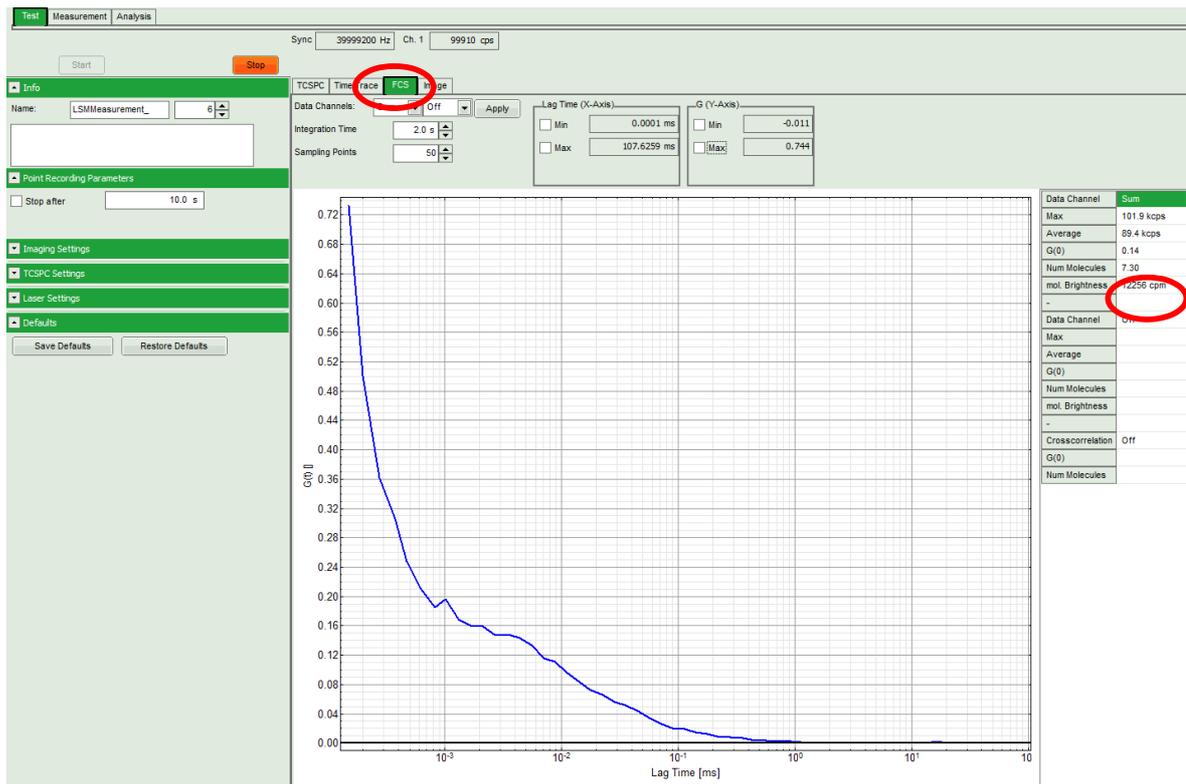
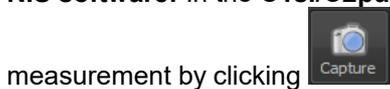


Fig. 2.10: Online monitoring of the of the FCS trace and photon count rate in the SymPhoTime preview window. On the right, the average count rate and the molecular brightness are displayed.

- NIS software:** In the **C1si/C2puls 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. 2.10). 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.

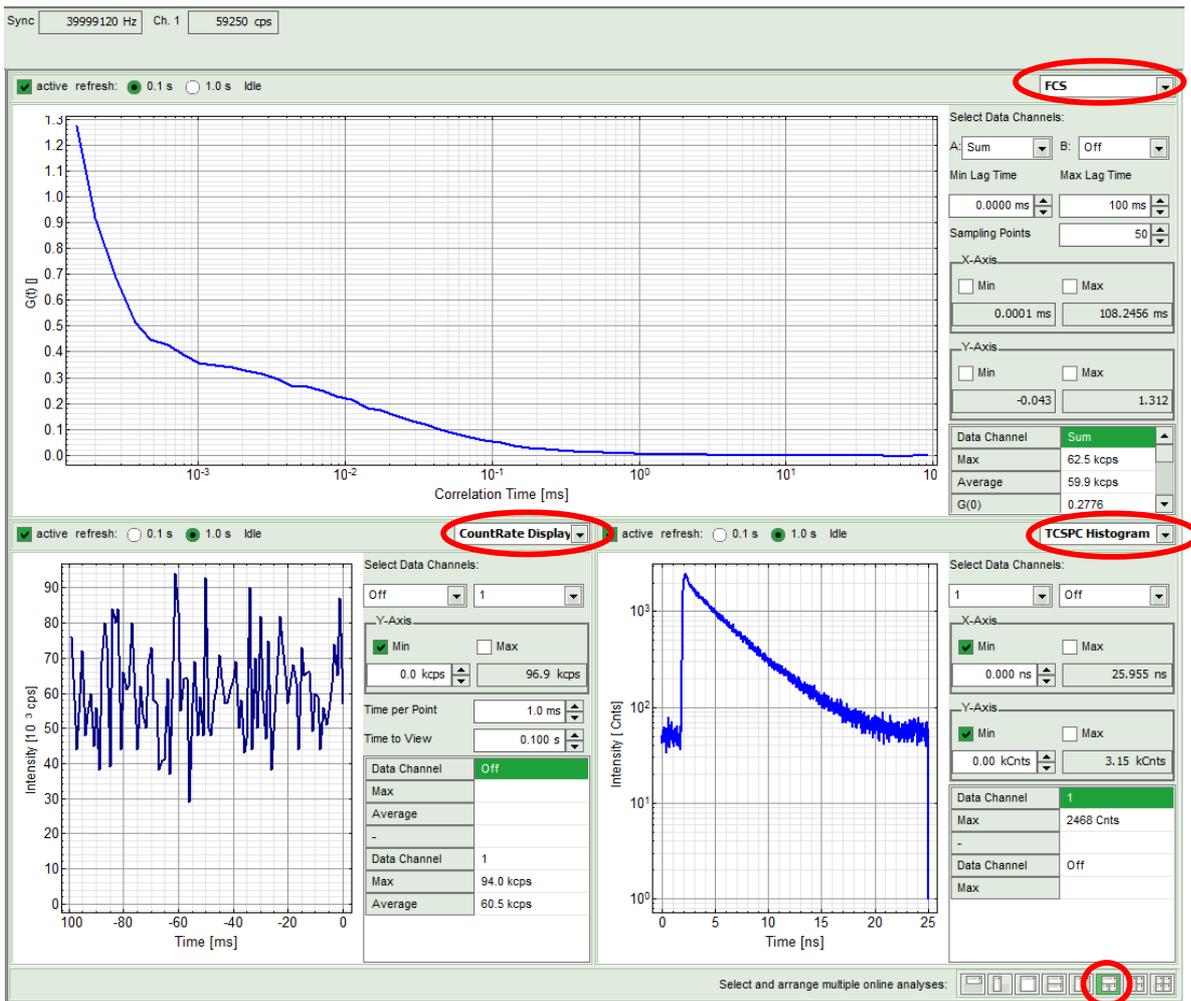


Fig. 2.11: Online Preview of the FCS measurement. In this configuration, the FCS curve is displayed in the upper panel, while the Time Trace is displayed simultaneously on the lower left and the TCSPC histogram on the lower right.

2.4.5. 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

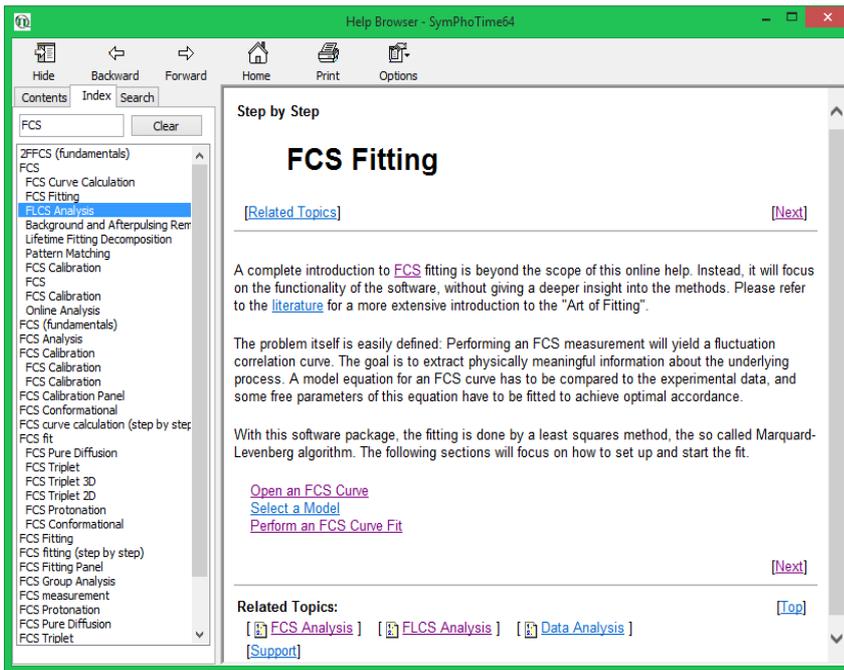


Fig. 2.12: Online Help with plenty of theory and technical information regarding analysis routines

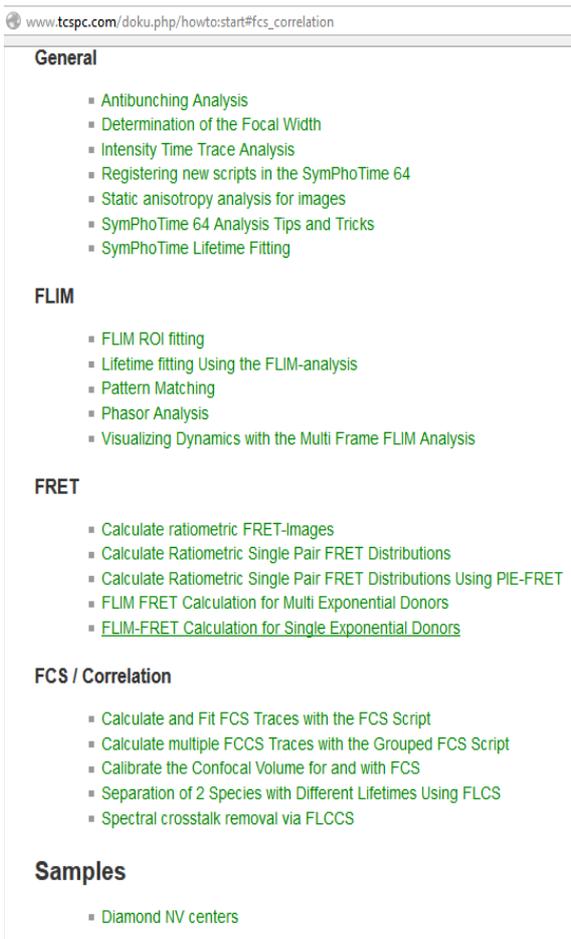


Fig. 2.13: "How to" section from the TCSPC wiki.

2.4.6. 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 C1si/C2 system as well as of the FLIM / FCS Upgrade are switched on.

3.1. 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”.

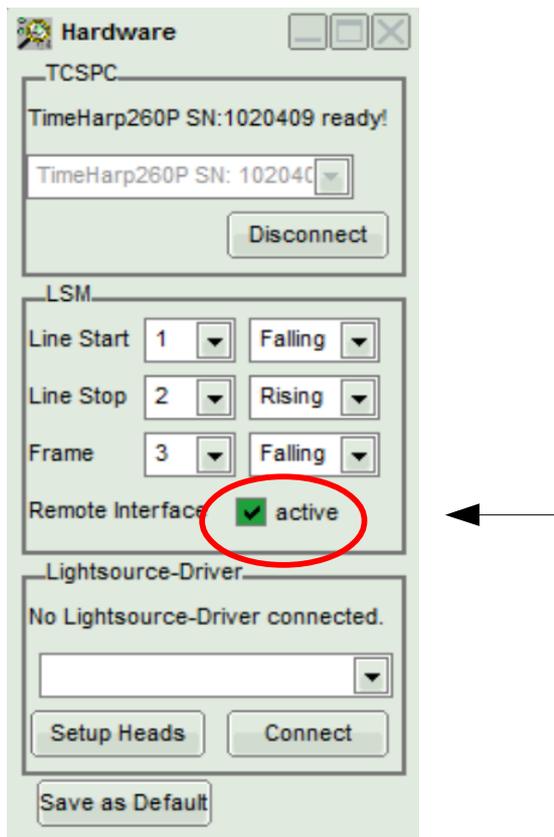


Fig. : Activation of the remote interface to allow the measurements to be initiated from the NIS software.

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”.

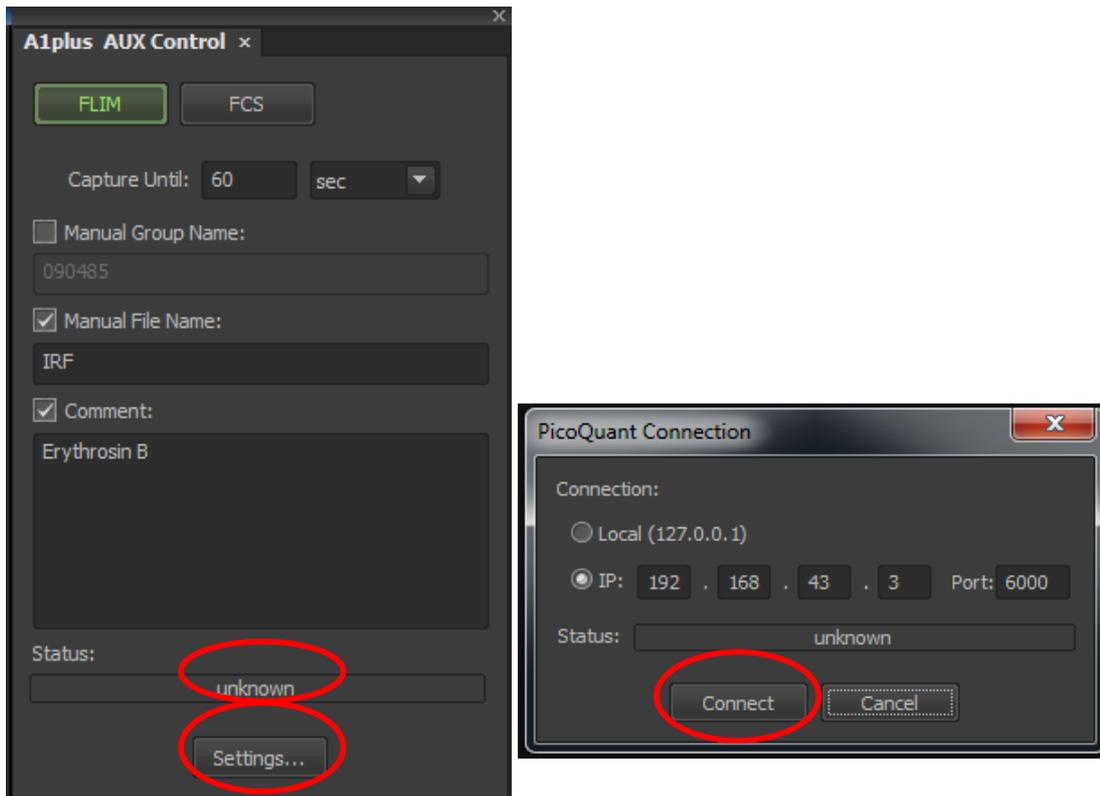


Fig. : Status and connection settings for Nikon and PQ computer communication.

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

The actual **hardware configuration** of your LSM FLIM / FCS Upgrade (LSM trigger signal configuration, name of the TCSPC device and its settings, number of detection channels, predefined view settings....) is saved in a **settings.pfs - file**. Restoring the configuration from the .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, the software can be started in the user mode and saved via “Settings” / “Save User Settings as”. This creates a *.pus – file, which contains the user specific settings. When changing from one user setting to another, the software needs to restart.

3.3. Instrument is loosing sensitivity

Note:

- It is recommended to purchase a **power meter** in the lab in order to measure the laser intensities for selected wavelengths. This is especially for FCS measurements of value since the laser power is a crucial parameter for FCS.
- If you have purchased a LSM FLIM / FCS Upgrade 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 so be found. A drop in system performance may be caused by:

1) Misaligned Pinhole

- Optimize the Pinhole Settings according 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.

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 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 PQ 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

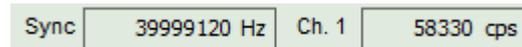
- Misalignment of the LSM itself: The first important step is to align the pinhole (see above). If this procedure does not help, contact your Nikon service representative.
- Misalignment of the detector(s): Refer to your detector manual.

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

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.
- Please make sure that the optical fiber to the PQ detector(s) is connected to the scan head.
- If the shutter is not the problem, take a FLIM image as described in the “acquire a FLIM image” section. Although no counts are displayed, a .ptu file is generated. **Calculate a time trace** from your FLIM - *.ptu - file by choosing the “*Intensity Time Trace*” analysis in the time-trace analysis drop-down menu (only available if you have a full software licence including point analysis). The displayed time trace should contain the photon counts. Select “*marker 1 - 3*” to show **line start, line stop and frame markers** that are passed from the instrument. You may have to select a suited display range in order to display the marker signals. If no marker signals are present, check cable connection between the Nikon C1si/C2 and the LSM FLIM / FCS Upgrade. Also try to start the Nikon C1si/C2 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.

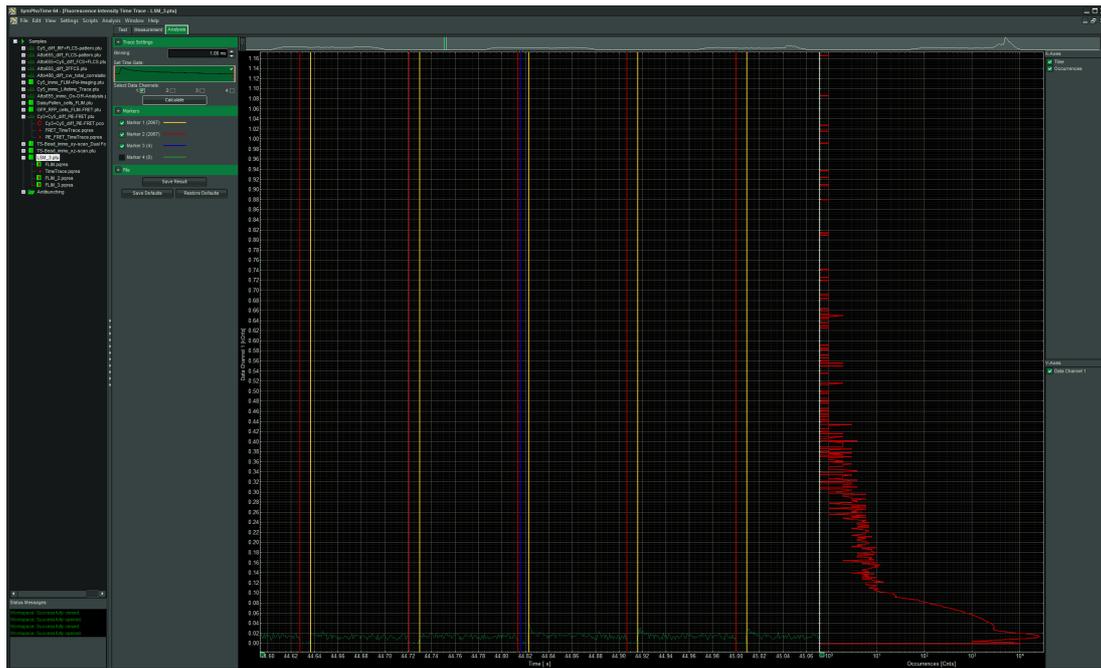


Fig. 3.1: The Time Trace analysis, where the line and frame - markers can be displayed.

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
- Adjust the pinhole as described above

3.6. 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>

4. 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

5. 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.

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