LSM Upgrade Kit

Compact FLIM and FCS
Upgrade Kit for **Nikon A1**
Laser Scanning Microscopes
with complete integration

User's Hardware Manual and Technical Data

Version 1.2

Table of Contents

1. Introduction

This manual describes the basic procedures to acquire fluorescence-lifetime imaging microscopy (FLIM) and point measurements for fluorescence correlation spectroscopy (FCS) using the laser-scanning miscroscope (LSM)-upgrade Kit in combination with your LSM.

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

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

For technical or software inquiries 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 (LCU), 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

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

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

- **MultiHarp 150, HydraHarp and TimeHarp 260 Manual:** Here you find all information about your Time Correlated Single Photon Counting (TCSPC) device. This manual also includes an introduction about single photon counting.

Fig. 1.3: MultiHarp150 and TimeHarp 260

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

Fig. 1.4: Laser driver for pulsed diode lasers: PDL 828 "Sepia" (left) and PDL 800-D (right)

- **Light Sources:** In the green folder you'll also find detailed information about the properties of your pulsed diode lasers.
- **Software Manual (SymPhoTime 64 - Manual):** Here you find all information about the software installation. The SymPhoTime-software contains a detailed, context sensitive online help function. Press F1 for accessing the online help. When placing the cursor into a number field and pressing F1, the help in the context of the field is opened.
- **Pre-Installation Requirements:** This is an important document that already should have been considered before installation. If you want to change the configuration of your LSM, you'll find detailed information if the desired change can be realized and whether additional parts are necessary. In case of doubt, contact a PicoQuant representative.
- **System Specifications:** The System Specifications contains specific information about your individual LSM FLIM / FCS Upgrade, including a description of the included parts, filter handling and a cable plan, which helps restoring the configuration after disassembly.

2. 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's hardware and software should be started in the following order:

1. Start both the NIS 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 **NIS** software.
4. 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.
5. If you want to work with user specific settings, load your **optical configuration (in NIS)**.
6. If you want to analyse your data in SymPhoTime, start **SymPhoTime in Analysis Mode (for FLIM) or in full SPT mode (FCS)**

   The upgrade kit is now ready to use.

2.3. Preparing NIS for FLIM

Start the NIS software with "PicoQuant (Nikon Confocal)" selected as a boot configuration. This selection only appears if the NIS hardlock has PicoQuant FLIM enabled. In case you can not find this configuration please contact your Nikon representative.

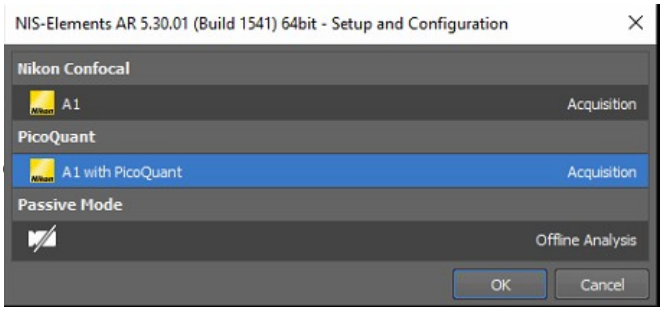


Fig. 2.1: Boot selection to perform FLIM measurements with NIS

Perform a right click and open the **A1 Pad** under acquisition controls. To access all necessary configurations for FLIM measurements select “FLIM” in the **A1 Pad**.

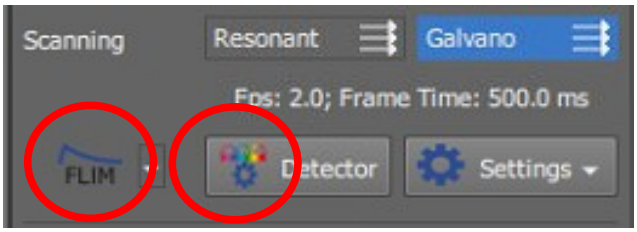


Fig. 2.2: Activate FLIM and open the optical settings to access the beam path configuration.

Click the settings button shown in Fig. 2.2 to select the active channels. Without an enabled channel on the DU4 detector no data aquistion is possible.

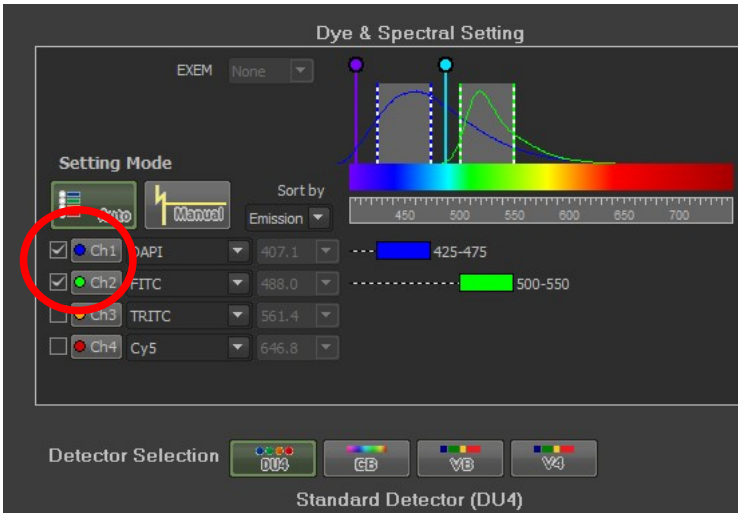


Fig. 2.3 Activation of channels in NIS software

Close the settings window and change to FLIM setup in **A1 Plus Pad** to continue.

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. It is selected at the top bar of the NIS software and buttons can be named e.g. “FLIM”.

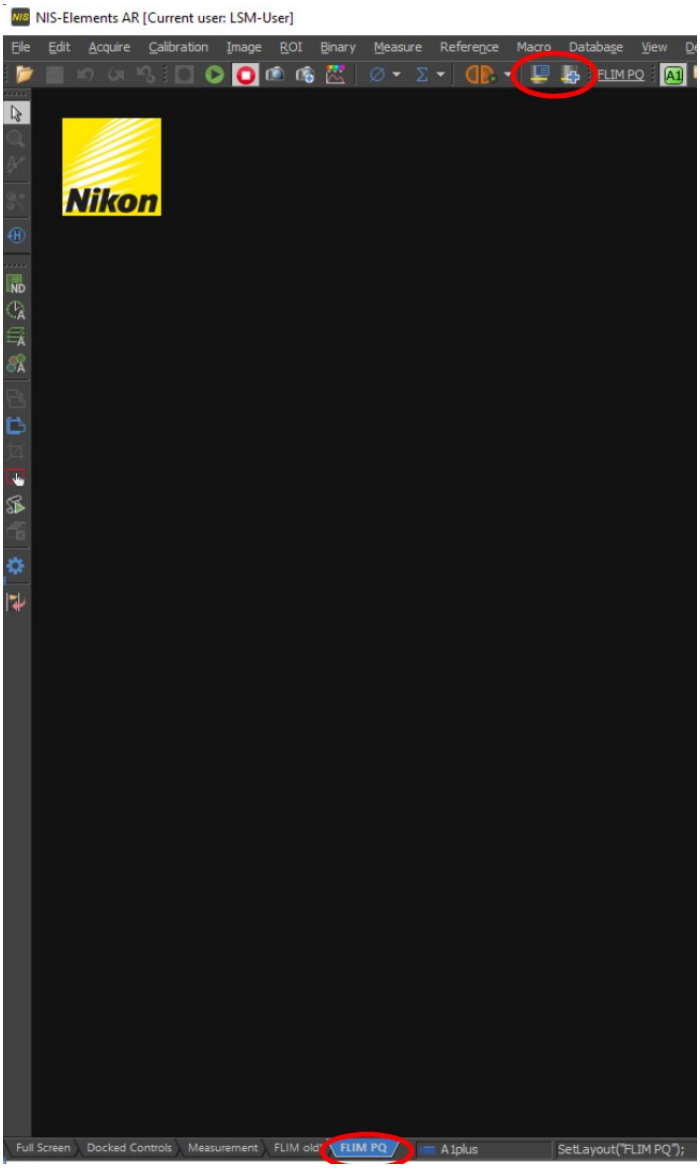


Fig. 2.4: Layout for FLIM measurements in NIS.

2.3.1. Creation of a Layout for FLIM measurements

In order to perform FLIM measurements the **FLIM Button** must be enabled. Furthermore, **A1 Pad**, **A1 Pinhole alignment** and **A1 Scan Area** should be open.

1. In the main NIS window do right click, go to **Acquisition Controls** and then Click in **A1 Pad**, **A1 Pinhole alignment** and **A1 Scan Area.**

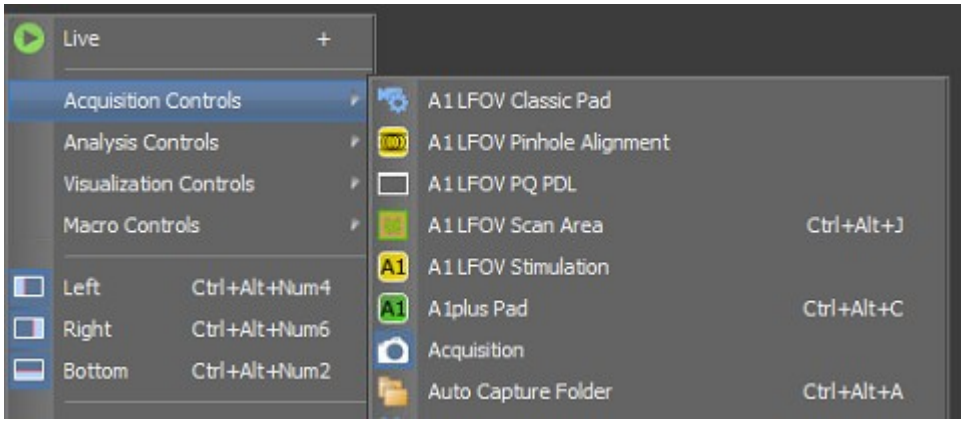
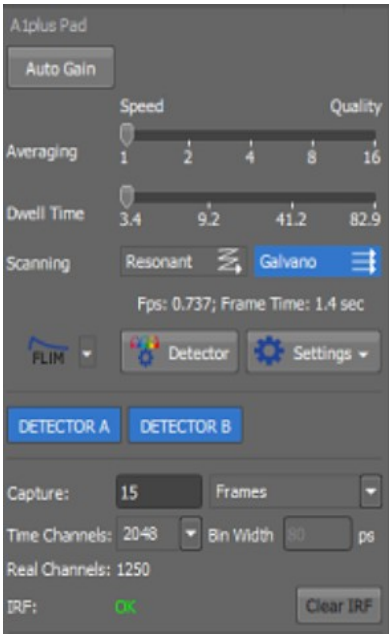


Fig. 2.5: Activation of the necessary windows to perform FLIM measurements in NIS.

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



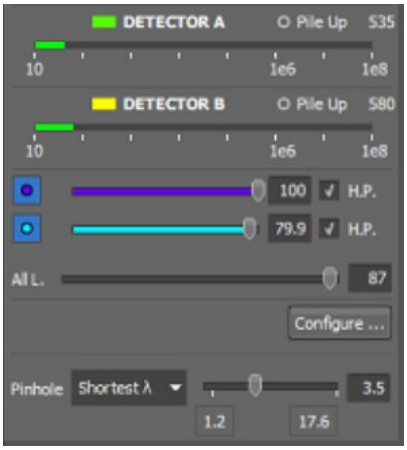


Fig. 2.6: Windows to perform FLIM measurementsin NIS.

2.3.2. PicoQuant Laser Control in NIS using the A1 Pad

It is possible to control the PicoQuant pulsed lasers via the NIS software using the A1 Pad. The appearance and features of the **laser control** depend on the LCU type that is used.

1. **LCU with motorized attenuators (AMOT)**

   In the main NIS window do a right click, go to **Acquisition Controls** and then select **A1 Pad** and **activate FLIM.** The Laser Control panel is located at the buttom of the window.

   It is possible to set the intensity of the lasers during a measurement using the sliders shown in Fig. 2.7. Pulse form and width are not changed. There is a slider “All L.” which controls the laser intensity if all pulsed lasers together.

   The electrical laser power is automatically set to an optimized value that corresponds to the shortest laser pulse. If “H.P.” (H.P. - High Pulse) is activated the electrical laser power goes to its maximum. This leads to a temporal broadening of the laser pulse.

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

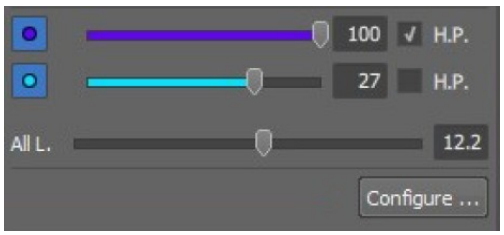


Fig. 2.7 PicoQuant laser driver control in NIS for the AMOT LCU.

2. **LCU without motorization (see Fig. 2.8):**

   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. The recommended pulse energy is set by clicking on "default". Please note that by changing the intensity, also the pulse form and temporal width will be changed.

Fig. 2.8: A1 Pad laser settings in NIS without AMOT LCU.

It is possible to select or deselect the lasers by clicking on one of the laser wavelength buttons. To set the **Pulse Mode (PIE (pulsed interleaved excitation) on/off)** and change the **Pulse Rate** click on **“Configure”**. (See Fig. 2.9)

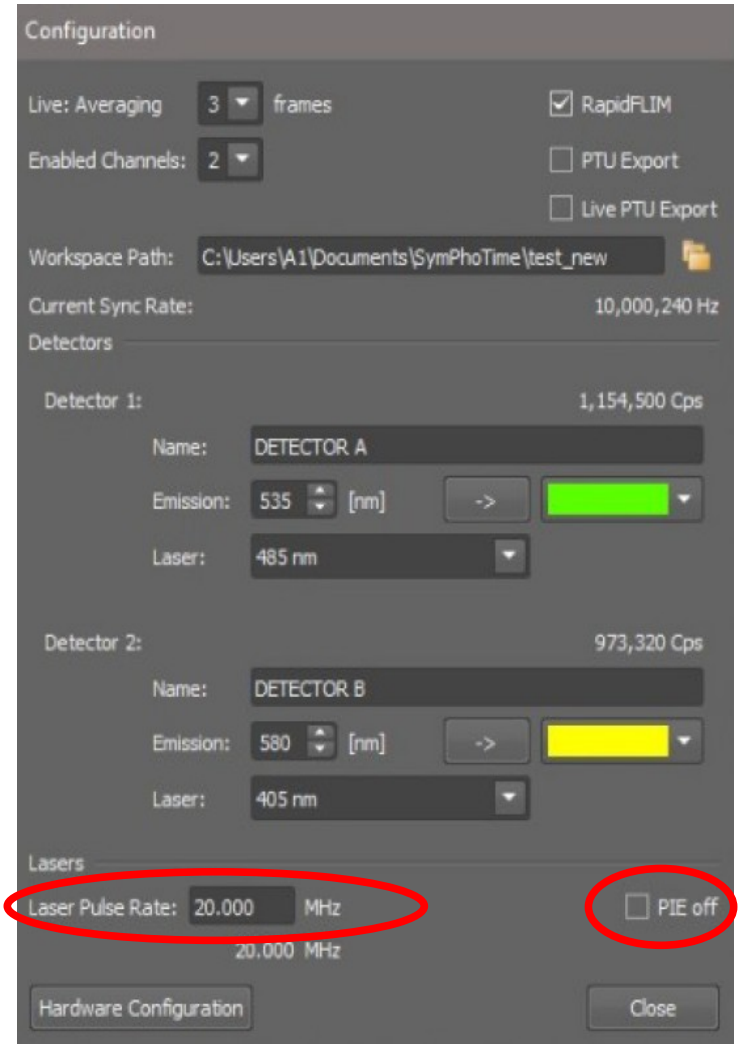


Fig. 2.9: Selection of Pulse Mode and Pulse Rate in NIS.

The given **repetition rate** describes the pulse rate of all laser pulses of different wavelengths together, while the resulting Sync Rate shows the Sync Rate of the generated pulse pattern. For example, when using three lasers with a pulse rate of 10 MHz, the distance between each laser pulse is 100 ns. The resulting sync rate is one third of the repetition rate, i.e. 3.33 MHz.

If in standard setting PIE is on: in this case, lasers of different wavelengths are pulsing not at the same time but one after another. By selecting the desired laser wavelength for each detector (e.g. 405 nm for DETECTOR B, as shown in Fig. 2.9), cross talk between channels can be minimized. Only the fluorescence detected in a specific detector channel is displayed, which is generated by the selected laser excitation wavelength.

2.3.3. Configuration for FLIM Measurements

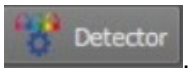
The configuration pre-sets the following optical and electronic settings in the Nikon A1 to perform the measurements:

- 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 to be performed.

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

1. In the **A1 Pad** click in the **Optical Path Settings** 

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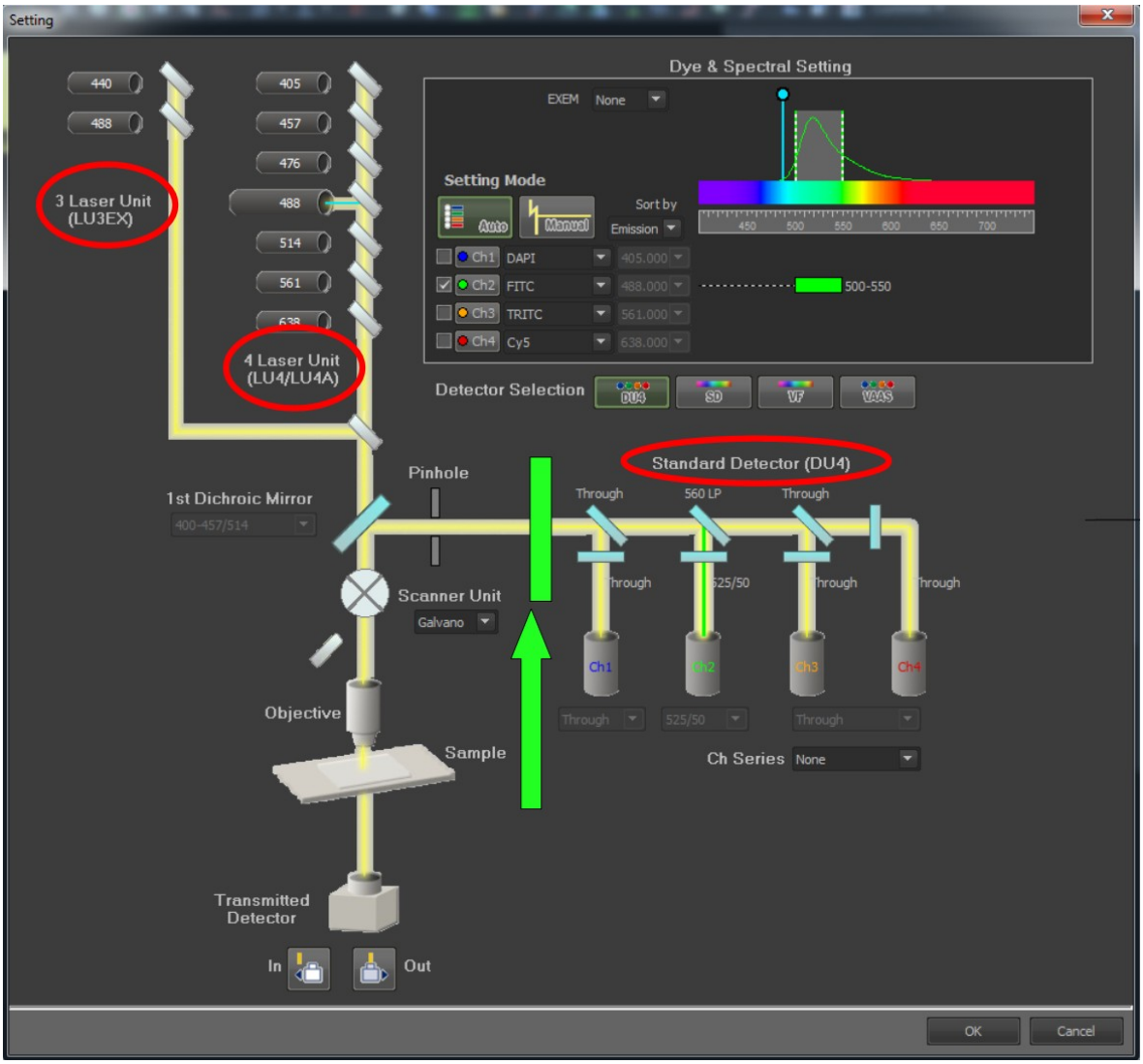


Fig. 2.10: Optical Path Settings. PicoQuant pulsed lasers are selected with the “port” selector, PicoQuant lasers are at port 2, Nikon lasers at port 1. Please be aware when using the Nikon laser combiner LUN-V that PicoQuant pulsed lasers cannot be displayed in the Settings window.

The window shows a diagram of the Optical Path. Pulsed lasers from **PicoQuant (LU3)** and CW lasers from **Nikon (LU4)** 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). PicoQuant lasers are selected automatically by selecting FLIM, even if they are not displayed in the Optical Path Settings. The laser entrance port switches for FLIM automatically to laser “Port 2” where the PicoQuant lasers are connected. Nikon lasers are connected to “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 Fig. 2.10 its position is marked with a green arrow.

2. Despite the fact 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 FLIM, respectively.

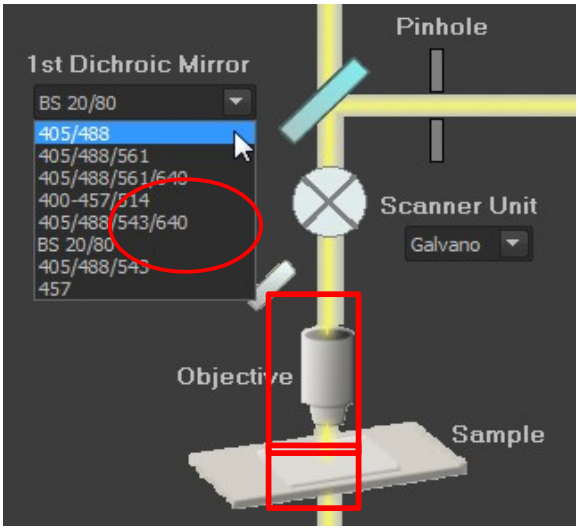


Fig. 2.11: Select a suitable excitation dichroic for the combination of excitation and detection..

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

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

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

4. In the **A1 Pad** window some settings also have to be selected. For standard FLIM measurements:
   - Scan with the Galvano mirrors (Resonant mode can not be used for FLIM).
   - Mono-directional scan (bidirectional sometimes leads to artefacts).
   - Image Size of 512 x 512 pixels is recommended.
   - No averaging (averaging can not be interpreted by SPT) → “Normal” must be selected.
   - Pinhole size of 1.2 Airy Units (AU) (you may increase it if the signal is too low, at the expense of axial resolution).
   - Select the proper excitation laser for the calculation of the pinhole size in AU.

- The “Time channels” should be set to the maximum value (2048 or 4096) for highest time resolution in FLIM image. If the data size of the recorded FLIM images becomes too big, you can decrease this value at the expense of a lower FLIM temporal resolution.

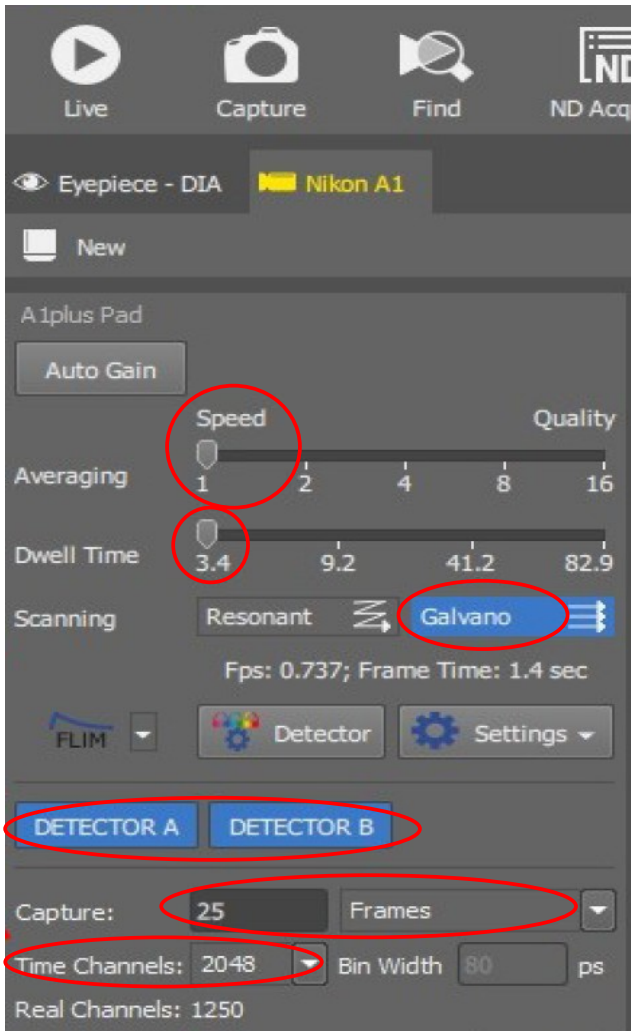


Fig. 2.12: Standard FLIM settings for the A1 Pad.

5. In the **A1 Pad** window select FLIM as well as the criterion to determine the duration of the FLIM measurement (number of frames, time or accumulated photon counts in the brightest pixel of the FLIM image).

Fig. 2.13: Selection of the duration of the measurement.

6. 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 nm) 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.

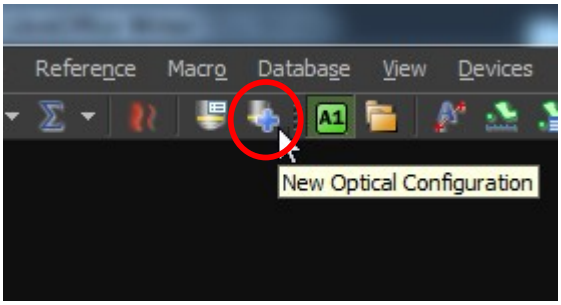


Fig. 2.14: Creation of a new Optical Configuration in NIS.

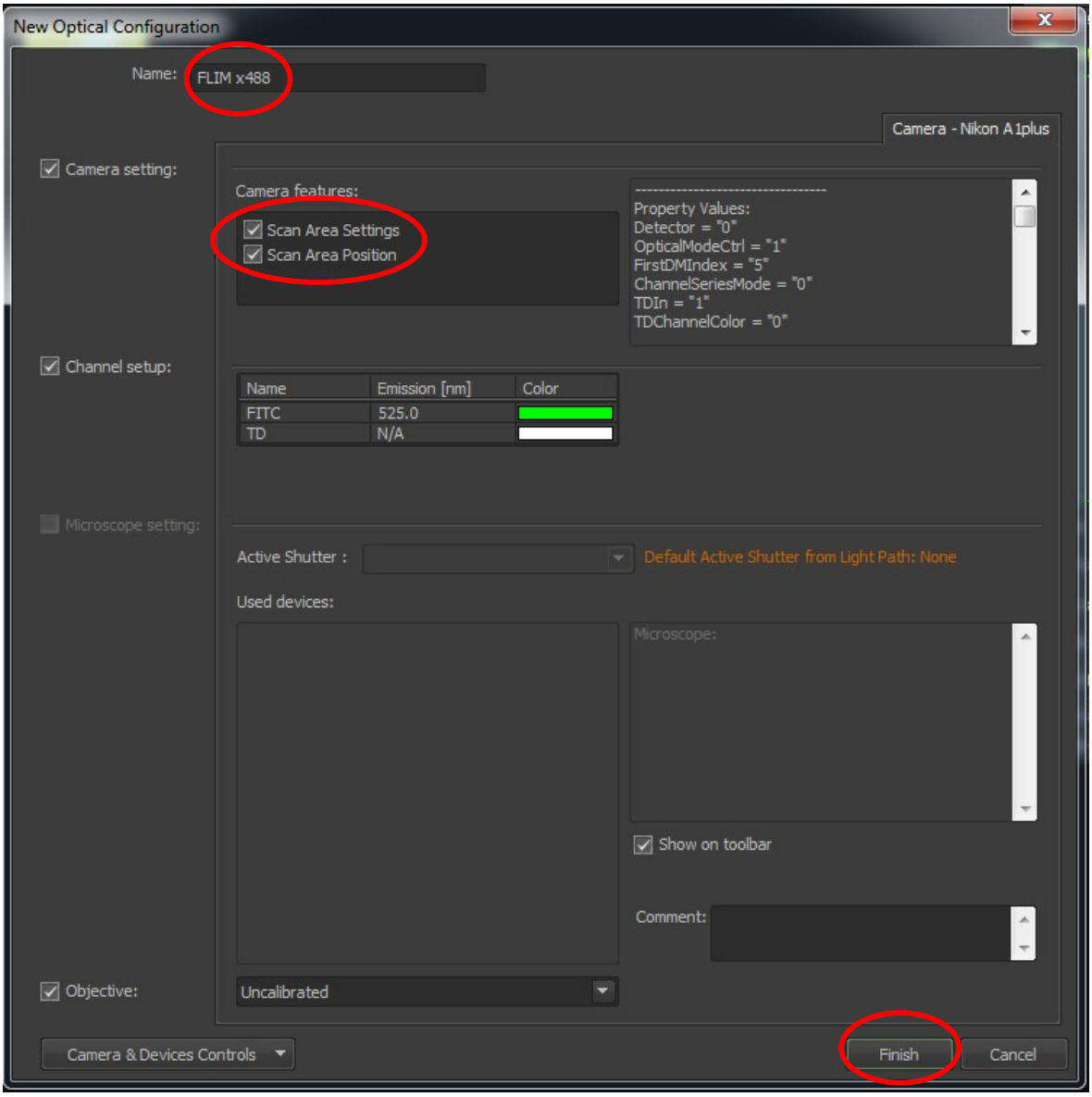


Fig. 2.15: Saving of an Optical Configuration. If “Scan Area Settings” and “Scan Area Position” are not checked, the “Optical Configuration” will not store these settings.

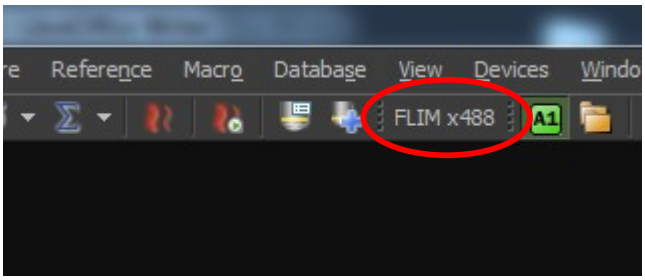


Fig. 2.16: User configuration “FLIM x488” in the top bar of the NIS main window.

2.4. Settings for FLIM Acquisition

2.4.1. Starting Point

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

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

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

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 "**Scan**" measurement.

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

. A test measurement will be automatically started.
3. After the scan has been activated, the image appears. Open the **PicoQuant Decay Curve Pad** by right clicking in the image area and observe the decay behavior (see Fig. 2.17). Tune the laser intensity with the attenuation sliders in **A1 Pad** until the observed decay curve has a peak between 10.000 and 100.000 counts.

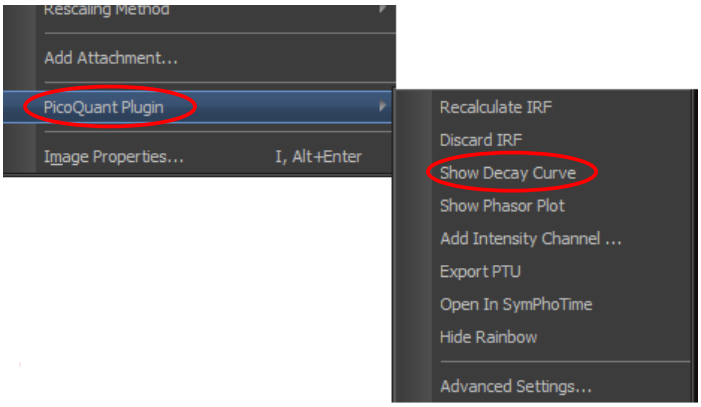


Fig. 2.17: Open the PicoQuant Plugin by right clicking on the image.

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

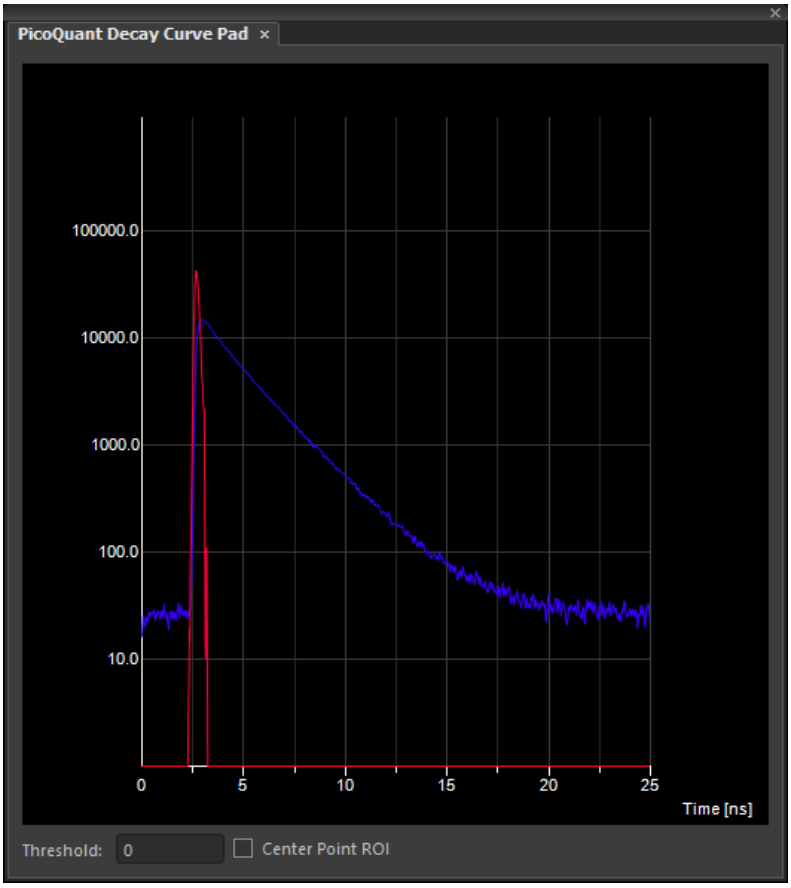


Fig. 2.18: Picoquant Decay Curve Pad showing fluorescent decay (blue) and calculated IRF (red) curves.

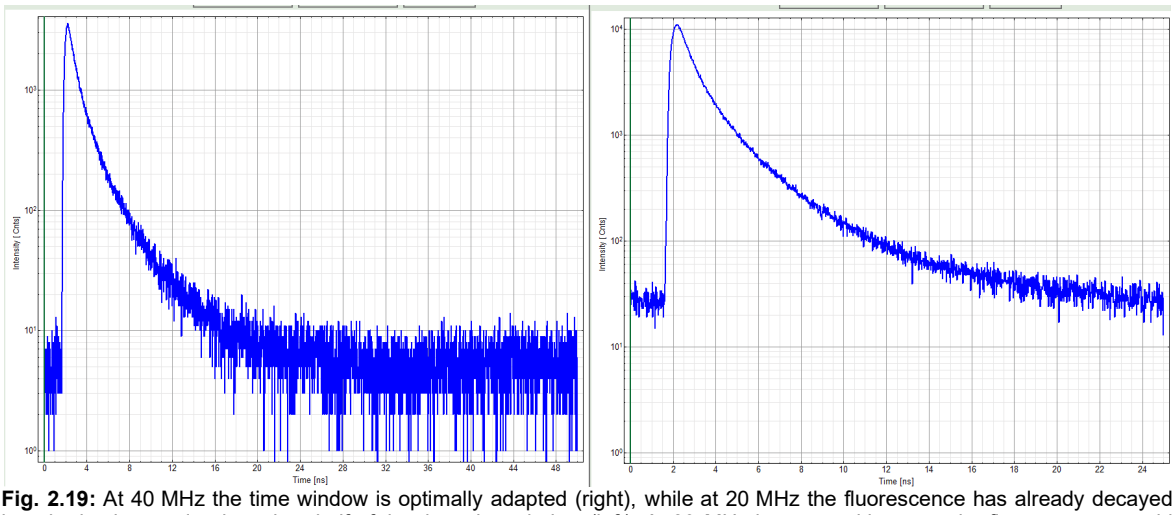


Fig. 2.19: At 40 MHz the time window is optimally adapted (right), while at 20 MHz the fluorescence has already decayed into the background at less than half of the detection window (left). At 80 MHz laser repetition rate, the fluorescence would not decay completely before the end of the time window is reached. Due to a "wrap around", the decay tail would be observed even before the actual pulse. In this case the "wrap around" correction of SymPhoTime should be used to analyse the FLIM image.

4. **The best repetition rate is the highest at which the fluorescence decay is complete**. In other words, the pulse sequence has to be as high as possible, in order to increase the overall count rate, but slow enough to allow the population of excited state to be completely depleted within two laser pulses[1]. The best way to quickly visualize this is by checking that at the beginning and end of the

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.

decay there is flat background, and that the background after the decay does not occupy more than 20-30% of the window.

5. For PicoQuant pulsed diode lasers operated with the Sepia II laser driver the **laser repetition rate** (Sync rate) can be set under **"Configuation"** in **A1 Pad** (see Fig. 2.20). In case a manual laser driver is used (e.g. PDL-800D), please set the repetition rate at the laser driver manually.

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

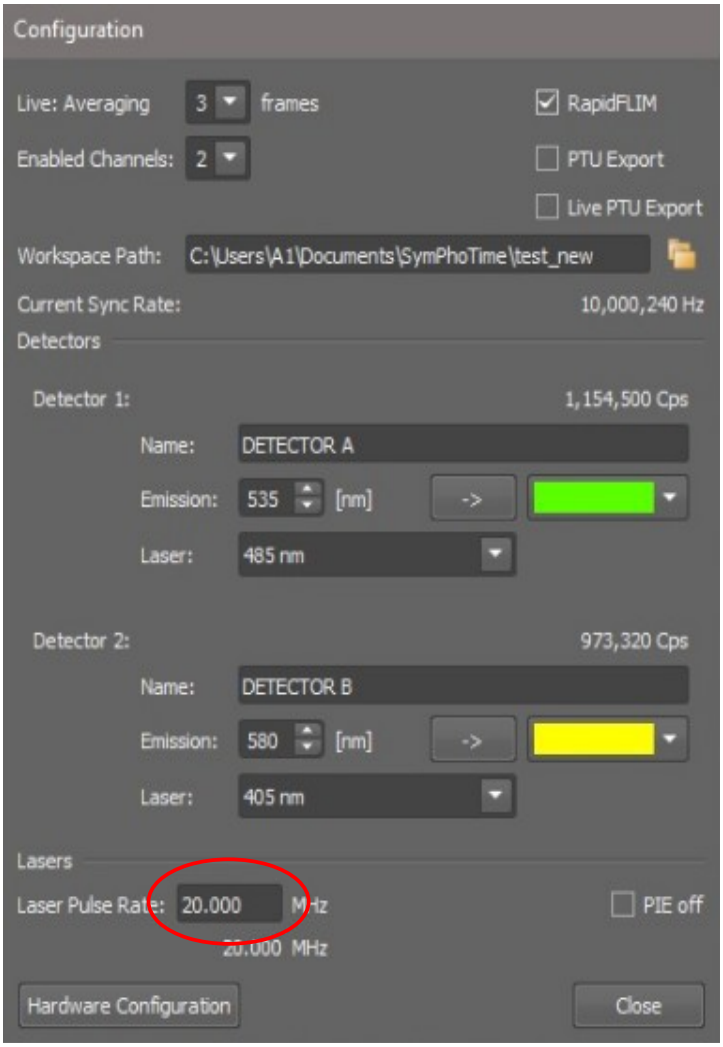


Fig. 2.20: Laser pulse rate settings in NIS.

6. The goal now is to **set the count rate in the brightest pixel at approximately 10% of the excitation rate**. For rapidFLIM systems, FLIM images at much higher count rates can be measured. High count rates can lead to distorted decays due to the pile up effect[1]. It is recommended to adjust the laser intensity in a way, that a high bleaching rate of the image is avoided and a detection count rate below the pile up limit is achieved. For detector count rates above the pile up limit, a red pile up indicator flashes (see Fig. 2.21). If it flashes red, the laser intensity should be reduced using the laser intensity settings. Count rates above the pile up limit lead to an error of the calculated lifetimes in the FLIM analysis.

   Systems equipped with the MultiHarp using the rapidFLIM technology are not restricted to a count rate limit of 10% of the repetition rate.

1 For more information about the pile effect and electronics dead-time please refer to the TCSPC card manual.

Fig. 2.21: Indication of Pile Up limit

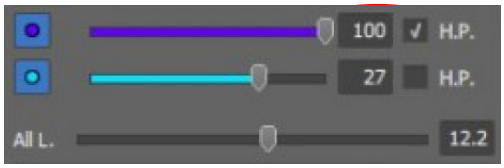


Fig. 2.22: Selection of the laser intensity

2.4.3. FLIM Measurement

1. Activate the PTU Export in **"A1 Plus Pad → Configuration".** Please specify a workspace Path, where all FLIM image data are to be saved for further analysis in the SymPhoTime software. The "PTU Export" feature allows NIS to generate and save automatically with each FLIM measurement a PTU file which contains the complete information of the recorded FLIM image. This PTU file export is especially useful for rapidFLIM applications since it can be used in conjunction with the rapidFLIM data fitting in SymPhoTime. This analysis method corrects for artifacts which come from the finite pulse width of the detectors leading to a so called "pulse pile up". By applying the pile up correction, acquisition at very high count rates in the range of the laser repetition rate and higher can be performed. Selection of the excitation laser or detection channels must be performed in SymPhoTime by using the PTU export feature.

   Please define a workspace path for FLIM data storage (see Fig. 2.23).

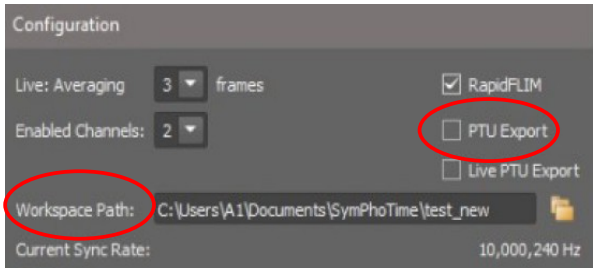


Fig. 2.23: When the PTU Export is activated, all scan data and each measurement will be saved in a workspace.

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

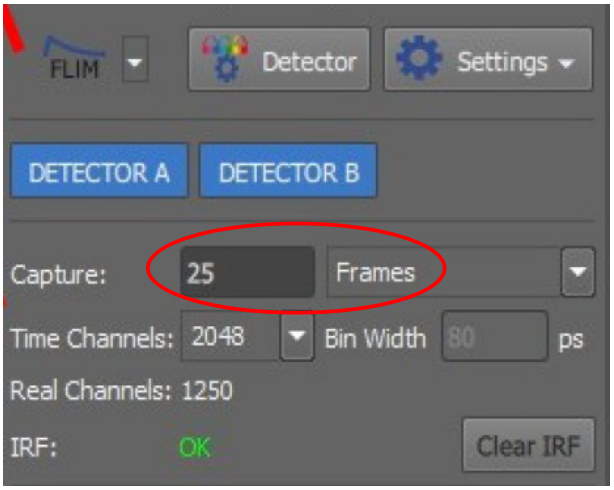


Fig. 2.24: Duration of a FLIM measurement. Choose between stop after frames, stop after photon counts or stop after time.

3. Press “Scan” to start a test measurement. The display parameters (LUTs) are available by clicking the blue arrow a the top of the window (see Fig. 2.25).

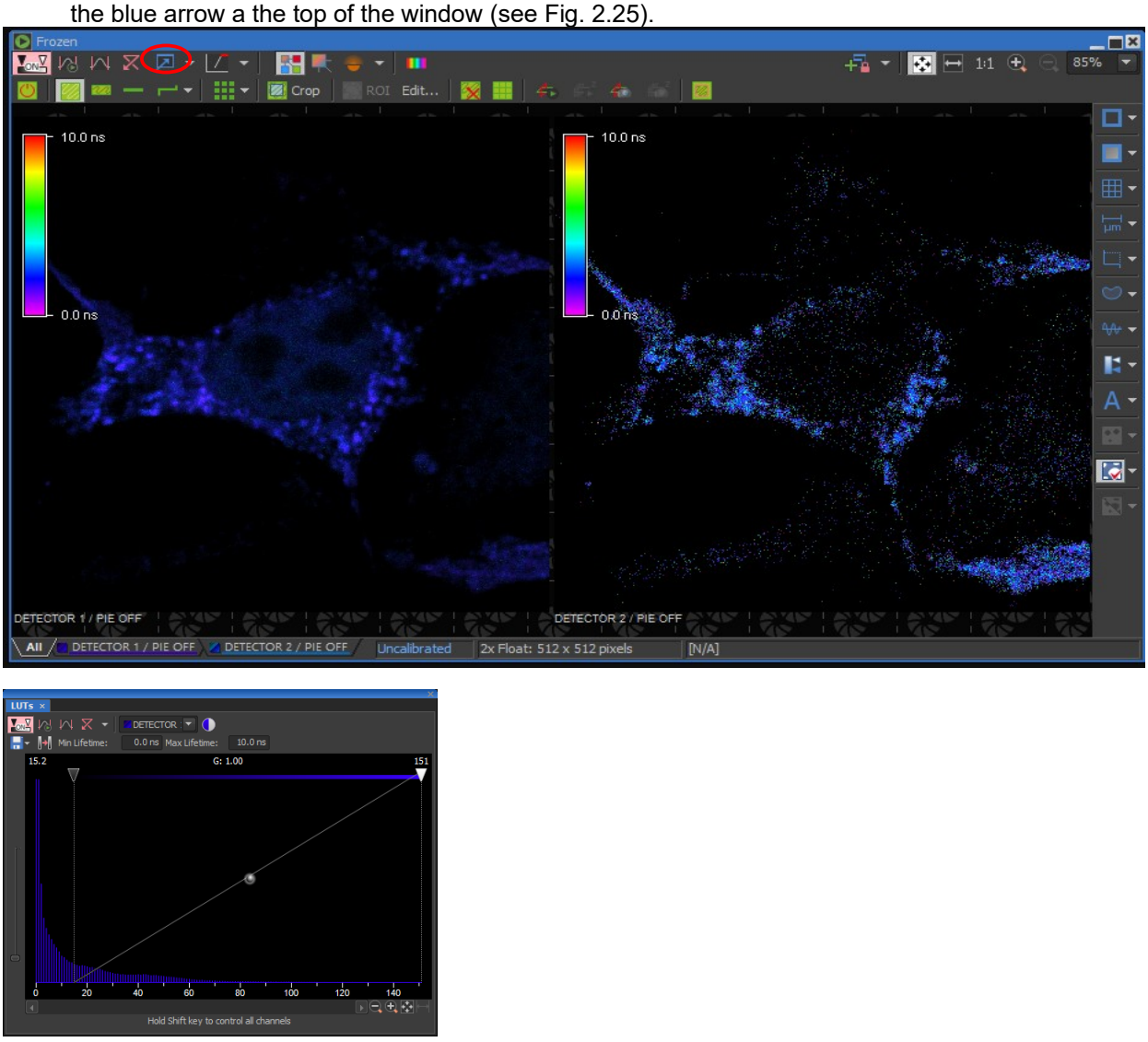


Fig. 2.25: Measurement preview displayed during acquisition. In the LUTs window the intensity scale as well as the lifetime display can be set (e.g. here the false color display shows 0 ns in magenta while lifetimes of 10 ns are depicted in red).

4. Press 

to start the final FLIM measurement.

5. The measurement comes to its end when the finishing criterion is reached. Alternatively it can be interrupted at any time by pressing again 

. Data will be automatically saved. As a rule of thumb for homogeneous samples, a FLIM measurement can be interrupted when the brightest pixel has acquired 1000 photons for an average lifetime calculation.

2.4.4. Data Analysis using SymPhoTime

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

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

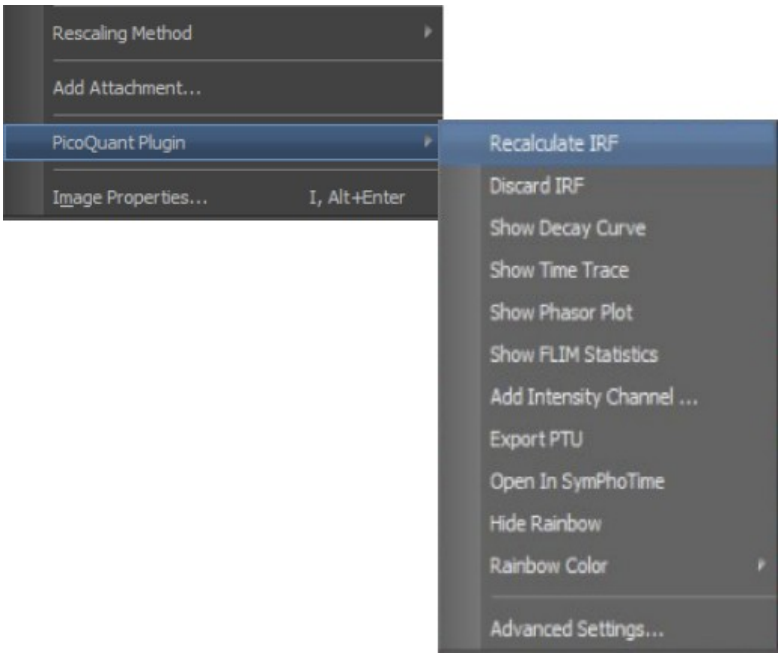


Fig. 2.26 „Open In SymPhoTime“ to analyze the FLIM image in SymPhoTime.

2. Open the workspace manually.

   NIS already creates the correct data format “.sptw “ under the path that was choosen in Fig. 2.23.

   Open SymPhoTime, click on “File” and select a workspace using “open Workspace”.

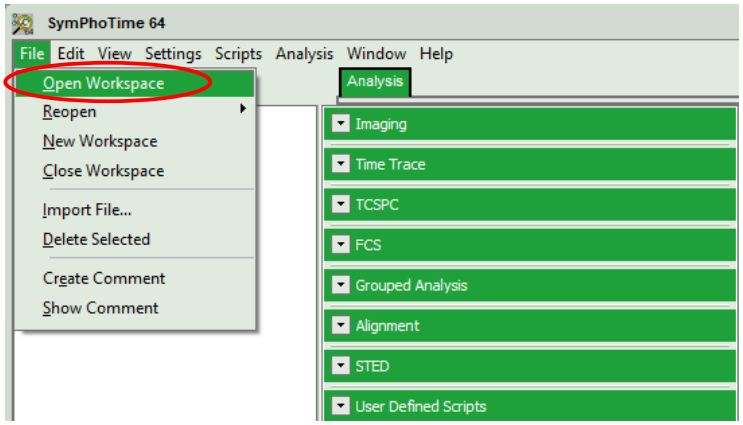


Fig. 2.27: How to a open a workspace in SymPhoTime.

Once the workspace has been loaded the user can select each scanned measurement and each captured measurement for the following analysis. New measurement data from NIS of the same day is loaded into this workspace and into SymPhoTime automatically.

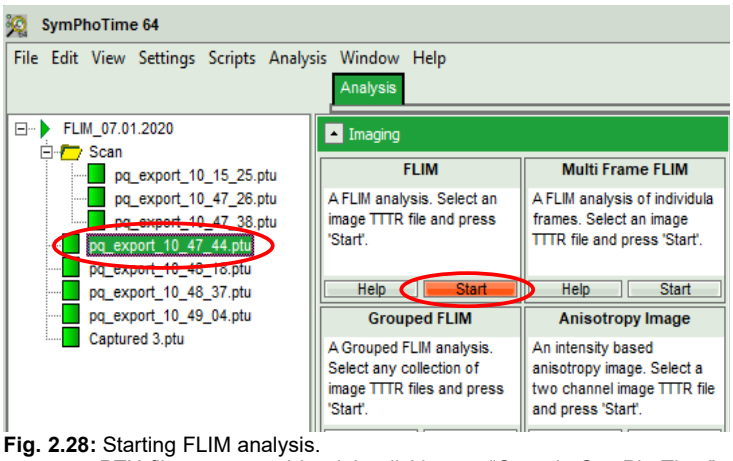


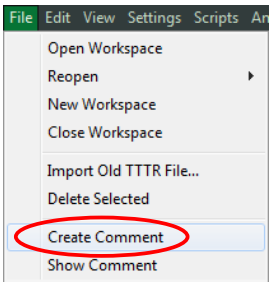
Fig. 2.28: Starting FLIM analysis.

- PTU files generated by right clicking on “Open in SymPhoTime” are listed as “Captured...ptu” files. No rapidFLIM correction is possible with these files. They contain only the selected detector channels excited by the selected laser line. The FLIM images contain only one frame (all frames during measurement are summed up).
- PTU file generated by the “PTU export” feature (see Fig. 2.23) are displayed in the workspace as “pq_export_hour_min_sec.ptu”. All ptu files generated by “Scan” imaging are listed in the “Scan” folder. RapidFLIM correction is possible, the PTU file contains all data channels. The correct laser line must be chosen by applying time gating in the TCSPC window in case of PIE excitation. Frame selection is possible.

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

2.4.5. Resulting Raw Data File and Documentation in SymPhoTime

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



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

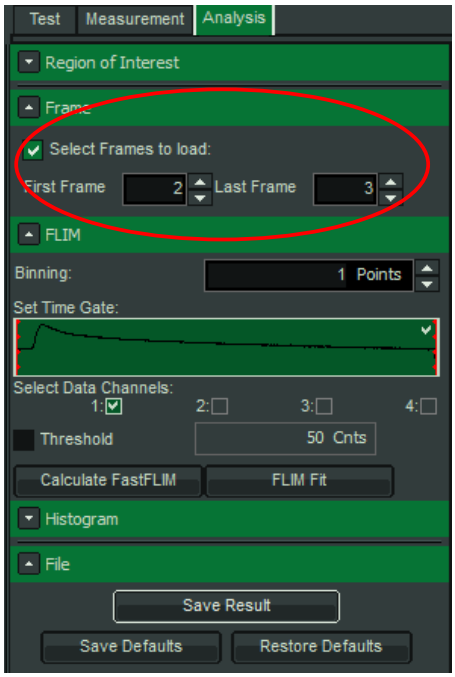


Fig. 2.29: Changing the selection of frame numbers for analysis in the SymPhoTime 64 Software (only for "PTU-export" files).

2.4.6. Measure an Instrument Response Function

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

1. Estimation of IRF in the Nikon NIS software

The NIS software estimates the IRF automatically from the decay measured during a FLIM “Scan” measurement. To check the IRF and observe its behavior make a right click inside the image and select **“PicoQuant Plugin → Show Decay Curve Pad”.**

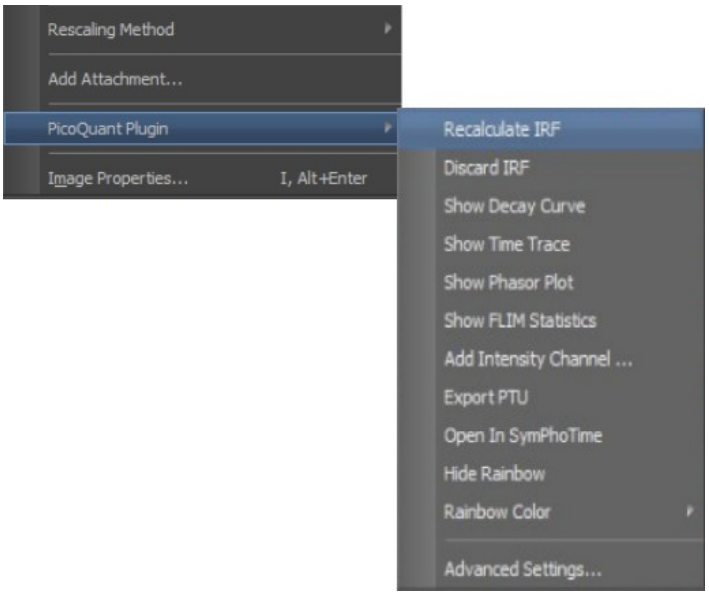


Fig. 2.30: Open the PicoQuant Decay Curve Pad: “Show Decay Curve“

If the settings are changed (e.g. repetion rate or the laser power is changed from optimal power to “H.P. - High Power”) the IRF should be recalculated. This is done automatically after clicking on “Recalculate IRF” with the next “Scan” measurement. The IRF is as well recalculated everytime the configuration is changed. For an optimal estimation of the IRF a monoexponential decay works best.

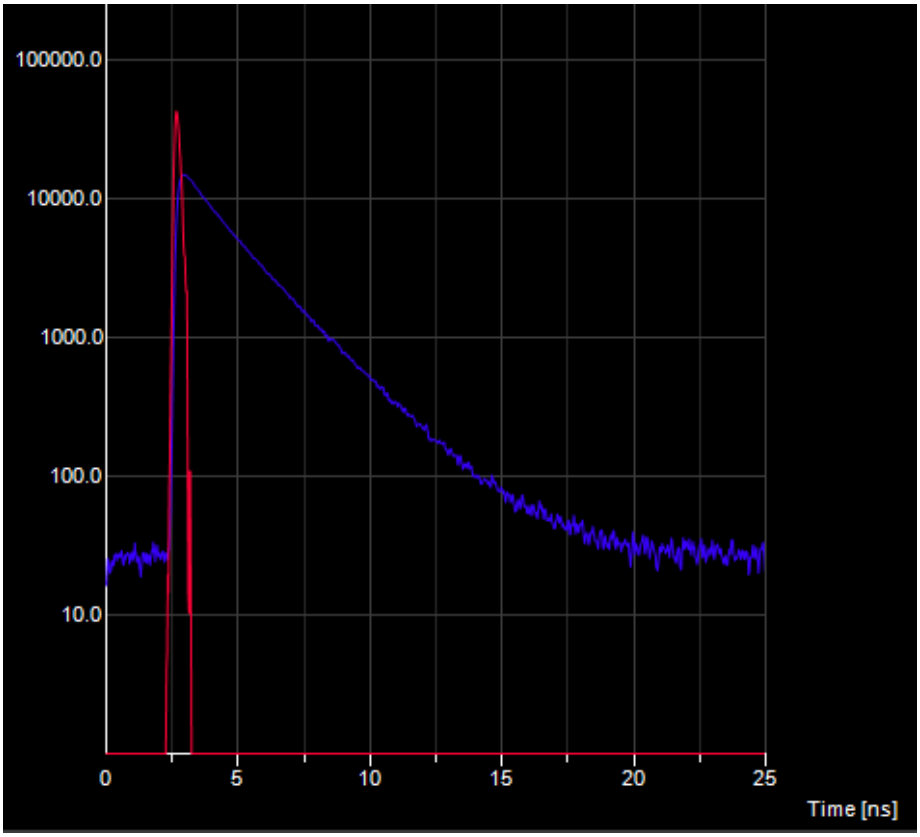


Fig. 2.31 PicoQuant Decay Curve Pad showing a calculated instrument response function (in red)

2. Measurement of the IRF and import into the SymPhoTime Software

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

a) Place a sample with a **negligibly short lifetime** in the laser focus on the microscope stage (e.g. Erythrosin B or Fluorescein, dissolved in a saturated KI-solution in concentration near to the saturation limit. For Fluorescein, a slight basic pH value is necessary to dissolve a sufficient amount of dye). The dye chosen should have a similar emission range as the sample that should be measured afterwards. Potassium iodide is a strong quencher and reduces the fluorescence lifetime to a few picoseconds. See e.g. Applied Spectroscopy, Vol.63, p.0363-0368 (2009). For 2-photon excitation, also second-harmonic generation may be used to measure the IRF (e.g. by using urea crystals).

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

c) Start the scanning process by pressing 

.

d) **PicoQuant Decay Curve Pad:** You can now check the decay behavior of your sample using the PicoQuant Decay Curve Pad, as shown in Fig. 2.30. Adjust the count rate to approximately 50 kCounts/s. The laser power can be changed optically by attenuating or electronically by changing the electrical power of the laser diodes. Optical attenuation does not change the IRF while electrical power changes will affect the IRF, which has in most cases a faster response at lower electrical laser powers. You can use the electrial laser power settings to optimize the pulse shape of the laser for shortest IRF width.

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

.
Steps d) and e) are only for checking of the IRF shape and settings, they do not need to be performed routinely.

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

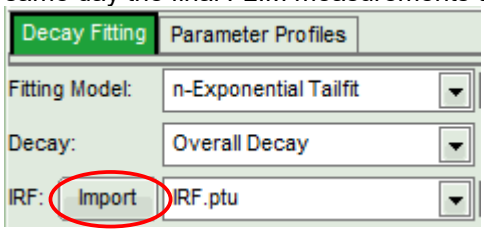
.

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

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

i) Replace the IRF solution with your sample again.

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



2.5. Settings for FCS Acquisition

Close NIS software if already open in FLIM mode.

Open SPT in data acquisition (standard) mode. PicoQuant hardware devices are now controlled by SPT.

To perform FCS the NIS software must be started in the “Nikon confocal” configuration.

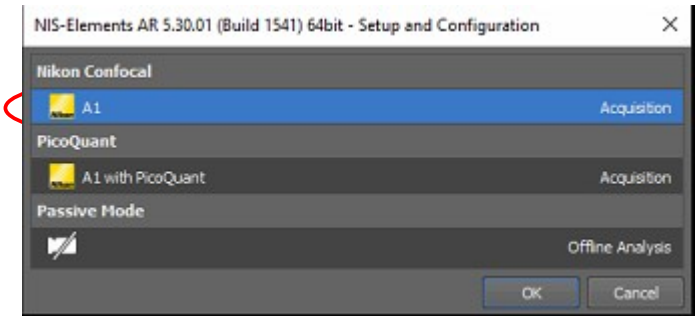


Fig. 2.32: Select this configuration to perform FCS measurements

2.5.1. Creation of a Layout for FCS Measurements

In order to perform FCS measurements the **AUX port** must be displayed and enabled. Furthermore, the **A1 AUX control**, **A1 Pad**, **A1 Pinhole alignment** and **A1 Scan Area** should be open.

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

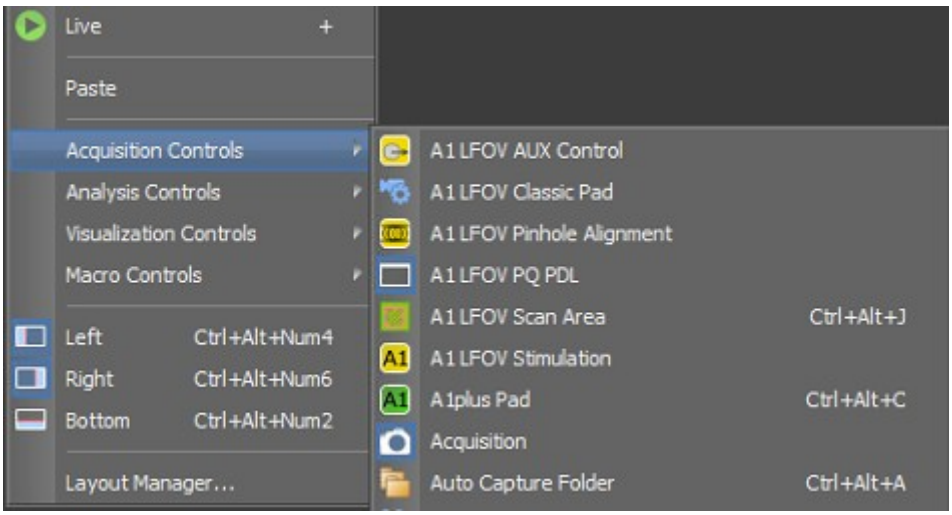


Fig. 2.33: Activation of the necessary windows to perform FLIM and FCS measurements in NIS.

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

For FCS measurements , the **“FCS-Button”** in the **A1 Plus AUX Control** must be enabled (see Fig. 2.34).

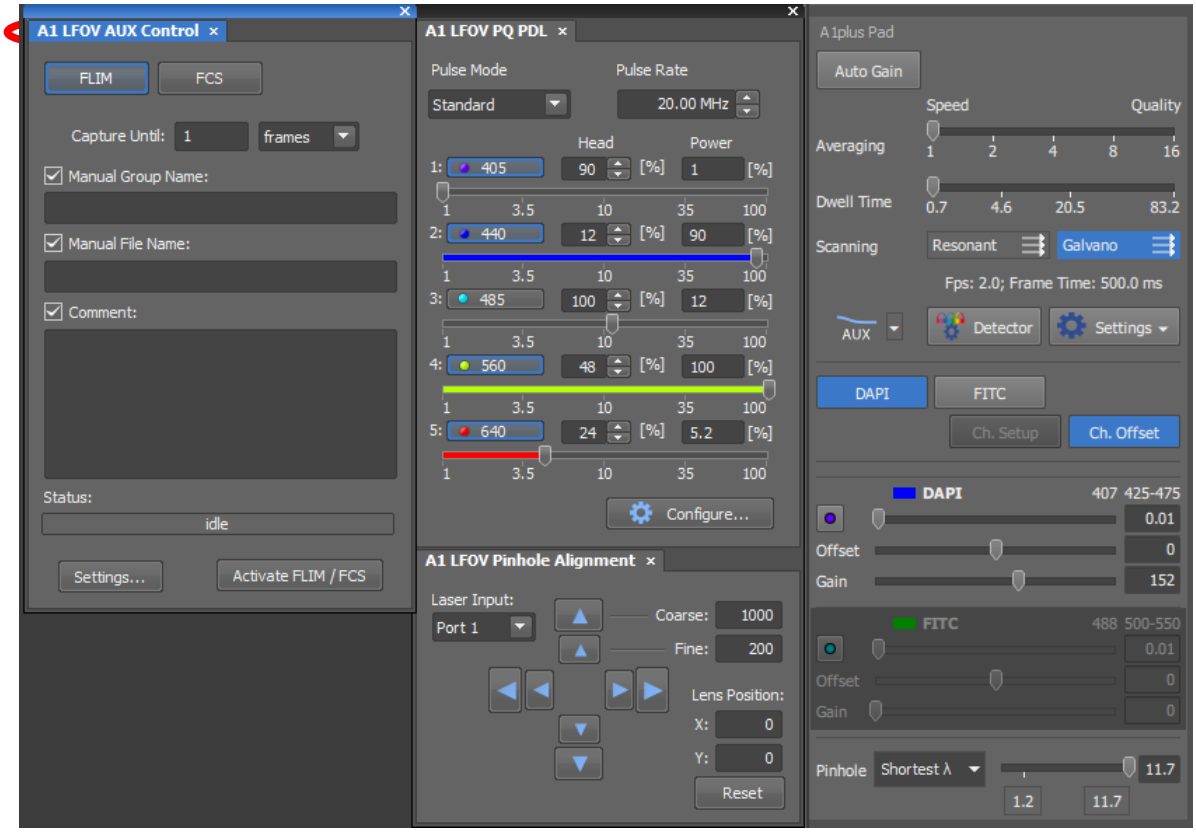
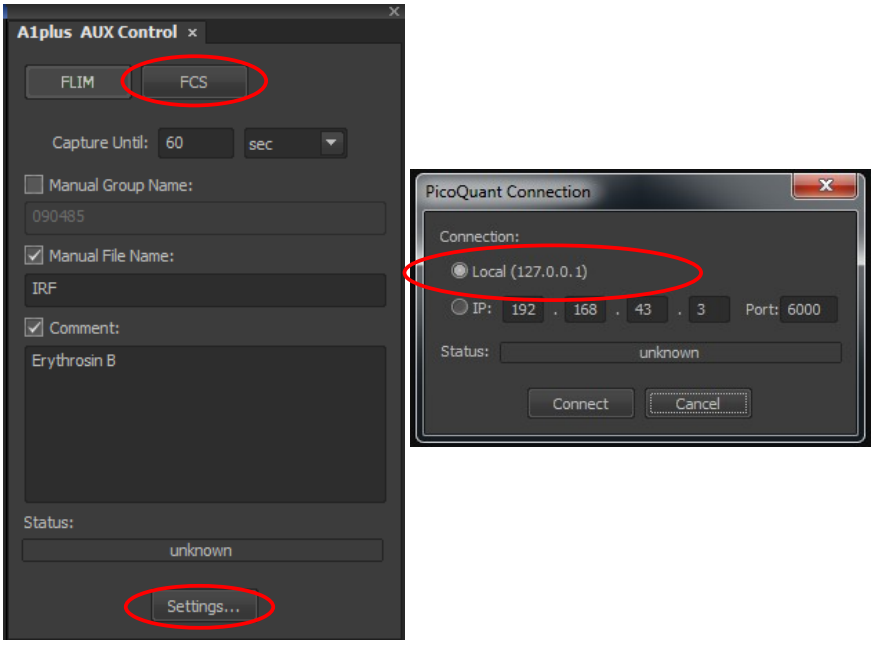


Fig. 2.34: Windows which should be opened to perform FLIM and FCS measurements.

2. Just to check, this should be set automatically: Go to the **A1 Plus AUX Control** and click **"Settings"** to open the **PicoQuant Connection** window and choose the local connection (127.0.0.1). The „Status" should be on „idle" while no measurement is performed.



3. **Fig. 2.35:** Status and connection settings for Nikon and PicoQuant computer comunication.

4. 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.

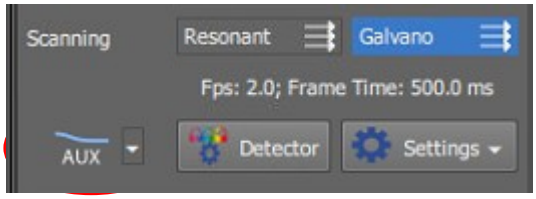


Fig. 2.36: AUX port should be selected.

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

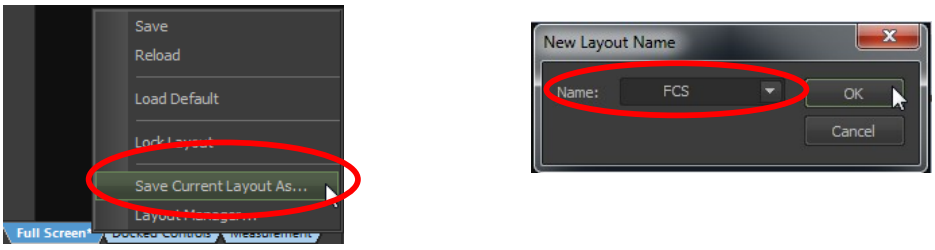


Fig. 2.37: Saving of the newly created FLIM-FCS layout.

The layout for FLIM and FCS measurements is now saved.

2.5.2. 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 **A1 PQ PDL**.

1. In the main NIS window do a right click, go to **Acquisition Controls** and then select **A1 PQ PDL.** Upon clicking on **A1 PQ PDL** its corresponding window will be opened.

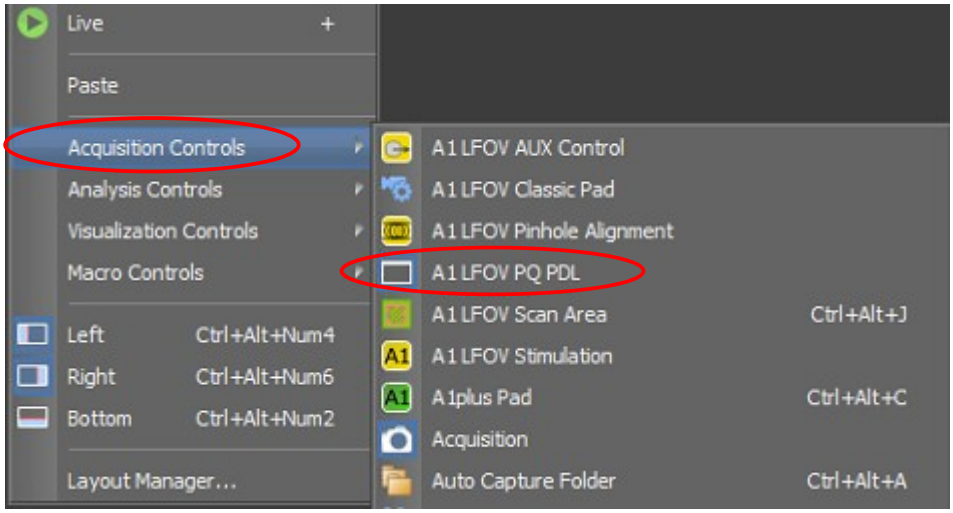


Fig. 2.38 Activation of the A1 PQ PDL window to control the PicoQuant Laser Driver in NIS Software.

2. The appearance and features of the **A1 PQ PDL** window depend on the LCU-Type that is used.

   A) **LCU without motorization** (see Fig. 2.39 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) **AMOT LCU with motorized attenuators** (see Fig. 2.39 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. 2.39 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.
   As a standard the system is already configured correctly. Only if there are problems, the system settings may be redone by clicking on “Configure”. The PicoQuant PDL Configuration window will pop up. 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.

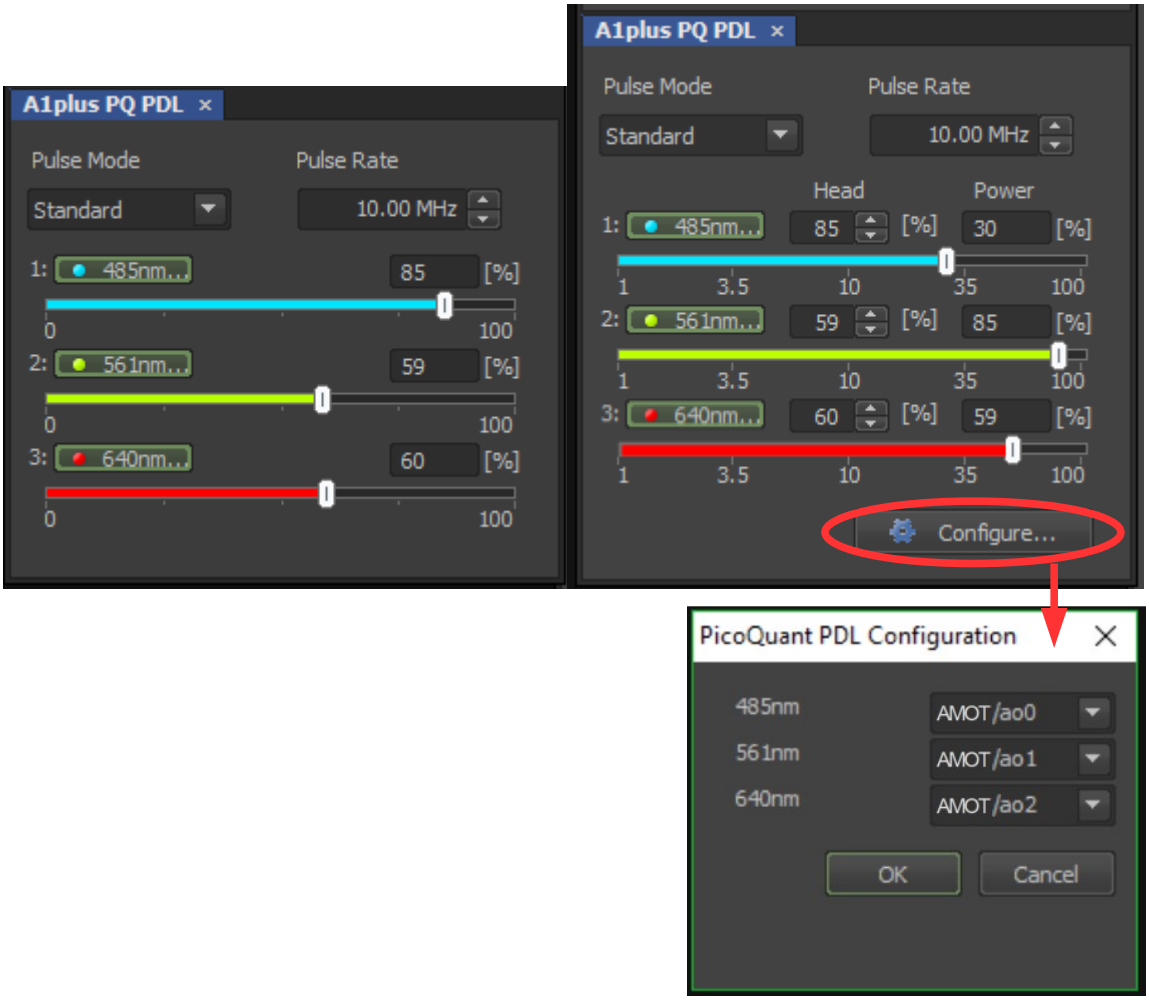


Fig. 2.39 A1 PQ PDL window, **left:** LCU without motorization The sliders can be used to change the electrical laser power. **right:** LCU with motorization. The electrical laser power can be set in the field named "Head", while the laser attenuation can be configured using the sliders or the field named "Power".
In the PicoQuant PDL Configuration window the analog 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 (see Fig. 2.12).

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 10 MHz, the distance between each Laser Pulse is 100 ns. The Resulting Sync is one third of the pulse rate, i.e. 3.33 MHz.

B) Once for example 3 lasers are selected in PIE-Pattern Mode with a Pulse Rate of 10 MHz and a measurement is started, the Pulse Pattern (Sync 3,33 MHz) 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 10 MHz, Sync 5 MHz) it is necessary to deselect all Lasers, start and stop a measurement and after that select the two lasers that should be used.

2.5.3. 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 **A1 Pinhole Alignment**, respectively).

Lets imagine we would like to excite with a PicoQuant laser at 488 nm.

1. In this case we select both lasers from the Optical Path Settings Menu and we choose a dichroic suitable for both excitation wavelengths.
2. In the **A1 Pinhole Alignment** we select **Port 2** and in the **A1 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...).

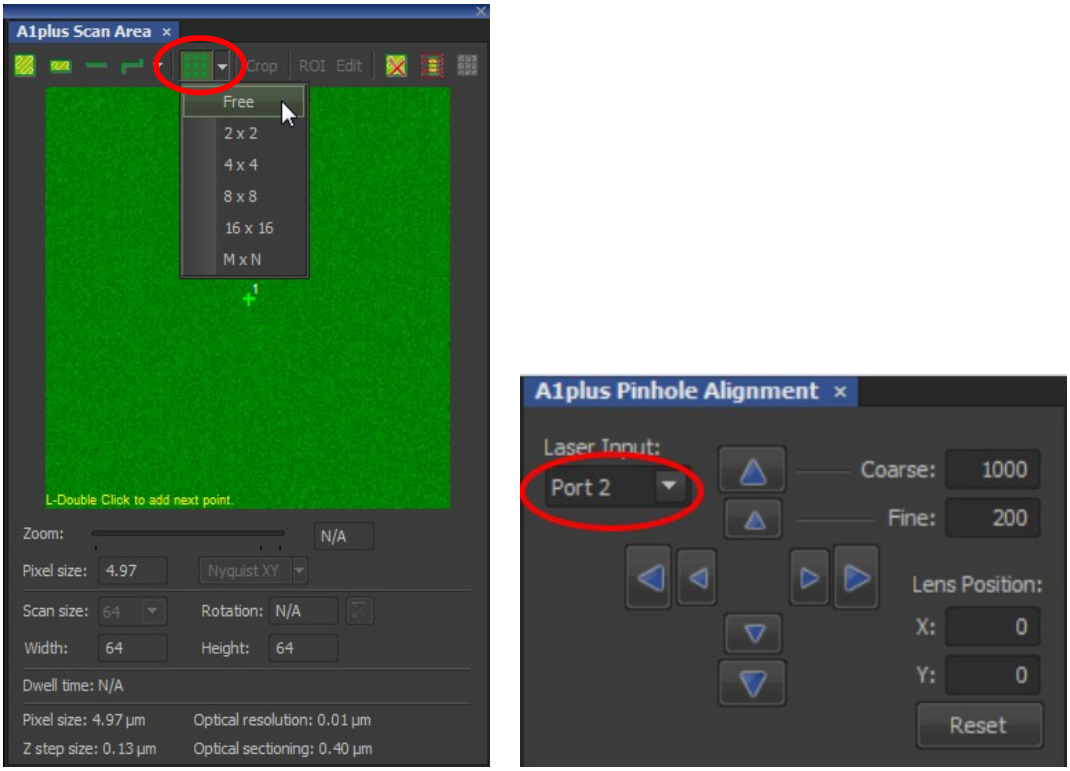


Fig. 2.40: Selection of the point location and excitation port.

3. In the **A1 Pad** select the AUX port and the pinhole size. Select the laser for which the pinhole size in Airy Units (AU) is calculated (in this case 488 nm). In the **A1 AUX Control** select FCS and to capture to a given number of seconds. Set the pinhole size to 1 AU.

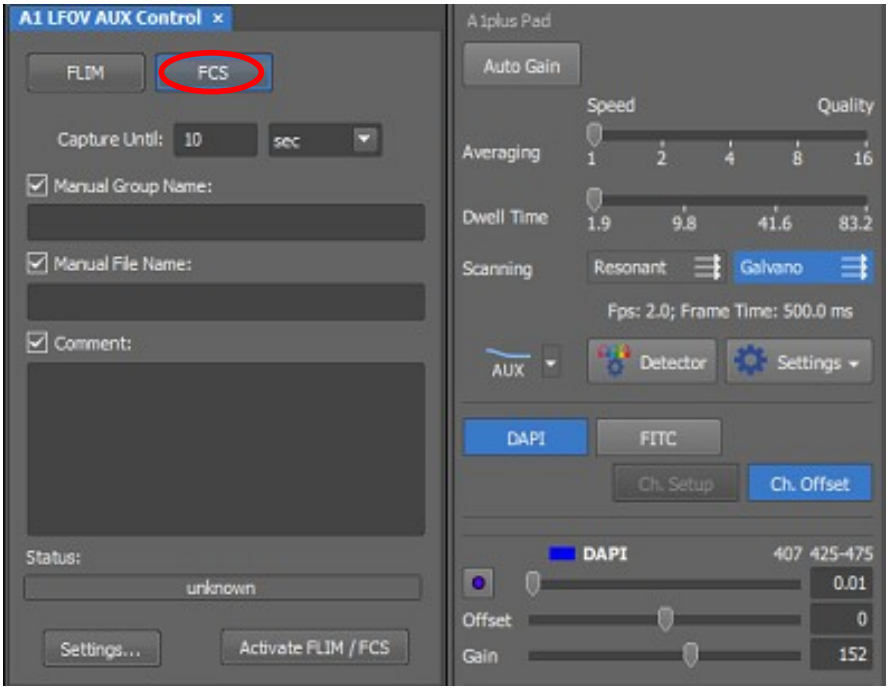


Fig. 2.41: Activation of the AUX port. Selection of FCS as well as the duration of the measurement. In addition the names for the measurements in the SPT can be set. The group name opens a folder in the SPT with the given name. Comments can be written in the section "comment". They will be displayed 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.

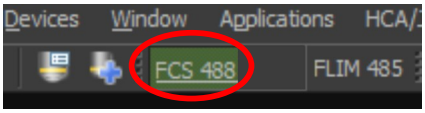


Fig. 2.42: Icons for the two newly created configurations for FLIM and FCS.

2.5.4. Requirements for FCS

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

Nikon A1	LSM Upgrade Kit	Sample
• 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.	• SPADs or PMA Hybrid PMTs. • SPT64 version with license for point measurements. • Clean optics and perfect alignment.	• Concentration in the range of 0.1 - 500 nM. • High Absorption Coefficient and Emission Quantum yield. • Photostability.

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

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 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.

2.5.5. 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 collar**. Both things can be monitored by maximization of the fluorescent signal.

1. Place a strongly fluorescent solution (>100 µM concentration) in focus. The color of such a dye solution is clearly visible. The solution has to have similar spectral properties to the target molecule to be investigated with FCS (for example, if you want to measure FCS with GFP, you can use ATTO488 or Fluorescein to check the optics). Likewise use a cover slip or microscope dish analogue to that in which the FCS sample will be measured. Important is an equal thickness of the glass between objective and sample.
2. **NIS software:** Select the optical configuration for FCS.
3. Introduce in the PicoQuant detection unit a filter adequate for the sample.
4. **NIS software:** Focus into the volume, being at least 20 µm away from the cover slide.
5. **NIS software:** In the **A1 scan Area** select a point in the center 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 **A1 pinhole alignment** window and note the XY position of the lens.

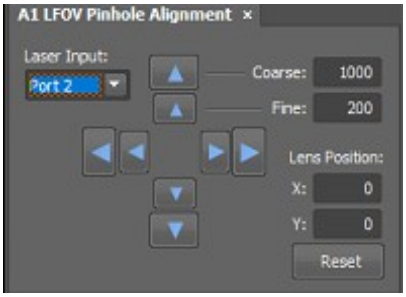


Fig. 2.43: Selection of Port 2 for pulsed excitation with PicoQuant lasers.

9. **NIS software:** Move the lens position with the arrows and observe the variation of the time trace signal. Move XY in all directions and note the XY 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. The system is now ready for a the FCS calibration measurement.

2.5.6. Perform a calibration measurement to determine the confocal Volume

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

1. There are several dyes that can be used as standard for calibration. Choose one which matches the spectral properties of your probe[1]. For example, for GFP, ATTO488 can be used as standard.
2. **NIS software:** Select the same FCS configuration used during the Optics Optimization in the previous section and make sure the XY 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 µm away from the coverslide.
4. **NIS software:** In the **A1 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/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 maximum[2].
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.

2.5.7. 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 **A1 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 focused into the solution. If you are measuring in live cells, take into account that FCS works best with very dim cells. Therefore cells appearing relatively dark in the manual epi-fluorescence view should be selected.

1 See application Note “Absolute Diffusion Coefficients: Compilation of Reference Data for FCS Calibration” on PicoQuant’s Website.

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

2. **NIS software:** Start a test measurement by clicking 

.

3. **NIS and SPT64 software/PicoQuant laser combiner:** especiallyfor 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 while not leading to a reduction in the diffusion time.

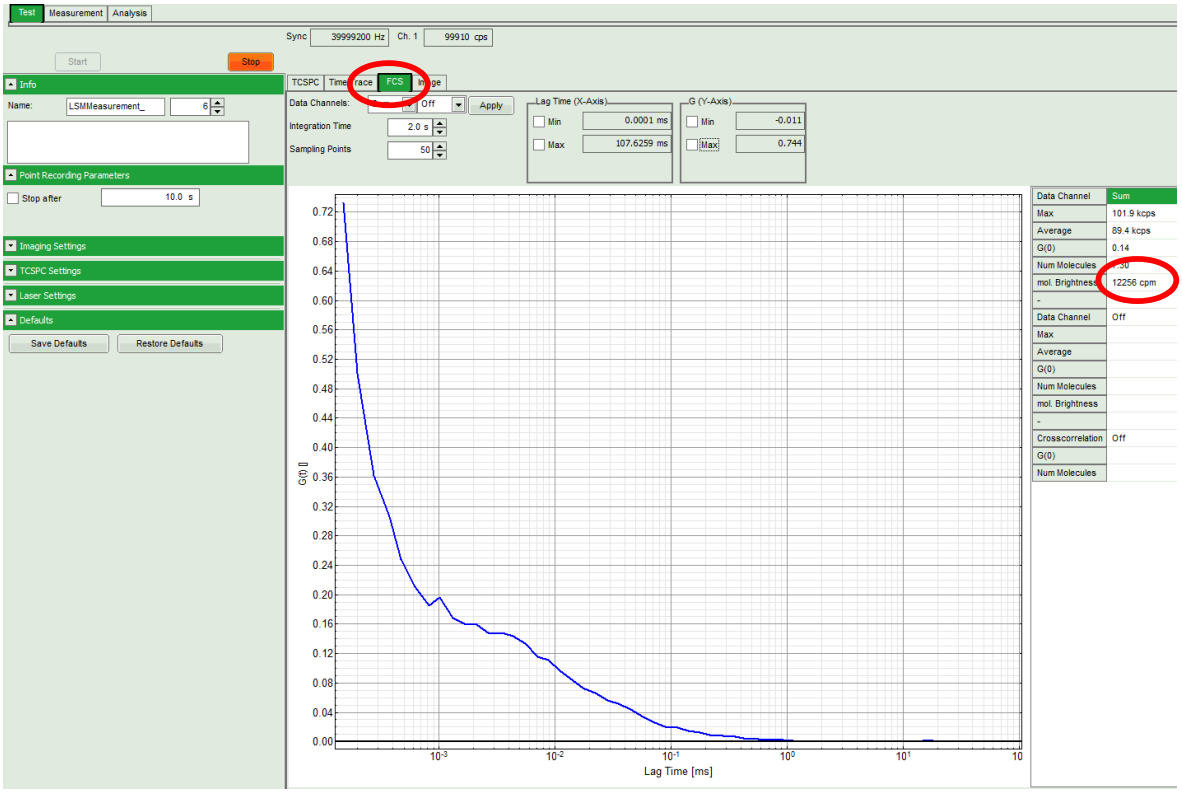


Fig. 2.44: Online monitoring of the of the FCS trace and photon count rate in the SymPhoTime preview window. On the right, the average count rate and the molecular brightness are displayed.

4. **NIS software:** In the **A1puls AUX control** select the desired acquisition time and start the measurement by clicking 

.

5. **SPT-64 software:** The FCS-curve is calculated during the measurement and displayed in the FCS tab (Fig. 2.44). 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.

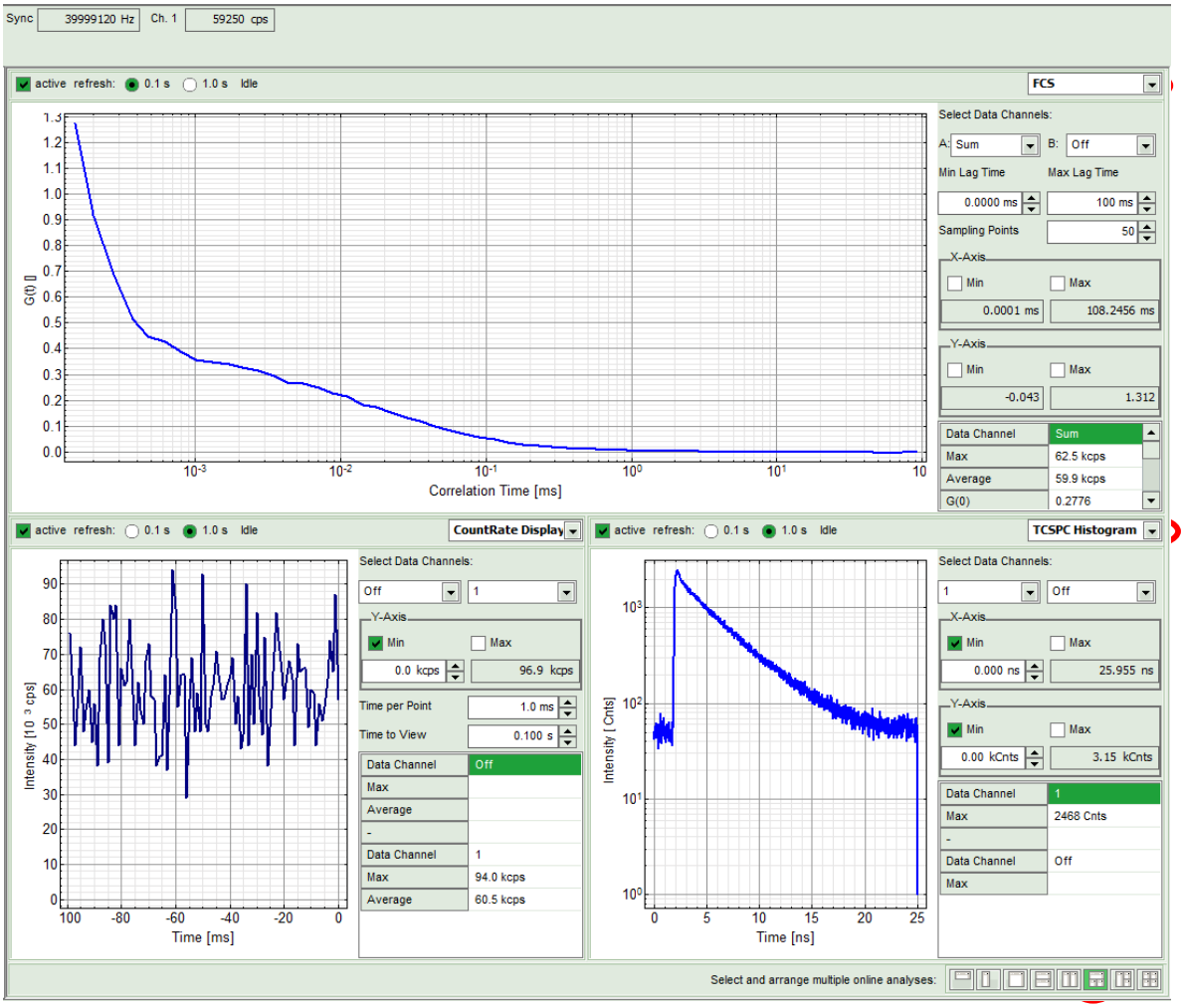


Fig. 2.45: Online Preview of the FCS measurement. In this configuration, the FCS curve is displayed in the upper panel, while the Time Trace is displayed simultaneously on the lower left and the TCSPC histogram on the lower right.

2.5.8. FCS Analysis

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

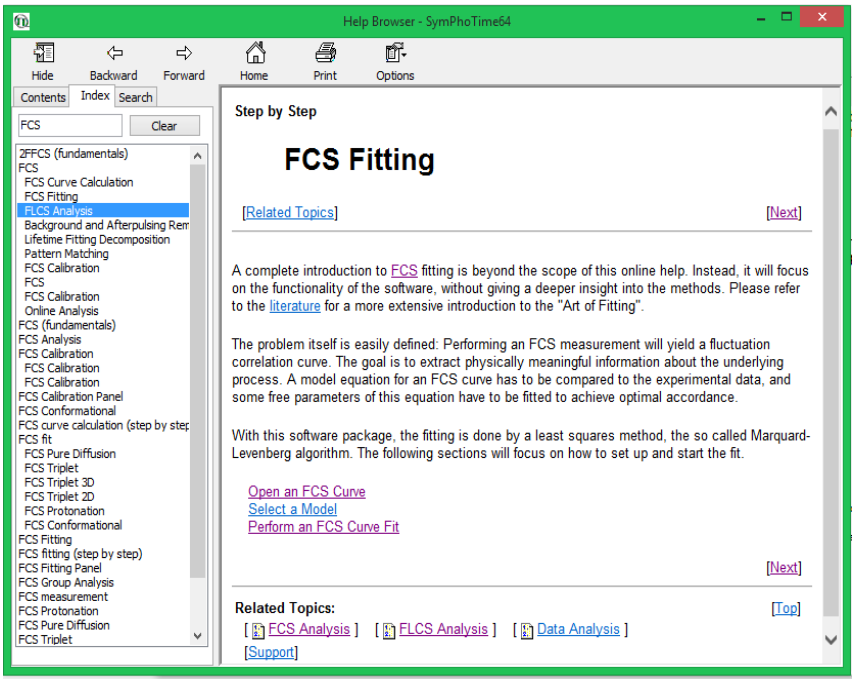


Fig. 2.46: Online Help with theory and technical information regarding analysis routines.

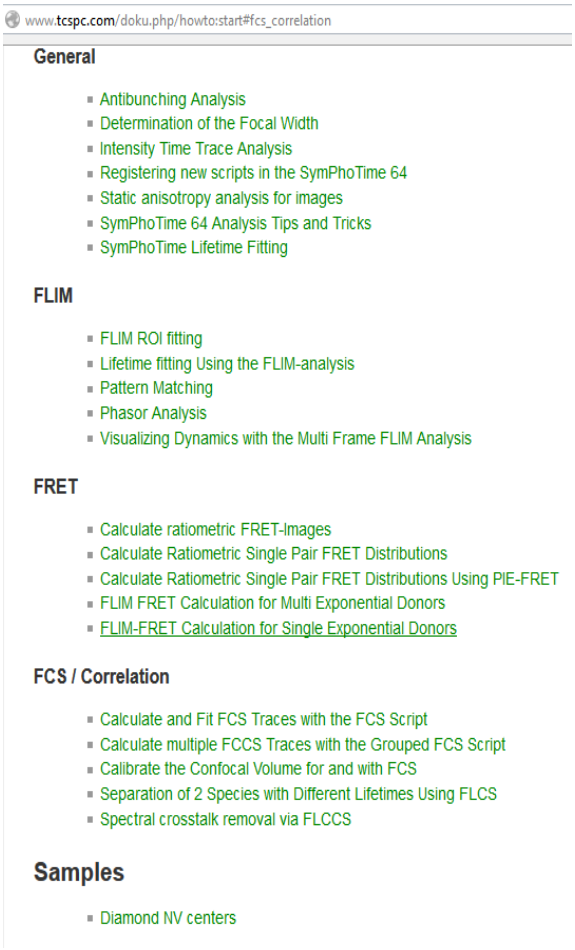


Fig. 2.47: “How to” section from the TCSPC wiki https//:TCSPC.com.

2.5.9. Remarks

- 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 PMT** 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 since the afterpulsing is very low for these detector types.
- For a solid FCS analysis, the **count rate** has to be **stable**. Signal decrease due to photobleaching may lead to an increasing FCS correlation amplitude at long lag times.
- The **calculation time** of an FCS curve 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 Cannot be Started From the NIS Software

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”.

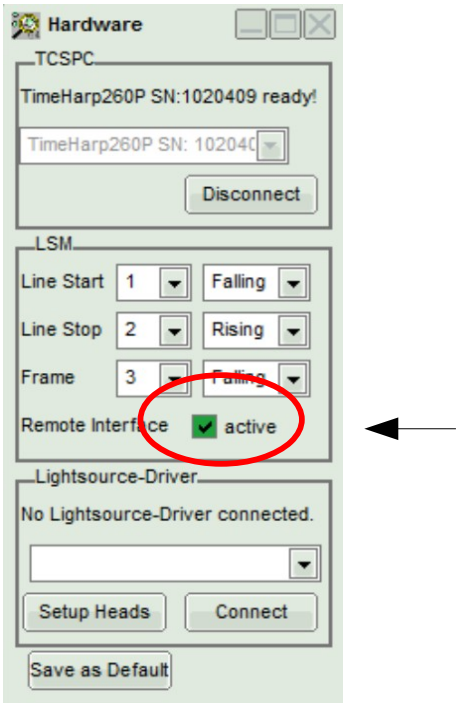


Fig. 3.1: Activation of the remote interface to allow the FCS measurements to be initiated from the NIS software.

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.**

- Settings of the NIS software are stored in the directory C:\ProgramData\Laboratory Imaging\Platform.
- SymPhoTime factory configuration is stored within the “Settings.PFS” file, which is located in the folder: C:\Program Files\PicoQuant\SymPhoTime 64.

- SymPhoTime: In order to restore the defaults saved during the installation of the system, 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.

Settings in NIS software are accessible after clicking on “FLIM” in the “Pad” via the button “Configuration” and then “Hardware Configuration”. A password is required to access these settings. Please ask PicoQuant or Nikon for advice if needed.

3.3. Instrument is Loosing Sensitivity

Note:

- It is recommended to purchase a **laser power meter** in the lab in order to measure the laser intensities for selected wavelengths. This is especially important for FCS measurements since the laser power is a crucial parameter for FCS.
- If you have purchased a LSM FLIM / FCS Upgrade with 1 or 2 SPAD or Hybrid-PMT detectors attached, a good way to check the performance of the instrument is the acquisition of **FCS-traces** of a **dye solution** with a dye that can be effectively excited. At a given laser intensity, filters and objective, and distance to the cover slide-surface, the **molecular brightness** 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 488 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.
- If a performance drop is noticed, first the origin of the performance drop needs to be found. Please first make sure that the objective lens is clean. A drop in system performance may be further caused by:

1) Misaligned Pinhole

- Optimize the Pinhole Settings as described above. Use a high concentrated dye sample (approx. 100 µM) and optimize the detected fluorescence count rate.
- Set the laser intensity to observe approx. 2 Mcps. This will result in a smooth time trace curve and even a small misalignment can be corrected. Please note the setting that you can refer to it for later measurements.

- Note down the pinhole settings in X and Y.
- Optimize the count rate by changing the pinhole settings.
  - In the **A1 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 and FCS measurements select **Port 2**.

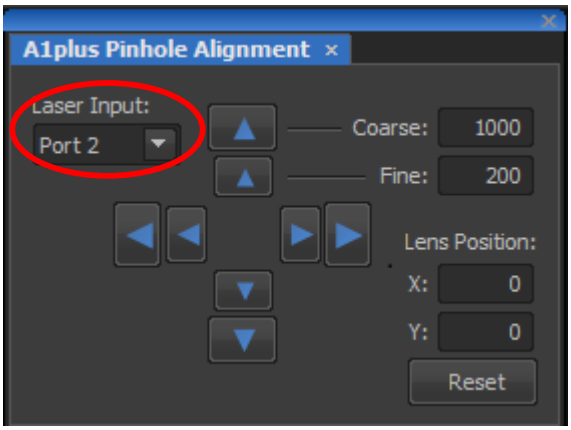


Fig. 3.2: Selection of Port 2 for pulsed excitation with PicoQuant lasers and pinhole alignment.

2) Correction Collar Setting

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

3) Decreasing Laser Performance

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

4) Decreasing Detection Sensitivity

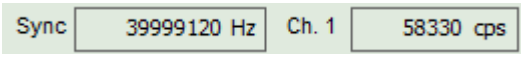
- Misalignment of the LSM itself: The first important step is to align the pinhole (see above). If this procedure does not help, contact your Nikon service representative.
- Misalignment of the detector(s): Refer to your detector manual.

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. 2.30). 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 and the laser is switched on.

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. In case of SPAD detectors, 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 anew. If no marker signals are displayed in spite of a present connection, contact PicoQuant. If you don't have the Point Measurement Mode included in your license, check cable connections and send the recorded FLIM file to PicoQuant.

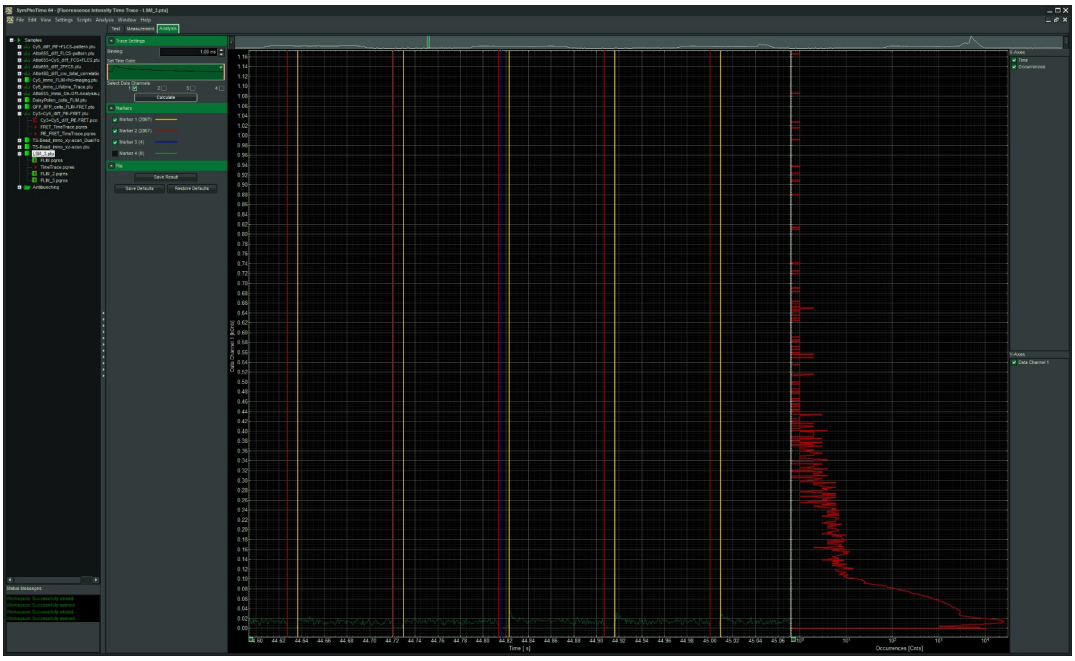


Fig. 3.3: The Time Trace analysis, where the line and frame markers can be displayed in yellow and red.

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

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.

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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