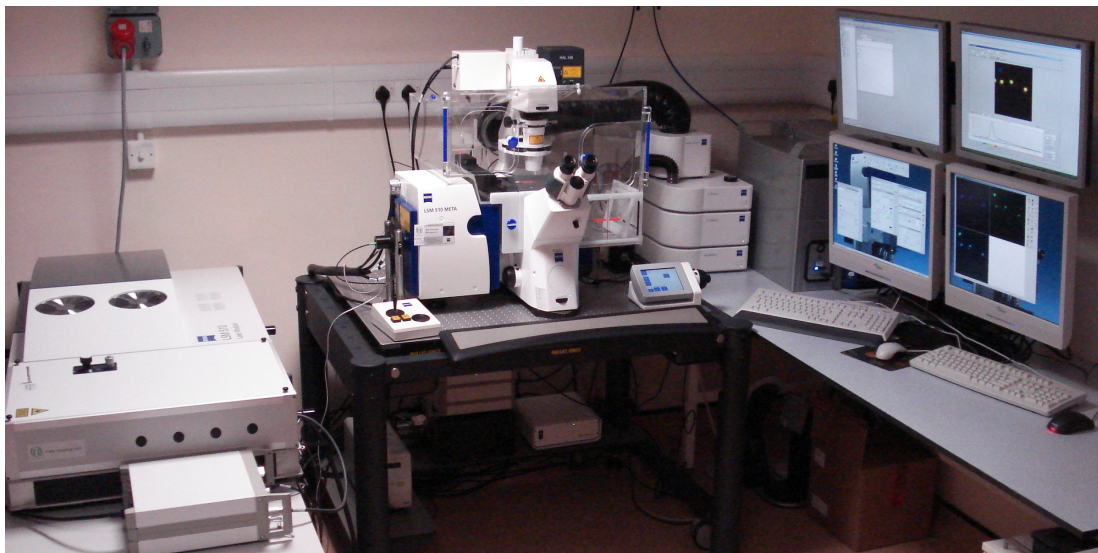


LSM Upgrade Kit

Compact Lifetime and FCS
Upgrade Kit for
Zeiss LSM510
Laser Scanning Microscopes



PICOQUANT GmbH
Unternehmen für optoelektronische
Forschung und Entwicklung



User's Hardware Manual and Technical Data

Version 1.2

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1. Introduction

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

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.

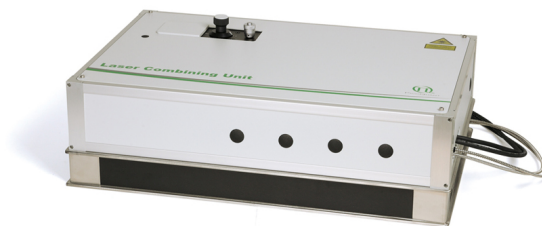


Fig. 1.1: Laser Combining Unit (LCU)

- **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 Upgrade Kits from left to right: Single Channel PMT 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.

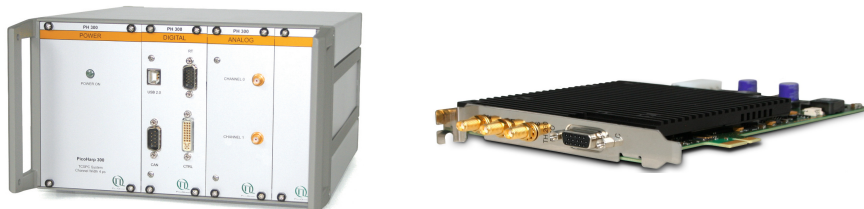


Fig. 1.3: PicoHarp 300 and TimeHarp260

- **Light Sources:** In the green folder you'll also find detailed information about the properties of your pulsed diodes lasers.

- **Router (PHR800):** The router is needed in systems with 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-B (right)

- **Software Manual (SymPhoTime - Manual):** Here you find all information about the software including data recording and analysis. The SymPhoTime-software also contains a detailed 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 Upgrade Kit, including a description of the included parts, filter handling and a cable plan, which helps restoring the configuration after disassembly.

2. Operation Using Classical LSM510 software

2.1. Safety Instructions



Laser Warning

The LSM upgrade kit is fitted 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.

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

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 101, the SPAD detector(s) must be switched off using the DSN 101 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 LSM510 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 can be started completely separate from the LSM510 itself.

The LSM upgrade kit 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", start the specific Sepia software and set the laser settings there.
2. Start the external **PicoHarp 300** TCSPC device, if applicable also the **PHR 800** router and the **DSN 101** SPAD power supply
3. Start the **PC**.
4. Start the **SymPhoTime 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. 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 plug in the compact power supply or if using a DSN 101 power supply start the respective detector by pressing the corresponding channel button on the DSN 101.

The Upgrade Kit is now ready to use.

The communication between the LSM itself and the LSM upgrade kit electronics starts just with the beginning TCSPC data acquisition.

2.3. FLIM Data Acquisition

Scope:

Acquire an FLIM image using a PicoQuant pulsed laser, the Zeiss LSM510 scanhead with appropriate scanning and dichroic settings and external PicoQuant TCSPC detectors.

2.3.1. Starting Point

1. **LSM510:** Choose and pre-scan with the LSM510 (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, a corresponding excitation dichroic mirror, a useful microscope objective and suitable detection filtering for the internal LSM510 detectors. Finally stop the scanning process.
2. All components (laser, detectors, software) of the PicoQuant upgrade kit are already running. For details about powering up see chapter 2.2

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). For details see chapter 2.3.6.
2. **PQ Pulsed Lasers:** If cw and pulsed excitation share the same fiber port at the LSM510 scan head **switch to pulsed excitation** by lifting the slider at the **LCU** (optional), as shown in Fig. 2.1. This allows the pulsed laser light of the laser heads inside the LCU to be guided into the single mode fiber which is attached to the LSM scanhead.



Fig. 2.1: LCU settings for standard confocal operation using the cw lasers of the LSM (left) and pulsed excitation for FLIM (right).

3. **LSM510 - software:** Choose a **suitable excitation dichroic mirror** (see Fig. 2.2) for the pulsed excitation wavelength. Recommended dichroics for PicoQuant pulsed diode laser wavelength can be found in table Tab. 2.1:

PQ Diode Laser	LDH 375	LDH 405	LDH 470	LDH 635
Recommended LSM510 excitation dichroic mirror	e.g. HFT 458, HFT 405/515/594, HFT 405/488/543/633	all major dichroics suited for the Zeiss 405 nm diode, e.g. HFT 405/488	HFT 470/635 (not standard)	all major dichroics suited for the Zeiss 633 nm HeNe-laser, e.g. HFT 488/543/633, HFT 488/633

2.3.3. Changing from internal LSM510 PMTs to external TCSPC detectors

1. **LSM510 - software:** Configure the detection beam path inside the LSM scan head. Guide the beam to the fiber exit port. This enables the fluorescence to reach the external TCSPC detectors via the attached Multi-mode fiber (Fig. 2.2). If you have the PicoQuant Upgrade using ConfoCor 3 detectors, configure the beam path as needed in the ConfoCor 3. If you are working with two-photon excitation and are using a PMT from PicoQuant connected to the NDD port, guide the light to the NDD detector position.

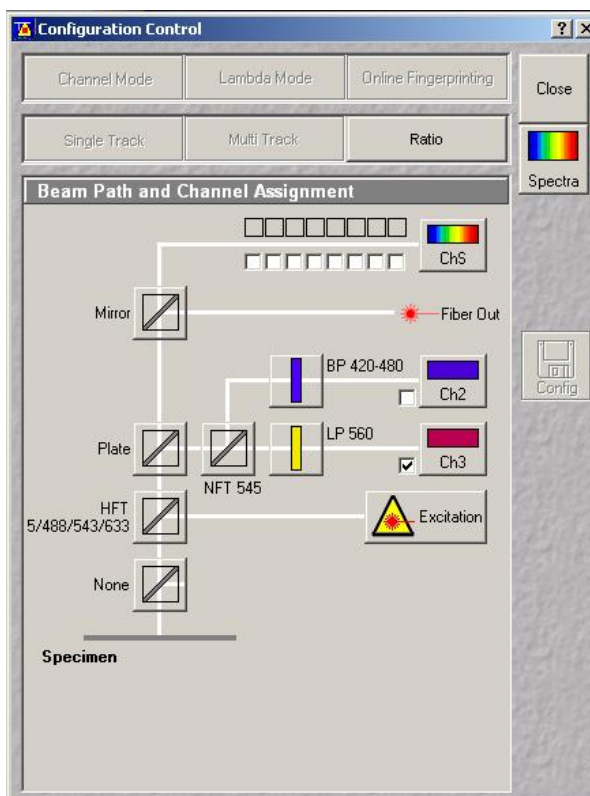


Fig. 2.2: Beam Path and Channel Control window of the LSM510 software: For FLIM data acquisition take care of the correct excitation HFT (major dichroic) and guide the light to the fiber out.

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

2.3.4. Set suitable scan parameters

1. In the **LSM510 - software**, set the correct scan pattern:
 - Use **max. 512x512 pixel** and **monodirectional scan** as indicated in Fig. 2.3. Using less pixel (e.g. 256x256) pixel is recommended, because in this way, more photons can be collected in each pixel. This results in better fits of the lifetime data.
 - **Averaging** can only be used in conjunction with the „Frame“ option. The „Line“ option will scan every line several times which cannot be correctly interpreted by the SymPhoTime software.

- Use a moderate scan speed since the calculation time for all FLIM analysis steps increases with an increasing number of acquired frames.
- **Switch on the 477 nm** line of the Argon ion-laser and set the intensity to the minimum value. This setting does not correspond to the respective laser line and the line is not switched on. The setting ensures that your LSM provides the correct line- and frame trigger signals (See Fig. 2.3). Whenever you acquire a FLIM – image, this laser line must be activated in the software.

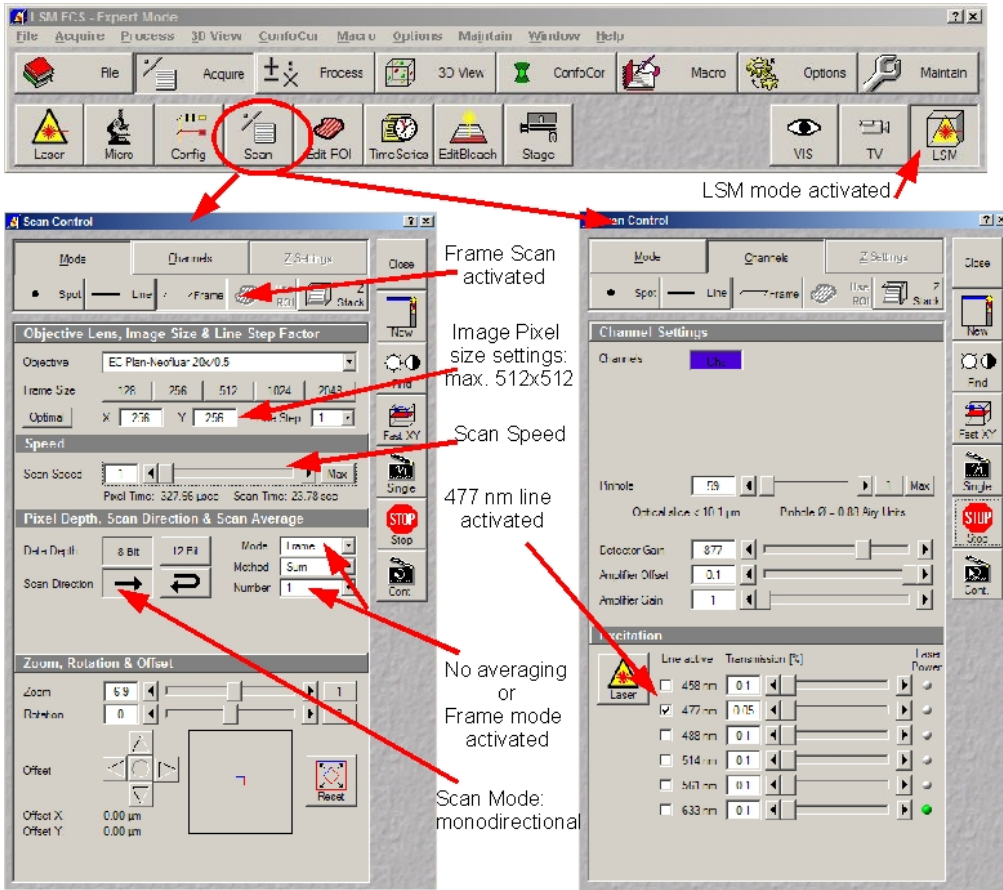


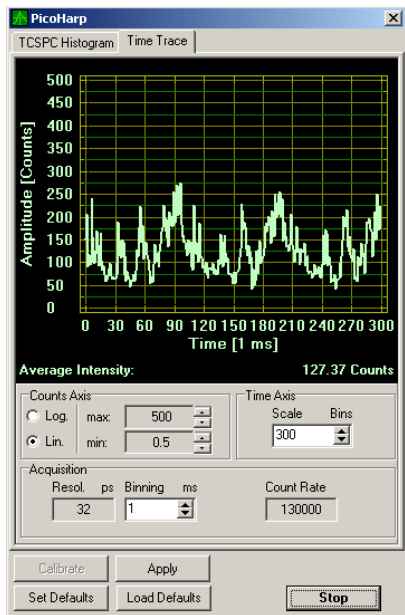


Fig. 2.3: LSM scanning settings in the LSM510 software

2.3.5. Optimise the detected photon count rate

1. **LSM510 software:** Start a repetitive xy – scan by pressing “Continuous” scan button ().
2. **SymPhoTime software:**
 - Open the Oscilloscope window (select ) and select the Time Trace Histogram (see Fig. 2.4). Make sure (acquisition time) binning is set to 1 ms and press “Apply”. Then press start in the oscilloscope window and **watch the count rate**. Change the counts axis display from “log” to “lin”.



PDL setting (Laser repetition rate)	Upper Count rate limit for different detectors, in [cts / ms]	
	PMA PMT or PDM SPAD	PE AQR SPAD
1 (40 MHz)	1000	700
2 (20 MHz)	1000	700
4 (10 MHz)	500	500
8 (5,0 MHz)	250	250
16 (2,5 MHz)	125	125

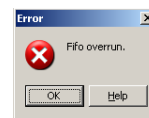
Fig. 2.4: Online monitoring of the of the photon count rate in the SymPhoTime oscilloscope window: The brightest image regions (peaks of the data curve) should not exceed the photon countrate given in the table (see y-axis: Amplitude [counts]). As scanning is fast, the binning time should be set to 1 ms.

- **Adjust the photon count rate** by changing the intensity of the pulsed laser at the laser combining unit LCU (Fig. 2.18) or – if you don't have a LCU, but a directly fiber coupled laser – in an analogous way at the lasers scaffold with the intensity regulation knob. For attenuation via changing the laser current see the laser driver manual. To read out the count rate use the y-axis (Amplitude [counts]). Do not use neither the count rate box display nor the average intensity values because both display not the actual count rate with the necessary 1 ms time resolution. For the brightest image regions the **photon count rate should not exceed the limit** given in the table (Fig. 2.4). Regions of the image recorded with photon count rates above the limit can display an inaccurate fluorescence lifetime.
- To ensure a shortest possible FLIM data acquisition time we recommend to **increase the photon count rate** if possible up to the TCSPC limit given in Fig. 2.4. Of course in case of weak fluorescing samples this upper limit cannot be reached even when using maximal laser power.

Supporting information:

Unnecessary small photon count rates extend the data acquisition, because one need a certain amount of photons for the FLIM analysis. On the other hand, too high photon count rates can lead to artefacts and falsify the FLIM analysis. The upper limit for the count rate is given by:

- TCSPC principle: To avoid **pile-up** artefacts (overestimation of early photons) the detected photon count rate should be in general less than maximum 5 % of the laser repetition rate. When using a laser repetition rate of 20 MHz this results in a photon count rate of 1 MHz (1.000 cts / ms).
- TCSPC detector: Some detectors introduce **artefacts** when being operated at **high count rates**. The AQR type SPAD from Perkin Elmer shows above 1 MHz photon count rate significant IRF broadening and a count rate dependent IRF shift.
- TCSPC electronics: the recorded photon data is permanently transferred via an FiFo-buffer from the TCSPC unit (TimeHarp or PicoHarp) to the PC. If photon **count rates above 5 MHz** are reached which cannot any more be handled by the buffer, the **data acquisition stops** and the error message displayed on the right occurs.




2.3.6. Choose the correct laser repetition rate

For PicoQuant pulsed diode lasers the **laser repetition rate** can be set at the PDL laser driver. In general the repetition rate should be as high as possible to achieve the highest possible photon count rate. However, the time window after a laser pulse should be still large enough to allow the population of excited dye molecules to deplete completely before the next laser pulse.

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.

This is illustrated in Fig. 2.5. At 80 MHz laser repetition rate, the fluorescence does not decay completely before the end of the time window is reached. Due to a “wrap around” the decay tail is observed even before the actual pulse (A). At 40 MHz the **time window is optimally adapted** (B), while at 20 MHz the fluorescence has already decayed at less than half of the detection window (C). Therefore 40 MHz would be the optimal repetition rate in this case.

You can check the decay behavior of your sample using the oscilloscope window of the SymPhoTime software (select: ). Activate the TCSPC Histogram-tab and monitor the fluorescence decay as shown in Fig. 2.5.

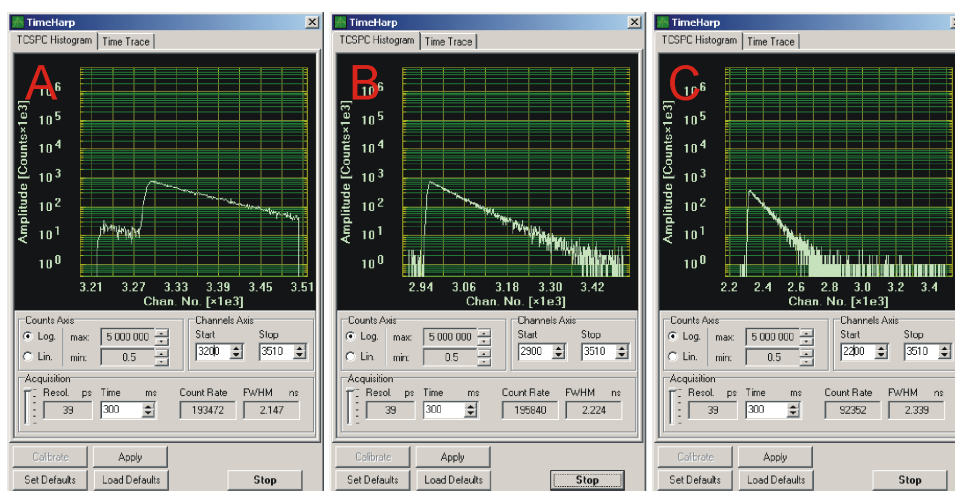



Fig. 2.5: Fluorescence decay at too high (A, 80 MHz), optimal (B, 40 MHz) and unnecessary low (C, 20 MHz) laser repetition rate.

2.3.7. Start FLIM data acquisition

1. **SymPhoTime software:** Start the LSM acquisition by pressing . When recording data, the SymPhoTime software should always be started before activating the scanning process in the LSM510 software, otherwise image information in the first frames can be lost.

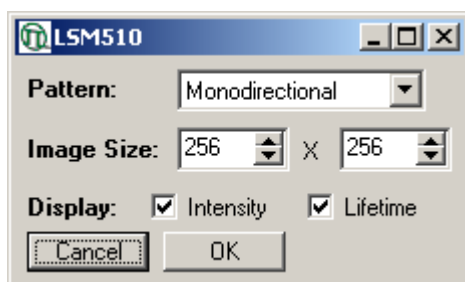



Fig. 2.6: SymPhoTime Pop-up window for LSM data acquisition.

2. **SymPhoTime software:** In the appearing pop-up window (see Fig. 2.6) you **set the scan pattern and the image size** in pixels analogue to the settings applied in the LSM510 software. Please note that the maximum image size is limited to 512 x 512 pixels. The “Display” settings let you choose which information should be displayed with the online image preview during data acquisition: Intensity information is displayed as brightness and lifetime as a special color, pixel per pixel. Finally press OK to start the LSM data acquisition.
3. **SymPhoTime software: During LSM data recording** a window pops up (Fig. 2.7) which gives you access to the preview FLIM image. To see the image during acquisition, press the button . When scanning several frames all the **information** is summed up pixel per pixel and displayed. Please note that the information is just summed up for the display, the stored raw data still allows you to treat the data separately frame by frame.

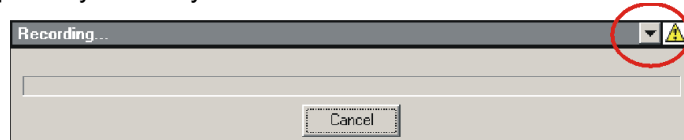



Fig. 2.7: SymPhoTime Pop-up-window being displayed during LSM data recording. To see the image during acquisition, press the button .

4. **LSM510 software:** After the LSM data acquisition, also start the scanning process in the LSM510 software (either in the “continuous” mode or via averaging a determined number of frames, see Fig. 2.8).

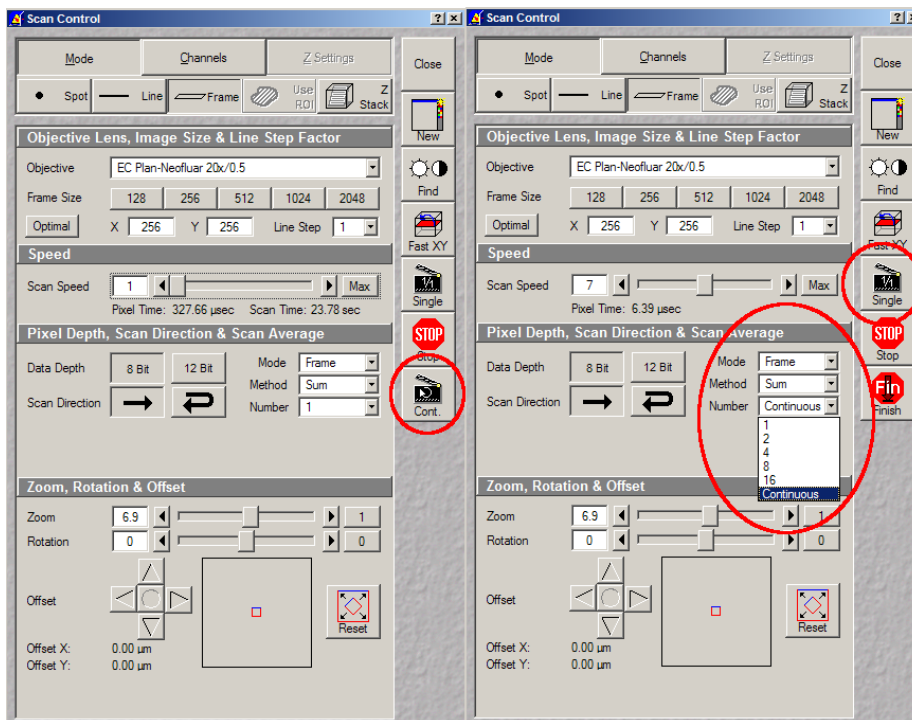



Fig. 2.8: Starting a Scan in the LSM510 software: **Left:** Using the “Continuous” mode for scanning. To start the Scan, click on the “Cont”-button indicated. Note that the Averaging mode should be set to “Frame” and the number of scans to average (“Number”) is set to 1 as shown. **Right:** For averaging a determined number of frames, select averaging a specific Number of frames using the “Number” pull-down menu. Clicking the “Single” button starts the measurement.

5. The **LSM scanning process stops** either after having scanned the pre - set number of frames, if the system is set for averaging a specific number of frames, or by just manually interrupting the continuous scan (“Cont”) by clicking the “Stop” - button. Of course you can always chose in the further data analysis with the SymPhoTime software which of the recorded frames you want to display. You can display also a sub ensemble of frames.

6. **SymPhoTime – software:** Press “cancel” in the LSM data recording window (see Fig. 2.7) in order to **stop data acquisition**. The TCSPC data acquisition works completely independent and can be stopped at any time without losing already recorded data.
7. The recorded FLIM image will be displayed in the SymPhoTime software.

2.3.8. Resulting raw data file and documentation

1. **SymPhoTime software:** The measurement result is a **FLIM image** together with a **T3R/PT3 raw data** file (dependent on the type of TCSPC device) containing for every detected photon the maximum available information (temporal, spatial, detection channel).
 - Every raw data file is stored together with an **annotation file** (indicated with a red dot icon  in the workspace overview, see Fig. 2.9) which contains all actual measurement parameters known by the SymPhoTime software, the possibility to type in manually individual information (measurement type, sample, image size, scanning velocity, etc) together with a preview image (as bitmap, to give you a quick impression about the scanned image).
 - Fig. 2.9 shows a typical **SymPhoTime workspace** overview on the left side. Derived from the raw data file “LSM_1.pt3” are the annotation file “LSM_1_cmt.bdf” which is displayed in detail on the right and the FLIM image “LSM_1_IMG.bdf” (not displayed).

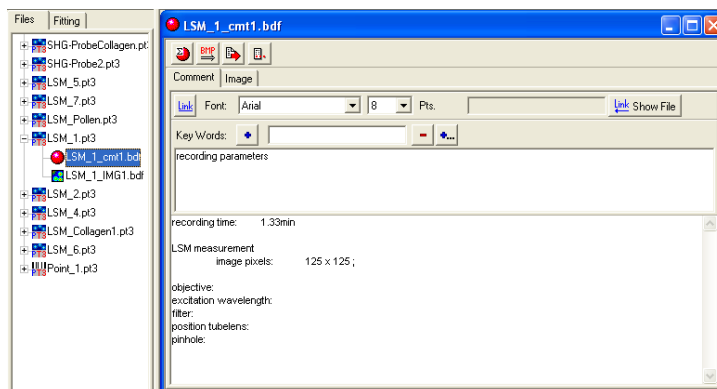


Fig. 2.9: SymPhoTime workspace showing on the left hand side the file history and on the right an annotation file with already known settings (recording time, image pixels) as well as space to enter additional information (objective, filter, pinhole,...).

2. **LSM510 software:** If you want to change in the SymPhoTime software the x,y-axis from pixel number to dimensions in microns you need in addition to the already known number of pixels per axis also the **pixel size (in microns)**. The latter is available if you click at the info-button of your scanned image in the LSM510 software (Fig. 2.10).

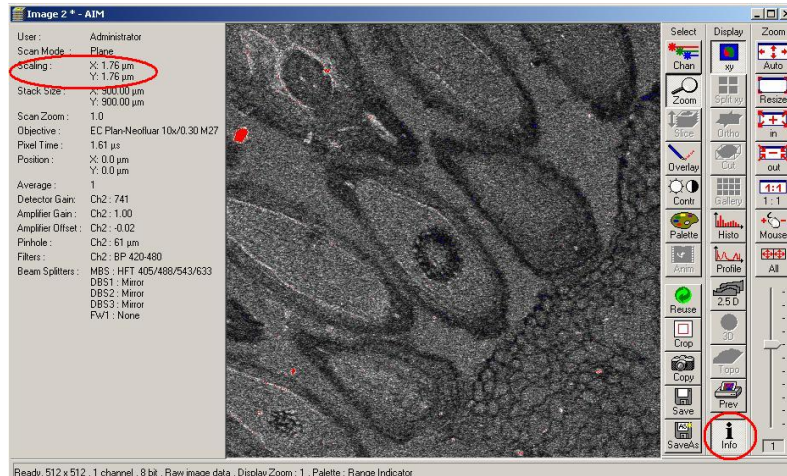



Fig. 2.10: The Image size information can be viewed after clicking the info button of the acquired image (bottom right). The scaling is then shown in the left panel, as marked in red.

3. **SymPhoTime software:** The **image dimensions** in the image can be changed using the **Stack Control** button (). A window with image specifications pops up, where the dimensions of the image can be entered. This affects all size measurements of the image.

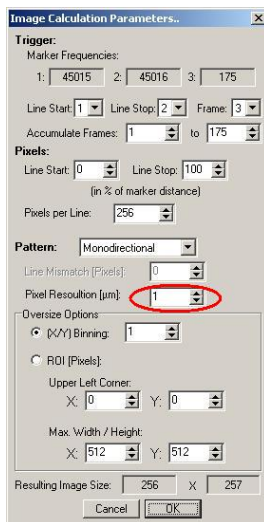



Fig. 2.11: Changing Image the image size in the SymPhoTime Software. Here the real Pixel resolution can be entered.

In this window also the frames can be selected (“Accumulate Frames”) which will be displayed in the FLIM image.

2.3.9. Measure an instrument response function (IRF)

For accurate measurements of lifetimes near to the timing resolution, the instrument response function (IRF) should be taken into account. The width of the IRF displays the timing resolution of the instrument. An IRF can be obtained following these steps:

1. Place a **scattering sample** (e.g. the surface of a cover slide or better a mirror) in the laser focus on the microscope stage. Reduce the laser power in order not to oversaturate the detectors. It is important that the laser power output is not changed at the laser driver itself, but only at the Laser Combining Unit (LCU) or attenuator at the laser head. Changing the laser driver settings typically changes the shape and temporal position of the laser pulse!
2. Place an **OD3 attenuating filter** in the filter holder in front of the detector. Look up your detector manual for locating the filter holder position.
3. Lift the **lever** at the **LCU** to direct pulsed laser light to the LSM (optional, Fig. 2.1).
4. Set the **LSM510** settings in the same way as for a **FLIM measurement**. This way, the back reflection of the cover slide can be monitored by the PicoQuant detectors.
5. Activate the **oscilloscope window** in the SymPhoTime software () and select the **Time Trace - tab**.

Important is the setting of a **linear Count Axis**. Start the oscilloscope measurement by clicking the Start - icon. The amplitude axis can be adapted by scrolling with the mouse wheel button in case the cursor is placed on the oscilloscope window. For IRF - measurements the collected countrate should be very small (in the range of a few kilocounts/second).

6. Select in the oscilloscope window the **TCSPC Histogram - tab** as shown in Fig. 2.12. Start the oscilloscope measurement by clicking the Start - icon. The amplitude axis can be adapted by scrolling with the mouse wheel button in case the cursor is placed on the oscilloscope window. The shape and width of the IRF curve is very much dependent on the laser and detector configuration.

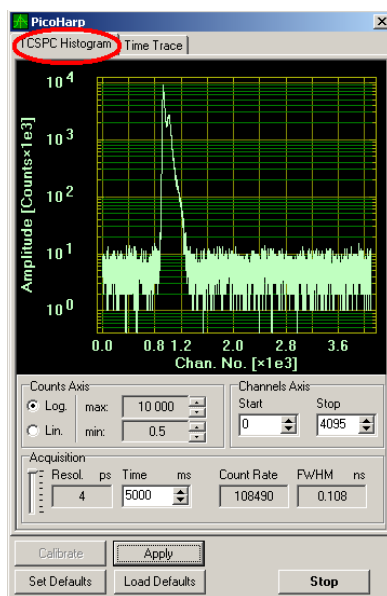


Fig. 2.12: IRF oscilloscope window, recorded with a PH300, MPD SPAD and PQ laser source.

7. When a satisfactory IRF appears on the oscilloscope window, stop the measurement in the oscilloscope window and start a **point measurement** as described in Fig. 2.17 and the corresponding chapter. Based on this IRF raw data file (in T3R or PT3 file format) an IRF TCSPC histogram can be build up (see following chapter for details).
8. **Stop the IRF point measurement**, replace the cover slide with your sample and insert the correct filters for your sample again.

2.3.10. Remarks

- For the ad hoc inspection of your samples you can also use the **pulsed lasers** even in conjunction with the **internal LSM510 PMT detectors**. Typically pulsed lasers have less power in comparison to the continuous wave (cw) lasers, therefore images of weak fluorescing samples should perhaps be recorded with slower scan speed.

- Your LSM510 allows **storing** defined **configurations**. Acquiring FLIM images is easier, when one configuration for pre-FLIM image inspection and one configuration for the FLIM measurement is stored. Storing and loading stored conformations is possible in the “Configuration Control” window of your LSM510 software. A configuration allows to pre-set the correct beam path and major dichroic as well as to switch on automatically the 477 nm line to provide the correct line trigger signals.

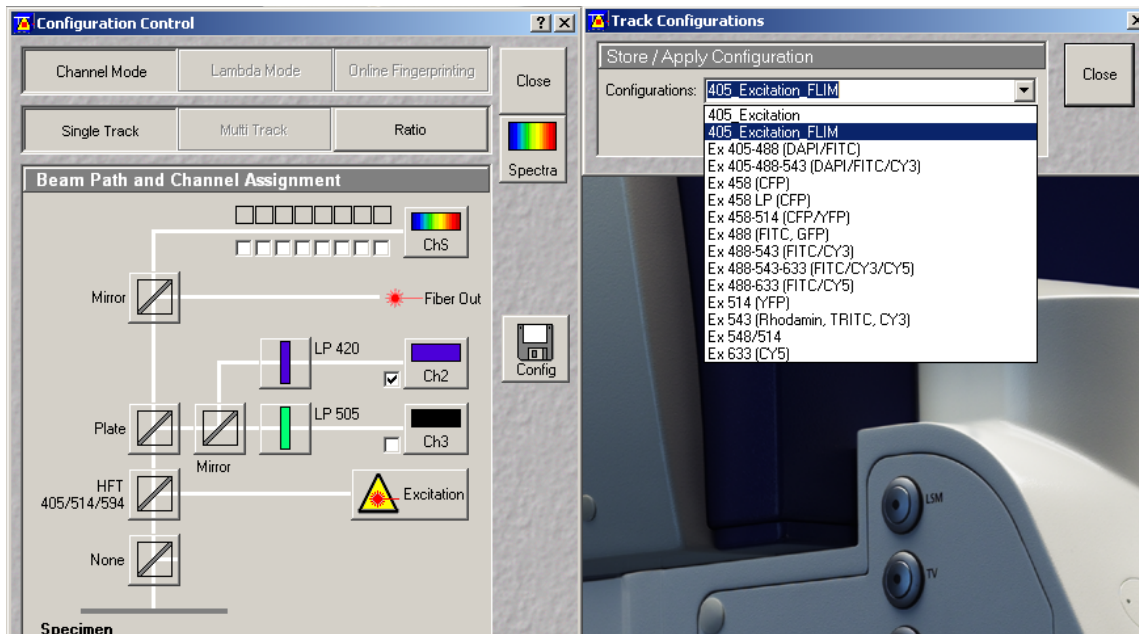


Fig. 2.13: Storing and applying stored configurations is possible via the Configuration Control. Clicking the "Config" button opens the "Track Configurations" window that allows storing the actual settings or uploading a stored configuration.

- The SymPhoTime software is **unable to compensate** for **hysteresis** effects that may be induced by bidirectional scanning, thus if such effects are noticed, it is preferable to perform monodirectional scans.
- The **laser intensity** of the diode lasers can be **adjusted** in two different ways:
On the one hand, the light intensity can be reduced at the LCU using the filter wheel and the scaffold (or only the scaffold at the direct fiber coupled laser heads), on the other hand, the intensity can be controlled at the PDL800 or the SEPIA controller. If the intensity is changed at the PDL800B or the SEPIA, this may change the pulse shape and therefore the instrument response function (IRF). Take care to record the IRF and the fluorescence decay with the same laser settings at the PDL800B or the SEPIA if you want to perform re-convolution. The time response of the instrument (width of the IRF) may be shorter at lower laser power settings at the laser driver.
- The **fluorescence detection sensitivity** of the optical system can also be increased by opening the pinhole in the LSM. On the other hand this may reduce the signal to background ratio.
- In order to obtain the **optimal lifetime information** it is in general preferable to scan FLIM images with less pixels than standard intensity "only" images, as the accuracy of the lifetime information is directly dependent on the number of photons per pixel. That may become crucial, if several lifetimes are present. Furthermore, the calculation time of a lifetime image increases linearly with the number of pixels in the image. The SymPhoTime software also allows to perform post-acquisition pixel binning for optimization of the best compromise between lifetime accuracy and resolution.

2.4. Data acquisition for point measurements as used in FCS

Scope:

Perform a Point measurement at a specific position. With sensitive detectors like single photon avalanche diodes, FCS measurements can be performed. In all point measurements, the fluorescence is recorded at a certain position in the sample and analysed later in the SymPhoTime software.

If lifetime information is not needed, also steady state lasers from the LSM might be used for the fluorescence excitation.

For performing and analysing Point measurements, your SymPhoTime license must cover these measurements.

2.4.1. Starting Point

The following description assumes that

- your **sample** is already placed onto the microscope and brought into **focus**. If you are measuring in solution, it is assumed that the laser is focussed into the solution.
- The **correct dichroic mirror** for your wavelength has been selected in the LSM510 software, and the **correct emission filter(s)** has/have been placed into your detection unit. The light is directed correctly to the fiber exit port as for a FLIM – image acquisition.

Measuring with pulsed excitation

- **Switch the laser on** and select the **correct repetition rate** (see 2.3.6).
- Set the lever at the Laser Combining Unit into the **upper position** (Fig. 2.1).

Measuring with cw excitation

- If measuring with cw lasers, still the **SYNC signal** of the driver unit must be **present**. In this case, best switch on laser driving unit (PDL800B or SEPIA) and set the laser to the standby mode by turning the key. This ensures that a trigger signal is provided, which is needed by the photon counting board. In the oscilloscope window of the SymPhoTime software, the repetition rate or a count rate different from 0 should be shown.
- Set the lever at the Laser Combining Unit into the **lower position** (optional, opposite as in Fig. 2.1).

2.4.2. Choose the location for the point measurement

1. **LSM510 software:** Enable the “Spot” measurement mode (Fig. 2.14). Clicking on “Spot Sel” results in scanning of an image where you can select a position for your point measurement. The scanner only moves to the indicated position, when the time series measurement is started and moves back to the standby position after the measurement has stopped (Fig. 2.15).

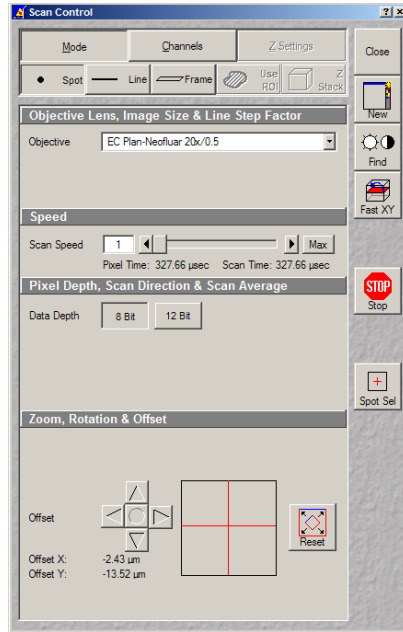


Fig. 2.14: Point Measurement Mode in the LSM510 software.

2. **LSM510 software:** Click the position for the point measurement in your already scanned image to select the position where you want to measure (Fig. 2.15).

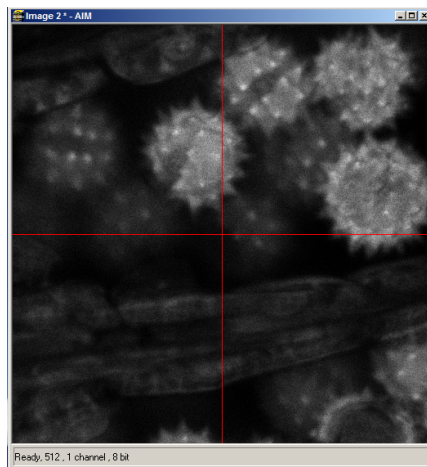


Fig. 2.15: Selecting the position of the point measurement in the Image window. Clicking at the desired position marks the area to be measured. The scanner moves to that position when the Time Series Scan is started.

2.4.3. Set the data acquisition time

3. **LSM510 software:** Open the Time Series window and set the number of iterations to maximum as shown in Fig. 2.16. The total scan time depends on the number of iterations and the scan speed (Fig. 2.14). Approximate times for different scan speeds are given in Tab. 2.1.

Scan Speed	4	5	6	7	8	9	10
approximate duration of the Point Scan [min]	50	25	12.5	6.2	5	3	2.5

Table 2.1.: Approximate Point Scanning Times as set with the Point Mode, using the maximum number of iterations as indicated in the Time Series Window (Fig. 2.16)

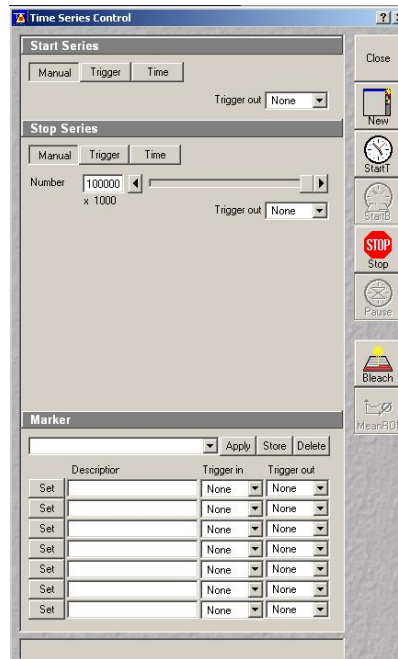


Fig. 2.16: Starting a Time Series in the LSM510 software: Set the "Stop Series" on "Manual" and to the maximum number of iterations. To start the measurement, click the "Start T" button.

4. SymPhoTime software:

- Open the **point measurement dialogue box** by clicking at the point measurement icon  indicated by the green circle **1** in Fig. 2.17.

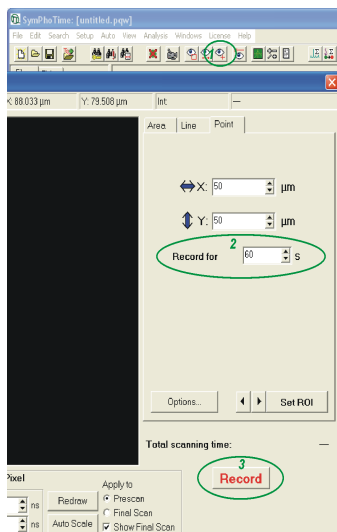


Fig. 2.17: Performing a Point Measurement with the SymPhoTime software: After clicking the icon indicated as **1**, the point measurement window pops up. Enter the recording time in the area marked as **2** and start the measurement by hitting the record button indicated as **3**. The other buttons in the menu are not used in LSM Upgrade Kits.


- **Enter** the desired **measurement time** (for example 60 s) in the area marked as **2** in Fig. 2.17. Make sure that the measurement time in the SymPhoTime software is **longer** than the measurement time entered in the LSM software and additionally accounts for the fact that the SymPhoTime software is started first.

2.4.4. Optimise the detected photon count rate

Similar guidelines as described in Chapter 2.3.5 apply. Additionally for certain analysis modes some special requirements are of interest:

- **Too high photon count rates** can lead to artefacts and falsify the fluorescence lifetime analysis.
- High count rates **increase calculation times** for FCS analysis.
- Crucial for FCS analysis is a **high countrate per molecule**. In order to achieve this, a balance between efficient excitation and photobleaching has to be found. A lower concentration with higher laser power is preferable, ideal concentrations for FCS span 0.1 - 10 nM.
- For **analysis of individual molecule transits**, the concentrations need to be sufficiently low, so that individual bursts can be recognized.

3. *SymPhoTime software:*

- **SymPhoTime – Software:** Open the Oscilloscope window (select ) and select the Time Trace Histogram (see Fig. 3.4). Make sure (acquisition time) binning is set to 1 ms. Then press start in the oscilloscope window and **watch the count rate**. If not already set change the counts axis display from “log” to “lin”.
- **LSM510 - software :** If the sample is sensitive to photobleaching, select a different area with similar brightness for optimization of the settings

Measuring with pulsed excitation

- **LSM510 software:** Set the “LSM mode” and select a suited dichroic. The laser beam can be seen at the objective (see Fig. 2.13). The count rate should increase at the detector. Make sure the sample is sufficiently shielded from room light, as this might increase background.
- **LCU: Adjust the count rate** by adapting the attenuation wheel and the scaffold at the Laser Combining Unit (see Fig. 2.18).



Fig. 2.18: Laser attenuation at the LCU: rough adjustment at the intensity can be performed with the filter wheel. the position correspond to the following attenuations: 0=no attenuation (max. power), 1 = 10x attenuation, 2 = 100x attenuation, 3 = 1000x attenuation. The upper knob controls the scaffold for intensity fine tuning. Turning the knob clockwise moves the scaffold into the laser beam and thereby attenuates the excitation intensity.

Measuring with cw excitation

- **LSM510 - software:** **Start a point measurement** at the LSM510. The count rate should increase at the detector. Make sure the sample is sufficiently shielded from room light, as this might increase background.
- **LSM510 - software: Adjust** the count rate by de- or increasing the **laser power** in the LSM510 software.

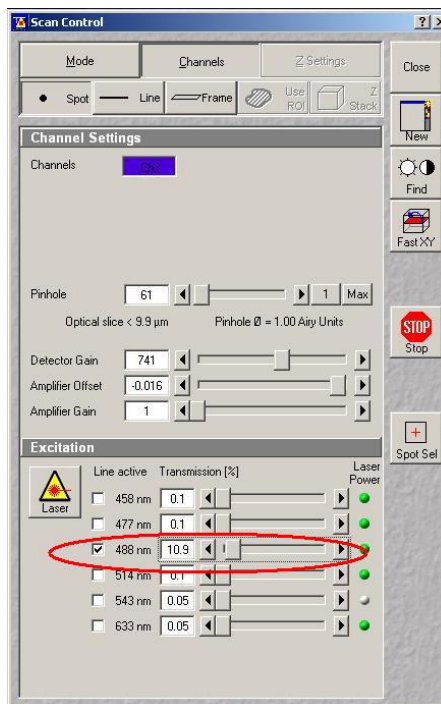


Fig. 2.19: Intensity regulation in the LSM510 software.

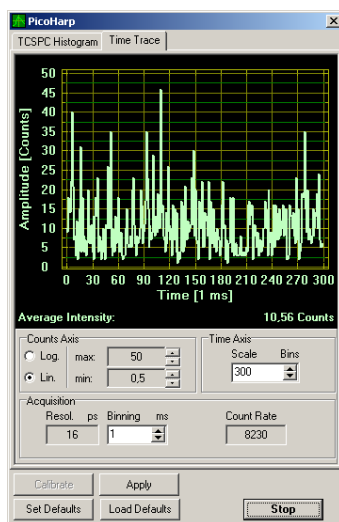


Fig. 2.20: FCS trace of diffusing dye molecules in solution. As FCS analysis spans several orders of magnitude of concentration, the count rate may vary significantly. Single molecule transits are displayed as bursts.

2.4.5. Start data acquisition

4. **SymPhoTime – Software:** Start the **point measurement** by clicking into the record box marked as **3** shown in Fig. 2.17. Take in consideration that there is a small delay between clicking the “Start” button and the actual start of the measurement.
5. **LSM510 – software:** Start the **point measurement** in the LSM software with “StartT”.

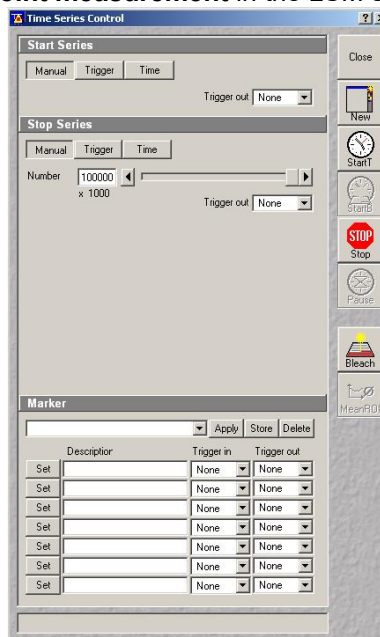


Fig. 2.21: Start of the point measurement in the LSM510 software. The measurement is started in the Time Series Control window by hitting "Start T"

6. **SymPhoTime – Software:** When **analysing** the data, take into consideration that the data stream is recorded **longer** than the time the sample has been illuminated. Restrict the data analysis of the time trace to the time the sample has been illuminated (see SymPhoTime manual).

2.4.6. Remarks

- **FCS measurements** can **only** be performed using **avalanche photodiodes** 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, FLCS can be used in order to suppress detector afterpulsing after pulsed excitation.
- For a solid FCS-analysis, the **count rate** has to be **stable**. Signal decrease due to photobleaching will result in an offset of the FCS curve.
- The **calculation time** of an FCS measurement depends on the **measurement time** and the detector **count rate**.

3. Operation Using LSM510 “Zen” software

The chapter describes the same procedures as Chapter 2, if the “Zen” version of the LSM510 software is used.

3.1. Safety Instructions



Laser Warning

The LSM upgrade kit is fitted 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.

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

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 101, the SPAD detector(s) must be switched off using the DSN 101 power supply unit BEFORE switching off the system by the main power button.

3.2. Starting Hardware and Software Equipment

To **start** the LSM itself, its detectors and cw lasers please refer to your LSM510 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 can be started completely separate from the LSM510 itself.

The LSM upgrade kit hard- and software should be started in the following order:

8. 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”, start the specific Sepia software and set the laser settings there.
9. Start the external **PicoHarp 300** TCSPC device, if applicable also the **PHR 800** router and the **DSN 101** SPAD power supply
10. Start the **PC**.
11. Start the **SymPhoTime software** .
12. **Open** an already existing **workspace** or create a new one.
13. Place correct **fluorescence filters** in the corresponding filter holder position of the external PicoQuant detector unit. 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).
14. **Make sure that no light is focussed onto the detector(s) and power** them on. Either plug in the compact power supply or if using a DSN 101 power supply start the respective detector by pressing the corresponding channel button on the DSN 101.

The Upgrade Kit is now ready to use.

The communication between the LSM itself and the LSM upgrade kit electronics starts just with the beginning TCSPC data acquisition.

3.3. FLIM Data Acquisition

Scope:

Acquire an FLIM image using a PicoQuant pulsed laser, the Zeiss LSM510 scanhead with appropriate scanning and dichroic settings and external PicoQuant TCSPC detectors.

3.3.1. Starting Point

1. **LSM510:** Choose and pre-scan with the LSM510 (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, a corresponding excitation dichroic mirror, a useful microscope objective and suitable detection filtering for the internal LSM510 detectors. Finally stop the scanning process.
2. All components (laser, detectors, software) of the PicoQuant upgrade kit are already running. For details about powering up see chapter 2.2

3.3.2. 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). For details see chapter 2.3.6.
2. **PQ Pulsed Lasers:** If cw and pulsed excitation share the same fiber port at the LSM510 scan head **switch to pulsed excitation** by lifting the slider at the **LCU** (optional), as shown in Fig. 3.1. This allows the pulsed laser light of the laser heads inside the LCU to be guided into the single mode fiber which is attached to the LSM scanhead.



Fig. 3.1: LCU settings for standard confocal operation using the cw lasers of the LSM (left) and pulsed excitation for FLIM (right).

3. **LSM510 - software:** Choose a **suitable excitation dichroic mirror** (see Fig. 3.2) for the pulsed excitation wavelength. Recommended dichroics for PicoQuant pulsed diode laser wavelength can be found in table Tab. 3.1:

PQ Diode Laser	LDH 375	LDH 405	LDH 470	LDH 635
Recommended LSM510 excitation dichroic mirror	e.g. HFT 458, HFT 405/515/594, HFT 405/488/543/633	all major dichroics suited for the Zeiss 405 nm diode, e.g. HFT 405/488	HFT 470/635 (not standard)	all major dichroics suited for the Zeiss 633 nm HeNe-laser, e.g. HFT 488/543/633, HFT 488/633

3.3.3. Changing from internal LSM510 PMTs to external TCSPC detectors

1. **LSM510 - software:** Configure the detection beam path inside the LSM scan head. Guide the beam to the fiber exit port. This enables the fluorescence to reach the external TCSPC detectors via the attached Multi-mode fiber (Fig. 3.2). If you have the PicoQuant Upgrade using ConfoCor 3 detectors, configure the beam path as needed in the ConfoCor 3. If you are working with two-photon excitation and are using a PMT from PicoQuant connected to the NDD port, guide the light to the NDD detector position.

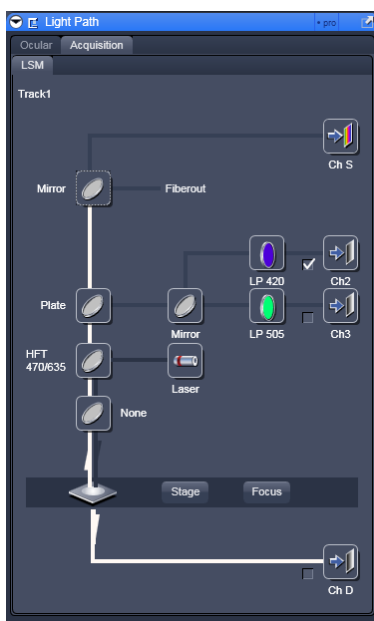


Fig. 3.2: Beam Path and Channel Control window of the LSM510 software: For FLIM data acquisition take care of the correct excitation HFT (major dichroic) and guide the light to the fiber out.

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

3.3.4. Set suitable scan parameters

1. In the **LSM510 - software**, set the correct scan pattern:
 - Use **max. 512x512 pixel** and **monodirectional scan** as indicated in Fig. 3.3. Using less pixel (e.g. 256x256) pixel is recommended, because in this way, more photons can be collected in each pixel. This results in better fits of the lifetime data.
 - **Averaging** can only be used in conjunction with the „Frame“ option. The „Line“ option will scan every line several times which cannot be correctly interpreted by the SymPhoTime software.
 - Use a moderate scan speed since the calculation time for all FLIM analysis steps increases with an increasing number of acquired frames.
 - **Switch on the 477 nm** line of the Argon ion-laser and set the intensity to the minimum value. This setting does not correspond to the respective laser line and the line is not switched on. The setting ensures that your LSM provides the correct line- and frame trigger signals (See Fig. 3.3). Whenever you acquire a FLIM – image, this laser line must be activated in the software.

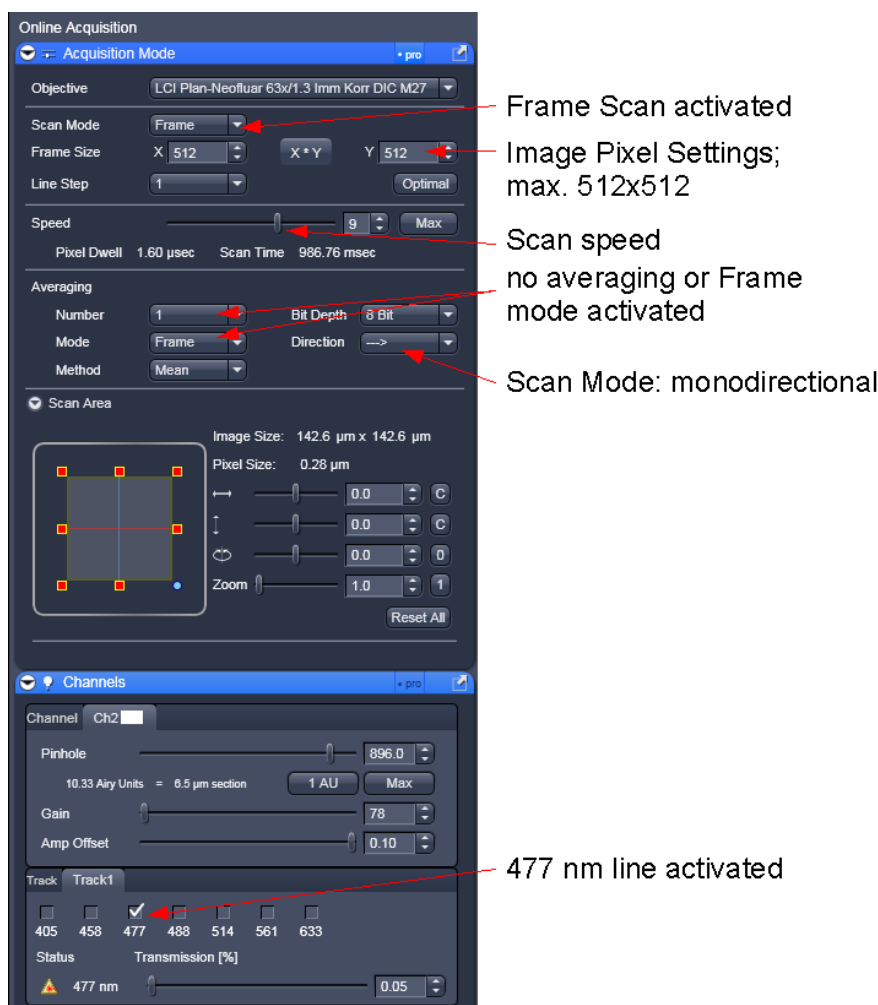


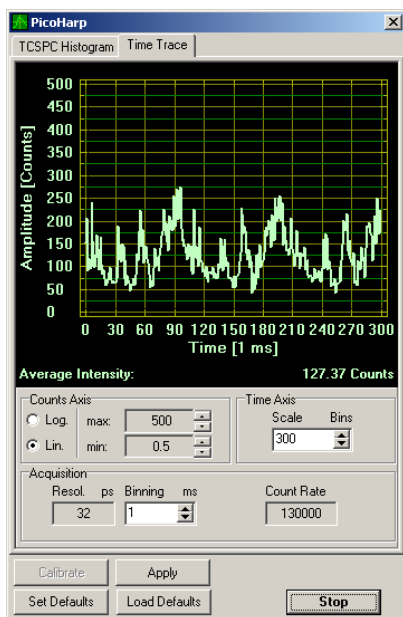


Fig. 3.3: LSM scanning settings in the LSM510 software

3.3.5. Optimise the detected photon count rate

1. **LSM510 software:** Start a repetitive xy – scan by pressing “Continuous” scan button ().
2. **SymPhoTime software:**

- Open the Oscilloscope window (select ) and select the Time Trace Histogram (see Fig. 3.4). Make sure (acquisition time) binning is set to 1 ms and press “Apply”. Then press start in the oscilloscope window and **watch the count rate**. Change the counts axis display from “log” to “lin”.



PDL setting (Laser repetition rate)	Upper Count rate limit for different detectors, in [cts / ms]	
	PMA PMT or PDM SPAD	PE AQR SPAD
1 (40 MHz)	1000	700
2 (20 MHz)	1000	700
4 (10 MHz)	500	500
8 (5,0 MHz)	250	250
16 (2,5 MHz)	125	125

Fig. 3.4: Online monitoring of the of the photon count rate in the SymPhoTime oscilloscope window: The brightest image regions (peaks of the data curve) should not exceed the photon countrate given in the table (see y-axis: Amplitude [counts]). As scanning is fast, the binning time should be set to 1 ms.

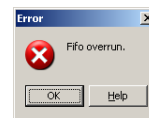
- **Adjust the photon count rate** by changing the intensity of the pulsed laser at the laser combining unit LCU (Fig. 3.18). For attenuation via changing the laser current see the laser driver manual. To read out the count rate use the y-axis (Amplitude [counts]). Do not use neither the count rate box display nor the average intensity values because both display not the actual count rate with the necessary 1 ms time resolution. For the brightest image regions the **photon count rate should not exceed the limit** given in the table (Fig. 3.4). Regions of the image recorded with photon count rates above the limit can display an inaccurate fluorescence lifetime.
- To ensure a shortest possible FLIM data acquisition time we recommend to **increase the photon count rate** if possible up to the TCSPC limit given in Fig. 3.4. Of course in case of weak fluorescing samples this upper limit cannot be reached even when using maximal laser power.

Supporting information:

Unnecessary small photon count rates extend the data acquisition, because one need a certain amount of photons for the FLIM analysis. On the other hand, too high photon count rates can lead to artefacts and falsify the FLIM analysis. The upper limit for the count rate is given by:

- TCSPC principle: To avoid **pile-up** artefacts (overestimation of early photons) the detected photon count rate should be in general less than maximum 5 % of the laser repetition rate. When using a laser repetition rate of 20 MHz this results in a photon count rate of 1 MHz (1.000 cts / ms).

- TCSPC detector: Some detectors introduce **artefacts** when being operated at **high count rates**. The AQR type SPAD from Perkin Elmer shows above 1 MHz photon count rate significant IRF broadening and a count rate dependent IRF shift.
- TCSPC electronics: the recorded photon data is permanently transferred via an FiFo-buffer from the TCSPC unit (TimeHarp or PicoHarp) to the PC. If photon **count rates above 5 MHz** are reached which cannot any more be handled by the buffer, the **data acquisition stops** and the error message displayed on the right occurs.




3.3.6. Choose the correct laser repetition rate

For PicoQuant pulsed diode lasers the **laser repetition rate** can be set at the PDL laser driver. In general the repetition rate should be as high as possible to achieve the highest possible photon count rate. However, the time window after a laser pulse should be still large enough to allow the population of excited dye molecules to deplete completely before the next laser pulse.

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.

This is illustrated in Fig. 3.5. At 80 MHz laser repetition rate, the fluorescence does not decay completely before the end of the time window is reached. Due to a “wrap around” the decay tail is observed even before the actual pulse (A). At 40 MHz the **time window is optimally adapted (B)**, while at 20 MHz the fluorescence has already decayed at less than half of the detection window (C). Therefore 40 MHz would be the optimal repetition rate in this case.

You can check the decay behavior of your sample using the oscilloscope window of the SymPhoTime software (select: ). Activate the TCSPC Histogram-tab and monitor the fluorescence decay as shown in Fig. 3.5.

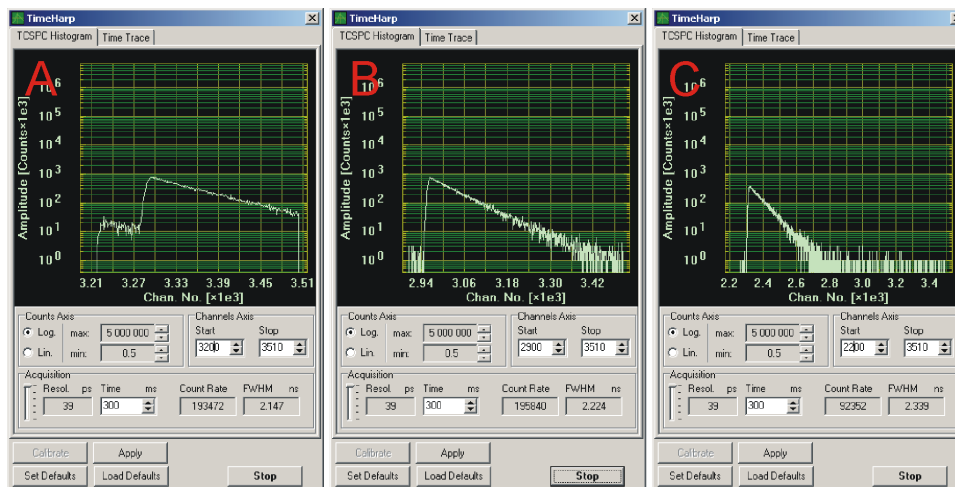



Fig. 3.5: Fluorescence decay at too high (A, 80 MHz), optimal (B, 40 MHz) and unnecessary low (C, 20 MHz) laser repetition rate.

3.3.7. Start FLIM data acquisition

1. **SymPhoTime software:** Start the LSM acquisition by pressing . When recording data, the SymPhoTime software should always be started before activating the scanning process in the LSM510 software, otherwise image information in the first frames can be lost.

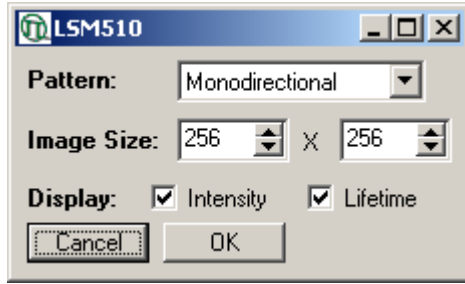


Fig. 3.6: SymPhoTime Pop-up window for LSM data acquisition.

2. **SymPhoTime software:** In the appearing pop-up window (see Fig. 3.6) you **set the scan pattern and the image size** in pixels analogue to the settings applied in the LSM510 software. Please note that the maximum image size is limited to 512 x 512 pixels. The “Display” settings let you choose which information should be displayed with the online image preview during data acquisition: Intensity information is displayed as brightness and lifetime as a special color, pixel per pixel. Finally press OK to start the LSM data acquisition.
3. **SymPhoTime software:** **During LSM data recording** a window pops up (Fig. 3.7) which gives you access to the preview FLIM image. To see the image during acquisition, press the button . When scanning several frames all the **information** is summed up pixel per pixel and displayed. Please note that the information is just summed up for the display, the stored raw data still allows you to treat the data separately frame by frame.



Fig. 3.7: SymPhoTime Pop-up-window being displayed during LSM data recording. To see the image during acquisition, press the button .

4. **LSM510 software:** After the LSM data acquisition, also start the scanning process in the LSM510 software (either in the “continuous” mode or via averaging a determined number of frames, see Fig. 3.8).

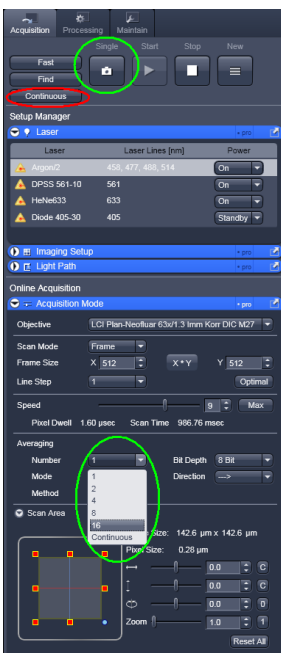



Fig. 3.8: **Starting a Scan** in the LSM510 software: **Marked in red:** Use the “Continuous” mode for scanning. To start the Scan, click on the “Cont”-button indicated in red. Note that the Averaging mode should be set to “Frame” and the number of scans to average (“Number”) is set to 1. **Marked in Green:** For averaging a determined number of frames, select averaging a specific Number of frames using the “Number” pulldown menu. Clicking the “Single” button starts the measurement.

5. The **LSM scanning process stops** either after having scanned the pre - set number of frames, if the system is set for averaging a specific number of frames, or by just manually interrupting the continuous scan (“Cont”) by clicking the “Stop” - button.
Of course you can always chose in the further data analysis with the SymPhoTime software which of the recorded frames you want to display. You can display also a sub ensemble of frames.
6. **SymPhoTime – software:** Press “cancel” in the LSM data recording window (see Fig. 3.7) in order to **stop data acquisition**. The TCSPC data acquisition works completely independent and can be stopped at any time without losing already recorded data.
7. The recorded FLIM image will be displayed in the SymPhoTime software.

3.3.8. Resulting raw data file and documentation

1. **SymPhoTime software:** The measurement result is a **FLIM image** together with a **T3R/PT3 raw data** file (dependent on the type of TCSPC device) containing for every detected photon the maximum available information (temporal, spatial, detection channel).
 - Every raw data file is stored together with an **annotation file** (indicated with a red dot icon  in the workspace overview, see Fig. 3.9) which contains all actual measurement parameters known by the SymPhoTime software, the possibility to type in manually individual information (measurement type, sample, image size, scanning velocity, etc) together with a preview image (as bitmap, to give you a quick impression about the scanned image).
 - Fig. 3.9 shows a typical **SymPhoTime workspace** overview on the left side. Derived from the raw data file “LSM_1.pt3” are the annotation file “LSM_1_cmt.bdf” which is displayed in detail on the right and the FLIM image “LSM_1_IMG1.bdf” (not displayed).

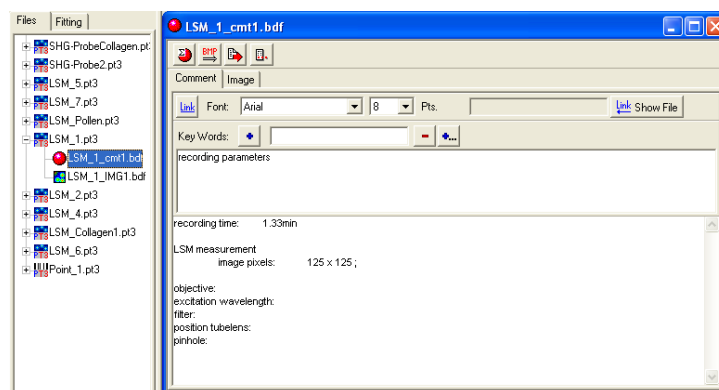


Fig. 3.9: SymPhoTime workspace showing on the left hand side the file history and on the right an annotation file with already known settings (recording time, image pixels) as well as space to enter additional information (objective, filter, pinhole,...).

2. **LSM510 software:** If you want to change in the SymPhoTime software the x,y-axis from pixel number to dimensions in microns you need in addition to the already known number of pixels per axis also the **pixel size (in microns)**. The latter can be taken from the acquisition settings (Fig. 3.10).

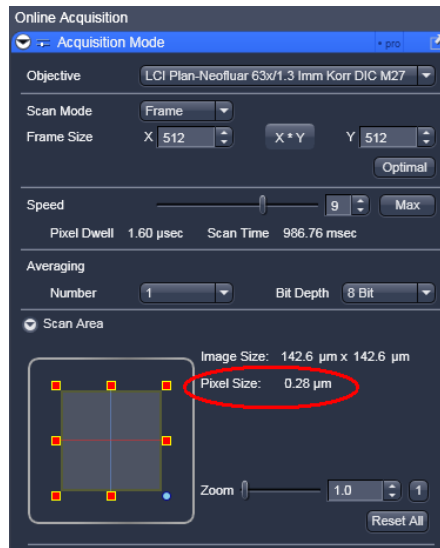



Fig. 3.10: The Image size information is shown in the online acquisition tab as indicated.

3. **SymPhoTime software:** The **image dimensions** in the image can be changed using the **Stack Control** button (). A window with image specifications pops up, where the dimensions of the image can be entered. This affects all size measurements of the image.

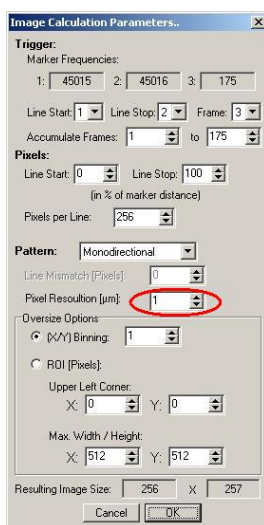



Fig. 3.11: Changing the image size in the SymPhoTime Software. Here the real Pixel resolution can be entered. Also, only a specific numbers of acquired frames can be used for display.

In this window also the frames can be selected (“Accumulate Frames”) which will be displayed in the FLIM image.

3.3.9. Measure an instrument response function (IRF)

For accurate measurements of lifetimes near to the timing resolution, the instrument response function (IRF) should be taken into account. The width of the IRF displays the timing resolution of the instrument. An IRF can be obtained following these steps:

1. Place a **scattering sample** (e.g. the surface of a cover slide or better a mirror) in the laser focus on the microscope stage. Reduce the laser power in order not to oversaturate the detectors. It is important that the laser power output is not changed at the laser driver itself, but only at the Laser Combining Unit (LCU) or attenuator at the laser head. Changing the laser driver settings typically changes the shape and temporal position of the laser pulse!
2. Place an **OD3 attenuating filter** in the filter holder in front of the detector. Look up your detector manual for locating the filter holder position.
3. Lift the **lever** at the **LCU** to direct pulsed laser light to the LSM (optional, Fig. 3.1).
4. Set the **LSM510** settings in the same way as for a **FLIM measurement**. This way, the back reflection of the cover slide can be monitored by the PicoQuant detectors.
5. Activate the **oscilloscope window** in the SymPhoTime software () and select the **Time Trace - tab**.

Important is the setting of a **linear Count Axis**. Start the oscilloscope measurement by clicking the Start - icon. The amplitude axis can be adapted by scrolling with the mouse wheel button in case the cursor is placed on the oscilloscope window. For IRF - measurements the collected countrate should be very small (in the range of a few kilocounts/second).

6. Select in the oscilloscope window the **TCSPC Histogram - tab** as shown in Fig. 3.12. Start the oscilloscope measurement by clicking the Start - icon. The amplitude axis can be adapted by scrolling with the mouse wheel button in case the cursor is placed on the oscilloscope window. The shape and width of the IRF curve is very much dependent on the laser and detector configuration.

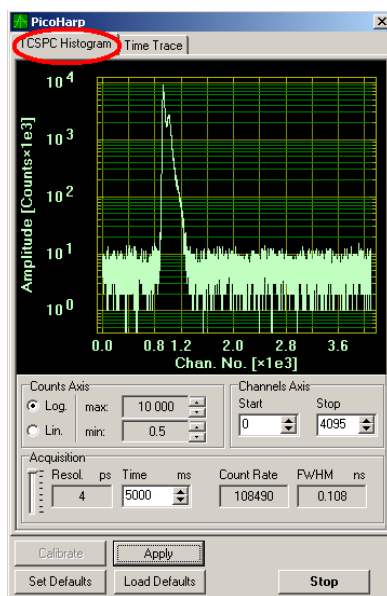


Fig. 3.12: IRF oscilloscope window, recorded with a PH300, MPD SPAD and PQ laser source.

7. When a satisfactory IRF appears on the oscilloscope window, stop the measurement in the oscilloscope window and start a **point measurement** as described in Fig. 3.14 and the corresponding chapter. Based on this IRF raw data file (in T3R or PT3 file format) an IRF TCSPC histogram can be build up (see following chapter for details).
8. **Stop the IRF point measurement**, replace the cover slide with your sample and insert the correct filters for your sample again.

3.3.10. Remarks

- For the ad hoc inspection of your samples you can also use the **pulsed lasers** even in conjunction with the **internal LSM510 PMT detectors**. Typically pulsed lasers have less power in comparison to the continuous wave (cw) lasers, therefore images of weak fluorescing samples should perhaps be recorded with slower scan speed.

- Your LSM510 allows **storing** defined **configurations**. Acquiring FLIM images is easier, when one configuration for pre-FLIM image inspection and one configuration for the FLIM measurement is stored. Storing and loading stored conformations is possible in the “Configuration Control” window of your LSM510 software. A configuration allows to pre-set the correct beam path and major dichroic as well as to switch on automatically the 477 nm line to provide the correct line trigger signals.

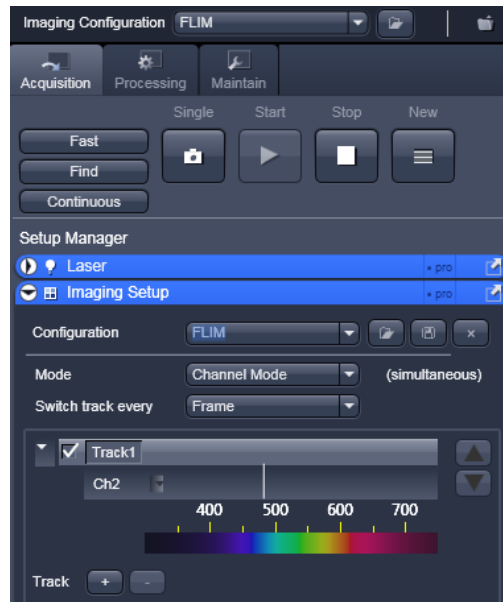


Fig. 3.13: Storing and applying stored configurations is possible via the “Imaging setup” tab. Clicking the “open” icon in the “Configuration” line opens a stored configuration. Entering a new configuration name and clicking on the “save” icon stores the actual configuration.

- The SymPhoTime software is **unable to compensate** for **hysteresis** effects that may be induced by bidirectional scanning, thus if such effects are noticed, it is preferable to perform monodirectional scans.
- The **laser intensity** of the diode lasers can be **adjusted** in two different ways:
On the one hand, the light intensity can be reduced at the LCU using the filter wheel and the scaffold (or only the scaffold at the direct fiber coupled laser heads), on the other hand, the intensity can be controlled at the PDL800 or the SEPIA controller. If the intensity is changed at the PDL800B or the SEPIA, this may change the pulse shape and therefore the instrument response function (IRF). Take care to record the IRF and the fluorescence decay with the same laser settings at the PDL800B or the SEPIA if you want to perform re-convolution. The time response of the instrument (width of the IRF) may be shorter at lower laser power settings at the laser driver.
- The **fluorescence detection sensitivity** of the optical system can also be increased by opening the pinhole in the LSM. On the other hand this may reduce the signal to background ratio.
- In order to obtain the **optimal lifetime information** it is in general preferable to scan FLIM images with less pixels than standard intensity “only” images, as the accuracy of the lifetime information is directly dependent on the number of photons per pixel. That may become crucial, if several lifetimes are present. Furthermore, the calculation time of a lifetime image increases linearly with the number of pixels in the image. The SymPhoTime software also allows to perform post-acquisition pixel binning for optimization of the best compromise between lifetime accuracy and resolution.

3.4. Data acquisition for point measurements as used in FCS

Scope:

Perform a Point measurement at a specific position. With sensitive detectors like single photon avalanche diodes, FCS measurements can be performed. In all point measurements, the fluorescence is recorded at a certain position in the sample and analysed later in the SymPhoTime software.

If lifetime information is not needed, also steady state lasers from the LSM might be used for the fluorescence excitation.

For performing and analysing Point measurements, your SymPhoTime license must cover these measurements.

3.4.1. Starting Point

The following description assumes that

- your **sample** is already placed onto the microscope and brought into **focus**. If you are measuring in solution, it is assumed that the laser is focussed into the solution.
- The **correct dichroic mirror** for your wavelength has been selected in the LSM510 software, and the **correct emission filter(s)** has/have been placed into your detection unit. The light is directed correctly to the fiber exit port as for a FLIM – image acquisition.

Measuring with pulsed excitation

- **Switch the laser on** and select the **correct repetition rate** (see 2.3.6).
- Set the lever at the Laser Combining Unit into the **upper position** (Fig. 3.1).

Measuring with cw excitation

- If measuring with cw lasers, still the **SYNC signal** of the driver unit must be **present**. In this case, best switch on laser driving unit (PDL800B or SEPIA) and set the laser to the standby mode by turning the key. This ensures that a trigger signal is provided, which is needed by the photon counting board. In the oscilloscope window of the SymPhoTime software, the repetition rate or a count rate different from 0 should be shown.
- Set the lever at the Laser Combining Unit into the **lower position** (optional, opposite as in Fig. 3.1).

3.4.2. Choose the location for the point measurement

1. **LSM510 software:** Enable the “Spot” measurement mode (Fig. 3.14). Clicking on “Spot Sel” results in scanning of an image where you can select a position for your point measurement. The scanner only moves to the indicated position, when the time series measurement is started and moves back to the standby position after the measurement has stopped (Fig. 3.15). Of course, for checking the position, the light has to be guided to an internal detector.

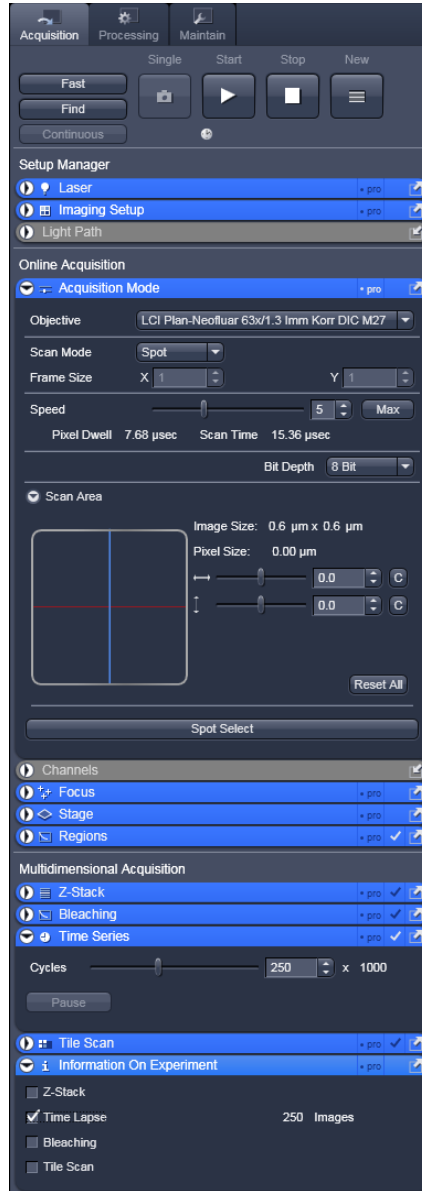


Fig. 3.14: Point Measurement Mode in the LSM510 software.

- 2. LSM510 software:** Click the position for the point measurement in your already scanned image to select the position where you want to measure (Fig. 3.15).

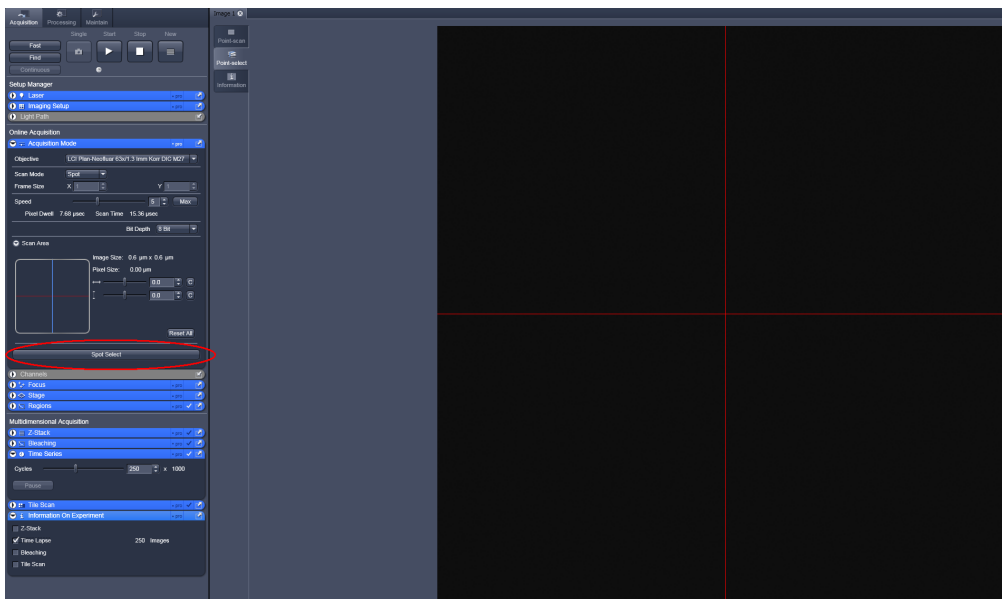


Fig. 3.15: Selecting the position of the point measurement in the Image window. Clicking “Spot select”, indicated in red, results in scanning of an image. Clicking at the desired position marks the area to be measured. The scanner moves to that position when the Time Series Scan is started.

3.4.3. Set the data acquisition time

3. **LSM510 software:** Open the Time Series tab and set the number of iterations to maximum as shown in Fig. 3.16. The total scan time depends on the number of iterations and the scan speed. Approximate times for different scan speeds are given in Tab. 3.1.

Scan Speed	4	5	6	7	8	9	10
approximate duration of the Point Scan [min]	50	25	12.5	6.2	5	3	2.5

Table 3.1.: Approximate Point Scanning Times as set with the Point Mode, using the maximum number of iterations using the slider shown in the Time Series Window (Fig. 3.16)

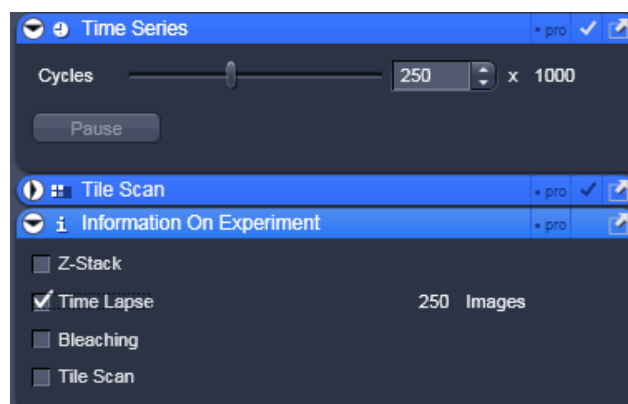


Fig. 3.16: Setting the number of iterations in the LSM software. Set the slider in “Cycles” to maximum. Check the “Time Lapse” button to allow starting the measurement.

4. SymPhoTime software:

- **Open the point measurement dialogue box** by clicking at the point measurement icon  indicated by the green circle **1** in Fig. 3.17.

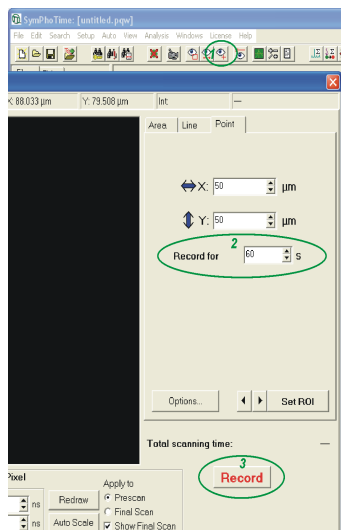


Fig. 3.17: Performing a Point Measurement with the SymPhoTime software: After clicking the icon indicated as **1**, the point measurement window pops up. Enter the recording time in the area marked as **2** and start the measurement by hitting the record button indicated as **3**. The other buttons in the menu are not used in LSM Upgrade Kits.


- **Enter the desired measurement time** (for example 60 s) in the area marked as **2** in Fig. 3.17. Make sure that the measurement time in the SymPhoTime software is **longer** than the measurement time entered in the LSM software and additionally accounts for the fact that the SymPhoTime software is started first.

3.4.4. Optimise the detected photon count rate

Similar guidelines as described in Chapter 2.3.5 apply. Additionally for certain analysis modes some special requirements are of interest:

- **Too high photon count rates** can lead to artefacts and falsify the fluorescence lifetime analysis.
- High count rates **increase calculation times** for FCS analysis.
- Crucial for FCS analysis is a **high countrate per molecule**. In order to achieve this, a balance between efficient excitation and photobleaching has to be found. A lower concentration with higher laser power is preferable, ideal concentrations for FCS span 0.1 - 10 nM.
- For **analysis of individual molecule transits**, the concentrations need to be sufficiently low, so that individual bursts can be recognized.

3. SymPhoTime software:

- **SymPhoTime – Software:** Open the Oscilloscope window (select ) and select the Time Trace Histogram (see Fig. 3.4). Make sure (acquisition time) binning is set to 1 ms. Then press start in the oscilloscope window and **watch the count rate**. If not already set change the counts axis display from “log” to “lin”.
- **LSM510 - software** : If the sample is sensitive to photobleaching, select a different area with similar brightness for optimization of the settings

Measuring with pulsed excitation

- **LSM510 software:** Set the “LSM mode” and select a suited dichroic. The laser beam can be seen at the objective (see Fig. 3.2). The count rate should increase at the detector. Make sure the sample is sufficiently shielded from room light, as this might increase background.
- **LCU:** Adjust the count rate by adapting the attenuation wheel and the scaffold at the Laser Combining Unit (see Fig. 3.13).



Fig. 3.18: Laser attenuation at the LCU: rough adjustment at the intensity can be performed with the filter wheel. the position correspond to the following attenuations: 0=no attenuation (max. power), 1 = 10x attenuation, 2 = 100x attenuation, 3 = 1000x attenuation. The upper knob controls the scaffold for intensity fine tuning. Turning the knob clockwise moves the scaffold into the laser beam and thereby attenuates the excitation intensity.

Measuring with cw excitation

- **LSM510 - software:** Start a point measurement at the LSM510. The count rate should increase at the detector. Make sure the sample is sufficiently shielded from room light, as this might increase background.
- **LSM510 - software:** Adjust the count rate by de- or increasing the laser power in the LSM510 software.

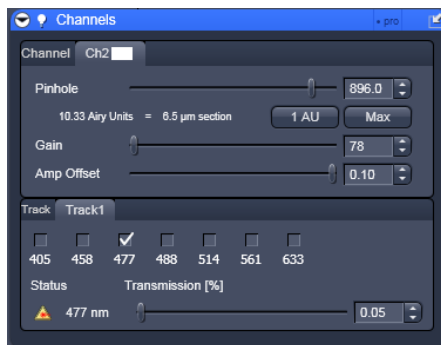


Fig. 3.19: Intensity regulation in the LSM510 software.

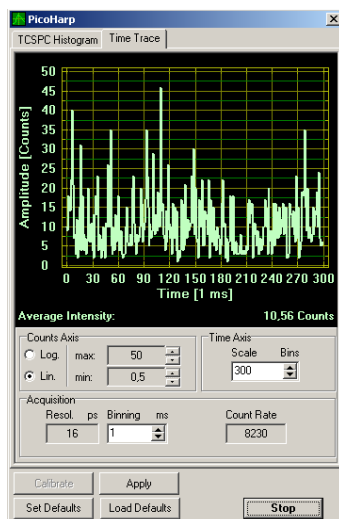


Fig. 3.20: FCS trace of diffusing dye molecules in solution. As FCS analysis spans several orders of magnitude of concentration, the count rate may vary significantly. Single molecule transits are displayed as bursts.

3.4.5. Start data acquisition

4. **SymPhoTime – Software:** Start the **point measurement** by clicking into the record box marked as **3** shown in Fig. 3.17. Take in consideration that there is a small delay between clicking the “Start” button and the actual start of the measurement.
5. **LSM510 – software:** Start the **point measurement** in the LSM software using the “Start” button.

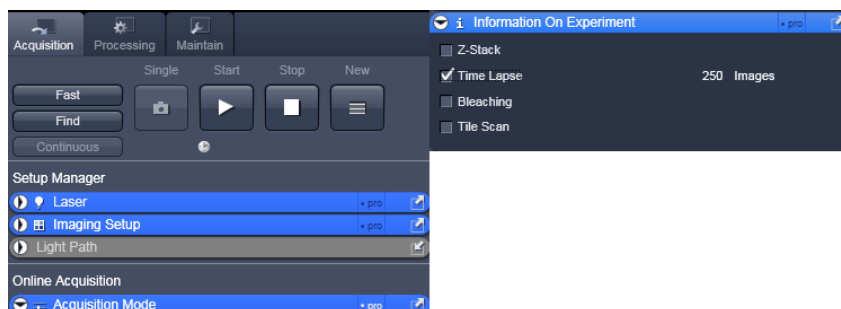


Fig. 3.21: Start of the point measurement in the LSM510 software. The measurement is started in the by hitting "Start". Note that the button is only active, if the "Time Lapse" Box is checked (right).

6. **SymPhoTime – Software:** When **analysing** the data, take into consideration that the data stream is recorded **longer** than the time the sample has been illuminated. Restrict the data analysis of the time trace to the time the sample has been illuminated (see SymPhoTime manual).

3.4.6. Remarks

- **FCS measurements** can **only** be performed using **avalanche photodiodes** 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, FLCS can be used in order to suppress detector afterpulsing after pulsed excitation.
- For a solid FCS-analysis, the **count rate** has to be **stable**. Signal decrease due to photobleaching will result in an offset of the FCS curve.
- The **calculation time** of an FCS measurement depends on the **measurement time** and the detector **count rate**.

4. Troubleshooting

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

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

The actual **hardware configuration** of your LSM upgrade kit (LSM trigger signal configuration, name of the TCSPC device and its settings, number of detection channels,...) is saved in a **.cfg-file**. Restoring the configuration from the .cfg-file is also useful, if the software has to be installed again.

- Use “restore from CFG-file” in the Set-up menu (Fig. 4.1). Apart from the designed folder “settings” in the computer, the original settings of the LSM-Upgrade Kit are stored in the “MicroTime 200.cfg” file on the external USB memory storage delivered with the instrument.

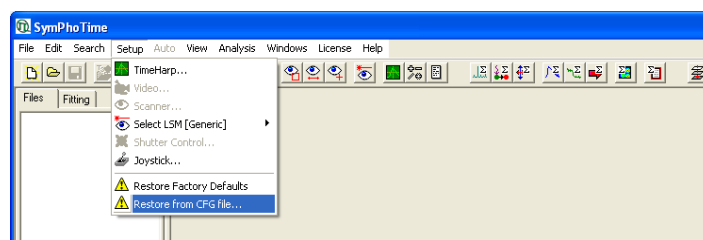


Fig. 4.1: SymPhoTime Menu for restoring the LSM Upgrade Kit setting

In case the **hardware equipment is changed**, the CFG file needs to be changed 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.

4.2. Instrument is losing sensitivity

Note:

- It is recommended to purchase a **power meter** in the lab in order to measure the laser intensities for selected wavelengths.
- If you have purchased a LSM Upgrade Kit with 1 or 2 SPAD 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. Suited dyes for different wavelengths are: ATTO655 for 635 nm, Rhodamine 6G for 532 nm or Fluorescein (in a solution with high pH) for 470 nm excitation. The molecular brightness is the **count rate** of the **detectors divided** by the **number of molecules** present in the detection volume. In general, a nM solution is used. 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) Decreasing Laser Performance

- The performance of the instrument decreases only for measurements where this laser is used. Check the **laser intensity** (a drop in the laser intensity is common with old gas lasers as HeNe lasers). Another cause of decreasing laser performance can be a sub-optimal alignment of the Laser Combining Unit (LCU).
- If the laser output is too low, either the LCU has to be readjusted or the laser needs to be repaired or replaced.

2) Deadjusted LCU Incoupling

- Follow the alignment procedure in the LCU manual

3) Deadjusted LSM510

- If the incoupling within the standard LSM is misaligned, refer to the LSM manual or contact your local LSM representative.

4) Deadjusted Detection Unit

- Refer to your detector manual


5) Misaligned Pinhole

- See section 4.4.


4.3. No image is displayed during measurement


Can laser light be seen over the objective during measurement?

If no:

- Is the **handle** from the **LCU** correctly lifted (see section: how to take a FLIM image)?
- Is the **pulse repetition rate** correct? This can be checked in the oscillator window (). The pulse repetition rate is displayed at the position of the count rate, when no measurement is activated. When starting a TCSPC measurement, dark counts should be displayed (see Fig. 2.12. 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 are correct. See PDL800B or SEPIA II manual, respectively.
- Is the 477 nm line activated?

If yes:

- Do you **see dark counts** when activating the **oscillator window** and start a measurement using the time trace tab ()? If not, check whether the detectors are still on (the DSN101 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 oscillator window, place a **fluorescent sample** on the Zeiss microscope, set all settings in the Zeiss software for FLIM and start scanning, with the time trace measurement in the Oscillator window running. An **increase in the count rate** should be observed. If the count rate does not increase, make sure that the **shutters** in front of the detectors are not closed (i.e. the top of the 2 channel detector box should be placed correctly, all filters should sit in the filter holder correctly. Shutters should clic 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 T3R file is generated. **Calculate a time trace** from your FLIM - T3R - file in the same manner as if it would be a point measurement by choosing the *Calculate Time Trace* button (). 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 LSM510 and the LSM Upgrade Kit. Also try to start the LSM510 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, send the file to PicoQuant.

4.4. Image looks blurry, or sensitivity is low – how to align the Pinhole

- The pinhole settings are crucial for image quality and results of FCS measurements
- If the Pinhole is:
 1. Completely open:
 - => the image resolution is not optimal, but the sensitivity is highest.
 - => In FCS the confocal volume is maximal, but the shape of the confocal volume will deviate from the assumed model and fits may be poor. Also, the noncorrelated background will be highest.
 2. Small but at wrong position:
 - => sensitivity will be poor in FCS and in imaging applications.
- Optimal Pinhole settings depend on the wavelength used.
- The pinhole in front of the fiber exit port used for FLIM and FCS is Pinhole 4.
- To align the Pinhole 4, proceed the following steps:
 1. In the SymPhoTime-software, select the oscilloscope window and monitor the intensity with a binning time of 30 ms (Time Trace tab).
 2. Place a fluorescent sample on the stage (e.g. a suited dye solution), select the right dichroics and detection filter, use the point mode and start a point measurement in the LSM510 software as in an FCS measurement. The count rate at the oscilloscope should be around 100 - 200 kcps.
 3. Go to the "Maintain" menu and select Pinhole 4 adjustment settings. If the sensitivity of the instrument is too low, open the pinhole completely. Does the intensity increase significantly? Set the desired pinhole diameter (see Fig. 4.2).

4. Move the "Position X", until the signal has maximally increased and repeat the same procedure for "Position Y" (see Fig. 4.2). Now, the Pinhole is optimally aligned.

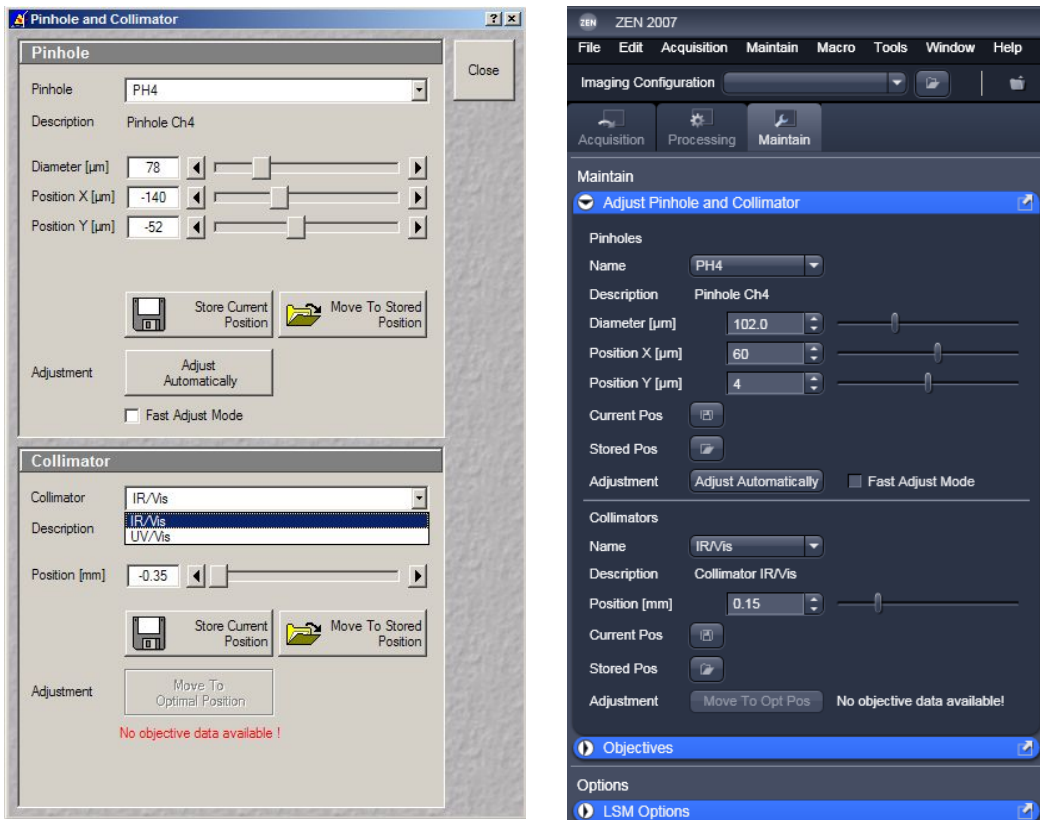


Fig. 4.2: Pinhole Alignment tab in the Zeiss LSM510 software (left) and in the Zeiss Zen software (right)

5. Recommended literature

Publications related to the LSM Upgrade Kit hardware, software, and underlying key technologies:

Wahl M., Koberling F., Patting M., Rahn H., Erdmann R.: *Time-resolved confocal fluorescence imaging and spectroscopy system with single molecule sensitivity and sub-micrometer resolution*. Current Pharmaceutical Biotechnology, Vol.05, p.299-308 (2004)

Koberling F., Wahl M., Patting M., Rahn H.-J., Kapusta P., Erdmann R.: *Two-channel fluorescence lifetime microscope with two colour laser excitation, single-molecule sensitivity, and submicrometer resolution*. Proceedings of SPIE, Vol.5143, p.181-192 (2003)

Ortmann U., Dertinger T., Wahl M., Rahn H., Patting M., Erdmann R.: *Compact TCSPC upgrade package for laser scanning microscopes based on 375 to 470 nm picosecond diode lasers* Proceedings of SPIE, Vol.5325, p.179 (2004)

Benda A., Hof. M., Wahl M., Patting M., Erdmann R., Kapusta P.: *TCSPC upgrade of a confocal FCS microscope*. Review of Scientific Instruments, Vol.76, 033106 (2005)

The following application and technical notes are available from PicoQuant upon request:

Koberling F., Schuler B.: FRET analysis of freely diffusing molecules using the MicroTime 200

Krämer B., Koberling F.: *Lifetime based hydrophobicity analysis of hepatocytes using the MicroTime 200*

Krämer B., Koberling F., Tannert A., Korte T., Hermann A.: *Lifetime based analysis of lipid organization in hepatocytes using the MicroTime 200*

Wahl M.: *Time-correlated single photon counting in fluorescence lifetime analysis*

Wahl M.: *Time tagged time resolved fluorescence data collection*

Numerous measurement examples are published on the PicoQuant website. Please visit the LSM Upgrade Kit section of http://www.picoquant.com/_systems.htm.

6. 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

7. Support and Warranty

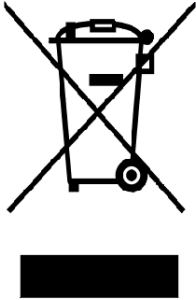
If you observe any errors or bugs, please try to find a reproducible error situation. E-mail a detailed description of the problem and relevant circumstances to photonics@pq.fta-berlin.de. In case of software problems, attach the log file of the measurement. The log - file to any measurement can be found under *View -> Log File* in the SymPhoTime Software. 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 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 Upgrade Kit or published scientific papers, we would also like to know! Please send us an e-mail to photonics@pq.fta-berlin.de containing the appropriate citation. Gain additional publicity! PicoQuant maintains a database of publications mentioning PicoQuant devices and/or written by us. It can be found at our website at http://www.picoquant.com/_scientific.htm. It is a valuable source if you want to know which laboratories are using PicoQuant products or how broad the field of various applications is.

Thank you very much in advance for your kind cooperation!

All information given here is reliable to the best of our knowledge. However, no responsibility is assumed for possible inaccuracies or omissions. Specifications and external appearance are subject to change without notice.

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