LSM Upgrade Kit

Compact Lifetime and FCS
Upgrade Kit for
Olympus FluoView 1000
Laser Scanning Microscopes

PicoQuant GmbH
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Forschung und Entwicklung

User's Hardware Manual and Technical Data

Version 2.1

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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.

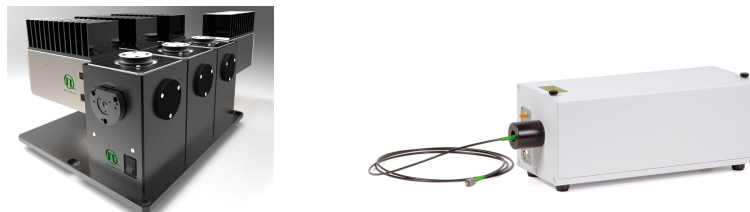**Fig. 1.2:** Detection Units for LSM FLIM / FCS Upgrades from left to right:
Multi channel PMA / SPAD detection unit, single channel SPAD detection unit

- **PicoHarp 300 and TimeHarp260 Manual:** Here you find all information about your Time Correlated Single Photon Counting (TCSPC) device. This manual also includes an introduction about single photon counting.
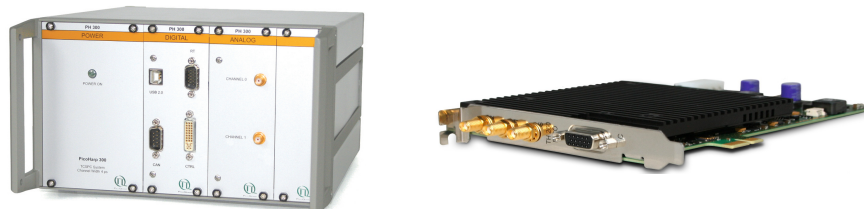
Fig. 1.3: PicoHarp 300 and TimeHarp 260

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

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

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

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

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

- **Software Manual (SymPhoTime 64 - Manual):** Here you find all information about the software installation. The SymPhoTime-software contains a detailed, context sensitive online help function.

- **Pre-Installation Requirements:** This is an important document that already should have been considered before installation. If you want to change the configuration of your LSM, you'll find detailed information, if the desired change can be realized and whether additional parts are necessary. In case of doubt, contact a PicoQuant representative.

- **Appendix:** The appendix contains specific information about your individual LSM FLIM / FCS Upgrade, including a description of the included parts, filter handling and a cable plan, which helps restoring the configuration after disassembly.

2. Operation

2.1. Safety Instructions

Laser Warning

The LSM upgrade kit is equipped with one or more pulsed diode lasers. To avoid hazardous radiation exposure you should carefully obey the safety instructions that are provided with your PDL diode laser operation manual. If your instrument uses another excitation system, follow the safety instructions of the relevant manual.

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

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

Protect the photon detectors (SPAD or PMT) as much as possible, particularly from excessive light intensities, e.g. the microscope illumination lamp, unattenuated backscattered excitation, etc.
In case of operation with the DSN 102, the SPAD detector(s) must be switched off using the DSN 102 power supply unit BEFORE switching off the system by the main power button.

2.2. Starting Hardware and Software Equipment

To **start** the LSM itself, its detectors and cw lasers please refer to your FluoView 1000 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 FluoView 1000 itself.

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

1. Start the **laser driver** and enable laser emission. Prior to data acquisition, the laser head should be operated for a few minutes to stabilize. If you use a PDL 828 "Sepia", you can set the laser settings in the SymPhoTime 64 software.

2. Start the external **PicoHarp 300** TCSPC device, if applicable also the **PHR 800** router and the **DSN 102** SPAD power supply

3. Start the **PC**.

4. Start the **SymPhoTime 64 software**.

5. **Open** an already existing **workspace** or create a new one.

6. Place correct **fluorescence filters** in the corresponding filter holder position of the external PicoQuant detector unit and the confocal microscope. If you have a Dual Channel SPAD Detector Unit, set the variable beam splitter unit to the correct position (see Dual - Channel Detection Unit manual for details).

7. **Make sure that no light is focussed onto the detector(s) and power** them on. Either power on the detector on its unit or, if using a DSN 102, start the respective detector by pressing the corresponding channel button on the DSN 102.

8. If you want to work with user specific settings, load your **User Configuration**.

    The FLIM / FCS Upgrade is now ready to use.

2.3. FLIM Data Acquisition

Scope:

Acquire a FLIM image using a PicoQuant pulsed laser, the Olympus FluoView 1000 scan head with appropriate scanning and dichroic settings and external PicoQuant TCSPC detectors.

2.3.1. Starting Point

1. **FluoView 1000:** Choose and pre-scan with the FV1000 (its cw lasers and internal PMTs) 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 FV1000 PMTs. Finally stop the scanning process.

2. All components (laser, detectors, software) of the PicoQuant upgrade kit are already running. For details about powering on see chapter 2.2.

3. **SymPhoTime 64 software:** The desired **User Profile** of the SymPhoTime 64 has been loaded. The SymPhoTime 64 allows configuring the view of the acquisition window. Thus the user can preconfigure the online analysis he wants to see during acquisition, e.g. images (also of the different spectral channels if more than one detector is present), a time trace, a decay (TCSPC) histogram. Several online analyses can be displayed simultaneously.

4. **SymPhoTime 64 software:** A workspace has been loaded or created. The workspace defines where the data should be stored to.

2.3.2. Changing from continuous wave to pulsed excitation

1. **Pulsed 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. If the lasers are driven by a PDL828 "SEPIA II" driver, the repetition rate can be set in the SymPhoTime 64 software.

2. **Pulsed Lasers:** If cw and pulsed excitation share the same fiber port at the FV1000 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 scan head.
If the pulsed laser head is attached directly to the fiber or situated in a LCU without switchable mirror, the laser light enters the microscope already by switching the lasers on.

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

3. **FluoView *1000 - software:*** Choose a **suitable excitation dichroic mirror** (see Fig. 2.2) for the pulsed excitation wavelength.

2.3.3. Changing from internal FluoView 1000 PMTs to external TCSPC detectors

1. **FluoView 1000 - software: Configure the detection beam** path inside the LSM scan head. Replace all dichroic mirrors (DM) in the detection beam path (FL Imaging) and replace them with glass (plates). This enables the fluorescence to reach the exit port and via fibre coupling the external TCSPC detectors (Fig. 2.2).
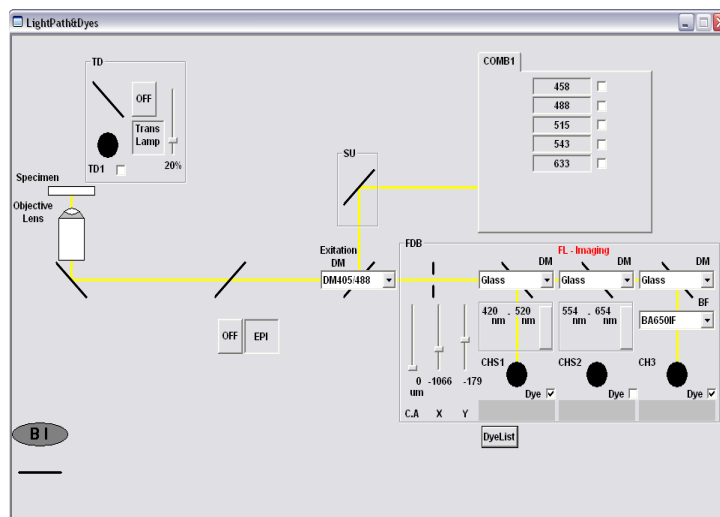

Fig. 2.2: Light Path & Dyes window of the FluoView 1000 software: For FLIM data acquisition take care of the correct excitation DM and glass (plates) in front of all internal detectors in the section FL Imaging.

2. **PicoQuant Detection Unit:** Make sure you have **suitable fluorescence detection filters** (bandpass / long-pass) 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 detection unit manual for details).

2.3.4. Set suitable scan parameters

1. In the **FluoView 1000 - software**, set the correct scan pattern:

   - Use a **monodirectional scan**. 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. However, you can also "bin" the pixels of the image in the analysis section later in order to calculate and analyse an image with less pixels. This will result in no loss of the acquired fluorescence. When using image sizes larger than 512x512 pixels, the online preview in the SymPhoTime 64 software might become slow. In this case, switch off the online previews in the SymPhotime 64 software. See Fig. 2.3 for how to set the pixel size and scanning mode in the FluoView software.

   - **Filter mode** (Kalman) can only be used in conjunction with the „Frame" option.
     „Line" option will scan every line several times which cannot be correctly interpreted by the SymPhoTime software.
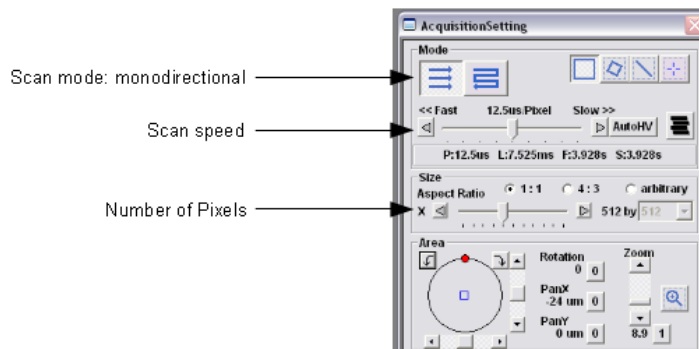
**Fig. 2.3:** LSM scanning settings in the FluoView 1000 software

2.3.5. Optimise the detected photon count rate

1. **SymPhoTime 64 software:**
    To set up the right parameters for data acquisition, go to the main "Test" - tab of the SymPhoTime 64 software. In the "Imaging" drop-down-menu, enter the selected image size and the selected scanning pattern, bi- or monodirectional scanning, and the image dimensions (in µm/pixel).
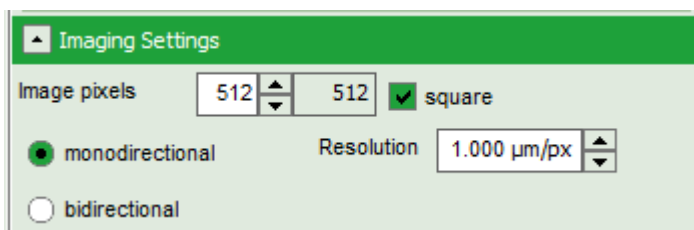
2. **SymPhoTime 64 software:** Press the Start-button.
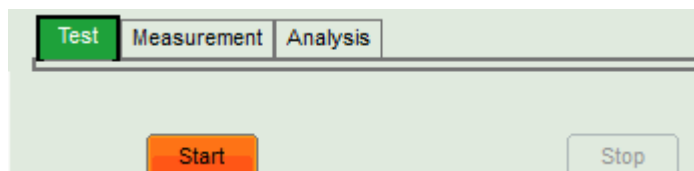
2. **FluoView 1000 software**: Start the scanning process.

3. **SymPhoTime 64 software**:
    The measurement preview is displayed, together with a time trace (Fig. 2.4). Make sure that the Peak Count Rate (marked in red) is below the values given in the table below. The Peak Count Rate is determined from the maximum in the time trace. The "Time trace binning" should be set to 0.001 s. Here you can watch the changes of the count rate over time. A red line additionally indicates a threshold of 10% of the repetition rate. The intensity should always remain below this value. If only very tiny lifetime-changes are expected, stay even well below this number.
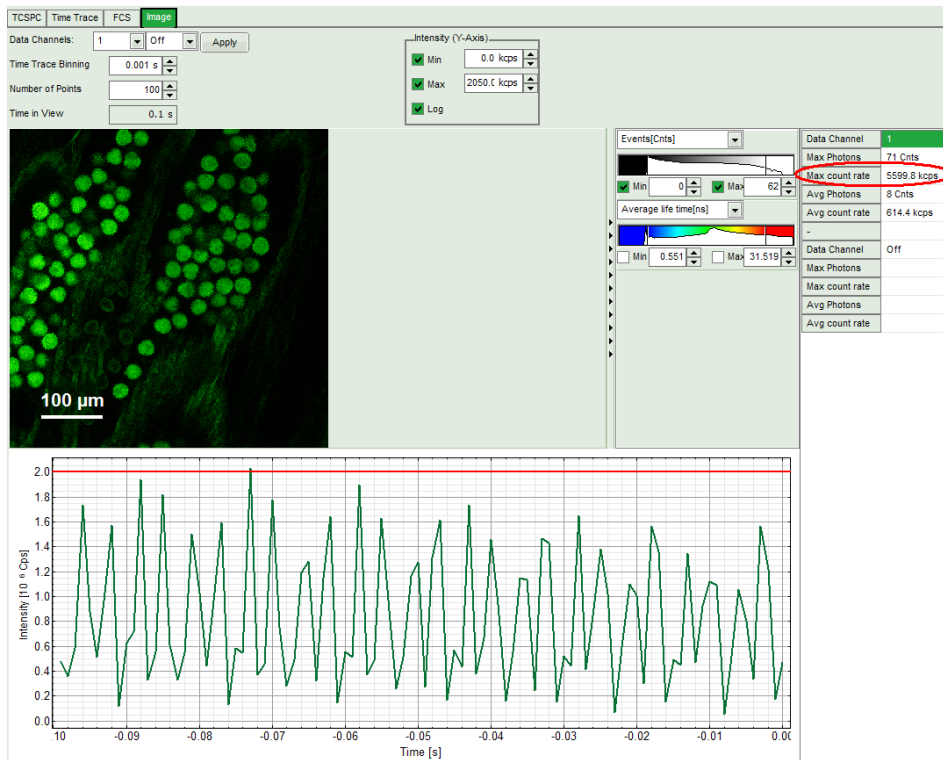
PDL setting (Laser repetition rate)	Upper Count rate limit for different detectors, in [KCounts / s]		
	PMA PMT, Hybrid PMT or PDM SPAD	PE AQR	τ-SPAD
1 (40 MHz)	2000	700	1000
2 (20 MHz)	1000	700	1000
4 (10 MHz)	500	500	500
8 (5,0 MHz)	250	250	250
16 (2,5 MHz)	125	125	125

Fig. 2.4: Top: Online monitoring of the of the photon count rate in the SymPhoTime 64 preview window. On the left, a FLIM is displayed. The intensity time trace is shown below. An intensity of 10% of the repetition frequency is indicated by the red line in the graph. The peak count rate is also indicated on the right (red circle) Bottom: Count rate limit for different combinations of excitation repetition rates and detectors.

- **SymPhoTime 64 software:** The Image **preview settings** can be pre-set, when no measurement is running. Defaults can be saved using the "Save Defaults" button in the Default drop down menu.
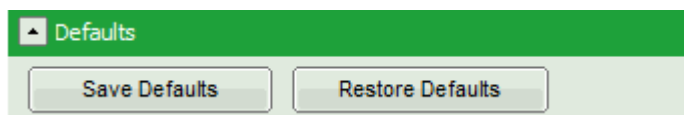
To finally save the settings in a User profile, select "Settings" => "Save as User Profile" in the main menu bar . This way, all settings can be saved in a user specific profile and loaded with the next start of the software.

- **Adjust the photon count rate** by changing the intensity of the pulsed laser at the laser combining unit (LCU) or – if you don't have a LCU, but a directly fiber coupled laser – in an analogous way at the laser scaffold with the intensity regulation knob.
  For attenuation via changing the **laser current** see the laser driver manual. If the pulsed lasers are controlled by a **SEPIA II**, the intensity can be regulated in the SymPhoTime 64 software.

Therefore, check the laser head you want to use in the "laser Settings"-drop-down menu and set the intensity. Keep in mind that changing the laser intensity via the laser current does alter the pulse shape of the laser. Setting the intensity close to the lower laser threshold will ensure the narrowest pulse width, but the intensity will also be lowest. If your system has been configured by PicoQuant, the lasers are preconfigured in the SymPhoTime 64 software, so that settings below the laser threshold are not possible. Note that the % values in the menu refer to the current that drives the laser and do not belong to a linear intensity scale.
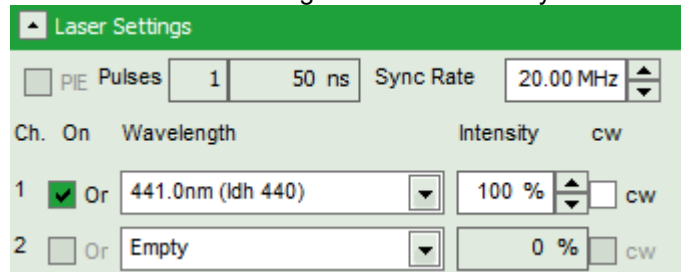
If the pulsed laser is not from PicoQuant, e.g. a TiSa-laser, the intensity can usually be regulated in the LSM software.

For adjustment use the **peak count rate**, since the average count rate does not give you any information about the count rate in the brightest parts of the image. Regions of the image recorded with photon count rates above the limit can lead to inaccurate fluorescence lifetimes.

- To ensure the shortest possible FLIM data acquisition time we recommend to **increase the photon count rate** up to the TCSPC limit given in the table from Fig. 2.4. Of course in case of weak fluorescing samples this upper limit cannot be reached even when using maximal laser power.
- Using the "Image" tab of the "Test" preview window you will see a **preview** of the **FLIM image**. The lifetime displayed in the image by the color code is calculated by the mean arrival time of the fluorescence photons after the last laser pulse. The image is updated roughly each second. It allows the optimization of the field of view and focus setting.

Supporting information:
Unnecessary small photon count rates extend the data acquisition, because of the need of 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** artifacts (overestimation of early photons) the detected photon count rate should be in general less than 10 % of the laser repetition rate. When using a laser repetition rate of 20 MHz this results in a photon count rate of 2 MHz (2.000 Cnts / 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 (PicoHarp) to the PC. If too high average photon **count rates above 4 MHz** are reached, the **data acquisition stops** and the error message displayed on the right occurs. The actual limit is determined by the data acquisition rate of the photon counting boards, the transfer rate to the computer and the processing speed of the computer.

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 or – if the lasers are controlled with a SEPIA II – in the "Laser Settings" drop-down – menu of the SymPhoTime 64 - software. 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.
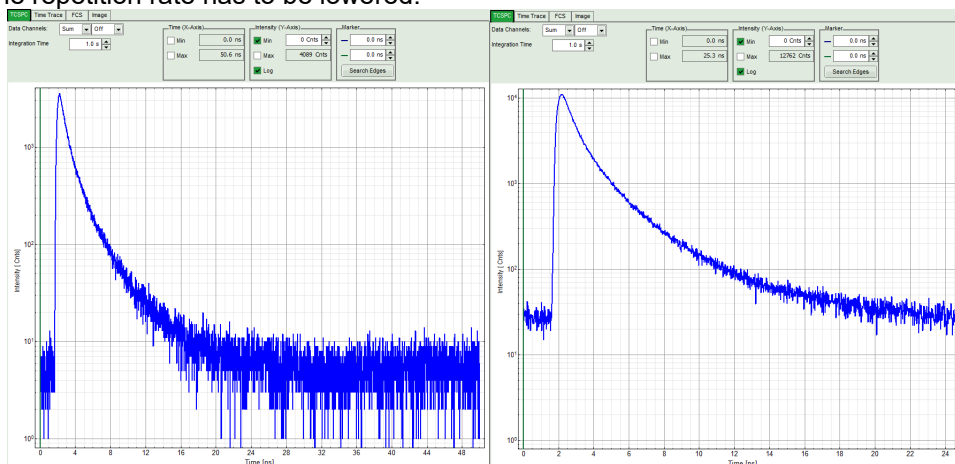

Fig. 2.5: Fluorescence decay at unnecessary low (left, 20 MHz) and optimal (right, 40 MHz) laser repetition rate. With an even higher repetition rate, a part of the fluorescence would appear at the beginning of the TCSPC-window.

Start the scanning in the FluoView software. Check the decay behavior of your sample using the TCSPC tab of the Test - window of the SymPhoTime 64 software. Activate the TCSPC Histogram-tab, press the "Start" button and monitor the fluorescence decay as shown in Fig. 2.5. At 40 MHz the **time window is optimally adapted** (right panel in Fig. 2.5), while at 20 MHz the fluorescence has already decayed at less than half of the detection window (left in Fig. 2.5). 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. Therefore 40 MHz would be the optimal repetition rate in this case. Stop the measurement and switch to the main "Measurement" tab for image acquisition.

2.3.7. Start FLIM data acquisition

1. **SymPhoTime 64 software:**
   To set up the right parameters for data acquisition, go to the main "Measurement" - tab of the SymPhoTime 64 software. In the "Imaging" drop-down-menu, enter the selected image size and the selected scanning pattern, bi- or monodirectional scanning and the image dimensions (in μm/pixel). Note that the entered dimensions become a part of the raw data file and therefore cannot be changed after image recording.
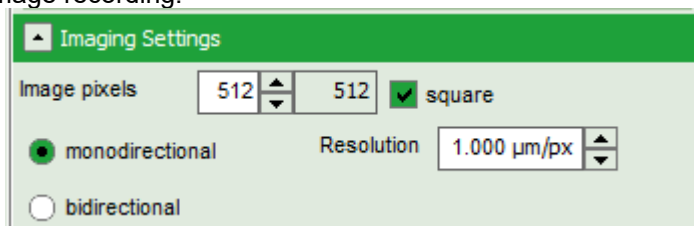
2. **SymPhoTime 64 software:** Make sure that the "Image" Measurement mode is selected and press the "Start" button.
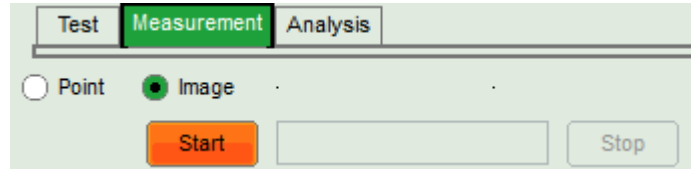
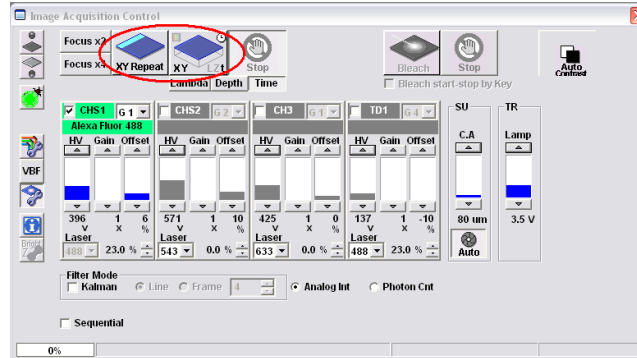3. **FluoView 1000 software**: Start the scanning process (either in the "X,Y repeat" or "X,Y,t" mode).

Fig. 2.6: Starting a Scan in the FluoView 1000 software:" XY Repeat" starts a xy-scan which is interrupted only by the user hitting the stop button, while the "XY-t" scan records a predefined number of scans. Setting the number of frames in the "XY-t"-mode is described in the Olympus FluoView 1000 manual.

4. **SymPhoTime 64 software: During LSM data recording** the chosen online analyses are displayed, e.g. one or – in case of several detectors - several FLIM -images. When scanning several frames, all frames are integrated over time. This display allows you to evaluate the quality of the FLIM data captured. Please note that the FLIM preview image is just integrated for the display, the stored raw data still allows you to analyse the data separately frame by frame.
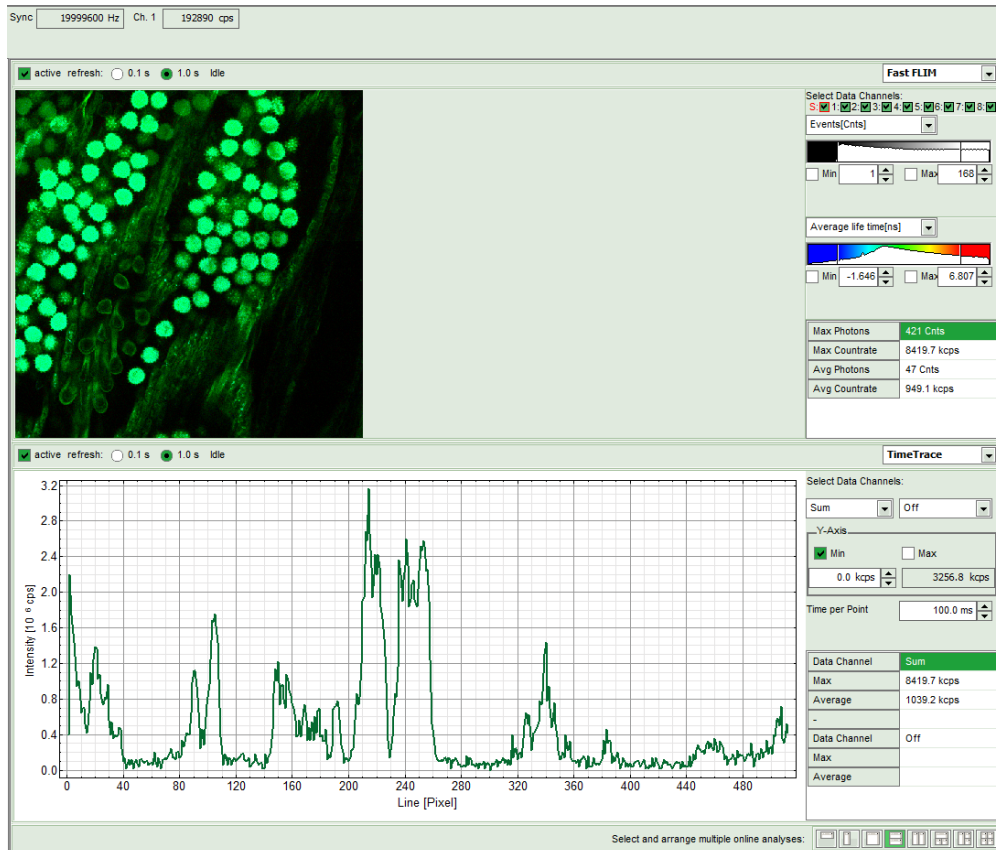

Fig. 2.7: SymPhoTime Measurement Preview being displayed during LSM data acquisition. In this preview, the FLIM image is displayed on the upper left. The display parameters are shown on the right. Below, a timetrace is shown, which represents a profile through lines of the image.

5. **FluoView 1000 software:** The **LSM scanning process stops** either after having scanned the pre - set number of frames ("X,Y,t") or by manually interrupting the "X,Y repeat" scan.

6. **SymPhoTime 64 software:** The data recording stops by just manually interrupting the data acquisition.

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

## 2.3.8. Resulting raw data file and documentation

- **SymPhoTime 64 software:** The measurement result is a .ptu-raw data file, containing for every detected photon the maximum available information (temporal, spatial, detection channel information), together with the online analysis files ".pqres". If e.g. a online Fast-FLIM-image is to be processed, highlight the raw data (.ptu) file and open this file with e.g. the FLIM-Analysis option in the Analysis-tab. If you double-click on the "OnlineFastFLIM_... .pqres-file", the FLIM analysis is directly opened.

- Every raw date file is stored together with the actual settings. It contains all actual measurement parameters known by the SymPhoTime software. To access, double click on the raw (".ptu")-file. The raw data file contains all actual measurement parameters known by the SymPhoTime software; individual information (measurement type, sample, etc) can be entered **before** the measurement via the "Info" dropdown-menu.
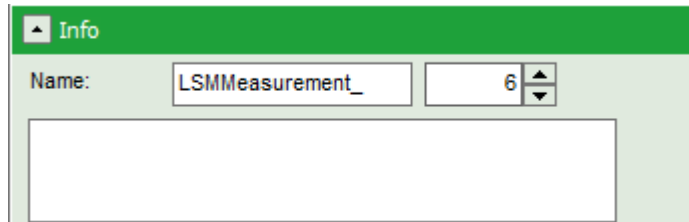
- **Note** that the raw data file **cannot be changed after** the measurement; if you need to add information after the measurement, you can add a comment file via the main menu bar.
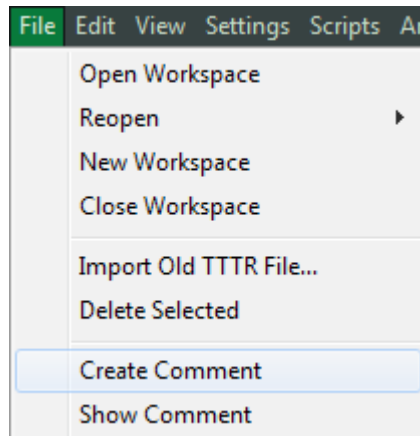
- Also the **image dimensions** that are used for internal distance calculation have to be entered as "Resolution" in the "Imaging Settings" of the **SymPhoTime 64 - software** before the start of the measurement .
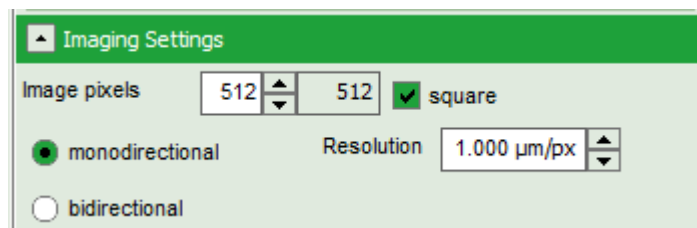
To find the image dimension in the **FluoView 1000 software**, open the information window in the FluoView1000 software (Fig. 2.8). If you want to change the x,y-axis dimensions from pixels to microns in the SymPhoTime 64 software enter the **pixel size (in microns) in the window of the SymPhoTime 64** software before the start of the measurement.
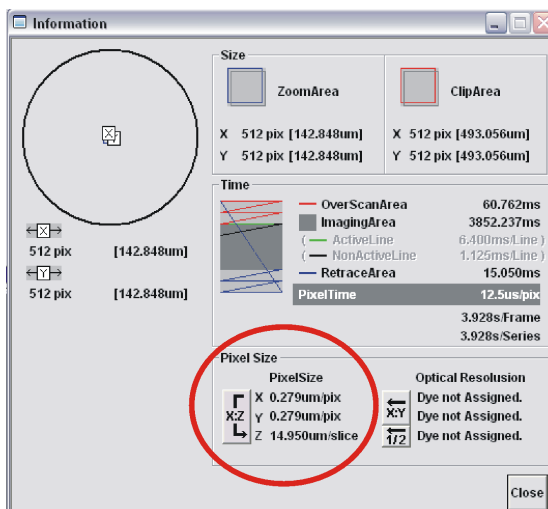
Fig. 2.8: FluoView 1000 scan information window giving the pixel size in microns (see red circle).

SymPhoTime 64 software: *S*pecific 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" dropdown 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. 2.9: Changing the selection of frame numbers for analysis in the SymPhoTime 64 Software.

2.3.9. Measure an instrument response function (IRF)

For accurate measurements of lifetimes close to the timing resolution of the instrument, the instrument response function (IRF) should be taken into account. The width of the IRF displays the timing resolution of the instrument. An IRF can be obtained following these steps:
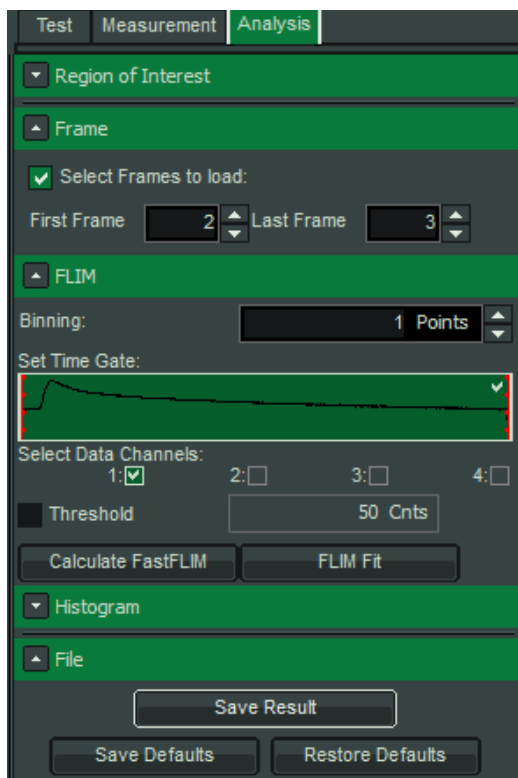
1. Place a sample with an **negligible short lifetime** (e.g. Erythrosin B or Fluorescein, either dissolved in a saturated KI-solution. For fluorescein, a slight basic pH value is necessary to dissolve a sufficient amount of dye) in the laser focus on the microscope stage. The dye chosen should have a similar emission range as the sample that should be measured afterwards. Potasium 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.

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

2. If present at your system, lift the **lever** at the **LCU** to direct pulsed laser light to the LSM (optional, Fig. 2.1).

3. Set the **FluoView 1000** settings in the same way as for a **FLIM measurement**. Start the scanning process.

4. **SymPhoTime 64 software:** You can now check the decay behavior of your sample using the TCSPC preview of the "Test" tab. Activate the TCSPC Histogram-tab, press the "Start" button and monitor the fluorescence decay as shown in Fig. 2.10. In the "TCSPC-settings" dropdown-menu, also the time width per channel can be set. Adjust the count rate to approximately 50 kCounts/s. It is important that the laser power output is not changed at the laser driver itself or in the SymPhoTime – software, if a SEPIA II is present at the system, but only at the Laser Combining Unit (LCU) or attenuator at the laser head. Changing the laser driver or software intensity settings changes the shape and temporal position of the laser pulse. You can use the driver settings to optimize the pulse shape of the laser for shortest IRF width.
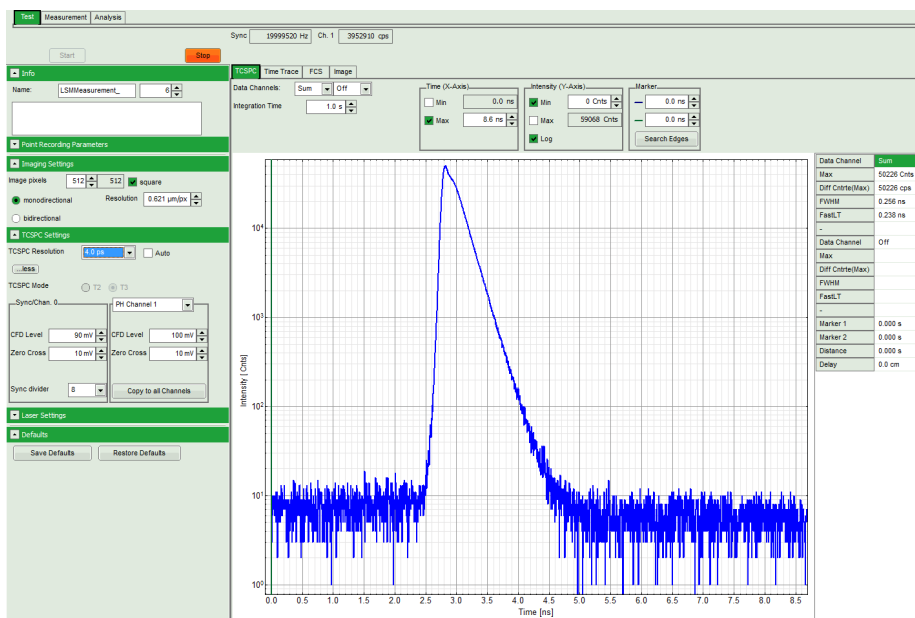
Fig. 2.10: TCSPC-preview window, recorded with a PH300, MPD SPAD and PQ laser source. The time width per channel can be set with the "TCSPC Settings" drop-down menu. The count rate is displayed over the preview graphs for each channel separately.

5. **SymPhoTime 64 software:** Stop the test measurement by pressing "Stop".
   Steps 4 and 5 are only for checking of the IRF shape and settings, they do not need to be performed routinely.

6. **SymPhoTime 64 software:**
   Switch to the "Measurement" tab and start the data acquisition pressing "start".
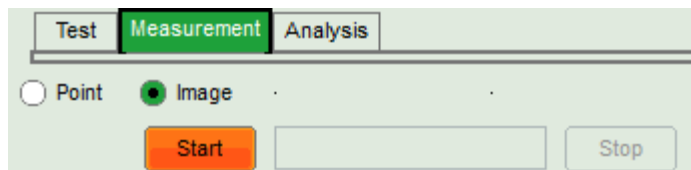
7. When the decay histogram in the TCSPC preview has reached 10000 counts in the peak channel, **stop** the image acquisition in the SymPhoTime and Olympus FluoView software. You can open the TCSPC histogram of the captured image, using the "TCSPC-Histogram" - analysis of the SymPhoTime 64 software. It contains the IRF.

9. Replace the IRF solution with your sample again.

2.3.10. FLIM Data acquisition using internal Olympus NDD-detectors

If you are using the internal Olympus NDD detectors for FLIM-imaging, the measurement procedure is the following:

Preparations:

− NDD detection is very background light sensitive. In general you need to cover the sample stage very well and/or keep the room dark.

− Make sure that in the NDD-Detection unit the right filters are inserted.

− In general, for the generation of the laser trigger signal of a TiSa-laser generally used with Olympus 2-Photon systems, the internal trigger diode of the TiSa is needed. Normally the system is already set to detect the trigger signal (SYNC) by PicoQuant. The internal diode is not always sensitive to all wavelengths, especially beyond 1000nm. The trigger settings of the laser pulse can be adapted to the real signal in the SymPhoTime 64 software. If only a TiSa laser is present at the system, change the CFD level of the SYNC input in the TCSPC dropdown menu.
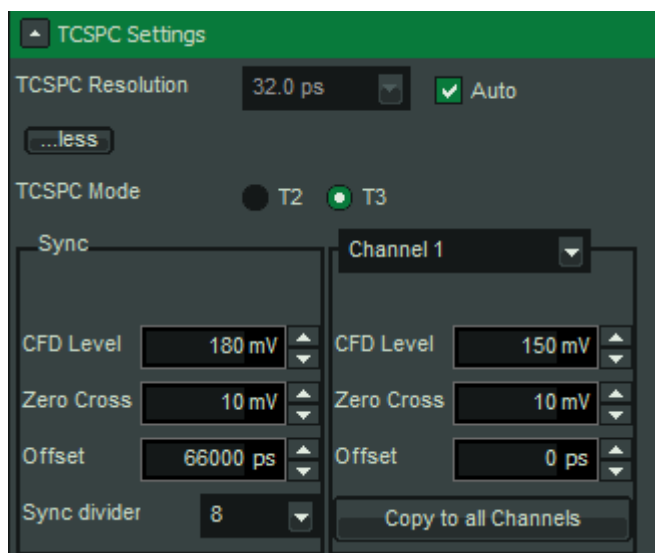

Fig. 2.11: Settings of the input trigger levels of the PicoHarp. On the left side, the laser settings are displayed, on the right side, the detector settings. These usually don't need to be modified.

If apart from the TiSa also pulsed laser diodes are present at the system, the TiSa trigger signal ("Sync") is not directly fed into the PicoHarp board, but into the laser driver and can be adapted either at the laser driver directly (for the PDL800-D, switch the driver to "external" triggering) or in the laser settings of the SymPhoTime 64. Set the oscillator to "rising edge" or "falling edge", depending on how the system has been set up. To use the pulsed diode lasers, the oscillator must be set to "internal", and adapt the Trigger level, until the right laser repetition rate is displayed.
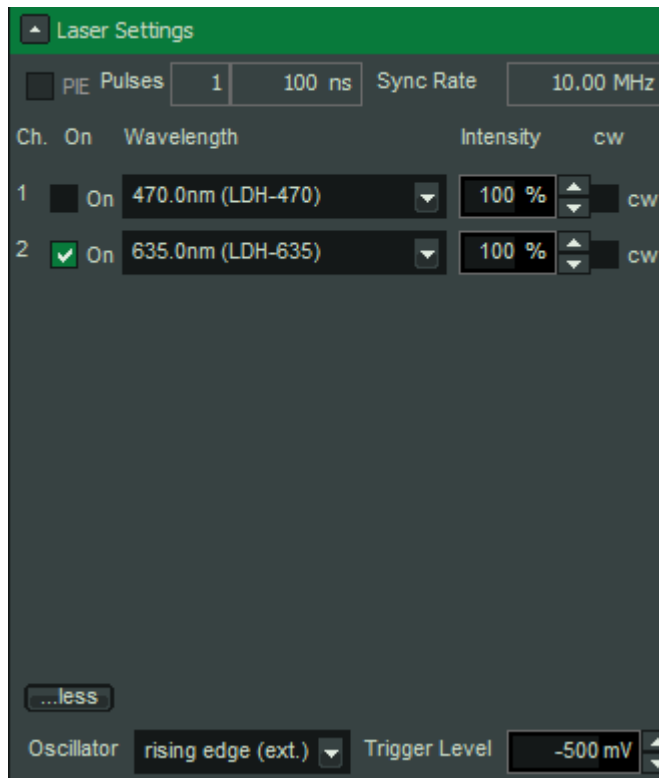

Fig. 2.12: Setting the trigger level to operation for 2 Photon excitation for systems with pulsed diode lasers and a TiSa laser. If you want to use the TiSa laser in this configuration, set the Oscillator from internal - for use of the diode lasers - to "rising edge" or "falling edge". The Sync rate displayed above the online analysis should switch to 80MHz. If it is switching stably to 10 or 11 MHz, check that the "Sync divider" is set to 8 (Fig. 2.11). If the Sync rate at Ch0 is 0, change the "Trigger level" settings, until a stable 80MHz Sync rate is measured.

−   make sure that the cable connection at the NDD detection unit transfers the signal to the detector. If you are not using FLIM, but the internal detectors in the standard configuration, The signal can be fed back into the Olympus NDD detection unit, either by manually swapping the cables (Fig. 2.13) or -if purchased with the system – using a computer controlled high frequency switch (Fig. 2.14).
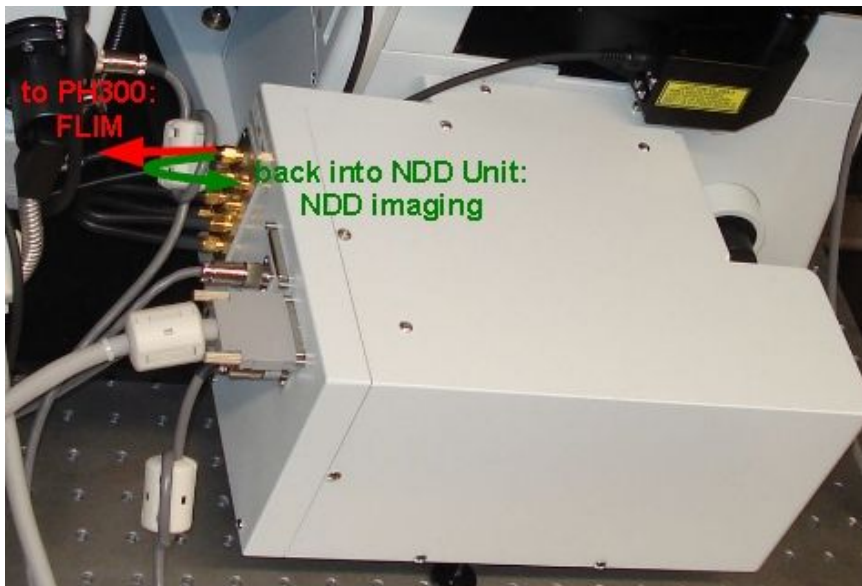
Fig. 2.13: Switching between NDD imaging and FLIM: The signal from the detectors used for FLIM must be guided into the PH300, in general via the PHR800 router for performing FLIM measurements (red). For using the detectors for normal NDD-imaging, the signal has to be fed back into the Olympus NDD-unit via the "back" connector at the NDD unit (green). It is possible to simultaneously use one or more detectors for FLIM and the remaining detectors for NDD-imaging using the FluoView FV1000 software. If your system is equipped with a software-controlled switch, changing the cables is not necessary, but the software of the high frequency switch can be used.
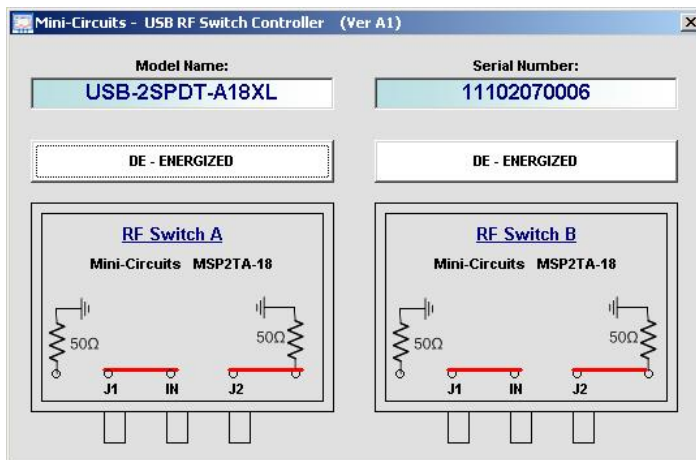
Fig. 2.14: Software of the high frequency switch to direct the NDD-signal either to the FV1000 or to the PicoHarp from FLIM. If such a switch is installed, the DE - ENERGIZED - positttion is directing the signal back to the FluoView. When not using the switch, the light is also automatically redirected into the FV1000. Only if switch is set to the ENERGIZED - position, the detector signal of that channel is directed to the PicoHarp.

Measurement Procedure:

1. In the Olympus software select the NDD-detectors you want to use. Set the Detectors to the Photon Counting mode in the FV1000-software. This automatically sets the driving voltage to 1050mV and ensures that single events are counted (Fig. 2.15). If you want to simultaneously use some channels in the Olympus softwareand record these with the analogue mode, you can also set manually the channels used for FLIM to a HV of 1050V.

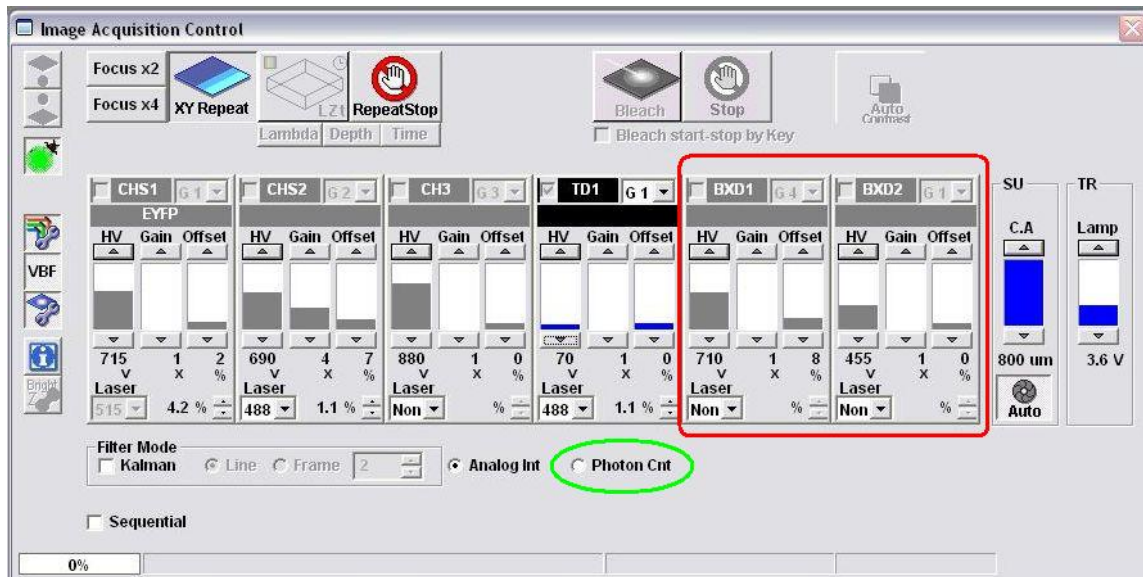**Fig. 2.15:** Olympus FV1000 software GUI for selecting NDD-detectors. Select the NDD detectors (in this case BXD1 & BXD2, indicated in red). Also switch from analogue mode to the photon counting mode (Photon Cnt, indicated in green).

2. Start the LSM-measurement in the SymPhoTime in the "Test" mode.

3. Start the measurement in the Olympus software. Set the laser intensity of the TiSa laser in the FV1000-software to a suited value until the detected count-rate is not too high (below 1Mcps as peak count rate).

4. Stop the measurement in the Olympus software as well as in the PicoQuant software.

5. For acquiring the final image, start the measurement in the SymPhoTime software in the "Record" mode.

6. Start the measurement in the Olympus software again.

7. When enough counts in the FLIM image have been acquired, stop the measurement in the FluoView software.

8. Stop the FLIM measurement in the SymPhoTime software.

2.3.11. Remarks

• For the ad hoc inspection of your samples you can also use the **pulsed lasers** even in conjunction with the **internal FV1000 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.

• **Bidirectional scanning:** With bidirectional scanning the images are often distorted, therefore monodirectional scanning is preferred.

• 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-D or the SEPIA controller. If the intensity is changed at the PDL800 or the SEPIA – the SEPIA II – control is integrated into the SymPhoTime 64 software -, this changes 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 PDL800 or the SEPIA II/SymPhoTime 64 if you want to perform reconvolution fit. The pulse width of the laser and therefore the time response of the instrument (width of the IRF) can be optimized with the 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 image 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 or Hybrid-PMTs, Fluorescence Correlation Spectroscopy (FCS) measurements can be performed. In all point measurements, the fluorescence is recorded at a certain position in the sample and analysed 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. If you are measuring in live cells, take into account that due to the high sensitivity of the SPAD or Hybrid-PMT detectors, cells appearing relatively dark in the manual epiluminescence view should be selected.

•   The **correct dichroic mirror** for your wavelength has been selected in the FV1000 software, and the **correct emission filter**(s) has/have been placed into your detection unit. Also the selected excitation lasers are switched on.

•   **SymPhoTime 64:** Usually, in the "TCSPC Settings"-dropdown menu, T3 should be selected. Only if no SYNC-source is present at your system and/or you are interested in the ns-dynamics, the T2 mode should be used.

•   Selected lasers are switched on. If pulsed lasers should be used, it is assumed that the correct repetition rate is already set (see 2.3.6). If cw-lasers are to be used, the PicoQuant laser driver must still be switched on in order to provide the correct trigger signal for the photon counting board. The lasers should be set to standby in this case. In the oscilloscope window, the SYNC rate should be displayed.

•   If your LCU is equipped with a switchable mirror (optional, Fig. 2.1), set it in the upper position for pulsed and in lower position for cw measurements.

•   If pulsed lasers are used and the pulsed lasers are not controlled via the Olympus software, close the shutter at the microscope in order minimize photobleaching.

Fig. 2.16: mechanical shutter at the Olympus IX81 microscope

- If cw lasers are used, take into consideration that the Olympus **FluoView 1000** by default does **not allow measurements** at one single position for a **long time** without **closing** a **shutter** for their continuous wave (cw) - lasers at determined time intervals. This is called beam blanking and disturbs any FCS measurement, due to sudden short drops in the signal count rate. For **switching Beam Blanking off**, go to "Tools" -> "maintenance" in the the FluoView 1000 software. A window pops up which allows you to switch off beam blanking (see Fig. 2.17) . The user has to be logged in as administrator to access this menu in the FV1000 software.

  Make sure to switch beam blanking later on again since the laser security may be affected and during image acquisition stronger bleaching may occur.
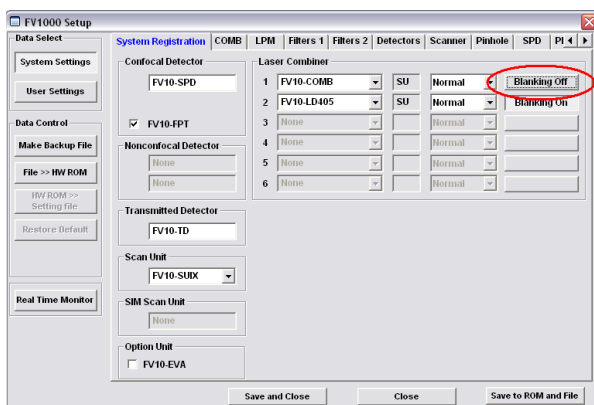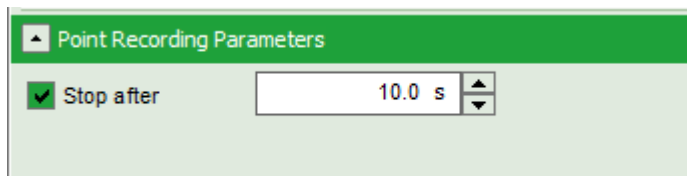

Fig. 2.17: Switching Blanking off in the FV1000 software. In this case, beam blanking for all lasers in the LSM combiner unit has been disabled, as necessary for FCS measurements.

- **SymPhoTime 64:** Enter the measurement time of the final measurement into the "Point Measurement Settings" tab. Check the "Stop after" - tick box.

Measuring with pulsed excitation	**Measuring with cw excitation**
- Select the pulsed laser and select the correct repetition rate	- If measuring with cw lasers, still the SYNC signal of the driver unit must be present. This can be ensured by switching on a pulsed laser at the laser driver (PDL).

2.4.2. Choose the location for the FCS measurement

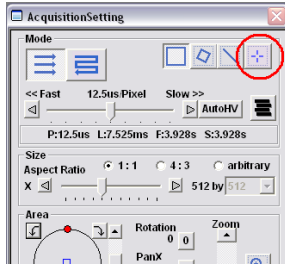1. **FluoView 1000 software:** Enable the point measurement mode (Fig. 2.18).

Fig. 2.18: Point Measurement Mode in the FluoView 1000 software

2. **FluoView 1000 software:** Click the position for the point measurement in your image to select the position where you want to measure (Fig. 2.19). When measuring with pulsed excitation and the pulsed lasers are not controlled by the Olympus software, open the shutter for image acquisition and close afterwards.
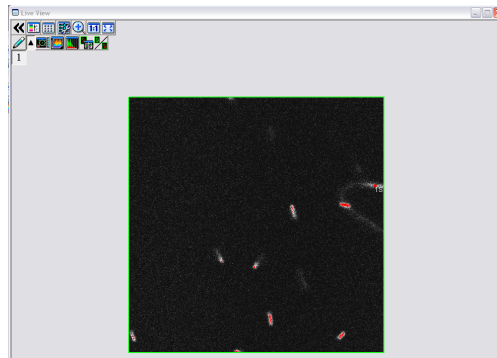

Fig. 2.19: Selecting the position of the point measurement in the Live View window. Clicking at the desired position marks the area by a red spot and moves the scanner to this position.

3. **FluoView 1000 software:** Start the point measurement in the Olympus software. The procedure depends on which laser you want to use and how they are coupled into the system.

Measuring with pulsed excitation

- **Microscope:** If closed, **open** the **shutter** at the Olympus IX81. The laser beam can be seen at the objective (see Fig. 2.16), if the pulsed lasers are not controlled via the Olympus LDIF – unit and registered in the Olympus software. 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. 2.20) or use the SymPhoTime 64 software to adapt the intensity, if the lasers are controlled via a PDL828 "Sepia II" unit.

Measuring with cw excitation

- **FluoView 1000 - software : Start** a **point measurement** at the FluoView 1000 (Fig. 2.22).The detector count rate should increase. Make sure the sample is sufficiently shielded from room light, as this might increase background.

- **FluoView 1000 - software : Adjust** the count rate by de- or increasing the **laser power** in the FluoView 1000 software.

Fig. 2.20: 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.
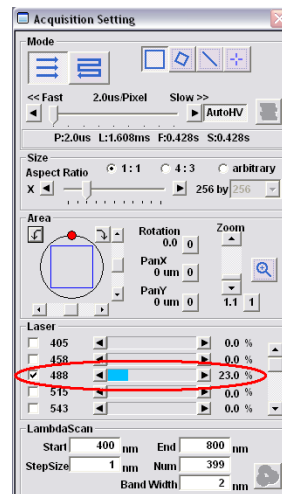

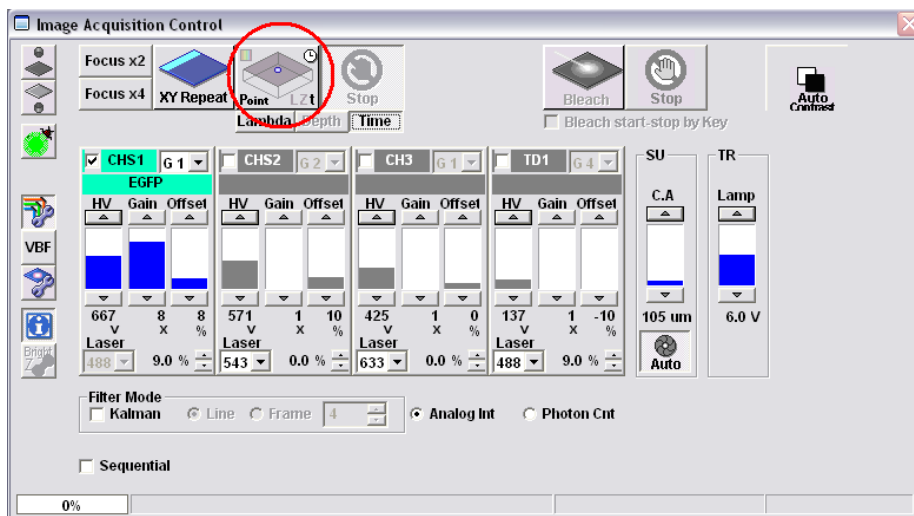**Fig. 2.21:** Regulation of the intensity of cw-lasers in the FluoView 1000 software

Fig. 2.22: Starting a Point measurement in the FluoView 1000 software.

4. **FluoView 1000 software:** The acquisition time is given by the scan speed and the number of iterations. The total measurement time is shown in the information indicated in red in Fig. 2.23.
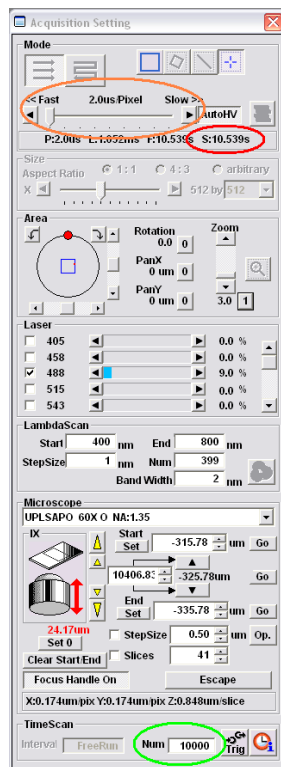

Fig. 2.23: Setting the measurement time in the FV1000 software. The total scanning speed can be seen in the measurement time indicated by the right circle. The scan speed (marked orange) and the number of iterations of the time scan (marked green) influence the total measurement time. The interval of the Time Scan must be set to "FreeRun".

4. **SymPhoTime 64:** Select the main "Test"-tab and open the FCS-view. Start the FCS data acquisition by pressing the "Start" button in the SymPhoTime 64 software.

5. **SymPhoTime 64:** The FCS-curve is calculated during the measurement and displayed in the FCS tab (Fig. 2.24). 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. The system is optimized (for example by adjusting the objective collar) if the molecular brightness is maximal. 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 1 ms.
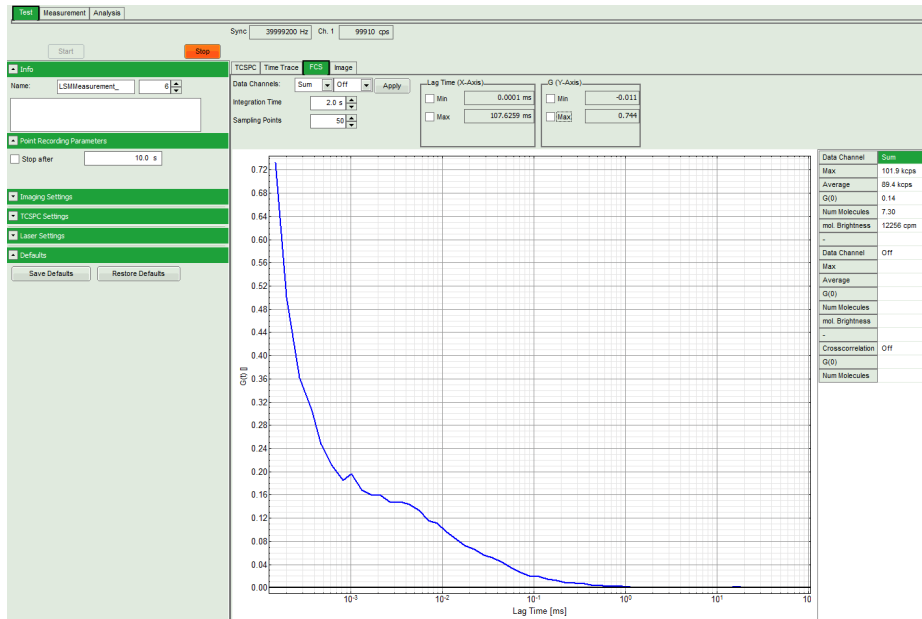
Fig. 2.24: Online monitoring of the of the FCS trace and photon count rate in the SymPhoTime preview window. On the right, the average count rate and the molecular brightness are displayed.

6. **SymPhoTime 64:** At very high count rates, depending on the used computer, a "Fifo overrun" can occur. In this case use a low refresh rate for the online display, decrease the laser power or the concentration.

7. **Optimize** the detected **photon count rate**:

   Similar guidelines as described for FLIM-measurements 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 could 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 - 100 nM.

   • For **analysis of individual molecule transits**, the concentrations need to be sufficiently low, so that individual bursts can be recognized.

   • **FluoView 1000 software:** If the sample is sensitive to photobleaching, select a different area with similar brightness for optimization of the settings

   • **FluoView 1000 software:** Adjust the count rate by de- or increasing the laser power if using cw lasers for FCS.

   • If the measurement is performed with **PicoQuant lasers**, adjust the intensity at the Laser combiner Unit (LCU) or at the scaffold at the single directly coupled laser, respectively, or – if the lasers are attached to a SEPIA II – regulate the intensity in the SymPhoTime 64 - software.
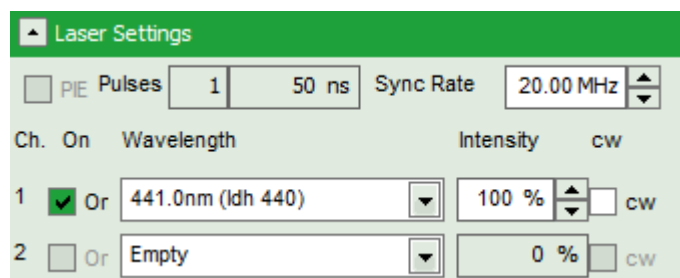
   • **SymPhoTime 64:** For FCS data acquisition, switch to the "Measurement" Tab and check "Point Measurement".

2.4.3. Start point / FCS data acquisition

1. **FluoView 1000 software:** Start a point measurement as described above (Fig. 2.22).

2. **Microscope:** Open the shutter at the Olympus IX81 if closed.

3. **SymPhoTime 64:** Start the FCS data acquisition by pressing the "Start" button in the SymPhoTime 64 – software. Make sure that the "Point" mode is selected in the SymPhoTime 64 -software.
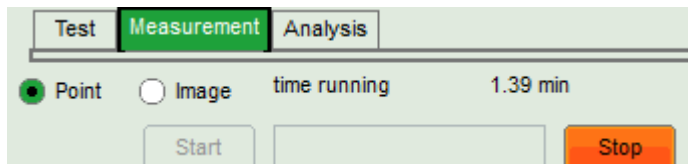
4. **SymPhoTime - software:** The measurement preview is displayed according to the chosen preview settings e.g. as set in Fig. 2.25. The FCS curve is now integrated over time.
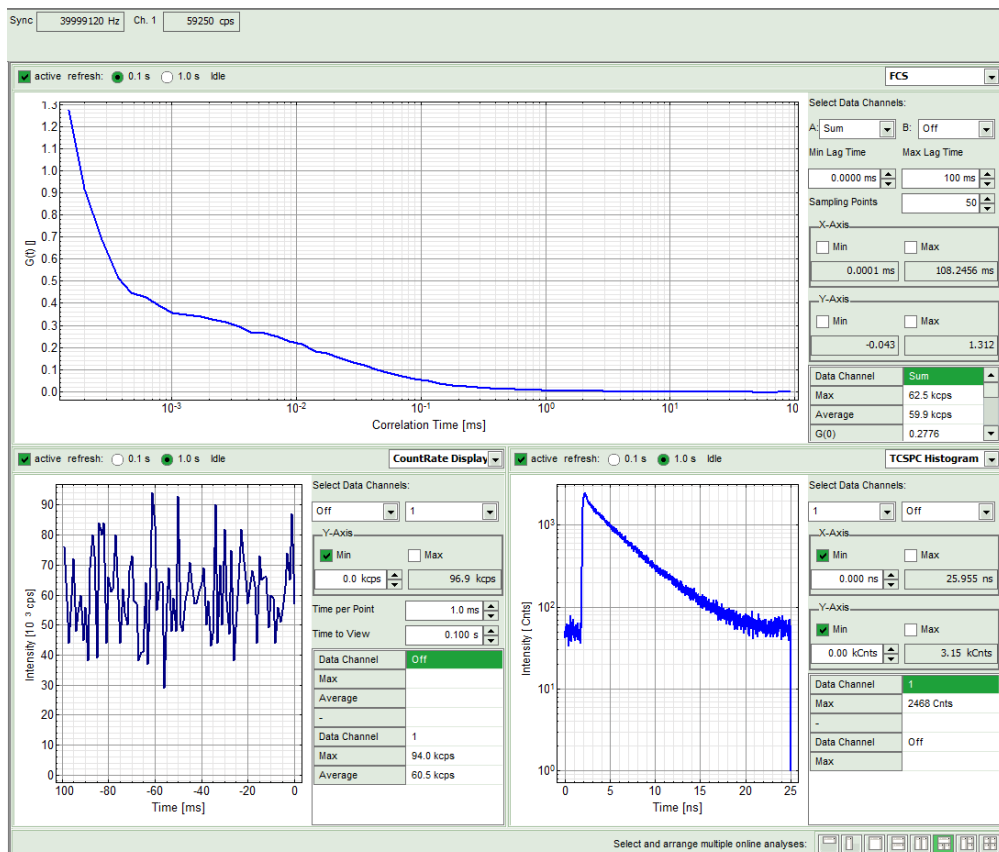

Fig. 2.25: Online Preview of the FCS measurement. In this configuration, the FCS curve is displayed in the upper panel, while the Time Trace is displayed simultaneously on the lower left and the TCSPC histogram on the lower right.

5. **SymPhoTime 64 software:** The data recording stops or when the set measurement time is over or by just manually interrupting the data acquisition.
6. **FluoView 1000 software:** Stop the point measurement, if it has not stopped automatically. If the lasers used are not controlled via the Olympus software, close the shutter at the microscope.

7. **SymPhoTime 64 software:** The recorded FLIM image - file is displayed in the workspace menu on the left and can now be opened for analysis. Therefore select in the "FCS " drop-down-menu of the "Analysis" – tab the FCS or FLCS analysis. The FCS and FLCS analysis scripts are described in detail in the SymPhoTime online help.

8. After the measurement is stopped:

Measuring with pulsed excitation	**Measuring with cw excitation**
• **Microscope: Close** the **shutter** at the Olympus IX81 after data acquisition to minimize photobleaching if the pulsed lasers are not controlled by the Olympus software.	• Nothing has to be done. Pulsed lasers that are controlled via an Olympus LDIF unit and registered in the FV1000-software, they also switch off automatically after the scan.

2.4.4. 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 PMTs** as detectors, as only these detectors are sufficiently sensitive. Using cross - correlation between two detectors allows complete suppression of detector afterpulsing effects on the correlation curve. If just one detector is present and pulsed excitation is used, FLCS can be used in order to suppress detector afterpulsing. This is not necessary for Hybrid PMTs.

- For a solid FCS-analysis, the **count rate** has to be **stable**. Signal decrease due to photobleaching may lead to an increasing FCS correlation amplitude at long lag times.

- The **calculation time** of an FCS measurement depends on the **measurement time** and the detector **count rate**.

3. Troubleshooting

In general, make sure that all components of the Olympus system as well as of the FLIM / FCS upgrade kit are switched on.

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

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

•   Use "restore factory defaults" in the "settings" main menu bar. Apart from the designed folder location of the SymPhoTime, the original settings of the LSM-FLIM / FCS Upgrade are stored on the external USB memory storage delivered with the instrument.

In case the **hardware equipment is changed**, the *.pfs file needs to be changed in order to adapt the software to the respective hardware changes. Please contact PicoQuant in this case. As the system is delivered already configured, it is not recommended to change settings without PicoQuant's supervision.

For storing individual user settings (e.g. incl. previews), the software can be started in the user mode and saved via "Settings" / "Save User Settings as". This creates a *.pus – file, which contains the user specific settings. When changing from one user setting to another, the software needs to restart.

3.2. Instrument is loosing sensitivity

Note:

•   It is recommended to purchase a **power meter** in the lab in order to measure the laser intensities for selected wavelengths. This is especially for FCS measurements of value since the laser power is a crucial parameter for FCS.

•   If you have purchased a LSM FLIM / FCS Upgrade with 1 or 2 SPAD or Hybrid-PMT detectors attached, a good way to check the performance of the instrument is the acquisition of **FCS-traces** of a **dye solution** with a dye that can be effectively excited. At a given laser intensity, filters and objective, and distance to the cover slide-surface, the **molecular brightness** is a stable, characteristic value which can be used to monitor the instrument's performance. It is displayed in the FCS-measurement – preview of the test-mode. Suited dyes for different wavelengths are: ATTO655 for 635 nm, Rhodamine 6G for 532 nm or Fluorescein (in a solution with high pH) and ATTO488 for 485 nm excitation. The molecular brightness is the **count rate** of the **detectors divided** by the **number** of **molecules** present in the detection volume. It is displayed in the FCS preview. In general, a 10 nM solution is used. For a day to day comparison, use the same laser power, best controlled with a power meter. A more detailed description for performance of FCS measurements is given in the point-measurement section.

•   If a performance drop is noticed, first the origin of the performance drop needs so be found. A drop in system performance may be caused by:

1) Correction collar setting

   •   For FCS measurements in biological sample, an apochromatic corrected water immersion objective with an NA of 1.2 has to be employed. This objective leads to only minimal optical aberrations inside the sample. Optimize the correction collar in order to achieve the highest fluorescence count rate. Use a high concentrated dye sample (approx. 1 µM). Set the laser intensity to observe approx. 500 kcps. 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.

2) Decreasing Laser Performance

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

3) Decreasing Detection Sensitivity

- Misalignment of the LSM itself. Contact your service representative.

- Misalignment of the fiber out-coupler for the fluorescence light. Contact PicoQuant.

- Misalignment of the detector(s): Refer to your detector manual

3.3. 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.

| Sync | 39999120 Hz | Ch. 1 | 58330 cps |
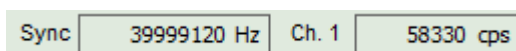
When starting a TCSPC measurement in the test mode, dark counts should be displayed (see Fig. 2.10). A peak must not necessarily appear, but at least background noise should be displayed. If the repetition rate is 0, check whether at the PDL800D the SYNC is set to internal or, if you use a SEPIA II, whether the pulse settings in the "Laser Settings" dropdown menu are correct.

- **Optional:** Is the **handle** from the **LCU** correctly lifted (see section: how to take a FLIM image)?

- Is the shutter at the FV 1000 in the open position?

If yes:

- Do you **see dark counts** when activating the **Time trace Tab** in the "Preview" window? If not, check whether the detectors are still on (the DSN102 (if applicable) shuts down a detector automatically if the count rate exceeds a limiting count rate).

- Check, whether **correct emission filters** are placed. If you have a 2 - Detector unit, make sure that the beam splitter is set to the correct position.

- When all detectors are on and background counts are displayed in the oscillator window, place a **fluorescent sample** on the Olympus microscope, set all settings in the Olympus 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 Olympus FluoView FV1000 and the LSM FLIM / FCS Upgrade. Also try to start the FluoView FV1000 and the PicoQuant system new. If no marker signals are displayed in spite of a present connection, contact PicoQuant. If you don't have the Point Measurement Mode included in your license, check cable connnections and send the file to PicoQuant.
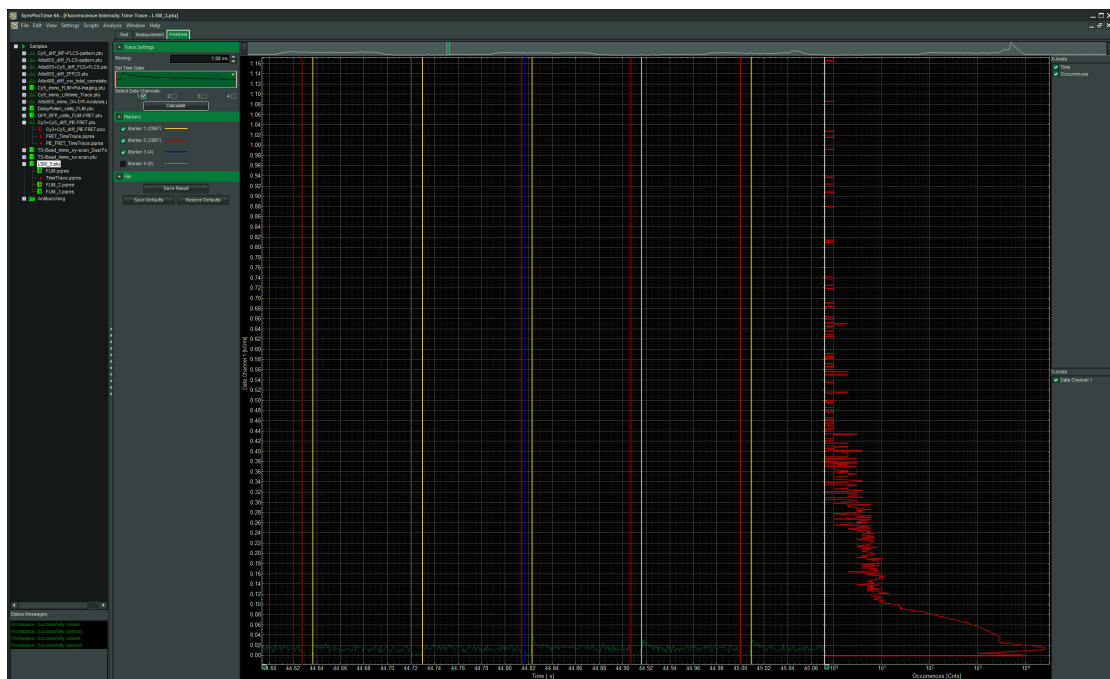

Fig. 3.1: The Time Trace analysis, where the line and frame - markers can be displayed.

3.4. 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 at wrong position:

    => sensitivity will be poor in FCS and in imaging applications.

- To align the pinhole, place a bright fluorescent sample on the microscope (e.g. a dye solution) and focus into the solution.
- Adjust the pinhole in the FluoView FV1000 software by moving in xy and maximizing the countrate on the PicoQuant detector. In the Maintenance settings, this position can also be saved.

4. Recommended literature

Publications related to the LSM FLIM / FCS upgrade 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)

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Further literature can be found in the help menu of the SymPhoTime software.

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

Buschmann V., Bleckmann A., Bülter A., Krämer B., Nikolaus J., Schwarzer R., Korte T., Ziomkowska J., Herrmann A., Koberling F.: *Polarization Extention Unit for LSM Upgrade Kits*

Buschmann V., Krämer B., König M., Koberling F., Orthaus S., Bülter A., Erdmann R.: *Compact FLIM NDD Upgrade Kit for Olympus FluoView FV1000MPE*

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

Krämer B., Buschmann V., Koberling F.: *Region of Interest (ROI) Scanning and Bleaching using Pulsed Lasers with the FluoView FV1000*

Krämer B., Buschmann V., Koberling F.: *Coupling of pulsed laser sources into the Olympus FluoView FV300 / FV1000 via the IR port using a polarization splitter*

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*

Orthaus S., Buschmann V., Bülter A., Fore S., König M., Erdmann R.: *Quantitative in vivo imaging of molecular distances using FLIM-FRET*

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 FLIM / FCS Upgrade section of `http://www.picoquant.com/_systems.htm`.

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.

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

In any case, we would like to offer you our complete support. Please do not hesitate to contact Olympus or PicoQuant if you would like assistance with your system.

Of course, we also appreciate good news: If you have obtained exciting results with the LSM FLIM / FCS Upgrade or published scientific papers, we would also like to know! Please send us an e-mail to **info@picoquant.com** containing the appropriate citation. Gain additional publicity! PicoQuant maintains a database of publications mentioning PicoQuant devices and/or written by us. It can be found at our website at http://www.picoquant.com/_scientific.htm. It is a valuable source if you want to know which laboratories are using PicoQuant products or how broad the field of various applications is.

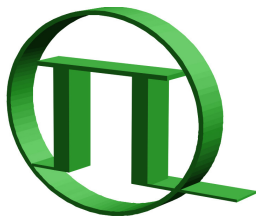Thank you very much in advance for your kind cooperation!

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