LSM Upgrade Kit

Compact Lifetime and FCS Upgrade Kit for **Scientifica Multiphoton** Laser Scanning Microscopes





User's Hardware Manual and Technical Data

Version 03

Table of Contents

1.	Introduction	3
2.	Operation	4
	2.1. Safety Instructions	4
	2.2. Starting Hardware and Software Equipment	4
	2.3. FLIM Data Acquisition	5
	2.3.1. Starting Point	5
	2.3.2. Optimize the detected photon count rate	7
	2.3.3. Start FLIM data acquisition	8
	2.3.4. Resulting raw data file and documentation	.10
	2.3.5. Measure an instrument response function (IRF)	.11
	2.3.6. Adjusting the threshold level for laser pulse detection	.12
	2.3.7. Remarks	. 13
3.	Troubleshooting	. 14
	3.1. Hardware configuration	. 14
	3.2. Instrument is loosing sensitivity	. 15
	3.3. No image is displayed during measurement	. 15
4.	Recommended literature	. 18
5.	Abbreviations	. 19
6.	Support and Warranty	. 20

1. Introduction

This manual describes the basic procedures to acquire NDD FLIM - images using the LSM Upgrade Kit in combination with a LSM.

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

• **Detection Unit - Manual:** This document contains information about the detector and how to change filters. The PMA Hybrid detectors are equipped with an automatic overload shutter to prevent too high detection count rates which could damage the detector. Overload is signalled with a beep sound. If an overload was detected, the shutter in front of the detector closes. After some seconds, the shutter opens automatically again. Please make sure that the cause for the over-illumination is eliminated as fast as possible.

Excessive light will reduce the sensitivity of the PMA Hybrid detector over time.





Fig. 1.1: Detection Unit for LSM NDD FLIM Upgrades: The Single (left) or Multi channel PMA detection unit (right).

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



Fig. 1.2: MultiHarp 150 and TimeHarp 260

- **Software Manual (SymPhoTime Manual):** Here you find all information about the software installation. The SymPhoTime 64 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.
- **System Specifications:** The System Specifications contain specific information about your individual LSM FLIM Upgrade, including a description of the included parts, filter handling and a cable plan, which helps to restore the configuration after disassembly.

2. Operation

2.1. Safety Instructions



LASER Warning!

Lasers for two photon excitation deliver infra-red emission which is in most cases not visible for the eye. These lasers emit light of laser class 4 / IV. Laser class 4 / IV requires to wear special laser safety glasses. Do not remove any items of the NDD adapter before switching the microscope and laser completely off. The installation room of the LSM Upgrade kit must be labeled as laser area. For class 4 / IV lasers, a laser safety officer has to be announced to meet the laser safety regulations and institutional and national guidelines and legislation.

Lasers can be hazardous and have unique safety considerations. Permanent eye injury and blindness is possible if lasers are used incorrectly. Pay close attention to each safety REMARK and WARNING statement in the user manual. Read all instructions carefully BEFORE operating this device.

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 (PMA Hybrid) as much as possible, particularly from excessive light intensities, e.g. room light, the microscope illumination lamp, unattenuated backscattered excitation, etc.

2.2. Starting Hardware and Software Equipment

To **start** the LSM itself, its detectors and lasers please refer to your multiphoton microscope 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 multiphoton microscope itself.

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

- 1. Start the external **MultiHarp 150** TCSPC device (if available)
- 2. Start the **PC**.
- 3. Start the **SymPhoTime software**.
- 4. **Open** an already existing **workspace** or create a new one.
- 5. Make sure that the correct **fluorescence filters** are set in the corresponding filter holder position of the external PicoQuant detector.
- 6. Make sure that no light reaching the detector(s) (room light off? Shielding closed?) and power them on. Prior to data acquisition, the detector should be operated for 5 minutes to stabilize.
- 7. If you want to work with user specific settings, load your User Configuration.

The FLIM Upgrade is now ready to use.

2.3. FLIM Data Acquisition

Scope:

Acquire a FLIM image using the Scientifica multiphoton microscope scan head with appropriate scanning and dichroic settings and external PicoQuant TCSPC NDD detectors.

2.3.1. Starting Point

- 1. **Open SymPhoTime Software** and select a workspace (File \rightarrow open or new workspace).
- 2. **NDD detection is very background light sensitive.** In general you need a suitable blackout box around the microscope and/or keep the room dark.
- 3. Make sure that in the NDD detection unit the right filters are inserted.
- 4. ScanImage Software: Start the software by entering "scanimage" in the Matlab edit field.

	📣 MAT	LAB R2	2015b						
ľ	HC	DME		PLOTS	APP	s			
	New Script	New	Open	Compare	s 🔛 Import Data	Save Workspace	New Var	iable riable 👻 rkspace 👻	Analyze Co
ļ			FILE				ARIABLE		CODE
•	+ +	1	2 🔋	► C: ► Us	ers 🕨 Tos	caDev1 🕨 Do	cuments 🕨 M	iatlab 🕨	
Γ	Currer	nt Fold	er					Commar	d Window
	Name △					ATLAB? See resourc			
	E L	Add-Or ConfigE file_000 file_000	ns Data D01.tif D02.tif				×	fx >> s	canimage
ľ				5elect a file to	view details	;	-		

Klick on continue after selecting the correct user settings file:

承 ScanImage Configuration	_ 🗆 🗵			
ScanImage Configuration Machine Data File C:\Users\ToscaDev1\Documents\MATLAB\machine_data_file RGG - GG - Browse New Modify User Settings File C:\Users\ToscaDev1\Documents\alex.usr Browse Browse				
C:\Users\ToscaDev1\Documents\MATLAB\machine_data_file RGG -	GG -			
Browse New Modify				
User Settings File				
C:\Users\ToscaDev1\Documents\alex.usr				
Browse				
Continue Cancel				

Select scan parameters like image size, Galvo / Resonant imaging in the CONFIGURATION window. For the beginning, 512 pixels/line, galvoimaging and monodirectional scan are recommended. For bidirectional scan the scan phase has to be optimized for the removing of bidirectional scanning artifacts.

In case of resonant scan **and** bidirectional scanning in addition the beam lead time in the POWER CONTROLS window has to be optimized, so that the bidirectional scanning artifacts vanish in the FLIMimage. However, the opening of the Pockels cell, which has some time-delay, will be still be visible at the left side of the resonant scan FLIM image. In addition vertical lines can become visible at high photon count numbers in the FLIM image due to the short pixel dwell times.

The settings for scan phase and beam lead time have to be reoptimized after changing the image size / zoom setting.

LSM FLIM Upgrade Kit Hardware Manual

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Active Imaging System: Galvolm	aging 🔄 Alignment	
Scan Type: Frame Scan 👱	Bidirectional Scan	
Pixels / Line 512 -	Scan Phase (us)	
Lines / Frame 512	0 Auto Adjust	
Pix=Lin 🔽 Square Pix		
Rate (Hz) 1.07 Measure	Pixel Dwell Time (ns)	
Advanced Timing Controls	3200.0	Beam Lead Time: 1.5 us 🔽 Blank Flyback

Use the MAIN CONTROLS window to select zoom and rotation and enter the filename for the mesurement. A test measurement is started with FOCUS while a measurement, which is saved to disk, is started with GRAB.

MAIN CONTROLS		🛃 Statu 📃 🗖 🕨
File Settings View		
User Settings alex Load Save	Rotate 0 0 Shift 0	Main Shutter
Configuration standard Load Save	Zoom Shift Slow 0	Photostim shutter
1 2 3 4 5 6 Fast Ctg		Imaging PC: 0%
Frames Done 6 of 20 0 Sices Done 0 of 18 Interval	Scan Angle Multiplier Fast 1 Slow 1 LS POINT	Line Clock: 0%
Acqs Done 0 of 1 10	Deta Logging	photostim: 0%
Enable MROI Imaging Enable Integration		
Edit ROIs Photostim Integration	# Frames/File Inf # Avg 4	I Stay on top
·····	Lock to # Frames	Raise Windows
		< IDLE
FOCUS GRAD LOOP	Ext Triggering Config >>	

The laser settings are controlled with the respective software from the laser company. The shutter status can be seen in the STATUS window.

5. Using the PicoQuant Control window FLIM measurements can be enabled. By checking "Enable FLIM" every measurement, FOCUS, GRAB and LOOP is automatically displayed in SymPhoTime. Only if "Save" is checked sime in the MAIN CONTROLS window the measurement will be saved in SymphoTime (save is however not active for FOCUS).

A group name can be specified. This creates a sub-folder in the workspace of SymphoTime where all subsequent measurements are stored.

The Comment window allows to add comments which will be visible in the SymPhoTime software by double-clicking on the recorded *.ptu file.

PICOQUANT CONTI	ROL		\times
Enable FLIM	meas	urement running	
Group			
Name z-stack			
Comment			
cell sample			^
			~

- 6. Choose the correct region where you want to perform a FLIM measurement. For the ad hoc inspection make sure that you are using a suitable excitation wavelength, a useful microscope objective and suitable detection filters. Finally stop the scanning process with ABORT.
- 7. All components (laser, detectors, software) of the PicoQuant upgrade kit are already running. For details about powering on see chapter 2.2.

- 8. **SymPhoTime 64 software:** The SymPhoTime 64 allows configuring the view of the acquisition window. Thus the user can preconfigure the online analysis he wants to see during acquisition, e.g. images (also of the different spectral channels if more than one detector is present), a time trace, a decay (TCSPC) histogram. Several online analyses can be displayed simultaneously. All settings can be saved by creating an **user profile**.
- 9. **SymPhoTime 64 software:** A workspace has to be loaded or created. The workspace defines where the data acquired should be stored to. In the workspace, all acquisitions and analysis steps can be opened by a double click on the file name.

2.3.2. Optimize 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 mono-directional scanning).

Imaging Settings	
Image pixels 512	512 🔽 square
monodirectional	Resolution 1.000 µm/px 📥
bidirectional	

2. SymPhoTime 64 software: Press the Start-button.



2. Scanimage software: Start the scanning process by klicking on FOCUS.



3. SymPhoTime 64 software:

The measurement preview is displayed, together with a time trace (Fig. 2.1). Make sure that the Peak Count Rate (marked in red) is below 8 Mcps by adjusting the laser intensity in ScanImage. The Peak Count Rate is determined from the maximum in the time trace. The "Time trace binning" should be set to 0.01 ms. Here you can watch the changes of the count rate over time. This indication is only valid for gavo imaging.

4. A red line additionally indicates a threshold of 10% of the repetition rate. TCSPC unit TimeHarp 260 Pico: The intensity should always remain below this value. By following this rule the so called "pile up" effect is reduced. Pile up can lead to a decrease of the determined lifetime. By staying below 8 Mcps the lifetime reduction will be less than 5%. If a MultiHarp 150 with rapidFLIM technology is used, the count rate can also be above this limit.



Fig. 2.1: Online monitoring of the of the photon count rate in the SymPhoTime 64 preview window. On the left, a FLIM image is displayed. The intensity time trace is shown below. An intensity of 10% of the repetition frequency is indicated by the red line in the graph.

- The peak count rate is also indicated on the right (red circle).
- 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
- 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.

2.3.3. 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 mono-directional scanning and the image resolution (in μ m/pixel). Note that the entered dimensions become a part of the raw data file and therefore cannot be

changed after image recording.

<u> </u>		
Imaging Settings		
Image pixels 512	512 🔽 s	quare
monodirectional	Resolution	1.000 µm/px
bidirectional		

2. **SymPhoTime 64 software:** Make sure that the "Image" Measurement mode is selected and press the "Start" button.

Test	t	Measurement	Analysis	
O Poin	t	🖲 Image		
		Start		Stop

3. **ScanImage software**: Enter the number of frames xx you want to scan in "Frames Done" ... of xx. Start the scanning process by clicking on GRAB.

MAIN CONTROLS		X
File Settings View		
User Settings alex	Load Save	Rotate 0 0 Shift 0
Configuration standard	Load Save	200m Shift Slow 0
1 2 3 4 5 6	Fast Cfg	
Frames Done 6 of 20	Timer	Scan Angle Multiplier
Slices Done 0 of 18	Interval	Fast 1 Slow 1 LS POINT
Acqs Done 0 Of 1	10	Data Logging
Imain contracts Imain contracts File Settings alex Load Save Configuration standard Load Save 1 2 3 4 5 6 Fast Cfg Frames Done 6 of 20 0 reterval Frames Done 6 of 70 0 reterval Acquistion 7 to 10 reterval Acquistion # 1 + Image Save Enable MROI Imaging Enable Integration Edt ROIs Photostim idle Loop 7 to 40 reterval FOCUS GRAB LOOP		
I Enable MROI Imaging I Enable	e Integration	Acquisition # 1 + Save
Edit ROIs Photostim	Integration	# Frames/File Inf # Avg 1
idle		Lock to # Frames
FOCUS GRAB L	.00P	Ext Triggering Config >>

4. SymPhoTime 64 software: During LSM data recording the chosen online analyses are displayed, e.g. one or – in case of two detectors - two 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 analyze the data separately frame by frame (using Multi Frame FLIM Analysis).

Sync 19999600 Hz Ch. 1 192890 cps		
Zactive refresh: 0.1 s 💿 1.0 s Idle	Fa	st FLIM 💌
	Select Data Channelle S 1 2 2 3 3 4 Events[Chts] Min 1 4 Average life time[ns	
	Max Photons	421 Cnts
	Max Countrate	8419.7 kcps
	Avg Countrate	949.1 kcps

Fig. 2.2: 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.

- 5. **ScanImage software:** The **LSM scanning process stops** either after having scanned the pre set number of frames or by manually interrupting the scan.
- 6. SymPhoTime 64 software: The data recording stops by just manually interrupting the data

acquisition (press "stop").

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.

2.3.4. Resulting raw data file and documentation

- SymPhoTime 64 software: The measurement result is a .ptu-raw data file, containing for every detected photon the maximum available information (temporal, spatial, detection channel information), together with the online analysis files ".pqres". If e.g. a online Fast FLIM image is to be processed, highlight the raw data (.ptu) file and open this file with e.g. the FLIM analysis option in the "Analysis" tab. If you double-click on the "OnlineFastFLIM_....pqres-file", the FLIM analysis is directly opened.
- Every raw date file is stored together with the actual settings. It contains all actual measurement parameters known by the SymPhoTime software. To access, double click on the raw (".ptu")-file. The raw data file contains all actual measurement parameters known by the SymPhoTime software; individual information (measurement type, sample, etc.) can be entered **before** the measurement via the "Info" drop down menu.

🔺 Info		
Name:	LSMMeasurement_ 6	

 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 ("Create Comment").

File	Edit	View	Settings	Scripts	An
	Оре	n Work	space		
	Reop	pen			F
	New	Works	space		
	Clos	e Work	space		
	Impo	ort Old	TTTR File		
	Dele	te Sele	cted		
	Crea	te Con	nment		
	Shov	w Com	ment		

If the size of one pixel in the image is known, it can be to be entered as "Resolution" in the "Imaging Settings" of the **SymPhoTime - software** before the start of the measurement .

Imaging Settings		
Image pixels 512	512 v square	
monodirectional	Resolution 1.000 µm/px	
 bidirectional 		

Unfortunately, this value can not be changed after image acquisition.

SymPhoTime 64 software: Specific recorded frames can be selected for analysis in the FLIM analysis. Highlight the raw data file, go to the main "Analysis" tab, and select the "FLIM"-analysis from the "Imaging" drop down menu. The FLIM analysis window pops up. The frames chosen for analysis can be entered in the field "Frame" (from "First Frame" up to "Last Frame"). More possibilities of frame-wise FLIM display can be found in the "Multi-Frame Analysis".

Test Measurement	Analysis
Region of Interest	
Frame	
Select Frames to loa	ad:
First Frame 2	Last Frame 3
FLIM	
Binning:	1 Points 🔺
Set Time Gate:	
· · · · · · · · · · · · · · · · · · ·	
Select Data Channels: 1:⊠	2: 3: 4:
Threshold	50 Cnts
Calculate FastFLIM	FLIM Fit
Histogram	
File	
s	ave Result
Save Defaults	Restore Defaults

Fig. 2.3: Changing the selection of frame numbers for analysis in the SymPhoTime 64 Software.

2.3.5. Measure an instrument response function (IRF)

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

- 1. Place a sample with an **negligible short lifetime** e.g. Erythrosin B or Fluorescein, 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. Potassium iodide is a strong quencher and reduces the fluorescence lifetime to a few picoseconds. See e.g. <u>Applied Spectroscopy, Vol.63, p.0363-0368</u> (2009). Also second harmonic generation by urea crystals 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. Set the **ScanImage** settings in the same way as for a **FLIM measurement**. Set the Zoom setting to 10 in order to scan only a small region. Start the scanning process with FOCUS.
- 3. **SymPhoTime 64 software:** You can now check the decay behavior of your sample using the TCSPC preview of the "Test" tab. Press the "Start" button and activate the TCSPC Histogram-tab, monitor the fluorescence decay as shown in Fig. 2.4. In the "TCSPC settings" drop down menu, also the time width per channel can be set. Adjust the count rate to approximately 50 kCounts/s.



Fig. 2.4: TCSPC-preview window. 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.

4. SymPhoTime 64 software: Stop the test measurement by pressing "Stop".

5. SymPhoTime 64 software:

Switch to the "Measurement" tab and start the data acquisition pressing "start".

Test	Measurement	Analysis
O Point	🖲 Image	
	Start	Stop

- 6. When the decay histogram in the TCSPC preview has reached 10000 counts in the peak channel, stop the image acquisition in the SymPhoTime and ScanImage 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.
- 7. Stop scanning and replace the IRF solution with your sample again.

2.3.6. Adjusting the threshold level for laser pulse detection

In general, for the detection of the laser pulse sequence of a femtosecond laser, the internal trigger diode of the laser is needed. Normally the system is already set to detect the trigger signal (SYNC) by PicoQuant. If the laser does not have an internal photodiode for pulse sequence detection, an external photodiode (like the TDA from PQ) is used. The photodiodes are not always sensitive to all wavelengths, especially up to 1064 nm. The trigger settings of the laser pulse can be adapted in the SymPhoTime 64 software. This CFD level of the SYNC input can be set in the TCSPC drop down menu.

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Fig. 2.5: Settings of the input trigger levels of the TCSPC Unit. On the left side, the laser synchronization threshold settings are displayed (CFD Level), on the right side, the detector settings. Zero Cross can always stay at 10 mV. Good starting values for all CFD levels for detection and sync are -100mV (in case of two TCSPC units: -50mV).

2.3.7. Remarks

• **Bidirectional scanning:** With bidirectional scanning FLIM images can be distorted if the scanner forward scan is not exactly adjusted to the backward scan. Therefore mono-directional scanning is preferred. Adjustment can be performed with the "Scan Phase" setting (GalvoImaging). In case of resonance scanning, careful manual adjustment of the Beam Lead Time is required.

Active Imaging System: Galvolm	aging 📩 Alignment	
Scan Type: Frame Scan 💌	Bidirectional Scan	
Pixels / Line 512 -	Scan Phase (us)	
Lines / Frame 512	0 Auto Adjust	
Pix=Lin 🔽 Square Pix		
Rate (Hz) 1.07 Measure	Pixel Dwell Time (ns)	POWER CONTROLS
Advanced Timing Controls	3200.0	Beam Lead Time: 1.5 us 🔽 Blank Flyback

 The SymPhoTime software also allows to perform post-acquisition pixel binning for optimization of the best compromise between lifetime accuracy and image resolution.

3. Troubleshooting

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

3.1. Hardware configuration

The actual **hardware configuration** of your LSM FLIM 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** at SymPhoTime software. 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 Upgrade are stored on the external USB memory storage delivered with the instrument.

In the ScanImage software, please make sure that the "Line Clock" entry is set to a Pwr (power) level of 100.

	-		
		Pwr	P/z Adj
Imaging PC	V	1	
Line Clock		100	
photostim		1	

This setting controls the "Line Clock" which is necessary for the FLIM image record.

If the PICOQUANT CONTROL window does not appear or the connection to SymPhoTime is not working, please check the "Machine Data File" by clicking on "Modify" at the startup of ScanImage.



At "General ScanImage Settings" the plugin "PicoQuant Remote Controller" should be activated:

	Plugins and Custom Components		
	Plugin/Component Name	lugin/Component Name	
	PicoQuant Remote Controller	~	
KoanImage Machine Configuration Editor	Thorlabs ECU 1 Thorlabs B-Scope 2 Analog PMT Controller Bruker Resonant Controller HooQuant Remote Controller		
General ScanImage Settings			
5 5	Custom		

At "PicoQuant Remote Controller" the host ip should be set to: "192.163.43.3" in case SymPhoTime is running on a different PC as ScanImage "local" in case SymphoTime is running on the same PC as ScanImage

PicoQuant Remote Controller	Delete	Variable Name	Valu	ie
	host		192.168.43.3	IP Address of listening PicoQuant Remote Interface

In case the **PicoQuant hardware equipment is changed**, the SymPhoTime *.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.

Individual user settings (e.g. incl. Previews) can be saved via "Settings" / "Save User Settings as". This creates a *.pus – file, which contains the user specific settings. When changing from one user setting to another, the software automatically restarts.

3.2. Instrument is loosing sensitivity

Decreasing Detection Sensitivity

 The PMA Hybrid detector can loose sensitivity with time, especially when exposed to high light conditions (e.g. room light) over longer time. This is a natural aging process which is induced by electron sputtering of the PMA Hybrid detector cathode. By this process the cathode gets thinner thus leading to a reduced sensitivity.

3.3. No image is displayed during measurement

• Is the pulse repetition rate correct? The Pulse frequency is displayed in a control window over the preview windows. It must display around 80000000 Hz (80 MHz), depending on the laser frequency.

When starting a TCSPC measurement in the test mode, dark counts should be displayed (see Fig. 2.4). A peak must not necessarily appear, but at least background noise should be displayed. If the repetition rate is 0, check whether the detector is switched on and the filter(s) inserted.

- Check, whether **correct emission filters** are correctly placed. If you have a 2 Detector unit, make sure that the beam splitter is set in its correct position.
- When all detectors are on and background counts are displayed, place a **fluorescent sample** on the Scientifica microscope, set all settings in the ScanImage 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.
- · Make sure that the emission light of the probe reaches the detector
- For the correct image synchronization, a line marker as to be generated by the ScanImage software. Therefore, an additional "Power Modulation Beam" has to be added in ScanImage. To control the settings, klick "File" and "Machine Configuration.."

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承 ScanImage Machine Configuration Edito	1.0.9.9 Idle
General ScanImage Settings	Power Modulation (Beams) Configure DAQs for beam power modulation. Each scanner can be assigned one beam DAQ. Multiple scanners can be assign the same beam DAQ, but cannot be used simultaneously in that case. To add a beam DAQ, select the desired DAQ and clicl
Shutter Configuration	the ">>" button. Then configure one or more beam channels below.
Power Modulation (Beams)	Available DAQ Devices Selected Beam DAQ Devices
Scanner Settings (ResScanner)	PXII Slot3 1: PXII Slot6 2: PXII Slot5
Scanner Settings (Galvolmaging)	¥ <<
Scanner Settings (GalvoPhotostim)	Beam DAQ 1: PXI1Slot6
Stage Controllers (Motors)	Configure a channel for each beam power modulation device (pockels cell, AOM, ect.) to be controlled by this beam DAQ. Note, the offset can be measured later.
FastZ Actuator Setup	Control Display Name Max Cmd Calibration Calibration Calibration Delete AO Channel Voltage AI Channel Offset Voltage Signal Source Signal Source
	A00 Imaging PC 1.5000 None Toransmitted Light X
Photostimulation	AO1 Line Clock 5 None 0 Transmitted Light X

Control AO Channel AO1 with the Display Name "Line Clock" should be entered with a Max Cmd Voltage of 5 V.

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 Scientifica ScanImage multiphoton microscope and the PicoQuant TCSPC unit. Also try to start the ScanImage multiphoton microscope and the PicoQuant. If you don't have the Point Measurement Mode included in your license, check cable connections and send the captured file to PicoQuant.



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

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• In case cables are disconnected or it is not clear how to connect them please contact Scienitifca or PicoQuant.

4. Recommended literature

Publications related to the LSM FLIM 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)

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)

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

Please have also a look to the technical and application notes on http://www.picoquant.com/

5. Abbreviations

British Naval Connector or Bayonet Nut Connector or Bayonet Neill Concelman
Charge-Coupled Device
Constant Fraction Discriminator
Counts per Second
Continuous wave (not pulsed)
Fluorescence Correlation Spectroscopy
First In, First Out (buffer type)
Fluorescence Lifetime Imaging
Förster Resonance Energy Transfer
Full-Width at Half-Maximum
Input-Output
Instrument Response Function
Laser Combining Unit
Light Emitting Diode
Laser Scanning Microscope
Multichannel Scaling
Optical Density
Personal Computer
Peripheral Component Interface
Pulsed Interleaved Excitation
Photomultiplier Tube
Red-Green-Blue (colour scheme)
Region of Interest
SubMiniature version A (connector type)
Single Molecule Detection
Single Photon Avalanche Diode
Synchronization (signal)
Time-Correlated Single Photon Counting
Transistor-Transistor Logic
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 \rightarrow Get Support* in the SymPhoTime 64 Software. Store the generated text as a text file and mail it to PicoQuant. Your feedback will help us to improve the product and documentation.

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

Of course, we also appreciate good news: If you have obtained exciting results with the LSM FLIM 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 <u>https://www.picoquant.com/scientific</u>. 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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