

## Combined Fluorescence and Phosphorescence Lifetime Imaging (FLIM / PLIM) with the Zeiss LSM 710 NLO Microscopes

The PLIM technique described here is based on modulating the titanium-sapphire laser of a multiphoton microscope by a signal synchronous with the pixel clock of the scanner, and recording the fluorescence and phosphorescence signals by multi-dimensional TCSPC [1]. Fluorescence is recorded during the on-phase of the laser, phosphorescence during the off-phase. Laser modulation is achieved by controlling the the AOM of the laser scanning microscope by a signal generated in the TCSPC system.

This document contains instructions of how to set the parameters of the bh SPC-150 or SPC-830 FLIM systems for combined fluorescence (FLIM) and phosphorescence lifetime imaging (PLIM). It should be considered a supplement to the bh TCSPC Handbook [1] and the bh Handbook of the FLIM systems for the Zeiss LSM 510 and 710 laser scanning microscopes [2].

### Principle and System Architecture

The general principle of combined fluorescence and phosphorescence lifetime imaging is shown in Fig. 1. A high-frequency-pulsed laser, in this case the Ti:Sa laser of the LSM 710 NLO microscope, is used for excitation. The laser is turned on for a short period of time,  $T_{on}$ , at the beginning of each pixel [1, 3]. For the rest of the pixel time the laser is turned off. Within the on-time the laser excites fluorescence, and builds up phosphorescence. Within the rest of the pixel dwell time,  $T_{off}$ , pure phosphorescence is obtained. Photon times are determined both with respect to the laser pulses and with respect to the modulation pulse. The times from the laser pulse,  $t$ , are used to build up the fluorescence decay. The phosphorescence decay is built up from times from the modulation pulse,  $T-T_0$ .

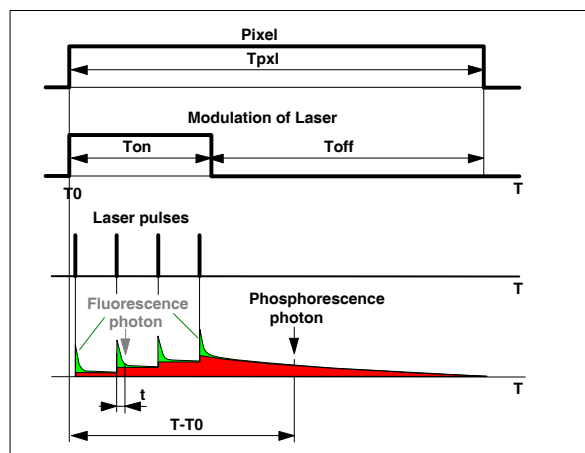


Fig. 1: Principle of Microsecond FLIM

Because the phosphorescence is built up over a large number of excitation pulses neither an extremely high laser peak power is required, nor an extremely high fluorescence peak intensity is generated. Saturation effects, both in the sample and in the detector, are therefore avoided. Moiré effects in the image do not occur because the laser-on/off modulation is synchronous with the pixel clock.



## FLIM / PLIM with the Zeiss LSM 710 NLO Microscopes

The system architecture is shown in Fig. 2. The system uses a standard bh TCSPC FLIM system that is connected to the LSM 710 NLO in the usual way [2]. To generate the laser modulation signal a bh DDG-210 programmable pulse generator card is added to the system. The DDG-210 is triggered by the pixel clock of the LSM 710. It delivers a laser modulation signal of programmable width, which is fed back into the beam blanking system of the microscope, see Fig. 2, left. The feedback of the modulation signal requires a change in the LSM 710 NLO laser controller. The changed controller is available via an ‘individual modification’ from Zeiss.

A second signal is generated to indicate to the TCSPC module whether the laser is on or off. It is used by the TCSPC module as a ‘routing signal’ to send fluorescence and phosphorescence photons into separate memory blocks [1]. The routing signal is slightly delayed with respect to the modulation signal to account for the delay in the opto-acoustic modulator (AOM) of the microscope, see Fig. 2, right.

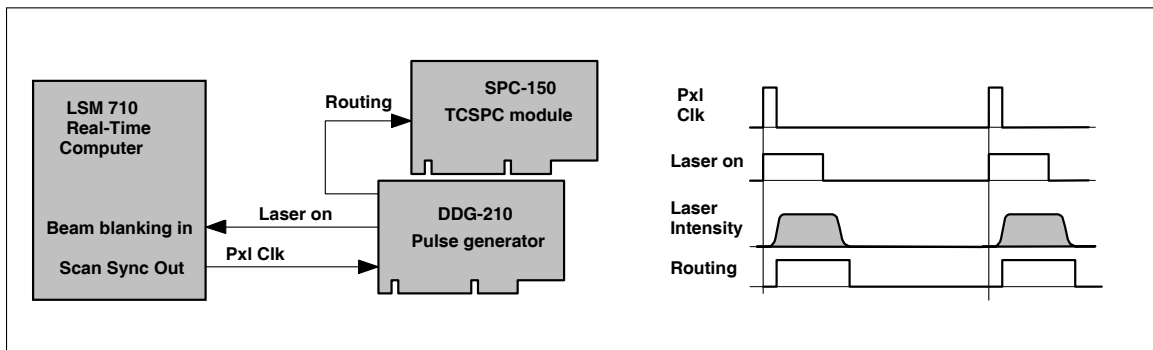


Fig. 2: On-off modulation of LSM 710 laser. The pixel clock of the LSM 710 triggers the generation of a laser-on pulse in the DDG-210 pulse generator module. The laser-on pulse controls the beam blanking in the LSM 710. The AOM of the LSM 710 responds to the beam blanking with a delay of a few 100 ns. A routing signal to the SPC-150 TCSPC module indicates when the laser is on. Connection diagram shown left, pulse diagram right.

### SPC System Parameter Setup

Combined FLIM / PLIM operation is obtained without any hardware changes in the TCSPC module. It only requires appropriate system parameter setup.

To set the parameters for FLIM / PLIM, open the ‘System Parameters’ panel of the SPCM software [1]. Click into the ‘Operation Mode’ field. Operation mode must be ‘FIFO Imaging’, see Fig. 3.

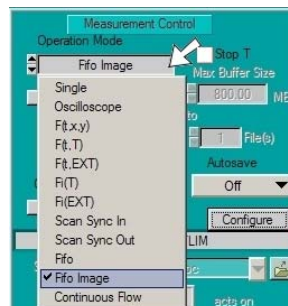


Fig. 3: Operation mode

Open the ‘Configure’ panel, see Fig. 4. Enable both Picosecond FLIM and MCS (multichannel scaler) FLIM. Select the source of the PLIM timing reference input. Timing reference for PLIM is

the pixel clock, which is identical with 'Marker 0'. Select the number of time channels. Recommended setting is 256. Select the desired time per point, or PLIM time channel width. Time channel width is a multiple of the macro time clock (25 ns) of the SPC module. The total time range of PLIM is the product of 'No. of points' and 'Time per point'.

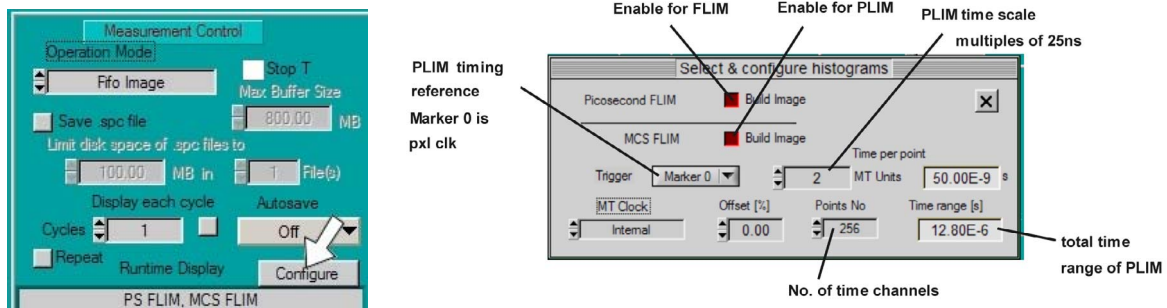


Fig. 4: Defining MCS imaging, and MSC timing parameters

In the 'Page Control section of the system parameters, set 'Routing Channels X' = 2. One channel is for fluorescence, the other for phosphorescence. 'Image pixels' is the size of the FLIM image. The typical value is 256 x 256. Larger and smaller images can be selected, see [1] or [2]. Please note that the correct binning factors must be used in the scan control parameters, see Fig. 6.

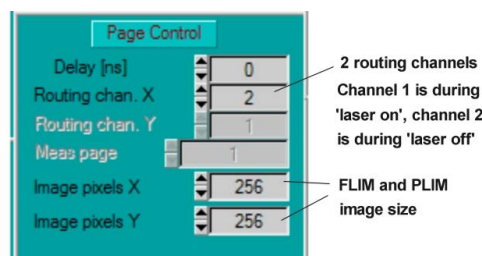


Fig. 5: Defining routing channels and FLIM / PLIM image size

To access the scan parameters, open 'More Parameters' in the system parameters panel. Define the scan parameters. Typical settings for the Zeiss microscopes are shown in Fig. 6. The Line Divider and Pixel Clock Divider values define the binning factor of the LSM scan into the FLIM image. For the standard LSM scan of 512x512 and a FLIM image of 256x256 the binning factors must be 2. Please see [1] or [2] for details.

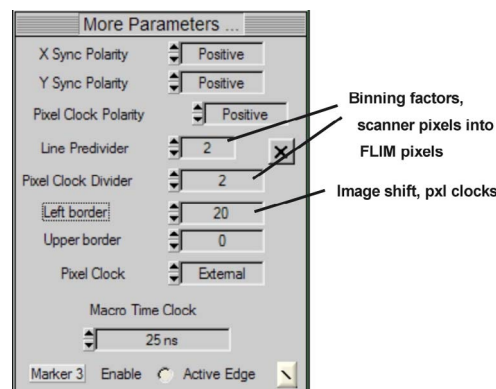


Fig. 6: Defining scan parameters and binning from LSM scan into FLIM / PLIM images

## Display setup

### Window Intervals

Open the ‘Window Intervals’ panel. Set the parameters are shown below. The ‘Routing X’ windows define three data channels. Window 1 contains the photons during the ‘Laser On’ time; window 2 contains the photons during the ‘Laser Off’ time. Window three contains all photons. Thus, an image displayed for Window 1 shows the essentially fluorescence, an image displayed for Window 2 shows phosphorescence. An image displayed for window 3 contains both fluorescence and phosphorescence.

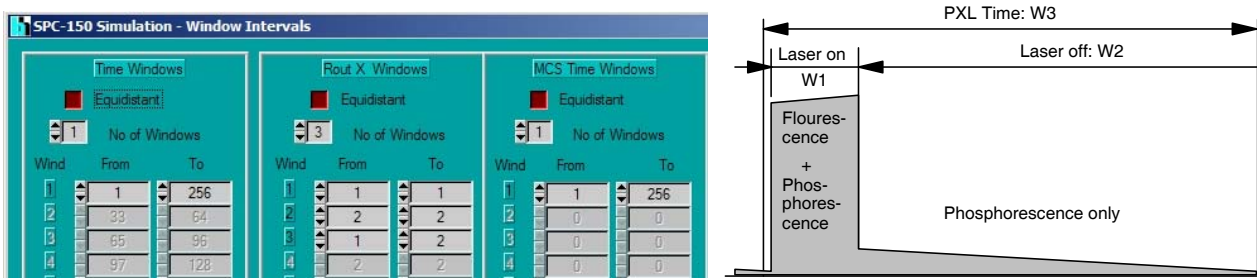


Fig. 7: Defining routing window intervals

### 3D Trace Parameters

Open the 3D Trace parameter panel. Set the parameters as shown below. The panel defines three images, one for Routing window 1 (fluorescence, recorded by ps FLIM), one for Routing Window 2 (phosphorescence, recorded by MCS imaging), and one for the total luminescence (Routing Window 3, recorded also by MCS imaging). The image for the total luminescence has been turned off. To activate it click on the ‘Window active’ button.

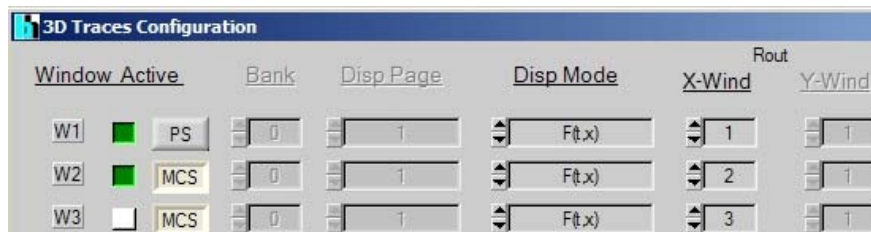


Fig. 8: Definition of display windows

### Display parameters

The display parameter setup is the same as for standard FLIM measurements [1, 2]. There are separate display parameters for the FLIM and the PLIM window. The figure below shows a display for the PLIM window. Note that you can change the ‘Routing X Window’ in the display parameters. With window intervals as shown above in Fig. 7 ‘Routing X Window’ 1 is for the laser-on time, ‘Routing X Window’ 2 is for the laser-off time, and ‘Routing X Window’ 3 is for the entire pixel time.

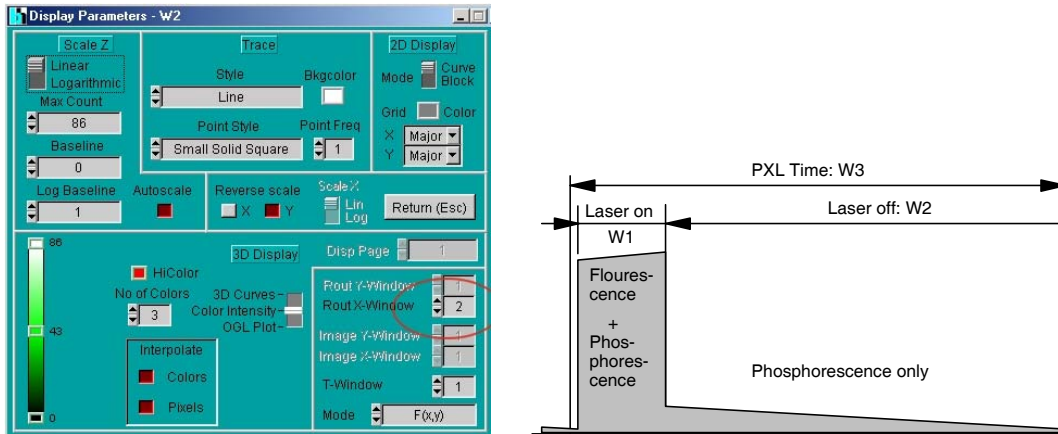


Fig. 9: Display parameters

### Saving the setup data

Please don't forget to save the setup data. Select the 'Save' panel and follow the instructions given in [1] or [2]. For frequently used setups we recommend to define an entry in the 'Predefined Setup' panel. Please see [1] for details.

### DDG Setup

Start the control software of the DDG-210 pulse generator card. The general settings are shown in the panel below. 'Repeat Sequence', 'Endless' and 'Trigger on Positive Edge' must be set. Channel polarity is '+'. If the cables are connected as suggested in the system cable plan the output of channel 1 controls the routing, the output of channel 2 the laser. You can use any other pair of outputs if you define the pulse parameters of the channels accordingly.

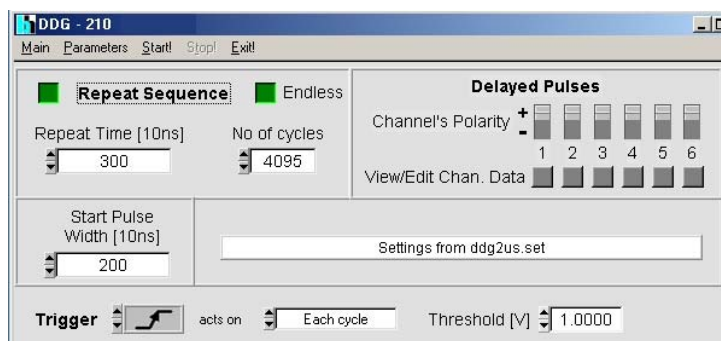


Fig. 10: DDG software panel

After starting the DDG software the DDG automatically comes up with settings as they were used in the previous session. If you want to run a PLIM experiment with the same excitation parameters as in the session before there is no need to change anything. All you have to do is to click the start button to start the DDG operation. See figure below.

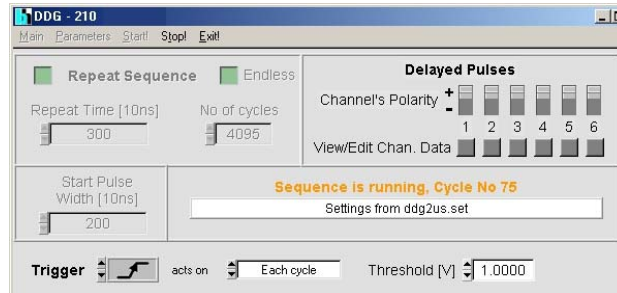


Fig. 11: DDG software panel, DDG is running

To change the ‘Laser-On’ duration, click on the View/Edit buttons of channel 1 and channel 2. Fig. 12 shows examples for a laser-on duration of 2 us (left) and 8 us (right). The numbers shown are the start times and the widths of the pulses in 10 ns units. Note that the routing signal is shifted by 800 ns with respect to the laser-on pulse. This compensates for the delay of the AOM (acousto-optical modulator) of the microscope, which is about 800 ns. Please see also Fig. 2.

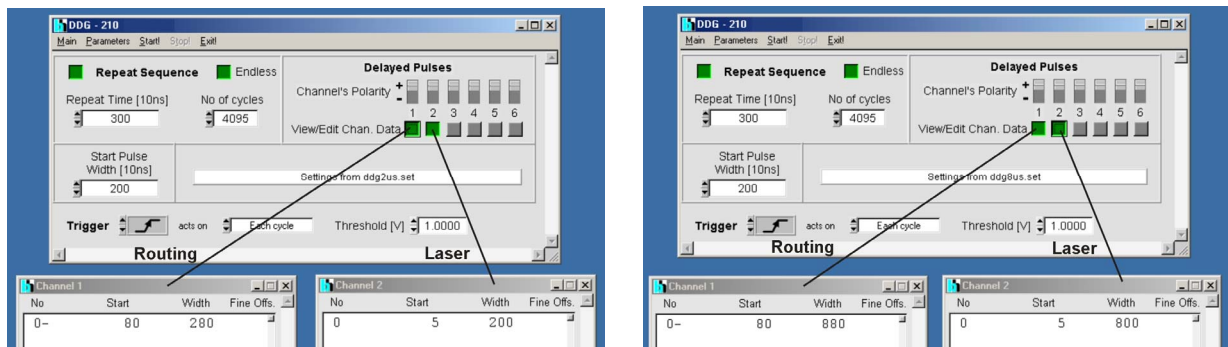


Fig. 12: DDG, defining the laser modulation time parameters for PLIM

Note that you need a pixel time of the LSM that is at least two times longer than the ‘Laser On’ interval defined in the DDG.

## Running the measurement

### Starting the measurement

The measurement is started as shown below.

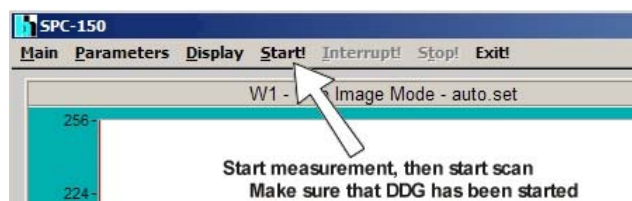


Fig. 13: Starting the measurement

*Important:* When the measurement is started the SPC hardware waits for the beginning of a new scan frame until it starts recording. The frame times used for PLIM can be up to a minute or more. If the scan is already running when the measurement is started it can take a long time until the recording begins. We therefore recommend to start the measurement first, and then the scan in the

LSM. The LSM scan starts with a frame clock, thus the recording starts with the start of the scan. Make sure that the DDG is running when you start the measurement.

*Interpretation of count rates*

The count rates present important information on whether the intensity is within reasonable limits and the recording process is running as expected.

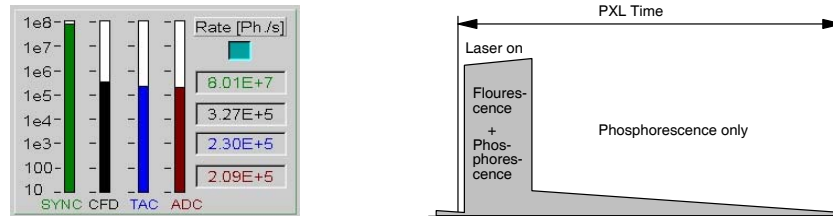


Fig. 14: Count rates

When running PLIM experiments, bear in mind that the count rates are determined over intervals of one second. Different than for FLIM, the excitation laser is only active for a short period of time at the beginning of each pixel. The sample may emit strong fluorescence during the laser-on time, but only weak phosphorescence for the rest of the pixel time. The fluorescence count rate (during the laser-on time) can therefore be considerably higher than the count rate displayed. If it exceeds a rate of about 5 MHz the fluorescence decay profiles may be distorted by counting loss and pile-up effects [1]. If the fluorescence count rates are so high that the detector overloads also the phosphorescence decay data may be impaired.

*Stopping the measurement*

There are two ways to stop a measurement: The measurement can be stopped after a defined acquisition time or by an operator command.

Stopping after a defined time is used when the intensities (photon numbers) in the data of several measurements must remain comparable. To stop after a given time, define the desired ‘Collection Time’ and activate the ‘Stop T’ button. Stop T and Collection Time can be defined either in the main panel or in the system parameters, see Fig. 15.

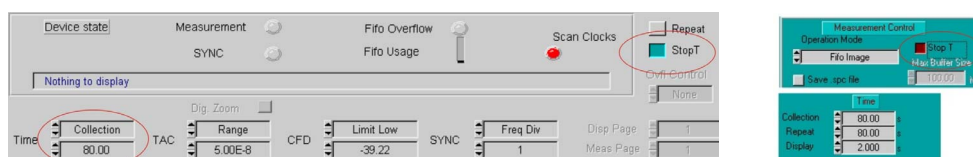


Fig. 15: Setup for stopping the measurement after Collection time

In most cases, however, intensity information in FLIM / PLIM data is not relevant. It is then more convenient to turn off ‘Stop T’ and stop the measurement manually when a reasonable signal-to-noise ration is reached. See figure Fig. 16.

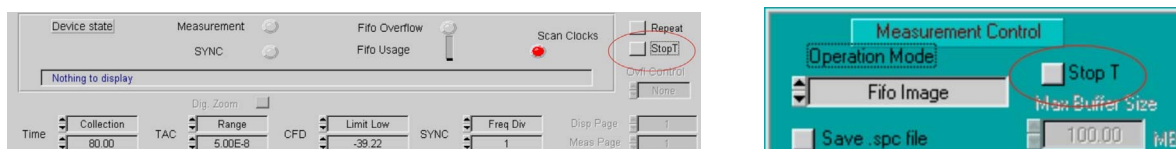


Fig. 16: Setup for stopping the measurement by the operator

## PLIM Data Analysis

### Sending data to SPCImage

There are two ways to load data into the SPCImage data analysis: An sdt file of the SPCM software can be imported via the 'Import' function of SPCImage, or the data can be sent to SPCImage by the 'Send data to SPCImage' function of the SPCM software [2].

Note that the 'send' function sends the data shown in the active display window. To send phosphorescence data, click into the top bar of the phosphorescence window, and then click on 'Send data to SPCImage', see Fig. 17.

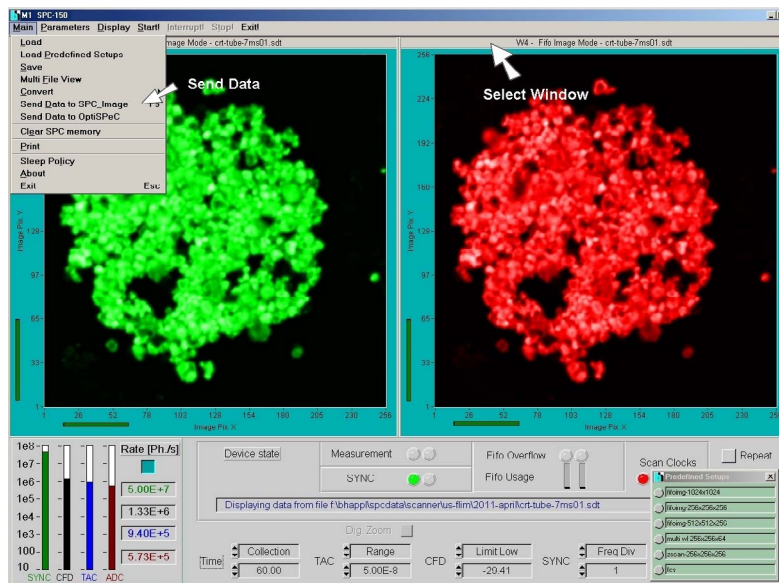


Fig. 17: Sending data to SPCImage data analysis

SPCImage will come up with the data contained in the selected window (defined by the corresponding window intervals). Depending on whether the selected display window was defined for the laser-off time only or for the whole pixel time SPCImage shows only the phosphorescence during the laser-off times, or the complete signal, see Fig. 18, left and right.

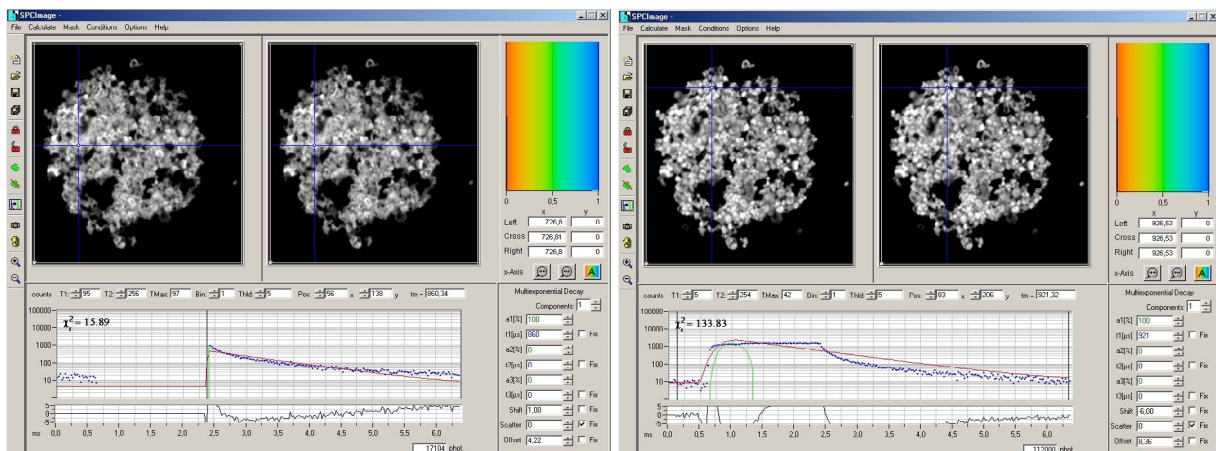


Fig. 18: SPCImage after sending data from SPCM. Left: From a display window containing data only within Laser-Off intervals. Right: From a display window containing data of the whole pixel time interval.

The phosphorescence data in Fig. 18, left, can be analysed without further preparation. However, analysis will be performed with an infinitely short instrument response (IRF) width. This will deliver the correct decay time of a single-exponential phosphorescence decay. However, if the phosphorescence decay is multi-exponential, the amplitude coefficients of the decay components cannot be determined correctly.

The data in Fig. 18, right, contain the combined fluorescence and phosphorescence data. To analyse these data, a reasonably correct IRF is required. This IRF can be measured, or created as shown in Fig. 19. Place the cursors of the decay window at the beginning and the end of the laser-on interval. Then click on the ‘Curve-to-IRF’ button. This declares the data in the cursor interval an IRF. The procedure works because (a) the phosphorescence originates from the S1 population (indicated by fluorescence) via intersystem crossing and (b) the intensity of the fluorescence during the laser-on interval is high compared to the intensity of the phosphorescence.

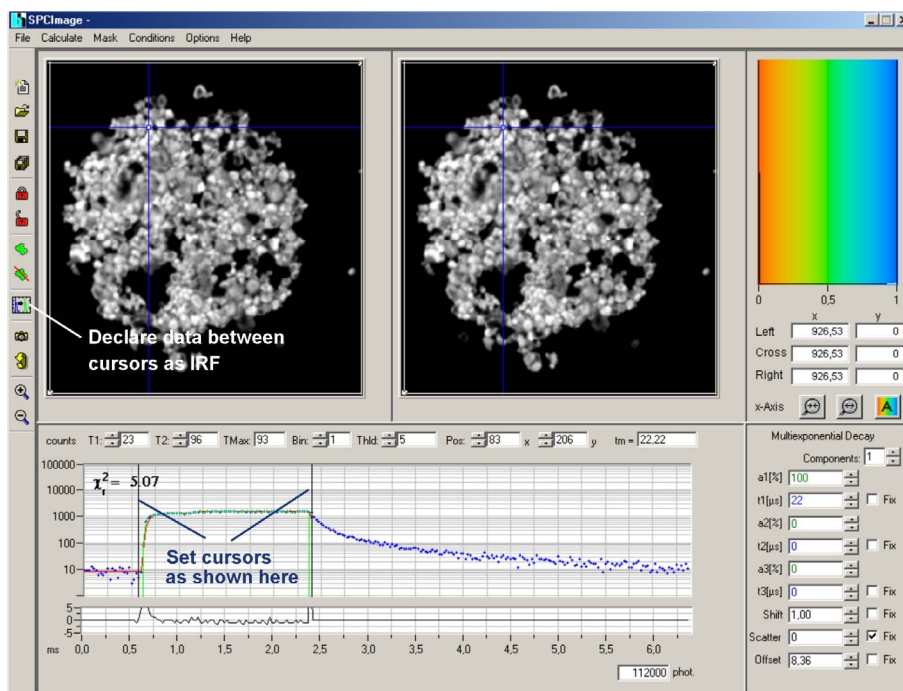


Fig. 19: Creating an IRF for PLIM analysis

When the IRF has been defined, pull the right cursor out to the last valid time channel. The rest of the procedure is the same as for FLIM data [2]. Select the appropriate model parameters on the right, and click on ‘Calculate’, ‘Calculate decay matrix’. This starts the analysis in all pixels of the image. The result is shown in Fig. 20.

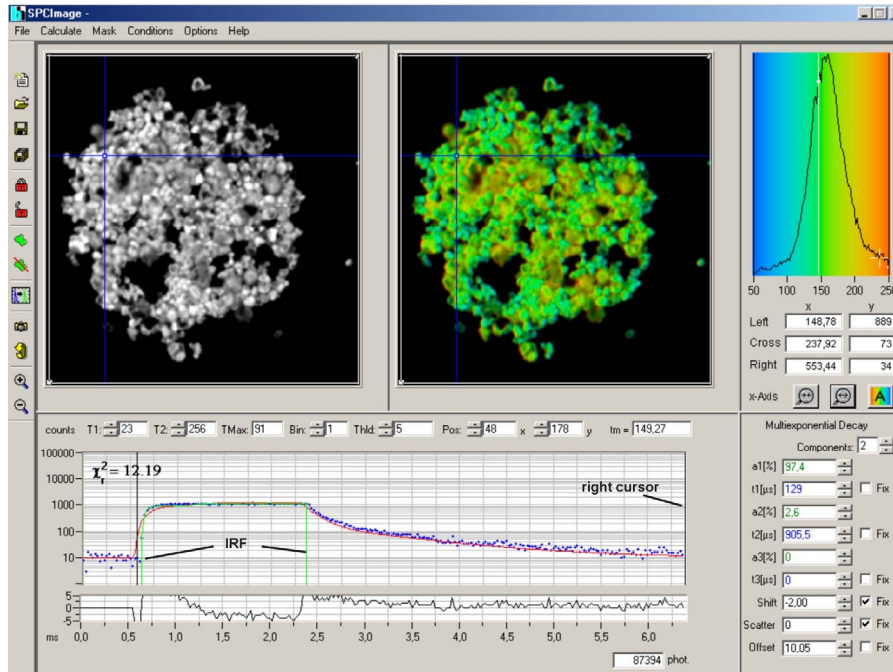


Fig. 20: Analysed PLIM data

## References

1. W. Becker, The bh TCSPC handbook. 4th edition, Becker & Hickl GmbH (2008), available on [www.becker-hickl.com](http://www.becker-hickl.com)
2. Becker & Hickl GmbH, Modular FLIM systems for Zeiss LSM 510 and LSM 710 laser scanning microscopes. User handbook, available on [www.becker-hickl.com](http://www.becker-hickl.com)
3. W. Becker, B. Su, A. Bergmann, K. Weissart, O. Holub, Simultaneous Fluorescence and Phosphorescence Lifetime Imaging. Proc. SPIE 7903 (2011)