

## Multiphoton FLIM with the Leica HyD RLD Detectors

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Leica have recently introduced hybrid detectors for the non-descanned (RLD) ports of their SP5 and SP8 multiphoton laser scanning microscopes. We have tested these detectors for FLIM with the bh TCSPC modules. We describe the TCSPC parameter setup and operating conditions for the detectors, and demonstrate the performance for typical samples.

Leica have recently introduced hybrid detector modules for the non-descanned ports of the multiphoton versions of their SP5 and SP8 laser scanning microscopes. The modules have outputs that deliver the single-photon pulses of the detectors to an external photon counting system. The detector modules have two channels detecting in different wavelength intervals. The detection intervals are selected by a standard microscope beamsplitter filter cube. The detector module attached to an SP5 MP microscope is shown in Fig. 1, left, the output connectors in Fig. 1, right. We have tested the HyD detector the with bh Simple-Tau TCSPC FLIM systems, see Fig. 2.

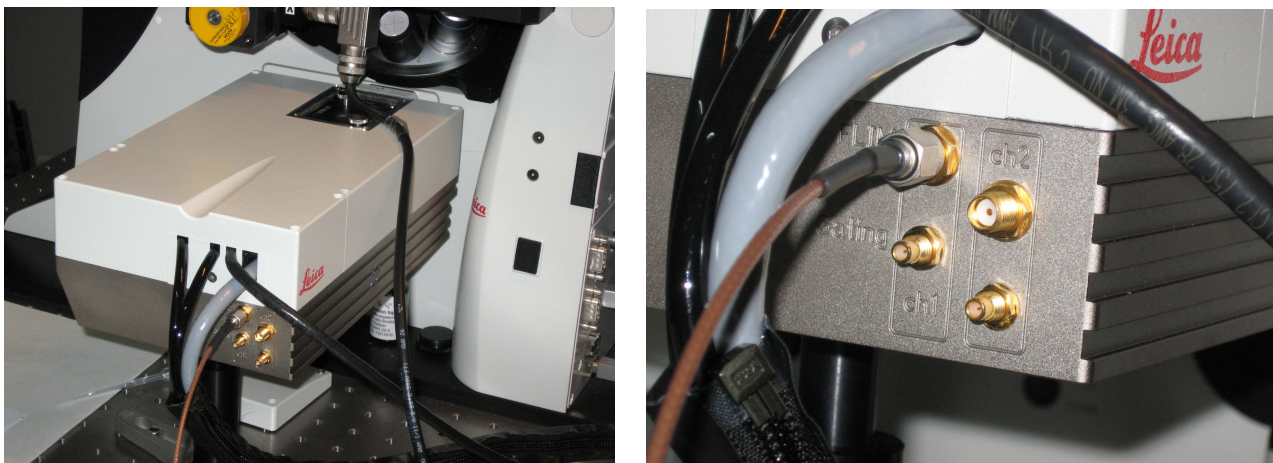


Fig. 1: Left: Leica HyD RLD dual-channel detector module at non-descanned port of SP5 MP microscope. Right: FLIM outputs of the detector.



Fig. 2: bh Simple-Tau system. Single-channel system (left) and dual-channel system (right). The systems contain one SPC-830 or one or two SPC-150 TCSPC modules.

The ‘FLIM’ outputs of the HyD detector were connected directly into the ‘CFD’ inputs of the TCSPC modules. For dual-channel TCSPC systems, such as the Simple Tau 152, the two outputs of the HyD module were connected to separate TCSPC channels. For single-channel TCSPC systems, such as the Simple-Tau 830 or 150, the output of detector channel one was connected to the CFD input. The SYNC (timing reference signal) was obtained from the synchronisation output of the Ti:Sa laser, the scan clock signals were derived from the clock breakout box of the SP5 via the standard bh scan clock cable for the SP5. Please see [1] and [2] for details.

## System Parameter Setup

### CFD Threshold

The amplitude of the single-photon pulses from the HyD detectors is about 200 mV, the polarity is negative. The signals from the detector outputs contain about 50 mV of noise. It is thus important that the CFD threshold of the SPC modules be set more negative than 50 mV. We found -100 mV to -200 mV appropriate to both suppress the noise and reliably trigger on the single-photon pulses. Please see Fig. 3.

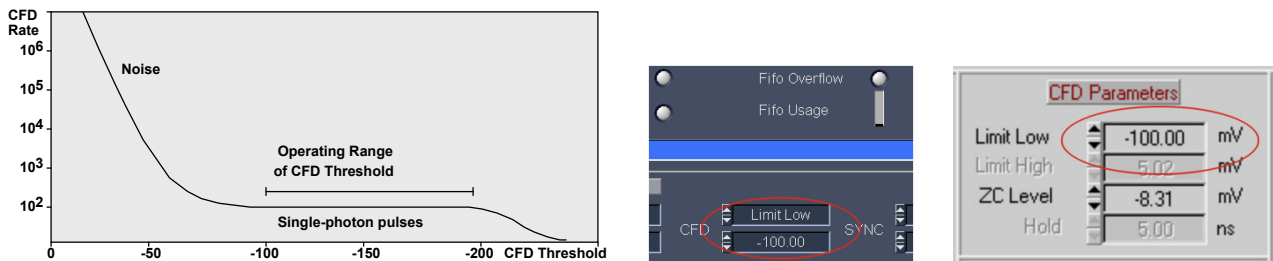


Fig. 3: General behaviour of the count rate versus CFD threshold (left, no light on the detector), and CFD threshold in the main panel (middle) and in the system parameters of the SPCM software (right).

Please note that the ‘Gain’ of the HyD detector in the Leica software has no influence on the effective gain of the detector itself, and no influence on the amplitude of the single-photon pulses. The setting is thus irrelevant for FLIM.

### Scan Control Parameters

Different than other LSMs, the SP5 uses a sinusoidal scan. The nonlinearity of the scan is compensated by a non-equidistant pixel clock. The pixel clock periods are shorter in the centre and longer at the outer parts of the line, see Fig. 4, left. The distance along the line then becomes a linear function over the pixel clock periods. Only if the FLIM system works with the pixel clock from the microscope it obtains a linearized X coordinate. This is no problem for the bh FLIM systems: They work routinely with external pixel clocks. The pixel clock source in the SPCM software must be set to ‘external’, Fig. 4, right. The scan parameters allow the user to define binning of pixels and lines, to shift the recorded part of the scan in X and Y, or to select between two different frame clock sources. The scan parameters shown in Fig. 4 were chosen to obtain a FLIM image of the same pixel number as the SP5 scan. Please see [1] for details.

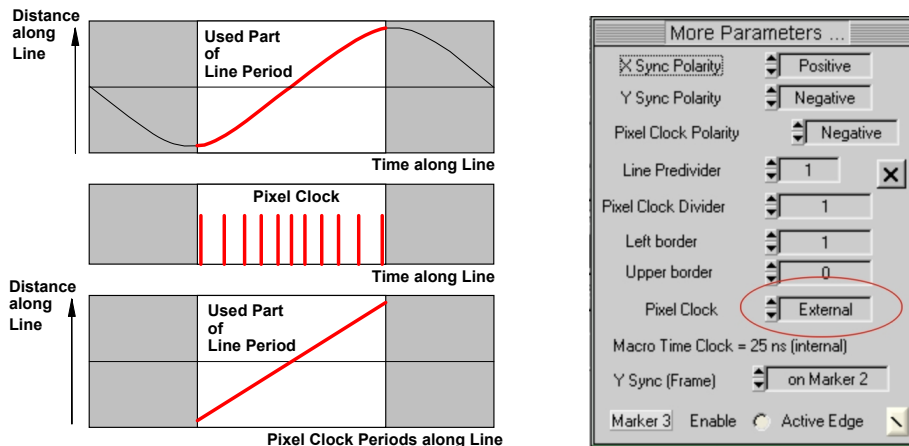


Fig. 4: Left: Sinusoidal scan and linearization by non-equidistant pixel clock. Right: Scan parameter setup in the SPCM software. The pixel clock must be 'external' to compensate for the nonlinearity of the sinusoidal scan.

### MP laser control in the SP5

The MP laser control parameters of the Leica software have significant influence on the image recording. The parameters, especially the 'Offset', must be set that the laser beam is turned on during the active part of the line period, and turned off during the rest of the line, see Fig. 5, left and middle. It seems that inappropriate settings can effectively reverse the laser-on and laser-off phases. In less severe cases, the microscope (and the FLIM system) may still acquire images, but the laser intensity may be higher in the flyback phase than in the active part of the scan, see Fig. 5, right. This can cause unnecessary load on the sample, massively increased photobleaching, and early bus saturation in the FLIM system.

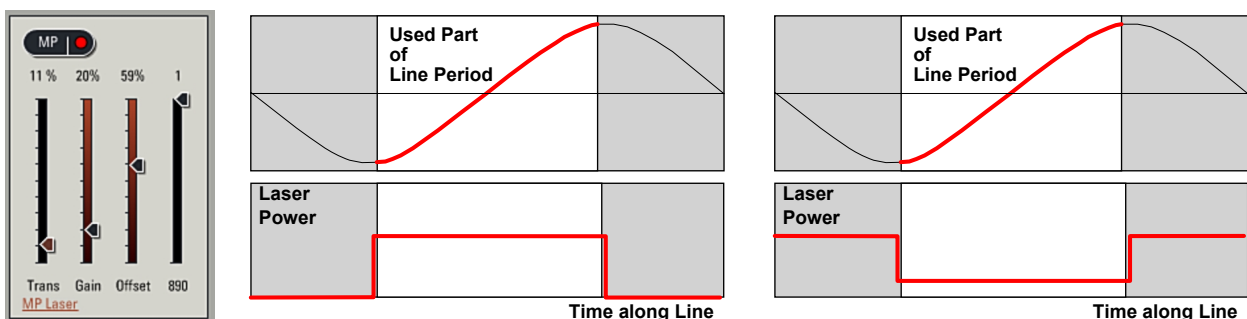


Fig. 5: Left: MP laser adjust panel in Leica Microscope Software. Middle: Laser power versus line period. Right: Laser power for inappropriately set MP laser adjust parameters.

## Results

Fig. 6 shows a lifetime image of a BPAE Cell labelled with Alexa 488 Phalloidin and Mito Tracker Red. The excitation wavelength was 860 nm. The laser wavelength was blocked by the standard 680 nm short-pass filter. No bandpass filter was used. The FLIM data were recorded in the 'FIFO Imaging' mode, with 512 x 512 pixels and 256 time channels. The usual precautions against room light pickup were used: All room lights were turned off, the back of the sample was covered by black paper, and the microscope was wrapped in black cloth. The decay data were fitted by a double-exponential decay model. A fluorescence decay curve at the position marked with the red cross is shown on the right. The decay data are clean, without any trace of reflections or laser leakage. Due to the long fluorescence lifetime there is some residual fluorescence from the previous

laser pulse periods. This signal component is, however, correctly fitted by the ‘incomplete decay’ model of the bh SPCImage data analysis software [1, 3].

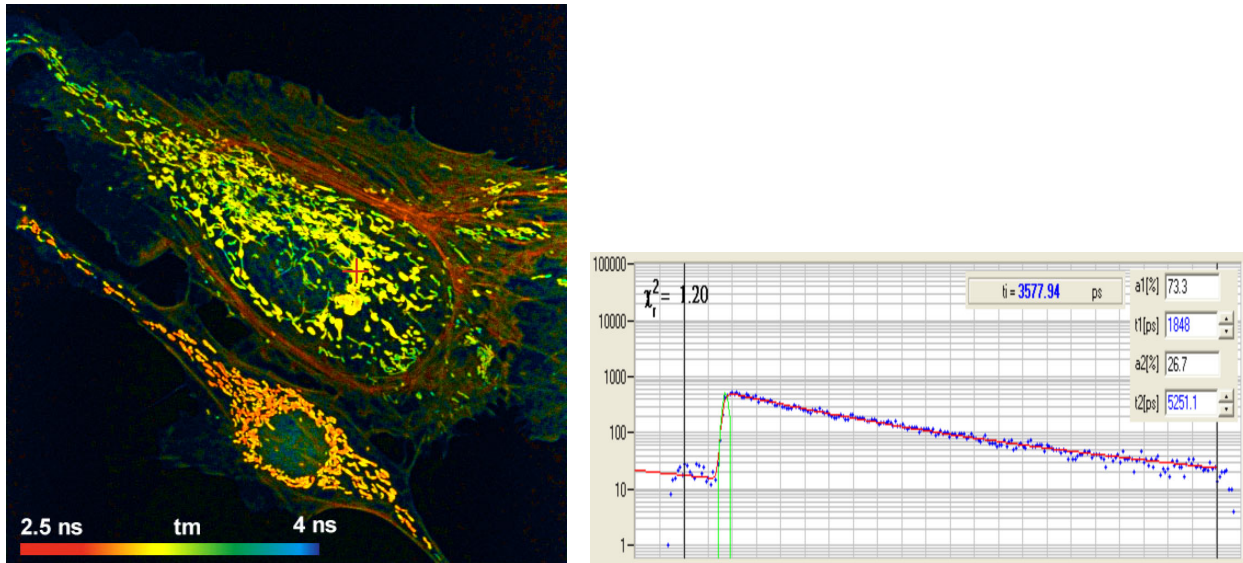


Fig. 6: Left: Lifetime image of a BPAE cell, labelled with Alexa 488 and Mito Tracker red. Amplitude-weighted lifetime of double-exponential decay. Right: Fluorescence decay curve at position marked with red cross. bh SPC-830 TCSPC module, image format 512 x 512 pixels, 256 time channels. Analysis by bh SPCImage, double exponential decay model with incomplete decay option.

Autofluorescence images of pig skin are shown in Fig. 7. The FLIM data format is 512 x 512 pixels, 256 time channels. The excitation wavelength was 800 nm. No bandpass filter was inserted, only the SP 680 laser blocking filter was used. Thus, the FLIM data contain both fluorescence and SHG components. The image on the left shows the intensity-weighted lifetime of a triple-exponential fit, the image on the right the relative intensity fraction in the ultra-fast (SHG) component.

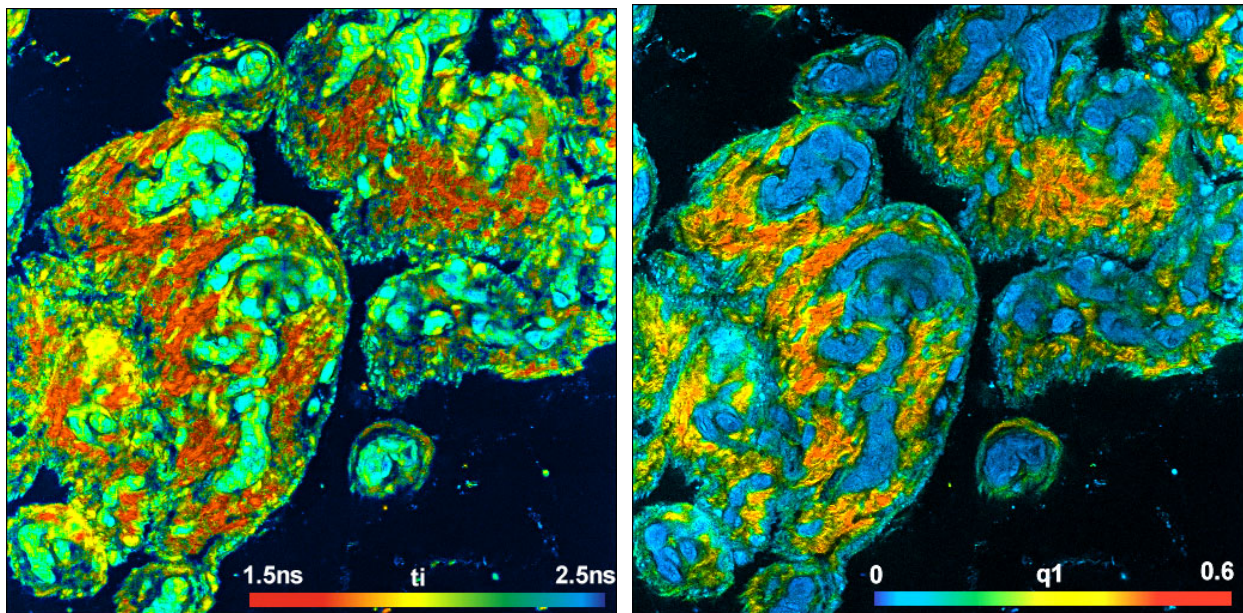


Fig. 7: FLIM of Pig skin, image format 512 x 512 pixels, 256 time channels. Left: Lifetime image, intensity-weighted lifetime of triple-exponential decay model. Right: Relative intensity of SHG component in total signal. bh SPC-830 TCSPC module, Analysis by bh SPC Image.

### *Instrument Response Function*

We did not explicitly record the temporal IRF of the HyD detector. It can, however, be estimated from the SHG components in the FLIM images shown in Fig. 7. Fig. 8, left, shows decay functions in a region dominated by SHG. The full-width at half maximum is about 130 ps, same as for the bh HPM-100-40 detectors [1, 4]. No intensity-dependent timing shift was observed, see Fig. 8, right.

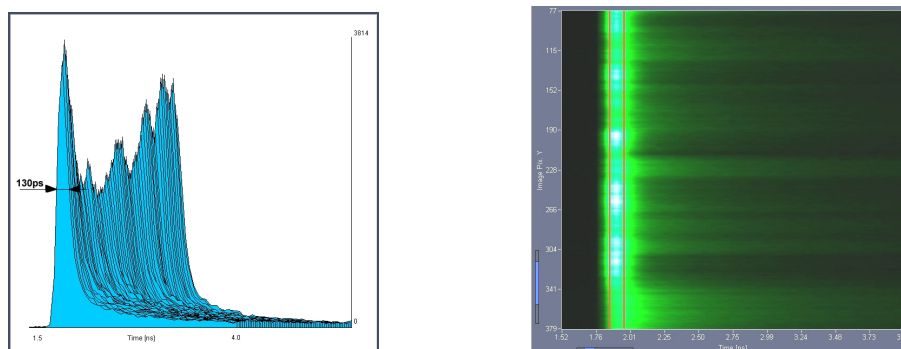


Fig. 8: IRF data extracted from the FLIM data in Fig. 7. Left: Decay functions in a region dominated by SHG. The Width of the curves is about 130 ps. Right: Decay functions over a vertical stripe of Fig. 7 displayed in colour-intensity mode. The distance between the vertical lines is 100 ps. No timing shift depending on the intensity is observed.

### *Sensitivity*

Sensitivity comparison of detectors in laser scanning microscopy is notoriously difficult. It would require measurements of the same area of a test sample in exactly the same focal plane with the same laser power for different detectors. We therefore did not attempt a direct comparison. However, the count rates obtained for a given sample and given laser power were about the same as they are observed with bh HPM-100-40 hybrid detectors on other SP5 microscopes.

### **Summary**

The HyD RLD detectors of the Leica SP5 and SP8 multiphoton microscopes are fully compatible with the bh TCSPC FLIM systems. We found an IRF width of about 130 ps. The decay curves recorded with the HyD detectors were clean, without bumps, ripple or reflections. The detectors should be used with a CFD threshold of -100 mV to -200 mV. The pixel clock of the TCSPC system must be 'external' to linearise the x coordinate of the scan. Moreover, we recommend to carefully adjust the MP laser control parameters in the Leica microscope software.

### **Acknowledgement**

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### **References**

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