The PZ-FLIM-110 Piezo-Scanning FLIM System

Abstract. The PZ-FLIM-110 Piezo Scanning FLIM system uses bh's multi-dimensional TCSPC technique in combination with a piezo scanner. The scanner is controlled via a bh GVD-120 scanner control card, the FLIM data are recorded by an SPC-150 or SPC-160 TCSPC / FLIM module. Data acquisition is controlled by 64 bit bh SPCM TCSPC software. The system is able to run X-Y scans, X-Z (vertical) scans, and to record simultaneously FLIM and PLIM data. Maximum FLIM data formats are 512 x 512 pixels, 4096 time channels, or 2048 x 2048 pixels, 256 time channels.

Stage Scanning

TCSPC FLIM systems use a combination of multi-dimensional TCSPC with optical scanning. TCSPC records fluorescence decay data at extremely good temporal resolution [1, 5, 6], near-ideal photon efficiency, and with the capability to resolve multi-exponential decay profiles into individual decay components [3]. Optical scanning delivers images that are free of out-of-focus haze and free of lateral and longitudinal scattering. TCSPC FLIM thus combines the most accurate and efficient electronic technique of fluorescence decay recording with the most accurate optical imaging technique [5].

Optical scanning for FLIM is normally performed by galvanometer scanners in combination with confocal detection or multiphoton excitation [5]. The high scan rate obtained by these systems is no problem for TCSPC FLIM. On the contrary, it the basis of advanced techniques like fast time-series recording [12], temporal mosaic imaging [11], and FLITS [4]. Moreover, it is required to run fast preview scans for selecting the desired focal plane, scan area, and sample position [6, 7].

Instead of scanning the laser and detection light beam the sample can be moved by a piezo scan stage. Sample scanning was used in the first confocal systems introduced by Marvin Minsky [13]. Moving the sample has the advantage that the optical system always works on the optical axis, and that the image area is not limited by the field of view of the microscope lens. Thus, high-NA lenses with high optical resolution and high light-collection efficiency can by used for imaging of large fields of view.

However, sample scanning has serious drawbacks. The most obvious one is that sample scanning is very slow. Typical scan rates are on the order of one to five lines per second, compared to 1000 lines per second for a galvanometer scanner. Another problem is that the scanning exerts forces on the sample. This is not acceptable for live-cell imaging. Nevertheless, sample scanning is often considered a cost-efficient alternative to beam scanning. This application note shows how sample scanning is integrated into the bh FLIM hardware and software.

Optical Principle

Two optical principles of a confocal system with sample scanning are shown in Fig. 1.

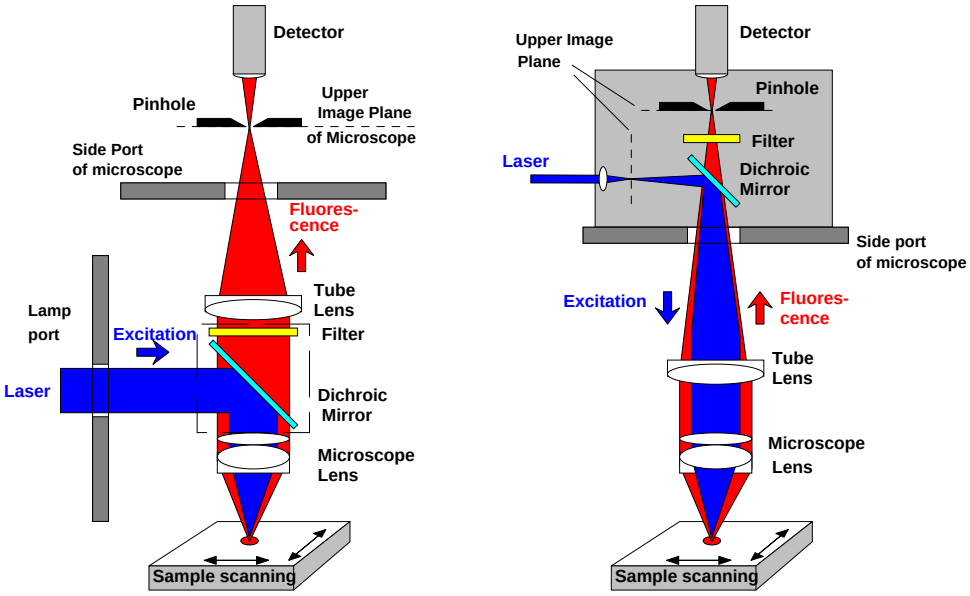

Fig. 1: Optical principle of a sample scanning system with confocal detection. Left: Beamsplitter and emission filter in microscope beamsplitter cube, pinhole and detector attached to the side port of the microscope. Right: Beamsplitter filter, pinhole and detector in compact optical assembly attached to a side port of the microscope.

A frequently used configuration is shown in Fig. 1, left. The laser beam is injected into the microscope via the lamp port of the microscope. The beamsplitter and the emission filter are inserted in one of the standard microscope filter cubes in the filter carousel of the microscope. The detector is attached to a side port of the microscope. A pinhole in the upper image plane of the microscope suppresses out-of-focus light. The setup is easy to implement but has serious disadvantages: The lamp port cannot be used for a fluorescence lamp, and the laser beam diameter must be increased to match the size of the back aperture of the microscope lens. The most significant problem is alignment. The laser must be focus in the sample must conjugate exactly with the pinhole. The angle of the dichroic mirror in the filter cube is therefore critical. Microscopy filter cubes are not designed for this level of precision. The setup is therefore not suitable for high-resolution confocal imaging.

The system in Fig. 1, right, has the laser input, the dichroic beamsplitter, the emission filter, the pinhole, and the detector integrated in one and the same compact optical building block. The entire block is attached to an optical port of the microscope. By placing the laser input, the dichroic mirror, and the pinhole on the same solid mechanical support the system stays in alignment much better than the system shown in Fig. 1, left. Further improvement in alignment stability can by achieved by fibre-coupling the laser and the detector. A problem of the setup can, however, arise from the fact that the upper image plane of the microscope is close to the side port. The dichroic mirror and the filter then do not fit between the port and the image plane.

The optical system available from bh therefore uses the setup shown in Fig. 2. A ps diode laser (bh BDL or BDS series [9, 10]) is coupled into the system via a single-mode fibre. A Qioptics (formerly Point-Source) fibre collimator is used to obtain a collimated beam out of the fibre. The beam is reflected down into the microscope beam path by a dichroic mirror. L1 focuses the laser into the upper image plane of the microscope. The laser thus forms a focused spot in the sample. The fluorescence light from the sample is collected back through the microscope lens, collimated by L1, and separated from the laser beam by the dichroic mirror. A bandpass or longpass filter in the collimated beam selects the detection wavelength range. The light passing the filter is focused into a

multi-mode fibre by a second lens, L2. The core of the fibre forms the confocal pinhole. The light collected by the fibre is transferred to the detector.

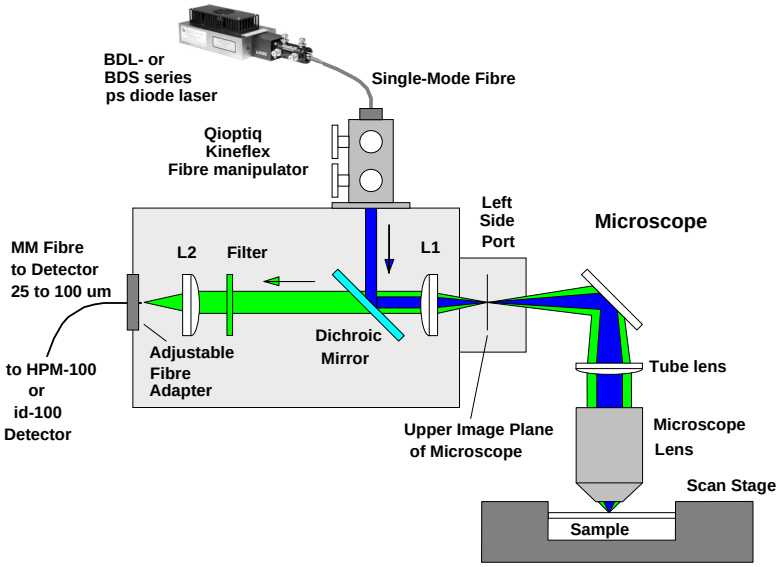

Fig. 2: Optical principle of the PZ-FLIM piezo stage scanning system. Beam path and optical elements not to scale.

Control of the Scan Stage

The scan stage is controlled by a bh GVD-120 scan controller card [6], see Fig. 3. The GVD-120 runs a linear raster scan in X and Y at a maximum resolution of 2048 x 2048 pixels. The x-y signals are generated by the hardware of the GVD module, the software is only used to load different waveforms or scan parameters in the scan controller. The waveforms are therefore independent of the computer speed and of software reaction times. The DCS-120 uses a cycloid trajectory for the fly-back of the scanner (see Fig. 3, right). This minimises mechanical resonances, and thus allows the scanner to be operated at the maximum possible line rate. Moreover, it minimises acceleration forces in the sample. The GVD-120 also generates the scan synchronisation pulses for bh TCSPC modules, and the beam blanking, multiplexing, and intensity control signals for two bh BDL-SMC, BDL-SMN, or BDS-SM ps diode lasers. The control of the GVD card is fully integrated in the SPCM software of the bh TCSPC systems [6, 7].

Fig. 3: GVD-120 scan controller card (left) an scan waveforms (right)

The GVD-120 scanner control panel of the SPCM software is shown in Fig. 4, left. The scan is started or stopped by the ‘Start Scan’ and ‘Stop Scan’ buttons at the upper left. Frame and line scans of different pixel numbers are defined via the ‘Scan’ field and the ‘Frame Size’ field. The scan speed is either selected via a slider, or the speed is automatically set to the maximum possible

scan rate for the scan amplitude used. The Scan area is either defined by the ‘Zoom’ and ‘Offset’ sliders, or by moving the scan area in a symbolic field of view. A point measurement is defined by clicking on the ‘Park Beam’ button. The beam park position can be defined in a previously recorded FLIM image, please see please see [6] or [7] for details.

The GVD-120 also controls two bh ps diode lasers. The laser on/off buttons and the laser power sliders are on the upper right. Beam blanking during the line and frame flyback is available to reduce photobleaching. The two lasers can be multiplexed synchronously with the pixels, lines, or frames of the scan. The multiplexing function is also used for phosphorescence lifetime imaging by bh’s dual-time base PLIM technique [2, 6, 7].

For operation with a scan stage, the GVD-120 hardware parameters can be adapted to scanners of different speed via the ‘GVD Adjustment’ parameters shown in Fig. 4, right. For details please see [6] or [7].

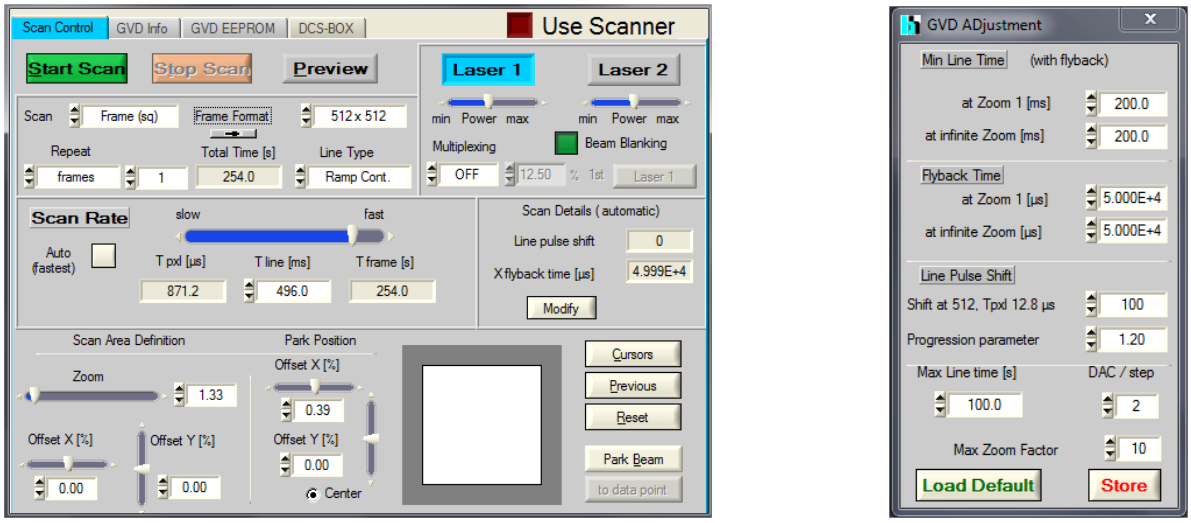

Fig. 4: Scanner control panel of the SPCM software (left) and setup of the scan speed parameters (right)

TCSPC Recording

TCSPC FLIM data are recorded by bh’s multi-dimensional TCSPC process [1, 5, 6]. Single photons of the fluorescence light are detected, the detection time, t, within the laser pulse period and the position of the laser beam in the sample in the moment of the photon detetction, x,y, are determined, and a photon distribution is built up over x,y,t. The result is an array of pixels with a full fluorescence decay curve in each pixel. With 64 bit SPCM software FLIM data formats of up to 2048 x 2048 pixels and 256 time channels can be used [8, 14]. Other formats can be defined, such as 512 x 512 pixels with up to 4096 time channels. Please see [6] for details.

System Architecture

The basic system architecture of a GVD-120-based piezo scanning system is shown in Fig. 5. The simplest system consists of an SPC-150, 160, or 830 TCSPC module, a GVD-120 scan control card, a piezo stage, a BDL or BDS series ps diode laser, and a Id Quantique, id100-50FP fibre coupled SPAD detector, see Fig. 5, left. Alternatively, an HPM-100-40 or -50 hybrid detector can be used. The HPM-100 is controlled by a DCC-100 detector controller card, see Fig. 5, right.

The ramp voltages generated by the GVD-120 scan controller are connected to the driver amplifier of the piezo stage as shown in Fig. 5.

The signal acquisition in the TCSPC board is synchronised with the scanning via frame, line, and pixel clock pulses generated by the GVD-120 card as usual [6, 7]. The scan clock pulses are connected to the 15 pin connector of the TCSPC module. The GVD-120 also controls the ps diode laser.

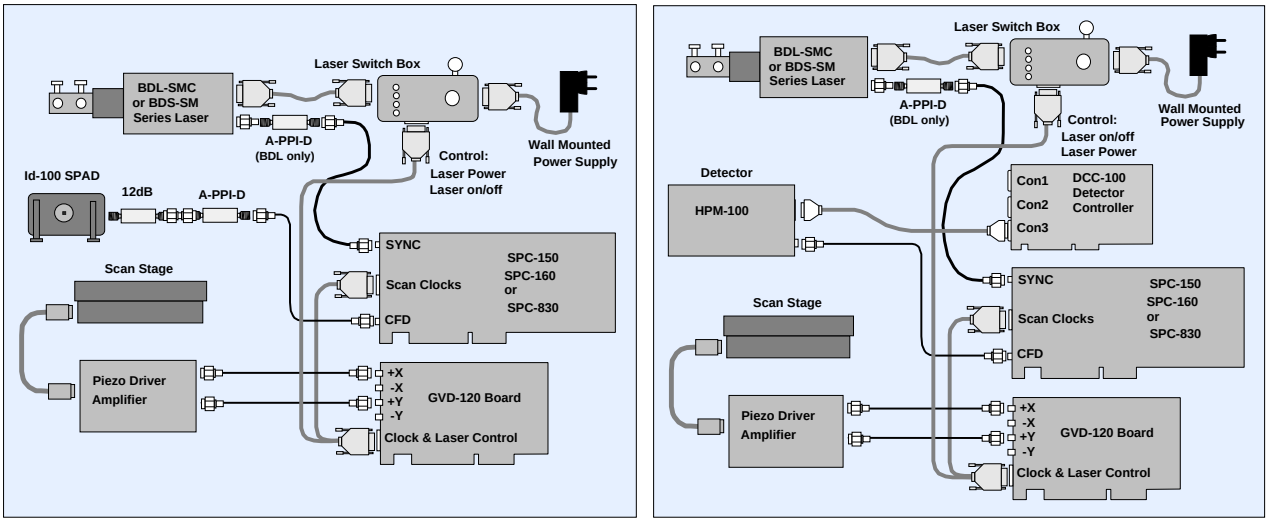

Fig. 5: Piezo stage scanning system controlled by the bh GVD-120 board. Left: With id100-50 SPAD detector. Right: With HPM-100 hybrid detector.

A photo of the entire piezo scanning system is shown in Fig. 6. It consists of a Nikon TE2000 microscope, a PZ-FLIM optical assembly connected to left side port, a BDL-SMN ps diode laser, a Mad City Labs Nano-View 200-3 piezo system, and a bh Simple-Tau TCSPC system. Other microscopes can be used if a side port is available.

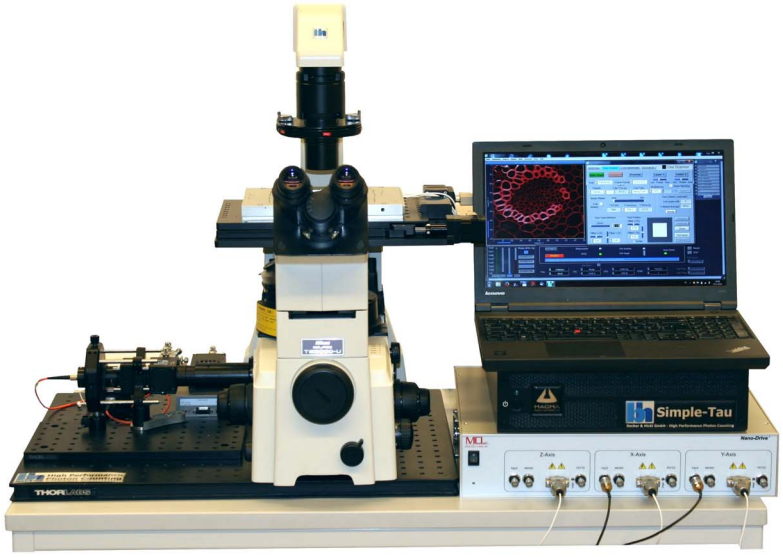

Fig. 6: Photo of the bh PZ-FLIM-110 piezo scanning system. Nikon TE2000 microscope, PZ-FLIM optical assembly connected to left side port, BDL-SMN ps diode laser, Mad City Labs Nano-View 200-3 piezo system, bh Simple-Tau TCSPC system.

Results

X-Y FLIM (Lateral Scan)

For recording the images shown below we used the system shown in Fig. 6, with a Mad-City-Labs Nano-View 200-3 piezo stage. (For an example with a PI scan stage please see [6]). A typical fluorescence lifetime image of a convallaria sample is shown in Fig. 7. Scanning was performed at a resolution of 512 x 512 pixels, and at a rate of 1 line, or 512 pixels, per second. The decay data were recorded at a temporal resolution of 1024 time channels, corresponding to 12.2 ps per time channel. Please note that up to 4096 time channels can be used without the need of decreasing the pixel number or compromising the recording speed or efficiency [6, 14]. The average count rate over the entire sample was 10^6 photons per second, corresponding to about $5{\cdot}10^6$ photons per second in the brightest pixels. The microscope lens was a Nikon 40x, NA=1.3 S Fluor oil immersion lens. The diameter of the detection fibre was 50 µm, corresponding to a pinhole diameter of about 3 Airy disk diameters, or 3 AU (Airy Units).

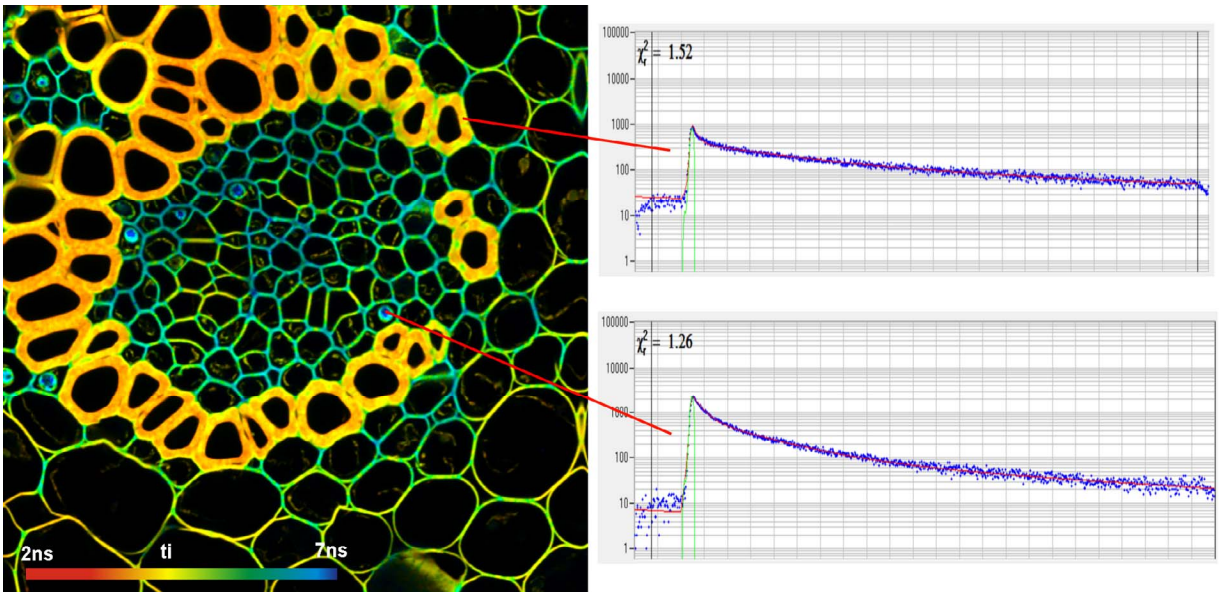

Fig. 7: Lifetime image of a convallaria sample. Intensity-weighted lifetime of triple-exponential decay. 512 x 512 pixels, 1024 time channels per pixel. 40x NA1.3 Oil immersion lens, effective pinhole size 3 AU. Right: Decay curves in selected pixels

The data shown in Fig. 7 feature high spatial resolution and clean decay data in the individual pixels. The problem is, as for any stage scanning system, the long acquisition time. Unless a substantial fraction of the line time is sacrificed for the line flyback the scanner cannot be run much faster than 5 lines per second. An image of 512 x 512 pixels therefore requires a scan time of at least 100 seconds. Recording an image of 2048 x 2048 pixels - easily achieved with the DCS-120 galvanometer scanning system [7, 14] - would require about 400 seconds, or 6.6 minutes. Such long acquisition times may be acceptable for acquisition of ultra-high quality decay data. In that case, the acquisition time is determined rather by the time required to record enough photons in the individual pixels than by the scan time. For standard FLIM applications the scan times are inconveniently long, and for time-series recording they are clearly unacceptable. The biggest problem caused by the long scan time is the impossibility of accurate focusing. Fine focusing, i.e. selection of the desired image plane within the longitudinal dimension of the sample, has to be done on the basis of confocal images. Even a perfectly parfocal eyepiece or camera does not solve the

problem - the features to be imaged by confocal scanning may simply not be visible this way. This is especially the case in thick samples of biological tissue with strong longitudinal and lateral scattering [5].

X-Z FLIM (Vertical Scanning)

The piezo system used for the experiments described here has a third axis which can be used to scan the sample vertically. All that has to be done to obtain a vertical scan is to swap the Y output from the GCD-120 from the Y input of the piezo amplifier to the Z input. A x-z scan (vertical scan) of the convallaria sample is shown in Fig. 8.

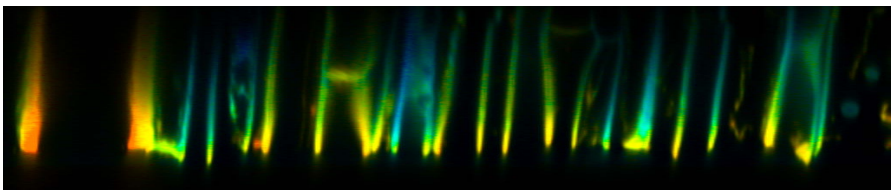

Fig. 8: Vertical (x-z) scan through a convallaria sample. Horizontal scan range 100 µm, vertical scan range 25 µm

For vertical scanning the speed disadvantage of piezo scanning compared with galvanometer scanning is less significat than for x-y scanning. What ever the line rate is - the time required for one Z step is determined by the speed of the Z drive. The Z drive is either the Z drive of a microscope or a piezo element and thus not faster than a few 10 ms per step. Also the focusing problem is less sinificant. The start position of the Z scan need not be defined as accurately as the image plane of an X-Y scan.

PLIM

In 2011 bh introduced a combined FLIM / PLIM techniques that is based on an additional on/off modulation of a pulsed laser, and recording photon times both within the laser pulse period and the laser modulation period [2]. In other words, the PLIM signal is excited by many laser pulses, not only by a single one. The result is a dramatic increase in PLIM sensitivity, and the possibility to record FLIM and PLIM simultaneously in the same scan. An example of a PLIM measurement with a palladium dye is shown in Fig. 9.

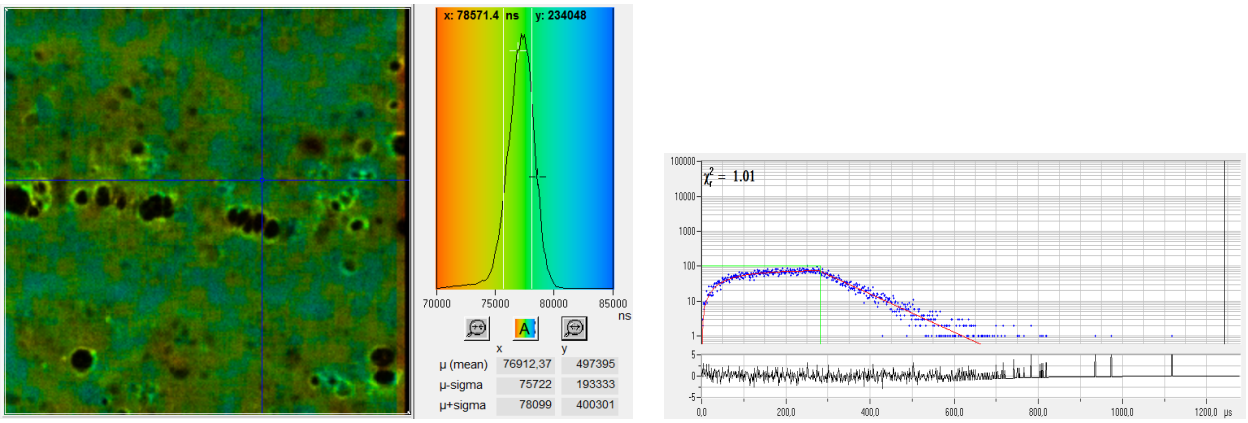

Fig. 9: PLIM measurement of a palladium dye. Phosphorescence lifetime image, histogram of lifetime over the pixels, decay curve in selected spot. 1024 time channels, time scale 1.3 ms. Decay time is 79 µs.

For PLIM or combined FLIM/PLIM the speed disadvantage of the piezo scanner is less significant than for FLIM. The pixel time of the scan must be selected a few times longer than the phosphorescence lifetime, which means a slow scan has to be used. Phosphorescence lifetimes are often on the order of 50 µs (for platinum dyes) or a few 100 µs (for palladium dyes), which means pixel dwell times on the order of 200 µs or 2 ms, respectively. This is close to or even within the reach of a piezo scanner.

Summary

The PZ-FLIM-110 Piezo-Scanning FLIM system records X-Y scans and X-Z scans of FLIM and FLIM/PLIM data at excellent spatial and temporal resolution. Problems do, however, arise from the slow scan speed of the piezo stage. Under typical imaging conditions, the acquisition time is determined by the speed of the scanner, not by the time needed to acquire the desired number of photons. Scan times for 512 x 512 pixel images are on the order of 100 seconds, scan times for 2048 x 2048 pixel images are at least 6.6 minutes. These scan times may be acceptable for high quality FLIM but not for imaging dynamic effects in a life sample. Moreover, the slow scan rate makes focusing into a defined image plane within a sample difficult. If these restrictions are taken into account the PZ-FLIM-110 is a cost-efficient alternative to a DCS-120 galvanometer scanner system.

References

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