Wide-Field TCSPC FLIM with bh SPC-150 N TCSPC System and Photek FGN 392-1000 Detector

Wolfgang Becker, Holger Netz, Becker & Hick GmbH, Berlin, Germany
Klaus Suhling, King's College London

Abstract: We present a wide-field TCSPC FLIM system consisting of a position-sensitive MCP PMT of the delay-line type, three SPC-150N TCSPC modules, a bh BDS-SM picosecond diode laser, an inverted microscope, and optics that projects a fluorescence image on the active area of the detector. The operation of the system is fully integrated in the bh SPCM TCSPC software, data analysis is performed by bh SPCImage. The system is able to record FLIM data with 1024 time channels and up to 1024 x 1024 pixels. The effective spatial resolution of the detector / TCSPC combination is about 250 x 250 pixels fwhm, corresponding to about 160 µm on the active area of the detector.

Detector Principle

Wide-field imaging by photon counting is around for more than a decade. Wide-field TCSPC techniques are based an single-photon detection, generation of a position signal for each photon, and building up the distribution of the photon number over the image coordinates. In case of FLIM also the time of the individual detection events is determined, and added as a coordinate of the photon distribution. The position information can be derived form the electric charge of the individual photon pulses at the outputs of a quadrant anode, a wedge-and-strip anode, or a resistive anode [1]. These principles require charge detection by low-noise noise charge-sensitive amplifiers, analog-to digital conversion, and calculation of quotients of the signals. These are time-consuming operations. The maximum count rate of such systems is therefore low. Another way to obtain position information is to couple the single-photon pulses into two crossed delay lines at the detector output. The position is then determined by measuring the arrival times of the photon pulses at the four outputs of the delay lines [1]. The delay lines can be placed inside the detector, or outside the detector and coupled capacitively to an inside resistive anode. The delay-line technique requires relatively complex recording electronics but works up to a count rate of about 1 MHz. The principle is shown in Fig. 1.

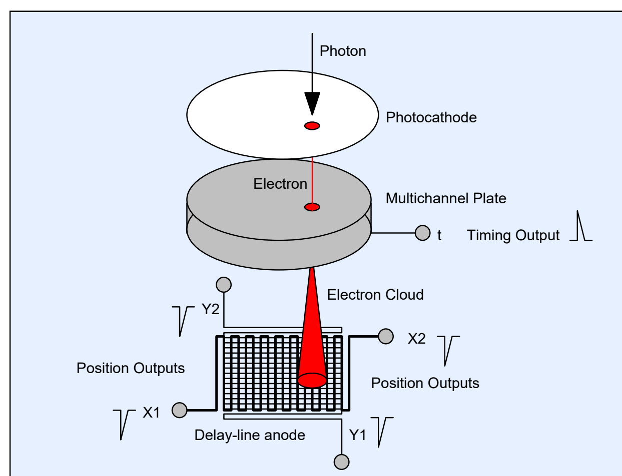

Fig. 1: Principle of position-sensitive detector: The detector has a delay-line structure as an anode. The X position of a photon is proportional to the delay between X1 and X2. The Y position of a photon is proportional to the delay between Y1 and Y2. The time of the photon is derived from a signal from the low-side of the channel plate, t.

TCSPC System

The TCSPC system consists of three parallel SPC-150N modules, see Fig. 2. The first module measures the times of the photons in the laser pulse period. The second and third module measure the times of the pulses at the outputs of the X and the Y delay lines. Delay cables in the stop lines guarantee that the start-stop times remain positive for all X and Y positions. The SPC modules are working in the FIFO (Parameter-TAG) mode [2]. That means the detection events, i.e. the times, t, and the positions, x and y, are transferred into the computer photon by photon together with a ‘macro time’. The macro time is an absolute time from the start of the acquisition. It is used by the software to assign the t, x, and y data delivered by different modules to a particular photon. From these data the software builds up a photon distribution over the coordinates x, y, and the times, t. This is the usual photon distribution of FLIM: It is an array of pixels, each of which contains a fluorescence decay curve consisting of photon numbers in consecutive time channels. To make this process possible the measurement in all three modules must be started at exactly the same moment, and the macro-time clocks in the modules must be synchronised. This is achieved by the ‘Trigger Master’ and ‘Clock Master’ functions of the SPC-150N modules [2].

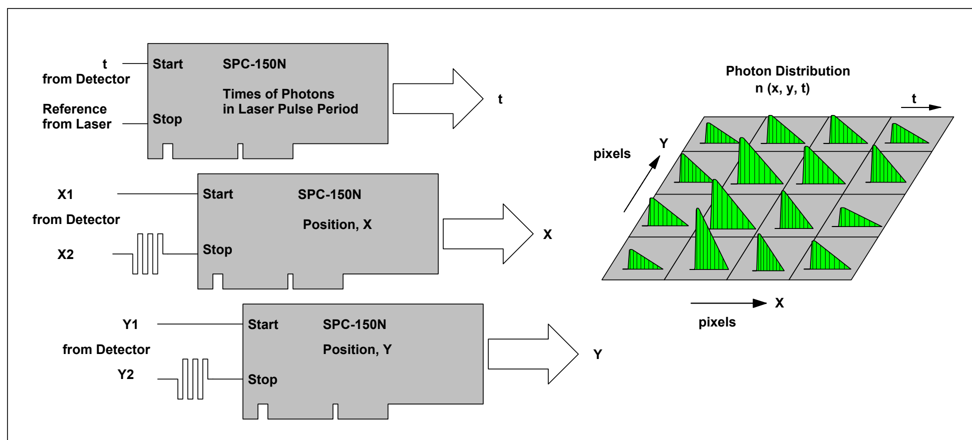

Fig. 2: TCSPC System

Constant-Fraction Discriminators

All bh SPC modules have constant fraction discriminators (CFDs) at their start and stop inputs. The CFDs not only reject noise and low-amplitude pulses but also prevent the amplitude jitter of the detector pulses from inducing timing jitter. For this purpose, the CFD circuitry shapes the detector pulses into a bipolar waveform. The zero-cross point of this pulse does not shift with the amplitude. A fast discriminator triggers on the zero-cross point and thus delivers the temporal position of the photon is independently of the pulse amplitude [1, 2]. If the pulse shaping network is adapted to the rise and fall time of the detector pulses this principle works well for all commonly used detectors. It also works for the single photon pulses at the timing output of the Photek FGN 392-1000 detector, see Fig. 3, left. A normal CFD does not work, however, for the signals delivered by the position outputs of a delay-line detector. The signals at these outputs have nothing in common with normal single-photon pulses, see Fig. 3, middle. They resemble of a burst of pulses rather than a single photon pulse. Trying to process a signal like this by a normal CFD is hopeless.

The reason of the strange signal shape is that the electron cloud of a single photoelectron simultaneously hits several parts of the delay line structure, see Fig. 1. Moreover, the subsequent steps of the delay line are electrically not perfectly de-coupled. The only way to obtain timing from

the position outputs is to determine the centroid of the entire burst. This can be achieved by sending the signal through a low-pass filter (pulse shape shown in Fig. 3, right) and processing the resulting pulse by a CFD that has an appropriately designed pulse shaping network [3]. The unavoidable side effect is that the filtered signal is slow, and that accurate timing on it becomes difficult. This sets a limit to the spatial resolution of the detector / TCSPC combination.

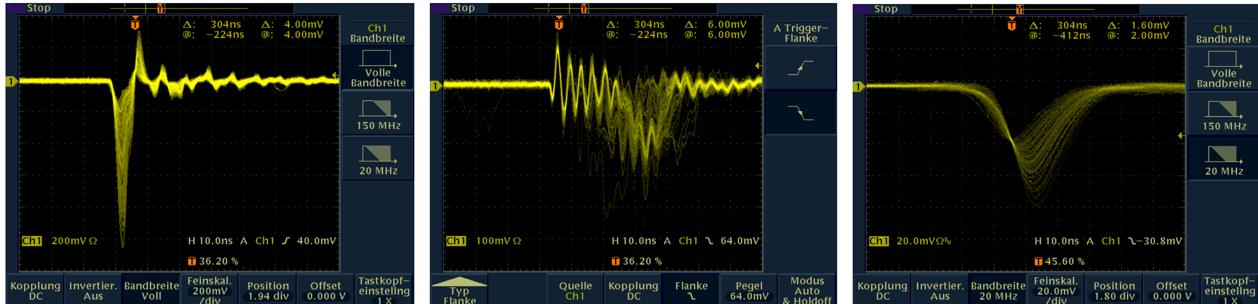

Fig. 3: Single photon pulses delivered by the Photek detector. Left: Timing output. Middle: Position output X1. Right: Signal of position output X1 after passing through a 20 MHz low-pass filter.

The SPC-150N modules in the two position channels have special CFDs (‘WF’ type). The WF CFDs have a pulse shaping network which simultaneously acts as a low-pass filter. The result is smooth timing on the position signals, and interpolation of the effective time of the signal over the discrete steps of the delay line structure [3].

Optical System

The optical part of the wide-field FLIM system is shown in Fig. 4. It consists of an inverted microscope, a BDS-SM 488nm picosecond diode laser, and a Photek FGN 392-1000 detector. The BDS-SM laser is used for excitation. A cleaning filter removes long-wavelength broadband emission from the laser beam. The light passing the filter is delivered into the microscope by a single-mode fibre. A standard microscope beam-splitter cube reflects the laser towards the microscope lens. The fibre output delivers a diverging beam of light into the back aperture of the microscope lens. The light is thus not focused into the sample, it illuminates the entire field of view. Due to the clean beam profile at the end of the single-mode fibre the illumination is homogeneous over the entire image area.

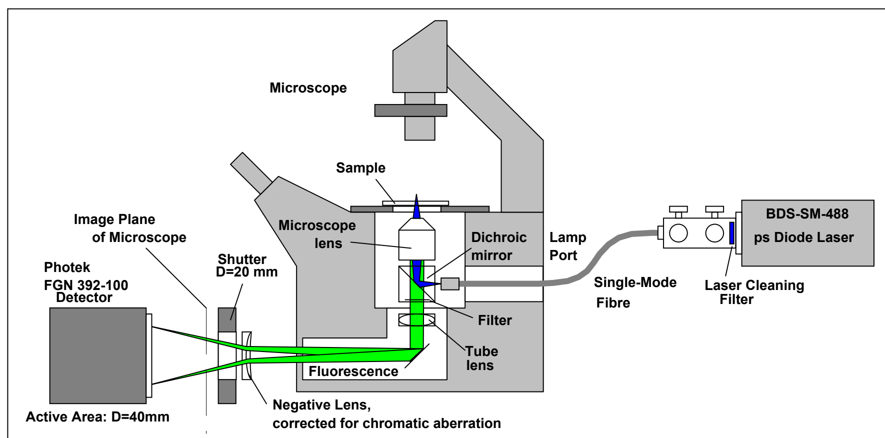

Fig. 4: Optical System

Fluorescence excited in the sample passes the dichroic mirror of the beamsplitter cube and an emission filter. It is directed out of the microscope via one of the side ports. An achromatic negative lens a few cm in front of the image plane magnifies the image to match the active area of the detector. A shutter is placed behind the lens to protect the detector and conveniently block the light path when the microscope lamp is used.

SPCM Parameter Setup

SPCM Main Panel Configuration

Data acquisition is performed by bh SPCM software [2]. The SPCM Main Panel is shown in Fig. 5. The image is shown on the left. The display parameter panel, the predefined setup panel, and the DCC-100 (detector, shutter, laser control) panel are open on the right. The display parameters define the colour, the intensity scale and the mode of the data that are displayed. Data can be displayed as intensity images, intensity images in several time gates, or as decay curves over selected areas of the image. For routine use we recommend the settings shown in Fig. 5. Please see SPCM software description in [2] for details.

The DCC-100 panel controls the intensity of the laser and operates the shutter. If an appropriate high-voltage power supply is used for the detector also the detector operating voltage can be controlled by the DCC.

The predefined setup panel is used to load different instrument configurations or system operation modes by a single mouse click. Please see [2].

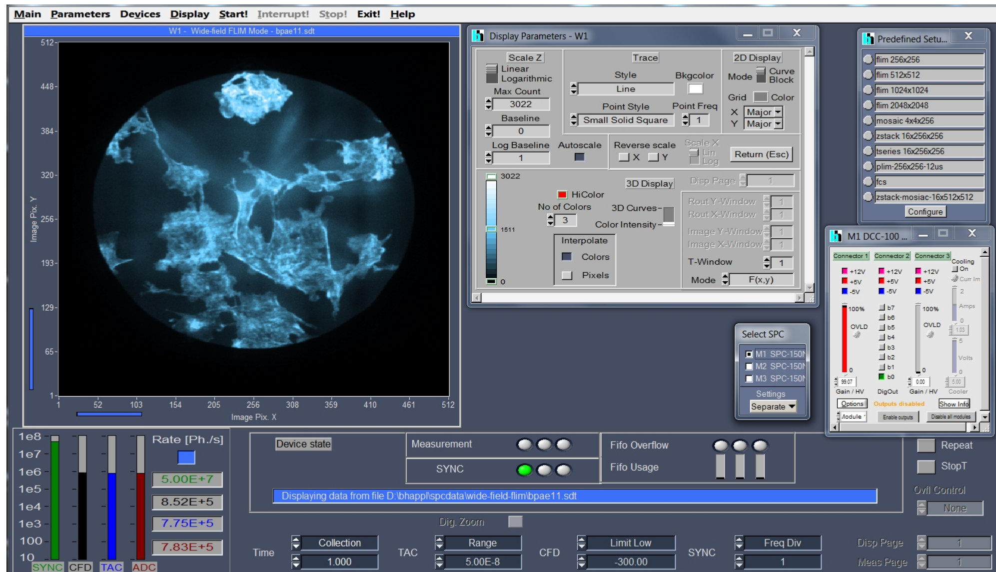

Fig. 5: Main panel of SPCM software, recommended configuration for wide-field FLIM. Image Invitrogen BPAE sample, acquisition time 1 minute.

The count rates are displayed on the lower left. The count rates have different meaning depending on which SPC module is selected in the ‘Select SPC’ panel. If module 1 is selected the Sync rate is the reference pulse frequency from the laser. The other bars are the photon rates in the at the CFD input, in the TAC, and in the ADC of the time-measurement module. For wide-field FLIM, we recommend not to exceed a photon rate of 1 MHz. This can cause detector saturation in bright parts of the image, or result in a large fraction of ‘unmatched events’ (unmatched time and position information) and, consequently, loss of photons. If M2 or M3 are selected the Sync and CFD rates

show the event rates in the X1 / X2 and Y1 / Y2 position channels. All rates should be approximately the same as the CFD rate in M1.

System Parameters

The SPCM system parameter panel with the recommended settings is shown in Fig. 6, left. Operation mode is 'Wide-Field FLIM'. Stop T is not set - the measurement is started and stopped by the operator. ADC resolution is 1024. That means, decay curves of 1024 time channels are recorded in the pixels. Image pixels Y and Image Pixels Y is 512, corresponding to an image of 512 x 512 pixels. The pixel number can be increased to 1024 x 1024 to obtain larger over-sampling factors as they are commonly used in microscopy.

To associate the events recorded in the three SPC-150N modules to the correct photons the modules must be started synchronously, and be operated from the same master clock. The definitions are shown in Fig. 6, right.

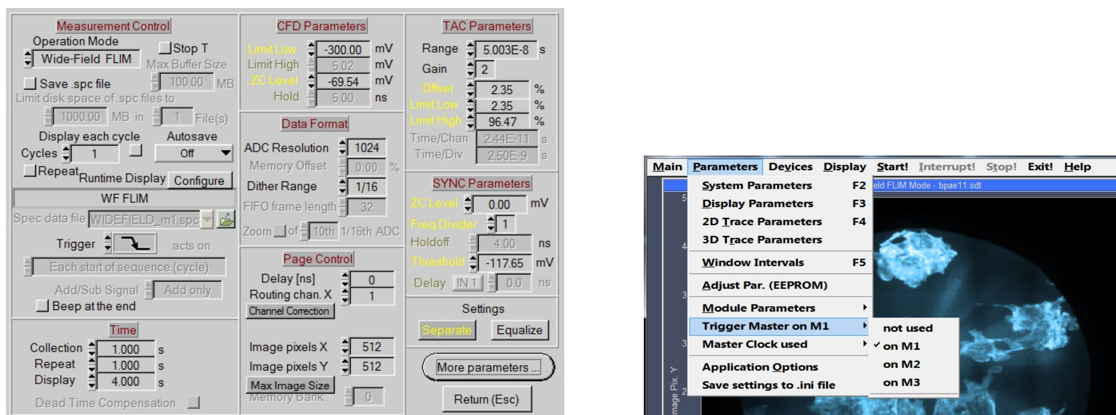

Fig. 6: System Parameters (left) and definition of Trigger Master and Master Clock function (right)

System-specific parameters are defined under 'More Parameters', see button in the system parameter panel, lower right. These parameters control the conversion of the times measured between the X1 and X2, and the Y1 and Y2 signals into spatial information. We recommend not to change these settings.

Running a Measurement

To run a measurement, click 'Enable Outputs' in the DCC-100 panel. Open the shutter (green button), and select an appropriate laser power (left slider). If you control the detector operating voltage via the DCC-100, select also the correct voltage. We recommend to set the voltage only once and then use 'Lock Con 3 Setup' in the 'Options'. The software then automatically uses the right voltage everytime it starts.

With the parameters shown above the system starts acquiring photons when the Start button is clicked. It continues to do so until the operator clicks the Stop button. Intermediate results are displayed in intervals of 'Display Time', i.e. 1 second with the parameters shown in Fig. 6.

A fast preview is run when the 'Repeat' and the 'Stop T' buttons are activated (Fig. 5, lower right) or Fig. 6, left, 'Measurement Control'. With the parameters selected the system runs 1-second measurements and displays the results periodically. The Fast Preview function is an excellent way to select the desired laser power, sample position, and focal plane.

Results

Data Analysis was performed by SPCImage [2] in the usual way, see Fig. 7. An intensity image build up from the TCSPC data is shown left, a lifetime image is shown right. A decay curve for the pixel at the cursor position is shown at the bottom. The decay curve is clean, without optical or electrical reflections. The residuals (shown below the decay curve) confirm the good quality of the temporal data.

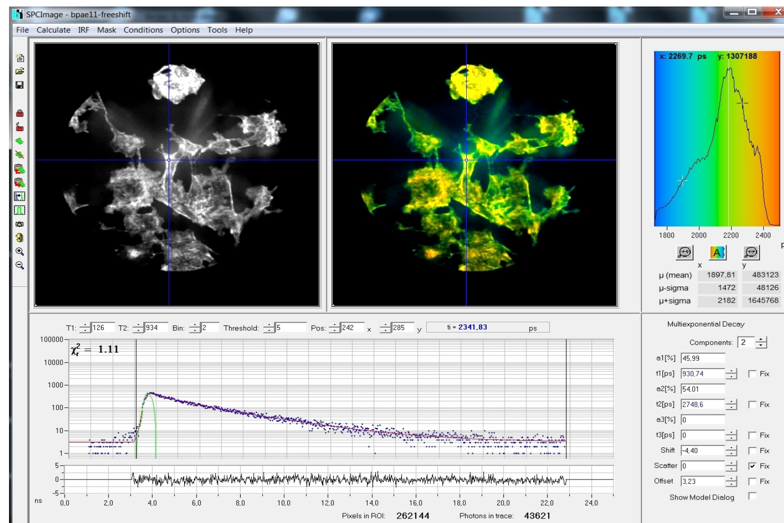

Fig. 7: Data analysis by SPCImage. Same data as shown in Fig. 5, 512x512 pixels, 1024 time channels per pixel. Sample Invitrogen BPAE cells.

A lifetime image at larger scale is shown in Fig. 8, left. The image was recorded with 512 x 512 pixels. This is more than the detector / TCSPC combination resolves. Fig. 8, right, shows a zoom into an area near the centre of the image. It can be estimated that the effective spatial resolution of the detector/TCSPC combination is about 250 x 250 pixels. This corresponds to about 160 µm on the cathode of the detector.

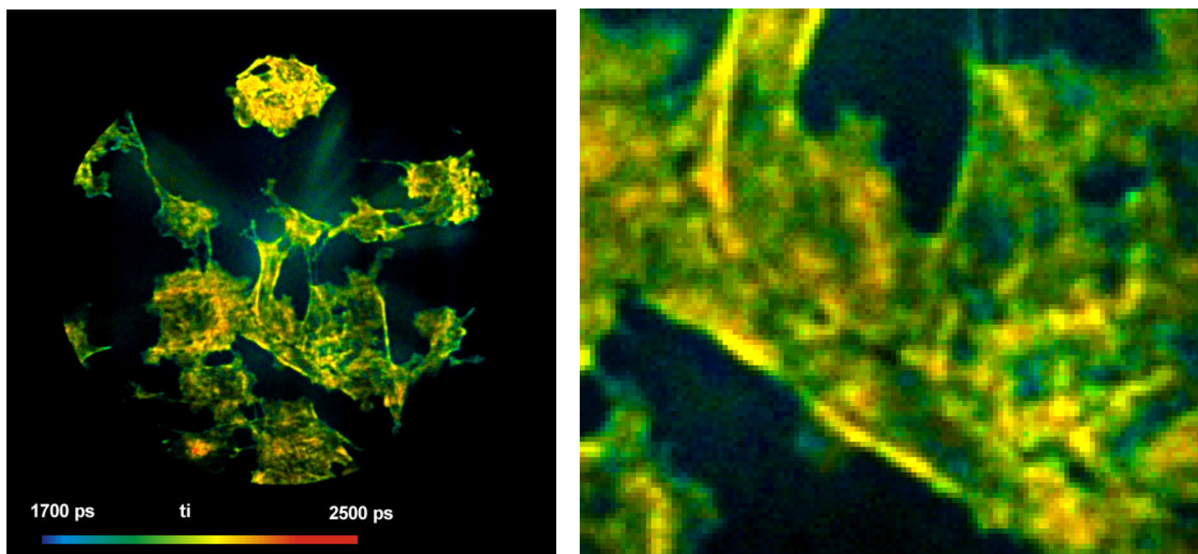

Fig. 8: Left: Wide-field FLIM image analysed with the parameters shown in Fig. 7. Right: Digital zoom into an area near the centre of the image.

The temporal instrument response function (IRF) of the detection system for different spots at the photocathode is shown in Fig. 9. To avoid saturation of the channel plates by high local intensity the IRF measurement was performed at a count rate of no more than 70 kHz.

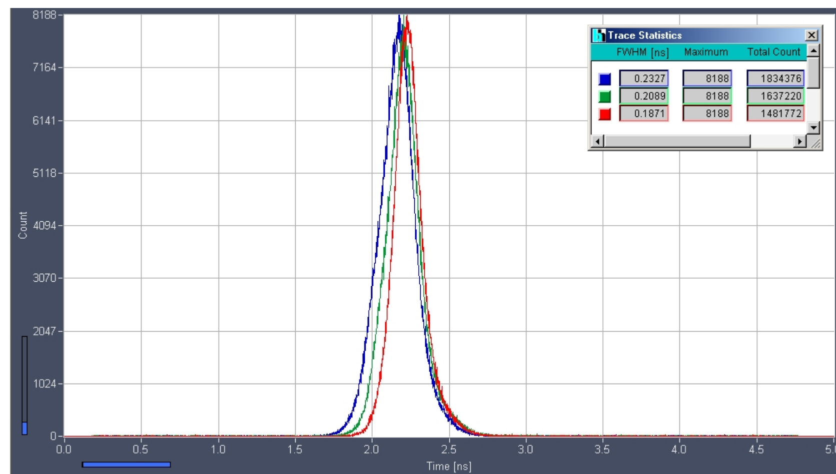

Fig. 9: Temporal instrument response function (IRF) for different x positions on the photocathode

The full-width at half-maximum (FWHM) is 187 to 233 ps. There is a slight change in the IRF shape and position with the position on the photocathode. The shift in the first moment of the IRF is about 75 ps. There are two reasons for this shift. The first one is that the timing signal is derived from one side of the microchannel plate. Therefore there is a non-zero propagation delay from the detection position to the output signal line. The second one is that there is a systematic variation in the shape of the electrical single-photon pulse with the detection position. The shape variation causes a shift of the zero-cross point in the CFD. Both effects result in a dependency of the IRF on the spatial position at which the photons are detected. A shift in the first moment of the IRF induces a shift of approximately the same size in the calculated fluorescence lifetimes. For the FLIM data analysis we therefore used a floating IRF [2].

Discussion

The setup described in this application note is a fully functional wide-field TCSPC FLIM system. The operation of the system is fully integrated in the bh SPCM TCSPC software. Data analysis is performed in the usual way by SPCImage. The data obtained with the system feature good time resolution, and reasonably good spatial resolution. Compared to scanning systems [2, 4], the system does, however, suffer from the general problems of wide-field imaging: Missing suppression of out-of-focus fluorescence and lateral scattering, and contamination by fluorescence and scattering in the optics [5]. These effects restrict the use of the system to thin samples with low internal scattering. Possible applications are TIRF and light-sheet microscopy which are inherently wide-field [6]. There may also be applications which forbid point scanning because of system complexity or temporarily high excitation power. Another application may be combined FLIM/PLIM with phosphorescence markers of millisecond lifetimes [7]. Such long lifetimes require extremely slow scanning but do not pose problems to wide-field FLIM. Wide-field FLIM may also by useful for recording fast physiological processes in cells, see, for example [8]. The time resolution for the physiological effect then would not be limited by the scan rate.

References

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Contact:

Wolfgang Becker
Becker & Hickl GmbH
Berlin, Germany
becker@becker-hickl.com