

# Lifetime Imaging with the Zeiss LSM-510

Wolfgang Becker<sup>a</sup>, Axel Bergmann<sup>a</sup>, Georg Weiss<sup>b</sup>

<sup>a</sup>Becker & Hickl GmbH, Nahmitzer Damm 30, D-12277 Berlin, Germany

<sup>b</sup>Zeiss Jena GmbH, Carl-Zeiss-Promenade 10, D-07745 Jena, Germany

## ABSTRACT

The Zeiss LSM-510 NLO laser scanning microscope can be combined with a new TCSPC (time-correlated single photon counting) lifetime imaging technique developed by Becker & Hickl, Berlin. This technique is based on a three-dimensional histogramming process that records the photon density over the time within the fluorescence decay function and the x-y coordinates of the scanning area. The histogramming process avoids any time gating and therefore yields a counting efficiency close to one. Upgrading the LSM-510 for TCSPC imaging does not require changes in the microscope hardware or software. A fast detector is attached to the fibre output of the scanning head, and synchronisation of the TCSPC module with scanning is achieved via the user I/O of the scan controller. With an MCP-PMT as a detector, fluorescence decay components down to 30 ps can be resolved. The capability of the instrument is shown for the separation of chromophores by their fluorescence lifetime and for CFP/YFP FRET.

## 1. INTRODUCTION

Laser scanning microscopes have initiated a revolution in the investigation of the spatial structure of cells or biological tissue [1]. High contrast fluorescence imaging, depth resolution, 2-photon excitation capability and deep tissue imaging are features beyond the reach of conventional microscopes [2]. Recent developments resulted in instruments that record the fluorescence spectrum in all pixels of the image and therefore allow efficient discrimination between different fluorescence markers [3]. However, to investigate energy transfer processes such as fluorescence quenching or fluorescence resonance energy transfer (FRET) fluorescence lifetime measurement is a more direct approach. Fluorescence lifetime imaging in a scanning microscope can be accomplished by modulation techniques [4,5], gated image intensifiers, gated photon counting [6,7], and time-correlated single photon counting (TCSPC) imaging [8]. At present, only TCSPC imaging is able to achieve a counting efficiency close to one, a time resolution below 50 ps, and a pixel dwell time below 1  $\mu$ s [9,10].

## 2. TCSPC IMAGING

Time-correlated single photon counting (TCSPC) is often believed to be an extremely slow method unable to reach short acquisition times. This ill reputation comes from older NIM based systems used in conjunction with low repetition rate light sources. State-of-the art TCSPC systems reach count rates in the MHz range and therefore are able to record a single decay function within a few ms [10,11]. Furthermore, the traditional one-dimensional histogramming process of TCSPC can be extended to three dimensions [9,10]. This allows to build up a histogram of the photon density over the time in the fluorescence decay function, and the coordinates of the scanning area. The principle of this TCSPC imaging technique is shown in fig. 1.

The recording electronics consists of a time measurement channel, a scanning interface, and a large histogram memory. The time measurement channel contains the usual TCSPC building blocks (CFDs, TAC, ADC) in the 'reversed start-stop' configuration. For each photon, it determines the detection time,  $t$ , referred to the next laser pulse [10].

The scanning interface is a system of counters which receive the scan control signals from the microscope, Frame Clock, Line Clock and Pixel Clock. It determines the current location,  $x, y$ , of the laser spot in the scanning area.

The obtained values of  $t$ ,  $x$ , and  $y$  are used to address the histogram memory. Thus, in the memory the distribution of the photons over the time and the image coordinates builds up. The result can be interpreted as a two-dimensional array of fluorescence decay curves over  $x$  and  $y$  or as a sequence of fluorescence images for different times ( $t$ ) after the excitation pulse.

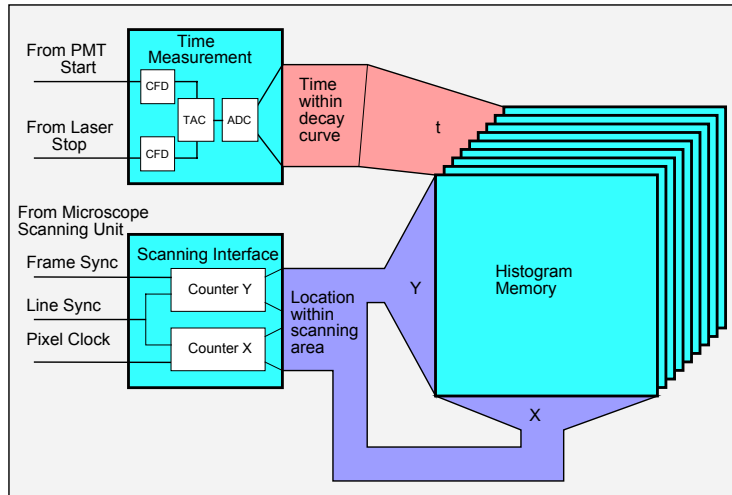


Fig. 1: Principle of TCSPC imaging

It should be pointed out that the histogramming process does not use any time gating. Therefore, the method yields a near-perfect counting efficiency and a maximum signal to noise ratio for a given acquisition time. Due to the short dead time of the TCSPC imaging electronics (180 ns) there is virtually no loss of photons for count rates up to a few  $10^5/s$  as they are typical for cell imaging.

The data acquisition runs at any scanning speed of the microscope. The acquisition can be run over as many frame scans as necessary to collect enough photons. Due to the synchronisation via the scan clock pulses, the zoom and image rotation functions of the microscope automatically act also on the TCSPC recording and can be used in the normal way.

### 3. MICROSCOPE SETUP

The complete instrument consists of a Zeiss LSM-510 NLO Laser scanning microscope, a femtosecond Ti:Sa laser, a fast photomultiplier (PMT), and a Becker & Hickl SPC-730 TCSPC imaging module. The setup is shown in fig. 2.

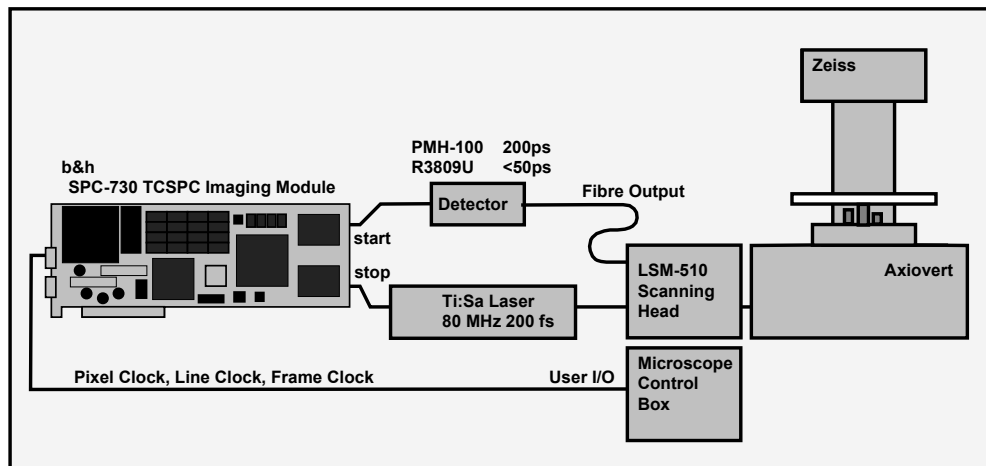


Fig. 2: Setup of the lifetime microscope

The fibre output option of the LSM-510 scanning head is used to feed the detected light into the photomultiplier. Depending in the required resolution we use either a PMH-100 detector head of Becker & Hickl or an R3809U MCP-PMT of Hamamatsu. For each photon, the detector delivers a start pulse to the SPC-730 TCSPC imaging module. The stop pulse for the time measurement is obtained from the monitor diode of the laser. The recording in the SPC-730 module is synchronised with the scanning via the Pixel Clock, Line Clock and Frame Clock signals from the control box of the microscope.

The system response for the PMH-100 and the R3809U is shown in Fig. 3 and 4. The instrument response width is 150 ps to 190 ps with the PMH-100 and 30 ps to 50 ps with the R3809U.

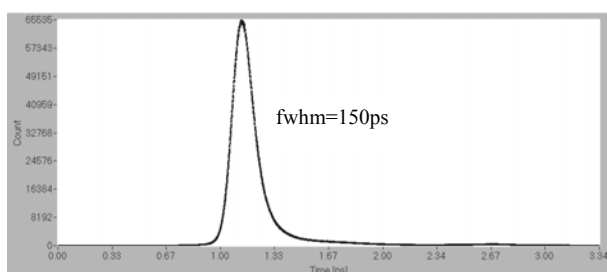


Fig 3: System response for the PMH-100 detector

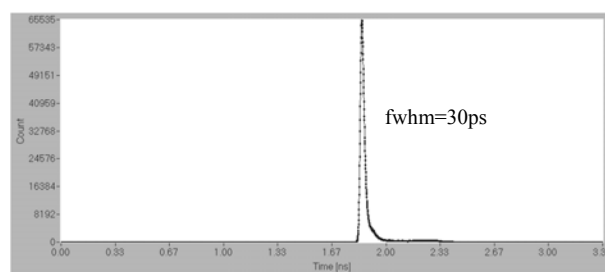


Fig 4: System response for the R3809U

Usually, deconvolution of the measured fluorescence decay from the system response delivers decay times 10 time shorter than the system response. Unfortunately recording the system response in a normal two-photon microscope is almost impossible because the excitation wavelength is blocked by filters, or detectors are used which are insensitive at the excitation wavelength. Furthermore, the sample is excited by a two-photon process while the detector sees the response via one-photon absorption. Therefore the detector need not necessarily see the same pulse shape as the sample. For standard applications we calculate a ‘best guess’ system response from the rising edge of the fluorescence decay functions themselves. The shortest lifetime that can reasonably be determined in this way is in the range of 20 ps to 30 ps.

#### 4. RESULTS

Fig. 5 shows a TCSPC image of a single cell layer (double staining with Hoechst 33342 for DNA and Alexa 488 for tubulin). The detector was a PMH-100 giving an instrument response function of 150 ps fwhm. The overall acquisition time was 60 seconds at a count rate of about  $10^5$  photons per second. The intensity image containing the photons of all time channels is shown left.

Data analysis delivers the fluorescence lifetime  $\tau$  in the individual pixels of the image. This allows to generate intensity- $\tau$  images that display the fluorescence intensity and the fluorescence lifetime as brightness and colour (fig. 5, right). The quality of the fit is shown for two selected pixels (fig.5, bottom). The decay times of 2.0 ns and 2.8 ns are clearly distinguished.

It should be pointed out that distinguishing different dyes is not the main application of lifetime imaging. For most dye combinations this can be done by imaging in different spectral ranges or by fitting a combination of the known fluorescence spectra of the constituents to the measured fluorescence spectrum [3]. The situation can, however, be different for autofluorescence measurements. Usually there are a lot of natural chromophores with ill-defined spectra so that the constituents are hard to distinguish. Furthermore, the fluorescence lifetime of the autofluorescence can be an indicator for essential environment parameters. Fig. 6 shows an autofluorescence intensity-lifetime image of human skin showing significant variations of the lifetime throughout the image.

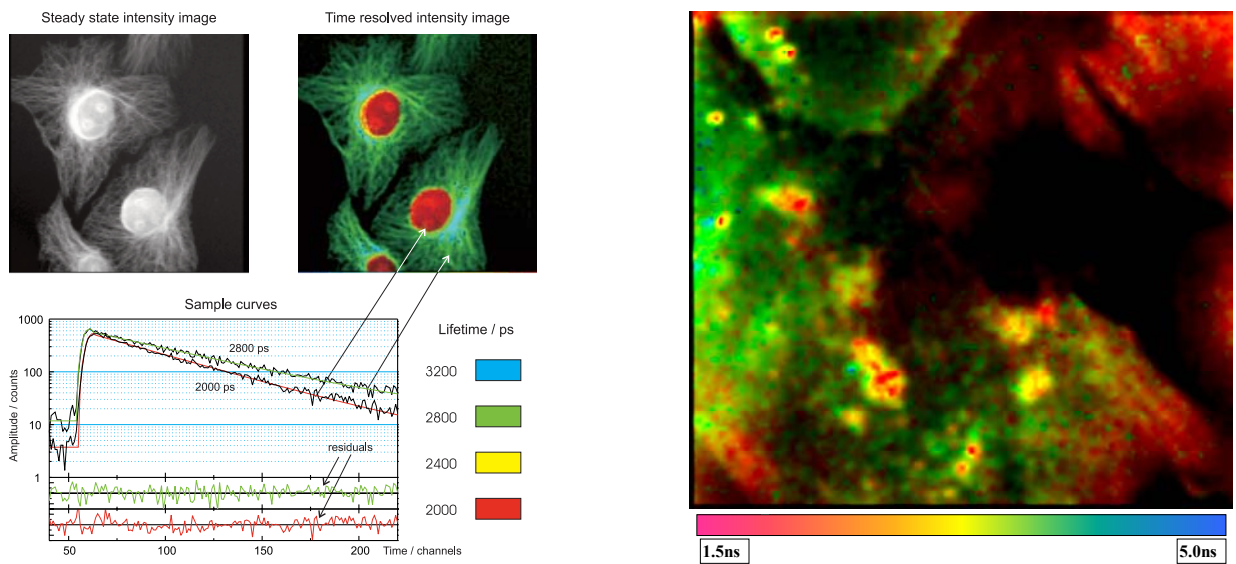


Fig. 5: Intensity image (top left), intensity-lifetime image (top right) and fitted curves. Double-stained cells, Hoechst 33342 and Alexa 488

Fig. 7 shows an HEK cell containing CFP and YFP in the  $\alpha$  and  $\beta 1$  subunits of the Na channels. Due to the overlap of the CFP emission with the YFP absorption FRET can be expected in regions where the Na channels are localised. To resolve the short lifetime of the quenched donor molecules we used the Hamamatsu R3809U-50 for detection. The image represents the lifetime obtained from a single-exponential approximation of the decay functions.

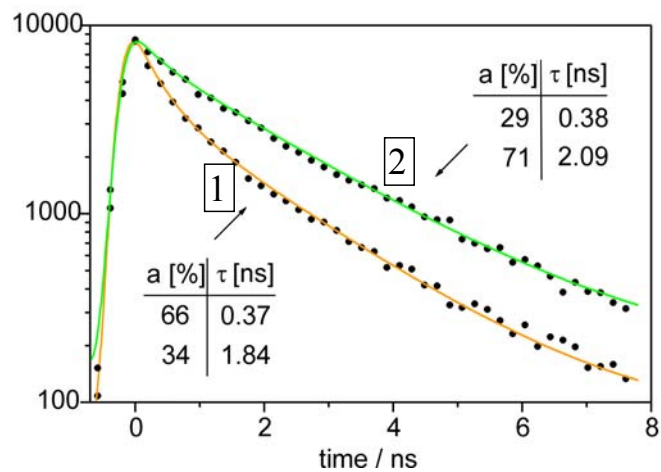
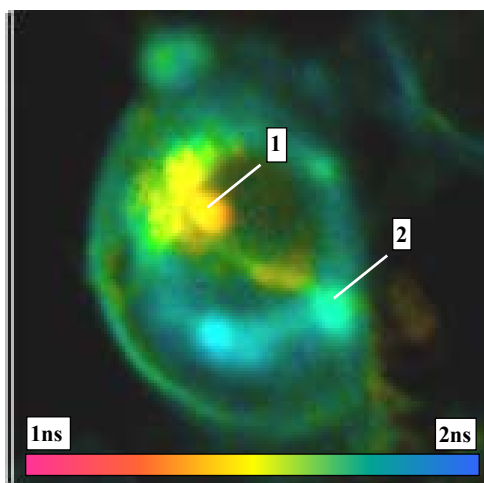


Fig. 7: Intensity-Lifetime Image of Donor (CFP)      Fig. 8: Fluorescence decay curves of selected pixels

Fig. 8 shows the decay functions of two selected pixels of fig. 7. The decay is clearly not mono-exponential. Double exponential analysis of the decay functions reveals a fast component of about 0.37 ns and a slow component of 1.84 ns to 2.09 ns. Both components are found anywhere in the image. However, the ratio of the intensity coefficients of the components differs considerably.

It is reasonable to assume that the fast decay component comes from the quenched CFP molecules and the slow component is fluorescence from the unquenched CFP. While the lifetime of the fast decay component is a measure of the distance of the FRET partners the ratio of the intensity coefficients is an indicator of the fraction of molecules participating in the FRET. Therefore, we build up FRET images showing this ratio as colour. The result is shown in fig. 9. It shows a lot of detail not visible in the pure intensity image and more contrast than the lifetime image obtained from the average lifetime.

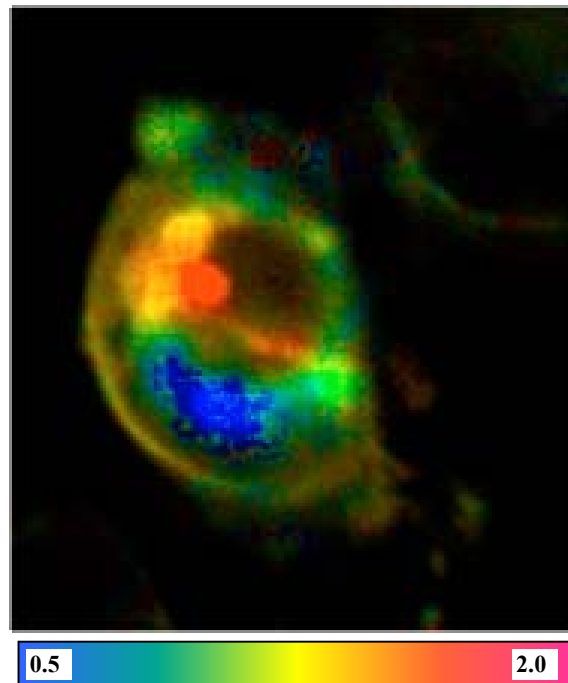


Fig. 9: FRET image built up from intensity (brightness) and ratio of coefficients of fast and slow decay component (colour)

## 5. CONCLUSION

Picosecond fluorescence lifetime imaging can be achieved by upgrading the Zeiss LSM-510 NLO laser scanning microscope with the Becker & Hickl SPC-730 TCSPC imaging module. The instrument is able to resolve double exponential decay functions and lifetimes down to 30 ps. Applied to FRET in living cells, the instrument delivers the decay components of the donor fluorescence in all pixels of the image. The data can be used to build up images showing the amount of FRET in the individual parts of the cell.

## 6. REFERENCES

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Author Information:

Wolfgang Becker  
[www.becker-hickl.de](http://www.becker-hickl.de)  
[becker@becker-hickl.de](mailto:becker@becker-hickl.de)

Axel Bergmann  
[www.becker-hickl.de](http://www.becker-hickl.de)  
[bergmann@becker-hickl.de](mailto:bergmann@becker-hickl.de)

Georg Weiss  
[www.zeiss.de](http://www.zeiss.de)  
[weiss@zeiss.de](mailto:weiss@zeiss.de)