

# FRET Measurements by TCSPC Laser Scanning Microscopy

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## ABSTRACT

We use a two-photon laser scanning microscope with a new Time-Correlated Single Photon Counting (TCSPC) imaging technique to obtain combined intensity-lifetime images for FRET measurements in living cells. Single photon pulses from a photomultiplier and signals from the scanning head are used to record the three-dimensional photon density over the time- and image coordinates. Double exponential decay analysis delivers the lifetime components of the quenched and the unquenched molecules in all pixels of the image. We use the ratio of the intensity coefficients of the fast and slow decay component to create images that show the size of the FRET effects in different parts of the cell.

**Keywords:** Fluorescence microscopy; Spectroscopy, time-resolved

## 1. INTRODUCTION

Fluorescence Resonance Energy Transfer (FRET) measurements are used to investigate the structure of cells on the molecular scale. The method makes use of the fact that the energy transfer rate between the different chromophores depends on the distance of donor and acceptor [1]. Therefore, the fluorescence quantum efficiency of the donor can be used as a distance indicator. Unfortunately, in intensity images quantum efficiency changes cannot be distinguished from concentration variations. Therefore, traditional FRET measurements are done by recording a fluorescence image of the donor, then photobleaching the acceptor, and recording the donor fluorescence again. The increase of the donor fluorescence then gives a measure of the FRET intensity. Of course, living cells barely survive this maltreatment without severe photodamage.

In contrast to simple intensity data, fluorescence lifetime data are a more direct approach to FRET images. The quenching of the donor causes a dramatic decrease of its lifetime so that the FRET shows up clearly. However, to obtain quantitative results the decay of the quenched molecules and the unquenched molecules must be distinguished. This requires high quality decay curves so that a double exponential decay can be resolved.

To record fluorescence lifetime images gated image intensifiers, modulation techniques [2], gated photon counting [3], and time-correlated photon counting (TCSPC) [4, 5] can be used. In conjunction with a scanning microscope, most of these methods have serious drawbacks. Simple gating of the detector electronics discards the majority of the fluorescence photons and thus yields a poor sensitivity. Gated photon counting and single channel modulation techniques have problems with the high scanning speed of the microscope. Although gated photon counting can be accomplished in several subsequent time windows simultaneously [3], it does not resolve the short decay of the quenched donor molecules on the background of the decay of the unquenched molecules.

In contrast, state-of-the art TCSPC systems reach count rates in the MHz range and therefore are able to record decay functions within a few ms [5]. The TCSPC method has a high detection efficiency, a time resolution limited only by the transit time spread of the detector and directly delivers the decay functions in the time domain. Furthermore, the TCSPC method can be combined with a multiplexed detection which is ideally suited for scanning applications [5]. We will show that TCSPC can be used to obtain images that clearly show the size of the FRET effect in different parts of living cells.

## 2. TCSPC LIFETIME IMAGING SETUP

Recording of time-resolved fluorescence images was achieved by combining a laser scanning microscope, a 150 femtosecond Coherent Titanium Sapphire Laser and an advanced Time-Correlated Single Photon Counting (TCSPC) imaging technique developed by Becker & Hickl, Berlin. The basic setup is shown in fig.1.

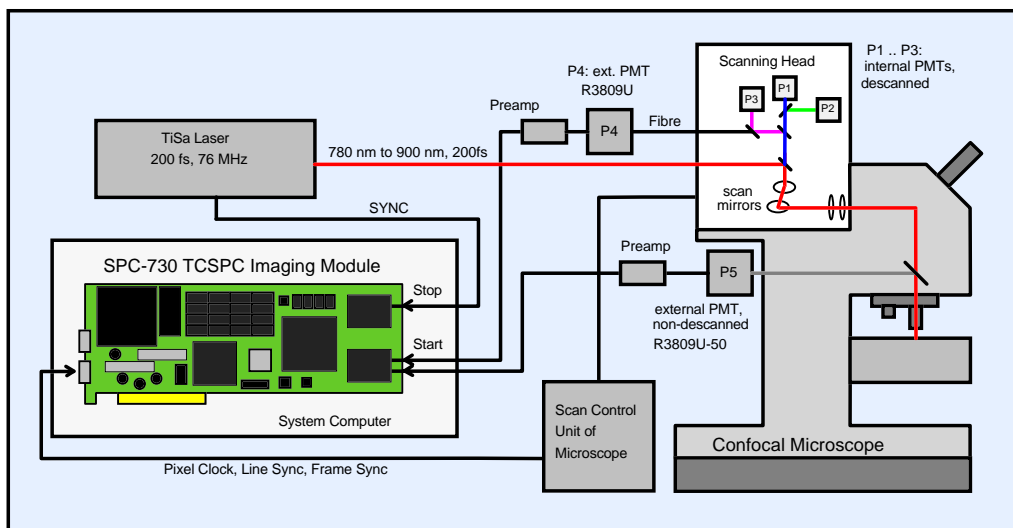


Fig.1: Basic setup of the lifetime microscope

The Ti:Sa laser delivers femtosecond pulses in the wavelength range from 780 nm to 900 nm. We use the laser without a frequency doubler and excite the sample by simultaneous two-photon absorption. Due to the short pulse duration of the laser and the small area of the laser spot in the focal plane of the microscope this works with a high efficiency. The average excitation power at the sample is less than 10 mW.

The microscope scans the sample in the x-y plane providing an image of the sample in the focal plane of the objective lens. We used the Zeiss LSM-510 as well as the older Zeiss LSM-410.

The scanning head of the microscope has several detection channels equipped with variable confocal pinholes, filters, and photomultipliers (PMTs). The PMTs are small side-window tubes which give good sensitivity but not the optimum time resolution in the TCSPC mode. Unfortunately, replacing these PMTs with faster ones is almost impossible.

The remedy is to place a fast detector at the non-descanned port (camera port) of the microscope. This solution has the benefit of high sensitivity, but it works only for two-photon excitation which does not require a pinhole in the detection path. Furthermore, non-scanned detection cannot take advantage of the sophisticated system of dichroics and filters in the scanning head.

To get a high time resolution from the detection channel of a LSM-510 scanning head we use the fibre output option offered by Zeiss. One detector in the scanning head is replaced with the fibre which is connected to a Hamamatsu R3809U-50 MCP PMT. This solution works for one- and two-photon excitation as well, takes advantage of the computer controlled filters of the scanning head and allows to run the lifetime module in parallel with the standard image recording electronics of the microscope.

Data acquisition is accomplished by the Becker & Hickl SPC-730 TCSPC Imaging module [5]. The CFD input receives the single photon pulses of the PMT. Synchronisation with the laser pulse sequence is achieved by the SYNC signal from the reference diode of the Ti:Sa laser.

The SPC-730 module gets the scan control signals, Pixel Clock, Line Sync, and Frame Sync, from the scan controller of the microscope. Connecting the TCSPC module does not require any modifications in the microscope hardware and software. The scan parameters are controlled in the usual way via the microscope software. The normal scan speed, the zoom function, region of interest setting, etc. can be used also when recording lifetime images. Furthermore, the SPC-730 can be run in parallel with the standard image recording electronics of the microscope. Basically, steady state images and lifetime images in different spectral ranges can be obtained at the same time.

The principle of the SPC-730 TCSPC Imaging module is shown in fig. 2. The module employs an advanced TCSPC technique featuring multi-decay recording, high count rate and low differential nonlinearity. It contains the usual building blocks (CFDs, TAC, ADC) in the 'reversed start-stop' configuration together with a scanning interface and a large histogram memory integrated on one board [5].

For each PMT pulse, i.e. for each photon, the TCSPC module determines the time of the photon in the laser pulse sequence and the beam location in the scanning area. These values are used to address the histogram memory in which the events are accumulated. Thus, in the memory the distribution of the photon density over X, Y, and the time within the fluorescence decay function builds up.

Interestingly, there is practically no loss of photons in the TCSPC imaging process. Due to the short dead time of the TCSPC imaging module (180 ns) nearly all detected photons are processed and accumulated in the histogram. This is a striking benefit compared to gated image intensifiers which gate off the majority of the photons.

The actual time resolution depends on the detector and is 300 ps to 500 ps (fwhm) with the PMTs typically built-in in the microscope, and 28 ps (fwhm) with the external Hamamatsu R3809U-50 MCP. The system response for the R3809U-50 is shown in fig. 3.

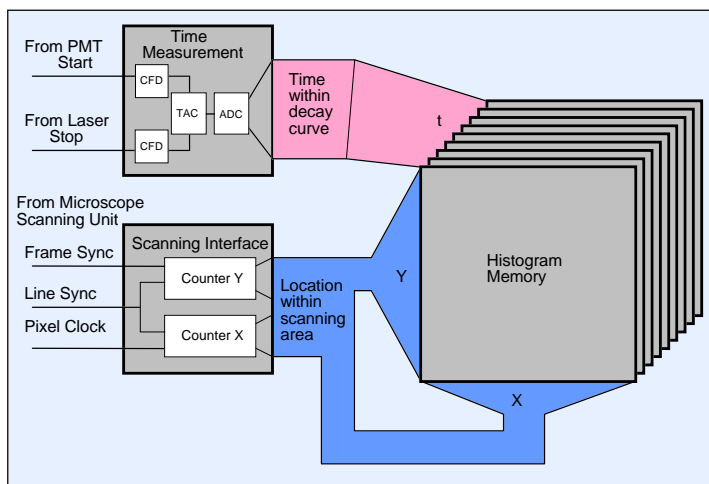


Fig. 2: Principle of the TCSPC Lifetime Imaging module

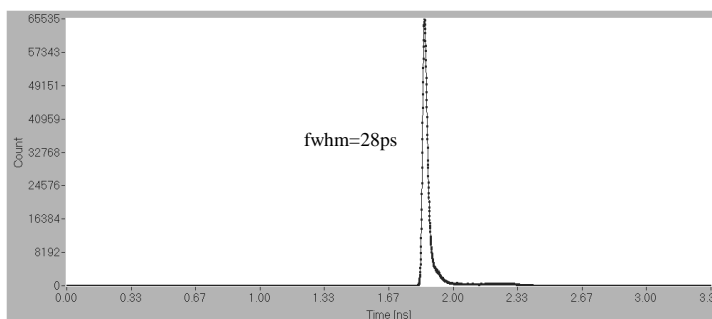


Fig 3: System response with the R3809U-50 MCP

### 3. RESULTS

Fig. 4 shows VERO cells containing CFP and YFP in different subunits of the cell. CFP acts as a donor and YFP as an acceptor. The microscope was a Zeiss LSM-410 with a Hamamatsu R3809U-50 MCP at the non-descanned port.

Fig. 4, left, shows an intensity image of the donor fluorescence obtained by summing the photons from all time channels of the individual pixels. Fig. 4 (centre) shows an intensity-lifetime image built up from the fluorescence intensity and the average decay time obtained by weighting the decay components with their relative intensity coefficients. The intensity was used as brightness and the lifetime as colour. The image clearly shows lifetime differences throughout the cell.

Fig. 5 shows the decay functions of selected pixels of fig. 4. The decay is clearly not single exponential. Double exponential decay analysis reveals a fast lifetime component of about 0.3 ns to 0.4 ns and a slow component of 1.8 ns to 2.0 ns. The fast component most likely comes from the quenched CFP molecules while the slow component is fluorescence from the unquenched CFP and perhaps some YFP fluorescence. Both components are found anywhere in the image. However, the ratio of the intensity coefficients of the components differs considerably between regions with strong FRET and 'normal' regions. This indicates that the ratio of the intensity coefficients of the lifetime components might be a better FRET indicator than the average lifetime. The result is shown in fig. 4, right. This image was created by using the intensity as brightness and the ration of the decay coefficients as colour. It shows a lot of detail not visible in the pure intensity image and higher contrast than the image obtained from the average lifetime.

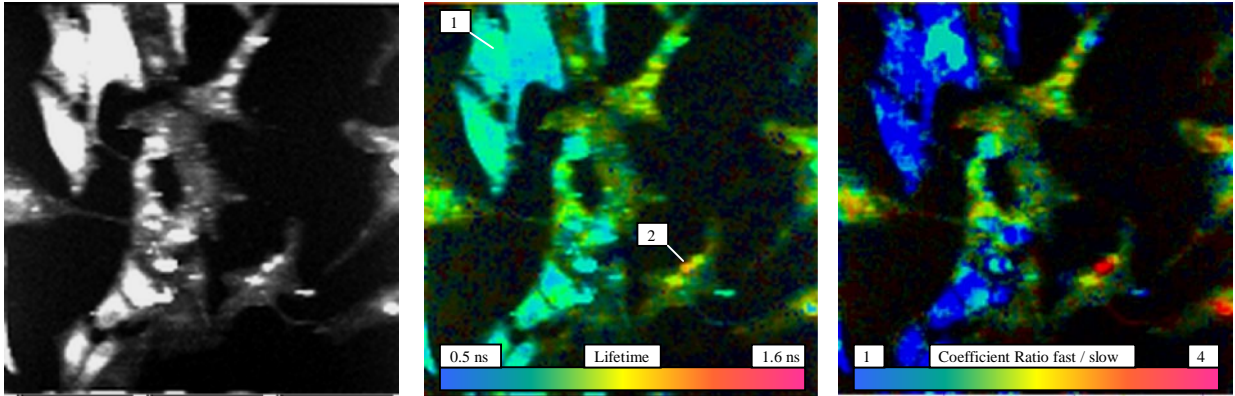


Fig. 4: VERO cells containing GFP and YFP in different subunits of the cell, LSM-410, R3809U-50 MCP, non-descanned detection. Left: Intensity image, Centre: Intensity / Lifetime image, Right: FRET image showing the ratio of the intensity coefficients of slow and fast lifetime component

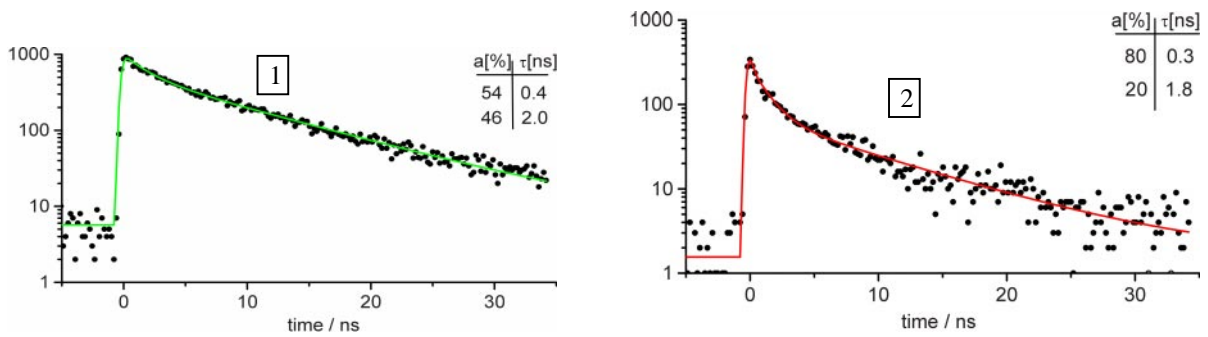


Fig. 5: Decay functions of selected areas of fig. 4. Left: Non-FRET region, Right: Region with strong FRET

The non-descanned setup used for the measurements shown above can be awkward if there is no proper laser blocking filter and no filter to select the emission wavelength in the microscope. Fig. 6 shows a result obtained through the descanned path of the LSM-510 and the fibre output option. The sample is an HEK cell containing CFP and YFP in the  $\alpha$  and  $\beta 1$  subunits of the Na channels.

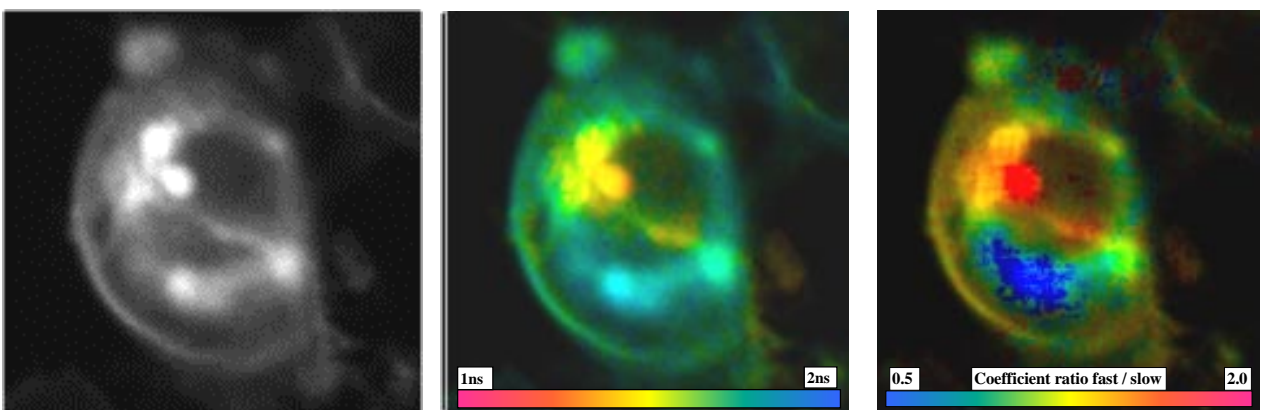


Fig. 6: HEK cells imaged via the fibre output of the LSM-510 scanning head. Left: Intensity image, Centre: Intensity / Lifetime image, Right: FRET image showing the ratio of the intensity coefficients of slow and fast lifetime component

Fig. 6 shows that good FRET results can also be obtained by using the descanned (confocal) detection path of the LSM-510 laser scanning head. There is probably some loss of intensity due to the fibre and the large number of optical components in the detection path, but the loss is not so substantial as to impair the quality of the results. The advantage of the confocal detection path is the simple selection of the detection wavelength, the uncomplicated suppression of scattered laser light and the ability to record several wavelength channels simultaneously.

#### 4. CONCLUSIONS

A new TCSPC imaging technique in conjunction with a scanning microscope yields high quality fluorescence lifetime data. Applied to FRET in living cells, the technique delivers the decay components of the quenched and unquenched donor molecules. The ratio of the intensity coefficients of the quenched and the unquenched fluorescence component can be used to build up images that show the size of the FRET effect in the individual parts of the cell. The images show detail not visible in pure intensity images and better contrast than images created from the average lifetime.

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