

Advanced time-correlated single photon counting technique for spectroscopy and imaging in biomedical systems

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Abstract

Time-correlated single photon counting (TCSPC) is based on the detection of single photons of a periodic light signal, measurement of the detection time of the photons, and the build-up of the photon distribution versus the time in the signal period. TCSPC achieves a near ideal counting efficiency and transit-time-spread-limited time resolution for a given detector. The drawback of traditional TCSPC is the low count rate, long acquisition time, and the fact that the technique is one-dimensional, i.e. limited to the recording of the pulse shape of light signals. We present an advanced TCSPC technique featuring multi-dimensional photon acquisition and a count rate close to the capability of currently available detectors. The technique is able to acquire photon distributions versus wavelength, spatial coordinates, and the time on the ps scale, and to record fast changes in the fluorescence lifetime and fluorescence intensity of a sample. Biomedical applications of advanced TCSPC techniques are time-domain optical tomography, recording of transient phenomena in biological systems, spectrally resolved fluorescence lifetime imaging, FRET experiments in living cells, and the investigation of dye-protein complexes by fluorescence correlation spectroscopy. We demonstrate the potential of the technique for selected applications.

Keywords: Time-correlated single photon counting, Time-resolved fluorescence, FLIM, FRET, Autofluorescence

1. Introduction

Optical spectroscopy techniques have found a wide range of applications in biomedical imaging and sensing because they are non-destructive and deliver biochemically relevant information about the systems investigated [1].

Typical applications are one- and two-photon fluorescence laser scanning microscopy, fluorescence endoscopy, control of drug delivery in photodynamic therapy, dynamics of protein-dye complexes on the single molecule level, chlorophyll fluorescence dynamics, and diffuse optical tomography of thick tissue.

Most of these techniques use the fluorescence of exogenous or endogenous fluorophores to obtain information about the systems investigated. In the majority of the applications the fluorescence intensity, fluctuations of the fluorescence intensity, or the fluorescence spectra are recorded. However, the fluorescence of organic fluorophores is not only characterised by its intensity or spectrum, it has also a characteristic fluorescence lifetime.

The fluorescence lifetime as an additional separation parameter is particularly useful to distinguish the fluorescence components of endogenous fluorophores in cells and tissues. These components often have poorly defined fluorescence spectra but are clearly distinguished by their fluorescence lifetime [2,3].

Moreover, the fluorescence lifetime is a direct indicator of the energy transfer rate from the excited molecules to the local environment or to other fluorophores [4]. Since the lifetime in a wide range does not depend on the concentration of the fluorophore its measurement is a direct approach to all effects that require the detection of changes in the quantum efficiency of a fluorophore. Typical examples are the mapping of cell parameters such as pH, ion concentrations or oxygen saturation by fluorescence quenching [5,6,7,8], and the probing of protein or DNA structures by lifetime sensitive dyes [9,10], and control of photodynamic therapy [45].

The distance between two different fluorophores can be probed by fluorescence resonance energy transfer (FRET). FRET occurs if the fluorescence emission band of one fluorophore, the donor, overlaps the absorption band of a second one, the acceptor. In this case the energy from the donor can be transferred directly into the acceptor, resulting in an extremely efficient quenching of the donor fluorescence. The energy transfer rate from the donor to the acceptor depends on the sixth power of the distance. FRET occurs on distances of the order of a few nm and therefore happens only if the

donor and acceptor are physically linked. With fluorescence lifetime imaging techniques, FRET results are obtained from a single lifetime image of the donor [11-14].

Single-molecule techniques usually record the fluctuations of the fluorescence intensity of a small number of emitters in a femtoliter sample volume [15]. Also here, simultaneous recording of the fluorescence lifetime helps to distinguish between different fluorophores, and to reveal quenching and FRET effects.

Optical tomography sends near-infrared light through thick tissue and investigates the diffusely transmitted or reflected light. With steady-state techniques, absolute scattering and absorption coefficients of the tissue and their variation throughout the sample cannot be obtained without presumptions about the sample parameters. Time-resolved measurements, i.e. illumination by pulsed or modulated light and detection of the waveform of the transmitted or reflected light, deliver the reduced scattering and absorption coefficients [16-18]. These, in turn, can be used to derive biochemically relevant parameters, such as oxygen saturation, or haemoglobin contents [19].

Biological systems often show dynamic changes in their fluorescence, absorption or scattering behaviour. Dynamic changes have to be recorded in living plants [20], flash photolysis, stopped flow experiments, experiments for photodynamic therapy [21], and brain imaging by diffuse optical tomography.

At the same time, the excitation power must be kept as low as possible to avoid light-induced changes, photobleaching or photodamage in the sample. A good fluorescence technique should therefore have a high recording efficiency, i.e. should not discard any photons, neither by gating off photons on the time axis, nor by blocking a part of the emission spectrum by filters. Thus, it should record the entire fluorescence decay simultaneously in several detection channels, covering the entire emission spectrum.

The time resolution must be high enough to resolve the fluorescence lifetimes of the typical endogenous and exogenous fluorophores, and the diffusion times of photons through tissue. Typical lifetimes and diffusion times are of the order of a few nanoseconds. However, the lifetimes in presence of strong fluorescence quenching, the lifetime of the quenched donor fraction in FRET experiments, and the lifetimes of short autofluorescence components can be as short as 100 ps [2,3,11]. Lifetimes down to 50 ps are found in dye aggregates [22] and complexes of dyes and metallic nano-particles [23,24]. Due to the mixture of different chromophores or non-uniform quenching the fluorescence decay functions found in cells and tissue are normally multi-exponential.

Therefore, a time-resolved spectroscopy technique for biomedical application should be able to record fast changes in the sample, resolve the components of multi-exponential fluorescence decay functions down to less than 100 ps, have imaging capability, multi-wavelength capability, and a near-ideal detection efficiency.

Time-resolved optical recording techniques are normally classified into frequency domain techniques, and time-domain techniques. Frequency domain techniques measure the phase shift between the high-frequency modulated or pulsed excitation and the fluorescence signal at the fundamental modulation frequency or its harmonics [25-30]. Time-domain techniques record the fluorescence decay functions directly [31-39]. Although the frequency-domain and the time domain are equivalent, the corresponding signal recording techniques may differ considerably in efficiency, i.e. in the lifetime accuracy obtained for a given number of detected photons [40,41].

For fluorescence lifetime imaging, both the frequency-domain and time-domain FLIM techniques can use either camera techniques or point-detector scanning techniques. The benefit of the camera techniques is that they are relatively easy to use. However, camera techniques do not exploit the benefits of the scanning technique, such as depth resolution, optical sectioning capability, and deep tissue imaging by two-photon excitation [42,43]. On the other hand, point-detector techniques can only be used in conjunction with scanning, but are fully compatible with commercial confocal or two-photon laser scanning microscopes.

Frequency domain camera techniques are based on modulated image intensifiers, frequency domain point-detector techniques on modulated photomultiplier tubes (PMTs) or on photomultiplier tubes with subsequent electronic mixers. Time domain camera techniques use gated image intensifiers, time-domain point-detector scanning techniques time-correlated single photon counting (TCSPC), or gated photon counting with several parallel time-gates.

Among all these techniques, TCSPC yields the highest recording efficiency and the highest time resolution [39-41].

Time-Correlated Single Photon Counting [39] is based on the detection of single photons of a periodical light signal, the measurement of the detection times of the individual photons within the signal period, and the reconstruction of the waveform from the individual time measurements. The TCSPC technique makes use of the fact that for low level, high repetition rate signals the light intensity is usually so low that the probability to detect one photon in one signal period is

much less than one. Therefore, the detection of several photons per signal period can be neglected and the principle shown in the fig. 1 be used.

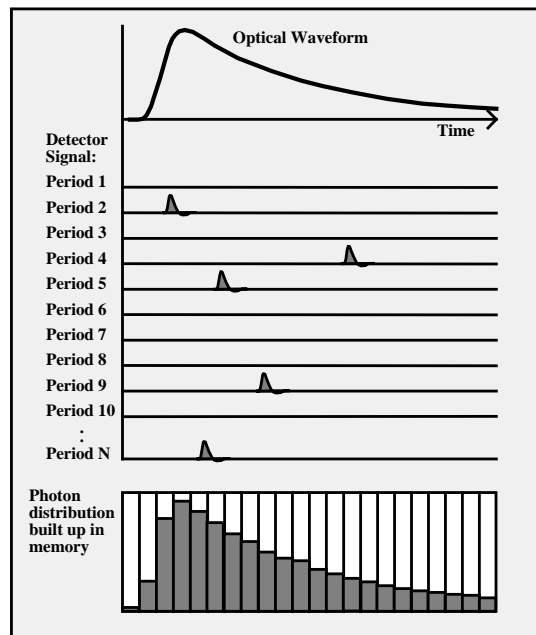


Fig. 1: Principle of classic time-correlated single-photon counting

There are many signal periods without photons, other signal periods contain one photon pulse. Periods with more than one photons are very rare. When a photon is detected, the time of the corresponding detector pulse is measured. The events are collected in a memory by adding a '1' in a location with an address proportional to the detection time. After many photons, in the memory the distribution of the detection times, i.e. the waveform of the optical pulse builds up.

The TCSPC technique does not use any time-gating and therefore, as long as the light intensity is not too high, reaches a counting efficiency close to one. The time resolution is limited by the transit time spread in the detector. With fast detectors, a width of the instrument response function (IRF) of 25 ps can be achieved. Moreover, the width of the time channels of the recorded photon distribution can be made as small as 1 ps. The small time bin width in conjunction with the high number of time channels available makes it possible to sample the signal shape adequately according to the Nyquist theory. Therefore standard deconvolution techniques can be used to determine fluorescence lifetimes much shorter than the IRF width and to resolve the components of multi-exponential decay functions.

The drawback of classic TCSPC instruments was that their counting capability was very limited, i.e. they were restricted to very low light intensities, which resulted in extremely long acquisition times. Moreover, the technique was intrinsically one-dimensional, i.e. only the waveform of the light signal in one spot of a sample and at one wavelength was recorded at a time.

2. Multi-dimensional TCSPC

Advanced TCSPC techniques are able to record a photon distribution not only versus the time in the fluorescence decay, but simultaneously versus the wavelength, the coordinates of a scanning area, or the time from the start of the experiment. Moreover, the applicable count rates have been increased by two orders of magnitude [38], with a corresponding reduction in acquisition time. The principle of multi-dimensional TCSPC is shown in fig. 2.

At the input of the detection system are a number of photomultipliers (PMTs), or a multi-anode PMT with 16 or 32 detection channels. Typically, the PMTs or PMT channels are detecting in different wavelength intervals. In the subsequent 'router' the photon pulses of the PMTs are combined into a common timing pulse line. Moreover, for each photon, the router delivers the number of the PMT channel in which the photon was detected [38]. TCSPC in general makes use of the fact that for low level, high repetition rate signals the light intensity is so low that the probability to detect one photon in one signal period is much less than one. That means, the combination of the photon pulses and the generation of the PMT channel number is possible without overlap of photons from different PMT channels.

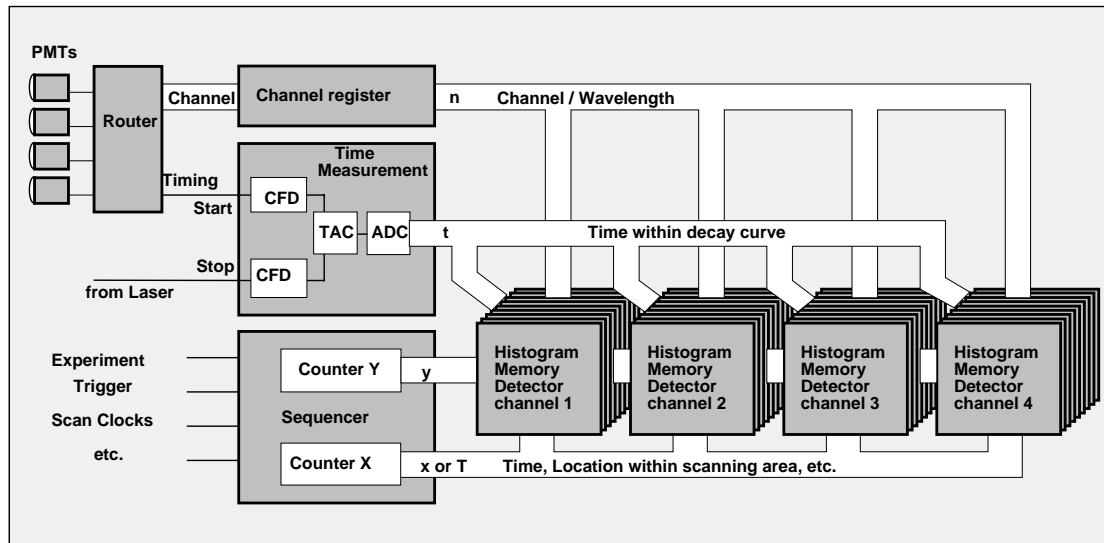


Fig. 2: Multi-dimensional time-correlated single photon counting

The timing pulse is sent through the normal time-measurement block of the TCSPC device. Two constant fraction discriminators, CFD, are receiving the photon pulses and the reference pulses from the light source. A time-to-amplitude converter, TAC, is started with the photon pulse and stopped by the next reference pulse. A subsequent analog-to-digital converter delivers the digital equivalent of the photon time. The architecture of the time-measurement channel is similar to a classic TCSPC device. However, in classic TCSPC only the time-measurement channel exists, and only a one-dimensional photon distribution over the time in the fluorescence decay is built up.

Multi-dimensional TCSPC uses the detector channel number of a photon as a second dimension of the photon distribution. Moreover, it generates a third or even a fourth dimension in a 'sequencer' block. The sequencer is a system of counters that are controlled by several external signals. The counters deliver one or two additional dimensions of the photon distribution. In the simplest way, the counters are driven by a fixed clock frequency and restarted by an external 'experiment trigger' signal. The result is a photon distribution over the time in the fluorescence decay, the wavelength, and the time since the experiment trigger pulse. It can be interpreted as a number of data sets for the individual wavelength intervals, each containing a sequence of fluorescence decay curves for consecutive time intervals.

The counters can also be controlled by line and frame clocks of an external optical scanner, i.e. a laser scanning microscope. The counters then deliver the location, X and Y of a photon within the scanning area. The recording process builds up the photon distribution over the time in the fluorescence decay, the wavelength, and the scanning coordinates. The result can be interpreted as a number of data blocks for the individual wavelength intervals, each containing a large number of images for consecutive times in the fluorescence decay. The recording works at any scan rate and can be continued over as many frames as required to reach the desired number of photons in the photon distribution. Because the multi-dimensional TCSPC technique avoids any time-gating and wavelength scanning the recording efficiency is close to one for each of the PMT channels.

3. Recording dynamic changes of fluorescence lifetime

A typical example of dynamic fluorescence lifetime changes are the chlorophyll transients found 1931 by Kautsky and Hirsch. When a dark-adapted leaf is exposed to light the intensity of the chlorophyll fluorescence starts to increase. After a steep rise the intensity falls again and finally reaches a steady-state level. The rise time is of the order of a few milliseconds to a second, the fall time can be from several seconds to minutes. The initial rise of the fluorescence intensity is attributed to the progressive closing of reaction centres in the photosynthesis pathway. Therefore the quenching of the fluorescence by the photosynthesis decreases with the time of illumination, with a corresponding increase of the fluorescence intensity. The fluorescence quenching by the photosynthesis pathway is termed 'photochemical quenching'. The slow decrease of the fluorescence intensity at later times is termed 'non-photochemical

quenching'. Non-photochemical quenching seems to be essential in protecting the plant from photodamage, or may be even a result of moderate photodamage.

Fig. 3 shows a sequence of fluorescence decay curves obtained from a leaf by TCSPC. A picosecond diode laser of 650nm wavelength, 50 MHz repetition rate, and 60 ps pulse width was used for excitation. The beam was focused to obtain a power density of about $1\text{mW}/\text{mm}^2$. The laser was repeatedly switched on for 13 ms in intervals of 0.5 s. A Hamamatsu H5773P-1 photosensor module and a Becker & Hickl SPC-630 TCSPC module was used for fluorescence detection. For each on-period, a sequence of 128 fluorescence decay curves was recorded with an acquisition time of 100 microseconds each. 10,000 off-on periods were accumulated to obtain enough photons in each of the curves.

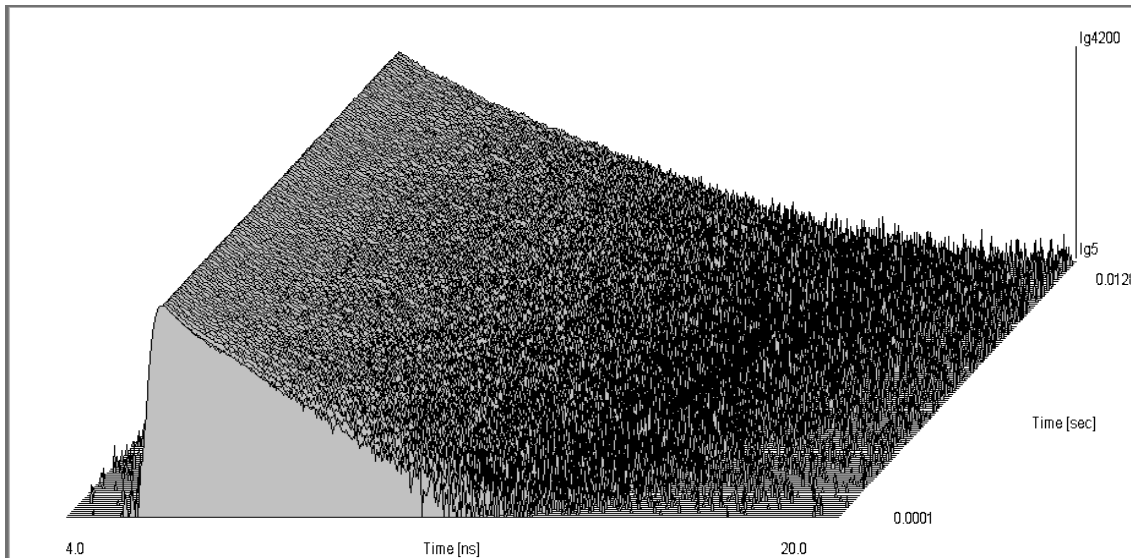


Fig. 3: Sequence of fluorescence decay curves obtained from a leaf after switching on the excitation laser. 10,000 off-on periods were accumulated

The result shows clearly the increase of the fluorescence lifetime with the exposure. The peak intensity is the same for all curves, which indicates that the intensity of photochemical quenching changes, not the concentration of the fluorescing chlorophyll molecules.

The non-photochemical quenching is shown in Fig. 4. A single sequence was started when the laser was switched on. 50 decay curves with a collection time of 2 s each were recorded. For better visibility, the sequence starts at the back. A considerable decrease in lifetime is observed in a fresh leaf (left) but not in a dry leaf (right). Also here, the change is mainly in the quenching intensity, not in the concentration of the chlorophyll.

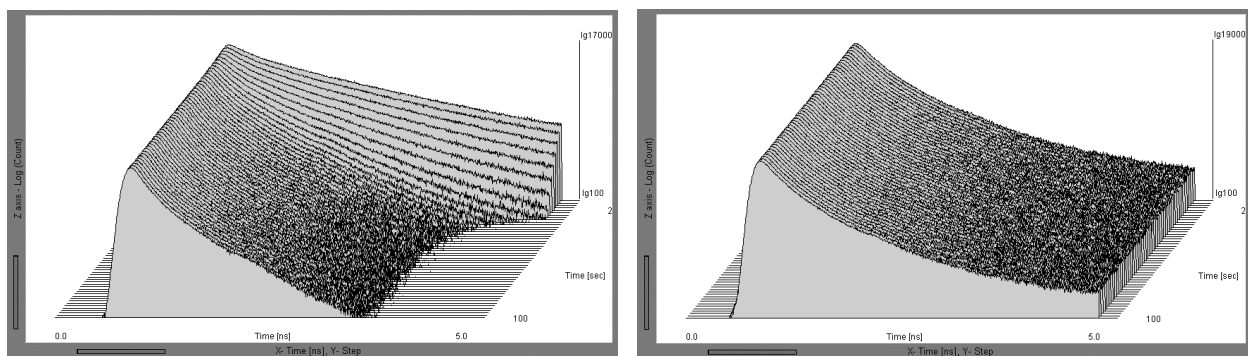


Fig. 4: Sequence of fluorescence decay curves obtained from a fresh leaf (left) and a dry leaf (right) after switching on the excitation laser. A single sequence of 50 curves was recorded with 2 s per curve. Sequence starts at the back.

4. Multi-wavelength recording

Multi-detector operation and multi-wavelength recording with multi-dimensional TCSPC technique has been described previously [12,38,44]. As an example, fig. 5 shows decay curves of a mixture of rhodamine 6G and fluorescein, both at a concentration of $5 \cdot 10^{-4}$ mol/l. The fluorescence was excited by a 405 nm ps diode laser at a repetition rate of 20 MHz. The detector was a R5900-L16 (Hamamatsu) in a PML-16 (Becker & Hickl) detector head. The detector head contains the routing electronics, i.e. delivers the detector channel number and the timing pulse to the TCSPC module. The fluorescence signal was spread spectrally by a polychromator (MS 125-8M, Polytec) over the cathode area of the R5900-L16. The high concentration causes strong re-absorption of the Rhodamin 6G fluorescence, with a clearly visibly change in the decay curves and an increase of the lifetime.

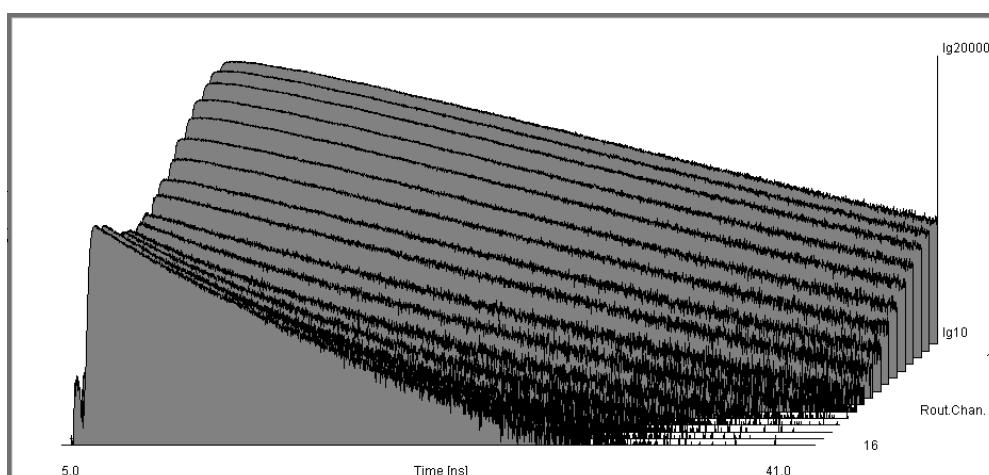


Fig. 5: Fluorescence of a mixture of Rhodamin 6G and fluorescein, simultaneously recorded over time and wavelength

Because the complete photon distribution is recorded simultaneously, the efficiency of the measurement is 16 times higher than for a wavelength scan of comparable resolution. This is an important benefit in all applications where sensitivity is an issue or sample exposure has to be minimised.

5. Time-resolved laser scanning microscopy

An application of the sequencing capability of multi-dimensional TCSPC to confocal laser scanning microscopy is shown in fig. 6 and 7. A Leica TCS SP2 D-FLIM confocal microscope was used to record the images. The sample is scanned by a 405 nm picosecond diode laser and the photons are detected by a Hamamatsu H5773P photosensor module. A TCSPC-module (SPC-830, Becker & Hickl) records the photon distribution over the fluorescence decay and the scanning coordinates.

Fig. 6, left, shows a fluorescence lifetime image of a plant tissue sample. The fluorescence decay function in a selected pixel of the recorded data array, and a double exponential Levenberg-Marquardt fit are shown in fig. 7. The decay is clearly double-exponential and cannot be reasonably approximated by a single exponential decay. For the lifetime image the lifetime components of a double exponential fit are weighted with their intensity coefficients and averaged. This mean lifetime is displayed as colour in the lifetime image.

Merging the components of the double exponential decay into a mean lifetime yields a single-exponential approximation of the data and therefore neglects useful information. An example for using the parameters of multi-exponential decay data is shown in fig. 6, right. It shows an image that displays the colour-coded ratio of the intensity coefficients of both lifetime components. Although the coefficient-ratio image has some similarity with the image of the mean lifetime it directly represents the concentration ratio of the molecules emitting the fast and slow decay component. It therefore can be used to separate different fluorescent species with more or less similar spectra in a single pixel. A typical application is FRET, where TCSPC lifetime imaging delivers the ratio of the quenched and unquenched fraction of the donor molecules [11-13].

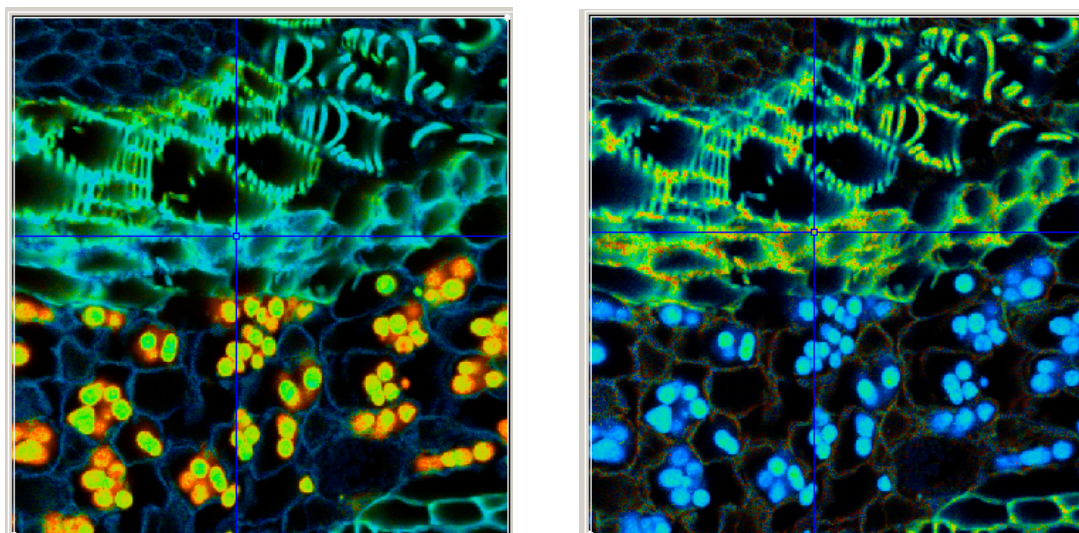


Fig. 6: TCSPC lifetime image recorded in a confocal laser scanning microscope. Left: Colour represents the mean lifetime of the double exponential decay, blue to red = 200 ps to 2 ns. Right: Colour represents the ratio of the intensity coefficients of the two fast and slow decay component, blue to red = 1 to 10.

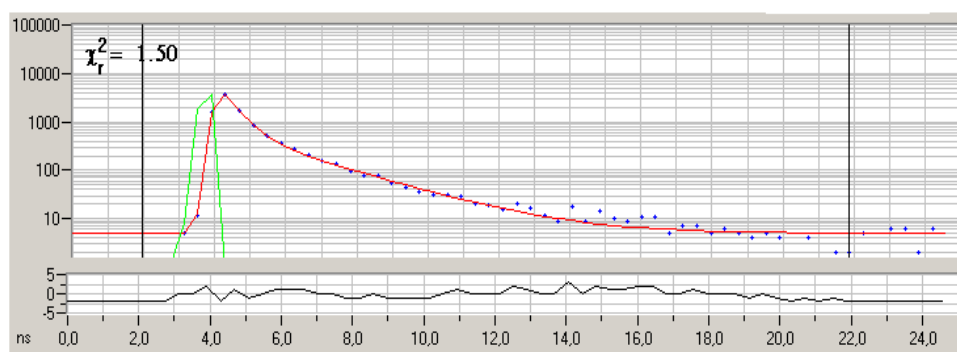


Fig. 7: Fluorescence decay function in a selected pixel of the data array, double exponential Levenberg-Marquardt fit and residuals of the fit. Data points are shown blue, the fitted curve red, and the instrument response function green. The selected pixel is marked in fig. 6.

6. Conclusions

Time-correlated single photon counting has developed from a slow and intrinsically one-dimensional fluorescence lifetime technique into a fast multi-dimensional optical recording technique. The photon distribution can be recorded simultaneously over the time within the excitation pulse sequence, the wavelength, the time from the start of an experiment, and over the coordinates of a scanning area. The multi-dimensional recording process does not use any time-gating or wavelength scanning and therefore works with near-ideal efficiency. This makes the technique suitable for a large number of biomedical spectroscopy applications, such as autofluorescence detection, diffuse optical tomography, and fluorescence lifetime microscopy.

5. References

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