Multi-dimensional time-correlated single photon counting

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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. 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 include 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, TCSPC, FLIM, FRET

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.

Typical applications are one- and two-photon fluorescence laser scanning microscopy [25], fluorescence imaging of tissue [19], in-vivo drug screening, monitoring of the photoconversion of sensitizers in photodynamic therapy [30], dynamics of protein-dye complexes on the single molecule level [12, 13, 28], chlorophyll fluorescence dynamics [15, 23], and diffuse optical tomography (DOT) of thick tissue [16, 21].

Optical techniques applied to biological objects are faced with the problem that only a limited number of photons is available from the sample. The reason may be a limited photostability of the sample, a limited excitation dose or excitation power due to sample stability or safety regulations, or a limited acquisition time. The efficiency (or photon economy) of a measurement technique is therefore important [14, 18].

Moreover, biological systems often show dynamic changes in their fluorescence, absorption or scattering behaviour. Examples are chlorophyll transients in living plants [15, 23], transport mechanisms in living cells, diffusion or conformational changes of labelled proteins [6, 12, 13, 28], internalization [17] or photoconversion [30] of photosensitizes, or haemodynamic changes in tissue [21]. Distortion of the recorded data by such effects either must be avoided, or the changes themselves are to be recorded. This not only sets additional constraints to the acquisition time but also requires spectral and temporal information to be recorded simultaneously.

The time resolution needed in biomedical spectroscopy ranges over many orders of magnitude. Chlorophyll transients effects occur on a time scale from milliseconds to seconds, diffusion effects and conformational changes of proteins are found on the microsecond and millisecond scale, and haemodynamic changes occur at a time scale of a few seconds. Simultaneously with these effects the fluorescence decay or the time-of-flight distribution of the photons through thick tissue has to be recorded. The width of time-of-flight distributions in DOT is on the order of 1 ns [16, 21]. Fluorescence decay times of the commonly used fluorophores are on the same order of magnitude [20]. However, the fluorescence lifetimes in presence of fluorescence quenching, the lifetime of the interacting donor fraction in FRET experiments [3], and the lifetimes of short autofluorescence components can be as short as 100 ps [6, 19]. Lifetimes down to 50 ps are found in dye aggregates [17] and complexes of dyes and metallic nano-particles [22]. Due to the mixture of different fluorophores or non-uniform quenching the fluorescence decay functions found in cells and tissue are normally multi-exponential. Recording these effects simultaneously with slower changes in the investigated objects not only requires a high time resolution but also some kind of ‘double-kinetic’ capability, i.e. fast sequential recording of fluorescence decay functions or time-of-flight functions. Moreover, fluorescence may be observed from a small number of molecules in a femtoliter volume. Signals obtained in such experiments show fluctuations by diffusion, conformational changes, rotation, and intersystem crossing. Resolving these effects requires to record relative and absolute time of the individual photons.

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2. TIME-CORRELATED SINGLE PHOTON COUNTING

Among all time-resolved optical detection techniques, TCSPC yields the highest recording efficiency and the highest time resolution for a given detector [1, 18]. Time-Correlated Single Photon Counting [24] 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 intensity, high repetition rate signals the probability of detecting one photon per signal period is much smaller than one. The detection of a second photon within one excitation period is so unlikely that it can be neglected. The principle of classic TCSPC is shown in Fig. 1.

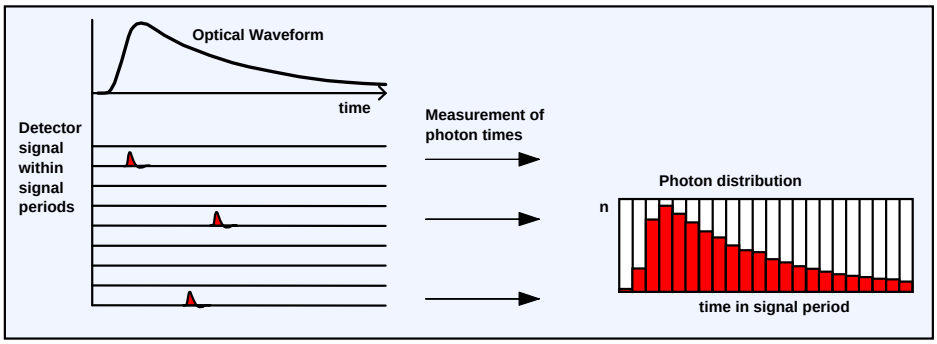

Fig. 1: Principle of classic time-correlated single-photon counting

There are many signal periods without photons, other signal periods contain one photon. When a photon is detected, the time of the corresponding detector pulse in the laser pulse period is measured. The times are used to build up the distribution of the photon numbers over the time in the pulse period. When a sufficiently large number of photons has been recorded the waveform of the optical signal is recovered. The distortion of the recorded waveform by the possible loss of a second photon is surprisingly small. It remains undetectable for detection probabilities less than P = 0.01 photons per laser period [24] and is tolerable up to P = 0.1 to 0.2 [4].

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 resolution of the time-measurement electronics of TCSPC is on the order of 1 ps for the classic time-to-amplitude conversion (TAC) principle and about 50 ps for direct time-to-digital conversion (TDC). With an electronic time resolution of 1 ps the optical resolution is only 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.

The small time-channel 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 (or other signal parameters) much shorter than the IRF width.

The drawback of classic TCSPC instruments was that their count rate was very limited. The technique was thus restricted to very low light intensities, which resulted in extremely long acquisition times. More important, 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.

3. MULTI-DIMENSIONAL TCSPC

Advanced TCSPC techniques are able to record photon distributions not only versus the time in the signal period, but also versus the wavelength, the time from the start of an experiment, or the coordinates of a scanning area [4]. Moreover, the applicable count rates have been increased by two orders of magnitude [2], with a corresponding reduction in acquisition time.

Multi-Wavelength TCSPC

Classic TCSPC is based on the presumption that the probability, P, that the detector detects a photon in one laser period is far smaller than one. Now consider an array of detectors over which the same photons flux is spread. Because it is

unlikely that the complete array detects several photons per laser period it is also unlikely that several detectors of the array will detect a photon in one signal period. This is the basic idea behind multi-dimensional TCSPC. Although several detectors are *active simultaneously they are unlikely to detect a photon in the same signal period*. The times of the photons detected in all detectors of the array can therefore be determined in a single TCSPC channel. To obtain multi-wavelength fluorescence decay data is sufficient to spread a spectrum of the fluorescence light over an array of detector channels, to determine the detection times and the channel number in the detector array for the individual photons, and to build up the photon distribution over these parameters. The principle of the recording electronics is shown in Fig. 2.

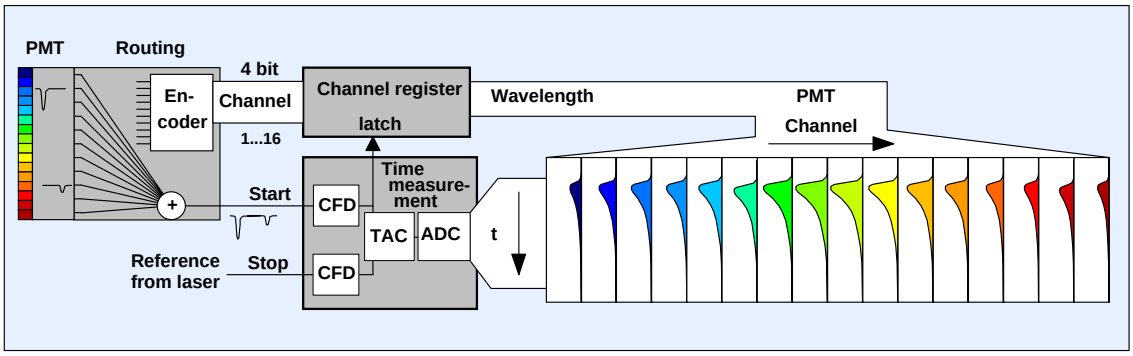

Fig. 2: Multi-wavelength TCSPC

At the input of the detection system are a number of single-photon avalanche photodiodes (SPADs), 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. The subsequent 'router' combines the photon pulses of the detectors into a single timing pulse line and delivers the number of the PMT channel in which the photon was detected.

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. Except for a different ADC principle [4] the architecture of the time-measurement channel is similar to that of classic TCSPC device [24]. However, multi-wavelength TCSPC uses the detector channel numbers of the photons as a second dimension of the photon distribution [4]. The result is a photon distribution over the time in the fluorescence decay and the wavelength, as indicated in Fig. 2.

An example of a multi-wavelength measurement is shown in Fig. 3. Luminescence of human skin was excited by a 405 nm picosecond diode laser. The fluorescent spot was projected on the entrance slit of a Becker & Hickl PML-SPEC multi-wavelength detection assembly [5]. The assembly contains an LOT MS 125-8M Polychromator, a Hamamatsu R5900-L16 16 channel multianode PMT, and the routing electronics. The signals were recorded by a Becker & Hickl SPC-830 TCSPC module [5].

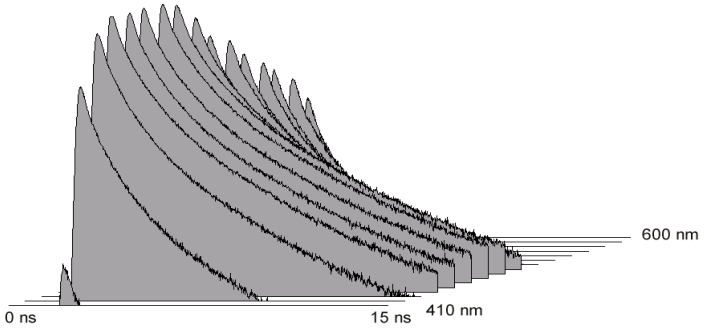

Fig. 3: Fluorescence of human skin, measured by multi-wavelength TCSPC. Excitation wavelength 405 nm, excitation power 60 µW, acquisition time 5 s.

Compared to a setup that scans the spectrum by a monochromator the efficiency of the measurement is improved by a factor of 16. The result shown in Fig. 3 was obtained at an excitation power of only 60 µW, and within an acquisition time of 5 seconds.

Sequential Recording

A third dimension of the photon distribution can be obtained from a counter that delivers the time from the start of an experiment, see Fig. 4.

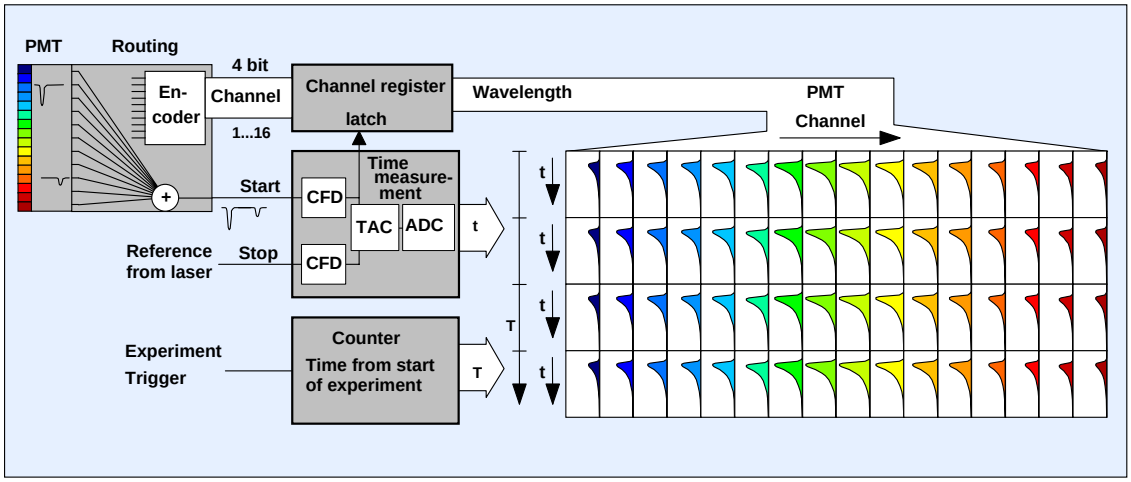

Fig. 4: Sequential recording

The result is a photon distribution over the time in the signal period, the wavelength, and the time from the start of the experiment. The result is a number of recordings for subsequent time intervals, each containing a number of optical waveforms for different wavelengths.

Fig. 5 and Fig. 6 demonstrate the technique for the 'fluorescence transients' of chlorophyll in living plants [15, 23]. When a dark-adapted leaf is exposed to light the intensity of the chlorophyll fluorescence changes. At the beginning the intensity rises steeply. Then the intensity drops slowly and finally reaches a steady-state level. The rise time is on the order of a few milliseconds, 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 in 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'. The intensity change is, of course, accompanied by a change in the fluorescence lifetime. Recording the change in the shape of the fluorescence profiles allows one to decide whether the intensity changes are due to a change in the quenching efficiency or to a change in the number of fluorescing molecules.

To record the non-photochemical-quenching fluorescence transients of a leaf after exposure to the excitation light the same 16-channel detection electronics as for Fig. 3 was used. The data of the individual wavelength channels were combined into in four wavelength intervals (Fig. 5, left to right). The fluorescence was excited by a Becker & Hickl BDL-405SM (405 nm) picosecond diode laser. This laser can electronically be switched on and off within about 1 µs. An external control signal was used to both switch on the laser and start the recording sequence in the TCSPC module.

Fig. 5 shows a sequence of fluorescence decay curves recorded at an acquisition time of 2 s per curve. For better display the sequence starts from the back. The decrease of the fluorescence lifetime with the time of exposure is clearly seen. Moreover, Fig. 5 does not show any significant change in the peak intensity of the decay curves. This indicates that the decrease in fluorescence intensity is due to an increase in quenching efficiency, not to a decrease in the number of functional chlorophyll molecules.

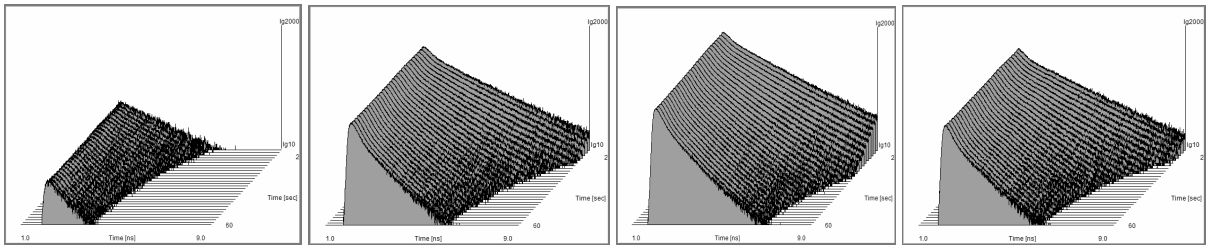

Fig. 5: Non-photochemical quenching of chlorophyll in a leaf, excited at 405 nm. Fluorescence decay curves in different wavelength channels versus time of exposure. 2 s per curve, sequence starts from the back.

The photochemical quenching transients are much faster. Recording these transients requires a resolution of less than 100 µs per step of the sequence. Of course, the number of photons detected in an interval this short is too small to build up a reasonable decay curve. Photochemical quenching transients must therefore be recorded over a large number of on/off cycles of the excitation laser. Each 'laser on' period initiates a photochemical quenching transient in the leaf; each 'laser off' period lets the leaf recover. Within each 'laser on' phase a fast sequence of decay curves is recorded in the TCSPC module, and the data of many such sequences are accumulated. Excessive non-photochemical quenching during the measurement is avoided by keeping the duty cycle of the 'laser on' periods low. A result is shown in Fig. 6.

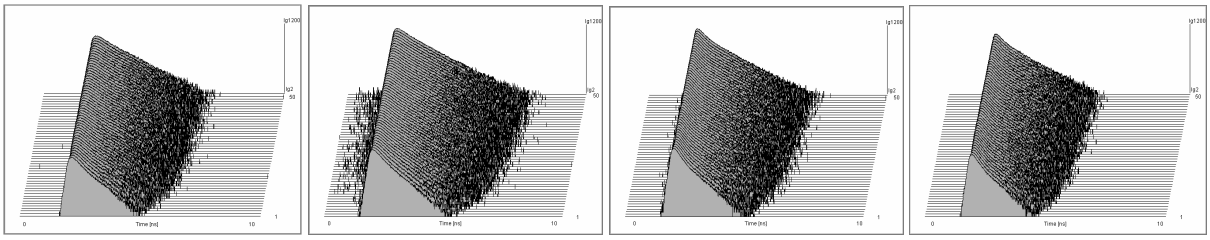

Fig. 6: Photochemical quenching of chlorophyll in a leaf. Fluorescence decay curves in different wavelength channels versus time of exposure. Triggered sequential recording, 50 µs per curve, 20,000 on-off cycles accumulated. The sequence starts from the front.

Combination with Laser Scanning Techniques

Multidimensional TCSPC can be combined with laser scanning. The combination of TCSPC with scanning a laser spot has been suggested earlier [10]. These applications used classic TCSPC. The photons were acquired for one pixel, the results were read from the TCSPC device, and then the photons for the next pixel were recorded. The technique was therefore restricted to slow scanning. The combination of scanning with multi-dimensional TCSPC has no such limitations. The principle is shown in Fig. 7.

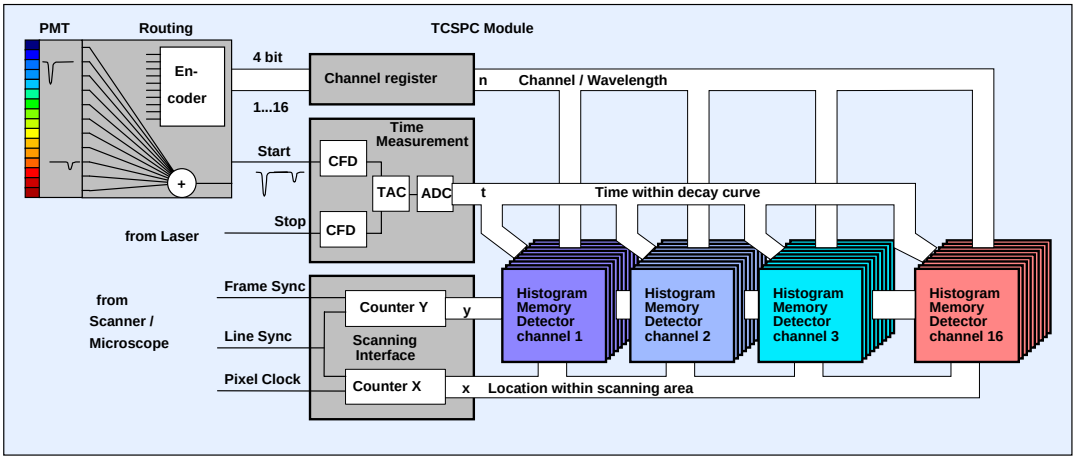

Fig. 7: Combination of multi-dimensional TCSPC with scanning

The counter used in Fig. 4 to measure the time from the start of the experiment is replaced with a scanning interface. In modern TCSPC modules such configuration changes can be performed by a software commend [5]. The scanning interface consists of two connected counters that count the pixels within the lines and the lines within the frames of a scan. The counters deliver the coordinates of the excitation laser spot within the scan area at any time within the scan. The coordinates are used as dimensions of the recording process. Thus, 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 principle shown in Fig. 7 has become a standard fluorescence lifetime imaging (FLIM) technique in confocal and two-photon laser scanning microscopes [3, 4, 6, 8, 11, 17, 19, 30]. These microscopes use optical beam scanning with pixel dwell times in the microsecond range and below [25]. Normally the pixel rate is higher than the photon count rate. This makes the recording process more or less random. In other words, when a photon is detected the TCSPC device measures the time of the photon in the laser pulse period, t, and determines the detector channel number, n, (i.e. the wavelength, λ, of the photon) and the current beam position, x and y, in the scanning area. These data are used to build up the photon distribution over t, λ, x, and y. The recording is continued over as many frames as necessary to obtain the desired number of photons per pixel.

Because the technique does not use any time-gating or wavelength scanning the counting efficiency is close to one. The high efficiency in combination with the high time resolution makes the technique especially attractive for FRET experiments in living cells [3, 8, 11, 26] and autofluorescence imaging of living tissue [19]. For the same reasons, the technique has found application in ophthalmic imaging [31, 32].

Fig. 8 shows multi-wavelength autofluorescence lifetime images of human epithelial cells. The sample was scanned by a Becker & Hickl DCS-120 scanning system connected to a Nikon TE 2000 U microscope. The excitation source was a Becker & Hickl BDL-405 SMC picosecond diode laser; the signals were recorded by Becker & Hickl SPC-830 TCSPC module.

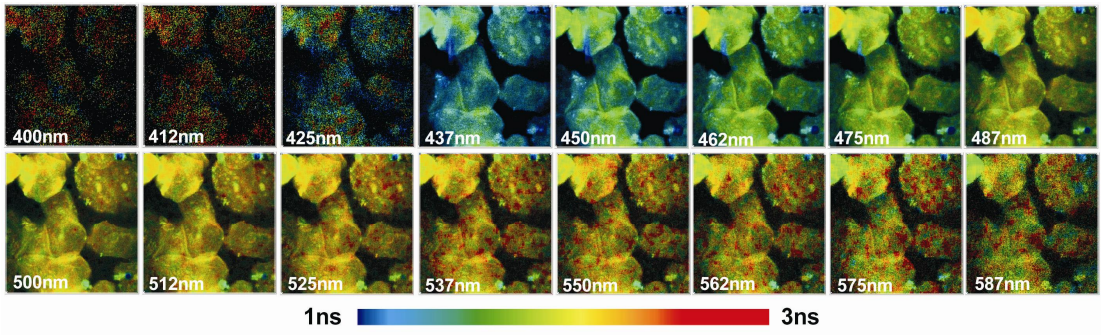

Fig. 8: Multi-wavelength autofluorescence images of human epithelium cells

Time-Tag Recording

The TCSPC systems described above build up multi-dimensional photon distributions in their own on-board memories. The advantage of this principle is that the recording process works independently of the computer and the control software. There is no limitation of the count rate by the data transfer rate of the computer bus or by software reaction times. The systems can therefore be operated at sustained count rates up to the saturated count rate of the signal processing electronics.

For single-molecule spectroscopy or similar experiments it is, however, desirable to have the information of each individual photon available. Modern TCSPC devices therefore have a 'Time-Tag' or 'FIFO' mode that does not build up photon distributions but stores information about each individual photon. The configuration can be changed by a software command so that both the photon distribution modes and the time-tag mode are available [5]. The structure of a TCSPC device in the time-tag mode is shown in Fig. 9.

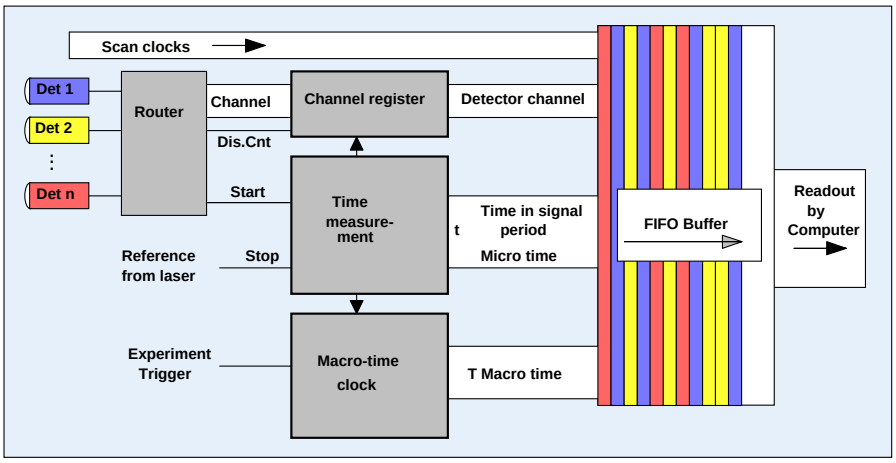

Fig. 9: TCSPC in time-tag mode

For each photon, the time in the signal period ('micro time'), the channel data word, and the time from the start of the experiment ('macro time') are stored in a first-in-first-out (FIFO) buffer. During the measurement the FIFO is continuously read, and the photon data are stored in the main memory or on the hard disc of a computer.

In principle, many of the multi-dimensional recording problems described above can also be solved in the time-tag mode. Synchronization with the experiment can be accomplished by starting the recording via a trigger pulse. Synchronisation with a fast scanning is obtained by recording the scan clock pulses in the photon data stream. The clock edges are tagged with the macro time and with a marker that identifies them either as a frame clock, a line clock, or a pixel clock. The FIFO data are used to build up images in the memory of the computer. The size of these images (i.e. the number of pixels, time channels and wavelength intervals) is not limited by the size of the on-board memory of the TCSPC module. Fig. 10 shows an example of a lifetime image obtained in the time-tag mode of a Becker & Hickl SPC-150 TCSPC module. A decay curve in a selected pixel and the decay parameters obtained by triple-exponential lifetime analysis are shown on the right.

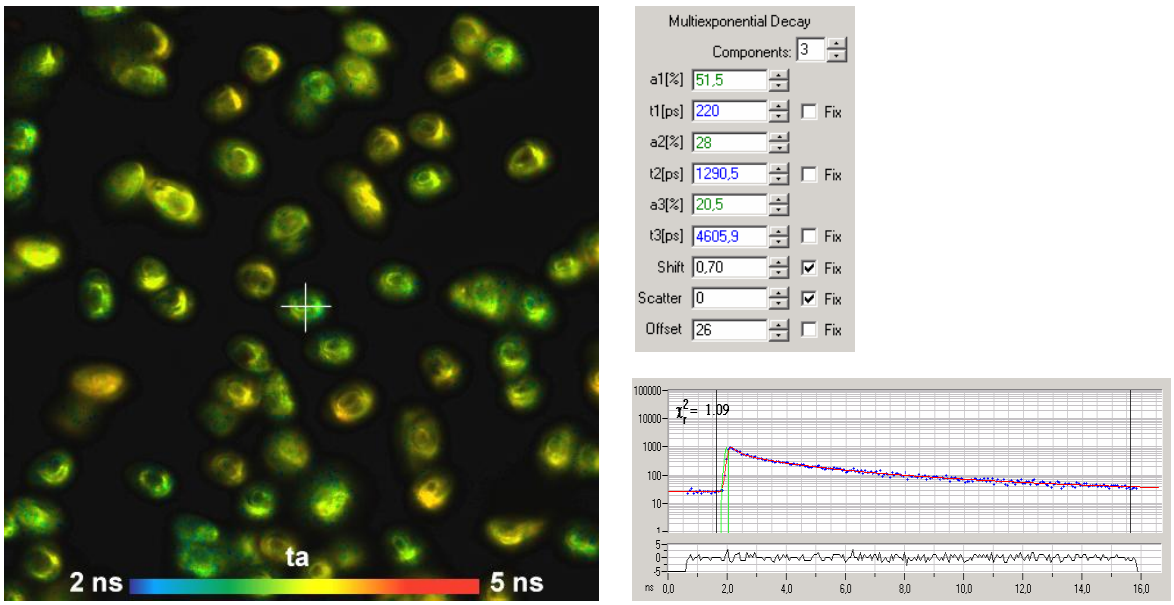

Fig. 10: Lifetime image built up in the time-tag mode. Amplitude-weighed lifetime of triple-exponential decay. Spores of amanita citrina, 512x512 pixels, 256 time channels, Becker & Hickl DCS-120 scanner and SPC-150 TCSPC module

The problem of the time-tag mode is the large amount of data that has to be transferred into the computer and processed or stored. A single experiment may deliver 10^8 to 10^9 photons and thus generate several gigabytes of data. At high count

rates the bus transfer rate into the computer may be insufficient, or the computer may be unable to process the data on-line or to write them to the hard disc. The transfer rate problem is even more severe in systems operating several TCSPC modules in parallel.

The benefit of the time-tag mode is that it delivers absolute photon times. The time-tag mode has therefore become a standard tool of single-molecule spectroscopy. From the time-tag data of a single experiment, fluorescence correlation and cross correlation (FCS and FCCS) data [29], combined FCS / FCCS and fluorescence lifetime data [6], fluorescence intensity distribution (FIDA) and lifetime data, or burst-integrated fluorescence lifetime (BIFL) data [12, 28] can be calculated. Thus, different techniques can be applied to the same sample, the same spot of a sample, and even to the same molecules of a sample.

Fluorescence correlation in one detector channel or between different detector channels connected to a single TCSPC channel can be obtained down to the dead time of the TCSPC module. Correlation below the dead time is possible by using two detectors connected to two synchronised TCSPC channels. It has been shown that such data can be correlated down to the picosecond range if both the macro and the micro times are used [13]. For TCSPC modules based on a TAC principle calibration is required to connect the time scales of the TAC and the macro-time counter. TCSPC modules based on multi-channel TDC chips do not require such calibration because the micro times and the macro times are derived from the same internal reference clock [7]. Fig. 11 shows a correlation curve obtained by a Becker & Hickl DPC-230 sixteen-channel TCSPC / Correlator board. A 10^{-9} mol/l Rhodamin 110 solution was excited by a continuous laser. A microscope and confocal detection with two SPAD modules were used to obtain photons from a femtoliter sample volume. With appropriate optics, correlation data like the curve shown in Fig. 11 deliver diffusion times, time constants of conformational changes, rotational relaxation times, triplet decay times, and singlet decay times from a single experiment.

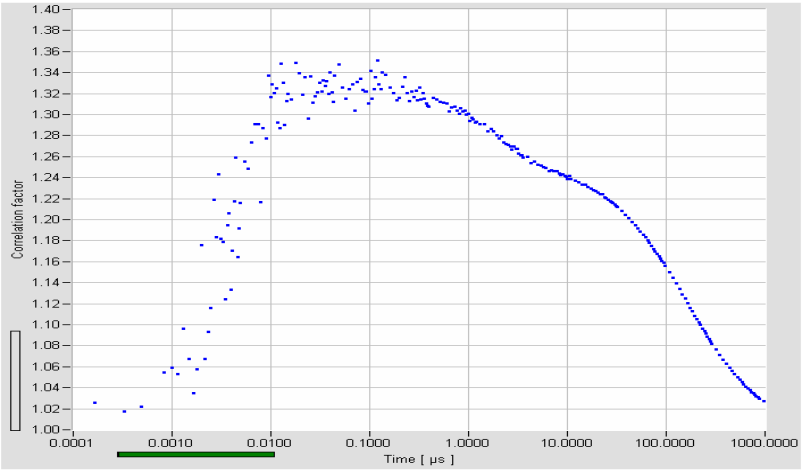

Fig. 11: Fluorescence correlation of a Rhodamin 110 solution over a range from 100 ps to 1 ms. Becker & Hickl DPC-230 TCSPC / Correlator board.

4. 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 at 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, and single-molecule spectroscopy.

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