Multi-dimensional time-correlated single photon counting

Wolfgang Becker, Axel Bergmann
Becker & Hickl GmbH, Nahmitzer Damm 30, D-12277 Berlin, Germany

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, TCSPC

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 [33, 47].

Typical applications are one- and two-photon fluorescence laser scanning microscopy [35, 46], fluorescence imaging of tissue [25], in-vivo drug screening [26], motoring of the photoconversion of sensitizers in photodynamic therapy [41], dynamics of protein-dye complexes on the single molecule level [15, 16, 39], chlorophyll fluorescence dynamics [20, 32], and diffuse optical tomography (DOT) of thick tissue [21, 22, 30].

Although the techniques and instruments used in these applications may differ in the detail, they have a number of features in common: Information is obtained both from spectral and from temporal resolution of the optical signals. Fluorescence spectroscopy records the fluorescence spectrum and the fluorescence decay function, diffuse optical tomography the absorption spectrum and the time-of flight distribution of the photons through the tissue.

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 [19, 24].

Moreover, biological systems often show dynamic changes in their fluorescence, absorption or scattering behavior. Examples are chlorophyll transients in living plants [20, 32], transport mechanisms in living cells [36], diffusion or conformational changes of labeled proteins [7, 15, 16, 39], internalization [23] or photoconversion [41] of photosensitizes, or haemodynamic changes in tissue [17, 30]. 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 [21, 30]. Fluorescence decay times of the commonly used fluorophores are on the same order of magnitude [29]. However, the fluorescence lifetimes in presence of fluorescence quenching, the lifetime of the interacting donor fraction in FRET experiments [4], and the lifetimes of short autofluorescence components can be as short as 100 ps [7, 25]. Lifetimes down to 50 ps are found in dye aggregates [23] and complexes of dyes and metallic nano-particles [18, 31]. Due to the mixture of different

Advanced Photon Counting Techniques, edited by Wolfgang Becker, Proc. of SPIE
Vol. 6372, 637201, (2006) · 0277-786X/06/$15 · doi: 10.1117/12.684742

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.

There are a number of additional requirements to the detection techniques, such as depth resolution in the specimen, excitation in and detection from femtoliter volumes, fluorescence excitation in and detection from deep tissue layers, compatibility with existing optical instruments, etc.

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. Time-domain techniques record the waveform of the investigated signals directly.

Frequency domain techniques are based on modulated image intensifiers [28], on modulated photomultiplier tubes (PMTs), or on photomultiplier tubes with subsequent electronic mixers [13]. Time domain techniques use gated image intensifiers [43, 44], streak cameras [27], gated photon counting within several parallel time-gates [12, 45], or time-correlated single photon counting (TCSPC) [4, 5, 34].

Although the frequency-domain and the time domain are mathematically equivalent, the corresponding signal recording techniques and, in a higher degree, the corresponding instruments, may differ considerably in efficiency, i.e. in the accuracy of the obtained signal parameters for a given number of detected photons [38].

2. TIME-CORRELATED SINGLE PHOTON COUNTING

It has been shown, that, among all time-resolved optical detection techniques, TCSPC yields the highest recording efficiency and the highest time resolution for a given detector [2, 24]. Time-Correlated Single Photon Counting [34] 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 probability of detecting one photon per signal period is much smaller than one. The situation is illustrated in Fig. 1.

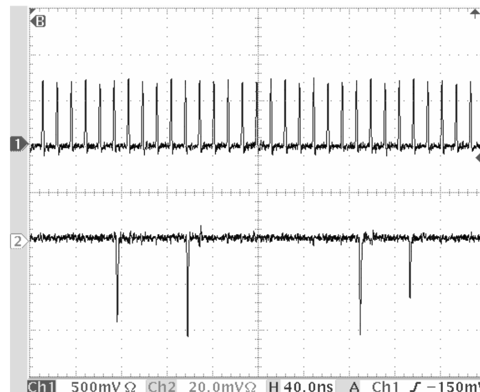

Fig. 1: Upper trace: Pulse train of a 80 MHz Ti:Sapphire laser. Lower Trace: Output signal of a PMT detecting a fluorescence signal excited by the same laser.

The figure shows the 80-MHz pulse train of a Ti:Sapphire laser (upper trace), and the output signal of a photomultiplier tube (PMT) detecting a fluorescence signal excited by the laser. One might expect that the PMT signal resembles a train of fluorescence decay functions, with each laser pulse initiating a new decay. Instead, the PMT signal is a random sequence of extremely short pulses. The signal shape, is, however, easily explained: On the one hand, the gain of the PMT is so high that the detection of each individual photon causes a distinct output pulse. On the other hand, the photon flux is so low that, on average, far less than one photon is detected per laser period. Thus, the distribution of the PMT pulses within the laser pulse period - not the shape of the pulses - resembles the optical waveform of the detected signal. Exactly this distribution is built up by TCSPC. The principle of classic TCSPC is shown in Fig. 2.

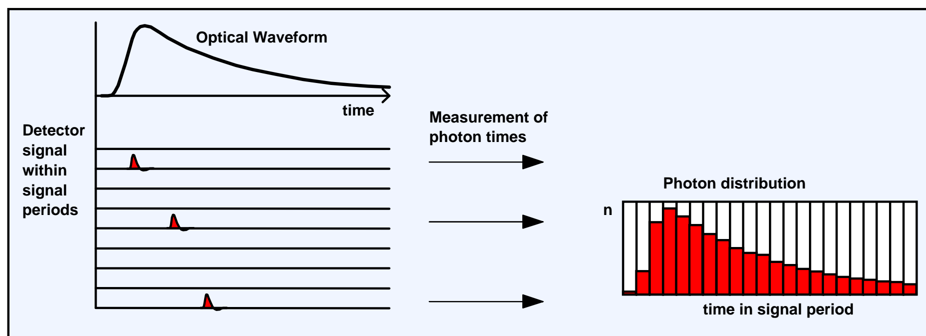

Fig. 2: Principle of classic time-correlated single-photon counting

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 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. After many photons, the waveform of the optical pulse is recovered.

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-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 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. 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 [5]. Moreover, the applicable count rates have been increased by two orders of magnitude [3], with a corresponding reduction in acquisition time.

Multi-Wavelength TCSPC

The photon detection rate in Fig. 1 was about 10^7 s^{-1}. Intensities this high already cause overload in detectors and photobleaching in most biological samples. Nevertheless, even at an intensity this high the detection of several photons per laser pulse period is a relatively unlikely event. Classic TCSPC makes use of this fact in that is considers only one photon to be detected per laser pulse period. 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 [34] and is tolerable up to P = 0.1 to 0.2 [5].

Now consider an array of PMT channels over which the same photons flux is spread. Because it is unlikely that the complete array detects several photons per period it is also unlikely that several channels 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 by 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. 3.

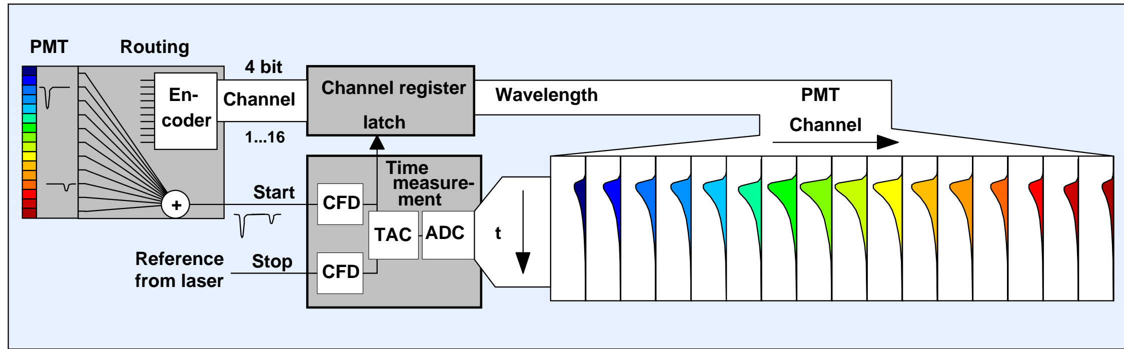

Fig. 3: Multi-wavelength TCSPC

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. This combination is possible because the pulses are unlikely to overlap. Moreover, for each photon, the router 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 [3, 5] the architecture of the time-measurement channel is similar to that of classic TCSPC device [34]. Multi-wavelength TCSPC, however, uses the detector channel numbers of the photons as a second dimension of the photon distribution [5]. The result is a photon distribution over the time in the fluorescence decay and the wavelength, as indicated in Fig. 3.

An example of a multi-wavelength measurement is shown in Fig. 4. 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 [6]. The assembly contains an LOT MS 125-8M Polychromator, a Hamamatsu R5900-L16 multianode PMT and the routing electronics. The signals were recorded by a Becker & Hickl SPC-830 TCSPC module [6].

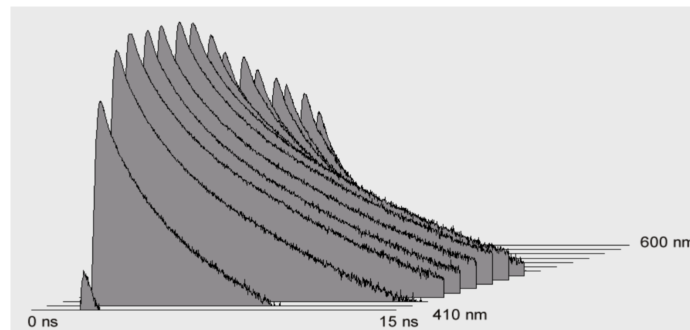

Fig. 4: Fluorescence of human skin, measured by multi-wavelength TCSPC. Excitation wavelength 405 nm, excitation power 60 µW, acquisition time 5 s.

Compared to a setup that scans the spectrum by a monochromator the efficiency of the measurement is largely improved. The result shown in Fig. 4 was obtained at an excitation power of about 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. 5.

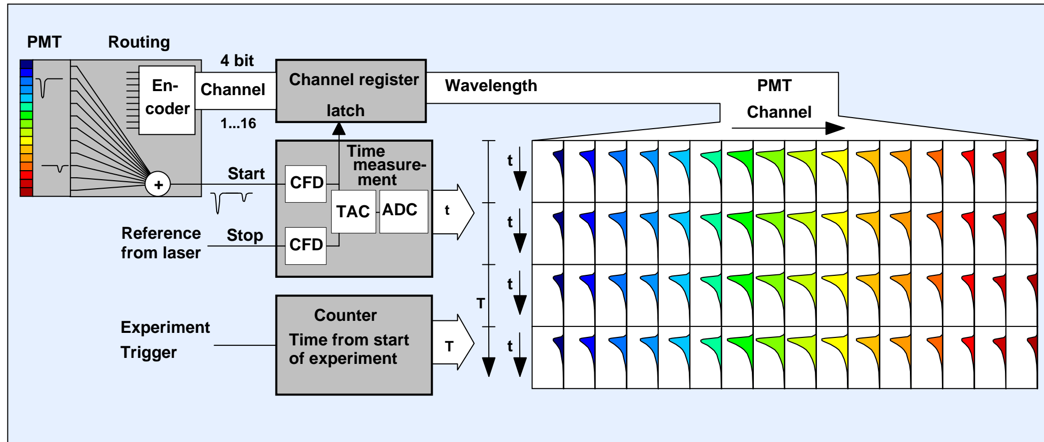

Fig. 5: Sequential recording

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 waveform (e.g. a fluorescence decay curve) for different wavelengths.

Fig. 6 and Fig. 7 demonstrate the technique at the example of the 'fluorescence transients' of chlorophyll in living plants [20, 32]. 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 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 centers 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.

Fig. 6 shows the non-photochemical-quenching fluorescence transients of a leaf after exposure to the excitation light. The same 16-channel detection electronics as for Fig. 4 was used. The data of the individual wavelength channels were combined into in four wavelength intervals (Fig. 6, 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. 6 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. 6 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.

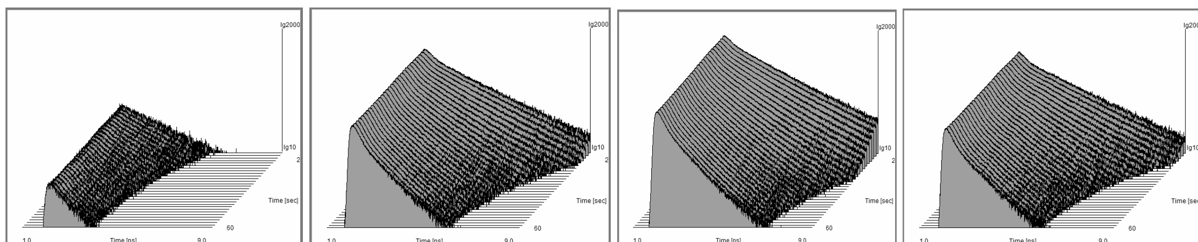

Fig. 6: Non-photochemical quenching of chlorophyll in a leaf, excited at 405 nm. Fluorescence decay curves in different wavelength channels versus time of exposure. 2 s per curve, sequence starts from the back.

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 a time this short is too small to build up a reasonable decay curve. Photochemical quenching transients must therefore be recorded by triggered sequential recording: The excitation laser is periodically switched on an off. 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. Excessive non-photochemical quenching during the measurement is avoided by keeping the duty cycle of the ‘laser on’ periods low. The measurement is continued for a large number of such on-off cycles, and the results are accumulated. A result is shown in Fig. 7.

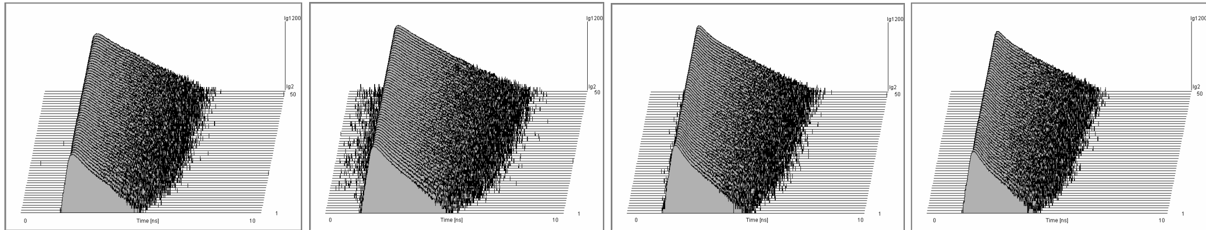

Fig. 7: Photochemical quenching of chlorophyll in a leaf. Fluorescence decay curves in different wavelength channels versus time of exposure. Triggered sequential recording, 50 µs per curve, 20,000 on-off cycles accumulated. The sequence starts from the front.

Scanning

Multidimensional TCSPC can be combined with laser scanning. The combination of TCSPC with scanning a laser spot has been suggested earlier [10, 11]. 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. 8.

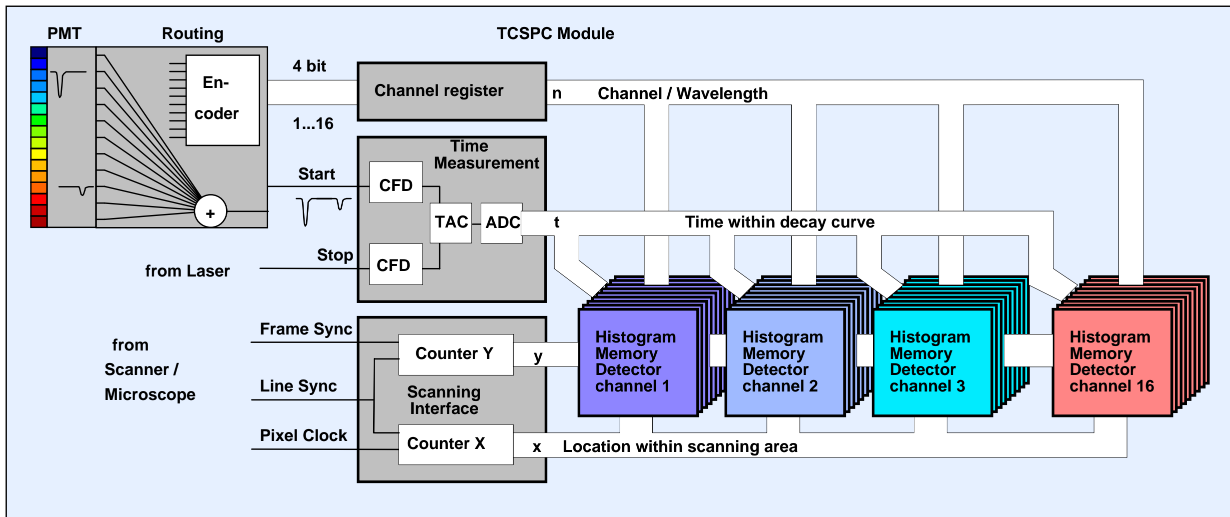

Fig. 8: Combination of multi-dimensional TCSPC with scanning

The counter used Fig. 5 is replaced with a scanning interface. In modern TCSPC modules such configuration changes can be performed by a software commend [6]. The 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. 8 has become a standard fluorescence lifetime imaging (FLIM) technique in confocal and two-photon laser scanning microscopes [1, 4, 5, 7, 8, 14, 23, 25, 41]. These microscopes use optical beam scanning with pixel dwell times in the microsecond range and below [35, 46]. The pixel rate is then 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 [1, 4, 8, 9, 14, 37] and autofluorescence imaging of living tissue [25, 26]. For the same reasons, the technique has found application in ophthalmic imaging [42].

Fig. 9 shows multi-wavelength autofluorescence lifetime images of a mouse kidney section. The sample was scanned by a Zeiss LSM 510 NLO multiphoton microscope. Two-photon excitation at 750 nm was used to excite the fluorescence. The same detecting and recording electronics as for Fig. 4, Fig. 6, and Fig. 7 were used.

The colors of the images represent the fluorescence lifetime obtained by a single-exponential fit to the photon numbers in the time channels of each pixel. Due to the various endogenous fluorophores present in the tissue the lifetimes in the individual wavelength intervals are different. The image of the 375 nm channel contains also light generated by SHG (second harmonic generation). SHG reveals itself as an infinitely short lifetime component, see the blue features in the image of the 375 nm channel.

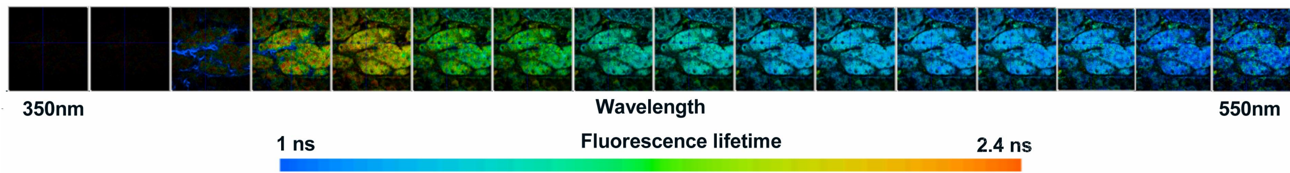

Fig. 9: Multi-wavelength autofluorescence image of mouse kidney tissue

Time-Tag Recording

For single-molecule spectroscopy or similar experiments it is 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 photon distribution modes and the time-tag mode are available [6]. The structure of a TCSPC device in the time-tag mode is shown in Fig. 10.

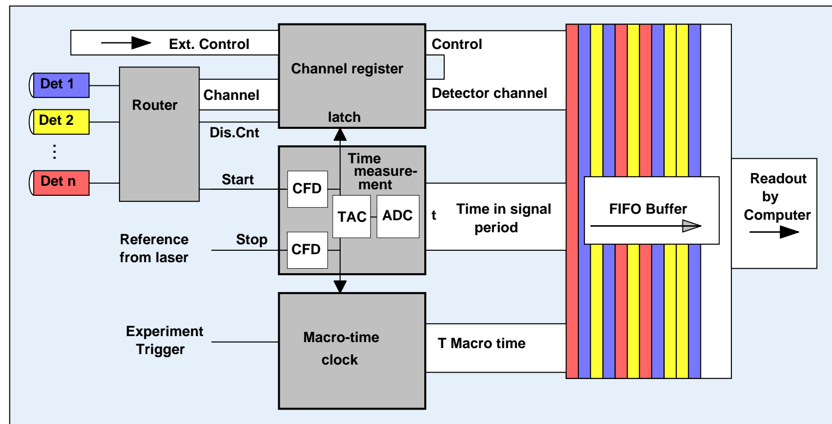

Fig. 10: TCSPC in time-tag mode

For each photon, the time in the signal period, the channel data word, and the time from the start of the experiment, or 'macro time,' is 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, e.g. with a scanner, can be accomplished via the experiment trigger, the macro time clock, and additional experiment control bits read into the channel register and stored in the photon data stream. The drawback 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 still be sufficient, but 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 one computer.

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 [40], combined FCS/FCCS and fluorescence lifetime data [7], fluorescence intensity distribution (FIDA) and lifetime data, or burst-integrated fluorescence lifetime (BIFL) data [15, 16, 39] 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.

FCS, FIDA and BIFL experiments require a femtoliter sample volume. A volume this small can is achieved in a microscope in combination with confocal detection or two-photon excitation. Laser scanning microscopes, do, in principle, meet these requirements. A modern TCSPC module connected to a laser scanning microscope can therefore be used both for fluorescence lifetime imaging and FCS, FIDA, and BIFL experiments [7]. FCS curves recorded in a Zeiss LSM 510 NLO multiphoton microscope is shown in Fig. 11. Two Hamamatsu H7422P-40 detectors were used; the signals were recorded in a Becker & Hickl SPC-830 TCSPC module.

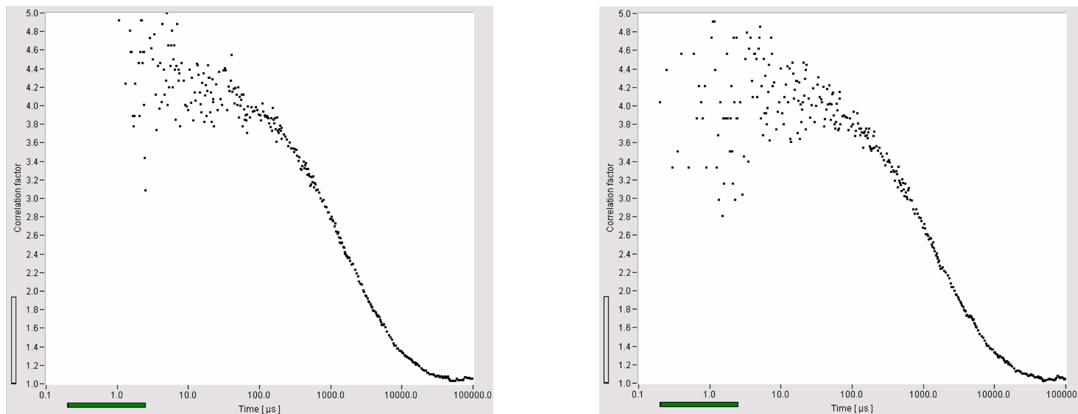

Fig. 11: FCS curves measured in a Zeiss LSM 510 NLO multiphoton microscope. SPC-830 FLIM module, two H7422P-40 detectors attached to the NDD module of the LSM 510 NLO. Left: Autocorrelation of one detector. Right: Cross-correlation between both detectors.

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.

5. REFERENCES

1. Bacskai, B.J., Skoch, J., Hickey, G.A., Allen, R., Hyman, B.T. Fluorescence resonance energy transfer determinations using multiphoton fluorescence lifetime imaging microscopy to characterize amyloid-beta plaques. J. Biomed. Opt. 8, 368-375, 2003.
2. R.M. Ballew, J.N. Demas, An error analysis of the rapid lifetime determination method for the evaluation of single exponential decays, Anal. Chem. 61, 30 (1989)
3. W. Becker, A. Bergmann, H. Wabnitz, D. Grosenick, A. Liebert, High count rate multichannel TCSPC for optical tomography. Proc. SPIE 4431, 249-254, 2001.
4. W. Becker, A. Bergmann, M.A. Hink, K. König, K. Benndorf, C. Biskup, Fluorescence lifetime imaging by time-correlated single photon counting, Micr. Res. Techn. 63, 58-66 (2004)
5. W. Becker, Advanced time-correlated single-photon counting techniques. Springer, Berlin, Heidelberg, New York, 2005
6. W. Becker, The bh TCSPC handbook. Becker & Hickl GmbH (2005). Available on www.becker-hickl.com
7. W. Becker, A. Bergmann, E. Haustein, Z. Petrasek, P. Schwille, C. Biskup, L. Kelbauskas, K. Benndorf, N. Klöcker, T. Anhut, I. Riemann, K. König, Fluorescence lifetime images and correlation spectra obtained by multi-dimensional TCSPC, Micr. Res. Tech. 69, 186-195 (2006)
8. C. Biskup, L. Kelbauskas, T. Zimmer, K. Benndorf, A. Bergmann, W. Becker, J.P. Ruppersberg, C. Stockklausner, N. Klöcker, Interaction of PSD-95 with potassium channels visualized by fluorescence lifetime-based resonance energy transfer imaging, J. Biomed. Opt. 9, 735-759 (2004)
9. C. Biskup, L. Kelbauskas, Th. Zimmer, S. Dietrich, K. Benndorf, W. Becker, A. Bergmann, N. Klöcker, Spectrally resolved fluorescence lifetime and FRET measurements, Proc. SPIE 5700, 188-196 (2005)
10. H. Brismar, B. Ulfhake, Fluorescence lifetime measurements in confocal microscopy of neurons labeled with multiple fluorophores. Nat. Biotech. 15, 373-377, 1997.
11. I. Bugiel, K. König, H. Wabnitz, Investigations of cells by fluorescence laser scanning microscopy with subnanosecond resolution. Lasers in the Life Sciences 3, 47-53, 1989.
12. E.P. Buurman, R. Sanders, A. Draaijer, H.C. Gerritsen, J.J.F. van Veen, P.M. Houpt, Y.K. Levine, Fluorescence lifetime imaging using a confocal laser scanning microscope. Scanning 14, 155-159, 1992.
13. K. Carlsson, A. Liljeborg, Confocal fluorescence microscopy using spectral and lifetime information to simultaneously record four fluorophores with high channel separation. J. Microsc. 185 37-46, 1997.
14. R.R. Duncan, A. Bergmann, M.A. Cousin, D.K. Apps, M.J. Shipston, Multi-dimensional time-correlated single-photon counting (TCSPC) fluorescence lifetime imaging microscopy (FLIM) to deetct FRET in cells, J. Microsc. 215, 1-12 (2004)
15. C. Eggeling, S. Berger, L. Brand, J.R. Fries, J. Schaffer, A. Volkmer, C.A. Seidel, Data registration and selective single-molecule analysis using multi-parameter fluorescence detection, J. Biotechnol. 86, 163 (2001)
16. S. Felekyan, R. Kühnemuth, V. Kudryavtsev, C. Sandhagen, W. Becker, C.A.M. Seidel, Full correlation from picoseconds to seconds by time-resolved and time-correlated single photon detection, Rev. Sci. Instrum. 76, 083104 (2005)
17. M.A. Franceschini, V. Toronov, M.E. Filiaci, E. Gratton, S. Fantini, On-line optical imaging of the human brain with 160-ms temporal resolution, Opt. Expr. 6, 49-57 (2000)
18. Geddes, C.D., Cao, H., Gryczynski, I., Gryczynski, Z., Fang, J., Lakowicz, J.R. Metal-enhanced fluorescence (MEF) due to silver colloids on a planar surface: Potential applications of indocyanine green to in vivo imaging. J. Phys. Chem. A 107, 3443-3449, 2003.
19. H.C. Gerritsen, M.A.H. Asselbergs, A.V. Agronskaia, W.G.J.H.M. van Sark, Fluorescence lifetime imaging in scanning microscopes: acquisition speed, photon economy and lifetime resolution, J. Microsc. 206, 218-224 (2002)
20. Govindjee, Sixty-three Years Since Kautsky: Chlorophyll α Fluorescence, Aust. J. Plant Physiol. 22, 131-160 (1995)
21. D. Grosenick, H. Wabnitz, H. Rinneberg, K.Th. Moesta, P. Schlag, Development of a time-domain optical mammograph and first in-vivo applications. Appl. Optics 38 (1999) 2927-2943
22. J.C. Hebden, E.W. Schmidt, M.E. Fry, M. Schweiger, E.M.C. Hillman, D.T. Delpy, Simultaneous reconstruction of absorption and scattering images by multichannel measurement of purely temporal data, Opt. Lett. 24, 534-536 (1999)
23. Kelbauskas, L., Dietel, W. Internalization of aggregated photosensitizers by tumor cells: Subcellular time-resolved fluorescence spectroscopy on derivates of pyropheophorbide-a ethers and chlorin e6 under femtosecond one- and two-photon excitation. Photochem. Photobiol. 76 686-694, 2002.

24. M. Köllner, J. Wolfrum, How many photons are necessary for fluorescence-lifetime measurements?, Phys. Chem. Lett. 200, 199-204 (1992)
25. König, K., Riemann, I. High resolution optical tomography of human skin with subcellular resolution and picosecond time resolution. J. Biom. Opt. 8, 432-439, 2003.
26. K. König, A. Ehlers, F. Stracke, I. Riemann, In vivo drug sceening in human skin using femtosecond laser multiphoton tomography. Skin Pharmakol Physiol 19, 78-88 (2006)
27. R.V. Krishnan, A. Masuda, V.E. Centonze, B. Herman, Quantitative imaging of protein-protein interactions by multiphoton fluorescence lifetime imaging microscopy using a streak camera, J. Biomed. Opt. 8, 362-267 (2003)
28. J.R. Lakowicz, K. Berndt, Lifetime-selective fluorescence lifetime imaging using an rf phase-sensitive camera. Rev. Sci. Instrum. 62, 1727-1734, 1991
29. Lakowicz, J.R. Principles of Fluorescence Spectroscopy. 2nd. Ed., Plenum Press,New York, 1999
30. A. Liebert, H. Wabnitz, J. Steinbrink, H. Obrig, M. Möller, R. Macdonald, A. Villringer, H. Rinneberg, Time-resolved multidistance near-infrared spectroscopy at the human head: Intra- and extracerebral absorption changes from moments of distribution of times of flight of photons, Appl. Opt. 43, 3037-3047 (2004)
31. Malicka, J., Gryczynski, I., Geddes, C.D., Lakowicz, J.R., Metal-enhanced emission from indocyanine green: a new approach to in vivo imaging. J. Biomed. Opt. 8 472-478, 2003.
32. Maxwell, K., Johnson, G.N., Chlorophyll fluorescence - a practical guide. J. Exp. Bot. 51 659-668, 2000.
33. Mycek, M-A., Pogue, B. W., Handbook of biomedical fluorescence, Marcel Dekker, New York, Basel, 2003.
34. D.V. O'Connor, D. Phillips, Time Correlated Single Photon Counting, Academic Press, London, 1984.
35. J. Pawley (ed.), Handbook of biological confocal microscopy, 2nd edn., Plenum Press, New York (1995)
36. A. Periasamy, Methods in Cellular Imaging. Oxford University Press, Oxford New York (2001)
37. M. Peter, S.M. Ameer-Beg, Imaging molecular interactions by multiphoton FLIM, Biology of the Cell 96, 231-236 (2004)
38. Philip, J.P., Carlsson, K. Theoretical Investigation of the Signal-to-Noise ratio in fluorescence lifetime imaging. *J. Opt. Soc. Am. A*, 20, 368-379, 2003.
39. M. Prummer, B. Sick, A. Renn, U.P. Wild, Multiparameter microscopy and spectroscopy for single-molecule analysis, Anal. Chem. 76, 1633-1640 (2004)
40. Rigler, R., Elson, E.S., Fluorescence Correlation Spectrosccopy. Springer, Berlin, Heidelberg, 2001.
41. A. Rück, F. Dolp, C. Hülshoff, C. Hauser, C. Scalfi-Happ, Fluorescence lifetime imaging in PDT. An overview. Medical Laser Application 20, 125-129 (2005
42. D. Schweitzer, M. Hammer, F. Schweitzer, R. Anders, T. Doebbecke, S. Schenke, E. R. Gaillard, In vivo measurement of time-resolved autofluorescence at the human fundus, J. Biomed. Opt. 9, 1214-1222 (2004)
43. M. Straub, S. W. Hell, Fluorescence lifetime three-dimensional microscopy with picosecond precision using a multifocal multiphoton mocroscope. Appl. Phys. Lett. 73, 1769-1771, 1998
44. K. Suhling, P.M.W. French, D. Phillips, Time-resolved fluorescence microscopy. Photochem. Photobiol. Sci. 4, 13-22 (2005)
45. J. Syrtsma, J.M. Vroom, C.J. de Grauw, H.C. Gerritsen, Time-gated fluorescence lifetime imaging and microvolume spectroscopy using two-photon excitation. J. Microsc. 191, 39-51, 1998.
46. J.G. White, W.B. Amos, M. Fordham, An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. J Cell Biol 105, 41-48, 1987.
47. T. Vo-Dinh (ed.) Biomedical Photonics Handbook, CRC Press (2003)