Fluorescence lifetime images and correlation spectra obtained by multi-dimensional TCSPC

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Abstract
Multi-dimensional time-correlated single photon counting (TCSPC) is based on the excitation of the sample by a high-repetition rate laser and the detection of single photons of the fluorescence signal in several detection channels. Each photon is characterised by its time in the laser period, its detection channel number, and several additional variables such as the coordinates of an image area, or the time from the start of the experiment. Combined with a confocal or two-photon laser scanning microscope and a pulsed laser, multi-dimensional TCSPC makes a fluorescence lifetime technique with multi-wavelength capability, near-ideal counting efficiency, and the capability to resolve multi-exponential decay functions. We show that the same technique and the same hardware can be used to for precision fluorescence decay analysis, fluorescence correlation spectroscopy (FCS), and fluorescence intensity distribution analysis (FIDA and FILDA) in selected spots of a sample.

Keywords: TCSPC, FLIM, FRET, FCS, FIDA, FILDA, BIFL

1. INTRODUCTION
Confocal and two-photon laser scanning techniques have caused a revolution in fluorescence microscopy [1,2,3]. In particular, multi-dimensional features like the optical sectioning capability in conjunction with the large penetration depth of two-photon excitation and the multi-wavelength capability [4] have resulted in a new quality of biological imaging. However, the fluorescence light emitted by organic molecules is not only characterised by its emission intensity and emission spectrum, it has also a specific lifetime. The fluorescence lifetime of an excited fluorophore is mostly independent of its concentration, but depends on the interaction with the local environment [5]. The fluorescence lifetime can therefore be used to map cell parameters such as pH, ion concentrations or oxygen saturation [6,7], aggregation effects [8], to probe protein or DNA structures by lifetime sensitive dyes [9,10], and to investigate the molecular proximity of proteins by Förster resonance energy transfer (FRET) [11,12,13,14,15,16,33,34]. Fluorescence lifetime imaging (FLIM) is also useful to distinguish the fluorescence components in autofluorescence measurements of tissue [17]. The fluorescence lifetimes of typical fluorophores used in cell imaging are of the order of a few nanoseconds. However, in the presence of quenchers fluorescence lifetimes can decrease to the sub-nanosecond range. The lifetimes of autofluorescence components can be as short as 100 ps [17]. Even shorter lifetimes are found in dye aggregates [8] and complexes of dyes and metallic nano-particles [18,19]. Due to the presence of several fluorophores with different lifetimes or due to non-uniform quenching and energy transfer rates the fluorescence decay functions observed in cells and tissues are normally multi-exponential.

The focal volume of a laser microscope is of the order of 100 fl and thus contains only a limited number of fluorophore molecules. Therefore, the fluorescence shows intensity and lifetime fluctuations due to diffusion, conformational changes, and intersystem crossing. The fluctuations of the fluorescence behaviour are the basis of the typical single-molecule spectroscopy techniques, like fluorescence correlation spectroscopy (FCS) [20,21,23], fluorescence intensity distribution analysis (FIDA) [24,25,26,27], and burst-integrated fluorescence lifetime (BIFL) techniques [28,29]. These techniques are a second approach to investigate the mobility, the size, and the conformation of proteins in cells. In practice it is a benefit if FLIM techniques and FCS, FIDA, or BIFL can be used in the same instrument and be applied to the same sample. A signal-acquisition technique for laser scanning microscopes should therefore record lifetime images at a resolution of better than 50 ps, resolve multi-exponential decay functions, and deliver FCS, FIDA, or BIFL data. A suitable technique does indeed exist in form of the recently introduced multi-dimensional time-correlated single-photon counting (TCSPC) technique.
2. MULTI-DIMENSIONAL TCSPC

Classic time-correlated single-photon counting is based on the detection of individual photons of a periodic light signal, the measurement of the photon detection times, and the build-up of the photon distribution versus the time in the signal period [30]. The technique delivers a near-ideal counting efficiency and an optimal time-resolution for a given detector. For a long time, TCSPC has been considered to be intrinsically slow and one-dimensional and therefore not very useful for fluorescence lifetime imaging. Certainly, this reputation came from early flashlamp-based TCSPC lifetime spectrometers which indeed needed extremely long acquisition times. Compared to early TCSPC setups advanced TCSPC devices can be used at count rates more than two orders of magnitude higher [31,32]. The acquisition times are correspondingly shorter. Moreover, advanced TCSPC techniques use a multi-dimensional recording process. The photon data can either be used to build up photon distributions or to store the data of each individual photon. Thus, modern TCSPC modules can be configured to record either multi-wavelength FLIM data [33,34,35], fast dynamic changes in the fluorescence decay behaviour of a sample [36,37], or combined fluorescence correlation and fluorescence lifetime data.

FLIM mode

The typical configuration of TCSPC for fluorescence lifetime imaging is shown in Fig. 1. At the input of the detection system are a number of photomultipliers (PMTs), detecting the fluorescence signal from the excited spot of the sample in different wavelength intervals. In the subsequent ‘router’ the single-photon pulses of the PMTs are combined into a common timing pulse line. Furthermore, the router delivers the number of the PMT in which a photon was detected. The routing technique can be used for several individual PMTs [31] and for multi-anode PMTs [35]. The TCSPC module receives the timing pulse, the PMT channel number, and the scan clock signals (frame sync, line sync and pixel clock) from the scanning unit of the microscope. For each photon, the TCSPC module determines the location within the scanning area, x and y, the time of the photon in the laser pulse sequence, t, and the detector channel number, n. These values are used to address a memory in which the detection events are accumulated. Thus, in the memory the distribution of the photon density over x, y, t, and n builds up.

The data acquisition runs at any scanning speed of the microscope. As many frame scans as necessary to obtain an appropriate signal-to-noise ratio can be accumulated. At the typical count rates obtained from living specimens the pixel rate is higher than the photon count rate. This makes the recording process more or less random. It should be noted that multi-dimensional TCSPC does not use any time gating, wavelength scanning, or detector multiplexing. For count rates up to several MHz virtually all detected photons contribute to the result. Consequently, a near-ideal signal-to-noise ratio for a given fluorescence intensity and acquisition time is obtained.

The time resolution is determined mainly by the transit time spread of the detectors. With multichannel PMTs the width of the instrument response function (IRF) is about 30 ps (fwhm) [33,34]. Moreover, the fluorescence decay curves in the individual pixels of the image are resolved into a large number - typically 64 to 1024 - time channels. Consequently, the signals are adequately sampled to satisfy the Nyquist criterion. Double-exponential decay profiles can therefore be resolved into their lifetime components and intensity coefficients [34].

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FIFO Mode

The configuration of multi-dimensional TCSPC for single-molecule spectroscopy is shown in Fig. 2. The router, the channel register, and the time-measurement block work in the same way as in the FLIM configuration. However, the scanning interface is configured as a clock that delivers the time of a photon measured from the start of the experiment. The memory of the TCSPC device is configured as a large first-in-first-out (FIFO) buffer. For each photon, the detector number, i.e. the wavelength, the ‘micro time’ in the signal period, t, and the ‘macro time’ from the start of the experiment, T, are determined. The data are buffered in the FIFO. The size of the FIFO is sufficient to buffer strong photon bursts or even a complete measurement. The host computer continuously reads the data from the FIFO and writes them into his own memory or on the hard disc.

![Diagram of FIFO Mode](image)

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**Measurement**

**Start**

**Stop**

**CFD**

**TAC ADC**

**CFD from Laser**

**Time**

**WL**

**Channel / Wavelength Channel**

**Detectors**

**Timing Start**

**Timing Stop**

**Routing**

**Channel register**

**Time Measurement**

**FIFO Buffer**

8 million photons

**Histogram of t:**
Fluorescence decay

**Autocorrelation of T:**
FCS

**Cross-Correlation of T:**
Fluorescence Cross Correlation

**Histogram of Photon No. within time intervals:**
PCH / FIDA / 2D FIDA

**Histogram of average t within time intervals:**
FILDA

**Analysis within the individual bursts:**
BIFL

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The obtained photon data can be analysed by a number of different algorithms. Fluorescence decay curves are obtained by building up histograms of the micro times, t. FCS curves of the individual detector channels are obtained by calculating autocorrelation functions [20,21,22] of the macro time, T. Cross-correlation of the macro times of different detectors delivers fluorescence cross correlation (FCCS) curves [23]. Histograms of the photon numbers within consecutive time intervals represent the photon counting histogram (PCH) [24] used for fluorescence intensity distribution analysis (FIDA) [25,26]. Fluorescence intensity and lifetime distribution analysis (FILDA) [27] is possible by building up additional histograms of the micro times within consecutive time intervals. Identification of the photon bursts of individual molecules and analysis within the bursts is the basis of the burst-integrated fluorescence lifetime (BIFL) technique [28,29]. The applicability of a large number of different techniques to one data set recorded in one spot of a sample is a considerable benefit of the TCSPC technique.

### 3. MULTI-EXPONENTIAL FLIM

**Autofluorescence of Skin**

Typical FLIM applications of TCSPC are autofluorescence imaging of tissue [17] and FRET experiments [32,34]. These applications benefit mainly from the high time resolution and the capability to resolve multi-exponential decay functions. Fig. 3, left, shows a two-photon lifetime image of the stratum corneum of human skin. The image was recorded by a Becker & Hickl SPC-830 TCSPC module [38] coupled to a Zeiss LSM 410. The decay time of a single-exponential approximation of the decay is used as colour. Blue to red corresponds to a lifetime range of 400 to 1500 ps. The fluorescence decay data in a selected spot of the image are shown right. The decay is clearly double exponential. The decay function can be described by a fast lifetime component, $\tau_{\text{fast}} = 208$ ps, a slow lifetime component, $\tau_{\text{slow}} = 2.1$ ns, and the corresponding intensity coefficients, $a_{\text{fast}} = 0.77$ and $a_{\text{slow}} = 0.23$.

It should be noted that the lifetimes and the ratio of the intensity coefficients, $a_{\text{fast}}/a_{\text{slow}}$, are independent parameters of the fluorescence decay. Consequently, a single TCSPC lifetime image can be considered to add three additional dimensions to multi-dimensional microscopy. Fig. 4 shows images of $\tau_{\text{fast}}$, $\tau_{\text{slow}}$, $\tau_{\text{slow}}/\tau_{\text{fast}}$, and $a_{\text{fast}}/a_{\text{slow}}$. The indicated parameter range corresponds to a colour range from blue to red. The images show significant differences. The biological interpretation of such data has to be subject of further investigation.
Fig. 3: Left: Two-photon lifetime image of the stratum corneum of human skin. Single-exponential approximation. Colour represents lifetime, blue to red corresponds to 400 to 1500 ps. Right: Fluorescence decay in selected spot of the image and decay components.

Fig. 4: Images of the double exponential decay parameters, left to right: $\tau_{\text{fast}}$, $\tau_{\text{slow}}$, $\tau_{\text{slow}}/\tau_{\text{fast}}$, and $a_{\text{fast}}/a_{\text{slow}}$. The indicated parameter range corresponds to a colour range from blue to red.

Fig. 5: CFP-YFP FRET. Left: Single-exponential lifetime image, CFP channel, 480 ± 15 nm. Blue to red = 1.5 to 2.2 ns. Right: Fluorescence decay in selected spot and decay components of a double-exponential fit.

Fig. 6: CFP-YFP FRET. Left: Colour represents $\tau_{\text{slow}} / \tau_{\text{fast}}$, blue to red = 2 to 4. The distribution of $\tau_{\text{slow}} / \tau_{\text{fast}}$ is shown for an area of weak FRET (top) and strong FRET (bottom). Right: Colour represents $a_{\text{fast}} / a_{\text{slow}}$, blue to red = 0.2 to 1
**FRET**

Fig. 5 and Fig. 6 show FRET in a human embryonic kidney cell expressing two interacting proteins labelled with CFP and YFP. The data were recorded by Becker & Hickl SPC-730 TCSPC module connected to a Zeiss LSM 510 NLO two-photon microscope. The detector was a Hamamatsu R3809U-50 MCP PMT.

In the regions where the proteins are physically linked the energy is transferred from the CFP to the YFP. The FRET results in a substantial decrease of the fluorescence lifetime of the CFP. Fig. 5, left, shows a single-exponential lifetime image calculated from the data recorded at the emission wavelength of the CFP. The lifetime is colour-coded and varies from 1.5 ns (blue) to 2.2 ns (red). The image gives a good indication of the distribution of the FRET in the cell.

However, a closer inspection of the fluorescence data in the individual pixels shows that the decay is double-exponential (Fig. 5, right). The reason of the double exponential decay is that only a fraction of the donor molecules are interacting with an acceptor molecule. Not all donor molecules may have their dipoles oriented parallel with the corresponding acceptor molecule. For conventional antibody labelling the double exponential decay profile may also be a result of incomplete labelling of the proteins [5]. However, in transfected cells complete labelling is expected. A double-exponential analysis of the decay data then disentangles the effects of the variable fraction of interacting proteins and of the distance of donor and acceptor. \( \tau_{\text{slow}}/\tau_{\text{fast}} \) is an indicator of the FRET distance, while \( a_{\text{fast}}/a_{\text{slow}} \) is the ratio of the interacting and non-interacting donor fractions. Fig. 6 shows that most of the lifetime variation found in the single-exponential lifetime image is due to a variable fraction of interacting donor, not to a change in the distance. The change in \( \tau_{\text{slow}}/\tau_{\text{fast}} \) is only 10%, corresponding to a distance variation of about 2%. A variation this small may not be real but be introduced by crosstalk between the lifetimes and the amplitudes of the two exponential components in the fitting routine. In particular, crosstalk may occur by the 1.3 ns-decay component of CFP as [33]. A double-exponential fit may partially merge the 1.3-ns contribution into the fast decay component.

A double-exponential decay is found in most TCSPC-based FRET experiments [13,15,16,34] and confirmed by streak camera measurements [14]. The finding has implications to FRET-distance determinations based on single-exponential donor lifetimes and possibly even to steady-state FRET techniques.

### 4. SINGLE-MOLECULE SPECTROSCOPY

FCS, FIDA, and FILDA experiments were performed by a Becker & Hickl SPC-730 TCSPC module [38] connected to a two-photon microscope with a home-made scanning setup. The scanner is based on galvanometer-driven mirrors. For FLIM experiments, the setup runs a repetitive scan of up to 512 x 512 pixels at a rate down to 4 us per pixel. For single-molecule experiments, the scan is stopped in a selectable spot of the image. Perkin Elmer SPCM-AQR modules were used as detectors. The FCS capability was tested at a 10^-9 mol/l GFP solution. Fig. 7 shows a trace of the intensity fluctuations, the FCS curve, and the fluorescence decay.

![Figure 7: 10^-9 mol/l GFP solution. Left to right: Intensity fluctuations displayed in time intervals of 1 ms/point, FCS curve calculated from 1 µs to 10 ms, fluorescence decay over three laser periods.](image)

Fig. 8 shows an application to a cell that contains a GFP-MK2 fusion protein [39]. The protein is located predominantly in the nucleus. Upon stress, it translocates to the cytoplasm. The upper row of Fig. 8 shows an intensity image, the fluorescence decay curve at the position where the beam was stopped, the FCS curve, a photon counting histogram for FIDA analysis, and a corresponding histogram of the photon arrival times, both within sampling time intervals of 1 ms. All curves were calculated from a single TCSPC recording. The lower row of Fig. 8 shows similar results for a cell that was stressed with a 0.4 mol/l solution of sorbitol. The treatment does not result in a noticeable change in the
fluorescence lifetime. However, aggregation of the proteins results in a substantial increase of the diffusion times, i.e. a shift of the FCS curves to longer times.

Fig. 8: Cell expressing a GFP-MK2 fusion protein. Upper row healthy cell, lower row cell stressed with sorbitol. Left to right: Intensity image, fluorescence decay curve, FCS curve, photon counting histogram, and histogram of photon arrival times in selected spot of the image.

5. CONCLUDING REMARKS

Multi-dimensional TCSPC is a promising way to combine high-resolution FLIM with FCS, FIDA, and BIFL techniques in a single instrument. Multi-dimensional TCSPC is frequently used for FLIM in commercial laser scanning microscopes. An extension to FCS, FIDA, and BIFL appears possible and may result in a unification of the currently used FLIM and single molecule techniques.

It should, however, be noted that currently available scanning microscopes are not necessarily optimised for these techniques. As an absolute requirement, a beam parking function of high reproducibility and low jitter is required. Many FLIM systems use two-photon excitation and non-descanned detection. However, two-photon excitation yields relatively fast photobleaching in the image plane [40,41]. Non-descanned detection is prone to collect background light from the environment. Both effects can impair the results of FCS, FIDA, and BIFL. Ideally, the microscope should also be equipped with a source for one-photon excitation, e.g. with a pulsed diode laser or a frequency-doubled titanium-sapphire laser. One-photon excitation, in turn, requires that a descanned confocal detection path is available for TCSPC recording.

Moreover, there is currently no detector which is equally suitable for FLIM and FCS. The best FLIM detectors are multichannel plate (MCP) PMTs. However, MCP PMTs use conventional photocathodes. Depending on the wavelength, the efficiency of these cathodes is by a factor of 2 to 8 lower than for the single-photon APDs typically used for single-molecule spectroscopy. On the other hand, the time resolution of the typical APDs is not satisfactory for FLIM. Thus, either different detectors must be implemented, or some tradeoff between the signal-to-noise ratio of FCS and the time-resolution of FLIM must be made. The best compromise between efficiency and time-resolution is currently obtained with the Hamamatsu H7422-40 detector module, with an efficiency of 40% at 500 nm and an IRF width of 200 to 300 ps.

6. REFERENCES

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