High-Speed FLIM Data Acquisition by Time-Correlated Single Photon Counting

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In this study, we describe a time-correlated single photon counting (TCSPC) technique for multi-wavelength lifetime imaging in laser-scanning microscopes. The technique is based on a four-dimensional histogramming process that records the photon density versus the time in the fluorescence decay, the x-y coordinates of the scanning area and the wavelength. It avoids any time gating or wavelength scanning and, therefore, yields a near-ideal counting efficiency. The decay functions are recorded in a large number of time channels, and the components of a multi-exponential decay can be resolved down to less than 30 ps. A single TCSPC imaging channel works with a high detection efficiency up to a photon count rate of about $5 \times 10^6$ s⁻¹. A modified version of the TCSPC fluorescence lifetime imaging (FLIM) technique uses several fully parallel detector and TCSPC channels. It operates at a count rate of more than $10^7$ photons per second and records double-exponential FLIM data within less than 10 seconds.

Keywords: Fluorescence lifetime imaging, FLIM, FRET, autofluorescence, time-correlated single photon counting, TCSPC

1. Introduction

Confocal¹ and two-photon laser scanning microscopes³ have considerably improved the image quality in fluorescence microscopy. The optical sectioning capability and the increased contrast make them superior to conventional wide-field fluorescence microscopes. Recent techniques exploit the spectral characteristics of the fluorescence emitted from the sample to better separate different fluorophores⁴. However, the fluorescence light emitted by organic molecules is not only characterised by its emission spectrum. It has also a specific lifetime, which can be used to distinguish fluorophores whose spectra are too similar to be separated spectrally. This approach has been particularly useful to distinguish the autofluorescence components in tissues. These components often have poorly defined fluorescence spectra but are clearly distinguished by their fluorescence lifetime⁵,⁶. The ability of FLIM to identify fluorophores has also been used to verify cell transfection⁷.

The fluorescence lifetime of an excited fluorophore is mostly independent of its concentration, but it can be influenced by its local environment⁸. Changes of the fluorescence lifetime can be used as an indicator of the energy transfer rate to the local environment. Thus, FLIM is a direct approach to measure all effects that change the quantum efficiency of a fluorophore. Typical examples are mapping of cell parameters such as pH, ion concentrations or oxygen saturation by fluorescence quenching⁹⁻¹², as well as probing protein or DNA structures by lifetime sensitive dyes¹³,¹⁴.

By exploiting fluorescence - or Förster - resonance energy transfer (FRET) fluorescence lifetime measurements can be used to prove the molecular proximity of proteins, which are labelled with appropriate donor and acceptor fluorophores. The rate of energy transfer from the donor to the acceptor depends on the inverse sixth power of the distance between the fluorophores. Because of this steep relationship FRET occurs only within distances of a few nm. Thus, FRET is a direct indicator for the molecular proximity of donor and acceptor fluorophores and an indirect indicator of an association of the protein molecules to which the fluorophores are attached¹⁵⁻¹⁹.

When FRET occurs, both the intensity and the lifetime of the donor fluorescence decrease, whereas the intensity of the acceptor emission is increased. The decrease of the fluorescence lifetime is related to the rate constant of the energy transfer and can be used to estimate the distance between donor and acceptor fluorophores. In contrast to steady-state FRET techniques, FLIM has the advantage of being independent of the local concentrations of donor and acceptor molecules. Therefore, FRET results can be obtained from a single lifetime image of the donor.

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\cite{6,15}. Even shorter lifetimes are found in dye aggregates\cite{20} and complexes of dyes and metallic nano-particles\cite{21,22}. Due to the presence of several fluorophores with different lifetimes or due to non-uniform quenching the fluorescence decay functions observed in cells and tissues are normally multi-exponential. Therefore, a lifetime imaging technique should be able to resolve the components of multi-exponential fluorescence decay functions down to less than 100 ps. Moreover, it should be compatible with two-photon and confocal laser scanning microscopes.

Rough single-exponential lifetimes can be derived from data containing a few hundred photons per pixel. This is not more than required for a mediocre steady state image. However, multi-exponential lifetime analysis requires much more photons\cite{23}. Due to prolonged excitation photobleaching\cite{24-26} can constitute a problem in precision FLIM experiments. Thus, recording efficiency is an important issue in FLIM experiments. A good FLIM technique should have a high recording efficiency, i.e. it 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. It should record the entire fluorescence decay simultaneously in several detection channels, covering the entire emission spectrum.

FLIM 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\cite{27-32}. Time-domain techniques record the fluorescence decay functions directly\cite{33-39}. 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\cite{40,41}.

Both the frequency-domain and time-domain FLIM techniques can use either camera techniques or point-detector scanning techniques. 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 techniques employ time-correlated single photon counting (TCSPC), or gated photon counting with several parallel time-gates.

Camera techniques are used in wide-field microscopes, but they cannot reasonably exploit the benefits of the scanning microscope. Point detector techniques cannot be used for wide field imaging but are compatible with laser scanning microscopes.

Therefore, a FLIM technique that can exploit the high spatial resolution of a laser scanning microscope should be a high-efficiency point-detector technique. For the reasons mentioned above it should record images with a time-resolution better than 100 ps, resolve multi-exponential decay functions, and record the fluorescence signal in several wavelength intervals simultaneously.

2. Multi-dimensional TCSPC technique

Time-correlated single-photon counting (TCSPC) delivers a near-ideal efficiency and an optimal time-resolution for a given detector. It acquires the signal in a sufficiently high number of time-channels so that multi-exponential decay functions can be resolved\cite{42}. For a long time, TCSPC has been considered to be intrinsically slow and one-dimensional and therefore not 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 which are two orders of magnitude higher and have correspondingly shorter acquisition times. Moreover, advanced TCSPC techniques are able to use a multi-dimensional histogramming process. They can built up a histogram for the photons emitted from the sample not only versus the time axis, but also versus the coordinates of the scanning area, and the wavelength.

The principle of multi-dimensional TCSPC is shown in Fig. 1. At the input of the detection system are a number of photomultipliers (PMTs), typically detecting the fluorescence signal in different wavelength intervals. The pulses of the PMTs are combined in the subsequent ‘router’ into a common timing pulse line. Furthermore, for each photon, the router delivers the number of the PMT in which the photon was detected. The routing technique can be used for several individual PMTs and for multi-anode PMTs.

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 with respect to the laser pulse sequence (t), and the
detector channel number (n). These values are used to address the histogram memory in which the detection events are accumulated. Thus, in the memory the distribution of the photon density over X, Y, t, and n is built up.

![Diagram of multidimensional TCSPC lifetime imaging](image)

**Figure 1:** Principle of multidimensional TCSPC lifetime imaging

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. Due to the synchronisation via the scan clock pulses, the regular zoom and image rotation functions of the microscope act automatically on the TCSPC recording and can be used in the normal way.

The recording process does not use any time gating, wavelength scanning, or detector multiplexing. Under ideal conditions all detected photons contribute to the result, and a maximum signal-to-noise ratio for a given fluorescence intensity and acquisition time is obtained. However, the signal processing of a recorded photon causes a ‘dead time’ during which the TCSPC electronics is unable to process another photon event. As long as the photon detection rate is small compared to the reciprocal dead time of the TCSPC module the counting efficiency is close to 100%. For a detector count rate of the reciprocal dead time the efficiency is 50%, which is still better than for most other signal recording techniques. The *recorded* count rate at an efficiency of 50% is defined as the ‘maximum useful count rate’. For the fastest TCSPC modules the dead time is 100 ns, corresponding to a ‘maximum useful count rate’ of $5 \times 10^6$ s$^{-1}$.

Another limitation of the count rate is the ‘pile-up’ effect. Pile-up results from the fact that a single TCSPC device can record only one photon per excitation pulse. Pile-up is negligible for a count rate of 1% of the laser pulse frequency and remains tolerable up to a count rate of 5% of the laser pulse frequency. For the lasers currently used in lifetime microscopy the pulse frequency is 80 to 90 MHz, corresponding to a pile-up limited count rate of $4 \times 10^6$ to $5 \times 10^6$ s$^{-1}$.

It remains questionable whether count rates this high can be obtained from living cells, especially under two-photon excitation. Nevertheless, cells stained with extremely stable fluorophores, or large-area tissue samples under one-photon excitation may yield count rates higher than the limits given above. To record count rates this high in diffuse optical tomography, packages of several TCSPC modules have been operated in a single Pentium PC. Here, we applied a similar setup of four TCSPC FLIM cards to microscopic samples. With four detectors, connected to four separate TCSPC channels, the total useful count rate of this system is $20 \times 10^6$ s$^{-1}$.

### 3. Single-Channel TCSPC System

The single-channel TCSPC system uses two ultra-fast Hamamatsu R3809U MCP-PMTs connected to the non-descanned detection (NDD) module of a Zeiss LSM 510 laser scanning microscope. The two R3809U MCPs are connected to a single Becker & Hickl SPC-830 TCSPC module via a HRT-41 routing module (Becker & Hickl, Berlin). The instrument response (FWHM) of the system is 28 ps$^{-1}$. Figure 2 shows an autofluorescence image of aorta tissue recorded in one detector channel. The figure shows the intensity image calculated from the photons in all time channels of the pixels, and a lifetime image. In the bottom panel the fluorescence decay recorded in a selected spot is shown. Obviously, this
fluorescence decay could not be approximated by a single exponential function, but had to be approximated by a bi-
exponential function. The parameters of the fit (i.e. lifetime components and intensity coefficients) are shown in the
lower right panel. To obtain a lifetime image from the decay data of the pixels, the lifetime components of the double
exponential fit are weighted by their intensity coefficients and averaged. In the lifetime image, the mean lifetime
obtained for each pixel is encoded by colour. The distribution of the mean lifetime over the entire image is shown in the
upper right panel.

Figure 2: Fluorescence lifetime image of an aorta tissue sample excited by two-photon absorption. Upper row: Intensity image,
lifetime image with mean fluorescence lifetime encoded by colour, distribution of the mean lifetime over the entire image.
Lower row: fluorescence decay curve in the selected spot and parameters recovered in the fit.

Merging the components of the double exponential decay into a mean lifetime yields a single-exponential approximation
of the data but certainly neglects useful information. Fig. 3 shows images of the short and the long lifetime component,
and of the ratio of their intensity coefficients, encoded by colour.

Figure 3: Images of the individual decay components of the sample shown in Fig. 2. Left: Fast lifetime component (blue to red =
0.1 to 0.5 ns), Centre: Slow lifetime component (blue to red = 1.8 to 3.0 ns), Right: Intensity ratio of fast to slow lifetime
component (blue to red = 1.5 to 5.0)

The image of the intensity ratio of the lifetime components shows more detail than the lifetime images of the
components. It represents directly the relative concentrations of the molecules emitting the fast and slow decay
components.
The high time-resolution and the multi-detector capability of the FLIM system makes it excellently suitable for FLIM-based FRET measurements. To get images from the donor (CFP) and the acceptor (YFP) simultaneously, the fluorescence light was split into a $480 \pm 15$ nm and a $535 \pm 13$ nm detection channel. Splitting can be achieved either via the Zeiss NDD switch box or via an external dichroic beamsplitter and bandpass filters. The filters were selected to detect the fluorescence of the CFP and the YFP, respectively.

Figures 4-6 show measurements of an HEK cell expressing two interacting proteins, which are labeled with CFP and YFP, respectively. Fluorescence was excited by two-photon absorption at 860 nm. The TCSPC images were recorded with 128 x 128 pixels, i.e. with a 4 x 4 binning of the pixels of the regular 512 x 512 LSM 510 scan. The total count rate was reduced to 60,000 s$^{-1}$ in order to avoid any detectable photobleaching or possible formation of photoisomers. To obtain enough photons for high accuracy data analysis the images were acquired for 20 minutes. Donor and acceptor intensity images of the cell are shown in Fig. 4. The fluorescence decay recorded in the indicated region is shown in Fig. 5.

Figure 4: HEK cell expressing two interacting proteins labeled with CFP and YFP. Left: Intensity image recorded in the donor channel. Right: Intensity image recorded in the acceptor channel. The marker indicates the position at which the fluorescence decay curves of Fig 5 are obtained.

Figure 5: Fluorescence decay curves in the spots indicated in Fig. 4. Left: Fluorescence decay recorded in the donor channel. Right: Fluorescence decay recorded in the acceptor channel. Donor fluorescence recorded in this channel has been subtracted.

The decay functions in the pixels of the donor image are double exponential, with a fast lifetime component representing the quenched and a slow lifetime component emerging from the unquenched donor fraction. A Levenberg-Marquardt fit delivers a fast lifetime component of $\tau_f = 0.68$ ns and a slow component of $\tau_s = 2.30$ ns. The intensity coefficients are $A_f = 0.44$ and $A_s = 0.56$, respectively. Due to the energy transfer from the CFP to YFP, the YFP fluorescence should be double-exponential as well, with a short lifetime component corresponding to the short lifetime component of the donor. However, $a_f$ should be negative, due to gradual transfer of energy from the donor$^{44}$. Unfortunately the fluorescence spectrum of the CFP has a long wavelength tail that extends far into the spectrum of the YFP. Therefore the YFP channel detects a considerable amount of CFP fluorescence, so that the true shape of the acceptor decay function is not visible in the raw data. The leakage was determined by imaging cells containing only CFP. It was found to be 60 % for the filter combination used$^{40}$. By subtracting the known percentage of CFP leakage from the YFP data, corrected decay curves of the YFP were obtained. The result for the indicated pixel is shown in the right panel of Fig. 5. The decay is double exponential, with a fast lifetime component $\tau_f = 0.66$ ns, and a slow component $\tau_s = 2.86$ ns. The intensity
coefficients are $A_f = -0.4$ and $A_s = 0.6$. Images of the intensity ratios of the lifetime components, $A_f / A_s$, for the donor and $-A_f / A_s$ for the acceptor are shown in Fig. 6. For the donor, $A_f / A_s$ represents the ratio of the number of quenched and unquenched molecules.

Figure 6: Image of the ratio of the intensity coefficients $A_f / A_s$. $|A_f / A_s|$ is encoded by colour, from 0.2 (blue) to 1.0 (red). Left: Intensity ratios calculated for the donor channel. Right: Intensity ratios calculated for the acceptor channel.

4. Parallel Four-Channel TCSPC System

To record images at a high count rate a package of four fully parallel TCSPC imaging cards (Becker & Hickl, SPC-144) was used. The fluorescence light was distributed into four detectors (Becker & Hickl PMH-100) without wavelength dispersion. Each detector was connected to an individual TCSPC channel. The image size in all TCSPC channels was 256 x 256 pixels x 64 time channels.

Fig. 7 shows a 16 µm section of a mouse kidney (Molecular Probes, F-24630) stained with Alexa Fluor 488, Alexa Fluor 568 phalloidin, and DAPI. The excitation wavelength was tuned to 860 nm. The count rate was about $3.5 \times 10^6$ s$^{-1}$ per TCSPC channel, corresponding to a total count rate of about $14 \times 10^6$ s$^{-1}$. Fig 7, left, shows a lifetime image of the combined photon data of all four channels. A double exponential Leveberg-Marquardt fit was applied to the decay data in the individual pixels. The colour of the image represents the amplitude-weighted mean lifetime of the double exponential decay.

In Fig. 7, middle and right, the relative amplitudes of the two lifetime components are encoded by colour. The images show that double exponential lifetime analysis of TCSPC data can be used to unmix fluorophores even if they are recorded in the same pixel. In the same way, the relative amounts of associated and free forms of proteins can be retrieved from FRET measurements.\textsuperscript{15,16,19}

Figure 7: Mouse kidney sample stained with Alexa Fluor 488 wheat germ agglutinin, Alexa Fluor 568 phalloidin, and DAPI, recorded with four detectors connected to separate TCSPC channels. Left: Lifetime image, the colour represents amplitude-weighted mean lifetime, blue to red = 0.7 to 1.7 ns. Middle and Right: Images of the intensity coefficients of the fast and slow lifetime components. The relative intensity of the lifetime components is encoded by colour, blue to red = 0.1 to 0.9.
The good quality of the images shows that TCSPC can be used to obtain double exponential lifetime images in 10 seconds. However, to obtain high count rates high excitation powers have to be used, which can destroy the sample by photodestruction or thermal effects. Fig. 8 shows a sequence of lifetime images obtained from the same specimen shown in Fig. 7. The upper row shows a series of intensity images, which were calculated from subsequent measurements by summing up fluorescence signals of the four detectors and of all time channels in each pixel. The acquisition time of each image was 20 seconds. The initial total count rate was $14 \times 10^6 \text{ s}^{-1}$. The lower row shows the distribution of the mean lifetimes over the image.

The sequence shows that the high excitation power, to which the sample had to be exposed to achieve high count rates, causes photobleaching and thermal destruction of the sample. Since not all fluorophores are equally susceptible to photobleaching the distribution of the mean lifetime changes during the exposure. The results show that such effects can bias lifetime measurements considerably.

4. Summary
TCSPC FLIM in conjunction with laser scanning microscopes delivers multi-exponential fluorescence lifetime images in several detector channels simultaneously. The detectors can either be connected to a single TCSPC card or to separate channels of a package of TCSPC cards operated in a single computer. The single-TCSPC system is efficient up to a count rate of a few $10^6 \text{ s}^{-1}$. Via double-exponential lifetime analysis, this system is able to distinguish between different fluorophores in a single pixel or to distinguish between associated and free donor molecules in FRET experiments. The four-channel TCSPC system can be operated at count rates in excess of $10^7 \text{ s}^{-1}$ and delivers double exponential FLIM data in less than 10 s. Imaging at a count rate this high in a two-photon microscope induces noticeable photobleaching and lifetime changes over an acquisition time of a few 10 s. Thus, in practice it is not the TCSPC technique which imposes limits to the maximum count rate. It is rather the sample itself which tolerates only a moderate excitation power.

5. References


