Fibre optic time-resolved spectroscopy using CMOS-SPAD arrays

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ABSTRACT

In vivo fibre optic fluorescence-based sensing is the use of synthesised fluorophores which interrogate the local environment via variation in their fluorescence emission, addressed through an optic fibre. However, the emission intensity is influenced by intrinsic factors such as photobleaching, quantitative factors like concentration dependency and background signals from autofluorescence of tissue and the delivery optical fibre. Many of these problems can be addressed by using time-resolved spectroscopy which measures variations in the fluorescent lifetime. We present a versatile fibre-based time-resolved spectrograph based on a CMOS SPAD line sensor capable of acquiring time and spectral resolved fluorescent lifetime data in a single measurement exploiting the time-correlated single photon counting (TCSPC) technique. It is shown that these TCSPC histograms enable the differentiation between autofluorescence of tissue and synthesized fluorophores, as well as the removal of unwanted fibre background through post-processed time-gating. As a proof-of-principle application the pH-dependent changes in fluorescent lifetime of 5-carboxyfluorescein (FAM) are measured.

Keywords: Time-resolved spectroscopy, CMOS line arrays, TCSPC, Laser-induced fluorescence, Lifetime-based sensing

1. INTRODUCTION

Fluorescence spectroscopy can be used for in vivo endoscopic point-detection of physiological parameters, e.g. pH,\textsuperscript{1} oxygen\textsuperscript{2,3} and glucose level.\textsuperscript{4} Fluorophores can be synthesised to exhibit high sensitivity to the structure and local environment of mammalian tissue and molecules. Classical approaches for fluorescence-based sensing focus on changes in signal intensity, spectral emission variation and polarization.\textsuperscript{5} While these approaches are based on steady-state illumination, they suffer from concentration-dependent signals due to encapsulation or diffusion, photobleaching and, especially for in vivo applications, additional autofluorescence signals from the surrounding tissue. There are several approaches to overcome these limitations such as dual-fibre or fibre bundles,\textsuperscript{6,7} multi-core fibres,\textsuperscript{8} tapered fibre ends\textsuperscript{6} and ratiometric approaches where two fluorophores are used, one is environmental sensitive and the other one not.\textsuperscript{9}

In contrast, fluorescence lifetime is largely independent from fluorophore degradation or diffusion into the environment. The applications for fluorescence lifetime techniques inside the human body include measurements of physiological parameters and environments on a molecular level and protein-interaction via the Förster resonance energy transfer (FRET).\textsuperscript{10} The lifetime of fluorophores depends on their binding target and their environment.

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Time correlated single photon counting (TCSPC) is often the preferred method for measuring accurate lifetimes of the most common fluorophores in low light environments because it has a nearly ideal counting efficiency which ensures a high time-resolution. As TCSPC measurements are inherently photon starved, measurements are usually not slowed down by photon statistic restraints due to pile-up and counting loss effects or the detector dead time and bandwidth limitations. When working with biological samples, it is often the high excitation power, rather than the acquisition time, that is the limiting factor due to photobleaching and other effects that cause changes to the lifetime.

This paper presents a CMOS SPAD line sensor which can be operated in two modes, single photon counting (SPC) and TCSPC. In TCSPC mode, the line sensor is enabled to collect the photon arrival time histograms for 256 pixels simultaneously with a readout of 700 frames per second, where one photon can be counted per pixel per frame. To gather spectral and time-resolved fluorescent decay data the line sensor is combined with a pulsed laser, an epi-fluorescence system and a spectrograph. All components are linked together through fibre patch cable which makes them easy exchangeable to match the fluorophore testing arrangement. The epi-fluorescence system couples the laser light into the delivery optical fibre and collects the emitted fluorescence; the spectrograph disperses the fluorescence and focuses it onto the line sensor. The pulsed laser triggers the line sensor with an electrical sync signal to correlate the arrival time from the fluorescence photons. As a proof-of-principle application for fluorescence-based sensing through an optical fibre, various fluorophores and tissue were measured, including the pH-dependent fluorescence decay curves of an off-the-shelf fluorophore, 5-carboxyfluorescein (FAM). For this demonstration, a pH buffer system from 2.0 to 10.5 in 0.5 steps was set up and each buffer combined with a 5uM solution of FAM. The fluorescent lifetime was derived through non-linear least-square fitting of the fluorescent decay curves.

2. MATERIALS AND METHODS

2.1 Optical setup

Fig. 1 shows a schematic of the time-resolved spectrometer which consists mainly of four parts, a pulsed laser, an epi-fluorescence light coupling and collecting system, a spectrograph and the CMOS SPAD line sensor. The first three parts are connected to each other via multimode fibres (50µm, NA 0.22, Thorlabs) to make the whole spectrograph versatile for numerous types of fluorophores. While the line sensor is placed in the exit focal plane of the spectrograph.

Multiple fibre-coupled pulsed laser sources were used and chosen depending on the absorption spectrum of the tested fluorophore. For the calibration and alignment as well as for the first fluorophore testing of FAM
and Cy7, a filtered supercontinuum (WhiteLase Micro and LLTF Contrast, NKT Photonics-Fianium) was used. The supercontinuum has a fixed repetition rate of 20MHz and a pulse width of 6ps. The peak power varies with wavelength, the output power at the delivery optical fibre is 15µW at 480nm for the FAM testing and at 630nm for the Cy5 11µW. For the pH-sensing, a 485nm LED laser head (P-C-485, PicoQuant) in combination with a pulsed laser driver (PDL 800-D, PicoQuant) was used. The laser produces a pulse width of < 500ps, the average power at the fibre tip of the delivery optical fibre is 320µW. The repetition rate can be varied but 20MHz was chosen here.

In TCSPC, the laser repetition rate determines the temporal duration of the measurement. If the repetition rate is too short, photons originating from a fluorescent decay longer than the repetition rate are detected during the next cycle. This decreases the accuracy of the lifetime measurement and increases the background. FAM has a fluorescent lifetime of around 5ns so a repetition rate of 20MHz was chosen for the experiments which equates to a measurement window of 50ns.

The laser light is filtered and redirected by a dichroic beam splitter into the delivery optical fibre, in our case a multimode fibre (50µm, NA 0.22, Thorlabs). Standard Thorlabs interchangeable matched epi-fluorescence filter sets are used, chosen for the specific wavelength of the fluorophores under test. During this measurement, the delivery optical fibre is used as an indirect sensor where the fibre tip is dipped into a fluorophore solution and the optical fibre acts solely to transmit light. The fluorescence of the excited fluorophore solution is collected and further filtered by a long-pass filter from the epi-fluorescence set.

The basic design of the spectrometer is a tuneable Czerny-Turner design. The first spherical mirror collimates the light from the fibre tip, which acts as a circular slit in our case, towards the grating. The light is diffracted at the grating and collected by the second spherical mirror which refocuses it onto the CMOS SPAD line sensor. Since each of the wavelengths arrives at a different point in the exit plane the wavelengths which are projected onto the detector can be selected by rotating the grating.

The spectrograph is equipped with two ruled gratings placed on a motorized rotational stage, one with 600 grooves per mm (g/mm) and one with 150 g/mm. The wavelength range, spectral resolution and efficiency was measured using the supercontinuum in combination with the LLTF Contrast filter which exhibits a laser linewidth of 2.5nm. The spectral range for the 150 g/mm grating was measured to be 120nm with a pixel resolution of 0.47nm and the spectral range for the 600g/mm grating is 30nm with a pixel resolution of 0.12nm. Because of the wider spectral range the 150g/mm grating was chosen. The spectral resolution and spectral range can be altered by using gratings with higher groove density as well as placing a focusing lens in the beam path.

Both gratings are blazed in the UV which makes them less suitable for measurements in the visible, but are used here for convenience in these proof of principle measurements. The efficiency is 16 ± 3% at 500nm and 11 ± 2% at 600nm, measured with a Photodiode (S120C, Thorlabs). The efficiency can be greatly improved by using gratings which are optimized for the tested spectral region as well as using an on-axis transmission spectrograph.

### 2.2 CMOS SPAD Line Sensor

The dispersed light from the spectrograph is detected with a line sensor based on 130nm CMOS technology which enables the acquisition of time-resolved fluorescence spectra. The sensor consists of two line arrays, each with 256 pixels, which can be chosen for different applications. The first line of SPADs are optimised for the wavelength range from 600nm to 900nm, later referred to as red SPAD. The second line is optimised for the wavelength range from 450nm to 550nm and are referred to as blue SPADs. Four SPADs in a column form one pixel, each with a fill-factor of 43.7% and a geometrical dimension of 100µm × 23.8µm. For these measurements, red SPADs were chosen because they exhibit a lower dark count rate and a more efficient photon detection efficiency in the region of interest.

Front end software with graphical user interface was written in Python, version 2.7, and enables operation of the line sensor in SPC and TCSPC mode. For TCSPC mode, the printed circuit board is triggered with an electrical synchronisation signal from the laser driver to correlate the arrival of the photon in the detector which is the start of the laser pulse. The sensor is running in reverse start-stop mode which means that the detection of a photon starts the counter and the synchronisation signal from the laser stops it.
During measurements, a trade-off between a higher sensitivity and a lower noise level has to be made by choosing an appropriate detector bias voltage. This choice depends on the background noise level and the signal strength from the sample. The best detector performance is achieved for a low bias voltage. A bias voltage of 20.30V was chosen.

The serial line readout per pixel operates at a 8MHz internal clock and the readout is 700 frames per second for each pixel, which is currently a limiting factor for our measurements. With a low excitation power and the low efficiency of the spectrograph, we operated predominantly in the photon starved regime and far away from any classical pile-up effect or signal distortion due to dead time counting losses. The exposure time per frame was chosen to be 250µs. For a total integration time of 1s, the measurement time was around 10 minutes due to the low readout rate. Readout overheads have been significantly improved subsequent to this work with further hardware, firmware and software development.

The time-to-digital (TDC) conversion bin resolution is measured for each pixel by quantising the number of time bins between two laser peaks with known externally applied delay. The average TDC bin resolution is 423±4ps. The instrument response function (IRF) of the whole system was measured with the supercontinuum source. The IRF is wavelength dependent and measured to have a Full Width Half Maximum (FWHM) around 1.3ns and exhibits a long tail due to a SPAD architecture designed for high sensitivity at long wavelengths.

The sensor and board architecture is described and characterised in a previous paper.

2.3 Data analysis

Measuring the spectral and temporal information of the fluorescent emission from the sample results in a 3-dimensional data cube (see Fig 2). Each pixel correlates to a certain wavelength and the arrival time of single fluorescent photons are measured and histogrammed for each pixel. Along the time trace there is a region which only exhibits photon counts from the detector noise, the lab background and fibre background and no photons from the fluorescence decay. This region can be seen in Fig. 3 from 15 to 30ns, with data summed across all pixels in the spectra. The average background counts per pixel were calculated from this time bin range and subtracted pixel-wise.

The fluorescent lifetime is then estimated from the fluorescent decay curves using a single exponential decay fitted onto the curve using a self-written routine in Python using least square curve fitting with Levenberg-Marquardt algorithm. The fitting region along the fluorescent decay curve was chosen to be as free from
distortions as possible, from the maximum up to a distortion in the decay curve due to reflection in the optical path.

The fitted exponential decay rate is the inverted average fluorescent lifetime we are looking for. As we are interested in the lifetime in nanoseconds, this value has to be multiplied with the time resolution of the sensor. An accurate lifetime is obtained by performing the fitting routine to each pixel and then applying an amplitude- or intensity weighted average. However, dealing with a low signal from the fluorophore solution and a median dark count rate in the order of a 1kHz, we are limited by the noise of the sensor and a pixel-wise determination of the fluorescent lifetime is not possible. Instead, the 3-dimensional data are binned along the spectral axis, summing up each pixel with the same weight. By binning the data along the spectral axis, we are summing up data from 256 individual histograms with varying histogram intervals. The one-dimensional decay curve is therefore broadened and the fitted lifetime will be longer than what is expected from literature. Using the average time resolution for the conversion between histogram intervals to time introduces an error to the lifetime which should average itself out.

The two main errors in this analysis are the average time resolution and the lifetime fitting. The time resolution is measured for each pixel, the average and the standard deviation are then calculated from this. The average time resolution is 423ps with a standard deviations of 4ps. The standard deviation of the least-square fitting is derived during the fit analysis as the square root of the diagonal entries of the covariance matrix. Using the error propagation formula, the following error is derived

$$\sigma_{\tau} = \tau \cdot \sqrt{\left(\frac{\sigma_t}{t}\right)^2 + \left(\frac{\sigma_k}{k}\right)^2},$$

where $t$ is the average time resolution, $\sigma_t$ the standard deviation of the time resolution, $k$ the fitted decay parameter and $\sigma_k$ the standard deviation of the least-square fit.

3. RESULTS AND DISCUSSION

3.1 Distinguishing between autofluorescence and fluorophores

Fig 3 compares time-resolved fluorescent decay measurements with steady-state fluorescent intensity spectra from FAM and skin (human finger). In an in vivo measurement, the autofluorescence from tissue may mask the presence of a fluorescent label by exhibiting a varying background dependent on exact tissue location. Here we demonstrate that in a fibre coupled system we are able to distinguish between fluorophore labels (e.g. FAM) and tissue autofluorescence by exploiting varying lifetime characteristics.
Tissue is highly heterogeneous and exhibits overlapping excitation and emission spectra. The autofluorescence from the skin excited in the blue region is believed to originate from structural proteins like collagen and elastin. Each endogenous fluorophore has very specific emission spectra and lifetime characteristics which alter with environmental changes during the disease process but also during aging and photoaging. Fluorescence lifetime of endogenous fluorophores excited in the blue are mostly in the order of sub-nanosecond up to 3ns. The system presented here allows exploitation of the intrinsic tissue lifetime information with the spectral profile in vivo, in addition to separating this autofluorescence from engineered probe signals.

3.2 Removing fibre background

A great limitation to overcome in fibre-based endoscopic measurement is both the inherent Raman scattering and background fluorescence of silica optical fibres which are seen as a fibre length- and wavelength-dependent background in fluorescence intensity measurements, see Fig. 4. While it is constant and can be subtracted from the data, the shot noise level introduced by the large background remains.

Fig. 4 shows the steady-state fluorescent intensity spectrum in comparison to the time-resolved measurement of Cy5. The top right plot, performed with a commercial spectrometer, demonstrates that a weak fluorophore signal (from Cy5 dye) is not resolvable to the eye from the optic fibre background. However, under the same experimental conditions, the left hand plot demonstrates that in the time correlated trace the fluorophore signal is clearly visible. Signal to background (not possible to estimate on the steady state data due to lack of signal visibility) is clearly much enhanced in the time resolved measurement, due to separation in time of the fibre background from the fluorophore signal.

Using the fact that the fibre background is also present during the time span the photon is travelling through the optical fibre before the fluorescent decay is taking place, the TCSPC histogram can be divided into ‘timegates’ of solely fibre background and fibre background along with fluorescent decay. The background from the timegate with solely fibre background can be determined and subtracted from the signal timegate and, hence, the fluorescence decay and the spectrum of Cy5 can be recovered from the time resolved data. This is plotted in Fig. 4 (bottom right).

3.3 pH sensing

Fibre based measurement of the properties of fluorophores introduced into otherwise inaccessible locations offers the possibility of measuring environmental conditions remotely. In Fig. 5 (left) it is seen that the fluorescent
Figure 5. (left) Fluorescent decay curves for FAM in different pH buffer. (right) Fitted fluorescent lifetime versus pH buffer from 2 to 10.5 FAM (red) and the countrate (black) of the signal as a measure of the fit quality. The exposure time per frame was 25µs with a total integration time of 1s. The peak power at the delivery fibre tip was 320µW for measurements in pH buffer 2 to 5 and 32µW for the ones in pH buffer 5.5 to 10.5.

decay of FAM gradually increases with higher pH level. The changes are more distinct at lower pH, until the decay curve reaches a certain lifetime in the higher pH region. In Fig 5 (right) the fitted lifetime is plotted versus pH. The lifetime of FAM shows a gradual increase from pH 5 up to pH 9. Overall it shows an increase of 0.44ns in this region. From pH 9 on, the lifetime levels-off due to OH saturation.

The physiological pH region for the human lung is between pH 6 and 8 with growing interest in developing technologies to measure this clinically. While signal amplitude is clearly seen to vary in this region, intensity is too susceptible to varying experimental conditions to be reliably used as a measure of pH. Here, distinct changes of fluorescent lifetime in this pH region measured through optic fibre offer a more robust pH sensor. Future work beyond the scope of this paper includes exploiting modified probes utilising FAM fluorophores as labelling dyes, with modified structures to be more sensitive to the pH of the environment.

The derived fluorescent lifetimes are longer than expected from literature due to systematic errors previously discussed (e.g. detector timing resolution). Comparing our fitted lifetimes with the lifetimes measured at a state-of-the-art confocal laser scanning microscope (Edinburgh Super-Resolution Imaging Consortium), we find that for the low pH buffer and low signal measurement we have an error of 35% which reduces to an error of 22% for higher pH buffer and a stronger signal. For sensing applications the variation of the fluorescent lifetime with pH is the feature of interest, rather than the knowledge of the absolute quantitative lifetime.

4. CONCLUSION

We presented a versatile time-resolved spectrograph for optical fibre-based measurements applicable to in vivo implementation, which can be used to simultaneously get time and spectral information about fluorophores. The capability and versatility of this technique have been demonstrated in three ways. The lifetime signal of fluorophores and tissue were measured and resolved, offering techniques for distinguishing fluorescent markers from tissue or examining intrinsic tissue lifetime signatures. A dominant optical fibre background was effectively removed through a time resolved measurement, allowing the spectrum of the fluorophore to be recovered where it was previously not visible. Finally, as a proof of principle, small changes in fluorescent lifetime dependent on environmental conditions, pH in our case, were successfully measured.

While the work presented here offers only brief investigation of each of these applications, the potential for this system combining and exploiting these features is vast. Optical fibre investigations in hard to access regions of the body are technically challenging, we believe exploitation of the system described helps overcome those challenges moving towards fibre based fluorescence spectroscopy in vivo.

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