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Wide-field TCSPC-based fluorescence lifetime imaging (FLIM) microscopy

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ABSTRACT

Time-correlated single photon counting (TCSPC) is a widely used, sensitive, precise, robust and mature technique to measure photon arrival times in applications such as fluorescence spectroscopy and microscopy, light detection and ranging (lidar) and optical tomography. Wide-field TCSPC detection techniques, where the position and the arrival time of the photons are recorded simultaneously, have seen several advances in the last few years, from the microsecond to the picosecond time scale. Here, we summarise some of our recent work in this field with emphasis on microsecond resolution phosphorescence lifetime imaging (PLIM) and nanosecond fluorescence lifetime imaging (FLIM) microscopy.

Keywords: Time-correlated single photon counting (TCSPC), fluorescence lifetime imaging (FLIM), phosphorescence lifetime imaging (PLIM), single photon detection, microchannel plate (MCP), delay line anode, single photon avalanche photodiode (SPAD), SPAD array

1. INTRODUCTION

Fluorescence microscopy is an important tool in the life sciences, as it is minimally invasive and allows the observation of cell dynamics and function in real time with negligible cytotoxicity. It can also provide high sensitivity, down to the single molecule level, and high specificity. In addition to localising fluorescent labels, the fluorescence can also be used for sensing and provide information about the environment of the fluorophore, via its spectral properties, its polarization or lifetime.[1]

The fluorescence decay is characterised by the fluorescence lifetime, which is the average time a fluorophore remains in the exited state. It is typically on the nanosecond time scale, whereas phosphorescence, which originates from the triplet state, is typically on the microsecond time scale, or longer. The fluorescence or phosphorescence decay can be a function of viscosity, temperature, pH, ion or oxygen concentrations, glucose, refractive index or polarity, and of interaction with other molecules, e.g. due to Förster Resonance Energy Transfer (FRET), a widely used technique to identify protein conformational changes or interactions. Moreover, autofluorescence lifetime measurements of intrinsic fluorophores are increasingly used in clinical diagnostics. Combined with imaging, fluorescence lifetime imaging (FLIM) or phosphorescence lifetime imaging (PLIM) can map the lifetimes in every pixel, thus providing image contrast according to the lifetime.[2] The resulting images can be viewed as viscosity maps, ion concentration maps and temperature maps, and they are independent of the fluorophore concentration, which is difficult to control in cells.

Time-correlated single photon counting (TCSPC) is a powerful delayed coincidence method that has long been used to measure fluorescence decays.[3] Its advantages stem from the digital nature of the technique, based on whether a single photon is detected after an excitation pulse, or not. It thus obeys well-defined Poisson statistics, which state that the counting error is the square root of the number of counts, and that the signal to noise ratio increases with the measurement time, i.e. the number of counts. It also has a large dynamic range in time, from picoseconds to

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microseconds, allowing PLIM, and affords an easy visualisation of fluorescence decays. TCSPC has the highest signal-to-noise ratio of the standard time-resolved imaging methods,[4-6] and is accurate enough to permit multi-exponential fluorescence lifetime fitting. FLIM or PLIM is often carried out with TCSPC-based confocal or multiphoton beam scanning. This approach employs single point or single pixel detectors, where the image is created pixel by pixel by raster scanning the focal spot over the sample, and provides intrinsic optical sectioning.[7]

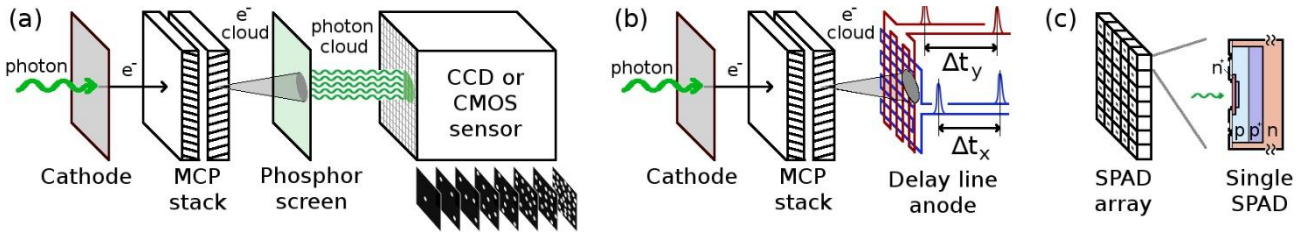

Figure. 1. Schematic overview of wide-field time-correlated single photon counting (TCSPC) methods. (a) Camera with a microchannel plate (MCP)-based image intensifier with a phosphor screen. (b) MCP-based image intensifier with a delay line anode. (c) Single photon avalanche diode (SPAD) array.

While TCSPC is straight-forward to implement with scanning microscopy, there are a number of fluorescence microscopy methods that employ a camera, for example lightsheet, total internal reflection fluorescence (TIRF) and supercritical angle fluorescence microscopy, super-resolution fluorescence microscopy methods based on localisation or re-scan confocal and structured illumination microscopy, as well as optical sectioning methods with speckle illumination, a spinning disk, temporal focusing, multifocal multiphoton microscopy or structured illumination. To harness the advantages of TCSPC for these microscopy methods, and to perform single photon sensitive picosecond resolution wide-field FLIM with these methods, a single photon sensitive camera with picosecond time resolution is required.[8,9] There are a number of position sensitive picosecond resolution read-out schemes for intensifiers, e.g. charge division and propagation time techniques, which can perform this task, as discussed below. Moreover, long decay times such as microsecond PLIM decays can require lengthy measurements due to the sequential nature of raster scanning. In this case, the process of data acquisition can be sped up significantly by the parallel acquisition of the photons in all pixels simultaneously, i.e. with a camera, perhaps at the expense of optical sectioning. Here, we summarise some of the wide-field TCSPC methods that have been described by us and others, and discuss some applications of these techniques.

2. TECHNIQUES

Traditionally, image intensifiers have been used for wide-field photon counting imaging, for example the Faint Object Camera on the Hubble Space Telescope was based on a cascade intensifier.[10] The prime consideration at the time it was designed and built was sensitivity, not photon arrival timing. In modern microchannel plate (MCP)-based intensifiers, an incoming photon hits a photocathode creating a photoelectron, which creates secondary electrons in the MCP pores when it is accelerated through a high voltage. The resulting electron cloud can either be converted back to photons with a phosphor screen and detected with a conventional camera, in this case the time resolution is given by the frame rate of the camera (Figure 1a),[11] or the electrons can be read out directly with a position-sensitive anode (Figure 1b), typically one photon at a time, but with picosecond time resolution.[8]

Another possibility for a position-sensitive detector is to build an array of single photon sensitive point detectors.[12-14] A single photon avalanche diode (SPAD, biased above the diode breakdown voltage) is a small all-solid state photon detector with a diameter of a few microns, and is capable of single photon detection with picosecond time resolution.[15] It does not require a high voltage or a vacuum, is not damaged by high light levels, and can be manufactured in arrays (Figure 1c); 256×256 pixel[16] and 240×320 pixel SPAD arrays[17] have been reported.

2.1 MCP-based intensifiers with a phosphor screen and camera readout

2.1.1 Timing obtained from the camera frame rate by direct imaging of photon events

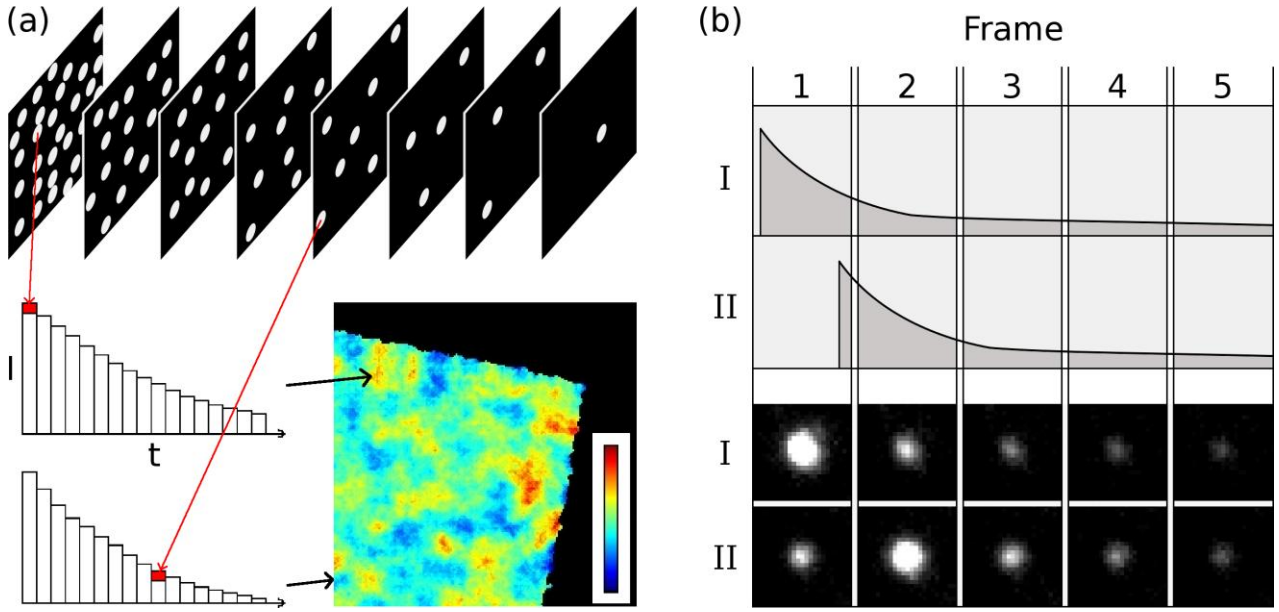

Figure. 2. (a) Principle of wide-field TCSPC data acquisition with an MCP intensifier and a CMOS camera. After each excitation pulse, a sequence of frames is acquired. Software searches the frames for position of the photons and their arrival time (i.e. their frame number). These are used to build a histogram of arrival times for each pixel. A lifetime image is obtained by fitting an appropriate exponential decay function to the arrival time histogram in each pixel, and encoding the lifetime in a pseudocolour scale.[18,19] (b) The time resolution can be improved by using an intensifier with a sufficiently long phosphor decay time so that the photon events are seen in consecutive frames. The sample is excited at the beginning of each frame exposure, and the arrival time is found from the relative intensities in the first two frames where the event is detected.[20] Photon I arrives at the beginning of frame 1 and most of the intensity from the phosphor decay is in this frame, whereas for photon II arriving at the end of the frame the second frame is brightest. Reproduced from reference [21]

Recent developments in sensor technology have allowed complementary metal oxide semiconductor (CMOS) cameras to reach MHz frame rates.[18] These cameras can be used in combination with a photon counting image intensifier (i.e. operated with saturated gain) for TCSPC. After each excitation pulse, a sequence of frames is acquired during the decay time of the probe, and this process is repeated until enough photons are collected so that a decay histogram is obtained for each pixel of the image, as shown in Figure 2a. The time resolution of these approaches is limited by the frame rate of the camera – currently 10 MHz with commercially available CMOS cameras – to the microsecond time scale. Although there is a trade-off between a high frame rate and the number of pixels that can be imaged, this technique enables the collection of up to hundreds of photons per frame, and even several photons after one excitation cycle per pixel as long as they arrive in different frames.[19]

2.1.2 Timing obtained from imaging the phosphor decay

Due to the phosphor decay, the image intensifier output screen has an afterglow that is usually undesired.[22] The phosphor decay time depends on the type of phosphor and can range from nano- to milliseconds, which can cause image artefacts with time-resolved measurements.[23] However, the afterglow, i.e. the invariant phosphor decay characteristics, can be exploited to find the photon arrival time within the frame exposure time.[24] The principle of obtaining photon arrival time information from the phosphor decay is similar to double exposure methods in time-of-flight mass spectroscopy.[25,26] It is based on matching the phosphor decay with the camera frame rate such that the photon events can be seen in several consecutive frames. The photon arrival time within the exposure time can then be found from the relative brightness of the photon event in different frames, as illustrated in Figure 2b. The measurement of the arrival time from the photon

event phosphor decay can improve the time resolution beyond the inverse frame rate of the camera, and the lower frame rate increases the number of recorded pixels, thus allowing bigger field of view.[20,27] Phosphorescent sample decays as short as 500 ns have been measured with a P20 phosphor and 300 kHz frame rate,[21] but a combination of a faster phosphor and a faster frame rate, or special cameras such as timepix[28] or pimms,[29] could allow the measurement of even faster sample decays.

Camera-based wide-field TCSPC is especially well suited for sensitive measurements of phosphorescence lifetimes in the micro- and millisecond time region – a technique termed phosphorescence lifetime imaging (PLIM).[2,30] Camera-based methods enable the collection of hundreds of photons per excitation cycle, shortening the data acquisition time with long lifetime probes compared to single point scanning measurements, but at the expense of optical sectioning. Lifetimes around 1 μs have been measured with several transition metal probes using these techniques, including a ruthenium-based oxygen sensor in living cells, with total image acquisition times of just a few seconds.[18,20] In fact, the best use of this approach is probably for long lifetime PLIM measurements due to the limited overall count rate of the MCP-based intensifier combined with its low noise compared to SPADs. 0.02 events/s/cm^2 have been quoted for MCP-based intensifiers.[31]

2.2 MCP-based intensifiers with an electronic anode readout

For nanosecond fluorescence lifetimes, picosecond timing accuracy is essential. While the MCPs themselves are capable of timing the photon arrival with a precision of a few tens of picoseconds,[7] they are not capable of recording the position without special read-outs. Different read-out architectures have been developed,[8] where the position of the electron cloud is determined via a charge division approach, or via the propagation time along a delay line, see Figure 1b. One of the first and simplest two-dimensional resistive anode read-out consists of a square sheet of insulating material with resistivity coating and a contact at each corner.[32] The pulse amplitudes and rise-times are proportional to the event distance from the contact, and the event location can be determined from either the charge division or the difference in the signal timing between opposing contacts. More sophisticated read-out architectures have been developed, including quadrant, wedge-and-strip, cross-strip and delay line anodes,[8,9] see Figure 3. The main drawback of these types of anodes is that the count rate is limited by the position readout electronics rather than the need to avoid overlapping events. Indeed, some of the read-out schemes can accommodate multiple photon events after one excitation cycle, i.e. the hexanode[33] or cross strip read outs.[8]

Whole-field data collection allows the tracking of individual molecules or particle trajectories, and single quantum dot tracking and FLIM has been demonstrated with a MCP and delay line anode detector.[34] However, the global count rate of most of these detectors has so far been limited by the position read-out electronics to a few 10s or 100s of kHz, which means image acquisition times of minutes are required.

2.3 SPAD arrays

A SPAD is a reverse biased semiconductor p-n junction operating with a bias voltage above the breakdown voltage where a single photon (or a single dark current electron) can set off a significant avalanche of electrons.[15] Single SPADs were first used for fast timing applications in the 1980s, and the implementation of SPADs in CMOS technology in 2003 enabled the development of SPAD arrays. In the past decade a number of SPAD array image sensors have been developed, which simultaneously deliver single photon sensitivity, tens of thousands of pixels spatial resolution and picosecond timing resolution.[35,36] The outstanding capability of enormous global count rates well into the gigahertz region,[37] which would allow the observation of fast cellular dynamics, is a big advantage of these devices. Although SPAD arrays currently suffer from a low fill factor, noise and non-uniformity of the pixels across the array, recent developments have shown significant improvements of these features, and this field continues to develop at a fast pace.[15,36]

2.4 Other methods

Single photon detection capabilities of several other detectors have been demonstrated, although they have not yet been applied to FLIM.[38] Superconducting tunnel junctions (STJ) arrays have been used for single photon detection in astronomy, and have been applied to the measurement of fluorescent spectra.[39,40] They have an intrinsic wavelength resolution, a high quantum efficiency over a very large wavelength range from x-rays to infra-red and low noise, but they

need to be operated at liquid helium temperatures, i.e. milli-Kelvin temperatures below -270°C. Superconducting nanowires[41] have also been used as single photon point detectors, with a good sensitivity in the red and infrared wavelength region and picosecond timing resolution,[42] and have been shown to be excellent detectors for time-resolved singlet oxygen luminescence at 1270 nm.[43] Arrays of STJs and superconducting nanowires have been made,[38] but the readout of a large number of pixels remains a challenge.

Single photon detection is also possible with electron-bombarded (EB) sensors, where the photoelectrons from the photocathode are accelerated directly into a CCD or CMOS sensor.[44-47] Unlike MCPs where the statistical electron multiplication process creates a broad pulse height distribution, in EB sensors the photon event brightness depends on the gain voltage. Thus, by sweeping the gain voltage during the exposure time, it could be possible to obtain photon arrival time information from the photon event brightness.[45] The concept has been proposed and simulated, but so far gain sweeping has not been possible with commercially available devices. In addition, line scanning streak cameras have been used for FLIM,[48] and a double pulse method similar to pump-probe techniques has been proposed,[49] which would be compatible with wide-field FLIM.

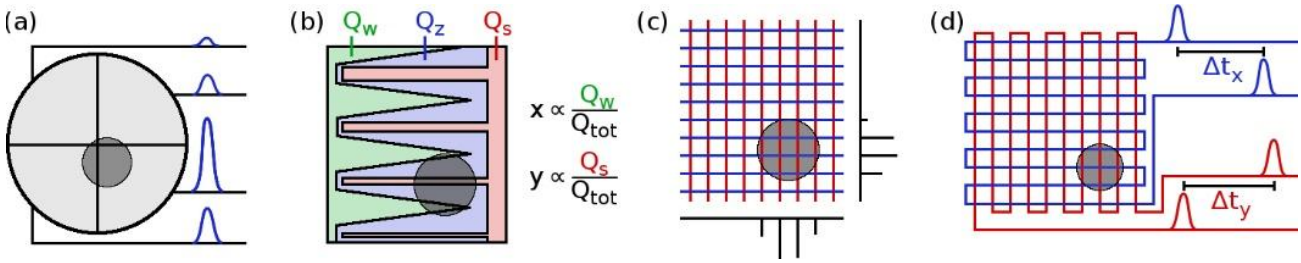

Figure 3. Different position-sensitive anode read-out schemes. (a) In a quadrant anode, the position of the photon event is obtained from the relative charge in the four quadrants. (b) In a wedge-and-strip anode the *x* and *y* position of the photon event is calculated from the relative charge of the components, Q_w, Q_z and Q_s. (c) In a cross-strip anode, all strips are read out and the position is obtained from the charge distribution. Multiple photon events can be accommodated with this scheme, as long as they are separated in time and space. (d) In a delay line anode, the position of the photon event is obtained from the signal propagation time difference to the ends of the delay line. Multiple photon events can be discarded by a run-time check, or timed with a third delay line (hexanode).[33]

3. APPLICATIONS

3.1 FLIM and PLIM microscopy

The low excitation power used in wide-field TCSPC can help to minimise photodamage in living cells which is especially beneficial for observing dynamics in living cells over long periods. Wide-field data collections allows particle tracking, and techniques that enable the collection of many photons per excitation pulse speed up the data collection time for long lifetime measurements. Quadrant anodes have been applied to FLIM of mitochondrial transport in neuronal processes and to the study of protein-protein interaction by FRET[50] and photosynthesis.[51] Single-quantum dot tracking and FLIM has been demonstrated with an MCP and delay line anode detector.[34] Camera-based methods that enable the collection of many photons per excitation pulse allow long lifetime measurements in PLIM with low excitation power and without lengthy scanning. We have imaged ruthenium based oxygen sensors in living cells with this method, with total image acquisition times of just a few seconds.[18,20]

Wide-field TCSPC is especially useful for microscopy methods where the whole field of view is illuminated with a technique that provides depth discrimination. One of these techniques is TIRF microscopy, where the sample is excited by an evanescent wave only near (up to 100 nm) the coverslip. Total internal reflection (TIR)-FLIM has been demonstrated with quadrant anodes[52] and SPAD detectors.[53] We have used a delay line anode detector (Photek) where the delay line is capacitively coupled to a resistive anode inside the tube and using an image charge technique,[9,54] to perform TIR-FLIM. Figure 4 shows wide-field TCSPC FLIM images of fixed HeLa cells acquired with this delay line anode detector, read out by conventional TCSPC timing boards (Becker & Hickl). The sample was excited with a Horiba DeltaDiode picosecond laser (375 nm) at 10 MHz, and the photon count rate was around 80 kHz, with an acquisition time of 100s. The measured intensity shows the cell membrane stained with membrane dye laurdan only under TIRF illumination (Figure 4a), while the whole cell is visible under wide-field illumination (Figure 4b). The laurdan fluorescence lifetime is also shortened under TIRF illumination (Figure 4c) compared to wide-field illumination (Figure

4d); one contributory factor here is the proximity of the high refractive index glass coverslip which consequently shortens the fluorescence lifetime.[55] Figure 4f shows the measured instrument response function (IRF), and the measured time decays in a small area in Figure 4c,d. The IRF full width at half maximum (FWHM) is 344 ps.

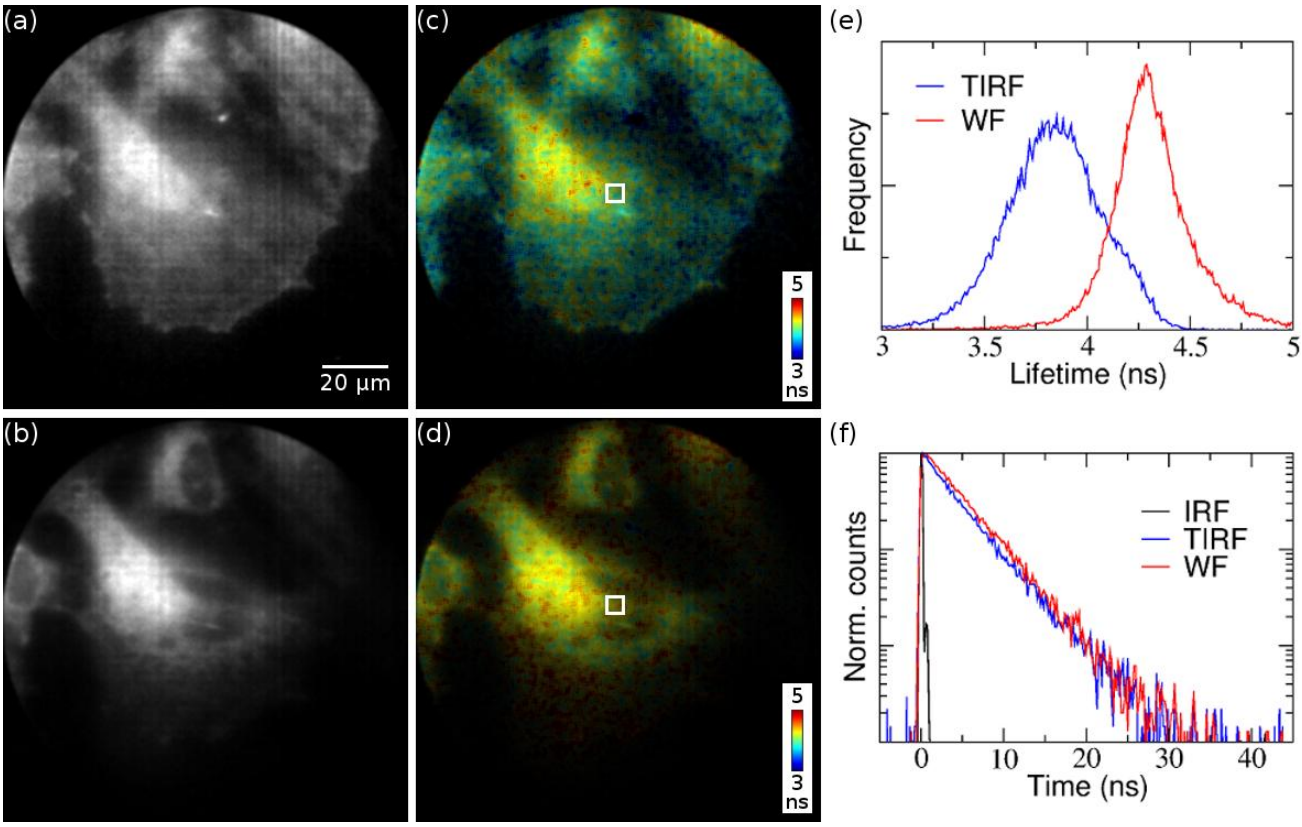

Figure 4. TCSPC images of fixed HeLa cells, stained with membrane dye laurdan, acquired with a delay line anode detector. The measured intensity shows the cell membrane only under TIRF illumination (a), and the fluorescence lifetime of laurdan is shorter (c), while the whole cell is visible under wide-field illumination (b) and the laurdan lifetime is longer (d). (e) Histograms of the individual pixel lifetimes in (c,d). (f) Fluorescence decays in the area indicated by a white rectangle are shown in (c,d). The instrument response function, measured with reflection, has a FWHM of 344 ns.

3.2 Applications in other fields

Apart from the visible light used in fluorescence microscopy, many single photon detectors can detect other types of electromagnetic radiation, including UV and infrared photons, x-rays and gamma rays, as well as particles, such as electrons, neutrons and ions. Infra red photons are used in photon time-of-flight measurements for light detection and ranging (lidar)[56] and optical tomography,[57] and MCPs are used in time-of-flight mass spectroscopy for detecting molecular ion fragments.[58] Moreover, MCPs have been used in autoradiography to detect decay emissions from radioactively labelled samples,[59] and boron doped MCPs have been used for neutron detection.[60,61] The development of wide-field detectors for FLIM may also benefit these applications.

4. CONCLUSIONS

FLIM is a widely used imaging technique in the life sciences which allows the monitoring of the microenvironment of fluorophores and their interaction. Wide-field TCSPC-based FLIM combines the advantages of single photon sensitivity and accuracy with wide-field data collection. This is important for implementation of FLIM microscopy methods that typically employ cameras, such as TIRF, lightsheet and others. The extremely low illumination intensity, distributed evenly over the field of view, is beneficial especially in life science applications where it allows long-term monitoring of

living cells and organisms, while wide-field data collection enables the observation of cell dynamics and single particle tracking.

We have shown that wide-field time-correlated single photon counting based on an image intensifier with a phosphor screen and a fast CMOS camera can be employed for PLIM to map phosphorescent decays on a microsecond time scale. To image nanosecond fluorescence decays, we show that a crossed delay line anode detector read-out with conventional TCSPC boards is feasible. This approach retains all the advantages of TCSPC, and extends them to wide-field detection, serving essentially as a single photon sensitive camera with picosecond time resolution. Application of this approach to TIR FLIM using the membrane dye laurdan shows lifetime contrast between the plasma membrane and the interior membranes of the cell. These approaches are particularly useful for fluorescence microscopy methods employing a camera to enable TCSPC-based FLIM, the FLIM method with the hightest signal to noise ratio, with these types of microscopy.

Apart from FLIM, single photon counting methods are used in other fields of science and technology. The development of wide-field TCSPC methods will benefit fields where time-of-flight measurements are required, for example lidar or ion velocity mapping. Wide-field TCSPC methods are currently mainly based on MCP-based detectors, a mature technology used especially in astronomy and medical imaging. However, the development of SPAD arrays and their application to FLIM with the prospect of huge photon count rates continues at an increasing pace, and the use of superconducting detectors for wide-field TCSPC, for example in the infra red, may also be feasible.

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