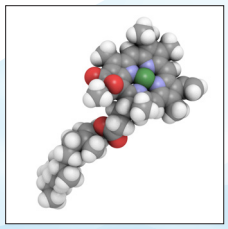
HORIBA Scientific

Fluorescence

FLASH-FLIM measurements with the FLIMera TCSPC Camera



Application Note
Life Sciences
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The HORIBA team in Glasgow, Scotland, UK

Abstract

The use of the fluorescence lifetime as a means to add contrast and extract molecular information has increased over the years. Fluorescence lifetime imaging (FLIM) using the sensitive time-correlated single-photon counting (TCSPC) technique has traditionally involved single point detection and scanning the sample. This has limited data acquisition speeds and led to the technique being thought of as slow. Developments in complementary metal oxide semiconductor (CMOS) technology has resulted in array detectors with in-pixel detection and timing to be fabricated. These recent developments allow widefield lifetime imaging, with the ability to measure FLIM in real time, to be employed to investigate mobile samples and kinetics, as well as remote imaging.

Introduction

The use of FLIM is usually associated with confocal microscopy systems, where a tightly focussed laser beam is scanned over a sample. When using TCSPC, which is regarded as extremely sensitive and provides time-resolutions to a few ps [1], the fluorescence decay at each small area of the sample is measured using a single TCSPC detector. The decay histogram is usually produced using a single set of timing electronics. The speed of image acquisition is limited by the scan rate, equipment deadtime as well as the photon flux. A widefield approach involves illuminating the whole of the sample, and the use of an array detector or camera allows all of the sample to be imaged at the same time, without scanning. Widefield illumination can be restrictive in terms of spatial resolution; dependent on the number of pixels and optical setup. Until recently, technology for recording widefield FLIM [2] has not enabled the full speed potential to be realized.

The recent advances in CMOS technology have now enabled arrays of single-photon avalanche photodiodes (SPADs) to be produced along with associated timing electronics [3]. As well as a SPAD detector, each pixel contains a time to digital converter (TDC) to time photon events (in-pixel timing using TCSPC). The array size has also increased such that the resolution is now useful for imaging applications. The ability to simultaneously process TCSPC data in individual pixels enables lifetime images to be displayed in real time.

In this note the “Fast Lifetime Acquisition by Simultaneous Histogramming” (FLASH) using the FLIMera TCSPC camera is described; with example applications of “FLASH-FLIM” reported.

The FLIMera TCSPC camera

This widefield TCSPC camera is based on a CMOS sensor with an imaging array of 192 x 126 pixels, with each pixel containing a SPAD and an associated TDC, see figure 1. Details of the sensor chip on which it is based have been previously reported [4].

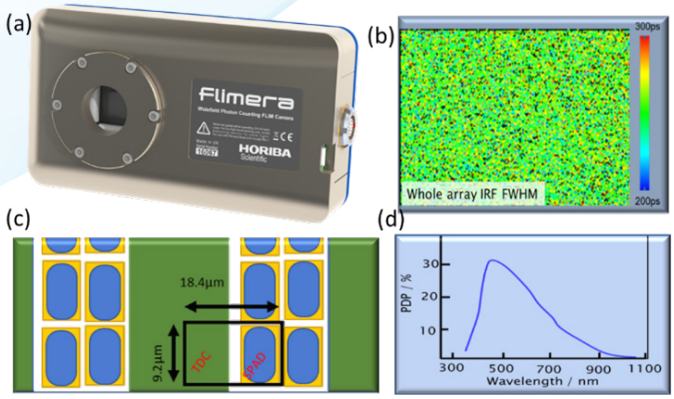

Figure 1: (a) The FLIMera camera, (b) IRF image over the whole array, (c) schematic of the sensor construct and (d) the wavelength response.

The FLIMera has a “C-mount” for easy attachment to an appropriate microscope or lens system, if it is to be used for MacroFLIM. A pulsed laser excitation source (such as a HORIBA Scientific DeltaDiode [5]) is required for the widefield illumination of the sample. The source repetition rate should be between 5 MHz and 100 MHz. Electronic synchronisation (TTL signal) is via the FLIMera base unit (FBU). Both data acquisition and analysis are performed using the simple to use, intuitive EzTime Image software package.

The electrical connections for the FLIMera are shown schematically in Figure 2.

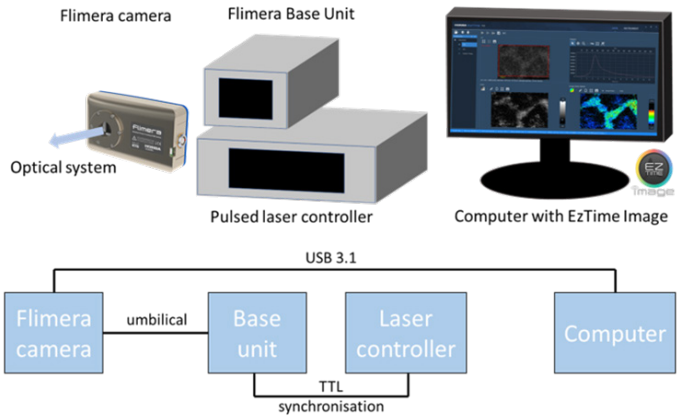

Figure 2: Schematic of the FLIMera electrical connections.

Measurement modes

When the FLIMera is turned on and the software launched, a photon counting intensity image is displayed. This simple intensity image (obtained by binning the native frame rate of ~12k fps), can be adjusted to provide image contrast, as well as the binning altered for responsiveness. This image can be used to help select the area for the lifetime measurement.

Since the FLIMera measures fluorescence lifetimes in all pixels simultaneously (FLASH-FLIM) it can image a sample in a widefield configuration very rapidly. Combined with the EzTime Image software this speed means that it is possible to present an average lifetime image, real time up to video rate (~30 fps) dependent on sample and measurement conditions. This “FLIMFlix” feature can save the average lifetime video as an “avi” file to be played back using third party software. Although useful to show contrast, the average lifetime value itself is only an approximation and should be treated as such. To gain more insight, a fuller analysis, fitting the data is required. The software enables a full global analysis, of up to five exponentials, to be performed on all pixels in the lifetime image as a tail fit. This cannot be done using the “FLIMFlix” data but only on data acquired in a normal timed measurement. If analysis of kinetic data is required, this can be accomplished by initially “streaming” the FLIM data to a HDF5 file format using EzTime Image. This file contains the complete records (photon stream data) for the photon events in each pixel, labelled with their macro- and microtime. This can be read using third party software or manipulated using EzTime Image.

Data representation

In a TCSPC measurement, both the intensity and average lifetime images are shown. EzTime Image can also display the “sum” histogram from either selected individual pixels or regions. EzTime Image can also display a phasor chart allowing selection of lifetime regions within the Intensity and Lifetime images to be shown.

Schematically these options are given in Figure 3.

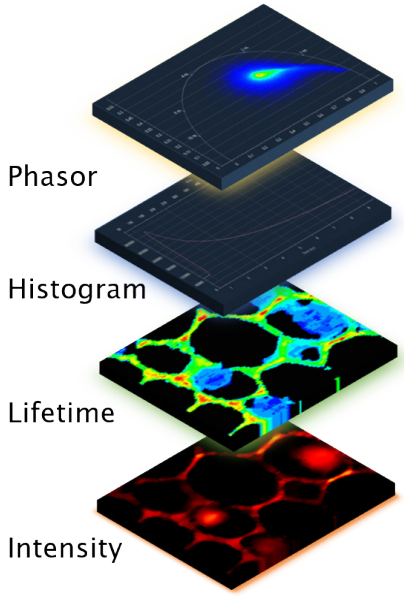

Figure 3: Schematic of the different types of data representation.

EzTime Image also displays analyzed data (in the TCSPC parameter window), giving the options of showing the average lifetime, the relative contributions of the normalised pre-exponential factors for the lifetimes and the goodness of fit (chi-squared) parameter. Figure 4 shows the user interface for the Data Page in the software.

Figure 4: Layout of the EzTime Image “Data Page”

As with most CMOS-based devices of this type there is the possibility of “hot” pixels. Their effect is to occupy data transfer bandwidth and can show up as bright spots on an image. The EzTime Image software can apply a “mask” to turn off these pixels, which in turn increases data transfer efficiency.

The effect of “hot” pixels or “screamers” is seen in Figure 5.

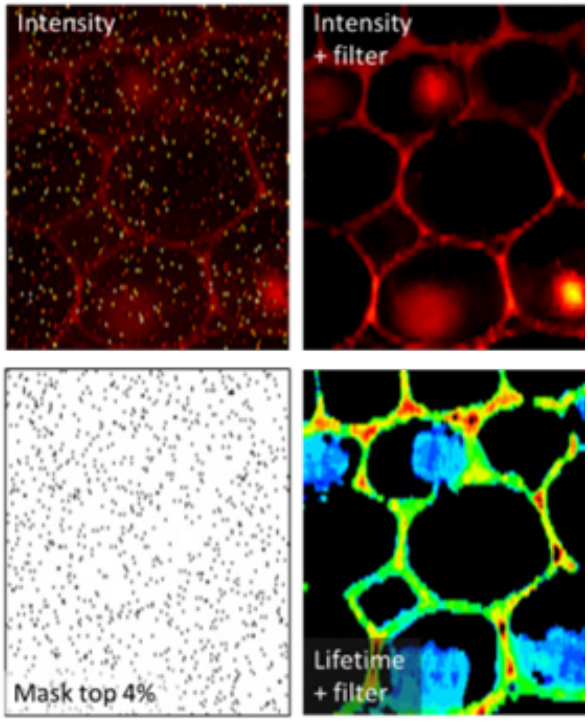

Figure 5: Effect of “hot” pixels on image quality and its mitigation using a pixel mask and noise reduction filter. The position of the noisiest 4% of pixels, which can be masked is also indicated.

These are spread across the sensor, so any gap in the image can be mitigated by use of the median filter available in EzTime Image.

Measurement examples

The FLASH-FLIM approach is exceptional at acquiring TCSPC data rapidly, in comparison to traditional scanning FLIM systems. This means that the application of fluorescence lifetimes to the investigation of mobile systems, such as live cells, and kinetic processes is now more readily available. This can assist in the study of metabolic and signalling pathways in and between cells. It can also be used to give lifetime capability to forms of microscopy where an intensity reading CCD camera is typically employed. This includes widefield, light sheet, total internal reflection fluorescence [2] and potentially single molecule [3] microscopy. Also by changing the optical system, e.g. replacing the microscope by a lens system, macroscale FLIM measurements can be made. Obviously, these require diffuse laser illumination of the subject or a very rapid scan.

Although having potential to collect image data very rapidly, as with all types of fluorescence microscopy, there has to be a sufficient number of photons in order to analyse the data [5]. Thus, in order to obtain good contrast and imaging up to video rates requires sufficient photons to be present.

In the following sections are some brief (but far from exhaustive) examples of the use of a FLIMera camera.

Widefield FLIM

The fact that the FLIMera can be easily attached to a microscope means that it is simple to add FLIM capability. Obviously there needs to be an appropriate optical path. The widefield approach, coupled with FLASH-FLIM, allows for rapid fluorescence lifetime collection. This is demonstrated by the measurement on a fixed sample of GHFT1 cells containing a Cerulean-5-Venus FRET pair which was acquired in 1 second and made use of HORIBA Scientific DeltaDiode pulsed laser excitation sources. The average lifetime image is obtained from the global fitting to a two exponential decay model (see Figure 6).

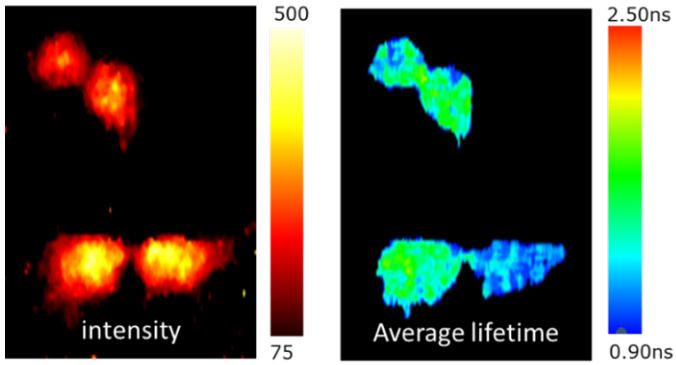

Figure 6: Intensity and lifetime from Cerulean-5-Venus labelled GHFT1 cells imaged in 1 second.

When looking at the image from the normalised pre-exponential functions, an increased prevalence of a 0.6 ns component is seen in the bottom right cell (Figure 7).

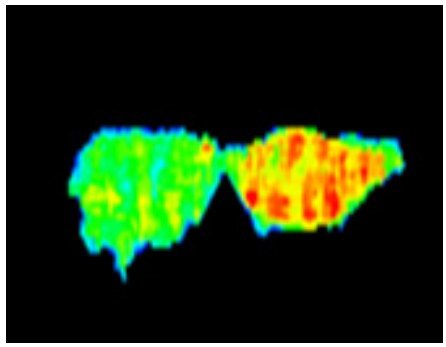

Figure 7: Pre-exponential contribution of the 0.6 ns component. Rainbow scale (blue = less, red = more).

This shows that contrast can be obtained using the fluorescence lifetime using a relatively short acquisition time, which can be important for FLIM-FRET studies.

Depending on the photon flux, it is possible to collect data even faster; in fact in real time. An example of where a FLIMera has been used for faster data acquisition has been reported in the study of a model tumour system [6]. Figure 8 shows a schematic representation of labelled yeast cells imaged at 20 fps. The yeast have been labelled with the viability stain FUN-1, whose time-resolved properties have previously been reported [7].

This demonstrates the ability to monitor flowing samples in real time.

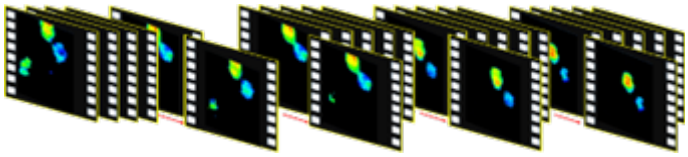

Figure 8: Schematic representation of a flowing sample of FUN-1 labelled yeast imaged at 20 fps (50ms per frame). Different colours relate to

The resolution of the FLIMera is 192 x 126 imaging pixels, but strategies have evolved in order to create higher resolution images (post processed) by combining images from other CCD cameras [8]. The upsampled image approach has also been employed with the FLIMera to study cancer cells [9]. It is also possible to obtain images larger than the FLIMera field of view. This can be achieved by using the FLIMera in conjunction with a scan stage. EzTime Image software can directly control certain stages and via scripting can call Python (for example) to enable other stages to be used. Using a scan stage enables a ”mosaic” image to be obtained. This is demonstrated imaging a section of convallaria, the central tile acts as the “anchor” point (the FoV of the FLIMera) and the stage positioned to give the selected number of tiles. These can then be arranged in EzTime Image. Although lifetime fitting of histograms is not available for the full mosaic image, contrast can be obtained using the thresholds and by the phasor plot, which is demonstrated in Figure 9.

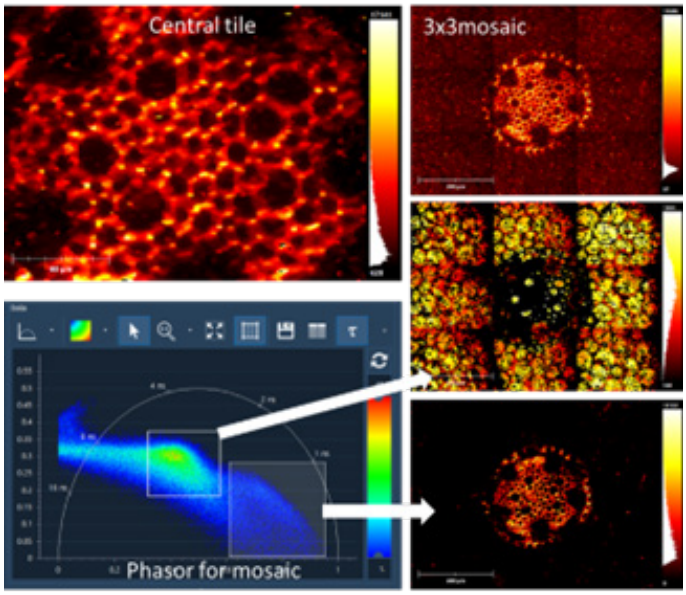

Figure 9: Outcome of a scripted mosaic measurement with differences in lifetimes obtained using the phasor plot to select different regions of the sample. Here the effect on the intensity image is displayed.

Light sheet microscopy

Several microscope-based measurement modalities make use of an intensity-based imaging camera. For some of these substituting the intensity based camera for the FLIMera offers the ability to add the extra dimension of time to the measurement. An example is with light sheet microscopy, which can also be used to obtain volume images. Light sheet microscopy utilising the FLIMera has been used to study in-vivo NAD(P)H in cells to rapidly determine perturbations in metabolism [10]. Digitally scanned light sheet microscopy has made use of the FLIMera coupled with two photon excitation. This form of excitation enables a greater penetration depth into the sample but is best scanned so that the photon flux is sufficiently high to excite the fluorophore in the sample. Linking the scan rate, along with novel optics, to the FLIMera enables efficient and rapid data collection [11]. An example of a volume image obtained using two photon excitation on a digitally scanned light sheet set up is shown in Figure 10. Here fluorescence labelled beads with different lifetimes can clearly be distinguished.

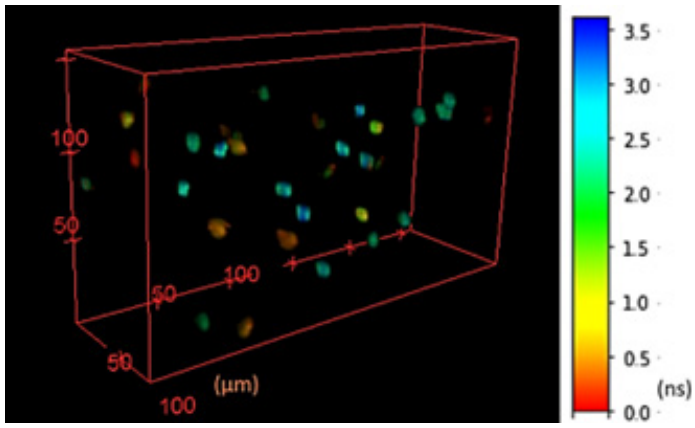

Figure 10: Volume image of fluorescent beads obtained using a digitally scanned light sheet microscope using a FLIMera.

MacroFLIM

It is not only in the field of microscopy that FLIM can be employed. Fluorescence imaging can be used on the macroscale. This can be achieved by simply using a lens system rather than a microscope, although diffuse, pulsed, illumination to cover the subject is required. An example of this type of measurement is shown in Figure 11, where a leaf from a variegated rubber plant (Ficus elastica variegata) has been imaged and contrast between the regions with and without chorophyll determined.

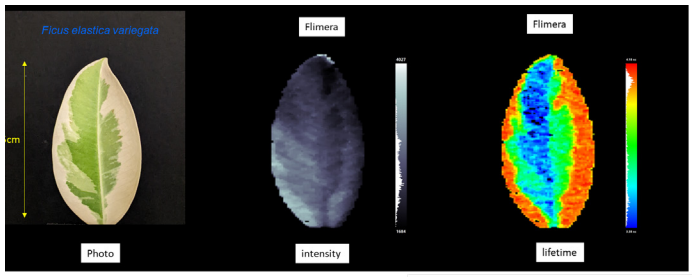

Figure 11: MacroFLIM of a variegated leaf, showing the intensity and average lifetime measurements taken using a FLIMera with diffuse DeltaDiode excitation.

In practice, each pixel of the FLIMera is a “time of flight” sensor as each collects its own fluorescence decay histogram. When looking at macroFLIM data, this opens up the opportunity to obtain both lifetime and distance information in one measurement. Figure 12 shows how the FLIMera can obtain this information by using data from different regions of interest selected in the EzTime Image software.

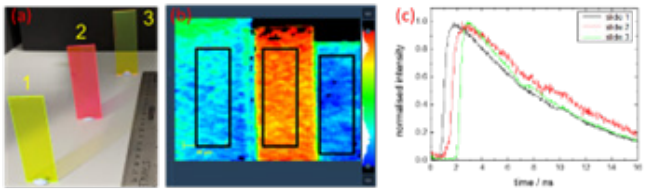

Figure 12: MacroFLIM of fluorescent slides placed at different distances from the FLIMera camera. (a) positions of slides (~10 cm separation), (b) FLIM data, with 3 regions of interest selected (black rectangles), (c) decay histograms form all the pixels within the different regions of interest.

In Figure 12 the FLIMera acquires the image in a reflection geometry and the fluorescence from the nearest slide arrives at the camera before that of the further away slides. Using this difference in time enables the distance between the slides to be measured. Figure 12c shows that the histograms from the different slides are shifted on the time axis. Considering the separation obtained from the midpoint of the rising edge of each decay, it is possible to estimate that each of the slides (shown in Figure 12a) is separated by approximately 9.4 cm. The distance resolution in the case of kind of measurement would be related to the bin width (~40 ps, ie ~1.2 cm or 0.6 cm in reflection).

Summary

FLASH-FLIM measurements made using the FLIMera TCSPC camera open up the possibility to fully apply fluorescence lifetimes to study both mobile systems and kinetic processes. The acquisition speed is significantly faster than that of traditional scanning systems and is based on the fact that each pixel both detects and times photon events enabling widefield FLIM acquisition in a “flash”. Real time average lifetime data can be displayed at video rate and post processing allows in-depth analysis of streamed data. As well as widefield microscope the FLIMera can be utilised both on and off the microscope for a variety of modalities. The in-pixel timing has also enabled this camera to find usage in non-FLIM related fields, such as Time domain near infra red imaging [12] and Depth imaging [13]. These features have earned the FLIMera an Institute of Physics Business Innovation award.

Business Innovation Award 2019

IOP Institute of Physics

Acknowledgement

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HORIBA Scientific

info.sci@horiba.com

USA: +1 732 494 8660
UK: +44 (0)1604 542 500
China: +86 (0)21 6289 6060
Taiwan: +886 3 5600606
France: +33 (0)1 69 74 72 00
Italy: +39 06 51 59 22 1
India: +91 80 41273637
Brazil: +55 (0)11 2923 5400

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Germany: +49 (0) 6251 8475 0
Japan: +81(75)313-8121
Singapore: +65 (0)6 745 8300
Other: +1 732 494 8660