

# Fast Confocal Fluorescence Lifetime Imaging

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## Introduction

Fluorescence lifetime imaging microscopy (FLIM) is a powerful and widely applied advanced microscopy technique, particularly in the biological sciences. The contrast in a FLIM image is provided by the fluorescence lifetime of fluorophores in sample which is largely independent of fluorophore concentration and has been shown to be sensitive to many local intracellular physical and chemical properties(1). Perhaps the most common use of FLIM is to measure protein-protein interactions via Förster resonance energy transfer (FRET) whereby energy transferred from a donor fluorophore on a protein of interest to an acceptor fluorophore on another protein results in a decrease in the donor fluorescence lifetime, yielding quantitative information about the location and efficiency of the interaction.

Whilst time-correlated single photon counting (TCSPC) employed in a laser scanning microscope is the most accurate method for measuring fluorescence lifetimes, acquisition times are typically on the order of 3-5 minutes dependent on the photon acquisition rate. This represents a significant limitation when dynamic information is sought in a biological experiment. We have recently shown that this limitation can be removed by the use of a scanning multiphoton multifocal arrangement (2, 3) in which a square array of 8x8 beamlets is scanned across a sample and the fluorescence generated at the focal plane of the beamlet array is imaged onto a single photon avalanche diode (SPAD) array detector (4). This parallelized multibeam scanning method leads to imaging speeds up to 64 times faster than single beam scanning. We have now constructed a one-photon excitation confocal multibeam system which, with firmware developments and concomitant increased data transfer rates, potentially allows for the use of more beamlets than the multiphoton system due to the reduced power requirements, and also further reductions in acquisition times even further.

In this report, we briefly describe the experimental arrangement for a time-resolved confocal multibeam microscope and present some preliminary data including a TCSPC FLIM image, for which current firmware implementations allow for an 8x8 detector region of interest (ROI). This demonstrates the efficacy of the system with acquisition times two orders of magnitude shorter than typical TCSPC experiments.

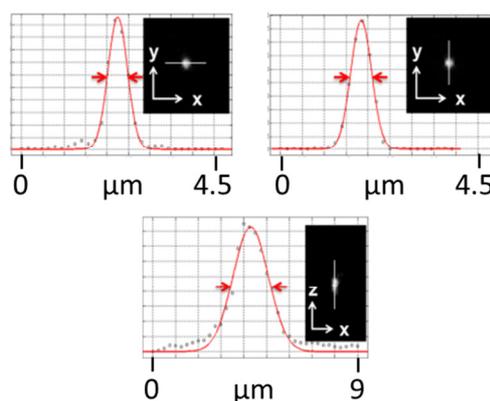
## Method

The system is based around an inverted microscope (Nikon Eclipse TiE). The frequency doubled output of a Ti:Sapphire laser (STFC loan laser, UFL3 Compact OPO system, Coherent) at 480 nm was incident on a phase-only liquid crystal on silicon (LCOS) spatial light modulator (Meadowlark, USA) which was used to generate an array of beamlets. The optimization of the

phase pattern for beamlet generation has been reported recently.(5) The beamlets were focused onto a sample using a Nikon Plan Fluor 40x NA 1.4 oil objective and raster scanned across the sample with the fluorescence descanned, passed through a dichroic beam splitter and a 500-560 nm bandpass filter, and detected using the MegaFrame 32 SPAD array detector which has 32x32 SPADs separated by 50  $\mu\text{m}$ , each with an active area of 6  $\mu\text{m}$  (4). The resulting time-resolved data consisted of fluorescence decays with 180 temporal bins of  $55 \pm 5$  ps in each pixel and was processed using TRI2 software(6).

## Optical performance

In order to determine the optical performance of the microscope we measured 175 nm green microspheres (Invitrogen) in ProLong Gold mounting medium on a glass coverslide (Fig. 1). We retrieve full width at half maximum values of  $\sim 520$  nm in the x- and y- directions and  $\sim 1750$  nm in the z-direction, as would be expected from our system, slightly underfilling the NA 1.4 objective lens.

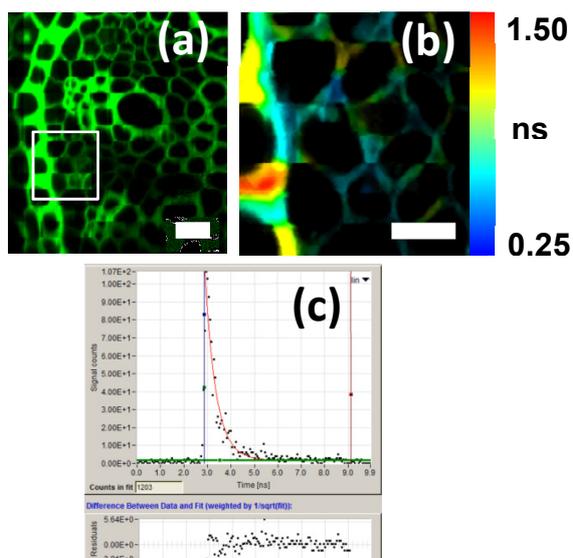


**Figure 1** Optical resolution measurements of a 175 nm green microsphere.

## Multibeam confocal laser scanning microscopy of convallaria

We demonstrate our system using a fluorescently labelled (Fast Green and Safranin) thin section of the rhizome of *Convallaria Majalis*. We measured a fluorescence intensity image using an array of 15x13 beamlets with a corresponding field of view of 75  $\mu\text{m}$  x 65  $\mu\text{m}$  (Fig. 2a). We then measured a TCSPC FLIM image from an 8x8 beamlet, 128x128 pixel ROI (Fig. 2b) with a corresponding field of view of 40  $\mu\text{m}$  x 40  $\mu\text{m}$  with an acquisition time of 2 s. The total laser power used in these

acquisitions was  $\sim 100 \mu\text{W}$ . A representative fluorescence decay from a 3x3 pixel region of the image is shown in Fig. 2c.



**Figure 2** (a) Fluorescence intensity image of convallaria section recorded using an array of 15x13 beamlets and (b) 8x8 beamlet TCSPC FLIM image measured in 2 s corresponding to the ROI shown by the white box in (a). Scale bar 10  $\mu\text{m}$ . (c) Representative decay curve from 3x3 pixels in the time-resolved image

### Future developments

We are currently working towards generating larger beamlet arrays with a view to filling the available 32x32 SPADs of the MF32 array detector. The frame rate for acquisition scales linearly with the number of detectors used. Recent improvements in firmware and data transfer via USB3.0 allow up to 0.5 billion photon counts per second across the detector array which will enable us to scale acquisition to the maximum extent (Krstajic et al. accepted for publication in Optics Letters).

Using the available hardware and optical arrangement sub-1s TCSPC FLIM imaging is achievable with Nyquist spatial sampling. In particular, the flexibility of the widely tuneable UFL3 laser source is essential for excitation of all commonly used fluorophores, and for future multiplexed and multi-wavelength experiments using multibeam confocal FLIM. Multi-detector acquisition is possible simultaneously without compromising data rate enabling highly complex multispectral experiments to be realized.

### Conclusions

We have demonstrated multibeam confocal laser scanning microscopy as a method for rapid FLIM with low laser powers and acquisition times two orders of magnitude shorter than conventional TCSPC-based laser scanning FLIM. This reduction in acquisition times will enable real-time monitoring of intracellular processes including protein-protein interactions.

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