Functional in vivo imaging using fluorescence lifetime light-sheet microscopy

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Light-sheet microscopy has become an indispensable tool for fast, low phototoxicity volumetric imaging of biological samples, predominantly providing structural or analyte concentration data in its standard format. Fluorescence lifetime imaging microscopy (FLIM) provides functional contrast, but often at limited acquisition speeds and with complex implementation. Therefore, we incorporate a dedicated frequency domain CMOS FLIM camera and intensity-modulated laser into a light-sheet setup to add fluorescence lifetime imaging functionality, allowing the rapid acquisition of volumetric data with concentration independent contrast. We then apply the system to image live transgenic zebrafish, demonstrating the capacity to provide fast, optically sectioned images.

In this Letter, we incorporate a CMOS camera (pco.flim [10], PCO AG) and modulated diode laser into a digitally scanned light-sheet microscope (DSLMM) setup. In this way, we combine the speed of DSLM for 3D imaging with the functional information obtained from FD-FLIM.

Previous implementations of fluorescence lifetime imaging in a light-sheet microscope configuration have been described in Greger et al. [11] and Weber et al. [12], to image a MDCK cyst and cell spheroids, respectively. Both studies used a cylindrical lens generated light-sheet, combined with a gated image intensifier (GII) in their setups. The digitally scanned sheet used in this Letter is less prone to shadowing and readily convertible from the standard Gaussian sheet to other sheets generated mechanisms such as the Airy [13] or lattice light-sheet [14] or the inclusion of two-photon illumination [15]. GII cameras may also suffer from low light throughput and well-known artifacts such as photo-bleaching and image distortion [16–19]. The pco.flim, by contrast, switches between two charge collection taps at the modulation frequency so, ideally, every photon incident on a pixel is detected, increasing the collection efficiency compared to a GII.

A schematic of the FL-DSLM system is shown in Fig. 1. Briefly, the output from a fiber-coupled 488 nm modulated
laser (PhoxX+ for pco.flim, Omicron–Laserage Laserprodukte GmbH, Germany) is directed into a galvanometer scanning system composed of two scanning mirrors separated by a 4f relay telescope. The beam is then magnified 4× before being conjugated onto the back of the excitation objective (EO). The beam was then magnified 4× (L5 = 50 mm, L6 = 200 mm) and entered the EO to illuminate the sample. The emitted fluorescence was collected by the DO, band-pass filtered (F1) to remove any scattered excitation light and focused onto the FLIM camera using a tube lens (TL = 200 mm).

Fig. 1. Optical design of the FL-DSLM system as (A) a schematic and (B) photograph. From (A), the laser is magnified (L1 = 75 mm, L2 = 50 mm) and passed through a 4f scan system (L3, L4 = 50 mm) to create the light-sheet (G1) and provide z-movement (G2, coupled with the PI-FOC). The beam was then magnified 4× (L5 = 50 mm, L6 = 200 mm) and entered the EO to illuminate the sample. The emitted fluorescence was collected by the DO, band-pass filtered (F1) to remove any scattered excitation light and focused onto the FLIM camera using a tube lens (TL = 200 mm).

Fig. 2. Quantification of a FL-SPIM system. Upon addition of a quencher, sodium iodide, the lifetime (modulation in black, phase in gray) of a fluorescent lake of FITC decreases according to the Stern–Volmer equation. The error bars denote standard deviation ($n = 5$).

Volmer equation Eq. (3) describes a linear change in inverse lifetime ($\tau$) upon the addition of a quenching agent at a concentration of $[Q]$ [20]:

$$1/\tau = k[Q] + 1/\tau_0.$$  

(3)

This linear change is observed in Fig. 2 with fitted values of the bimolecular constant $k$ calculated to be 3.67 and 3.76 ns$^{-1}$ M$^{-1}$ and $\tau_0$ of 2.17 and 2.94 ns using the calculated phase and modulation, respectively. The measured lifetime is shorter than the published value of 4.0 ns [20], but was consistent with data obtained on a multiphoton TCSPC microscope [21]. This shorter lifetime, coupled with a higher measured bimolecular constant (expected to be 2 ns$^{-1}$ M$^{-1}$ [20]) may be due to a low ionic strength FITC solution [22].

The lifetime calculated using the modulation shows a larger variance than the phase-based lifetime calculation. The modulation lifetime accuracy could be improved by applying the corrections described in Chen et al. [23].

After calibration of the lifetime, the system was able to measure relative phases in lifetime (Fig. 2). The system was then applied to an in vivo system: zebrafish (danio rerio). Two transgenic fish lines were used in this Letter: NFKB:EGFP [24] and α-actin:EGFP [25], both of which can be observed expressed in muscle fibers.

Fish were reared under standard conditions [26] as per UK Home Office regulations. In the case of the NFKB:EGFP fish, the larvae were injected with 20 μg · μl$^{-1}$ 2 MDa FITC-dextran 24 h preceding imaging to perfuse the vasculature. At five days post-fertilization (dpf), larvae were embedded in low-melt
agarose (Sigma-Aldrich Ltd.) in plastic 60 mm dishes (Nunc, Sigma-Aldrich Ltd.) with the dorsoventral axis at 45° to the base of the dish to optimally expose the muscle to the DO and immersed in an E3 medium with 0.01% MS-222 (Sigma-Aldrich Ltd.) and imaged immediately. Fluorescence lifetimes were calculated using Eq. (1). Intensity and intensity-mediated lifetime stacks were generated in MATLAB, and post-processing was performed in Imaris (Bitplane AG, Switzerland).

Images of the NFKB:EGFP and α-actin:EGFP fish are displayed in Figs. 3 and 4, respectively. In Fig. 3(B), the vasculature labelled in FITC-dextran (τ<sub>expected</sub> = 4.0 ns [20], τ<sub>measured</sub> = 3.5 ns) can be clearly discriminated from the EGFP-labelled (τ<sub>expected</sub> = 2.4 ns [27], τ<sub>measured</sub> = 2.6 ns) muscle by their different lifetimes, which is almost impossible by use of the intensity image only. [Figure 3(B) can only be seen where the FITC intensity is at its brightest.] This type of optical barcoding is useful to image fluorophores with similar emission wavelengths [Fig. 3(C)]. The dorsal aorta and intersegmental vessels can then be segmented from the phase lifetime image [Fig. 3(D), threshold = 2.9 ns] and appear as expected in a 5 dpf larvae [28].

Figure 3 was imaged using a long (1 s) camera exposure to remove FLIM artifacts introduced by the fast-moving blood cells. To show fast volumetric FLIM imaging, we therefore imaged α-actin:EGFP fish which expresses strongly in all the muscle fibers (Fig. 4). The volume displayed in Fig. 4 (280 μm x 280 μm x 100 μm before cropping) was collected in less than 7 min which is comparable to a commercial

multiphoton or confocal system, but additionally capturing lifetime information.

Skin autofluorescence across a breadth of wavelengths presents a confounding factor when imaging transgenic zebrafish larvae in intensity-based approaches [29]. Utilizing FLIM, the short lifetime autofluorescence can easily be distinguished from the longer lifetime EGFP (Fig. 4).

In summary, we have presented the first in vivo demonstration of FL-DSLM using commercial FD-FLIM-enabled components. Using this system, we have been able to demonstrate fast volumetric FLIM imaging of zebrafish larvae muscle and to distinguish between spectrally similar fluorophores, using their different lifetimes.

The deployment of a digitally scanned light-sheet opens the options for beam shaping for improved field-of-view and resolution. The speed of acquisition could also be improved further by decreasing the number of phases acquired, potentially reaching speeds of up to 10 s per volume [30]. Such speeds of acquisition, coupled with low phototoxicity of the light-sheet microscope, make this configuration suitable for probing lifetime signals from rapidly moving cells in vivo.

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