Adaptive optics for a time-resolved Förster resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM) in vivo

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Förster resonance energy transfer (FRET) and fluorescence lifetime imaging (FLIM) have been coupled with multiphoton microscopy to image in vivo dynamics. However, the increase in optical aberrations as a function of depth significantly reduces the fluorescent signal, spatial resolution, and fluorescence lifetime accuracy. We present the development of a time-resolved FRET-FLIM imaging system with adaptive optics. We demonstrate the improvement of our adaptive optics (AO)-FRET-FLIM instrument over standard multiphoton FRET-FLIM imaging. We validate our approach using fixed cellular samples with FRET standards and in vivo with live imaging in a mouse kidney.

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intermediate slope contributions which increase the wavefront reconstruction accuracy. The overlapping approach also reduces the bias of modal fitting towards the periphery of the pupil [14]. The intermediate slopes contribute equally to the Zernike polynomial reconstruction algorithm. The reconstructed Zernike polynomial phase pattern is used to compensate for the aberrations. AO corrections are performed by adding the calculated pattern to the SLM.

In this Letter, we apply AO to multiphoton fluorescence lifetime imaging microscopy (FLIM). FLIM permits high spatiotemporal resolution imaging of dynamic processes, including Förster resonance energy transfer (FRET). Many questions related to cellular dynamics, such as protein interactions and conformational changes, can be addressed by FRET-FLIM [15-17]. To achieve high temporal precision, we used time-correlated single-photon counting (TCSPC). TCSPC permits picosecond time resolution conferring the most accurate lifetime determination [18].

The AO-FRET-FLIM optical layout is shown in Fig. 1. Laser light from a Ti:sapphire laser system (Spectra-Physics, DeepSee Mai Tai) was expanded to overfill a phase-domain SLM (Hololoe Photonics AG, Pluto-2 NIR). The SLM displays an eight-level blazed grating which efficiently projects the illumination pattern into the first order. The remaining orders are removed using an aperture in the image plane of the SLM. The SLM pattern is conjugated to the back pupil of a ×25 1.1 water dipping NA objective (Nikon Instruments, Ltd.) via a xy galvanometer scanning system (VM1000C, Cambridge Technology, Ltd.). Fluorescence is collected by the same objective lens, separated from the excitation using a dichroic (Semrock, Inc., FF670-SDi01), spectrally split using a second dichroic (Semrock, Inc., FF560-FDi02), and imaged onto two separate hybrid photomultiplier tubes (PMT) (Becker & Hickl GmbH, PMH-100) capable of TCSPC. Fluorescence is acquired in the non-descanned detection path. In contrast to a confocal microscope, a multiphoton AO microscope requires that the corrective optical element is placed only in the excitation optical path [9].

to determine aberrations induced by the optical components of the microscope, known as system aberrations, we imaged isolated fluorescent microspheres deposited on a cover slide. Imaging of red (emission > 561 nm) 0.2 μm diameter beads show a full width at half-maximum (FWHM) of 0.65 ± 0.2 and 1.85 ± 0.1 μm, in the lateral and axial directions, respectively (Fig. 2). Performing AO correction reduces the FWHM to 0.56 ± 0.02 and 1.1 ± 0.07 μm, in the lateral and axial directions, respectively. In addition, we demonstrate a ×1.6 increase in signal under the same experimental conditions. The amplitude of the various Zernike polynomials determined shows that the AO pattern primarily corrects for astigmatism, a common deformation of a SLM microdisplay [19]. The optical system corrective phase pattern is then added to the initial SLM blazed grating. This constitutes a pre-compensation for the optical train, and further experiments shown include this correction.

To evaluate the lifetime imaging performance of our AO imaging system for time-resolved FRET-FLIM, we used enhanced green fluorescence protein (EGFP) expressing cells [20]. Our FRET standards consist of two fluorescent proteins (EGFP and mRFP1) linked by a 7 amino acid linker, and thereby demonstrating high FRET efficiency. The FRET standards were expressed in HEK293 cells and placed under 50 μm mouse liver tissue slices (Fig. 3). The cells were transfected using Effectene Transfection Reagen (Qiagen, Ltd.) and, after 24 h, fixed in 4% paraformaldehyde. Fixed cells
were placed on an imaging slide and dried in a laminar hood. A mouse liver of a C57BL/6 (B6) mouse was prepared by fixation in 4% paraformaldehyde for 24 hours, dehydrated in 30% sucrose for an additional 24 hours and subsequently frozen in optimal cutting temperature compound (Leica Biosystems). Cryostat sections of 50 µm thickness were placed on top of the cells and sealed under a coverslip with Vectra-Shield mounting media (Vector Laboratories, Inc.).

The mouse liver tissue placed between the cells and the objective introduces refractive index mismatches, which reduces the fluorescent intensity and broadens the FLIM histograms (Fig. 3). Performing AO correction reduces the influence of aberrations and demonstrates an increased accuracy of determining FRET-FLIM [Figs. 3(b)–3(d)]. This is evidenced by a reduced spread of the histogram. The FWHM of the FRET histogram was reduced from 0.12 to 0.06 with AO correction, a ×2 improvement. FRET efficiencies were calculated as $E = 1 - \tau/\tau_D$, where $\tau$ is the fluorescence lifetime of the probe in the presence of an acceptor and $\tau_D$ the control donor lifetime. The lifetime data were analyzed with 5 × 5 binning and Levenberg–Marquardt fitting in TRI2 lifetime analysis software. We determined the lifetime values of green fluorescence protein (GFP) to be 2.3 ± 0.06 and 2.27 ± 0.03 ns with AO correction. The lifetime values of the FRET standards were determined to be 1.67 ± 0.2 and 1.59 ± 0.07 ns with AO correction. The FRET efficiencies of the ruler constructs were determined as 27 ± 6.8% and 29.7 ± 3.3% with AO correction. These values are equivalent to previously reported values [20].

To assess the benefit of AO to image FRET-FLIM of fine structures in highly aberrating media, we imaged a fixed mouse liver stained with Alexa-488 at a depth of 100 µm (Fig. 4). Figure 4 demonstrates the potential of AO-FLIM to detect protein conformations in highly aberrated environments. Figure 4(a) shows a side-by-side comparison between FLIM images acquired for 300 s without AO and an AO corrected FLIM image acquired for 200 s. Thus, we also demonstrate that in highly aberrated environments our AO-FLIM is a significant improvement over prolonged acquisitions. The improvement in FLIM due to AO is highlighted in the zoomed-in area shown in Fig. 4(b). The highlighted feature would have been lost without AO. Figure 4(c) shows the lifetime histogram for Fig. 4(b) before and after AO correction. In Fig. 4(c), we demonstrate a ×4 improvement in the amplitude of the lifetime histogram counts and a ×1.5 reduction in standard deviation.

To evaluate the AO-FLIM capability in a challenging in vivo environment, we imaged macrophages within the kidney of a live mouse (Fig. 5). Cx3Cr1-GFP Rag2-/-Il2rg-/- mice were anaesthetized with a combination of ketamine (50 mg/kg),
Fig. 5. AO applied to FRET-FLIM in vivo. Intravital imaging of tissue resident macrophages in a live Cx3Cr1-GFP Rag2−/−Il2rg−/− mouse. Multiphoton intensity, lifetime, and Z-projection using (a) standard imaging and (b) AO correction. (c) Intensity line profile comparison between system corrected (No AO) and AO corrected shows a ×3 signal improvement. (d) Normalized FLIM histogram using standard imaging (gray curve) and AO correction (red curve). (e) Corrective phase pattern. (f) Zernike modes and coefficients used in (e).

xylazine (10 mg/kg), and acepromazine (1.7 mg/kg) injected intraperitoneally. During imaging, anesthesia was maintained by the inhalation of 0.5% isoflurane in oxygen [21]. The kidney was surgically exposed, and the mouse was positioned on a custom-made stage insert with a glass coverslip. The temperature was maintained at 37°C using an environmental chamber.

We demonstrate a ~3-fold improvement of the fluorescence intensity in three dimensions at a 200 µm depth over system corrected imaging [Figs. 5(a)–5(c)]. Normalized FLIM histograms also show an improvement due to AO correction [Figs. 5(d)–5(f)]. The FWHM of the lifetime histogram was reduced from 0.37 to 0.21 ns, an approximated ×1.8 improvement.

In conclusion, we demonstrate a proof-of-principle application of AO to two-photon FRET-FLIM microscopy. We demonstrate an improvement in fluorescence lifetime measurement uncertainty as a direct result of increasing both the resolution in aberrating media and photon count due to an improved Strehl ratio. We demonstrate improved FRET-FLIM imaging both in vitro using fixed cellular samples and in vivo by live mouse imaging.

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