Frequency-multiplexed in vivo multiphoton phosphorescence lifetime microscopy

Scott S. Howard¹,² *, Adam Straub³, Nicholas G. Horton¹, Demirhan Kobat¹ and Chris Xu¹*

Multiphoton microscopy (MPM) is widely used in vivo for optical sectioning deep inside scattering tissue.¹,² Phosphorescence lifetime imaging microscopy (PLIM)³ is a powerful technique for obtaining biologically relevant chemical information through Förster resonance energy transfer and phosphorescence quenching.⁴,⁵. Point-measurement PLIM⁶ of phosphorescence quenching probes has recently provided oxygen partial pressure measurements in small rodent brain vasculature identified by high-resolution MPM⁷,⁸. However, the maximum fluorescence generation rate, which is inversely proportional to the phosphorescence lifetime, fundamentally limits PLIM pixel rates. Here, we demonstrate experimentally a parallel-excitation/parallel-collection MPM–PLIM system that increases the pixel rate by a factor of 100 compared with conventional configurations, while simultaneously acquiring lifetime and intensity images at depth in vivo. Full-frame, three-dimensional, in vivo PLIM imaging of phosphorescent quenching dye is presented for the first time and defines a new platform for biological and medical imaging.

Current technologies for overcoming the fundamental pixel rate limitation of serial-acquisition multiphoton microscopy (MPM) require parallel excitation and imaging a sample onto multielement detectors (typically a charge-coupled device, CCD⁹,10). Although satisfactory for thin tissue slices or non-scattering samples, thick, scattering samples, such as those typically encountered in in vivo applications, cause crosstalk between excited pixels when imaged onto a detector array, resulting in smeared images.¹¹. State-of-the-art fast fluorescence lifetime imaging microscopy systems utilize parallel excitation (for example, light-emitting diode (LED) arrays or pulsed diode excitation) or collection (for example, gated CCD, single-photon avalanche diode array, or multichannel photomultiplier tube (PMT))¹² for videorate acquisition.¹³,¹⁴ These systems have been applied to oxygen sensing,¹⁵,¹⁶ but are similarly limited by excitation and emission crosstalk and cannot resolve three-dimensional optical sections.

Phosphorescence lifetime imaging microscopy (PLIM) acquisition rates in phosphorescence quenching systems are further limited by long dye lifetimes (τ > 1 μs), and therefore require low-repetition-rate lasers and long pixel dwell times (T ≫ τ). Lifetime measurement rates of phosphorescent dyes, as often used in oxygen sensing,⁷,⁸,¹⁷–¹⁹, are thus limited to <10 Hz at depth.⁶ Although these systems provide valuable measurements at specific points within tissue (for example, partial pressure of oxygen within specific vascular compartments), full-frame optical sectioning measurements of phosphorescent quenching at depth are impractical.

This Letter demonstrates experimentally a parallel-excitation/parallel-collection MPM–PLIM system that achieves high-pixel-rate, simultaneous lifetime and intensity imaging at depth, in vivo. Our method, unlike previous parallel image-acquisition approaches, does not require imaging of signal photons, thus eliminating the impact of signal scattering. Furthermore, in MPM, ballistic photons dominate the nonlinear excitation²⁰ until the imaging depth is many times the scattering length (for example, ~1 mm in biological tissues)²¹. Therefore, cross-talk from scattered excitation photons between neighboring pixels is eliminated. In this technique, called multifocal multiphoton modulation microscopy (M4), each point in the sample is uniquely intensity-modulated. The entire emitted signal is collected onto a single-element detector. Spatial information is recovered by means of signal frequency demodulation, and pixel location is independent of sample-induced scattering. The magnitude and phase of each modulation frequency are mapped to fluorescence intensity and lifetime, respectively, for each pixel in the sample. Similar imaging techniques have been used when detector arrays are impractical²² and in fluorescence imaging.²³,²⁴

Assuming perfect sinusoidal modulation for simplicity, the modulated illumination excites a two-photon fluorescence response:

\[
S(t) = \sum_i S_i \left( 1 + \frac{4}{3\sqrt{1 + (\omega_i \tau_i)^2}} \sin(\omega_i t + \theta_i) \right) + \frac{1}{3\sqrt{1 + (2\omega_i \varphi_i)^2}} \sin(2\omega_i t + \varphi_i) \right)
\]

where \(S_i\) is the cycle averaged signal strength, \(\omega_i\) is the modulation angular frequency, \(\theta_i\) and \(\varphi_i\) are, respectively, the phase delay due to the finite fluorescence lifetime (\(\tau_i\)) at the fundamental modulation frequency \(\omega_i\) and its second harmonic 2\(\omega_i\), at the \(i\)th pixel. All fundamental modulation frequencies \(\omega_i\) are within a single octave to avoid crosstalk between the fundamental and second-harmonic terms. Thus, the number of resolvable pixels is limited by the number of resolvable frequencies, given by \(N = (\omega_i/2\pi)T\), where \(\omega_i\) is the lowest modulation frequency and \(T\) is the dwell time.

Complex demodulation of the emission signal yields the complete set of \(S_i\) and \(\theta_i\) for all \(\omega_i\). The value of \(\omega_i\) corresponds to the position of the \(i\)th pixel, while \(S_i\) gives the pixel intensity and \(\theta_i\) can be used to calculate the fluorescence lifetime:

\[
\tau_i = \frac{\tan(\theta_i)}{\omega_i}
\]

For optimum measurement of fluorescence lifetimes near \(\tau_{\text{target}}\), the angular modulation frequencies should be near \(\omega_{\text{target}} = 2\pi (0.1/\tau_{\text{target}})\) (refs 25,26). M4 is capable of variable modulation frequency to increase dynamic range. Furthermore, by restricting the modulation frequencies to one octave, the entire range of modulation frequencies across the sample is within the flat optimum in photon efficiency.²⁵

The experimental setup is described in Fig. 1. A mode-locked Ti:sapphire laser was used as the excitation source (\(\lambda = 780\,\text{nm},\))
pulsewidth \( \approx 100 \text{ fs} \), repetition rate = 80 MHz, power in the range 80–150 mW at the sample). We first focused the beam to a line using a 10 cm cylindrical lens. This line illumination then impinged onto a one-dimensional spatial light modulator (SLM), generating a linear array of point sources with each point source modulated by a different frequency (this process is described in the Methods, ‘Custom reflection-based linear SLM’ and the top dashed box in Fig. 1). The linear point source array was imaged onto the sample to excite fluorescence, forming a one-to-one mapping between the modulation frequency and the pixel, that is, the spatial information along the focused line was encoded in the frequency domain by modulating the excitation light intensity. The excited nonlinear signal was epi-collected through the objective and reflected off a dichroic mirror (for example, a Hamamatsu H7422P-40). The dichroic mirror also reflected a linear point source array onto a large-area PMT (for example, a photomultiplier tube; OL, objective lens; SLM, spatial light modulator). The fluorescence lifetime of each pixel was determined by the difference in measured phase between the signal and reference spectrograms. The fluorescence/phosphorescence lifetime of each pixel was determined by the difference in measured phase between the signal and reference spectrograms. Data were collected on a 10 MHz National Instruments DAQ (PCI-6110) card and calculations were performed in real time in LabView (National Instruments Corporation). Post-processing and further data analysis were performed with custom scripts in MATLAB (Mathworks).

An intuitive understanding of our technique can be obtained by considering subcarrier multiplexing, a standard communication technique. Each data channel is encoded in its distinct carrier radio-frequency (RF). A simple RF tuner then selects the desired band and filters out the rest. In the M4 PLIM system, each pixel in space acts like an independent data channel emitting at a distinct frequency that can be decoded to form a spatial image using the frequency-to-space correlation.

The M4 excitation and collection scheme is similar to that of line-scanning MPM. However, there is a significant difference in that each excitation point along the line is intensity-modulated at a unique frequency by a custom, reflection-based linear SLM capable of modulating up to \( \sim 1 \text{ MHz} \) and corresponding to emission lifetimes of \( \sim 100 \text{ ns} \). For faster fluorescence lifetimes, commercially available polygon scan mirrors \(^{27,28}\) (SA24 & 72 sided polygon, Lincoln Laser) can further increase the modulation rates to 60 MHz, corresponding to a lifetime resolution of \( \sim 50 \text{ ps} \) and suitable for a wide range of biological fluorescent lifetime measurements. Alternatively, a two-dimensional digital micromirror device (DMD) or digital microelectromechanical system (MEMS) array can replace our linear SLM to achieve simultaneous full-field imaging by uniquely modulating and imaging a two-dimensional array of pixels onto the sample. Commercially available DMD kits (DLP4X00KIT, Texas Instruments) can already achieve 16 kHz modulation rates, suitable for wide-field M4 PLIM imaging of oxygen-sensing dyes.

Figure 2 demonstrates the M4 concept by imaging a 1951 USAF Resolution Test Chart and collecting the reflected light signal on a
The signal at the diode is divided into consecutive time windows corresponding to adjacent image lines with spatial information encoded in the frequency domain. Signal demodulation reconstructs the original image.

M4 axial and lateral resolution of two-photon excited fluorescence were measured by imaging 0.5-μm-diameter fluorescent (505/515) beads (Invitrogen) in a 3% agarose gel with a NA = 0.75, ×20 objective. The lateral extent (full-width at half-maximum, FWHM) of the 0.5 μm bead was measured to be 1.13 μm and 0.73 μm along the frequency and time axes, respectively. The axial extent measured 4.0 μm. Lateral resolution along the frequency-resolved dimension was limited by the demagnification of the modulator onto the sample.

Although multiplexing significantly increases the pixel rate of M4 versus serial point-scanning PLIM systems, M4 suffers additional noise. Shot noise generated at a single pixel (that is, the modulation frequency) is shared among all simultaneously imaged pixels. Because two-photon microscopy eliminates cross-talk from the excitation and collection of photons, shot noise is the dominant noise term. To address this concern, the microscope was adapted to perform both M4 and standard serial-acquisition point-scanning PLIM. Ru(dpp3)2+-dyed tissue fibres (ref. 18) were imaged using both techniques at equivalent sample illuminations (that is, equivalent fluorescence generation rate per pixel). This criterion was chosen because the maximum fluorescence generation rate is the ultimate limitation on fluorescence imaging pixel rate for a large class of problems where the generation rate of signal photons poses the fundamental limit to imaging speed. Therefore, the fair comparison is to excite samples in both systems close to the maximum generation rate and determine the relative error. The results shown in Fig. 3 demonstrate two orders of magnitude improvement in pixel acquisition rate for M4 over point-scanning MPM–PLIM.

There is significant interest in the functional imaging of pO2 in brain vasculature during stimulation.8-10 Recent work has demonstrated functional imaging measurements at depth11. However, no oxygen-sensing system produced three-dimensional fluorescence/phosphorescence lifetime imaging of a heterogeneous lifetime system in a scattering medium (for example, in vivo).

To demonstrate M4 in vivo, we injected Ru(dpp3)2+-pluronic-nanomicelle probes into FVB/n mouse vasculature, retro-orbitally. Optical access to the brain was achieved via a cranial window. Simultaneous intensity and lifetime optical sections were acquired by M4 (Fig. 4). Blood vessels are clearly present in the intensity M4 image (Fig. 4a), and phase is mapped to fluorescence lifetime with increasing averaging (Fig. 4b–d; Supplementary Movie S1). Lifetime histograms of the interior vascular compartments

![Figure 3](image-url)  
**Figure 3** | Lifetime measurement uncertainty versus pixel integration time in Ru(dpp3)2+-dyed tissue fibre, comparing M4 with point-scanning PLIM.

![Figure 4](image-url)  
**Figure 4** | Mouse brain vasculature containing Ru(dpp3)2+-pluronic-nanomicelle probes imaged by M4. a. Phosphorescence intensity. b–d. Phosphorescence lifetime after 20 s (b), 1 min (c) and 5 min (d) of integration. e. Top to bottom: lifetime histograms of 800 pixels from the interior of the blood vessel from images b–d, respectively. f–i. Optical sections separated by 10 μm through brain vasculature. Images b–d and f–i map lifetime to hue and intensity to value to simultaneously illustrate lifetime and intensity acquisition.
Figure 5 | M4 and conventional two-photon microscopy of DsRed-labelled mouse brain vasculature, in vivo. a, Conventional point-scanning MPM of mouse-brain blood vessel labelled with DsRed, before Ru(dpp3) injection. b, c, M4 intensity (b) and lifetime (c) maps of DsRed-labelled mouse brain after injection of phosphorescent Ru(dpp3)2+ dye. d, Lifetime sections taken in 10 μm steps through the blood vessel after injection of Ru(dpp3)2+ dye.

(800 pixels) displayed in Fig. 4b–d are presented in Fig. 4e from top to bottom, respectively. Image frames are 500 × 160 pixels with 85 mW optical power at the sample; modulation rates range from 32 to 64 kHz. Figure 4f–i and Supplementary Movie S2 demonstrate simultaneous intensity and lifetime optical sections at 10 μm steps and 3 min averaging per frame.

A heterogeneous in vivo sample was prepared by injecting Ru(dpp3) Pluronic-nanomicelle probes into a fluorescent protein (DsRed)-labelled mouse, retro-orbitally. Conventional two-photon microscopy revealed that DsRed is expressed predominately in the blood vessel walls in the absence of dye (Fig. 5a). Optical sections of Ru(dpp3) in DsRed-labelled mouse vasculature were obtained in 10 μm steps through a blood vessel. The simultaneously acquired intensity and lifetime images are presented in Fig. 5b and c, respectively. It is clear that the blood vessel walls contain fast-lifetime (τ ≲ 1 μs) DsRed fluorescence while Ru(dpp3)-nanomicelle dye (τ ≈ 2.5 μs) is localized in the vessel interior. The M4–PLIM optical stack of 11 full frames through 110 μm is presented in Fig. 5d and Supplementary Movie S3, and represents, to our knowledge, the deepest in vivo PLIM imaging of phosphorescence quenching dye. The modulation frequencies were between 64 kHz and 128 kHz, the frame period was 3 min 20 s, and the average photon rate was less than 500 kHz (corresponding to a low photon generation rate per pixel of 1 kHz). The frame size is 256 × 500 pixels, yielding a pixel rate of ≈ 1 Hz, ref. 8). M4 pixel rates can be greatly improved by increasing the photon generation rate with improved dyes or higher excitation pulse energy at the sample.

In summary, we have experimentally demonstrated a parallel-excitation/parallel-collection MPM–PLIM system that achieves a high pixel rate while simultaneously acquiring lifetime and intensity images at depth, in vivo. By mapping spatial positions to modulation frequencies, image smearing due to signal scattering is avoided. M4 provides a two orders of magnitude improvement in pixel rate over serial-acquisition PLIM and allows, for the first time, full-frame, three-dimensional lifetime imaging of a heterogeneous sample containing a phosphorescence quenching probe.

Methods

Custom reflection-based linear SLM. High modulation rates are required (for example, ∼100 kHz, as used in our experiments) to resolve distinct points along the line and to optimally match the desired fluorescence/phosphorescence lifetime. Because commercially available linear SLMs with the required pixel numbers cannot modulate at such high speeds, we created a free-space optical chopper (Fig. 1, upper dashed box) that can modulate an array of point sources at megahertz rates by scanning a line-focused laser beam over a small (period, 10–20 μm) mirror grating on a photolithography mask (Fig. 1). Each horizontal line on the photolithography mask has a different spatial frequency. The reflected light is then descanned by the same scan mirror, and is imaged onto the sample by the line-scanning microscope. The spatial frequencies are limited to one octave to avoid crosstalk between the fundamental modulation frequency and higher harmonics generated by the nonlinear response of the dye and deviations from a perfect sinusoidal modulation.

Serial-acquisition two-photon PLIM microscope. A serial-acquisition frequency-domain PLIM microscope was built to compare the pixel rate improvement of M4 over serial-acquisition PLIM microscopy. The same M4 microscope was used for both parallel and serial acquisition. The microscope was modified for serial acquisition by removing the first cylindrical lens. A conventional point focus was then obtained on both the sample and the SLM. The SLM thus acts as an optical chopper and intensity-modulates the excitation beam. The input optical power was adjusted so that the excitation intensity at the sample was identical in both parallel and serial acquisition.

The comparison of pixel rate was performed by measuring the relative lifetime uncertainty of identical regions of an Ru(dpp3)-dyed lens paper with both M4 and serial point-scanning MPM–PLIM in the frequency domain as a function of pixel dwell time.

Lifetime measurement calibration. The lifetime measurements were calibrated by measuring the phase delay of a signal produced by reflecting the excitation beam off a mirror surface at the sample. Because optical delay is negligible (<1 ns), any measured delay can be attributed to latent delays in the system electronics (detectors and amplifiers). This calibration was performed before PLIM imaging and included in the lifetime calculations.

Preparation and demonstration of phosphorescence quenching Ru(dpp3)2+, encapsulated nanomicelles. Water-soluble Ru(dpp3)2+–pluronic nanomicelles were prepared30. For the solution, 4 mg of Ru(dpp3)Cl2 (Alfa Aesar) was dissolved in 100 μl of chloroform and added to 5 ml of Pluronic F68 or F127 (2 wt%) aqueous solution. The solution was pre-emulsified by stirring for 15 min, followed by 15 min agitation in an ultrasonic bath (150 W). After producing the microemulsion, the chloroform was evaporated off by stirring the mixture while heating in a regulated 50 °C bath for 30 min.

Phosphorescent quenching of Ru(dpp3)2+ encapsulated in pluronic nanomicelles was demonstrated by comparing the measured phosphorescence lifetimes of pools of oxygenated and deoxygenated dye. Two 100-μl well slides were filled with the dye solution. An excess of enzymatic system for oxygen removal (glucose/glucose oxidase/catalase) was added to one of the wells before both were sealed. The measured lifetimes for the oxygenated and deoxygenated solutions were 1.5 μs (oxygen quenching at atmosphere) and 2.0 μs (oxygen depletion, respectively). The lifetime values are within a factor of 2 of the lifetime of Ru(dpp3)2+ dissolved in ethylene glycol31; however, additional work is needed to properly
characterize this dye nanomicelle dissolved in water if it is to be used as an oxygen-sensing probe.

**Animal preparation and surgery.** All animal procedures were reviewed and approved by the Cornell Institutional Animal Care and Use Committee. FVB/n (Charles River Laboratories) and Tg(ActB-DsRed*MST)1Nagy/J mice were anaesthetized using isoflurane (MWI; 4% in O2 for induction, 1.5–2% in O2 for surgery and imaging) and were hydrated with 50 μl h−1 subcutaneous injections of 5% glucose (Sigma) in saline (MWH). Body temperature was maintained with a feedback-controlled heat blanket (Harvard Apparatus). Before surgery, glycopyrrolate and dexamethasone were administered by intramuscular injections to quadriiceps. After the skull was exposed and cleaned with ethanol (70%) and ferric chloride (10%), (VWR), a custom-built metal plate was attached to the skull using a cyanoacrylate-based glue (Loctite-495) and dental cement (A-M Systems). A 4-mm-diameter craniotomy was centred 2 mm posterior and lateral to the Bregma point. The dura was left intact. A 5-mm-diameter glass coverslip (Electron Microscopy Sciences) was used to seal the craniotomy.

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**Author contributions**

S.H. coordinated the project, designed and built the microscope, designed and fabricated the linear SLM, wrote control and image-processing software, performed simulations, wrote analysis algorithms, analysed data, carried out animal preparation and surgery, prepared dye and calibrations, and wrote the manuscript. A.S. greatly assisted with microscope design and assembly, performed simulations and analysis, wrote analysis algorithms, significantly contributed to the content of the manuscript, and performed experiments verifying pixel rate increase. N.H. and D.K. assisted in animal preparation and surgery for imaging. C.X. initiated the project, significantly contributed to the design of M4 and experimental design, and greatly contributed to the theoretical and experimental discussions. All authors contributed to editing the manuscript.

**Additional information**

Supplementary information is available in the online version of the paper. Reprints and permission information is available online at http://www.nature.com/reprints. Correspondence and requests for materials should be addressed to S.S.H. and C.X.

**Competing financial interests**

The authors declare no competing financial interests.