



Published in final edited form as:

Nat Photonics. 2013 January 1; 7(1): 33–37. doi:10.1038/nphoton.2012.307.

Frequency Multiplexed *In Vivo* Multiphoton Phosphorescence Lifetime Microscopy

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Abstract

Multiphoton microscopy (MPM) is widely used for optical sectioning deep in scattering tissue, *in vivo* [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 PLIM [6] of phosphorescence quenching probes has recently provided oxygen partial pressure measurements in small rodent brain vasculature identified by high-resolution MPM [7, 8]. However, the maximum fluorescence generation rate, which is inversely proportional to the phosphorescence lifetime, fundamentally limits PLIM pixel rates. Here we experimentally demonstrate a parallel-excitation/parallel collection MPM-PLIM system that increases 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.

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AUTHOR CONTRIBUTIONS

S.H. coordinated the project, designed and built the microscope, designed and fabricated the linear spatial light modulator, wrote control and image processing software, performed simulations, wrote analysis algorithms, analyzed data, performed animal preparation and surgery, prepared dye and calibrations, and wrote the paper. A.S. greatly assisted with microscope design and assembly, performed simulations and analysis, wrote analysis algorithms, significantly contributed to the content of the paper, 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 manuscript editing.

Current technologies for overcoming the fundamental pixel rate limitation of serial-acquisition MPM require parallel excitation and imaging a sample onto multi-element detectors (typically CCD) [9–10]. While satisfactory for thin tissue slices or non-scattering samples, thick scattering samples 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 (e.g. LED arrays or pulsed diode excitation) or collection (e.g. gated CCD, single photon avalanche diode array, or multichannel PMT) [12] for video rate 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 3D optical sections.

PLIM acquisition rates in phosphorescence quenching systems are further limited by long dye lifetime ($\tau > 1\mu\text{s}$), which require low repetition rate lasers and long pixel dwell times ($T \gg \tau$). Lifetime measurement rates of phosphorescent dyes often-used in oxygen sensing [5, 7, 8, 17, 18, 19] are thus limited to $< 10\text{Hz}$ at depth [8]. Although these systems provide valuable measurements at specific points within tissue (e.g. partial pressure of oxygen within specific vascular compartments), full-frame optical sectioning measurements of phosphorescent quenching at depth is impractical.

This paper experimentally demonstrates 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 prior 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 excitation [20] until the imaging depth is many times the scattering length (e.g., $\sim 1\text{ mm}$ in biological tissues) [21]; therefore, crosstalk 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 via signal frequency demodulation and pixel location is independent of sample-induced scattering. Each modulation frequency's magnitude and phase is mapped to fluorescence intensity and lifetime, respectively, for each pixel in the sample. Similar imaging techniques have been employed when detector arrays are impractical [22] and fluorescence imaging [23, 24].

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

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

where S_i is the cycle averaged signal strength; ω_i is the modulation angular frequency; θ_i and ϕ_i are, respectively, the phase delay due to the finite fluorescence lifetime (τ_i) at the fundamental modulation frequency ω_i and its second harmonic $2\omega_i$ at the i^{th} -pixel. All fundamental modulation frequencies (ω_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 $N = \frac{\omega_0}{2\pi} T$, where ω_0 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 θ_i for all ω_i . The value of ω_i corresponds to the position of the i^{th} -pixel, while S_i gives the pixel intensity and θ_i can be used to calculate the fluorescence lifetime [3]:

$$\tau_i = \frac{\tan(\theta_i)}{\omega_i} \quad (2)$$

For optimum measurement of fluorescence lifetimes near τ_{target} , the angular modulation

frequencies should be near $\omega_{\text{target}} = 2\pi \frac{0.1}{\tau_{\text{target}}}$ [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 set-up is described in Fig. 1. A mode-locked Ti:Sapphire laser is used as the excitation source ($\lambda = 780$ nm, ~ 100 fs pulse width, and 80 MHz repetition rate with power in the range of 80 to 150 mW at the sample). We first focus the beam to a line using a 10 cm cylindrical lens (CL). This line illumination then impinges onto a one-dimensional spatial light modulator (SLM), generating a linear array of point sources with each point source modulated by different frequency (this process is described in the Methods section “Custom Reflection Based Linear Spatial Light Modulator” and top dashed box in Fig 1). The linear point source array is imaged onto the sample to excite fluorescence, forming a one-to-one mapping between the modulation frequency and the pixel, i.e., the spatial information along the focused line is encoded in the frequency domain by modulating the excitation light intensity. The excited nonlinear signal is epi-collected through the objective and reflected off a dichroic mirror onto a large area photomultiplier tube (PMT) (e.g. Hamamatsu H7422P-40). The dichroic mirror also reflects a small amount ($< 1\%$) of the 780 nm excitation light onto a Si photodiode, which acts as a reference channel for fluorescence/phosphorescence lifetime measurements. The signal and reference channels are then processed as complex spectrograms to reconstruct, respectively, the reference image and sample image: The modulation frequency is mapped to the y-axis, and time is mapped to the x-axis. The brightness of each pixel is determined by the magnitude measured in the signal spectrogram. The fluorescence/phosphorescence lifetime of each pixel is determined by the difference in measured phase between the signal and reference spectrograms. Data is collected on a 10 MHz National Instruments DAQ (PCI-6110) card and calculations are performed in real-time in LabView (National Instruments Corporation). Post processing and further data analysis was performed with custom scripts in MATLAB (The Mathworks Inc.).

An intuitive understanding of our technique can be obtained by considering sub-carrier 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 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, the prominent difference is each excitation point along the line is intensity modulated at a unique frequency by a custom, reflection-based linear spatial light modulator capable of modulating up to ~1 MHz and corresponding to emission lifetimes of ~100 ns. For faster fluorescence lifetimes, commercially available polygon scan mirrors [27, 28] (SA24 & 72 sided polygon, Lincoln Laser Corp) can further increase modulation rates to 60 MHz, corresponding to a lifetime resolution of ~50 ps and suitable for a wide range of biological fluorescent lifetime measurements. Alternatively, a 2D digital micromirror device (DMD) or digital MEMs array can replace our linear SLM to achieve simultaneous full-field imaging by uniquely modulating and imaging a 2D array of pixels onto the sample. Commercially available DMD kits (DLP4X00KIT, Texas Instruments) already can achieve 16kHz modulation rates, suitable for wide-field M4 PLIM imaging of oxygen sensing dyes

Fig. 2 demonstrates the M4 concept by imaging a 1951 USAF Resolution Test Chart and collecting the reflected light signal on a large-area silicon photodiode. 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 0.75NA 20x objective. The lateral extent (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 is 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 (i.e. modulation frequency) is shared among all simultaneously imaged pixels. Since two-photon 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⁺ [18] dyed tissue fibers were imaged using both techniques at equivalent sample illumination (i.e. equivalent fluorescence generation rate per pixel). This criterion was chosen since 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.

Significant interest exists in functional imaging of pO_2 in brain vasculature during stimulation [16, 29]. Recent work demonstrated functional imaging measurements at depth [8]. However, no oxygen sensing system produced 3D fluorescence/phosphorescence lifetime imaging of a heterogeneous lifetime system in a scattering medium (e.g., *in vivo*).

To demonstrate M4 *in vivo*, we injected Ru(dpp₃)-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 are 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; Supplemental Video 1). Lifetime histograms of interior vascular compartments (800 pixels) displayed in Fig. 4(b–d) are presented in (e) from top to bottom, respectively. Image frames are 500×160 pixels with 85 mW optical power at the sample; the modulation rates range from 32–64 kHz. Fig. 4f and Supplemental Video 2 demonstrates simultaneous intensity and lifetime optical sections at 10 micron steps and 3 minute averaging per frame.

A heterogeneous *in vivo* sample was prepared by injecting Ru(dpp₃)-pluronic-nanomicelle probes into a fluorescent protein (DsRed) labeled mouse retro-orbitally. Conventional two photon microscopy shows that DsRed is expressed predominately in the blood vessel walls in the absence of dye (Fig. 5a). Optical sections of Ru(dpp₃) in DsRed labeled mouse vasculature were obtained in 10 micron steps through a blood vessel. The simultaneously acquired intensity and lifetime images are presented in Figs. 5b and 5c, respectively. It is clear that the blood vessel walls contain fast lifetime ($\tau \ll 1 \mu\text{s}$) DsRed fluorescence while Ru(dpp₃)-nanomicelle dye ($\tau \sim 2.5 \mu\text{s}$) is localized in the vessel interior. The M4-PLIM optical stack of 11 full-frames through 110 μm is presented in Fig 5(d) and Supplemental Video 3, and represents, to our knowledge, the deepest *in vivo* PLIM imaging of phosphorescence quenching dye. The modulation frequencies are between 64 kHz and 128 kHz, the frame period is 3 minutes 20 seconds, 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 PLIM pixel rate of 640 Hz. This is significantly faster than previously reported long lifetime two-photon PLIM imaging in a similar system (pixel rates of ~ 1 Hz [8]). M4 pixel rates can be greatly improved by increasing the photon generation rate with improved dyes or higher excitation pulse energy at sample.

In summary, we experimentally demonstrated a parallel-excitation/parallel-collection MPM-PLIM system that achieves 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 phosphorescence quenching probe.

METHODS SECTION

Custom Reflection Based Linear Spatial Light Modulator

High modulation rates are required (e.g., ~ 100 kHz used in our experiments) to resolve distinct points along the line and to optimally match the desired fluorescence/phosphorescence lifetime. Since commercially available linear SLMs with the required pixel numbers cannot modulate at such high speeds, we created a free-space optical chopper (Fig. 1a, dashed box) that can modulate an array of point sources at MHz rates by scanning a line-focused laser beam over a small (10–20 μm period) mirror grating on a photolithography mask (Fig. 1b). 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 described in Methods is used for both parallel and serial acquisition. The microscope is modified for serial acquisition by removing the first cylindrical lens. A conventional point focus is then obtained on both the sample and spatial light modulator. The spatial light modulator thus acts as an optical chopper and intensity modulates the excitation beam. The input optical power is adjusted so that the excitation intensity at the sample is identical in both parallel and serial acquisition.

The pixel rate comparison 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 of a mirror surface at the sample. Since optical delay is negligible (<1ns), any measured delay can be attributed to latent delays in the system electronics (detectors and amplifiers). This calibration is performed before PLIM imaging and included in the lifetime calculations.

Preparation and demonstration of phosphorescence quenching Ru(dpp₃)²⁺ encapsulated nanomiscelles

Water soluble Ru(dpp₃)²⁺-pluronic nanomiscelles were prepared [30]. 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 minutes.

Phosphorescent quenching of $\text{Ru}(\text{dpp3})^{2+}$ 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 lifetime for the oxygenated and deoxygenated solution 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 $\text{Ru}(\text{Dpp3})^{2+}$ dissolved in ethylene glycol [18], 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 anesthetized using isoflurane (MWI) (4% in O₂ for induction, 1.5–2 % in O₂ for surgery and imaging) and was hydrated with 50 $\mu\text{L}/\text{hour}$ subcutaneous injections of 5% glucose (Sigma) in saline (MWI). Body temperature was maintained with a feedback-controlled heat blanket (Harvard Apparatus). Prior to surgery, glycopyrrolate and dexamethasone were administered by intramuscular injections to quadriceps. 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 centered 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research project is supported in part by NSF/DBI (Grant No. DBI-0546227) and NIH/NCI grant number R01-CA133148. Nicholas G. Horton is supported by NSF Graduate Research Fellowship (Grant No. DGE-0707428). The authors would like to thank Prof. Chris Schafer, Cornell University, and Prof. Sergei Vinogradov, Department of Biochemistry and Biophysics, University of Pennsylvania, for their helpful discussions on brain imaging and phosphorescence quenching dyes for in vivo oxygen sensing, respectively.

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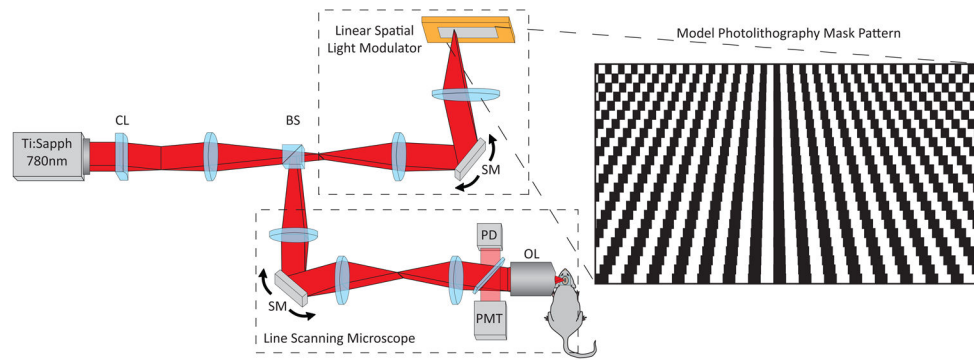


Fig. 1. Schematic of the M4 microscope. CL: cylindrical lens; BS: beam splitter; SM: scan mirror; PD: photodiode; PMT: photo-multiplier tube; OL: objective lens. Inset: Illustration of the spatial light modulator

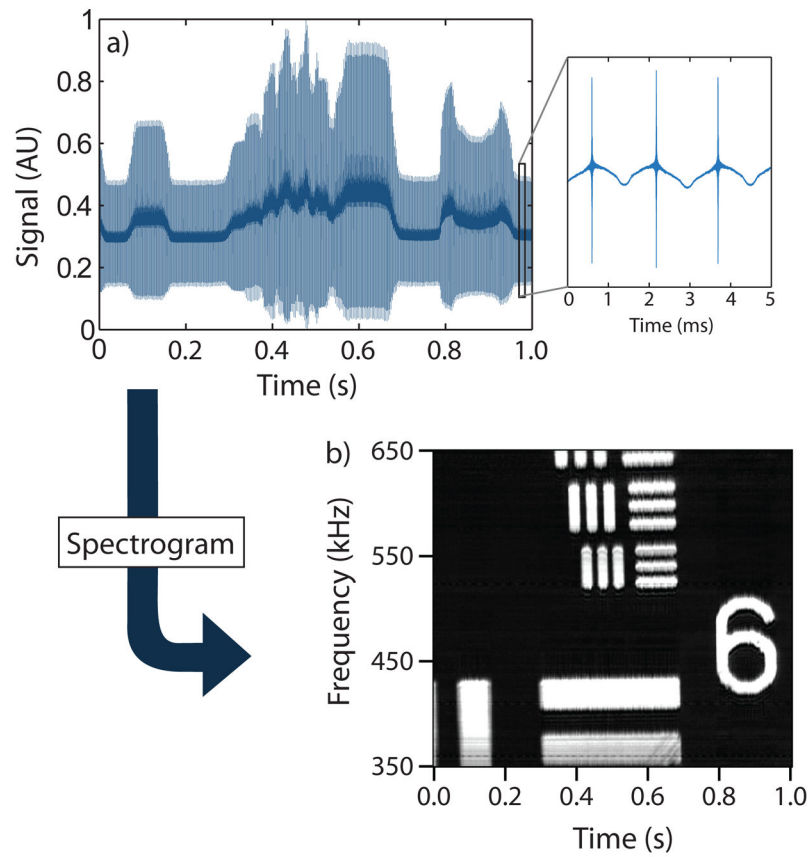


Fig. 2. M4 data processing and image reconstruction. Original (a-top) and processed (b-bottom) data of a reflection image of a 1951 Air Force test target with a 60x 1.10NA water immersion objective. The smallest features are $2.2 \mu\text{m} \times 11.0 \mu\text{m}$. The inset of (a): raw data corresponding to three lines in the image.

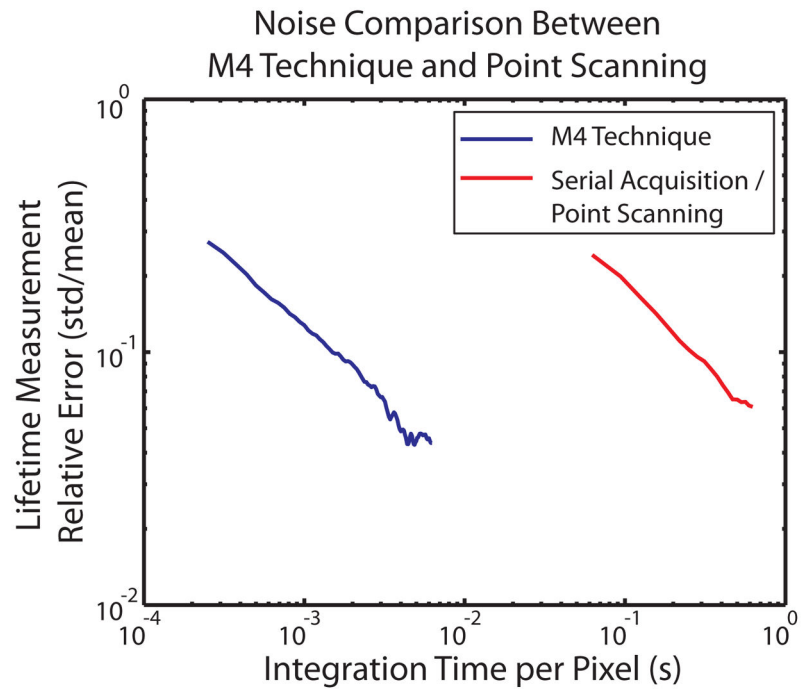


Fig. 3. Lifetime measurement uncertainty vs. pixel integration time in Ru(dpp3) dyed tissue fiber, comparing M4 against point scanning PLIM.

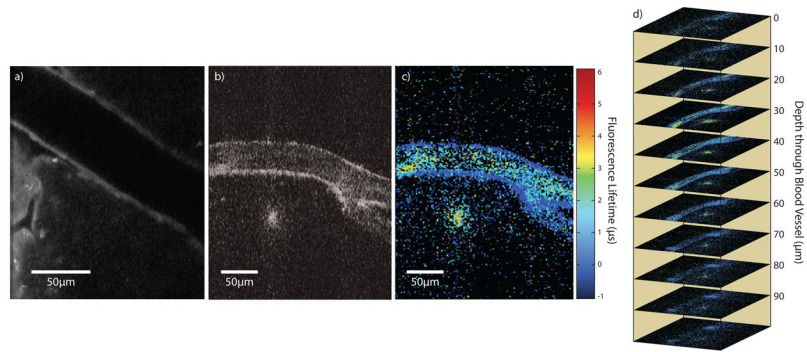


Fig 4. Mouse brain vasculature containing Ru(dpp₃)-pluronic-nanomicelle probes imaged by M4. Phosphorescence intensity (a); phosphorescence lifetime after 20 s (b), 1 min (c) and 5 (d) of integration; lifetime histograms (e), from top to bottom, of 800 pixels from the interior of the blood vessel from images (b–d), respectively; and optical sections separated by 10 μm through brain vasculature (f) are presented. Images (b–i) map lifetime to hue and intensity to value to illustrate simultaneous lifetime and intensity acquisition. Images (f–i) are optical sections of brain vasculature each separated by 10 μm.

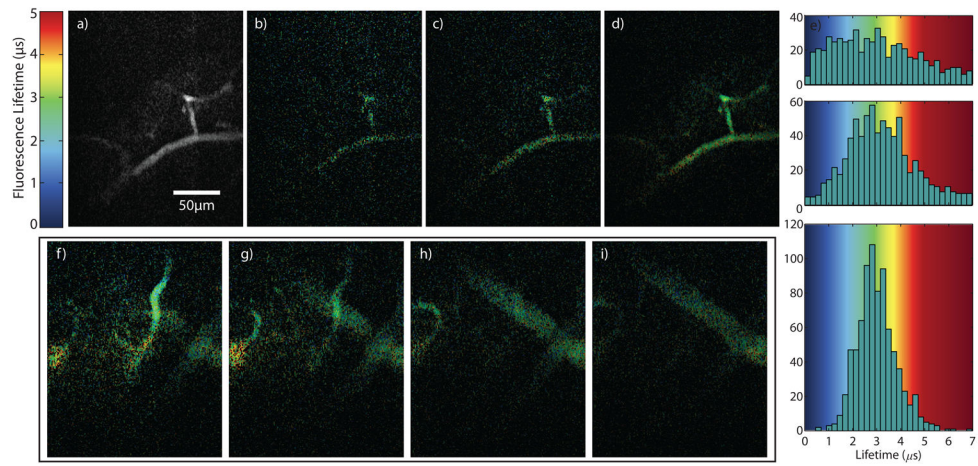


Fig. 5. M4 and conventional two-photon microscopy of DsRed labeled mouse brain vasculature, *in vivo*. Conventional point scanning MPM of mouse brain blood vessel labeled with DsRed (a) prior to Ru(dpp₃) injection. M4 intensity (b) and lifetime (c) maps of DsRed labeled mouse brain after injection of phosphorescent Ru(dpp₃)²⁺ dye. Lifetime sections were taken at 10 μm steps through the blood vessel (d) after injection of Ru(dpp₃)²⁺ dye.