Selective plane illumination microscopy (SPIM) with time-domain fluorescence lifetime imaging microscopy (FLIM) for volumetric measurement of cleared mouse brain samples

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We have developed an imaging technique which combines selective plane illumination microscopy with time-domain fluorescence lifetime imaging microscopy (SPIM-FLIM) for three-dimensional volumetric imaging of cleared mouse brains with micro- to mesoscopic resolution. The main features of the microscope include a wavelength-adjustable pulsed laser source (Ti:sapphire) (near-infrared) laser, a BiBO frequency-doubling photonic crystal, a liquid chamber, an electrically focus-tunable lens, a cuvette based sample holder, and an air (dry) objective lens. The performance of the system was evaluated with a lifetime reference dye and micro-bead phantom measurements. Intensity and lifetime maps of three-dimensional human embryonic kidney (HEK) cell culture samples and cleared mouse brain samples expressing green fluorescent protein (GFP) (donor only) and green and red fluorescent protein [positive Förster (fluorescence) resonance energy transfer] were acquired. The results show that the SPIM-FLIM system can be used for sample sizes ranging from single cells to whole mouse organs and can serve as a powerful tool for medical and biological research. *Published by AIP Publishing.* <https://doi.org/10.1063/1.5018846>

I. INTRODUCTION

There has been an increasing interest in medical and biological studies to achieve fast three-dimensional (3D) volumetric fluorescence imaging of centimeter-sized biological tissue with both microscopic (submicrons to microns) and mesoscopic resolution (several tens of microns). Light-sheet fluorescence microscopy (LSFM),^{1,2} where the sample is illuminated by a sheet-shaped focused laser beam that is perpendicular to the detection axis so that only an in-focus single plane is illuminated, has been explored as a possible imaging technique for high resolution imaging of small organs because of low photo-bleaching of fluorescence and fast image acquisition with image sensors such as charge-coupled device (CCD) cameras.^{3–7} With this method, optically sectioned images are obtained by the camera without the need for tomographic reconstruction. To reduce or completely suppress the effect of light scattering, transparent gel or cleared tissue prepared by tissue clearing techniques is used.^{8,9} There are two main techniques for generating light-sheets for sample illumination. A static light sheet is created by beam expansion, a slit, and a cylindrical lens [e.g., selective plane illumination microscopy (SPIM)].⁴ A dynamic light-sheet can be created by scanning a thin light beam with galvanometer mirrors [e.g., digitally scanned light-sheet microscopy (DSLM)].^{10,11}

Although light-sheet illumination is typically used with time-accumulated (continuous wave, CW) fluorescence, it can

be extended to time resolved fluorescence as a way to extract more information from a biological sample. Time resolved fluorescence allows us to measure fluorescence lifetime, which is a sensitive indicator of local environment, such as pH, temperature, polarity, and ion concentration. Lifetime can also be an indicator of Förster (fluorescence) resonance energy transfer (FRET) caused by molecular interactions or conformation change^{12,13} and is a useful contrast mechanism for the removal of background fluorescence (e.g., auto-fluorescence). Fluorescence lifetime imaging microscopy (FLIM) has been used for acquiring microscopic images of fluorescence lifetime maps in cells or tissue sections.¹⁴ As discussed previously,¹⁵ FLIM-FRET has several advantages over ratiometric FRET when the donor fluorescence significantly overlaps the acceptor emission wavelengths or when the donor is prone to photobleaching. Fluorescence lifetime has also been applied for whole body tomographic imaging in thick scattering tissue and small animals.^{16–21} An important advantage of fluorescence lifetime is that when two or more fluorophores that have the same excitation wavelength are mixed within the diffuse medium, they can be completely separated tomographically using lifetime multiplexing if their lifetimes are sufficiently different and known.²² Lifetime multiplexing can be performed in time-domain or frequency-domain configurations. The time-domain and frequency-domain FLIM techniques are complementary methods. The time-domain FLIM with the single-photon timing technique has a very high sensitivity for measurements with very low levels of fluorescence (from low-concentration samples such as mouse brains with low-level expression of

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target molecules),²³ whereas in the frequency-domain FLIM fluorescence intensity must generally be higher. For a single exponential decay model, frequency-domain FLIM has a higher speed, but for multi-exponential decay, the time of data collection is about the same for both techniques.²⁴

A combination of light-sheet and FLIM techniques can offer important benefits for imaging biological tissue. Greger *et al.* reported a combination of SPIM and frequency-domain FLIM and demonstrated the high precision with latex bead samples and live Madin-Darby canine kidney cysts that are embedded in gel and mounted in small Polytetrafluorethylene (PTFE) chambers with a 100× objective lens.²⁵ In this paper, we present a light-sheet microscope system in combination with time-domain FLIM technology to achieve fast 3D fluorescence lifetime image acquisition on centimeter-sized cleared tissue that includes mouse brain. The light-sheet microscope is capable of imaging whole mouse organs using a 10× objective lens. Here we demonstrate the performance of a custom-built system using a FRET-based assay with green and red fluorescent protein (GFP-RFP) fusion of 3D cell culture gel samples and cleared mouse brain samples.

II. MATERIALS AND METHODS

A. System description of SPIM-FLIM

Our custom-built selective plane illumination microscopy with time-domain fluorescence lifetime imaging microscopy (SPIM-FLIM) system consists of frequency-doubled laser-generation, light-sheet illumination, and time-domain FLIM detection modules. The optical setup of the frequency-doubled laser generation with a near-infrared laser source and a photonic crystal is shown in Fig. 1. A Ti:sapphire laser (Spectra-Physics, Mai Tai HP 1020; 770-nm excitation; 100-fs pulses; 80-MHz repetition rate; 690–1040-nm wavelength range) was used to generate 946-nm laser output (~1.3 W). Frequency doubled blue (473-nm) laser was generated with a BiBO (BiB₃O₆) nonlinear crystal (size: 3 × 3 × 10 mm, Newlight Photonics, Inc.). After filtering the output of the BiBO crystal with a 650-nm short-pass filter (FES0650, Thorlabs, Inc.), the 473-nm light (~400 mW) was launched into a single mode fiber (P1-460B-FC-5, Thorlabs, Inc.) with a bi-convex lens ($f = 100$ mm, LB1187-A-ML, Thorlabs, Inc.). The output power at the end of the single mode fiber was around 5 mW. Since the BiBO crystal can be used over a broad wavelength range, the wavelength can be flexibly changed using a wavelength-tunable source such as the Ti:sapphire laser.

The experimental setup for light-sheet generation and the sample chamber and holder of the SPIM-FLIM system are shown in Fig. 2. The output of single mode fiber is collimated with a collimator lens (A240TM-A, $f = 8.0$ mm, NA = 0.50, Thorlabs, Inc.) and expanded with a two-lens beam expander (focal lengths: 15 and 150 mm). A light sheet was generated with a mechanical slit (VA100, Thorlabs, Inc.) and a cylindrical lens ($f = 100$ mm, ACY254-100-A, Thorlabs, Inc.).²⁶ The theoretical thickness ($1/e^2$ beam width) of the light sheet in the water was 13 μm with a slit width of 6 mm (medium refractive index: 1.33, wavelength: 473 nm).

A home-made liquid chamber with a size of 70 × 70 × 90 mm³ was made from 2-mm-thick acrylic panels. The liquid used in the chamber was the water in all the measurements in this study. The chamber can serve to minimize the effect of changes in focal length when the cuvette is positioned at different locations in the chamber. The refractive indices of the liquid in the cuvette and the chamber should ideally be the same, but any differences can be corrected by an electrically focus-tunable lens (ETL) described below. A slight change of illumination focus can occur depending on the difference of refractive indices between the liquid in the cuvette and chamber, but we did not correct for this since its effect on image quality is small compared with the detection focus change.

The experimental setup (horizontal view) of the detection system for the SPIM-FLIM system is shown in Fig. 3. The fluorescence signal is introduced through a detection objective (10×, NA = 0.28, working distance = 33.5 mm, 10× EO M Plan Apo Long Working Distance Infinity Corrected, Edmund Optics) and a tube lens ($f = 200$ mm, AC508-200-A-ML, Thorlabs, Inc.). The focal position of the detection system can be adjusted by an electrically tunable lens (ETL, EL-10-30-Ci, Optotune Switzerland AG). The 4-f relay system²⁷ is adopted so as not to change the magnification or field of view (FOV) even when the focal distance of the ETL is changed. Between the tube lens and 4-f system lenses an electrically controllable filter wheel (with 500-nm long-pass filter) was placed. The ETL and filter wheel are controlled via serial communication from a computer. The 3D position of cuvette holder is controlled by a micro-manipulator system (MP-285, Sutter Instrument Company) with a smallest step of 0.04 μm . The movement of the cuvette holder is synchronized with the measurement and it is controlled via serial communication from the computer.

The 80-MHz trigger signal from the Ti:sapphire laser is relayed by a constant fraction discriminator (CFD) unit

FIG. 1. Optics setup of frequency-doubled laser generation with Ti:sapphire laser and a BiBO photonic crystal and launch of it into a single mode optical fiber.

FIG. 2. Experimental setup of light-sheet generation and the sample chamber and holder of the SPIM-FLIM system.

(PicoHR12-OCF2, LaVision) and connected to a fast high rate imager (HRI) trigger delay unit (Kentech Instruments Ltd.) which was used to delay the trigger output (80 MHz) from the laser to the intensifier unit. The delay unit is connected to the HRI unit (Kentech Instruments Ltd.) that is connected to a time gated image intensifier (Picostar HR-12, LaVision GmbH, Goettingen, Germany) with a 500-ps gate width, which was used to intensify the image and transmit it to the CCD camera (Imager QE, Picostar HR-12 CAM 2, LaVision GmbH, Goettingen, Germany). The full resolution of the CCD camera was 1376×1040 pixels. For a higher signal-to-noise ratio and frame rate, the camera images were 4×4 binned (averaged) at the hardware level to a reduced size of 344×260 pixels (pixel size: $5.5 \times 5.5 \mu\text{m}$ with a $10\times$ objective lens). The FOV of this system using this camera with a $10\times$ objective lens is $1.9 \text{ mm} \times 1.4 \text{ mm}$. The delay and gain of the intensifier were controlled by a serial communication from a control personal computer.

To validate the accuracy of lifetime measurements of the system, coumarin 6 (546 283, Sigma-Aldrich) in ethanol at a

TABLE I. Specifications of SPIM-FLIM system.

Parameter	Value
Excitation wavelength	473 nm (tunable by a Ti:sapphire laser and SHG crystal)
Light-sheet thickness	$13 \mu\text{m}$ (theoretical, in water)
Lateral resolution (x - y)	$7.0 \pm 1.9 \mu\text{m}$ [horizontal (y)], $7.8 \pm 3.4 \mu\text{m}$ [vertical (x)] (experimental, $n \sim 1.4$)
Axial resolution (z)	$9.9 \pm 2.0 \mu\text{m}$ (experimental, $n \sim 1.4$)
Emission filter	500-nm long pass
Gate width	400 ps–1 ns
Number of pixels	256×344 (4×4 binning of 1024×1376)
Field of view (FOV)	$1.4 \times 1.9 \text{ mm}^2$
Maximum sample size	$10 \times 10 \times 20 \text{ mm}^3$

concentration of $100\text{-}\mu\text{M}$ was used as a lifetime standard. The lifetime of the reagent is 2.4 ns from the literature.²⁸ It was measured by both a commercial confocal-based FLIM system (LSM 510 META, Carl Zeiss) and our system developed in this study. The obtained lifetimes were both around 2.4 ns (commercial system: $2.4 \pm 0.1 \text{ ns}$, our system: $2.4 \pm 0.1 \text{ ns}$). This validated the lifetime accuracy of the SPIM-FLIM system. The specifications of the developed SPIM-FLIM system are shown in Table I.

B. Microbeads' phantom measurement

To evaluate the performance of the developed system, a polydimethylsiloxane (PDMS) phantom (refractive index: ~ 1.4) that includes three kinds of microbeads (microspheres) was created. The home-made microbeads were created following a previously described procedure^{29,30} using coumarin 1 (546 283, Sigma-Aldrich) and fluorescein (32 615-25G-R, Fluka Analytical) dyes and $3\text{-}\mu\text{m}$ polystyrene latex beads (LB30, Sigma-Aldrich).

The spatial resolution of the system was validated by measuring the point spread function of the microbeads.³¹ The typical diameters of microbeads are 3, 3, and $2 \mu\text{m}$ for coumarin 1 (home-made), fluorescein (home-made), and flash red (FS05F, lot no. 12 137, Bangs Laboratories), respectively, which are sufficiently small so that the FWHM of the imaged microbeads can be regarded as the spatial resolution. In the measurement, 30 image planes with a $2\text{-}\mu\text{m}$ interval in the z -axis were obtained. In the analysis, the intensity spatial profile (with an intensity threshold) was fitted to a Gaussian curve in each axis and the FWHM of the Gaussian curve was calculated. The minimum FWHM (i.e., spatial resolution) of the point spread functions was determined for all microbeads in the imaged volume. The spatial resolutions in the x , y , and z axes, defined by the FWHM of the point spread function of the fluorescent beads, were $7.8 \pm 3.4 \mu\text{m}$, $7.0 \pm 1.9 \mu\text{m}$ (among 30 images), and $9.9 \pm 2.0 \mu\text{m}$ (among 10 y - z slices that have pixels with $0.2 \times$ maximum intensity), respectively.

We embedded beads in PDMS in the following way: 1. We mixed a SYLGARD® 184 silicone elastomer curing agent and SYLGARD 184 silicone elastomer base with weight ratios of 1 and 10. 2. We poured microbeads in the material and

FIG. 3. (a) Experimental setup (horizontal view) of the detection system of the SPIM-FLIM system. BFP denotes the back focal plane. (b) Picture of the cuvette and cuvette holder.

mixed it further. 3. We removed air bubbles by vacuum over several hours. 4. We heated the material at 80 °C for several hours to harden the mixture. We acquired an instrument response function (IRF) by measuring temporal profile of photon counts through the microbeads' phantom without emission filter. Measured pulse width (FWHM) of frequency doubled light was 316 ± 41 ps.

C. 3D cell culture measurement

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) in a 37 °C, 5% CO₂ incubator. The cells were transfected with plasmids encoding either green fluorescent protein (GFP) or green and red fluorescent protein fusion (GFP-RFP) using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's protocol. 24 h after the transfection, the cells were removed from the culture dish with Trypsin-EDTA (0.25%) (Thermo Fisher Scientific), centrifuged at 350× g, washed with FluoroBrite DMEM, and 10⁶ cells were resuspended in 500-μl Matrigel Matrix, Phenol Red Free (BD Biosciences). The gel with the cells was transferred into the imaging cuvettes (3-G-5, Starna Cells, Inc.) and incubated in the tissue culture incubator at 37 °C, 5% CO₂ for 30 min. After that FluoroBrite DMEM was added to the cultures. Before imaging, the cultures were fixed for 1 h in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) in phosphate-buffered saline (PBS) (Thermo Fisher Scientific) and washed three times with PBS. The refractive index of Matrigel is 1.34 which is the same as that of water (1.33) at 20 °C.

D. Cleared mouse brain measurement

GFP or GFP-RFP expression in wild-type 3-month-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) hippocampi was achieved using stereotaxic injection of adeno-associated viruses serotype 2/8 (AAV2/8) carrying GFP or GFP-RFP expression plasmids (Fig. 4). The stereotaxic injection coordinates from bregma were 3.1 mm posterior, 2.8 mm lateral, and 2.5 mm in-depth from the brain surface. The AAVs were produced at Penn Vector Core, Perelman School of Medicine, University of Pennsylvania. One month after the AAV2/8 injection, the mice were perfused intracardially with ice-cold PBS (Thermo Fisher Scientific) followed by fixation solution [4% PFA (Electron Microscopy Sciences) and 1% glutaraldehyde (GA) (Electron Microscopy Sciences) in PBS]. Then the brains were removed, incubated for 3 days at 4 °C in the fixation solution, and further processed according to the SWITCH protocol.³² Briefly, the fixative was inactivated by two 6-h washes at room temperature with PBS-T [PBS, 0.1% (v/v) Triton X-100, 0.02% (w/v) sodium azide] followed by one overnight wash at 37 °C with inactivation solution [PBS, 4% (w/v) acetamide, 4% (w/v) glycine]. Next, the samples were washed twice for 6 h at room temperature with freshly prepared thermal clearing solution [200 mM sodium dodecyl sulfate (SDS), 20 mM sodium sulfite in water] and incubated for one month

at 37 °C in the thermal clearing solution. Finally, the samples were equilibrated in a refractive index-matching solution by two 6-h and one overnight washes at 37 °C with optical clearing solution [23.5% (w/v) *N*-methyl-D-glucamine, 29.4% (w/v) diatrizoic acid, 32.4% (w/v) iohexanol in water]. All the reagents, unless stated otherwise, were purchased from Sigma. We put the sample (entire brain) submerged in the optical clearing solution (refractive index: ~1.47) in a 3.5-ml cuvette.

All *in vivo* procedures complied with the National Institutes of Health (NIH) guidelines for the use of animals in experiments and were approved by the Massachusetts General Hospital Animal Care and Use Committee.

E. Measurement procedure

The measurement was controlled by custom-built software based on MATLAB (The Mathworks, Inc.). Instrumental response function (IRF) was measured with no emission filter (i.e., only reflected light) condition. Images obtained under a dark (i.e., no excitation laser) condition were subtracted from the data. The time gate width (adjustable from 200 ps) for the image intensifier was set to 500 ps and the interval of sampling time was set to 100 ps. The exposure time of the CCD camera and the intensity gain of the intensifier were adjusted depending on the fluorescence intensity. It took, for example, about 100 min to complete the measurement for acquiring a lifetime image of 6 mm × 4 mm (12 × 8 imaging positions) single plane.

F. Analysis

Decay curves were fitted to a single exponential function. A nonlinear parameter fitting routine was conducted using the following equation:

$$f(t) = a_0 + a_1 \exp(-t/\tau), \quad (1)$$

where t denotes time, τ denotes fluorescence lifetime, and a_0 and a_1 denote constants.

For reconstructing images, the step size of x - and y -scanning was set to 500 μm. For each image, only lifetimes at each pixel in the same region of interest (ROI) (91 × 91 pixels, 500 × 500 μm) were calculated and stitched. Basic

FIG. 4. Introduction of AAV2/8 carrying GFP or GFP-RFP into mouse hippocampi.

FIG. 5. Fluorescence lifetime maps of HEK cell culture expressing (a) GFP and (b) GFP-RFP. (c) Mean decay curves of samples with GFP and GFP-RFP. (d) Histograms of lifetime images for samples with GFP and GFP-RFP.

image processing was performed using ImageJ (National Institute of Health)³³ and MATLAB (The Mathworks, Inc.). For displaying images of fluorescence intensity and lifetime of mouse brain samples, pixels around stitching boundaries (at 10 pixels for CW intensity and 15 pixels for lifetime from stitching boundaries) were filtered with moving averages (± 10 pixels for intensity and 15 pixels for lifetime) for eliminating stitching noise by custom-made software in MATLAB.

III. RESULTS

A. 3D HEK cell culture measurement

Fluorescence lifetime maps of maximal fluorescence of HEK cell culture expressing GFP and GFP-RFP are shown in Figs. 5(a) and 5(b), respectively. A color bar indicates lifetime (ns). The lifetimes of the top 400 highest-intensity pixels are shown in color, while those of the other pixels are

FIG. 6. Multi-z-slice (with a z -interval of $400 \mu\text{m}$) lifetime maps of transverse sections of a mouse brain expressing GFP. The double-headed arrow indicates orientation, OB denotes olfactory bulb, and Cereb denotes cerebellum. The color map indicates lifetime (ns). The slices shown are for displacements of (a) $z = 0 \mu\text{m}$ (start position), (b) $z = 400 \mu\text{m}$, (c) $z = 800 \mu\text{m}$, and (d) $z = 1200 \mu\text{m}$.

shown in black. Mean decay curves of samples expressing GFP and GFP-RFP and histograms of fluorescence lifetime for samples expressing GFP and GFP-RFP using the top 400 highest-intensity pixels are shown in Figs. 5(c) and 5(d). They are single-plane images without stitching (number of pixels: 256×344 , FOV: $1408 \times 1892 \mu\text{m}$). The width of cell-like areas varies from 6 to $72 \mu\text{m}$. Mean ($\pm$ S.D.) fluorescence lifetimes of higher intensity (top 400 highest) pixels for sample expressing GFP and GFP-RFP were 2.28 ± 0.10 and 1.98 ± 0.09 ns, respectively. The lifetime of the sample expressing GFP-RFP was significantly lower than that expressing GFP alone (*t*-test, $p < 0.001$).

B. Mouse brain sample measurement

As a demonstration of 3D lifetime imaging, multi-z-slice lifetime maps of a mouse brain sample expressing GFP with a z-interval of $400 \mu\text{m}$ (imaging volume: $3.5 \times 4.0 \times 1.2 \text{ mm}^3$) are shown in Fig. 6. For quantitative analysis for FRET effects, single planes of lifetime maps for the mouse brain samples were acquired. Fluorescence intensity images, fluorescence lifetime images, and mean decay curves for GFP or

GFP-RFP-expressed in mouse brain samples are shown in Fig. 7. GFP and GFP-RFP fluorescence was easily detectable close to the injection sites (hippocampi). In the case of GFP alone, 10×13 images were stitched (number of pixels: 910×1183), and the recorded lifetime using 10 000 high-intensity pixels varied from 2.02 to 2.13 ns. The mean ($\pm$ S.D.) lifetime in the higher intensity site was 2.08 ± 0.02 ns. As noted from Fig. 7(e), the semilog plots of the decay curves are not perfect straight lines, suggesting the presence of non-exponential decays, possibly caused by auto-fluorescence. However the influence of auto-fluorescence was still sufficiently small so that the decays could be fit reasonably well using single exponential fitting. When auto-fluorescence is significant, a basis function analysis can be used to separate the auto-fluorescence contribution from the total signal.³⁴ In the GFP-RFP case, 12×8 images were stitched (number of pixels: 1092×728), and the recorded lifetime using 10 000 high-intensity pixels varied from 1.3 to 2.0 ns. The mean ($\pm$ S.D.) lifetime was 1.83 ± 0.11 ns and was significantly shorter when compared to the GFP lifetime (*t*-test, $p < 0.001$), indicative of the occurrence of FRET within the GFP-RFP fusion protein.

FIG. 7. (a) Fluorescence intensity image, (b) lifetime image of virally transduced GFP-mouse brain sample. (c) Intensity image, (d) lifetime image of virally transduced GFP-RFP-mouse brain sample. They are transverse sections and a double-headed arrow indicates orientation, where OB denotes olfactory bulb and Cereb denotes cerebellum. (e) Mean decay curves of the GFP and GFP-RFP mouse brain sample. (f) Histograms of lifetime for GFP and GFP-RFP mouse brain samples.

IV. DISCUSSION

We demonstrated a SPIM-FLIM system by measuring the microbeads' phantom, 3D cell culture samples, and mouse brain samples. The water chamber performed well in this study even when a liquid with a different index of refraction was used in the sample holder because the ETL could be used to correct focal length changes caused by differences in indices of refraction. The focal length could be changed without a change in field of view (FOV) or magnification. We found that the focal length of the illumination light also changes, but the effect on image quality is minimal compared to that caused by detection focal length changes. Ideally, a matching liquid with the same refractive index as the clearing solution should be used, but chemical effects on chamber windows and walls should be considered for long term maintenance. Since distilled, non-ionized water is chemically stable, the maintenance of the chamber for our experiments was straightforward.

We performed lifetime calculations with or without consideration of IRF and found that the effect of IRF on the calculated lifetime was less than 0.1%. Hence, the width of the IRF was sufficiently short so that it did not impact the detection of the nanosecond lifetimes that were relevant for our study. The effect of IRF was thus ignored. The limiting factor which dictates the theoretical x-y spatial resolution of our system is the CCD pixel size of 5.5 μm . Although the theoretical lateral resolution from detection optics at the representative emission wavelength of 520 nm is $\sim 1.1 \mu\text{m}$ (Rayleigh's criterion), the effective lateral resolution is limited by the larger CCD pixel size (5.5 μm). The theoretical axial resolution, on the other hand, was calculated to be 8.0 μm (medium refractive index: ~ 1.4).³⁵ The experimentally obtained values for lateral resolution $\{7.0 \pm 1.9 \mu\text{m}$ [horizontal (y)], $7.8 \pm 3.4 \mu\text{m}$ [vertical (x)] $\}$ are larger than the pixel size (5.5 μm), which is probably caused by the variations of microbeads' locations (center or edge of pixels). The axial resolution ($9.9 \pm 2.0 \mu\text{m}$) is a little worse than the expected theoretical value (8.0 μm), which might be due to the deviations from the ideal conditions (e.g., optical aberration and slight scattering in the beads' phantom).

As a result of the FRET effect, images with significantly shorter lifetime were obtained, which demonstrates that our system can be used for investigation on 3D volumetric samples with a detectable FRET effect. Figure 6 also shows that our SPIM-FLIM system allows 3D fluorescence lifetime imaging. The absolute lifetimes for mouse brain samples are shorter than those of the cells in Matrigel samples. This can be attributed to the small amount of auto-fluorescence in mouse samples and effective lifetime in higher-intensity fluorescence sites decreased by mixing the target fluorescence and auto-fluorescence [lower lifetime, clearly seen in a non-injection site in Fig. 7(b)]. It is known that fluorescence lifetimes decrease with increasing refractive index of the local environment.^{36,37} The refractive index of the clearing solution we used was 1.47, which is a little larger than that of Matrigel samples ($n = 1.33$). The mean ($\pm$ S.D.) differences between lifetimes of GFP and GFP-RFP conditions are 0.30 ± 0.10 and 0.25 ± 0.11 ns for Matrigel and mouse samples, respectively. To exclude the influence of refractive index on fluorescence

lifetime, the FRET effect was evaluated under the same refractive index conditions.

As mentioned in Sec. II E, it takes about 100 min to complete the measurement (for a temporal profile of detected photon counts) for acquiring a lifetime image of 6 mm $\times$ 4 mm (12×8 imaging positions, number of pixels: 1092×728) single plane. To improve the imaging speed, higher optical power (shorter exposure time) and higher efficiency of optical components and higher resolution camera with larger FOV are needed. Additionally, to acquire the lifetime image from the measurement data, lifetime calculation at each pixel is needed. It took about 2 h to calculate lifetimes of all pixels with the same size (CPU: 2.9 GHz, RAM: 8 GB). The calculation time can be significantly improved using faster computers or parallel processing.

There are many mouse models for neurological diseases (e.g., Alzheimer's disease), and imaging system for mouse brain is a basic infrastructure in medical research for such diseases. Fluorescence lifetime provides more biological information than just fluorescence intensity for the detection of protein interactions.³⁸ Furthermore, the technique demonstrated here can be applied to any cleared organs such as mouse kidney, heart, lung, and liver.³⁹ SPIM-FLIM for centimeter sized samples can be applied to all samples that are currently used with light-sheet microscopes with static light-sheet illumination (e.g., SPIM) at the expense of longer acquisition times. Such measurements, however, will shed new light into the previous intensity images of light-sheet microscopes and will pave the way for acquiring new insights in biological research.

V. LIMITATION

The efficiency of coupling SHG light (473 nm) into a single mode fiber was 1.25% (400 mW input and 5 mW output) in our configuration. The efficiency is low because the SHG crystal generates frequency-doubled light with a walk-off angle. The amount and variance of the walk-off angle depend on the thickness of the SHG crystal (10 mm for our system). Since the SHG efficiency also depends on the thickness of the crystal (i.e., more SHG light is generated when a thicker crystal is used), the total usable output power after the fiber could be further optimized. More generally, the signal-to-noise ratio of the lifetime measurements could be further improved if a higher intensity pulsed light source was used for light sheet generation.

VI. CONCLUSION

We developed a SPIM-FLIM system for acquiring 3D volumetric images of larger samples like mouse brain. For pulsed light source generation, a Ti:sapphire laser and an SHG crystal were used which allowed for tunability of the excitation wavelength. Lifetime maps for HEK cell culture samples and mouse brain samples were successfully obtained and FRET signals were clearly observed. The system is applicable for a variety of biomedical investigations using cleared mouse brain (i.e., centimeter size) or other cleared organ samples.

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