Thesis Summary

Development of *in vivo* volumetric imaging method for mouse brain utilizing multibeam scanning two-photon microscopy

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Introduction

Two-photon microscopy (2PM) is a laser scanning fluorescence microscopy that uses a two-photon excitation process and has been widely used in the neuroscience field as a robust tool for *in vivo* observation of neuronal activities in the mouse brain. For single-beam scanning 2PM, galvanometer-based scanning mirrors are usually equipped and the focal plane is raster-scanned by a single focus. Meanwhile, for multibeam scanning 2PM, a confocal spinning-disk scanner has been implemented for higher temporal resolution (up to 333 frame/second). This scanner splits a single excitation beam into hundreds of foci at the focal plane through the microlens-array (MLA). The fluorescence signals at the focal plane are relayed to the image plane and captured by a two-dimensional detector. In addition, a 40–100-fold increase in imaging speed compared with a single-beam scanning 2PM has been demonstrated. Moreover, volumetric imaging approaches based on the 2PM with galvanometer-based scanning mirrors were recently proposed for elucidating neuronal computations *in vivo.*

Although the technologies for imaging over a large volume have advanced in recent years, volumetric imaging with a multibeam scanning 2PM approach using a spinning-disk or MLA instead of galvanometer-based scanning mirrors has rarely been reported. Indeed, there is a principal issue of low photon density at hundreds of foci in the multibeam scanning 2PM. However, since volumetric imaging requires the acquisition of a large number of voxels, the multibeam approach is advantageous because of the higher pixel (voxel) rate compared to the single-beam approach. For these reasons, this

study proposed a volumetric imaging system using the multibeam scanning 2PM with a spinning-disk scanner, a high-peak power excitation laser, and an electrically tunable lens (ETL) as an axial scanner for visualizing the living mouse brain.

Materials and Methods

Multibeam lateral scanning by a spinning-disk scanner

A confocal spinning-disk scanner (CSUMPΦ100, Yokogawa Electric Corp.) was employed for scanning the focal plane. The rotation speed of the spinning-disk was set up to 10,000 rpm. The excitation beam was expanded to use the entire area of the detection window and split into several hundreds of beamlets by an MLA. Each beam was focused on multiple points through the objective lens. The fluorescence emission was passed through the pinhole-array disk to block the residual out-of-focus fluorescence signals and a long-pass dichroic mirror (750-nm cutoff wavelength).

Continuous axial scanning by an ETL

Axial scanning of MCAS-2PM was implemented by adjusting the divergence angle of the excitation beam before introducing it into the objective lens. A convergence beam makes the focal spot closer to the objective lens than the nominal working distance. Inversely, a divergence incident beam makes the focal spot far from the objective lens for achieving axial scanning. An ETL enables the axial scanning based on this scheme, and is a suitable axial scanner because the light weight of the moving part results in a faster response time compared to inertia-limited z-scanners, such as a piezo objective scanner (Grewe *et al.*, 2011; Chen *et al.*, 2021). The ETL (EL-16-40-TC-VIS-5D, Optotune Switzerland AG) was placed just before the objective lens to change the divergence of the incident beam. The focal

length of the ETL was controlled using a lens driver (Lens Driver 4i, Optotune Switzerland AG) connected to the data acquisition (DAQ) board (USB-6353, National Instruments Corp.).

4D image reconstruction and visualization

A virtual 3D (*xyz*) space with uniform voxel grids was prepared on the internal memory, and a 4D (*xyz*-*t*) image file with OME-TIFF format was created on the high-speed storage of a computer. The driving voltages and corresponding timings recorded by the DAQ boards containing the information on tick counts, ETL currents, and camera triggers (i.e., elapsed time, axial positions, and frame numbers, respectively) were integrated to determine the axial range of each frame (*xy*-image). Using this information, the area of 3D space corresponding to each frame was updated frame-by-frame and the entire 3D information at the time was output as a part of the 4D image at a certain interval.

The image reconstruction code was written by Python3 and powered by the tifffile package (Gohlke, 2022) to create OME-TIFF image files. 3D and *z*-projection views were created using image analysis software (NIS-Elements AR, Nikon; ImageJ/Fiji, Schindelin *et al.*, 2012; Schneider *et al.*, 2012).

Parameters for volumetric imaging

Because the focal plane is continuously imaged by a camera and owing to the high simultaneity of lateral scanning by a spinning-disk, the imaging parameters of axial range and pitch can be configured

freely. The relational expression of the key parameters for volumetric imaging with MCAS-2PM can be written as follows:

$$f_{xy} = f_z \cdot \frac{z_{range}}{z_{pitch}},$$

where f_{xy} [Hz] denotes the frame rate, f_z [Hz] denotes the volume rate, z_{range} [m] denotes the length of the depth of view, and z_{pitch} [m] denotes the axial range for each frame (*xy*-image). Notably, $f_z \cdot z_{range}$ means that the total travel distance per second along the *z*-axis, and f_{xy} separates the total distance into each frame. Therefore, these parameters determine that z_{pitch}. f_{xy} is a constraint on the other parameters because f_{xy} is limited by the maximum frame rate of a camera or the minimum exposure time needed to detect the fluorescence.

Animals

Male and female Thy1-EYFP-H (H-line; Feng *et al.*, 2000)) and GLT-1-G-CaMP7 (G7NG817; Monai *et al.*, 2016) transgenic mice (7–12-week-old) were used for *in vivo* imaging experiments (Subsections 3.1–3.3). H-line mice that express an enhanced yellow fluorescence protein (EYFP) in the neocortical and hippocampal neurons (Porrero *et al.*, 2010) were used for the deep brain imaging of the fine cortical structures. G7NG817 mice that express a green Ca^{2+} probe G-CaMP7 in astrocytes and neurons were used for *in vivo* Ca^{2+} imaging. Male wild-type C57BL/6J mice (6–9-week-old) were used to perform *in vivo* volumetric Ca^{2+} imaging with a synthetic Ca^{2+} indicator. All mice were housed under

a 12 h/12 h light/dark cycle.

Cranial window surgery

The mice were anesthetized with 0.5%–1.5% isoflurane, and their body was warmed by a disposable heat pad during experiments. Local anesthesia with 2% xylocaine was applied to the surgical field, and their skin was incised to expose their skull. A custom-made head chamber (made from a plastic dish or a stainless-steel plate) was attached to the skull centered on the right parietal bone. To decrease the intracranial pressure and loosen the dura mater, 20% mannitol (Terumo Corp.) or glycerol (Taiyo Pharma Co., Ltd.) was administered via intraperitoneal injection (15 μL/g). About 15 min after the administration, the skull overlying the parietal lobe was partially removed for a ~4.2-mm diameter circle using a dental drill. The exposed brain was sealed by a Φ4.2-mm glass coverslip (0.17-mm thickness; Matsunami Glass Ind., Ltd.) with instant glue (Aron Alpha, Toagosei Co., Ltd.) or an ultraviolet curable resin (Luxa Flow Star, Yoshida Dental Trade Distribution Co., Ltd.).

For specific labeling of astrocytes *in vivo*, a red fluorescent dye, sulforhodamine 101 (SR101), was permeated into the brain (Nimmerjahn *et al.*, 2004). After removing the skull, 50-μM SR101 in saline was exposed to the brain surface where the dura mater remained for 10 min to avoid causing seizure-like neuronal activity (Rasmussen *et al.*, 2016).

For multicell bolus loading of the Ca^{2+} indicator, the dura mater was removed for smooth insertion of

a glass pipette, and the brain was left unsealed. After injecting the Ca^{2+} indicator into the cortex, the exposed brain area was sealed as described above.

Bolus loading of synthetic Ca^{2+} indicator

The multicell bolus loading approach (Stosiek *et al.*, 2003; Tischbirek *et al.*, 2019) was employed to introduce synthetic Ca^{2+}-sensitive acetoxymethyl (AM) dyes into the cortex. The AM ester groups of Ca^{2+}-sensitive AM dyes are cleaved from the Ca^{2+}-sensitive dye molecules by esterases inside the cell, and the dye becomes fluorescent. In this study, because of its compatibility with a 1,042 nm excitation laser source, a red-shifted Ca^{2+} indicator, Cal-590 AM, that was effectively excited by 1,050 nm (Tischbirek *et al.*, 2015) was used.

Cal-590 AM dye (50 µg, AAT Bioquest, Inc.) was dissolved in DMSO + 20% pluronic F-127 (i.e., 10-mL DMSO + 2-g pluronic F-127). The dye was dissolved to 1.5 mM in a solution containing 150-mM NaCl, 2.5-mM KCl, and 10-mM HEPES at pH 7.4. Dextran conjugated Alexa Fluor 488 (25 µM, 10,000 MW; Thermo Fisher Scientific, Inc.) was also added to the solution for fluorescence guidance during bolus loading. A glass pipette was made from borosilicate glass capillaries (GD-1.5, Narishige) using a micropipette puller (P-1000, Sutter Instrument Co.). The diameter of the pipette tip was adjusted to approximately Φ20–30 µm under the bright field microscope (SMZ25, Nikon). The glass pipette was backfilled with ~10-µL dye solution.

Dye loading was performed via conventional single-beam scanning 2PM (A1R-MP, Nikon) with 16× 0.8 NA objective (N16XLWD-PF, Nikon) and a Ti:Sa laser (MaiTai eHP, Spectra-Physics, Inc.) tuned to a 920-nm wavelength. The glass pipette was angled ~30° and slowly inserted into the neocortex at 150–300-μm depth from the surface using a 3-axis manipulator. To eject the dye from the pipette, 0.01–0.025-MPa air pressure was applied for ~6 min using a custom tool (made from a syringe and F-clamp) with a pressure meter. About 30–45 min after the injection, spontaneous Ca^{2+} activity was briefly confirmed by the same microscope setup using 1,000–1,040-nm excitation wavelength. An *in vivo* Ca^{2+} imaging experiment with MCAS-2PM was started 1 h after the dye loading, and it lasted up to 6 h.

Chirped pulse amplification

A custom-made YDFA (Kanazawa *et al.*, 2014; Kawakami *et al.*, 2015) was introduced to the excitation optical system. A pulse stretcher and compressor were designed using two pairs of diffraction gratings (reflective gratings: 25 × 25 mm^2, 1,200 grooves/mm, 900 nm blaze wavelength, 067R, Newport, Corp.; transmission gratings: 31.8 × 12.3 mm^2, 1,000 grooves/mm, 1,040 ± 20 nm designed wavelength, T-1000-1040-31.8 × 12.3-94, II-VI, Inc.). A Yb-doped double-clad fiber (Φ40-μm core diameter, 3-m length, DC-200/40-PZ-Yb, NKT Photonics) was used as a laser gain medium. A continuous wave (CW) laser diode (915-nm wavelength, 55 W, K915FA5RN-55.00W, BWT Beijing, Ltd.) was used for pumping the Yb^{3+} ions. An optical isolator (IO-5-1030-HP, Thorlabs, Inc.) was

placed before the YDFA to block the pumping light in the backward direction to protect the laser source and other devices. To construct a stretcher and compressor, the propagation distance of two diffraction gratings to introduce a group delay dispersion (GDD) was derived.

The stretcher was designed to stretch the seed light to a pulse width of ~10 ps from ~350 fs (L = 97 cm, GDD ~1,225 × 10^3 fs^2). The compressor was designed to compress the amplified light to a pulse width of ~350 fs from ~10 ps (L = 103 cm, GDD~ −1,284 × 10^3 fs^2).

Results

To assess the applicability of the multibeam scanning 2PM for *in vivo* volumetric imaging, first, the penetration depth limitation in living mouse brains was experimentally confirmed. As a result, dendritic fibers were visualized at a depth of over 300 μm. Second, in vivo multiplane Ca^{2+} imaging was performed with a piezo z-scanner, and Ca^{2+} transients were recorded at depths of 140 μm (single-plane) and 80–100 μm (tri-plane).

Next, using an ETL, continuous axial scanning mechanics was introduced to improve the proposed volumetric imaging system; this improved imaging system is called the multibeam continuous axial scanning 2PM (MCAS-2PM) system. Using the MCAS-2PM system, a 1-μm bead phantom was observed and clearly resolved in the 3D volume as a z-projection stack with negligible axial spatial gaps. *In vivo* volumetric Ca^{2+} imaging was also performed with a synthetic Ca^{2+} indicator, Cal-590 AM, in the primary visual cortex of a mouse. As a result, spontaneous Ca^{2+} transients were successfully recorded in neurons up to a 155 μm depth from the brain surface with a 200 × 200 × 36 μm^2 field of view.

Finally, to improve the brightness of the fluorescence image, a CPA system with diffraction gratings and a previously reported Yb-doped fiber amplifier were incorporated. The CPA output had an average power of 12.0 W and a pulse width of ~1 ps. It is expected that, compared with using the original excitation light source, a roughly 3-fold brighter fluorescence image can be obtained.

Discussion

In this study, the penetration depth of multibeam scanning 2PM was examined and dendritic fibers were observed at a depth of over 300 μm from the surface. This result is equivalent to or slightly better than that of a previous report using multibeam 2PM (≦300 μm; Zhang *et al.*, 2019). In the *in vivo* Ca^{2+} imaging, Ca^{2+} activities were observed at a depth of 140 μm with single-plane, 80–100 μm with tri-plane, and 120–155 μm with volume. Recent studies that performed multibeam one-photon Ca^{2+} imaging with a confocal spinning-disk scanner reported penetration depths of 160 μm (Iwasaki & Ikegaya, 2018) and 120 μm (Yoshida *et al.*, 2018). These results indicate that although continuous axial scanning was implemented in MCAS-2PM, it is still can be improved. To increase the penetration depth of Ca^{2+} imaging up to 200–300 μm or the mouse cortical layer 2/3, a higher peak power of excitation laser pulses is required. The excitation laser source with a low repetition rate might be effective in multibeam scanning 2PM. Nevertheless, there are several techniques to enhance the imaging system and specimen, such as optimizing the detection system, efficiency of illumination, and localization of fluorescence probe.

The continuous axial scanning approach arose from a simple idea that reduces the dead time occurring in axial scanning, which is generally adapted for multiplane imaging. Multiplane volumetric imaging is frequently implemented by repeating two steps: 1) depth-to-depth shift of the focal plane and 2) capturing the *xy*-image while the focal plane is stationary. However, such an approach costs the settling time for the damped oscillation of the focal plane in a few tens of milliseconds with a piezo *z*-scanner

or ETL; hence, the frame rate is limited (Han *et al.*, 2019). In this study, the continuous axial scanning approach enabled the scanning of a 3D volume with no settling time. Notably, 3D volume was reconstructed as a *z*-projection stack with negligible axial spatial gaps. This feature enables the observation of all neurons in a specific region of the brain.

In conclusion, a multibeam scanning 2PM-based volumetric imaging system for observing living mouse brains is proposed. To realize 3D scanning, lateral scanning with a spinning-disk and continuous axial scanning with an ETL were combined. With the proposed system, *in vivo* volumetric Ca^{2+} imaging was performed in living mouse brains up to a 155 μm depth from the surface. With further improvements, the proposed system can be a practical volumetric imaging system with a simple design and efficient scanning capability. Such features may make volumetric imaging widespread and thereby open new neuroscience pathways for many researchers. For example, the cortical representation of perceptual and cognitive states of the behaving animals can be elucidated by visualizing a set of neurons, or ensembles, that are coordinately active within a large 3D volume.