Plug-and-play adaptive optics for two photon high-speed volumetric imaging

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Abstract

To understand brain functions, it is important to study functional connectivity among stereoscopically distributed neurons. Since the brain is composed of 3D neuron networks, volumetric imaging with high spatiotemporal resolution is highly desirable. Two-photon microscopy (2PM) conveniently offers 3D tissue imaging with sub-micrometer resolution based on its intrinsic optical sectioning and deep penetration capabilities. However, the main challenge lies in the volumetric imaging speed and contrast reduction in deep tissue due to aberration. In this study, we integrate a tunable acoustic gradient lens and a plug-and-play adaptive-optics lens into 2PM. The former provides ~100 kHz axial scan rate, achieving volumetric imaging rate in 1–10 Hz range, while the latter enhances image contrast by nearly two-fold in deep brain regions via correcting both systematic and sample aberrations. The combination offers a practical approach toward high-speed, high-contrast optical volumetric imaging of brain tissues.

1. Introduction

The amalgamation of optical microscopy and neuroscience has brought new insights into how the brain functions over the past decade [1, 2]. When considering optical microscopy, there are four fundamental parameters, namely contrast, resolution, speed, and imaging depth [3]. To enable observation of neuronal activities in a living brain, we need calcium or voltage sensitive indicators as functional contrast sources, at least subcellular spatial resolution to clearly resolve the neurons, sub-second to millisecond temporal resolution to capture in vivo neuron ionic or action potential activities, and enough penetration capability to allow 3D volumetric visualization of neuron network communications.

For thick-tissue volumetric observation, two-photon microscopy (2PM) has been widely used in recent years. The main advantages of 2PM [4] are intrinsic optical sectioning ability and deep penetration compared to single photon microscopy. Optical sectioning is due to its nonlinear excitation nature, which is beneficial for reaching high contrast in thick samples. Deep penetration depth is because 2PM typically utilizes near-infrared light, which is less affected by tissue scattering than visible light in biological tissue [5]. Tight focusing is often required for efficient two-photon excitation, so 2PM naturally provides sub-micrometer spatial resolution. Nevertheless, in most cases, 2PM is based on a point scan geometry, thus significantly limiting the imaging speed. Various high speed volumetric 2PM techniques have been developed recently, including light sheet [6], wide-field [7, 8], spatio-temporal multiplexing [9–12], and inertia-free high-speed axial scanning [13–16].

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Full-sampling techniques such as light-sheet and wide-field two-photon imaging enable high-throughput imaging, but require camera-based detection, which suffers from crosstalk in scattering tissues. Spatially multiplexed systems, such as patterned illumination via spatial light modulator or Bessel beam, improve scanning speed by increasing the number of foci or length of focus to reach high volumetric acquisition and a large volume size, but might suffer from crosstalk between fluorescence signals from individual foci or axial planes. Spatiotemporally multiplexed system thus introduces time delay in each foci to avoid crosstalk and has achieved a 30 Hz volumetric imaging rate [10]. However, current spatiotemporal multiplexing is mostly sparse sampling techniques and is susceptible to sample movement. For inertia-free high-speed axial scanning techniques, electrically and acoustically tunable lenses are the main choices. The frequency range of the former is typically below 1 kHz, and of the latter is much higher. Here we adopt a tunable acoustic gradient (TAG) lens which offers full axial sampling at frequency above 100 kHz, which potentially enables millisecond temporal resolution [15, 17–19], as our volumetric imaging strategy.

Having tackled the issues of resolution, depth, and speed with 2PM, the remaining challenge is to improve contrast in a thick brain sample, which induces significant aberration from tissue inhomogeneity. To enhance deep-tissue contrast, combining 2PM with adaptive optics (AO) is one of the major strategies [20–23]. AO techniques are generally divided into direct wavefront sensing and sensorless methods. The speed of AO correction in direct wavefront sensing [24] is usually faster; however, the light path is complicated compared to sensorless methods and it typically requires a 'guide star' inside the biological sample. In recent years, a significant amount of efforts have been placed toward sensorless AO, which requires only a wavefront shaping instrument, i.e. a deformable mirror [25], a spatial light modulator (SLM) [26, 27], or digital micromirror device [28]. Based on iteratively manipulating the wavefront, not only the image sharpness is maximized, but the tissue-induced aberrations are quantitatively backward acquired. However, current volumetric 2PM combining sensorless AO are mostly either random-access scanning [28] or multiplane scanning [29], which is not able to offer full sampling to cover the whole specimen. In addition, the conventional AO components need a long relay beam path, thus significantly increasing the space requirement when integrated with a microscope.

In this study, we combined a plug-and-play AO lens with a TAG lens into a 2PM setup, to demonstrate contrast enhancement for full volume sampling. The AO lens is designed to be conveniently installed at the objective nosepiece directly [30], thus significantly reducing the space requirement and installation complexity. The TAG lens exhibits a resonant frequency of nearly 200 kHz. Via combination with a set of galvo mirrors and a high-speed electronic data acquisition system, 1–10 Hz volumetric imaging rate is demonstrated. Our results indicate that the TAG + AO (TAO) combination effectively corrects both system aberration due to optical alignment and specimen aberration due to tissue inhomogeneity, allowing significant contrast enhancement during sub-second volumetric imaging. The TAO 2PM would be an ideal tool for studying 3D neuronal dynamics toward understanding the function of the brain.

2. Materials and methods

2.1. Microscope setup

The high-speed volumetric TAO 2PM system consists of a home-built two-photon microscope, a TAG lens, and an AO lens, as shown in figure 1. A tunable femtosecond Ti:Sapphire laser (Chameleon Vision II, Coherent) was used as a light source for two-photon excitation at 940 nm. The laser beam was sent vertically up to the TAG lens (TAG lens 2.5β, TAG Optics, dioptic power ranging from $+2$ to $-2$ m$^{-1}$), through a beam expander lens pair (AC254-060-B-ML & AC254-040-B-ML, Thorlabs), and was relayed onto a set of galvo scanners (6215 H, Cambridge Technology) to achieve raster scan. The raster scan pattern went through a scan lens (SL50-CLS2, Thorlabs) and a tube lens (TL200-CLS2, Thorlabs), and transferred onto a plug-and-play AO lens (Dynamic Optics srl, Italy) (see section 2.2) that was attached onto an objective (XLPLN25XMP2, Olympus). The scanned laser beam was focused on the mouse brain slice to excite two-photon fluorescence, which was epi-collected by the same objective, and was extracted via a dichroic beamsplitter (FF573-Di01, Semrock) and a bandpass filter (FF01-520/15-25, Semrock) to a photomultiplier tube (PMT) module (H7422A-40, Hamamatsu). More details on the AO lens and the TAG lens are given in the subsequent sections.

2.2. Adaptive lens and wavefront correction

The main component of the AO setup was a transmissive multi-actuator adaptive lens (MAL, AOL1810, Dynamic Optics srl) capable of correcting aberrations up to 4th radial order Zernike polynomial; the MAL design and operation has been fully described [31]. The main benefit of using a transmissive element to correct for wavefront aberrations is the possibility to place it directly in the back aperture pupil of the objective lens thus avoiding the need of extra optical relay.

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The wavefront correction has been performed using a sensorless modal approach. For the mathematical representation of the wavefront, we chose the Zernike polynomial basis. This of course was done for convenience since the deformable lens has a circular domain. For each Zernike polynomial till the fourth order, with the exception of piston, tip, tilt and defocus, one positive and one negative bias were induced and an image-based sharpness metric was computed for each offset. The sharpness metric used was the variance of the considered sample region. We then approached the aberration estimation with a model-based algorithm where we expect that near its maximum the sharpness can be approximated by a parabola and with a fit, we can estimate the corrected mode amplitude. This procedure was repeated in sequence for all the aberrations up to the 4th order till we saw no further increase in the merit function.

With respect to the algorithm used in [32], this algorithm is faster and more robust. In fact, the main issue to assess during the correction process is the hysteresis of the piezoelectric actuators (about 10%). This effect is mitigated in [32] by using a ‘de gauss’ operation for both the scan directions of each Zernike mode and a total of 10 images are acquired for every Zernike scan (five images for the positive bias scan and five images for the negative bias). The de gauss operations take 6 s, so, assuming 1.3 s for each acquisition, we have a total of 19 s for scanning each mode. The algorithm used here instead has a different approach: for each Zernike mode we just acquire three images (one with positive bias and one negative), and after all Zernike modes are scanned, we iterate the procedure until the merit function stops increasing. Usually, three iterations are enough, so globally we need 11.7 s for each Zernike. Moreover, we observed that the iterative process tends to obtain more reliable results and to be more robust to any variation of the image. To avoid the saturation in case of high aberration magnitude the actuators commands are generated using a linear least-squares solver with bounds given by the maximum actuator’s voltages.

2.3. Data streaming and volumetric image reconstruction

The electronic processing and image reconstruction of volumetric 2PM based on the TAG lens has been reported [17]. Briefly, the PMT output signal was first amplified with 50 dB current-to-voltage gain (C6438-01, Hamamatsu), subsequently converted into digital signals through a digitizer (PXIe-5122, National Instruments), and then streamed to an embedded hard drive (PXIe-8840, National Instruments). To reconstruct 3D volumetric images, spatial coordinates were determined by the trigger Transistor-Transistor Logic (TTL) signals of the TAG lens (z-axis) and the galvo scanners (xy), which were synchronized, i.e. each axial scan of the TAG lens was equal to one galvo pixel dwell time. Between two TTL triggers (188 kHz, the TAG lens driving frequency), the laser focus scanned axially twice. With a 100 MHz data acquisition (DAQ) (PXIe-5122, National Instruments), the maximal number of pixels in the z-direction would be ~5320 data points. Considering the axial optical resolution and to improve the signal-to-noise ratio, we resampled them to 53 voxels (customized software, Southport, Taiwan).

For volume rate calculation, since one pixel dwell time is 1/188 k second, the volumetric imaging speed thus depends on the number of pixels in the xy plane. For example, 128 × 128 pixels requires $128^2/188 000 = 0.09$ s, and 512 × 512 pixels requires $512^2/188 000 = 1.39$ s. In most of the images below, we
use $512 \times 512$ pixels to analyze the pixel-based aberration correction. With the aid of a custom MATLAB program, a volumetric image with $512 \times 512 \times 266$ voxels at rate 0.72 Hz could be reconstructed.

2.4. Mouse sample preparation

Adult male Thy1-GFP (Jackson Laboratory, #007788) and Pcp2-GCaMP6f mice of eight- to ten-weeks age were used in this study. The Pcp2-GCaMP6f mice were generated by crossing Pcp2-cre (Jackson Laboratory, #004146) female mice to Ai148D male mice (Jackson Laboratory, #030328). The mice were housed with a 12 h light/12 h dark cycle, and fed with an ad libitum food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee of National Taiwan University (approval number: NTU110-EL-00023).

The mice were euthanized with 5% isoflurane in a chamber for 5 min via an anesthetic vaporizer to induce death. After the breath had stopped, the mice were transcardially perfused with 30 ml phosphate-buffered saline and followed with 30 ml 4% paraformaldehyde. When the perfusion was completed, the brain was dissected and postfixed in 4% paraformaldehyde for two days at 4°C. The brain was cut into half from the midline and mounted on the stage with super glue. A vibratome was used to cut the brain into 500 µm sagittal sections. The sections were immersed in RapiClear solution (SunJin Lab Co., Taiwan) overnight at room temperature. The clarified brain sections were mounted on a chamber composed of 0.5 mm plastic spacer and cover glass. For volumetric image demonstration, the dense Purkinje cells in the Pcp2-GCaMP6f mice were adopted, while for AO correction, we used the Thy1-GFP mice to quantitatively analyze the contrast enhancement among soma and fibers.

3. Results

In the following we first demonstrate the feasibility of the high-speed volumetric 2PM system by applying it on a mouse brain sample. Next, we combine the system with a plug-and-play AO lens, and demonstrate TAO 2PM observation of superficial and deep regions, where optical system aberration and sample aberration play major roles, respectively.

3.1. Volumetric imaging of TAG lens

Figure 2 shows two types of two-photon volumetric imaging; (b)–(e) are slow-scan results acquired with sample holder translation, while (f)–(i) are fast-scan images by adding a TAG lens. The samples are fixed Pcp2-GCaMP6f mouse brain slices, showing the neuronal architecture within the cerebellar lobe.

Figure 2(b) is the z-projection of the slow-scan 3D image stack which covers a $400 \times 400 \times 80$ µm³ volume. Figures 2(c)–(e) are representative layers in the stack, corresponding to 0 µm, 40 µm, 80 µm depths, respectively. The arrows point out the matching neurons between figures 2(b) and 2(c)–(e). Here the voxel dwell time is 5 µs, each 2D image contains $512 \times 512$ voxels, and there are 80 frames in the stack, so the total acquisition time of the whole 3D stack is 105 s.

Figure 2(f) is the fast volumetric image which covers the same 3D region of figure 2(b), but acquired with the TAG lens on, whose resonant frequency is 188 kHz, corresponding to 5.3 µs per axial line scan, and voxel dwell time becomes 5.3/53 = 100 ns. With $512 \times 512$ pixels in the lateral 2D scan, the volumetric acquisition time is 1.39 s, which is two orders faster than that of figure 2(b). Figures 2(g)–(i) are the representative layer images extracted from figure 2(f), and the arrows point out the matching neuron. Note that the laser power used in figures 2(f)–(i) was 60.8 mW, which is about three times stronger than the 21.8 mW used in figures 2(b)–(e), and these figures have been normalized to enhance visibility. We observe that the contrast of the image decreases in figures 2(f)–(i) compared to figures 2(b)–(e), as a typical tradeoff for high-speed imaging. Nevertheless, all soma and major fiber distributions are still recognizable in the fast scan images, manifesting the potential of this high-speed platform for studying neuroscience.

3.2. Volumetric aberration correction in superficial region (0–20 µm)

Figure 3 shows the effect of adding the AO lens into the volumetric 2PM. Images of a fixed Thy1-GFP mouse brain slice were acquired at the surface, and the field of view (FOV) of the image is ~500 × 500 (µm) at 512 by 512 pixel resolution. In figure 2, the 100 µm depth z-extension causes overlapping of the neurons and fibers. To clearly demonstrate the ability of the TAO volumetric imaging system, in figure 3 we choose a smaller z-extension of 20 µm. Figures 3(a) and (b) are the results before and after AO correction. It is obvious that the 2PM intensity is enhanced in the latter. To quantify the aberration source, figure 3(c) presents the Zernike mode analysis of AO lens correction, manifesting that astigmatism and trefoil are dominant terms. Since the imaging depth is shallow, the aberrations might mainly come from system misalignment. In figures 3(d) and (e) we quantitatively analyze the intensity enhancement of several randomly selected neurons and fibers in figures 3(a) and (b). The intensity enhancement was 55% for neurons and 31% for
Figure 2. Volumetric two-photon imaging of pcp2-GCaMP6 labeled neurons in a fixed mouse brain tissue. (a) Schematic of the comparison between slow scan and fast scan (b) Slow-scan 3D image acquired with sample holder translation. (c)–(e) Individual z-planes from the region shown in (b) at the depth of 0 \( \mu m \) (c), 50 \( \mu m \) (d) and 100 \( \mu m \) (e), respectively. Arrows mark selected cell bodies that appear at these depths (f) Fast-scan 3D image acquired with the TAG lens. (g)–(i) Individual z-planes from the region shown in (f) at 0 \( \mu m \), 50 \( \mu m \) and 100 \( \mu m \) depths.

Figure 3. Aberration correction effect of the TAO 2PM system in the superficial region (0–20 \( \mu m \) depth). (a), (b) High speed volumetric image before correction (a), and after correction (b). (c) The reconstructed Zernike aberration modes. (d), (e) Examples of quantitative fluorescence intensity improvement of neurons (d) and fibers (e), which correspond to the N line and F lines in (a) and (b), respectively. (f) Quantitative contrast enhancement of the whole image. The brain sample was from a Thy1-GFP mouse.

fibers. Figure 3(f) is the overall analysis by subtracting the intensity of figure 3(a) from figure 3(b) then dividing over figure 3(a), i.e. \( \Delta F/F \), revealing the distribution of intensity enhancement in the whole FOV. The analysis shows that the \( \Delta F/F \) intensity enhancements are 20%–60% for most of the regions and 40% on average, manifesting the effectiveness of the plug-and-play AO in correcting the optical system aberrations.

3.3. Volumetric aberration correction in deep region (270–290 \( \mu m \))

Figure 4 is the counterpart image of the same mouse brain slice in the deep region, approximately 270 \( \mu m \) under the surface. We choose the same volume size, i.e. \( 500 \times 500 \times 20 \mu m^3 \), with figure 3 for comparison. Figures 4(a) and (b) shows the volumetric image before and after AO correction, manifesting significant
Figure 4. Aberration correction effect of the AO + TAG system in the deep region (270–290 µm depth). (a), (b) High speed volumetric image before correction (a), and after correction (b). (c) The reconstructed Zernike aberration modes. (d), (e) Examples of quantitative fluorescence intensity improvement of neurons (d) and fibers (e), which correspond to N line and F line in (a), (b) in (a), (b), respectively. (f) Quantitative contrast enhancement of the whole image. The brain sample was from a Thy1-GFP mouse.

contrast enhancement in the deep brain region. The measured Zernike mode of the correction is given in figure 4(c), and it shows that the aberration was mostly concentrated on two terms, i.e. oblique and vertical astigmatism. Comparing the superficial aberrations in figure 3(c) to deep region aberrations in figure 4(c), oblique astigmatism flips from negative to positive, vertical astigmatism emerges as the dominant term, and vertical trefoil vanishes. The difference might come from the non-uniform tissue distribution in the brain sample. To quantify the AO enhancement, we highlighted some neurons and fibers in figures 4(d) and (e). The intensity has increased 140% for neurons and 150% for fibers. Then, we performed an overall analysis of the intensity enhancement in figure 4(f), manifesting that the intensity has increased in the range of 20%–150% for most of the region and 62% on average. Combining the results of figures 3(f) and 4(f), the AO lens can correct both system and sample aberrations.

4. Discussion

In this study, we construct a TAO 2PM by combining a TAG lens and a plug-and-play AO lens. The TAG lens provides ~100 kHz axial scan rate, achieving volumetric imaging rate in the 1–10 Hz range, while the AO lens significantly enhances image contrast via correcting both systematic and sample aberrations. In figure 2, we have shown the volumetric two-photon images of pcp2-GCaMP6 labeled neurons in a fixed brain tissue with ~1 Hz volume rate and 400 × 400 × 100 µm³ in size, and we are able to separate neurons at different depths. Compared to Bessel-beam volumetric imaging, which is more appropriate in sparse samples, the dense distribution of Purkinje cells in cerebellum requires TAG lens 2PM to distinguish individual layers in the axial direction. In figures 3 and 4, we demonstrate volumetric imaging with aberration correction in superficial and deep regions of Thy1-GFP mouse brain slice, respectively, with peak fluorescence intensity enhancement above 100e%. On average, the enhancement is more pronounced in deep regions, validating the potential of brain tissue imaging.

In figure 4(f), it is interesting to note that the enhancement was not uniform in the whole FOV, where the white rectangle region enhances far more than the others. One potential reason is that different regions in the deep sample may exhibit various aberrations, and the AO algorithm tends to maximize overall image variance. Therefore, the blind iteration of the sensorless AO system might not be able to compensate for all aberrations in a large area image simultaneously, resulting in the non-uniform contrast enhancement. The resulting non-uniform contrast enhancement had also been reported in other literature [33], and further research efforts are required to avoid the potential problem in functional live brain imaging.
When considering volumetric imaging and aberration correction, there are several different strategies to set a reference plane. Potential candidates are to monitor the contrast enhancement at only the central plane \[34\], at multiple planes \[26\], or at whole volume projection \[35\]. The advantage of the single-plane or multi-plane methods is to achieve optimal contrast enhancement in selected depths. However, it is suboptimal for aberration correction of the whole volume, and suffers from lower temporal resolution with increasing number of planes. On the contrary, AO correction based on whole volume projection, such as our work here or Bessel beam techniques, offers better volumetric correction and temporal resolution, though it may not be optimal for each layer. The volumetric projection strategy works best in sparse samples, to avoid signal overlapping during projection. Here we choose to use 20 µm z-extension, which may be further extended to 60 µm based on the recent results of Bessel beam aberration corrections. Note that compared to Bessel beams that acquire projected images, the TAG lens offers 3D full sampling, so it is possible to reinforce the sensorless AO algorithm to assess the 3D images directly and maximize the image sharpness across the whole volume.

An interesting future technical exploration is to use both AO lens and TAG lens as plug-and-play components \[15\], i.e. to place both lenses directly before the objective. In our current design, the TAG lens is placed in a conjugate plane away from the objective lens because its effective aperture is only 3 mm when operating at 188 kHz, while the objective back aperture is much larger. Other TAG lens designs that offer larger effective aperture may be adopted to enable plug-and-play function. Nevertheless, the main challenge is to determine where to place the conjugate back-aperture plane. In a conventional laser scanning microscope, the conjugate plane is located at the back aperture of an objective. In our current TAO 2PM system, the AO lens aperture is 1 cm, smaller than the objective aperture, so the conjugate plane is located at the back aperture of the AO lens to maximize the transmission efficiency. It would be an interesting experiment to understand what is the optimal arrangement in a serial AO-TAG-objective lens combination.

In summary, high-speed volumetric optical microscopy that offers subcellular spatial resolution and sub-second temporal resolution is an indispensable tool to unravel the 3D functional connections among neurons and toward understanding the function of the brain. Here we demonstrated a TAO 2PM to not only enhance volumetric imaging speed through a TAG lens, but also improve contrast via a plug-and-play AO lens in both superficial and deep brain tissues. This work opens up future potentials of true 3D volumetric aberration correction as well as convenient 2PM expansion. We envision that the continuous technical explorations in this direction shall enable a practical tool toward high-speed, high-contrast optical volumetric mapping of brain functional connectomics.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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