Large-field-of-view imaging by multi-pupil adaptive optics

Jung-Hoon Park1,5,6, Lingjie Kong1,5,6, Yifeng Zhou1 & Meng Cui1-4

Adaptive optics can correct for optical aberrations. We developed multi-pupil adaptive optics (MPAO), which enables simultaneous wavefront correction over a field of view of 450 × 450 μm² and expands the correction area to nine times that of conventional methods. MPAO’s ability to perform spatially independent wavefront control further enables 3D nonplanar imaging. We applied MPAO to in vivo structural and functional imaging in the mouse brain.

The refractive index inhomogeneity within biology tissue makes in vivo optical imaging challenging1-3. Adaptive optics (AO) has been used to correct the optical wavefront distortion caused by this inhomogeneity1,4-14. However, previous AO implementations exhibit a constrained field of view (FOV) or reduced imaging speed across larger areas, as wavefront distortion varies spatially, and wavefront correction has to be updated accordingly7,8,11,15,16. Thus a wavefront correction that works well in one location may fail to improve or sometimes degrade the image quality for another region in the sample. When imaging slow dynamics, researchers can afford the time-consuming procedure of switching the wavefront to image multiple small FOVs sequentially11,13. However, this method may be ineffective in applications with faster dynamics (Supplementary Note 1). Approaches that provide simultaneous wavefront correction over a large FOV and hence enable imaging at a speed limited only by the imaging hardware are needed. Here, we present one such solution, MPAO, and demonstrate its performance for in vivo imaging of the mammalian brain.

At the core of MPAO is simultaneous position-dependent wavefront correction. In conventional AO, a single spatial light modulator (SLM) is placed on the pupil plane of the objective lens, and the wavefront correction measured from one region is applied to the entire image (Fig. 1a); this, however, improves imaging performance only within a limited FOV. If the wavefront correction is based on averaging aberration over a larger region, the resulting correction is incomplete throughout this larger region (Fig. 1b). In MPAO, we position a multifacet prism array on the sample plane; this prism applies angular tilt to the optical beam (Fig. 1c). Because of the Fourier transform property of the lenses, angular tilt at the sample plane becomes pupil image translation. Thus, we construct an array of pupil images (plane 2 in Fig. 1c), each corresponding to one region on the sample plane. We use an SLM to display a corresponding array of wavefront profiles and achieve simultaneous independent correction for all regions. Using a second prism array of complementary angular tilt, we cancel the applied tilt and obtain a fully corrected image.

We implemented MPAO with two-photon microscopy (TPM) by using a nine-facet prism array forming a 3 × 3 array of pupil images on an SLM (Supplementary Fig. 1). Each prism segment corresponded to a 150 × 150 μm² area, and thus the 3 × 3 prism array provided a 450 × 450 μm² FOV (Supplementary Fig. 2). The prism segment’s dimension was determined experimentally to provide a compromise between FOV and correction quality (Supplementary Fig. 3 and Supplementary Note 2). Before imaging, we employed Zernike-mode-based optimization to determine the correction wavefront1,8 (see Online Methods). Using MPAO, we measured the wavefront for all nine regions simultaneously and then performed resonant Galvo-based TPM.

In our first application of MPAO for in vivo mammalian brain imaging, we imaged microglia dynamics. For the wavefront measurement, we used the fluorescence of GFP in the microglia of CX3CR1–GFP mice as the feedback. We compared the images obtained by either correcting only the system-induced aberration (system correction, Fig. 1d) or by correcting both the system- and the tissue-induced aberration (full correction, Fig. 1e and Supplementary Fig. 4). The independent and parallel full correction (Fig. 1f) improved both image brightness and resolution (Fig. 1g–j). To quantify the resolution, we measured the cross-section of the microglia’s fine processes (Fig. 1j), whose full width at half maximum was 0.54 ± 0.20 μm (n = 40, mean ± s.d.) with system correction and 0.36 ± 0.02 μm (n = 40) with full correction, close to the theoretical limit (0.34 μm for 935-nm laser and 1.04 excitation NA).

We compared the image qualities obtained with MPAO with the two implementations of conventional AO shown in Figure 1a,b. In one implementation (named ‘full single’), we used the image within one of the nine segments of MPAO for wavefront measurement and applied the same correction to the other eight segments. The wavefront was identical to the one employed by MPAO for the same segment and provided improvement only within a limited FOV (Supplementary Fig. 4 and Supplementary Video 1).

1School of Electrical and Computer Engineering, Purdue University, West Lafayette, Indiana, USA. 2Department of Biological Sciences, Purdue University, West Lafayette, Indiana, USA. 3Integrated Imaging Cluster, Purdue University, West Lafayette, Indiana, USA. 4Bindley Bioscience Center, Purdue University, West Lafayette, Indiana, USA. 5Present addresses: J.-H.P (Department of Biomedical Engineering, Ulsan National Institute of Science and Technology, Ulsan, Republic of Korea) and L.K. (Department of Precision Instrument, Tsinghua University, Beijing, China. 6These authors contributed equally to this work. Correspondence should be addressed to M.C. (mengcui@purdue.edu).

RECEIVED 4 DECEMBER 2016; ACCEPTED 5 APRIL 2017; PUBLISHED ONLINE 8 MAY 2017; DOI:10.1038/NMETH.4290

NATURE METHODS | VOL. 14 NO. 6 | JUNE 2017 | 581
In the other implementation (named ‘full average’), we used the image over the entire FOV as the feedback to optimize the wavefront. Compared with the individually optimized wavefront in MPAO, the wavefront obtained in this implementation was smoother (Fig. 1f); however, this smoother wavefront provided only minor improvement in image quality (Supplementary Fig. 4, Supplementary Video 1, and Supplementary Note 3).

Next, we demonstrated volumetric imaging of microglia dynamics during resting (Supplementary Fig. 5 and Supplementary Video 2) and activated cellular states. To activate microglia, we introduced tissue damage by laser ablation and monitored microglia cells’ response by time-lapse volumetric imaging, which revealed coordinated movement of the fine processes around the damage site (Fig. 1k, Supplementary Fig. 6, and Supplementary Video 3).

The plasticity of dendritic spines is the foundation for memory and learning. High-resolution in vivo imaging of dendritic spines is therefore important in neuroscience. On account of aberration induced by brain tissue, most studies are constrained to imaging at depths of 200–300 µm. Using MPAO, we can perform large-FOV imaging at a depth of up to ~650 µm. We employed Thy1–YFP mice which express yellow fluorescence protein (YFP) in a subset of layer 5 neurons. Using MPAO, we recorded volumetric images of the neuronal network (Supplementary Fig. 7 and Supplementary Video 4). We showed the maximum intensity projections from two stacks, each of 50-µm thickness, and zoomed-in images of the dendritic structures resolved by MPAO (Supplementary Fig. 7b,c; centered at the depth of 75 µm (panel b) and 325 µm (panel c)). When we applied the correction wavefront optimized for one MPAO region to all other regions, the FOV with improved image quality was reduced even at moderate depth (Supplementary Fig. 8). With full correction, we resolved spines on the basal dendrites of layer 5 neurons at large depths (623 µm in Supplementary Fig. 7d and 653 µm in Supplementary Fig. 9)—these spines were blurrier with only system correction applied.

Calcium imaging has been widely employed for large-scale recording of neuronal activity. Here we applied MPAO to image calcium activity in Thy1–GCaMP6s (line GP 4.3) mice, which express calcium indicator GCaMP6s in subsets of neurons. We monitored the spontaneous neuron activity in the primary visual cortex. As the fluorescence from GCaMP inherently fluctuates on account of spontaneous neuron activity, we introduced a stable calcium imaging and system correction (Fig. 2i, Supplementary Video 4).
Supplementary Note 4

Supplementary Fig. 11). With the mice under mild anesthesia, we observed synchronized dilation of the vasculature network at 7.65 Hz (Supplementary Fig. 12). To perform wavefront correction using the YFP-expressing neural dendrites, we shifted the focal planes and corrected tissue-induced aberration. Rad, radian. (e) Synchronized dilation of three blood vessels at different depths. Their corresponding positions are marked in c, with the dotted lines indicating the position of measurement. Vessel III is a vertically penetrating vessel originating from vessel II as shown by the volumetric view in a. Scale bars, 50 µm. The data shown in this figure are representative of three experiments.

red fluorescence signal for wavefront measurement by staining astrocytes with SR101, which can be excited at the same wavelength (935 nm) as GCaMP. After the wavefront measurement, we performed calcium imaging at a 15-Hz frame rate. For comparison, we show the imaging of neurons and astrocytes both with full and system wavefront correction (Fig. 2). With full correction, the fine processes of astrocytes (Fig. 2a–d) and neurites (Fig. 2f) are resolved. We extracted the calcium transients from several astrocytes with SR101, which can be excited at the same wave length (935 nm) as GCaMP. After the wavefront measurement, we performed wavefront correction using the YFP-expressing neural dendrites. (d) Wavefront applied to the pupil array, which shifted the focal planes and corrected tissue-induced aberration. Rad, radian. (e) Synchronized dilation of three blood vessels at different depths. Their corresponding positions are marked in c, with the dotted lines indicating the position of measurement. Vessel III is a vertically penetrating vessel originating from vessel II as shown by the volumetric view in a. Scale bars, 50 µm. The data shown in this figure are representative of three experiments.

The independent wavefront control also enables nonplanar microscopy. We believe that simultaneous large-FOV correction will be helpful for high-spatiotemporal-resolution measurements in various biological systems (Supplementary Note 4).

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

**ACKNOWLEDGMENTS**

This work was funded by NIH grant no. 1U01NS094341-01 and Purdue University. The authors thank W. Gan for valuable discussion and advice; G. Holton and B. Wei for their help on manuscript preparation; and the Howard Hughes Medical Institute for equipment support. J.-H.P. thanks the NRF (grant no. 2016R1C1B2015130) for support during the manuscript preparation; and L.K. thanks the NSFC (grant no. 61327902) for support during manuscript revision.

**AUTHOR CONTRIBUTIONS**

M.C. invented MPAO, developed the experimental schemes of wavefront correction and nonplanar imaging, designed the liquid-immersion-based tunable prism array, and supervised the project. J.-H.P. and M.C. designed the MPAO-based two-photon imaging system. J.-H.P. developed the wavefront measurement and correction algorithm, implemented the imaging system, and performed structural imaging of neurons and dynamic imaging of microglia. L.K. designed and performed structural imaging and calcium imaging of neurons, dynamic imaging of microglia, and nonplanar imaging of blood vessels and neurons (data shown in Figs. 1–3 and Supplementary Figs. 6–9, 12). Y.Z. supported the system development, performed structural imaging of neurons and microglia, and assisted with figure preparation. M.C. and L.K. wrote the manuscript with input from all authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Booth, M.J., Neil, M.A.A., Juskaits, R. & Wilson, T. Proc. Natl. Acad. Sci. USA 99, 5788–5792 (2002).
2. Vellekoop, I.M. & Mosk, A.P. Opt. Lett. 32, 2309–2311 (2007).
3. Popoff, S., Lerosey, G., Fink, M., Boccara, A.C. & Gigan, S. Nat. Commun. 1, 81 (2010).
4. Roorda, A. & Williams, D.R. Nature 397, 520–522 (1999).
5. Paterson, C., Munro, I. & Dainty, J. Opt. Express 6, 175–185 (2000).
6. Marsh, P., Burns, D. & Girkin, J. Opt. Express 11, 1125–1130 (2003).
7. Kam, Z., Kner, P., Agard, D. & Sedat, J.W. J. Microsc. 226, 33–42 (2007).
8. Zeng, J., Mahou, P., Schanne-Klein, M.-C., Beaurepaire, E. & Debarré, D. Biomed. Opt. Express 3, 1898–1913 (2012).
9. Jäätelä, M., Logg, D.C. & Betzig, E. Nat. Methods 7, 141–147 (2010).
10. Tao, X. et al. Opt. Lett. 36, 3389–3391 (2011).
11. Tang, J., Germain, R.N. & Cui, M. Proc. Natl. Acad. Sci. USA 109, 8436–8439 (2012).
12. Adie, S.G., Graf, B.W., Ahmad, A., Carney, P.S. & Boppart, S.A. Proc. Natl. Acad. Sci. USA 109, 7175–7180 (2012).
13. Wang, K. et al. Nat. Methods 11, 625–628 (2014).
14. Cizmár, T. & Dholakia, K. Nat. Commun. 3, 1027 (2012).
15. Wang, K. et al. Proc. Natl. Acad. Sci. USA 109, 9236–9241 (2015).
16. Mertz, J., Paudel, H. & Bifano, T.G. Appl. Opt. 54, 3498–3506 (2015).
17. Yasuda, R. et al. STKE 2004, p5 (2004).
ONLINE METHODS

System design. We designed the imaging system by combining MPAO with TPM (Supplementary Fig. 1). The laser source was a tunable femtosecond laser (Coherent Chameleon Vision and Discovery) with built-in dispersion compensation capability. The excitation wavelength was 935 nm. We expanded the laser beam to overfill the 5-mm aperture of the dual-axis resonant Galvo scanning mirror system (Cambridge Technology) and aligned the polarization along the working axis of the SLM (Hamamatsu, X10468-07). We used a pair of telecentric relay lenses (focal length 150 and 110 mm) in a 4-f configuration to image the Galvo mirror onto the SLM. In the common focal plane of the two relay lenses, we positioned the first prism array, which established nine (3 × 3) pupil images on the SLM. With the 5 mm aperture resonant Galvo scanner, the size of each pupil image was 3.67 mm in diameter on the SLM (183 SLM pixels across the pupil diameter). It is worth noting that the prism array did not split the pupil into nine images. Instead, there was only a single pupil image on the SLM for any point on the sample plane. The second pair of telecentric relay lenses (focal length 110 and 500 mm) imaged the SLM onto the back focal plane of the objective lens (Nikon 25× NA 1.1). We also positioned a second prism array that offered complementary angular tilt at the common focal plane of the second relay lens pair. The complementary prism array canceled the angular tilt applied by the first prism array and recombined the 3 × 3 pupil array back into a single pupil image (16.7 mm in diameter).

Prism design. The key component of MPAO is the element that can apply angular tilt to the light beam, which can be achieved by either reflection, such as by a multifacet mirror ball, or transmission, using the multifacet prism array of this work. The reflective configuration is independent of wavelength and is completely free from chromatic angular chirp, making it most appropriate for applications involving very large optical bandwidth such as fluorescence emission in widefield recording. However, the reflection design allows no adjustment of the beam-tilting angles. To permit matching the requirement for objectives of different NA, we developed the transmission design with customized multifacet prisms (CPG Optics, segment size = 9.375 mm, facet angle = 0, ±23.4, ±31.5 degree) and immersed the prism in refractive index liquid (Cargille Labs, Supplementary Fig. 2). With such a design, we gained the flexibility of adjusting the tilt angle. In fact, we used the same fused silica (n = 1.451) prism array and selected different immersion liquids (n = 1.529 and 1.374) to form both of the two complementary prism arrays. It is worth noting that the spectral angular chirp induced by the prism array was negligible for the ~100 fs duration laser pulses for two reasons. First, the applied angular tilt was a very small angle equal to the angular spread of the optical beam. This is very different from the dispersion prism used in pulse compressors, which produces a huge angular deviation. Second, we chose low dispersion materials, fused silica, and low-refractive-index immersion liquid. To hold the prism array and the immersion oil, we attached the prism array to an optical window using UV-epoxy, fixed the optical window to the bottom of a lens tube (SM2L10, Thorlabs), filled in the immersion oil, and covered the oil with the second optical window. We applied UV-epoxy to seal the interface between the lens tube and the two optical windows (one on each side of the prism array).

Sensorless wavefront measurement. A variety of wavefront measurement methods can be applied to the MPAO system. In this work, we employed the Zernike-mode-based sensorless method for its compatibility with the resonant Galvo-based TPM and the update rate of liquid-crystal SLM. Essentially, we adjusted the coefficient of each Zernike mode and observed the variation of certain image metrics. In this work, we chose image intensity as the optimization metric, and we found that this robustly yielded the aberration correction wavefront. Typically, we used seven to nine data points (i.e., changed the amount of each Zernike mode to seven to nine different values), extracted the image metric at each data point, and applied Gaussian fitting to the metric curve (metric value versus amount of Zernike mode) to find the center of the Gaussian curve, which returned the coefficient of the needed Zernike mode correction (see Supplementary Software). As an example, we show the wavefront measurement result (Supplementary Fig. 13) obtained during the microglia imaging (Fig. 1). Typically, we measured up to 60 orders of Zernike modes.

The employed objective lens (Nikon 25× NA 1.1) can provide spherical aberration correction. Experimentally, we tuned the correction collar such that the coverglass aberration was minimized. We then performed a system aberration measurement to determine the remaining aberration. After this step, the overall imaging system (including the coverglass) was fully corrected. For in vivo measurements, we encountered additional aberrations caused by the biological tissue. The full correction therefore contained both the system correction and the tissue correction. When we made image comparisons, we compared the results with full correction and with system correction. We also compared MPAO-enabled full correction with that of other conventional AO methods (Supplementary Fig. 4 and Supplementary Note 3). For 3D volumetric imaging, the wavefront aberrations were measured at different depths in 50 μm steps (Supplementary Fig. 14).

Nonplanar imaging. Using MPAO, we could flexibly add defocusing wavefront to each pupil and axially shift features from different depths onto a single 2D image plane for high-speed recording. Experimentally, we first added the defocusing wavefront to reach the desired depth and then measured the correction wavefront at that depth. Thus, each region was still optimally corrected while being at different depths. The defocusing wavefront, dcos(θ)n, is a function of the axial shift (d), the incident beam angle (θ), and the refractive index of the immersion medium (n).

Animal preparation and in vivo imaging. All procedures involving mice were approved by the Animal Care and Use Committees of Purdue University. We used female mice of 8 weeks to 6 months old without randomization or blinding.

We used Thy1-YFP (Hline) (JAX No: 003782) and CX3CR1GFP/GFP (JAX No: 005582) transgenic mice for structural imaging of neurons and microglia, respectively. During the craniotomy above S1 cortex (circular; diameter (Φ) = 3 mm), we placed the mice on a soft heating plate and kept them under isoflurane anesthesia (2–2.5% for induction, 1.5–2% during surgery). After that, we applied 1% agarose on the cranial windows, and we cemented the coverslips (circular; Φ = 5 mm, 170 μm thick) and the custom titanium head posts to the skull. Then we placed the anesthetized (1–1.5% isoflurane) mice under the microscope for imaging
while keeping the mice warm. For the dynamic imaging of activated microglia, we introduced laser ablation as described in the literature\textsuperscript{18}.

We used Thy1-GCaMP6s\textsuperscript{19} (line GP4.3) (JAX No.: 024275) transgenic mice for functional imaging of neural activity. We prepared the mice using the same procedures as those used for structural imaging, except that (i) we made the craniotomy above V1 cortex (centered 2.7 mm left and 0.2 mm anterior to the Lambda suture); (ii) before placing agarose and installing the window, we applied SR101 solution (10 µM) onto the cerebral cortex for 5–8 min to stain the astrocytes\textsuperscript{20}; and (iii) during imaging, we kept the mice under low anesthesia (~0.5% isoflurane) to have more active spontaneous neural activities.

We used Thy1-YFP (H line) (JAX No.: 003782) for the non-planar microscopy of neurovasculature regulation. We prepared chronic windows above S1 cortex using the same procedures as those used in structural imaging. Before imaging, we performed retro-orbital injection of Texas-Red to label the blood plasma for monitoring vasculature dilations. During the imaging, the mice were under mild anesthesia (~1% isoflurane).

For each type of imaging experiment, we typically used three or more mice. The variation in the effectiveness of wavefront correction between different mice is minor. The surgical procedure of craniotomy is important to minimize damage to the dura, which ensures a consistent imaging result.

Detailed imaging parameters are summarized in Supplementary Table 1.

Data analysis. We rendered the 3D data using Amira (FEI), and we displayed the 2D images with ImageJ (NIH). For functional imaging data, we selected regions of interest using a semiautomated algorithm\textsuperscript{21}.

Statistics. To quantify the spatial resolution in Figure 1, we measured the FWHM from 40 locations and reported the mean value and the s.d.

Code availability. The custom code we used for wavefront measurement is available as Supplementary Software.

Data availability statement. The data that support the findings of this study are available from the corresponding author upon request. Source data are available for Figures 1–3.

18. Davalos, D. et al. Nat. Neurosci. 8, 752–758 (2005).
19. Dana, H. et al. PLoS One 9, e108697 (2014).
20. Nimmerjahn, A., Kirchhoff, F., Kerr, J.N. & Helmchen, F. Nat. Methods 1, 31–37 (2004).
21. Chen, T.-W. et al. Nature 499, 295–300 (2013).