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A spherical aberration-free microscopy system for live brain imaging

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ABSTRACT

The high-resolution *in vivo* imaging of mouse brain for quantitative analysis of fine structures, such as dendritic spines, requires objectives with high numerical apertures (NAs) and long working distances (WDs). However, this imaging approach is often hampered by spherical aberration (SA) that results from the mismatch of refractive indices in the optical path and becomes more severe with increasing depth of target from the brain surface. Whereas a revolving objective correction collar has been designed to compensate SA, its adjustment requires manual operation and is inevitably accompanied by considerable focal shift, making it difficult to acquire the best image of a given fluorescent object. To solve the problems, we have created an objective-attached device and formulated a fast iterative algorithm for the realization of an automatic SA compensation system. The device coordinates the collar rotation and the Z-position of an objective, enabling correction collar adjustment while stably focusing on a target. The algorithm provides the best adjustment on the basis of the calculated contrast of acquired images. Together, they enable the system to compensate SA at a given depth. As proof of concept, we applied the SA compensation system to *in vivo* two-photon imaging with a 25 × water-immersion objective (NA, 1.05; WD, 2 mm). It effectively reduced SA regardless of location, allowing quantitative and reproducible analysis of fine structures of YFP-labeled neurons in the mouse cerebral cortical layers. Interestingly, although the cortical structure was optically heterogeneous along the z-axis, the refractive index of each layer could be assessed on the basis of the compensation degree. It was also possible to make fully corrected three-dimensional reconstructions of YFP-labeled neurons in live brain samples. Our SA compensation system, called Deep-C, is expected to bring out the best in all correction-collar-equipped objectives for imaging deep regions of heterogeneous tissues.

1. Introduction

Spherical aberration (SA), an optical effect that is produced by the spherical form of a lens, gives different foci for marginal and central light rays. In principle, SA is most pronounced in high NA objective lenses, and the margin of the lens has a shorter focal length than the center (positive SA). Basically, correction of SA is made for commercially available objectives. For example, the high NA water-immersion objective lens for neurophysiology applications is designed so that all incoming light rays converge on the focal point in the water (Supplementary Fig. 1A). However, another kind of SA is generated when concentrated light rays coming out of the objective pass through media having different refractive indices, such as glass coverslips. When observing a specimen in the water underneath a coverslip, the margin of the lens has a longer focal length than the center (negative SA) (Supplementary Fig. 1B, left), and it is instructed that a lens equipped with a revolving objective correction collar be used [1].

As far as an observed object is located beneath the coverslip, SA can be corrected manually depending on the coverslip's thickness (generally 0.17 mm) (Supplementary Fig. 1B, right). Although an

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automatic method for correction collar adjustment was proposed on the basis of light reflection at the coverslip surfaces [2], its application is limited to very thin samples.

Two-photon excitation microscopy [3–5], together with the expression of fluorescent proteins, enables deep, high-resolution imaging of neuronal structures *in vivo*. A chronic cranial window made by replacing a bone flap with a coverslip [5,6] provides optical access to rodent cerebral cortex for long-term imaging of synaptic structures, such as dendritic spines [7–11]. The Olympus 25 ×, NA 1.05 water-immersion objective lens is often used for such imaging experiments. As brain tissue has a higher refractive index than water, however, a significant amount of negative SA is generated additionally when observing a deep region of brain tissue below a coverslip window (Supplementary Fig. 1C, left).

Although such an under-correction in deep imaging can be compensated by rotating the correction collar further (Supplementary Fig. 1C, right), it is difficult to acquire the best image of a given fluorescent object due to the following three problems. First, the manual operation of a conventional correction collar does not ensure precise and reproducible adjustment. Particularly, two-photon excitation observation requires that the peri-objective space be fully dark, which makes it difficult for an experimenter to gain access to the correction collar. Second, the rotation of the correction collar is inevitably accompanied by considerable focal shift, resulting in the loss of fluorescent targets. Third, because of the presence of heterogeneous architectures having distinct refractive indices in tissue, it is impossible to know if the current adjustment is an under-correction or an over-correction.

To overcome these problems, in this study, we have developed an automatic SA compensation system (Deep-C) that is composed of an objective-attached device (Zlin-C) and a fast scanning algorithm (Peak-C). Deep-C enables precise and reproducible determination of the optimum position of the correction collar for minimizing SA, and is useful for not only focusing on a specific structure at a given depth but also obtaining volumetric images that produce optimized and accurate 3D reconstructions.

2. Materials and methods

2.1. Surgical procedures

Male and female Thy1-YFP-H (YFP-H) [12] and Thy1-ChR2-YFP (ChR2-YFP) [13] transgenic (Tg) mice (8–21 weeks old) were used. Mice were housed under a 12-h:12-h light:dark cycle and raised in a group of up to five. Mice were anesthetized with urethane (1.6 g/kg) and their body temperature maintained at 37 °C with a heating pad (BWT-100A, Bio Research Center, Nagoya, Japan; or TR-200, Fine Science Tools, Foster City, CA, USA) during surgery and recording. After skull exposure, a metal frame was attached to the skull by using dental acrylic (Fuji LUTE BC, GC Corporation, Tokyo, Japan; Super Bond C&B, Sunmedical, Shiga, Japan). For two-photon imaging, a craniotomy (4 mm in diameter) was made above the visual cortex (AP −2.0 mm, ML +3.0 mm) and motor cortex (AP +3.0 mm, ML +1.5 mm). The dura mater was surgically removed. The craniotomy was gently sealed with a thin glass coverslip (4 × 4 mm, thickness: 0.12–0.17 mm, Matsunami Glass, Osaka, Japan). The cranial window was secured with dental cement (Fuji LUTE BC, GC Corporation, Tokyo, Japan; Super Bond C&B, Sunmedical, Shiga, Japan).

2.2. Fluorescent bead imaging

A fluorescent bead ($\phi = 0.5\,\mu m$, Fluoresbrite® YG Carboxylate Microspheres 0.50 μm, Polysciences, Inc., Warrington, PA) fixed onto a coverslip (thickness: 0.17 mm, Matsunami Glass, Osaka, Japan) with 1% agarose gel. The coverslip was set in a dish filled with pure water ($n_e = 1.33$), and the fluorescent bead was observed by two-photon excitation microscopy (see below) across the coverslip. In simulated deep imaging of brain tissue with inhomogeneity of refractive index, three coverslips were assembled with spacers (500 μm thick) and fluorescent beads ($\phi = 0.5\,\mu m$) on the coverslips were observed. Pure water was used as an immersion medium, and the space between two coverslips was filled with silicone oil ($n_e = 1.4$) or pure water.

2.3. ScaleA2

Sample preparation (YFP-H line) and tissue clearing with Sca*l*eA2 reagent (Sca*l*eView-A2, Olympus) were performed as described previously [14]. The cleared brain was immobilized in a plastic cup with 0.35% agarose in water. Sca*l*eA2 reagent was used as an immersion medium.

2.4. Two-photon excitation imaging

Two-photon excitation imaging was performed to observe fluorescent beads, Sca*l*eA2-treated transparent brains, and urethane-anesthetized adult mouse brains. An FVMPE-RS laser scanning microscope (Olympus) equipped with an InSight laser system (Spectra-Physics, wavelength 960 nm) and an Olympus objective (XLPLN 25× WMP, NA 1.05, working distance 2 mm, immersion medium: water) was used. Images were acquired with a size of 1024 × 1024 pixels (12-bit resolution), and at a frame rate of 1 Hz, every 0.5 μm (for fluorescent bead imaging) and 4.0 μm (for Sca*l*e and *in vivo* imaging) along the z-axis.

2.5. Data analysis

Calculation of contrast: Acquired images were converted to 1024 × 1024-pixel (8-bit-resolution), bitmap-format images for contrast calculation using a LabVIEW-based system. Contrast (F_{wide}) was calculated based on the well-known Brenner gradient [15] with the following modification (1).

$$F_{wide} = \sum_{n}\sum_{xy} \{f(x,y) - f(x+n,y)\}^2$$

where $f(x,y)$ is the intensity of 2-D images with discrete values ($n = 2, 3, 5, 10$ and 20) ensuring the robust nature of the calculation at various spatial frequencies (Supplementary Fig. 2). Contrast was calculated at each correction collar position (from $-\theta_m$ to θ_n) at a certain Z-position of an objective. If the contrast value is noisy, the same operation was repeated multiple times to give an average value. The maximum contrast value was estimated by Lagrange interpolation, and the corresponding optimum correction collar position ($\theta_{opt}(Z)$) was determined (Fig. 1).

2.6. Compensation of focus shift

Focus shift was defined as a linear function, which was determined by fitting the focus position shift at each correction collar position in advance by using the least-squares method. The objective was driven automatically in accordance with the focus shift function at the corresponding correction collar position.

2.7. Automatic correction collar driving

For 3D imaging, the correction collar was driven automatically in accordance with the linear function, which was determined by

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