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Continuously tracing brain-wide long-distance axonal projections in mice at a one-micron voxel resolution

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ABSTRACT

Revealing neural circuit mechanisms is critical for understanding brain functions. Significant progress in dissecting neural connections has been made using optical imaging with fluorescence labels, especially in dissecting local connections. However, acquiring and tracing brain-wide, long-distance neural circuits at the neurite level remains a substantial challenge. Here, we describe a whole-brain approach to systematically obtaining continuous neuronal pathways in a fluorescent protein transgenic mouse at a one-micron voxel resolution. This goal is achieved by combining a novel resin-embedding method for maintaining fluorescence, an automated fluorescence micro-optical sectioning tomography system for long-term stable imaging, and a digital reconstruction-registration-annotation pipeline for tracing the axonal pathways in the mouse brain. With the unprecedented ability to image a whole mouse brain at a one-micron voxel resolution, the long-distance pathways were traced minutely and without interruption for the first time. With advancing labeling techniques, our method is believed to open an avenue to exploring both local and long-distance neural circuits that are related to brain functions and brain diseases down to the neurite level.

Introduction

The study of neural circuits that are distributed in the brain through neurites, i.e., axons and dendrites, is essential to understanding how the brain operates when healthy, injured, or diseased. However, current knowledge about the intricate patterns of neural circuits is extremely sparse. To address these issues, it would be ideal for researchers to be able to visualize local and long-distance connections across the brain (Miyamichi et al., 2011). Currently, local connections can be mapped by neural branch reconstruction (Cowan, 1998; DeFelipe, 2010) and electrophysiology (Luo et al., 2008). However, long-distance branches often extend through several nuclei or even span nearly the entire brain. For example, some axonal branches of pyramidal neurons in the cerebral cortex project to the spinal cord. The difficulty of investigation is also increased because of the fine architecture of neurites, which are approximately one micron in diameter. For general techniques, it is still a formidable obstacle for brain-wide tracing of long-distance connections at the single neurite resolution (Lichtman and Denk, 2011).

To confront such an obstacle, optical imaging combined with genetically encoded fluorescence labeling techniques (Feng et al., 2000; Luo et al., 2008) is the most promising approach for whole-brain imaging with a sub-micron resolution. To date, specific labeling of the fine structures of individual circuits in large-sized tissue volume has been achieved (Miyamichi et al., 2011; Smith, 2007). However, observing deep into the brain volume remains a problem.

Two types of strategies have been proposed to image fluorescence-labeled projections deep into a large-sized volume of mouse brain. One strategy involves physical sectioning to examine deeper tissue structures. Miyamichi et al. adopted conventional manual sectioning and confocal imaging to observe cortical long-distance neural projections of the olfaction system (Miyamichi et al., 2011). Serial two-photon imaging (STP) combined with block sectioning has achieved intermittent sampling of evenly spaced coronal sections of whole mouse brain with high lateral resolution (Ragan et al., 2012). The other strategy adopts a clearing agent to render a biological sample optically transparent (Dodt et al., 2007; Hama et al., 2011), which reduces the light scattering in the tissue. Chemical clearing (notably, Scale's approach) greatly extends the imaging depth to several millimeters (Hama et al., 2011). However, to date, the uninterrupted brain-wide tracing of long-distance axonal projections has not been reported.

In fact, this type of tracing requires imaging the fine structure of neurites across the whole brain (centimeter size for a mouse brain) with a one-micron voxel resolution ($1\times1\times1\ \mu m^3$). Combining ultra-thin sectioning with confocal imaging ensures high-contrast imaging and highly three-dimensional spatial resolution, which facilitates uninterrupted axonal tracing. However, imaging an entire mouse

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brain using this approach would require weeks. The challenge is to automatically achieve a stable fluorescence image. Moreover, ultra-thin physical sectioning requires a sample of adequate hardness, which is commonly achieved by plastic tissue embedding. However, plastic embedding tends to significantly decrease fluorescence intensity (Becker et al., 2012; Ragan et al., 2012). Therefore, an additional challenge is to plastically embed the fluorescent brain and maintain its fluorescence property at the same time.

In the present study, we demonstrated an unprecedented brain-wide tracing of long-distance axonal projections. We introduced a fluorescence micro-optical sectioning tomography (fMOST) method. In fMOST, stable and high-precision fluorescence imaging was achieved with a custom-built acousto-optical deflector (AOD)-based scanning microscope, and ultra-thin sectioning was achieved as described previously (Li et al., 2010). We also introduced a novel resin-embedding method that was optimized for the fluorescent protein-labeled mouse brain to provide adequate specimen hardness and to maintain fluorescence intensity. With these techniques, we acquired completed brain datasets of fluorescence protein transgenic mice (Thy1-eYFP-H and Thy1-GFP-M) at a 1-μm voxel resolution. Then, we depicted the detailed information of anatomical locations of brain-wide neural pathways using a dedicated pipeline designed for neuron reconstruction and nuclei level annotation. Building upon this system, we have successfully achieved the continuous tracing of brain-wide long-distance axonal projections in the true sense.

Methods

Tissue processing

Thy1-eYFP-H and Thy1-GFP-M transgenic mice (Jackson Laboratory, Bar Harbor, US) were used in this study (Feng et al., 2000). The mice were anesthetized with a 1% solution of sodium pentobarbital and were intracardially perfused with 0.01 M PBS (Sigma-Aldrich Inc., St. Louis, US) followed by 4% paraformaldehyde (Sigma-Aldrich Inc., St. Louis, US) and 2.5% sucrose in 0.01 M PBS. The entire brain was removed from each mouse and post-fixed in 4% paraformaldehyde at 4 °C for 24 h. After fixation, each intact brain was rinsed overnight at 4 °C in a 0.01 M PBS solution that contained 2.5% sucrose and was subsequently dehydrated via immersion in a graded series of ethanol mixtures (50%, 70%, and 95% ethanol solutions for 1 h each at 4 °C). After dehydration, the whole brains were impregnated with Glycol Methacrylate (GMA, which is water-soluble and low acid, Ted Pella Inc., Redding, CA) by means of sequential 2 h immersions in 70%, 85%, and 100% solutions of GMA water-soluble resin (Ted Pella Inc., Redding, CA) (70% and 85% GMA solutions were prepared in a 95% ethanol solution) followed by an overnight immersion in a 100% GMA solution and immersion in pre-polymerized GMA for 3 days at 4 °C. Finally, each whole brain was embedded in a gelatin capsule that had been filled with pre-polymerized GMA and polymerized at 60 °C for 60 h. To improve the preservation of the fluorescence signal, we added 1 M NaOH:GMA = 0.3% (vol:vol) to all of the GMA infiltrate solutions. The 100% GMA solution contained 67 g of GMA monomers, 3 g of water, 30 g of butyl methacrylate, and 0.6 g of benzoyl peroxide. All of the animal experiments followed procedures approved by the Institutional Animal Ethics Committee of Huazhong University of Science and Technology.

fMOST system and image acquisition

The principle of the fMOST system is shown in Fig. 1. Automated high-resolution imaging was conducted in synchronization with ultra-thin sectioning. The sectioning scheme has been described previously (Li et al., 2010). Briefly, a sub-micron resolution in the z dimension was achieved by ultra-thin sectioning using a diamond knife with a three-dimensional nano-precision translation stage. We adopted a laser scanning microscopy scheme to provide highly sensitive fluorescence imaging with sub-micron lateral resolution (x–y resolution: 0.6 μm × 0.8 μm). A confocal slit was used to suppress background fluorescence interference. To ensure stable imaging during the weeks-long sectioning of an entire mouse brain, a non-mechanical acousto-optical deflector (AOD) was utilized as the scanner, which provided stable, fast, and flexible scanning capability (Saggau, 2006). A 515-nm laser (Fandango 50 mW, Cobolt) beam was passed through an AOD (DTS.XY, AA), a correcting cylindrical lens (CL), and a microscope system, after which an objective lens (LUMPLFLN 40XW, NA = 0.8, Olympus Inc., Tokyo, JP) was used to focus the laser on a sample strip over the surface of a diamond knife used for sectioning. Fluorescence that had been excited by laser exposure was collected by the objective lens and was then passed through a tube lens, a dichroic mirror (Di01-R514-25 × 36, Semrock Inc., Illinois, US), an emission filter (FF01-515/LP-25, Semrock Inc., Illinois, US), and a slit before being measured by a photomultiplier tube (R1924A, Hamamatsu Inc., Iwata City, JP).

To satisfy the imaging speed requirement, the AOD was set to work in a rapid frequency sweep (frequency chirp) mode (Fig. 1B). In this case, the AOD produced a convergent output beam in a single direction (Kaplan et al., 2001). A correcting concave cylindrical lens was introduced to avoid the subsequent deterioration of the spatial resolution of the system. The scan range was set to be larger than the width of the strip to ensure the acquisition of a complete sample dataset. The location of the scan line was adjusted such that it was 10 μm from the knife blade, and a slit with a 50-μm width was used to suppress out-of-focus fluorescence and improve the fiber contrast and connectivity (Qi et al., in submission) (Fig. 1C).

Data acquisition and AOD scan control were accomplished using a high-speed digitizer (PXI-5122, National Instruments Inc., Austin, US) and a multifunctional data acquisition module (PXIe-6363, National Instruments Inc., Austin, US), respectively. The sampling rate was approximately 14 MSamples/s, and the fluorescence intensity of each pixel was obtained by taking the average of every five samples to improve the signal-to-noise ratio.

Image acquisition was triggered and synchronized by the movement of the tissue sample. Because this synchronization was combined with high-precision stage movement (ABL20030-ALS130-AVL125, Aerotech Inc., Pittsburgh, US; with 20-nm feedback accuracy and 100-nm programmable precision), all of the strip images in the dataset are naturally aligned with each other. The system software was written in LabVIEW (National Instruments Inc., Austin, US). As shown in Fig. 1D, the strip images were the basic unit for image file storage. In other words, the complete dataset was composed of many image files, and each file contained an image of one strip. The strip width for the dataset from the adult Thy1-eYFP-H mouse brain was 300 μm, and the pixel dwell time was 0.4 μs. For the adult Thy1-GFP-M and the Thy1-eYFP-H mouse brain at postnatal day 14 (P14), the strip width was 150 μm, and the pixel dwell time was 0.4 μs.

Image pre-processing

The original images were collected and saved in a lossless TIFF format. Because the total strip width in the collected image was slightly wider than the actual width of the section (and thus the part of the image that contained valid information), the redundant or the invalid part of the image had to be removed prior to the image alignment. The uneven illumination along the Y-axis that existed in each original image (Figs. 2A and C) was calibrated according to a curve that was computed from the mean projection of the image background along the X-axis (Figs. 2B and D).

Long-distance neuron reconstruction

The reconstructions of all of the projection neurons were completed interactively in Amira visualization and data analysis software (Visage