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Abstract. Neural circuits in the central nervous system are the substrate of various high-order brain functions. However, little is known about the mechanisms underlying neuronal information processing in the brain. Anatomical and functional graph structures of neural networks with actual connections will provide us with perspectives to elucidate the brain complexity. Here, we aim to develop a three-dimensional mouse brain atlas of neural circuits using nano-resolution x-ray tomography by synchrotron radiation. In addition to identifying a large number of synapses, our research will also clarify the structure of neuronal networks for understanding the most complex organ in the body. In this study, we observed metal-stained biological tissues of the mouse brain using hard x-ray Zernike-type phase-contrast microscopy with 60-nm resolution at SPring-8. As a result, the nano-resolution hard x-ray phase-contrast microscope revealed nerve fibers and organelles including mitochondria and synapses in the neural tissue. In the near future, this information will be utilized to begin deciphering the wiring diagram of the brain by using nano-resolution x-ray tomography.

Keywords: Synaptic connections, neuronal networks, x-ray phase contrast imaging, brain, mouse
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INTRODUCTION

The human brain is composed of complex neural networks that include tens of millions of neurons connected by billions of synapses. Detailed maps of synaptic connectivity will be needed if we are to understand how the brain underlies normal behavior and how brain malfunctions underlie behavioral disorders. Thus, detailed images of neurons with their subcellular features that would display their interactions with other neurons should be acquired. Our purpose is identifying and quantifying the network morphology and connections, neuron by neuron, and synapse by synapse, namely "The Connectome Project" [1-3]. The project aims to decipher all synaptic connections between all neurons and to clarify wiring diagrams of the neuronal networks.

Recent developments in optics research [4] have enabled x-ray microscopy to produce high spatial resolution of the synapse, so much so that a spatial resolution of 15 nm has been achieved. X-ray imaging has higher spatial resolution than light microscopy, since the x-ray wavelength is much shorter than that of visible light. A neuronal connection, called a "synapse," could be observed by using an x-ray microscope and similarly with an electron microscope [5-7]. We will be able to identify a great number of "synapses" in the brain. On the other hand, hard x-ray microscopy can be transmitted through thick materials, allowing observation of inner structures without destruction, similar to MRI, and making a number of ultra-thin slices (50 nm) to observe large-scale brain regions unnecessary. Thus, x-ray computed tomography (CT) combined with the microscope can clarify the three-dimensional (3D) neuronal structures (axons, dendrites, and soma) and synapses with isometric high resolution. This

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should enable the identification of complicated neural circuits and synaptic connections at the same time. Using hard x-ray CT with nanometer resolution, we can expect to decipher wiring diagrams in large volumes of the brain.

MATERIALS AND METHODS

Tissue Preparation

All experiments were performed in accordance with the guidelines of The Physiological Society of Japan: 8-week-old C57BL/6J mice were fixed by perfusion in 2.5% glutaraldehyde and 2% paraformaldehyde in HEPES buffer under tribromoethanol anesthesia and their brains quickly removed and placed in the same fixative solution. The brains were then kept in the fixative overnight at 4°C. Blocks were dissected from the cerebral cortex. The blocks were washed with HEPES buffer and immersed in a mixture of 1% osmium tetroxide and 1.5% potassium ferrocyanide in the buffer for 1 hr at 4°C, and then *en bloc* stained by lead citrate for 1 hr at room temperature. The blocks were dehydrated through ascending degrees of ethanol concentration and propyleneoxide, and then embedded in epoxy resin. The resin blocks were hardened for 48 hrs at 70-90°C. Sections of the neural tissue were cut with an ultramicrotome; slice thickness was 70-200 nm. The rod-shaped samples (diameter approximately 25 μm) for CT measurement were fabricated by micro-lathe and set on the rotary stage for observation with an x-ray tomography system.

X-ray Microscopy

Full-field x-ray micrographs were obtained using a transmission hard x-ray microscope at the BL47XU of SPring-8 in Japan. Figure 1 shows the setup of the optical system. A Fresnel zone plate (FZP, NTT-AT) with the outermost zone width of 50 nm is used as an objective. Zone material of the FZP pattern is 0.5-μm-thick, 155-μm-diameter tantalum. A circular condenser plate (NTT-AT), designed to adapt the matching with the numerical aperture of the objective, is used for Kohler's illumination. The x-ray energy is chosen to be 8 keV. In order to eliminate speckle noise, a rotating beam diffuser is installed in front of the condenser. The sample is mounted on a high-precision rotation stage with slide-guide translation (Kohzu Precision). A cooled CCD camera (Hamamatsu Photonics) coupled with relay lens and scintillator (P43) screen is used as an imaging detector. The CCD camera is used under the 2 × 2 binning mode, and in this case the camera format is 2000 × 1312 pixels. The total length of the optical system is about 7 m, magnification is about 140, and the pixel size at the object plane is approximately 22 nm. Zernike phase-contrast mode, especially sensitive for the light material samples, is selected by installing a phase plate onto the back focal plane.

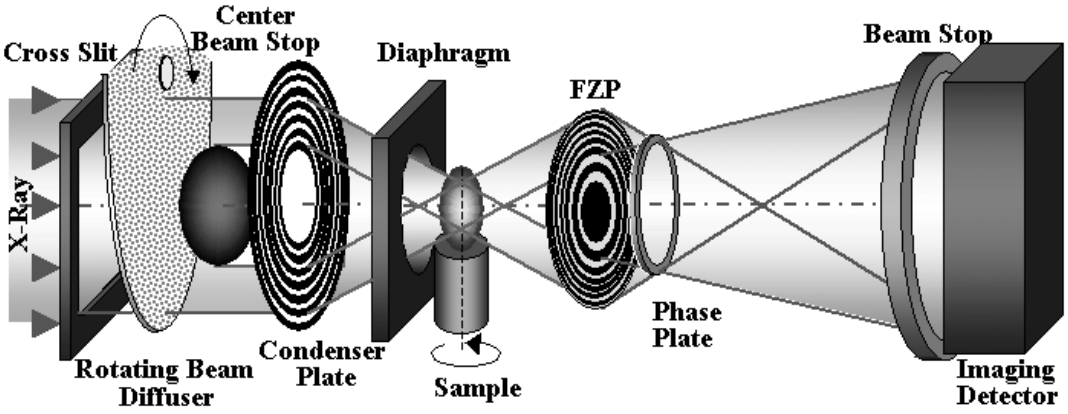

FIGURE 1. Schematic diagram of Zernike-type hard x-ray microscope optical system. The system consists of a beam diffuser, a condenser zone plate, an objective FZP, and a phase plate used to measure samples. When the x-ray energy is 8 keV (wavelength = 0.155 nm), the magnification of the system is about 140. The pixel size is estimated to be approximately 22 nm.

RESULTS

We acquired hard x-ray Zernike-type phase-contrast micrographs for the mouse neural tissue in the cerebral cortex stained with osmium, lead, and uranium (Fig. 2(a)). Exposure time was 90-120 seconds for a single picture. The spatial resolution of the images was about 60 nm, and the field of view was about 50 μm. Transmission electron micrograph (Fig. 2(b)) was taken in the same tissue slice as Fig. 2(a). The magnification of the electron microscope was about 8000; the pixel sizes of both kinds of micrographs were about the same (approximately 22 nm). Arrows indicate the synapses, i.e., the connection sites of neurons, well-stained with heavy metals because the electron density is high at the neural structure of "Post Synaptic Density." Although we tried reconstructing 3D neural tissue of the mouse brain by using the phase-contrast images, the x-ray phase contrast was weak for imaging because of insufficient dynamic range of the x-ray CCD detector. The measuring time for a CT scan was about 40 minutes a sample, and the total data size was about 9 GB.

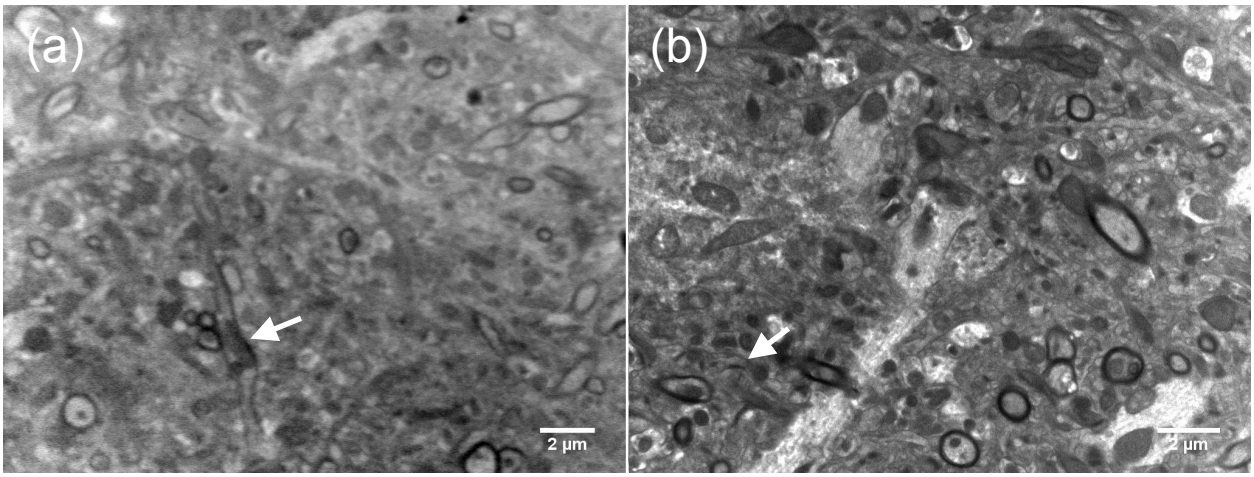

FIGURE 2. Hard x-ray and electron micrographs of mouse neural tissue in the cerebral cortex. (a) Micrograph taken by phase-contrast hard x-ray microscope at BL47XU in SPring-8 (Japan). Spatial resolution is approx. 60 nm, and the field of view is about 50 μm. The scale bar is 2 μm. (b) Transmission electron micrograph of the same tissue slice as (a). Arrows indicate the synapses.

DISCUSSION

Recent research shows that the best spatial resolution is higher than 15 nm using a full-field soft x-ray microscope [4] and that a laterally graded multilayer mirror allows focusing of hard x-ray to 7 nm [8]. Neuronal connections of the mammalian brain can be observed with the same proficiency as by electron microscopy. For the first time in this study, x-ray microscopy was applied to nervous system tissue slices or blocks to identify the synapses. This work demonstrated that it is possible to decipher the wiring diagram of the brain by using the nano-resolution x-ray tomography. Moreover, testing a practical high-resolution unit (nearly 10 nm) by utilizing better optics using both soft and hard x-ray microscopy is necessary to observe synapses clearly. In fact, we observed thin slices of the neural tissue with a full-field soft x-ray microscope with 25-nm resolution at beamline 6.1.2 of the Advanced Light Source. Although we acquired more resolved images of the mouse neural tissue, focal depth is also a critical parameter in determining the sample thickness with nano-tomography. Higher energy (hard x-ray) has the appropriate focal depth for thick biomaterials in CT measurement. To develop suitable staining methods for x-ray high-contrast imaging, *en bloc* electron staining is useful for neuron tracing and synapse identification at higher resolutions. However, it would be desirable to find methods that would further increase accumulation of heavy metals in neurons, or, possibly, even accumulate preferentially in those neurons actively engaged in neuronal activity at the time of tissue harvest. Field of view depends on the x-ray CCD camera performance. A higher-performance camera with more than 64×10^6 pixels and 10^5 dynamic range is needed.

We tested 3D phase-contrast imaging of the rod-shaped neural tissue for CT measurement. Although we acquired cross-sectional images reconstructed by a CT algorithm of the convolutional back-projection method, the x-ray phase contrast was weak for imaging because of insufficient dynamic range of the x-ray imaging detector, and spatial resolution of the 3D image was worse than that of the 2D image because samples drifted during a measurement.

Extraction of all neurons from 3D images—the analysis of anatomical structures and synaptic connections by computer vision—will require algorithms for 3D image processing—such as feature extraction, region growing, and principle component analysis—in order to identify and quantify neurons and synapses from large-scale 3D images. We are currently unable to elucidate information processing mechanisms of the mammalian brain or its component parts. One of the critical reasons is due to the fact that anatomical neural wiring diagrams and connection topology are still unclear in synapse-resolution. Functions and characteristics of real neuronal networks are calculated and compared with neural circuits in various brain areas. We will develop a database containing all wiring diagrams of the mouse brain. Anyone can access the database and acquire the wiring data in areas of interest. Our ultimate purpose is to simulate mammalian brain activities and behaviors with spatiotemporal information such as neural circuits and firings.

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