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To cite this article: Haruo Mizutani et al 2009 J. Phys.: Conf. Ser. 186 012092

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X-ray microscopy for neural circuit reconstruction

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Abstract. Neural circuits in the central nervous system build our various higher brain functions. However, little is known about mechanisms underlying neuronal information processing in the brain. Anatomical graph structures of real neural networks will provide us with fundamental views to elucidate them. We aim at developing a three-dimensional atlas of neural circuits using high resolution hard X-ray microscopy by synchrotron radiation. We stained neurons of a complete brain from a mouse by the Golgi-Cox method. The heavy metals used in our procedure enhanced X-ray absorption and phase contrast. 3D images of fibriform axons and dendrites of various neurons were reconstructed by back projection. X-ray microscopy with a Talbot interferometer demonstrated finer histological structures of pyramidal neurons in the hippocampus and the cerebral cortex. This observation probably serves as a foundation for achieving a mammalian Connectome Project (identifying complete wiring diagrams of the brain) with X-ray nano-tomography in the near future.

1. Introduction

The human brain is composed of complex neural networks that include millions of neurons connected with billions of synapses. Detailed maps of synaptic connectivity will be needed if we are to understand how the brain underlies behavior and how brain malfunctions underlie behavioral disorders. However, there is no evidence of what kinds of neuronal networks are built on the cellular and molecular level. Our purpose is to identify and quantify the network morphology and connections, neuron by neuron, and synapse by synapse namely "The Connectome Project" [1, 2, 3]. The project aims at deciphering all synaptic connections between all neurons and clarifying wiring diagrams of the neuronal networks.

X-ray microscopy performs such high spatial resolution of the synapse, that it has achieved a spatial resolution of 15 nm due to recent developments in optics research [4]. 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 X-ray microscopy and similarly with an electron microscopy [5]. 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. It is not necessary to make a number of ultra-thin slices (50 nm) to observe large-scale brain regions. Thus, X-ray computed tomography combined with microscopy can clarify the three dimensional neuronal
fibriform structures (axons and dendrites) and synapses with isometric high resolution. And we will enable the identification of complicated neural circuits and synaptic connections at the same time. We can expect to decipher wiring diagrams in large volumes of the brain.

2. Materials and Methods

2.1. 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 4% paraformaldehyde and 1% glutaraldehyde under halothane anesthesia and their brains quickly removed and placed in cacodylate buffer. The tissue was then left in the fixative for overnight at 4°C. Several neurons of a complete brain were stained by the Golgi-Cox method [6]. After rinsing in the buffer, the tissue was then dehydrated in a graded series of ethanol and propylene oxide. The tissue was then embedded in Epon 812 resin and polymerized at 70°C for 2 days. Rods 200 μm on a side were cut with a saw microtome and set on the rotary stage to observe with the X-ray micro-tomography system.

2.2. X-ray microscopy
X-ray projection images were acquired using a transmission hard X-ray microscope at the BL20XU of SPring-8. The microscope was equipped with Fresnel zone plate condenser and objective with 100 nm outer zone width and 320 μm diameter. Data were collected using X-rays with an energy of 12.4 keV (0.1 nm), and images were recorded using an X-ray CCD camera. The magnification of the system was about 19. Since the pixel size of the detector was 4.34 μm, converted pixel size was estimated to be approximately 230 nm. An X-ray Talbot interferometer for X-ray phase imaging and tomography was constructed using an amplitude grating of gold pattern, 8 μm in pitch and 30 μm in height developed by X-ray lithography and gold electroplating [7]. The effective area of the grating was 20 mm x 20 mm. Figure 1 shows a setup layout of the optical system.

3. Results
We collected X-ray projection images of several neurons stained with mercury. The spatial resolutions of the images are 500 nm with absorption contrasts (Figure 2-A) and 800 nm with the phase contrasts (Figure 2-B). Images produced with phase contrast have much sharper outlines of neurons than those produced by absorption contrast. We reconstructed 3D neurons of the mouse brain by using phase contrast images (Figure 2-C), that showed neuronal somata, axons and dendrites in various areas of the brain; such as the hippocampus CA1 region (Figure 2-A, B) and the cortical layer 2/3 (Figure 2-C). The measuring time was about 3 hours a sample and the data size was about 1.1 GB.
Figure 2. A, B, X-ray microscopy images of absorption contrast and phase contrast of mouse hippocampal pyramidal neurons in the CA1 region respectively. One image was taken by the X-ray micro-tomography system with a Talbot interferometer (right, B) and the other without it (left, A). Spatial resolution is about 800 nm, pixel size is 230 nm. The field of view is about 320 μm max. The scale bar is 20 μm. C, a three-dimensional rendering view of the pyramidal neurons in the cortical layer 2/3 of the mouse brain. This image was reconstructed by CT algorithm and rendered as a 3D view. This was taken by the X-ray micro-tomography system with a Talbot interferometer.

4. Discussion

Recent research shows that the best spatial resolution is better than 15 nm using soft X-ray microscopy. It can, in principle, observe neuronal connections of the mammalian brain with the same proficiency as electron microscopy. However, the X-ray microscopy has still not been applied to nervous tissue slices or blocks to identify the “Synapses” of the brain. We need to test a practical high resolution unit (nearly 10 nm) by utilizing better optics using both soft and hard X-ray microscopy. Focal depth is also a critical parameter to determine the sample thickness with the micro-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 imaging, en bloc electron staining is expected to be useful for the synapse identification at higher resolutions. We also need the uptake of heavy metals into all neurons or the ones with neuronal activity. Field of view depends on the X-ray CCD camera performance. There is a need for a higher performance camera with more than 64 x 10⁶ pixels and 10⁴ dynamic range.

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 not currently able 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 of 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 the mammalian brain activities and behaviors with spatiotemporal information such as neural circuits and firings.

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