Applied Microscopy
Korean Society of Microscopy

pISSN 2287-5123 · eISSN 2287-4445
https://doi.org/10.9729/AM.2016.46.4.171

Review Article

Nano-Resolution Connectomics Using Large-Volume Electron Microscopy

Gyu Hyun Kim, Ja Won Gim[1], Kea Joo Lee*

Laboratory of Synaptic Circuit Plasticity, [1]Laboratory of Computational Neuroscience, Department of Structure and Function of Neural Network, Korea Brain Research Institute, Daegu 41062, Korea

*Correspondence to:
Lee KJ,
Tel: +82-53-980-8320
Fax: +82-53-980-8339
E-mail: relaylee@kbri.re.kr

Received December 21, 2016
Revised December 24, 2016
Accepted December 24, 2016

A distinctive neuronal network in the brain is believed to make us unique individuals. Electron microscopy is a valuable tool for examining ultrastructural characteristics of neurons, synapses, and subcellular organelles. A recent technological breakthrough in volume electron microscopy allows large-scale circuit reconstruction of the nervous system with unprecedented detail. Serial-section electron microscopy—previously the domain of specialists—became automated with the advent of innovative systems such as the focused ion beam and serial block-face scanning electron microscopes and the automated tape-collecting ultramicrotome. Further advances in microscopic design and instrumentation are also available, which allow the reconstruction of unprecedentedly large volumes of brain tissue at high speed. The recent introduction of correlative light and electron microscopy will help to identify specific neural circuits associated with behavioral characteristics and revolutionize our understanding of how the brain works.

Key Words: Synapse, Neuron, Circuit, Electron microscopy, Brain mapping

INTRODUCTION

The brain is organized into functionally and topographically specific areas. This functional distinction among brain regions is mainly achieved by different types of neurons (e.g., excitatory and inhibitory), their relative abundance in each area, and their specific local and long-distance connectivity. In the central nervous system, synapses are specialized junctions that permit a neuron to send chemical signals to another neuron. The human brain is estimated to have a hundred billion neurons and a thousand trillion synaptic connections. This vastly interconnected network of neurons is highly plastic in structure and function during development, learning, and memory, and is believed to make us unique individuals.

More than a century ago, Santiago Ramón y Cajal demonstrated that neurons communicate with each other through synapses using a light microscope and a silver staining technique discovered by Camillo Golgi. In recognition of their pioneering work on the structure of the nervous system, the 1906 Nobel Prize in Physiology or Medicine was awarded jointly to Cajal and Golgi. Although Cajal's neuron doctrine eventually became the foundation of modern neuroscience, its acceptance came very slowly due to the lasting debates between the two scientists about the basic structure of the nervous system as well as the lack of a sufficiently advanced microscopic technology to resolve subsynaptic elements. Indeed, the first indisputable morphological evidence for synaptic contacts came in the 1950s with the newly-developed transmission electron microscopy (TEM) (Blackstad, 1965).

Since then, EM has been established as a valuable tool for examining ultrastructural characteristics of neurons, synapses, and subcellular organelles. Subsequent technical advances in specimen preparation and instrumentation made it possible to explore the small-scale three-dimensional (3D) composition of nervous system using serial section EM (Harris et al., 1992; Sorra & Harris, 1993; Toni et al., 2001). However,

large-scale circuit reconstruction has been challenged by the daunting tasks of acquiring thousands of serial sections and manually tracing the aligned stack images. Indeed, the technical difficulties, demanding a considerable amount of experience and expertise, led neuroscientists to understand profiles of only fragments of the brain's complexity.

In order to understand how the brain works, the mapping of complete neuronal networks is necessary. To this end, nano-resolution connectomics has emerged to produce comprehensive maps of neuronal networks. This ambitious approach will significantly increase our understanding of physiological brain functions, including perception, learning and memory, decision making, and emotion. It will also provide insight into how the structural substrate of the brain is changed in neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases as well as mental disorders such as depression, autism, schizophrenia, and posttraumatic stress disorder. The main tool used for nano-resolution connectomics research is volume EM. Fortunately, recent breakthroughs in the automation of volume EM have enabled neuroscientists to undertake an unprecedentedly large-scale reconstruction of complicated neural circuits (Denk & Horstmann, 2004; Knott et al., 2008; Micheva et al., 2010; Micheva & Smith, 2007). The intense attention for large-scale brain circuit reconstruction seems to have inspired a renaissance in 3D EM (Krijnse Locker & Schmid, 2013; Kuwajima et al., 2013). In this review, we focus on introduction and application of this emerging EM technique.

BRAIN MAPPING PROJECTS ACROSS THE WORLD

There is an increasing interest in brain mapping projects as international big science, including the USA, Europe, Japan, and China. The USA BRAIN (Research through Advancing Innovative Neurotechnologies) initiative announced by President Barack Obama in 2013 (http://www.braininitiative.nih.gov) strives to develop innovative technologies for the precise mapping of molecules, cell types, and circuits in the human brain. The ambitious goal of this big project can best be attained by collaboration across disciplinary and geographic boundaries with continuous support from different funding agencies including the National Institutes of Health, National Science Foundation, and Defence Advanced Research Projects Agency. On the other hand, the Human Brain Project (http://www.humanbrainproject.eu), a European Flagship research initiative launched in 2013, aims at providing researchers with Information and Communication Technological tools and building detailed mathematical models of neural circuitry in order to understand how the human brain works. In 2014, Japan also joined in this international fellowship of brain mapping. The Brain Mapping by Integrated Neurotechnologies for Disease Studies (Brain/MINDS) seeks to understand the vast complexity of the human brain by taking advantage of a unique non-human primate model, the common marmoset (http://www.brainminds.jp). The rationale for using a marmoset model is that the marmoset brain shares some aspects of the developmental, anatomical, and social characteristics of the human brain; moreover, the marmoset has a small body size and its reproduction is efficient (Okano & Mitra, 2015). Taken together, these brain projects with different approaches plan to map the structure and function of neural circuits, and ultimately identify the neuronal circuits responsible for devastating human brain diseases.

TECHNICAL ADVANCES IN VOLUME ELECTRON MICROSCOPY

Manual serial sectioning and imaging of individual sections with TEM (serial-section TEM) was introduced in the 1950s (Birch-Andersen, 1955) and the technique has been used for 3D ultrastructural observation of biological tissues at sub-nanometer resolution. Typically, 50~100 nm thick sections are cut with a diamond knife and manually mounted onto a grid coated with a thin film (Harris et al., 2006). Then, the images acquired under TEM are manually aligned to obtain a 3D representation of the objects. However, serial sections can easily be distorted or lost during collection or heavy metal staining. To overcome these disadvantages of manual sectioning, automated volume imaging methods were developed for faster and more reliable imaging of serial sections. Indeed, technological advances in scanning EM (SEM) are now driving a paradigm shift in large-volume electron imaging. There are three major SEM-based 3D approaches: the serial block-face (SB)-SEM, the focused ion beam (FIB)-SEM, and the automated tape-collecting ultramicrotome (ATUM)-SEM.

In the SB-SEM system (Fig. 1), a sharp diamond knife installed in the vacuum chamber removes an ultrathin surface layer of the specimen at periodic intervals. Between sectioning, the detector for back-scattered electrons produces a top surface image of the tissue block (Denk & Horstmann, 2004). This first and fully-automated volume EM method allows the acquisition of thousands of serial EM images by significantly reducing the need for the alignment and ordering stages of serial-section TEM. The main drawback of this system is that it is not possible to reacquire regions of interest at a different magnification because specimens are lost after serial sectioning.

SEM imaging combined with the FIB milling technique has presented remarkable outcomes for biological specimens (Knott et al., 2008). The FIB-SEM method is conceptually analogous to the SB-SEM system except that the removal

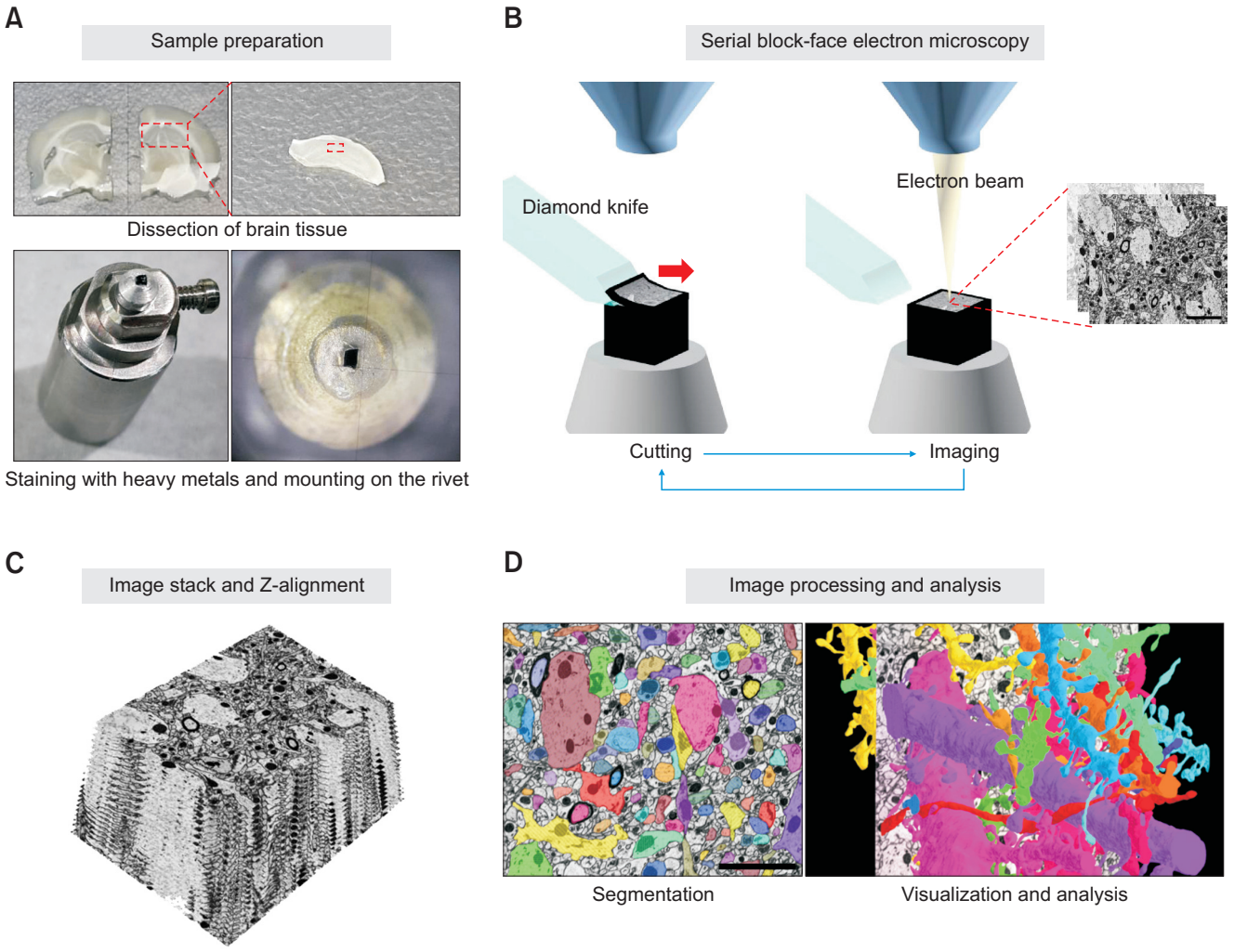

Fig. 1. Serial block-face scanning electron microscopy (SEM) analysis workflow. (A) Sample preparation of hippocampal tissues (150 μm-thick slices). (B) Removal of a thin surface layer with the built-in diamond knife in the SEM chamber and acquisition of serial EM images. (C) Alignment of the acquired stacked images. (D) Segmentation and three-dimensional reconstruction of neuronal profiles. Scale bar=5 μm.

of the surface layers is done with a focused beam of gallium ions. Compared to SB-SEM, FIB-SEM can perform thinner sectioning for high Z-axis resolution and is more appropriate for examining hard specimens such as teeth and bone. However, this approach has the smallest field of view of all three approaches, making it unsuitable for large-scale neural circuit reconstruction.

The ATUM-SEM technique was introduced by Dr. Jeff Lichtman and his colleagues at Harvard University to solve the technical limitations mentioned above. Similar to other SEM-based methods, it takes advantage of the surface imaging capabilities of SEM after collecting serial ultrathin sections on a much more durable substrate than can be used for TEM (Hayworth et al., 2014). The serial sections cut with an ultramicrotome are automatically transferred onto a reel of transparent plastic tape. This tape collecting device can generate ultrathin section libraries that are made up of thousands of slices mounted onto silicon wafers. In this way, it enables the acquisition of larger-area images (several square-millimeters) and allows rare and hard-to-acquire samples such as human brain tissues to be re-used multiple times. One disadvantage of the ATUM-SEM is that the rotation of sections or an irregular interval between sections on the plastic tape can hinder automated acquisition of sequential images.

Despite substantial advances in volume EM, the human brain is still hard to tackle with volume EM due to its huge size. To further enlarge the imaging area (centimeter scale) and boost up acquisition speed, a new type of SEM featuring a column with 61 electron beams and 91 beams was recently developed (Eberle et al., 2015; Kemen et al., 2015). This multi-beam SEM will allow previously inconceivable large-scale neural circuit reconstruction to be made at high speed.

APPLICATION OF VOLUME ELECTRON MICROSCOPY

The nematodes *Caenorhabditis elegans* has been considered as an ideal model organism to study neural circuitry because of its simple structure, transparent body, and tractable genetic system (Brenner, 1974). A series of *C. elegans* connectome studies in the 1970s used manual reconstruction of EM images to visualize the wiring diagram of various areas of the *C. elegans* nervous system. The first structural connectivity map of *C. elegans* was realized by genetic screening and reconstruction of serial EM images (White et al., 1986). However, it took more than a decade to completely map the network of all 302 neurons in the *C. elegans* nervous system because of the bottleneck of manual reconstruction. Intriguingly, the chemical synapses and gap junctions between neurons are stereotypical and highly reproducible among animals (Bargmann, 1993; Hall & Russell, 1991). The wiring diagram of *C. elegans* has been made freely available to the community through a website called WormAtlas (www.wormatlas.org). Some scientists might anticipate that this type of multi-investigator megaproject would reduce individual investigator's research. However, sharing the results with the scientific community seems to promote and inspire individual projects.

In comparison with the *C. elegans* connectome, 3D-EM reconstructions of neural circuits in the mammalian brain have been conducted on a smaller scale for specific functional systems due to technical challenges (Helmstaedter et al., 2013; Kasthuri et al., 2015). Recently, based on a 100-trillion-voxel EM dataset, it was identified that cohorts of retinal ganglion cell axons innervated each of a diverse group of postsynaptic thalamocortical neurons (Morgan et al., 2016). This pioneering work revealed that neural circuits in the mouse thalamus are formed by experience-based mixing of different kinds of inputs onto individual postsynaptic cells, but not by a canonical set of connections between intrinsically different neuronal types. We expect a progressive increase in the number and size of large-scale reconstructions of neural circuitry in the mammalian brain in the near future.

CONCLUSIONS

The tools for large-volume EM imaging are currently available, but some obstacles remain in the processing and analyzing steps of EM data sets. For instance, alignment of huge numbers of EM images and manual segmentation of neuronal structures are now major bottlenecks. There is consensus that more automation in the data analysis and easier access to computational tools are necessary to accelerate the reconstruction of densely packed neuropils in a wider volume (Helmstaedter et al., 2013; Knott & Genoud, 2013). It is also important to create an optimal specimen preparation protocol for volume EM. This may include finding optimal imaging parameters for each sample type, improvement of detector sensitivity, and the selection of highly conductive resins to reduce charging artifacts.

More importantly, it is critical to combine ultrastructural data with functional information. As it is now possible to record the activity pattern of neuronal populations in freely behaving animals with genetically engineered calcium indicators combined with multi-photon and in vivo confocal imaging, correlative light and electron microscopy (CLEM) techniques will help to identify specific neural circuits associated with certain behaviors and to decode how the brain perceives internal and external stimuli, integrates multiple sensory stimuli, stores information, controls emotion, and monitors motor executions.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

This research was supported by the KBRI basic research program funded by the Ministry of Science, ICT & Future Planning (No. 2231-415), the Basic Science Research Program funded by the Ministry of Education (2014R1A1A2058740), and the R & D Convergence Program of the National Research Council of Science & Technology of the Republic of Korea.
The authors thank Bo Gyeom Bae for 3D work.

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