Volume electron microscopy for neuronal circuit reconstruction
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The last decade has seen a rapid increase in the number of tools to acquire volume electron microscopy (EM) data. Several new scanning EM (SEM) imaging methods have emerged, and classical transmission EM (TEM) methods are being scaled up and automated. Here we summarize the new methods for acquiring large EM volumes, and discuss the tradeoffs in terms of resolution, acquisition speed, and reliability. We then assess each method's applicability to the problem of reconstructing anatomical connectivity between neurons, considering both the current capabilities and future prospects of the method. Finally, we argue that neuronal 'wiring diagrams' are likely necessary, but not sufficient, to understand the operation of most neuronal circuits: volume EM imaging will likely find its best application in combination with other methods in neuroscience, such as molecular biology, optogenetics, and physiology.

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Introduction
Vertebrate and invertebrate nervous systems are densely packed with intertwining neuronal axons and dendrites and the synapses between them. The small physical size of these structures, as thin as 40–50 nm in diameter [1,2], requires imaging by electron microscopy (EM), particularly when the goal is the dense reconstruction of neuronal circuits. By imaging volumes of brain using 3-dimensional EM, the details of neuronal shape and connectivity can be reconstructed. Importantly, and in contrast to fluorescence-based labeling approaches that require sparse labeling [3] or super-resolution optical imaging [4,5] to resolve densely packed neurites, standard EM stains result in a relatively unbiased staining of all membranes and synapses in the neuropil [6]. This means that EM volumes can, in principle, be used to reconstruct the complete connectivity of a neuron with all its presynaptic and postsynaptic partners. Furthermore, this operation can be repeated for all the neurons in the volume, such that the connectivity of the neurons comprising a circuit — its wiring diagram or 'connectome' — can be extracted.

The main challenge in volume EM imaging is to acquire a data set of sufficient size, resolution, and completeness that the tortuous trajectories of axons and dendrites can be followed, and the chemical (and, ideally, electrical) synaptic connections identified. The necessary volume depends on the anatomical extent of the circuit to be characterized. For example, the volume of an entire adult nematode, Caenorhabditis elegans (approximately 50 μm × 50 μm × 1000 μm, [7]), is about 1% that of a single mouse cortical column (400 μm × 400 μm × 1000 μm, [8]). The required 3D voxel resolution depends on how fine the processes are that must be traced: the finer the process, the greater the required resolution to reliably follow it over a long distance. For example, dendritic spine necks in the mammalian central nervous system can be as fine as 40 nm in diameter [2], and the fine neurites of the fruit fly Drosophila melanogaster can be as thin as 50 nm in diameter [1]. The required completeness of the EM-imaged volume is related to required resolution: when image data are lost due to staining artifacts, a missed section, or some other glitch in the imaging process, the probability of ambiguities in the dataset increases, resulting in fine processes becoming lost or mixed up during tracing.

There is currently no ‘best’ volume EM imaging method. Rather, each of the available methods involves tradeoffs in size, resolution and completeness, and which method is most appropriate depends on the scientific questions under investigation. The field, however, is changing rapidly. Here we summarize current volume EM methods, focusing on those designed to answer questions about neuronal circuit structure, and offer suggestions about the types of circuit questions that each method is currently well suited to answer.

Acquisition techniques
The primary dichotomy between modern volume EM methods lays in the choice of widefield transmission electron microscopy (TEM)-based or scanning electron microscopy (SEM)-based techniques. Because TEM-based approaches rely on the imaging of those electrons that pass through a specimen, a requirement is the use of thin sections cut before imaging. SEM, in contrast,
typically used to image electrons backscattered from the surface of samples, allowing the surfaces of both thin sections and block-faces to be imaged. Both types of instrument are capable of high lateral (x–y) resolutions down to a few nanometers, a resolution sufficient for circuit reconstruction.

However, the z-resolution of all techniques (with the exception of electron tomography [9]) depends on the ability to remove thin sections from a block of plastic embedded tissue. The majority of the new technical developments we focus on are aimed at improving this minimum z-resolution or, at least, improving the reliability of thin sectioning. Since the lateral resolution is typically several-fold finer than the section thickness, z-resolution imposes one important limit on the usefulness of datasets.

**TEM-based methods**

In modern serial section TEM (ssTEM [10**]) individual thin sections, typically between 40 and 90 nm in thickness (e.g. [11*12,13]) are cut in an ultramicrotome with a diamond knife (Figure 1a). Sections are picked up by hand onto a 3 mm diameter metal support grid. The grid typically has a slot of length 2 mm, and width 0.2–1 mm. An electron-transparent support film is suspended across the slot, and the thin sections are picked up onto the film. To enhance contrast, the sections are usually post-stained with heavy metals. The grid is then manually inserted into a TEM, and a small (typically 10–20 μm) region of interest is selected for imaging. Electrons are accelerated using an 80–120 kV potential, permitting them to pass through the sample and underlying support film. Some electrons are scattered by the heavy metals in the sample, generating contrast. After passing through a series of electromagnetic enlarger lenses, the transmitted electron image reaches a layer of phosphor, and a digital camera is used to acquire EM images (Figure 1a). TEM imaging is inherently parallel, with each pixel on the camera corresponding to a location in the section. With digital TEM cameras, large image mosaics can be conveniently collected through the use of a motorized x–y stage to translate the sample between image acquisitions, allowing the entire section area to be imaged.

The upper limit for section size in routine ssTEM is 1 mm × 2 mm, the dimensions of the slot in the support grid. Although custom grids with slots up to 25 mm long have been used (e.g. [14], p. 60), the prospects for increased section width in ssTEM are limited by the fragility of the support film underlying the cut sections and the narrowness of the gap in the objective lens pole piece which admits the sample holder.

**SEM-based methods**

In a SEM, a finely focused electron beam is raster scanned across the surface of a sample and backscattered electrons are collected with a detector positioned above the sample (Figure 1b–d). Unlike TEM-based imaging, SEM-based imaging is therefore inherently serial. The use of low electron energies (typically 1–3 kV) limits the depth of the back-scattered electron signal to the upper tens of nanometers of the sample [15]. Therefore, both thin sections and block faces can be imaged with high z-resolution. As in TEM-based methods, following the acquisition of one field of view (typically tens of microns on a side), an x–y stage translates the sample within the SEM, allowing large millimeter-sized mosaics to be imaged.

An automated tape-collecting ultramicrotome (ATUM) has been recently developed to automate the pickup of sections onto a spool of support tape (Figure 1b) [16*]. This technique obviates the specialized and error-prone process of manual sectioning, and provides the additional benefit of cutting sections thinner (30 nm, K. Hayworth, personal communication) than what is routinely possible in conventional ssTEM. Additionally, much wider and longer sections than normal — up to 2.5 mm × 6 mm — can be cut (K. Hayworth, personal communication), permitting unusually large brain volumes to be thin-sectioned. The use of an electron opaque support tape, however, means that sections collected with ATUM cannot be imaged within a TEM, but rather must use the secondary or backscattered electron signal in a SEM (Figure 1b) [17**].

Alternatively, the surface of tissue blocks can be imaged in SEMs and sectioned in situ within the SEM vacuum chamber [18**,19]. Ideally, the energy-dependent electron sampling depth is matched to the thickness of tissue that is then removed. Tissue can be removed either mechanically using a diamond knife, as with serial block-face SEM (SBEM, Figure 1c [18**]) or by milling with a focused ion beam (FIB-SEM, Figure 1d [20**,21*]). The cycle of obtaining block-face images and then cutting/milling is fully automated, with no interaction from the experimenter. Unlike ssTEM and ATUM-SEM, block-face SEM methods are destructive; the sections are lost as soon as they are removed from the block face.

**Resolution and reliability**

The lateral resolutions obtainable in TEMs remain unparalleled, with sub-nanometer resolutions easily achieved in modern TEMs. In practice, sub-nanometer imaging is overkill for the purposes of circuit reconstruction; a common pixel resolution in TEM is ∼2–4 nm (e.g. [11*,12,13]). Such resolutions are also achievable in SEMs where the size of the electron probe ultimately limits resolutions to 1–2 nm [22]. What resolution do we need for circuit reconstruction? If the minimum diameter of a neurite, such as a spine neck, can be expected to be as thin as 40–50 nm [1,2], the maximum voxel size in any
Figure 1

Simplified schematics (not to scale) of the volume EM techniques described in this review. (a) Serial section transmission electron microscopy (ssTEM). Sections are cut by hand with an ultramicrotome, floated onto a water bath, and picked up onto grids. In ssTEM, electrons transmitted through the sample are focused with electron optics (not pictured) to form an image on a phosphor plate and the image is recorded digitally with, in this case, a CCD camera array (TEMCA). (b) Automated tape-collecting ultramicrotome scanning electron microscopy (ATUM-SEM). Sections are cut automatically on an ultramicrotome and collected from the water bath using a custom designed tape-collection conveyor belt. Because the support tape is electron opaque, ATUM sections are mounted on an imaging plate and imaged in a SEM. Images are formed by collecting back-scattered electrons with an electron detector mounted above the sample (not shown). (c) Serial block-face scanning electron microscopy (SBEM). Automated sectioning with a diamond knife and imaging are performed within the vacuum chamber of a SEM using a custom designed microtome and specimen stage. (d) Focused ion beam milling scanning electron microscopy (FIB-SEM). Automated milling with a focused ion beam and imaging are performed within a dual-beam SEM.

dimension should ideally be at least half as thick (i.e. 20–25 nm) to reliably follow every neurite. However, much higher resolutions of 3–5 nm are in principle required for the reliable detection of some types of subcellular structures such as gap junctions [23].

The new techniques are distinguished by improved z-resolutions (Figure 2). FIB-SEM [20**] currently offers the highest z-resolution of 5 nm (Figure 3). Automated serial sectioning using diamond knives, either with SBEM or ATUM-SEM, can lead to repeatable sectioning of 20–30 nm [24*]. Manual sectioning using an ultramicrotome as in ssTEM is typically limited to sections of 40–50 nm [10**]. z-Resolution in TEM can be improved to a few nanometers through tomographic reconstruction, in which the same field of view is imaged at multiple small tilt increments [9]. The number of tilts typically used for EM tomography would be prohibitively slow for volume EM imaging of circuits. However, a recent method of sparse tomographic reconstruction [25*], in which z-resolution is moderately improved using only a few tilt angles, may permit ssTEM methods to achieve z-resolution comparable to that of SBEM or ATUM-SEM. Sparse tilts in combination with re-imaging at high magnification
(0.2 nm x-y resolution) can also be used to disambiguate gap junctions from chemical synapses and other membrane appositions [13].

There is currently a spectrum of opinion regarding the importance of high z-resolution. Some researchers have suggested that a high enough x-y resolution allows most neurites to be followed unambiguously even with modest z resolutions of 45–50 nm [11*,12,26]. Others stress the importance of achieving nearly isotropic voxels, not only for the ability to follow every neurite within a dataset, but also for the development of automated image segmentation algorithms [27]. To date, a comprehensive assessment of the degree to which neuron traceability is compromised by the limited z resolution of all techniques has not been performed.

A limitation of manual sectioning remains that some sections inevitably suffer from folds or warping during cutting or post-staining. These errors in the final dataset can be minor (many neurite continuities are traceable across a single missed section [11*]), although a rigorous analysis of the effects of missed or damaged sections has not been published. Manual sectioning methods also generally suffer from variability in section thickness, making it difficult to directly compare average z-resolutions. The automated sectioning methods (ATUM-SEM and SBEM) were designed with an increase in the repeatability of section thickness in mind. Again, however, a quantitative comparison of section thickness variability between the various methods has not yet been performed.

**Acquisition speed**

Because local neuronal circuits can span 3D volumes of at least hundreds of microns on a side, acquisition speed has become an increasingly important parameter (Figure 3). A TEM camera array (TEMCA, Figure 1a) enabled high acquisition rates through the use of multiple high frame rate cameras in combination with optimized stage motion and on-line image processing software [11*]. System throughput is largely determined by how quickly image frames can be read out from the cameras in the array, and how quickly the sample can be moved between image frame acquisitions. Saturation the camera sensors in the short period of a single frame exposure can require a large electron dose (electrons/nm²). To avoid rupturing the thin section and its underlying support film with the intense electron beam, various optimizations in sample...
preparation are important, including carbon coating of the support film, minimization of post-staining artifact, and elimination of any inhomogeneity in the support film. Electron dose must also be monitored with SBEM and FIB-SEM, to prevent too much electron-beam induced damage to the block-face before section removal (although the tolerable dose may be different for SBEM and FIB-SEM). The imaging speed of SEM-based approaches is limited by the bandwidth of electron detectors and the available electron beam current leading to a tradeoff between the acquisition rate and the signal-to-noise ratio of images. In addition to raw image acquisition speed, the duration of an experiment is extended by time spent sectioning, loading and unloading specimens from vacuum, and translating samples under the electron beam to construct image mosaics (Figure 3). For large volumes,
the duration of these steps can sum to substantial over-
head (see actual time estimates, Figure 3).

**Post-acquisition alignment**

Once a series of images has been acquired, an alignment
step is necessary to stitch the images into a 3D volume.
An advantage of block-face SEM imaging is the inherent
registration that comes with acquiring images before
sectioning; typically only a simple translational shift of
images is required [24*]. Alignment is more complicated
for ssTEM and ATUM-SEM sections, often requiring
local warping algorithms to compensate for section
stretching, folds in sections, and distortions that occur
during imaging. While such alignment procedures were,
historically, daunting, the use of high-performance com-
puting makes automating complex alignment algorithms
possible [11*,28,29].

**Tissue preparation and correlative techniques**

The electron dense staining of tissue primarily still relies
on chemical compounds first described decades ago
during the early years of biological EM. Stains based
on high-Z number elements such as osmium, uranium,
and lead are most common for each of the techniques
described [30]. The use of *en bloc* staining methods [30] is,
in particular, essential for the block-face methods in
which post-staining of sections is not possible. But the
art of staining is by no means dead. The development of
indicators to highlight structures of interest, such as
membrane-targeted HRP [31,32] or genetically encoded
fluorescent reactive oxygen generators for photoconver-
sion [33] is geared toward selectively increasing contrast
in genetically controllable ways.

While electron micrographs provide structural informa-
tion at resolutions unreachable by light microscopy,
correlative light microscopy can provide the labeling
necessary to bridge the gap to both circuit function and
patterns of molecular expression and localization.
Array tomography was developed to take advantage of
the z resolution of ultrathin sections combined with the
ability to multiplex the labeling of antigens [34]. By
sequentially exposing a section to fluorescently labeled
antibodies, a number of different synaptic proteins (for
example) can be localized to the same synapse [35]. The
tissue processing steps needed to label endogenous anti-
gens are, however, often incompatible with good ultra-
structural preservation; nevertheless, the labeling of
experimentally introduced antigens has been demon-
strated with good preservation of ultrastructure [36,37].
Additionally, near-infrared branding can be used to place
a high-resolution fiducial mark near a fluorescent object of
interest, for visualization using both light and EM [38].

Another approach to connect circuit function to structure
is to directly measure the activity of populations of
neurons from the same piece of tissue to be reconstructed.

Methods for 2-photon laser scanning calcium imaging [39]
allow signals from neurons hundreds of microns deep
within the brain to be recorded without the risk of
damaging ultrastructure with recording electrodes. When
combined with subsequent volume EM acquisitions,
patterns in wiring diagrams can be directly correlated
with neuronal activity [11*,24*].

**Current best applications and future outlook**

Each volume EM method has a maximum section size.
ATUM-SEM sections are supported by a tape substrate,
and can therefore be as large as 2.5 mm × 6 mm (K.J.
Hayworth, pers. comm.). Sections cut for TEM can be as
large as 1 mm × 2 mm, with the possibility of longer
sections if custom support grids (e.g. [14], p. 60) are used.
SBEM, in its current implementation, is limited to tissue
blocks of about 1 mm on a side [18**], although there is no
fundamental technological limit to acquiring larger
volumes. For a variety of technical reasons, the spatial
extent of FIB-SEM is limited to 50–100 μm per side.

Given these section dimensions and the resolutions
described above, the techniques are currently best suited
for different applications. Because FIB-SEM currently
obtains the highest 3D resolution but is limited to small
volumes, it is best suited for questions of local synaptic
circuity. SBEM is currently best suited for moderately
sized (several hundred microns on a side) volumes con-
taining circuits such as those within brain nuclei or
perhaps cortical columns. ssTEM and ATUM-SEM,
due to the current ability to cut and image the largest
area sections, are best suited to multi-millimeter scale
connectivity questions, perhaps even of whole brains of,
for example, fruit flies or zebrafish larvae.

We anticipate that, as more labs begin to adopt these new
techniques, a number of technical advances will follow.
We anticipate further increases in z-resolution for each of
the described techniques. For ssTEM, a more reliable
sectioning and mounting procedure, possibly combined
with ATUM technology, will likely aid data reliability.
We also anticipate that the TEMCA approach offers
substantial headroom for increased imaging throughput.
The time estimates for each of the techniques provided in
Figure 3 should therefore be taken only as current best
estimates and are expected to significantly improve over
the next few years.

Parallelizing acquisition across multiple microscopes is an
obvious way to further increase throughput. When sections
are cut before imaging as in ssTEM or ATUM-SEM,
parallelization is straightforward: sections can be imaged
in separate microscopes. Parallelizing block-face imaging
between multiple microscopes is more difficult because
the block would need to be cleaved in such a way to not lose
any material along the cut. However, this may be possible
using a recently described ‘hot-knife’ method [40].
Is it worth it?

As neuroscientists interested in the structure of circuits, a common question we are asked is something along the lines of: ‘Will wiring diagrams tell us how circuits work?’ Our answer is, ‘Not on their own.’ We argue that wiring diagrams, in the absence of any other information about cell type, synapse type, firing dynamics, etc. are likely insufficient to define circuit function. However, we think wiring diagrams may be necessary to understand circuit function. At a minimum, wiring diagrams in conjunction with complementary data will continue to generate, constrain, and falsify hypotheses about the functional organization of neural circuits.

We have mainly focused on methods for acquiring image data prerequisite to the determination of wiring diagrams, but wish to point out that data acquisition times, while daunting, are still miniscule compared to data analysis times for complete dense reconstruction [27]. In the short term, sparse reconstruction of a subset of the connections contained in vast image datasets will likely continue to yield new insights [11*,24*]. In the medium-to-long term, we anticipate that the recent progress in automating and speeding the acquisition of volume EM data will carry over to automation of the analysis of these large datasets [41,42], eventually permitting complete reconstruction of mammalian circuits across millimeter scales.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest


10. Harris KM, Perry E, Bourne J, Feinberg M, Ostroff L, Hurlbut J: ** Uniform serial sectioning for transmission electron microscopy. J Neurosci 2006, 26:12101-12103. This review summarizes the current state of the art in ssTEM. Additionally, the authors provide essential advice to outsiders attempting to master the technique. Although newer methods have significant advantages, ssTEM remains a core technique for volume EM data acquisition, both in terms of potential imaging throughput and practical accessibility to a large population of neurobiologists.

11. Bock DD, Lee WC, Kerlin AM, Andermann ML, Hood G, * Wetzel AW, Yungerson S, Soucy ER, Kim HS, Reid RC: Network anatomy and in vivo physiology of visual cortical neurons. Nature 2011, 471:177-182. This article shows how volume EM and in vivo two-photon calcium imaging can be combined in mouse primary visual cortex. A custom high-speed TEM camera array was used to acquire a 450 μm × 360 μm × 50 μm EM volume. The EM data were used to determine the post-synaptic targets of the calcium-imaged neurons in the volume. Inhibitory interneurons in the volume were found to sample uniformly from the available surrounding excitatory pyramidal cells, with regard to their orientation selectivity.


16. Hayworth KJ, Kathuri N, Schalek R, Lichtman J: Automating the collection of ultrathin serial sections for large volume TEM reconstructions. Microsc Microanal 2006, 12(Suppl. 1):86-87. The authors introduce a method for automated sample collection in which serial thin sections cut by a conventional diamond knife are picked up from a water boat onto a thick, electron-opaque support tape. The tape is then spaced into a reel, which is cut into easily handled segments. The method is able to cut much larger and thinner sections than is routinely possible in ssTEM.


18. Denk W, Horstmann H: Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure. PLoS Biol 2004, 2:e39. The authors introduce an innovative method for volume EM data acquisition. A conventionally embedded block is placed in an SEM with a custom automated microtomate. The surface of the block is scraped away with an oscillating diamond knife, and an SEM image of the resulting block face is acquired, in an iterative, completely automated fashion. This obviates error-prone manual pickup of serial sections (as for TEM) and simplifies the registration of volume EM image stacks, since image distortions are usually less than result from ssTEM.


This paper demonstrates the successful application of the method of Heymann et al. [21], FIB-SEM, for automated volume EM imaging of neural tissue. Although the volumes generated by this method are currently relatively small, the available x, y, and z resolutions are unsurpassed by any method besides conventional TEM tomography.


The authors present focused ion beam SEM (FIB-SEM), a new method for volume EM imaging of biological tissue. A dual-beam electron microscope is used to alternately ablate a thin layer from the surface of a sample block with a beam of gallium ions, and then to image the surface of the block by SEM. Sectioning and imaging of the sample are completely automatic.


This article combined volume EM and two-photon calcium imaging in the mouse retina. SBEM was used to acquire a 350 μm × 300 μm × 60 μm volume, and the connections between starburst amacrine cells (SACs) and physiologically characterized direction-selective retinal ganglion cells (DSGCs) were reconstructed. SAC dendrites were found to inhibit DSGCs selectively; when the preferred direction of the SAC dendrite was aligned along the null direction of a DSGC, the probability of a SAC-DSGC synapse was greatly increased.


The authors demonstrate that the z resolution of conventional sTEM can be increased by acquiring four additional tilt images at +45° and -45° on two perpendicular axes, and then processing the image stack using a set of basis functions which model membrane structure.


