ELSEVIER

Towards neural circuit reconstruction with volume electron microscopy techniques

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Electron microscopy is the only currently available technique with a resolution adequate to identify and follow every axon and dendrite in dense neuropil. Reconstructions of large volumes of neural tissue, necessary to reconstruct even local neural circuits, have, however, been inhibited by the daunting task of serially sectioning and reconstructing thousands of sections. Recent technological developments have improved the quality of volume electron microscopy data and automated its acquisition. This opens up the prospect of reconstructing almost complete invertebrate and sizable fractions of vertebrate nervous systems. Such reconstructions of complete neural wiring diagrams could rekindle the tradition of relating neural function to the underlying neuroanatomical circuitry.

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Introduction

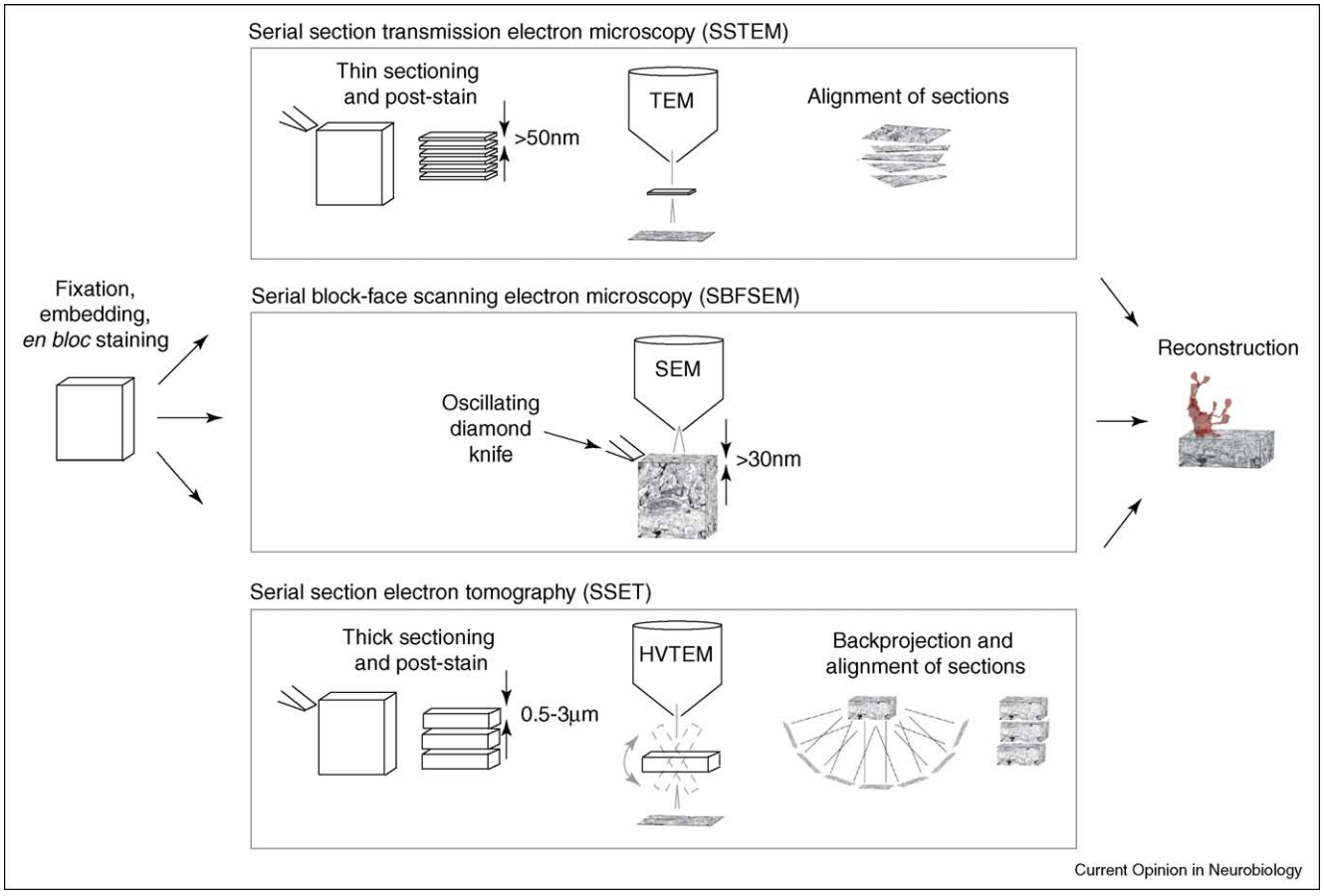
Advances in optical and electrical recording technologies now enable systems neuroscientists to record activity in relatively large populations of neurons with single cell resolution and without averaging across trials [1–5] and then search for activity patterns related to computational processes [6,7]. A key quality to these experiments is the simultaneous recording of many cells, enabling one to take advantage of information contained in co-varying activity. A profound limitation to the interpretation of these datasets is the lack of a detailed wiring diagram relating the observed signals to circuit connectivity. In the absence of anatomical connectivity information, statistical connectivity rules formed on the basis of proximity (e.g. Peters' rule; [8,9]) are used; attempts have also been made to infer 'functional connectivity' from activity correlations in populations [10]. However, knowing the circuit diagram of the actual network from which one has recorded could fundamentally change the way in which population data are analyzed and interpreted and might ultimately be necessary to decode neural algorithms.

We learn little about the computations performed by local neural circuits by recording from one neuron at a time, and instead need the ability to cross correlate the activity of multiple — ideally all — neurons involved. Similarly, the knowledge of the morphology of an individual neuron is of limited use to determine circuit connectivity. In other words, the morphology of a single neuron might provide details useful for understanding computations in single cells [11], but knowledge of all the pre- and postsynaptic synaptic connections of a cell is necessary to understand its role in a network. The reconstruction of all neurons in a sufficiently large volume with adequate resolution to identify synaptic connections would constrain models enormously. For both invertebrate and vertebrate nervous systems, it is widely accepted that the connections between neurons are specific, based on neuron type, and not randomly distributed [12–14]. Elucidation of the complete connectivity matrix for a particular piece of tissue might reveal high-order connection statistics and specific patterns of connectivity that are crucial for understanding neural computations. We, therefore, believe that the connectivity matrices representing specific anatomical patterns must be derived from reconstructions of large volumes of neural tissue and not inferred from generalized statistical descriptions of cell and synapse densities.

The techniques available for the reconstruction of large tissue volumes are dictated by the required resolution. Some of the narrowest neuronal processes in the mammalian brain are unmyelinated axons (~100 nm in diameter; [15]) and the thinnest necks of dendritic spines (~50 nm in diameter, [16]). These structures are too small to be resolved by 3D light-microscopy techniques such as confocal [17] or two-photon microscopy [18]; only electron microscopy (EM) has sufficient resolution to reveal them.

In this review, we focus on EM techniques suitable for the reconstruction of neural circuits in their entirety (Figure 1). Traditionally, three dimensional reconstruction of neural tissue has been achieved by serial section transmission electron microscopy (SSTEM) of ultrathin sections [19] (Figure 2). An alternative is serial block-face scanning electron microscopy (SBFSEM; [20]), in which data acquisition is automated and subsequent-section alignment is no longer an issue. We also discuss serial section electron tomography (SSET; [21]).

Figure 1

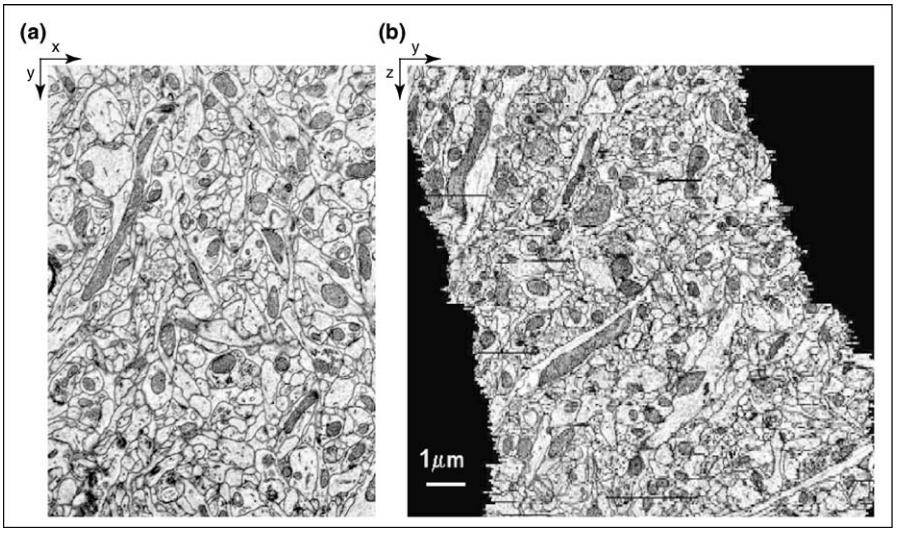


A schematic diagram of the steps involved in the acquisition of tissue volumes using SSTEM, SBFSEM and SSET. The main differences between these techniques are how sections are cut from embedded tissue blocks, the process of image acquisition and the subsequent alignment of images. Sections are cut prior to imaging in SSTEM and SSET, but after imaging in SBFSEM. Transmission electron microscopy (TEM) and high-voltage transmission electron microscopy (HVTEM) are imaging techniques that require 'transparent' samples; scanning electron microscopy (SEM), however, is a surface imaging technique. Image stacks collected in the SBFSEM need no further alignment prior to reconstruction. See text for a more detailed description of each technique.

Staining

Before discussing imaging techniques *per se*, we need to address the issue of selective staining, which is essential for all reconstruction efforts. Contrast in electron micrographs depends on the accumulation of heavy electron-dense (heavy metal) atoms on the structures of interest. For the purposes of circuit reconstruction, a stain selective for neuron plasma (but not internal) membranes, synaptic vesicles, and post-synaptic densities would be ideal. Standard EM staining protocols rely on various combinations of osmium tetroxide, uranyl acetate, lead citrate and a number of other compounds to stain subcellular structures [22], but these techniques are not selective for the plasma membrane. The identification of single neurons within tissue sections historically relied on the Golgi-EM method [23•]. Modern techniques enable the intracellular filling of neurons that were first characterized electrophysiologically by injecting biocytin [24], biotinylated dextran amine (BDA; [25]) or horseradish peroxidase (HRP; [26]). In all cases it is ultimately HRP that catalyses, through the creation of free oxygen radicals from hydrogen peroxide, the oxidation-assisted polymerization of a chromogen, usually diaminobenzidine (DAB; [27]). Subsequent heavy-metal intensification of DAB yields an electron density [28]. DAB polymerization has also been used to 'photo-convert' fluorescent probes into electron-dense products. Photoconversion of chromophores such as resorufin-based arsenical hairpin binder (ReAsh) [29] thus enables correlation between structures observed in living tissue with the same structures in electron micrographs. Quantum dots of different size and shape can also be discriminated in the transmission electron microscope (TEM), further aiding correlations between light microscopy and EM studies [30•]. A complementary approach is to stain the extracellular space, which, in particular with suppressed staining

Figure 2



Serial section transmission electron microscopy. Ultrathin 50 nm sections of rat neocortex imaged with TEM at 7 nm lateral resolution. **(a)** TEM image of a single section (xy plane). **(b)** Registered (aligned) stack of 239 sections resectioned *in silico* (yz plane). Data courtesy of G Knott, registration by DB Chklovskii.

of intracellular organelles, would substantially aid reconstruction. Interstitial staining has been attempted using HRP–DAB (Figure 3a), lanthanum nitrate [31] or transgenic expression of HRP on the cell surface [32].

Serial section transmission electron microscopy

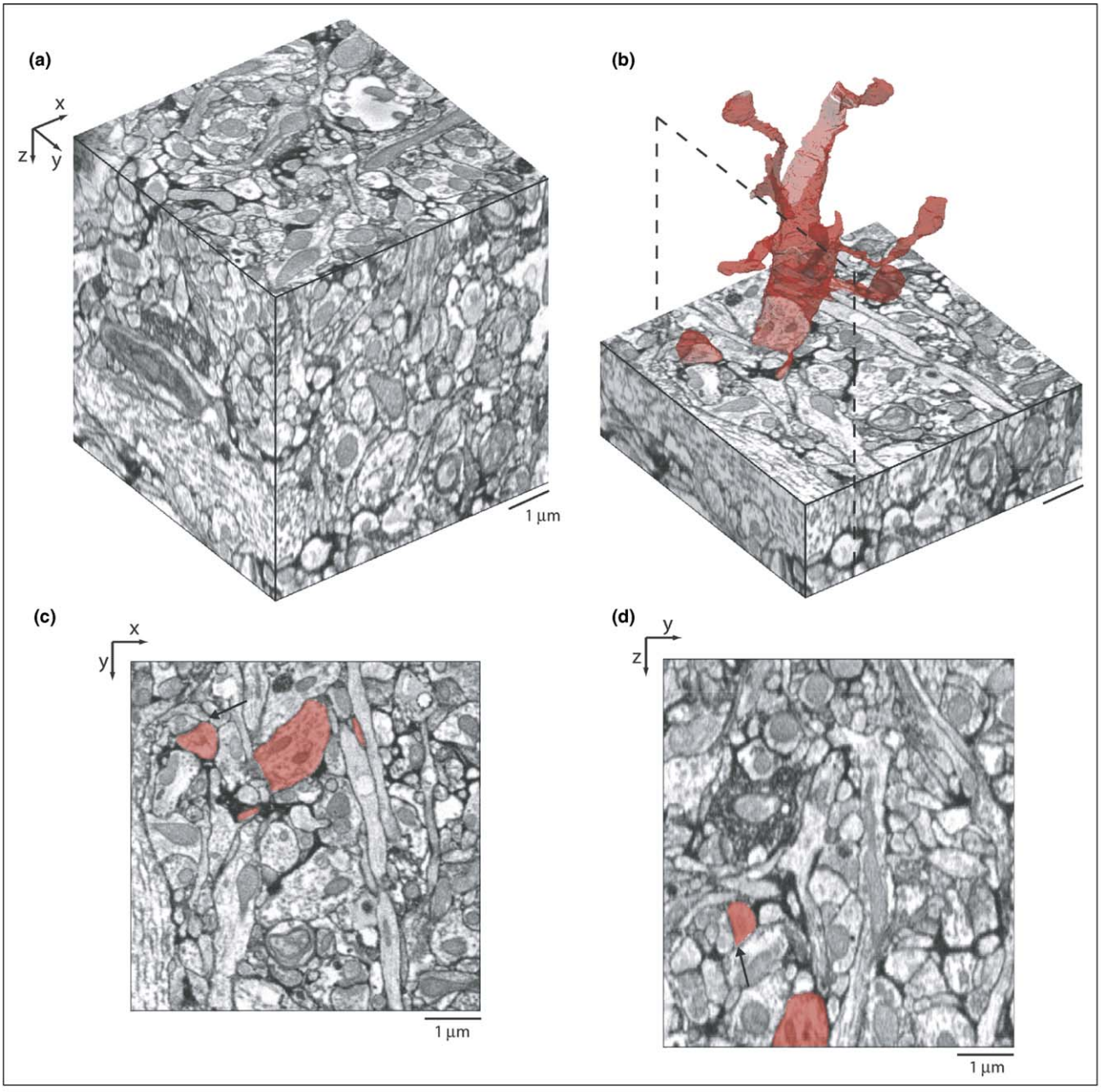
The well-established technique of SSTEM [33–35] is responsible for the vast majority of studies of neuronal circuitry at EM resolution during the past 30 years. SSTEM is a conceptually simple technique (Figure 1), but the sectioning process is tedious and prone to error. Thick blocks (several hundred μm) of tissue are first fixed, usually in some type of aldehyde, and then embedded in a polymeric material, usually a type of epoxy resin. The embedded block is then serially sectioned with a diamond knife on an ultramicrotome, yielding ribbons of thin sections. Staining can be performed *en bloc* or, after sectioning, on the thin sections. It was recently demonstrated that both neuronal ultrastructure and immunoreactivity in thin sections is better preserved through the process of high-pressure freezing than by conventional fixation [36,37].

Ribbons of sections are transferred onto grids for imaging with TEM. In the TEM image, contrast is due mainly to the increased elastic scattering of electrons in areas containing the heavy metal stain as they travel through a thin section [38]. The sectioning and transfer processes are susceptible to many problems —even for experienced practitioners — including loss of sections, uneven section thickness, debris on sections and geometrical distortion. Even if the sections can be successfully imaged, distortions, which vary locally and between sections, hamper automated approaches for the alignment and reconstruction of fine neuronal processes (but see Figure 2).

In TEM, the lateral resolution of ultra-thin sections can be better than 10 nm, depending somewhat on the type of stain employed. Although high-energy electrons can penetrate sections as thick as 10 μm [39], albeit at some loss of resolution, merely projections rather than three-dimensionally resolved data are obtained, because of the large depth of field of electron microscopes. Ultra-thin sections are, therefore, used to generate resolution perpendicular to the sectioning plane. Thick sections can, however, be virtually sectioned using electron tomography (discussed below). Although section thicknesses as thin as 25 nm have been claimed [35], most studies have not been able to achieve long series of sections thinner than 50 nm, partly because of poor formation of section ribbons (JG White, pers comm). A resolution of 50 nm along the depth (Z) axis does not enable the reliable reconstruction of, for example, thin dendritic spine necks. The difference between the lateral and the depth resolutions is apparent when comparing xy and xz images in Figure 2.

Although SSTEM has successfully been used to reconstruct small lengths of dendrites [16] and axons [15], we focus here on attempts to reconstruct neuronal circuitry, which, in most cases, requires reconstructing dendritic arborizations in their entirety. The largest such reconstruction performed to date is that of the entire nervous system of the nematode *Caenorhabditis elegans* [40]. This

Figure 3



Serial block-face scanning electron microscopy. **(a)** A 350 μm^3 volume from adult rat barrel cortex sampled at 13.2nm/pixel in the xy plane consisting of 253 sections 30 nm thick. **(b)** Manually traced spiny dendrite. The dotted line indicates the location of the slice shown in panel d. **(c)** A sample xy plane. The arrow points to a spine head and a synaptic density. **(d)** A sample yz plane. The arrow points to the same structure as in (c). Unpublished data of KL Briggman, RM Bruno, W Denk, T Euler and H Horstmann.

seminal study identified the morphologies of all 302 neurons and their 5000 chemical synaptic connections using approximately 8000 serial sections of 50 nm section thickness. This achievement has profoundly influenced subsequent studies of the function of neural circuits in this species. One example is the neural circuit for touch sensitivity, the anatomy, function and development of which was elucidated by using the circuit diagram to first guide single-cell laser ablation studies and then to interpret their results [41].

SSTEM has also been applied to the study of retinal circuitry [42] and to the mammalian hippocampus [43] and cortex [44]. Partial reconstructions of major cell

classes in the vertebrate retina have helped to reveal distributions of synapses along dendrites and the identity of pre- and post- synaptic partners (e.g. [35,45–47]). However, insufficient Z-axis resolution made tracing the axons of some bipolar cells impossible because of their ''extremely fine and tortuous'' nature [46]. Importantly, SSTEM studies of the retina demonstrated that the complexity of circuit connectivity exceeds what had previously been suspected [48]. Reconstructions of synapses along lengths of dendrites and axons (ranging from 10–100 μm) have also been obtained from the visual [49,50] and somatosensory [51] areas of the cortex. A major thrust of these studies has been to search for patterns of synaptic input into layer 4 spiny stellate neurons [52].

A cursory search in the primary literature on the use of SSTEM for the purpose of neuronal circuit reconstruction reveals a rapid decrease in the number of published studies towards the end of the 1980s. This perhaps coincides with the introduction of high-resolution optical sectioning techniques [53] and genetically targeted fluorescence tracer molecules [54]. Such techniques offer a clear advantage in time and effort to identify connections between stained pairs of neurons. However, although super-resolution techniques [55,56] might be able to do so in the future, current optical methods are not capable of resolving axon and dendrite trajectories or unambiguously identifying synaptic contacts among thousands of neurons in a dense neuropil. For example, assuming that single synapses occur only at axon varicosities might overlook whole classes of subcircuits [57].

With the exception of the *C. elegans* reconstruction ($\sim 10^6$ μm^3; [58]), in which only neurons have been traced and which constitute only a small fraction of the body volume, the volumes of neural tissue that have been reconstructed from individual specimens are in the order of 10^3 μm^3. These volumes are far smaller than, for example, the volume necessary to reconstruct a complete *Drosophila* brain ($\sim 10^8$ μm^3; [58]) or a single cortical column from a mouse ($\sim 10^8$ μm^3; [59]). Given that the *C. elegans* reconstruction took about 15 years to complete (JG White, pers commun), manual SSTEM reconstructions of such large volumes appears impractical.

Serial block-face scanning electron microscopy

The SBFSEM automates the process of sectioning and imaging blocks of tissue by incorporating a custom microtome into a low-vacuum SEM chamber [20,60,61]. Unlike a TEM, the images in an SEM are generated from electrons scattered off the surface of an embedded tissue sample, making the imaging of block faces possible [62]. Existing *en bloc* stains, such as uranyl acetate and osmium, provide adequate contrast, and a lateral resolution of better than 30 nm is possible [20]. Sections are cut from the surface of the block with an oscillating diamond knife. It is possible to cut series of hundreds if not thousands of sequential sections [20] with a section thickness of as little as 30 nm (KL Briggman, W Denk, unpublished, Figure 3).

There are several crucial advantages of SBFSEM over traditional SSTEM. Because the images are taken directly from the block face prior to each cut, the problems of sections being distorted or lost during handling are completely avoided. Furthermore, the images in raw SBFSEM datasets are already aligned and are, therefore, amenable to fully automated analysis techniques. Because the sectioning process is fully automated, large volumes can be imaged without significant operator involvement.

In most cases the area to be imaged is many times larger than the field of view of the SEM at the required resolution; it is, therefore, necessary to take multiple images to cover block faces as large as 500 μm. The microtome, therefore, needs to be mounted on a translation stage with a mechanical reproducibility better than the lateral resolution (10 nm) in order to maintain the alignment within and between subimage stacks.

The SBFSEM has been used to section successfully small volumes of cortex, cerebellum, retina, zebrafish brain, and fly brain. Examples of such datasets are available online [20]. The quality of recent datasets enables single retinal processes to be followed manually through image stacks >100 μm along the Z-axis. Current efforts are underway to further reduce the section thickness below 30 nm and to improve intra- versus extra-cellular staining contrast.

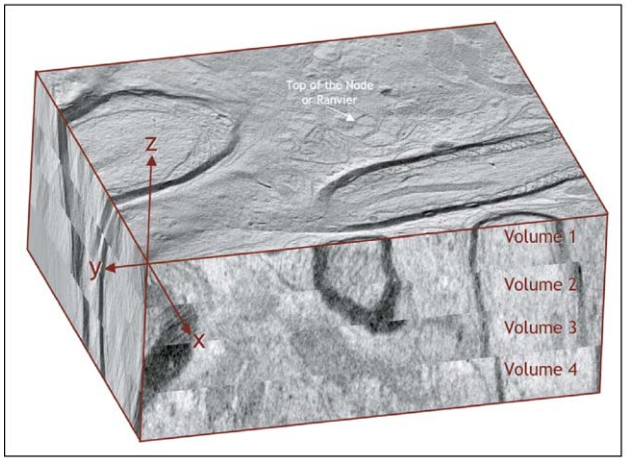
The speed of SBFSEM is ultimately limited by the pixel dwell time necessary to achieve a reasonable signal-to-noise ratio, with rates of slightly above 100 kHz currently being used (KL Briggman, W Denk, *et al.* unpublished, see also Figure 3). At this rate the time needed to collect 10^7 μm^3 (at 30 nm^3 resolution) would be about a month.

Serial section electron tomography

One alternative to SSTEM is SSET [21], which is based on the principle of reconstructing a 3D structure from multiple 2D projections at varying angles, a method similar to computed tomography (CT) scans in medical radiology [37,63,64]. The 3D structure can be reconstructed from the 2D tilt-series of images by a variety of back projection techniques [65]. SSET reduces the number of sections that need to be cut for a given total sample thickness and also improves the Z-axis resolution below that of the thinnest sections that can currently be cut by a microtome. SSET has been used to reconstruct a number of subcellular structures, including dendritic spines [66] and nodes of Ranvier [67•] (see Figure 4).

Blocks of tissue are stained and embedded, similar to the process in SSTEM, and then sectioned into thick

Figure 4



Serial section tomographic reconstruction of a CNS node of Ranvier. This reconstruction contains 90–95% of the entire width of the node of Ranvier and includes the axon, myelin layers and astrocytic processes (for details see [67•]). Each thick section was ~1.5 μm thick. The faces of this cube represent orthogonal xy, yz, and xz slices of the reconstruction. Data courtesy of GE Sosinsky and MH Ellisman.

(0.5–3 μm) sections. Note that in SSET (as in SBFSEM) post-section staining methods (such as post-embedding immuno-staining) cannot be used. Tilt series are obtained by imaging a section at 1–2° increments in a high-voltage TEM (HVTEM). The specimen is ideally tilted along both the X- and the Y- axes through angles as large as ±70°, which optimizes Z-axis resolution and reduces the range of missing spatial frequencies (missing wedge and pyramid), which are responsible for reconstruction artifacts. Because at such extreme angles the effective electron path length is almost three times the section thickness, energy filtering techniques are essential for thicker sections to reach resolutions of better than 10 nm along all dimensions [68,69].

The appeal of SSET is a large reduction in the number of sections that need to be cut for an equivalent volume compared with the number needed for either SSTEM or SBFSEM, reducing the risk of section damage and loss. Although for some applications the loss of material between sequential thick sections, estimated to be 15–25 nm in a SSET reconstruction of the Golgi complex in rat kidney cells [70] is a concern, it might not seriously affect the ability to reconstruct neural processes. However, distortion and shrinkage of tissue caused by the large electron dose needed for the acquisition of a full tilt-series, which comprises as many as 150 images [71], needs to be detected and corrected.

Reconstruction

As computer processing power and storage capacity have increased, efforts have been made to automate the tedious task of manually tracing neuronal processes through dense neuropil. One of the earliest uses of computer-assisted tracing was a reconstruction of a portion of the optic ganglion in the small crustacean *Daphnia magna* [72,73]. There is now a plethora of freely or commercially available semi-automated reconstruction software packages providing morphometric operations, region growing techniques, and interfaces for digitizing tablets (e.g., [74–76]). Given the estimate that computer-assisted tracings by humans can be performed at a rate of ~1 μm^3/hour [58], a manual reconstruction of a mouse cortical column would take >10 000 person years. Indeed, the most time-consuming step in the *C. elegans* reconstruction was the manual tracing and tracking of neuronal processes (JG White, pers commun). Thus, for even modestly large volumes, the amount of user interaction per volume to be traced needs to be rather small and reconstructions will need to be almost fully automated, perhaps involving the development of new machine-learning algorithms [77–79]. The complexity of this task can be appreciated, for example, by viewing the available SBFSEM datasets [20]. Automated reconstruction will greatly benefit from staining protocols that clearly distinguish between the neuronal plasma membranes (or the extracellular space) and the intracellular membranes.

Correlating structure with function

In almost all areas of biology the need to find the detailed connection between biochemical function, such as synaptic vesicle fusion, and (macromolecular) structure is undisputed. (A, by now, classical example being the electron tomographic reconstructions of the neuromuscular synapse [80].) In systems neurobiology, the utility of having structural (connectivity) information is undisputed as well, and was the main motivation behind the complete reconstruction of the *C. elegans* nervous system, which has demonstrated how knowledge of the circuitry can be combined with selective ablations and genetic manipulations [41,81,82]. The need for having the complete connectivity map for a particular instantiation of the nervous system in order to discover and understand the neural algorithms is more in dispute, possibly partly out of desperation in the face of the enormity of the task [83]. For more complex nervous systems, such as a single mouse cortical column or a complete mouse brain, the number of neurons will be 10^4 and 10^7 times larger; the number of synaptic connections will be 10^7 and 10^{11} times larger; and the volumes will be 10^2 and 10^5 times larger, respectively [9,58,84]. One advantage that mammals, in particular, have over the nematode is the availability of physiological data acquired by both electrical [1,85] and optical [2–4] means.

Although the reconstruction of even a single cortical column appears daunting, it appears just feasible. Initial experiments (KL Briggman, RM Bruno, W Denk, *et al.*, unpublished) show that thousands of multi-tiled slices (each more than 10^8 pixels) can be acquired using the

SBFSEM technology within a matter of months. In particular, the prospect of combining physiological population recordings with a post-hoc determination of the underlying neuronal circuitry studies might turn out to be the missing link in finding neural algorithms. At the very least, such detailed knowledge will enable us to validate hypotheses about the geometrical statistics of neural wiring [86••].

Conclusions

Detailed neuroanatomy could well be in for a serious comeback as the technology needed to reconstruct entire local circuits, and perhaps (for *Drosophila*) entire brains, at the resolution needed to follow each and every neural 'wire' is becoming available at the level of both data acquisition and data analysis. Although the interpretation of the data (in the form of, say, a matrix with 10 000 rows and 10 000 columns and synaptic weights as the 10^8 matrix elements) will be another challenge, every theory of how any neural computation works will have to be consistent with the measured connectivity.

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