Toolbox

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Uniform Serial Sectioning for Transmission Electron Microscopy

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The transmission electron microscope (TEM) was first used approximately half a century ago to answer important neurobiological questions, showing unequivocally that neurons communicate via synaptic junctions (Palay and Palade, 1955; Gray, 1959). TEM usually requires that biological specimens be <100 nm thick. These thin sections allow electrons to pass and stop only where stains delineate objects of interest. From the beginning, serial thin sections were visualized and reconstructed to provide a three-dimensional context (Birch-Anderson, 1955; Bang and Bang, 1957; Spacek and Lieberman, 1974). These efforts were considered heroic because of the difficulty in collecting and photographing the serial sections and producing realistic three-dimensional reconstructions. In neurobiology, there is a renewed interest in serial section TEM (ssTEM) (Stevens et al., 1980) to understand how synaptic structure is modified during changes in brain state (Ostroff et al., 2002; Knott et al., 2006). The resolution of ssTEM is needed to distinguish and measure accurately dendrites, axons, glial processes, synapses, and subcellular organelles such as synaptic vesicles, polyribosomes, and endosomal compartments. ssTEM is a labor intensive enterprise; hence, choosing when to use it is important. Confocal and multiphoton microscopy provide lower resolution images from living cells to assess whether labeled structures have changed location or size; however, the resolution is too low to identify, quantify, and measure the dimensions and composition of structures in brain neuropil. ssTEM is required for accurate identification and measurement of objects smaller than 250 nm. A sample volume of 500–1000 μm^3 spanning 250–500 serial thin sections is practical with sSTEM. Photomontaging can be used to enlarge these volumes but at a marked increase cost in time. Here, we describe methods optimized to produce uniform ribbons of serial thin sections.

We aim for a section thickness of 45–50 nm to minimize overlap among small structures, such as synaptic vesicles (~35 nm) or narrow axonal or astroglial processes (~50 nm) that would be obscured by neighboring structures in thicker sections. We routinely collect 200–300 serial electron microscopic (EM) sections; however, much longer series can be collected with these methods. A review of fixation and processing methods is beyond the scope of this paper; as an example, we describe the procedure for hippocampal slices fixed rapidly in mixed aldehydes in the presence of microwave irradiation (Jensen and Harris, 1989). Within 1 d, the slices are embedded in 7% agarose, dissected to a region of interest, and sliced with a vibrating blade microtome (Leica, Nussloch, Germany) at 70 μm (Fig. 1*a–c*) to permit osmium, uranyl acetate, or other en bloc stains to penetrate uniformly, while allowing visualization of regions of interest in the Epon block later (Fig. 1*d*). Epon is shaved off in 1–3 μm increments to a region near the stimulating electrode indentation on a neighboring section (Fig. 1*h*, dotted line). A test thick section is used to guide the trimming of the series trapezoid with a diamond trim tool to a height of ~30 μm, a width of ~100–200 μm, and a depth of 20–30 μm for stability, and with one side slanted for orientation (Fig. 1*f*,*g*). Ribbons of serial thin sections are cut and then retrieved on pioloform-coated slot grids. Lowicryl and some other resins are notoriously difficult to ribbon (Fig. 1*h*) because the hydrophilic sections fall apart;

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however, a few quick sprays of salon-quality hair spray and overnight drying produces uniform continuous ribbons on the same trapezoid (Fig. 1*i*).

The goal always is to have perfectly uniform section thickness along fold-free ribbons for accurate quantitative analyses (Fig. 2*a*). A 35° diamond knife minimizes section compression (Figs. 2*b*,*c*). The grids are coated within 24 h before section pickup to avoid having the Pioloform sag under the weight of the sections during pickup, which can create folds after drying (Fig. 2*b*). Pioloform that is coated too thickly or with an uneven flow rate altered apparent section thickness (Fig. 2*e*). Small holes in the Pioloform (Fig. 2*f*) are avoided by drying the coated slide in a small jar with desiccant. Saturated aqueous uranyl acetate and freshly prepared Reynolds lead citrate are both filtered through a 0.22 μm syringe filter, and grids were stained in a CO_2- free environment to avoid dark precipitate over the surface of the sections (Fig. 2*g*). Microwave-enhanced infiltration of epoxy resins had been used to speed the process, however, infiltration was not always uniform, which resulted in cracks (Fig. 2*h*); hence, tissue blocks are now infiltrated in epoxy resins overnight and then cured for 48 h in a 60°C oven. The ribbon curved if the north and south edges of the trapezoid were not parallel (Fig. 2*i*). Uneven section thickness (Fig. 2*j*) is easily corrected by enclosing the ultramicrotome (Fig. 2*k*) to prevent air drafts and local temperature changes. Stopping and restarting the ribbon also produces a change in section thickness (e.g., third section from the edge of the knife in Fig. 1*j*). A video camera is used to monitor progress, and the enclosure doors are not opened until the desired ribbon length is achieved. These methods produce long, fold-free, and clean ribbons of serial thin sections with uniform section thickness. Dry grids are then loaded into grid cassettes and stored in gelatin capsules for repeated viewing and photography in a TEM. For detailed methods, refer to supplemental methods, movies, and figures (available at http://www.jneurosci.org as supplemental material). In addition, consult http://SynapseWeb.org, http://synapses.bu.edu, or http://clm.synapses.utexas.edu for updates on these methods and detailed methods for photography, alignment, reconstruction, and measurement in three dimensions.

New approaches are sought to achieve the information currently available only with ssTEM more rapidly. Recently, serial block-face-scanning EM (Briggman and Denk, 2006) has been added to the EM toolkit. The advantage of this approach is that fragile, serial thin sections do not need to be handled; instead, the remaining block face is photographed after each section is removed. Theoretically, larger sample areas could be photographed without montaging; however, the resolution achieved at this lower magnification does not currently allow deciphering of individual axons, astroglial processes, dendritic spines, synapses, and their composition of organelles in brain neuropil. Automatic montaging, alignment, and

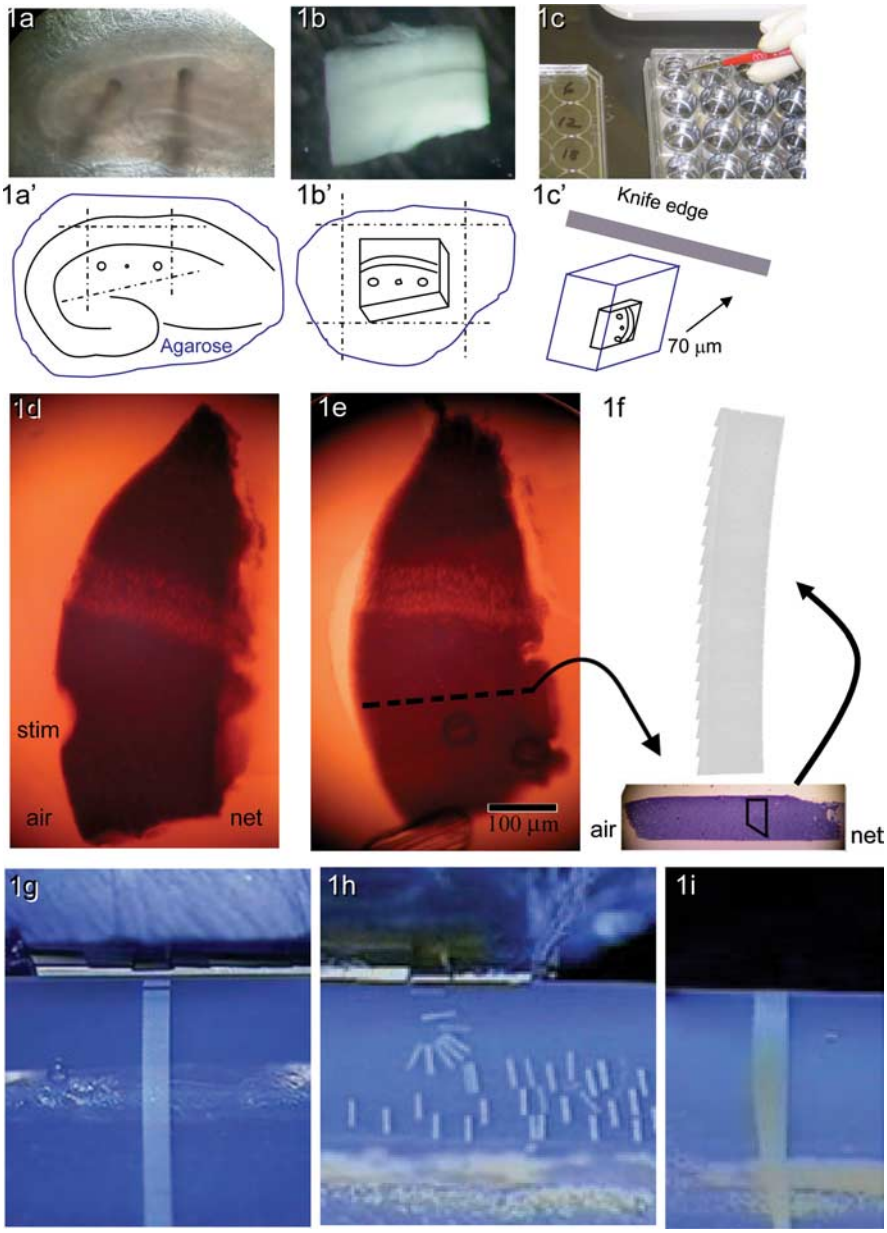

Figure 1. Preparation of serial thin sections from a specific region of interest. ***a***, Stimulating electrodes positioned in a hippocampal slice. ***a′***, Diagram of a region of interest surrounding the indentations left by the stimulating electrodes on the slice, which is embedded in agarose for stability. ***b***, Fixed region of interest illustrated diagrammatically in ***b′*** and turned on edge to obtain slices using a vibrating blade microtome to produce "vibra-slices" as illustrated in ***c′***. ***c***, The 70-μm-section vibra-slices are gently transferred into a 24-well tissue culture plate containing 0.1 M phosphate buffer using a small brush at the corner of the surrounding agarose to avoid mechanically induced dark artifacts in the tissue. ***d***, On-edge view of a vibra-slice through the depth of the hippocampal slice from the air to the net surface; this vibra-slice is through the indentation left by the stimulating electrode (stim). ***e***, Neighboring vibra-slice used to obtain series near the stim, by shaving off the Epon to the dotted line. The scale bar in ***e*** is for ***d*** and ***e***. ***f***, Toluidine blue-stained thick section at the bottom is used to guide where to place the trapezoid on the face of the Epon block. The goal is to have a long ribbon of uniform section thickness. ***g***, Uniform ribbon sectioning, except where the section arm was stopped and restarted. ***h***, Routine Lowicryl embedded tissue does not ribbon well. ***i***, After hair spray, the same trapezoid sections well.

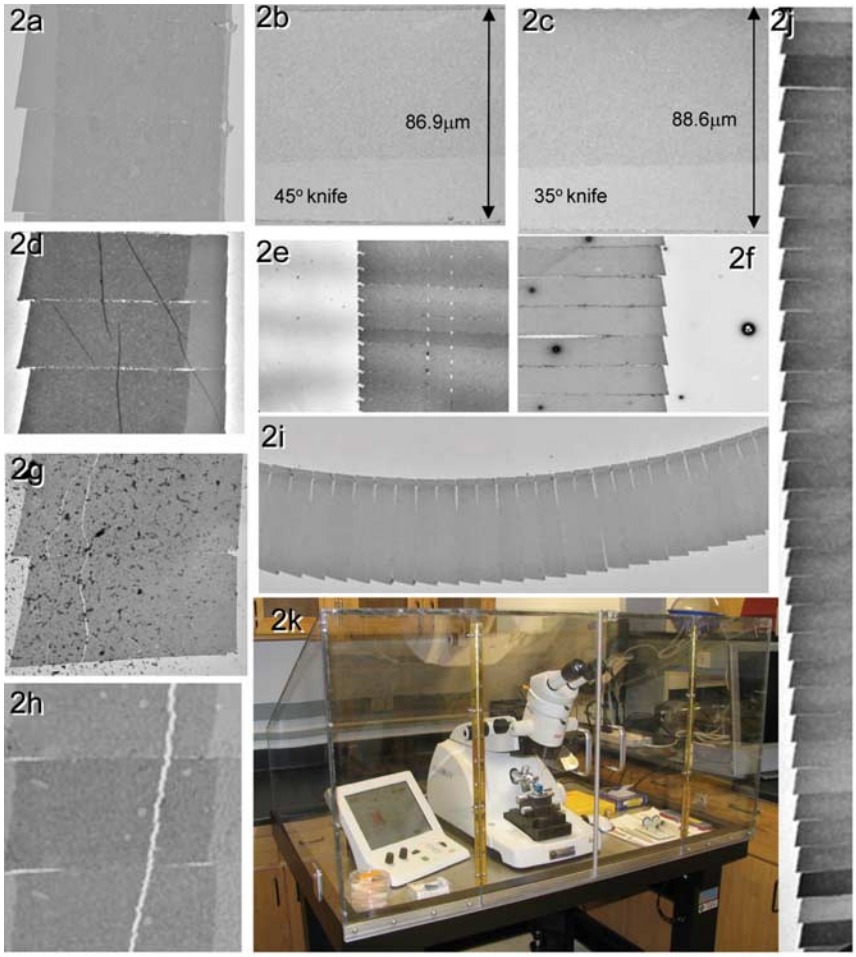

Figure 2. Problems to overcome during sSTEM. ***a***, Goal is a set of clean serial sections of uniform thickness. ***b***, ***c***, Section compression is minimized by using a 35° diamond knife. ***d***, Folds in the Pioloform film. ***e***, Uneven Pioloform film thickness. ***f***, Holes in Pioloform film. ***g***, Dirty grid stain. ***h***, Poorly infiltrated tissue has cracks. ***i***, Curved ribbon. ***j***, Uneven section thickness. ***k***, Enclosure of the ultramicrotome usually solves most issues that cause uneven section thickness.

segmentation are desired to speed the reconstruction process. As these and other approaches are optimized, the time required to analyze brain ultrastructure and connectivity should decrease markedly. sSTEM is unsurpassed for high resolution three-dimensional reconstruction in brain neuropil; hence, the effort to produce high-quality serial thin sections will be rewarded with beautiful images of brain ultrastructure that can be accurately quantified.

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