Reconstructing What Was: Software Applied To Serial Section TEM

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The well-known recipe for serial reconstruction in microscopy consists of the following six steps: fix and embed specimen, cut and mount sections for microscopy, image sections, align images in the computer, identify and trace profiles in images, generate three-dimensional (3D) visualizations. The free software program *Reconstruct* [1] facilitates the final steps of this process for many types of microscopy, including scanning electron or ion beam microscopy applied to material and light microscopy applied to biological samples. We describe here how serial sectioning and transmission electron microscopy (TEM) can be combined with the Reconstruct software to generate large volumes of soft tissue ultrastructure for analysis.

With regard to fixation and embedding, we defer to a number of excellent sources [2, 7, 8, 10]. We emphasize, however, that epoxy resin-embedded biological tissues should be made hard enough to minimize compression during the cutting phase [3]. Our method of cutting and mounting serial thin sections for TEM begins with precision trimming of the block face. We use the Diatome diamond 45°-angled Cryo Trim tool for mechanized trimming on an ultramicrotome. This trim tool is ideal for carving a pyramidal-shaped mesa on a block surface, with perfectly parallel north and south edges that ensure consistent ribboning. The thin sections of the ribbon do become sequentially larger, unfortunately, but one can start with a smaller mesa to offset this affect.

Begin by securing an epoxy resin block in the ultramicrotome and setting both the segment arc (block angle) and knife stage angle at 0° on the ultramicrotome. Maintaining these angle settings throughout the process keeps things simple, but the procedure can be modified if tissue orientation is a critical factor. Cut preliminary semi-thin (1µm) sections from the block face, stain with toluidine blue and determine a site for serial sectioning. Decide on the dimensions of the sections, keeping in mind that the wider dimension will be parallel to the knife edge during sectioning [3

Insert the diamond trim tool securely in the knife holder (clearance angle 0° to 4°). Set the cutting speed to 1mm/s and section thickness to 0.5µm. Rotate the block 90° clockwise and set the length of the cutting window. Align straight edge of the trim tool to block face; (a small deviation from 0° in the knife stage angle is acceptable). Position the left corner of trim tool to the proposed north edge of the mesa. Note that the 45° trim tool removes more tissue to the side as it cuts deeper. Trimming 30µm deep, will remove 30µm more from the width of the top of the mesa, so factor this offset into the measurements for the position of the mesa.

Use the microtome to trim away 20-40µm, and blow away trimmings with can air. Move the knife stage over to the left side of the mesa site and position the right corner of the trim tool to the proposed south edge, measuring from the newly created north edge. Trim away 20-40µm to form the south edge of the mesa (Fig. 1a). Rotate the block 90° counterclockwise, and align the trim tool edge to the face of the mesa as needed. Also adjust the length of the cutting window as needed. Position the left corner of the trim tool

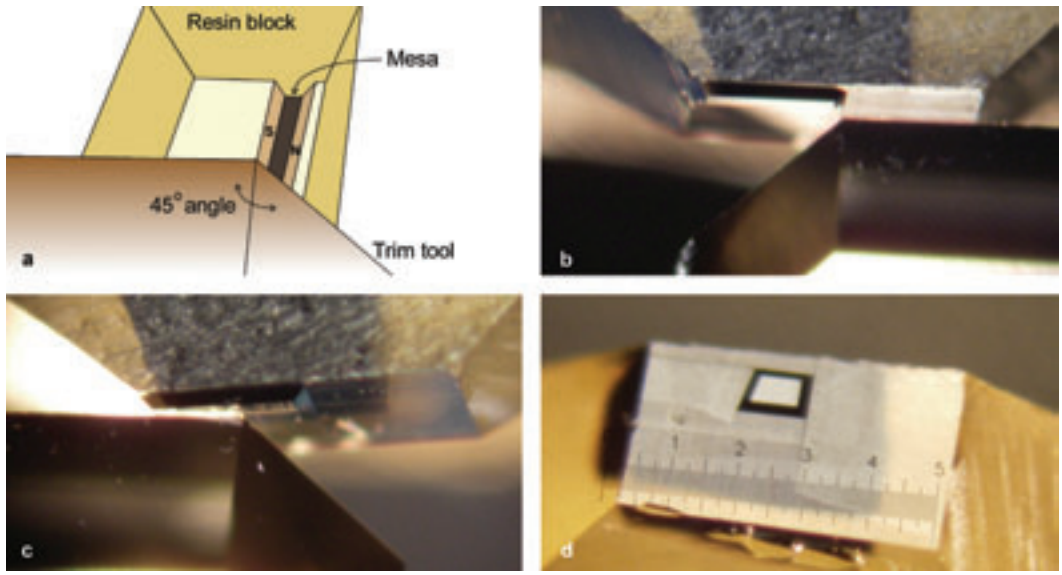

Figure 1. *Trimming a pyramidal-shaped mesa with a diamond trim tool.* **a.** *Trimming of the south edge (S) is done parallel to the north edge (N) to ensure sections stick together in a ribbon. These trims are performed with the block rotated 90° from the position in which sectioning will be done, so that the south edge is where the sectioning knife first contacts the block face.* **b.** *The east edge trim is performed after rotating the block back 90° to the original position.* **c.** *The west edge trim is performed at a slight angle relative to the already trimmed east edge. This angle gives the sections a trapezoidal shape that helps determine the serial order of sections in the TEM.* **d.** *The final trimmed mesa ready for sectioning.*

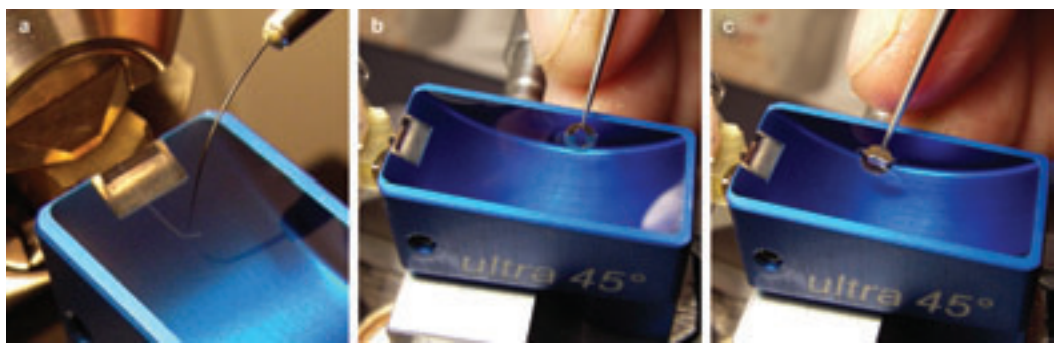

Figure 2. *Collecting ribbons of sections.* **a.** *A super fine hair tool is used to separate a segment of ribbon for collection.* **b.** *Segments of ribbon are collected onto slot grids coated with polyvinyl support film. In this image a grid is held in the 12 o'clock position relative to the slot.* **c.** *Collecting a ribbon with a slot grid held in the 3 or 4 o'clock position.*

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to the proposed east side and trim off 20-40µm (Fig. 1b). Move the knife stage to the left side of the mesa, and rotate the block about 15° counterclockwise. (This will give the west side of the sections a slight angle to help indicate their serial order.) Align trim tool edge to mesa face as needed. Place the right corner of the trim tool at proposed west trim line, measuring from the newly created east edge. Trim off 20-40µm as before (Fig. 1c). Rotate the block 15° clockwise, back to the proper position for cutting sections from the tiny, pyramidal-shaped mesa (Fig. 1d).

Replace the trim tool with a diamond ultra knife and adjust the knife clearance angle accordingly. Reset the length of the cutting window, set the microtome to cut 40-50nm sections, and maintain the same cutting speed. Fill the diamond knife boat with clean, filtered water to the appropriate level. Align the knife edge to south edge of mesa, adjusting block rotation as needed. Align the knife edge to mesa face and adjust the knife stage angle as needed. Have ready 6 to 10 pairs of clean fine forceps, a super fine eyelash hair (Ted Pella, Inc), support-film coated Synaptek slot grids, grid box, notebook, and draft shield.

Position the knife edge close to the mesa face, put the draft shield in place, and turn on the microtome to begin thin sectioning. Once full-faced sections appear, turn on the counter to count sections. When a sufficient length of ribbon is obtained, turn off the microtome, and separate the ribbon into shorter segments (about 1.75 mm or less) for collection onto grids. To do this, wet the tip of eyelash hair in the boat water to prevent sticking to sections, then lightly tap near the interface of two sections to break the ribbon (Fig. 2a). Once a segment has broken off, waft the water with the hair to draw the segment to an isolated area in the boat.

Pick up a coated slot grid by either positioning the forceps tips at the top (12 o'clock, Fig. 2b) or on the side (4 o'clock, Fig. 2c). Use the edge of the grid to gently waft water to draw the ribbon segment closer. Then partially submerse the grid, film side toward ribbon, at a slight angle Once a ribbon appears to contact the support film, lift the grid (and hopefully the ribbon) out of the water and set the forceps aside to let the grid air dry for several minutes before storing it in the grid box. Continue to separate and collect ribbon segments, keeping track of the the order of the sections and making notes on the number of sections in each segment and their orientation on the grid. We recommend mounting the ribbons in a consistent orientation to make serial microscopy easier. One can stain the grids containing the ribbons if more contrast is required [see *e.g.* 11].

When photographing the series of thin sections in the TEM, we recommend using a rotating specimen holder that allows the grid to be rotated while in the microscope. This type of holder allows the exact photo field to be maintained across all the sections (and grids), with perfect orientation of each section. A rotational holder was used, for example, in a study of axons in cerebral cortex in which a large area was investigated by photographing three overlapping fields at 1000× on each of 100 sections (Fig. 3). To maintain such photographic fields during photography, detailed drawings and descriptions are invaluable.

The Reconstruct software provides a user-friendly, Windows-based editor for reconstructing serial sections [4]. The software creates a series of blank sheets (called *sections*) on which images are positioned. Each section can hold any number of images taken at different magnifications, and these images (called *domains*) can be arranged to make a mosaic or montage. Sections can be aligned to each other to reconstruct the structure of tissue as it was before sectioning. The user can then draw and position any number of outlines (called *traces*) on top of the images for the purpose of labeling, counting, measuring, or visualizing the three-dimensional structure.

Reconstruct facilitates section-to-section alignment of images by displaying adjacent sections in rapid alteration (*flickering*) or by showing one section overlaid on the other (*blending*). The user can then adjust the alignment of sections using mouse or keyboard movements. More automatic methods, such as alignment through image correlation or by correspondence points, are also provided. When correspondence points are used, points are added to each section in alteration to indicate which parts of the sections should

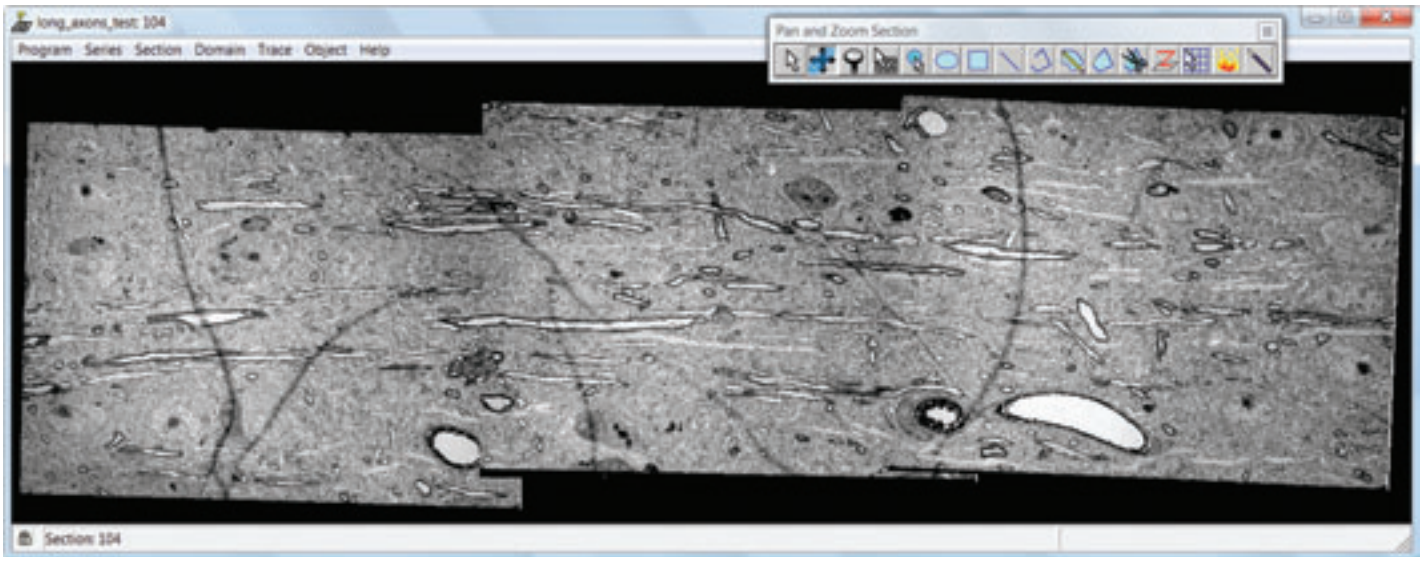

Figure 3. *The main window of the Reconstruct software showing a section composed of a montage of three images. Each image is one photographic field of cortical brain tissue, imaged at 1000X onto 3.25x4inch film at the TEM and then digitized at 1200dpi on an Epson flatbed scanner with transparency adaptor. The final section of aligned images contains about 53 megapixels of data covering an area of 253x60 microns. This data can be zoomed to a magnification of about 25,000X on a typical computer monitor, and quickly examined through serial sections by moving the mouse wheel.*

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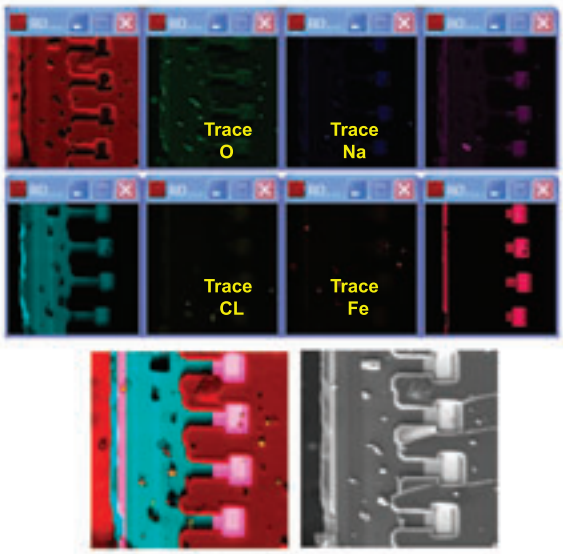

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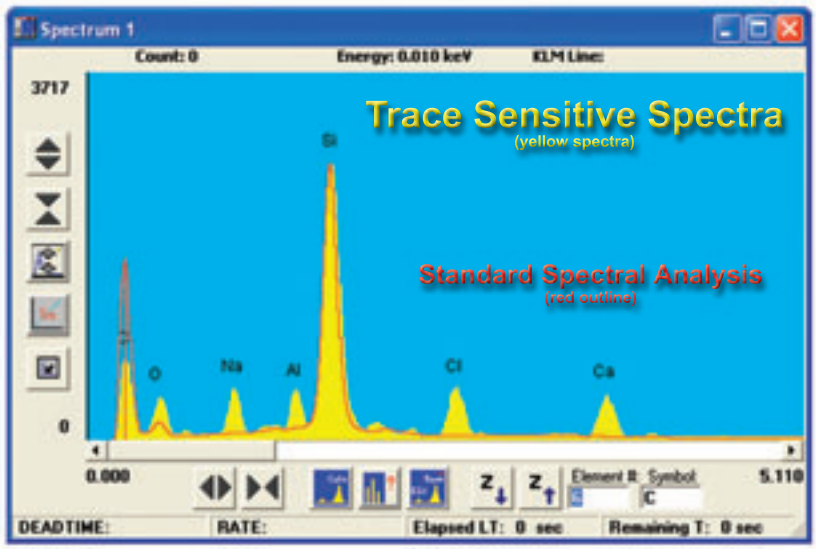

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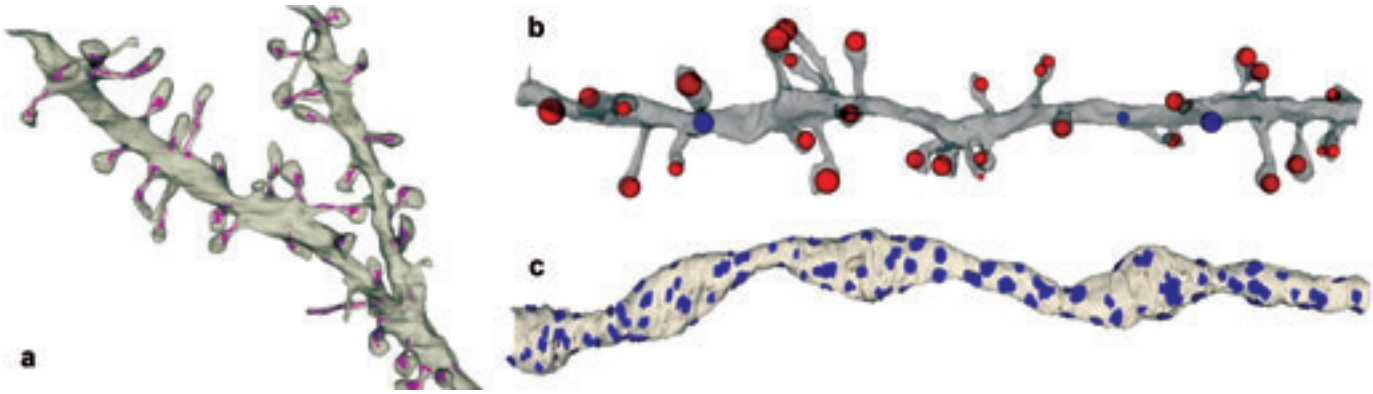

Figure 4. *Some examples of reconstructed dendrites from primate prefrontal cortex.* **a.** *A branched segment of a spiny dendrite is visualized with semi-transparency to show the tubules of spine endoplasmic recticulum (pink). This reconstruction demonstrates that more than 80% of dendritic spines in cerebral cortex contain extensions of the smooth endoplasmic reticulum.* **b.** *A spiny dendrite segment visualized with semi-transparency has excitatory synapses denoted by red spheres and inhibitory synapses denoted by blue spheres. In the characteristic pattern typical of pyramidal neurons, excitatory synapses contact the heads of spines, but inhibitory synapses reside on the shaft of the dendrite.* **c.** *A long segment of dendrite from an inhibitory interneuron reconstructed from a series of over 300 sections. This dendrite has no spines, and is densely covered with excitatory synapses (blue).*

be aligned. The software then computes a transformation for one section that puts it into alignment with the adjacent section. This process can be applied to sections consisting of single images or to sections containing montages of images.

The process of aligning a montage of images begins by choosing a starting section for which the images are free of distortion and the montage is easy to piece together. Import the images for this section one at a time, and use the *Domain Selection Tool* to select the image from the section and drag it into position. Final adjustments are made by moving the image with keyboard commands in blend mode so that both the domain image and the rest of the images in the montage can be seen. Move each image into the correct position to form the montage of the first section. All other sets of section images will be aligned to this initial montage.

After the first section is correctly montaged (Fig. 3), create a new section in Reconstruct to hold the images of the adjacent section. Import one image into this new section, select it, move it into the approximate correct position, and then merge it back into the section. The exact alignment will be determined by making point correspondences between sections. Using an appropriate drawing tool and trace name, create numbered traces on the new image and the adjacent section to indicate points where the images should match up. Select the *Align section* command to align the new image to the first section. When the alignment is satisfactory, delete the correspondence points from both sections and lock them to prevent further movement of the whole sections. Import the next image in the montage and move it into position. Create new correspondence traces for alignment, but instead of aligning the entire section just align the new image. This is done by first selecting the *Align traces* command (which only aligns the traces), then selecting the domain image and repeating the movement on the image. This technique allows each image in the new section to be aligned to the first section's montage. The process continues on adjacent sections until the entire series is aligned.

Once a series is aligned, the process of drawing traces to identify profiles of objects and generate a 3D visualization is relatively simple. The software is capable of automatically surfacing the traces to create a 3D model that is displayed on the computer screen. This object can be rotated and zoomed, and additional objects can be easily added to the scene, to observe the full 3D structure. The final scene can be exported from the software for more detailed rendering using production quality rendering packages such as 3D Studio MAX. Figure 4 shows several 3D renderings of brain structure obtained by exporting scenes from Reconstruct in this way.

The recipe for 3D reconstruction from electron microscopy, while once popular, seemed to have fallen out of favor with many laboratories due to the perceived difficulty and time-consuming nature of the process in comparison with computer-scanning light microscopic imaging. We feel that the basic techniques for serial section electron microscopy are relatively easy to learn and apply, and these time-tested protocols have been indispensable in understanding the structure, plasticity and pathology of the brain [e.g. 5,6,11]. Technical advances will no doubt continue to improve methods of 3D reconstruction and allow even larger data sets to be more easily generated and analyzed. ■

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