

Uniform Serial Sectioning for Transmission Electron Microscopy

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Introduction

The transmission electron microscope (TEM) was first used approximately half a century ago to answer important neurobiological questions and showed 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 of collecting and photographing the serial sections and producing realistic three-dimensional reconstructions.

In neurobiology, there is a renewed interest in serial section electron microscopy (ssEM) (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 ssEM is needed to distinguish and accurately measure dendrites, axons, glial processes, synapses, and subcellular organelles such as synaptic vesicles, polyribosomes, and endosomal compartments. ssEM 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. ssEM 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 ssEM. Photomontaging can be used to enlarge these volumes but at a markedly increased 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, Wetzlar, Germany) at 70 μm (Fig. 1a–c) to permit osmium, uranyl acetate (UA), or other en bloc stains to penetrate uniformly, while allowing visualization of regions of interest in the Epon block later (Fig. 1d). Epon is shaved off in 1–3 μm increments to a region near the stimulating electrode indentation on a neighboring section (Fig. 1h, 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. 1f,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. 1h) because the hydrophilic sections fall apart; however, a few quick sprays of salon-quality hair spray and overnight drying produce uniform continuous ribbons on the same trapezoid (Fig. 1i).

The goal always is to have perfectly uniform section thickness along fold-free ribbons for accurate quantitative analyses (Fig. 2a). A 35° diamond knife minimizes section compression (Figs. 2b,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. 2b). Pioloform that is coated too thickly or with an uneven flow rate altered apparent section thickness (Fig. 2e). Small holes in the Pioloform (Fig. 2f) are avoided by drying the coated slide in a small jar with desiccant. Saturated aqueous UA and freshly prepared Reynolds lead citrate are both filtered through a 0.22 μm syringe filter, and grids were stained in a CO₂-free environment to avoid dark precipitate over the surface of the sections (Fig. 2g). Microwave-enhanced infiltration of epoxy resins had been used to speed the process; however, infiltration was not always uniform, which resulted in cracks (Fig. 2h). 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. 2i). Uneven section thickness (Fig. 2j) is easily corrected by enclosing the ultramicrotome (Fig. 2k) 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. 1g). 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

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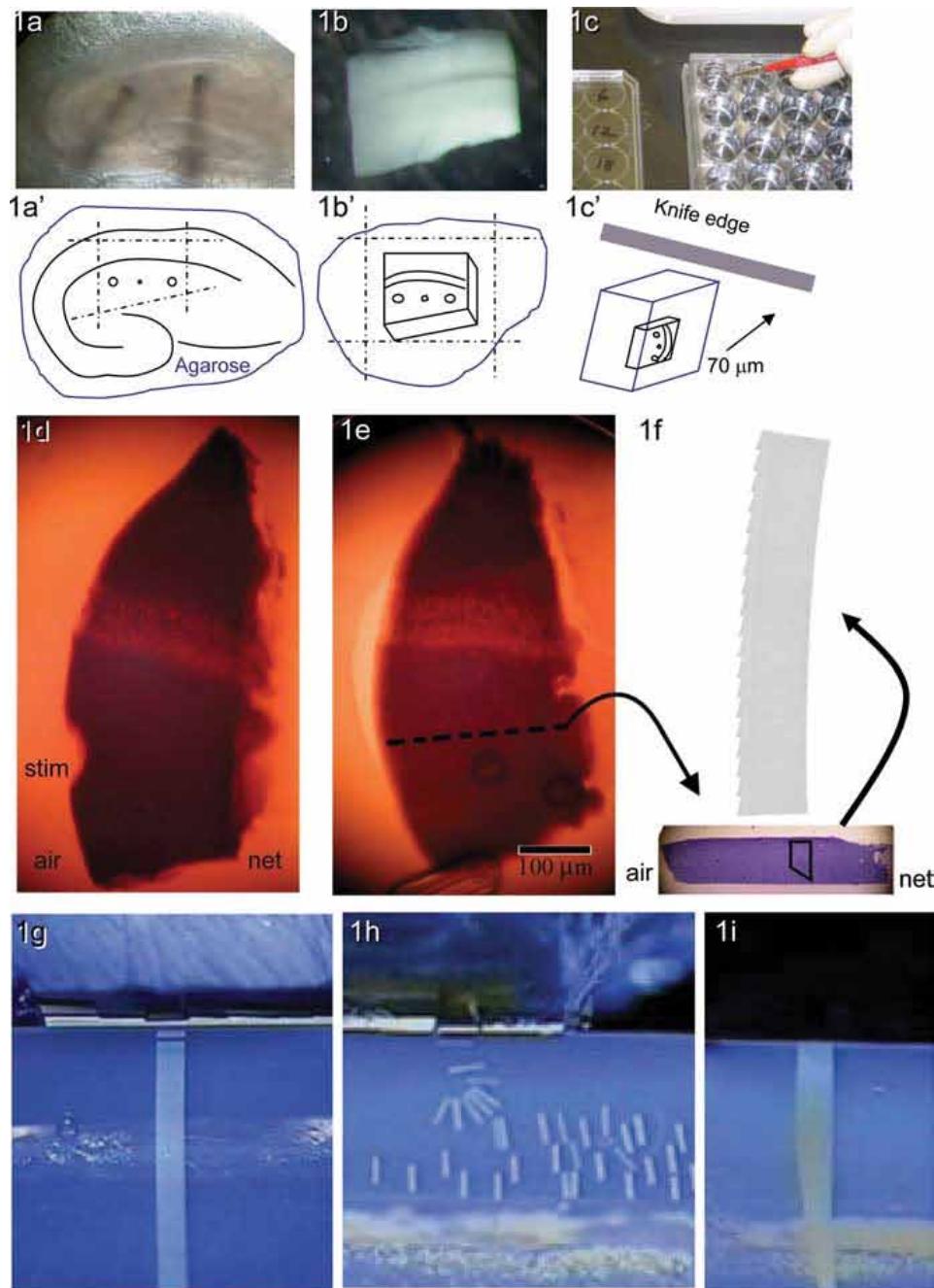


Figure 1. Preparation of serial thin sections from a specific region of interest. **a**, Stimulating electrodes positioned in a hippocampal slice. **a'**, Diagram of a region of interest surrounding the indentations left by the stimulating electrodes on the slice, which is embedded in agarose for stability. **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 occurs 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 placement of 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. Scale bar, 100 μm for **d** and **e**.

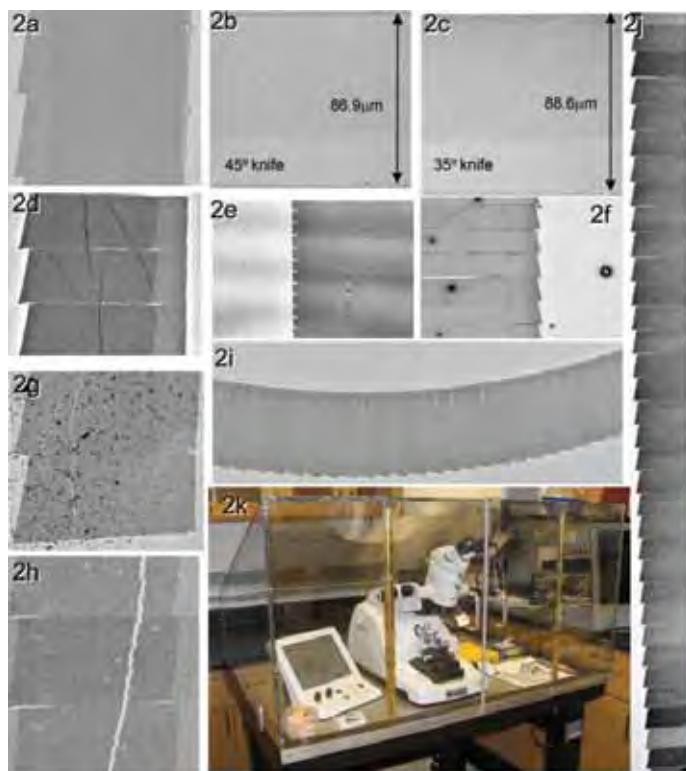


Figure 2. Problems to overcome during ssEM. *a*, Goal is a set of clean serial sections of uniform thickness. *b, c*, Section compression is minimized by using a 35° diamond knife. *d*, Folds in the Pioloform film. *e*, Uneven Pioloform film thickness. *f*, Holes in Pioloform film. *g*, Dirty grid stain. *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.

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://synapses.clm.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 ssEM 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 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. ssEM 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.

Extended Tissue Preparation Methods

The slice fixative contains 6% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer with 2 mM CaCl₂ and 4 mM MgSO₄. The fixed slice may be processed immediately or stored in fixative at room temperature overnight. The next day, the slices are first rinsed in cacodylate and then transferred to 0.1 M phosphate buffer and processed as described below. Storage of the 70 μ m vibra-slices in buffer for more than 1 day

can have detrimental effects, usually resulting in a blurring of membranes and darkening of cytoplasm.

Microwave-enhanced staining and dehydration followed by manual resin infiltration appear to be optimal from the perspective of saving time and having sufficient infiltration. Vibra-slices surrounding the region of interest, e.g., the indentation from the stimulating electrode (Fig. 1g), are postfixed in reduced osmium (1% OsO₄/2% KFeCN in 0.1 M cacodylate) for 5 min, rinsed in cacodylate buffer, and transferred to 1% OsO₄ in 0.1 M cacodylate buffer in the microwave at 175 W under vacuum, with the microwave on for 1 min, off for 1 min, and then on again, for a total of 3 min. Sections are then dehydrated at 250 W under vacuum through a series of ethanol dilutions from 50% to 3 \times 100% for 40 s each, with all dilutions containing 1% UA to enhance polyribosome

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staining. Alternatively, if staining is too intense, try 1 h of aqueous UA with no microwave irradiation followed by the same dehydration schedule without UA. Infiltration with epoxy resins is done outside the microwave oven on a rotator in 1:1 and 1:2 ethanol to propylene oxide (PO) for 10 min each; 2 changes of 100% PO for 15 min each; 1:1 PO:Epoxy resin for 1 h and 1:2 PO:epoxy resin overnight. The next morning, the blocks are transferred through 3 changes of 100% Epoxy resin for 1 h each and then properly oriented in coffin molds with fresh 100% Epoxy resin and cured for 48 h in a conventional oven at 60°C. All mixtures with Epoxy resins contain the DMP-30 accelerator. These procedures produce Epoxy blocks of uniform infiltration and of sufficient hardness to obtain thousands of serial ultrathin sections.

Casting Pioloform Coated Grids

Serial thin sections are picked up on Pioloform-coated slot grids, which were prepared within 24 h prior to cutting the series (supplemental Figs. 1, 2; supplemental movies 1–3). Longer delays can result in folds owing to sagging of the Pioloform film under the weight of the sections during pickup (Fig. 2c). A film-casting device (Electron Microscopy Sciences [EMS] #71305-01) is used to pump 1.2% Pioloform (Ladd Research Industries, #19244) in chloroform into a cylindrical chamber containing a Gold Seal (EMS #63718-05) slide (supplemental Fig. 1). Clean Synaptek notched slot grids (EMS #S2010 or Pella #4514) should be cleaned in advance (supplemental Fig. 2). The Pioloform is forced into the chamber by squeezing the bulb, and then allowed to drain slowly down the slide into the flask. The drain rate is controlled by a stopcock on the side of the caster device. The slower the solution drains, the thinner the Pioloform film; the faster it drains, the thicker the film.

Immediately after the slide is drained, it is placed for 1 min in a desiccant jar with the base of jar filled to a 5 cm depth with silica gel to facilitate drying. Both sides of the slide are then scored with a single-edged razor blade, and the slide is vertically submerged in a flat-bottomed glass bowl and slowly raised from the water (supplemental movie 1). The slide is again slowly lowered vertically into the water while the surface tension of the water causes the films on both sides of the slide to gently float off, onto the water's surface. Films are visually examined to determine whether they are suitable for grid coating. The ideal film thickness is approximately 50–60 nm and is silver/platinum in interference color. The film should be uniform in color, with no wave patterns or folds. If there are any imperfections in the film, it should be discarded and another slide should be coated.

Synaptek notched slot grids (EMS #S2010) are gently dropped, notched side up, onto the film (supplemental movie 2). A perfect film can hold up to 30 evenly spaced grids. The edges of the grid are gently tapped to secure the grid to the film surface (supplemental movie 3). A dry glass slide is then wrapped with Parafilm and positioned directly over the floating film. The tip of the slide is pressed down on one end of the film and then "scooped" up through the water, with the coated grids lying atop the slide, film side up (supplemental movie 2). The slides with grids are then stored in a Petri dish in a dust-free environment until they dry completely and are ready for use within 24 h.

Trapezoid Placement

A trapezoid with one straight side and one slanted side makes it easy to cut and orient the serial sections, minimize chatter, and conserve tissue for future analysis. Smaller trapezoids allow more serial sections to fit on a single-slot grid. Therefore, trimming the trapezoid to a specific location is important to ensure that the structures of interest will present in the serial thin sections. A Diatome Cryotrim 45 tool (Diatome, Biel, Switzerland, #CryoTrim) is used to create the mesa and final series trapezoid in the region of interest (supplemental Fig. 2a).

First, test thick and thin sections are cut across the entire width of the slice from the air to the net surface and viewed in the light and electron microscope to evaluate tissue quality. If tissue quality is deemed suitable, a mesa is trimmed to the approximate region of interest (supplemental Fig. 2b). A test thick and thin section are obtained in the region of the mesa and measured to position the final series trapezoid (supplemental Fig. 2c). The series trapezoid is trimmed to achieve 200–300 ultrathin sections. The east–west sides of the trapezoid are trimmed first to a width of about 100 µm, spanning the 70 µm thickness of the vibra-slice, and a depth of 20–30 µm (supplemental movie 4). Then the north–south sides are trimmed to a height of about 30 µm and depth of 15–20 µm for stability (supplemental movie 5).

Cutting Serial Thin Sections

The base of the series trapezoid is carefully aligned to the edge of the diamond knife. To reduce compression, a Diatome Ultra 35° knife (Diatome) is used instead of the traditional 45° knife. Once the block is aligned, the knife is retracted 22–23 µm (slightly more than the 20 µm height of the trapezoid) so that the top of the trapezoid will be clear of the knife edge at the start of approach. The block can approach the knife rapidly, at 45–50 nm increments, without worry of damaging the knife edge. As soon

as the first section is cut, the speed is adjusted to 1 mm/s and remains at that speed for the entire series (supplemental movie 6). Once the first sections come off the knife, the microtomist can tell whether the series ribbon is going to be curved or straight. A very slight curve of the series is acceptable for pickup and future photography, but a deep curve will render the series hard to break apart into segments that can be placed on the slot grid.

After determining that the ribbon is cutting straight, the microtomist sets the counter to zero, closes and latches the Plexiglas enclosure doors, and leaves the room. The sectioning process is viewed on a monitor in the adjacent room so as not to disturb the sectioning through any vibration, air currents, or body heat. If the sectioning is proceeding smoothly, a periodic look at the counter on the Leica UC6 Ultramicrotome (Leica) is necessary to see how many sections have been cut. A perfectly straight ribbon derived from a trapezoid that is 20 μm high will produce approximately 300 serial sections.

After the series is cut, the doors of the enclosure are opened and the arm advance is stopped. The number of sections is recorded. Ideally, the series will be one long, unbroken ribbon of 300 sections. Usually, the ribbon is broken into 35–50 sections using a very fine eyelash (The Perfect Eyelash Set [EMS #70616-10]). The tip of the eyelash is dipped into the water next to the floating ribbon to be sure that it is clean, which is determined by viewing the surface tension of the water and looking at the edges of the eyelash. Then, the intersection between two sections is gently tapped with the tip of the eyelash. The sections should slowly separate upon tapping, and the segment of ~30 sections is isolated into an area of the boat away from the main ribbon (supplemental movie 7). A pair of closed-curved number 7 forceps is used to poke tiny holes around each grid to separate the grid from the surrounding Pioloform film (supplemental movie 8). Once the grid edge is perforated, the grid is lifted from the film using forceps. Alternatively, the grid can be picked up using the shimmy method (supplemental movie 9). Occasionally, there will be tiny “shards” of Pioloform hanging off the side of the grid. If the grid is gently tapped on the slide or some other very clean surface, the shard will adhere to the side of the grid, will not be visible, and will not interfere with section pickup.

The slot grid is carefully inspected for holes and any other imperfections, lowered into the water (with Pioloform three-fourths submerged and slot horizontally oriented) beside the ribbon segment,

and gently wafted back and forth, drawing the ribbon segment toward the center of the film slot. When the ribbon segment is almost touching the film, the grid is carefully lifted out of the water, with the ribbon segment adhering to the film in the middle of the slot (supplemental movie 7).

After ribbon pickup, the forceps are fastened with rubber O-rings (EMS #72903-12) and placed in a covered dust-free Petri dish to dry before staining. Subsequent ribbon segments are tapped away from the main series, isolated, oriented, and picked up on slot grids, then placed in the Petri dish and allowed to dry thoroughly.

Staining, Rinsing, and Drying the Grids

Dry grids are stained with 6.25% aqueous UA and Reynolds’ lead citrate. Both of these reagents can be prepared in advance and stored at 4°C in a refrigerator in syringes with all of the air extracted from them. The base of a 55 mm glass Petri dish is placed on a hot plate, filled with dental wax, and heated until all the wax is melted. The Petri dish is then set aside to cool completely. After cooling, individual shallow slits are carved in the dental wax using a razor blade or scalpel to provide indentations for the grids to be inserted vertically on edge. Dental wax is chosen for its slightly sticky quality. The entire series can then be stained uniformly.

Each grid is first placed in the slot (supplemental movie 10) and then gently pushed down using the back side of the forceps until secure, thereby immobilizing each grid during the staining process. Once all the grids are loaded into the dental wax, a drop of filtered 6.25% UA is positioned with a blunt-tip needle onto the section side of each grid surface and allowed to remain for 5 min. The top of the Petri dish covers the staining chamber to prevent dust accumulation or stain evaporation. After 5 min, the Petri dish is tilted, allowing the UA stain to be collected in a nearby tri-pour beaker. A gentle stream of double-distilled or 18° water is directed down the side of the Petri dish until all of the grids are thoroughly covered in water. Once the grids are covered, the Petri dish is tilted, allowing the rinse water to be collected in the tri-pour beaker. This rinsing procedure is repeated at least 10 times to completely rid the grids of any remaining UA precipitate. Each grid is then “wicked” dry of residual water using the tip of an angled piece of Whatman #1 filter paper (Whatman, Kent, UK), carefully touched near the base of the grid. Care should be taken not to poke the film of the grid with the filter paper or else the grid can pop and sections will be lost.

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A round 55 mm piece of filter paper is saturated with a 0.02 N NaOH solution and inserted into the lid of the Petri dish to prevent lead precipitation with carbon dioxide while staining with lead citrate. Lead citrate stain is applied on the section side of the grids through a syringe needle. The grids are then covered and left to stain for 5 min. The lead citrate is then poured off into a separate tri-pour and the grids rinsed 4 times with a stream of 0.02 N NaOH from a squirt bottle, followed by 8 rinses with distilled (18 $^{\circ}$) water. Each grid is again thoroughly wicked with filter paper and allowed to dry thoroughly (usually overnight) prior to loading it into grid cassettes for viewing in the electron microscope.

Loading Grids into Grid Cassettes and Storing Them in Gelatin Capsules

Load grids into copper and brass grid cassettes before loading them in the specimen holder of the electron microscope (supplemental movie 11). These grid cassettes provide additional security against breaking the Pioloform film. The grid cassettes are designed to fit into a rotational specimen holder of the electron microscope for proper orientation during photography. We use the Gatan rotational holder (Gatan, Pleasanton, CA) in a JEOL electron microscope (JEOL, Tokyo, Japan), but similar grid cassettes could be designed for other electron microscopes. The grid cassette has two parts: a round copper base with a shallow ledge inside on which the grid rests, and a brass insert that slides down inside the base when it is compressed with forceps and springs open when the forceps are released to hold the grid securely. Each grid cassette is stored in a 00 gelatin capsule (EMS #70100) to provide a dust-free environment. The gelatin capsules are labeled and stored in a Plexiglas dessicator cabinet.

Overcoming Nonribboning in a Lowicryl Series

Lowicryl and some other resins are notoriously difficult to ribbon because the hydrophilic sections fall apart (supplemental movie 12). A few quick sprays of salon-quality hair spray (supplemental movie 13) and overnight drying produce uniform continuous ribbons on the same trapezoid (supplemental movie 14).

Reviewing the Series Ribbon in the TEM

Each ribbon of sections is inspected in the electron microscope to ensure that the grids for each series are

in order. First, a low-magnification (50 \times) picture is taken of the entire grid to check that the orientation of the sections is correct and to count the number of sections on the grid. Next, the upper-right corner of the first and last sections are photographed at high magnification (5000 \times). The next grid in the series is imaged in the same way, and the upper-right corner of the last section of the previous grid is compared with the upper-right corner of the first section of the current grid. If the sections do not match, the grids may be out of order, or a few sections may have been lost. By photographing the first and last section of every grid, the images can then be matched up and the grids placed in the proper sequence. If a few sections are missing, either a shorter continuous series will be imaged or a new series should be cut.

Selecting a Region of Interest and Imaging a Series

After the sections have been counted and the grids determined to be in the proper order, a region of interest is selected for imaging of the series. Start with a low-magnification setting (50 \times) and allow the beam to stabilize (etch) the entire Pioloform film for about 5 min. This strategy will minimize image drift during subsequent photography. Photograph a low-magnification (500 \times) image of the entire first section on the first grid of the series. Then obtain about 10–15 higher magnification (5000 \times) images at potential sample locations across the entire section. The region of interest is selected from these sample images to optimize analysis of target structures located within the photographic area, e.g., the total number of cross-sectioned dendrites available for analysis or the presence of an astroglial cell body that one wants to examine. The region of interest is then photographed on all the subsequent serial sections, usually retaining a single structure (e.g., a cross-sectioned dendrite) in the middle of the image. Although neighboring structures may migrate out of the image across hundreds of serial sections, this strategy ensures that at least some of the target structures remain within the photographic series. Periodically check to determine that the top and bottom edges are oriented parallel to the top and bottom of the photographic fields; this is best done by moving the top right-angle corner of the section into a corner of the photographic field. If the ribbon curves, you will need to rotate the section periodically to ensure that most of the region of interest remains within the field of view.

Materials and Instrumentation

Leica UC6 Ultramicrotome; VT1000S Leica, Vibrating Blade Microtome; Synaptek grids (EMS #S2010); Film Caster (EMS #71305-01); Diatome Ultra 35° (Diatome #Ultra) thin sectioning diamond knife; and The Perfect Eyelash Set (EMS #70616-10 or Ted Pella #113).

Supplemental Methods

This paper was previously published in *The Journal of Neuroscience* (2006;26:12101-12103). All methods are posted at synapses.clm.utexas.edu. Figures and movies can be viewed at <http://www.jneurosci.org/content/26/47/12101/suppl/DC1>.

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