Supplemental Methods:

All methods are posted at our website: www.synapse-web.org or www.synapses.clm.utexas.edu and updated as needed.

Extended Tissue Preparation Methods:

The slice fixative contains 6% glutaraldehyde and 2 % paraformaldehyde in 0.1 M cacodylate buffer with 2 mM $CaCl_2$ and 4 mM $MgSO_4$. 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 micron vibra-slices in buffer for more than a 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 (Figure 1g), are post fixed in reduced osmium (1% OsO_4/2% KFeCN in 0.1 M cacodylate) for 5 minutes, rinsed in cacodylate buffer and transferred to 1% OsO_4 in 0.1 M cacodylate buffer in the MW at 175 W under vacuum with the MW on for 1 min off for 1 min and then on again for a total of 3 min. Sections are then dehydrated at 250W under vacuum through a series of ethanol dilutions from 50% to 3x100% for 40 seconds each with all dilutions containing 1% uranyl acetate (UA) to enhance polyribosome staining. Alternatively, if staining is too intense, try 1 hour of aqueous UA with no microwave irradiation followed by the same dehydration schedule without UA.

Infiltration with Epoxy resins is done outside the MW oven on a rotator in 1:1 and1:2 ethanol to propylene oxide (PO) for 10 min each; 2 changes of 100% PO for 15 minutes each; 1:1 PO:Epoxy resin for 1 hour and 1:2 PO:Epoxy resin overnight. The next morning the blocks are transferred through 3 changes of 100% Epoxy resin for one hour each and then properly oriented in coffin molds with fresh 100% Epoxy resin and cured for 48 hours in a conventional oven at 60^{o}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 1000's of serial ultrathin sections.

Casting Pioloform Coated Grids

(Supplemental Figures (SF) 1,2; Supplemental Movies (MV) 1-3)

Serial thin sections are picked up on Pioloform-coated slot grids which were prepared within 24 hours prior to cutting the series. Longer delays can result in folds due to sagging of the Pioloform film under the weight of the sections during pickup (e.g. Figure 2c). A film casting device (EMS# 71305-1) is used to pump 1.2% Pioloform (Ladd # 19244) in chloroform into a cylindrical chamber containing a Gold Seal™ slide (SF1). Clean Synaptek notched slot grids (EMS# S2010 or Pella #4514) should be cleaned in advance (SF2). 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 stop-cock 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 one minute in a desiccant jar with the base of jar

filled to a 2 inch 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 (MV1). 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 to 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 (MV2). A perfect film can hold up to 30 grids evenly spaced. The edges of the grid are gently tapped to secure the grid to the film surface (MV3). 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 (MV2). 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 hours.

Trapezoid Placement (SF2, MV 3-6)

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 be sure the structures of interest will present in the serial thin sections. A Diatome 45^{o} Cryotrim tool is used to create the mesa and final series trapezoid in the region of interest (SF2a).

First, test thick and thin sections are cut across the entire width of the slice from the air to 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 (SF2b). A test thick and thin section are obtained in the region of the Mesa and measured to position the final series trapezoid (SF2c). The series trapezoid is trimmed to achieve 200 to 300 ultrathin sections. The east-west sides of the trapezoid are trimmed first to a width of about 100 microns spanning the 70 micron thickness of the vibra-slice and a depth of 20-30 microns (MV4). Then the north-south sides are trimmed for a height of about 30 microns and depth of 15-20 microns for stability (MV5).

Cutting Serial Thin Sections (MV6-9)

The base of the series trapezoid is carefully aligned to the edge of the diamond knife. A Diatome 35° Ultra knife is used instead of the traditional 45° knife to reduce compression. Once the block is aligned, the knife is retracted 22-23 microns (this is slightly more than the 20 micron height of the trapezoid) so 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 1mm/sec and remains at that speed for the entire series (MV6).

Once the first sections come off the knife, the microtomist can tell if the series ribbon is going to be curved or straight. A very slight curve of the series is acceptable for pick up 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 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 UC6 is necessary to see how many sections have been cut. A perfectly straight ribbon derived from a trapezoid that 20 microns high will produce approximately 300 serial sections.

The doors of the enclosure are opened and the arm advance is stopped after the series is cut. The number of sections is recorded. Ideally, the series will be one long unbroken ribbon of 300 sections. Usually, the ribbon is broken 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 intersection between two sections is gently tapped with the tip of the eyelash. The sections should slowly separate upon tapping and the 30 or so section segment is isolated into an area of the boat away from the main ribbon (MV7).

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 (MV8). Once the grid edge is perforated, the grid is lifted with forceps from the film. Alternatively, the grid can be picked up using the shimmy method (MV9). 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 pick up.

The slot grid is carefully inspected for holes or any other imperfections, lowered into the water (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 (MV7).

After ribbon pick up, 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, placed in the Petri dish, and allowed to dry thoroughly.

Staining, rinsing, drying the grids (MV10)

Dry grids are stained with 6.25% aqueous Uranyl Acetate and Reynolds Lead Citrate. Both of these reagents can be prepared in advance and stored in syringes with all of the air extracted from them at 4° in a refrigerator. The base of

a 55 mm glass Petri dish is placed on a hot plate, filled with dental wax, and heated until all of 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 (MV10) and then gently pushed down using the back side of the forceps until secure immobilizing each grid during the staining process. Once all the grids are loaded in 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 minutes. The top of the Petri dish covers the staining chamber to prevent dust accumulation or stain evaporation. After 5 minutes 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 ohm 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 ten 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 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 the grid can pop and sections will be lost.

A round 55 mm piece of filter paper is saturated with a 0.02N 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 minutes. The lead citrate is then poured off into a separate tri-pour; the grids rinsed 4 times with a stream of 0.02 N NaOH from a squirt bottle, followed by eight rinses with distilled (18 ohm) water. Each grid is again thoroughly wicked with filter paper and allowed to dry thoroughly (usually overnight) prior to loading it in grid cassettes for viewing in the electron microscope.

Loading grids into a grid-cassettes and storing in gelatin capsules (MV11)

Load grids into copper and brass grid cassettes before loading them in the specimen holder of the electron microscope. These grids cassettes provide additional security from 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 in a JEOL electron microscope, but similar grid cassettes could be designed for other electron scopes. 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 a 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 non-ribboning Lowicryl series (MV12-14)

Lowicryl and some other resins are notoriously difficult to ribbon because the hydrophilic sections fall apart (MV12). A few quick sprays of salon quality hair spray (MV13) and overnight drying produces uniform continuous ribbons on the same trapezoid (MV14).

Reviewing the Series Ribbon in the TEM

Each ribbon of sections is inspected in the electron microscope to ensure that the grids for a series are in order. First, a low magnification (50x) 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 (5,000x). 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 to 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

A region of interest is selected for imaging of the series after the sections have been counted and the grids are determined to be in the proper order. Start with a low magnification setting (50x) and allow the beam to stabilize (etch) the entire Pioloform film for about 5 minutes. This strategy will minimize image drift during subsequent photography. Photograph a low magnification (500x) image of the entire first section on the first grid of the series. Then obtain about 10 - 15 higher magnification (5000x) 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 analyis, or the presence of an astroglial cell body that one wants to examine etc.. The region of interest is then photographed on all of 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 will 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 - EM EMS# 71305-1S # S2010

Film Caster –EMS# 71305-1

Diatome 35°Ultra Thin Sectioning Diamond Knife

The Perfect Eyelash Set – EMS# 70616-10