Detailed protocol for conjugate IF-EM AT using freeze substitution and embedding in Lowicryl HM-20

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1. Tissue Fixation: Chemical fixation by perfusion

1A. Required materials and equipment:

Anesthetic
Paraformaldehyde (reagent grade, crystalline; Sigma-Aldrich #P6148)
Glutaraldehyde (50%, EM grade; Electron Microscopy Sciences #16310)
Sodium phosphate, monobasic, monohydrate
Sodium phosphate, dibasic, anhydrous (or dibasic heptahydrate)
Sodium chloride
Sodium heparin
Hazardous waste collection containers
Fume hood
Surgical tools
Gloves, safety glasses, coats (PPE)
Storage vials or tubes (20 ml scintillation vials or culture/centrifuge tubes)

1B. Preparations:

0.1M phosphate buffer, pH 6.8 (PB 6.8):

6.93 g sodium phosphate, monobasic, monohydrate
7.06 g sodium phosphate, dibasic, anhydrous (or 13.32 g dibasic heptahydrate)
Distilled or deionized water up to 1 L, titrate as needed

Fixative: 2% paraformaldehyde/2% glutaraldehyde in PB 6.8. Prepare same day, keep at room temperature,

For 100 ml (per mouse):
2 g paraformaldehyde
Dissolve in warm, but not boiling:
100 ml PB 6.8
Cool solution to room temperature, add:
4 ml 50% glutaraldehyde
Filter (we use vacuum filtration with Whatman #40 filter paper)

Heparinized normal saline

9 g sodium chloride
1 L distilled or deionized water
Add 1000 units/100 ml of sodium heparin

1C. Procedures:

Deeply anesthetize mice (e.g. sodium pentobarbital 80-100 mg/kg IP, no response to firm forepaw pinch), surgically open the chest to expose pericardial region, cut the right atrium, and insert a perfusion cannula into the left ventricle. We use gravity (i.e. pressure-based) flow, aiming for ~ 3 feet hydrostatic pressure. A peristaltic pump also works; most important is technical success, getting fix to the brain reasonably quickly so the body and brain harden properly. After a brief (~30 second) flush with normal saline, run fixative for ~10 minutes. Remove the brain, put it in the same fixative, store overnight at 4°C, then replace with buffer and keep in the refrigerator. Fixed brains are stable in the refrigerator for > 1 week.

Collect fixative for hazardous waste disposal. Procedure should take place in a hood or with personnel-protecting ventilation, as fumes are hazardous.

2. Tissue blocking, mounting, sectioning

2A. Required materials and equipment:

Double-edged razor blades
Tweezers/forceps
Cyanoacrylate cement (we use "Crazy Glue")
70-95% ethanol in dispensing bottle (for cleaning blades and Vibratome)
Vibratome and mounting blocks
Saline and saline ice cubes
Paint brushes, or other section pickers
Collection trays (24-well cell culture trays work well)
Tray sealers (adhesive plastic film) minimize buffer loss and tissue dehydration
0.025% sodium azide in 0.01 M PBS (only for long storage, do not allow contact with metal, collect for disposal as hazardous waste)
Petri dish and/or dental wax

2B. Preparations:

0.01 M PBS (store in refrigerator)
- 0.2 g sodium phosphate monobasic, monohydrate
- 2.6 g sodium phosphate dibasic, heptahydrate
- 8.8 g sodium chloride
- 1 L distilled or deionized water

Sodium azide storage solution (collect used solutions as hazardous waste)
- 0.25 g sodium azide (use non-metal spatula)
- 1 L 0.01 M PBS

2C. Procedures:

Remove brain from storage vial and place in a petri dish containing buffer or PBS. Use tweezers or fine forceps under a dissecting microscope to peel and remove as much remaining vasculature and pial membrane as you can. Using a razor blade, make a block of tissue and glue it to a mounting block using Crazy Glue (not too much or it will rise into tissue gaps and cause sectioning problems). Avoid getting

glue on fingers; if skin glues to block, remove glue with acetone. Re-immerse in buffer to keep tissue moist.

Set up Vibratome with saline and saline ice cubes or other chilling means. Have collection trays filled with buffer +/- azide on a chilled surface (we often use shipping cold packs, which we store in a freezer); mounted tissue blocks should be kept wet and cold until sectioned. For freeze-substitution processing, cut sections 100-200 µm thick. We typically cut 200 µm for capsule embedment of small pieces, but if processing an entire section it should be no more than 100 µm to ensure proper resin cure. It is usually wise to cut some 50 µm sections between sets of 200's for Nissl-stained reference or other LM/EM purposes. If possible, the block surface glued to base should be larger than the portion to be cut.

3. Freeze-substitution and embedment in Lowicryl HM-20

3A. Required materials and equipment:

Processing shell vials with caps (1 dram EMS/Fisher) and vial holders (aluminum, EMS #72640), and/or scintillation vials (20 ml)
Fine scalpel blades (#11 or #15)
Small, fine-tipped paint brush
Styrofoam box(es), +/- foil lining
Dry ice
100% ethanol (200 proof)
Cold packs
Disposable polyethylene transfer pipets (Fisher #13-711-9CM)
Glycerol
$CaCl_2$
Sodium acetate trihydrate (ACS)
Uranyl acetate (depleted, available from EMS, Ted Pella, Fisher)
Methanol, (absolute, certified ACS, Fisher Scientific #A412-500)
Lowicryl HM-20 embedding kit (EMS #14340, or Ted Pella)
Freeze-substitution unit (Leica AFS or equivalent) with flow-through capsules and gelatin capsules for small chunks (1 mm x 1-2 mm x 200 µm)
For whole sections (100 µm thick), use baskets (Leica #G3314A)
For wafering and curing of sections: glass slides, scoring tool, ACLAR, and upside-down metal cans for wafer support
ACLAR embedding film (7.8 mil thickness, EMS #50425)
Oven capable of 60° C for drying things before use
Independent temperature probe for AFS unit (Omega HH800A with Omega 5SC-TT-K-36-36 precision fine wire thermocouple, insulated, self-adhesive)
Acetone (can be used as substitution medium, but must be strictly anhydrous and doesn't work as well as methanol in humid climates or conditions); we use acetone mainly for cleaning.

Day 1:

Preparations:

0.1 M sodium acetate (NaA): 1.36 g sodium acetate trihydrate/100 ml distilled water, store at 4°C

0.1% $CaCl_2$: Make a 10% stock solution in water, store at 4° C; dilute 1:100 with NaA as needed

Glycerol Dilutions (keep at 4° C):
10% in NaA
20% in NaA
30% in NaA

Substitution solution: 1.5 - 4% (varies, depending on lot) uranyl acetate in anhydrous methanol, make day before needed, store tightly capped at 4° C, filter (0.2 µm PES 13 mm syringe filter); standard run uses 15 ml (5 ml/can)

Pre-clean Flo-through capsules (put capsules in a can with absolute ethanol or methanol, swirl, then remove one by one with forceps and force solvent through capsule with pipet before setting on Kimwipe to dry). Do the same for capsule holders, ACLAR spears (2 cm x 2 mm), and cans (for sections: baskets and ACLAR strips—2 cm x 6-8 mm), dry overnight in 60° C oven.

Procedures:

Select sections and put in labeled shell vials with 0.1 M NaA, keep all chilled, we set the vials in aluminum vial holders on shipping cold packs in a Styrofoam box on a gently rotating shaker.

Pretreatment:
3 x 10 min changes of NaA
1 h in 0.1% $CaCl_2$/NaA
3 x 5 min rinses in NaA

Cryoprotection, keep chilled throughout:
30 min in 10% glycerol/NaA
30 min in 20% glycerol/NaA
30 min in 30% glycerol/NaA
Overnight in 30% glycerol/NaA

Day 2

Procedures:

Prepare AFS unit by filling with liquid nitrogen, insert black-topped tube, turn on and set the following program (can be modified):
T1= -90° C for 32 h
S1= +4° C /h

T2= -45° C for 50 h
S2= +5° C/h
T3= 0° C for 40 h

Hit “Start” and then immediately “Pause”. Machine will go to T1 setting and hold. The instrument is subject to defective temperature monitoring; accordingly, when it displays the T1 temperature, the temperature should be independently verified using an independent probe or solutions of known freezing point. If necessary, adjust settings to achieve desired temperature. When all samples are in the machine, hit “Pause” again and program will start. *Troubleshoot—things freezing when they shouldn’t—if chamber temperature is verified, check that TF control knob isn’t set too high (too far right).*

Set up a freezing box (foil-lined Styrofoam box with about 1" 100% ethanol and dry ice bath), load capsules into labeled holders, fill each holder with about 5 ml absolute methanol and place in freezing bath to cool (cover when not transferring samples).

Under a dissecting microscope, place a section with a modest amount of cold 30% glycerol in a petri dish or on dental wax. Use a scalpel blade to excise desired areas of a size suitable for ultramicrotomy from cryoprotected sections (remainder of section can be returned to vial and stored for future use). Use a fine brush to transfer each sample to the tip of an ACLAR spear (2 cm long x 2 mm wide tapered at one end), blot the brush and use it to wick off excess glycerol. Then transfer the loaded spear to the first capsule (refer to capsule numbering scheme in manual, #1 is marked by dots) and drop it in. As soon as one holder is loaded (8 samples), transfer it to the AFS chamber. Continue until all holders are loaded and transferred to AFS. Hit “Pause” to start the programmed run.

** If using 100 µm sections for flat embedding, mount the cryoprotected sections on 2 cm x 6-8 mm ACLAR strips (both sides if necessary), and insert upright into mesh-bottomed baskets in cans. Sections must be small enough to be immersed while upright. Baskets (4 /can) can be notched to identify and cans tape-labeled. Sections can be wafer-embedded after substitution and infiltration and either cured in the AFS or removed to a chilled, external UV equipped chamber.

Once the program is started:

Chill any new solution in a separate can in the AFS for 5 - 10 min; also provide a can to receive waste solutions.

Briefly chill 2 disposable pipets (1 for removal of solutions, 1 to transfer new solutions).

2 x 10 min changes of anhydrous methanol (3 changes if there are moisture concerns).

After each change, use the new solution pipet to gently suck up and down in the central tube in each capsule holder, to mix the solution.

Replace the final methanol rinse with uranyl acetate/methanol. After mixing in each holder, close the machine, lower the glass plate, and detach it from the lifter.

Collect ethanol and methanol for hazardous waste disposal.

Clean used cans.

Day 3

Procedures (T1, S1):

Chill a pipet and gently mix solutions. Using a forceps carefully raise an ACLAR spear, if there is no sample attached to it, remove it to a rinsing container. If a sample is still attached, lower it back into its capsule and gently tap the spear against the bottom and flex it a bit, then check again to see if the sample has detached. Repeat if necessary, or move on and come back. Continue until all spears are removed. If desperate, chill a tool and use it to dislodge the sample. Do a final round of gentle solution mixing, then close lid, lower glass, and detach it from the lifter.

Day 4

Preparations:

Lowicryl HM-20	20 ml	15 ml	10 ml
Crosslinker D	2.98 g	2.24 g	1.49 g
Monomer E	17.02 g	12.76 g	8.51 g
Mix gently, add			
Initiator C	0.1 g	0.075 g	0.05 g

Mix by swirling gently, do not incorporate air bubbles.

5 ml of solution are needed for each holder. Dedicated 20 ml vials with caps for full strength Lowicryl and for mixtures with methanol are useful. Keep solutions in dark as much as possible.

For 1:1 Lowicryl/methanol— Make up 20 ml as above, take 8 ml and combine with 8 ml methanol in mixing bottle, swirl gently to mix.

For 2:1 Lowicryl/methanol—Take remaining 12 ml from the 20 ml above and combine with 6 ml methanol in mixing bottle.

Dry overnight at 60° C:

The larger parts of 24 gelatin capsules (Leica 16702745) size 1
Spider covers (to pick up and transfer capsules)
Stem holders for spider covers
Bottom plate (spacer) for cans
4-5 universal cans
Chamber for gelatin capsules

Procedures (T2):

Chill solutions and pipets as in beginning.
Remove uranyl acetate solution (collect for hazardous waste disposal).
3 x 15 min rinses in methanol, with gentle mixing as before (collect for disposal).

2 h 1:1 Lowicryl/methanol
2 h 2:1 Lowicryl/methanol
2 h Lowicryl (need 5 ml/capsule holder)
Overnight Lowicryl (need 5 ml/ capsule holder)

Collect all for hazardous waste disposal.

Day 5

Required materials:

100% Ethanol
Oven-dried gelatin capsules, spider covers, stem holders, bottom spacer plates, cans, chamber for gelatin capsules
Cryomanipulator with M4 thread
Red temperature tube

Preparations:

10 ml Lowicryl (see recipe above)

Set a stem holder in each of 3 curing containers (label cans) and place a bottom spacer plate on top of its base.

Procedures (T2, S2, T3):

Chill Lowicryl, pipets and chamber for gelatin capsules.

Gently jiggle/tap capsule holders to encourage tissue to settle at the bottom of capsules.

Insert gelatin capsules in chamber.

Fill gelatin capsules with Lowicryl.

Screw cryomanipulator into socket of spider cover.

Align arrow of spider cover with position #1 of capsule holder (between 2 dots).

Push spider cover down into capsules, lift slightly and carefully check that all capsules are attached (you may have to try more than once; don't lose your alignment). When possible, move the spider cover with capsules to the gelatin capsule chamber and insert tissue capsules into gelatin capsules, pushing down to attach the gelatin capsules to the spider cover. Again, lift slightly and check for attachment. Unscrew the cryomanipulator, but leave the spider cover in place. Remove the empty capsule holder, mop up any spilled Lowicryl, and insert a labeled curing container (prepared as above) to which 10 ml 100% ethanol has been added. Chill. When chilled, use the insulated tweezers to transfer the loaded spider cover (again, lift slightly to check attachment and push down to re-engage if necessary) to the stem holder. The bottom spacer plate is used to help ensure that even if a gelatin/tissue capsule detaches in the curing container it will remain in place and not fall over into the ethanol. Insert and fill the next set of gelatin capsules (transfer the Lowicryl

within the gelatin chamber as needed). Then repeat as above until all spider cover/capsule sets are seated on their stem holders. Remove gelatin chamber and Lowicryl can. Mop the cryochamber floor and temperature tube top with a Kimwipe so no Lowicryl puddles remain.

Replace the black temperature tube with the red one.

Close cover, lower and release glass plate, re-open cover and insert UV lamp apparatus, connect to AFS and plug into power. Turn on the UV power switch. The UV indicator light on control panel should show a steady light. If it is flashing, the UV light is not lit. Check connections and seating of apparatus.

Days 6, 7: No procedures

Day 8

Materials and Equipment:

Acetone for cleaning
Styrofoam box, foil-lined, with UV lamp (BLAK-RAY Lamp, longwave UV366nm, UVP, Upland, CA) embedded in lid to shine into box, and raised interior platform, or commercial chillable UV curing box
Dry ice

Procedures:

To ensure that capsules are sufficiently cured, set up Styrofoam UV box with dry ice and platform at about 2" away from UV lamp (or use commercial box).
Remove curing cans from AFS, remove spider covers with attached capsules (labeling each set), blotting dry on paper towel, making sure capsules are stable.
Set the spider covers on the platform with the capsules facing up.
Replace the UV lid and turn on.
Let cure overnight.

When curing is complete, remove one capsule at a time, noting position, slit the gelatin and Flo-through capsule with a razor blade and peel them away from the Lowicryl block; label block if no label was inserted.
Repeat for remaining capsules.
Empty ethanol from curing cans (dispose as hazardous waste).
Place cans with stem holders and bottom spacer plates in acetone for cleaning, rinsing off residual resin debris, then air dry.
Spider covers should have cured resin residue gently removed with a dissecting needle, then clean in acetone and air dry.

4. Ultrathin sectioning and preparation of arrays

4A. Required materials and equipment:

Silanized coverslips (24 x 65 mm; Aratome, Menlo Park, USA), or High performance Schott Nexterion coverslips coated with Aminosilane (Schott, Jena, Germany)
Carbon rods (e.g. Ted Pella, #93010)
Carbon evaporator (e.g. Cressington Carbon Coater 308R)
Ultra Jumbo Diamond Knife or Histo Jumbo Diamond Knife (Diatome): The choice of knife depends on the size of the block to be cut. The Ultra Jumbo Knife has a 3 mm diamond edge. For larger block faces, the Histo Jumbo knife comes with 2 diamond size options – 6 and 8 mm. Even though its cutting range is specified as 0.2 – 5 μm, a new Histo Jumbo knife will cut ultrathin sections (70 nm).
Cryotrim 45 Diamond Knife (Diatome) – for trimming the block face
Ultramicrotome
Weldwood Contact Cement
Xylene
Thin paint brush
Slide warmer (Set at ~60°C)
Tween-20 (Electron Microscopy Sciences 25564)

4B. Preparations:

Carbon coated coverslips: Coat the coverslips with carbon (medium gray color). Follow the instructions for the particular carbon evaporator. Store in dust free boxes.
Note: If the coat is too light, this can cause problems with charging of the sample in the SEM. If the coat is too dark, the immunofluorescence signal will be significantly attenuated. The coverslip will be very hydrophobic, however we don't recommend glow discharge to increase hydrophilic properties, because this decreases the adhesion of sections to the coverslips.

Glue: Dilute contact cement with xylene (~1:3 until easily applicable with a paintbrush).
Tween water: 0.005% Tween 20 in water. Dissolve 10 μl Tween 20 in 200 ml distilled water.

4C. Procedures:

Prepare the block for ultrathin sectioning: Trim the block around the tissue using a razor blade. A smaller blockface (2 mm or less width) with the width of the trapezoid greater than its height works best. Cut semithin sections until you reach the tissue. Trim the block again at this point, to ensure that the block face is not too big and the leading and trailing edge of the block face are parallel. The cryotrim 45 diamond knife can be used for precision trimming (Figure 10A). Using a paint brush, apply the diluted glue to the leading and trailing sides of the block pyramid. Let dry (5 min) and apply again.

Ultrathin sectioning: Fill the boat of the Jumbo Diamond knife with Tween water and insert a coverslip. The carbon coated coverslips are very hydrophobic and it is difficult to keep them submerged in pure water in the knife boat. The use of Tween water helps with wetting the coverslips and the stainless steel rod prevents the water from receding (Figure 10B). The water level in the knife boat should be kept

very low or water will jump onto the block face. After the glue has dried (up to 30 min), start cutting ribbons of serial sections (70 nm).

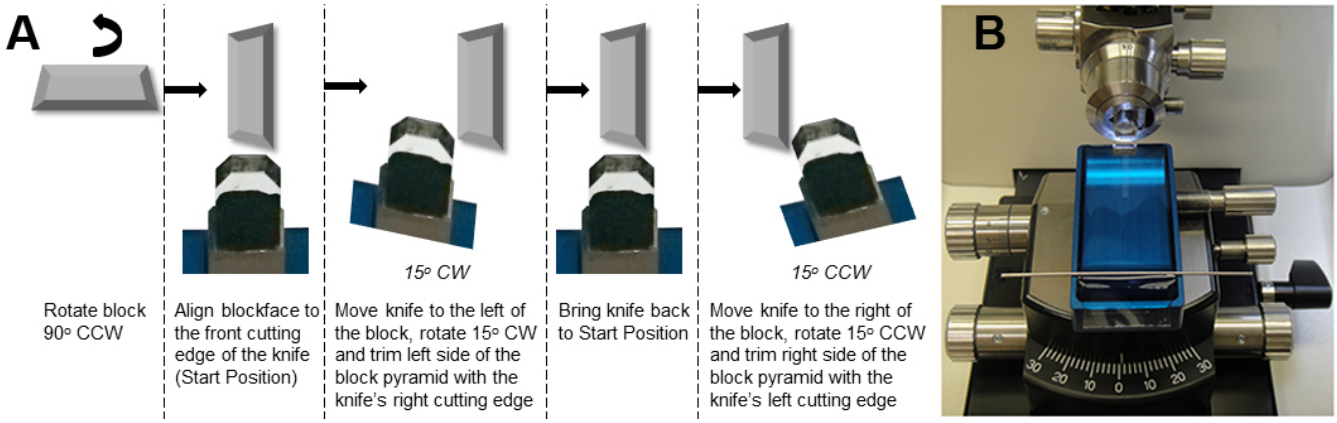

Figure 10. Trimming of the block and ultrathin sectioning setup.

Picking up the sections on the coverslip: When the desired length of the ribbon is achieved, carefully detach it from the knife edge using an eyelash. Remove the rod and with the eyelash gently push the ribbon towards the coverslip, so that the edge of the ribbon touches the glass at the interface of the glass and the water. The edge of the ribbon will then stick to the coverslip. Using a syringe, slowly lower the water level in the knife boat until the entire ribbon sticks to the coverslip. Take out the coverslip. The coverslips have to be left flat to dry, because the sections may still move while wet with the Tween water. The position of the ribbon can be marked using a permanent marker on the coverslip side opposite of the sections. After the water has dried out, place the coverslip on the slide warmer (~ 60°C) for 30 min. The coverslips can be stored at room temperature for at least 3 months.

Note: Slight variations in the Tween concentration can cause problems in sectioning and picking up of ribbons. If the sections sink or the ribbons start to break up easily, decrease the Tween concentration by adding several drops of water in the knife bath. If the water recedes abruptly or unevenly from the coverslip increase the Tween concentration by adding several drops of 0.01% Tween in water.

5. Immunostaining

5A. Required materials:

PAP pen (ImmmEdge Pen, Vector Laboratories)
Tris Buffered Saline tablets (Sigma #T5030)
Sodium borohydride (Sigma #S9125)
Glycine
Bovine serum albumin (e.g. AURION BSA-C, Electron Microscopy Sciences 25557)
Tween-20 (Electron Microscopy Sciences 25564)
Primary antibodies
Secondary antibodies: the appropriate species of Alexa Fluor 488, 594 and 647, IgG (H+L), highly cross-adsorbed (Invitrogen)
Transfer pipets, extra-fine tip polyethylene (Fisher Scientific 13-711-31)
Mounting medium: SlowFade Gold antifade reagent with DAPI (Invitrogen)
Glass Slides, precleaned Gold Seal Rite-On micro slides (Fisher Scientific 12-518-103)

5B. Preparations:

Tris buffer: dissolve 1 tablet in 15 ml of ultrapure water

1% Na borohydride: 10 mg of Na borohydride in 1 ml of Tris. Prepare immediately before use. Do not close the Eppendorf tube or it may burst from the building pressure.

50 mM Glycine: 4 mg glycine in 1 ml of Tris

Blocking solution: Make a 1% stock of Tween-20 (10 µl Tween in 1 ml of ultrapure water). Then add 50 µl of the 1%Tween stock solution to 0.94 ml Tris. Add 10 µl of Aurion BSA.

5C. Procedures:

Encircle the sections with a PAP pen leaving some extra space at the two ends of the ribbon. Put the coverslips in a Petri dish or box, and add wet KimWipes on the side to prevent evaporation of solutions. Keep closed during incubation times. All incubations except primary antibodies are done at room temperature.

Cover the sections with 1% Na borohydride for 3 min. Remove big bubbles that form during the incubation by gently tapping the box to the bench. Wash with Tris for a total of 20 min. Washing of the sections is done in several periods (3-4 periods of about 10-15 s) of continuous flow of buffer, by adding buffer with a plastic transfer pipet on one side of the sections and removing buffer with another pipet (or a pipet tip connected to a vacuum line) on the opposite side of the sections.

Remove Tris and add 50 mM glycine for ~ 5 min.

Remove glycine and apply blocking solution for ~ 5 min (no need to wash).

Remove blocking solution and apply primary antibody in Blocking Solution for 2 hours at room temperature or overnight at 4ºC (no need to wash). Spin down the antibody solution at 13,000 rpm for 2 min before applying to sections.

Note: *After this step, it is important not to let the sections dry at any point!*

Wash several times with Tris for a total of 15 min.

Apply secondary antibody (e.g. Alexa, 1:150) in blocking solution for 30 min. Spin down the antibody solution at 13,000 rpm for 2 min before applying to sections. Keep in the dark.

Wash with Tris several times for a total of 15 min.

Wash with filtered ultrapure water: wash the sections once, and then wash the whole coverslip. Note: *At this point it is very easy for the sections to dry out, so be careful to always leave some water behind.*

Mount with SlowFade Gold antifade reagent with DAPI (Invitrogen). The DAPI stain helps find the sections and focus. For mounting: remove some, but not all of the water from the array, then add a couple of drops of mounting medium and remove more of the water. Image with a fluorescence microscope.

6. Elution

6A. Required materials:

NaOH (10N)
SDS (20% w/v; Roche 1666924)
Tris buffer

6B. Preparations:

Elution solution (0.2M NaOH and 0.02% SDS in ultrapure water): Add 200 µl of NaOH (10N) and 10 µl SDS to 10 ml of ultrapure water. Store at room temperature for up to 6 months.

6C. Procedures:

Add water around the edge of the coverslip to help detach it from the microscope slide. Wait ~ 1 min. The coverslip will float up. Pick it up with tweezers and wash away mounting medium with ultrapure water. *Note: It is best to perform this step as soon as finished with imaging. Leaving the sections too long with the mounting medium will damage the sections.*

Apply the elution solution for 20 min at room temperature (add the solution gently to the sections, do not wash with the elution solution). Elution time may vary for different antibodies; can be tested by applying only the secondary antibody after elution and checking for remaining fluorescence. Some antibodies cannot be sufficiently eluted, for example GABA, and such antibodies are best left for the last cycle of immunostaining.

Wash with Tris buffer (15 min). The initial wash should be slow.

A new immunostaining can be performed following the same procedure as described in Section 4.

Alternatively, to store the coverslip for possible staining at a later time, after the 15 min Tris wash briefly wash with filtered ultrapure water. After the sections have dried, the coverslips can be stored for a long period of time.

7. Poststaining for electron microscopy

7A. Required materials:

$KMnO_4$
H_2SO_4 1N
Uranyl acetate
Lead citrate
NaOH 2N, carbonate free

7B. Preparations

Acidified permanganate staining solution (0.1% $KMnO_4$ in 0.1 N H H_2SO_4 [49]): Prepare a stock solution of 1% $KMnO_4$ by adding 0.1 g $KMnO_4$ in 10 ml of distilled water. Immediately before staining, add 100 µL of the $KMnO_4$ stock solution and 100 µL of 1 N H_2SO_4 to 800 µL of distilled water. The $KMnO_4$ stock solution can be stored for several months at room temperature.

5% aqueous uranyl acetate: Weigh 5 g of uranyl acetate under the fume hood and add to 100 ml of ultrapure water. Cover the bottle with foil and dissolve overnight on a shaker in a warm room (37ºC). Can be stored for several months in the dark. Filter before use.

Lead citrate solution: Prepare fresh, during the uranyl acetate staining period. Under the fume hood weigh out 0.01 to 0.04 g lead citrate in a 10 ml tube. Add 1 ml of carbonate free 2N NaOH to dissolve lead and then add 9 ml of water. Filter before using. The lead citrate powder can be weighed out in tubes ahead of time and stored at room temperature for convenience.

7C. Procedures

Dip coverslip with sections in a Coplin jar filled with ultrapure water (wet sections stain better). Remove and put into petri dish with filter paper or gauze underneath. Cover the sections with freshly made acidified permanganate staining solution and leave for 1 min. Quickly remove stain and flood coverslip with ultrapure water. Wash extensively with water.

Put coverslip in a clean petri dish and add a small amount of filtered 5% aqueous uranyl acetate over the sections. Cover the petri dish and let stain for 30 min. While coverslip is staining, make up fresh lead solution.

Remove UA staining solution and flood coverslip with water. Wash extensively and then store in Coplin jar while getting lead stain ready. Add NaOH pellets to staining dish.

Add freshly made and filtered lead citrate over the sections. Immediately close the petri dish. Stain for 1 min ONLY! Remove lead and flood coverslip with water. Wash extensively with water.

Let dry, then mount with carbon paint onto SEM stub.

Ribbons are imaged in an FESEM microscope using the backscatter detector at 5-8 keV.

Further clarifications from Kris Phend:

I work with the original AFS system from Leica, which they have discontinued (replaced with the AFS2 or EMAFS2) and no longer support (provide parts, expendables, service). I'm going to put my answers (if I have any) embedded below.

Kris P

I'm planning for the Lowicryl AT according to the article you sent me. For some of the AFS accessories I can't figure out what we need (see Leica AFS equipment attached).

For embedding small chunks of tissue I use the plastic capsules D5 x H15 mm (#1, on page 11)(we call them flo-through capsules), inserted in the chambers for capsules (#11, on that page, and with displacement bodies #4 in their centers) for carrying out the freezing and substitution phases and resin infiltration. When ready for the UV cure stage, a chamber for gelatin capsules (#12) is set up with gelatin capsules (#2) (the large portion only), which are filled with fresh resin, then a spider cover (#9) screwed on to a cryo tool with M4 thread (16701958) is pushed onto the plastic capsules (making sure there is an alignment reference), and the spider cover with attached capsules is transferred to the gelatin capsule chamber and the plastic tissue capsules are inserted into the gelatin capsules firmly enough to seal. Meanwhile, a universal container (#13) with a stem holder for spider cover (#6) and a bottom plate for universal container (#5) is set up and filled with absolute ethanol (and chilled) as a heat sink. Then, the spider cover with the attached plastic in gelatin capsules is transferred to the stem holder in the universal container (the cryo tool should be removed beforehand, and the unit transferred by forceps). This transferral can be difficult if gelatin capsule and plastic capsule attachment is weak. The procedure is repeated as needed by placing and filling fresh gelatin capsules, transferring a set of plastic capsules with tissue, preparing another heat sink universal container with stem holder, etc. When all are full/ready, UV curing can proceed.

The baskets #G3314A (4/can) from Leica are not available and Leica even doesn't know what is meant by that order number.

These look sort of like the Plastic capsules D13 x H18 with mesh bottom (#3), although a little taller and the bottom may be different (may not require bottom plate for complete liquid exchange). I use these for processing sections (100μm thick) or portions of sections of tissue.

I also don't know what flow-through capsules are

Plastic capsules D5 x H15 (#11) mentioned above for chunk embedment.

and what wafer-embedding means.

It refers to the flat embedment of a tissue section or portion thereof between 2 generous pieces of ACLAR sandwiched between 2 pieces of glass slide for support. One could use the "Flat embedding unit" (#10) and its cover (#8), but we find the blocks too thick to excise areas of interest for cutting for EM. We prefer our wafer or sandwich approach, which brings us to the last line...

Also, I don't know what scoring upside-down metal cans for wafer support and

I think you are referring to some things done when we work with sections instead of chunks.

When processing sections, the fluids, etc. are the same but the containers and curing set-up is different. The cryoprotected sections are transferred to a piece of ACLAR cut so it will fit in a basket (Leica's Plastic capsule D13 x H18 with mesh bottom, #3)(make the ACLAR just slightly taller than the basket for easy removal). The sections should be flat on the ACLAR and be of a size to eventually fit comfortably in the basket and stay covered by fluids. To start, universal cans (#13) with bottom plates (#5) and 4 baskets each are set up with initial freezing liquid. ACLAR pieces with attached sections are inserted in the baskets for the freezing, substitution, and resin infiltration steps. Section should release from the ACLAR before resin infiltration.To proceed to curing, a universal can is placed upside down in the cold area, so there is a flat surface upon which to set a square piece of glass slide (made by "scoring" or running a glass cutting tool on a slide so it will break where you want it to), upon which you set a similar sized piece of ACLAR, a small amount of fresh resin is added on that, and then the sections from the 4 baskets of one universal can are placed/arranged there—plan ahead for sizes, shapes, and ability to identify if needed, as the sections move—, a second piece of ACLAR is placed on top of the sections, followed by another piece of glass, and you have a "wafer" or sandwich. When all the sections have been transferred to their respective wafers, UV curing can proceed.

bottom plate for cans (4-5 universal cans) are.

(#5) used in the UV curing set-up for capsules of chunks (if a capsule comes loose, the plate prevents it from coming completely off the spider cover and falling over in the heat sink liquid), or as an aid to more complete fluid changing in tissue section processing for wafer embedding (may not be needed with new style baskets—#3).