

Array Tomography

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1. Tissue fixation.

Note: Below follows the protocol for chemical fixation by immersion, but tissue prepared in different ways can also be used – for example, fixed by perfusion. It should be dissected into small pieces of < 1 mm in at least one dimension. If you already have your tissue fixed, continue from section 2.

Required materials and equipment:

Isoflurane
Paraformaldehyde (8%, EM grade; Electron Microscopy Sciences #157-8)
Optional: Glutaraldehyde (8%, EM grade; Electron Microscopy Sciences #16020)
PBS (Sigma #P3813)
Guillotine: for adult rats
or
Sharp scissors: for young rats and mice
Dissection instruments: handling forceps, small scissors, bone rongeur, forceps #5; small spatula; scalpel, glass pipette (tip broken off) with a bulb to transfer the tissue pieces, small paint brush.
Red biohazard bag
Petri dishes (35 mm)
Glass scintillation vials (20 ml)
Optional: PELCO 3451 laboratory microwave system with a ColdSpot set at 12°C; Ted Pella: speeds up sample preparation. In the protocols below two options are provided: **BT** - bench top processing, or **MW** - microwave processing.

To prepare:

PBS (2x): Dissolve 1 packet in 500 ml to prepare 0.02M PBS.

Fixative (prepare the same day, keep at room temperature; alternatively, freeze for long-term storage):

A: Paraformaldehyde (PF)

4% PF in 0.01M PBS

For 10 ml: 5 ml PF (8%)
 5 ml PBS (0.02M)

or

B: PF and Glutaraldehyde (GA)

2% PF/2% GA in 0.01M PBS

For 10 ml: 2.5 ml PF (8%)
 2.5 ml GA (8%)
 5 ml PBS (0.02M)

Procedure (for rodent brain):

- ▶ Anesthetize the animal with isoflurane.
- ▶ Remove head using the guillotine for adult rats or sharp scissors for young rats and mice.
- ▶ Quickly remove the brain out of the skull and put in a petri dish with fixative (room temperature). In the hood, dissect out the general region of interest (don't cut into the final size small pieces yet).
- ▶ Transfer the tissue to a scintillation vial with fixative solution

Note: **BT** is the currently preferred method (no differences were observed between **BT** and **MW** tissue processing). For **MW**, use approximately 1ml of fixative per vial, or just enough to cover the tissue; excessive liquid volume will cause overheating in the microwave.

BT: ▶ Fix first @ room temperature for 2-3 h and then leave overnight at 4°C.

MW: ▶ Microwave using a cycle of 1 min on – 1 min off – 1 min on at 100 – 150W. **Note:** After this and each following cycle feel the glass vial to check for overheating. If solutions are getting too warm (>37°C), decrease the amount of liquid added.

▶ Microwave using a cycle of 20 s on – 20 s off – 20 s on at 350-400W: repeat 3 times.

▶ Leave at RT for about 2-3 h.

2. Dehydration and embedding.

Required materials and equipment:

PBS (Sigma #P3813)
Glycine
Ethanol (200 proof)
LR White resin (hard grade, SPI supplies #2645)
Gelatin capsules (Size 00, EMS#07010)
Capsule holder (e.g. EMS #70161)
Oven (set at ~ 53°C)
Optional: PELCO 3451 laboratory microwave system with a ColdSpot set at 12°C; Ted Pella: speeds up sample preparation. In the protocols below two options are provided: **BT** - bench top processing (preferred) or **MW** - microwave processing.

To prepare:

PBS: Dissolve 1 packet in 500 ml to prepare 0.02M PBS.

Wash buffer: 50 mM glycine in 0.01M PBS (can be prepared in advance and stored at 4°C for up to 1 month; discard if appears cloudy):

To make 50 ml:
25 ml PBS (0.02M)
25 ml H₂O
187.5 mg glycine

Ethanol dilutions (keep at 4°C):

50% ethanol
70% ethanol
95% ethanol

Procedure:

▶ Wash in wash buffer (cold from fridge) and keep the samples at 4°C. 2-3 times for up to 30 min. Dissect the tissue further, to obtain small pieces (< 1 mm in at least 1 dimension).

BT: ▶ 50% ethanol (4°C): 10 min at 4°C.
▶ 70% ethanol (4°C): 10 min at 4°C.
▶ 70% ethanol (4°C): 10 min at 4°C.

Stop here if processing samples with GFP or other fluorescent proteins (see below).

▶ 95% ethanol (4°C): 10 min at 4°C.
▶ 100% ethanol (4°C): 10 min at 4°C.
▶ 100% ethanol and LRWhite resin (1:1 mixture): 10 min at room temperature (RT).
▶ LRWhite (100%): 10 min at RT.
▶ LRWhite (100%): 10 min at RT.
▶ LRWhite (100%): 10 min at RT.

For preservation of fluorescence omit the 95% and 100% ethanol. Instead, place samples into a mixture of 70% ethanol and LRWhite (1 part 70% ethanol and 3 parts LRWhite; if it turns cloudy add 1-2 extra drops of LRWhite): 10 min at RT. Then do 100% LRWhite 3 times for 10 min at RT.

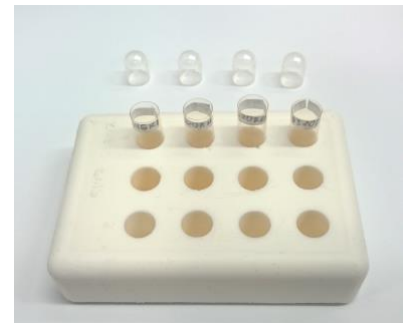
MW: ▶ Do each of the above steps for 45 s @ 350W. Use just enough liquid to cover the tissue; excessive liquid volume will cause overheating.

▶ Change with new LRWhite and leave overnight in fridge. **Note:** *At this point, if needed, the sample can be left for several days at 4°C in LRWhite.*

▶ Next day, put tissue at the bottom of gelatin capsules (paper labels can also be added inside the capsule) – *see Figure*, and fill to the rim with LRWhite.

▶ Close the capsules well. **Note:** *Oxygen inhibits LRWhite polymerization and that is why gelatin capsules, which completely exclude air, are used. The little bubble of air that will remain at the top of the capsule will not interfere with the polymerization.*

▶ Put capsules into oven at approximately 53°C for 24 h. **Note:** *If the oven temperature falls below 50°C, the resin may not polymerize. It is better to set the oven temperature just above 50°C (~53°C) to ensure consistent polymerization.*



3. Preparation of test arrays and arrays planned for up to 3-4 restains (for arrays planned for a large number of restains, see #4):

Required materials and equipment:

Coverslips, VWR Micro Cover Glasses, 24x60 mm, No.1.5, 48393-252; or, for quantitative studies: Bioscience Tools High Precision Glass Coverslips CSHP-No1.5-24x60
Gelatin (300 Bloom)
Chromium potassium sulphate
Jumbo Histo Diamond Knife (Diatome)
Cryotrim 45 Diamond Knife (Diatome) - optional
Ultramicrotome
Weldwood Contact Cement
Xylene
Thin paint brush
Slide warmer (Set at ~60°C)

To prepare:

Subbed coverslips:

What you need:

1. Coverslips (must be clean and dry)
2. Staining rack and dish (Pacific Southwest Lab Equipment, Inc. 37-4470 and 4456)
3. Subbing solution. Dissolve 1.5 g gelatin in 290 ml ultrapure water by heating to <60°C. **Note:** *Do not overheat!* Dissolve 0.15 g chromium potassium sulphate (chrome alum; CrK(SO₄)₂.12H₂O) in 10 ml ultrapure water. Combine the two solutions, filter and pour into the staining dish. Use fresh.

What to do:

1. Put the clean coverslips in the staining rack.
2. Immerse in the subbing solution for 30 to 60 seconds, with occasional gentle agitation.
3. Lift out and drain off excess liquid, then leave the rack of coverslips in a dust-free place until dry.

The dry subbed coverslips are stored in boxes until they are needed.

Glue: dilute contact cement with xylene (~ 1:3 until easily applicable with a paintbrush).

Procedure:

▶ Trim the block around the tissue. A smaller blockface (1mm or less width) with the width of the trapezoid greater than its height works best - *see Figure A.*

▶ Cut semithin sections until you reach the tissue. Trim the block again at this point, to ensure that the blockface is not too big and the leading and trailing edge of the blockface are parallel. The cryotrim 45 diamond knife is very good for this purpose.

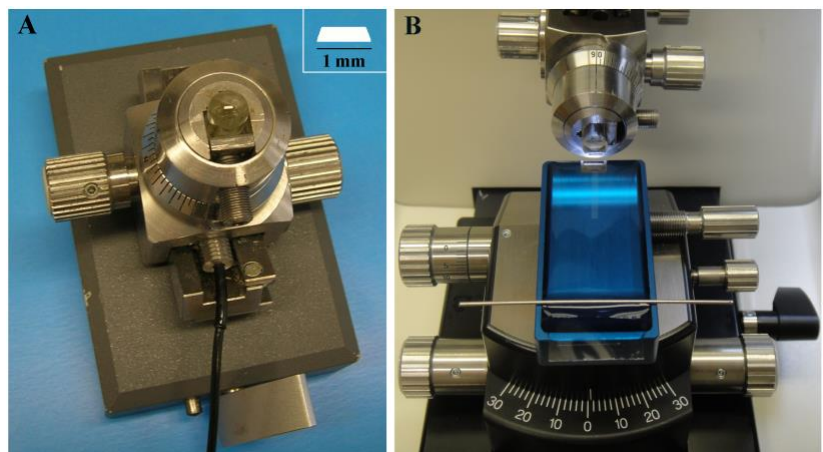
▶ Using a paint brush, apply the diluted glue to the leading and trailing sides of the block pyramid. Blot excess glue with a KimWipe.

▶ Insert a subbed coverslip into the knife boat of the Jumbo Histo diamond knife – *see Figure B.* The stainless steel rod helps prevent the water from receding.

▶ After the glue has dried (~ 2 min), start cutting ribbons of serial sections (60–200 nm) with the diamond knife. In general, thinner sections stick better to the glass.

▶ 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 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 (~ 55°C) for 30 min. The coverslips can be stored at room temperature (in the dark) for at least 3 months. **Note:** *It is important that the sections are completely dry before placing on the slide warmer to prevent the formation of folds.*



4. Preparation of arrays planned for a large number of restains:

Required materials and equipment:

Subbed coverslips, prepared as described in #3
Carbon rods (e.g. Ted Pella, #93010)
Carbon evaporator (e.g. Cressington Carbon Coater 308R)
Jumbo Histo Diamond Knife (Diatome)
Cryotrim 45 Diamond Knife (Diatome) - optional
Ultramicrotome
Glue, prepared as described in #3
Thin paint brush
Tween-20 (Electron Microscopy Sciences 25564)
Slide warmer (Set at 60 – 65°C)

To prepare:

Carbon coated coverslips:

What to do:

1. Put the subbed coverslips in the carbon coater.
2. Following the instructions for the particular carbon evaporator coat the coverslips to a light grey color.
3. Store in dust free boxes. **Note:** *It is better to use older (several days or more) carbon coated coverslips. The freshly made ones are very hydrophobic.*

0.005% Tween in dH₂O: dissolve 10 µl Tween in 200 ml dH₂O

Procedure:

► Follow the procedure described in #3, except that now use the carbon coated coverslips. The carbon coat ensures better adhesion of the sections to the coverslips. However, these coverslips are much more hydrophobic than the regular subbed coverslips and it may be difficult to keep them submerged in water in the knife boat. If this happens, add 0.005% Tween to the water to help with wetting the coverslips. The water level in the knife boat should be kept very low or water will jump onto the blockface. The coverslips should be left flat to dry before putting on the slide warmer, because the sections may still move while wet with the Tween water. **Note:** *The Tween may start dissolving the glue, which will result in breaking of the ribbons. If this occurs, slightly decrease the concentration of Tween and/or add two coats of glue to the sides of the block.* **Note 2:** *Glow discharge decreases the hydrophobicity of carbon coated coverslips, however it also decreases the adhesion of sections and is not recommended.*

5. Immunostaining:

Required materials:

PAP pen (ImmmEdge Pen, Vector Laboratories)
For glutaraldehyde fixed tissue only: Sodium borohydride (Sigma S9125)
Tris Buffered Saline tablets (Sigma #T5030)
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 Diamond antifade reagent with DAPI (Invitrogen S36964)
Glass Slides, precleaned Gold Seal Rite-On micro slides (Fisher Scientific 12-518-103)

To prepare:

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

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

Blocking Solution: 0.05% Tween and 0.1% BSA in Tris: First prepare 1% Tween stock (10 μ l Tween in 1 ml of H₂O). Then add 50 μ l of the 1% Tween stock and 10 μ l of 10% BSA solution to 0.94 ml Tris buffer.

Primary antibodies: Higher concentration than used for immunolabeling on Vibratome or cryostat sections, usually 1:50 to 1:100 from a stock of 1mg/ml, diluted in Blocking Solution

Secondary antibodies: Alexa 1:150 (from a stock of 2 mg/ml) in Blocking Solution

Note: *The Alexa 647 channel is weaker. Do not use to test new antibodies.*

Note: *Approximately 150 μ l of solutions will be enough for a ribbon of about half the coverslip length. Spin down all antibody solutions at 13,000 rpm for 2 min.*

For glutaraldehyde fixed tissue only: Sodium borohydride, 1%: 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.

Procedure:

► 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.

► *If glutaraldehyde was included in the fixative, pretreat with Na borohydride for 3 min, then wash with Tris for a total of 20 min. Remove big bubbles that form during the incubation by gently tapping the box to the bench.*

If tissue was fixed with paraformaldehyde only, omit this step and begin with the glycine incubation below.

► Glycine in Tris for ~ 5 min, room temperature.

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

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

► Remove blocking solution and apply primary antibody in Blocking Solution for 2 hours at room temperature or overnight at 4°C (no need to wash). Spin down the antibody solution at 13,000 rpm for 2 min before applying to sections. **Note:** *For the majority of antibodies, both incubations give similar results. A few antibodies give lower background labeling with the 2h incubation (e.g. VGAT mouse from SySy).*

► Wash several times with Tris for a total of 15 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 on the opposite side of the sections – *see Figure.*



- ▶ Secondary antibody (e.g. Alexa, 1:150) in blocking solution for 30 min at room temperature. 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 Diamond 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 on one end of the array. The mounting medium will push the remaining water away – remove it from the other end. Leave just enough mounting medium to have the array covered and then turn the coverslip over and slowly lay over a glass slide so as not to get bubbles. If bubbles form, the coverslip can be removed, washed with water and mounted again. If there is too much mounting medium (comes out at the edges and the coverslip easily slides around) then blot out some of it.
 - ▶ Clean the back of the coverslip from the marker and any other dust by wiping out with 70% ethanol. Hold the coverslip and the glass slide together, so that the coverslip doesn't move. Wipe also the glass slide side – big dust even from there can be visible under the microscope.
- Note:** *It is better to image as soon as possible after staining or at least the same day. For some antigens, the staining may be very weak and not visible with low magnification objectives. Use a high mag objective (e.g. 63x or 100x) and longer exposures (up to several seconds) if necessary.*

6. Antibody elution.

Required materials and equipment:

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

To prepare:

Elution solution:

0.2 M NaOH and 0.02% SDS in ultrapure water
 To prepare, add 200 µl of NaOH (10N) and 10 µl SDS (20%) to 10 ml of ultrapure water.
 Store at room temperature (up to 6 months).

Procedure:

- ▶ 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 dH₂O. **Note:** *It is better to perform this step as soon as finished with imaging. Leaving the sections too long with the mounting medium will decrease the quality of subsequent immunolabeling. If needed, the sections can be left in Tris overnight, then the next day washed with water and eluted as below.*
- ▶ Apply the elution solution for 20 min at room temperature (add the solution gently to the sections, do not wash with the elution solution; if the sections are not well attached to the glass they may start detaching at this point; works better on the thinner, 70 nm sections). Elution time may vary for different antibodies; can be tested by applying only the secondary antibody after elution and checking for remaining fluorescence).
- ▶ Wash with Tris buffer (15 min). The initial wash should be slow.
- ▶ Rinse the entire coverslip with water. After the water has dried out, place the coverslip on the slide warmer (~55°C) for 30 min. After this, the coverslips can be stored for a long period of time (RT, in the dark).
- ▶ A new immunostaining can be performed following the same procedure as described in Section 4.