Institute of Pharmacology and Toxicology

Section of Neuromorphology

Main protocol
for electron microscopy

J.-M. Fritschy, Patrizia Panzanelli
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Contents:

General comments ... 3
1. Fixation ... 3
1.1) Routine Fixative ... 3
1.2) pH shift fixatives (recommended by Zoltan Nusser) ... 3
2) Tissue block preparation ... 4
3) Osmification (tissue blocks) ... 4
4) Dehydration and embedding ... 5
5) Pre-embedding immunohistochemistry ... 6
5.1 Tissue preparation ... 6
5.2 Immunoperoxidase ... 6
5.3 Fluonanogold labeling ... 7
5.4 Dehydratation and embedding ... 7
6) Contrasting ultrathin sections ... 8
7) Post-embedding immunohistochemistry ... 9
7.1 Epon-embedded sections ... 9
7.2 Lowicryl-embedded sections ... 10
Annex I: Resins ... 12
1) Epon ... 12
2) Epon - Araldite ... 12
3) Durcupan ... 12

General comments

Note: all solutions should be made fresh with cell culture grade dH_2O (do not use dH_2O stored in plastic containers)

Caution: many reagents are highly toxic (fixative; cacodylate buffer, osmium tetroxide, solvents, resins); wear protective equipment (lab coat, gloves) and work in a ventilated hood; discard waste appropriately

1. Fixation

1.1) Routine Fixative

4% PFA and 15% saturated picric acid solution in 0.1 M Na-phosphate buffer, pH 7.4; add 0.1% - 1% glutaraldehyde just before the perfusion

For 0.5 liter:

– Dissolve 20 g PFA in ~200 mL dH_2O; add 6 drops of NaOH (8 N); warm up to max. 60°C while stirring continuously until entirely dissolved
– In a separate Erlenmeyer on ice, filter 125 mL 0.4 M Na-phosphate buffer, 75 mL saturated picric acid, and 50 mL dH_2O
– Filter the PFA solution and add dH_2O to make up 500 ml; mix well
– Cool the solution to room temperature, adjust pH to 7.4, and cool it further to 4°C
– Just before the perfusion, add the appropriate amount of glutaraldehyde (from 25% stock solution) (0.1% = 2 ml; 0.5% = 10 ml; 1% = 20 mL)

The perfusion has to start as fast as possible; rinse the blood with ice cold saline (0.9% NaCl in dH_2O; contained in the plastic tube of the pump) and proceed without pause with the fixative (flow rate set at 80-90 for adult mice; about 7 mL/min).

Remove the brain ~15 min after the perfusion; postfix in the same fixative (in 50 mL Falcon tubes) for 4-6 hours at 4°C.

Rinse well with 0.1 M Na-phosphate buffer (3 x 10 min)

Store in 0.1 M Na-phosphate buffer at 4°C (up to 2-3 days)

Go to section 2

1.2) pH shift fixatives (recommended by Zoltan Nusser)

Two separate fixative solutions (2% paraformaldehyde and 0.1-1% glutaraldehyde) are being prepared (solution A, pH 6.0; solution B, pH 8.5); start with solution A (rapid penetration, poor fixation) and continue with solution B (strong fixation). The brain is left in the skull for postfixation.

Perfusion buffers (for one adult mouse):
Dissolve 12 g paraformaldehyde in 150 mL dH_2O containing 4 drops of NaOH (8 N) by warming up to 60°C; filter and make up to 300 mL with dH_2O.
Solution A: Dissolve 0.82 g sodium acetate in 50 mL dH_2O; add 50 mL of 4% paraformaldehyde solution; adjust pH to 6.0 with 1-2 drops glacial acetic acid. Cool the fixative to 4ºC. Before perfusion add glutaraldehyde (from 25% stock) as required (0.1% - 1%).
Solution B: Dissolve 3.1 g boric acid and 4.75 g borax in 250 mL dH_2O; add 250mL of 4% paraformaldehyde solution; adjust pH to 8.5 with 1 M NaOH.

Cool the fixative to 4ºC. Before perfusion add glutaraldehyde (from 25% stock) as required (0.1% - 1%)

Perfuse transcardially first with ice-cold 0.9% NaCl followed by the ice-cold solution A for 2 min (pump setting 250) followed by ice-cold solution B for 1 hour (pump setting 40-50).

After the perfusion, store the head in a Falcon tube overnight at 4ºC and remove the brain the following day.

Rinse well with 0.1 M Na-phosphate buffer (3 x 10 min)

Store in 0.1 M Na-phosphate buffer at 4°C (up to 2-3 days)

Go to section 2

2) Tissue block preparation

- Tissue blocks can be prepared with a sharp razor blade or scalpel (use the binocular) or with a vibratome if the region of interest is small and should be precisely embedded
- Tissue blocks should be given a shape that allows orienting them during the inclusion; the size should not exceed 2x3x1 mm

Collect tissue blocks in 12-well cell culture plates filled with Na-cacodylate buffer (pH 7.4). Caution: toxic

Change Na-cacodylate at least twice (total time > 60 min)
To make Na-cacodylate buffer:

(sodium-dimethylarsenate trihydrate; $C_2H_6AsNaO_2$ – 3 H_2O); Merck 820670

Stock solution (0.2 M): dissolve 21.4 g in 500 mL dH_2O; store at 4ºC

Working solution (0.1 M, pH 7.4): mix 50 mL stock solution and 2.8 mL HCl 0.1N; add dH_2O to 100 mL (check pH)

3) Osmification

Caution: Osmium tetroxide (OsO4) is **highly reactive** and **extremely** toxic. All objects that come in contact with it have to be discarded appropriately (not in the waste basket!). Never use metal tools (only plastic or wood).

Work with the hood has closed as possible and always wear good gloves.

Stock solutions (2%) are stored at 4ºC in aliquots (sealed glass vials)

Tissue blocks:
- Make a 1% solution by diluting stock with dH_2O; break the glass vial open by hand, transfer the solution into a 5 mL container and add the same volume of dH_2O
- Aspirate the Na-cacodylate buffer and immerse tissue blocks in a small volume of OsO_4 solution
- Close with a lid and incubate for 60 min in the hood on ice

- Aspirate the OsO_4 solution using a plastic Pasteur pipet and wash twice rapidly with Na-cacodylate buffer; discard fluid in hermetic container (e.g. Falcon tube; fill up to maximally half the volume)
- Fill the wells with Na-cacodylate buffer and wash for >30 min
- Transfer the tissue blocks in Polyethylene (PE) tubes containing cacodylate buffer; using wooden sticks

Go to section 4

Sections (after pre-embedding staining):

- Aspirate the Na-cacodylate buffer and add 2 mL 0.5% OsO4 solution
- Close with a lid and incubate for 15 min in the hood on ice
- Aspirate the OsO4 solution using a plastic Pasteur pipet and wash twice rapidly with Na-cacodylate buffer; discard fluid in hermetic container (e.g. Falcon tube; fill up to maximally half the volume)
- Transfer sections in a new well, using a wooden stick and wash in Na-cacodylate buffer 2x10 min

Return to section 5

- Inactivate the liquid waste by adding the same volume of vegetable oil; label clearly and store in the ventilated cabinet below the hood)
- Collect all solid waste in the yellow waste container (including your gloves); keep it in the hood until collected

4) Dehydration and embedding

Work in the hood and avoid inspiring fumes; wear gloves

- A successful inclusion depends critically on a complete dehydration
- Tissue can be dehydrated with ascending series of ethanol, methanol or acetone (preferred), using PE tubes
- Acetone is kept water free with beads of “molecular sieve” (Merck 5704)
- Be careful that the tissue blocks do never dry while changing the solvent solution
- Use plastic Pasteur pipets to transfer solutions
- Solvent solutions (30-95%) can be reused, as well as the first 100% solution; for second and third wash, use only clean 100% solvent

See Annex for preparation of resin I and II

Start dehydration:

Concentration	30%	50%	70%	80%	95%	100%
Time	5 min	5 min	2x 5 min	2x5 min	2x10 min	10 + 20 + 30 min

Propylene oxide	2 x 5 min
Propylene oxide + Resin I	60 min
Resin I	overnight in the hood (keep tubes open to allow evaporation of residual propylene oxide)

Make sure the incubator is turned on and set to 37°C

Resin I	37°C, 60 min
Resin II	37°C, 60 min

- Change the incubator temperature to 60°C
- Transfer the tissue carefully into PE inclusion molds using a wooden stick; include in each mold a label (pencil) describing its content
- Fill the mold with resin II
- Incubate all molds at 60°C for at least 48 h; also put in the oven all containers and objects in contact with resin II (including gloves) to polymerize. Do no open to incubator during this time
- Once polymerized, let the mold cool down; extract the resin blocks and store them

Discard all liquid waste appropriately

5) Pre-embedding immunohistochemistry

5.1 Tissue preparation

- Cut 80 μm sections with a vibratome and collect them in ice-cold Na-phosphate buffer 0.1 M in 7 mL PE tubes
- Cryoprotect the sections by immersion in 30% sucrose solution in phosphate buffer overnight; caution: do not put too many sections or overfill the container with sucrose
- Freeze by plunging the PE tubes into liquid nitrogen and thaw at RT; repeat the procedure three times
- Wash in phosphate buffer 3x10 min
- Wash in Tris-buffered saline, pH 7.5 (TBS) 3x10 min
- Pre-incubate the sections in TBS containing 10% NGS for 2 h at RT
- Transfer sections in primary antibody solution (TBS containing 3% NGS and 0.05% Na-Azide) and incubate at 4º on a shaker for at least 72 h.
- Wash 3x15 min with TBS

Proceed either with immunoperoxidase (section 5.2) or with Fluonanogold (section 5.3) labeling

5.2 Immunoperoxidase

- Dilute biotinylated secondary antibodies (Jackson Immunoresearch) 1:250 in TBS containing 3% NGS
- Incubate sections overnight at RT in secondary antibodies
- Wash 4x10 min with TBS
- Incubate in ABC-Elite kit (1:100) in TBS for 60 min at RT
- Wash in TBS 4x10 min
- Wash in TBS pH 7.7 for 10 min
- Proceed with DAB staining; incubate sections first in DAB without H_2O_2 for 10 min, and with H_2O_2; do not make a strong reaction (typically 1-3 min instead of 5-15 min)
- Stop the reaction with ice-cold TBS and wash 3 x 10 min with TBS
- Wash the sections 2 x 10 min in 0.1 M Na-cacodylate buffer
- Postfix in 2.5% glutaraldehyde in Na-cacodylate buffer for 2 h at 4º
- Rinse in Na-cacodylate buffer and store overnight at 4ºC

Proceed with gold intensification:

Caution: use only clean glassware dedicated for this purpose; do not use detergents for washing this glassware

- Wash the sections in Na-cacodylate buffer + 50 mM glycine for 15 min at RT
- Wash the sections 3x10 min in dH_2O
- Prepare an aqueous solution, diluting the following components in this order:
    - 5 mL of 3% hexamethylenetretramine ($C_6H_{12}N_4$) in dH_2O (Merck 4343)
    - 250 μl of 5% silver nitrate ($AgNO_3$) in dH_2O (Merck 1512)
    - 50 μl of 2.5% di-Na tetraborate (Borax; $Na_2B_4O_7$) in dH_2O (Merck 6310)
- The solution should become transparent; transfer into glass vials and put them in a water bath at 60ºC. When they are warm, add the sections and incubate for 10 min
- Wash 5x3 min in dH_2O at RT
- Prepare a solution of 0.05% gold chloride [$H(AuCl_4)4H_2O$] in dH_2O (from a 1% stock solution; Merck 1582)
- Incubate the sections for 2 min at RT
- Wash 3x3 min in dH_2O
- Incubate the sections in 2.5% Na-thiosulfate ($Na_2S_2O_3$ $5H_2O$) for 2 min (Merck 6590)
- Wash 3x3 min in dH_2O
- Wash in Na-cacodylate buffer 2x15 min and transfer in 7 mL PE tubes
- Osmificate with 0.5% OsO_4 (small volume; 1-2 mL) on ice 15 min (see section 3)
- Wash in Na-cacodylate buffer 2x10 min
- Wash in dH_2O 2x5 min
- Transfer in new 7 mL PE tube in dH_2O
- Incubate in 1% uranyl acetate made up in dH_2O for 25 min in dark (see section 6)
- Wash in dH_2O 2x5 min

Proceed with dehydration (in acetone) and inclusion in Epon (section 5.4)

5.3) Fluonanogold labeling

- Dilute secondary antibodies coupled to Fluonanogold 1:50 in TBS containing 3%NGS and incubate overnight at RT
- Wash the sections 2 x 10 min in 0.1 M Na-cacodylate buffer
- Postfix in 2.5% glutaraldehyde in Na-cacodylate buffer for 2 h at 4º
- Rinse in Na-cacodylate buffer and store overnight at 4ºC

Proceed with gold intensification:
Caution: use only clean glassware dedicated for this purpose; do not use detergents for washing this glassware

- Wash the sections in Na-cacodylate buffer + 50 mM glycine for 15 min at RT
- Wash the sections 3x10 min in dH_2O
- Change vials with rod or glass Pasteur
- Wash in dH_2O 2x5 min
- Gold enhance EM (Nanoprobes) 7 min
- Wash in dH_2O 2x5 min
- Wash in Na-cacodylate buffer 2x15 min and transfer in 2.5 mL PE tubes
- Osmificate with 0.5% OsO_4 on ice 15 min (see section 3)
- Wash in Na-cacodylate buffer 2x10 min
- Wash in dH_2O 2x5 min
- Transfer in new 2.5 mL PE tube in dH_2O
- Incubate in 1% uranyl acetate made up in dH_2O for 25 min in dark (see section 6)
- Wash in dH_2O 2x5 min

Proceed with dehydration (in acetone) and inclusion in Epon (section 5.4)

5.4) Dehydratation and embedding

Start dehydration with Acetone:

Concentration	50%	70%	90%	100%
Time	10 min	10 min	10 min	3 x 15 min

Make sure the incubator is turned on and set to 37°C

Resin II + Acetone (1:1) Room Temperature, 60 min
Resin II + Acetone (3:1) Room Temperature, 60 min

Resin II Room Temperature, 60 min

Resin II 37°C, 60 min

- Change the incubator temperature to 60°C
- Cut Aclar film to the size of a glass slice (4 x 1 cm) and put one film on a glass slide
- Transfer the sections carefully on Aclar Film using a home-made copper ring mounted on a stick and make sure they are flat
- Put another Aclar Film on top and a second glass slide; put a label (indicating content) on the second glass slide
- Put all "Aclar sandwiches" on a cardboard and incubate at 60°C for 24-48 h; also put in the oven all containers and objects in contact with resin II (including gloves) to polymerize. Do no open to incubator during this time
- Once polymerized, let the slides cool down; carefully extract the "Aclar sandwiches", transfer the label on the top film, and store them in small plastic bags

Discard all liquid waste appropriately

6) Contrasting ultrathin sections

This step should be performed with grids that are ready to be seen in the microscope (i.e. after all staining procedures are completed). Skip the uranyl acetate step if the tissue was already exposed to it (Section 5).

Solutions:

NaOH: 0.1 N NaOH in dH_2O. Adjust pH to 12 with HCl (make fresh)

Uranyl acetate:

- Prepare a saturated solution by dissolving 1-2% uranyl acetate in dH_2O
- Place overnight on a shaker. Store in 50 mL Falcon tube, protected from light, at 4ºC
- When using, pipet carefully the solution, avoiding to mix the undissolved pellet

Caution: radioactive; to be used only in dedicated laboratory.

Lead staining solution:

- Weigh 500 mg lead citrate, 500 mg lead nitrate, 50 mg lead acetate and 1 g Na-citrate and pour into a clear 50 mL clean glass balloon.

- Add exactly 41 mL dH_2O and shake vigorously for 1 min. Add 9 mL of a freshly prepared 1N NaOH solution and mix well. The solution should become clear and contain only a few crystals.
- Transfer into a brown bottle, close hermetically and protect from light (with aluminum foil). Store at 4ºC.
- Immediately wash the glass balloon with dH_2O and NaOH 1N. Use only for this purpose.

Procedure:

- Put drops dH_2O on clean parafilm and transfer the grids onto the drops.
- Put drops of uranyl acetate solution on second parafilm and transfer the grids
- Cover with dark lid and incubate for 30 min.
- Transfer the grids on dH_2O drops on third parafilm.
- Wash the grids one per one by energetically dipping them in 100 mL beaker containing dH_2O.
- Let them dry for 60 min under cover.
- Put drops of lead citrate solution on fourth parafilm and add 2-3 pellets of NaOH.
- Transfer grids onto the drops, cover with dark lid, and incubate for 7 min
- Transfer the grids on dH_2O drops on fifth parafilm.
- Wash the grids one per one by energetically dipping them in 100 mL beaker containing dH_2O.
- Store the grids in appropriate storage box and let them dry fully.
- Dispose of uranyl acetate and lead citrate in appropriate waste bottles.

7) Post-embedding immunohistochemistry

The immunolabeling can be increased by:
1- using the pH-shift fixation protocol (see section 1.2)
2- reducing the concentration of NaCl in the buffer (from 0.9% to 0.3% or 0.1%).
3- using Fab-fragments as secondary antibodies and small gold particles.

TBST:

	1000ml	200 mL	500 mL
-	100 mL 0.05M Tris, pH 7.4	20 mL	50 mL
-	900 mL dH_2O with **0.9%** (8.1 g) NaCl	180 mL (NaCl 1.62g)	450 mL (NaCl 4.05g)
-	1 g Triton X-100	0.2 g	0.5 g

NB: TBST can be stored in the fridge. All the other solutions should be freshly prepared.

7.1 Epon-embedded sections

1a Etching

Dip the grids in a solution of 10% $NaIO_4$ in dH_2O (w/v) for 15 min. Rinse in three Bechers containing dH_2O and put on a drop of dH_2O on a parafilm for 10 min.

1b Reduction

Dip the grids in a solution of 1% $NaBH_4$ in dH_2O (w/v) for 5 min. Rinse in three Bechers containg dH_2O and put on a drop of dH_2O on a parafilm for 10 min.

2 Blocking

Incubate the grids with TBST containing 2% (20 mg/ml) human serum albumin (HSA) for 10 min.

3 Primary antibody incubation

Carefully remove TBST-HSA with a pipet and with filter paper (but do not let the grids dry completely!) and incubate in the primary antibodies, diluted in TBST with 2% HSA.
The grids can be incubated 2 hours or overnight at room temperature (depending on the antibody).Use 30-50 µL per grid.
– Rinse the grids with TBST (3 times for a few seconds).
– Rinse the grids with TBST (10 min).
– Rinse the grids with TBST (3 times for a few seconds).
– Rinse the grids with TBST (10 min).
– Rinse the grids with TBST containing 2% HAS (10 min).

4 Secondary antibody incubation (colloidal gold)

Incubate the grids with the secondary antiserum coupled to colloidal gold particles. The antiserum should be diluted 1:20 in TBST with 2% HSA and polyethyleneglycol (PEG). PEG prevents the aggregation of gold particles.
– Dissolve 5 mg PEG in 1 mL of TBST. (Take 100 µLl of this solution and 900 µL of TBST with 2% HSA).

– 30 µL are sufficient per one grid.
– Rinse the grids in dH_2O (3 times).
– Repeat these rinsing steps.
– Dry sections (with filter paper)

Proceed with contrasting (Section 6)

7.2 Lowicryl-embedded sections

(modified from van Lookeren et al., 1991, J. Histochem. Cytochem. 39:1267-1279).

1a Etching

The grids are dipped for up to 3 sec into a saturated solution of NaOH in ethanol (in a small Becher). They are then rinsed into three different Becher containing dH_2O and immersed into a drop of dH_2O (on parafilm in a Petri dish) for 10 min. The grids are then dried with filter paper and put on a parafilm sheet in a Petri dish. Check out if the sections are still on the grids!

Note: The Na-ethanolate solution (100 g NaOH in 700 mL absolute ethanol) should be used after a couple of days (when it becomes brown) and then for about three months.

1b Reduction

Transfer the grids to grid holder plates (Leica). Each plate can hold 20 grids (or more according to the type) and different plates should be used to incubate the grids with different antibodies. Put the plates in a Petri dish (with wet filter paper below). Incubate the grids in 50 µL drops of TBST, containing 0.1% (w/v, 10 mg/10 mL) sodium borohydride and 50 mM glycine (37 mg/10 mL) for 10 min. Rinse 3 times 10 sec in dH_2O.

Go to Section 7.1 for immunostaining.

Annex I: Resins

Avoid skin contact and inspiring fumes; work in the hood; wear gloves and lab coat

1) Epon

Solution A: Epon 812 with DDSA
Solution B: Epon 812 with MNA
Catalyst: DMP-30
Resin I is a mixture of Solution A and B
Resin II is made by adding 1.5% catalyst to Resin I

Prepare the solutions inside the hood (if necessary take the balance into the hood); resins are viscous; it is easier to weight them than to measure volumes

To make solution A:
Mix 75.7g (62 mL) Epon 812 with 100 g (100 mL) DDSA (or 18.95 g Epon 812 with 25 g DDSA) in a PE container using a plastic spatula; be careful not to make bubbles
Final density: 1 mL = 1.08 g
Can be stored frozen at -20°C

To make solution B:
Mix 122.2 g (100 ml) Epon 812 with 108.5 g (89 ml) MNA (or 30.55 g Epon 812 with 27.1 g MNA) in a PE container using a plastic spatula; be careful not to make bubbles
Final density: 1 mL = 1.22 g
Can be stored frozen at -20°C

To make resin I:
The hardness of Epon depends on the proportion of solution A and B
Soft Epon: A:B = 1.7/1
Hard Epon (preferred): A:B = 3:7

Soft Epon (1.7:1)		**Hard Epon (3:7)**		**Approx. volume**
Sol. A (g)	Sol. B (g)	**Sol. A (g)**	**Sol B (g)**	
13.6	9.0	**6.5**	**17.1**	20 mL
27.2	18.1	**13.0**	**34.2**	40 mL
40.8	27.1	**19.4**	**51.2**	60 mL
54.4	36.1	**25.9**	**68.3**	80 mL
68.0	45.1	**32.4**	**85.4**	100 mL

To make resin II:
Mix 1.5% (v/v) DMP-30 to resin I (0.78 g/40 mL or 1.95 g /100mL)

2) Epon - Araldite

Under construction

3) Durcupan

Under construction