

Staining Protocol for Zhang *et al.*, 2019

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This is the staining protocol for **Multiplexed peroxidase-based electron microscopy labeling enables simultaneous visualization of multiple cell types**. *Nature Neuroscience* **22**, 828-839 (2019).

Treat all samples and solutions as light-sensitive (especially the ones indicated in the list of reagents) and cover plates and vials with aluminum foils at all times. Most organic reagents are moisture sensitive and therefore containers should be kept closed as much as possible and in desiccators. Most reagents used in this protocol are highly toxic, and therefore appropriate personal protective equipment should be worn at all times and exposures should be minimized by using a fume hood.

DAB staining:

All steps are done at room temperature (r.t.) unless otherwise specified.

1. Sacrifice the animal 2-3 weeks after AAV injection by transcardial perfusion with warm (37 °C) Ames' medium with heparin (equilibrated with carbogen: 95% O₂, 5% CO₂) to clear blood, followed by warm (37 °C) 2.5% glutaraldehyde, 2% paraformaldehyde in cacodylate buffer. Post-fix the sample using the same fixative at 4 °C overnight. If transcardial perfusion is known to not fix your particular type of samples well, fix your samples with an alternative protocol, but use the fixative described above. Note if no blood clearing is done you will see strong endogenous peroxidase labeling of red blood cells, which may interfere with the observation of peroxidase staining in LM but does not usually cause any issue in EM analysis.
2. Wash the sample with cacodylate buffer, and section the sample with a vibratome at 100-200 μm in cacodylate buffer. Use low melting point agarose for embedding if possible. Trim the sections to include only the area expected to have staining. This and the following DAB staining steps should be done as soon as the post-fix is done, as we have noticed loss of enzyme activity over time in storage.
3. Use a shaker or rotator for all staining steps to ensure even staining. Place one section per well in 12-well or 24-well plates. Wash the sections for 2×10 min with 50 mM glycine in cacodylate buffer to quench aldehyde fixatives, and then wash the sections for 1×10 min with cacodylate buffer.
4. Incubate the sections in 1 mL of 0.3 mg/mL 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB, corresponds to ~ 0.16 mg/mL of the free base, assuming 9.5% water content) in cacodylate buffer for 30 min. Add 10 μL of 0.3% H₂O₂ in cacodylate buffer directly into the DAB solution (to a final H₂O₂ concentration of 0.003%) and mix well to initiate the peroxidase staining reaction. Allow the reaction to proceed for 1 hr. If higher staining solution volume is needed, scale up volumes of reagents proportionally.
5. Wash the sections with cacodylate buffer for 4×10 min. Post-fix in 3% glutaraldehyde in cacodylate buffer at 4 °C overnight.
6. Wash sections in cacodylate buffer for 1×10 min, and then in 50 mM glycine in cacodylate buffer for 1×10 min, and finally in cacodylate buffer for 2×10 min. Samples can be stored at 4 °C for a few days before further processing without apparent issues.

EM sample preparation:

Use a shaker or rotator for all staining steps to ensure even staining. Use glass scintillation vials for EM preparation.

1) Normal-contrast TEM:

Day 1:

1. Stain in 1% osmium tetroxide/1.5% potassium ferrocyanide in cacodylate buffer at r.t. for 1 hr. Place filter paper and weights on top of sections for the first 15 min to flatten the sections if necessary.
2. Wash sections in ddH₂O for 4×5 min.
3. Stain in 1% uranyl acetate in 0.05 M maleate buffer at 4 °C overnight.

Day 2:

1. Wash sections in ddH₂O for 4×5 min.
2. Dehydrate the sections with 50 % EtOH at 4 °C for 5 min.
3. Dehydrate the sections with 70 % EtOH at 4 °C for 5 min.
4. Dehydrate the sections with 90 % EtOH at 4 °C for 5 min.
5. Dehydrate the sections with 95 % EtOH for 3×10 min, with the first wash warm to r.t. from 4 °C, and second and third washes at r.t.
6. Dehydrate the sections with 100 % EtOH at r.t. for 3×20 min.
7. Exchange the sections into propylene oxide for 2×30 min.
8. Infiltrate the sections with 25% resin mix in propylene oxide for 2 hrs.
9. Infiltrate the sections with 50% resin mix in propylene oxide overnight.

Day 3:

1. Infiltrate the sections with 75% resin mix in propylene oxide for 2 hrs.
2. Infiltrate the sections with 100% resin mix for 2 hrs.
3. De-gas the samples under vacuum for 1 hr.
4. Embed the sections in 100% resin mix and cure at 60 °C for 48-72 hrs. Samples can be stored indefinitely at r.t. after curing.

Sample imaging considerations:

1. Section to 30-40 nm for best resolution.
2. We pick up sections on Formvar or Pioloform films coated with carbon. Other types of sample support may work but may have different properties under the electron beam.
3. Do not stain with lead or uranyl on sections as extra staining could obscure the DAB signal. Sample should have sufficient contrast when imaged with a digital camera.

2) SEM and High-contrast TEM (modified from Hua et al., 2015):

Day 1:

1. Stain in 2% osmium tetroxide in cacodylate buffer at r.t. for 1 hr. Place filter paper and weights on top of sections for the first 15 min to flatten the sections if necessary.
2. Stain in 2.5% potassium ferrocyanide in cacodylate buffer at r.t. for 1 hr.
3. Wash sections in ddH₂O for 4×5 min.

4. Stain in 1% thiocarbohydrazide in ddH₂O at 40 °C for 15 min.
5. Wash sections in ddH₂O for 4×5 min.
6. Stain in 2% osmium tetroxide in ddH₂O at r.t. for 1 hr.
7. Wash sections in ddH₂O for 4×5 min.
8. Stain in 1% uranyl acetate in 0.05 M maleate buffer at 4 °C overnight.

Day 2:

1. Heat sections in uranyl acetate solution to 50 °C for 2 hrs.
2. Wash sections in ddH₂O for 4×5 min.
3. Dehydrate the sections with 50 % EtOH at 4 °C for 5 min.
4. Dehydrate the sections with 70 % EtOH at 4 °C for 5 min.
5. Dehydrate the sections with 90 % EtOH at 4 °C for 5 min.
6. Dehydrate the sections with 95 % EtOH for 3×10 min, with the first wash warm to r.t. from 4 °C, and second and third washes at r.t.
7. Dehydrate the sections with 100 % EtOH at r.t. for 3×20 min.
8. Exchange the sections into propylene oxide for 2×30 min.
9. Infiltrate the sections with 25% resin mix in propylene oxide for 2 hrs.
10. Infiltrate the sections with 50% resin mix in propylene oxide overnight.

Day 3:

1. Infiltrate the sections with 75% resin mix in propylene oxide for 2 hrs.
2. Infiltrate the sections with 100% resin mix for 2 hrs.
3. De-gas the samples under vacuum for 1 hr.
4. Embed the sections in 100% resin mix and cure at 60 °C for 48-72 hrs. Samples can be stored indefinitely at r.t. after curing.

Special considerations for using Matrix-dAPEX2 and IMS-dAPEX2:

1. This protocol leads to strong staining in the IMS for mitochondria labeled with Matrix-dAPEX2 (see **Supplementary Figure 5**). This is preferred because this IMS staining pattern is usually not obscured with heavy counterstaining required for SEM. Do not use IMS-dAPEX2 with this protocol, as it has a smaller range of optimal expression level than Matrix-dAPEX2 and often not distinguishable from Matrix-dAPEX2 under this staining condition.
2. Consider examining ultrathin sections with TEM first to determine the percentage of mitochondria with IMS staining when using Matrix-dAPEX2. If the percentage is too low, you can increase the DAB concentration to 0.5 mg/mL and see if IMS staining becomes more prevalent.

Sample imaging considerations:

1. Section to 30-40 nm for best resolution. We use the same sectioning and pickup setup as in Kasthuri *et al.*, 2014.
2. Stain with lead citrate on sections to increase contrast.
3. Ultrathin sections can be imaged with SEM using either backscattered electrons or secondary electrons. Postsynaptic densities tend to be more prominent using backscattered electrons, but imaging using secondary electrons can be substantially faster.

Catalog # for reagents:

Ames' medium: Sigma A1420
Sodium bicarbonate: Sigma-Aldrich 792519
Heparin: Sigma-Aldrich H3393
Glutaraldehyde: Electron Microscopy Sciences 16316
Paraformaldehyde: Electron Microscopy Sciences 15712
Sodium cacodylate: Electron Microscopy Sciences 12310
Hydrochloric acid: Fisher A144SI-212
Calcium chloride: Sigma-Aldrich 793639
Magnesium chloride hexahydrate: Sigma-Aldrich M9272
Glycine: Sigma-Aldrich 410225
3,3'-diaminobenzidine tetrahydrochloride hydrate: Sigma D5637, **light sensitive**
Hydrogen peroxide: Sigma-Aldrich 216763, **light sensitive**
Osmium tetroxide: Electron Microscopy Sciences 19190
Potassium ferrocyanide: Sigma-Aldrich P3289, **light sensitive**
Uranyl acetate: Electron Microscopy Sciences 22400, **light sensitive, radioactive**
Maleic acid: Sigma-Aldrich M0375
Sodium hydroxide: Fisher SS266-1
Ethanol: Sigma-Aldrich 459836
Propylene oxide: Electron Microscopy Sciences 20411
LX-112: Ladd Research 21310
DDSA: Electron Microscopy Sciences 13710
NMA: Electron Microscopy Sciences 19000
DMP-30: Electron Microscopy Sciences 13600

SEM and High-contrast TEM only:

Thiocarbohydrazide: Electron Microscopy Sciences 21900, **light sensitive**

Protocols for solution preparation:

Ames' medium with heparin (10 U/mL)

Dissolve the Ames' medium powder in appropriate amounts of ddH₂O. Gas the solution with 100% CO₂, and then slowly add sodium bicarbonate at 1.9 g/L. Make sure no precipitates are formed. Dissolve heparin in ddH₂O to make a 10 kU/mL solution. Add 1 mL of 10 kU/mL heparin solution per 1 L of Ames' medium. Filter the solution with a 0.22 µm filter and store at 4 °C.

Cacodylate buffer (0.15 M sodium cacodylate, 4 mM CaCl₂, 4 mM MgCl₂, pH 7.4)

Dissolve sodium cacodylate (64.21 g for 1 L), calcium chloride (0.89 g for 1 L) and magnesium chloride hexahydrate (1.63 g for 1 L) in ddH₂O to make a 0.3 M sodium cacodylate, 8 mM calcium chloride, 8 mM magnesium chloride solution. Adjust pH to 7.4 with 12 M hydrochloric acid. Filter the solution with a 0.22 µm filter and store at 4 °C. Mix equal volumes of 0.3 M sodium cacodylate, 8 mM calcium chloride, 8 mM magnesium chloride solution (pH 7.4) and ddH₂O to make cacodylate buffer. Store at 4 °C.

DAB stock solution

Dissolve 3,3'-diaminobenzidine tetrahydrochloride hydrate in ddH₂O to make a 50 mg/mL solution. Aliquot and store the solution at -20 °C. Thaw the solution just before use and vortex to dissolve all solids. The solution can be refrozen for repeated uses but should be discarded when significant color appears in the solution.

1% uranyl acetate in 0.05 M maleate buffer (pH 5.15)

Dissolve 11.6 g of maleic acid with 100 mL 1 N sodium hydroxide solution and bring up the volume to 500 mL with ddH₂O to make a 0.2 M sodium maleate solution. Combine 100 mL of 0.2 M sodium maleate solution, 10.76 mL 1 N sodium hydroxide solution, bring up the volume to 400 mL with ddH₂O and verify that pH is 6.0, and then add 4 g of uranyl acetate to make 1% uranyl acetate in 0.05 M maleate buffer. Filter the solution with a 0.22 µm filter and store at 4 °C.

Resin mix

	~60 g total	~30 g total
Resin (WPE = 143)	30.00 g	15.00 g
DDSA	16.38 g	8.19 g
NMA	15.21 g	7.61 g
DMP-30	0.78 g	0.39 g

Mix the first three components together thoroughly for 5 min by hand. Then add DMP-30 and mix for another 5 min. Use the freshly prepared resin mix immediately for infiltration and do not store and reuse the resin mix, as polymerization occurs immediately after all the components are mixed in leading to increasing viscosity, even at r.t.

This resin mix is equivalent to the 5 mL/5 mL Mixture A/Mixture B combination in Luft, 1961. We used LX-112 as the resin. We have not tried other types of resins but they may also give comparable results. Different infiltration/embedding schedules may need to be used if using other types of resins or hard to infiltrate sample types.

For consistent results, adjust the amounts of components using the WPE specific to your batch of resin. To calculate the amounts of components, multiply the amounts of DDSA and NMA in the table above by 143/WPE. Then, for each gram of increase/decrease of total anhydrides (DDSA + NMA), increase/decrease the amount of DMP-30 by 0.0132 g. For example, if the WPE of your resin is 160, then for 30.00 g of resin, you need $16.38 * 143 / 160 = 14.64$ g of DDSA, $15.21 * 143 / 160 = 13.59$ g of NMA, and $0.78 - (1.74 + 1.62) * 0.0132 = 0.74$ g of DMP-30.

Normal-contrast TEM only:

1% osmium tetroxide/1.5% potassium ferrocyanide in cacodylate buffer

Mix equal volumes of 4% osmium tetroxide and ddH₂O to make a 2% osmium tetroxide solution. Dissolve potassium ferrocyanide in 0.3 M sodium cacodylate, 8 mM calcium chloride, 8 mM magnesium chloride solution (pH 7.4) to make a 3% potassium ferrocyanide solution. Add equal volumes of the 3% potassium ferrocyanide solution dropwise to the 2% osmium tetroxide solution, and then mix well to make 1% osmium tetroxide/1.5% potassium ferrocyanide in cacodylate buffer.

SEM and High-contrast TEM only:

2% osmium tetroxide in cacodylate buffer

Mix equal volumes of 4% osmium tetroxide and 0.3 M sodium cacodylate, 8 mM calcium chloride, 8 mM magnesium chloride solution (pH 7.4) to make 2% osmium tetroxide in cacodylate buffer.

2.5% potassium ferrocyanide in cacodylate buffer

Dissolve potassium ferrocyanide in cacodylate buffer to make 2.5% potassium ferrocyanide in cacodylate buffer.