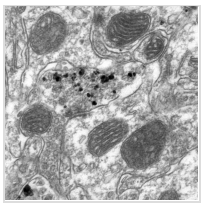

Sep 12, 2022

Quantitative analyses of the ultrastructural features of dopaminergic axon terminals. Protocol #1: Tissue preparation for electron microscopy

In 1 collection

Natalie M Doig[1], Peter J Magill[1,2]

[1]Medical Research Council Brain Network Dynamics Unit, Nuffield Department of Clinical Neurosciences, University of Oxford, Mansfield Road, Oxford, OX1 3TH, United Kingdom;

[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD

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Cláudia C. Mendes

ABSTRACT

The release of dopamine from axons is critical for normative brain function and behaviour. Impaired or otherwise inappropriate dopamine release often correlates with changes in the ultrastructure of dopamine neuron axons that can assessed with electron microscopy. Here, we provide two protocols that can be used serially to, first, help the user process animal brain tissue for electron microscopy and, secondly, help the user undertake quantitative analyses of the ultrastructural features of dopaminergic axon terminals in the brain.

This Protocol #1 describes how to prepare brain tissue, carry out pre-embedding immunohistochemistry for tyrosine hydroxylase as a marker of dopaminergic axons, and then make tissue sections ready for electron microscope.

The partner Protocol #2 details how to examine and image ultrathin sections of tissue using a transmission electron microscope and then how to analyse the digital images.

Protocol #1 and #2 are part of the collection "Quantitative analyses of the ultrastructural features of dopaminergic axon terminals".

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COLLECTIONS

Quantitative analyses of the ultrastructural features of dopaminergic axon terminals

KEYWORDS

Dopamine, Axon terminal, Striatum, Tyrosine hydroxylase (TH), Transmission Electron Microscopy (TEM), Electron microscopy (EM), Substantia nigra pars compacta (SNc), Ventral Tegmental Area (VTA), Vesicle, Synapse, Ultrastructure, Immuno-EM, Immunogold

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PARENT PROTOCOLS

Part of collection

Quantitative analyses of the ultrastructural features of dopaminergic axon terminals

MATERIALS TEXT

Perfusion-Fixation

Reagents:

- Paraformaldehyde
- Glutaraldehyde (25% aqueous solution, EM grade)
- Sodium Hydroxide (0.5 M NaOH)
- 0.2 M phosphate buffer, pH=7.4 (PB)
- Phosphate buffered saline, pH=7.4 (1 X PBS)
- Anaesthetic

Equipment:

- Peristaltic pump
- Dissection Instruments
- Plastic or blunt needle to insert into the heart

To make PFA-glutaraldehyde fixative solution:

This protocol makes 1 litre of 4% w/v PFA in 0.1 M phosphate buffer (PB), pH 7.4, with 0.1% v/v glutaraldehyde. All procedures should be carried out in a fume hood. The finished paraformaldehyde solution can be stored at 4°C for 1-2 weeks. Note that you can purchase methanol-free ready-made 4% PFA.

1. Heat approximately 400 mL of distilled water (dH_2O) to 65-70°C on a hotplate stirrer in a fume hood.
2. Weigh and add 40 g PFA, stir continuously.
3. Add 0.5 M NaOH dropwise until the solution clears, the solution will clear slowly at about 60°C. There is a lag so pause between adding NaOH.
4. Filter the solution.
5. Add 500 ml 0.2 M PB. Make up to 1 L with dH2O.
6. Just before starting the perfusion procedure, add the glutaraldehyde to make up to a final concentration of 0.1% v/v. For 1 L of fixative solution, to achieve a final concentration of 0.1% v/v glutaraldehyde (starting with a solution of 25% v/v glutaraldehyde) you would need to add 4.0 ml.

Tissue Preparation

Reagents:

- Superglue
- Phosphate buffered saline, pH=7.4 (1 X PBS)
- Agar (4% in dH2O)
- Cryoprotectant (25% sucrose w/v and 10% glycerol v/v in 0.05 M PB)
- 0.2 M phosphate buffer, pH=7.4 (PB)

Vibrating Microtome Sectioning

Equipment:

- Vibrating microtome, Leica VTS1000 S, Leica Biosystems

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- Labelled glass vials
- Paint brushes
- Brain matrix for mouse (stainless steel), Ted Pella, Catalog #15003
- 50 ml centrifuge tubes
- Razor blades (wipe clean with 70% ethanol prior to use)
- Filter paper
- Liquid nitrogen
- Extra strong aluminium foil

Immunohistochemistry

Reagents:

- Normal goat serum (NGS; Vector Laboratories Catalog # S-1000-20; RRID:AB_2336615)
- Rabbit anti-tyrosine hydroxylase antibody (Millipore Catalog # AB152; RRID:AB_390204)
- Nanogold IgG goat anti-rabbit IgG (H+L) Antibody (Nanoprobes Catalog # 2003; RRID:AB_2687591)
- HQ silver enhancement kit from Nanoprobes (#2012-45ML; use at RT; reagents are light sensitive, store at -20°C, make sure to thaw completely before use)
- Sodium acetate buffer (0.1 M, pH 7.0-7.5). To make 250 ml: 3.4 g sodium acetate tri-hydrate dissolved in 250 ml dH2O, adjust pH to 7.0-7.5 with dilute acetic acid.

Equipment:

- Glass embryo dishes (or similar) with foil to cover
- Glass Pasteur Pipettes
- Microcentrifuge tubes

Preparation of Sections for EM

Reagents:

- 1% solution of osmium tetroxide in 0.1 M PB pH 7.4 (bought as a 4% solution, EM grade)
- Graded series of v/v dilutions of ethanol in dH2O (50%, 70%, 90%, 100%).
- Dry absolute ethanol (stored over anhydrous copper sulphate).
- Propylene oxide
- 1% w/v solution of uranyl acetate in 70% v/v ethanol. This solution should be covered or stored in the dark and must be filtered before use.
- EM epoxy resin (Durcupan ACM)

Equipment:

- Stereomicroscope
- Double- and single-edged razor blades
- Scalpel
- Hot plate
- Glass Pasteur Pipettes
- Glass petri dishes with lids
- Pre-made resin blocks (these are made with the same Durcupan resin as the tissue is embedded in; the resin is cured in a suitable mold/capsule to create a cylindrical block with a flat top).

Re-sectioning for EM

Equipment:

- Ultramicrotome, Leica UC6, Leica Biosystems

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- Pioloform coated single-slot copper grids (you can coat these yourself or you can purchase readymade grids)
- Glass knives
- Diamond knife (e.g., ultra 45), Diatome
- Cocktail sticks

Staining of ultrathin sections with Reynold's lead citrate

Reagents:

- Lead nitrate
- Tri-sodium citrate
- 4 M NaOH
- NaOH pellets
- dH2O (use double distilled if you can)

Equipment:

- Petri dish
- Parafilm
- Filter paper
- Hair dryer
- Pasteur Pipettes (glass?)

To make lead citrate:

1. Weigh out 0.27 g of lead nitrate into a glass vial. Weigh 0.35 g tri-sodium citrate in a separate vial or weigh boat.
2. Add 9.6 ml dH2O to the lead nitrate. Put cap on and shake to dissolve.
3. Add the tri-sodium citrate to the lead nitrate. Shake to dissolve. The solution will become milky in appearance.
4. Add 0.4 ml of 4 M NaOH. Replace the lid quickly and shake. It is important to prevent contact with the air as much as possible as the lead citrate reacts with atmospheric carbon dioxide. The solution should be clear.
5. You can aliquot and store the solution in syringes – make sure that all air is expelled from the syringe. The solution can be stored at 4 °C for a few weeks (store in appropriate toxic fridge).

SAFETY WARNINGS

Safety Note – Paraformaldehyde and glutaraldehyde

Paraformaldehyde and glutaraldehyde are toxic and harmful if swallowed, inhaled, or absorbed through the skin. Wear appropriate personal protective equipment. All manipulations should be carried out in a fume-hood. Dispose of waste in accordance with your institution's safety guidelines.

Safety Note – Osmium tetroxide

Osmium tetroxide is a very powerful oxidizing agent and is highly toxic. Osmium tetroxide is volatile and can cause irreversible damage to lungs and eyes at low concentrations. All manipulations should be carried out in a fume hood. Wear appropriate personal protective equipment. Osmium tetroxide should be stored at low temperature (2-8°C). It can penetrate plastics and packaging; therefore, stock solutions should be stored in accordance with your institute's safety guidelines; we routinely use double ground glass stoppered bottles to store solutions of osmium tetroxide.

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Inactivate and dispose of waste according to your Institution's safety guidelines.

Safety Note – Propylene Oxide

Propylene oxide is a volatile and extremely flammable liquid. It is highly toxic and a potential mutagen and carcinogen. It is toxic via ingestion, inhalation, or absorption through the skin. All manipulations must be carried out in a fume hood. Wear appropriate personal protective equipment. Please note that propylene oxide requires specific gloves for handling and should not be handled with standard nitrile gloves. Store, handle and dispose of propylene oxide in line with your institution's safety guidelines.

Safety Note – Uranyl acetate

Uranyl acetate is toxic as it is both mildly radioactive and a heavy metal. Uranyl acetate is very toxic if ingested, inhaled as dust, or by skin contact if skin is cut or abraded. Wear appropriate personal protective equipment and only handle in a fume hood. It must be handled, stored, and disposed of in line with your institution's safety and radiation guidelines.

Safety Note – Durcupan Epoxy Resin

Durcupan ACM epoxy resin contains toxic components. Wear appropriate personal protective equipment and only handle in a fume hood. Polymerize all excess resin and all contaminated materials at 60°C for 48h before disposal.

Safety Note – Reynold's Lead Citrate

Lead salts are extremely toxic so exercise care and wear suitable personal protective equipment. Store, handle and dispose of lead salts in accordance with your institution's safety guidelines.

BEFORE STARTING

Animal use should be carried out in accordance with the institutional and national guidelines on the use of animals in research.

Note that many of the chemicals used in the preparation of tissue for electron microscopy (EM) are toxic. We recommend discussing all protocols and chemicals with your institute's safety officer before beginning.

Perfusion-Fixation

1 A successful perfusion is not only key to ensuring good ultrastructure of tissue when assessed in the electron microscope, but also is a requirement for successful sectioning, staining, and embedding. The following steps are written for an adult mouse but can be adapted for a rat (Lapper, S. R. & Bolam, J. P. , 1992).

Here, we briefly outline perfusion-fixation, see Gage et al., 2012; Zhang and Xiong, 2014 for more details.

The main adaptation for EM, compared to light microscopy (LM), is that glutaraldehyde

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should be added to the paraformaldehyde (PFA) fixative solution to help optimally preserve for tissue ultrastructure.

Note that glutaraldehyde is essential for preserving tissue ultrastructure for EM, as it is a more effective cross-linker than PFA; but it is slower to penetrate the tissue and thus a combination of both aldehydes is needed. There is, however, a balance to be found between immunogenicity and tissue preservation – higher concentrations of aldehydes will give you better preservation but may block antigenic sites; therefore, you might need to reduce the concentration of glutaraldehyde if you do not get good immunolabeling with your chosen antibodies.

We routinely use 0.1% v/v glutaraldehyde, the working range is from 0.01% up to 1%; if no immunolabeling is to be carried out at all (i.e., exanimation of ultrastructure alone) you can use a much higher concentration. The object of the protocol described here is to carry out immunohistochemistry for tyrosine hydroxylase (TH) so ~0.1% glutaraldehyde achieves a good balance.

1.1 Deeply anaesthetize the animal. We induce anaesthesia using isoflurane (4% v/v in O_2), then give an overdose of injectable anaesthetic e.g., pentobarbitone (1.5 g/kg, i.p.).

1.2 Open up the thorax and put a cannula into the left ventricle and gently push into left atrium towards the ascending aorta.

1.3 Using a peristaltic pump, exsanguinate the mouse by transcardial perfusion with 20-50 ml of PBS. Once all blood has been removed, switch to the PFA-glutaraldehyde fixative solution. It is beneficial to limit the PBS rinse time to ~100 s or less to avoid significant changes to synaptic structure (Tao-Cheng et al., 2007). A good indicator of when to switch to fixative is when the liver becomes clear of blood and is a pale cream colour.

1.4 Run through the fixative solution for a fixed time or volume; an average would be about 100 ml over 20 mins, but this should be determined experimentally. Rapid stiffening of muscles (often following transient muscle twitching) is often an indicator of a successful perfusion with fixative.

1.5 Remove brain carefully to avoid damage to your area of interest. The brain should be firm to the touch and a creamy-white colour. Any pinkness is generally an indicator of a poor perfusion.

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1.6 Immerse brain(s) in the fixative solution for an additional 12 h at 4°C.

Tissue Preparation

2 The most common method for preparing sections for EM is a vibrating microtome; the reason being that frozen sections made with a cryostat or a freezing microtome have poor ultrastructure (Lapper, S. R. & Bolam, J. P. , 1992).

The aim of this protocol is to obtain coronal sections of the mouse brain from anterior to the striatum to posterior of the midbrain; in order to allow the experimenter to examine dopamine neuron axon in all parts of the striatum and preserve sections containing the midbrain dopaminergic cell bodies for any other investigations. For EM, it is beneficial to process the tissue as soon as possible after the 12h immersion in fixative.

3 **Protocol for sectioning a mouse brain with a vibrating microtome**

3.1 In the fume-hood, rinse the brain thoroughly in PBS to get rid of any excess fixative solution. Trim/remove any meninges.

3.2 Block the most anterior part of the brain i.e., the olfactory bulbs, making sure to leave the entire striatum intact. Trim the very caudal part of the cerebellum to create an even, flat surface. Use a brain matrix to help keep cuts straight and perpendicular to the direction of cutting. It is a common error to trim unevenly which results in the hemispheres within one section being at different coronal planes (in relation to Bregma); this is especially problematic if one hemisphere is the control for the other hemisphere.

3.3 When using the vibratome to section the majority of the mouse brain, we recommend embedding in agar to improve support of the tissue.

3.4 To embed in agar, make a 4% w/v solution of agar in dH_2O, heat the solution in a glass beaker until it boils and then allow to cool until it is safe to the touch but not yet thickened.

3.5 Whilst the agar is cooling, dry the brain with filter paper and place it flat, cerebellum side down within a container (we use a trimmed 50 ml centrifuge tube on a plastic surface secured by Blu Tack™).

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3.6 Pour the cooled agar over the brain ensuring that the brain remains in the centre and does not float. Once the agar has set, remove it from the container and trim a safe distance around the brain to create a block for sectioning.

3.7 Dry the bottom of the agar-embedded brain with filter paper and glue it to the vibratome stage with cyanoacrylate adhesive (e.g. superglue).

3.8 Set-up the vibratome and fill the cutting chamber with PBS.

3.9 Cut sections; a thickness of 50-70 µm is recommended.

3.10 Start collecting sections at about the level where the forceps minor of the corpus collosum appears, approximately 2 mm anterior to Bregma (Paxinos and Franklin, 2019).

3.11 Using a paint brush, carefully collect sections into a 1 in 6 series into glass vials containing PBS. Keep collecting sections until you have cut past the entire midbrain.

3.12 Wash sections 3 × 10 min in PBS.

4 **Protocol for freeze-thawing of mouse brain sections:** Freeze-thawing is a technique used to increase the penetration of immunochemicals by mechanically disrupting the tissue. For EM, it is preferred over the use of detergents as it is less detrimental to the ultrastructure. The aim is to produce very small ice crystals that cause restricted damage to the tissue. There are multiple methods to freeze-thaw tissue; here we describe one that we routinely use.

4.1 Select the sections to be processed for EM and place these in a separate container.

4.2 To make 100 ml cryoprotectant: add 25 g sucrose, 10 ml glycerol and 25 ml 0.2 M PB into a glass bottle and make up to 100 ml with dH_2O.

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4.3 Place chosen sections into cryoprotectant for a minimum of 4 h. Gently shake until the sections have sunk. You can leave them overnight on a shaker at 4°C.

4.4 Prepare liquid nitrogen in a Dewar flask or other suitable container (take appropriate safety measures when using liquid nitrogen including the use of appropriate personal protective equipment).

4.5 The aim is to freeze the sections very rapidly on the surface of the liquid nitrogen. To enable this, the sections are placed in a holder that can be readily applied to the nitrogen. A 6-well plastic plate or another holder that will not break when frozen rapidly can be used. You can also create such a holder out of extra strong aluminium foil and then make one end into an 'envelope' to hold the sections and use the other end as the handle.

4.6 Remove section(s) from cryoprotectant using a paintbrush. Place the section(s) into one of the wells of the 6-well plate and remove all excess cryoprotectant using filter paper; no more than 3 sections should be processed at any one time. Make sure sections are flat and not touching each other or the sides of the well-plate.

4.7 Hold the well plate using plastic forceps and place onto the top of the liquid nitrogen for 30 s, in the vapor just above the liquid surface. After 30 s, sections should be clearly frozen and become opaque.

4.8 Remove the well-plate from the liquid nitrogen and quickly add room temperature cryoprotectant to the well to thaw the sections rapidly.

4.9 Repeat the freeze and thaw process twice more – to give a total of 90 s of freezing.

4.10 After the final freeze, you can thaw the sections with room temperature PBS instead of cryoprotectant.

4.11 Repeat for all your sections.

Immunohistochemistry

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5 In this protocol we use a primary antibody for tyrosine hydroxylase (TH) as a marker of dopaminergic axons in the striatum; followed by a gold-conjugated secondary antibody (See Figure 1). These antibodies listed under Materials/Reagents have been tried and tested (Kosillo et al., 2019), but could be replaced by other suitable antibodies if needed. Silver-intensification is then used to increase the size of the colloidal gold particles so that they can be seen at the LM level and more easily located at lower magnifications at the EM level (Bolam, 1992).

6 **Primary and Secondary Antibody Incubation**

6.1 Wash sections thoroughly in PBS.

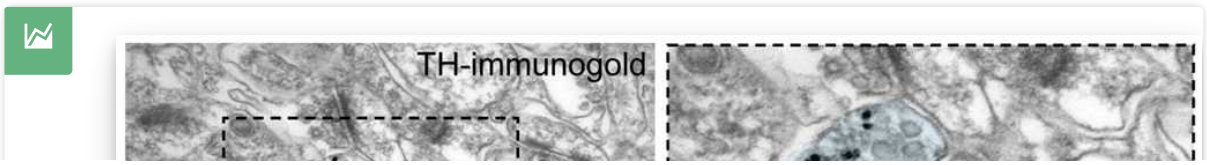
6.2 Block sections by incubation in PBS containing 10% v/v normal goat serum (NGS) for 1 h. All incubations should be carried out with constant gentle shaking.

6.3 Incubate in primary rabbit anti-TH antibody (1:1000 dilution) in PBS with 2% v/v NGS overnight at room temperature (RT). Alternatively, sections can be incubated for 72 h at 4°C.

6.4 Wash sections 3 × 5 min in PBS.

6.5 Incubate in gold-conjugated goat anti-rabbit secondary antibody (1:400) in PBS with 1% v/v NGS for a minimum of 4 h at RT (can be incubated overnight at 4°C).

7 **Silver Intensification of Gold Conjugated to Secondary Antibody:** Silver intensification or enhancement is the process by which growing shells of silver are deposited over the gold particles that are conjugated to the secondary antibody. We use commercial kits as they are easy to use and reliable. Here we use the HQ silver enhancement kit from Nanoprobes (Nanoprobes #2012-45ML) but there are other commercial kits available. Detailed instruction protocols come with the kits; here we describe our routinely used method (See Figure 1).



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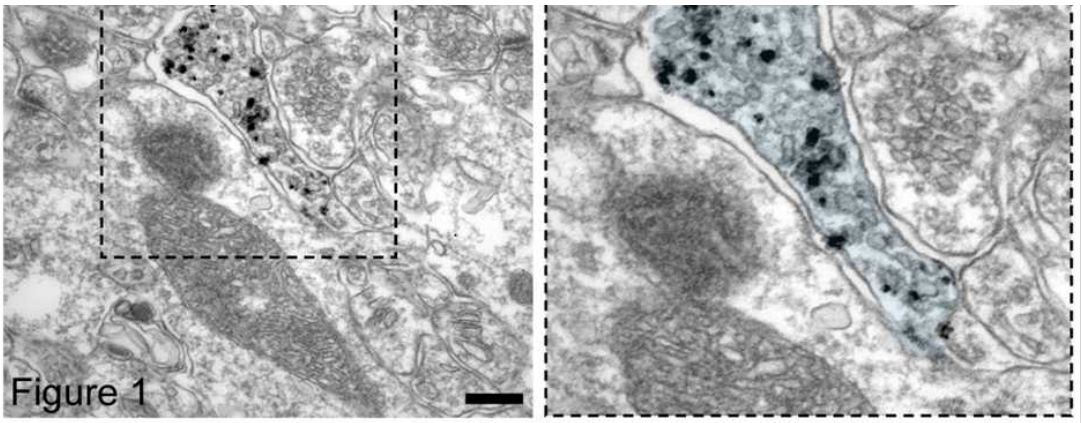

Figure 1. An example electron micrograph of a dopaminergic axon profile in the striatum. Left, Axon terminal showing enriched silver-intensified immunogold labelling for tyrosine hydroxylase (TH) as a marker of dopaminergic structures. Right, enlarged view pseudocolored to highlight the axon terminal. Note the presence of multiple synaptic vesicles. This image was acquired and analysed as part of a previous study (Kosillo et al., 2019). Scale bar, 200 nm.

7.1 Wash sections 2 × 10 min in PBS.

7.2 Wash in dH_2O (2 × 5 min). Sections must be thoroughly rinsed with deionized water before silver enhancement reagents are applied. This is because the buffers used for antibody incubations and washes contain chloride ions and other anions which form insoluble precipitates with silver (Bolam, 1992).

7.3 Wash in sodium acetate buffer (2 × 5 min; see Materials for preparation). This is optional, but sodium acetate can speed up the reaction and reduce background.

7.4 Place a section into a small glass embryo dish (or similar), remove excess sodium acetate buffer and make sure section is flat. Ensure the tissue section does not dry out.

7.5 Mix solutions A and B from HQ silver enhancement kit in equal volumes (i.e., 5 drops of each) in a microcentrifuge tube. Add an equal volume of C into the tube and mix.

7.6 Remove any remaining buffer from the dish and add the mixed silver enhancement solution to start the intensification process.

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7.7 React in the dark for 2-10 min. Stop reaction with acetate buffer.

7.8 Wash with dH_2O (2 × 5 min). Wash with PBS (1 × 10 min).

7.9 More or less reaction time controls silver-gold particle size. You will need to determine the optimal reaction timing dependent on the antibody and tissue; to do this, react and wash the first section and check it under a light microscope to assess whether reaction time needs to be altered. It can be useful to react a section containing midbrain dopaminergic neurons as you will very clearly see if the reaction has worked if the TH-immunoreactive cell somata become a dark brown colour. To assess the extent of the reaction in the striatum focus on the border between striatum and cortex; the cortex should remain a very pale cream colour and the striatum should look yellow-gold in comparison. Note that the gold particles are on the surface of the tissue and do not penetrate into the middle of the section.

7.10 Repeat for remaining sections.

Preparation of Sections for EM

8 **Post-Fixation with Osmium Tetroxide:** After immunohistochemical procedures have been carried out, sections that have been chosen for EM are post-fixed in a buffered osmium tetroxide (OsO_4) solution. Osmium, a heavy metal, will embed directly into the lipid membranes and myelin and thereby create necessary contrast for EM (Bolam, 1992; Zhang and Xiong, 2014). During the osmium treatment, the sections will turn dark brown, almost black (depending on length of treatment), and will become very brittle. Any folds in the tissue before osmium treatment will become fixed irreversibly; it is therefore very important to make sure your sections are very flat and even before applying the osmium solution.

All manipulations involving osmium tetroxide must be carried out in a fume hood. See Materials for how to prepare a working 1% v/v solution of osmium tetroxide.

8.1 Wash sections 2 × 10 min in 0.1 M PB.

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8.2 Transfer sections to a small glass Petri dish containing 0.1 M PB (make sure that dish has a suitable glass lid).

8.3 Remove the buffer slowly, using a paintbrush to ensure that the sections remain flat on the bottom of the dish. Pipette off as much of the PB as possible.

8.4 Carefully pipette 1-2 ml of the 1% v/v osmium tetroxide solution onto the sections using a glass Pasteur pipette. Replace lid. Make sure section is well covered but not floating.

8.5 Incubation time depends on the thickness of the sections. The osmium reaction time is also reduced when it is done following silver intensification as osmium tetroxide can cause some loss of silver. For 50 µm sections we immerse them in osmium tetroxide solution for 7-12 min, depending on the level of silver-intensified gold labelling.

8.6 Pipette off the osmium solution and place in a waste container (dispose according to your institution's safety guidelines).

8.7 Wash section 3 × 15 min in 0.1 M PB.

8.8 It is preferable to dehydrate and embed the sections in resin immediately following osmium treatment.

9 **Dehydration and Embedding in Resin:** It is necessary to embed the sections in an electron microscopic resin before ultrathin sectioning. The resin provides support for re-sectioning but also allows high resolution LM prior to sectioning. Most resins are not miscible with water; therefore, the tissue must be fully dehydrated with ethanol before embedding. Once all water has been removed, the tissue sections are equilibrated in a solution that is miscible with both the ethanol and the resin; in our protocol this is propylene oxide (Bolam, 1992). During the dehydration, the tissue sections are also exposed to a solution of uranyl acetate, another heavy metal used to increase contrast.

9.1 Remove the PB from the petri dishes and replace with 50% v/v ethanol in dH_2O. Wash 2 × 15 mins. Use a large volume to remove phosphate, as phosphate can react with uranyl acetate to form an electron dense precipitate.

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9.2 Replace 50% v/v ethanol solution with 1% w/v uranyl acetate dissolved in 70% v/v ethanol. Incubate for 20-45 mins in the dark (cover with foil).

9.3 Replace with 95% v/v ethanol for 15 min.

9.4 Replace with 100% ethanol for 10-15 min.

9.5 Incubate in dry absolute ethanol 2 × 10 min.

9.6 Carefully replace ethanol with propylene oxide (2 × 10 min). Note that propylene oxide is extremely volatile, so ensure sections do not dry out when changing solutions. There are safer alternatives to propylene oxide that are commercially available.

9.7 During the dehydration, prepare the resin according to the manufacturer's instructions. For Durcupan ACM epoxy resin, we mix the components A:B:C:D in the ratio: 10: 10: 0.3: 0.2, by weight. Mix the resin in a plastic beaker with a wooden spatula. Note that small changes to these ratios can result in drastic changes to the polymerization of the resin.

9.8 Transfer a few ml of resin into a shallow container such as a weigh-boat. Make sure containers are well labelled (note that ethanol and propylene oxide will wash away permanent marker pen).

9.9 Using a paintbrush, carefully transfer the sections from the propylene oxide into the resin. Gently make sure that the sections are covered in resin and leave overnight in the fume hood.

9.10 Gently warm boats on a hot plate in the fume hood, to reduce the viscosity of the resin. Using your resin paintbrush and some forceps, carefully transfer the sections to uncleaned slides. Allow the sections and resin to settle on the slide.

9.11 Carefully manipulate the sections using cocktail sticks or a paintbrush to make sure they are orientated correctly and not overlapping; it is helpful to do this using a dissection stereomicroscope (protected from resin using

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Parafilm).

9.12 Gently place a coverslip over the sections, take care not to press the coverslip down too hard. Try and remove any bubbles that are on or near the sections using a cocktail stick, but do not press down the coverslip too hard on top of the sections as they are still brittle and liable to shatter.

9.13 Getting the amount of resin just right is done by trial and error; enough resin is needed so that there is no air under the coverslip but not so much that it is seeping out the sides of the coverslip. Absorb excess resin with filter paper and add extra resin using the end of a cocktail stick, place small drops along the edge of the coverslip. If it is not necessary to do high resolution LM prior to EM you can use acetate foils to cover the sections rather than a glass coverslip, the benefit of this is that the acetate is easier to remove than a coverslip. If using coverslips, it is helpful to carefully rub them on your skin so that they have a thin layer of grease on them, this aids greatly when trying to remove them at a later stage.

9.14 Place slides in a container lined with foil. To polymerize the resin, place the container with the slides in it into an oven at 60°C for 48 h. Make sure the container is level. All materials that are contaminated with resin can be placed in the oven to polymerize and then be disposed of safely.

9.15 The slides and sections are now suitable for LM prior to EM and can be stored indefinitely.

10 **Re-embedding for EM:** Once you have examined your slides in the light microscope (LM), you can select specific regions of the sections to be further processed for examination in the electron microscope. It is very useful to take some LM images of these areas prior to re-sectioning the tissue for EM. We recommend taking a few low- and high-magnification images to help with the next few stages. In the re-embedding protocol, superglue is used to affix the dissected-out resin-embedded tissue to the top of a pre-made resin block; we use this method as it is quick, but the drawback is that you cannot take high magnification LM images of the tissue in the block once re-embedded.

10.1 Examine your slides and sections. Identify which section(s) from each slide you will want to examine tissue from. It is useful to take pictures of the whole slides for reference (you can do this using a regular scanner or photocopier). Using a light microscope, identify the region(s) of the striatum to be examined at the EM level; take LM photos for correlation and orientation. The region of interest from each section will then be cut out and attached to a resin cylinder suitable for ultrathin sectioning for the electron microscope.

10.2 If you are using acetate instead of glass coverslips, you can remove the acetate prior to imaging but take care not to damage the underlying tissue. If

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you need higher magnification images you can apply a drop of immersion oil and temporarily place a glass coverslip over the top of the tissue.

10.3 If using a glass coverslip, remove your coverslip from the slide; to do this remove all the resin from around the coverslip using a single or double-edged blade. Then gently insert a double-edged razor blade between the coverslip and the resin and gently push the blade under the coverslip until it becomes free. Wear eye protection.

10.4 Warm the slide on a hot plate; the polymerized resin is brittle at room temperature and will crack or split if not warmed enough.

10.5 Make guide cuts into the resin-embedded tissue using a scalpel or double-edged razor blade, then warm again and make cuts under dissecting microscope to free the piece of tissue in your region of interest. Keep warming the slide between cuts to prevent cracking of the tissue.

10.6 Place a small drop of superglue on top of a resin block, gently remove your section of resin-embedded tissue from the slide using forceps and place onto the superglue, adjusting with a cocktail stick if needed. You can flatten the piece of tissue on a razor blade on the hotplate if needed.

10.7 Label the resin block and allow the glue to set for 24 h before re-sectioning.

11 **Re-sectioning for EM:** To examine tissue in the transmission electron microscope (TEM), you will need to cut ultrathin sections; these need to be in the region of 50-70 nm thickness. This sectioning process is carried out using an ultramicrotome (we use Leica EM UC6). We collect ultrathin sections on pioloform-coated copper grids. It is beyond the scope of this protocol to describe ultramicrotomy in detail (and the method is dependent on the model of ultramicrotome), but we will point the reader to some useful resources and give our most useful tips.

Resources Videos are a helpful way to learn and improve skills. Suppliers of ultramicrotomes often have videos of their models. There are also several instructional videos on YouTube. Some protocol papers also have supplemental movies (Harris et al., 2006).

11.1 **Useful tips for Ultramicrotomy:**

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1. To get a good ribbon of serial sections (e.g., trapezoid shaped sections joined together), the top and bottom edges of your re-embedded tissue block must be clean and parallel. You can achieve good parallel edges to your trapezoid block using a glass knife. This can also be achieved with a 'trim tool' (more details can be found in the following sub-steps).

1.1. Orientate and line up the block with the glass knife so that the long edges at the top and bottom (as usual), cut a few semi-thin (500-1000 nm) full sections. Rotate the block 90° (either clockwise or counter-clockwise), line up the glass knife with the surface of the block, cut until you get a whole section.

1.2. Change the glass knife block angle (and only this angle) so that it is at the maximum (~ 30°) in one direction (e.g., left).

1.3. Set your cutting window and start cutting until you have cut a clean edge. Move the knife to the other extreme i.e., 30° to the right if you started with the left. Repeat the cutting. You can then examine the block under a microscope – you should have a very neat pyramid with clean parallel edges at the top and bottom of your block. This method is also detailed here (Yamaguchi and Chibana, 2018). The bonus to having straight edges, apart from sections cutting well and forming a nice ribbon, is that the ribbon will not curve. Once the block is trimmed and you are at/approaching tissue you can switch to the diamond knife for cutting thin section (50-70 nm).

2. A smaller block will cut and form ribbons better. If you are having any issues when cutting with the diamond knife, then the first trouble-shooting step is to make your block smaller.

3. Keep everything meticulously clean during ultramicrotomy, clean everything with ethanol prior to use and filter your dH_2O. Carefully clean the diamond knife before cutting and replace the water in the bath often to avoid the build-up of any dirt or dust.

12 **Staining of ultrathin sections with Reynold's lead citrate:** Although sections can be examined in the electron microscope as soon as they have been collected onto copper grids, you can enhance contrast by staining with lead citrate. With microscopes that are equipped with a digital screen camera instead of eyepieces, this step is not essential but is often beneficial, nonetheless. You can purchase 'readymade' lead citrate, but it is relatively easy and economical to make it yourself; if you do purchase commercial lead citrate solutions, make sure that they are 'EM grade'.

Here, we describe preparing (see Materials) and using Reynold's lead citrate (below) which is formed as a product from the reaction between sodium citrate and lead nitrate (Reynolds, 1963). Lead citrate will precipitate easily in the presence of CO_2 in the air or in water, therefore we recommend not staining all your grids at once – stain a third or half of them to be sure that you do not have any issues with the lead stain precipitating. Note that it is very important when you are using immunogold particles to make sure that there is no lead precipitate as it can be confused with silver intensified gold particles.

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12.1 Line a large petri dish with Parafilm (clean side up). Carefully place some sodium hydroxide pellets into one section of the petri dish to absorb CO_2.

12.2 Carefully take up some of the lead citrate into a Pasteur pipette, discard the first drop, and then place separate drops onto the Parafilm.

12.3 Place a copper grid, sections down, onto each drop. Take care not to leave the petri dish lid off for long.

12.4 Leave sections to stain for 1-5 mins.

12.5 Wash each grid in distilled water; have ~4 vials of filtered distilled water and carefully dunk the grid several times in each before removing excess water with filter paper.

12.6 Completely dry grid with a hairdryer, ensuring that there is no fluid between the tips of the forceps, before replacing in the grid box.It is preferable to carefully dry off the drop of fluid on the grid with filter paper to avoid any contaminants in the water drying onto the sections.

12.7 Dispose of lead waste in accordance with your Institute's safety guidelines.

12.8 When you image your grids in the EM, check to see if the lead staining has worked well and that there is not an issue with lead precipitate.

13 Your sections are now ready to be imaged with the TEM – please see Protocol #2 for imaging and quantitative ultrastructural analyses.

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