IMMUNOELECTRON MICROSCOPY TISSUE PROCESSING PROTOCOL:

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BEFORE YOU START:

It is important to check in with the bio-imaging facility staff at least a week before you want to start sample processing to make sure they have all the supplies you need. Talk to Garnet (garnet.martens@botany.ubc.ca), Brad (bradford.ross@botany.ubc.ca) and Derrick (derrick.horne@botany.ubc.ca). If this is your first time doing ImmunoEM tissue processing, email Garnet before you start. He will make you a login account so you can book the equipment ahead of time.

Things to ask about: These are items/reagents supplied by the Bioimaging Facility, and it's a good idea to make sure they have these things before you start.

- Baskets
- Snap-fit Gel capsules (size 1)
- Liquid Ethane
- HM20 resin
- Make sure that no AFS pieces have gone missing since the last experiment

It is also important to check with the Naus lab to make sure that the vibratome is free when you need it. Talk to Sin (wun-chey.sin@ubc.ca).

DAY 1: PERFUSION + POSTFIX

Prepare Fixative:

Recipe for fixative for one mouse (filter sterilize all solutions):

- 4ml 10x PBS
- 10 ml 16% Formaldehyde (10 ml in one glass vial, **CAT: 15710, EMS Diasum**)
- 26 ml MilliQ water
- **TOTAL** – 40 ml

Also prepare 1 x PBS (filter sterilize)

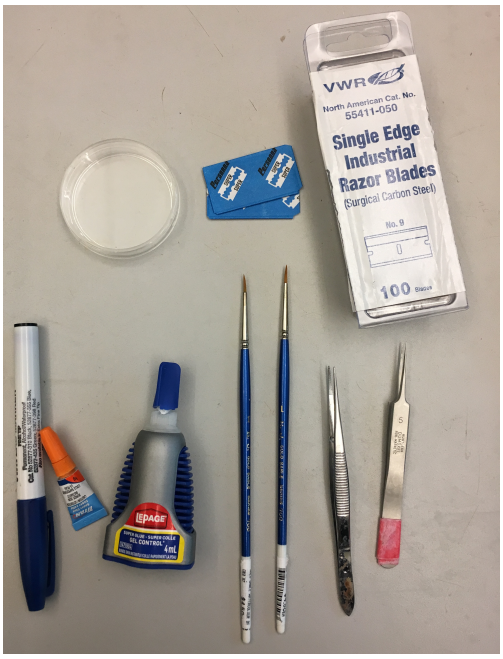
1. Transcardially perfuse mice with ~35 mLs 1x PBS (made fresh) [0.1 M sodium phosphate buffer (PB; pH 7.4)] (room temperature) followed by 30 mls fix.

2. Post-fix brains overnight in ~10ml **4% EM grade paraformaldehyde** at 4˚C
OVERNIGHT POSTFIX (O/N ONLY; 2-3 hrs = no good)

DAY 2: VIBRATOME + CRYOPROTECTION

Be careful when using the Vibratome. It is very old, and certain buttons do not work. Make sure to check with Sin regarding any changes for its use.

Fill the tank with 1xPBS. To cut horizontal sections, glue the dorsal side of the brain (cortex side) onto the metal block, and cut 200-250 um horizontal slices on the vibratome. Sometimes it's helpful to slice off the very top of the cortex so that you have a flat surface to glue to the block. (note: if you would prefer coronal sections, you can slice off the cerebellum and glue the caudal end of the brain to the block. However, this will be less stable). Dry the side you are gluing to the block with a kimwipe before attaching so it adheres (but make sure to keep the brain submerged in PBS until you're ready to slice so it doesn't dry out).



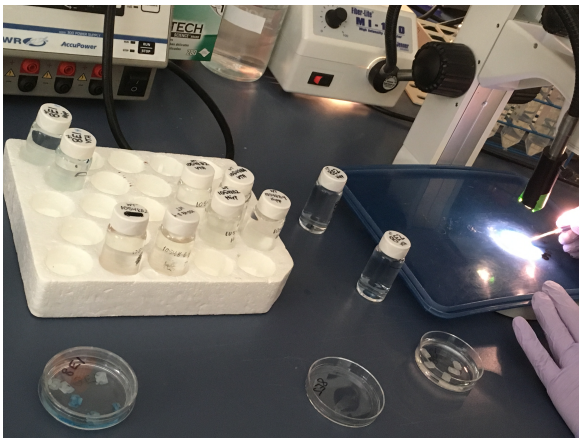
Supplies needed:

- small petri dishes (for slices)
- double edge razor blades (for vibratome)
- single edge razor blades (for prepping brain)
- forceps
- paint brushes
- crazy glue (from Staples)
- marker to label dishes
- 1L 1xPBS (not shown)

Vibratome Controls:

(Note: 'on' switch is at the back of the machine)
Direction: Up – blade moves towards sample Down – blade backs up
Speed knob: adjust as necessary
Amplitude: keep at 10
Thickness: set to desired slice thickness (200-250)

Do not press pause, do not change from single mode!!



Collect all sections in the midbrain area (or whatever your brain region of choice is). Save sections from each mouse in a separate mini petri dish, labelled with the mouse's ID. To clean up – use pipette gun to remove all PBS from chamber, rinse 1-2x with water. Otherwise the vibratome will be salty!

Bring the samples back downstairs. Place on a plastic lid and examine under the dissecting scope. Cut out ~1x1 mm brain samples (as small as possible – larger samples tend to have ice damage when plunge frozen). Dye the samples with Chicago sky blue 6B (**CAT: C8679-25G, Sigma**; it is not electron dense, but makes it much easier to keep track of the samples through the processing.)

1. 10% Glycerol (in PBS) → 30 mins (note: we often skip to 30% glycerol, no difference)
2. 20% Glycerol (in PBS) → 30 mins
3. 30% Glycerol (in PBS) → 30 mins
4. 30% Glycerol (in PBS) → OVERNIGHT

For tomorrow: Prepare solutions – 1.5% Uranyl acetate (UA; **CAT: 22400, EMS Diasum**) in 100% Methanol. Store at 4˚C.

DAY 3: PLUNGE FREEZE

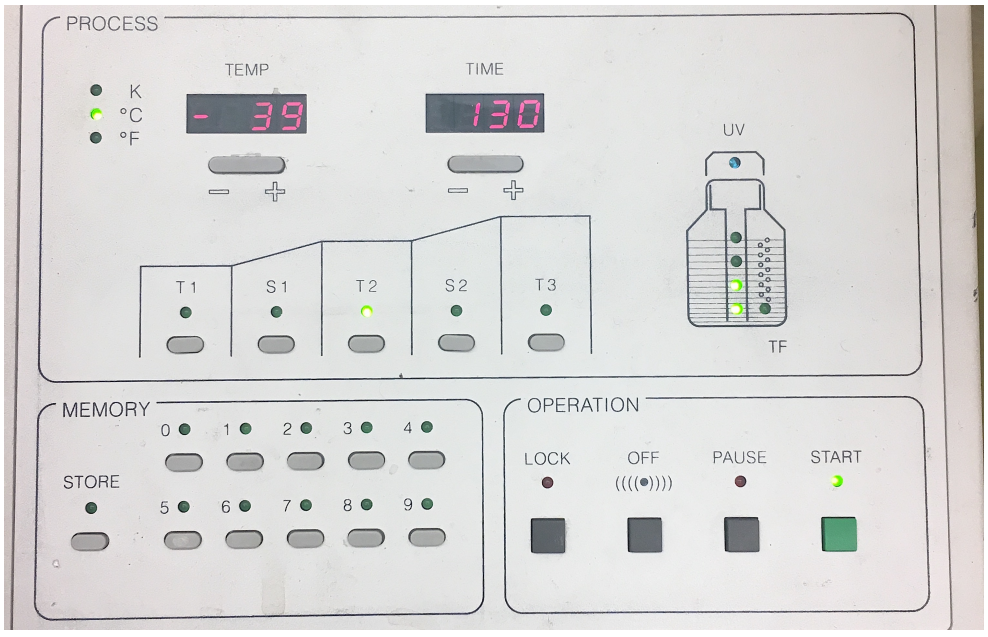
Bring the 1.5% UA in MeOH, as well as samples in 30% glycerol, methanol, plunge freezing pins (obtained from bio-imaging facility, but we have a set), and paint brushes, forceps to bio-imaging facility (Cunningham building basement).

The Plunge Freeze Apparatus: long grey tube on right of cart connects to LN2 tank (blue lid). This pumps LN2 into the small chamber on the left. Once cooled, you must fill he tiny metal chamber in the middle with liquid ethane. When you click the small black button on the top tall piece above the chamber, samples are plunged into the ethane to freeze instantly.

1. Set up plunge freeze equipment (fill small dewar with liquid nitrogen (LN2), connect tube and turn on so that it starts to cool the chamber (it pumps the LN2 to surround the plunge freezing chamber). It should be set to cool to -170˚C (This will take at least 1 hour).

The AFS:

2. Fill AFS with LN2. First, you need to run the “bake” cycle so the chamber does not frost up. This is usually present program 1 (Heats up to 60 ˚C for 1 hour). After this cycle, set up program.

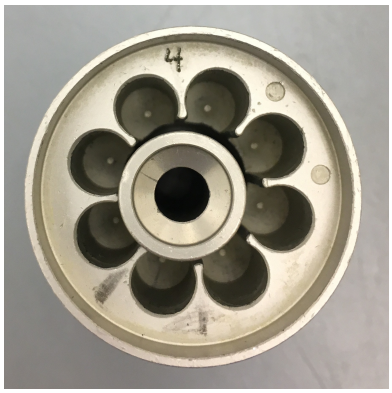


AFS PROGRAM:
T1: -90 30h
S1: T 11h
T2: -45 75h
S2: T 9h
T3: 0 48

As the AFS cools, put the long, black cap into the hole in the middle hole, and fill the chamber with a small amount of ethanol. If you have 24 samples, 3 of the 4 cartridges are placed in the ethanol-filled chamber, there should be about 1 cm depth of ethanol surrounding the cartridges so that the cold-transfer is efficient.

Fill the cartridges with the 1.5% UA in Methanol solution.

Next you need to place the plastic 'baskets' into the circular-shaped slots in the cartridges. However, they cannot be numbered/labelled with a marker, so you need to use a sharp tool (usually the tip of a razor blade) to carefully carve numbers into the baskets. You will label the baskets in cartridge #1 with the numbers 1-8, in cartridge 2 with the numbers 9-16, and in cartridge #3 with the numbers 17-24. Note the dots on either side of one of the slots on top of the cartridge. We usually put the lowest number basket between these two dots, and then put them in order clockwise around the cartridge. The baskets have mesh-like slots along the edge of the bottom so as you drop them into the slots, they will fill with the UA/methanol solution. The UA/methanol solution should not flow over the top of the baskets, otherwise your samples may float out and away.



3. When the AFS and the Plunge freezing apparatus have both cooled (Plunge freeze to -170˚C, AFS to Plunge Freeze in liquid Ethane (-170°C), you can prepare to plunge freeze the samples. Fill the chamber in the Plunge freezer with liquid Ethane (from a canister, ask the bioimaging staff for help if you can't find it). Carefully transfer 2-3 samples onto a pin that has been cleaned off by rinsing in Methanol. We usually use a piece of Styrofoam to hold the pin upright, and a dissecting scope to see what we're doing as we transfer the sample.

Make sure you arrange the samples so that they are not right beside each other on the pin. Once the samples are on the pin, dry your paintbrush and touch the top of the pin to soak up any excess methanol (we think the freezing happens quicker – and with fewer ice crystals – when there is less material to be frozen).

Use forceps to transfer the pin to the plunge freezing apparatus. Click the button on the top to quickly drop the samples into the chilled liquid ethane

4. Quickly draw up the plunger and use two forceps to remove the pin from the apparatus. Transfer pins with plunge frozen tissue to the baskets in the 1.5% UA solution in 100% MeOH (in AFS).

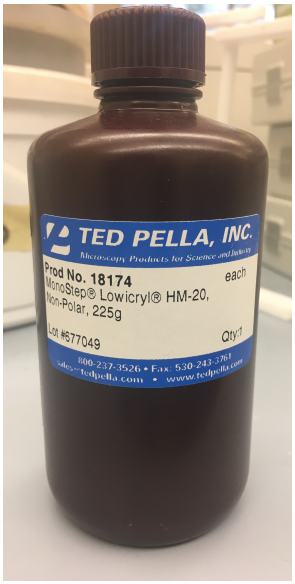
<u>IMPORTANT: Keep detailed notes about which samples are put into which basket number!! Also note how many samples were in each basket. This is the only way to know which sample is which at the end of processing.</u>

DAY 4: FREEZE SUBSTITUTION (We don't have to do anything)

Incubate 24hrs. (note the AFS should be set up for the following settings)
@ -90°C 30 hours
THEN increase to -45°C over 11 hr period

DAY 5: INFILTRATION

First, you must get all the samples off of the pins. They are frozen on. We don't want to remove the pins because they will frost up and the samples will be damaged. So we've found the best way to deal with this is to grab onto the end of each pin with forceps, and aggressively shake the pin (while the end with the samples on it stays submerged in the basket) so that the samples come loose. Often, shaking each pin like this is enough to loosen the samples. If not, you can shake it and then gently scrape the pin against the edge of the basket so that the sample falls back into the basket. Once the samples are off the pin, the pins can be removed and collected (to be saved for next time).



After removing the pins, this day is a series of washes, followed by 2 hour infiltrations with increasing amounts of the HM20 resin (we don't order, but **CAT: 18174**, Ted Pella). HM20 resin is located in the fridge in the bio-imaging facility. It comes pre-mixed and is in a brown bottle. Ask Garnet/Derrick/Brad if you can't find it!

The way we do these wash steps if we have 24 samples (3 cartridges full) is one cartridge at a time – put the 4th cartridge in the AFS chamber, filled with Methanol. Wait for the AFS to cool back to temperature, and then slowly move each basket from cartridge 1 to the new cartridge. Then remove Cartridge 1, pour out the UA solution into UA waste. Rinse the cartridge in Methanol and make sure it's warmed up so there is no frost. Then fill with Methanol, put in AFS, wait for it to cool, and then move cartridge 2 samples into this cartridge.

It can be useful to keep track of which samples have moved to which cartridge on a sheet of paper (ie. First #1 moves to #4, then #2 moves to #1, then #3 moves to #2, etc.) Keep track of the time that you move samples, so you can make sure the incubations are the right length of time.

Note: if you have less than 16 samples, the washes/resin changes are much quicker, as you can use the 2 spare cartridges to switch with the ones holding the samples.

Also – as you get up to the pure HM20 infiltrations, it is important to make sure the cartridge is **completely dry** before filling with HM20 and starting the next wash step.

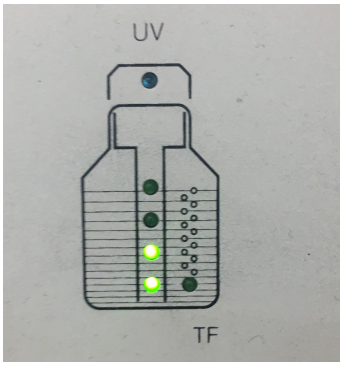
The washes and infiltrations are as follows:

1. Rinse with 3x change of 100% Methanol @ -45°C
2. 1:1 HM20:MeOH -45°C 2h
3. 2:1 HM20:MeOH -45°C 2h
4. Pure HM20 -45°C 2h
5. Pure HM20 -45°C OVERNIGHT

Note: you need about 15-20 ml for each step (~5 ml in each cartridge).
Note: Make sure that as you do the infiltrations, you save the resin after each step. Tomorrow, we will polymerize it, so you can dispose of it in solid form (rather than more volatile/toxic liquid form).

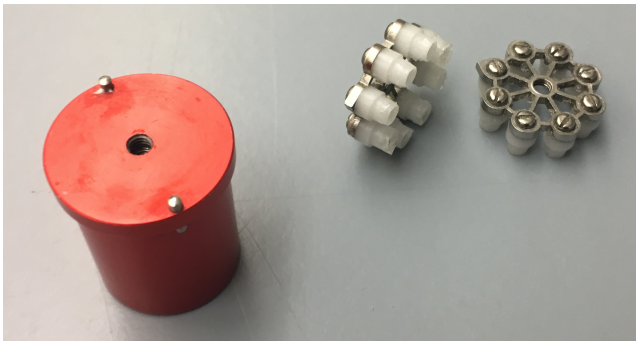
DAY 6: POLYMERIZATION

Usually at the start of this day, the AFS needs to be refilled with LN2. Ask Brad, Derrick or Garnet for help with this process. You can check the indicator on the AFS to see how full the tank is (but we usually do have to refill before polymerization).



This is a relatively short day – when refilling the AFS, you need to remove the black plug. Afterwards, replace it with the red plug (see image below, left). This one is used for UV polymerization.

There are these three 'spider' holders that are used for polymerization (see image below, right). They likely have a layer of polymerized resin on the edge of them (on the white plastic part), which means the baskets won't lock into place as easily. Take a razor blade and clean them up, scraping off excess resin.



Take the spare cartridge and put the gel capsule halves in the slots. Fill each capsule with HM20, as close to the top as possible. Put in the AFS chamber and wait for it to cool. Once cool, slowly transfer each basket to a gel capsule. Don't press them into place too fast, as samples can spill out this way.

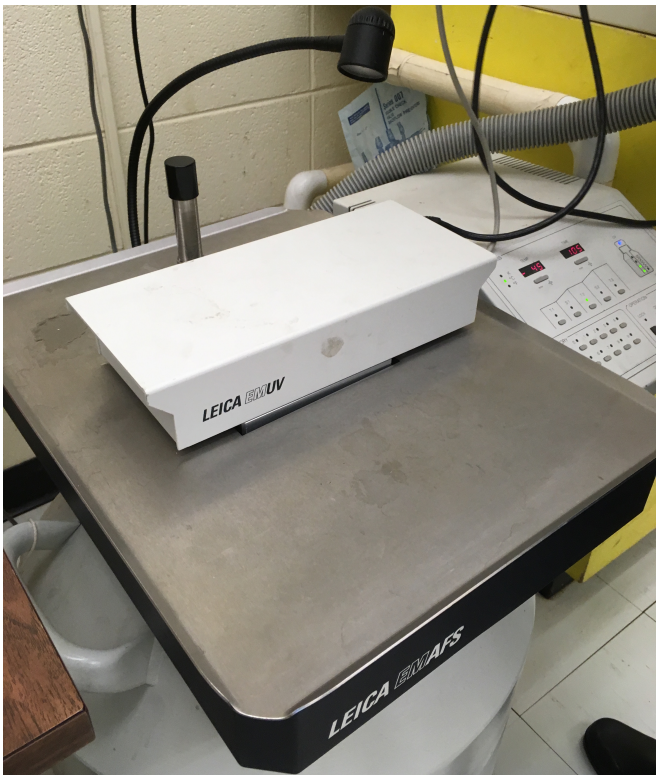
You also have to prepare the holders for the 'spiders'. Place these in the AFS chamber and move some of the chilled ethanol into the basin in the holder (this will allow cold transfer directly to the gel capsules when they are in place).

Once the baskets have more or less sunk into place, press them down with forceps. Put the spider over top of the basket/gel caps, and press down forcefully onto the spider, pressing over each of the samples so that each basket is properly pressed into place. Carefully pick up the spider with the forceps, and move it onto the holder. **These are delicate. Do not bump or move the spider/holder once the spider is in place. Move other cartridges rather than the spider with samples.**

Repeat for each set of samples**. Ones these are all in place, make sure to remove the other cartridges from the AFS chamber (otherwise you will polymerize the resin in the cartridges!! Bad news.)**

Place the crystal cover over top of the samples (it is cracked, try to arrange it so that the cracks aren't overtop of the samples).

Plug in the mini UV light that fits overtop of the AFS chamber. This UV light also attaches to the AFS. There is a blue light on the indicator on the AFS that tells you if the light is on. If the blue light is flashing, flip the switch on the UV light on. Make sure it is before you leave!



1. UV Light	-45°C	24 h
2. THEN increase to	0°C	9 hours
3. Hold at	-0°C	35 h

Don't forget to polymerize the waste resin – there is a spare UV light that fits over a styrofoam container that is lined with tin foil. Put the plastic cups with your waste resin in the container, plug in the light, and switch it on. **It's UV – so be careful and only turn on when it's in position over the Styrofoam container!**

DAY 9: SAMPLE PICK-UP ☺
When you pick up the samples, make sure you clean up after yourself, and log the equipment time used on the computer (talk to Garnet if you need help with this).

See next protocol for cutting EM samples.