Epoxy Embedding of Fixed Brain Tissue

K. Harris lab, Ctr for Learning and Memory, UT-Austin.

0. Safety Precautions:
1. Reagents and supplies (for up to 24 pieces of tissue)
   2. Tissue processing basket
   3. Microwave
   3. Epoxy resin (can be done the day before)
   4. Reagents for osmium fixation, dehydration, and UA en bloc staining
      4.1. Handling sodium cacodylate buffer
      4.2. Making stock solution of uranyl acetate, 2% in ethanol
      4.3. Dispensing osmium tetroxide solution from glass ampule
      4.4. Reagents for processing up to 12 pieces of tissue
      4.5. Reagents for processing 13 to 24 pieces of tissue
   5. Reagents for infiltration
      5.1. Handling propylene oxide
      5.2. Reagents for infiltration
3. Processing (4 days)
   - Day 1 (estimated work duration: 3 hrs + enough time for dissection and agarose embedding)
   - Day 2 (estimated work duration: 4 hrs)
   - Day 3 (estimated work duration: 0-1 hr)
   - Day 4 (estimated work duration: 1-2 hrs)
4. Clean-up
   - 4.1. Waste containers
   - 4.2. Used supplies and equipment

0. Safety Precautions:

- You must complete required lab safety training before starting this procedure.
- If this is your first time doing this procedure, ask to be trained by an experienced lab member. If you have not done this in a while, you should ask for a refresher.
- Before starting, even if you have done this procedure before,
  - read this protocol entirely
  - review relevant Safety Data Sheets and Harris Lab SOP (also see below)
  - ensure you have all reagents and supplies listed below
  - ensure all equipment is in good working order
  - have all waste containers ready (also see Clean-up)
  - plan your schedule well so that you wouldn’t have to rush
- Review SDS and Harris Lab SOP for the following hazardous chemicals used in this procedure:
  - Ethanol: flammable; irritant (eye)
  - LX-112 epoxy resin kit
    - LX-112: irritation (eye, skin, respiratory, oral)
  - DDSA: irritant (eye, skin, respiratory)
  - NMA: irritant (eye, skin, respiratory)
  - DMP-30: permanent eye damage, irritant (skin, respiratory, oral)
  - Osmium tetroxide: acutely toxic; mutagenic
  - Potassium ferrocyanide: contact with acid releases very toxic gas
  - Propylene oxide: flammable; acutely toxic (skin, respiratory, oral); serious eye damage; mutagen; carcinogen
  - Sodium cacodylate: carcinogen; irritant (skin, eye); skin permeator
  - Uranyl acetate: fatal (inhalation, ingestion); flammable
  - Osmium tetroxide, propylene oxide, sodium cacodylate, and uranyl acetate must be handled only under a chemical fume hood.
- The following Personal Protective Equipment is required for this procedure:
  - Lab coat
  - Nitrile gloves (double-layer required; regularly check for holes)
  - Eye goggles
  - Also recommended when using osmium tetroxide and uranyl acetate: plastic apron and shoulder-length gloves
  - Place a piece of absorbent sheet on the work surface before starting the procedure. When done, discard into the “Solid Waste – UA” bag
- Read the following papers:
1. Reagents and supplies (for up to 24 pieces of tissue)

- Worksheet for this procedure [1-12 pieces of tissue | 13-24 pieces of tissue]
- Vibraslices (70-100 µm thickness) of chemically fixed brain tissue, embedded into 7-9% agarose
- H₂O (purified water: double-distilled, ASTM type I, WFI grade, or equivalent; e.g., Fisher 9150-25 or VWR RC915025)
- 0.2M sodium cacodylate buffer (SCB; EMS 11652)
- 0.1M SCB (diluted from 0.2M SCB)
- Ethanol (EtOH; EMS 15056); use a fresh bottle for each processing.
- K₃Fe(CN)₆·3H₂O (potassium ferrocyanide, or KFeCN; Sigma P3289-100G)
- 2% uranyl acetate (UA; EMS 22400): See 2.4.1
  - Dissolve 1 g UA in 50ml EtOH, stored in dark at 4°C.
- 4% aqueous OsO₄ (osmium tetroxide; stored at 4°C; EMS 19190): See 2.4.2
  - Use a fresh ampule for each processing; discard excess
- Propylene oxide (PO; EMS 20411)
- Epoxy resin (LX-112 embedding kit; Ladd Research 21210)
- LX-112 (glycerol polyglycidyl ether; Ladd Research 21310)
- DDSA (dodecenyl succinic anhydride; Ladd Research 21340)
- NMA (nadic methyl anhydride; Ladd Research 21350)
- DMP-30 ([2.4.6-tri(dimethylaminomethyl) phenol; Ladd Research 21370)
- Absorbent paper with plastic backing
- Disposable pipets (some with their tips trimmed – e.g., for LX-112 components, for transferring tissue)
- Kimwipes
- 12- or 6-well processing basket (Ted Pella 36157-1 or 36158-1) × 1 or 2.
  - These are stored in glass jars filled with acetone, in a fume hood.
- 50-mm polypropylene processing dishes (Ted Pella 36135) × 25 or 50.
- 20-ml syringes and syringe filters (PES membrane; 0.2-µm pore size) × 4 each.
- 20-ml borosilicate glass scintillation vials with caps (e.g., EMS 72634) × 10, labeled as below:
  - OsO₄
  - 1
  - 2
  - KFeCN
  - 50% EtOH
  - EtOH/UA: 1 each for 50%, 70%, 90%, 100%
  - 100% EtOH
- A Pasteur pipet and bulb, a 20-ml borosilicate glass scintillation vial without cap (labeled “OsO₄”)
- Ice packs: one for keeping OsO₄ solutions cold and another for cooling tissue during processing.
- Shell vials (EMS 72631-10 [3.4 ml]), 1 per tissue.
- Embedding mold (e.g., Chien mold, EMS 70140)
- Applicator sticks
- Razor blade
- Block labels
  - Suggested label format:
    - Font: Tw Cen MT Condensed, 9 pt., with 8 pt. spacing between lines
    - Width = 0.5 in. max; Height = 2 lines max
    - Label information should at least contain: Animal ID (e.g., MK01) and Tissue ID (e.g., R42CA1, for right hemisphere, vibrasection 42, area CA1)
- Aluminum foil for preparing LX-112, as well as for wrapping open OsO₄ ampoule and used Pasteur pipet
- Tri-pour beakers:
  - 6 × 250-ml beakers for mixing LX-112 components (see 2.3)
  - 1 × 250-ml for collecting open OsO₄ ampoule and used Pasteur pipet
  - 1 × 250-ml for washing the processing basket in 0.1 M Na cacodylate buffer, and then for collecting liquid waste during the procedure (labeled “Waste cacodylate, osmium, UA-ethanol”).
  - 1 × 100-ml beaker for infiltration reagents (labeled “ethanol, propylene oxide, and LX-112”)
- Solid waste bags for UA and non-UA chemical-contaminated waste.Liquid waste bottles:
  - Formaldehyde, glutaraldehyde, and cacodylate
  - OsO₄, KFeCN, and cacodylate
  - Uranyl acetate, ethanol
  - Flammables
  - Resin and propylene oxide
2. Reagent/Equipment Preparation (the day before, or on the day of processing)

2.1. Tissue processing basket

- Remove and air-dry 12- or 6-well processing basket from acetone, then wash in a disposable beaker filled with SCB (0.1M, pH 7.4).

2.2. Microwave

1. Fill load cooler with fresh deionized water and attach tubing to cold spot device. Make sure pump is on.
2. Set microwave power level to 1 (175W).
3. Run microwave for 2 minutes to warm up magnetron.

2.3. Epoxy resin (can be done the day before)

1. Place stir bars (kept in acetone) in three tri-pour beakers and label "A", "B", and "A+B".
2. Check the weight per epoxide (WPE) of LX-112 indicated on the bottle and record the value in worksheet.
3. Refer to Table 1 to determine the correct amounts of DDSA and NMA. Record the amounts in worksheet.
4. Measure resin components with the scale in fume hood, using disposable pipettes with their tips cut off.
   1. Beaker A: mix DDSA and LX-112 (see Table 2)
   2. Beaker B: mix NMA and LX-112 (see Table 2)
   3. The recipe here should make enough resin for embedding up to 24 pieces of tissue.
5. Into beaker A+B, add the contents of beakers A and B (see Table 3 for the amounts). Cover with foil and mix for another 15 min.
6. Add DMP-30 (see Table 3 for the amount) and mix thoroughly for another 15 min. If mixing resin the day before, wait until the day of use to add DMP-30.

| Table 1: Amounts of DDSA and NMA to be used for different WPE values |
|---------------------------------|-------------------|-------------------|-------------------|-------------------|
| Weight Per Epoxide of LX-112    | Weight of DDSA (g) | Weight of NMA (g) | Weight of DDSA (g) | Weight of NMA (g) |
| 140                             | 9.31              | 16.02             | 18.62             | 32.04             |
| 141                             | 9.24              | 15.91             | 18.49             | 31.81             |
| 142                             | 9.18              | 15.79             | 18.36             | 31.59             |
| 143                             | 9.11              | 15.68             | 18.23             | 31.37             |
| 144                             | 9.05              | 15.58             | 18.10             | 31.15             |
| 145                             | 8.99              | 15.47             | 17.98             | 30.94             |
| 146                             | 8.93              | 15.36             | 17.85             | 30.72             |
| 147                             | 8.87              | 15.26             | 17.73             | 30.51             |
| 148                             | 8.81              | 15.15             | 17.61             | 30.31             |
| 149                             | 8.75              | 15.05             | 17.50             | 30.10             |
| 150                             | 8.69              | 14.95             | 17.38             | 29.90             |

| Table 2: Amounts of LX-112 for
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of LX-112 (g) for:</td>
<td>Beaker A (g)</td>
<td>Beaker B (g)</td>
</tr>
<tr>
<td>Day 1</td>
<td>7.00</td>
<td>18.00</td>
</tr>
<tr>
<td>Day 2</td>
<td>14.00</td>
<td>36.00</td>
</tr>
</tbody>
</table>

| Table 3: Composition of the final resin mixture |
|-----------------------------------------------|-------------------|-------------------|
| Beaker A+B: Weight of A (g) | Weight of B (g) | Weight of DMP-30 (g) |
| Day 1                          | 13.50             | 31.50             | 0.63              |
| Day 2                          | 27.00             | 63.00             | 1.26              |
2.4. Reagents for osmium fixation, dehydration, and UA *en bloc* staining

2.4.1. Handling sodium cacodylate buffer

**under construction**

2.4.2. Making stock solution of uranyl acetate, 2% in ethanol

1. Wear appropriate PPE (see above). Place a piece of absorbent pad on work surface in fume hood.
2. Retrieve the following:
   1. Sonicator (stored in a cabinet under the lower fume hood), filled with some water
   2. A clean 100-ml graduated cylinder
   3. 100% ethanol (in flammables cabinet)
   4. Solid uranyl acetate (in the desiccator cabinet)
   5. Glass bottle containing 2% uranyl acetate solution in ethanol (stored in a secondary container in the fridge)
   6. Kimwipes
   7. A small piece of Parafilm (~1 in. x 4 in.)
3. Place a large plastic weighing boat on a scale in fume hood.
4. Open bottle of uranyl acetate powder in the fume hood.
5. Gently tap out approx. 1 g of uranyl acetate onto the weighing boat, then carefully pour into the glass bottle. Keep the weighing boat and note the exact amount of uranyl acetate.
6. Based on the amount of uranyl acetate, measure out the volume of ethanol necessary to make the final concentration of 2% (weight-by-volume; e.g., 52 ml ethanol for 1.04 g of UA).
7. Pour a small volume of ethanol from the graduated cylinder into the weighing boat to collect any remaining uranyl acetate. Pour this into the bottle.
8. Pour the remaining ethanol into the bottle.
9. Cap the bottle (but do not tighten), wipe the bottle exterior with wet Kimwipes, and sonicate for at least 15 min.
10. Tighten the cap, remove the bottle from sonicator, and wipe the bottle exterior with Kimwipes.
11. Wrap around the cap with a piece of Parafilm and place the bottle in a secondary container before storing in the fridge.
12. Dispose of the weighing boat and other supplies contaminated with uranyl acetate (e.g., used Kimwipes and outer layer of gloves) in "Solid Waste – UA" bag.
13. Clean and return supplies and equipment to their storage locations.

2.4.3. Dispensing osmium tetroxide solution from glass ampule

1. Wear appropriate PPE (see above). Place a piece of absorbent pad on work surface in fume hood.
2. Retrieve the following:
   1. A Pasteur pipet and bulb
   2. A 20-ml borosilicate glass scintillation vial without cap (labeled “OsO₄” and placed in the vial rack)
   3. A 250-ml tri-pour beaker
   4. A piece of Aluminum foil
   5. A glass serological pipet
   6. A 10-ml ampule of 4% aqueous solution of osmium tetroxide (stored encased in a plastic sleeve in a metal can in the fridge)
   7. A pair of forceps (located in fume hood)
3. Open the metal can to retrieve a 10-ml ampule of osmium tetroxide solution. Keep the ampule encased in the plastic sleeve.
4. Close and return the metal can to the fridge.
5. In fume hood, while still in the plastic sleeve with the red cap on, break open the ampule.
6. Remove the red cap and discard into "Solid Waste – No UA" bag.
7. Remove the broken ampule top with forceps and place on the foil. Keep the ampule bottom (with osmium tetroxide solution) in the plastic sleeve.
8. Use Pasteur pipet to dispense osmium tetroxide solution into a 20-ml borosilicate glass scintillation vial.
9. Once empty, keep the pipet tip in the ampule bottom, remove the pipet bulb, place the ampule top into plastic sleeve, and loosely wrap them in the foil. Place the foil-wrapped waste into a 250-ml tri-pour beaker and discard into "Solid Waste – No UA" bag.
10. Dispense osmium tetroxide solution out of the scintillation vial for tissue processing using a glass serological pipet.
11. Discard any remaining osmium tetroxide solution into "Waste OsO₄-KFeCN" bottle.
12. Discard the scintillation vial into "Solid Waste – No UA" bag.

2.4.4. Reagents for processing up to 12 pieces of tissue

- In 50-ml conical tubes, add:
  - ddH₂O: 50 ml
  - Na cacodylate buffer (SCB; 0.2M): 15 ml
  - Na cacodylate buffer (0.1M): 50 ml (~150 ml total will be needed)
  - In the labeled vials (Mix well by shaking):
1. Add 3 ml of KFeCN solution to vial 2.
2. Place vials 1 and 2 on ice.
3. When ready, pour vials 1 and 2 back and forth to mix well before adding to tissue.

***1% OsO₄: Keep vial on ice until use.

2.5. Reagents for infiltration

2.5.1. Handling propylene oxide

**under construction**

2.5.2. Reagents for infiltration

* Prepare ~2 ml per tissue of the following:
  1. 1:1 = EtOH : propylene oxide (PO)
  2. 1:2 = EtOH : PO
  3. 100% PO
  4. 1:1 = PO : Resin
  5. 1:2 = PO : Resin
3. Processing (4 days)

Day 1 (estimated work duration: 3 hrs + enough time for dissection and agarose embedding)

1. Place a piece of absorbent paper on work surface in the fume hood.
2. Prepare reagents and equipment, as described in Section 2 above.
3. Place 12- or 6-well processing basket in 50-mm processing dish filled with Na cacodylate buffer (SCB; 0.1M, pH 7.4).
4. With a disposable pipet, transfer the tissue (embedded in 7-9% agarose) to the processing basket.
5. SCB washes, 5 min × 5.
6. Osmium fixation/en bloc staining
   1. Reduced osmium (1% OsO₄ + 1.5% K₄Fe(CN)₆ in SCB), 5 min.
   2. SCB washes, 5 min × 5.
   3. 1% OsO₄ in SCB, in microwave (175 W, or power level 1) under vacuum, 1 min on 1 min off 1 min on.
   4. Place the tissue processing basket in dish on an ice pack, cool to about 15°C.
   5. Repeat microwave (175 W, or power level 1) under vacuum, 1 min on 1 min off 1 min on.
   6. SCB washes, 2 min × 5.
   7. H₂O washes, 2 min × 2.
7. Dehydration and en bloc UA staining
   1. 50% EtOH, 5 min.
   2. Dehydration and en bloc UA staining in microwave (250 W, or power level 2) without vacuum, 40 sec each. UA-EtOH mixtures must be filtered through the syringe filter.
      1. 50% EtOH with 1% UA.
      2. 70% EtOH with 1% UA.
      3. 90% EtOH with 1% UA.
      4. 100% EtOH with 1% UA.
      5. 100% EtOH.
8. Infiltration on rotator. With a disposable pipet (with the tip cut off), transfer the tissue into shell vials containing 1:1 = EtOH : PO. After each exchange, make sure the tissue is not stuck on the bottom of vial.
   1. 1:1 = EtOH : PO, 10 min.
   2. 1:2 = EtOH : PO, 10 min.
   3. 100% PO, 15 min × 2.
   4. 1:1 = PO : Resin, 1 hr.
   5. 1:2 = PO : Resin, overnight.

Day 2 (estimated work duration: 4 hrs)

1. Make a fresh batch of resin (See 2.3.).
2. Infiltration with 100% Resin, 1 hr × 3 on rotator.
3. Place block labels writing side up in embedding molds and cover with a small amount of fresh resin.
4. Bevel a wooden applicator stick with a razor blade. Using the stick, transfer the tissue from shell vials into the mold.
5. Under a stereomicroscope, move the tissue to desired position.
   • Small blocks of polymerized resin can be used to support the tissue inside the mold.
6. Make sure that the resin is slightly convex from top of the mold.
7. Polymerize resin for 48 hr at 60°C.

Day 3 (estimated work duration: 0-1 hr)

1. Continue resin polymerization. You may check and adjust the positioning of tissue at this point.

Day 4 (estimated work duration: 1-2 hrs)

1. Take the resin-embedded tissue blocks out of oven. Remove the blocks out of the mold immediately and store in the cardboard pill boxes labeled with block identification numbers.
2. Take solidified resin waste into “Solid Resin” waste drum.
3. Examine the blocks under the stereomicroscope and record their images before trimming and cutting.
4. Scan worksheet into a pdf file.

4. Clean-up

4.1. Waste containers

• Hazardous Liquid Waste: Pour all waste into the proper waste collection bottles available in fume hood.
- Aldehyde-Cacodylate (fixative solution, cacodylate buffer)
- Osmium-Ferrocyanide (reduced osmium/osmium tetroxide): Discard excess $K_4Fe(CN)_6$ and OsO$_4$.
- Uranyl acetate-Ethanol (ethanolic UA solution)
- Flammable Solvents (propylene oxide and alcohols that do NOT contain other chemicals such as uranyl acetate and Epon)
- Resin-PO (mixture of LX-112 and propylene oxide)
  - One resin beaker should be used to collect all excess LX-112 resin and aluminum foil, plastic pipets, applicator sticks, and vials (uncapped) used with it. This should be cured in the oven with the blocks and discarded in hazardous solid waste container labeled “solid LX-112 resin”.
- Hazardous Solid Waste: Place all contaminated solid waste (e.g., gloves, pipets, vials, processing dishes, etc.) into hazardous waste bags in fume hood. All vials must be uncapped and empty. Make sure to separate all waste contaminated with UA.

4.2. Used supplies and equipment

- Supplies (e.g., vials and dishes) that came in contact with UA should be rinsed with ethanol or water into the UA waste bottle and disposed of in the solid UA waste bag. All vials must be uncapped and empty. Make sure to separate all waste contaminated with UA.
- Used absorbent paper should be discarded into the UA solid waste bag.
- Wipe inside vacuum chamber of the microwave with wet Kimwipe (discard into the UA waste bag).
- Stir bars (for resin mixing) and processing baskets should be placed in jars of acetone in the fume hood.
- Monitor the area for radioactivity (see Harris lab SOP for uranyl acetate).