O.T.O Enhanced Contrast Procedure for Transmission Electron Microscopy

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It is very important that the size of sample is sufficiently small to ensure proper penetration of all components of this protocol. Tissue pieces should not be larger than 2 mm on any one side.

1. Fix tissues in 2.5% glut in **0.15 M** Na Cacodylate and 2% paraformaldehyde, plus 2 mM calcium chloride **2-3 hours** at 4°C (tissue may be left in fix longer).
2. Wash **5 x 3 min** in cold 0.15 M Sodium Cacodylate buffer with 2 mM Ca Chloride ($CaCl_2 \cdot 2\,H_2O$ FW=147.01; make .2M stock and add 1 ml per 100 ml of buffer for .002 M [2 mM] concentration). 0.1 M sucrose may be added to the buffer.
3. **Right before use**:
    - Combine a solution of 3% potassium ferrocyanide in **0.3 M** cacodylate buffer with 4 mM Ca Chloride

      Prepare as follows

      5 ml 0.6 M Na Cacodylate buffer **(HAZARDOUS CHEMICAL; put about 1 tsp in pre-weighed . weighboat under the hood, cover with another boat and , weigh/extrapolate for amount of water to use for a 0.6M ` solution)**
      (w/ 8 mM Ca Cl_2)

      3 ml 10% Postassium Ferrocyanide

      2 ml dd H_2O

      Total volume : 10 ml
    - Equal volume of 4% aqueous OsO_4 (e.g. 2 ml to 2 ml). **(ACCUTELY HAZARDOUS CHEMICAL!!. Osmium tetroxide is an acutely hazardous chemical. It can cause eye damage, and is toxic to liver and kidneys. All use should be under a fume hood with proper protective clothing, gloves and eye protection. It is important to familiarize yourself with the SDS for this chemical.)**
    - Incubate **1 hr** on ice
4. While this is happening prepare thiocarbohydrazide (TCH) solution. It must be fresh and available right at the end of step 5:
    - 0.1g TCH and 10 ml ddH_2O, 60° oven for 1 hr.
    - Agitate by swirling **every 10 min**.
    - Filter through 0.22 µm syringe filter

5. **BEFORE TCH**: wash with (**filtered**) ddH_2O at RT, **5 x 3 min**
6. Place Tissues in *filtered* TCH, **20 min**, RT
7. Rinse **5 x 3** min in ddH_2O, RT
8. 2% OsO_4 in ddH_2O, **30 min**, RT
9. **For Tomorrow**: Make 100 ml of 0.03 M aspartic acid (MW 133; (0.4 g/100 ml)) for the next day. Let go into solution overnight on stir plate.
10. **For tomorrow** make **FRESH** 1 N KOH (MW 56)**.**
11. Wash **5 x 3 min** in ddH_2O, RT
12. Place samples in 1% uranyl acetate overnight at 4°C (aqueous, **filtered with .22 um syringe tip filter.**)

    Stock 5% U.A.: 2.5 g UA to 50 ml ddH_2O, cover with foil, stir overnight. Add 10 drops of glacial acetic acid. (Store at 4°C, stable at least 6 months.)

13. **Next Day**: Walton's lead aspartate.

0.33 gm lead nitrate to 50 ml of 0.03 M aspartic acid (MW 133) solution. If lead clumps, weigh out as close to .33 gm as possible and calculate the volume of aspartic acid by proportion.

Bring to pH 5.5 adjusted with 1 N KOH (see below for pipetting instructions)

IMPORTANT: When pipetting KOH into solution, do so in small increments. The pH changes very quickly. Use 1 ml micro pipeter until you get to 4.5, then switch to 200 µl. Once you get to 5.4, switch to 0.5N KOH. With single drops you will get to 5.5.

14. Place lead aspartate (**NO TISSUE YET)** 60° for **30 min** (no precipitate should form).
15. Wash tissue in ddH_2O **5 x 3** min at RT.
16. Place tissue in lead aspartate solution in 60°oven for 30 min.
17. Wash in ddH_2O **5 x 3 min**
18. Dehydrate graded ethanol as follows: 20%, 50%, 70%, 90%, 95%, 20 minutes each. From this point on, use *super dry* ethanol only (pint bottle kept in a desiccator after opening); 100% ethanol 3 x 10 minutes.
19. 2 x propylene oxide, 20 min each.
20. U**se** propylene oxide to infiltrate as listed below:

Prepare **Epon (EMS cat. # 13940)** as manufacturer suggests:

Mixing instructions:

Embed-812	27.5 g
Araldite 502	17.0 g
DDSA	55.0 g
DMP-30	1.45 g (or BDMA between 2.15 and 2.51g; may allow better penetration per EMS)

Prior to measuring and mixing, the resin (Araldite) and anhydride (DDSA) should be warmed (60^oC) to reduce their viscosity.

Thorough mixing is imperative to be able to achieve uniform blocks.

Infiltration:

1:3 Epon:propylene oxide, on rotator, 6 h to overnight (depending on the size of the tissue), RT.
1:1 Epon:propylene oxide on rotator 6 h to overnight,
3:1 Epon:propylene oxide on rotator 6 h to overnight
100% embedding medium on rotator overnight, RT.
Fresh embedding medium, rotate for at least 2 hours.

Embed in appropriate mold.