Staining Ultrathin Sections with Uranyl Acetate and Lead Citrate

The lead citrate stain was first described by Reynolds (1963). Assume room temperature unless otherwise specified.

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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.
- Also review the following papers:
- 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:
  - Nitric acid: strong oxidizer; corrosive; causes severe skin burns and eye damage; irritant (respiratory)
  - Lead nitrate: oxidizer; toxic (ingestion, inhalation); carcinogen; reproductive toxin; causes serious eye damage; environmental toxin
  - Sodium hydroxide: corrosive; causes severe skin burns and eye damage; irritant (respiratory)
  - Uranyl acetate: fatal (inhalation, ingestion); flammable
- The following Personal Protective Equipment is required for this procedure:
  - Lab coat
  - Nitrile gloves (double-layer required; regularly check for holes)
  - Eye goggles
  - Mask
  - OPTIONAL: plastic apron, shoulder-length gloves, lead glass shield
- Place a piece of absorbent sheet on the work surface before starting the procedure. When done, discard in the “Solid Waste – UA” bag.
  - This procedure involves the use of hot plate. Do not leave the hotplate on unattended.

1. Reagents and Supplies

1.1. For Saturated Aqueous Uranyl Acetate solution:

- uranyl acetate (EMS 22400)
- wide-mouth amber bottle with cap (PTFE-lined), 120 ml capacity
- scale (in fume hood in NHB 3.360E)
- large weighing dish (140 x 140 mm)
- Purified water (double-distilled, ASTM Type I, or equivalent)
1.2. For Reynold’s Lead Citrate solution:

- RO/deionized water
- concentrated nitric acid (usually 65-70%; Fisher A200)
- glassware dedicated for lead citrate solution (kept in a box):
  - 1000-ml Erlenmeyer flask (or Fleaker) × 1
  - 50-ml volumetric flask with PTFE stopper × 2 sets
  - 100-ml glass beaker × 2
  - 20-ml borosilicate scintillation vial with cap × 3 sets
  - 100-ml Kimax (or equivalent) glass bottle × 2
  - magnetic stirrer × 1 (small enough for 100-ml beaker)
- Purified water (double-distilled, ASTM Type I, or equivalent)
- Hot plate
- scale (in fume hood in NHB 3.360E)
- top loading balance (on benchtop in NHB 3.360E)
- large weighing dish (140 × 140 mm), weighing paper (4 × 4 in.)
- sodium hydroxide (Fisher S318)
- lead nitrate (Ladd Research 23603)
- sodium citrate (EMS 21140)
- 10-ml borosilicate glass pipette × 2
- 10-ml syringe × 5
- syringe filter (0.22-µm pore) × 5
- 20-gauge hypodermic needle × 5
- small rubber stopper × 1
- 500-ml tri-pour plastic beaker × 1

1.3. For Grid Staining:

- glass Petri dish set (60-mm diameter; dish plus the lid), containing about 4 mm of hardened dental wax melted in the bottom dish.
  You should have TEM grids containing your ultrathin sections wedged onto surface of the dental wax.
- 100-ml tri-pour plastic beaker × 2; label one as “UA waste” and the other as “lead waste” (They are used to collect UA and lead waste temporarily.)
- 5-ml syringe
- 0.22-µm pore syringe filter
- Saturated aqueous uranyl acetate solution (see 1.1. and 2.1.)
- Parafilm (~1/2 in. × 2 in.)
- Purified water in a squeeze bottle
- fine forceps
- Whatman #2 filter paper (55-mm diameter)
- paper points and/or Whatman filter paper cut into small wedges (use a pair of dedicated scissors)
- Reynold’s lead citrate solution (see 1.2. and 2.2.)
- 20-ml borosilicate glass scintillation vial
- 5-ml borosilicate glass pipette
- Micropipette with a 200-µl tip
- 1 N NaOH (aq) solution
- A small boat made of Parafilm (~1/2 in. square)
- NaOH pellets
- timer

1.4. Waste containers:

- Liquid waste bottles
  - “Nitric acid”: in the corrosive cabinet in NHB 3.360E
  - “Lead-NaOH”: bench top in NHB 3.360E
  - “UA-water”: bench top in NHB 3.360E
- Solid waste containers
  - “Solid NaOH” bottle: in “Base” storage container in NHB 3.360E
  - “Solid Waste – No UA” bag: in fume hood in NHB 3.360E
  - “Solid Waste – UA” bag: in fume hood in NHB 3.360E

2. Stain Solutions

2.1. Saturated Aqueous Uranyl Acetate Solution (estimated work duration: ~1 hr)
2.2. Reynolds’s Lead Citrate Solution (estimated work duration: 2+ days)

2.2.1. Day 1: Cleaning glassware

1. Wear appropriate PPE: lab coat, eye goggles, and double layers of nitrile gloves.
2. Place a piece of absorbent sheet on the work surface before starting the procedure.
3. Place a large plastic weigh boat on a scale in the fume hood. Then place a glass amber bottle on the weigh boat.
4. Carefully tap out about 6.25 g of uranyl acetate powder into the bottle. If the amount is not exactly 6.25 g, then adjust the volume of purified water.
5. Measure out purified water in a graduated cylinder and add to the bottle. 100 ml water for 6.25 g UA (i.e., 16 ml water per 1 g UA).
6. Add about 750 ml of purified water in the 1000-ml Erlenmeyer flask (or Fleaker) and heat to rapid boil on hotplate. Maintain rapid boiling for at least 30 min to de-gas CO₂. Continue to boil throughout procedure.
7. Weigh out 1.33 g of lead nitrate (in the fume hood) and carefully add to 50-ml volumetric flask. Then weigh 1.76 g of sodium citrate.
8. Using a 10-ml borosilicate glass pipette, add 30 ml of the boiled water to the 50-ml volumetric flask. Place the stopper and shake to dissolve completely.
9. Place a large plastic weigh boat on a scale in the fume hood. Then place a glass amber bottle on the weigh boat.
10. In a 100-ml beaker, add 30 ml of purified water. This water must be at RT – NEVER add NaOH into hot water.
11. Add 4.0 g of NaOH to the beaker and use a small magnetic stirrer to completely dissolve.
12. Transfer the NaOH solution from the beaker into another 50-ml volumetric flask.
13. Use the same beaker to dispense purified water and bring the volume to 50 ml. Mix well. Excess water should be disposed into the “Lead - NaOH” waste bottle.
14. Dispense NaOH solution into the cleaned 20-ml scintillation vials. Make sure to label the vials and store at RT in the “Base” storage container.
15. Old batch of 1N NaOH solution can be used for adjusting pH, or otherwise discarded into “Lead - NaOH” waste bottle.
16. Into the lead citrate mixture, add 8.0 ml of the freshly made 1 N NaOH (aq). Place the stopper and mix by inversion. The solution should become clear. If it does not, start over.
17. Bring up the volume to 50 ml with the boiling purified water and mix by inversion.
18. Transfer the stain solution into another cleaned 100-ml glass beaker. Dispense the lead citrate solution into 10 ml portions into each of the syringes.
19. Place a syringe filter and cap with a hypodermic needle.
20. Expel any air in syringe.
21. Place the syringes, hypodermic needle down into the rubber stopper and place all in a 500-ml plastic tri-pour beaker for storage at 4°C.
22. Label the beaker with today’s date.
23. Dispense boiled water into the 100-ml glass bottles and label with today’s date. Boiled water remaining in the flask can be used to rinse grids after staining.
24. Wipe any spill with a moistened Kimwipe. Discard all contaminated items into “Solid Waste – no UA” bag.

2.2.2. Day 2: Making lead citrate solution

1. Wear appropriate PPE: lab coat, eye goggles, and double layers of nitrile gloves.
2. Place a piece of absorbent sheet on the work surface before starting the procedure.
3. Add about 750 ml of purified water in the 1000-ml Erlenmeyer flask (or Fleaker) and heat to rapid boil on hotplate. Maintain rapid boiling for at least 30 min to de-gas CO₂. Continue to boil throughout procedure.
4. Weigh out 1.33 g of lead nitrate (in the fume hood) and carefully add to 50-ml volumetric flask. Then weigh 1.76 g of sodium citrate.
5. Using a 10-ml borosilicate glass pipette, add 30 ml of the boiled water to the 50-ml volumetric flask. Place the stopper and shake to dissolve completely.
6. Remove the stopper and add 1.76 g of sodium citrate to the volumetric flask.
7. Place the stopper and shake vigorously for 2 min. Solution will become milky white.
8. Let stand for 30 min with occasional mixing by inversion.
9. Make 1 N NaOH solution (aq):
   1. In a 100-ml beaker, add 30 ml of purified water. This water must be at RT – NEVER add NaOH into hot water.
   2. Add 4.0 g of NaOH to the beaker and use a small magnetic stirrer to completely dissolve.
   3. Transfer the NaOH solution from the beaker into another 50-ml volumetric flask.
   4. Use the same beaker to dispense purified water and bring the volume to 50 ml. Mix well. Excess water should be disposed into the “Lead - NaOH” waste bottle.
   5. Dispense NaOH solution into the cleaned 20-ml scintillation vials. Make sure to label the vials and store at RT in the “Base” storage container.

2.3. Grid Staining (estimated work duration: 30-60 min)

1. The TEM grids should be loaded into the pre-made razor slits in the wax bottom of the staining dish in an orderly array.
2. Wear appropriate PPE: lab coat, eye goggles, and double layers of nitrile gloves.
3. Place a piece of absorbent sheet on the work surface before starting the procedure.
4. Make 0.02 N NaOH (aq) solution:
   1. Use a 5-ml glass pipette to add 4.9 ml of purified water in a 20-ml glass scintillation vial.
   2. Use a micropipette with a 200-µl tip to add 100 µl of 1 N NaOH to the vial. Close it with a cap and gently shake to mix.
5. Draw the saturated aqueous uranyl acetate solution from the middle of the stock bottle into the 5-ml syringe (UA precipitates accumulate at the bottom and liquid surface). Attach a 0.22-µm pore syringe filter. Close the stock bottle and seal it with a new piece of Parafilm.
6. Dispense filtered uranyl acetate solution onto the section side of grids only (one drop per grid).
7. Cover the dish and stain for 5 min. Dispose of the uranyl acetate contaminated filter-syringe unit into “Solid Waste – UA” bag.
8. Pour off the stain into a 100-ml tri-pour plastic beaker labeled “UA waste”. Then, rinse the grids by directing a gentle stream of purified water from squeeze bottle against the side of the interior glass dish, not on the grids directly. Pour off the rinse water into the waste beaker. Repeat the water rinses 5 to 10 times.
9. Use the filter paper wedges to wick the grids dry of water. To do this, carefully place the paper to the side of each grid. Make sure to remove water from both sides of the grid, as well as the slot on wax surface. Dispose of these filter papers into “Solid Waste – UA” bag.
10. Cover the staining dish for now. Pour all waste into “UA-water” waste bottle. Dispose of the uranyl acetate contaminated items, and change the outer layer of gloves.
11. Make a boat with a small (~1/2 in. square) piece of Parafilm. Place this into the staining dish away from the grids, and add ~3 pellets of sodium hydroxide into it.
12. Place a 55-mm diameter filter paper into the underside of the glass lid cover. Apply ~300 µl of 0.02 N NaOH solution to moisten the paper so that it will adhere.
13. Remove a syringe filled with lead citrate stain solution from the rubber stopper and expel the first few ml of the solution into a 100-ml tri-pour plastic beaker labeled “lead waste”.
14. Dispense filtered lead citrate solution onto the section side of grids (one drop per grid).
15. Cover the dish and stain for 5 min. Place the syringe back onto the rubber stopper.
16. Discard the NaOH pellets into “Solid NaOH” waste container. The Parafilm boat should be disposed in a solid waste bag.
17. Pour off the lead stain into the lead waste beaker.
18. Add the 0.02 N NaOH solution as a first rinse to grids (10-15 sec). Pour this into the lead waste beaker.
19. Apply rinses with purified water 5 to 10 times, in the same manner as done for uranyl acetate above. Make sure to remove water from both sides of the grid, as well as the slot on wax surface.
20. Wick the grids dry using the filter paper wedges. Dispose of the filter paper adhered to the underside of the glass lid cover into “Solid Waste – No UA” bag.
21. Rinse the lid with purified water and wipe dry with Kimwipe. Place the lid back onto the staining dish and let the grids air dry in the dish overnight or longer.
22. Pour all liquid waste into “Lead citrate-NaOH” bottle. Dispose of lead-contaminated items in “Solid Waste – No UA” bag. The absorbent sheet on the work surface should be discarded in “Solid Waste – UA” bag.
23. Make sure to record the grid storage location (i.e., grid box ID and position in the box). Also make note of any damages to the grid made during staining.

3. Clean-up

3.1. Waste containers:
- Hazardous Liquid Waste: Pour all waste into the proper waste collection bottles available on bench top or in the corrosive cabinet in NHB 3.360E.
  - “Nitric acid” (10% nitric acid and first rinse)
  - “Lead-NaOH” (lead nitrate, sodium citrate, sodium hydroxide, water)
  - “UA-water” (aqueous UA solution and rinse)
- Hazardous Solid Waste: Place all contaminated solid waste (e.g., gloves, pipets, vials, processing dishes, etc.) into hazardous waste bags in fume hood. Vials must be uncapped. Make sure to separate all waste contaminated with UA (including plastic-backed absorbent pad).
- Solid NaOH pellets used during lead citrate staining should be disposed in a designated container (a bottle labeled "NaOH" in NaOH box).

3.2. Glassware and equipment:
- All items that came in contact with UA must be rinsed with water into the “UA-water” waste bottle and disposed of in the “Solid Waste – UA” bag. Make sure to separate all waste contaminated with UA.
- All items that came in contact with lead nitrate or lead citrate must be rinsed with water into the “Lead-NaOH” waste bottle.
- Wipe any small spill with a moistened tissue (e.g., Kimwipe).
- Monitor the area for radioactivity, as described in the Harris Lab SOP for uranyl acetate.

4. Tips and Troubleshooting
- If you are using Poloform-coated grids, Coat fresh grids the day before you plan to use them. Otherwise the film will trampoline and cause sections to have ‘pockets’ where stain can collect.
- Good clean handling technique from the block trimming steps and forward all contribute to a final clean stain result:
- Knives with nicks, both Cryotim and Ultra, can add plastic particles and shavings to surface, later causing stains to pool, aggregate, or precipitate.
- Clean freshly cut styrofoam stick with 100% ethanol before cleaning knife.
- Clean knife and boat (with purified water and then ethanol) after each cutting session. Inspect the diamond under dissecting scope before session for stubborn contamination that may not have been resolved by routine boat rinsing and drying.
- Use clean boat water for sectioning (filtered through a syringe-filter unit).
- Clean forceps and eyelash (ethanol and ultrasonication).
- Dry grids in dust-free location (big glass or plastic Petri dish).
- Keep blocks free of dust when trimming with air gun. Store blocks dust-free (e.g., gelatin capsules and small zippered bags).
- Use the "air-gap within tubing" method if peristaltic pump is used to level boat. This helps to keep old line water out of the boat when filling or leveling. Or, for critical work (e.g., series cutting), consider using a separate syringe to level boat.
- Before each staining session, re-melt wax and clean wax surface. For cleaning, rinse thoroughly with 70% alcohol --> purified water --> 0.02 N NaOH solution --> purified water, and air-dry completely. Make sure this dish is absolutely clean and dry before and after use, keep it covered when not in use.
- Do clean the razor blade with ethanol or high-purity acetone before making slots on wax surface.
- Always stain your grids on the same day that you cut them. Waiting until the next day will markedly reduce the quality of the stain.
- AVOID getting any stain on the 'back side of the grid' where it is very hard to clean due to the depth of the Synaptek grid.
- For rinse after lead citrate staining, use purified water that was boiled and cooled right before rinsing. Final cooling should be done in a sealed bottle. Use Parafilm to seal when the bottle is just cool enough to handle comfortably.
- The original factory-cut outside edges of filter paper are cleaner. Try to use the factory-cut edges if possible.
- Drying as much as possible the wax slots used to hold the grids may reduce contamination. Paper points are good for this, or long thin triangles of filter paper. A decent spacing between the grids in the wax-filled dish should make it easier to dry them well.
- When you have only a small number of grids to stain, use a gentle stream of purified water on the section side of the grid for several seconds. Avoid getting the backside wet, but if you do, then be sure to rinse and dry both sides between stains.