

## PRIMARY FIXATIVES

### GLUTARALDEHYDE (glutaric acid dialdehyde)

#### *NOTES:*

- a. Most widely used primary fixative for EM
- b. To minimize extraction of cellular components by autolysis, fix at low temperatures (e.g. 4 C in fridge).
- c. Concentration for usage: below 2%, extraction may occur. above 4%, shrinkage may occur. Usually prepared at 2.5% for biological specimens.
- d. Purified GA is good at around pH 3-6. If below 3% then discard. You can purchase in ampoules of 25 or 8% from EM vendors which will last several months unopened). Biological grade GA contains impurities such as glutaric acid, acrolein glutaradozamine, ethanol, methanol and various polymers and products of oxidation and photochemical degradation.
- e. Osmolarity: purified – around 300 mosmols; biological – around 500-600 mosmols
- g. Glutaraldehyde/formaldehyde mixtures provide faster penetration and good crosslinking of proteins. Structures are stabilized with formalin, then crosslinked with GA.

### Basic EM primary fixation

#### To mix 2 - 2.5% Glutaraldehyde:

2.5% buffered GA - 10 ml 25% GA into 90 ml buffer.

2% buffered GA - 1 ml 8% GA into 2 ml double strength buffer and 1 ml water (or 1 part GA, 2 parts buffer, 1 part water)

### GLUTARALDEHYDE/PARAFORMALDEHYDE MIXTURES

*Note:* Lower concentrations of GA (0.02 – 0.2%) mixed with formaldehyde are preferred when preparing samples for immunolocalization.

### **Karnovsky's (1965)**

Extremely hypertonic (2010 mosmols). Not often used.

5% glutaraldehyde, 4% formaldehyde in cacodylate buffer (cacodylic acid).

### **2.5% GA, 2% paraformaldehyde**

-Most widely used fixative for EM

Cacodylate (0.2M)	25 ml
pForm (10%)	10 ml
GA (25%)	5 ml
distilled H <sub>2</sub> O	to 50 ml

The mixture results in 0.1M buffered solution.

### **McDowell and Trumps (1976)**

-Used mainly in pathological labs where both EM and light microscopy are required from the same tissue.

GA (50%)	2 ml
Formaldehyde (40%)	10 ml
NaH <sub>2</sub> PO <sub>4</sub> H <sub>2</sub> O	1.16 g
NaOH	0.27g
dH <sub>2</sub> O	88 ml

### **GA/FA mix**

- The high concentration of formaldehyde in this mix is effective in localizing amines by formation of fluorescent products and for fixing CNS for EM. Fluorescent intensity is enhanced if dried, but lost if rehydrated.

GA 0.5-1%  
FA 4%  
0.1M Cacodylate or PBS (pH 7.0)

### **SPECIALIZED FIXATION**

#### **GA- Ruthenium red**

Preserves and defines membranes and myofilaments. Will stain acidic mucopolysaccharides.

*CAUTION! Ruthenium red is toxic.*

<b>Soln A:</b> GA (4% aqueous)	5 ml	<b>Soln B:</b> OsO <sub>4</sub> (5% aqueous)	5 ml
cacodylate (0.2M, pH 7.3)	5 ml	cacodylate buffer	5 ml
ruthenium red (stock)	5 ml	ruthenium red (stock)	5 ml
(1,500 ppm in H <sub>2</sub> O)			

Fix in solution A for 1 hr at RT  
Rinse 3x10 min in cacodylate buffer  
Fix in solution B for 3 hr at RT  
No post staining necessary

#### **GA – Alcian blue**

To obtain staining of a cell coat and intercellular substances. Its behavior is identical to ruthenium red except alcian blue is not toxic. The GA-mucosubstance-alcian blue complex formed is osmiphilic.

Fix in 4% GA, 1% alcian blue in buffer for 1-18 hours.  
wash  
post-fix in OsO<sub>4</sub>  
buffer both GA and OsO<sub>4</sub> to pH 6.5

### **GA- digitonin (Okros 1968)**

Helpful in retaining fine structural localization of free cholesterol and cholesterol esters. The complex is osmiphilic and insoluble in lipid solvents.

Fix at RT and dehydrate only 70 and 95% ethanols. Infiltrate with 95% ethanol/Epon mix. Crystal artifacts are not uncommon. The 1% digitonin causes complete disruption of lysosomal membranes in intact tissues but has little effect on peroxisomes.

GA (2.5%)	5.0 ml	fix at RT for 2 hr
FA (2%)	5.0 ml	The mixture is stable for two days
digitonin in buffer (0.2%)	5.0 ml	

### **GA – hydrogen peroxide**

Used to enhance quality of fixation. The possible increase in oxygen during fixation is thought to be needed for irreversible protein crosslinking.

**CAUTION!** Hydrogen peroxide cannot be mixed with formaldehyde/formalin as explosive compounds may result.

Cacodylate buffer (0.1M)	25 ml
GA (25%)	5 ml
Hydrogen peroxide (15%)	5-25 drops

Add drops while continuously stirring. The final concentration of GA will be between 3 and 6%.

Fix for 1-2 hr at RT or 3-4 hr at 4 C. If you fix at the cooler temp, fix for one hour in 3-5% GA after the initial fix.

### **GA- Lead acetate**

Used to preserve soluble inorganic phosphate (nucleolar orthophosphate). Osmium and UA are not used in this prep as they will remove the lead precipitate.

Rxn: GA-lead acetate and inorganic phosphate will form lead hydroxyapatite.

GA 2%  
Lead acetate 2%  
0.1 M Cacodylate

fix at pH 7.0 at RT for 3 hr

### **GA-malachite green**

Used to preserve lipids containing granules in mammalian sperm. Enhances lipid opacity under the “big eye”. The overall ultrastructural preservation when using this combination is not satisfactory.

GA 2%  
Malachite green 0.1%

Fix as usual.

**GA – trinitro compounds (Picric acid, trinitrocresol.)**

Preserves smooth ER in testicular interstitial cells and other steroid secreting cells. Preserves peroxidase activity. **CAUTION!** Trinitro compounds are unstable when crystallized and can explode.

Buffer (0.2M, pH 7.2)	45 ml
GA (25%)	5 ml
FA (4%)	50 ml
trinitrocresol (2%)	50 ml

fix for 2 hr at RT

**GA – potassium dichromate**

For visualizing biogenic amines. Useful for demonstrating norepinephrine and argentaffin cells.

a. Fix tissue in 3% GA in Cacodylate buffer (pH 7.2) for 4 hr followed by incubation in 2.5% potassium dichromate in Caco buffer (pH 4.1) for 4 hr.

b. Mix: GA 1%  
FA 0.4%

0.01 M Na chromate-potassium dichromate buffer (pH 7.2)

Fix tissue in above formulation for 1 to 10 minutes. Store in Na chromate-K dichromate buffer (pH 6.0) for 18 hr at 4 C. Do not stain with UA.

**Champy's fluid**

3% potassium dichromate	7 parts
1% chromic acid	7 parts
2% OSO <sub>4</sub>	4 parts

**GA- potassium ferrocyanide – OsO<sub>4</sub> (Elbers 1965)**

Preserves labile lipids, surfactants in the lung and increases visualization of lipids. Used for the demonstration of liposome particles.

Fix in 1% acrolein and 2.5% GA in 0.067M cacodylate (pH 7.4) containing 1mM CaCl<sub>2</sub> for 24 hr at RT. Store overnight in buffer.

Postfix in 1% OsO<sub>4</sub> in same buffer containing K<sub>3</sub>Fe(CN)<sub>6</sub> (0.05M) and CaCl<sub>2</sub> (0.05M) for 3-4 hr in dark at 4 C.

### **GA – Tannic acid**

Tannic acid is a mordant for heavy metal staining of mucins and complex carbohydrates. Treatment prior to OsO<sub>4</sub>: Provides enhanced cyto-membranes and cytoskeletal features. Prevents disruption of MTs by OsO<sub>4</sub>. Reacts with phospholipids and phosphatidyl cholines. Treatment after OsO<sub>4</sub>: High density staining of secretory bodies.

Preserves intercellular glycosaminoglycans (Singer & Solursh 1980).

Prepare all solutions in buffer

#### Treatment 1:

GA (5 %)	10 ml
tannic acid (8%)	10 ml
Adjust pH with NaOH	

#### Treatment 2:

GA (5%)	20 ml
FA (16%)	10 ml
tannic acid (16%)	10 ml
sucrose	0.05 M

Postfix in OsO<sub>4</sub>

### **GA- Uranyl acetate**

Recommended for fixing bacteria containing intracellular phages (Sechaud and Kellenberger 1972). Gels the DNA. See the article for concentrations.

### **Acrolein**

Allows for increased penetration with large or impermeable tissues. This is a component or impurity found in biological grade glutaraldehyde. **CAUTION! Acrolein is highly volatile and toxic. Very dangerous and should only be used when absolutely necessary.**

Acrolein formulations:

#### 1% acrolein – 2.25% GA in 0.1 M buffer

cacodylate buffer (0.2 M)	50 ml
Acrolein	1 ml
GA (25%)	10 ml
dH <sub>2</sub> O to make 100 ml	

GA (25%)	12 ml
acrolein	3 ml
Millonigs Phosphate buffer	85 ml

Either formulation; Fix for 1-3 hr, start in cold and allow to come to RT.

Wash

Postfix in 2% buffered OsO<sub>4</sub> at 4 C for 2 hr.

### **Osmium Vapor fix**

Primarily used with specimens that are in an aqueous solution or very delicate and addition of one of the above fixatives would disrupt or be osmotically detrimental.

Take a lid or cover and tape a filter paper to it. Put a drop of 4% aqueous osmium on the filter paper and cover the sample for at least 1 hr. Another drop of 4% osmium into the sample directly can be done if required for good fixation. Wash completely. Do not use glutaraldehyde after as precipitation will occur and you will have a mess.

## **SECONDARY FIXATION**

### **OsO<sub>4</sub> (osmium tetroxide, osmic acid)**

OsO<sub>4</sub> acts as both a stain and a fixative. Most commonly used post-fixative. Has a high molecular weight (254.2) in a water soluble crystalline form that will not change the pH. It is a noncoagulant fix that stabilizes proteins further. It is a non-polar compound which oxidizes aliphatic double bonds. It is thought that one molecule of osmium reacts with one double bond in the lipid molecule and forms glycol osmates stabilizing the molecule.

Fixation in OsO<sub>4</sub> should be kept to a minimum to avoid leaching and damage to cellular components. Also, longer times causes the lipids to “over stain” decreasing the information gained from staining. Optimal concentration is between 1-2%. Higher concentrations cause cleavage of protein molecules resulting in loss of peptide fragments. Usually obtained in ampoules of 4% aqueous or in solid form. If solid, make aqueous stock solution of 4% that can be frozen until used. Tissue size should not exceed 0.5 mm for optimal fixation and penetration of sample.

One method used by Dr. Farmer and others is a method of using the osmium vapors to initially fix aquatic specimens (e.g *Euglena* and other protists). See above in Primary fix for details.

Crystalline OsO<sub>4</sub> can be mixed in acetone or methanol for freeze substitution protocols. If used in this manner, extra precautions are needed to keep the solution at -80 C as it will oxidize rapidly. If either aqueous or other osmium solution is dark brown to black then discard and prepare fresh. Fresh solution should appear light tan or “weak tea” colored.

Cryofixation and freeze substitution are covered in detail in Bozzola EM text. Briefly, one rapidly freezes the sample by plunging into liquid propane at liquid nitrogen temperature and then transferring the sample to the osmium/acetone or methanol substitution fluid which is kept at -80 C. This substitution fluid will remove the water over time, usually 48 hrs while kept at -80. The sample in substitution fluid is then warmed very slowly by periodically moving the sample from -80 to -20 freezer for several hours, then to the 4 C fridge and finally washing the sample with several changes of fresh acetone or methanol. Infiltration can begin immediately.

**CAUTION! Osmium vapors will fix tissue! ALWAYS use in hood and with appropriate safety precautions.**

## **BUFFERS**

Most buffers used in electron microscopy are effective in the physiological range: pH 7.2 - 7.4

### **PHOSPHATE BUFFERS**

Non-toxic to cells in culture and the pH is stable at various temperatures. They can be stored for several weeks in the refrigerator and is the most widely used buffer for many cellular methods and EM. Forms a precipitant when contaminated and has a tendency to decrease the nuclear mass. Will extract non-chromosomal proteins in nucleus and can cause swelling on organelles. Will precipitate polyvalent cations, lead and uranium salts. Not good for negative staining.

May inhibit certain enzymes. Phosphate ions are thought to precipitate in concentrations of ethanol above 50%, adhere to cellular structures and attract uranyl and lead ions. Rinsing in lower concentrations of ethanol reduces much of the phosphate.

Osmolarity is around 290 mosmols.

### **Millonig's**

#### 1. Phosphate buffer (1961)

Soln A: 2.26% monobasic sodium phosphate in water  
Soln B: 2.52% NaOH in water

Buffer final concentration: 0.13 M

Mix 41.5 ml of Soln A with 8.5 ml Soln B

Remove 5 ml of mix and add 5 ml of 4% sucrose solution.

Add 25g of MgCl<sub>2</sub> or CaCl<sub>2</sub> to each 100 ml of buffer

Adjust pH to 7.3 with Soln B

Stable for several weeks at 4 C

#### 2. Phosphate buffer (1964)

In 500 ml dH<sub>2</sub>O:

Monobasic sodium phosphate 1.8 g  
Dibasic sodium phosphate 23.25 g  
NaCl 5 g

Ph to desired value then add

d H<sub>2</sub>O to make 1,000 ml

### **Karlsson and Schultz Phosphate (1965)**

Monobasic sodium phosphate 3.31 g

Dibasic sodium phosphate 33.77 g

dH<sub>2</sub>O to make 1.0 L

pH 7.4, Osmolarity 320 mosmols (equivalent to cerebrospinal fluid in rats).

### Maunsbach Phosphate (1966)

Monobasic sodium phosphate 2.98 g  
Dibasic sodium phosphate 30.40 g  
dH<sub>2</sub>O to 1.0 L

### Sorenson's Phosphate (0.1 M)

Solution A: 0.2 M

Na HPO<sub>4</sub> · 2H<sub>2</sub>O 35.61 g  
or Na<sup>2</sup>HPO<sub>4</sub> · 7H<sub>2</sub>O 53.65 g  
or Na<sup>2</sup>HPO<sub>4</sub> · 12H<sub>2</sub>O 71.64 g  
then dH<sub>2</sub>O to make 1.0 L

Solution B: 0.2 M

Na HPO<sub>4</sub> · H<sub>2</sub>O 27.6 g  
or Na<sup>2</sup>HPO<sub>4</sub> · 2H<sub>2</sub>O 31.21 g  
Then dH<sub>2</sub>O to make 1.0 L

Prepare by mixing two solutions as given here and diluting to 100 ml with distilled water.

<u>pH at 25 C</u>	<u>Soln A</u>	<u>Soln B</u>
6.4	13.25	36.75
6.6	18.75	31.25
6.8	24.5	25.5
7.0	30.5	19.5
<b>7.2</b>	<b>36.0</b>	<b>14.0</b>
<b>7.4</b>	<b>40.5</b>	<b>9.5</b>
7.6	43.5	6.5
7.8	43.75	4.25
8.0	47.35	2.65

Osmolarity of 0.1 M buffer (pH 7.2) is 226 mosmols. Addition of 0.18 M sucrose raises it to 425 mosmols.

### Phosphate Buffered Saline (PBS)

For immunofluorescence, immunocytochemistry – pH 7.0

For 1.0 L

NaCl 6.8 g  
Na HPO<sub>4</sub> (dibasic) 1.5 g  
Na<sup>2</sup>HPO<sub>4</sub> (monobasic) 0.43 g

## Dulbecco's Phosphate buffered saline

In 800 ml water:

Soln A:        8.0g    NaCl  
                  0.2 g    KCl  
                  1.15 g   Na<sub>2</sub>HPO<sub>4</sub> (dibasic)  
                  0.2 g    KH<sub>2</sub>PO<sub>4</sub> (monobasic)

In 100 ml water each:

Soln B:        0.1 g    CaCl<sub>2</sub>  
Soln C:        0.1 g    MgCl<sub>2</sub>

Dissolve separately, then combine.

## **CACODYLATE BUFFER** (cacodylic acid)

Made at 0.2 M and then diluted accordingly. Effective in pH range of 6.4 to 7.4. Avoids interference of extraneous phosphates in cytochemical localization. Does not increase nuclear mass, little removal of acid soluble proteins from nuclei. Desirable for auto-radiography and enzyme localizations. Resistant to bacterial contamination. Calcium can be added without precipitation. The buffer is incompatible with UA, so enbloc staining is not recommended. Membrane permeability may be altered due to toxicity of the buffer leading to a redistribution along the osmotic gradient of changed chemical activity. This impairment will affect the quality of fixation.

**CAUTION! Contains arsenic – contact with acid produces arsenic gas.**

Cacodylate formulation: (0.05M)

Prepare in fume hood and wear gloves.

Solution A:    Sodium cacodylate trihydrate    42.8 g  
                  Add dH<sub>2</sub>O to 1.0 L

Solution B:    0.2 M HCl  
                  Concentrated HCl (36-38%)    10 ml  
                  dH<sub>2</sub>O                                    603 ml

The desired pH can be obtained by adding soln B to 50 ml of soln A and diluting to a total volume of 200 ml with dH<sub>2</sub>O, according to the following schedule:

<u>Soln B</u>	<u>pH</u>	<u>0.2 M Cacodylate</u>
18.3	6.4	Molecular Wt in grams into 1.0L H <sub>2</sub> O
13.3	6.6	Adjust pH accordingly.
9.3	6.8	
6.3	7.0	
4.2	7.2	
2.7	7.4	

## **COLLIDINE BUFFER**

Efficient at pH 7.4 when half neutralized with HCl (range of buffer 6.0 – 8.0)

Does not react with OsO<sub>4</sub> and is stable at RT indefinitely. Great for lung tissue but collidine is a pyridine derivative and therefore extracts phospholipids. Tissue sections very easily, probably due to this extraction. Extraction facilitates penetration of fixative into larger specimens. It is not recommended for EM.

**CAUTION! TOXIC**

Stock: s-collidine (pure)            2.67 ml  
      dH<sub>2</sub>O to make 50 ml

Buffer: Stock soln                    50 ml  
      1.0 M HCl                        9.0 ml  
      dH<sub>2</sub>O to make                    100 ml  
adjust pH to 7.4 with HCl

## **TRIS BUFFERS**

As a primary amine, it reacts with GA. It has poor buffering capacity below 7.5. Biological inhibitor and used mostly in enzyme localizations.

Tris (0.05 M)

## STAINS

### **THICK SECTION STAINING**

#### Procedure

1. Transfer sections to small drop of water on slide.
2. Heat slide gently until section adheres to slide – evaporate all water but do not boil.
3. Cover section with drop of staining solution and heat gently for thirty sections to one minute.
4. Drain off excess stain, and wash well in two changes of dH<sub>2</sub>O
5. Dry with heat.

### STAINS

#### **Toluidine blue**

Most commonly used stain for epoxy sections

1 part dH<sub>2</sub>O

1 part 5% toluidine blue

1 part 2% sodium borate

Mix well, then filter. Store at RT

#### **Methylene Blue**

1% Azure blue or Azure II in 1% borax

#### **Paragon stain** (Martin et al. 1966)

**1% aqueous p-phenylene diamine** (Estable-Puig 1965)  
for black and white photography.

## **THIN SECTION STAINING**

Usually thin sections for EM are sequentially stained with 4% uranyl acetate followed by Reynold's lead citrate.

### **URANYLACETATE**

Most widely used stain for thin sections. Provides high contrast by staining nucleic acids, proteins, free amino groups. Solutions are photolabile and should be kept dark. Alcohol solutions produce better contrast and require shorter staining times. Aqueous solutions must be used when working with supportive films or when enzymes have been localized. Concentrations may be varied so it is suggested that you use the one that provides your material with best contrast.

#### **Common formulations**

1-4% aqueous	2% in methanol or ethanol
saturated aqueous	saturated in 50% methanol
saturated in methanol	

#### **Staining procedure:**

1. Staining should be performed at RT. Filter solution.
2. Place one drop onto parafilm in Petri dish and float grids section-side on solution.  
Do **not** allow drop to evaporate or precipitated UA will result.
3. Stain for 15 to 45 minutes in dark. This can be done by covering the Petri cover with aluminum foil. 30 minutes is typical.
4. wash grids in three changes of dH<sub>2</sub>O or rinse with a continuous flow over the grid. If alcohol based, wash with progressively lower concentrations of alcohol.

### **En Bloc Staining with UA**

When UA is used en bloc, it has a fixative effect giving the fine structural preservation of DNA filaments, membranous structures and cell junctions. But it can also cause extraction of cellular components.

#### **Procedure:**

Apply stain to tissue either before or after OsO<sub>4</sub>. It is thought that application of UA after OsO<sub>4</sub> leads to better retention of phospholipids.

1% UA in buffer or 50% ethanol.

**CAUTION!** UA is a uranium salt and is slightly **radioactive**. It is not advisable to come in contact with UA. Dispose of waste in appropriate receptacles.

## **LEAD STAINING**

Stains most intensely at high pH, more intense staining occurs when preceded by OsO<sub>4</sub> fixation – especially membranes because phosphate, sulfhydryl, tyrosyl and carboxyl groups become more ionized after osmium resulting in increased binding of lead. Stains glycogen, membranes and the ground substance of cells. Insoluble crystals of lead may form while staining by precipitating in the presence of CO<sub>2</sub>. NaOH pellets around the staining chamber will chelate lead hydroxide and prevent precipitation.

### **Precautions:**

Maintain a clean work area.

Use NaOH around grids and keep lid on Petri dish.

Hold your breath when transferring or washing

Filter stain before using

Wash initially with 0.02M NaOH before washing well with dH<sub>2</sub>O. Some prefer to boil water to drive off CO<sub>2</sub>, allowing the water to cool before using for wash.

### **Reynold's Lead Citrate (1963)**

Boil 100 ml of dH<sub>2</sub>O to remove dissolved CO<sub>2</sub>. Let cool to RT.

Lead Nitrate 1.33 g

Sodium citrate 1.76 g

dH<sub>2</sub>O 30 ml in 50 ml volumetric flask

Shake vigorously for 1 minute and then every 5 minutes or so over 30 minutes time period.

Add 8 ml of 1N NaOH to clear solution and dilute to 50 ml.

Dispense into syringes with filter and store at 4 C. Can be stored up to 1 month.

Stain grids in similar fashion as described above for UA. It is advisable to place NaOH pellets around the area of stain in the Petri dish. Discard first few drops from syringe and then use drops for grids.

Stain for 1 minute. Longer periods will decrease UA staining, as the UA bleaches from the high pH.

First wash in 0.02N NaOH, then three thorough washes in water.

Dry grid by wicking water away with filter paper. Allow to dry completely before viewing in scope (approx. 1 hr minimum)

### **Venable and Coggesall Lead Citrate (1965)**

dH<sub>2</sub>O 100 ml

Lead citrate 0.4 g

10 N NaOH 1.0 ml

Shake vigorously in screw-capped vial. Do not expose to atmospheric CO<sub>2</sub>. Stain as described above.

### **Sato's Lead Citrate (1967)**

Lead nitrate	1.5 g
Lead acetate	1.5 g
Lead citrate	1.5 g
dH <sub>2</sub> O	90 ml

Heat to 40 C while stirring for one minute.

Add 3 g Sodium citrate to this mixture and stir for 1 min

Add 24 ml 1N NaOH and 24 ml dH<sub>2</sub>O to this mix

This solution can be stored for one year at RT.

1. Stain sections by immersion with concentrated solution or dilute with dH<sub>2</sub>O 1:7  
Stain for 10 minutes
2. Wash with water and air dry

### **Lever Lead Hydroxide (1960)**

Add 1 g lead hydroxide to 100 ml dH<sub>2</sub>O and bring to boil.

Cool and filter

Add drop by drop, 2N Potassium hydroxide until the solution clears completely.

Stain the sections by floatation for 5 min

Wash in 1% aqueous potassium hydroxide followed by thorough water washes.

### **Potassium Permanganate**

Stains membranes, myelin sheaths, tonofibrils, glycogen and desmosomes. Reactive with NMA, a component of EPON 812 resin.

Used aqueous at 1% for section staining

For en bloc, 1% in 100% acetone.

### **Bismuth**

Has a strong affinity for nucleic acids. Great for chromosome studies and is a general enhancer of contrast.

An alkaine bismuth subnitrate solution is used

See Ainsworth & Karnovsky (1972) J.Histochem. Cytoschem. 20:995.

### **Thorium**

Specific for mucosubstances. Radioactive, used at low pH (2 –2.5). Stains outside of plasma membrane.

Colloidal thorium 0.5 g

3% acetic acid (pH2.5) 50 ml

Wash glutaraldehyde –osmium fixed tissue with dH<sub>2</sub>O. Immerse the tissue in staining solution for 24 hr at RT.

Rinse the blocks in 3% acetic acid

Dehydrate and embed as usual.

### **Indium trichloride**

For staining of nucleic acids. The staining is attributed to binding of indium by phosphate groups in the nucleic acids. Other groups that may react are first blocked by acetylation and borohydride reduction. The method is not completely specific as keratohyalin granules, mammalian sperm tails and mast cell granules stain as well. Produces good contrast and ultrastructural staining.

Fix tissue in buffered aldehyde. Do not use osmium at any stage.

Dehydrate in an acetone series at 0-5 C.

Once tissue in absolute acetone, add pyridine gradually and bring tissue to pure pyridine in 15 minutes. wash in pyridine 3x 10 min at 4 C.

Reduce for 2 hr with pyridine saturated with lithium borohydride prepared just before.

Wash 3x 10 min with cold pyridine.

Acetylate overnight at RT with freshly prepared mix of 6 parts pyridine// 4 parts acetic anhydride saturated with sodium acetate.

Wash 3x 10 min with 100% acetone at RT

Stain en bloc for 2 hr at 4 C in 25 mg anhydrous indium trichloride in 1 ml acetone.

Wash 2x 10 min in acetone at RT

Embed in methacrylate or epoxy. Do not use araldite.

### **Phosphotungstic acid (PTA)**

Has an affinity for polysaccharides, used as a precipitation agent in the localization of amino acids. Reacts with serotonin, histamine, epinephrine; proteins rich in lysine, histidine, and arginine. Stains lysosomes and plasma membrane.

Used in acidic environment (pH 1-3). Very popular as a negative stain, has a finer grain than UA.

Oxidize sections for 20 min in 1% periodic acid  
stain in 5% PTA for 1-2 hr

Always use PTA in an ethanolic solution. Store the reagent in freezer.

### **NEGATIVE STAINING**

The method of negative staining described by Brenner & Horne (1959) is essentially a simple one. Its original purpose was to enable large numbers of specimens prepared by other physical and chemical methods to be examined by EM. Their original procedure was to prepare a 2% solution of phosphotungstic acid (PTA) in water or ammonium acetate and adjust the pH to a neutral value between 6.4 – 7.4, by adding small drops of N-KOH. The potassium phosphotungstate (KPT) was then added to the virus suspension and the mixture sprayed onto carbon film supports by one of the methods described. Droplet patterns were formed by the electron-dense KPT enclosing or surrounding the virus particles, thus producing a reversal in the contrast seen in the final image.

A method also used is to dry the sample suspension (bacteria, flagellar suspension, viral) onto the coated grid and then put a drop of either PTA or 4% UA onto the grid, then wait a period of time determined previously to provide best results. Wipe off the excess stain and allow to dry before viewing in the TEM.

## PHOTOGRAPHY SOLUTIONS FORMULATIONS

For Film Development:

Microdol-X:

Gives the finest grain with a minimum speed loss. Produces a very low fog level. When possible use a 1:3 dilution over full strength – the grain will be finer.

HC-110:

Rapid developer for processing most B&W films. Produces sharp negs with normal contrast. Great for TRI-X film. Use dilution B for better grain. Stored at “full strength” until diluted.

D-11

High contrast developer used in graphic arts. use this developer for Ortho film. 2.5 min at full strength at RT.

D-19

Rapid developer that yields high contrast negs. Used to develop EM negs. Use 1:1 with H<sub>2</sub>O

Change after approx. 200 negs

Dektol

Yields high contrast negs and used to develop cold-tone papers. Used to develop LPD IV and B&W papers.

D-76

Produces maximum emulsion speed and great shadow detail. Produces a normal contrast.

Common dilutions:

B&W 35 mm film

Microdol-X 1:3

Dektol 1:2

D-11 Straight

HC-110 1:7 (dil.B)

B&W paper:

Ektaflo type I: 1:9 for cold tone papers

Dektol: 1:2

## MISCELLANEOUS PROCEDURES

### Preparing mixtures and volumes

When preparing mixtures, use the following calculations to minimize solutions used and decrease waste production.

(original concentration of starting solution) x (amount of original solution to add) = (the final amount required) x (the working concentration).

For example:

8% glut (X) = 20 ml (2% glut)

8% is the original concentration of glut in ampoule      2% is the working concentration

$8X = 40$

$X = 40/8$      $X=5$     so 5 ml of 8% glut added to 15 ml buffer (20ml - 5ml) to provide 2% final.

### Preparing solutions from dry ingredients

When preparing any solutions, such as a buffer, it is important to read the instructions and know the molecular weight (or formula weight) of the reagent and the final pH of the solution. A 1 molar solution (1.0 M) is equivalent to adding the m.w. in grams to a liter.

A "normal" solution is usually used for pH reagents and is the same as molar as long as there is only 1 hydrogen involved in the formula (e.g. HCl or NaOH). e.g. 1N HCl = 1M HCl

Always begin with less water than the final volume, as adding the dry ingredients and pH reagents will add volume. Usually about ½ to ¾ will be sufficient.

Measure each chemical out onto the balance by tapping the chemical out of the bottle if possible to avoid contamination by a potentially dirty spatula. If you overshoot the amount needed, use the spatula to remove the excess and discard into the trash or other receptacle (depending on the toxicity or hazard of the chemical used).

### pH

If pH adjustment is required, make sure the solution is being mixed with a stir bar on a stir plate while taking measurements. If needed, calibrate the pH meter using the solutions provided. If the final solution you are making is to be acidic (low pH values), then calibrate using the 7 and 4 solutions. Similarly, if making a basic solution (high pH), then calibrate using the 7 and 10. See someone in charge on how to use the pH meter if you are unfamiliar with it.

**Always wash the pH electrode thoroughly with dH<sub>2</sub>O prior to placing in any solution: calibration or prepared solution.**

Add the appropriate acid or base to change the pH the desired direction. Remember to use an appropriate acid or base. For example, if the solution is to be used in a reaction where you don't want to introduce sodium, then raise the pH with KOH rather than NaOH.

If you overshoot the pH in any direction, one or two readjustment will be OK. Too many adjustments back and forth will eventually ruin the solution and you should start over making the solution. **Always replace the electrode back into the storage solution when finished.**

## RESINS

### **EPON 812**

Fairly hard epoxy resin. Commonly used for EM. Not as easy to cut with glass knife as Spurr's or maraglass. Supposedly provides a higher contrast to tissues than other resins.

#### **Polymerize at 60 C**

To be measured in 30 ml syringe:

DDSA	7.5 ml
Epon 812	12.1 ml
NMA	6.7 ml
total:	26.3

DMP-30        10 drops

Or can be made in sample jar by weighing:

Embed 812	6.25 g
NMA	4.9 g
DDSA	7.3 g

Mix ingredients well, then  
DMP-30        10 drops while stirring

### **Spurr's resin (Spurr 1969)**

Low viscosity resin. Infiltrates better than Epon – used more with plant tissues and specimens with walls or hard to penetrate outer matrices. **Polymerize at 70 C**

Vinylcyclohexene dioxide (VCD or ERL)	10 g	5 g
Diglycidyl ether of Polypropyleneglycol (DER)	6.3 g	3.15 g
Nonenyl succinic anhydride (NSA)	26 g	23 g
Dimethy aminoethanol (DMAE)	0.4 g	0.2 g (8 drops)

mix well but do not stir vigorously or air bubbles will occur

### **LR WHITE**

Used for Immunolocalization and enzyme protocols. Hydrophobic and difficult to section. Low contrast. Increased contrast may be obtained by UA if the antigenic sites are not disrupted. Tannic acid can be used to increase contrast during fixation. High heat will also disrupt antigenic sites.

Used straight. Can be infiltrated from 70% ethanol.

Polymerizes when exposed to UV light at any temperature. Also polymerizes at 50 C without UV.

Oxygen inhibits polymerization, so use either gelatin capsules with minimal air space or a vacuum oven that has been flushed several times with N<sub>2</sub> gas.