**TEM staining**

Cytochemical localization of peroxisomes

Microbodies - a distinctive organelle, bound by a single membrane, and closely related to the E.R. contain catalase and other hydrogen peroxide (H2O2) producing oxidases. The term “peroxisome” associated with this structure emphasizes its role in hydrogen peroxide (H2O2) metabolism.

3-3’ diaminobenzidine (DAB) in the presence of hydrogen peroxide (H2O2) is used to demonstrate peroxidatic activity of microbodies containing catalase. Under specific conditions, i.e., lower pH, a positive reaction is also observed on the inner and outer membranes of mitochondria. The reaction product is probably osmium black formed by the reaction of oxidized DAB and OsO4.

**Procedure**

1. Fix: 3% Glut. in 0.1M cacodylate buffer, pH 7.4 for 3-6 hours
2. Cut: 25-40µ sections on Sorvall chopper
3. Rinse: in 0.1 M cacodylate buffer/0.22 M sucrose, pH 7.4
4. Incubate: in media at 37OC for 30 - 60 min.
5. Rinse: in 0.1 M cacodylate buffer/o.22 M sucrose for 15 - 20 min.
6. Post fix: 2% OsO4, 0.1 M cacodylate buffer for 2 hours
7. Dehydrate and embed.

**Media for peroxisome localization: (Catalase)**

1. 20 mg 3-3 diaminobenzidine DAB. (tetra-HCL Sigma)
2. 9.8 ml 0.05M propanediol buffer, \*pH 10
3. 0.2 ml 1% hydrogen peroxide (H2O2), Fresh
4. 0.22M sucrose
5. adjust pH 9.0 and filter

Note: The inhibitor for catalase is 0.01M 3 amino 1,2,4, triazole.

\*Propanedial Buffer - 2 amino - 2 methyl 1-1, 3 propandio

\* Novikoff, A.B. and S. Goldfischer, *Visualization of Microbodies for Light and Electron Microscopy*, Journal of Histochemistry and Cytochemistry, [16(8):507 (1969)](http://www.jhc.org/cgi/issue_pdf/frontmatter_pdf/16/8.pdf)

Grid staining for plant and animal tissues

**Things you need**

* 2 medium petri dishes
* 1 self-closing tweezer
* parafilm
* flter paper (cut into small triangles and 1 whole sheet)
* glass pipettes
* 2 washing 50 ml beakers
* timer
* grid staining cover
* NaOH pellets
* 2 % Uranyl acetate
* Lead citrate

**Method**

1. Put a square of parafilm on each top lid of petri dish. Use the back of tweezer to flatten the edges of the parafilm.
2. Put 8-9 NaOH pellets into one of the petri dish.
3. Put droplets of uranyl acetate onto parafilm in another petri dish with a metal cover to keep out the light. Only use uranyl acetate from the middle of the bottle so that precipitates would not be introduced to the grids. Droplets should be far apart on the parafilm.
4. Put one grid facing down into the uranyl acetate droplets (i.e. shiny side up and specimen on the dull side into the uranyl acetate).
5. Set timer going up from zero.
6. Record the exact time each grid is placed in the uranyl acetate stain. For plant tissue, staining time is 30 min; for animal tissue, staining time is 12 min.
7. Put the grid staining metal cover on because uranyl acetate is light sensitive.
8. Put lead citrate into a microcentrifuge tube and centrifuge it for while staining with UA.
9. Fill up 2 washing beaker with d.d.w.
10. Set subsequent grids onto the UA, one minute apart.
11. Once the time is up, pick up grid gently and dip grid into washing beaker. 1st washing beaker for 26 times, 2nd washing for 20 times.
12. Use small triangles of filter paper to dry the grids. Remember to use filter paper to dry the grid between the tines of the tweezer and push the grid gently onto a large piece of filter paper.
13. In the petri dish with NaOH pellets, put droplets of lead citrate.
14. Put grids facing down into the lead citrate droplets as in step 4.
15. Repeat from step 5 and 6. Stain grids in lead citrate for half the time it takes to stain in uranyl acetate (i.e. 15 mins for plant tissue and 6 mins for animal tissue).
16. Refill the 2 washing beakers with d.d.w. after rinsing them 3 times with d.d.w.
17. Put a tiny NaOH pellet into the 1st washing beaker.
18. Repeat step 11 after the staining time is over.
19. Repeat step 12.
20. Put grids back into grid box.

Grid staining for LR White resin tissues

**Things you need**

* 3 medium petri dishes
* 1 self-closing tweezer
* parafilm
* filter paper (cut into small triangles and 1 whole sheet)
* glass pipettes
* 2 washing 50 ml beakers
* timer
* grid staining cover
* NaOH pellets
* 4 % Uranyl acetate
* Lead citrate
* 2% Aq OsO4

**Method**

1. Put a square of parafilm on each top lid of petri dish. Use the back of tweezer to flatten the edges of the parafilm.
2. Put the grids onto the parafilm in order.
3. In the fume hood, put four drops of 2% osmium tetroxide into the petri dish at the sides of the parafilm. Leave for 30 minutes.
4. Meanwhile set up the UA and lead. Put 8-9 NaOH pellets into one of the petri dish.
5. Put droplets of uranyl acetate onto parafilm in another petri dish with a dense cover to keep out the light. Only use uranyl acetate from the middle of the bottle so that precipitates are not introduced to the grids. Droplets should be far apart on the parafilm.
6. Put one grid facing down into the uranyl acetate droplets (i.e. shiny side up and specimen on the dull side into the uranyl acetate).
7. Set timer going up from zero.
8. Record the exact time each grid is placed in the uranyl acetate stain.
9. Put the dark cover over the petri dish because uranyl acetate is light sensitive.
10. Put lead citrate into a microcentrifuge tube and centrifuge it for while staining with UA.
11. Fill up 2 washing beaker with d.d.w.
12. Set subsequent grids onto the UA, one minute apart.
13. Once the time is up, 5 mins each, pick up grid gently and dip grid into washing beaker. 1st washing beaker for 26 times, 2nd washing for 20 times.
14. Use small triangles of filter paper to dry the grids. Remember to use filter paper to dry the grid between the tines of the tweezers and push the grid gently onto a large piece of filter paper.
15. In the petri dish with NaOH pellets, put droplets of lead citrate.
16. Refill the 2 washing beakers with d.d.w. after rinsing them 3 times with d.d.w.
17. Put grids facing down into the lead citrate droplets as in step 6.
18. Repeat from step 6 to 9. Stain grids in lead citrate for 2 minutes.
19. Repeat steps 13, 14 after the staining time is over.
20. Put grids back into grid box.

Reynold's lead citrate

**Prepare CO2-free water**

1. Put approximately 200 mls ddw in conical flask in microwave to boil for 5 minutes. Cover and allow to cool.

or

take approximately 200 mls ddw as it comes off the still and put into the vacuum oven to degass.

**Prepare 1N CO2-free sodium hydroxide**

1. Weigh 1 g sodium hydroxide.
2. Make up to 25 mls with CO2-free water

**Lead citrate**

1. Weight 1.33 g lead acetate and 1.76 g sodium citrate on small weighing boats.
2. Wash into 50 ml volumetric flask with 30 mls CO2-free water.
3. Shake vigorously for 1 minute then intermittently for 30 minutes. Lead turns into lead citrate. It should be a milky white solution.
4. Add 8 mls 1N sodium hydroxide (carbonate free). This would change the solution to colourless.
5. Make up to 50 mls with CO2-free water.

Shelf life 2 months. Discard previously if slightly milky. Sodium citrate and sodium hydroxide should be less than 6 months opened (Hyat).

Preparing 2% aqueous uranyl acetate

* Mix 1 g uranyl acetate in 50 ml C02-free water

Keep in dark bottle in dark.

Shelf life is 1 week.

Negative staining - for studying subcellular components

**Stain materials**

* 1-2% uranyl acetate (fine-grained, fixative-preserves viruses, radioactive, precipitates at physiological pH and in presence of many salts)
* 1-2% Na or K phosphotungstate (not a fixative, destroys some viruses, not radioactive, can be used at physiological pH, more forgiving with salts and biological medis)
* 1-2% ammonium molybdate, (fine grained)
* Others: Gold thioglucose, lanthanum acetate, lithium tungstate, methylamine tungstate, sodium silicotungstate, sodium zirconium glycollate, tungstoborate, uranyl acetate aluminium formate, uranyl formate,uranyl oxalate, uranyl sulfate.

**Support films**

* Most used:
	+ Formvar/carbon (mechanically strong-good for virus specimen, must be cast on glass)
	+ Collodion (Parlodion)/carbon (not as stable, easy to cast)
	+ Carbon (a must for high resolution work, easy to break - not good for heavy samples, e.g. stool).
* Others:
	+ Butvar
	+ Graphite oxide
	+ Quartz
	+ Pioloform

**Specimen deposition**

* Direct: Place grid onto specimen drop, drain, place grid into stain, drain
* Spray: Atomize specimen onto grid (frequently with glycerol or sugar), stain; used with proteins/crystals; DO NOT USE WITH VIRUSES/OTHER PATHOGENIC ORGANISMS!
* Carbon Film: Add specimen to mica, coat with carbon, float off onto negative stain or onto water and then negatively stain.

**Treatments to facilitate sample spreading**

* Grid Treatment:
	+ Glow discharge, 10-60 seconds
	+ UV light exposure, 30 min (collodion)
	+ Bacitracin, 50 ml/ml 20 sec
	+ Cytochrome c, 1 ml/ml, 30 sec
	+ Poly-L-lysine, 1000 ml/ml, 10 sec-10 min
	+ Alcian blue 1%, 5 min, for nucleic acids: 0.001% in 0.003% HOAc
	+ Ethidium bromide, 30 mg/ml, 15 min
	+ Octyl glucoside, 0.01-0.1 mM
	+ Polyethylene glycol, 0.01-0.05%

**Specimen or stain Additives**

* Bovine serum albmin, 0.005-0.05%
* Bacitracin, 50 mg/ml
* 1-octadecanol
* Glycerol, up to 30%
* Trehalose, 1%
* Glucose
* Sucrose
* Benzyldimethyl ammonium chloride, 0.05 g/ml, several hrs

**Examples**

* Routine:
	+ Subcellular components: membranes, mitochondria, ribosomes. Proteins/cystals/filaments, bacteria, pili, flagella, cell walls
	+ Virology: used to ID viruses in liquid specimens
* IEM:
	+ Virus identificatin/serotyping (clumping, coating, Au-antibody labeling)
	+ Membrane & subcellular component Au-antobody
	+ Other probe labeling
* Computer enhancement:
	+ Rotation
	+ Modeling

**Problems**

* Uniform spreading: Grid treatment (glow discharge, 10-60 sec, preferred treatment UV light exposure, 30 min, for collodion; bacitracin 50 mg.ml; 30 sec; cytochrome c, 1 ml/ml, 30 sec; poly-L-lysine 100 1/ml, 30 sec-10 min; Alcian blue, 1%, 5 min, for nucleic acids 0.001-0.003% in HOAc; ethidium bromide 30 mg/ml, 15 min. Specimen/stain additives (bovine serum albumin, 0.005-0.05%; bacitracin 50 g/ml; 1-octadecanol; benzyl dimethyl ammonium chloride, 0.05 mg/ml); sugars-trehalose, glucose, sucrose). Bubbles: confuse virus ID. Hayat & Miller, 1990; Miller, 1986.

**A negative staining technique by Dr. Frank Mayer**

1. Evaporate about 20-40Å carbon on freshly cleaved mica.
	* Coating should be done rapidly with short pump down time.
	* Very high vacuum is not necessary.
2. Cut mica into small squares, i.e. 2-3 mm.
3. Pick up small squares with forceps. Touch to drop of molecular material.
4. Approach from side

Allow carbon and mica to remain attached at forceps end.

1. Use drops

Protein wash, stain, or Protein Fix Wash Stain

1. Float carbon film with specimens on stains
2. Pick up carbon from stain with grid from top. Do not submerge grid in UR.

**Notes**

* Protein – dilute to 10-50µg/ml

mw about 300,000/conc. 30µg/ml = 15-20 sec. dip time larger size – bacteria 1 min. dip time

* Remember, small particles will tend to predominate; therefore, if coating, be sure to take this into consideration.
* Spray particles. If particles are sprayed with nebulizer, use 20-50% glycerol.
* Stain. 4% uranyl acetate in triple distilled H2O – pH will be ca. 4.9 – 5.2.
* Microscope should be well stigmated and never focus on specimens for long periods of time. Crystalize uranyl acetate.

Staining of thick sections

Thick sections are cut and stained to help one orient the block, to roughly judge the quality of the fixation, to sample the tissue until the specific area of interest is observed.

Elaborate procedures and techniques have been worked out, but the following methods are designed for prompt observation of your material.

**Stains most commonly used:**

* 1% methylene blue in 1% sodium borate and % azure II in distilled water. Working solution, equal parts of both stains. If this is found to stain too intensely, dilute the working solution with distilled water.
* Paragon stain
* 1% toluidine blue in 1% sodium borate
* 1% para-phenylenediamine in distilled water (useful only for phase optics)

**General method for all of the above stains**

1. Transfer 0.25-1.0 um thick sections to a small drop of water on a clean glass slide. Transfer of the sections from the glass knife to the slide can be done in several ways using a fine camel’s hair brush, a wooden applicator stick, a hypodermic needle, a hair loop, a transfer loop, a single hair or any means you can think of that works.
2. Place the slide on a warming tray or a hot plate and allow the water to dry. The low setting on the hot plate will be adequate.
3. Add a few drops of the staining solution. Wait until the edges of the staining solution just barely begin to dry.
4. Remove the slide from the hot plate and rinse well in distilled water and blot off any excess water.
5. Return the slide to the hot plate to dry.
6. The slide can then be mounted with a coverslip and permount or the sections can be viewed directly with or without immersion oil.