

Laboratory for Advanced Electron and Light Optical Methods

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Calcium Staining Methods Utilizing Pyroantimonate

Calcium Staining (General Concepts)

Calcium in tissues and cells may be found as relatively insoluble products (calcium oxalate crystals in kidneys, hydroxyapatite crystals in bone) or may be extremely soluble. In the former case, calcium can be demonstrated by primary fixation followed by post-fixation in osmium containing 2-4% potassium pyroantimonate with good results. In the latter case, the calcium is solubilized during primary fixation and subsequent washing so that an osmium-pyroantimonate staining solution will no longer reveal calcium in the cells or tissue.

The pyroantimonate staining method has the potential to localize sodium, potassium, calcium and magnesium, but the general consensus (Wick and Hepler, 1982) is that the specificity of the pyroantimonate staining methods utilized today is sufficient to conclude that a positive staining reaction demonstrates the presence of calcium. If any doubt remains, electron dispersive spectroscopy (EDS), electron energy loss spectroscopy (EELS) or an energy filtration system as offered with the Zeiss EM-902 can be used to confirm the presence of calcium.

Wick and Hepler (1982) suggest, however, that the pyroantimonate is actually more sensitive to the presence of calcium than typical microanalytical techniques.

Reference:

Wick, S.M. and P.K. Hepler. 1982. Selective localization of intracellular Ca^{2+} with potassium antimonate. *J. Cytochem. Histochem.* 30:1190-1204.

Pre-Fixation Calcium Staining in Muscle Tissue

1. Applications and Objectives: This technique is utilized to demonstrate soluble calcium in muscle (Oberc and Engel, 1977). Tissues are initially placed into the staining solution containing potassium pyroantimonate (KPA), which interacts with and stabilizes calcium. Unfortunately, it is not actually a fixative for any other cellular constituents and few tissues and cells other than muscle can be expected to be well-preserved following the procedure. Muscle is highly proteinaceous and highly resistant to autolysis. For this reason, it is possible to incubate muscle in the pyroantimonate solution for hours before aldehyde fixation and still have good ultrastructural preservation (as well as excellent calcium staining). Unfortunately, the stabilizing mixture of formaldehyde and pyroantimonate used after the primary pyroantimonate incubation does not appear to produce adequate calcium stabilization and localization if utilized as the first incubation medium.

2. Materials Needed:

2% KPA:

add 2 g potassium pyroantimonate (KPA) to distilled H₂O (previously heated until steaming) while stirring; cool and adjust pH to 9.4 with 0.1N KOH

KPA/Formaldehyde:

add 7 parts KPA mixture (above) to 1 part 37% Fisher formaldehyde; adjust pH to 9.4 if needed

10 mM EGTA:

0.038 g EGTA/10 ml distilled H₂O, adjust pH to 9.4 with 0.1 N KOH (the EGTA will not go into solution until the pH is raised; do this slowly so as not to overshoot the pH desired)

2% aqueous osmium

3. Procedure:

3.1. Staining Procedure:

3.1.1. Gently stretch muscle and make incision sufficient to insert sterilized hair clip. After a hairclip is attached to the muscle at a right angle to the fiber orientation, use a scalpel to cut muscle just outside the hairclip jaws. Place the clamped muscle into the KPA staining solution at room temperature.

- 3.1.2. After 24-48 hr at room temperature, place tissue into KPA:formaldehyde mixture and leave at room temperature for an additional 24-48 hours.
- 3.1.3. Rinse tissue in distilled H₂O and post-fix in aqueous 2% osmium for 1 hr at room temperature.
- 3.1.4. Remove the tissue from the hairclip and cut it into mm-thin strips so that the longitudinal orientation can still be seen.
- 3.1.5. Dehydrate, embed, and polymerize tissue samples as described for routine TEM sample processing.
- 3.1.6. Post-stain sections only with uranyl acetate to provide contrast.

3.2. Control:

Incubate tissue 4-16 hr in 10 mM EGTA and then rinse thoroughly in distilled H₂O prior to KPA incubation (this should remove calcium from the tissue and result in a negative result for KPA staining).

4. Results Expected: Calcium should be demonstrated by the presence of electron-dense granular deposits in sarcoplasmic reticulum, mitochondrial matrices, and cytoplasmic domains.

5. Cautionary Statements: Do not post-stain sections with both uranyl acetate and lead citrate, because the stain product will be more difficult to see against the overall electron density of the cellular components. Uranyl acetate alone produces enough contrast in the sections to focus and produce good photographs.

The EGTA control does not seem to work consistently. Since the purpose of the control is to demonstrate that the material stained with KPA can be chelated and removed from the tissue by EGTA (thus suggesting its calcium identity), a more conclusive method would be to utilize microanalytical techniques (alluded to above) on a sub-sample to characterize the deposits.

The 4% KPA solution is supersaturated, so there may be some residual precipitate that refuses to go into solution. If the pH is allowed to drop below 7.6, the pyroantimonate will precipitate from the solution. When making pH adjustments, add reagents slowly with stirring to prevent precipitation. If visible precipitation occurs, discard the mixture and start again.

To raise pH, use KOH, and to lower pH, use acetic acid (not HCl, which could complex with Ca to produce calcium chloride).

References:

Oberc, M.A. and W.K. Engel. 1977. Ultrastructural localization of calcium in normal and abnormal skeletal muscle. *Lab. Invest.* 36:566-577.

Post-Fixation Calcium Staining with Pyroantimonate

1. Applications and Objectives: Pyroantimonate may be used to demonstrate largely insoluble or partially insoluble calcium in a variety of tissues and cells. Potassium pyroantimonate is mixed with osmium so that post-staining and calcium staining take place simultaneously. Calcium not solubilized during primary fixation and subsequent washing with buffers will be revealed.

2. Materials Needed:

4% potassium pyroantimonate:
add 4 g potassium pyroantimonate to 100 ml of steaming
distilled H₂O with stirring; adjust pH to 7.6 with
0.1 N KOH

4% aqueous osmium

3. Procedure:

- 3.1. Fix samples as per routine TEM processing instructions.
- 3.2. Rinse in distilled H₂O (3 X, 10 min each)
- 3.3. Incubate in pyroantimonate/osmium mixture prepared by mixing equal volumes of the 4% pyroantimonate solution and 4% osmium solution just before adding tissues/cells) for 1 hr at room temperature.
- 3.4. Rinse in distilled water 3 X (5 min each).
- 3.5. Dehydrate and embed as per routine TEM procedures.

4. Results Expected: As with the technique for soluble calcium staining, calcium deposits will be demonstrated as electron-dense, granular deposits. Large insoluble crystals containing calcium such as those of calcium oxalate are typically difficult to section because they damage glass and diamond knife edges and the crystals are often ripped from the plastic during sectioning, leaving large holes. After staining with pyroantimonate, the electron-dense product is much easier to section, leaving far fewer holes in the sections.

5. Cautionary Statements: As a control, incubation in EGTA prepared as explained for calcium staining in muscle is recommended. When mixing the supersaturated solution of potassium pyroantimonate with osmium, mix gradually to avoid precipitation of the

pyroantimonate. Maintain pH at 7.6 or above to prevent precipitation and discard the mixture if any precipitate forms.

Reference:

Dykstra, M.J., and R.L. Hackett. 1979. Ultrastructural events in early calcium oxalate crystal formation in rats. *Kid. Internat.* 15:640.