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Practical Solutions to Frequent Problems Encountered in Thin Sections Electron Microscopy

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Abstract: Based on many years of practical experience with techniques for thin section electron microscopy, we propose several minor procedural modifications that have given us satisfactory results. The purpose of this article is to clarify the advantages and disadvantages of procedural variants in relation to specific experimental requirements. The final aims are good structural preservation, high contrast, and a minimum effort.

Introduction

The most common fixing and embedding techniques for preparing cells/tissues prior to thin section TEM involve an initial fixation by a cross linking agent (glutaraldehyde with or without addition of paraformaldehyde); post-fixation and contrast enhancement by osmium tetroxide (OsO_4), with possible addition of other agents; a second "en-bloc staining" usually with uranyl acetate and a final dehydration, resin infiltration and embedding. Extensive washes between the treatments eliminate problems with incompatibility of solutions. Further contrast is added by "staining" the thin sections (for example, see [1]). An excellent generic guide to embedding procedures is found in Google under "Standard fixation and embedding protocol for resin section TEM" [2]. Here, we deal with often-encountered problems and/or requirements during these procedures and offer useful observations and some easy, practical solutions.

Saving precious solutions. All procedures can be performed with the cells/tissue in 1.5 ml Eppendorf centrifuge tubes that are resistant to all solvents and that seal well, avoiding humidity contamination from the air. Reagents 100% ethanol and acetone are kept in disposable 10 ml syringes from which small volumes can be readily delivered through a long needle. Both approaches result in considerable savings of time and precious fluids. We thank Dr. Simona Boncompagni (CeSi, Chieti, Italy) for these suggestions.

Avoiding small dense deposits. Most electron microscopists have, at some point, found disfiguring electron dense artifactual deposits in images of thin sections that are not due to section staining procedures. Osmium tetroxide (OsO_4) (for post fixation) and uranyl acetate (*en bloc* stain) are the obvious culprits for contamination within the block because they contain the two high-atomic-number atoms involved in the procedures. In the presence of glutaraldehyde OsO_4 is reduced (and thus it precipitates out of solution), but problems are avoided by extensive rinsing between primary and secondary fixations. A preventive drop of H_2O_2 in the glutaraldehyde fixative helps in avoiding inappropriate reduction of the OsO_4 [3], but usually it is not necessary if the stock OsO_4 solution is stable. If small bottles are repeatedly used for preparing/storing the stock OsO_4 solution, it is important to clean of the bottles with H_2O_2 between uses in order to oxidize and solubilize any reduced osmium adhering to the glass. With these cautions it is relatively easy to obtain a clean sample if osmium is the only heavy metal used *en bloc*.

Staining "dots."

Additional *en bloc* staining with uranyl acetate greatly enhances the contrast of the tissue to the point that single

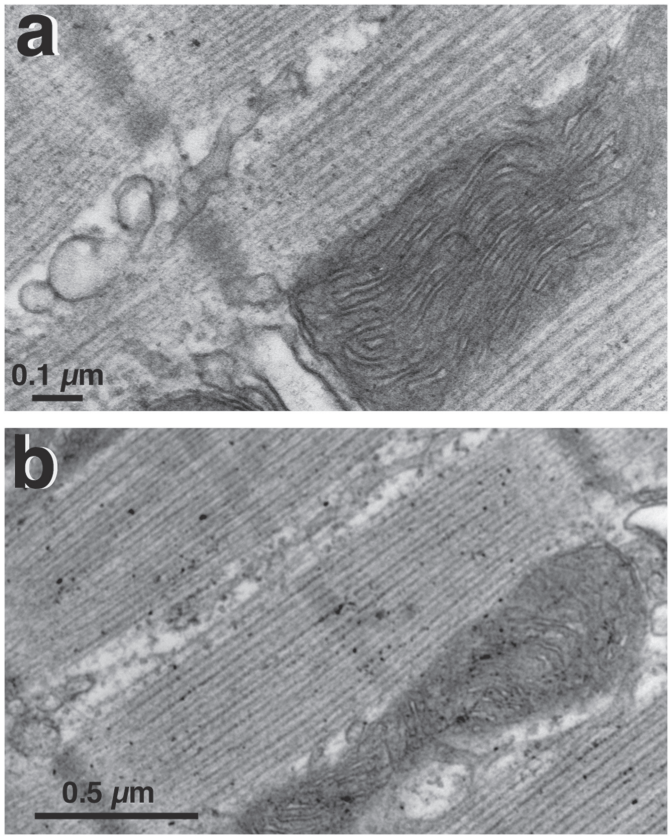

Figure 1: Longitudinal sections of isolated cardiomyocytes from the same embedded block showing small uranyl acetate precipitations. (a) Few small dots are present. (b) More frequent dots in a different cell.

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staining of the sections with lead salts is sufficient to obtain excellent images. However, this procedure is the main culprit for the relatively frequent and ubiquitous presence of small "dots" often affecting only part of an otherwise perfect specimen. Figure 1 shows two examples from the same embedding illustrating variability of the effect even within the same bloc. Small dots frequently occur even after extensive water washes between OsO_4 and uranyl exposure and have been a continuous source of frustration. We have found a satisfactory solution to the problem of avoiding dots by considering that uranium precipitation is not due to an incompatibility of osmium and uranium, but rather to the fact that uranyl acetate is not soluble at the neutral pH of the heavily buffered glutaraldehyde and osmium fixatives. Even extensive water washes after the tissue is exposed to buffered OsO_4 may not reduce the pH to the required acidity, particularly within the depth of a slightly bigger tissue block. This problem can be solved by stabilizing the overall pH to an acidic level after OsO_4 post fixation by using 2–3 10-minute washes in 0.1 M acetate buffer pH 4.2 (diluted from a 0.2 M buffer; available from Electron Microscopy Sciences). An alternative solution is to acidify the aqueous uranyl acetate solution to a low pH before use or even better to dissolve the uranyl acetate in a low-pH buffer, such as the acetate buffer indicated above.

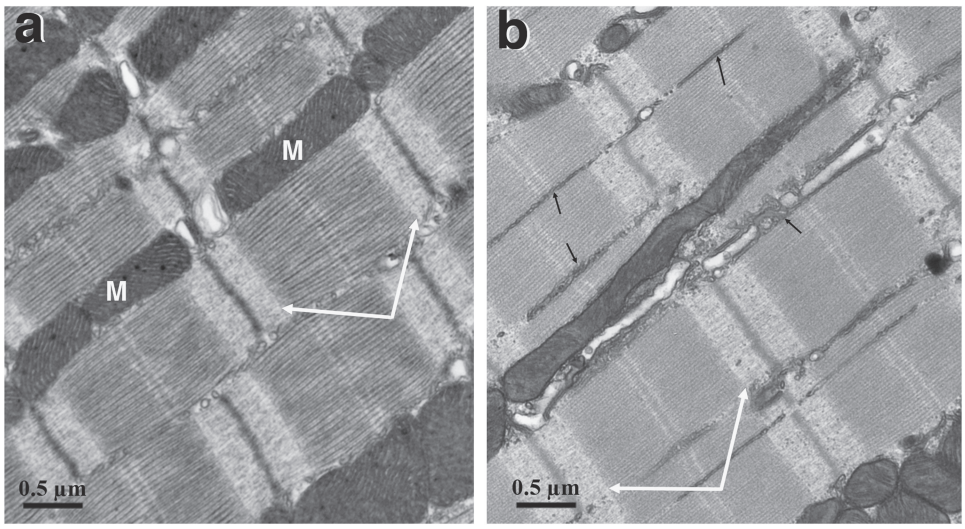

Figure 2: Mouse cardiac muscle stained *en bloc* either with uranyl acetate (a) or OsO_4-$K_3Fe(CN)_6$ (b). (a) Myofilaments, particularly the A band (indicated by two white arrows) and the matrix of mitochondria (M) have a high contrast. (b) The use of $K_3Fe(CN)_6$ highlights mitochondria (M) and sarcoplasmic reticulum (small arrows) membranes, while protein elements and myofilaments (between white arrows) have a lower contrast.

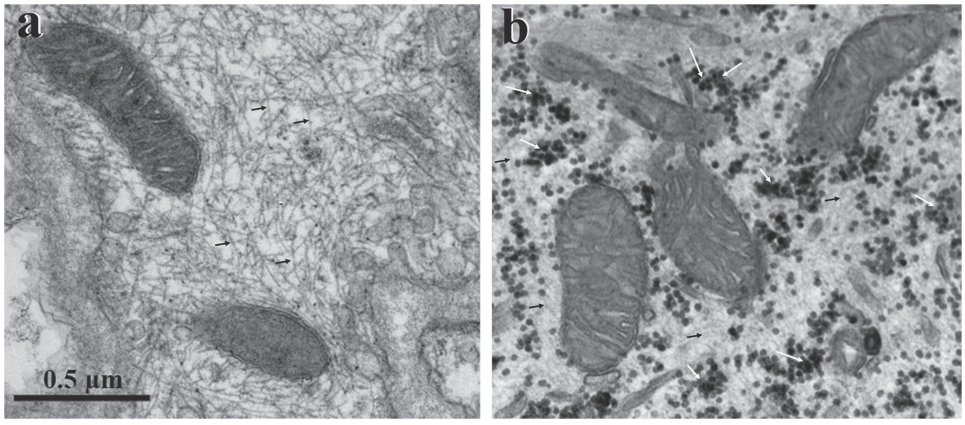

Figure 3: Sinoatrial node cells from rabbit heart stained *en bloc* either with uranyl acetate (a) or OsO_4-$K_3Fe(CN)_6$ (b). (a) Numerous intermediate filaments (black arrows) are clearly visible, but glycogen granules are apparently absent. (b) Intermediate filaments (black arrows) are hardly detected, but clusters of glycogen granules (white arrows) fill the "empty spaces" observed in (a).

"Staining" without uranyl acetate. Some groups opt out of using uranyl acetate altogether and instead add potassium ferricyanide to the osmium solution: this reduces OsO_4 and stains structures with metallic osmium, but without producing "dots" [4–6]. It is, however, not often appreciated that uranyl acetate and potassium ferricyanide have different properties. The former strongly enhances the contrast of proteins, while membrane lipids are less well contrasted, and vice versa for the latter. Figure 2 shows examples of cardiac myocytes stained *en bloc* using the two different methods. Uranyl acetate (Figure 2a) enhances the protein elements, so that myofibrils are in strong contrast and the mitochondria matrix is dark but membranes delimiting the sarcoplasmic reticulum (SR) and mitochondria are less strikingly visible. Potassium ferricyanide $K_3Fe(CN)_6$ added to the OsO_4 solution enhances membrane contrast, so the outlines of mitochondria and SR elements are clearly visible, but the protein filaments in myofibrils are pale and not well defined (Figure 2b). An additional difference between the two approaches arises from the fact that after *en bloc* staining with uranyl acetate, glycogen granules are not visible (Figure 3a), while in the K ferrocyanide treatment they are in good contrast (Figure 3b). Figure 3 also shows that intermediate filaments are well visible after uranyl acetate staining (Figure 3a), but hardly detectable after K ferrocyanide (Figure 3b) even in the same type of cell. Other protein-based cell organelles, for example, microtubules, are obviously better visualized after uranyl staining. Therefore, selection of the alternate methods should be based on experimental requirements rather than being imposed by the need to avoid dots.

Handling single cells, particularly elongated ones. Two approaches can be used for fixing suspensions of isolated cells; both are best implemented using eppendorf tubes through the whole procedure: (A) Pellet the cells hard and then exchange the supernatant for the fixative (for example, buffered glutaraldehyde) without disturbing the pellet, and (B) lightly centrifuge the cells and re-suspend them in the fixative.

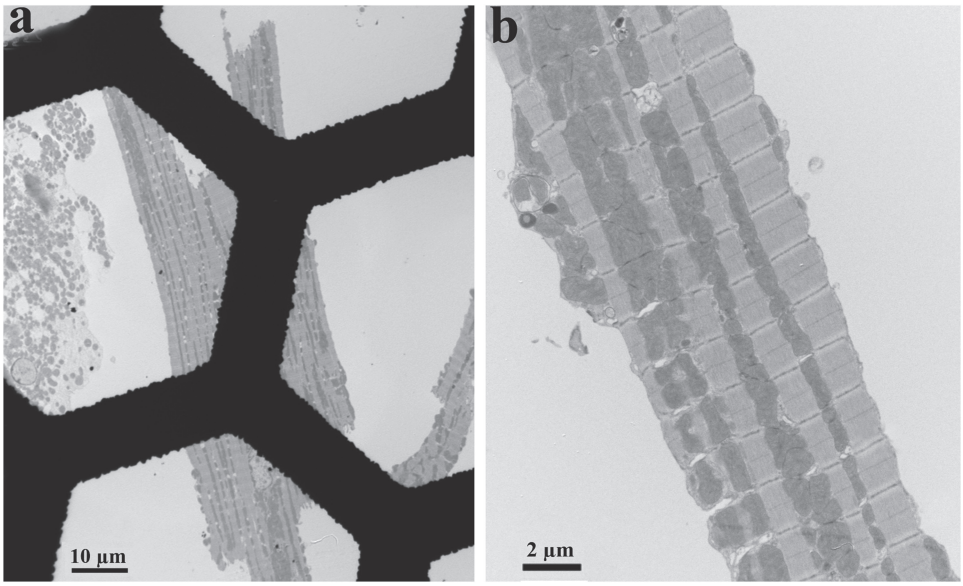

Figure 4: Isolated cardiac myocytes, illustrated at low magnification (a) and higher magnification (b) are well oriented longitudinally following the protocol described in the text. The 400-mesh thin bar grid allows both good support and good view of the cells, without need of a supporting either formvar or collodion membrane.

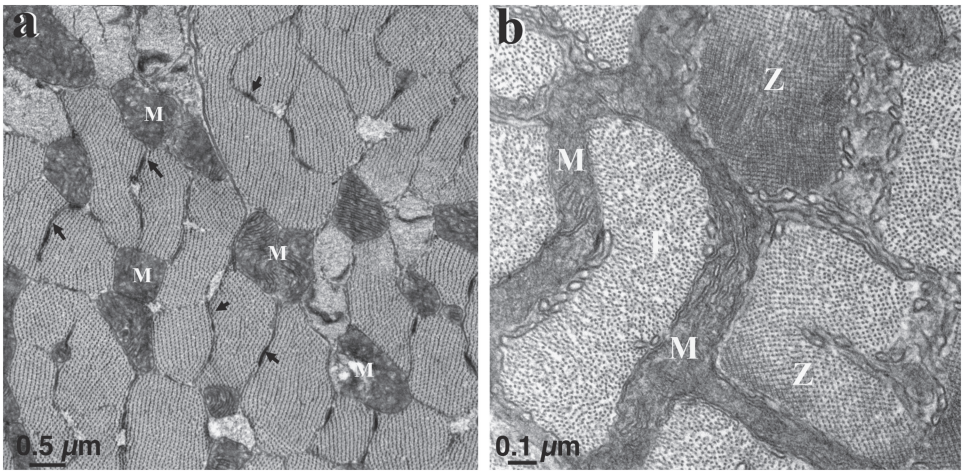

Figure 5: (a) Cross section of two muscle fibers from the leg of a primitive arthropod, commonly called "daddy long legs" at low magnification. The dotted regions are cross sections of the myofibrils, and large and small dots represent sections of thick and thin filaments. Mitochondria (M) and elements of the sarcoplasmic reticulum (small arrows) separate the myofibrils. (b) High magnification illustrating the cross section of a mouse EDL fiber at the level of the Z line (Z) and I bands (I). Elongated mitochondria (M) are present between the myofibrils.

Following fixation (A), the pellet behaves essentially as a piece of tissue. No centrifugation is needed between solution changes. The solid pellet can be taken out after fixation and used for freeze fracture [7] or postfixed and dehydrated for embedding while kept in the original tubes through the whole procedure. One difficulty is met at the stage when the pellets are infiltrated in the 1:1 Epon/acetone mixture. Acetone must be completely eliminated to avoid polymerization problems. Removal of the pellets from the bottom of the tubes would result in their disintegration. The best policy is to decrease the amount of 1:1 mixture on top of the pellet to a minimum and let the acetone evaporate overnight or longer under vacuum, with the tubes open. A very small amount of fresh Epon can be gently introduced and layered over the pellet and then polymerized. After polymerization, the tube can be filled with Epon for later sectioning, or the pellet can be re-embedded in a flat mold. This method is appropriate for a cell with no specific orientation.

Method (B) requires centrifugation at each solution change, because the cells float at each step, but offers the advantage of allowing orientation of the cells in the last step. After some infiltration time in the 1:1 Epon/acetone (1/2 to 1 hr) the cells are centrifuged into a light pellet. Supernatant is discarded, and droplets of the dark cell-rich pellet are pipetted into the surface of a piece of aclar plastic (available at Electron Microscopy Sciences). As the acetone evaporates the cells tend to settle flat on the aclar surface. This is aided by incubation at room temperature for some hours before polymerizing in the embedding oven. In the meantime, small gelatin capsules are filled with Epon, which is then polymerized. After cells and capsules are polymerized, the Epon blocks in the capsules are glued to the cell-rich spots. After a second polymerization aclar is detached, and the cells at the surface of the bloc are ready for immediate sectioning. In the case of elongated cells such as muscle (Figure 4), a perfect orientation is obtained. The method is also useful for obtaining grazing views of flat (for example, epithelial) cells.

Mix Epon in relatively large batches. Epon (as well as Araldite) mixture ready for embedding, including the catalyst, can be prepared in fairly large batches and then stored for use in 10 ml syringes covered by a plastic sleeve, at -20 ºC for several months. Thaw only the amount needed just before use because the resin polymerizes fairly fast at room temperature. Be careful however not to open the sleeve around the syringe until it is equilibrated to room temperature to avoid water condensation. Note: in contrast to common practice, there is no need to use propylene oxide after dehydration in acetone. Acetone is far less dangerous than propylene oxide, and it is an excellent solvent for Epon. If a problem is encountered, infiltration can be encouraged by a brief period at low pressure in a vacuum chamber.

Practical approach to collecting thin sections. Some laboratories/EM facilities collect the sections on grids with a single large slot or with very large spacings between the grid bars (for example, 100-mesh grids). This allows view of the entire section (or most of it) without interruptions, but the advantage is obtained at the expense of having to cover the openings with

a membrane, which requires considerable time and money and also results in a clear reduction of image contrast.

Alternatively, all except the thinnest sections are adequately supported on "naked" grids with sufficiently small openings, and this maintains higher image contrast. The 300– to 400–mesh "thin bar" grids (available at Electron Microscopy Sciences) offer good support and extended views (Figure 4a). The grids can be used directly from the small box in which they are delivered, without need to clean off the minor amount of oxidation that accrues with time. The problem of section adhesion to the slightly oxidized grid is solved by incubating the grid-attached sections for 10–15 minutes at ~65 ºC before using them for staining. Sections settle on the grid very tightly during the heat exposure and do not detach during staining.

Obtaining the best lead staining of sections. T. Sato published in 1968 [8] a recipe for a lead stain that is extremely stable (several months if appropriately prepared and stored) and extremely effective because it stabilizes an unusually high concentration of lead ions. However, Sato's stain has apparently faded from the collective minds of electron microscopists. The original reference is not easily obtainable, but a slightly revised method was published in 1986 [9]. This new version offers a reliable guide to an excellent, durable stain solution. The following suggestions further enhance the stability and usefulness of Sato's stain: (1) Boil the water, (2) "calcinate" the lead citrate by heating it dry in a small aluminum dish until it turns light brown, (3) use only freshly diluted 4% NaOH, (4) mix in a volumetric flask with a narrow neck, (5) store solution in 10 ml syringes avoiding air bubbles and (6) attach a millipore filter to the syringe in use.

Figure 5 illustrates the level of contrast that can be routinely achieved in striated muscles by a combination of *en bloc* staining with uranyl acetate and staining of the sections with "Sato" lead.

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