Electron microscopic double staining method with hematoxylin followed by lead citrate

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Method Article

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Abstract
In this study, a new staining method for electron microscopic specimens is used. The method is based on the double staining of hematoxylin, which is widely used as a staining agent for light microscopy, with lead solution, and is expected to replace the conventional double staining method of uranyl acetate and lead solution in terms of safety, cost, and ease of handling.

Introduction
The electron microscopic staining technique for biological specimens using uranyl acetate (UA)\(^1\) has been applied in electron microscopy facilities worldwide due to its simplicity and optimal staining results. In recent years, however, international regulations on the use, acquisition, storage, and disposal of uranium compounds have become increasingly strict because uranium is used in the production of nuclear weapons\(^2\). In this context, alternative staining methods have long been needed in the field of biomedical electron microscopy. Although several have been proposed, none has been an effective alternative.

To develop a safe and easy-to-handle alternative staining method to UA for ultrathin sectioning in electron microscopy, we examined various commercially available dyes for light microscopy\(^3\). As a result, we found that double staining with Mayer’s hematoxylin (MH) and Reynold’s lead citrate solution\(^4\) (RPb) (Figure. 1), which is commonly used as a staining agent in light microscopy, exhibited staining properties equivalent to those of conventional electron microscopic double staining with UA and RPb in various tissues and cells.

Reagents
Glutaraldehyde, Grade 1, 25% aqueous solution (Merck, G5882)
Osmium tetroxide, 4% aqueous solution (Taab Laboratory Equipment, 0014)
Phosphate buffer tablets, pH 7.2 (Merck, 109468)
Ethanol (≥99.5%)
Propylene oxide (≥99%)
Epoxy resin (Taab Laboratory Equipment, T024)
Mayer’s hematoxylin (Merck, MHS1, 16, 32 or 80)
Lead nitrate (Merck, 467790)
Sodium citrate (Merck, W302600)
**Equipment**

Millipore filter (Merck, SLHV033NB, 0.45 μm)

Embedding mold

Embedding oven

Ultramicrotome

Transmission electron microscope (TEM)

Field-emission scanning electron microscope attached to a backscatter electron detector (FE-SEM)

Thin section supporting grid for TEM

Semi-thin section supporting glass slide or silicon substrate for FE-SEM

Osmium plasma coater

**Procedure**

Figure 1 shows the flow of the sample preparation procedure for TEM and FE-SEM observations.

- **Fixation and embedding**

1. Pre-fixation: 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h at 4 °C.

2. Rinse: 0.1 M phosphate buffer (pH 7.2) for 10 min three times at 4 °C.

3. Post-fixation: 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4 °C.

4. Dehydration: ascending ethanol series, 60%, 70%, and 80% at 4 °C, followed by 90% and 95% at room temperature (RT) for 10 min each, and 100% at RT 2 times for 10 min each.

5. Substitution: propylene oxide for 10 min, two times at RT.

6. Substitution: 1:1 Epon812 epoxy resin and propylene oxide overnight at RT

7. Embedding: Epon812 for 3 h at RT, and then Epon812 for 2 days at 60 °C.

8. Thin sections: 80-nm thin sections on the grids for TEM and 200- to 500-nm semi-thin sections on the slide glasses or silicon substrate for FE-SEM, prepared with an ultramicrotome.
- **EM staining**

1. Pretreatment: MH was filtered through a 0.45-μm Millipore filter before use.

2. Pre-staining: Samples were stained with MH for 10 min for TEM and 20 min for FE-SEM at RT\(^3\).


4. Post-staining: RPb solution\(^4\) for 5 min at RT.

5. Wash: Double-distilled water for 1–3 min.

6. Osmium conductivity coating for FE-SEM

- **Observations**

The samples were observed and recorded using a TEM or an FE-SEM attached to a backscatter electron detector.

**Troubleshooting**

Although typical MH staining times are between 5 and 10 min for TEM and 20 min for FE-SEM, the optimal times varied depending on the sample. Contamination on the sections increased after 15 and 20 min of staining. Contamination tended to increase as the staining time increased.

To prevent contamination formation on TEM and SEM samples, (1) use freshly prepared solutions, (2) after staining with MH, allow samples to thoroughly wash with double-distilled water and then dry before staining with RPb, and (3) ensure RPb is at the proper pH. If a precipitate is present before lead citrate staining, then it could be caused by a contaminated buffer, fixative, or MH.

**Time Taken**

Fixation, dehydration, and embedding: 3–4 days

Sections: 30 min per sample block

Staining for TEM: 15 min per grid

Staining for FE-SEM: 25 min per slide

Observation and image recording: 30 min to 1 h per sample

Total time: 5–7 days
Anticipated Results

Following this protocol, various tissues and cell organelles, such as nuclear chromatin, plasma membrane structures, ribosomes, glycogen, lipid droplets, cell adhesion apparatus, and cytoskeletal systems, were stained with high contrast using the MH-RPb staining method. Plasma membrane staining in all samples was also satisfactory (Figure 2a - e). Backscattered electron images of 200 nm semi-thin sections of mouse kidney observed by a FE-SEM also showed that the MH-RPb staining techniques provided a wide-area and high-quality image of the renal cortical tubules and the renal glomerulus (Figure 2e).

References


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Figures
Figure 1

Preparation procedure of samples for electron microscopic observation.
Figure 2

Electron microscope images of various types of cells and tissues stained with Mayer’s hematoxylin and then with lead solution. Scale bars: (a and b) 3 µm; (c and d) 300 nm; (e) 10 µm.