Preparation of biological specimens for Electron Microscopy
Introduction

There are several methods to prepare biological specimens depending on the nature of the specimen and the purpose of the study. This presentation introduces some commonly used methods. Please, be careful in handling chemicals used in these methods as some are toxic.
## I–1 Preparing a biological sample for ultramicrotomy

Tools and chemicals listed here are necessary for preparation.

<table>
<thead>
<tr>
<th>(Procedure)</th>
<th>(Instruments/Chemicals)</th>
</tr>
</thead>
</table>
| 1. Extraction of tissue | • Tweezers  
|                    | • Glass bottles  
|                    | • Razor                                                                 |
| 2. Fixation       | • Glutaraldehyde  
|                    | • Osmium tetroxide  
|                    | • Phosphate buffer                                                                 |
| 3. Dehydration    | • Ethanol  
|                    | • Propylene oxide                                                                 |
| 4. Embedding      | (• Disposable syringes)  
|                    | (• Disposable beakers)  
|                    | • Epoxy resin                                                                 |
| 5. Polymerization | • Embedding plate (Top photo),  
|                    | Embedding capsule (Middle photo)  
|                    | • Oven for polymerization (Bottom photo)                                                                 |
| 6. Sectioning     | • Ultramicrotome (Top photo)  
|                    | • Grid                                                                 |
| 7. Staining       | • Diamond knife (Middle photo)  
|                    | (• Glass knife)  
|                    | (• Knife maker) (Bottom photo)                                                                 |
| 8. Observation    | • Uranyl acetate  
|                    | • Lead citrate                                                                 |
**I - 2 Fixation of biological tissue**

**I - 2 - 1 Extraction of tissue**

**I - 2 - 2 Fixation**

1. Pre-fixation (fixes proteins) ……1–2 hr.
   2.5% glutaraldehyde in Phosphate buffer

2. Post-fixation (Fixes lipids) 1–2 hr. (4°C)
   1–2% OsO₄ in Phosphate buffer

3. Washing in phosphate buffer. …… 10 min

**I - 2 - 3 Dehydration**

1. 50% ethanol 10 min.
2. 70% ethanol 10 min.
3. 80% ethanol 10 min.
4. 90% ethanol 10 min.
5. 95% ethanol 10 min.
6. 100% ethanol * 20 min.
7. 100% ethanol * 20 min.

*Water is removed through a molecular sieve

**Methods of washing & dehydration**

- Liquid is quickly replaced before the sample can dry.
I - 2 - 4 Substitution

① Only propylene oxide (PO) …… 10 min × 2-3 times
(Change Liquid 2 or 3 times every 20 minutes.)

② Mixed-solution of PO and resin

PO : Epoxy resin = 2:1 30 min.
PO : Epoxy resin = 1:1 1 hr.
PO : Epoxy resin = 1:2 1 hr.

③ Only Epoxy resin …… 2hr. or overnight

I - 2 - 5 Embedding (The freshly mixed resin should be used.)

Epoxy resin

[In the case of TAAB EPON 812 resin]

① EPON 812 48g
② DDSA 19g
③ MNA 33g
④ DMP-30 2g

The specimen is placed in a well of a silicone embedding plate.
Resin is poured over the tissue. Ensure no air bubbles are trapped within the resin.

*For a specimen with various orientations, use a silicone embedding plate.

**Preparation of Epoxy resin**

[In the case of TAAB EPON 812 resin]

① Put EPON 812 · DDSA · MNA in beaker.

*The use of disposable syringes and beakers facilitates the post washing.

② Mix it well.

③ Add DMP-30 and stir well.

*Since an added volume is small, the use of syringes for tuberculin is recommended.
I - 2 - 6 Polymerization
(35°C  1 day)
45°C  1 day
60°C  1 day

Appendix
Preparing solutions used in microtomy

How to prepare 2.5% glutaraldehyde solution
1. Add 40mL of distilled water to 10mL of 25% glutaraldehyde solution (commercially available: GA). (⇒ 5% GA solution is prepared.)
2. Add 50mL of 0.2M phosphate buffer. ⇒ 100mL of 2.5% GA-0.1M phosphate buffer (pH 7.4) is prepared.

How to prepare 0.2M phosphate buffer (Sorensen’s phosphate buffer)
Solution A: Phosphate sodium (NaH₂PO₄) 27.6g/L
Solution B: Phosphate sodium (Na₂HPO₄) 53.6g/L
A : B = 28mL : 72mL ⇒ pH 7.2
A : B = 19mL : 81mL ⇒ pH 7.4

How to prepare 1% osmium solution
1. Dissolve 1g of osmium crystal in 25mL of distilled water. ⇒ 4% osmium solution (kept in a dark place and in a refrigerator)
2. Add 5mL of distilled water and 10mL of 0.2M phosphate buffer to 4% osmium solution (kept in a dark place and in a refrigerator) prepared in Step 1. ⇒ 20mL of 1% osmium-0.1M phosphate buffer (pH 7.4) is prepared.
*Preparation must be performed in a fumehood.
I – 3 Steps in sectioning with an ultramicrotome

I - 3 - 1 Trimming

Expose the tissue.
Trim the tip using a razor to form a pyramidal shape.

A cutting surface is made into a square, rectangle or trapezoid.

Examples of trimming

Square  Rectangle  Trapezoid

Square : Ideal for when ultra-low magnification images are needed.
Rectangle : Suitable when sequential sections are cut and when the block contains both hard and soft tissue.
Trapezoid : Ideal for keeping track of the order of sections in a ribbon.

I – 3 – 2 Sectioning

① Setting a specimen
A sample block and a knife are set on a microtome.

Ultramicrotome (Leica EM UC6)
Making of ultrathin sections
Sectioned thin specimens float and are flattened on water in the knife boat.

A) The sections floated in the surface.

B) A actual section

Thickness of ultrathin section
The thickness of thin specimens may vary depending on the hardness of resin and room temperature.
It is necessary to judge the thickness by color of interference.

The interference color and thickness of an ultrathin section

<table>
<thead>
<tr>
<th>The optimal thickness</th>
<th>Thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gray</td>
<td>&lt; 60 nm</td>
</tr>
<tr>
<td>Silver</td>
<td>60 – 90 nm</td>
</tr>
<tr>
<td>Gold</td>
<td>90 – 150 nm</td>
</tr>
<tr>
<td>Purple</td>
<td>150 – 190 nm</td>
</tr>
<tr>
<td>Blue</td>
<td>190 – 240 nm</td>
</tr>
<tr>
<td>Green</td>
<td>240 – 280 nm</td>
</tr>
<tr>
<td>Yellow</td>
<td>280 – 320 nm</td>
</tr>
</tbody>
</table>

The optimal thickness for observation in a 120 kV TEM.

Picking up sections
Pick up thin specimens on water using a TEM grid.

A) Press Method
Place a grid on a thin specimen with a support film side down.

B) Pull up Method
Place a TEM grid under thin specimens with a support film on the top. Bring thin specimens on the grid by using an eyelash probe.
5 Grid types

Grids of various shapes are available; circular holes, square holes, hexagonal holes, slit holes, single hole and marked holes. Grids with large square or hexagonal holes, or with a single slot are often used to pick up ultrathin sections. The grid sizes range from 50 to 2000 mesh, which indicates the number of holes per inch. Thus, the larger the value, the smaller the grid square is.

Examples of grids

- Grating
- Hole
- Circle holes
- Square holes
- Marked holes
- Slit holes
- Single hole

It is advantageous to select an appropriately shaped grid depending on the research.

Example) Slit holes: For sequential sections

Single hole: For ultra-low magnification observation

Material: Cu, SS, Ti, Mo, Pt, Al, etc.

Cu is used in many cases, but for chemical analysis by EDS, the grid should not contain any elements targeted for analysis.

Be careful when using Ni grids, as this material is paramagnetic.

6 How to make glass knifes

1) Place a glass bar (6 to 10 mm (T), 25 mm (W), 400 mm (L)) on the knife maker and equally divide this rod into two portions.

2) Repeat equal division to make glass squares 25 mm on edge.

A) Lock the glass bar.
B) Score the glass surface.
C) Fracture along the score line.
3) Place a 25 mm square glass block in the knifemaker with the fresh cut to the right.
4) Score the block along a diagonal line and by applying pressure fracture the glass along the score line.

Typical diamond knives

Ultra
Used in room temperature.
For thin sectioning.

Cryo dry
Used in low temperature.
For thin sectioning

Ultratrim
For preparation of resin block

Diamond knife

Two knifes from one 25 mm square block
I - 4 Conventional staining of ultra-thin sections

I - 4 - 1 Various staining solutions

1. Uranyl acetate (stains nucleic acids)
   Usually, 2–5% (w/v) uranyl acetate in water is used.
   *Since it easily decomposes in light and high temperatures, it should preferably be kept in a refrigerator.

2. Lead citrate (stains protein and glycogen granules)
   Method of Reynolds (There are several other methods.)
   ① 1.33 g of lead nitrate, 1.76 g of sodium citrate, and 30 mL of distilled water are mixed.
   ② 8 mL of 1N NaOH is added. (Transparent liquid)
   ③ Then, distilled water is added so that the whole quantity is 50 mL.
   *Lead forms crystal by reacting with CO₂. Put the mixed liquid in a syringe. Place silicon plug on the tip of needle and store it in a refrigerator.

I - 4 - 2 Procedure of staining

1. Uranyl acetate staining  10 minutes
   Place a droplet of uranyl acetate solution on Parafilm. Float the grid on the droplet with the tissue down.

2. Washing
   Run water (very gently!) parallel to the surface of a grid or place the grids on droplets of distilled water.

3. Lead staining  10 minutes
   Drop Lead citrate liquid on Parafilm. Float a TEM grid on it.
   *Place NaOH grains around liquid.

4. Washing
   Run water (very gently!) parallel to the surface of a grid.

5. Drying
   Wick excess water by blotting with a piece of filter paper.
II Observation of single particles and viruses by negative staining

II-1 Contrast in negatively staining

Negative staining solutions (uranyl acetate, phosphotungstic acid, ammonium molybdate, etc.) envelope the specimen and upon drying form a glass-like, electron dense film that increases the contrast of the specimens, i.e. the specimen is viewed against a dark background.

II-2 Staining procedures

1. Apply the specimen to a TEM grid

2. Mount specimen on a TEM grid
   Clamp a grid with support film in a tweezer. With a pipette, apply the specimen. Remove excess liquid with a filter paper.

3. Negatively staining
   Apply a droplet of uranyl acetate liquid on the grid before the specimen dries. Remove excess liquid with a filter paper.

4. Dry the specimen
III Preparation of supporting film

### III–1 Preparation of collodion supporting film (wet method)

1. Prepare distilled water.
   - Put distilled water (temperature 30 to 40°C) in a Büchner funnel (diameter: 10 to 15 cm).

2. Arrange grids.
   - Place a wire mesh in the funnel and arrange grids on the mesh.

3. Using a glass pipet, apply a drop 0.5-2% (w/v) of collodion solution (in iso-amyl acetate) on the water solution.
   - After applying, the droplet quickly expands on the water and forms a thin collodion film after the iso-amyl acetate evaporates.

4. Pull out distilled water.
   - Slowly drain the distilled water at the bottom of the Büchner funnel, and allow the thin film to settle on the grids.

5. Dry the thin film.
   - Place the wire mesh with grids and the collodion support film on a filter paper and allow to air dry.

### III–2 Preparation of formvar supporting film (dry method)

1. Clean a glass slide with lens paper.

2. Immerse the glass slide in 2% (w/v) formvar solution.

3. In a smooth motion, gently pull the glass slide up.
   - The quicker you pull the glass slide up, the thicker the film will be.
④ Score the edge of the collodion film.
To help floating off the collodion film on water, score the edges of the glass slide with a razor.

⑤ Float the collodion film.
Slowly immerse the glass slide in water with the slide at a 20° to the water surface.

⑥ Arrange the grids.
Arrange the grids on the film floating on the water.

⑦ Lift the supporting film.
Place a piece of Parafilm on the collodion with the grids, and then slowly lift it up.

⑧ Allow the grids with the collodion support film to air dry.
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