Biological Specimen Preparation

How might one prepare biological specimens for TEM?

- Fix, embed and section
- Metal shadow
- Negative stain
- Positive stain
- Unstained, frozen-hydrated
- Freeze-fracture
- Freeze-etch
- Freeze-dry
- Cryo-section
- Etc., etc., etc.

BIO595S - Microscopy of Macromolecules (M. Sherman)
BIO595T - Microscopy of Cells and Tissues (D. Sherman)
Electron Microscopy of Biological Macromolecules: Specimen preparation
Specimen preparation: Why bother?

- Specimens are in aqueous medium – to protect from column vacuum
- Weak electron scattering – extremely low contrast
- Low radiation tolerance
Specimen preparation

✓ To increase scattering contrast (both, amplitude and phase) – to see better
✓ To preserve specimen structure at highest resolution possible
✓ To improve radiation stability of the samples
✓ Time resolved microscopy – to capture dynamic changes in the structure
✓ To label parts of the structure (e.g. gold or Fab labeling)

[part of Debby Sherman’s lecture but labeling is used in macromolecular TEM as well although with smaller probes]
Specimen preparation techniques

- **Surface replicas**
  - Metal shadowing
    - Unidirectional
    - Rotary
  - Freeze drying
  - Freeze fracturing

- **Negative staining**

- **Cryo microscopy**
Metal Shadowing, Negative-Staining and Electron Cryomicroscopy

- Metal Shadowing
  Bacteriophage T4 polyheads

- Negative Staining
  Maize Streak Virus

- Cryo-EM
  Maize Streak Virus
Metal shadowing

From H. S. Slayter 1976 (p. 17 of class reference list)
\[ t_{\text{calc}} = k_\alpha \frac{W \sin \theta}{4 \pi R^2 \rho} \]
Specimen application by spraying

From H. S. Slayter
Apparatus for deposition of coatings by electron gun onto a cold tilted specimen

From H. S. Slayter
Backing replicas with C and detaching them on water

From H. S. Slayter
Figure 9. Extension of present visibility limits by the use of tungsten coating and darkfield electron microscopy.

A: Type VI collagen molecule prepared with $10^{-7}$ g/cm$^2$ tungsten, close to focus in brightfield (frame 2) vs. darkfield (frame 1). Frame 3 is the same area slightly under focus showing the critical dependency upon exact focus of the image in darkfield. Magnification 196 650×.

B: Thrombospondin molecule in brightfield (left) vs. darkfield (right) prepared by tungsten coating with $10^{-7}$ g/cm$^2$. Magnification 242 000×.

C: Thrombospondin molecule in brightfield (left) vs. darkfield (right) prepared by platinum coating with about $10^{-7}$ g/cm$^2$. Magnification 242 250×.
Rotary shadowed closed loop DNA molecule prepared according to Kleinschmidt. The DNA was spread on water in presence of cytochrome c, absorbed on a carbon film and rotary shadowed at angle of 8° with Pt-C (from A. B. Maunsbach & B. A. Afzelius 17. Freeze fracturing and shadowing, p. 429)
Freeze-drying
Both adsorbed and unadsorbed particles are present in the droplet (air-drying); only adsorbed particles in case of freeze-drying (A); Unadsorbed particles aggregate or overlap (air-drying) (B); Collapse or disruption of particles during air-drying caused by surface tension forces

Freeze-drying of cells. Drying should be carried out for a reasonable time (1-2 hr) to uncover upper half of cells; otherwise the replica becomes discontinuous.

From M. V. Nermut
An avian adenovirus freeze-dried and shadowed with Pt-C

From M. V. Nermut
Freeze-fracturing
Frozen samples fracture usually by membranes where present.

Fracturing (a) and etching of frozen thylakoid membranes. Deep etching (b) exposes the surfaces hidden before in ice.

Freezing methods and cryogens used in freeze-fracturing and etching

Cryoprotectants are used to prevent ice crystals formation

Cryogens used: propane, ethane, freon

From Robinson et al.
From S. Bullivant

Fig. 5. Diagram of Moor-Balzers freeze-etch unit. 1: specimen, 2: specimen stage, 3: microtome stand, 4: microtome arm, 5: knife, 6: advance mechanism, 7: microtome drive, 8: liquid nitrogen in arm, 9: thermo-couple lead, 10: liquid nitrogen supply, 11: lens, 12: lamp, 13: electrical feed-through, 14: carbon electrodes (platinum-carbon not shown), 15: metal bell jar. The diagram is taken from Moor (1965) and is by courtesy of Dr. H. Moor and Balzers AG.
• Pure liposomes (A);
• Liposomes with Na,K-ATPase (B);
• Liposomes with increasing concentration of aquaporin (D-F)

(from A. B. Maunsbach & B. A. Afzelius in: 17. Freeze fracturing and shadowing, p. 447)
A cartoon showing the topography of a mitochondrion fracture face as it would appear in profile view in a vertical cut through the fracture plane.

The cristae drawn in vertical planes and arranged to fit as close as possible the way they appear in the fracture face.

From F. S. Sjstrand
Freeze-fracturing devices:

b – multi-disc table;
c - complementary replica device

From Robinson et al.
Complementary freeze-fracture replicas of an apyrene snail spermatozoon
(from A. B. Maunsbach & B. A. Afzelius)
Contamination artefacts:

A.: Freeze-fracture replica of pure water droplet. Crystals should not be there. Evaporation of Pt-C was initiated a few seconds after the last cut with the knife;

B.: Microvilli freeze-fractured at -100 °C; evaporation started 1 min later;

C.: A higher magnification of a similar to B. specimen. Small and big particulate elevations are seen.

(from A. B. Maunsbach & B. A. Afzelius)
Negative staining
Purpose of Negative Staining

- Embed the sample in a layer of heavy metal salt
  - Contrast arises from “cavities” in the embedding matrix (specimen “casting”).
  - Supposed to be chemically inert
  - Penetrates hydrophilic areas and replaces water
- Increases scattering contrast
  - “Negative Staining” - the sample appears light on a dark background
- Preserves the structure from dehydration and radiation damage
Positive Staining

- Stain molecules react to ions on the surface of the sample
- Some positive staining always occurs
  - pH of uranyl acetate (UA, pH4.5) close to isoelectric point of positively charge proteins (pI5.0)
  - UA has a strong affinity for carboxyl and phosphate groups in nucleic acids, lipids and some proteins
- Positive staining may be more apparent when the embedding layer of stain is improperly washed away

Phage 2037/1

<table>
<thead>
<tr>
<th>Phosphotungstic acid</th>
<th>Uranyl acetate</th>
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<tbody>
<tr>
<td>Negative staining</td>
<td>Positive staining</td>
</tr>
<tr>
<td></td>
<td>Negative staining</td>
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</table>
## Negative stains frequently used

<table>
<thead>
<tr>
<th>Negative Stain</th>
<th>Chemical Formula</th>
<th>pH for use</th>
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<tbody>
<tr>
<td>Uranyl acetate</td>
<td>$\text{UO}_2[\text{CH}_3\text{COO}]_2$</td>
<td>2 – 4.5</td>
</tr>
<tr>
<td>Sodium phosphotungstate</td>
<td>$\text{Na}_3\text{PO}_4\text{12WO}_3$</td>
<td>5 - 8</td>
</tr>
<tr>
<td>Sodium silicotungstate</td>
<td>$\text{Na}_3\text{SiO}_2\text{12WO}_3$</td>
<td>5 - 8</td>
</tr>
<tr>
<td>Ammonium molybdate</td>
<td>$[\text{NH}_4]_6\text{Mo}<em>7\text{O}</em>{26}$</td>
<td>5 - 8</td>
</tr>
</tbody>
</table>

From R. Harris & R. Horn
Choice of Stain

- Try, try again

- Choice of anionic or cationic stain and pH
  - UA (cationic) – pos. charged at low pH, proteins also pos. charged
  - PTA (anionic) – neg. charged at neutral pH which is above pI of most proteins which are also neg. charged
  - Stains soluble at different pH’s (UA pH 4.5, PTA in buffer at pH 7.0)

- Osmolarity of the stain and effect on sample

- Granularity of stain (UA 0.4-0.5nm PTA 0.8-1.5nm)

- Are samples disrupted in stain
  - Rotavirus sometimes disrupted in PTA, not in UA
  - Will the stain handle high concentrations of buffer salts (PTA yes, UA no)

- How stable is the stain in the electron beam
  - Stain recrystallization and redistribution when hit by beam
  - Aurothioglucose preserves sample with less flattening but extremely beam sensitive and bubbles when hit by beam
Negative Staining Procedure
Glow Discharging the Carbon Support Film

High voltage glow discharge in a reduced atmosphere makes carbon films hydrophilic and negatively charged.
Negative Staining Procedure

Applying Sample
Negative Staining Procedure

Washing with dH$_2$O
Negative Staining Procedure
Apply Stain and Blot with Filter Paper
Some Problems of Negative Staining

- Unpredictability and uneven staining
- Sample flattening
- Sample collapse
- Beam-induced stain redistribution
- Cannot be used for quantitative determination of sample concentration
Negative staining problems

Cowpea chlorotic mottle virus
Negative staining problems
Sample collapse
Negative staining problems

UA-stained *Parmecium bursaria Chlorella virus-1* (left)
Frozen-hydrated (right)
Low Density Lipoproteins

Negative stain does not penetrate inside. Result: surface staining

Negative staining (UA)  Frozen-hydrated
Negative Staining
Maize Streak Virus
- Cryo TEM
- Labeling using molecular probes
- Time-resolved cryo TEM
Cryo-EM

✓ Embedding unstained macromolecules in vitreous (noncrystalline) water
✓ Sample density appears with positive contrast
✓ Contrast primarily the result of phase or interference contrast
✓ Preserves the native structure of the sample without flattening or drying
Sample is Vitrified ("frozen") Over Holes in a Carbon Support Film
Sample Preparation Equipment

- Gaseous Ethane
- Freeze-slamming Device
- Self-closing Forceps
- Dewar with Nitrogen and Liquid Ethane Cups
- Holey Carbon Films
- Pipette and tips
- Filter Paper
- Nitrogen Bath and Grid Box
Dewar with Liquid Nitrogen and Ethane Cups

Liquid nitrogen (-196°C)
Addition of Sample and Blotting
Cold Holder and Cryotransfer Station
Cryotransfer Station

Clipring Tool

Grid Box

Cryoshield
Holder in Microscope
Images Need to be Recorded with “Low Dose” Techniques
Reovirus Core Particles
Ordered specimen: Acrosomal Bundle
Results: Geminivirus Study

Maize Streak Virus: UA-Stained, Frozen-Hydrated and 3D Reconstruction from Cryo-EM Data
Figure 1: Schematic showing UNDECAGOLD labeling of Fab' fragment via reaction of a sulphydryl and a maleimide group. 50 nmol of reagent is supplied: this is sufficient to label up to 0.5 mg of Fab' fragments, or up to 1.5 mg of IgG molecules.

Monomaleimido Undecagold™ labeling agent

Nanoprobe Inc.
Actomyosin complex

3D maps and difference maps calculated by cryo-EM and image analysis. F-actin is red, tropomyosin is blue, the myosin motor domain is yellow, the essential light chain is blue-green, the regulatory light chain is dark red, the SH3 domain at the N-terminus of the heavy chain is purple, an **undecagold label** attached to Cys374 pf actin **is gold**, the N-terminal portion of the A1 light chain is white.

*R. Milligan’s web site*
Diagram of the spray freezing apparatus. 
H. D. White at el. 1998, JSB 126, 306
Photograph of the spraying apparatus. The piston carrying the forceps is shown fully extended and the tips of the forceps are in the ethane thimble on the left rim of the nitrogen dewar. Immediately above the thimble is the brass spray nozzle; this is connected to the vertically mounted stainless steel block of the atomizer (marked S) by a T pipe connector.

H. D. White at el. 1998, JSB 126, 306
Actin sprayed with S1 with a delay of 50 ms between spraying and freezing.
M. Walker et al. 1998 PNAS 96, 465
Electron micrograph of actin sprayed with S1 5 ms before freezing. The S1 bound to the actin is disordered. The intensely black round particles are 5-nm colloidal gold that was included in the spray to aid in searching for areas of the grid that had received spray droplets. M. Walker at el. 1998 PNAS 96, 465