Basics of Electron Microscopy

Liguo Wang
Department of Biological Structure
The University of Washington
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Biological structures and investigation techniques

Wang lab at UW (lw32@uw.edu)
Light microscope vs TEM
Major difference between light microscopy and electron microscopy

Wave length:
- Visible light: $\lambda=370-720$ nm
- Electrons: $\lambda=0.0025$ nm (200keV)

Image formation
- Light microscopy: amplitude contrast
- Transmission electron microscopy: phase contrast

Images are the clearest at focus for light microscopy, but have the minimum contrast for TEM.
TEM

http://www.matter.org.uk/tem/lenses/beam_convergence.htm

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C1 condenser and spot size

Focal length \( f \): as small as 2 mm. Object distance \( u \): 20 cm >> \( f \)

\[
\frac{1}{f} = \frac{1}{u} + \frac{1}{v}
\]

\( v \approx f = 2 \text{ mm} \)

Magnification factor:
M = \( \frac{v}{u} = \frac{(2 \text{ mm})}{(20 \text{ cm})} = 1/100 \)

For a W-filament electron source, \( ds \) 40\ um,

\( d_1 = M \cdot ds = (0.01)(40 \text{ um}) = 0.4 \text{ um} \).

C1 lens current can be adjusted to give several different values of \( d_1 \), using a control that is often labeled “spot size”.

http://www.matter.org.uk/tem/lenses/beam_convergence.htm
C1 lens controls spot size

Strong C1 lens produces small probe size and weak beam

Weak C1 lens produces larger probe size and bright beam

This is known as “spot size”

C1 crossover produces image of source and Mag = u/v for C2 lens

Williams and Carter
C2 condenser and intensity

The convergence angle of the illumination is always defined in terms of the variation in angle of the electrons that arrive at a **single point in the specimen**.

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C2 aperture
Objective lens

(A) Overfocused lens
(B) Focused lens
(C) Underfocused lens

Lens

Object

Image plane

$\alpha_1$

$\alpha_2$

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In focus or out of focus?

![Images of samples at different focal distances: 5.0 μm, 1.0 μm, -5.0 μm, -1.0 μm. The image on the right is marked as "In focus." ]

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The field in the back focal plane is the Fourier transform of the field in the front focal plane.
Pre-processing: Comparison of lowpass filters

99.11%

99.7%

98.74%

99.37%

96.44%

98.65%

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Sample preservation: negative staining

EM requires a vacuum - an environmental constraint that is incompatible with unprotected biological material.

**Negative staining** exploits that salts of heavy metals are relatively insensitive towards electrons and form a stable “cast” around the molecules when dried down. Salts such as uranyl acetate or phosphotungstic acid titrated to neutral pH, vanadates and molybdates have and are still being used.

- Sample appears “white” and the electron-dense stain is “black”.
- Helps to reduce dehydration and radiation damage effects.
- Attainable resolution is ~ 15-25 Å.

Hayat & Miller (1990), *Negative Staining*
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TEM grids

An EM grid coated with a thin carbon film (10-30 nm).

400 mesh: 400 squares in 1 inch  ➞ 2.54cm/400 = 63.5 microns
208C High Vacuum Turbo Carbon Coater

- Grow carbon film on mica sheet, then float the continuous carbon onto TEM grids
- Coat commercially available TEM grids with fresh carbon

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Glow discharge to change surface hydrophobicity

Applying a potential difference (of a few 100 V to a few kV) between two electrodes. Electrons that are emitted from the cathode are accelerated away from the cathode, and give rise to collisions with the gas atoms or molecules in a chamber (a few mTorr to 1atm). The collisions give rise to excited species, which can decay to lower levels by the emission of light. This process makes that a gas discharge plasma typically emits a characteristic glow (and is therefore also called “glow” discharge).
### Staining solutions

<table>
<thead>
<tr>
<th>Stain</th>
<th>pH range</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (K) phosphotungstate</td>
<td>5-8</td>
<td>Significant disruptive effect on many membrane systems. Interact with lipoproteins. Less likely to precipitate with salts and biological media</td>
</tr>
<tr>
<td>Uranyl acetate (1-3%)</td>
<td>4.2 – 4.5</td>
<td>Highest electron density and image contrast</td>
</tr>
<tr>
<td>Sodium silicotungstate (1-5%)</td>
<td>5-8</td>
<td>Good contrast; Good for small particles and individual molecules</td>
</tr>
<tr>
<td>Ammonium molybdate (1-2%)</td>
<td>5–7</td>
<td>Best results for many types of specimen; Lower electron density than other stains</td>
</tr>
<tr>
<td>Methylamine tungstate (2%)</td>
<td>6-7</td>
<td>Contrast is not as good as uranyl acetate. Resolution is good.</td>
</tr>
<tr>
<td>Uranyl formate (0.75-1%)</td>
<td>4.2-4.5</td>
<td>Best for small molecules, but only stable for 1-2 days.</td>
</tr>
</tbody>
</table>

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Radiation damage and low temperature imaging

Figure 1.8 Comparison of the rate of fading of electron diffraction intensities at room temperature and at low temperature. A series of electron diffraction powder patterns of glucose-embedded purple membrane were recorded after specified periods of previously accumulated electron exposure (Hayward and Glaeser, 1979). The results show that about 5 to 7 times greater electron exposure can be tolerated at low temperature than at room temperature, for the same extent of specimen damage.
Vitrification of water

- Vitreous ice:
  An amorphous solid state in which water was frozen without adopting any crystalline structure.

![Diagram showing the vitrification of water](image)

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Sample Vitrification

Need high relative humidity in environment because at wind velocities of ~1m/s and 4°C the rate of natural water evaporation is: ~52Å/second (99% humidity) but ~1000Å/second at 80% humidity. At room temperature, this rates are about 4-fold higher!
Fig. 37. Bubbling on a carbon-coated formvar film c. 10 nm thick, covered with a layer of condensed vitreous water. Fields (a)-(h) correspond to irradiations by 5, 20, 40, 80, 120, 240, 340 and 450 ke/nm² respectively. The total thickness of the specimen is 160 nm.
Appearance of trehalose dried down on a carbon film (left). The sugar allows to demonstrate how “low-dose” microscopy is done (right). Let X be the area of interest (for instance a crystal or virus/single particle). Prior to taking a picture some parameters such as “defocus” and “astigmatism” need to be adjusted. To avoid destruction of the specimen, any adjustments are made on small areas (Focus 1 and 2) located adjacent to the area that will be photographed. In the example, the trehalose burned as it was exposed at high magnification (220kx, Focus 1 and 2). Similarly, by exposing the area to be captured for about 30 seconds at 52,000 fold magnification.
Electron optics of Low-Dose imaging

SEARCH

FOCUS

Beam shift

Image shift

EXPOSURE

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Summary of specimen preservation techniques

**Negative Staining (embedding in salts of heavy metals [e.g. Uranyl acetate, tungsten])**

- Easy & fast
- Good reproducibility
- Radiation damage can be seen, but not so critical
- Resolution limited to ~15Å
- No internal feature can be seen
- No time resolution (unless very slow)
- Specimen may be denatured by stain (Drying and flattening artifact)

**Vitrification (embedding in vitreous water or small organic substances [e.g. trehalose, glucose])**

- See protein directly including internal features
- No drying / flattening artifact
- Resolution higher than negative stain
- Time resolved studies possible (ms - minute range)
- Not so easy to do
- Lower contrast
- More radiation sensitive

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Why CryoEM?

- Can determine structures at different chemical or biological conformation states
- Can work with large complex of mixed/dynamic conformations
- Can determine Structure that cannot be tackled readily by NMR or crystallography
- Need only low concentration (<1mg/ml) in less than 100 µl sample
- Resolution can be reached to ~3.5 Å in favorable cases

Negative Stain vs Frozen-Hydrated