1. Some background and theory to electron microscopy
2. Imaging with electrons
3. Preparing samples for electron microscopy

1897: discovered “corpuscles”, small particles with a charge/mass ratio more than 1000 times greater than that of protons, swarming in a sea of positive charge ("plum pudding model").

=> Discovery of the ELECTRON

Sir Joseph John Thomson (1856 – 1940)
Nobel prize in 1906
De Broglie's doctoral thesis (1924):
Application of the idea of particle–wave duality (only known for photons up to then) for any kind of matter.

"With every quantum of matter, there is an associated wave..." characterized by the equation:

\[ \lambda = \frac{h}{m \cdot v} \]

where \( \lambda \) is the wavelength, \( h \) is the Planck constant, \( m \) is the rest mass, \( v \) is the velocity and \( c \) is the speed of light in a vacuum.

De Broglie's ideas were tested experimentally. Observed electrons forming diffraction patterns when passing through a thin film of metal.

Sir George Paget Thomson (1892 – 1975)
Nobel Prize: 1937 (shared with C.J. Davison)

Electrons = wave

Electron optics was born in 1927, when Hans Busch showed that the elementary lens equation is applicable to electron lenses.
The resolving power of a microscope is proportional to $\frac{\lambda}{NA}$.

$\lambda$ = wavelength

$NA = n \sin \theta$

$n = \text{index of refraction}$

In electron microscopy, the refractive index cannot exceed 1.0, the half angle is very small, and thus the only thing that can be adjusted is decreasing the wavelength of illumination.
Planck’s constant ($h = 6.624 \times 10^{-27}$ erg/second)
m = mass of an electron ($9.11 \times 10^{-28}$ gram = 1/1837 of a proton)
v = velocity of the electron

A beam of 100 KeV has a wavelength of 0.0389 Å

Theoretical resolution of 0.1 Å,

In reality most TEMs will only have an actual resolution of around 2.4 Å at 100 KeV.

**Light microscopy and TEM analogy**

**Transmission electron microscope**

**Scanning electron microscope**
Helmut Ruska, 1939, imaged the tobacco virus

**Technique**  
- negative staining  
- virus dried to a thin film  
- solution of electron dense stain applied  
- dried  
- imaged

### Scale of biological structures

Why we need electron microscopes to see biological structures

- Light Microscopy  
- Electron Microscopy

### Vapour Pressure of Different Types of Vacuum Pump

- Turbomolecular pump
- 20 - 10⁻⁷ mbar
- Rotary pump
- Oil diffusion pump
- 10⁻⁷ - 10⁻⁷ mbar
- Ion getter pump
- 10⁻⁷ - 10⁻¹⁰ mbar
Light microscopy and TEM Analogy

- Specimen
- Immersion lens
- Objective lens
- Image
- Magnifying lens
- Film

Typical EM grids for holding sections

- Single slot grid
- Multi hole grid

Thin sectioning

- Specimen size:
  - 3 mm in diameter
  - Ca. 100 nm in thickness
  - Electron transparent
High energy electrons transmitting through a sample

Electrons passing through the sample are deviated depending on the composition of the sample - amplitude contrast imaging

Phase contrast imaging - frozen hydrated sample
Microscopes - for biological samples

SEM

TEM

TEM 120 kV - Tecnai Spirit
Cryo and ambient temperature

TEM 200 kV - Tecnai F20
Cryo and ambient temperature

TEM 200 kV - Jeol 2200 FS
Cryo energy filtered

HR SEM - Zeiss Merlin

HR FIB/SEM - Zeiss NVision 40

Considerations for EM of biological structures

- Samples contain elements of low molecular weight
- Most biological samples contain water
- Intense heat of the e beam
- Size of Specimen - only thin or very small samples can be imaged without sectioning

Jellyfish - aequoria victoria

Helmut Ruska, 1939, imaged the tobacco virus

- Technique: negative staining
  - Virus dried to a thin film
  - Solution of electron dense stain applied
  - Dried
  - Imaged

Support film

Solution of heavy metal ions

Cast of dried metal stain

Jellyfish - aequoria victoria
General scheme for preparing biological samples for EM

Fixation

- Cryofixation
- Chemical fixation

Sectioning

- Embedding: Epoxy/acrylic resins
- Thin sectioning: 40 - 200 nm sections

EM Imaging

- Ambient temperature TEM
- Cryo TEM

Staining

- Dehydration
- Substitution
- Radioimmunoelectrophoresis
Fixation

A process used to preserve the structure of freshly killed material in a state that most closely resembles the structure and composition of the original living state.

Chemical - coagulative/noncoagulative

- Coagulative: original killing agents (alcohols, Bouin's), low pH, unbuffered, coagulates cellular components.
- Non-coagulative: formaldehyde, glutaraldehyde, osmium tetroxide

Freeze Fixation

- Egyptian mummy - few thousand years old
- Baby mammoth, 37,000 years old

Chemically fixing biological samples

- Pieces should be small to allow rapid and complete penetration of the fixative.
- The volume of fixative should exceed the volume of tissue by a factor of 10.
- Plants: small pieces may be excised and dropped directly in fixative. Very small plants and algae may be fixed whole.
- Insects, other invertebrates have very impermeable coatings.
- Larger tissues should be perfused with the fixative.

Chemical fixation of biological samples - considerations

- pH (isoelectric point)
- Total ionic strength of reagents
- Osmolarity
- Temperature
- Length of fixation
- Method of application of fixative

0.2% glutaraldehyde
2% glutaraldehyde
8% glutaraldehyde
in 0.1M phosphate buffer
TEM images of kidney tubules

Interpreting EM images of fixed tissue

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Typical fixatives - aldehydes

Formaldehyde

- Notable for its role in the fixation of all of the cytoplasmic and intracellular proteins, enzymes, and nucleic acids.
- A high-molecular-weight formaldehyde with weak cross-links at 4°C, which results in a low penetration rate.

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Mechanism of action of aldehydes

- Glutaraldehyde
  - Glutaric acid dialdehyde, a 5-carbon dialdehyde, is the most widely applied in EM work, with high specificity and low penetration rate.
  - Cross-links at room temperature; no polymerization.

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Glutaraldehyde

- Glutaraldehyde: the most widely applied to EM work, with high specificity and low penetration rate.
- Cross-links at room temperature; no polymerization.

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Both aldehyde groups of a single glutaraldehyde molecule react with proteins to form cross-linkage. Glutaraldehyde introduces free aldehyde groups to the fixed tissue. Glutaraldehyde readily cross-links proteins.

Effects of aldehydes fixatives

Over-fixing – artefacts

Muscle tissue fixed for 3 days at room temperature in 2.5% glutaraldehyde.

Other fixatives, and stains
**DISADVANTAGES OF STEM FOR 3D STRUCTURAL ANALYSIS**

- labour intensive
- slow
- section loss and damage
- thickness of section

**Serial thin sectioning**

- 50nm thick sections collected on water

**Typical EM grids for holding sections**

- Single slot grid
- Multi hole grid

**Grid staining on silicon plate**

**Embedding in resin**

- Epoxy resin – araldite
- Acrylic resin – methyl methacrylate
cytoplasm of a neuron in the mouse brain