Introduction to Electron Microscopy
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Preparation

Physical demands of electron microscopy

Biology
- Aqueous/hydrated
- Soft
- Light elements (C, O, H, N, S, P etc.)
- “Large”

Electron microscope
- High vacuum
- Electron beam
- Sensitive to vibration/motion (High magnifications)

Biological samples need to be transferred into a solid state...
...which preserves the structures as a function of the living state...
...and not as a function of specimen preparation

Not suitable for EM

Resistant to high vacuum
Resistant in electron beam
Thin – permeable for electrons (for TEM)
Contrast
**Physical demands of electron microscopy**

**Biology**
- Aqueous/hydrated
- Soft
- Light elements (C, O, H, N, S, P etc.)
- “Large”

**Electron microscope**
- Not suitable for EM
- High vacuum
- Electron beam
- Sensitive to vibration/motion (High magnifications)

Any treatment changes the specimen!

Resistant to high vacuum
Resistant in electron beam
Thin – permeable for electrons (for TEM)
Contrast

**Physical demands of electron microscopy**

What is (was) this?

![Image of a unidentified object] 2 cm
Main preparation pathways for TEM

1. Fixation
   - Requires solid specimen (embedding in plastic)
   - Chemical fixation (cross-linking) with Aldehydes, OsO₄, Ur²⁺...
   - Glutaraldehyde (CH₂ = CH₂ = CH₂ = CO)

2. Dehydration
   - Solvents dissolve biological matter
   - Plastic only soluble in solvents (e.g. acetone)

3. Embedding
   - Plastic only soluble in solvents (e.g. acetone)

4. Thin sectioning
   - Requires solid specimen (embedding in plastic)

5. Staining
   - Requires thin specimen: 70 nm

   ————

Room temperature processing for TEM

1. Fixation
   - Stabilization of biological material
   - Chemical fixation (cross-linking) with Aldehydes, OsO₄, Ur²⁺...
   - Glutaraldehyde

2. Dehydration

3. Embedding

4. Thin sectioning

5. Staining

6. TEM

   ————

   Maximum size for good preservation

   ————
Room temperature processing for TEM

Fixation
- Post-fixation with OsO₄
  - Osmiumtetroxide
  - Cross linker mainly of unsaturated lipids
  - some proteins & phenolic compounds
- Provides contrast
- Can solubilise some proteins

Dehydration
Embedding
Thin sectioning
Staining
TEM

Room temperature processing for TEM

Fixation
- Well preserved
- Not well preserved

Dehydration
Embedding
Thin sectioning
Staining
TEM

Liver tissue
Room temperature processing for TEM

1. Fixation
2. Dehydration
3. Embedding
4. Thin sectioning
5. Staining
6. TEM

Substitution of water with solvent (ethanol, acetone) Usually performed with gradient of different concentrations.

Infusion with “plastic” formulation followed by polymerisation

- Plastic formulations consist of monomers, hardener, accelerator
- Polymerization by heat or UV light
- Epoxy resins, acrylic resins
- Note: Resins are toxic and allergenic

Specimen embedded in Epon
Room temperature processing for TEM

- Fixation
- Dehydration
- Embedding
- Thin sectioning
- Staining
- TEM

Cutting sections of ca. 70 nm -> electron transparent

Ultramicrotomy
Room temperature processing for TEM

- Fixation
- Dehydration
- Embedding
- Thin sectioning
- Staining
- TEM

Cutting sections of ca. 70 nm -> electron transparent

Contrast enhancement with heavy metals

- Uranium ions: phosphate groups of lipids (membrane contrast)
- Lead ions preferably bind to proteins
Room temperature processing for TEM

Fixation → Dehydration → Embedding → Thin sectioning → Staining → TEM

Interpretation/orientation

HEP2 cells infected with Chlamydia pneumoniae

Aldehydes: Slow (seconds to minutes), lots of artefacts like shrinkage, osmotic effects, conformational changes of proteins, loss of ions and small molecules. OsO₄: Depolimerisation of proteins

Shrinkage
Conformational changes of proteins
Loss of lipids

Mechanical effects
Loss of Lipids
Shrinkage during polymerisation

Compression, knife marks

Staining artefacts (precipitation of heavy metals)

Interpretation mistakes
Cryo preparation for TEM

- Fixation
- Dehydration
- Embedding
- Thin sectioning
- Staining
- TEM

Cryo-Immobilization
Stabilization of biological material by freezing

- Liquid water and vitrified water
- Frozen water with ice crystals

Well frozen mouse cerebellum

Not well frozen mouse cerebellum
Cryo preparation for TEM

Fixation

Dehydration

Embedding

Thin sectioning

Staining

TEM

- Plunge freezing in liquid ethane/propane:
  - Only suspensions (< 1 µm) or thin tissues containing anti-freeze

- Slam freezing:
  - Suspensions and thin tissues (few µm, only front well frozen ca. 1 µm)

- Propane jet freezing (JFD):
  - Adequate freezing of suspensions not thicker than 15 µm
  - Thicker specimen require anti-freeze

- High pressure freezing (HPM)
  - Freezing under high pressure (2100 bar)
  - Adequate freezing of samples up to 200 µm thickness without anti-freeze

Cryo preparation for TEM

Fixation

Dehydration

Embedding

Thin sectioning

Staining

TEM

- Plunge/slam freezer
- Propane jet freezer
- High-pressure freezer

Relative sizes
Cryo preparation for TEM

Freeze-substitution
Substitution of water/ice with solvent (ethanol, acetone)
Usually combined with simultaneous fixation with chemicals (OsO₄, Uranyl-acetate…)

Temperature (°C)

-100
-90
-80
-70
-60
-50
-40
-30
-20
-10
0

Time (h)

0 5 10 15 20 25 30 35

-90°C acetone

Embedding
Fixation
Dehydration
Thin sectioning
Staining
TEM

Infusion with "plastic" formulation followed by polymerisation at low or room temperature
Cryo preparation for TEM

Fixation

Dehydration

Embedding

Thin sectioning

Staining

TEM

Same procedure as RT

Fixation

Dehydration

Embedding

Thin sectioning

Staining

TEM

No RT fixation artefacts
Ice crystal damage possible

Reduced extraction of cell constituents
Reduced shrinkage

Mechanical effects
Loss of Lipids
Shrinkage during polymerisation

Compression, knife marks

Interaction of heavy metals with biology provides electron density

Interpretation/orientation
Room temperature vs. cryo preparation

Mouse cerebellum

Conventionally fixed (glutaraldehyde)  High pressure frozen

500 nm

High-pressure frozen, freeze-substituted mouse cerebellum

5 μm

Specimen courtesy of Bettina Sobottka, Neurologische Klinik, University of Zurich
Dehydration
Critical Point Drying
Freeze-fractured/etched specimen
Freeze-dried specimen
Freeze-fracturing/Freeze-drying/Coating
RT-SEM
Low temperature processing
Low temperature embedding
RT-TEM
Cryo-Ultramicrotomy
Cryo-TEM
Cryo thin section
Freeze-substitution
Cryo-SEM
RT specimen processing
Chemical fixation
Dehydration
Embedding
Cryo Ultramicrotomy
Immunolabeling
Replica
Freeze-fractured/Freeze-drying/Coating
Plunge freezing
High pressure freezing
Propane jet freezing
Critical Point Drying
Coating
Ultramicrotomy
Staining
Chemical fixation
Freeze-substitution
Replica
Freeze-fractured/Freeze-dried specimen
Cryo thin section
WARM SPECIMEN
Chemical fixation
Room temperature processing for SEM
Fixation
Same as RT preparation for TEM
Sample finally in solvent like ethanol or acetone
Dehydration
Critical point drying
Coating
SEM
Critical point drying
Fixation
Dehydration
Critical point drying
Coating
SEM

Critical point of H₂O: 374°C and 221 bar
Critical point of CO₂: 31°C, 74 bar

Room temperature processing for SEM

Fixed point of H₂O: 374°C and 221 bar
Critical point of CO₂: 31°C, 74 bar

Temperature
Pressure
solid
liquid
gas
S Starting point
E End point
C Critical point

Phase diagram of CO₂

Room temperature processing for SEM

Air drying

Fixation
Dehydration
Critical point drying
Coating
SEM

Air drying

Evaporation
Surface tension
Structural collapse
Room temperature processing for SEM

- Fixation
- Dehydration
- Critical point drying
- Coating
- SEM

Critical point drying
Air drying

Surface of rose blossom
Spider mite

Room temperature processing for SEM

Critical point drying
Air drying

Surface of rose blossom
Spider mite

Electron Microscopy ETH Zurich
Critical point drying
Fixation
Dehydration
Critical point drying
Coating
SEM

Thin heavy metal layer applied to the specimen surface
- Sputter coating
- Resistance evaporation

Primary electron beam
Platinum/Gold (1-10 nm)

Interpretation/orientation
Room temperature processing for SEM

Critical point dried, fractured liver tissue

Cryo processing for SEM

Fixation

Freeze-fracturing

Sublimation (partial freeze-drying)

Coating

Cryo-SEM

Cryo-Immobilization (same as for TEM)
Cryo processing for SEM

Fixation

Freeze-fracturing

Sublimation (partial freeze-drying)

Coating

Cryo-SEM

...under high vacuum and at low temperature

-120°C...-150°C

2 cm

Fixation

Freeze-fracturing

Sublimation (partial freeze-drying)

Coating

Cryo-SEM

...under high vacuum and at low temperature

-120°C...-150°C

2 cm
Cryo processing for SEM

Fixation

Freeze-fracturing

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Coating

Cryo-SEM

Fixation

Freeze-fracturing

Sublimation (partial freeze-drying)

Coating

Cryo-SEM

Revealing the ultrastructure by removing the ice embedding the biological material (under high vacuum)

Heating (for example: -100°C for 5 minutes)
Cryo processing for SEM

Freeze-fractured Vero cell: NO sublimation

Freeze-fractured mouse intestine: with sublimation
Cryo processing for SEM

- Fixation
- Freeze-fracturing
- Sublimation (partial freeze-drying)
- Coating
- Cryo-SEM

Thin heavy metal layer applied to the specimen surface
...at low temperature

Primary electron beam

Platinum/Gold (1-10 nm)

Interpretation/orientation
Cryo processing for SEM

High-pressure frozen, freeze-fractured brain tissue

Critical point dried, dry-fractured brain tissue