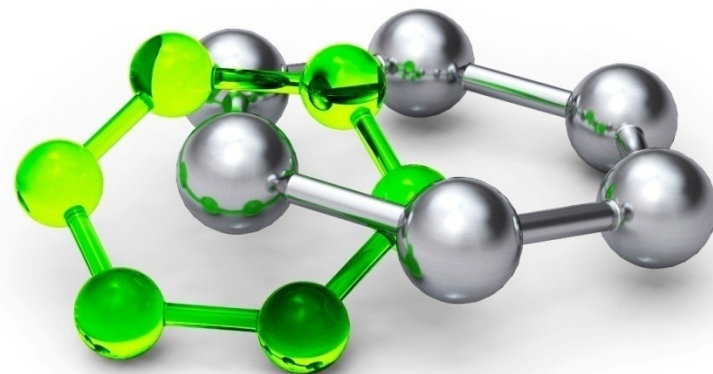




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S1007 Doing structural biology with the electron microscope

Lecture 2: Sample preparation

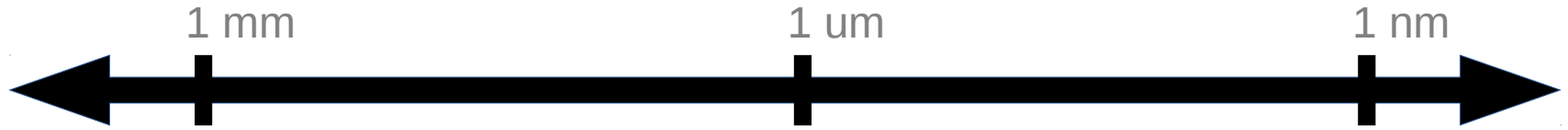


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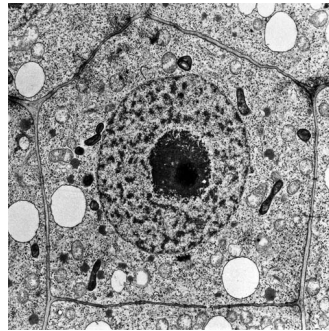


OP Výzkum a vývoj
pro inovace

Samples in electron microscopy



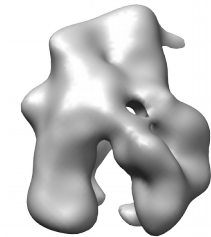
Tick (ESEM)



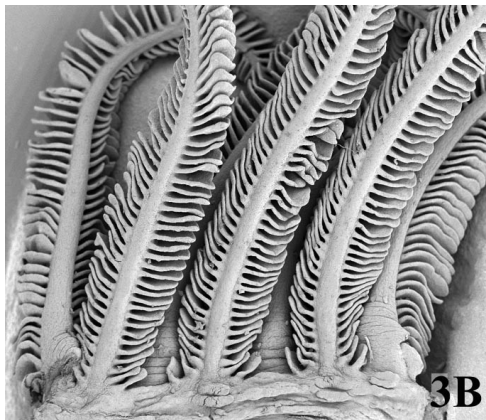
Plant cell (TEM)



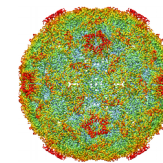
Bacteria (SEM)



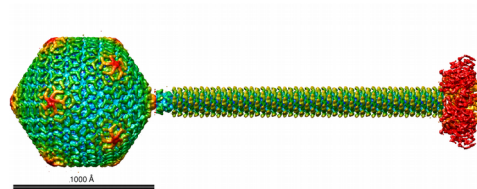
RNA polymerase (TEM)



Plant (SEM)

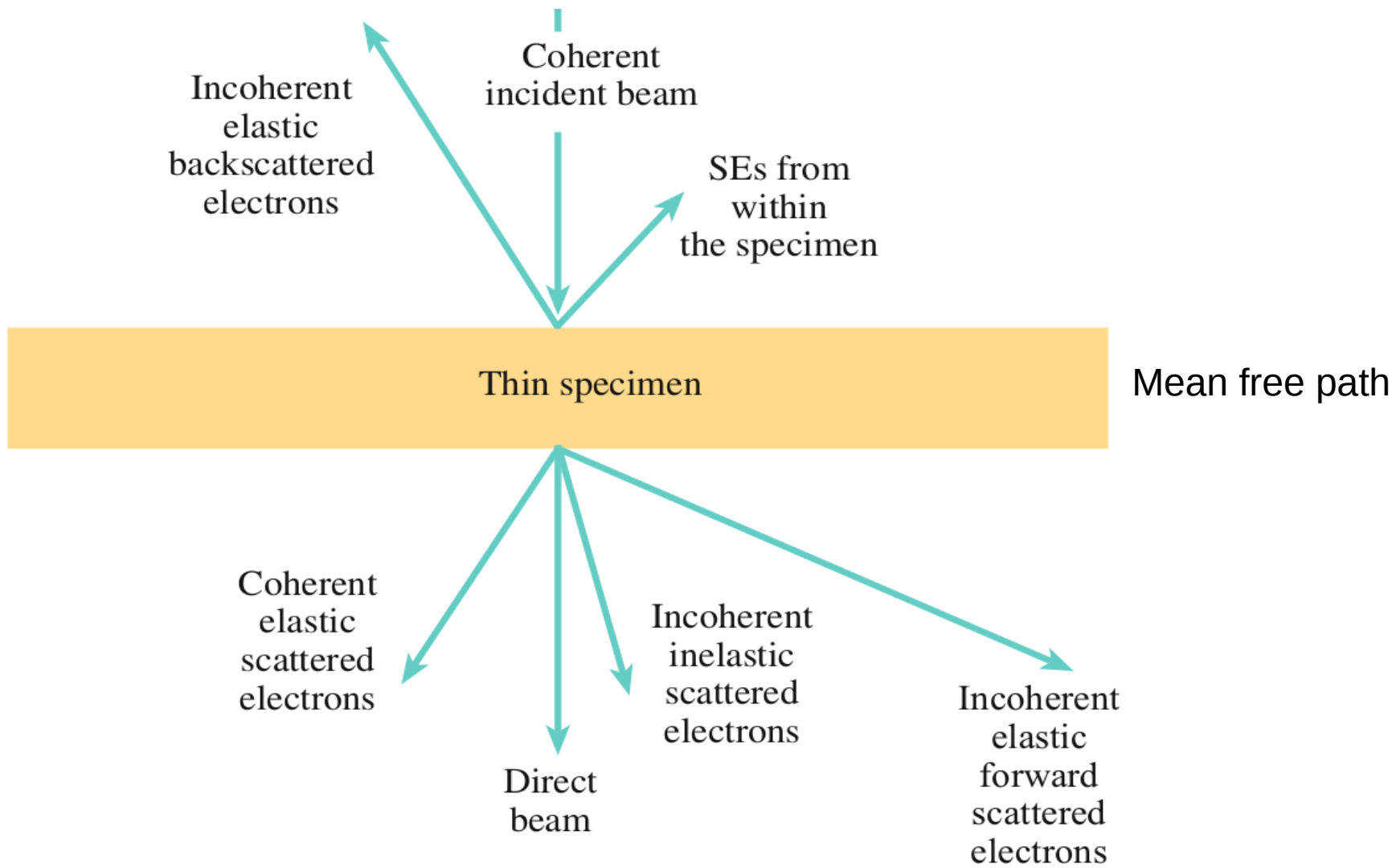


Virus (TEM)

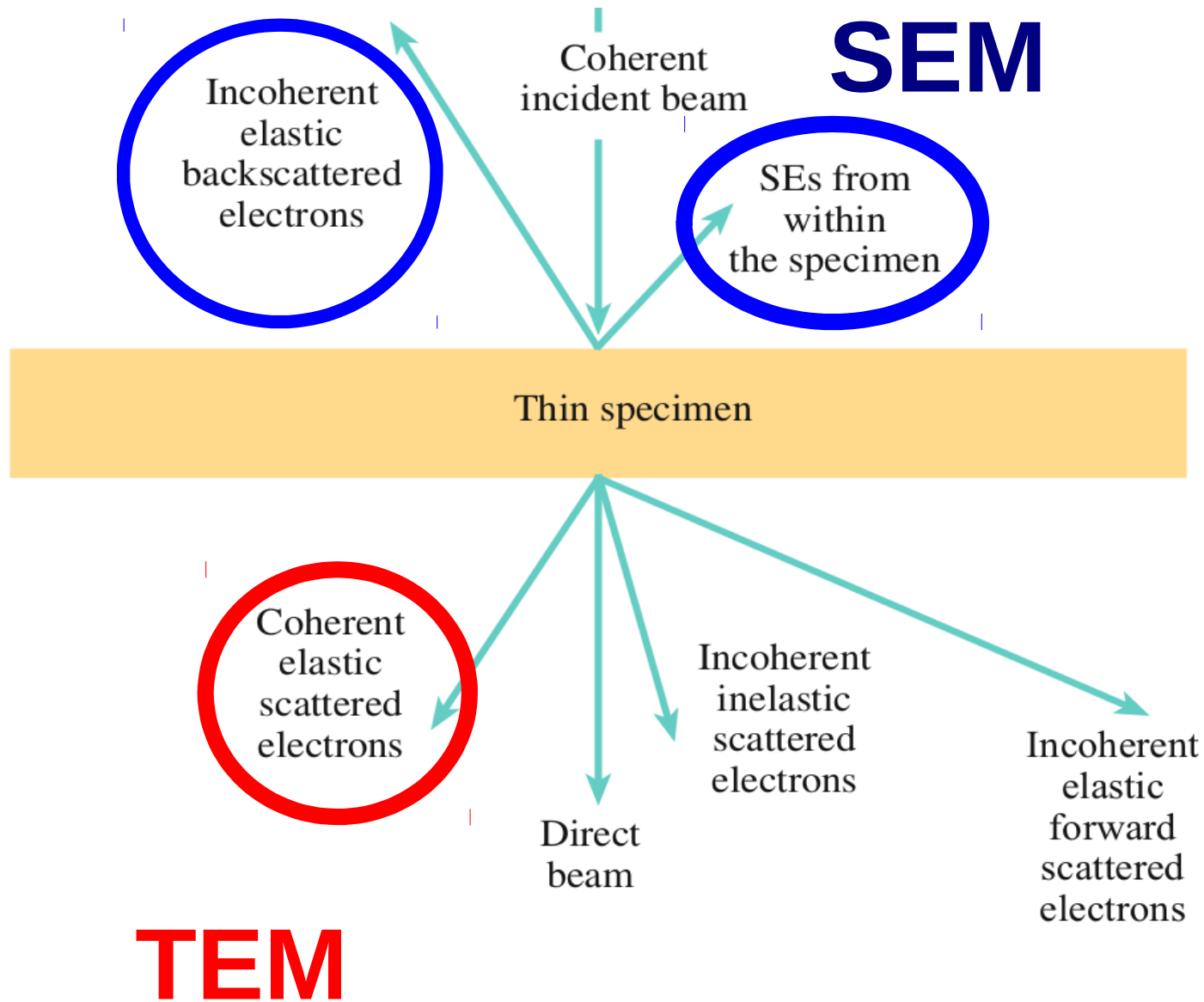


Bacteriophage (TEM)

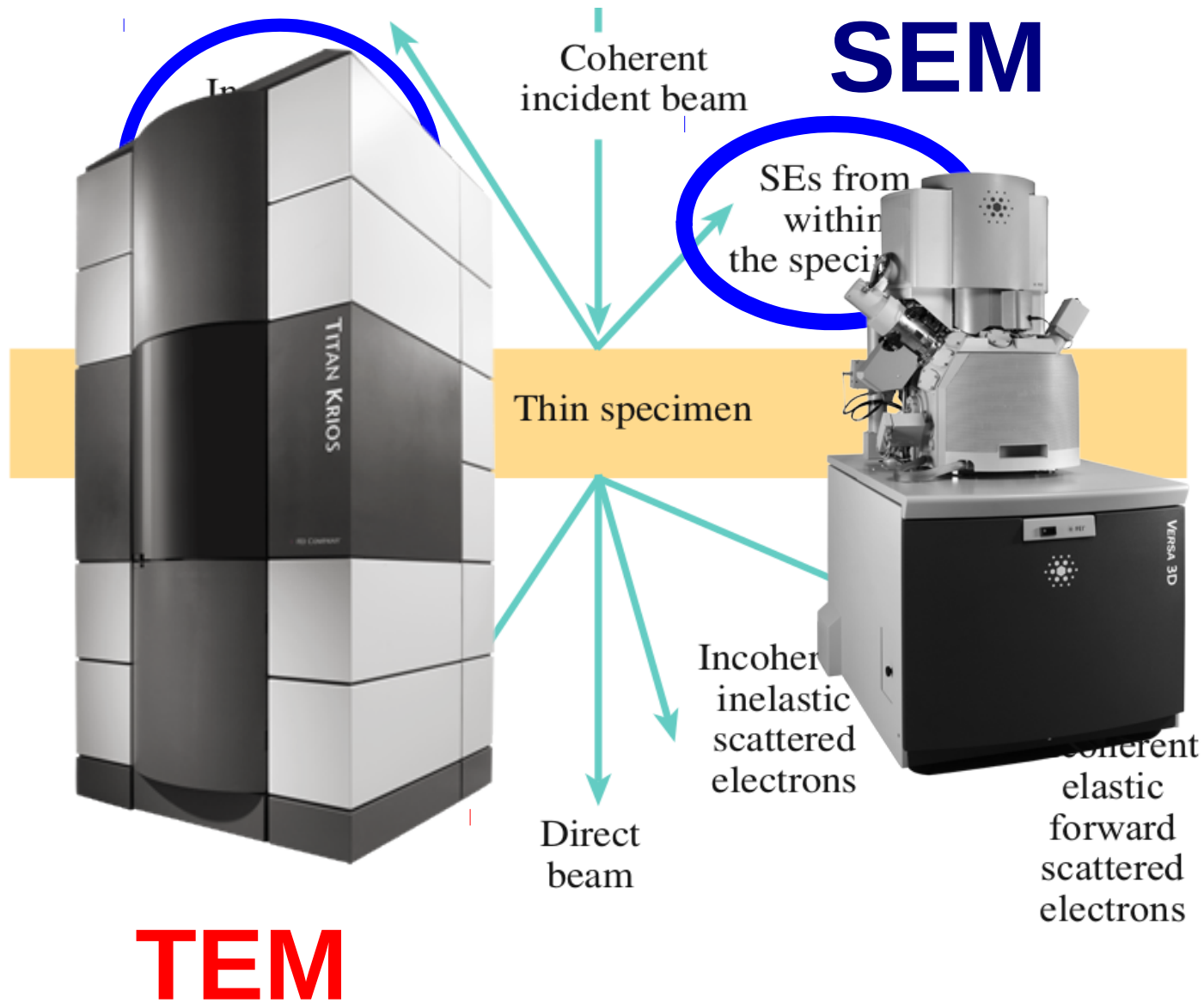
Interaction of electrons with matter



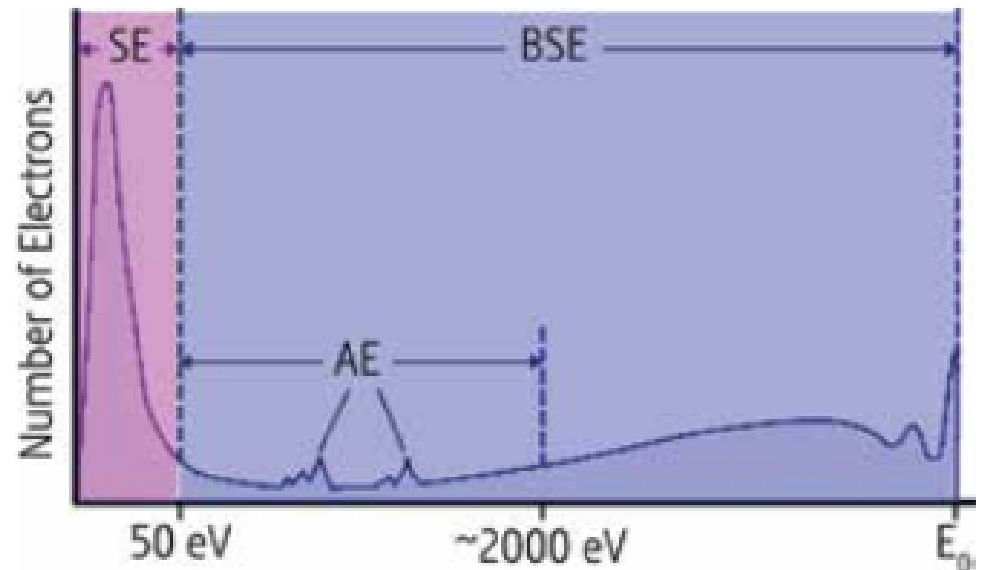
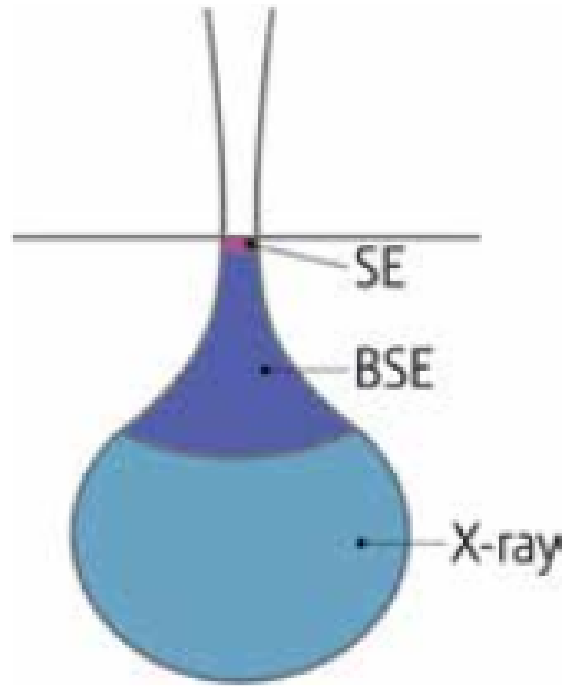
Interaction of electrons with matter



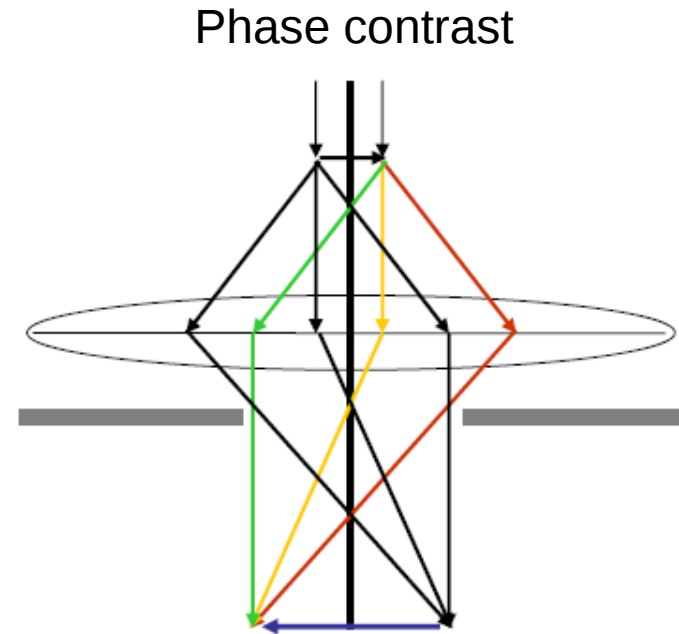
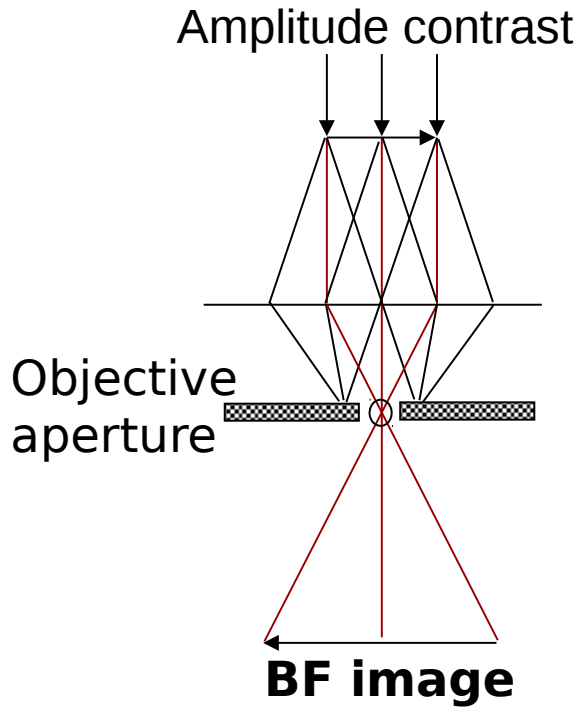
Interaction of electrons with matter



Scanning electron microscopy



Transmission electron microscopy



- difference in intensity in two adjacent area

- Transmitted and diffracted waves travel through different distances

Sample preparation techniques



● Thin section methods

● Heavy metal staining and shadowing

● Plunge freezing

● High pressure freezing

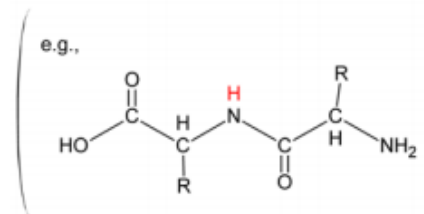
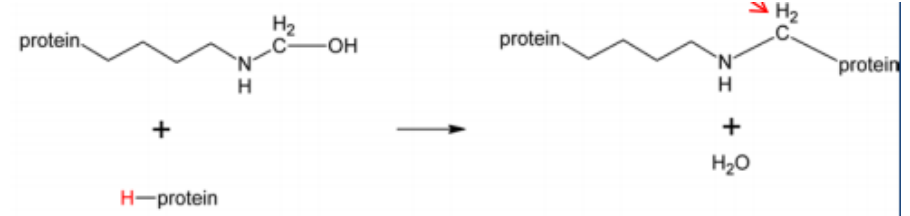
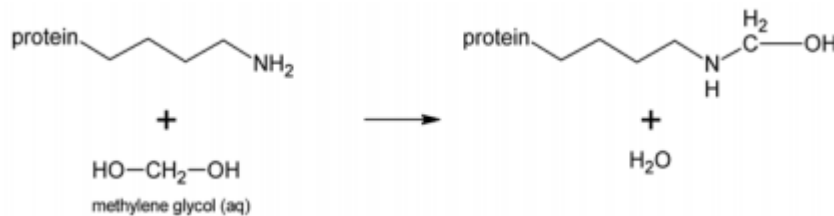
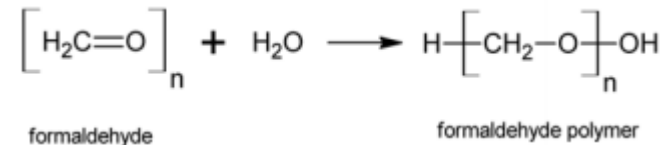
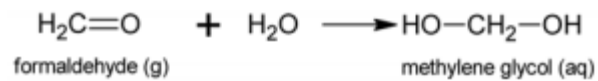


Thin section methods

- 1 Chemical fixation (formaldehyd, glutaraldehyde, osmium tetroxide)
- 2 Dehydration (EtOH, acetone)
- 3 Plastic embedding
- 4 Sectioning

Thin section methods

Chemical fixation



- 2% solution in buffer or water
- variable duration – sample thickness (2-24hours)

Thin section methods

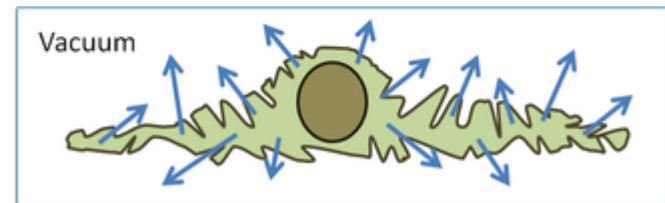
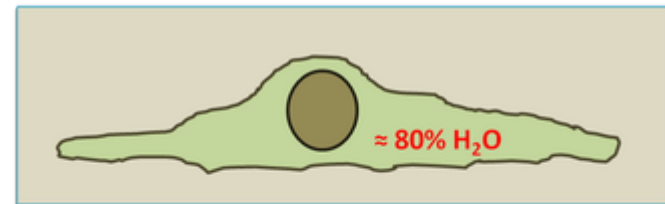
Dehydration

- high vacuum in the microscope
- EtOH, acetone
- successive increase of dehyd. agent concentration

- 30% acetone - 15 mins
- 50% acetone - 15 mins
- 70% acetone - 15 mins
- 90% acetone - 15 mins
- 100% acetone - 3 changes

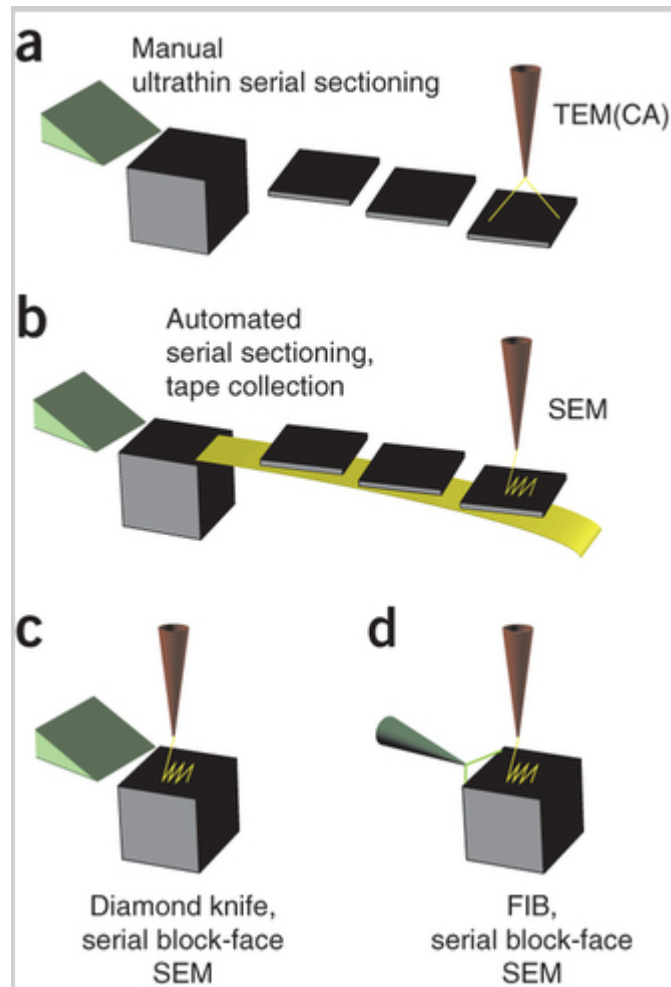
Drawbacks:

- contraction of protein lipids
- sample shrinking up to 40%
- formation of various artefacts



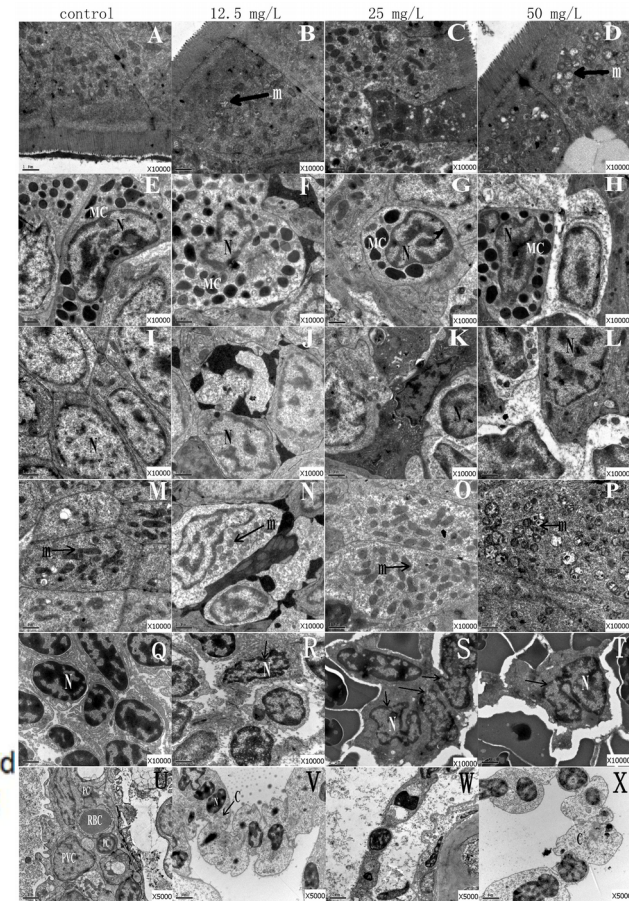
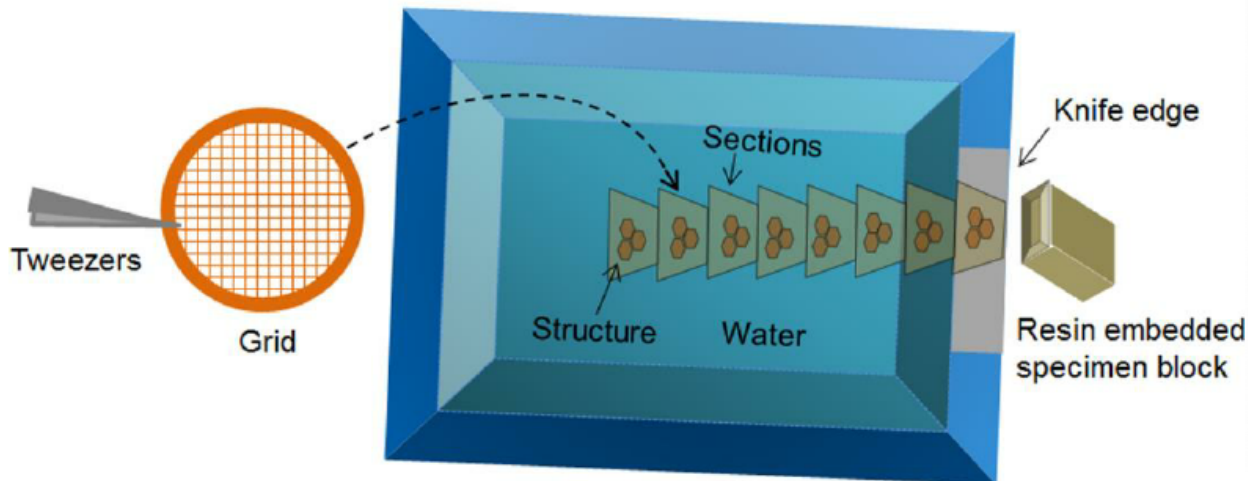
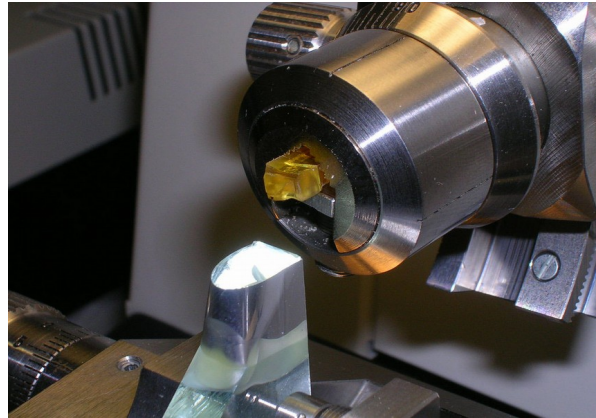
Thin section methods

Sectioning



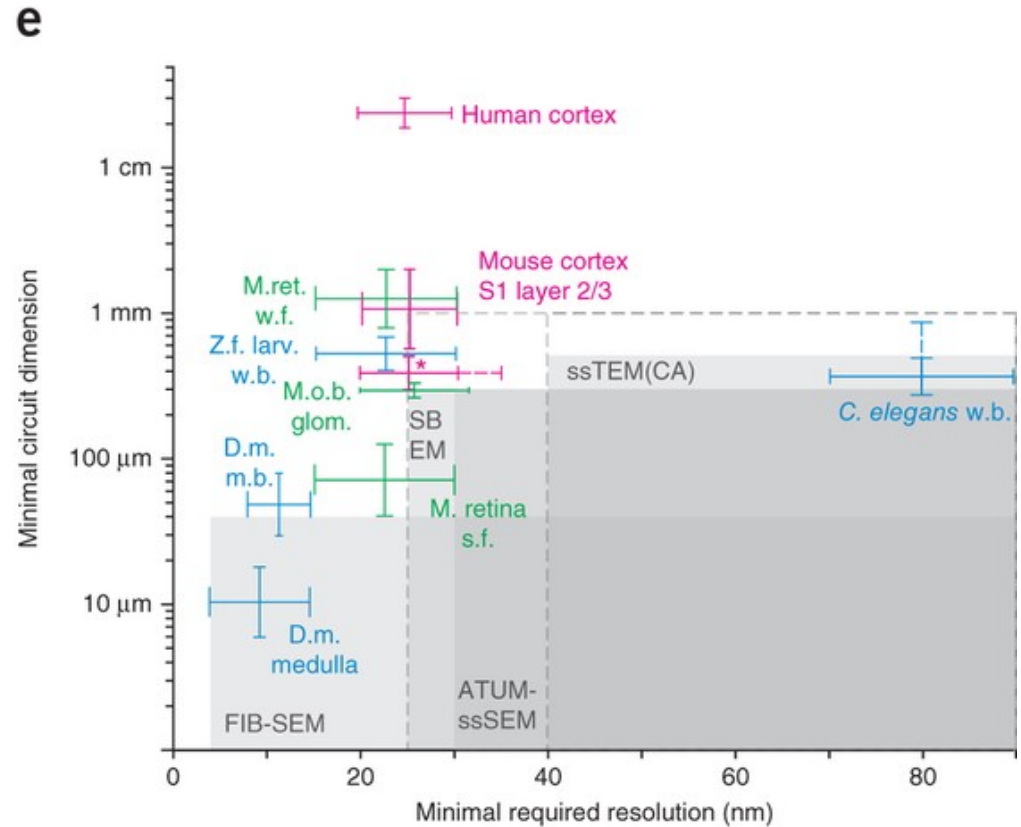
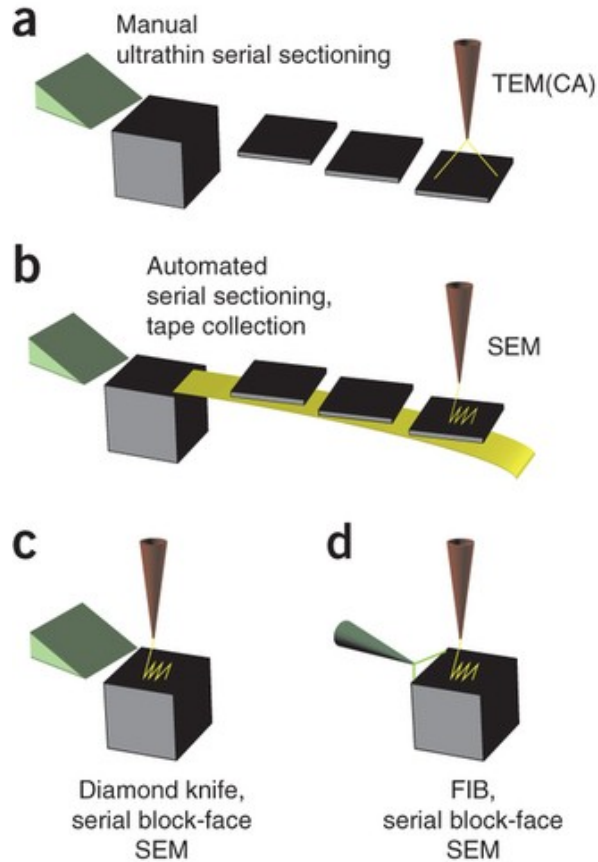
Thin section methods

Mechanical sectioning for TEM



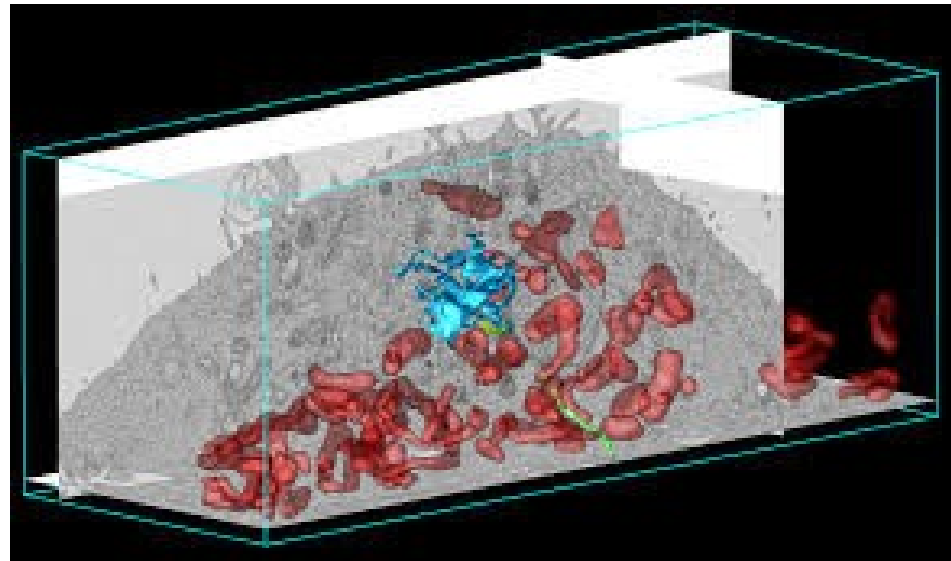
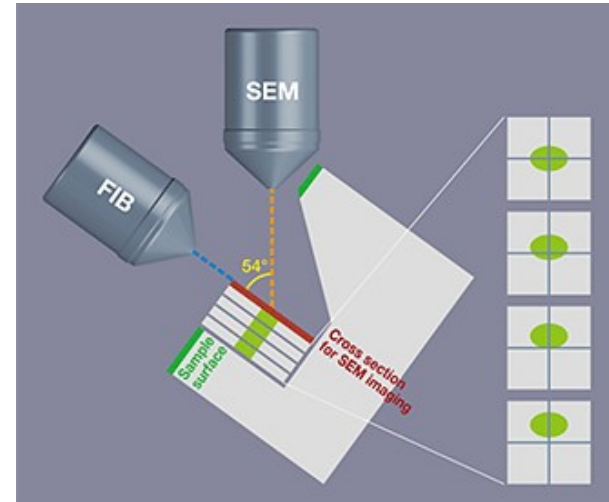
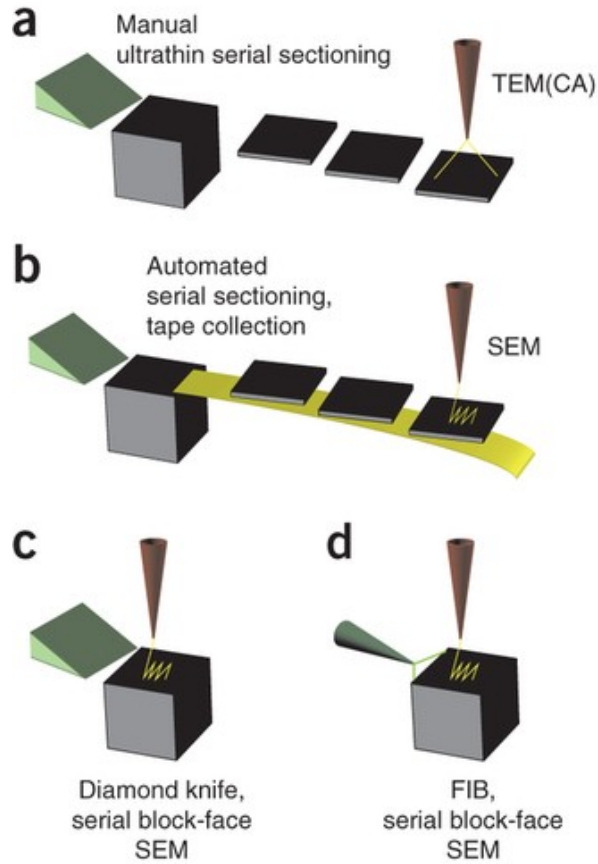
Thin section methods

Mechanical sectioning/block-face for SEM



Thin section methods

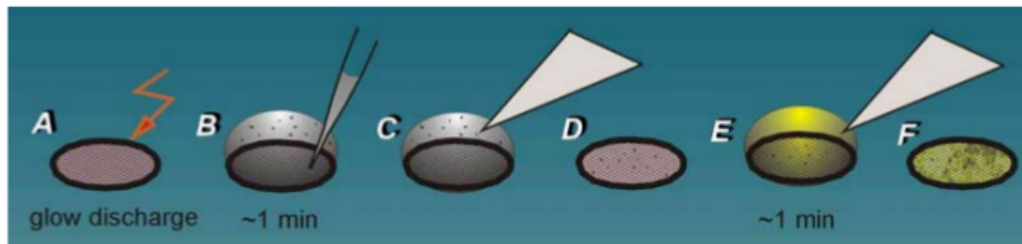
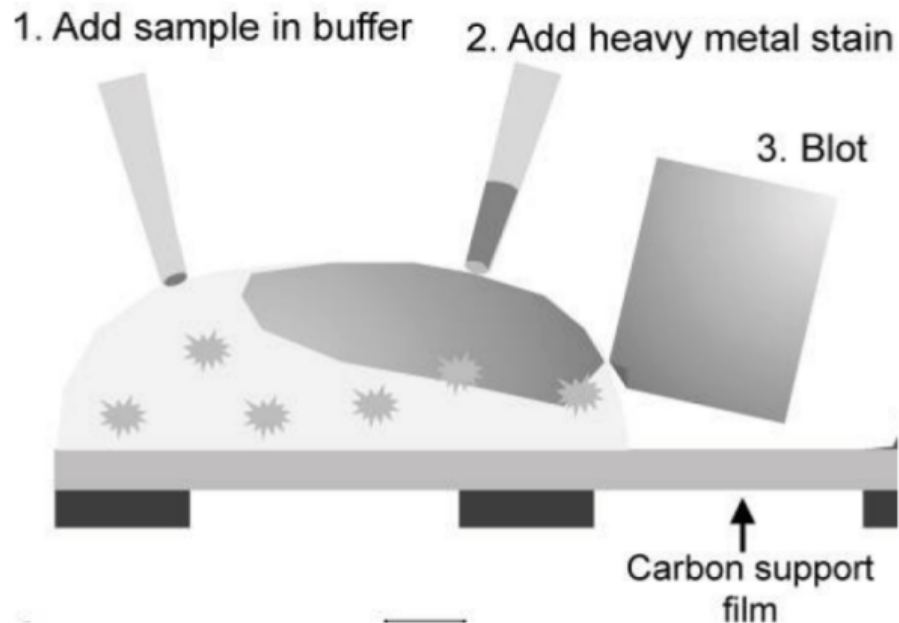
Focused ion beam block-face for SEM



Heavy metal staining

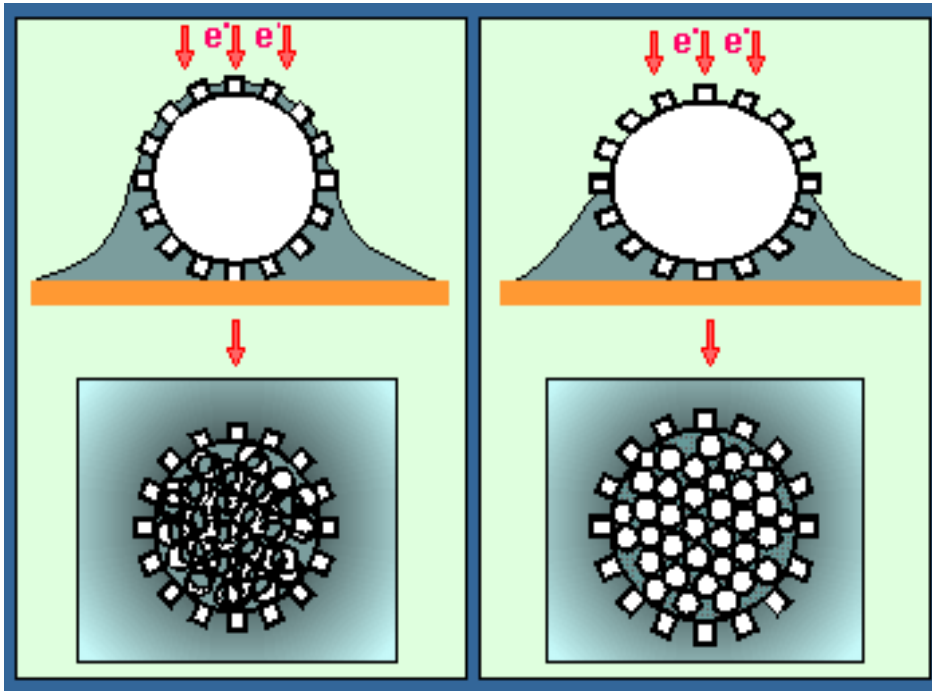
Negative staining

Stains: uranyl acetate (pH=4)
uranyl formate (pH=4)
ammonium molybdenate (pH=7)
phosphorus tungstanate (pH=7)



Heavy metal staining

Negative staining

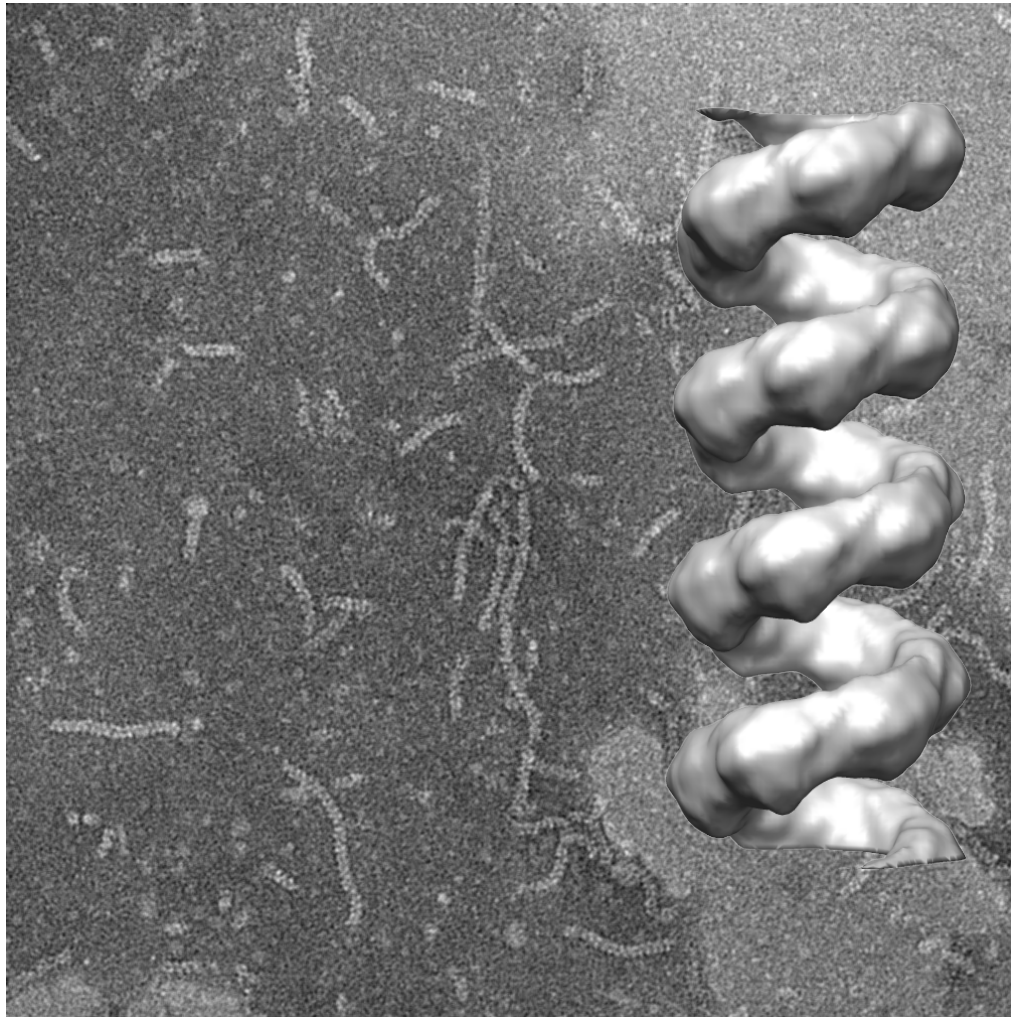
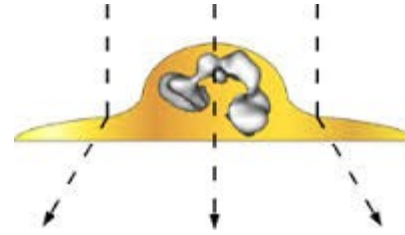


Pros: quick sample screening
high amplitude contrast
less prone to beam damage

Cons: limited resolution (20Å)
flattening artefacts
denaturation of proteins

Heavy metal staining

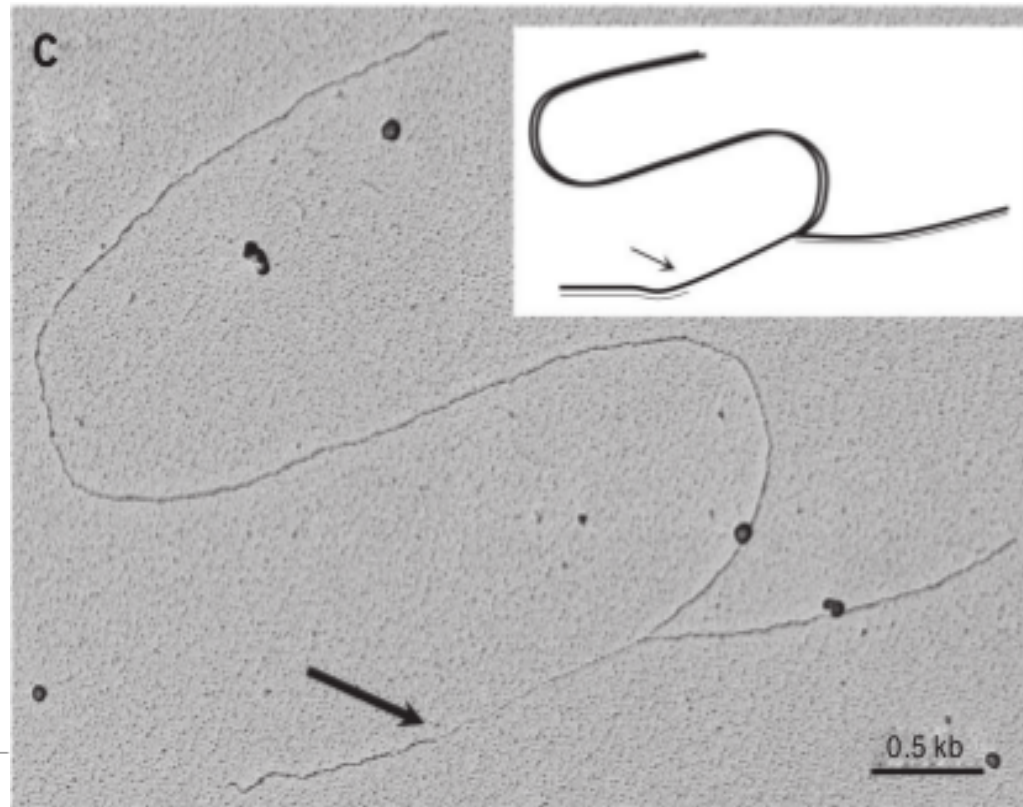
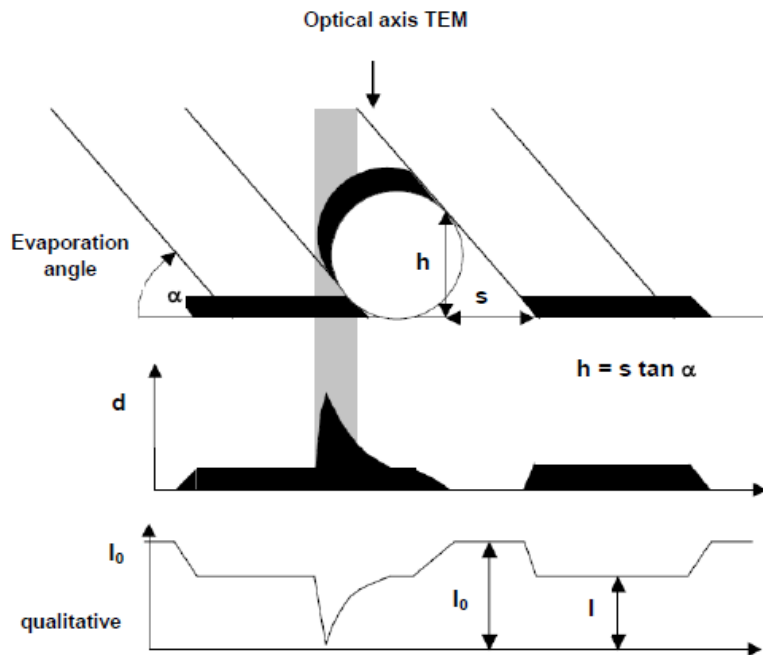
Negative staining



Heavy metal staining

Metal shadowing

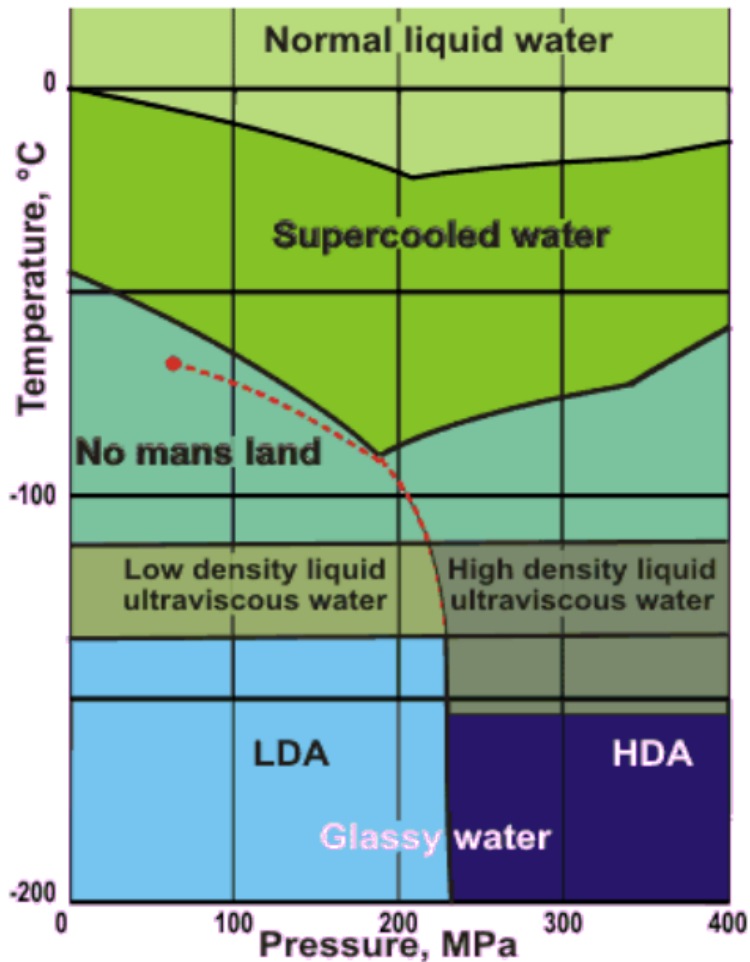
- DNA visualization



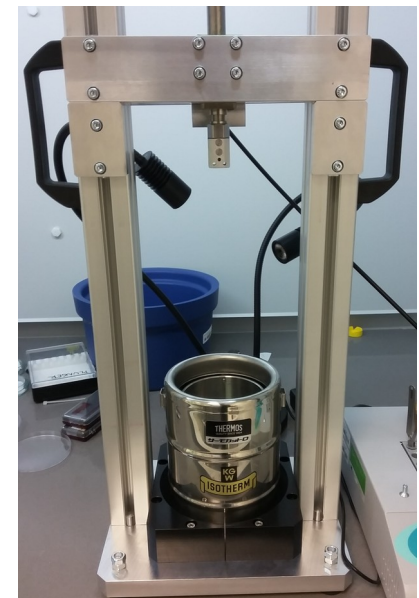
Thin section methods and heavy metal staining

- non-physiological conditions during sample preparation
 - artefacts (changes in cell structure, depression of proteins)
 - extremely toxic chemicals used during sample prep (OsO₄)
 - obtainable level of detail limited
-
- + high signal to noise
 - + low dose sensitivity
 - + robust (easy sample handling)

Plunge freezing

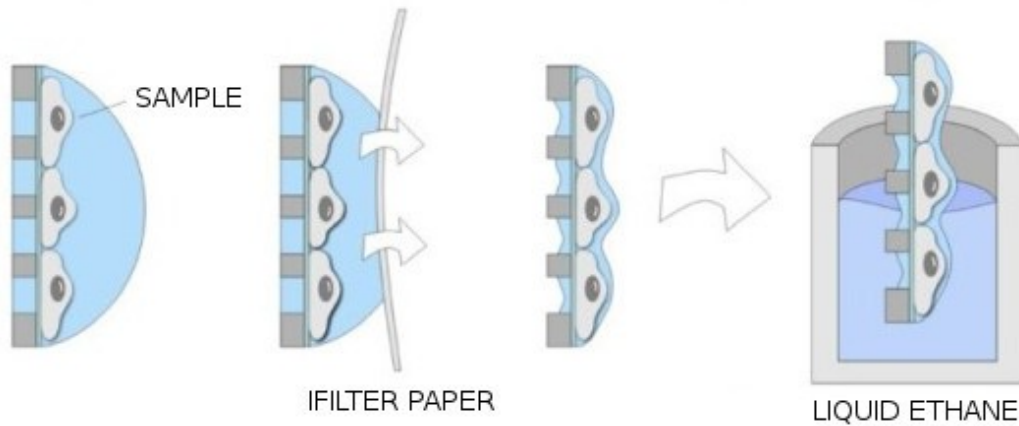
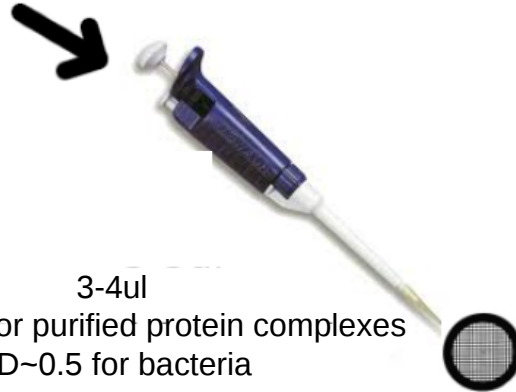


- Rapid immersion of buffered sample into cryogen
 - Cryogens: liquid ethane
 - ethane:propane mixture
- Vitrification has to be fast ~ 1000 K/s
- Possible only for samples with thickness $\sim < 10\mu\text{m}$
- => amorphous ice
- => thin layer (200-600nm)

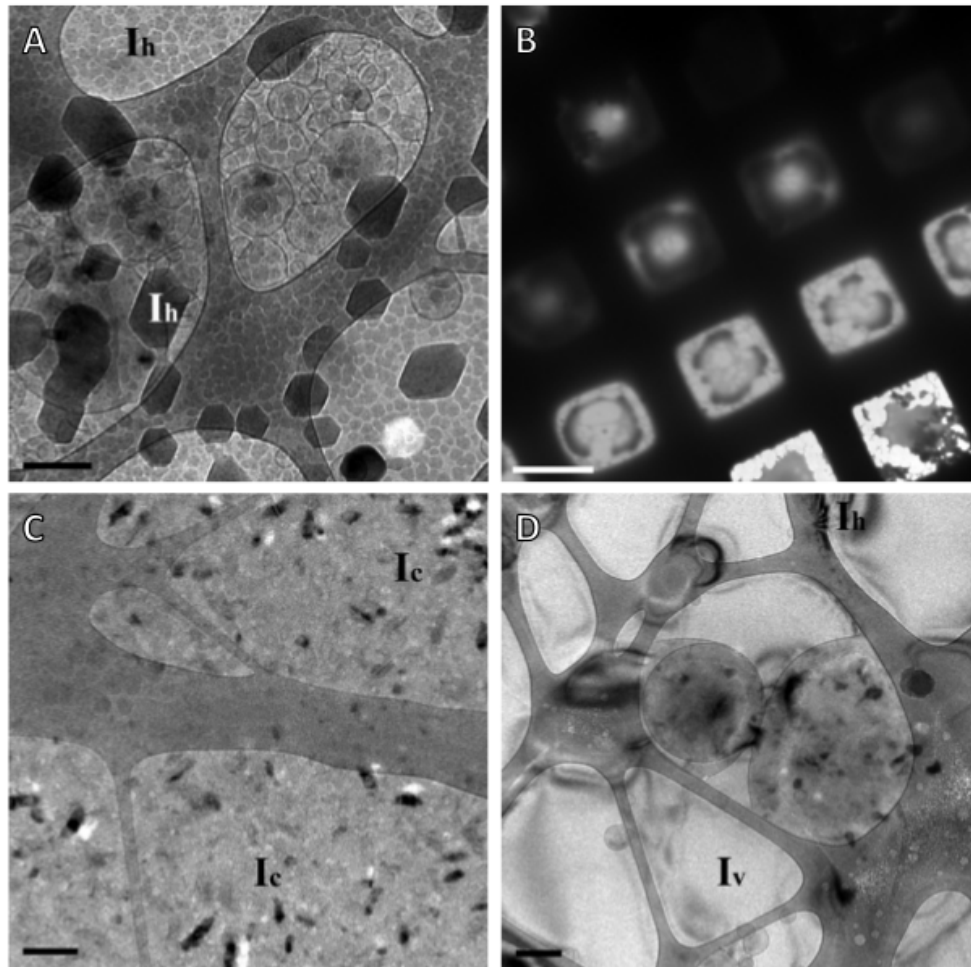


Plunge freezing

Cryogen	Melting point (°C)	Boiling point (°C)	Cooling rate (10^3 °C/s)	Relative cooling efficiency*
Ethane	-183	-89	-260 – -258	1.3
Liquid nitrogen	-210	-196	-272	0.1
Propane	-189	-42	-263 – -261	1.0
Freon 22	-160	-41	-267 – -265	0.7

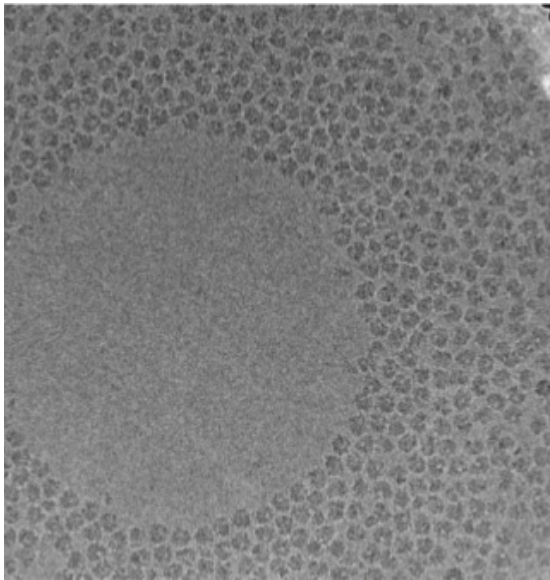
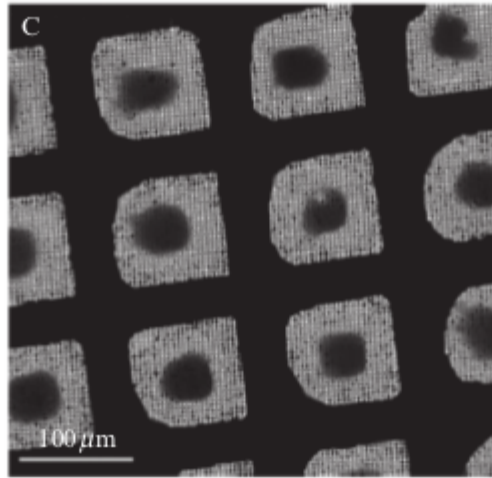


Plunge freezing

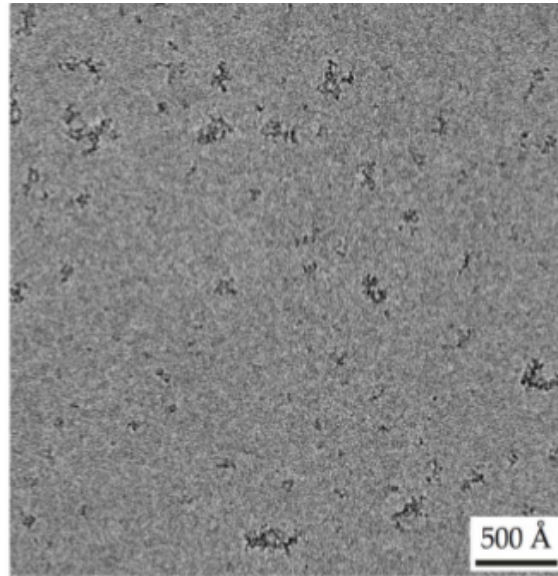


- Sample frozen in hydrated state
- Amorphous ice
- Sample has to be kept at temperatures above devitrification point (~-135C)
- Internal structures can be visualized
- High resolution information is retained
- Possible problems: ice thickness
- hexagonal ice, cubic ice

Plunge freezing

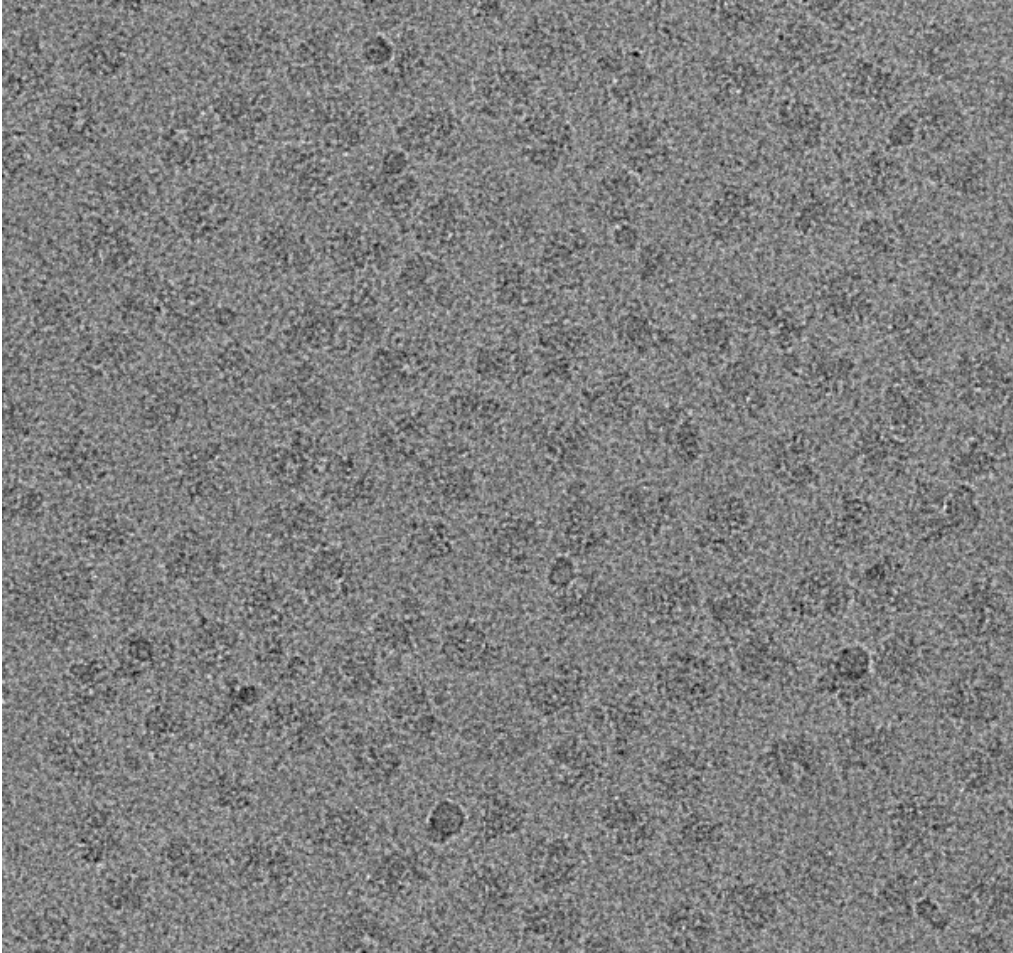


Extrusion of particles from thin ice



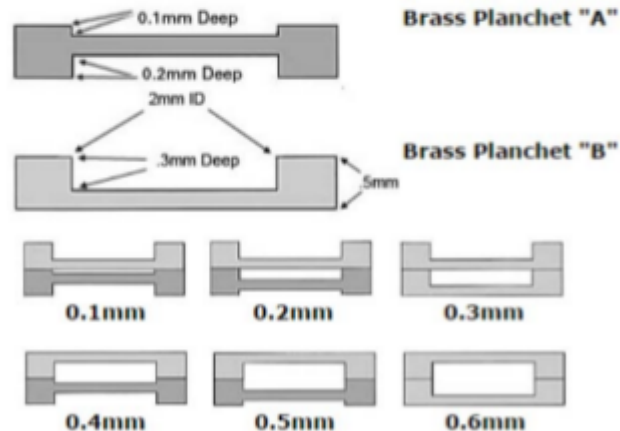
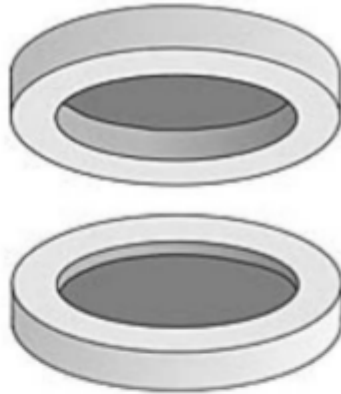
Denaturation at air water interface

Plunge freezing



- Cons:
-
- Low signal to noise
-
- Prone to radiation damage
-
- More delicate sample handling required
-

High pressure freezing



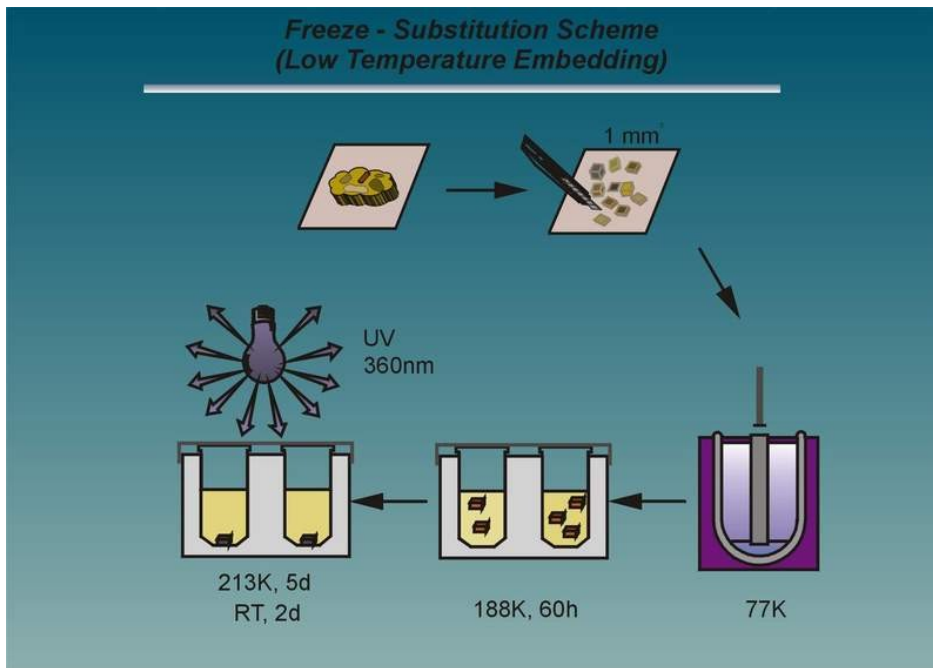
- Vitrification of samples with thickness
- 10 – 200 μm
-
- 2000 bars, liquid nitrogen, 20 ms
- Eukaryotic cells, tissues,...
- Coupled with freeze substitution and sample thinning using cryo-ultramicrotomy, ultramicrotomy, or focused ion beam milling

High pressure freezing

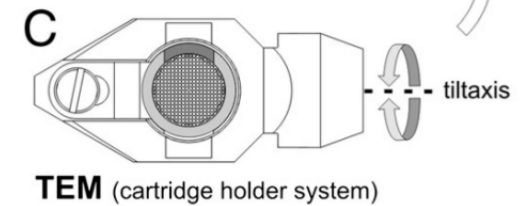
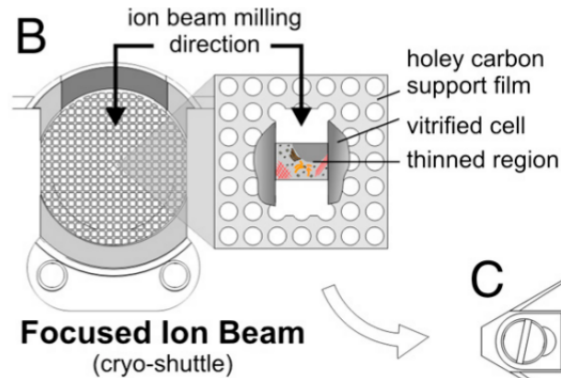
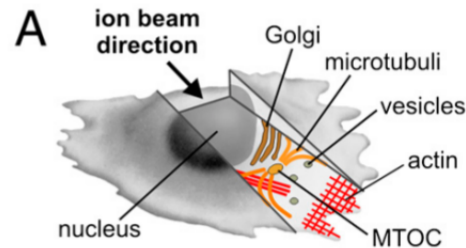
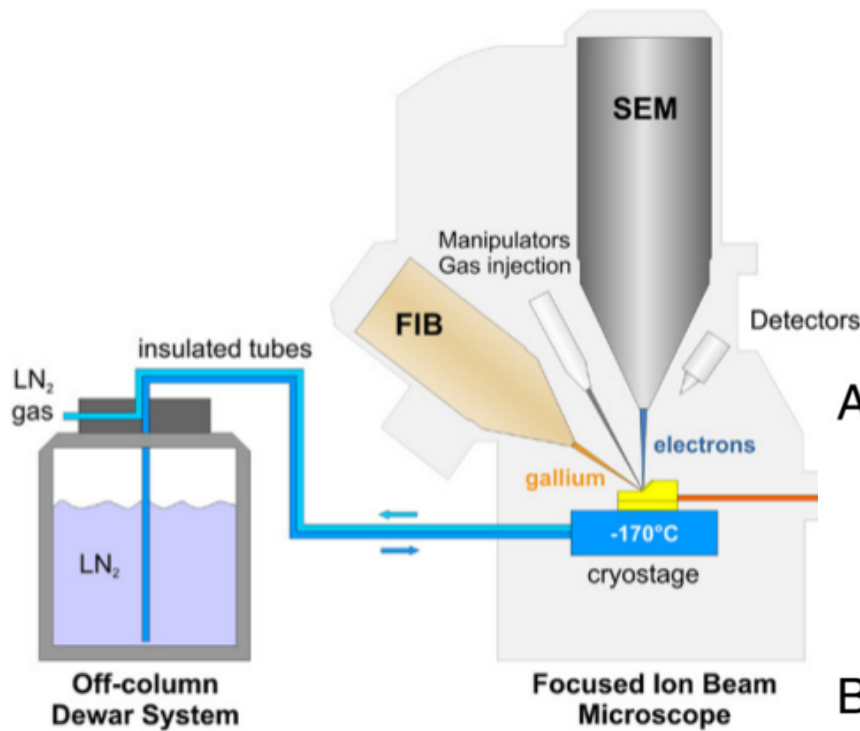


Freeze substitution

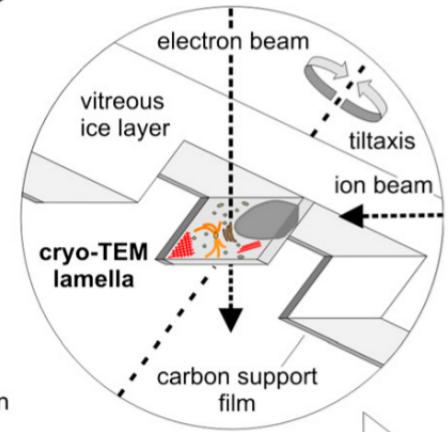
- Reduce ultra-structure changes at room temperature
- due to dehydration as seen at ambient temperature
-
- Dehydration at temperatures below -70°C
- (acetone typically -90°C)
-
- Fixatives are evenly distributed before cross-linking at ambient temperature
-
- Resin embedding for ultramicrotomy
- at room temperature



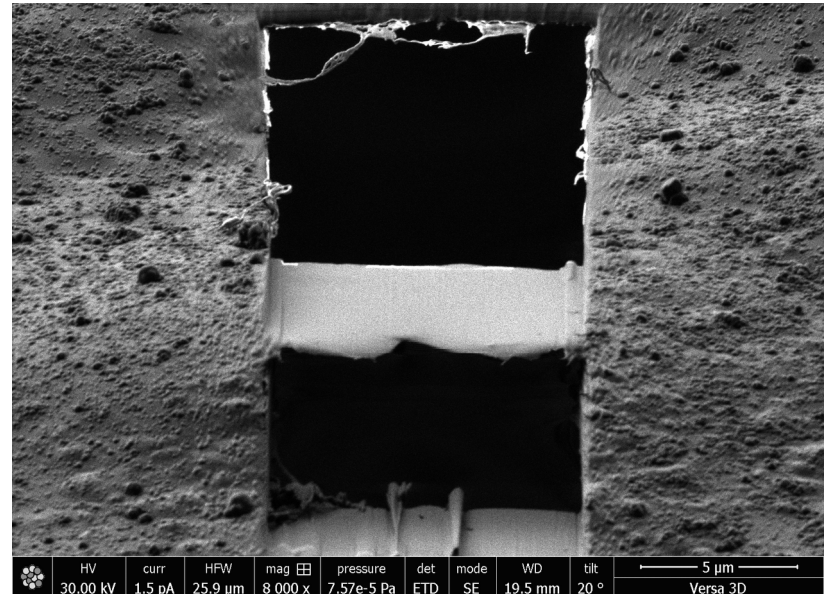
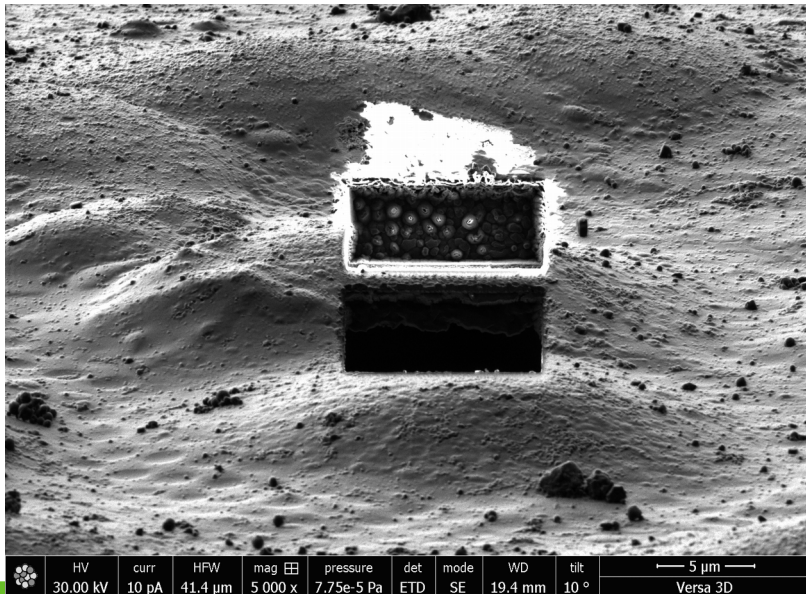
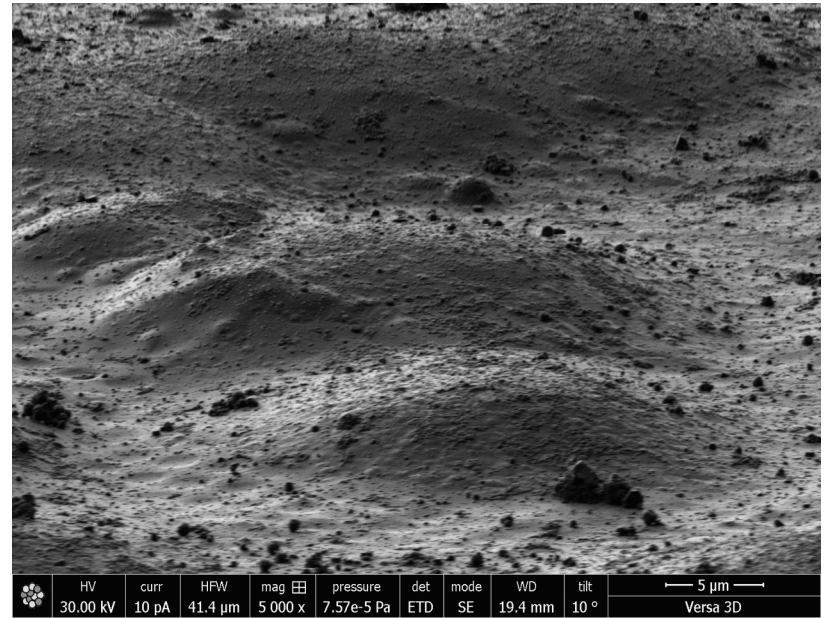
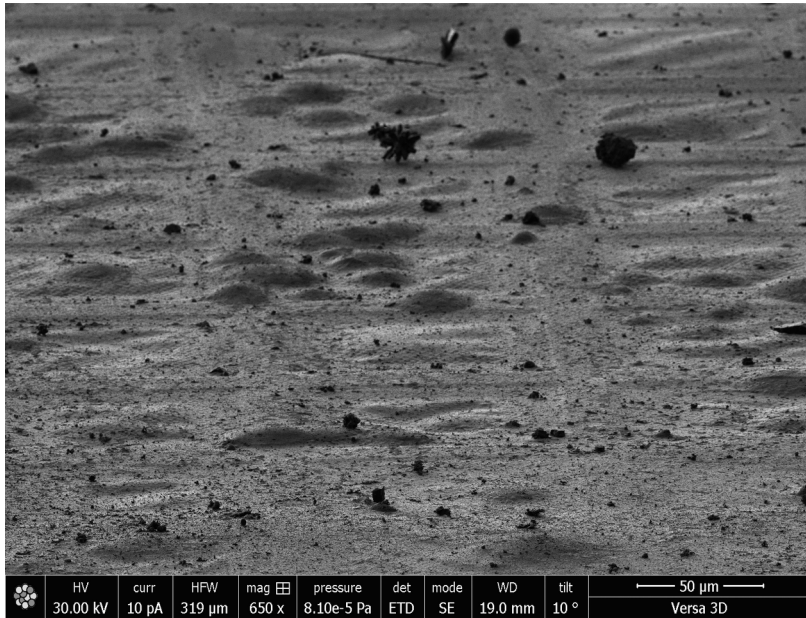
Focus ion beam milling



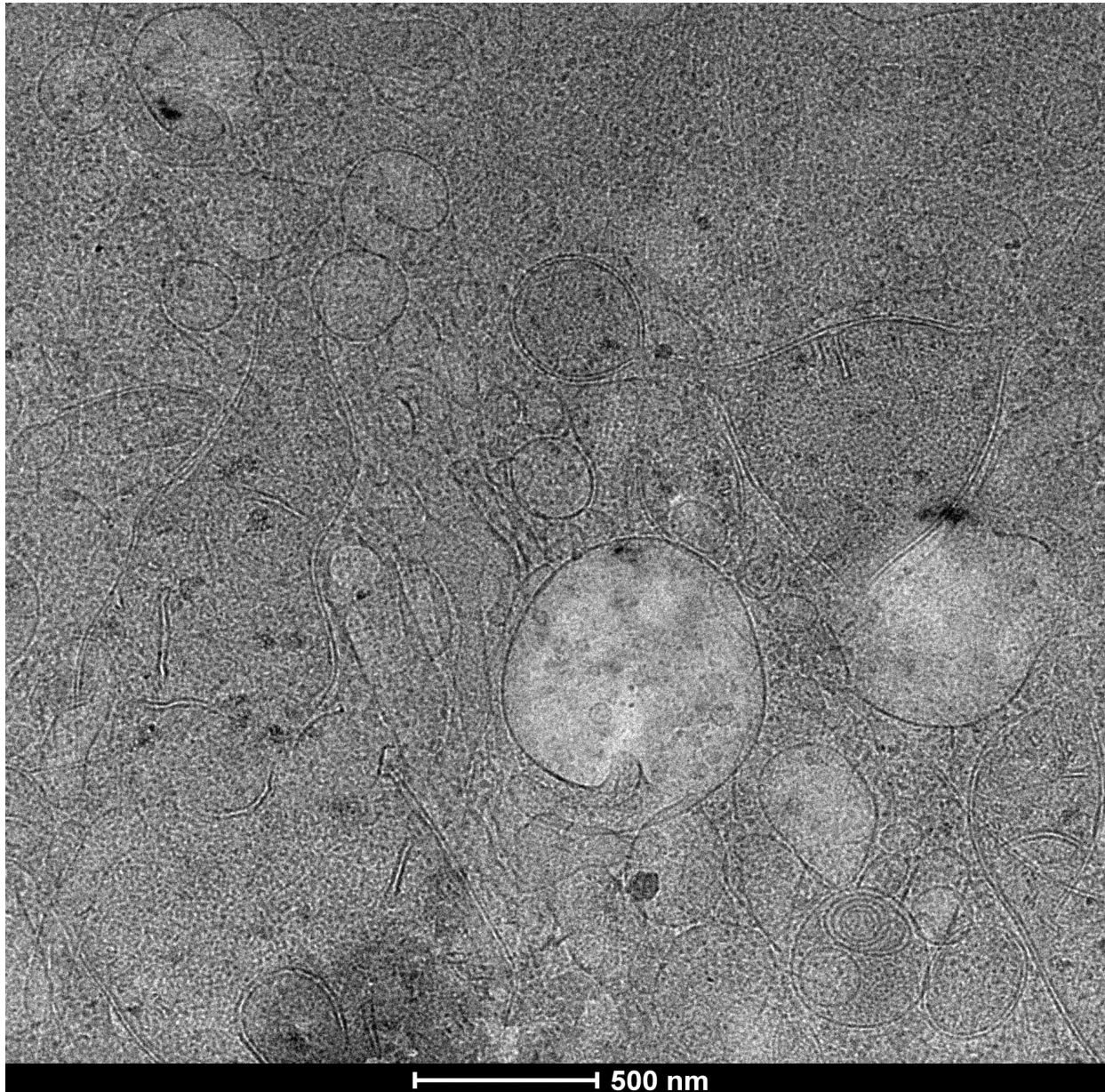
D Cryo-Electron Tomography



Focus ion beam milling



Focus ion beam milling





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