CEITEC

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FB820

Lecture 4

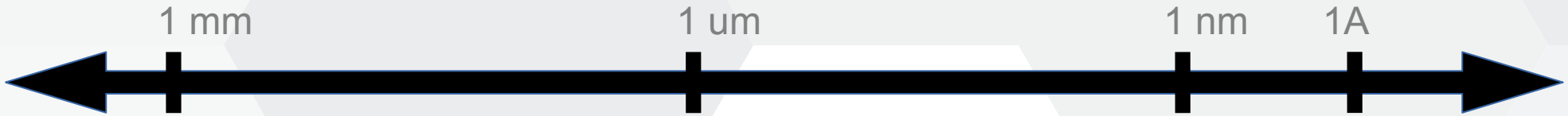
Sample Preparation

Jiri Novacek

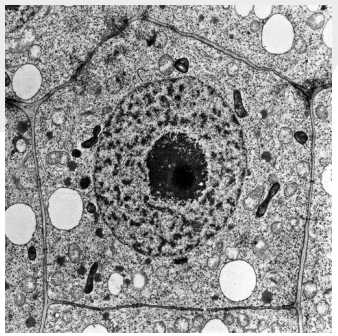
Content

- sample preparation for SEM (2D imaging)
- structural TEM sample preparation
- volume EM sample prep.

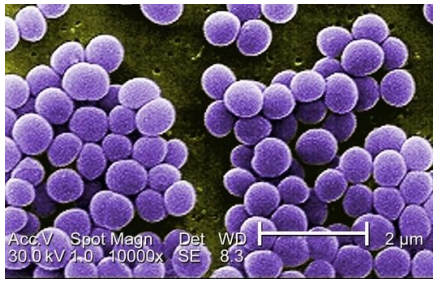
Scales attainable with electron microscopy



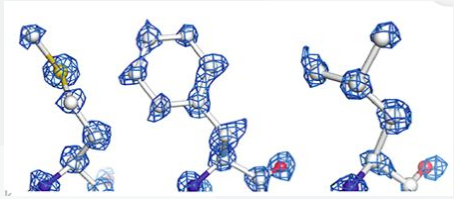
Tick (ESEM)



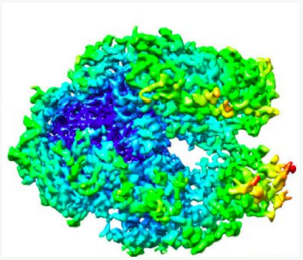
Plant cell (TEM)



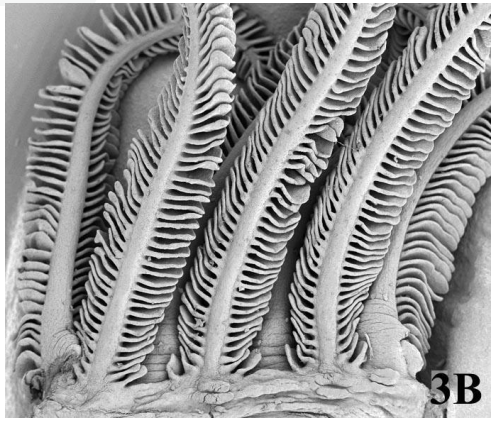
Bacteria (SEM)



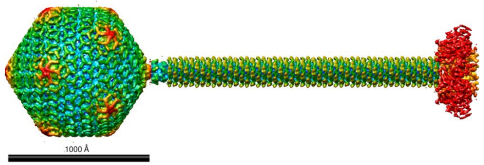
apoferritin @1.2A (TEM)



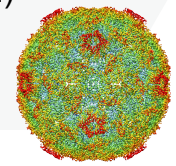
RNA polymerase (TEM)



Plant (SEM)

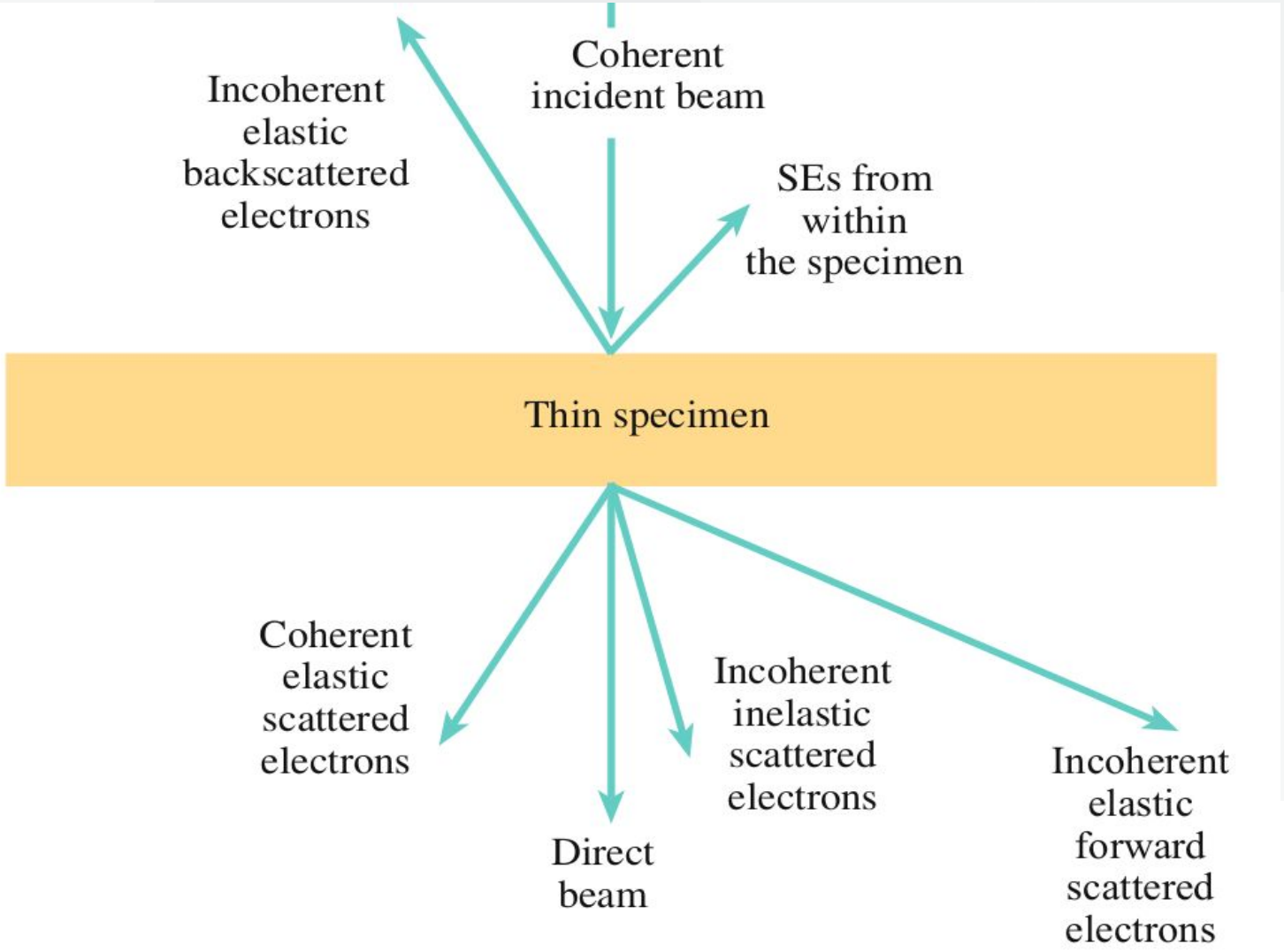


Bacteriophage (TEM)

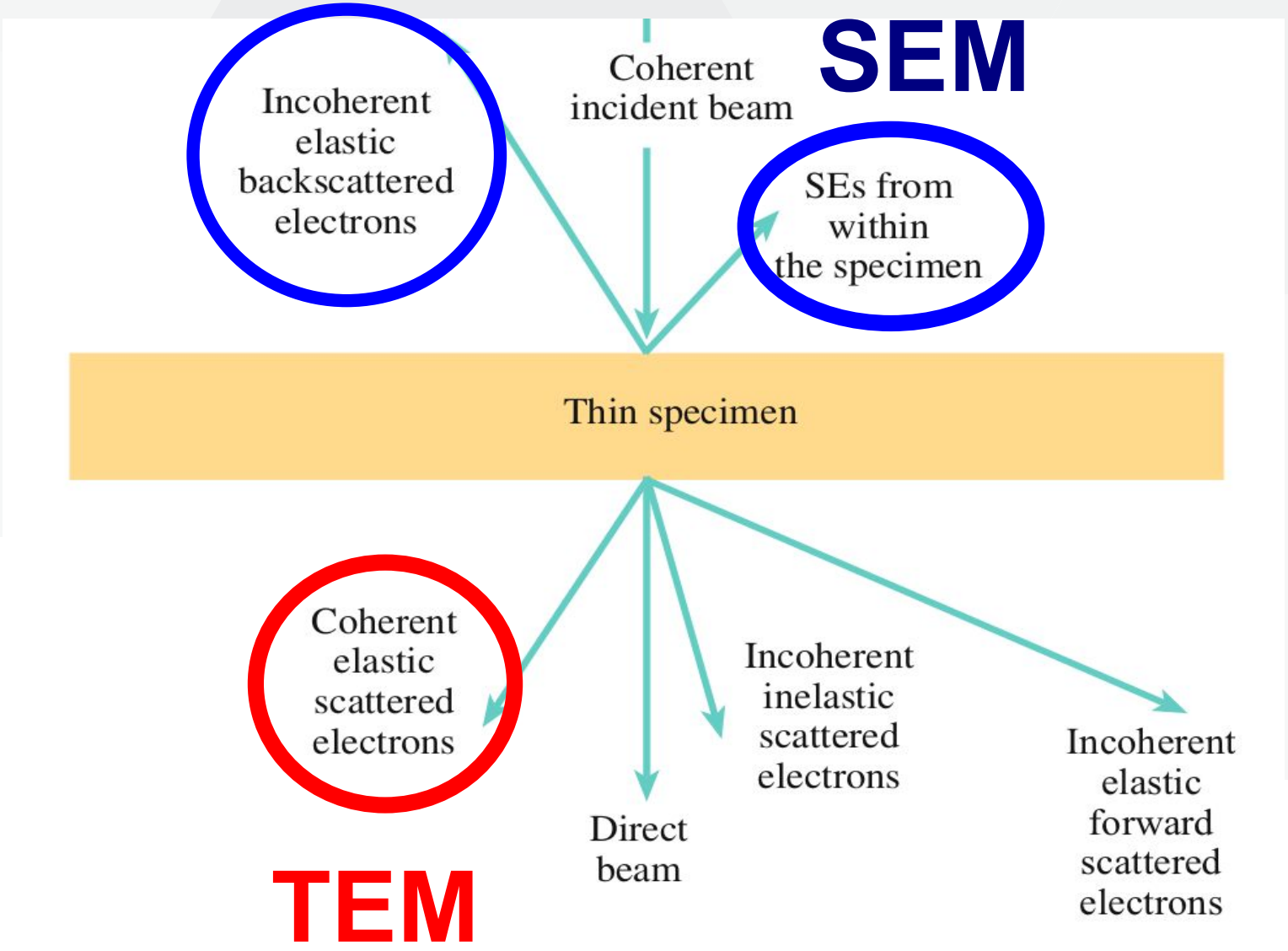


Virus (TEM)

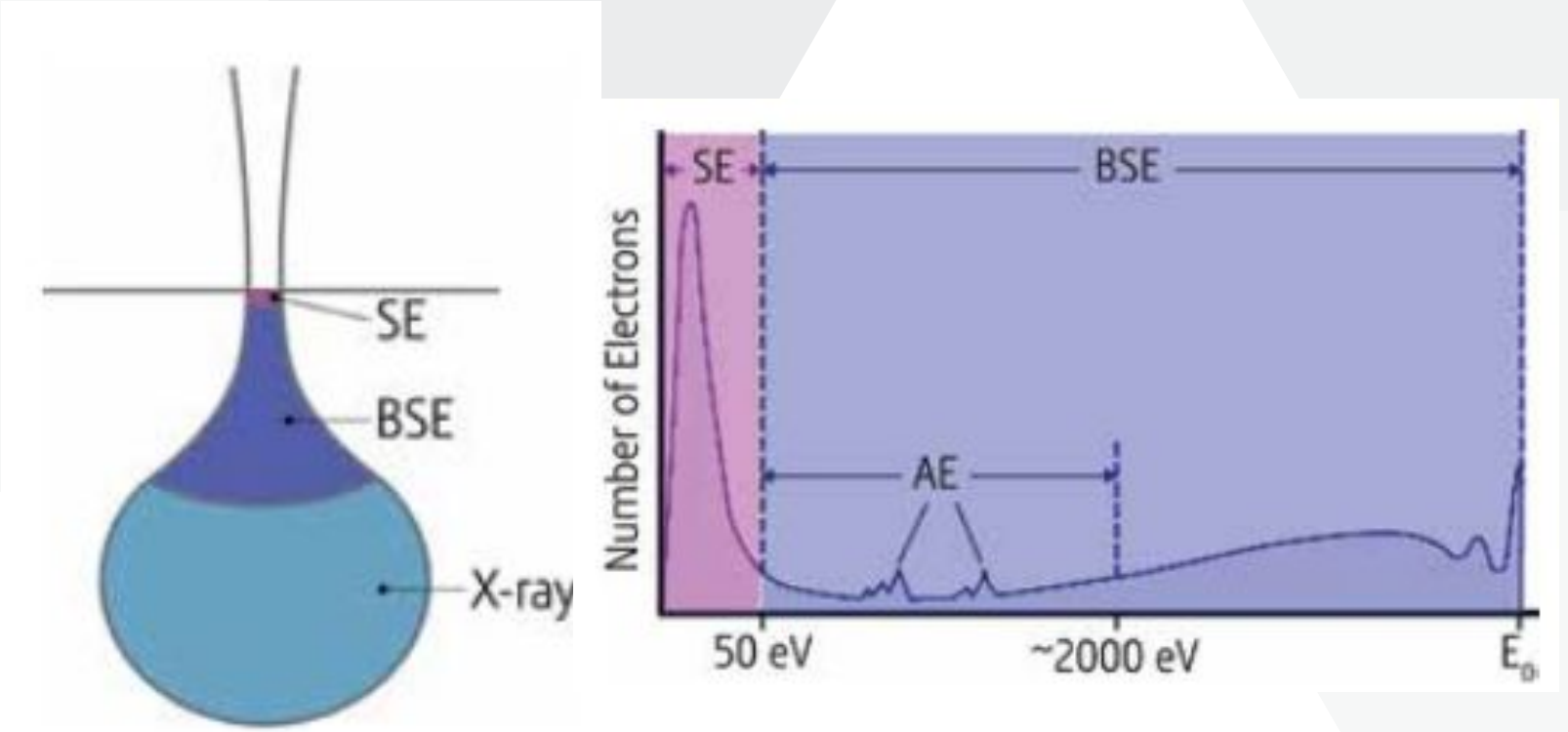
Interaction of an electron with a matter



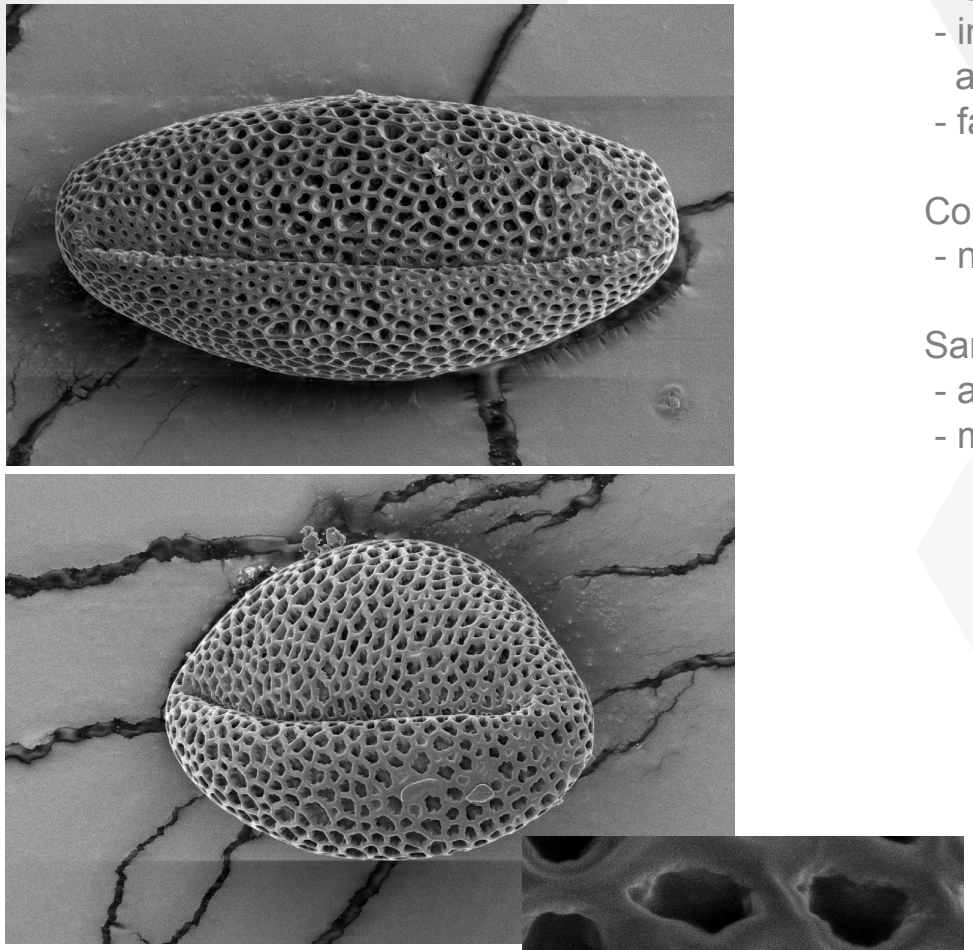
Interaction of an electron with a matter



Scanning electron microscopy



SEM imaging

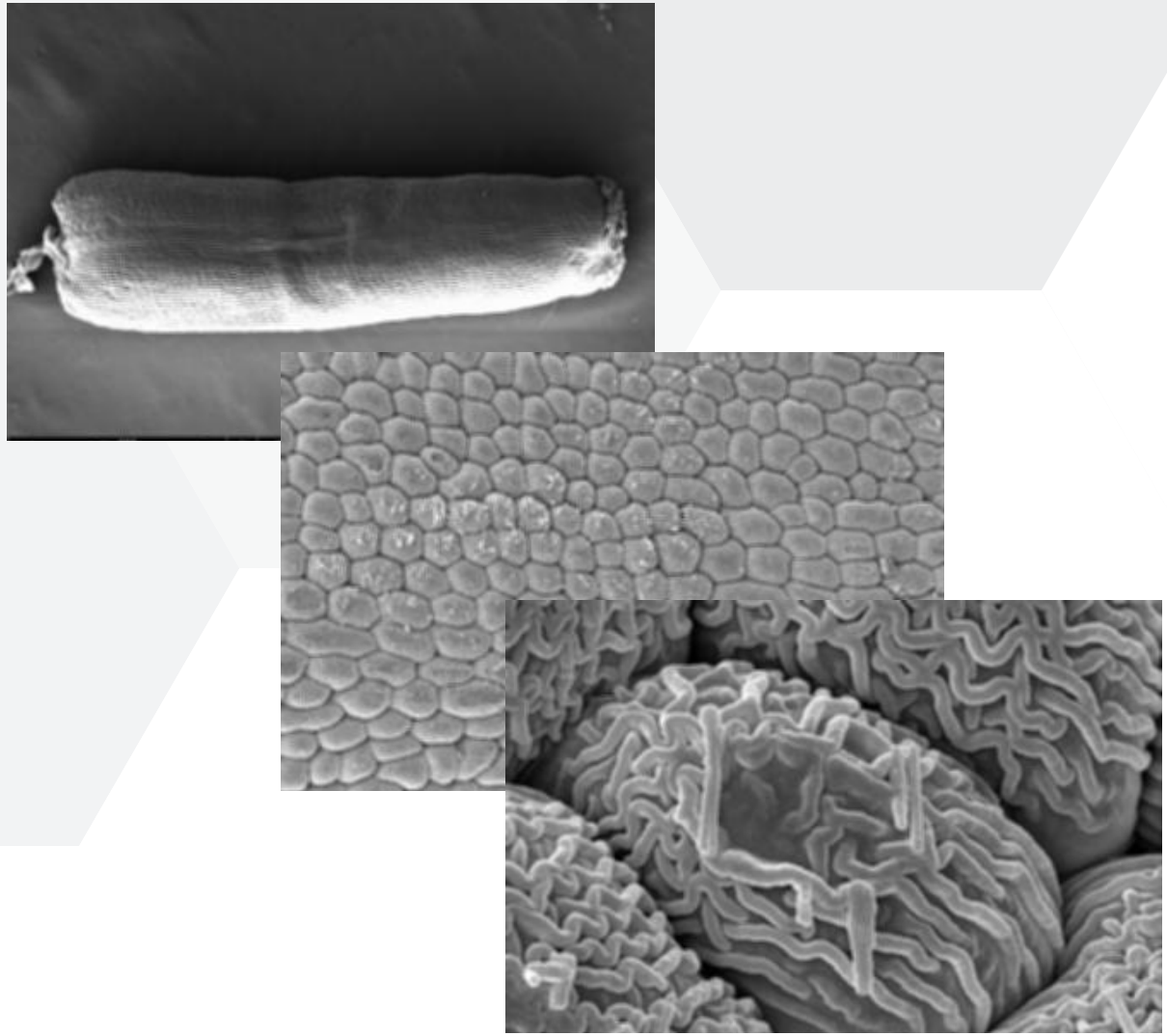


Pros:
- imaging of sample morphology
  at significant scale difference(1mm - 10nm)
- fast sample preparation

Cons:
- non-native (sample dehydrated)

Sample preparation:
- air drying
- metal sputtering (Pt, Au, Ir)

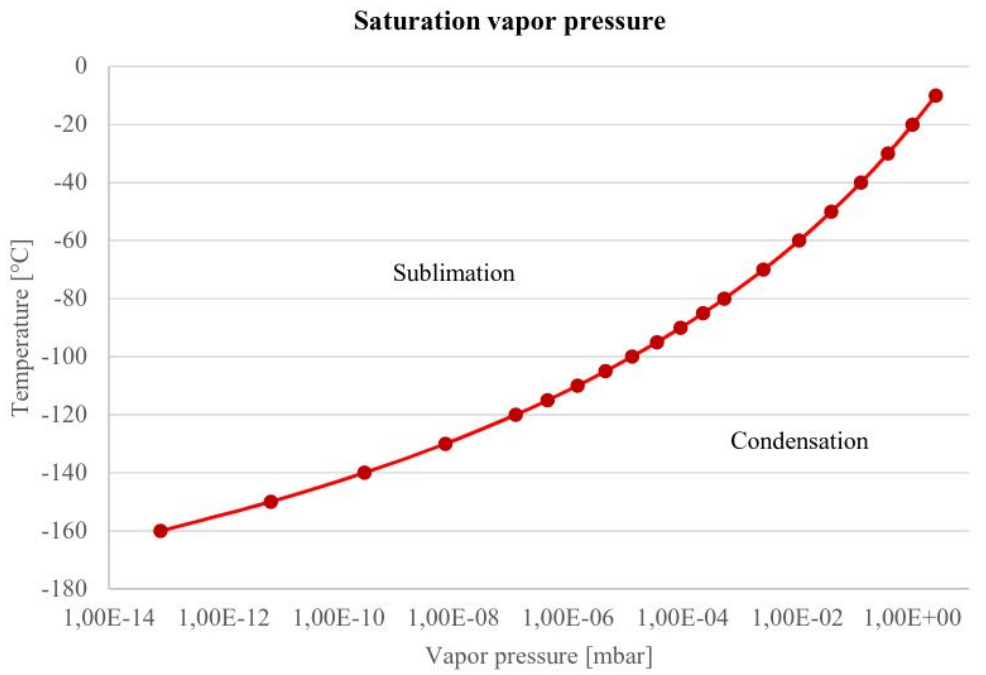
SEM imaging



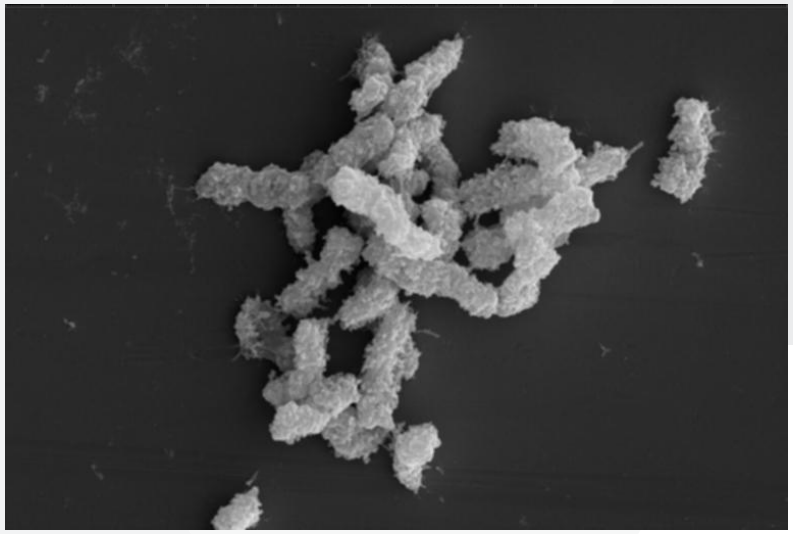
Pros:
- imaging of sample morphology at significant scale difference(1mm - 10nm)
- fast sample preparation

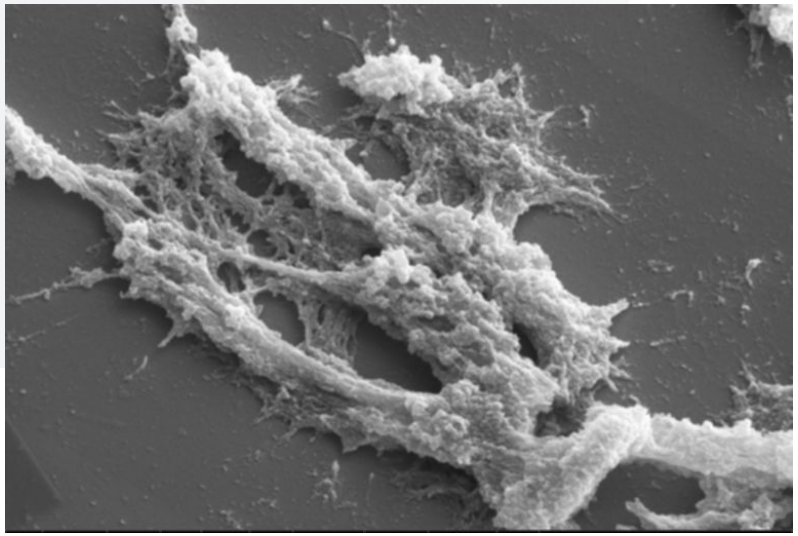
Cons:
- non-native (sample dehydrated)

Sample preparation:
- freezing into LN2
- sublimation
- metal sputtering (Pt, Au, Ir)



SEM imaging

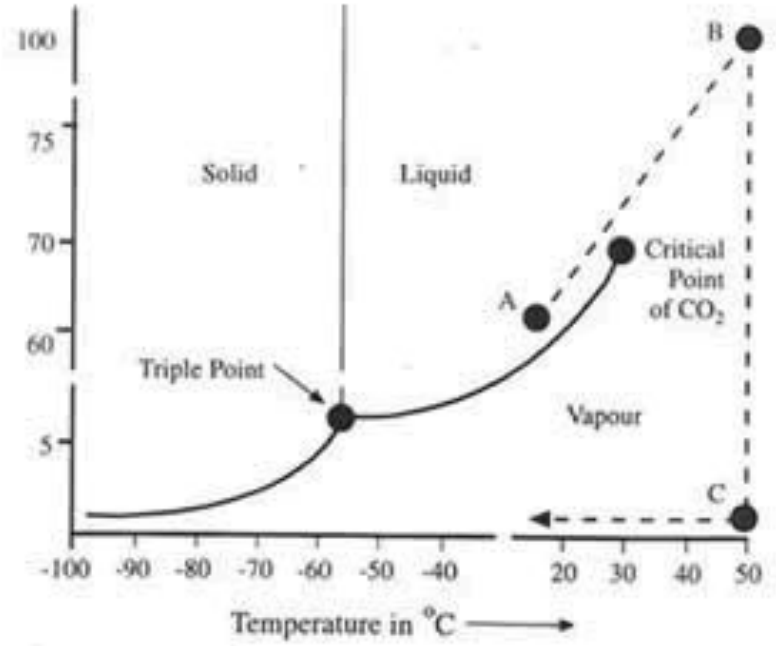




Pros:
- imaging of sample morphology
  at significant scale difference(1mm - 10nm)
- fast sample preparation

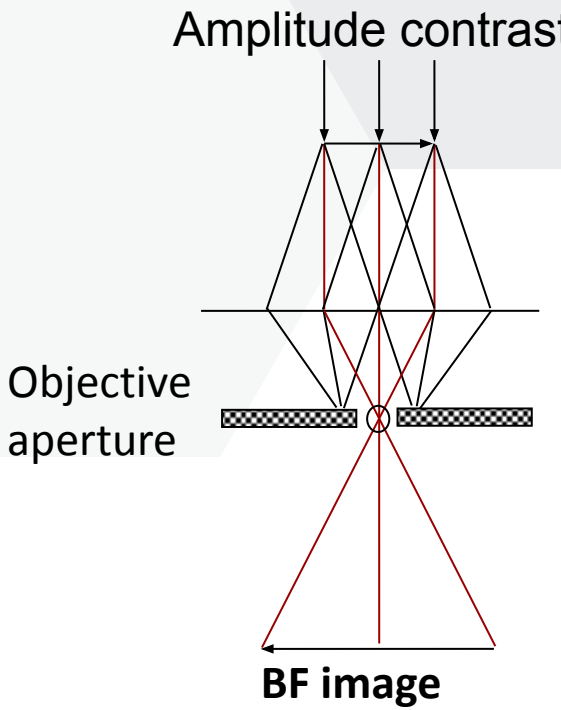
Cons:
- non-native (sample dehydrated)

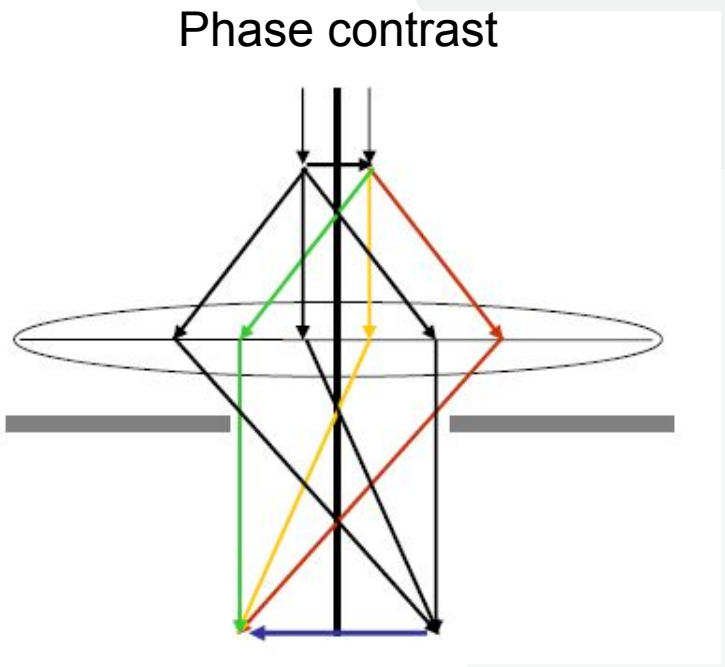
Sample preparation:
- chemical fixation
- contrasting (Pt,U)
- dehydration (EtOH,aceton,HMDS)
- **critical point drying**
- metal sputtering (Pt, Au, Ir)



Structural TEM sample preparation

Transmission electron microscopy

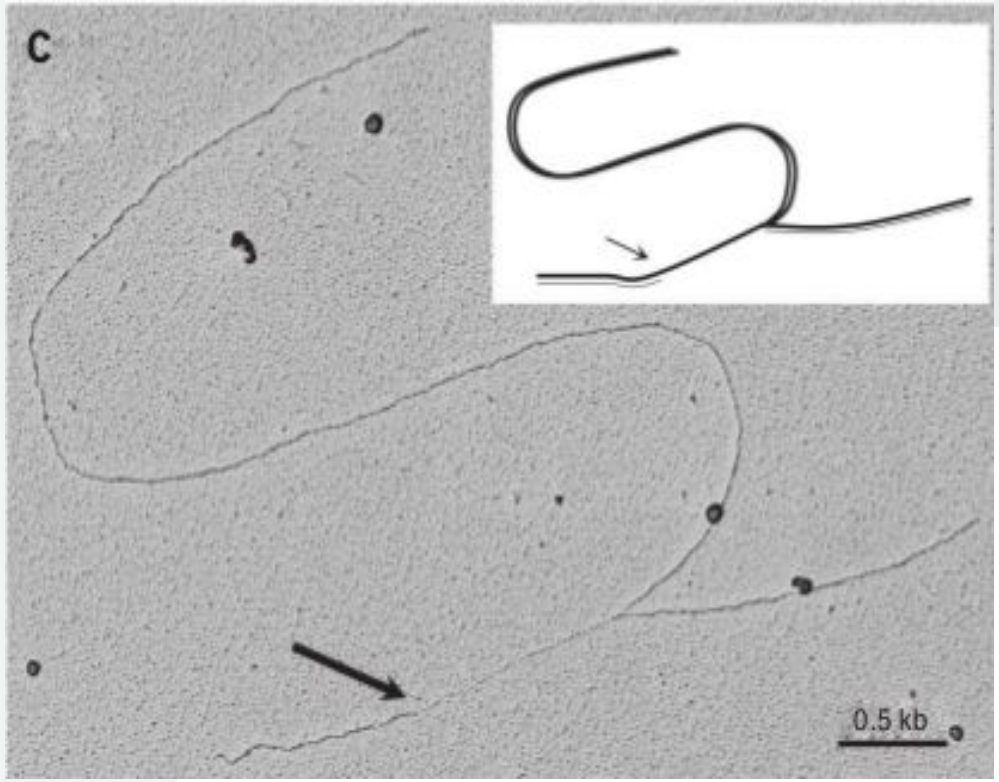




- intensity difference in two adjacent area
- minor contribution in life-science TEM

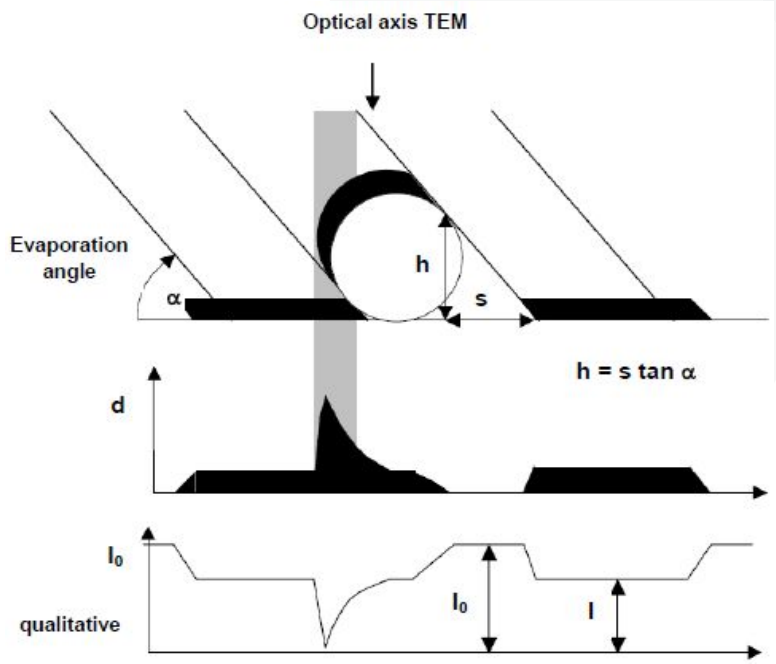
- phase shift between transmitted and diffracted wave
- primary source of contrast in life-science TEM

Rotary shadowing

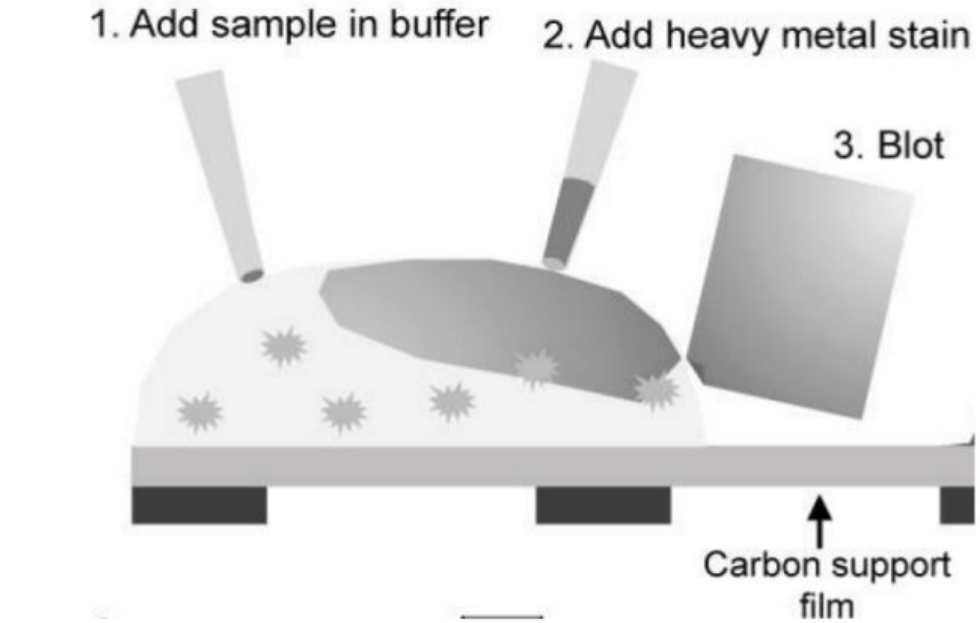


Pros:
- high signal to noise
- fast sample preparation
- potentially high-resolution - single vs. double stranded nucleic acid

Cons:
- non-native (sample dehydrated on surface)
- limited applicability (primarily filamentous structure)
- limited information content (imaging thickness of metal layer not the studied molecule)



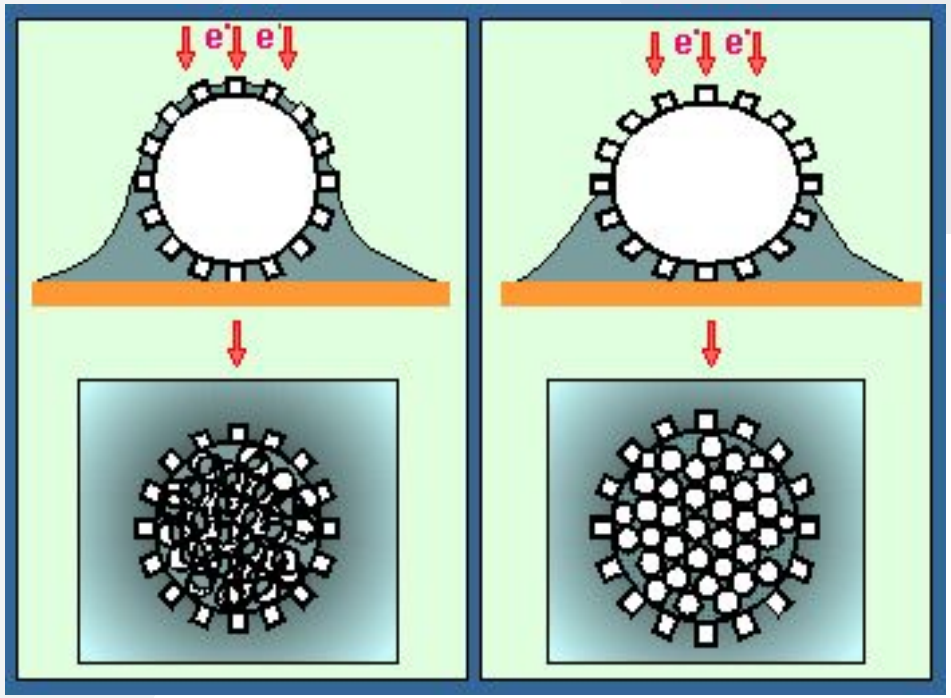
Negative staining



contrasting with heavy metal stains (typically 0.5-2.0% water solution)

- uranyl acetate (pH~4)
  - Pros:
    - high contrast
    - fixative effect
  - Cons:
    - disintegration of sensitive samples (e.g. enveloped viruses)
- uranyl formate (pH~4.5)
  - Pros:
    - high contrast
    - fixative effect
    - smaller grain (suitable for smaller proteins)
  - Cons:
    - low stability
    - soluble in very narrow pH range
    - disintegration of some sample
- ammonium molybdenate, phosphorus thungstanate
  - Pros:
    - pH~7
    - more suitable for fragile complexes (e.g. enveloped viruses)
  - Cons:
    - slightly lower contrast than UAc
    - low fixative effect (fragile complexes may be disassembled)

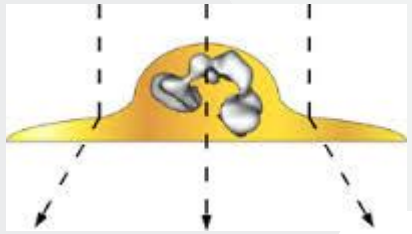
Negative staining

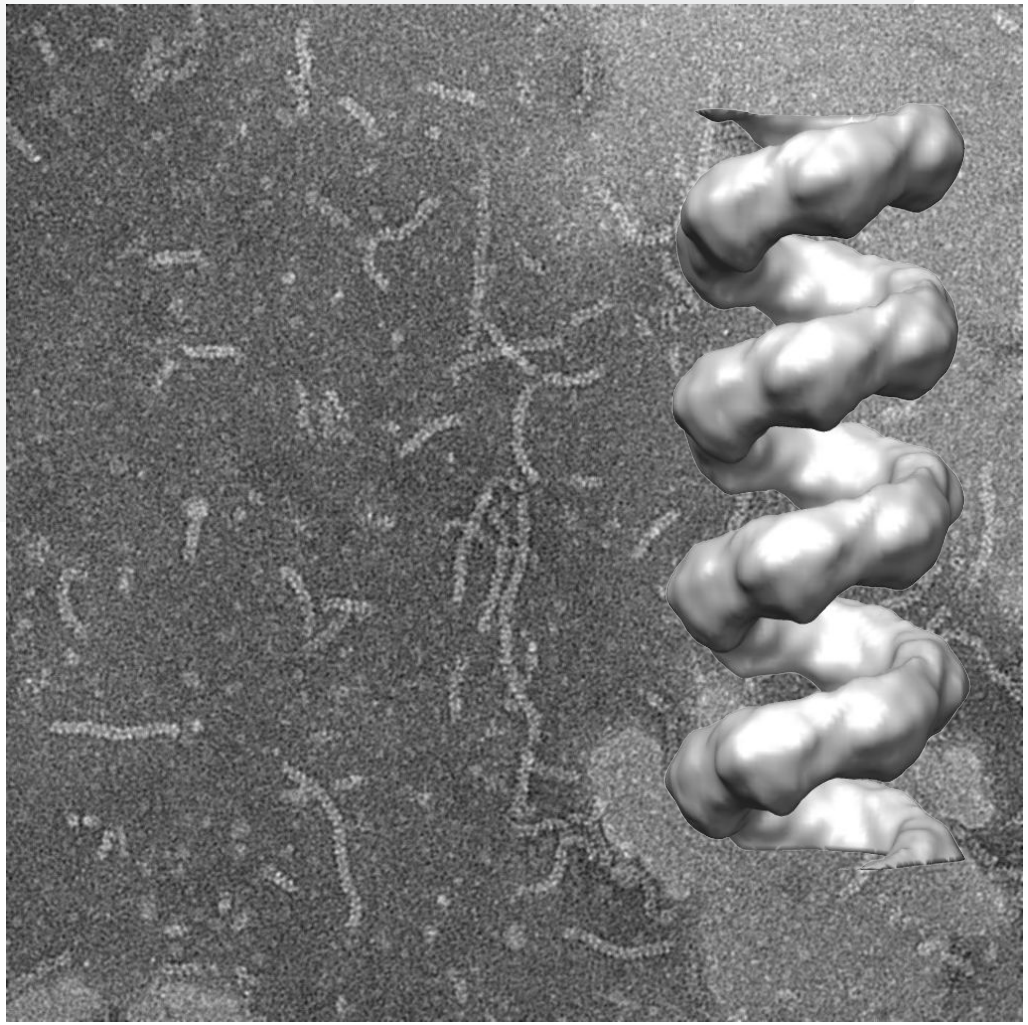


Pros:
- sample preparation quick and robust
- high contrast
- efficient method for sample quality control
- initial structural data
- low sensitivity to radiation damage

Cons:
- resolution limited (10-20A)
- non-native conditions (air drying, high salt)
- flattening artifacts
- denaturation of proteins and NA

Negative staining

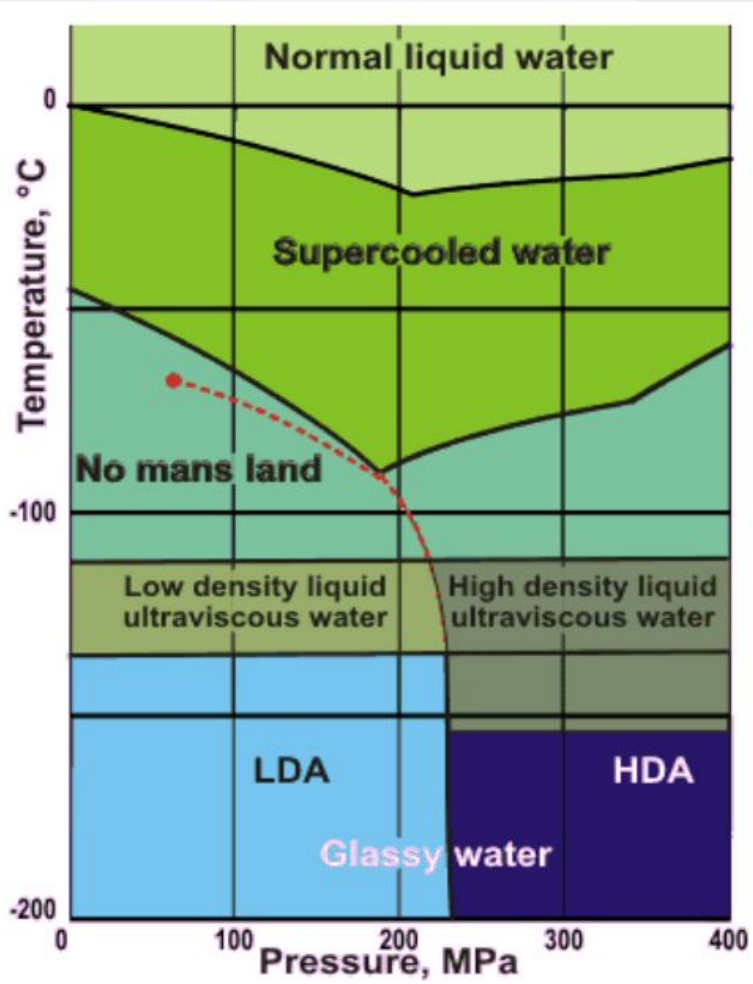




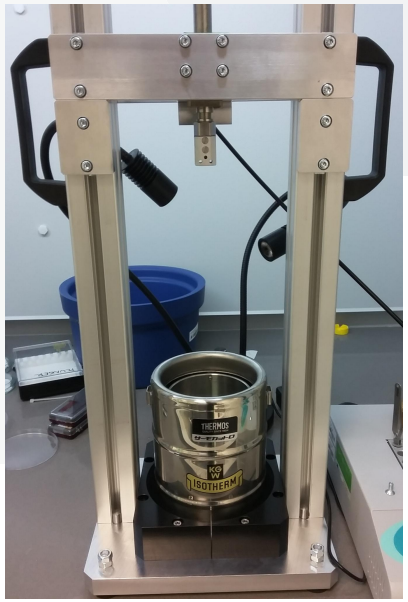
Pros:
+ high signal to noise
+ low dose sensitivity
+ robust (easy sample handling)

Cons:
- non-physiological conditions during sample preparation
- artefacts (changes in cell structure, depression of proteins)
- usually toxic chemicals used during sample prep
- obtainable level of detail limited

Plunge freezing - electron cryo-microscopy

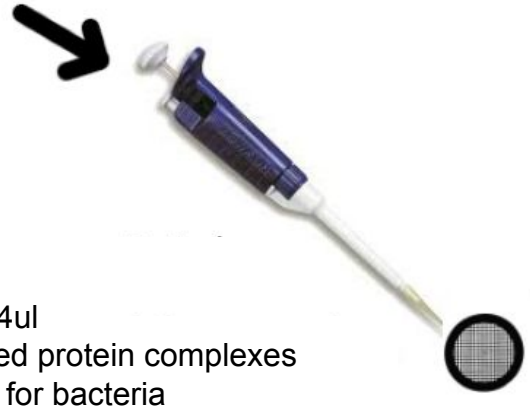


- Rapid immersion of buffered sample into cryogen
- Cryogens:
  - liquid ethane
  - ethane:propane mixture
- Vitrification has to be fast ~1000 K/s
- Possible only for samples with thickness ~<10um
- => amorphous ice
- => thin layer (50-400nm)
- **near-native conditions**
  - difficult to reverse the process and defrost the sample back to functional state
  - LDA water - 0.94 g/l; HDA - 1.17 g/l



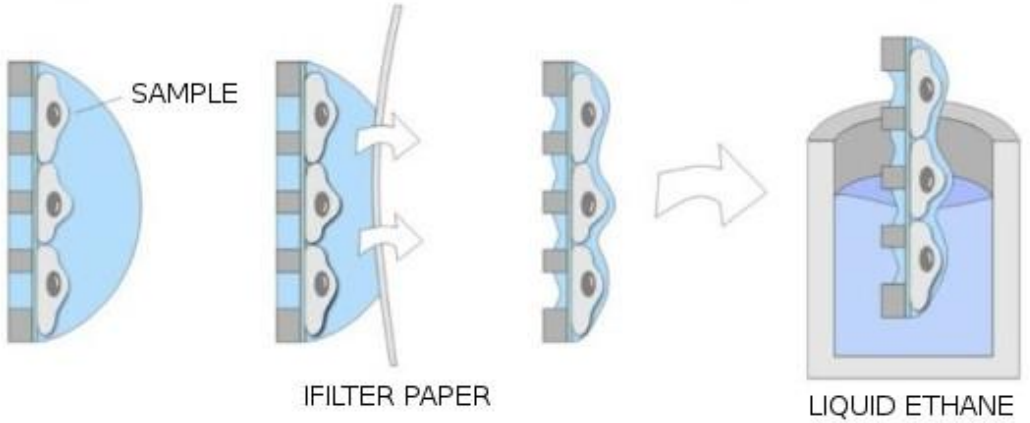
Plunge freezing

Cryogens	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

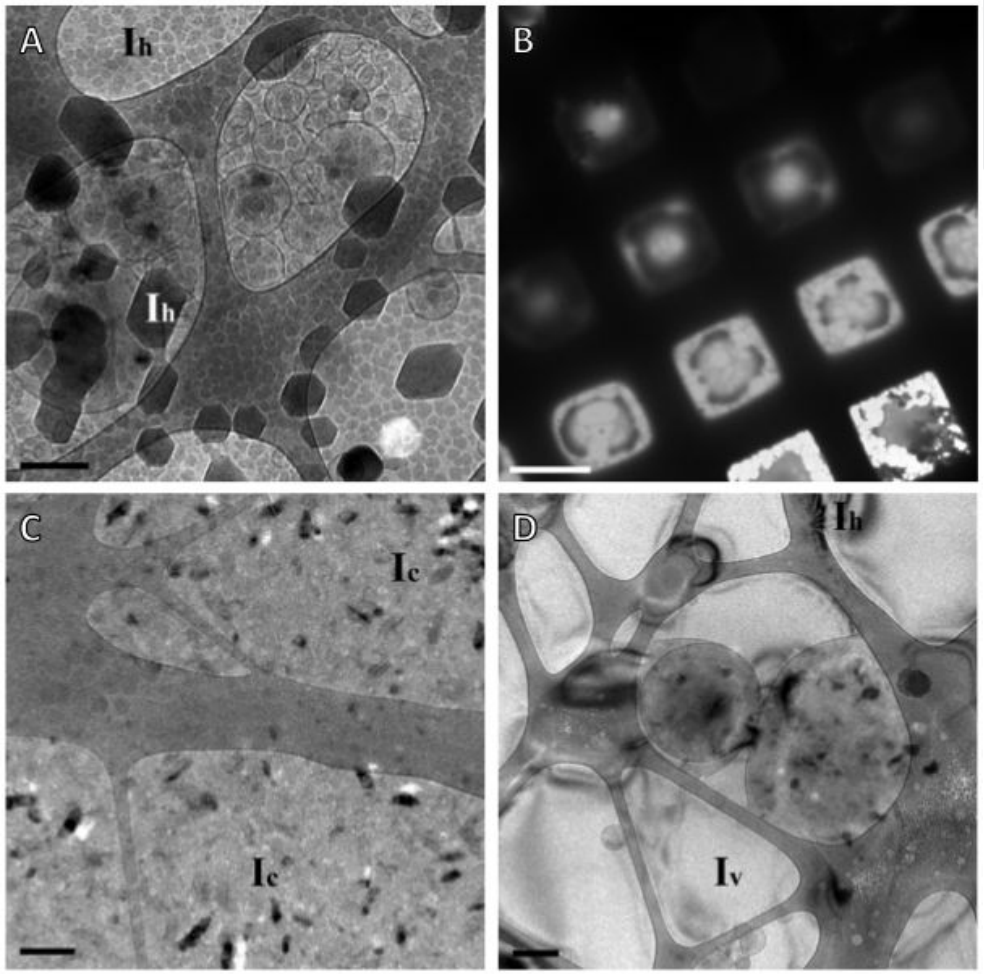


3mm diameter

3-4ul
0.1-1mg/ml for purified protein complexes
OD~0.5-20 for bacteria

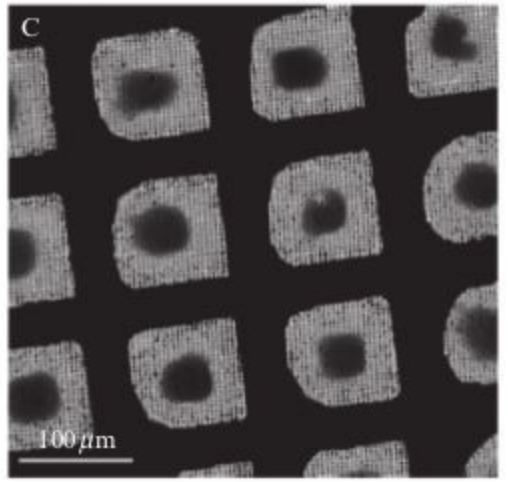


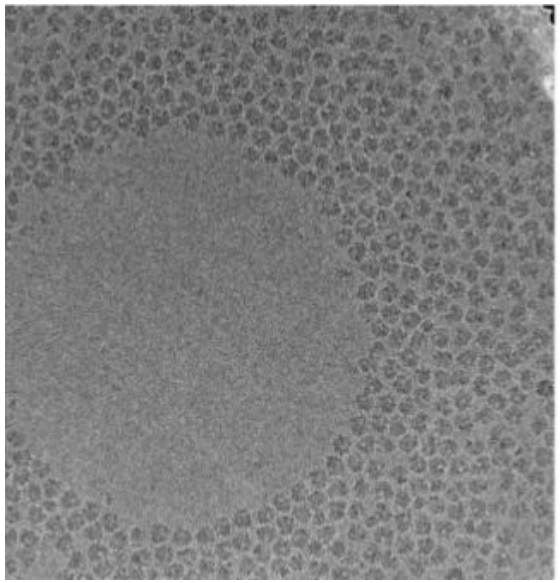
Plunge freezing



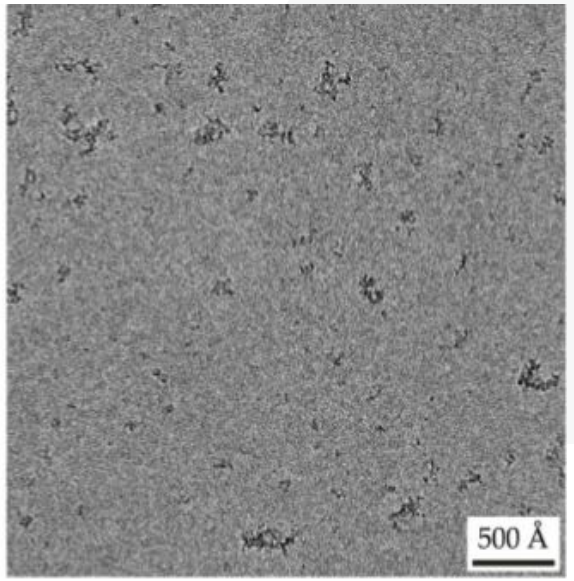
- Sample frozen in hydrated state
- Amorphous ice
- Sample has to be kept at temperatures above devitrification point (~-135C)
- Internal structures can be visualized
- High resolution information is retained
- Possible problems: ice thickness
- hexagonal ice, cubic ice

Plunge freezing



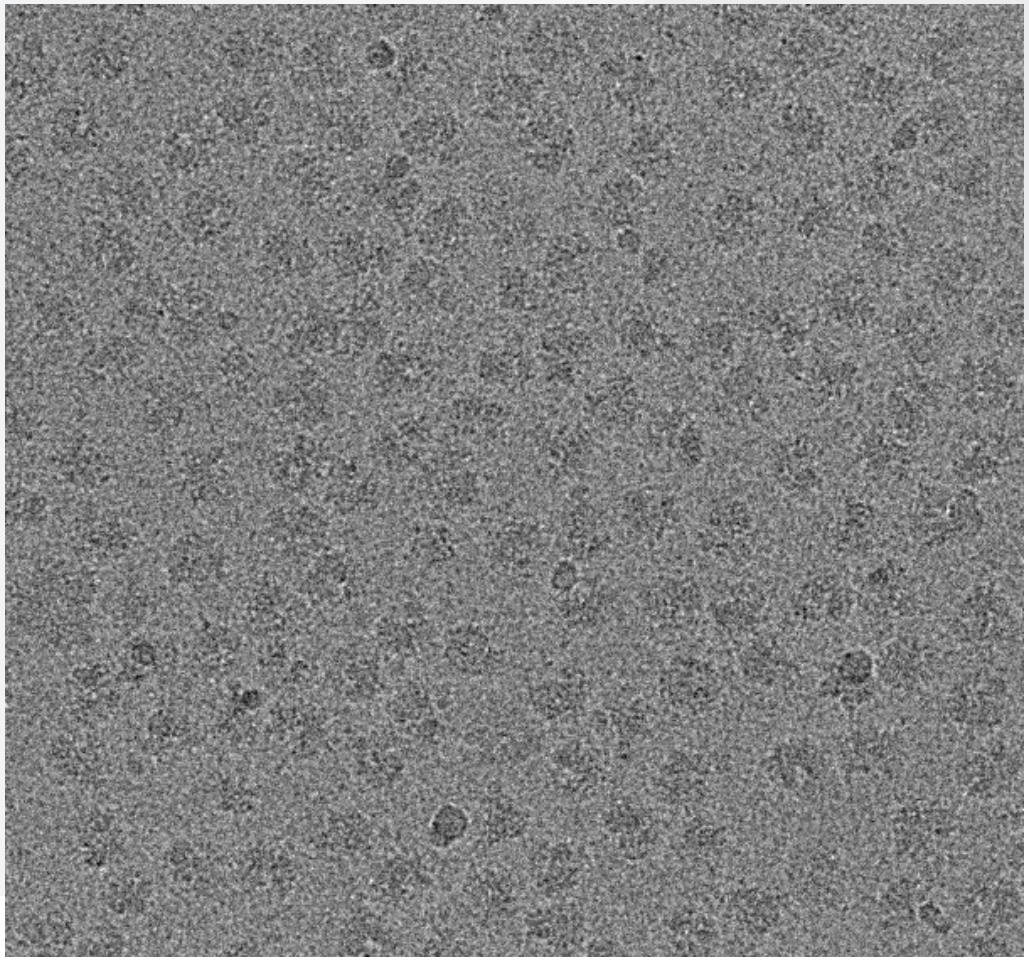


Extrusion of particles from thin ice



Denaturation at air water interface

Plunge freezing



Pros:
+ near-native state of molecule
+ attainable resolution not limited by sample prep.
+ no toxic chemicals in the process
+ applicable not only to protein but usually also to cellular monolayer

Cons:
- low signal to noise
- sample handling only under LN2 conditions (risk of devitrification and sample surface contamination)
- prone to radiation damage (sample is insulator)
- obtainable level of detail limited

Volume EM - block face imaging

Workflow

- Chemical fixation (formaldehyd, glutaraldehyde, osmium tetraoxide)
- Dehydration (EtOH, aceton)
- Resin embedding
- Sectioning

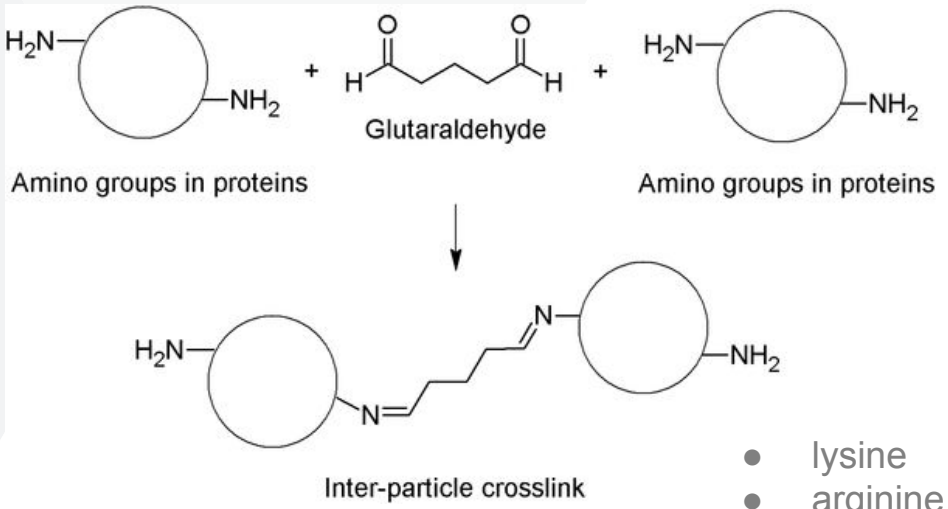
Block face imaging

Pros:
- 3D volume reconstruction at ultrastructural level of detail
- high signal to noise
- low dose sensitivity
- robust (easy sample handling)

Cons:
- non-physiological conditions during sample prep
- artefacts (changes in cell structure, depression of proteins)
- extremely toxic chemicals (OsO_4)
- attainable level of detail limited

Block face imaging

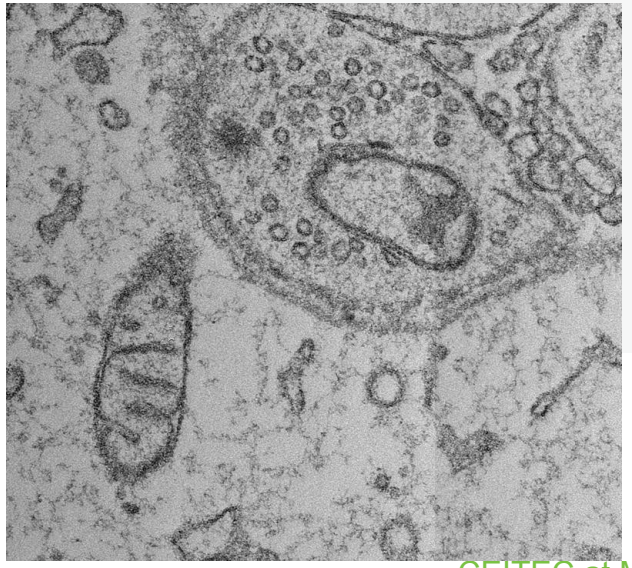
Sample preparation 1:
- formaldehyde, glutaraldehyde
- chemical fixation - ~2% solution in water or buffer
- variable duration – 2-24 hours (sample thickness)
- contrasting (OsO4, UAc, Pb)



- lysine
- arginine

Pros:
- 3D volume reconstruction at ultrastructural level of detail
- high signal to noise
- low dose sensitivity
- robust sample preparation

Cons:
- non-physiological conditions during sample prep
- artefacts (changes in cell structure, depression of proteins)
- extremely toxic chemicals (OsO4)
- attainable level of detail limited



Block face imaging

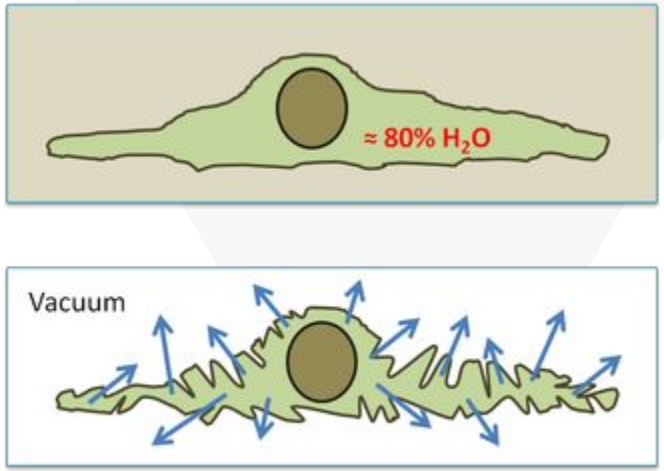
Sample preparation 2:

Dehydration – EtOH or aceton series
(30% for 15mins, 50% for 15min, 70% for 15mins, 90% for 15mins, 100% - 3x)
- shrinking of protein and lipids
- sample shrinking up to 40%
- formation of various artefacts

Resin embedding – resin infiltration (2:1 propylen oxide: resin for 1h, 1:1 for 1h, 1:2 for 1h, 100% resin overnight
- polymerazation 24-72h at 60-70C

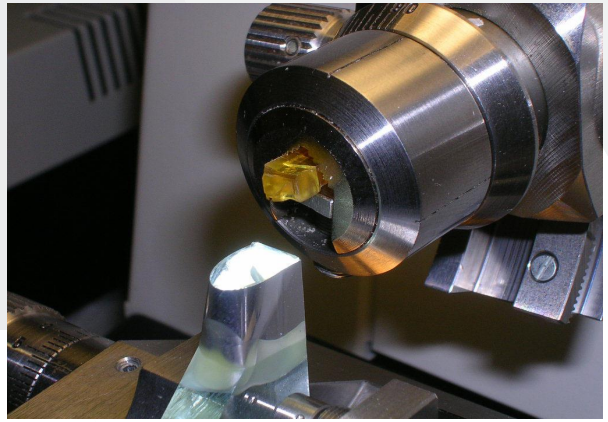
Pros:
- 3D volume reconstruction at ultrastructural level of detail
- high signal to noise
- low dose sensitivity
- robust sample preparation

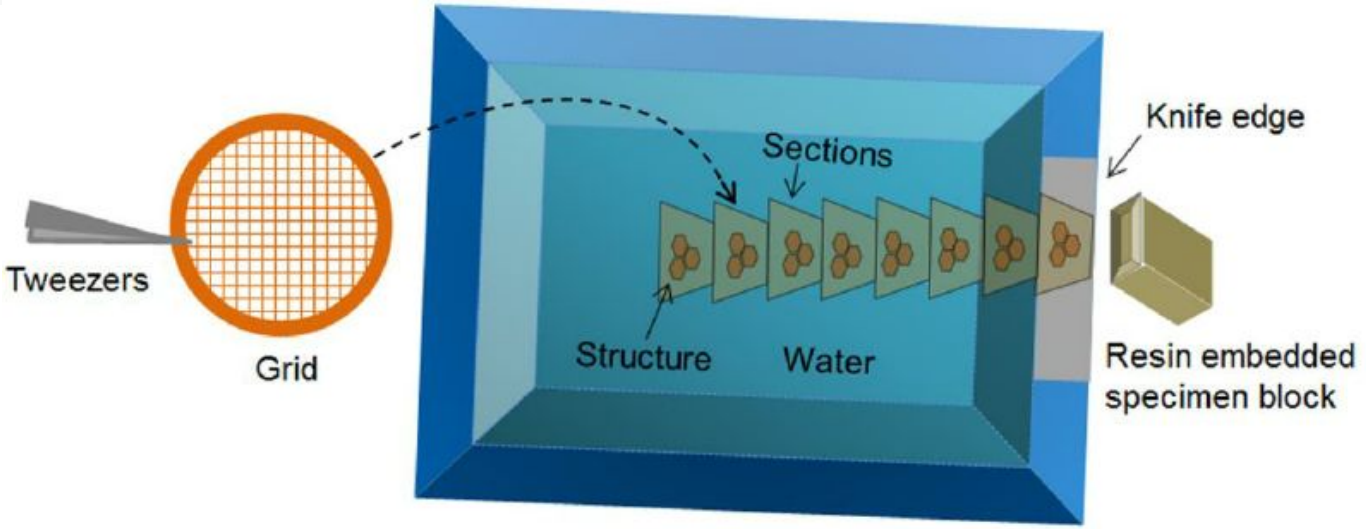
Cons:
- non-physiological conditions during sample prep
- artefacts (changes in cell structure, depression of proteins)
- extremely toxic chemicals (OsO4)
- attainable level of detail limited



Block face imaging

Mechanical sectioning for TEM

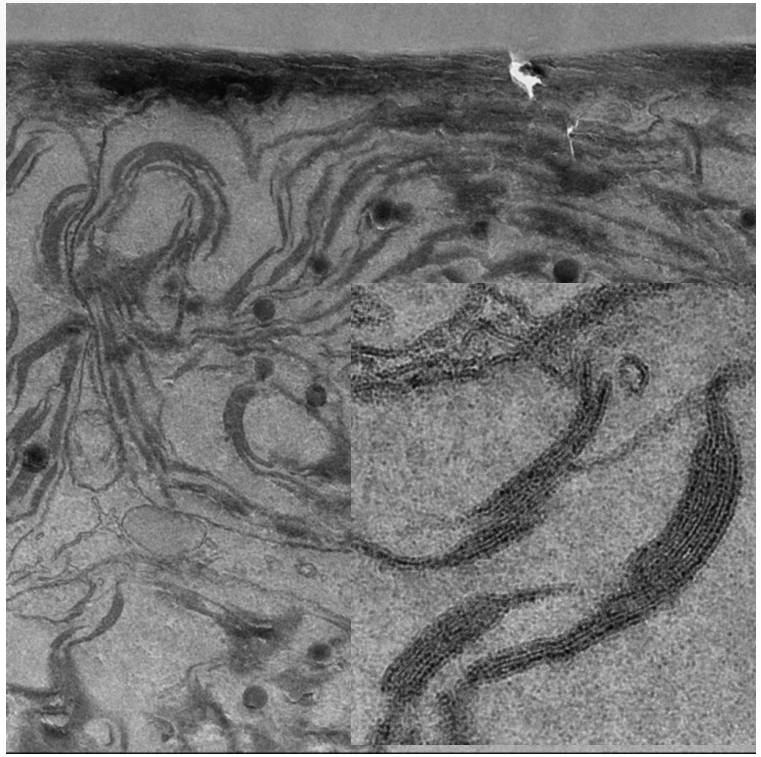


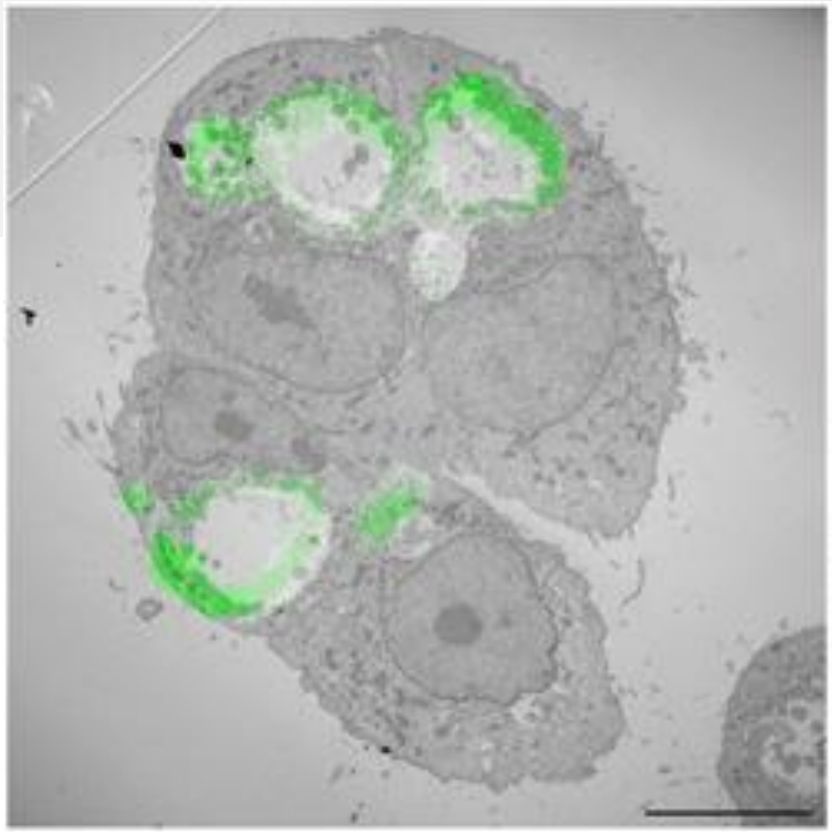


Block face imaging

Mechanical sectioning for TEM

- 50 – 70 nm thick sections
- high-resolution imaging in TEM (tomography)
- 3D volume reconstruction
- resolution limited by sample preparation
- staining with EM contrasting agents (nanoparticles) or fluorescent markers (CLEM) for targetting



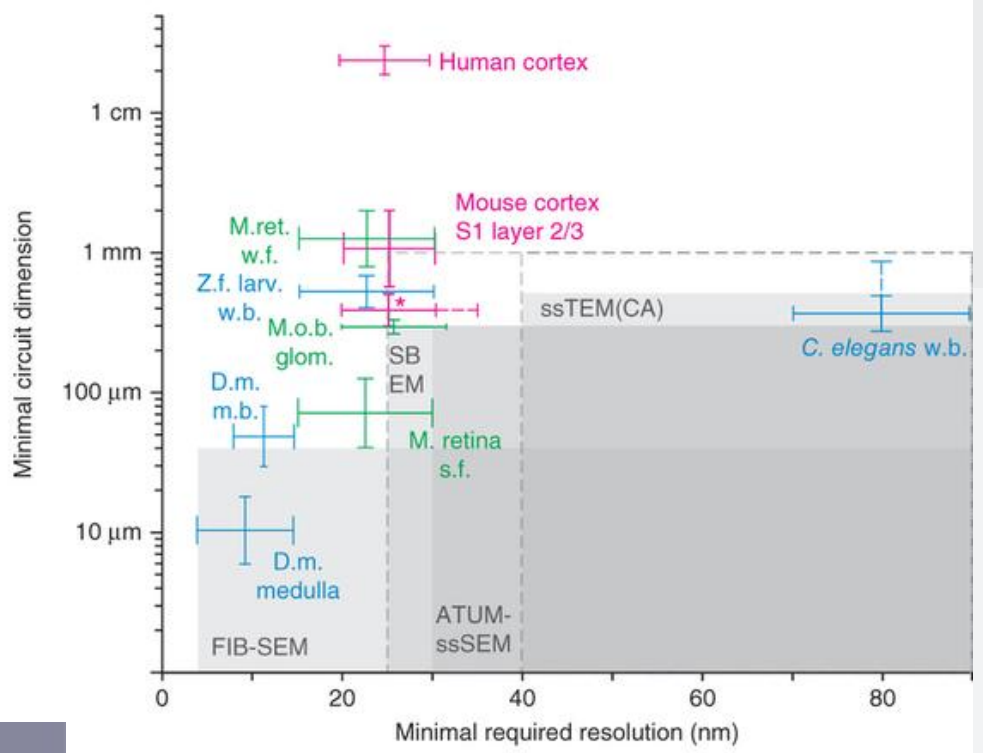


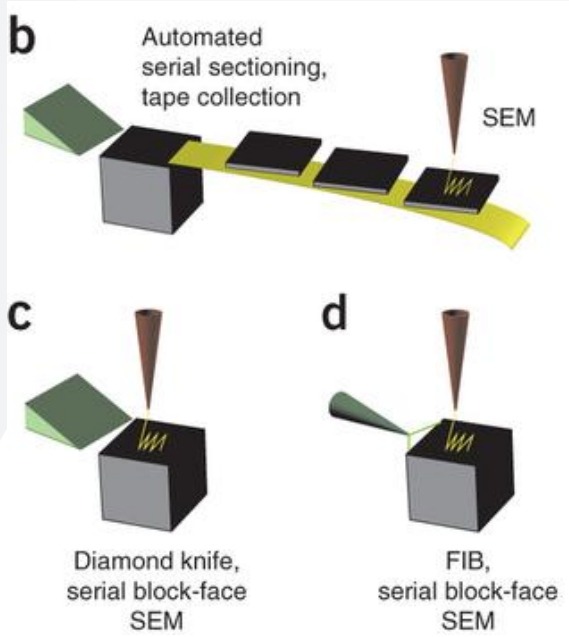
NIH el. mic. facility

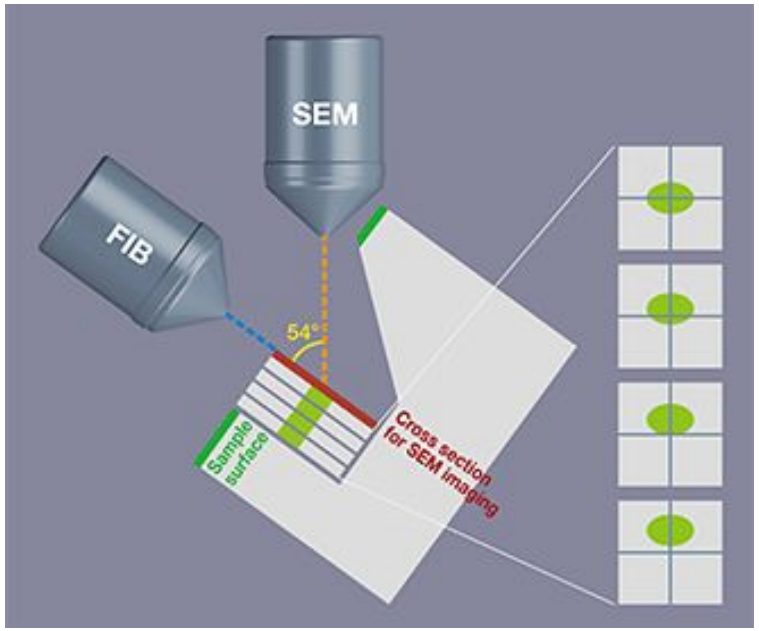
Block face imaging

Mechanical sectioning of FIB sectioning for SEM

- detection of back scattered electrons
- mechanical sectioning either inside or outside SEM
- FIB sectioning (10nm)
- FIB-SEM tomography – correlative studies limited
- FIB sectioning - destructive vs. mechanical sectioning - non-destructive
- FIB sectioning - easier image registration vs. mechanical sectioning - image registration may become cumbersome







Block face imaging

Mechanical sectioning of FIB sectioning for SEM

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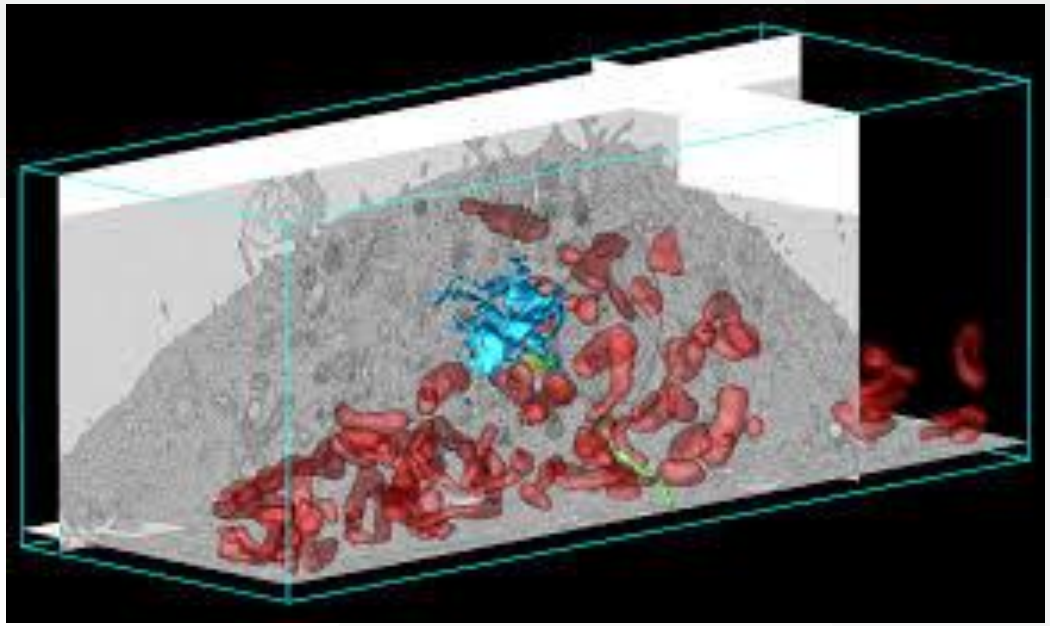
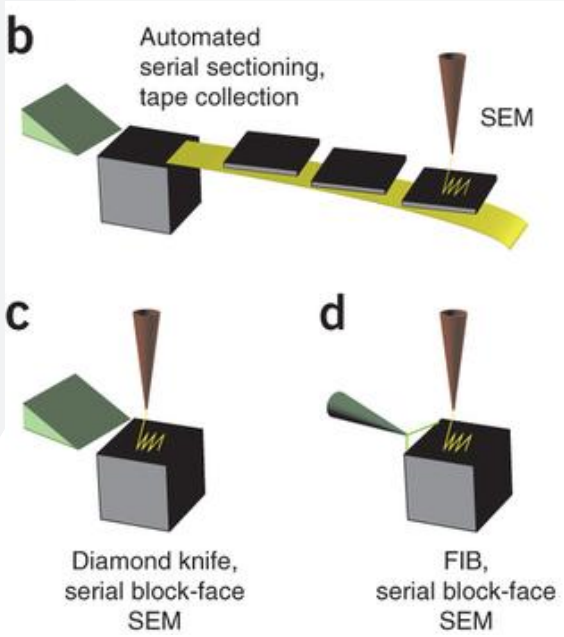
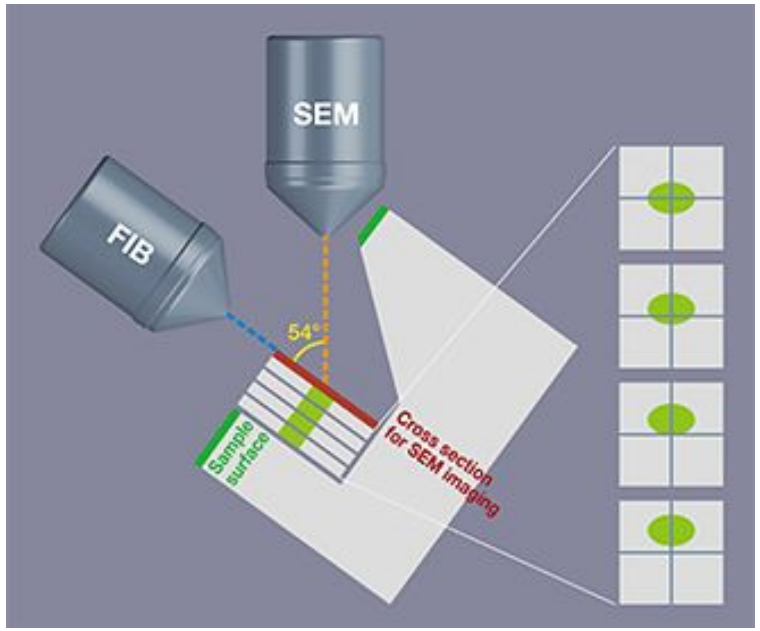
- FIB sectioning - destructive vs. mechanical sectioning - non-destructive
- FIB sectioning - easier image registration vs. mechanical sectioning - image registration may become cumbersome

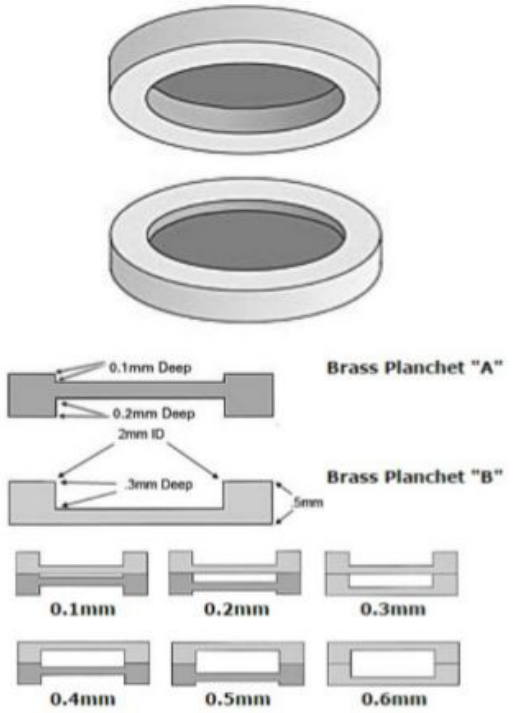






Volume EM - cryo-EM techniques

High pressure freezing



Plunge freezing:
- rapid immersion of buffered sample into cryogen (liquid ethane, ethane:propane mix)
- vitrification has to be fast 10e4-10e5 K/s
- available only for samples ~<10um thick

High pressure freezing
- sample thickness <200um
- freezing with liquid nitrogen
- 2000 bars, 20 ms

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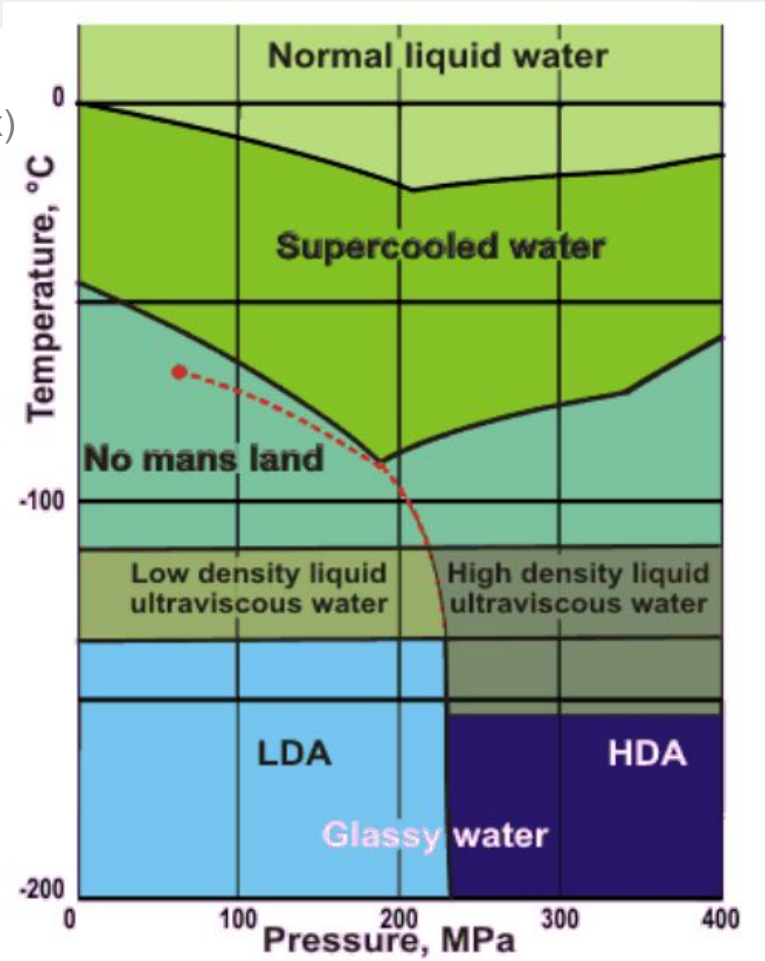
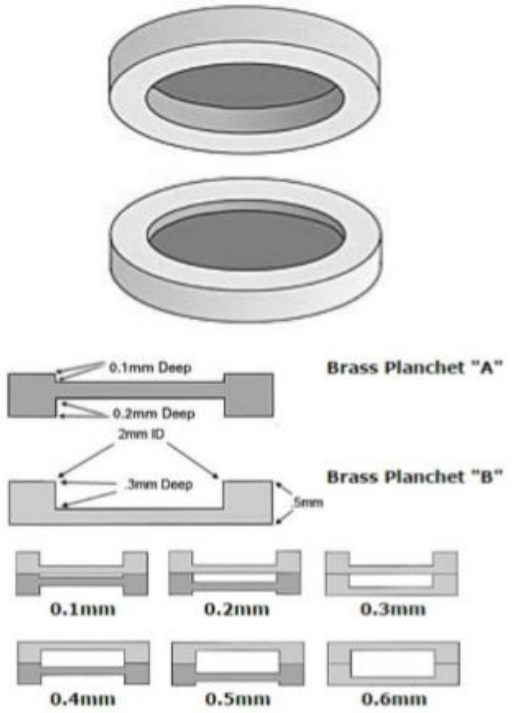


Volume EM - cryo-EM techniques

High pressure freezing & freeze substitution



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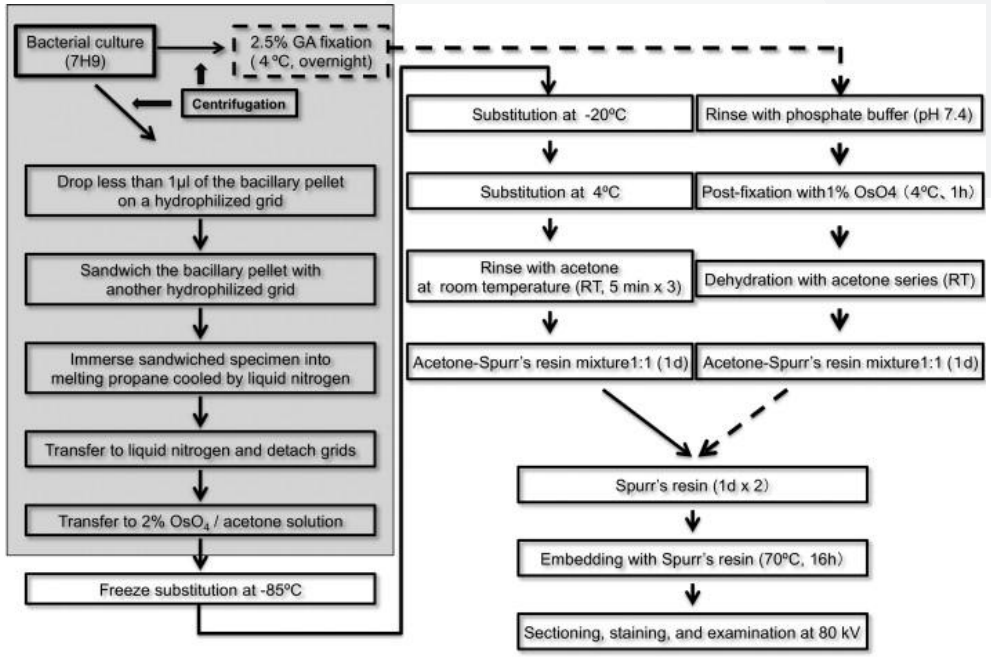
Freeze substitution
- reduction of ultrastructure changes compared to dehydration at ambient temperature
- dehydration at temperatures <-70C (aceton typically -90C)
- fixatives are evenly distributed before cross-linking at ambient temperature
- resin embedding for ultramicrotomy at room temp.

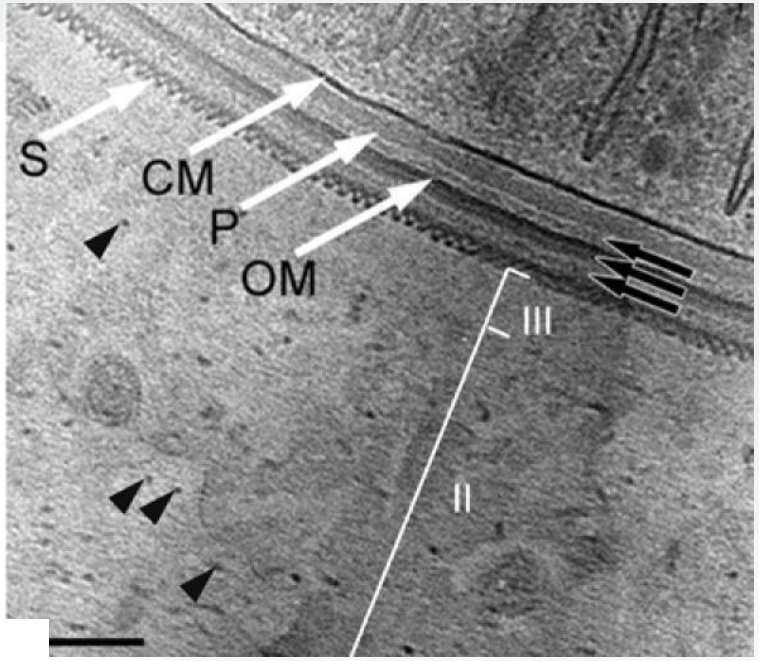


Yamada et al. JMM 2010

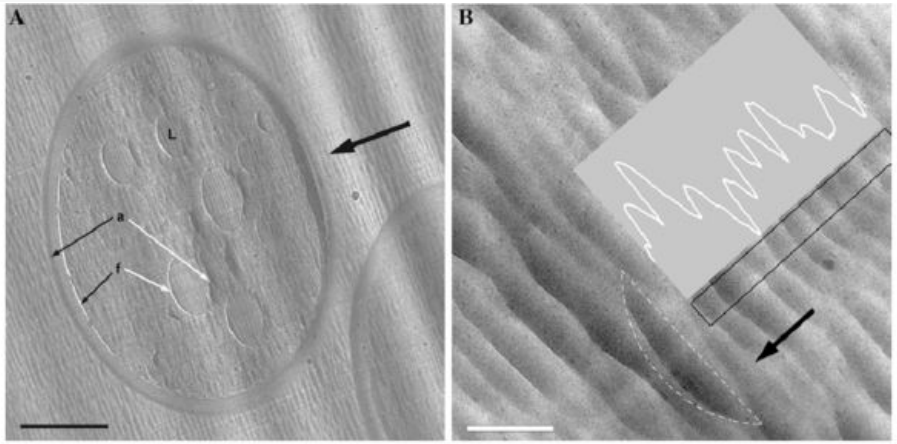
Volume EM - cryo-EM techniques

CEMOVIS - cryo-EM of vitreous sections

- sectioning for TEM (tomography)
- section thickness ~70nm
- no chemical fixation, dehydration or contrasting
- low contrast
- preservation of the sample in near-native conditions
- mechanical sectioning by ultramicrotome at LN2 conditions
- sectioning artefacts



Al-Amoudi et al. EMBO J 2004

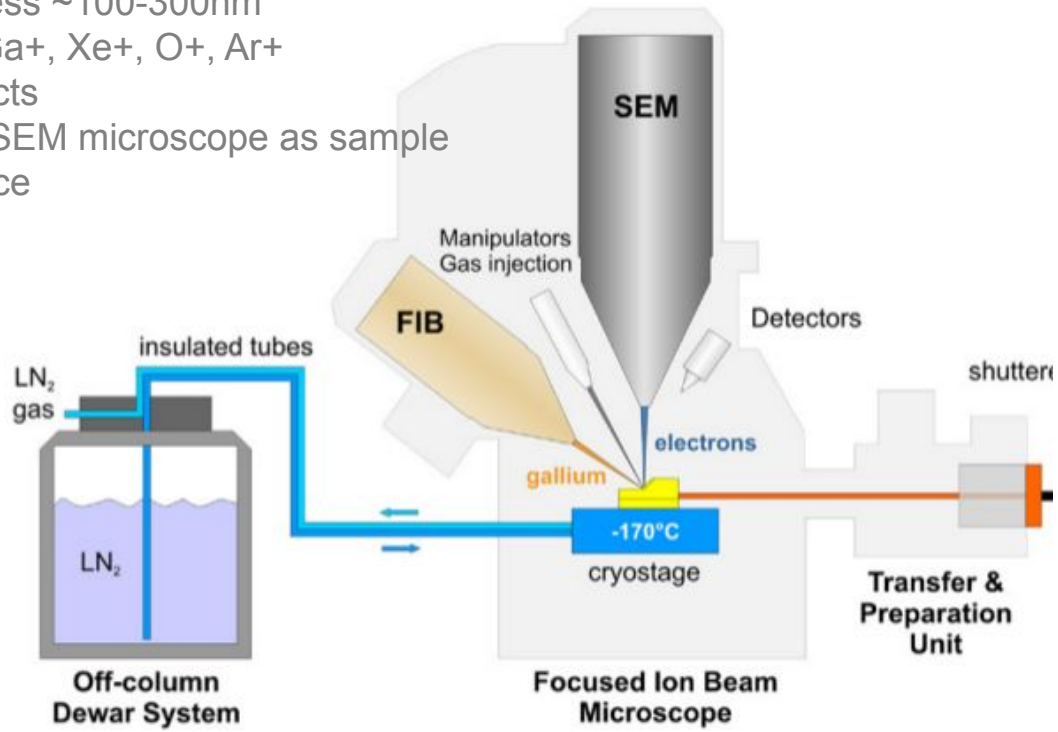


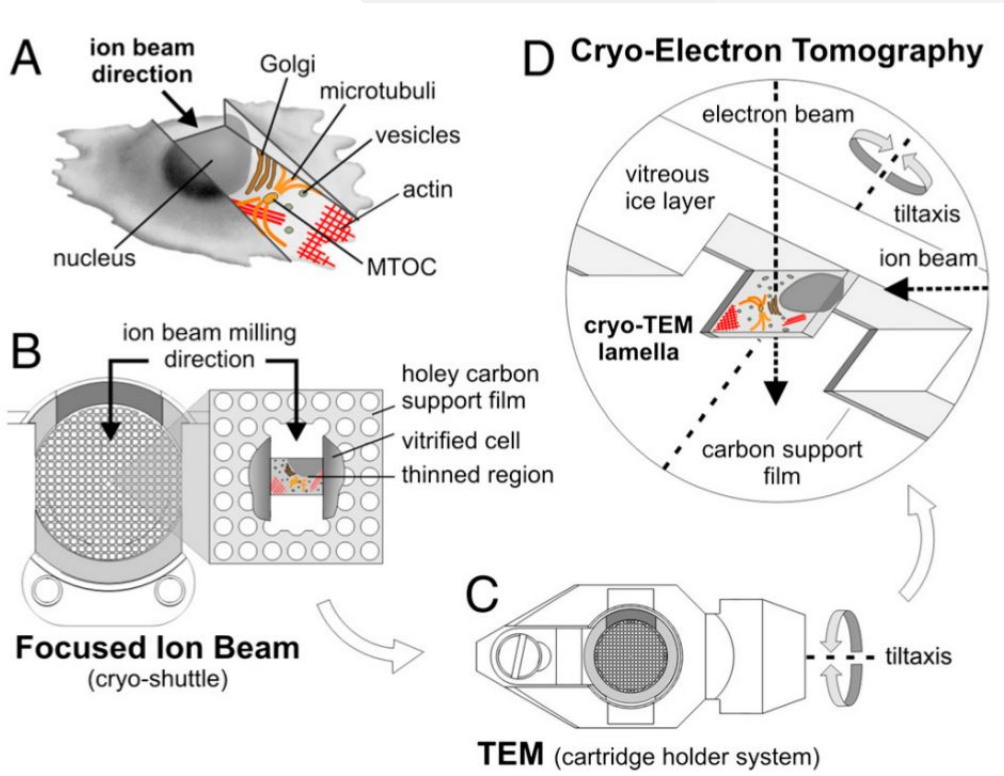
Al-Amoudi et al. JSB 2005

Volume EM - cryo-EM techniques

Focused ion beam micromachining of cellular lamellae

- only single section per cell
- section thickness ~100-300nm
- ablation with Ga+, Xe+, O+, Ar+
- minimal artefacts
- complex (FIB/SEM microscope as sample preparation device





Volume EM - cryo-EM techniques

Focused ion beam micromachining of cellular lamellae