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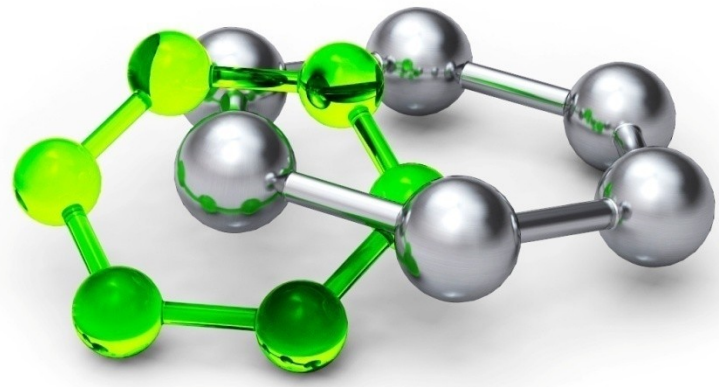
Středoevropský technologický institut

BRNO | ČESKÁ REPUBLIKA

C9940 3-Dimensional Transmission electron microscopy

Lecture 2: Sample preparation

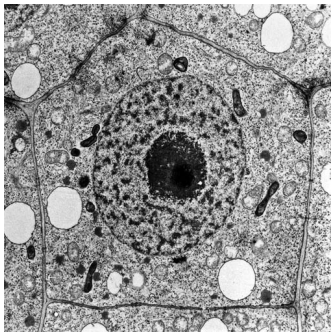


EVROPSKÁ UNIE
EVROPSKÝ FOND PRO REGIONÁLNÍ ROZVOJ
INVESTICE DO VAŠÍ BUDOUCNOSTI

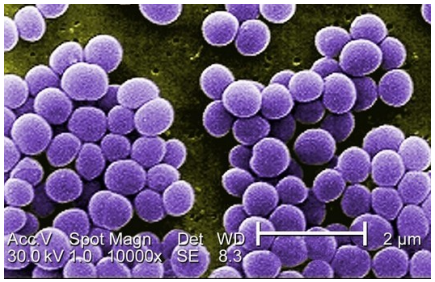
2007–13
OP Výzkum a vývoj pro inovace

Samples in electron microscopy

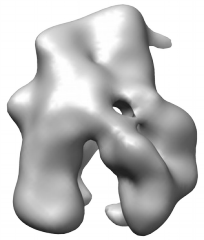
Tick (ESEM)



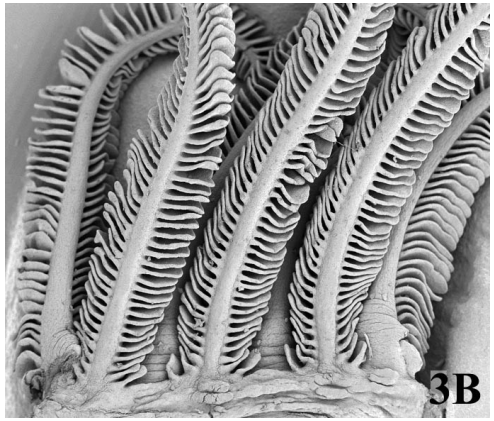
Plant cell (TEM)



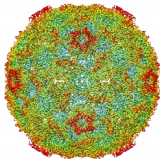
Bacteria (SEM)



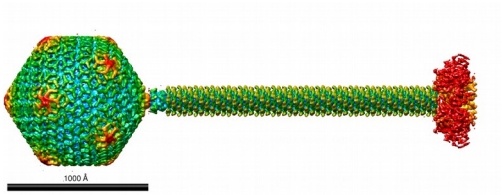
RNA polymerase (TEM)



Plant (SEM)



Virus (TEM)



Bacteriophage (TEM)

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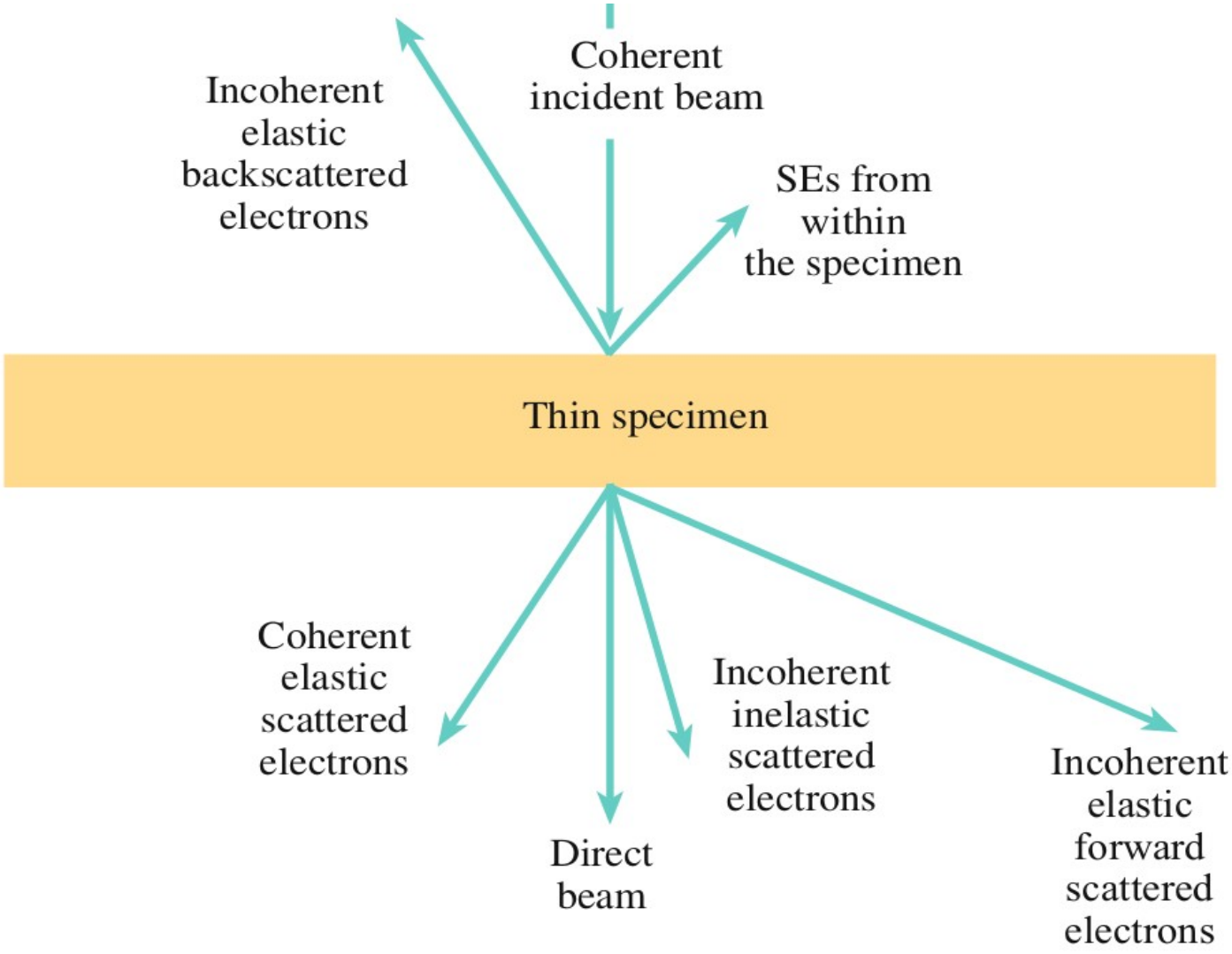
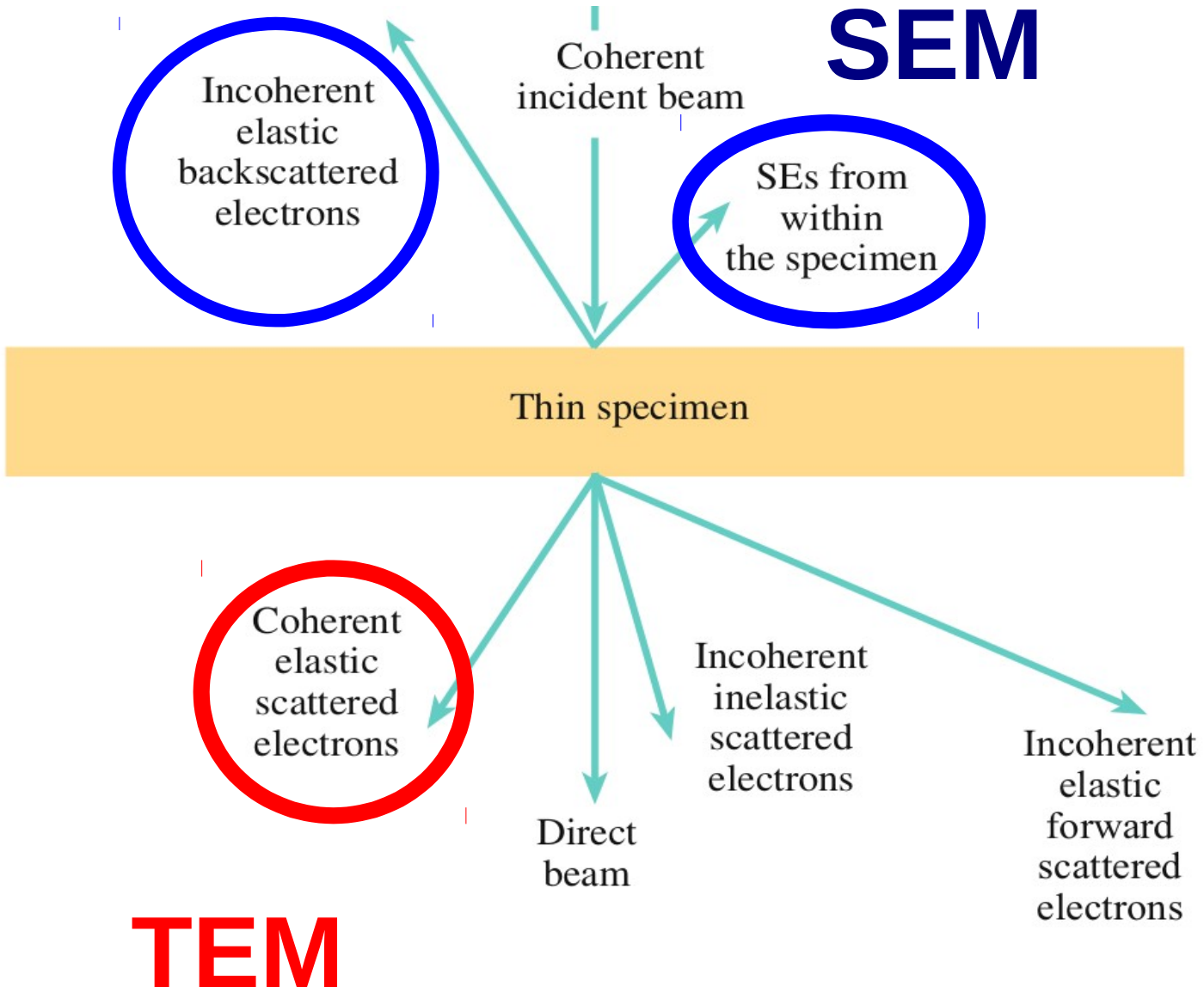
Interaction of electrons with matter

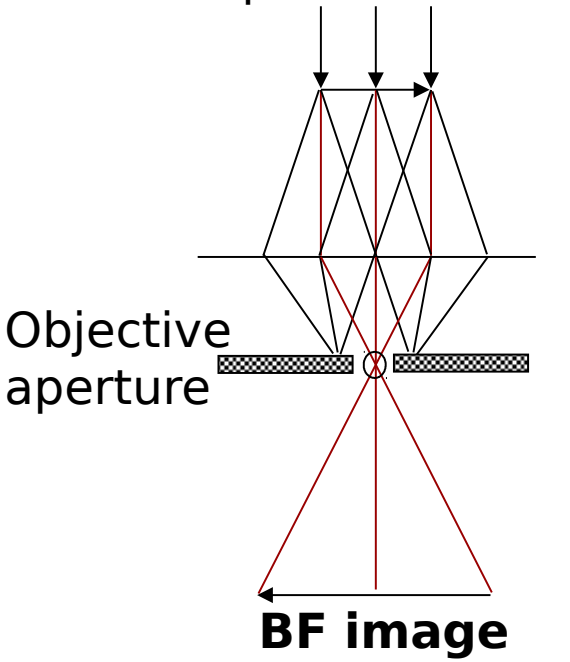


Interaction of electrons with matter

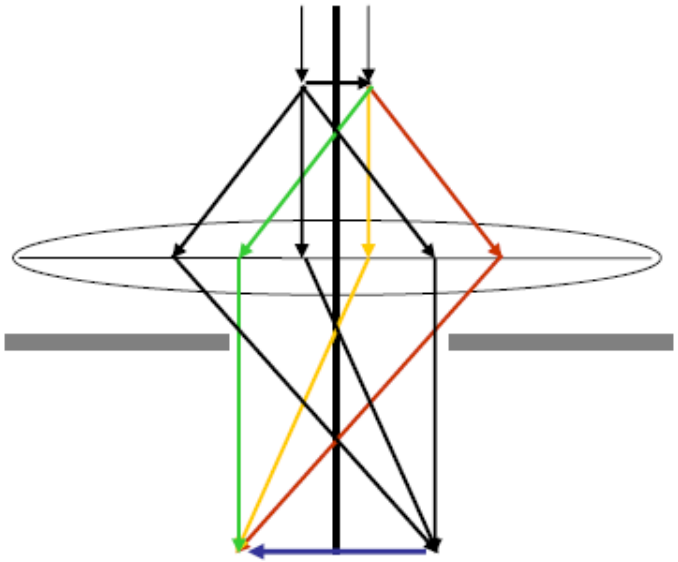


Transmission electron microscopy

Amplitude contrast



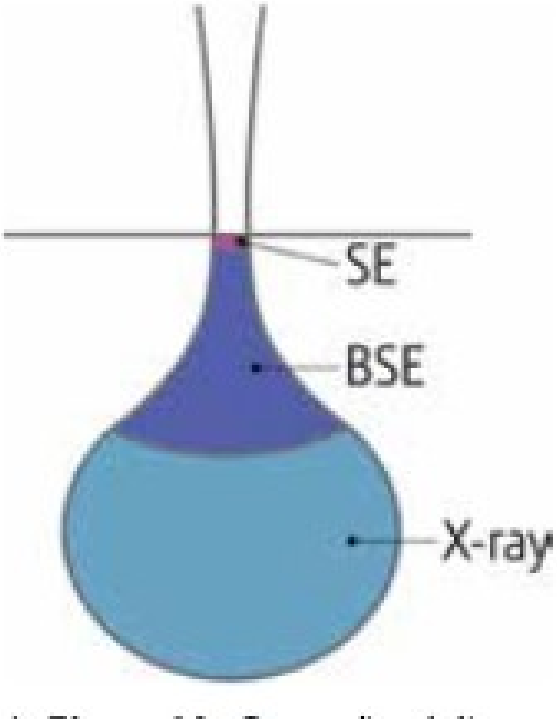
Phase contrast

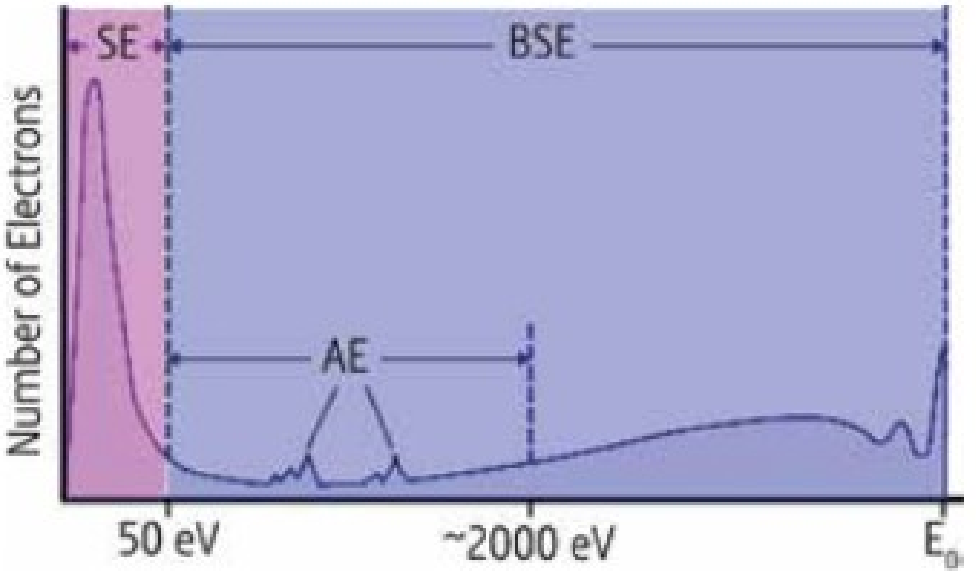


- difference in intensity in two adjacent area

- Transmitted and diffracted waves travel through different distances

Scanning electron microscopy

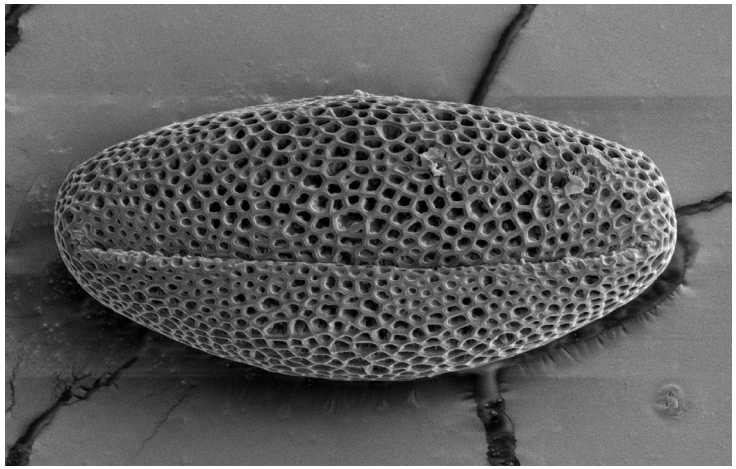


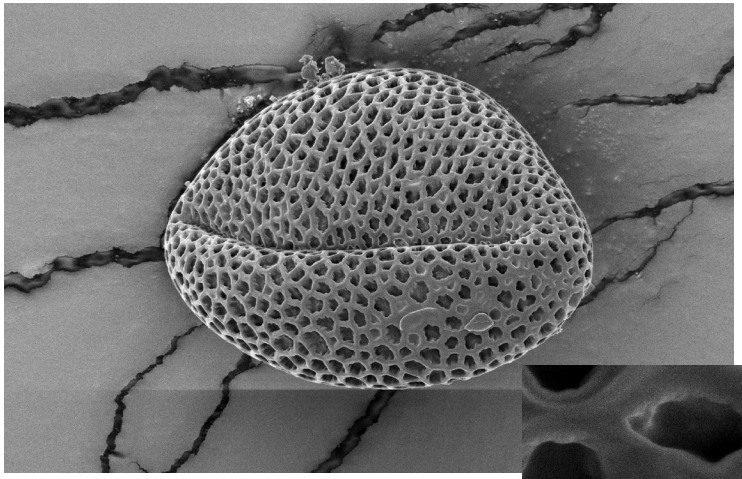


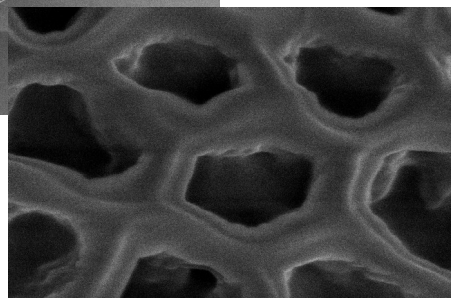
Applications in life-sciences

- **SEM imaging**
- **Block face imaging**
- **Negative staining**
- **Cryo-EM techniques**

SEM imaging







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

Cons:
- non-native (sample dehydrated)

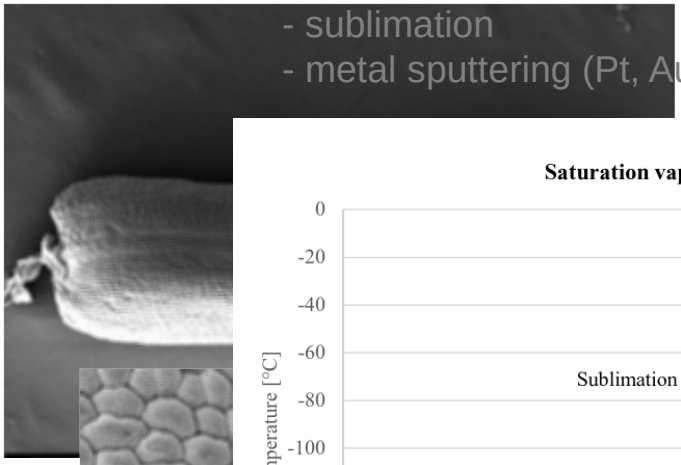
Sample preparation:
- air drying
- metal sputtering (Pt, Au, Ir)

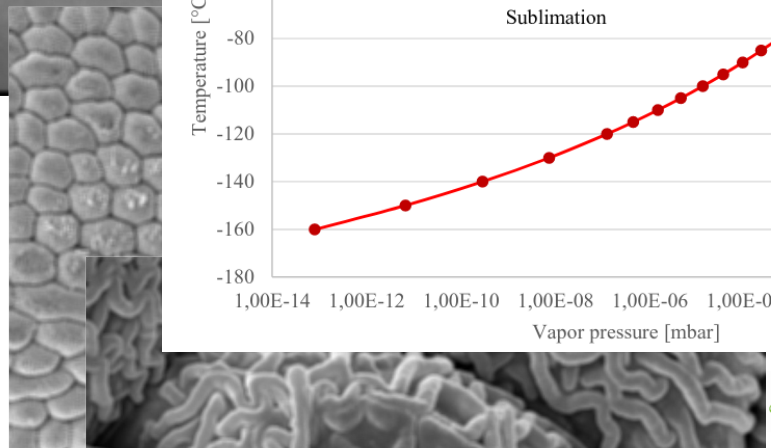
SEM imaging

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)





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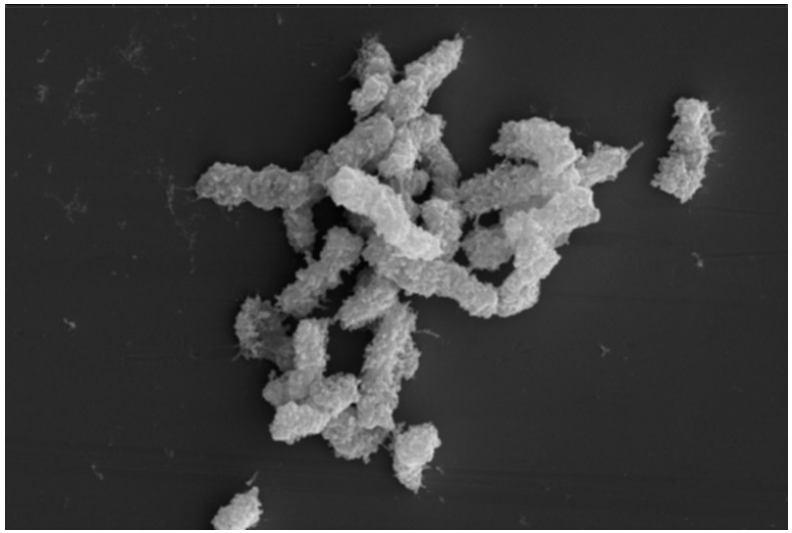
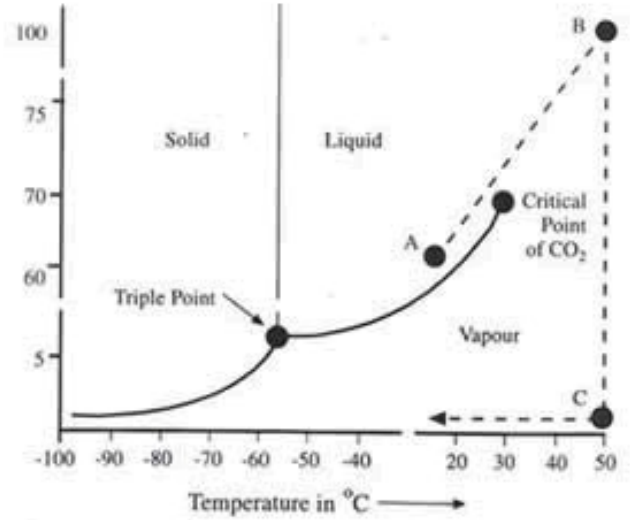
SEM imaging

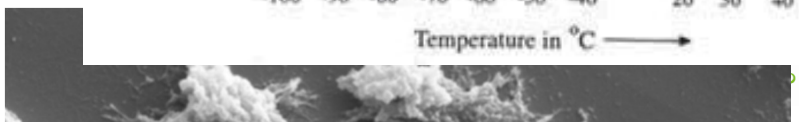


Pros:
- imaging of sample morphology
  at significant scale difference(1mm - 10nm)
- fast sample preparation

Cons:
- non-native (sample dehydrated)

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





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Block face imaging

- Chemical fixation (formaldehyd, glutaraldehyde, osmium tetraoxide)
- Dehydration (EtOH, aceton)
- Plastic 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)

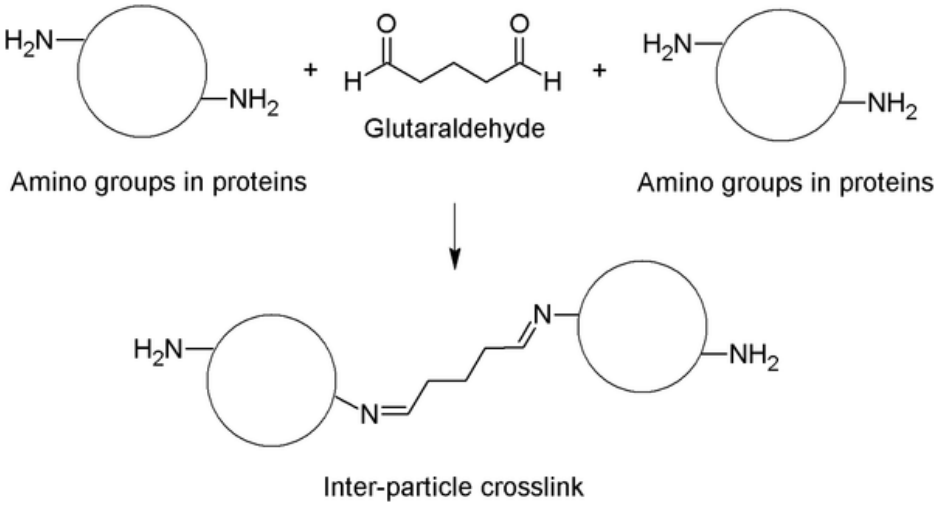
Cons:
- non-physiological conditions during sample prep
- artefacts (changes in cell structure, depression of proteins)
- extremely toxic chemicals (OsO_4)
- attainable level of detail limited

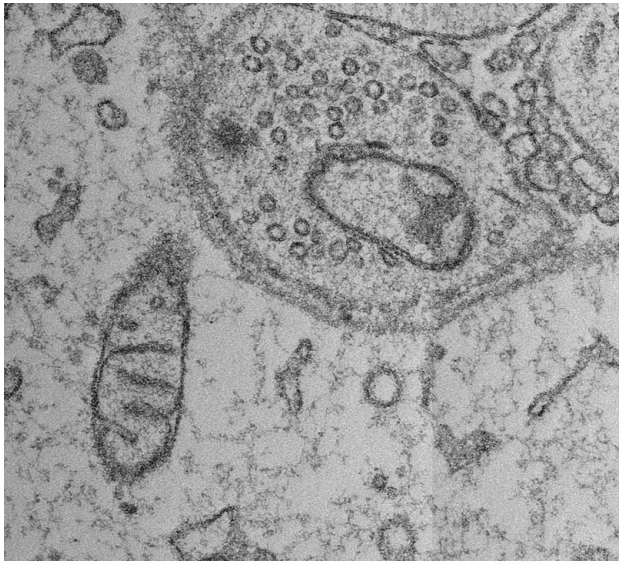
Block face imaging

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

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





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Block face imaging

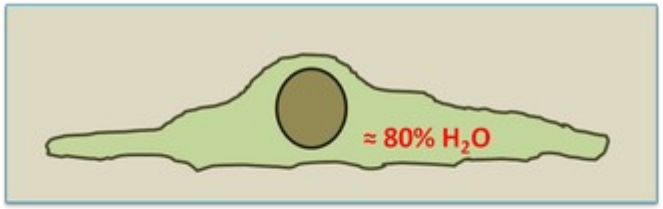
Sample preparation 2:

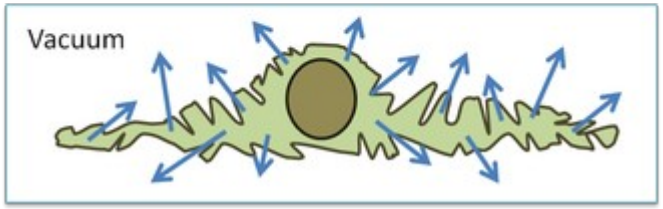
Dehydration – EtOH or aceton series
(30% for 15mins, 50% for 15min, 70% for 15mins, 90% for 15mins, 100% - 3x)
- shrinking of protein and lipids
- sample shrinking up to 40%
- formation of various artefacts

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

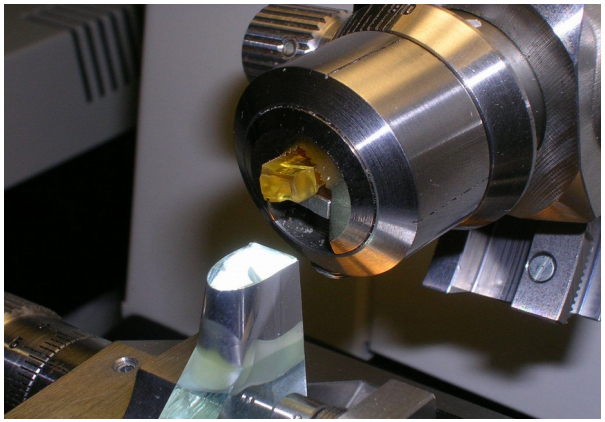
Cons:
- non-physiological conditions during sample prep
- artefacts (changes in cell structure, depression of proteins)
- extremely toxic chemicals (OsO4)
- attainable level of detail limited

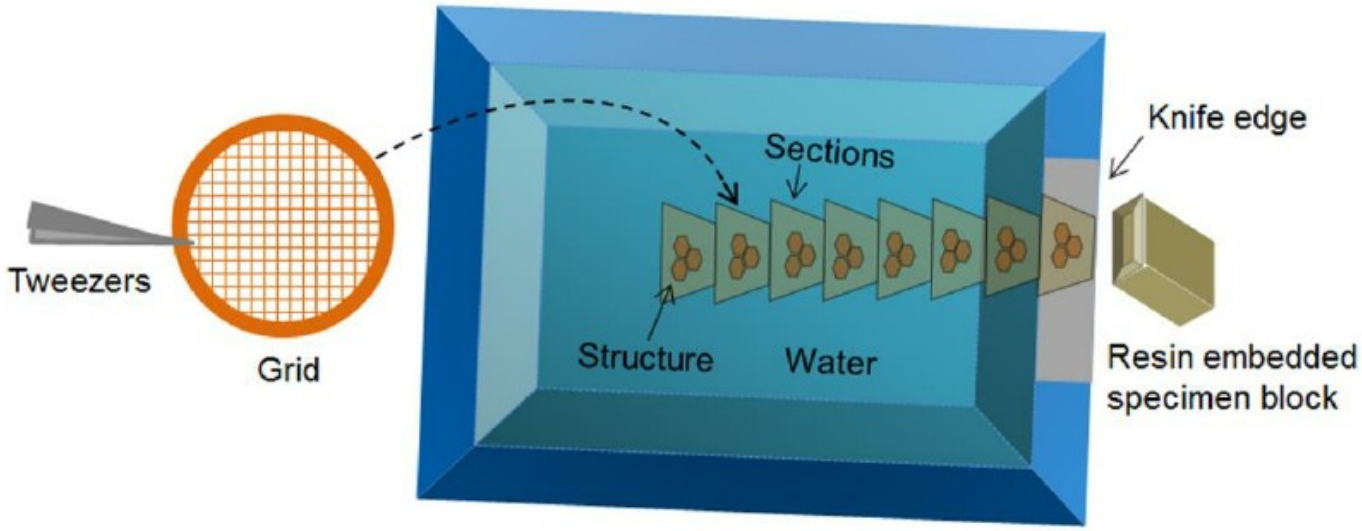




Block face imaging

Mechanical sectioning for TEM

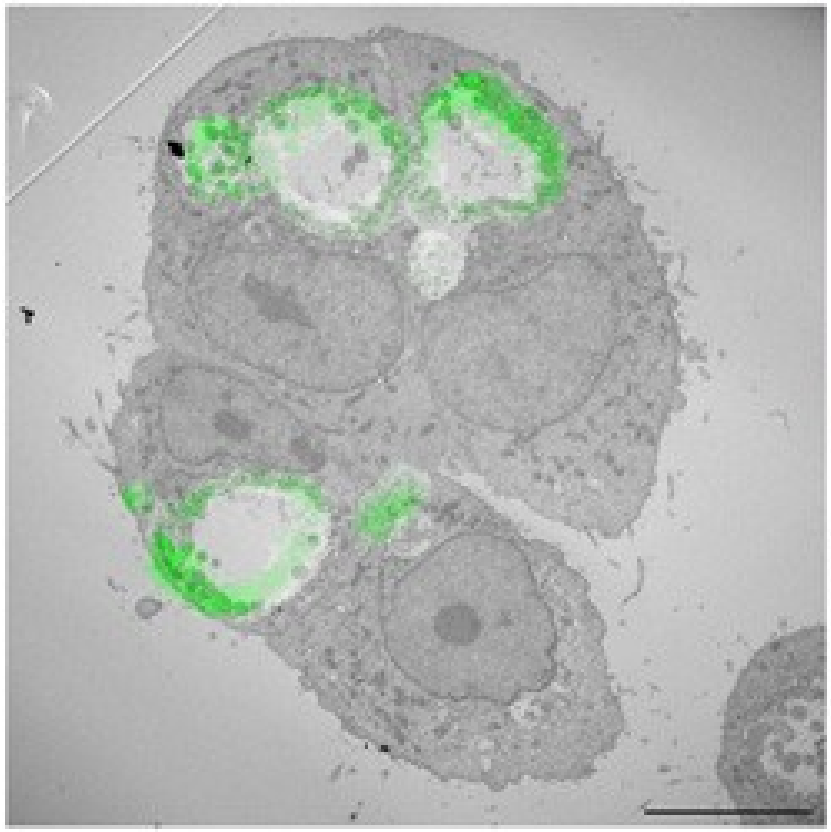




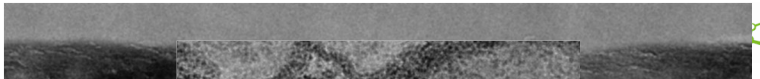
Block face imaging

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



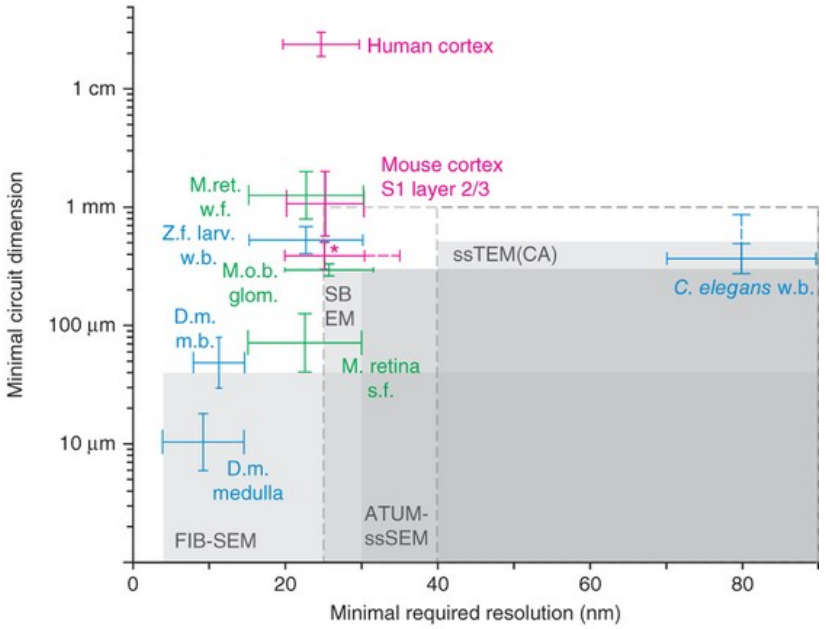
NIH el. mic. facility

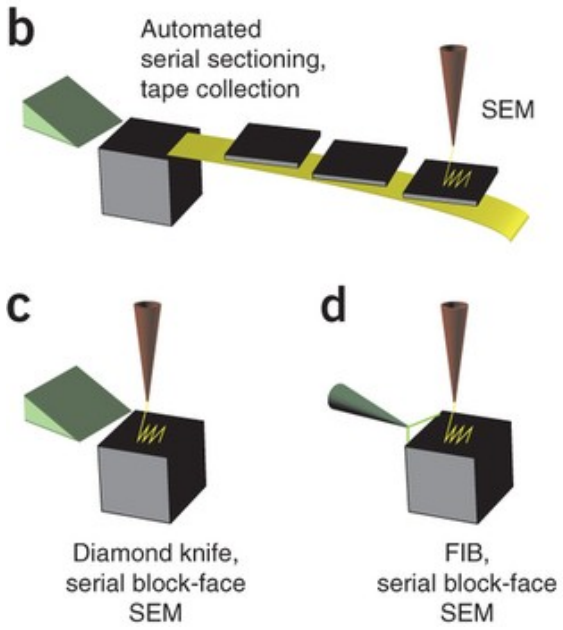


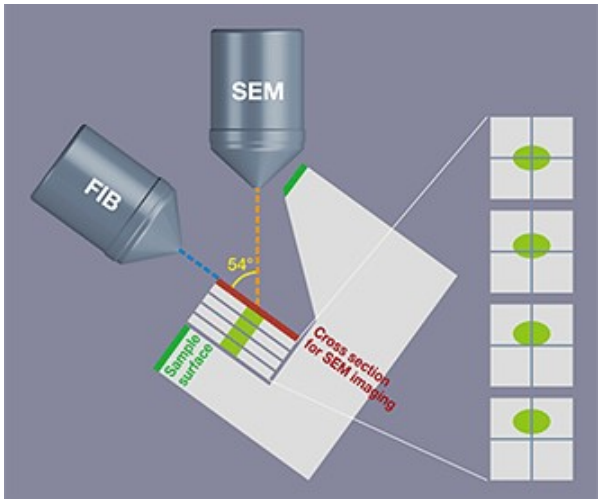
Block face imaging

Mechanical or 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

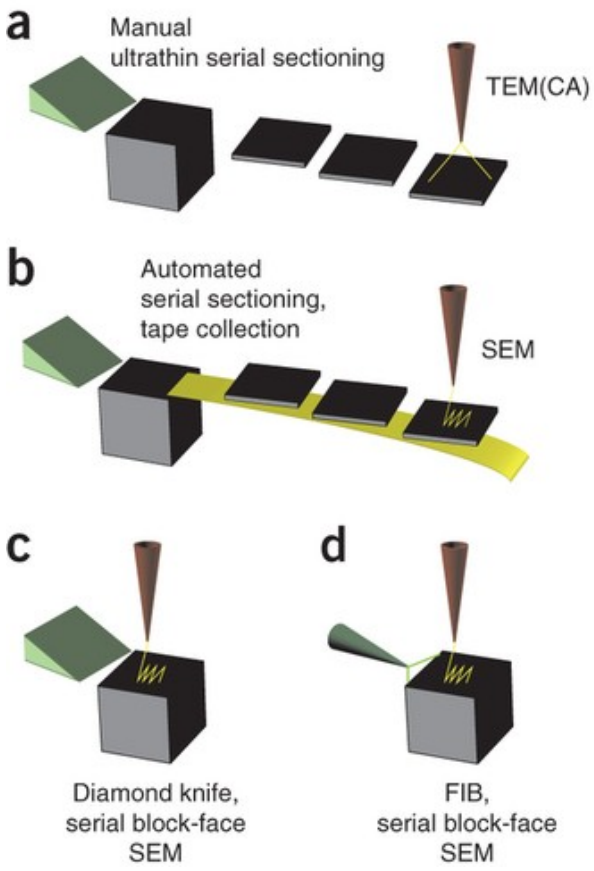


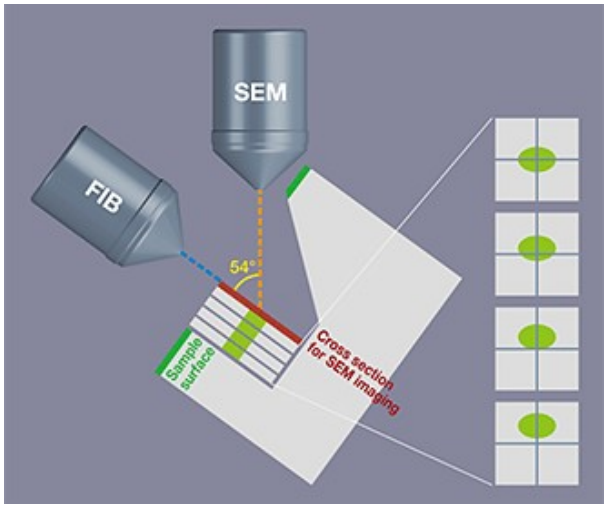


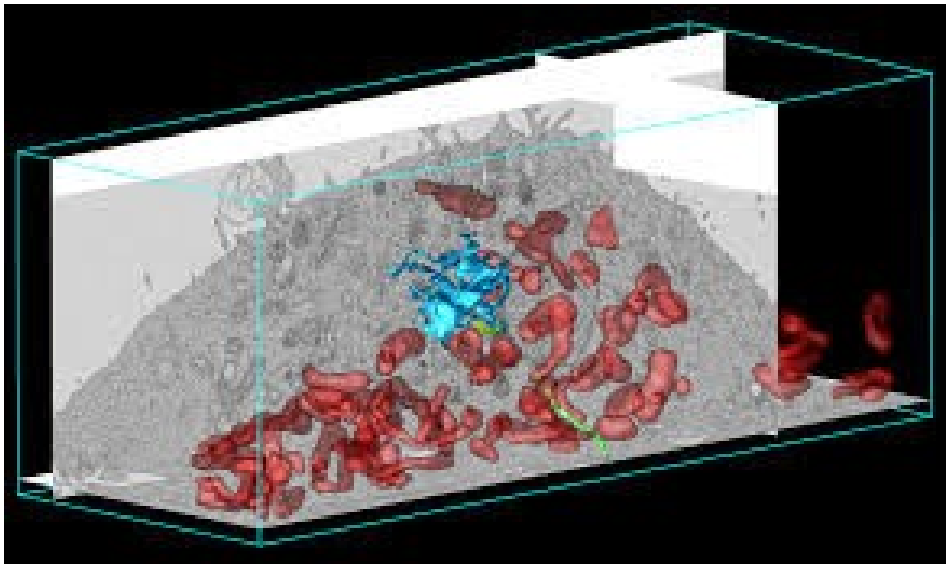


Thin section methods

Focused ion beam block-face for SEM



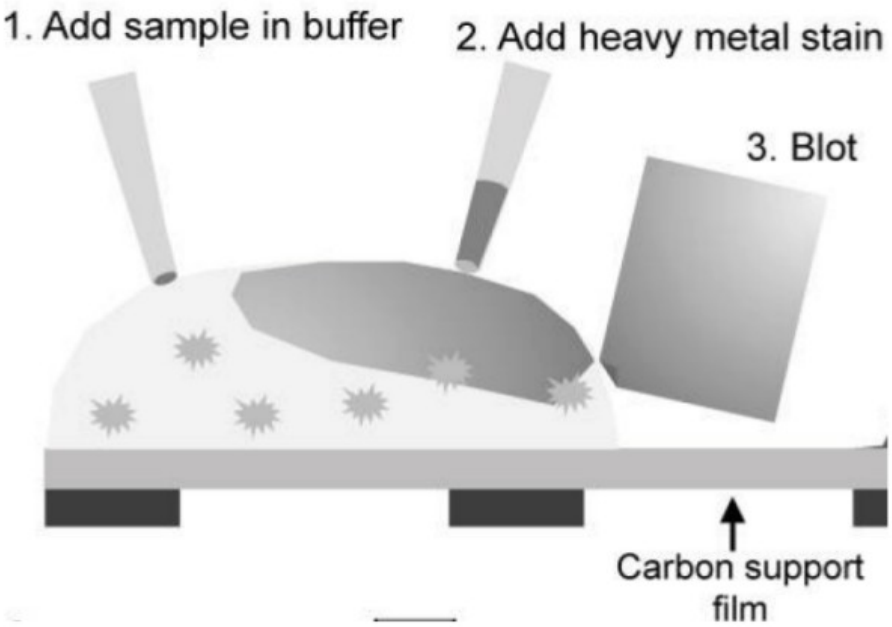


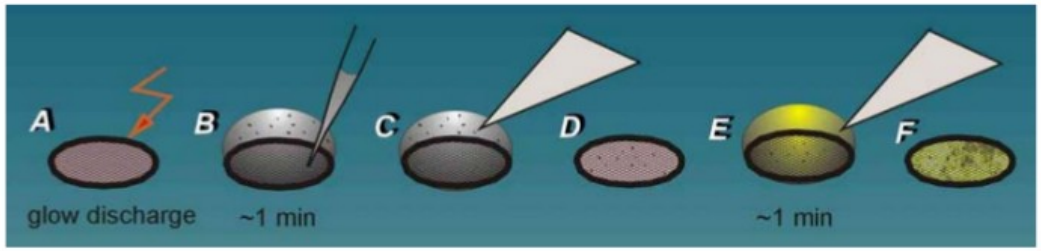


Heavy metal staining

Negative staining

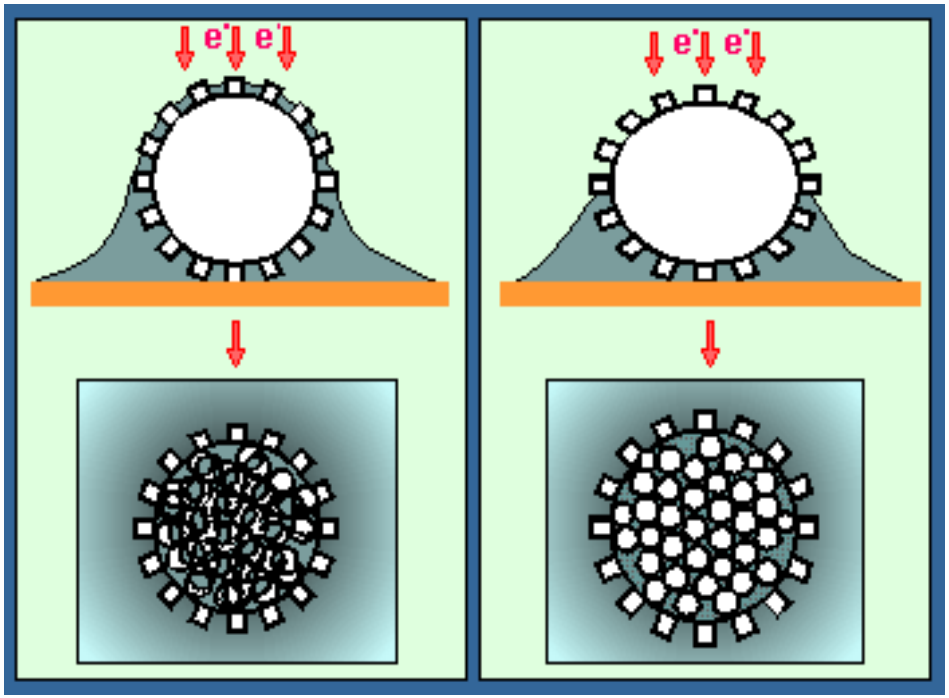
Stains: uranyl acetate (pH=4)
uranyl formate (pH=4)
ammonium molybdenate (pH=7)
phosphorus thungstanate (pH=7)





Heavy metal staining

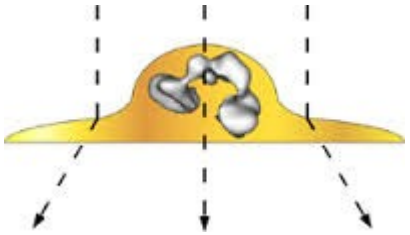
Negative staining



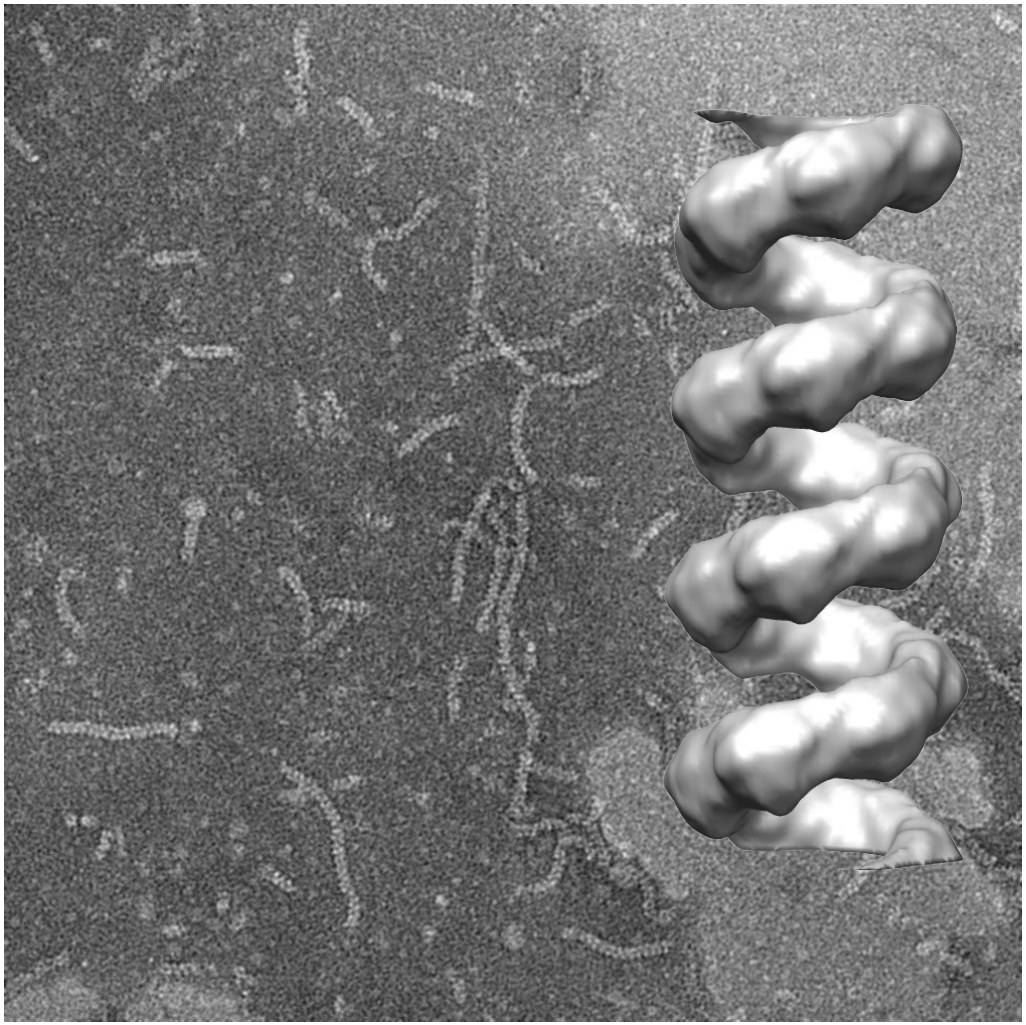
Pros: quick sample screening
high amplitude contrast
less prone to beam damage

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

Heavy metal staining



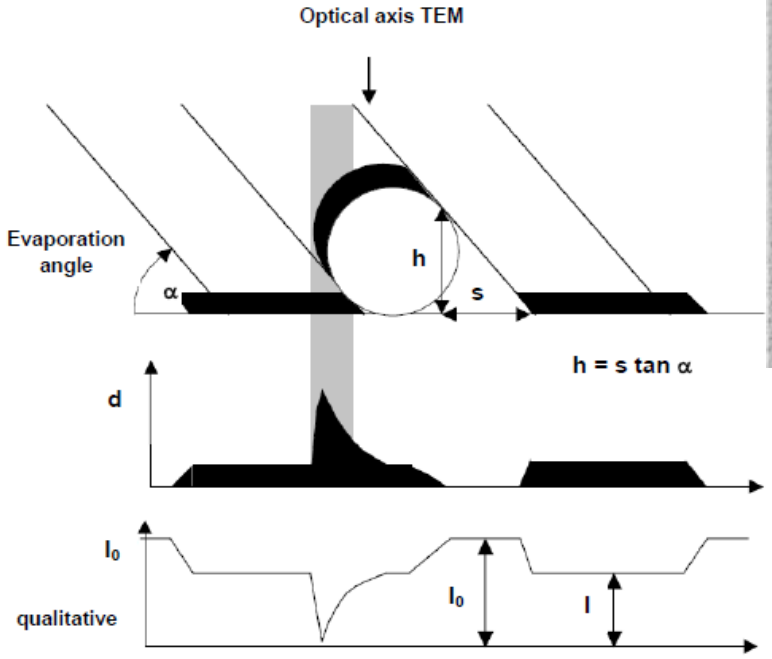
Negative staining

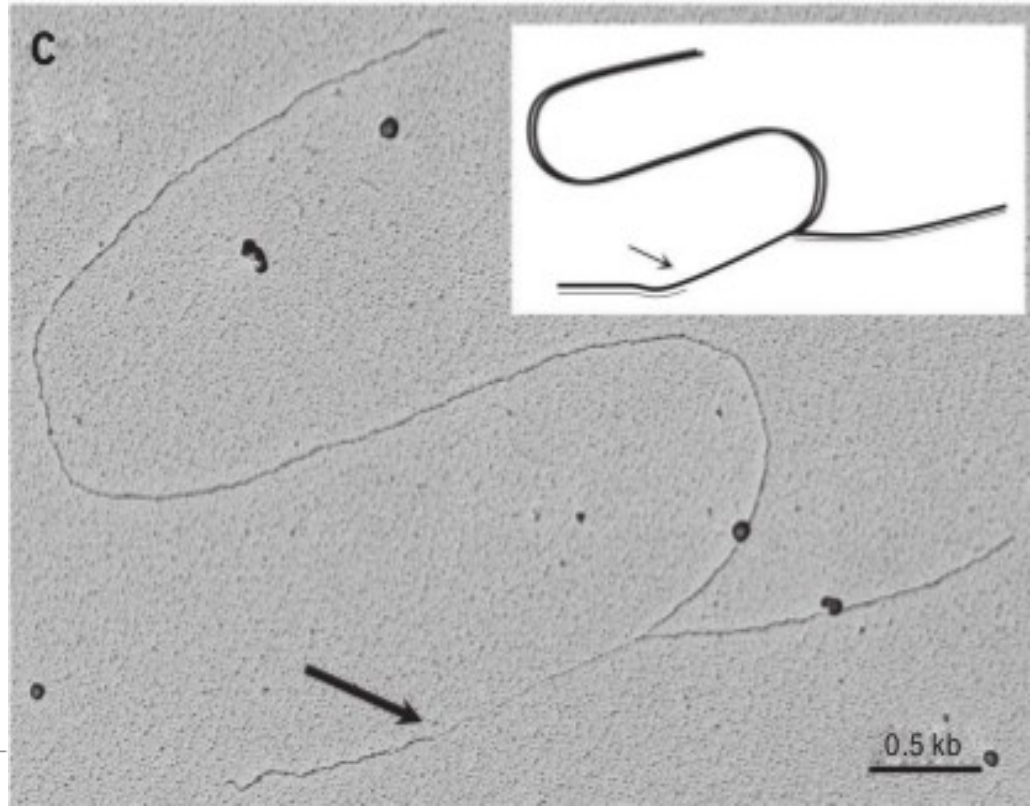


Heavy metal staining

Metal shadowing

- DNA visualization



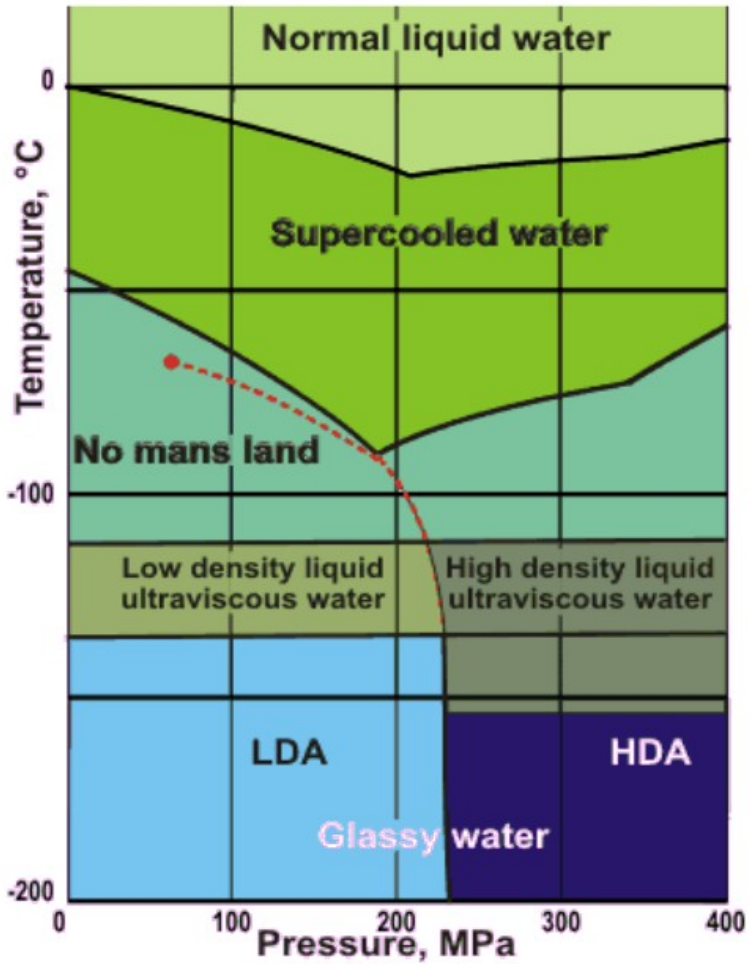


Heavy metal staining

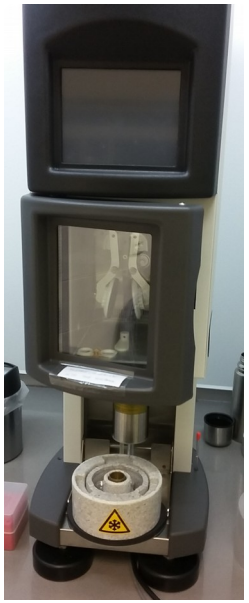
- non-physiological conditions during sample preparationd
- artefacts (changes in cell structure, depression of proteins)
- usually toxic chemicals used during sample prep
- obtainable level of detail limited

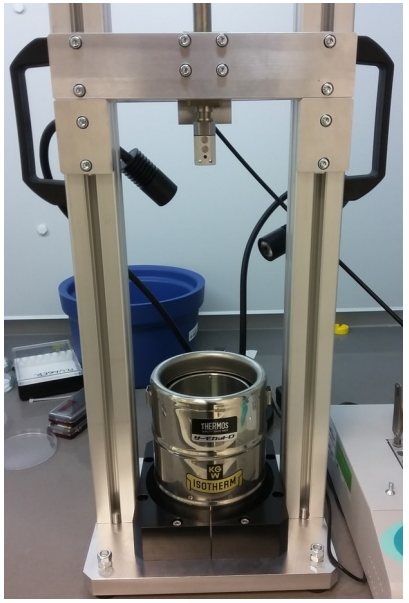
+ high signal to noise
+ low dose sensitivity
+ robust (easy sample handling)

Plunge freezing



- Rapid immersion of buffered sample into cry
- Cryogens: liquid ethane
  - ethane:propane mixture
- Vitrification has to be fast ~1000 K/s
-
- Possible only for samples with thickness ~<
- => amorphous ice
- => thin layer (200-600nm)





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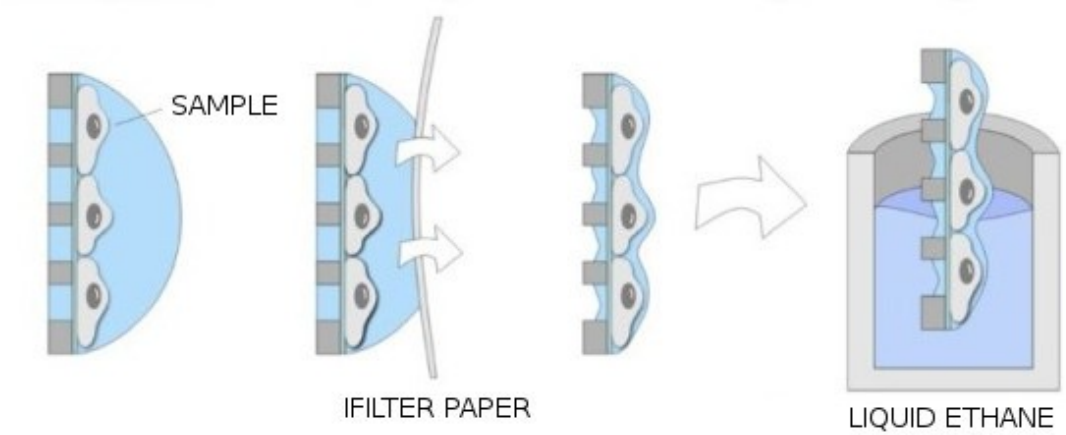
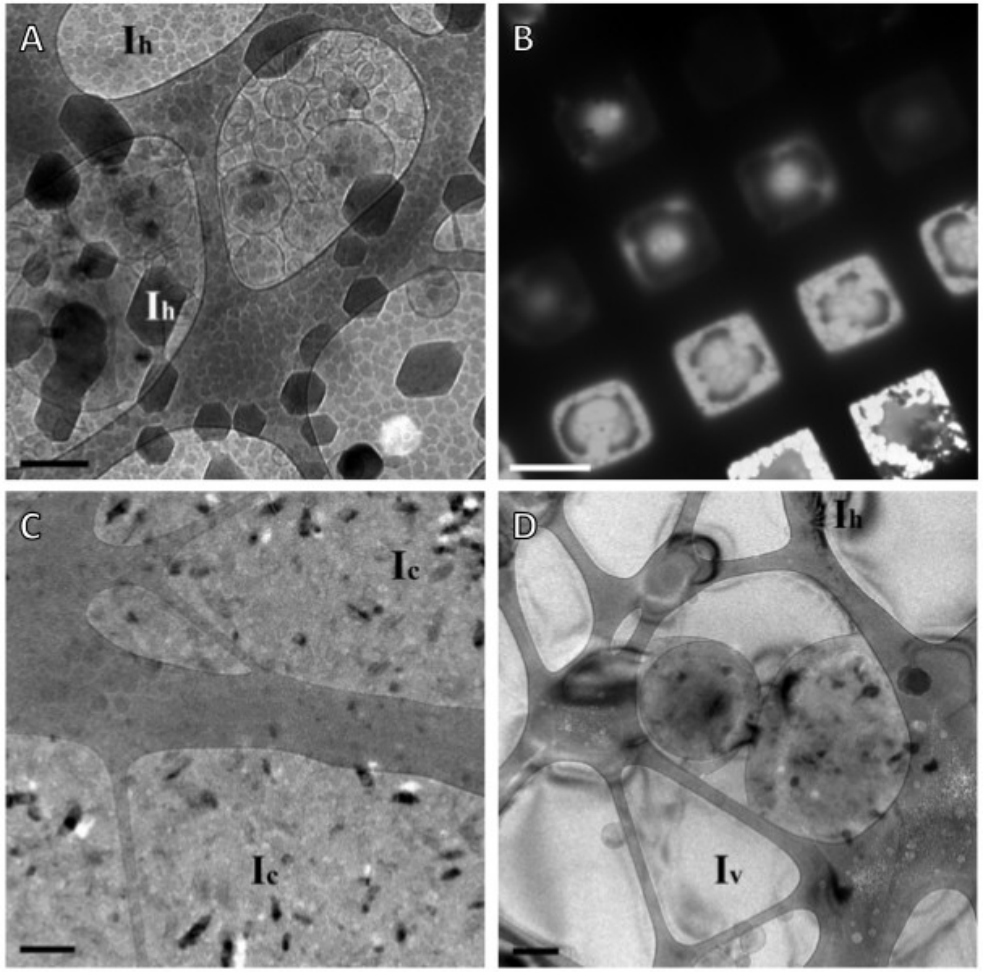
Plunge freezing

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

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

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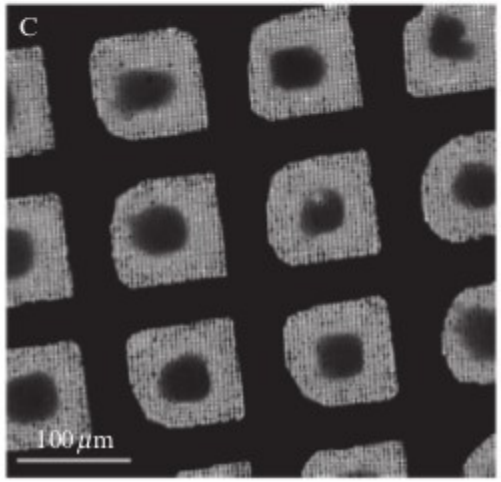
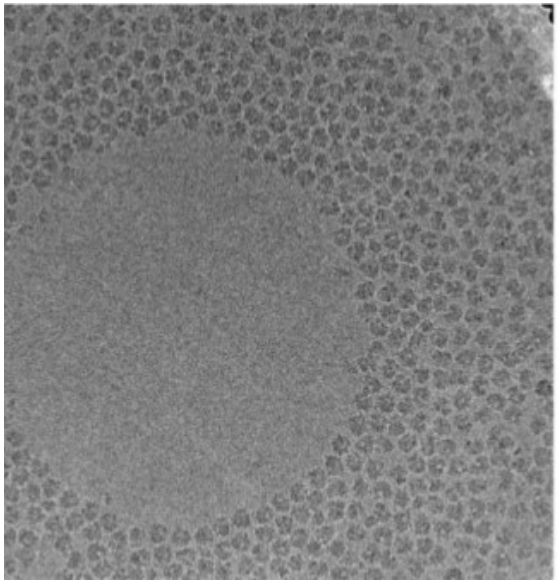
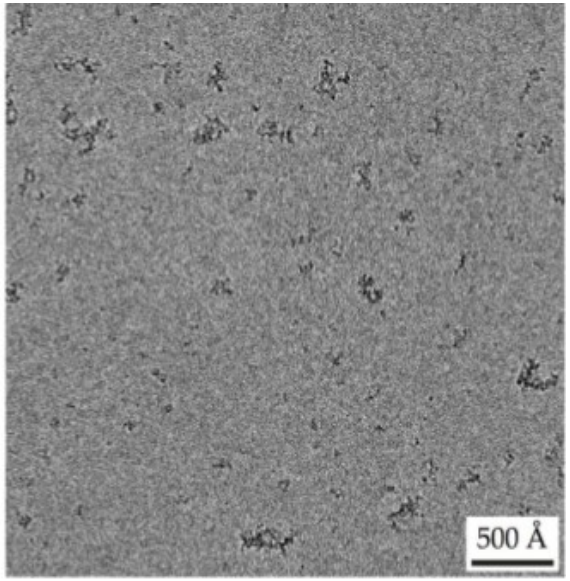
Plunge freezing



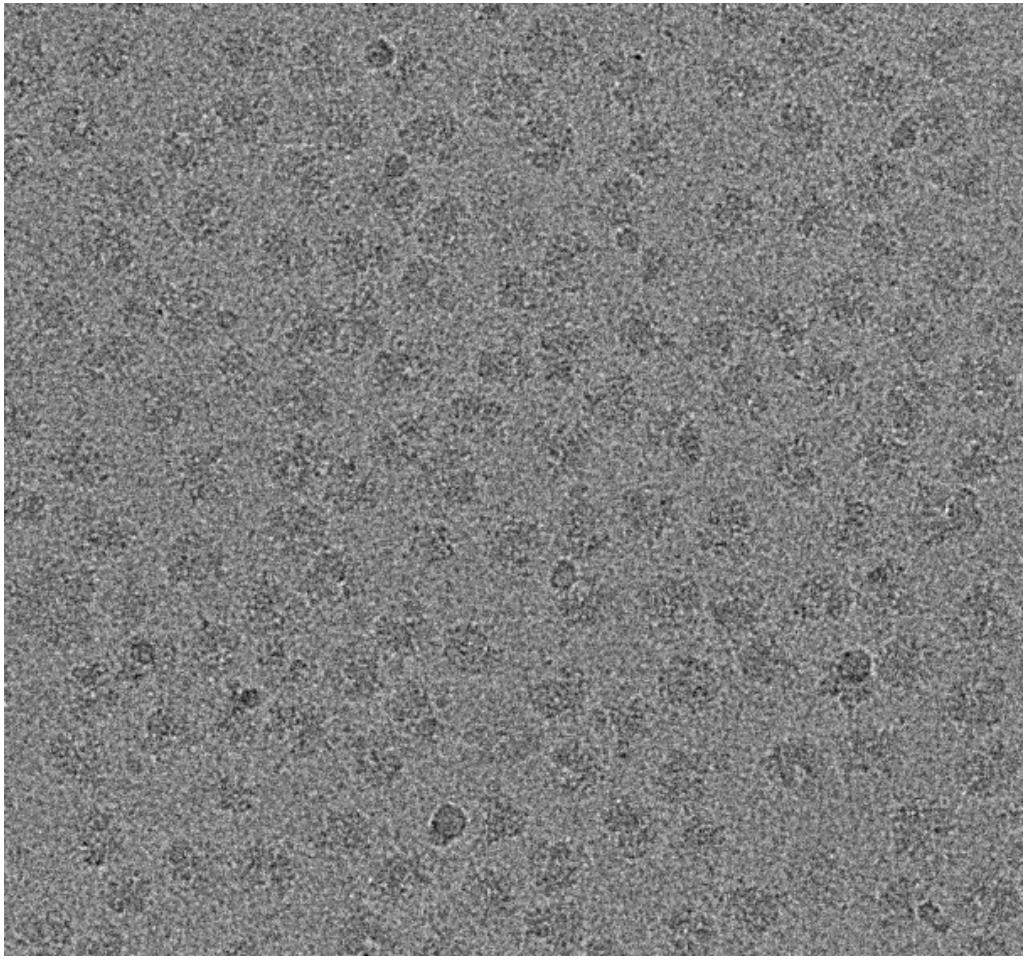


Extrusion of particles from thin ice



Denaturation at air water interface

Plunge freezing



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

Cellular cryo-EM techniques

Plunge freezing:

- rapid immersion of buffered sample into cryogen (liquid ethane, ethane:propane mix)

- vitrification has to be fast 10e4-10e5 K/s

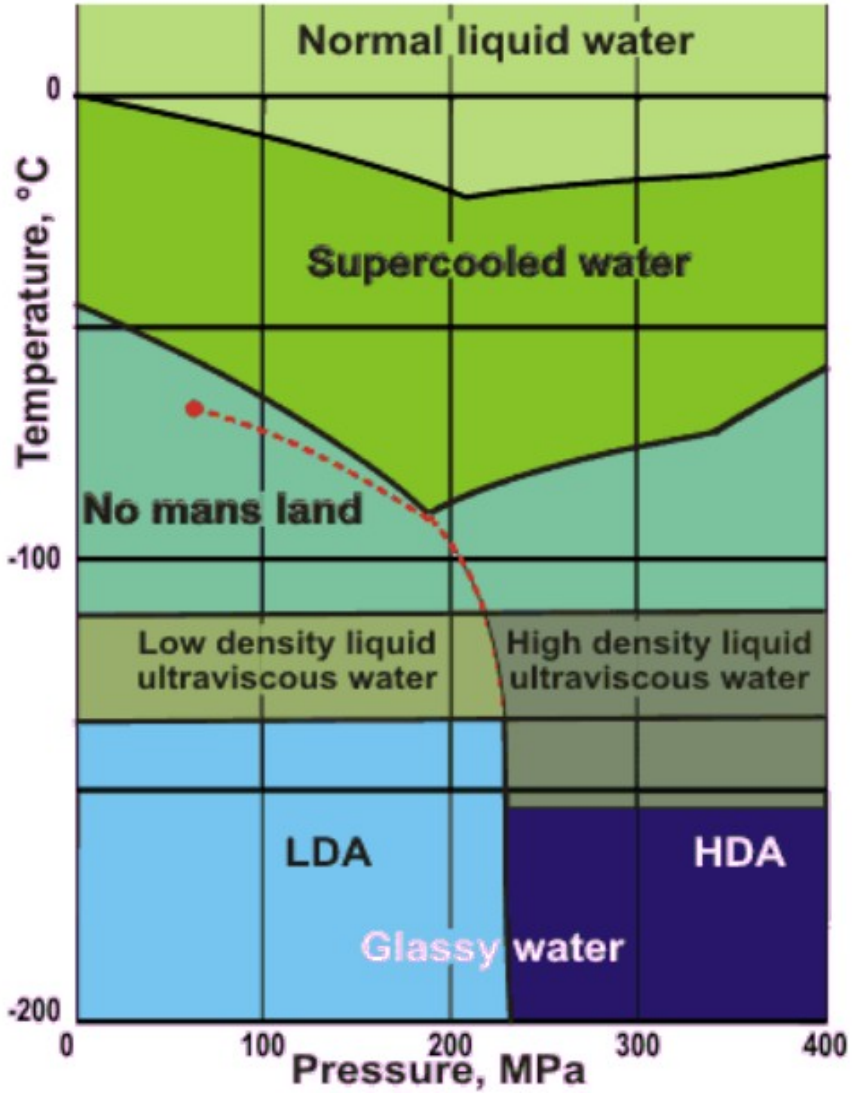
- available only for samples ~<10um thick

High pressure freezing

- sample thickness <200um

- freezing with liquid nitrogen

- 2000 bars, 20 ms



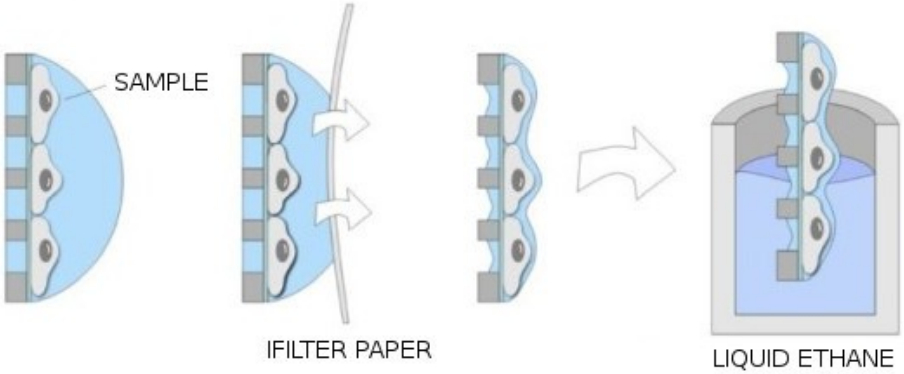
Cellular cryo-EM techniques

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

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

3mm diameter



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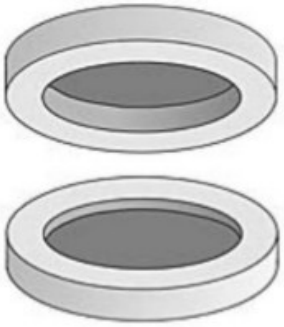
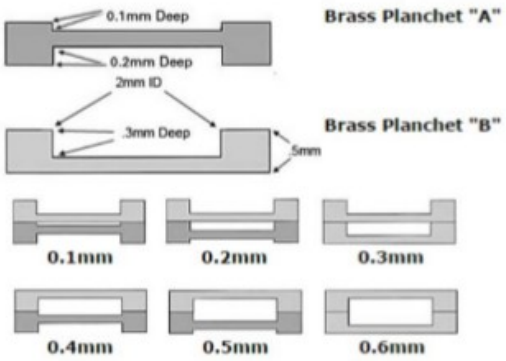
Cellular cryo-EM techniques

High pressure freezing, freeze substitution





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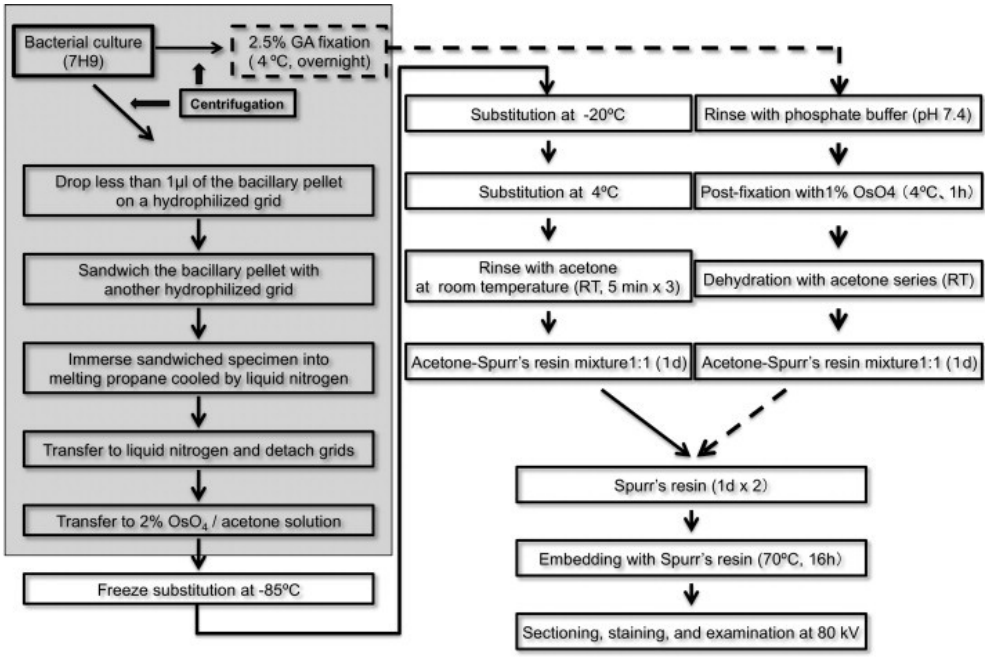
Freeze substitution
- reduction of ultrastructure changes compared to dehydration at ambient temperature
- dehydration at temperatures <-70C (aceton typically -90C)
- fixatives are evenly distributed before cross-linking at ambient temperature
- resin embedding for ultramicrotomy at room temp.



Yamada et al. JMM 2010

CEITEC

Cellular cryo-EM techniques

CEMOVIS – cryo-EM of vitrous sections

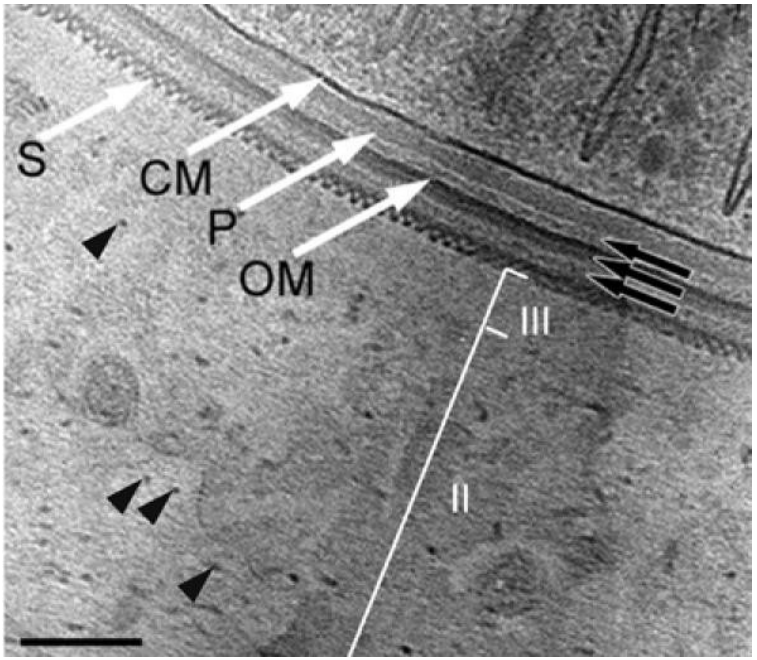
- no chemical fixation, dehydration or contrasting

- low contrast

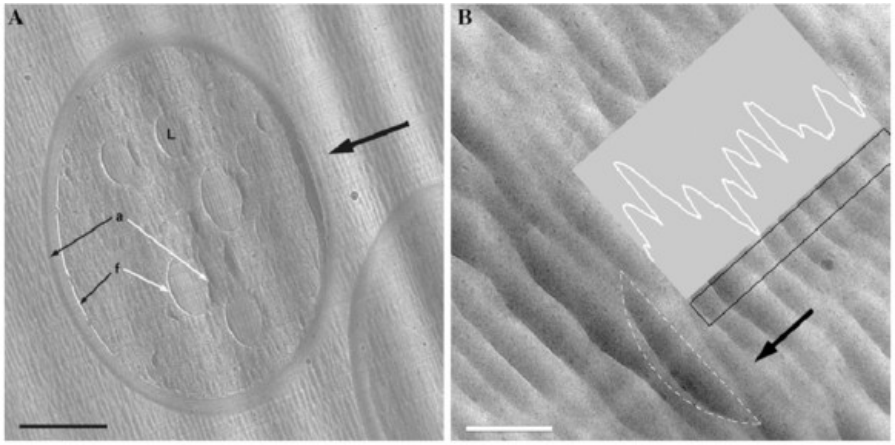
- preservation of the sample in near-native conditions

- mechanical sectioning by ultramicrotome at LN2 conditions

- sectioning artefacts

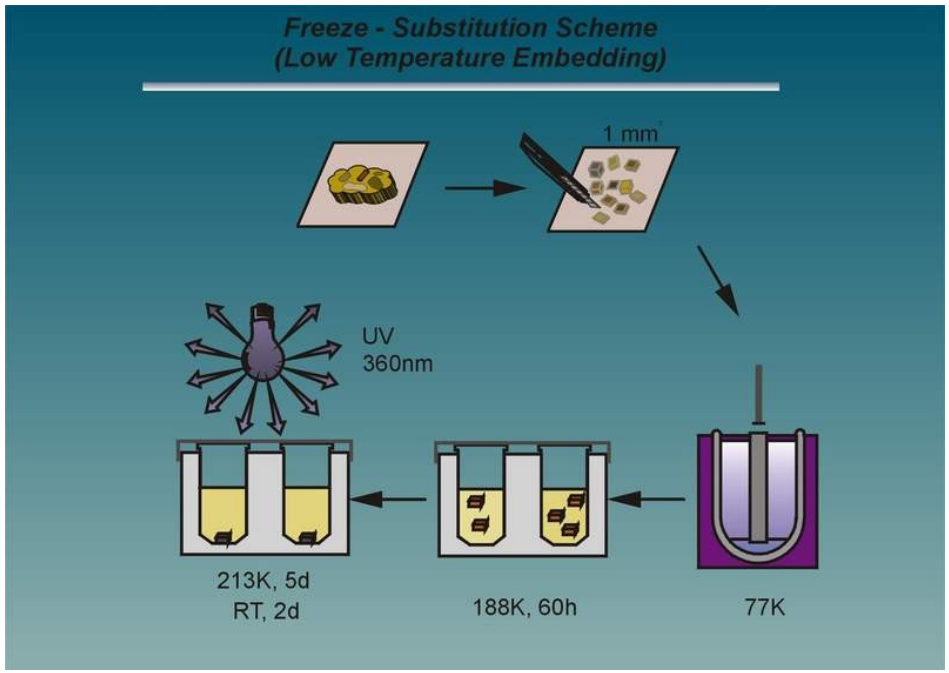


Al-Amoudi et al. EMBO J 2004



Al-Amoudi et al. JSB 2005

Thin section methods - note



Freeze substitu

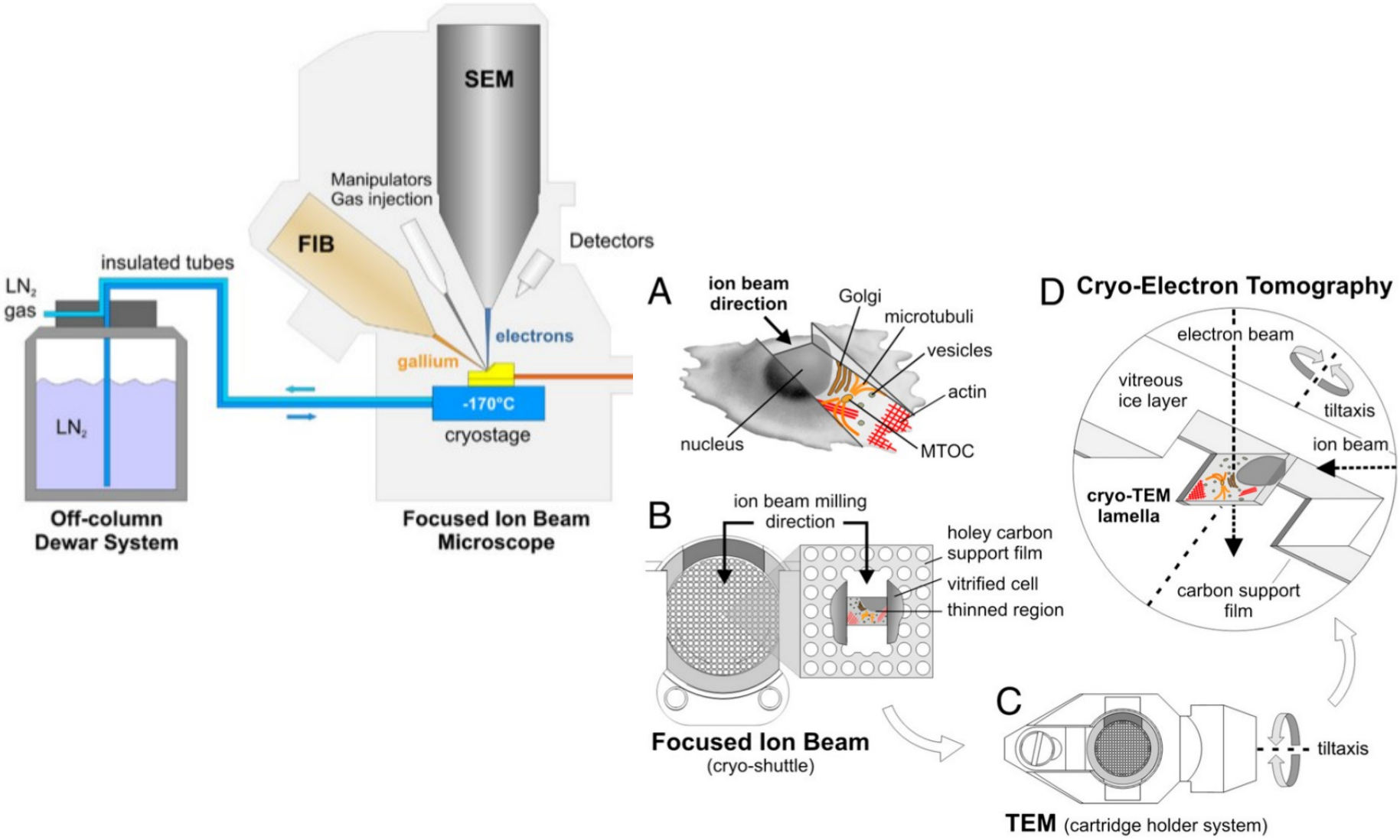
- Reduce ultra-structure changes at
- due to dehydration as seen at amb
- temperature
-
- Dehydration at temperatures belo -
- (aceton typically -90C)

- Fixatives are evenly distributed bef
- cross-linking at ambient temperatu

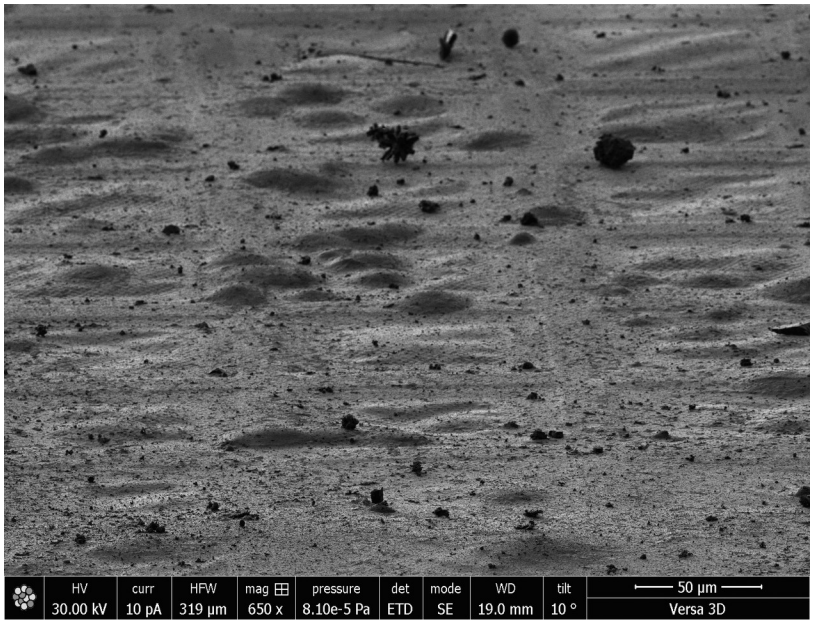
- Resin embedding for ultramicrotom
- at room temperature

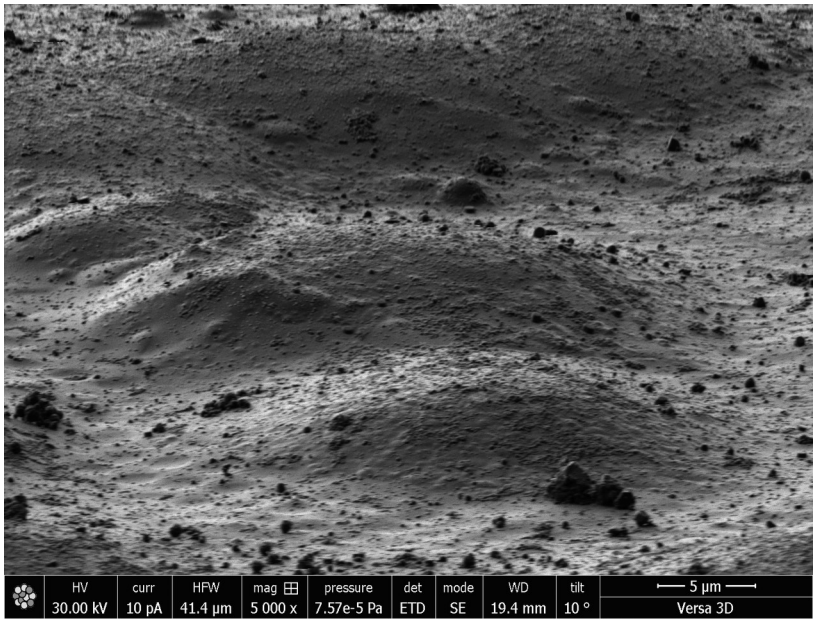
Cellular cryo-EM techniques

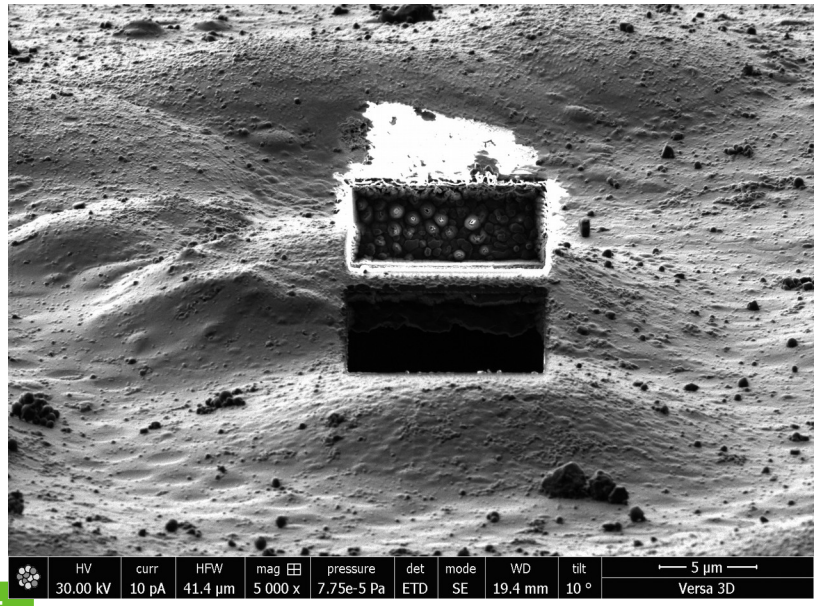
Focused ion beam milling of cellular lamellas

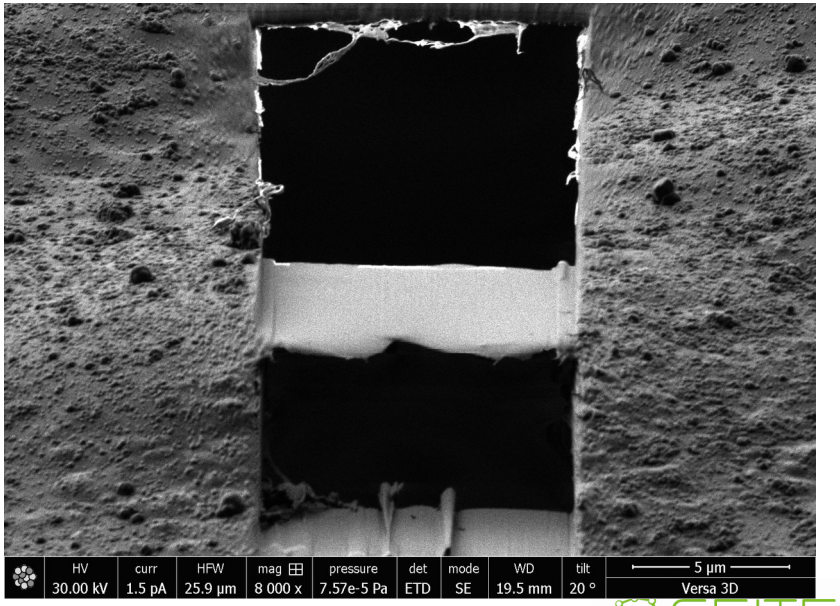


Cellular cryo-EM techniques



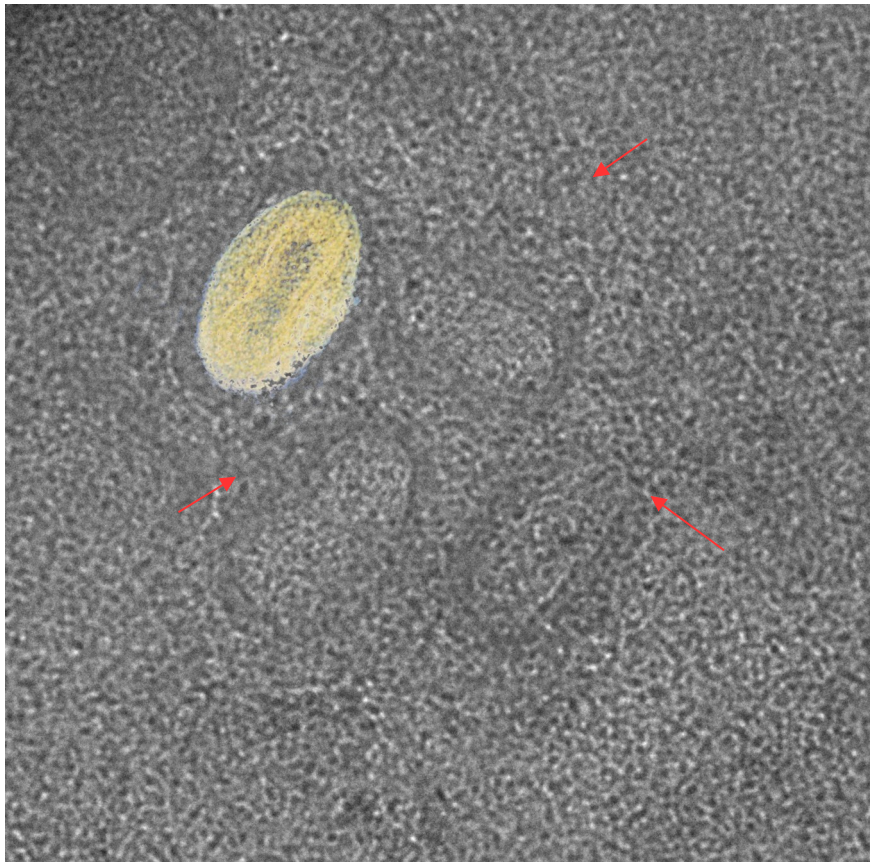






Cellular cryo-EM techniques

Vaccinia virus inside cell



Pavel Plevka group

HeLa cells