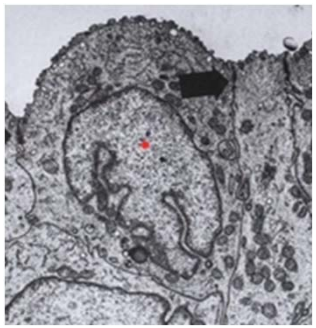
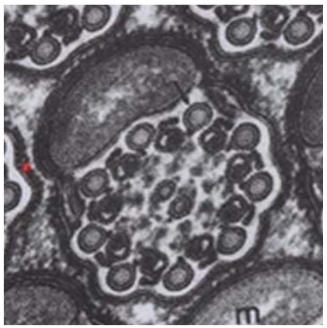
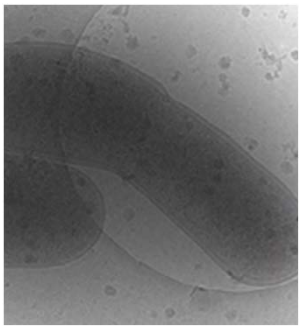
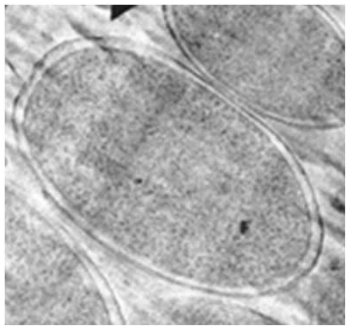
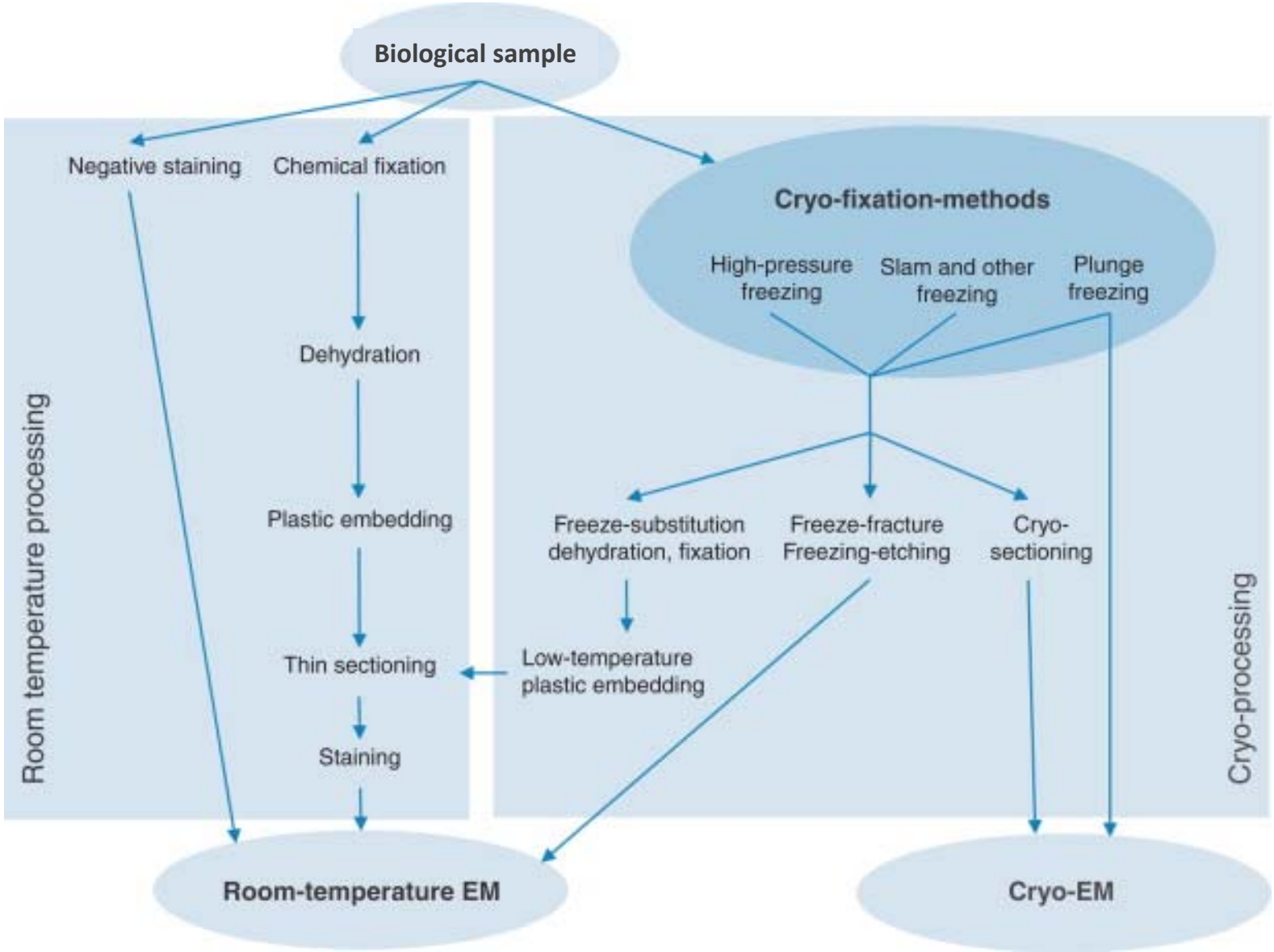
Lecture 3: Sample Preparation

HOW TO PRESERVE A BIOLOGICAL SAMPLE IN HIGH VACUUM ??

1. Traditional Thin Section Techniques
2. Staining / Shadowing Techniques
3. Plunge Freezing Techniques
4. High Pressure Freezing Techniques
5. Focus-Ion-Beam Milling Techniques

Sample Preparation Techniques



Pilhofer et. al (2010) *Methods Cell Biol.* 96, 21

Thin Sectioning Techniques

A. Chemical fixation (aldehydes, osmium)

B. Sample dehydration (EtOH, acetone)

C. Plastic embedment (epon)

D. Sectioning (ultramicrotome)

E. Staining (uranyl acetate, lead citrate)

Chemical Fixation

Chemical fixatives:

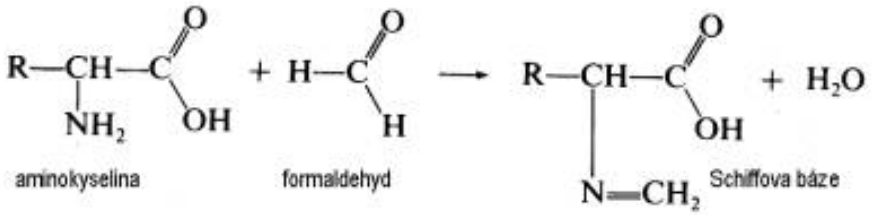
a) Coagulators : cause protein denaturation and aggregation (MetOH, EtOH, HCl)

b) Non-coagulators: polymerization of macromolecules (aldehydes, osmium oxide)

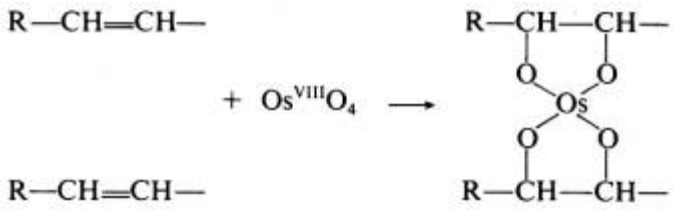
Factors affecting fixation:

- fixative reagent and sample size
- fixation procedure (fixative concentration, additives)
- external conditions (pH, temperature, duration, osmosity)

1) Primary aldehyde fixation (proteins, nucleic acids): 1-3% solution



2) Secondary OsO_4 fixation (membranes, proteins): 1-2% solution



Dehydration and Plastic Embedding

DEHYDRATION: successive washing with 30, 50, 70, 80, 90, 95% solutions

EtOH: most common, least extraction of cellular material

reactive with OsO_4, immiscible with epoxy resins

Acetone: more extraction of cellular material than EtOH

less shrinking artifacts, miscible with epoxy resins

Common artifacts due to dehydration:

- extraction of proteins, lipids, etc.
- sample shrinking up to 40 %
- formation of various precipitates

Plastic Embedding: epoxy, acryl or polyester resins

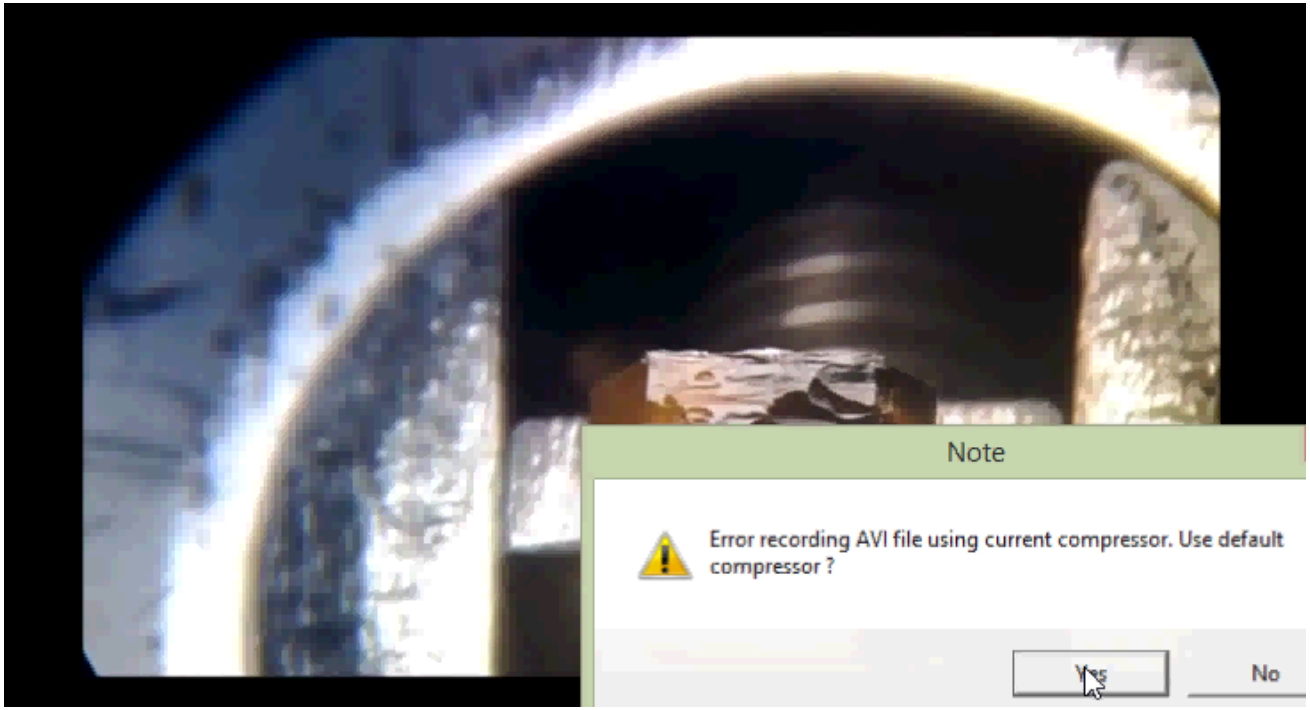
Penetration: successive washes with incresing concentrations of resin

Polymerization: initiated by heat (60°C), UV radiation, catalysts

NB: ultrastructure observed in EM is highly affected by the choice of resin !!!

Ultramicrotomy / Sectioning

1) Initial trimming of the plastic block
2) Initial slicing of 500 nm sections
3) Thin sectioning of 50-100 nm sections
4) Recovery of sections and transfer to EM



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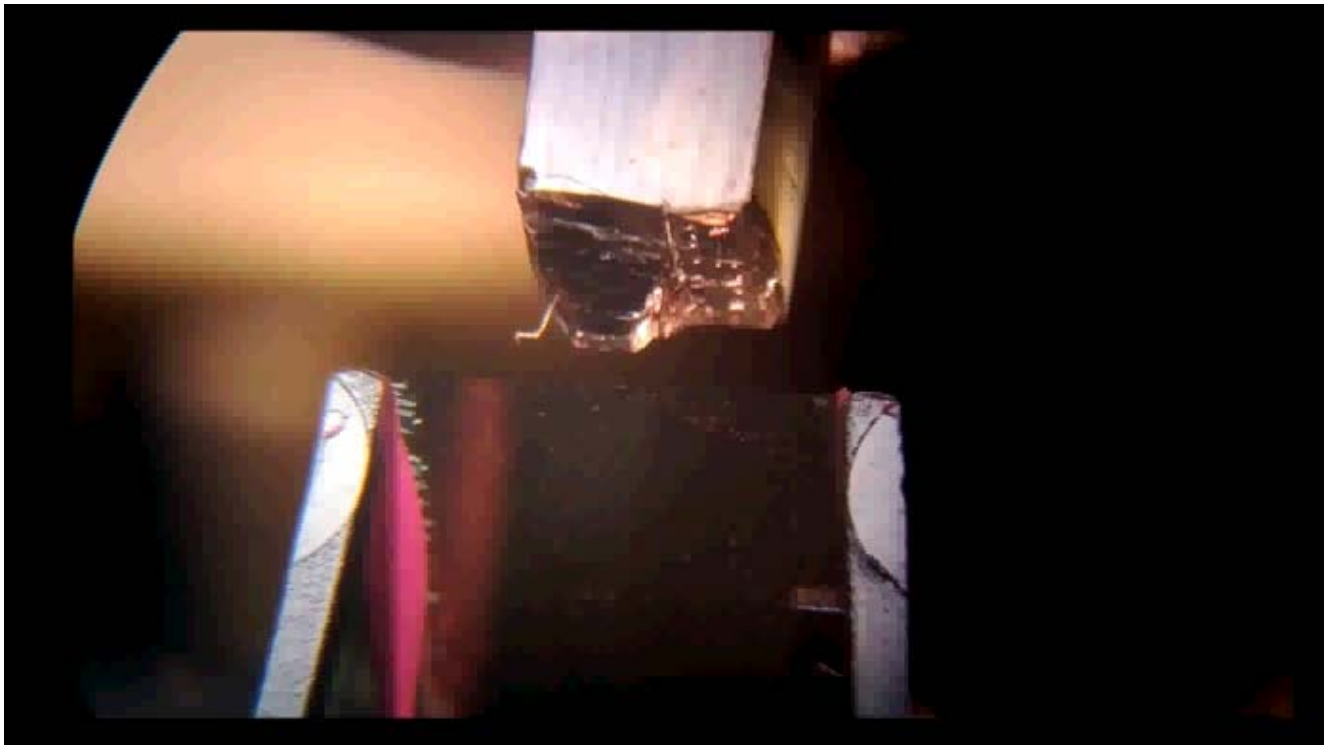


Staining and Immuno-labeling

Staining:

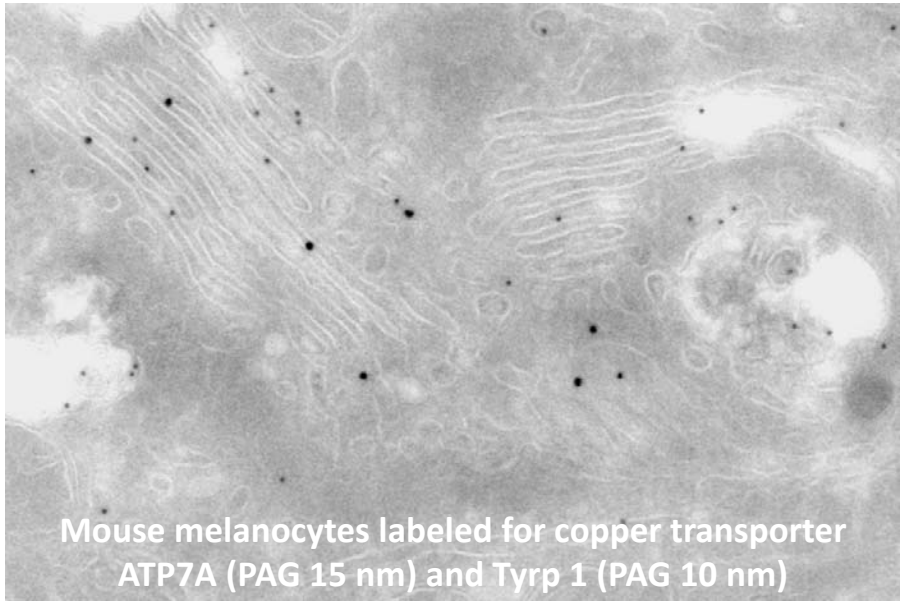
a) uranyl acetate in alcohol solution: staining of proteins and nucleic acids

b) lead citrate in aqueous solution: staining of membranes and lipids

Immuno-labeling:

a) pre-embedding protocols (labeling of 50-um sections before fixation)

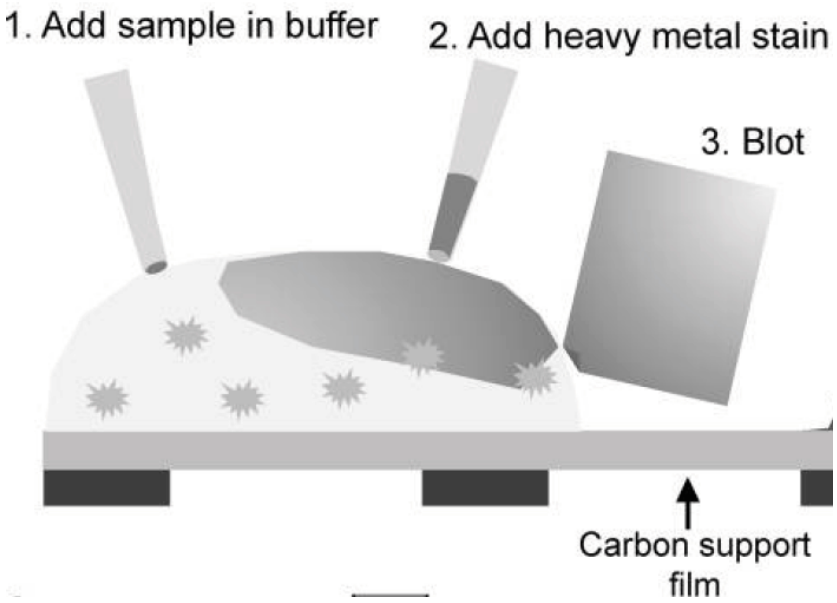
b) post-embedding protocols (thin sections on a EM grid before staining)

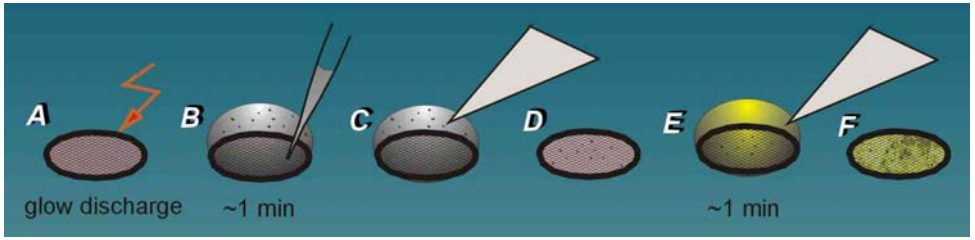


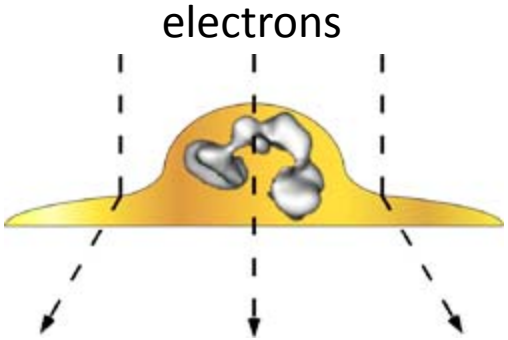
Mouse melanocytes labeled for copper transporter ATP7A (PAG 15 nm) and Tyrp 1 (PAG 10 nm)

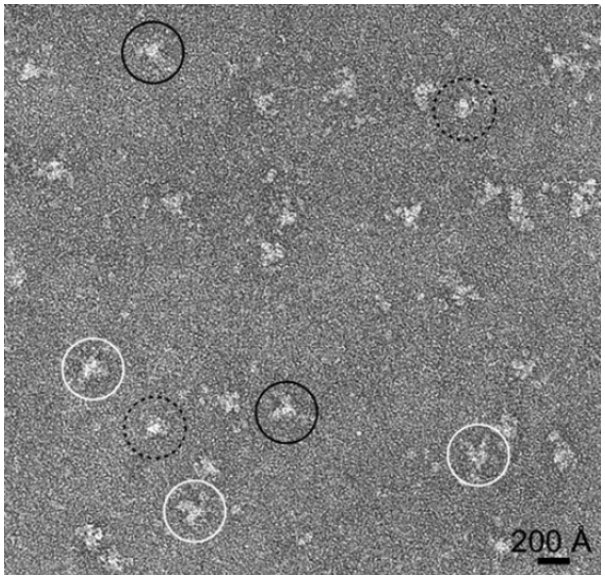
Negative Staining of Proteins

- **sample is embedded in a layer of heavy metal salts**
- **reveals overall shape and solvent excluded surface**





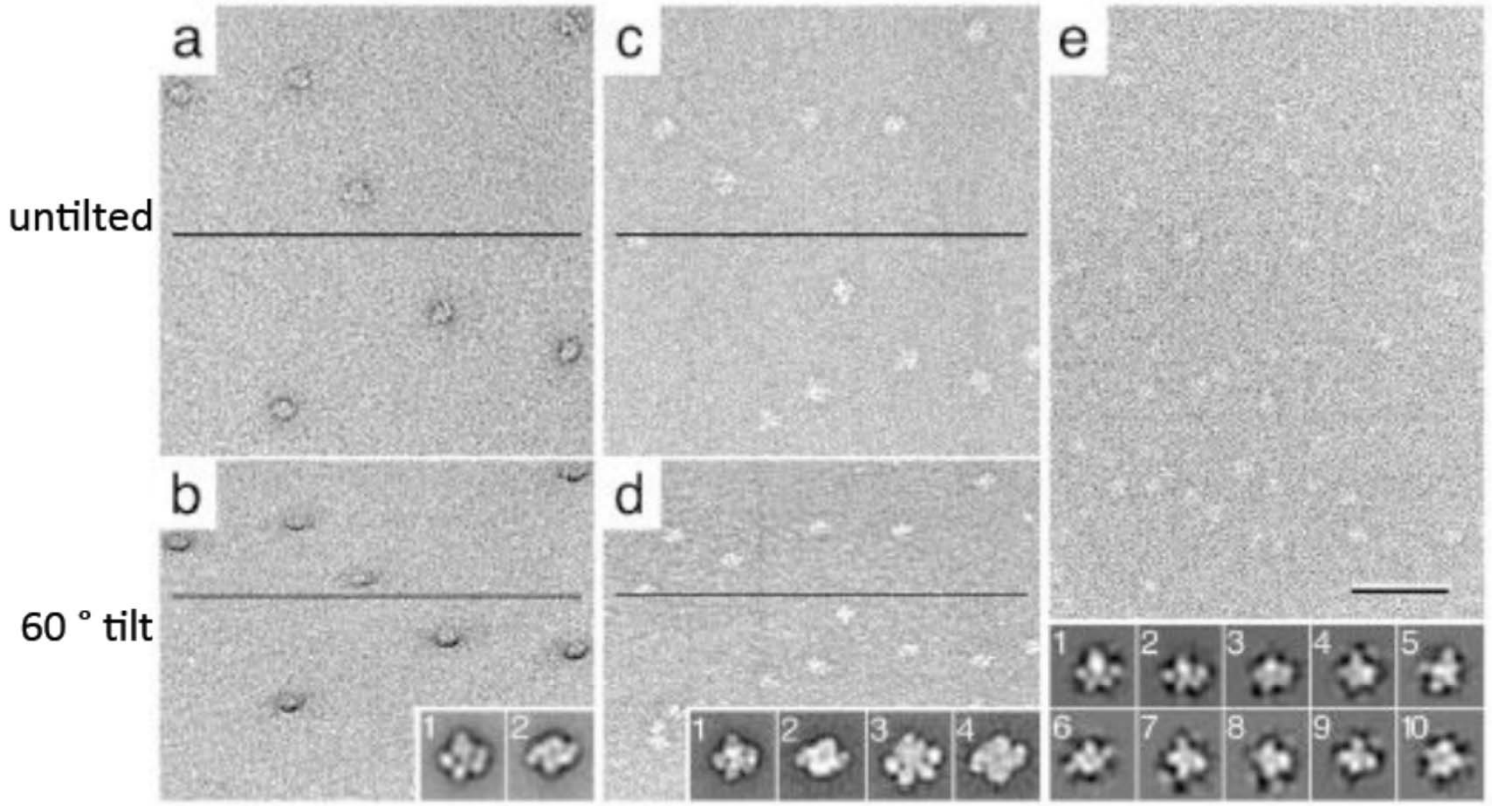




Negative Staining of Proteins

Challenges:	even and uniform layer of stain good adsorption of sample to carbon stability of the protein sample
Advantages:	quick method to screen sample conditions very high amplitude contrast stain protects the sample from beam damage
Disadvantages:	limited resolution due to stain grain size (20 Å) flattening and denaturation of proteins uneven staining complicates image processing
Typical stains:	uranyl acetate (stable, high contrast, pH 4) uranyl formate (fine grain, precipitates, pH 4) ammonium molybdate (neutral pH, unstable) phosphorus tungstate (neutral pH, fine grain)

Negative Staining of Proteins



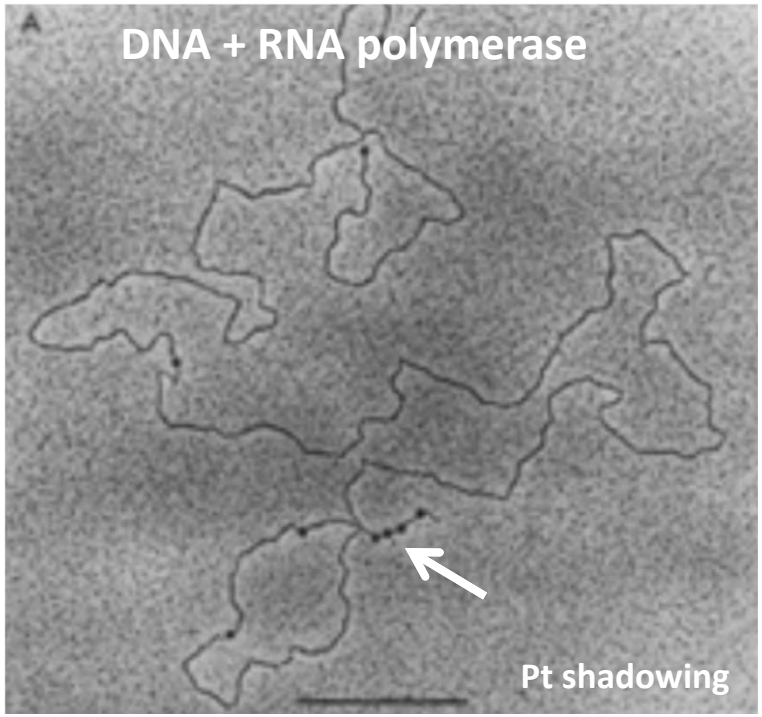
Conventional negative stain (uranyl formate)

Carbon sandwich (uranyl formate)

Glucose and ammonium molybdate (image at liquid N_2)

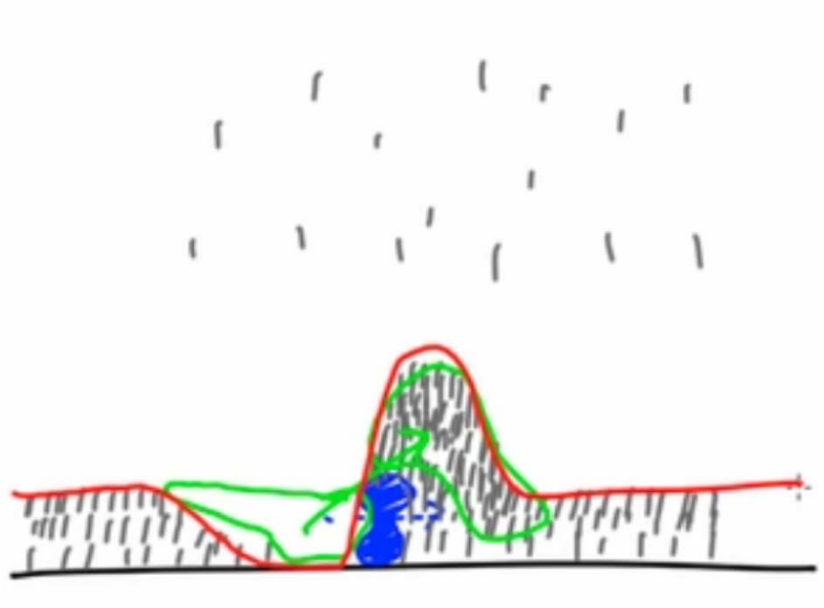
Ohi, Cheng, Walz (2004)

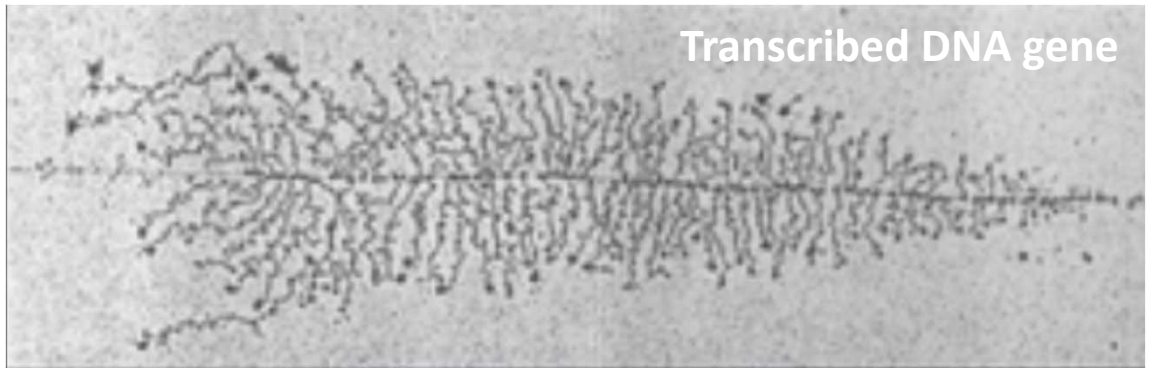
Metal Shadowing



Annu. Rev. Biophys. Bioeng. (1978) 7, 19

Principle of rotary shadowing



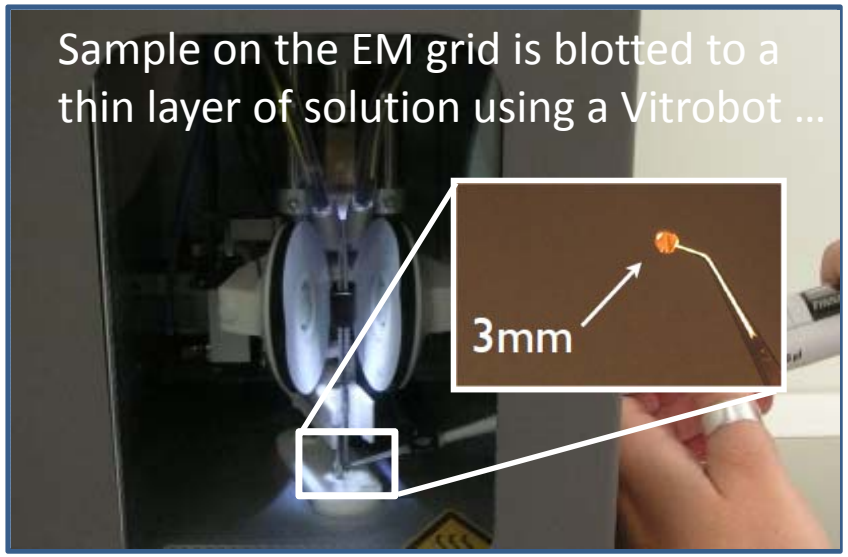


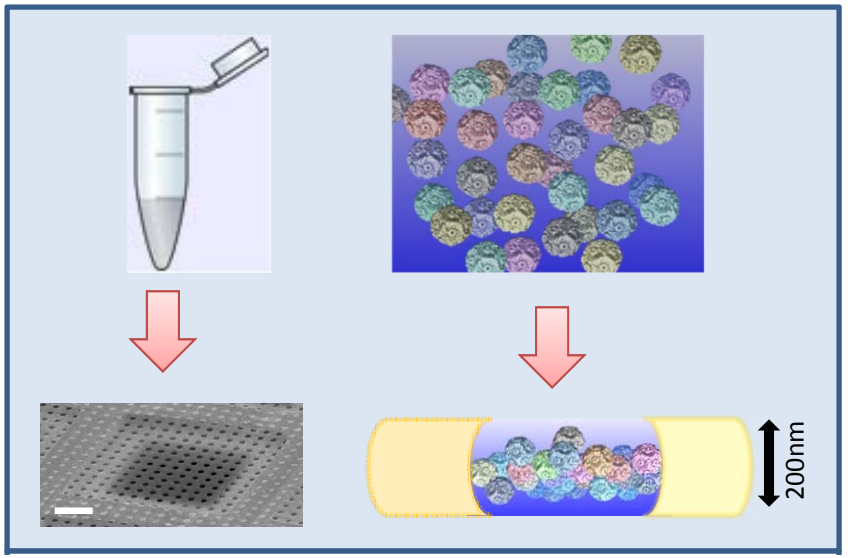
Trendelenburg, MF et al, *Histochem. Cell Biol*. (1996) 106, 167

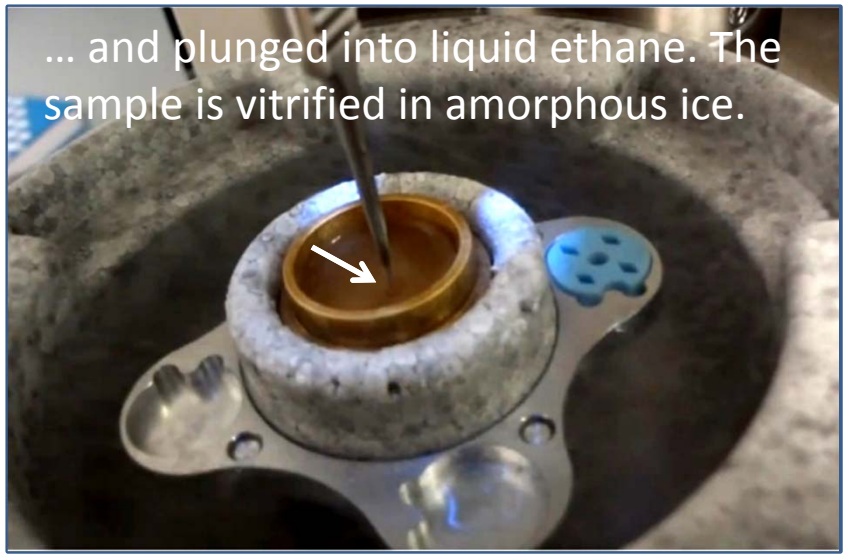
Cryo Plunging Techniques

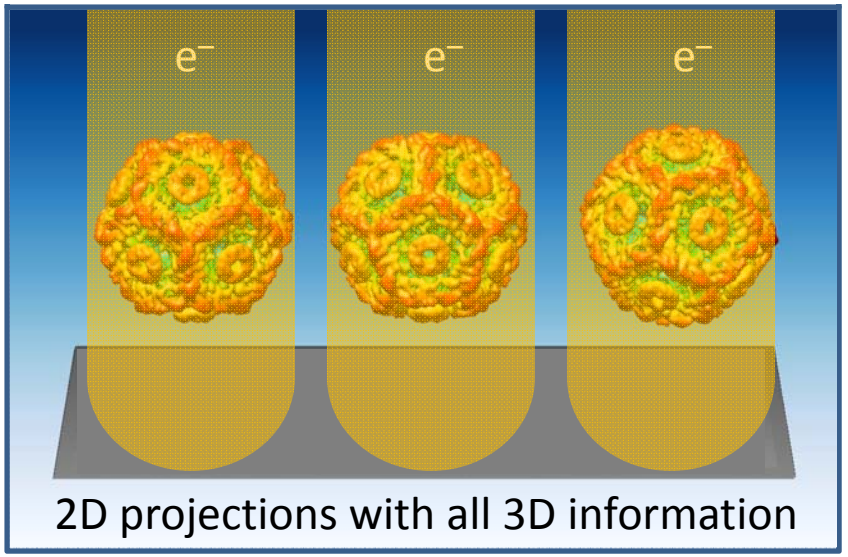
- Sample is rapidly frozen in buffer => direct imaging in near-native conditions
- Amorphous water prevents sample damage and is transparent to electrons
- Vitrification is a fast (10^{-4} s) process => freezing rates of 10^5-10^6 K/s
- Liquid nitrogen is not suitable due to low heat conductivity => ethane
- Aqueous samples are properly frozen only up to 1 um thickness
- Plunge freezing using automated or manual plungers

Cryo Plunging Techniques



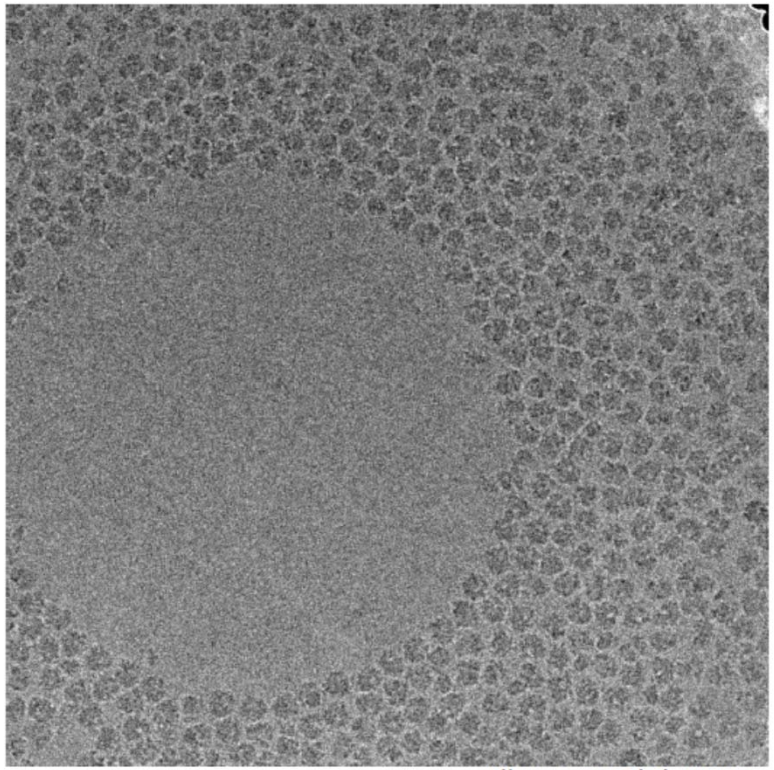






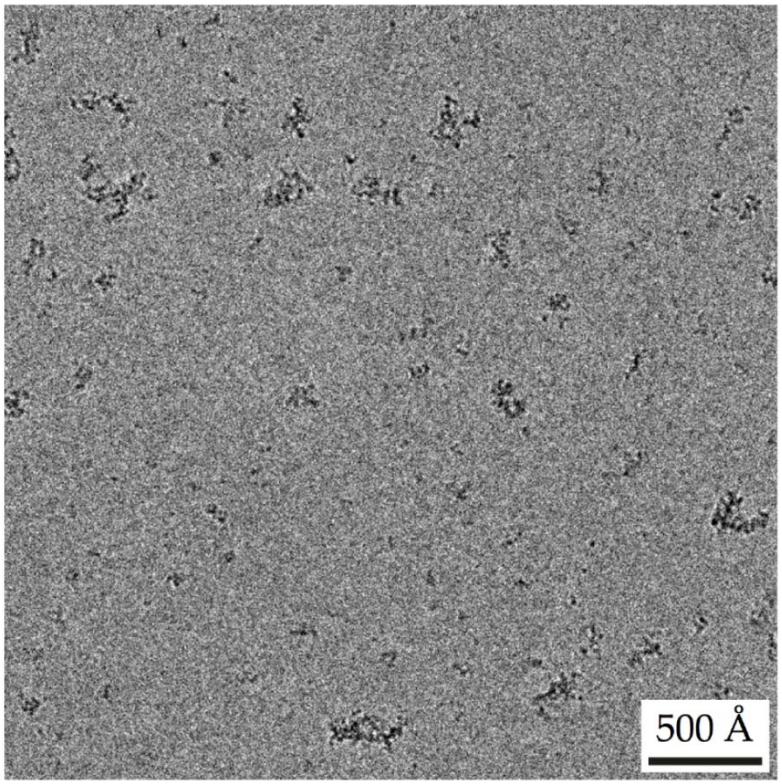
Cryo Plunging Techniques

Ice thickness



Ribosomes, Chris Russo

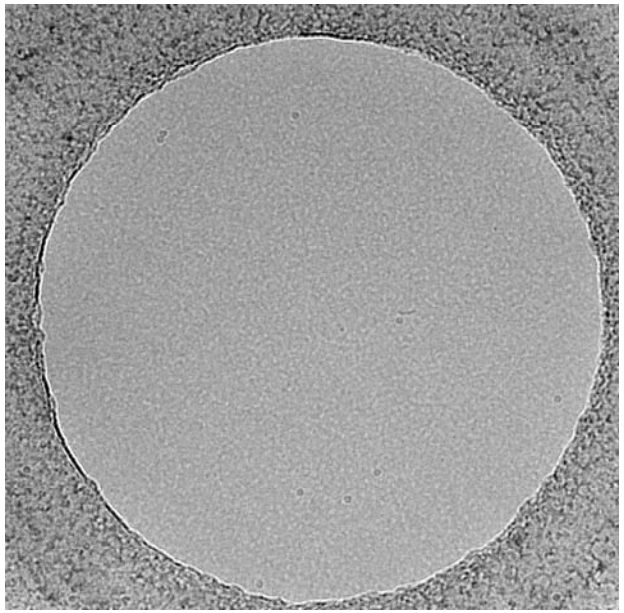
Extrusion of particles from thin ice

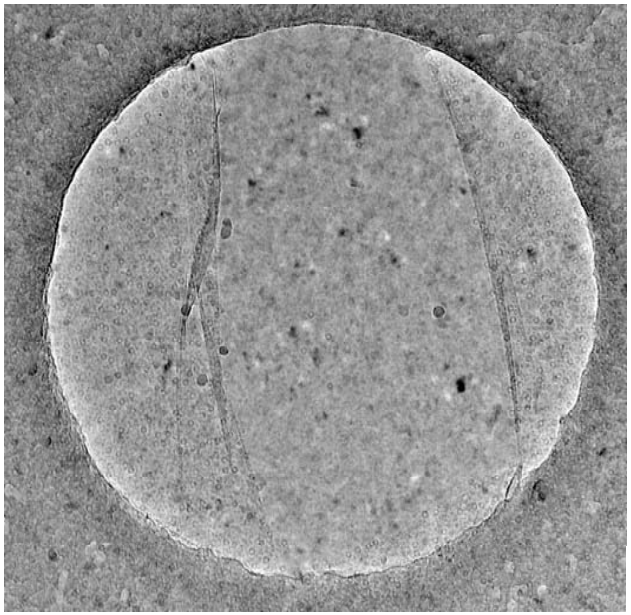


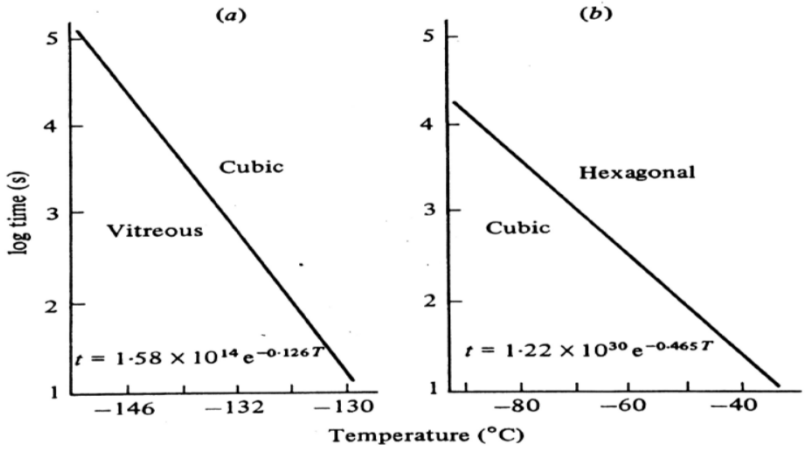
Denaturation at water-air interface

Cryo Plunging Techniques

Defitrification

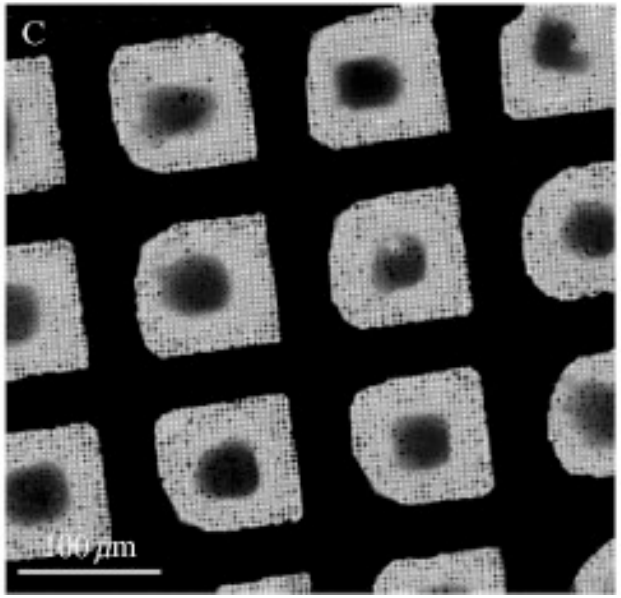


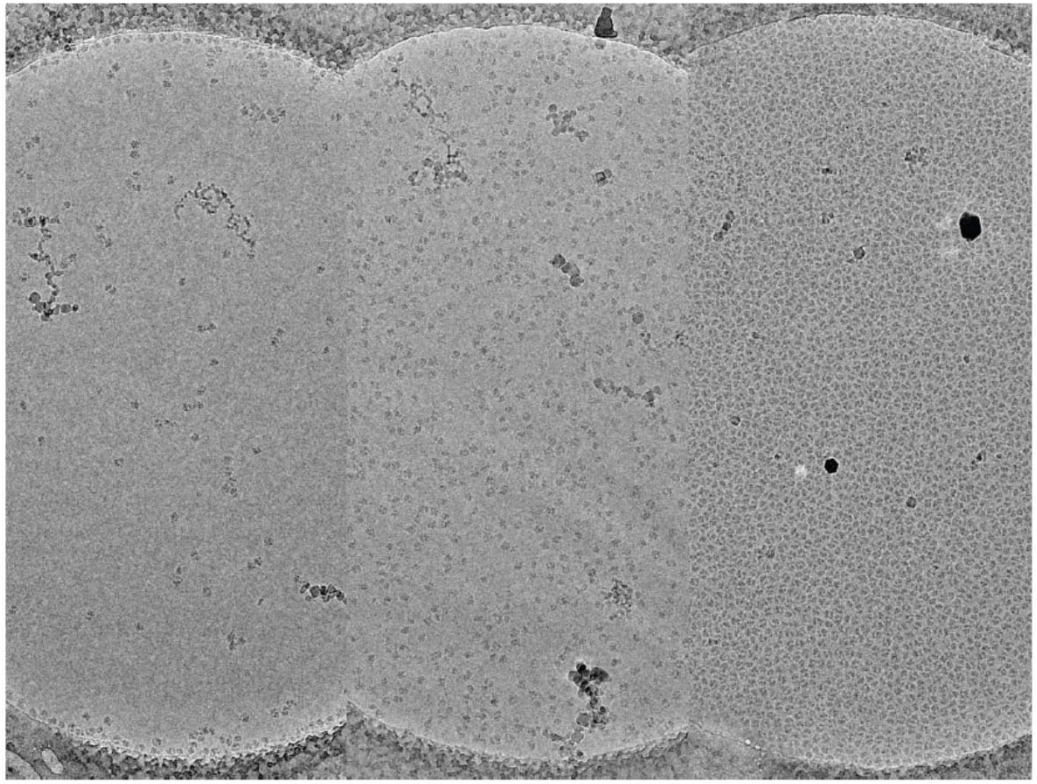




Cryo Plunging Techniques

Grid hydrophilicity



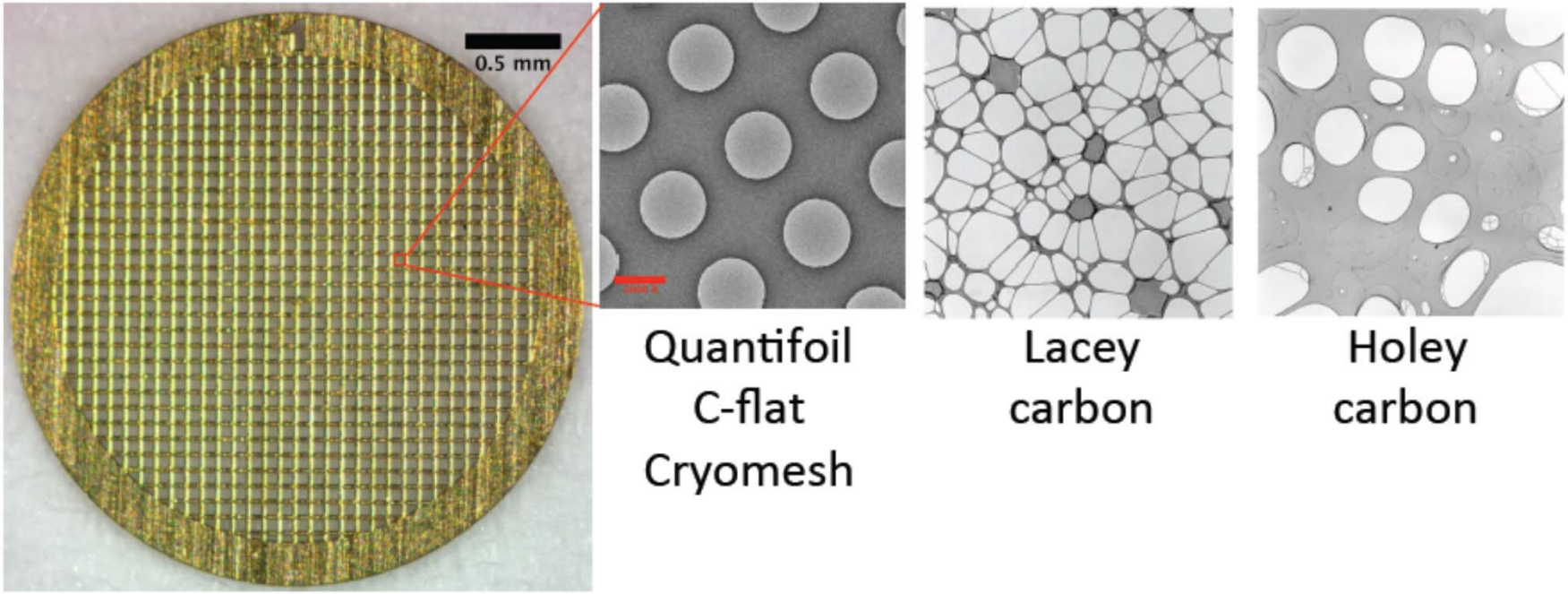


no graphene

graphene +
20 s hydrogen

graphene +
40 s hydrogen

Types of EM grids



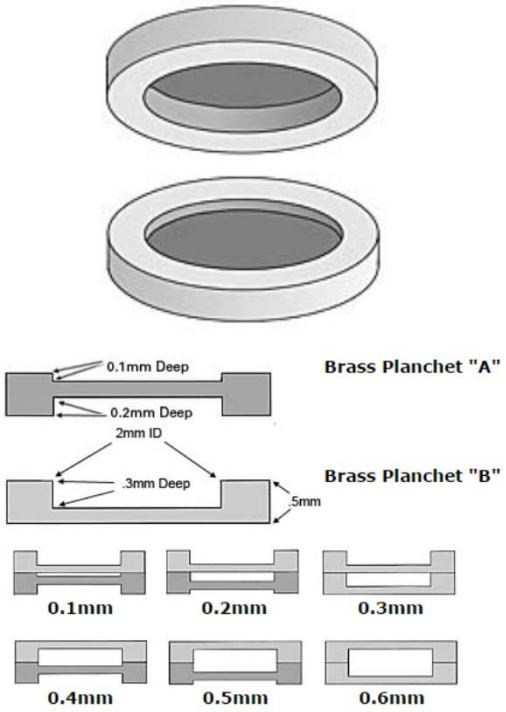
EM grid material:	copper, gold, molybdenum
Mesh sizes:	200, 300, 400 grid-bars per inch
Support film:	continuous carbon, graphene, gold
Support film:	C-flat, Quantifoil, lacey, holey
Hole size:	1-2 um

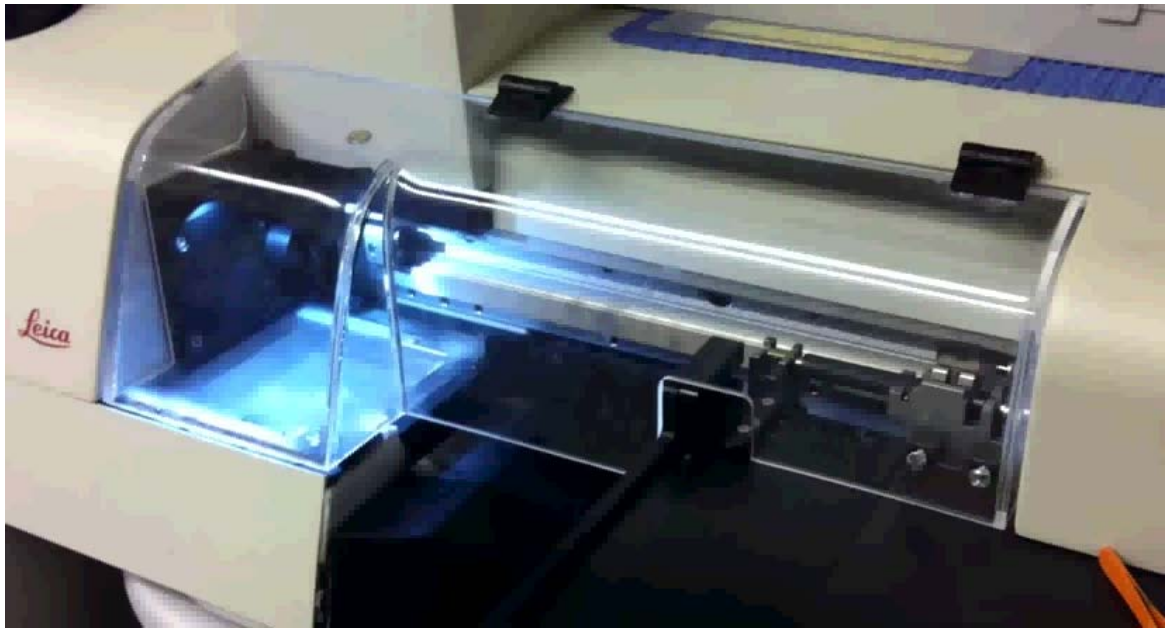
Cryo Plunging Techniques

Challenges:	prevent devitrification at increased tempartures avoid ice contamination during transfers prepare grids with the right ice thickness
Optimization:	sample concentration on the grid minimize preferred orientations ice thickness and quality
Advantages:	sample is preserved in hydrated state internal structures are imaged high resolution information is preserved
Disadvantages:	low dose imaging due to radiation damage low signal-to-noise ratio in images laborious and prone for error only few samples can be examined a day

High Pressure Freezing Techniques

- **High pressure freezing and freeze substitution**
- **High pressure freezing and cryo-ultramicrotomy**



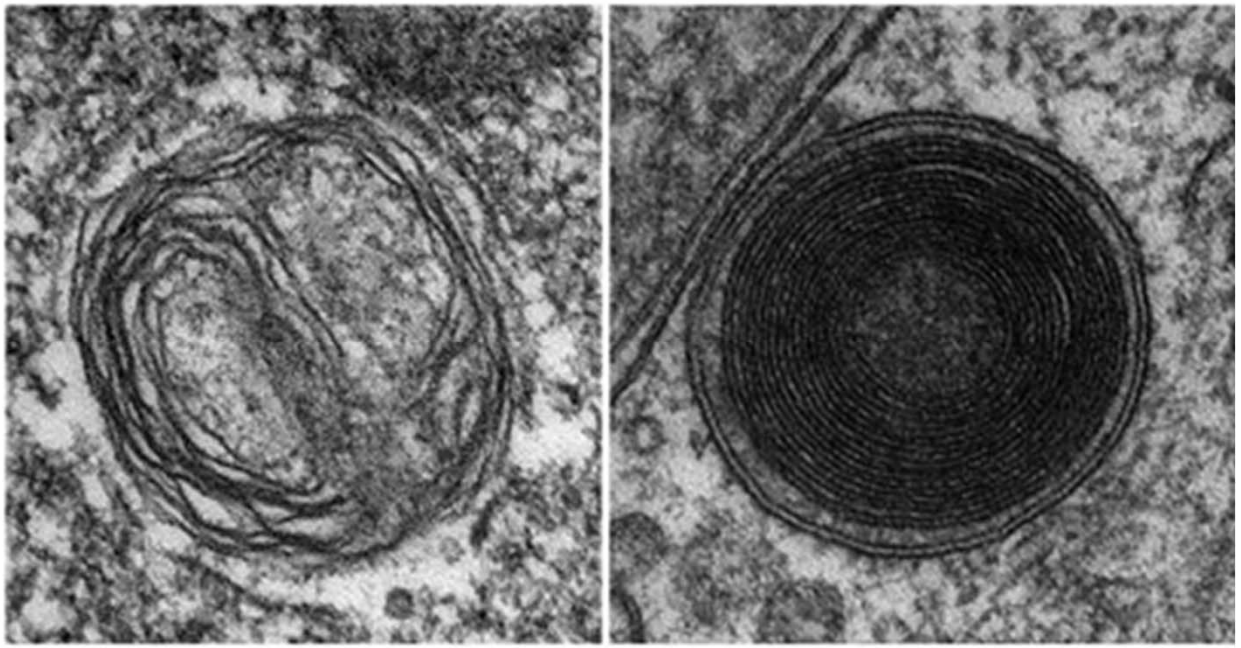


Freezing in 20 ms at 2000 bars (samples up to 200 um)

High Pressure Freezing Techniques

- Freezing cell tissue at high pressure in liquid nitrogen
- Dehydration of frozen sample at low temperatures
- Plastic embedding at room temperature
- Staining at room temperature

Multivesicular body images courtesy Mark Ladinsky



Traditional
Chemical Fixation

Ultra-Rapid Freezing
and Freeze-Substitution

High Pressure Freezing Techniques

Freeze substitution (below -70°C)

- reduced ultra-structural changes due to dehydration as seen at room temperature
- fixatives are evenly distributed before crosslinking occurs at elevated temperatures
- embedding at low temperature may better preserve epitopes for immunolabeling

Typical freeze substitution protocol

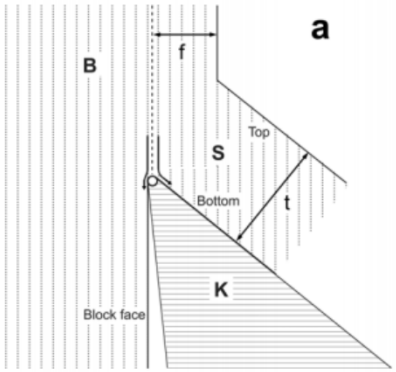
- 1% osmium oxide in anhydrous aceton at -90°C substituted for 3 days
- 0.1–0.5% gluteraldehyde in acetone at -90°C substituted for 3 days
- warm to room temperature and rinse with acetone
- plastic embedding at room temperature using standard protocols

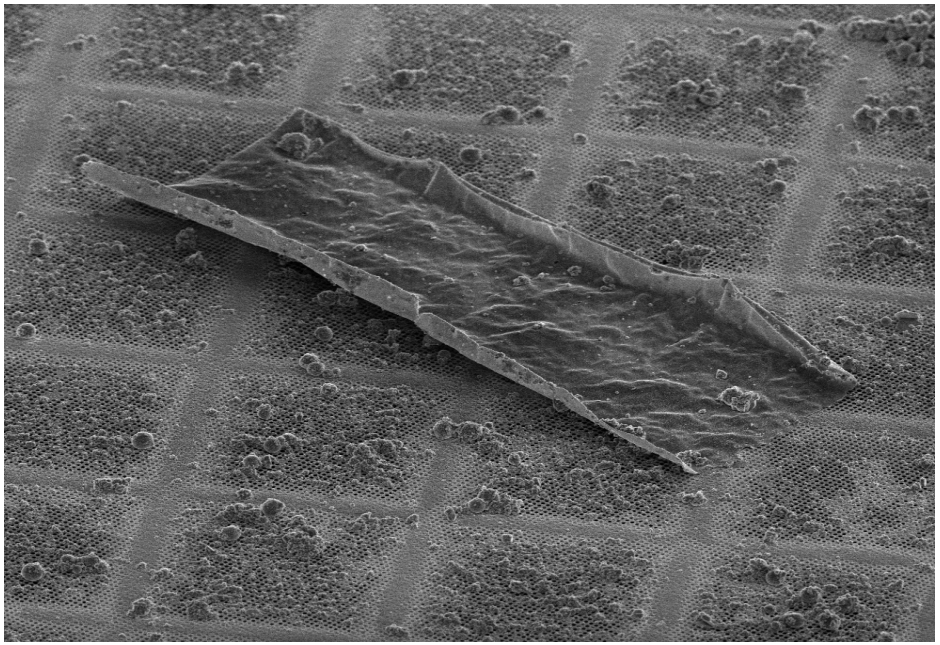
CEMOVIS

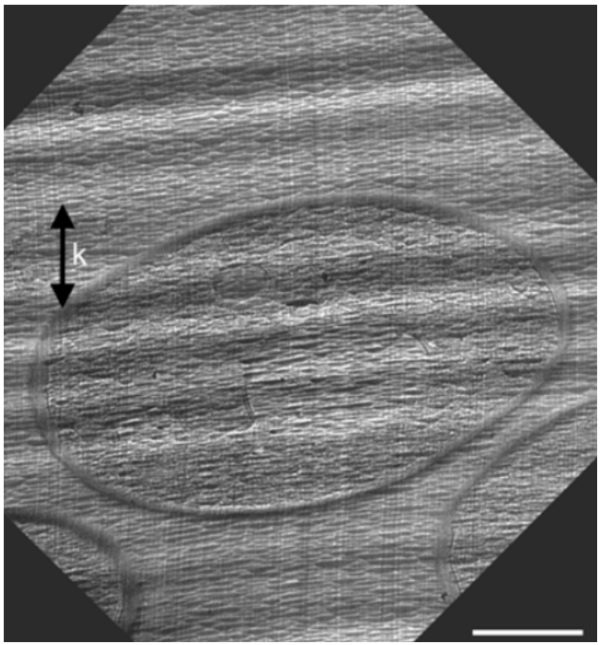
Cryo-sectioning of high-pressure frozen samples

Advantages: no chemicals or fixatives
imaging of unstained structures

Artifacts: compression, crevasses

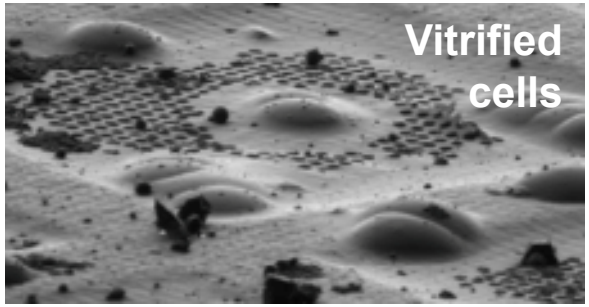


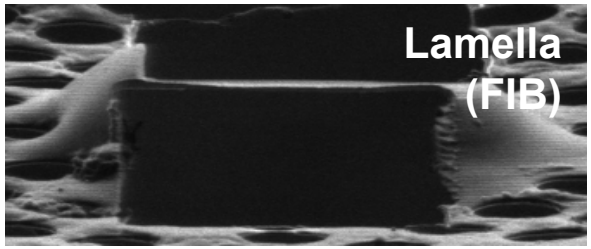


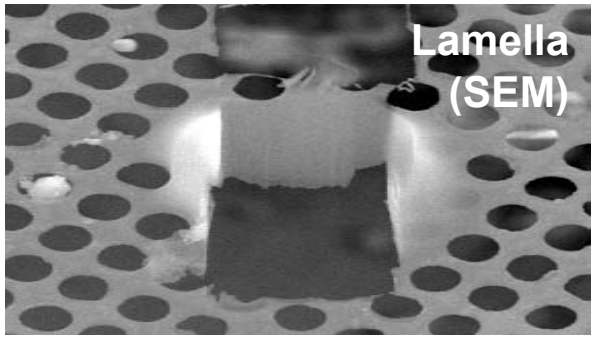


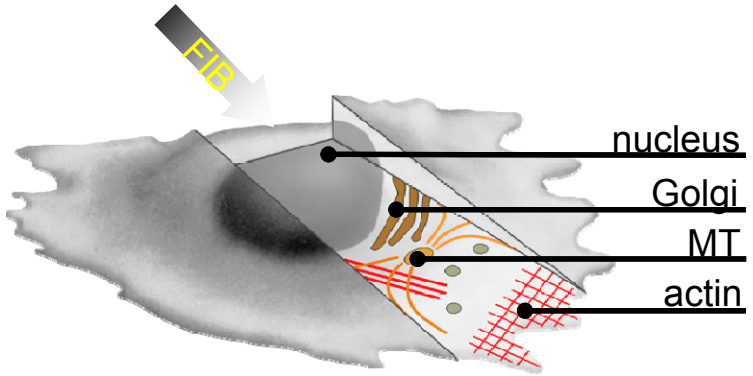
vitreous section of yeast cells (SEM and TEM)

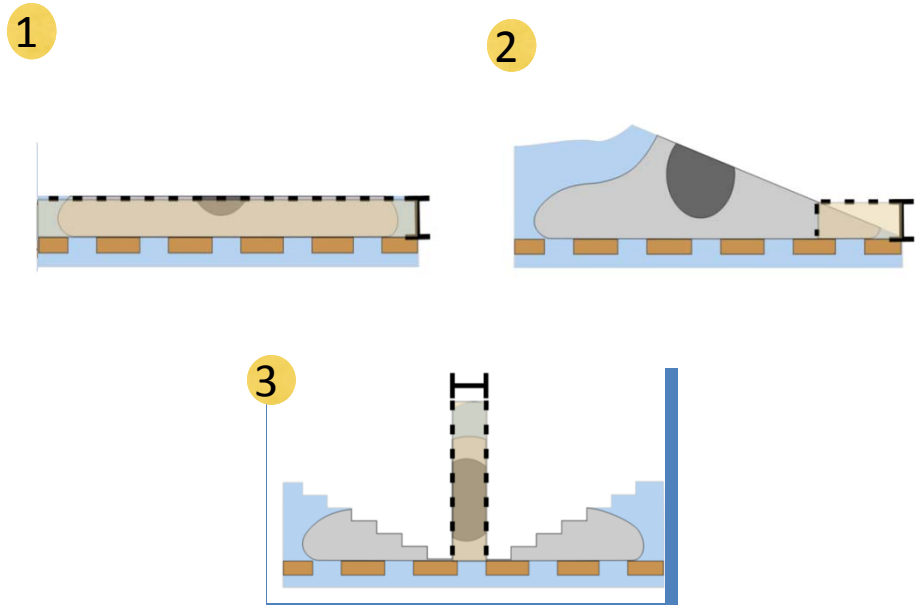
Cryo-FIB Milling











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