Preparation of Frozen-Hydrated Specimens

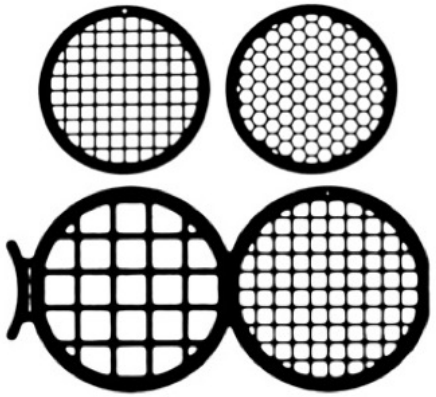
1. Support films & Grids
2. Cryo-Crinkling
3. Properties of Negative stains
4. Effect of the air-water interface
5. Radiation Damage
6. cryoEM of suspensions

Support Films & Grids

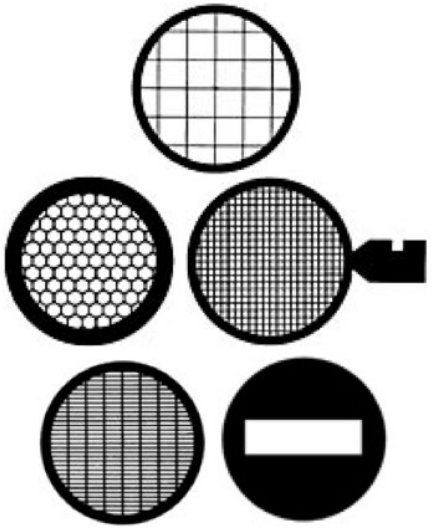
- The specimen & supporting film require the mechanical support of a metal grid which is necessarily electron opaque.
- Most EM grids are made of copper
  - non-ferromagnetic
  - good heat conductor preventing thermal expansion & drift
- Also Ni, Mo, Au, Al, Ti, stainless steel
  - Mo preferred for protein electron crystallography
    - Preferred because its coefficient of thermal expansion is nearly equal to that of carbon
    - Avoids the problem of “cryocrinkling”
  - Ni & Au for live cells (Cu grids are toxic)
- It is usual practice in high resolution studies to avoid recording images of specimens positioned near the grid bars.
- Modern grids are generally made by a photographic electrodeposition

Grids Come in a Wide Range of Designs

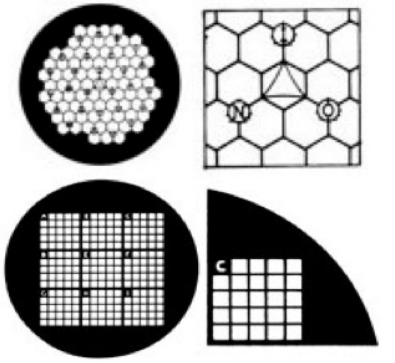
- Many different kinds of grids
- 50-400 mesh (lines/in) grids are commonly used.
- Grid bar thickness is another variable
- Square, hexagonal, slot holes
- Finder grids
- Folding grids



StrataTek™ Grids
Affordable, sturdy, medium mesh grids.
Mesh, Hexagonal, Slot and Hole



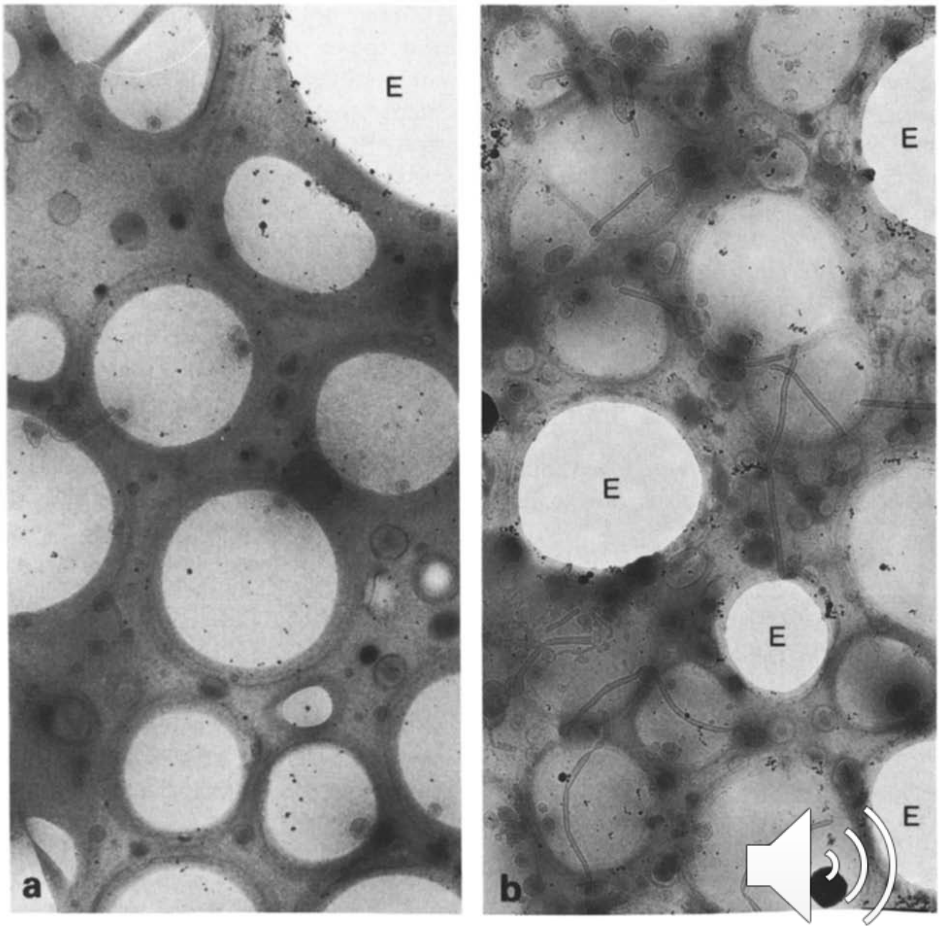
Veco Grids
Selection of rigid grids.
Mesh, Hexagonal, and Slot



Reference / Finder Grids
Wide selection of reference, locator and micron index grids.

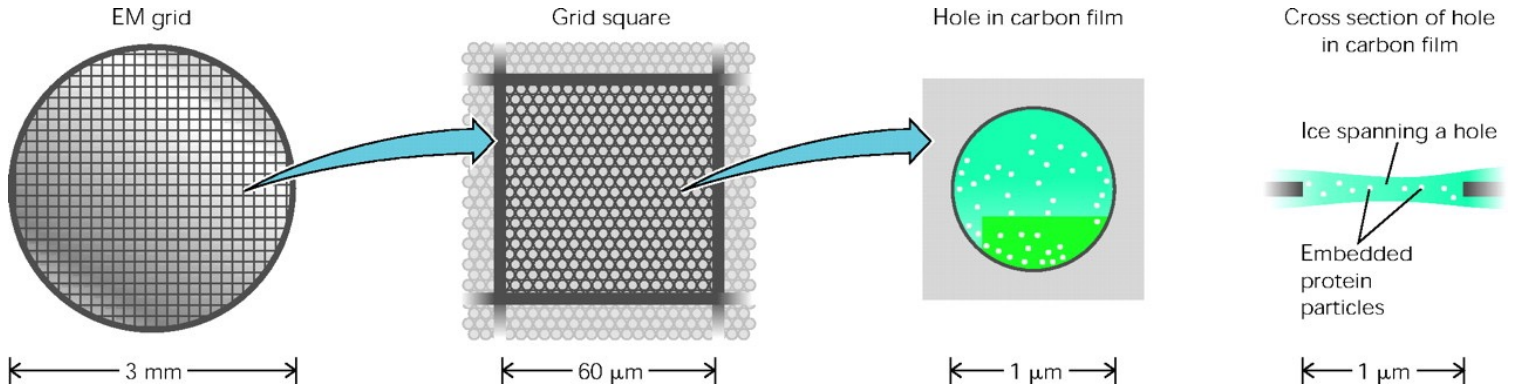
Support Films

- Support films are generally one of four types:
  - plain plastic such as **collodion** (nitrocellulose) or **Formvar**
    - coated (stabilized) with evaporated carbon to avoid charging
  - plain carbon
    - may be formed by removal of a plastic substrate
    - may be formed by floating a carbon film off of a freshly cleaved mica substrate
    - Hydrophilic initially
  - reticulated (holey) grids
    - non-uniform holes – made usually in-house
    - pattern holes – Quantifoil, C-flats
  - latest trend is to use gold films on a gold grid or variations on this idea using other materials



Toyoshima, C. 1989. *Ultramicroscopy*.

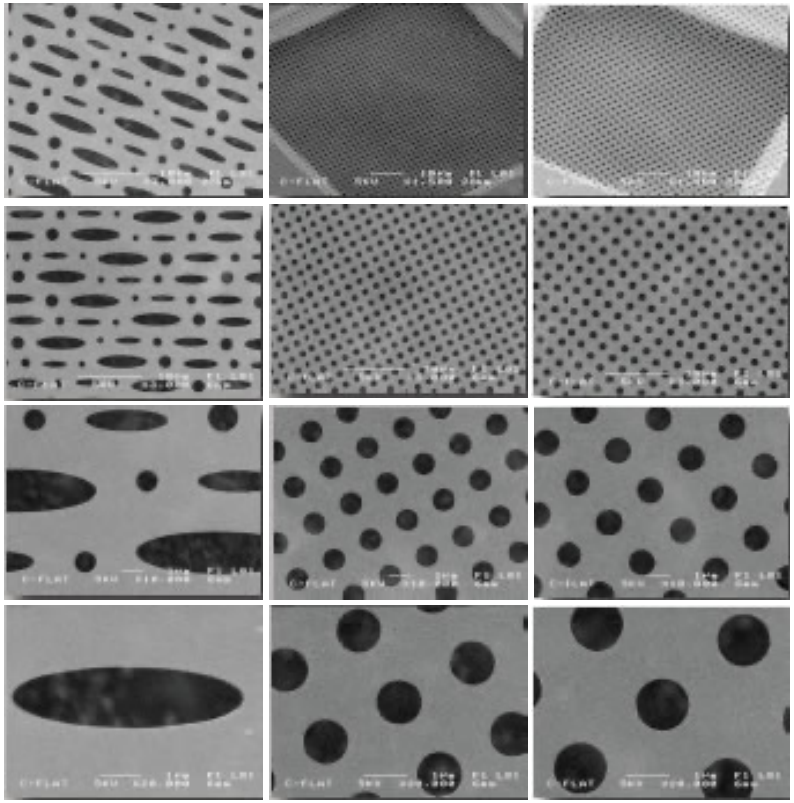
Patterned Support Films on EM Grids



Holey (Reticulated) Carbon Support Films

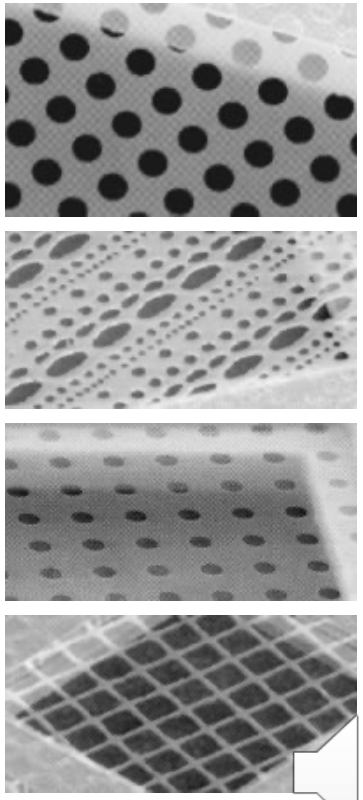
- Reticulated support films consist of holes embedded on a carbon matrix
- Ideally the holes have a pattern

C-flat



http://www.emsdiasum.com/microscopy/products/grids/cflat.aspx

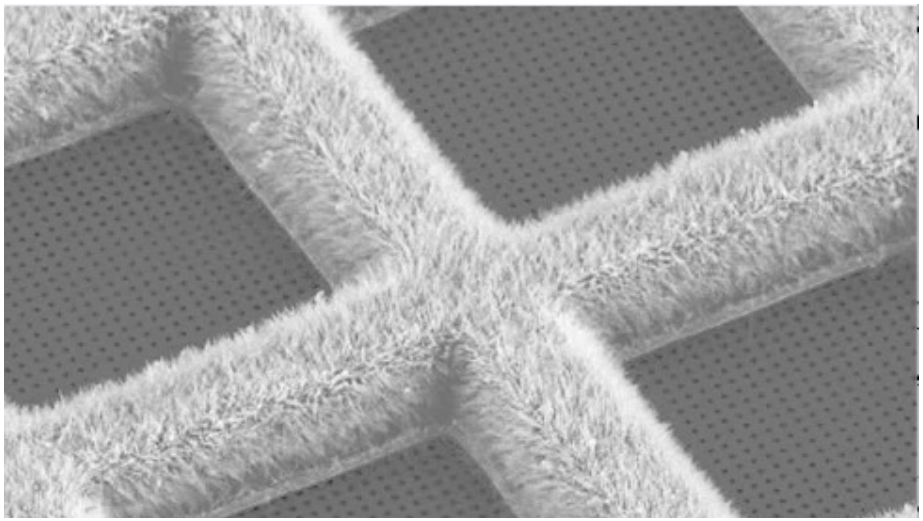
Quantifoil

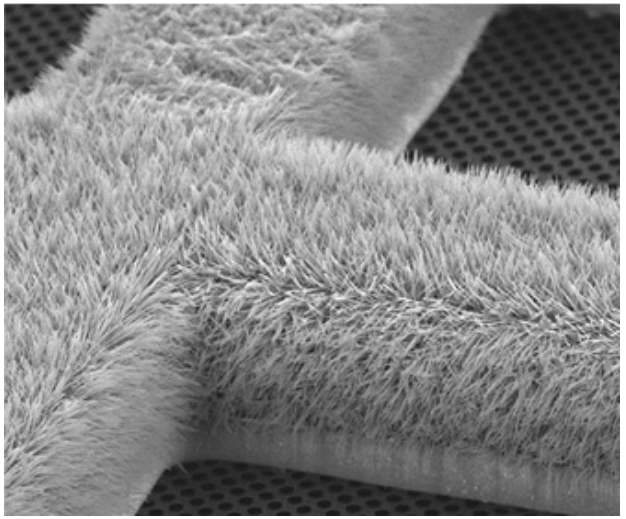


http://www.emsdiasum.com/microscopy/products/grids/quantifoil.aspx

Self-wicking Grids

- For use in the SPT Labtech Chameleon
- The holes have a pattern
  - ~1.2 μm diameter with 0.8 μm edge to edge spacing
- Copper nano-wires do the wicking

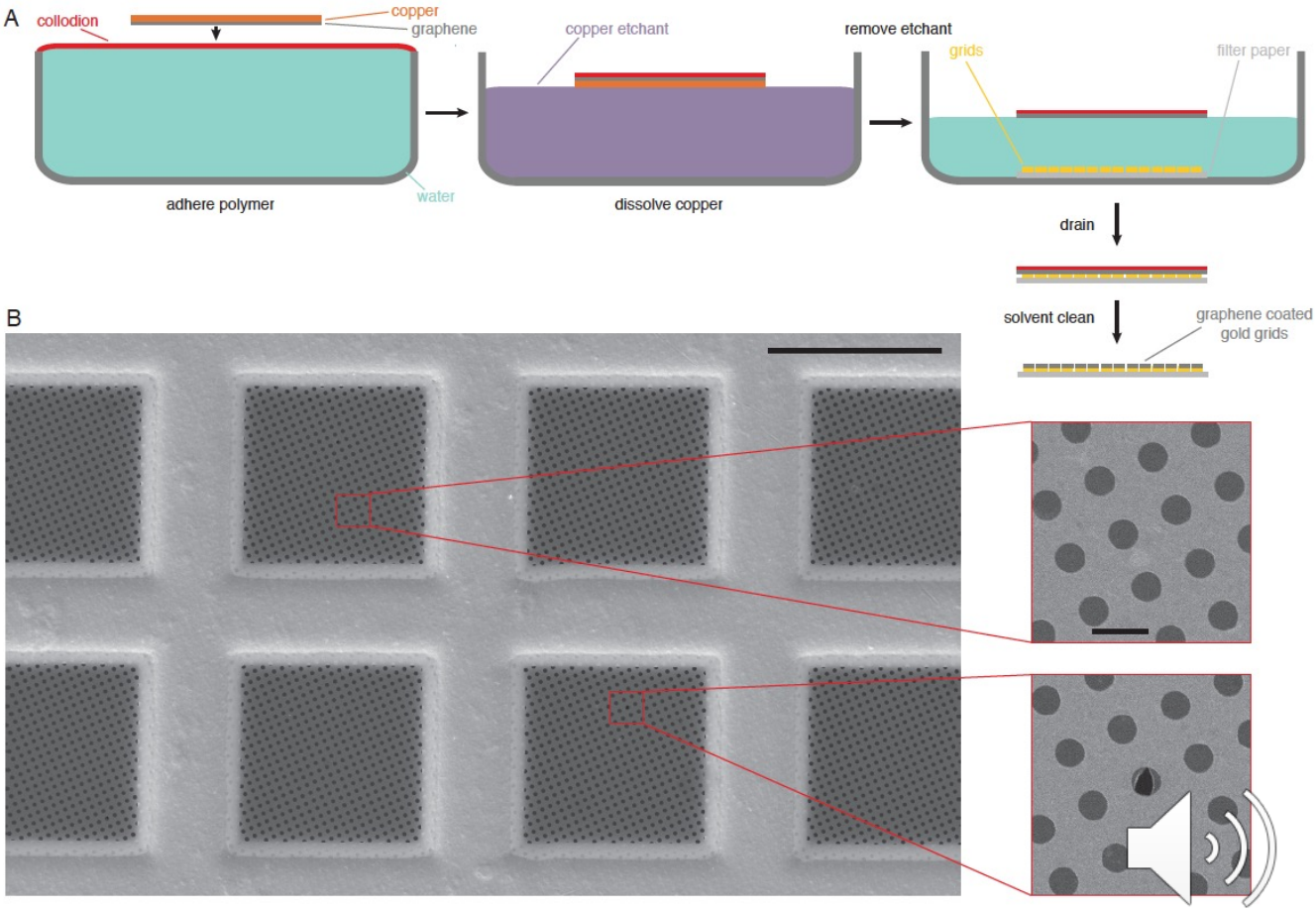




https://www.quantifoil.com/products/quantifoil-active

Graphene Support Films

- Graphene is just a layer of graphite one atom thick, ~3.4 Å
- Pure graphene is hydrophobic but there are procedures that can derivatize graphine to give is a (+), (-) or neutral hydrophilic surface
- Graphene is an excellent conductor of electricity and so the specimen does not charge



Naydenova et al.,2019,PNAS

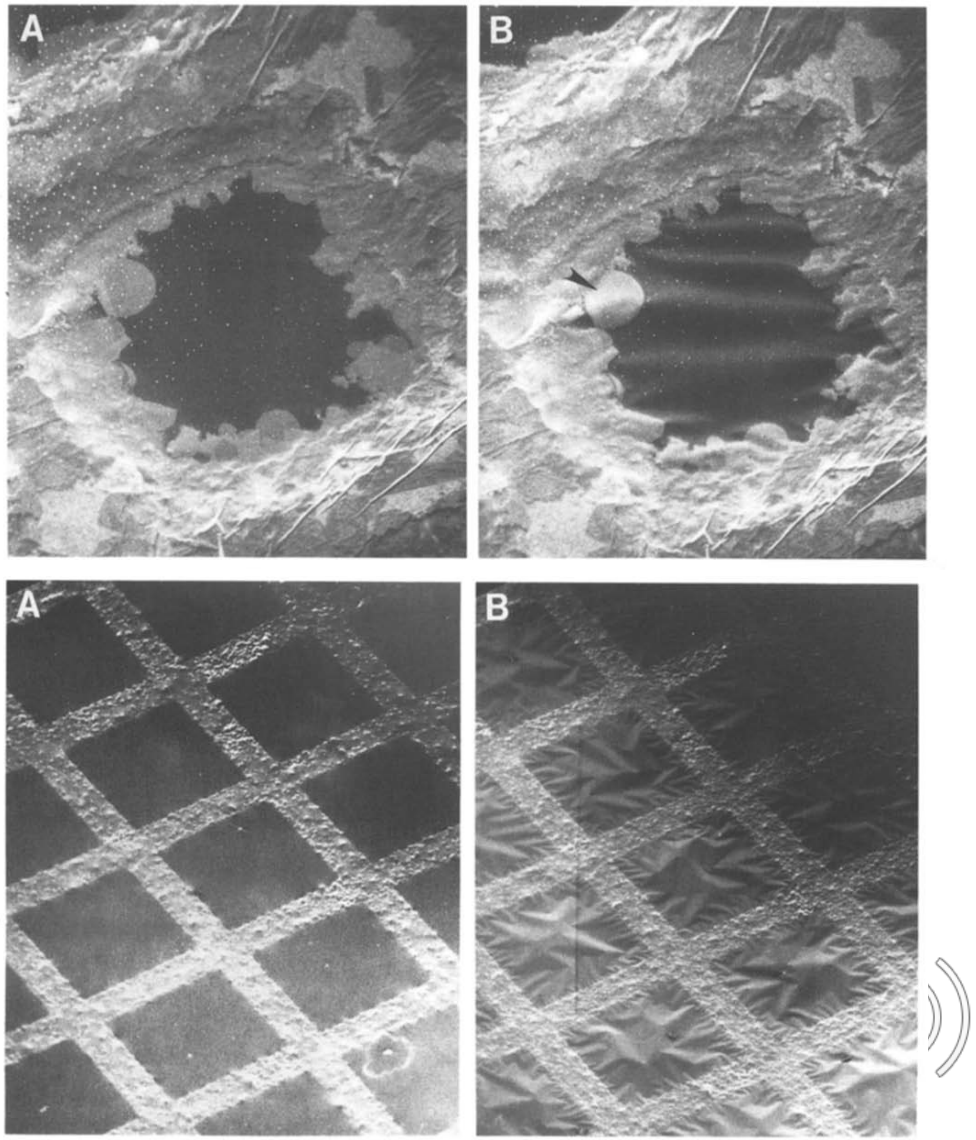
Cryo-Crinkling

- Occurs when the coefficient of thermal expansion of grid material & support film material are too different
  - Copper 16.6
  - Graphite 7.9
  - Molybdenum 5.0
  - Amorphous Carbon 2.0
- Can be reduced by reducing the difference between grid & support film
- Currently best solutions are
  - Carbon film on molybdenum
  - Gold film on gold grid

Material	Coefficient of thermal expansion x 10^6/ºC	Degree of Crinkling
Aluminum	25	Not measured
Gold	14.2	
Amorphous ice	?	
Brass	18.0	Not measured
Copper	16.6	Severe
Platinum	9	Not measured
Titanium	8.5	Slight
Graphite	7.9	
Zirconium	5.6	Slight
Molybdenum	5.0	Little/none
Amorphous carbon	2.0	
Tungsten	4.5	Little/none

Cryo-Crinkling

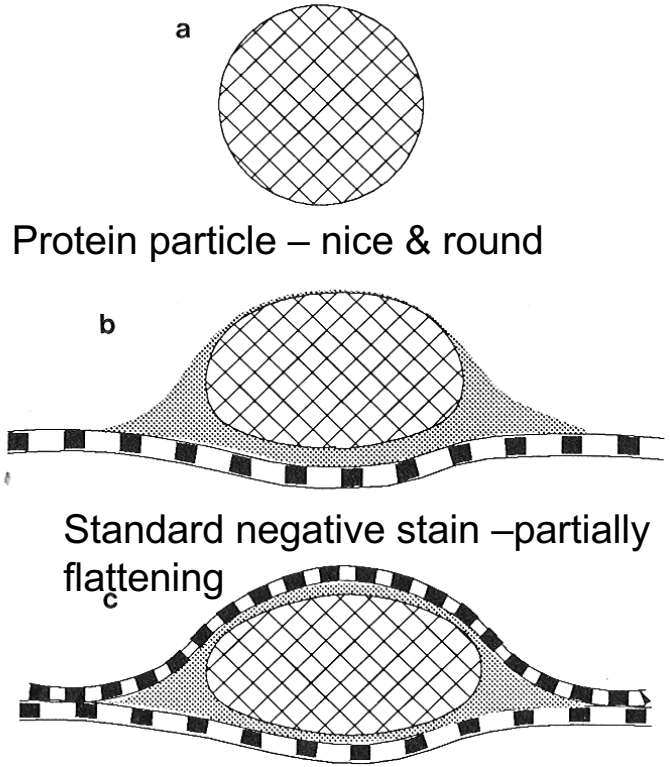
- Originally discovered as a major explanation for why images of tilted 2-D crystals were so much poorer than ones at 0º of tilt
- Caused by a large(2-3x) difference between the coefficient of thermal expansion between film & grid material
- Easily visualized in images of tilted specimens

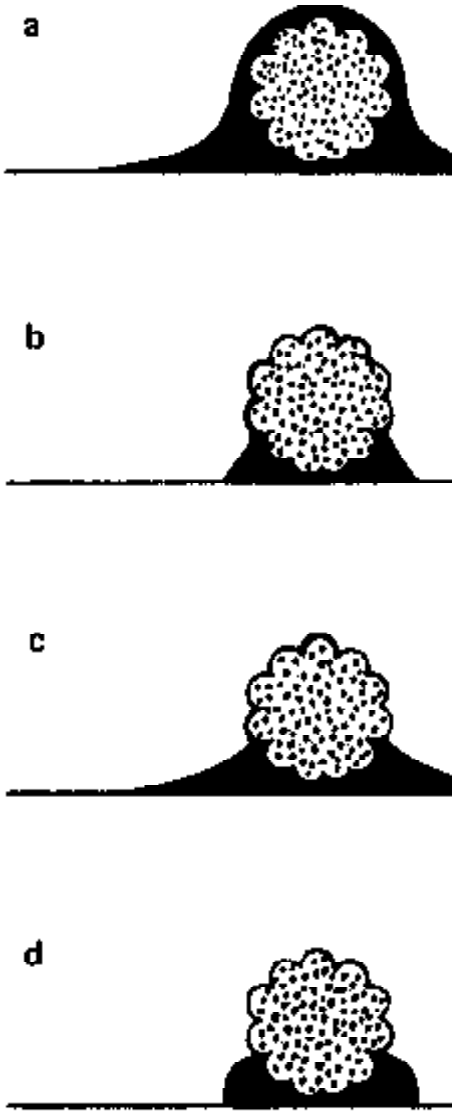


Cryo-crinkling: what happens to carbon films on copper grids at low temperature. F. P. Booy and J. B. Pawley. Ultramicroscopy (1993) 48(3), 273-80.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?

Properties of ideal negative stain

- Each of the commonly used stains fails in some respect to meet the "ideal" specifications outlined belo
- An ideal stain should have:
  - **High density**: Ability to protect specimen against dehydration effects
  - **High solubility**: Non-chemicall reactive with the specimen
  - **High melting & boiling points**
  - **Uniform spreading** on the support film
  - **Amorphous structure** (*i.e.* structureless) when dry

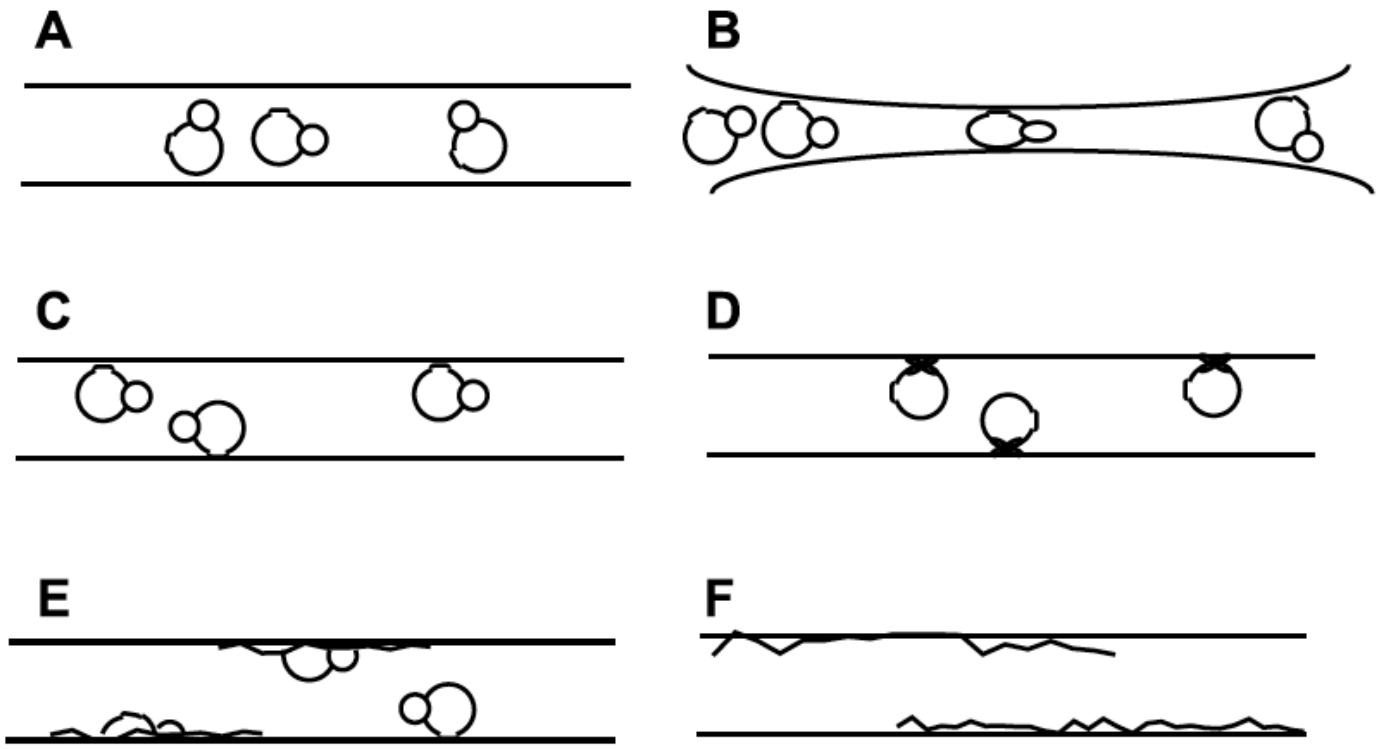




Common Negative Stains

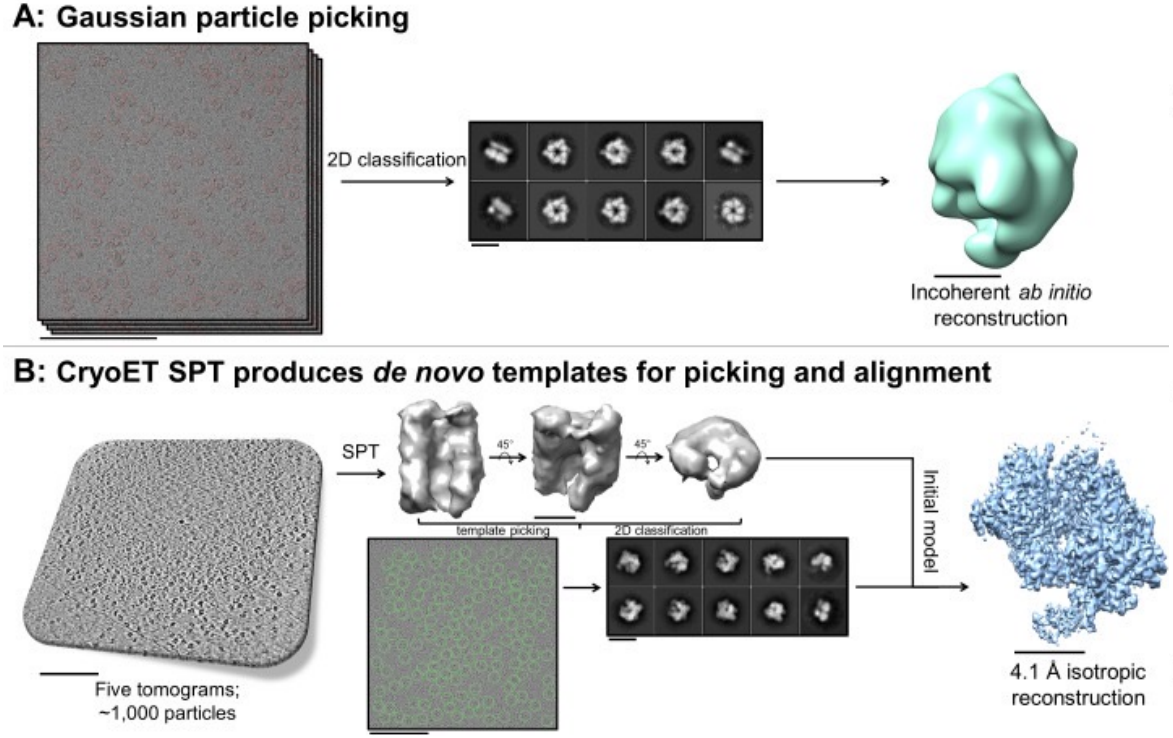
STAIN	FORMULA	Solubility (g/100ml H2O)	Anhydrous Density (g/cc)
ANIONIC STAINS			
Ammonium molybdate	$NH_4Mo_7O_{24} \times 4H_2O$	44	2.5
Sodium phosphotungstate	$Na_3PO_4 \times 12WO_3$	?	3.8
Sodium tungstate	Na_2WO_4	90	4.2
CATIONIC STAINS			
Uranyl acetate	$UO_2(C_2H_3O_2)_2 \times 2H_2O$	8	2.9
Uranyl formate	$UO_2\ (CHO_2)_2 \times H_2O$	7	3.7
NONIONIC STAINS			
Methylamine vanadate	$CH_3NH_2VO_3$		
Methylamine tungstate	$(CH_3NH_2)_2H_2WO_4$		

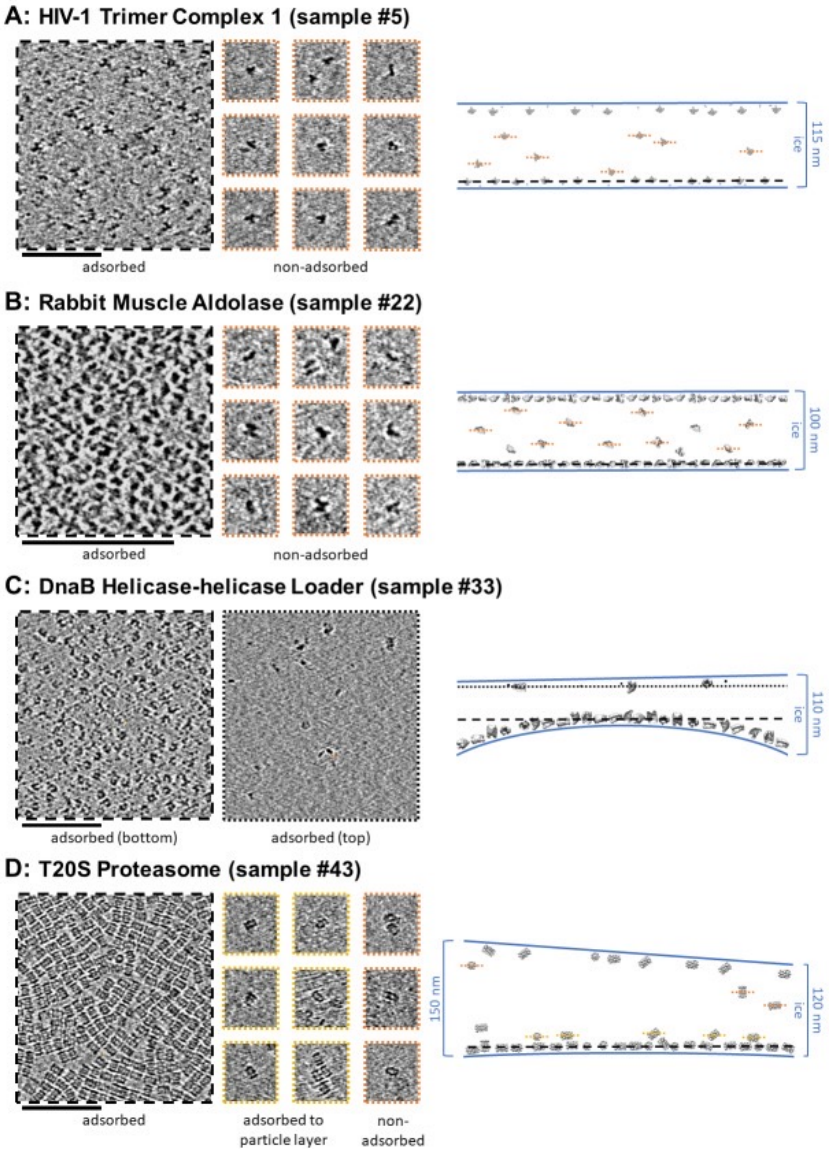
Effect of the Air-Water Interface



Taylor & Glaeser J Struct Biol (2008) 163(3), 214-23

Effect of the Air-Water Interface





Noble AJ et al. Routine single particle CryoEM sample and grid characterization by tomography. *Elife*. 2018;7:e34257.

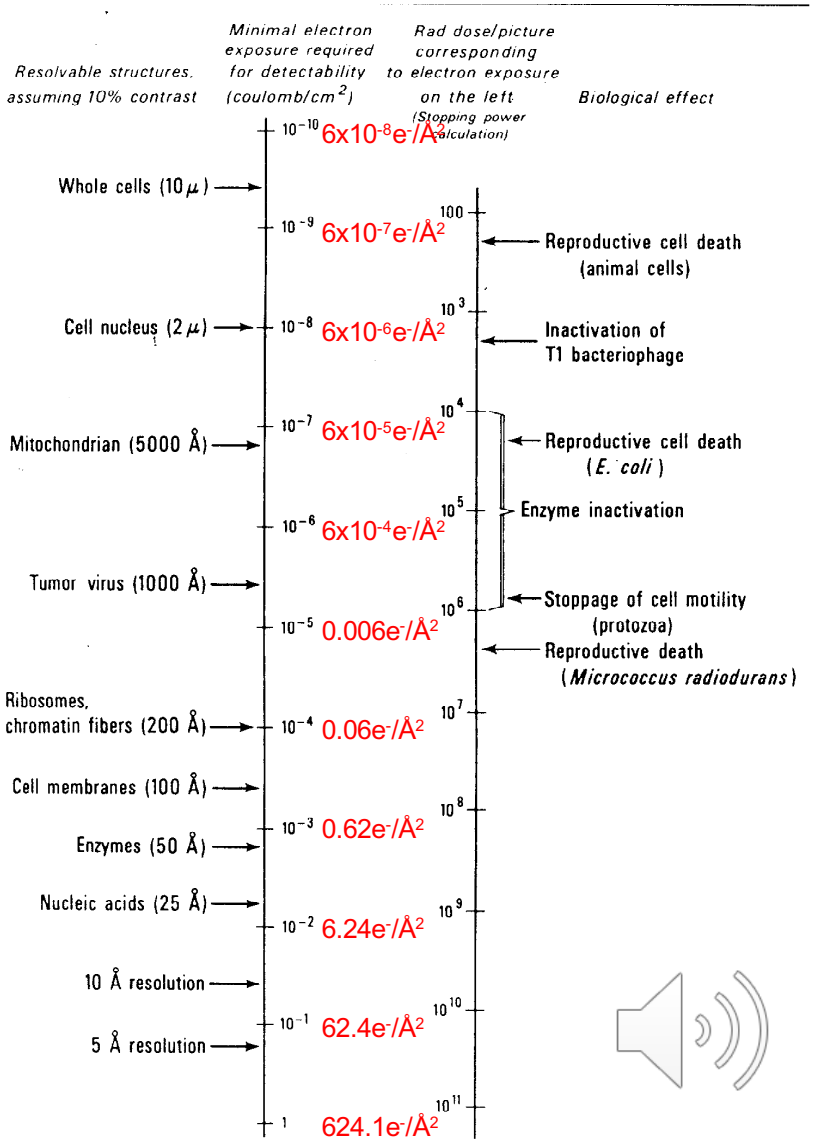
Radiation Damage

- Damage ~ TOTAL DOSE
  - product of the beam current density (dose rate) and the exposure time
  - most specimen damage not dose-rate dependent.
- Conventional unit is the Gray, abbr. Gy. One Gy = 1 Joule/kg =100 rads. One **rad**, is 100 ergs absorbed/gm. 1 joule equals 0.239 calories
- In TEM → flux in coulombs/cm^2 or e$^-$/Å^2 (recall that 1e$^-$ = 1.6x10^{-19} coulomb).
- For carbonaceous materials a rough conversion is
  - 1 coulomb/m^2 (or ~0.06 e$^-$/Å^2) at 100 kV = 4x10^7 rads.
  - 10^9 rads → destroy the original properties of most organic materials
  - 10^6 rads will kill the all living things & inactivate of most enzymes.
- The electron dose that completely disorders an *unstained molecule* is ~100 e$^-$/Å^2 at 80 kV and ~250 e$^-$/Å^2 at 500 kV.
  - Negatively stained molecules reach a stable state of radiation damage that still reveals molecular structure
- *Minimum* current density to barely visualize an object at the fluorescent screen is ~1 e$^-$/μm^2/sec which equals 4 e$^-$/Å^2/sec @ 20,000X.
  - Insufficient time to visualize the specimen let alone focus and record an image.
- 2 second exposure (gives an optical density ~2.0 with a film speed of 1.0 e$^-$/μm^2
  - energy at the specimen = 5x10^9 rads
  - approximately equivalent to the energy delivered from a 10 megaton Hydrogen-bomb exploding 30 meters away!

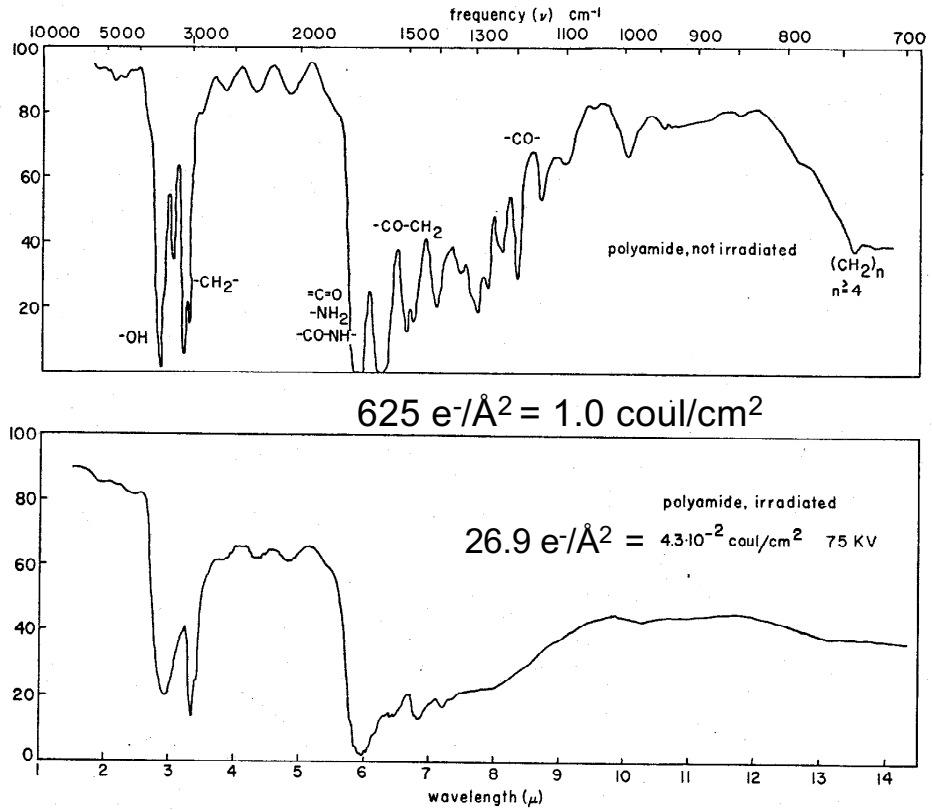
Radiation Damage at 1MeV

Ions or **radicals** result when enough energy is absorbed by an orbital electron to become free or reach a higher energy (non-bonding) orbital. Subsequently that molecule has either a charge (ion) or an unpaired electron (radical).

The fates of these species are known as the secondary events of radiation, and it is these reactions, not the primary events, that cause material damage.



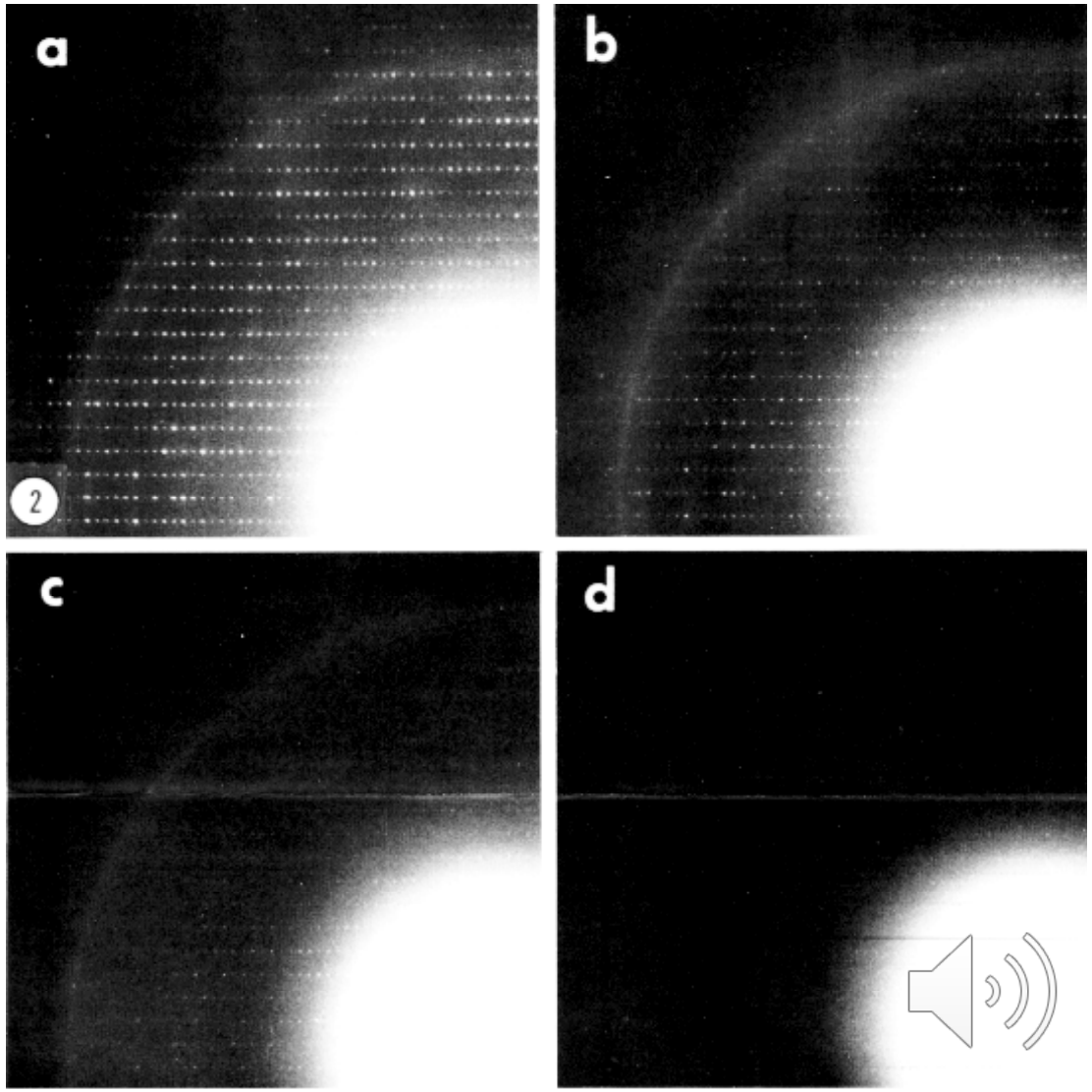
Chemical Structure Changes



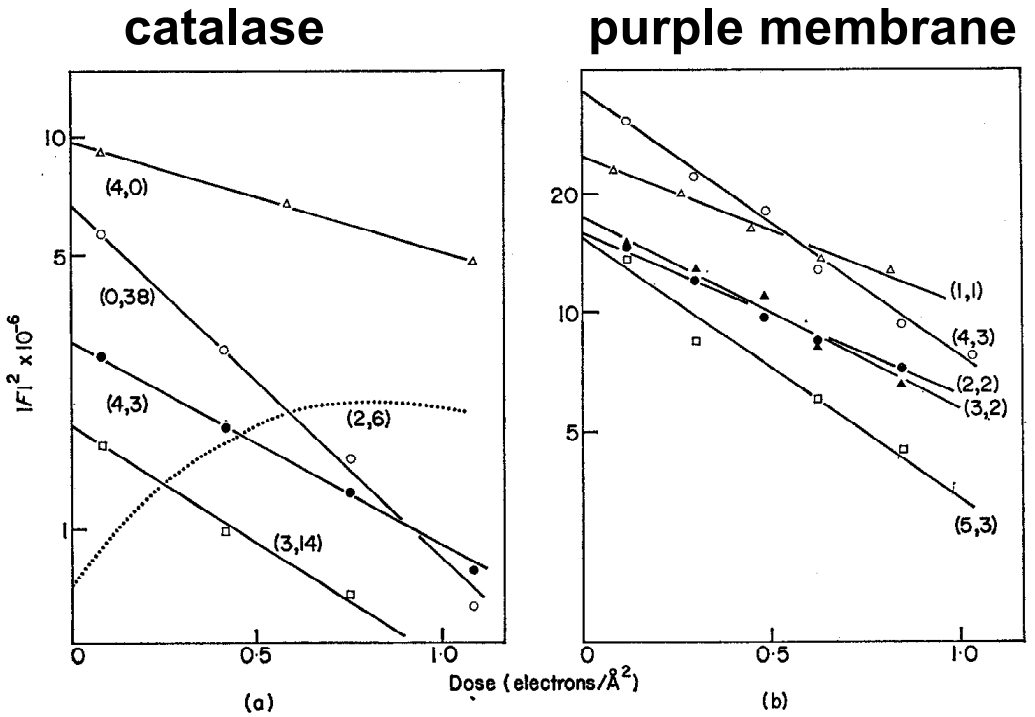
Infrared absorption spectra of non-irradiated (*top*) and irradiated (*bottom*) polyamide. (From Bahr, Johnson, and Zeitler [1965] *Lab. Invest.* **14:377**)

Degradation of e^- Diffraction Pattern

Changes in the electron diffraction pattern of frozen-hydrated catalase crystals resulting from radiation damage. (a) The initial diffraction pattern extends to 2.8 Å. The ring present in the pattern at a spacing of 3.67 Å is due to ice condensed on the surface of the specimen from water vapor present in the column of the microscope. (b) The pattern recorded after an exposure of 2.5 $e^-/Å^2$. (c) The pattern recorded after an exposure of 5.0 $e^-/ Å^2$. (d) The pattern recorded after an exposure of 11 $e^-/ Å^2$; the pattern still extends to 8.5 Å resolution. (From Taylor and Glaeser [1976] *J. Ultrastruc. Res.* **55:448**)



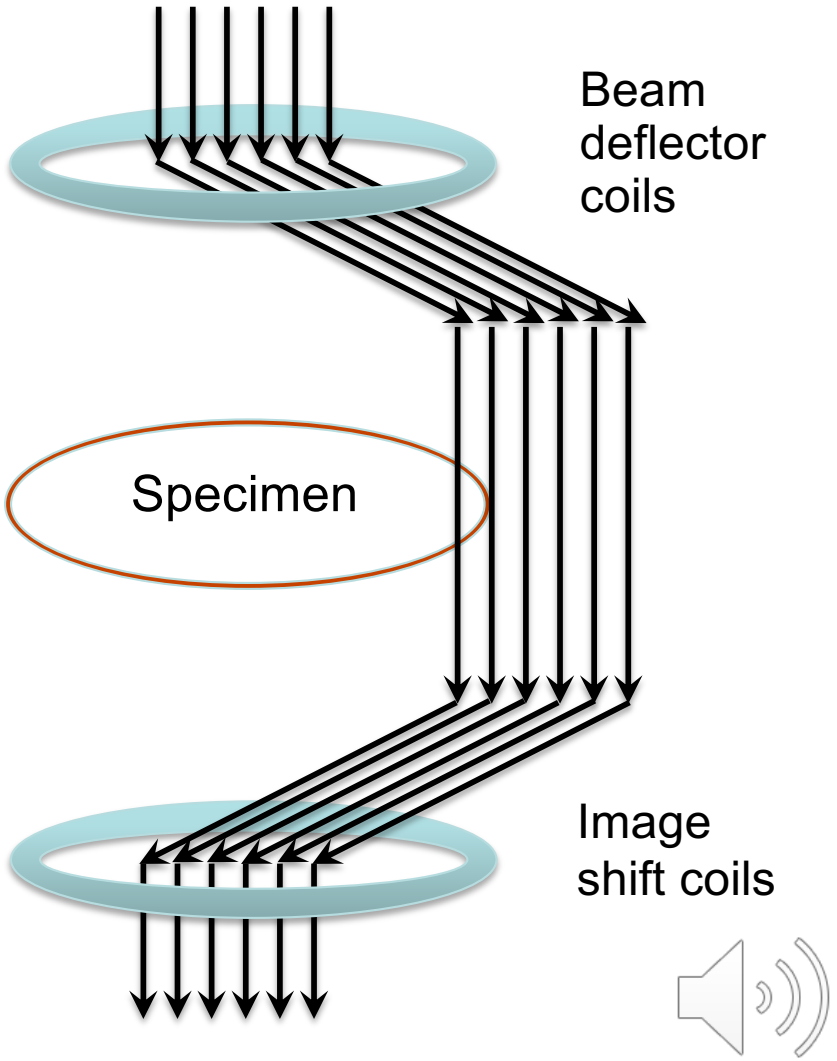
The radiation damage of stained catalase is of a fundamentally different nature than for unstained valine or adenosine. The critical dose corresponds to about **2** ionizing events/Å^2 or **6000** per molecule. A significant portion of the matter within the unit cell of the structure changes to some more stable configuration as a result of the irradiation. This would account for the changes in the relative intensities of the diffraction maxima that remain after irradiation. The distribution of matter becomes more disordered as well, since only the lower-resolution diffraction reflections remain. This redistribution of matter probably includes a significant redistribution or aggregation of stain molecules.



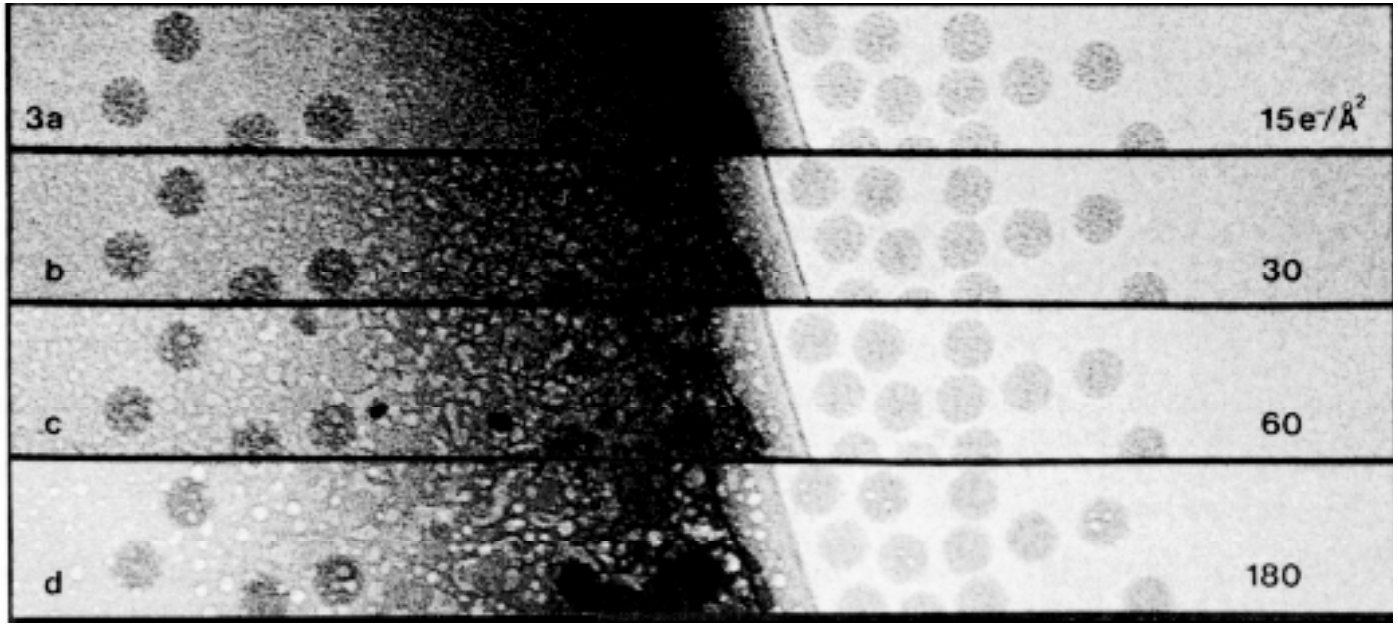
The intensities (on a logarithmic scale) of some typical electron diffraction reflections in (a) unstained catalase and (b) unstained purple membrane, plotted as a function of electron dose at room temperature. The dotted curve is for one of the few reflections which actually increases in intensity during the intitial exposure period. (Such reflections are at variance with the more typical behavior, over the dose range shown, of exponential decay.) (From Unwin & Henderson [1975] *J. Mol. Biol.***94:425**)

Minimize Number of Electrons

- A basic rule : use the minimum mag. that will reveal detail of a given size determined by the resolution of the recording medium
- Minimal exposure techniques involve focusing on one specimen region but photographing another.
- Modern techniques use off-axis focusing with the specimen in position for recording so no further stage movements are necessary.
- Off-axis focusing utilizes beam deflection circuitry
  - The beam is aligned so it it illuminates a region a few microns from the specimen.
  - After passing through the specimen off-axis, the beam is redeflected back on axis so the image of the area used for focusing appears in the center of the viewing screen.
  - When focusing and astigmatism corrections are made to satisfaction, and the exposure button is pushed, the beam is automatically recentered and expanded to a predetermined amount and an image of the desired area is recorded.
  - After the exposure is finished, the beam is again automatically redeflected away from the specimen to prevent further irradiation.



Radiation Effects in Frozen-Hydrated Specimens



Dose series of frozen-hydrated SV40 virus showing how bubbles form first over the carbon film and then in the virus particles over the hole. The water over the hole forms bubbles after continued irradiation. (From Baker *et al.*, [1985] *Proc. Elec. Microsc. Soc. Amer.* **43:316**)

Fig. 1. Dose series of intact, frozen-hydrated bacterial cells. *Me. florum* cells were plunge frozen and imaged in the Polara cryomicroscope. Seventy images were recorded of each cell with 10 $e^{-}/Å^2$/image.

The 1st and 2nd columns show images recorded at 82 and 12 ° K, using liquid nitrogen and liquid helium as cryogen, respectively. One particular cluster of density is circled in each image of the first column to facilitate comparison.

The 3rd and 4th columns show images recorded again at 12 ° K, but where the sample was briefly warmed up in the liquid nitrogen cooled multispecimen holder, as if for rotation in the flip-flop rotation stage, and then re-cooled in the column to 12 ° K.

In the 3rd column, the sample was warmed once after a cumulative dose of 30 $e^{-}/Å^2$.

In the 4th column the sample was warmed and re-cooled after every 30 $e^{-}/Å^2$. Note how the membrane contrast fades and is then replaced by bubbles at 12 ° K but not 82 ° K. This effect is delayed by one warming cycle, and prevented indefinitely by iterative warming cycles. (Scale bar 250 nm.)

Iancu et al., A comparison of liquid nitrogen and liquid helium as cryogens for electron cryotomography. J Struct. Biol. 153(3), 231-240 (2006)

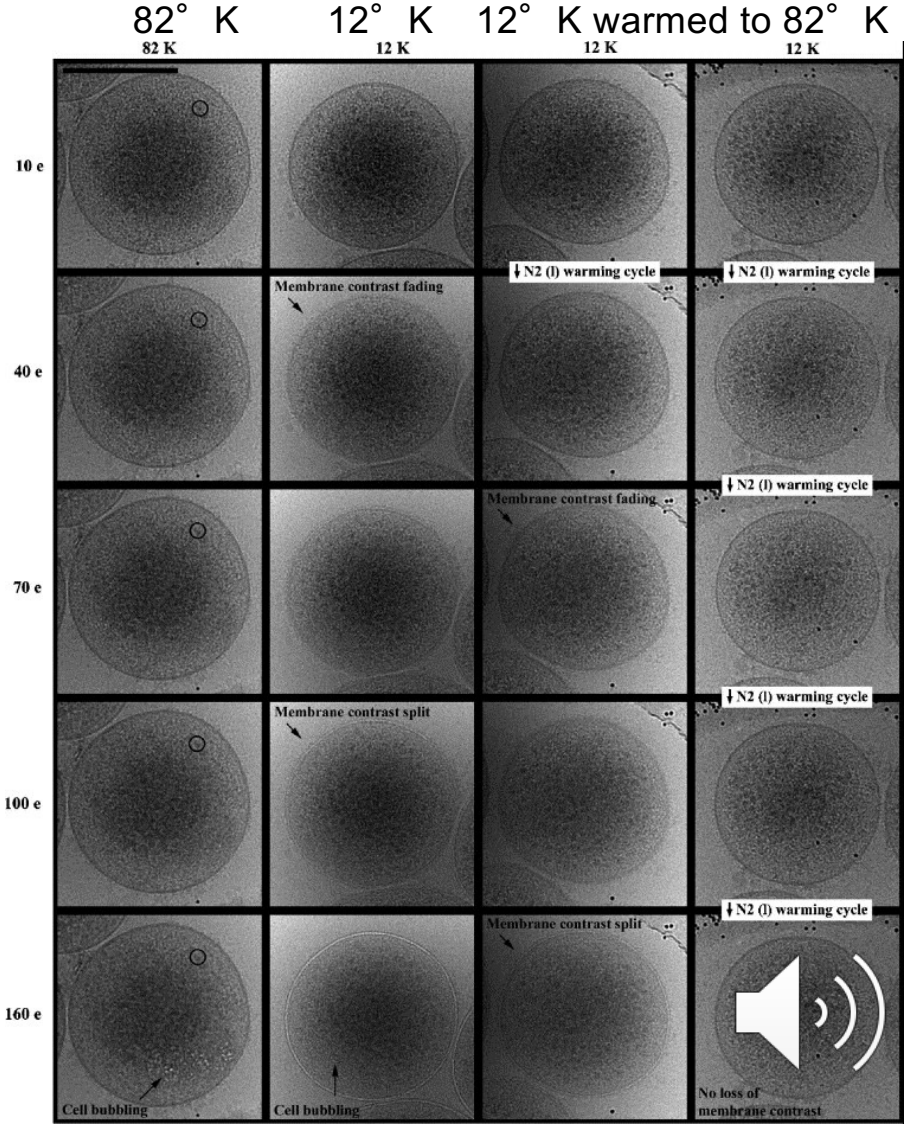
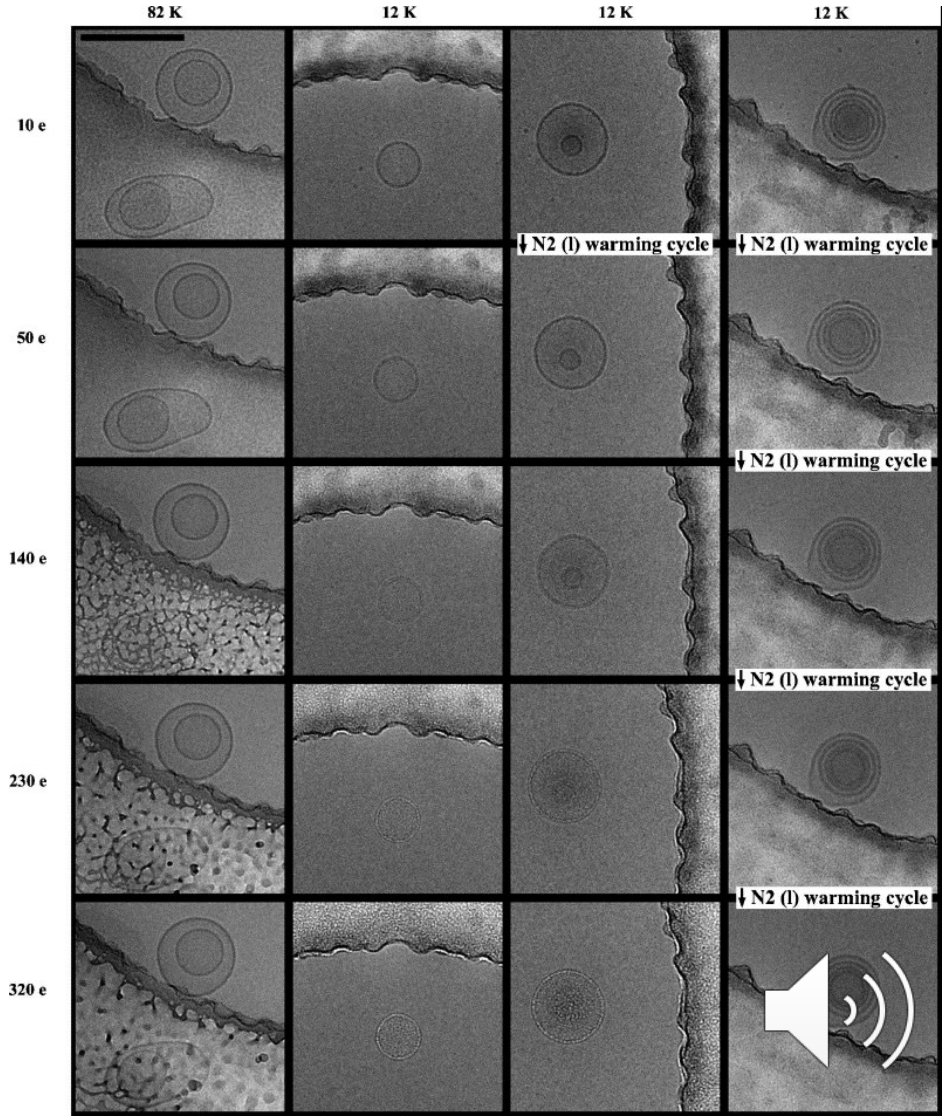


Figure 2. Dose series of liposomes. Dose series were recorded as in Figure 1, but this time of liposomes. Pure lipids exhibit the same contrast effects as the cell membrane, but at approximately twice the dose. While bubbles form on the carbon support at both temperatures, they are much larger and coalesce to a greater extent at 82 ºK than 12 ºK. Bubbling on the carbon is apparently prevented by iterative warming cycles. (Scale bar 200 nm.)



Iancu et al., A comparison of liquid nitrogen and liquid helium as cryogens for electron cryotomography. J Struct. Biol. 153(3), 231-240 (2006)

Cryo-electron microscopy of vitrified specimens.

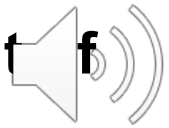
Adrian, M., Dubochet, J., Lepault, J., McDowall, A.W. (1984) *Nature* 308, 32-36.

Dubochet, J., Adrian, M., Chang, J.J., Homo, J.C., Lepault, J., McDowall, A.W., Schultz, P. *Q Rev Biophys* 21, 129-228 (1988)

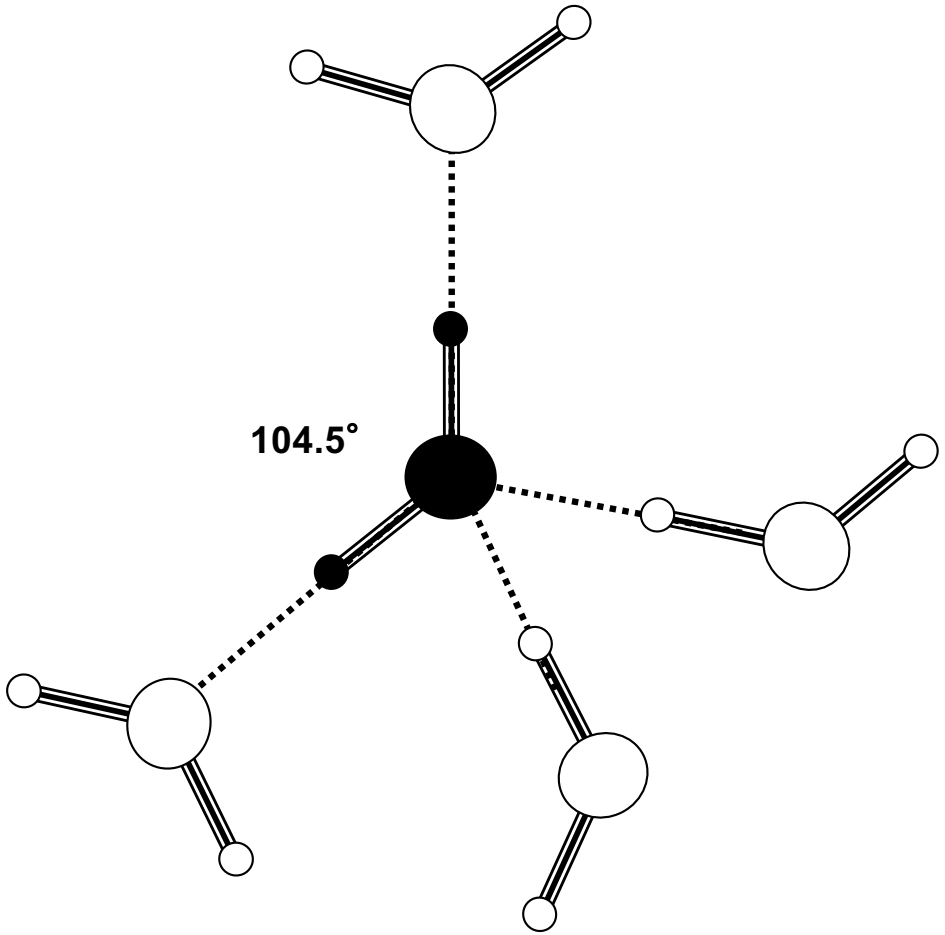
Dubochet, J. Cryo-EM--the first thirty years. *J Microsc* 245, 221-224 (2012)

Structure of Water – H_2O

- **Liquid water has some unusual properties**
  - **a negative volume of melting**
  - **highest density under normal pressure is achieved at 4 ºC**
  - **for its molecular weight, water has a high boiling point & melting point**
    - **Water – M.W. 18 ; melting point 0 ºC ; boiling point 100 ºC ; Δ = 100 ºC**
    - **Methane – M.W. 16 ; melting point -182.5 ºC ; boiling point -162 ºC ; Δ = 20.5 ºC**
    - **Nitrogen – M.W. 28 ; melting point -209.9 ºC ; boiling point –-195.8 ºC ; Δ = 14.1 ºC**
    - **Ethane – M.W. 30 ; melting point -182.8 ºC ; boiling point –88.5 ºC ; Δ = 94.3 ºC**
    - **Propane – M.W. 44.1 ; melting point -187.7 ºC ; boiling point –42 ºC ; Δ = 145.7 ºC**
  - **high surface tension & dielectric constant**
  - **viscosity increases with pressure**
  - **forms at least 10 solid polymorphs**
- **Most of these properties derive from the high dipole moment of water**



Structure of Water – H_2O



- Each water molecule can form 4 hydrogen bonds
  - at 20 kJ/mole, these 4 are almost as strong as an ionic interaction
- Water's dipole moment is 1.8 Debye
  - each Debye corresponds to one electron displaced 0.2 Å from the nucleus

Structure of Water – H_2O

- In liquid water, the molecules have great mobility
  - exchange times are ~10^{-12} sec
  - same global symmetry as water vapor
- Because some H-bonds are broken, liquid water is denser than ice
- Dubochet suggests that ~13% of the H-bonds are broken in liquid water
- The most generally accepted model is that of a random network of H-bonds based on a locally distorted tetrahedral network
- Water clathrates
  - these form around hydrophobic molecules
  - all the H-bonds are satisfied within an "icosahedral" shell around the insoluble molecule with one H-bond pointing outward
- This is why "oily" molecules are insoluble in water
  - the formation of many H-bonds gives a high enthalpy of dissolution
  - counterbalanced by high negative entropy caused by the excessive ordering of the H-bond lattice

Structure of ice

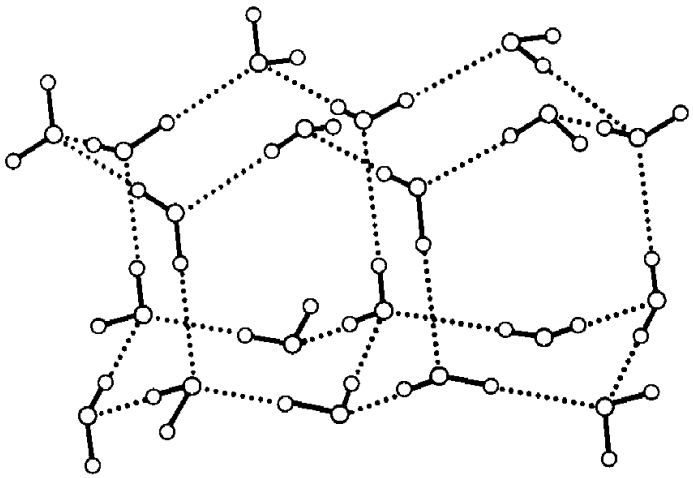


Fig. 2. Schematic view of a small part of a hexagonal ice crystal.

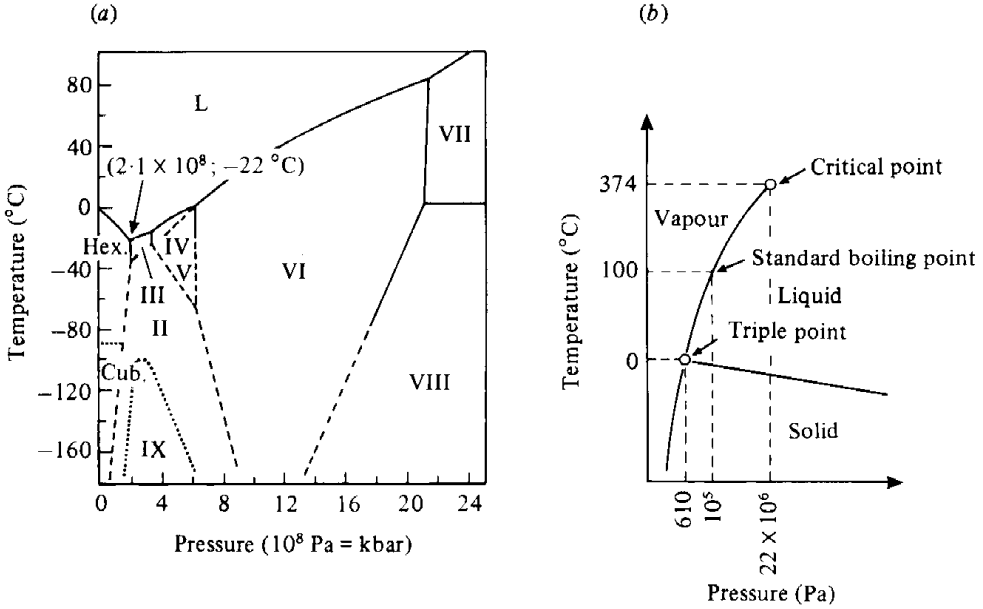


Fig. 3. (*a*) Part of the phase diagram of water. (*b*) Phase diagram around the triple point.

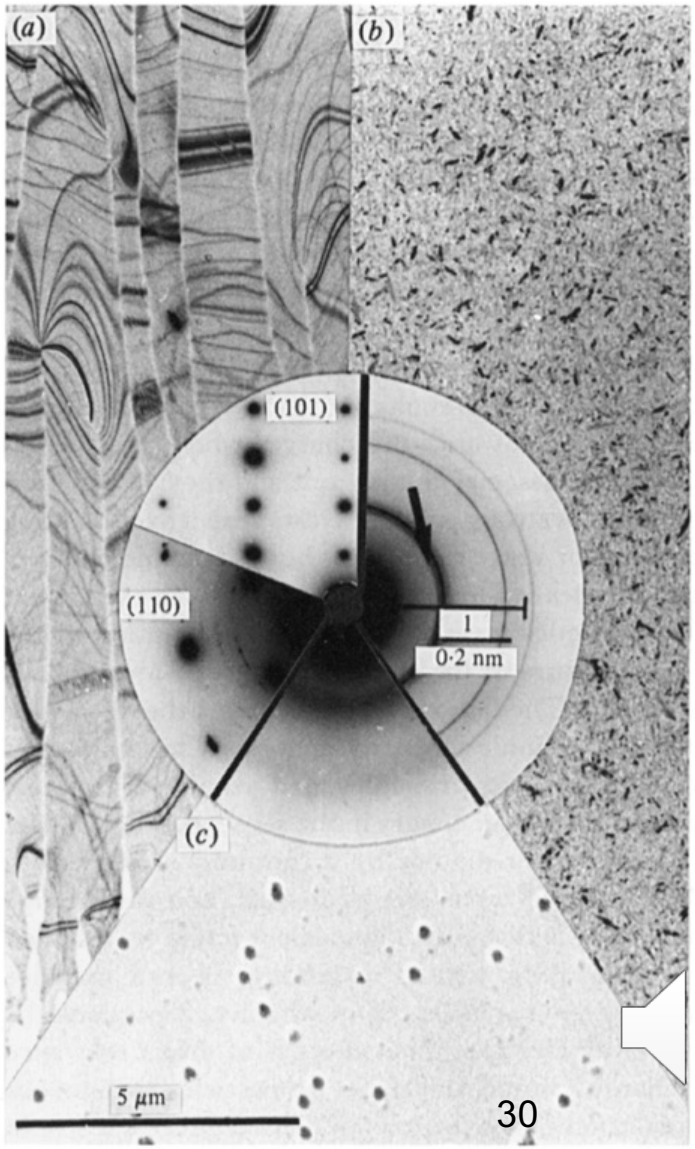
- **Common ice is hexagonal ice**
- **Each water molecule has 4 nearest neighbors**

Structure of ice

- Common ice is hexagonal ice
- Cubic ice, another common form is only stable at below -70° C
- All others crystalline forms are observed at high pressure
  - Ice II has an additional water molecule approaching the hydrogen bonded neighbors
  - Ice III and IX have 4 member rings
  - Ice VII and VIII have two interpenetrating networks of cubic ice
  - Ice X has the water molecules pushed so close together that the H atom is midway between oxygen atoms

Structure of ice

- **In the EM, the different kinds of ice take on characteristic appearances**
  - **hexagonal ice appears with numerous "bend" contours & gives a single crystal diffraction pattern**
    - **Those black lines are called "bend contours"**
    - **If the objective aperture is large enough, they appear as paired white and black lines of identical shape**
  - **cubic ice appears granular & gives a "powder" diffraction pattern**
  - **vitreous ice is relatively featureless & gives a diffraction pattern characteristic of an amorphous solid**

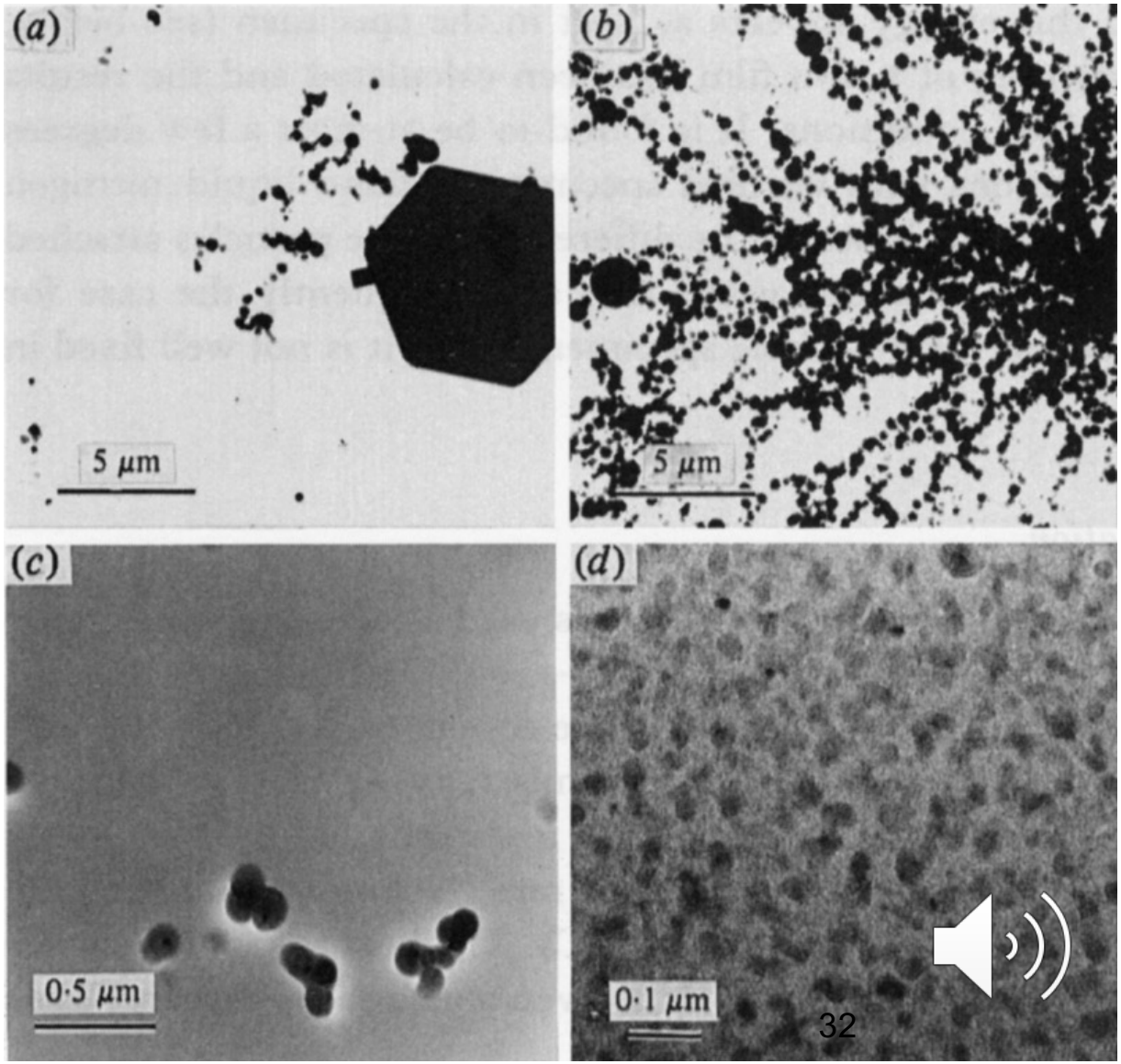


What Does the Type of Ice Tell You About Your Experiment?

- Presence of hexagonal ice tells you that your freezing was too slow or the specimen is way too warm in the microscope
- Presence of cubic ice, as a contaminant, indicates that the specimen is too warm in the microscope
- Vitreous ice means that your freezing was fast and that your specimen is cold enough in the microscope
  - Any contamination that forms at this temperature will be vitreous

Types of Ice Contamination

Different forms of ice contamination (a) hexagonal ice contamination formed during specimen preparation, (b) ice formed by condensation in liquid nitrogen, (c) vitreous ice contamination in the microscope, (d) cubic ice contamination in the microscope

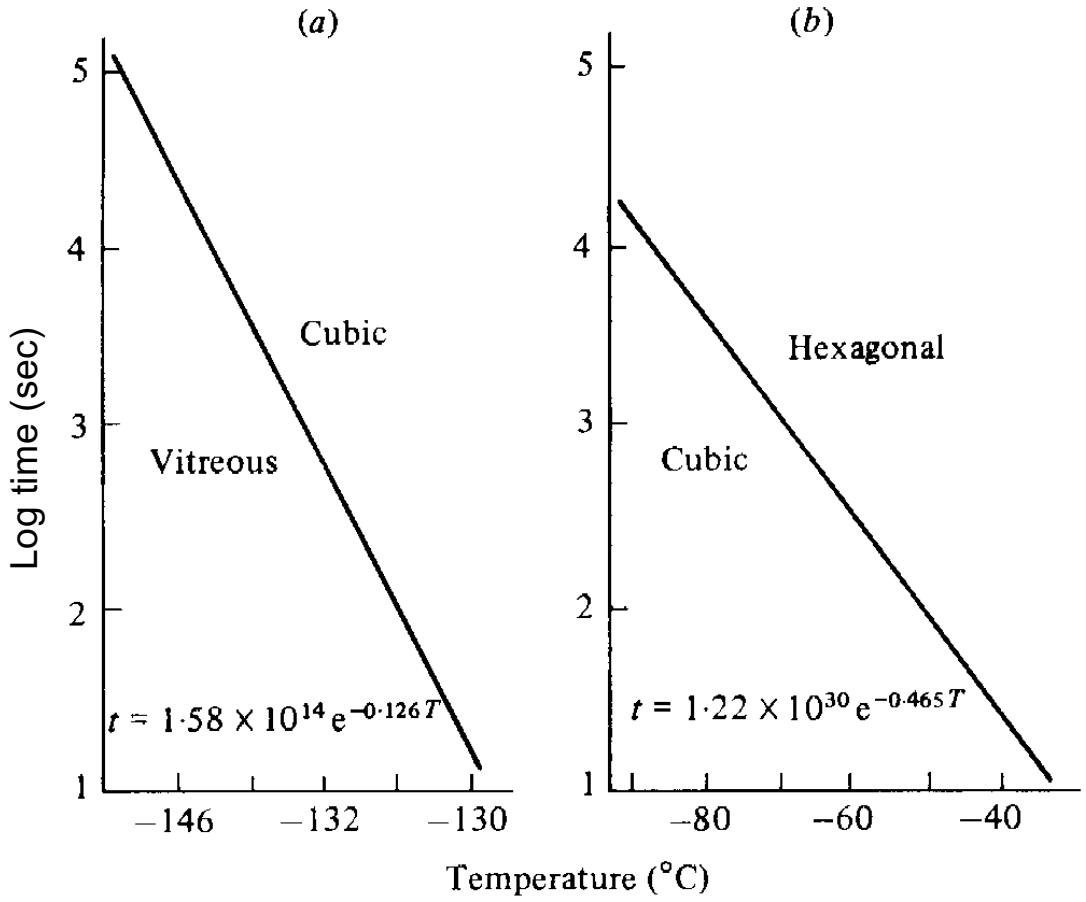


Structure of ice

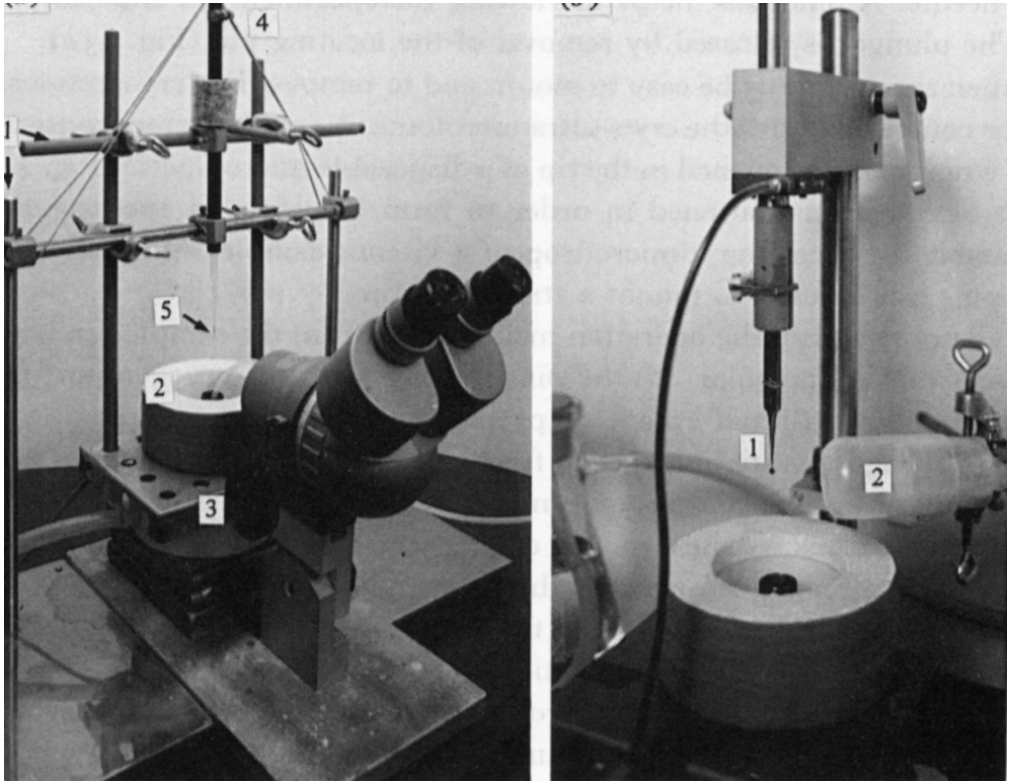
Table 2. *Main reflections in the electron diffractogram of the various forms of ice at −160 °C*

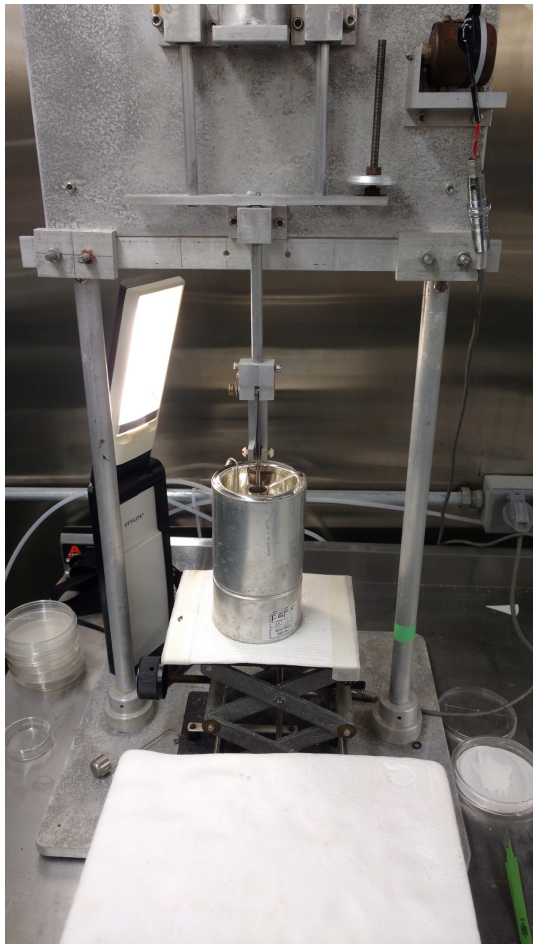
Hexagonal	Cubic	Vitreous	*d* (nm)	Intensity
100	—	—	0·389	Very strong
	—	First maximum	0·370	Very strong
002	111	—	0·366	Strong/very strong
101	—	—	0·343	Strong
102	—	—	0·266	Weak
110	220	—	0·224	Medium/medium
	—	Second maximum	0·214	
103	—	—	0·207	Medium
200	—	—	0·194	Very weak
112	311	—	0·191	Weak/weak
201	—	—	0·188	Very weak
202	—	—	0·172	Very weak

The stability of vitreous & cubic ice depends on temperature



Freezing





Examples of guillotine freezing devices

http://www.jove.com/video/1943/electron-cryotomography-of-bacterial-cells

ThermoFisher Vitrobot

Gatan CP-2

Leica GP-2

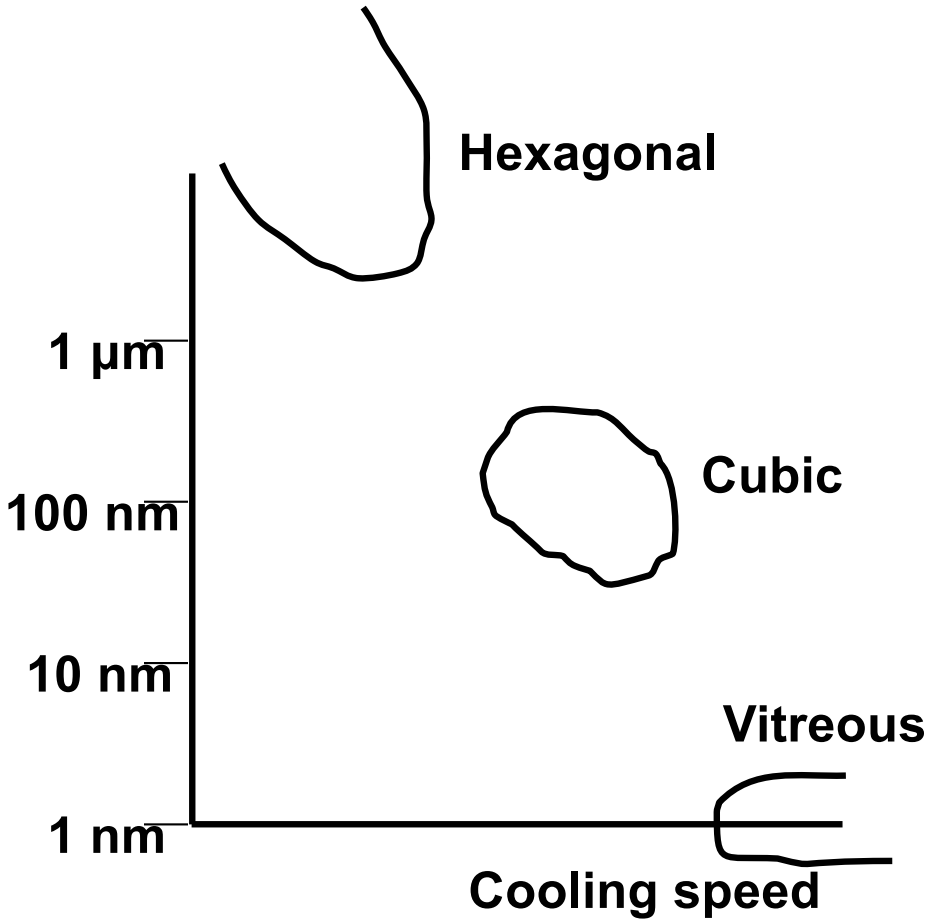
Examples of guillotine freezing devices

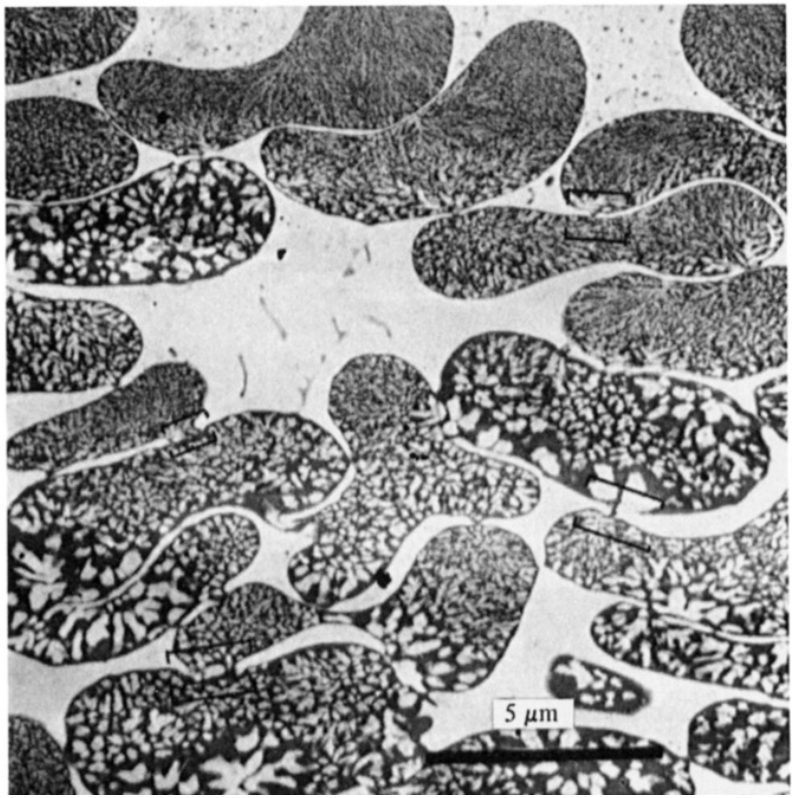
http://www.jove.com/video/1943/electron-cryotomography-of-bacterial-cells

Freezing

- **What happens if freezing is too slow**
  - **solutes & specimen are ejected from the growing hexagonal ice phase**
  - **ejected solutes cannot equilibrate with surrounding liquid and so inhibit growth of the crystals**
  - **as ejected solute increases, it further inhibits crystal growth; makes crystal look polycrystalline**
  - **although the crystals appears disrupted, it is nevertheless a single hexagonal ice crystal**

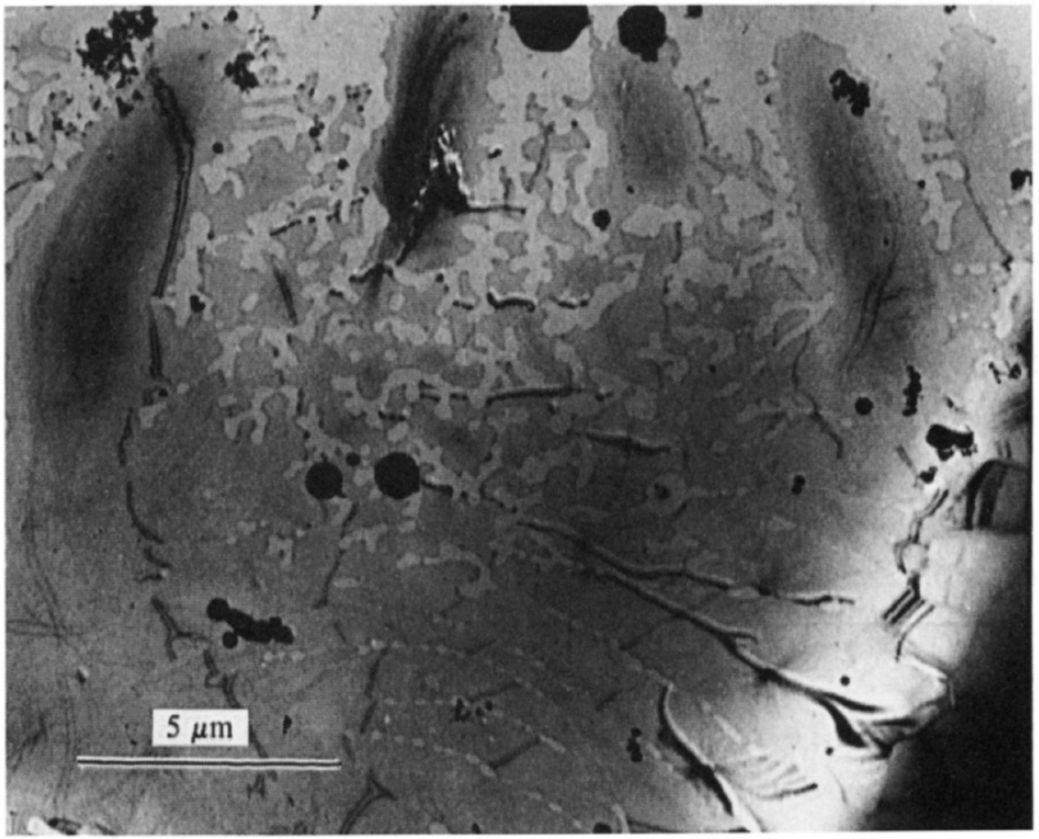
Ice Crystal size as a function of cooling rate





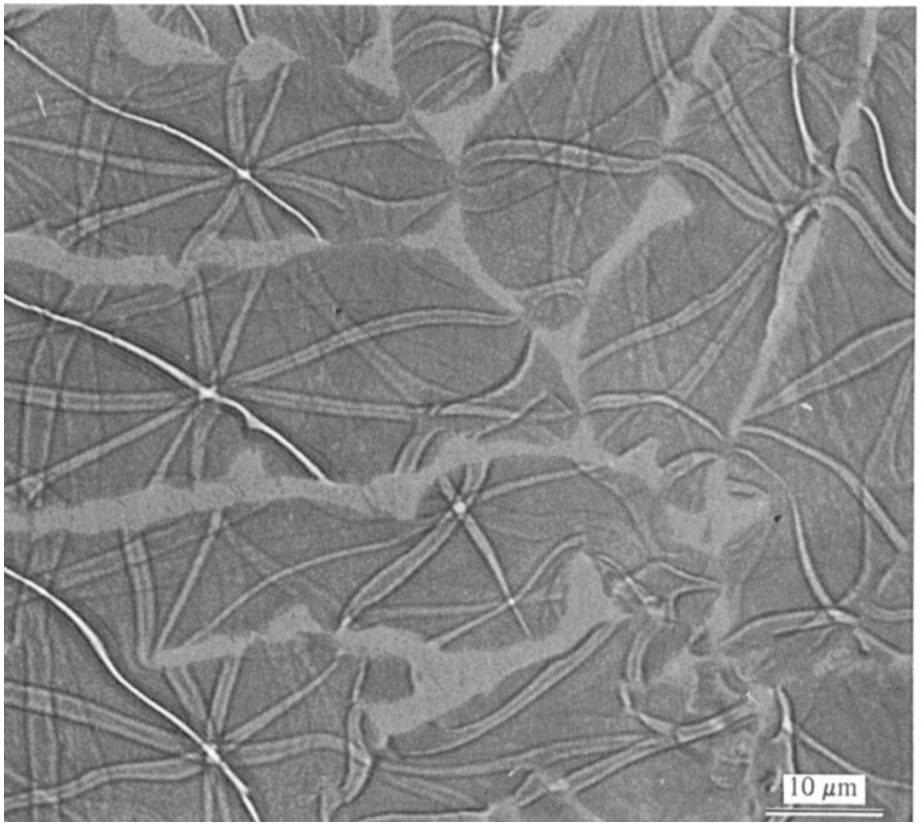
This is a thin, plastic section of frozen RBCs, not a cryoEM section.

Freezing

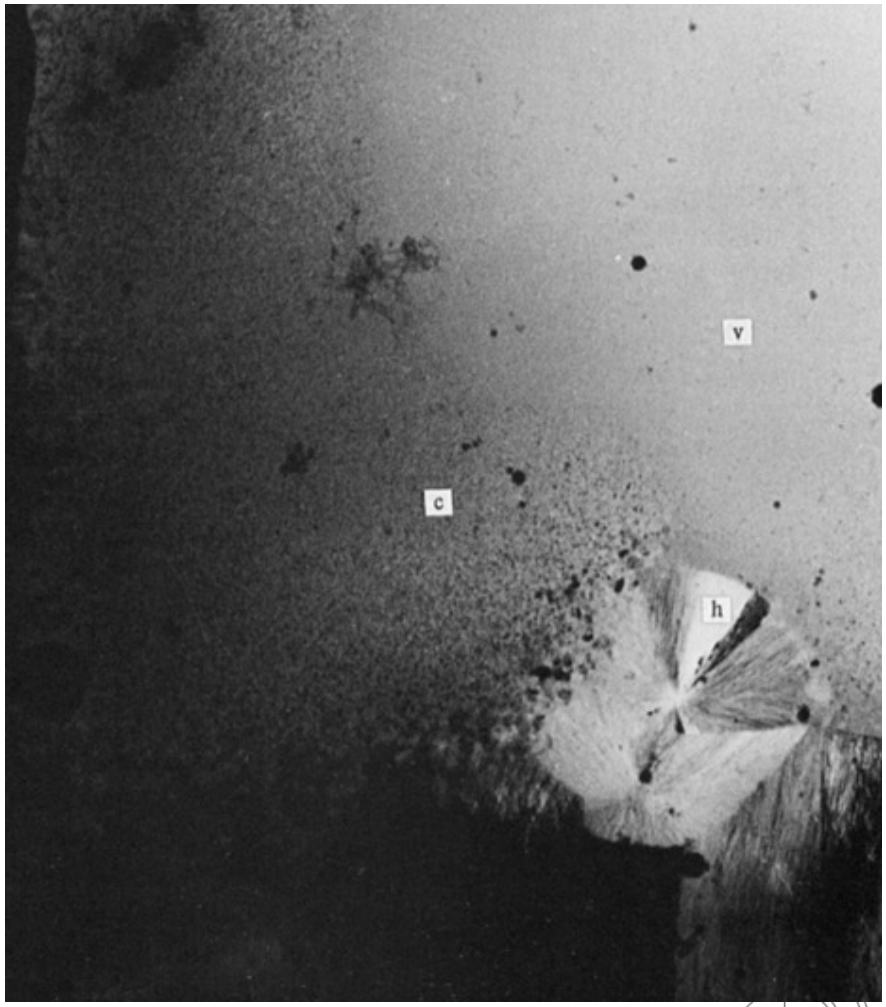


Water droplet spreading on a hydrophilic surface

Freezing



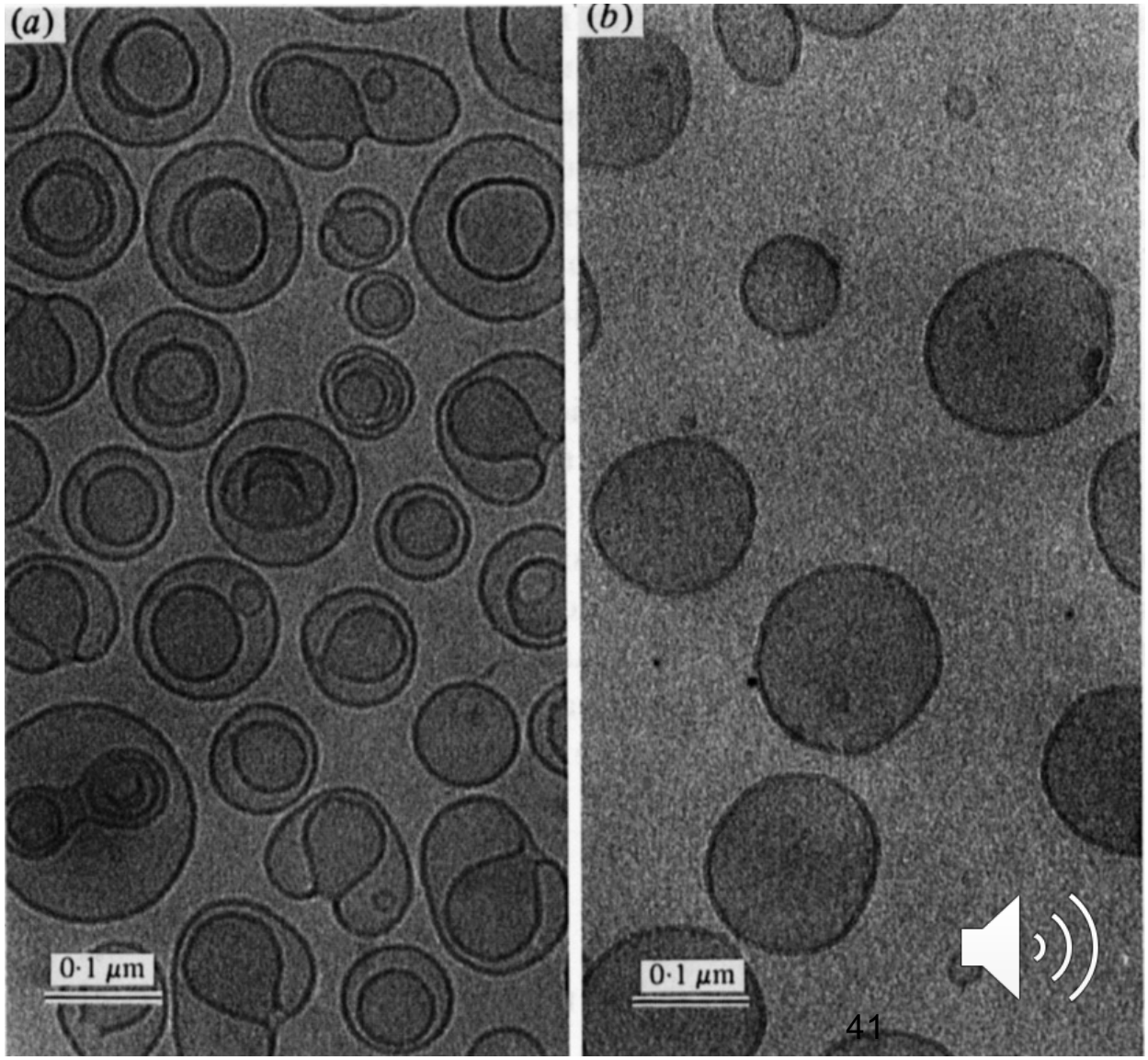
Thin crystals of hexagonal ice



Three kinds of ice coexisting in the microscope

Freezing

Evaporation during freezing. (a) shows vesicle merging & distorting due to changes in solution ionic strength. (b) shows freezing where evaporation was inhibited

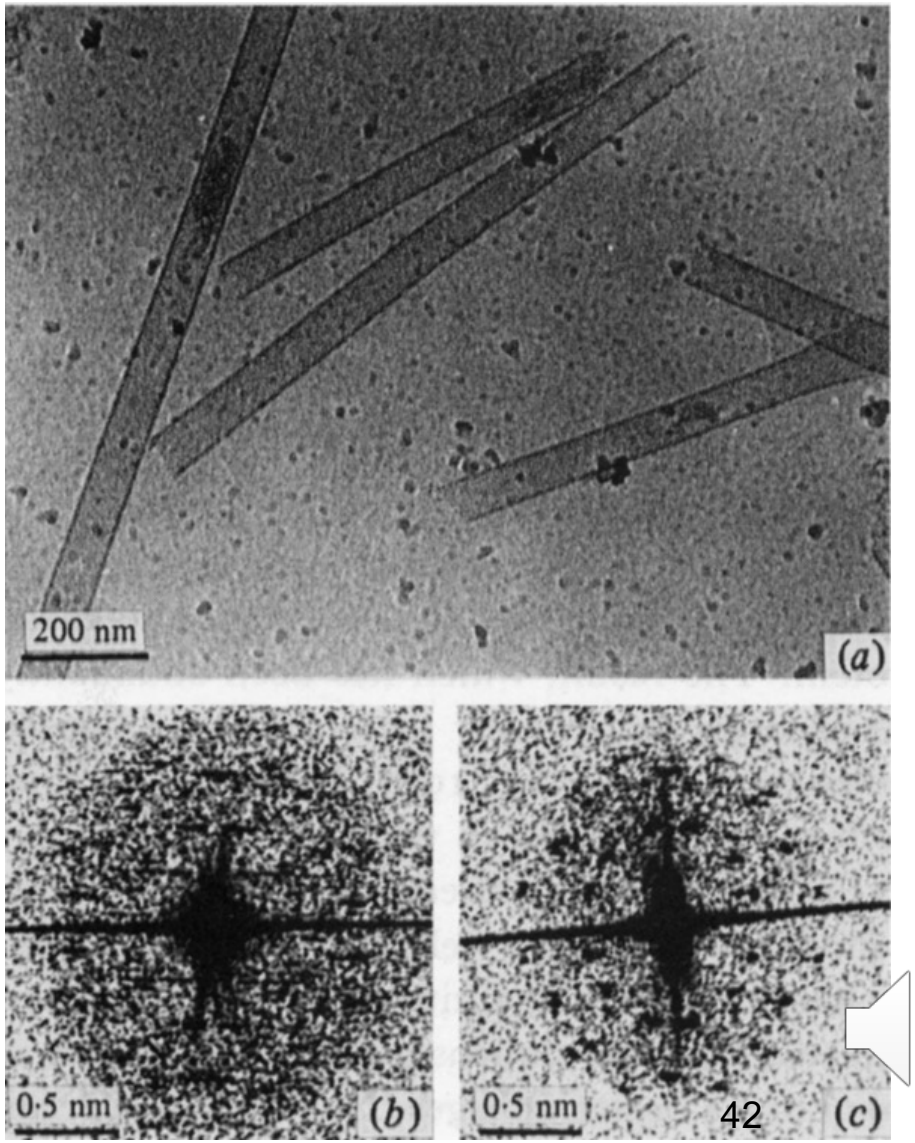


Freezing

- Flattening
- Cylindrical objects can be flattened if water film gets too thin

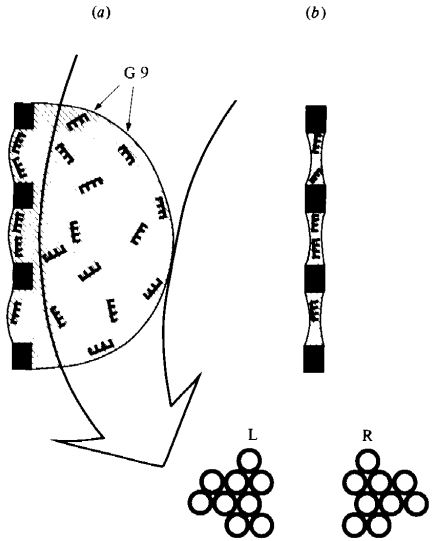
(b) Is the optical diff^{n} pattern for a cylindrical tube

(c) Is the optical diff^{n} pattern for a flattened tube

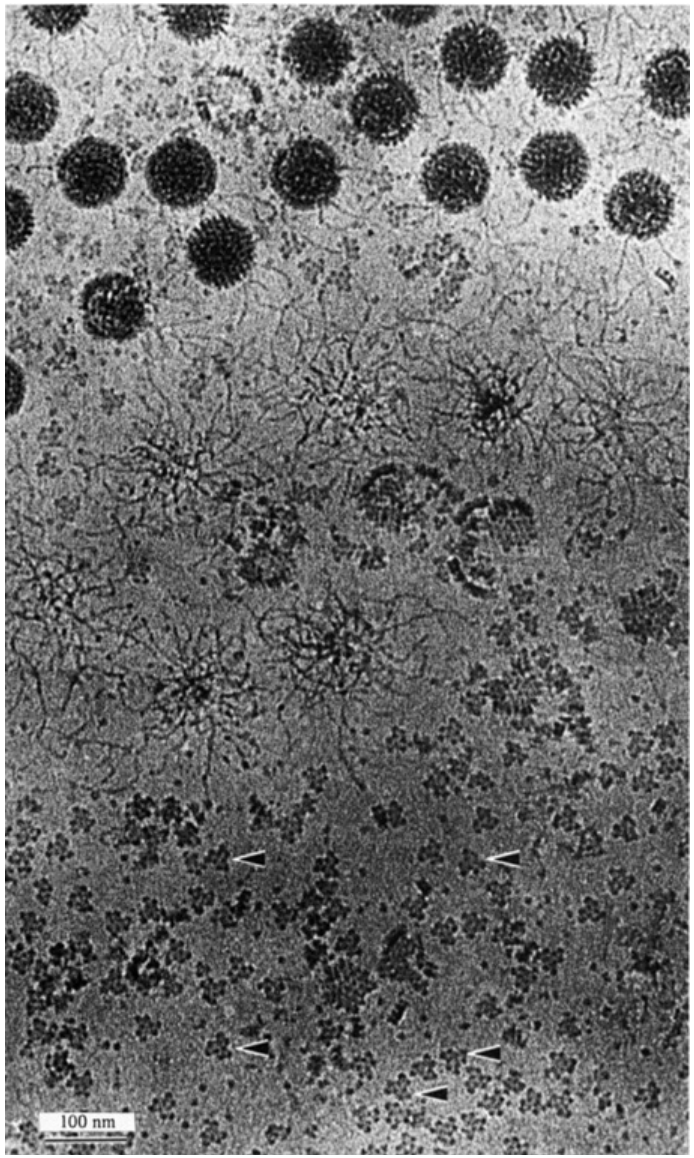


Freezing

Molecules may adsorb in preferred orientations to the air-water interface

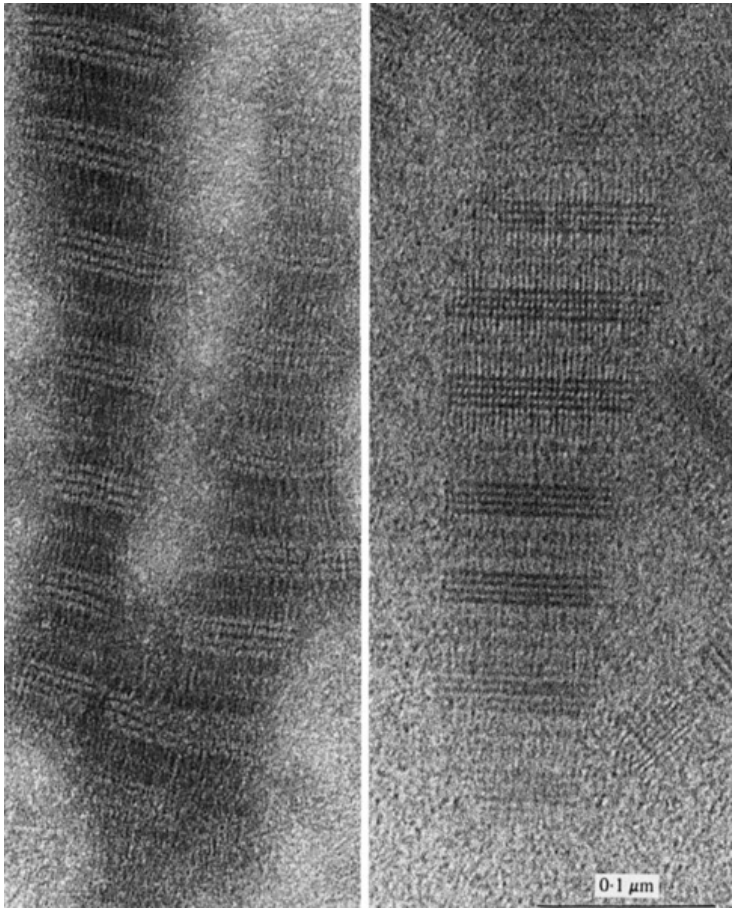


Molecules denaturing at the air-water interface is the greatest technological barrier to high resolution cryoEM

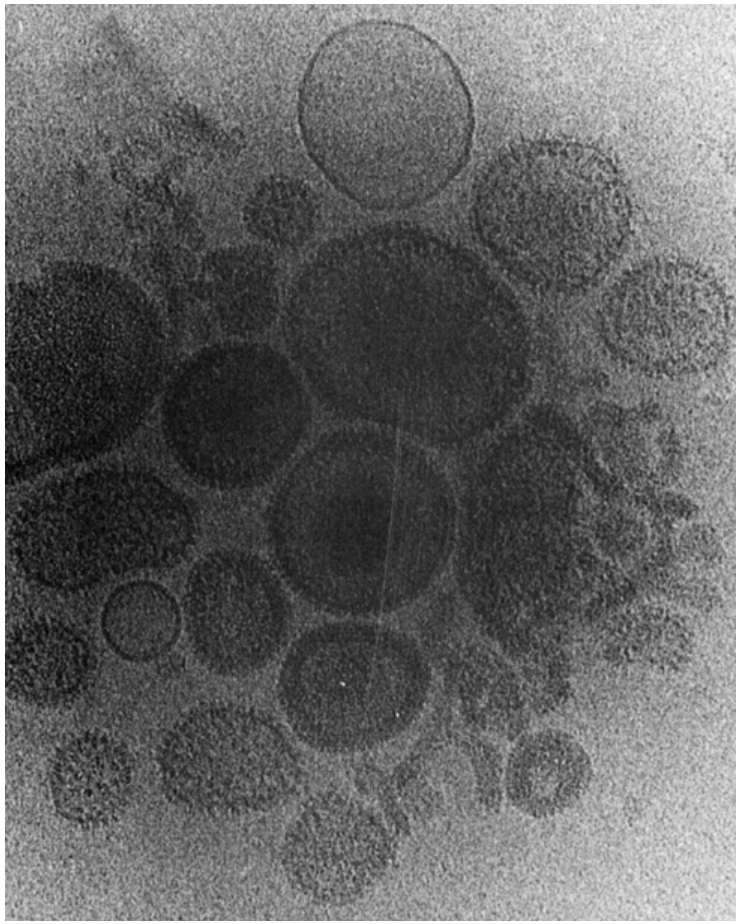


This is a virus that has fallen apart at the air-water interface spreading out its capsomeres & nucleotide in the thin ice film

Examples

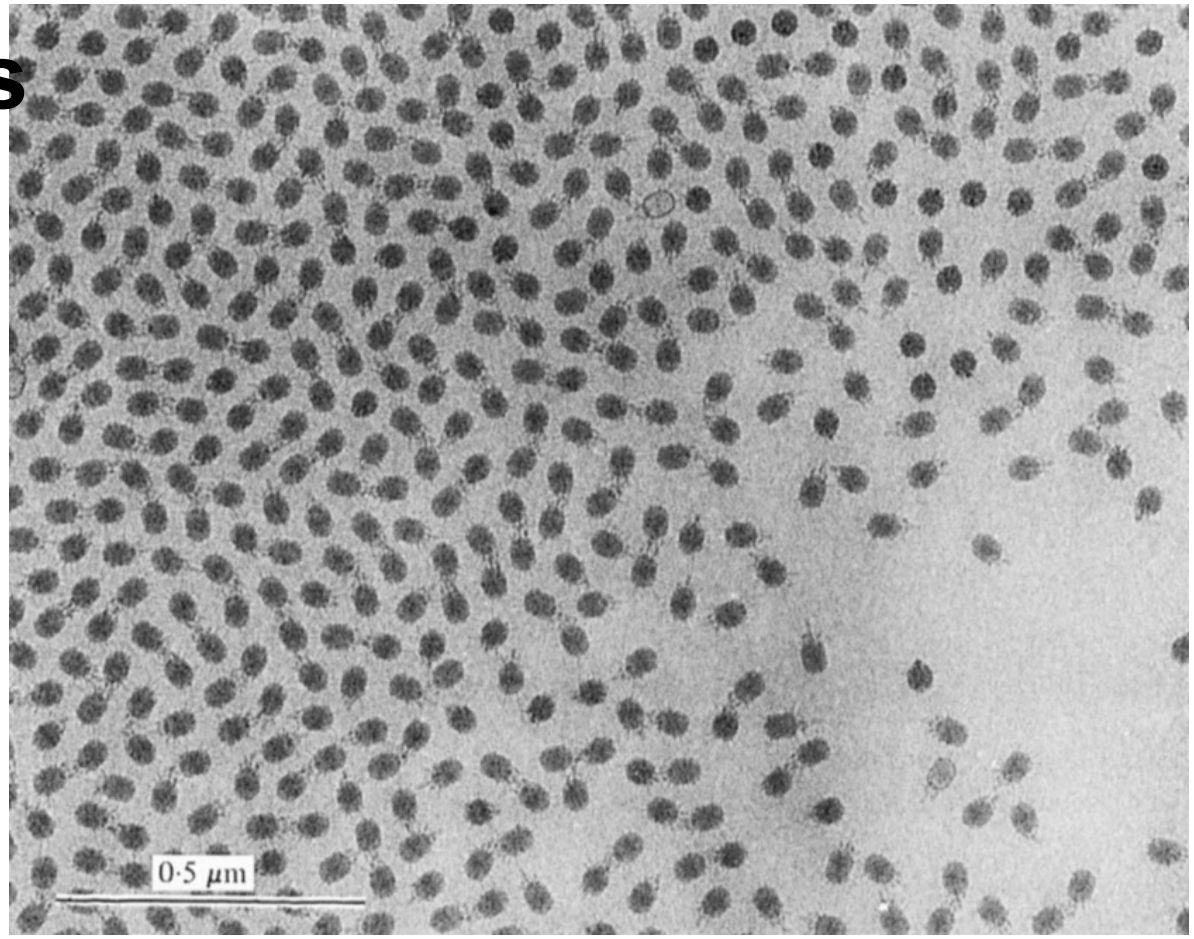


Adenovirus spikes



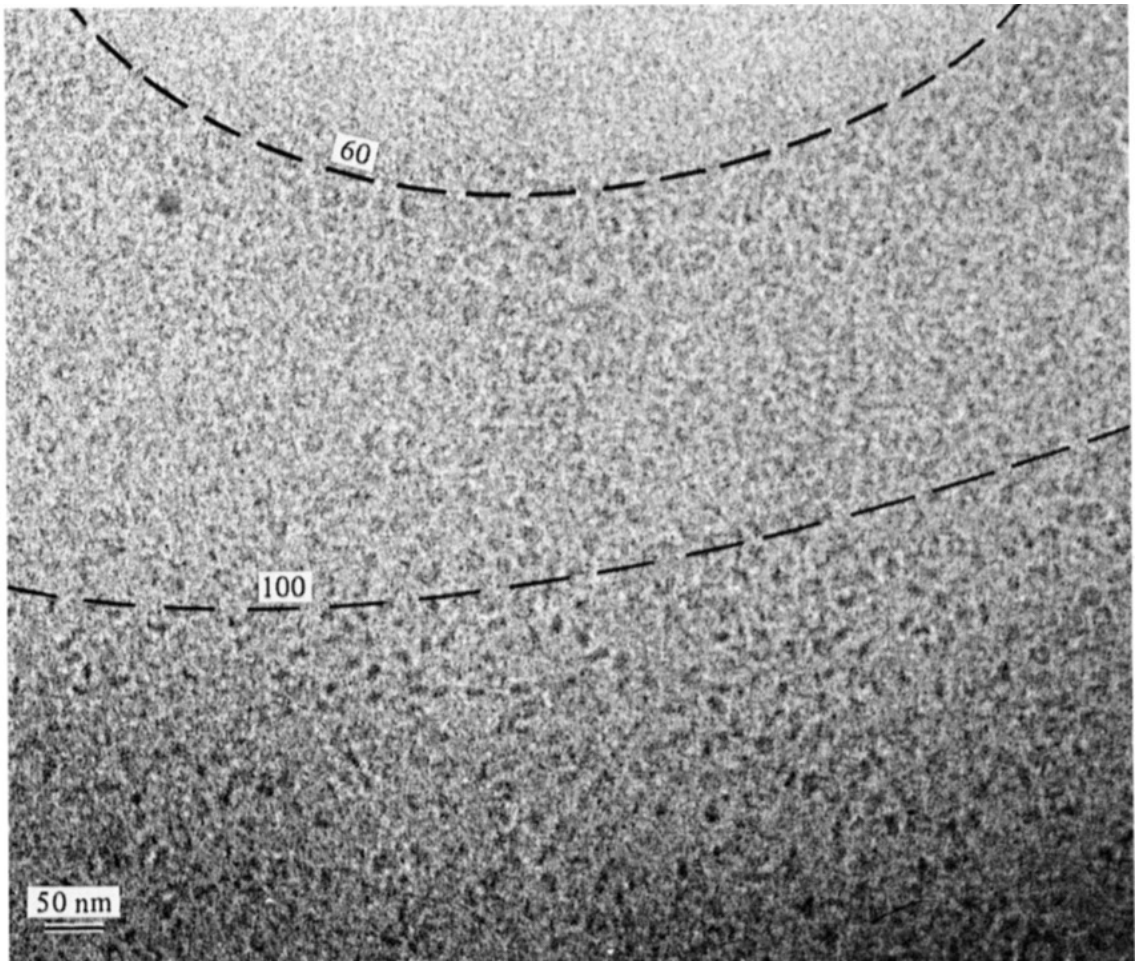
Virosomes formed by lipid vesicles containing spike proteins

Examples



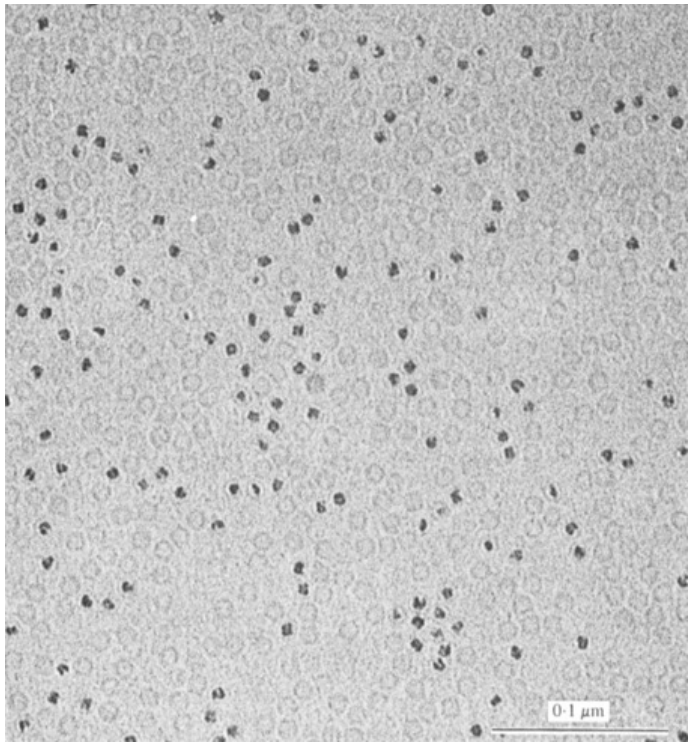
Thin vitrified layer of bacteriophage f29 prepared on a holey carbon film. Thinnest ice is in the lower right-hand corner.

Examples



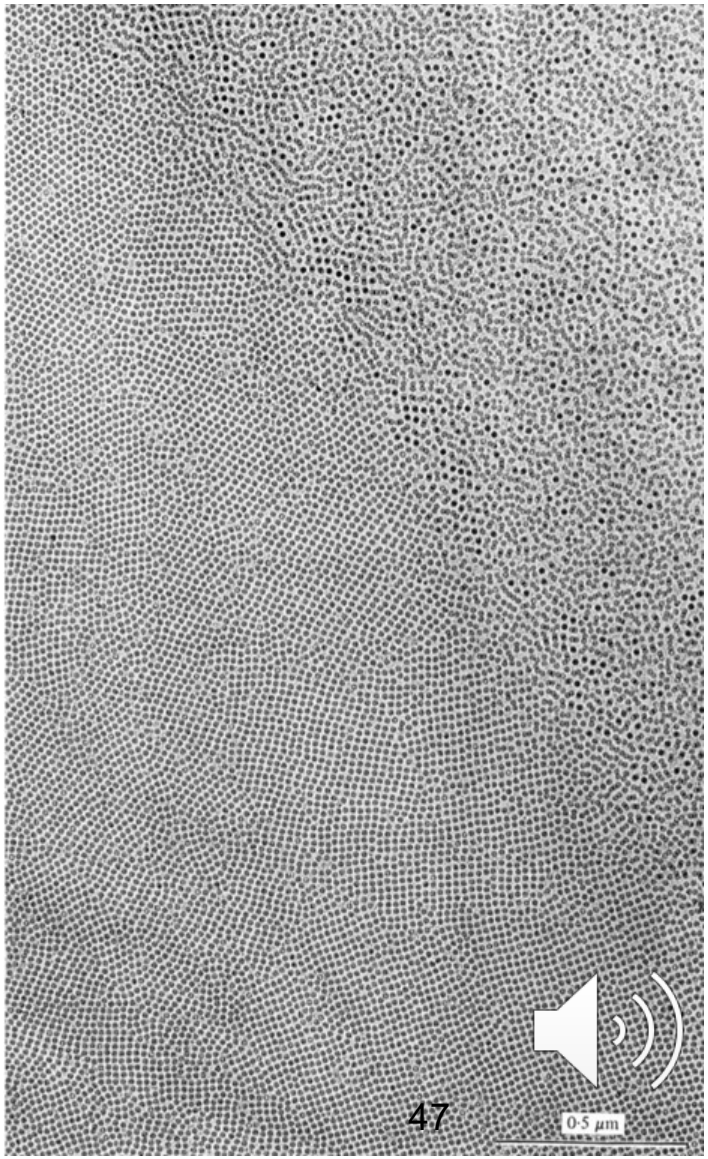
Isolated nucleosomes prepared in the presence of negatively charged lipids in 100 mM NaCl.

Examples

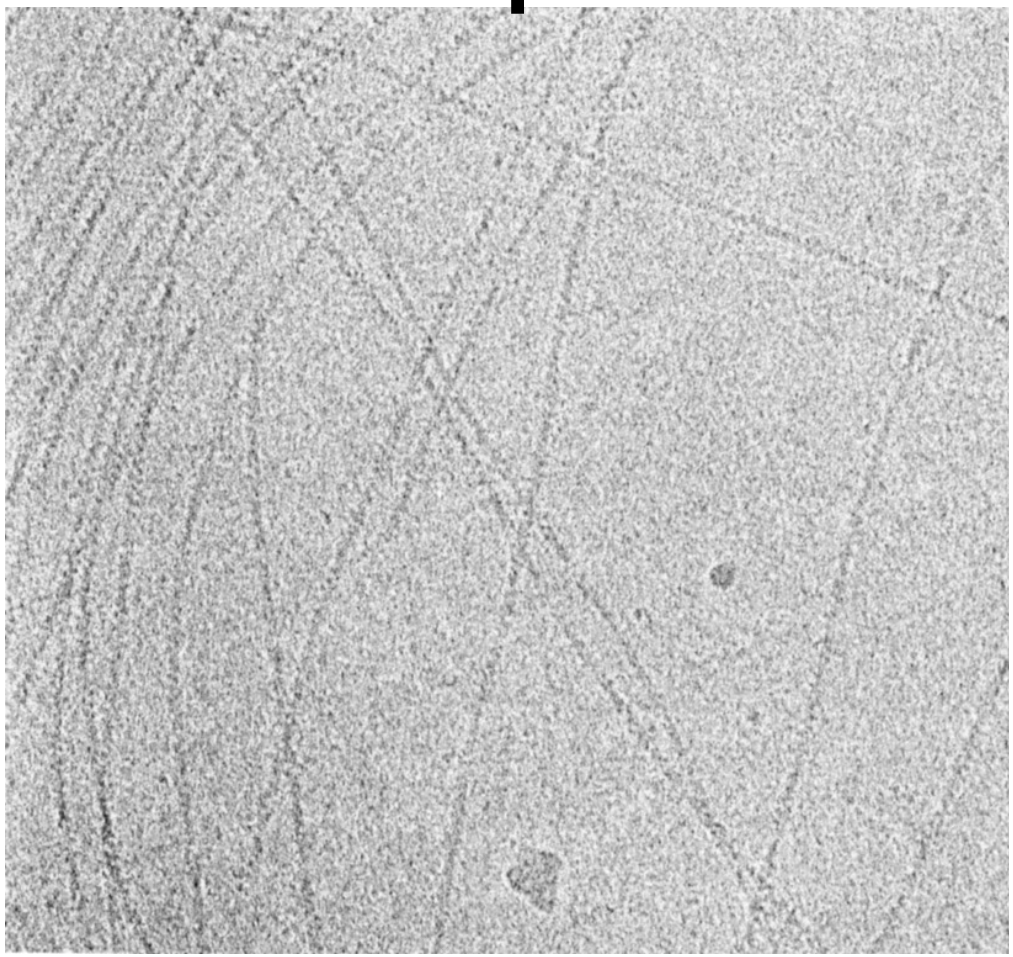


Ferritin & apoferritin prepared on a holey carbon film.

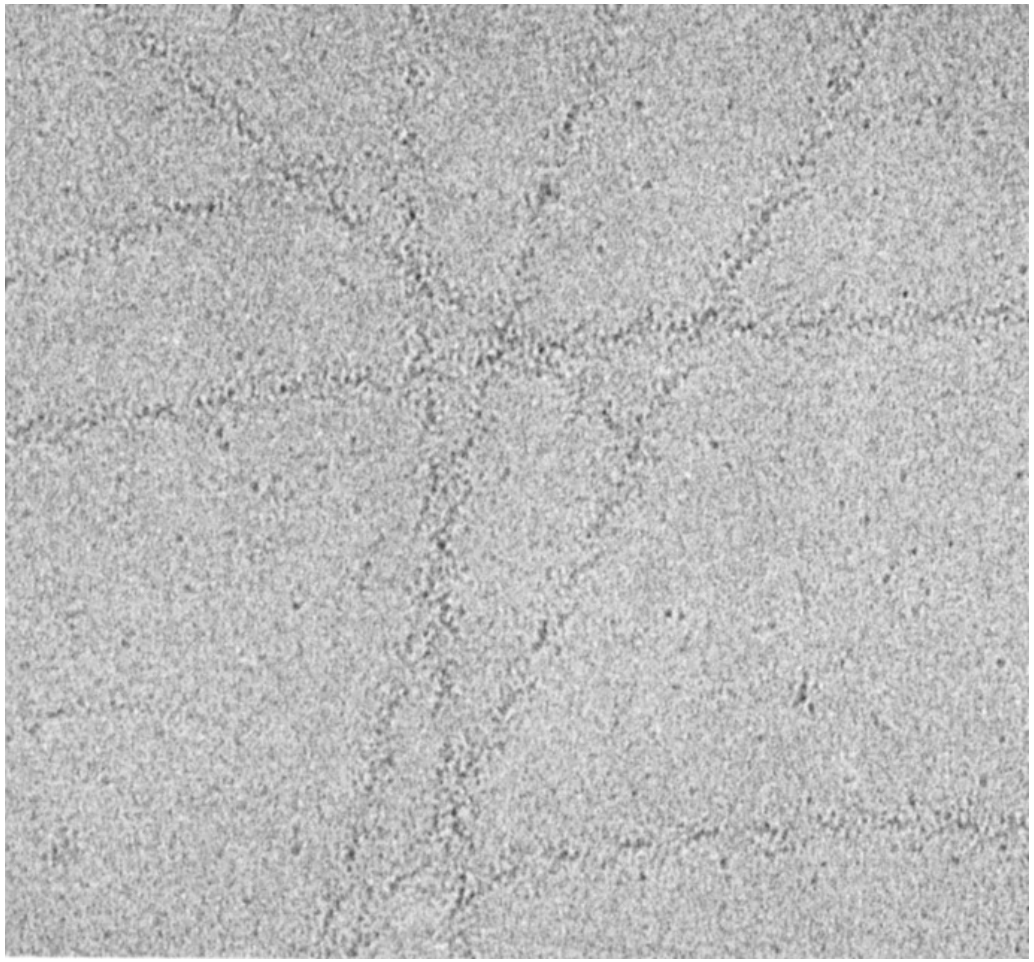
Thin layer of brome grass virus prepared by the bare grid method. Thinnest ice is at the top right corner.



Examples

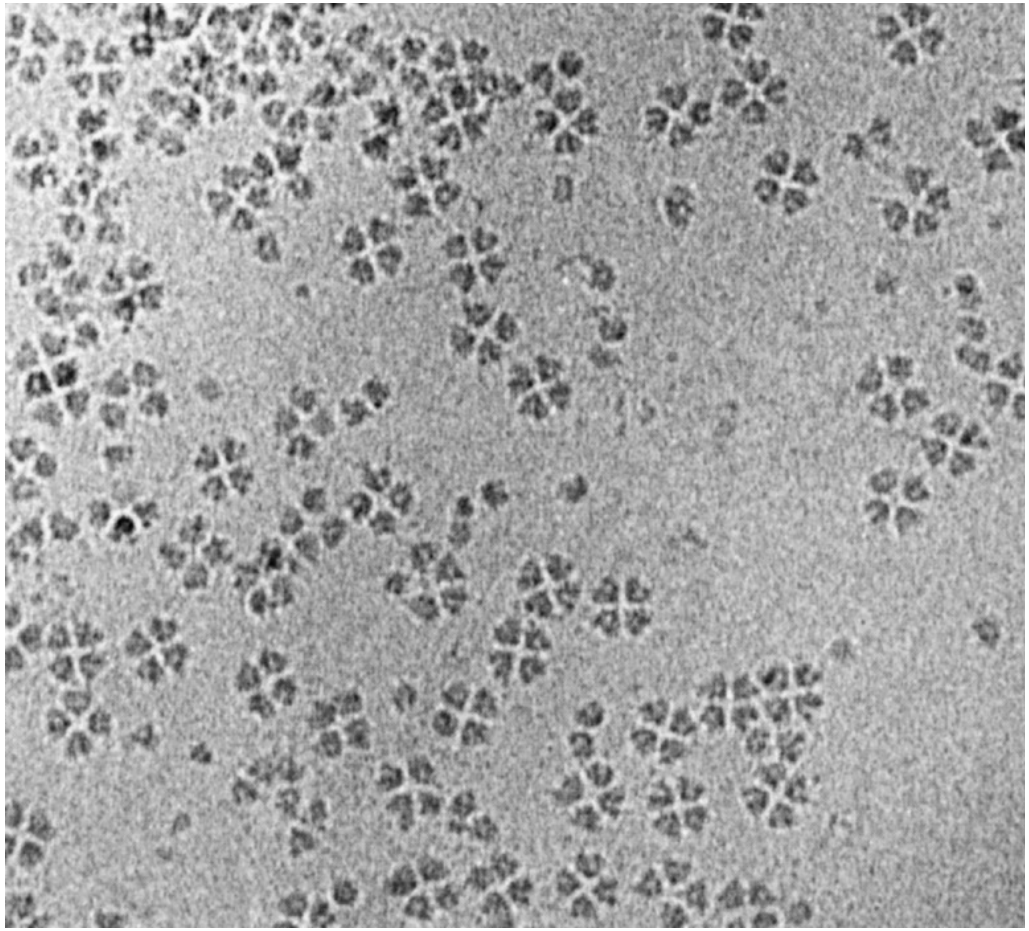


Actin filaments in vitreous ice

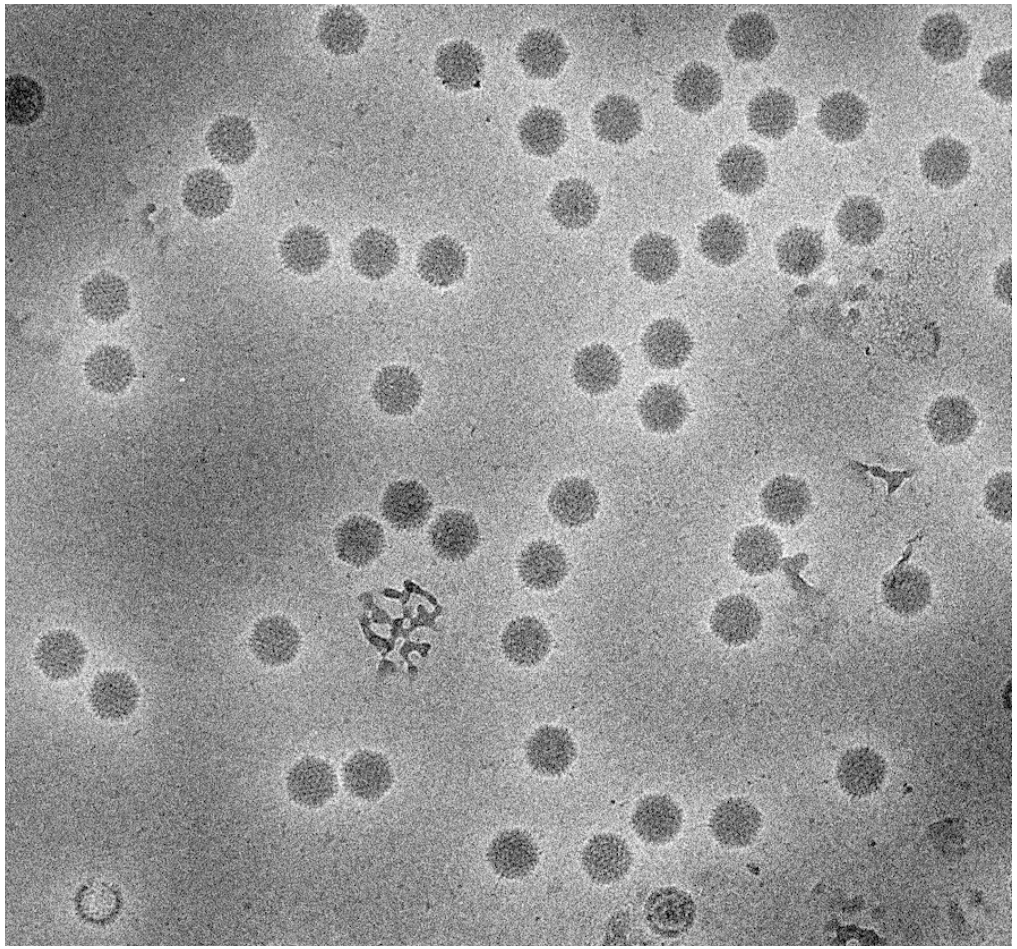


Acto-S1 filaments in vitreous ice

Examples



Ribosome tetramers in vitreous ice



Icosahedral viruses in vitreous ice

From Adrian et al. (1984) *Nature* 308, 32-36.