# Biological Specimen Preparation

How might one prepare biological specimens for TEM?

- Fix, embed and section
- Metal shadow
- Negative stain
- Positive stain
- Unstained, frozen-hydrated
- Freeze-fracture
- Freeze-etch
- Freeze-dry
- Cryo-section
- Etc., etc., etc.

BIO595S - *Microscopy of Macromolecules* (M. Sherman)

BIO595T - *Microscopy of Cells and Tissues* (D. Sherman)

# Electron Microscopy of Biological Macromolecules:

## Specimen preparation

# Specimen preparation: Why bother?

- ✓ Specimens are in aqueous medium – to protect from column vacuum
- ✓ Weak electron scattering – extremely low contrast
- ✓ Low radiation tolerance

# Specimen preparation

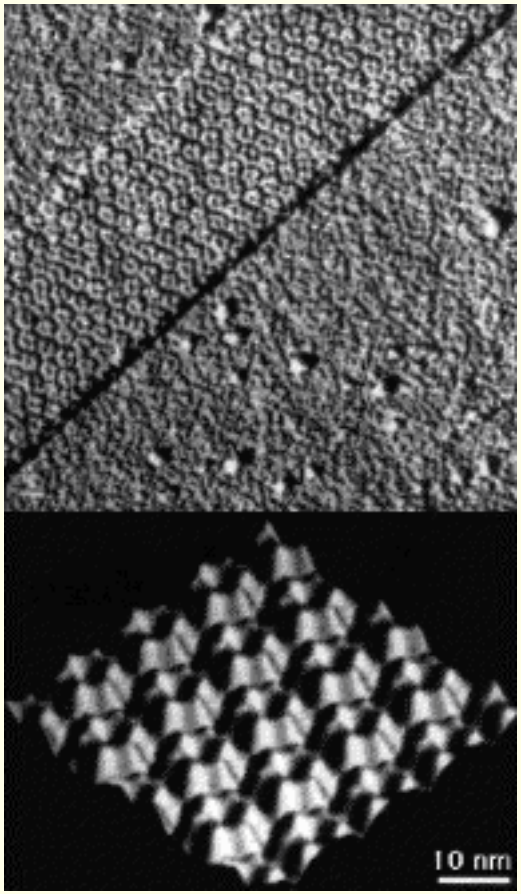
- ✓ To increase scattering contrast (both, amplitude and phase) – to see better
- ✓ To preserve specimen structure at highest resolution possible
- ✓ To improve radiation stability of the samples
- ✓ Time resolved microscopy – to capture dynamic changes in the structure
- ✓ To label parts of the structure (e.g. gold or Fab labeling)

  [part of Debby Sherman's lecture but labeling is used in macromolecular TEM as well although with smaller probes]

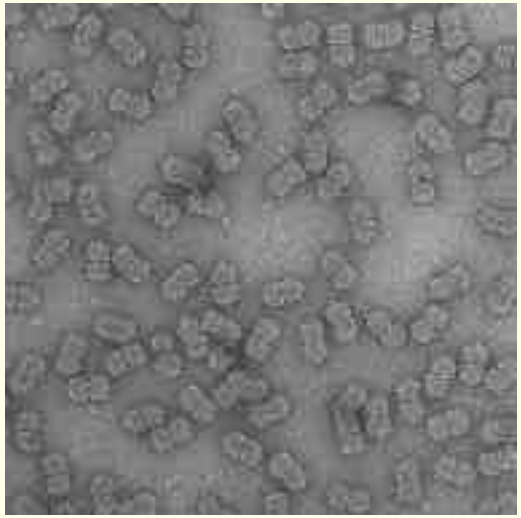
# Specimen preparation techniques

- ✓ **Surface replicas**
  - Metal shadowing
    - Unidirectional
    - Rotary
  - Freeze drying
  - Freeze fracturing
- ✓ **Negative staining**
- ✓ **Cryo microscopy**

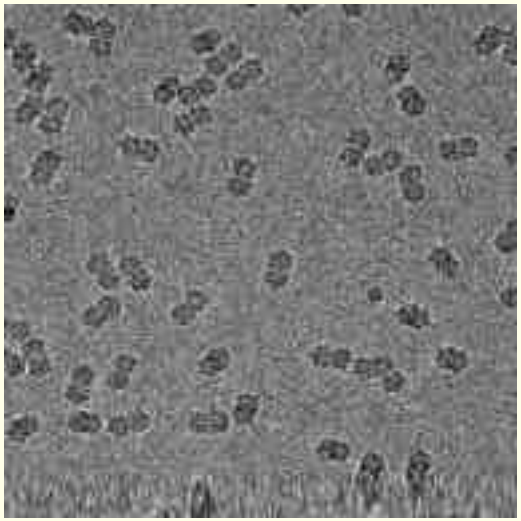
# Metal Shadowing, Negative-Staining and Electron Cryomicroscopy




Metal Shadowing
Bacteriophage T4 polyheads

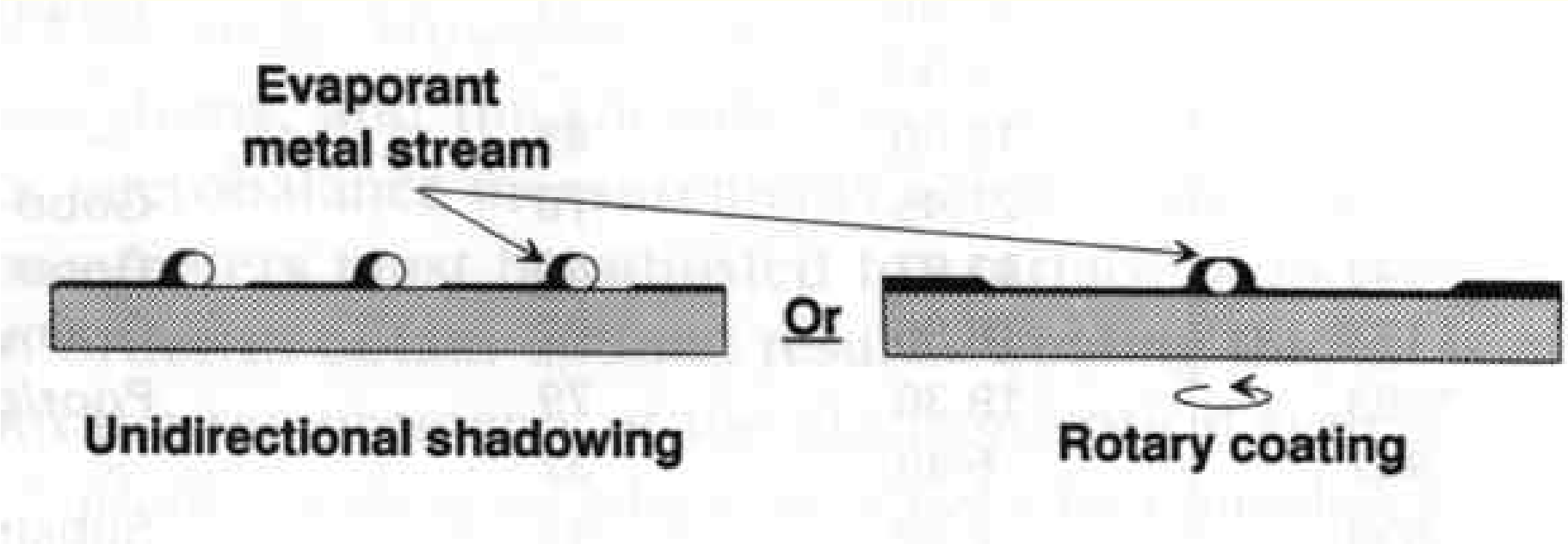


Negative Staining
Maize Streak Virus



Cryo-EM
Maize Streak Virus

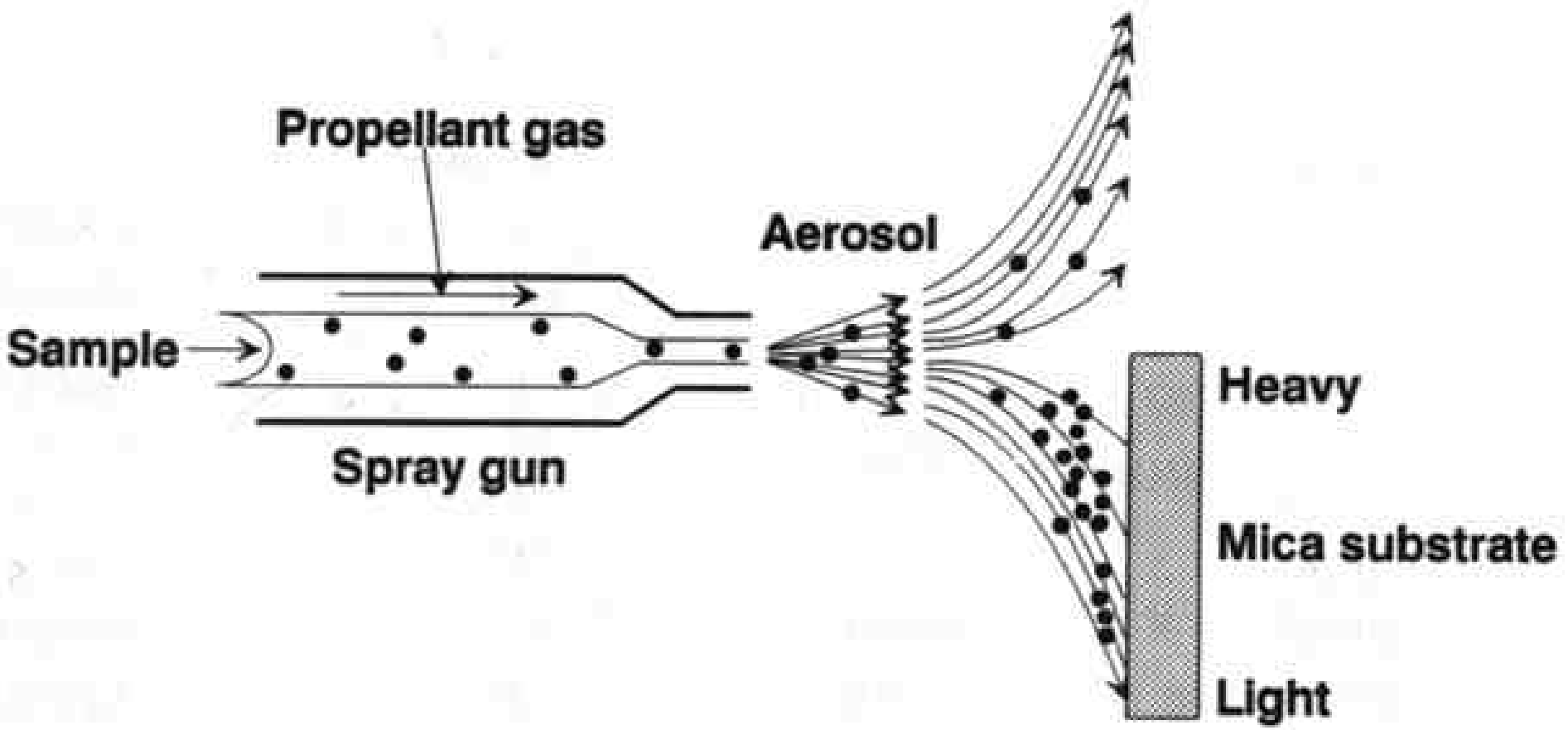
# Metal shadowing




From H. S. Slayter 1976 (p. 17 of class reference list)

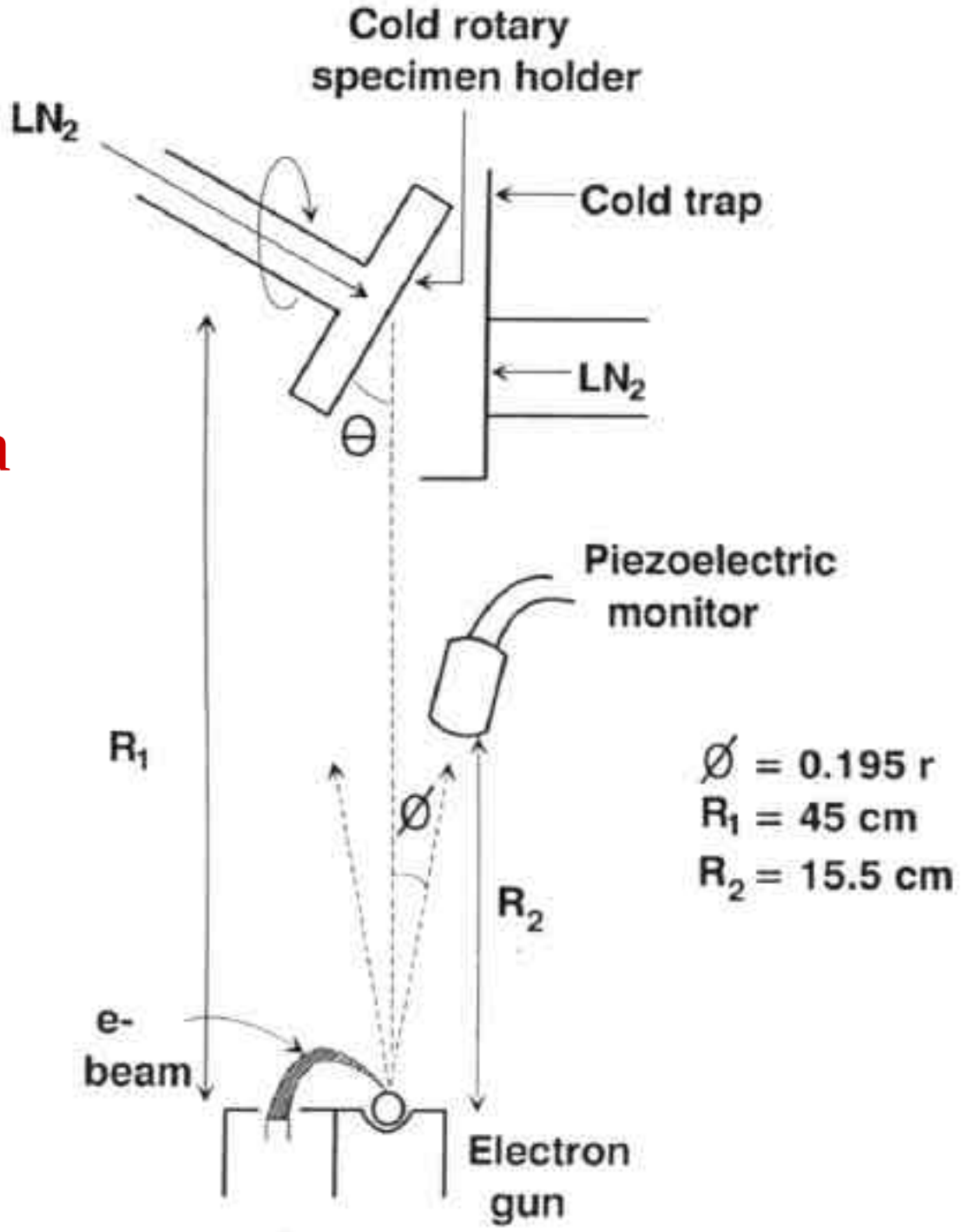
$$t_{\text{calc}} = k_{\alpha} \frac{W \sin \theta}{4 \pi R^2 \rho}$$

# Specimen application by spraying



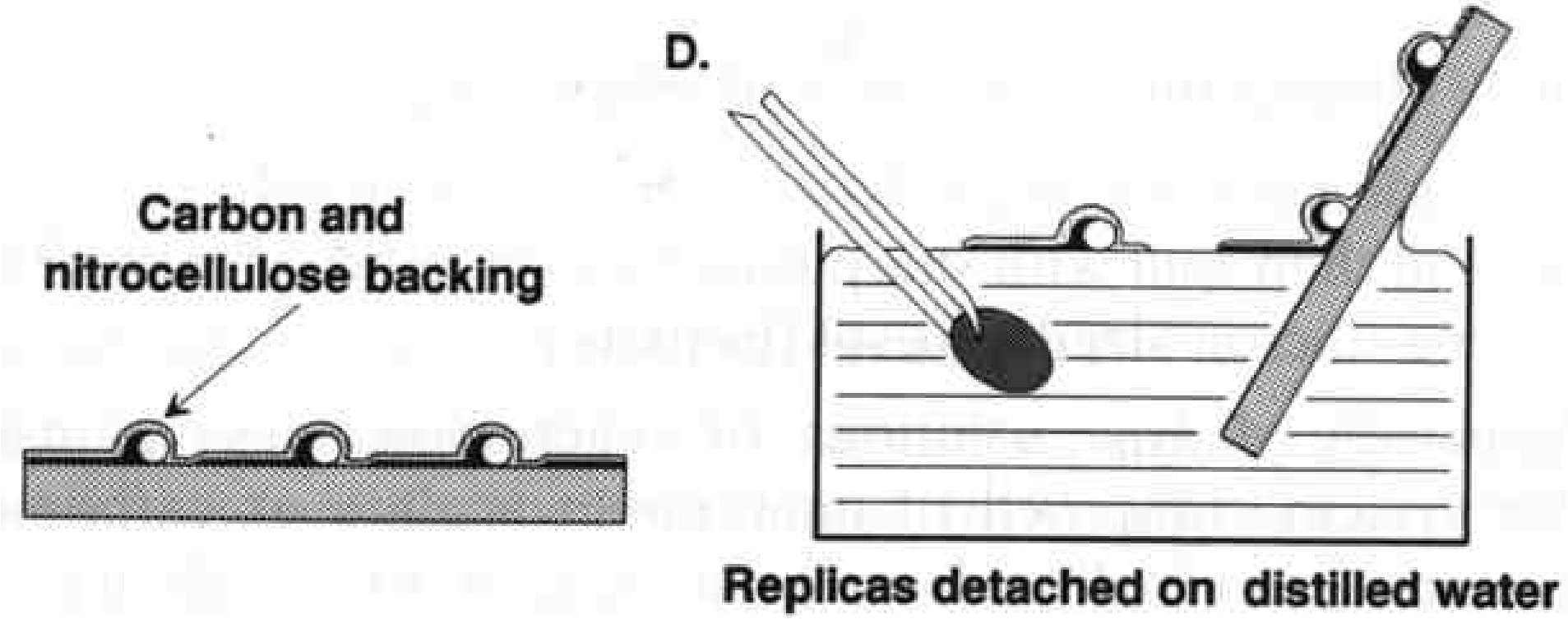

From H. S. Slayter

# Apparatus for deposition of coatings by electron gun onto a cold tilted specimen




From H. S. Slayter

# Backing replicas with C and detaching them on water




From H. S. Slayter

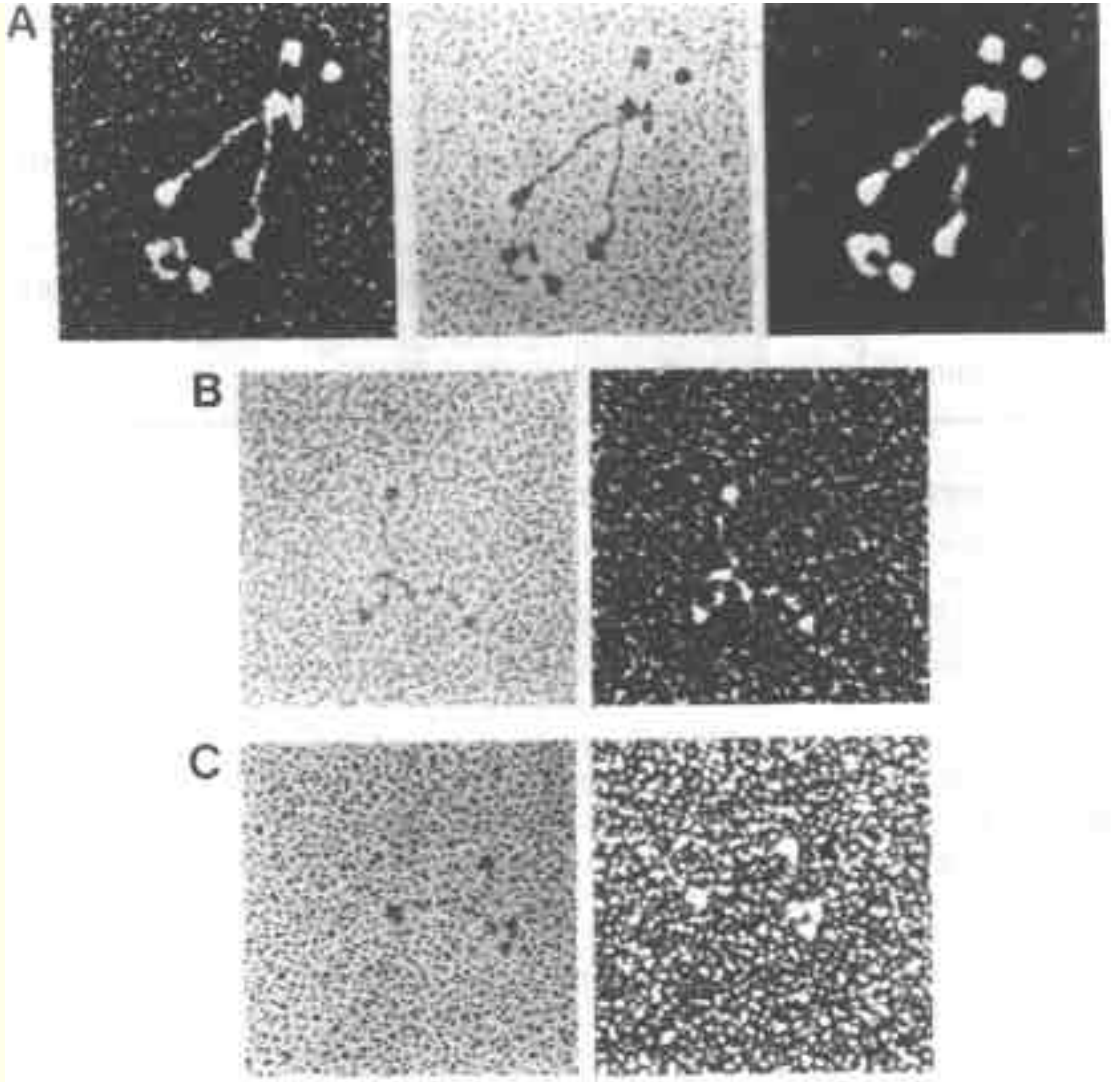



**Figure 9**. Extension of present visibility limits by the use of tungsten coating and darkfield electron microscopy.

**A**: Type VI collagen molecule prepared with $10^{-7}$ g/cm$^2$ tungsten, close to focus in brightfield (frame 2) vs. darkfield (frame 1). Frame 3 is the same area slightly under focus showing the critical dependency upon exact focus of the image in darkfield. Magnification 196 650×.

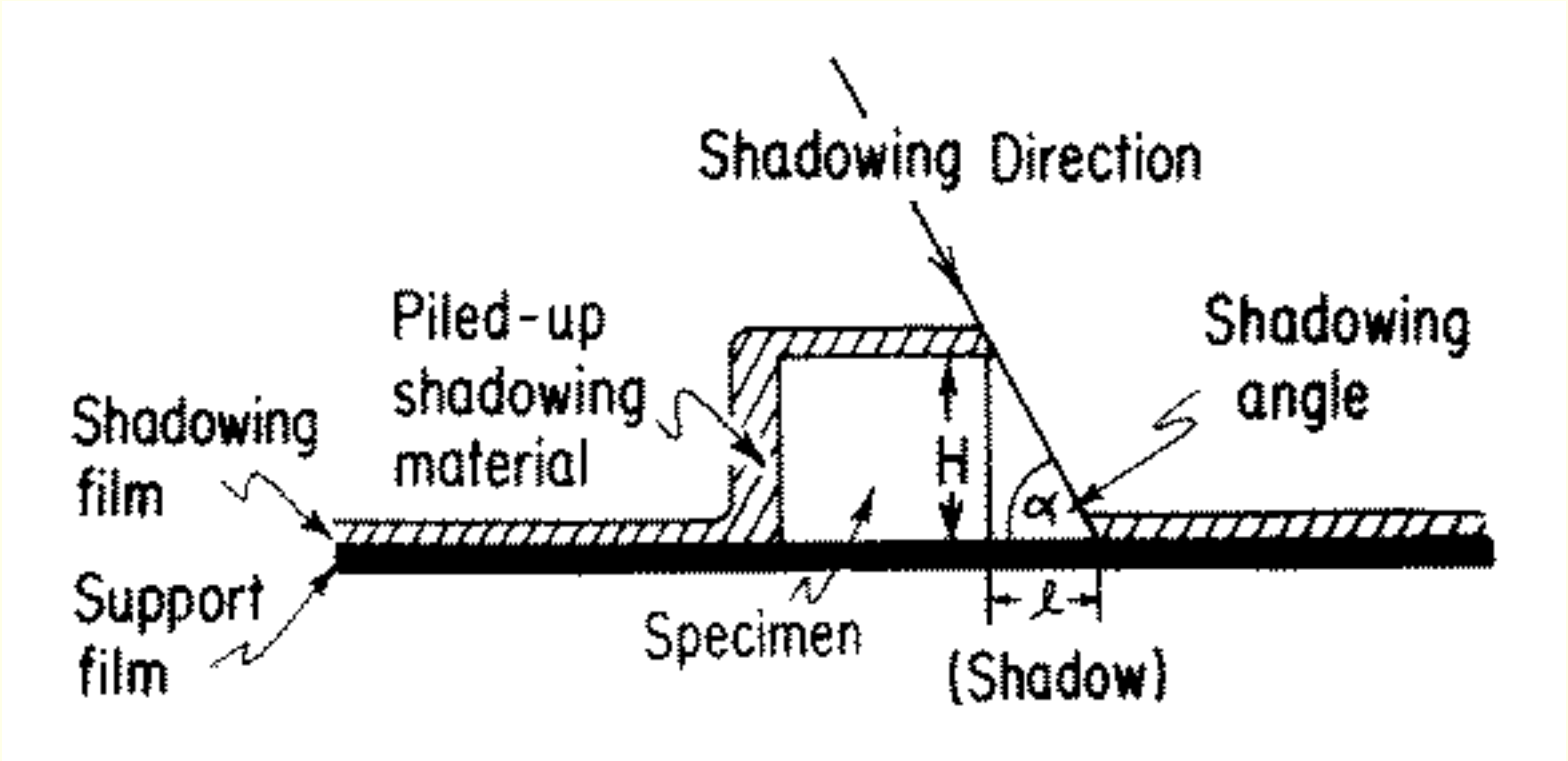
**B**: Thrombospondin molecule in brightfield (left) vs. darkfield (right) prepared by tungsten coating with $10^{-7}$ g/cm$^2$. Magnification 242 000×.

**C**: Thrombospondin molecule in brightfield (left) vs. darkfield (right) prepared by platinum coating with about $10^{-7}$ g/cm$^2$. Magnification 242 250×.

From H. S. Slayter

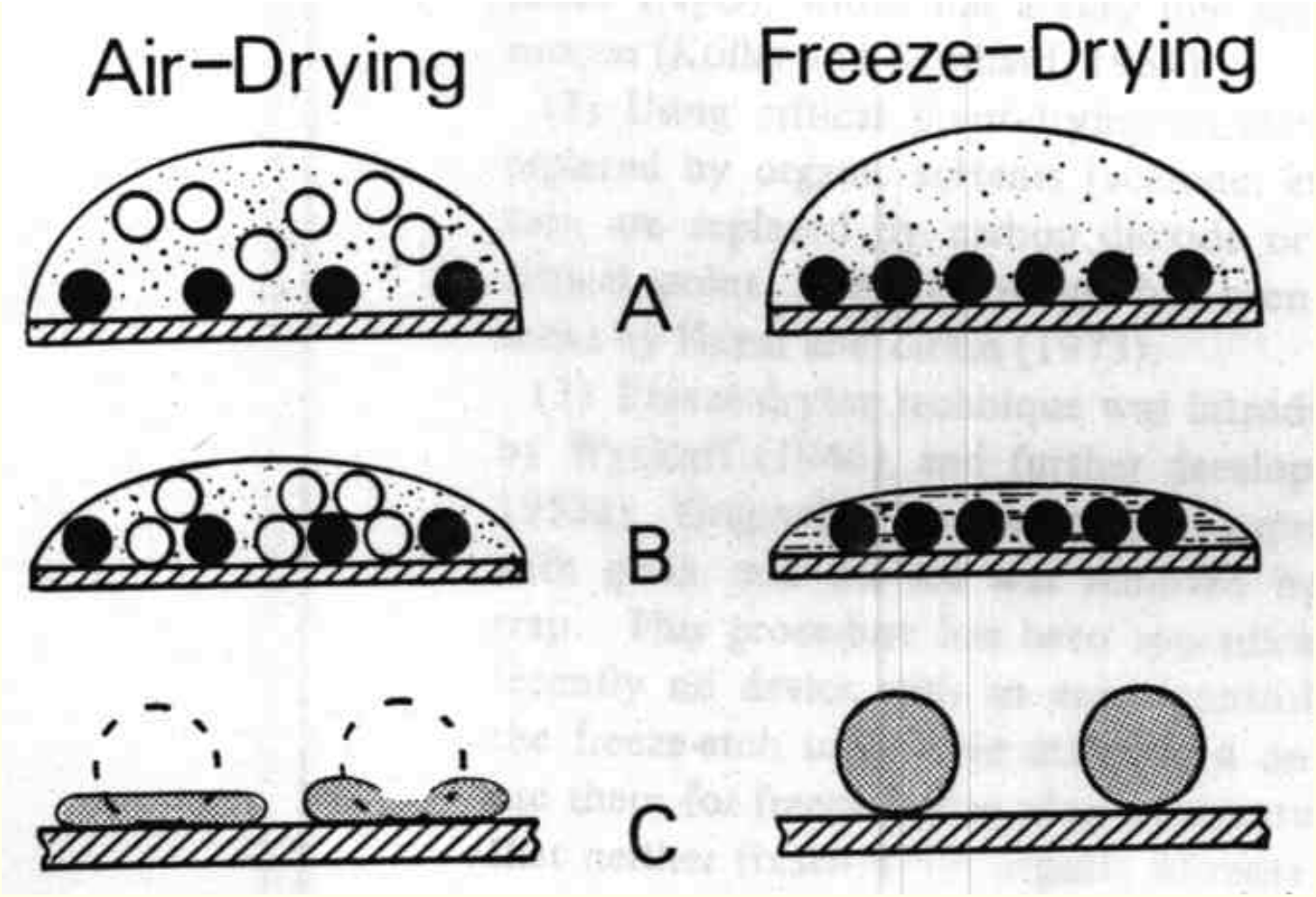
Rotary shadowed closed loop DNA molecule prepared according to Kleinschmidt. The DNA was spread on water in presence of cytochrome c, absorbed on a carbon film and rotary shadowed at angle of 8° with Pt-C

(from A. B. Maunsbach & B. A. Afzelius 17. Freeze fracturing and shadowing, p. 429)



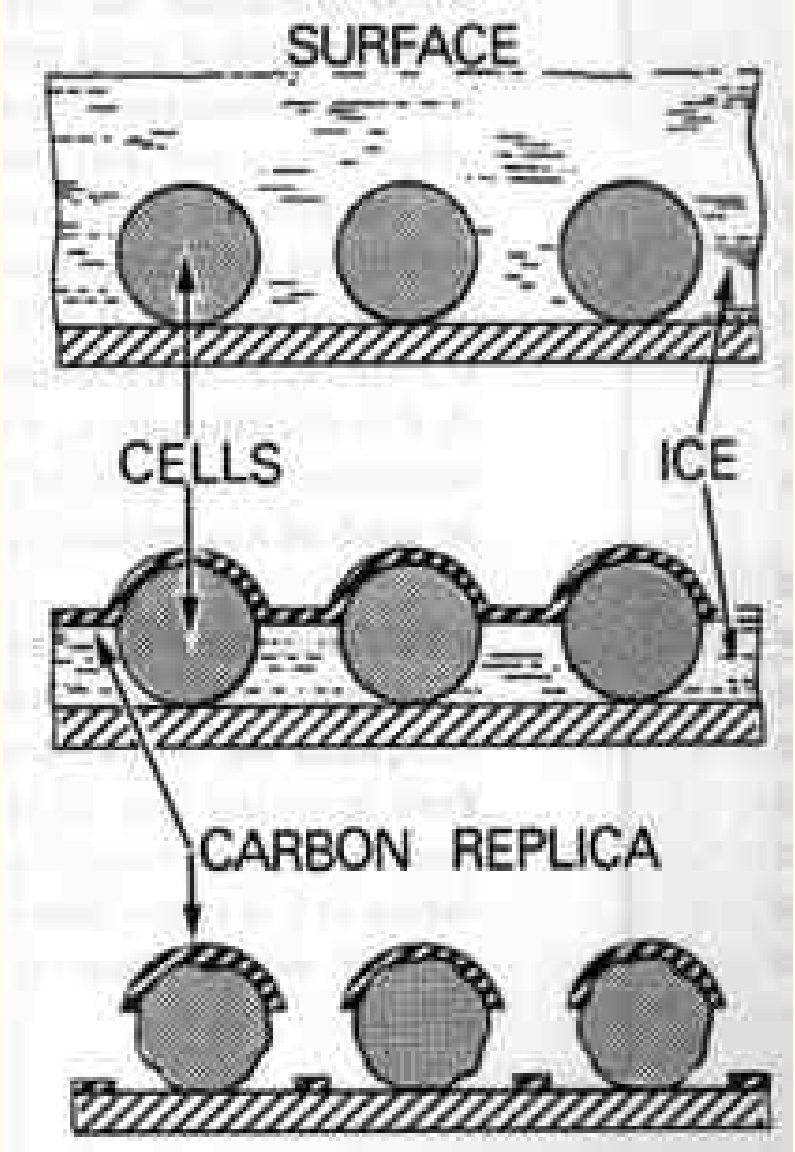

Wishnitzer 2nd edition p. 244

# Freeze-drying



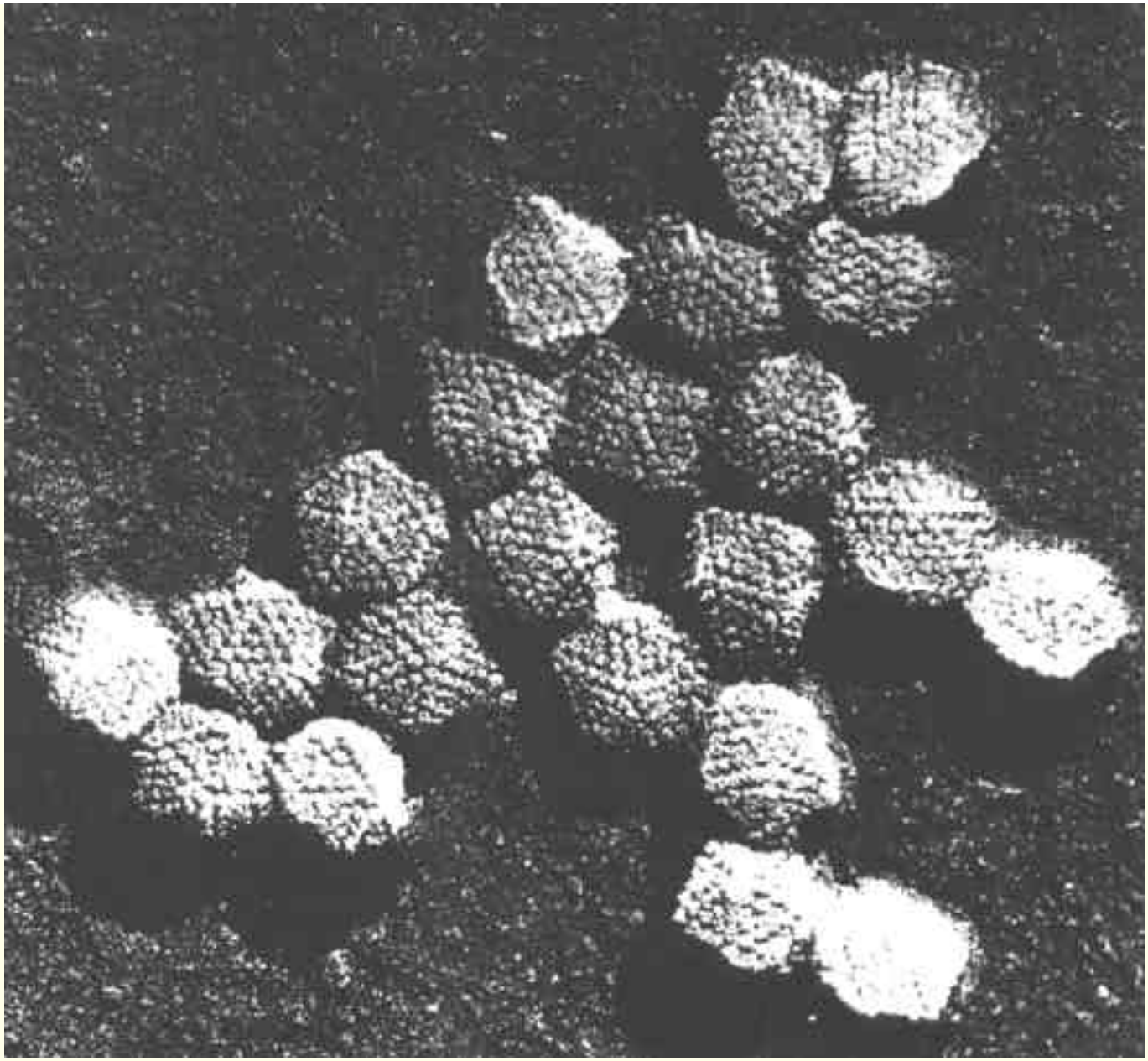

Both adsorbed and unadsorbed particles are present in the droplet (air-drying); only adsorbed particles in case of freeze-drying (A); Unadsorbed particles aggregate or overlap (air-drying) (B); Collapse or disruption of particles during air-drying caused by surface tension forces

From M. V. Nermut 1977, in Princ. Tech. Elec. Microsc. **7**,79




Freeze-drying of cells. Drying should be carried out for a reasonable time (1-2 hr) to uncover upper half of cells; otherwise the replica becomes discontinuous.

From M. V. Nermut

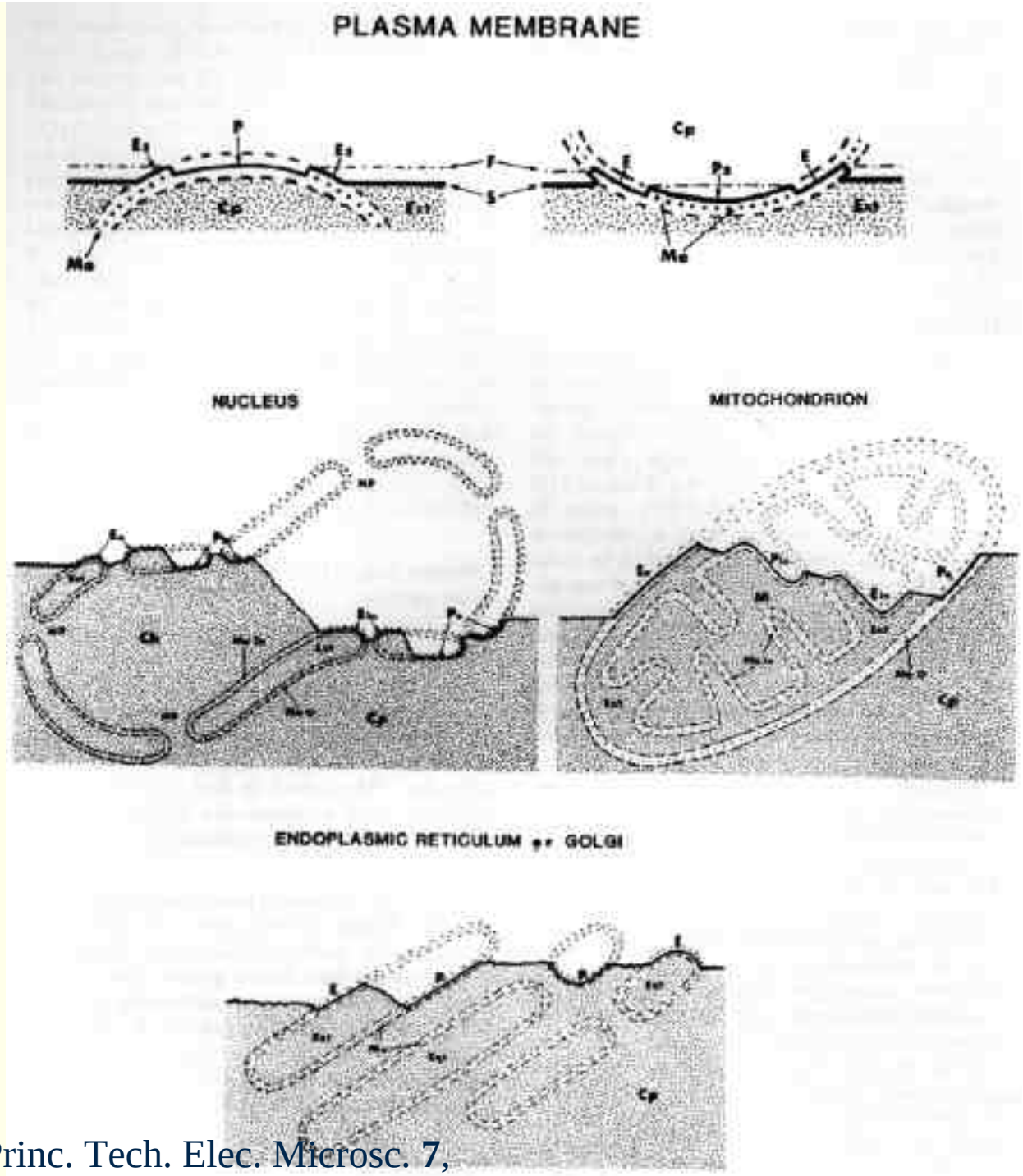


An avian adenovirus freeze-dried and shadowed with Pt-C

From M. V. Nermut

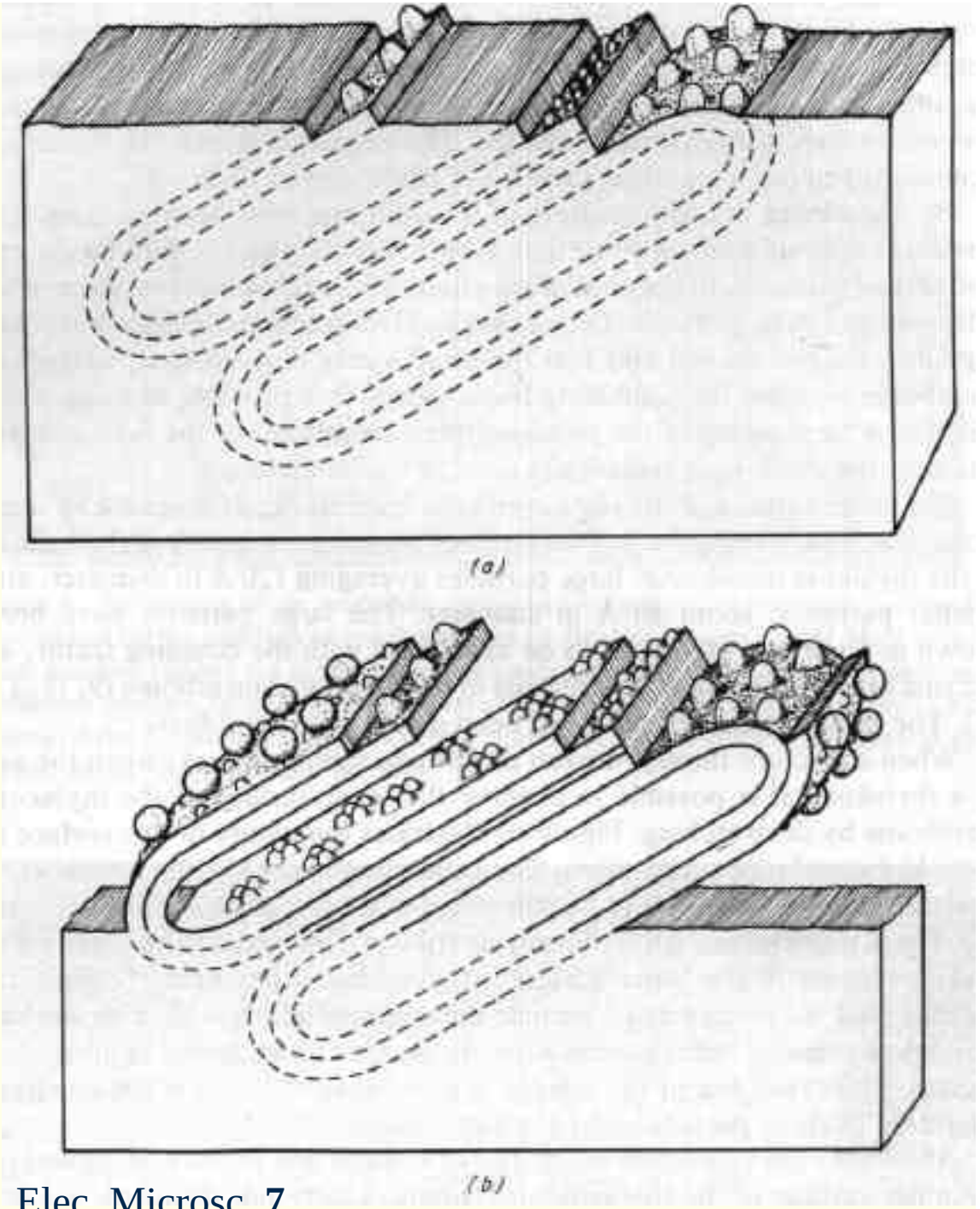
# Freeze-fracturing

Frozen samples fracture usually by membranes where present




From C. Stolinski 1977 , in Princ. Tech. Elec. Microsc. 7,

Fracturing (a) and etching of frozen thylakoid membranes. Deep etching (b) exposes the surfaces hidden before in ice.



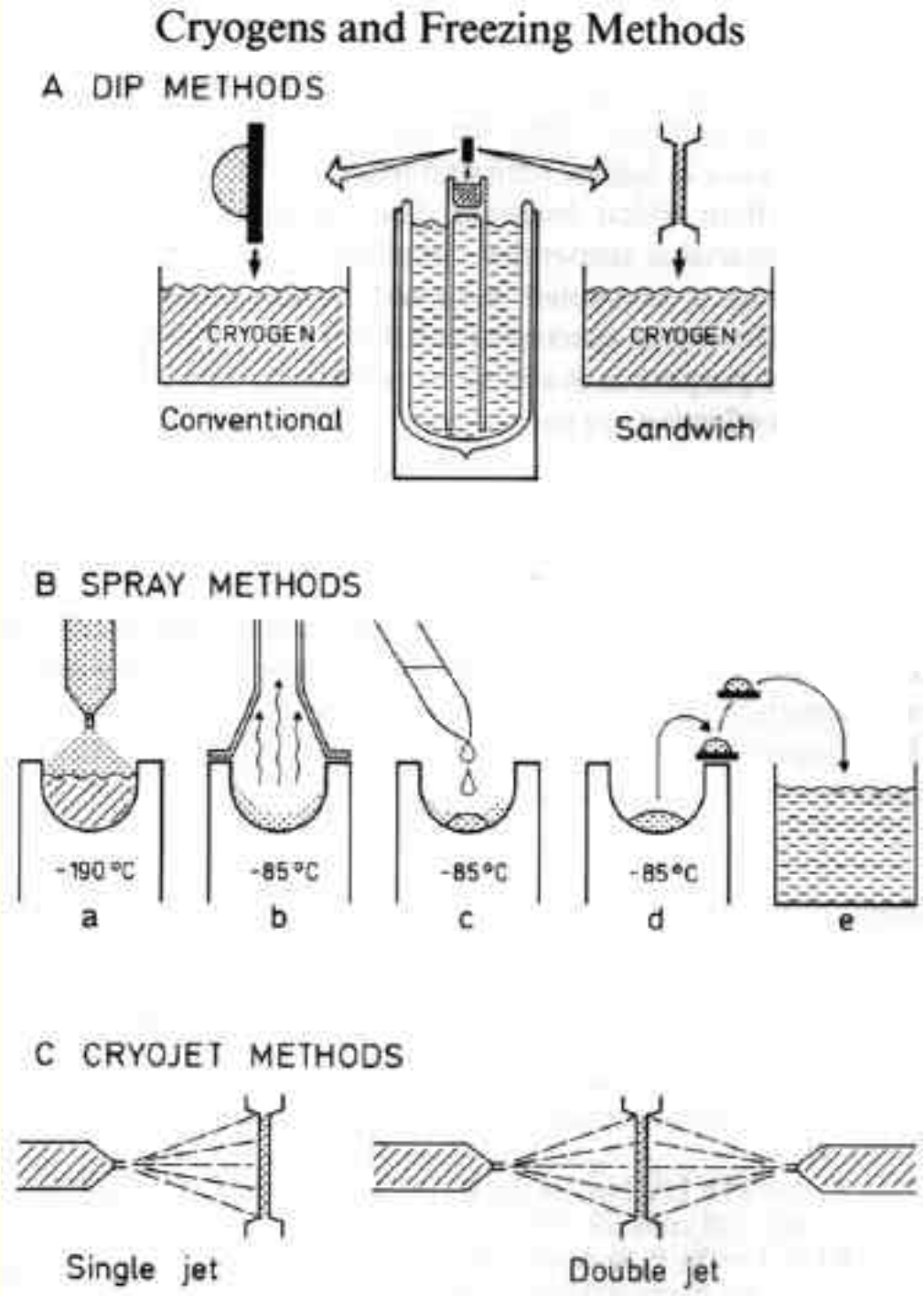

From K. R Miller 1977 , in Princ. Tech. Elec. Microsc. **7**,

Freezing methods and cryogens used in freeze-fracturing and etching

Cryoprotectants are used to prevent ice crystals formation

Cryogens used: propane, ethane, freon

From Robinson et al.



From S. Bullivant

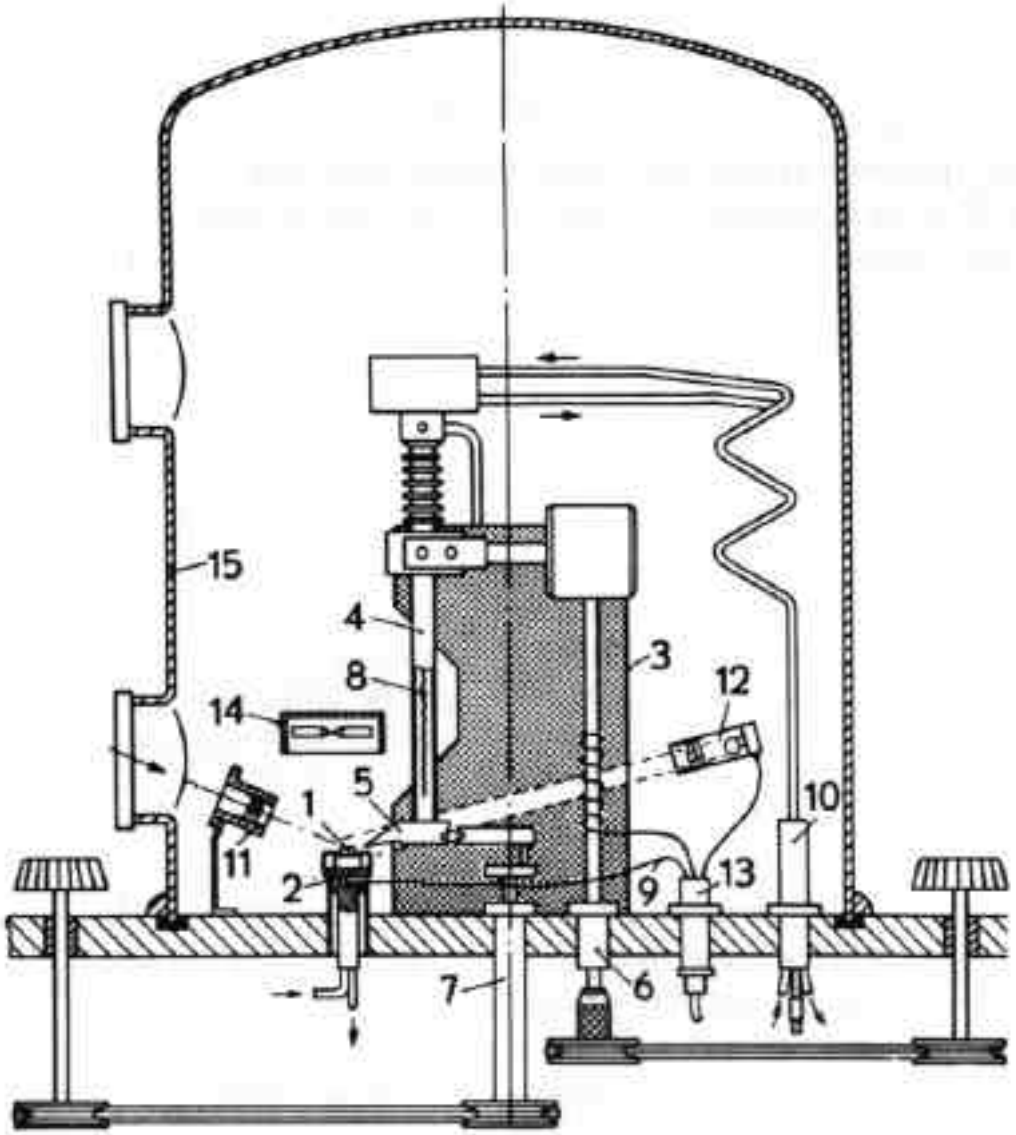
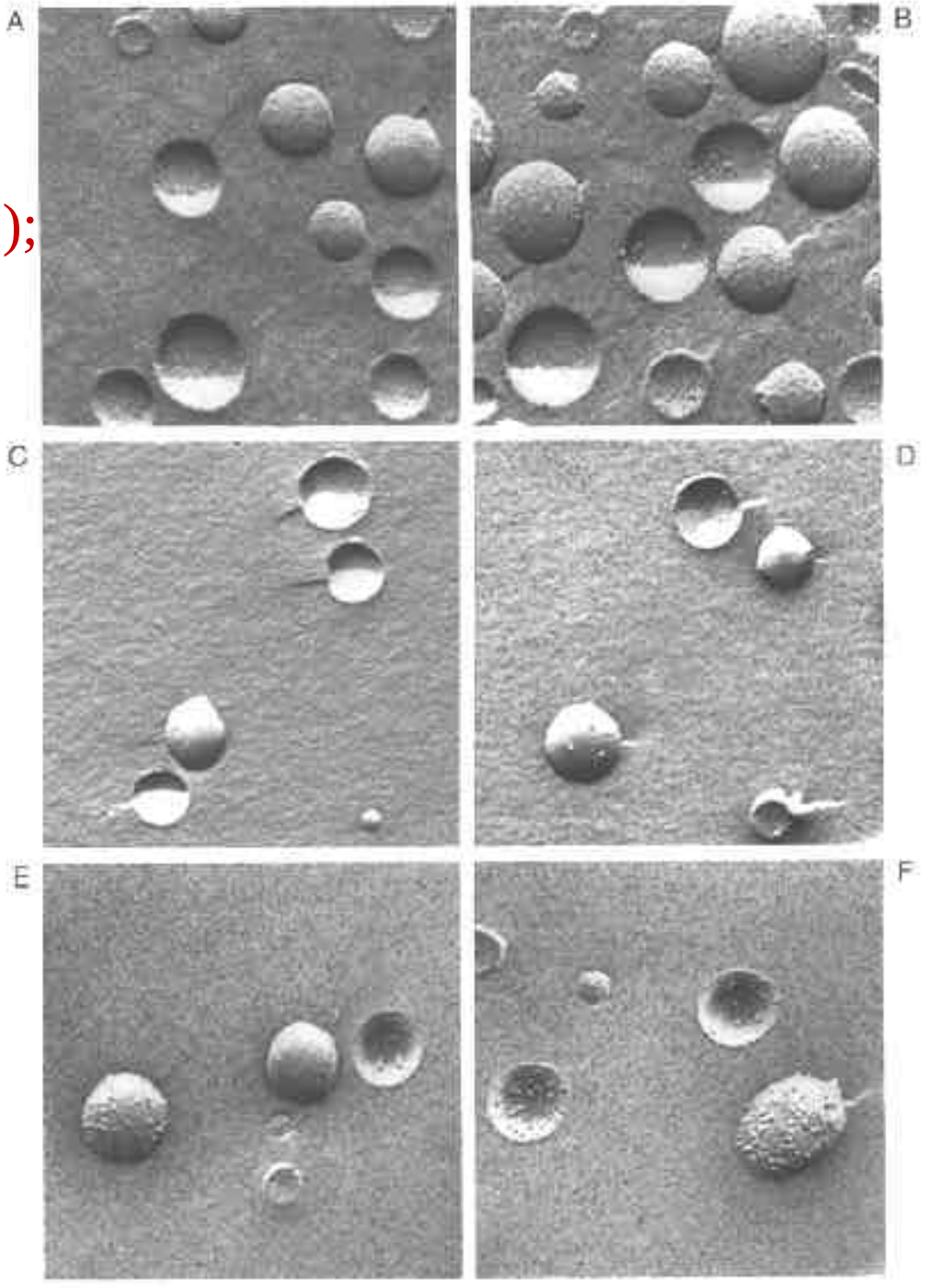



Fig. 5. Diagram of Moor-Balzers freeze-etch unit. 1: specimen, 2: specimen stage, 3: microtome stand, 4: microtome arm, 5: knife, 6: advance mechanism, 7: microtome drive, 8: liquid nitrogen in arm, 9: thermo-couple lead, 10: liquid nitrogen supply, 11: lens, 12: lamp, 13: electrical feed-through, 14: carbon electrodes (platinum-carbon not shown), 15: metal bell jar. The diagram is taken from Moor (1965) and is by courtesy of Dr. H. Moor and Balzers AG

- Pure liposomes (A);
- Liposomes with Na,K-ATPase (B);
- Liposomes with increasing concentration of aquaporin (D-F)

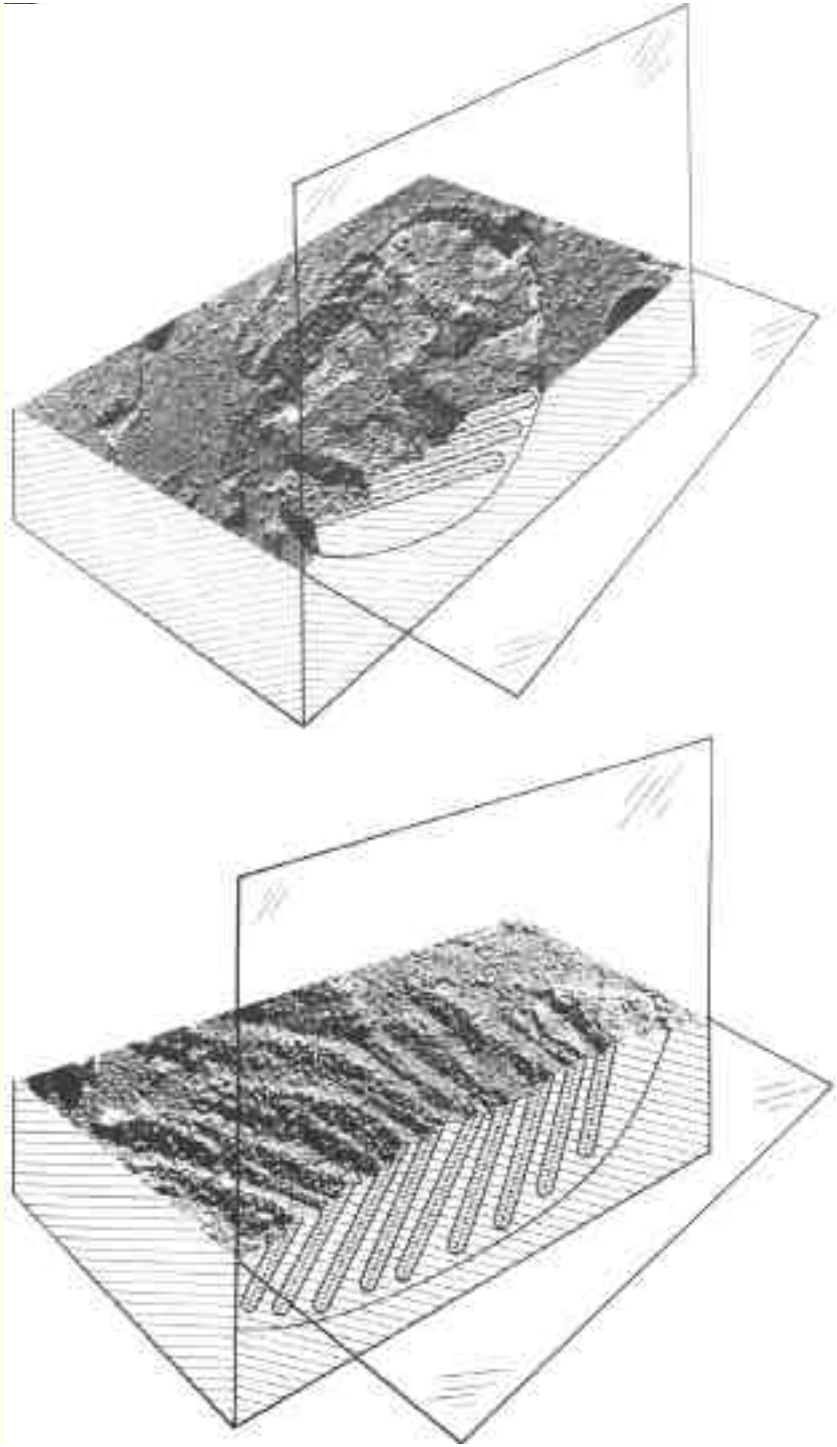
(from A. B. Maunsbach & B. A. Afzelius in: 17. Freeze fracturing and shadowing, p. 447)



A cartoon showing the topography of a mitochondrion fracture face as it would appear in profile view in a vertical cut through the fracture plane.

The cristae drawn in vertical planes and arranged to fit as close as possible the way they appear in the fracture face

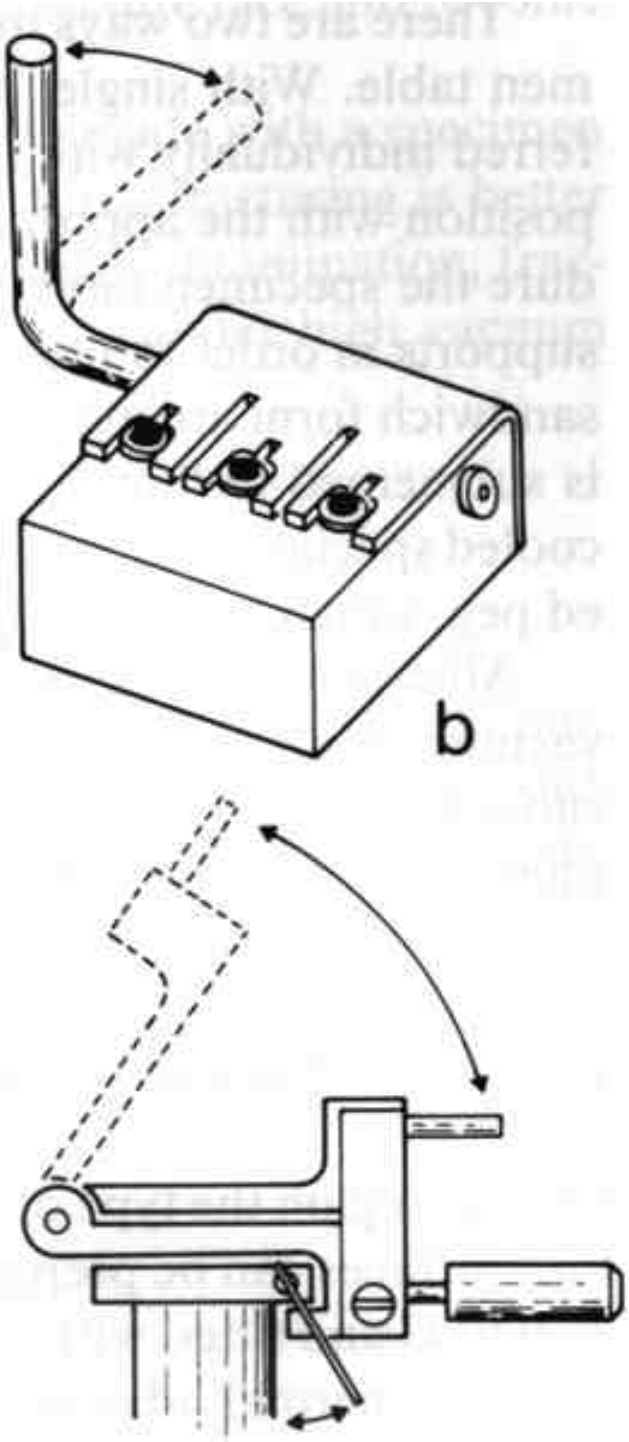
From F. S. Sj_strand



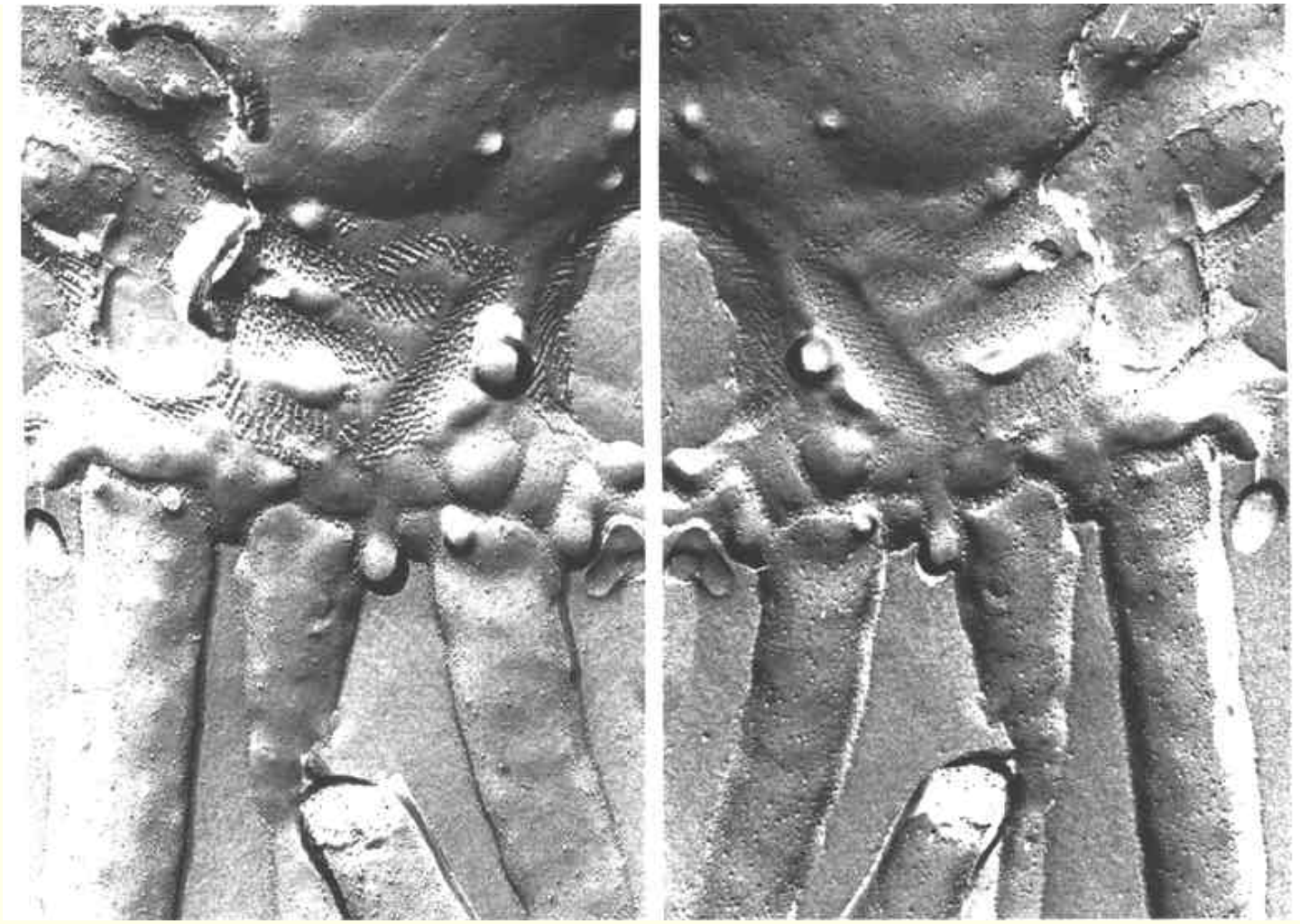
# Freeze-fracturing devices:

**b** – multi-disc table;

**c** - complementary replica device




From Robinson et al.



Complementary freeze-fracture replicas of an apyrene snail spermatozoon

(from A. B. Maunsbach & B. A. Afzelius)

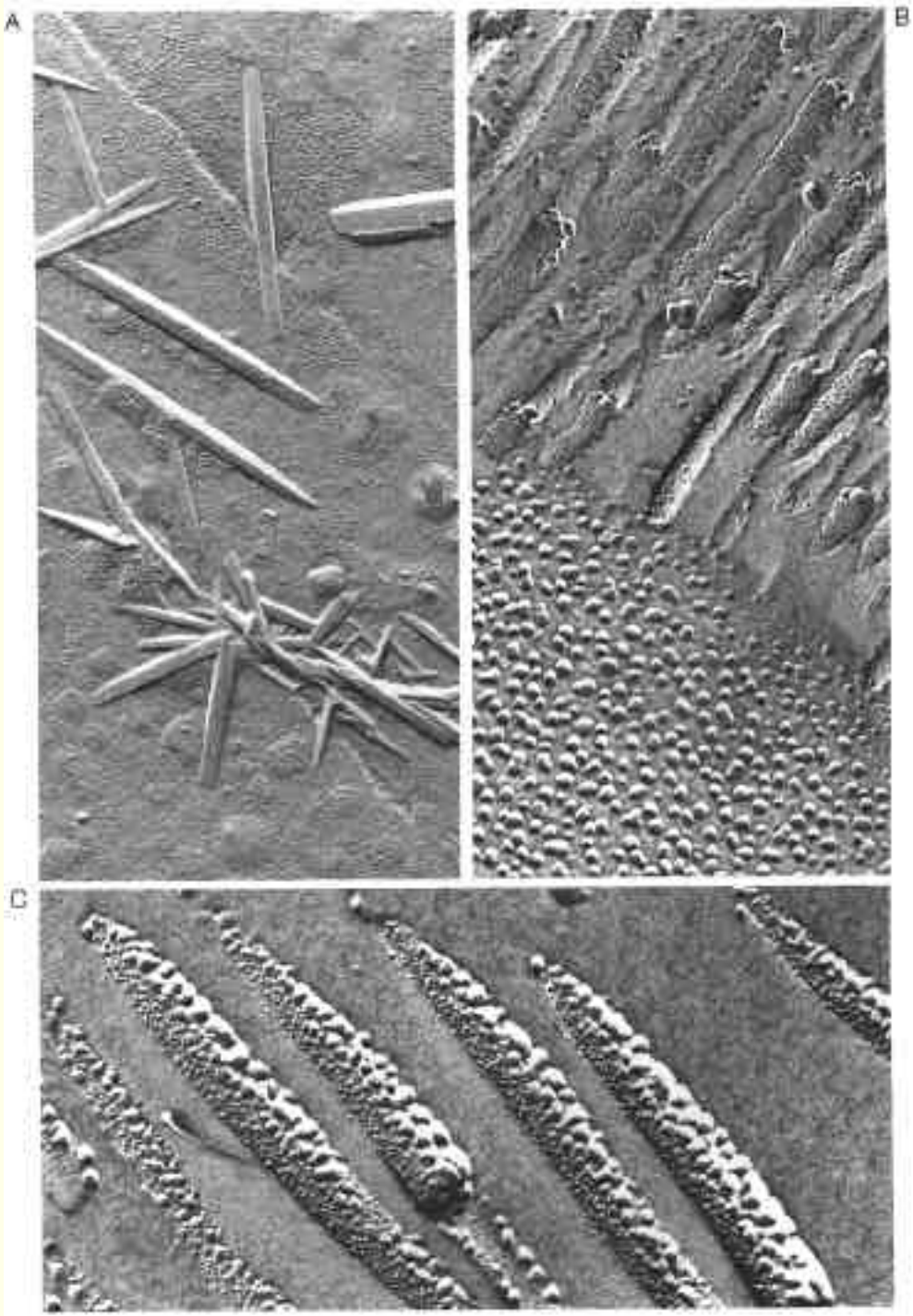
# Contamination artefacts:

**A**.: Freeze-fracture replica of pure water droplet. Crystals should not be there. Evaporation of Pt-C was initiated a few seconds after the last cut with the knife;

**B**.: Microvilli freeze-fractured at -100 °C; evaporation started 1 min later;

**C**.: A higher magnification of a similar to **B**. specimen. Small and big particulate elevations are seen.

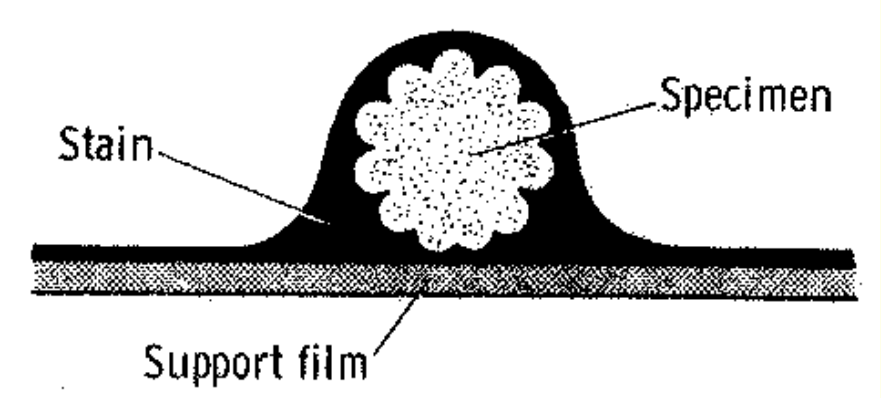
(from A. B. Maunsbach & B. A. Afzelius)



# Negative staining

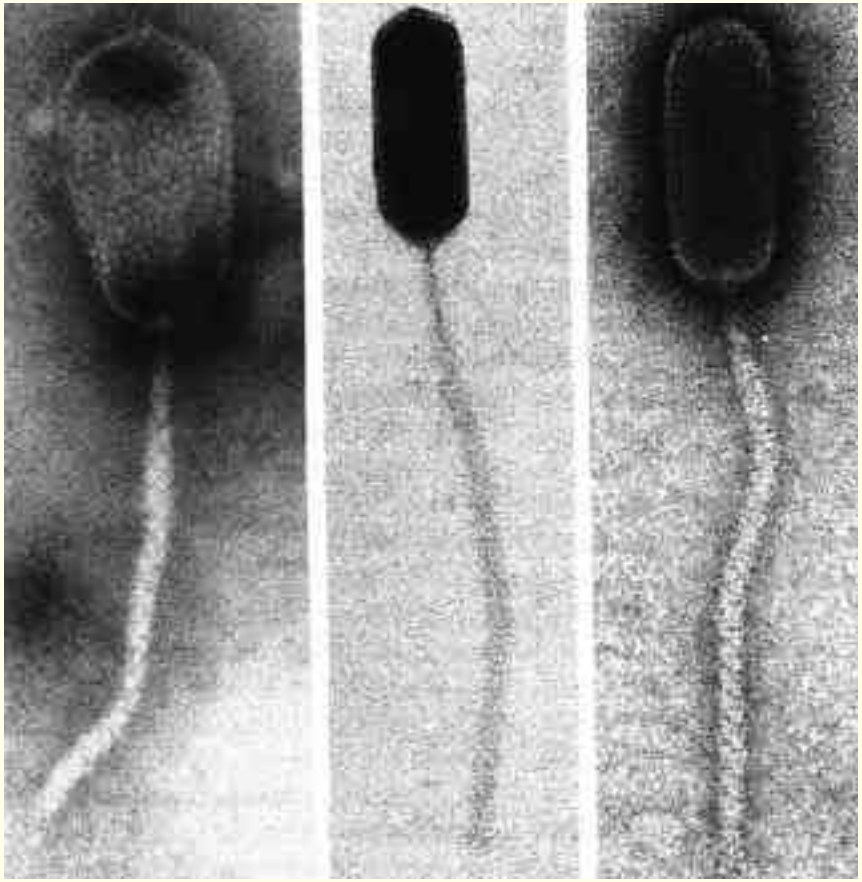
# Purpose of Negative Staining

- Embed the sample in a layer of heavy metal salt
  - Contrast arises from “cavities” in the embedding matrix (specimen “casting”).
  - Supposed to be chemically inert
  - Penetrates hydrophilic areas and replaces water
- Increases scattering contrast
  - “Negative Staining” - the sample appears light on a dark background
- Preserves the structure from dehydration and radiation damage



# Positive Staining

- Stain molecules react to ions on the surface of the sample
- Some positive staining always occurs
  - pH of uranyl acetate (UA, pH4.5) close to isoelectric point of positively charge proteins (pI5.0)
  - UA has a strong affinity for carboxyl and phosphate groups in nucleic acids, lipids and some proteins
- Positive staining may be more apparent when the embedding layer of stain is improperly washed away



Phage 2037/1

| Phosphotungstic acid<br>Negative staining | Uranyl acetate<br>Positive staining | Uranyl acetate<br>Negative staining |
|---|---|---|

# Negative stains frequently used

| Negative Stain | Chemical Formula | pH for use |
|---|---|---|
| Uranyl acetate | $UO_2[CH_3COO]_2$ | 2 – 4.5 |
| Sodium phosphotungstate | $Na_3PO_412WO_3$ | 5 - 8 |
| Sodium silicotungstate | $Na_3SiO_212WO_3$ | 5 - 8 |
| Ammonium molybdate | $[NH_4]_6Mo_7O_{26}$ | 5 - 8 |

From R. Harris & R. Horn

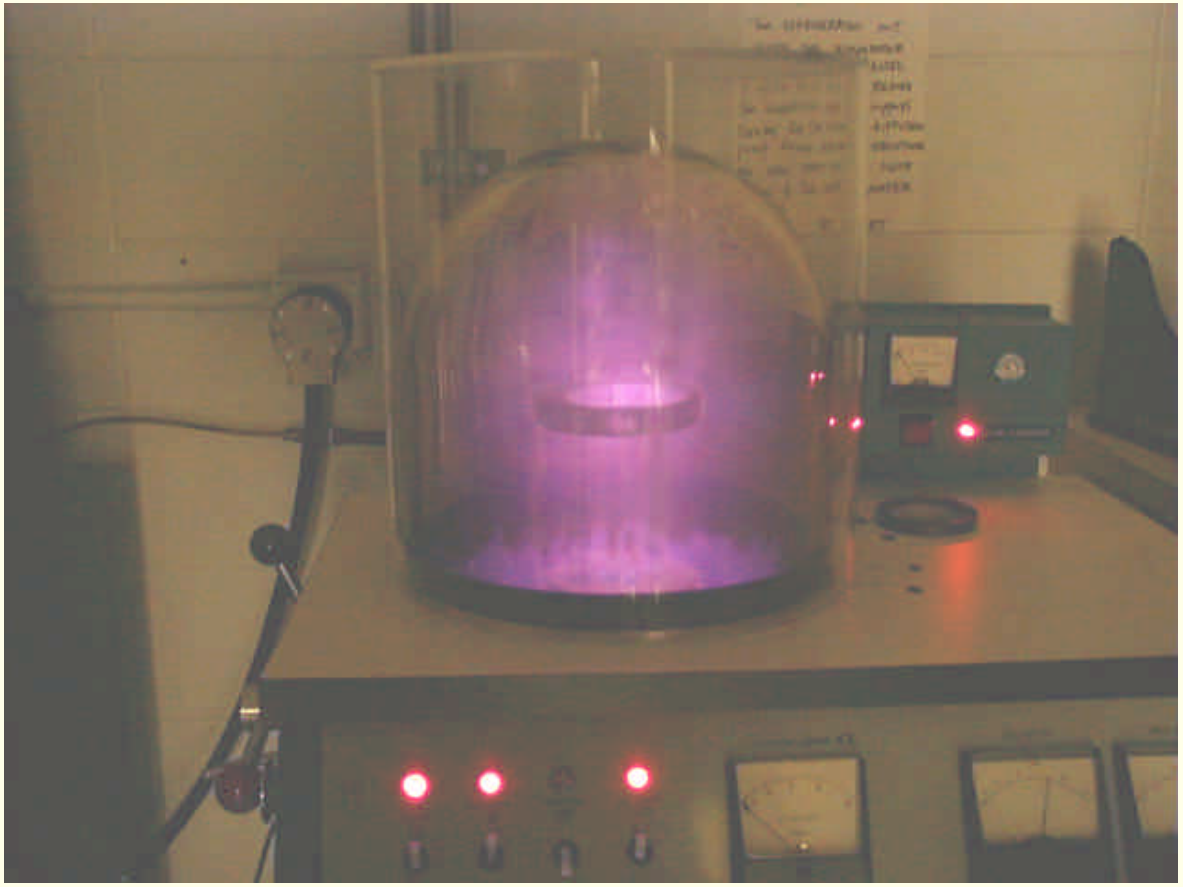
# Choice of Stain

- **Try, try again**

- Choice of anionic or cationic stain and pH
  - UA (cationic) – pos. charged at low pH, proteins also pos. charged
  - PTA (anionic) – neg. charged at neutral pH which is above pI of most proteins which are also neg. charged
  - Stains soluble at different pH's (UA pH 4.5, PTA in buffer at pH 7.0)
- Osmolarity of the stain and effect on sample
- Granularity of stain (UA 0.4-0.5nm PTA 0.8-1.5nm)
- Are samples disrupted in stain
  - Rotavirus sometimes disrupted in PTA, not in UA
  - Will the stain handle high concentrations of buffer salts (PTA yes, UA no)
- How stable is the stain in the electron beam
  - Stain recrystallization and redistribution when hit by beam
  - Aurothioglucose preserves sample with less flattening but extremely beam sensitive and bubbles when hit by beam

# Negative Staining Procedure

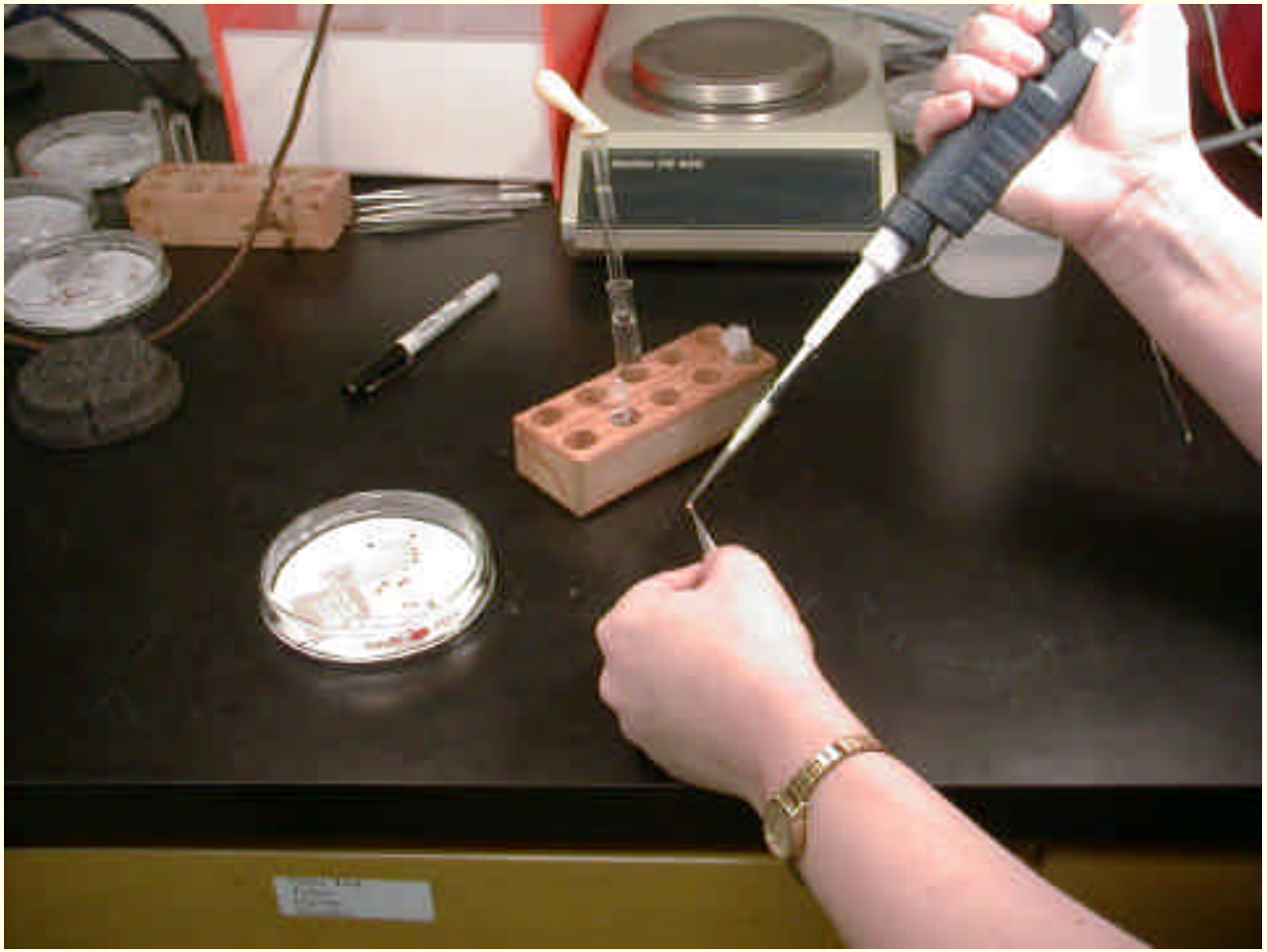
## Glow Discharging the Carbon Support Film



High voltage glow discharge in a reduced atmosphere
makes carbon films hydrophilic and negatively charged

# Negative Staining Procedure

## Applying Sample



# Negative Staining Procedure

## Washing with $dH_2O$

# Negative Staining Procedure

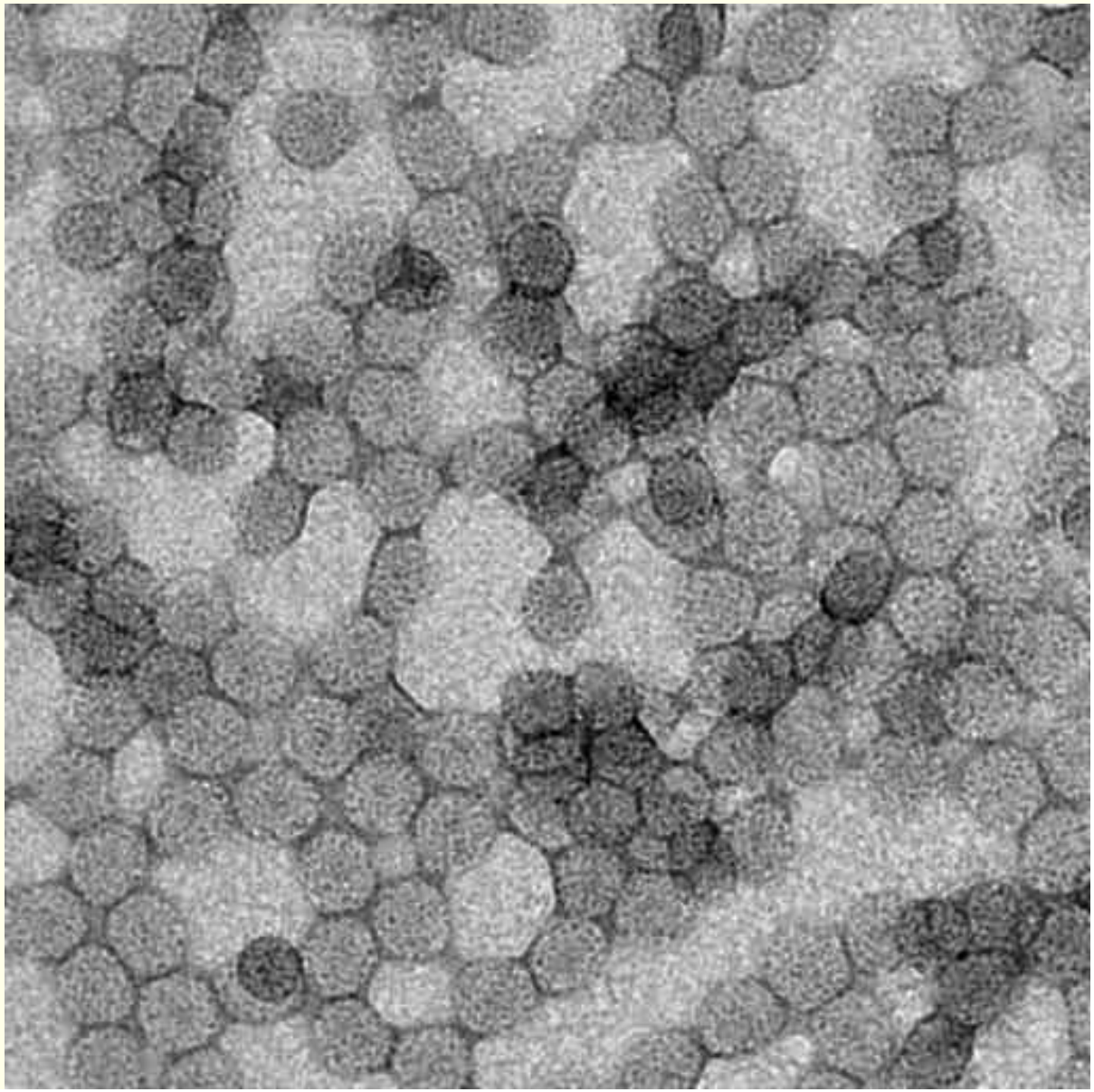
## Apply Stain and Blot with Filter Paper

# Some Problems of Negative Staining

- Unpredictability and uneven staining
- Sample flattening
- Sample collapse
- Beam-induced stain redistribution
- Cannot be used for quantitative determination of sample concentration

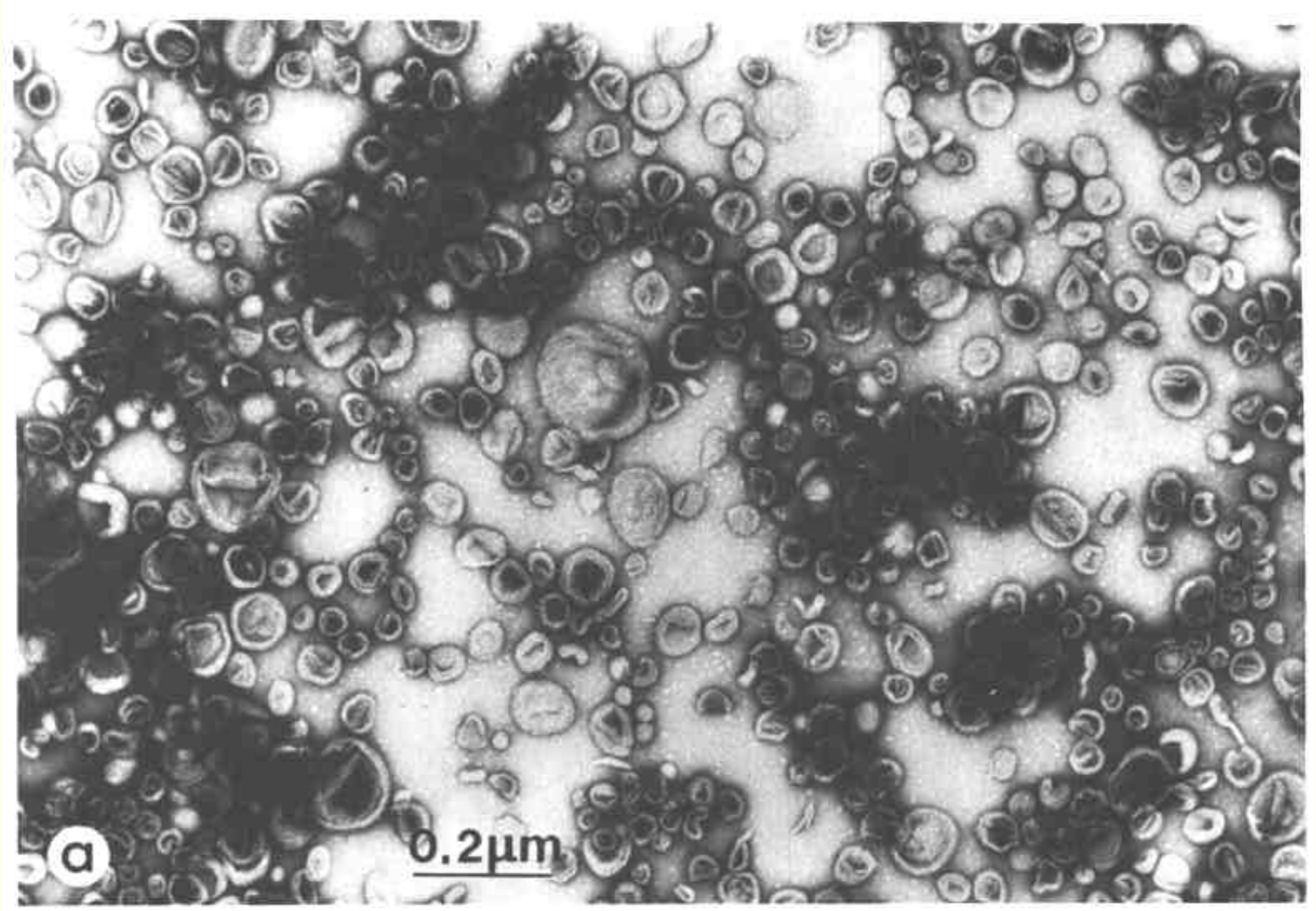
# Negative staining problems

Cowpea chlorotic mottle virus



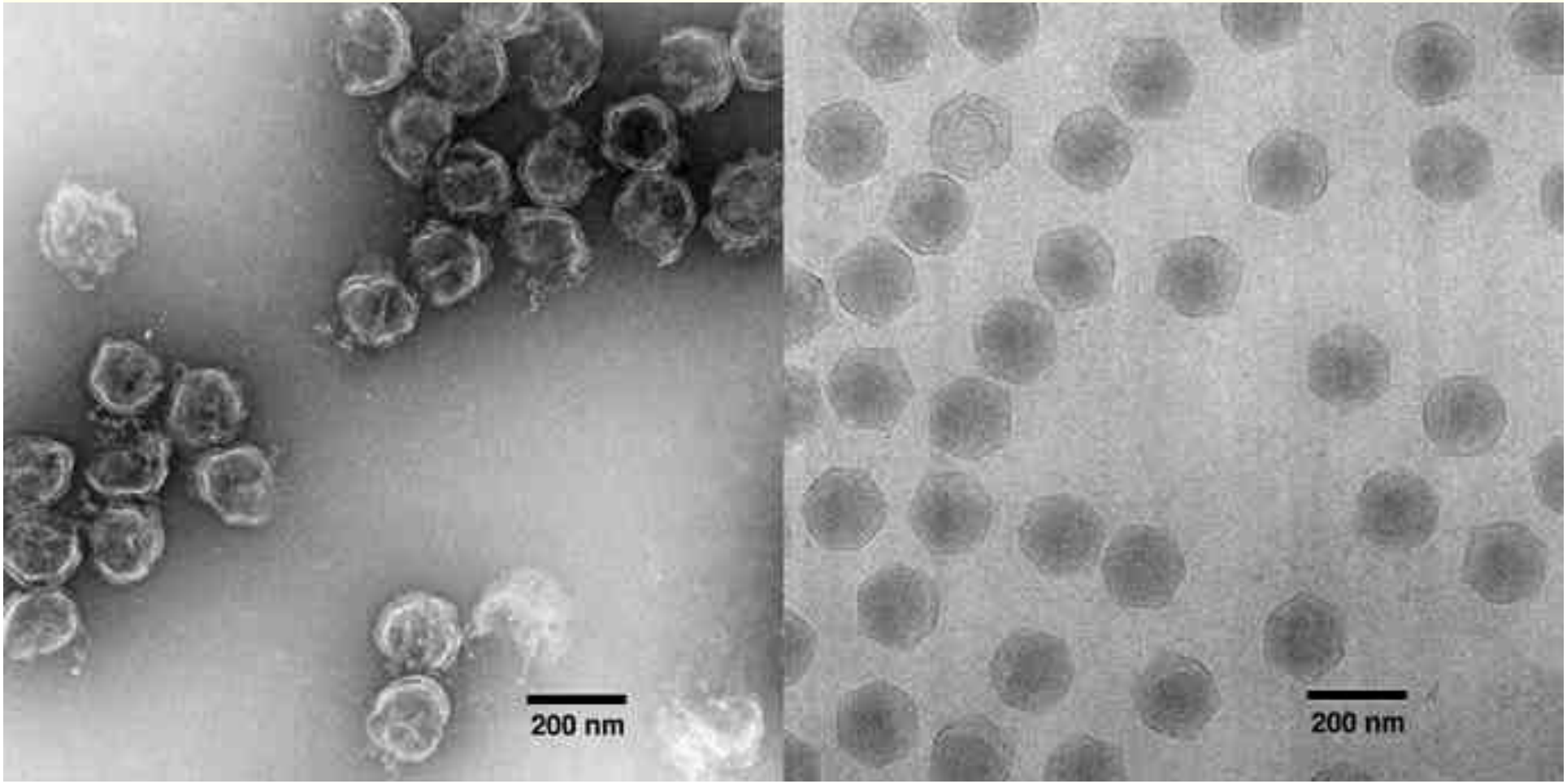
# Negative staining problems

## Sample collapse



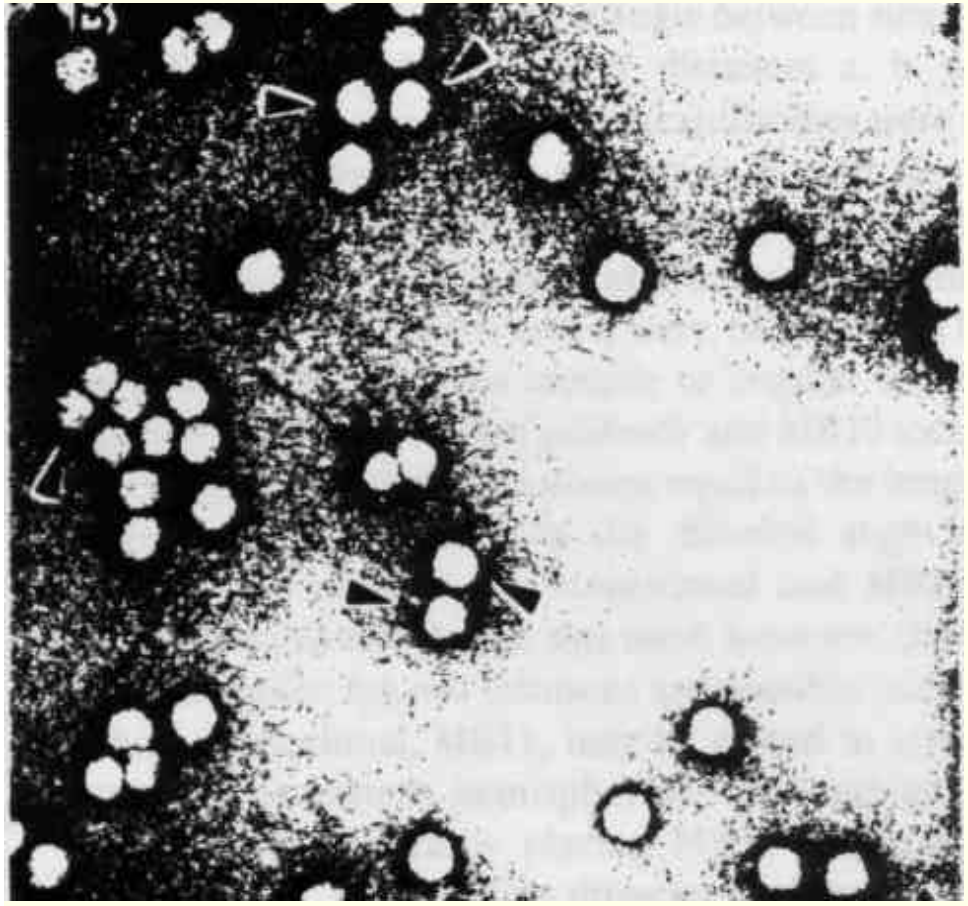
# Negative staining problems

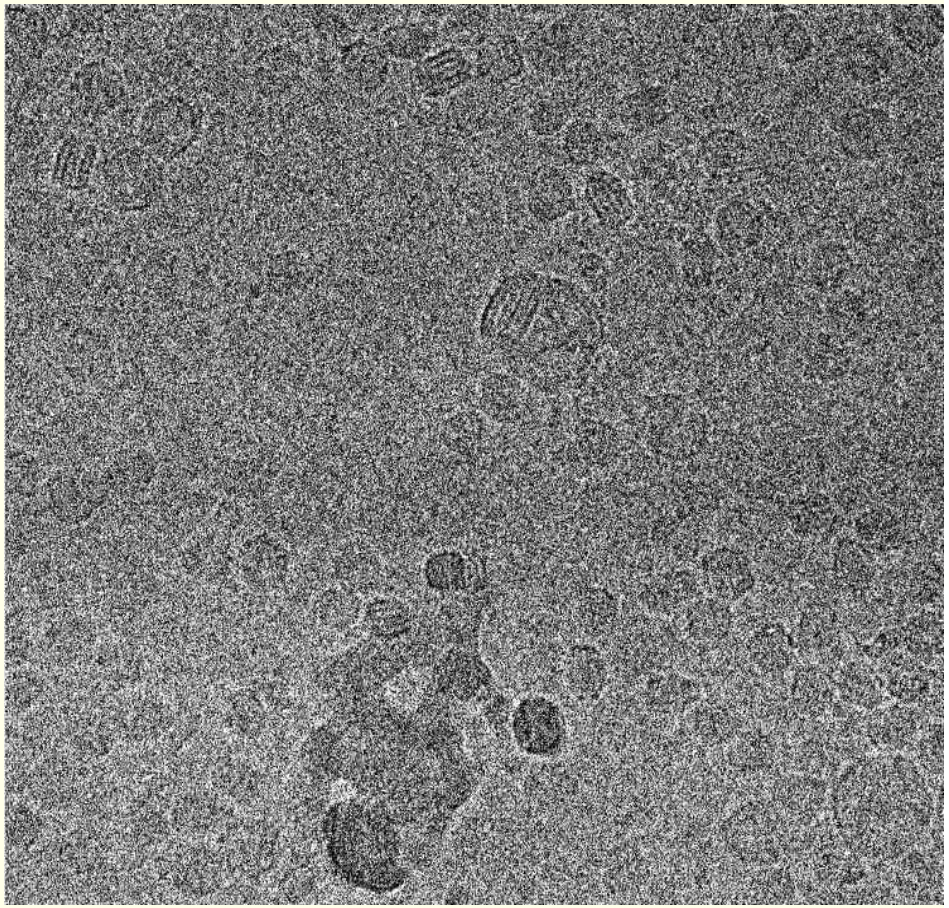
UA-stained *Parmecium bursaria Chlorella* virus-1 (left)
Frozen-hydrated (right)



# Low Density Lipoproteins

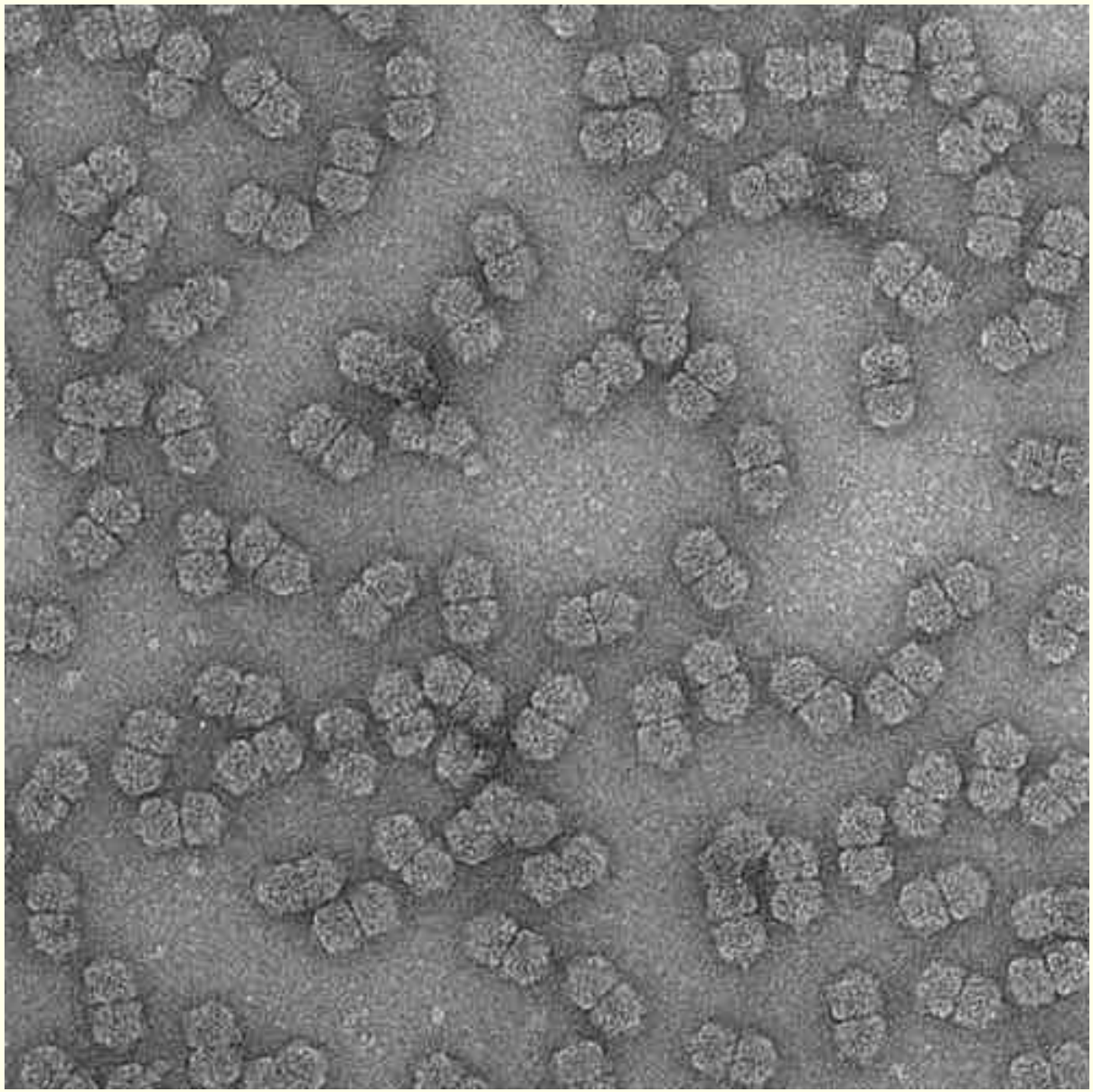
Negative stain does not penetrate inside. Result: surface staining





Negative staining (UA)

Frozen-hydrated



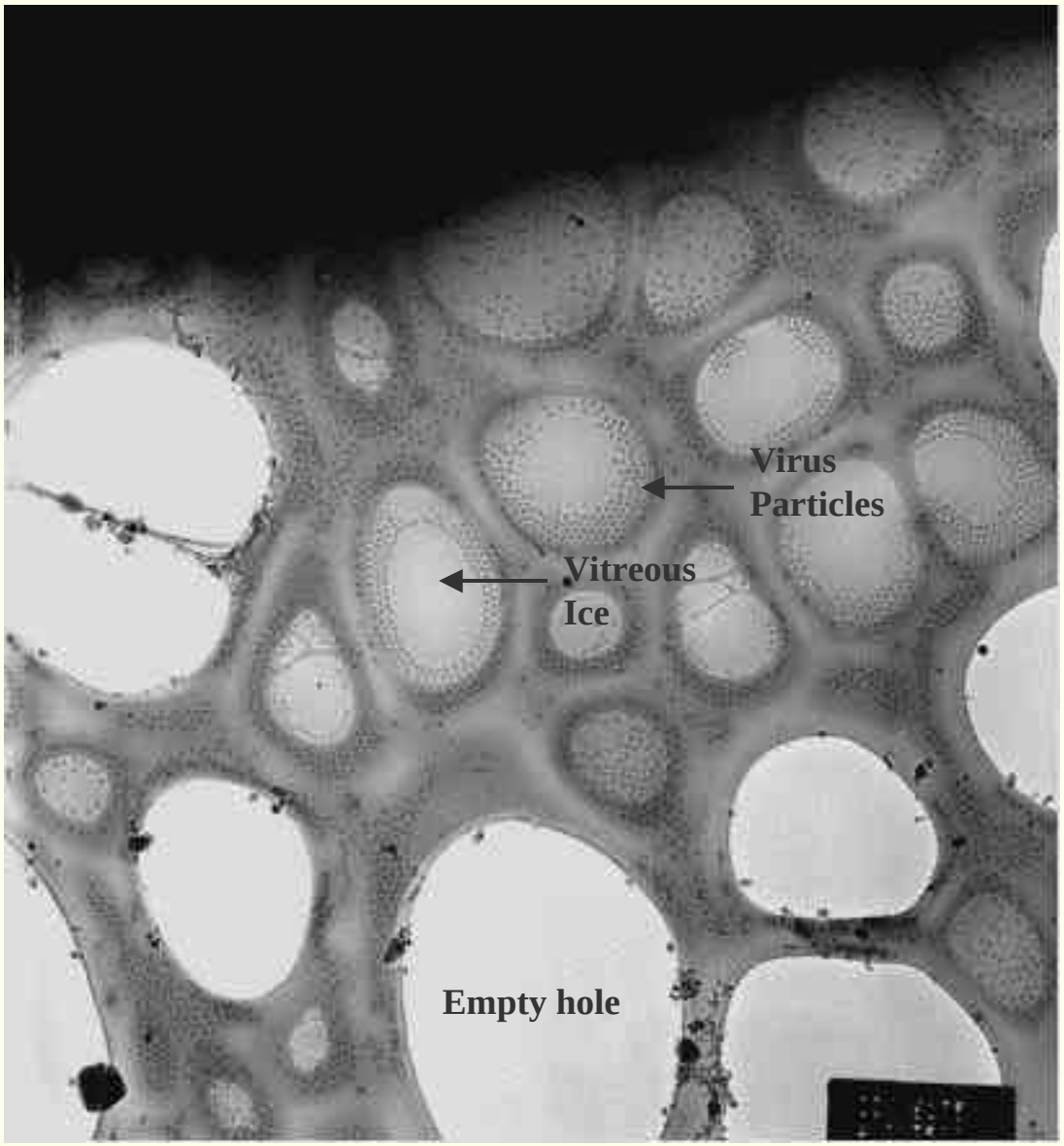
Negative Staining
Maize Streak Virus

- **Cryo TEM**
- **Labeling using molecular probes**
- **Time-resolved cryo TEM**

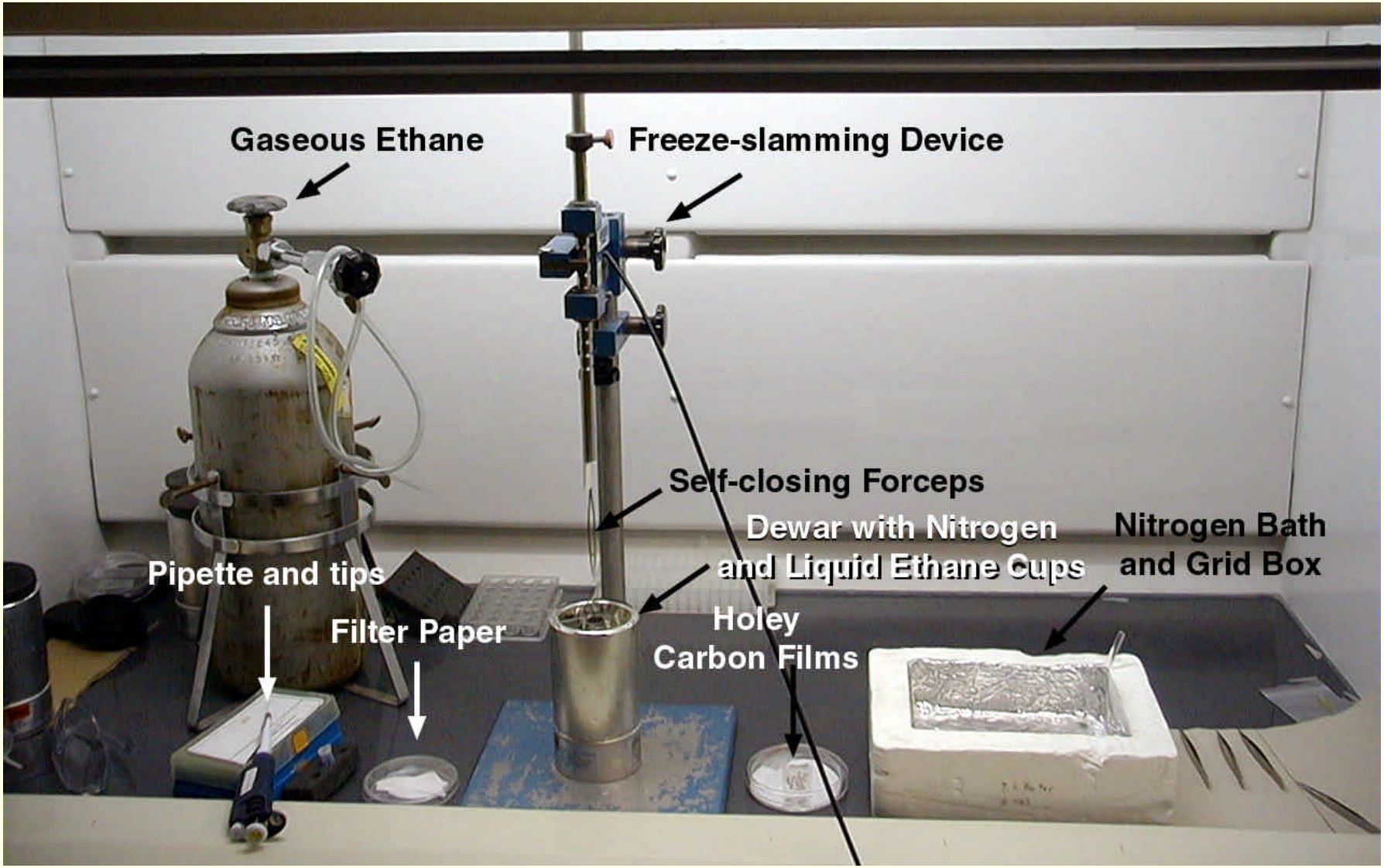
# Cryo-EM

- ✓ Embedding unstained macromolecules in vitreous (noncrystalline) water
- ✓ Sample density appears with positive contrast
- ✓ Contrast primarily the result of phase or interference contrast
- ✓ Preserves the native structure of the sample without flattening or drying

# Sample is Vitrified (“frozen”) Over Holes in a Carbon Support Film

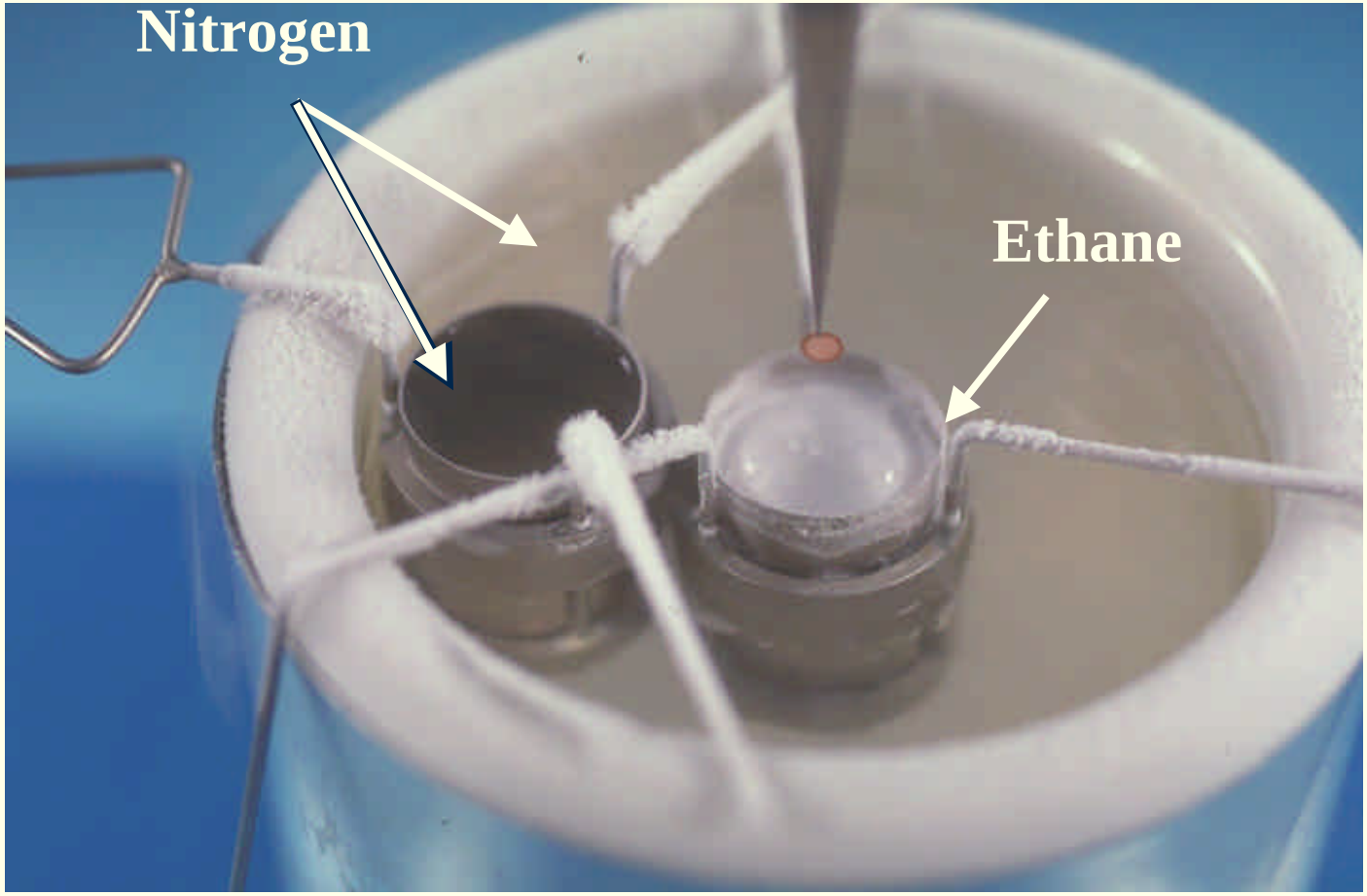


# Sample Preparation Equipment

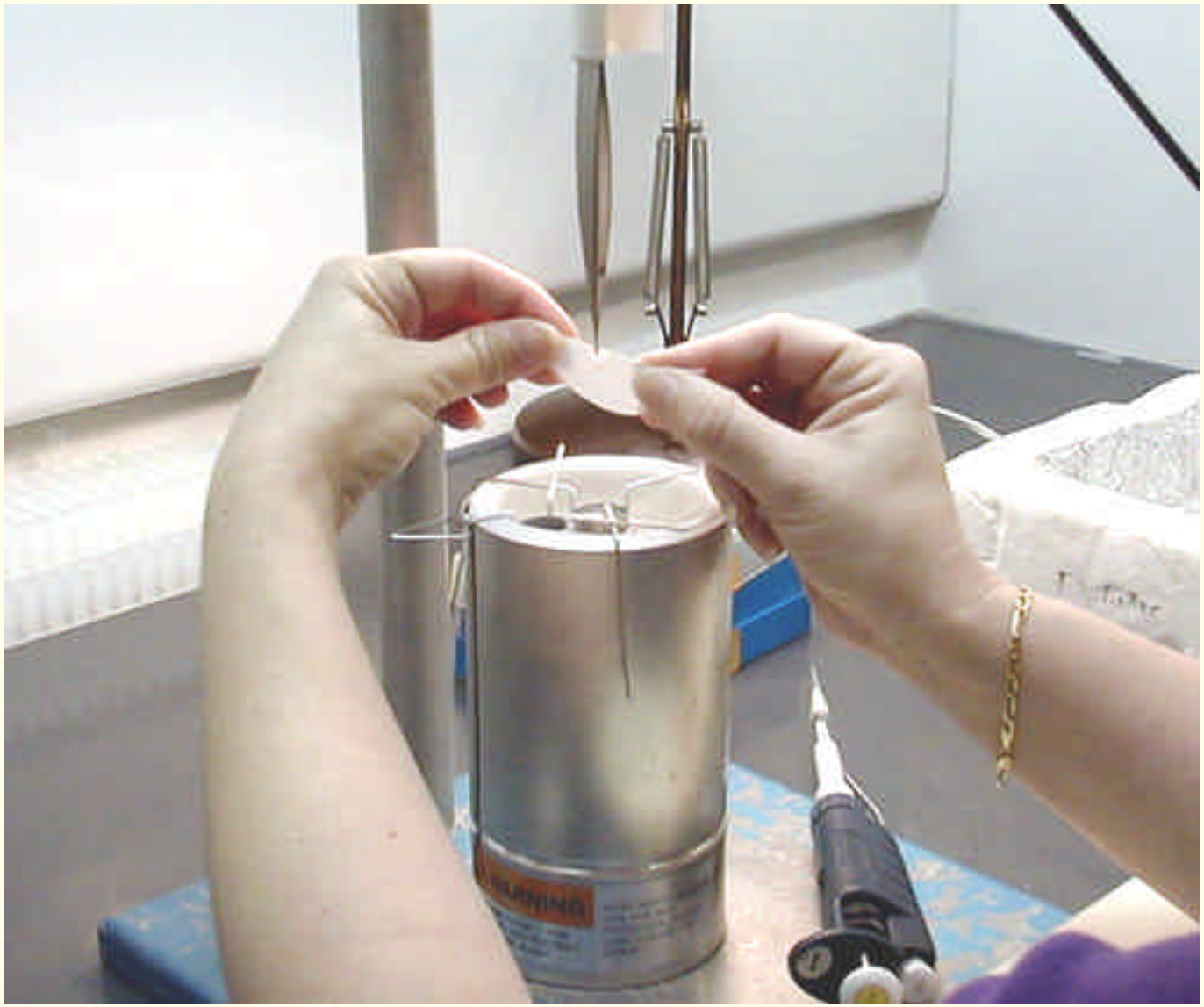


# Dewar with Liquid Nitrogen and Ethane Cups

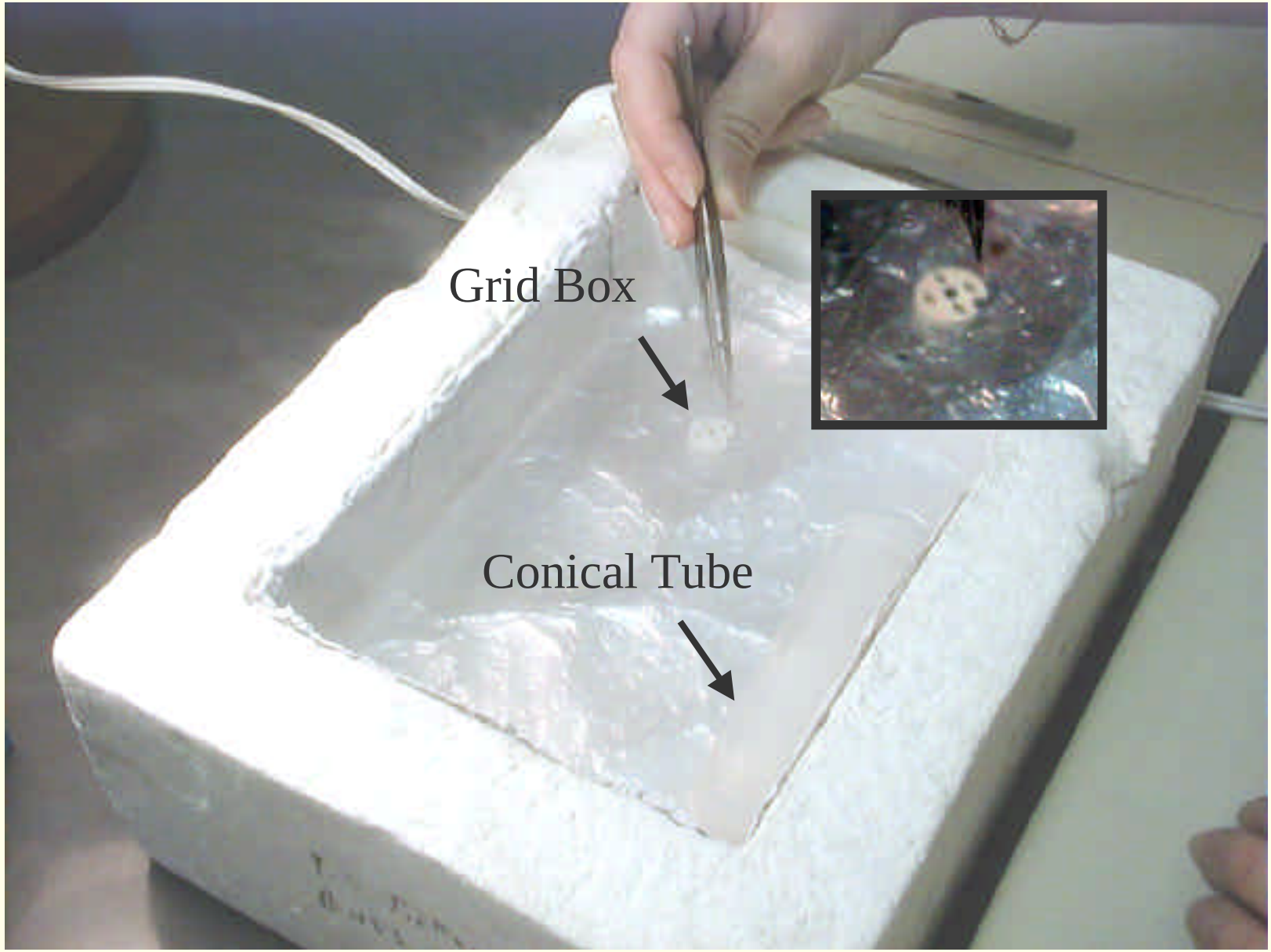
## Liquid nitrogen (-196C)



# Addition of Sample and Blotting

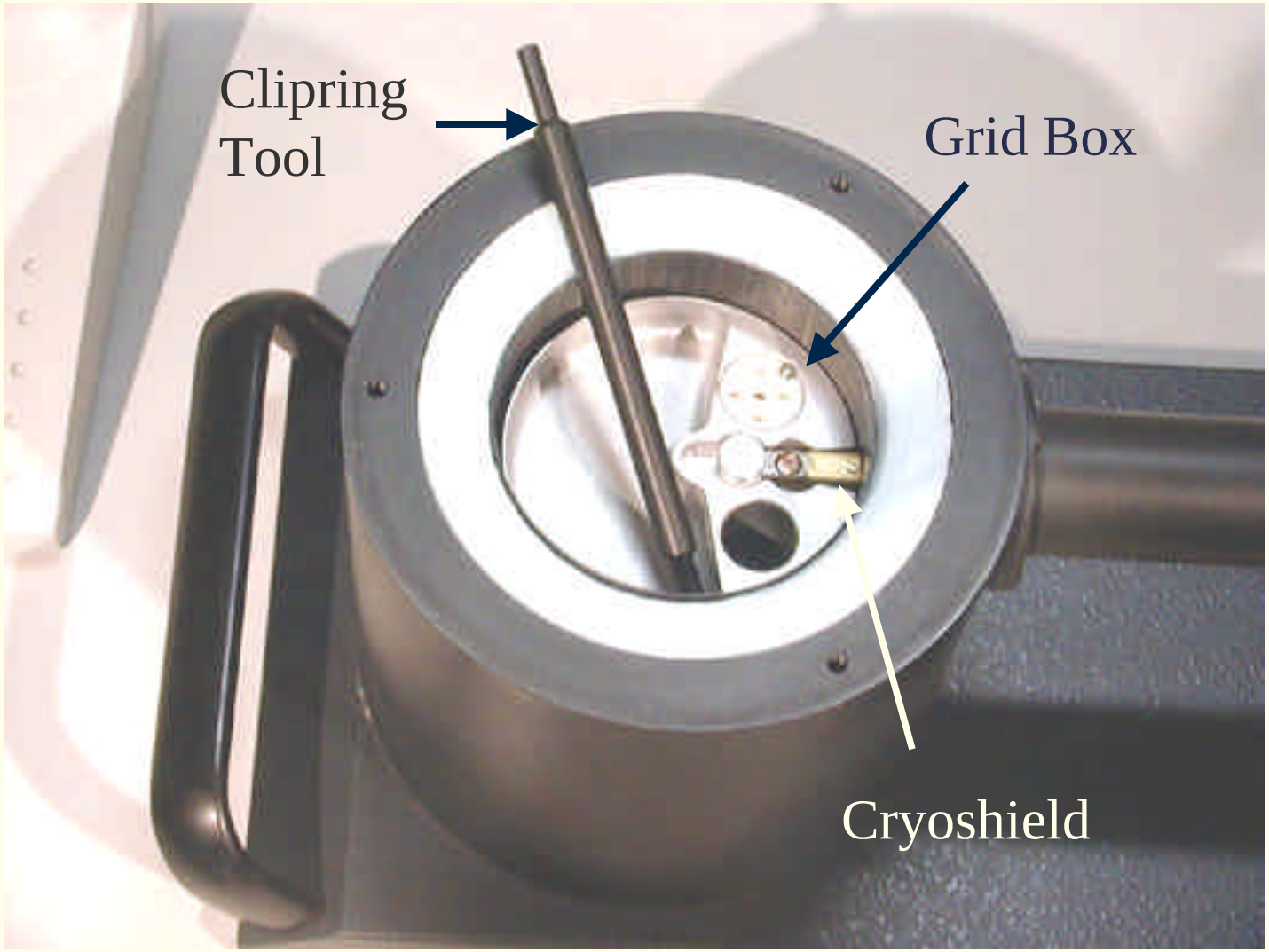


# Grid Storage



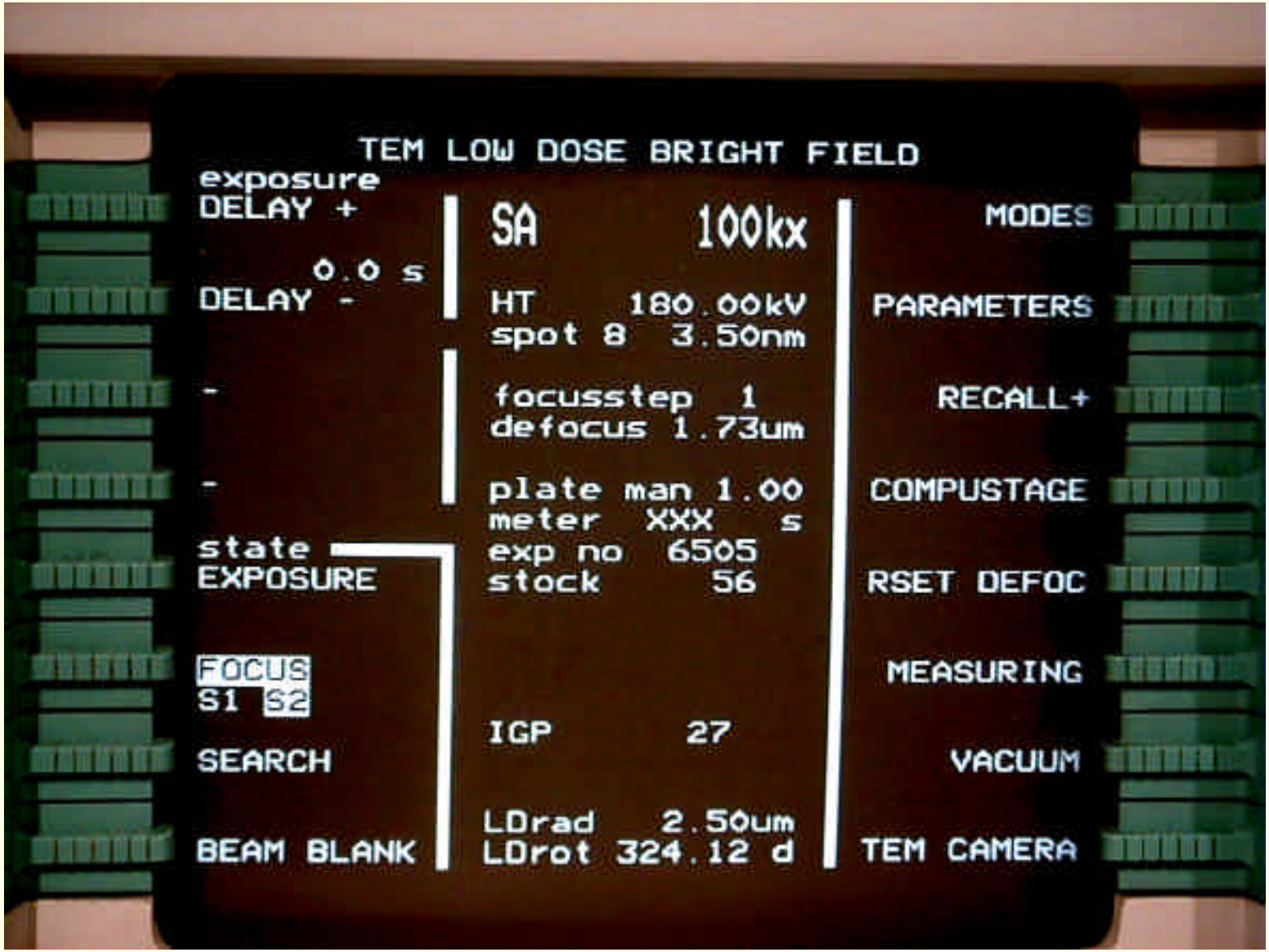
# Cold Holder and Cryotransfer Station

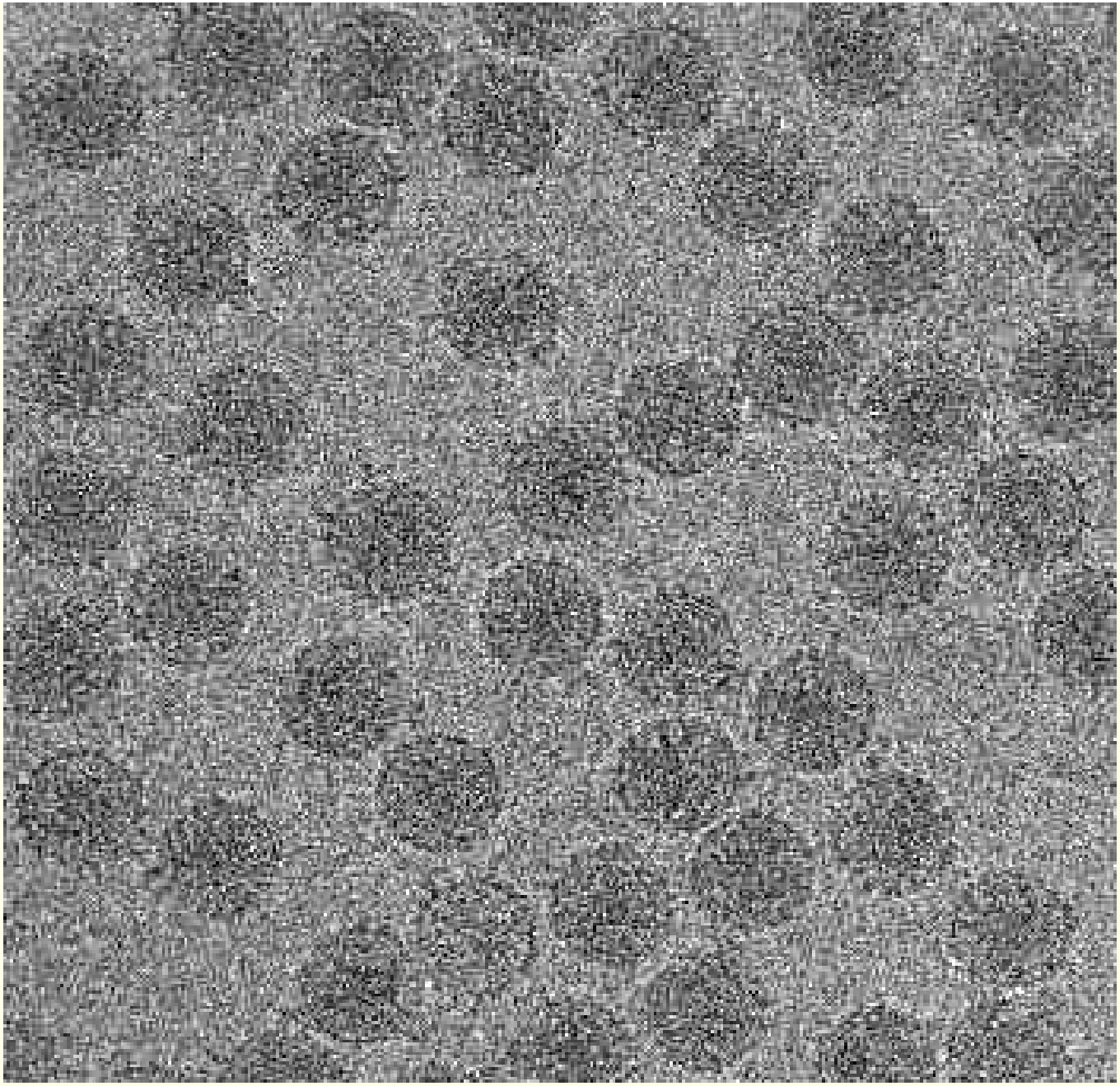
# Cryotransfer Station



# Holder in Microscope

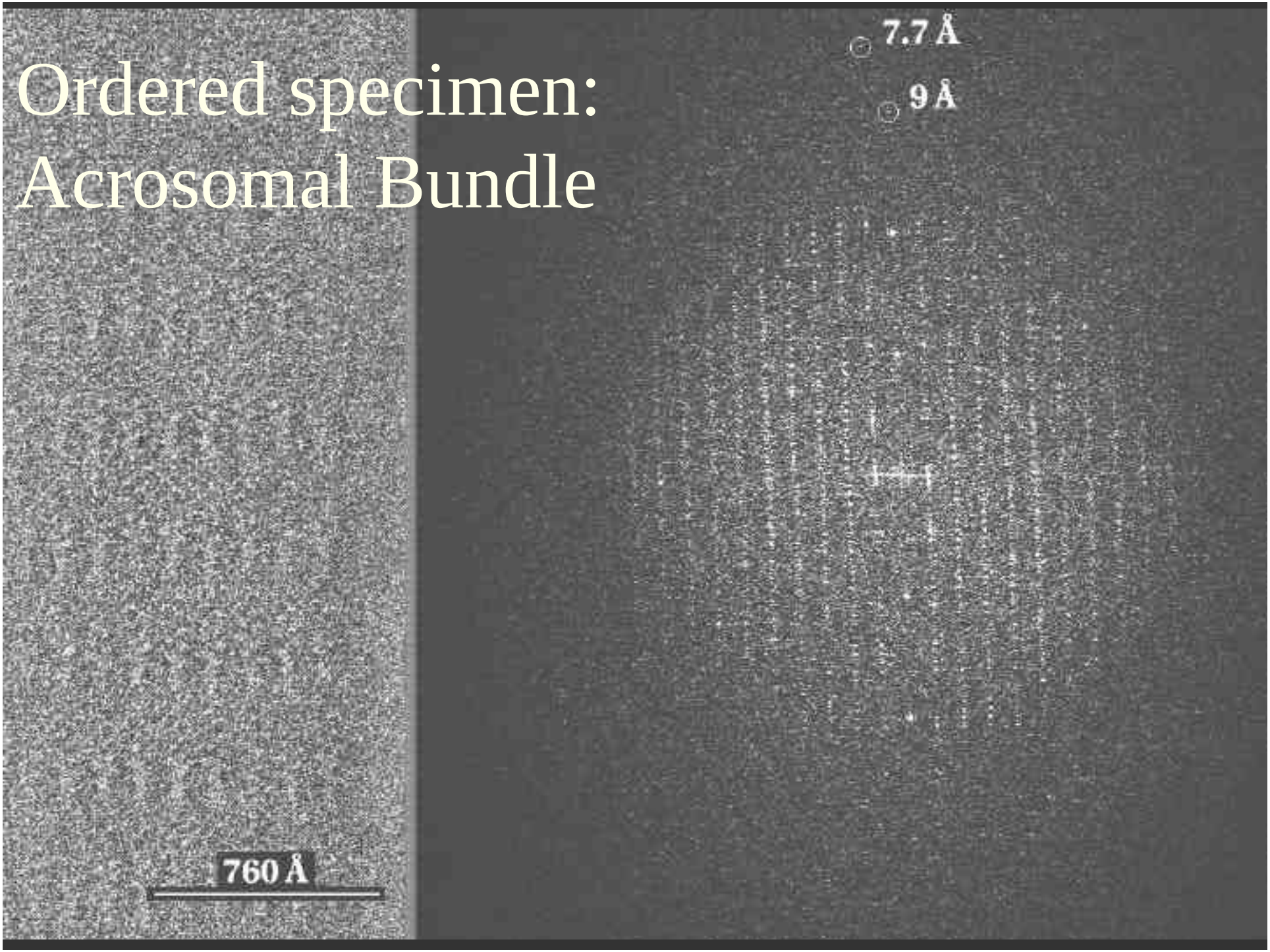
# Images Need to be Recorded with “Low Dose” Techniques



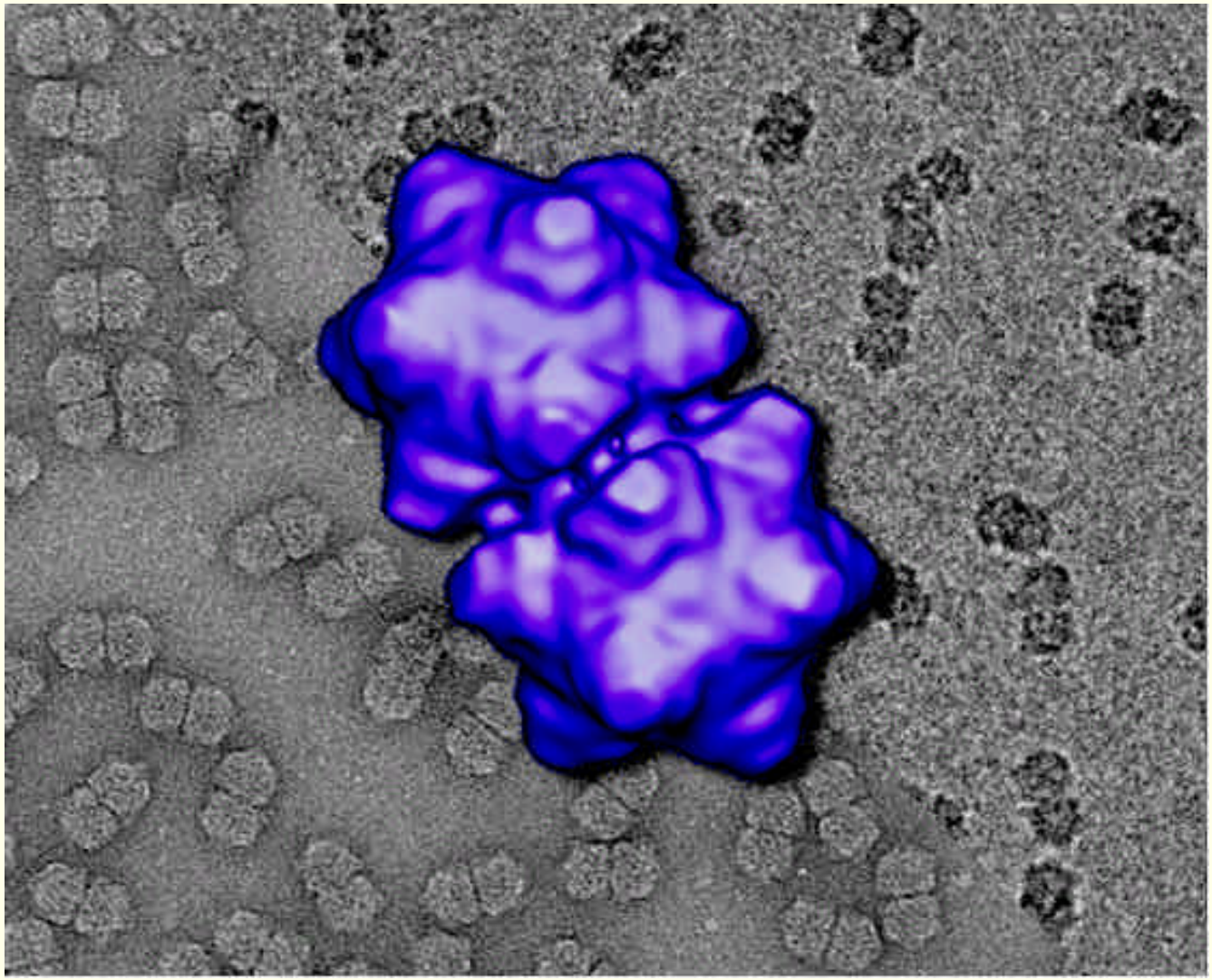


Reovirus Core Particles

# Ordered specimen: Acrosomal Bundle



# Results:  Geminivirus Study



Maize Streak Virus: UA-Stained, Frozen-Hydrated
and 3D Reconstruction from Cryo-EM Data

# Labeling

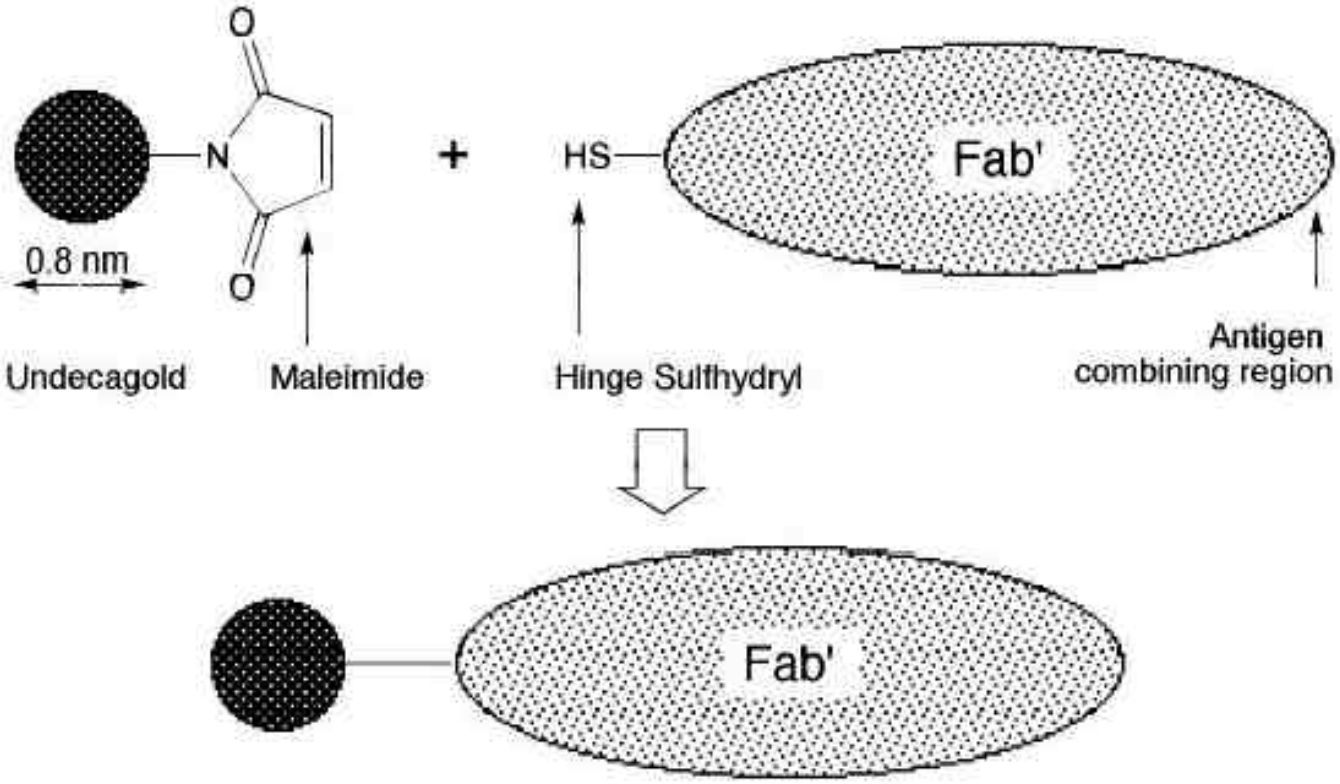
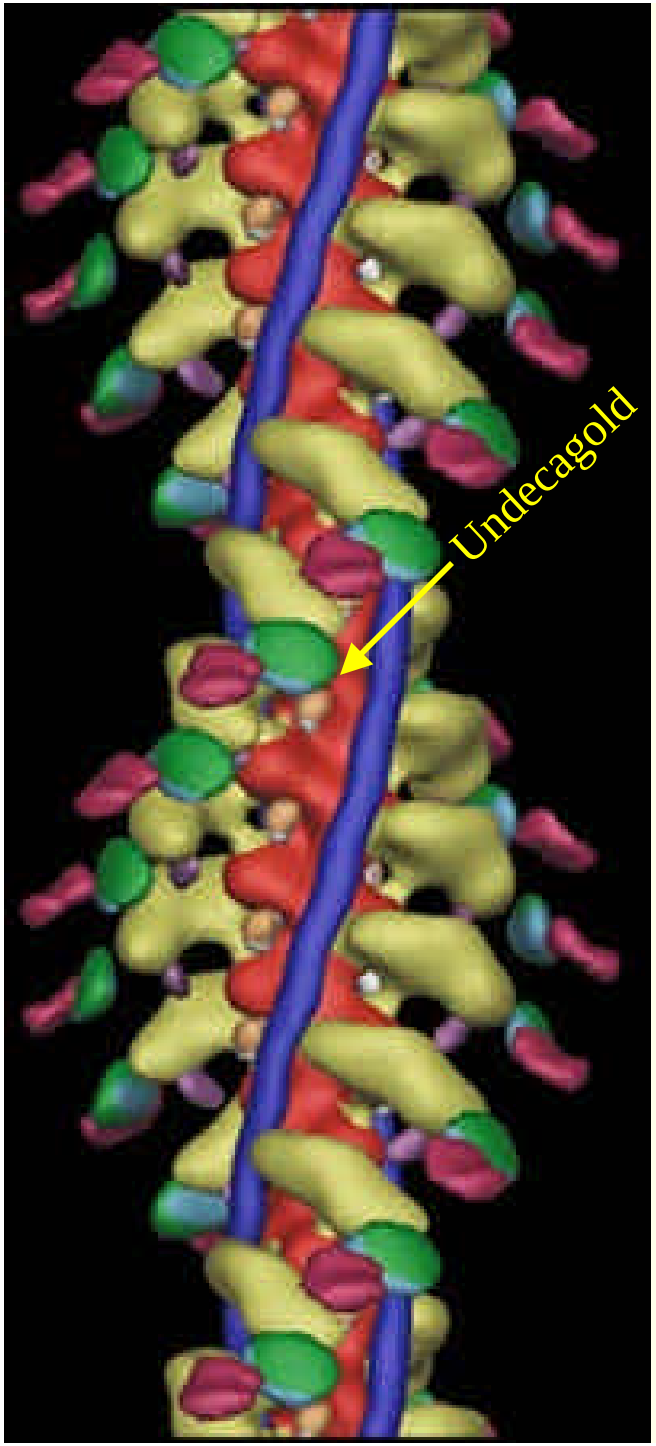



**Figure 1**: Schematic showing UNDECAGOLD labeling of Fab' fragment via reaction of a sulfhydryl and a maleimide group.

50 nmol of reagent is supplied: this is sufficient to label up to 0.5 mg of Fab' fragments, or up to 1.5 mg of IgG molecules.

Monomaleimido Undecagold™ labeling agent
*Nanoprobes Inc.*




# Actomyosin complex

3D maps and difference maps calculated by cryo-EM and image analysis. F-actin is red, tropomyosin is blue, the myosin motor domain is yellow, the essential light chain is blue-green, the regulatory light chain is dark red, the SH3 domain at the N-terminus of the heavy chain is purple, an **undecagold label** attached to Cys374 pf actin **is gold**, the N-terminal portion of the A1 light chain is white.

*R. Milligan's web site*

# Time-resolved TEM

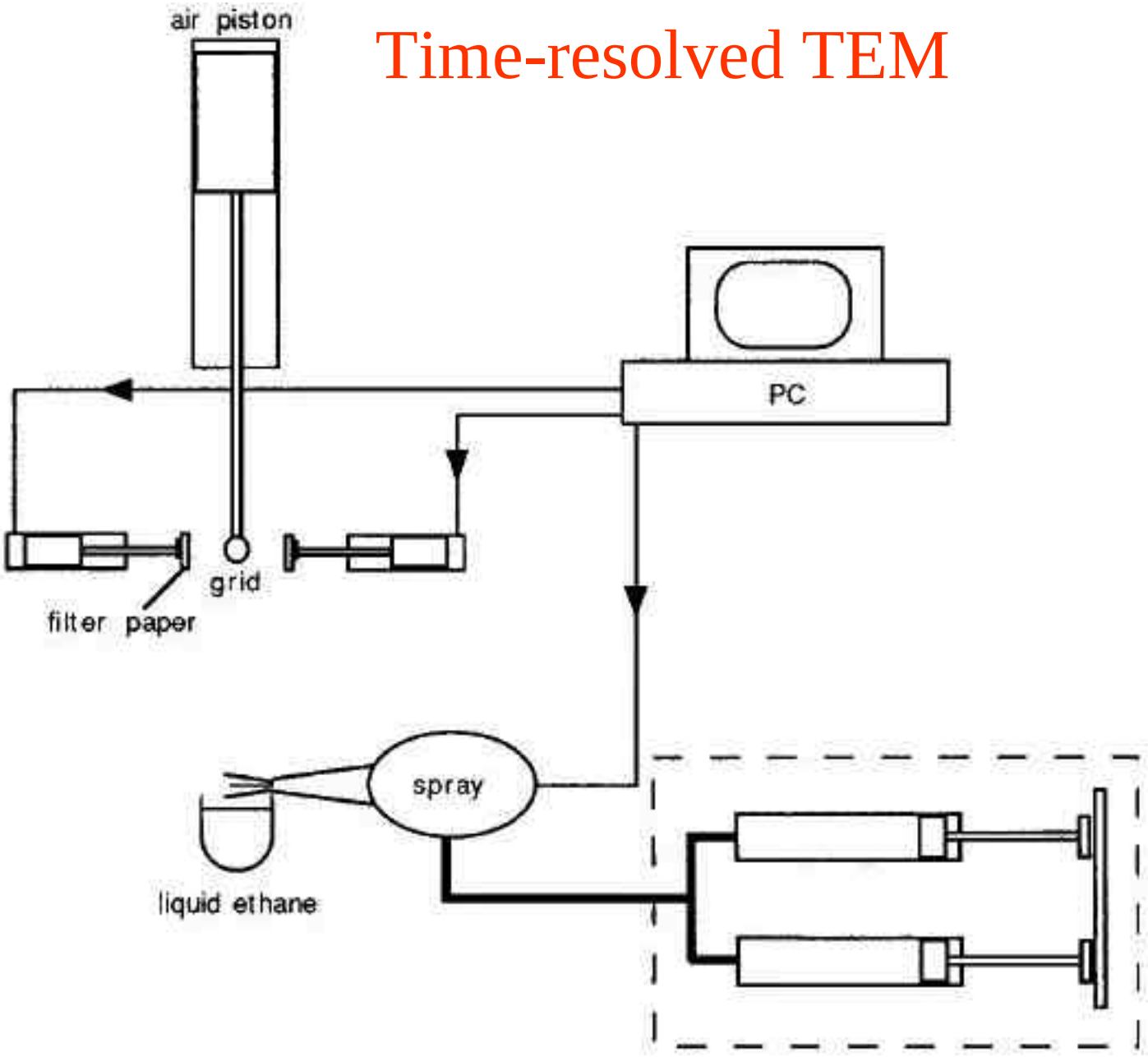
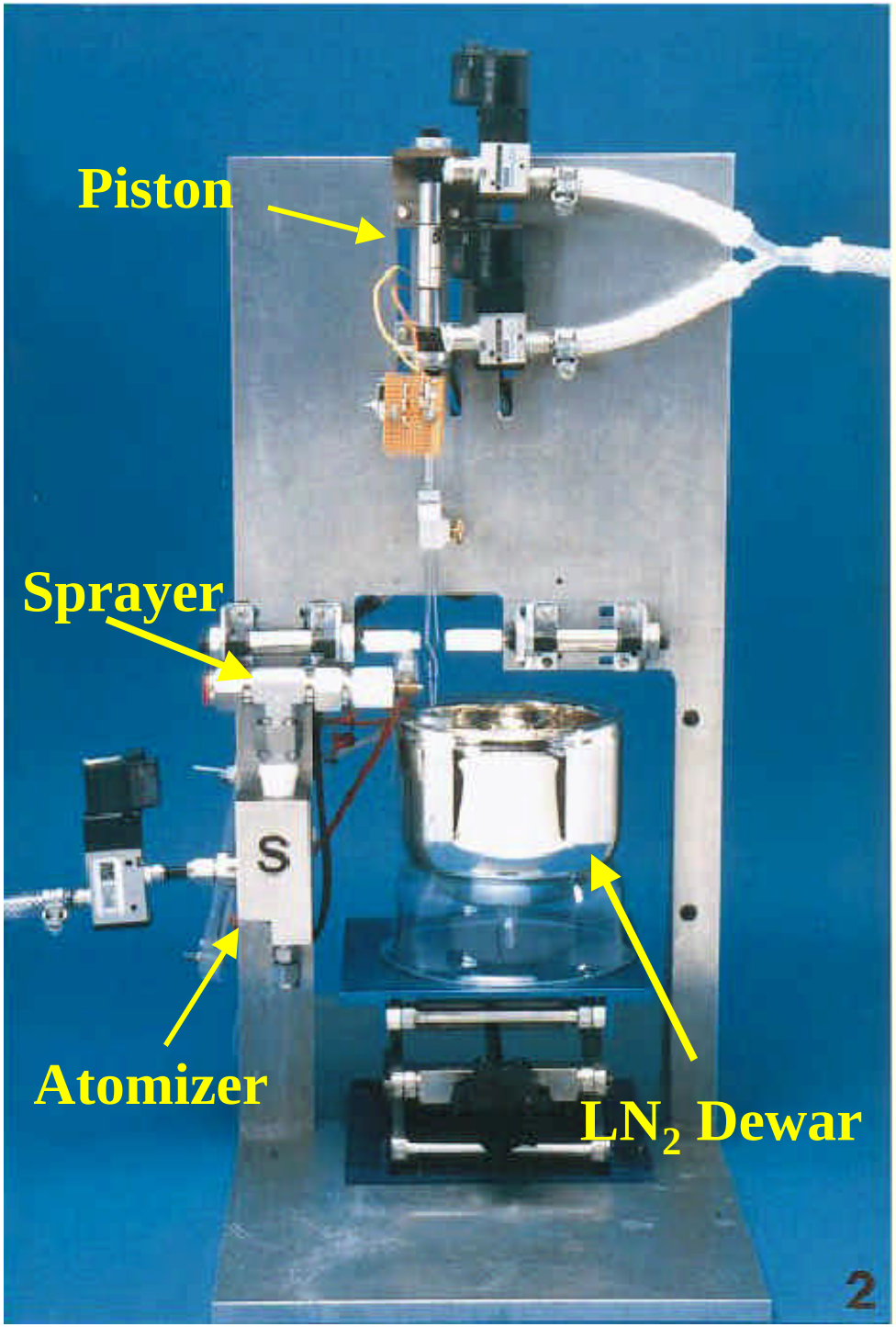
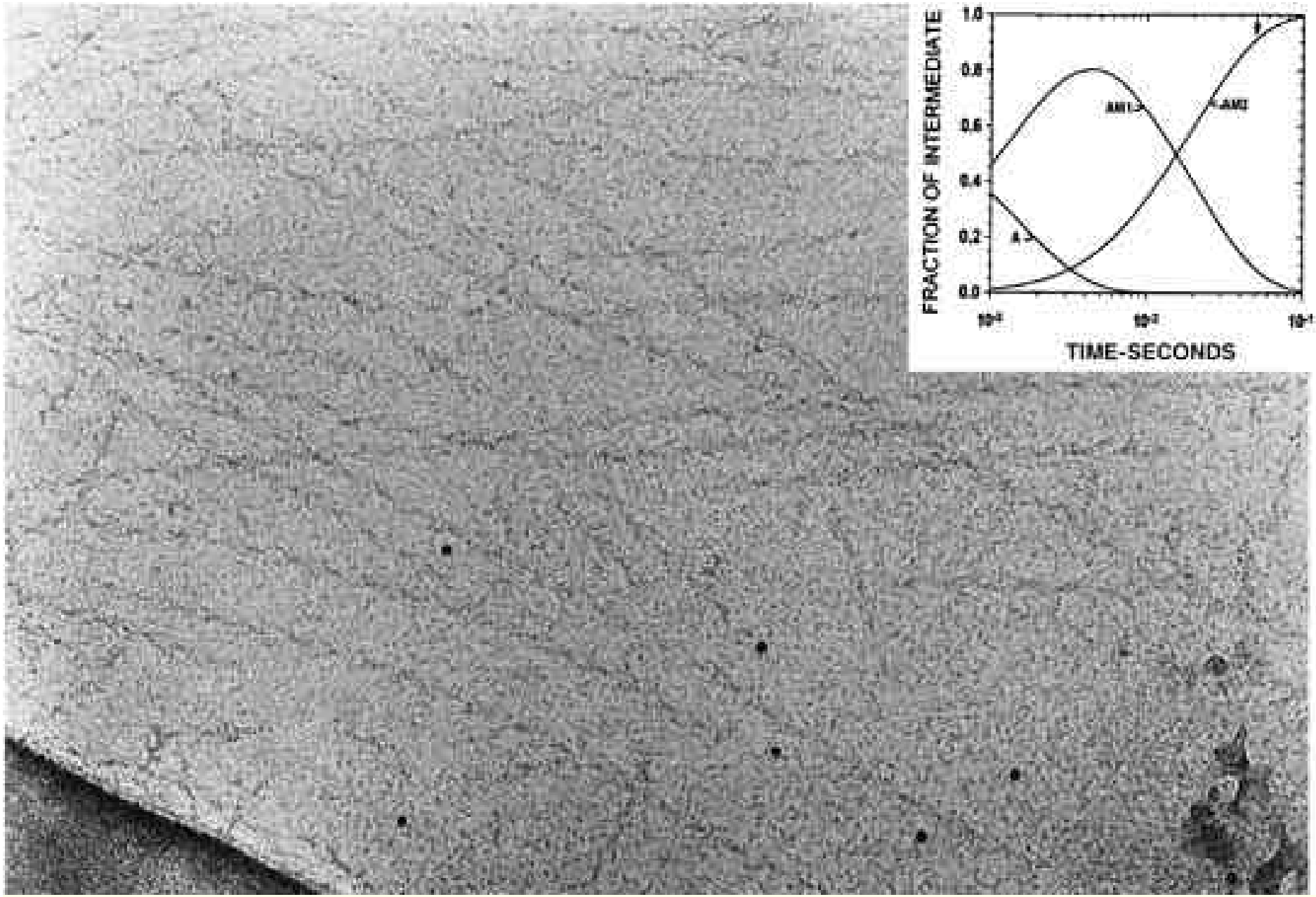



Diagram of the spray freezing apparatus.
H. D. White at el. 1998, JSB **126**, 306

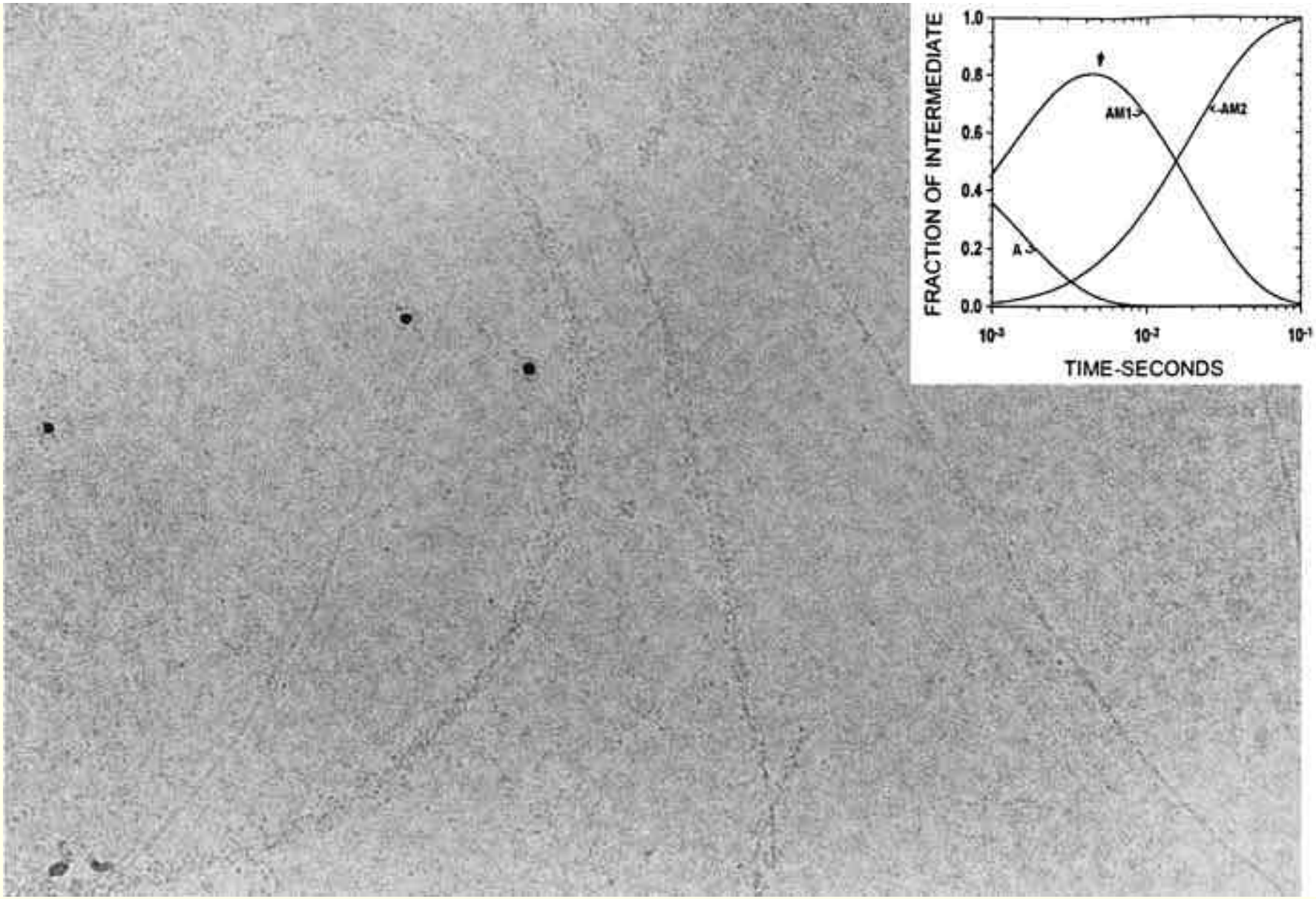



Photograph of the spraying apparatus. The piston carrying the forceps is shown fully extended and the tips of the forceps are in the ethane thimble on the left rim of the nitrogen dewar. Immediately above the thimble is the brass spray nozzle; this is connected to the vertically mounted stainless steel block of the atomizer (marked S) by a T pipe connector.
H. D. White at el. 1998, JSB **126**, 306




Actin sprayed with S1 with a delay of **50 ms** between spraying and freezing.

M. Walker at el. 1998 PNAS **96**, 465




Electron micrograph of actin sprayed with S1 **5 ms** before freezing. The S1 bound to the actin is disordered. The intensely black round particles are 5-nm colloidal gold that was included in the spray to aid in searching for areas of the grid that had received spray droplets. M. Walker at el. 1998 PNAS **96**, 465