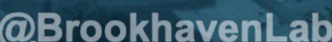
Negative staining and cryo-EM sample preparation

Liguo Wang

June 20th 2023

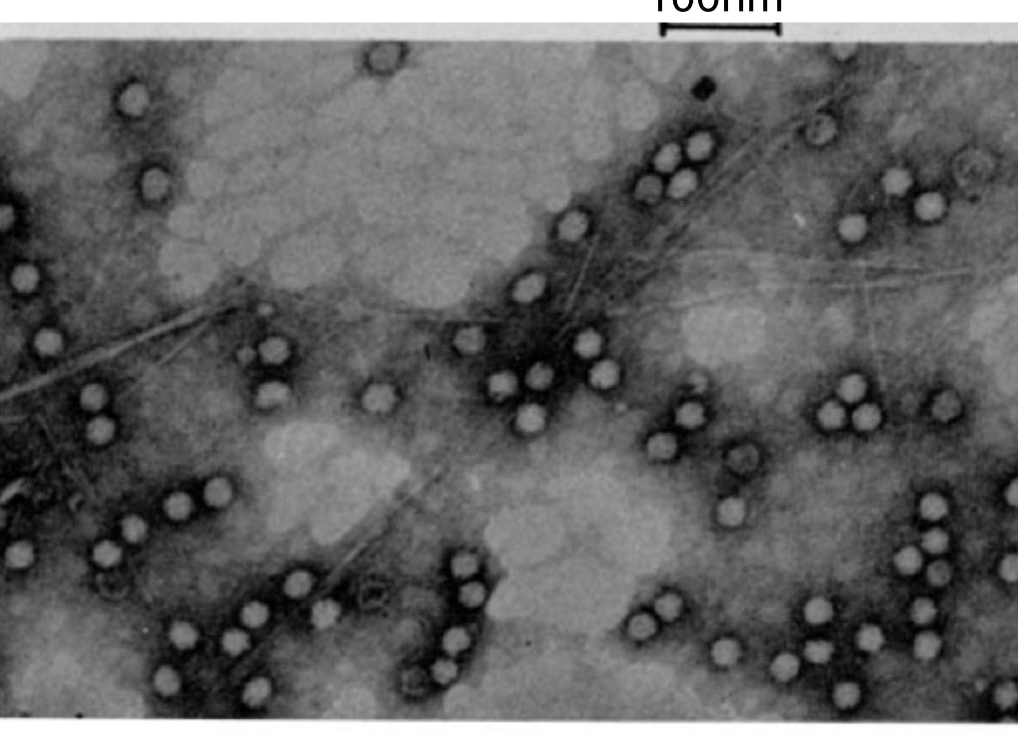


Negative staining

EM requires a vacuum - an environmental constraint that is incompatible with unprotected biological material.

BSV stained with 1% 12 PTA (Phosphotungstic acid) at pH 7.0 followed by 1% 12PTA at pH 0.7

BSV stained very lightly with 5% PTA at pH 4.6 and insufficiently washed



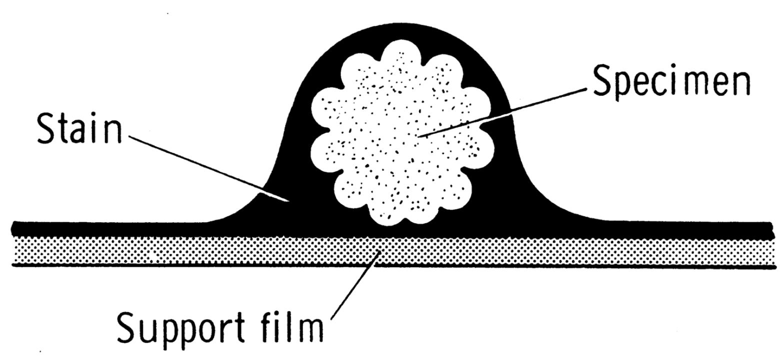
In the pH range above 2 where the **tomato bushy stunt (BSV) viruses** are stable, the amount of stain absorbed is too small to produce adequate contrast in the electron microscope. Maximum stain absorption was achieved at pH about 1.

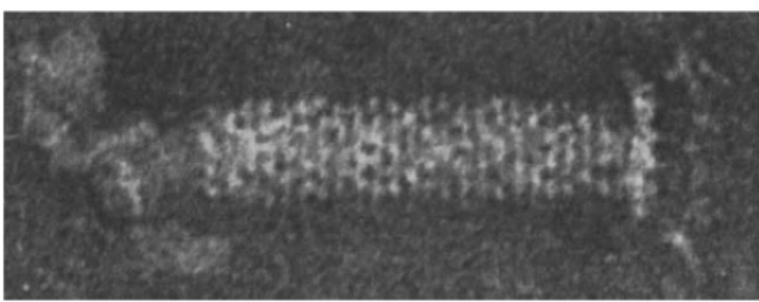
Hall, C. E. (1955). The Journal of Biophysical and Biochemical Cytology, 1(1), 1-12.

Negative staining

EM requires a vacuum - an environmental constraint that is incompatible with unprotected biological material.

Negative staining exploits that salts of heavy metals are relatively insensitive towards electrons and form **a stable "cast" around the molecules** when dried down. Salts such as uranyl acetate or phosphotungstic acid titrated to neutral pH, vanadates and molybdates have and are still being used.





- Sample appears "white" and the electron-dense stain is "black".
- Helps to reduce dehydration and radiation damage effects.
- Attainable resolution is ~ 15-25 Å.

➔ Mainly used as a sample screening method

De Rosier, D.J. and A. Klug. Nature, 1968
Hayat & Miller (1990), *Negative Staining*

Radiation damage and resolution limit

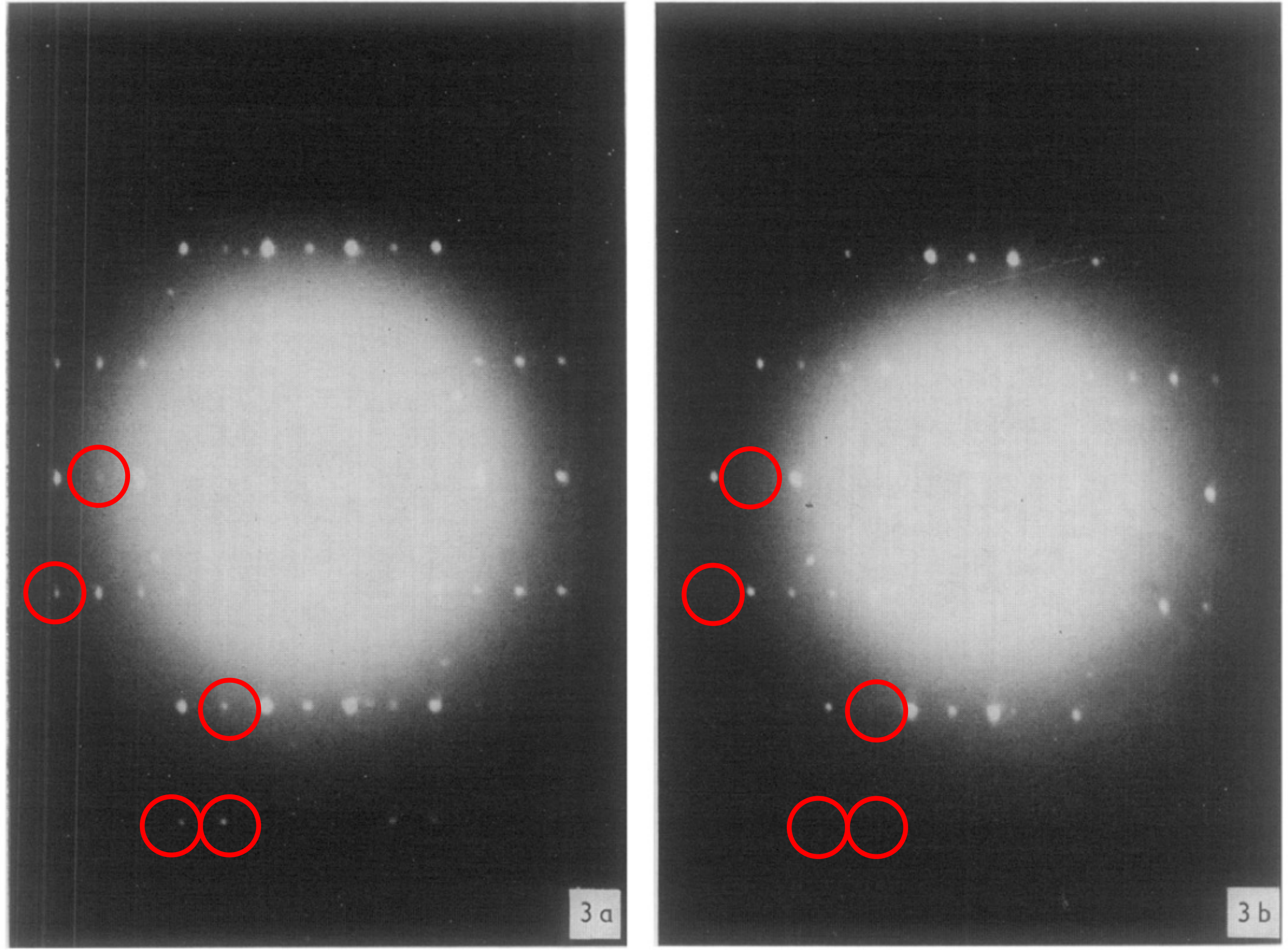


FIG. 3. The diffraction pattern of uranyl-acetate stained catalase is shown (a) before any significant changes have occurred and (b) after irradiating to a degree that no further changes occur. Reflections at Bragg spacings of less than 25 Å to 30 Å are no longer visible after so extensive an irradiation. Data were taken at 75 kV by the three-lens method (7) from a field approximately 10 μ in diameter.

Glaeser, R. M. (1971). Journal of Ultrasructure Research 36(3-4): 466-482.

Electron Diffraction of Frozen, Hydrated Protein Crystals

Abstract. *High-resolution electron diffraction patterns have been obtained from frozen, hydrated catalase crystals to demonstrate the feasibility of using a frozen-specimen hydration technique. The use of frozen specimens to maintain the hydration of complex biological structures has certain advantages over previously developed liquid hydration techniques.*

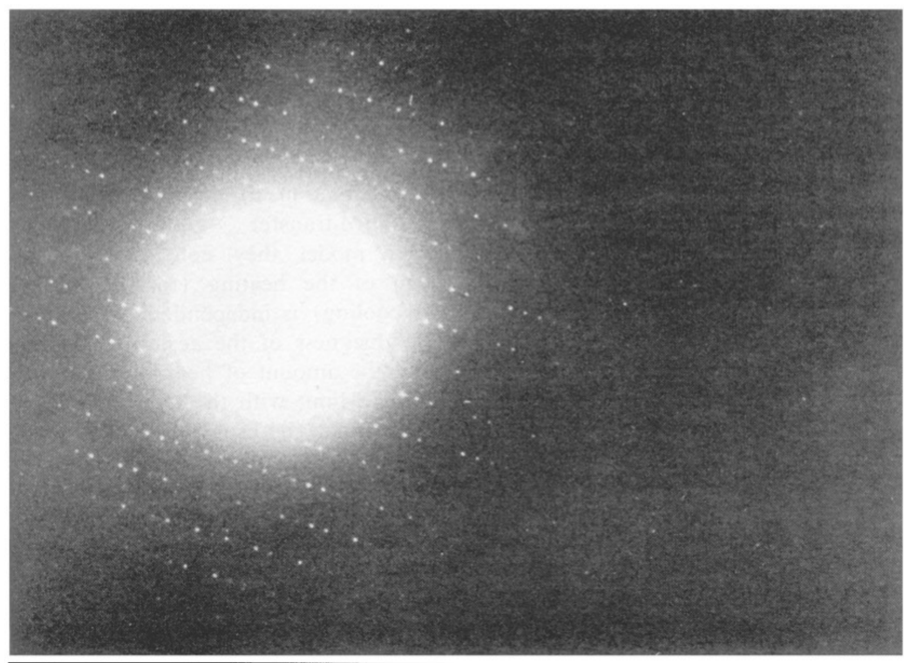


Fig. 1. Electron diffraction pattern of a catalase crystal which was frozen in liquid nitrogen and observed on a specimen stage cooled with liquid nitrogen. The resolution of the photographic reproduction is 4.5 Å, although that of the diffraction pattern on the original plate was 3.4 Å.

A drop was placed between two halves of a folding grid coated with hydrophilic film (SiO) and plunge frozen in LN2.

In parallel to the work of Henderson and Unwin, Taylor and Glaeser discovered that biological specimen can be observed in a **frozen-hydrated state**. This discovery was not only key to advancing 2D-crystallography (and later being adopted by the X-ray community as well), but also made possible the study of single particles of large macromolecular complexes.

Resolution to 3.4 Angstrom!

13 December 1974, Science 186:1036-37

Radiation damage and low temperature imaging

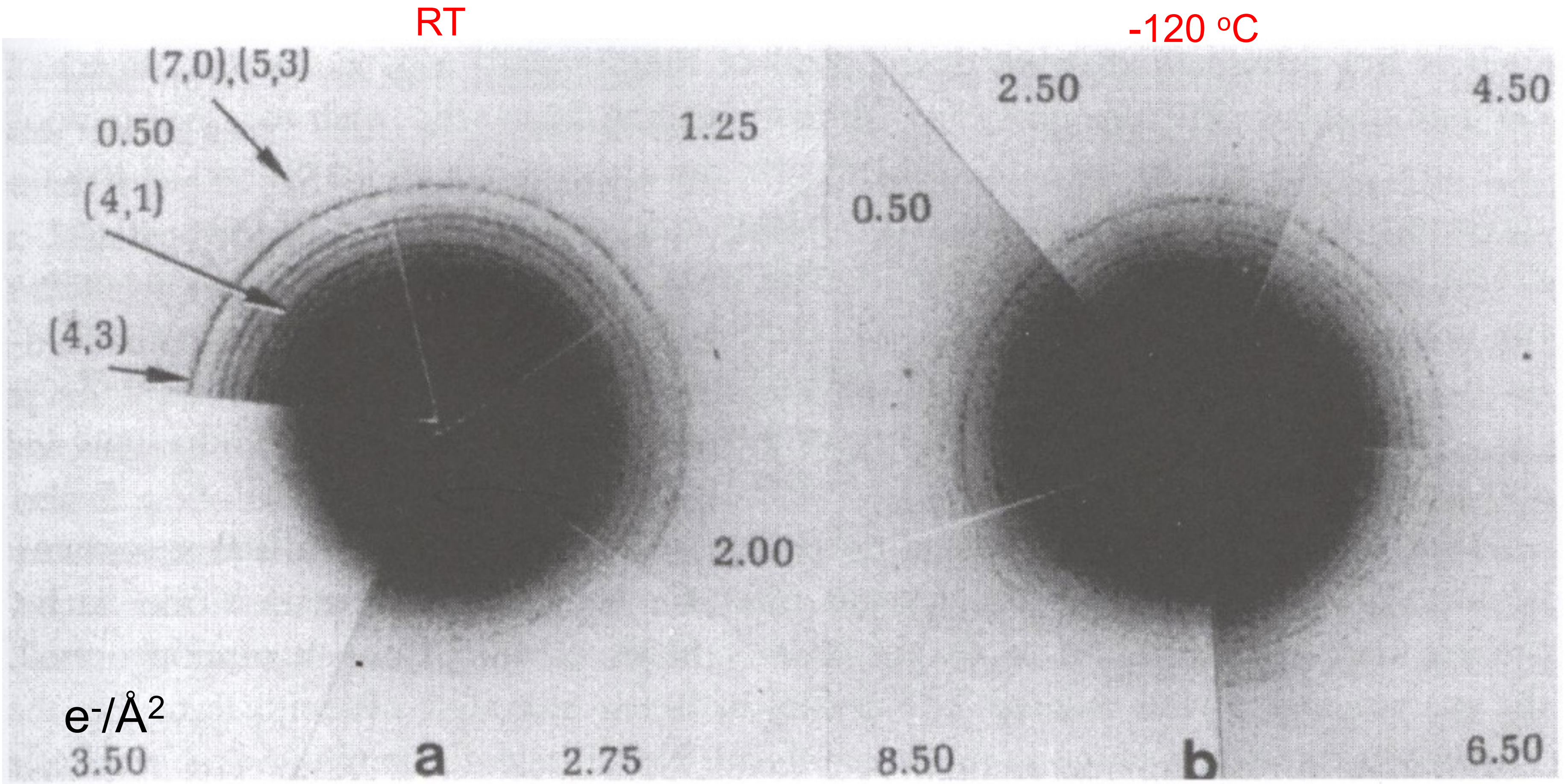


Figure 1.8 Comparison of the rate of fading of electron diffraction intensities at room temperature and at low temperature. A series of electron diffraction powder patterns of glucose-embedded purple membrane were recorded after specified periods of previously accumulated electron exposure (Hayward and Glaeser, 1979). The results show that about 5 to 7 times greater electron exposure can be tolerated at low temperature than at room temperature, for the same extent of specimen damage.

Sample is preserved in water!

History of water vitrification

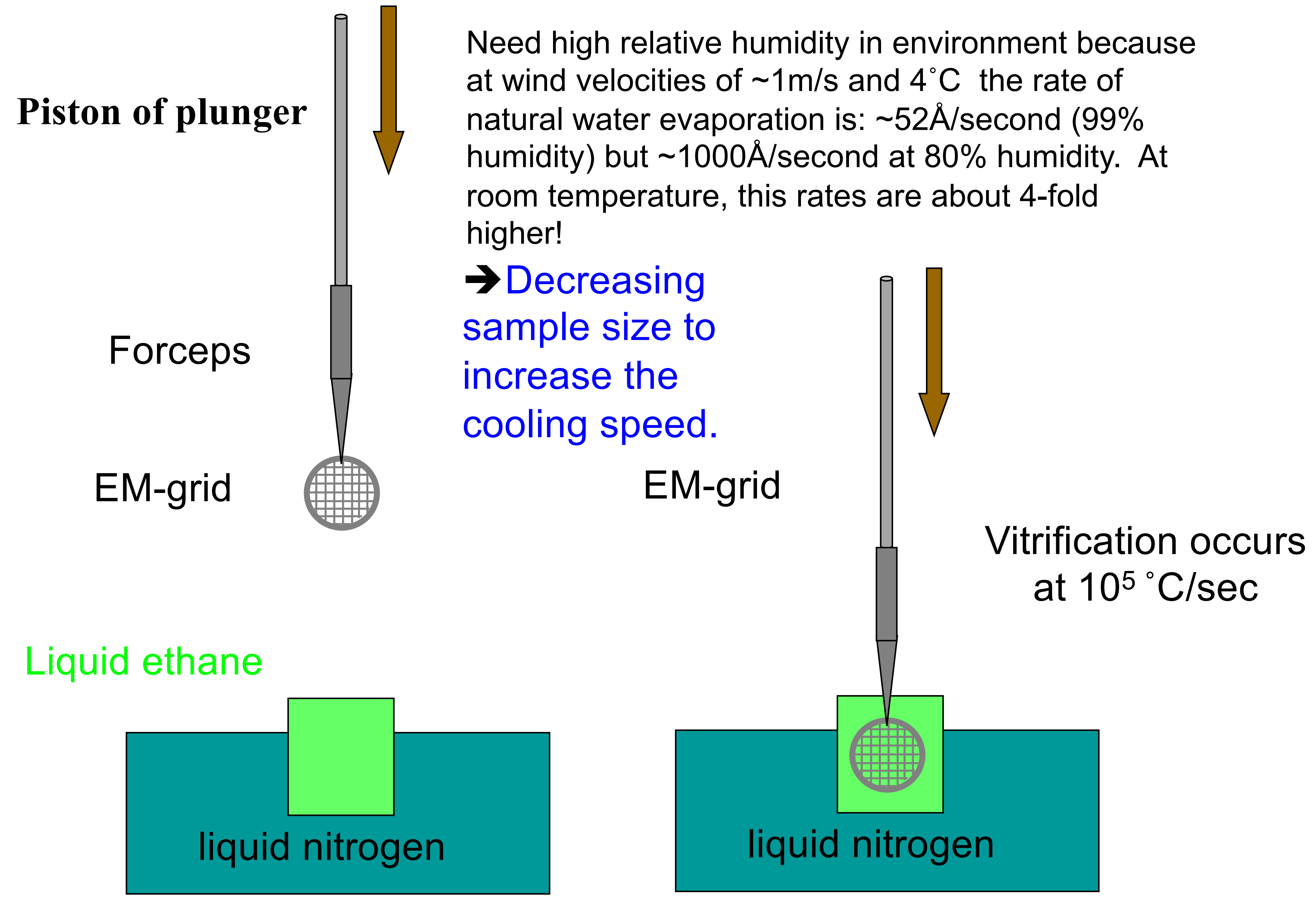
- Idea was proposed in late 1930s: Father B. Luyet proposed to cool a liquid so rapidly that molecules have no time to crystallize.
- Idea was not favorable: due to the discovery of the cryoprotecting effect of glycerol, ice crystals are allowed to grow but under controlled conditions.
  ➔ The vitrification of water was thought to be fundamentally impossible.
- Rapid development in 1980-1983
  - In 1974: Taylor and Glaeser, frozen catalase crystal (a drop between two grids was blotted and plunged into liquid nitrogen)
  - In 1981, Duboche group vitrified thin water layers, obtained by spreading on a support, by immersion in liquid ethane (Dubochet & McDowall, 1981)
  - In 1983, EMBO course to teach the vitrification method.

Why liquid ethane instead of liquid nitrogen?

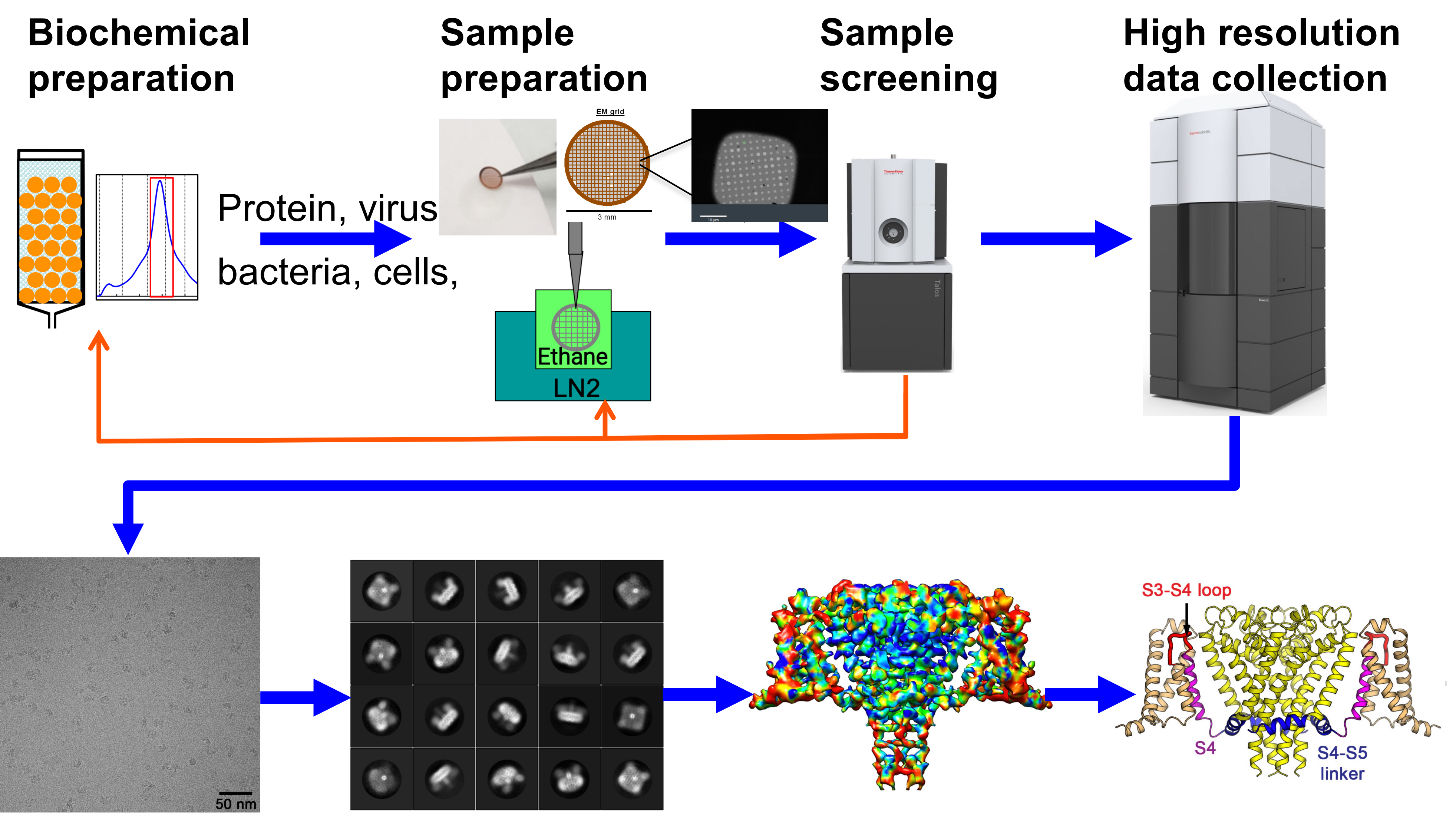
	Melting Point (°C)	Boiling Point (°C)	Heat of vaporization (kJ/kg)	Heat capacity (kJ/(kg·K)	Heat to boil (kJ/kg)	Heat to evaporate (kJ/kg)	Liquid density (kg/m3)
Nitrogen	-210	-196	6	0.9-1.6	13-22	19-28	809
Ethane	-183	- 89	489	2.3-3.5	216-329	705-818	546
Water	0	100	2257	4.185	418.5	2675.5	1000

$Q=C*\Delta T$

Sample Vitrification

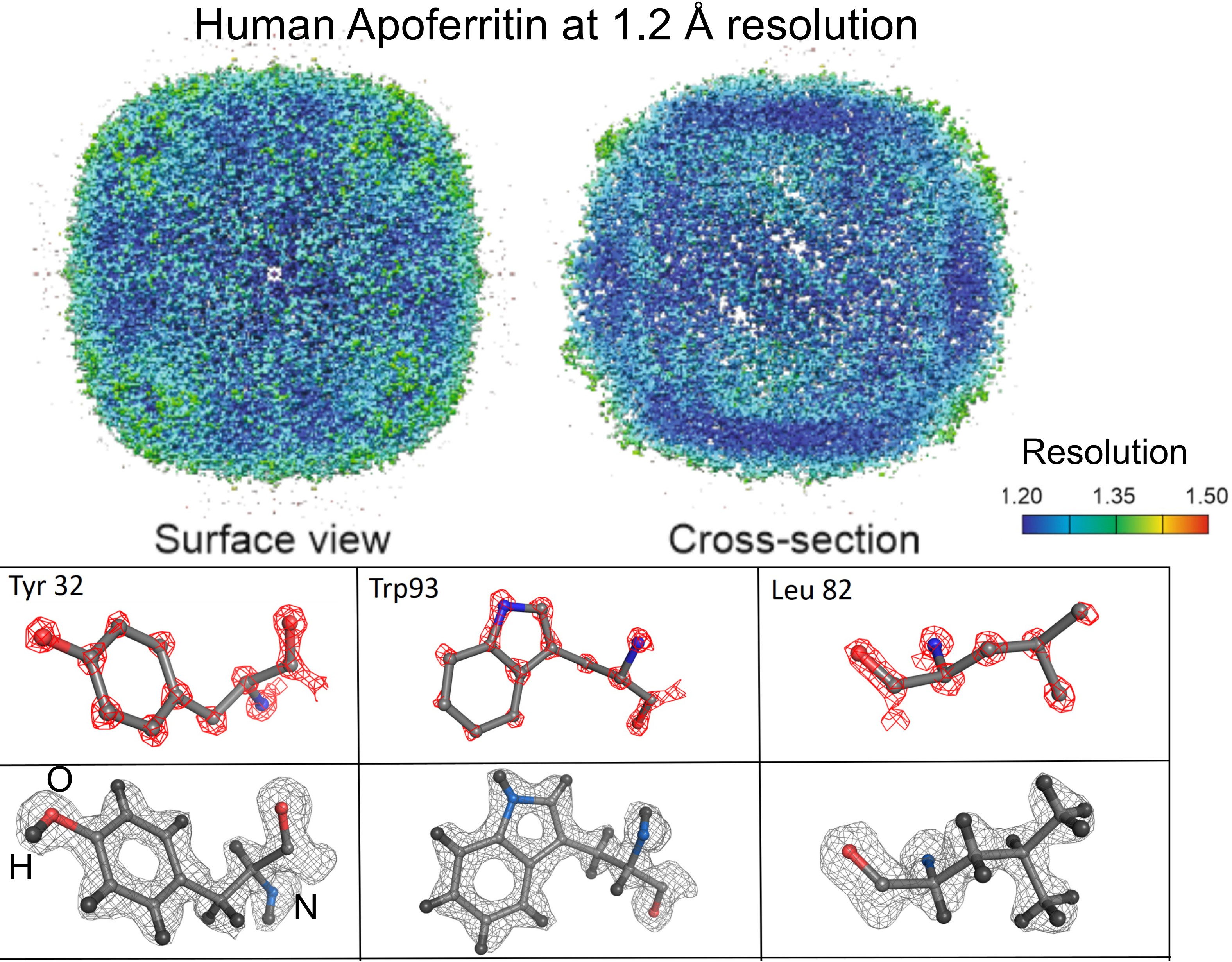


Workflow of cryo-EM Single Particle Analysis (SPA)



Cryo-EM images Image processing Reconstruction Structural analysis

SPA cryo-EM: individual atoms are resolved!

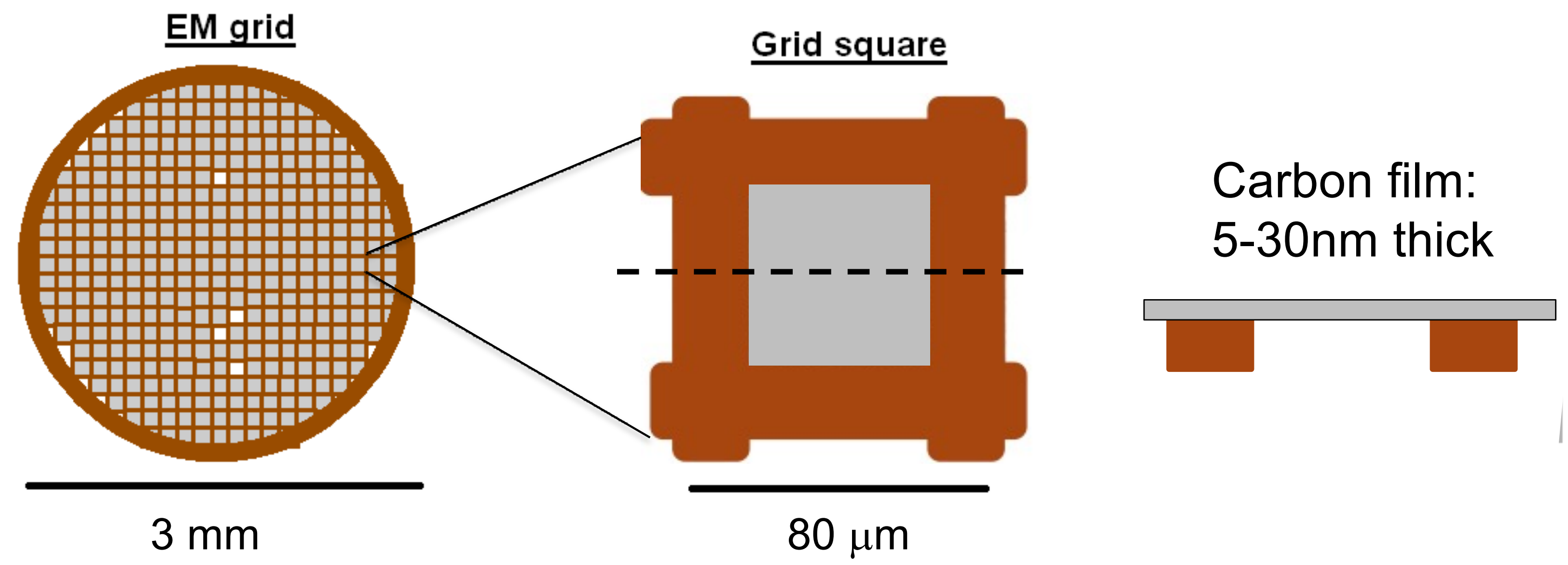


K. M. Yip et al. Preprint at bioRxiv http:// doi.org/dx3w; 2020
T. Nakane et al. Preprint at bioRxiv http://doi.org/dx3x; 2020

Sample preparation

- Negative staining
- Cryo-sample

Continuous carbon grids for negative staining



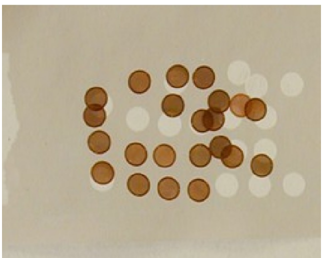
An EM grid coated with a thin **continuous carbon film** (5-30 nm).

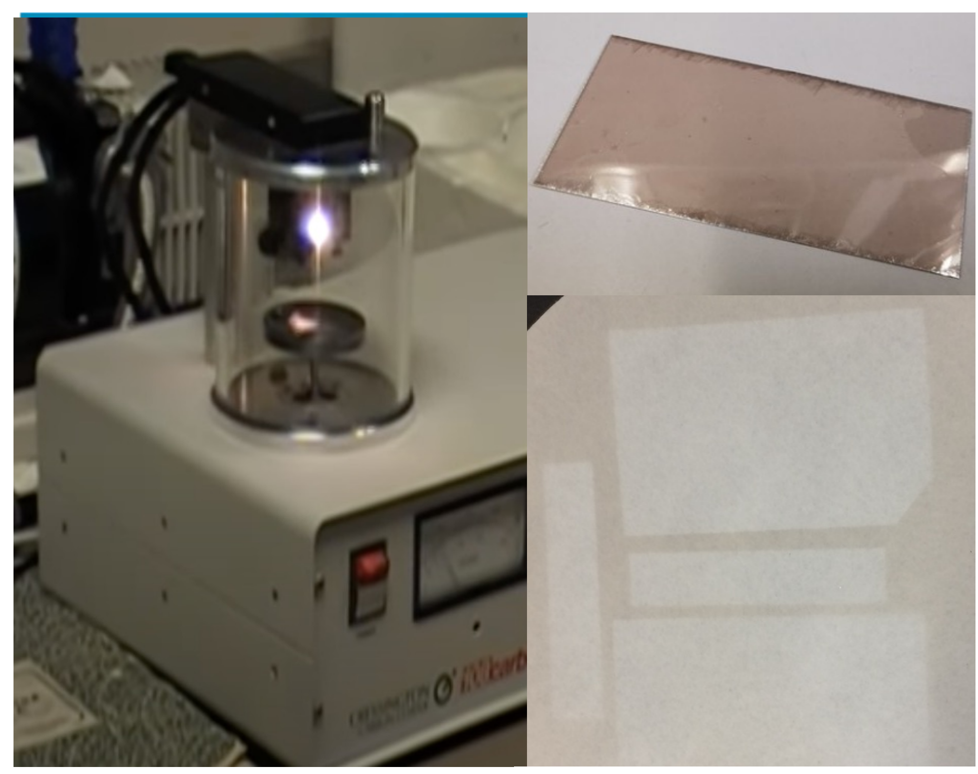
400 mesh: 400 squares in 1 inch➔2.54cm/400=63.5 microns

➔ Can be made in the lab or purchased from companies

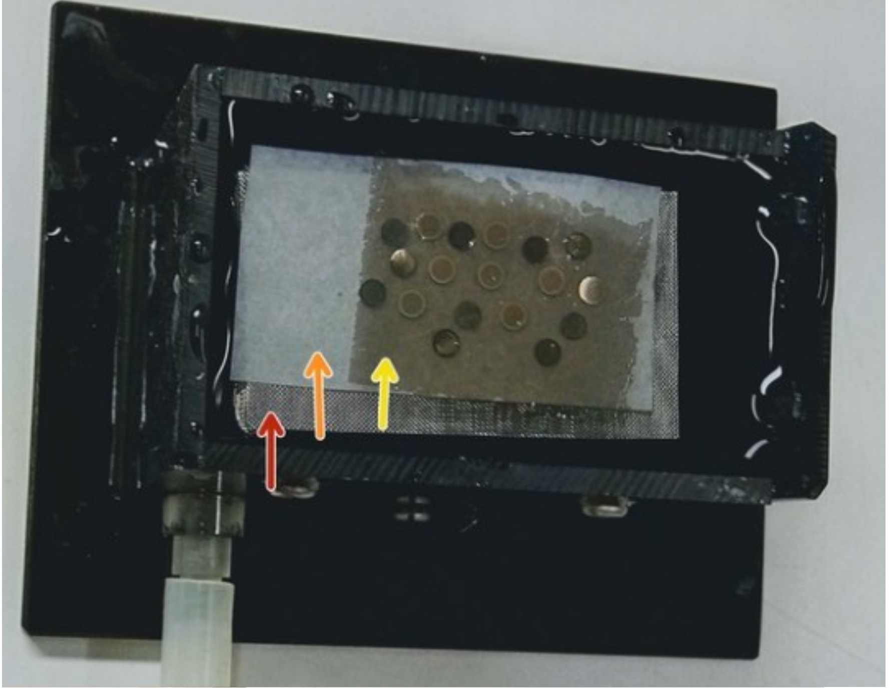
Carbon film growth and transfer

- Grow carbon film on mica sheet, then float the continuous carbon onto TEM grids
- Coat commercially available TEM grids with fresh carbon





Carbon Coating Grids Electron Microscopy SJDC Dr. Jon Krupp - YouTube



How to coat EM grids with thin carbon films using mica - ZMB UZH (dozuki.com)

Hydrophobicity of carbon films

γ_L = surface tension of the liquid

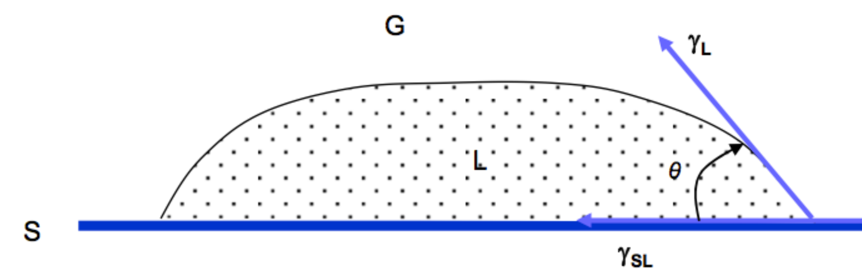
γ_S = surface tension of the solid

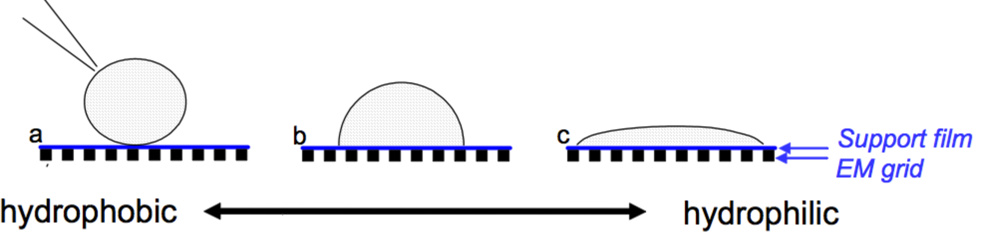
γ_{SL} = interfacial tension between the liquid and solid

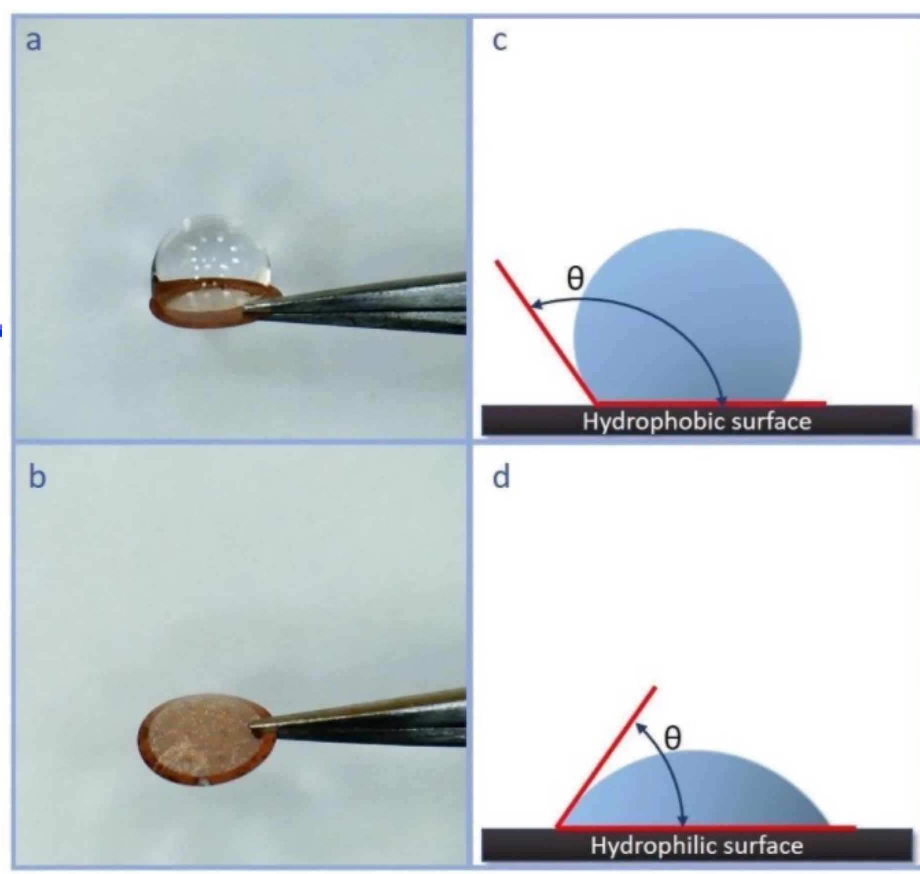
θ = wetting angle

At equilibrium: $\gamma_S = \gamma_L \cos\theta + \gamma_{SL}$

Perfect wetting conditions occur when $\theta = 0$



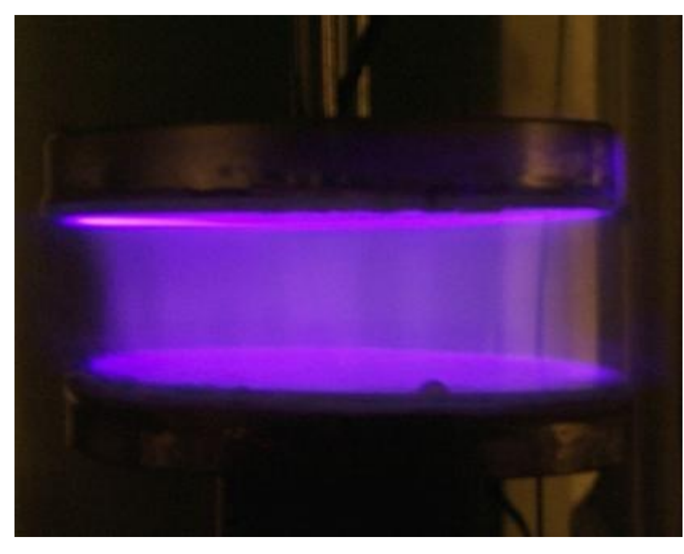




https://www.azom.com/article.aspx?ArticleID=19884

Glow discharge to change surface hydrophobicity

Applying a potential difference (of a few 100 V to a few kV) between two electrodes. Electrons that are emitted from the cathode are accelerated away from the cathode and give rise to collisions with the gas atoms or molecules in a chamber (a few mTorr to 1atm). The collisions give rise to excited species, which can decay to lower levels by the emission of light. This process makes that a gas discharge plasma typically emits a characteristic glow (and is therefore also called "glow" discharge).



Pelco Easiglow Glow Discharge Cleaning System

- 20-30mA, 20 s for carbon
- 20-30mA, 120 s for gold
- 10 mA, 5s for ultrathin carbon coated holey grids
- Use withing 30 minutes

Staining solutions

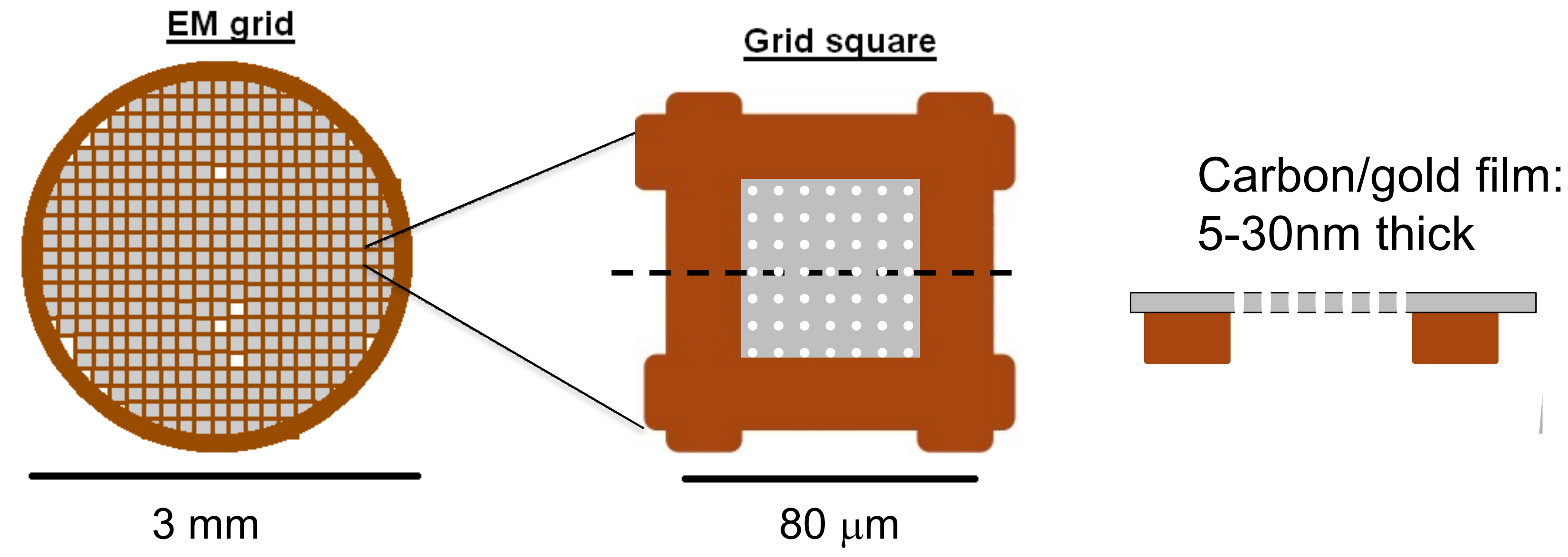
Stain	Density (g/ml)	Useful pH range	Radiation sensitivity	Contrast	References	Comments
Uranyl acetate	2.89	3– 4	Moderate	High	Van Bruggen et al. [101]	Fixative effect
Uranyl oxalate	2.50–3.07 [b)]	3– 7	Moderate	High	Haschemeyer and Meyers [10]; Mellema et al. [102]	Very light sensitive, store frozen
Uranyl nitrate	2.81	3– 4	Low	High		
Uranyl formate	3.70	3– 4	Moderate	High	Haschemeyer and Meyers [10]; Leberman et al. [103]	Fixative effect, smallest grain size
Uranyl sulfate	3.28	3– 4	Low	High	Estis et al. [104]	Reported not to recrystallize upon irradiation with electrons
Na/K-phosphotungstate	1.69 [c)]	4– 9	Low	High	Brenner and Horne [105]	Positive staining, increases with lowering the pH; destructive effect on phospholipid membranes
Na silicotungstate	2.84 [d)]	4– 8	High	High	Sherman et al. [106], Terry [107] Haschemeyer [108]	
Methyl-phosphotungstate		4– 9.5	Low	Medium	Oliver [109]	
Methylamine tungstate	3.88	3–10	Low	High	Faberge and Oliver [110], Shaw and Hills [111]	Supposed not to be a positive stain at any pH. With glycoproteins, add tannic acid
Ammonium molybdate	2.28	5– 8	Moderate	Medium	Bohonek [112], Manella and Frank and [113]	Good for membranes, some fibrous proteins
Aurothioglucose	2.92	4–10	High	Low	Kühlbrandt [114], Kühlbrandt and Unwin [13]	Yields Au-crystallites upon electron irradiation
Cadmiumthio-glycerol	2.0	4–10	Moderate	Low	Jakubowski et al. [15]	No crystallite formation upon electron irradiation, possibly useful with undecagold
Vanadate	2.85 [e)]		Low	Low		Very light stain, can be used with undeca-gold labelling

Bremer, A. et al Ultramicroscopy 46 (1992) 85-111

Staining solutions

Stain	pH range	Note
Sodium (K) phosphotungstate	5-8	Significant disruptive effect on many membrane systems. Interact with lipoproteins. Less likely to precipitate with salts and biological media
Uranyl acetate (1-3%)	4.2 – 4.5	Highest electron density and image contrast
Sodium silicotungstate (1-5%)	5-8	Good contrast; Good for small particles and individual molecules
Ammonium molybdate (1-2%)	5–7	Best results for many types of specimen; Lower electron density than other stains
Methylamine tungstate (2%)	6-7	Contrast is not as good as uranyl acetate. Resolution is good.
Uranyl formate (0.75-1%)	4.2-4.5	Best for small molecules, but only stable for 1-2 days.
Nano-W® (methylamine tungstate)	6.8	excellent spreading qualities and a high density for high contrast

Holey TEM grids for cryo-samples

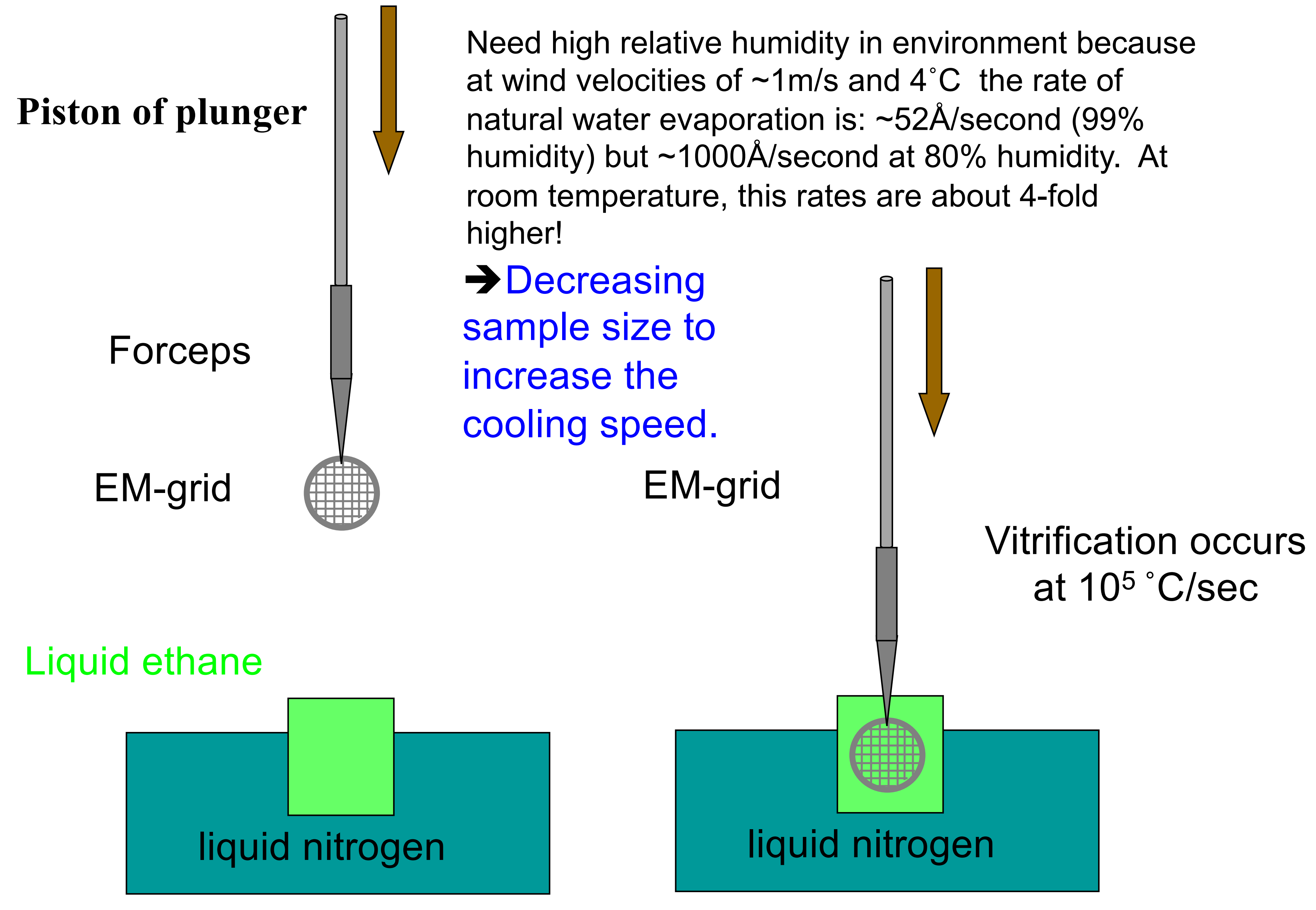


An EM grid coated with a thin carbon film (5-30 nm).

400 mesh: 400 squares in 1 inch➔2.54cm/400=63.5 microns

➔ Mainly purchased from companies

Sample Vitrification



Water evaporation during freezing

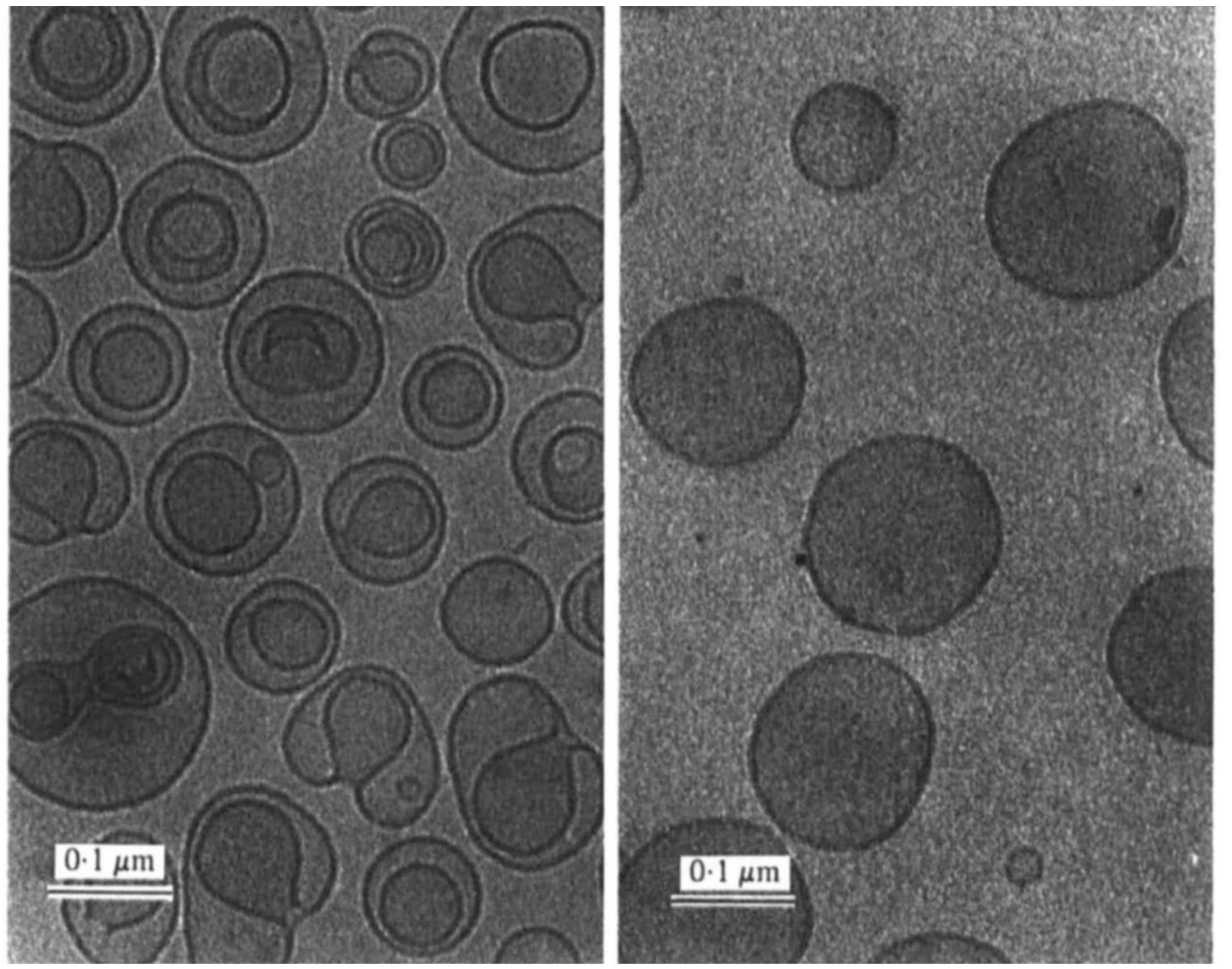
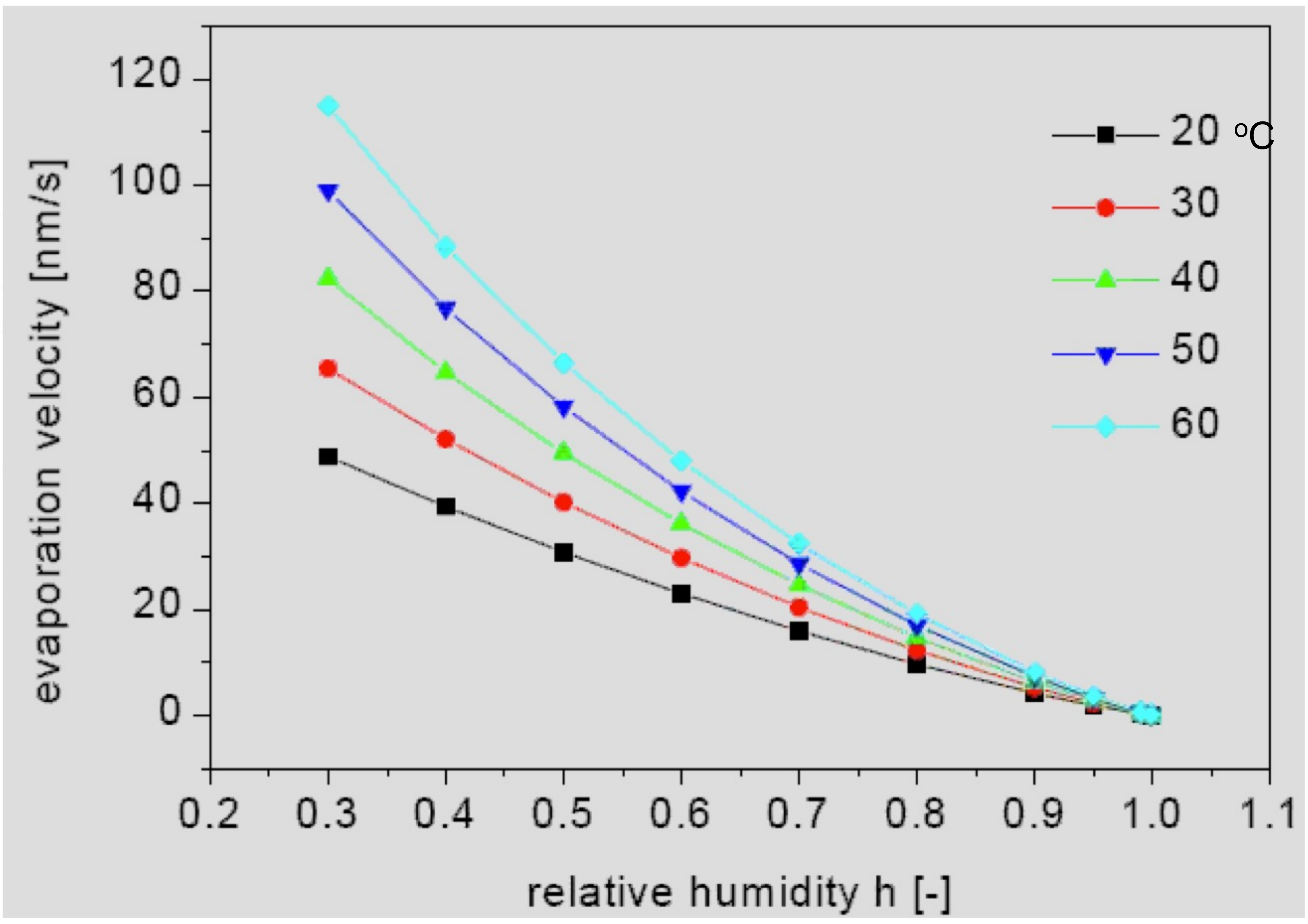


Fig. 24. Solution of lipid vesicles in 100 mM-NaCl. *(a)* The specimen has been prepared by the bare-grid method under conditions where part of the water evaporates before the thin film is vitrified. **Invagination of the vesicles and formation of concentric vesicles** reveal the osmotic effect due to the rapidly changing salt concentration in the liquid, *(b)* The same sample prepared in saturated humidity does not show osmotic effects.

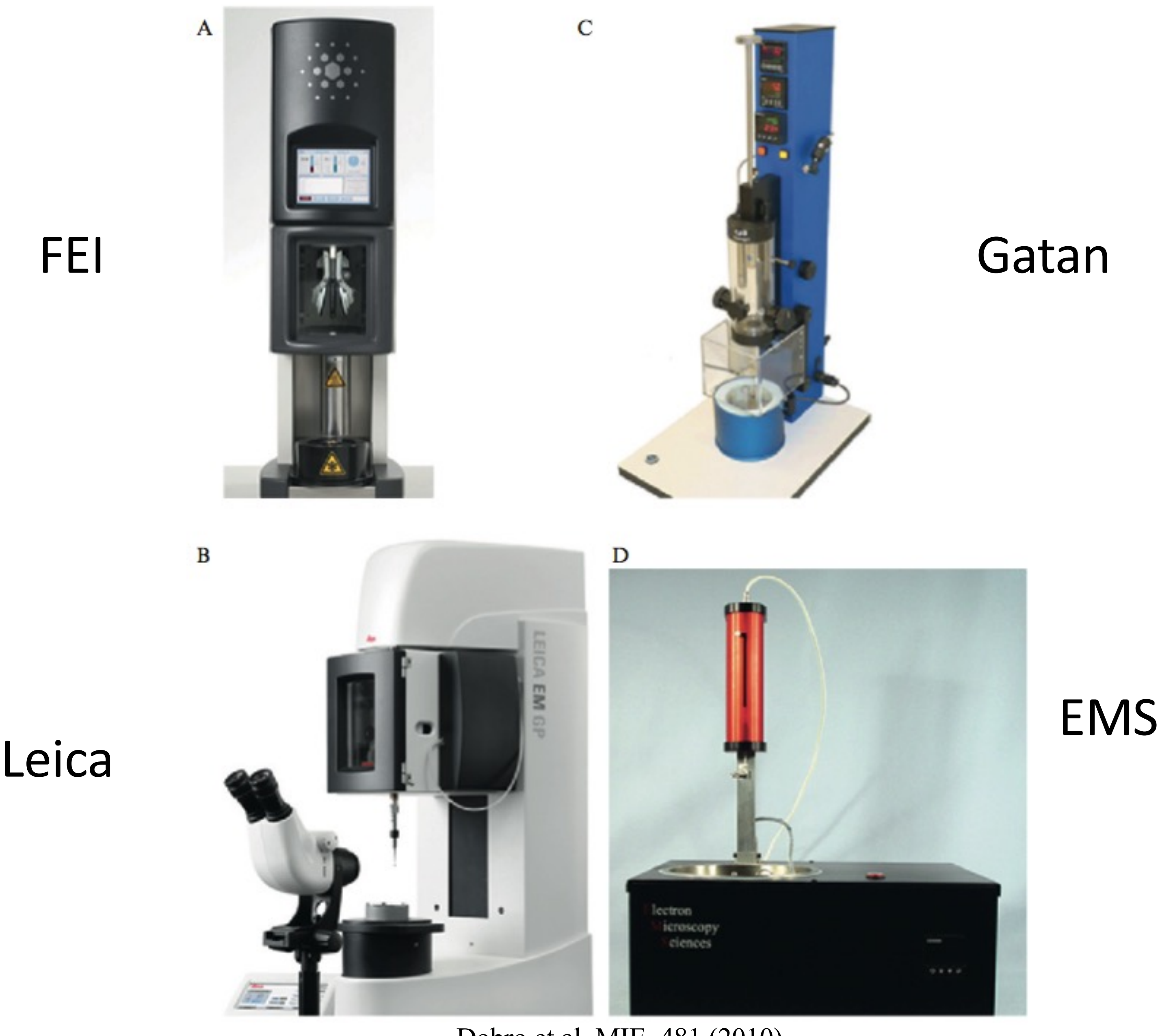
Dubochet J, et al. 1988. Q Rev Biophys 21:129-228.

Water evaporation depends on humidity and temperature



Frederik, P.M. and D.H.W. Hubert, *Cryoelectron Microscopy of Liposomes*, in *Methods in Enzymology*, D. Nejat, Editor. 2005, Academic Press. p. 431-448.

Automated plunge freezers



Dobro et al, MIE, 481 (2010)

Automated plunge freezers

Table 1 | Deposition techniques

Deposition techniques	Devices	Methodology	Sample carrier compatibility	Stock volume	Volume per grid	Dewpoint control	Layer inspection	Time from deposition to vitrification	Grid coverage
Ultrasonic spray	Back-It-Up[83]	High-frequency droplet generation with through-grid wicking	All	0.5–1 µl	200 nl–1 µl	No	No	±130 ms	25–35%
	Shake-it-off[77]	High-frequency droplet generation with self-wicking grids	Self-wicking nanowire grids	0.5–1 µl	50 nl	No	No	<100 ms	5–10%
	De Marco group[102]	Surface acoustic waves through microfluidic device	All	0.05–5 µl	1.5–2 nl	No	No	10–1,000 ms	5–10%
Gas pressurized spray	TED (Muench group)[74]	Gas sheath around nozzle using optional high voltage to steer droplets	All	33 µl	4 µl	No	No	2–200 ms	1–5%
	Frank group[72]	Gas sheath around nozzle	All	>30 µl	9 µl	No	No	10–1,000 ms	5–10%
Electrostatic spray	Trinick group[80]	High potential difference between nozzle and grid	All	5–10 µl	1–2 µl	No	No	>1 s	5–10%
Inkjet	Spotiton[81], chameleon[85]	Droplets formed by a piezoelectric dispenser deposited onto self-wicking grids	Self-wicking nanowire grids	3–5 µl	2–16 nl	No	Yes	50–2,500 ms	10–15%
Scribing based									
Pin printing	VitroJet[89]	Dip pen deposition while maintaining dewpoint	All	0.5 µl	1 nl	Yes	Yes	1–5 s	15–25%
Capillary writing	Cryowriter[92]	Capillary deposition with controlled evaporation or reaspiration	All	15–25 nl	0.1 nl	Yes	Yes	1–3 s	10–20%

Weissenberger, et al, Nature Methods, 18, 2021, p463–471

Ice forms

Cubic
3.66 /2.24
/ 1.91A

Amorphous ice
3.7A/2.1A

(a) (b) (101) (110) 1 0·2 nm (c) 5 µm

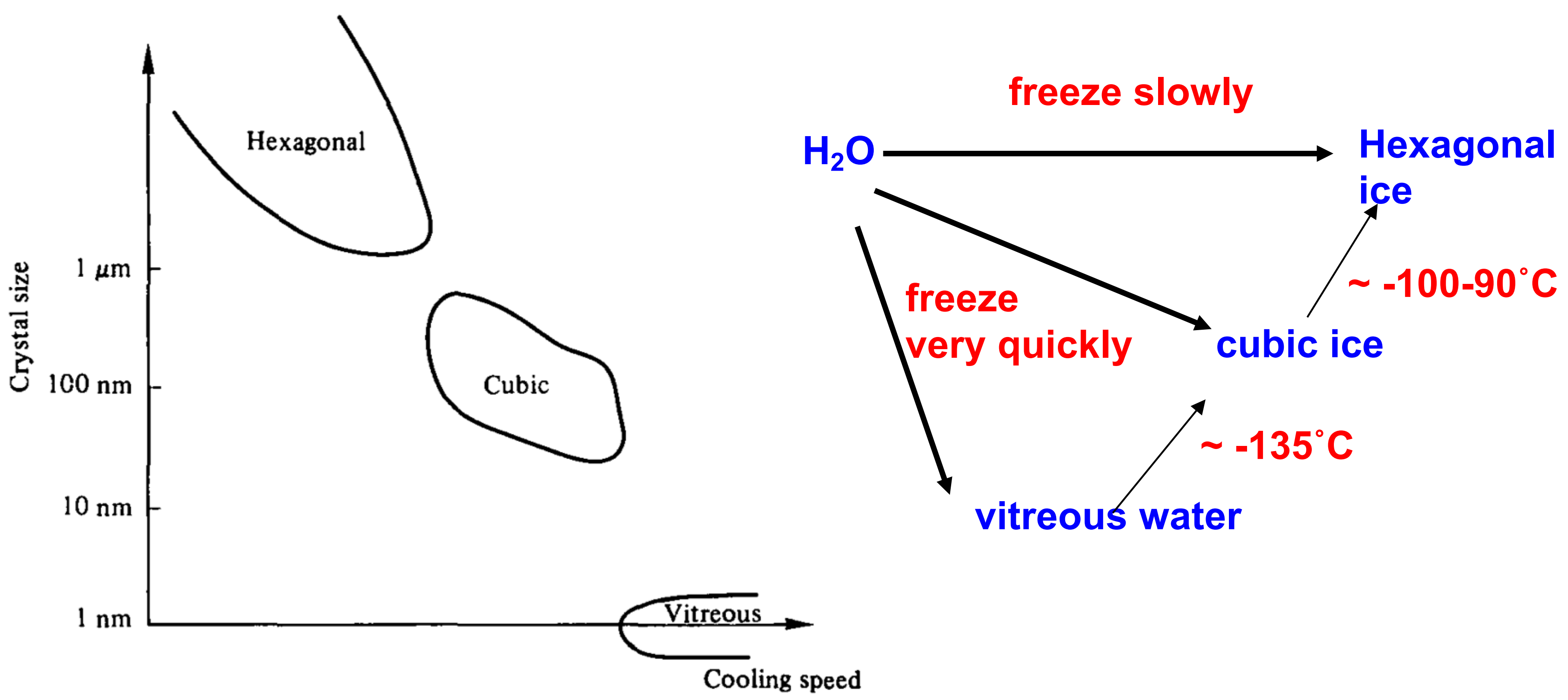
Fig. 5. Typical images and electron diffractograms of three forms of solid water observed in the electron microscope. The direct images and their diffractograms are all printed at the same scale, (a) Hexagonal ice obtained by **rapid freezing** of a water layer on a carbon film. The diffractograms, obtained from other specimens, show the (110) and (101) plane, (b) Cubic ice obtained by **warming a layer of vitreous water** obtained by condensation. The shoulder on the (111) reflection, possibly indicating the presence of a small amount of hexagonal ice, is marked by an arrow, (c) Vitreous water obtained in the microscope, by **condensation of vapour on a cold carbon film** supporting **polystyrene spheres**.

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

Cooling speed is the key for amorphous ice

- Vitreous ice:
  An amorphous solid state in which water was frozen without adopting any crystalline structure.



Maintaining low temperature is critical

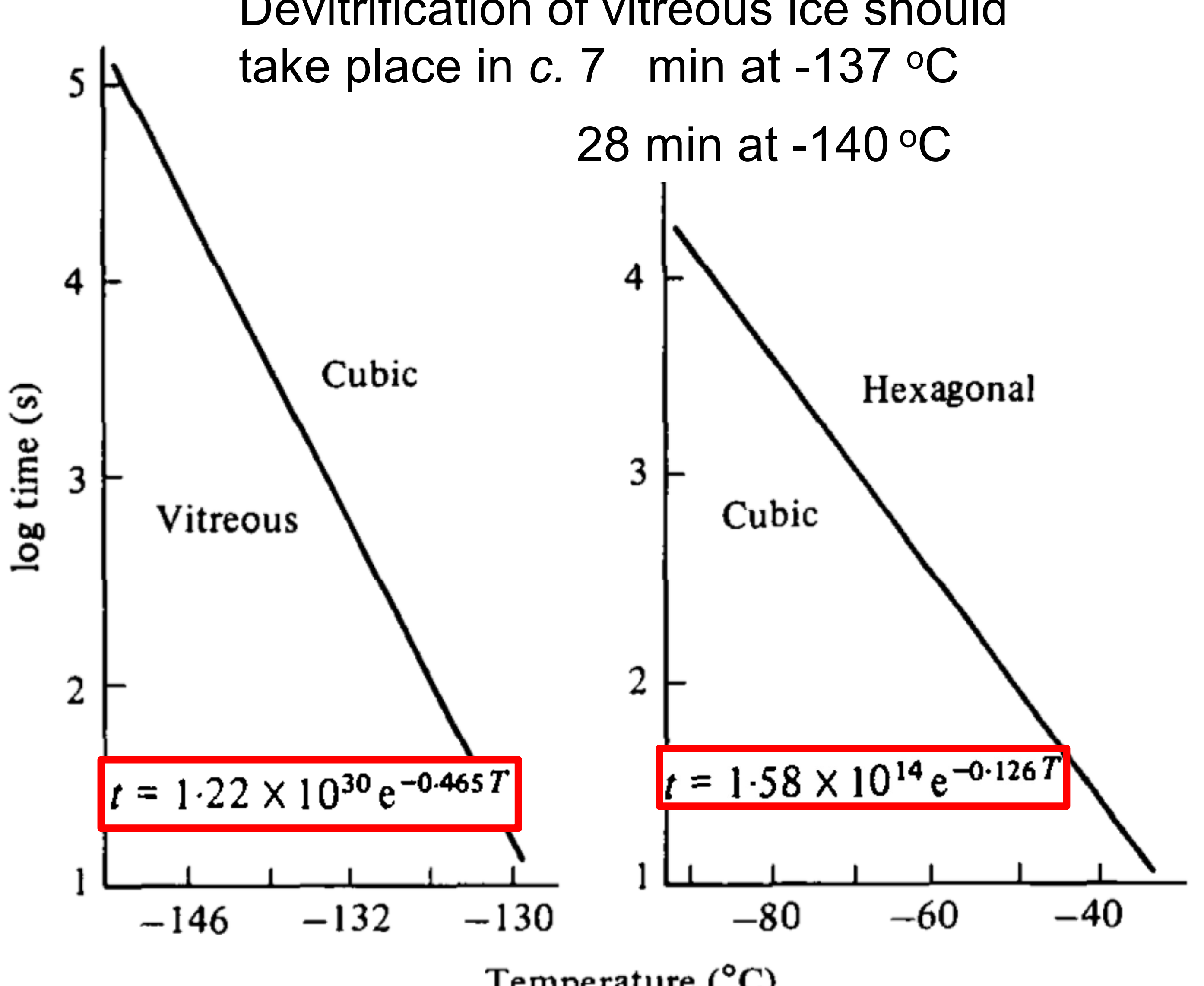


Fig. 6. Time t required, at the temperature T (° C), for the phase transition from (a) vitreous water to cubic ice and (b) cubic to hexagonal ice to take place (Dowell & Rinfret, 1960). Recent results suggest that curve (a) should be displaced by 10-20 ° C towards the higher values (see text).

Dubochet, J., Adrian, M., Chang, J. J., Homo, J. C., Lepault, J., McDowall, A. W. & Schultz, P. (1988). Cryo-electron microscopy of vitrified specimens. Quarterly Reviews of Biophysics, 21(2), 129-228.

Ice contamination

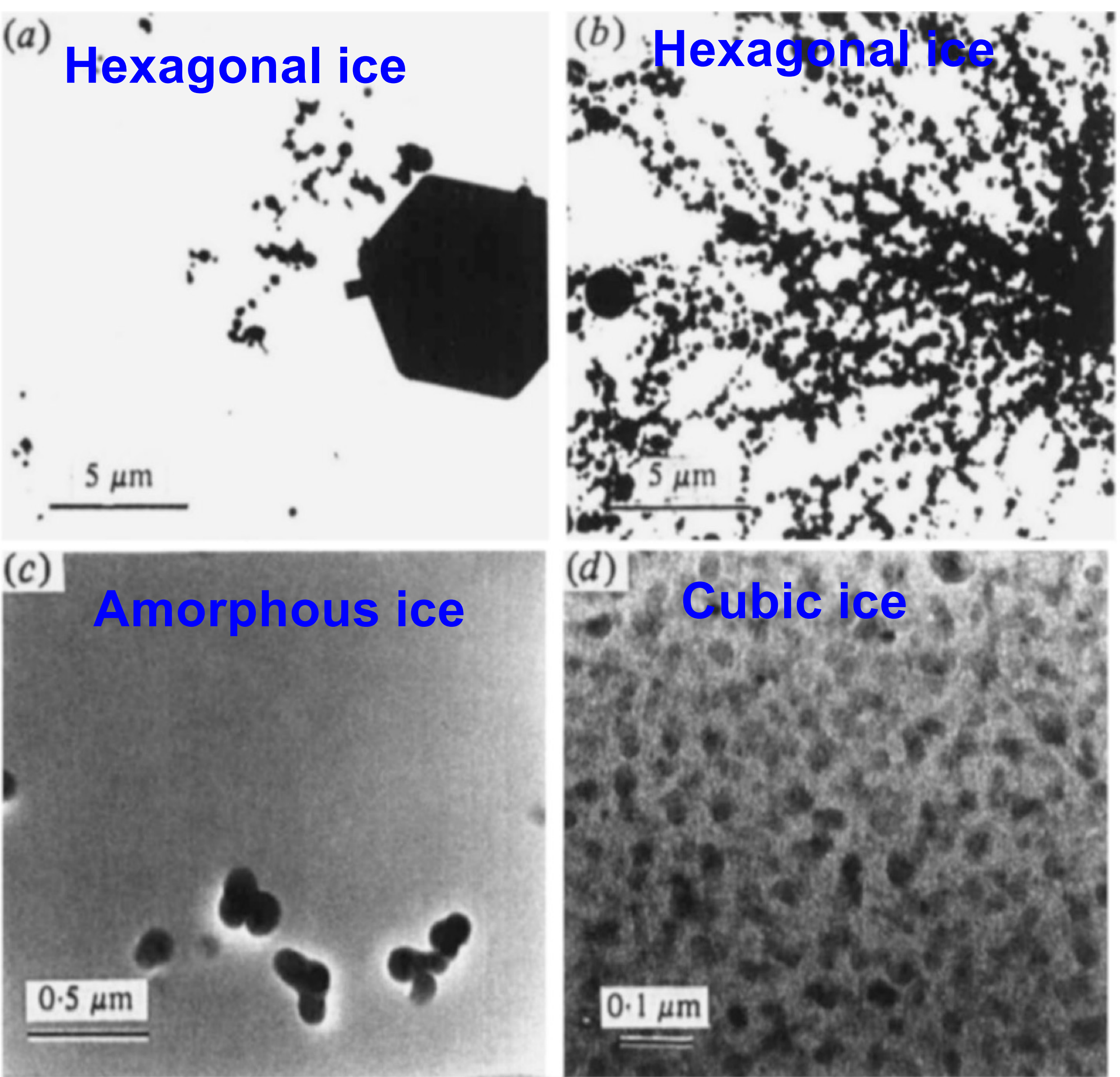
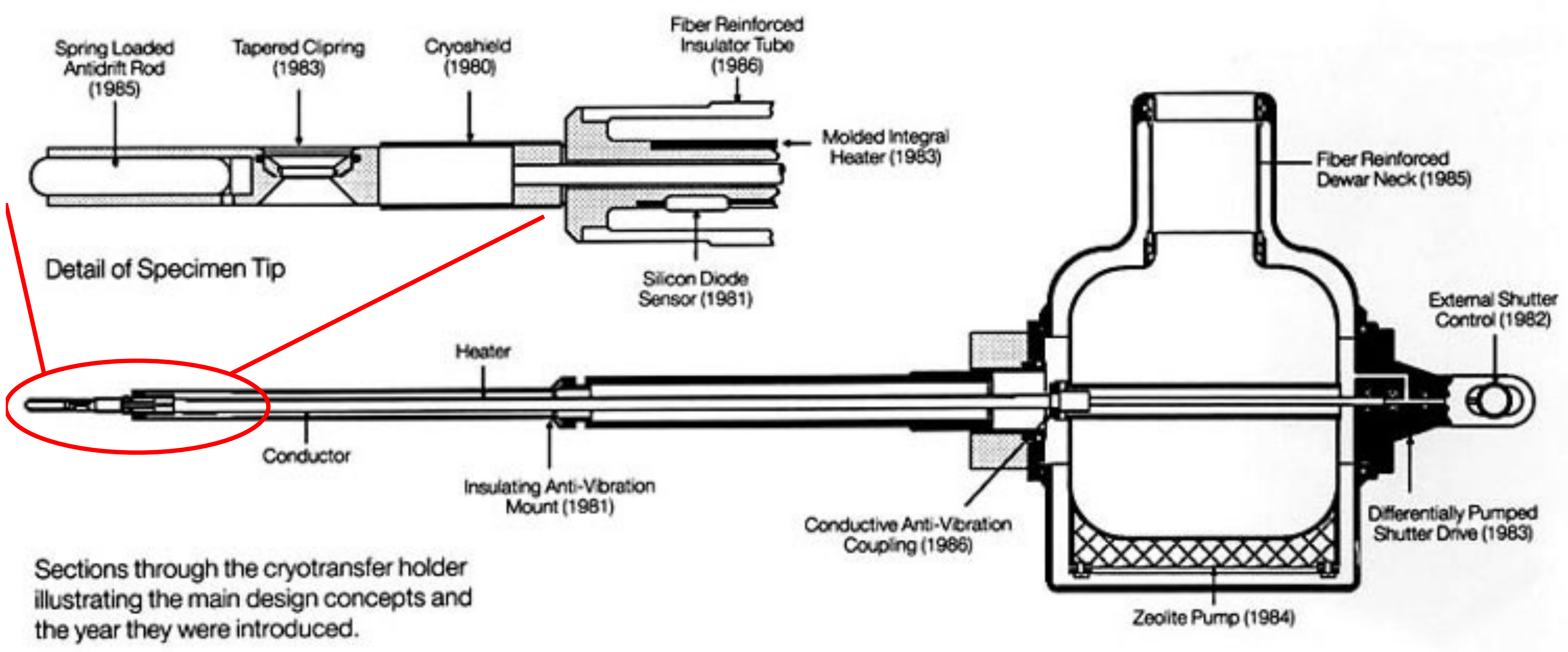


Fig. 32. Various forms of contamination on the specimen, *(a)* **Hexagonal ice** crystals **formed in humid air** and deposited during preparation of the specimen (in particular in the cryochamber of the microtome) and during transfer, *(b)* Agglomerate of **hexagonal ice** crystals **formed by humid air condensing on liquid nitrogen**, *(c)* Layer of **vitreous water deposited in the microscope**, on a thin vitrified film. **Hexagonal ice** crystals deposited on the specimen have been shadowed, thus revealing the contaminating layer and demonstrating that the water molecules came predominantly from one direction, *(d)* Crystals of **cubic ice** formed by deposition of water vapour in the microscope in a similar but **more rapid way** than in *(c)*.

Dubochet, J., Adrian, M., Chang, J. J., Homo, J. C., Lepault, J., McDowall, A. W. & Schultz, P. (1988). Cryo-electron microscopy of vitrified specimens. Quarterly Reviews of Biophysics, 21(2), 129-228.

Cryo-holder to keep samples cold

Cryo-holder to keep sample cold



Sections through the cryotransfer holder illustrating the main design concepts and the year they were introduced.

Cryo holder transfer station

Bubbling of ice during imaging

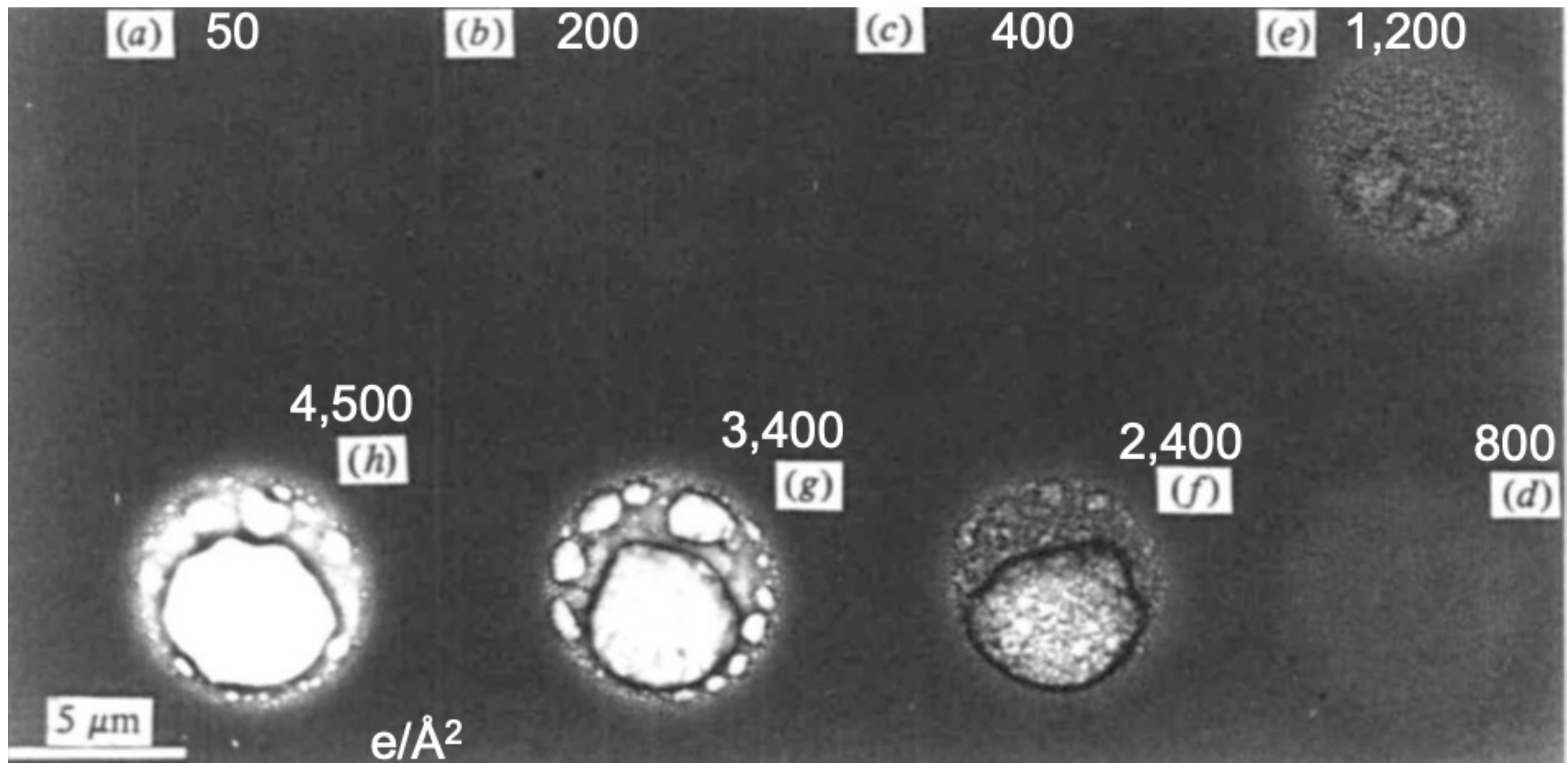
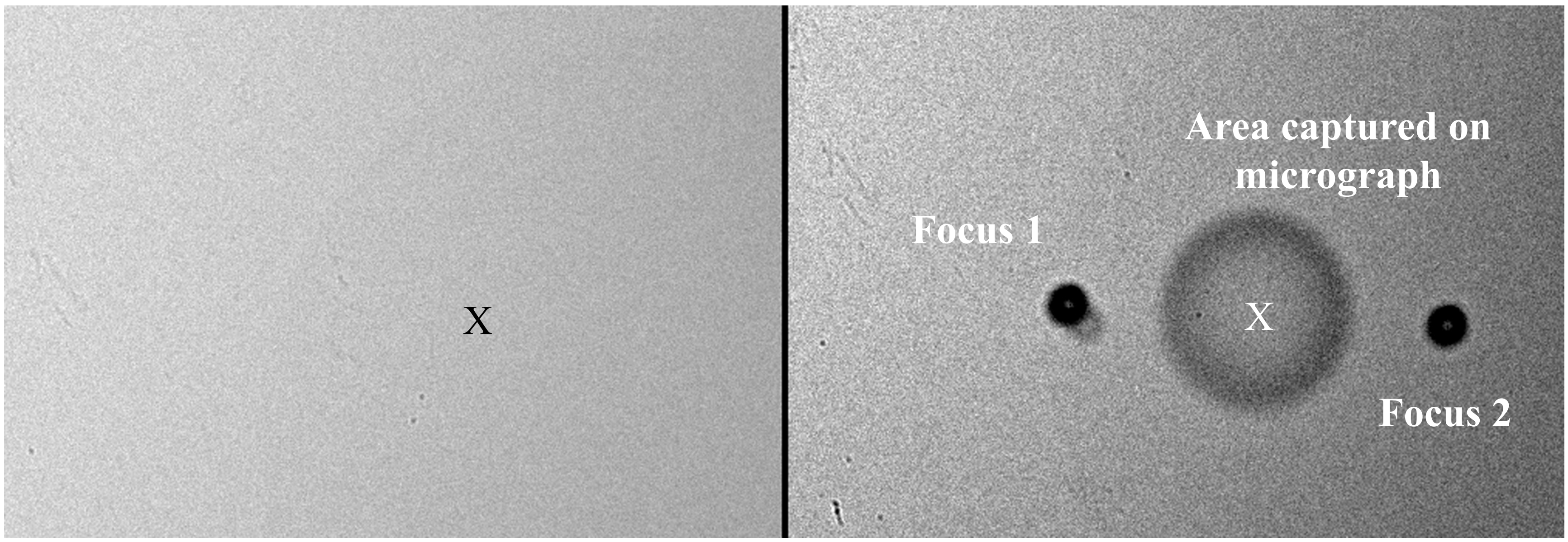


Fig. 37. Bubbling on a **carbon-coated formvar film** c. 10 nm thick, covered with a layer of condensed vitreous water. Fields (a)-(h) correspond to irradiations by 5, 20, 40, 80, 120, 240, 340 and 450 ke/nm2 respectively. The total thickness of the specimen is 160 nm.

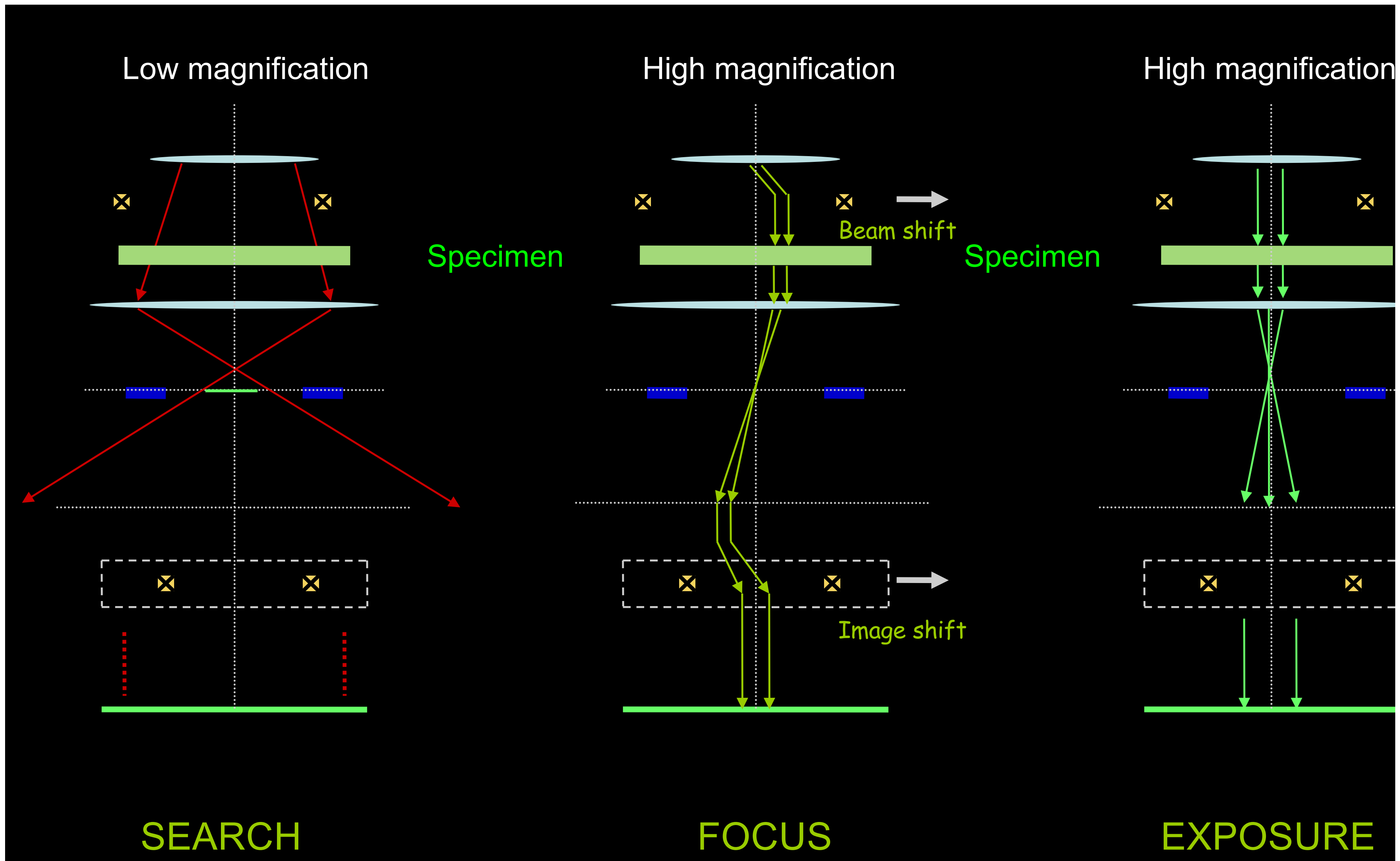
Dubochet, J., Adrian, M., Chang, J. J., Homo, J. C., Lepault, J., McDowall, A. W. & Schultz, P. (1988). Cryo-electron microscopy of vitrified specimens. Quarterly Reviews of Biophysics, 21(2), 129-228.

Low dose to reduce radiation damage



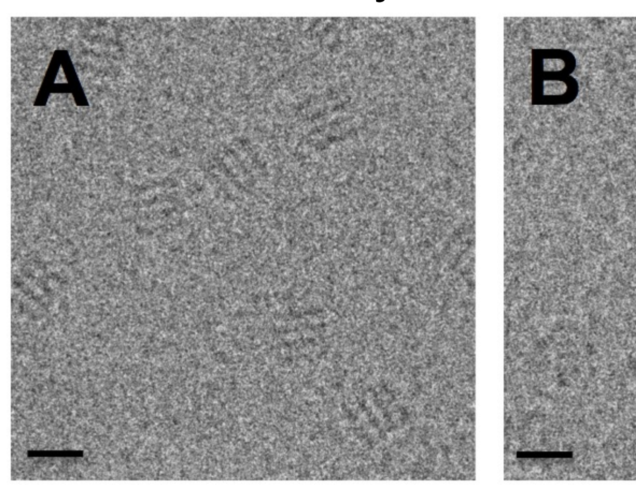
Appearance of trehalose dried down on a carbon film (left). The sugar allows to demonstrate how "low-dose" microscopy is done (right). Let X be the area of interest (for instance a crystal or virus/single particle). Prior to taking a picture some parameters such as "defocus" and "astigmatism" need to be adjusted. To avoid destruction of the specimen, any adjustments are made on small areas (Focus 1 and 2) located adjacent to the area that will be photographed. In the example, the trehalose burned as it was exposed at high magnification (220kx, Focus 1 and 2). Similarly, by exposing the area to be captured for about 30 seconds at 52,000 fold magnification.

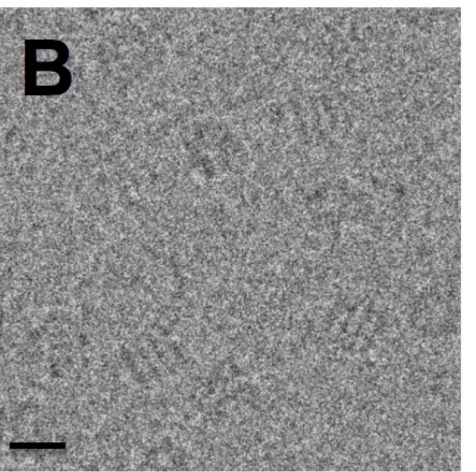
Electron optics of Low-Dose imaging

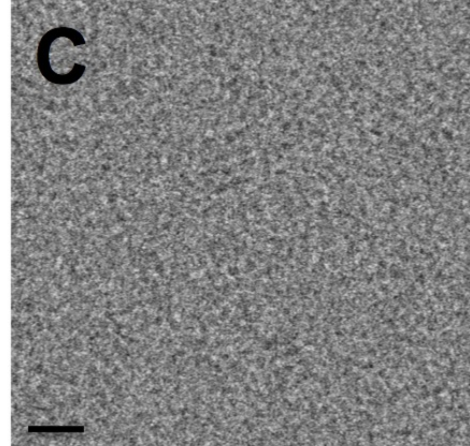


Ice thickness affects image contrast significantly

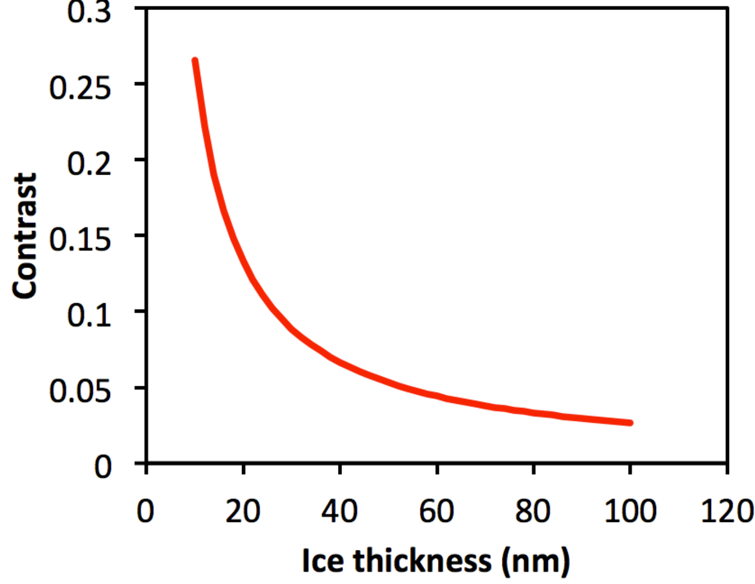
Cryo-EM images of GroEL







The reported ice thickness shown in B and C are 150% and 200% of the ice thickness shown in A.

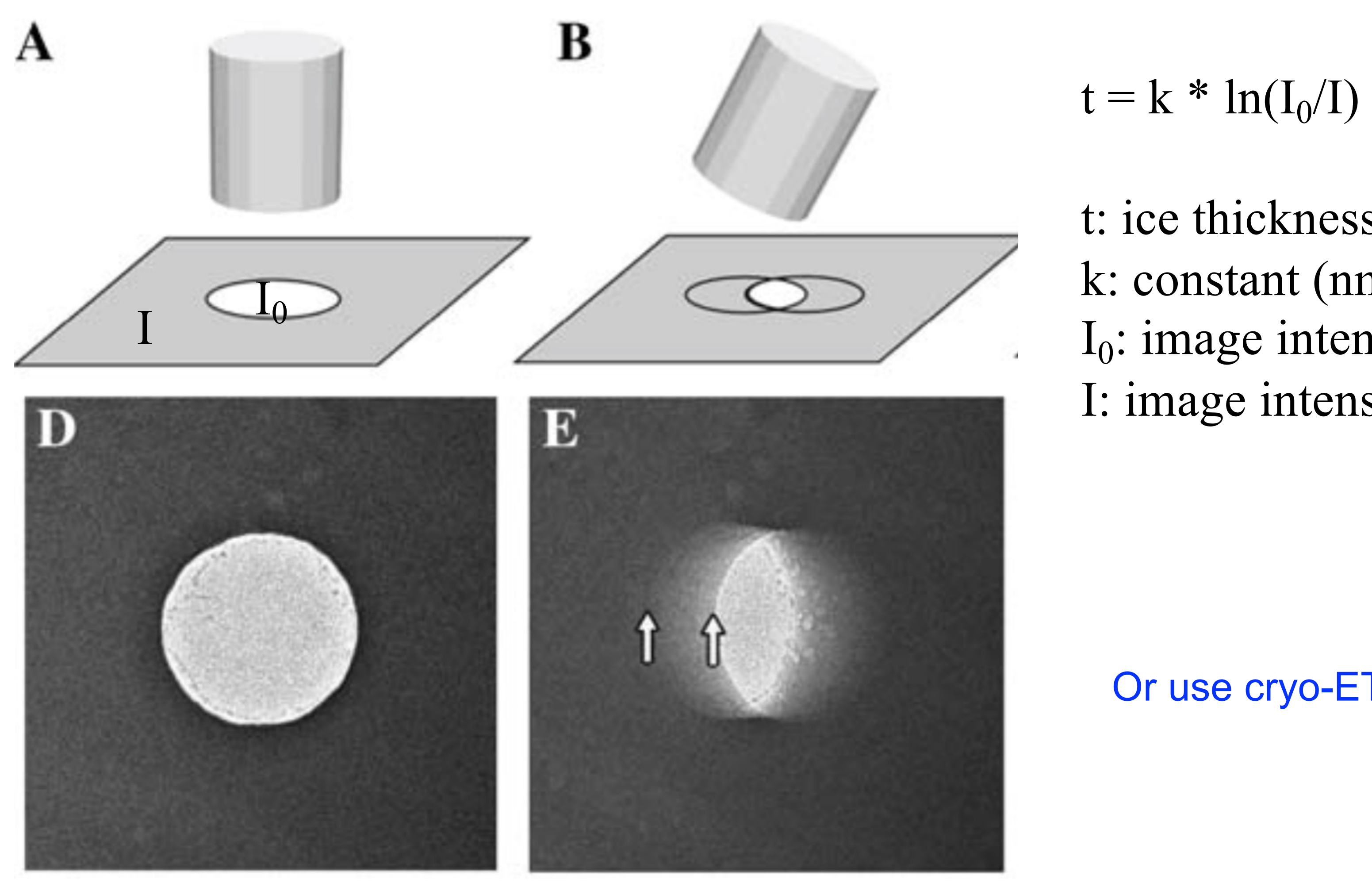


$$C = \frac{\Delta I}{I_s} = \frac{(\phi_{protein} - \phi_{water}) \bullet t_{protein}}{\phi_{water} \bullet t_{ice}}$$

where $\phi_{protein}$ and ϕ_{water} are phase shifts of electrons passing through protein and water regions, and $t_{protein}$ and t_{ice} are thickness of protein molecules and ice layer, respectively

http://nramm.scripps.edu/data/05may19a.

Method to measure ice thickness



$t = k * \ln(I_0/I)$

t: ice thickness (nm)
k: constant (nm)
I_0: image intensity of a hole
I: image intensity

Or use cryo-ET!

Note: important to use really thick ice

A. Cheng et al. / Journal of Structural Biology 154 (2006) 303–311

Measure ice thickness with tomography

Apparent mean free path for inelastic scattering $d = \Lambda \ln \frac{I}{I_{zlp}}$ with and without GIF

Microscope	Voltage (keV)	Slit Width (eV)	Apparent MFP for inelastic scattering (nm)	Method
Titan Krios	300	15	395 +/– 11	Tomography
Titan Krios	300	20	435 +/– 30	Compare with 15 eV

ALS coefficients

$d = \lambda \ln \frac{I_0}{I}$ with and without sample

Microscope	Voltage (keV)	Obj. Aperture diameter (μm)	Lambda (nm)	Method
Titan Krios 20 eV slit	300	100	322	compare with EF determination
Titan Krios (no EF)	300	100	3,329	Aldolase thickness
Titan Krios (no EF)	300	none	78,788 *	Aldolase thickness
Tecnai F20	200	100	392	Tomography
Tecnai F20	200	70	302	Compare with 100 μm
Tecnai T12	120	100	319	Tomography
Tecnai T12	120	70	247	Compare with 100 μm

W. Rice, et al. J Struct Biol. 2018 October ; 204(1): 38–44

Ice thickness measurement on Krios at LBMS

y = 390.51x + 0.0095
$R^2 = 0.9999$

Ice thickness (nm)

0
50
100
150
200
250
300
350
400

0
0.2
0.4
0.6
0.8
1
1.2

ln(I0 / I_15eV_noOA)

$t = k * \ln(I_0/I)$

t: ice thickness (nm)
k: constant (nm)
I_0: image intensity of a hole
I: image intensity

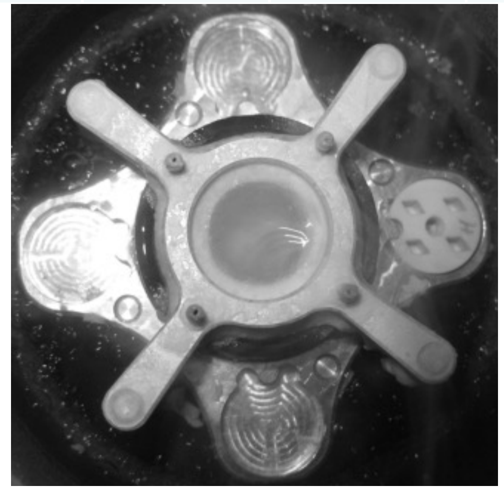
Advanced topics

- Ethane and propane mixture
- Glycerol in sample
- Air-water interface

Ethane and Propane mixture

- **Do not solidify** when at the temperature of liquid nitrogen
  63% propane and 37% Ethane: -196 ^{o}C melting temperature

	Melting Point (^{o}C)	Boiling Point (^{o}C)	Heat of vaporization (kJ/kg)	Heat capacity (kJ/(kg·K)	Heat to boil (kJ/kg)	Heat to evaporate (kJ/kg)	Liquid density (kg/m3)
Nitrogen	-210	-196	6	0.9-1.6	13-22	19-28	809
Ethane	-183	-89	489	2.3-3.5	216-329	705-818	546
Propane	-188	-42	428	1.63	238	666	580
Water	0	100	2257	4.185	418.5	2675.5	1000



- Ethane cooled directly with LN2: solidify completely
- Ethane insulated from LN2: solid ethane melts and at unknown temperature

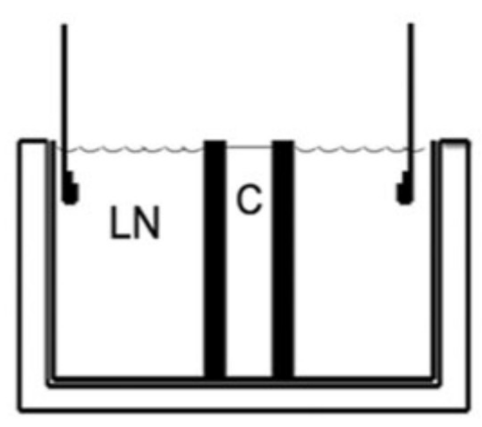
Cheng, D., Mitchell, D., Shieh, D.-B., & Braet, F. (2012). Practical Considerations in the Successful Preparation of Specimens for Thin-Film Cryo-Transmission Electron Microscopy.

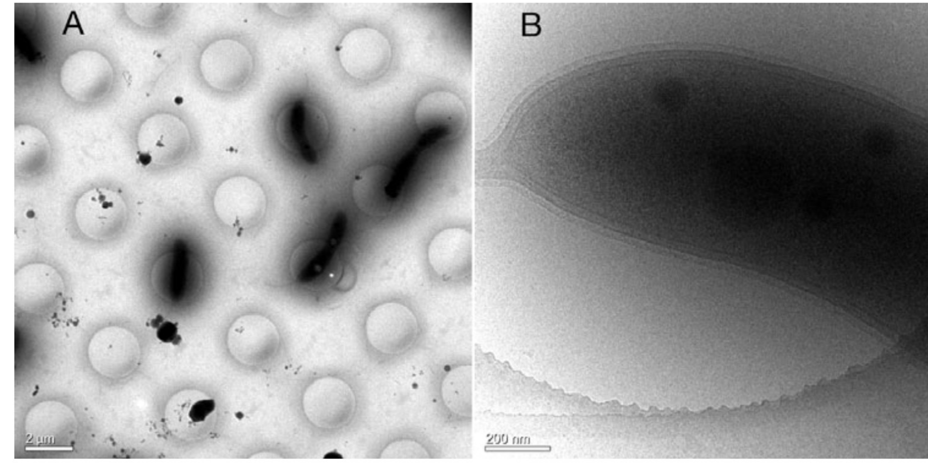
Ethane and Propane mixture

- **Do not solidify** when at the temperature of liquid nitrogen
  63% propane and 37% Ethane: -196 °C melting temperature

Thermally isolated cup

Direct contact of the cup with LN2





Freezing with Pr-Et using Vitrobot. bacterium embedded in amorphous ice.

Tivol, W. F., Briegel, A., & Jensen, G. J. (2008). An Improved Cryogen for Plunge Freezing. Microscopy and Microanalysis, 14(5), 375-379. doi:10.1017/S1431927608080781

Glycerol?

- Why is it strongly discouraged by cryo-EM community?
  * Decrease image contrast
  * Increase beam-induced motion
  * Increase sensitivity to radiation damage (”bubbling”)

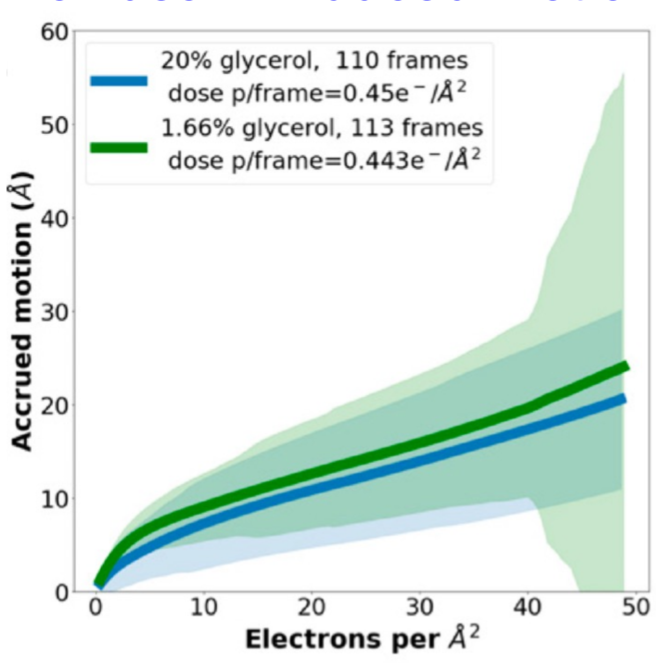
- Recent investigation:
  - With up to 20% glycerol, high resolution structure can be determined: 2.3 Å apoferritin, 3.3 Å aldolase
  - Some disadvantages exist

Similar beam-induced motion

20% glycerol

1.66% glycerol

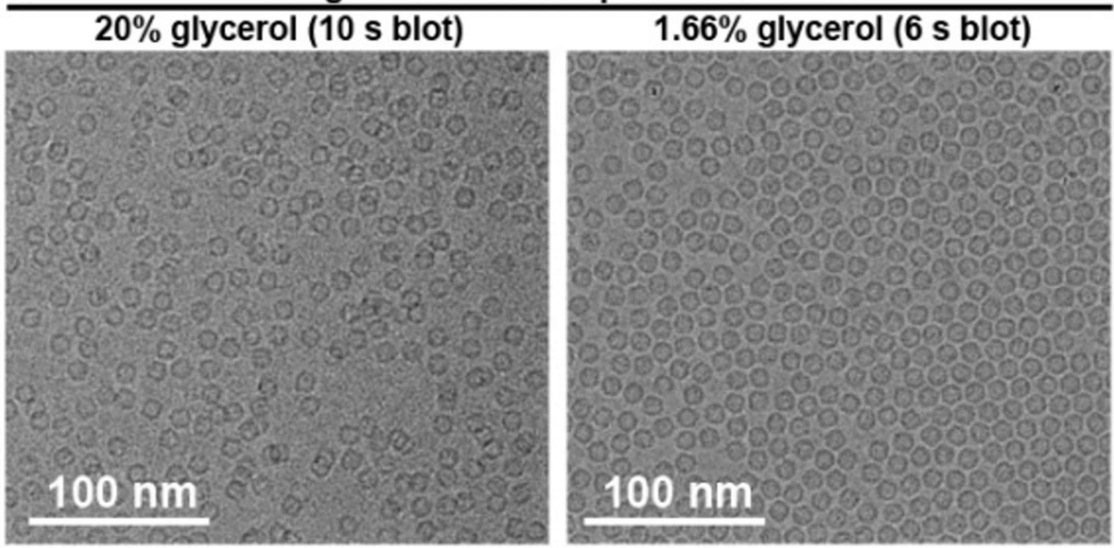
Residues 78-94 of PDB 6V21 fit into density



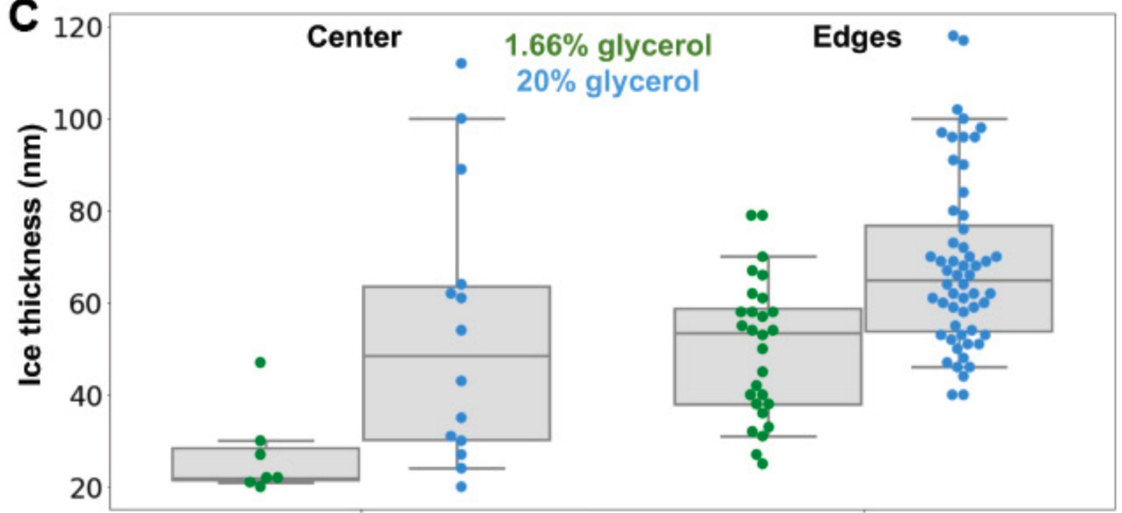
bioRxiv preprint doi: https://doi.org/10.1101/2021.09.10.459874

Glycerol: disadvantages

- Longer blotting time: nearly two-fold for the 20% glycerol sample



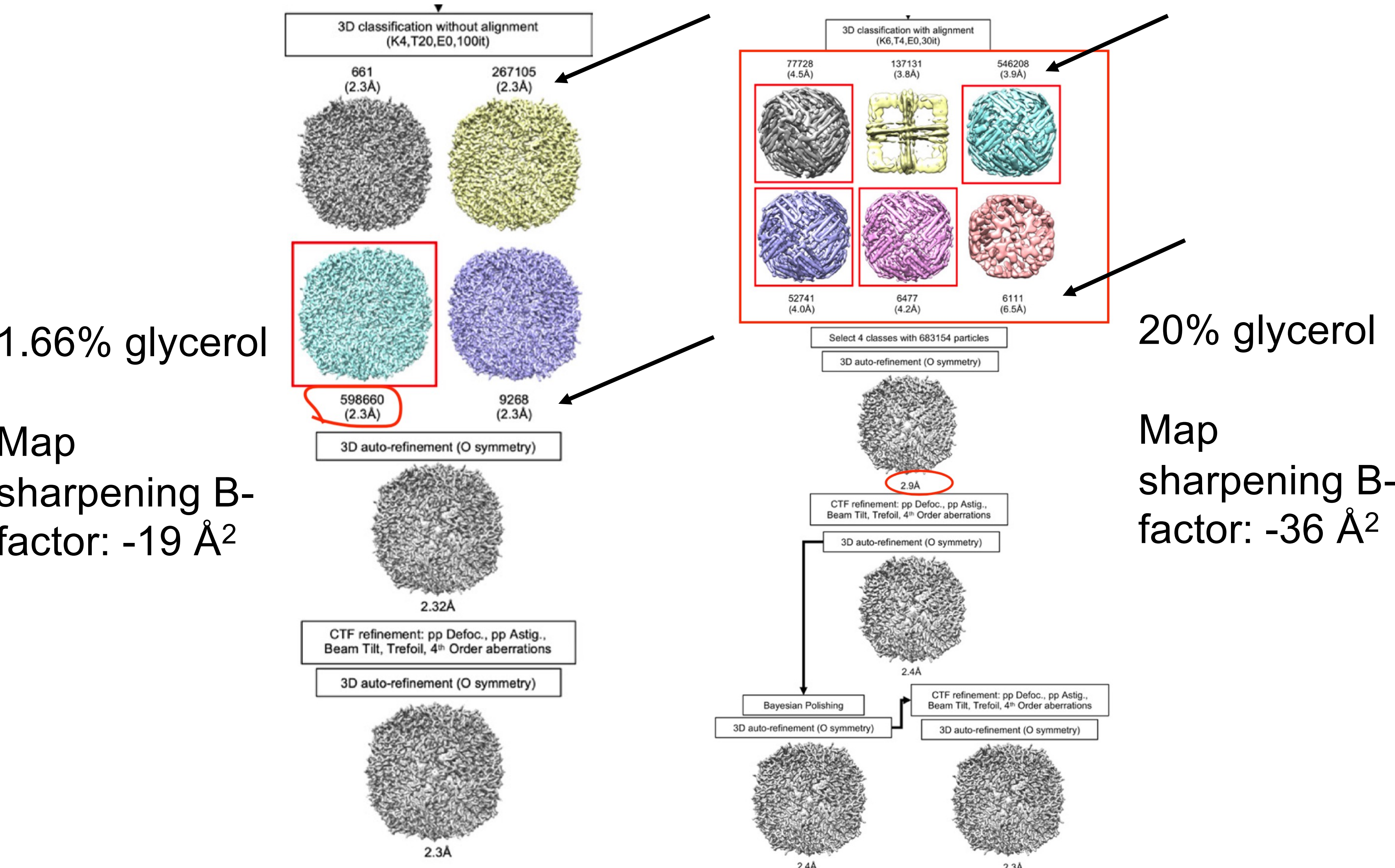
- Thicker ice even with longer blotting time



bioRxiv preprint doi: https://doi.org/10.1101/2021.09.10.459874

Glycerol: disadvantages

- Complicated data processing and larger B-factor: apoferritin @200keV



bioRxiv preprint doi: https://doi.org/10.1101/2021.09.10.459874

Air-water interface

Sample # Name	Example cross-sectional schematic diagram	Sample # Name	Example cross-sectional schematic diagram	Sample # Name	Example cross-sectional schematic diagram	Sample # Name	Example cross-sectional schematic diagram
1* 32 kDa Kinase		14* Neural Receptor		27* IDE		38*† Apoferritin (0.5 mg/mL)	
4*† Hemagglutinin		17* Protein with Bound Lipids (deglycosylated)		30*† GDH		39*† Apoferritin with 0.5 mM TCEP	
5* HIV-1 Trimer Complex 1		18 Protein with Bound Lipids (glycosylated)		31*† GDH		40 Protein with Carbon Over Holes	
6* HIV-1 Trimer Complex 1		19* Lipo-protein		32*† GDH + 0.001% DDM (2.5 mg/mL)		41 Protein and DNA Strands with Carbon Over Holes	
7* HIV-1 Trimer Complex 2		20 GPCR		33*† DnaB Helicase-helicase Loader		42*† T20S Proteasome	
10* Stick-like Protein 1		21*† Rabbit Muscle Aldolase (1mg/mL)		34*† Apoferritin		43*† T20S Proteasome	
12* Stick-like Protein 2		22*† Rabbit Muscle Aldolase (6mg/mL)		35*† Apoferritin		44*† T20S Proteasome	
13* Neural Receptor		25* Protein in Nanodisc (0.58 mg/mL)		36*† Apoferritin		45*† Mtb Proteasome	
				37*† Apoferritin (1.25 mg/mL)		46 Protein on Streptavidin	

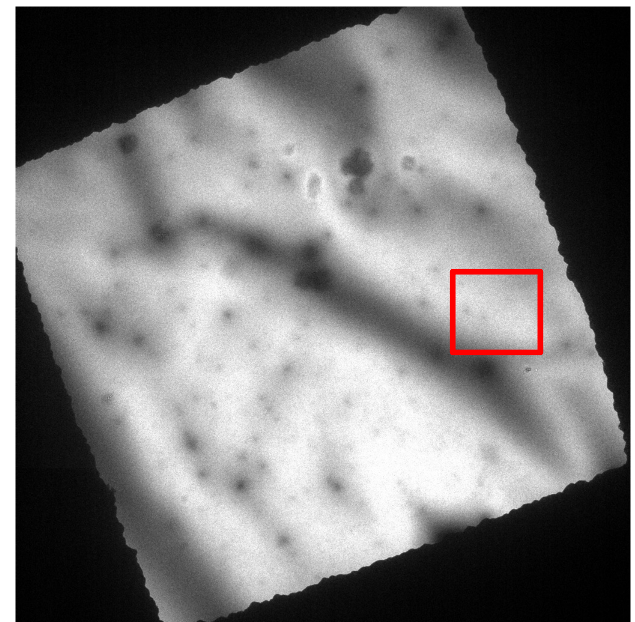
Noble et al. eLife 2018;7:e34257. DOI: https://doi.org/10.7554/eLife.34257

Experience and examples

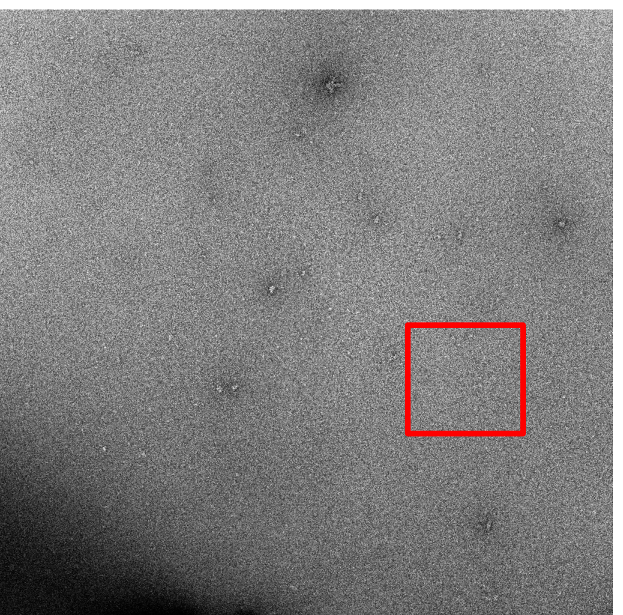
- Good NS sample
- Bad NS sample
- Good cryo-EM samples
- Bad cryo-EM samples
- Weird ice
- Denatured-particle “skin”
- Tips to reduce ice contaminations

Good NS examples: continuous carbon grids

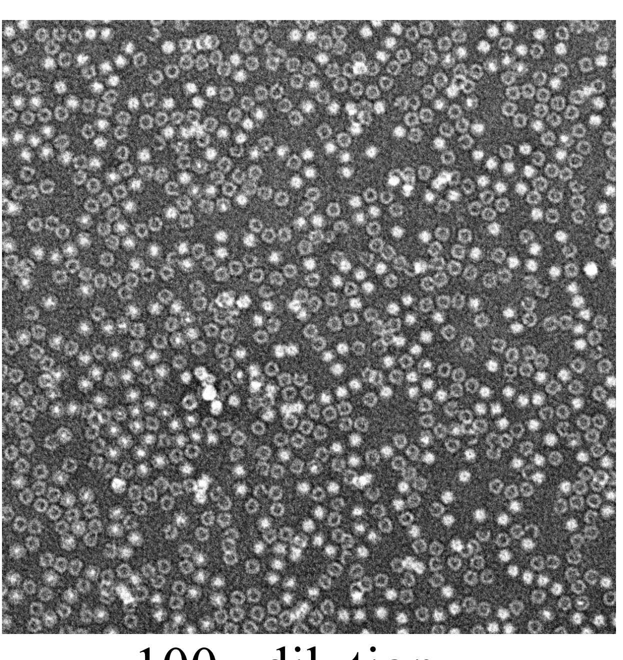
550x



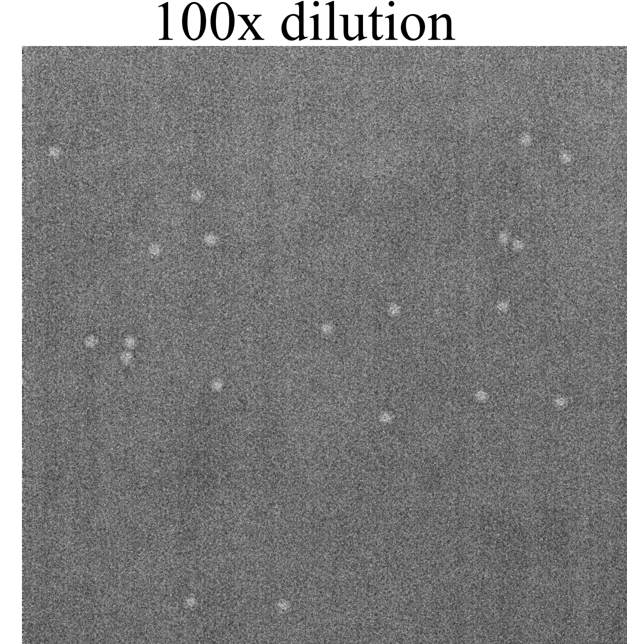
8500x



120kx

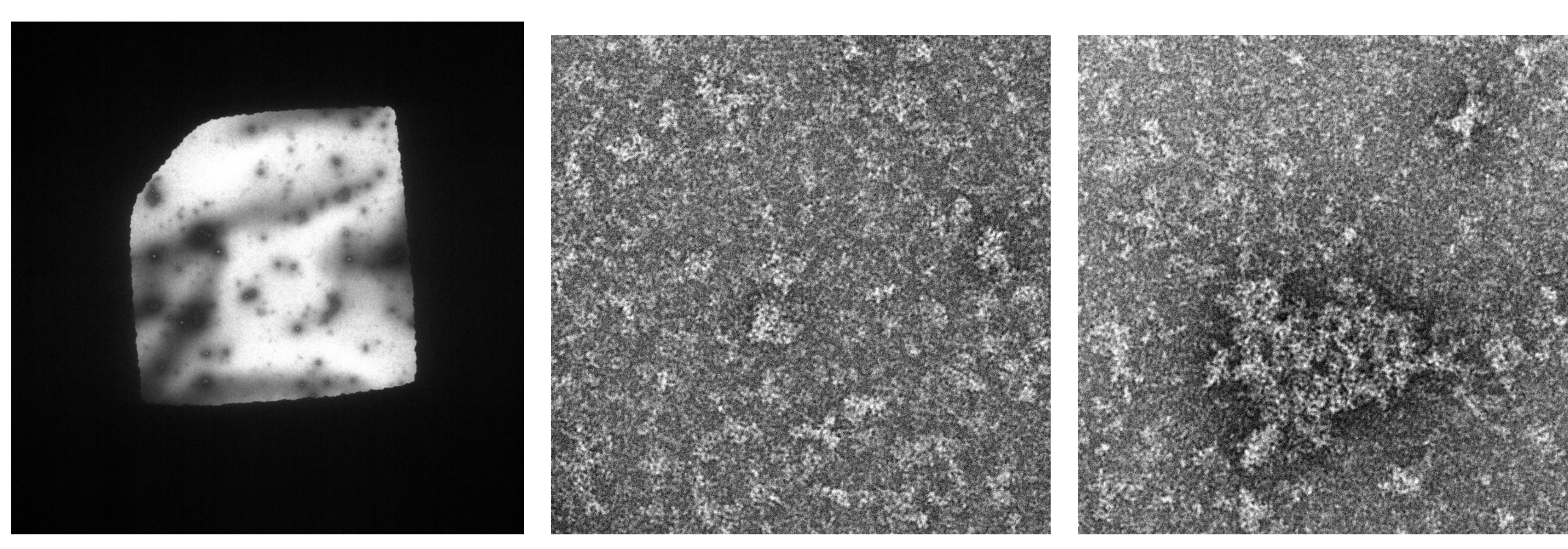


100x dilution



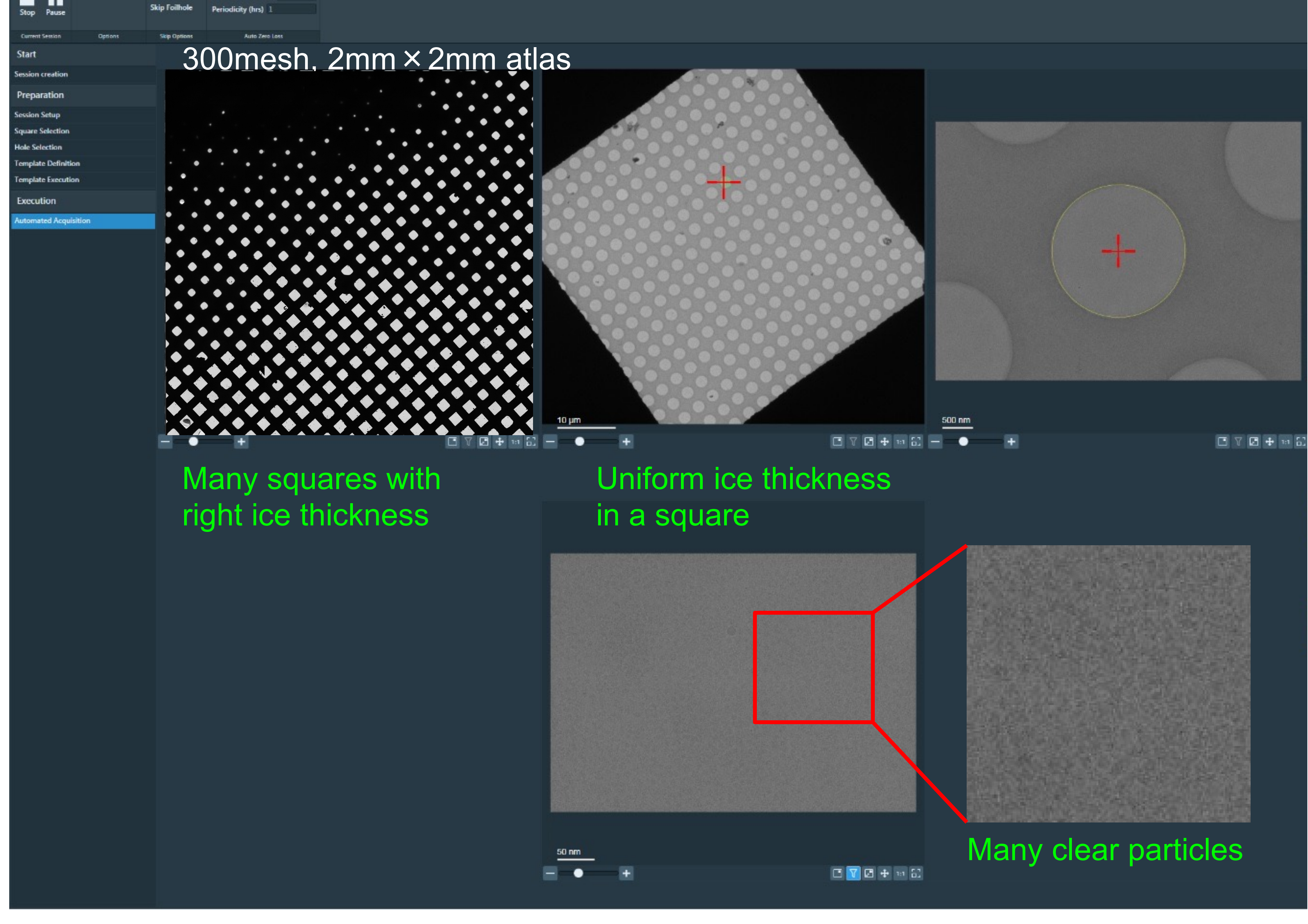
- 400 mesh continuous carbon (recommended)
- 20 mA, 20s glow discharge
- 60s 6uL sample + blot,
  60s Nano-W + blot,
  60s Nano-W + blot to dry

Bad NS examples

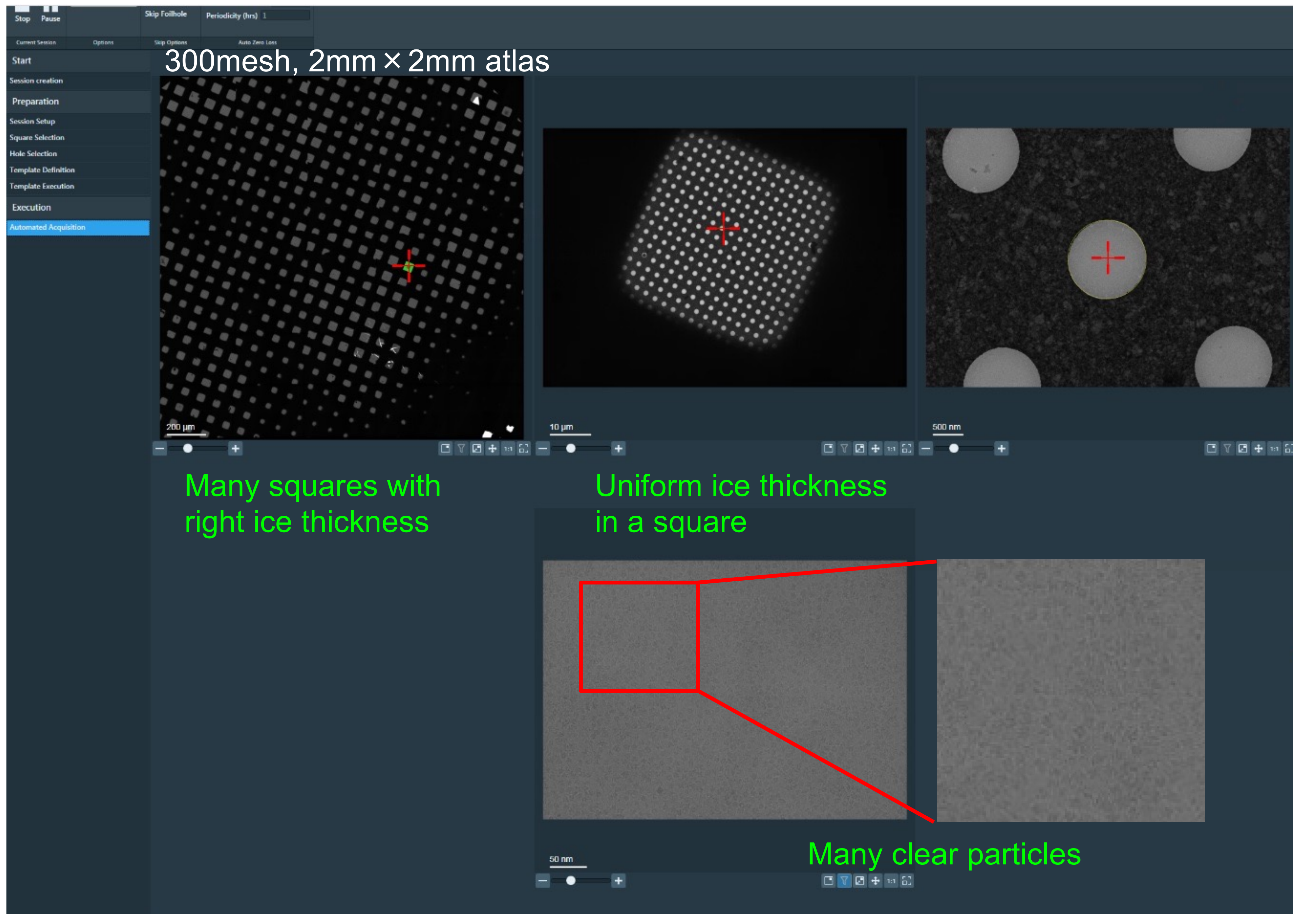


- Heterogenous particles
- Aggregates

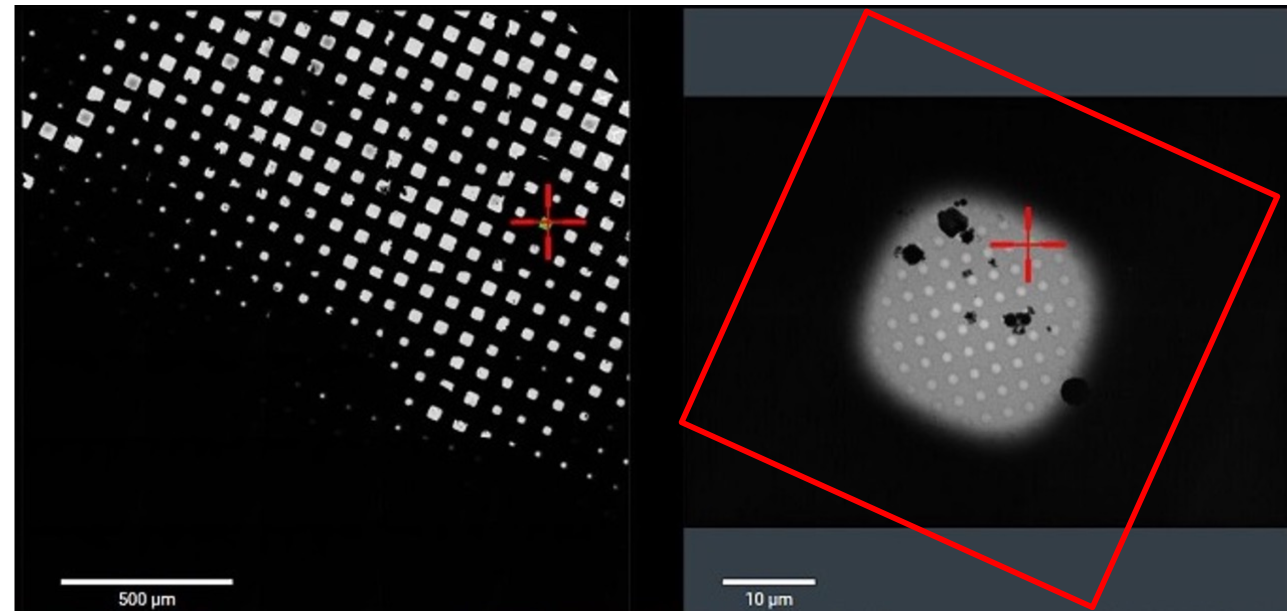
Good examples: holey carbon grids



Good examples: holey gold grids

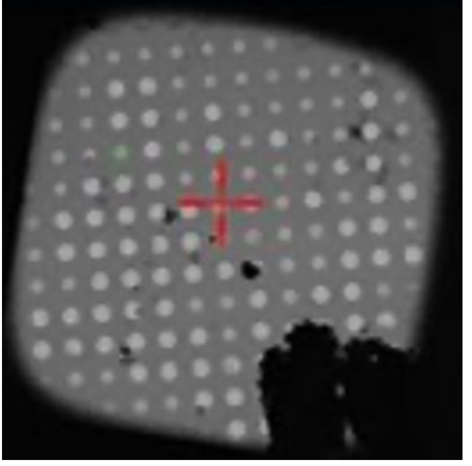


Bad cryo-examples

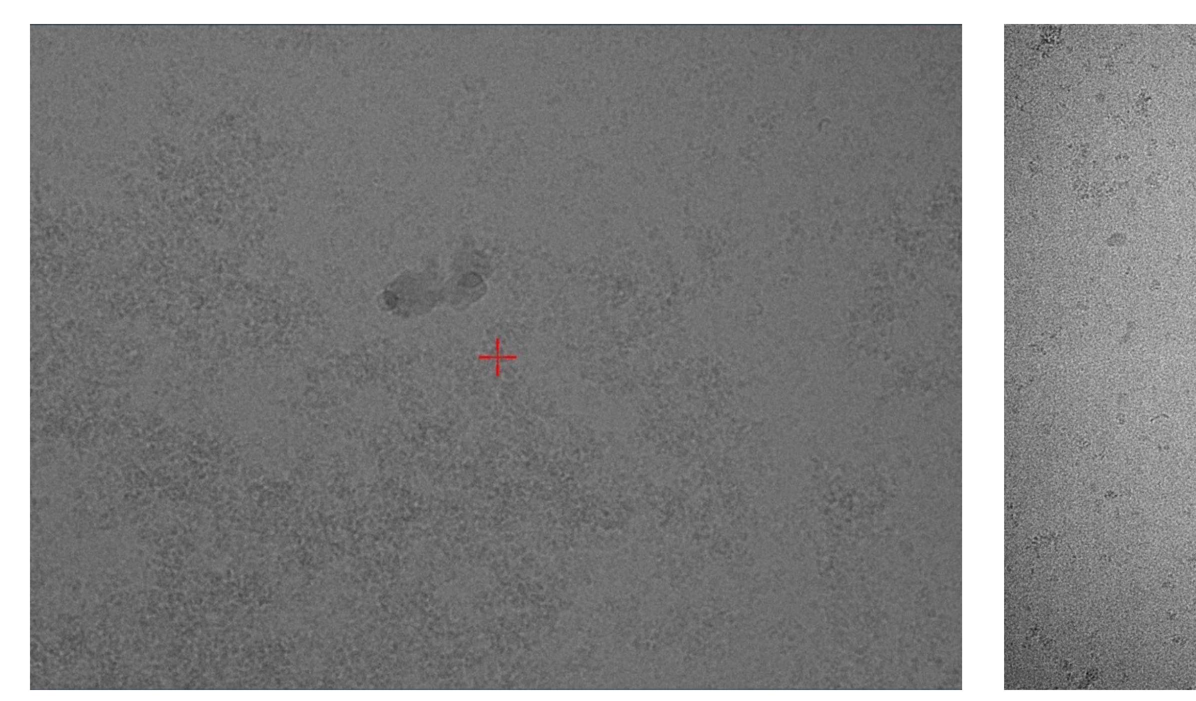


2mmx2mm atlas

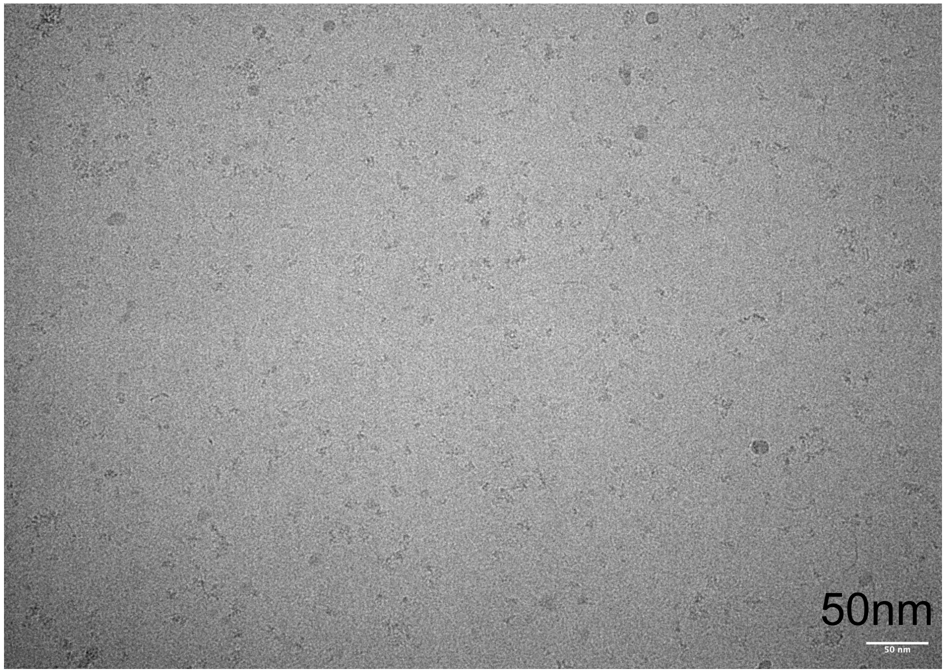
No-uniform ice thickness



Bad cryo-examples

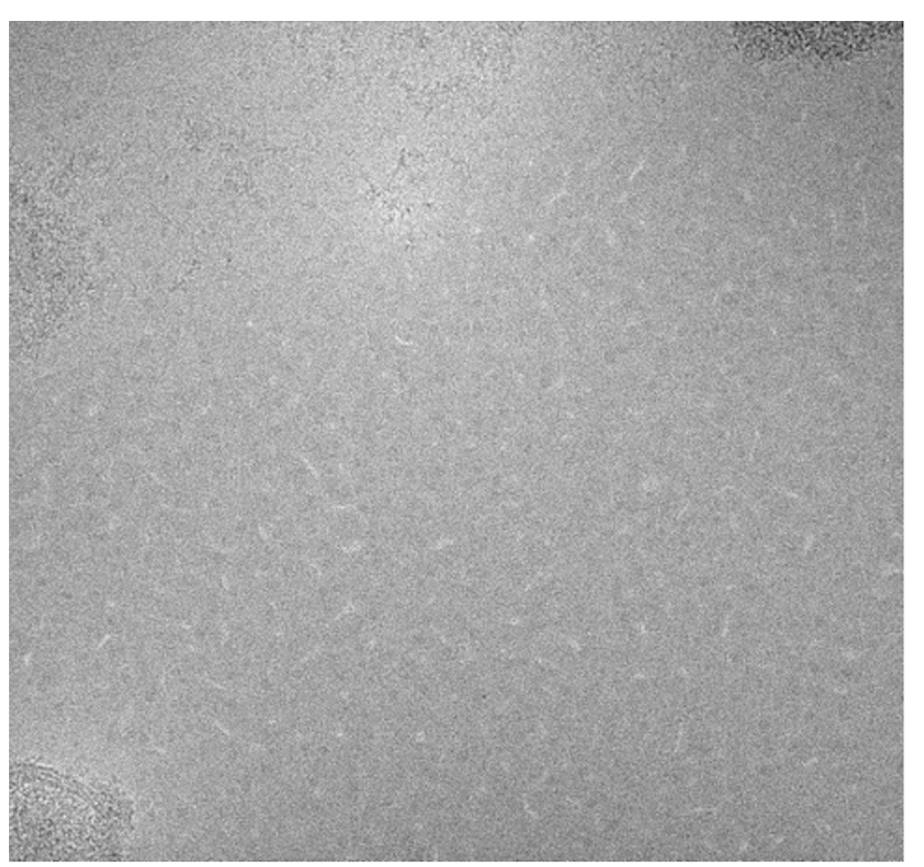


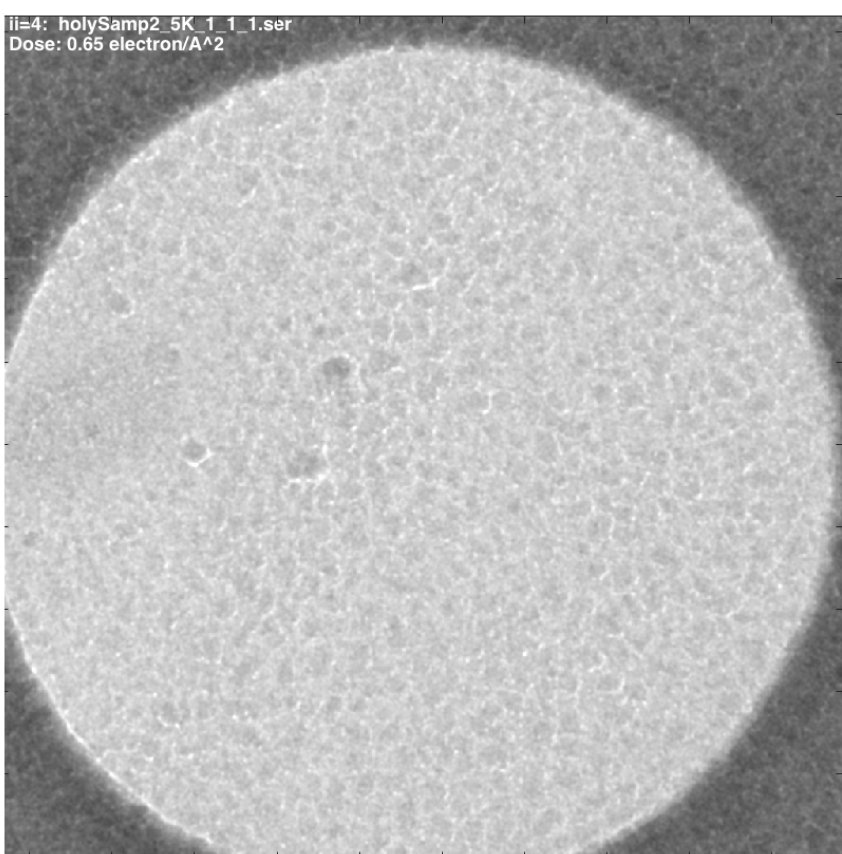
Protein aggregates



Low particle density

“Leopard ice”, "turtle-ice”, “alligator ice”, "dried mud"





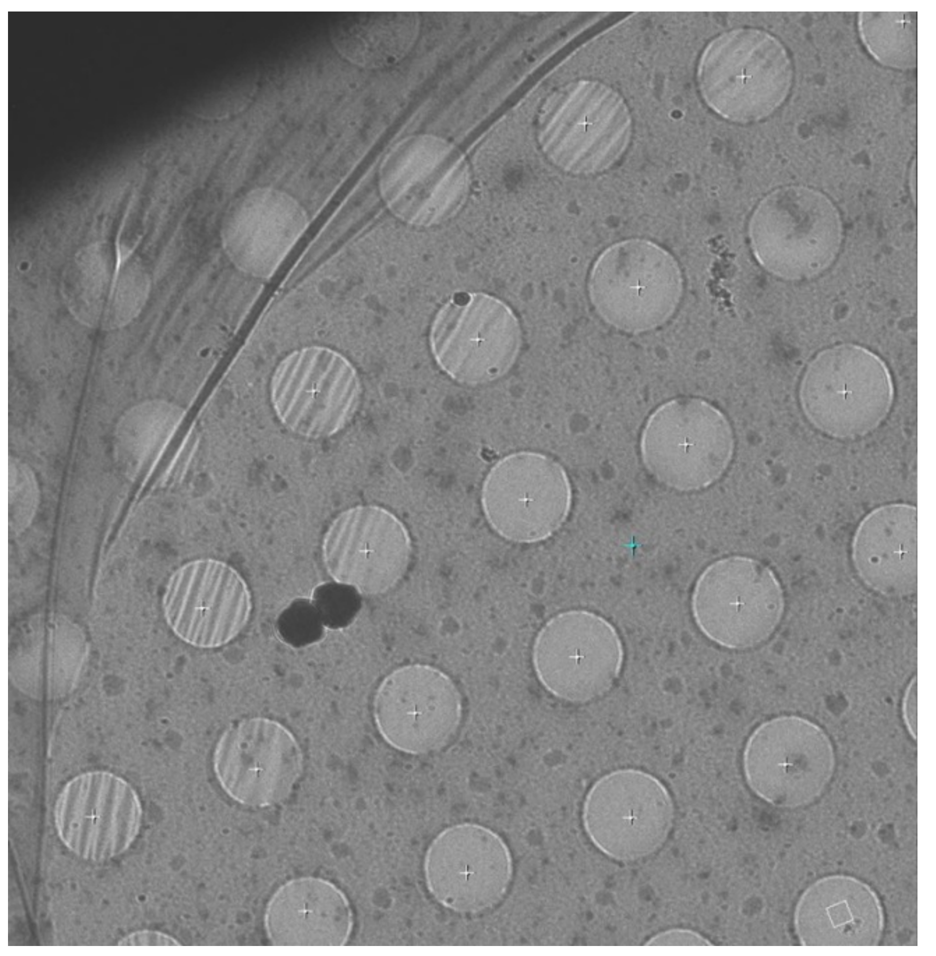
More than one source of the problem and usually not reproducible

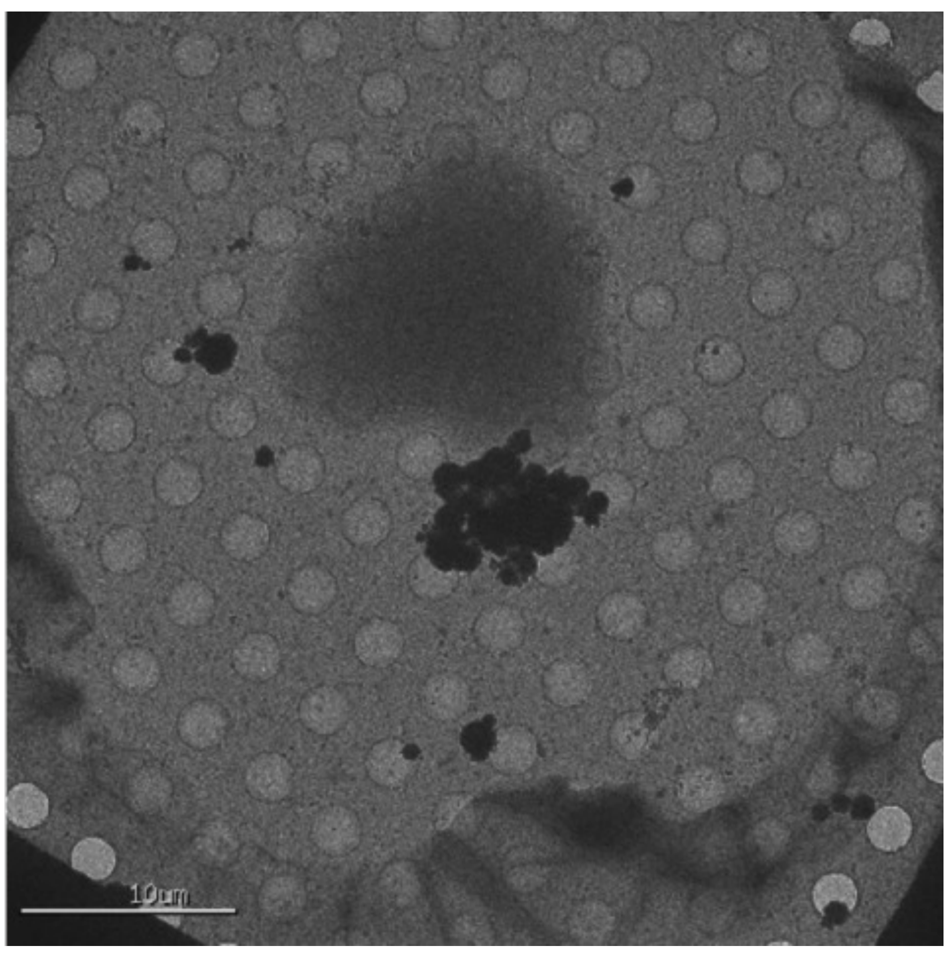
- Transfer of the cryo-holder is likely the most common one
- Not having filled the nitrogen high enough in the dewar
- Not cooling the ethane long enough
- Sample in the EM is not cold enough. Water sublimates and recrystallizes nearby.

Courtesy: Yutong Song

Features observed in cryo-samples

Denatured-particle “skin”: some types of protein can rapidly form a monolayer “skin” of denatured protein at the air-water interface,





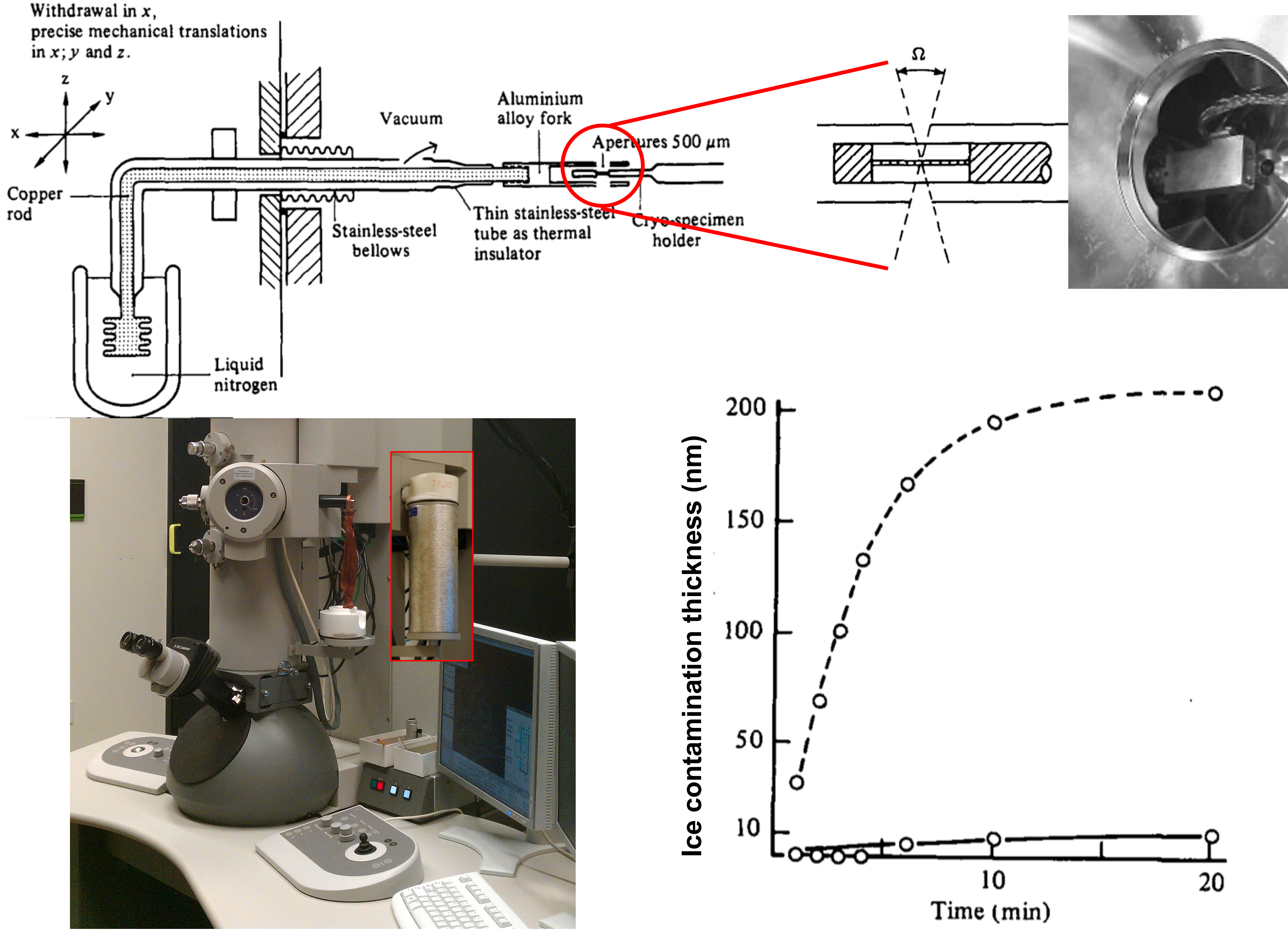
Han, BG et al. J Struct Biol. 2016 August ; 195(2): 238–244. doi:10.1016/j.jsb.2016.06.009.

Cryo-sample preparation tips

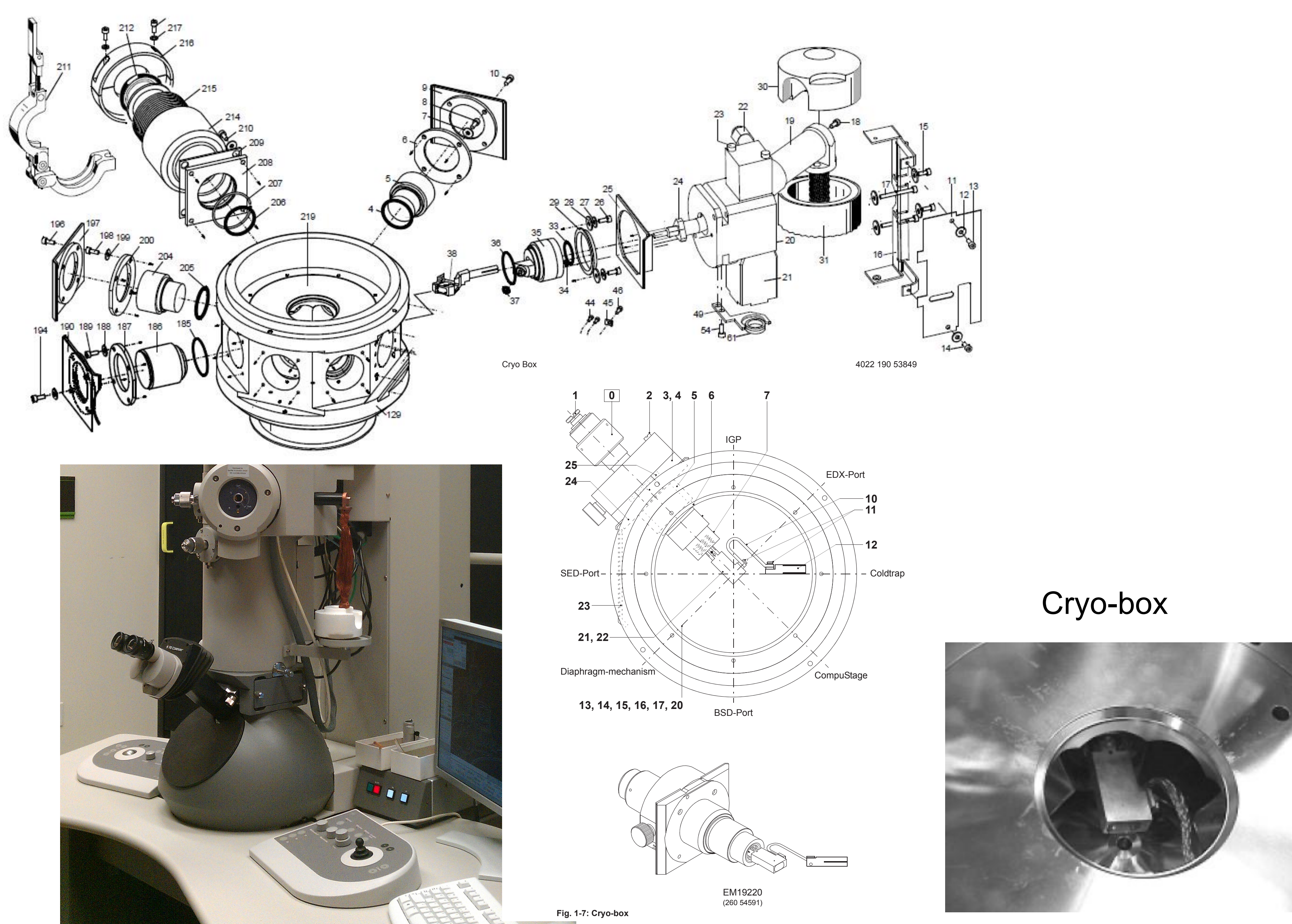
- Really plan your experiment!
- Get everything ready before pouring LN2
  e.g. grid box, storage tube, 1L dewar, tweezers (sample number+1), grids, plunger (tested), filter paper, samples, pipette, pipette tips, timer, long tweezer, screw driver, etc
- Check ethane level to ensure immersion of the grid
- Cover flask containing grid box after topping off the flask, and don’t cover it after starting freezing samples.
- Grids: 400 mesh grids for NS
  300 mesh grids for cryo-samples
- Glow discharge:
  20-30mA, 20 s for carbon / 120 s for gold;
  10 mA, 5s for ultrathin carbon coated holey grids

Thanks!

Anticontaminator

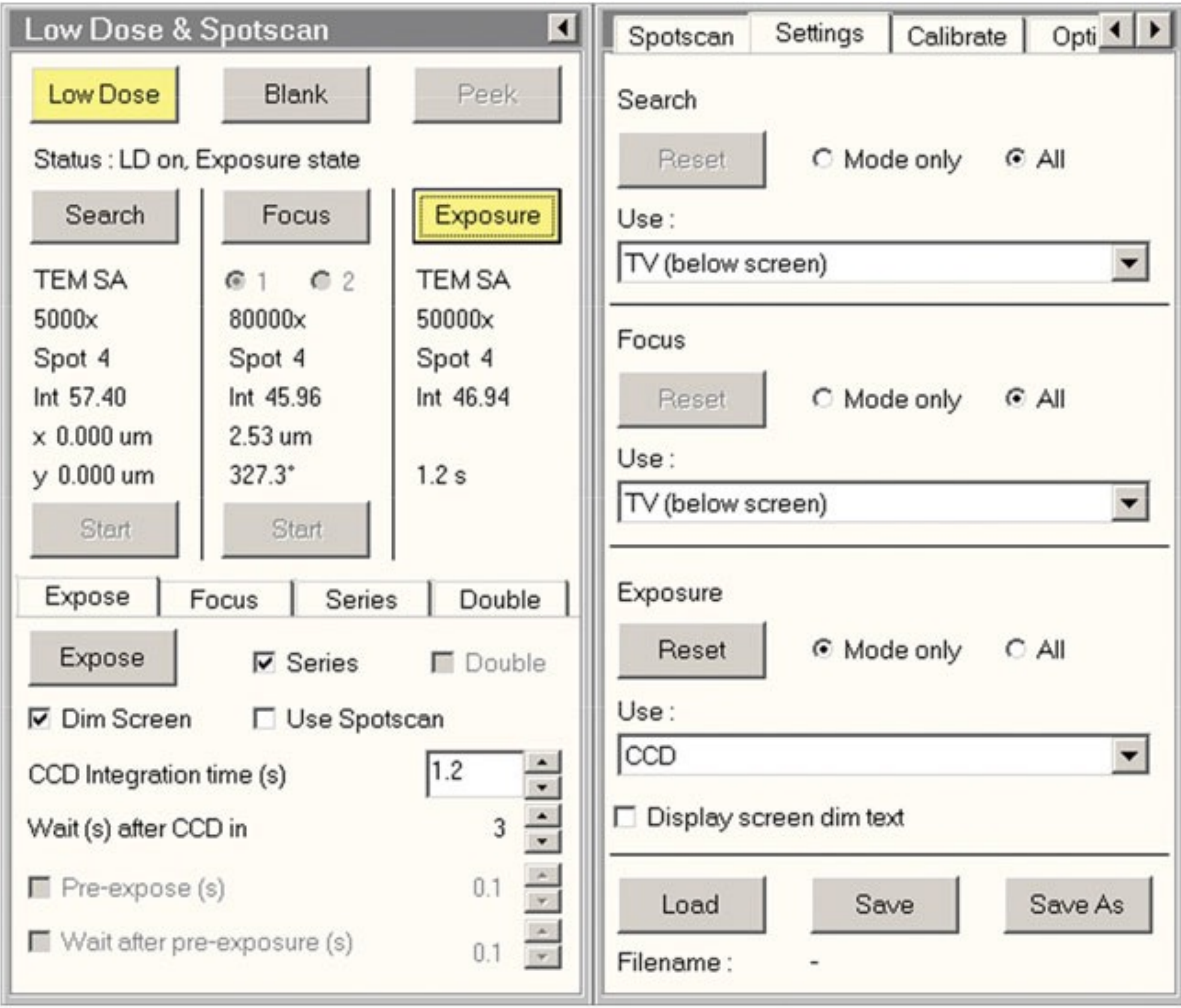


Anticontaminator



Cryo-box

Principle of low-dose microscopy



Glow discharge to change surface hydrophobicity

Atmosphere	Surface Condition	Charge
Air	Hydrophilic	Negative
Air (with subsequent magnesium Acetate treatment)	Hydrophilic	Positive
Amylamine (Pentylamine)	Hydrophobic	Positive
Methanol	Hydrophobic	Negative

*Dubochet, J., Groom, M. and Mueller-Neuteboom, S. (1982), Mounting of macromolecules for electron microscopy with particular reference to surface phenomena and treatment of support films by glow discharge. Advances in optical and electron microscopy, Barrer, R. and Cosslett, V. E. (eds.), Academic Press, London, New York. 107-135.

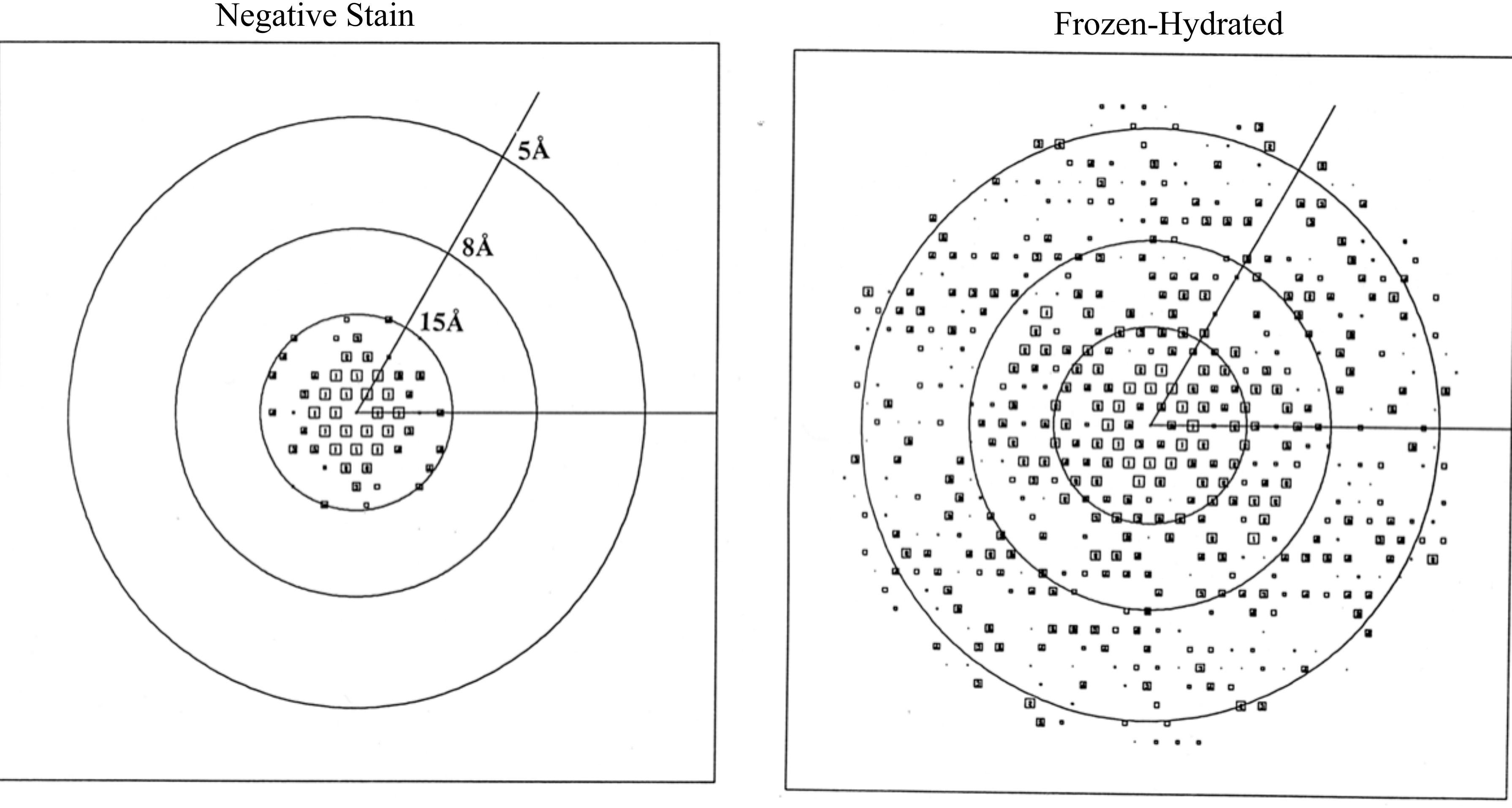
Glow discharge to change surface hydrophobicity

"Concerning the hydrophilic/hydrophobic nature of the amyl-amine (pentylamine) glow-discharged film, we had the following observation. Simple tests for the hydrophobiciy/hydrophilicy of a grid consist in
- touching the edge of the dry grid with a drop of water. If the surface is very hydrophilic, the drop spread immediately over the whole surface.
- touching the edge of the grid covered with a drop of water with a blotting paper. If the surface is very hydrophilic the water goes in the blotting paper but a thin layer of water always remains on the whole grid.

The strange thing with amyl-amine treated film (as I was used to do it) is that the film was hydrophobic according to the first test but hydrophilic according the second test, once the drop was forced on the surface. We proposed some explanations but they have never been seriously tested as far as I know. Complicated!"

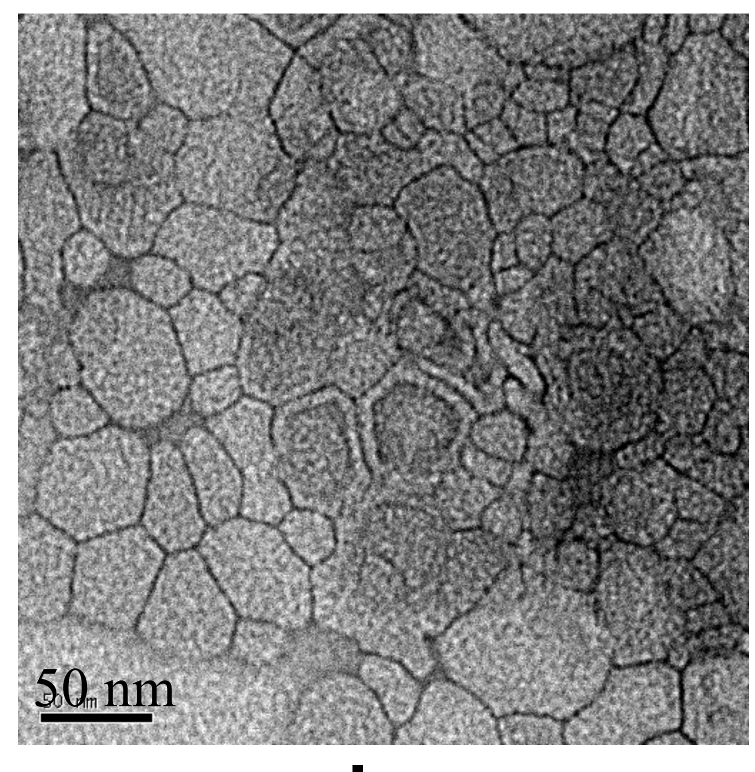
Jacques Dubochet, Feb 12, 2009

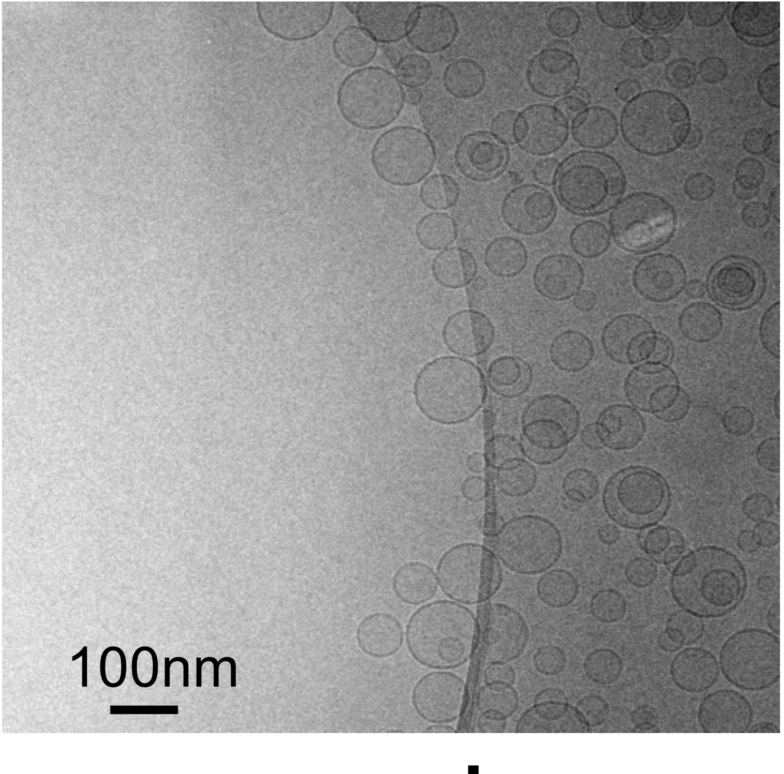
Comparison: Negative Stain vs Vitrification

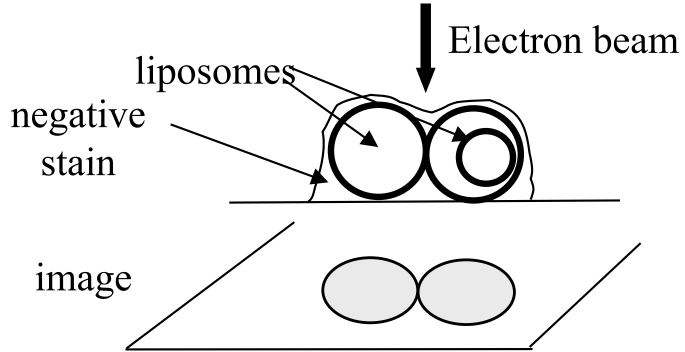


Data shown were obtained from 2D-crystals of gap-junction channels

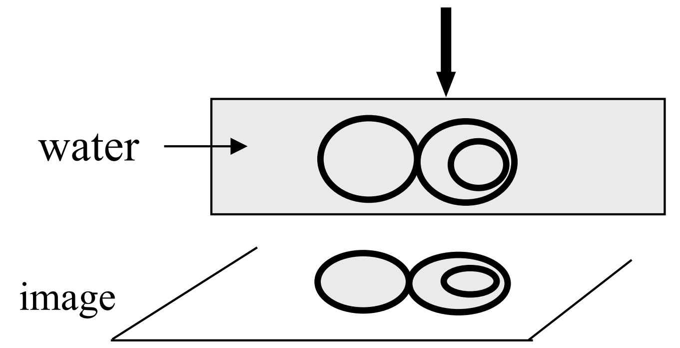
Negative Stain vs Frozen-Hydrated







No internal detail/features

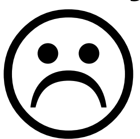


Internal detail/features are revealed

Summary of specimen preservation techniques

Negative Staining (embedding in salts of heavy metals [e.g. Uranyl acetate, tungsten])

- Easy & fast
- Good reproducibility
- Radiation damage can be seen, but not so critical



- Resolution limited to ~15Å
- No internal feature can be seen
- No time resolution (unless very slow)
- Specimen may be denatured by stain (Drying and flattening artifact)

Vitrification (embedding in vitreous water or small organic substances [e.g. trehalose, glucose])

- See protein directly including internal features
- No drying / flattening artifact
- Resolution can be 1.2 Å
- Time resolved studies possible (ms - minute range)

- Not so easy to do
- More radiation sensitive