NU*ANCE* Tech Talk, 06-24-2021

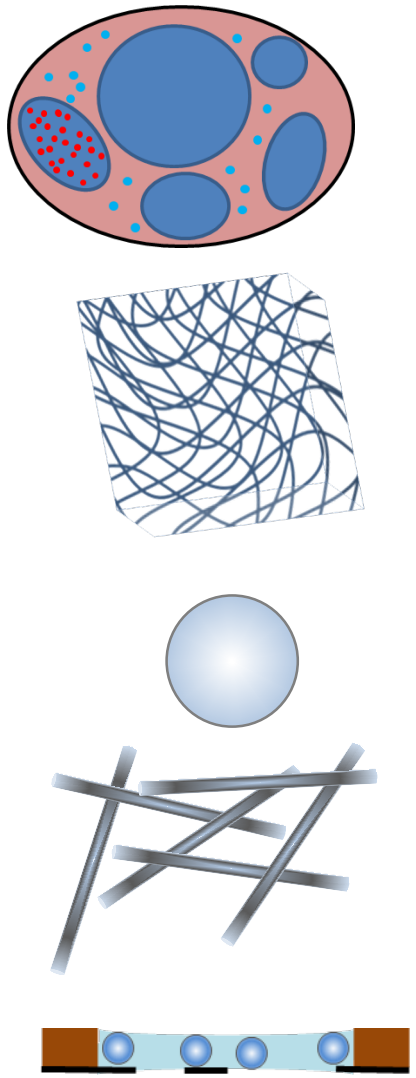
Cryogenic sample prep for electron microscopy: considerations and techniques

Reiner Bleher

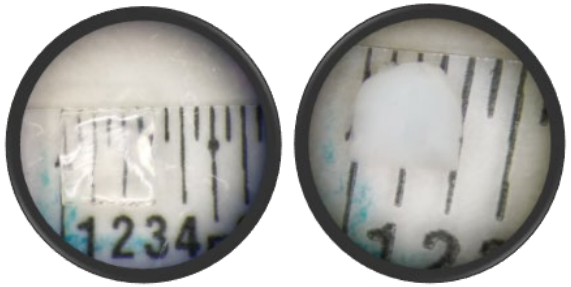
Cryogenic vs. conventional processing

High-pressure freezing

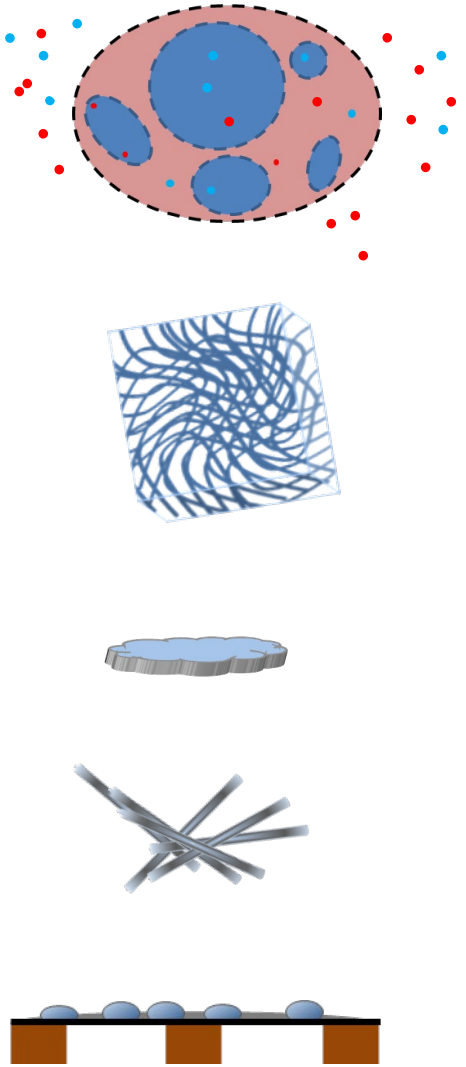
Plunge freezing



permeability changes, redistribution/loss of diffusible ions and small molecules and extraction of lipids



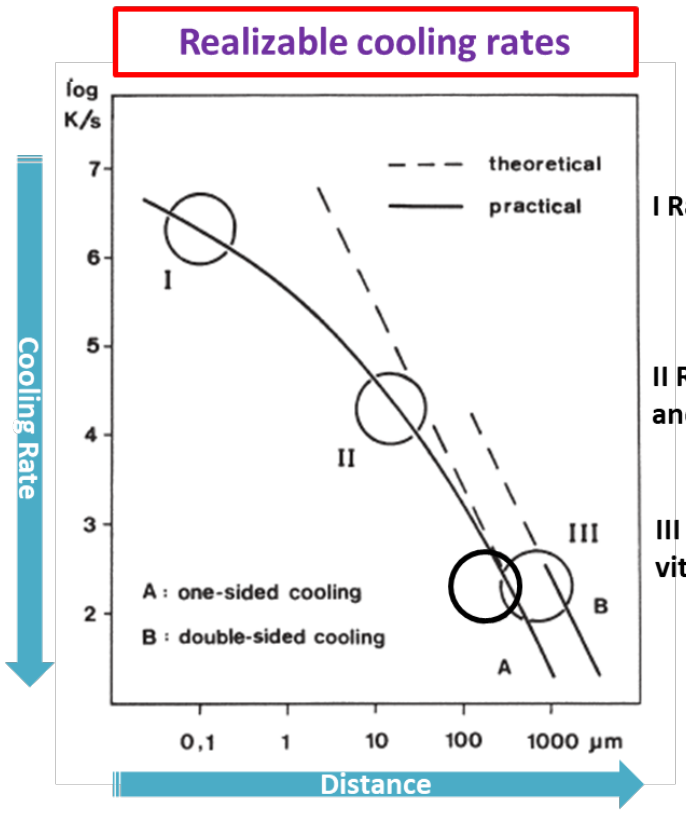
shrinkage/ distortion/ collapse



Chemical fixation, dehydration, resin embedment or CPD

Cryofixation without ice crystal formation

Realizable cooling rates



I Range of vitrified pure water

II Range of vitrified animal cells and tissues

III Range of specimens vitrified with high pressure

Which cryogens are suitable?

Cryogen	Melting Pt. [°C]	Boiling Pt [°C]	Freezing Rate [°C]
Freon 13 (CClF3)	-181	- 81	98000
Propane	**-188**	**- 42**	**98000**
Ethane	**-183**	**- 89**	**97000**
Isopentane	-160	28	45000
Nitrogen	-209	- 196	16000

Steinbrecht, Rudolf A., and Karl Zierold, eds. Cryotechniques in biological electron microscopy. Springer Science & Business Media, 2012.

Cryofixation

Achievable vitrified sample thickness

Device	Freezing depth (µm)
Plunge Freezer	≤ 10
Spray Freezer	≤ 15
Slam Freezer	≤ 15
Propane Jet	≤ 40
High-Pressure Freezer	≤ 500

Moor, Hans. "Theory and practice of high pressure freezing." In *Cryotechniques in biological electron microscopy*, pp. 175-191. Springer Berlin Heidelberg, 1987.

Basic cryogenic workflows

frozen-hydrated

ambient temp. dehydrated

Plunge freezing

Cryo-EM
(TEM, STEM, SEM)

Plunge freezing variant:
Flash freezing
Requires the use of cryoprotectants
(Sucrose, Glycerol, OCT)
used for

- immuno-EM (Tokuyasu method)
- immunohistochemistry

Plunge freezing
High-pressure freezing

Freeze drying → **Coating** → **SEM**

Freeze fracture/ etching/coating → **Cryo-SEM**

Freeze substitution → **Resin embedment** → **Ultra microtomy** → **EM (TEM, STEM, SEM)**

Cryo ultra-microtomy or cryo planing → **Cryo-EM (TEM, STEM, SEM)**


Cryo-TEM/STEM/SEM


Cryo-SEM


TEM/STEM


SEM


Light Microscopy

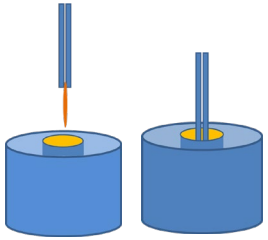

AFM

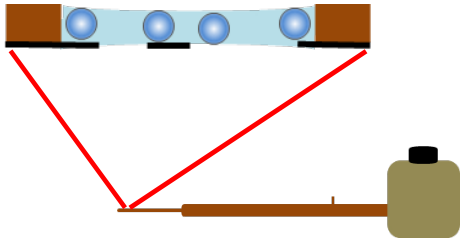

Laser ablation

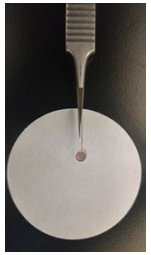

XRF

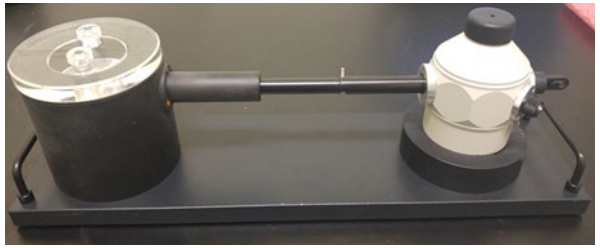
...

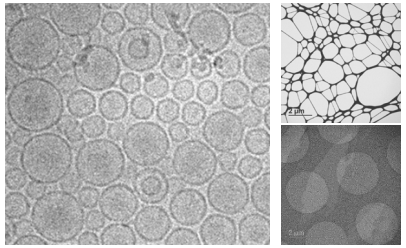
Plunge freezing



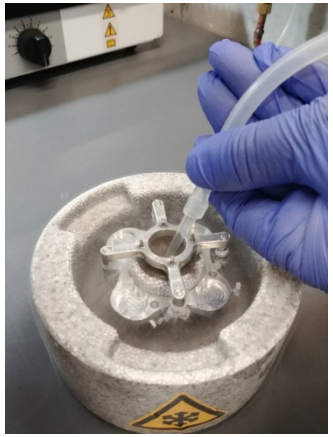








Plunge freezing



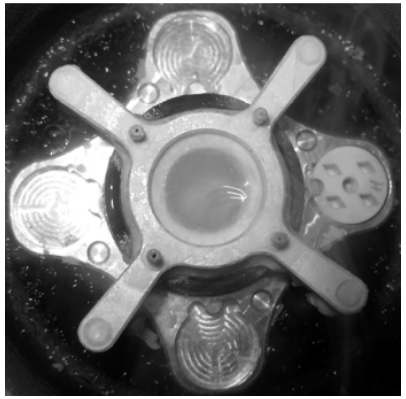


Table 2 Guideline for blotting parameters for a range of specimens, determined by Iancu *et al.* (2005) [11] and Frederik *et al.* (2009) [12]. Blot time: duration the grid is blotted against the blot pads. Drain time: waiting time after the blotting and before the plunge freezing. Blot total: number of blots. Blot offset: angle between the grid and the blot pads.

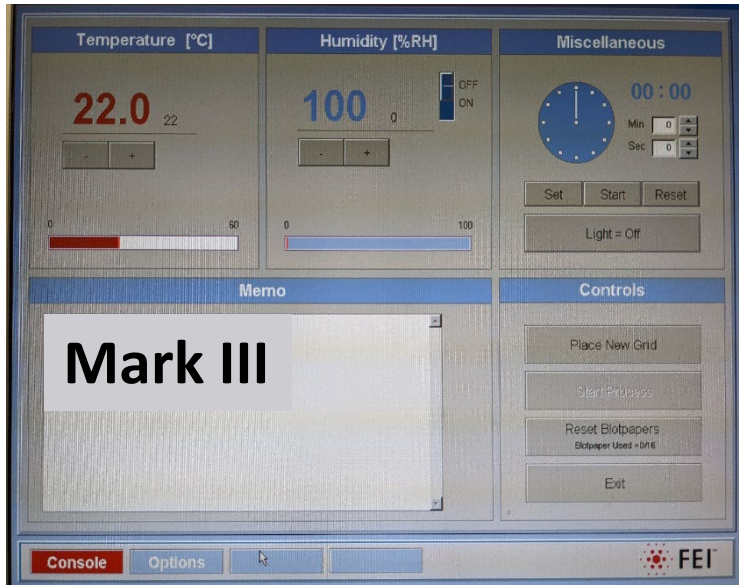
Samples	Blot time / drain time (s)	Blot total	Blot offset (mm)
Large protein complexes	3 – 4	1 – 2	2 – 3
Viruses	2 – 3 + drain time 0.5 – 1	1 – 2	2
Organelles	1 – 2	1 – 2	1 – 2
Cells	1 – 2 + drain time 1	1 – 2	1 – 2
Gels (low viscosity)	5 – 6 + drain time 1	1 – 2	2 – 3
Gels (high viscosity)	5 – 6 + drain time 1	2 – 3	2 – 3
Liquid emulsions	2-3	1	2

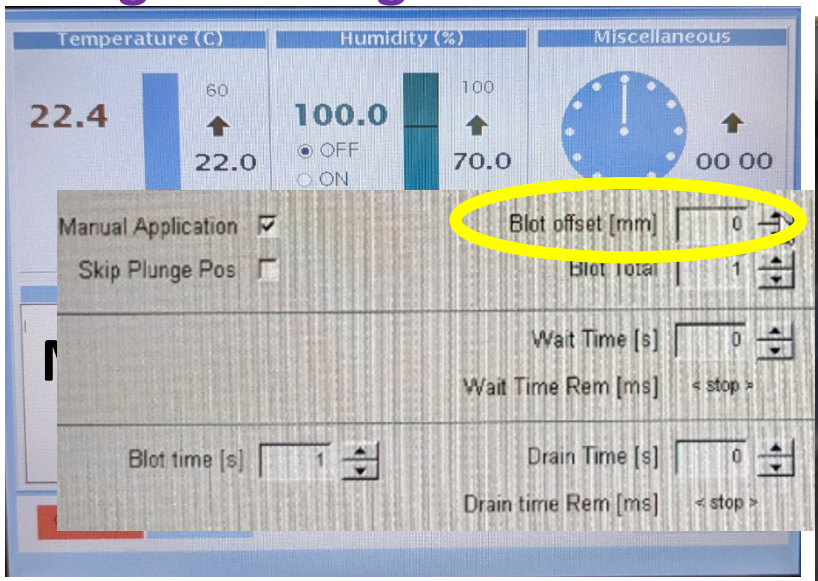
Mark III

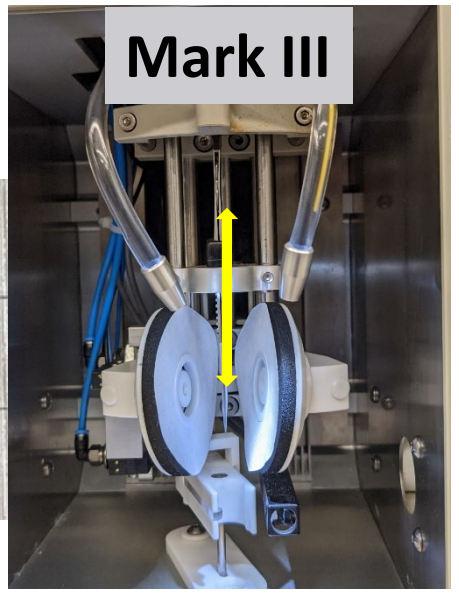
Mark IV

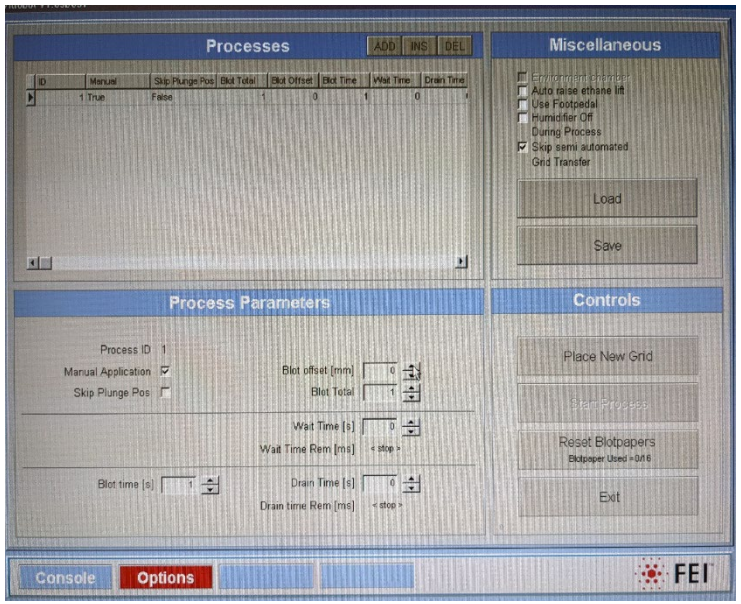
Cheng, D., D. R. G. Mitchell, D. B. Shieh, and F. Braet. "Practical considerations in the successful preparation of specimens for thin-film cryo-transmission electron microscopy." Current Microscopy Contributions to Advances in Science and Technology. A. Mendez-Vilas, editor. FORMATEX, Badajoz, Spain (2012): 880-890.

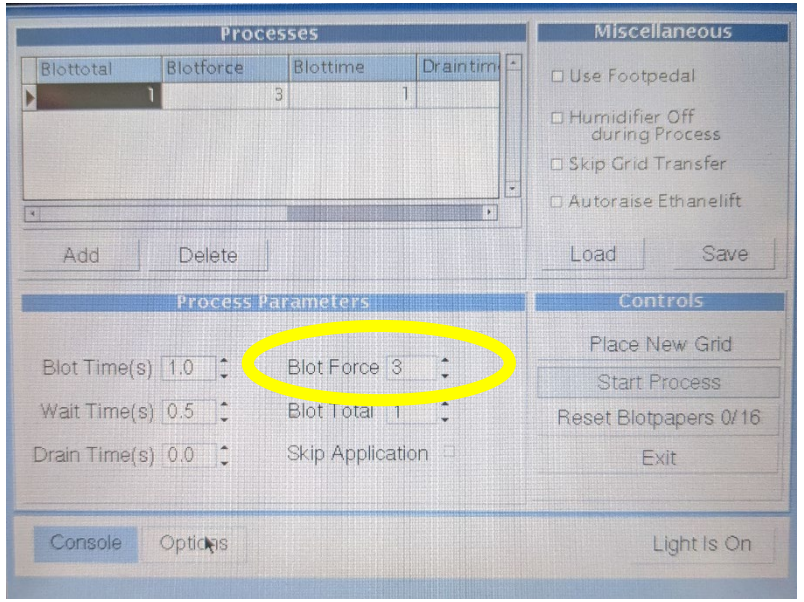
Plunge freezing



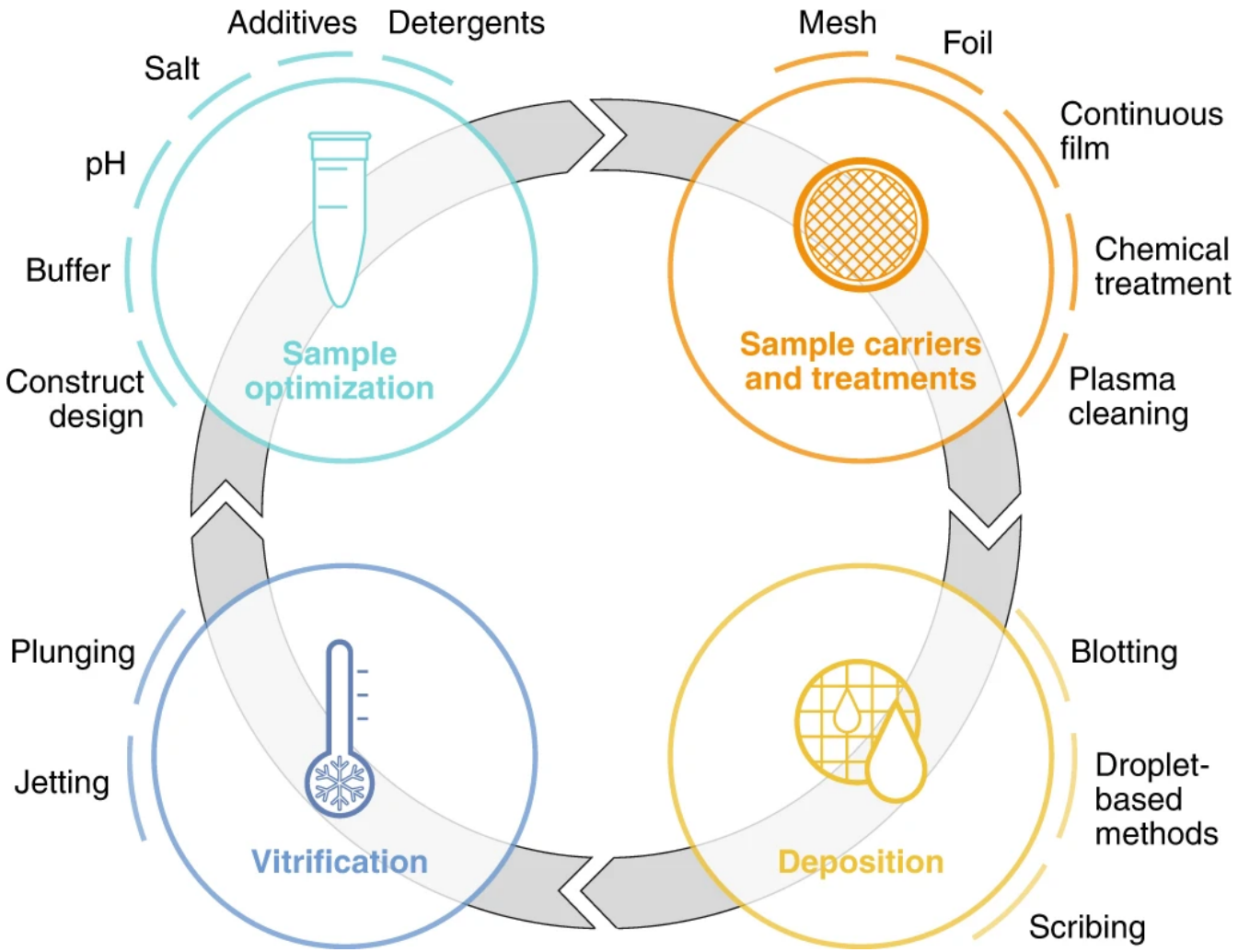








Plunge freezing



4 stages:

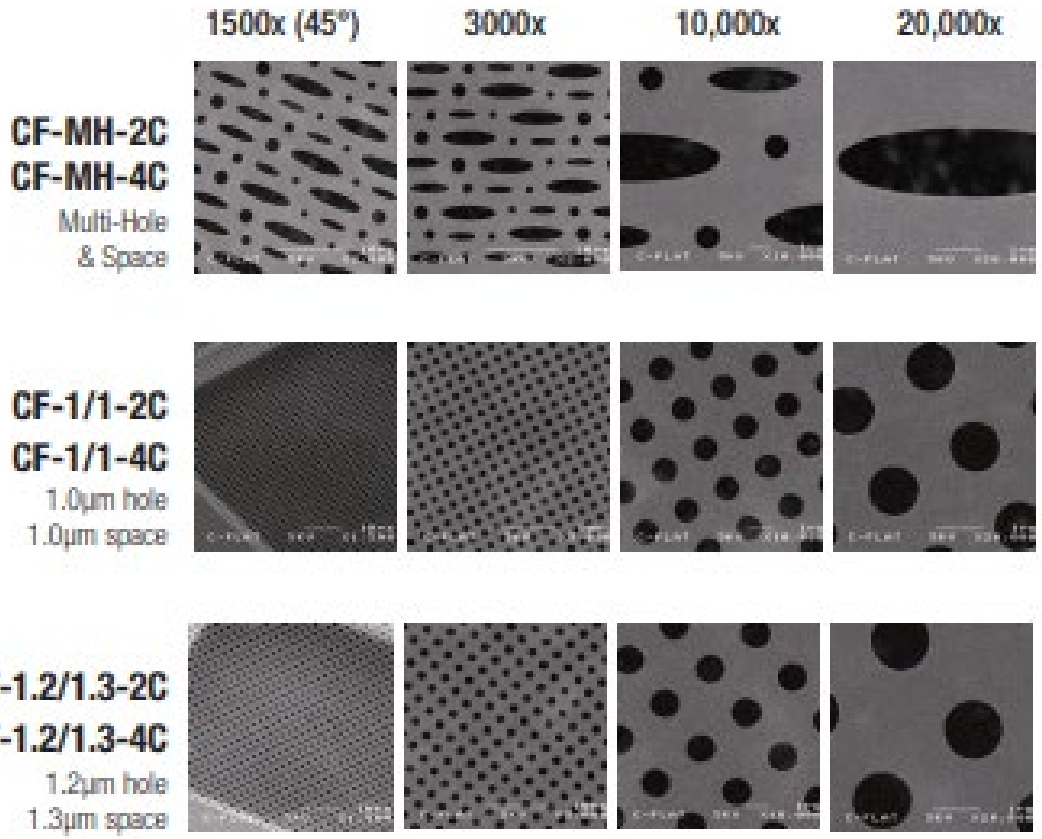
1. Sample optimization
2. Sample carriers and treatments
3. Deposition
4. Vitrification

Each of these stages is subdivided into options users are faced with in the workflow.

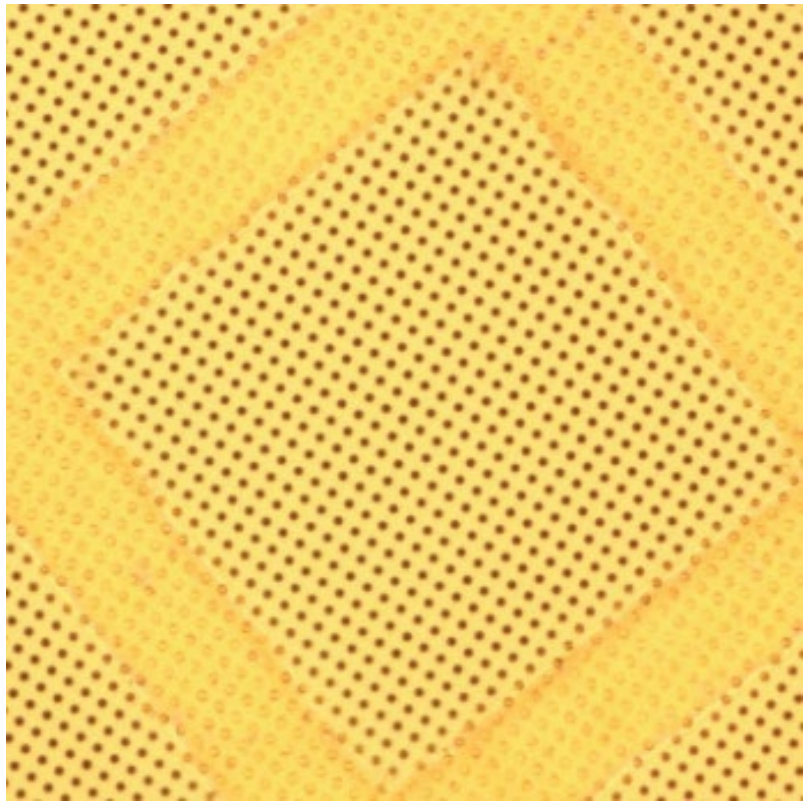
Weissenberger, G., Henderikx, R.J.M. & Peters, P.J. Understanding the invisible hands of sample preparation for cryo-EM. *Nat Methods* **18,** 463–471 (2021). https://doi.org/10.1038/s41592-021-01130-6

Plunge freezing

C-flat™



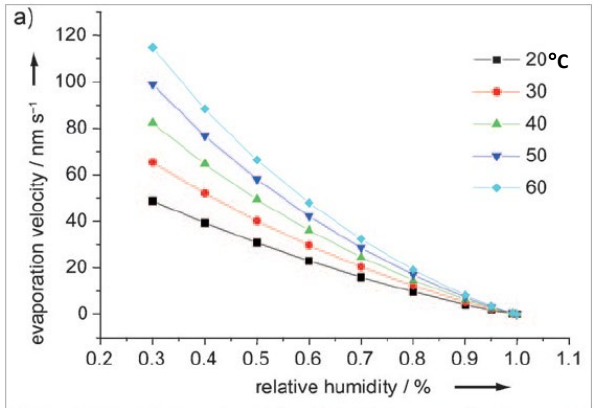
UltrAuFoil™ Holey Gold Films



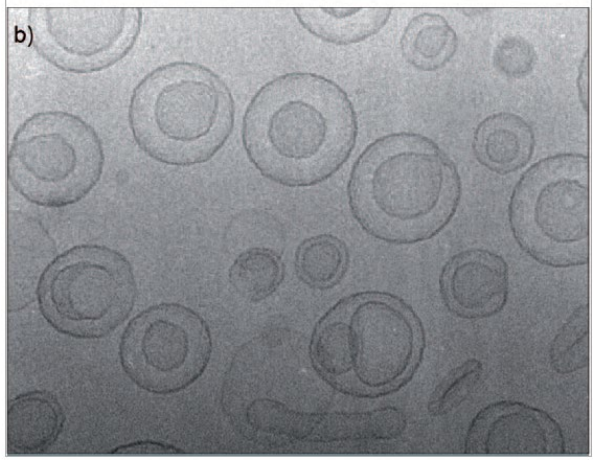
Thickness of Gold Foil about 500 Å Structure of Gold Foil
regular square array of micrometer-sized circular holes

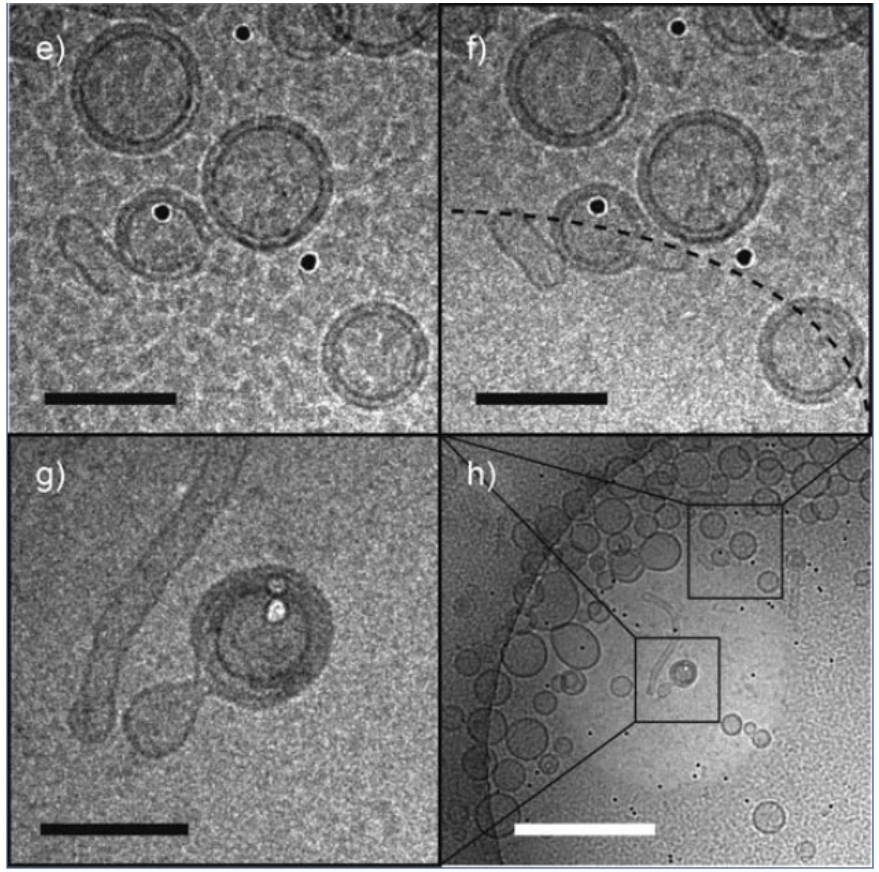
Plunge freezing - artifacts

Evaporation rates at different temperatures and humidities



Osmotic collapse of spherical liposomes into "vaselike" structures

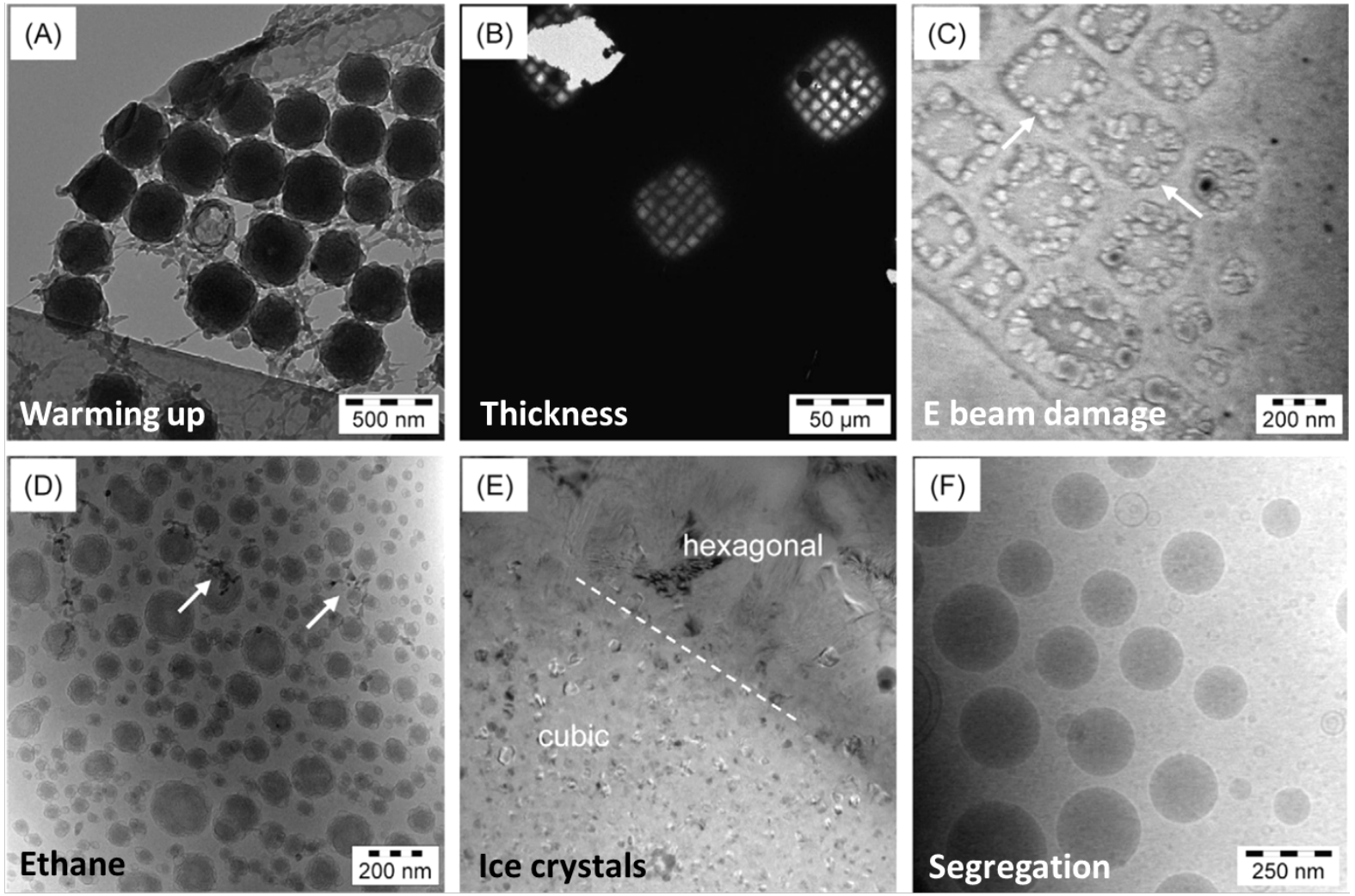




Ice contamination as a result of a high partial water vapor pressure in the microscope column.

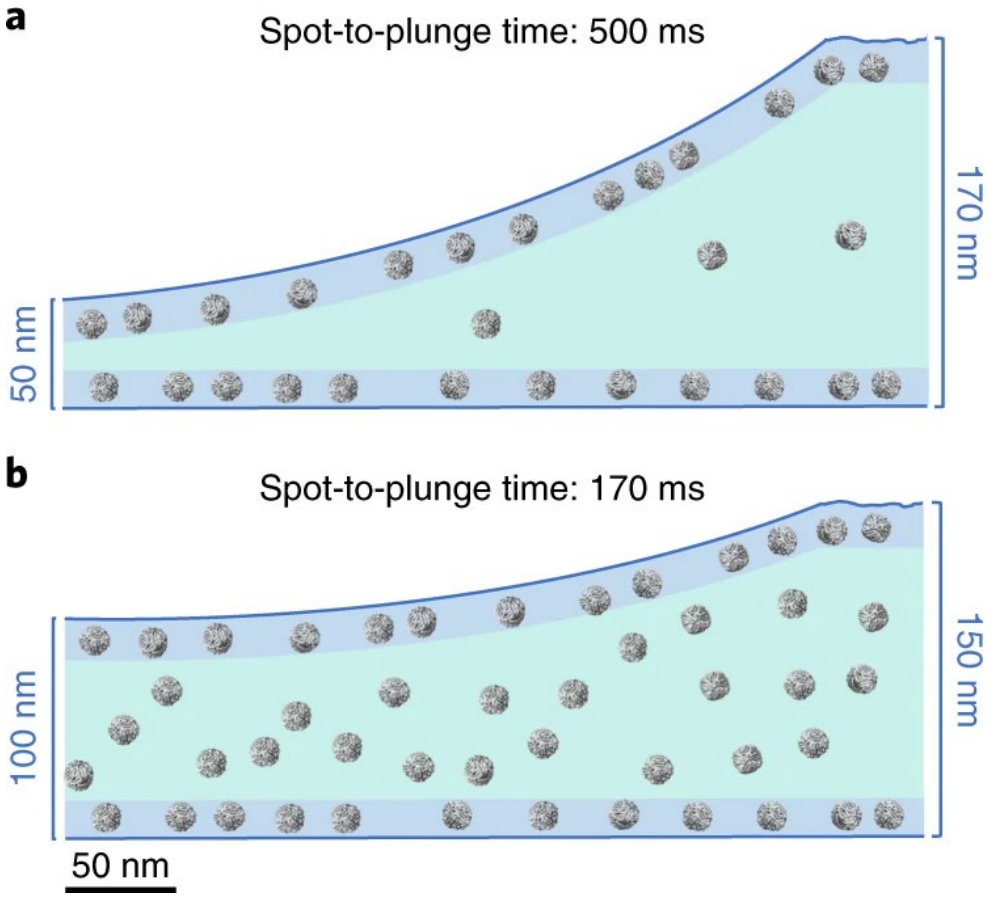
Friedrich, Heiner, Peter M. Frederik, Gijsbertus de With, and Nico AJM Sommerdijk. "Imaging of self-assembled structures: interpretation of TEM and Cryo-TEM images." Angewandte Chemie International Edition 49, no. 43 (2010): 7850-7858.

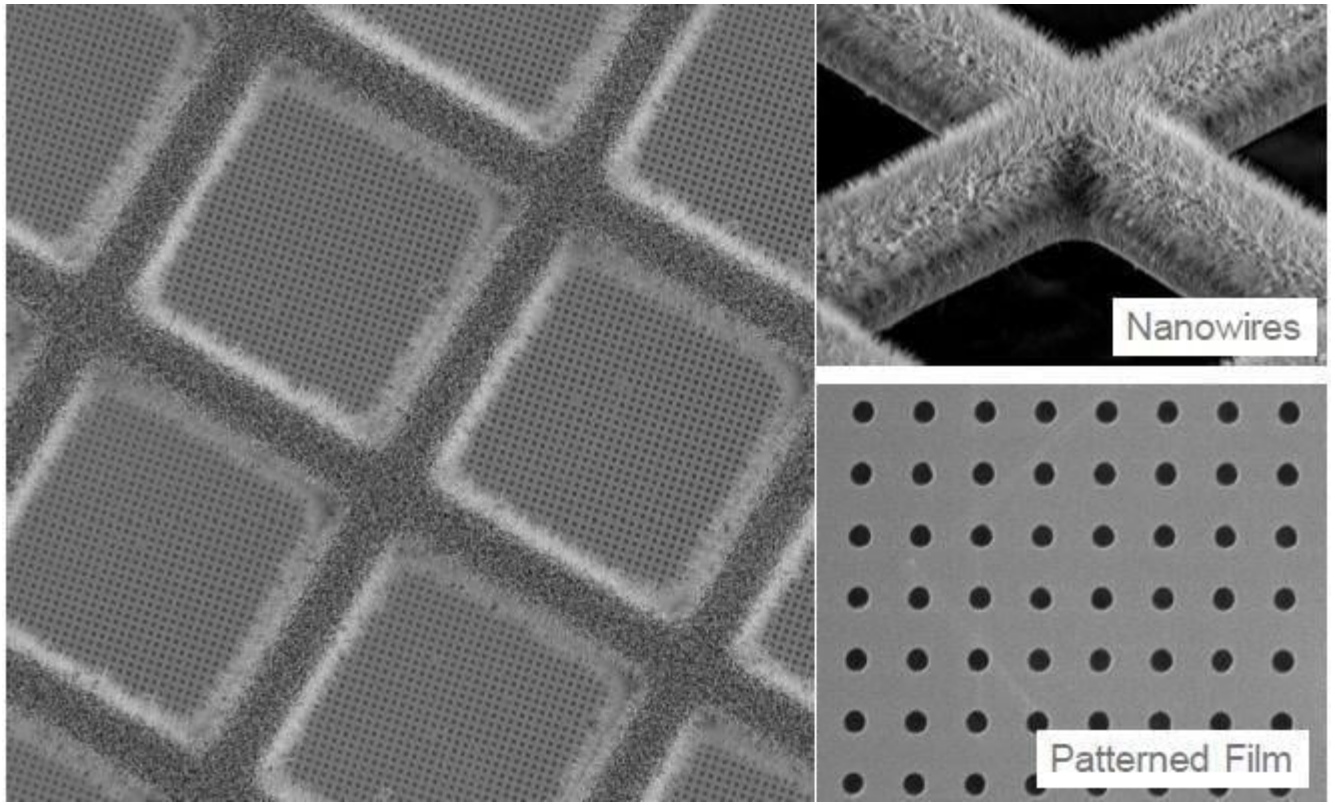
Plunge freezing - artifacts



Kuntsche, Judith, Jennifer C. Horst, and Heike Bunjes. "Cryogenic transmission electron microscopy (cryo-TEM) for studying the morphology of colloidal drug delivery systems." *International journal of pharmaceutics* 417, no. 1-2 (2011): 120-137.

Plunge freezing – new developments





https://www.sptlabtech.com/products/sample-preparation/chameleon/

Noble, Alex J., Hui Wei, Venkata P. Dandey, Zhening Zhang, Yong Zi Tan, Clinton S. Potter, and Bridget Carragher. "Reducing effects of particle adsorption to the air–water interface in cryo-EM." Nature methods 15, no. 10 (2018): 793-795.

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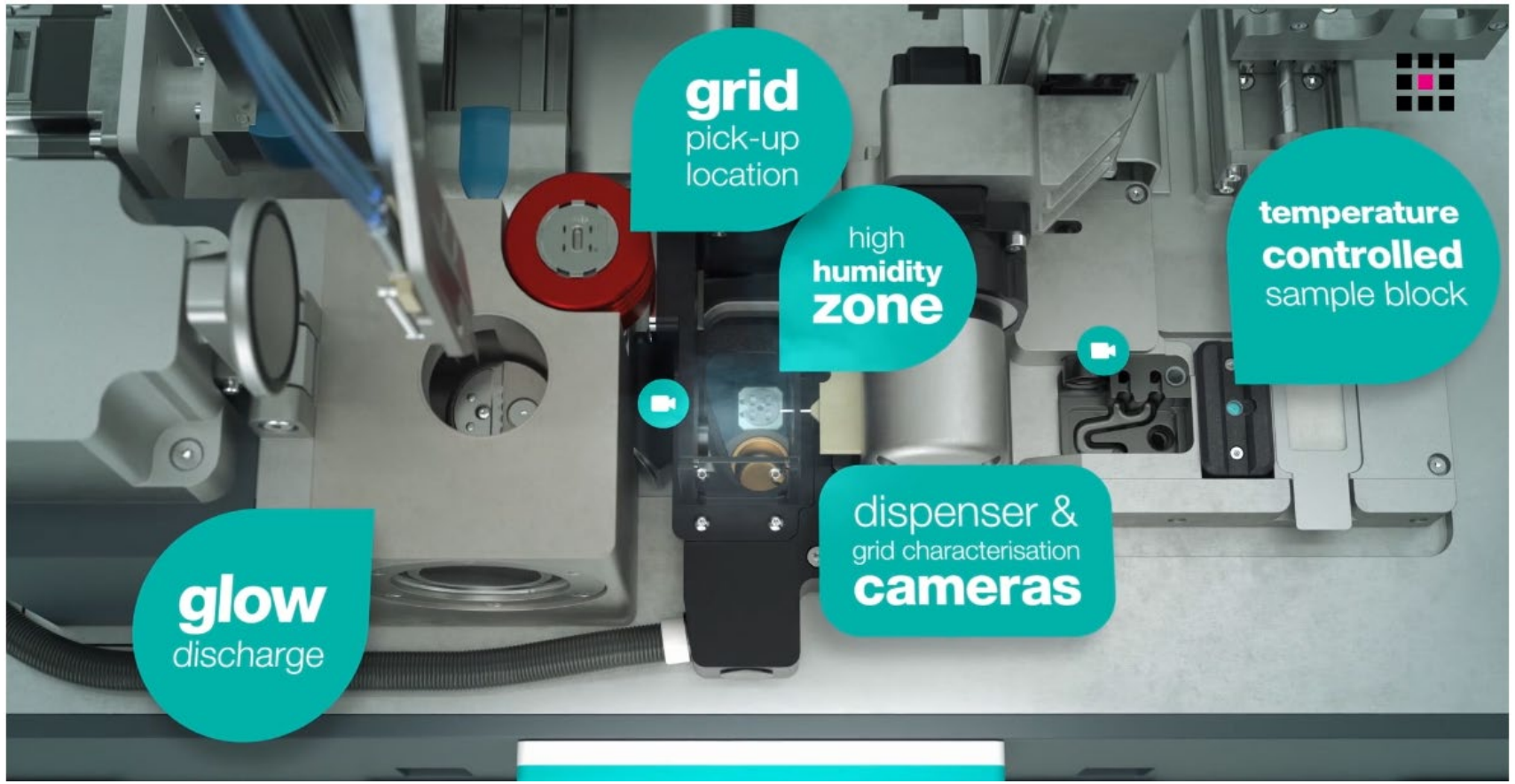

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High-pressure freezing

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Cryo-TEM/STEM/SEM


Cryo-SEM


TEM/STEM


SEM


Light Microscopy


AFM


Laser ablation


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...

High-Pressure Freezing

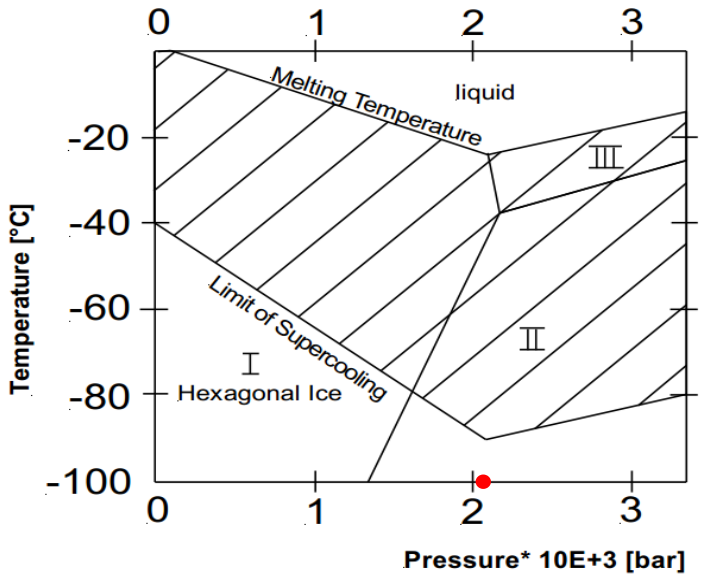
When water freezes, its volume increases

(Chatelier's Principle)

High pressure (~2050 bar):

1. **inhibits volume expansion**
   and
2. **reduces the critical freeze rate to a range between 100 and 500 °/s**

Phases of water



How?

1) **Lowering of the freezing point**
2) **Lowering the supercooling temp. limit**
3) **Reduction in the rate of ice crystal nucleation**
4) **Slowing the growth of ice crystals**

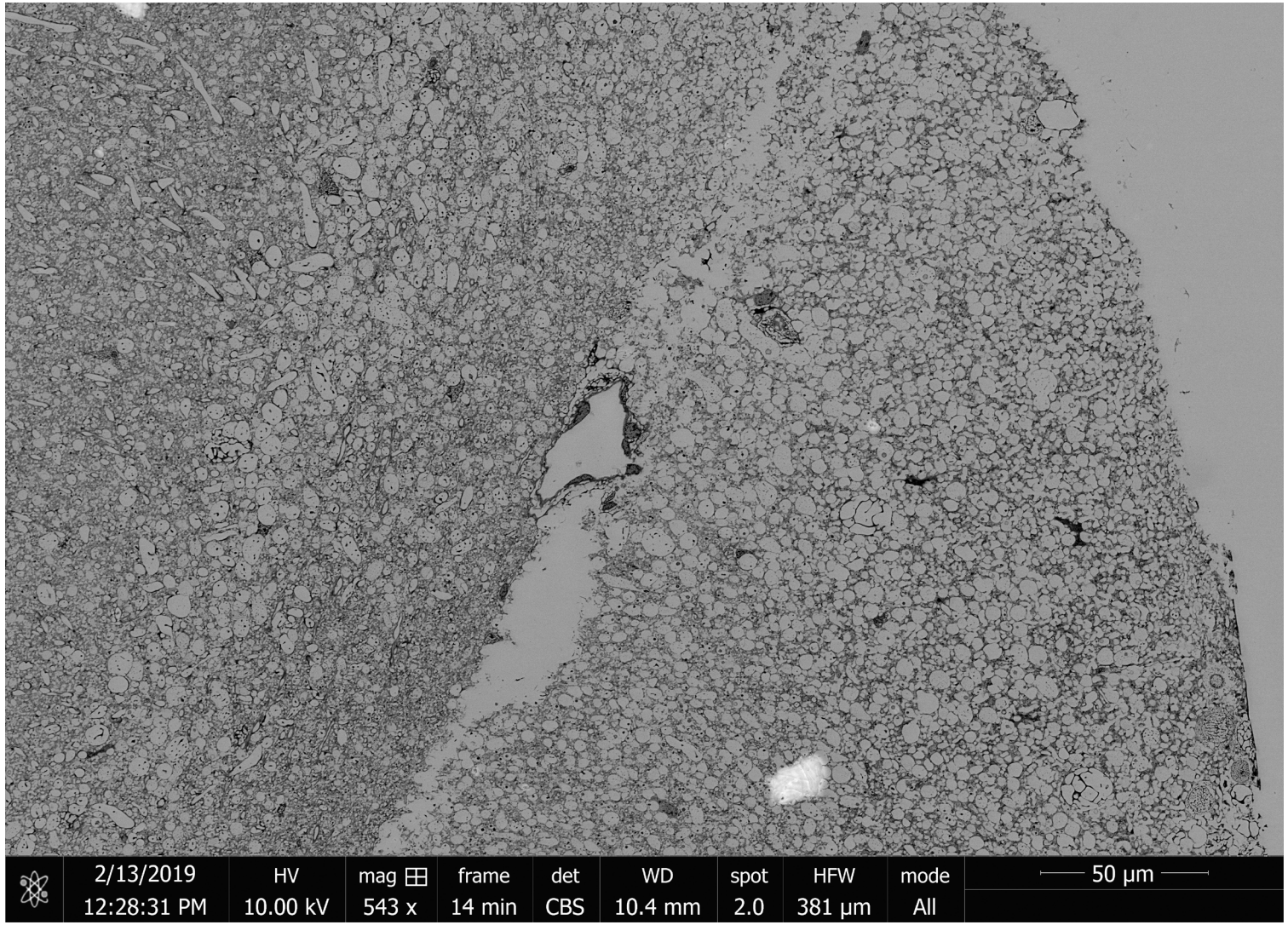
Temperature ranges of water crystallization

	melting point	devitrivication temp.
pure water	-0.15°C (273 K)	-133.15°C (140 K)
physiologically active cells and tissues	-2.15°C (271 K)	-80.15°C (193 K)
frost-hardy cells with reduced water content	-13.15°C (260 K)	-43.15°C (230 K)

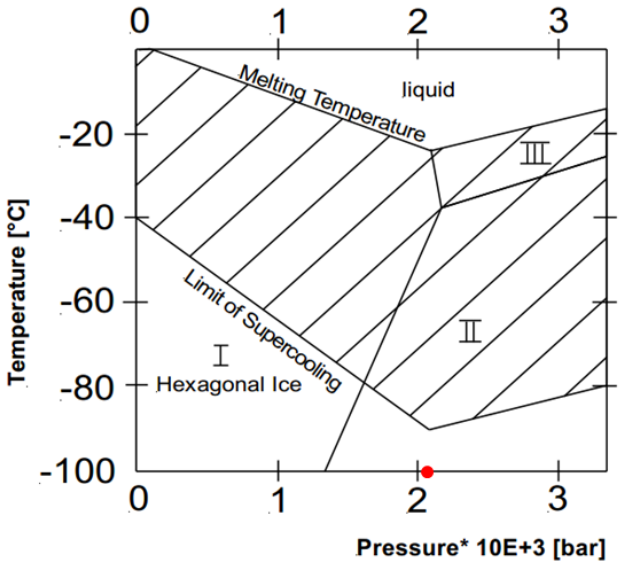
Dahl R, and Staehlin AL, Journal of electron microscopy technique, 1989 vol:13 iss:3 pg:165 -174.

Freezing damage

A brain slice (ms) of 300 µm thickness was frozen in liquid nitrogen



High-Pressure Freezing

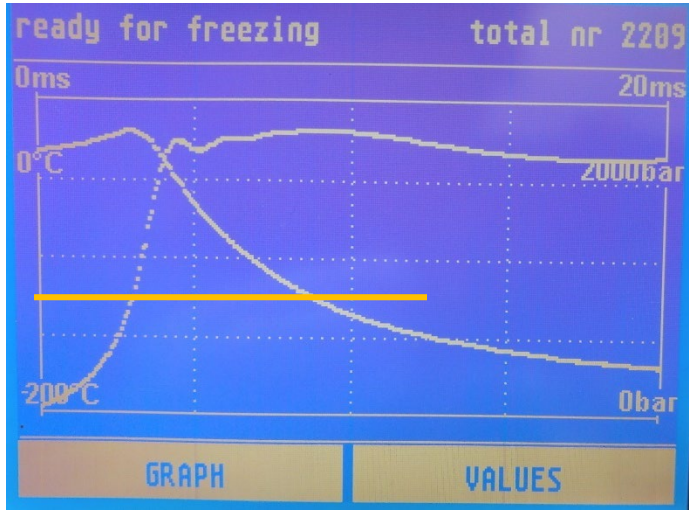


H_20 Phase Diagram

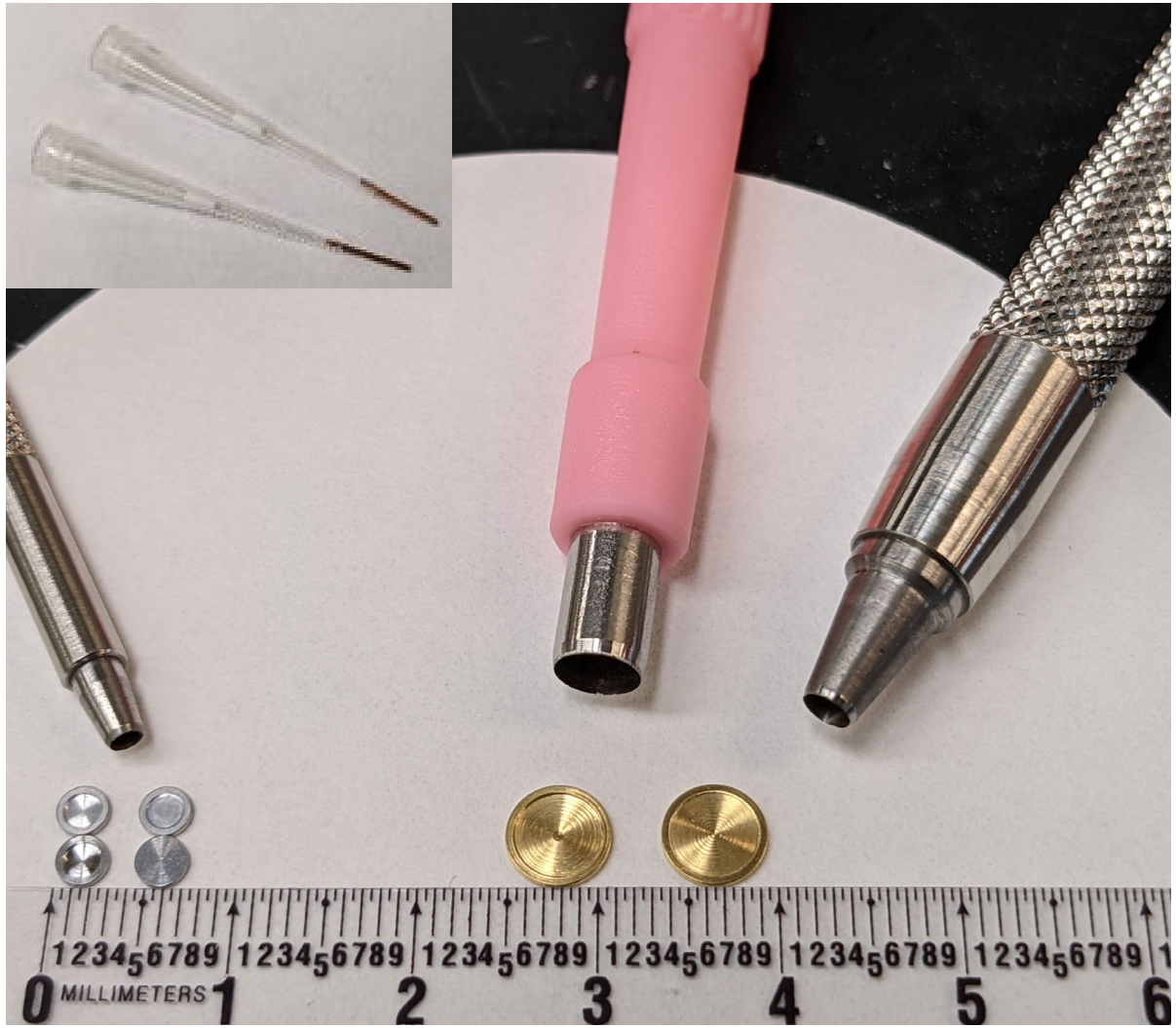
1 Supercooling capability curve
2 Melting point curve

HPM100

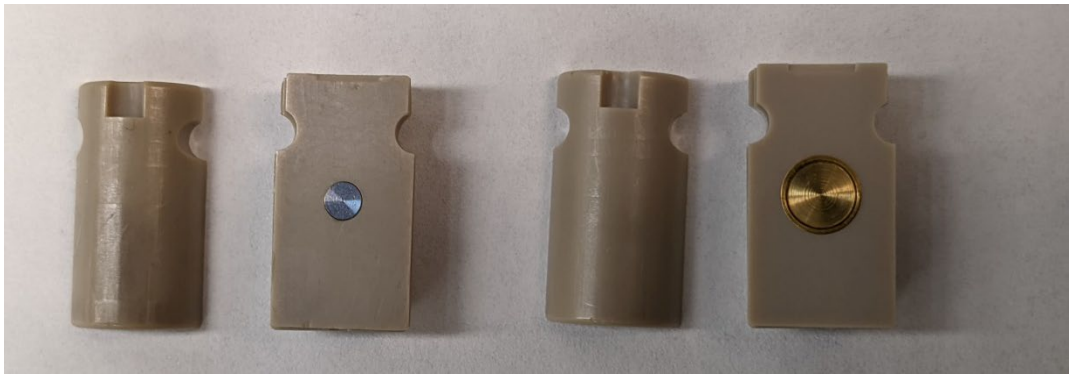
HPM100: temperature and pressure dynamics during a freeze.

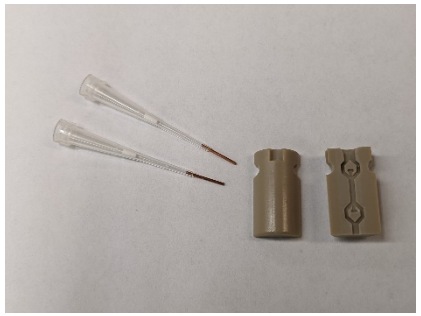


High-pressure freezing



VT1200S



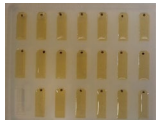


Freeze substitution

High-pressure freezing

Freeze substitution

Resin embedment



Ultra microtomy

EM (TEM, STEM, SEM)

frozen-hydrated

ambient temp. dehydrated

Freeze substitution involves replacing the frozen water of the cell with an organic solvent at low temperature, thus avoiding the damaging effects of dehydration that occur at ambient temperature (Steinbrecht and Müller, 1987).

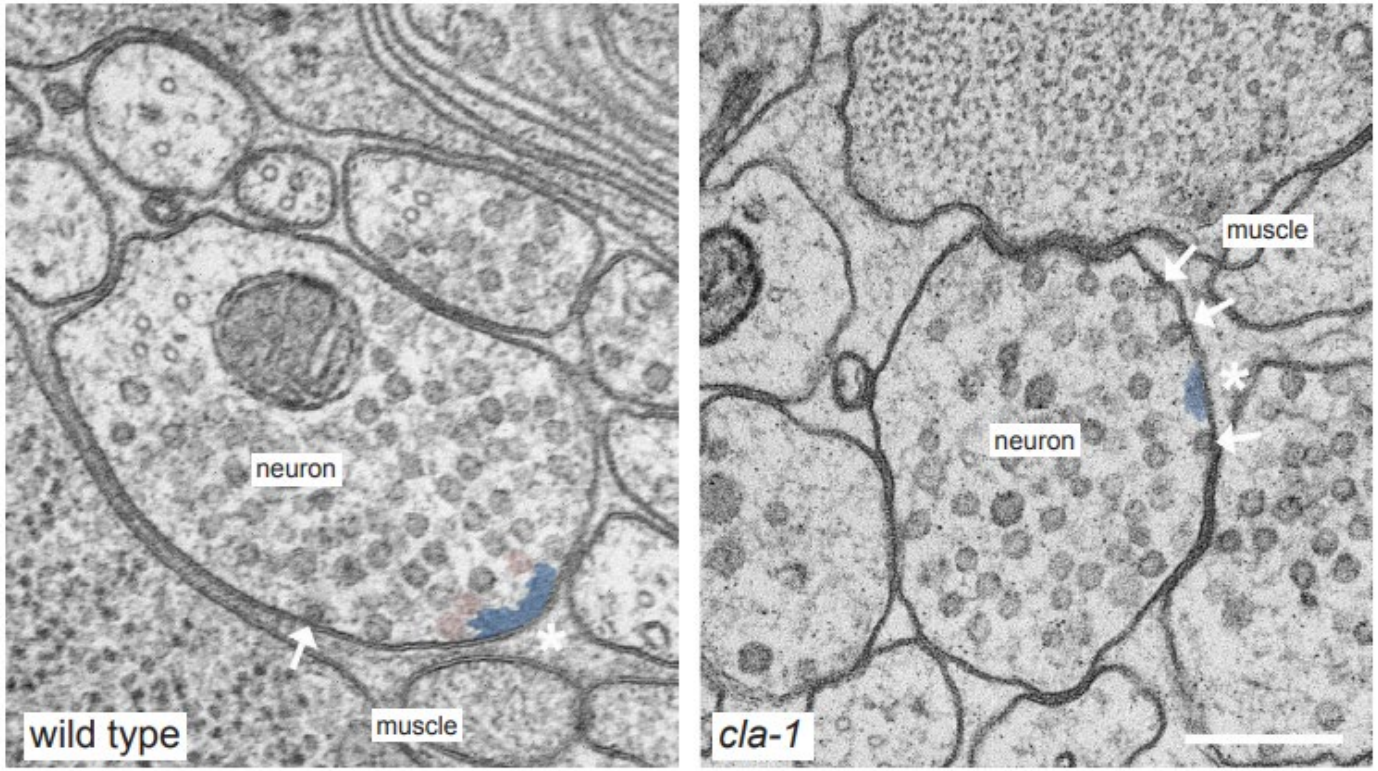
FS is fully automated with the Leica AFS2

- FS medium: UA, OsO_4 or GA in acetone
- From ~-90°C to RT in several days
- Steps with holding and ramping up (e.g., 5°C per hour) temperature
- Ending at RT, then rinsing with acetone and resin embedment

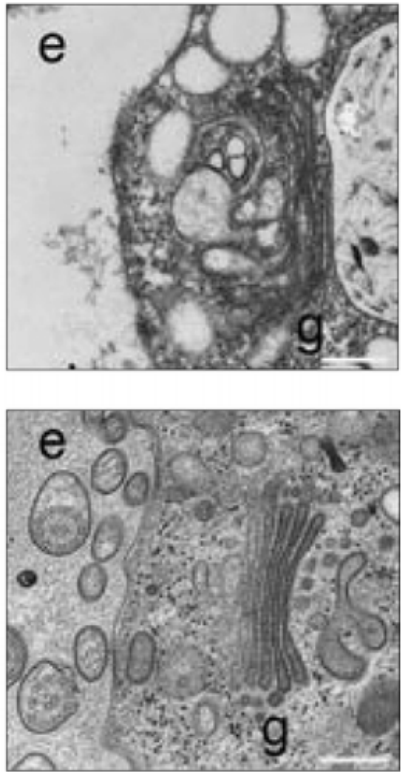
or (mainly for immuno-EM or fluorescent samples)

- Ending at low temp. then automated rinses and resin infiltration at low temp., usually -50, -40, or -20 °C, depending on resin used (e.g., GMA, LR Gold, K4M, HM20)
- Polymerization with UV light at low temperature

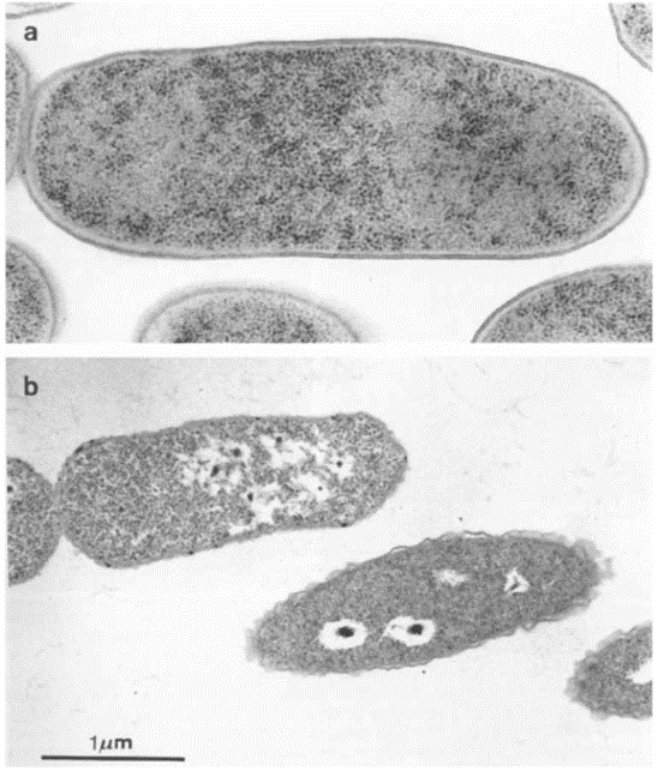
Freeze substitution



Xuan, Zhao, Laura Manning, Jessica Nelson, Janet E. Richmond, Daniel A. Colón-Ramos, Kang Shen, and Peri T. Kurshan. "Clarinet (CLA-1), a novel active zone protein required for synaptic vesicle clustering and release." Elife 6 (2017): e29276.



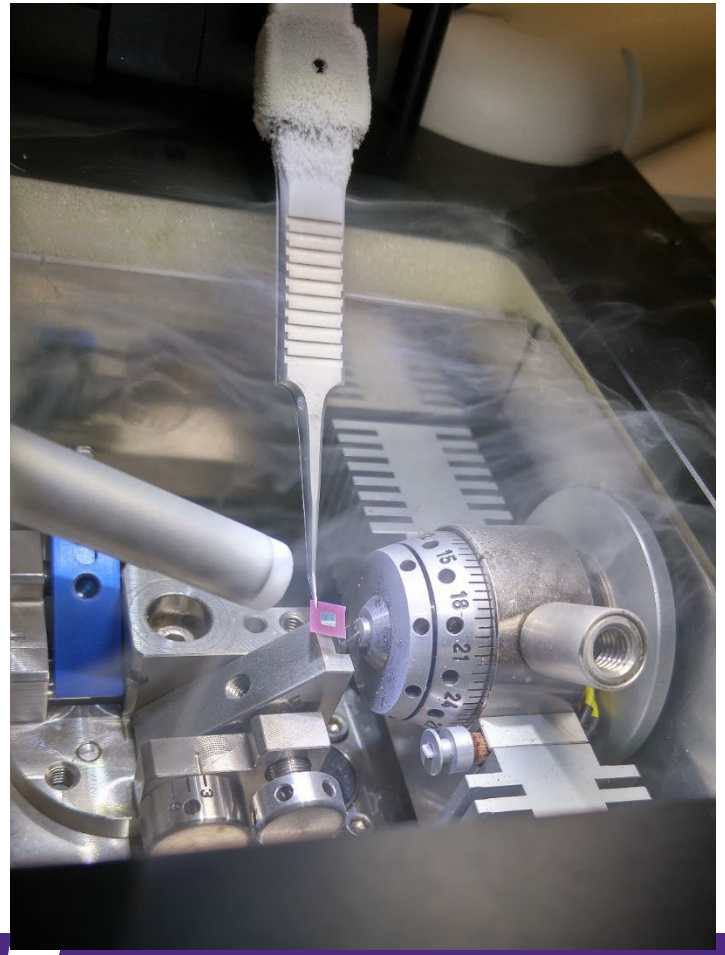
McDonald, Kent L., and Manfred Auer. "High-pressure freezing, cellular tomography, and structural cell biology." *Biotechniques* 41, no. 2 (2006): 137-143.
(scale is 300nm)

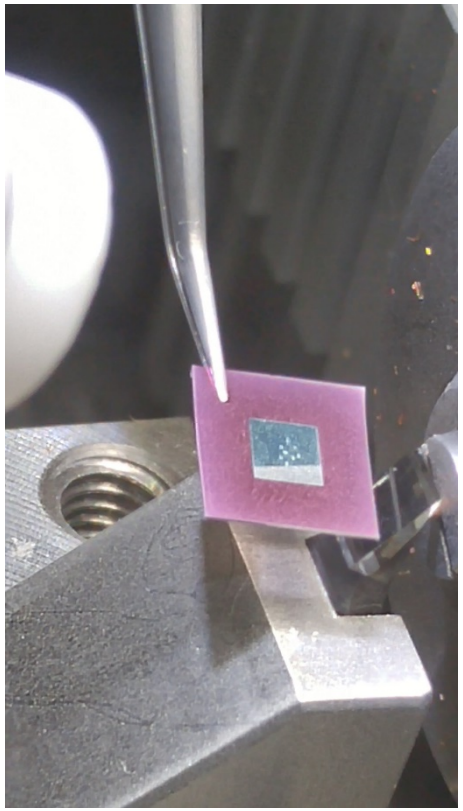


Kellenberger, E. "The potential of cryofixation and freeze substitution: observations and theoretical considerations." *Journal of microscopy* 161, no. 2 (1991): 183-203.

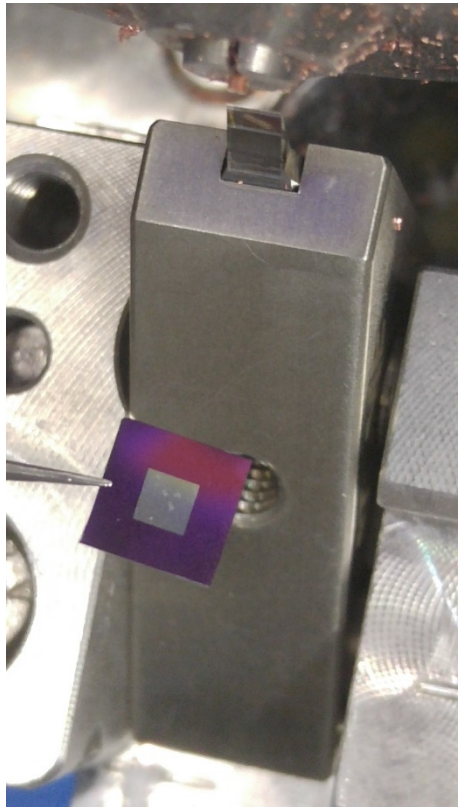
HPF and cryo-ultramicrotomy for cryo-XRF at ANL

Cryosections of high-pressure frozen E. coli ΔcusR on silicon nitride windows





Window #1 with sections



Window #2 with sections

Thanks for your Attention!

Q & A

Contact: r-bleher@northwestern.edu