Cryo Electron Microscopy

John Mitchels

Thanks to Miloš Hovorka and Alex Rigort

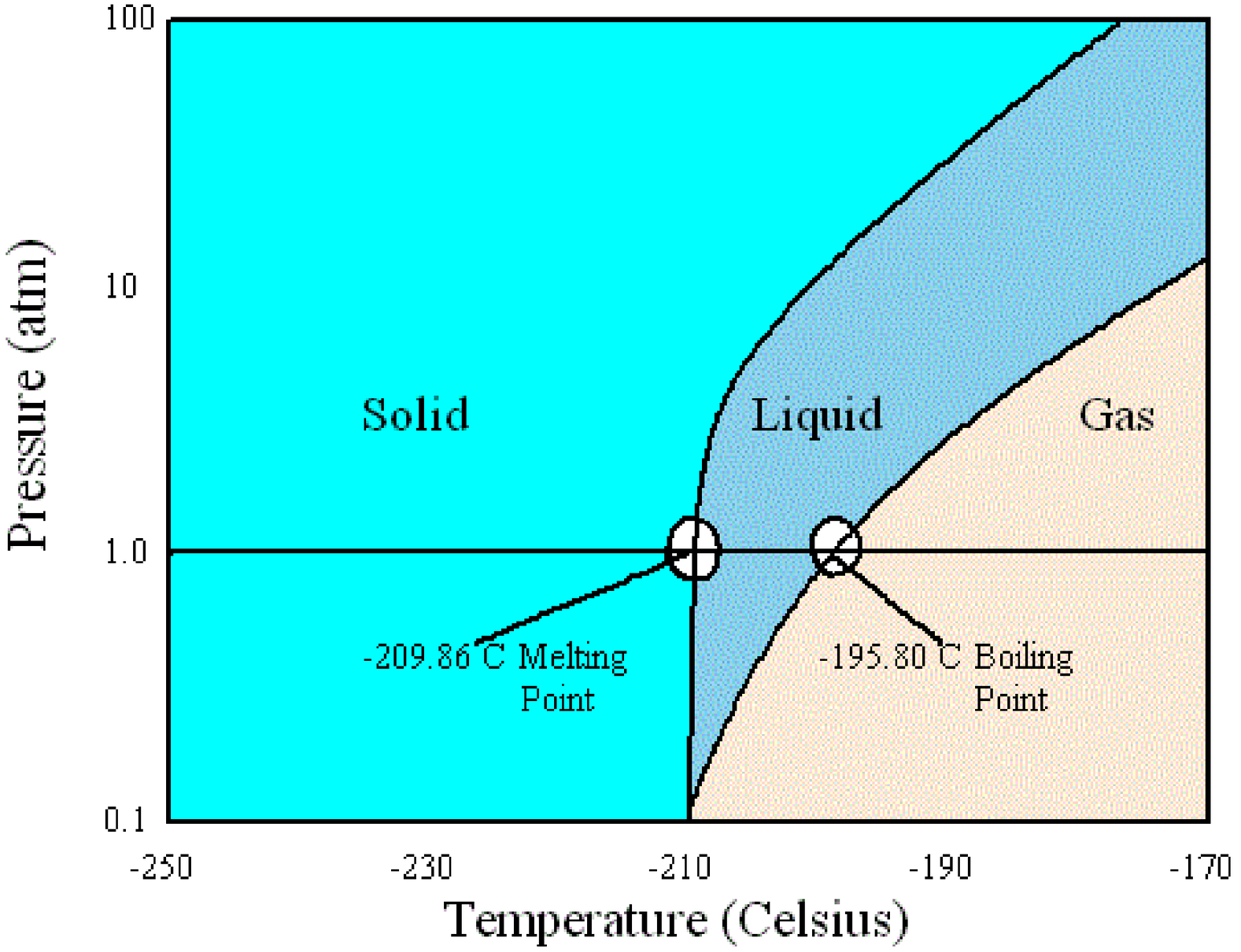
Define cryo…

The word **cryo** or **cryos** (κρύο) is Greek and means "icy cold" (from crystallos)

Typically temperatures lower than -100 Centigrade, but hardware generally allows temperatures lower than room temperature.

- (N) Nitrogen -196
- (He) Helium -269
- (C6H6) Ethane -88

Nitrogen Phase Diagram



FEI Product Line - Technology Leadership

Transmission Electron Microscopes (TEM)

Scanning Electron Microscopes (SEM)

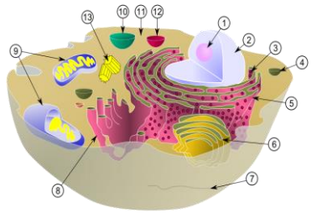
Dual Beams (FIB/SEM)

Optical solutions, data processing, etc.

Bio samples (or other soft water)

- **Biological samples are full of water.** This has the implications for the sample preparation and observation (body water content around 60%, brain 73%, cell 70%).
- Most/less abundant elements: H, C, N, O/Na, Mg, P, S.
- Biological sample = poor scattering = poor contrast in electron microscope.
- In EM context low sample conductivity = charging.
  - coating by metal layer, low dose, low kV, scanning strategy
  - HiVac x LoVac/ESEM

Specimen preparation



- **Fixation** = to stop the biological activity and to preserve the tissue structure for subsequent treatments.
- “The objective is the process tissues and cells without significant change in size, shape, positional relationship of the cellular components and to preserve as much of the biological activity and chemical nature of cellular components…”

The problem with Fixation

Normal Strawberry

Freeze Dried

Good for general morphology at tissue level; at macro level lots of distortion, very easy method and easy to image in SEM.

CPD

Better preservation of the macro structure; loss of components via solvent extraction; very easy and quick.

Chemical Fixation

Good macroscopic control; ultra structure can be well preserved but soft and squishy; needs support for sectioning; a lot of extraction; quick easy.

Heat Preservation

Well you can see it does not work for strawberries unlike eggs.

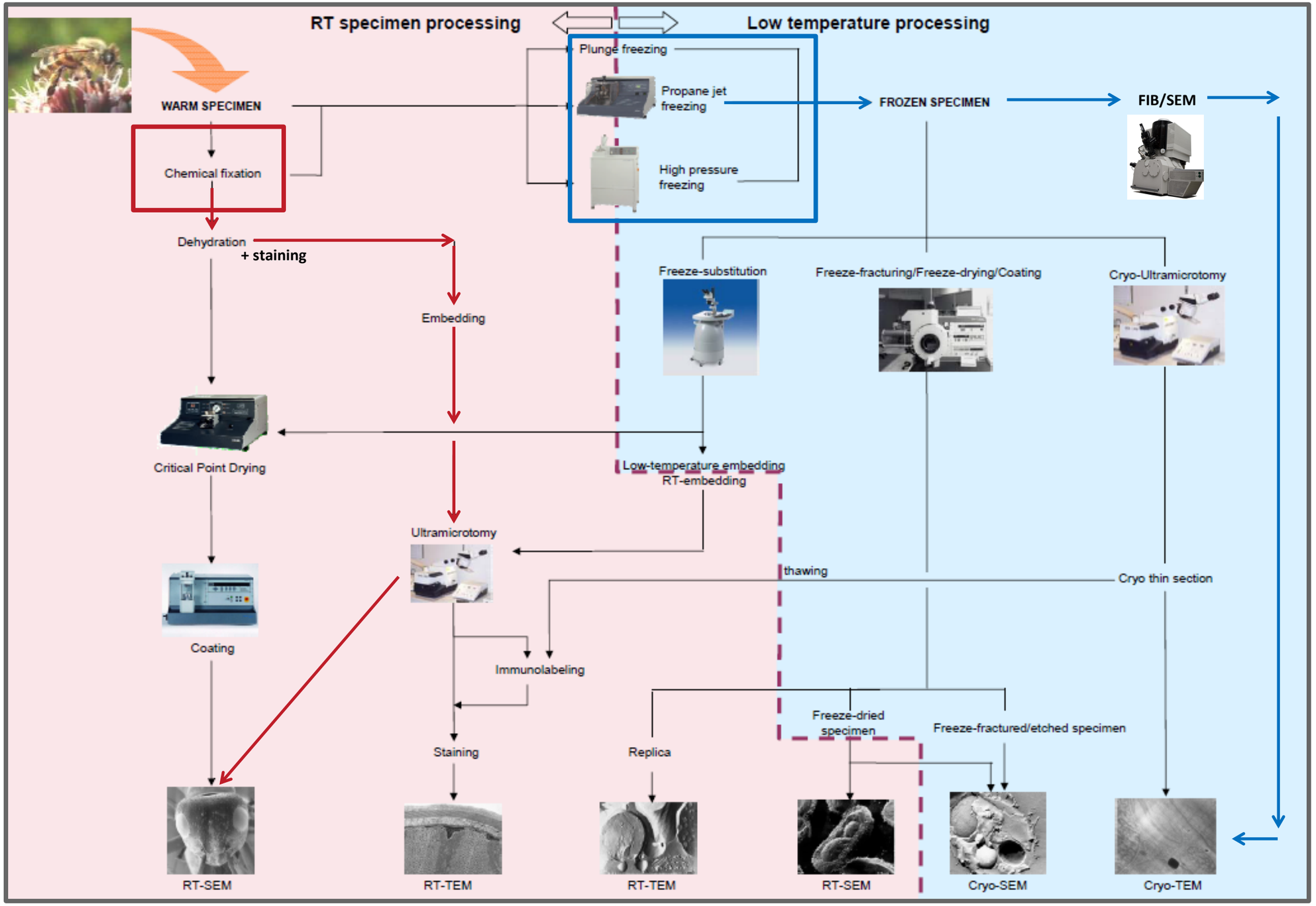
Cryo Preservation

Good macro and micro, and ultra structure and tissue support when frozen.

Bad when thawed!

Sample preparation flow chart

(selected techniques)



Adapted, image by Andres Kaech, University of Zürich.

Tissue preparation (using chemicals)

Sample preparation flow.

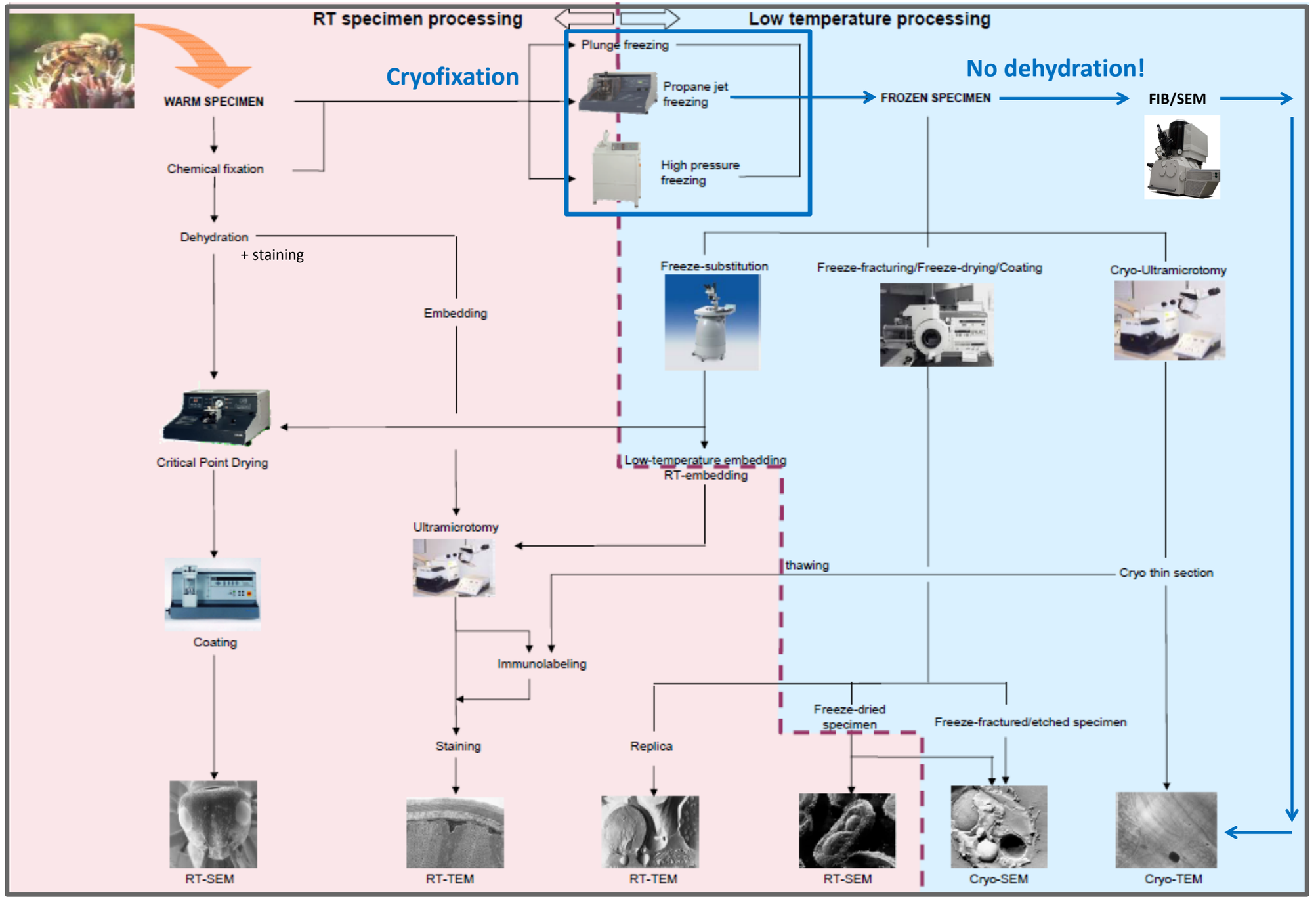
- **chemical fixation**
  - e.g. glutaraldehyde, formaldehyde, osmium tetroxide, …
  - result is influenced by sample size, a way how to fix, concentration of solution, speed of penetration of fixative, temperature, time etc.
- **dehydration** = to remove water → to enable infiltration (ethanol/aceton replaces water)
- **infiltration/embedding** into suitable medium (resin - EM, paraffin - LM)
  - examples of resins: acrylic (Lowicryl, LR White), epoxy (Epon (1956), Araldites, Spurr, Durcupan, etc.)
  - ideally well soluble in dehydration agents, low viscosity, minimal shrinkage, stability under e-beam, minimal granularity
- **block observation / cutting into sections → post staining**

Cryofixation

- **Why, advantages**
  - The best method of preservation. Rapid freezing in milliseconds = near perfect preservation (minimal chemical and physical changes if done well).
  - Offers a SnapShot at a particular time, very important when studying function.
- **Sample vitrification**
  - Cool the specimen so rapidly that there is not time for ICE (crystalline water) to form! → ICE is what does the damage as it rips structures apart.
  - Increase cooling speed by the reducing size of the specimen.
- **Methods**
  - High pressure freezing (HPF, up to 200 µm), plunge freezing, slam (metal-mirror) freezing, spray freezing, double jet propane freezing (all up to units or tens of µm)

Sample preparation flow chart

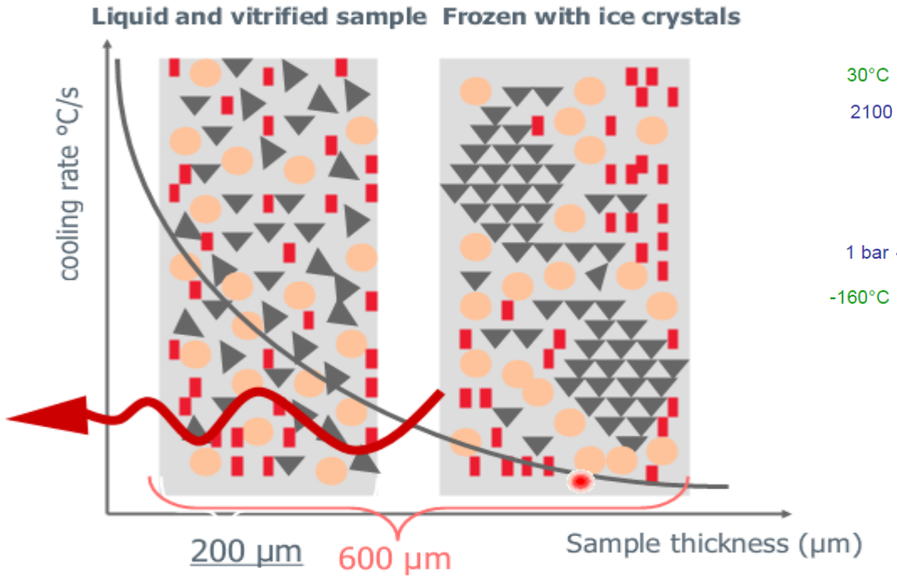
(selected techniques)

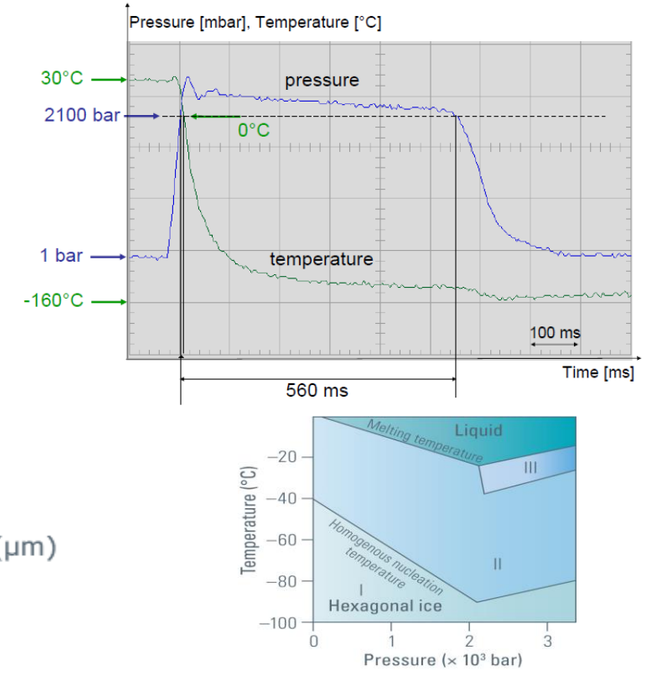


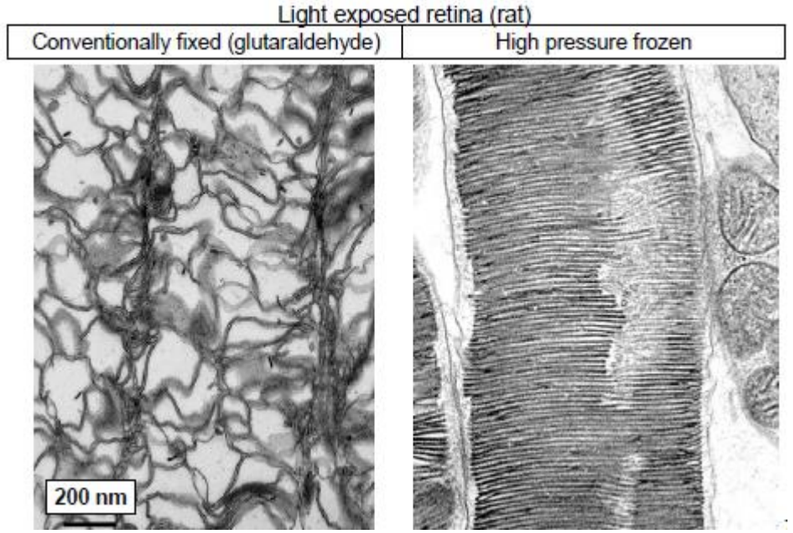
Adapted, image by Adres Kaech, University of Zurich.

High Pressure Freezing

- freezing of aqueous specimens up to approx. 200 µm thickness
- high pressure (2100 bars) + rapid cooling (>10^4 K/s) = vitrified specimen
- Water increases its volume upon freezing → great pressure applied during cooling makes ice formation difficult.



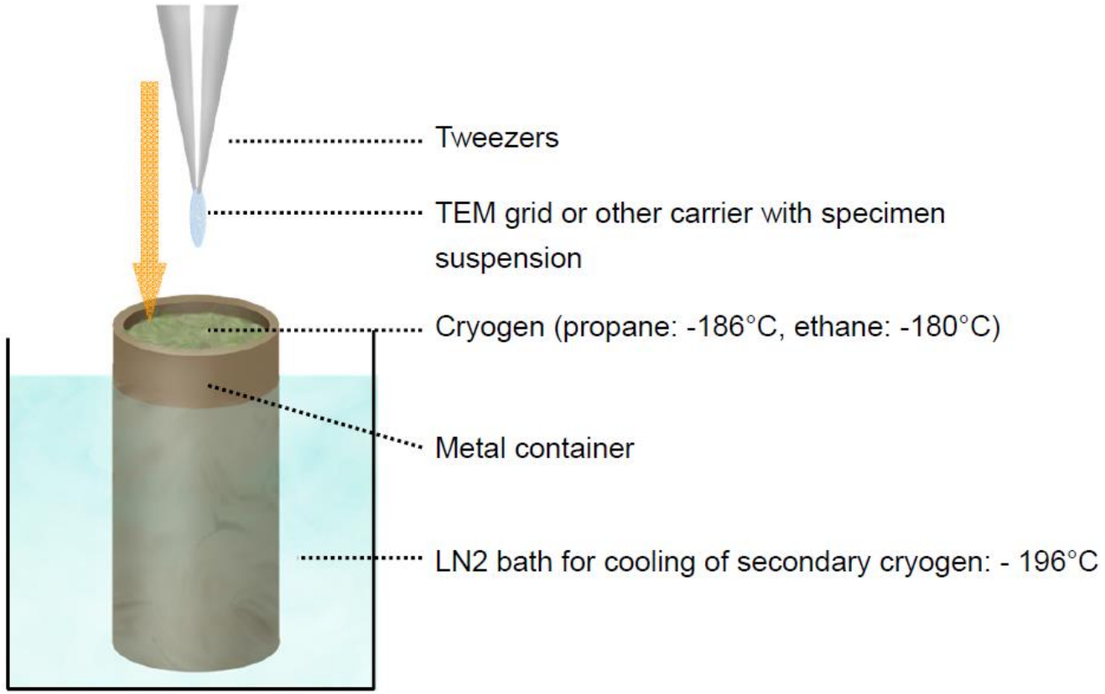




Adopted from www.leica.com; Adres Kaech, University of Zurich; Kanno H, et al. Science 189: 880–881 (1975)

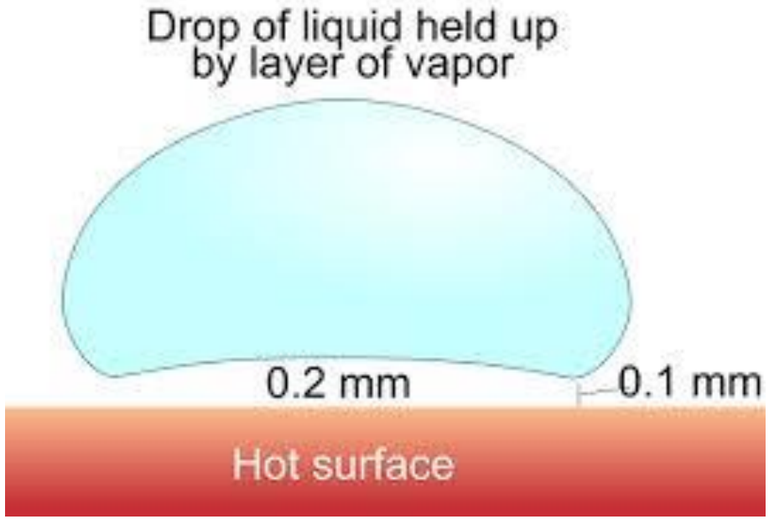
Plunge Freezing

- freezing of aqueous specimens: cell suspensions or thin slices (< 10 µm)
- plunging blotted specimens into melting e.g. ethane at high velocity
- high freezing velocity + coolant with high thermal conductivity needed

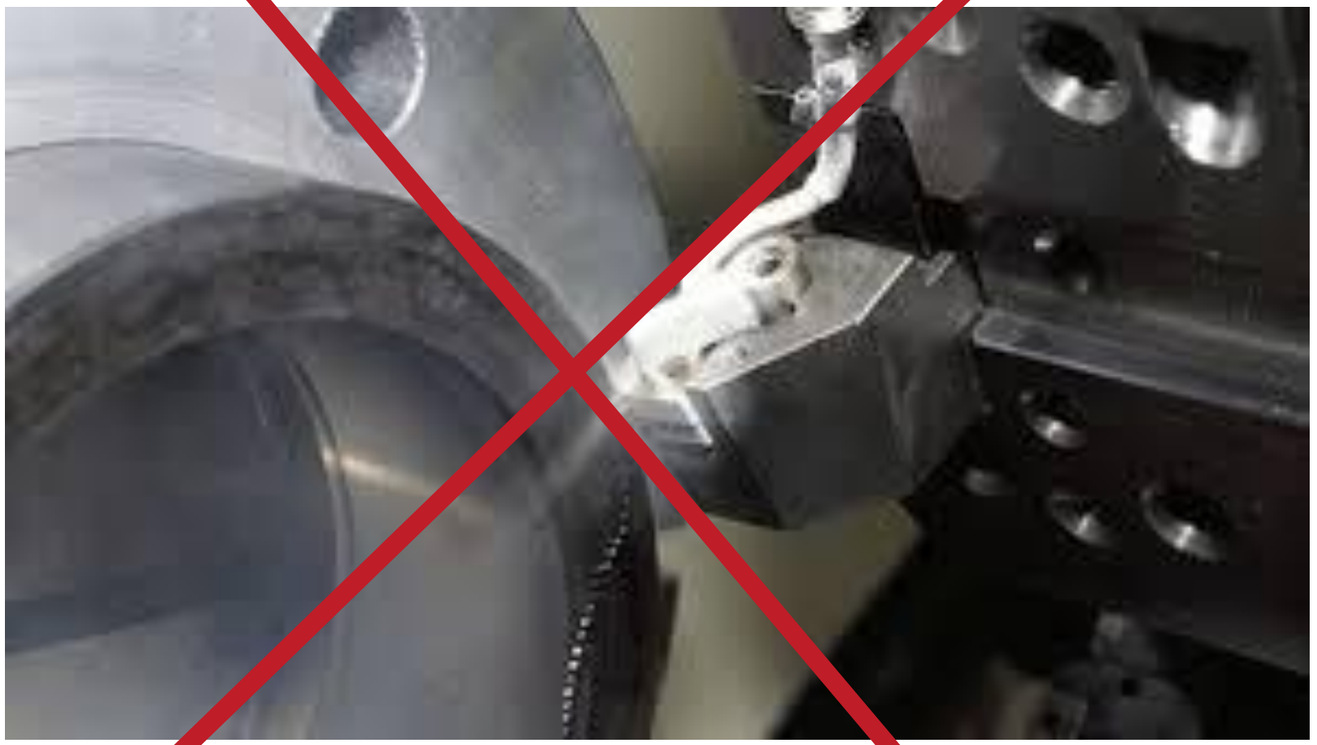


Adopted from presentation of Adres Kaech, University of Zurich.

Why ethane at -88?

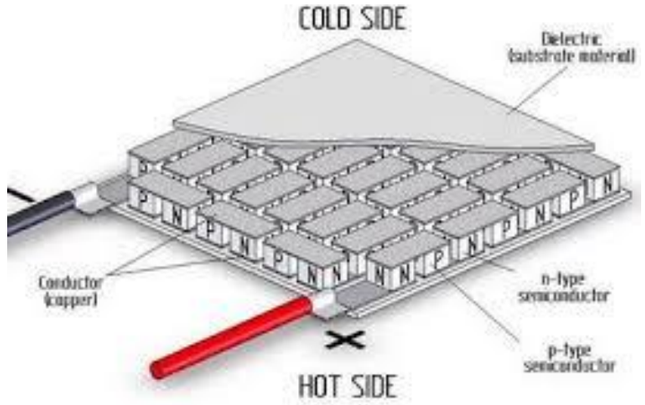


Methods of cooling



Direct Liquid/Gas Cooling

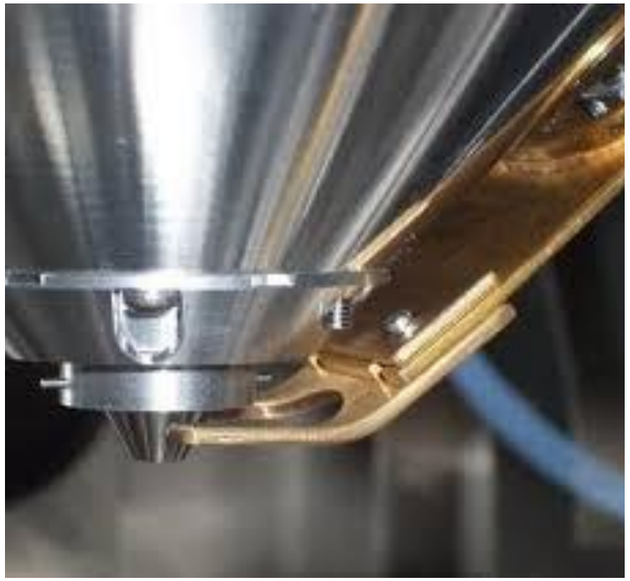
Methods of Cooling

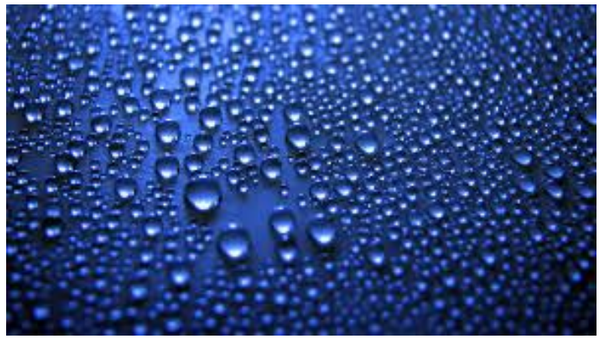


Passive and Closed Loop Flow cooling

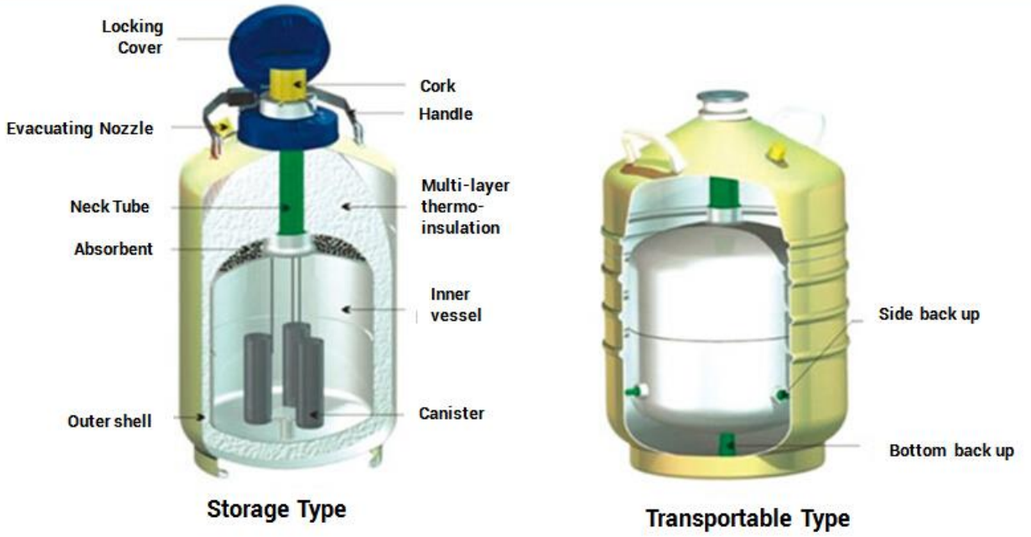
Peltier? -90

Methods of cryo pumping

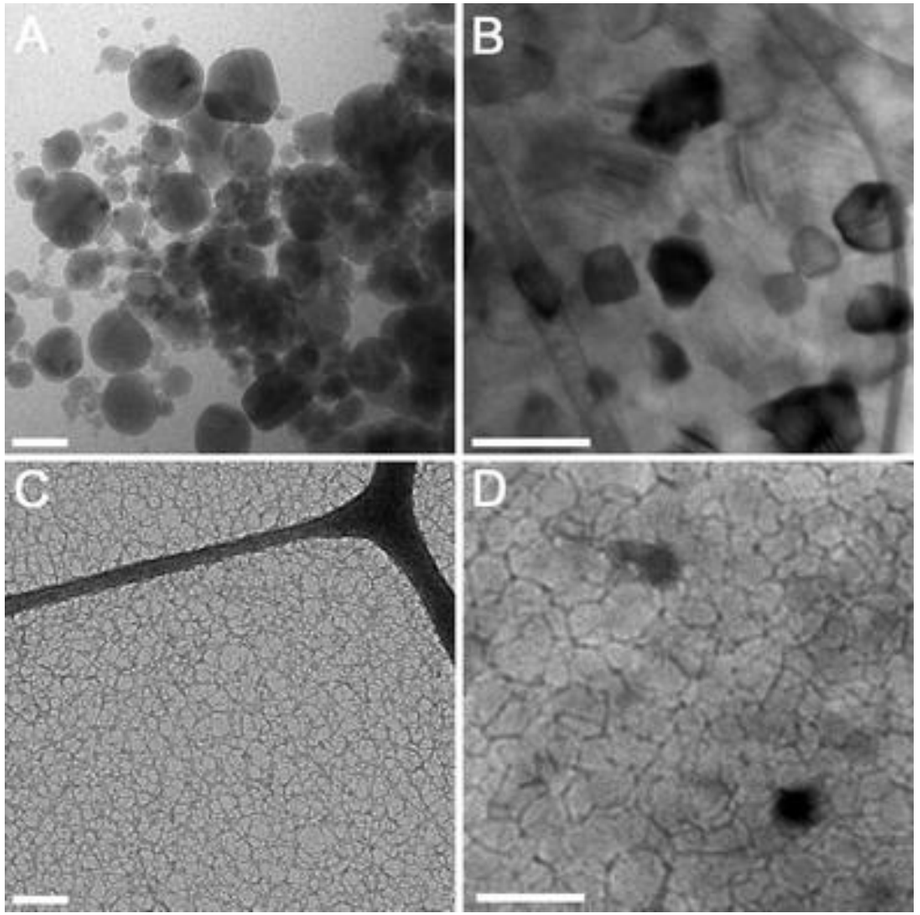




Cryogen storage and safety



Problems with contamination



Elucidating the assembled structure of amphiphiles in solution *via* cryogenic transmission electron microscopy

Honggang Cui,[a] Travis K. Hodgdon,[b] Eric W. Kaler,[b] Ludmila Abezgauz,[c] Dganit Danino,[c] Maya Lubovsky,[d] Yeshayahu Talmon[d] and Darrin J. Pochan*[a]
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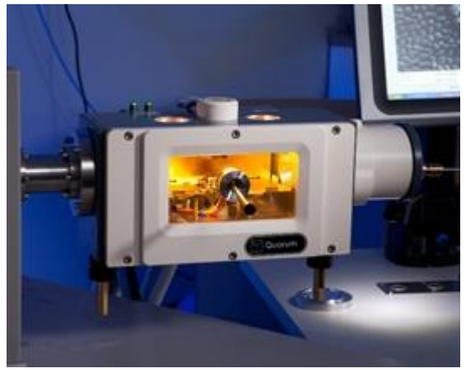
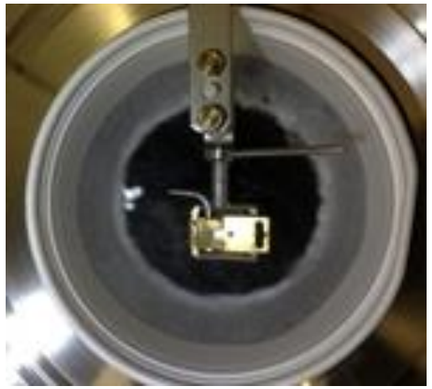
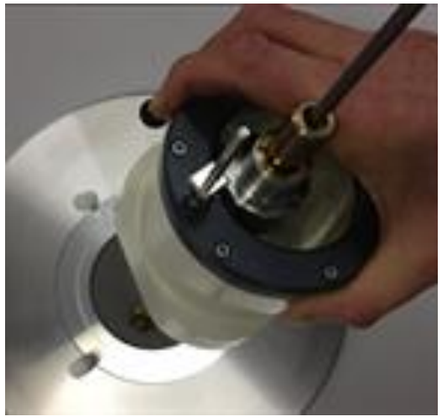
https://www.emsdiasum.com

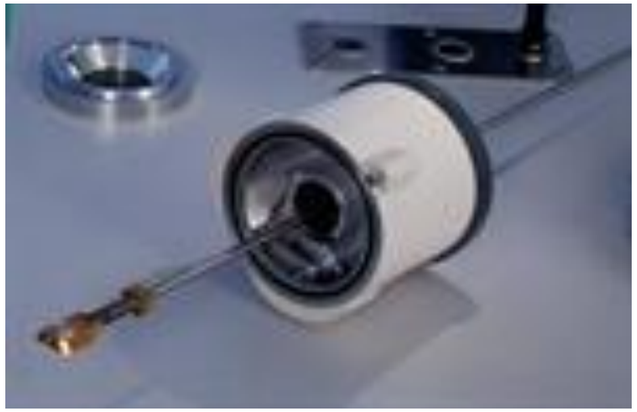
Cryo SEM

Features



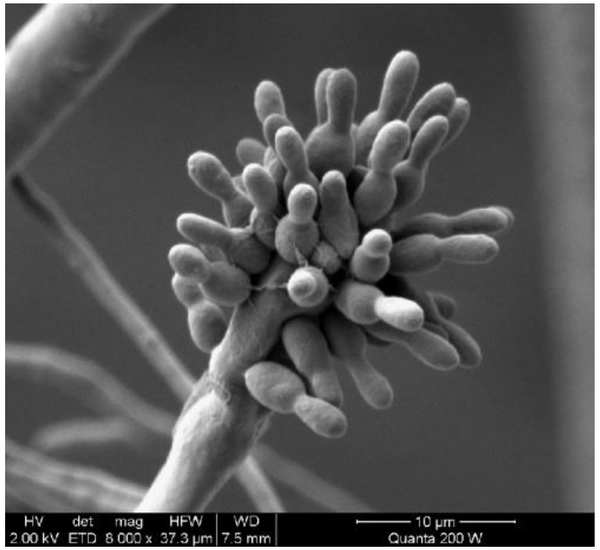


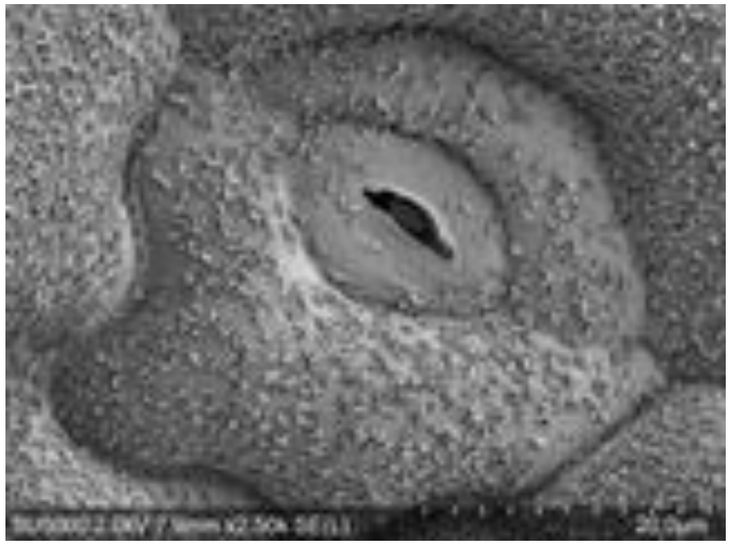


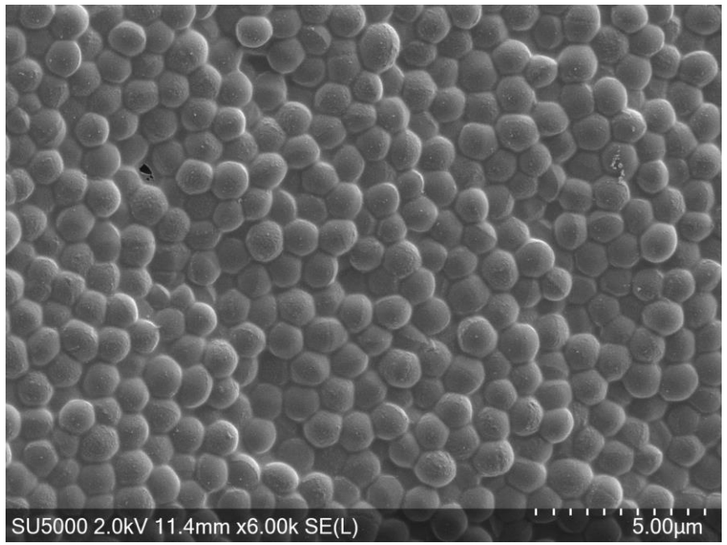


Images

Bio - Macro

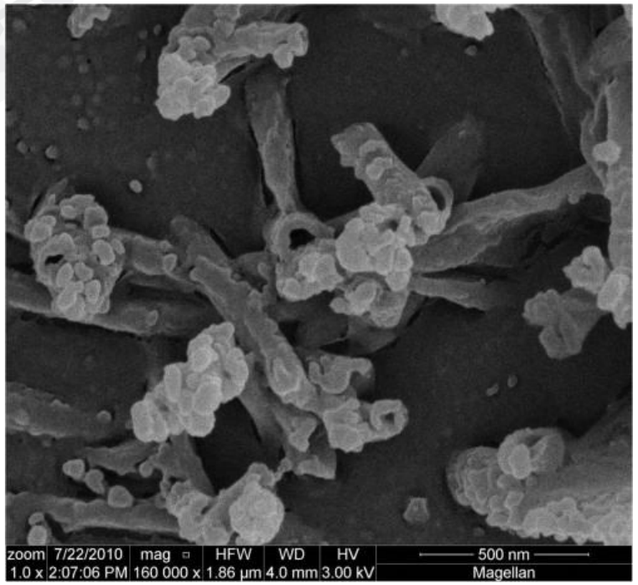


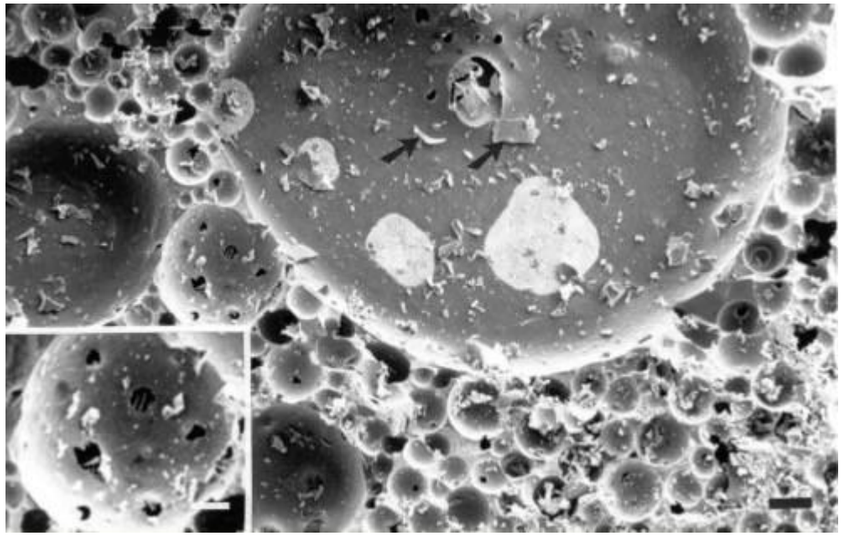


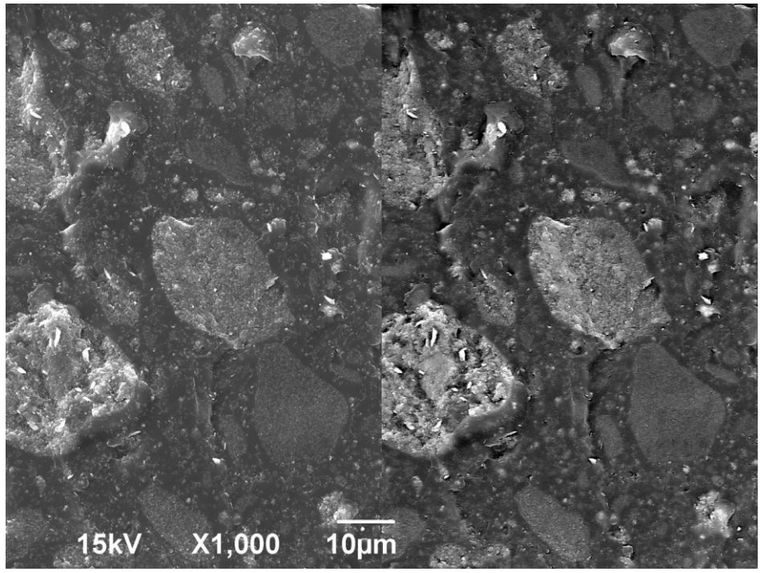


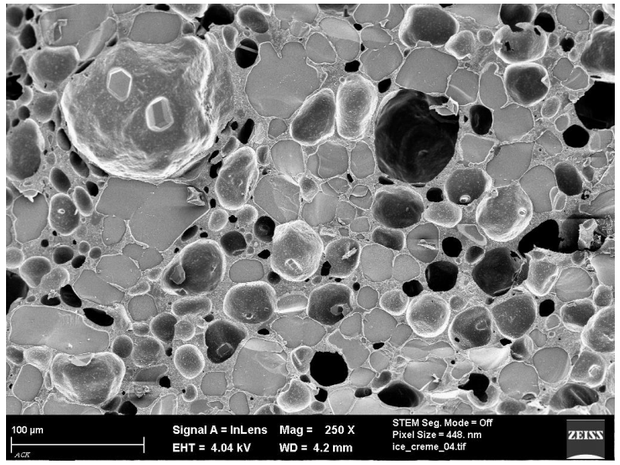
Explore. Discover. Resolve.

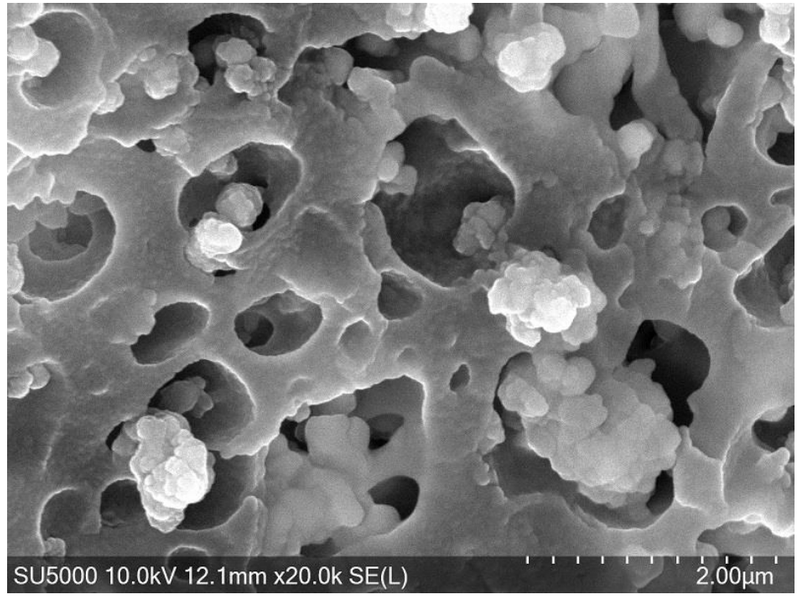
Images

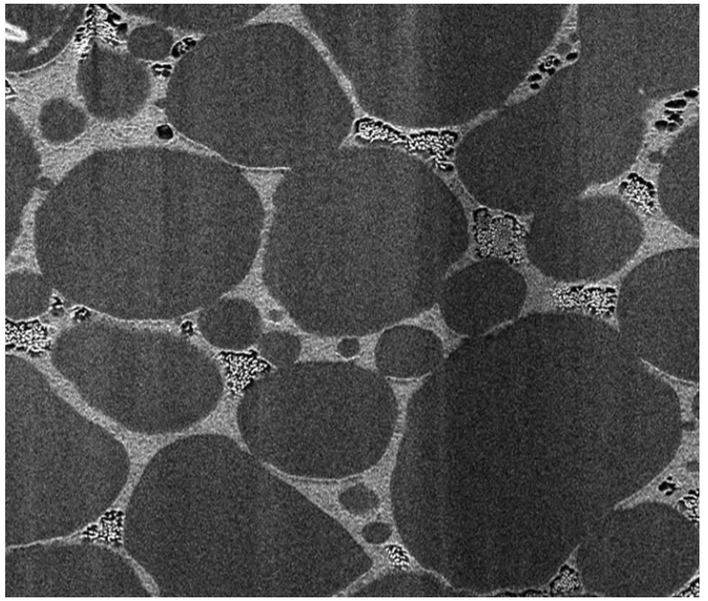

zoom 7/22/2010 mag HFW WD HV 500 nm
1.0 x 2:07:06 PM 160 000 x 1.86 µm 4.0 mm 3.00 kV Magellan



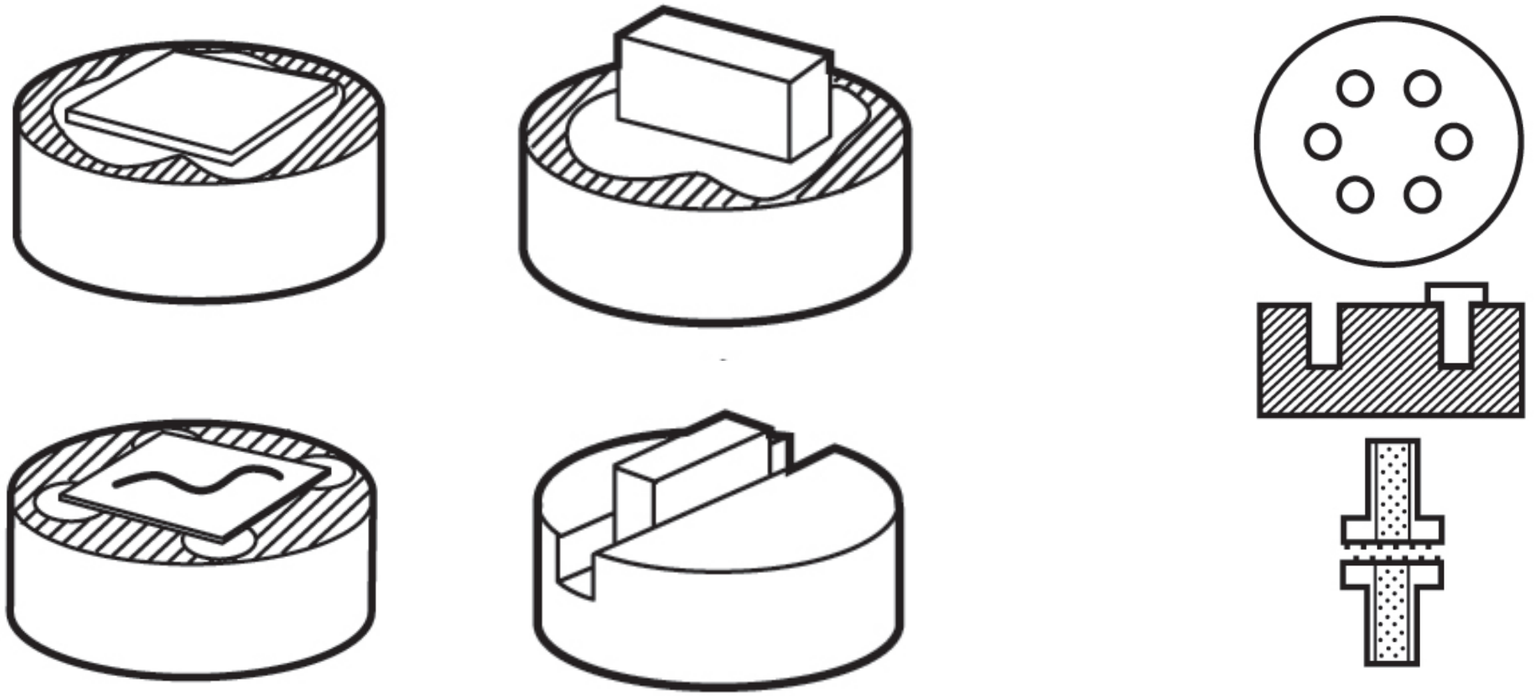

15kV X1,000 10µm


100 µm
Signal A = InLens Mag = 250 X
EHT = 4.04 kV WD = 4.2 mm
STEM Seg. Mode = Off
Pixel Size = 448. nm
ice_creme_04.tif
ZEISS

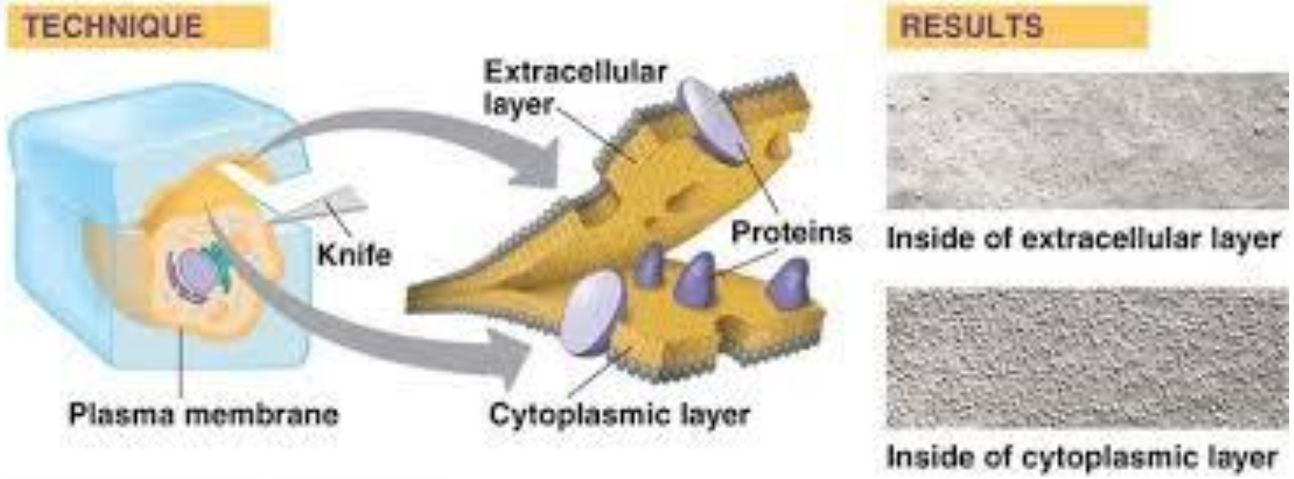

SU5000 10.0kV 12.1mm x20.0k SE(L)
2.00µm

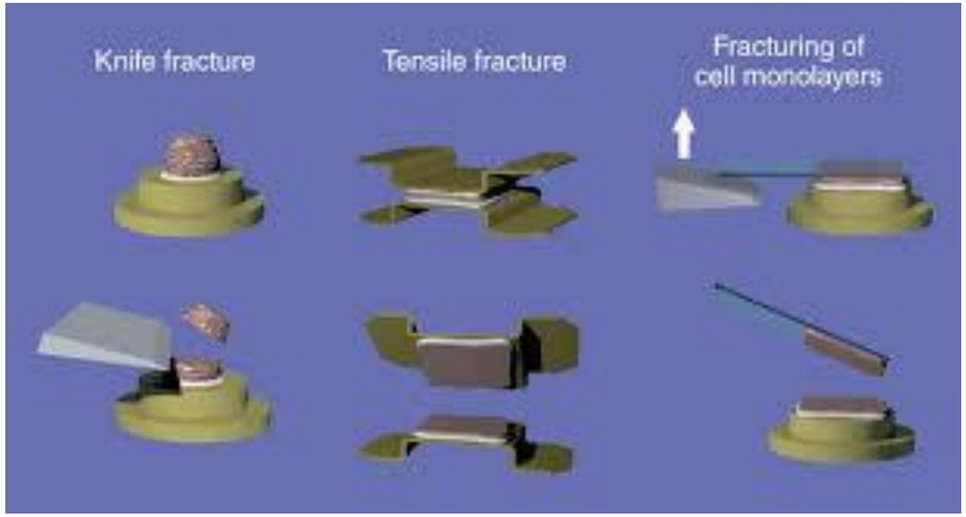


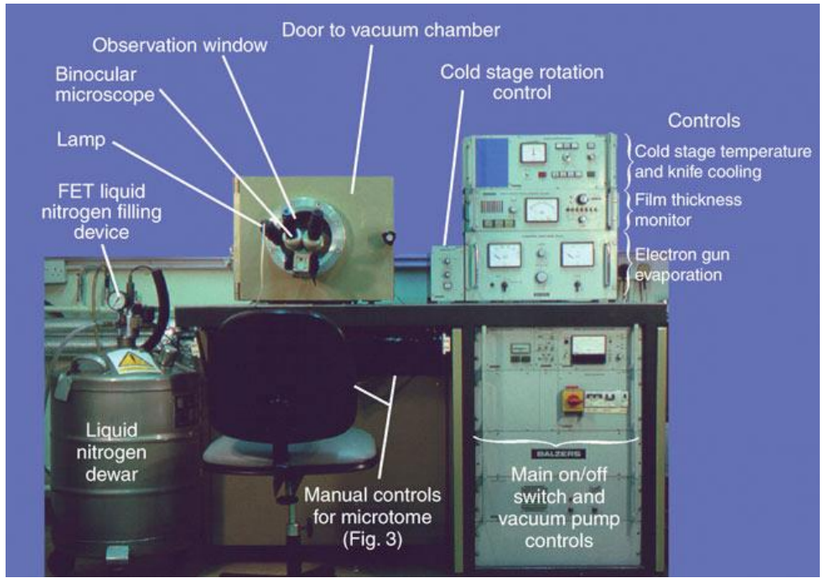
Mounting Methods for Cryo SEM

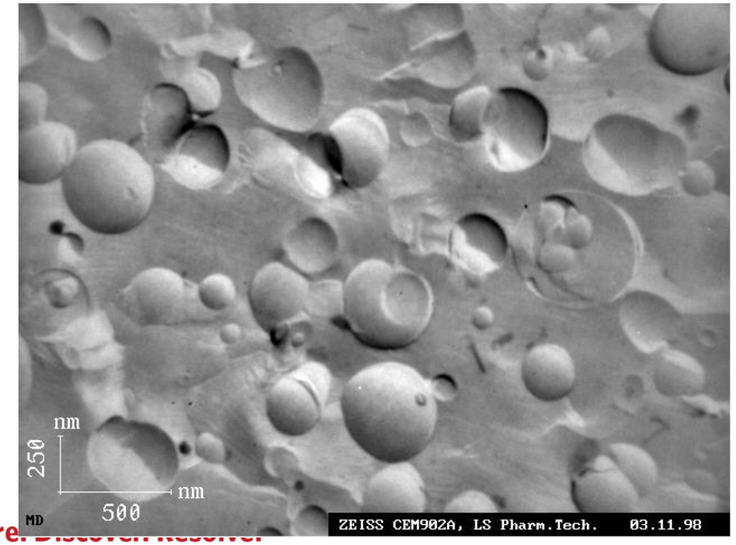


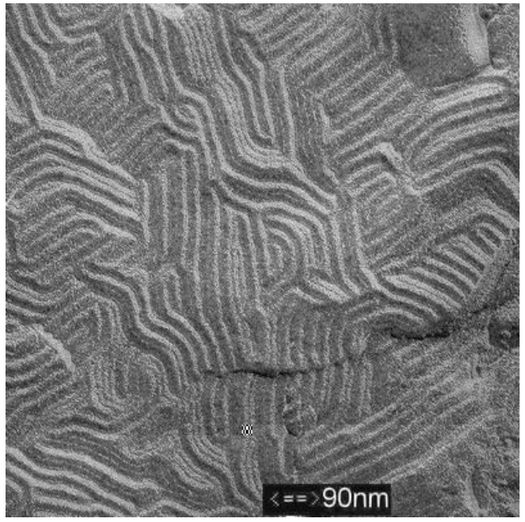
Freeze Fracture



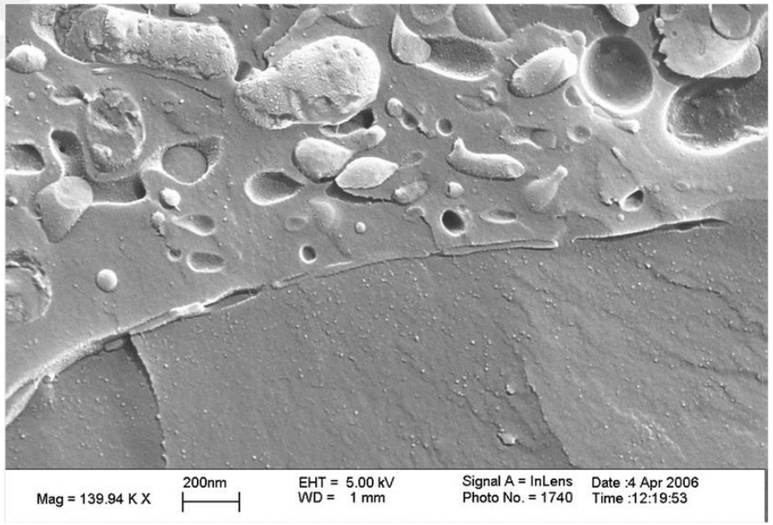


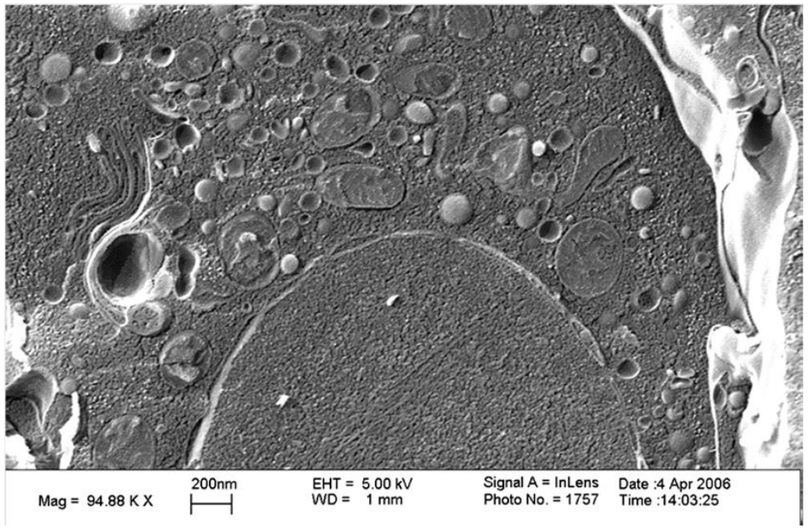




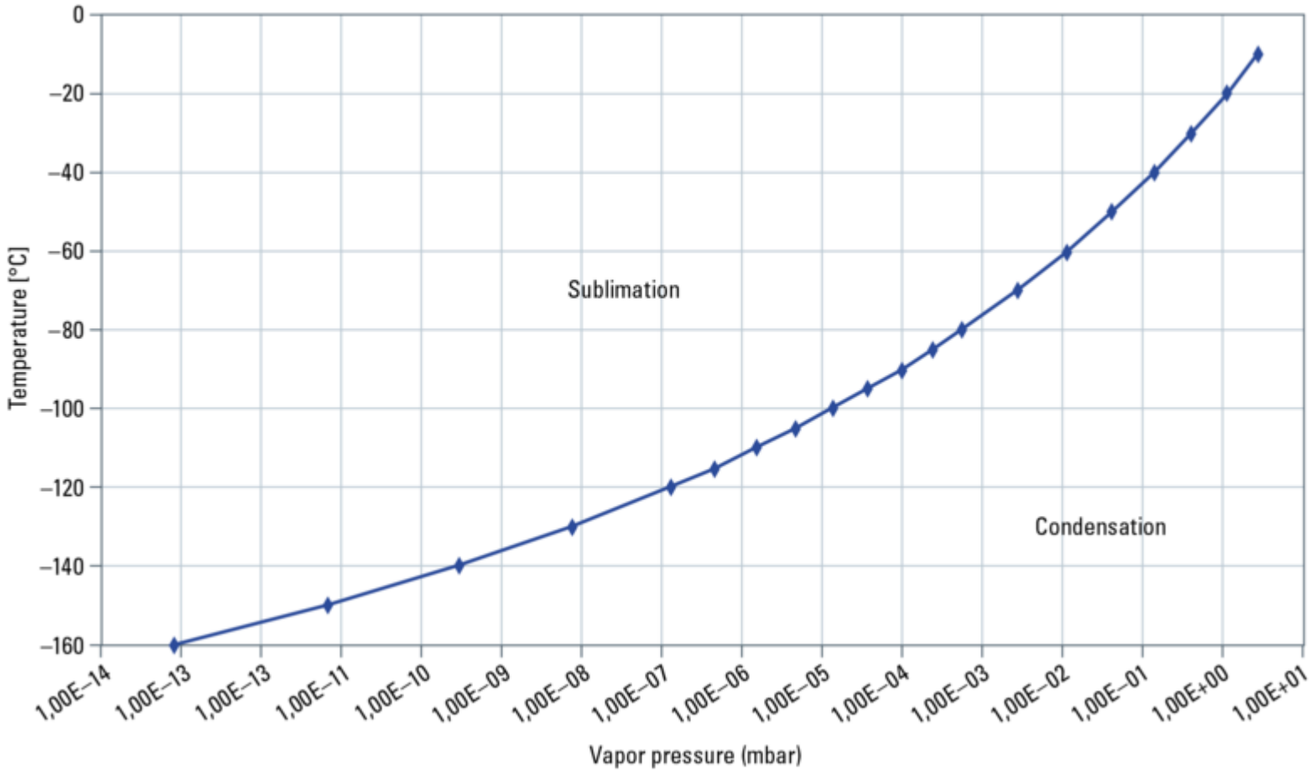


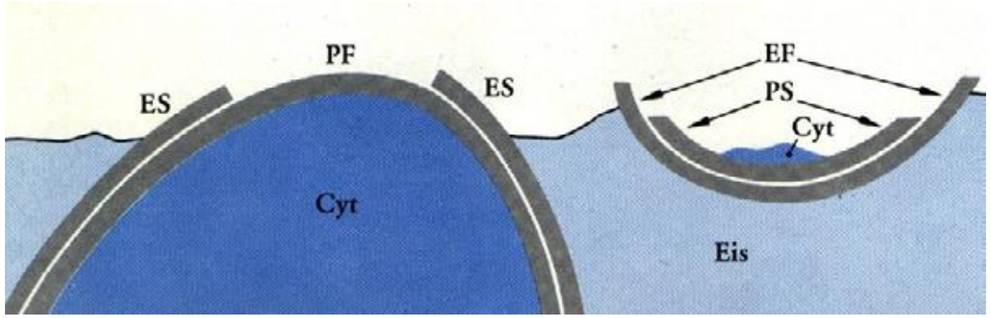
Sublimation Etching





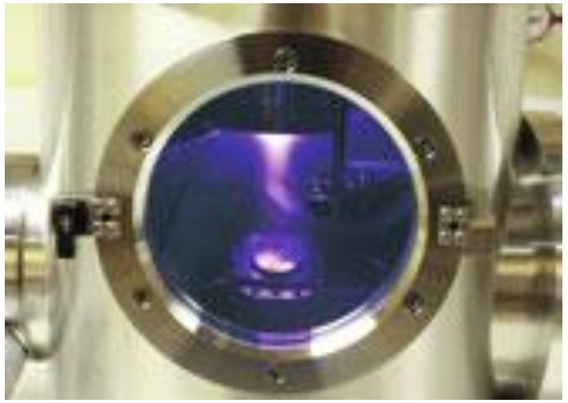
From ZTH, Zurich

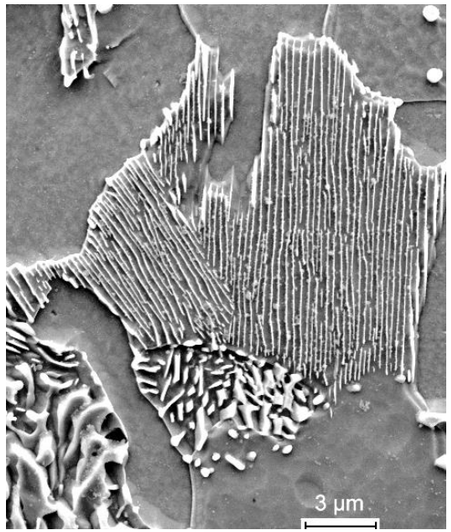


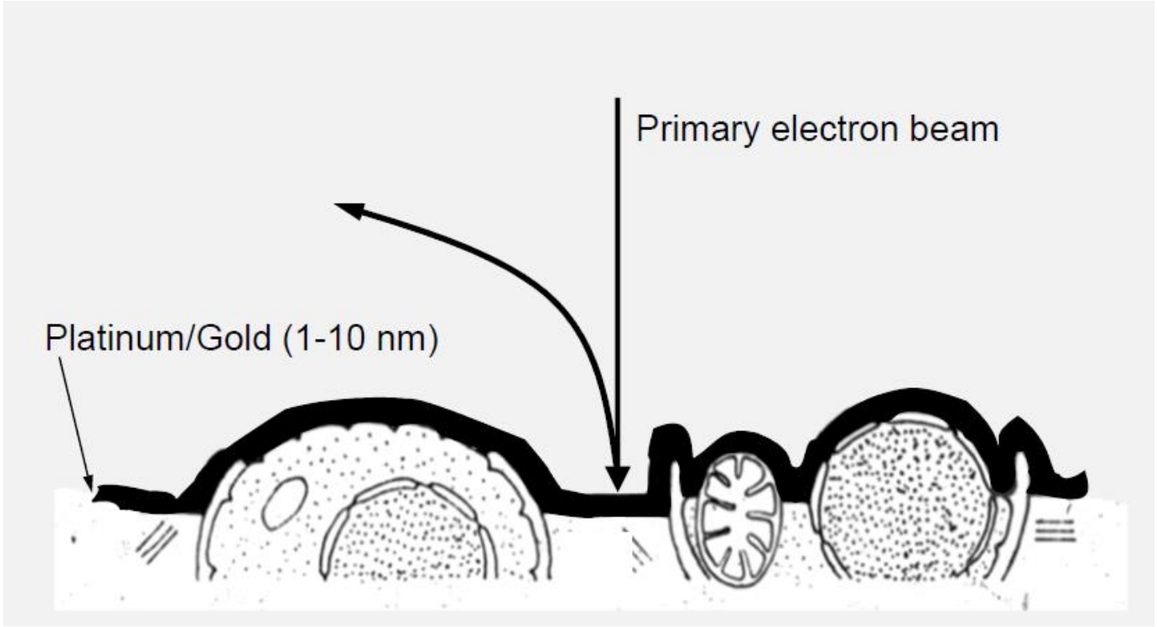


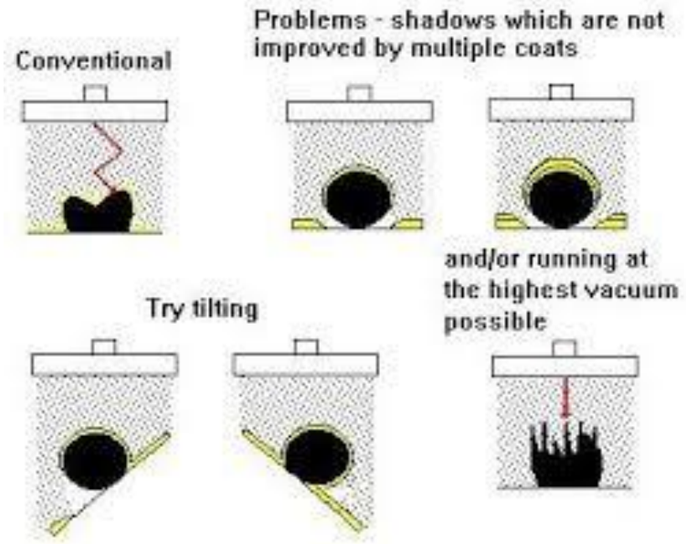
From Leica

Metal Coating in Cryo









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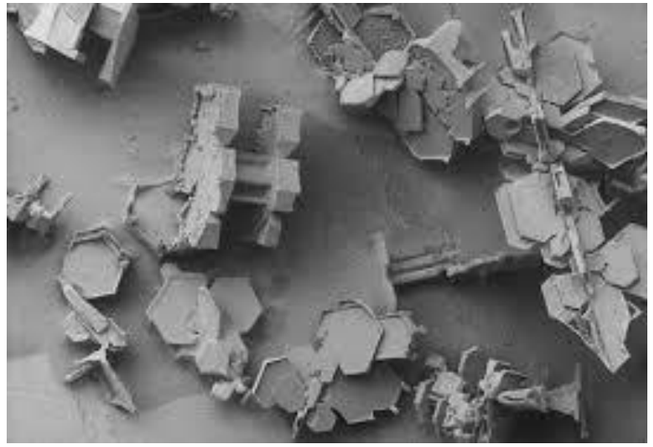
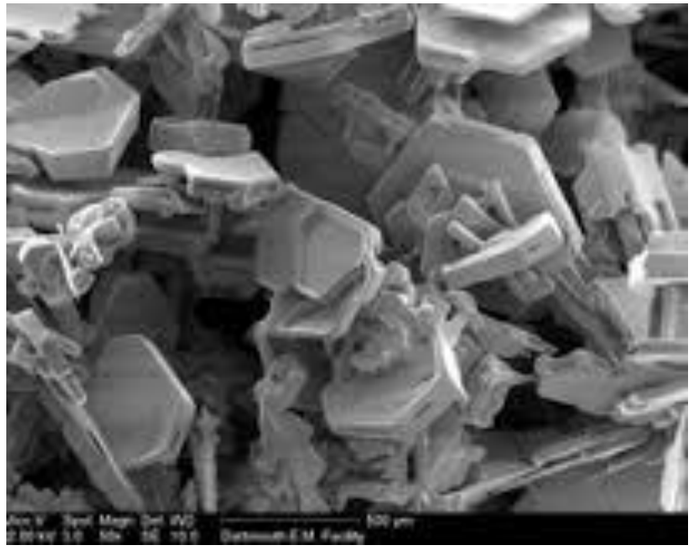
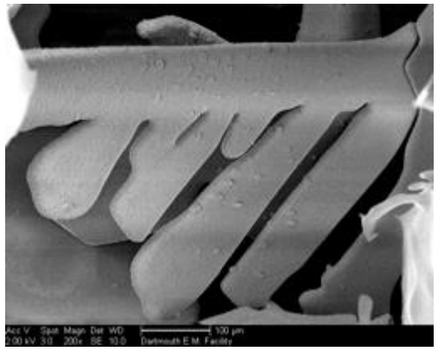
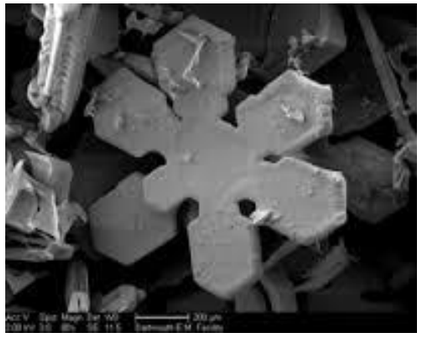
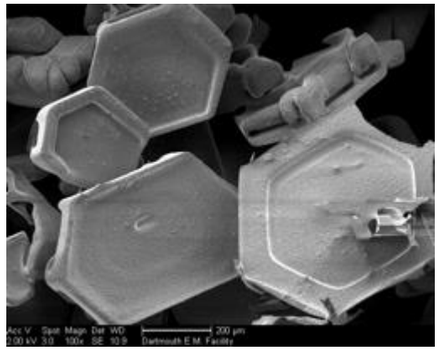
Cryo SEM Fun



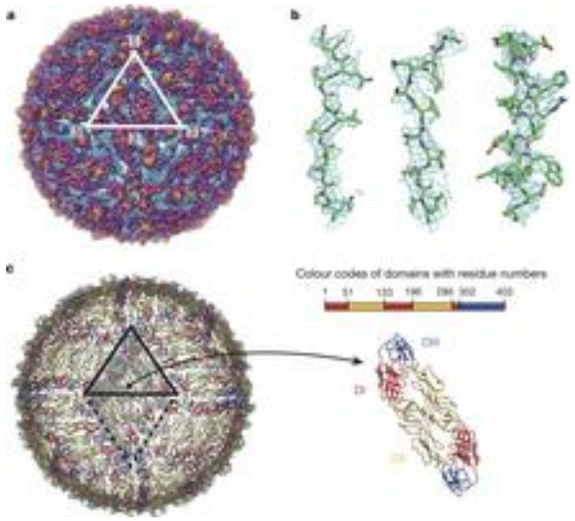




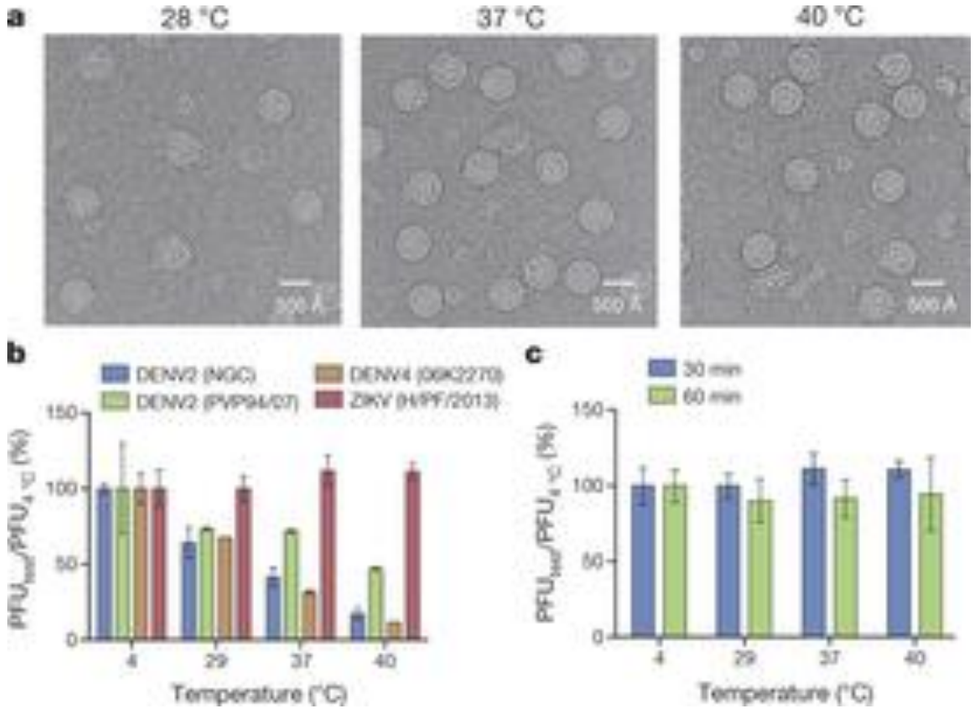




TEM

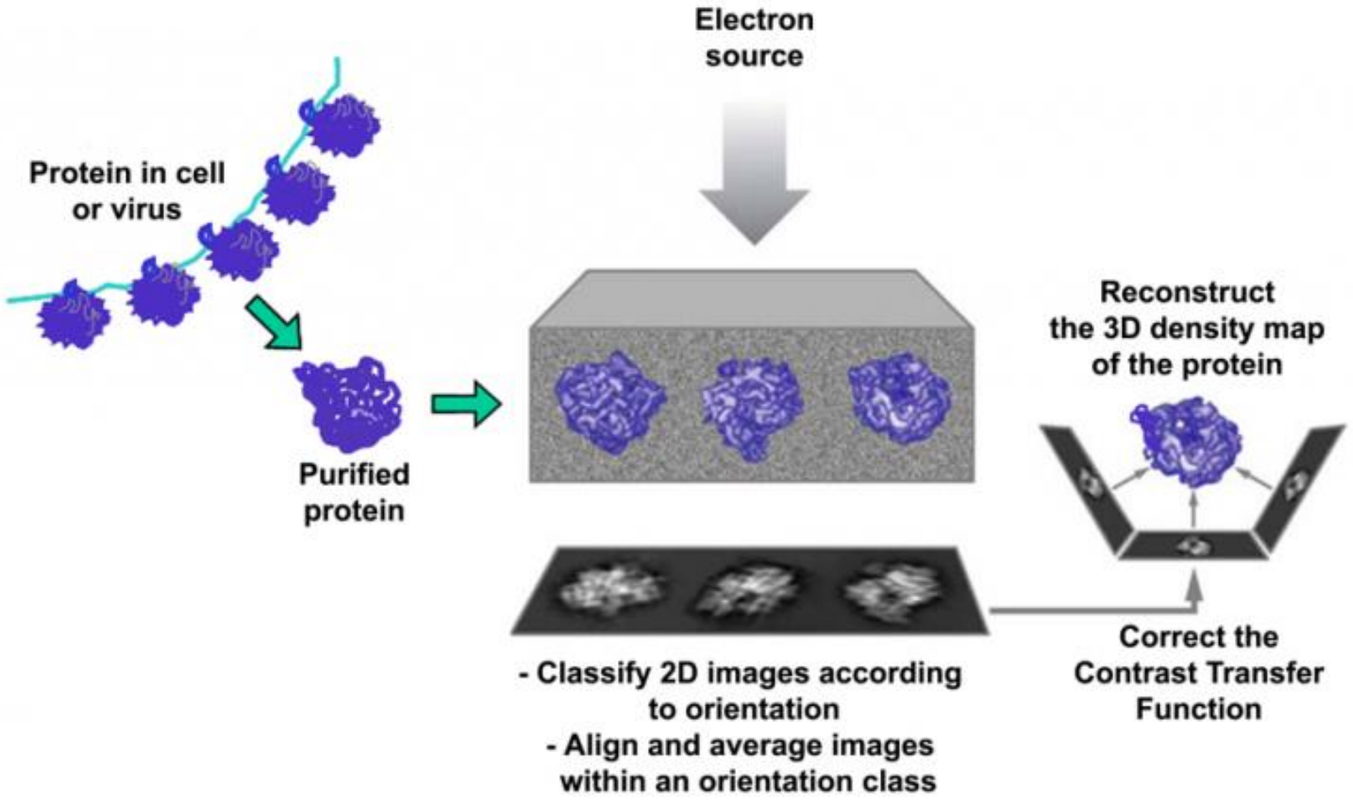


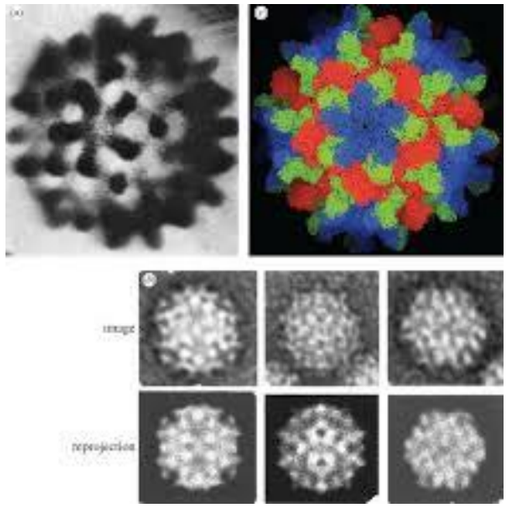
Structure of the thermally stable Zika virus
Nature (2016) doi:10.1038/nature17994



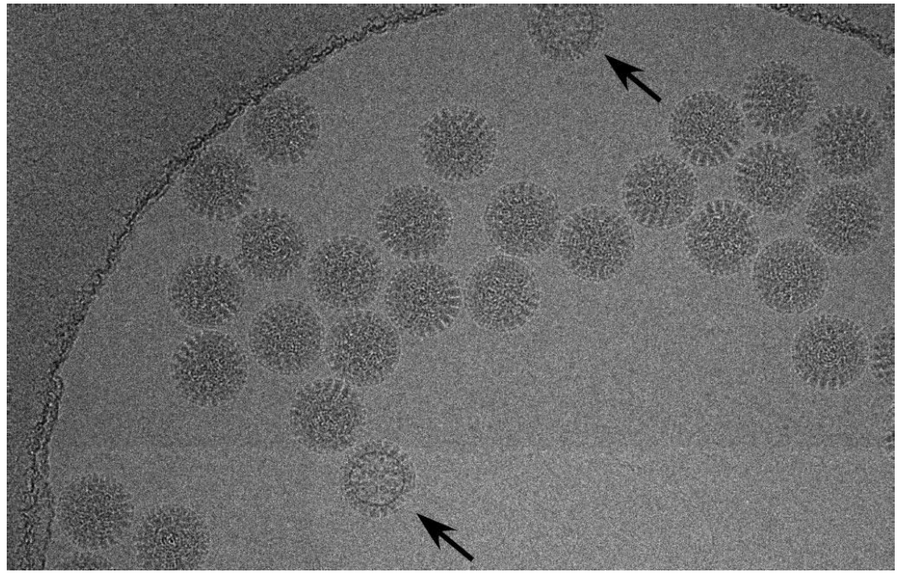
Explore. Discover. Resolve.

Single Particle Analysis



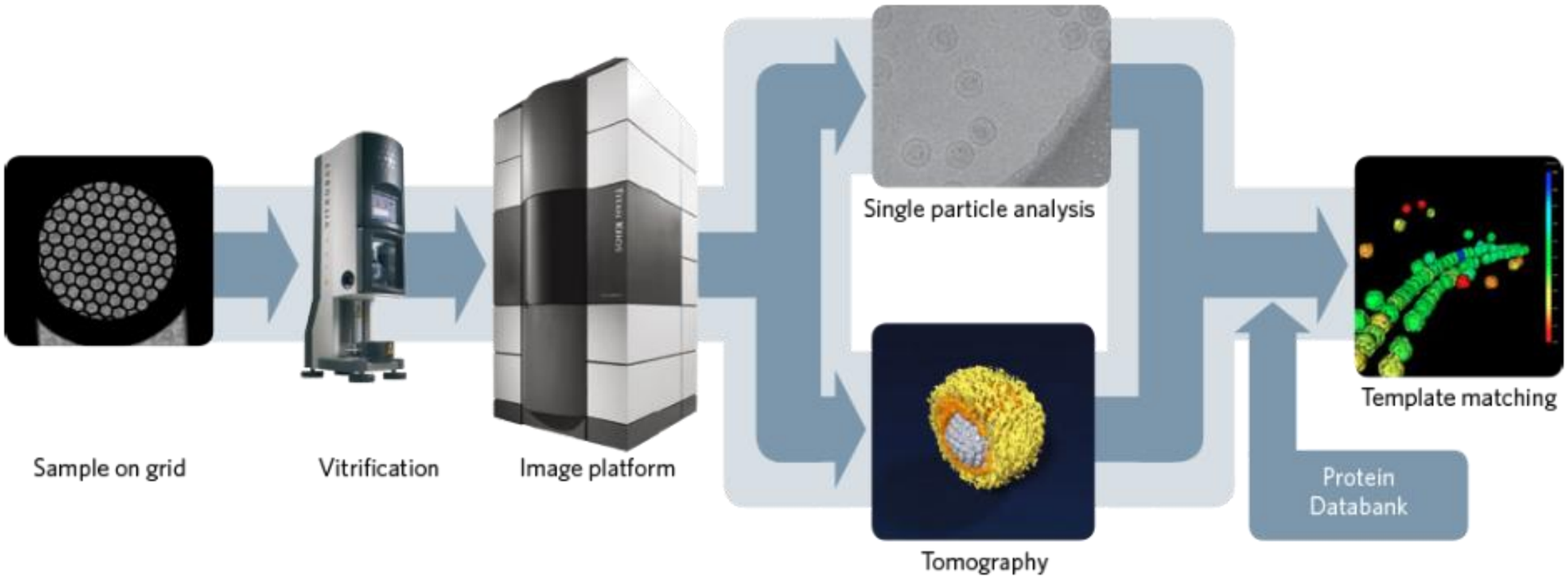


http://cns.fas.harvard.edu/CryoEM

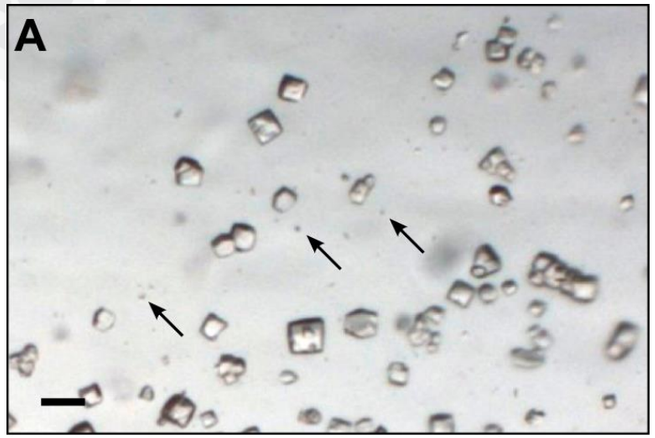


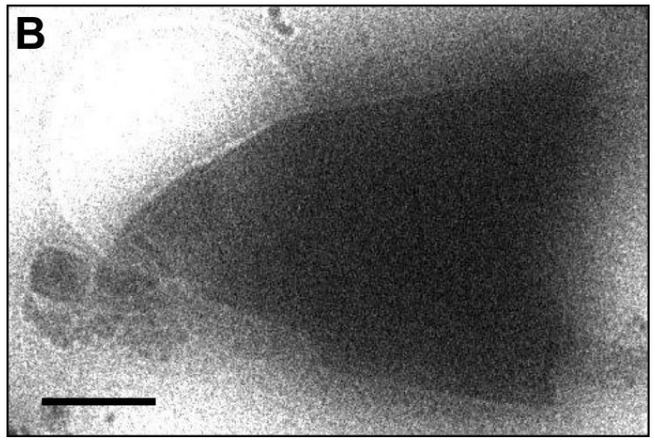
www.pnas.orgcgidoi10.1073pnas.0711623105

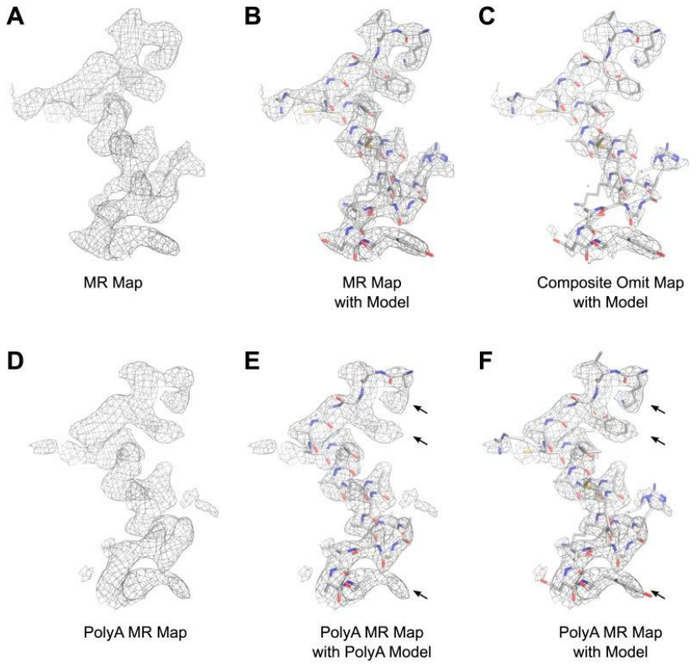
Spa workflow

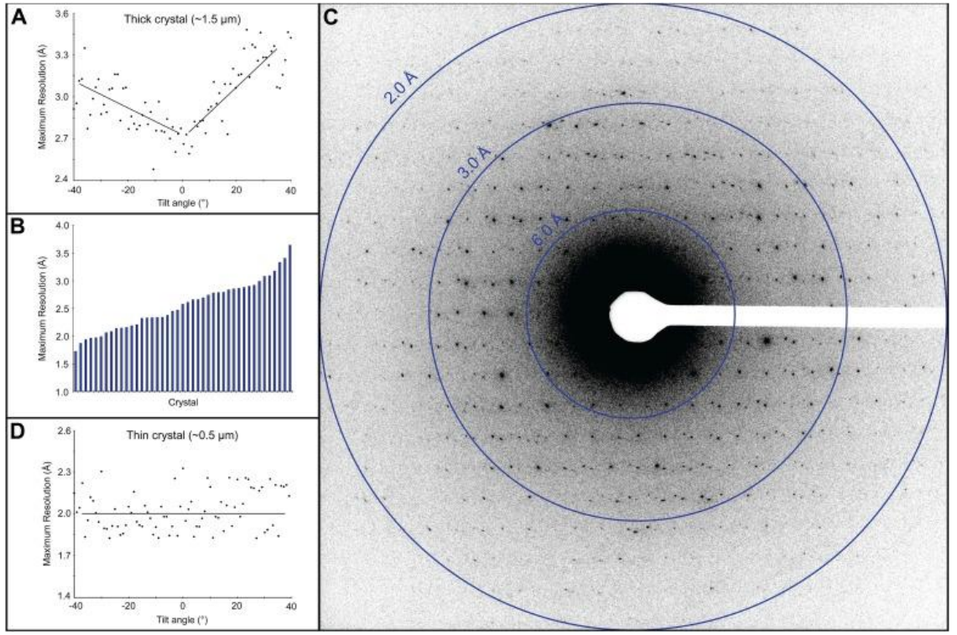


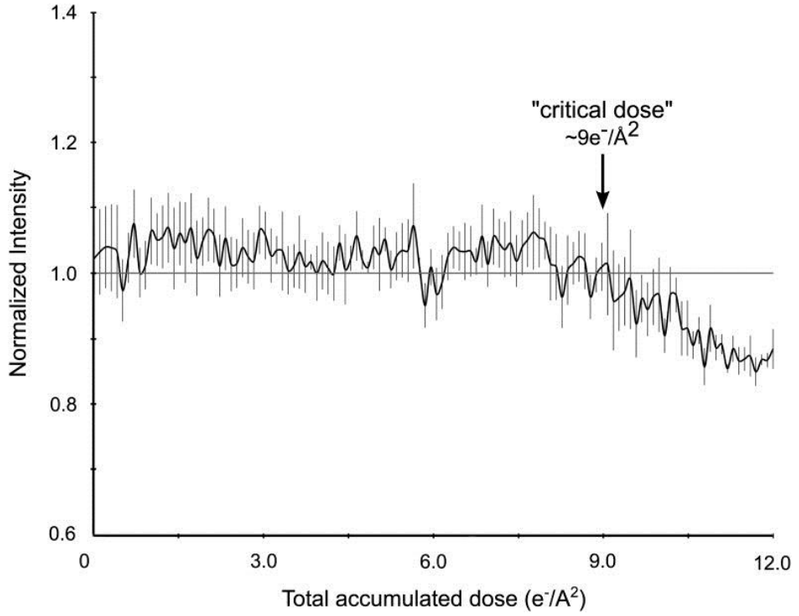
Crystallography









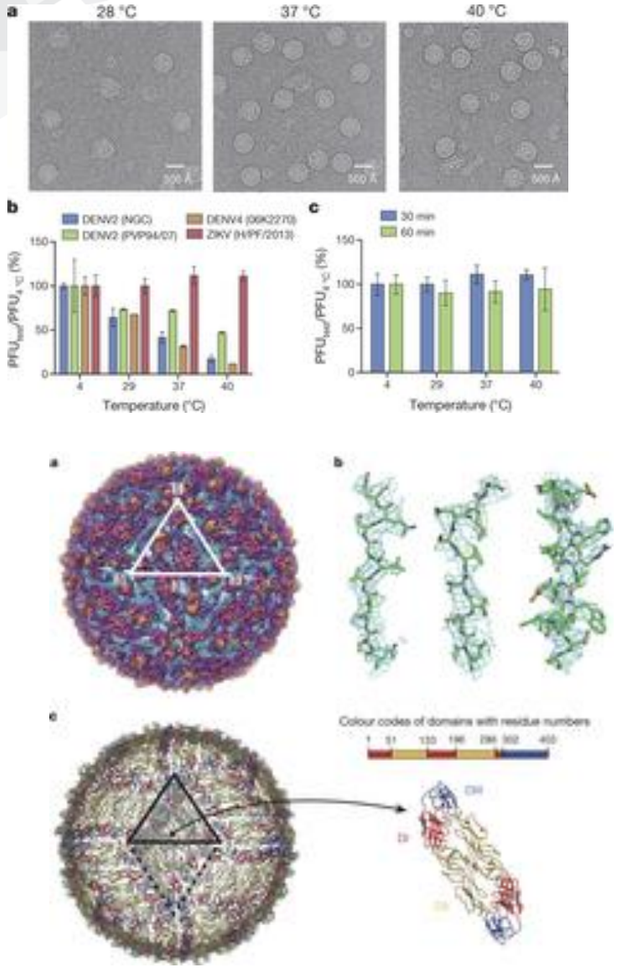


Three-dimensional electron crystallography of protein microcrystals

Dan Shi,[1,†] Brent L Nannenga,[1,†] Matthew G Iadanza,[1,†] and Tamir Gonen[1,*]

Explore. Discover. Resolve.

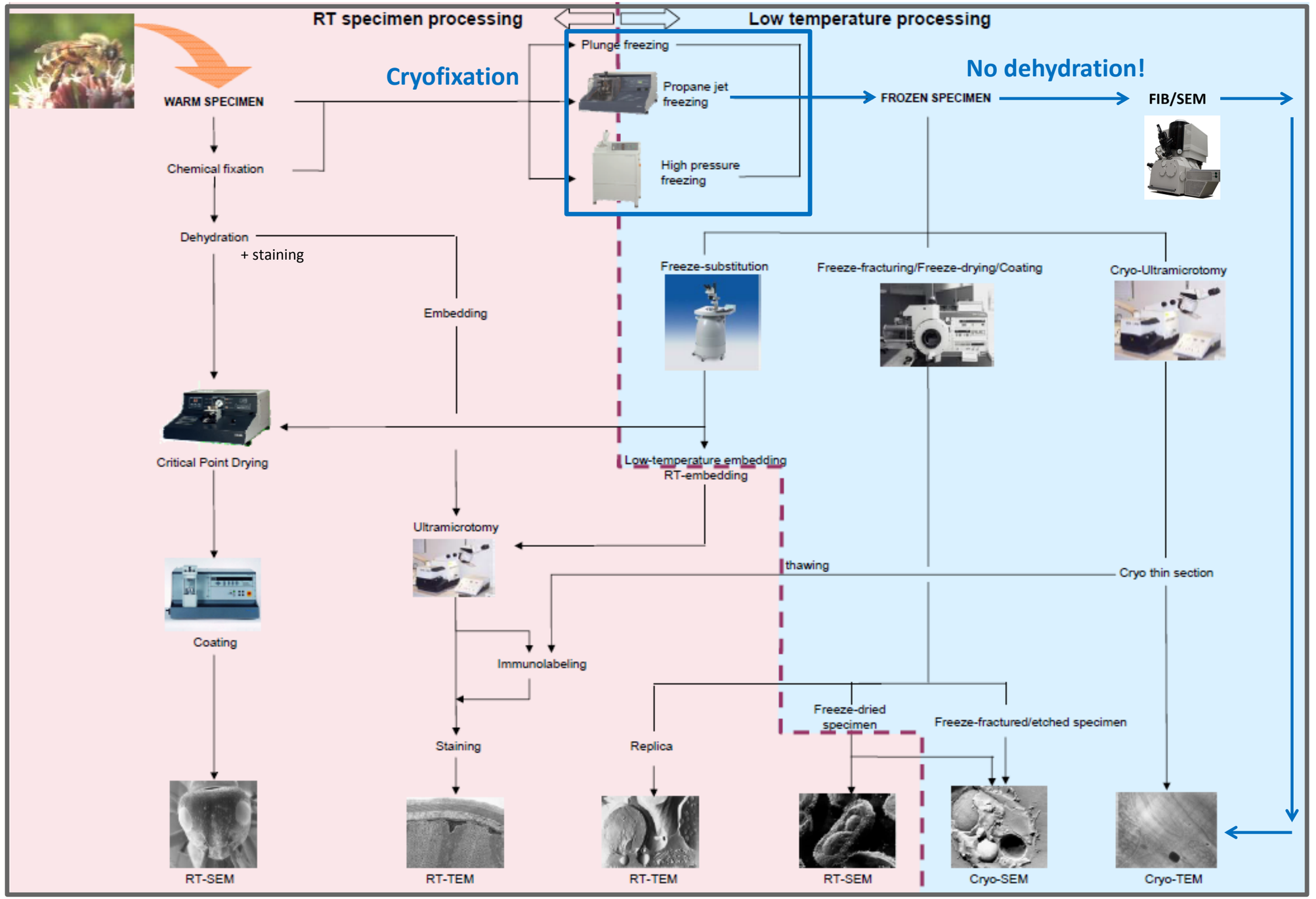
Data – use zika and ebola



Structure of the thermally stable Zika virus
Nature (2016) doi:10.1038/nature17994

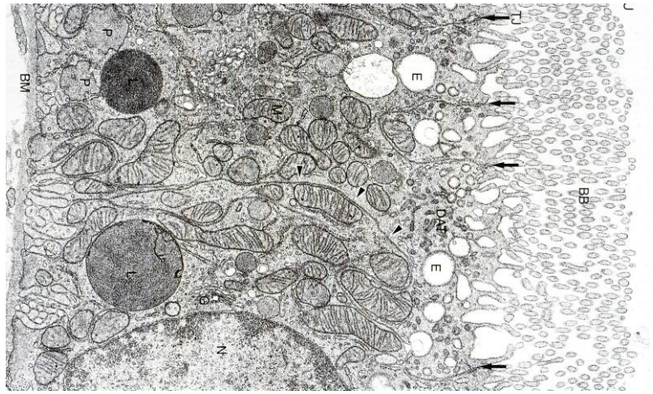
Sample preparation flow chart

(selected techniques)



Adapted, image by Adres Kaech, University of Zurich.

Infiltration and embedding

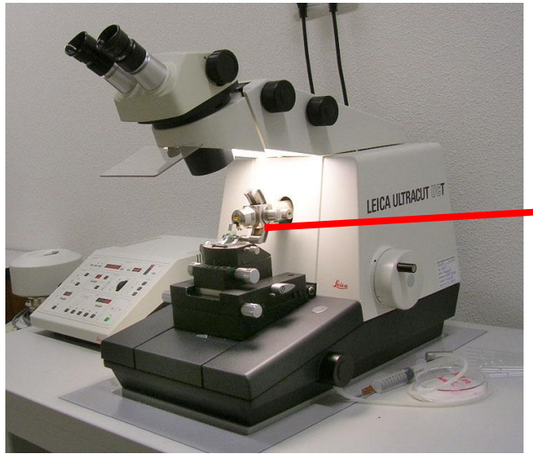


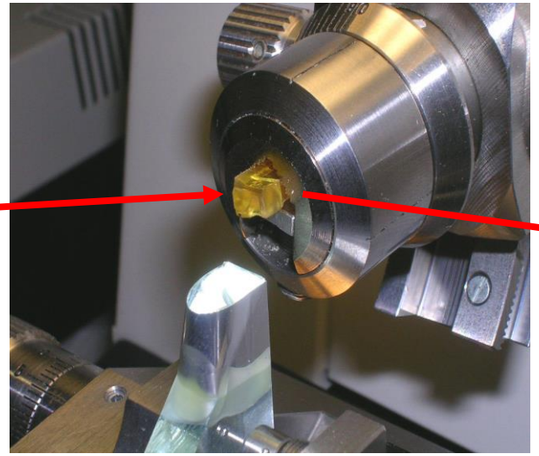
- To replace all water in the sample with liquid resin.
- To form the tissue into a hard block to allow the sectioning of thin (< 100 nm) sections.

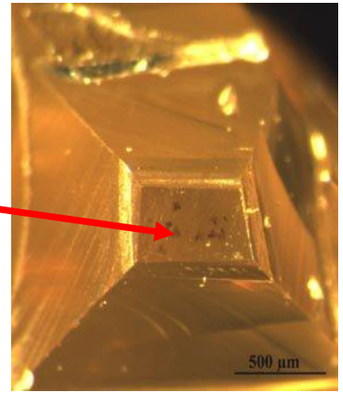
Resins

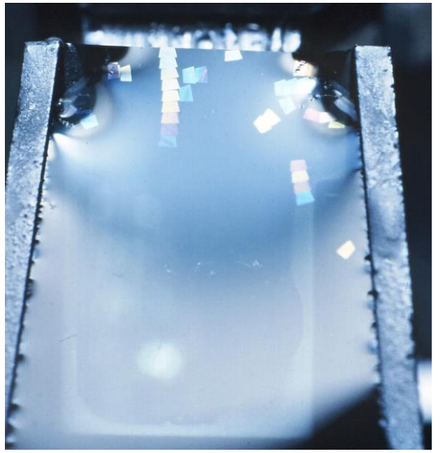
- Epoxy, cured with heat at 60-70^{o}C.
- Acrylic, can be cured at 50^{o}C or at low temps of -20 to -30^{o}C with UV.

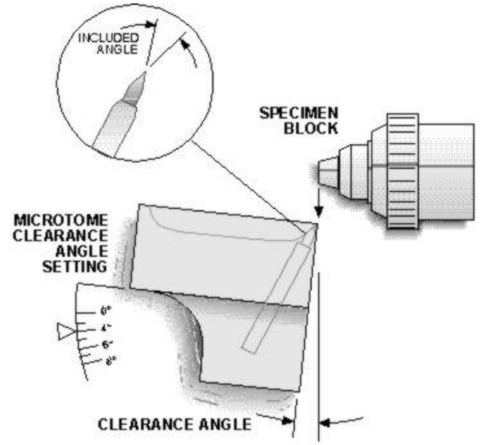
Normal sectioning of resin blocks

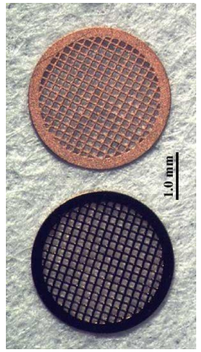


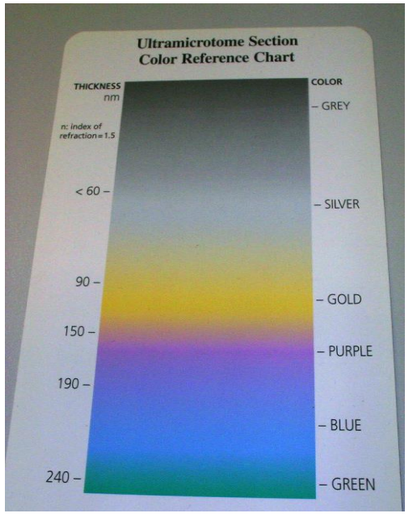




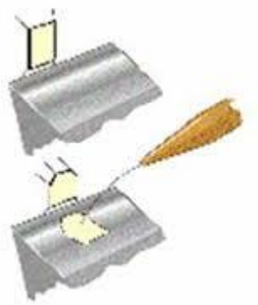


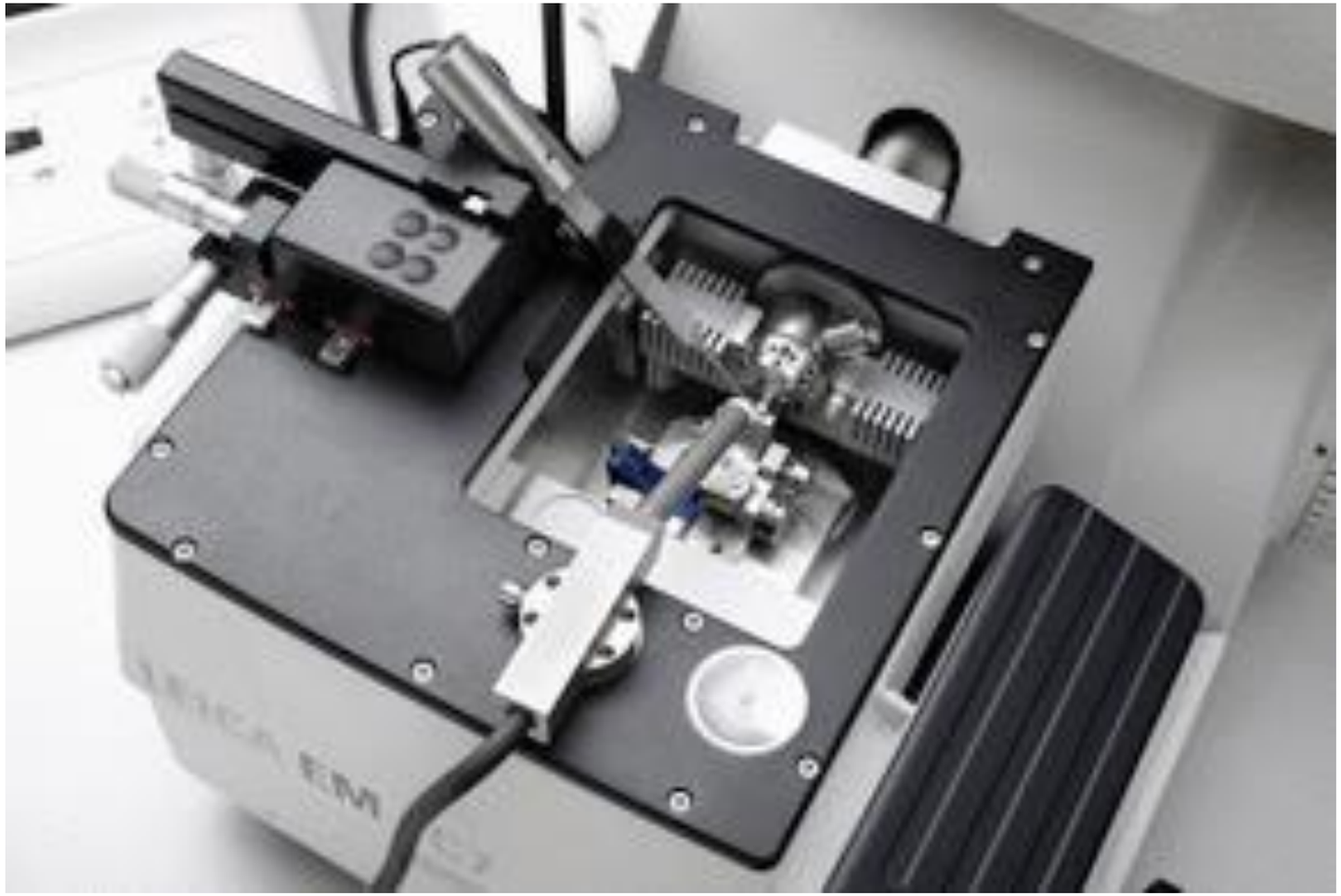






Cryo Ultra Microtomy





Cryo-FIB sample preparation and observation

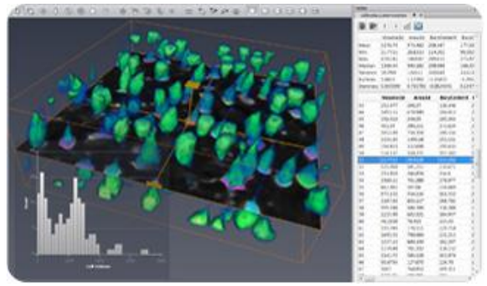
“A great challenge for cell biology is to study the complex interplay of molecular assemblies in cellular systems in situ and at different scales of resolution (e.g. from ‘cells to molecules’).”…

“Especially for ultrastructural imaging in cell biology this (FIB) preparation route is becoming increasingly important, similar to the impact micro- and nanomachining techniques had on the progress in materials science. A key instrument in this context is the FIB system.”…

(Rigort A., Plitzko J. M., ABB 581 (2015), 122-130.)

- **basic idea** = to prepare cellular samples for TEM investigations directly on the EM grid
- hardware and protocols adapted to cryogenic conditions (first successful cryo-FIB experiment on bio sample in 2003)
- frozen samples (plunge freezing, HPF) in situ thinned
- native status/context → image is generated by the density of the biological material itself, no artifacts generated by sample preparation

Cryo TEM prep workflow



Vitrobot

Sample Plunge Freezing.

High quality sample preparation.

CorrSight

Correlative Light and Confocal Imaging of Cryo Samples.

Scios

Correlative Electron/Ion imaging and FIB milling of Lamella, Slice and View.

Krios

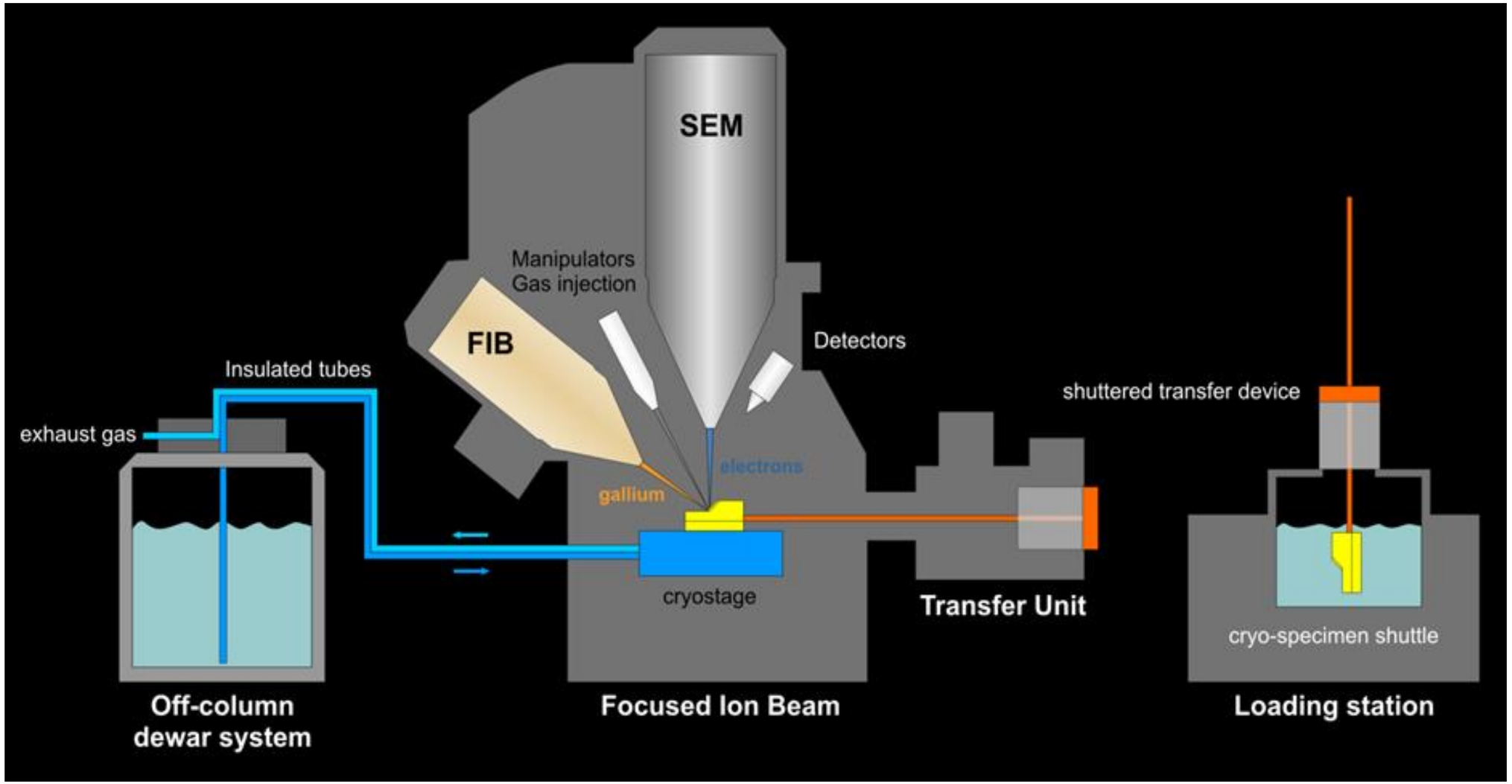
Correlative TEM imaging, tomography, diffraction and automation.

Amira & Maps

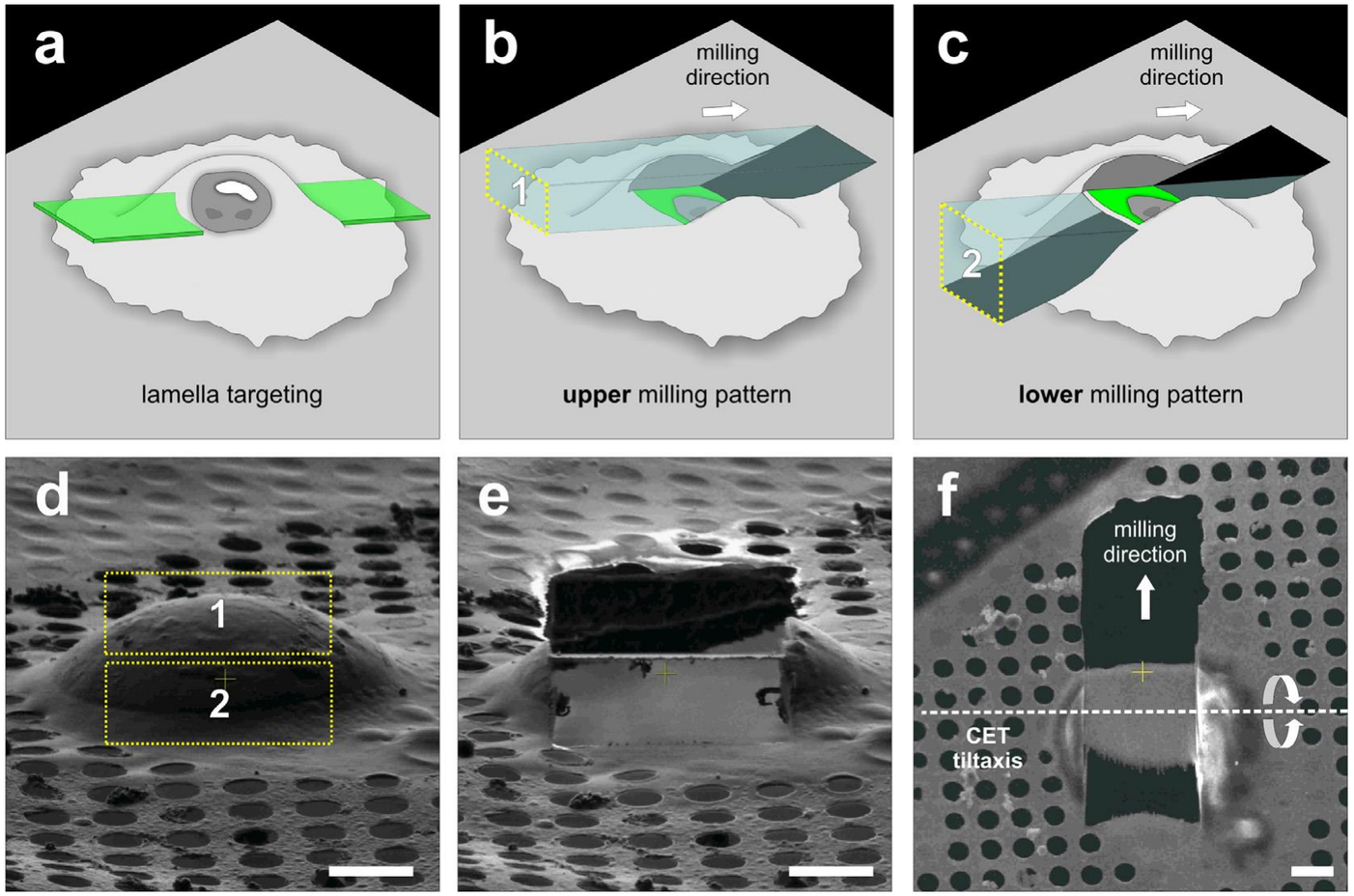
Extraction of the data from the Krios and the correlation with the light and electron.

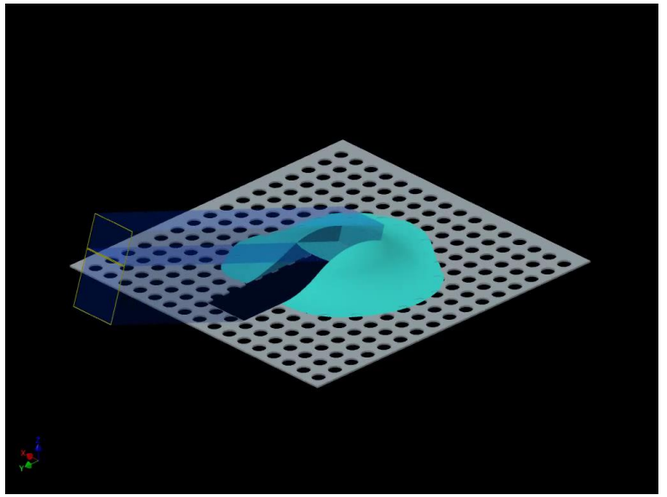
FEI is the only manufacturer to offer a ***complete*** workflow solution.

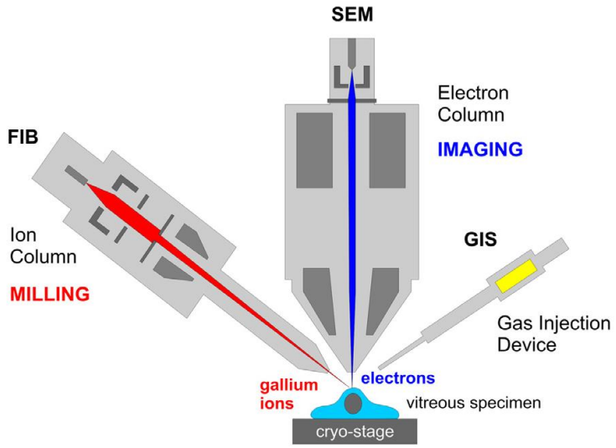
The Scios FIB transfer system



In situ cryo lamella milling



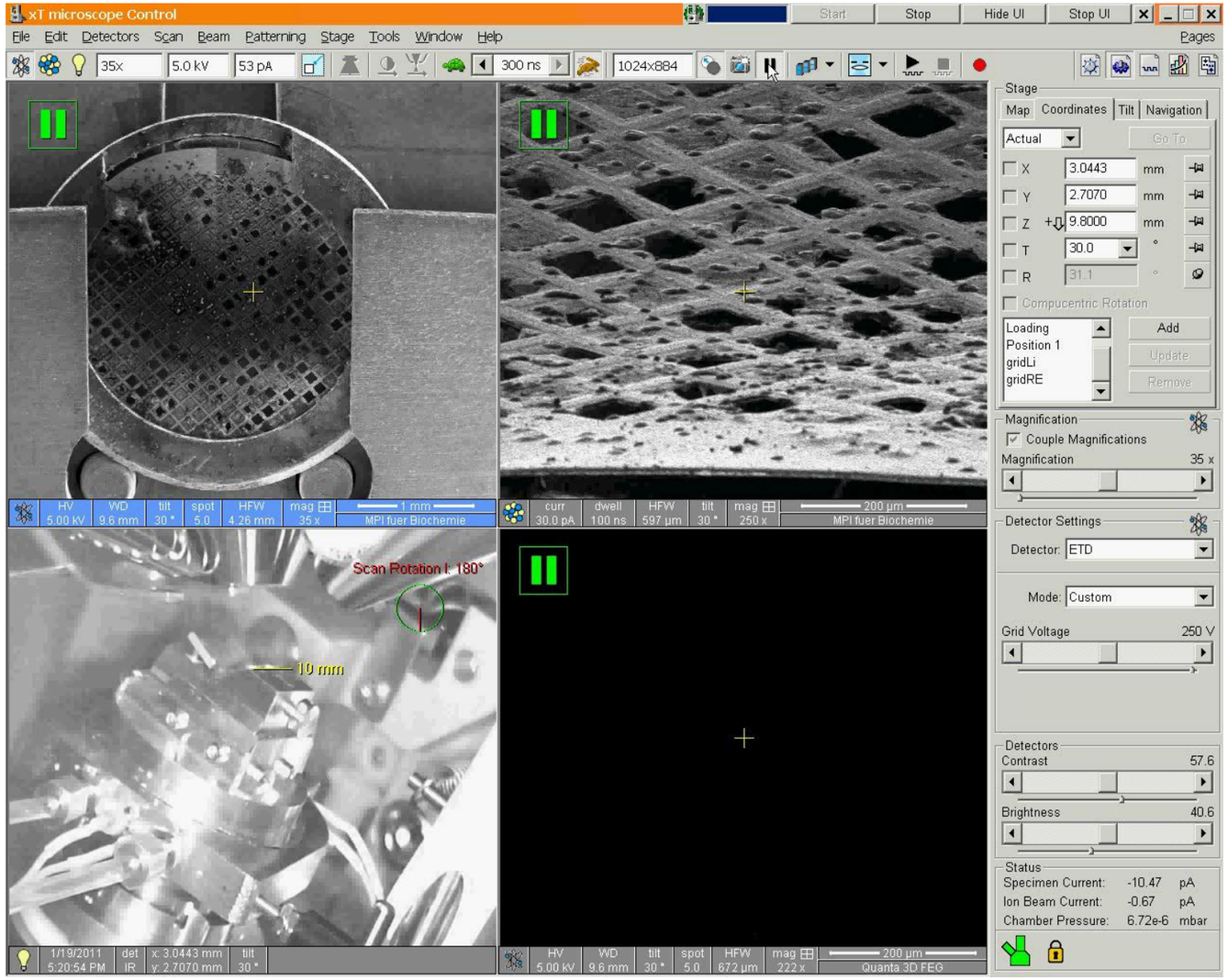




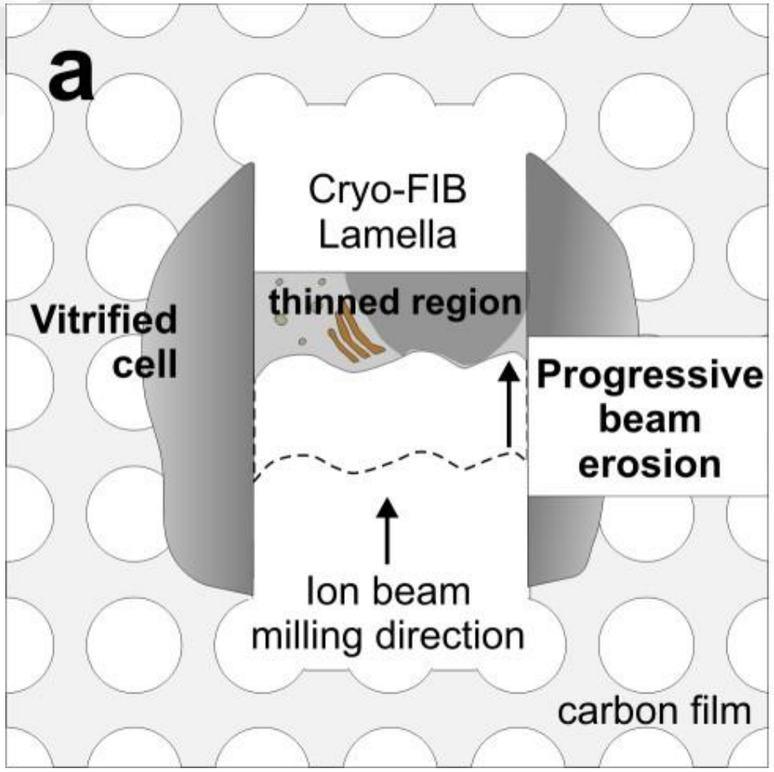
Multiple in-situ cryo-FIB lamellae can be prepared before transfer into TEM.

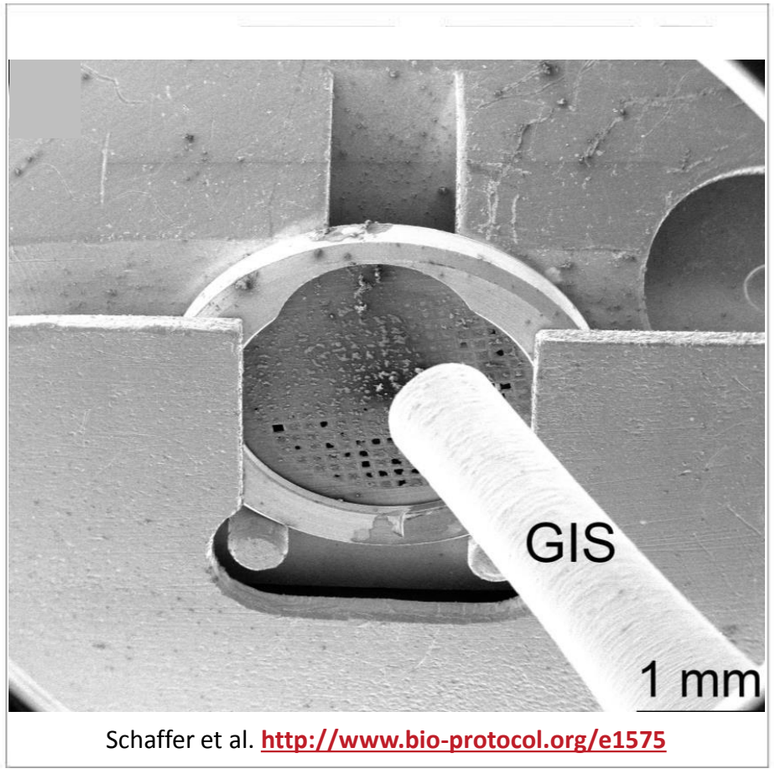
A. Rigort, J.M. Plitzko; Archives of Biochemistry and Biophysics 581 (2015) 122-130.

Cryo FIB process

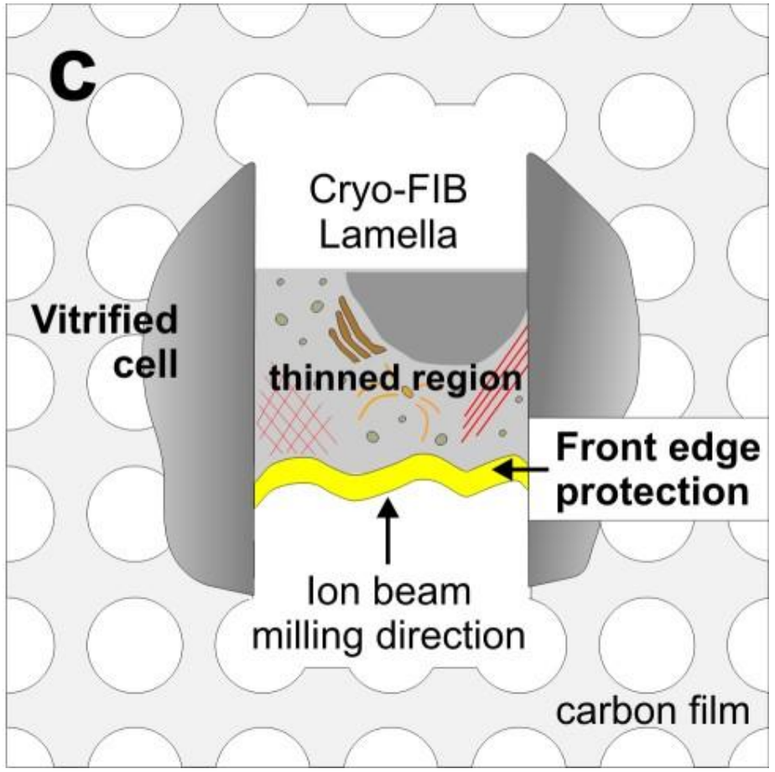


Protective Coating Preventing Beam Erosion





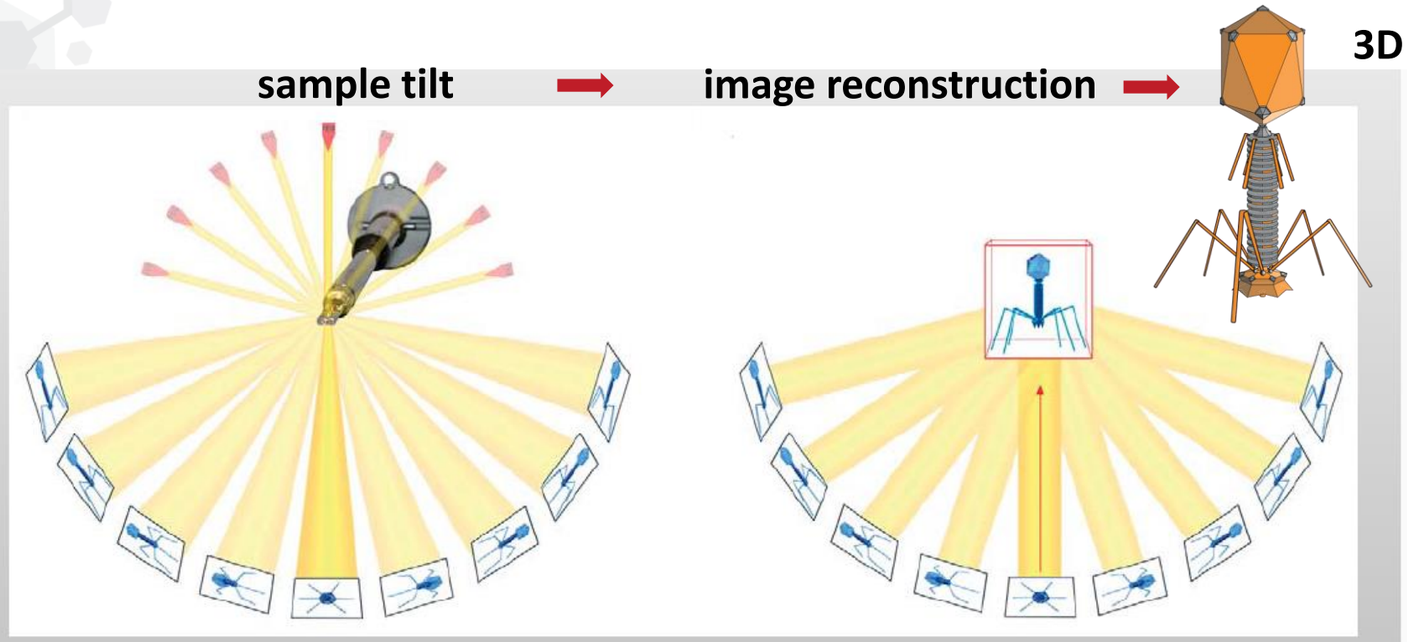
Schaffer et al. http://www.bio-protocol.org/e1575



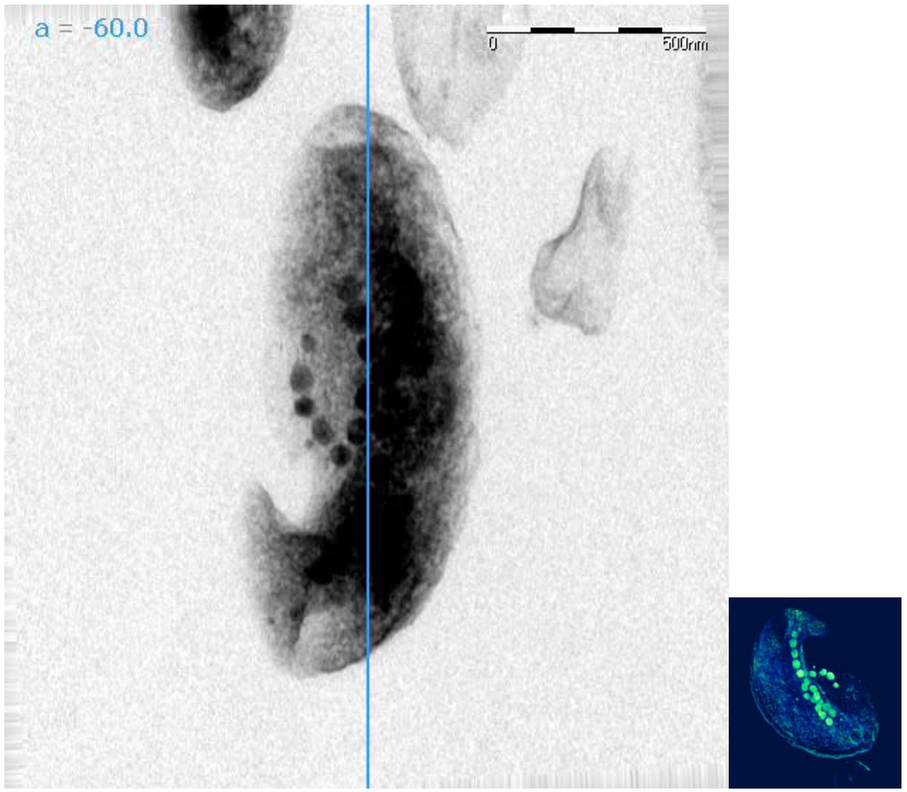
Cryo-focused-ion-beam applications in structural biology.
Rigort A, Plitzko JM. Arch Biochem Biophys. 2015 Sep 1;581:122-30.

Electron tomography

sample tilt → image reconstruction → 3D image

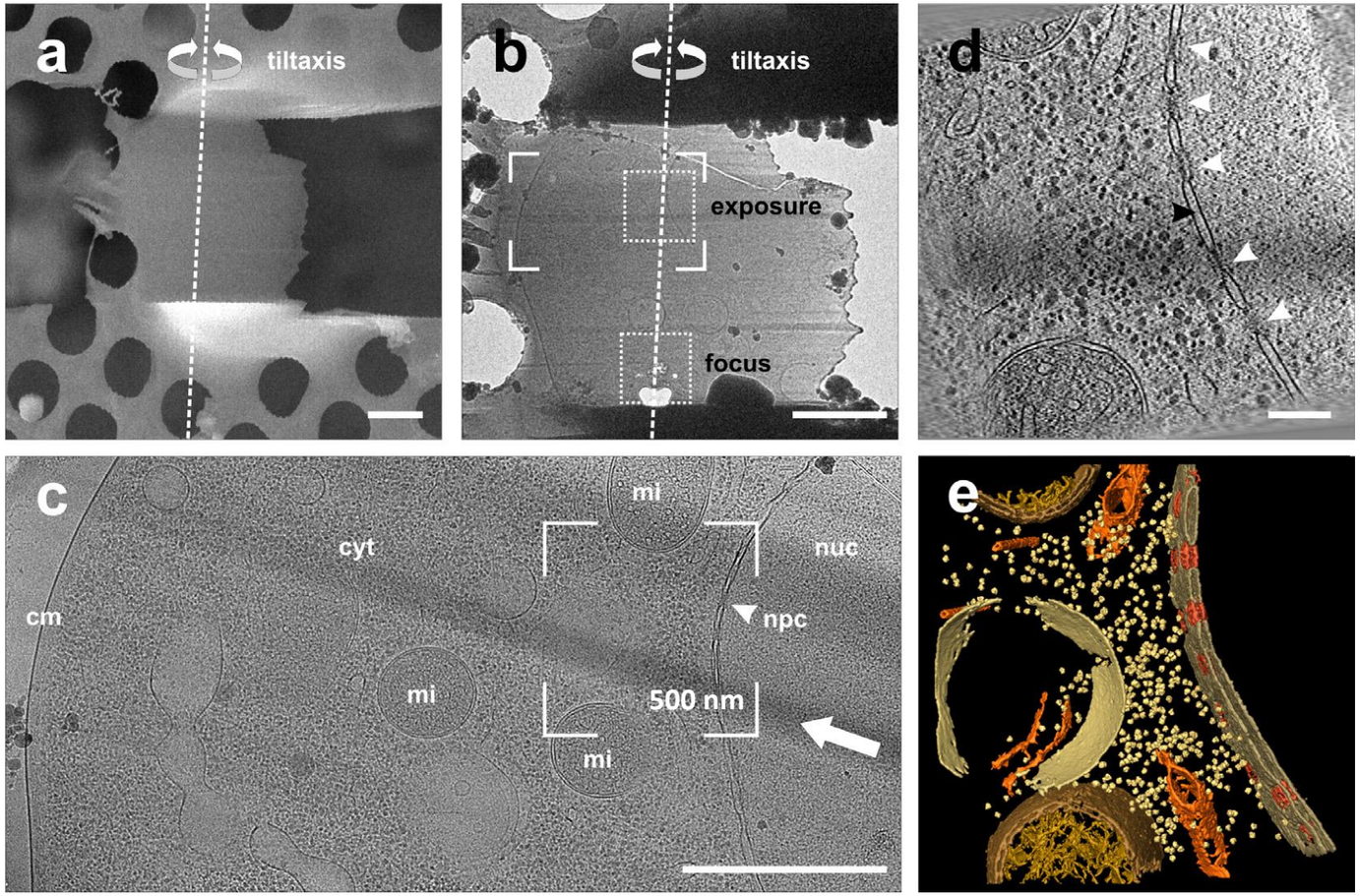


Sběr dat pro ET představuje záznam obrazu při náklonu preparátu okolo jedné osy. Obvykle se snímají data v rozsahu -70° až +70°, pokud není vzorek příliš tlustý. Kompletní záznam dat by vyžadoval náklon vzorku v rozsahu -90° až +90°, to však není z praktických důvodů možné. Při náklonu ±60° je vzorek 2x silnější než při náklonu 0°, při ±70° je téměř 3x silnější.

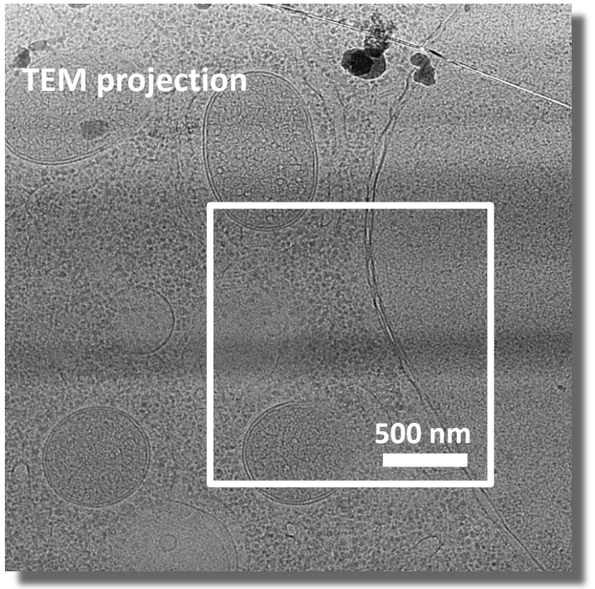


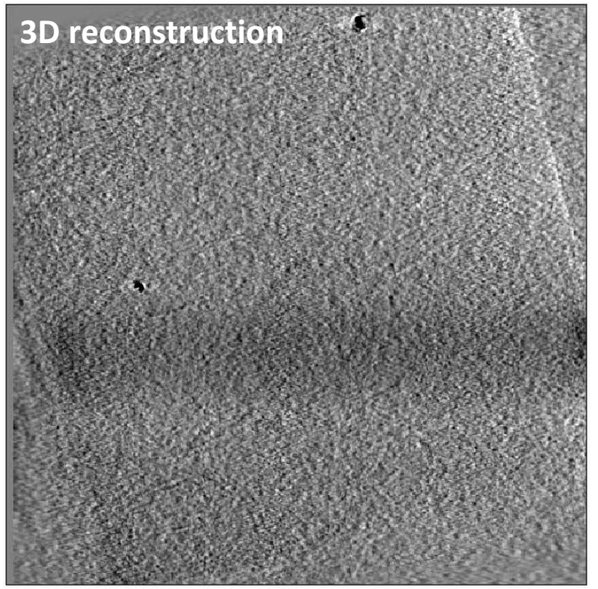
Magnetotactic bacteria
(Courtesy: Dr. Kobayashi, National Institute of Advanced Industrial Science and Technology, Osaka, Japan.)

Cryo lamella inspection

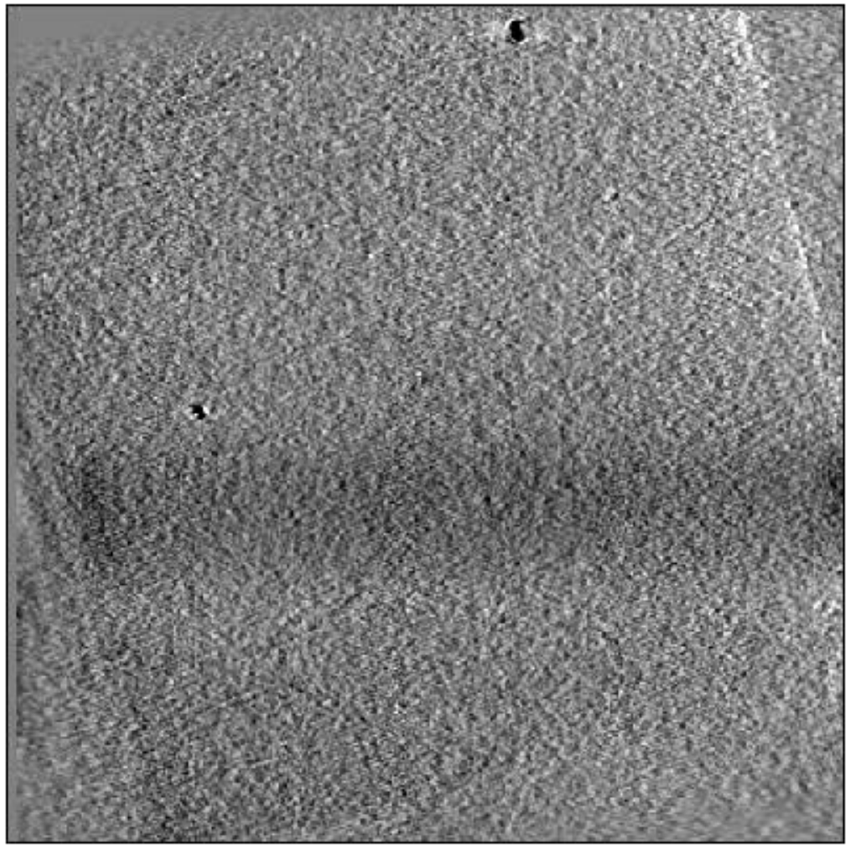


A. Rigort, F. J. B. Bäuerlein, et al. J.M. Plitzko; Proc. Natl. Acad.Sci. U.S.A. 109 (12) (2012) 4449-4454..





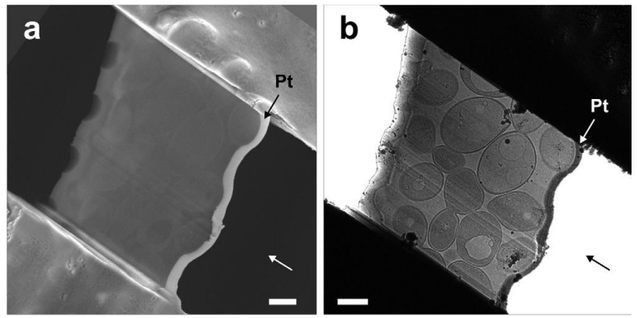
Cryo-Electron Tomography FIB Lamella

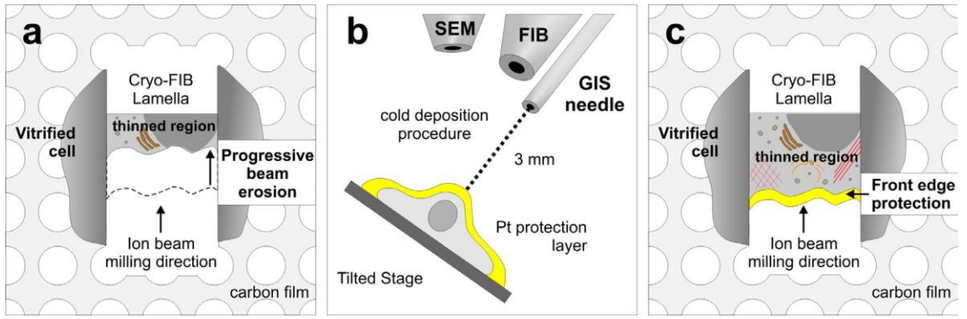


VOLUME RENDERING

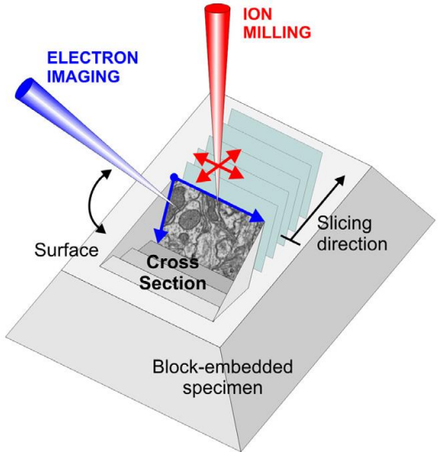
Remarks

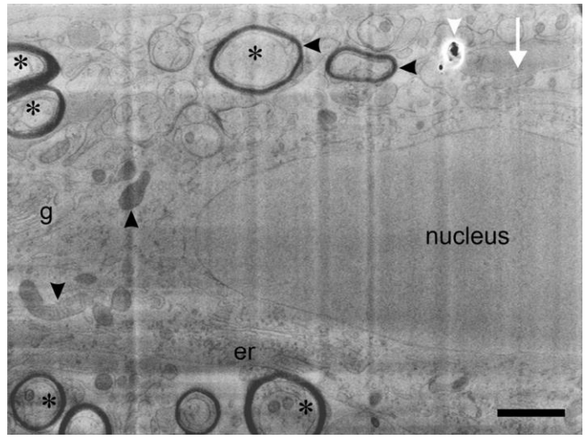
Protective coating on milling.

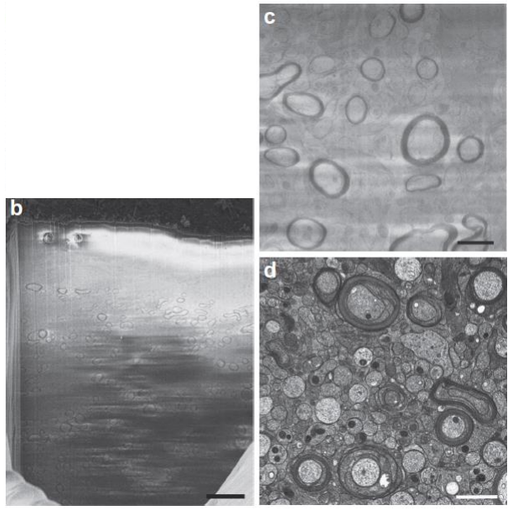




FIB /SEM tomography vs. vitrified samples

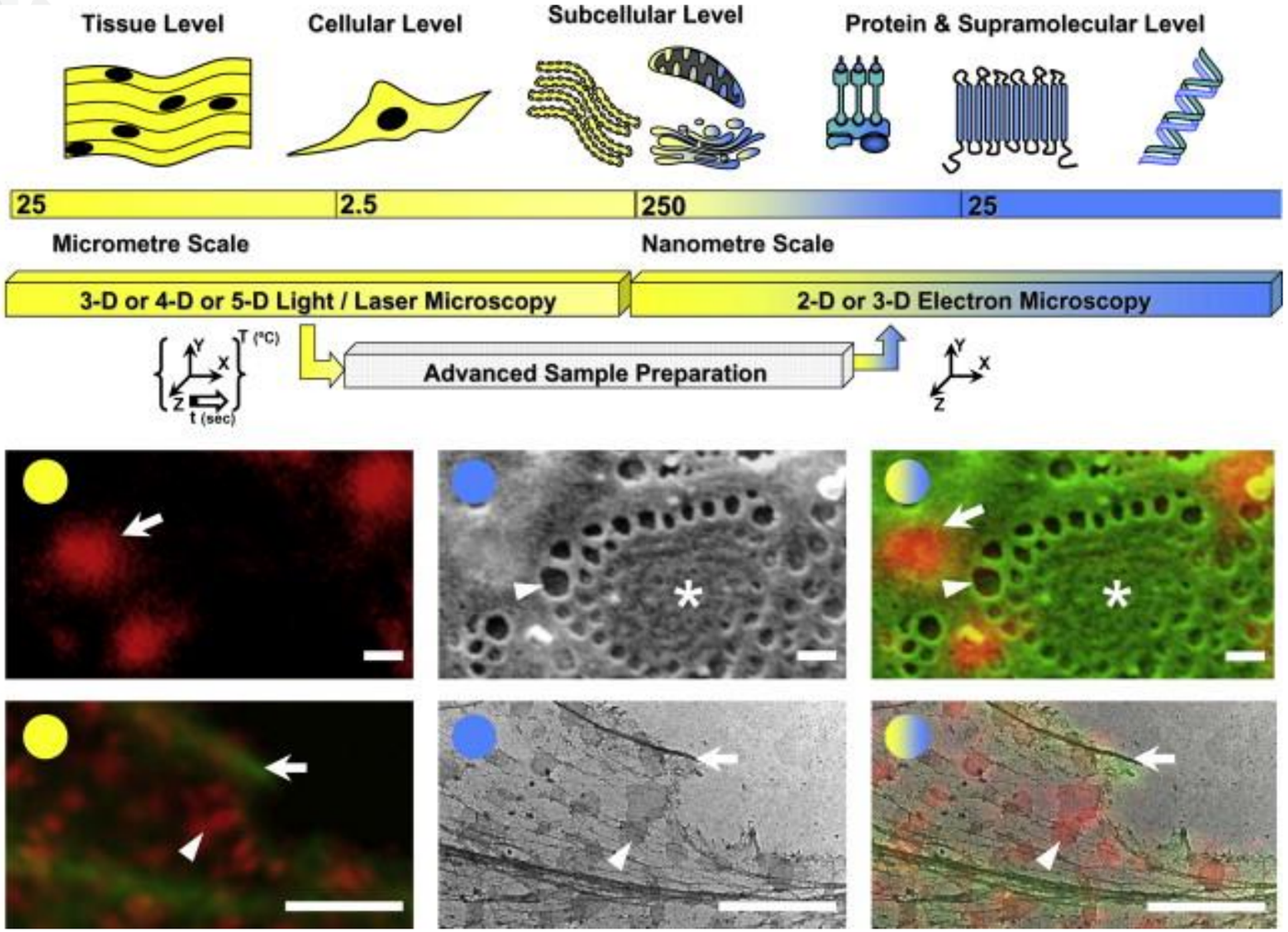


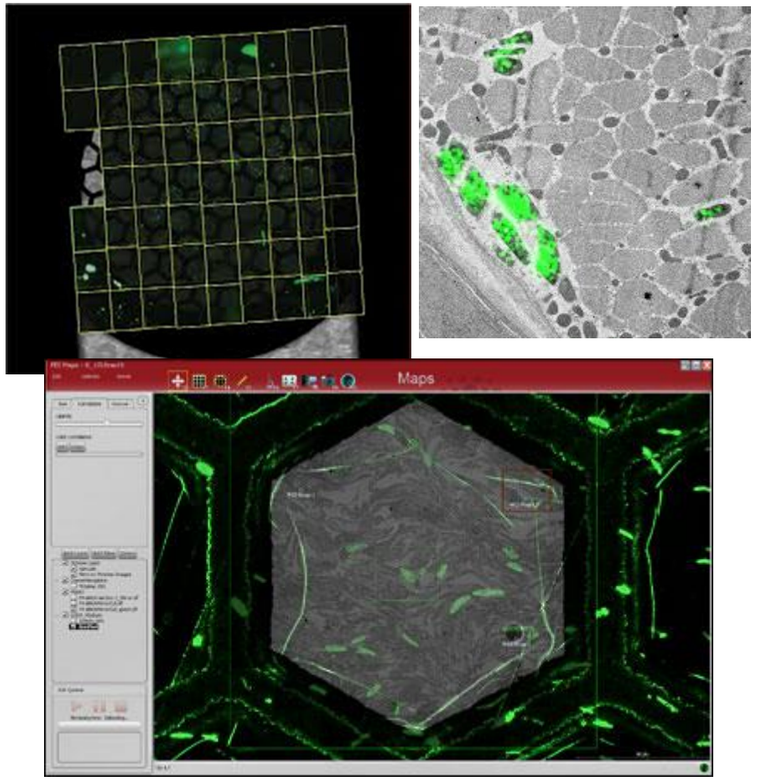




A. Rigort, J.M. Plitzko; Archives of Biochemistry and Biophysics 581 (2015) 122-130. Schertel A., Journal of Structural Biology 184 (2013) 355–360.

Correlative microscopy





K.A. Jahna, et al., Correlative microscopy…, Micron 43, p.5 (2012).

Correlative microscopy

High-pressure Freezing → Freeze substitution & low temp. embedding → Determination of ROI & 3D imaging → 3D FIB-SEM tomography

e.g.
cell culture
microorganisms
tissue (biopsy)
plants material …

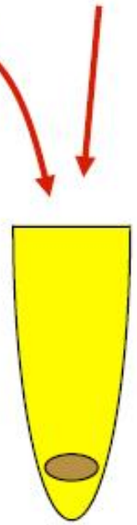
+ UAc and/ or OsO_4
+ Fluorescent dyes
e.g. DiIC18

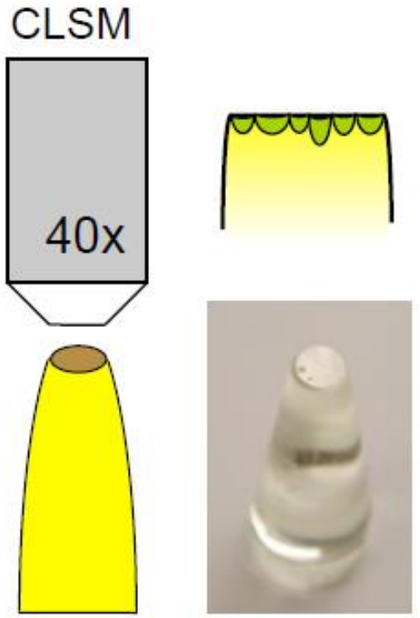

Soy bean plant

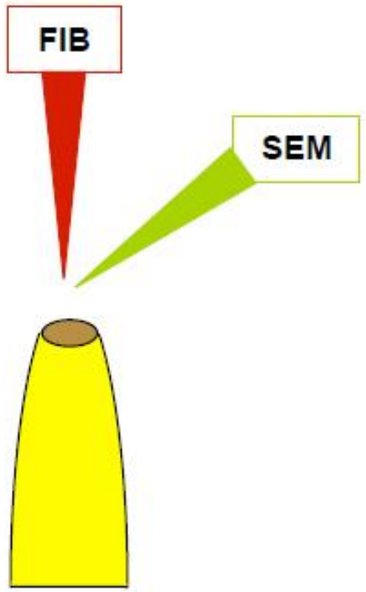

C. elegans


Cell culture
on sapphire disc


Tissue…



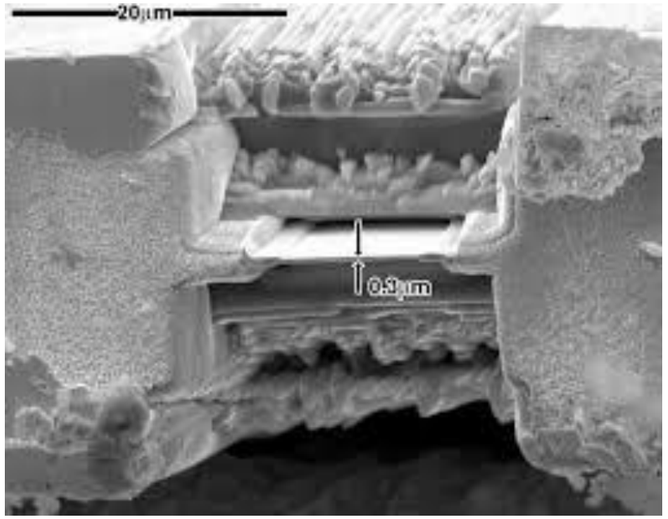


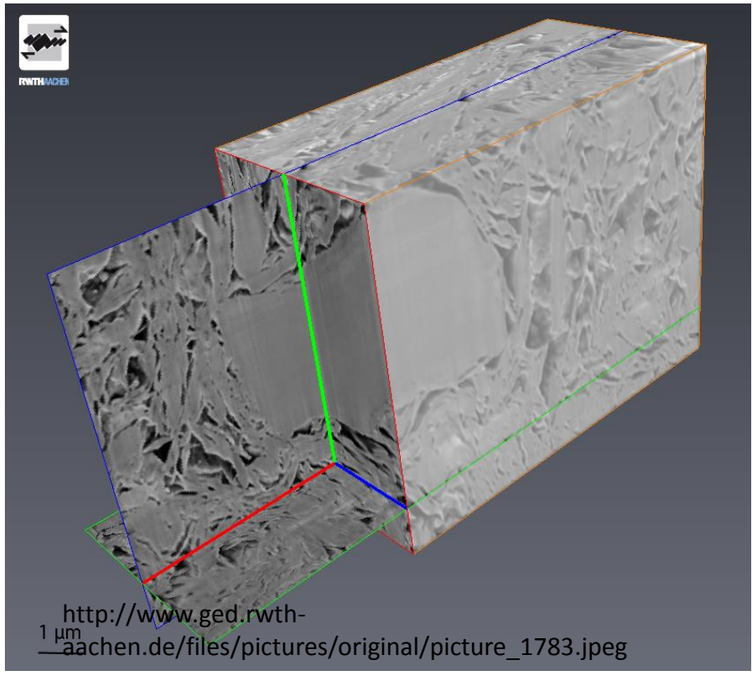


Katja Kawaschinski; *2000* ; Biel et al., *J Microsc* **212** (2003)

Adapted from R. Wepf (ETH Zürich) presentation.

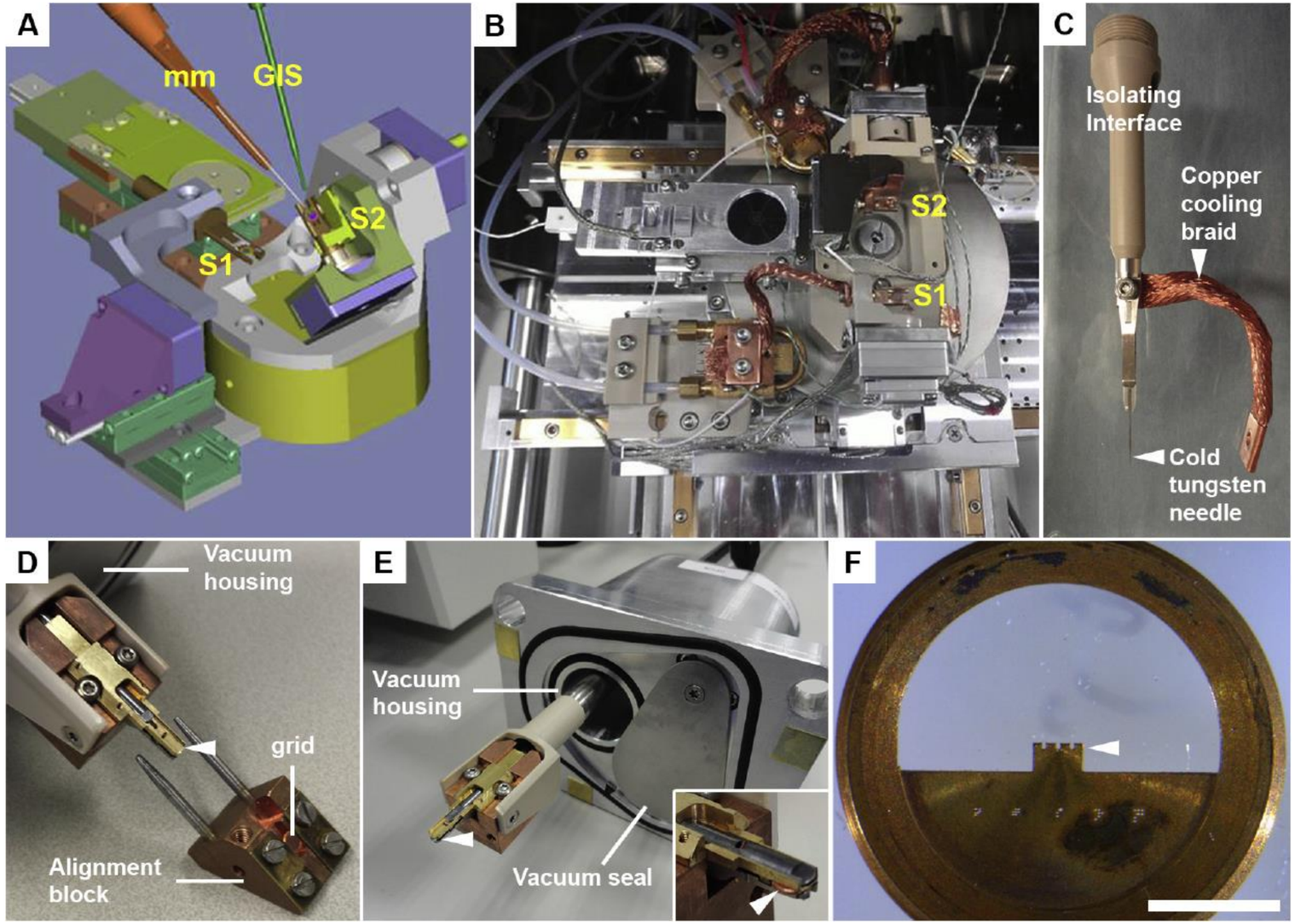
Cryo FIB Traditional





https://youtu.be/XfmFmeLU0Sg

Bulk Specimens: Cryo-Lift Out



Mahamid et al. Journal of Structural Biology 192 (2015) 262–269

- Create a thick lamella at stage position S1
- Pick up the lamella with a cold needle
- Transfer the lamella from stage position S1 to stage position S2
- Thin the lamella
- STEM image

So why/why not Cryo?

Pros

- Rapid
- No chemicals
- Easy (…ish)
- Versatile
- Allows in situ etching
- Light correlation possible

Cons

- Requires Cryogens/specialised equipment
- Artefact if incorrect
- Some materials don’t freeze well
- Contamination
- Contrast?!

Which method?

- Basically required magnification/resolution

- >10mm – uCT under or Light Microscopy (can be used for higher magnification also)
- 10-0.2mm – Standard SEM cryo but artifact at high magnification
- 200um-20nm – Cryo TEM prep (vitrification)
- 5um-0.5A – TEM (SPA, crystallography, tomography and/or lamella)

Thank you for your attention!

Questions? Or email john.mitchels@fei.com