Common Cell and Tissue Embedding Protocols at BioCryo: HPF-FS and Chemical Fixation

NU*ANCE* July Tech Talk: BioCryo

July 17, 2025

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NU*ANCE* BioCryo & EPIC-FIB

NUANCE Northwestern University Atomic and Nanoscale Characterization Experimental Center

BioCryo Electron Microscopy

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Please Note: BioCryo has a new RRID. When publishing papers that used BioCryo facilities or assistance, please include this text in your acknowledgements:

This work made use of the BioCryo facility (RRID:SCR_021288) of Northwestern University's NUANCE Center, which has received support from the SHyNE Resource (NSF ECCS-2025633), the IIN, and Northwestern's MRSEC program (NSF DMR-2308691).

The acknowledgment text is available on the NUANCE *website under Publication Acknowledgements for each of the* NUANCE *centers*

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You have a biological or soft matter sample that you want to characterize.

However, because it is hydrated, you can't put it into an electron microscope or other vacuum system very easily.

What can you do?

Ask ▸BioCryo◂ for help!

Electron Microscopy

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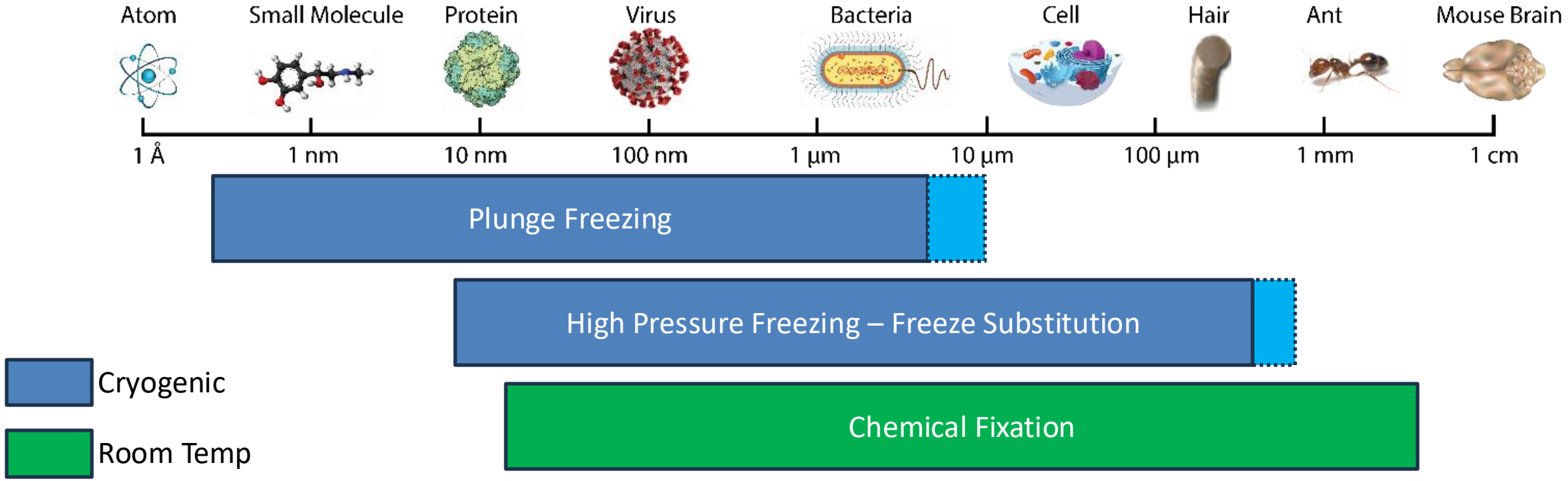
Let's start at the very beginning (a very good place to start)

Three important questions:

- How big is your sample and how big is your feature of interest?
- Will your sample have inherent contrast, or does it need to be stained?
- Is your sample sensitive to dehydration, osmotic effects, pH, polyvalent ions, and/or shear?



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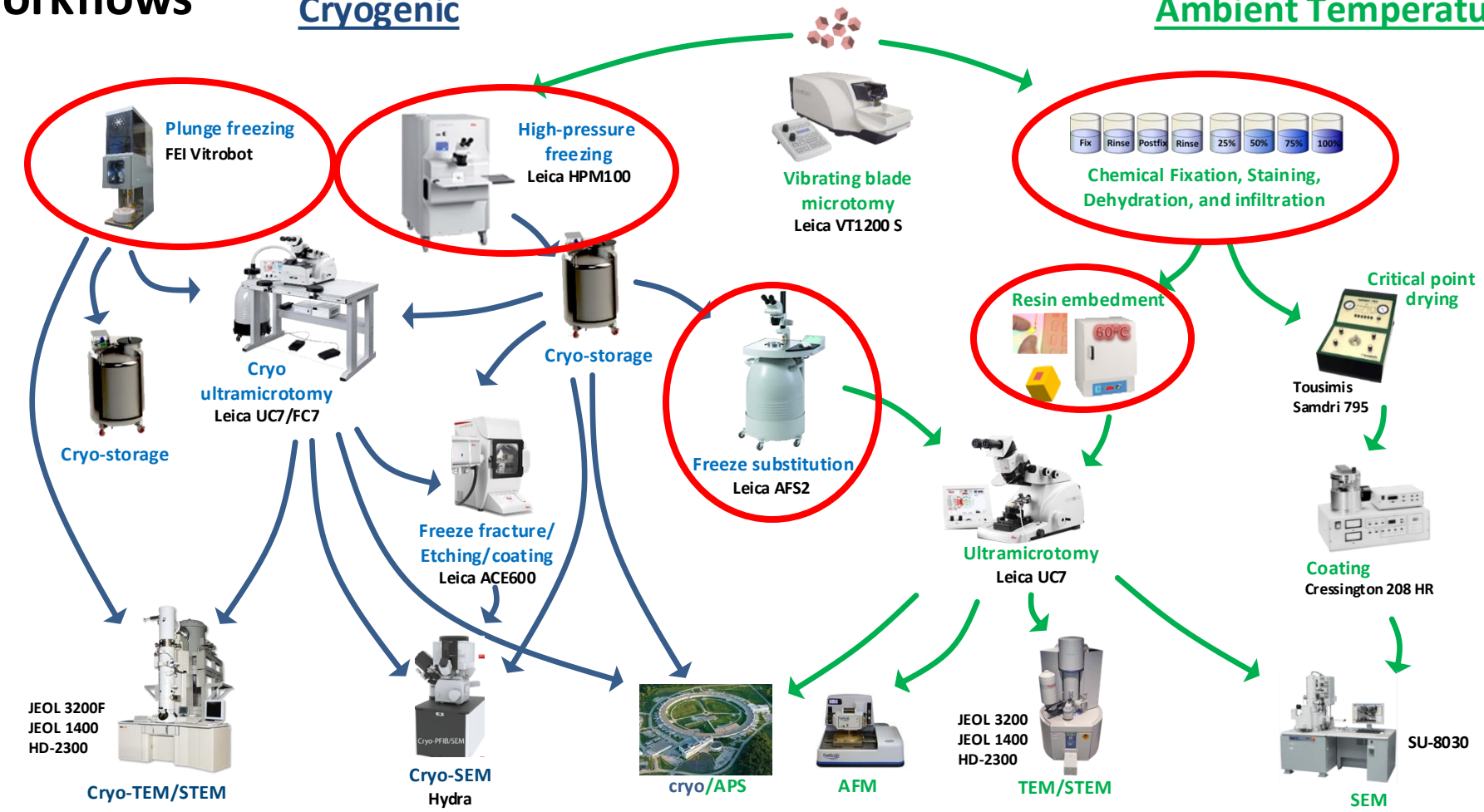
Richard Rodgers, Oscar Hammerstein II, Howard Lindsay, & Richard Crouse, "Do-Re-Mi," *The Sound of Music*
Moor, Hans. "Theory and practice of high pressure freezing." In *Cryotechniques in biological electron microscopy*, pp. 175-191. Springer Berlin Heidelberg, 1987.
Studer, D., Humbel, B.M. & Chiquet, M. Electron microscopy of high pressure frozen samples: bridging the gap between cellular ultrastructure and atomic resolution. *Histochem Cell Biol* **130**, 877–889 (2008). https://doi.org/10.1007/s00418-008-0500-1

BioCryo Workflows



BioCryo Workflows

Cryogenic

Plunge freezing
FEI Vitrobot

High-pressure freezing
Leica HPM100

Freeze substitution
Leica AFS2

Vibrating blade microtomy
Leica VT1200 S
(soft samples only)

Ambient Temperature

Chemical Fixation, Staining, Dehydration, and infiltration

Plunge Freezing

- Aqueous samples only
- ~ 0.1-10 wt.% (1-100 mg/mL)
- Fairly uniform, well-dispersed samples only
- Thin bacteria monolayers

Length Scales:
Sample: <1 µm thick
Feature: <1-10+ nm
Staining: *not required*

Bad for shear-sensitive samples

High Pressure Freezing – Freeze Substitution

- Aqueous or hydrated samples
- Almost any concentration of sample (but higher is better)
- Avoids most artifacts

Length Scales:
Sample: <200 µm thick
Feature: *variable*, <1-10+ nm
Staining: *depends*
SEM/TEM: needs staining via Freeze Substitution
Freeze Fracture SEM: no staining
Cryo-FIB-TEM: no staining

Chemical Fixation & Resin Embedding

- Aqueous, organic, or dehydrated samples up to 1 mm thick

Length Scales:
Sample: <1 mm thick
Feature: >10 nm
Staining: *necessary*

Many potential issues: osmotic effects, loss of small molecules, lipid removal, protein conformation changes & depolymerization, shrinkage/collapse of tissues (especially hydrogels)

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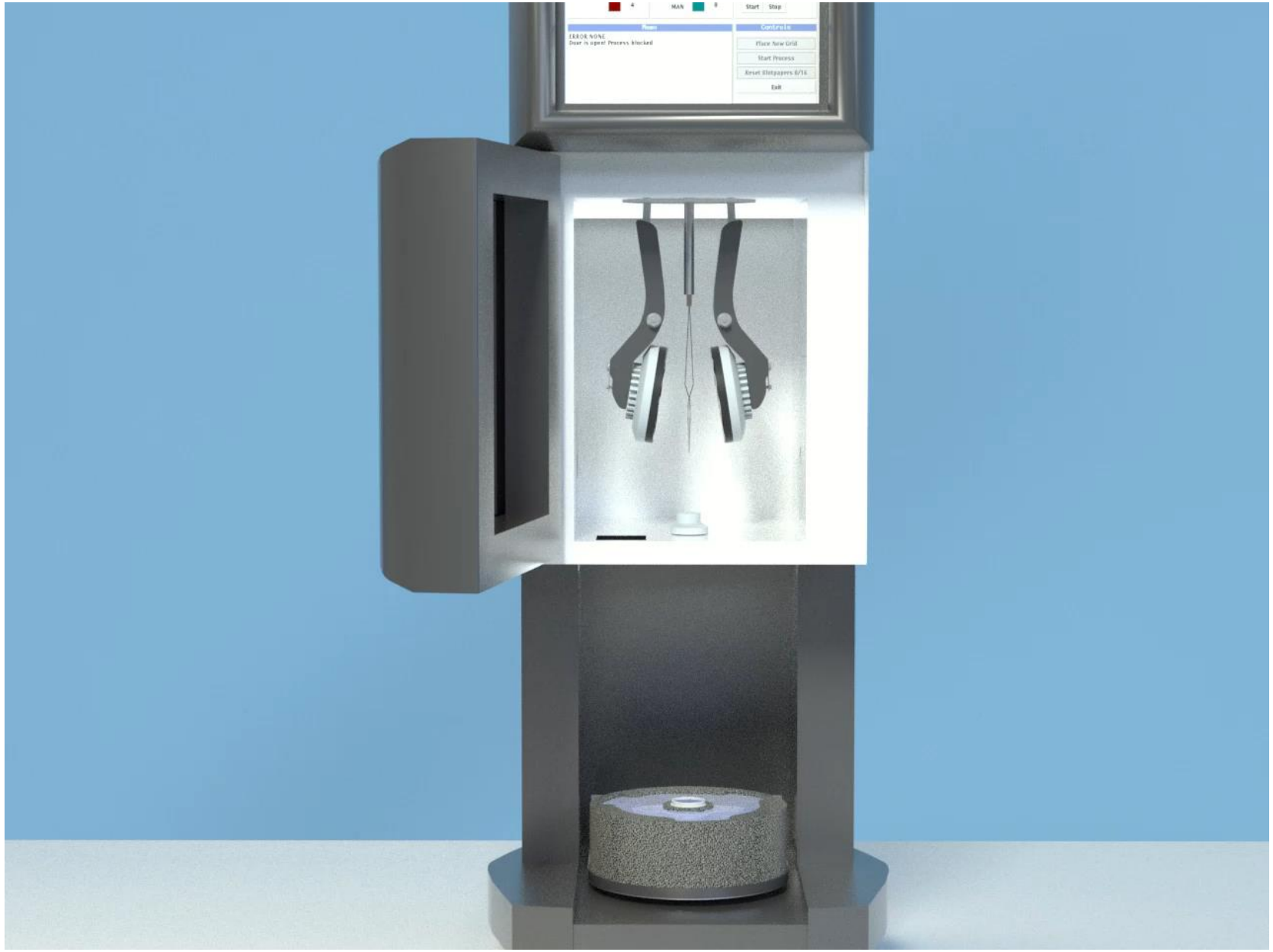
Plunge Freezing

To learn more about plunge freezing, see the Tech Talk: "Cryogenic Sample Prep for Electron Microscopy: Considerations and Techniques" on the NUANCE Center YouTube page!



Dr. Yu Chen, "Cryo-TEM sample prep animation with clapping"

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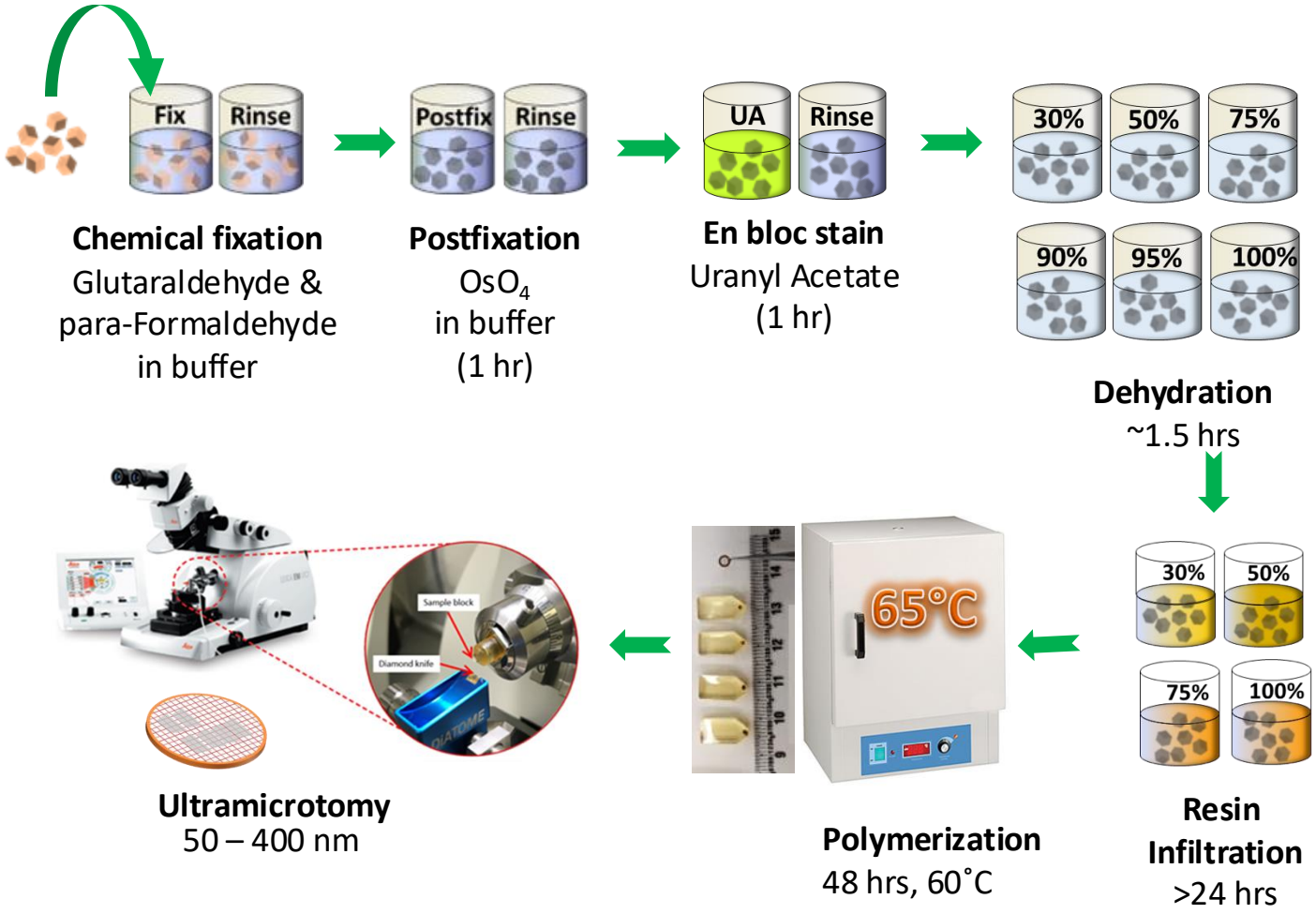
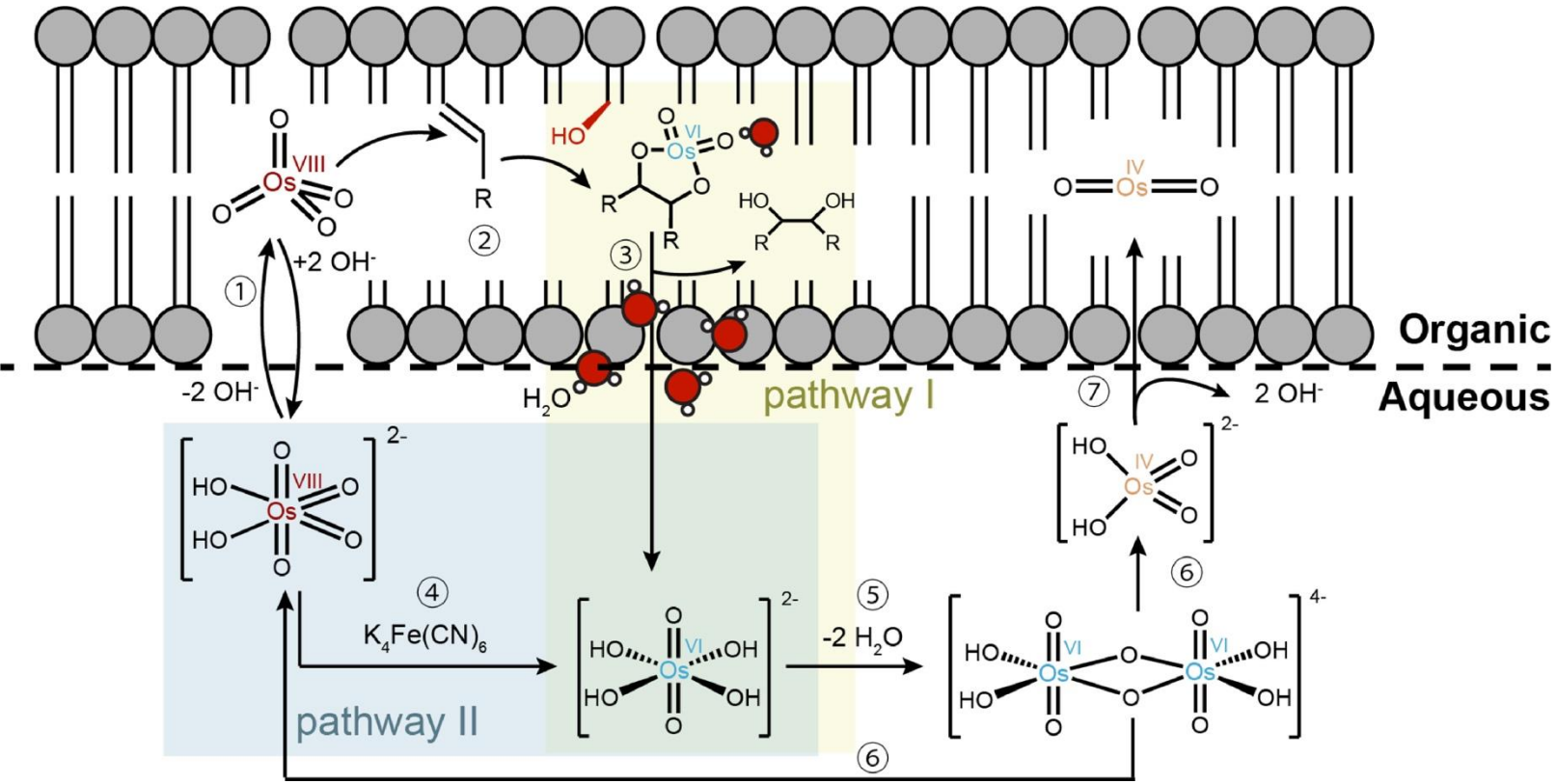
Chemical Fixation & Resin Embedding



1. Chemically crosslink proteins with glutaraldehyde & para-formaldehyde in a buffer solution (phosphate or cacodylate)
2. Postfix & stain lipids with OsO_4
3. Stain DNA & proteins with Uranyl Acetate
4. Dehydration series (acetone or ethanol)
5. Resin infiltration series (epoxy or acrylic), duration depends on size & resin viscosity
6. Resin Polymerization
7. Ultramicrotomy sectioning to generate TEM-thickness sections (70-400 nm)
8. (Optional) Post-staining with UA and lead citrate for additional contrast

Osmium Staining Mechanism

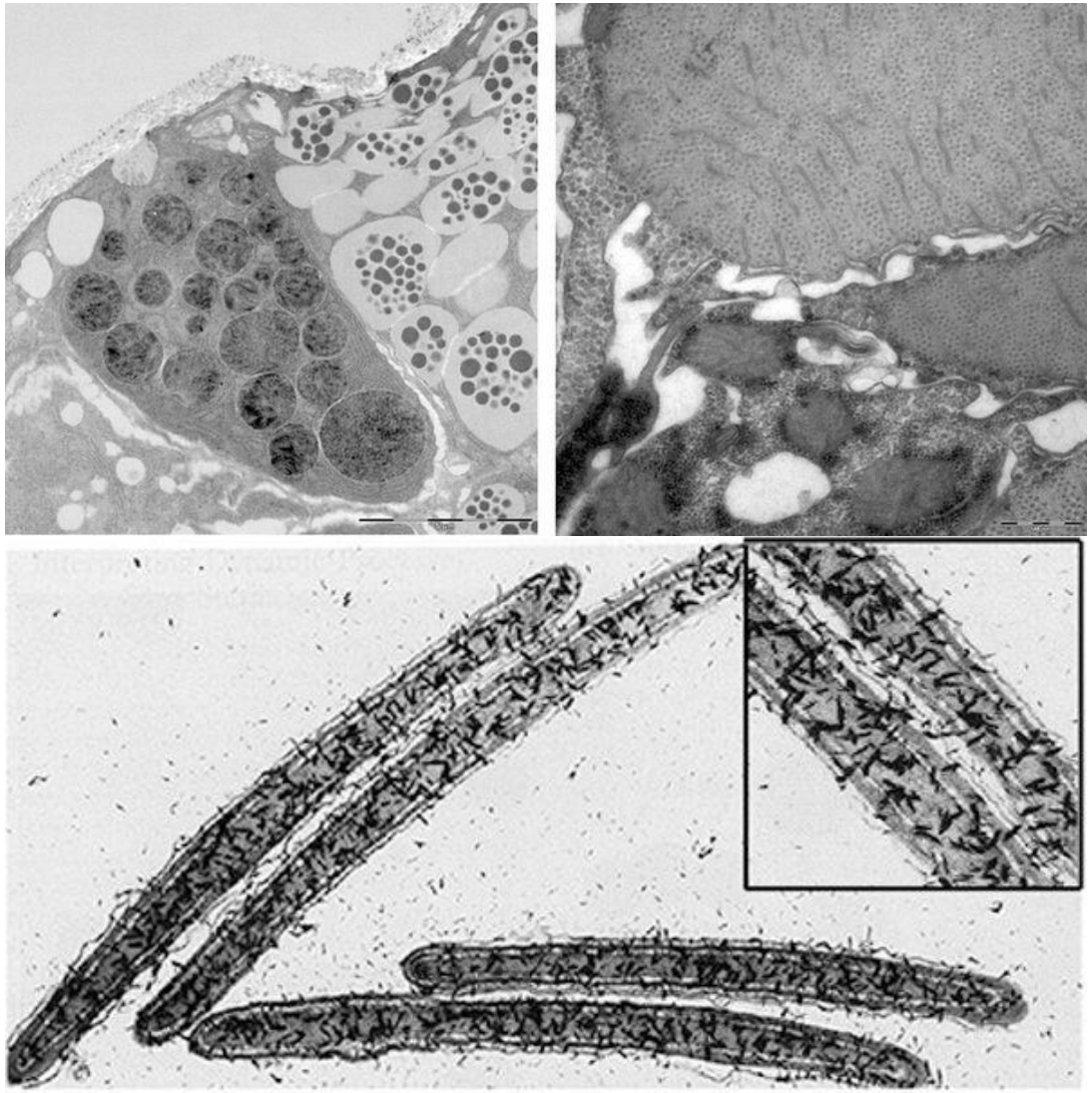


1. Tetrahedral Os(VIII) partitions into the lipid bilayer, where it reacts with unsaturated lipids, ultimately forming a cis-diol and Os(VI)
2. Alternatively, Fe(II) can be used to aqueously reduce octahedral Os(VIII) to octahedral Os(VI)
3. Octahedral Os(VI) forms a dimer, then self-reacts to produce octahedral Os(VIII) and tetrahedral Os(IV)
4. The octahedral Os(VIII) continues back at steps 1 and 2 to react with more lipids/Fe(II)
5. The tetrahedral Os(IV) decomposes to linear Os(IV), which is lipophilic; it partitions into the lipid bilayer and forms nano-aggregates. This is the Os we see by EM.

OsO_4 is both extremely volatile and incredibly toxic. Use exclusively in a well-functioning hood while wearing full PPE. Collect all OsO_4 waste in sealed containers. The first sign of OsO_4 exposure is often your cornea turning permanently black.

R. Li, G. Wildenberg, K. Boergens, Y. Yang, K. Weber, J. Rieger, A. Arcidiacono, R. Klie, N. Kasthuri, S.B. King. "OsO_2 as the Contrast-Generating Chemical Species of Osmium-Stained Biological Tissues in Electron Microscopy." *ChemBioChem,* 25 (*20*), e202400311.

Uranyl Acetate Staining Mechanism



- Thanks to its high atomic number, UA provides excellent EM contrast
- Uranyl Acetate binds to negatively charged groups in the cell (proteins, lipids, glycoproteins, DNA, and RNA)
- Excessive UA and/or insufficient rinsing can lead to the deposition of needles of UA (below; compare to above)
- Due to its poor solubility and proclivity to precipitate, UA is often used as a post-stain after sections have been generated and placed onto TEM grids for extra contrast.

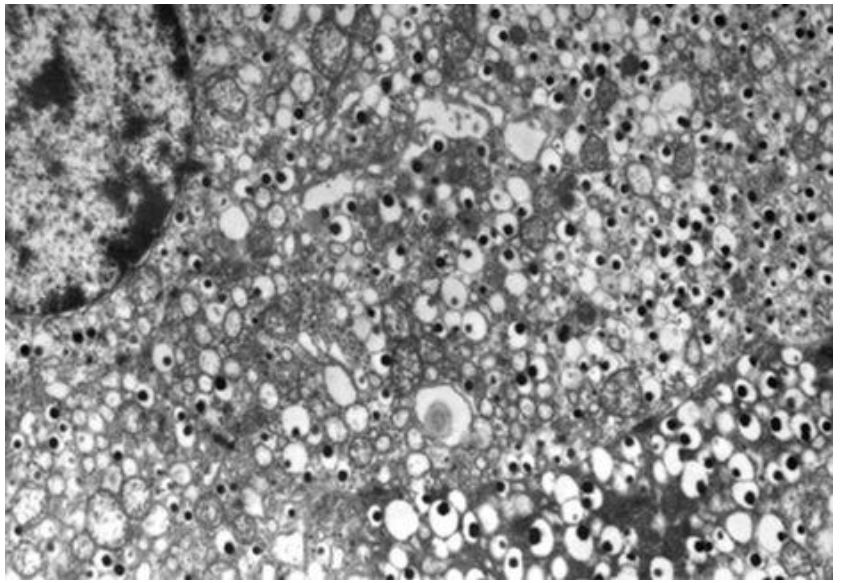
Uranyl acetate is slightly radioactive and severely nephrotoxic. Handle it carefully (with gloves!), and be sure to collect all UA waste as radioactive waste. When storing UA, keep it wrapped in aluminum foil to limit radiation exposure and to protect it from UV light, which will cause it to degrade.

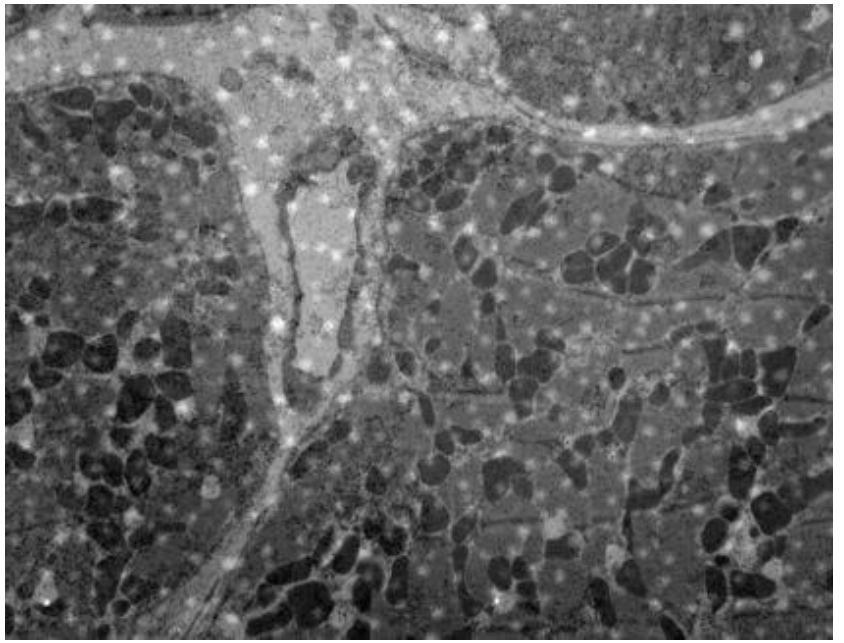
R. Pandithage, Leica Microsystems. "Brief Introduction to Contrasting for EM Sample Preparation." 02 October 2013, Leica Microsystems. *https://www.leica-microsystems.com/science-lab/life-science/brief-introduction-to-contrasting-for-em-sample-preparation/*

Lead Citrate Post-Staining

- Lead citrate is also commonly used for additional contrast after sectioning.
- Similarly to UA, it binds to a variety of structures in the cell: ribosomes, lipid membranes, cytoskeleton components, and a variety of cytoplasm compartments
- It is used as a post-stain because it binds to reduced Os and UA
- However, lead citrate is finicky: it is water insoluble and precipitates when exposed to carbon dioxide
- Post-staining yields either large dark grains (above) or a thin coating across the whole section (below)

Lead citrate is also extremely toxic, so be careful when preparing and handling it. Avoid exhaling onto lead citrate solutions, as the carbon dioxide in your breath will cause it to precipitate.





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Ambient Temperature

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Plunge Freezing

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Feature: <1-10+ nm
Staining: *not required*

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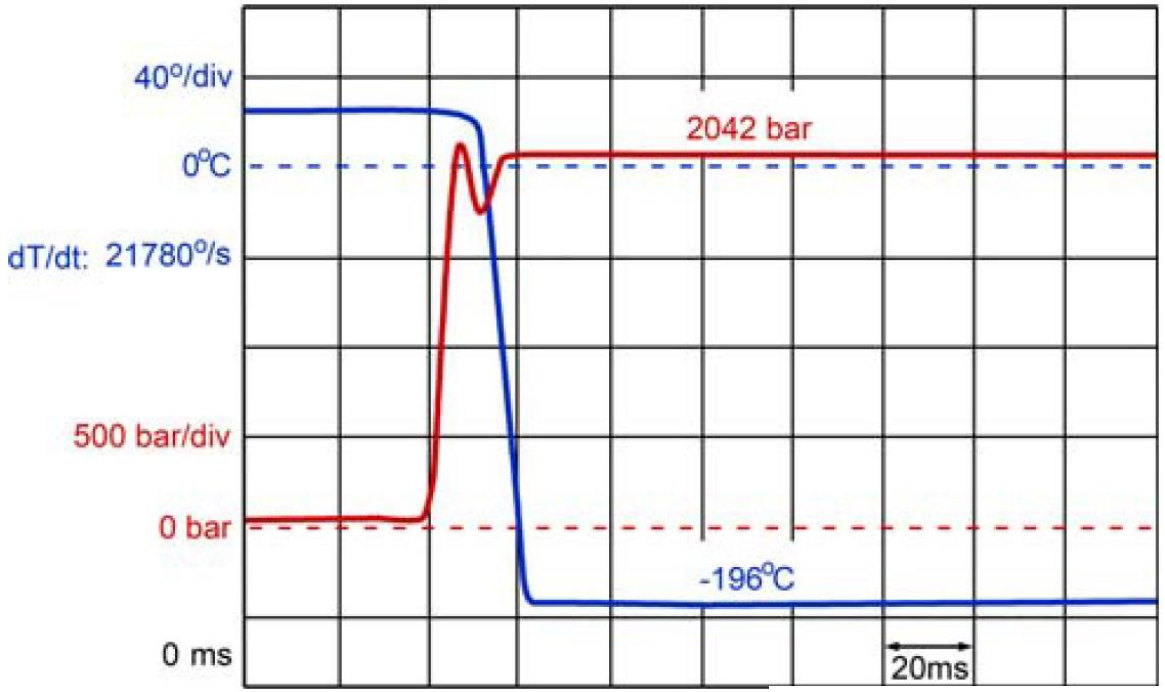
High Pressure Freezing – Freeze Substitution (HPF-FS)

- HPF-FS combines cryogenic flash freezing with chemical fixation to yield resin-embedded samples for sectioning
  - Vitreous freezing of thick samples is possible only thanks to water's volume expansion upon freezing
  - By applying high pressure (2072 atm), the melting and supercooling points of water can be depressed
  - This allows for slower cooling rates to still produce amorphous, vitreous ice throughout samples up to 200 µm thick (400 µm with cryoprotectants)
- The chemical fixation processes begin at cryogenic temperatures after HPF; by etching out the solid water with acetone at these temperatures, most osmotic effects are prevented *and* the chemical fixatives are already present once they warm to their reactive temperatures.

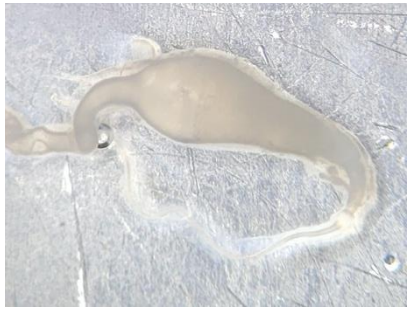
Temperature ranges of water crystallization

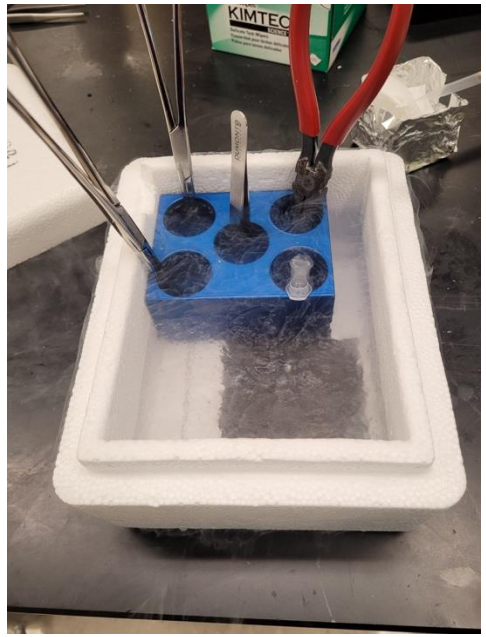
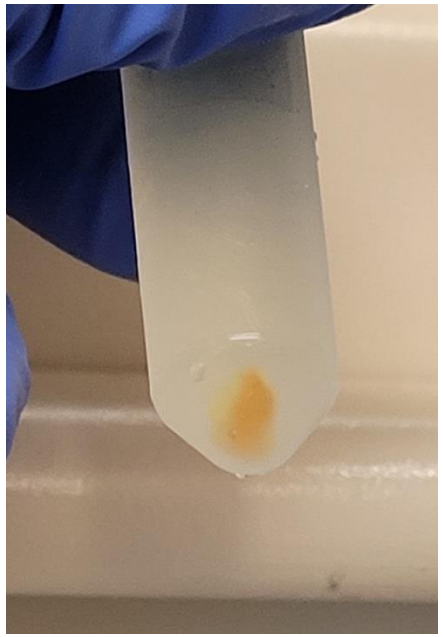
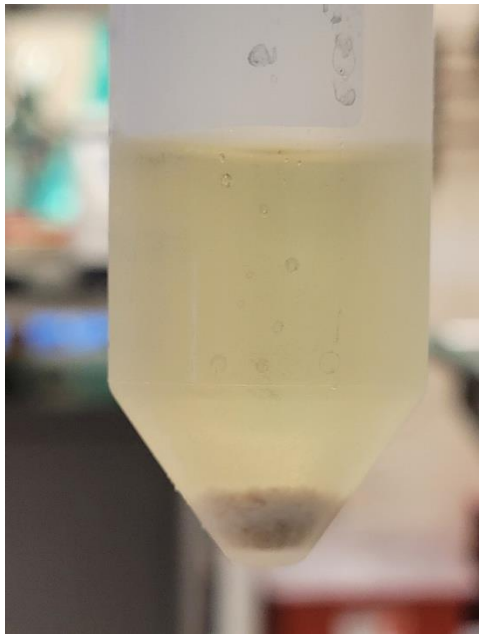
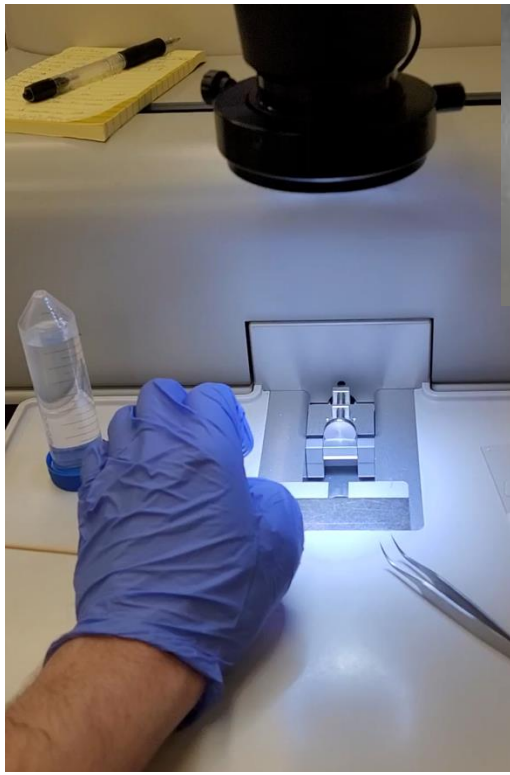
	Melting Temp	Supercooled Temp
Ambient (1 atm)	0˚C	-42˚C
2072 atm	-22˚C	-92˚C

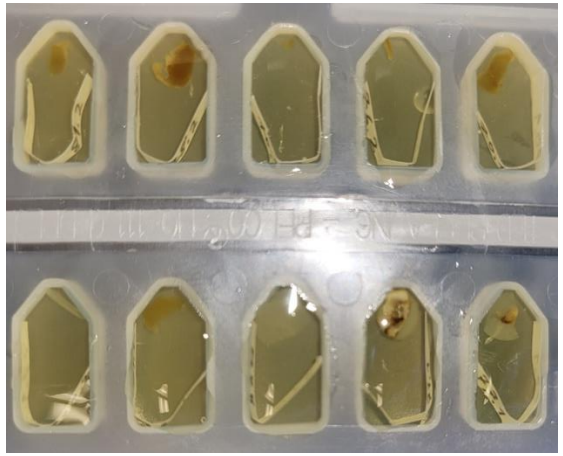


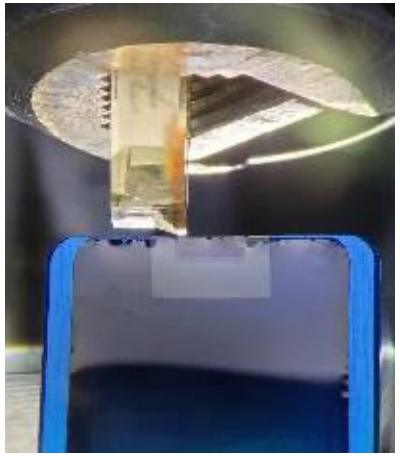
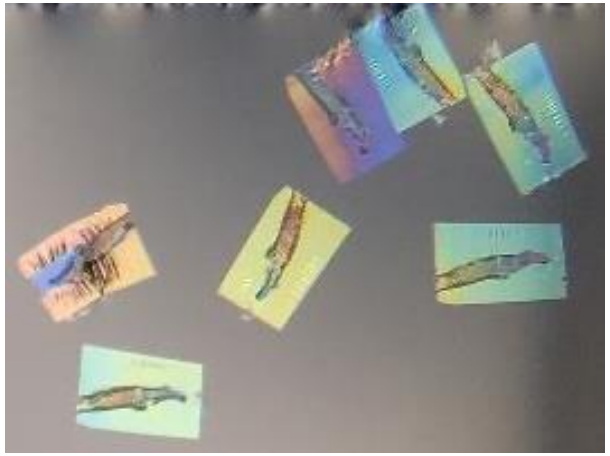
D. Studer, B.M. Humbel, M. Chiquet, *Histochem. Cell Biol.*, **2008**, 130, 877-889

High Pressure Freezing – What does it look like?





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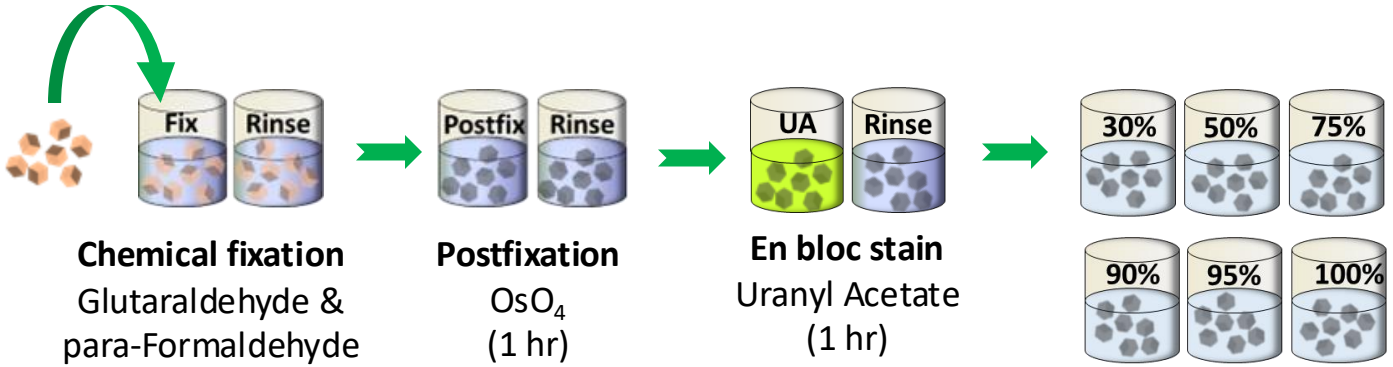
Chemical Fixation vs HPF-FS

Chemical Fixation

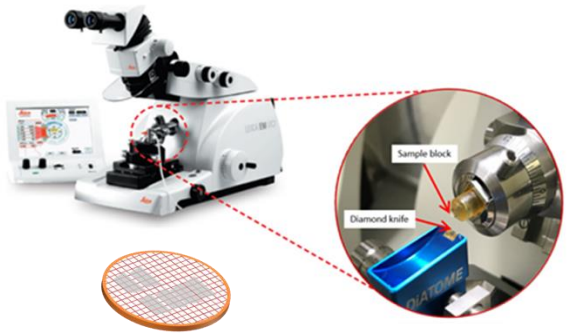


Chemical fixation
Glutaraldehyde & para-Formaldehyde

Postfixation
OsO_4 (1 hr)

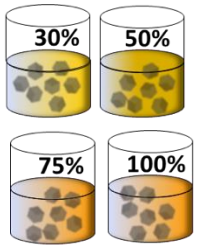
En bloc stain
Uranyl Acetate (1 hr)

Dehydration
~1.5 hrs



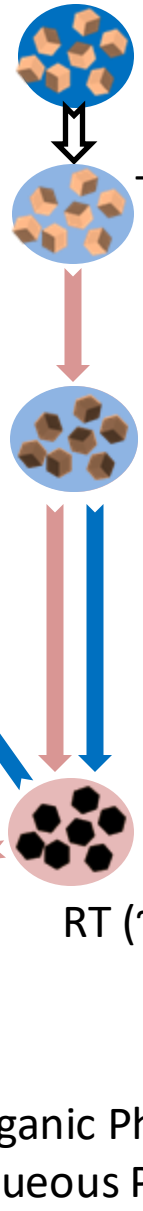
Ultramicrotomy
50 – 400 nm

Polymerization
48 hrs, 60˚C



Resin Infiltration
>24 hrs

HPF-FS



HPF (with filler – often BSA, agarose, or similar phase)
Freeze Substitution: Acetone with either Glutaraldehyde & Uranyl Acetate or OsO_4, warming from -90 to 0˚C
Post-fixation (optional) Glutaraldehyde or OsO_4, aqueous or organic
(Optional) Rehydration, antibody labeling, & fluorescence microscopy
En bloc Staining: Osmium Tetroxide & Ferricyanide or UA
EtOH Dehydration (if rehydrated)
Resin Infiltration
Cure @ 60-65ºC for 2 days

Organic Phase
Aqueous Phase

Which of these techniques is right for me and my sample?

Plunge Freezing

- Dilute, aqueous, and/or very thin samples
- Very high resolution possible
- Keeps sample in its native state
- Fastest sample turnaround

- Requires lots of practice to do well
- Limited to very thin samples (<1 µm)
- Highly shearing

High Pressure Freezing – Freeze Substitution

- Almost any aqueous/hydrated samples
- Avoids most artifacts

- Limited to samples <200 µm thick
- Takes a long time to prepare samples (~2 weeks)
- Extremely toxic metal stains

Chemical Fixation & Resin Embedding

- Large samples (up to 1 mm)
- Aqueous, hydrated, organic, or dried samples
- Easily applied to most samples
- ~1 week of sample prep

- Highly toxic metal stains
- Many potential artifacts: osmotic effects, loss of small molecules, protein conformation changes & depolymerization, lipid removal, shrinkage/collapse of tissues (especially hydrogels)

Please reach out to BioCryo if you want to try one of these techniques!
We are happy to help you pick/design and test a protocol for your sample

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If you have any questions, please do not hesitate to reach out!

BioCryo is happy to schedule a meeting to discuss your samples or any technical questions you have.

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Eric Roth, *eric-roth@northwestern.edu*

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