Cryo sample preparation for cryoET investigations

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2025 Tomo Shortcourse - NYSBC

Goals

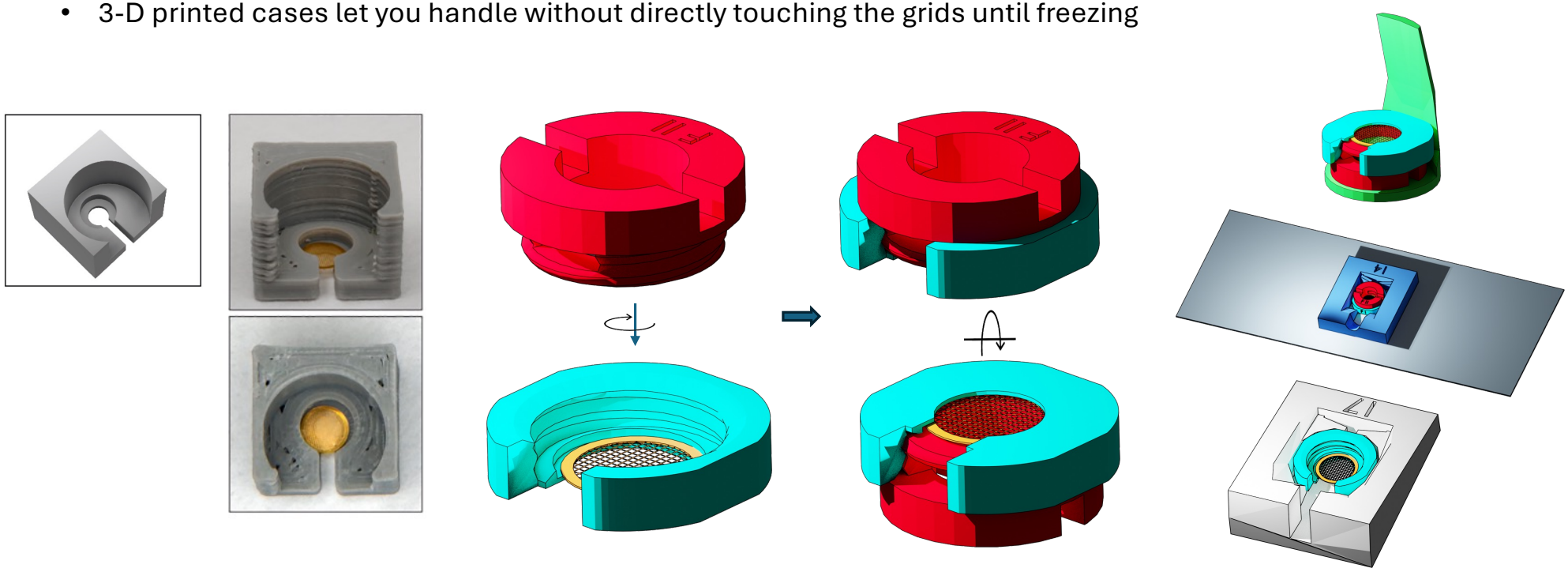
1. Get the cells on grids
   - Do the cells need to be milled (thin enough already)?
   - Do the cells need to be positioned on the grid (between bars)?
   - Do the cells need to be shaped (patterns of adhesive molecules)?

2. Freeze the samples
   - Do the samples have the right geometry to plunge-freeze?
     - Thickness and total mass
   - Do the samples need cryoprotectants?

3. Locate the targets for milling/CryoET
   - Labeling prior to freezing if needed

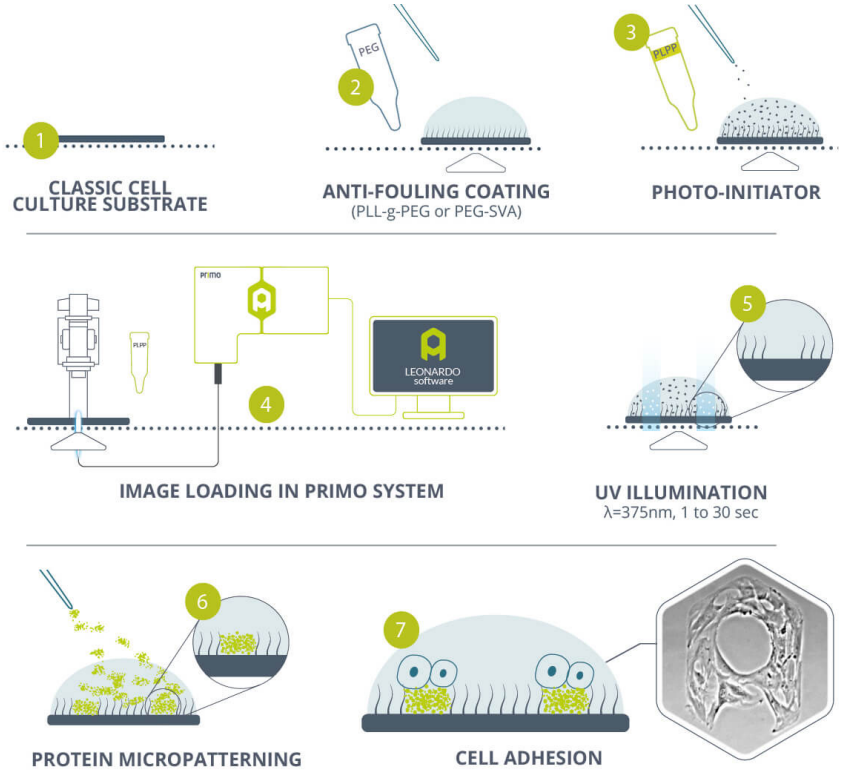
Preparing Grids for cell growth

- If cells are to be grown directly on the grid, the normal carbon support might not be sufficiently stiff or resilient
  - Evaporate ~20-25 nm carbon on top, and baked overnight at 60º C
- Handling the grids prior to and during culturing can lead to breaking and bending
  - 3-D printed cases let you handle without directly touching the grids until freezing



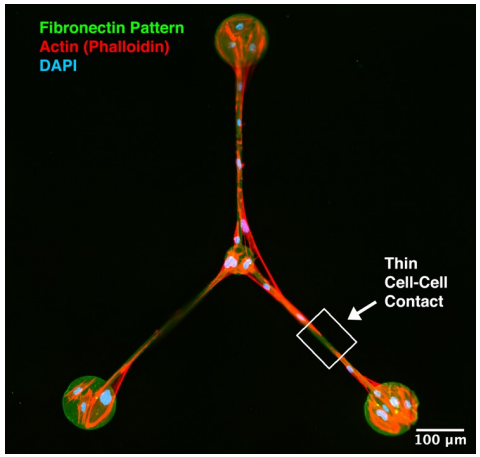
Fäßler, F., Zens, B., Hauschild, R., & Schur, F. K. M. (2020). **3D printed cell culture grid holders for improved cellular specimen preparation in cryo-electron microscopy**. *Journal of Structural Biology*, *212*(3), 107633.

Positioning cells for milling: PRIMO



- PRIMO system uses a DMD (digital micromirror device) to illuminate an arbitrary pattern with UV light using the microscope's objective lens

1. Coat the grid in a non-cell adhesive layer (e.g. PLL-PEG)
2. Add the UV-sensitive photo-activator (PLPP)
3. Illuminate the grid using the user-supplied pattern
4. Add cell-adhesive molecule (fibronectin, PLL, etc)
5. Seed the cells
    - May need to play with the concentration



Source: https://www.alveolelab.com/technology/micropatterning-surface-functionalization/

Vitrification of Cells

- Need to cool down the entire sample fast enough to prevent crystalline ice formation
  - 290 K to 90 K: ~20 μs
  - ~10^6 degrees/second

- Heat needs to transfer from the sample to the cooling medium, and away then from the sample to allow for more heat to move from more interior in the sample.
  1. Transfer within sample to interface
  2. within cooling medium away from sample

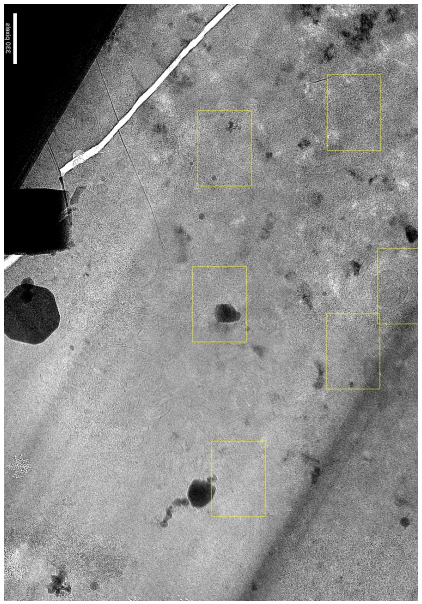
- Limits the thickness of plunge-frozen samples to less than 1 μm
  - Total mass on grid also affects freezing

- Once ice starts, excess heat from heat of formation also needs to be removed!

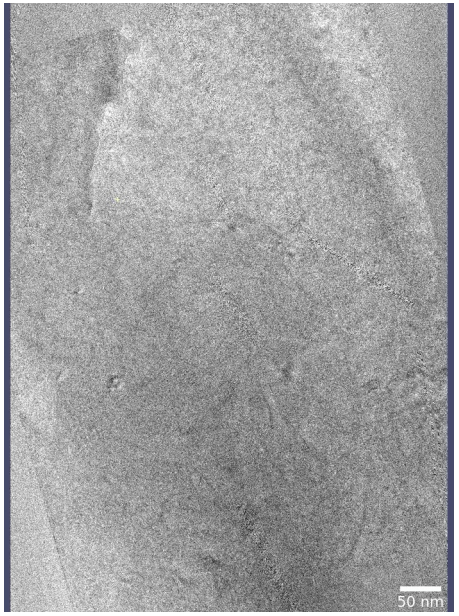
- Below ~ -40º C, homogenous ice formation begins
  - Doesn't need nucleation centers, spontaneously forms
  - The small ice crystals form don't affect freeze-substitution, but cryo-ET is harmed

Incomplete vitrification of cells

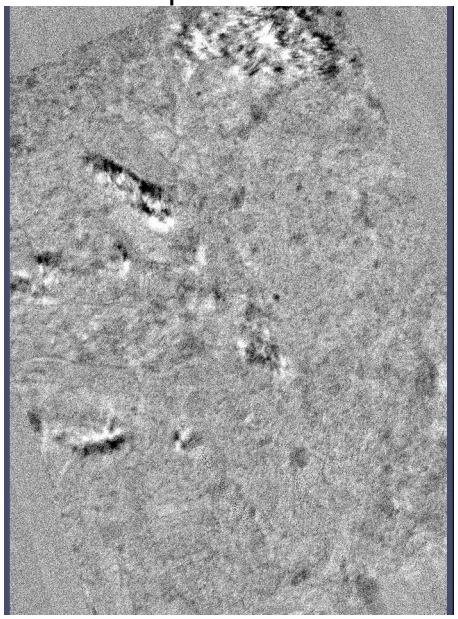
Zoomed-out TEM



"Stripe" patterned cells



"Donut" patterned cells

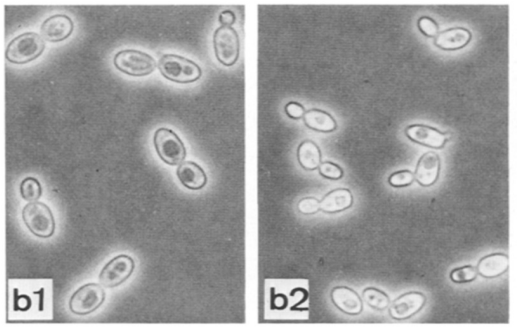


- "Burning" pattern indicative of incomplete vitrification can be seen zoomed-out TEM
- Appearance changes with tilt angle due to crystal planes of the crystalline water
- Lowers the S/N of the back-projected tomogram
  - Distorts the structure of embedded proteins?
- Counter with
  1. Cryoprotectants
  2. High-pressure freezing

Cryoprotectant agents

1. Cell permeable
   - Most commonly used is glycerol – can affect cellular structure

2. Cell impermeable
   - Only affects freezing in media surrounding cells
   - Heat of ice formation doesn't need to be removed

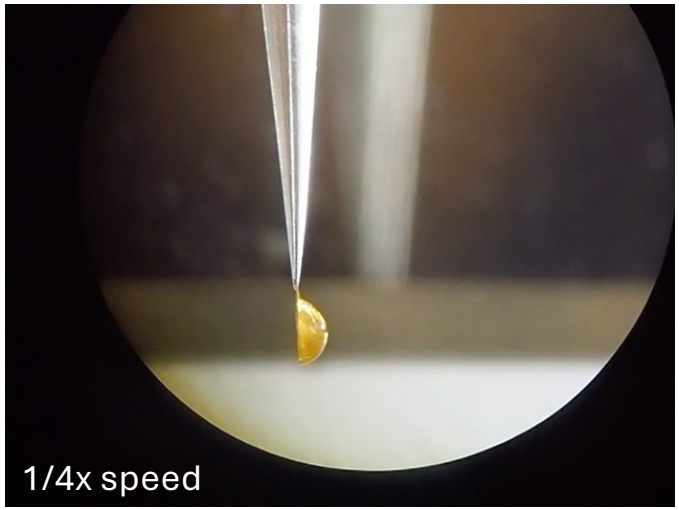


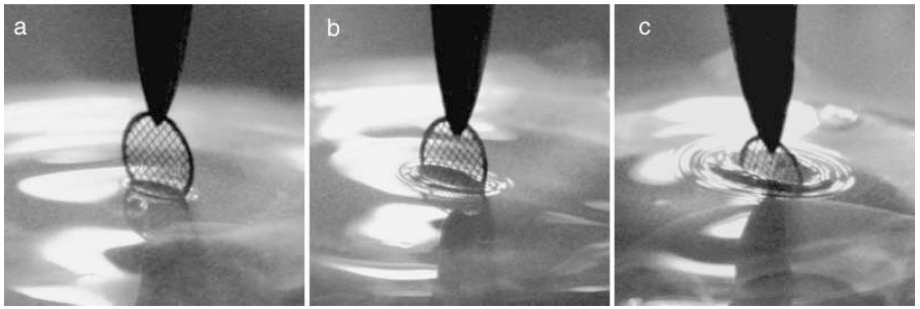
0% Glycerol 9% Glycerol

Cryoprotectant	Degassed	Vitrification	Background
Native	-	Incomplete	None
15% PVP in PBS	-	Incomplete	Acceptable
15% PVP in 0.1 M PB	-	Incomplete	Acceptable
15% BSA in 0.1M PB	-	Incomplete	High
20% Dextran/5% Sucrose in PBS	-	Complete	High
20% Dextran/5% Sucrose in PBS	+	Complete	High
20% Dextran in PBS	+	Incomplete	Acceptable
10% Dextran in PBS	+	Incomplete	Acceptable
10% Dextran in PB	+	Complete	Acceptable
5% Dextran in PB	+	Incomplete	Acceptable
Native	+	Incomplete	None
10% BSA in medium	+	Incomplete	Acceptable
20% BSA in medium	+	Complete	High

Niedermeyer, W., Parish, G. R., & Moor, H. (1977). **Reactions of yeast cells to glycerol treatment alterations to membrane structure and glycerol uptake.** *Protoplasma*, *92*(3–4), 177–193.
Zens, B., Fäßler, F., Hansen, J. M., ..., & Schur, F. K. M. (2024). **Lift-out cryo-FIBSEM and cryo-ET reveal the ultrastructural landscape of extracellular matrix**. *Journal of Cell Biology*, *223*(6).

Liquid-Ethane Plunge-freezing





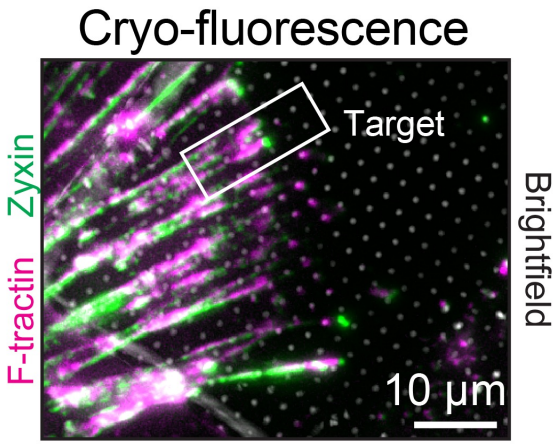
0 µs 500 µs 1000 µs

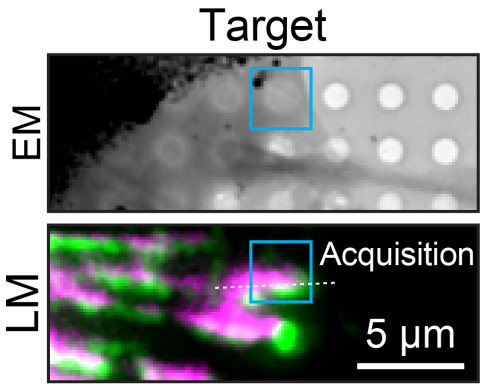
- One sided blotting does not disturb the sample on the grid (Only method on Leica GP)
- Blotting from one side means the liquid has to move through the layer of carbon using capillary action
- The entire sample needs to cool within ~10-20 ms to ensure that ice is vitreous (not crystalline)
- Highly dynamic cooling
  - Liquid ethane touches bottom of grid before top
  - Movement of liquid ethane?
  - Kinetics of heat transfer within cellular sample?
  - Kinetics of transport of heat within liquid ethane?
  - Grids bars as radiators or just extra mass?

Kasas, S., Dumas, G., Dietler, G., Catsicas, S., & Adrian, M. (2003). **Vitrification of cryoelectron microscopy specimens revealed by high-speed photographic imaging**. *Journal of Microscopy*, *211*(1),

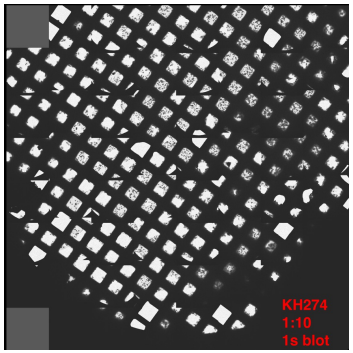
Test Case: Plunge-Freezing with No Milling

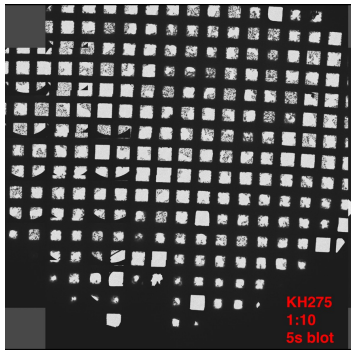
- If area of interest is thin enough to be transparent without milling
  - Will ice will be vitreous after plunge freezing?
  - Positioned in center of grid square?
  - Blot time?
- Blotted 7s and was able to acquire good tomograms in edge of the cell

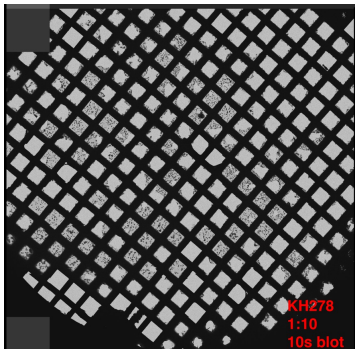


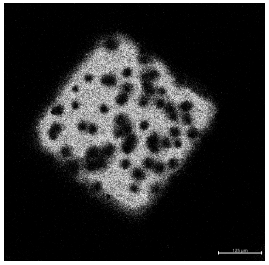


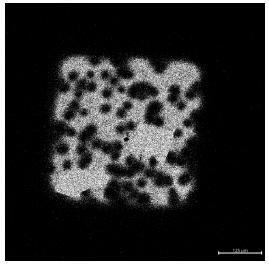
Test Case: Unpatterned Bacteria for Plunge-Freezing

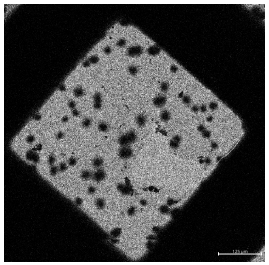


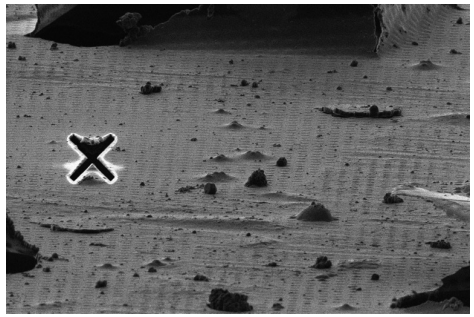




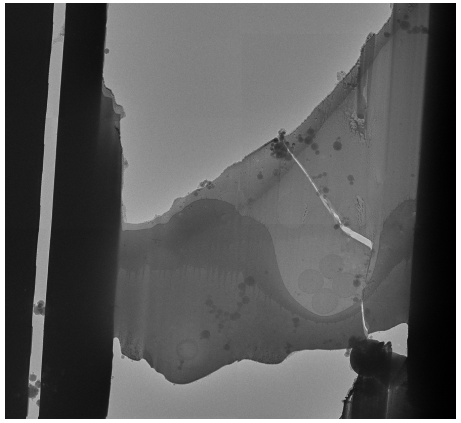


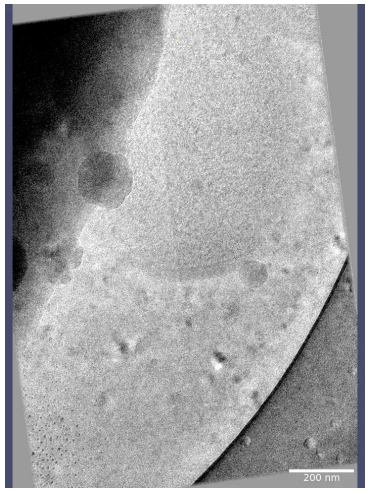




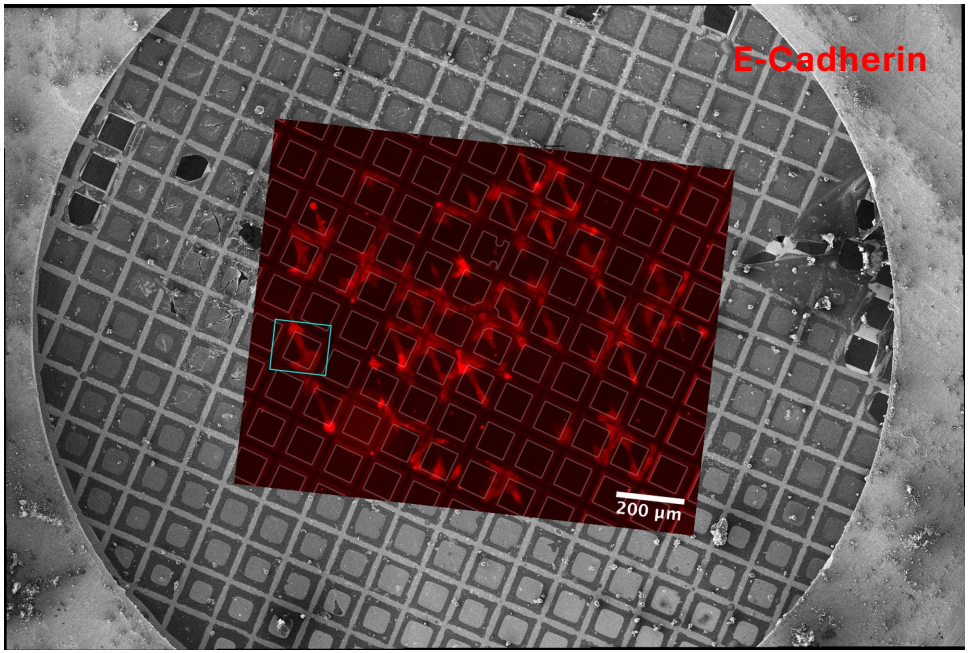


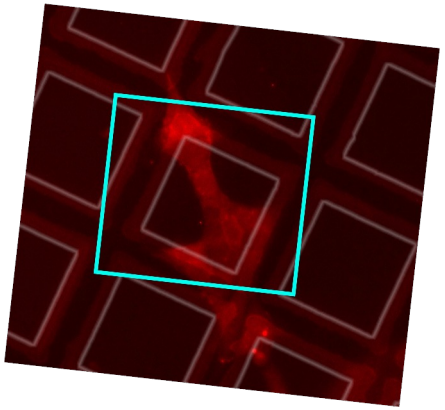
- Bacterial samples were added and different blotting times tested
  - No patterning, just plasma cleaning and direct addition
  - No cryoprotectant
  - 10s blotting removed almost all the surrounding buffer
- Cells visible in the SEM as mounds
- All the cells were vitreous
- Where buffer remained, some non-vitreous ice

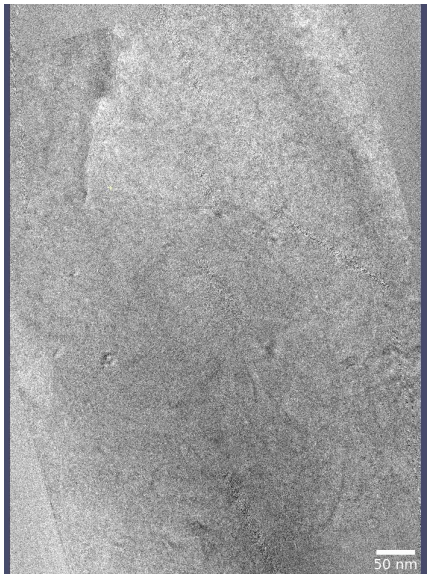




Test Case: Patterned Stripes of Cells for Plunge-Freezing

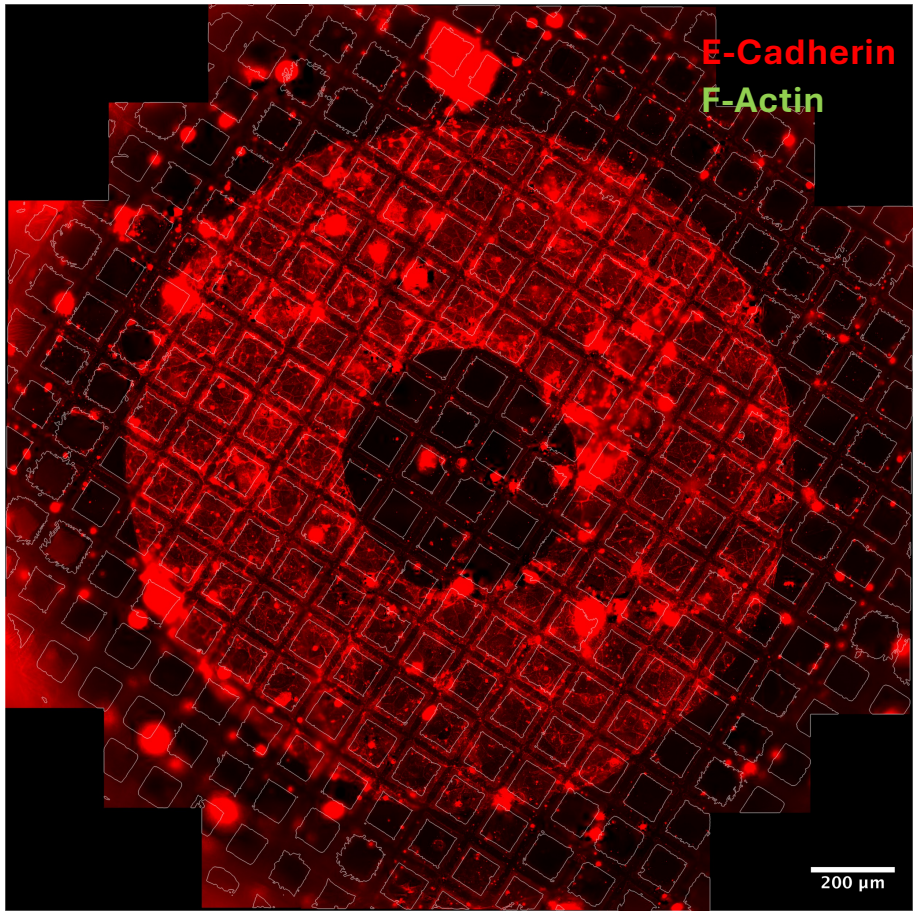


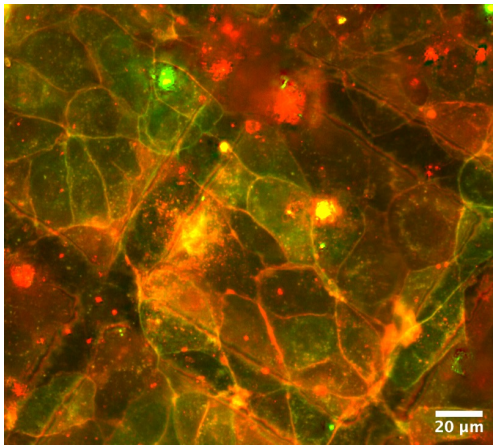


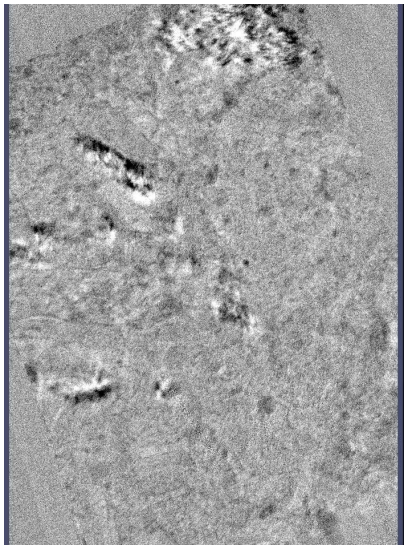


- “Stripes” of fibronectin were patterned with PRIMO, and EPH4 (mouse mammary epithelial) cells were seeded and allowed to grow 24h
- Plunge-frozen on Leica GP
  - 15s blot time
- Cells had non-vitreous ice, although tomogram reconstructions were usable
- Not physiologically relevant, cells didn’t form mature cell:cell junctions

Test Case: Patterned “Donuts” of Cells for Plunge-Freezing



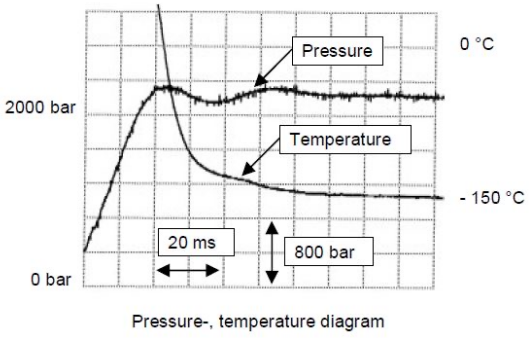




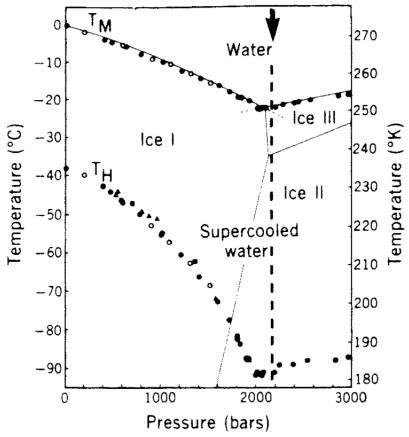
- Patterned a ”donut” to let the EPH4 cells grow in a sheet, while still allowing the water the drain
  - Added 0.05% detergent (FOM) prior to plunge freezing on GP
  - Blotted 15s
  - ~0.5 µm water left on top of the cells
- Inside of cells was not vitreous!

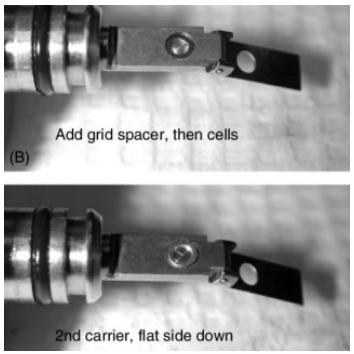
High Pressure Freezing

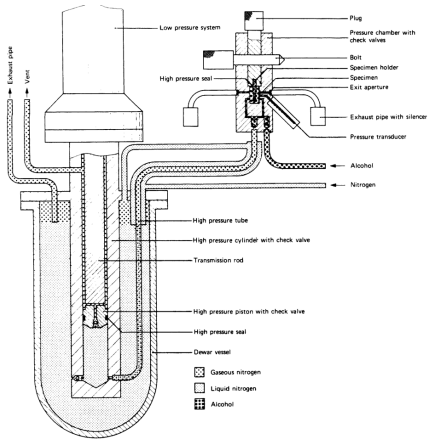
- Increase the pressure to ~ 2000 Mbar prior to freezing
- This slows the formation of crystalline ice
  - Increases viscosity of water
  - Prevents the expansion needed to create crystalline ice
- Put the sample between two brass “planchettes”
- Planchettes are squeezed by high-pressure LN_2, cushioned by a layer of alcohol
- After pressurizing (~15 ms), the alcohol is pushed out and the LN2 directly contacts the planchettes
- Cools at ~ 10^3-10^4 degree/s



Pressure-, temperature diagram

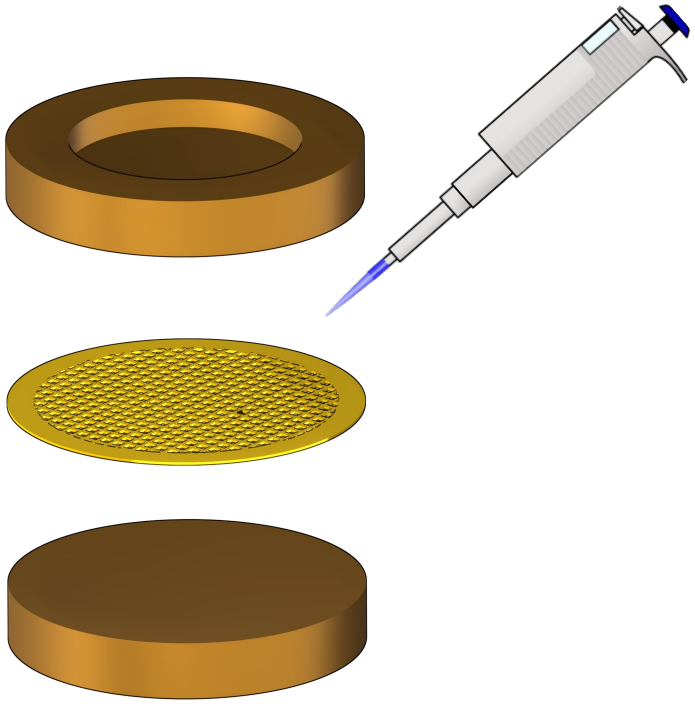




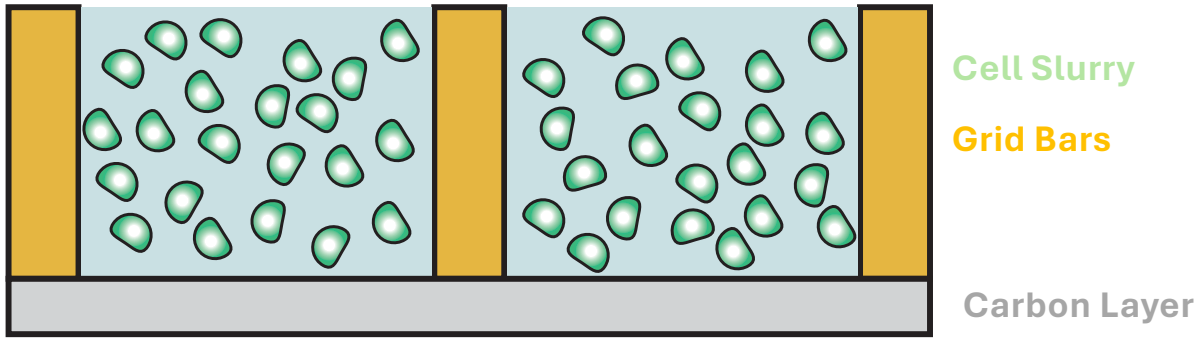


Dahl, R., & Staehelin, L. A. (1989). **High-pressure freezing for the preservation of biological structure: Theory and practice**. *Journal of Electron Microscopy Technique*, *13*(3), 165–174

High-pressure freezing – Suspended Sample

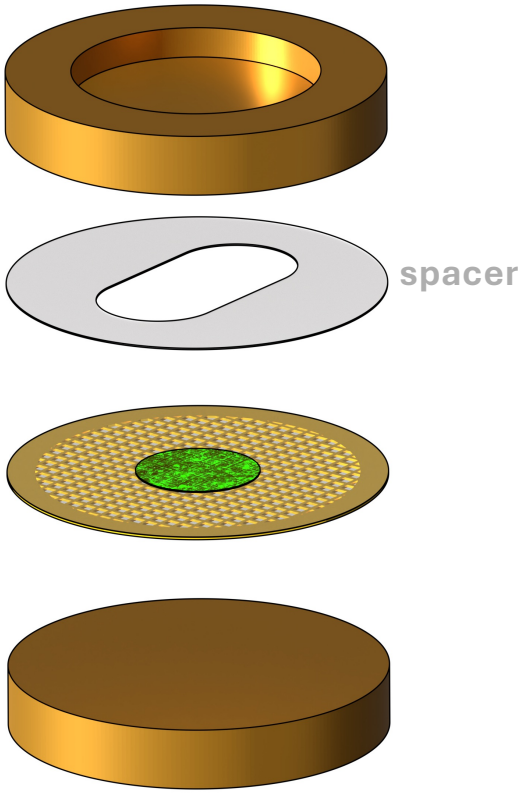


- Concentrate sample with cryoprotectant into a “slurry”
- Deposit sample onto the upside-down grid
  - Space between bars act as small wells
  - Gold grids ~ 25 µm thick
- Sandwich between two brass planchettes
  - Polished and 1-hexadecene coated to facilitate removal
- High-pressure Freeze
- Sample should be everywhere, can mill top part with “Waffle Method”

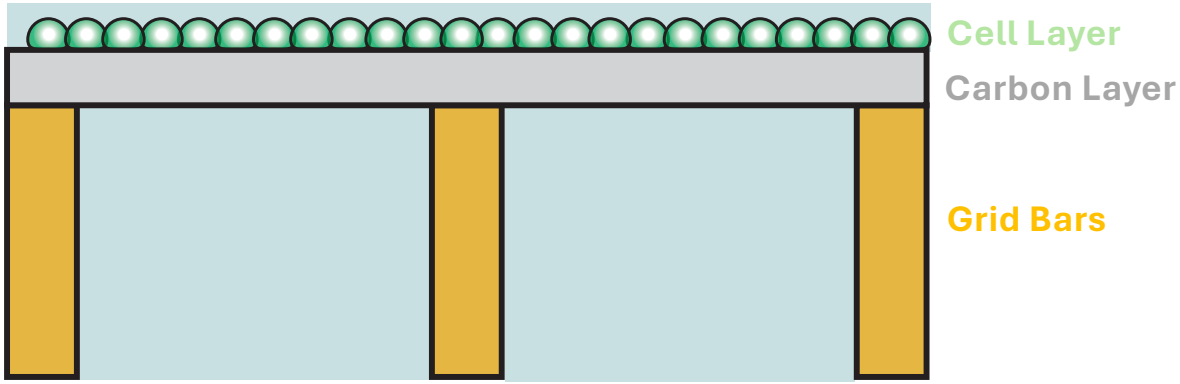


Kelley, K., Raczkowski, A. M., ..., G., Potter, C. S., Carragher, B., & Noble, A. J. (2022). **Waffle Method: A general and flexible approach for improving throughput in FIB-milling**. *Nature Communications*, *13*(1), 1857

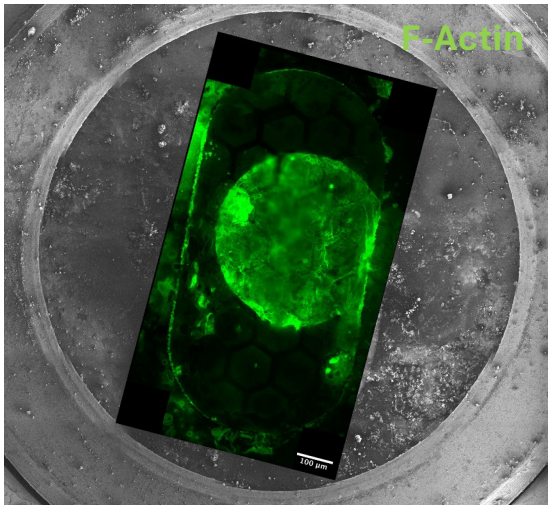
High-pressure freezing – Adhered Sample

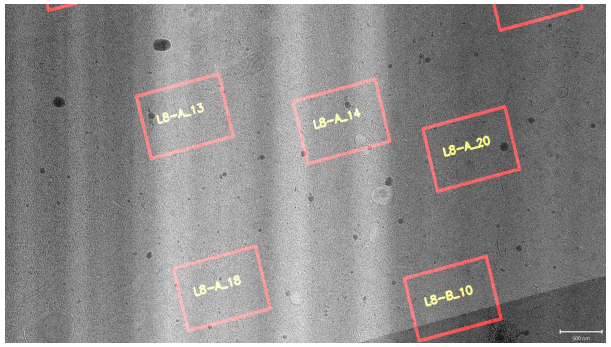


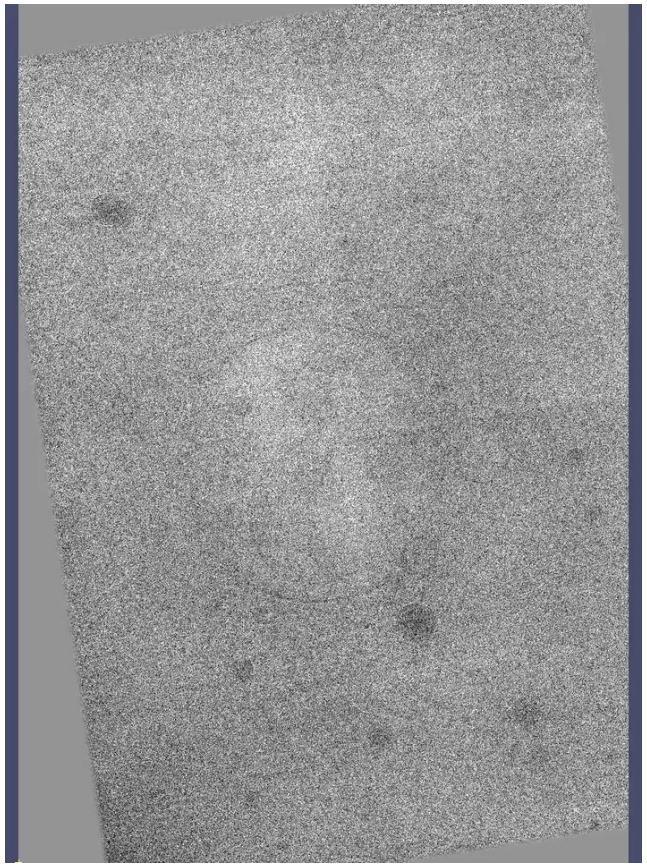
- Grow cells directly on the grid
- Add an aperture grid as a spacer to keep the cells from being squeezed
  - Copper spacer: ~ 35 µm
  - gold spacer: ~ 25 µm
  - Steel spacer ~ 10 µm
- Sandwich between two brass planchettes
  - Polished and 1-hexadecene coated to facilitate removal
- High-pressure Freeze
- Thickness of the media layer is dependent on the spacer type



Test Case: Patterned Sheets of Cells for High-Pressure Freezing



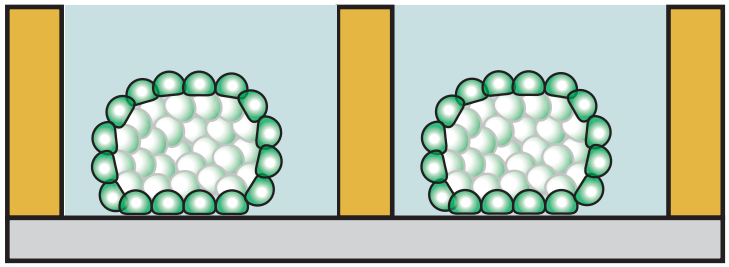


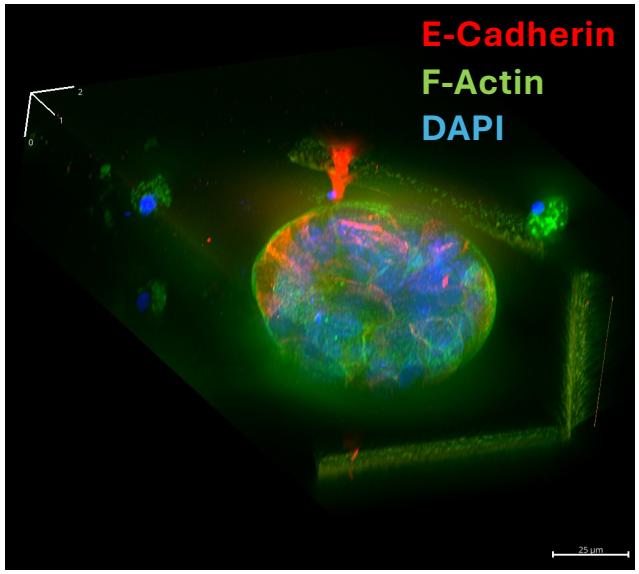


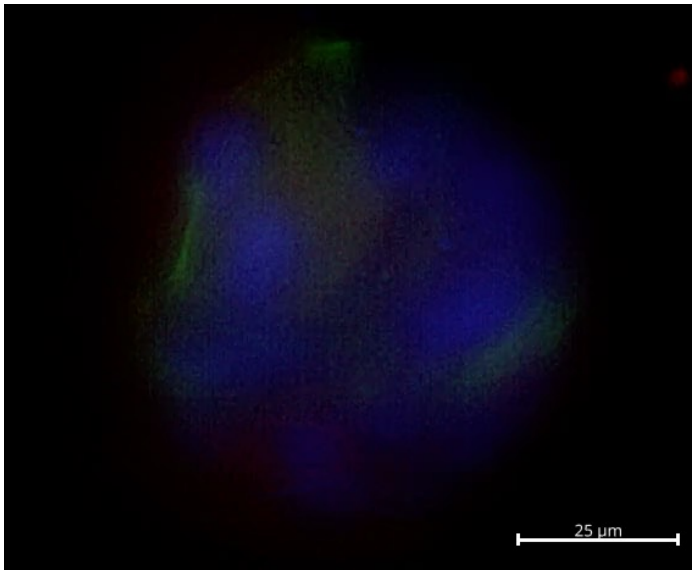
- Used high-pressure freezing to freeze a sheet of EPH4 cells
- Patterned a “button” to confine the cells within the spacer center
- Lamellae showed very little non-vitreous ice
- Tilt-series also had small to no areas non-vitreous ice

Test Case: Confined Cysts for High Pressure Freezing

- Used the ”fill in the grid wells” method to grow cysts of epithelial cells (MDCK)
- PRIMO patterned 1 mm circle, added PLL and 2% matrigel
- Use larger hexagon shaped grids (100 mesh)
- Fixed some grids to make sure they grew properly
- High-pressure froze the rest
- Currently imaging on CLEM in preparation for FIB-milling







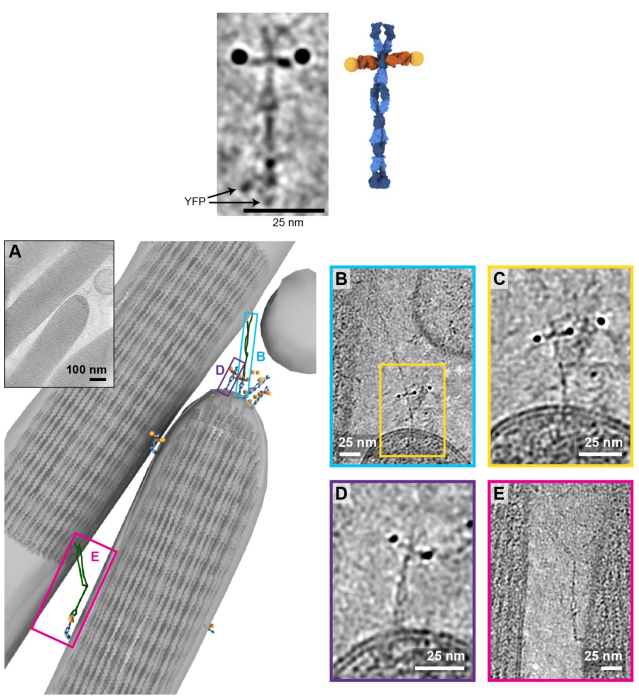
Labeling targets for CLEM and TEM

- Labeling with a fluorescent tag allows the localization of the target molecules within the sample
  - Bleaching is not much of an issue at cryogenic temperatures
  - Membranes (especially multimembrane vesicles) auto-fluoresce at cryogenic temperatures, strongest in GFP channel
  - Can’t localize more than ~0.1 um area

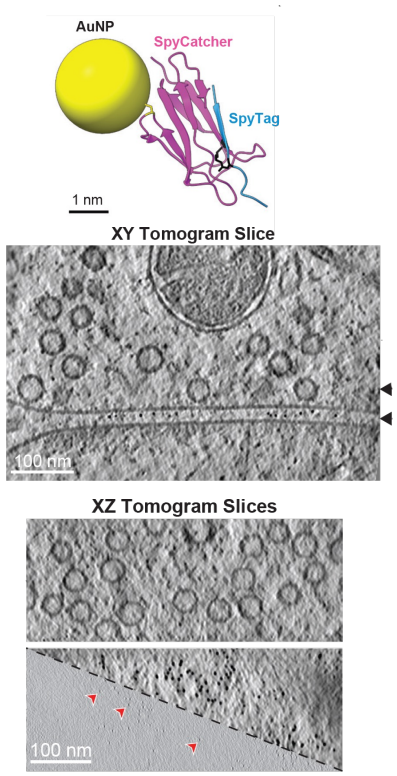
- Labeling protein of interest with a small (~3-5 nm) gold nanoparticle (AuNP) allows precise localization within the tomogram
  - Not easy to get nanoparticles inside of cells, so normally this is limited to extracellular targets
  - Need to conjugate the AuNP to a molecule that would bind to the target

- Other methods include genetically encoded multimeric tags (GEMS) or Ferritags to label inside of cells

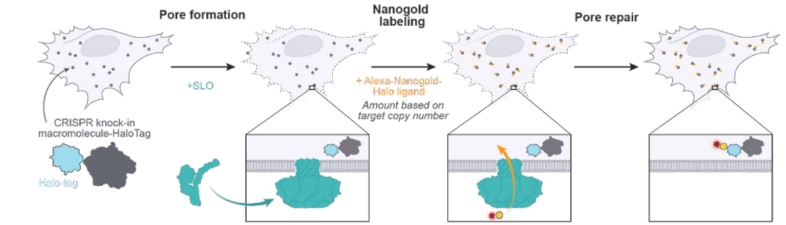
AuNP labeling for Localization in Tomograms: Examples

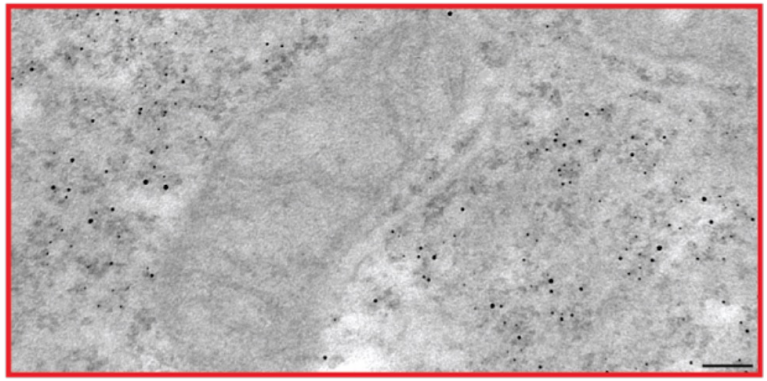


FAB-conjugated AuNP



Spycatcher





Halo-Tag In-cell!

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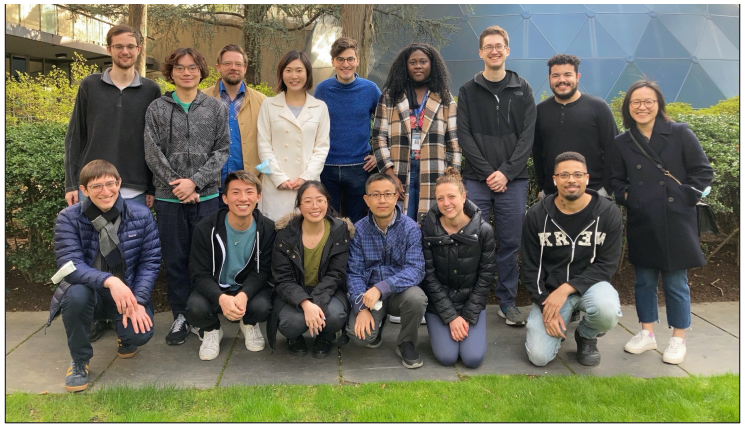

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