

# Cryo-FIB Milling of Cells for Electron Tomography

Workshop on Advanced Topics in EM Structure Determination  
NRAMM, TSRI  
November 2014

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UCSD

SD: EM Mecca by the Sea



# Frontiers in Cryo-EM

1. Higher resolution
2. Small complexes
3. Conformational/compositional heterogeneity (dynamics!)
4. How do complexes look like and behave in their natural environment :  
Structural Cell Biology

# My Job Today

**1:30 pm**      **FIB milling**

**Elizabeth Villa**

*[What is the advantage of in situ cellular EM? Where are we now? What are the challenges going forward? What are the practical issues involved in FIB milling? Is it ready for prime time? Is it time consuming? How much skill is required? Will any lab be able to do it?]*

... and a few others raised here so far

# What are the advantages of *in situ* cellular EM?

Complexes in their natural environment

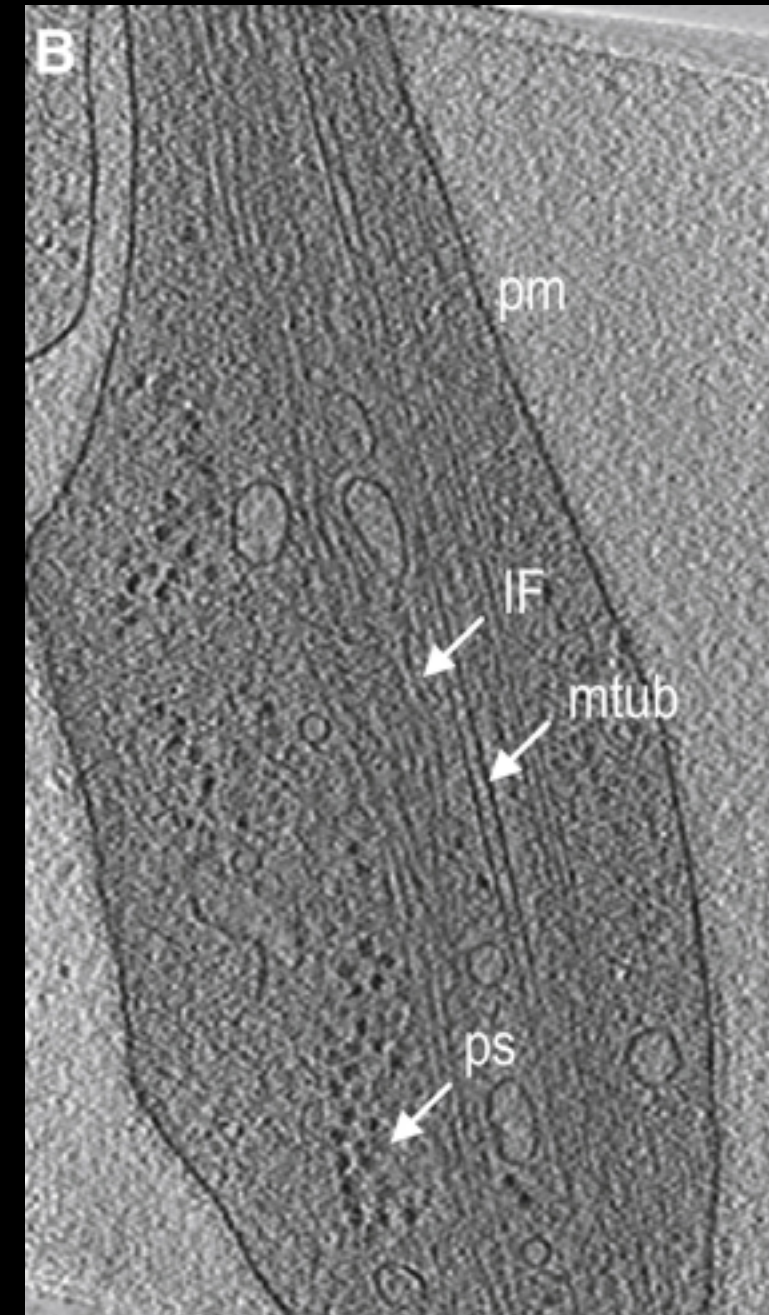
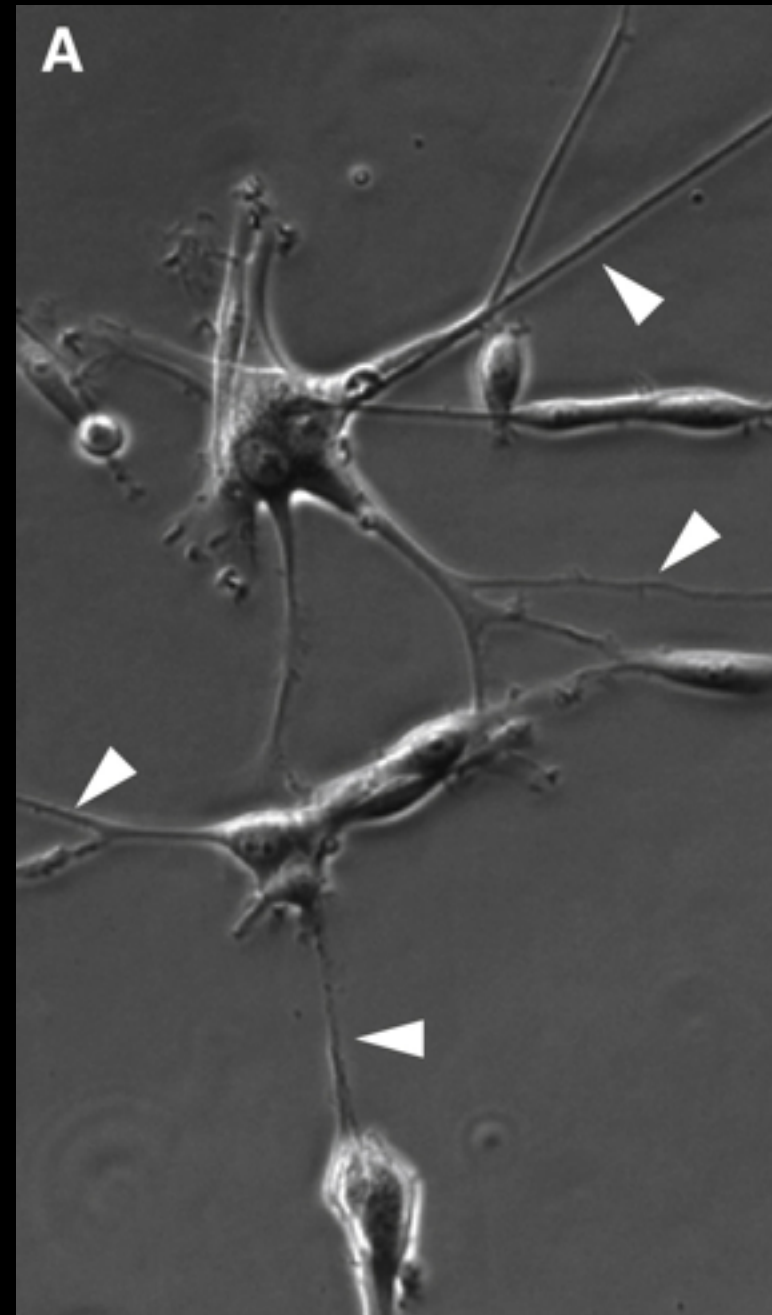
Transient and rare complexes

Neighborhoods and molecular census under different cellular conditions

## Structure vs. Story

# Where are we now?

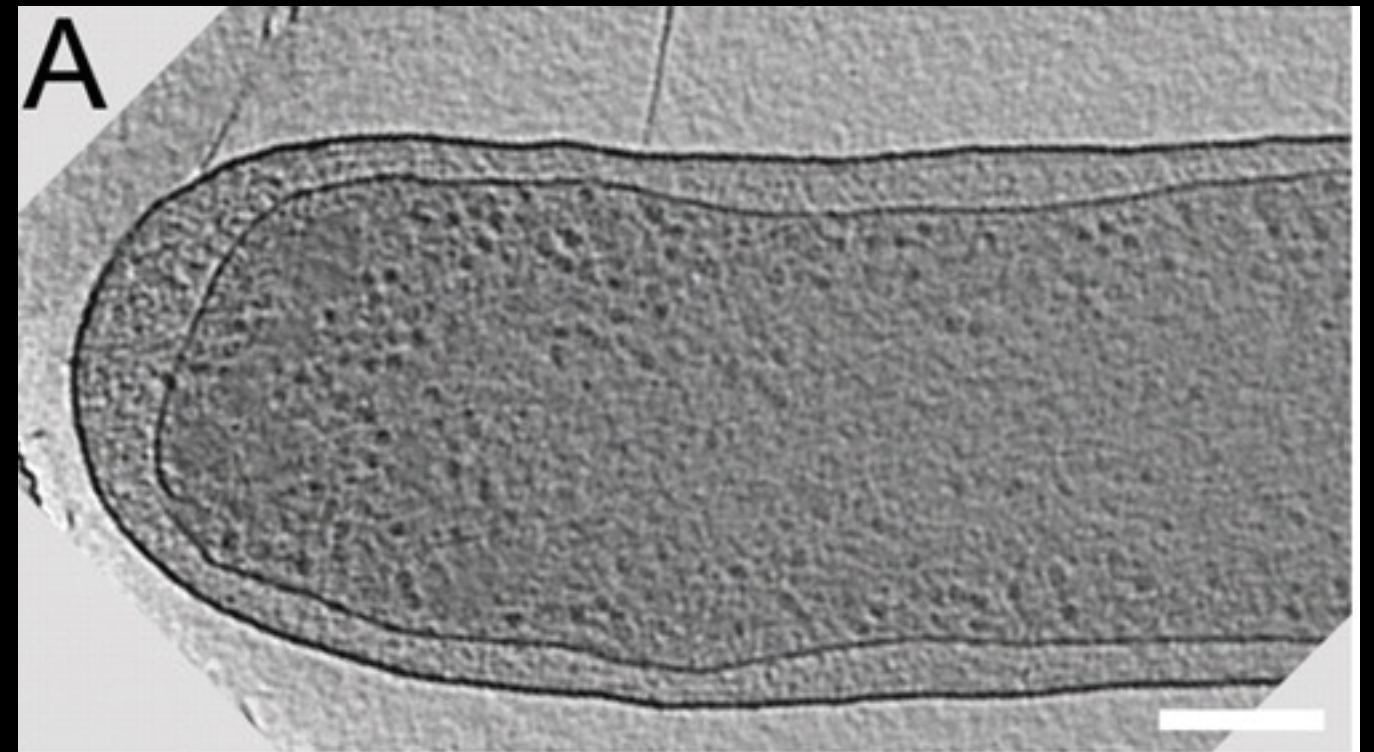
- **Peripheral regions of cells**
- Thin cells (starved, mutant)
- Isolated or reconstituted systems
- Cryo-sections
- FIB milling



Brandt, Carlson et al. Mol Cell 2009

# Where are we now?

- Peripheral regions of cells
- **Thin cells (starved, mutant)**
- Isolated or reconstituted systems
- Cryo-sections
- FIB milling

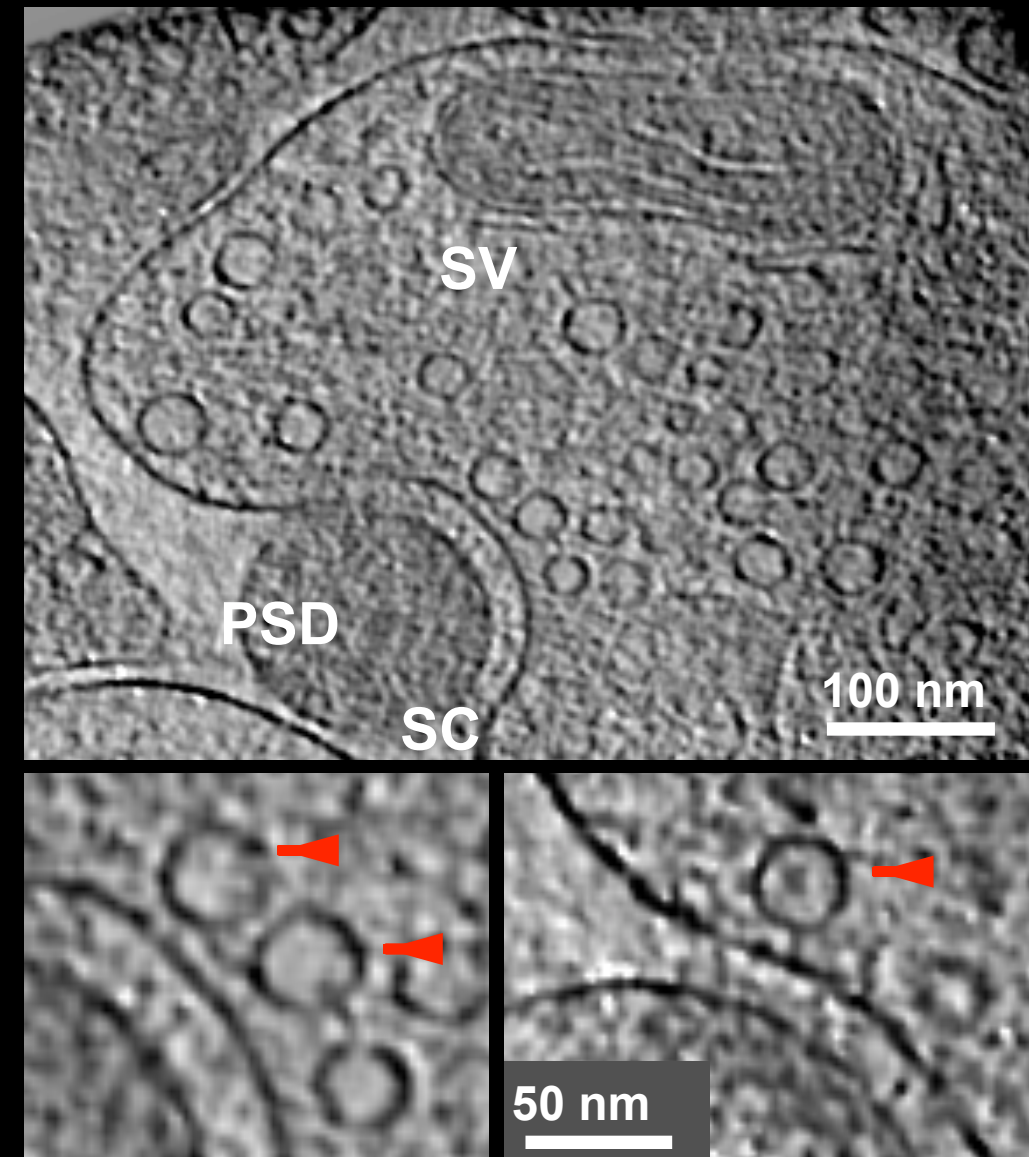


*E. coli* in minimal media. Ortiz et al., JCB 2010

# Where are we now?

- Peripheral regions of cells
- Thin cells (starved, mutant)
- **Isolated or reconstituted systems**
- Cryo-sections
- FIB milling

Synaptosomes (Fraction)

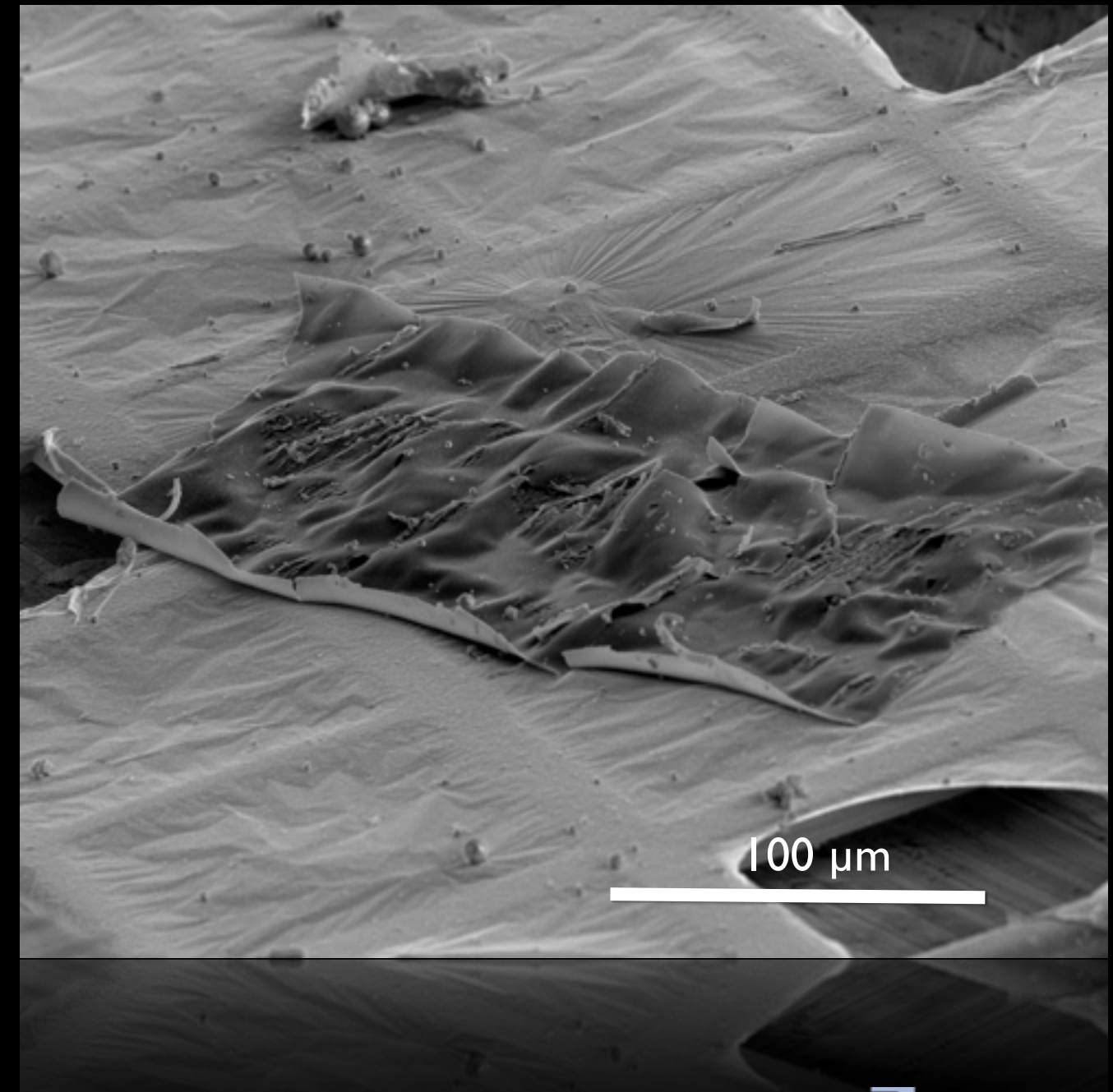




# Where are we now?

- Peripheral regions of cells
- Thin cells (starved, mutant)
- Isolated or reconstituted systems
- **Cryo-sections**
- FIB milling

Hippocamal Cryo-Section



# Cryo-Sectioning

(with a little help from some friends)

It used to be exotic... now it's **golden!**

“In our last CEMOVIS course in August, every participant could prepare good grids after a day of practice (some got it after an hour or so).”

## Compression

Unavoidable, but not relevant to all scales?

## Quality of section for data acquisition

Some experience is still useful to judge

## Throughput

Half a morning, on a single grid you have about 300k  $\mu\text{m}^2$  of sample to analyze (yay?)

Serial sections possible, cool labeling

## Lower price

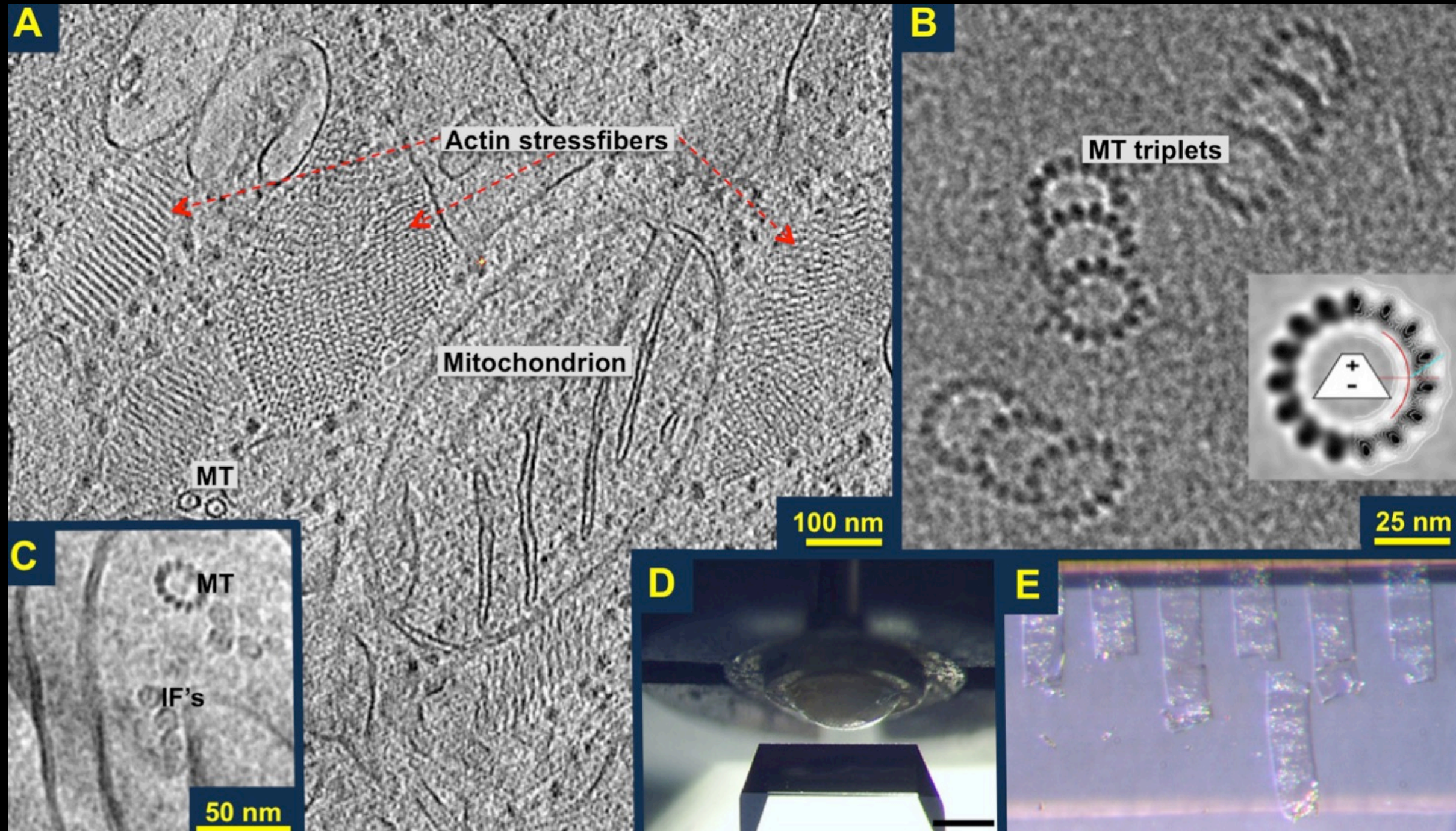
Need high-pressure freezer

Can be applied to HPF samples — tissue!



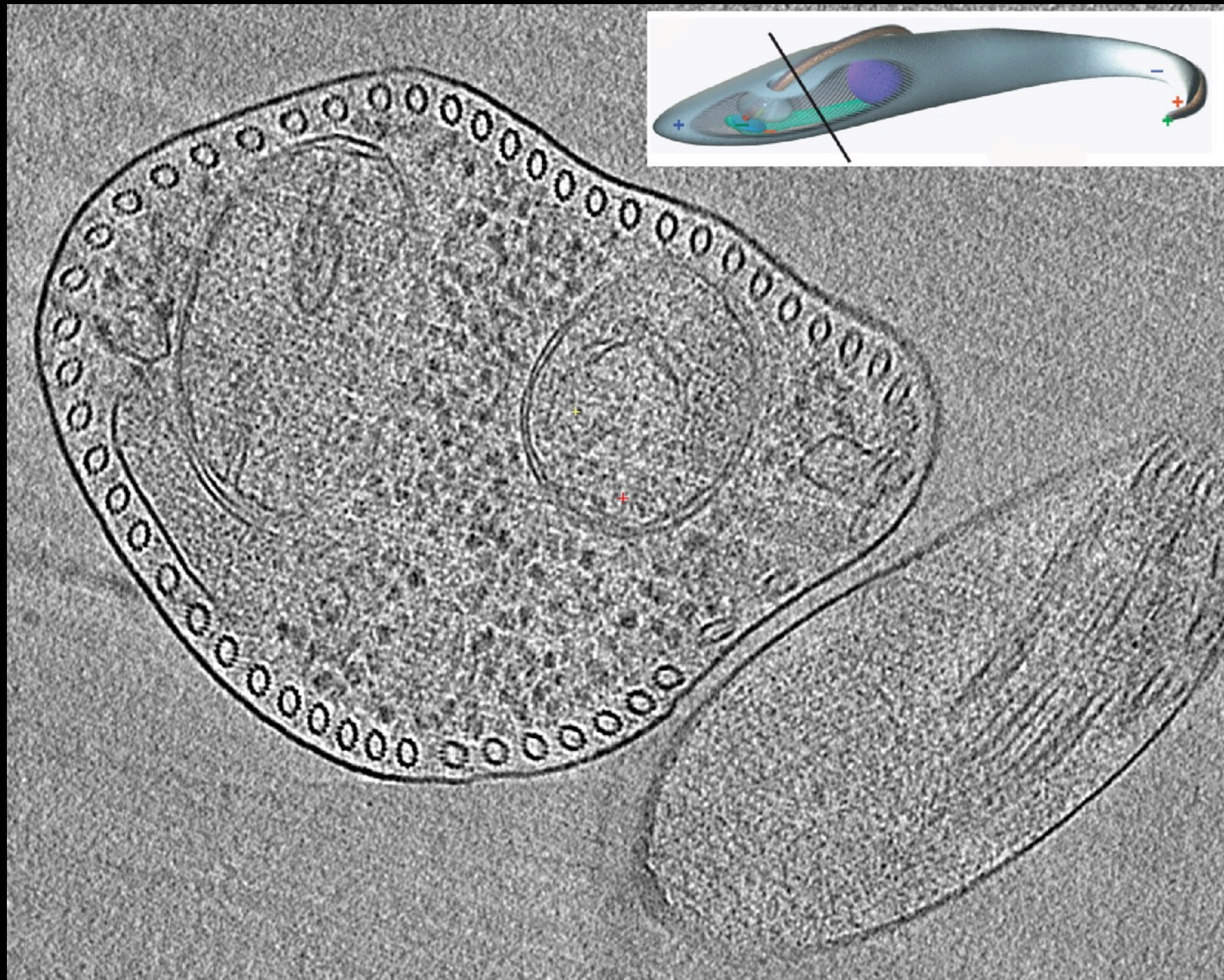
# Cryo-Sectioning

(with a little help from some friends)

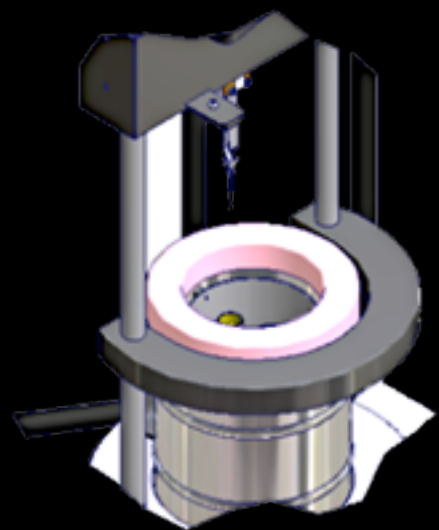


# Cryo-Sectioning

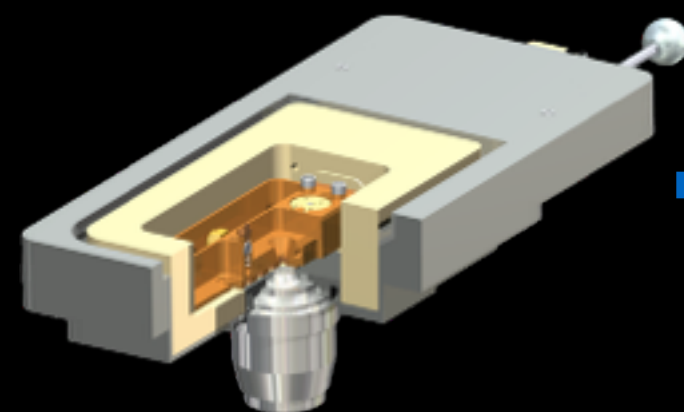
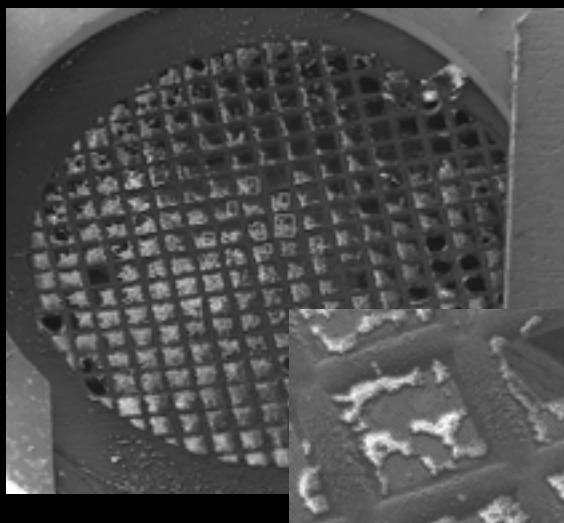
(with a little help from some friends)



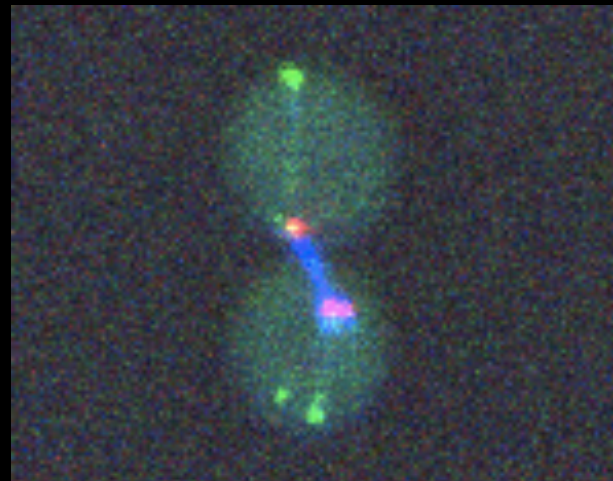
# Where are we now?



Vitrification



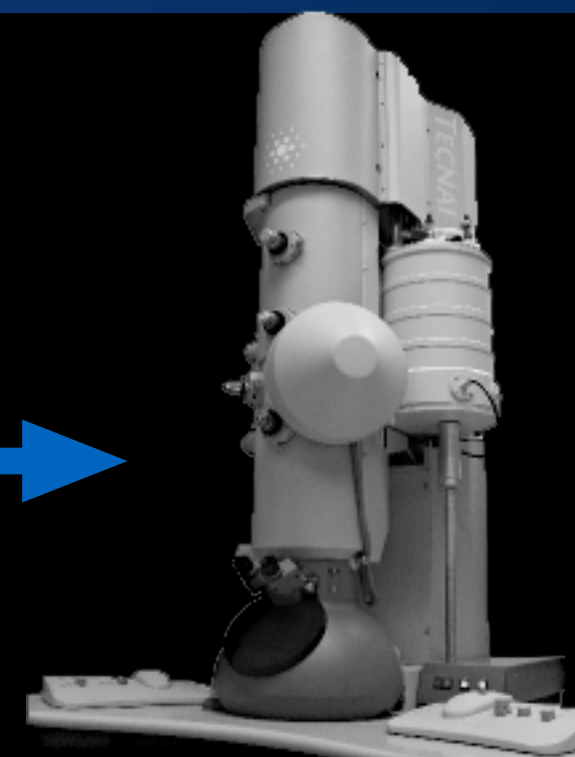
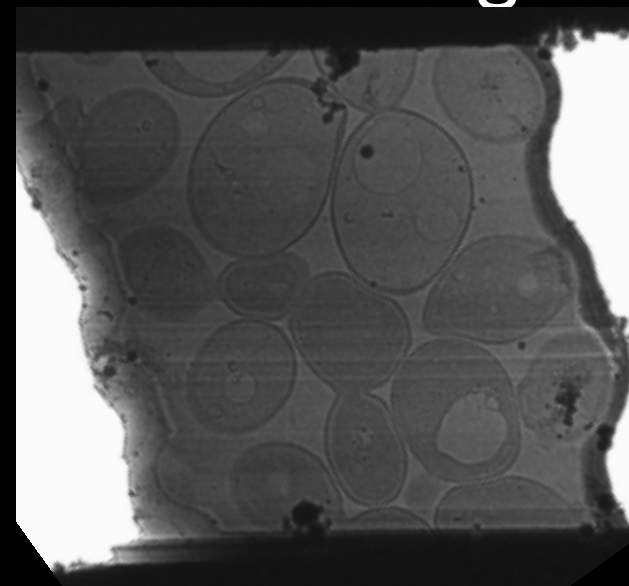
Light Microscopy



Strain from Reck-Peterson Lab (HMS)



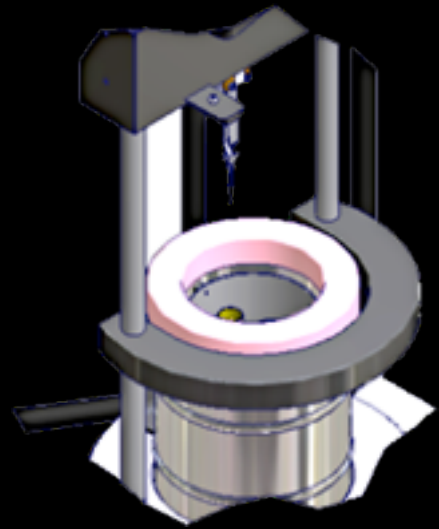
FIB milling



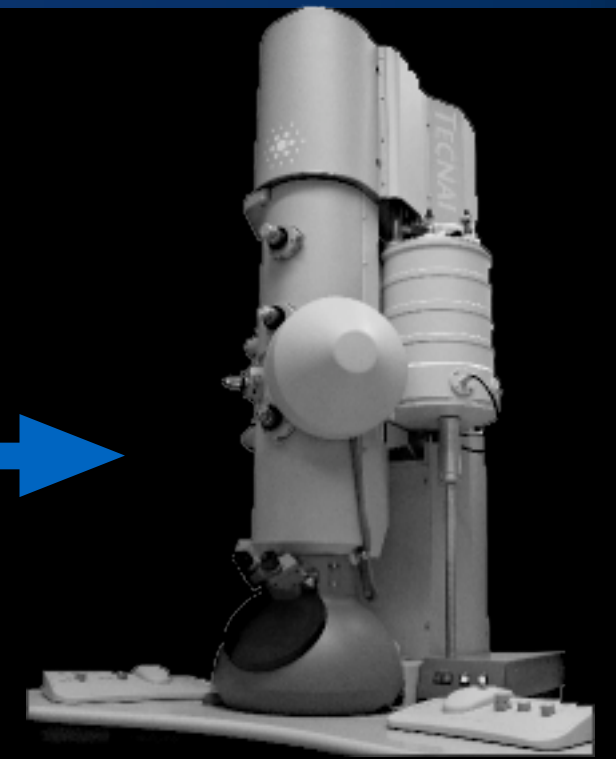
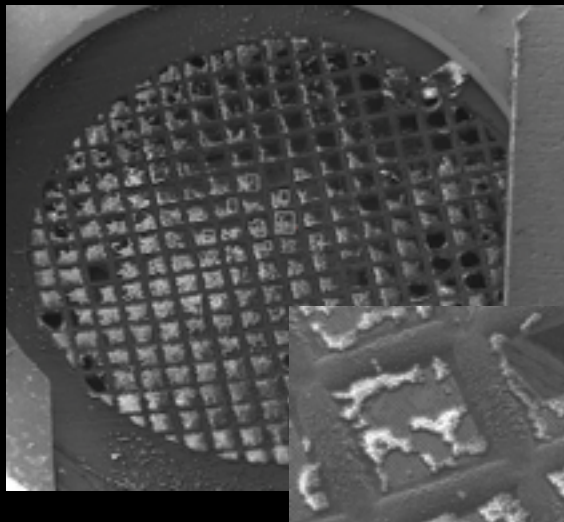
Tomography



# Cryo-electron Tomography Workflow

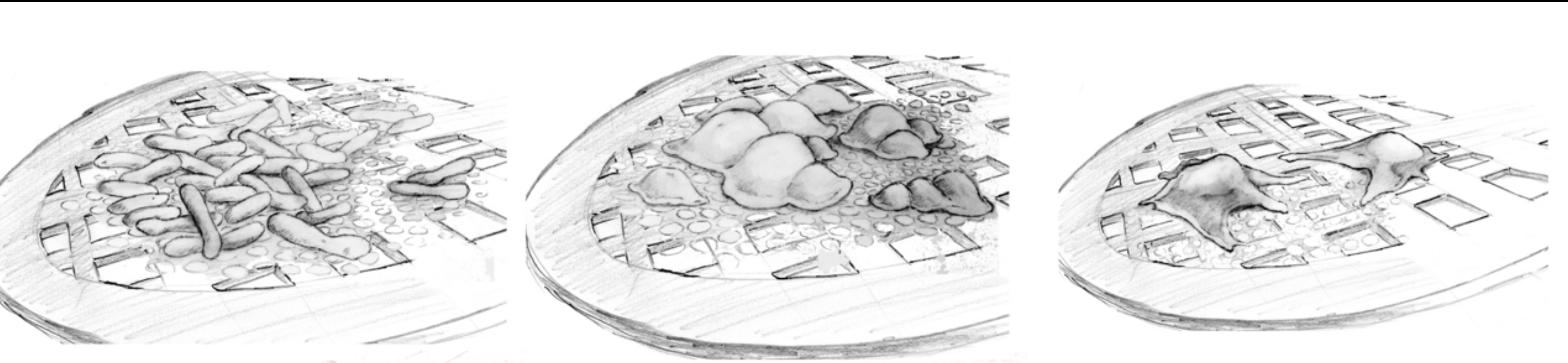


Vitrification



Tomography

# What can we freeze on the grid?

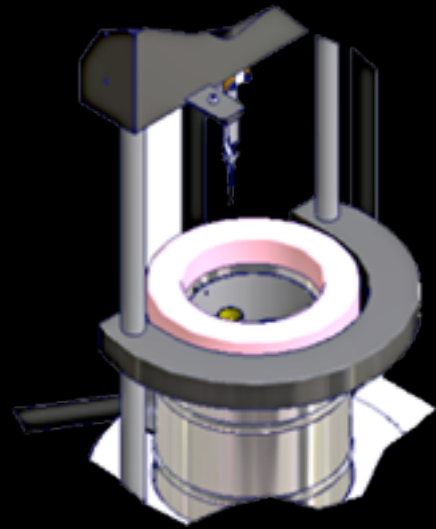


Bacterial Cells  
~0.5  $\mu\text{m}$

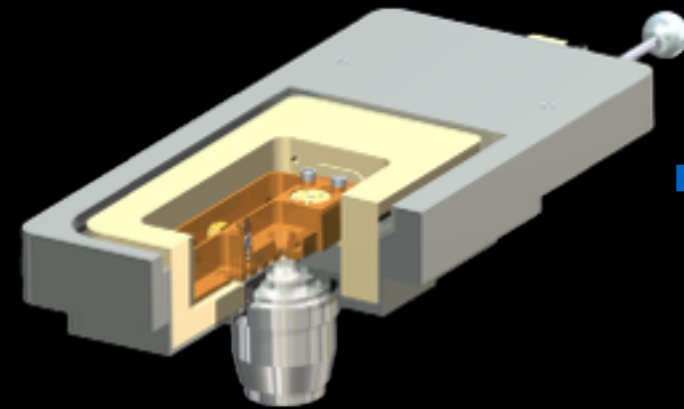
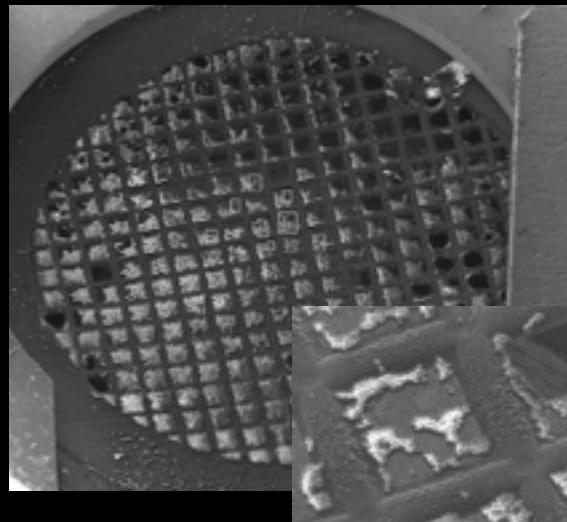
Small Cells  
~2  $\mu\text{m}$

Mammalian Cells  
~5-10  $\mu\text{m}$

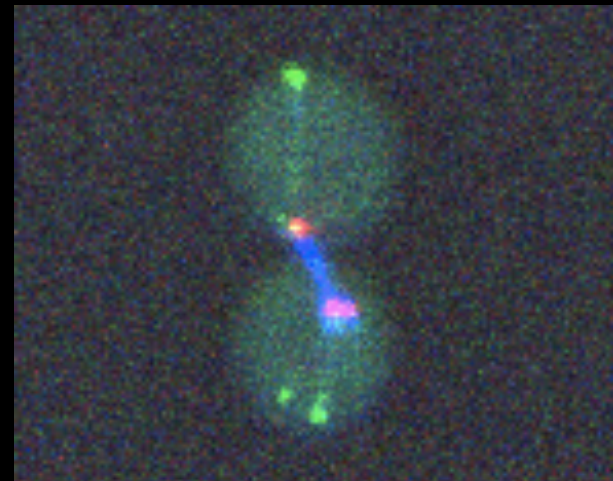
# Cryo-electron Tomography Workflow



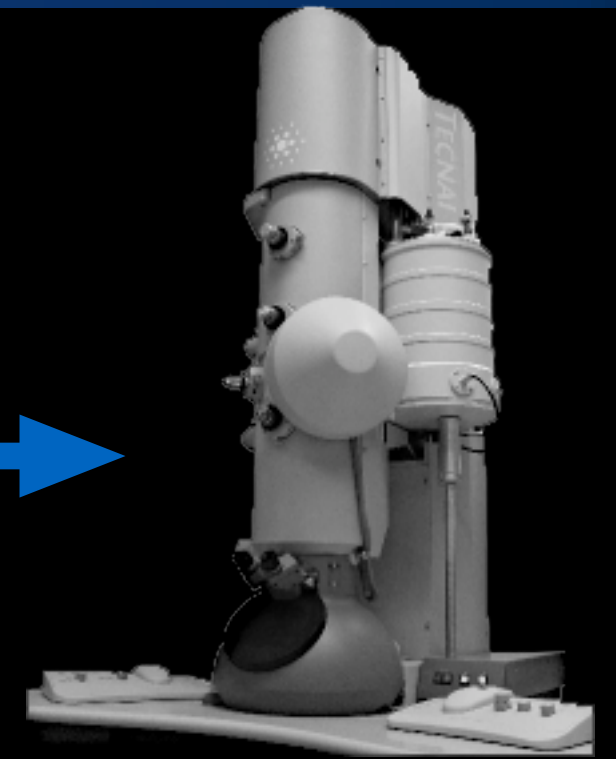
Vitrification



Light Microscopy



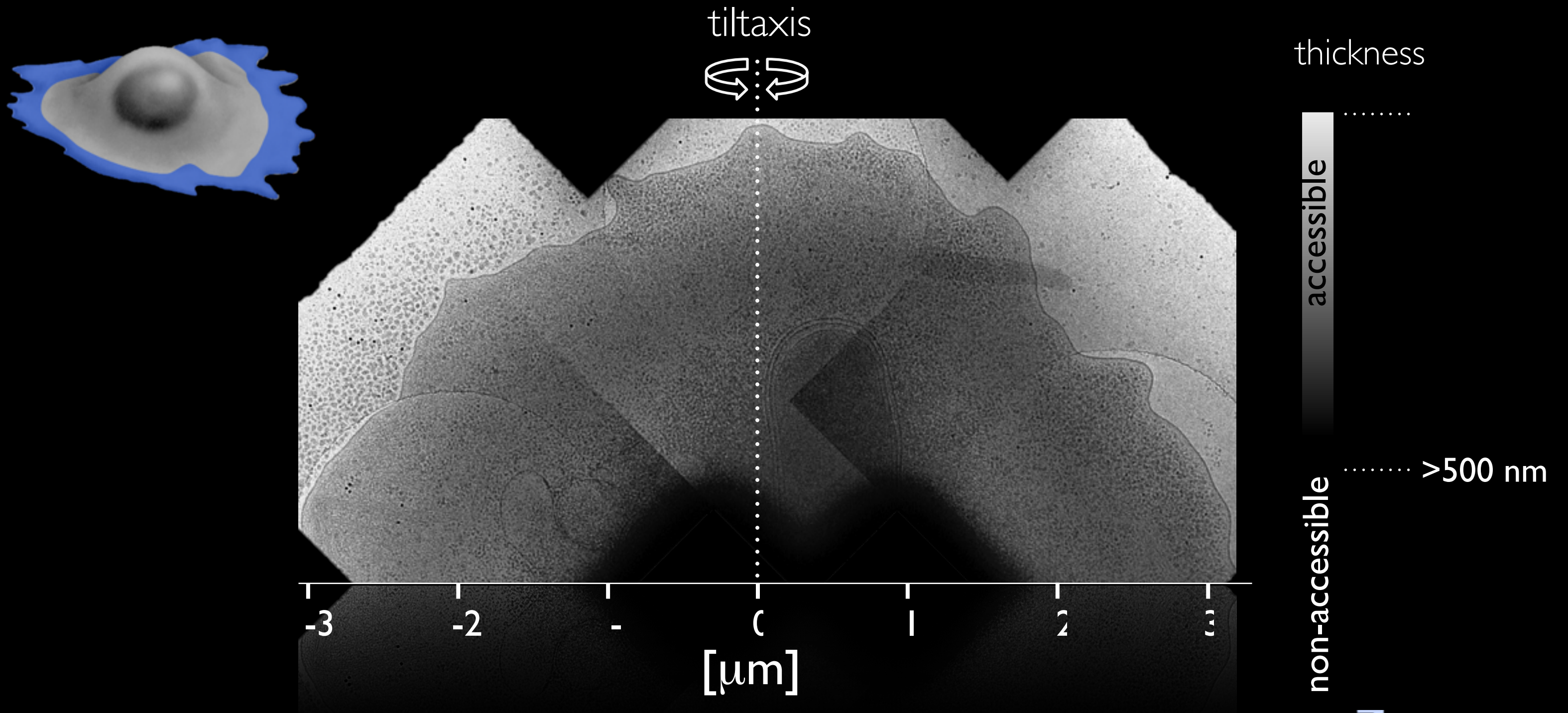
Strain from Reck-Peterson Lab (HMS)



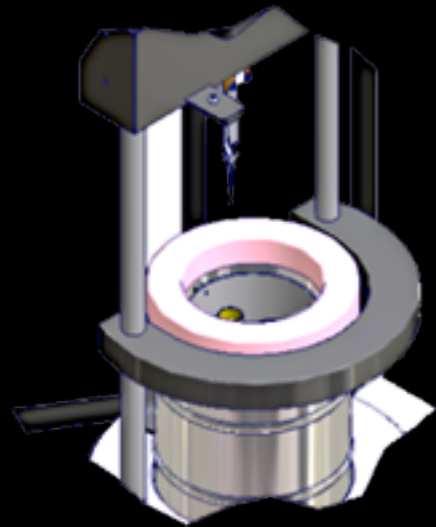
Tomography



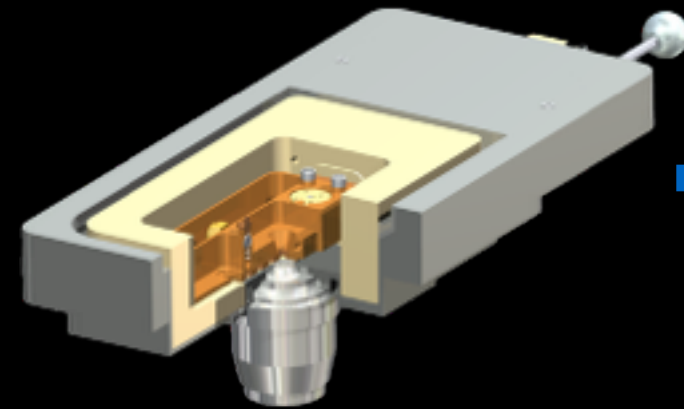
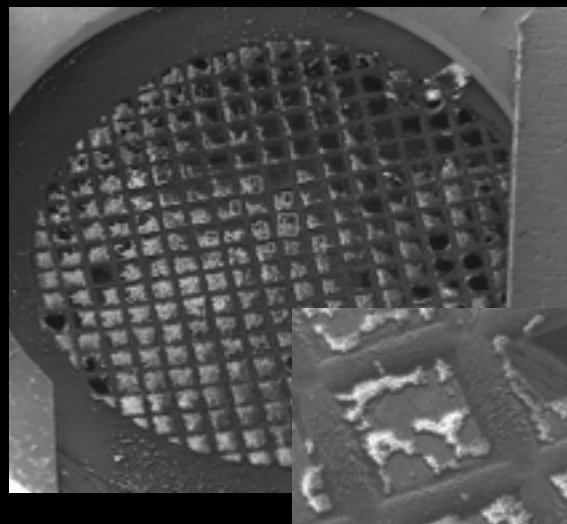
# Regions Accessible to CET



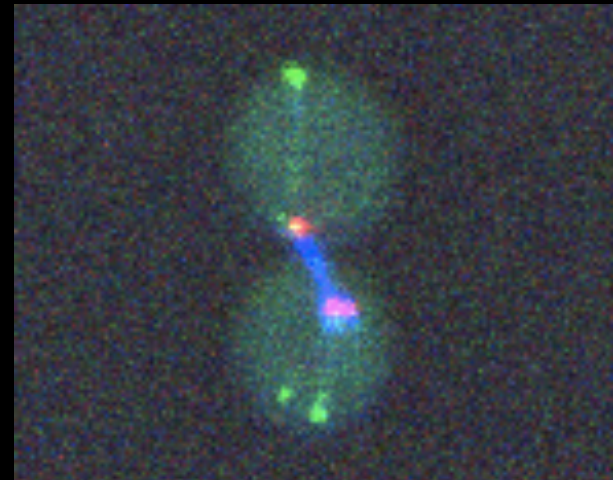
# Cryo-electron Tomography Workflow



Vitrification



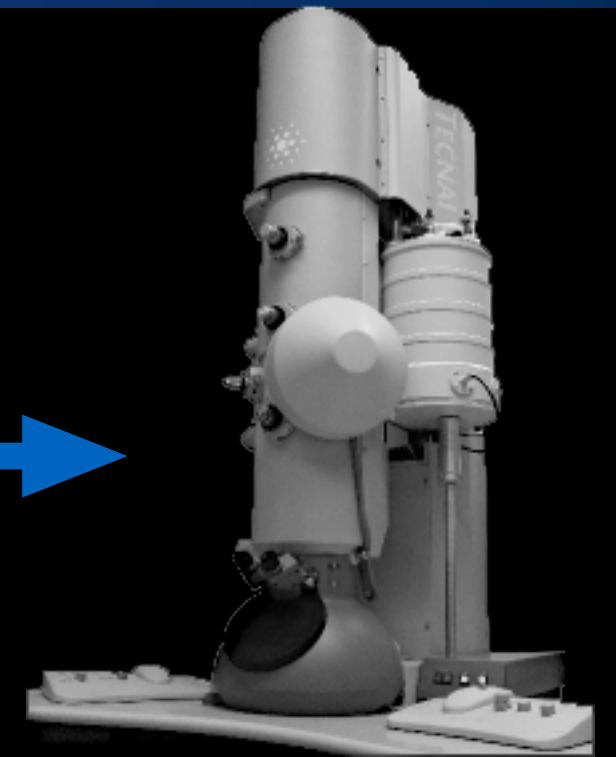
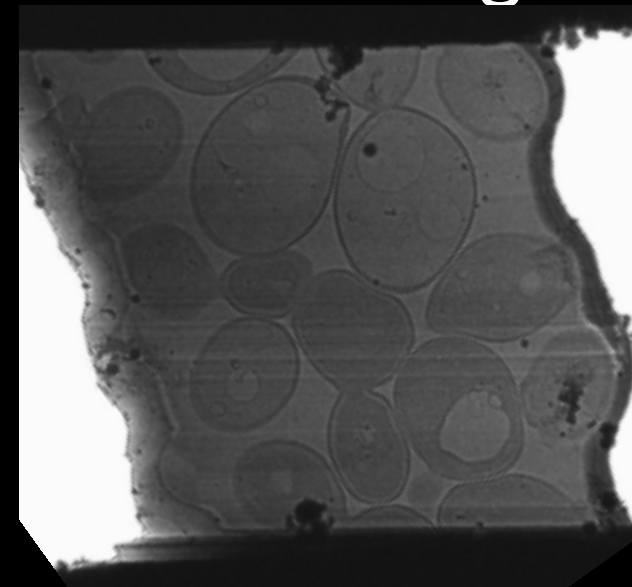
Light Microscopy



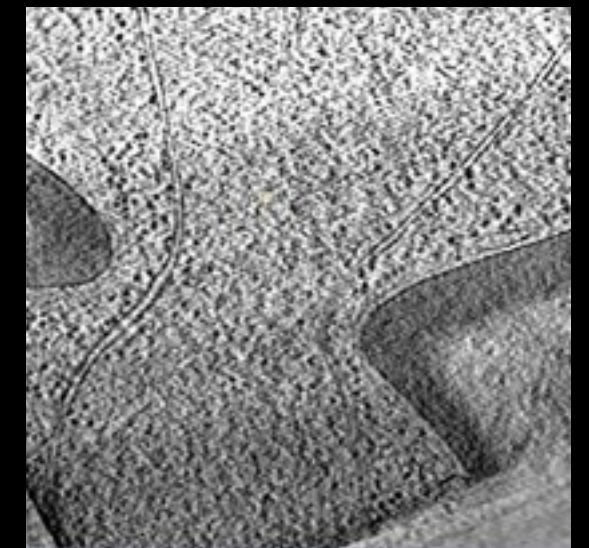
Strain from Reck-Peterson Lab (HMS)

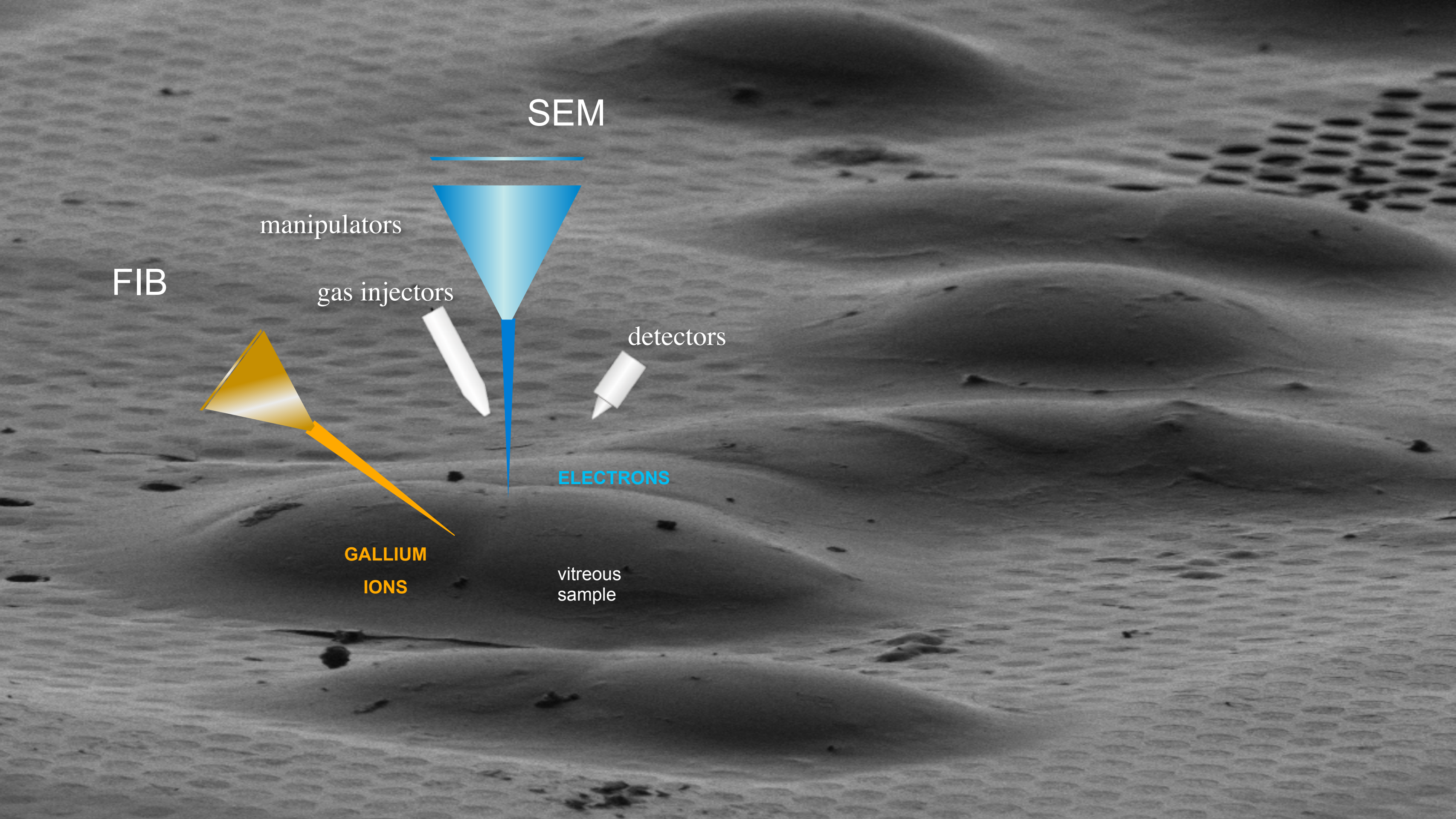


FIB milling



Tomography





SEM

manipulators

FIB

gas injectors

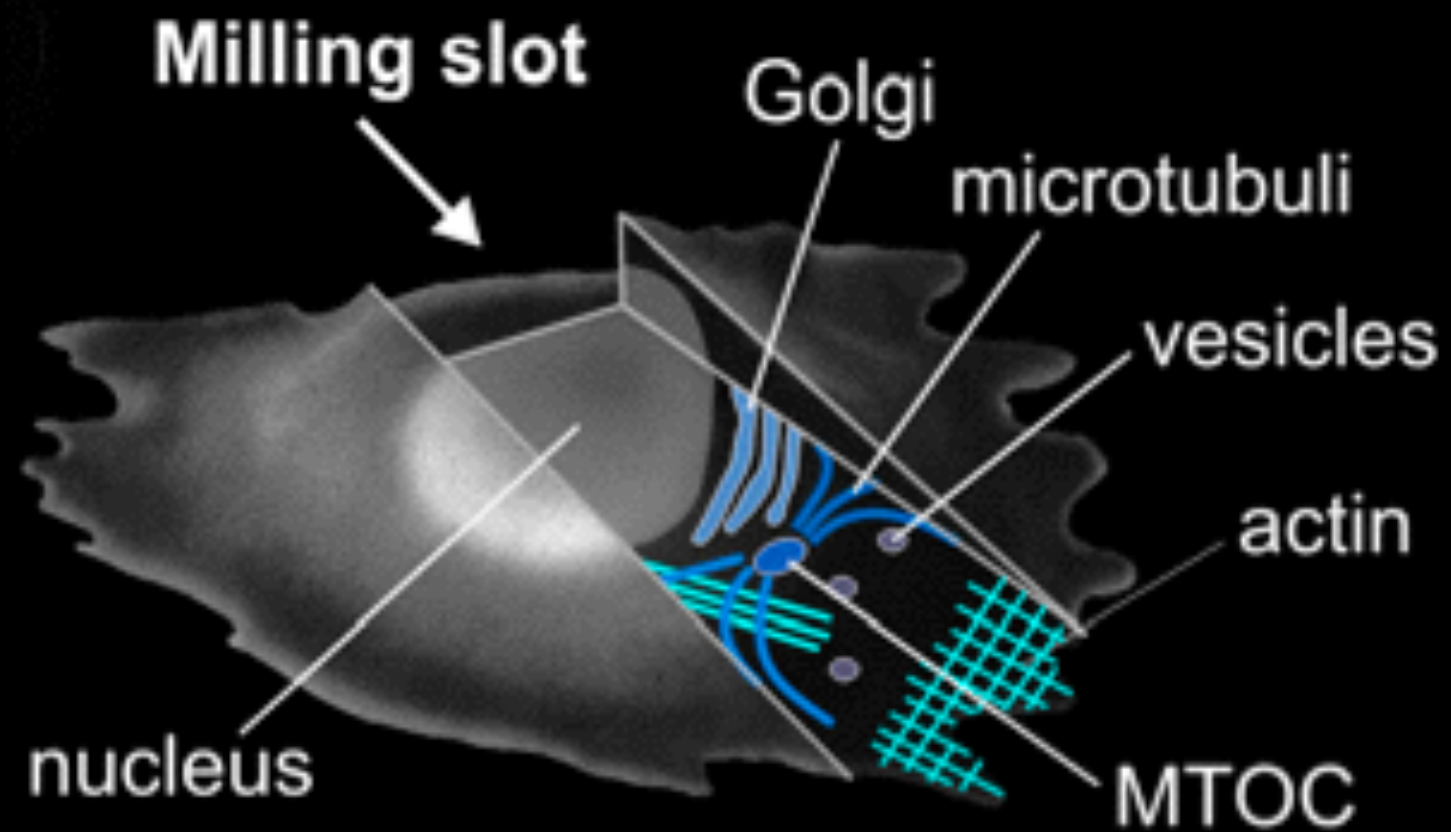
detectors

ELECTRONS

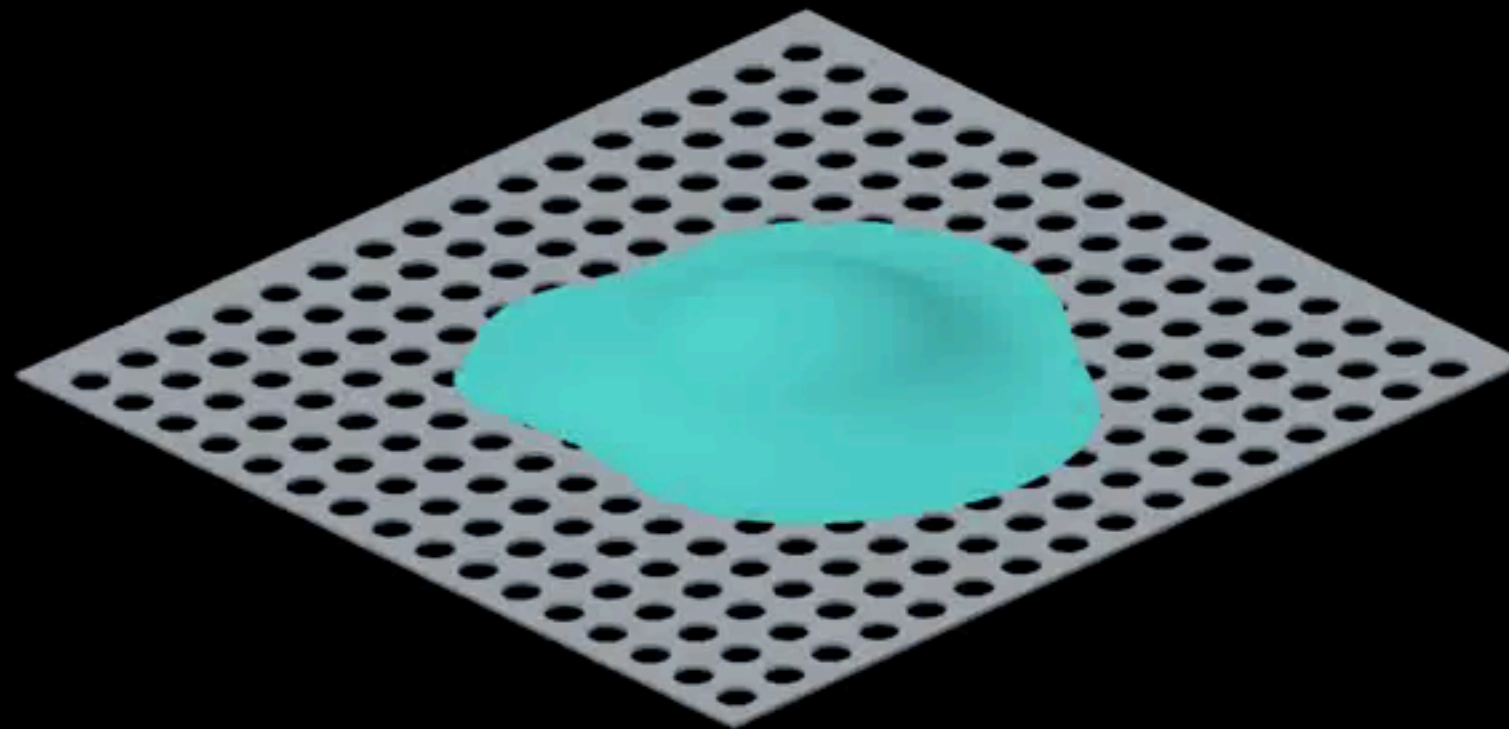
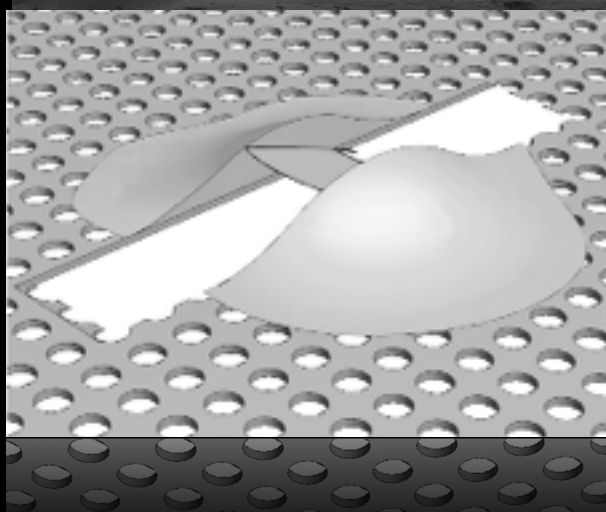
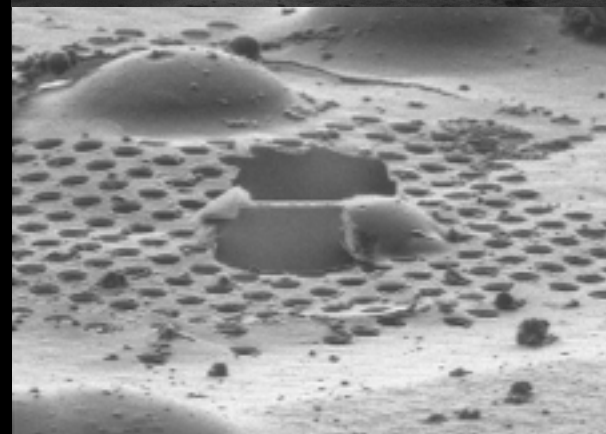
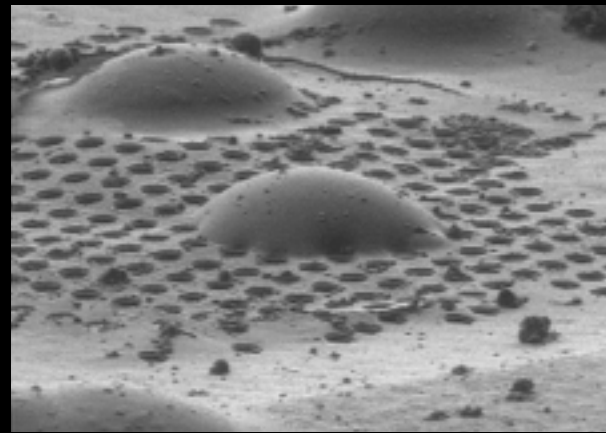
GALLIUM  
IONS

vitreous  
sample

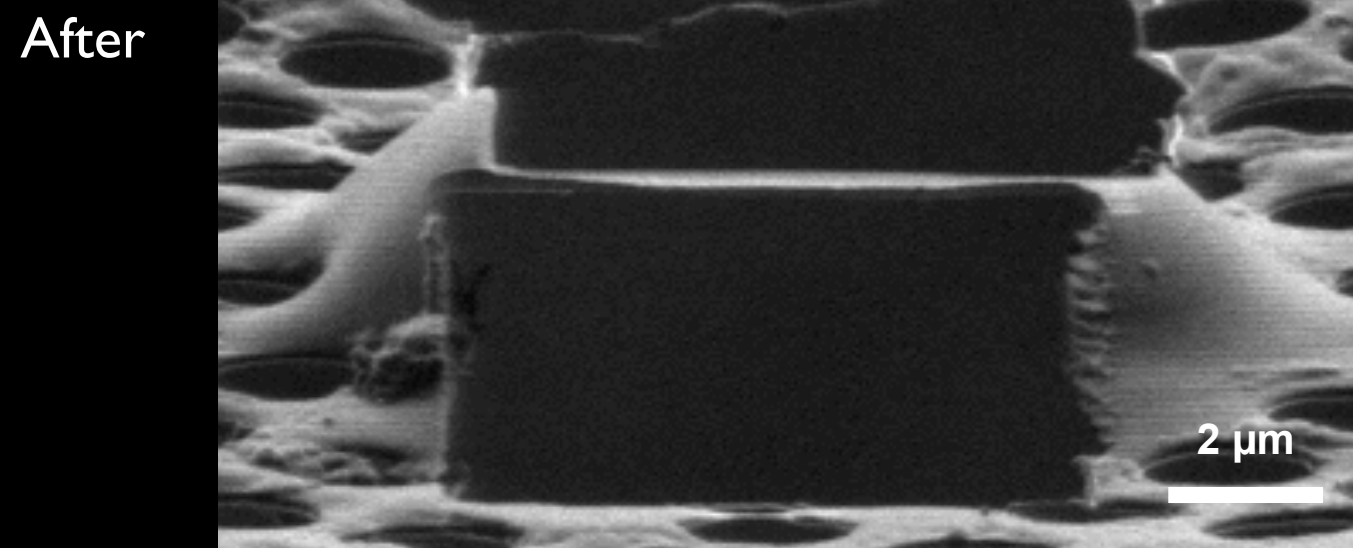
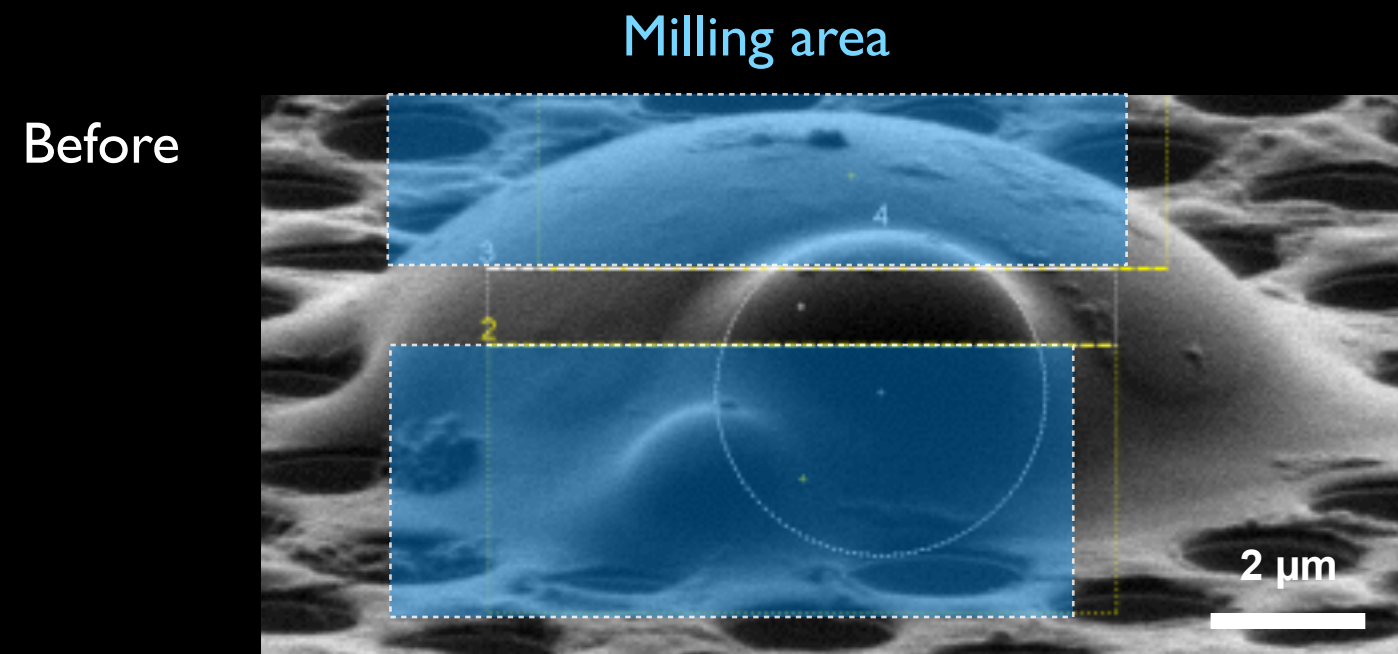
# Opening Windows into the Cell



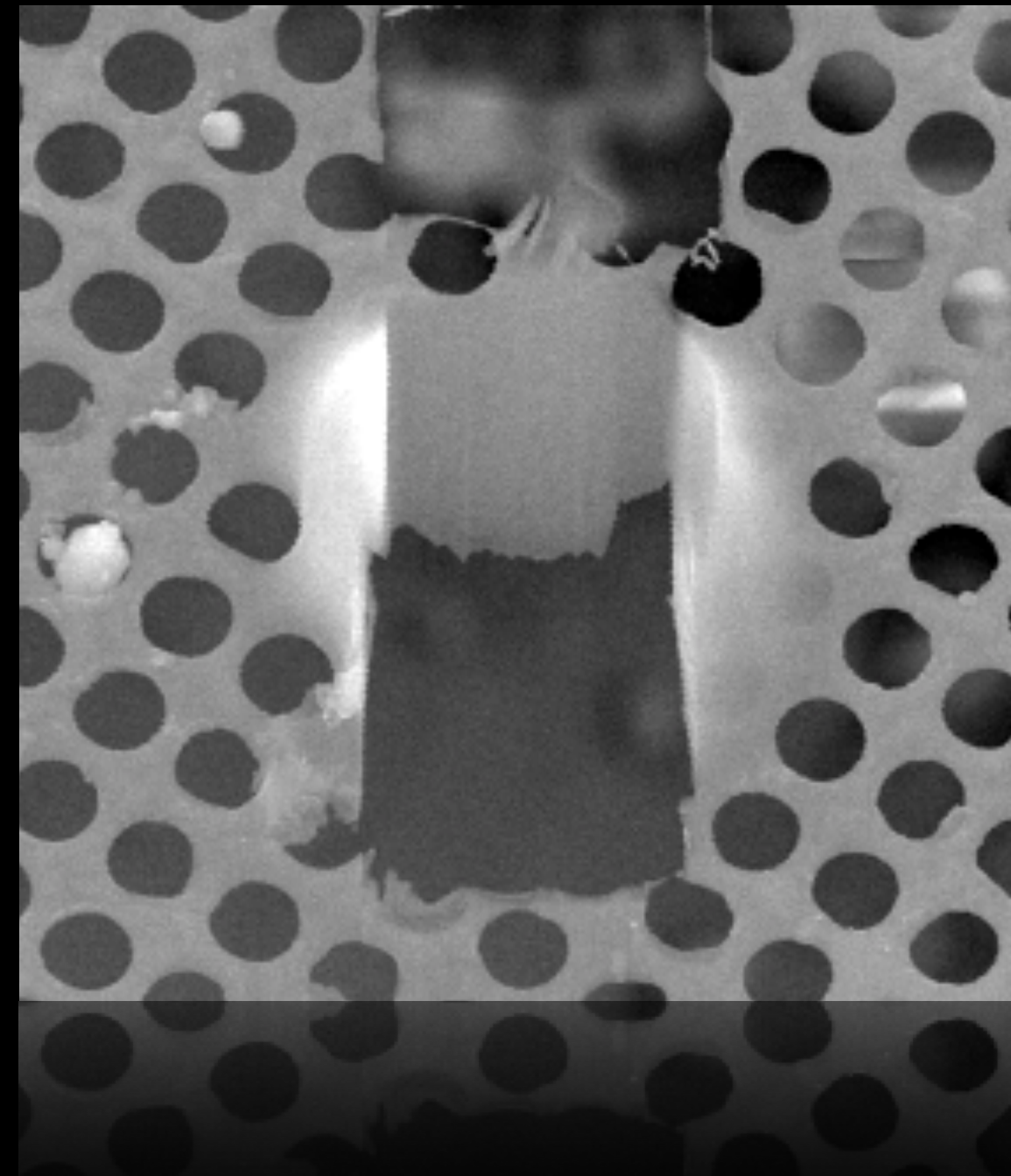
# Opening Windows into the Cell



# Opening Windows into the Cell

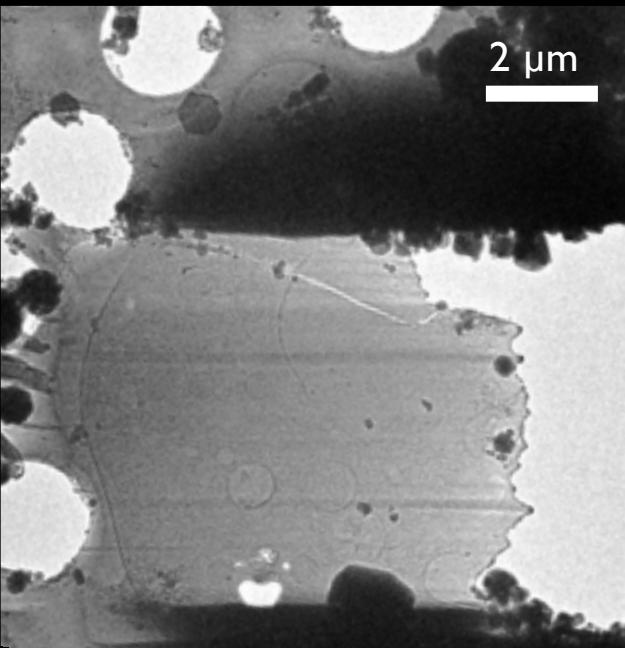
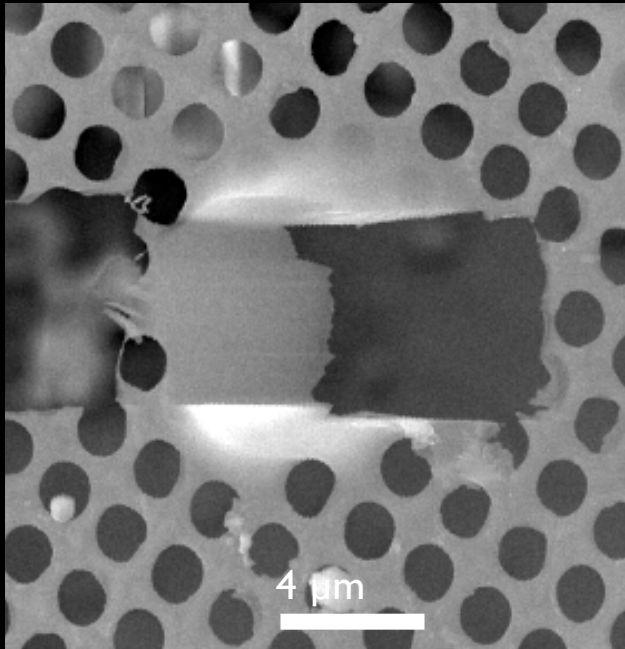


Thinned region: Cryo-SEM (top view)

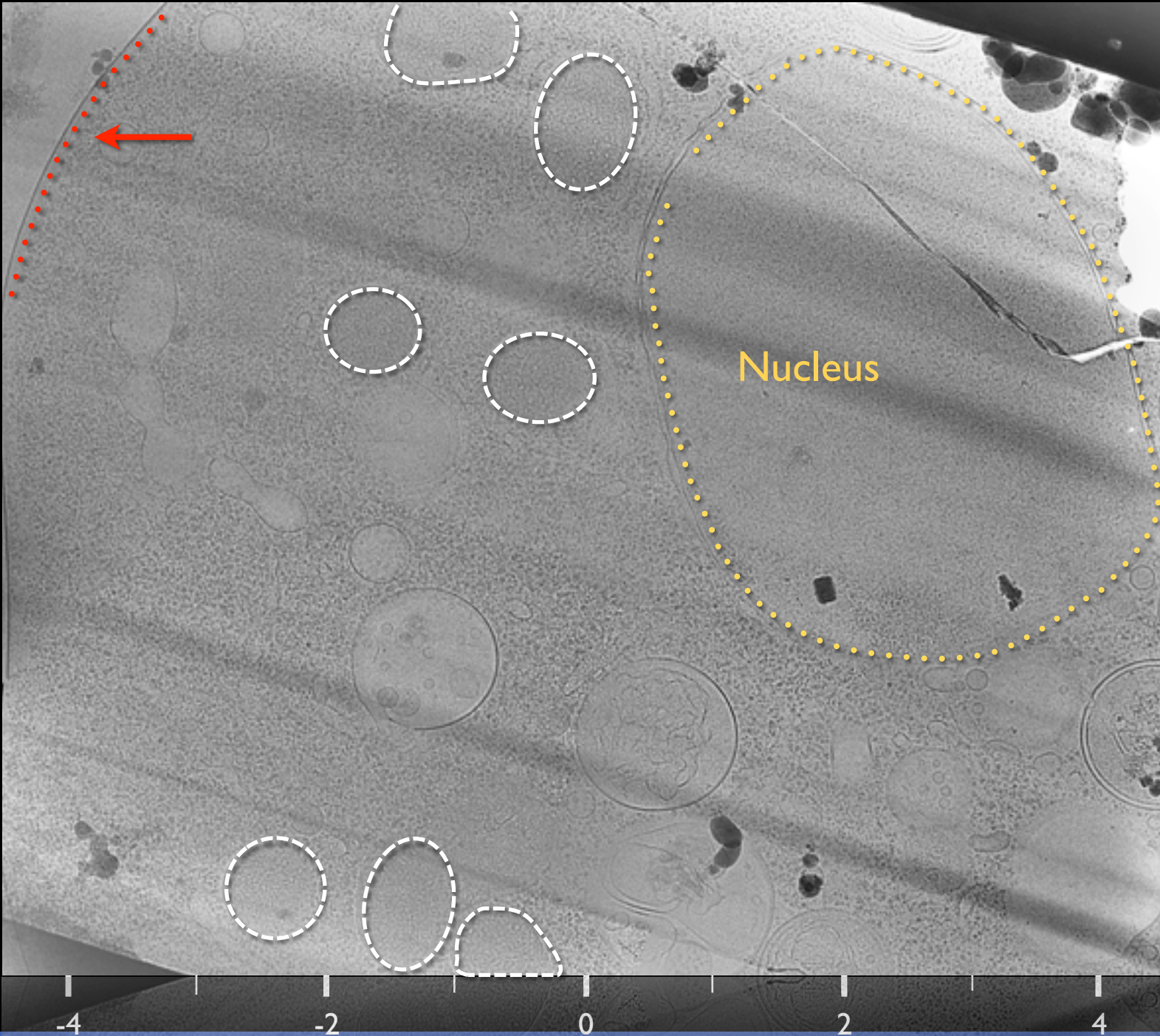


# FIB: Lamella Preparation

cryo-SEM

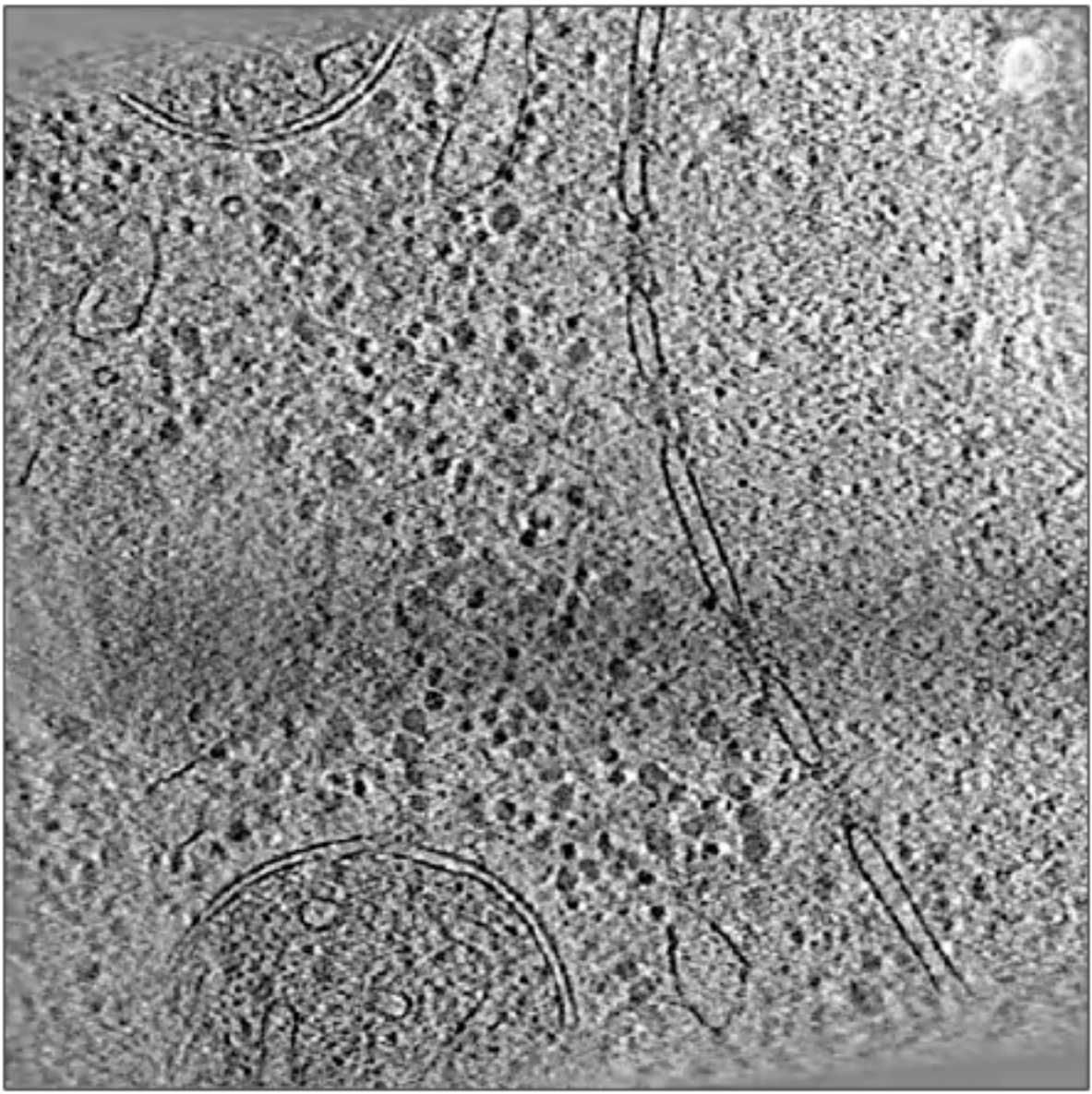
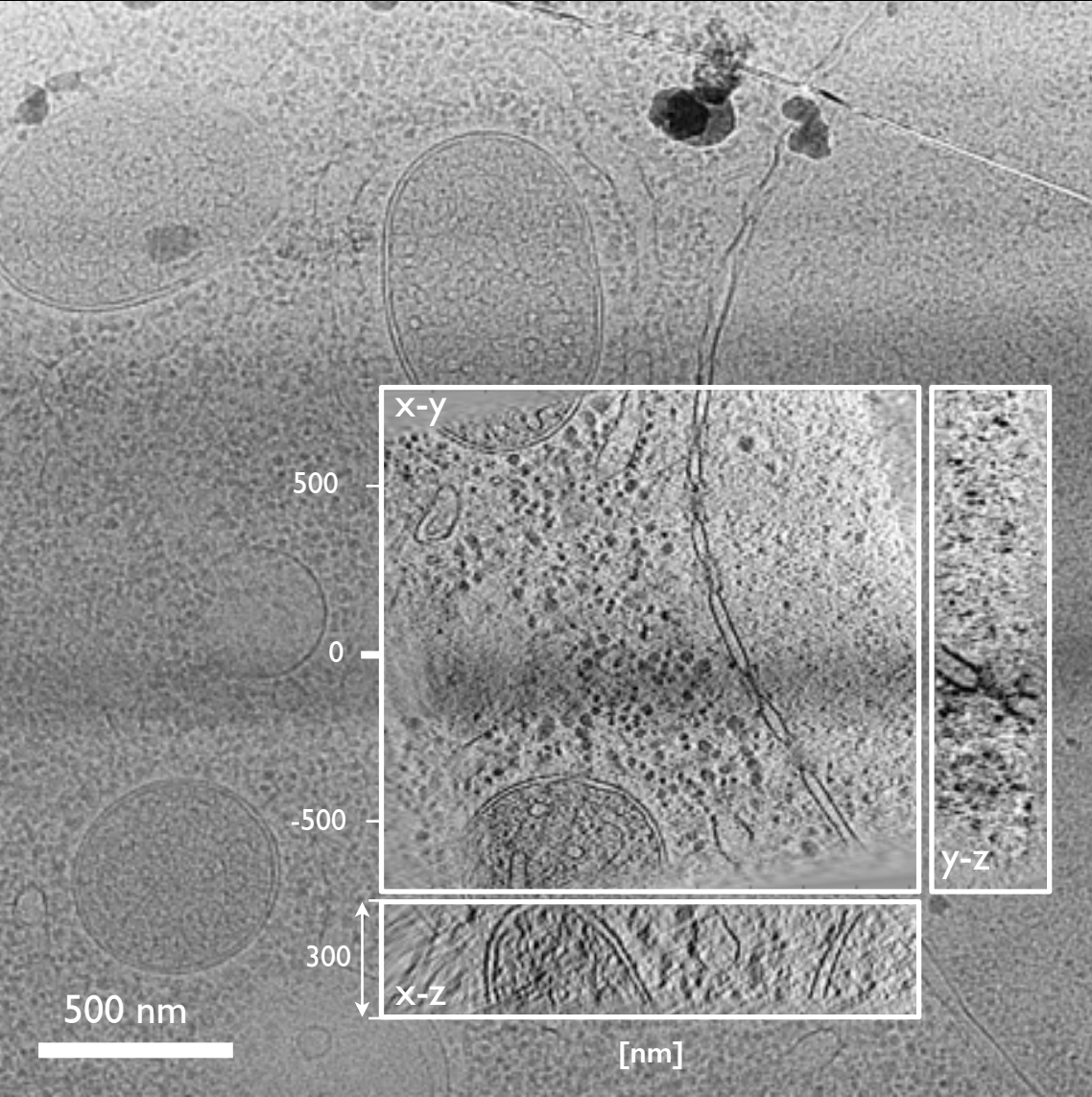


cryo-TEM

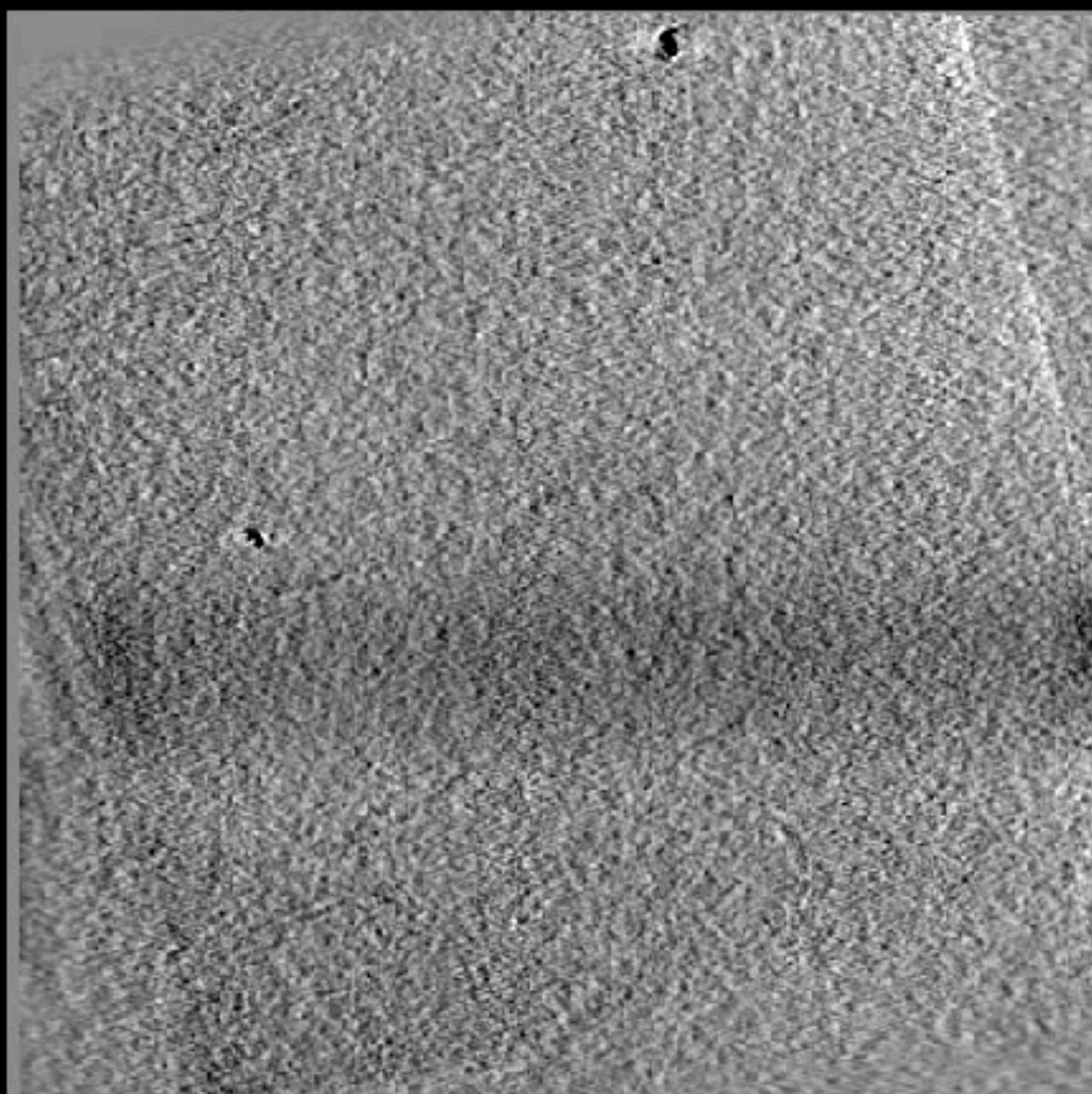


# FIB: Lamella Preparation

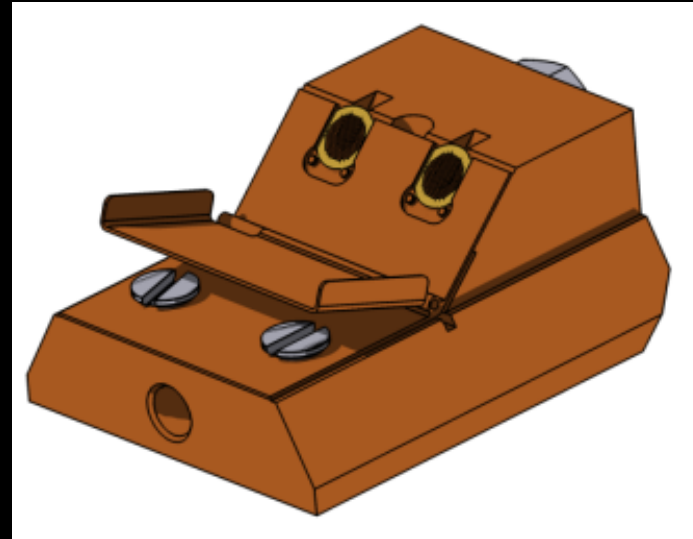
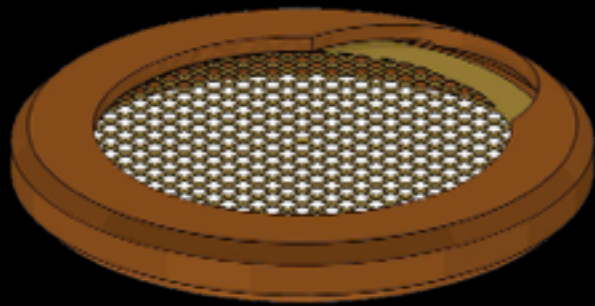
TEM 2D projection (high-pass filtered)



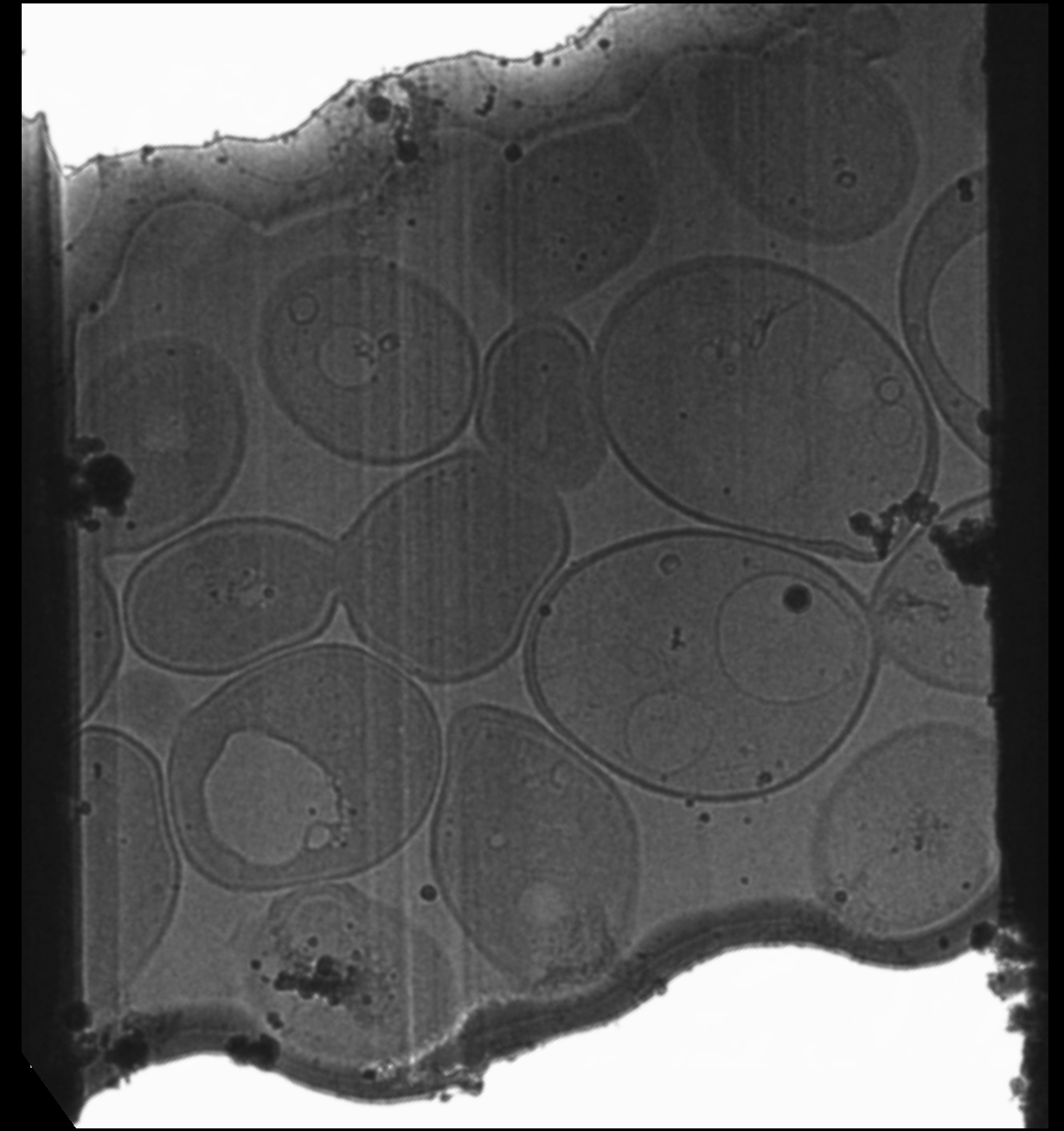




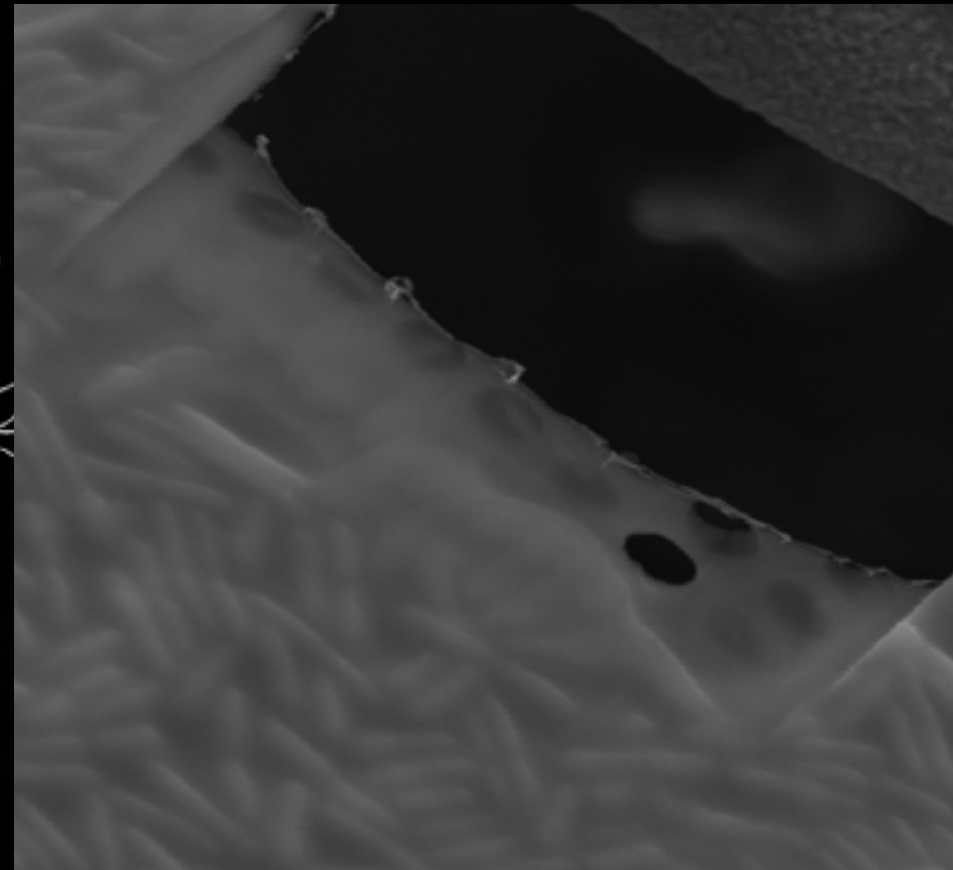
# Practical Issues: Typical cryo-FIB milling session



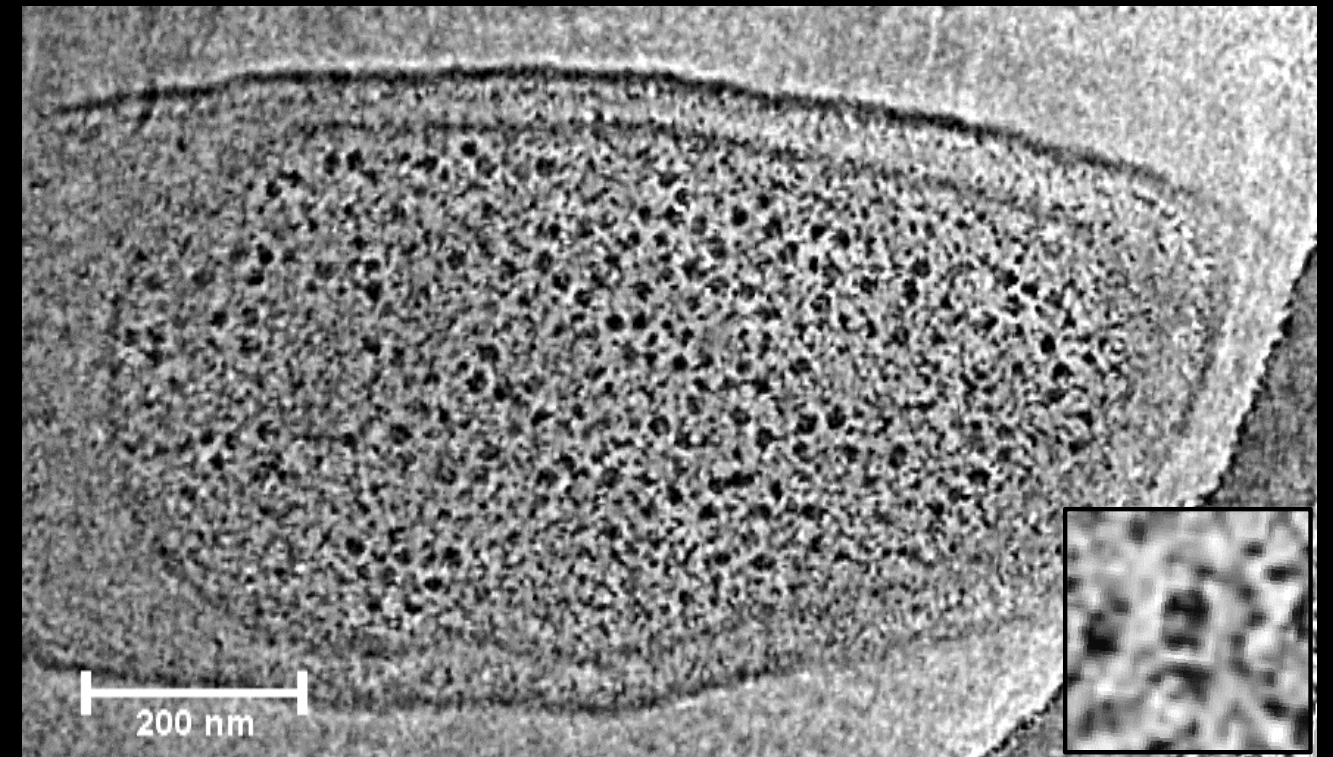
- Load 2 grids clipped in modified AutoGrids
- Platinum coating on the EM grids using GIS
- 5-10 lamella in a 3-6 h session
- Lamellae are made at varying currents
- Area:  $\sim 100 \mu\text{m}^2$
- Thickness: 80-350 nm
- 1-4 tomograms per lamella



# Bacterial Cells - Wedges

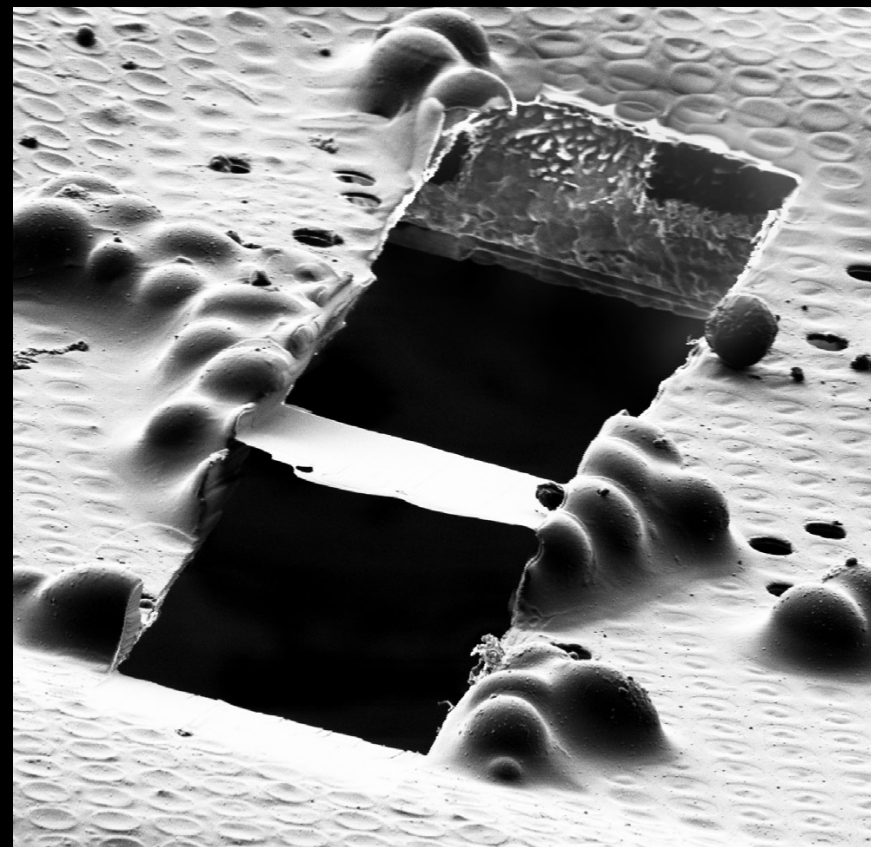
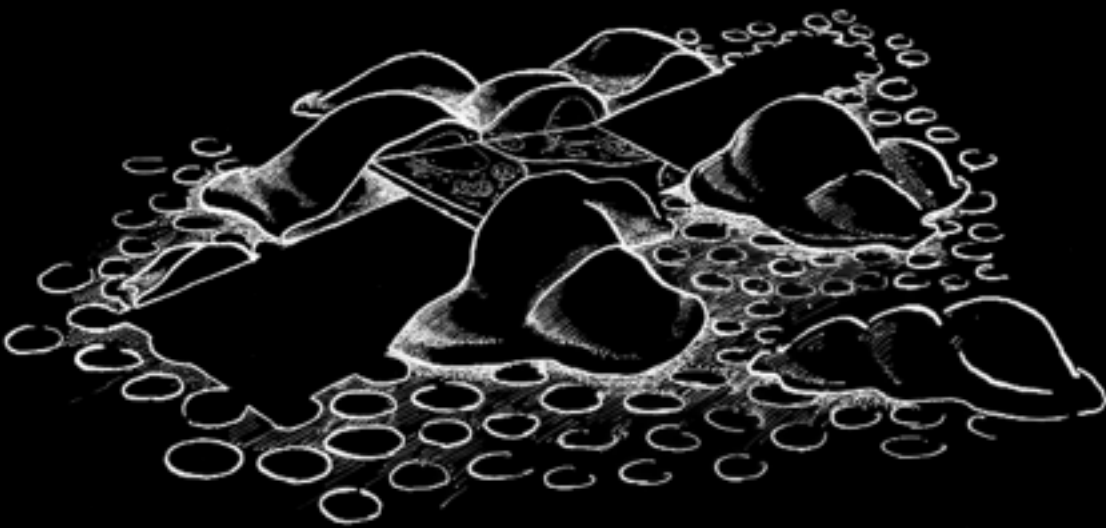


*E. coli*



Thomas Hoffman  
~150 nm  
Dual Tilt, K2, GIF, Titan 2

# Eukaryotic Cells - Lamella

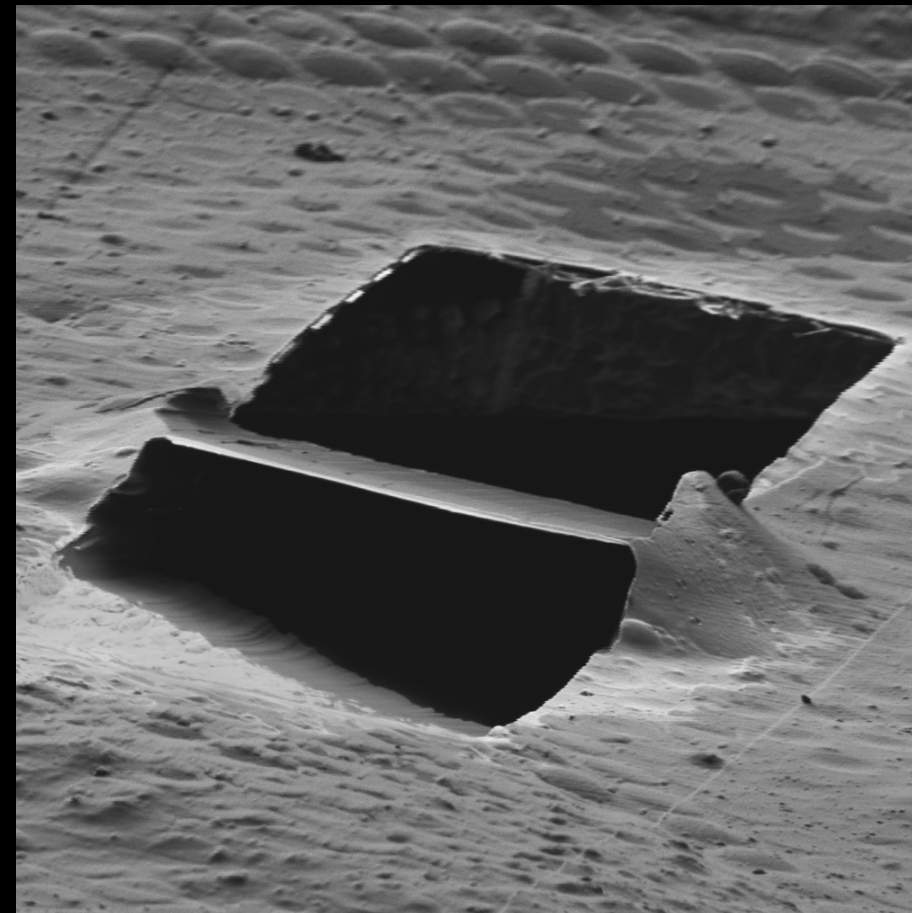
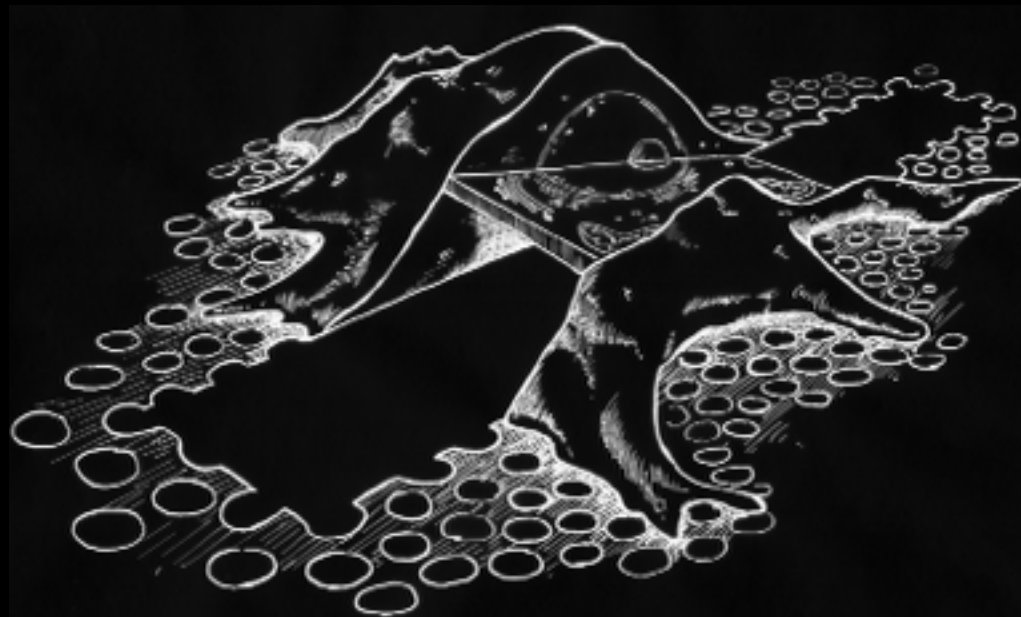


*S. cerevisiae*

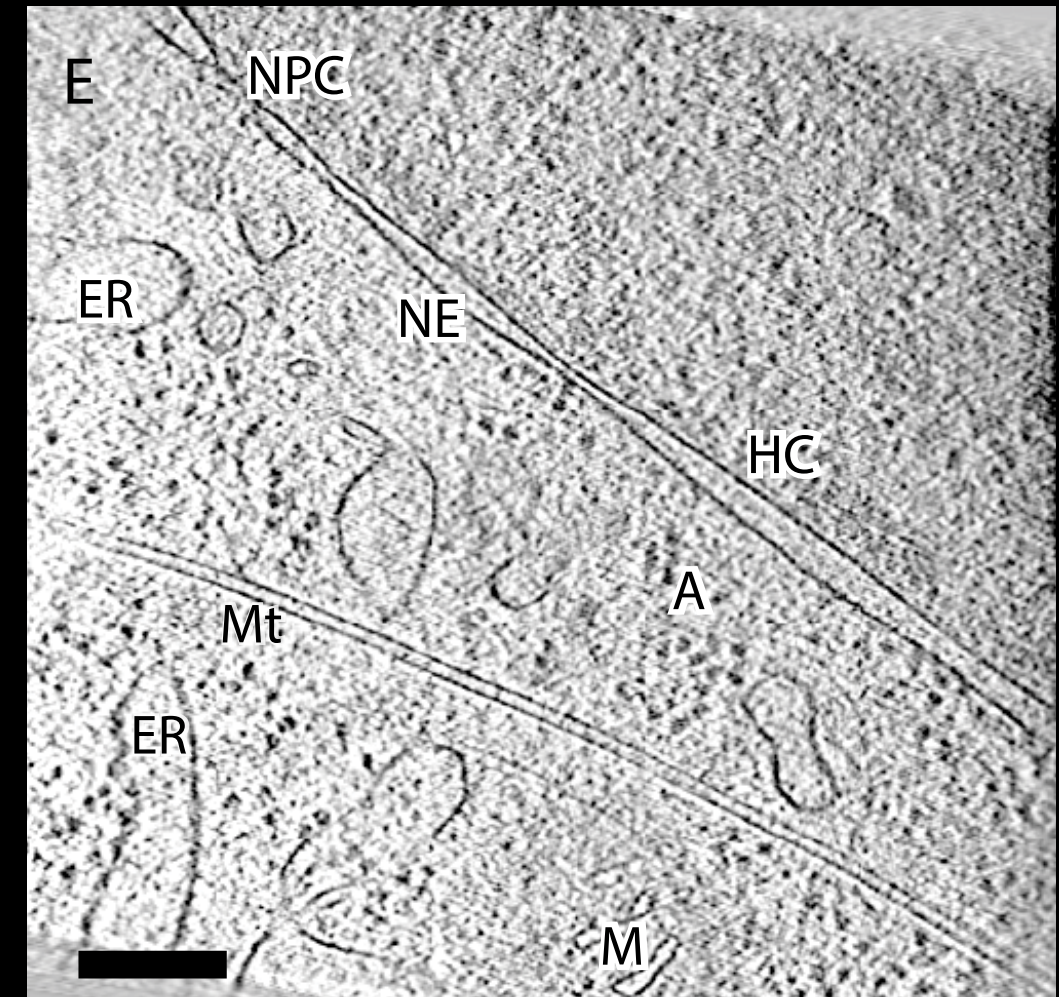


~300 nm  
K2, GIF, Titan 2

# Eukaryotic Cells - Lamella

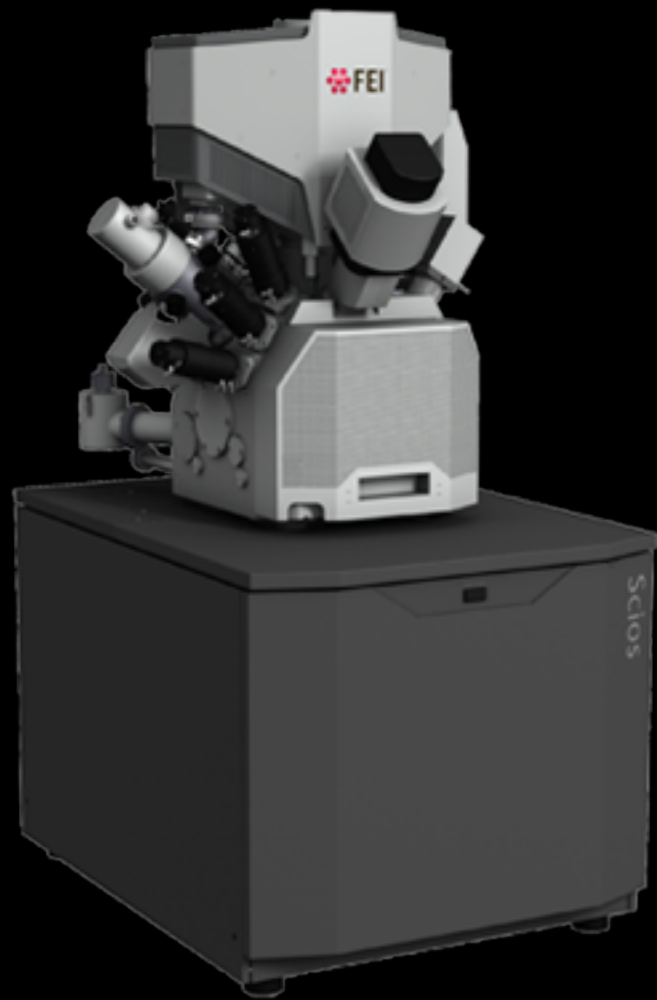


*HeLa*



~350 nm  
CCD GIF, Polara

# Cryo-EM set up at UCSD

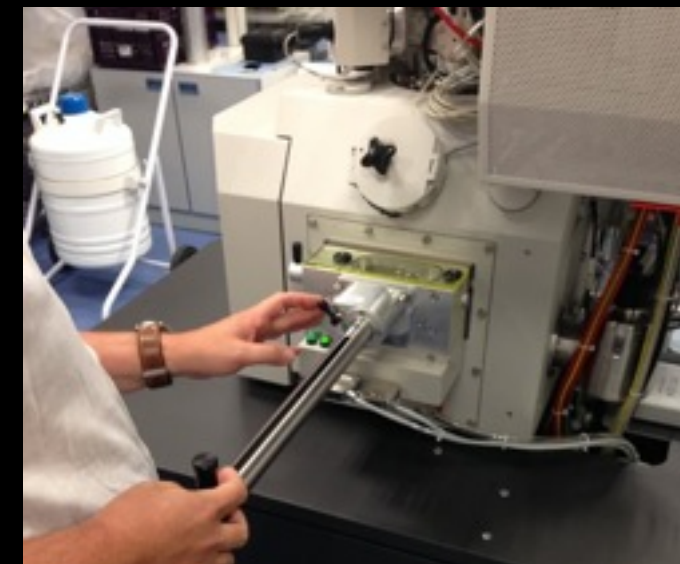
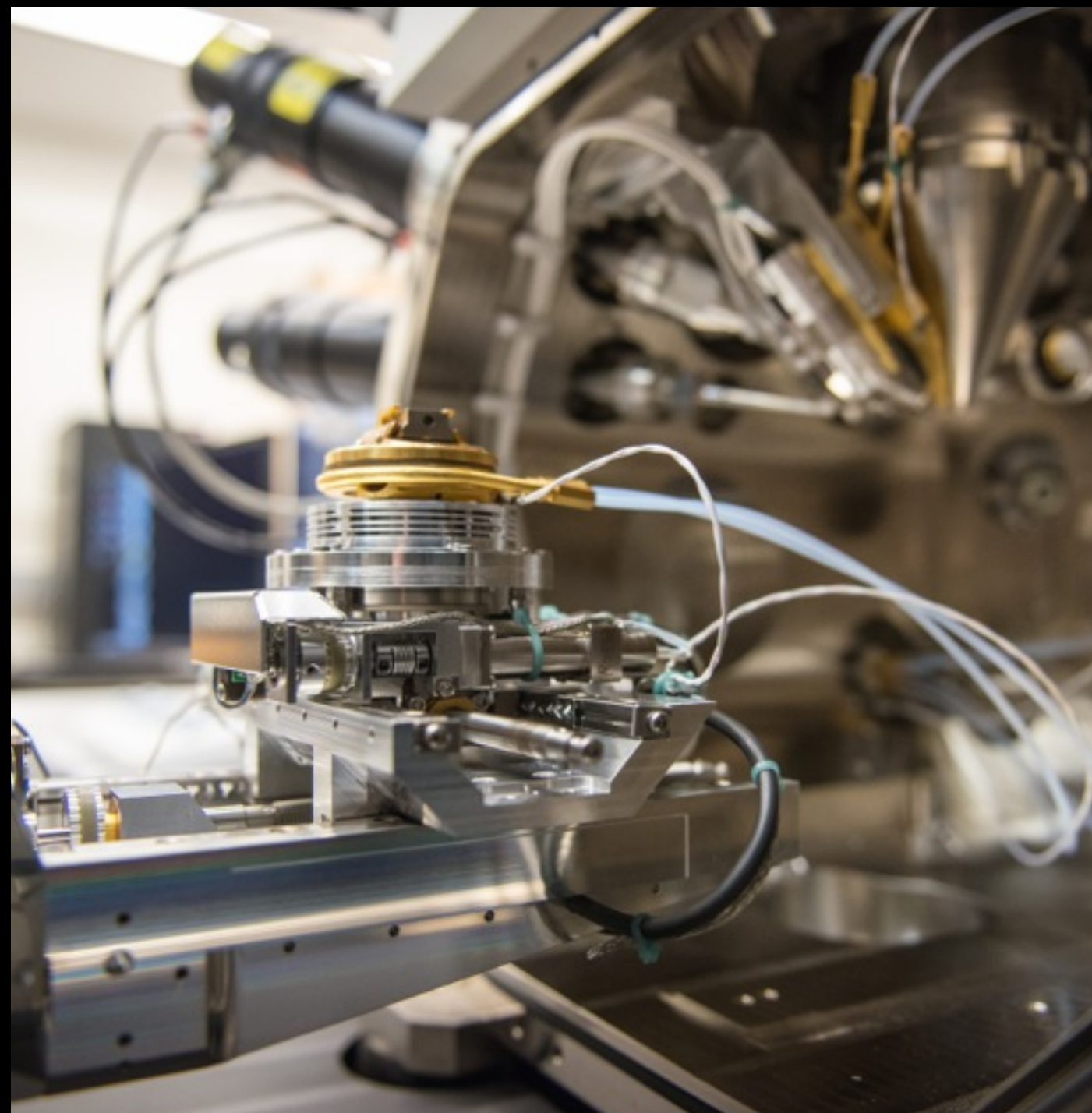
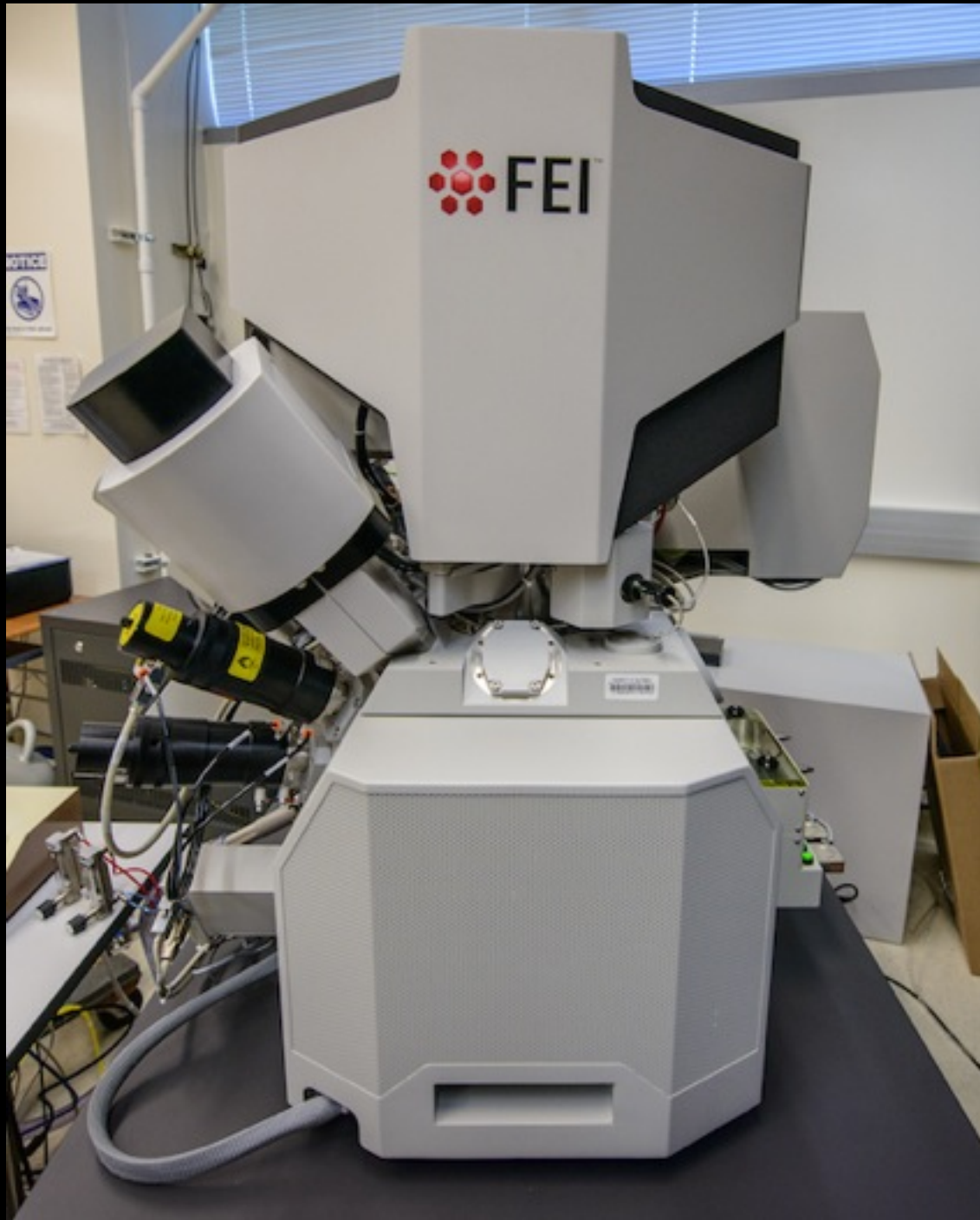


FEI Scios Dual Beam  
Prototype for cryo-sample preparation  
Collaboration with FEI

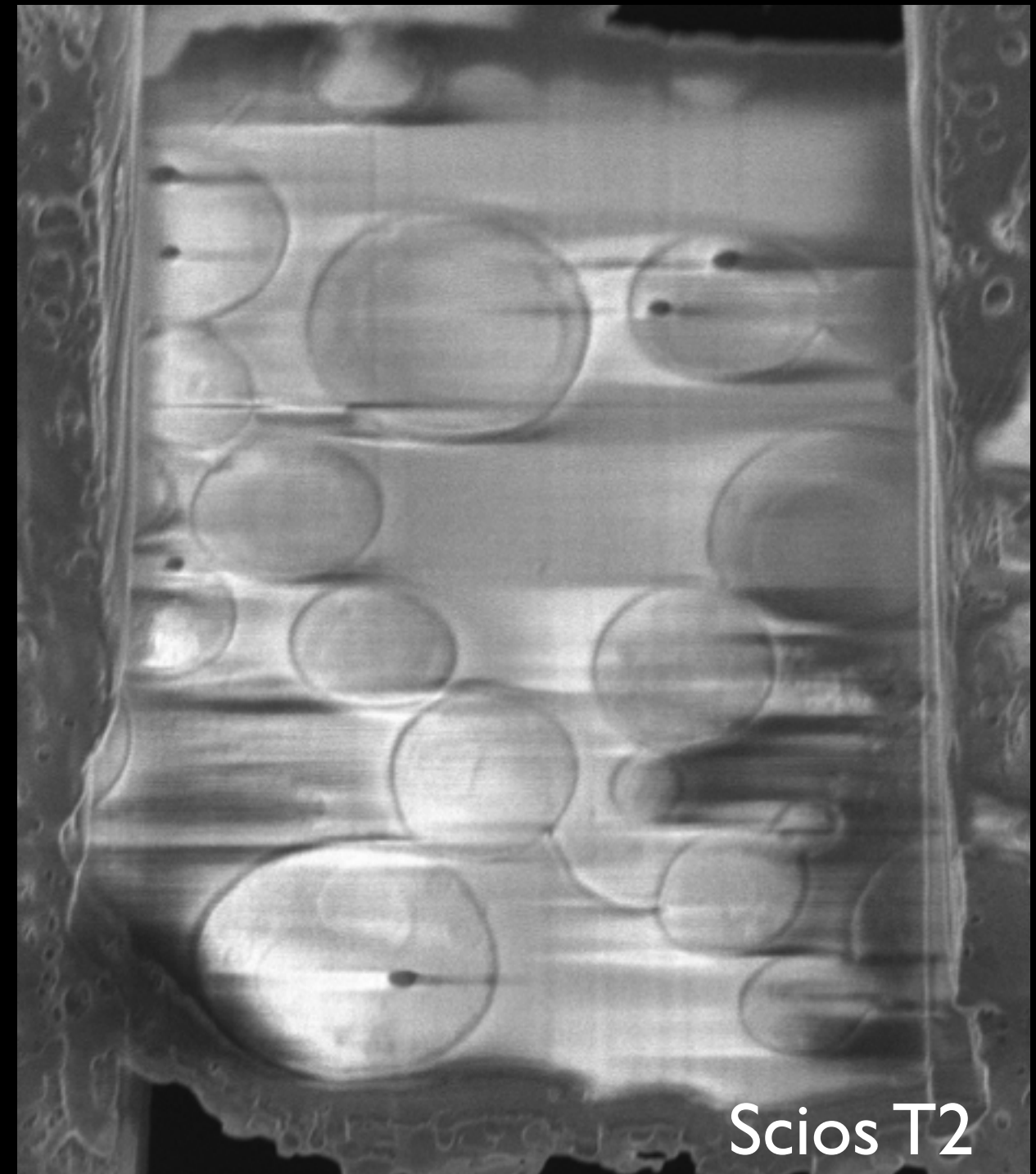
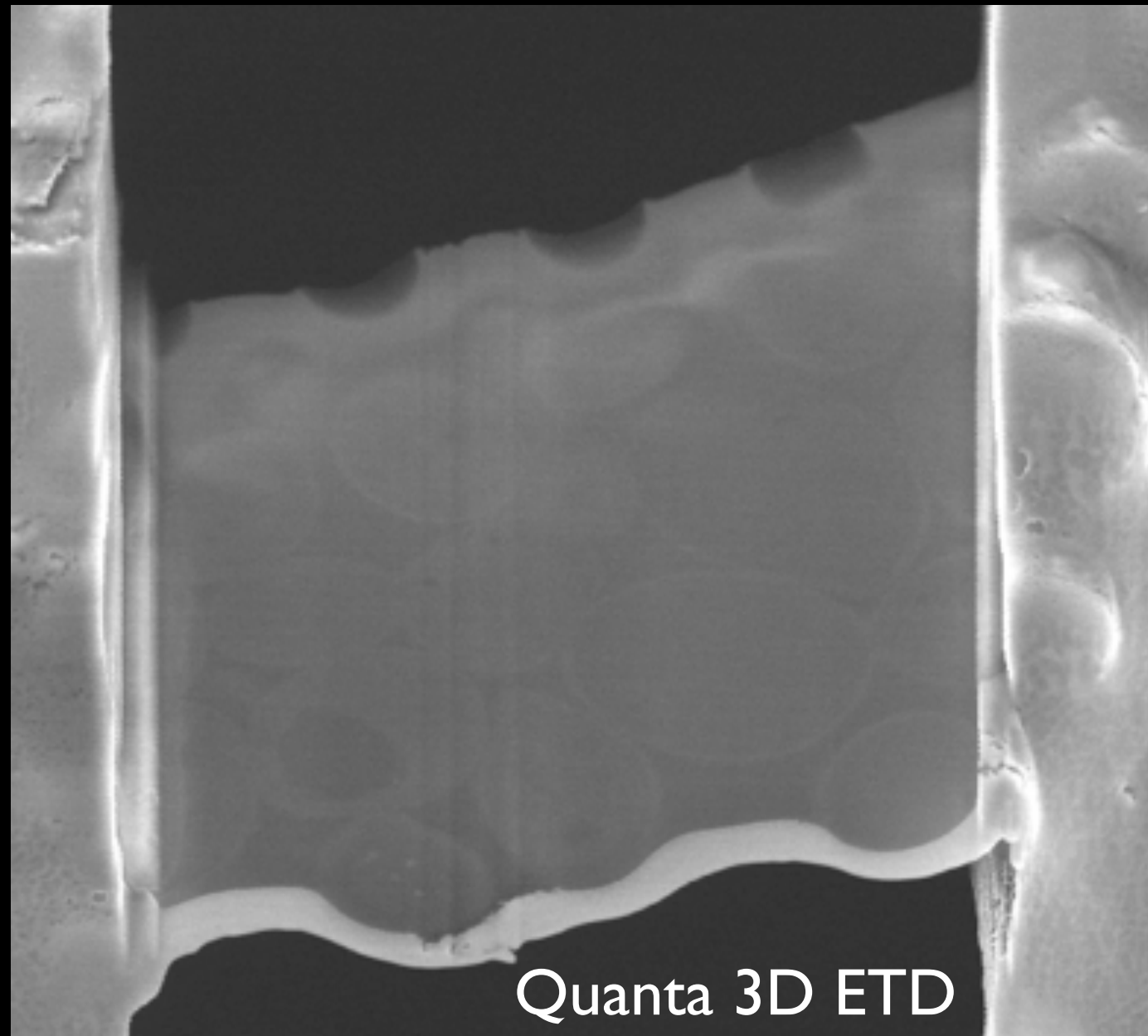


FEI Polara @ 300 keV  
Gatan Quantum energy filter  
Gatan K2 summit detector

# Installation of cryo-Scios at UCSD (July 2014)

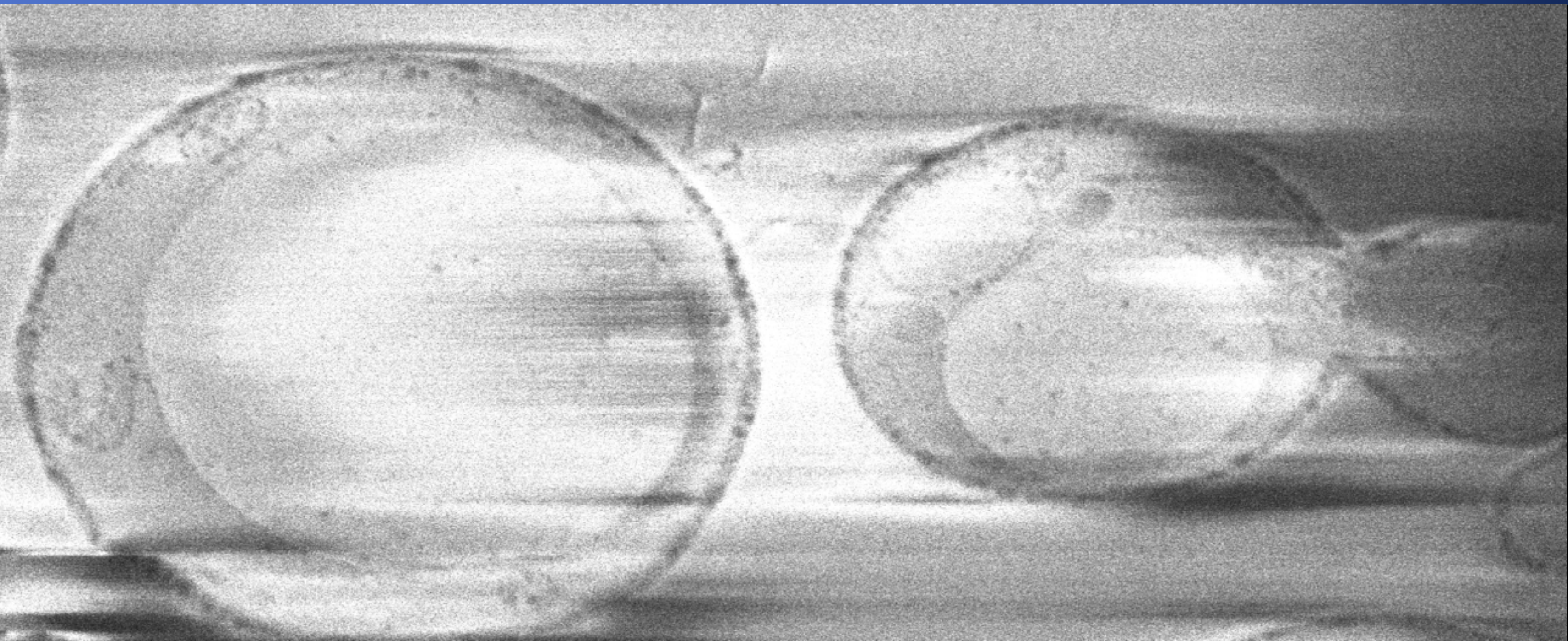


# Scios @ UCSD

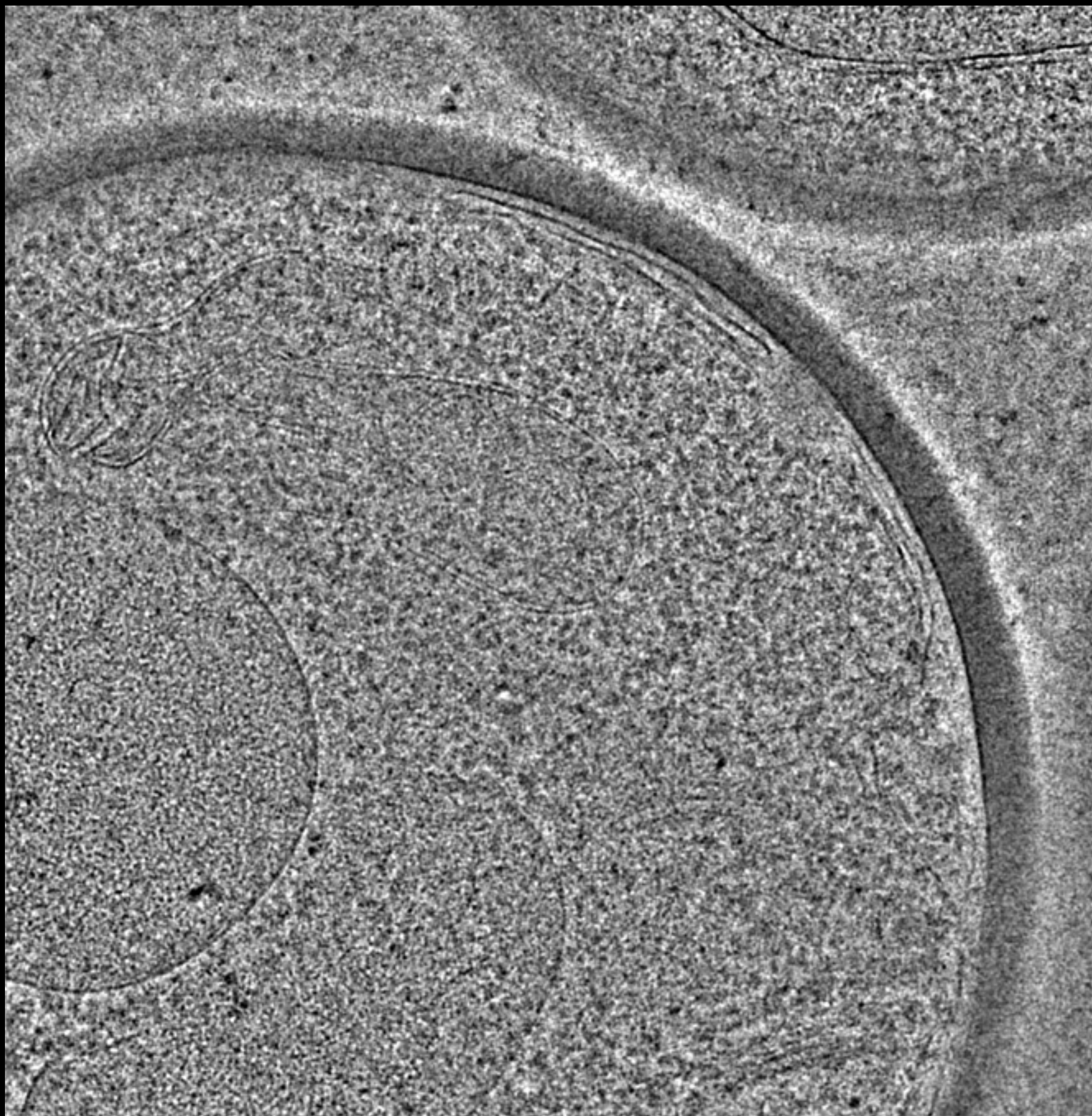




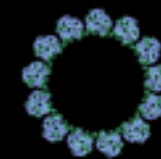
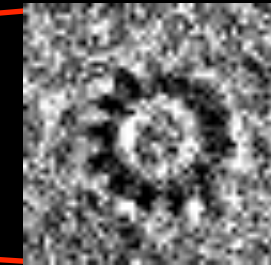
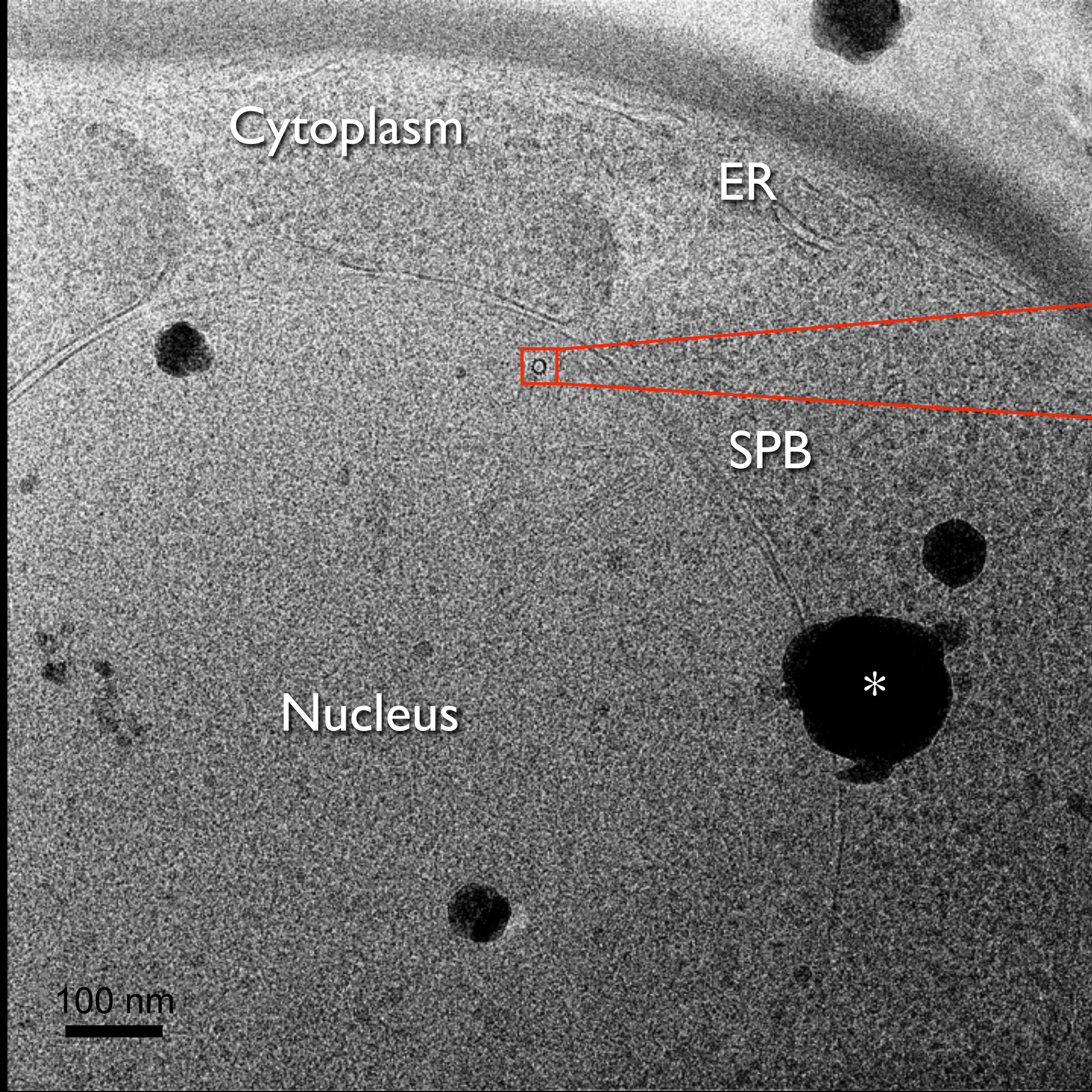
# cryo-contrast in SEM!



Scios, 2 keV, 25 pA, T2 (in-lens) detector



Polara @ 300 keV  
Gatan K2 summit detector  
Gatan Quantum energy filter  
Dose = 1.5 e/A<sup>2</sup>  
Defocus = 4.5  $\mu\text{m}$   
~300-nm FIB yeast lamella



FEI Titan Krios @ 300 keV  
Gatan K2 summit detector  
Gatan Quantum energy filter  
Dose = 1 e/A<sup>2</sup>  
Defocus = 5  $\mu$ m  
~300-nm FIB yeast lamella

# Future of sample prep with FIB milling

Is it ready for prime time?

Not yet out-of-the-box

Handful of labs painstakingly implementing/reinventing

*Product prototype very promising*

How much skill is required?

When it works, it's easy (and boring!)

With more robust tool, automation should be possible to a large extent

Is every lab going to be able to do it?

Yes!

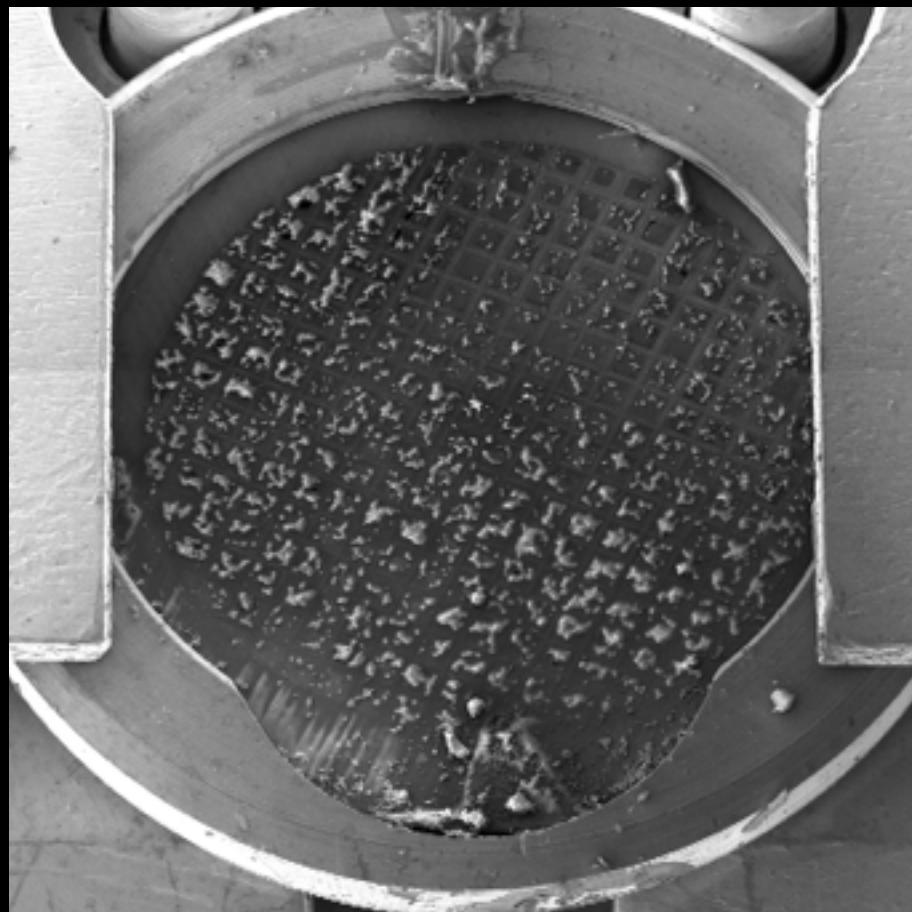
Right. But, how do we avoid paying for another service contract?

Multi-use tool

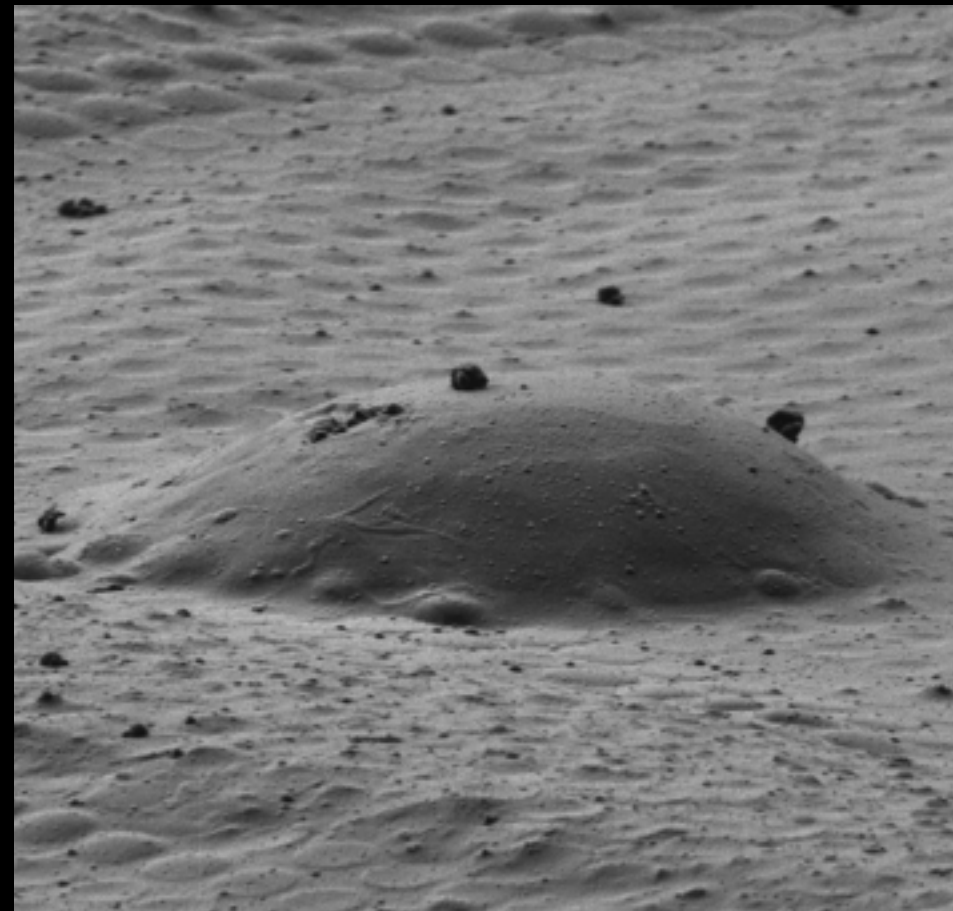
# Future of sample prep with FIB milling

One must:      Locate the areas of interest  
                         Make them accessible to TEM

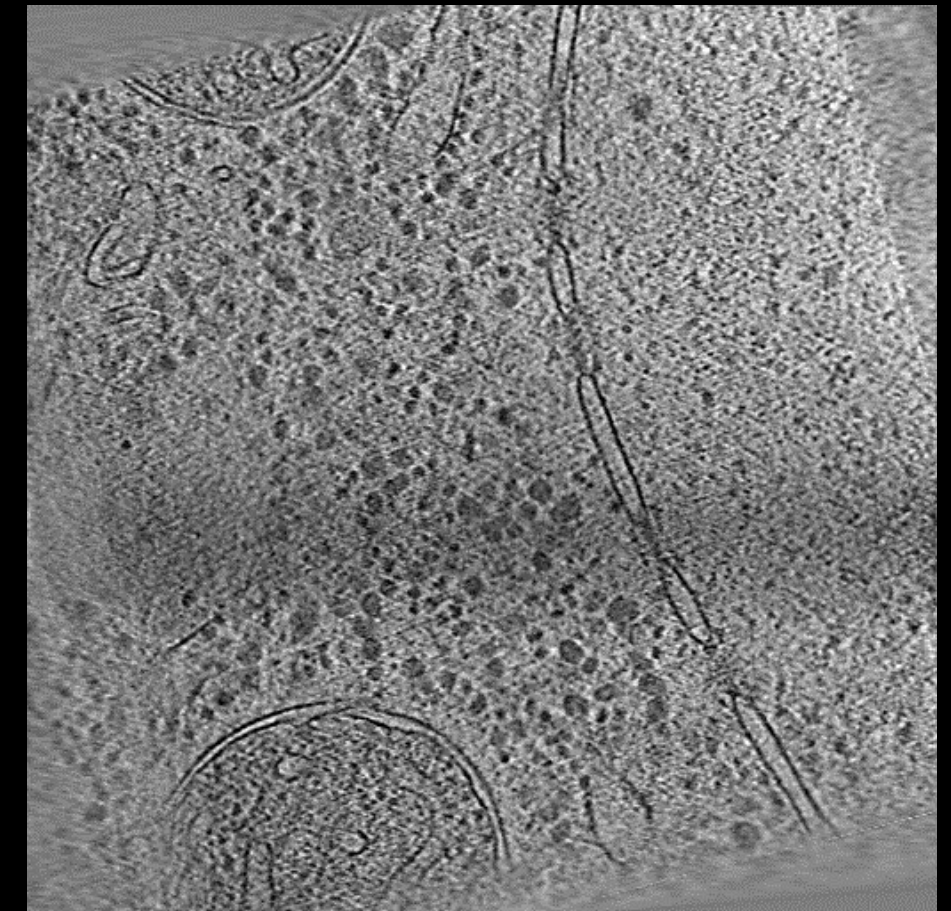
A tomogram only covers 0.1 - 0.3 % of the cell volume!



Grid: 3 mm



Cell:  $\sim 300 \text{ um}^3$



Tomogram:  $\sim 0.4 \text{ um}^3$

# Challenges ahead

Establish and ensure acceptable standards (yield, contamination rates, etc)

Serviceable multi-use tool

Targeting

Charging

Software adaptation for tomography acquisition schemes

Alignment of tilt series (no fiducials)

Statistics

Will it be applicable to high-resolution?

Tissue (readily adaptable for milling geometries!)

# Future of CET

*EM single-handedly launched the field of Cell Biology..*

The Promises:

High-resolution structural determination *in situ*

Visual Proteomics

Quantitative Cell Biology

Integrate with light microscopy:

CET Targets

Functional Assays / Molecular Identification (not only our targets!)

CET as a scaffolding assay in the lab integrated with other techniques

Single particle cryo-EM, X-ray crystallography, nanoSIMS, volume imaging, super-resolution LM, proteomics



**MPIB**

Wolfgang Baumeister  
Jürgen Pitzko

Julia Mahamid  
Miroslava Schaeffer  
Tim Laughs & Workshop



Peter Fruhstorfer  
Ruud Schampers  
Hans Peerson  
Wim Voorhout



Felix Wagner  
Reika Castillon  
James Bouwer