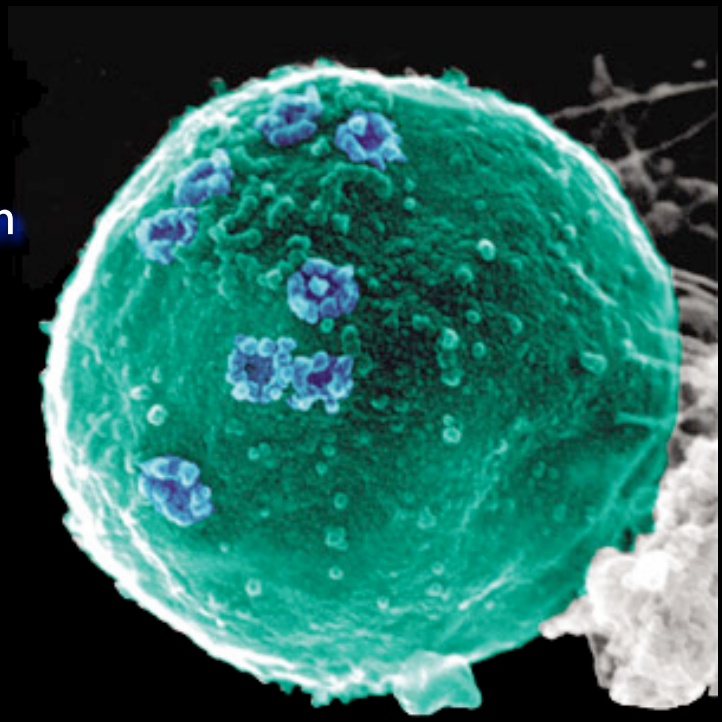
Opening Windows into the Cell

or:

How I Learned to Stop Worrying and Love the Beam



Elizabeth Villa

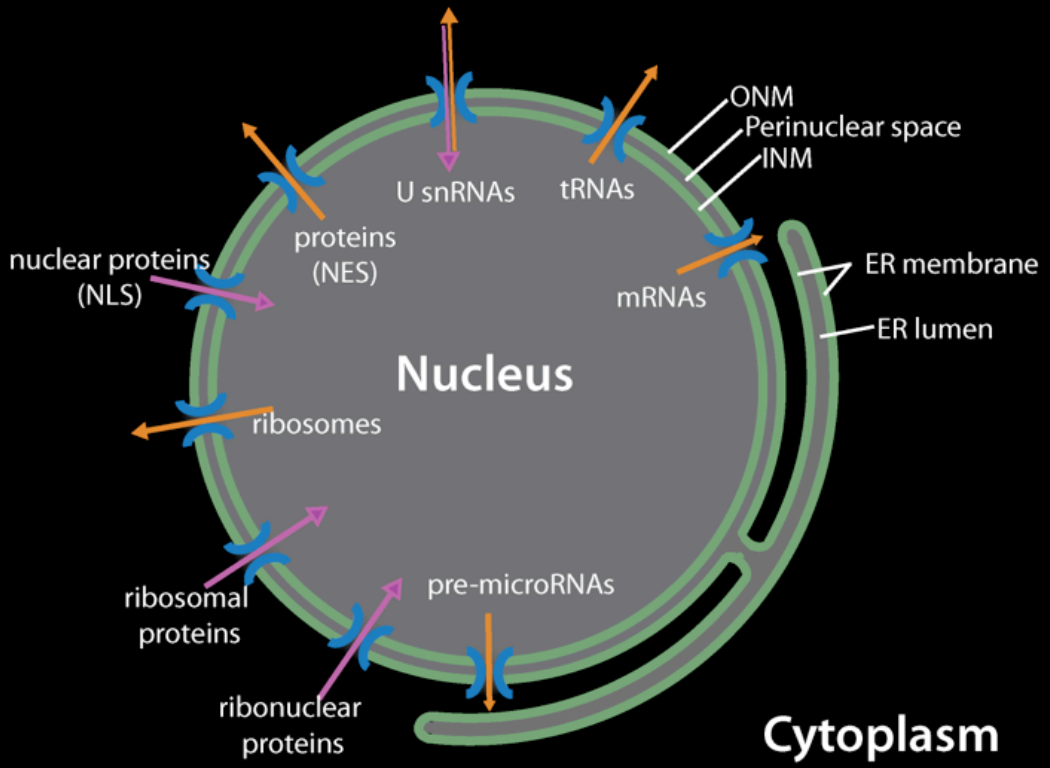
Department of Molecular Structural Biology

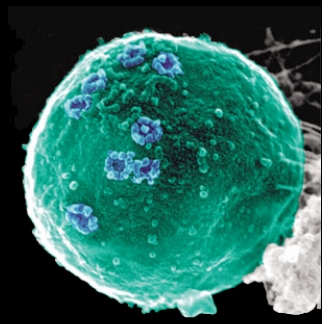
MPI Biochemistry, Martinsried

NRAMM Worksop on Advanced Topics in EM Structure Determination

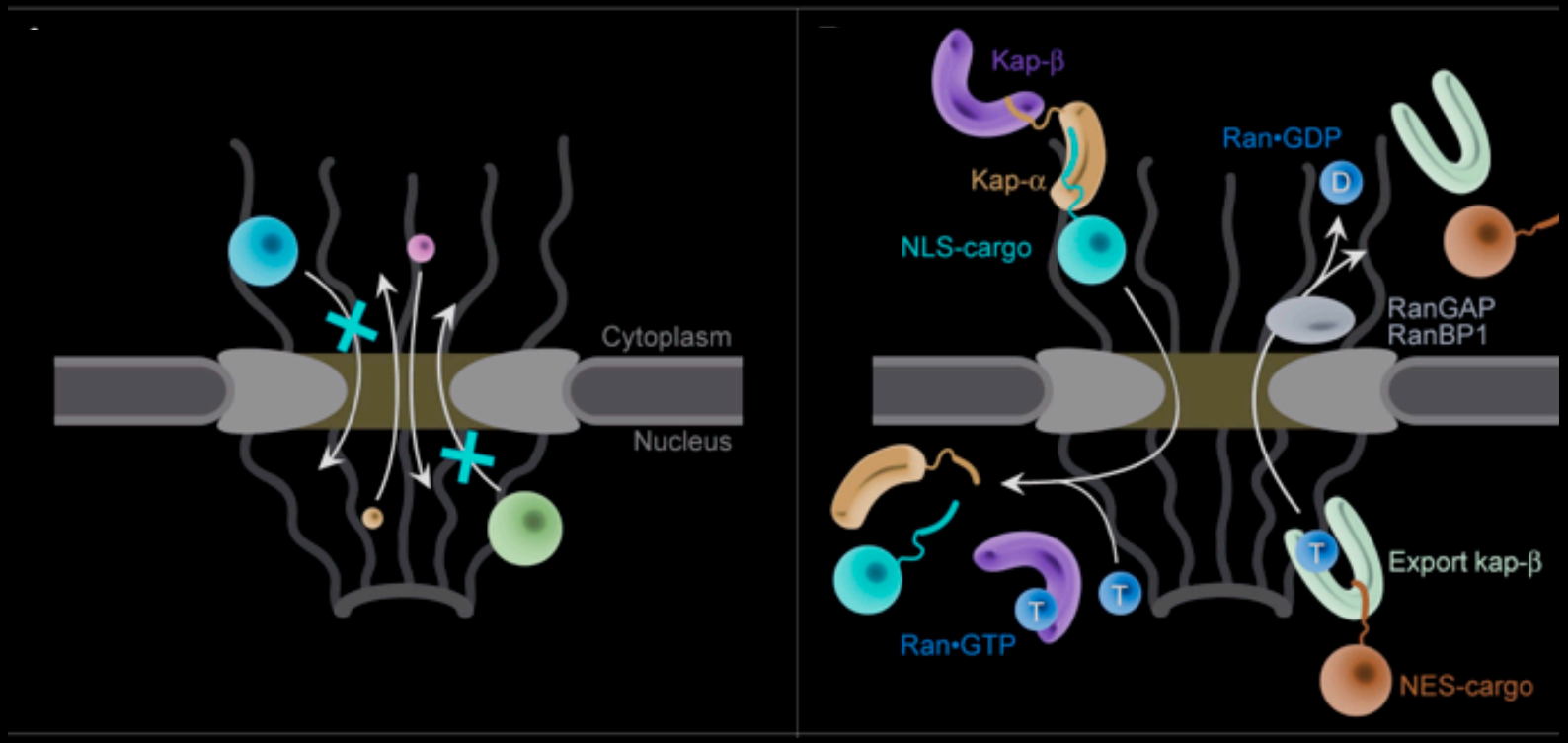
La Jolla, November 2012

NPCs are the mediators of exchange between the nucleus and the cytoplasm



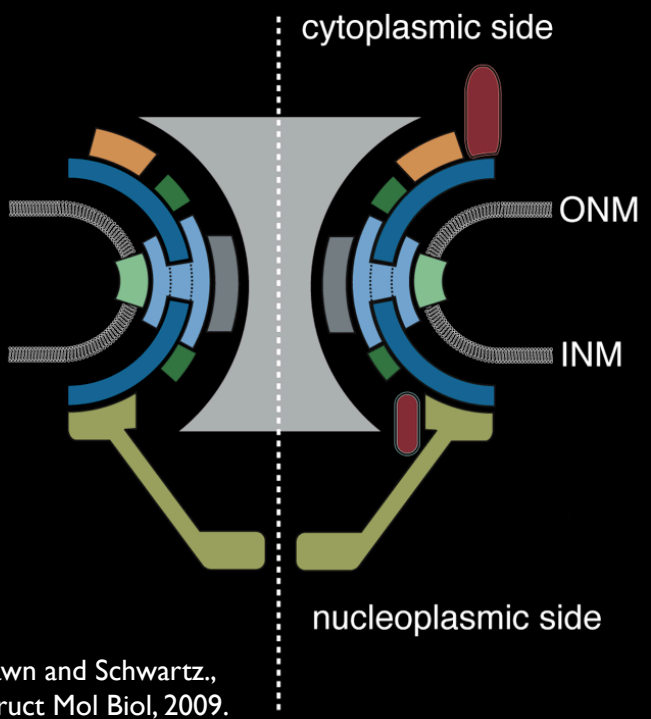


NPCs are the mediators of exchange between the nucleus and the cytoplasm



The NPC has a modular architecture

S.cerevisiae *Metazoa*



Brohawn and Schwartz.,
Nat Struct Mol Biol, 2009.

Largest macromolecular complex in the cell

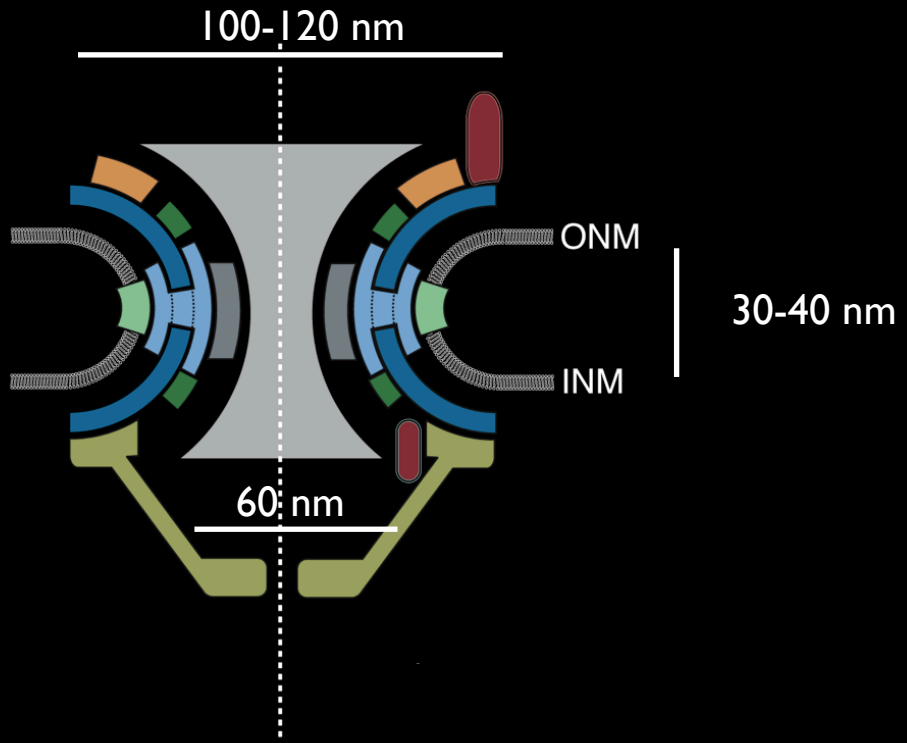
MW: ~60 MDa in yeast, ~120 MDa in Metazoa

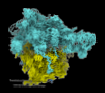
Composed 30 different nucleoproteins (Nups)

Multiple copies of each Nup

Arranged in subcomplexes

The NPC has a modular architecture





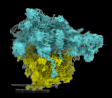
Ribosome (30 nm)

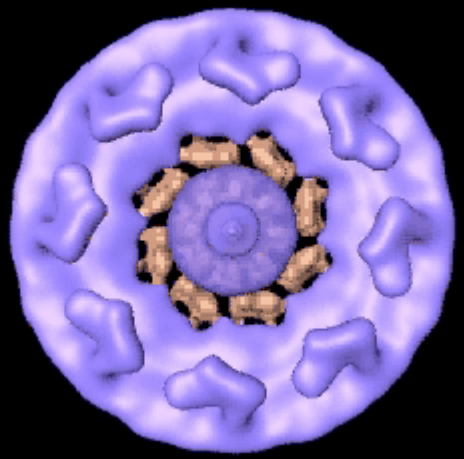
Aquaporin (5 nm)

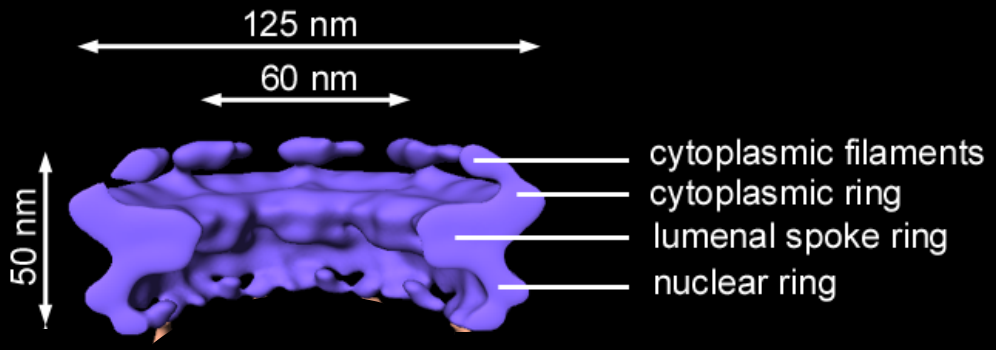
Cryo-ET can reveal the overall architecture

Two 3-D structures of the Nuclear Pore Complex

Highest resolution: ~6-7 nm
12-15 µm defocus
523 NPCs







Beck et al. *Science* 306, 1387-90 (2004)
Beck et al. *Nature* 449, 611-615, (2007)

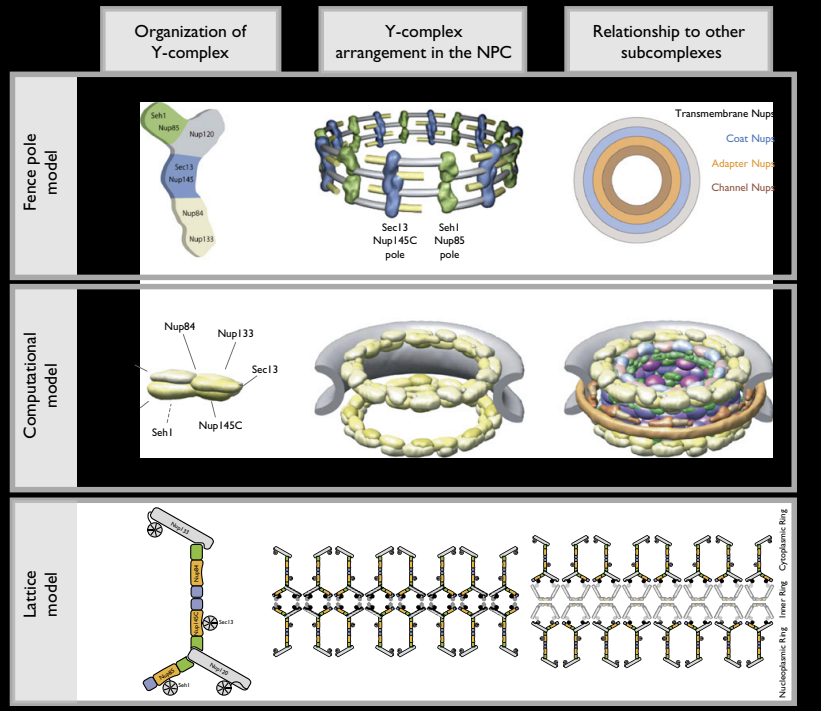
Higher Resolution: the NPC has reached the atomic age

Why?

- Wealth of available structural and "-omics" data
- EM structure will serve as a scaffold for hybrid modeling
- Discern between different models
- Structural Dynamics

How?

- Cryo-electron tomography
- *S. cerevisiae*
- Thinner samples
- High throughput
- Computational classification of states

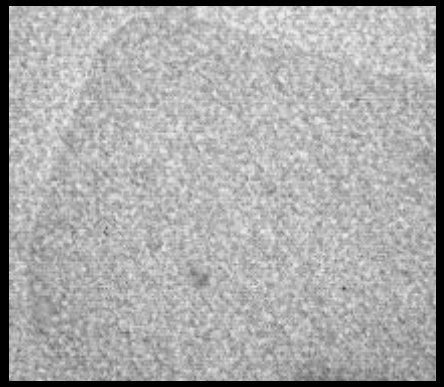


Brohawn and Schwartz., Nat Struct Mol Biol, 2009.

Three-Dimensional Cryo-Electron Microscopy

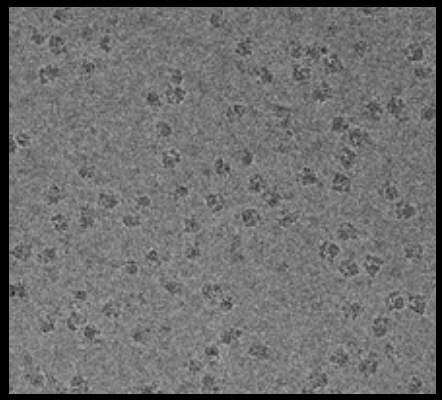
Electron crystallography

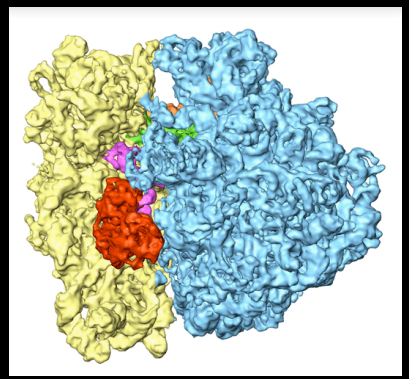
2-D crystals of membrane proteins in their native environment



Single-particle analysis

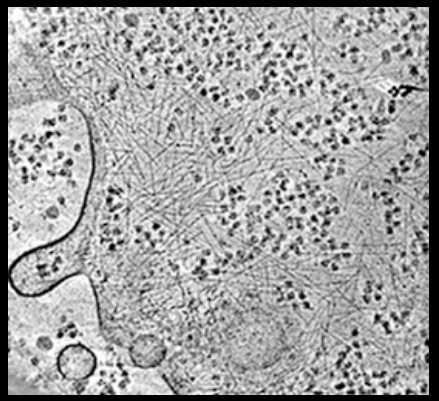
Purified molecules in solution ~200-10 MDa

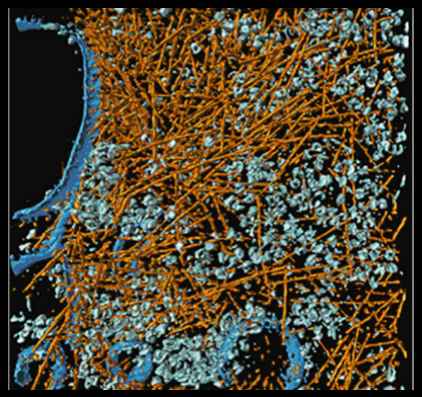




Electron tomography

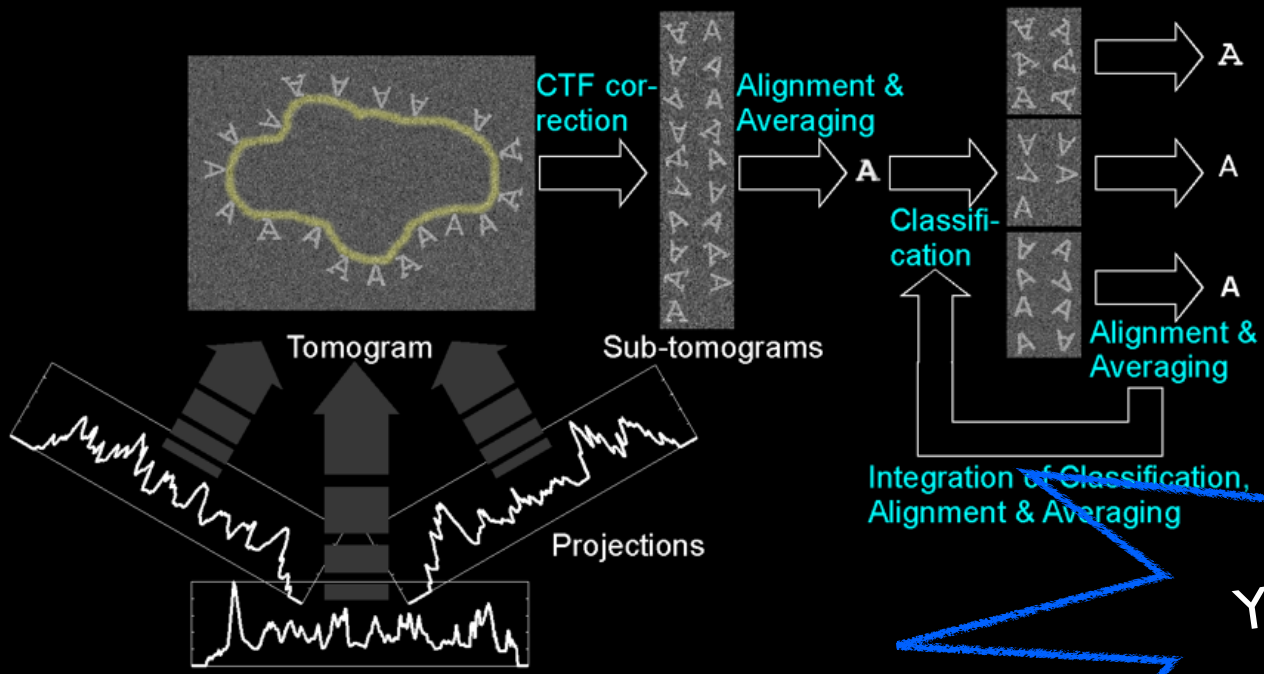
Pleomorphic samples, e.g., cells and organelles





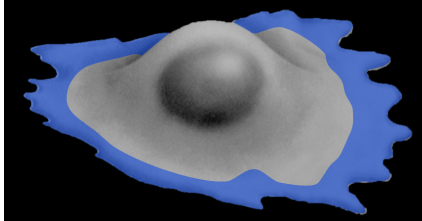
Subtomogram Averaging

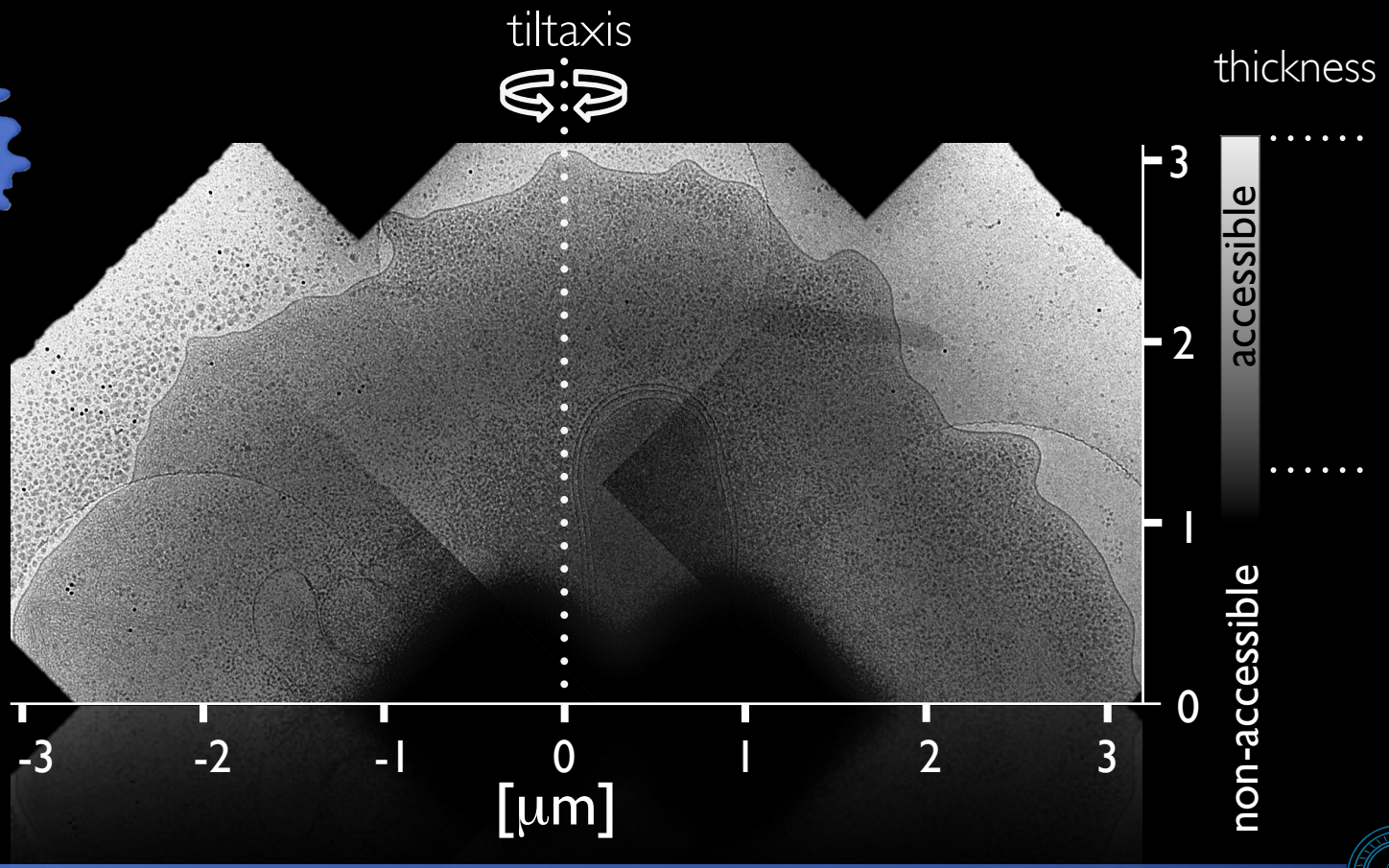
Particles of interest can be extracted from the tomograms. Expectation-maximization algorithm to obtain structure.



PyTom:
Yuxiang Chen's
poster

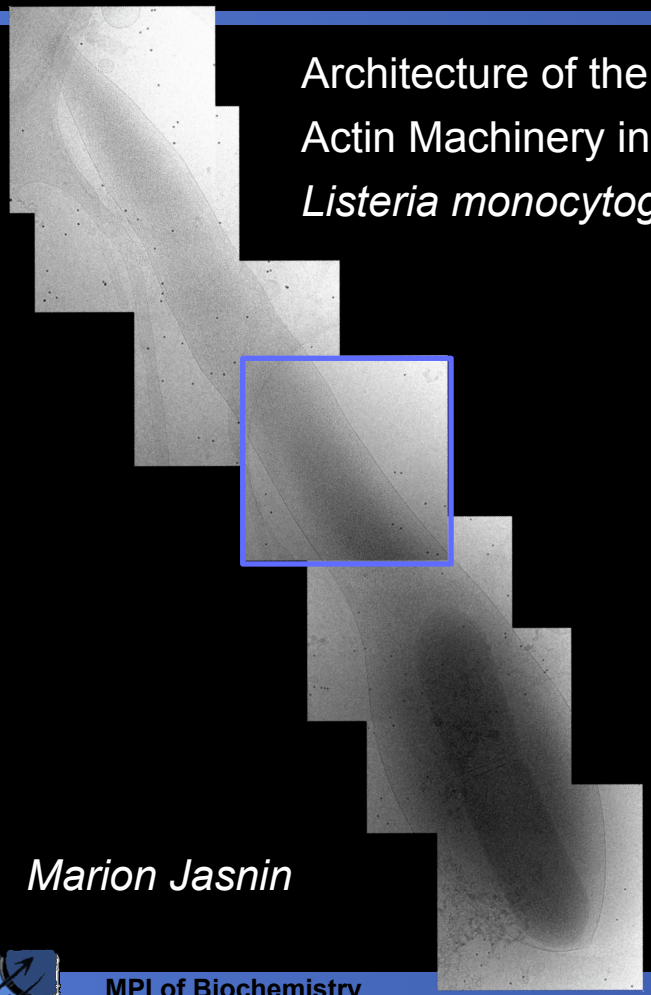
Regions Accessible to CET

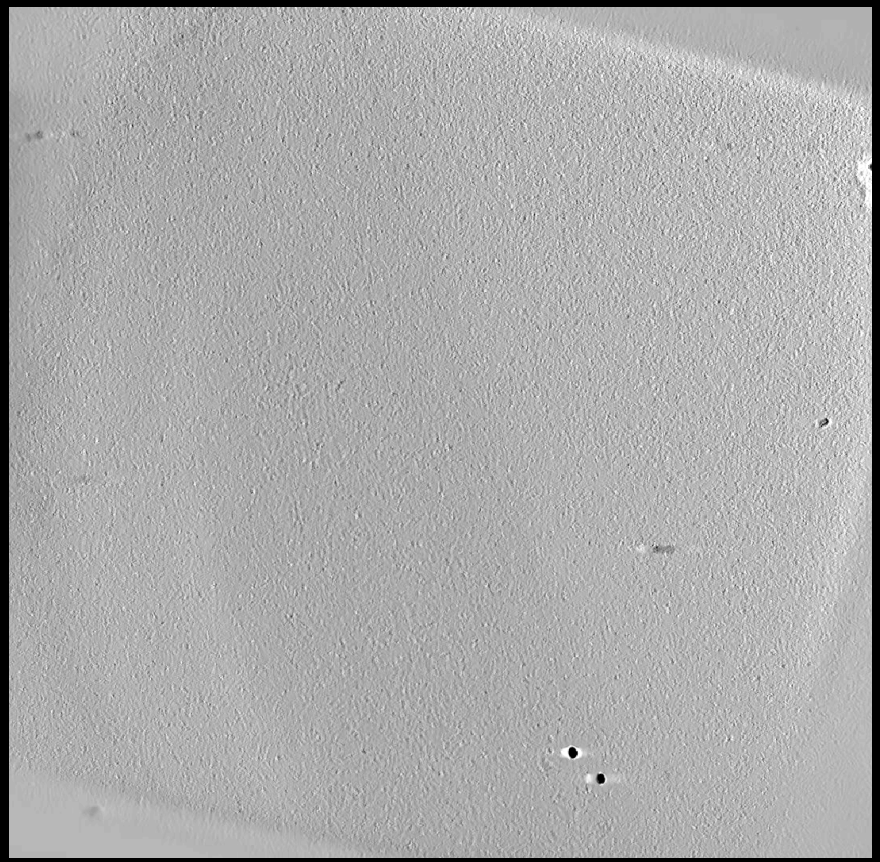




But some cool examples...

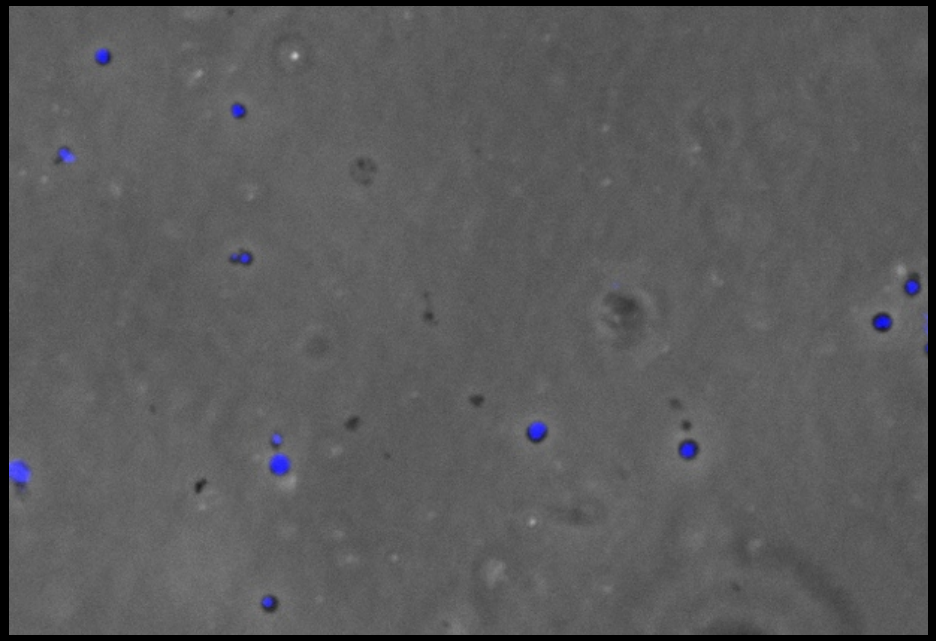
Architecture of the
Actin Machinery in
Listeria monocytogenes



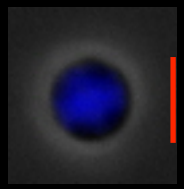


Marion Jasnin

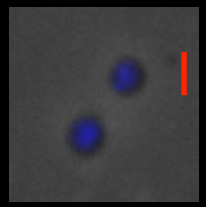
Alternative I:
Isolating organelles (intact nuclei from *S. cerevisiae)*



Enriched nuclear fraction from W303a strain.
Overlay of phase and fluorescence images DAPI-stained nuclei.

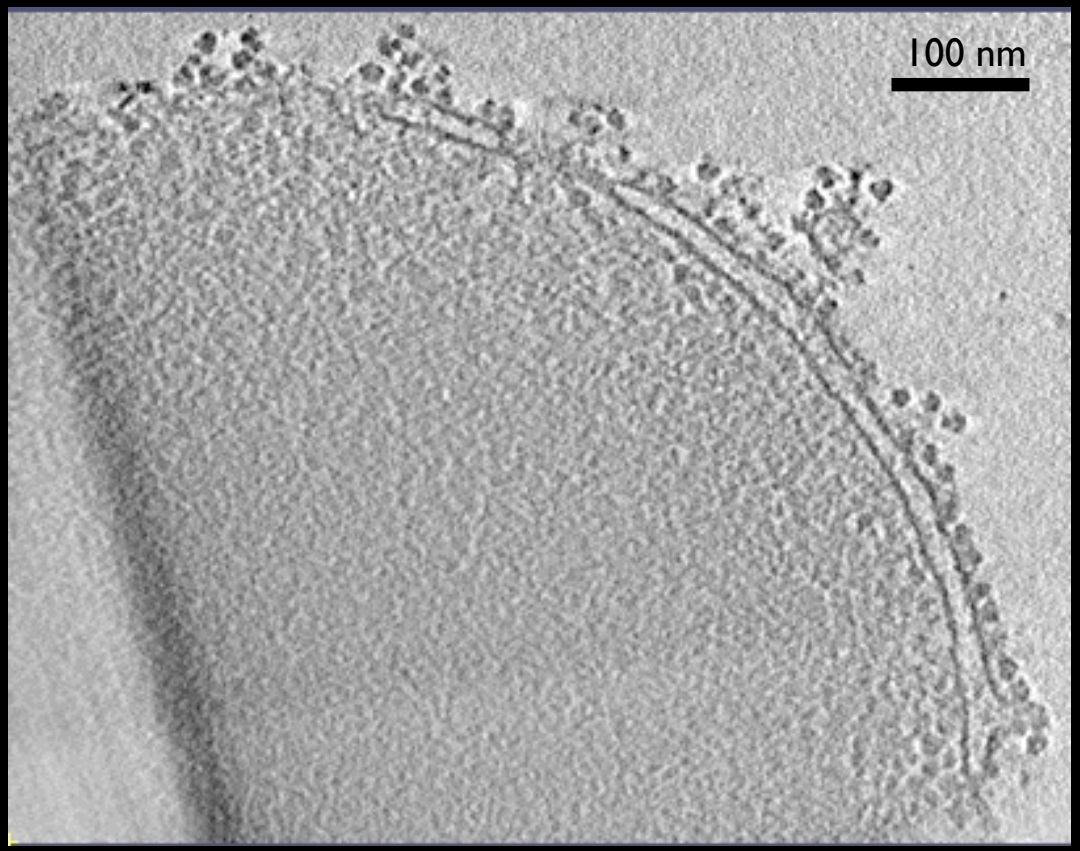


2-4 um in Dicty

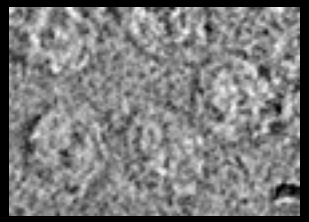


1-1.5 um in yeast

Tomography of Yeast Nuclei



side view
(thinner areas)

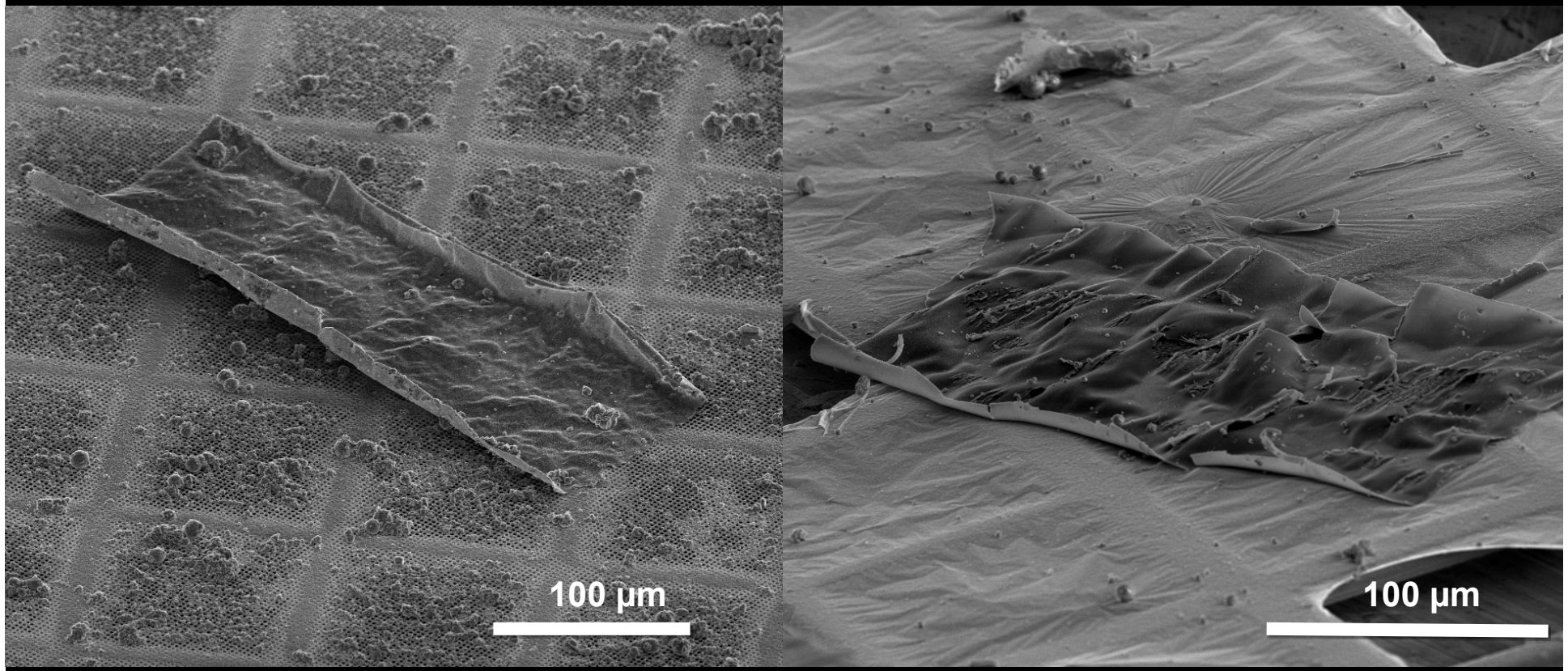


grazing view
(thicker areas)

FEI Polara G2 @ 300 keV
-6 to -8 um defocus
-64° to 64°, 2° increment
0.57 nm/pixel

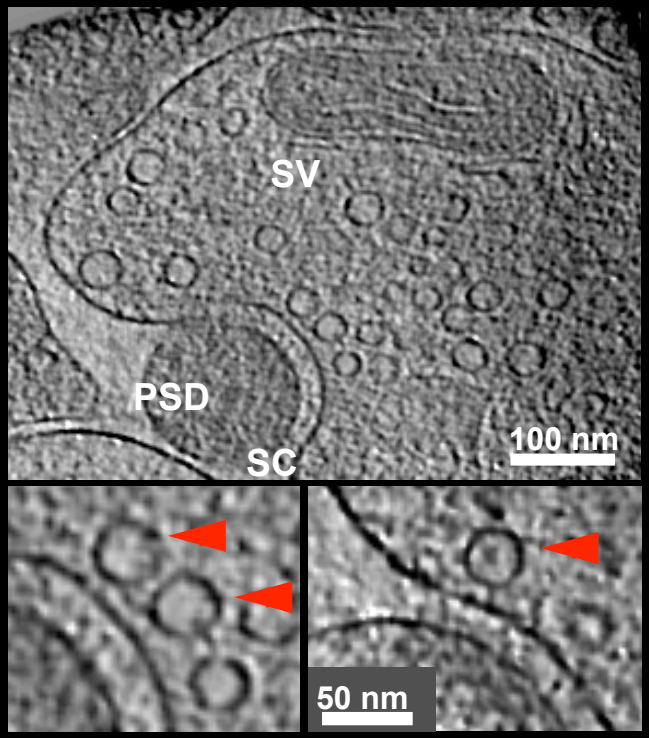
Sample thickness: 400-600 nm
~35 NPC/s per tomogram

Alternative II:
Cryo-ultramicrotomy

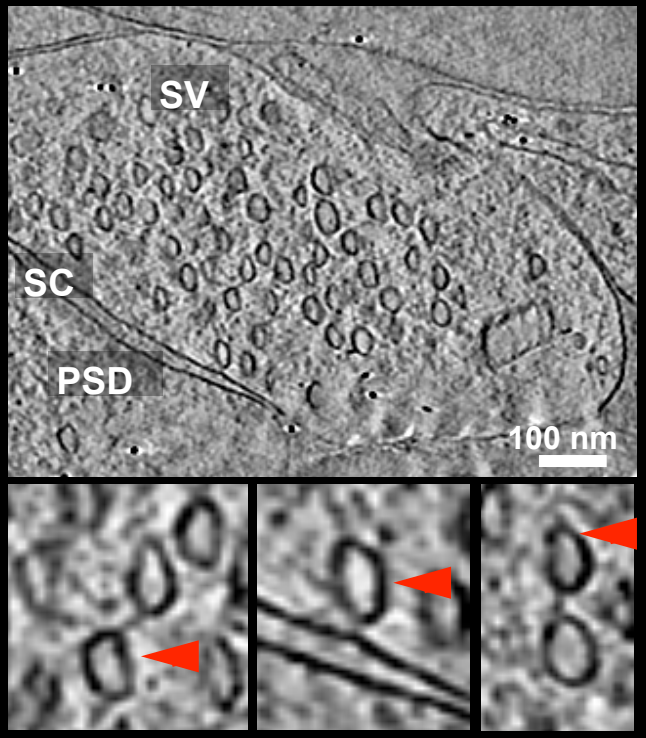


Alternative II:
Cryo-ultramicrotomy

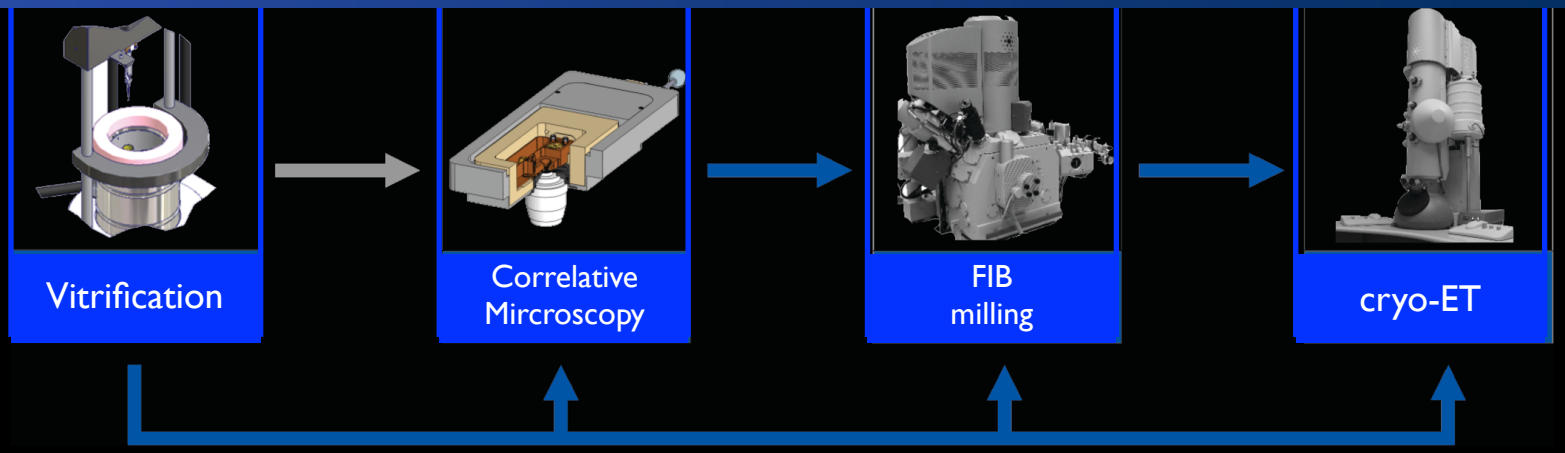
Synaptosomes (Fraction)

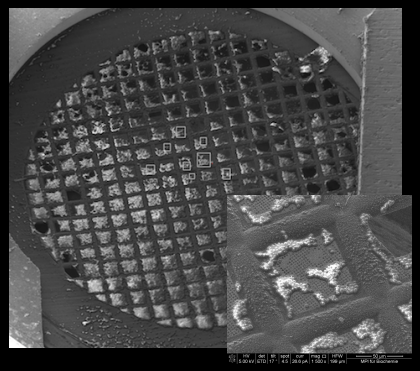


Hippocamal Cryo-Section

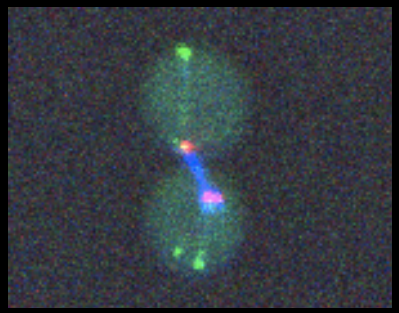


Cryo-electron Tomography Workflow

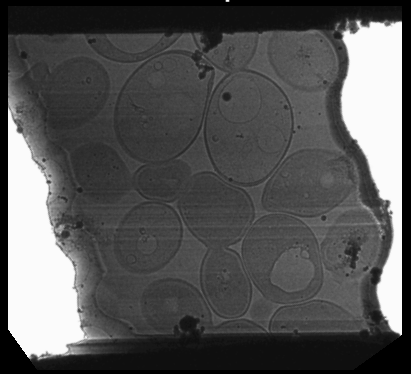




Identify regions of interest with cryo-light microscopy

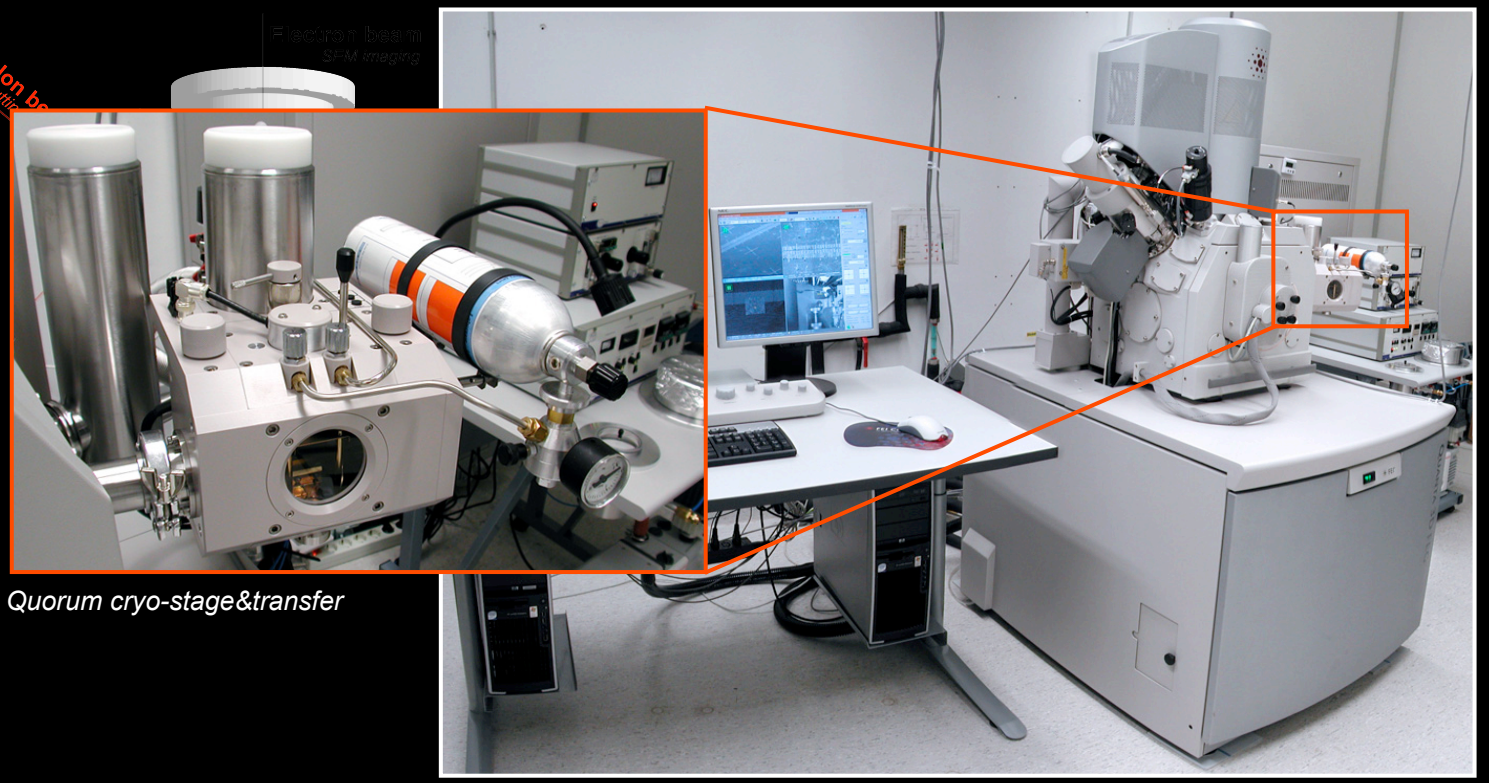


Thin samples



Tomography

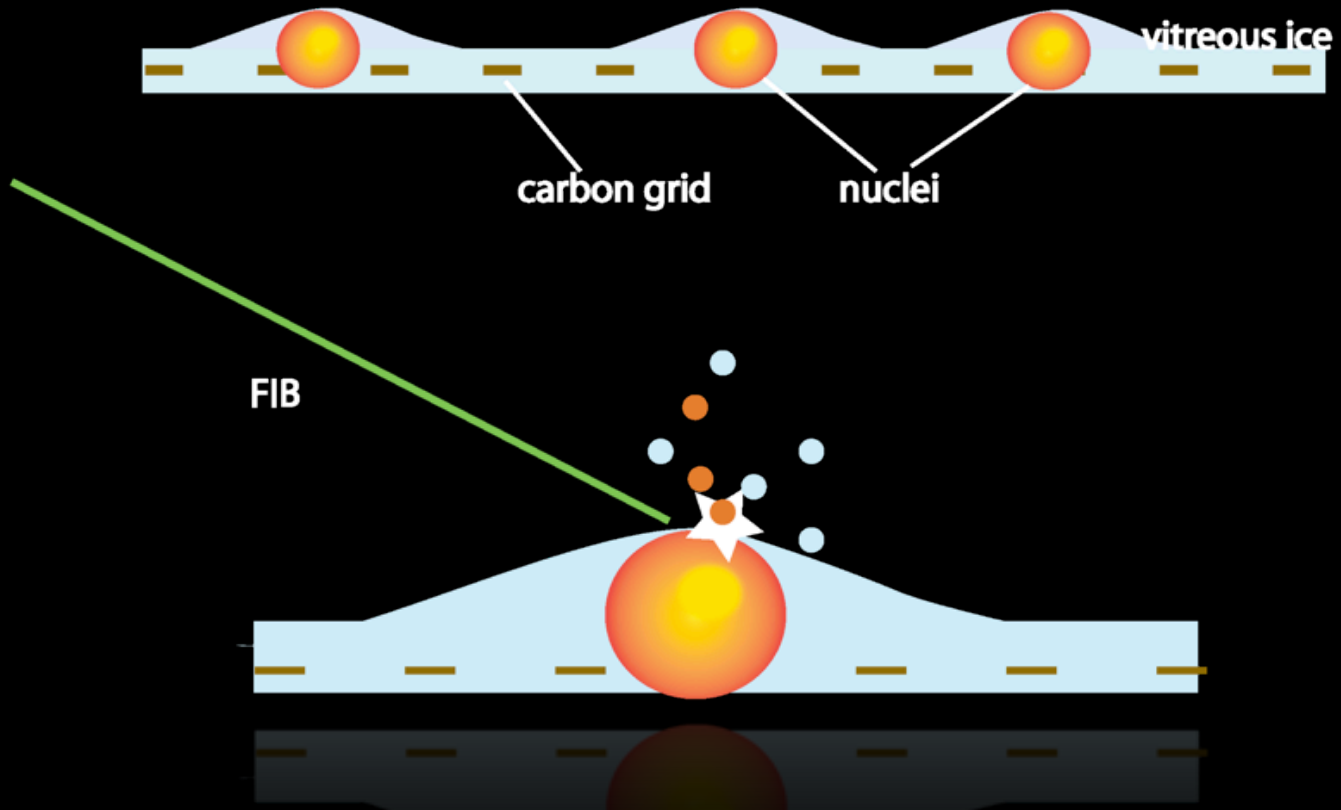
Sample thinning through FIB milling



Quorum cryo-stage&transfer

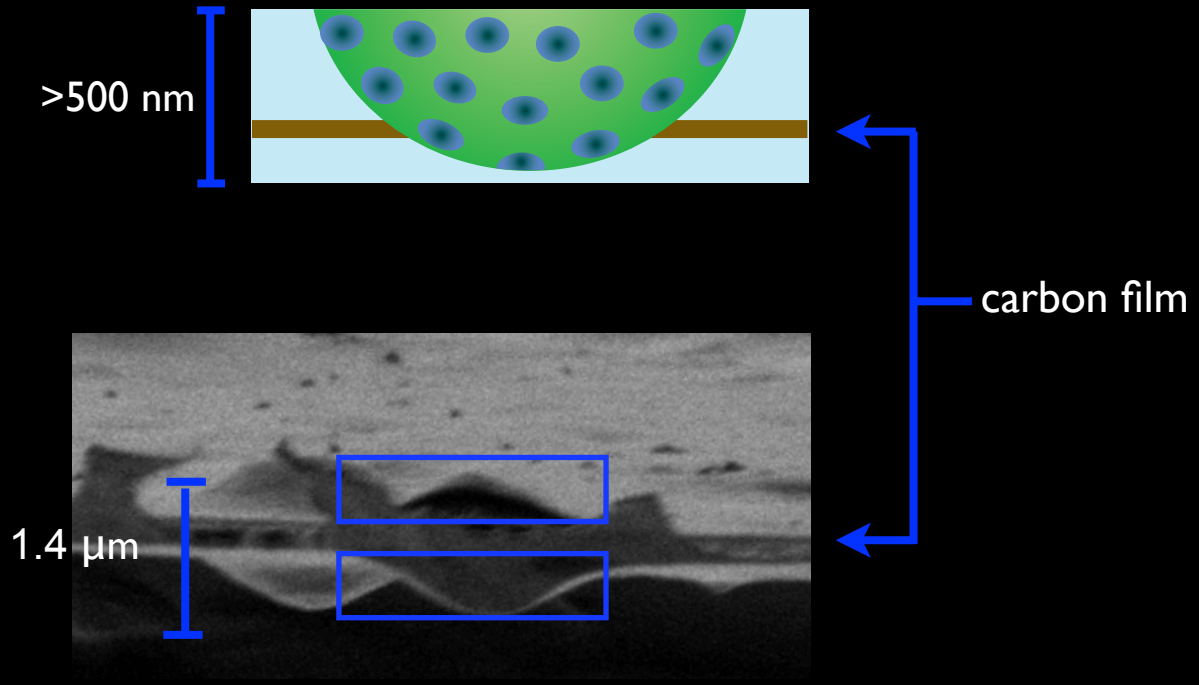
FEI Quanta 3D FEG dual beam FIB/SEM instrument as installed at the MPIB

Sample thinning through FIB milling

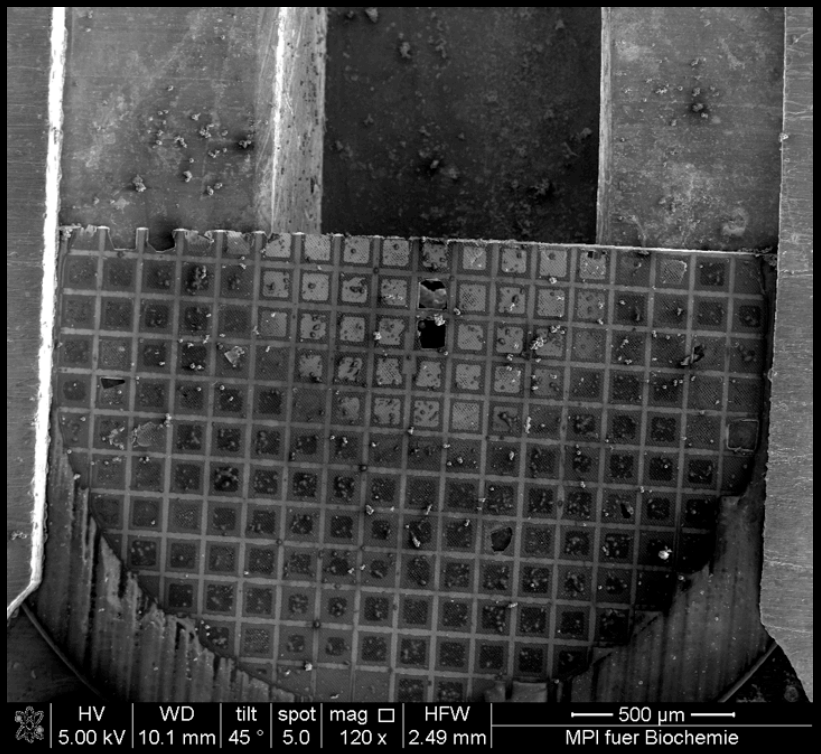


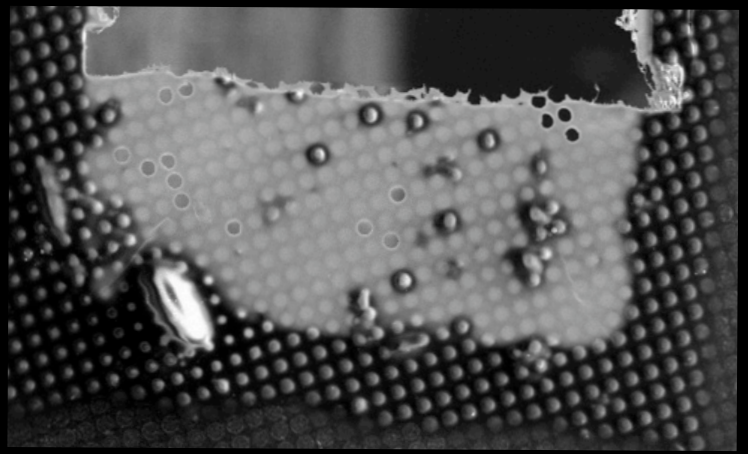
First attempt: Isolated nuclei

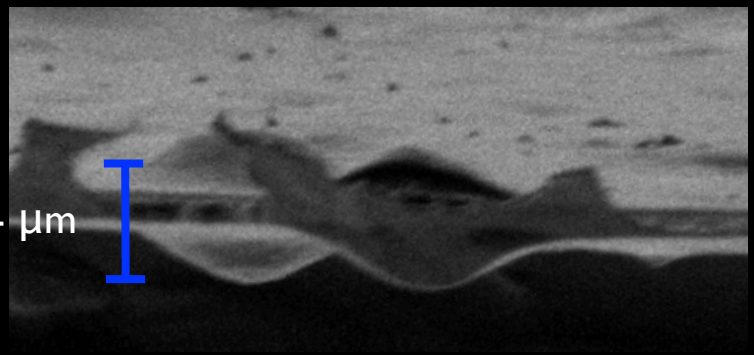
Milled samples will have ideal thickness for tomography and contain NPCs in all orientations



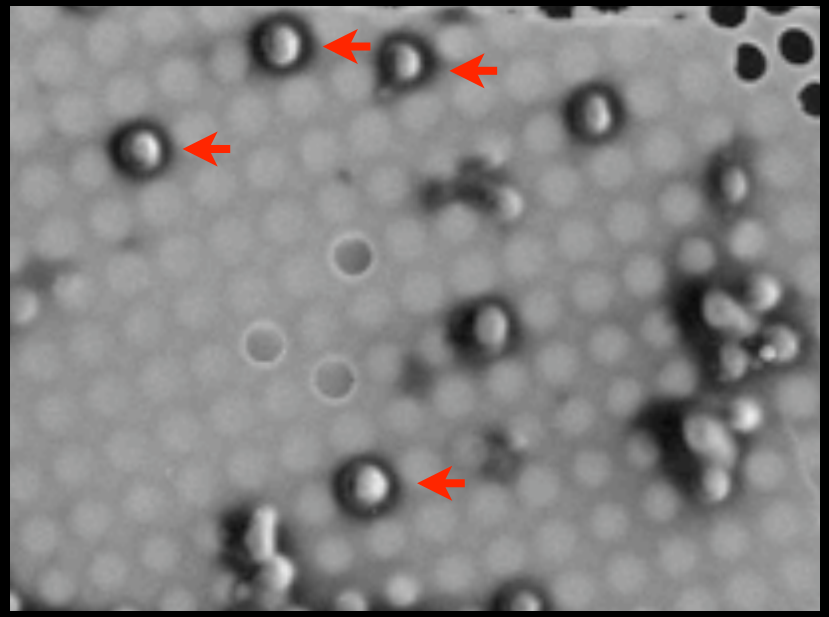
First attempt: Isolated nuclei

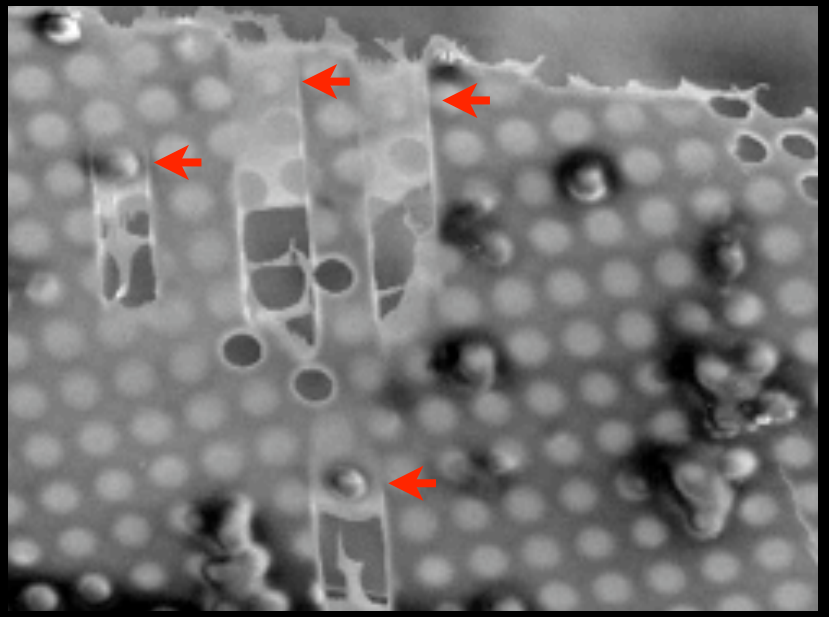






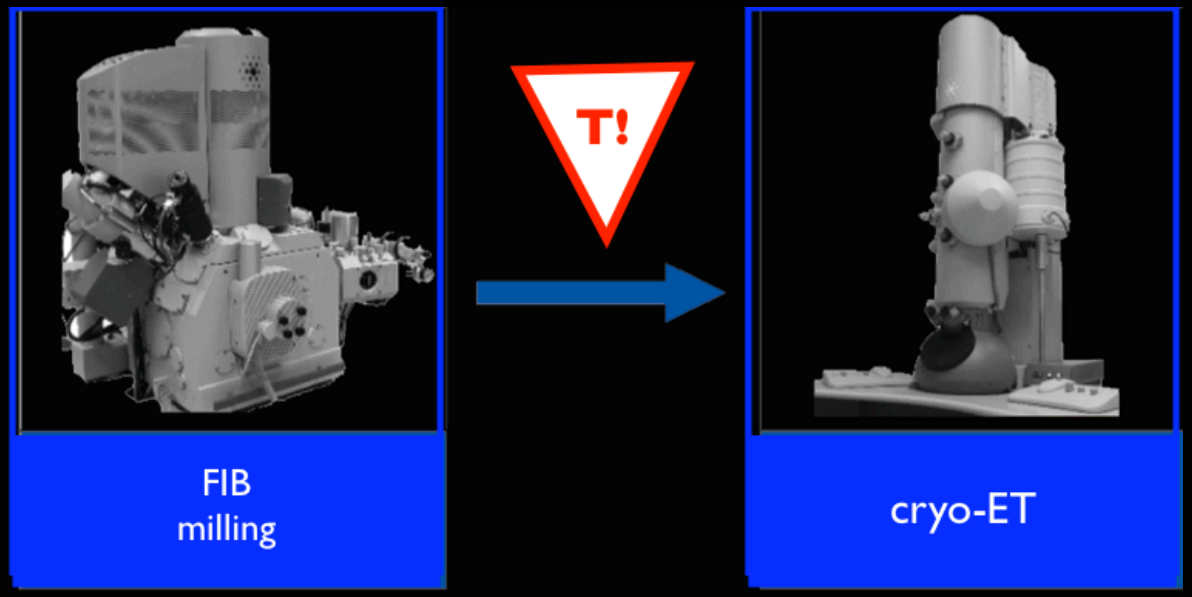
First attempt: Isolated nuclei





Fragile Specimen: Transfers between microscopes

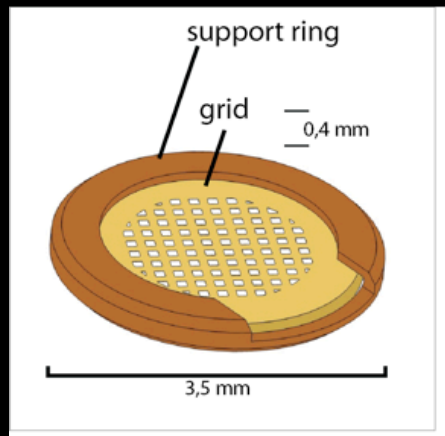
Transfers between different steps of the workflow: what happens when you multiply a handful of small probabilities

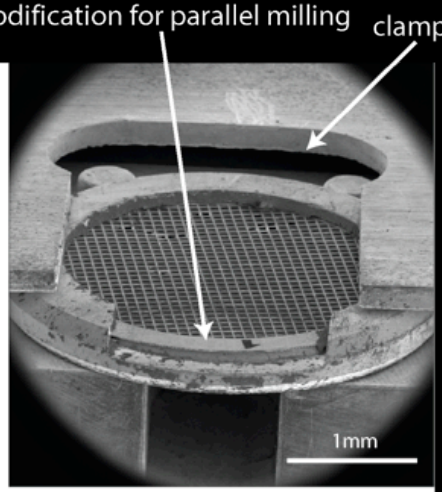


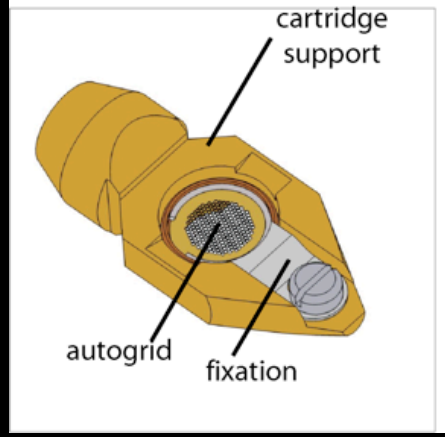
Modified Autogrid

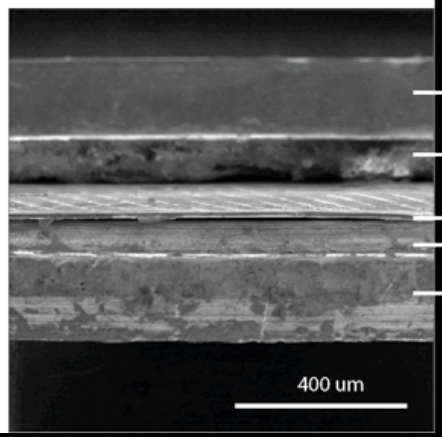
Grid reinforcement using “auto grids” provides mechanical stability during cryo-transfers.

The slot modification allows milling at parallel ion beam incidence.

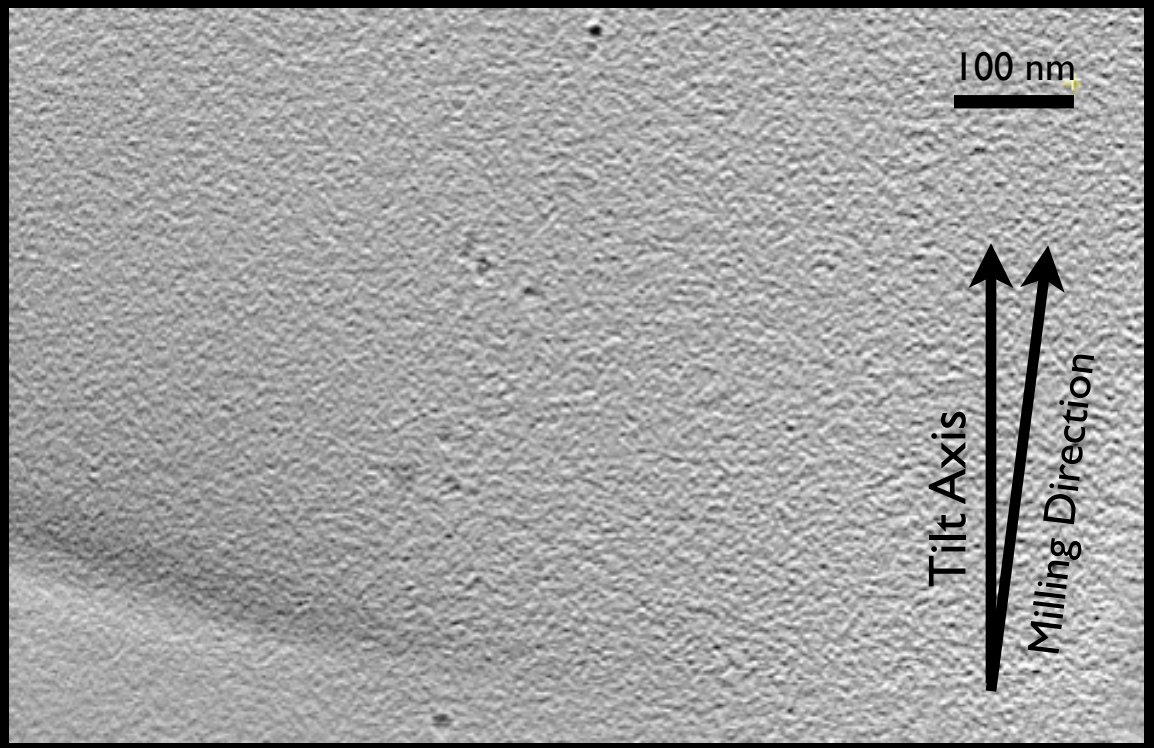








First attempt: Isolated nuclei

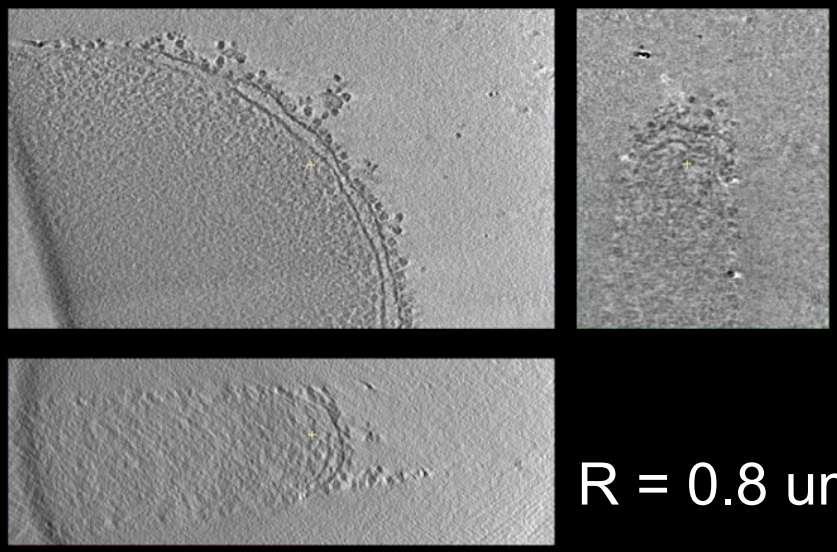


FEI Dual Beam SEM/FIB
(Quanta 3D FEG)
~30 sec/nuclei
Gallium, 30 keV, 50 pA

FEI Polara 300 keV
-8 um defocus
-60° to 60°, 2° increment
0.71 nm/pixel

Sample thickness: ~300 nm

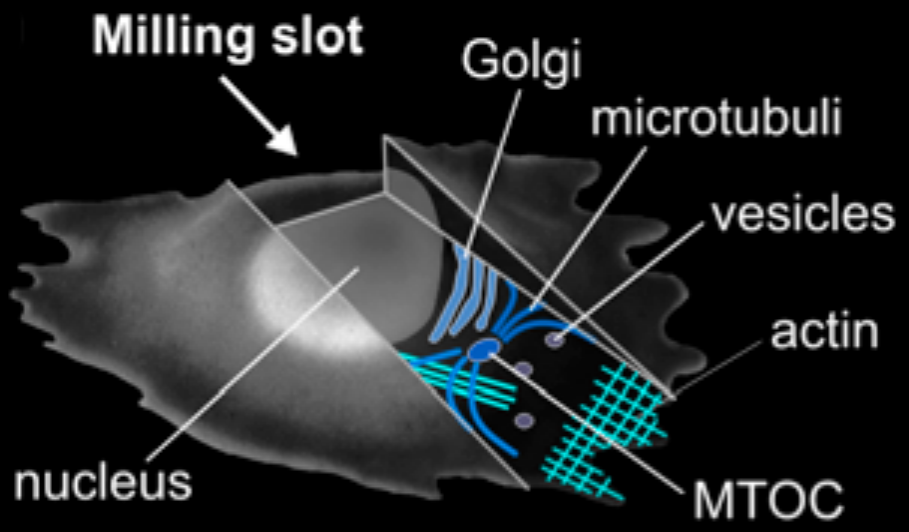
Sample Distortions



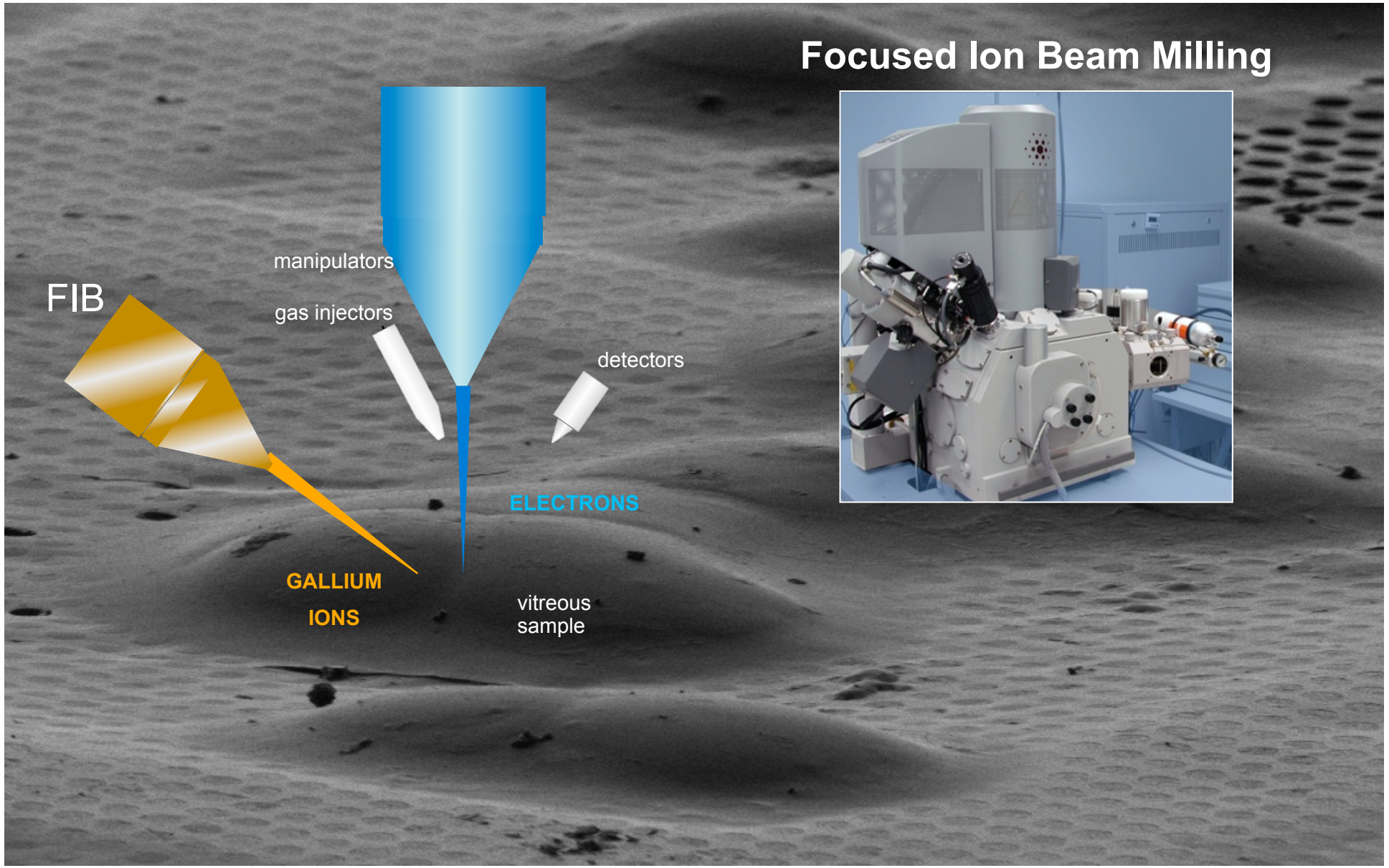
R = 0.8 um

compressed to 30% its original thickness

Opening Windows into the Cell



Focused Ion Beam Milling

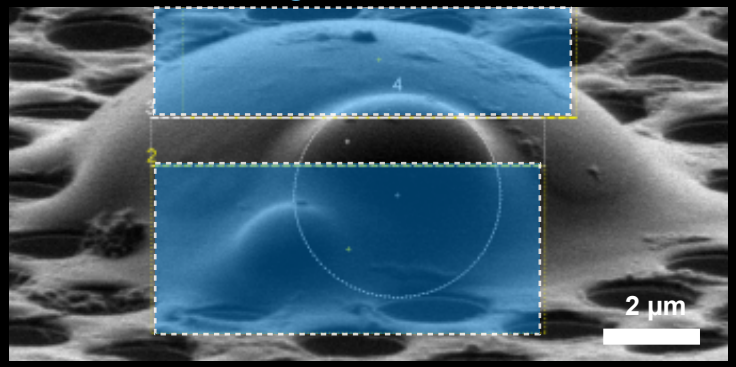


Opening Windows into the Cell

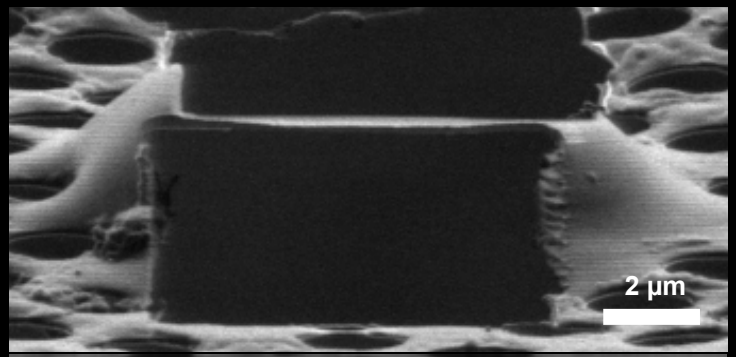
Milling area

Thinned region: Cryo-SEM (top view)

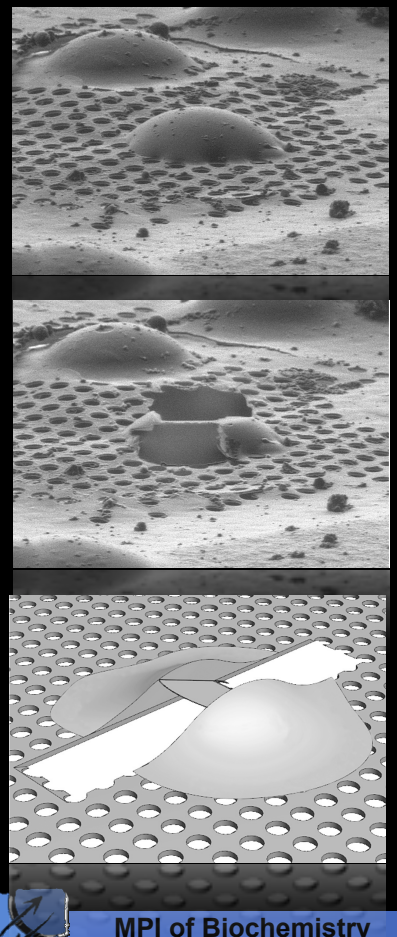
Before

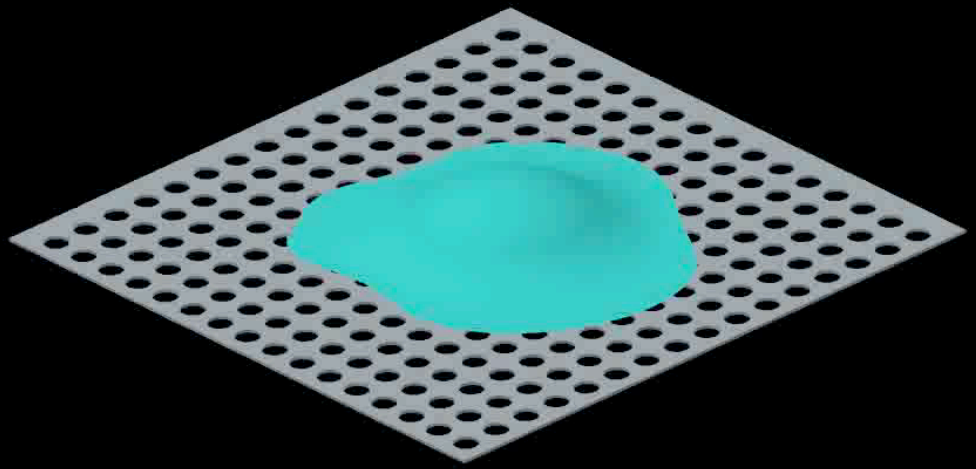


After

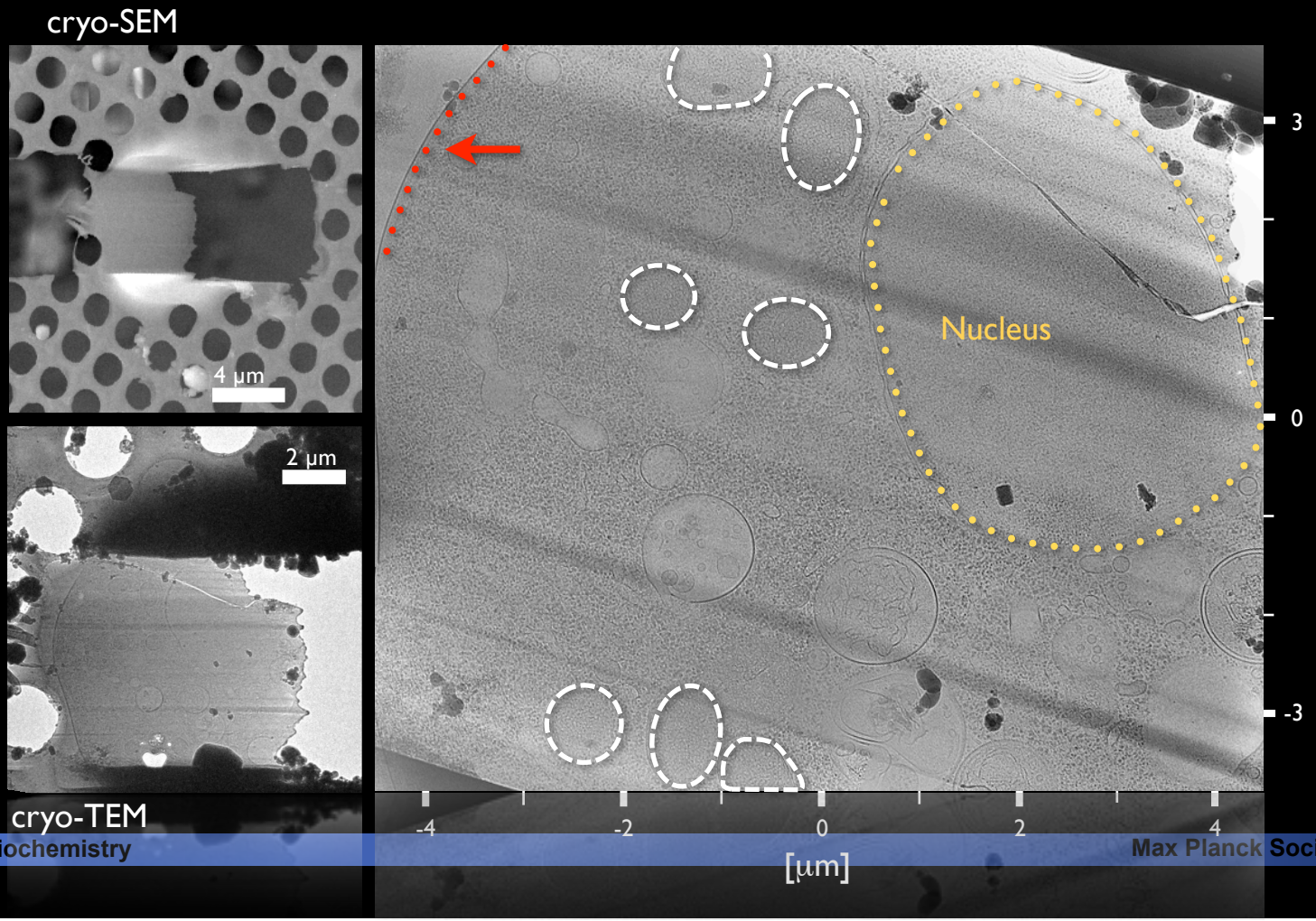


Opening Windows into the Cell



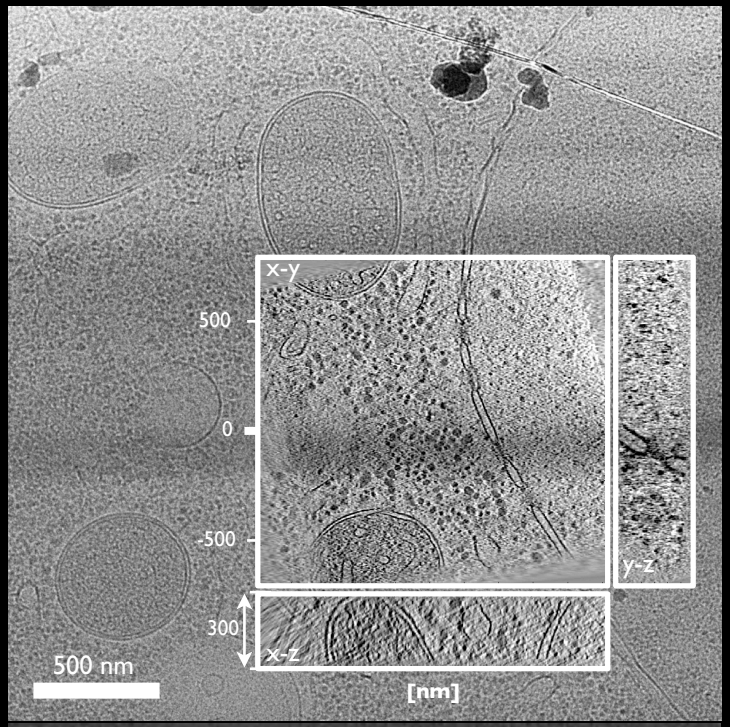


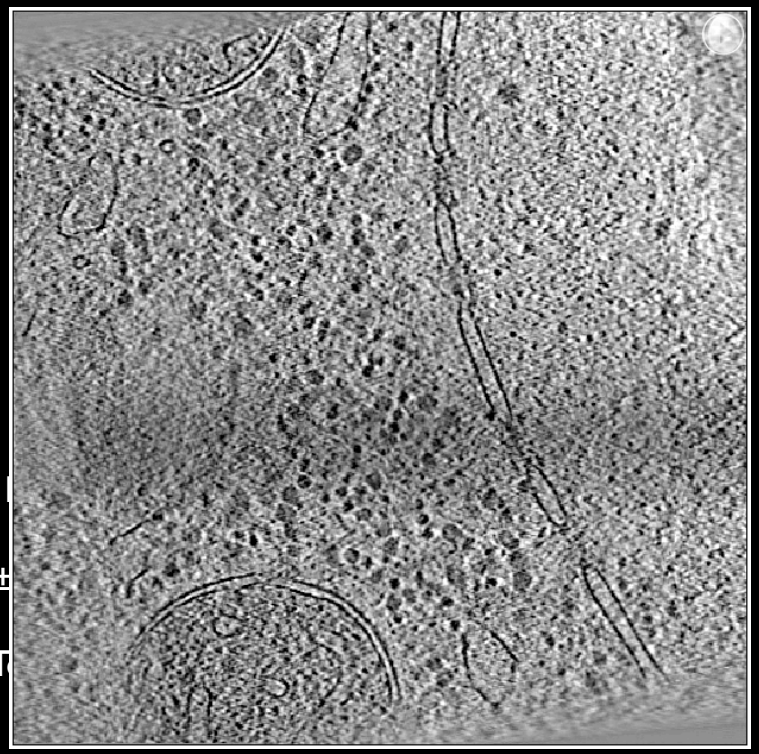
FIB: Lamella Preparation



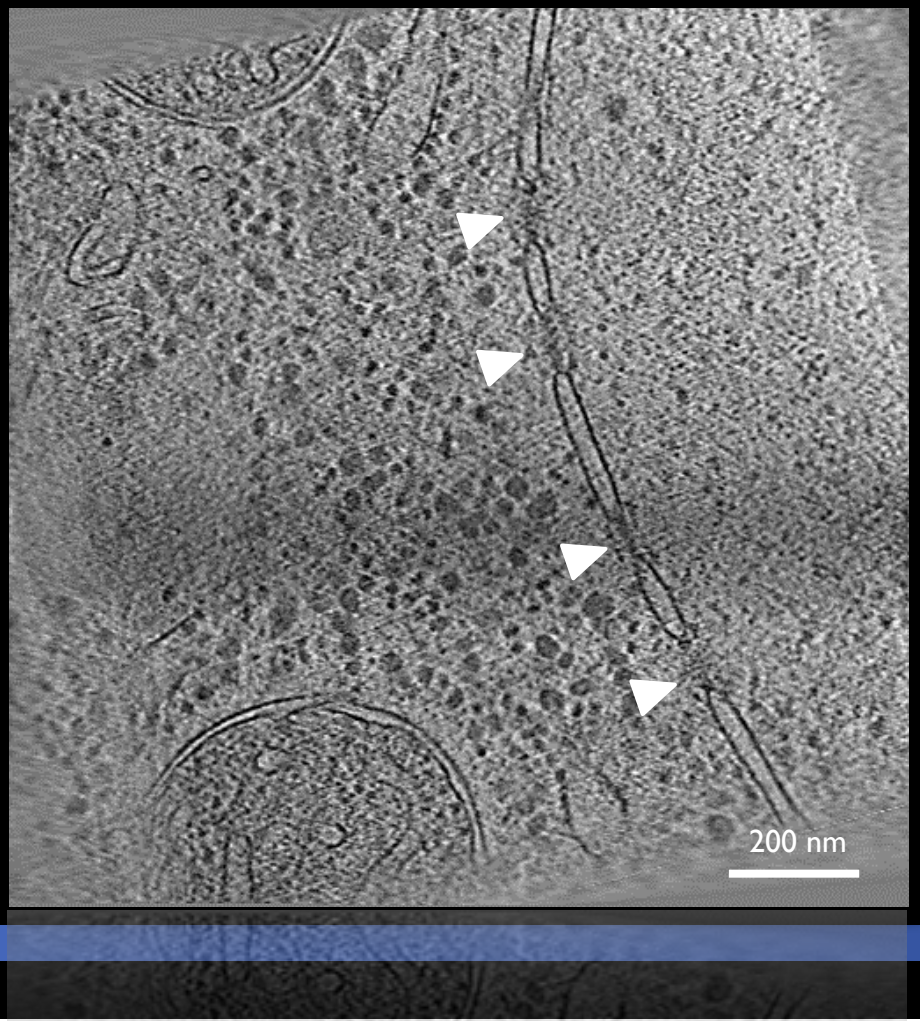
FIB: Lamella Preparation

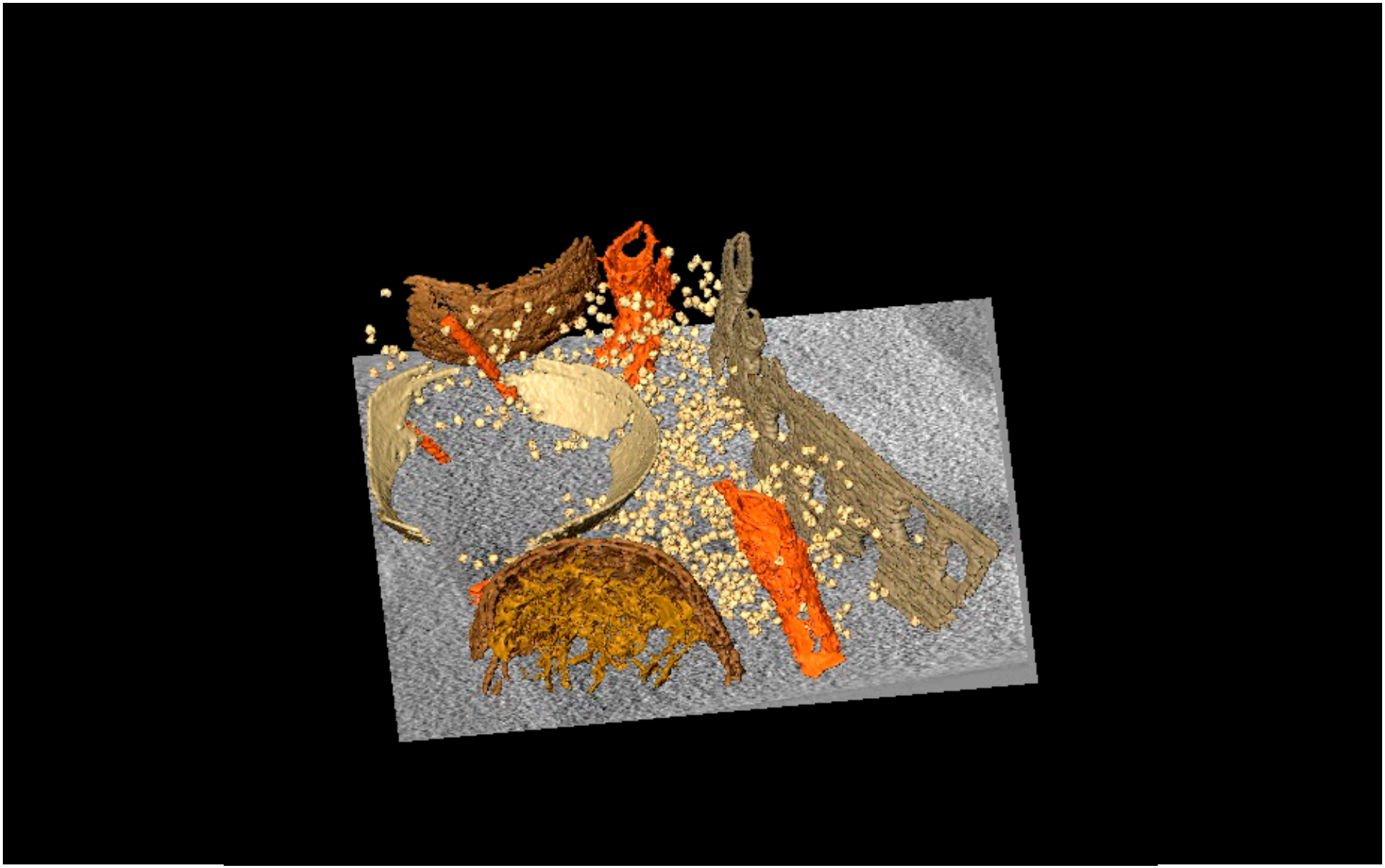
TEM 2D projection (high-pass filtered)



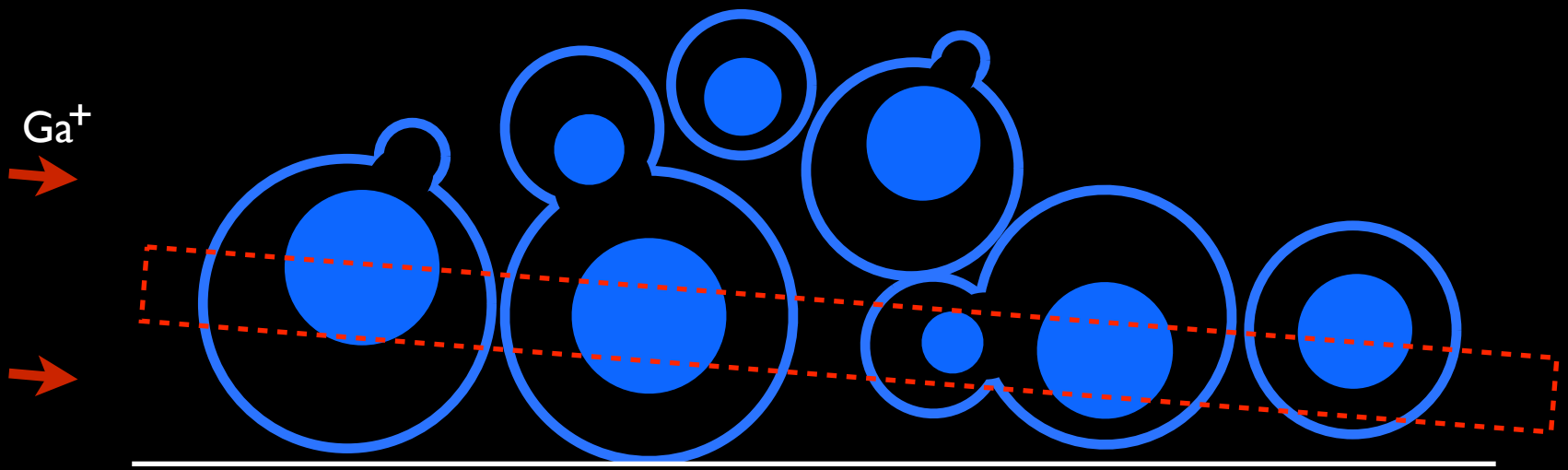


FIB: NPCs in situ



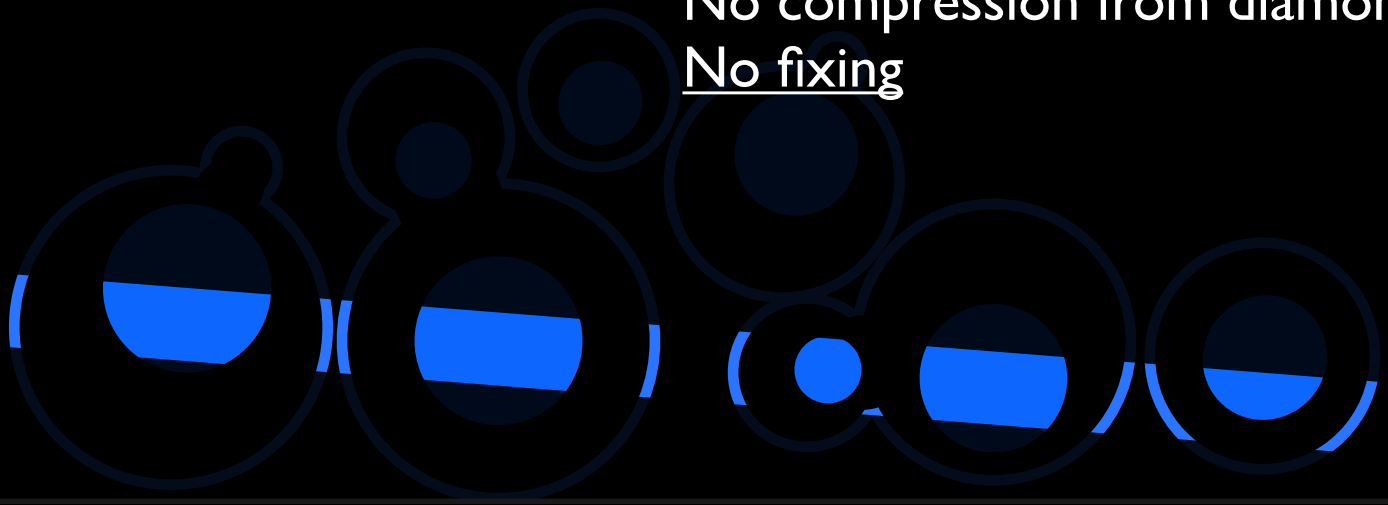


Focused Ion Beam Milling of Yeast Cells

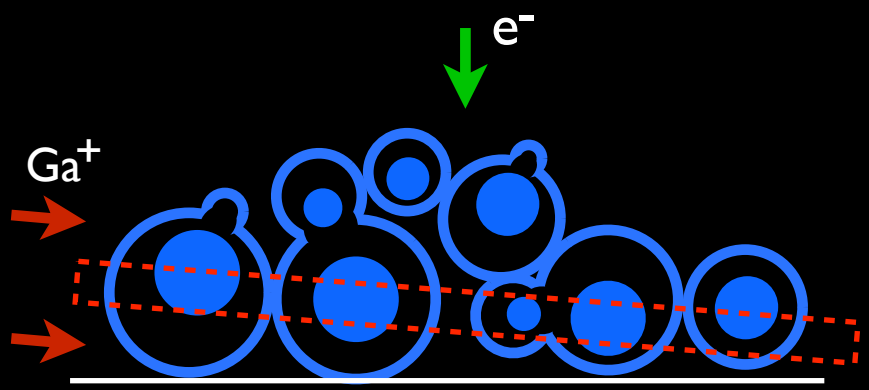


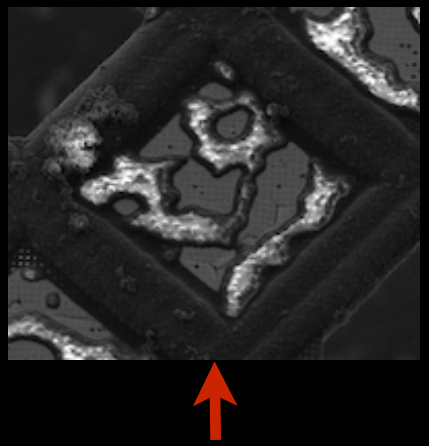
Focused Ion Beam Milling

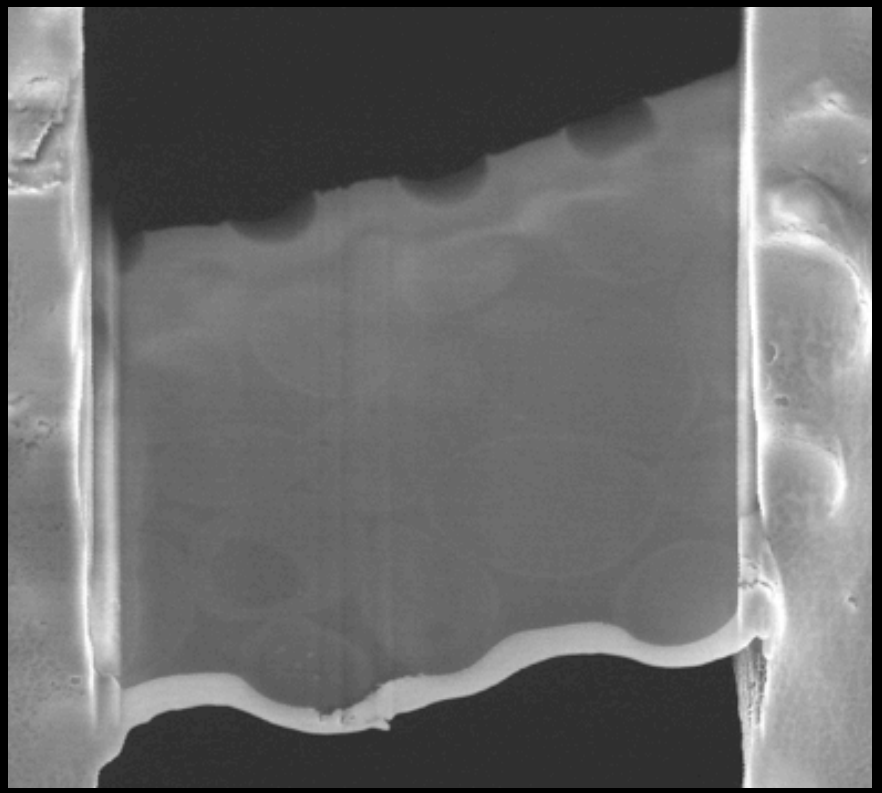
Cellular processes *in situ*
No compression from diamond knife
No fixing



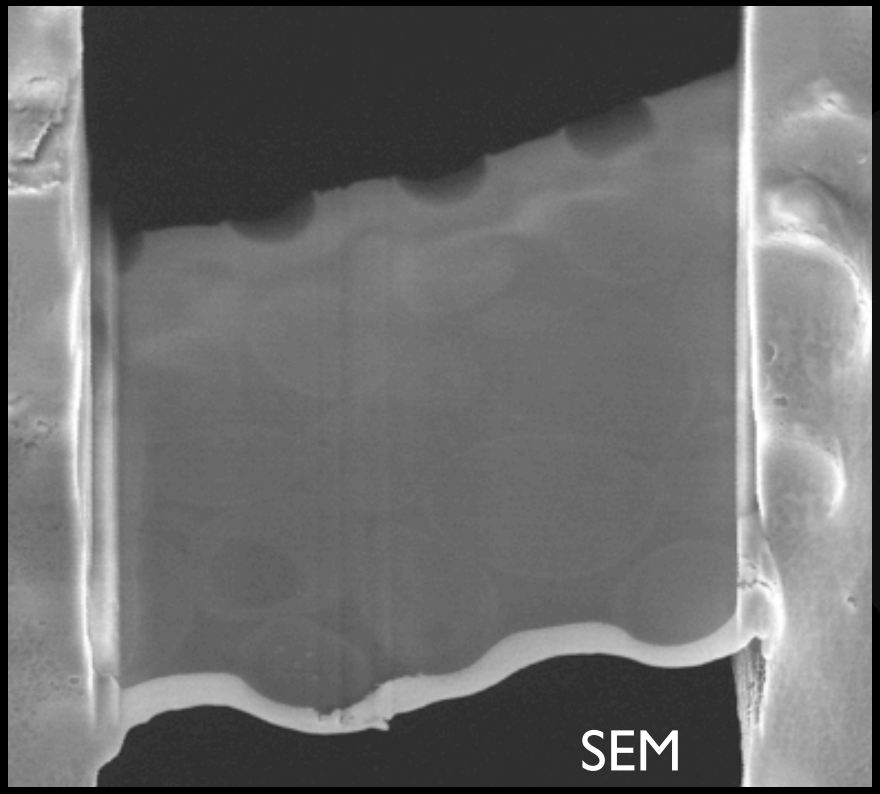
Opening Windows into the Cell

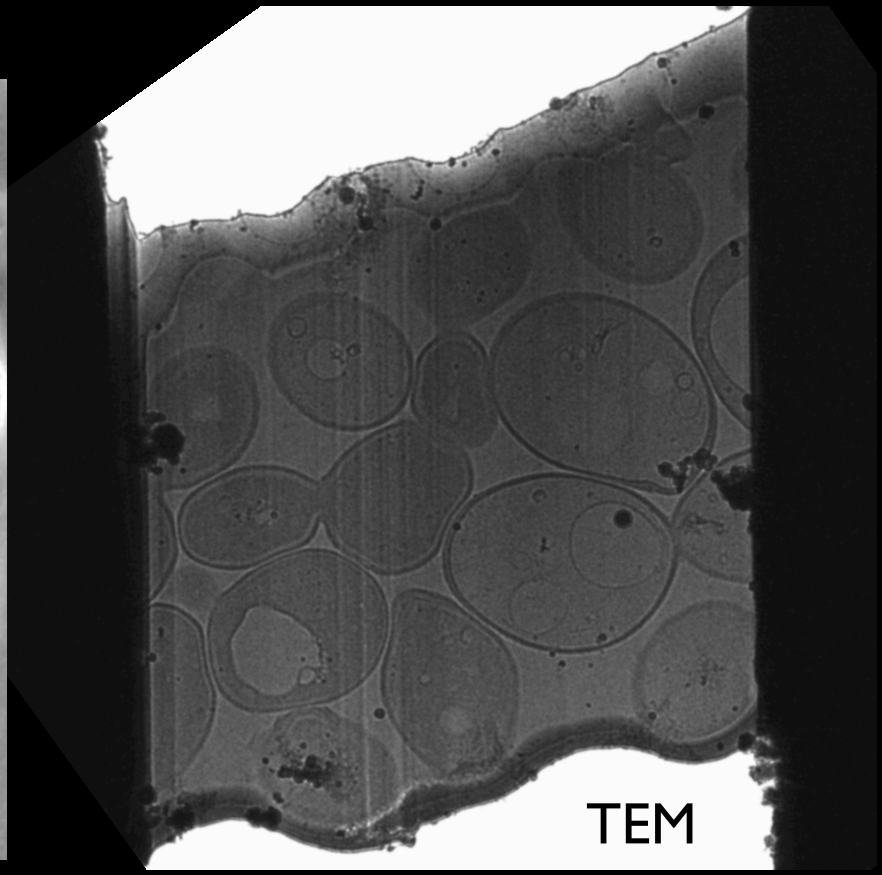




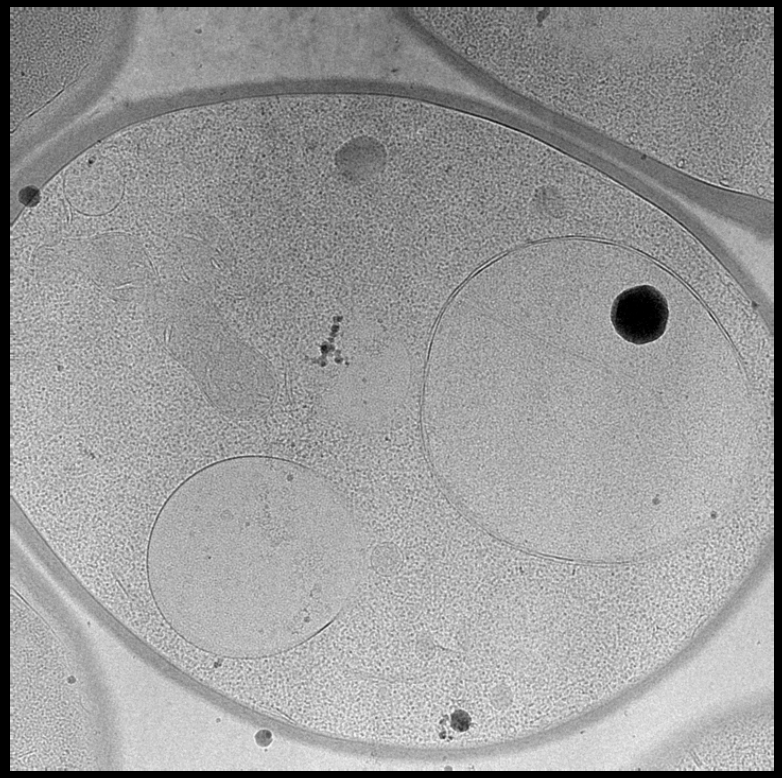


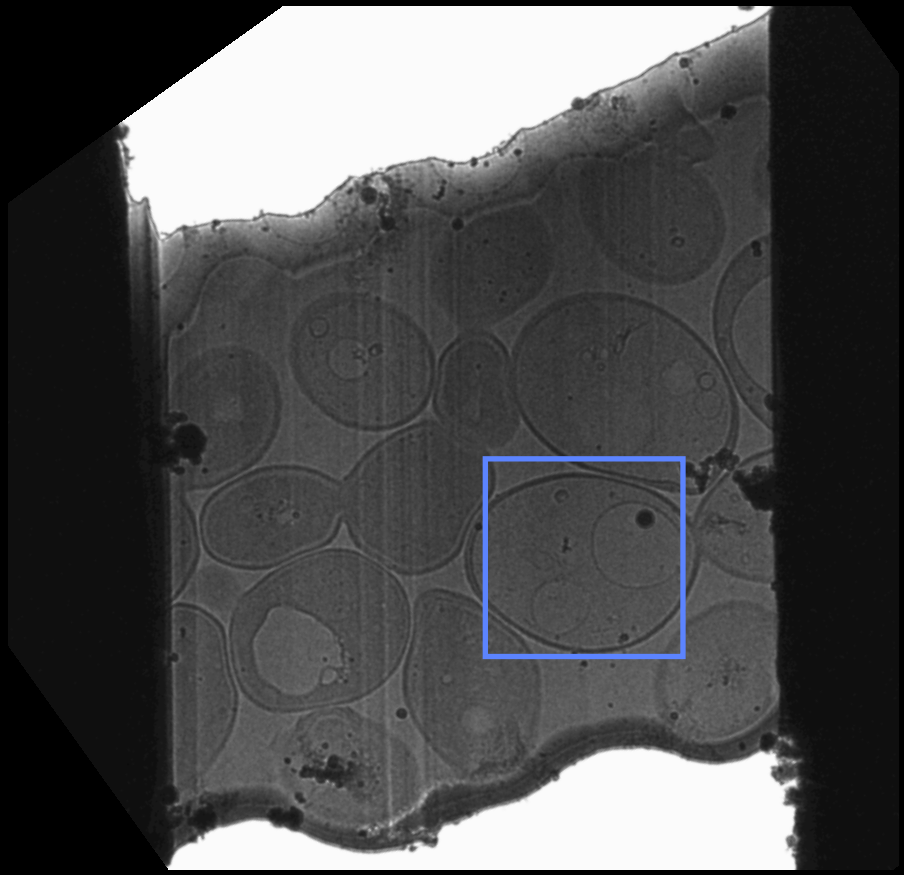
Opening Windows into the Cell



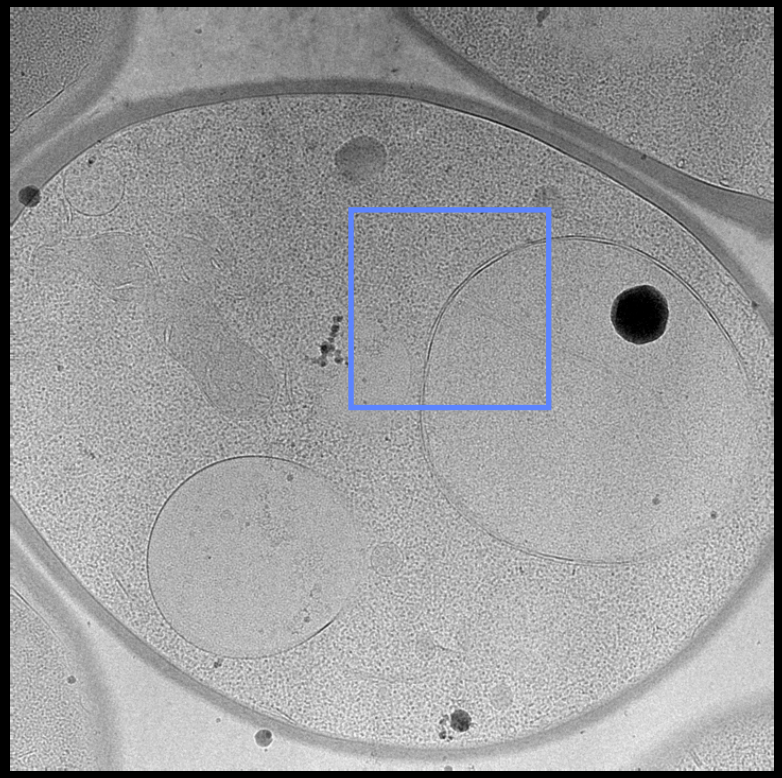


Opening Windows into the Cell

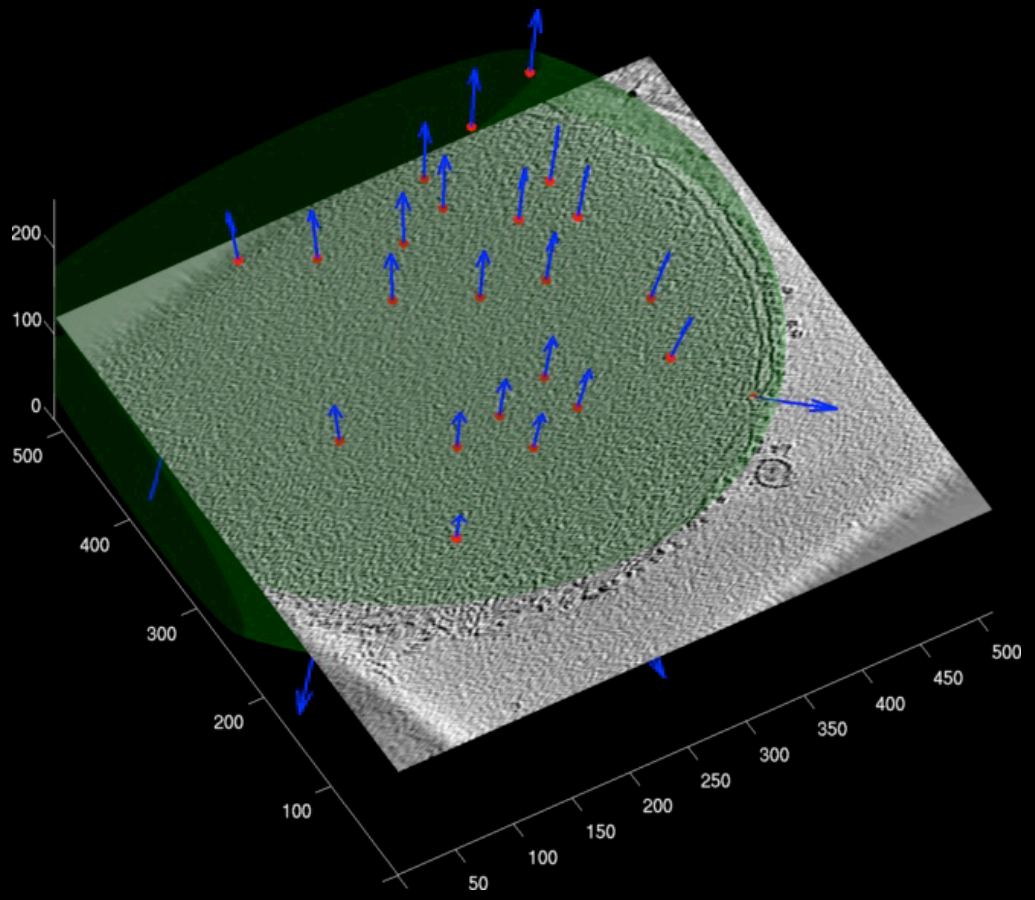


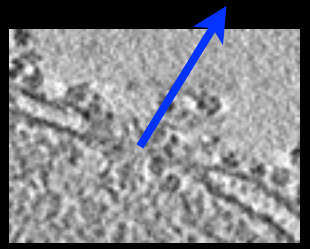


Opening Windows into the Cell



Particle Picking



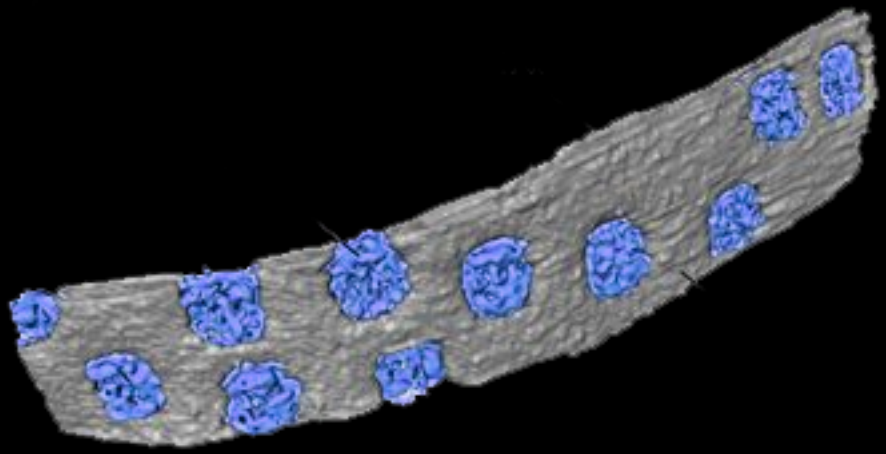


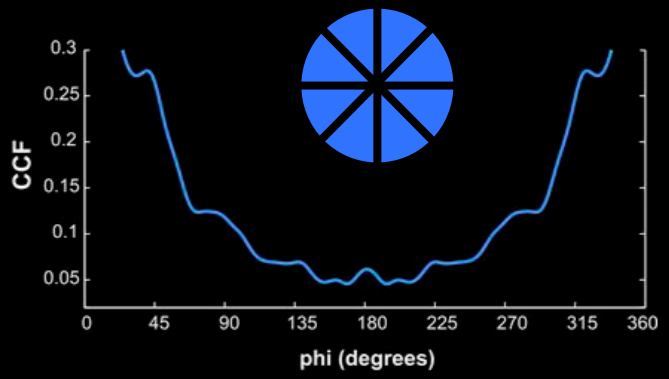
>A nuclear surface is approximated

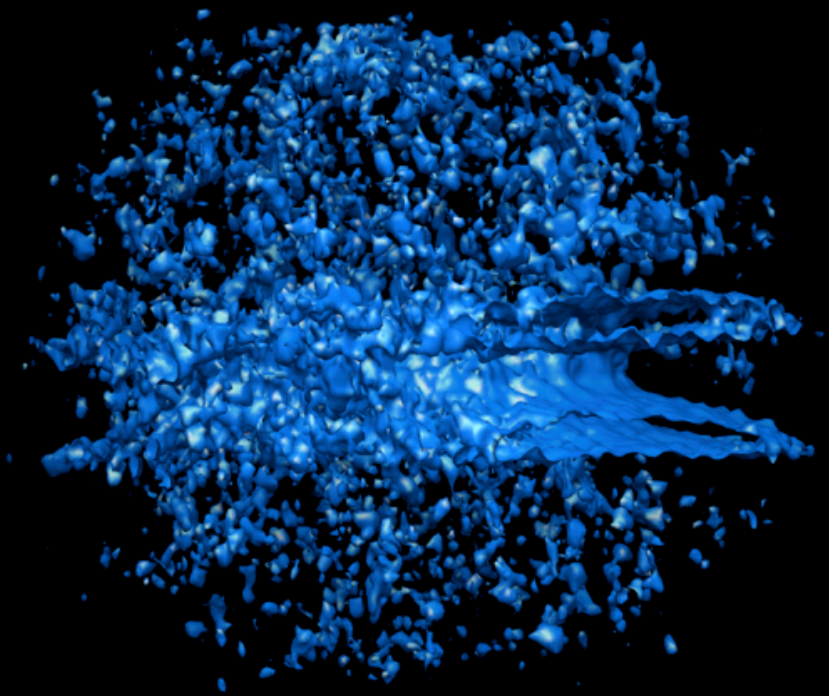
>The normals of the NPCs are determined.

>Subtomograms are extracted, aligned, and averaged

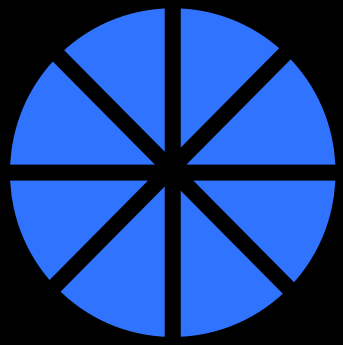
Nuclear Pore Complex Structure

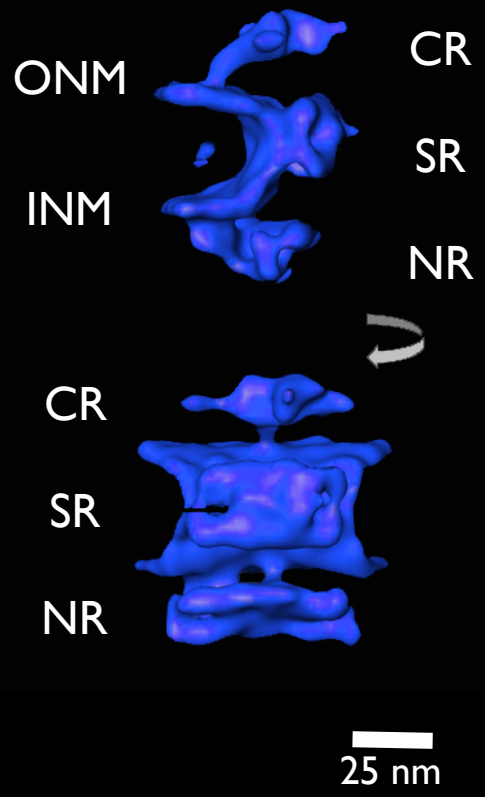


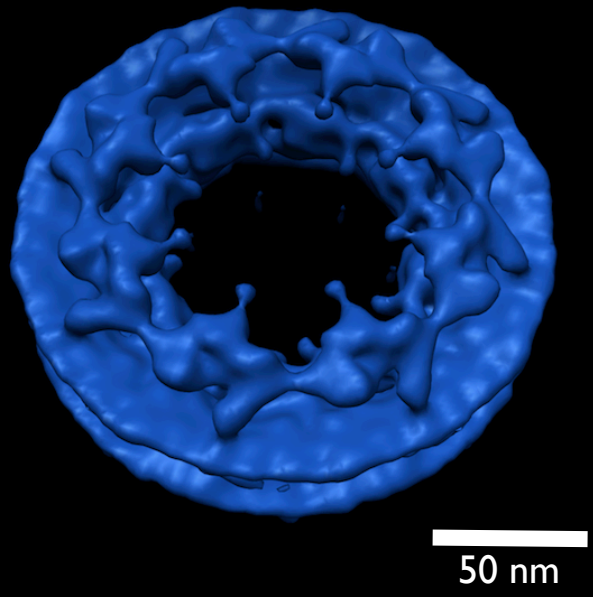




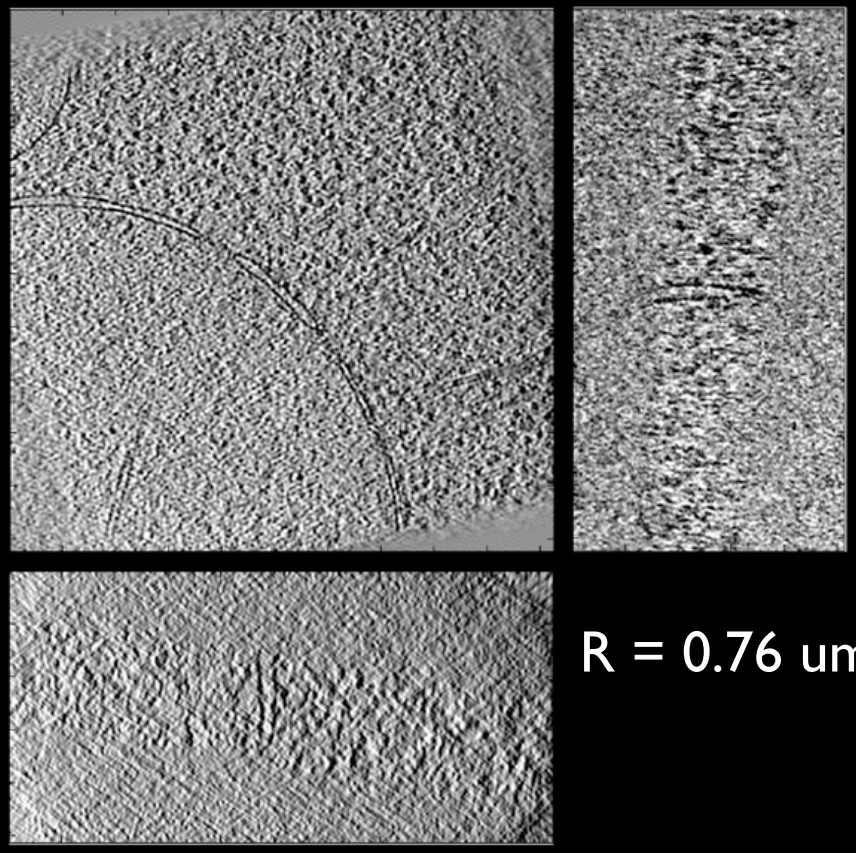
Architecture of the Nuclear Pore Complex





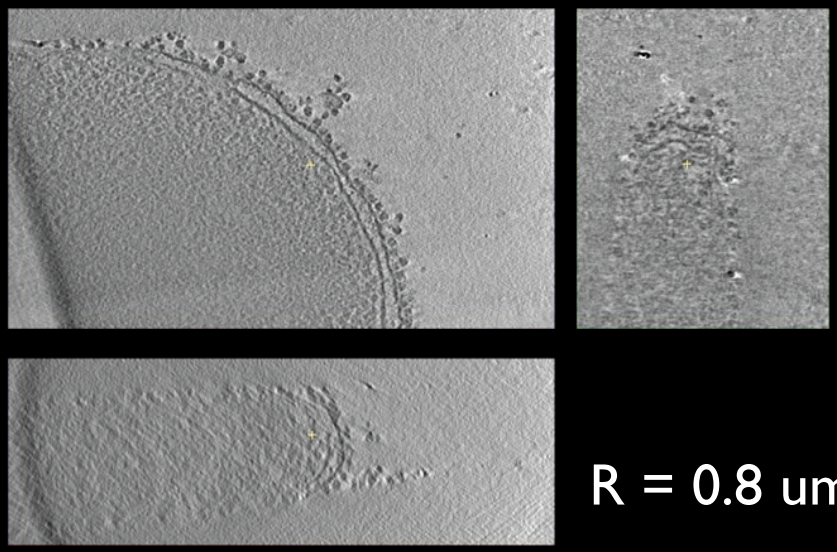


Sample Distortions



R = 0.76 um

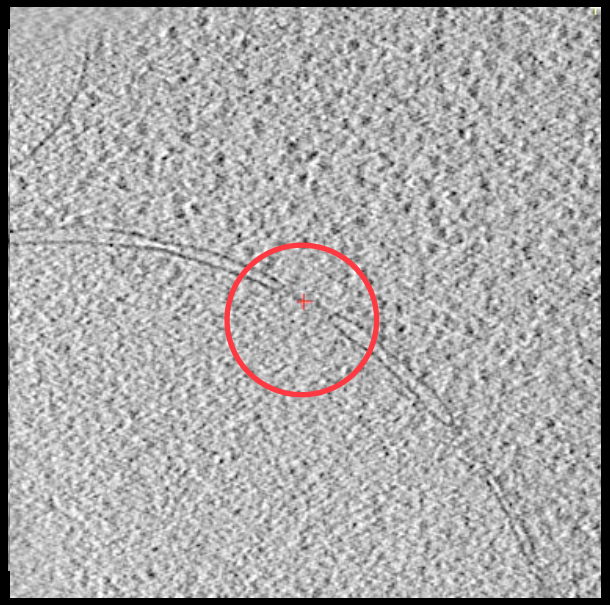
Sample Distortions

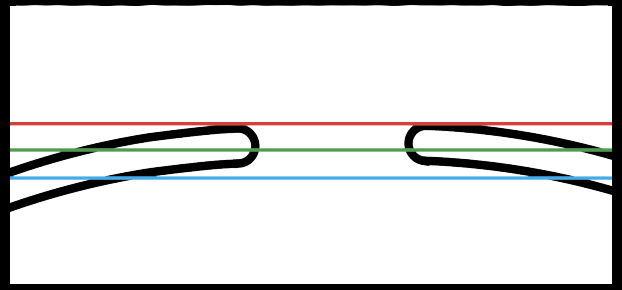


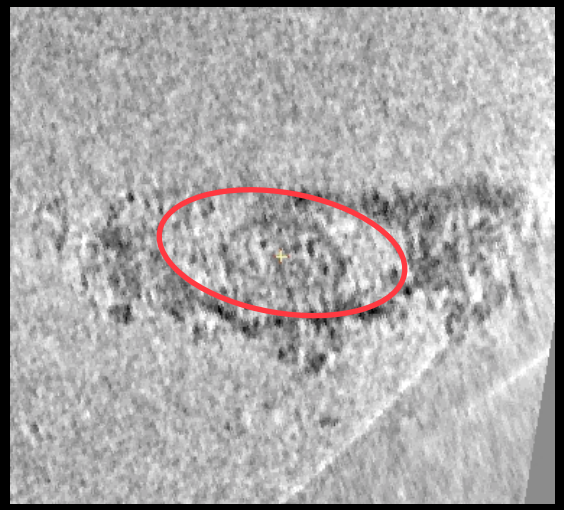
R = 0.8 um

1/3 compression

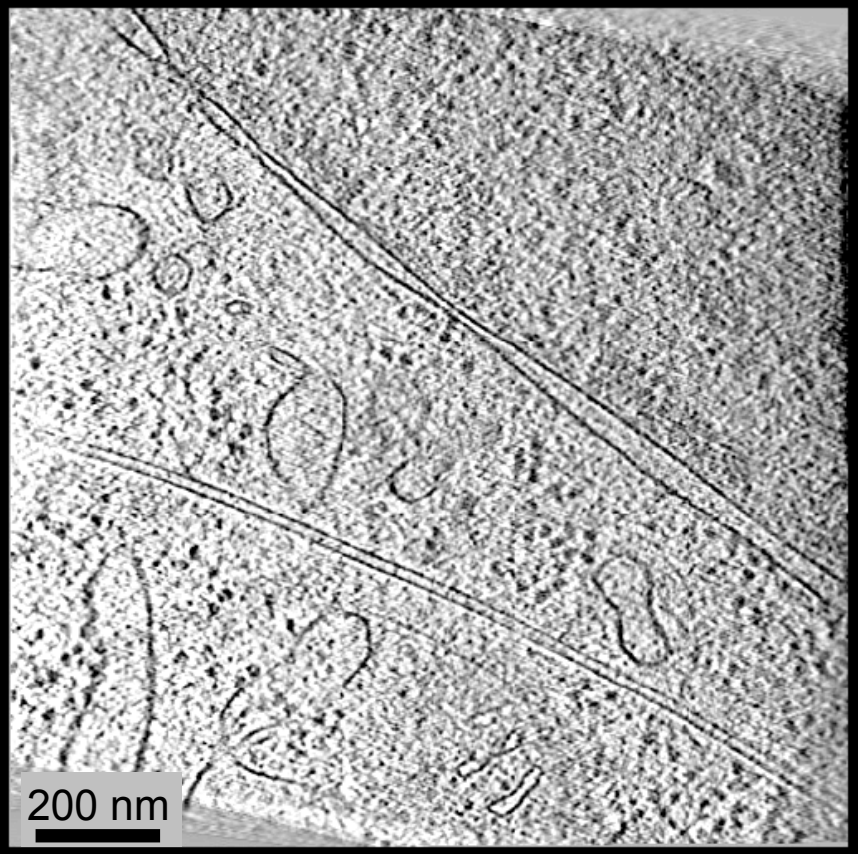
Sample Distortions



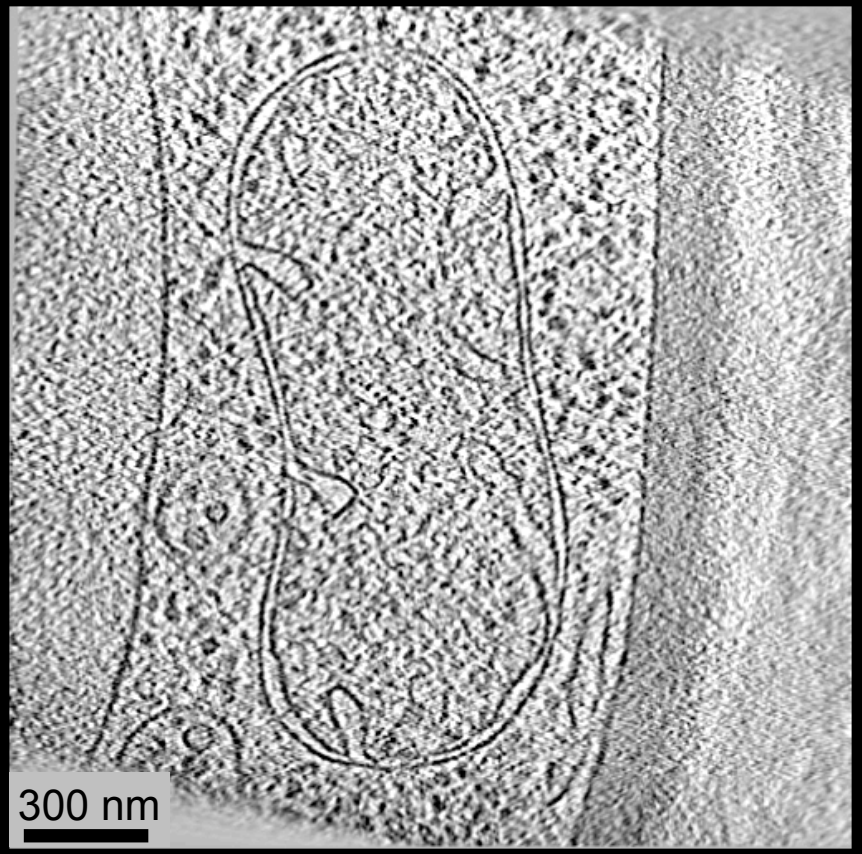




Other Applications



Mammalian Cell



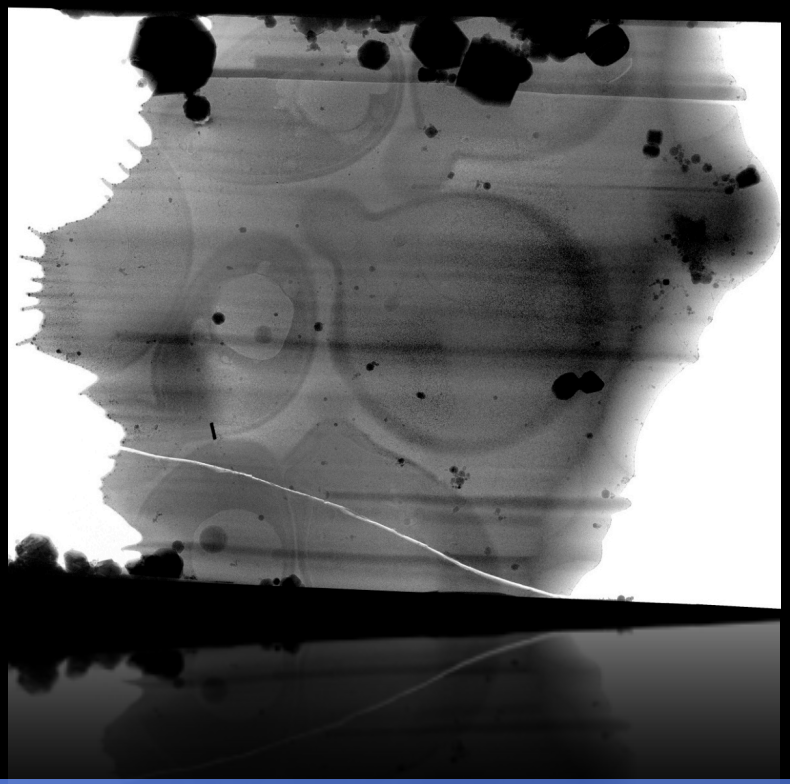
Mithocondria

What can go wrong?

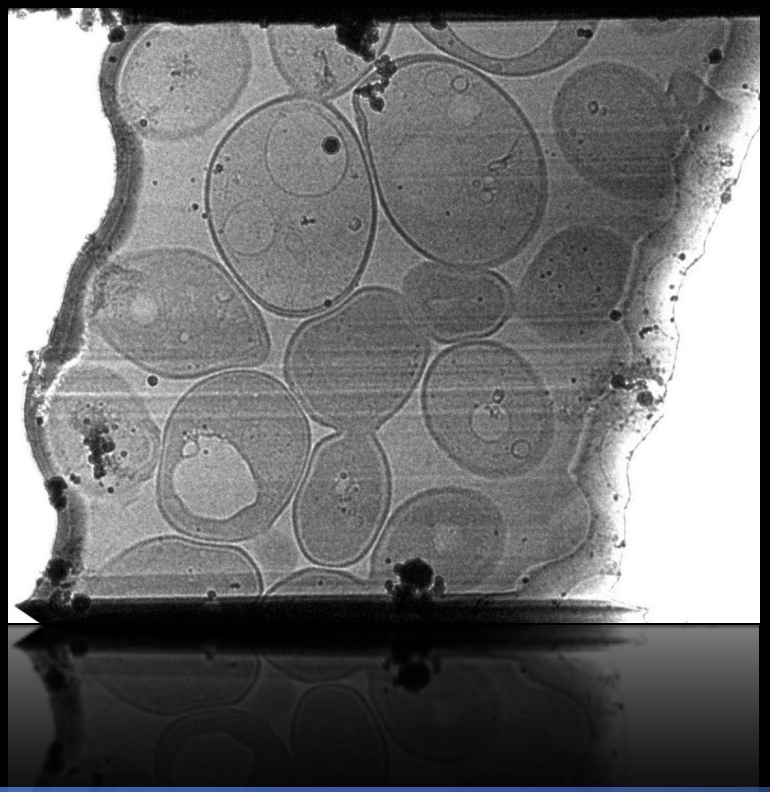
- Working distance
- Vitrification
  - Size of cells
  - Plunging conditions
  - High-pressure freezing
- Transfers
  - Autogrid
  - Polara cartridge
- Curtaining
  - Platinum coating
  - (need rotational stage)
- Goo
  - Leaks
- Charging
  - Don’t see it.. yet
- Alignment Problems
  - Correlation-based
  - Hopefully no gold
- Locate sample
  - 3D Correlative LM-EM

Platinum coating to avoid beam erosion

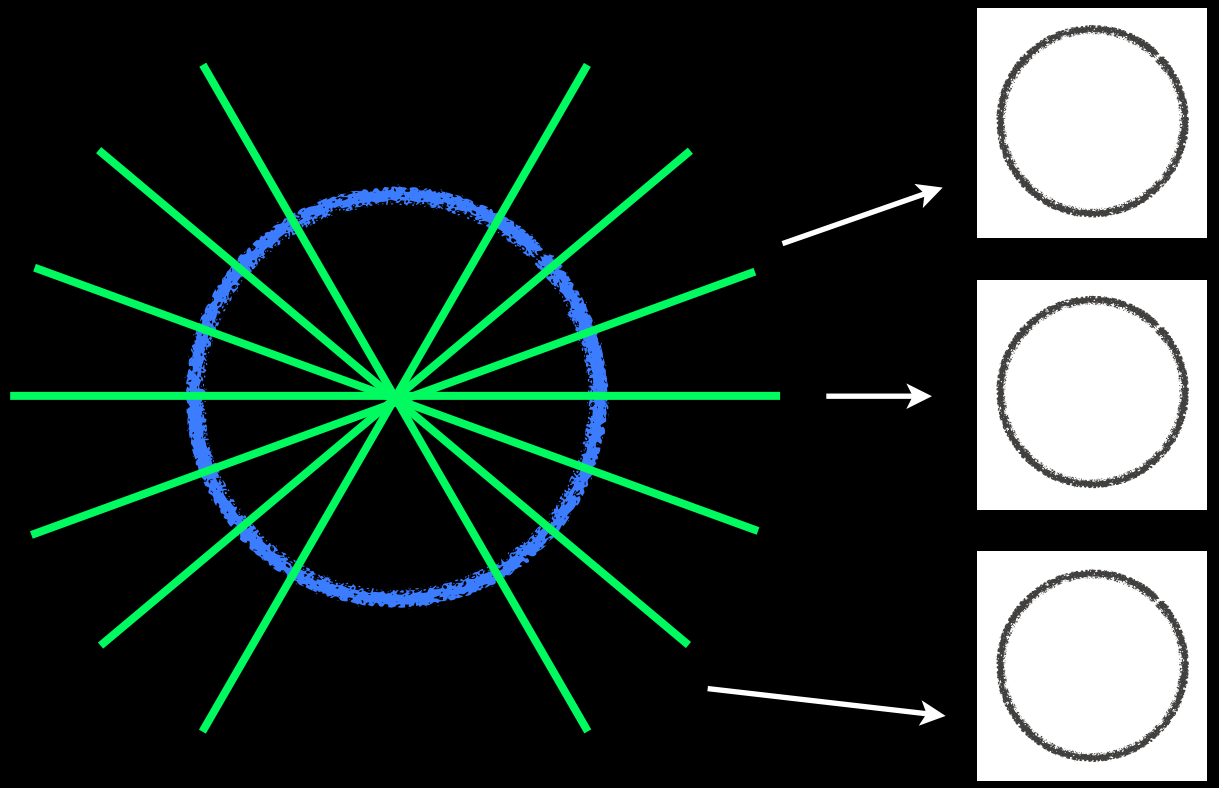
Progressing Beam Erosion



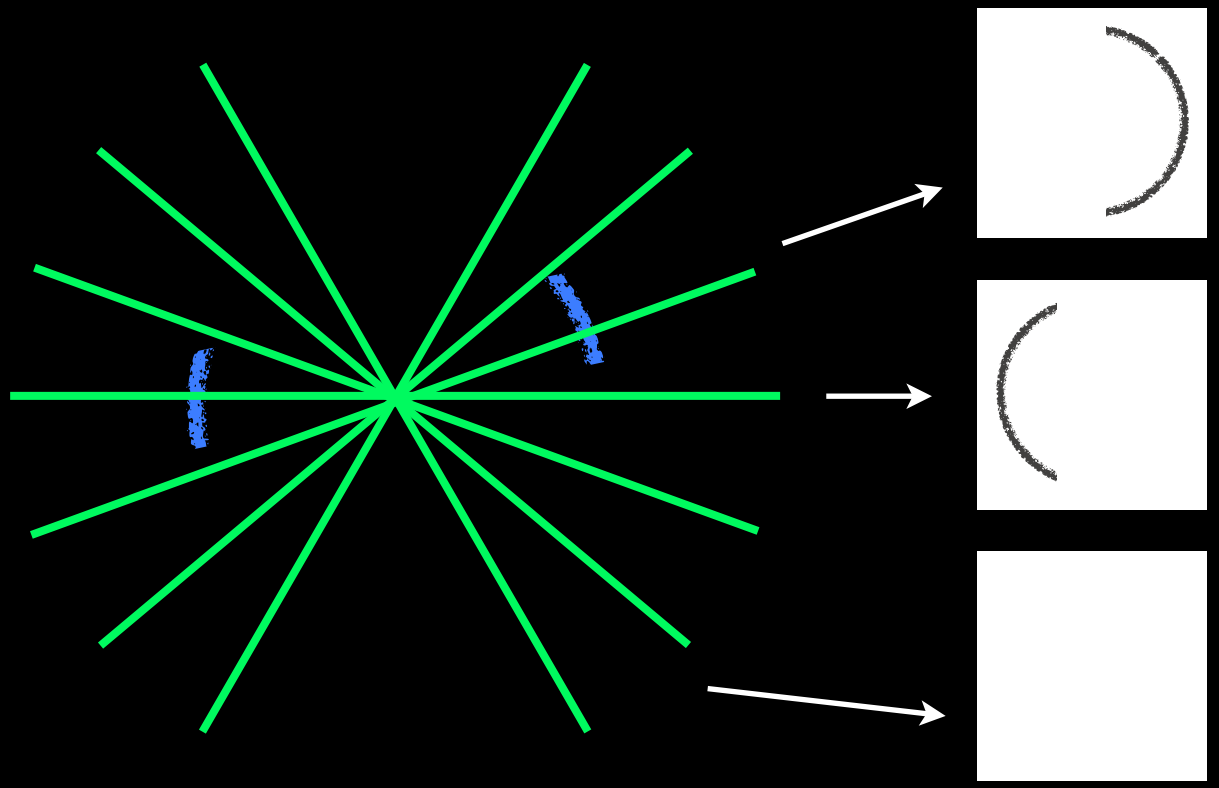
Protective Pt coating



Feature-tracking alignment blues

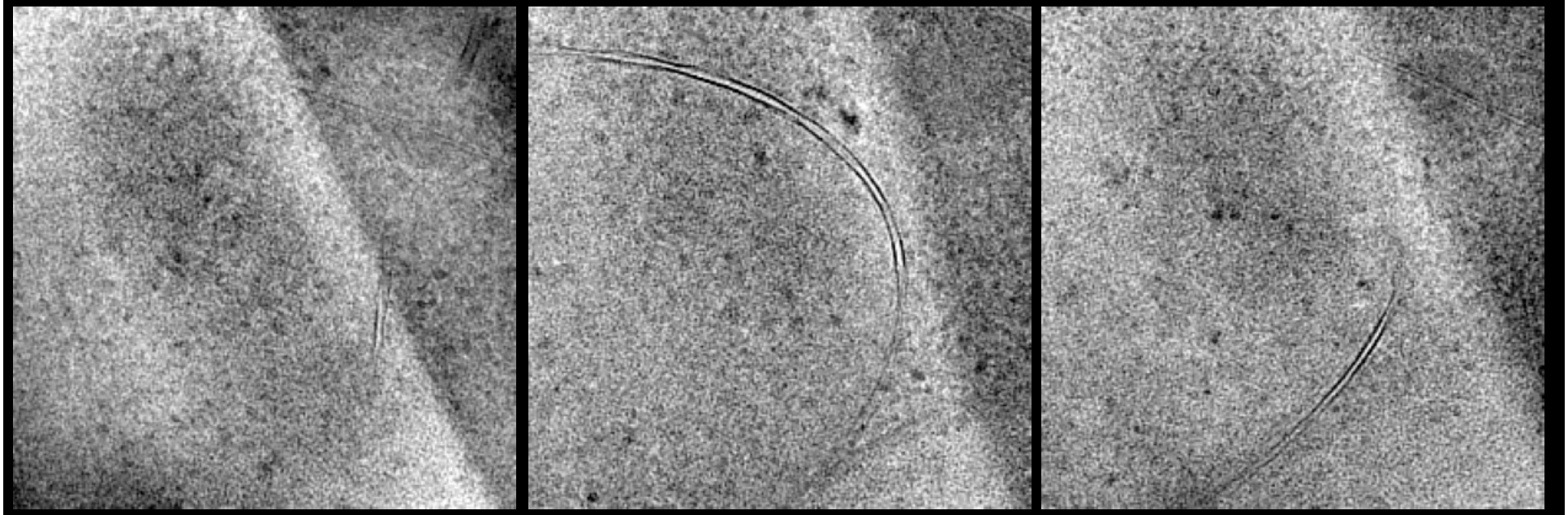


Feature-tracking alignment blues



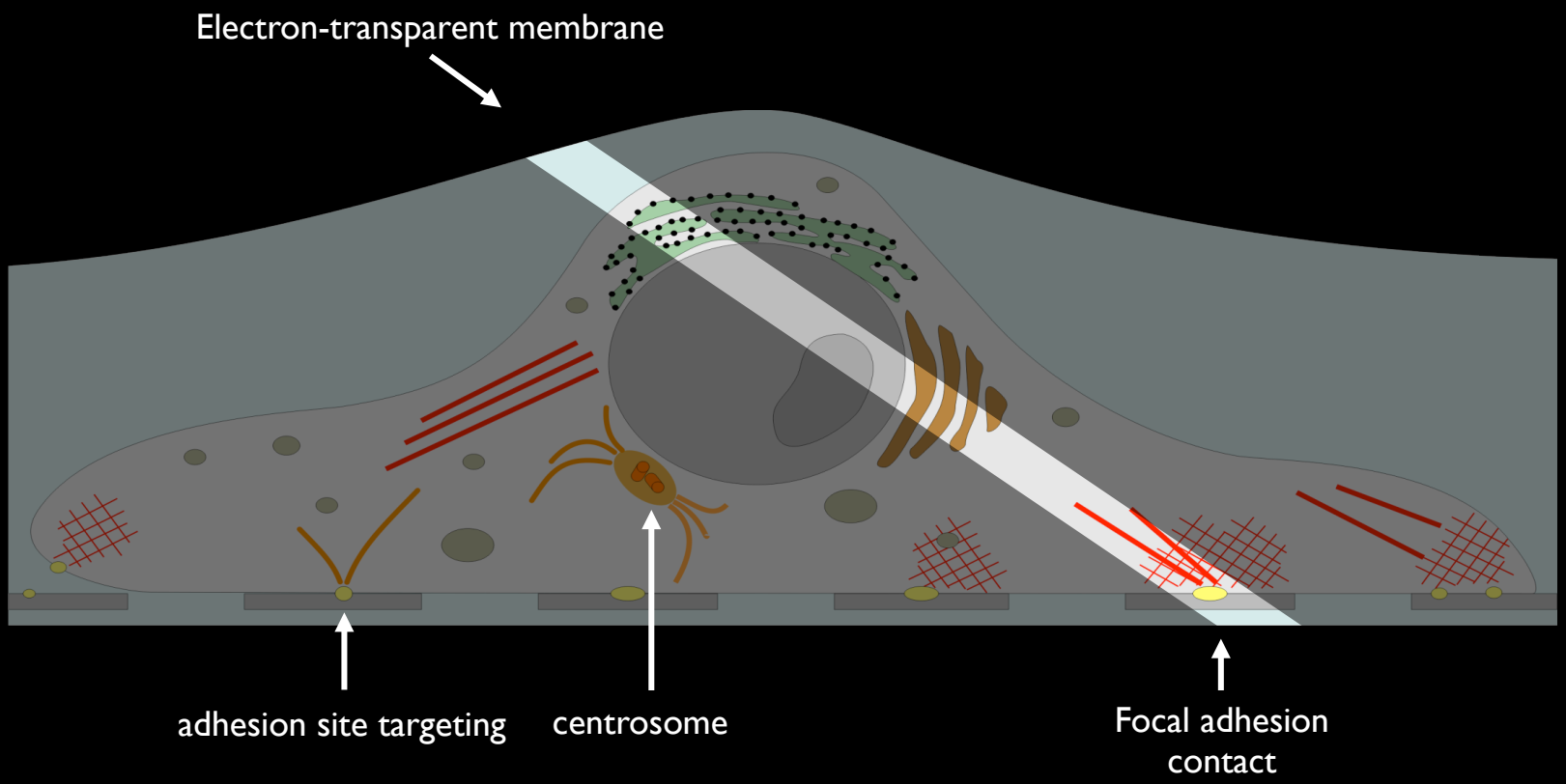
Feature-tracking alignment blues

Uber-blues: Goo, crystalline ice, curtaining



-30° 0° 40°

Challenge: 3-D Correlation



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Curtaining
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- (need rotational Stage)

Goo
- Leaks

Charging
- Don’t see it.. yet

Alignment Problems
- Correlation-based
- Hopefully no gold

Locate sample
- 3D Correlative LM-EM

But when it works.. it’s all worth it

Acknowledgements

Wolfgang Baumeister

Jürgen Plitzko (soon Utrecht!)
Felix Bauerlein
Tim Laughs
Alexander Rigort (now Zeiss)
Workshop

Lena Fitting Korkoutis
Miroslava Schaeffer

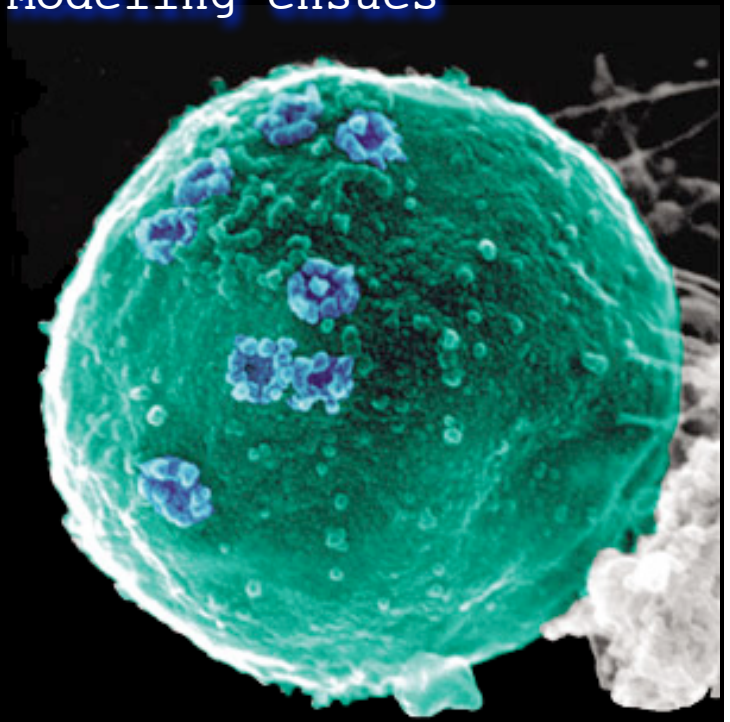
Friedrich Förster
Eri Sakata (now Yale)
Ohad Medalia (now Zurich)

DFG

SEVENTH FRAMEWORK PROGRAMME

Max Planck Society

Fat, concealed nucleus
Ion beam reveals the gate
Modeling ensues



Yeast nucleus imaged by SEM . Courtesy of Elena Kiseleva