SEMC @ NYSBC Winter EM Course 2019

Tomography

Introduction and Overview

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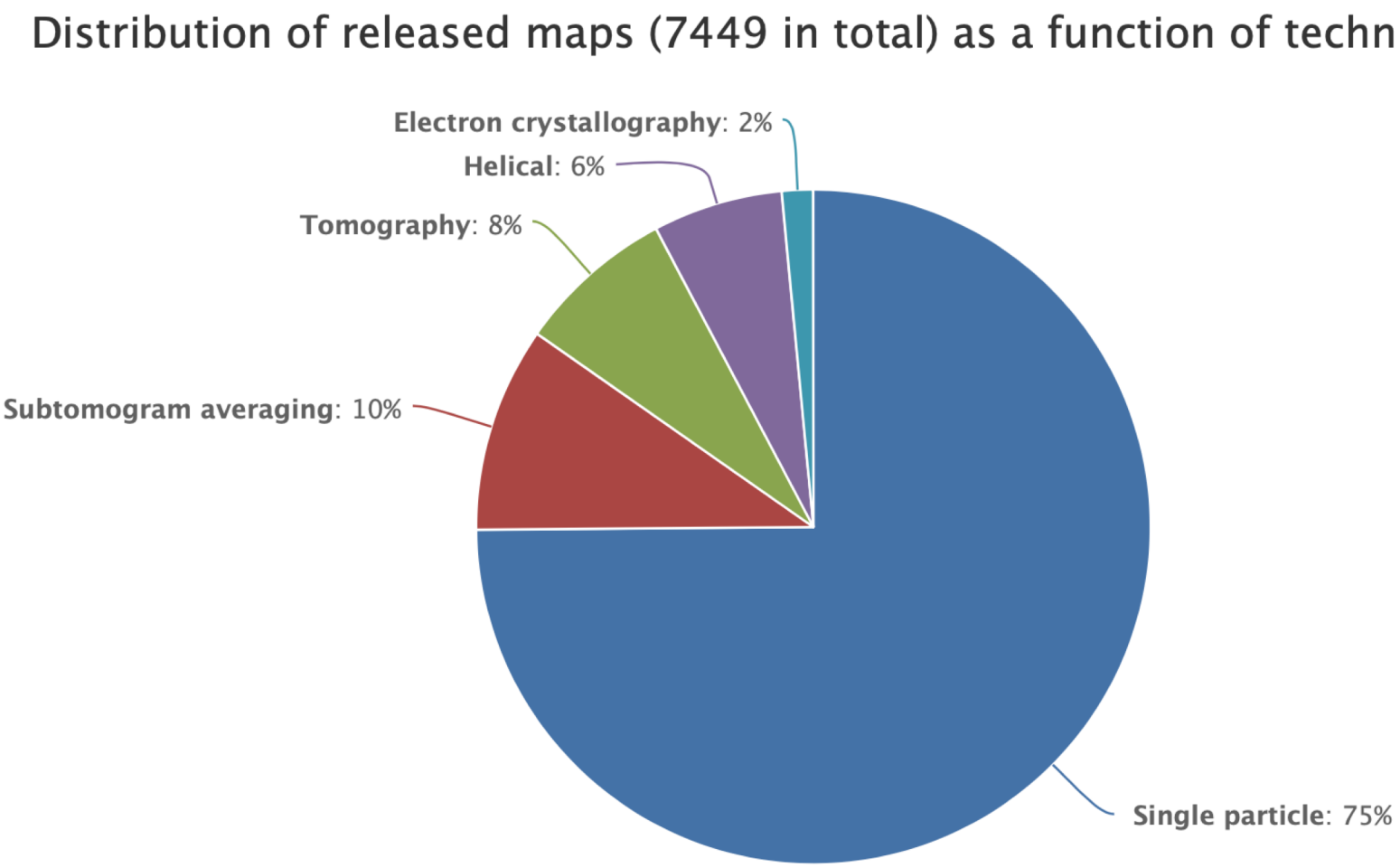
Outline

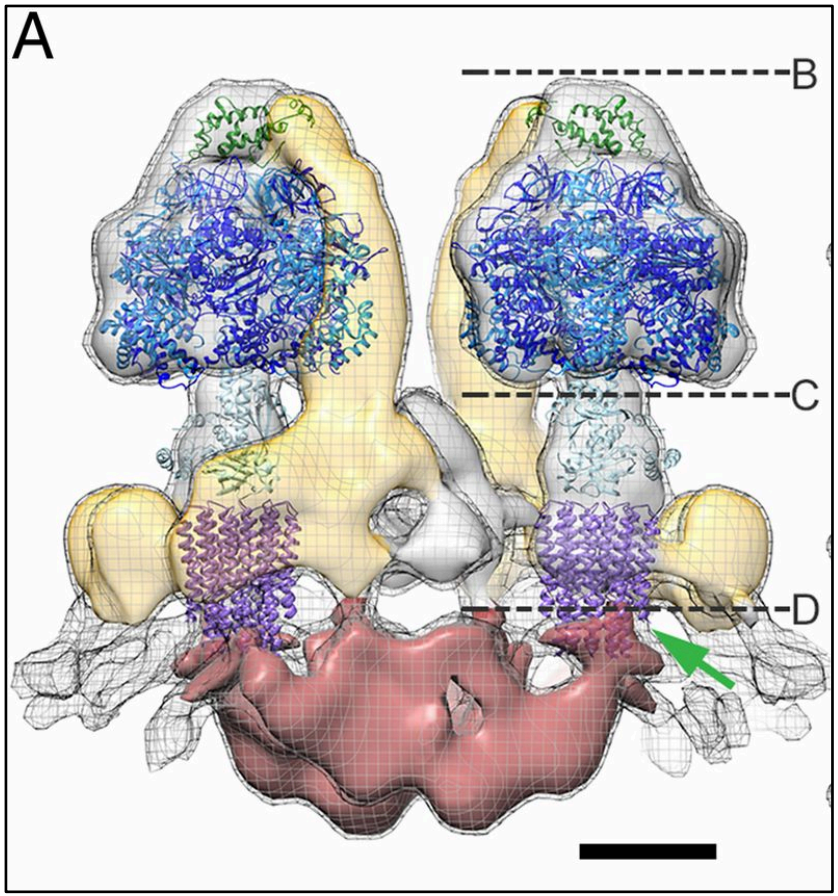
- What samples are for cryo- cryo-electron tomography?
- Sample preparation for cryo-electron tomography
- A case study: Correlated cryo-PALM-CryoET

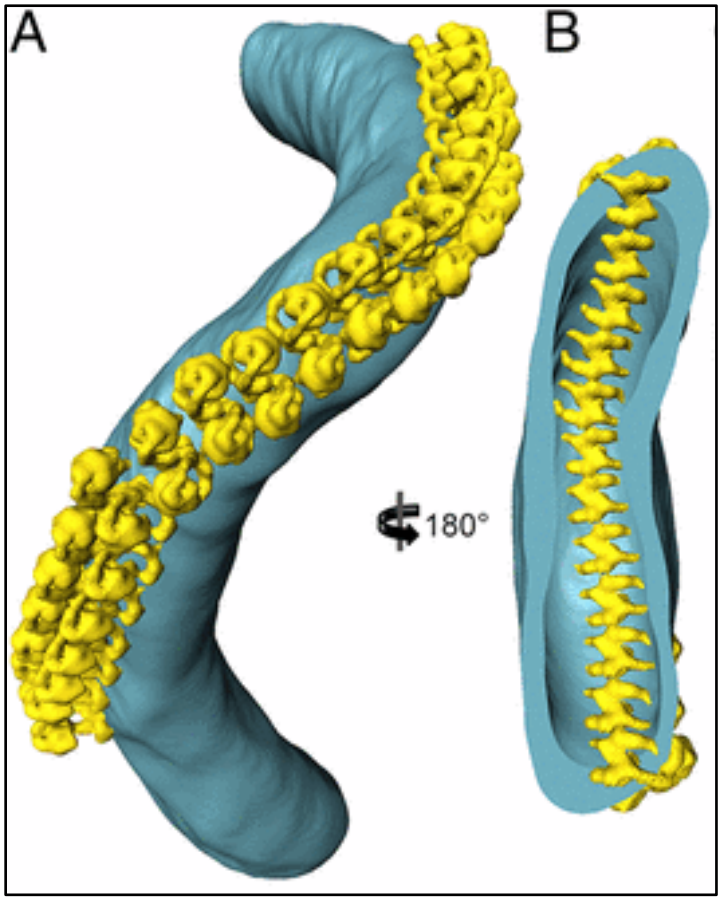
Structures Resolved by Tomography in EMDB



Cryo-Electron Tomography

- Large protein complexes: mitochondria ATP synthase dimer

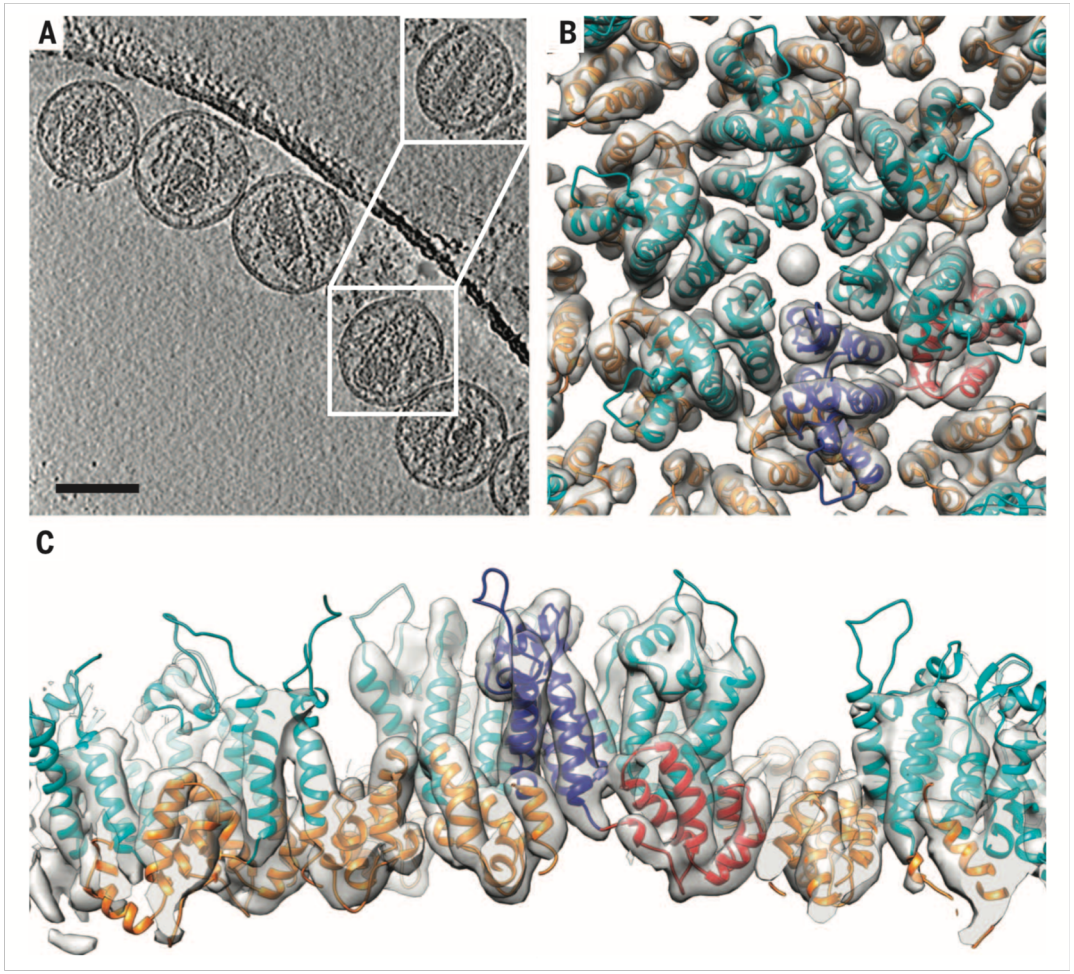


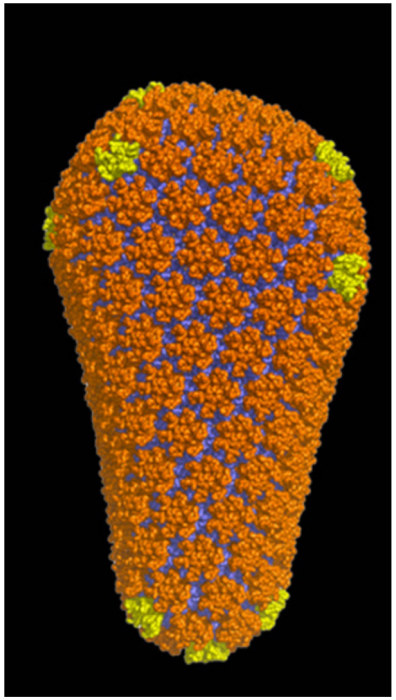


Alexander W. Mühleip *et al.* PNAS 2016;113:8442-8447

Cryo-Electron Tomography

- HIV CA hexamer



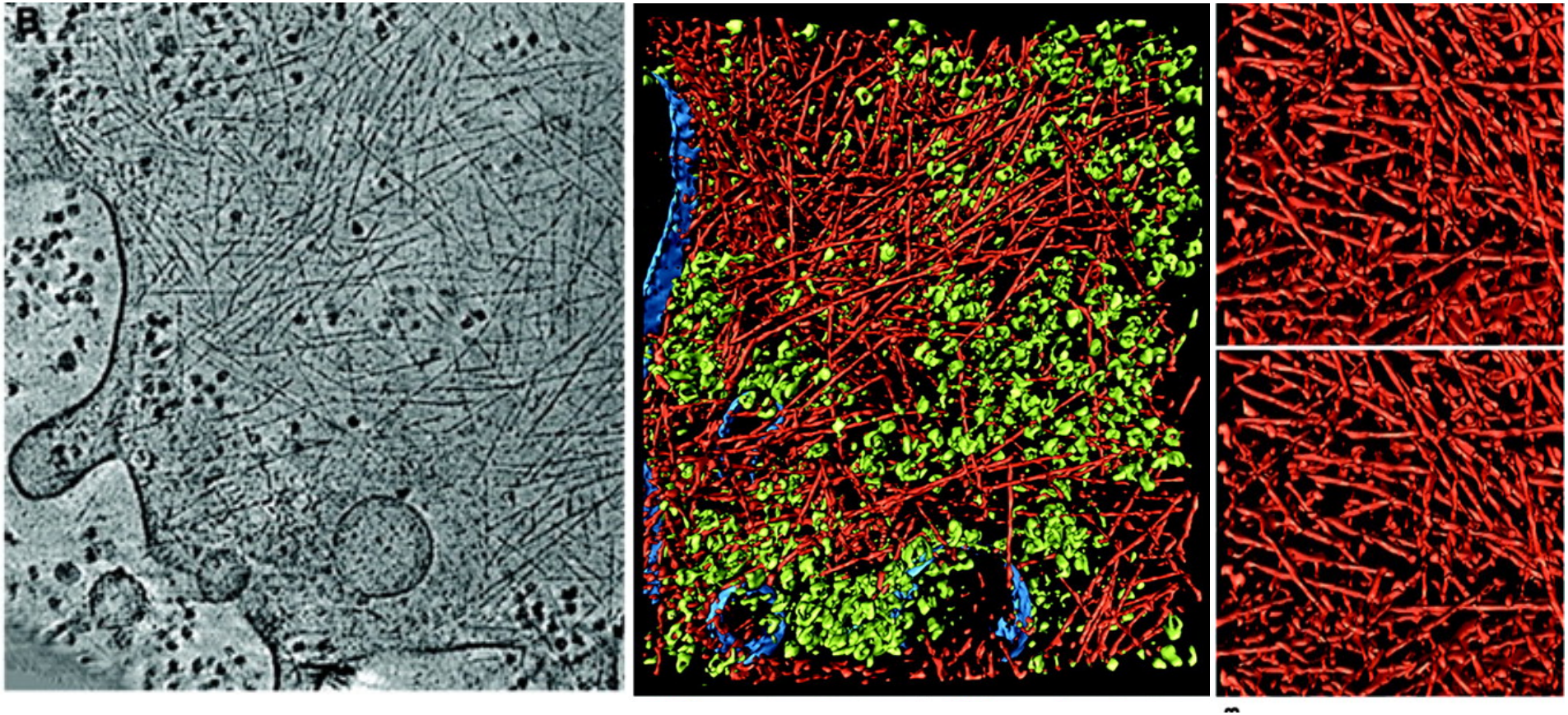


Conical fullerene cone

S. Mattei *et al*. Science 2016;354:6318

Cryo-Electron Tomography

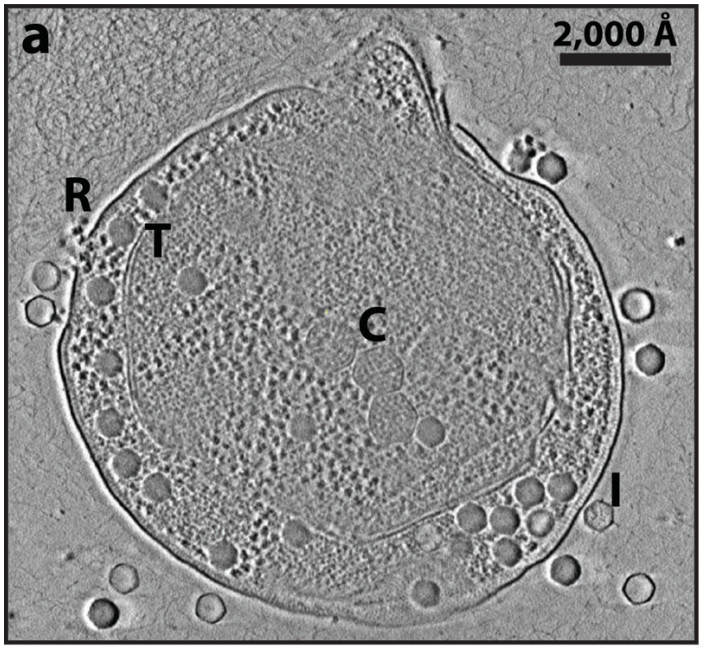
- Edge of eukaryotic cells

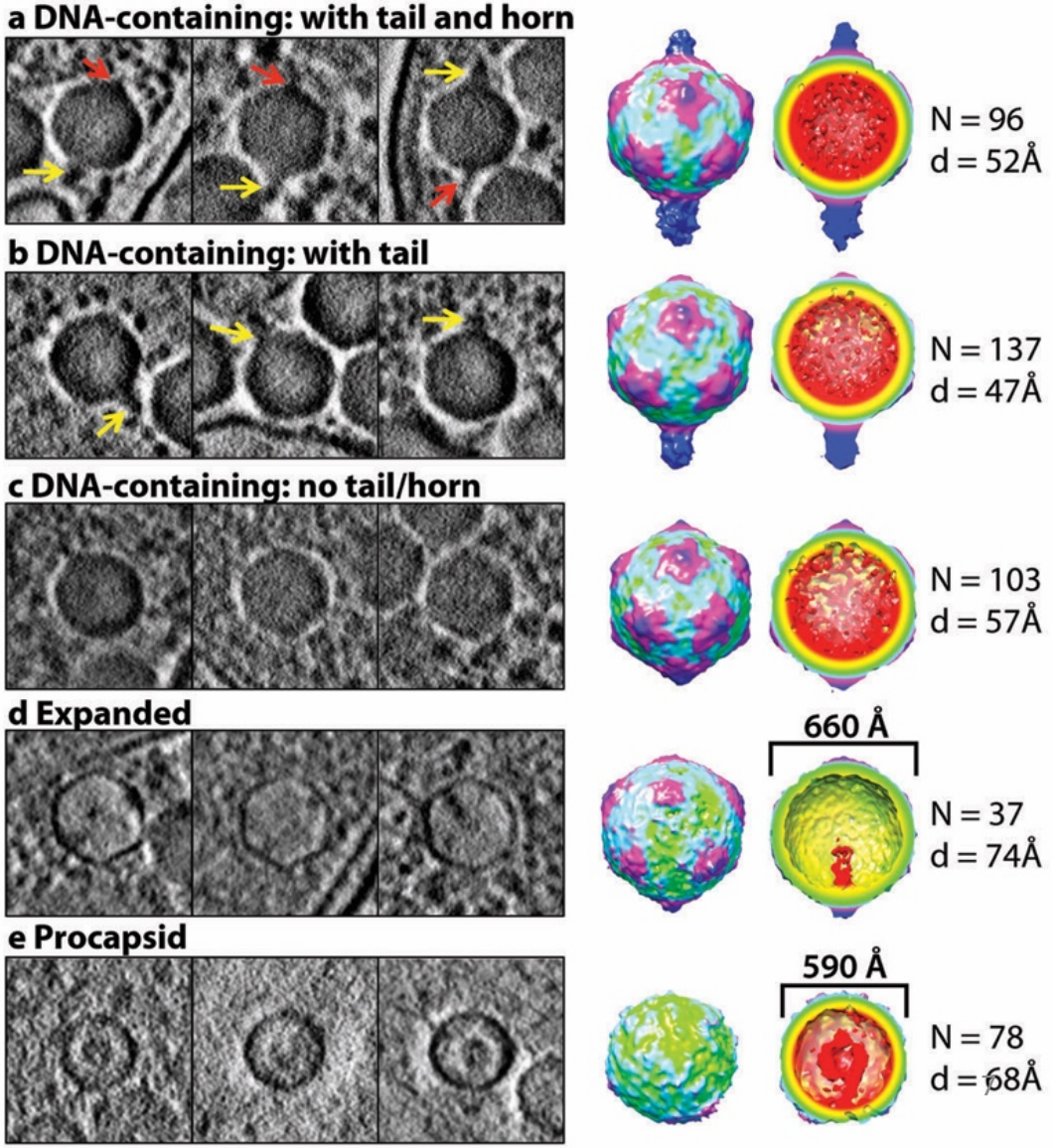


Medalia et al. Science 298, 2002

Cryo-Electron Tomography

- Phage/Viruses





Dai et al 2013 Nature

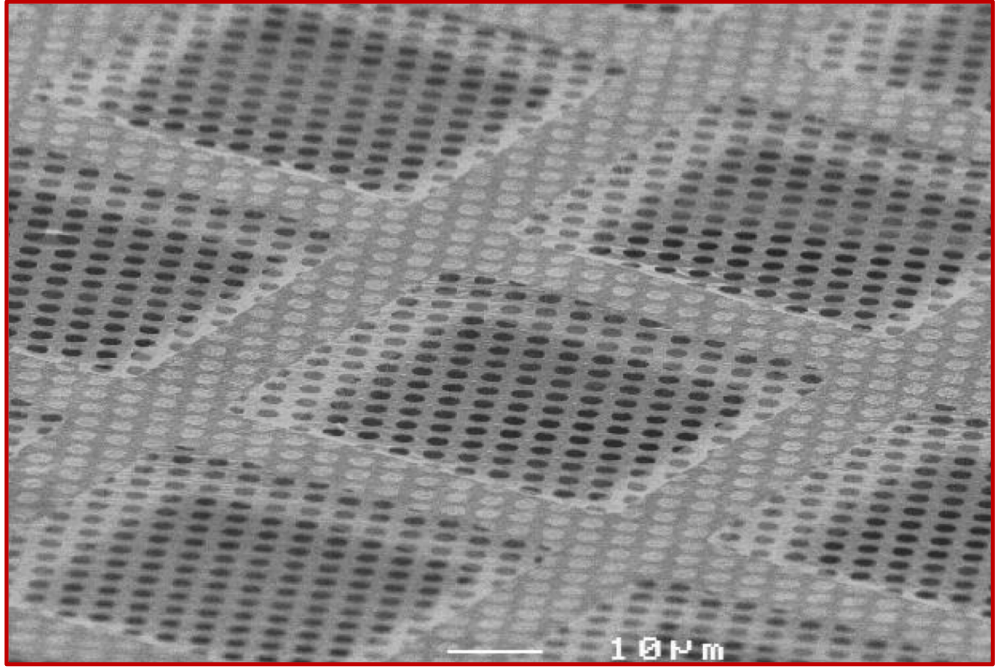
Why Tomography?

- Sample has a unique structure or heterogenous
  - Single particle tomography and subtomogram classification and averaging
- Sample in a complex environment
  - Cellular tomography

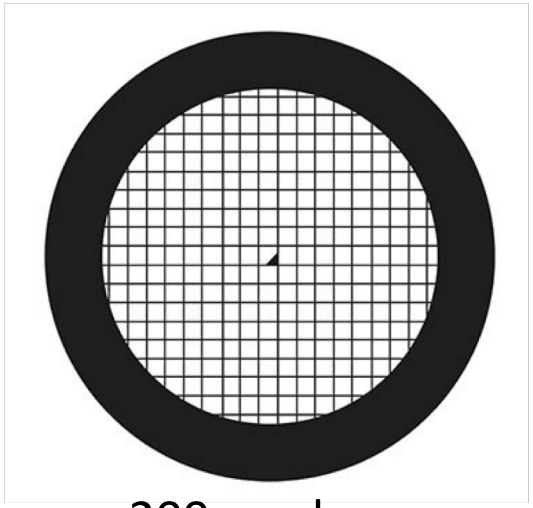
Sample Preparation

- Preserving various structural elements of the specimen in native structure in aqueous solution
- Good concentration
- Good thickness & good contrast

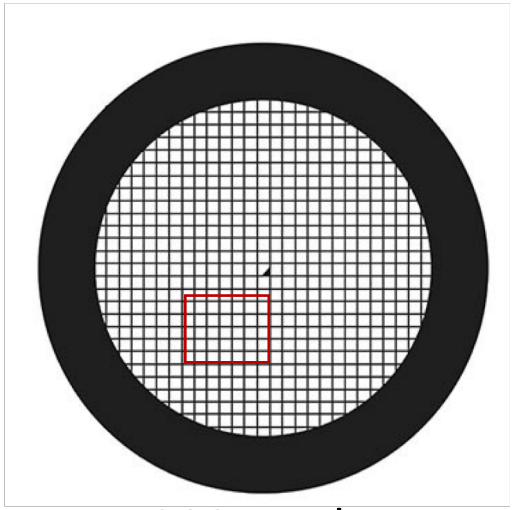
EM Grids



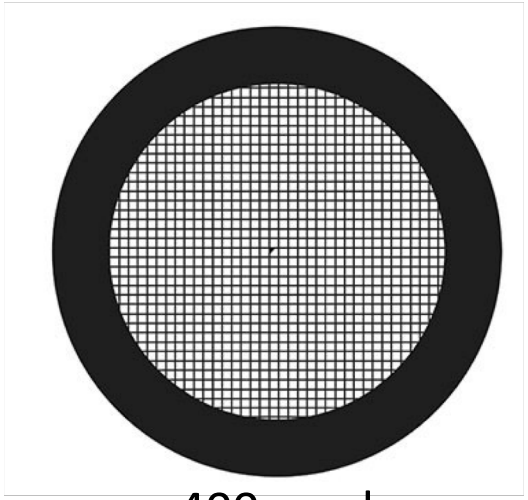
- Material:
  - Copper
  - Gold
  - Molybdenum
- Thickness: 10 – 25 um
- Usually have an additional layer of
- Mesh: define the square number on the grid



200 mesh



300 mesh



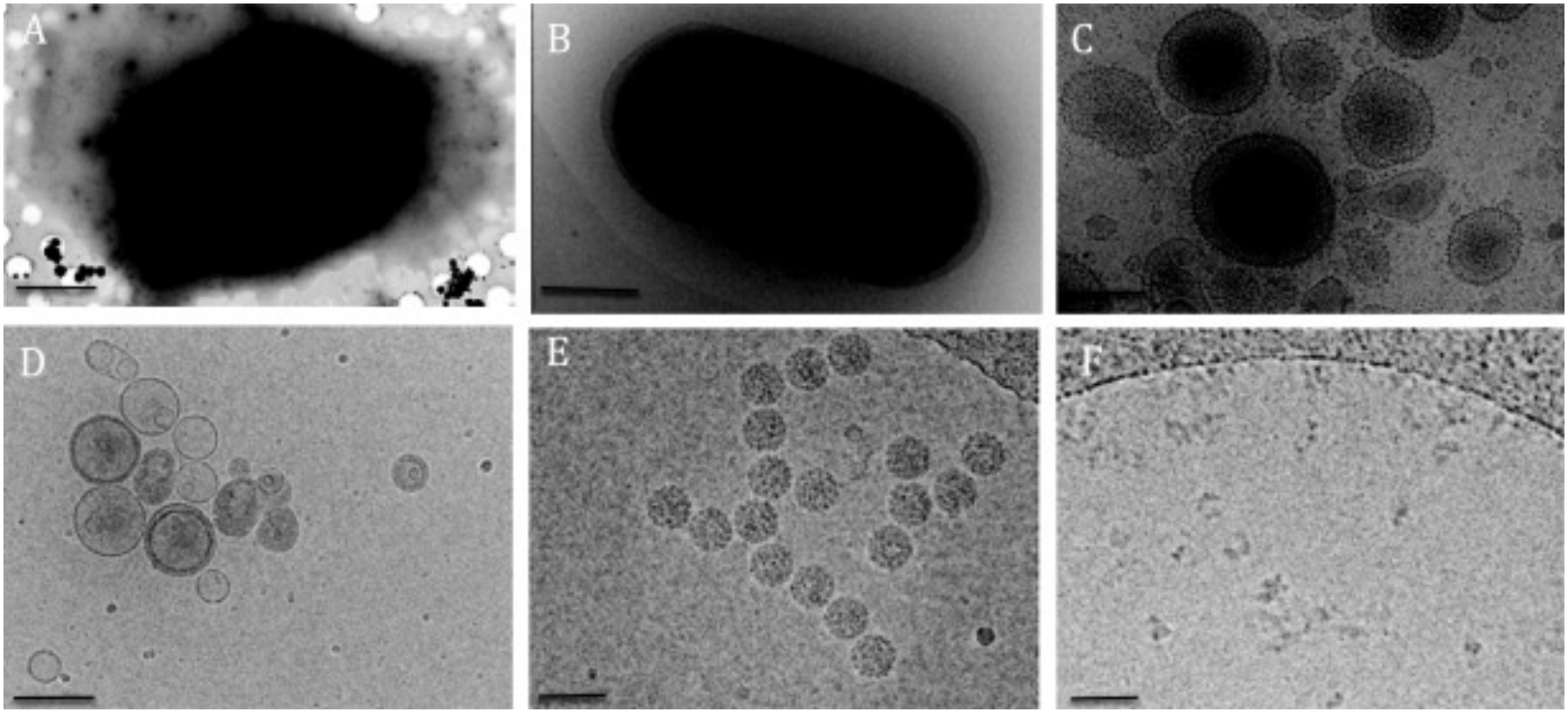
400 mesh

Sample Preparation in EM

- Conventional fixation and staining
  - Negative staining
  - Chemical fixation and dehydration

- Cryo-preservation
  - Plunger freezing (5 µm)
  - Freeze slamming (10 µm)
  - Propane jet freezing (40 µm)
  - High pressure freezing (100 - 200 µm)

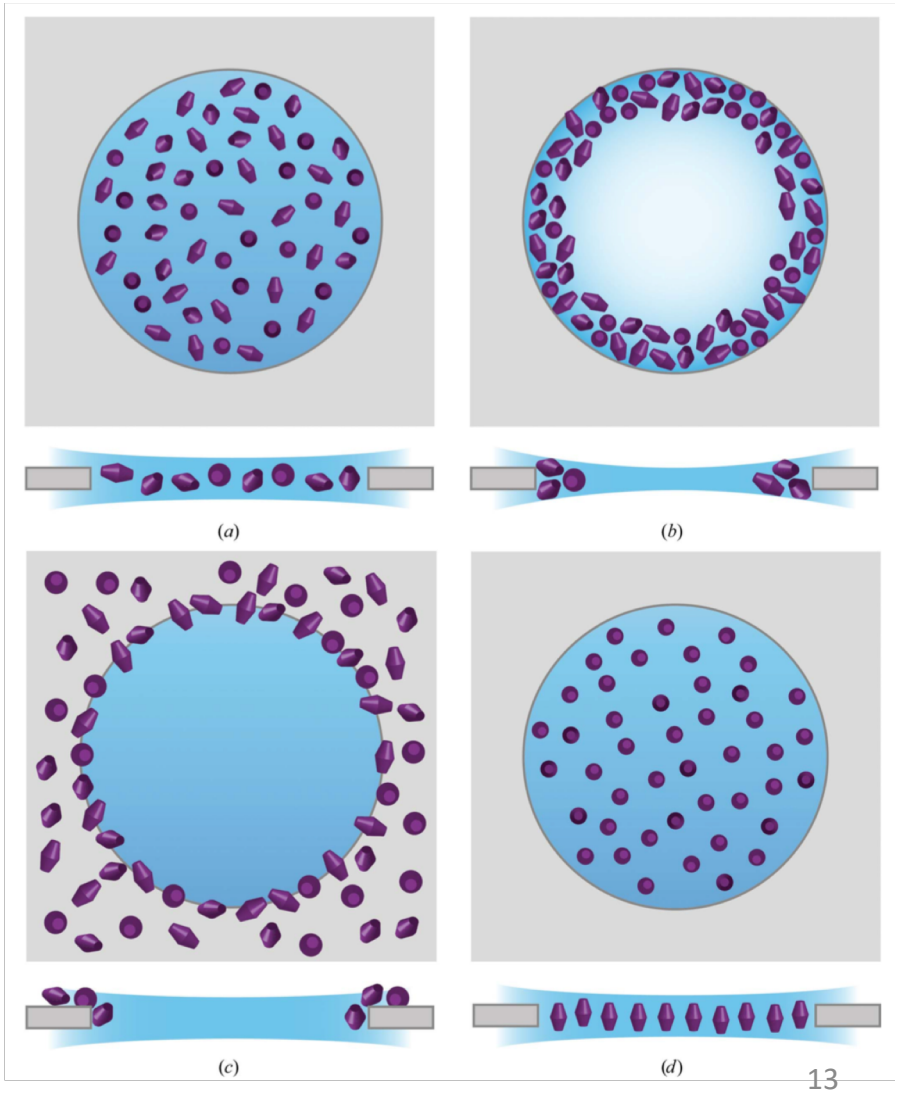
Specimen Preserved by Plunge Freezing



R. F. Thompson, *et al.*, 2016
Methods, Vol. 100 , 3-15

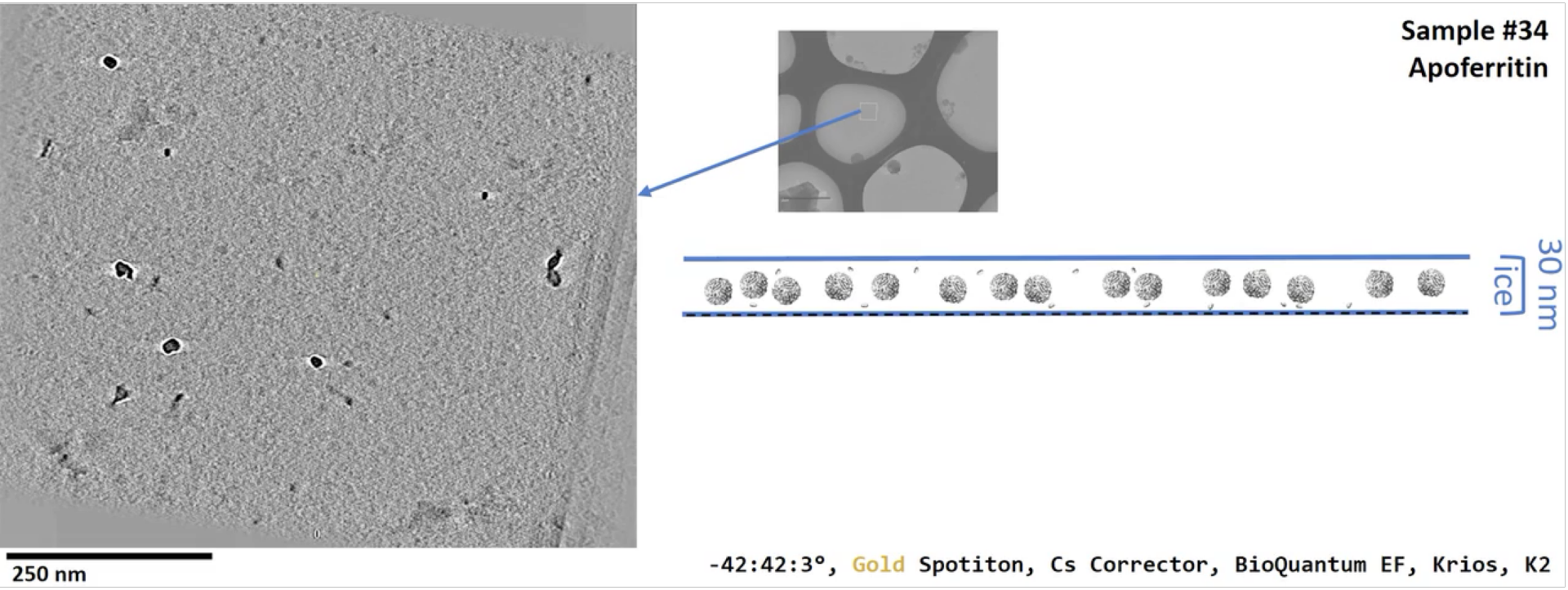
Particle Density, Distribution and Orientation

- Specimen concentration
- Volume used
- Buffer
- EM grid type
- Supporting film
- Glow discharge/plasma cleaning parameters
- Plunger
- Blotting conditions



L. Drulyte, *et al.*, 2018 *Acta. Cryst.*

Particle Density, Distribution and Orientation



A. Noble, *et al.*, 2018 Nature Methods

Preparing Intact Mammalian Cells For Cellular Tomography

Prepare EM Grids
- Material: gold
- Mesh: 200 mesh
- Supporting film: carbon or SIO_2
- Hole size: support *vs* contrast

↓

Plate Cells on EM Grids
- Coating protein
- Plating density

↓

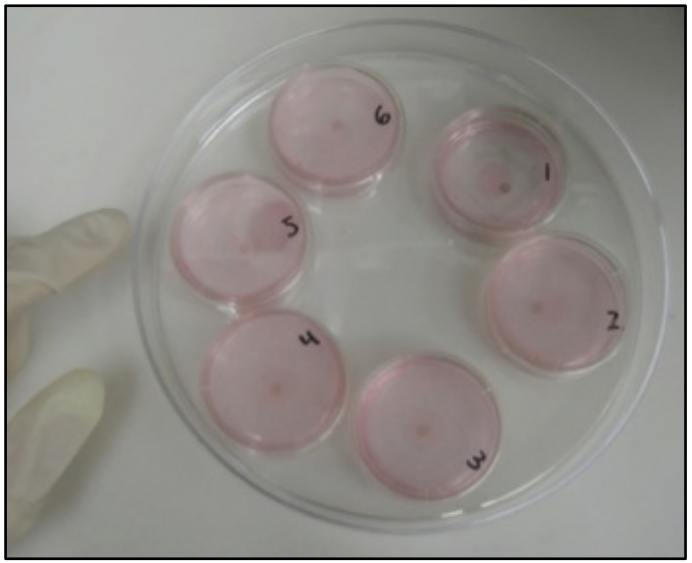
Allow Cells to Adhere/Grow

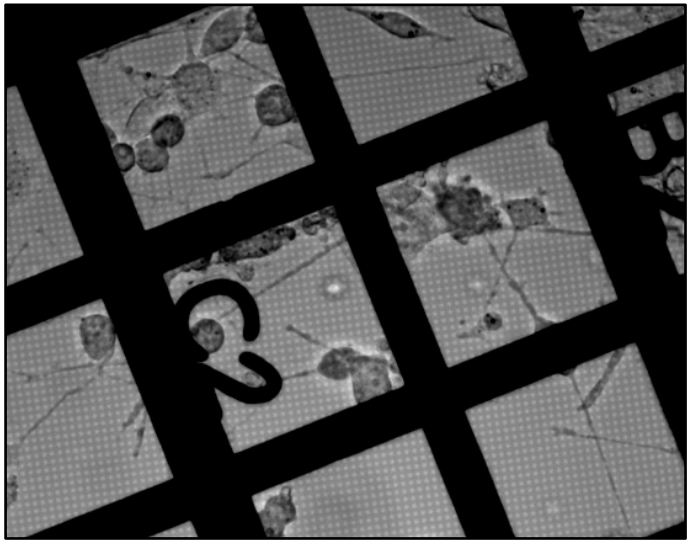
↓

Plunge Freeze Grids

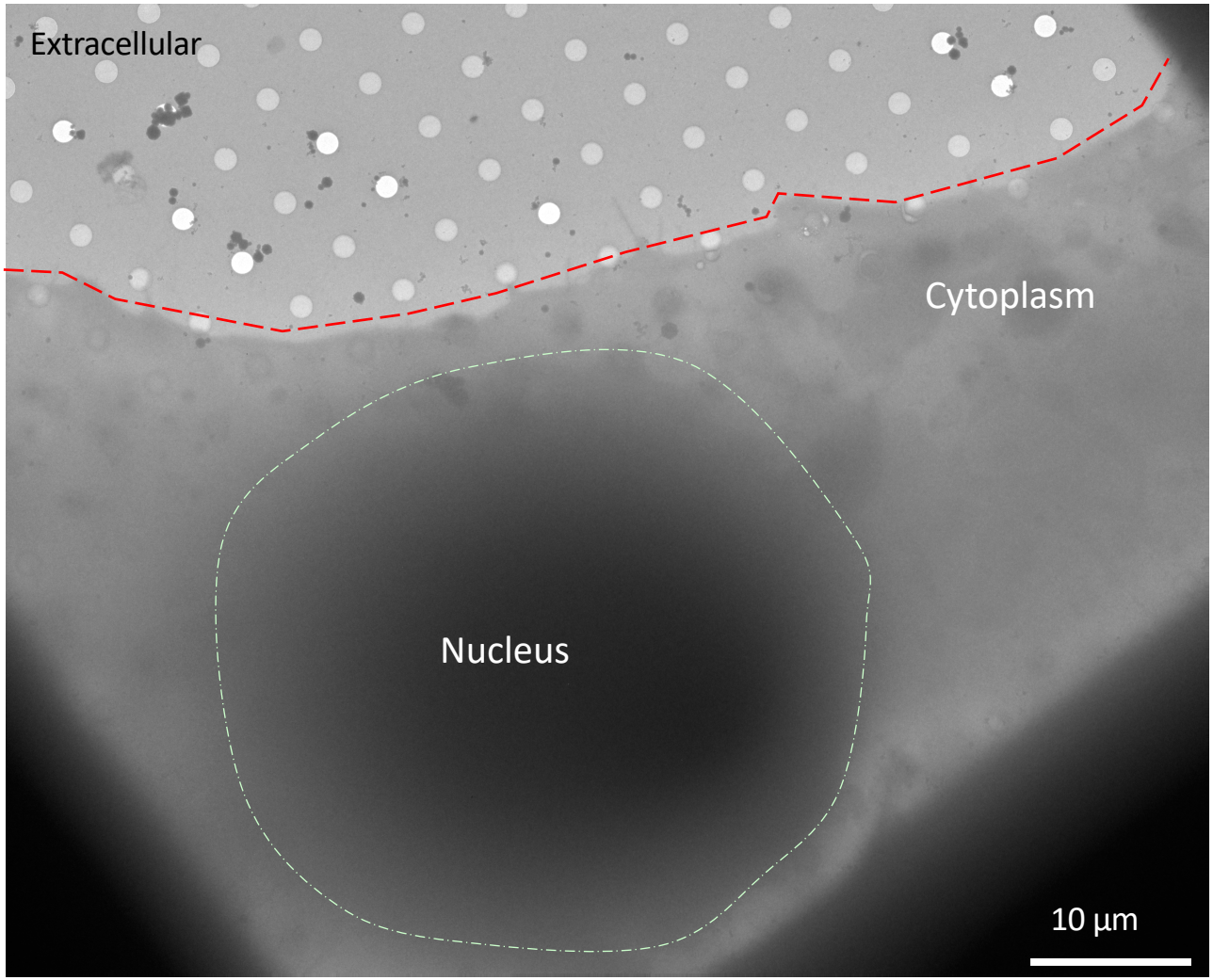
↓

Imaging



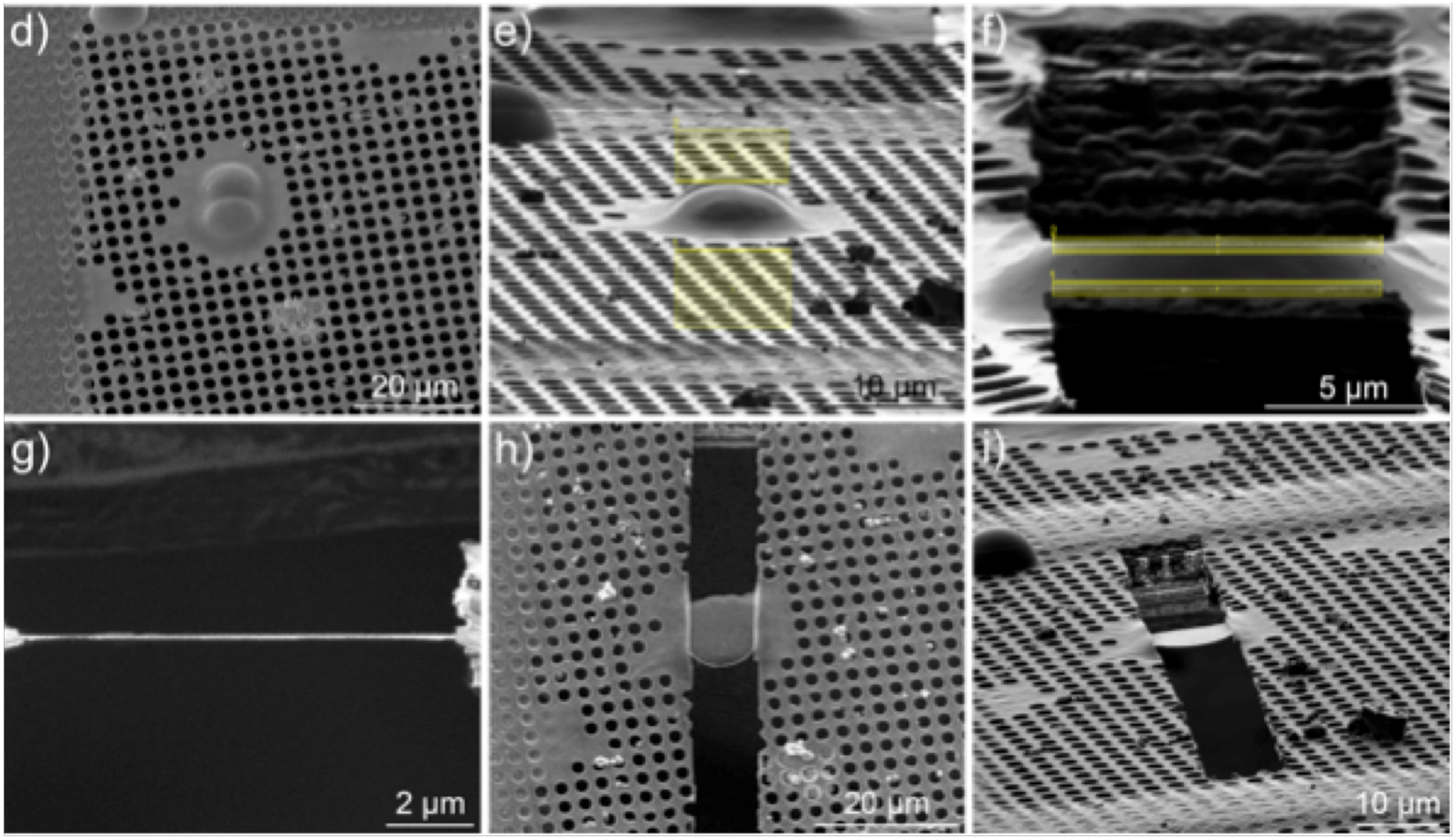


Imaging Intact Mammalian Cells



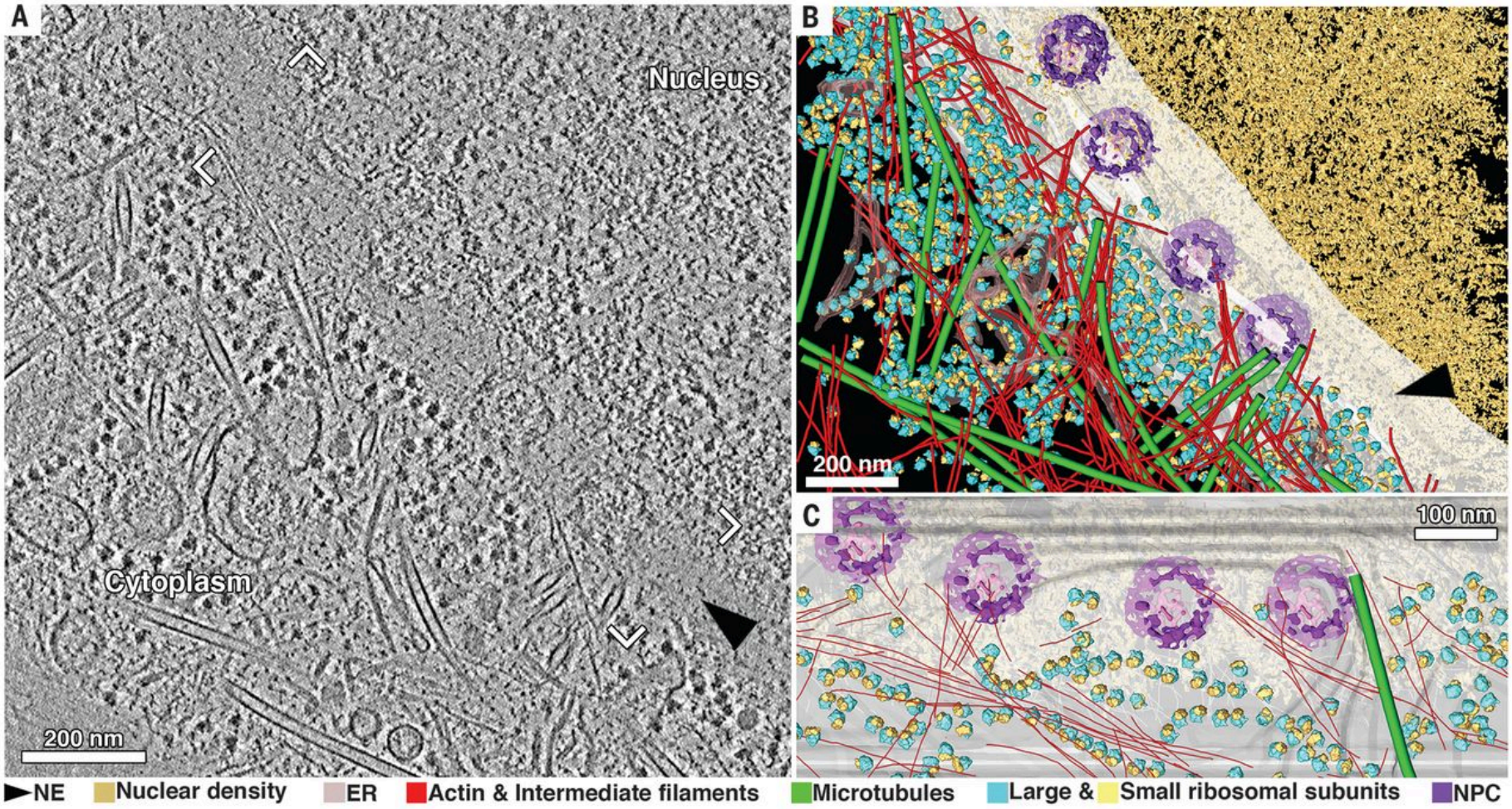
Corey Hecksel

Focused Ion Beam



M. Schaffer, et al 2015, Bio-protocol, 5 (17)

Nuclear Periphery Revealed by FIB and Tomography



J. Mahamid, et al 2016, Science, 351 (6276) 969-972

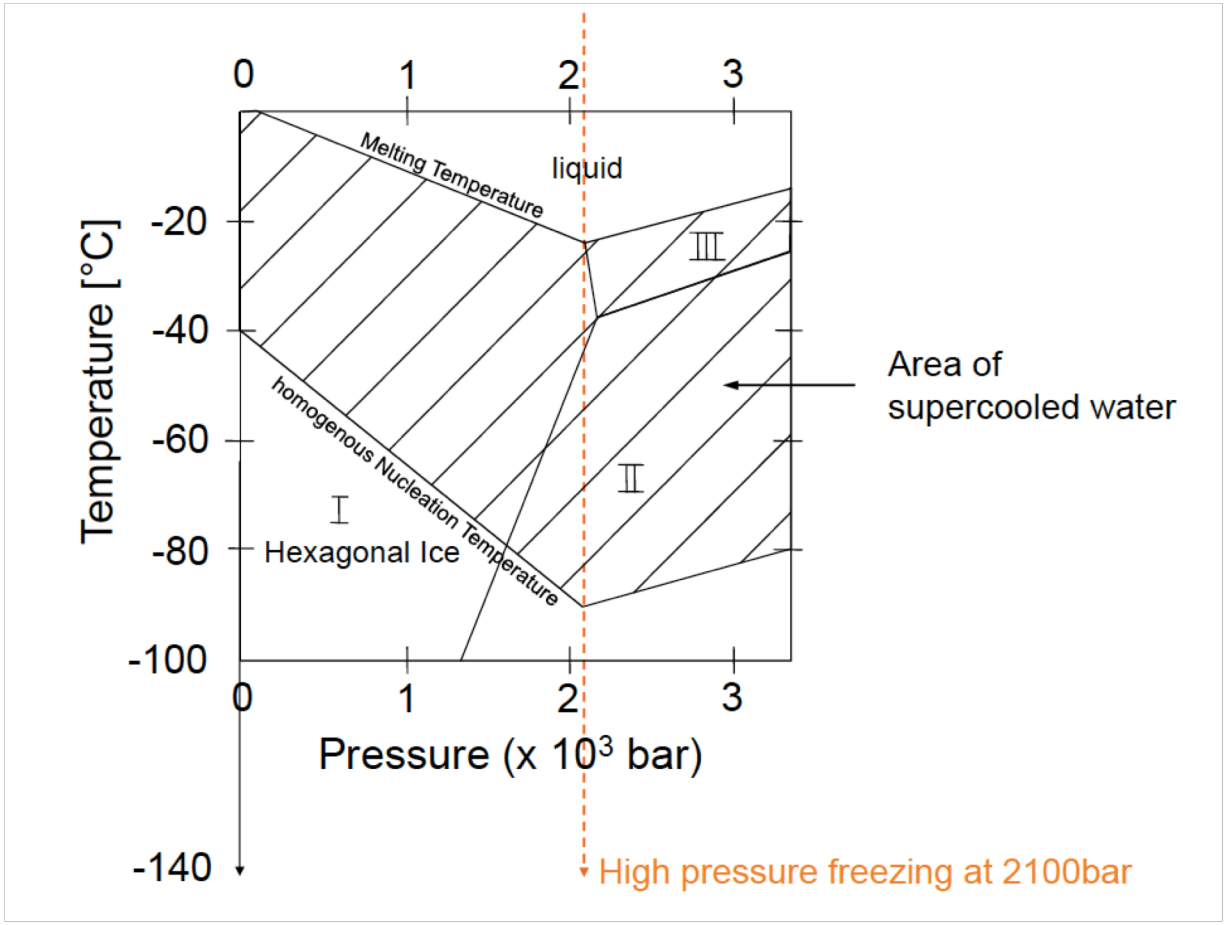
Sample Preparation in EM

- Conventional fixation and staining
  - Negative staining
  - Chemical fixation and dehydration

- Cryo-preservation
  - Plunger freezing (5 µm)
  - Freeze slamming (10 µm)
  - Propane jet freezing (40 µm)
  - High pressure freezing (100 - 200 µm)

High-pressure Freezing and Cryo-sectioning

- High pressure delays ice crystal nucleation



Kanno H, 1975, Science 189:880-881

High-pressure Freezing and Cryo-sectioning

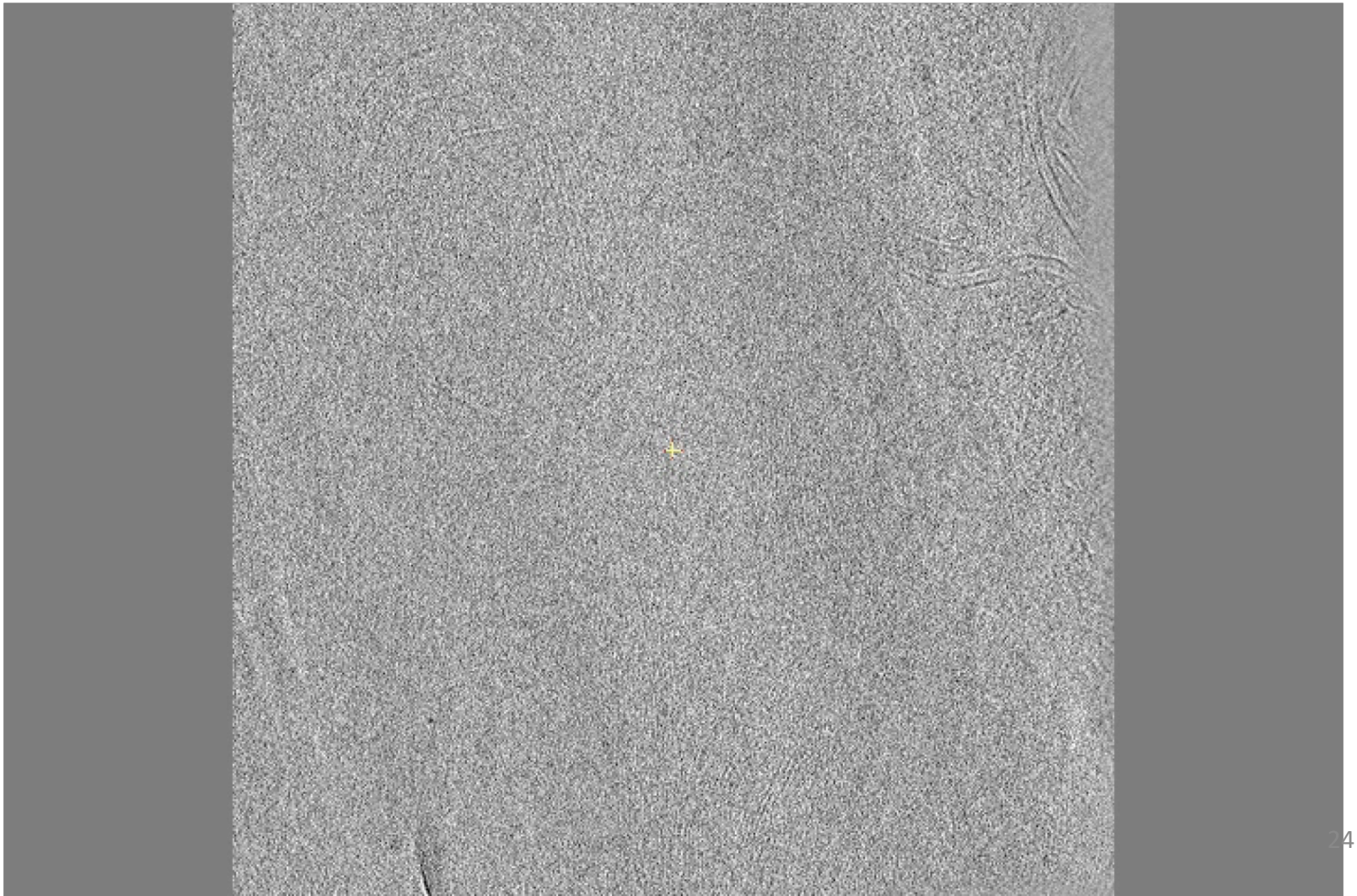
High-pressure freezer

Cryo-microtome

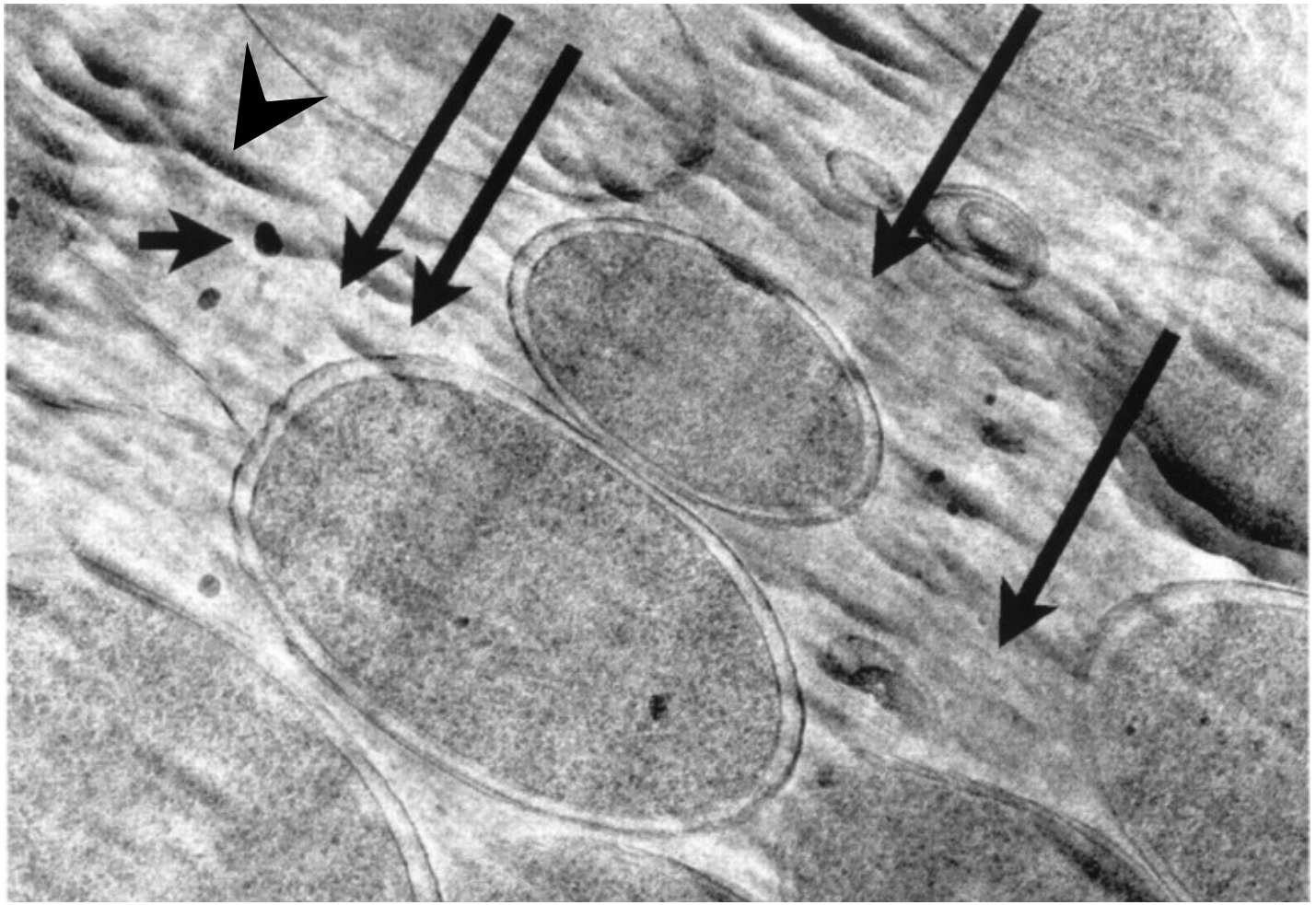
Cryo-sectioning

https://www.youtube.com/watch?v=q4Ydgv9dm00

Tomogram of Cyanobacteria Cryo-sections

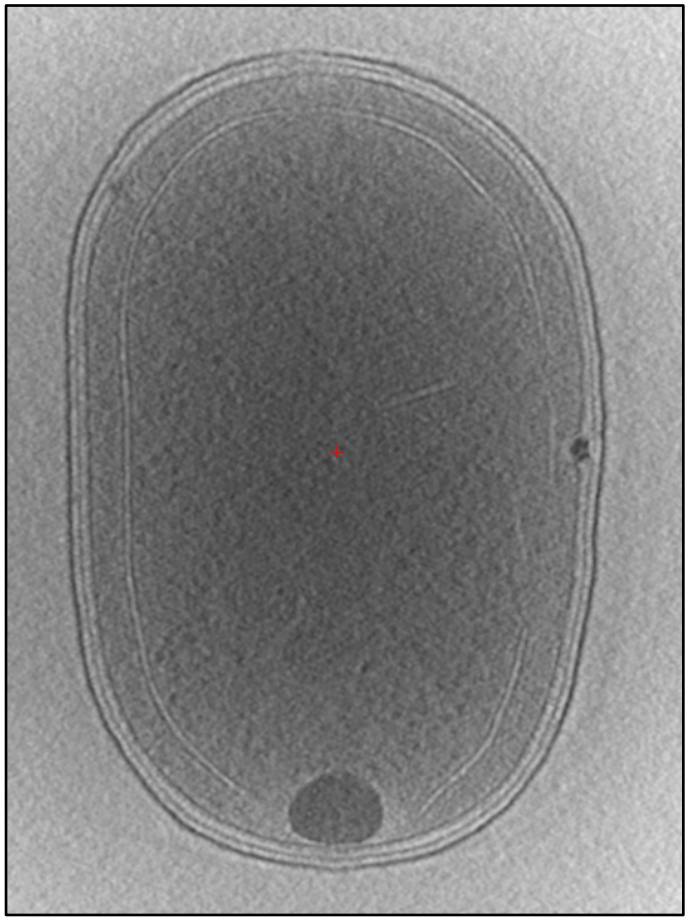


Knife Marks, Crevasses, and Contaminations

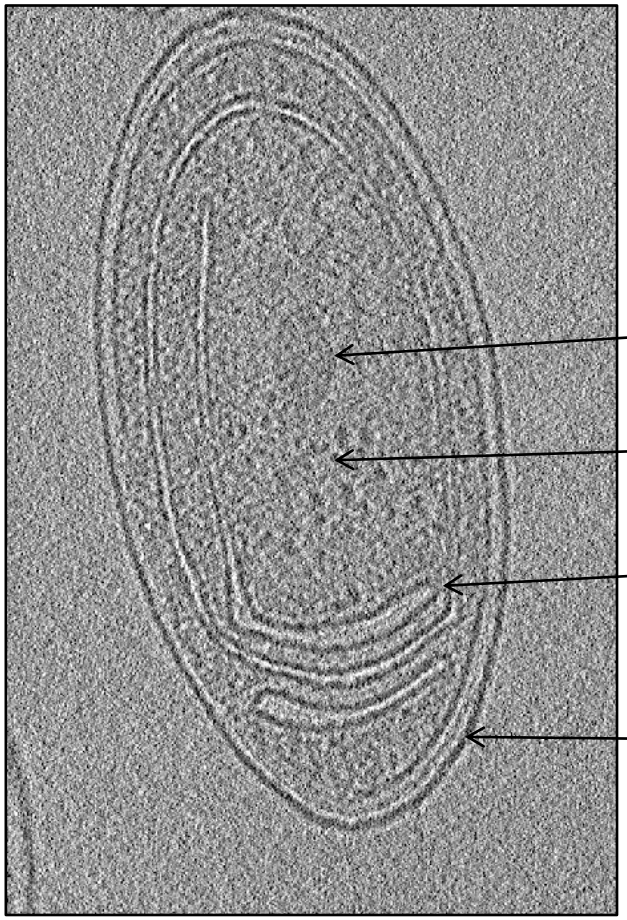


Valério R. F. Matias et al. J. Bacteriol. 2003;185:6112-6118

Compression



Plunge freezing



High pressure freezing
Cryo-sectioning

Outline

- What samples are for cryo- cryo-electron tomography?
- Sample preparation for cryo-electron tomography
- A case study: Correlated cryo-PALM-CryoET

A Case Study: now we have this sample…

- *Myxococcus Xanthus*
- Goal: location and architecture of type VI secretion system
- Experiment design
  - Sample preparation
  - Correlated cryo-PALM
  - Tilt series data Collection
  - Image processing
  - Annotation

Correlated cryogenic photoactivated localization microscopy and cryo-electron tomography

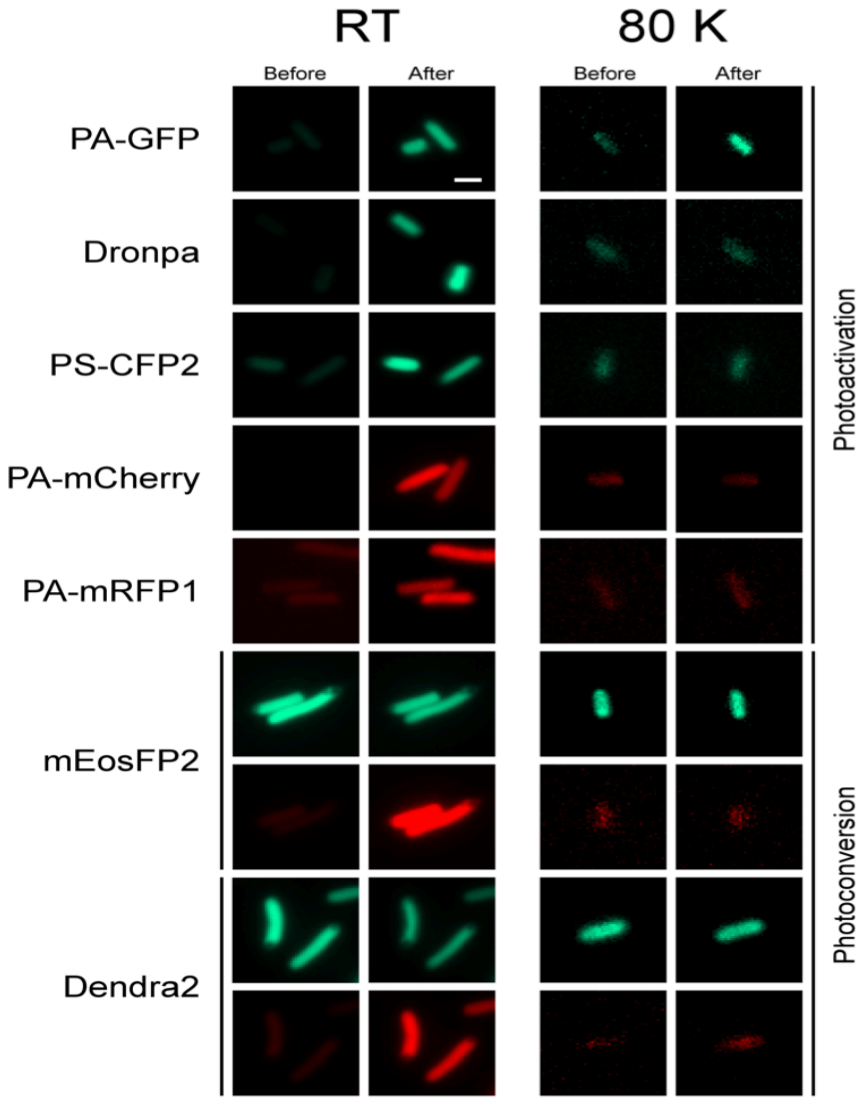
Yi-Wei Chang[1,2], Songye Chen[1,2], Elitza I Tocheva[1], Anke Treuner-Lange[3], Stephanie Löbach[3], Lotte Søgaard-Andersen[3] & Grant J Jensen[1,2]

Cryo-electron tomography (CET) produces three-dimensional images of cells in a near-native state at macromolecular resolution, but identifying structures of interest can be challenging. Here we describe a correlated cryo-PALM (photoactivated localization microscopy)-CET method for localizing objects within cryo-tomograms to beyond the diffraction limit of the light microscope. Using cryo-PALM-CET, we identified multiple and new conformations of the dynamic type VI secretion system in the crowded interior of *Myxococcus xanthus*.

Sample Preparation

- Cell culture
- Plasmid construction – tag protein of interest with a photoactivatable fluorophore

Photoactivatable Fluorophore at 80K



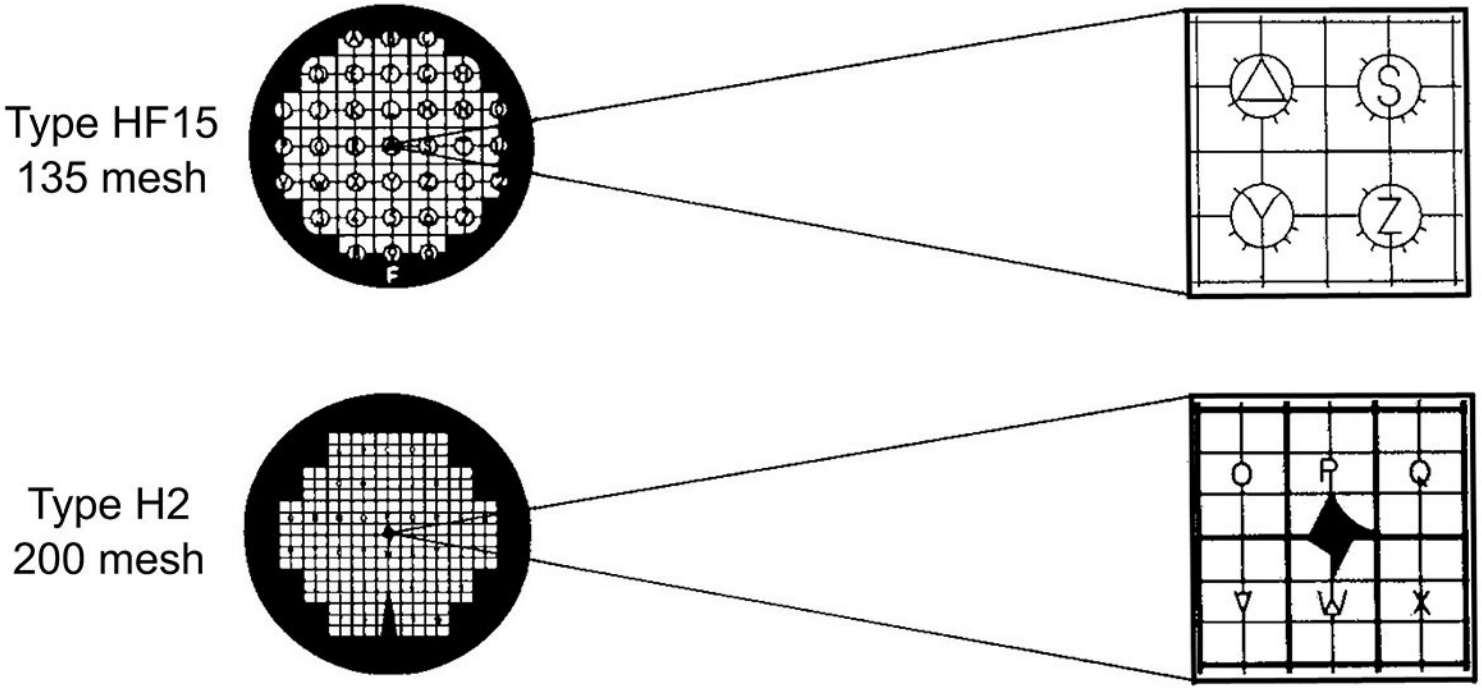
Chang, Yi-Wei, et al., Correlated cryogenic photoactivated localization microscopy and cryo-electron tomography. Nature methods. 2014

Sample Preparation

- Cell culture
- Plasmid construction – tag protein of interest with a photoactivatable fluorophore
- Functional analysis
- Plunge freezing of cells

Correlative Light and Electron Microscopy (CLEM)

- Targets are fluorescence-labeled
- Use special finder grids



Sample Preparation

- Cell culture
- Plasmid construction – tag protein of interest with a photoactivatable fluorophore
- Functional analysis
- Plunge freezing of cells
- Cryo-PALM

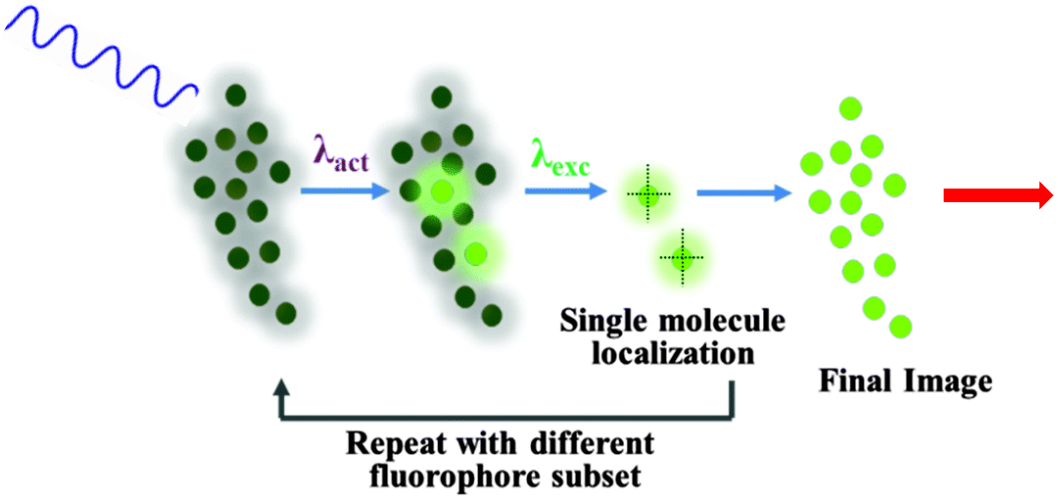
Identifying objects in tomograms concept check questions:

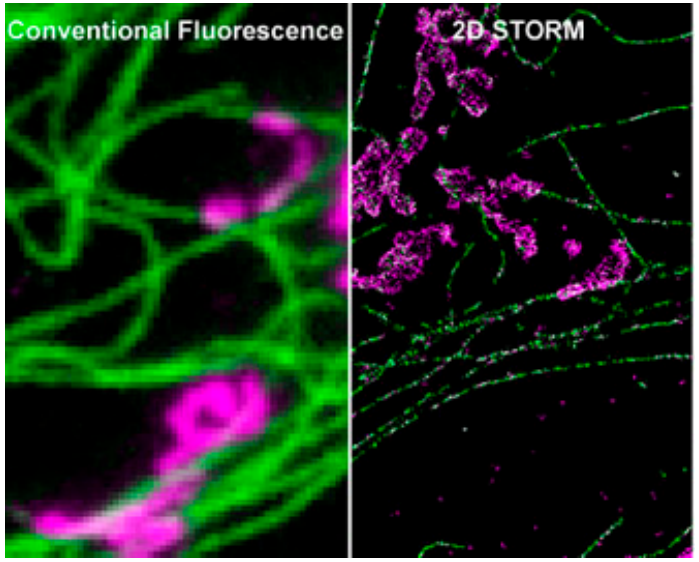
- What is “cryo-PALM”? Bonus exercise and question: Estimate the localization precision of cryo-PALM from the example shown. Do you know what factors limit the localization precision in this experiment?

- PALM: photoactivated localization microscopy

Concept Check Questions

PALM (Photo Activated Light Microscopy)



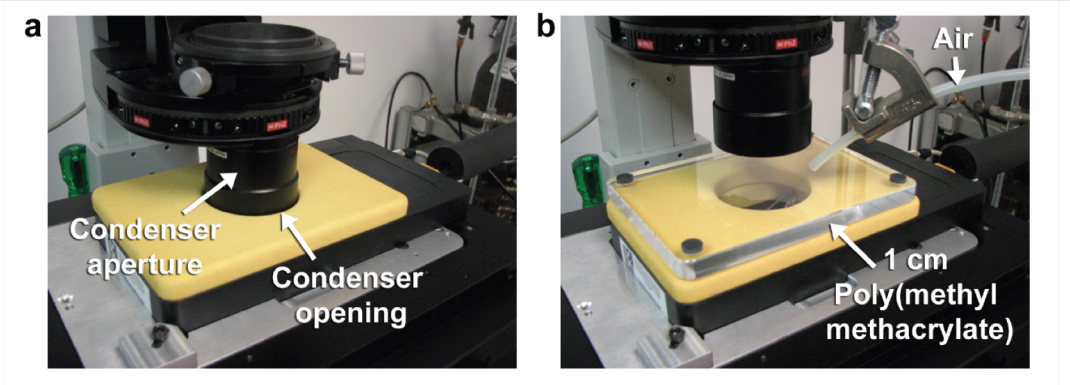


In PALM, small bursts of UV light are used to excite subsets of fluorophores. Since these fluorophores are excited in small sets, their diffraction patterns are less likely to overlap, allowing the location of the actual molecules to be located through back calculation

http://huanglab.ucsf.edu/STORM.html

Cryo-PALM

- Cells are embedded in ice. Ice thickness (<500 nm)
- Cold stage to maintain the sample at low temperature during imaging



- Long working distance (NA 0.7) air objective lens to avoid heat transfer
- Need control laser power and exposure to achieve good image while minimizing damage to cells

Chang, Yi-Wei, et al.,. Nature methods. 2014

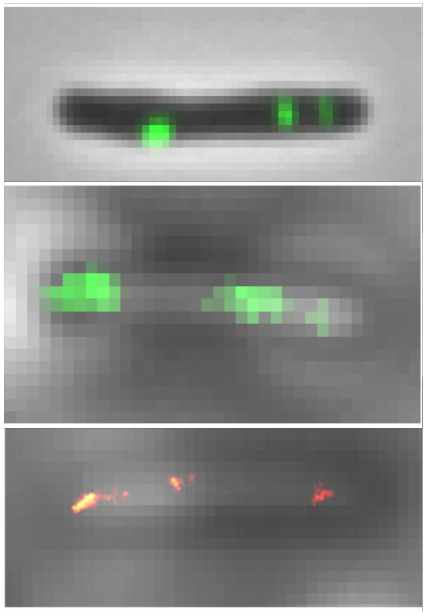
CLEM: RT vs Cryo

Concept Check Questions

- What are the advantages and disadvantages of doing light microscopy at room temperature and cryo-temperature?
  - Room temperature
    - oil immersion objective lens, large NA
    - better fluorophore performance
  - Cryo temperature
    - samples immobilized

Do you know what factors limit the localization precision?

Concept Check Questions

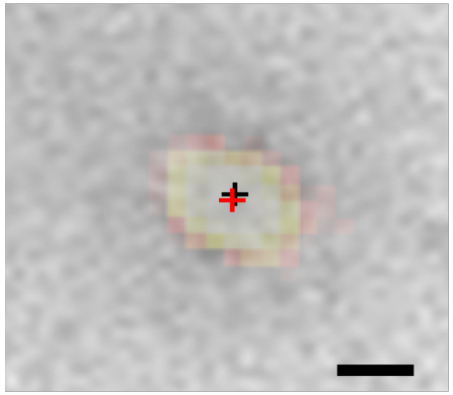


GFP RT
100x NA1.4
oil immersion objective lens

GFP 80K
60x NA 0.7
air objective lens

PA-GFP 80K
cryo-PALM

Fluorophore & objective lens



Distance between EM/cryo-PALM bead centers around 9±2 nm

Correlation of images

Chang, Yi-Wei, et al., Correlated cryogenic photoactivated localization microscopy and cryo-electron tomography. Nature methods. 2014

Data Collection – overview (1)

- FEI Tecnai G2 Polara™ : 300 kV FEG transmission electron microscope equipped with a Gatan energy filter
- Camera: K2

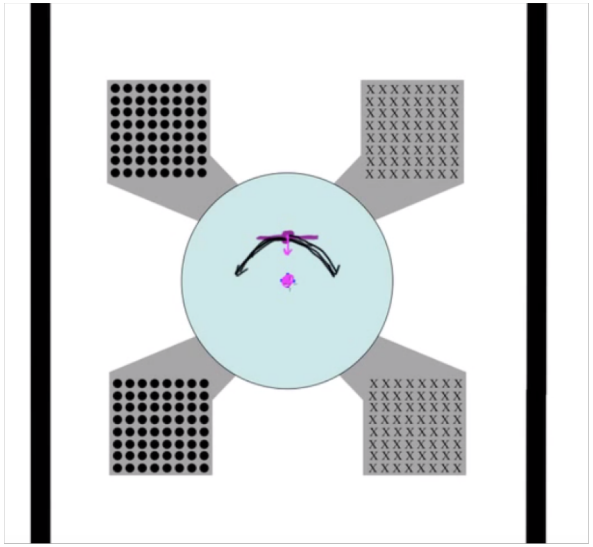
CryoET Data Collection

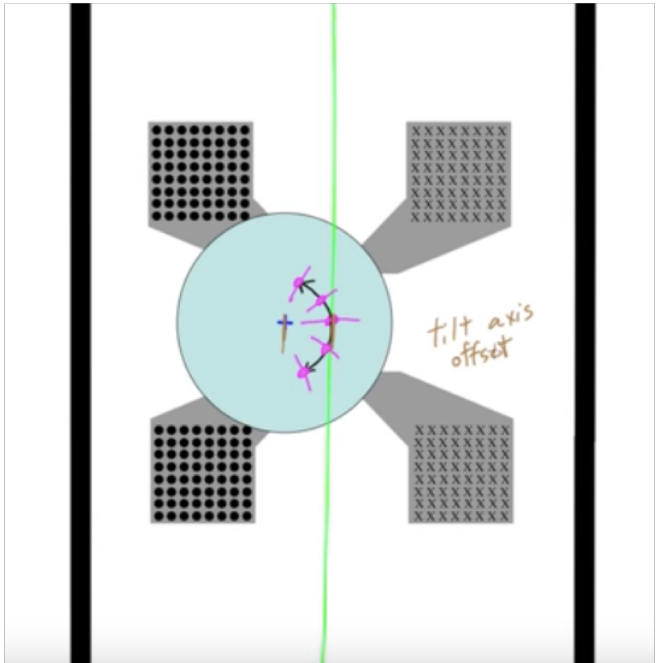
Concept Check Questions

- What is a “tilt-series”?
  - Images taken when the sample is tilting about the tilt axis.
- What range of angles is typically imaged?
  - Two limiting factors: sample geometry, goniometer mechanical limit (-70 <->+70)
- What is the “missing wedge”?
  - Missing data that are not recorded because of limited tilt range

Data collection

- What is “eucentric height”? Is it different for every sample?
  - When the sample is aligned with goniometer axis.
- What is the “tilt axis offset”? Is it different for every sample?
  - When goniometer tube is not coincident with optical axis of the objective lens





Concept Check Questions

Data Collection – overview (2)

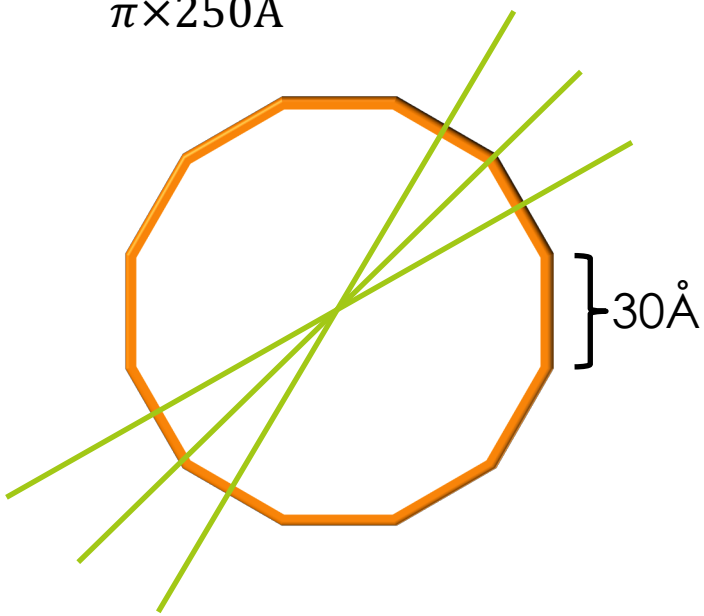
- There are many choices involved in the design of a tomography experiment. Each is a balance between opposing considerations. Explain the compromises involved in the choice of magnification, total dose, tilt-increment, exposure time per image, and defocus.
  - Defocus: -10µm
  - Total dose: 180 $e^{-}/Å^{2}$
  - Tilt range and increment: tilt-series from −60° to 60° with an increment of 1°

Tilt step / angular sampling

- "Crowther criterion":

$$\frac{180° \times resolution}{\pi \times diameter} = angular\ step$$

$$\frac{180° \times 3Å}{\pi \times 250Å} = 0.69°$$



radius (Å)	res (Å)	# views	angular step (°)
125	3	259	0.69
125	10	79	2.292
125	40	20	9.167
10000	3	20944	0.009
10000	10	6283	0.029
10000	40	1571	0.115

Concept Check Questions

Courtesy of Jason Kaelber

Data Collection – overview (3)

- FEI Tecnai G2 Polara™ : 300 kV FEG transmission electron microscope equipped with a Gatan energy filter
- Camera: K2
- Total dose: 180 $e^{-}/Å^{2}$
- Tilt range and increment: tilt-series from −60° to 60° with an increment of 1°
- Defocus: -10µm
- Semi-automatically using predictive UCSF-Tomo package

Data collection – automated data collection

- When speaking about automatic sequential tilt-series acquisition, what is “tracking”? What is “targeting”?
  - Tracking: Keeping the object of interest in the field of view and image on the detector throughout the tilt series
  - Targeting: the process going from one imaging target to the next
- How is the “predictive” tracking method different from the “focus position” method? Which is faster? Why would the slower method ever be used?

Concept Check Questions

“Predictive method”

center object (with low dose/low mag)
record 0° image (at desired mag)
tilt, record first tilted image
    determine shifts
tilt, record next tilted image
    determine shifts
fit shifts to model of tilt axis offset, specimen height
predict and apply beam shift, image shift, and focus changes needed

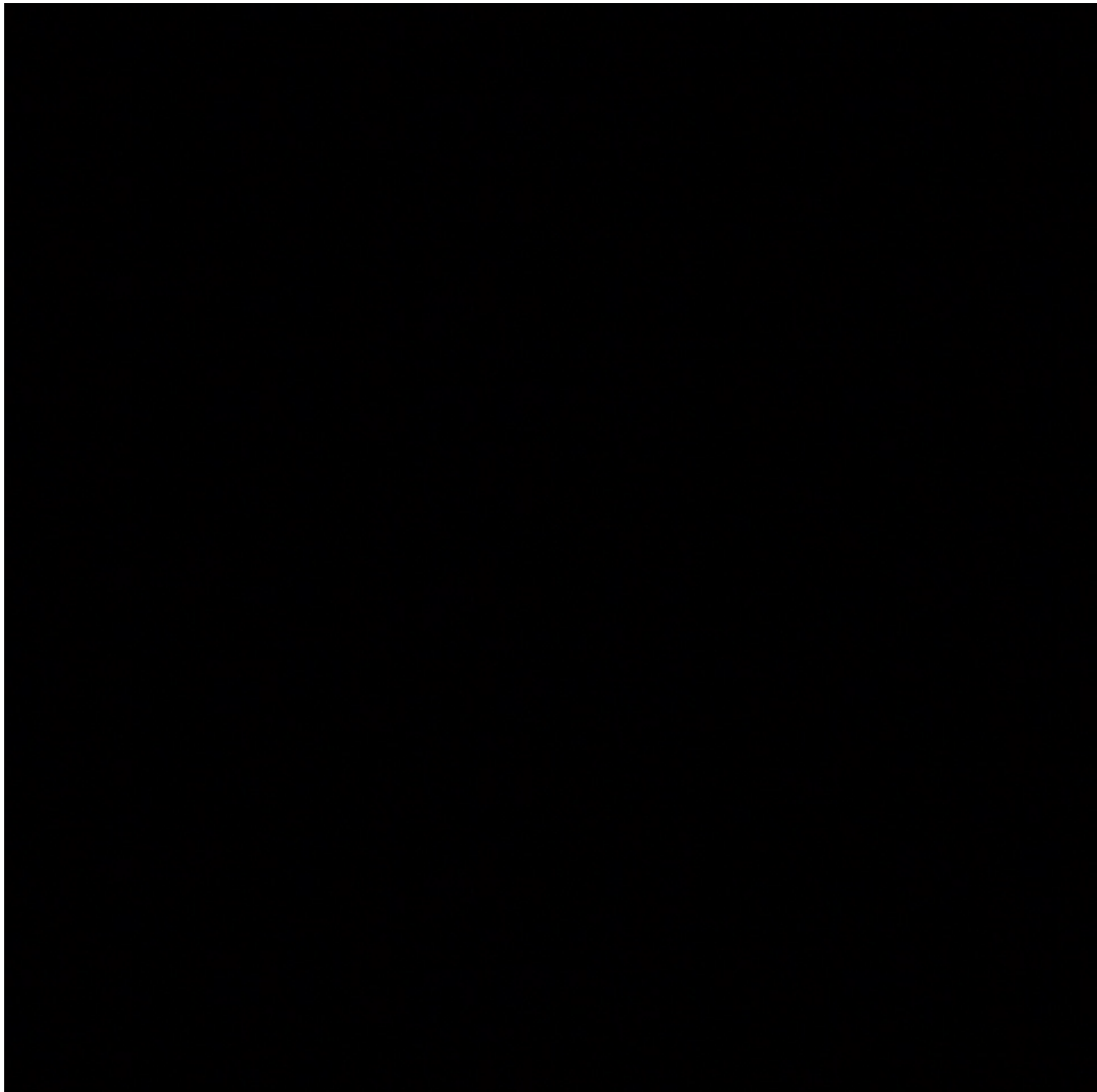
“Focus position method”

- center object (with low dose/low mag)
  beam shift to focus position, focus, record reference image
  blank beam, unshift beam (back to object), record image
- tilt
  beam shift to focus position, re-focus, record image
    determine x,y shifts needed

Data alignment and reconstruction

Concept Check Questions

- What about each image has to be determined to “align” a tilt-series? How are these parameters found?
  - x, y shift
  - Rotation (position of tilt axis)
  - Tilt angle
  - magnification
  - defocus



Annotation and Visualization

Concept Check Questions

- How is “volume rendering” different than showing an “isosurface” or single slice?
  - Volume rendering: see through the 3D object, all voxels.
  - Isosurface: one surface based on a threshold.

Automation

Concept Check Questions

- What steps of data collection and 3-D reconstruction have to be done by the investigator, and which are typically automated?

Annotation and Visualization

Concept Check Questions

- How large or small a sample can be imaged by electron tomography?
  - Large: human cell or organelle by serial section montage tomography
  - Small: individual macromolecular protein complex

Annotation and Visualization

Concept Check Questions

- What is “template matching”? In the slide on template matching, four variables were listed as arguments in the cross-correlation function - what were they and why was each present?
  - Template matching uses known structure as template to search for densities that look like the template.
  - Three orientation parameters and template
- What kinds of macromolecular complexes are likely to identifiable within a cell by template matching?
  - With known structure
  - Large
- What is “visual proteomics”?
  - The effort to discover which macromolecules exist in a cell, and their spatial relationship by direct imaging

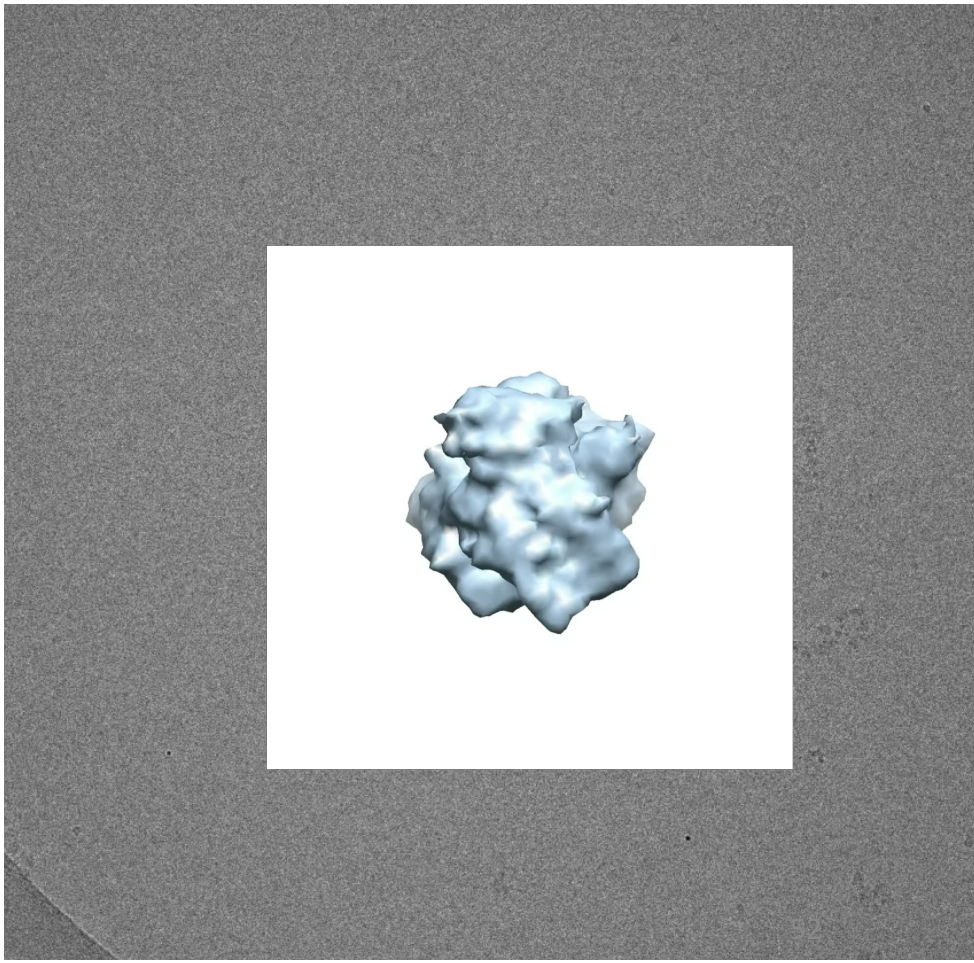
Tomography - Limitations

Concept Check Questions

- What is the fundamental resolution limitation in tomography for native samples?
  - Radiation damage
- What is the fundamental resolution limitation for stained samples?
  - Fidelity of the stain
- Name and explain four other resolution limitations in tomography.
  - Tilt increment
  - Tilt range : missing wedge
  - Defocus
  - Precision of image alignment
  - Magnification – pixel size
- What is the “missing wedge”? Why is it missing? What effect does it have on reconstructions?
  - A wedge of missing data because of limited tilt range
  - Shape the point spread function: a point → a football blurred in z direction

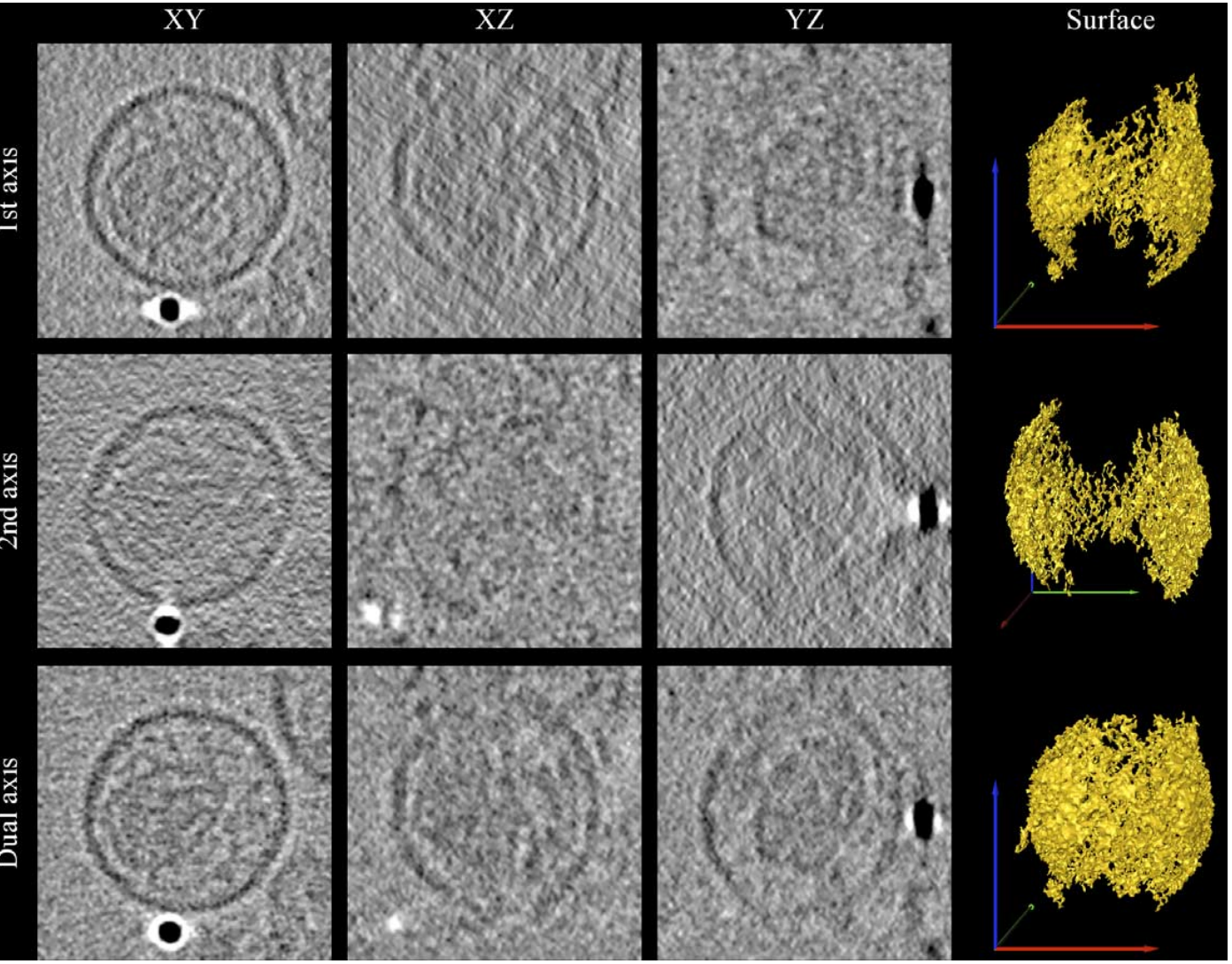
Missing wedge

- Anisotropic resolution due to missing wedge



Courtesy of Jason Kaelber

Dual tilt reduces missing wedge



A holder that “flips” in the microscope so x-tilt will image a second axis

Iancu, Wright &al. *JSB* 2005

End

- Thank you!