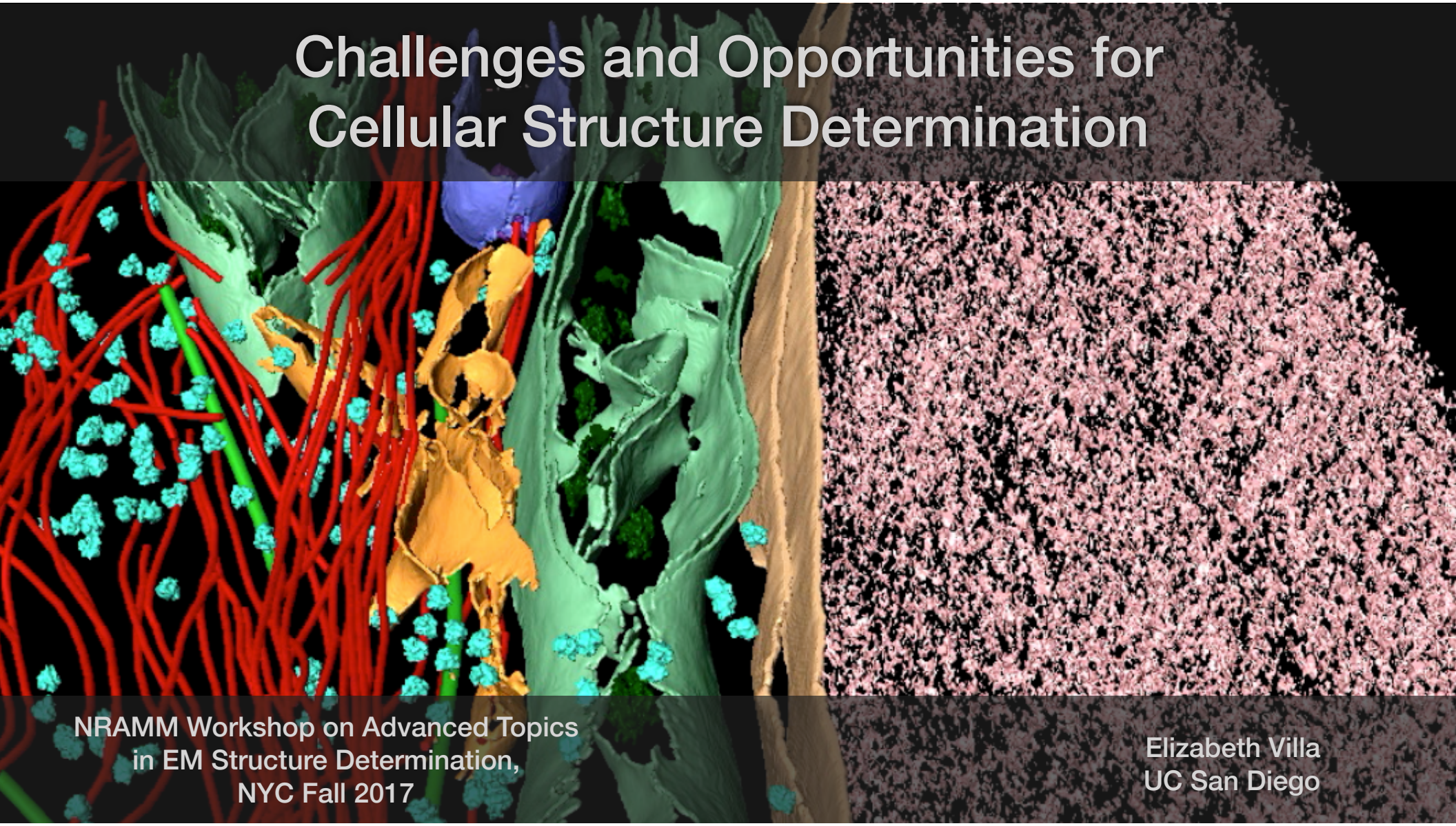
Challenges and Opportunities for Cellular Structure Determination



NRAMM Workshop on Advanced Topics
in EM Structure Determination,
NYC Fall 2017

Elizabeth Villa
UC San Diego

This introductory talk will describe the big picture: what have we achieved and what do we still need to do. How important is it to visualize complexes in their biological environment? What classes of biological questions are best addressed this way? What are the problems associated with imaging large cellular complexes in situ? How do we locate and identify what we are interested in? How important is correlative LM-EM? Has this approach reached a stage of development where it is easily and generally applicable? What are the challenges associated with recording high-resolution information from cells? What are the general strategies currently in use? What is the role of FIB milling and other advanced specimen preparation methods? What technologies do we still need? Is tomography coupled with sub tomogram averaging the best way to extract the highest resolution information? This approach has recently yielded near atomic resolution structures; could this become routine? Are there theoretical limitations to what can be achieved? What is the current state of the art and the future prospects? What should we pay attention to and what should we NOT do?

Yikes.

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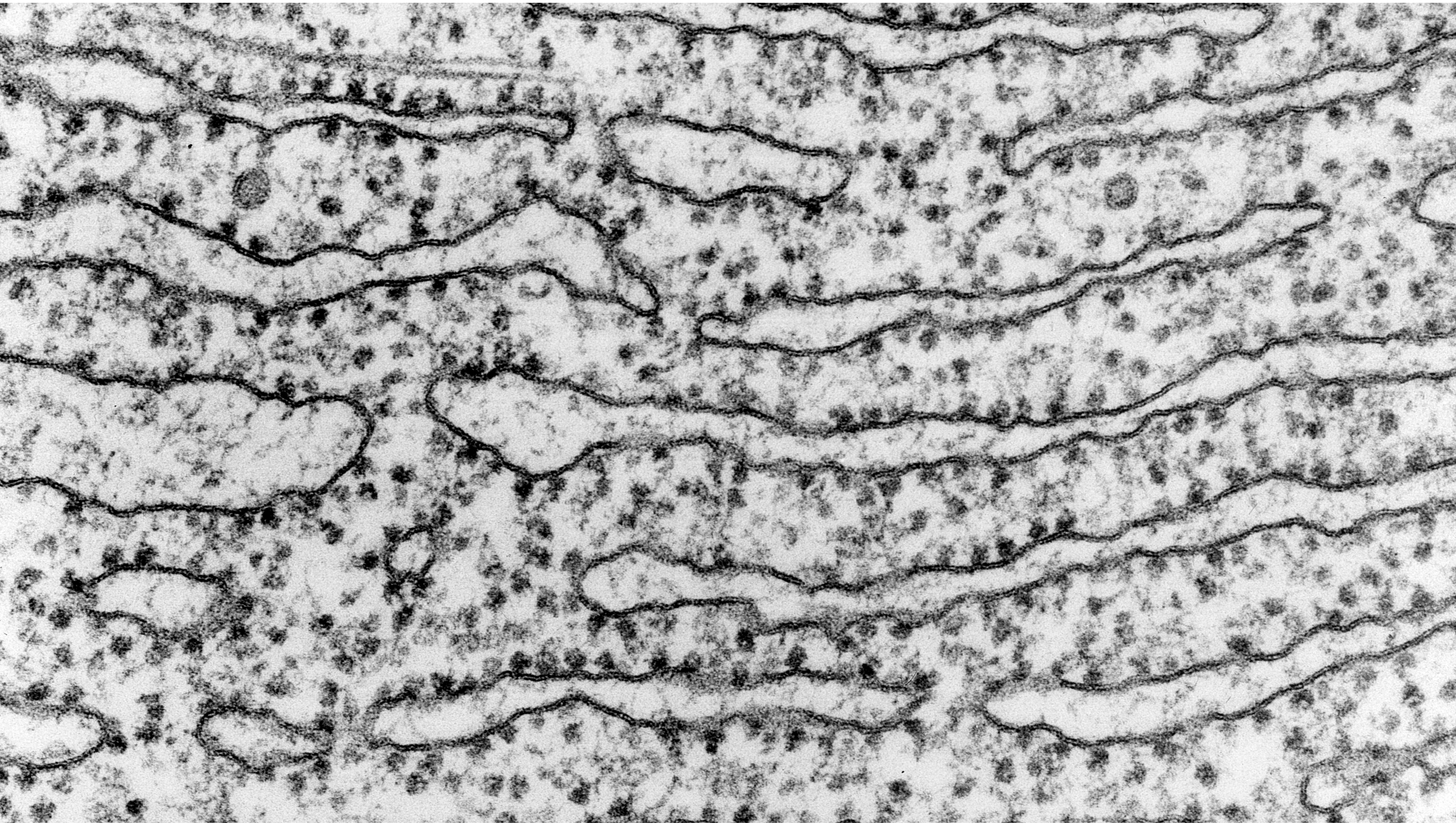
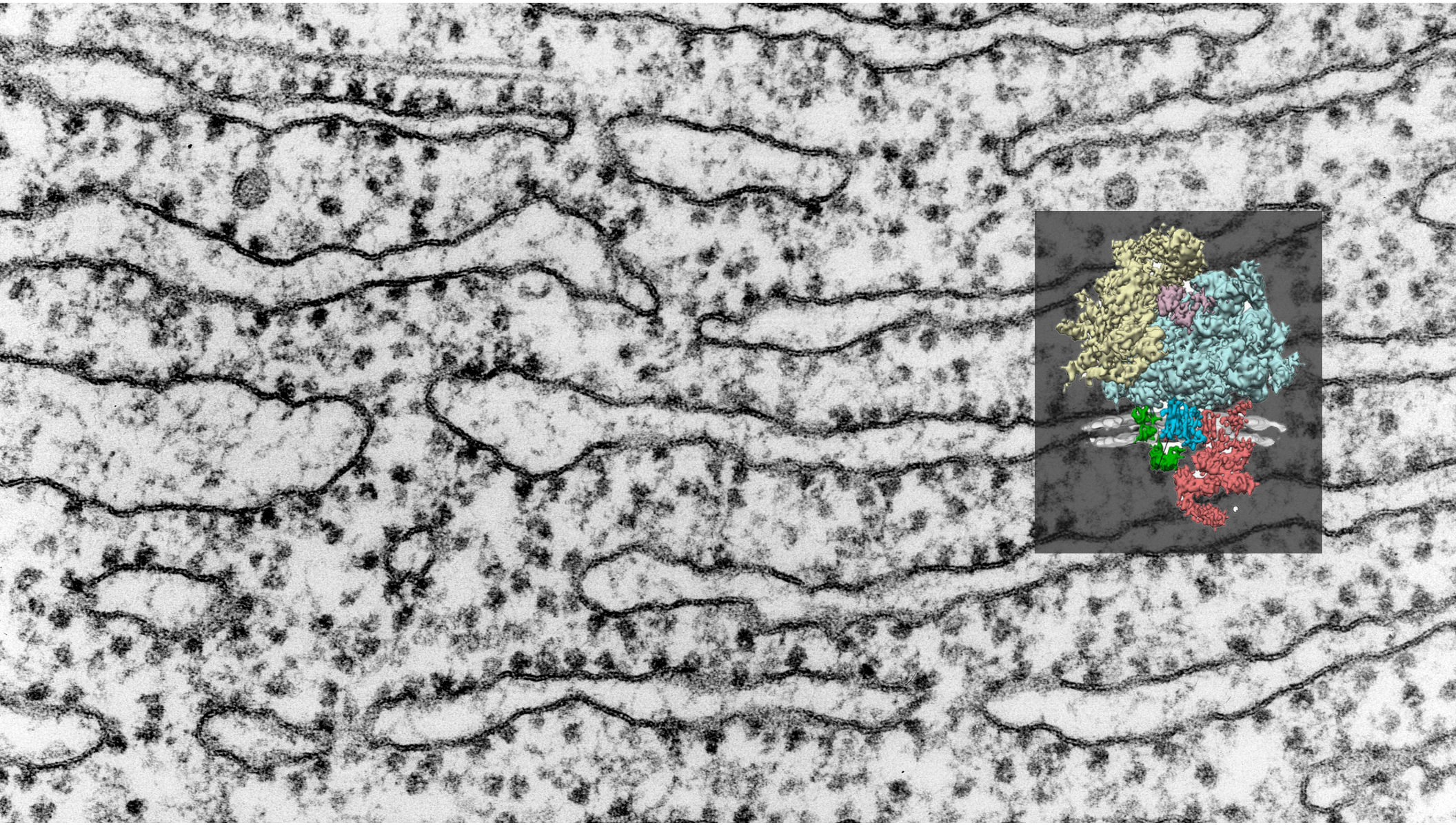
Yikes.

Non-exhaustive collection of ideas and results from many of you



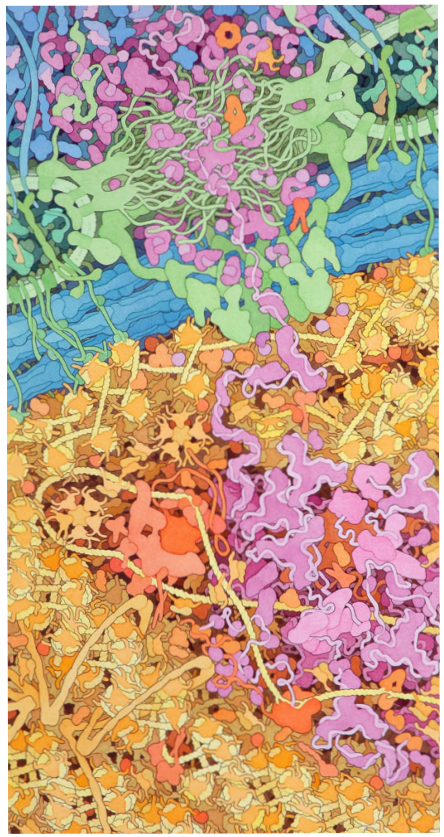


Outline

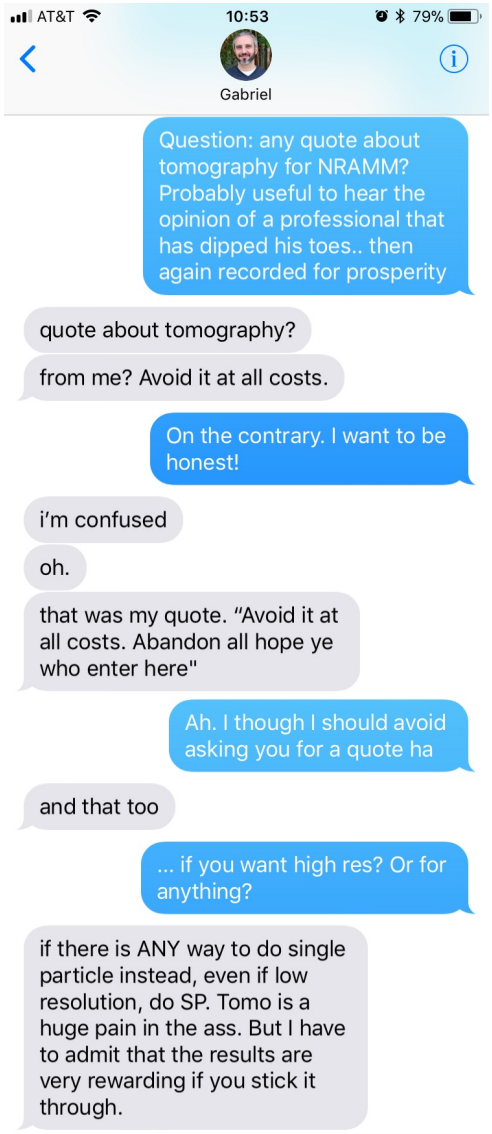
- Why Tomography?
- Overview of Sample Preparation
- Data Acquisition and Processing for Subtomogram Averaging
- Revisiting Sample Preparation
- CLEM
- A swift example from work in my lab
- Other ways to Identify Macromolecules
- What technologies are still needed?
- Future Prospects

How important is it to visualize complexes in their biological environment?

- Most proteins exist in multiprotein complex at any given time
- Conformation/composition depend on their environment
- Entangled in their environment
- Transient
- We don’t know what the look at
- Cell Biology!



When to use tomography?


AT&T
10:53
79%
Gabriel
Question: any quote about tomography for NRAMM? Probably useful to hear the opinion of a professional that has dipped his toes.. then again recorded for prosperity
quote about tomography?
from me? Avoid it at all costs.
On the contrary. I want to be honest!
i'm confused
oh.
that was my quote. "Avoid it at all costs. Abandon all hope ye who enter here"
Ah. I though I should avoid asking you for a quote ha
and that too
... if you want high res? Or for anything?
if there is ANY way to do single particle instead, even if low resolution, do SP. Tomo is a huge pain in the ass. But I have to admit that the results are very rewarding if you stick it through.

When to use tomography?

When your biological question requires it, e.g.:

- When you cannot purify or isolate the object of interest (OOI)

- When you have a pleomorphic OOI

- When you want to know the
    - location
    - interaction partners
    - occurrence
    - conformational states

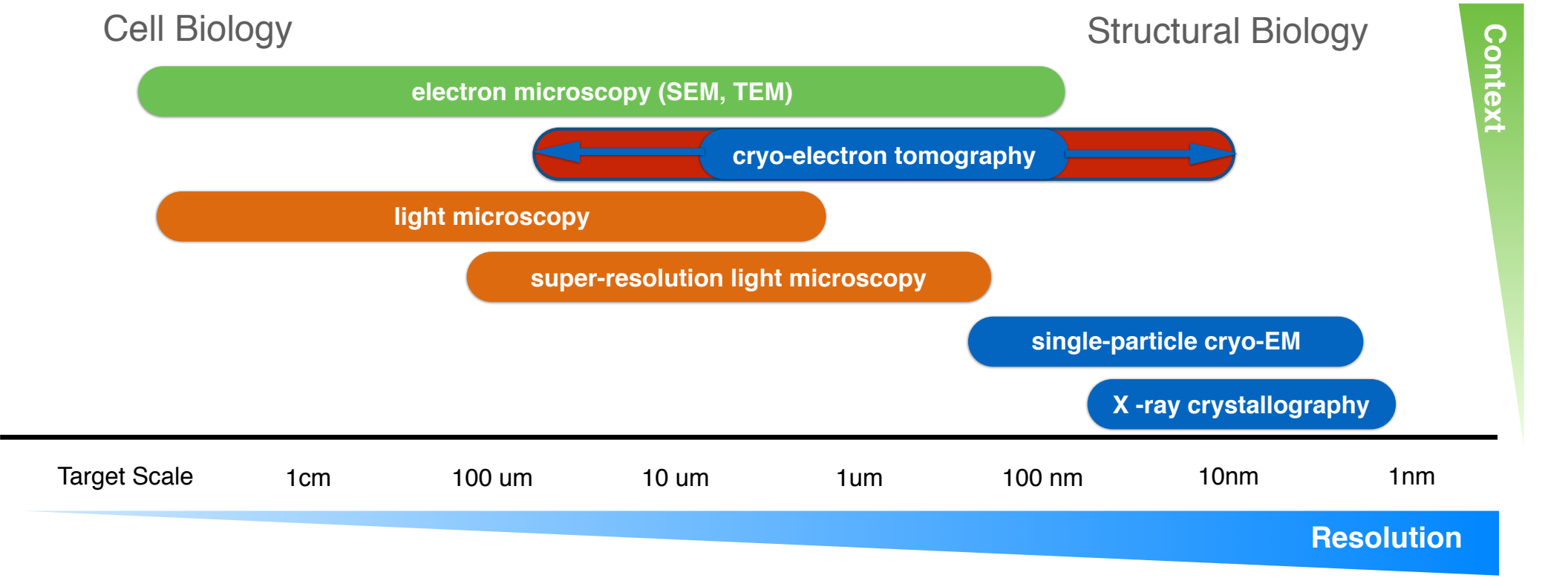
  of your OOI within a larger environment

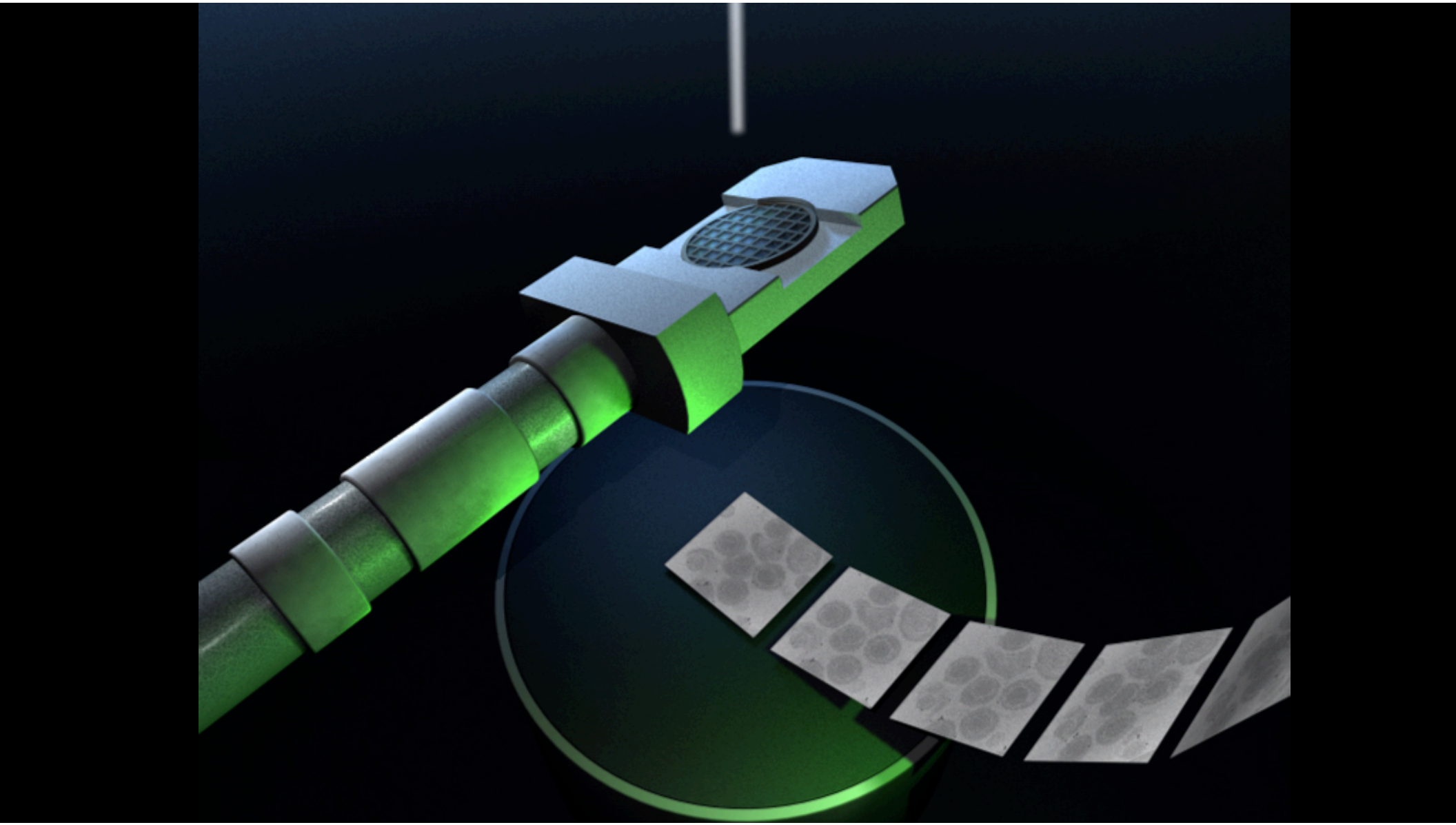
- When you want to look at cells in the highest possible resolution, in a near-native form, able to observe or count single molecules and do other quantitative analysis

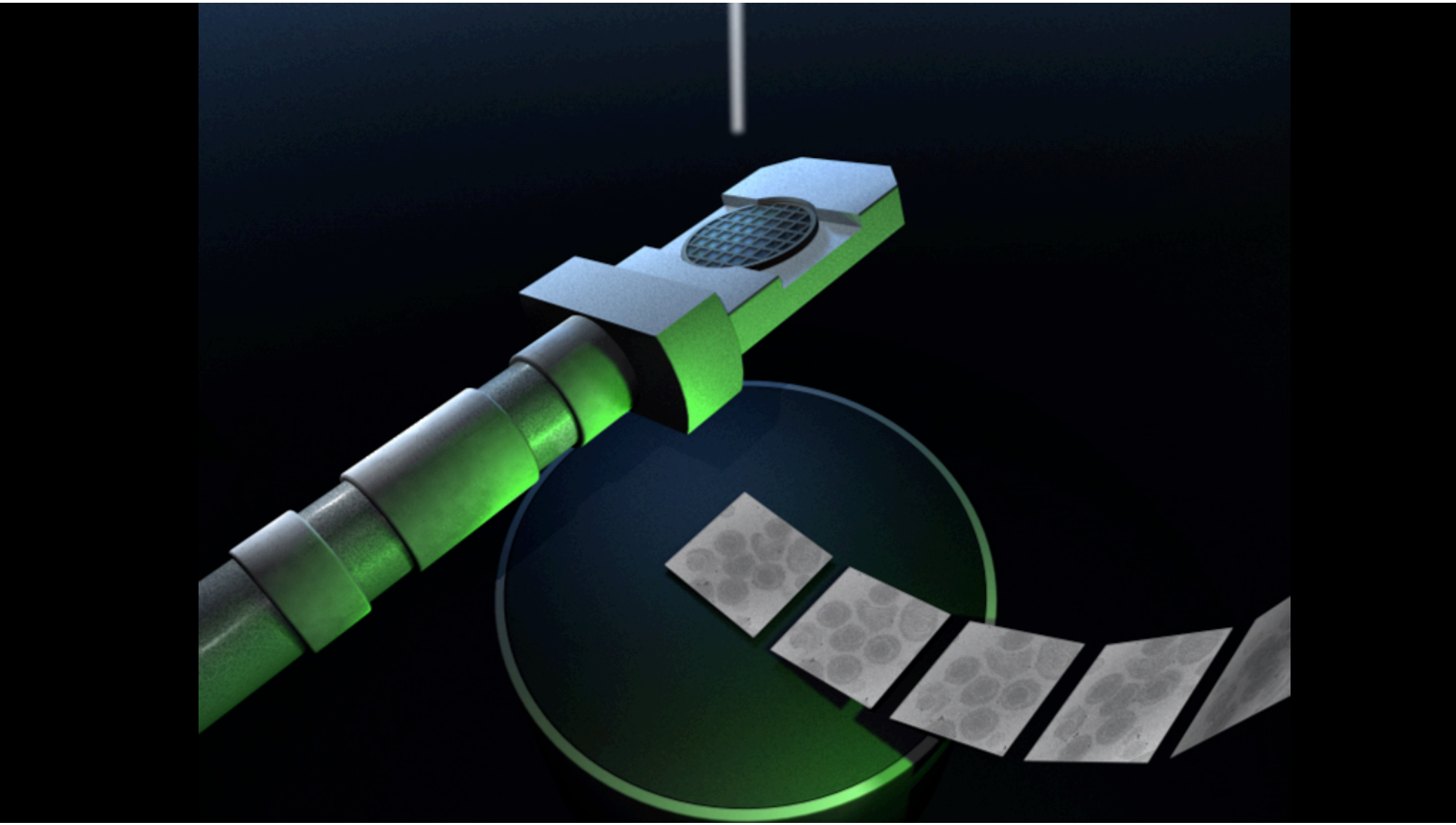
What classes of biological questions are best addressed this way?

- Questions about localization of molecular complexes
- Molecular census
- Structures deeply entangled in their environment
- Quantitative questions - persistence lengths, surfaces, crowding
- Existence of supramolecular structures
- Initial structural studies that make testable predictions and inform future experiments (structural, dynamic, biochemical)
- No other way to get the structure!

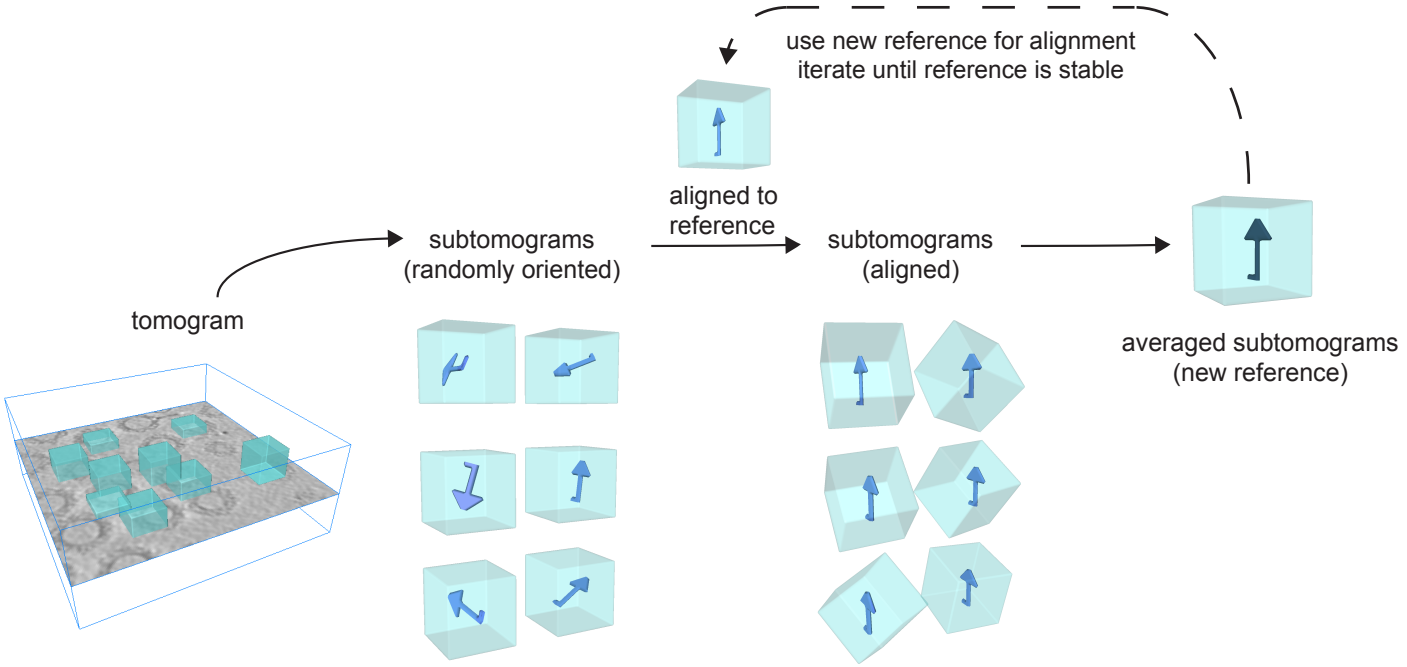
Imaging Biomolecules







Subtomogram Averaging



Briggs 2013, *Curr. Opin. Struct. Biol.*

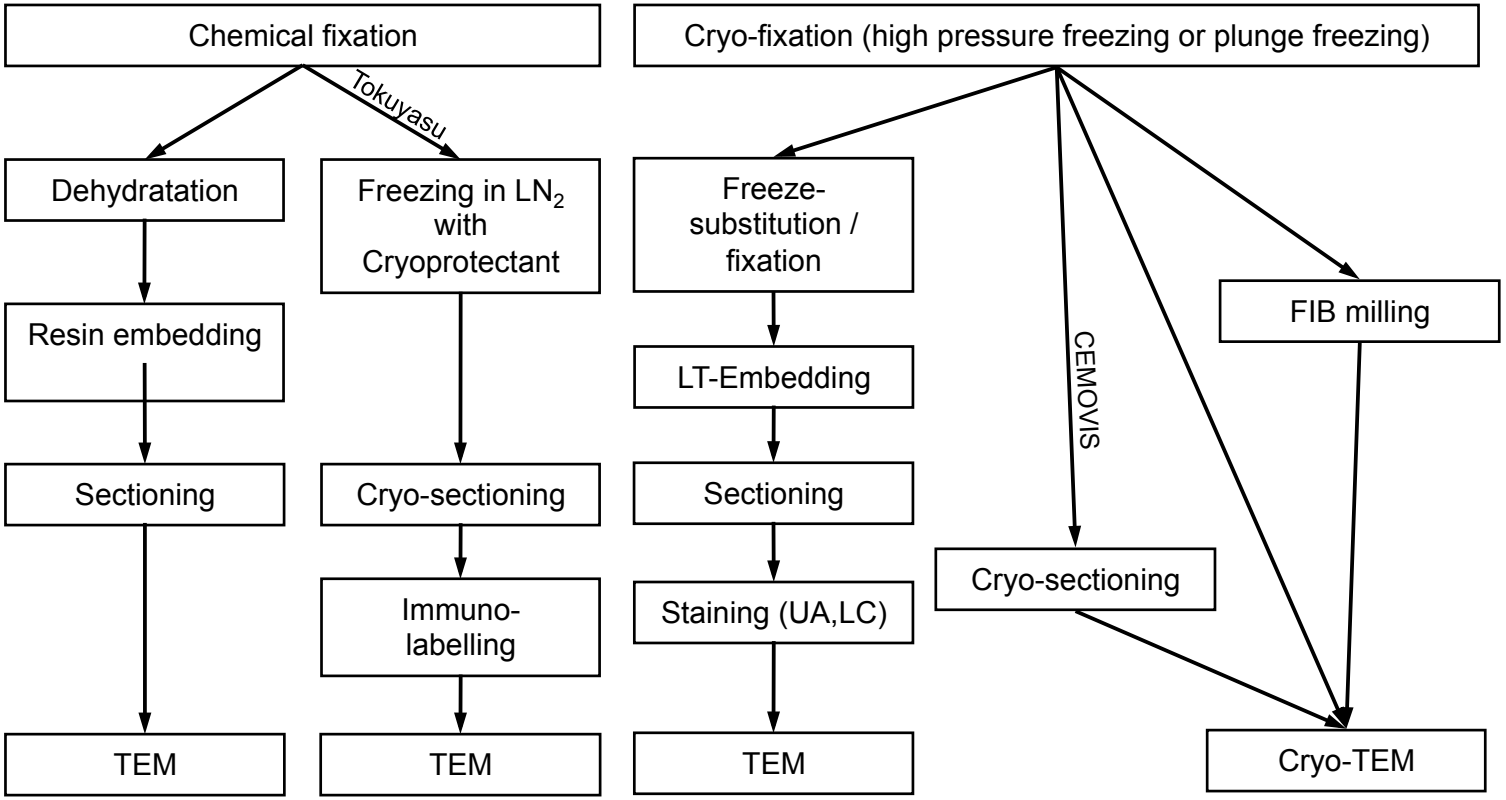
Cryo-Electron Tomography

Sample Preparation

Data acquisition

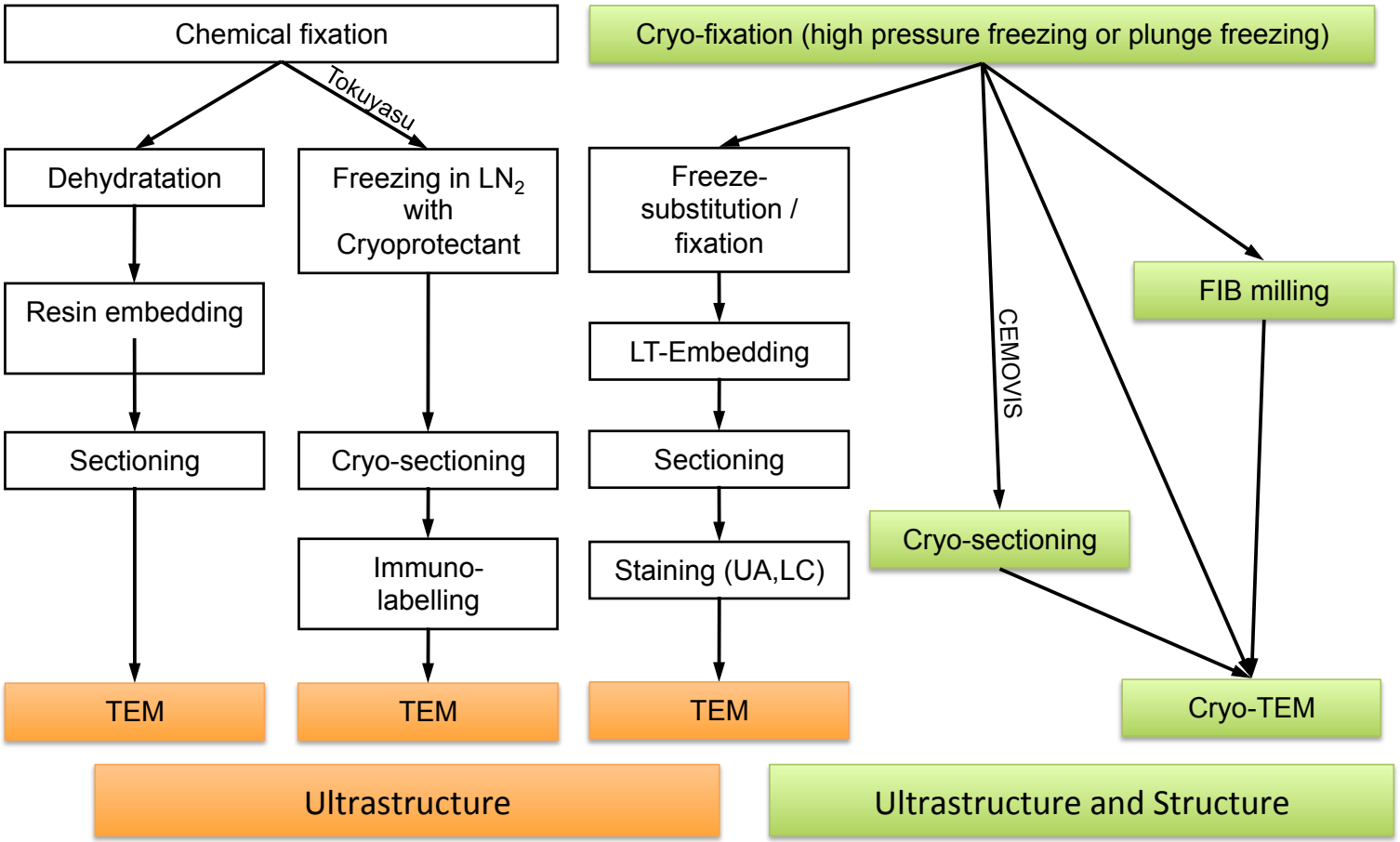
Image Processing / Data Analysis

Cellular Sample preparation methods



Claude Antony

Ultrastructure or Structure?



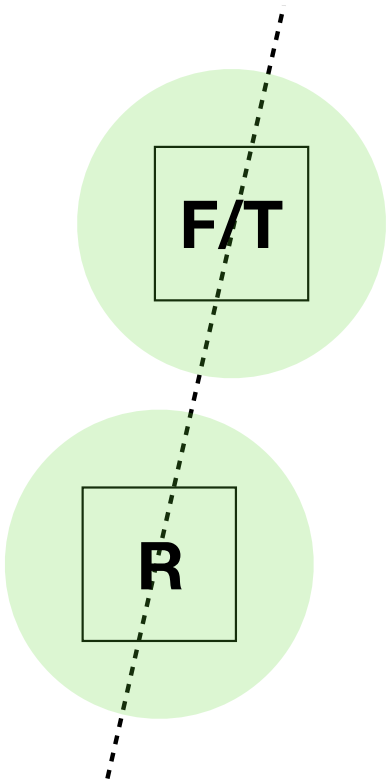
prêt-à-observer cryo-ET

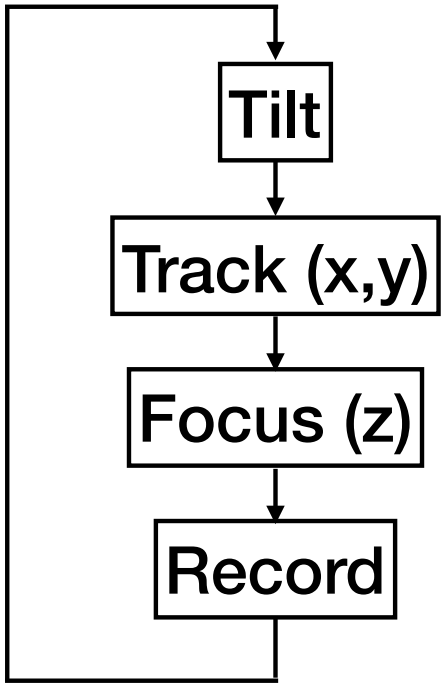
- Sample Preparation: Plunge Freezing
- Data Acquisition: Optimize for high-resolution
- Data Analysis: Subtomogram averaging, focus on high-resolution

Data Acquisition Considerations

- Sample Thickness
- Radiation Damage (higher doses over many images)
- Low S/N in each image (hard to align title series and correct CTF)
- Tilt range and tilt sampling: Missing Wedges (can I find objects in various orientations)
- Pixel Size (target resolution, dose radiation)
- Number of Particles (time consuming to acquire and/or hard to find)

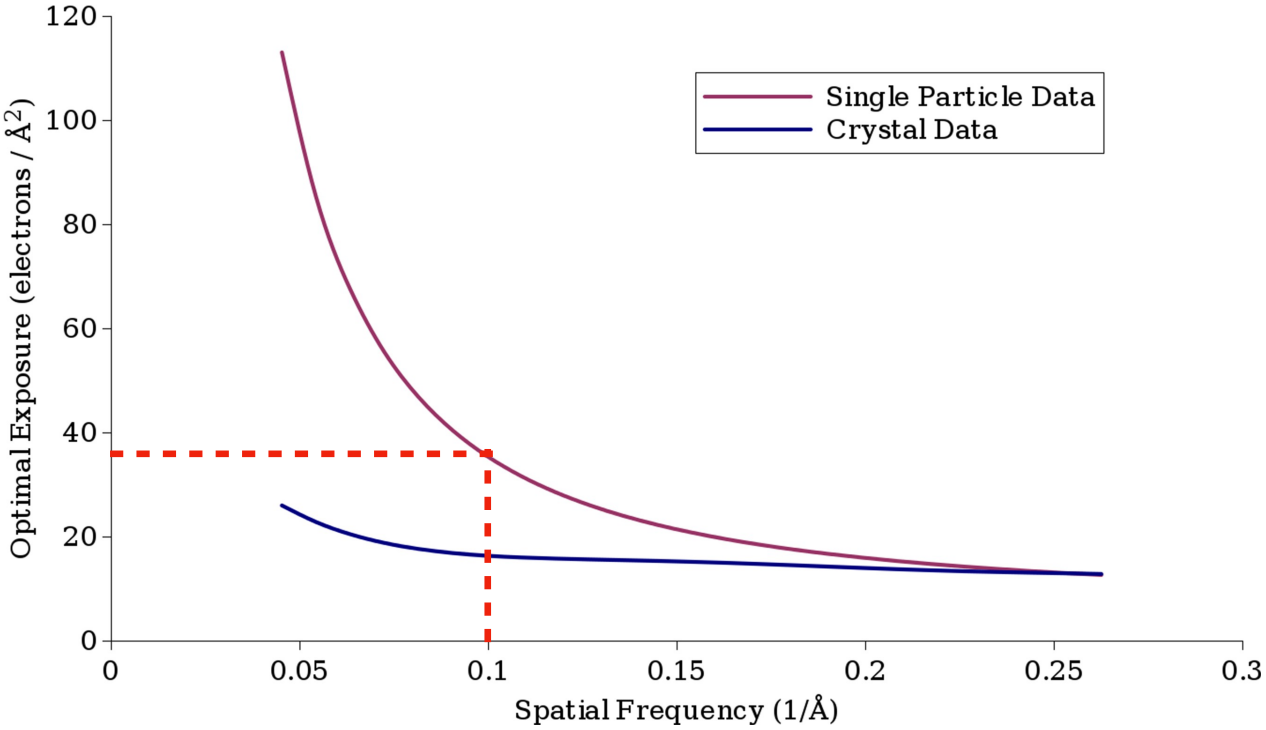
Low-Dose Automated Tomography Scheme





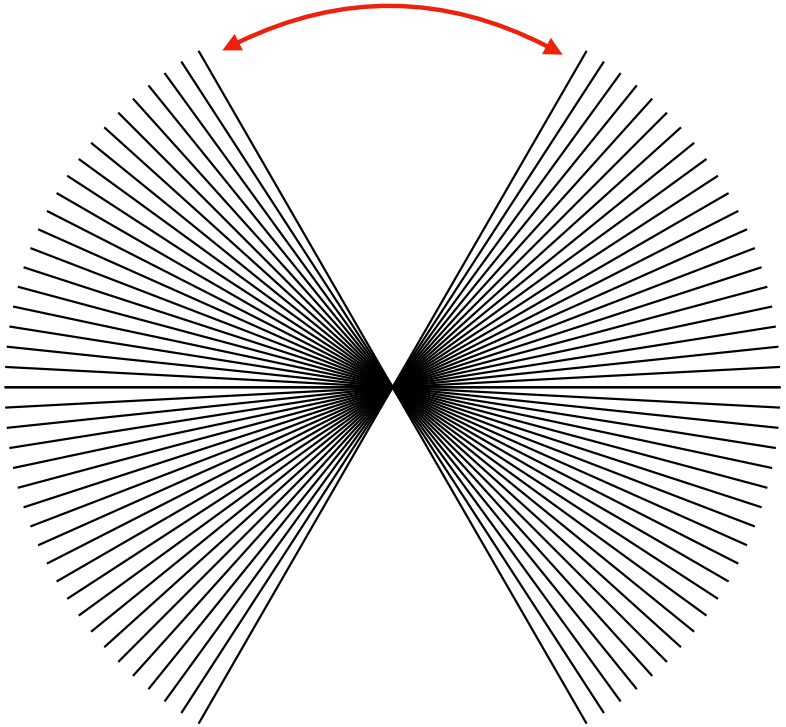
Automated tomography implemented in many softwares and works
Batch tomography possible

Radiation Damage

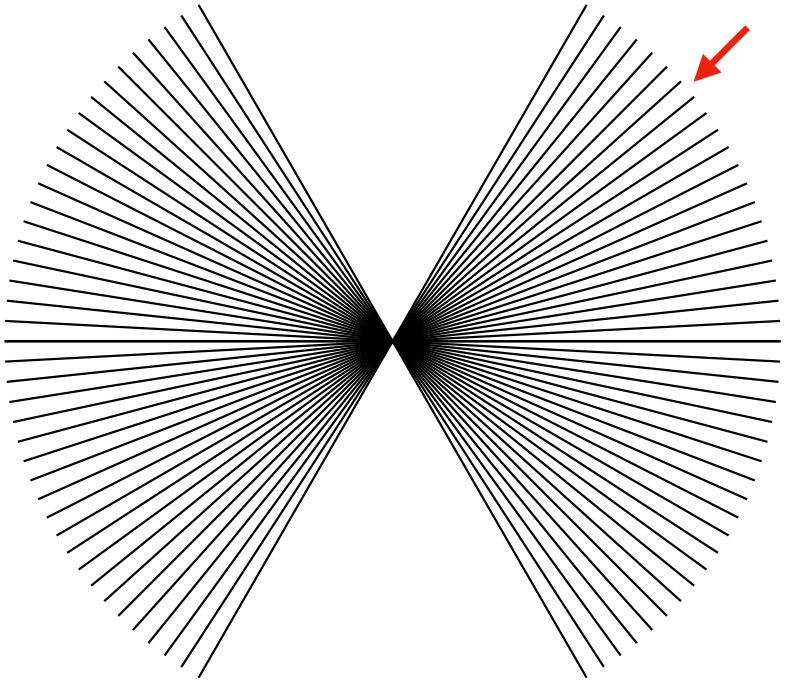


Grant and Grigorieff, 2015

Missing Wedge
(The big wedge)



Tilt Sampling
(the little wedges)



Crowther Criterion

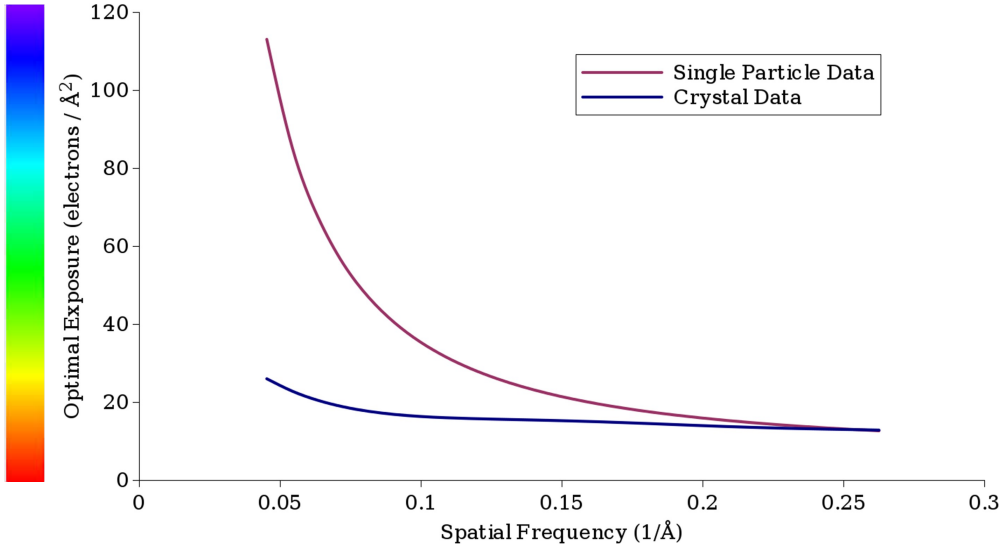
$$R = \pi D/N$$

R: resolution limit
N: Number of projections
D: Object thickness

Target Resolution

- Pixel size (Nyquist) vs. Radiation damage
- 2x change in magnification results in 4x more dose!
- Number of particles (field of view)

Tilt Scheme



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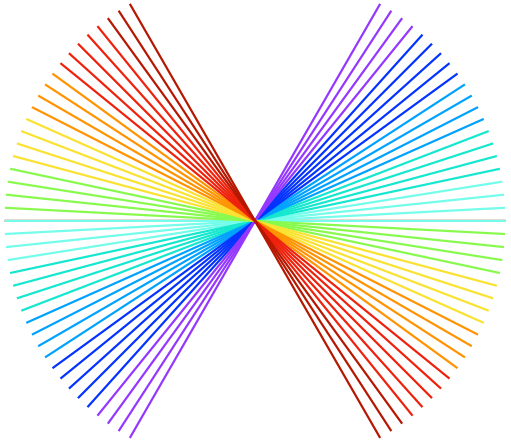
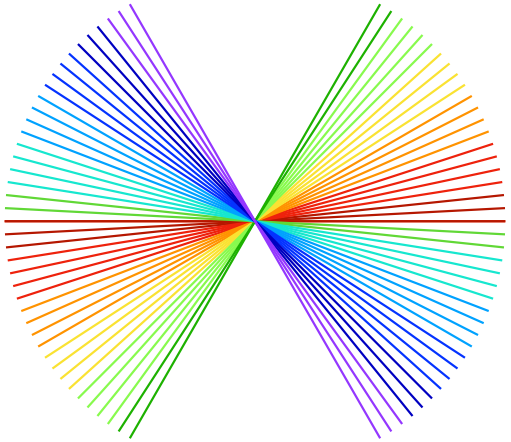


Continuous



Bidirectional

jump

State of the Art for High-Resolution

- Use a tilt scheme that takes advantage of “good dose” at “good tilts” (Hagen Scheme)
- Correct for beam induced motion
- Weight frequencies according to dose accumulated
- Estimate and correct CTF
- Use Phase Plates

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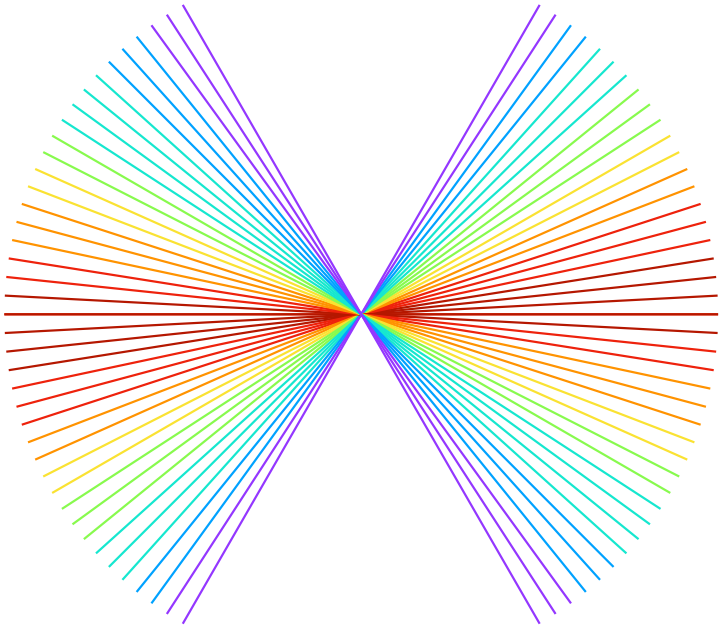

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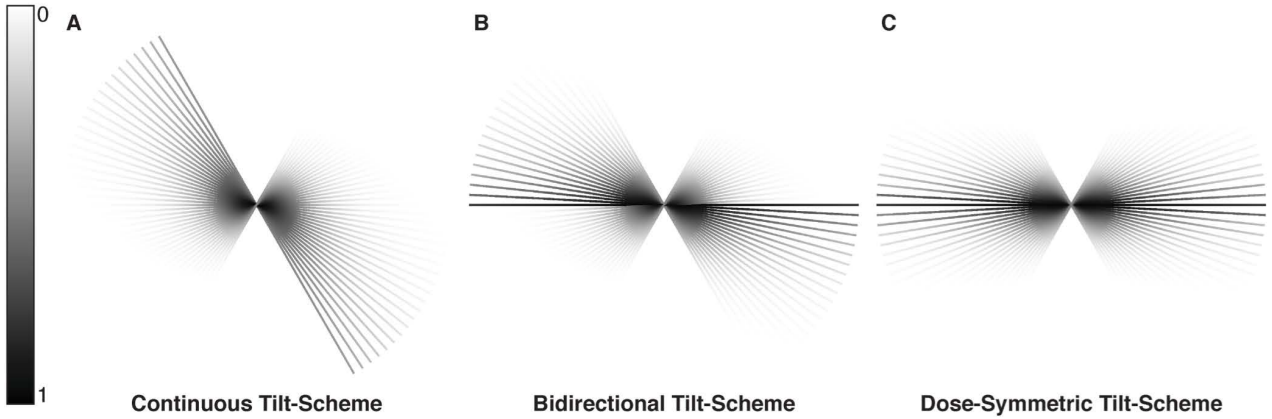

Hagen et al., 2016

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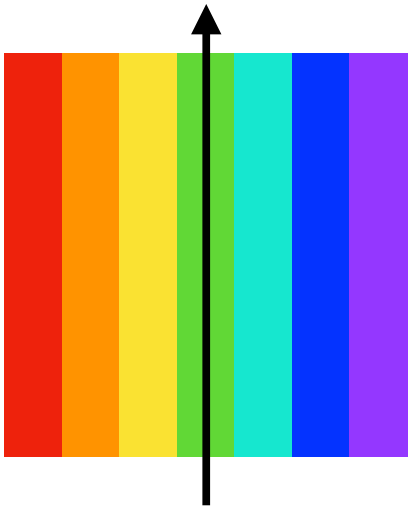
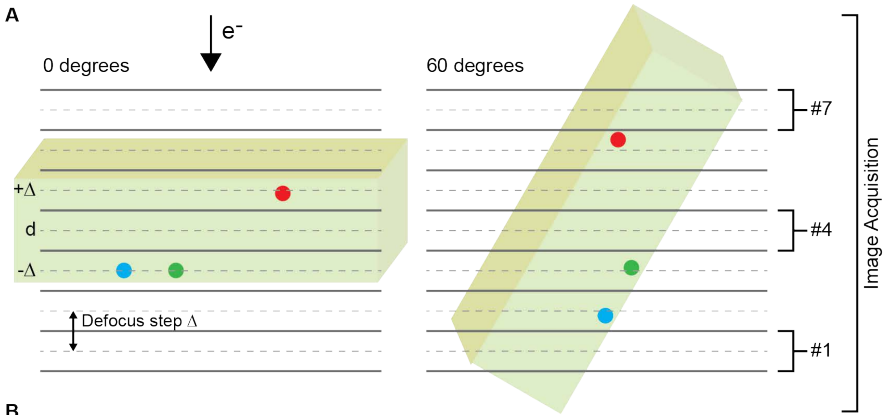
State of the Art for High-Resolution

2D CTF Correction



3D CTF Correction



Turonova et al., 2017

2D CTF correction considers only the gradient due to tilt; 3D CTF also considers the gradient through the thick sample

State of the Art for High-Resolution

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- **Use Phase Plates**

Subtomogram Averaging

- Find the particles and extract subtomograms
- Assign initial angles
- Align subtomograms (2nd alignment!)
- Average subtomograms
- Iterate, classify
- Structure building (integrative modeling)

Many software packages available: Dynamo, PEET, bSoft, PyTom, RELION

Subtomogram Averaging

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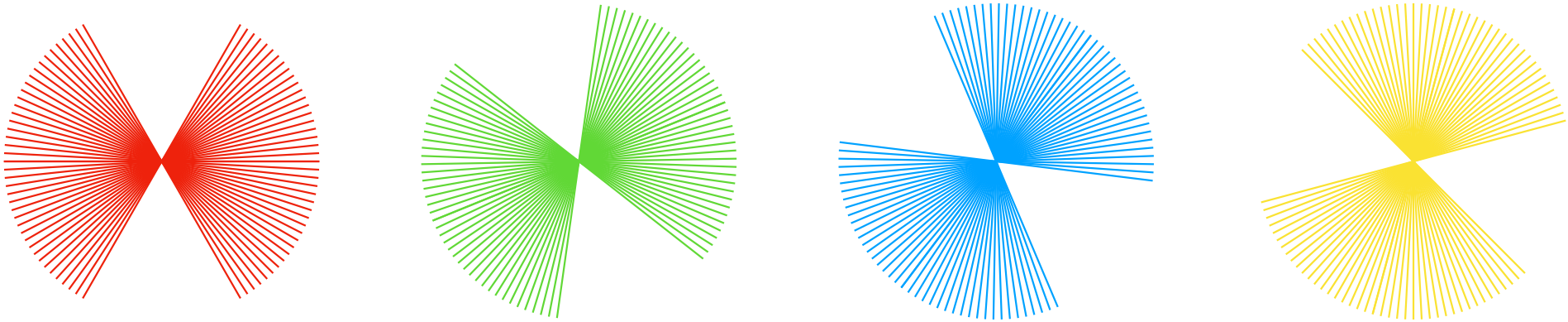
Subtomogram Averaging

Can eliminate the missing wedge if you have particles in all orientations:



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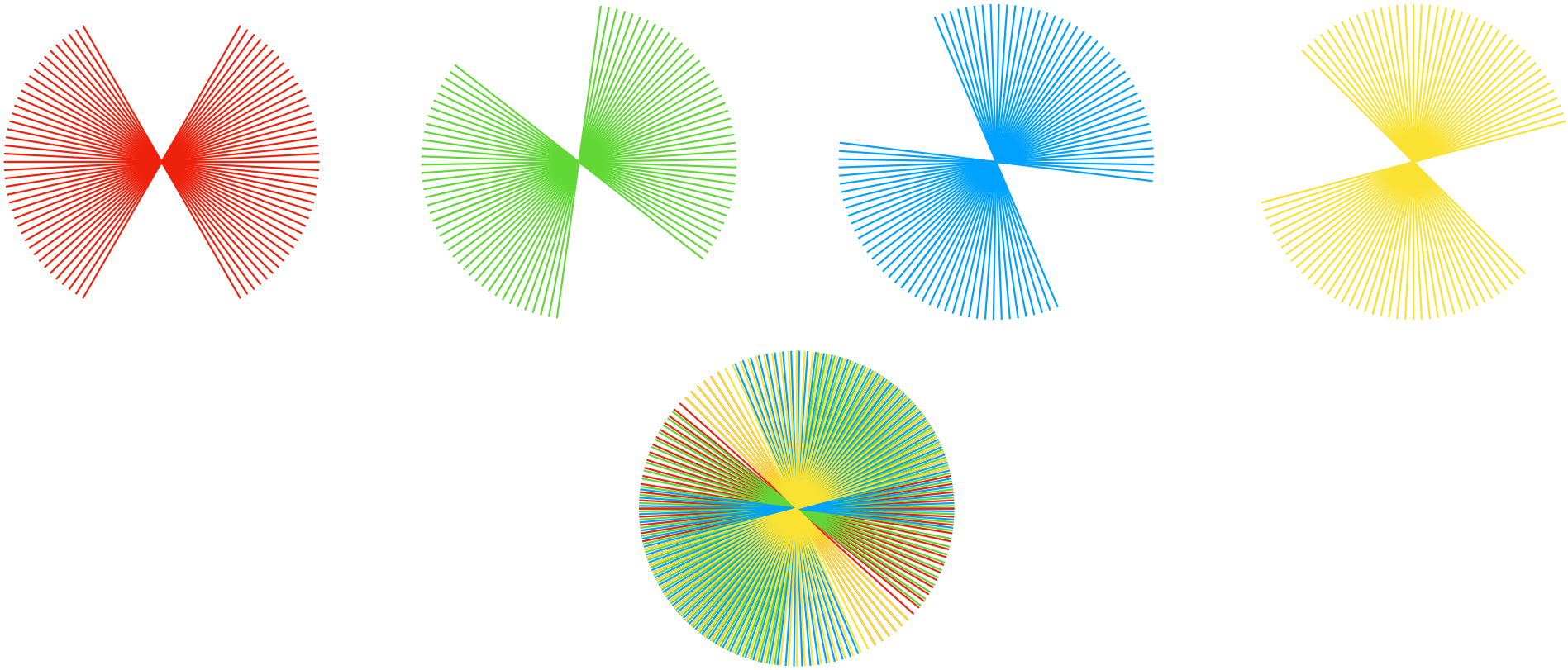
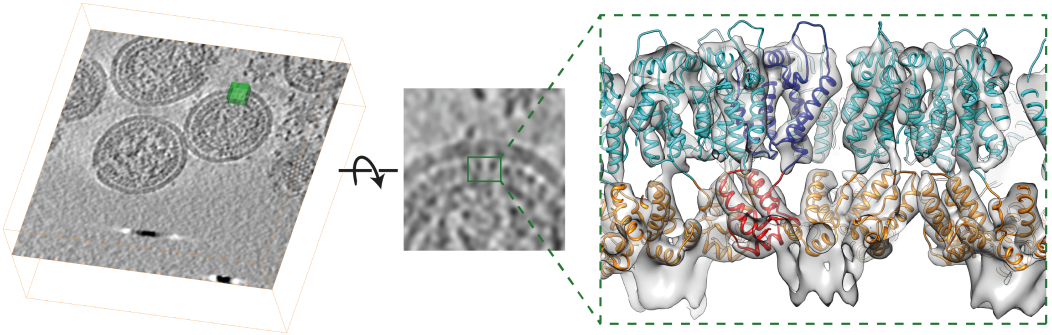


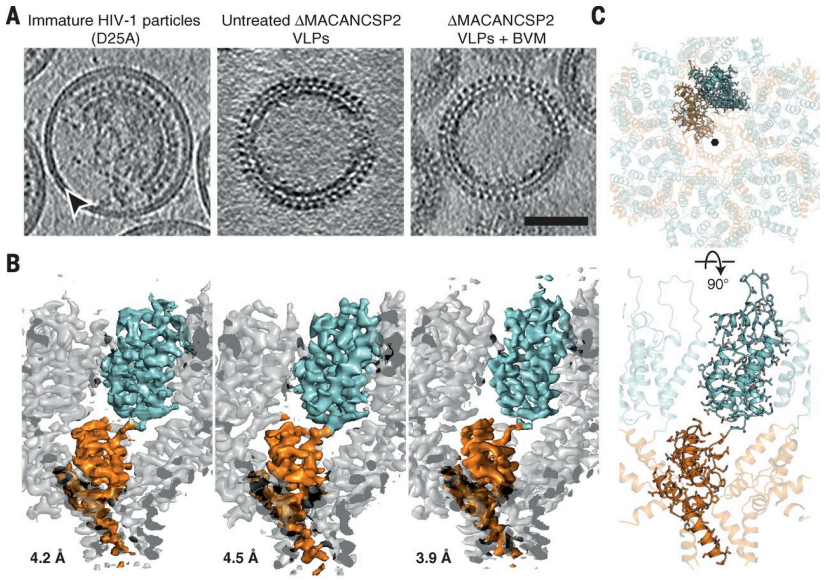
What are the challenges associated with recording high-resolution information ~~from cells~~?

- Radiation damage (sample changes affect alignment and resolution)
- Two alignment steps (tomogram reconstruction, subtomogram analysis)
- Sample thickness at high tilts (lower S/N and higher apparent BIM due to buckling)
- Identification of particles
- Finding enough particles in all orientations
- Low yield - less particles than in SPA (data acquisition time consuming)
- Many images with low dose - hard to correct CTF
- Classification (affected by many of the above)

Notable Success Story:
Structure of the immature HIV-1 CA-SP1 lattice (Briggs Lab)



Schur, et al., Nature 2015 (8.8 Å)



Schur, et al., Science 2016 (3.9 Å)

Turoňová et al., JSB 2017 (3.4 Å)

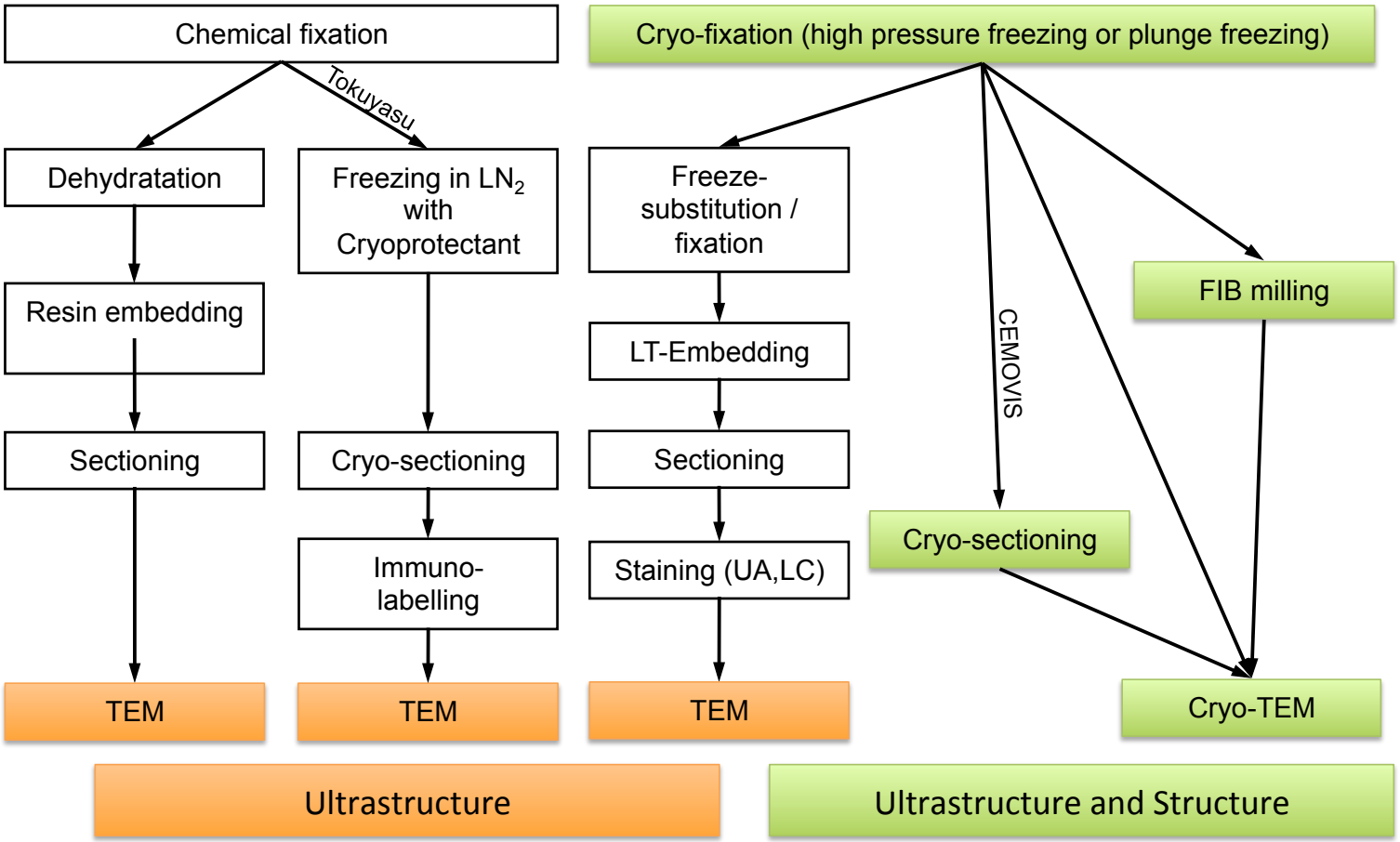
- CCD to K2
- BIM correction
- Dose weighting
- Hagen Scheme
- CTF correction

From 8.8 Å to 3.4 Å

What is the role of FIB milling and other advanced specimen preparation methods?

We’re not in Kansas anymore.

Ultrastructure or Structure?

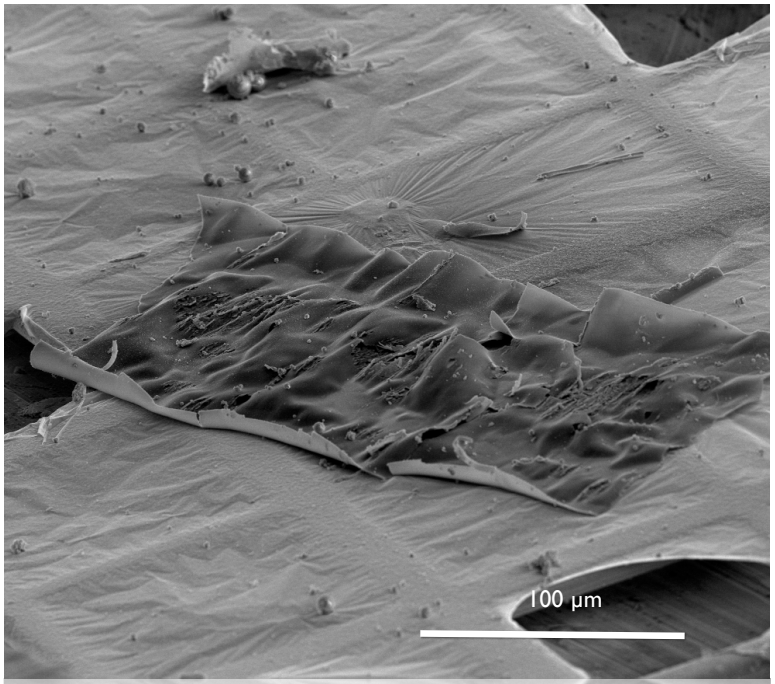


Cryo-Sectioning: CEMOVIS

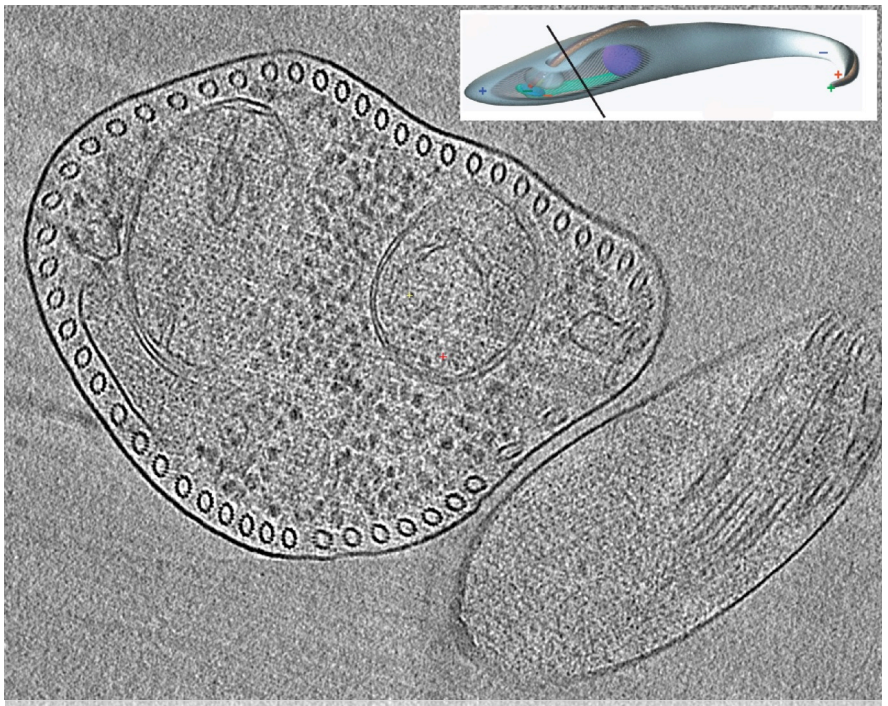
High Pressure Freezer

Microtome

Cryo-Sectioning: CEMOVIS

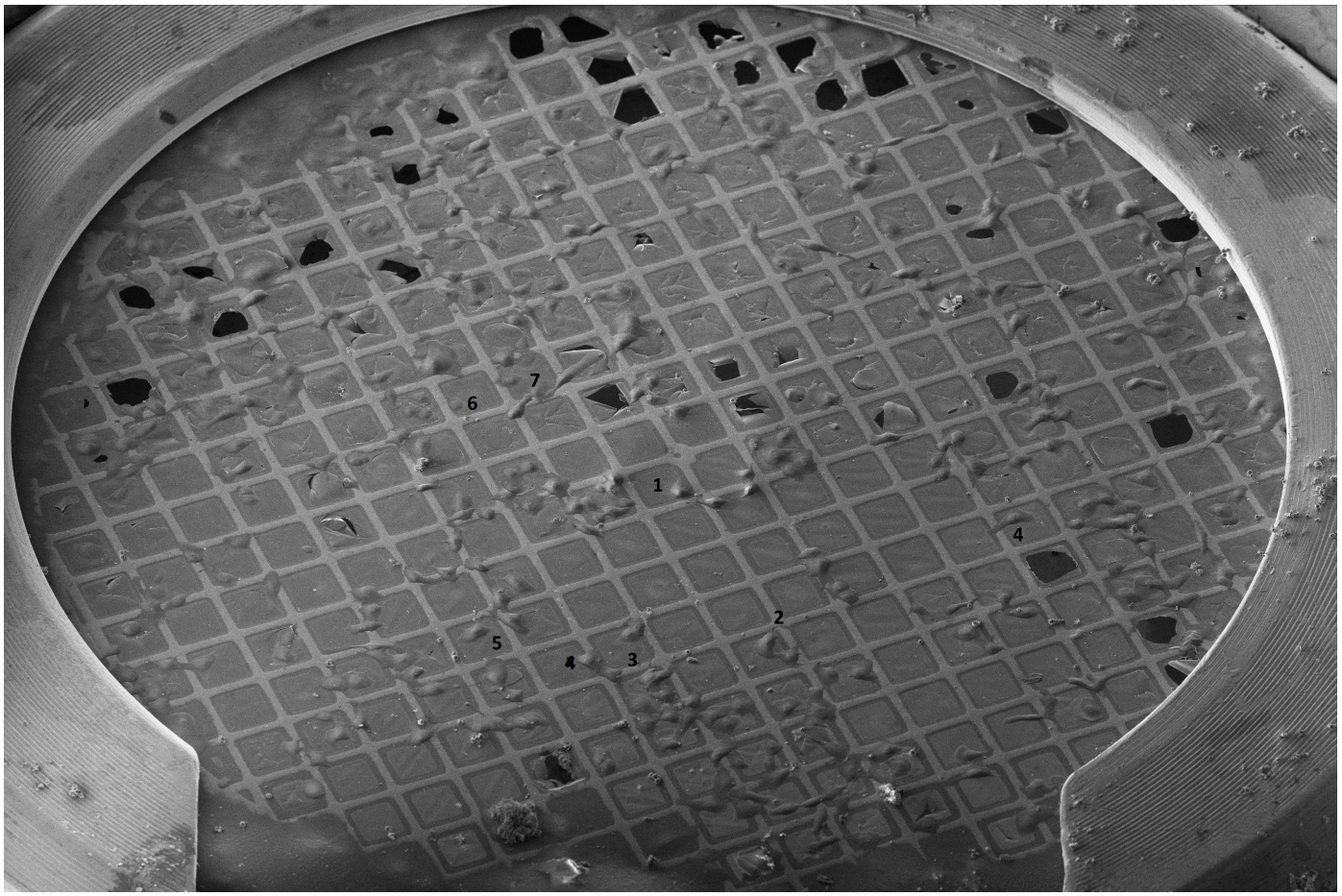


Mouse Hyppocampus, Andrew Leis



Trypanosoma brucei: *Johanna Höög, Cédric Bouchet-Marquis*

Cryo-FIB Milling



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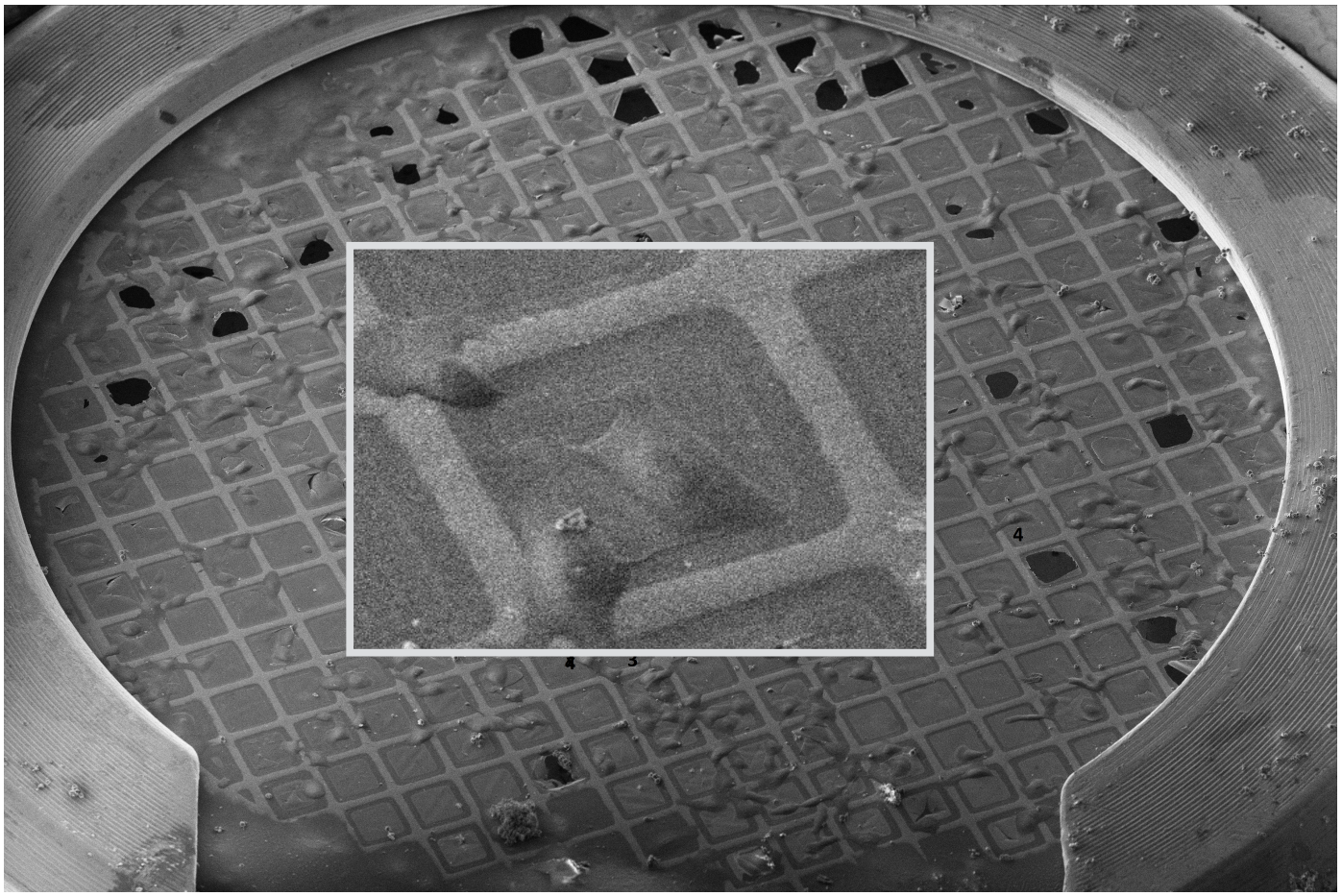

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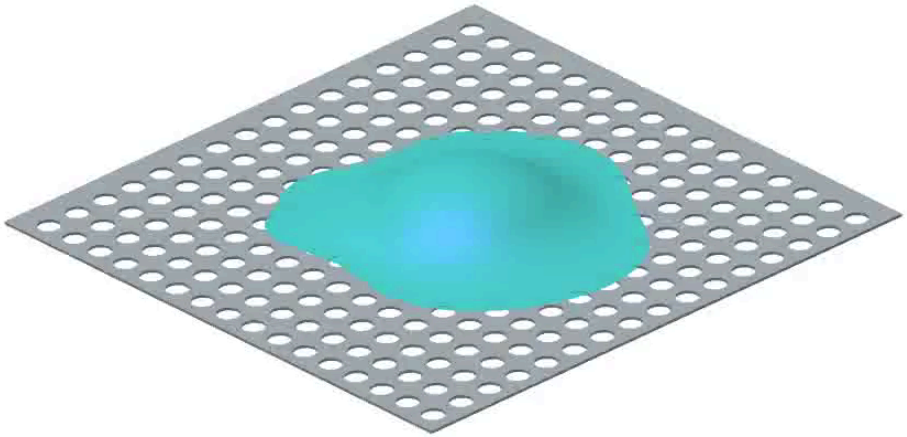

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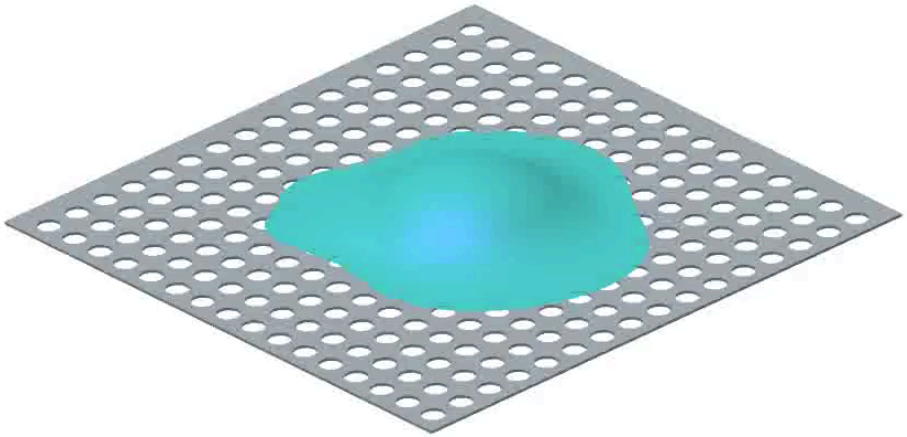

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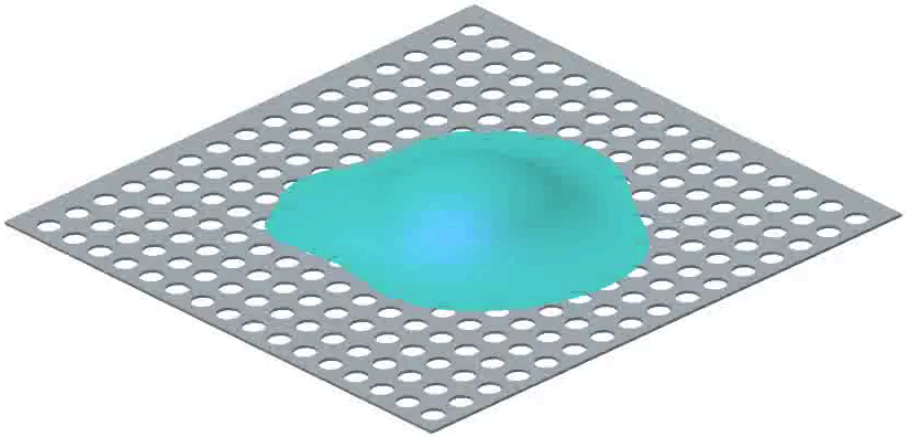
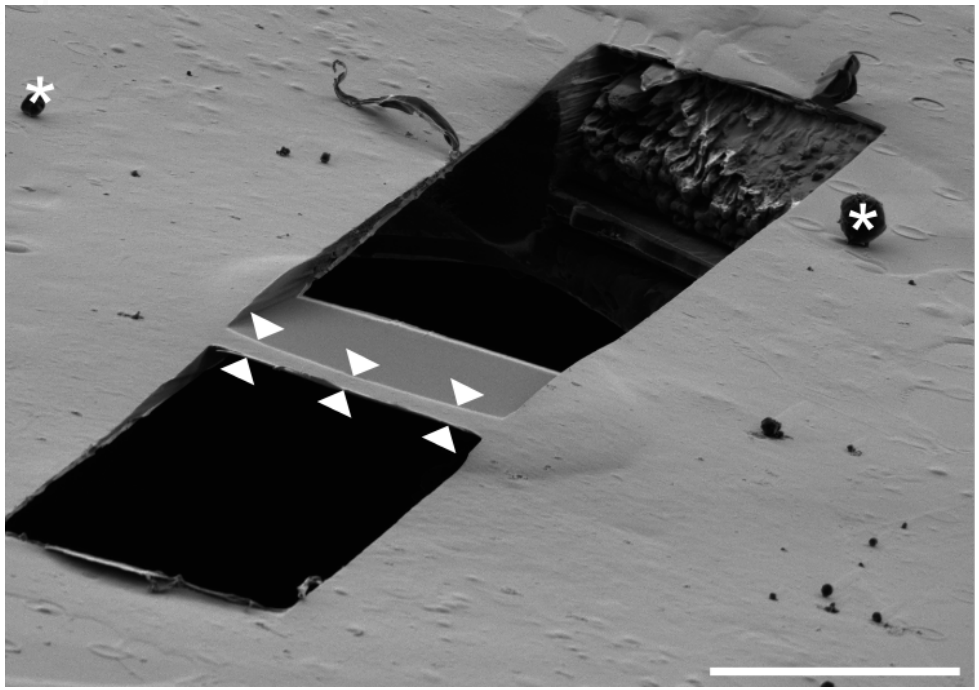
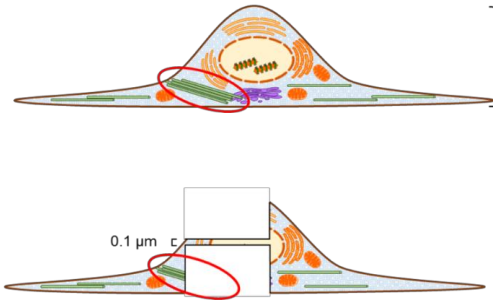




Cryo-FIB Milling: Challenges

- Where to cut? (need localization)
- Freezing of cells not always works with plunge freezing
- Low yield, even when everything works
- Not cheap
- Charging and use of phase plates
- Dog that caught the car (now what?)



How important is correlative LM-EM?

Right now, game changing to find regions of interest in a grid if looking for specific cellular events or phenotypes

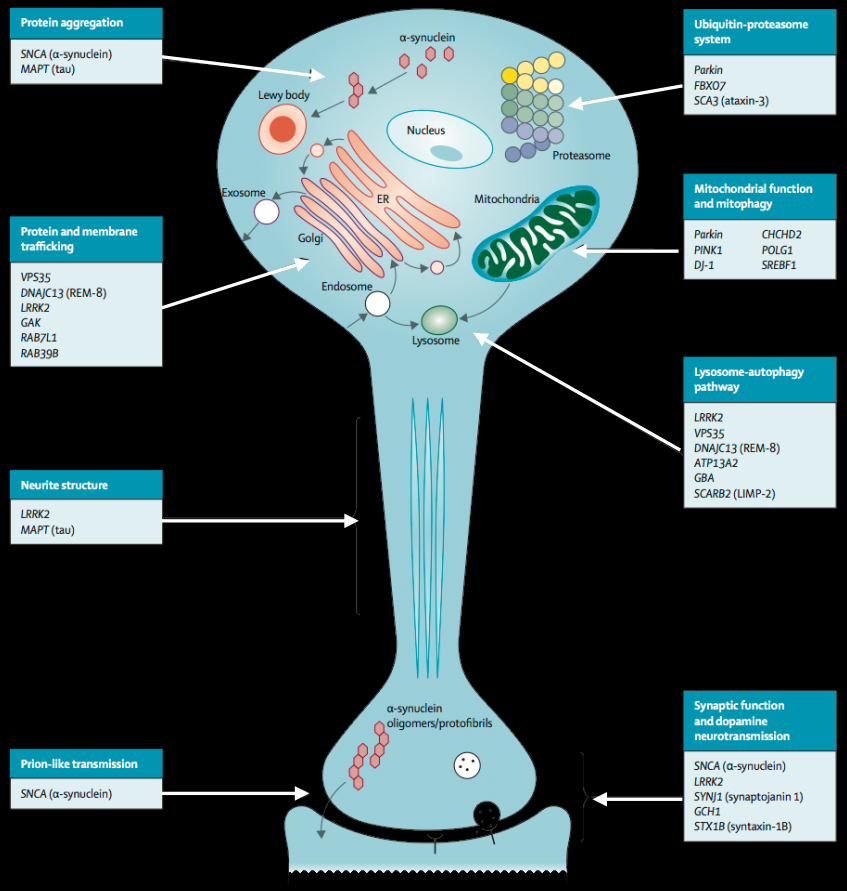
Parkinson's Disease

Protein Aggregation

Protein and Membrane Trafficking

Neurite Structure

Prion-like transmission



Ubiquitin Proteasome System

Mitochondrial Function and Mitophagy

Lysosome-Autophagy Pathway

Synaptic Function and Dopamine Neurotransmission

Kalia, L. V., & Lang, A. E. Parkinson's disease. *Lancet, 2015.*

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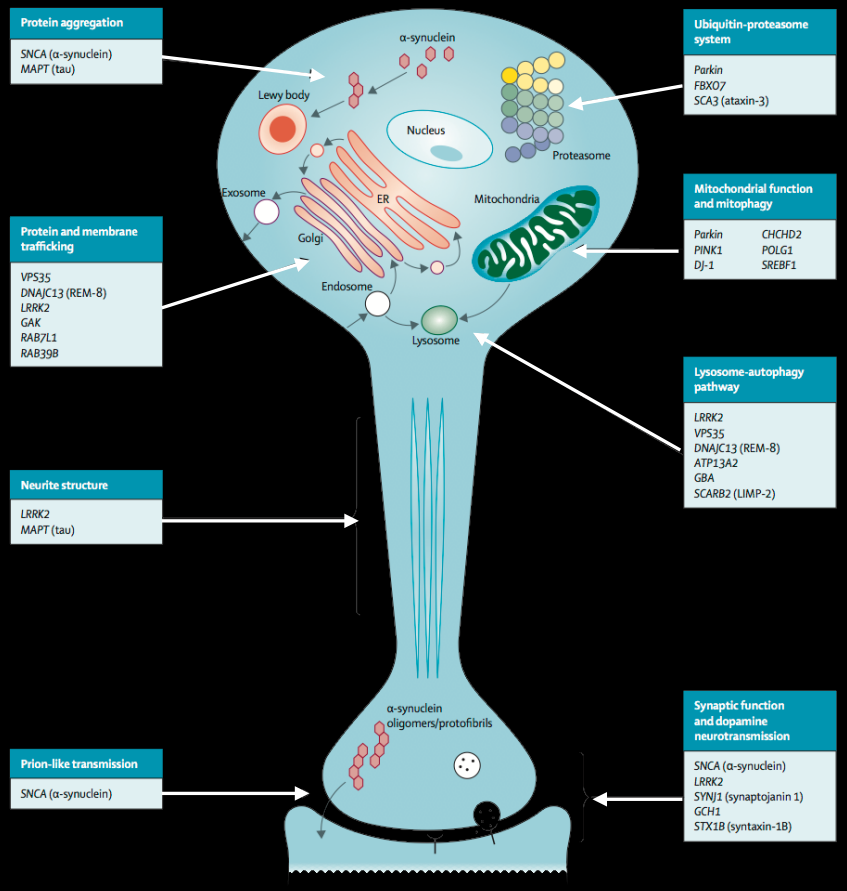

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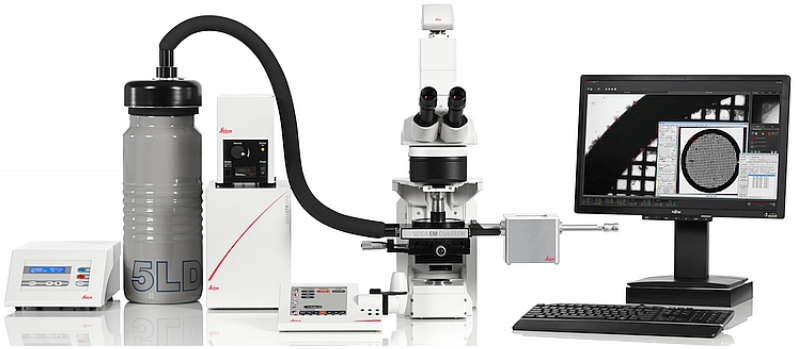
Has CLEM approach reached a stage of development where it is easily and generally applicable?

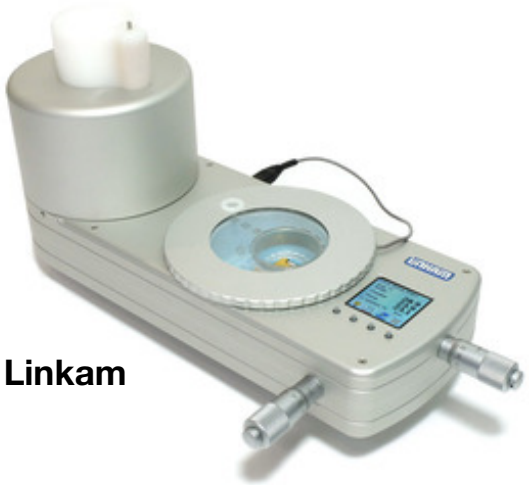
- Depends if you have a working system
- Added transfer step— lack of contamination is not a foregone conclusion
- Coordinate transfer between microscopes not straight forward
- 3-D is crucial: no commercial system available
- Super-resolution set-ups are to date home built
- Thin samples don’t give enough photons

Has CLEM approach reached a stage of development where it is easily and generally applicable?

FEI Corrsight



Leica



Linkam

How do we locate and identify what we are interested in?

- Visual Inspection
- CLEM (general neighborhood, not yet suitable for identifying individual molecules)
- Image analysis (hacky, stitched solutions, exciting prospects).
- Tags (find/build the GFP for cryo-EM)
  - Genetically encoded
  - Introduced

ALL OF THESE AREAS NEED MAJOR THRUSTS

What are the problems associated with imaging large cellular complexes in situ?

- Sample Preparation
- Low throughput - How many particles?
- Low resolution
- Molecular Identification is hard in most cases
- Crowding makes molecular identification and image processing harder

What technologies do we still need?

- Automate everything
- Reduce risk of contamination during transfers (better cryo-stages in FIB, LM)
- Better freezing strategies for single cells and tissue — vitreous ice, time-resolved freezing.
- More compatibility between cell culture and our substrates
- Routine (automated?) FIB milling (cells and tissue)
- More stable TEM stages - faster acquisition
- CLEM stages at higher resolution
- Tags for molecular identification
- Tomogram reconstruction: avoid two alignment steps, iterative reconstructions
- Image analysis algorithms tailored to tomography:
  - Segmentation
  - Molecular identification (visual proteomics)
  - Subtomogram classification on amphetamines

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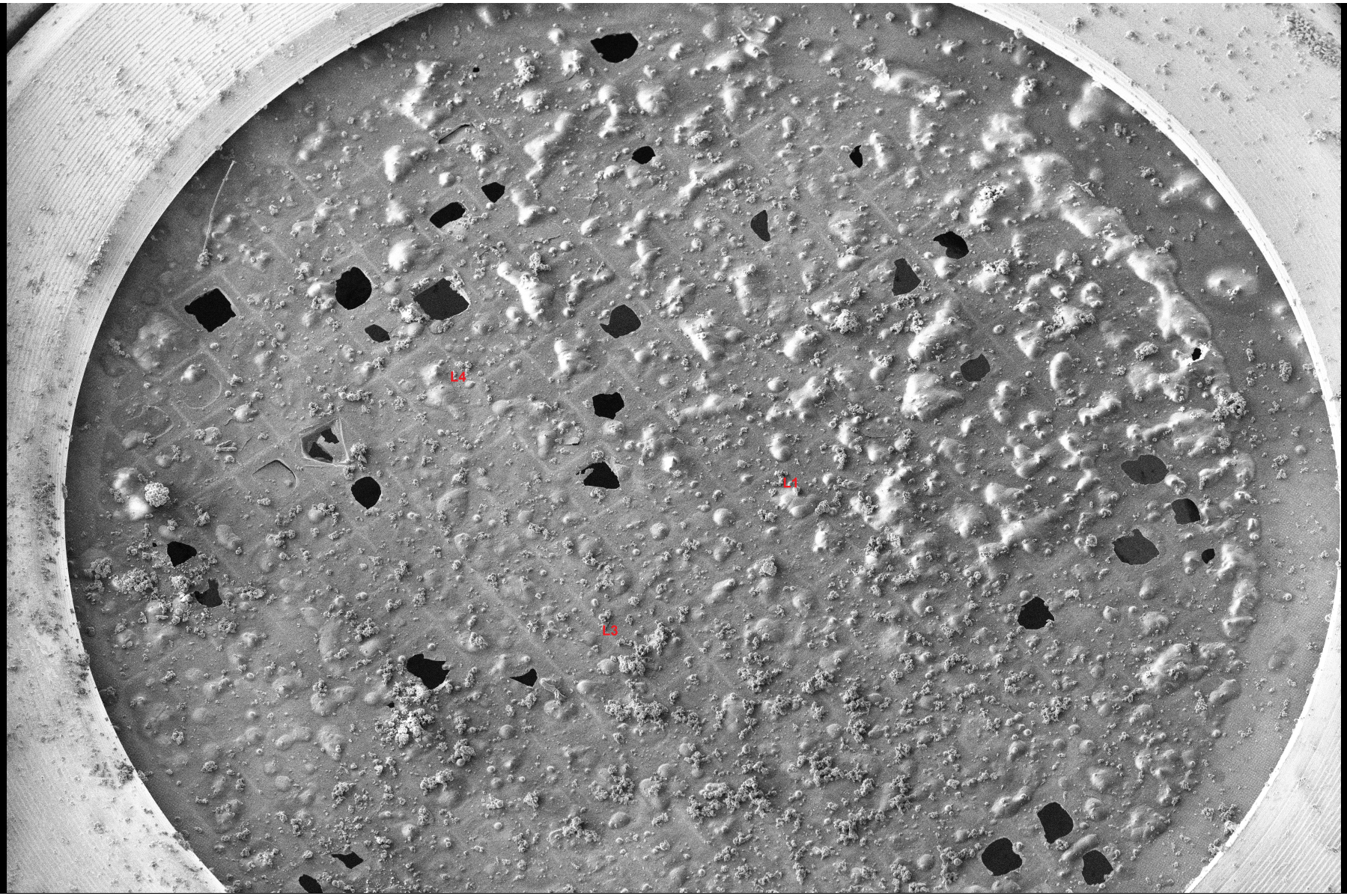
L4
L1
L3

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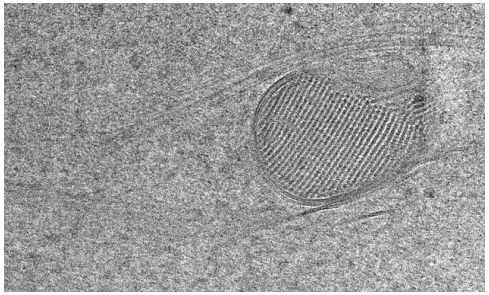
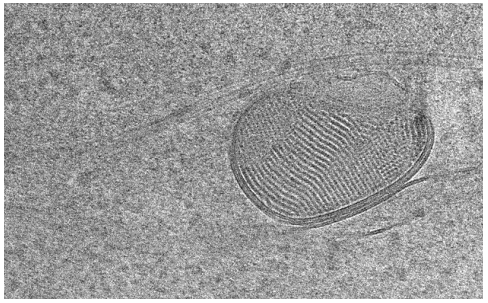
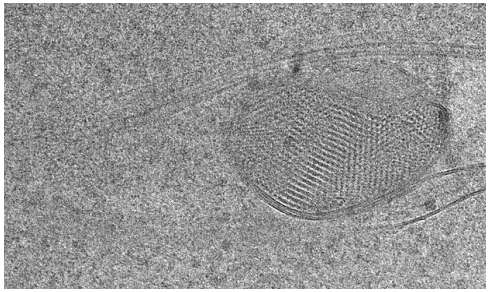
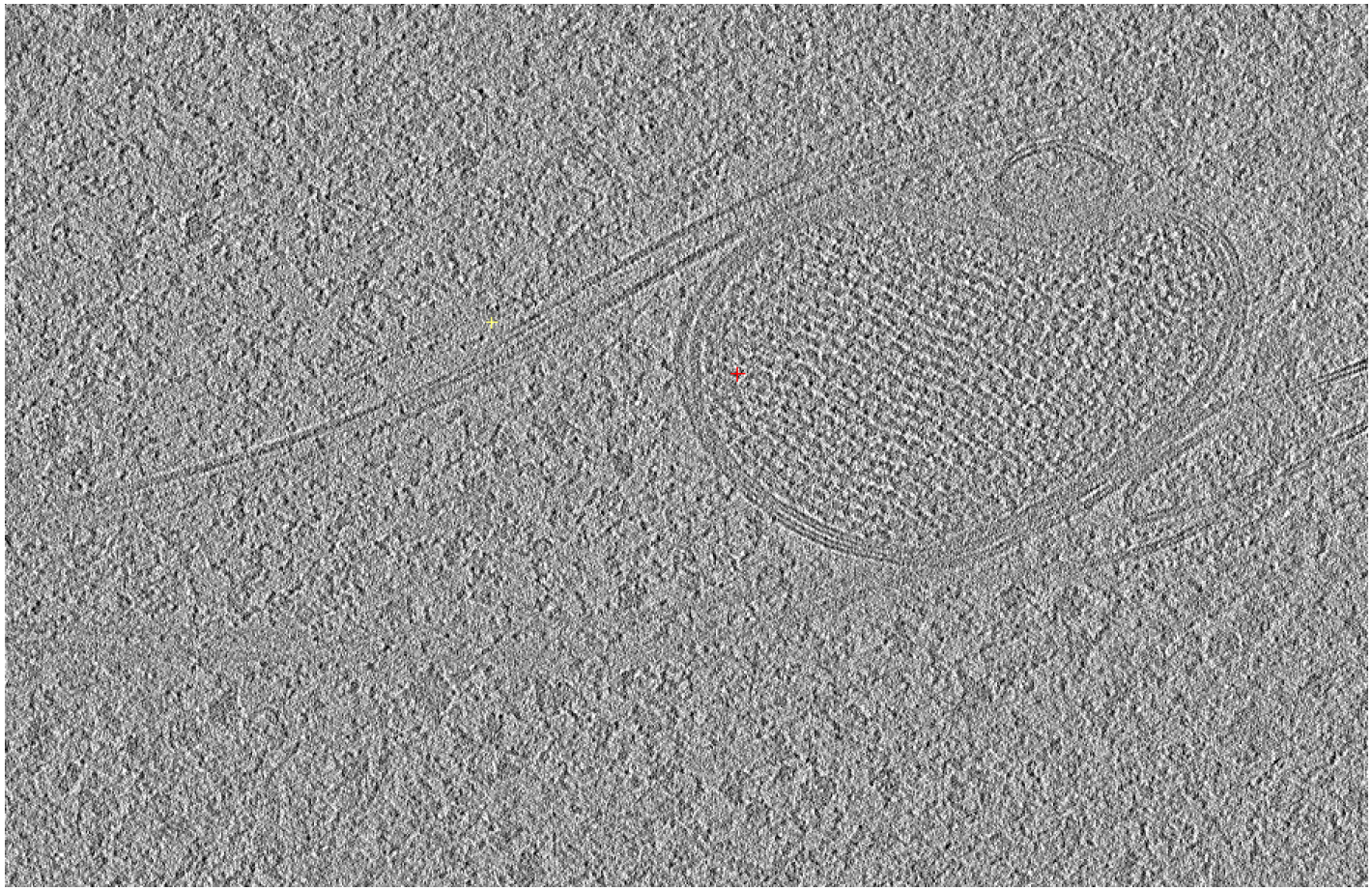
Sneak Peek: Deconvolution



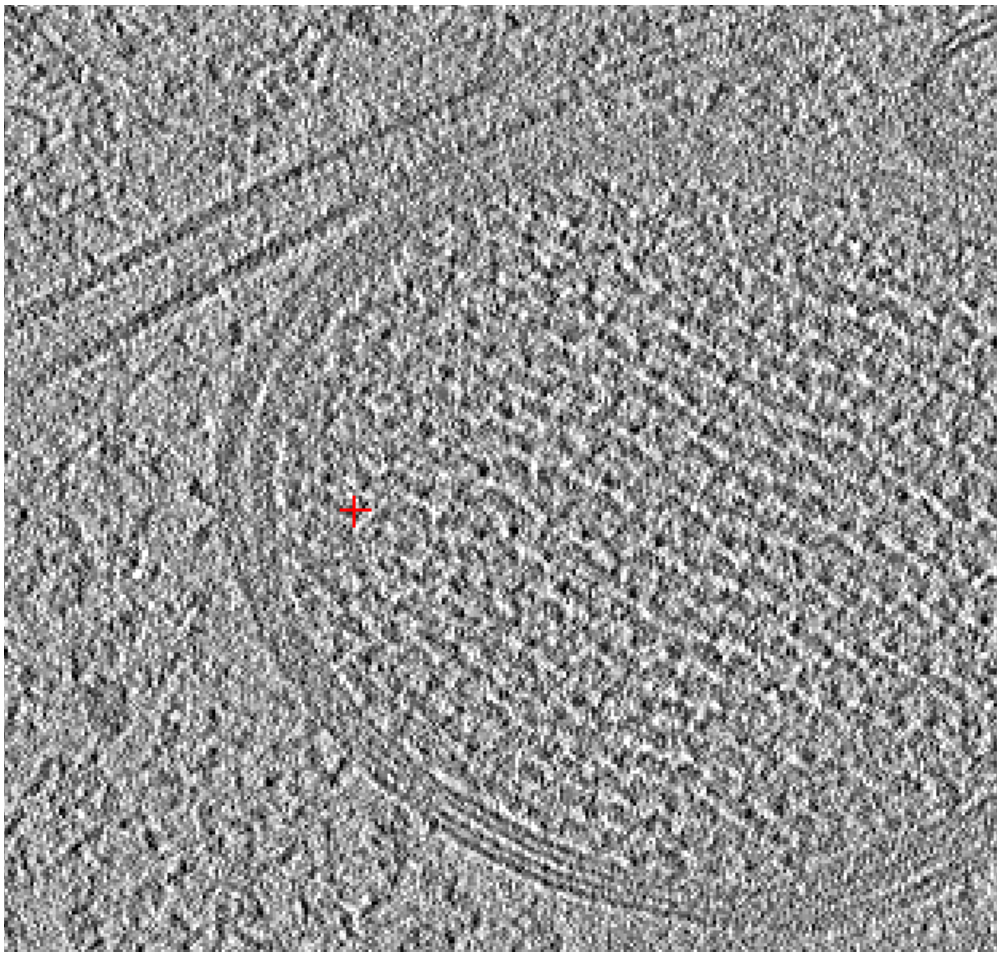


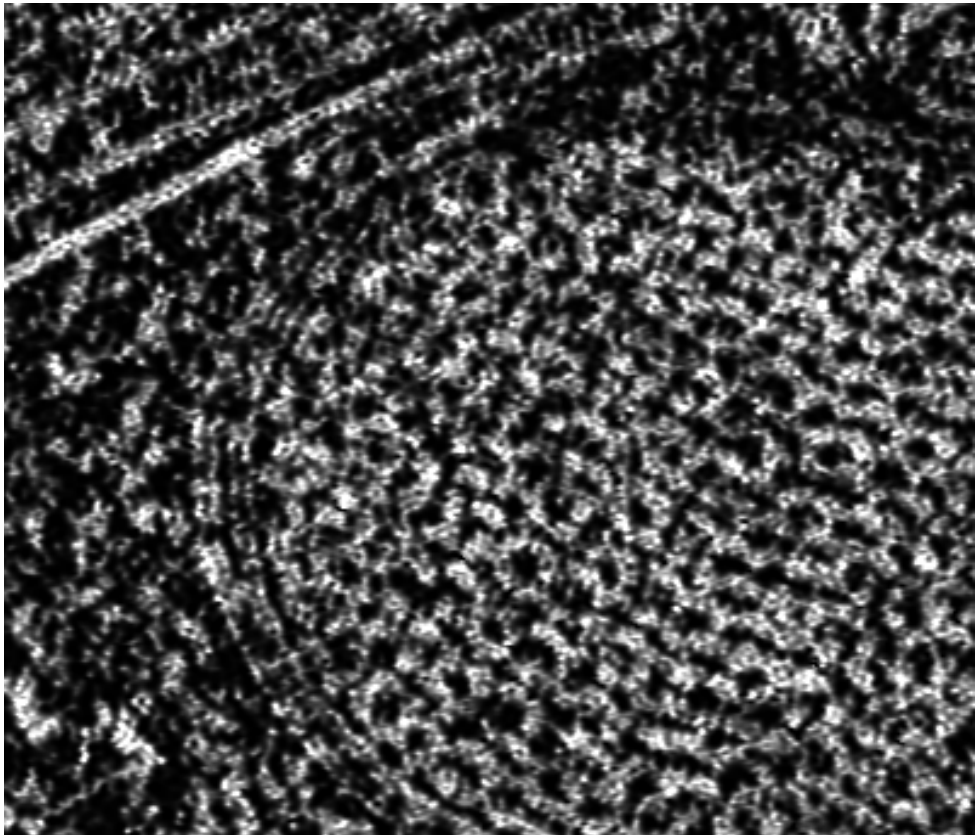




John Sedat

Sneak Peek: Deconvolution





John Sedat

Is tomography coupled with sub tomogram averaging the best way to extract the highest resolution information?

Other interesting approaches have emerged:

- Use tomography for determining location of particles (TYGRESS, Nicastro Lab)
- Use tomography for determining helical parameters (EMBL)
- Do not use tomography! Find particles from high resolution features within 2-D projections (Grigorieff Lab)

Approaches we hope for:

- New detectors and phase plates may allow for treating projections independently (get rid of two separate tilt series alignment and subtomogram alignment)

This approach has recently yielded near atomic resolution structures; could this become routine?

Very likely, for well behaved samples that have many copies of our target of interest.

Possibly, but requires a larger effort towards developing methods (physical and computational) for cryo-ET.

The one inescapable limitation is radiation sensitivity.

Compositional and conformational heterogeneity

What should we pay attention to and what should we NOT do?

We all want to see our molecules in action, but:

Don't forget that in blobology, an image doesn't tell the story alone. Getting a structure at low resolution, even *in situ*, needs a precise biological question.

Ask yourself what concentration does this molecular have on the cell — what are your chances of:

- it being in your sample? (think nM concentration vs. the volumes we can access)
- you finding it?

Now, imagine this for tissue.

What happens if you find 10 events?

Don't underestimate the challenges — but do jump in with both feet!
For in situ cryo-EM, it still holds true that there's a reason that the word CRY is included in CRYO

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HAPPY HUNTING!

There’s a lot of room at the bottom

- Richard Feynman

There’s a lot of room at the bottom

(of the resolution barrel)

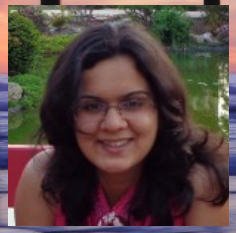
- Richard Feynman

Martina Audagnotto

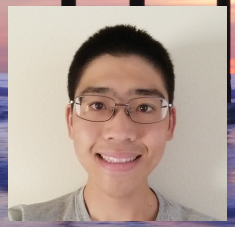
Jan Böhning

Robert Buschauer

Matt Croxford

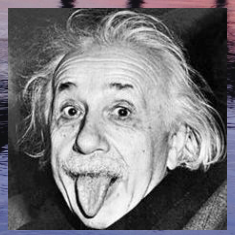


Kanika Khanna



Vinson Lam

Reika Watanabe



YOU!

Thanks to…

Bridget and Clint for the mandate

everyone contributing great ideas and results to the next big thing for our field!

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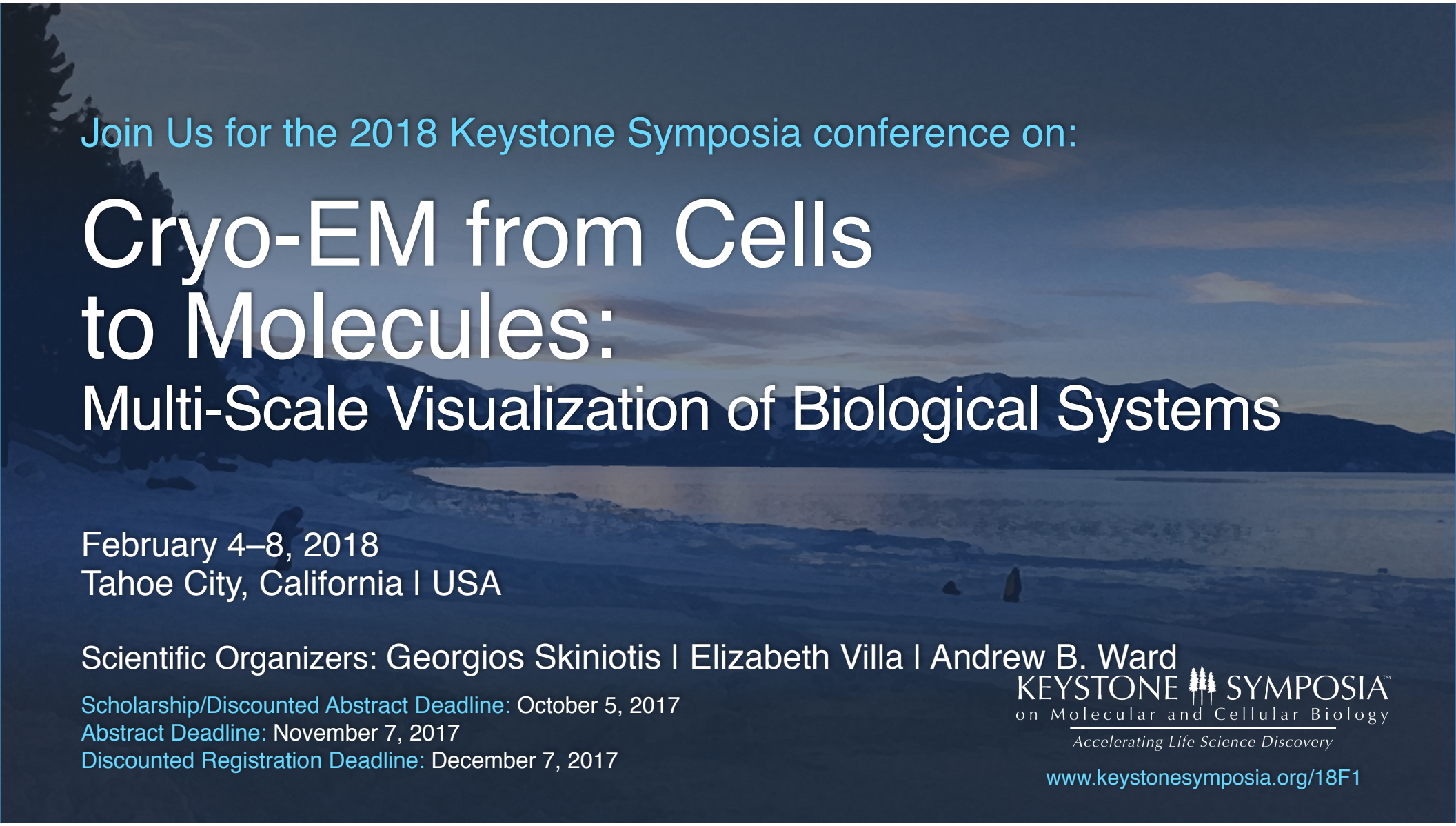
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