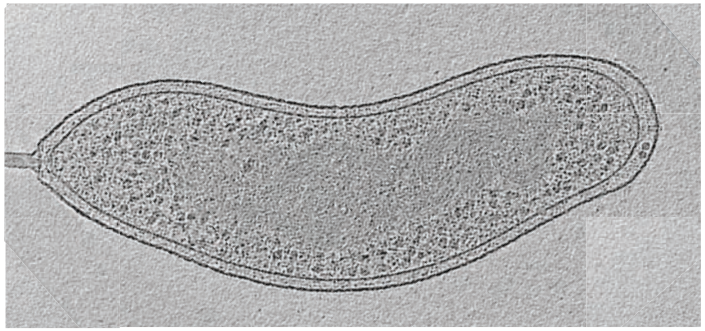
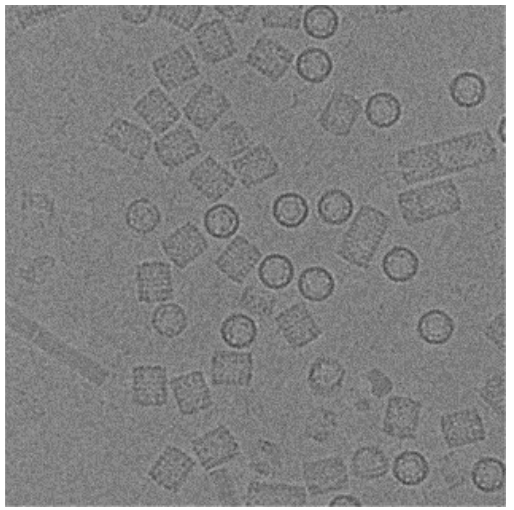
Part 5 - Tomography

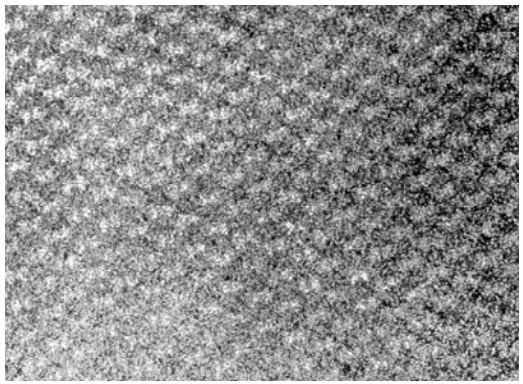
Basic approaches in cryo-EM



Tomography

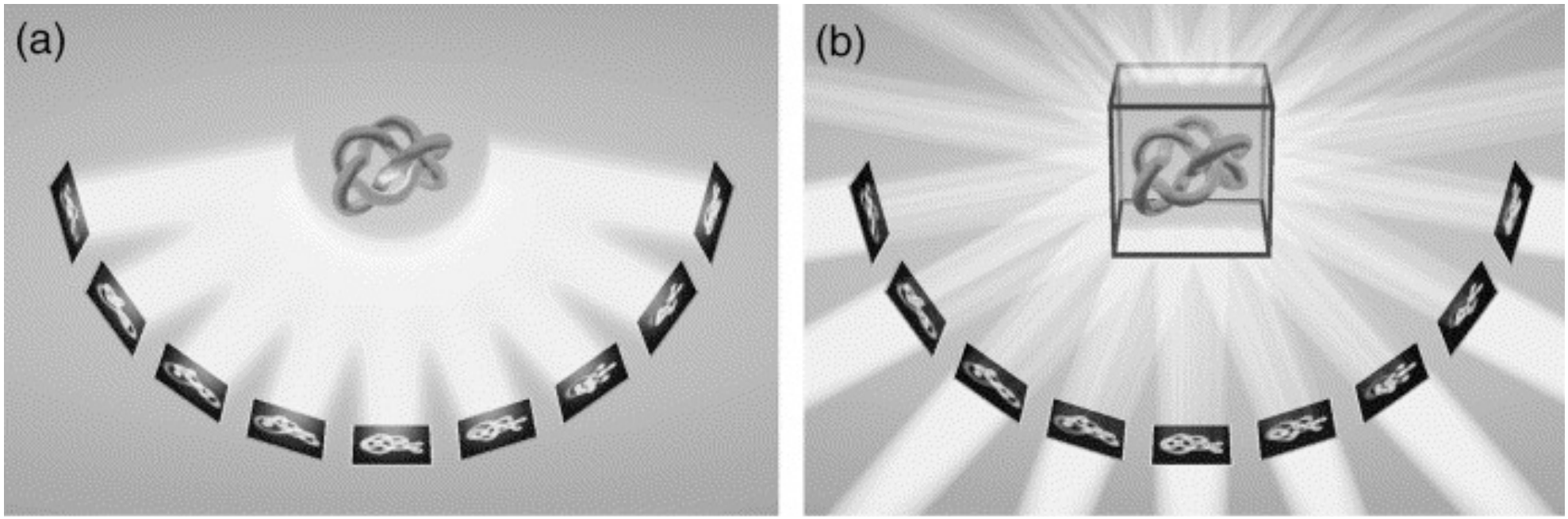
Single particle analysis





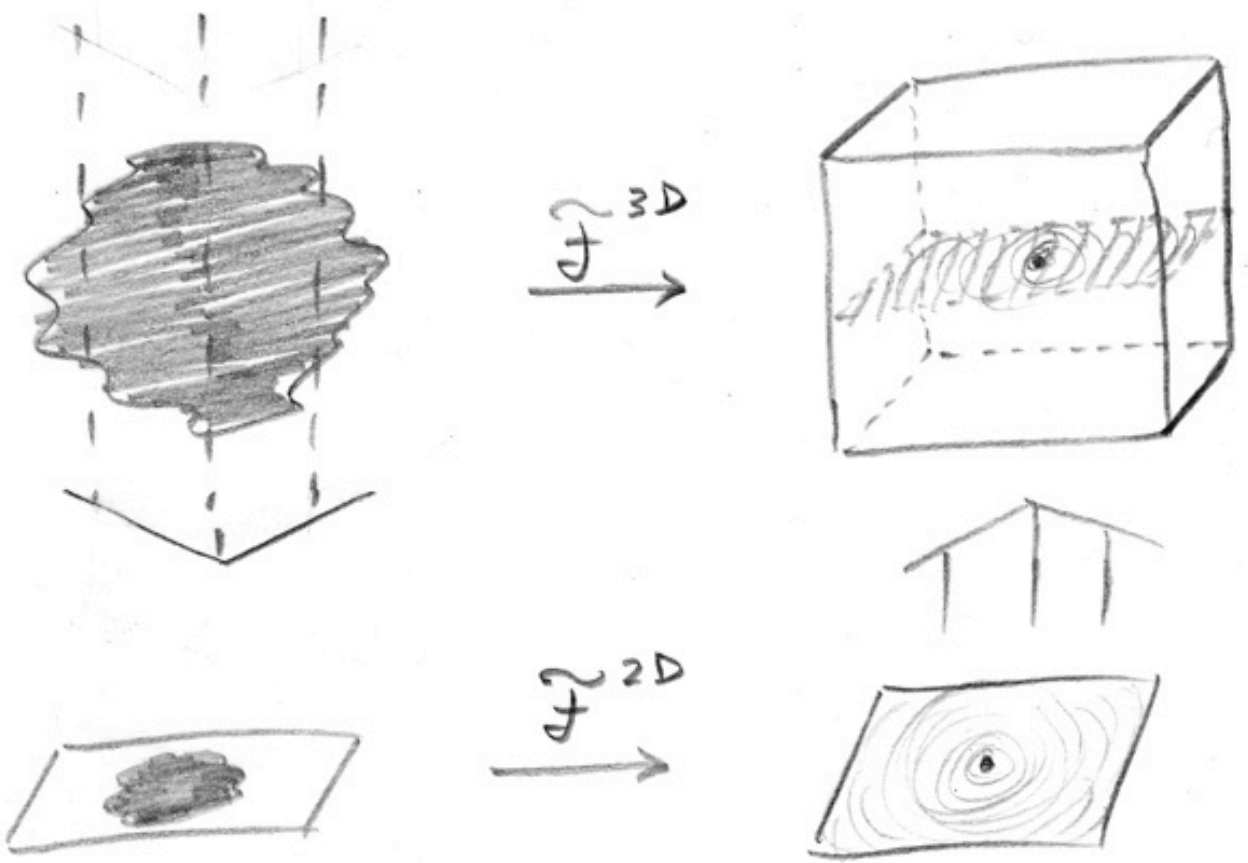
2D crystallography

Back-projection

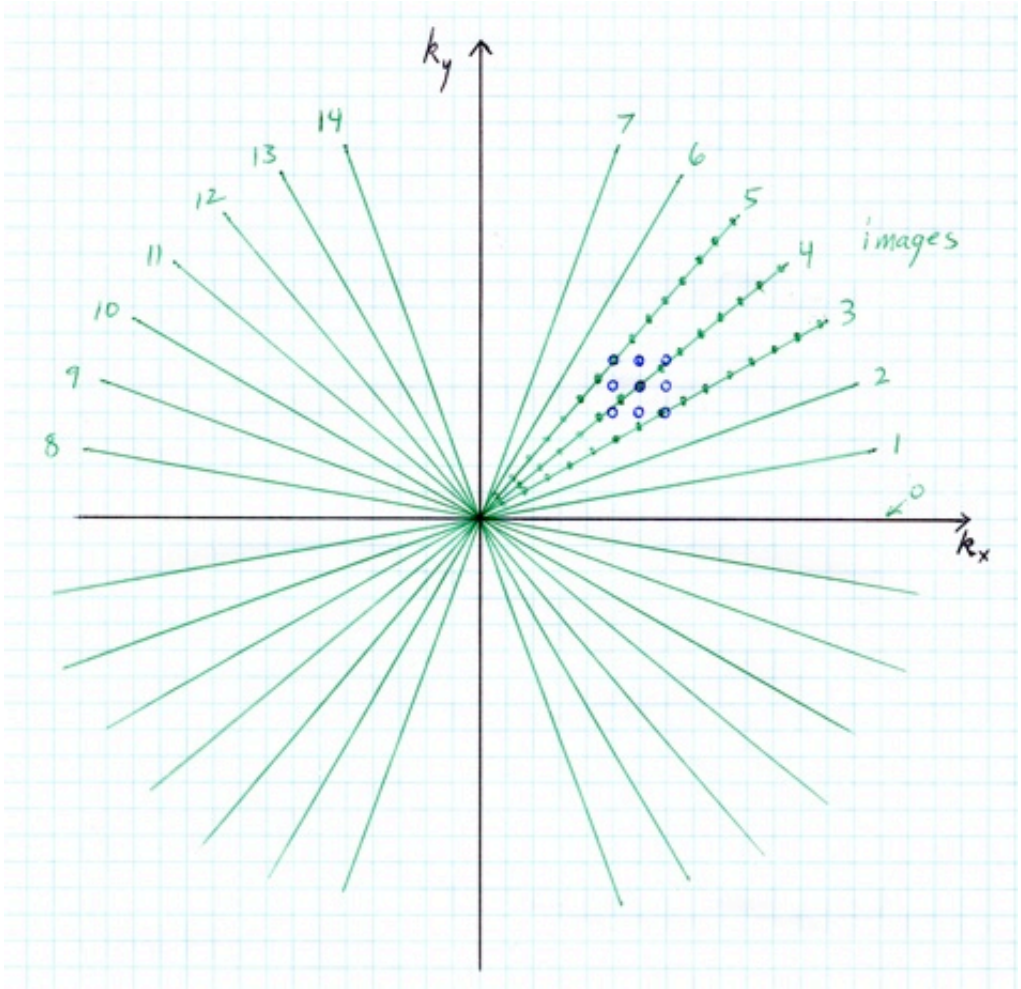


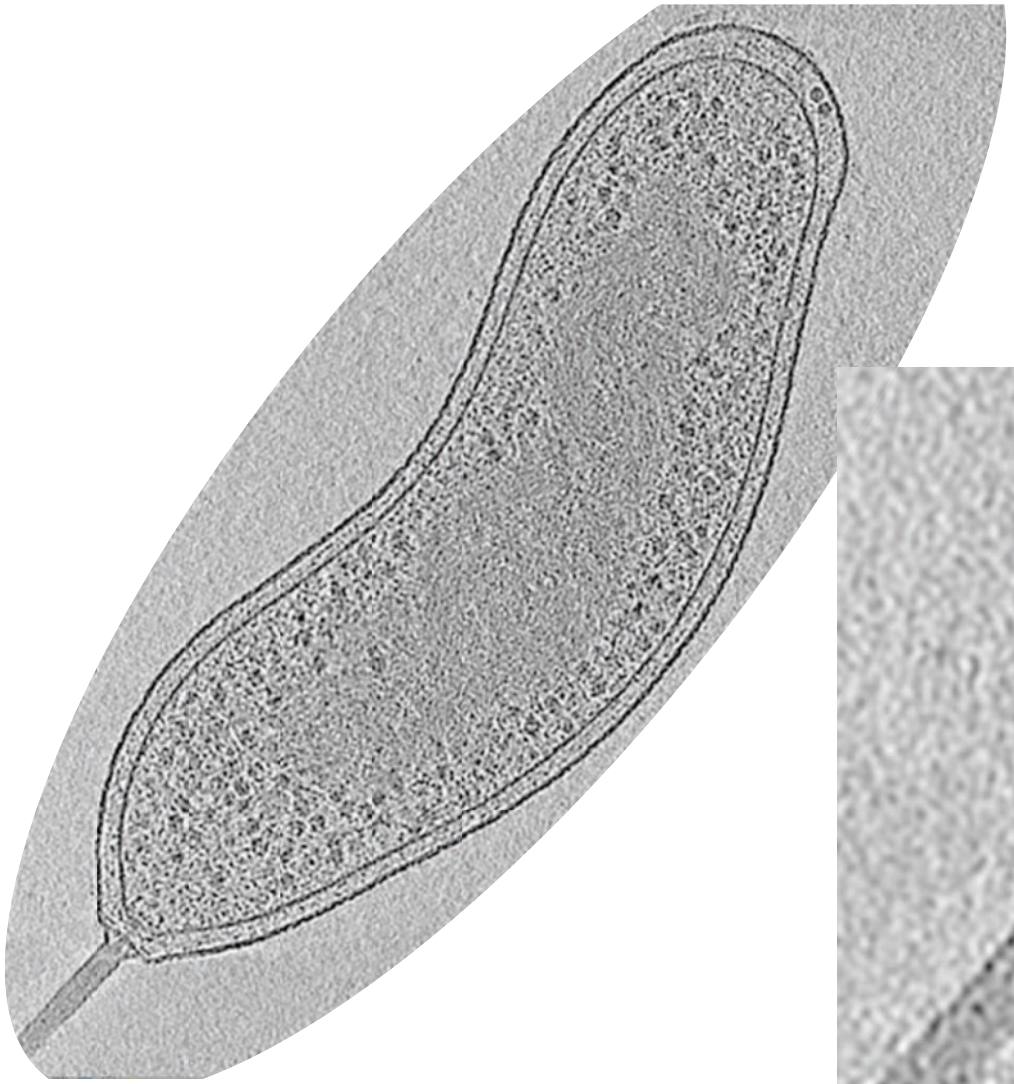
Baumeister et al., Trends in Cell Biology 9:81

The projection theorem

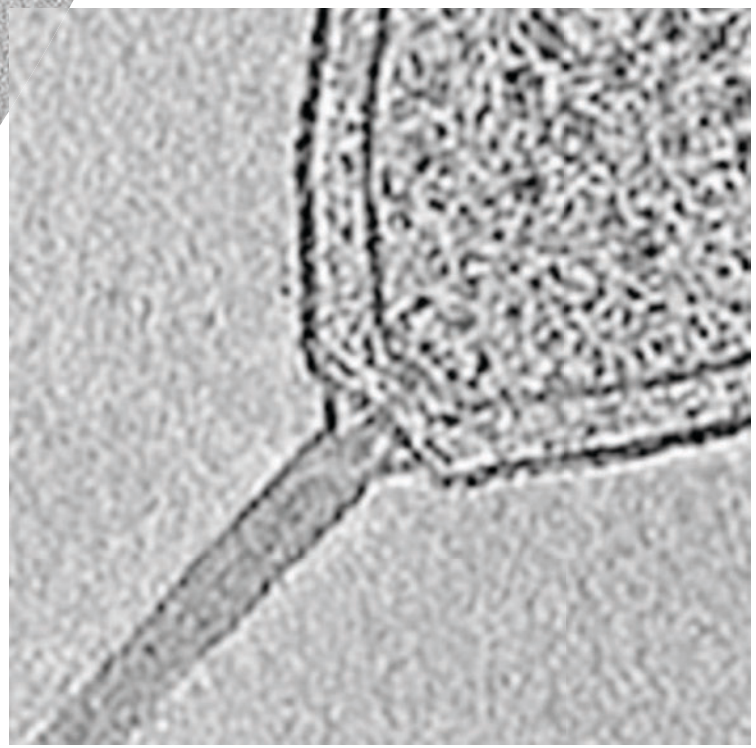


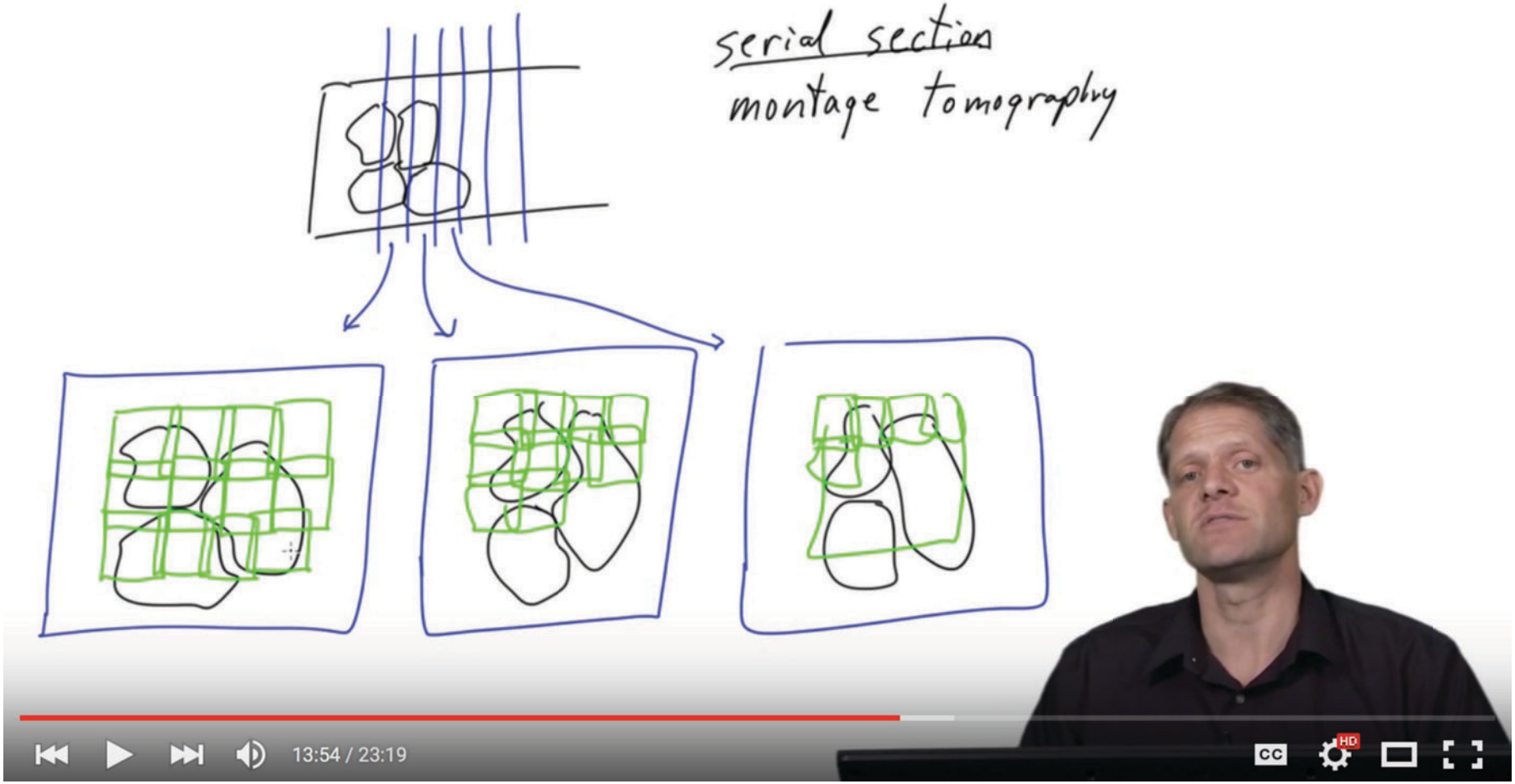
Tilt series are merged in reciprocal space



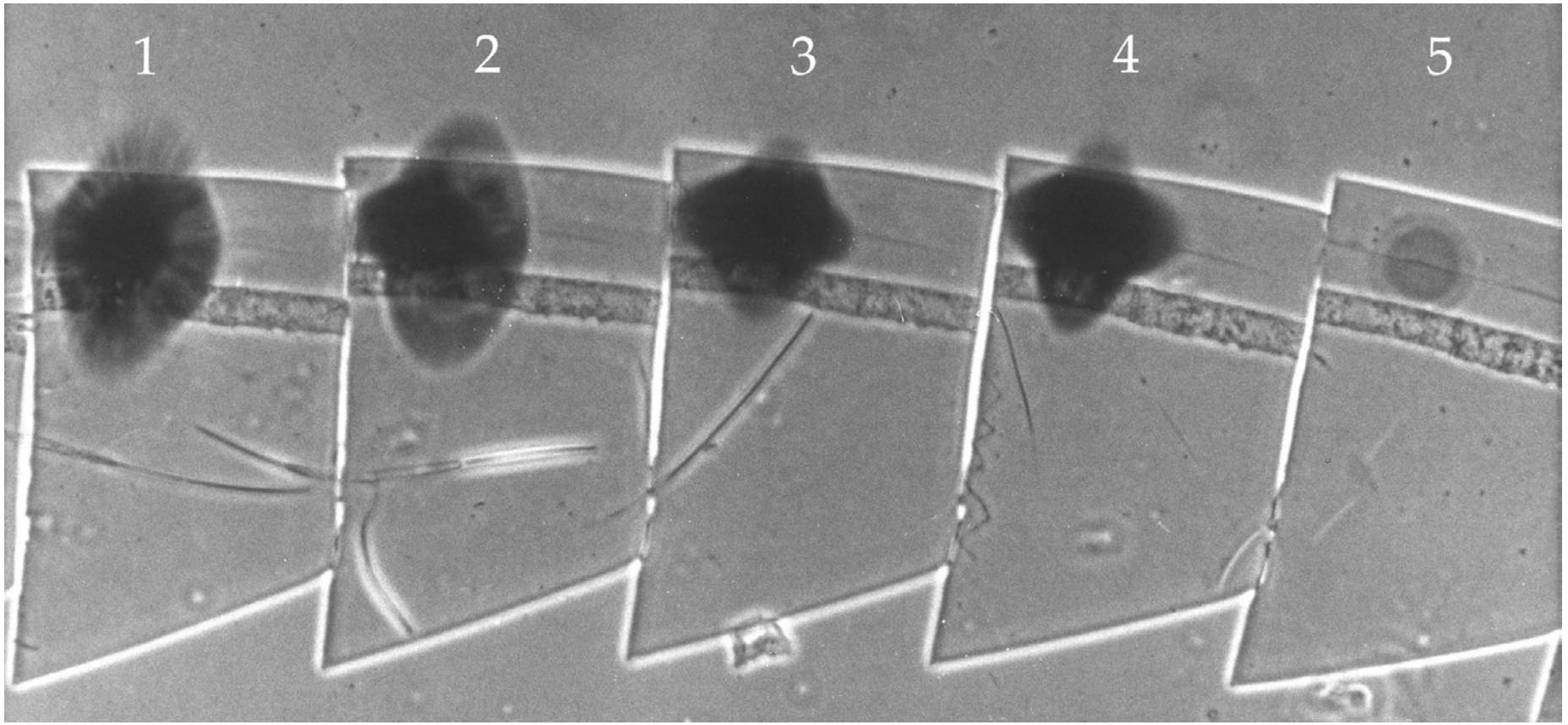


Example images showing
detail visible by ECT

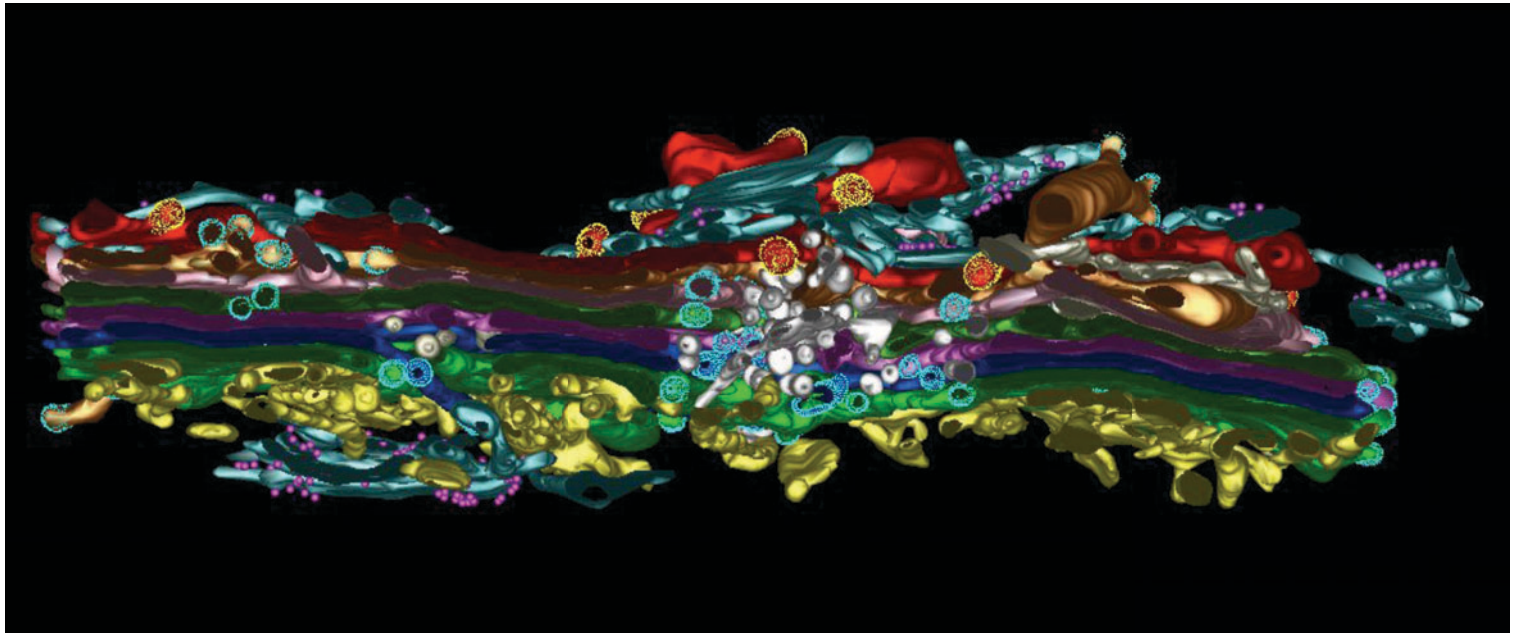



serial section
montage tomography
13:54 / 23:19
CC
HD

“Ribbon” of sections

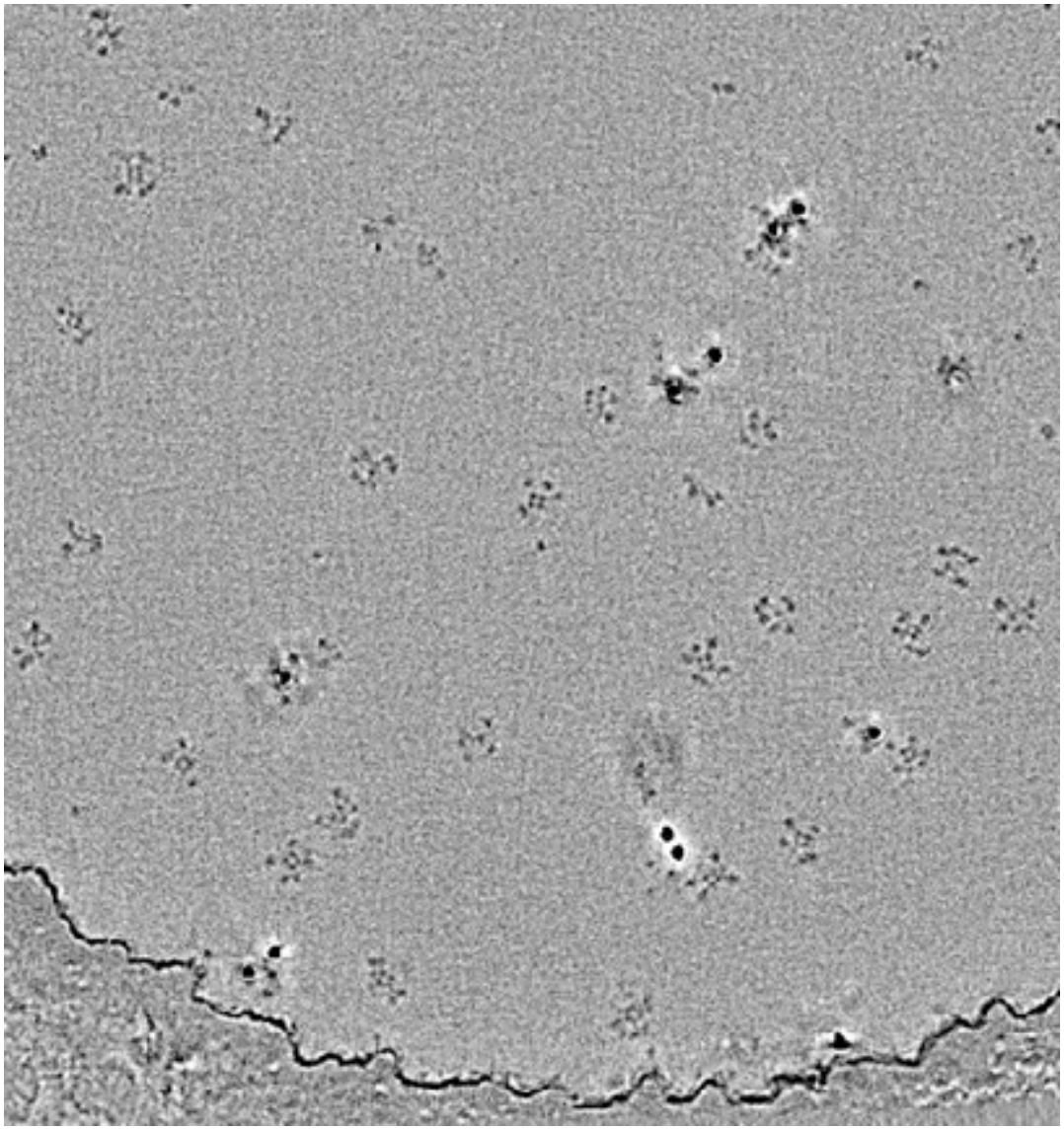


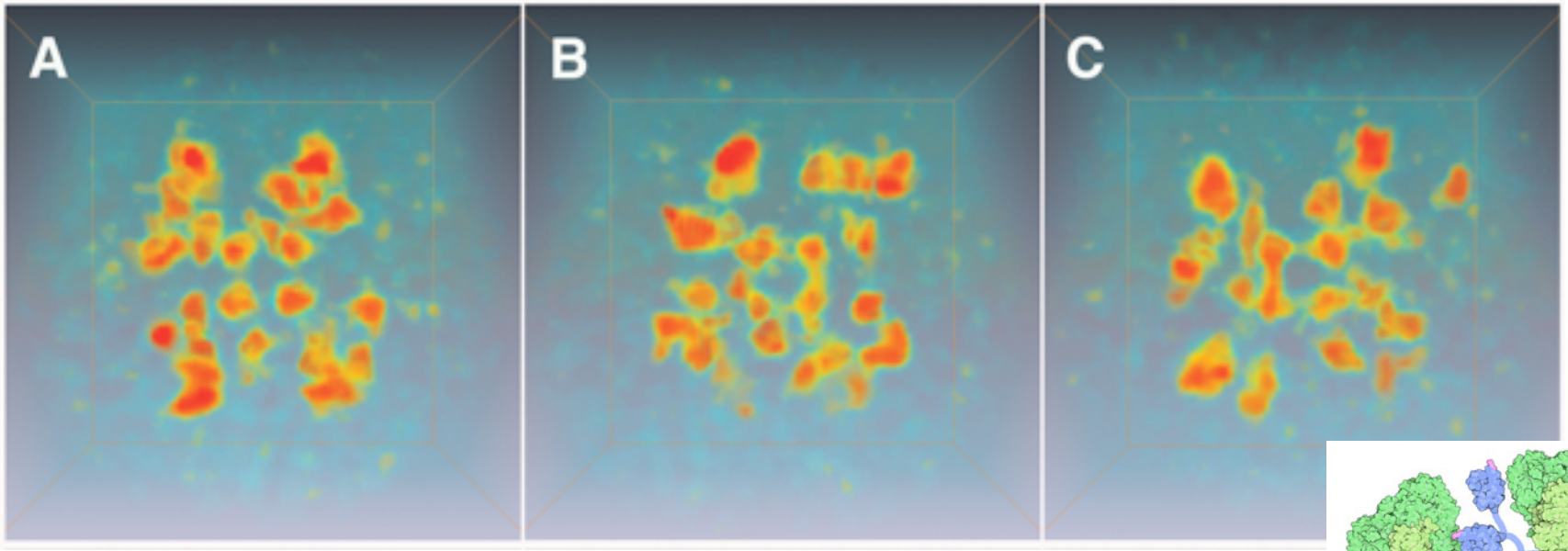
Example serial section montage tomography
(of high-pressure-frozen Golgi)



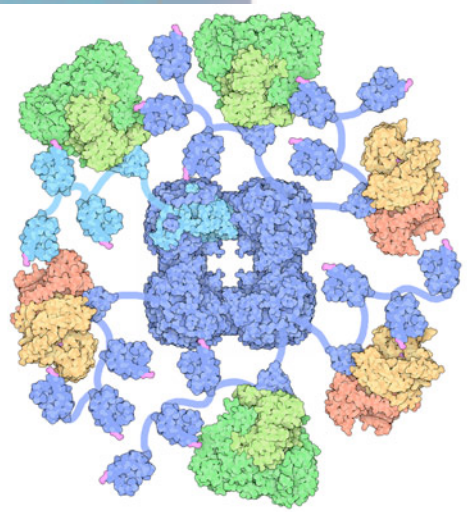
From the Boulder Lab for 3D EM of Cells.
http://bio3d.colorado.edu/

Example tomographic slice through a purified protein complex (pyruvate dehydrogenase multienzyme complex)





"volume rendering"

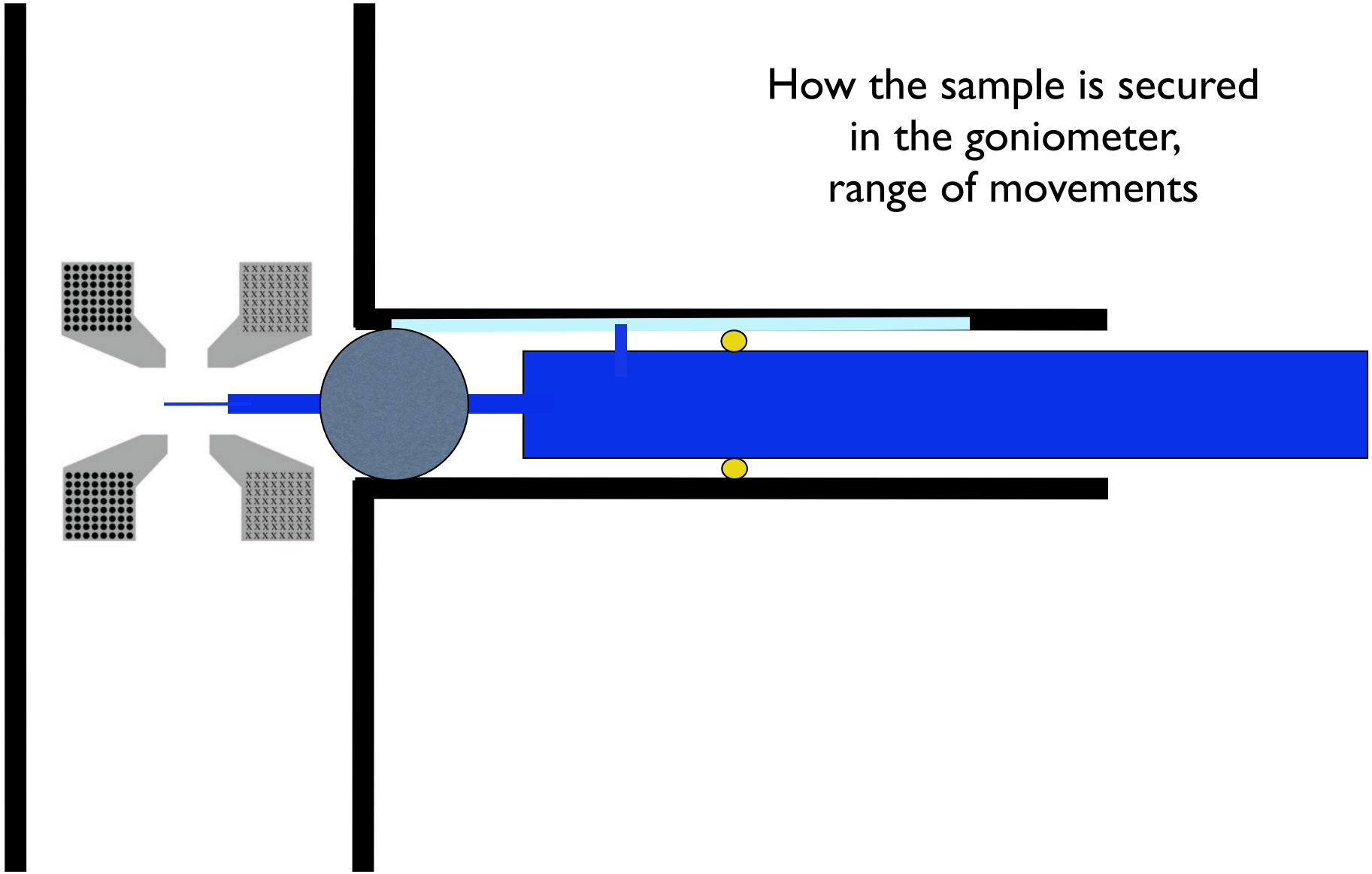


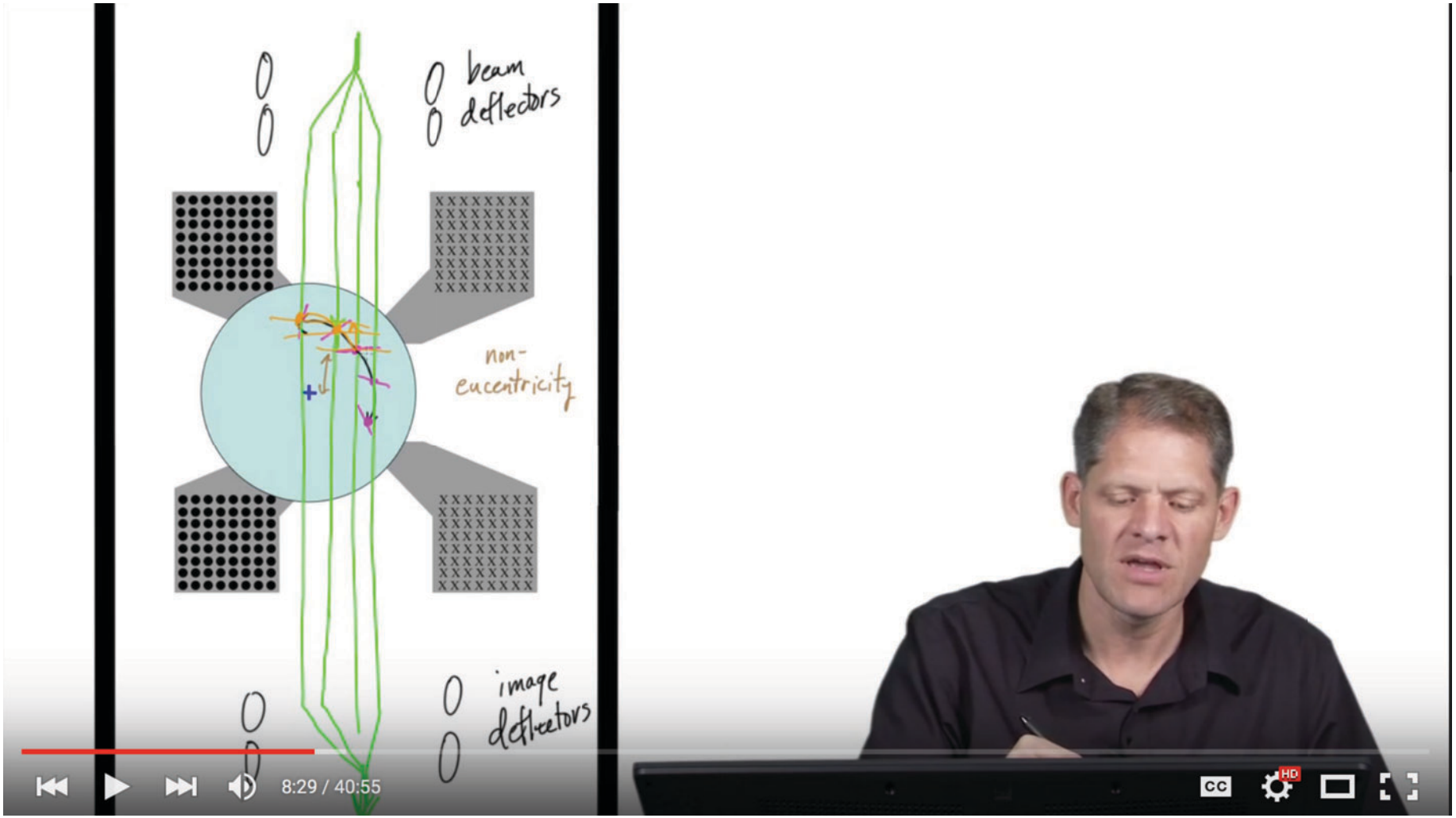
http://www.rcsb.org/pdb/101/

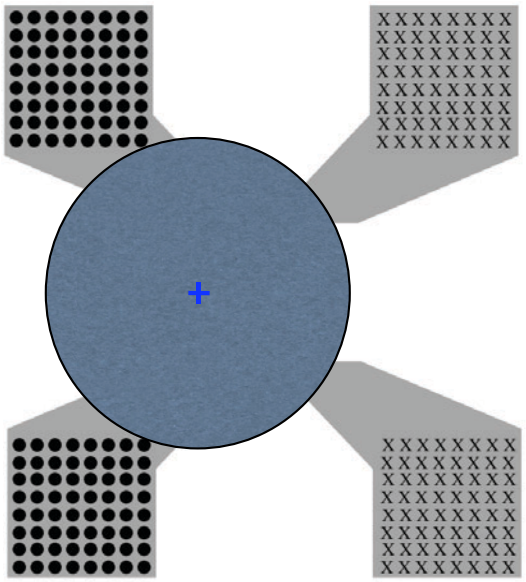
Tomography - Intro
Concept check questions:

- What is a "tilt-series"?
- What range of angles is typically imaged?
- What is the "missing wedge"?
- How are interpolations involved in the calculation of a tomogram?
- What does each word in "serial section montage tomography" signify?
- How large or small a sample can be imaged by electron tomography?
- How is "volume rendering" different than showing an "isosurface" or single slice?

How the sample is secured
in the goniometer,
range of movements




beam
deflectors
non-
eccentricity
image
deflectors
8:29 / 40:55
CC
HD



“Predictive method”

center object (with low dose/low mag)

record 0° image (at desired mag)

tilt, record first tilted image

determine shifts

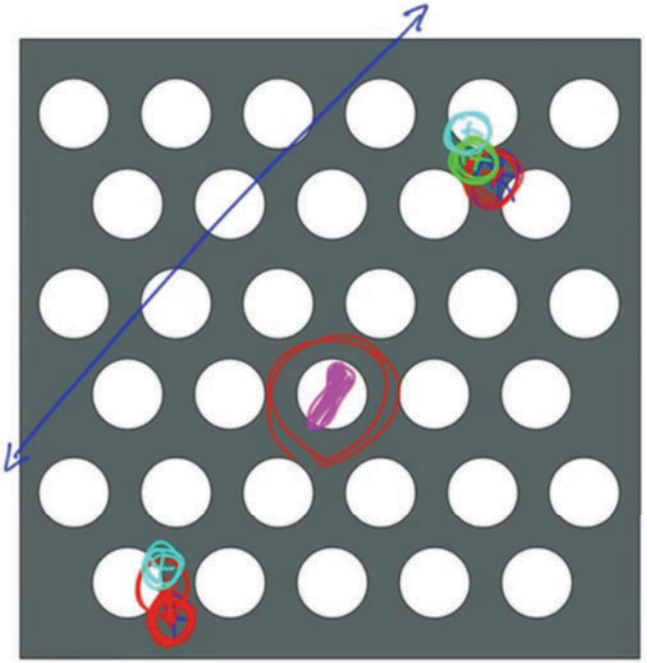
tilt, record next tilted image

determine shifts

fit shifts to model of tilt axis offset, specimen height

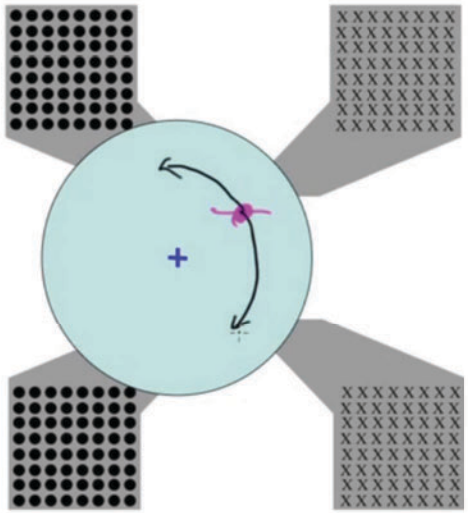
predict and apply beam shift, image shift, and focus changes needed

“Tracking”



"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

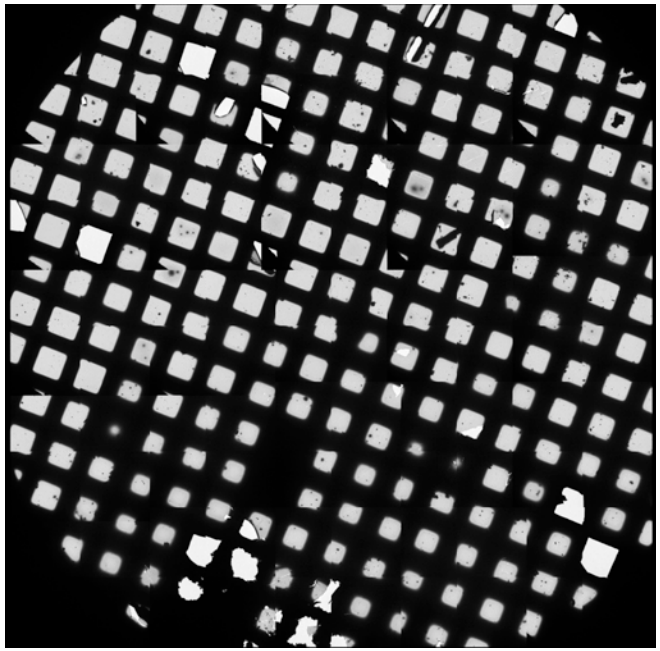


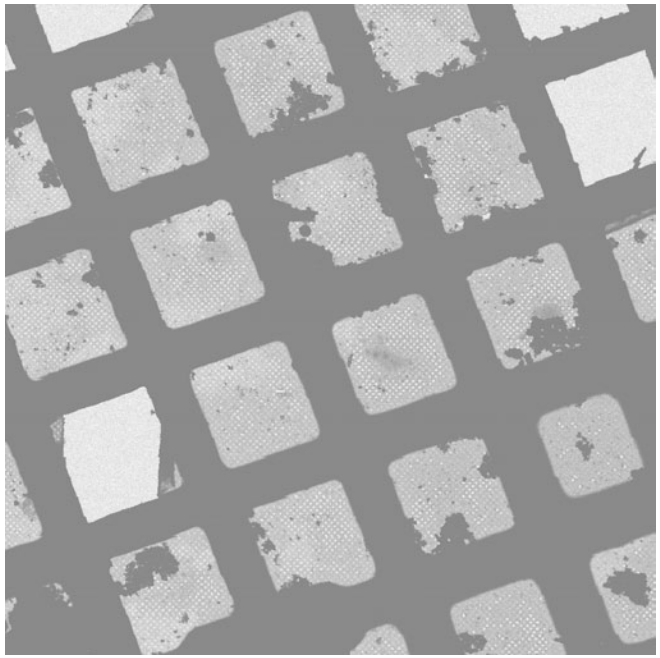
"Predictive method"

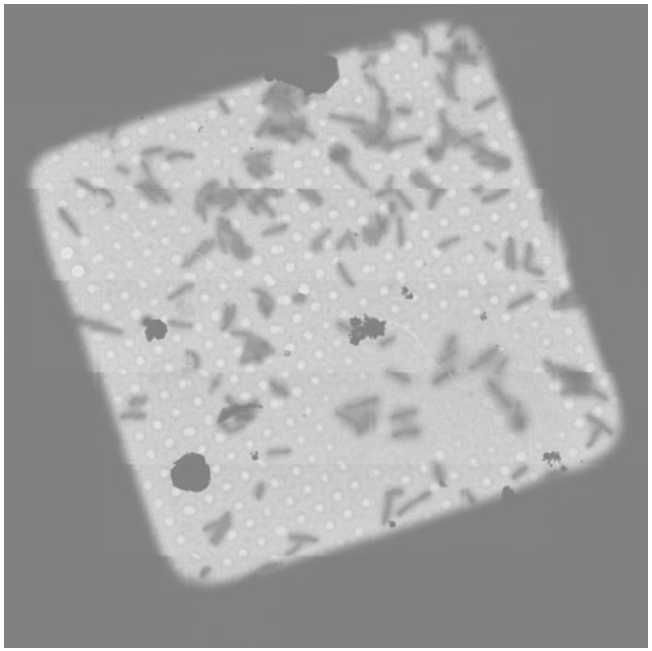
center object (with low dose/low mag)
record 0° image (at desired mag)
tilt, record first tilted image
    determine shifts
tilt, record next tilted image
    determine shifts
fit shifts to model of tilt axis offset, specimen height
predict and apply beam shift, image shift, and focus changes needed

"Tracking"

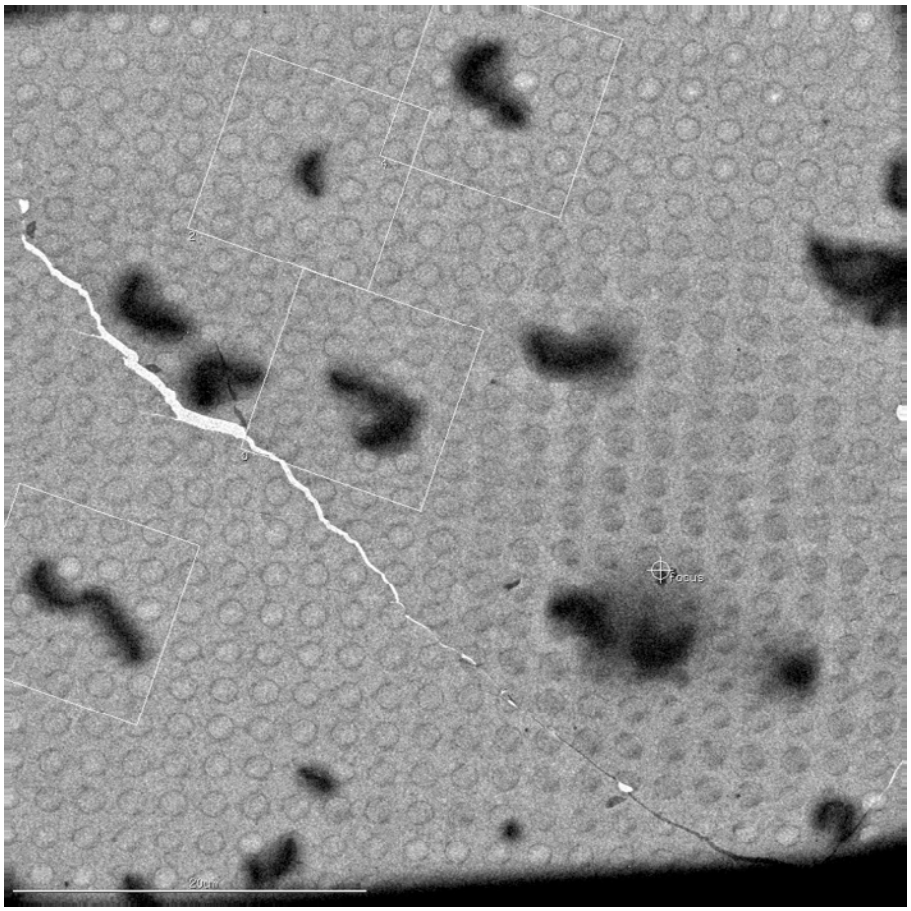
13:24 / 40:55







“atlas series”

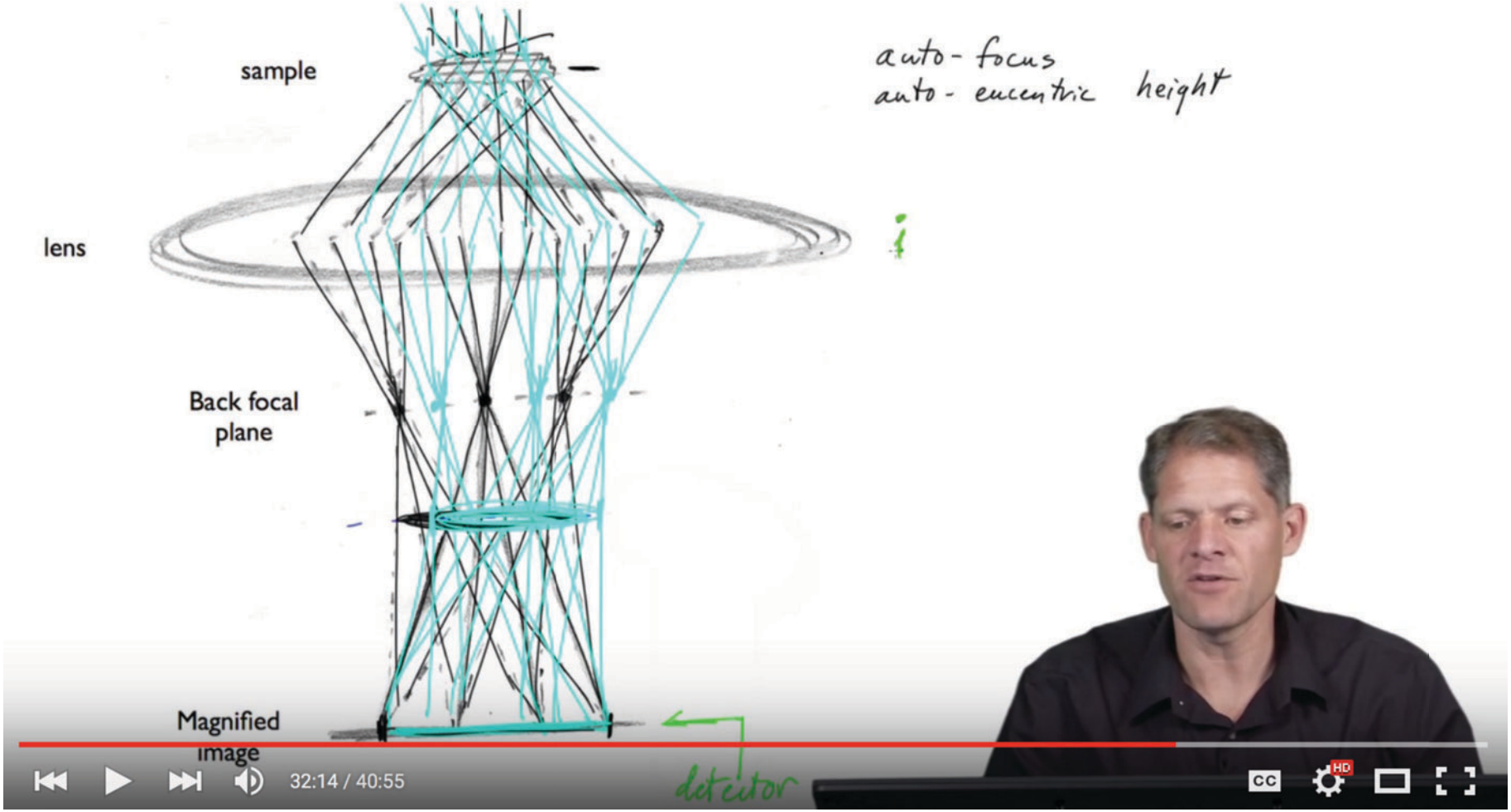


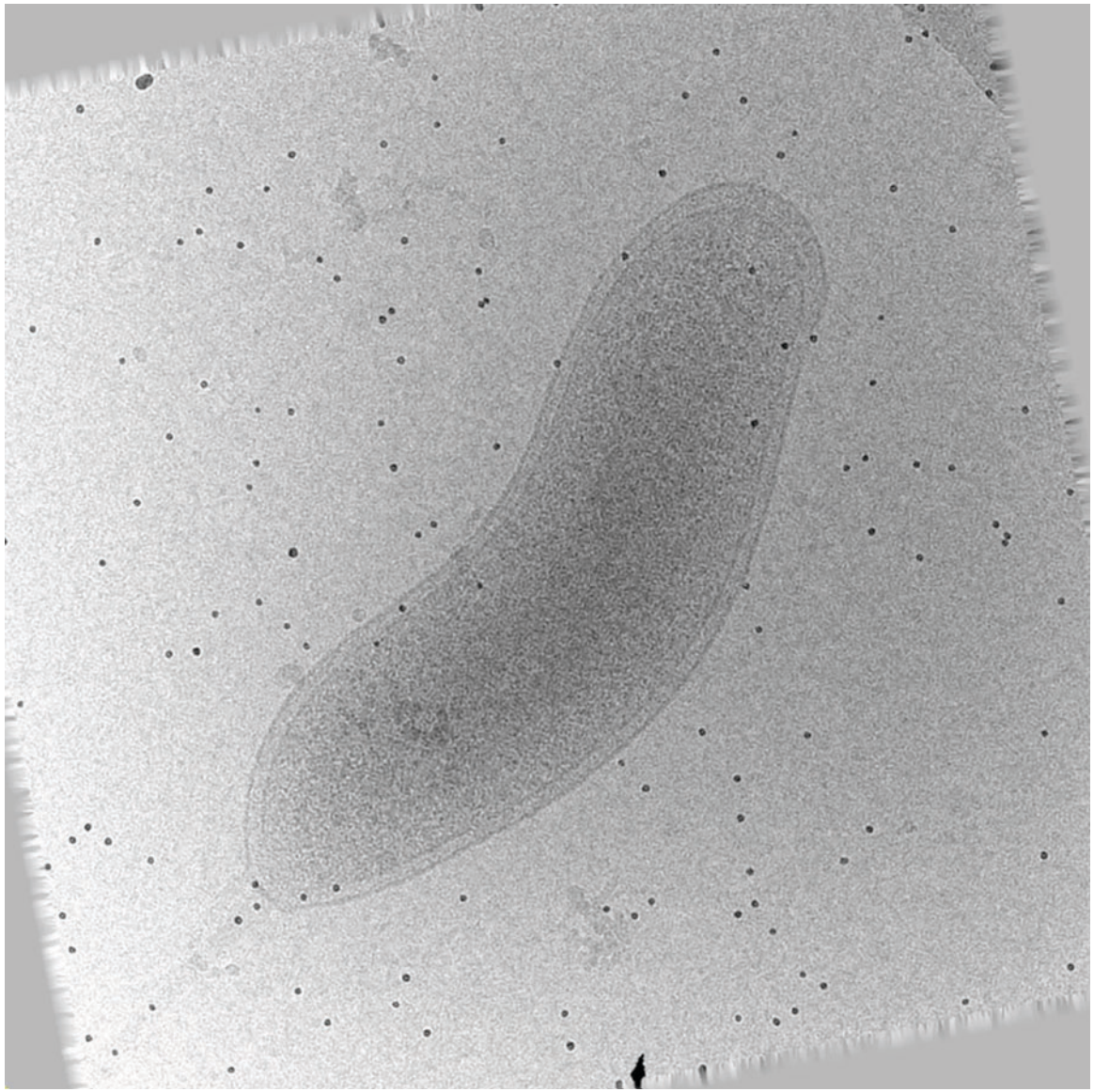
Example automatic sequential tilt-series acquisition protocol

mark targets and focus positions on low mag "atlas" image
stage shift (potentially far) to first target
record low dose, low mag image
    determine shifts, re-shift stage as necessary to center on object
stage or beam shift to nearby focus position
auto-focus, auto-eucentric height, (center slit?)
unshift back to target
record tilt series

"Targeting"

auto- eccentric height
25:14 / 40:55
CC
HD


sample
lens
Back focal plane
Magnified image
auto-focus
auto-eucentric height
detector
32:14 / 40:55



Aligning tilt series

For each image, determine

- x,y shifts
- rotation (position of tilt axis)
- tilt angle
- magnification
- defocus?

Fiducials, Fiducial-less

Weighted back-projection,
iterative real-space reconstruction

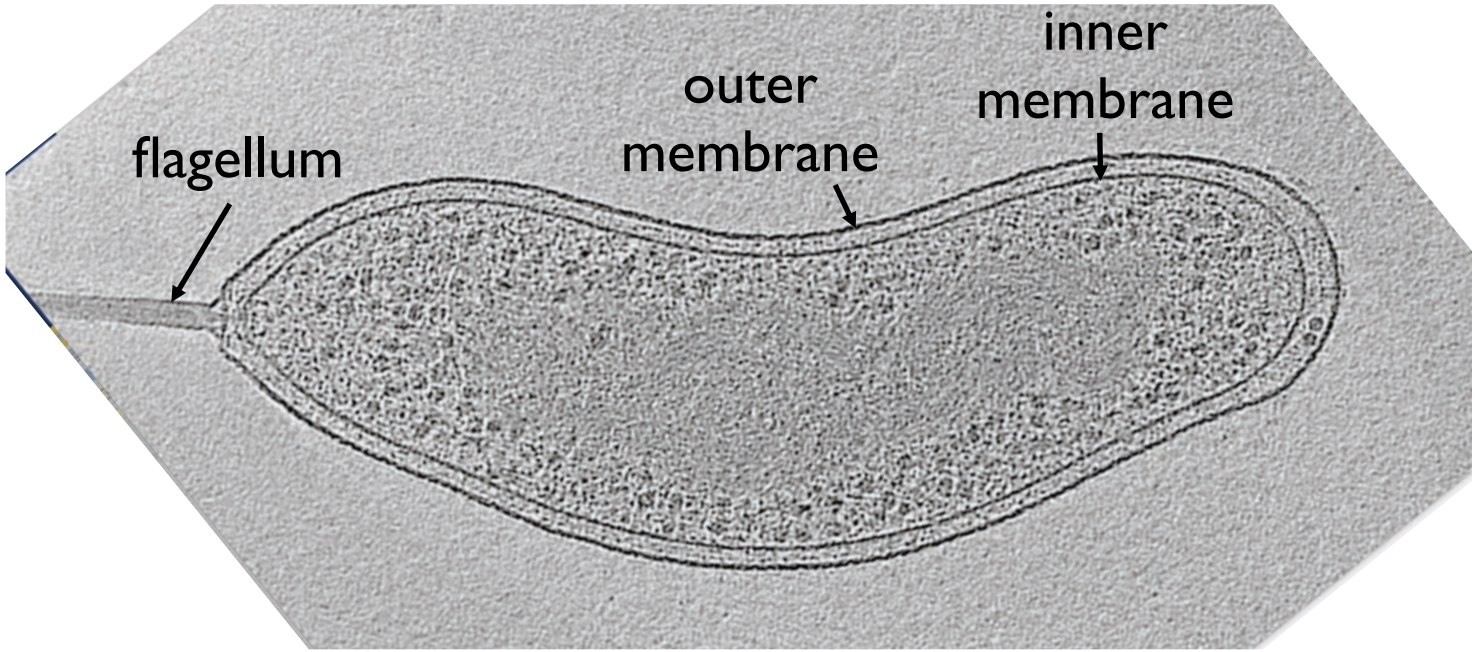
Tomography - Data collection and reconstruction Concept check questions:

- What is "eucentric height"? Is it different for every sample?
- What is the "tilt axis offset"? Is it different for every sample?
- When speaking about automatic sequential tilt-series acquisition, what is "tracking"? What is "targeting"?
- How is the "predictive" tracking method different from the "focus position" method? Which is faster? Why would the slower method ever be used?
- What is a "low mag atlas" and why would one be recorded?
- What microscope operations are used to set specimen height automatically? Why?
- What microscope operations are used to focus objects automatically? Why?
- What about each image has to be determined to "align" a tilt-series? How are these parameters found?
- What steps of data collection and 3-D reconstruction have to be done by the investigator, and which are typically automated?

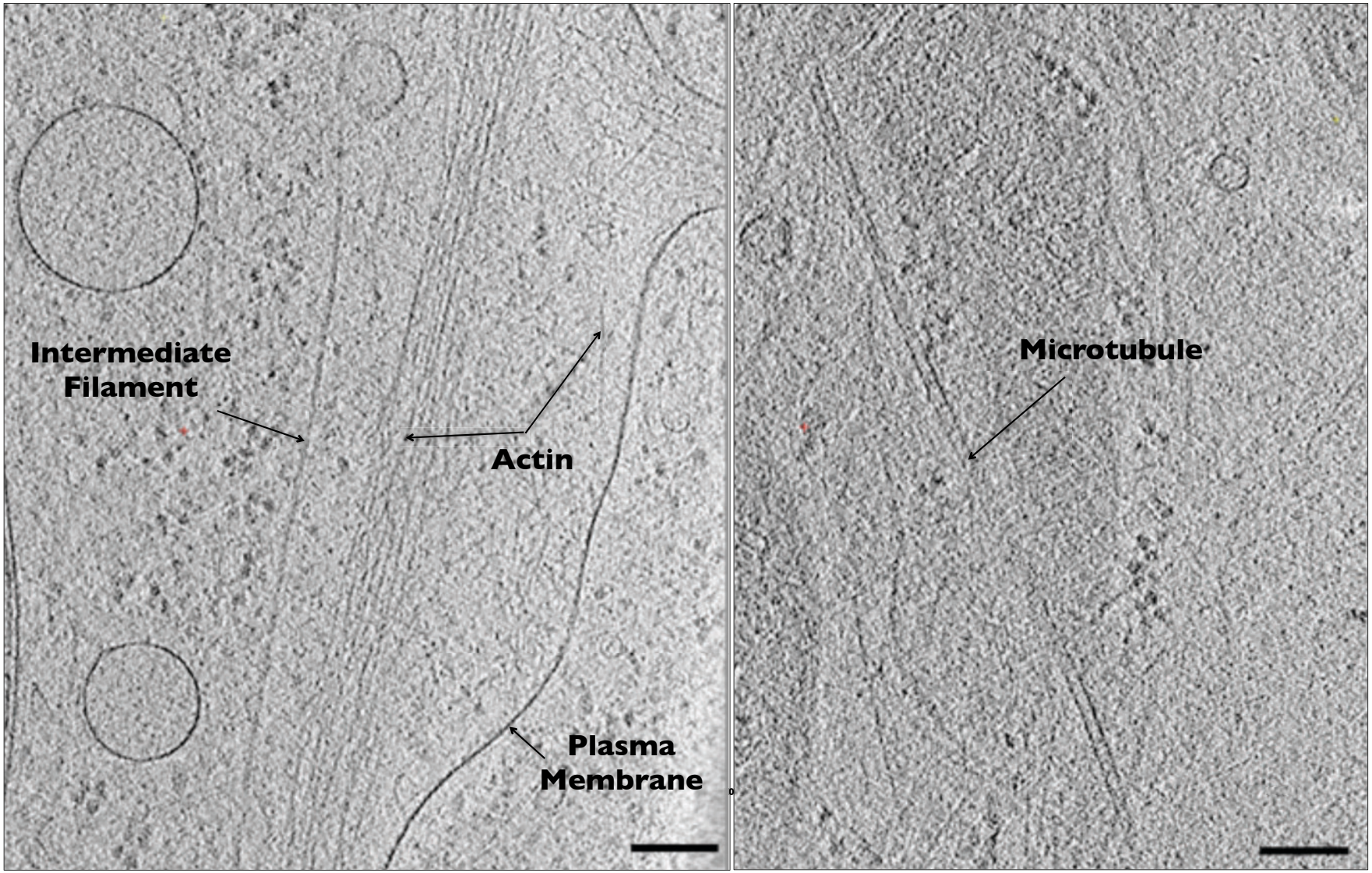
How can objects in tomograms be identified?

1. Some things are obvious
2. Correlated light and electron microscopy (CLEM)
3. Perturbing abundance or structure
4. Template matching
5. Heavy metal tags?

How can objects in tomograms be identified?



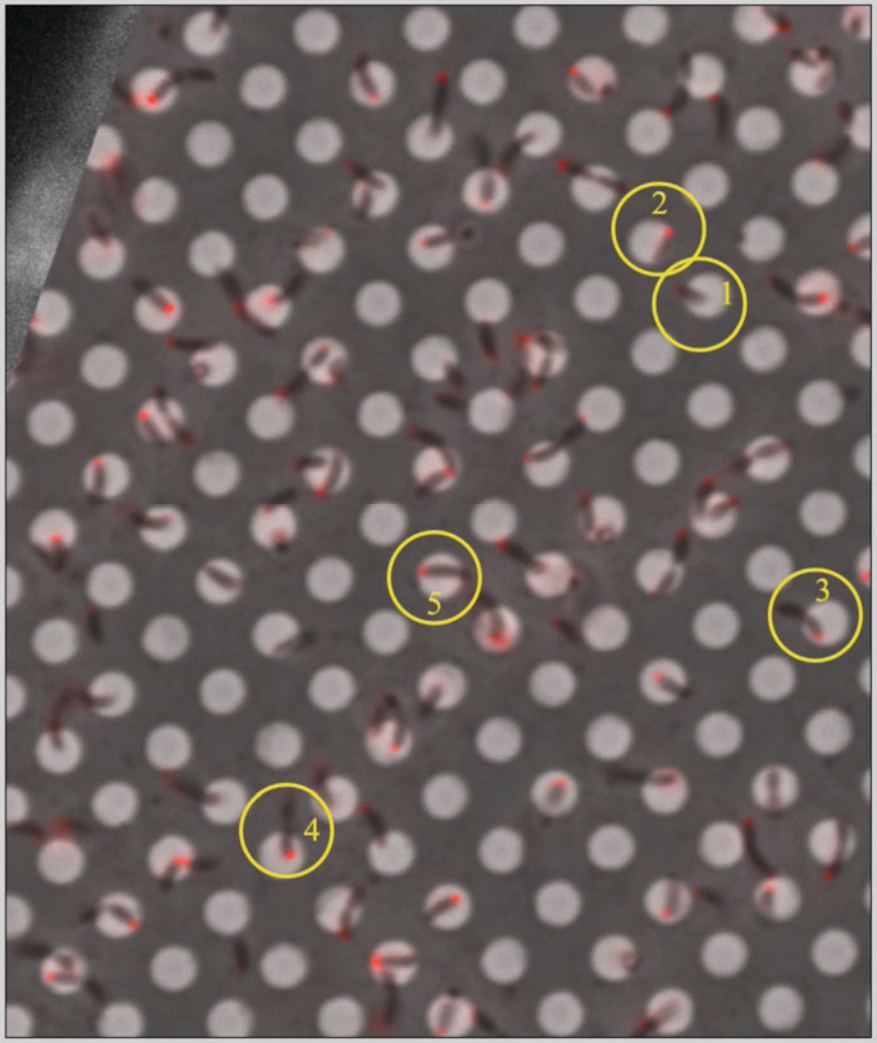
Some things are obvious

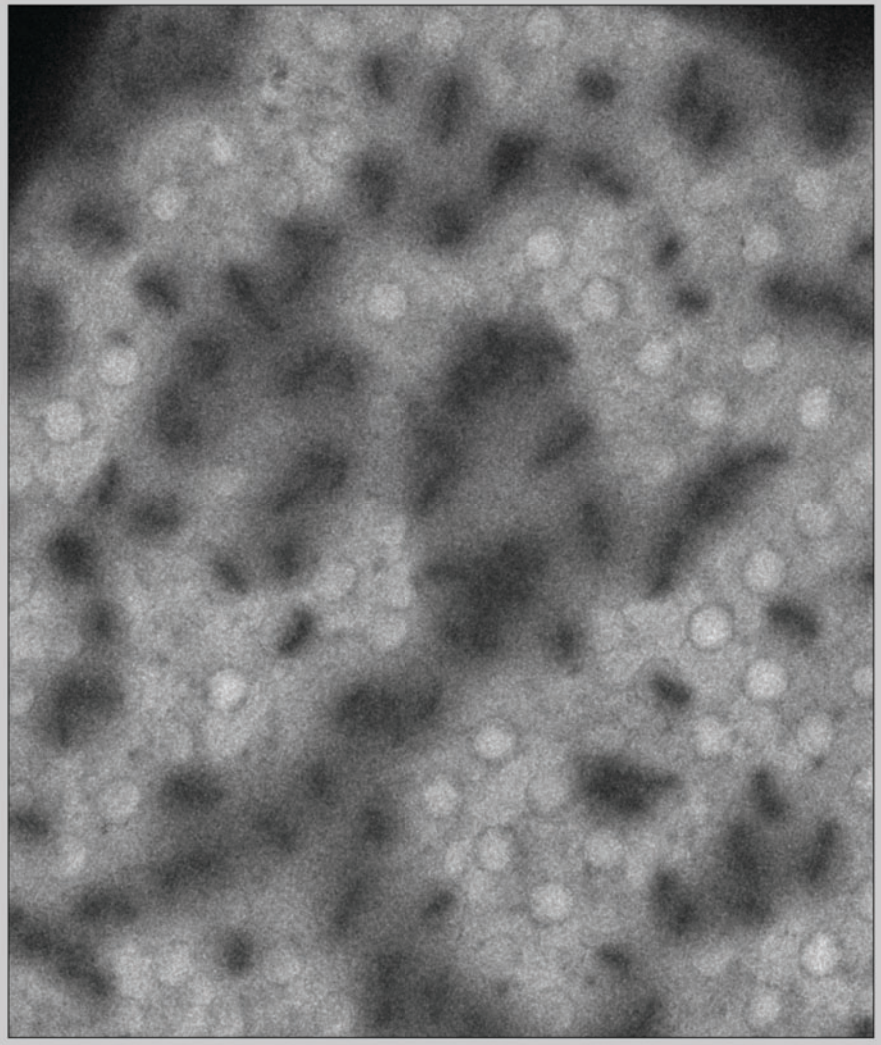

Intermediate
Filament
Actin
Plasma
Membrane
Microtubule

Fluorescence image

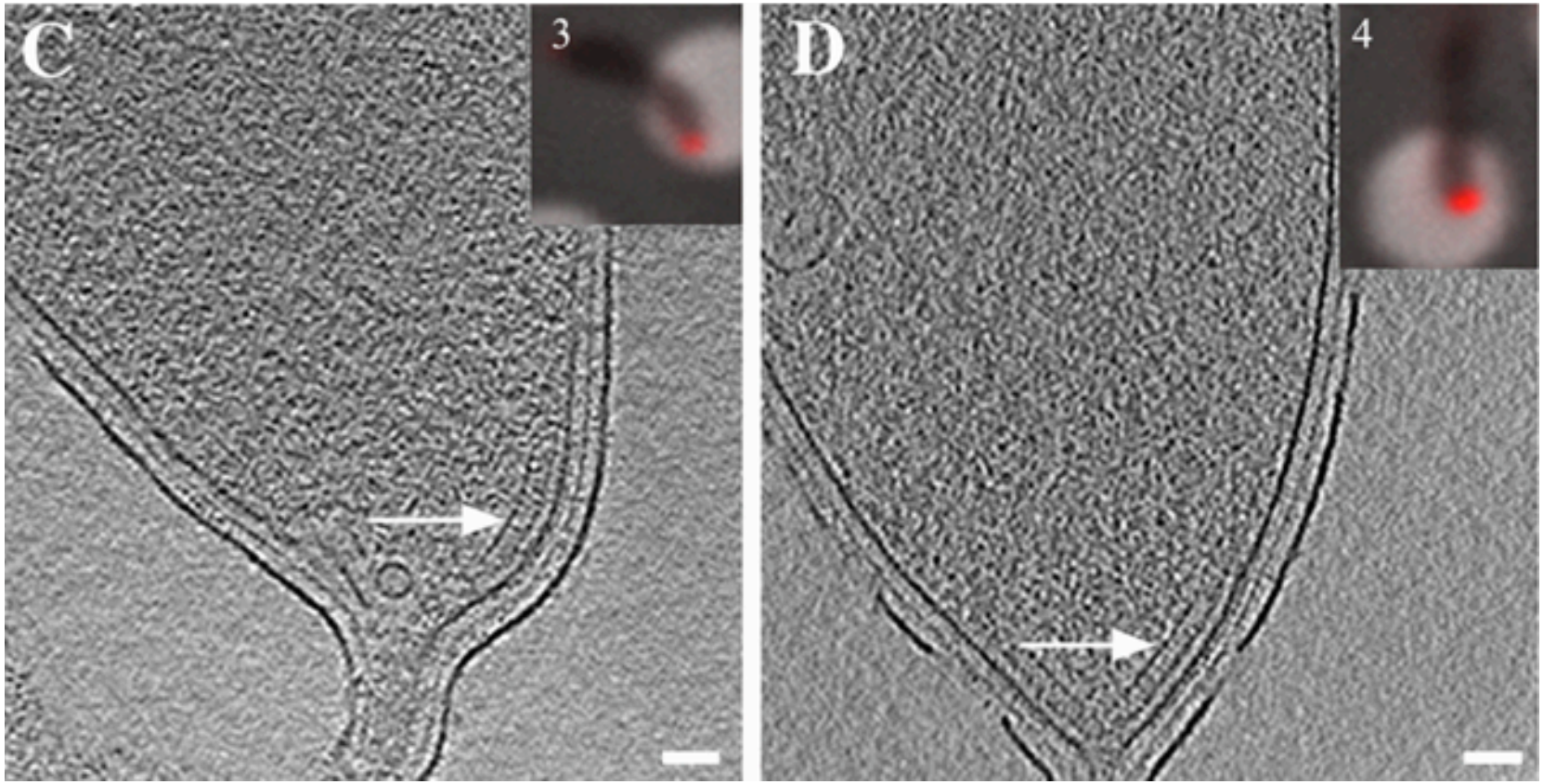
CLEM

Low-mag EM image

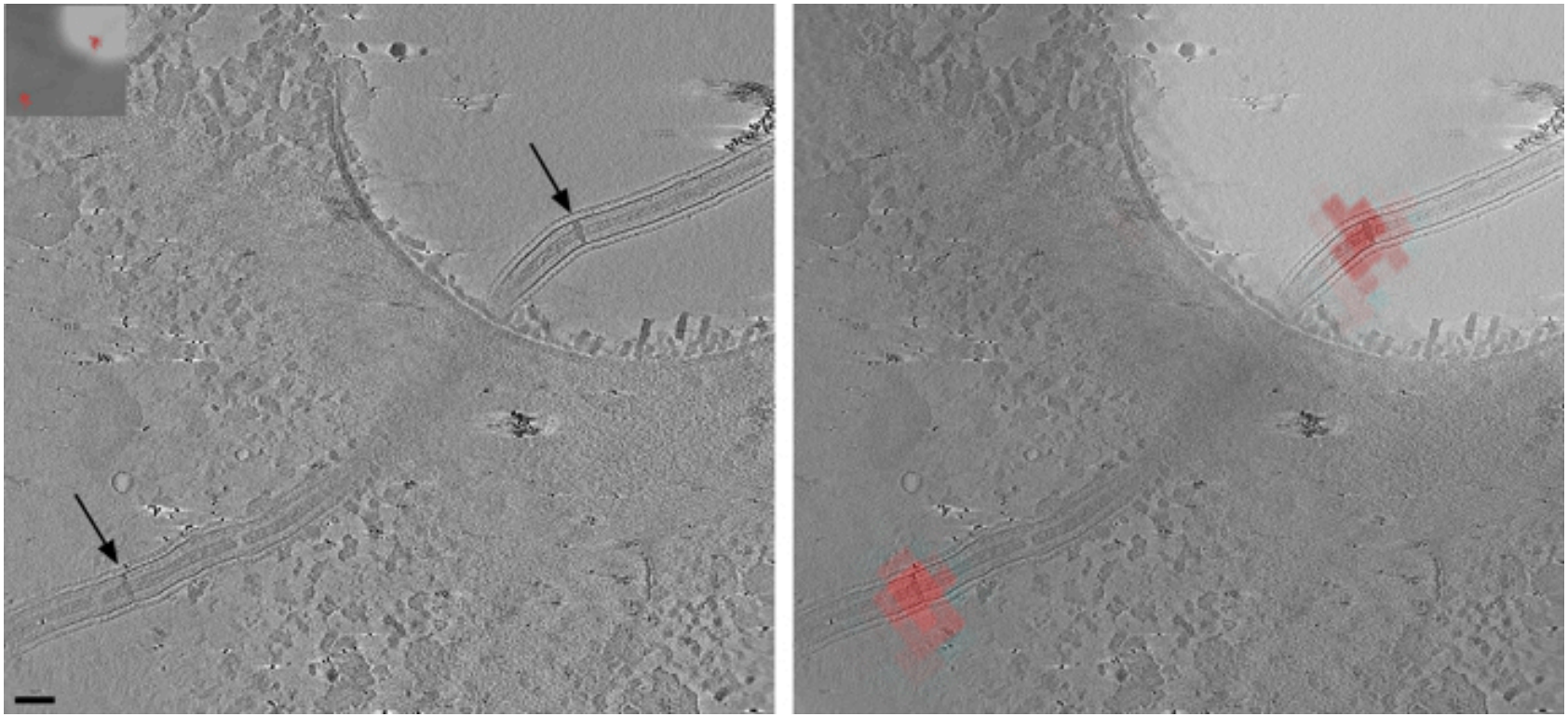




Example of correlated light and electron microscopy (CLEM): identifying chemoreceptor arrays within *Caulobacter crescentus*

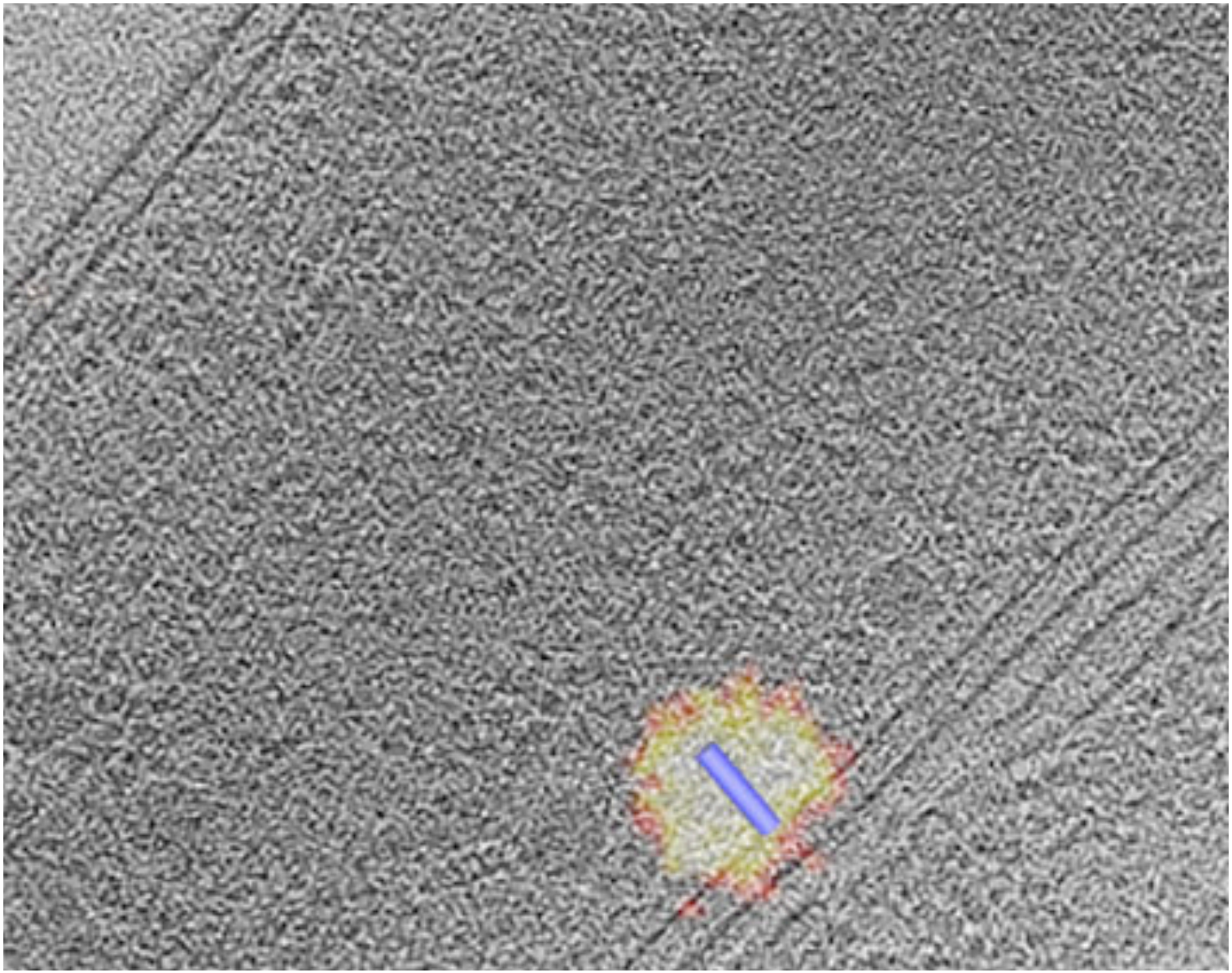


Example of correlative light and electron microscopy (CLEM):
Identifying StpA-D as components of *Caulobacter crescentus* stalkbands

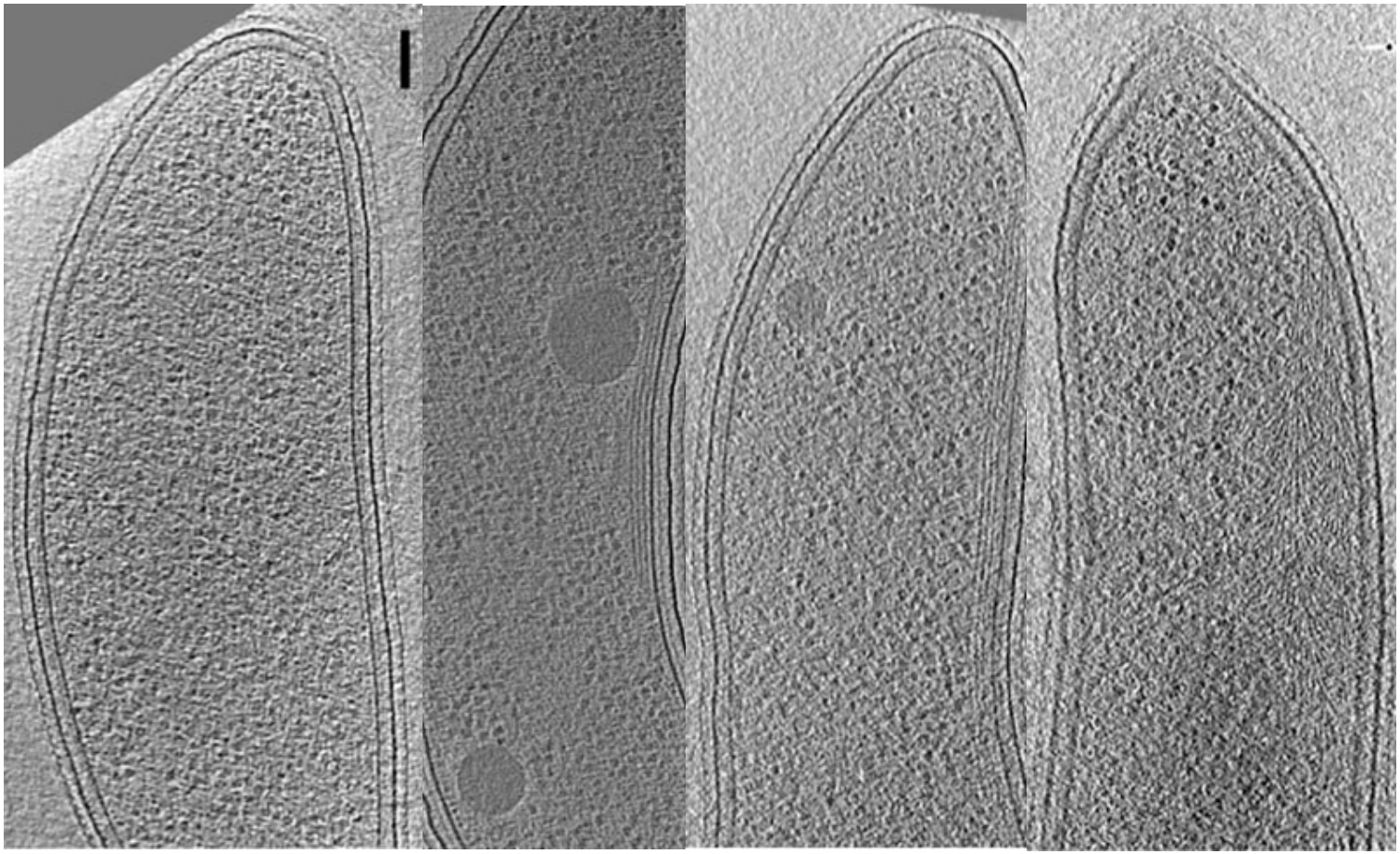


Example of "super-resolution" CLEM (cryo-PALM + ECT):

Identifying type VI secretion systems within *Myxoccocus xanthus*

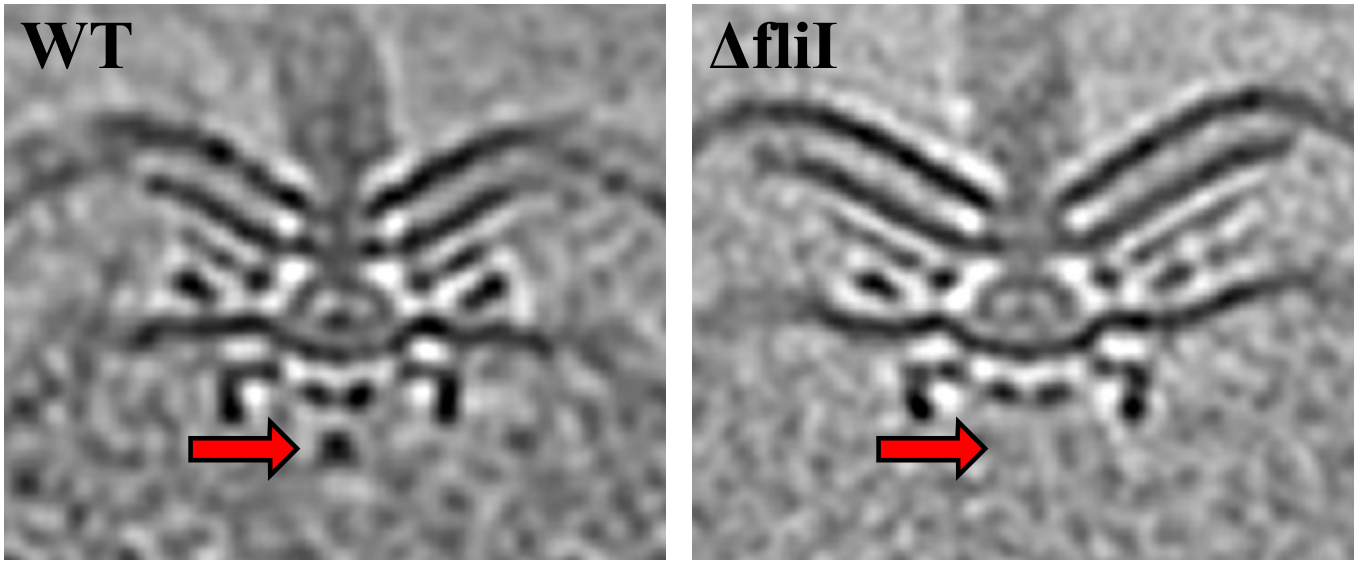


Example of identifying objects by perturbing their abundance:
CTP synthase filaments within *Caulobacter crescentus*

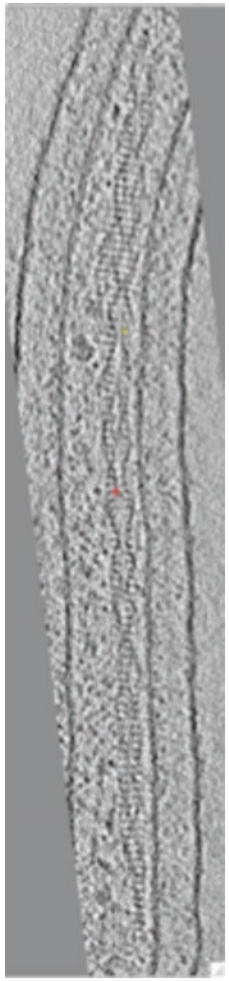


+DON | WT | Mild overexpression | Extreme overexpression

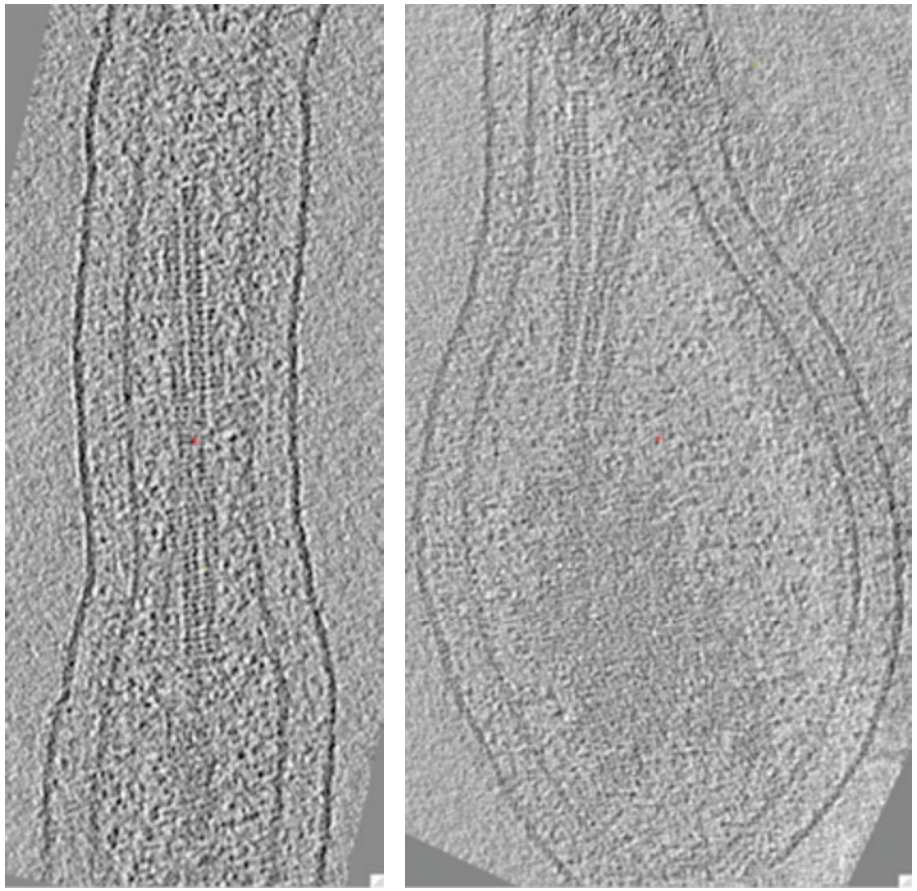
Example of identifying objects by perturbing their abundance:
Finding FliI within the bacterial flagellar motor



Example of identifying objects by perturbing a structure:
adding an intercalator to perturb a hypothesized DNA supercoil

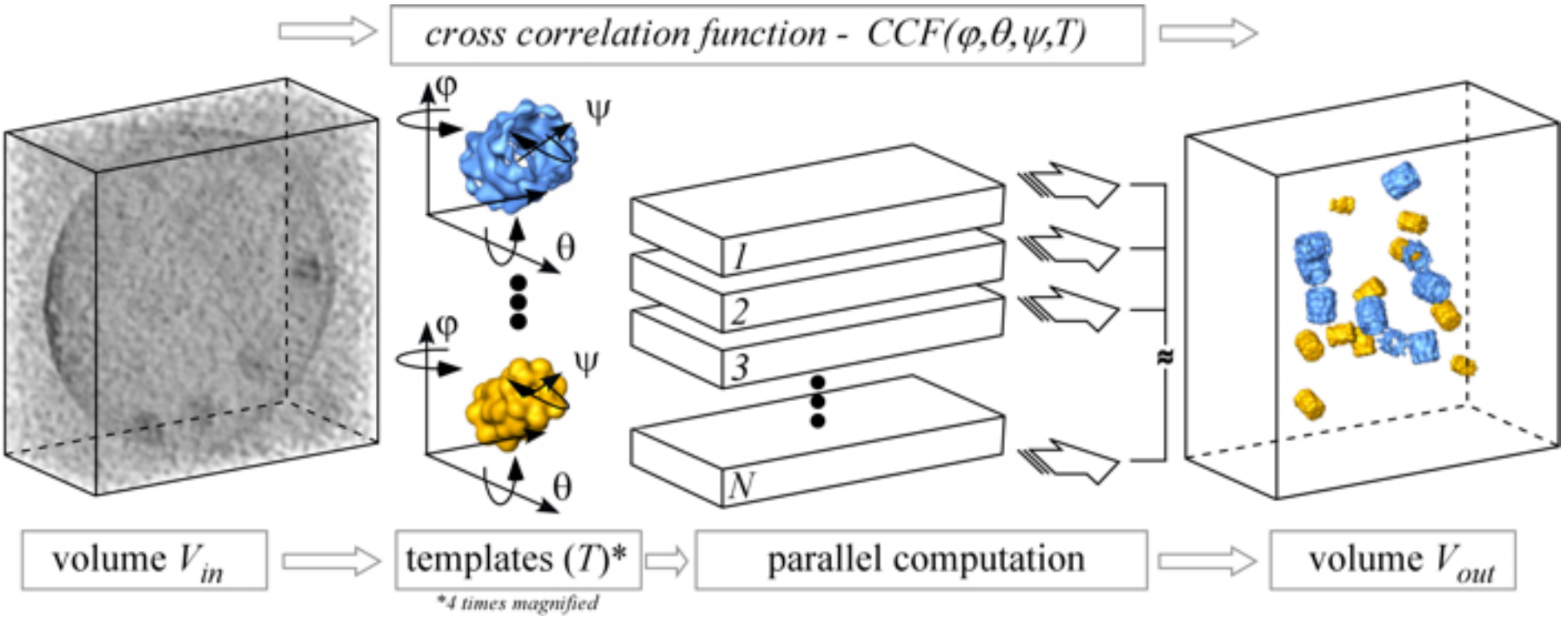


normal

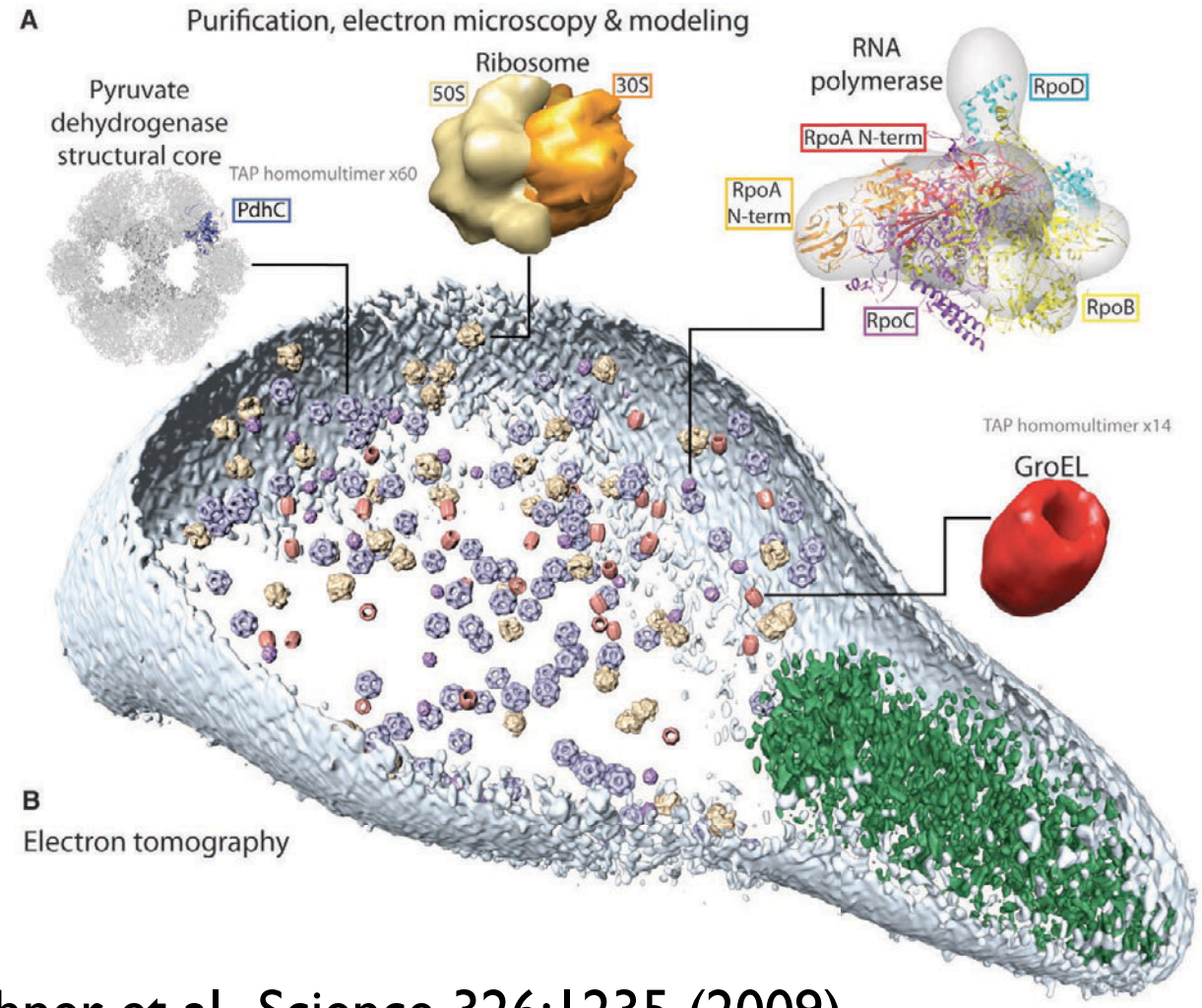


+ ethidium bromide

Identifying structures by template matching



Frangakis et al., PNAS 2002



Kuhner et al., Science 326:1235 (2009)

Tomography - Identifying objects in tomograms
Concept check questions:

- What does "CLEM" stand for?
- What are the advantages and disadvantages of doing the light microscopy at room temperature and cryo-temperatures?
- 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?
- How can small molecule inhibitors or genetic manipulations be used to identify objects in tomograms?
- 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?
- What kinds of macromolecular complexes are likely to identifiable within a cell by template matching?
- What is "visual proteomics"?
- What ideas have been tried so far to label objects of interest inside cells with electron dense tags?

Resolution limitations in tomography

- Radiation damage (if frozen-hydrated)
- Quality of sample preservation, fidelity of stains (if chemically fixed and stained)
- Tilt increment
- Size of missing wedge (or pyramid for dual-axis tomography)
- Defocus
- Alignment errors (translation, rotation, tilt-angle, tilt-axis, magnification)
- Magnification (pixelsize)

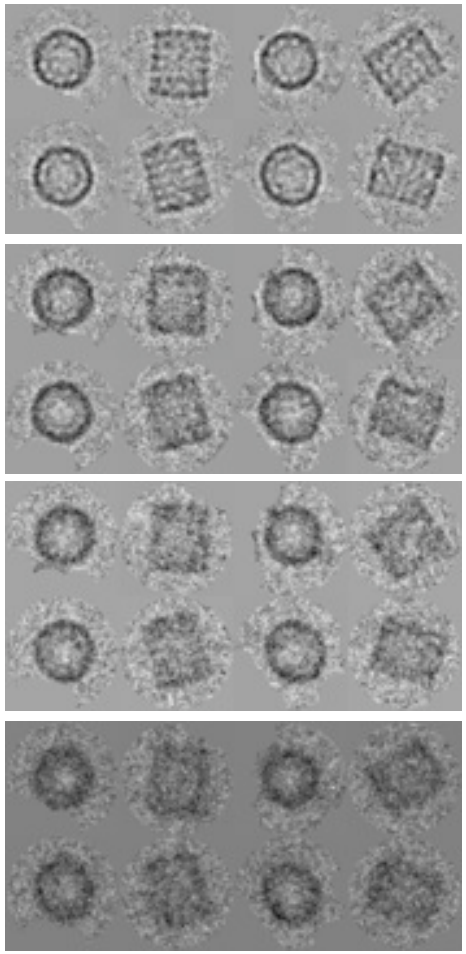
For native samples, the ultimate resolution limit is radiation damage

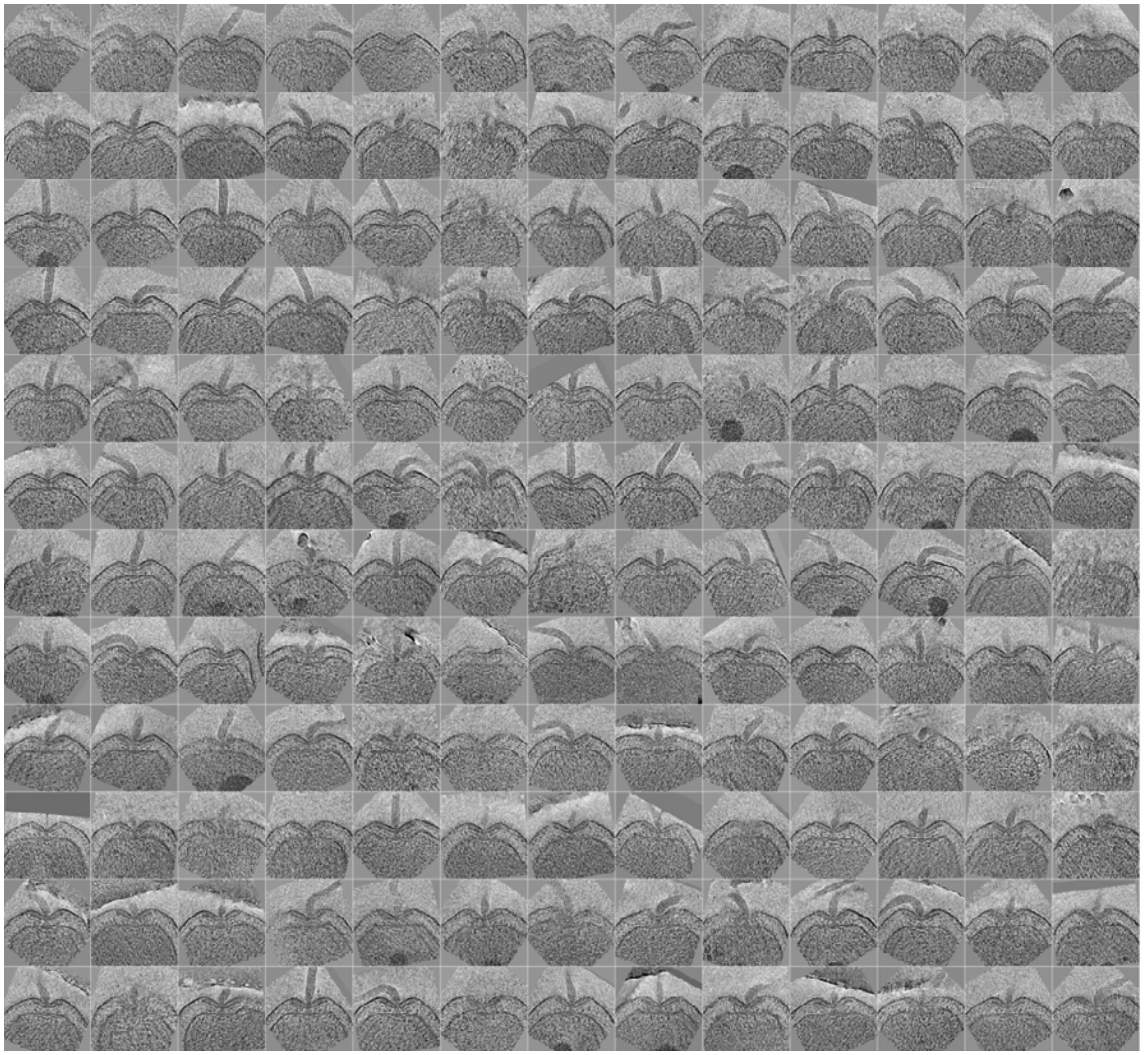
10 or 20 e^-/A^2

120 e^-/A^2

200 e^-/A^2

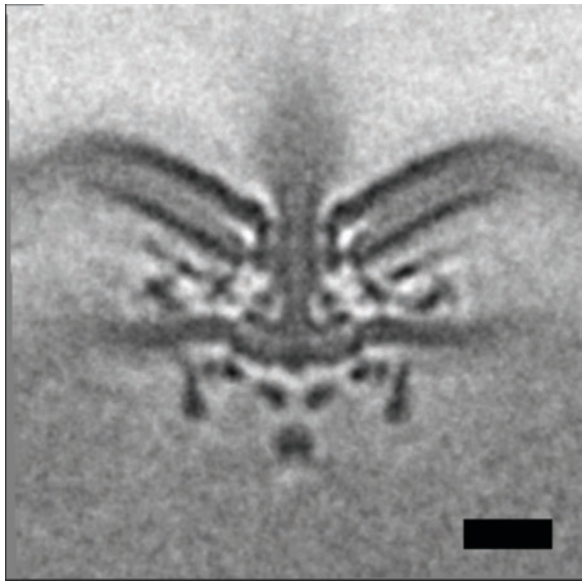
350 e^-/A^2



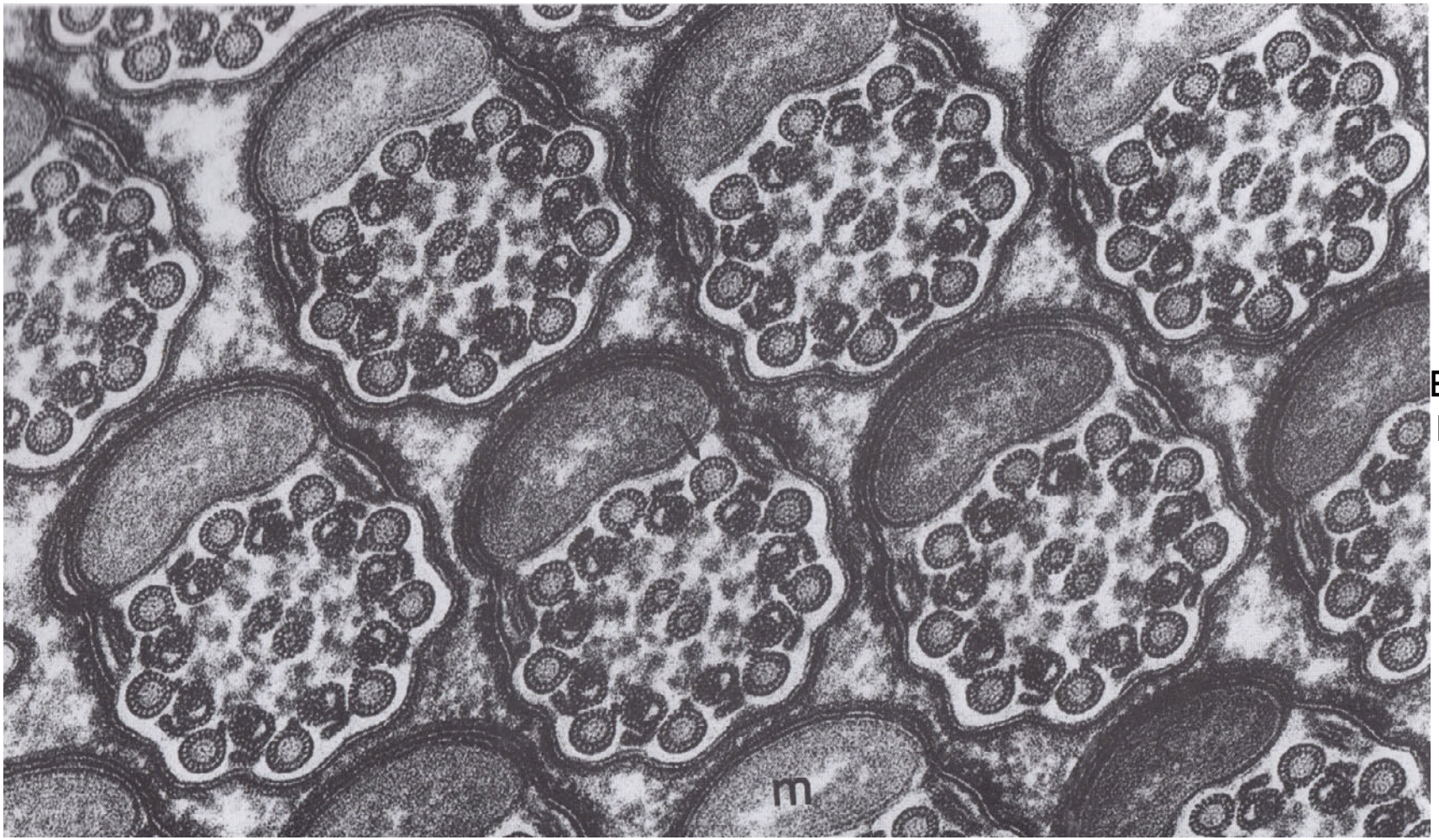


Data: Morgan Beeby

"Sub-tomogram averaging"

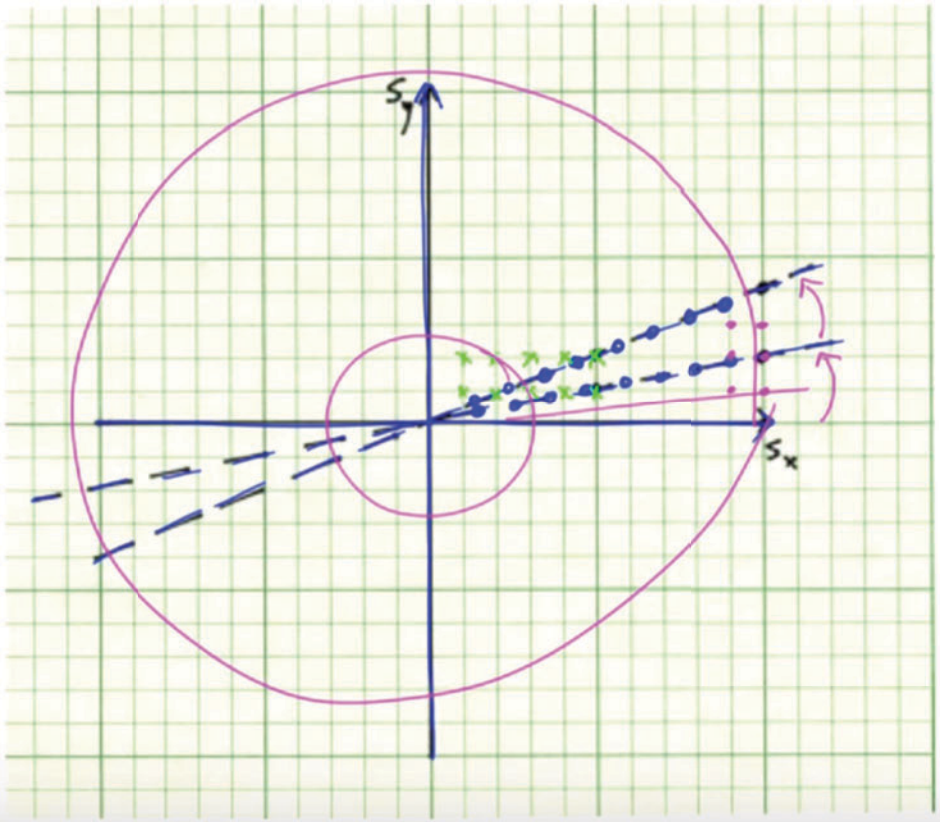


For fixed and stained samples, the ultimate resolution limit is the fidelity of the stain



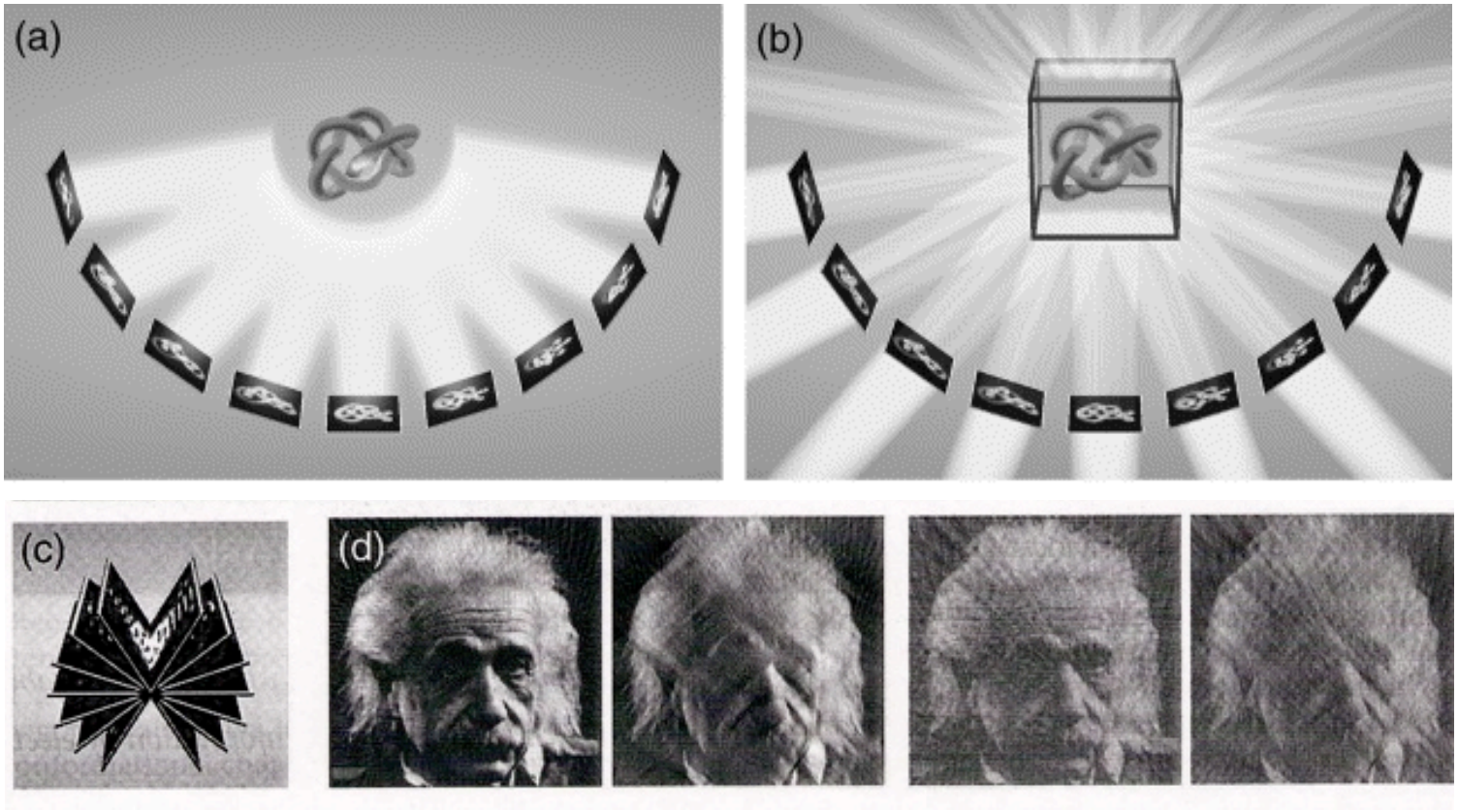
Bozzola and Russells pg. 553

Other resolution limits: tilt increment



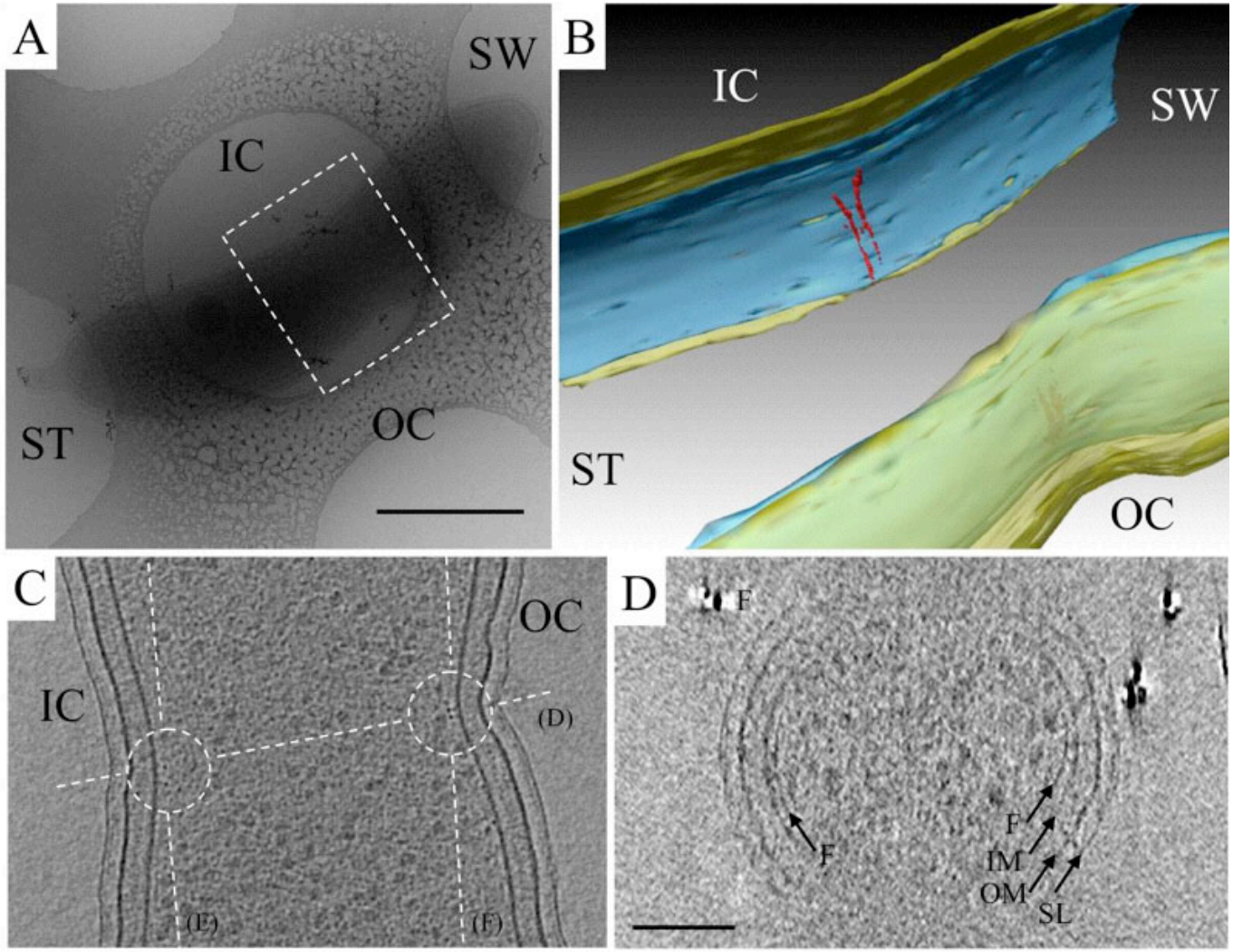
$N \cong \pi D s$ (DeRosier and Klug, 1968)

Effects of tilt increment and missing wedge

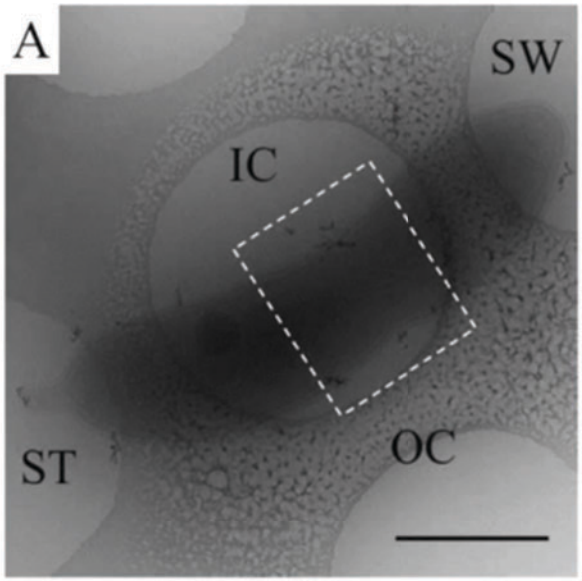


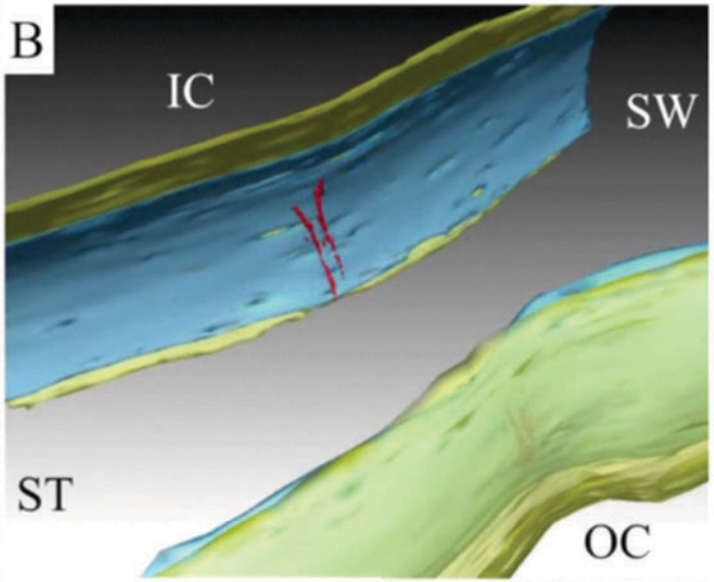
+/- 90˚ 2˚ steps | +/- 60˚ 2˚ steps | +/- 90˚ 5˚ steps | +/- 60˚ 5˚ steps

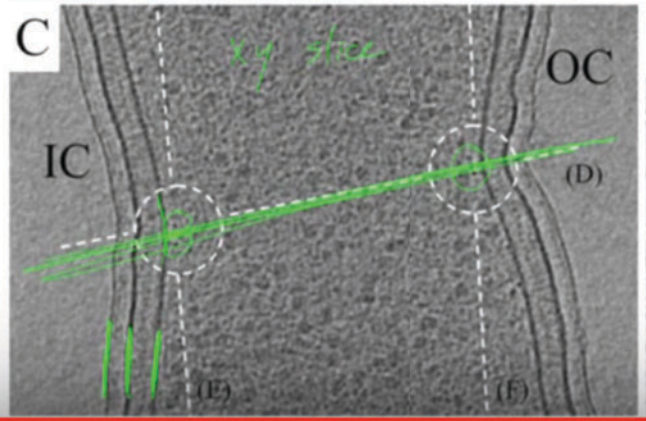
Baumeister et al., *Trends in Cell Biology* **9**:81

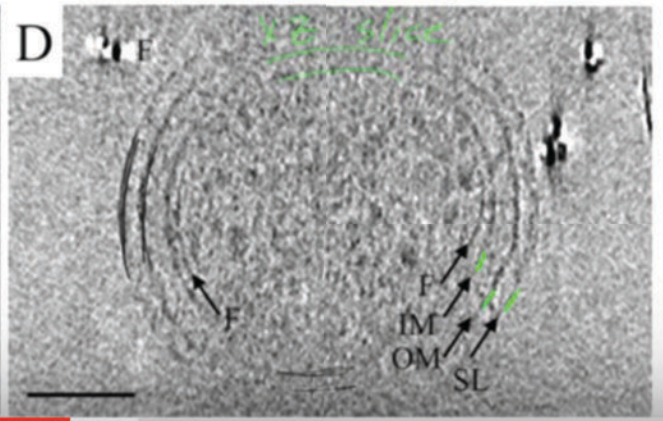


Li et al., EMBO 2007



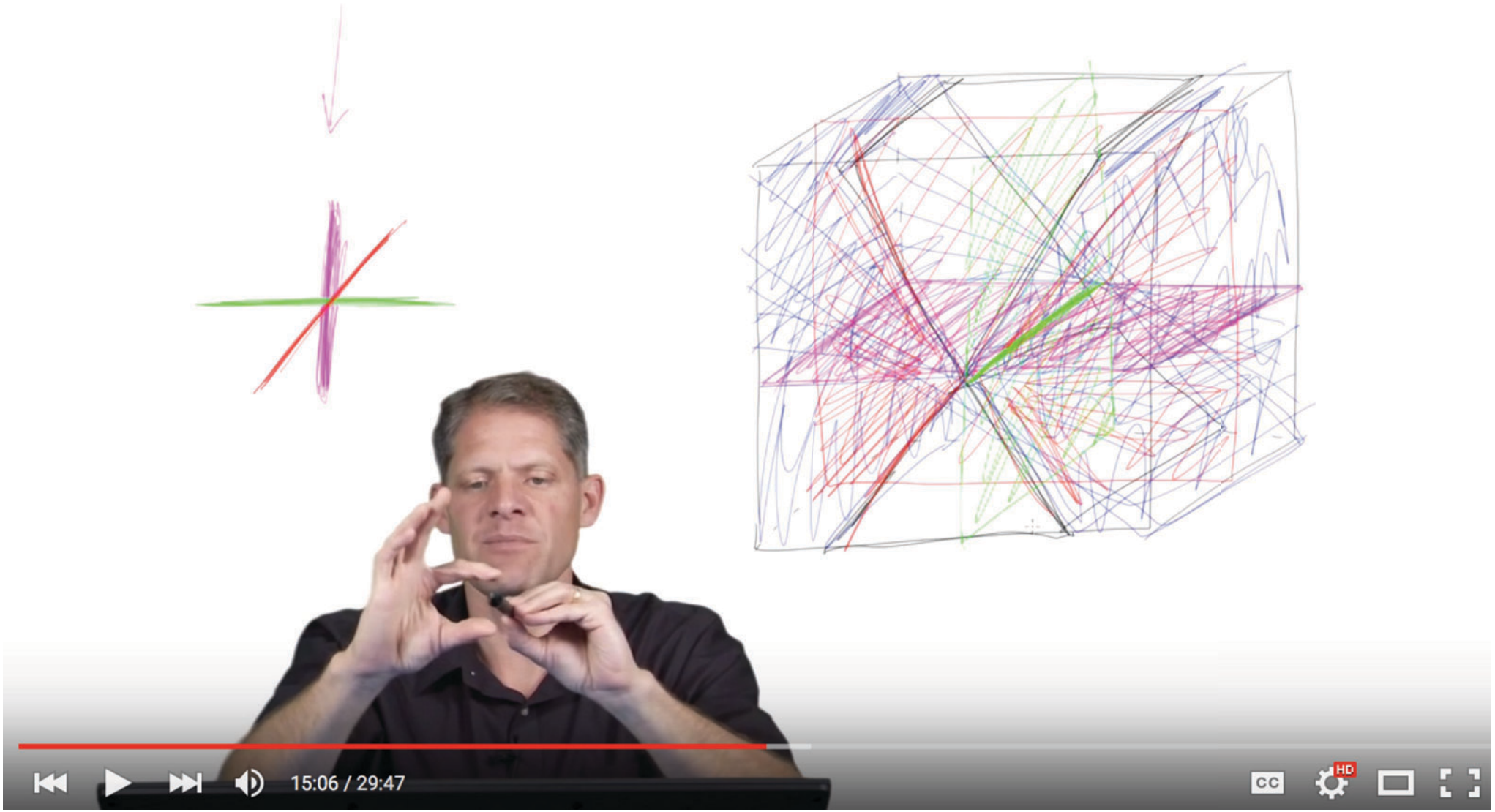




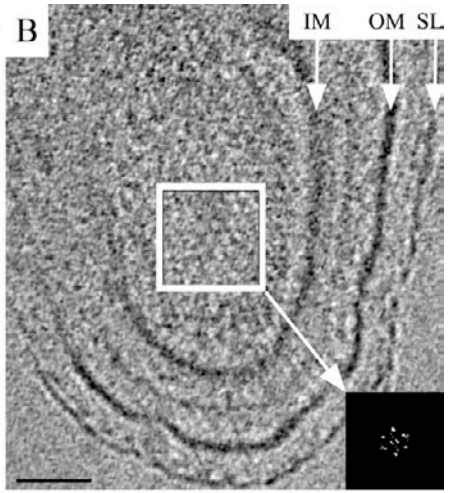


20:44 / 29:47

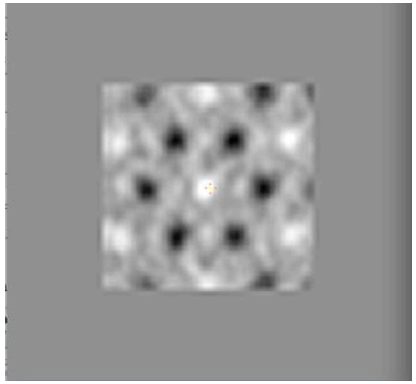
Li et al., EMBO 2007


15:06 / 29:47
CC
HD

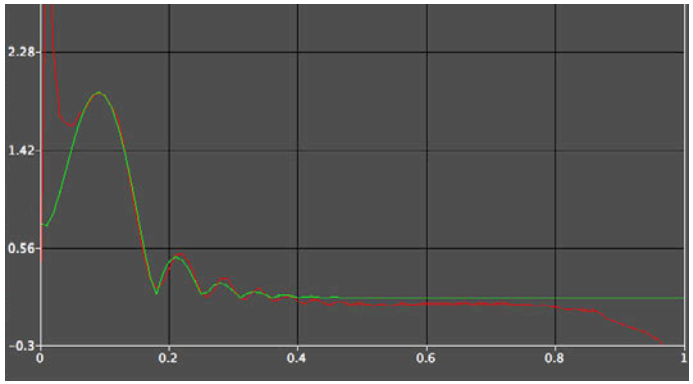
Other resolution limits: defocus



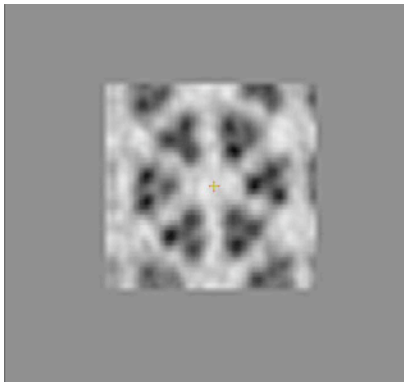
Sub-tomogram averaging



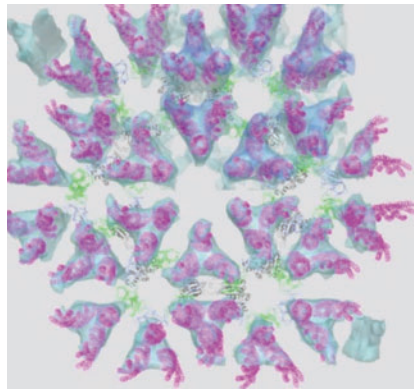
CTF-correction



Sub-tomogram averaging



Modeling



Data: Ariane Briegel

Magnification

- Shannon's sampling theorem says pixels should be at least 2x smaller than resolution target. In practice use 3 or even more times smaller.
- Smaller pixelsizes result, however, in smaller fields of view.
- To operate direct detectors in counting mode, the electron hits must be separated in either space (higher mags) or time (longer exposures).

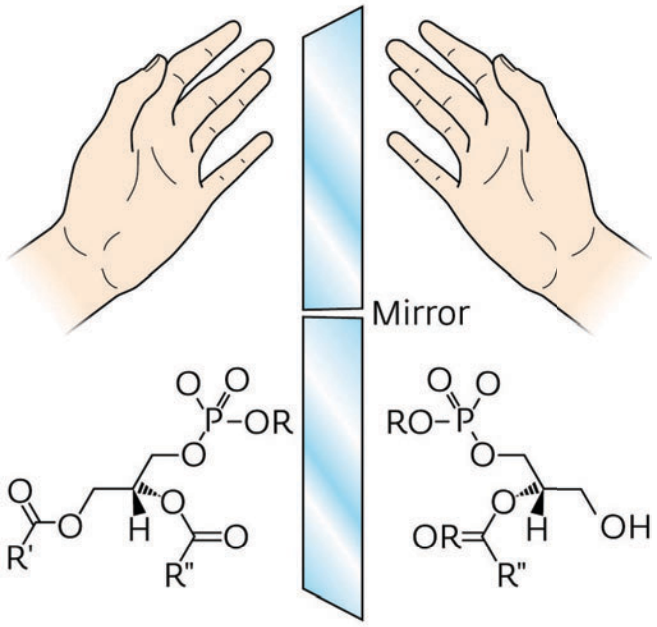
Tomography - Limitations
Concept check questions:

- What is the fundamental resolution limitation in tomography for native samples?
- What is the fundamental resolution limitation for stained samples?
- Name and explain four other resolution limitations in tomography.
- What is the "missing wedge"? Why is it missing? What effect does it have on reconstructions?

How to choose data collection parameters in a tomography project

- Choose resolution target (5 nm? 4 nm? will you average sub-tomograms?)
- Choose a magnification that will give you a pixel size ~3-4 times smaller than your target resolution.
- Record and inspect several dose series - choose target dose as half of that which first produces visible damage or even less if you are targeting higher resolution. Remember the energy filter may be removing electrons, so be careful that you are measuring total INCIDENT dose!
- Choose a tilt increment that will give you a resolution just better than that.
- To a first approximation, the dose per image is then just the total dose you want to use divided by the number of images you will include. In fact you will want to increase the dose with increasing tilt angle (Saxton, exponential, 1/cos schemes).
- Choose an exposure time and beam intensity that will deliver the dose you want per image in as short as time as possible (to reduce drift) but that still separates individual electron hits well if you are using a direct detector.
- If you are going to low pass filter everything past the first zero of the CTF, choose a defocus whose first zero is beyond your resolution target. If you are going to CTF-correct, choose a defocus where the spacings of interest are in CTF-maxima.

Handedness

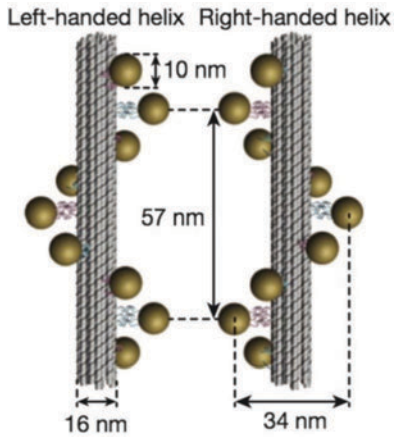


http://cdn.phys.org/newman/gfx/news/hires/2012/oddlipidout.jpg

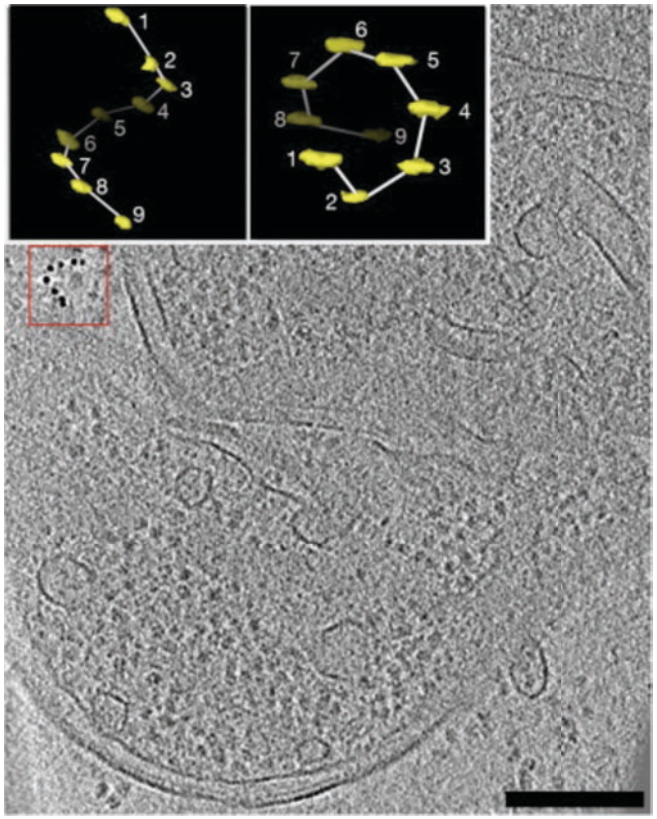
Factors in tomography that influence handedness

- *Convention for tilt angles (+/-)*
- *Rotation of image through lenses*
- *Digital images presented/stored from above or below?*
- *Conventions in reconstruction software*
- *Conventions in visualization software*

DNA origami gold nanoparticle helices can be used as molecular handedness standards



Briegel et al., JSB 2013



Tomography - Parameters and handedness
Concept check questions:

- There are many choices involved in the design of a tomography experiment. Each is a balance between opposing considerations (more/higher/smaller is good, but only up to some point). Explain the compromises involved in the choice of magnification, total dose, tilt-increment, exposure time per image, and defocus.

- Which steps of a tomography project influence the handedness of the final reconstruction?

- What can be done to ensure that the handedness of chiral features in tomograms is interpreted correctly?