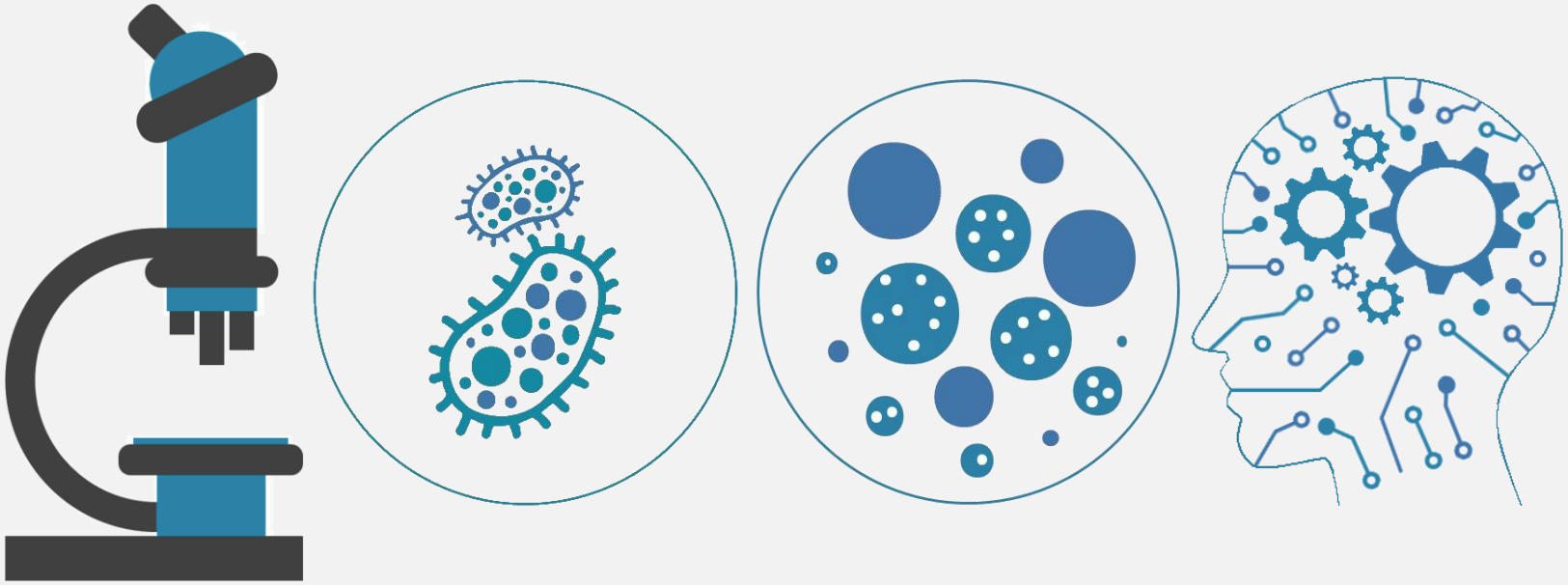
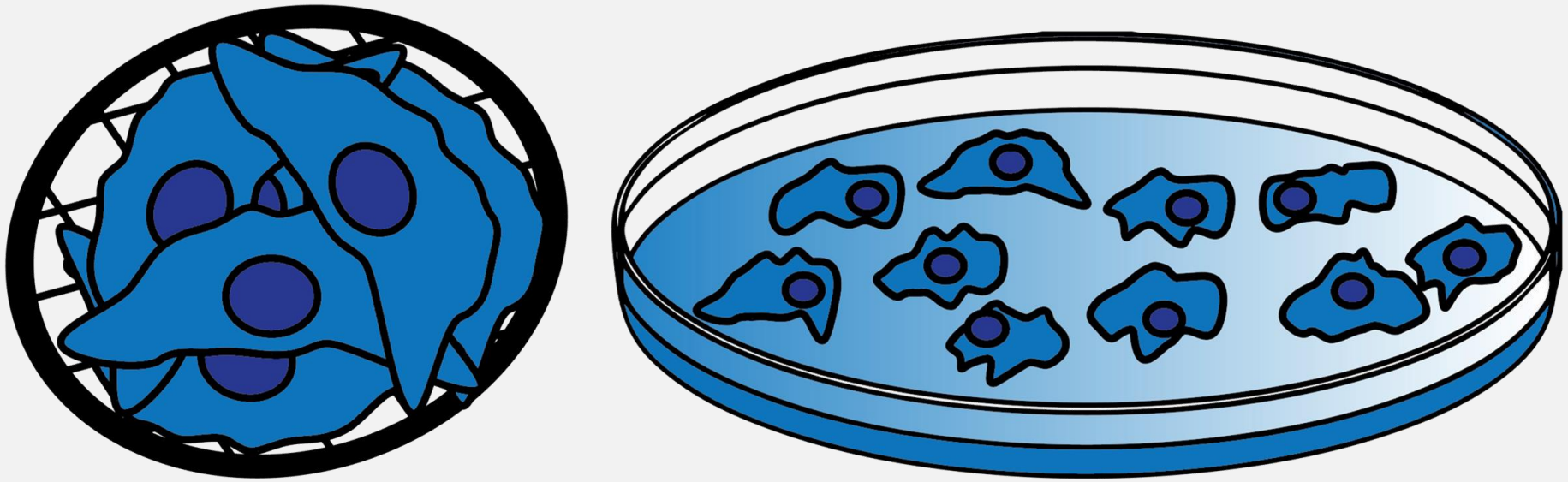
Introduction and Overview of Tomography

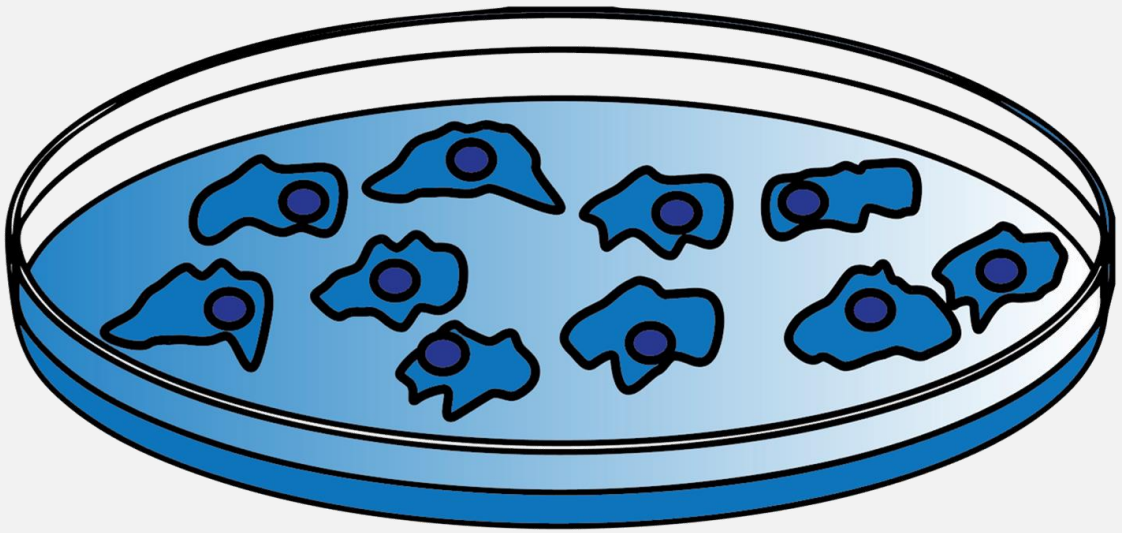


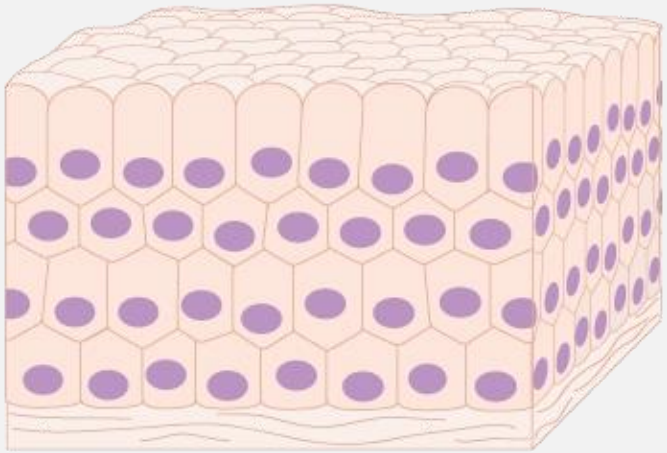
Tomography Short Course!

4-10-23

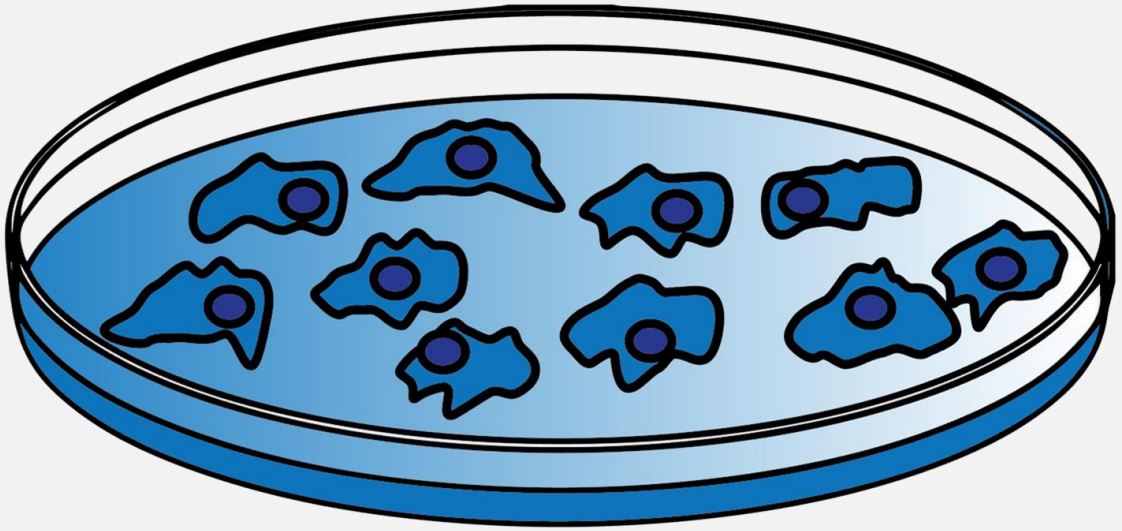
Alex Noble

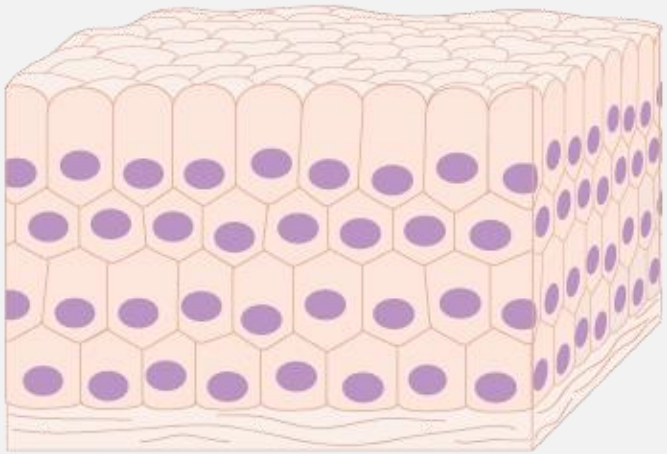




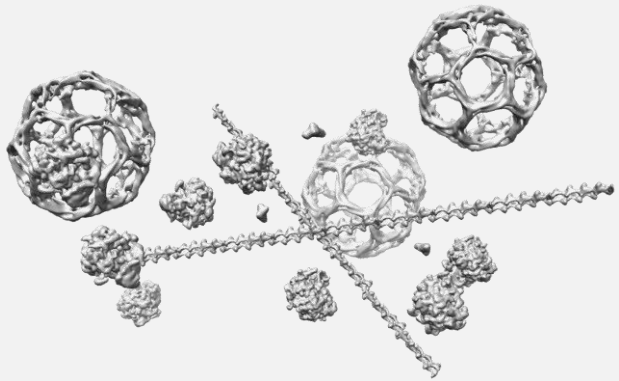


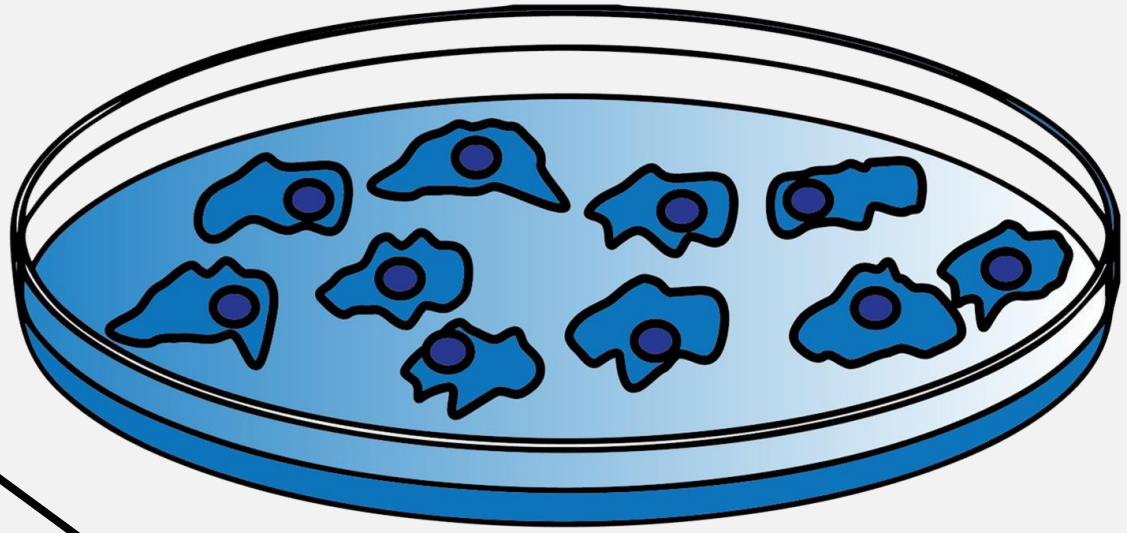
Cancer Research UK

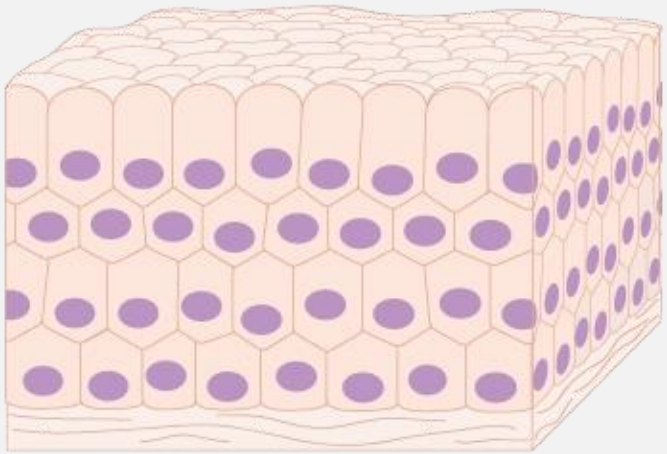




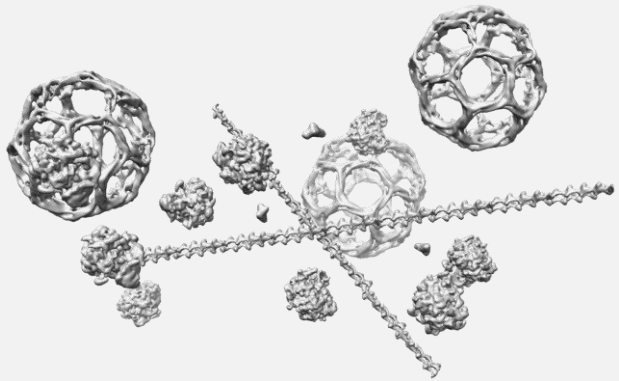
Cancer Research UK

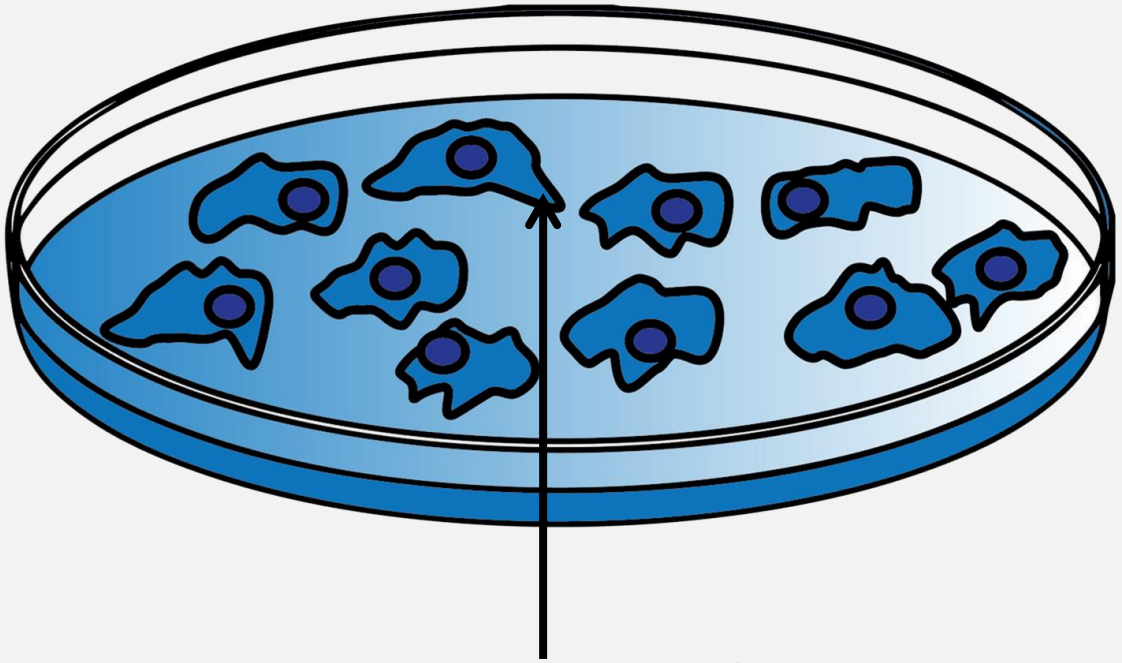


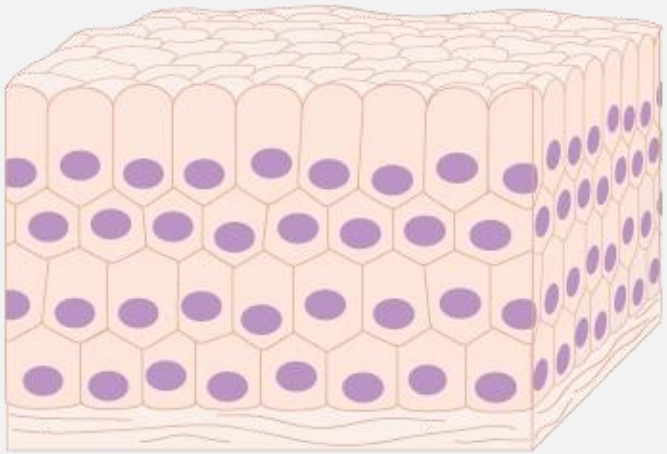




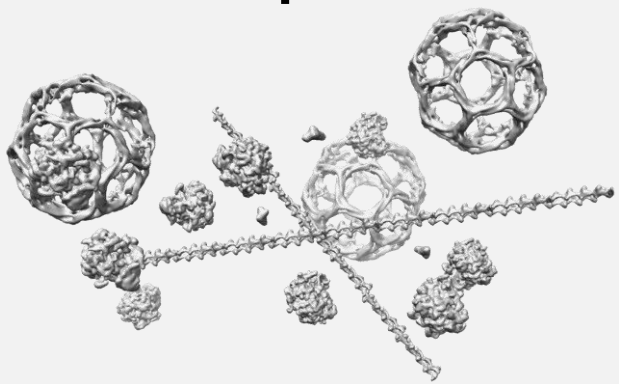
Cancer Research UK

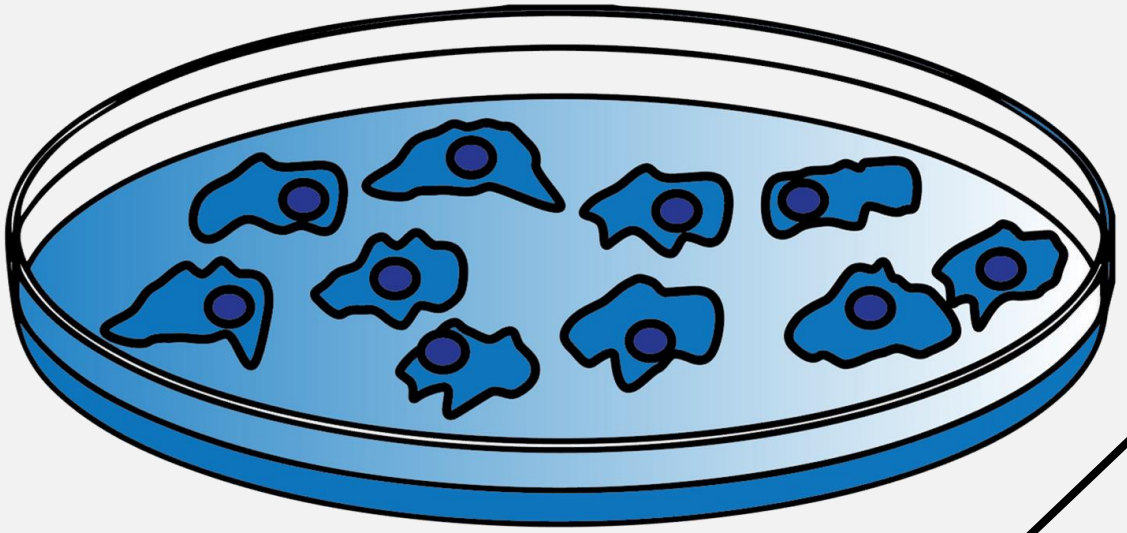


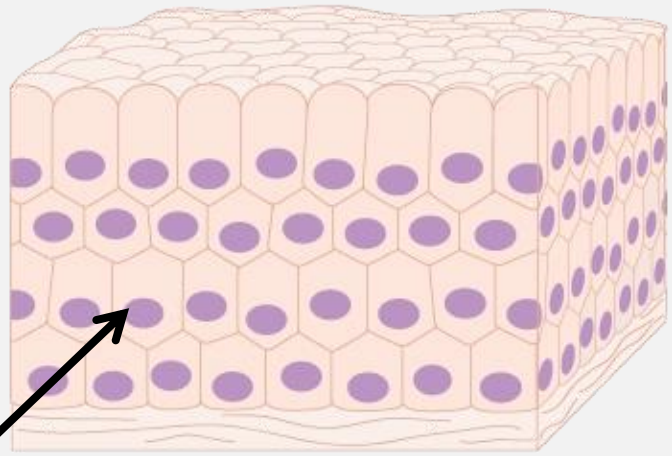




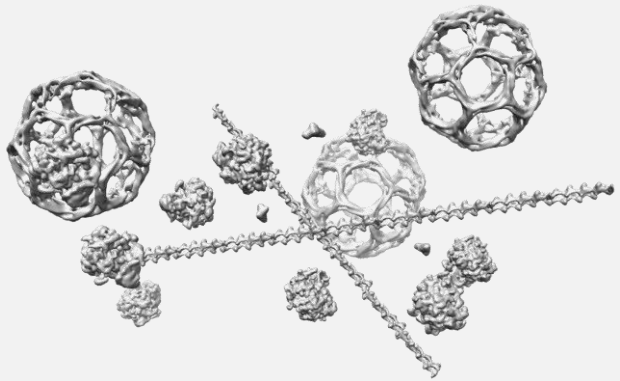
Cancer Research UK

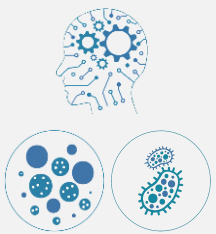


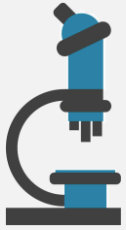


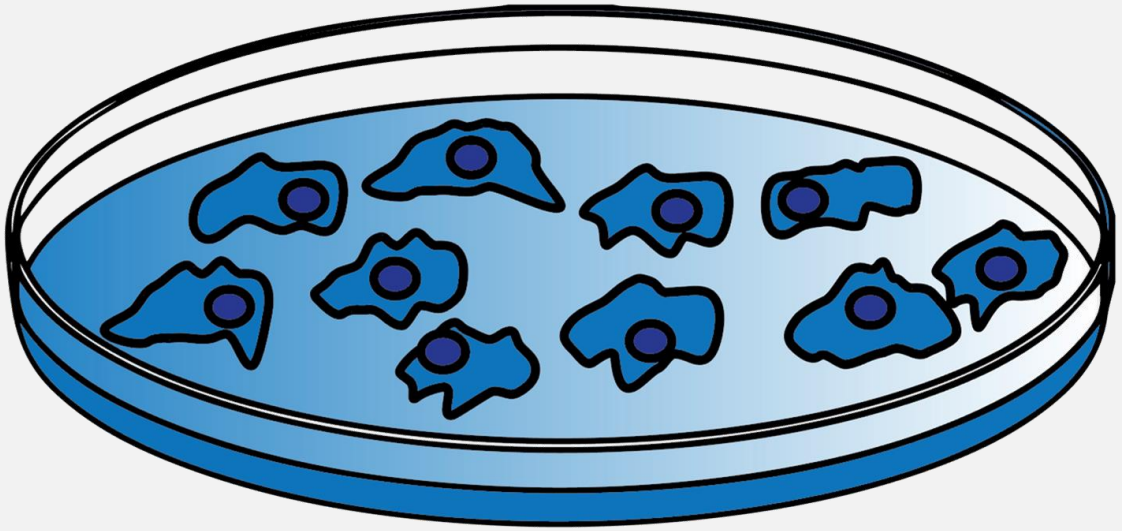


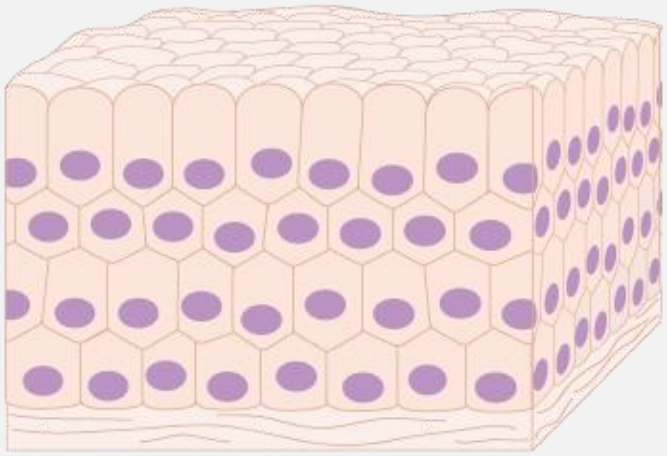
Cancer Research UK



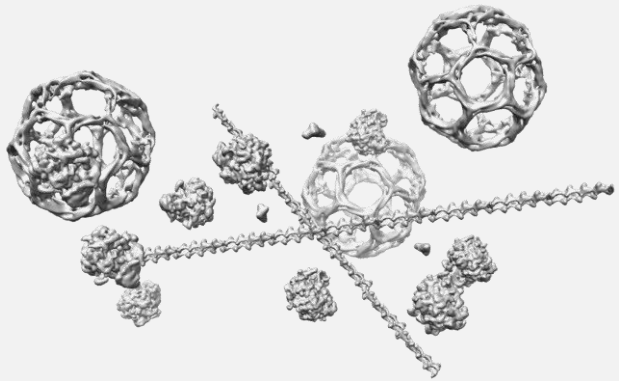


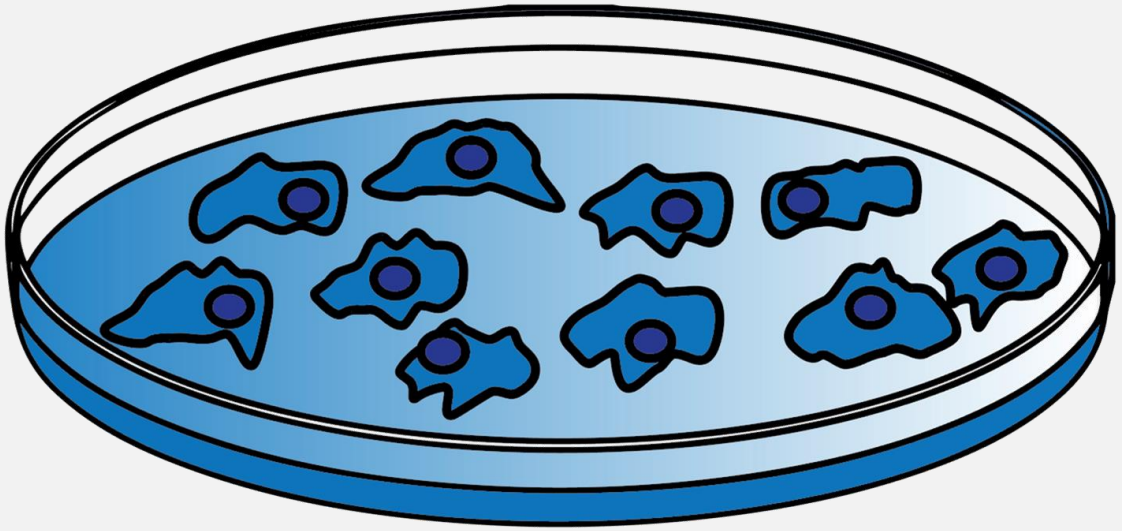


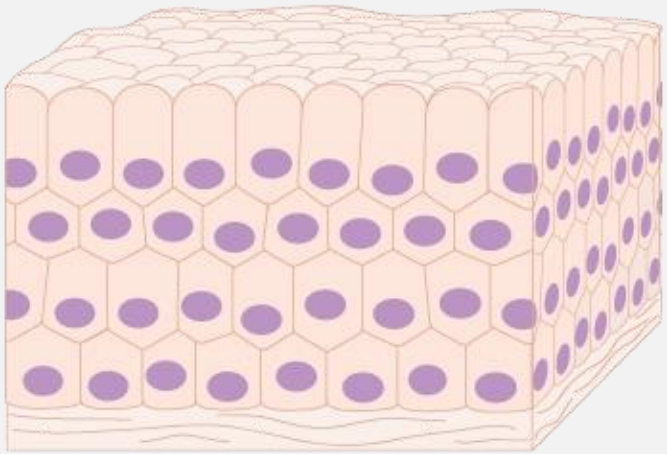




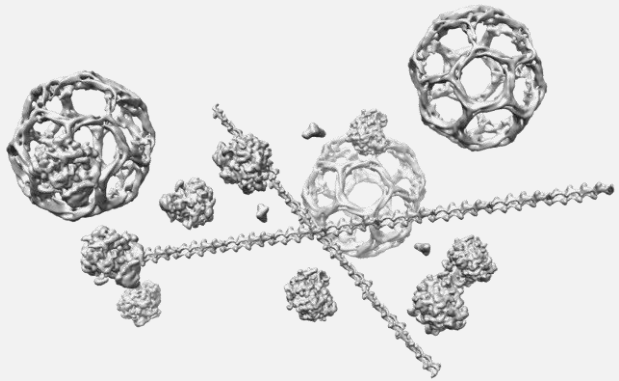
Cancer Research UK

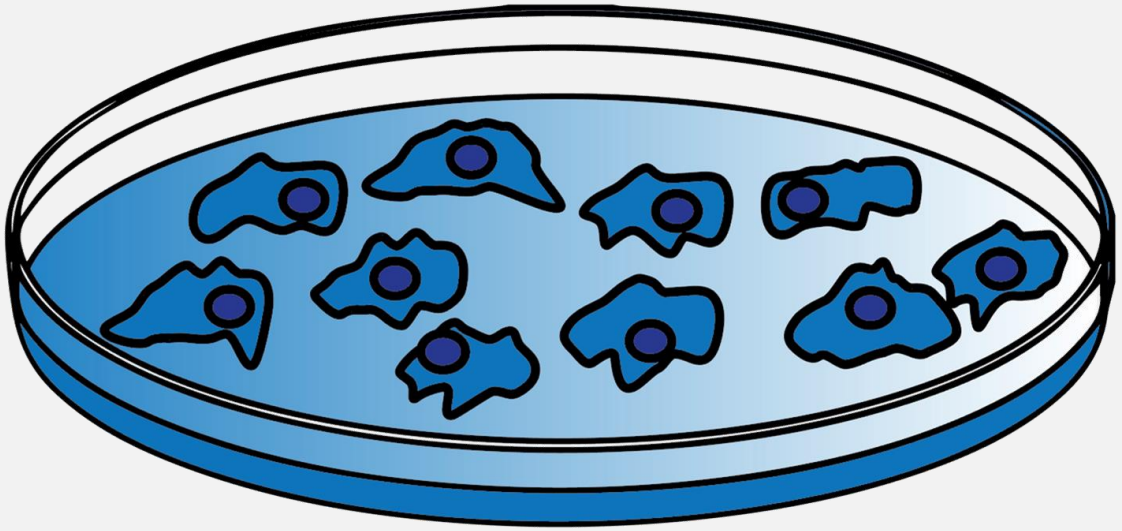


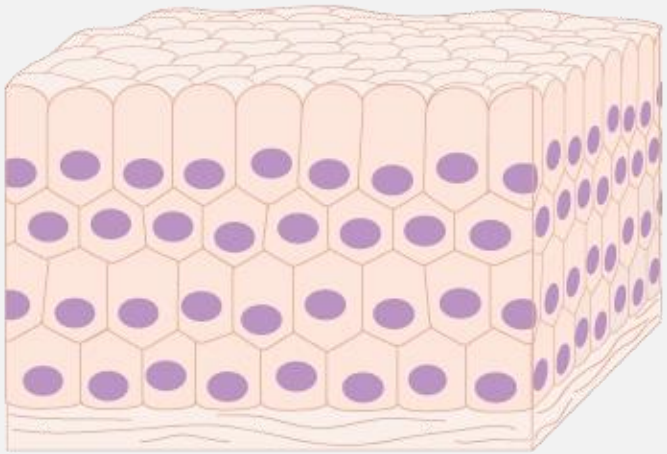




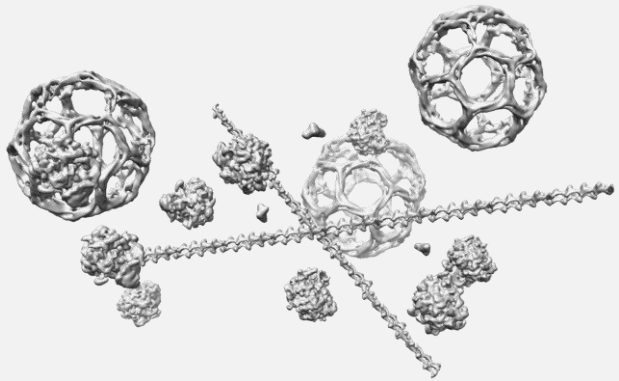
Cancer Research UK

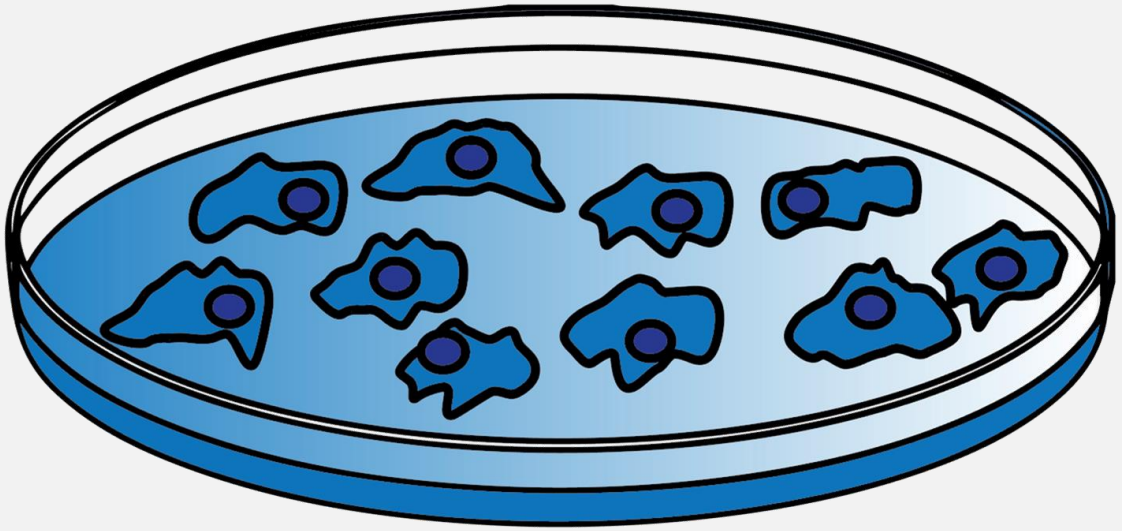


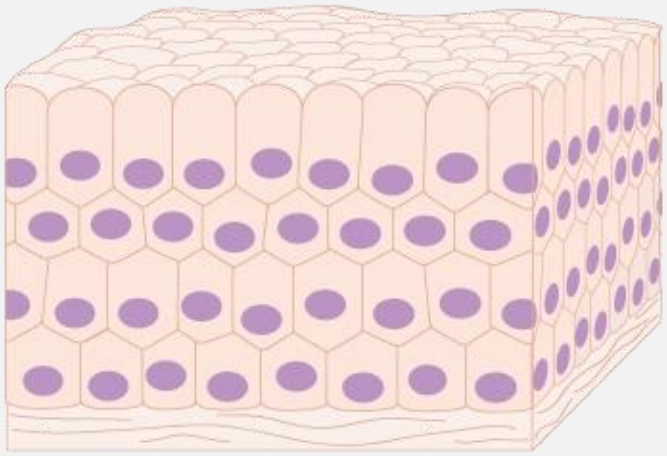




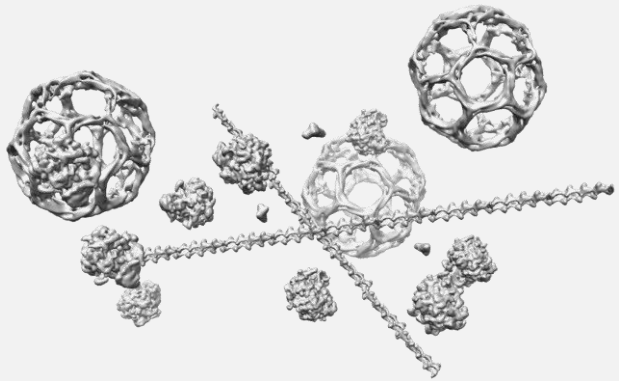
Cancer Research UK

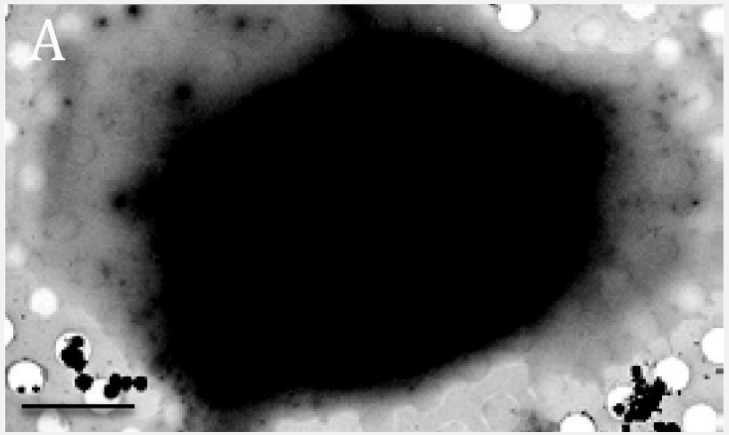




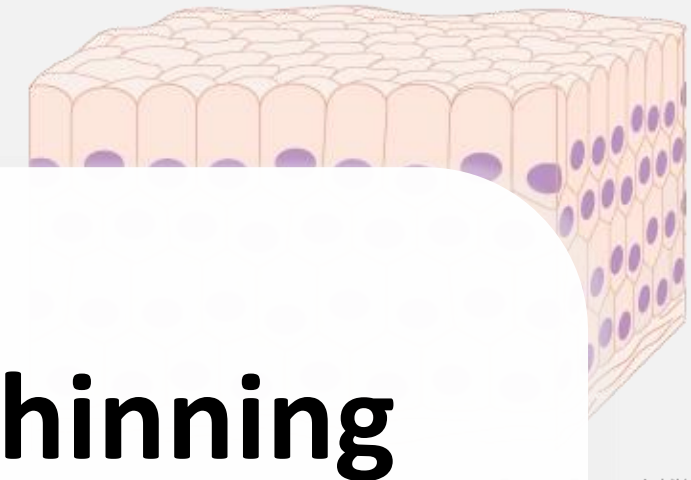


Cancer Research UK

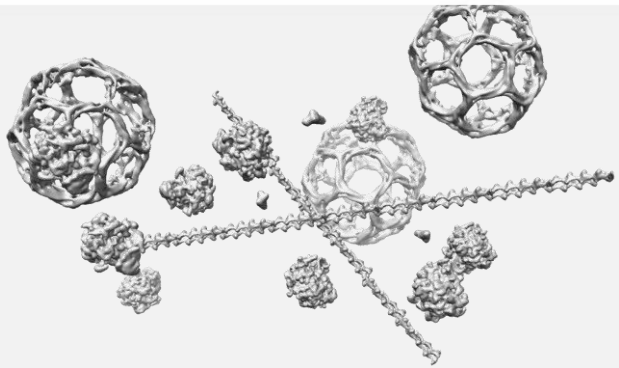


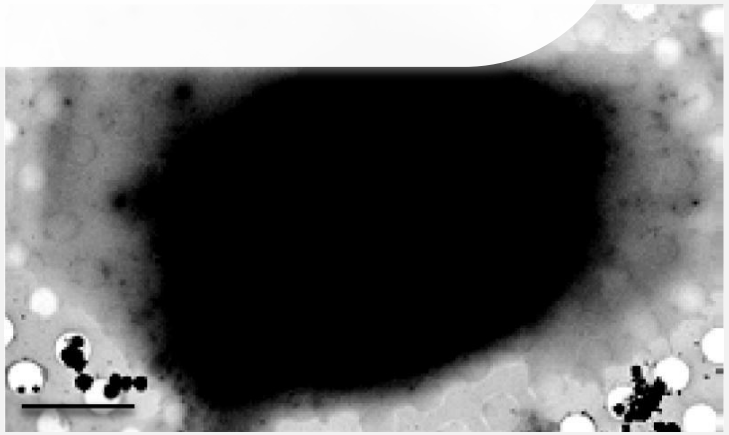


Thompson et. al., 2016

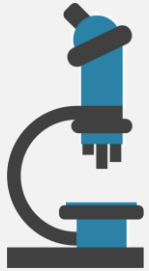


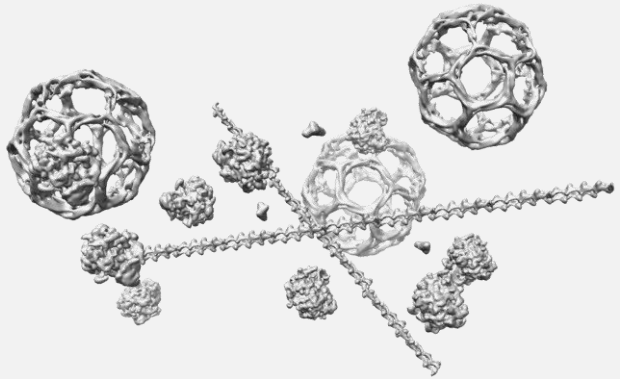
Step 1 **after freezing** is often **thinning**



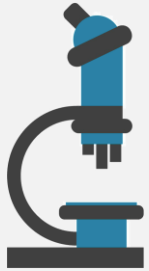


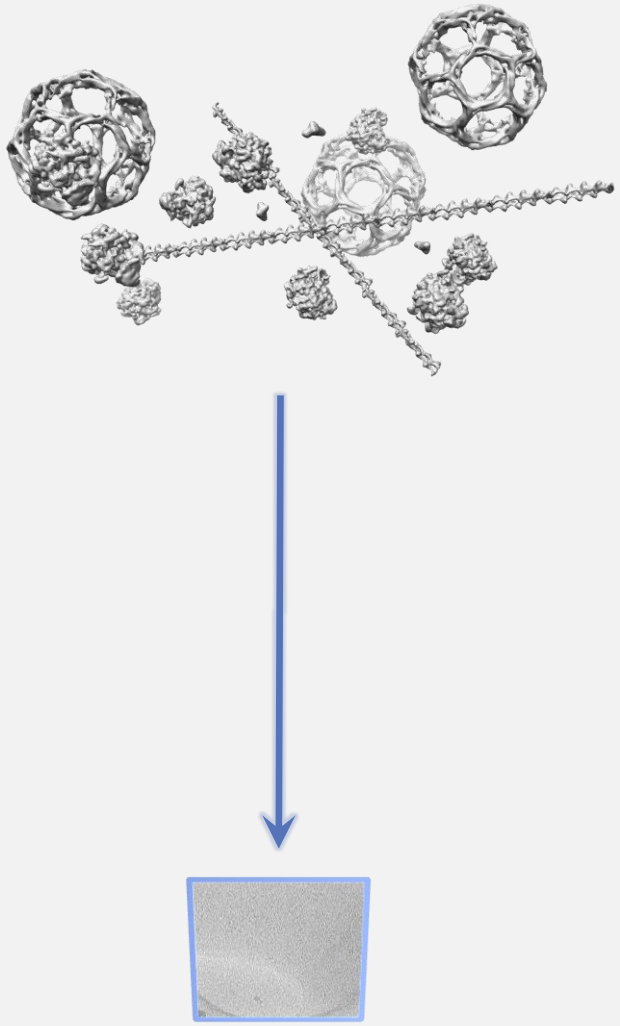
Thompson et. al., 2016





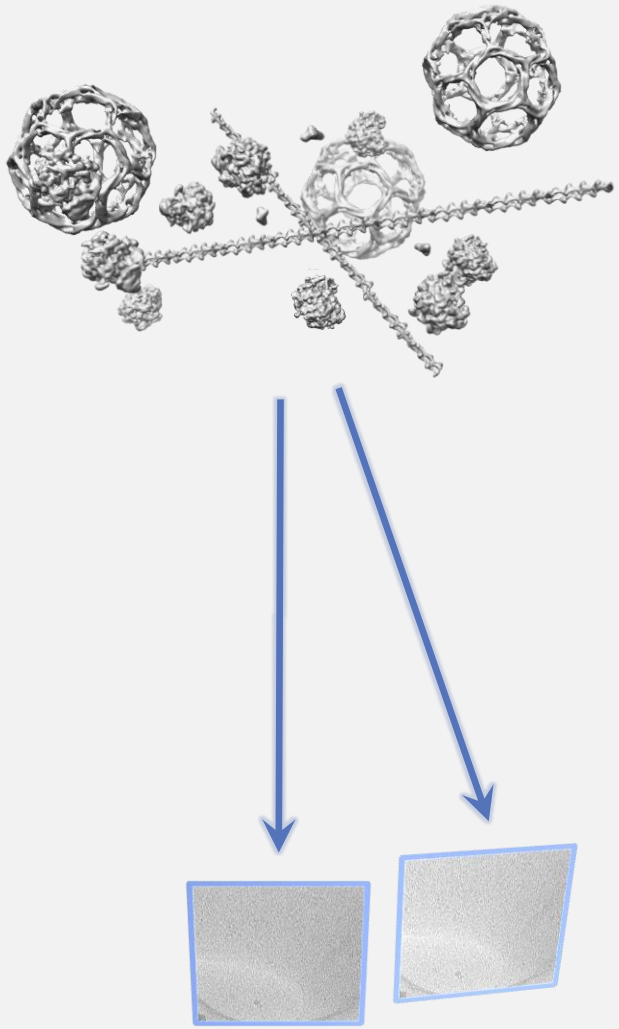
3D specimen movement during collection





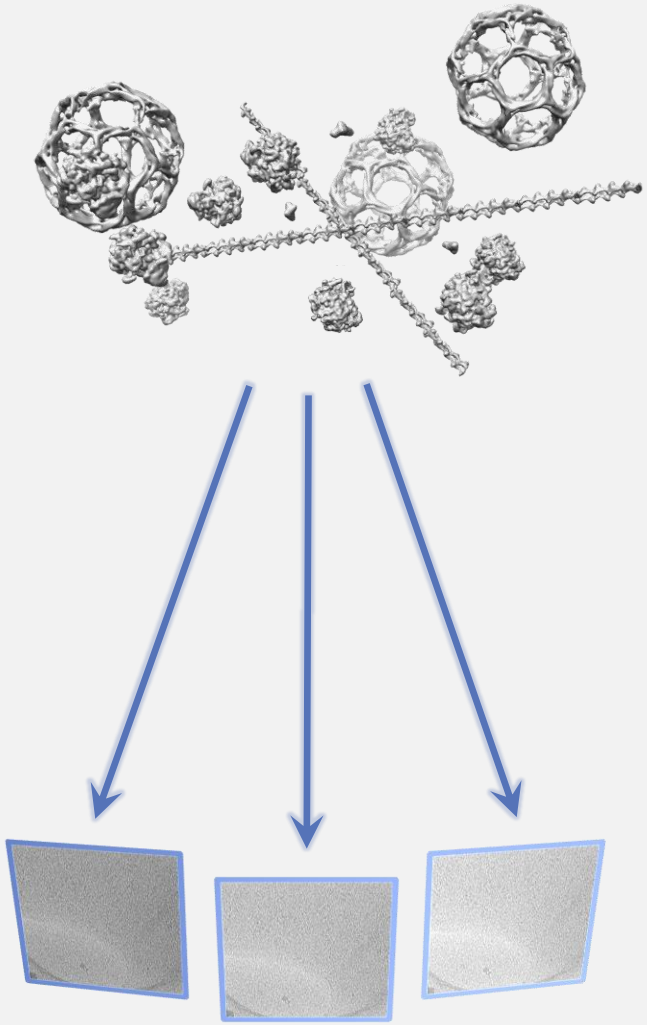
(movements are exaggerated)

3D specimen movement during collection



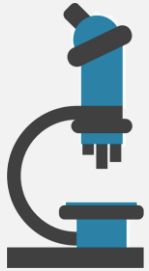
(movements are exaggerated)

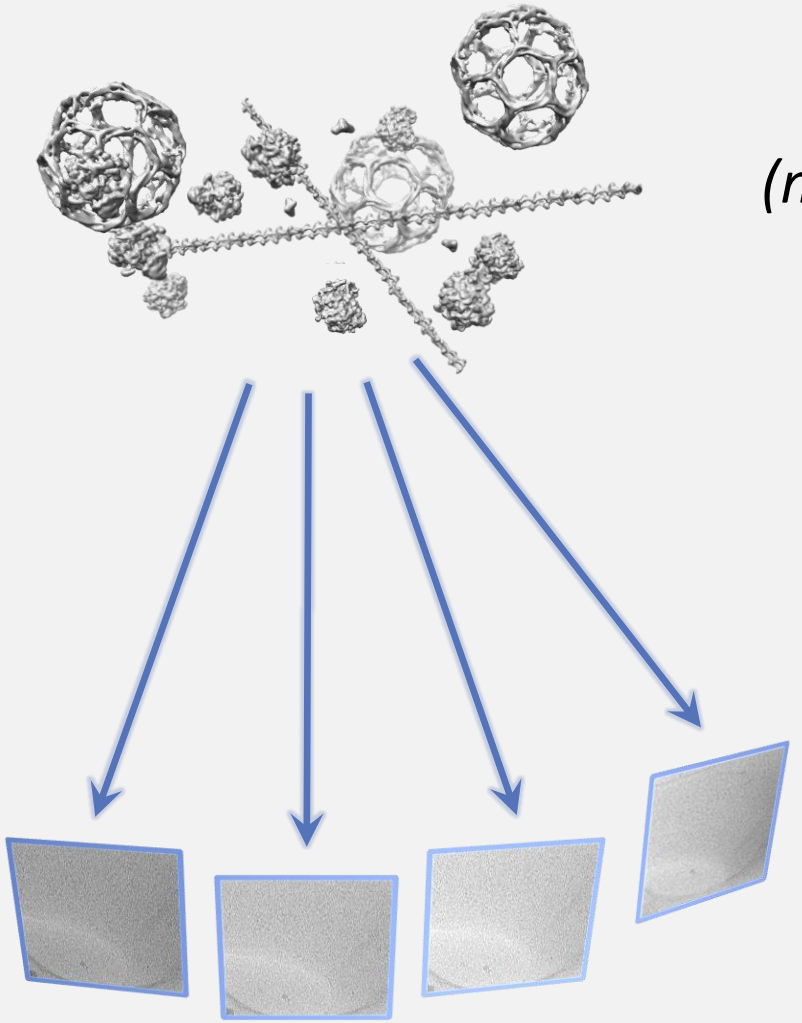
3D specimen movement during collection



(movements are exaggerated)

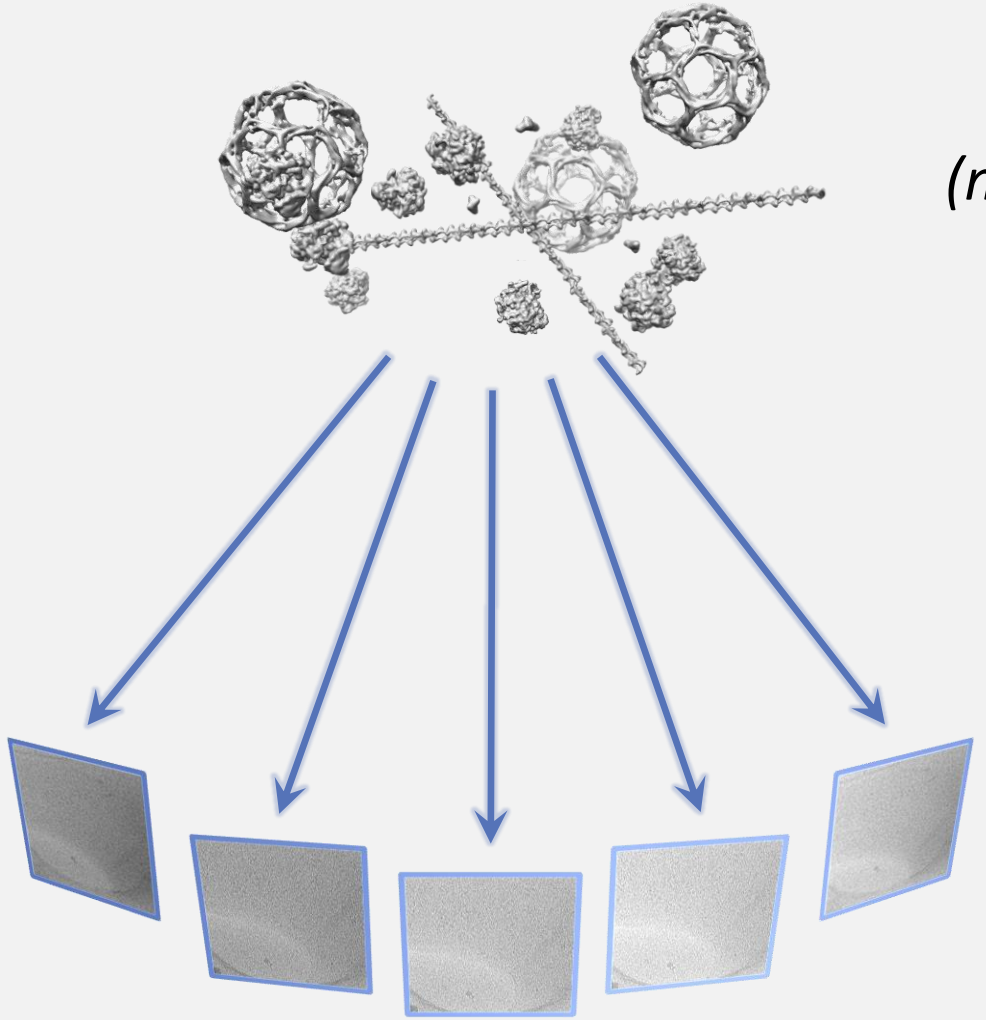
3D specimen movement during collection





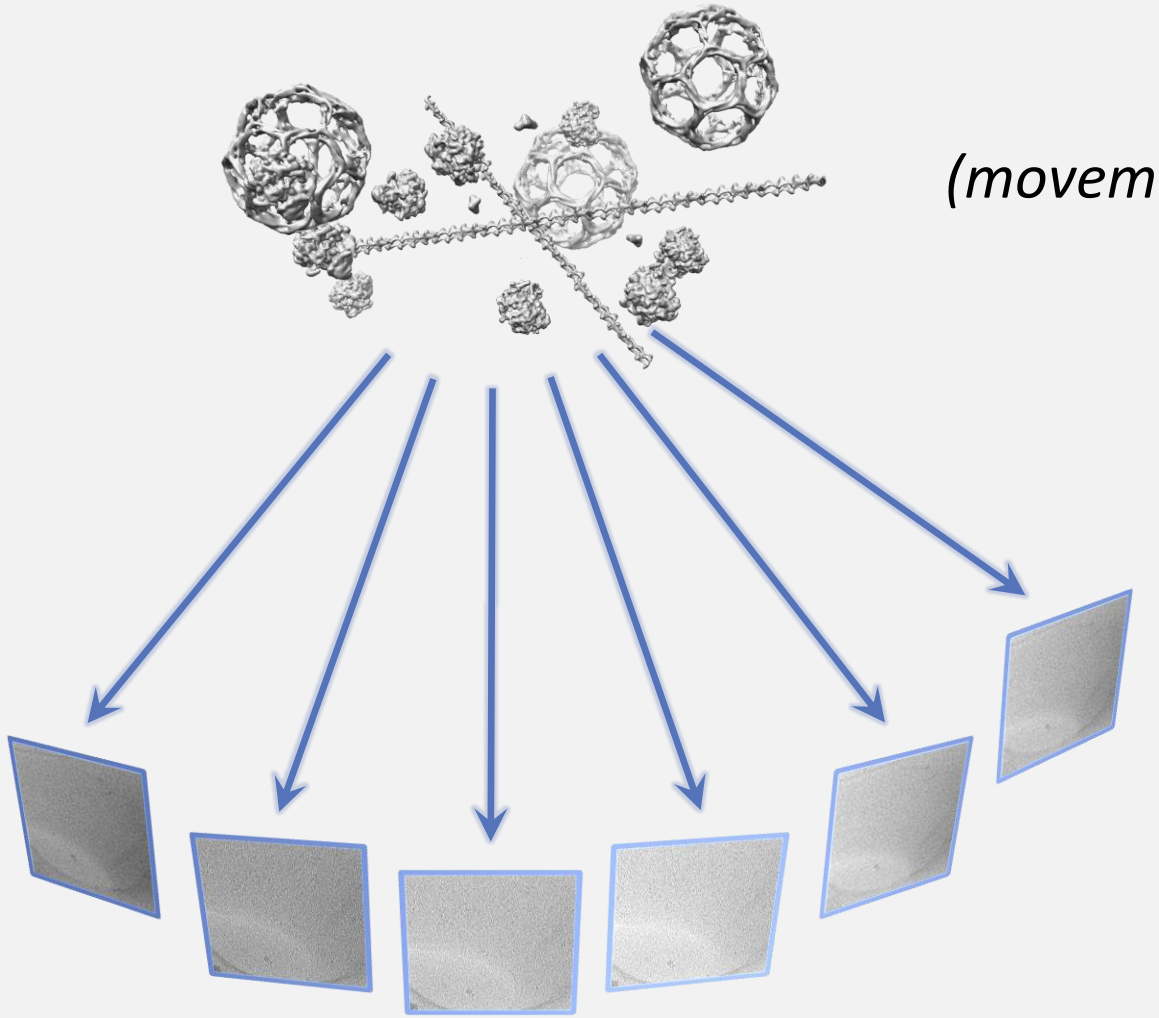
(movements are exaggerated)

3D specimen movement during collection



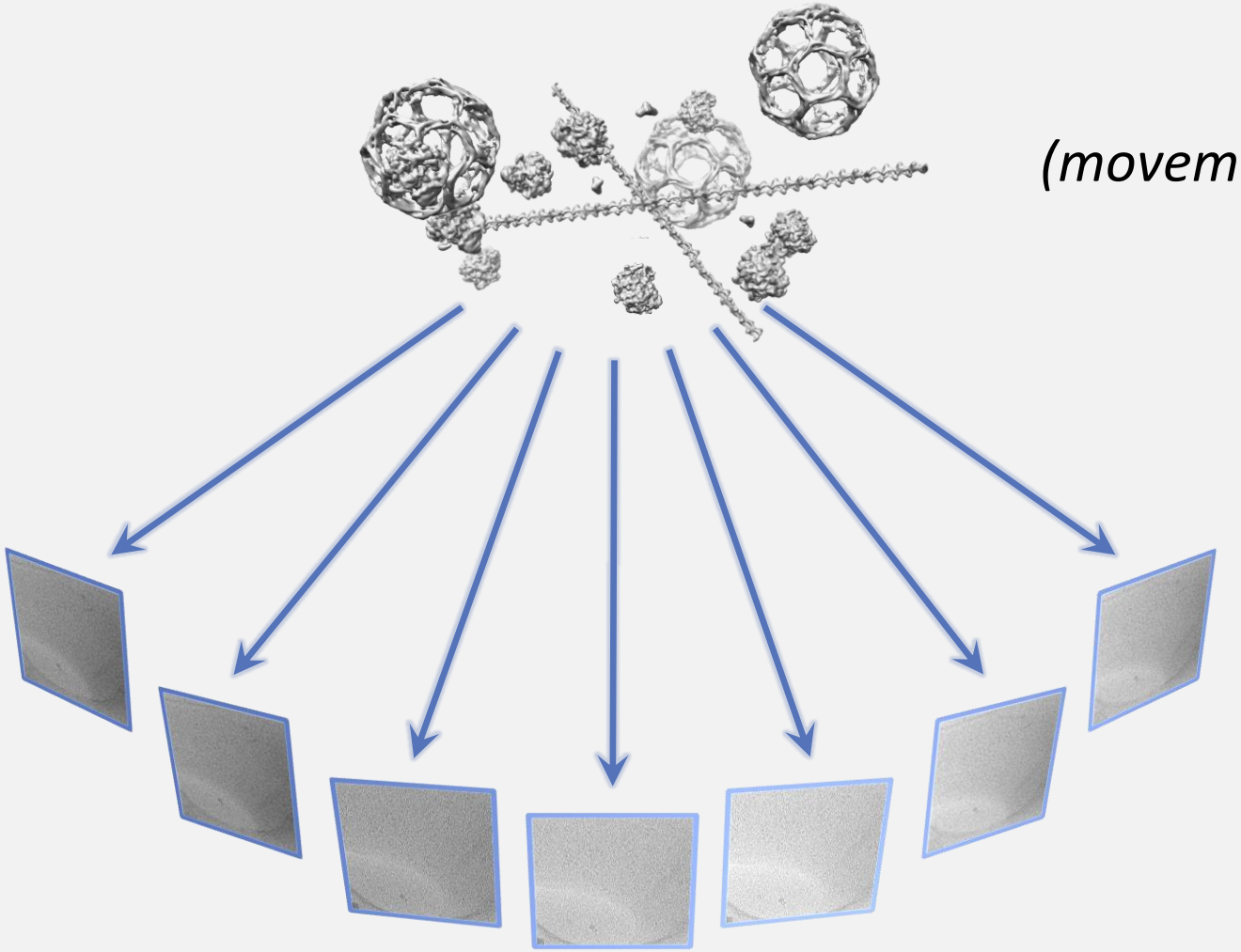
(movements are exaggerated)

3D specimen movement during collection



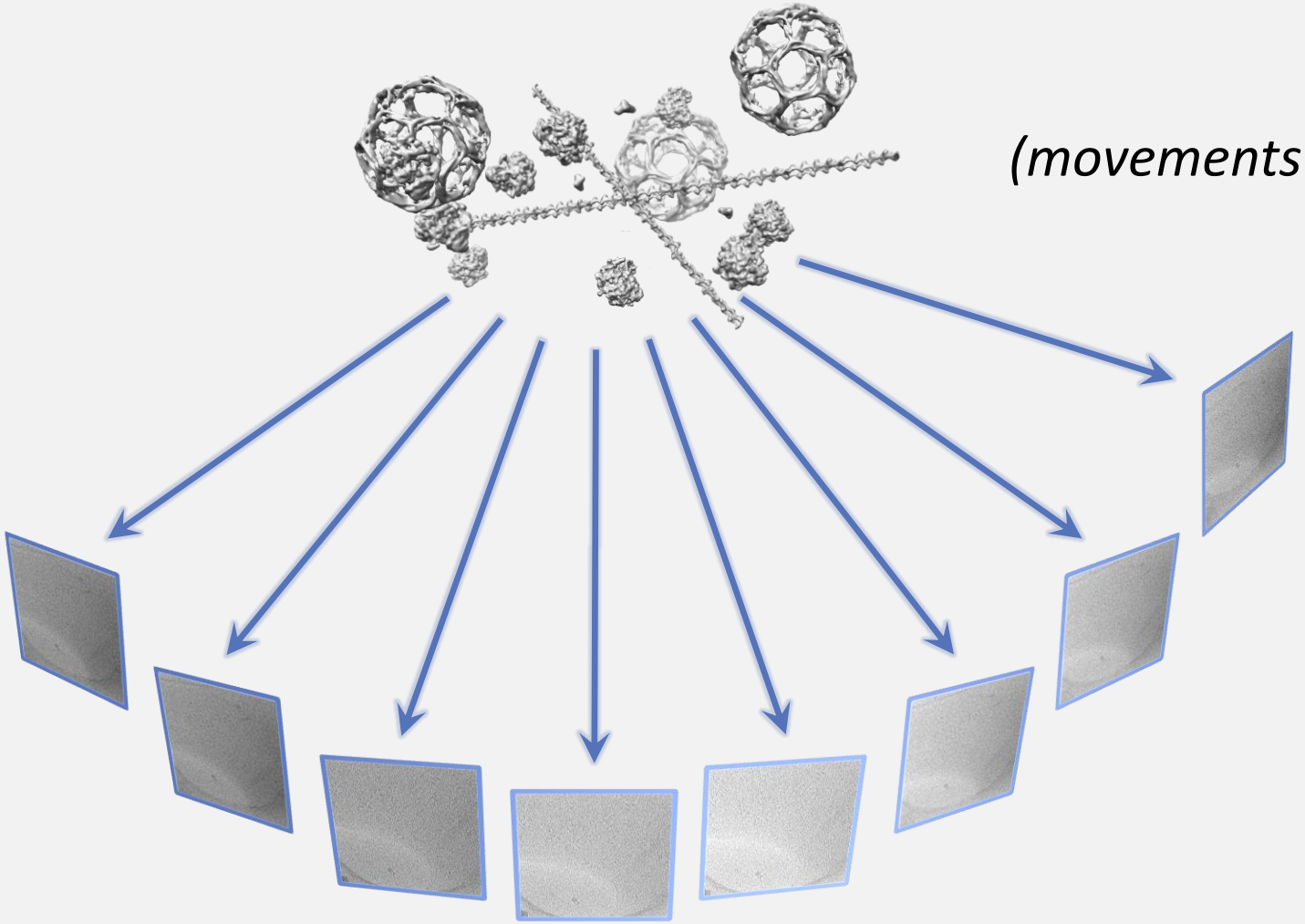
(movements are exaggerated)

3D specimen movement during collection



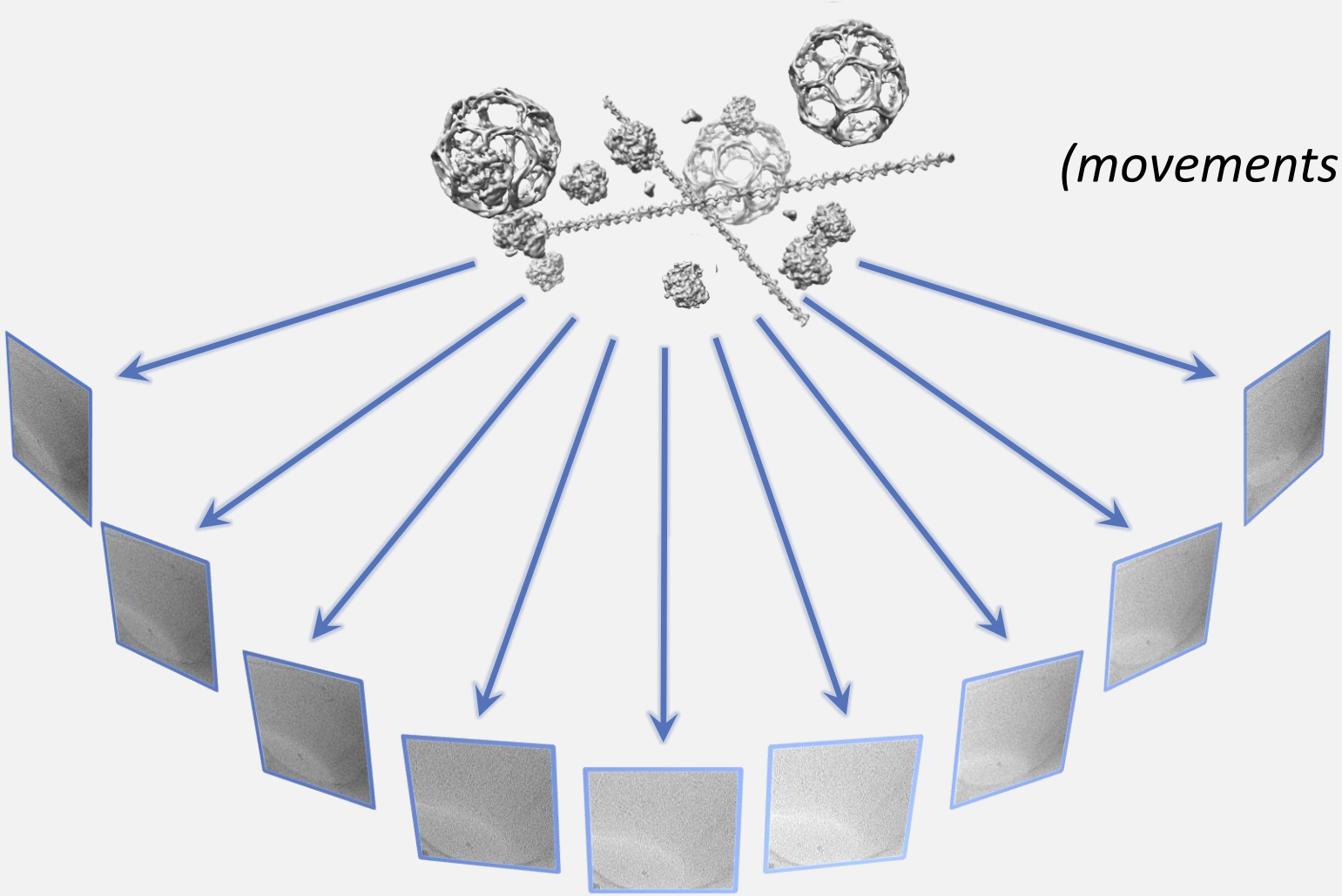
(movements are exaggerated)

3D specimen movement during collection



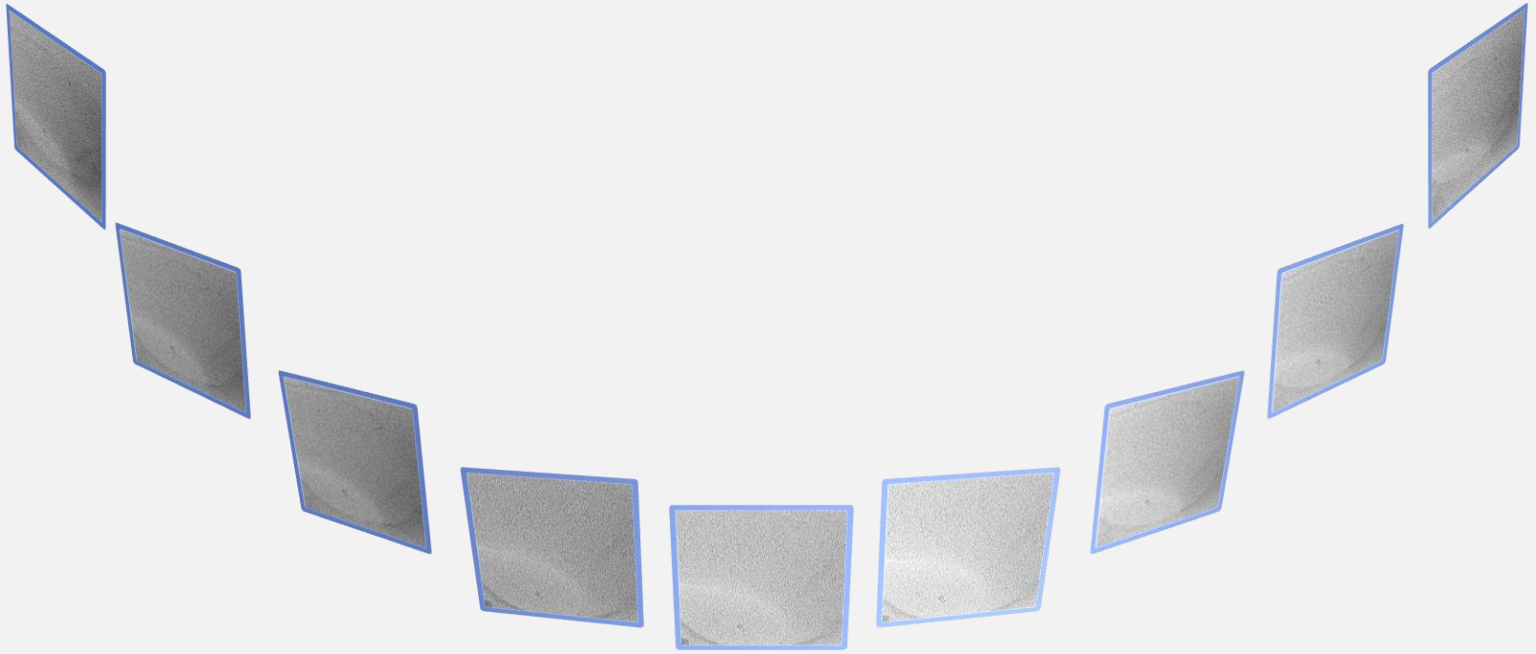
(movements are exaggerated)

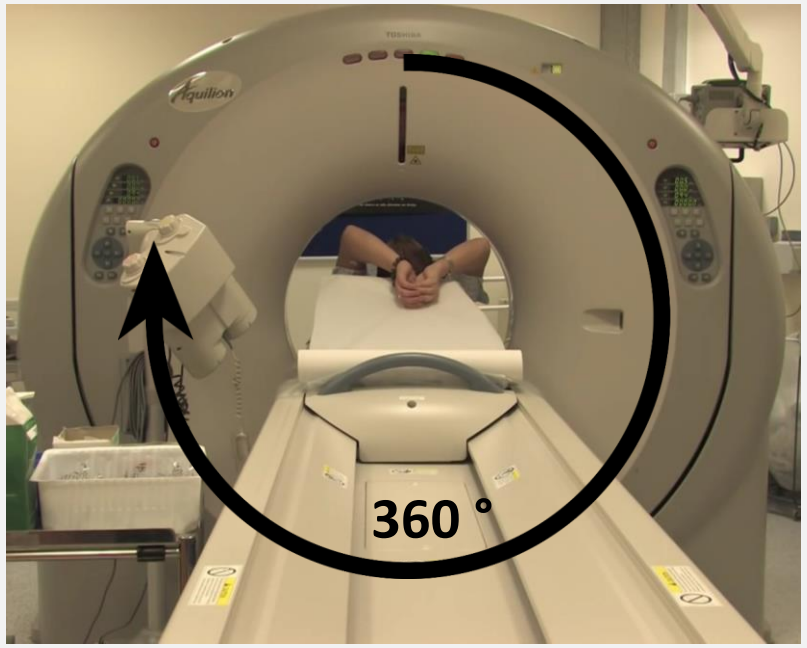
3D specimen movement during collection

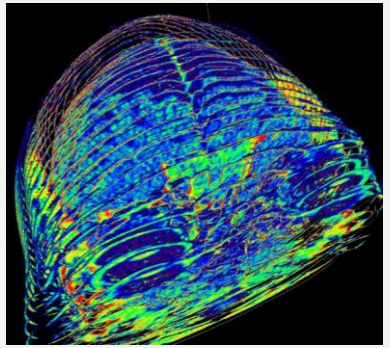


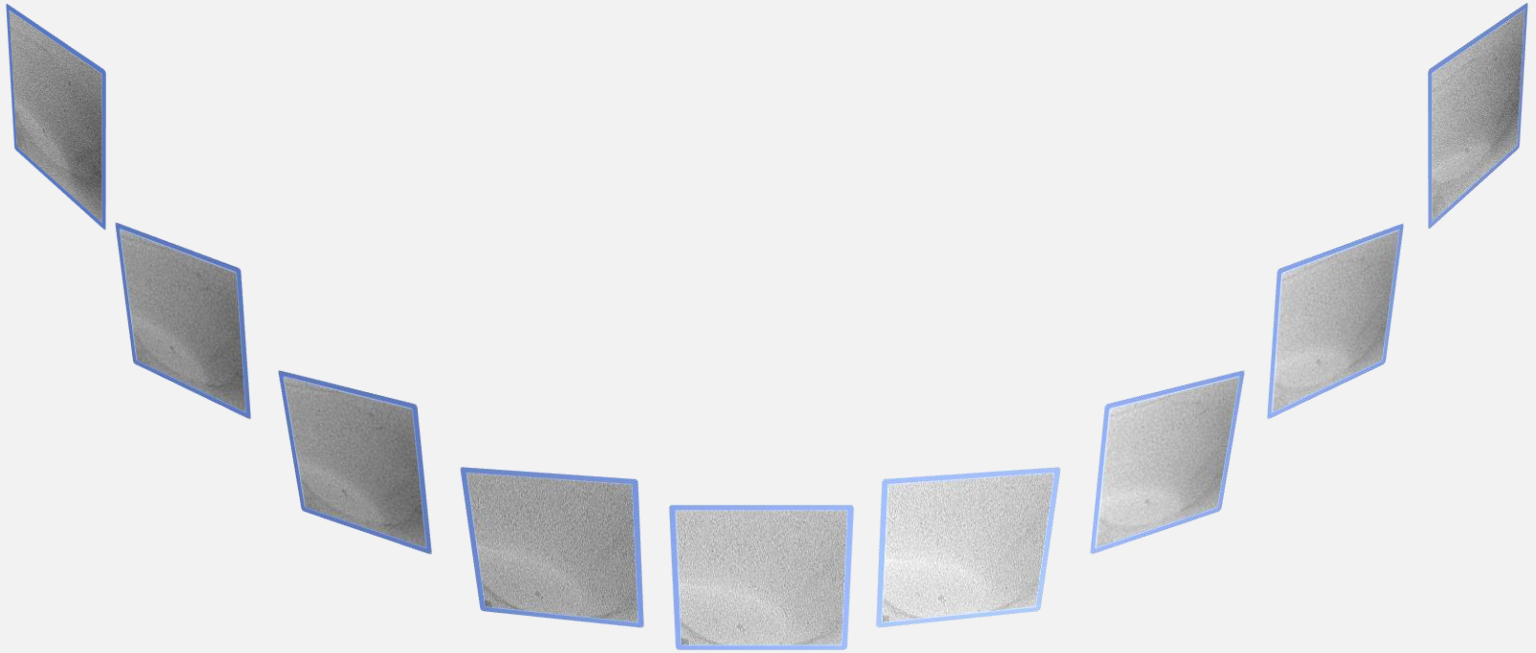
(movements are exaggerated)

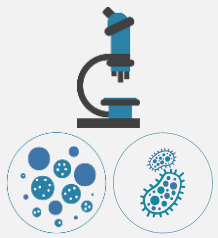
Tilt-series

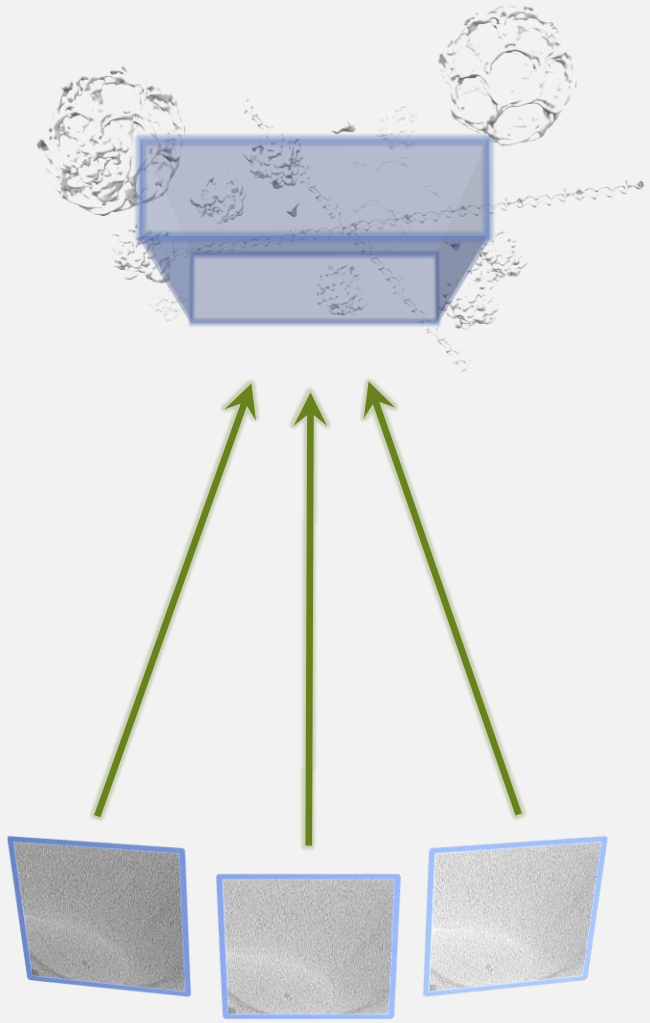


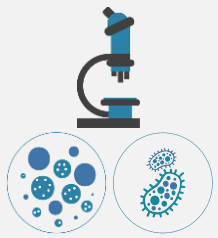

360 °

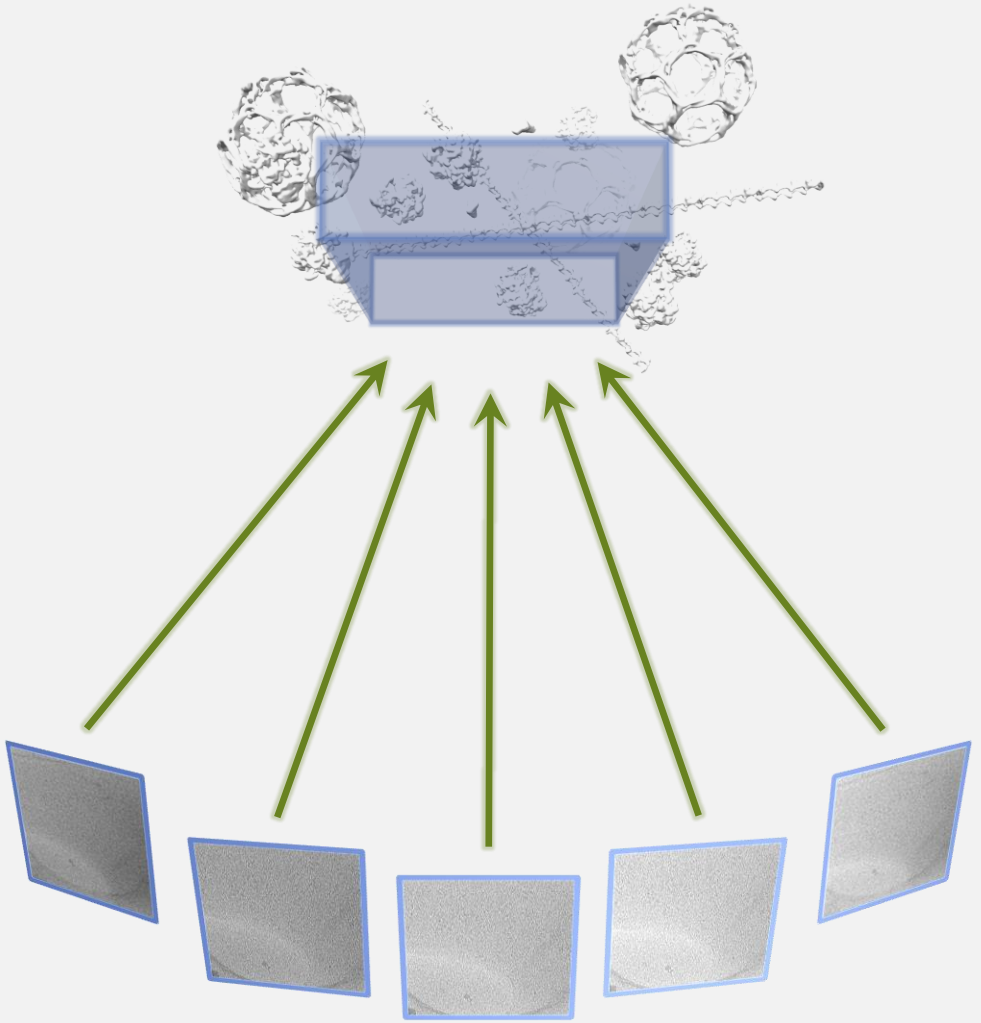


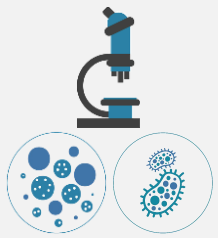


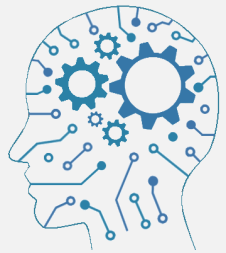


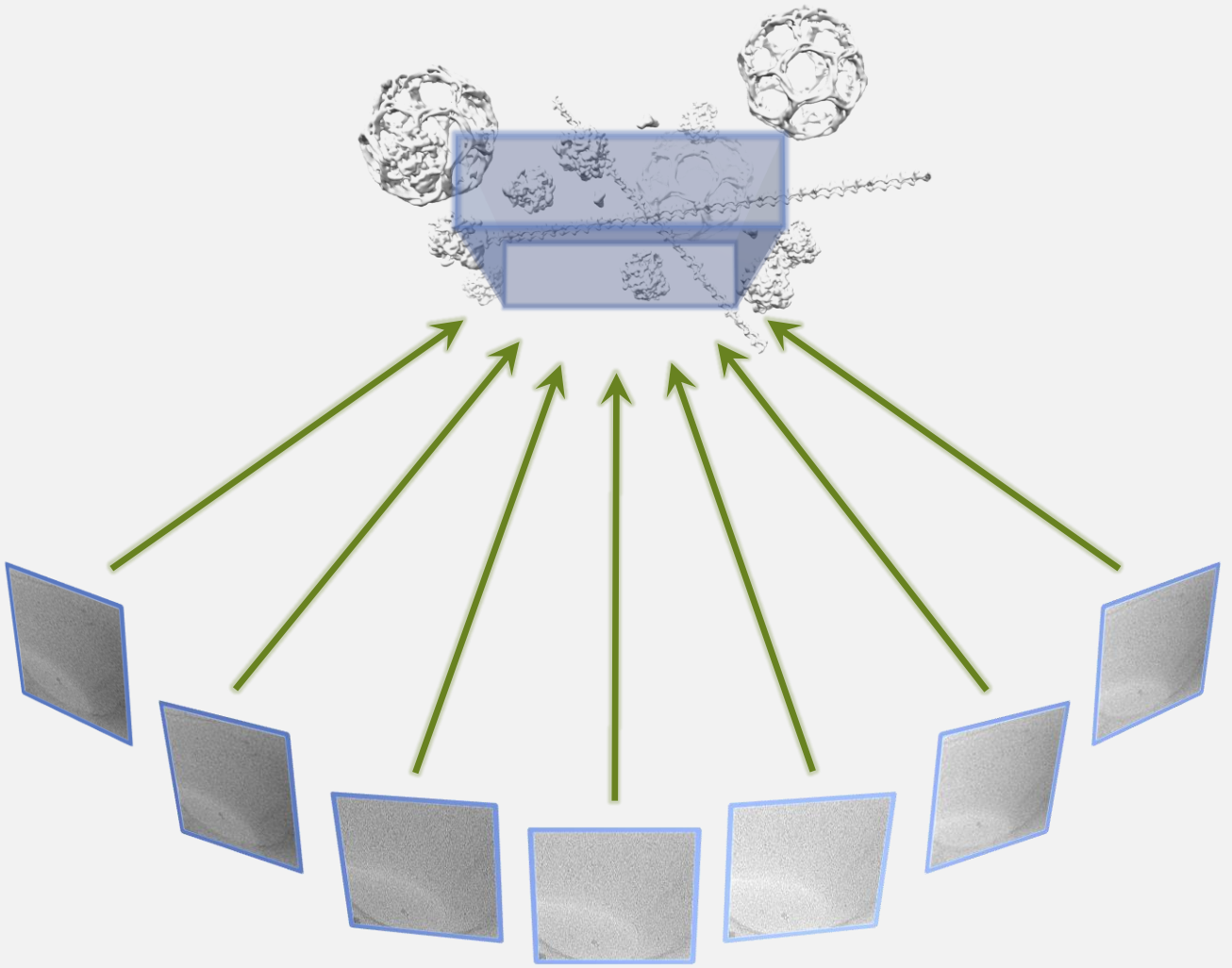


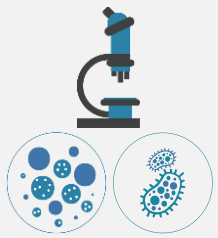


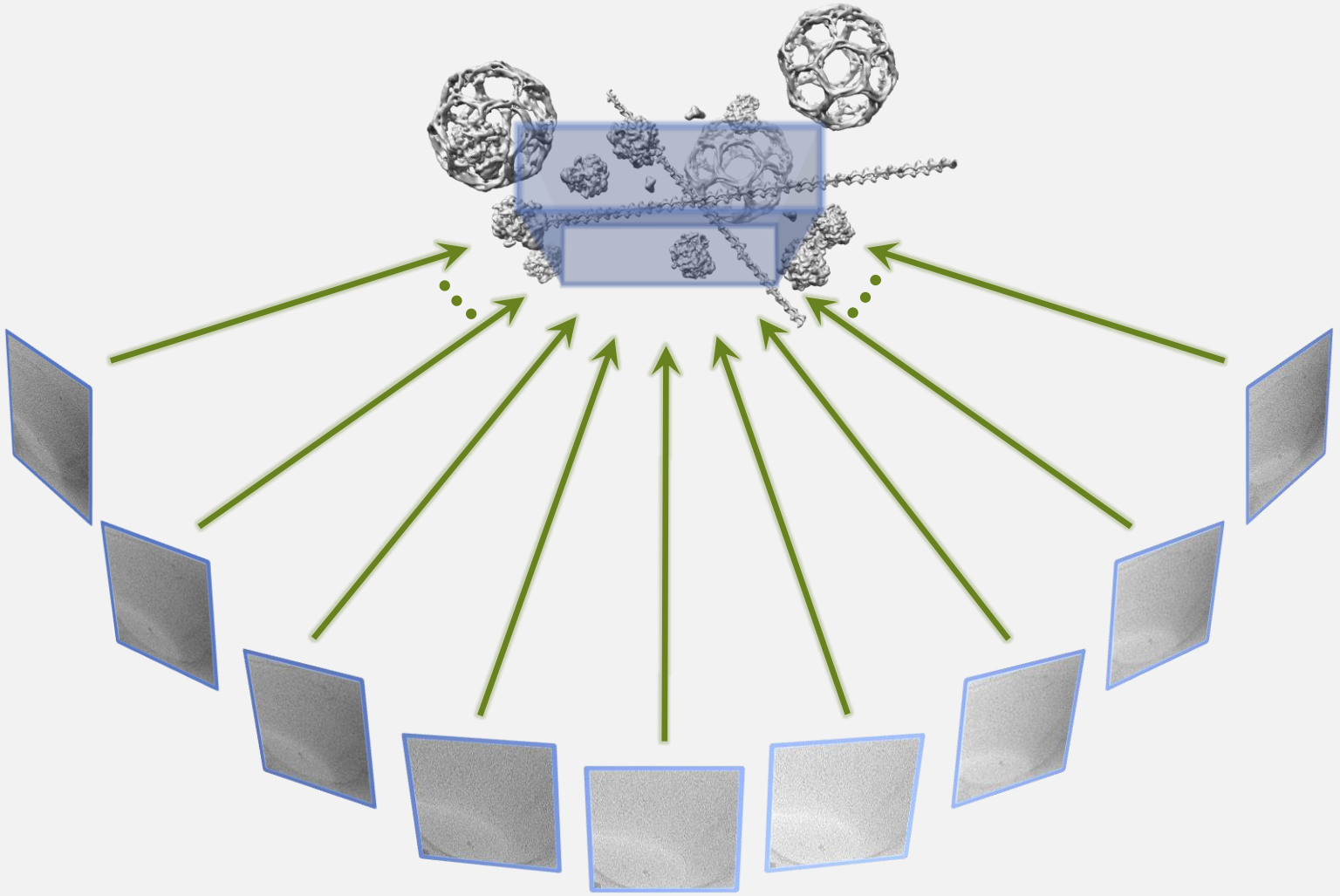




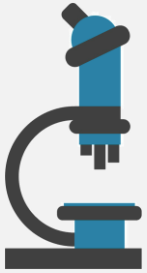


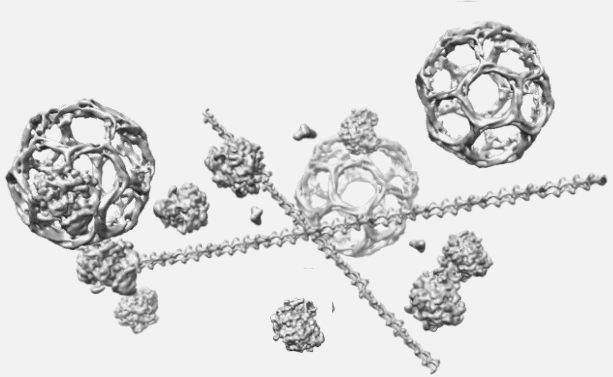




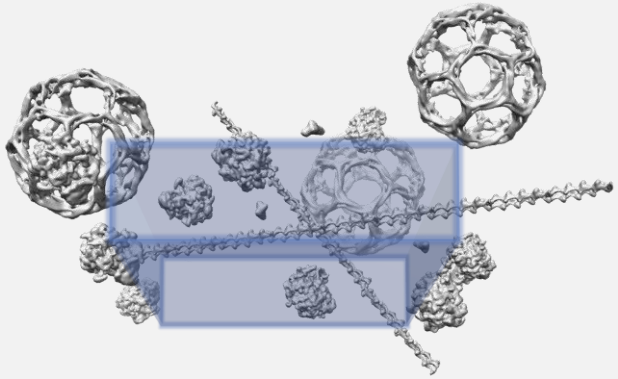


Before collection

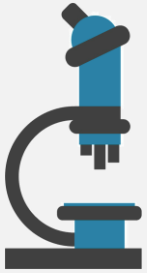


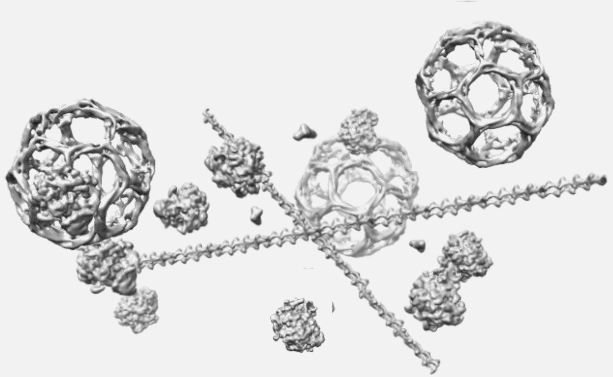


After collection

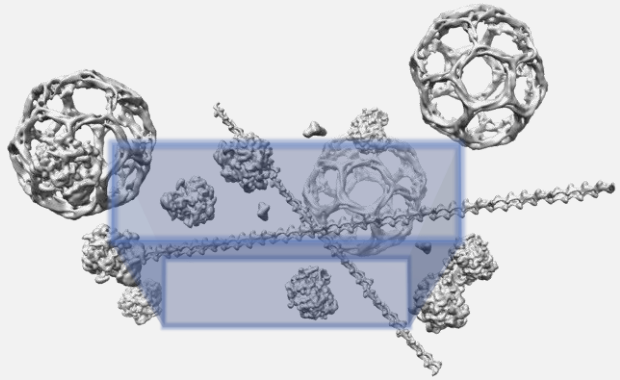


Before collection





After collection

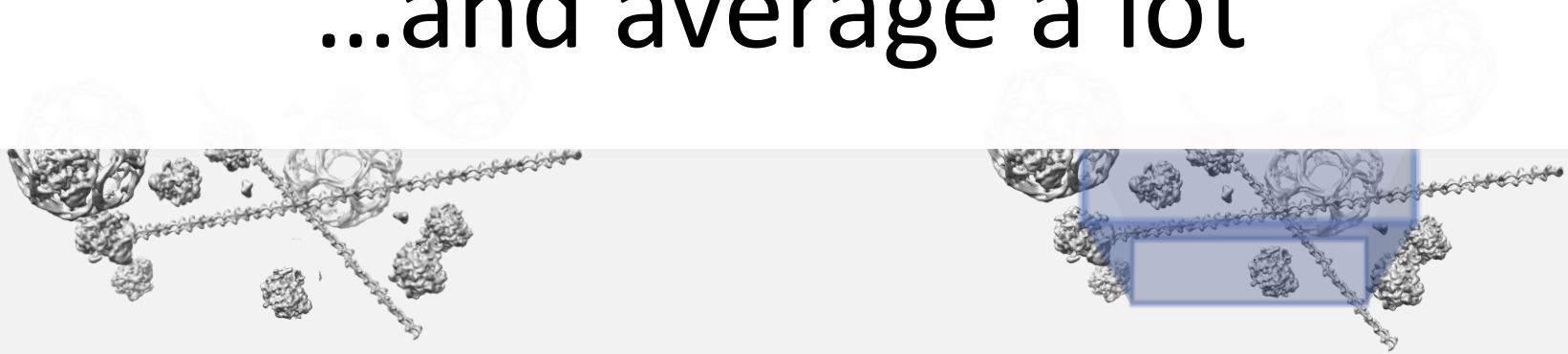


The sample has moved in 3D
…and is damaged

Before collection

After collection

Solution: Correct for local 3D movements
…and average a lot

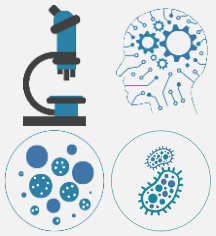


The sample has moved in 3D
…and is damaged

Solution: Correct for local 3D movements …and average a lot

Software exists for this:

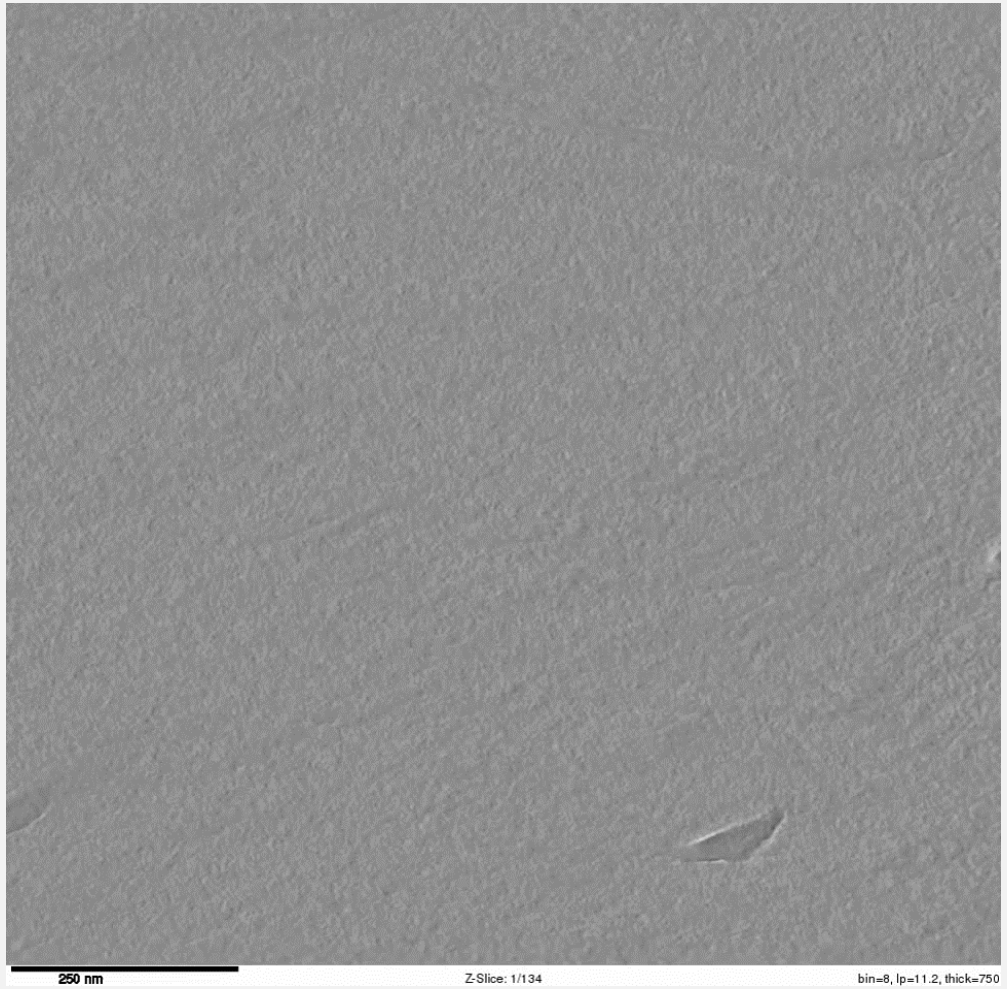
- Warp/M
- Relion 4
- EMClarity
- EMAN2

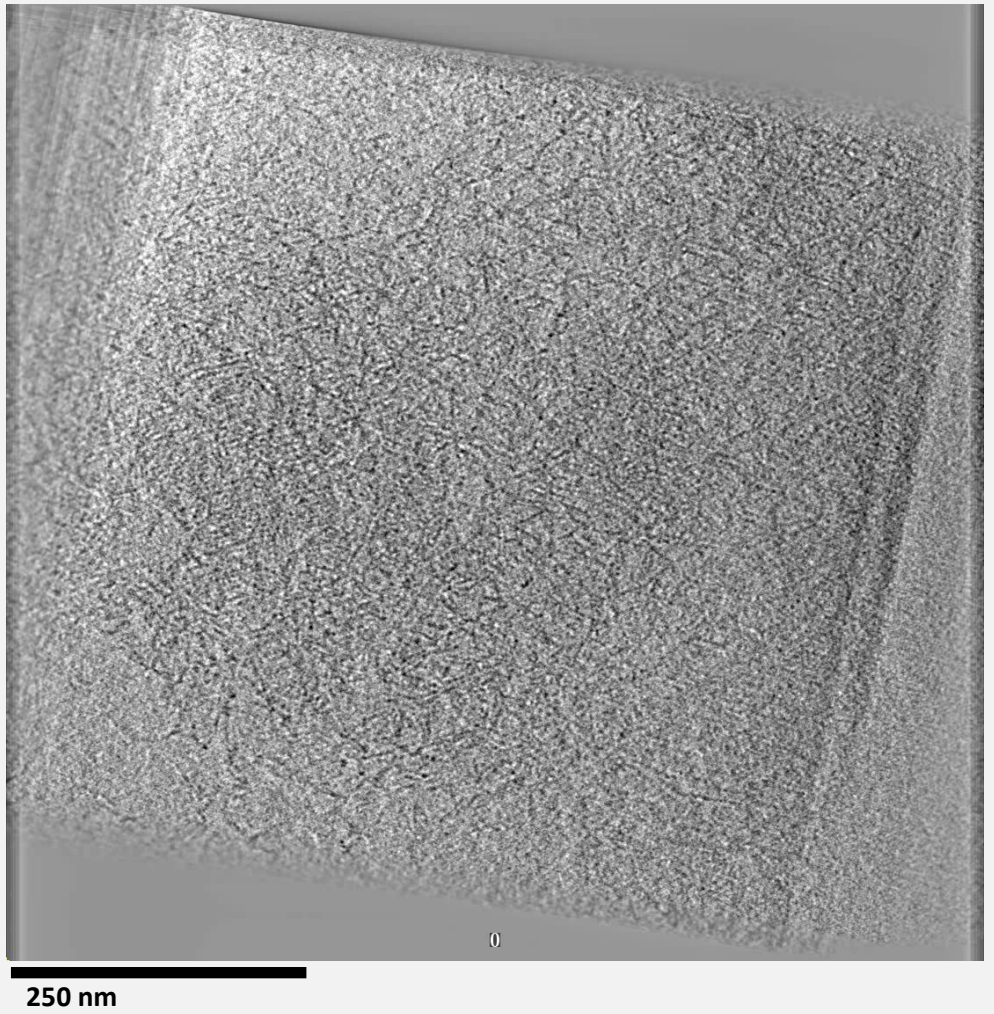


…But we’re getting ahead of ourselves=)

Why CryoET?

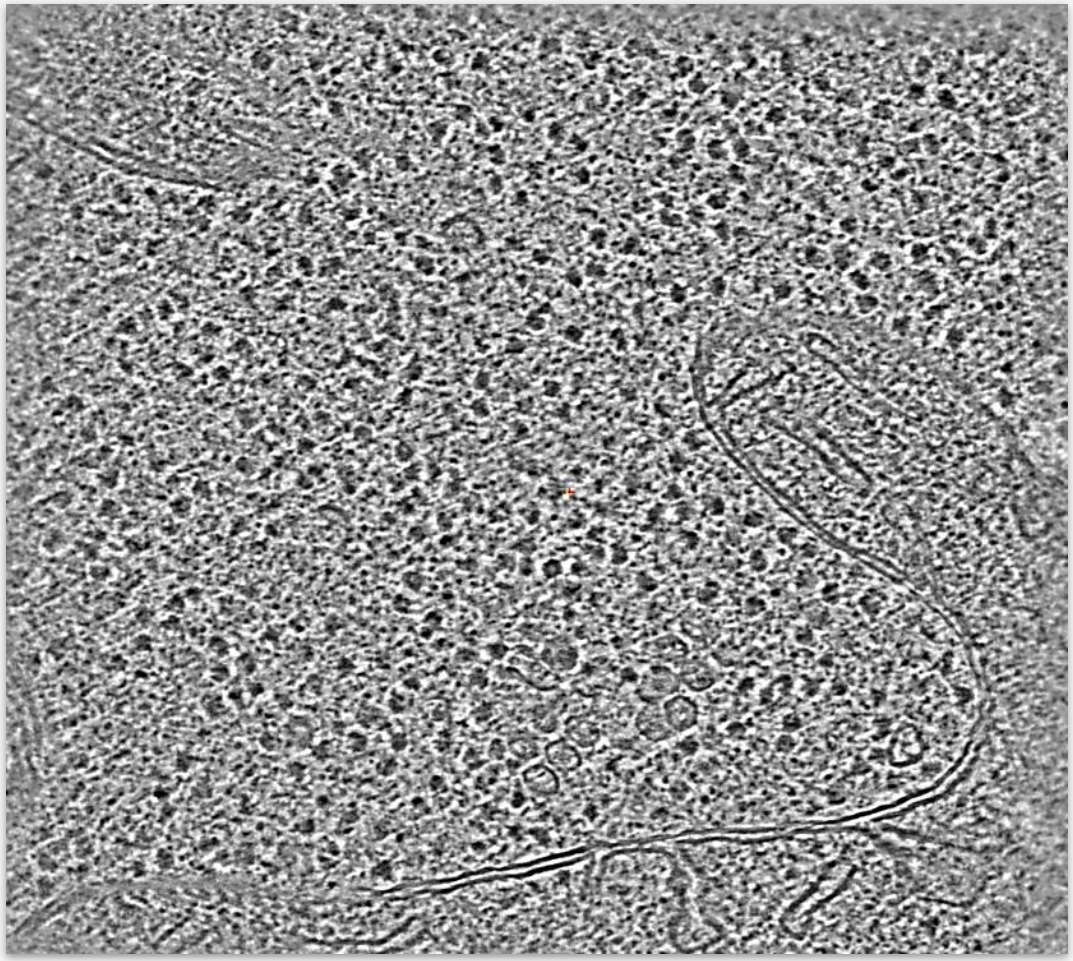
>> CryoET is the **highest resolution method** for **native specimen**

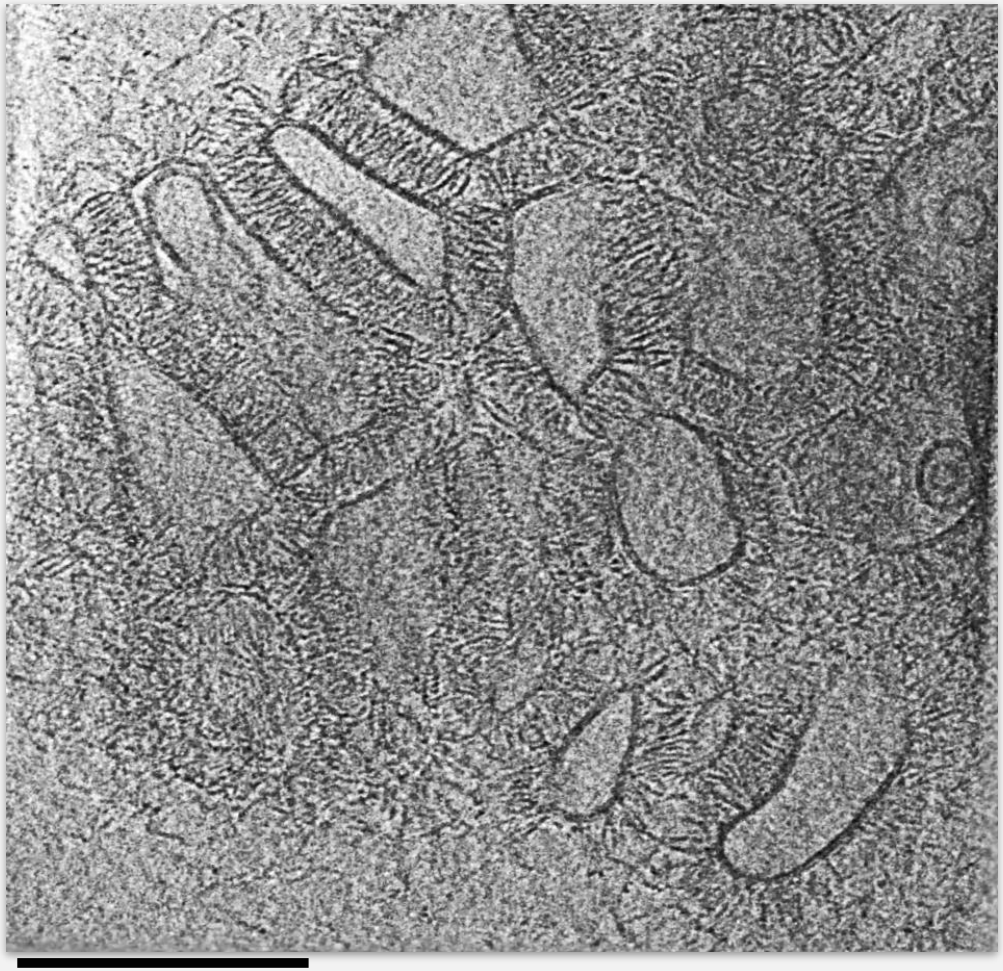




Why CryoET?

>> CryoET is the **highest resolution method** for **native specimen**





Why CryoET?

Why **cryo**?

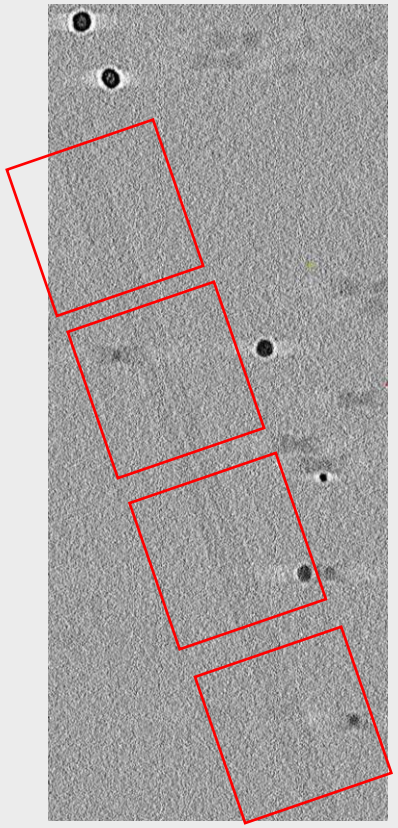
- Specimen preservation in **native or near-native** environments

Why **tomography**?

- Some combination of:
  - Sample is **unique**; e.g. cells, tissues
  - Sample is too **heterogeneous** (structurally or morphologically)
  - **Contextual information** is desired
  - **Sub-nanometer** information is usually **not** required, but may be possible

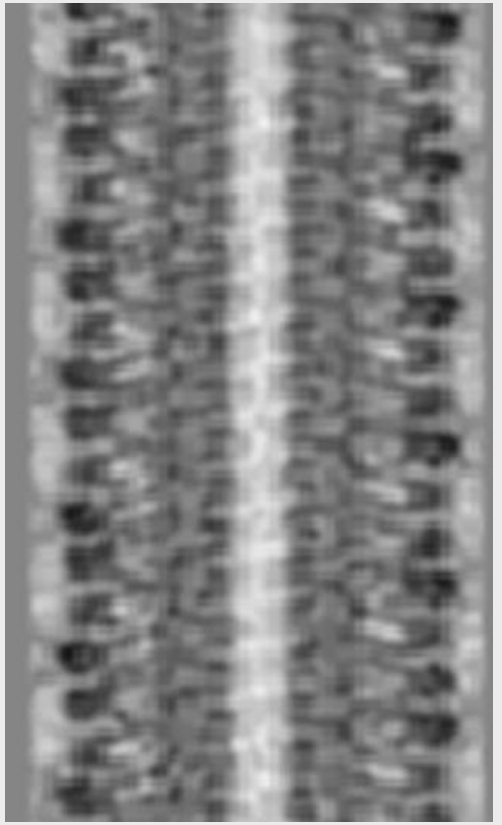
Why subtomogram averaging?

- Some amount of structural **repetition**,
- Repeating subunit preferred **orientation** overcome by **tilt range**



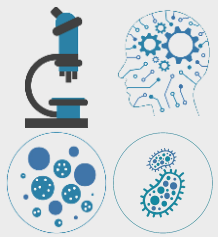
particles

align



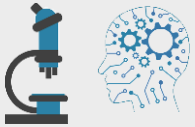
reconstruction

Courtesy of Misha Kudyashev



Overview

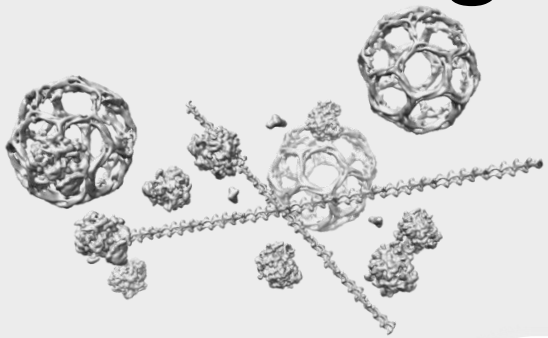
- Sample freezing and prep
- CryoET limitations
- Tilt-series collection
- Tilt-series alignment
- Defocus estimation and CTF correction
- Sub-tomogram localization
- Sub-tomogram alignment and averaging
- Processing limitations
- Future directions and improvements



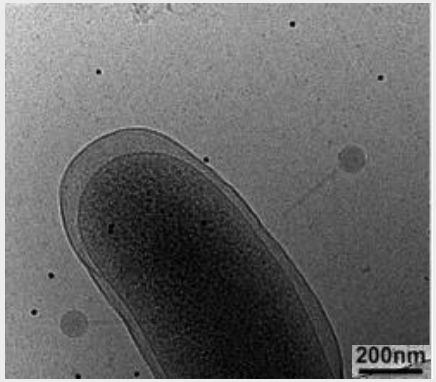
Sample freezing and prep

Overview – Sample freezing and prep

- Reconstituted samples

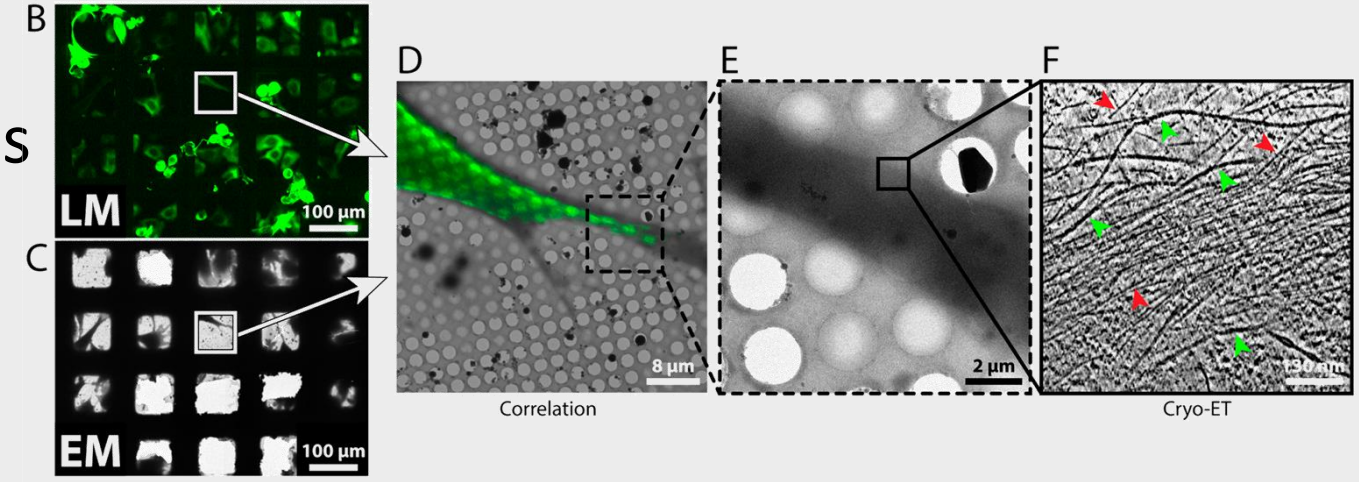


- Small cells



Farley et. al., 2016

- Thin cell edges

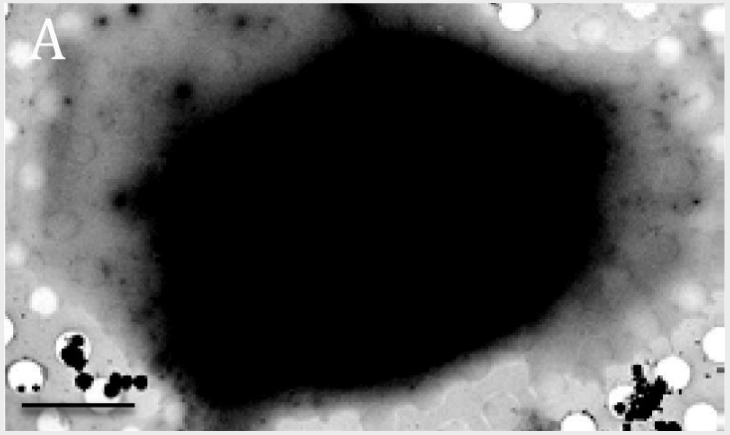


Weber et. al., 2019

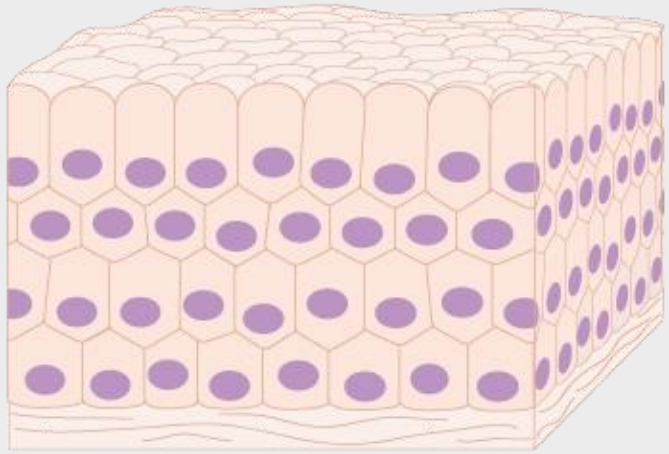
>> Plunge freezing

Overview – Sample freezing and prep

- Thicker cells or tissues



Thompson et. al., 2016



Cancer Research UK

>> High-pressure freezing (HPF)

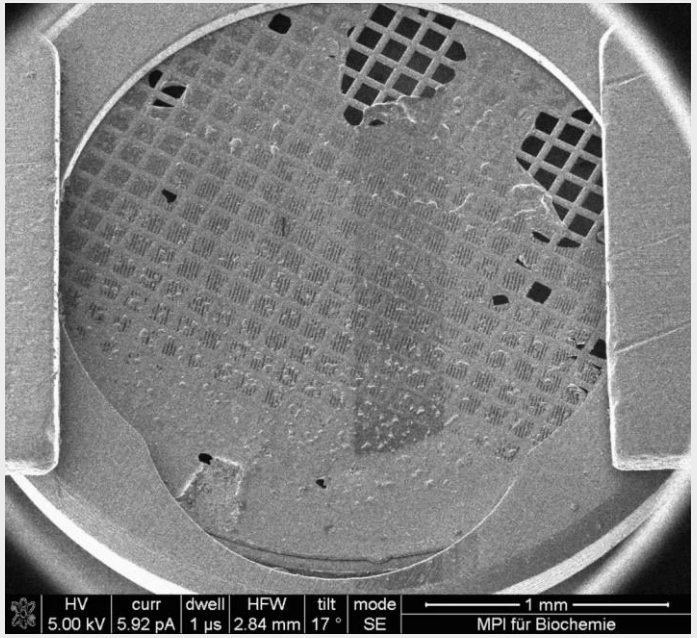
Overview – Sample freezing and prep

- For thinner samples, you can:
  - Pipette onto grid
  - Grow cells on grid
  - Micropattern cells on grid

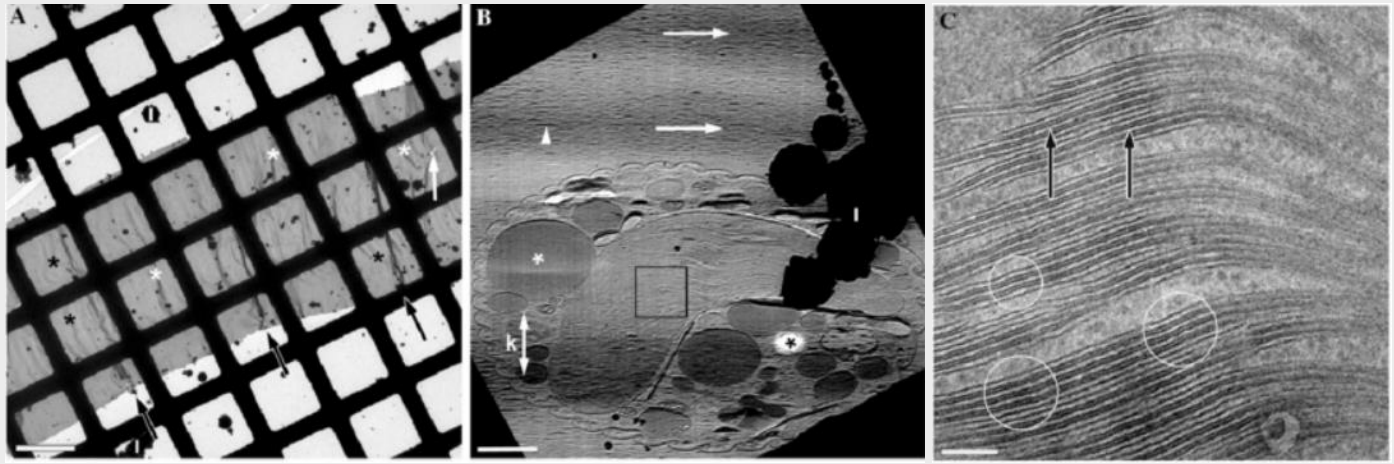
- For thicker samples, you can:
  - HPF directly
  - Tissue laser microdissection at room-temperature before HPF

Overview – Sample thinning methods

- Two options for thinning thick samples:
  1. Cryo-FIB-milling
     - Surfaces will be damaged 20-50 nm
  2. Cryo-sectioning
     - May create large-scale deformations



Baumeister et al., MPI



Al-amoudi et al., 2006

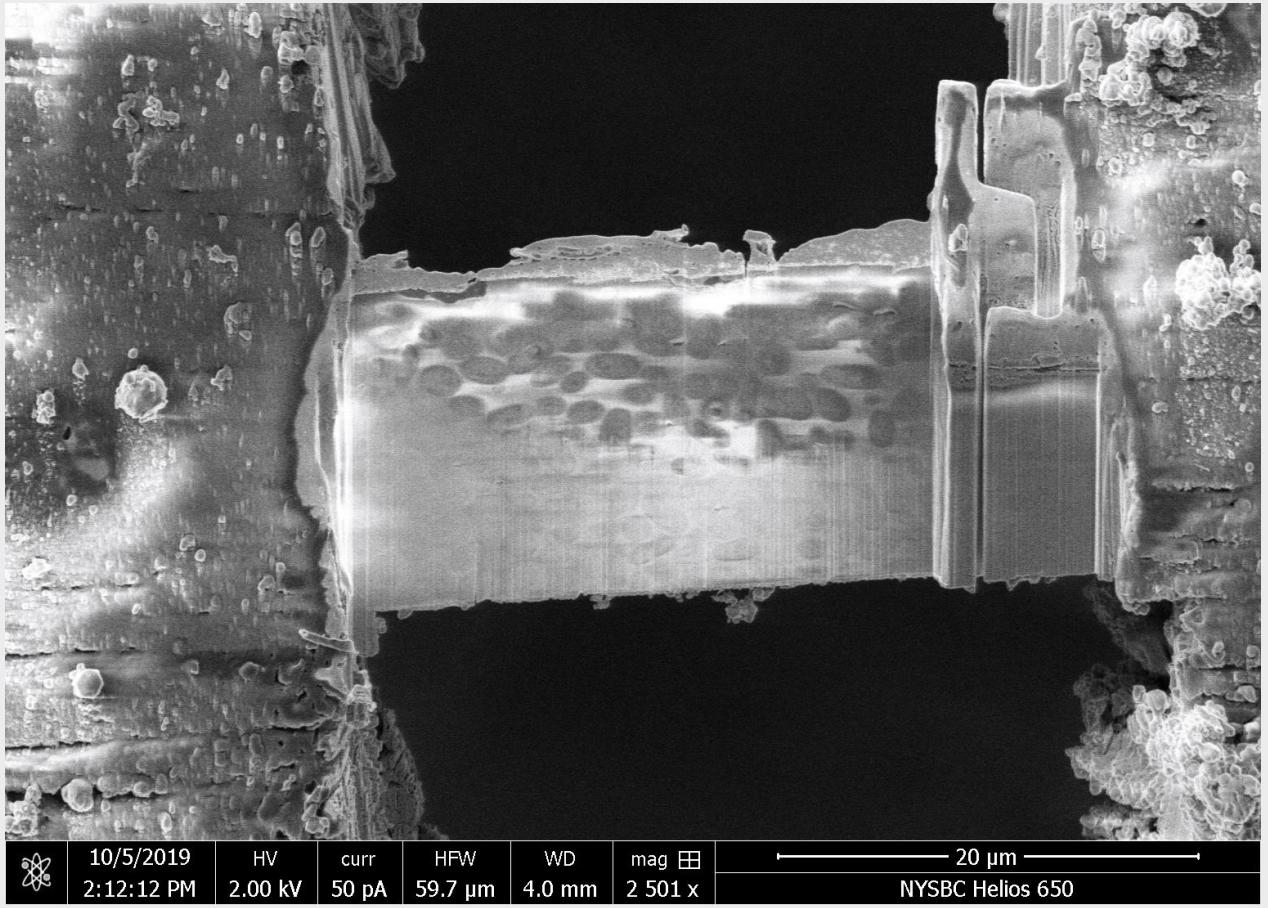
Overview – Sample thinning methods

- Sample prep methods can be combined, e.g.

  - Tissue microdissection 100x100x100 µm
  > HPF
  > Cryo-sectioning
  > cryo-glue on grid
  > cryo-FIB-milling

Overview – Sample thinning methods

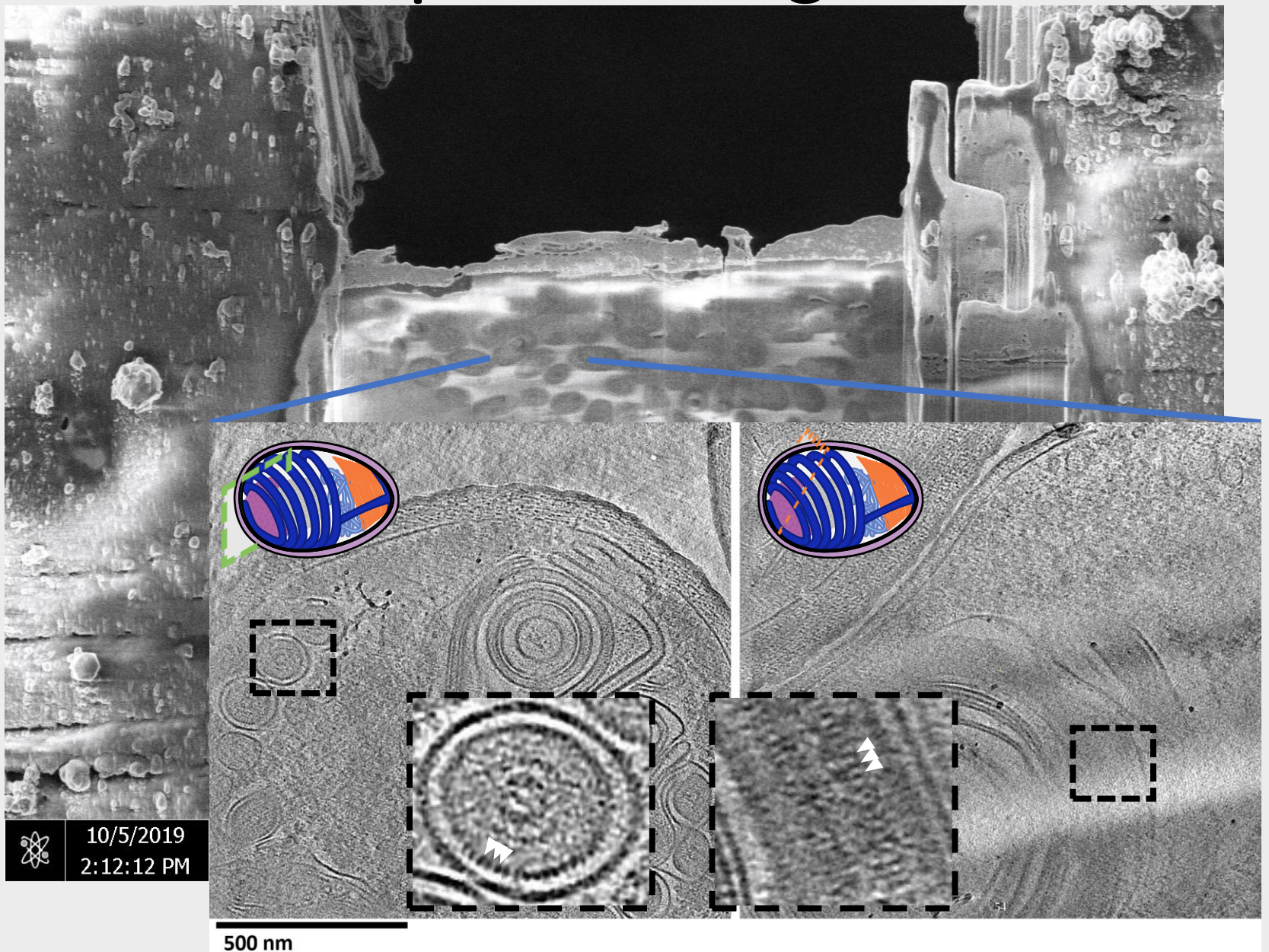
- In our experience, most samples can be **waffle milled**



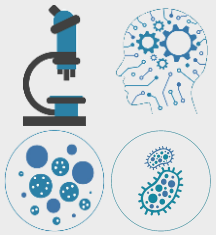
Waffle method

Kelley et al., 2020

Overview – Sample thinning methods



Kelley et al., 2020

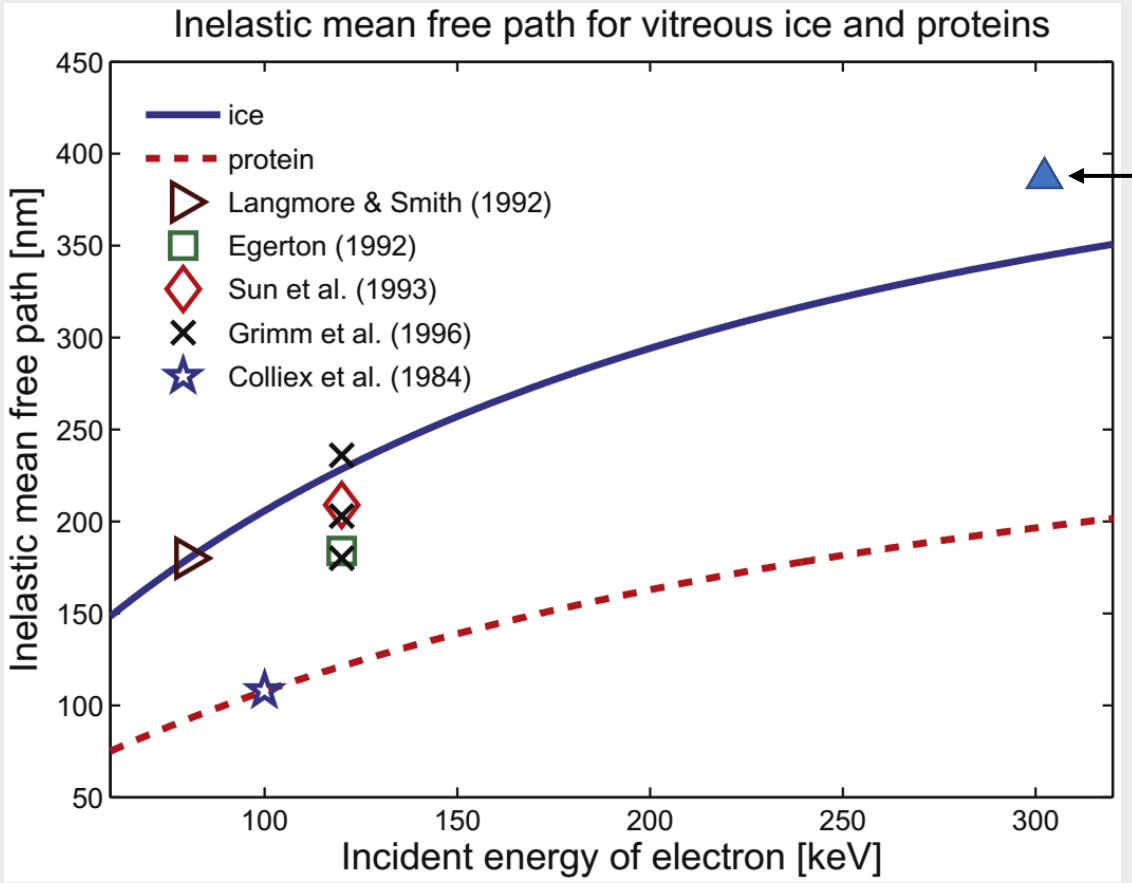


CryoET Limitations

Overview – Limitations

Limitation: Specimen/Ice **thickness**

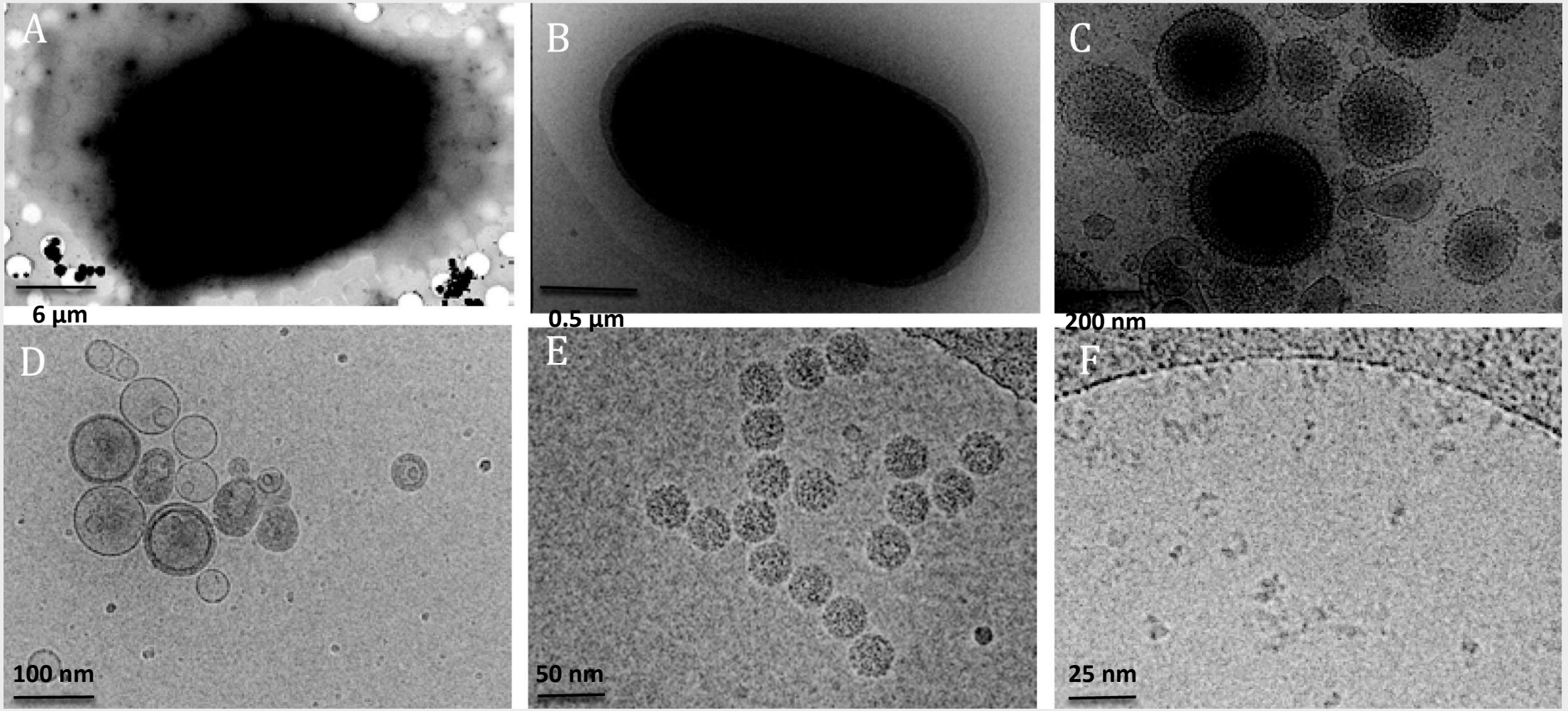
Vulović, 2013



William J. Rice, NYSBC, 2017
300 keV Krios

Overview – Limitations

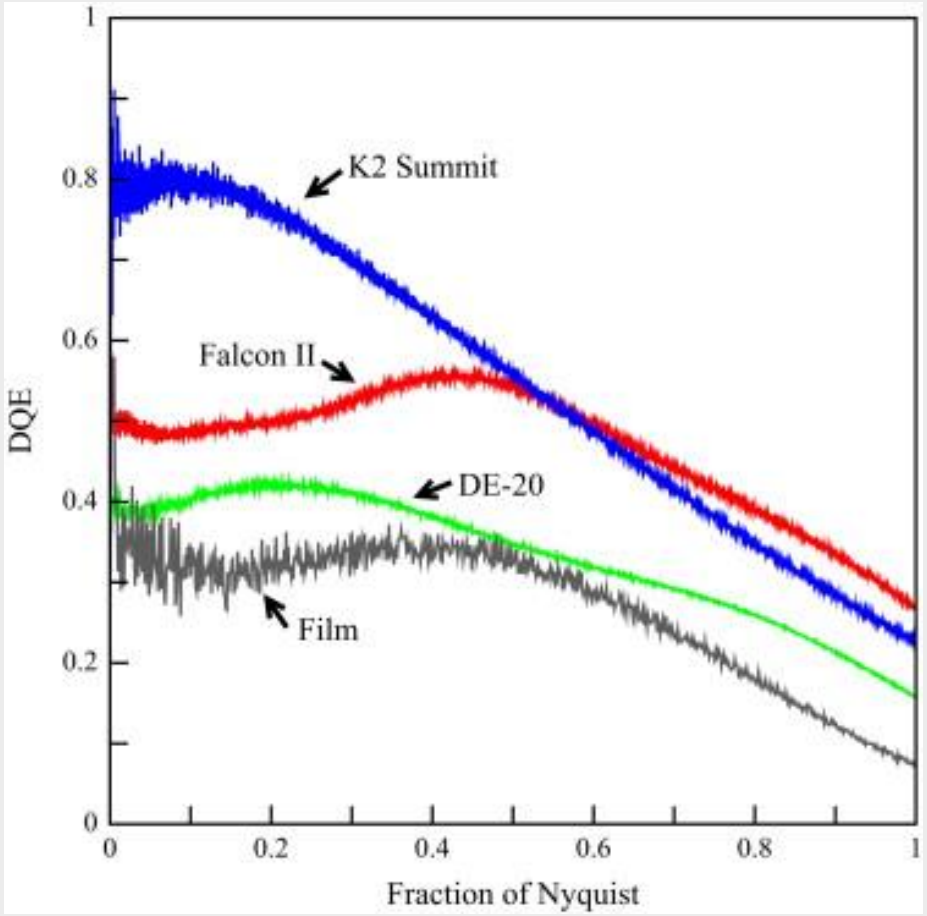
Limitation: Specimen/Ice **thickness**

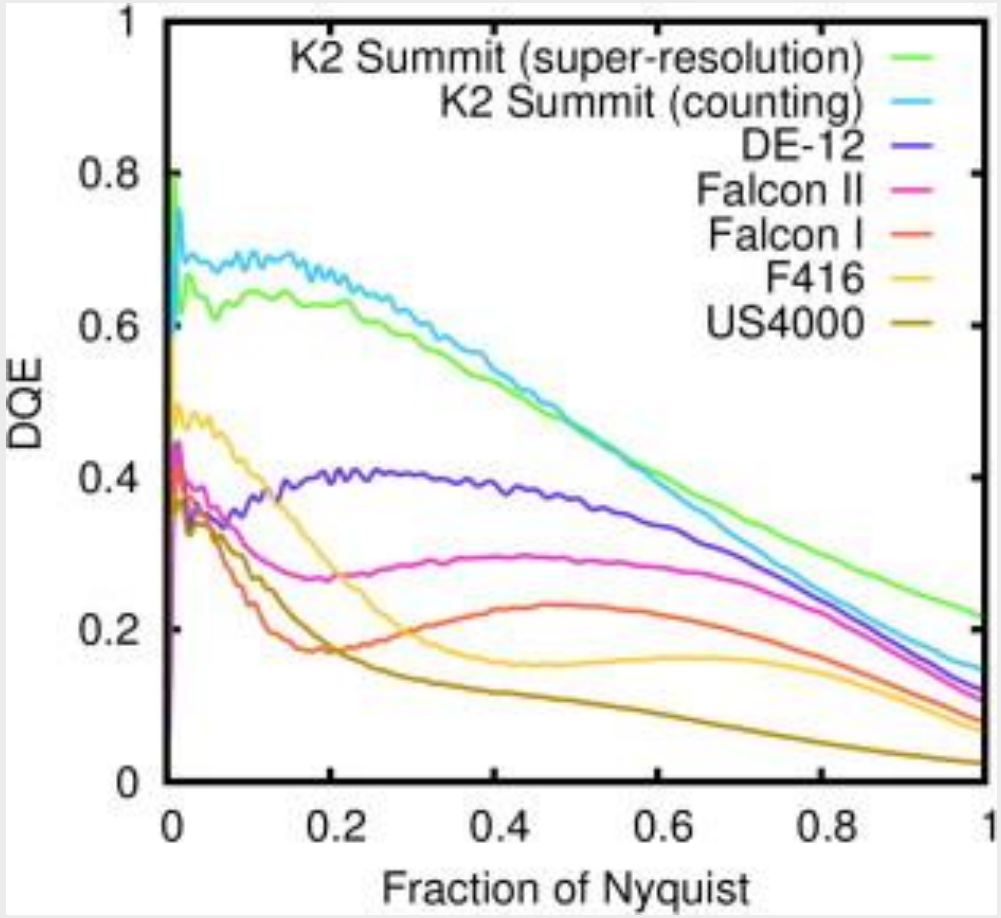


Thompson et. al., 2016

Overview – Limitations

Limitation: Camera fidelity



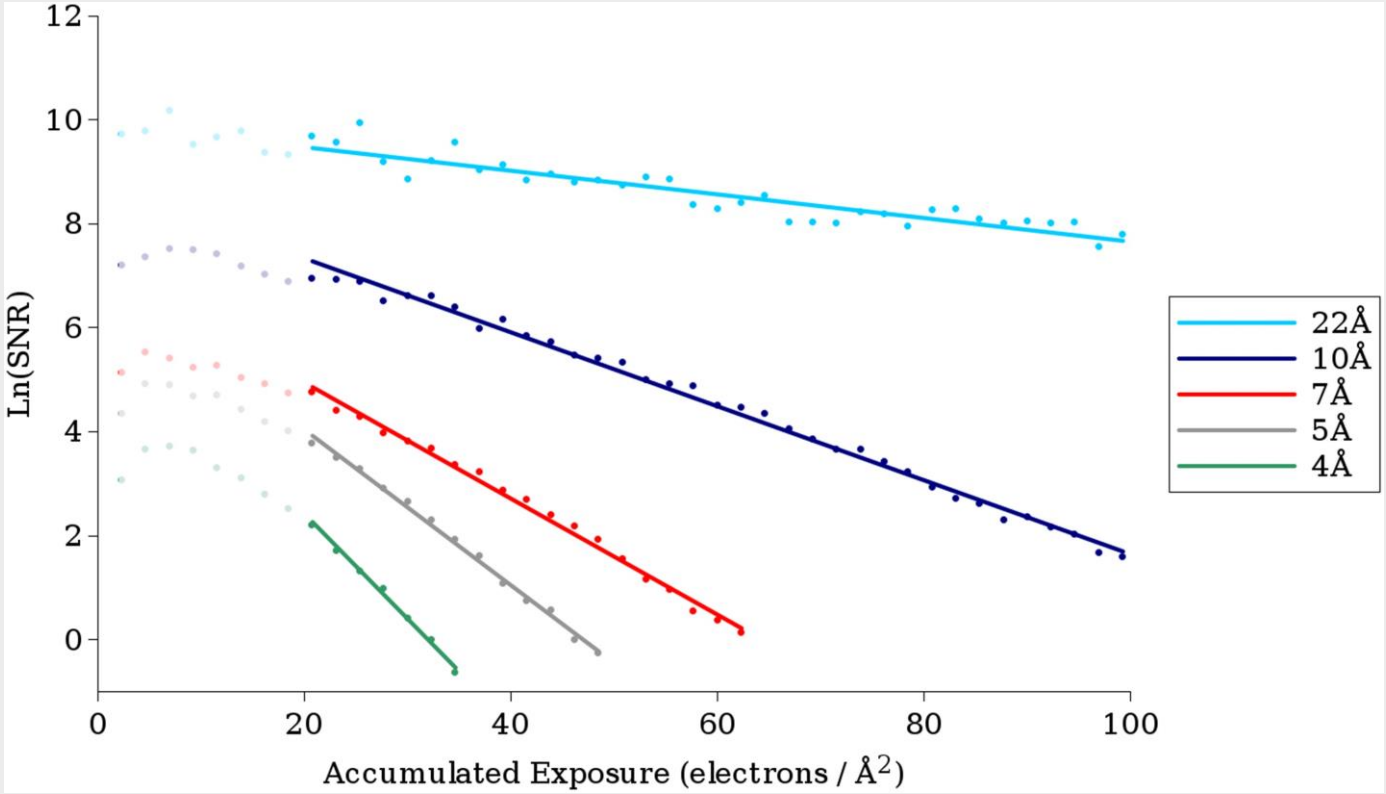


McMullin, 2014 &
Ruskin, 2013

Overview – Limitations

Limitation: **Electron damage** of the specimen

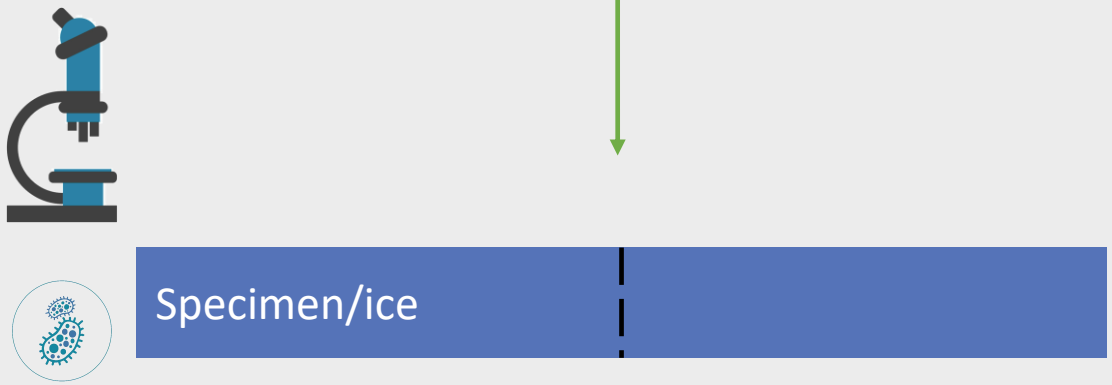
- **High resolution information is lost first**.



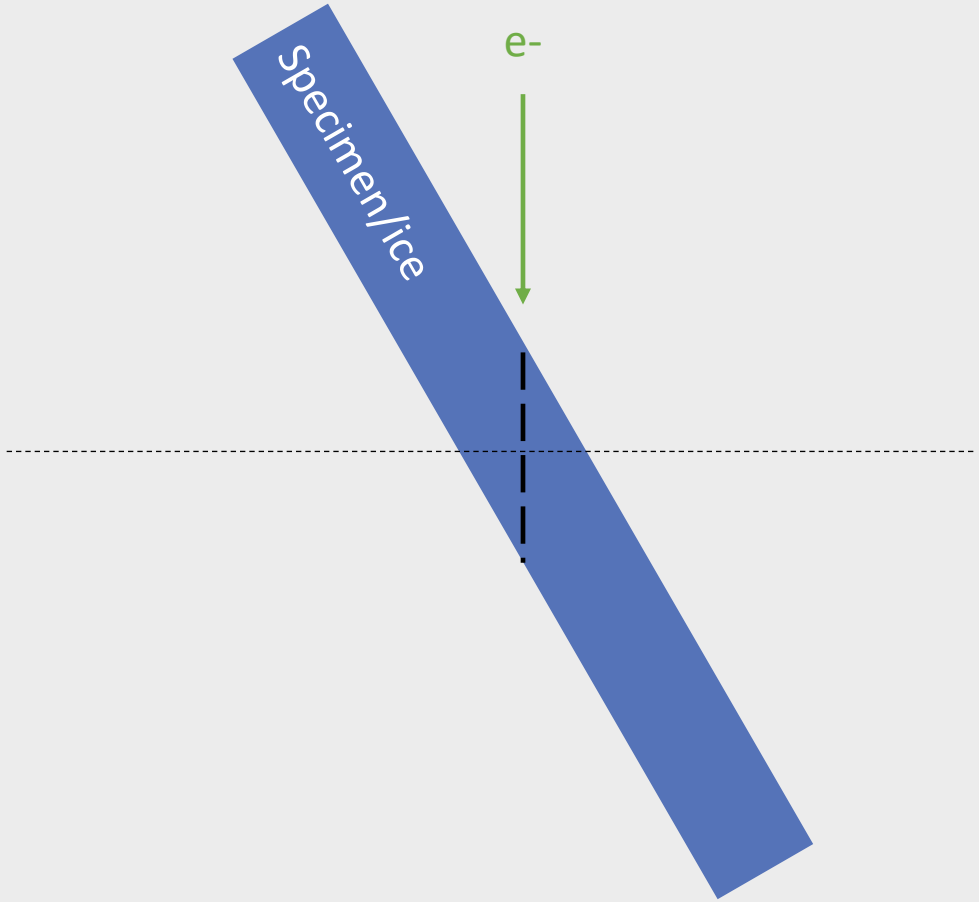
Grant & Grigorieff, 2015

Solution:
Remove damaged information from image frames

Grid tilting increases thickness

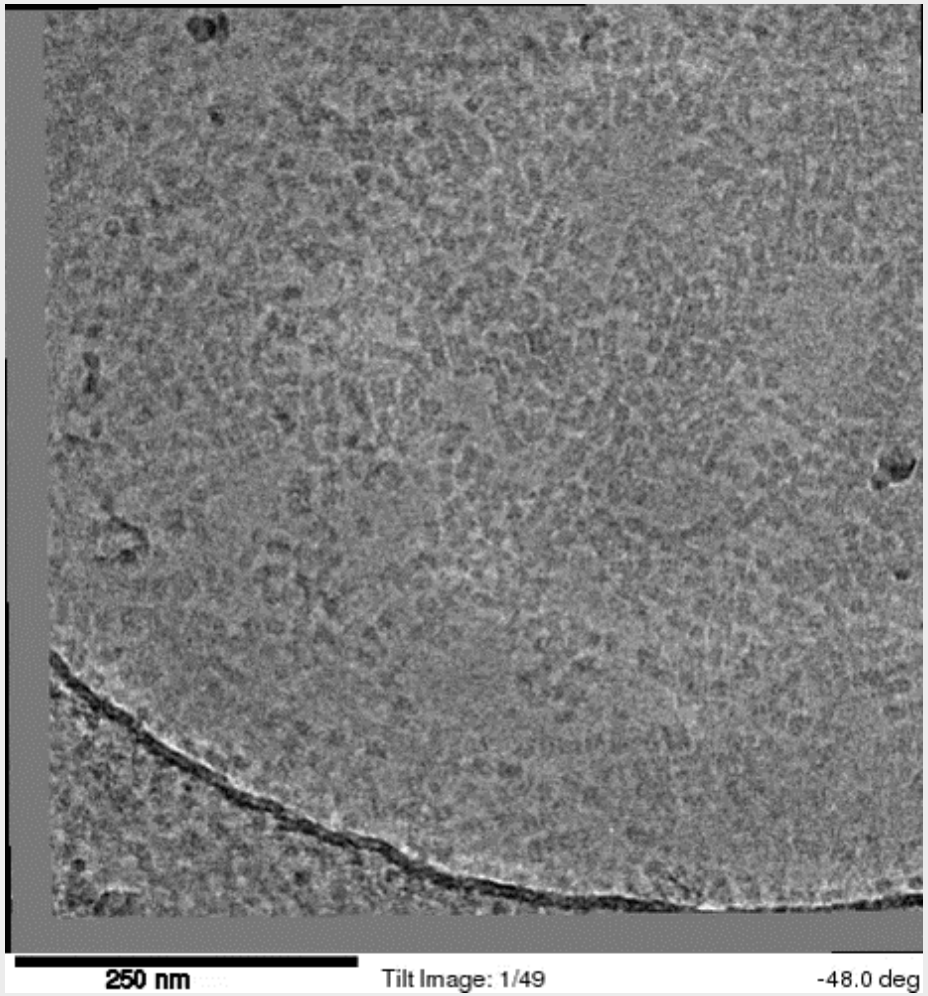


untilted grid



grid tilted 60° = 2x thickness

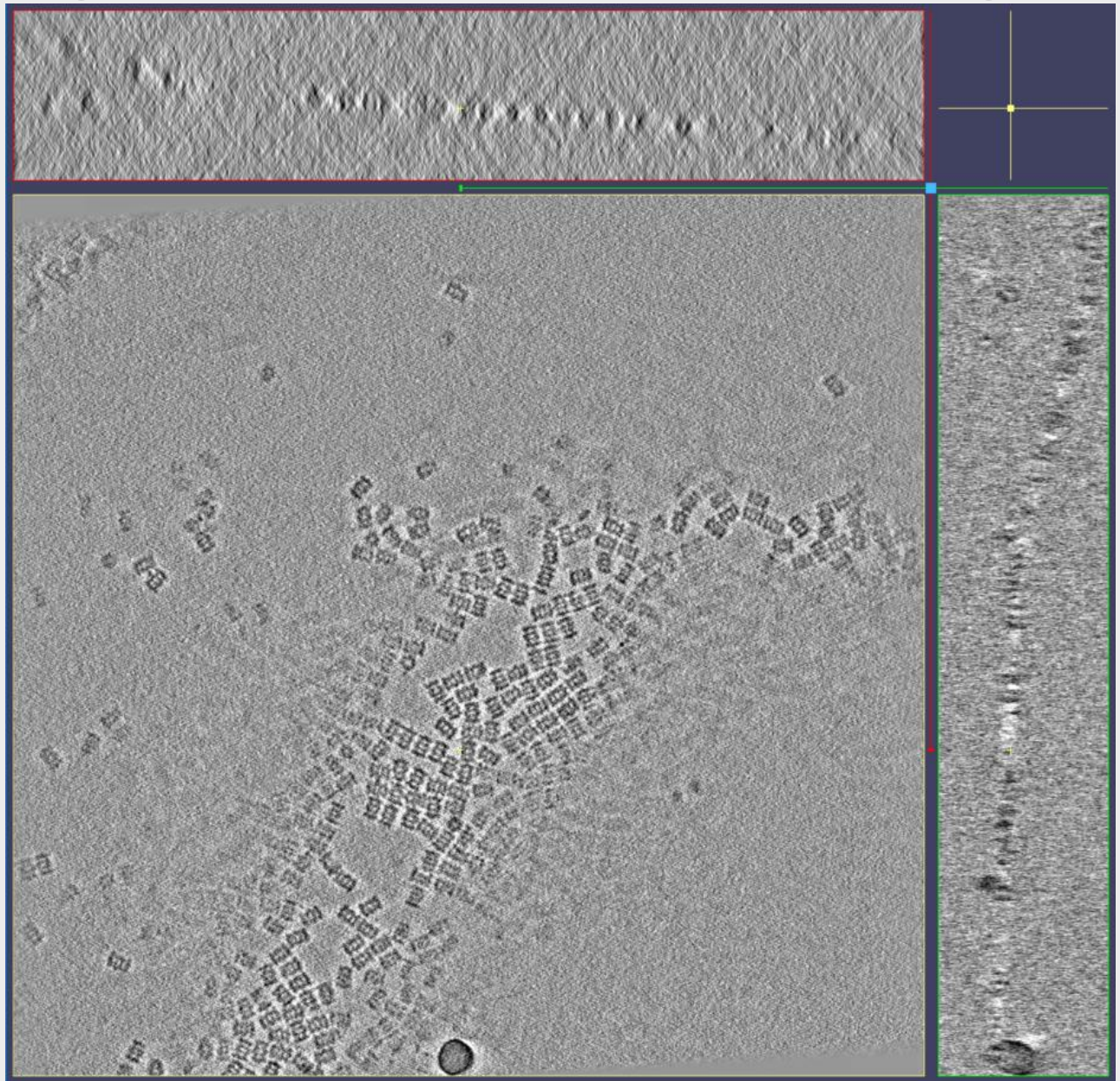
Grid tilting thickness increase limits tilting



Noble et al., 2018

- Phase plate tilt-series of T20S Proteasome
- Tilt axis is **horizontal**

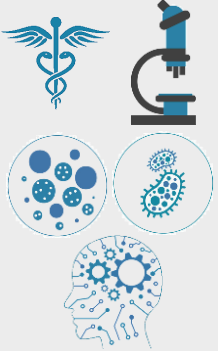
Grid tilting limit results in missing information



Phase plate tilt-series of T20S Proteasome.

Tilt axis is **vertical**

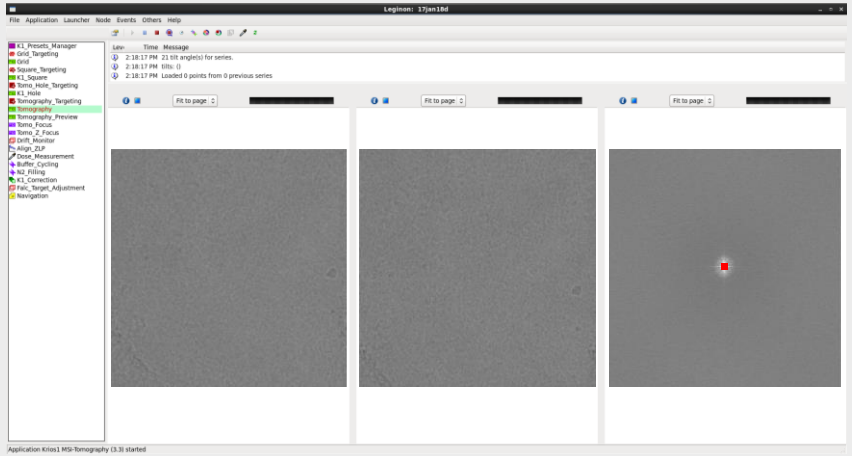
Noble et al., 2018

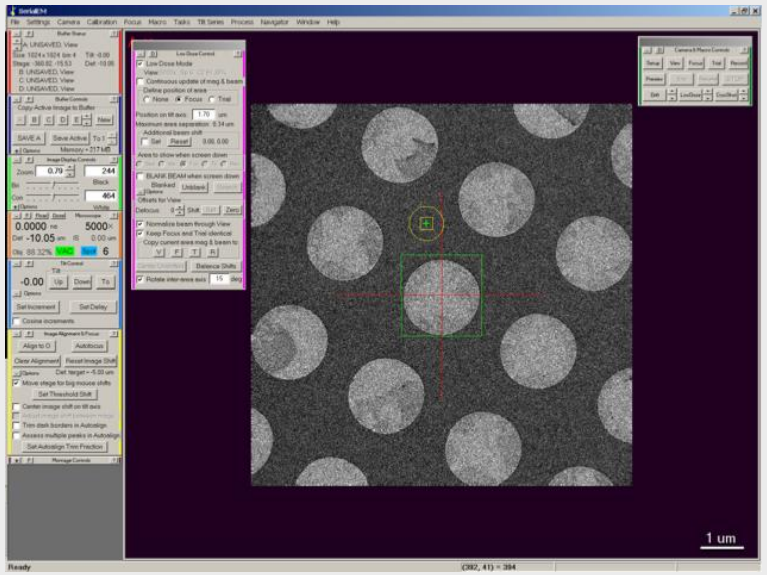


Tilt-series collection

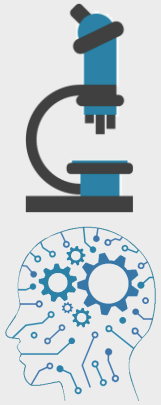
Tilt-series collection software

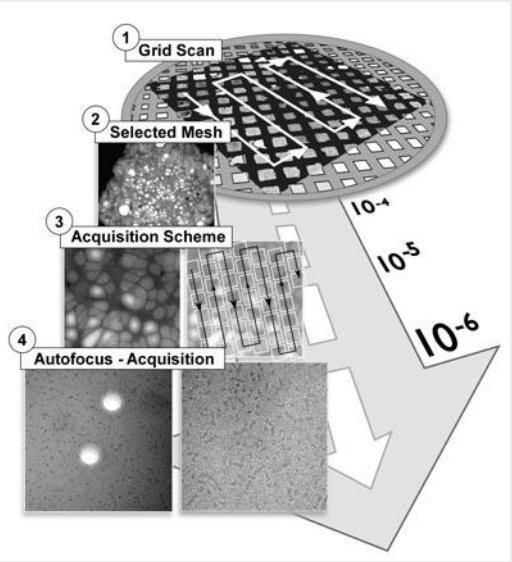
Leginon





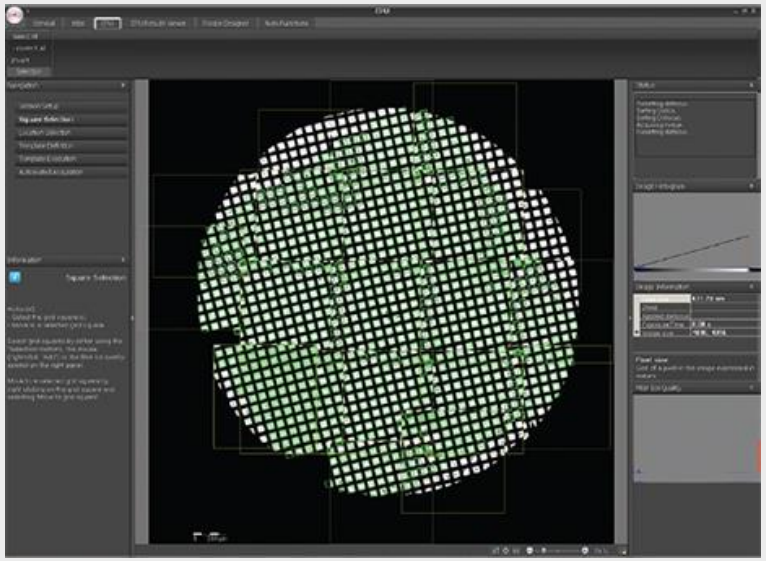
SerialEM

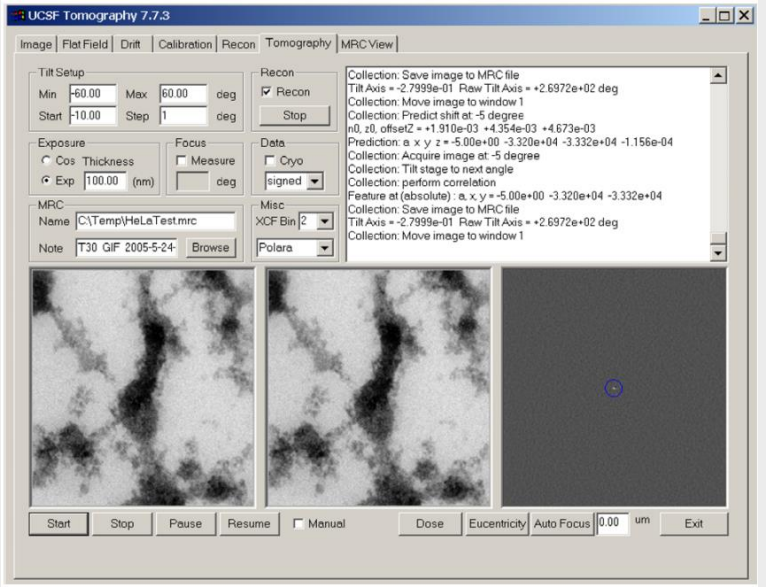




TOM Toolbox

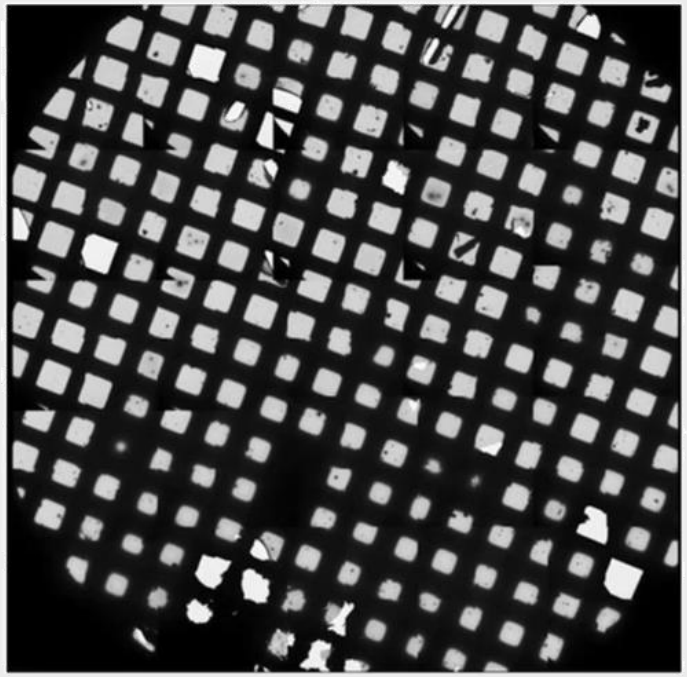
EPU

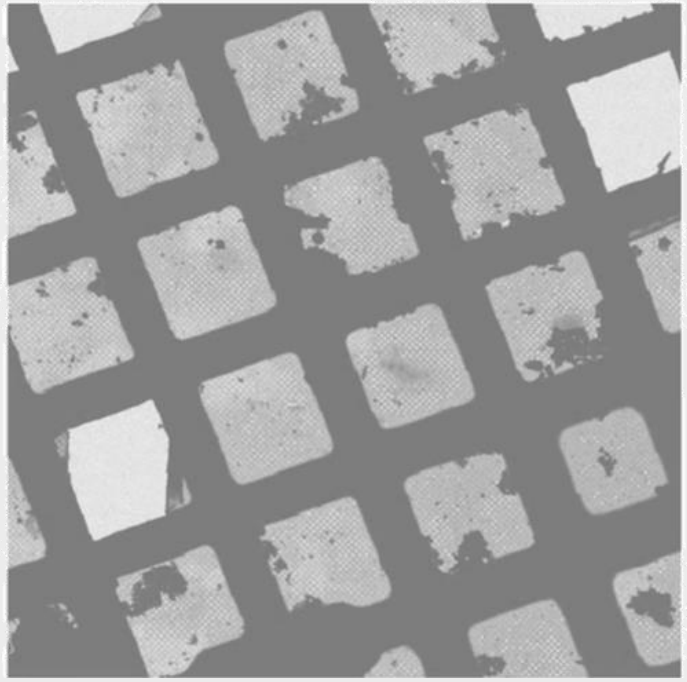


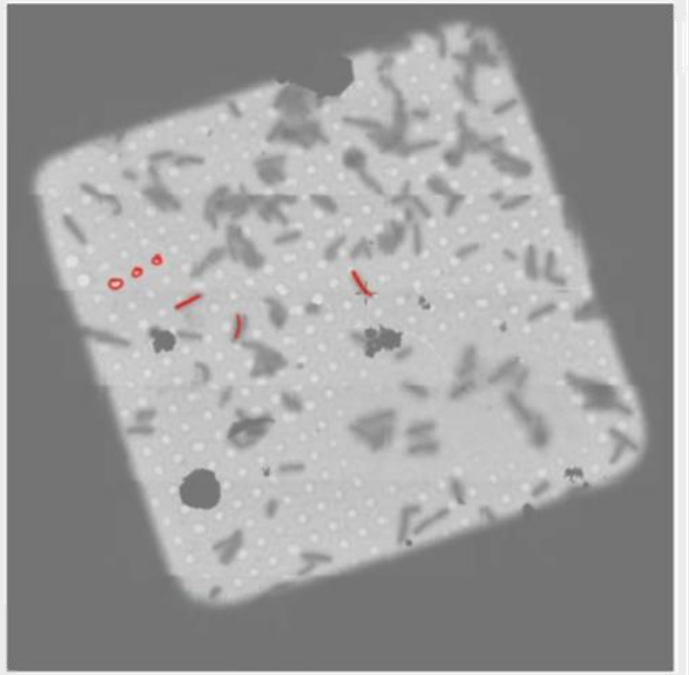


UCSF Tomography

Automated tilt-series collection







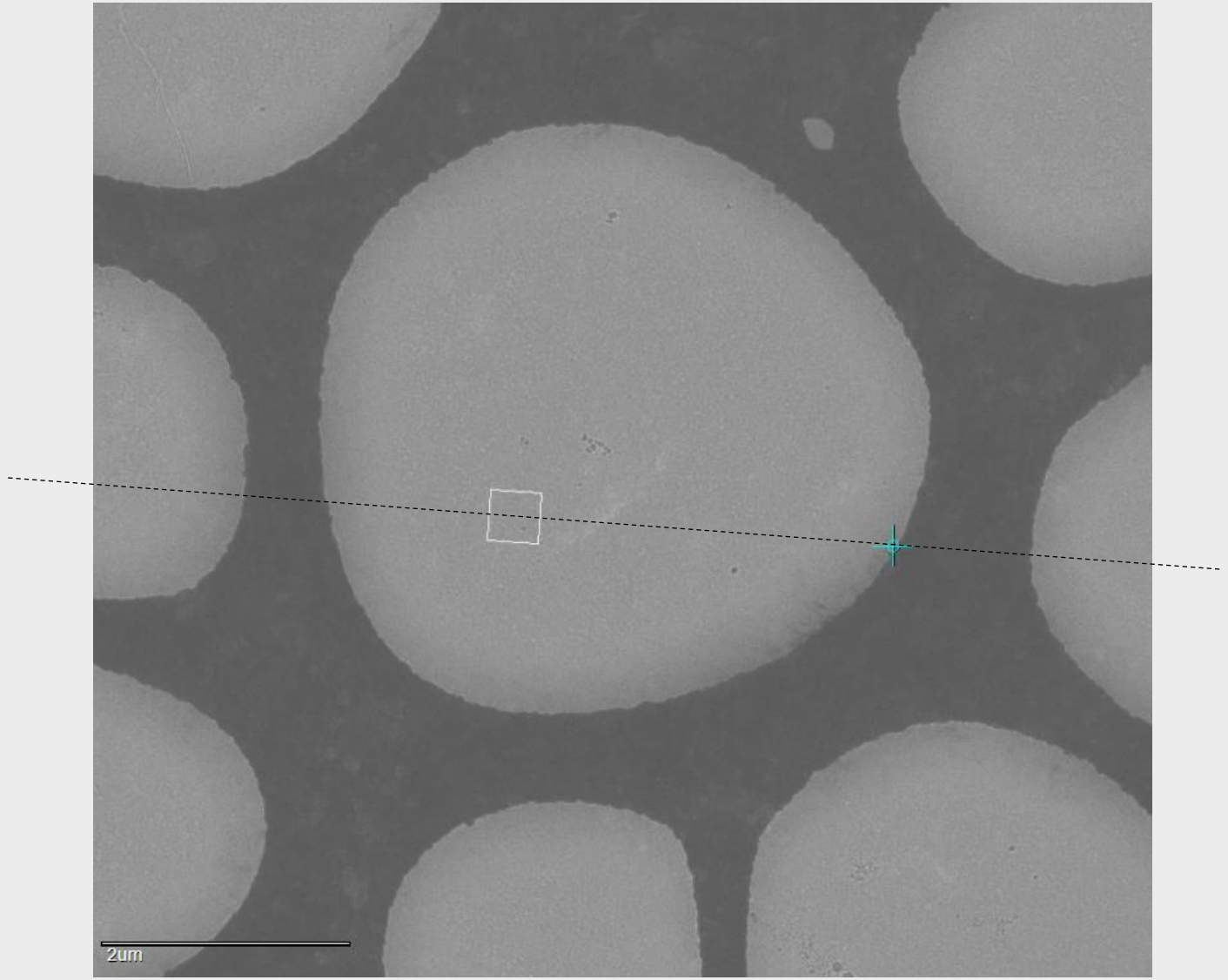
Automated tilt-series collection is currently **routine**

- From an atlas, select multiple squares, and from each square select holes,
- For each hole place an exposure target along with one or more focus targets,
- Set up dose, defocus range, tilt model, etc. appropriately,
- Collect!

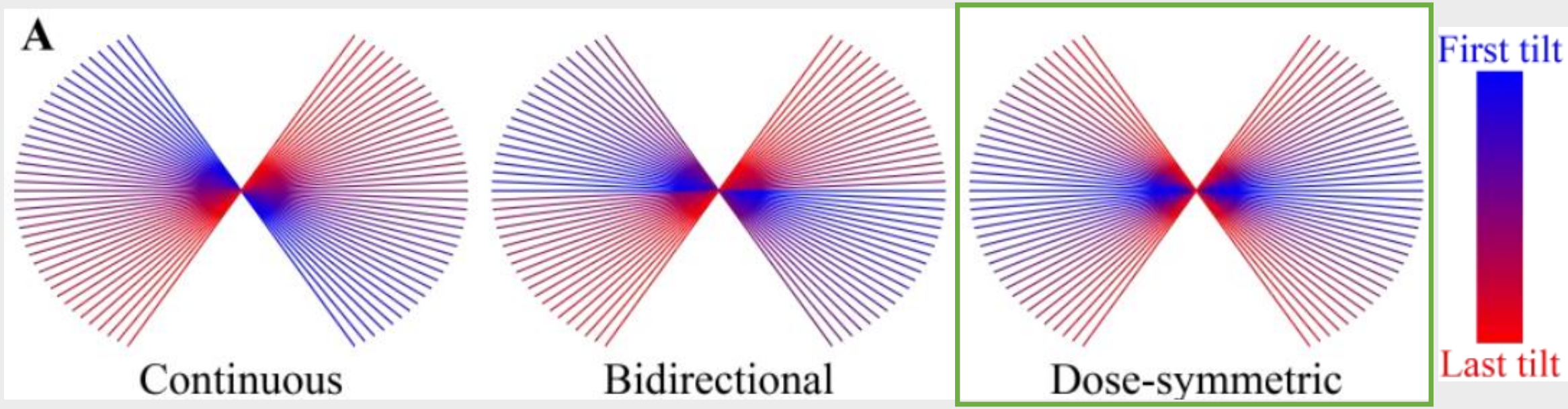
Automated tilt-series collection

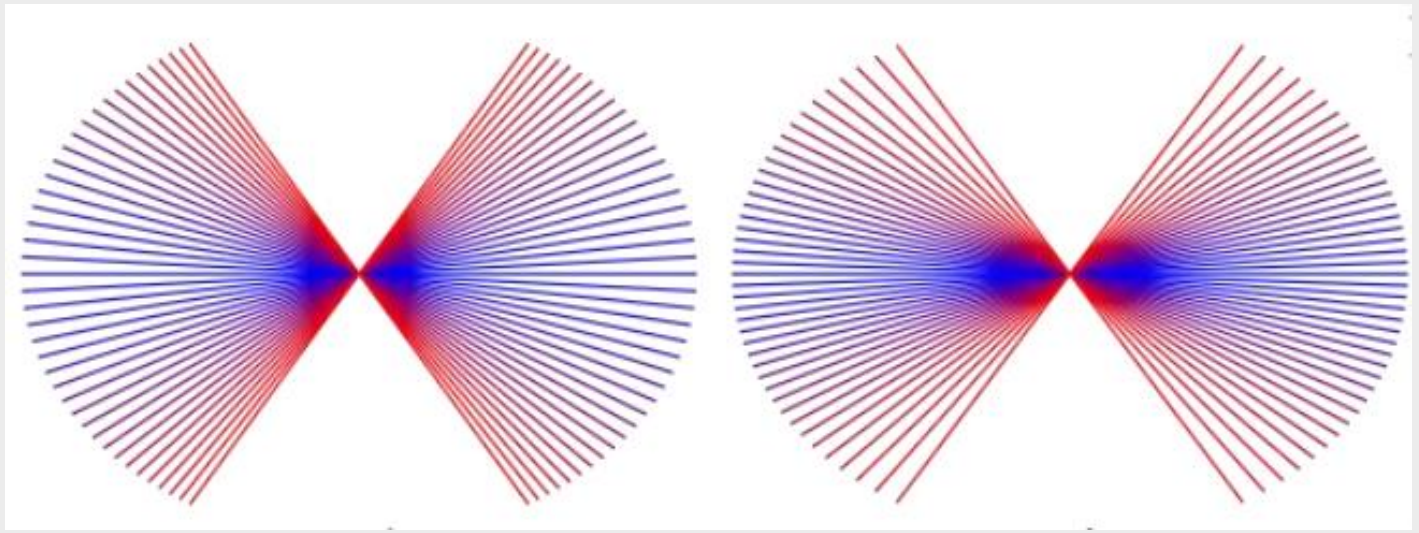
Focus on the tilt axis!

- You want to minimize the amount of tracking error
  - ➢ Tilting should not change the x,y,z target location
- This is called getting **eucentric height**.



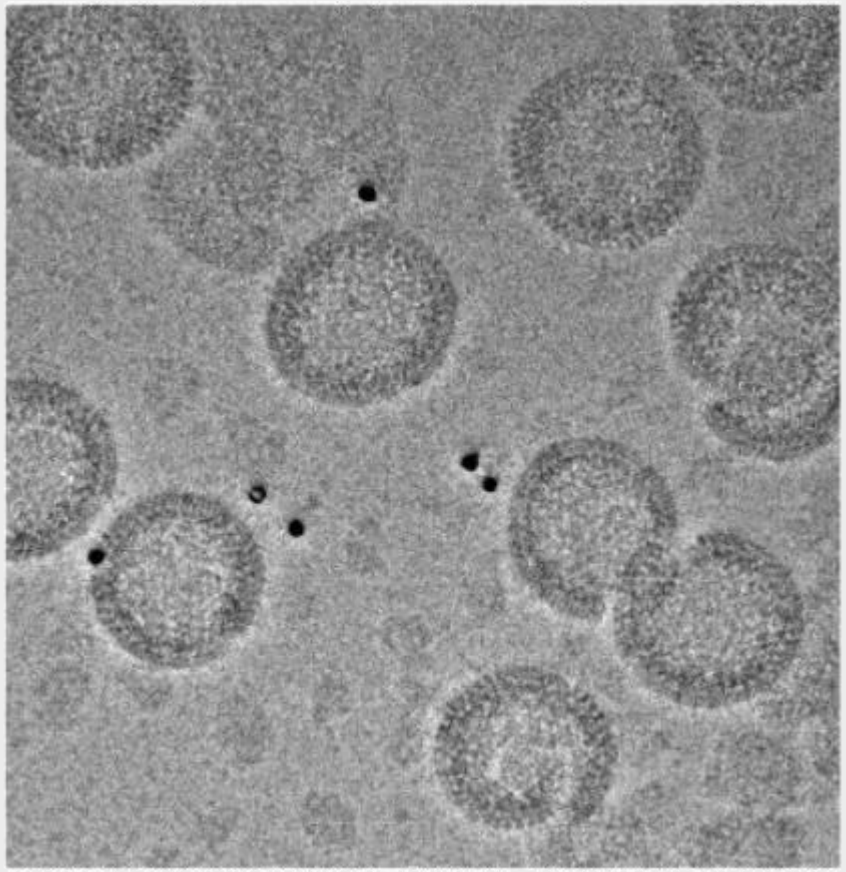
Some Collection Schemes

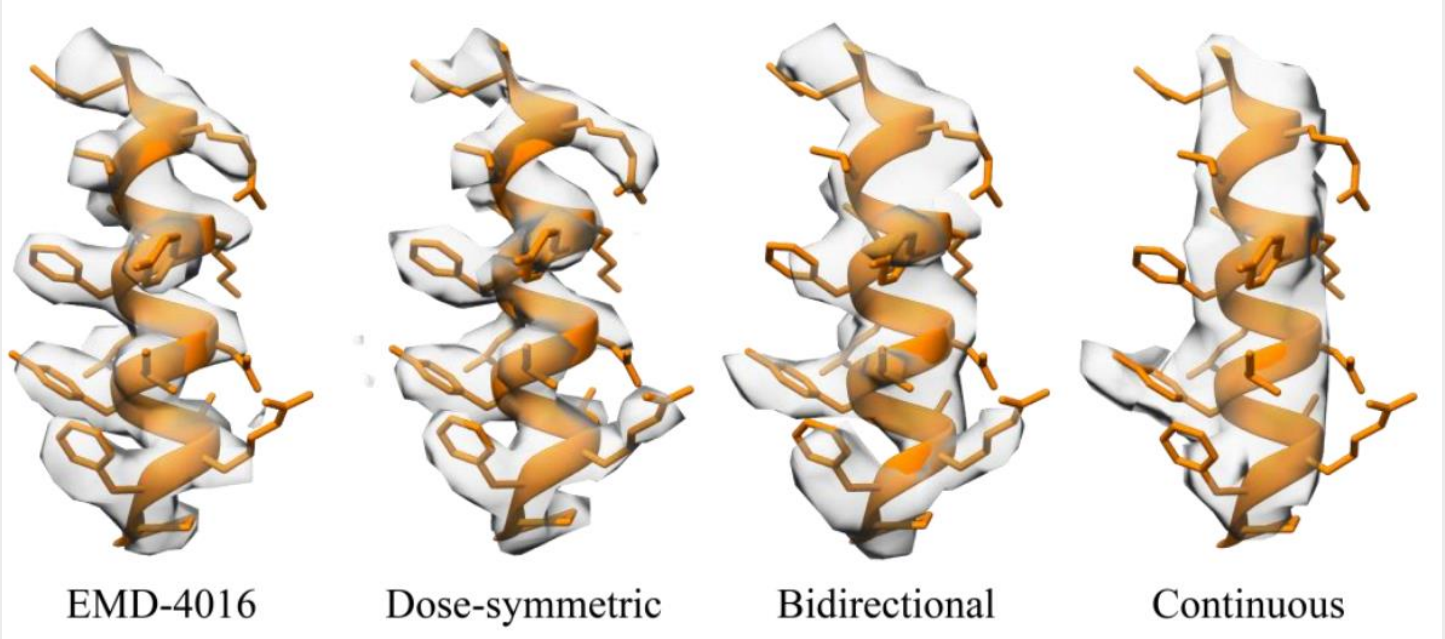




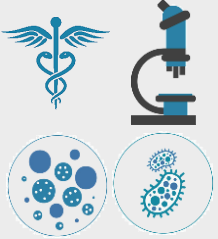
Turoňová et al., 2019 & 2020

Some Collection Schemes on an *Isotropic* Sample





Turoňová et al., 2019



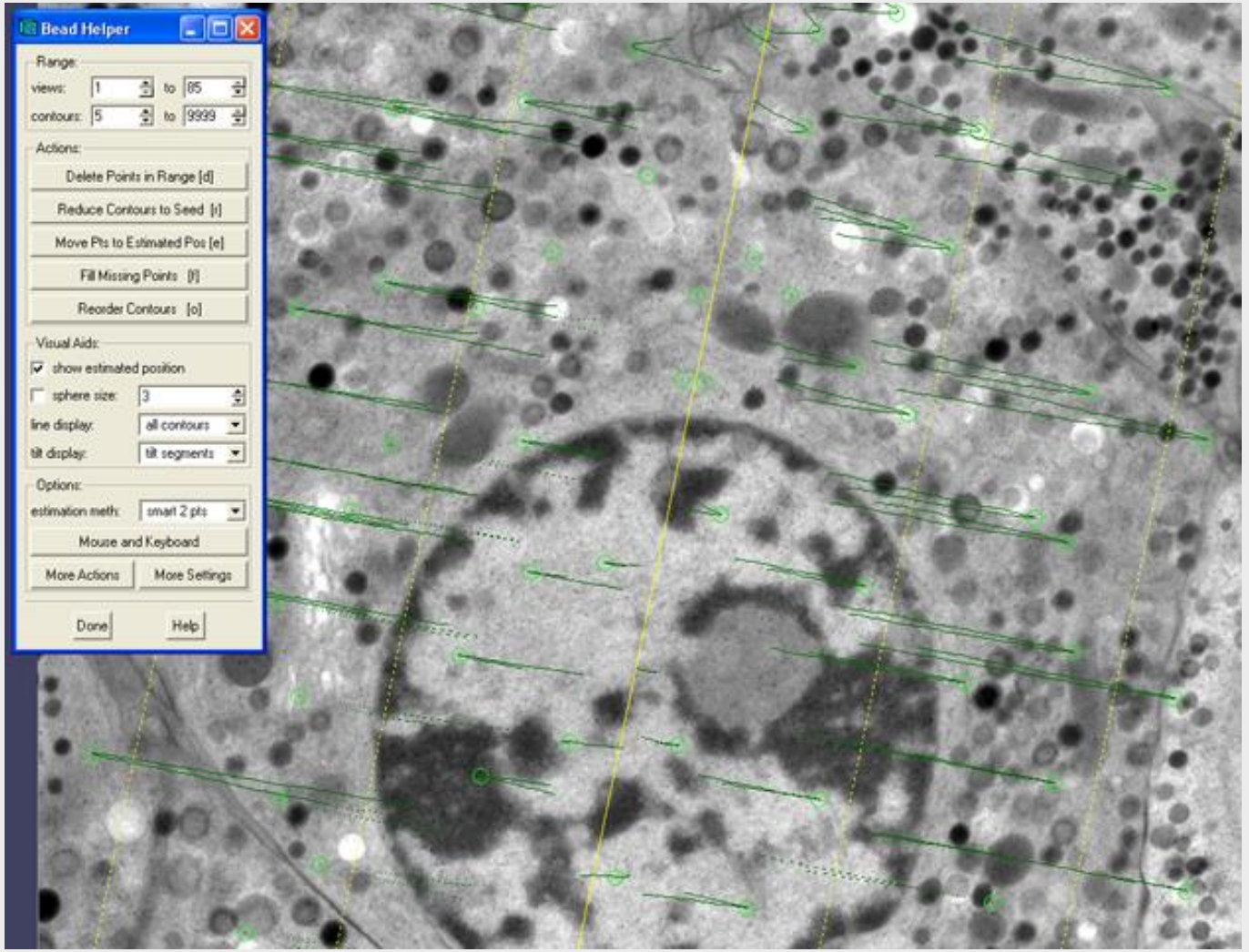
Tilt-series alignment

Tilt-series alignment

- **Software:**
  - AreTomo – **Fiducial-less** alignment with GPU
  - ETomo in IMOD – **Fiducial-based** alignment (also **patch tracking**)
  - Markerauto and AuTom – Automated **fiducial-based** alignment
  - Protomo – **Fiducial-less** alignment
  - Alignator – **Patch tracking** alignment, GPU-accelerated
  - Dynamo – **Fiducial-based** alignment
- **Must refine** most or all of the following:
  - Tilt image shifts, rotations, defocus changed, & magnification changes
  - Tilt axis location
  - Tilt angles

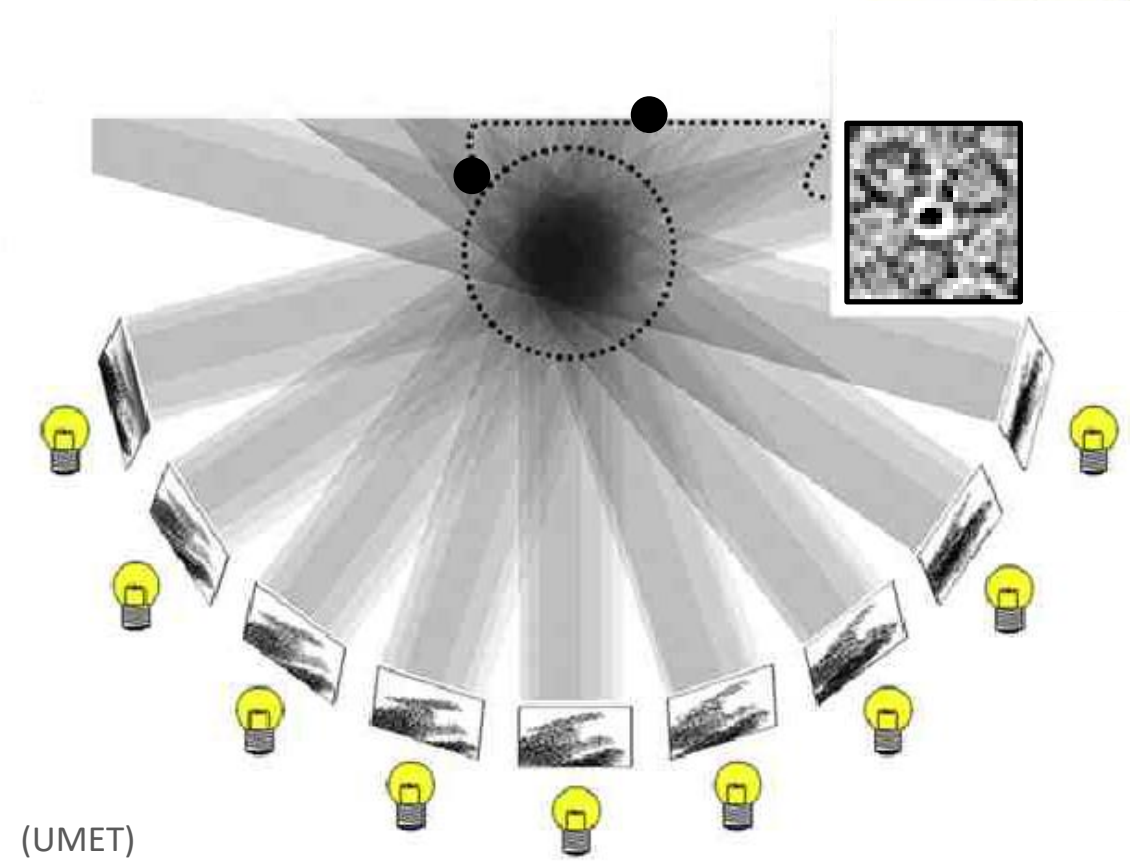
Fiducial-based tilt-series alignment

- Requires a **sufficient number of well-behaved gold beads**
- Semi-automated (IMOD, Dynamo) or automated (AuTom/markerauto, IMOD) processing



http://bio3d.colorado.edu

Fiducial-based tilt-series alignment issues

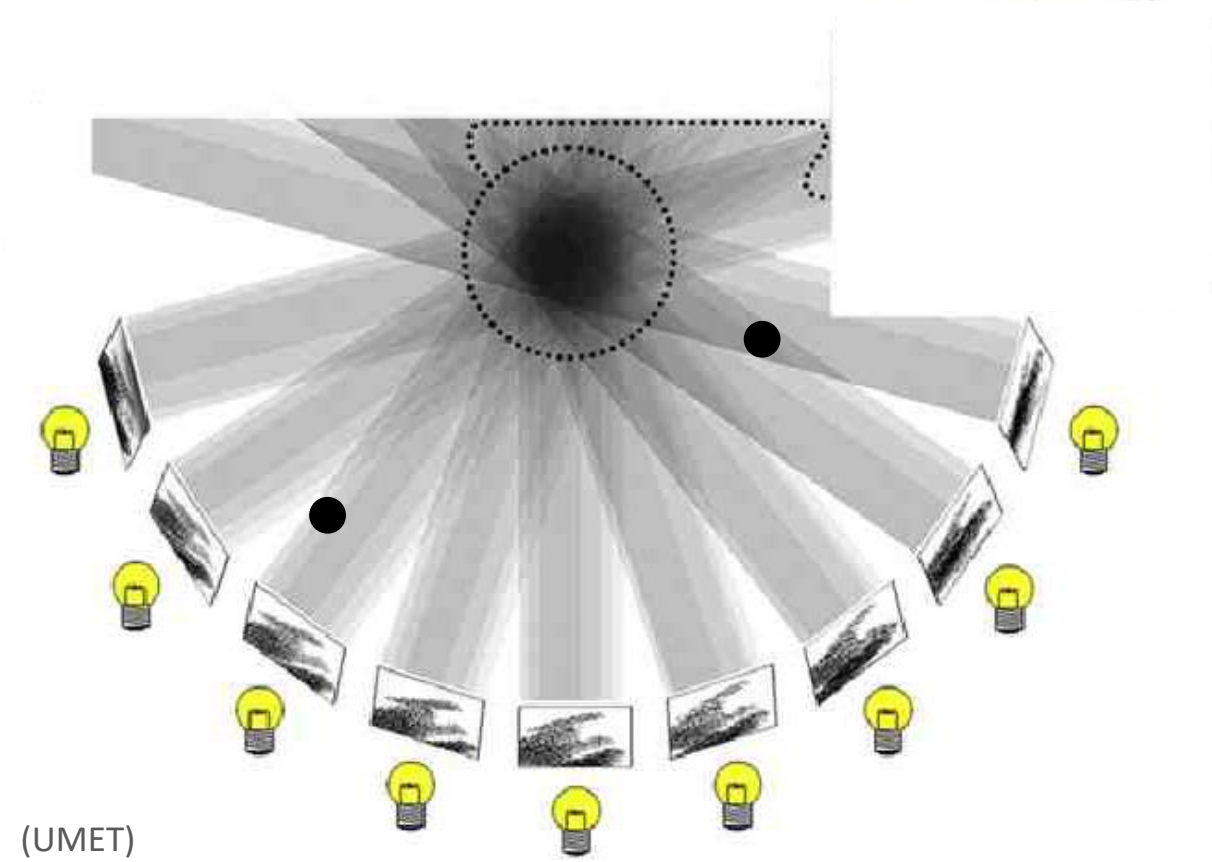


(UMET)

Nearby Fiducials Affect Signal and Contrast

- **Fiducial fringes** change the **power spectrum** of your reconstructed object.

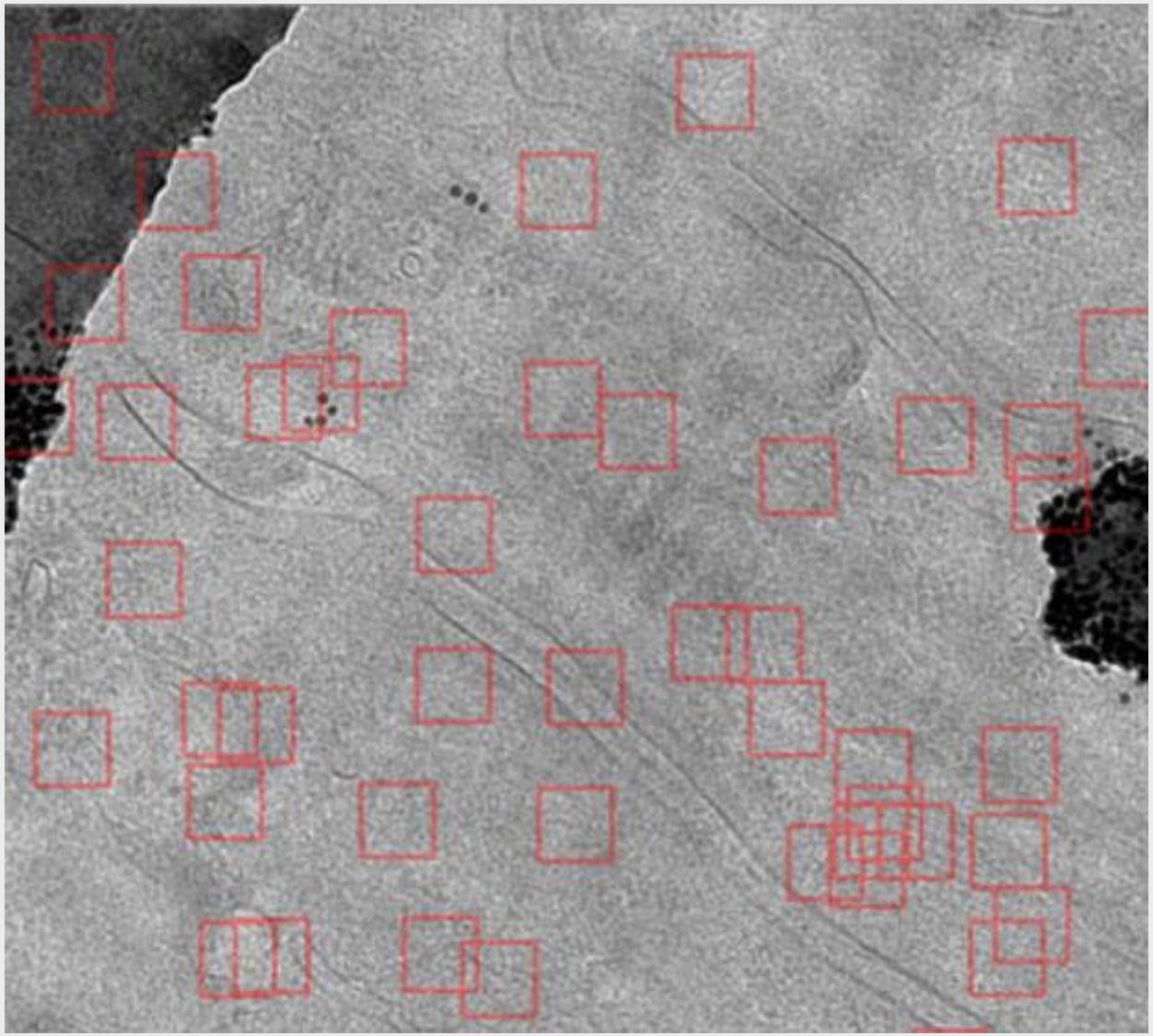
Fiducial-based tilt-series alignment issues



Fiducials are in the reconstruction,
Even if You Can't See Them!

- **Distant fiducials** can be in the **projection direction** of your extracted object of interest.
- Erasing fiducials isn't perfect.

Patch tracking tilt-series alignment



Castaño-Díez, 2010

Identify featureful objects with contrast in all tilt images and track them.

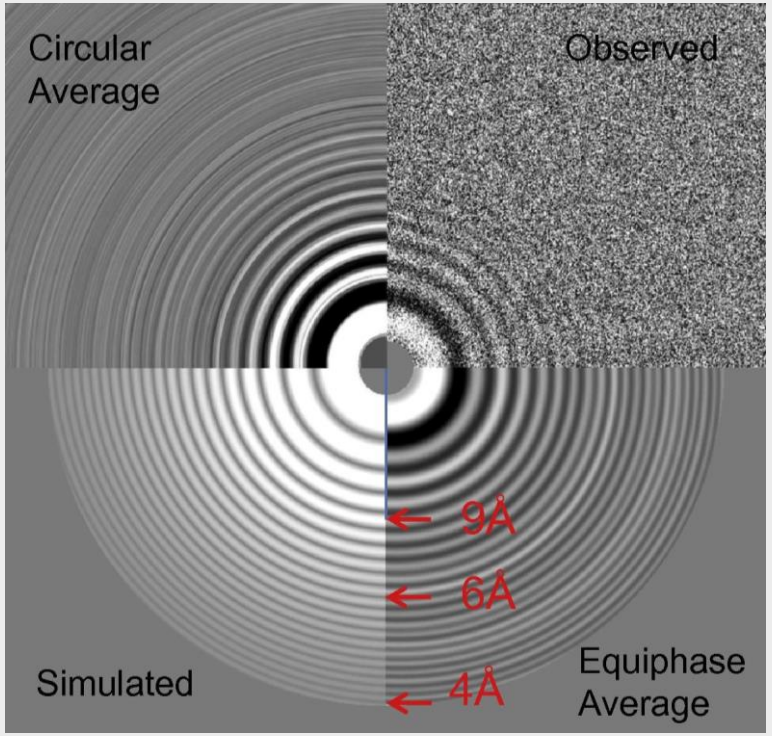
- Semi-automated (IMOD, Alignator)

Defocus estimation

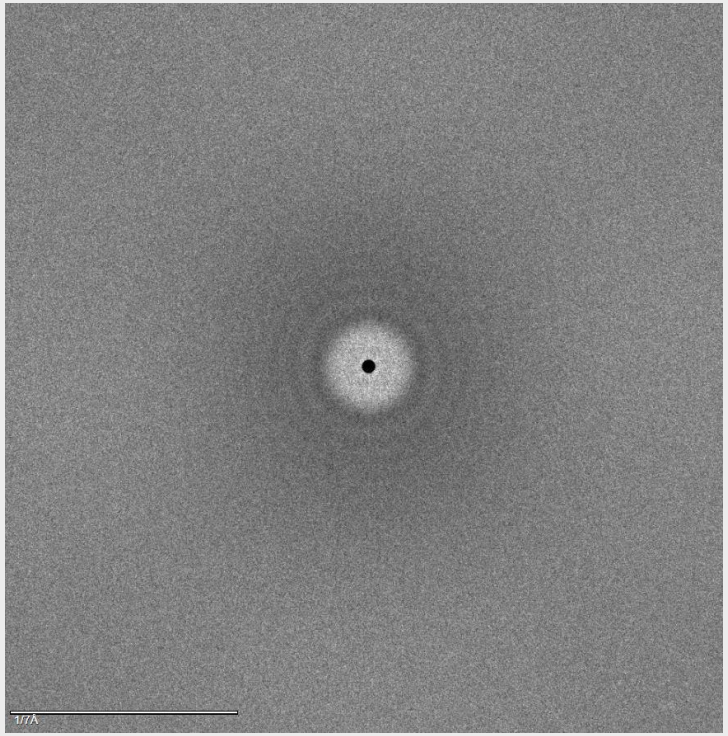
Goal: Find the **height of your objects** of interest to correct for microscope aberrations (CTF)

Problem: **Low** per-image **SNR** and potential poor tracking

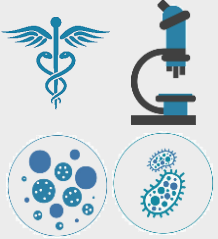
Zhang, 2016



High dose single particle image



3 e^{-}/Å^2 single particle tilt image



CTF estimation and correction for tilt-series or tomograms

Defocus estimation methods

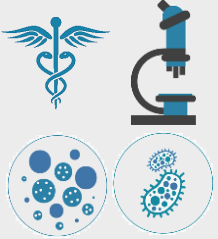
Current best workflow:

- First estimate **per-image** defocus and account for tilts (CTFFIND4, GCTF, etc.)
- Then after particle picking, perform **local CTF estimation** (M, Relion)

CTF correction methods

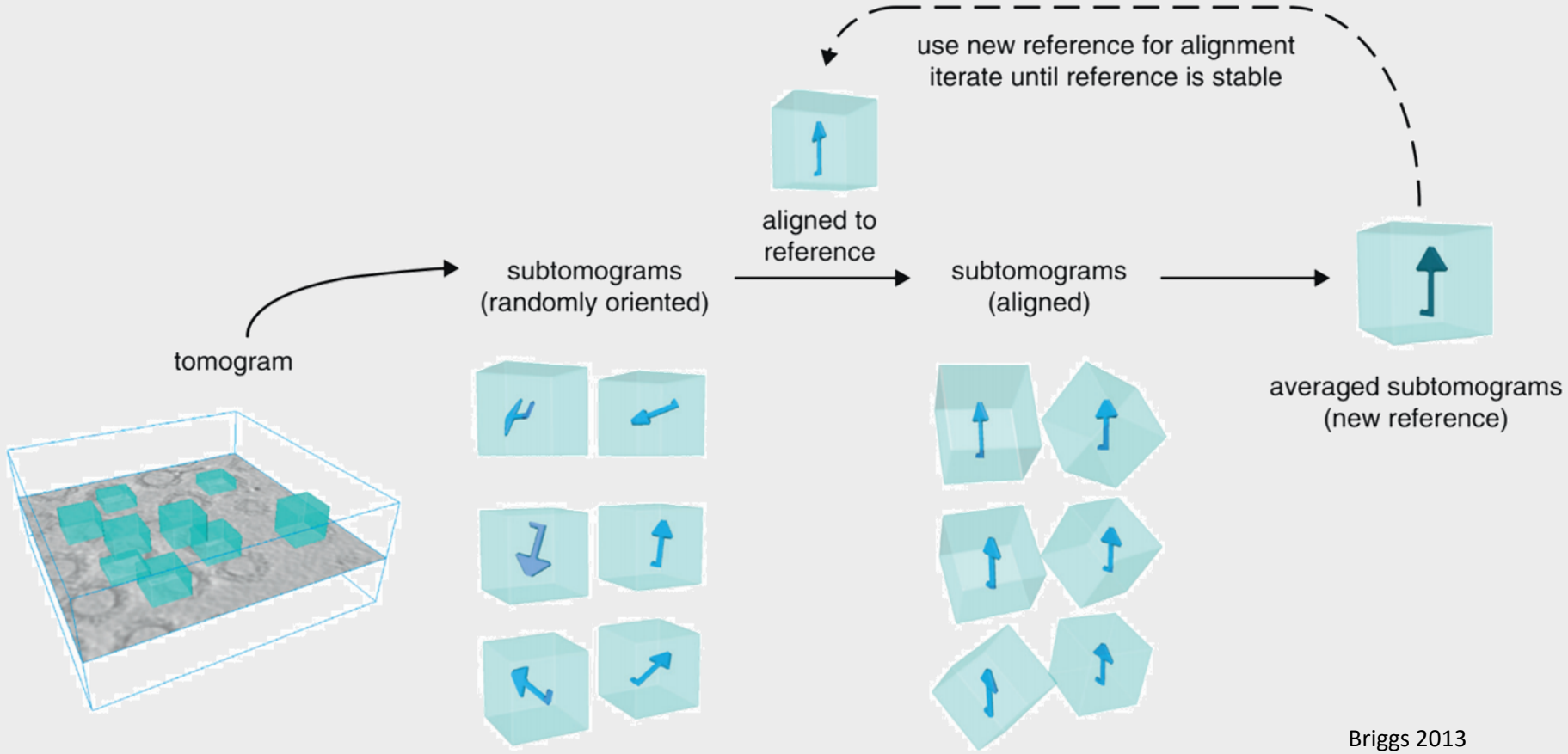
Current best workflow:

- **Per-particle** correction (M, Relion, EMAN2, EMClatity)
- Tomogram correction (NovaCTF)



Sub-tomogram processing

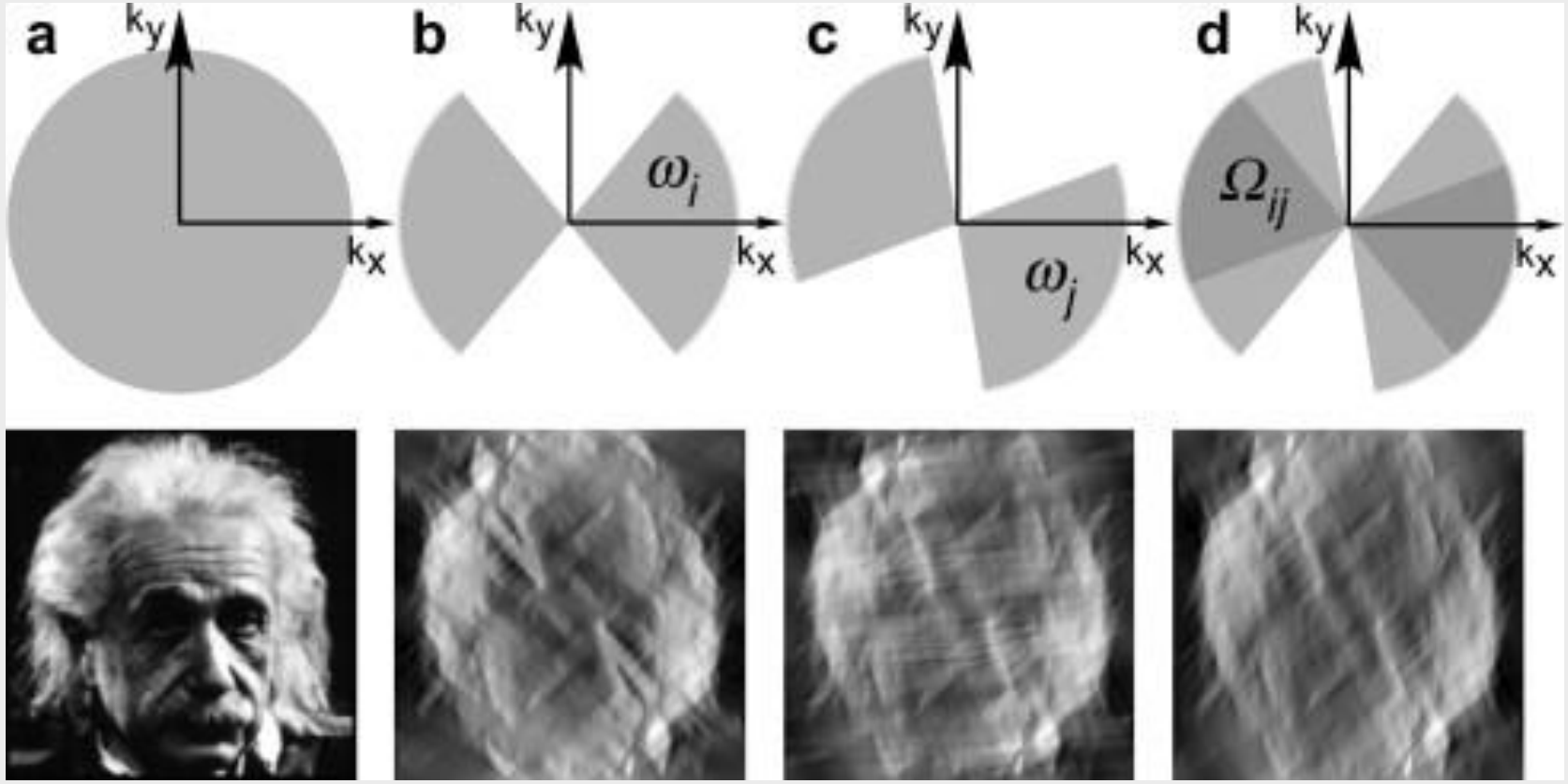
Sub-tomogram processing workflow



Briggs 2013

- **Missing wedge** must be taken into account for each sub-tomogram

Must take into account subtomogram missing wedges

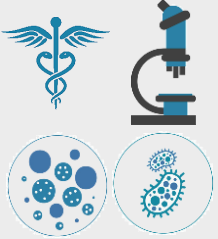


Forster et al, J. Struct. Biol, 2008

- Effectively align volume in common in Fourier space

Sub-tomogram processing software

- Warp/M – Local motion and CTF, 3D deformation model
- Dynamo – GPU accelerated, tomogram database, extensive picking abilities
- Relion – Local motion and CTF
- EMAN2 – Sub-tilt-series refinement and defocus estimation/correction
- emClarity – Sub-tilt-series refinement and defocus estimation/correction
- TYGRESS – Intended for use w/ high dose 0 degree image (Nicastro group)
- PyTom
- PEET
- Jsubtomo
- TOM & AV3
- XMIPP

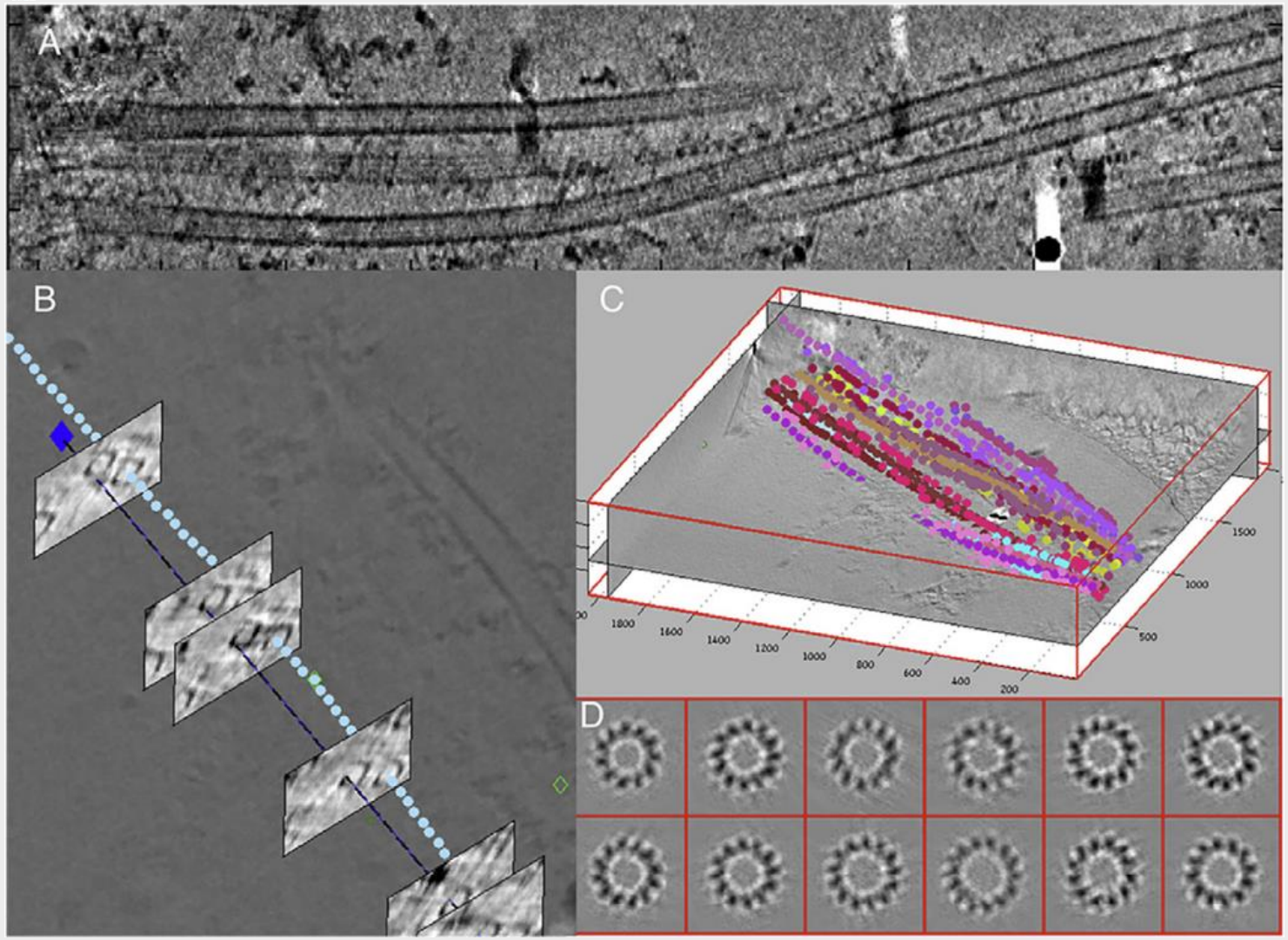


Tomogram annotation

Tomogram/sub-tomogram annotation and segmentation software

- Dynamo – Annotate membranes, tubes, helices, crystal structures, vesicles, etc.
- EMAN2 – Neural network segmentation
- MemBrain – Flat membrane and membrane protein finder
- Surface morpheometrics – Membrane curvature and context quantification
- Dragonfly – Train deep models
- Amira – Interactive segmentation and filtering suite
- UCSF Chimera w/ Segger - Interactive segmentation
- Template picking – MolMatch, Dynamo
- Deep picking: CrYOLO, EMAN2, Topaz 3D (soon)

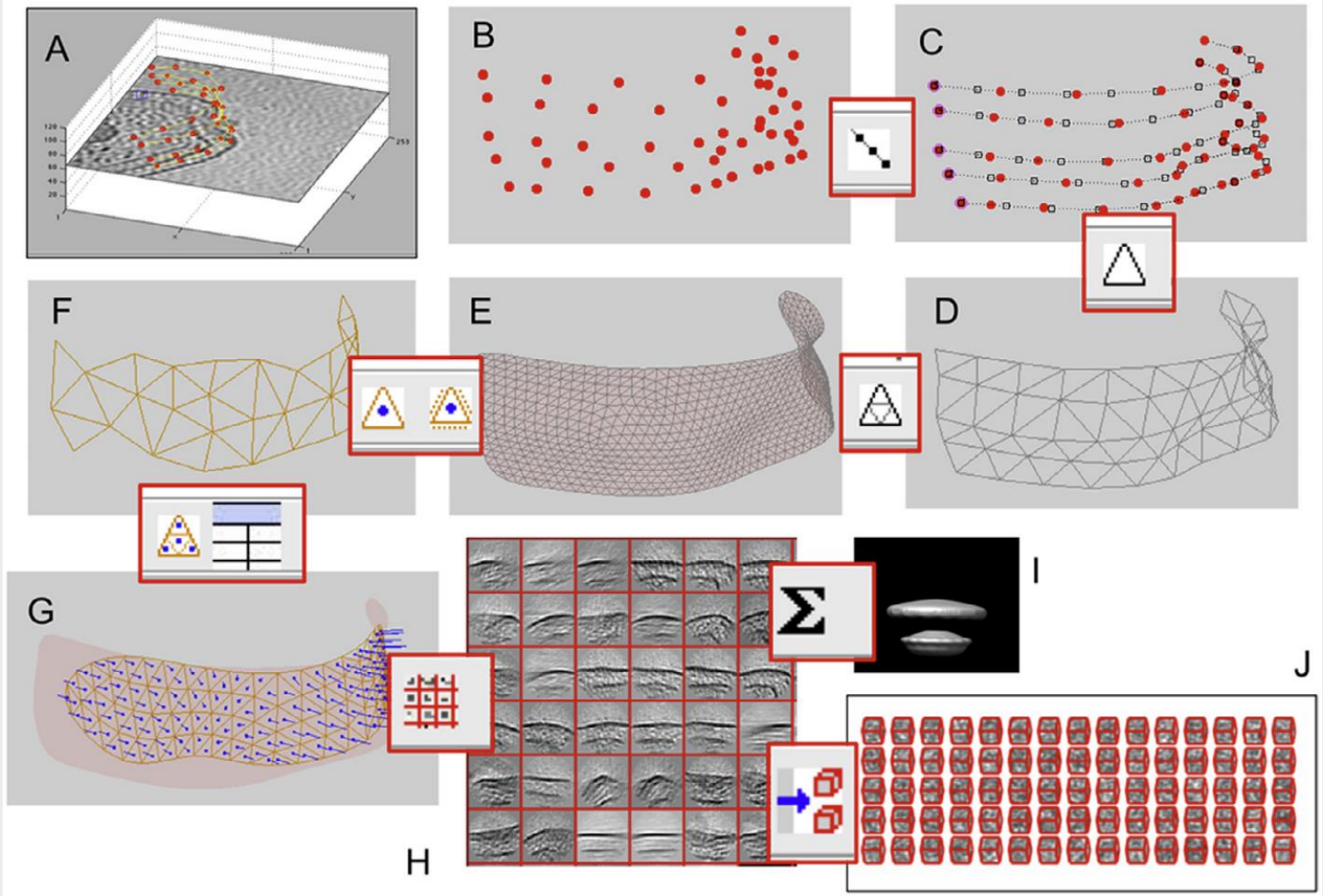
Sub-tomogram annotation processing in Dynamo



- Backbone, helical, and circumferential picking
- Helical symmetry determination

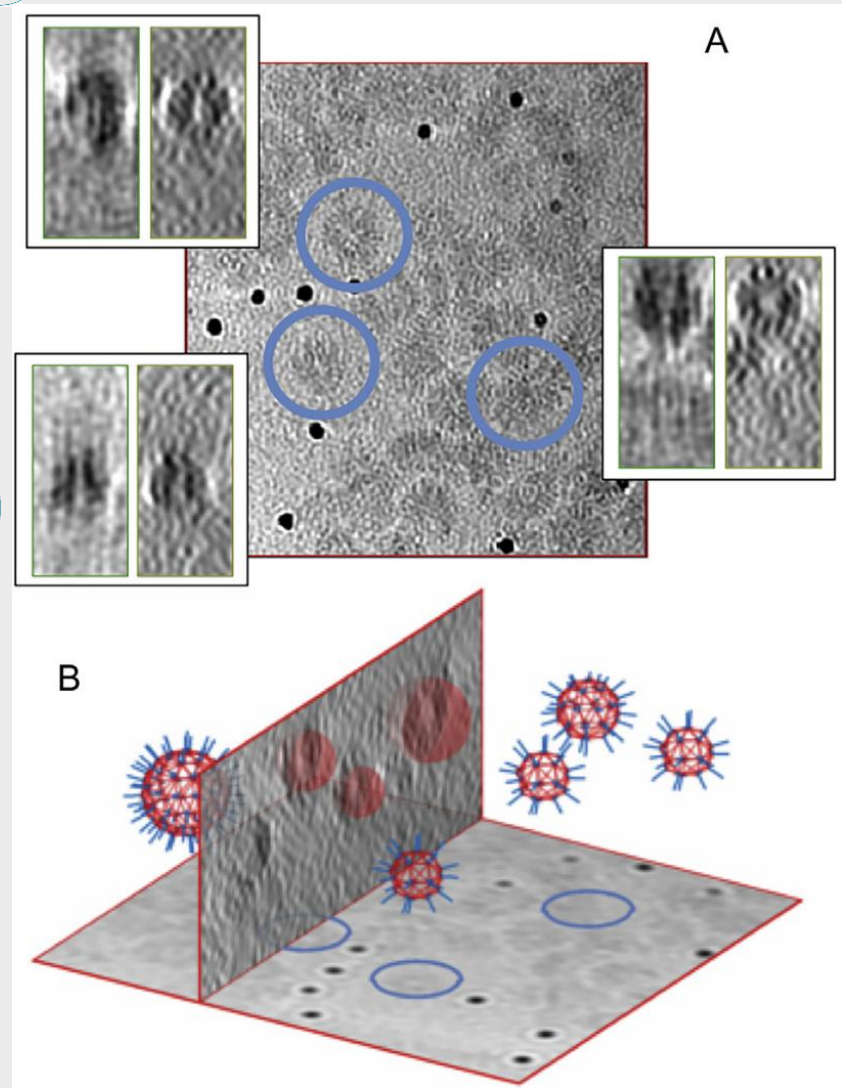
Castaño-Díez et. al., JSB 2012 & 2016

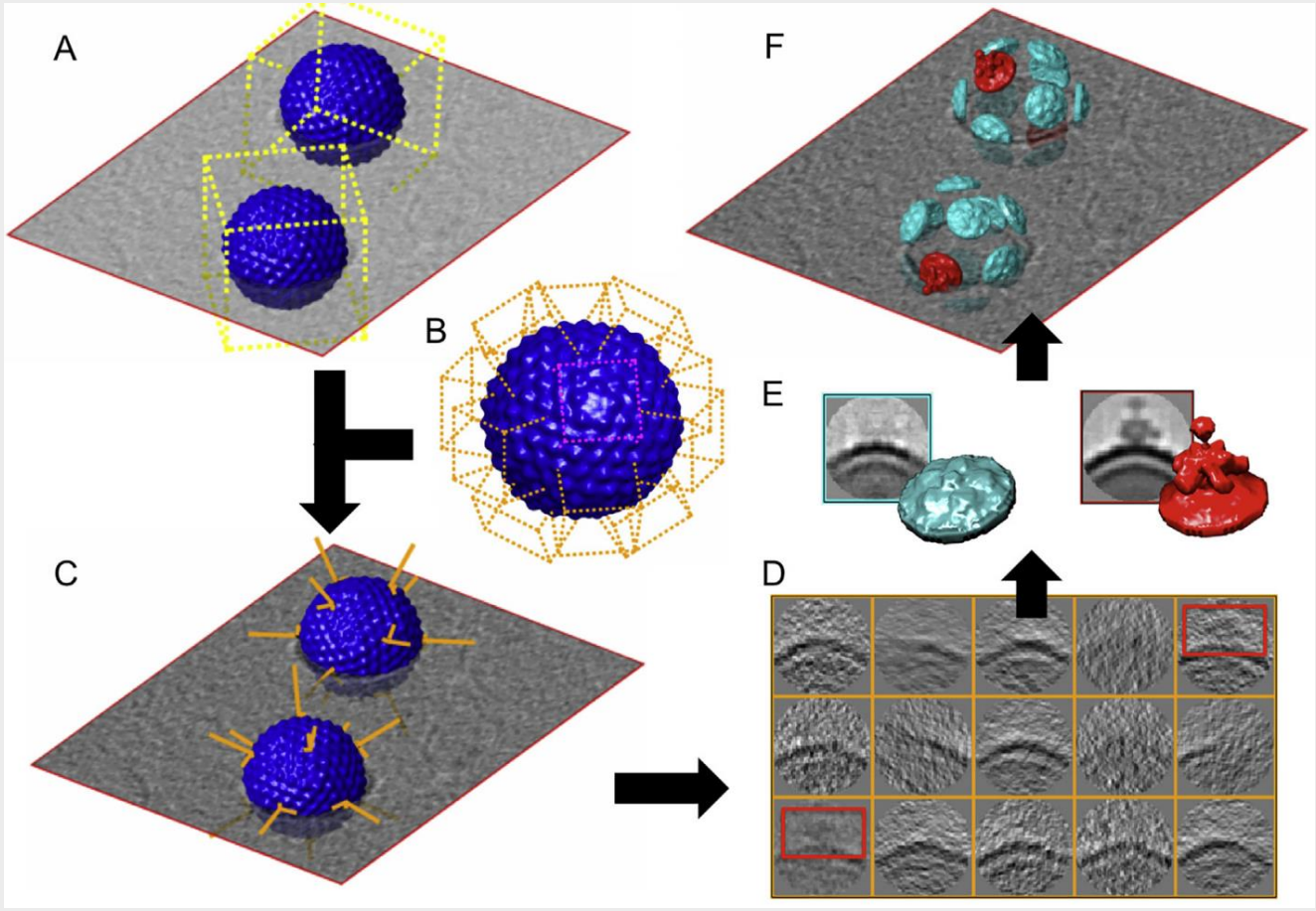
Sub-tomogram annotation processing in Dynamo



Castaño-Díez et. al., JSB 2012 & 2016

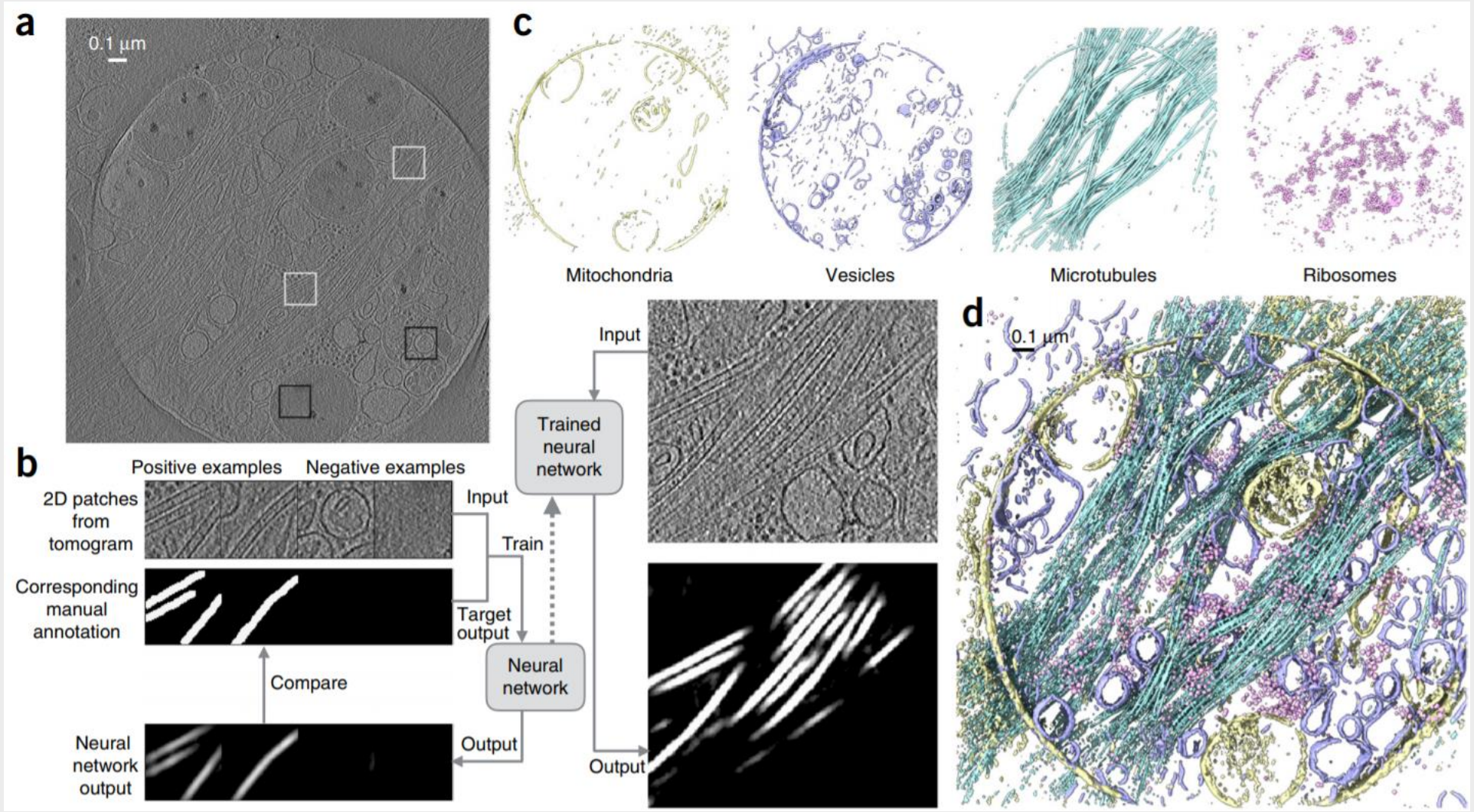
Sub-tomogram annotation processing in Dynamo





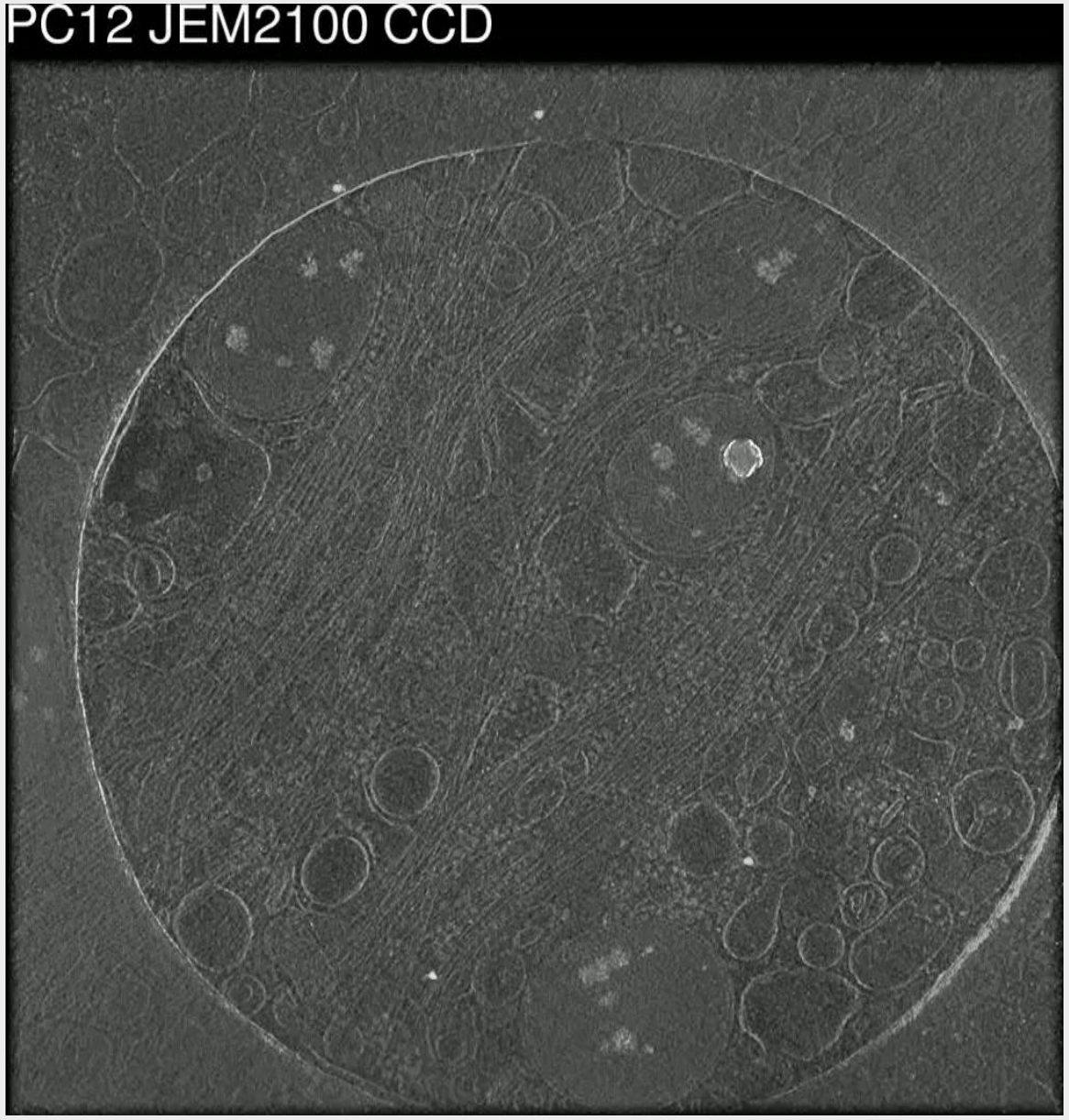
Castaño-Díez et. al., JSB 2012 & 2016

Sub-tomogram segmentation with CNNs in EMAN2



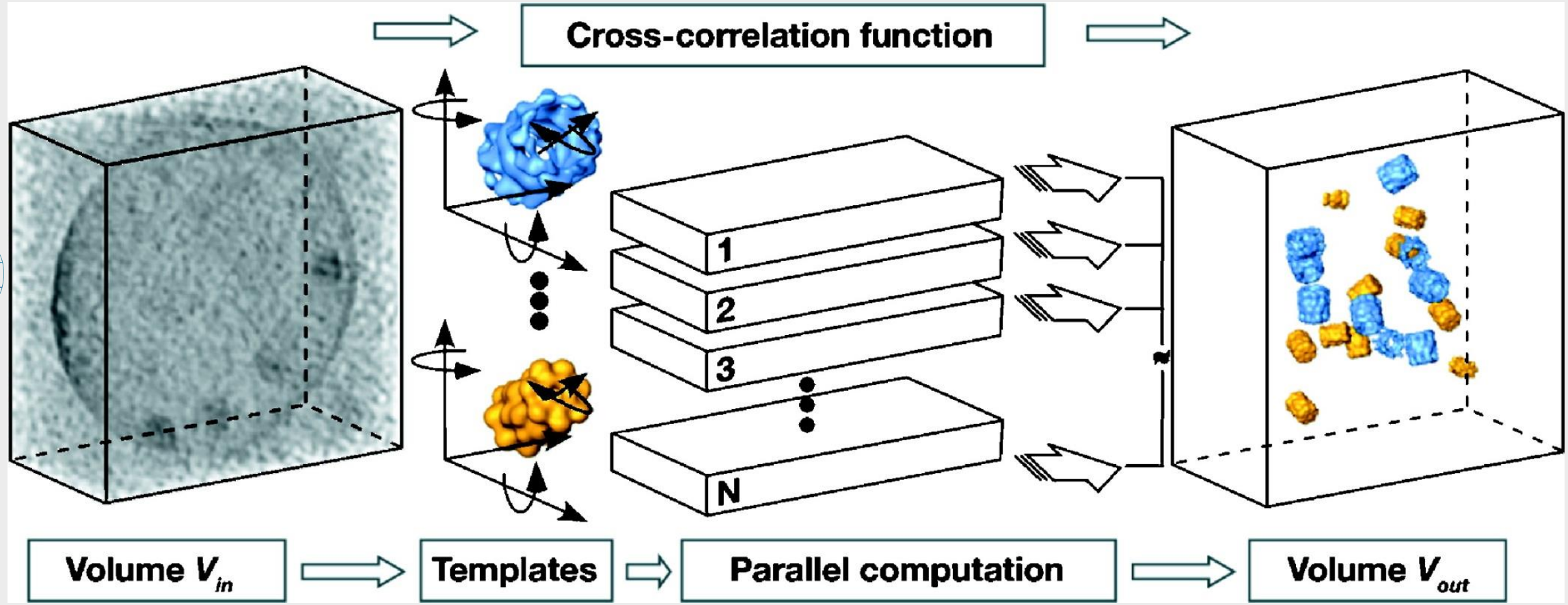
Chen et. al., Nat. Meth. 2017

Sub-tomogram segmentation with CNNs in EMAN2



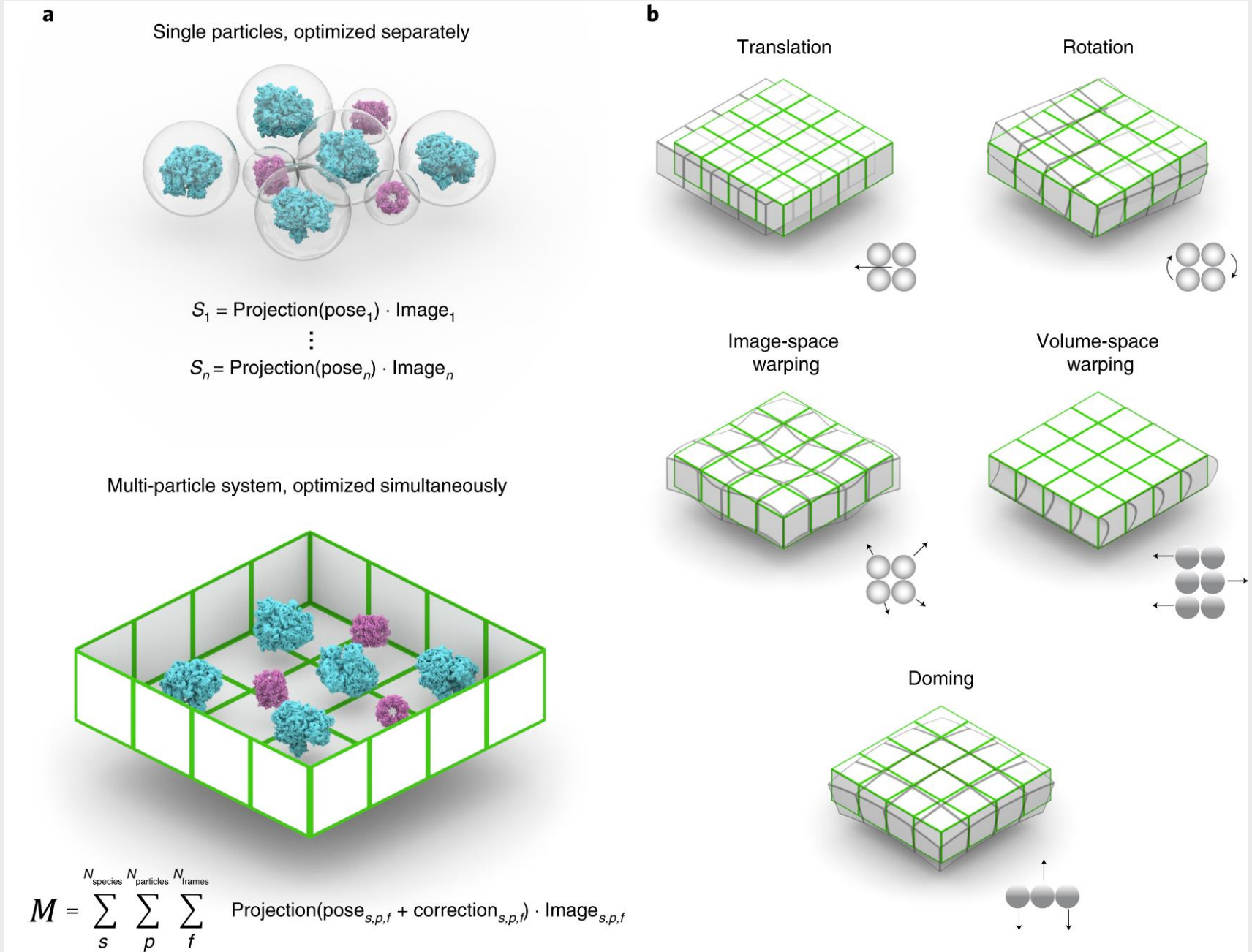
Chen et. al., Nat. Meth. 2017

Template matching



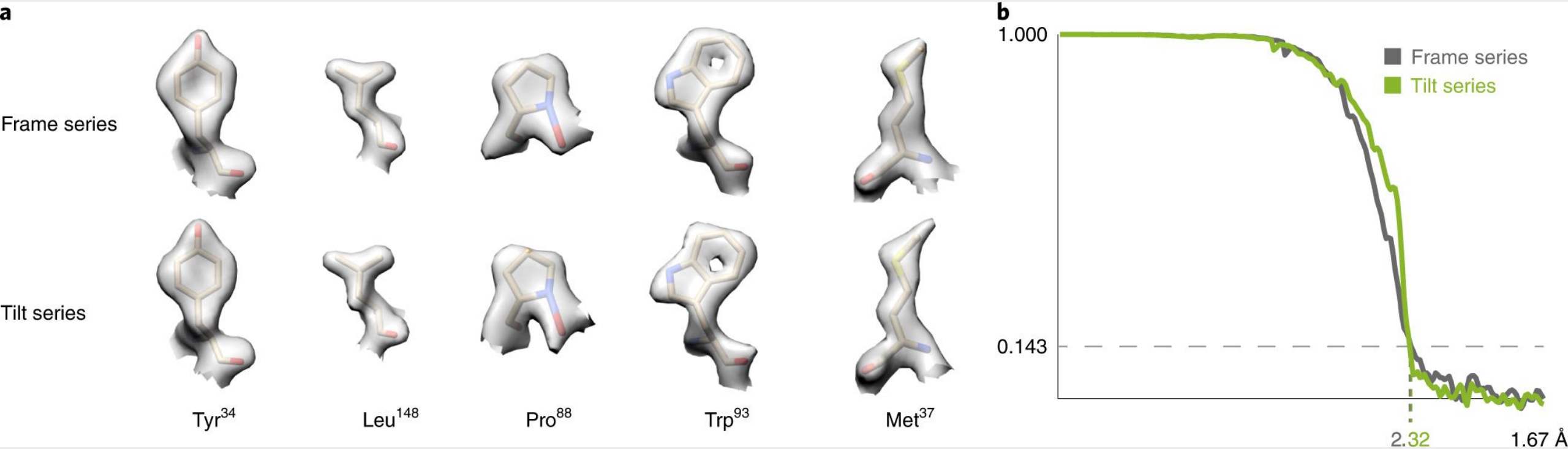
Lucic et al, 2005, Annu. Rev. Biochem

Warp/M Co-sub-tilt-series refinement



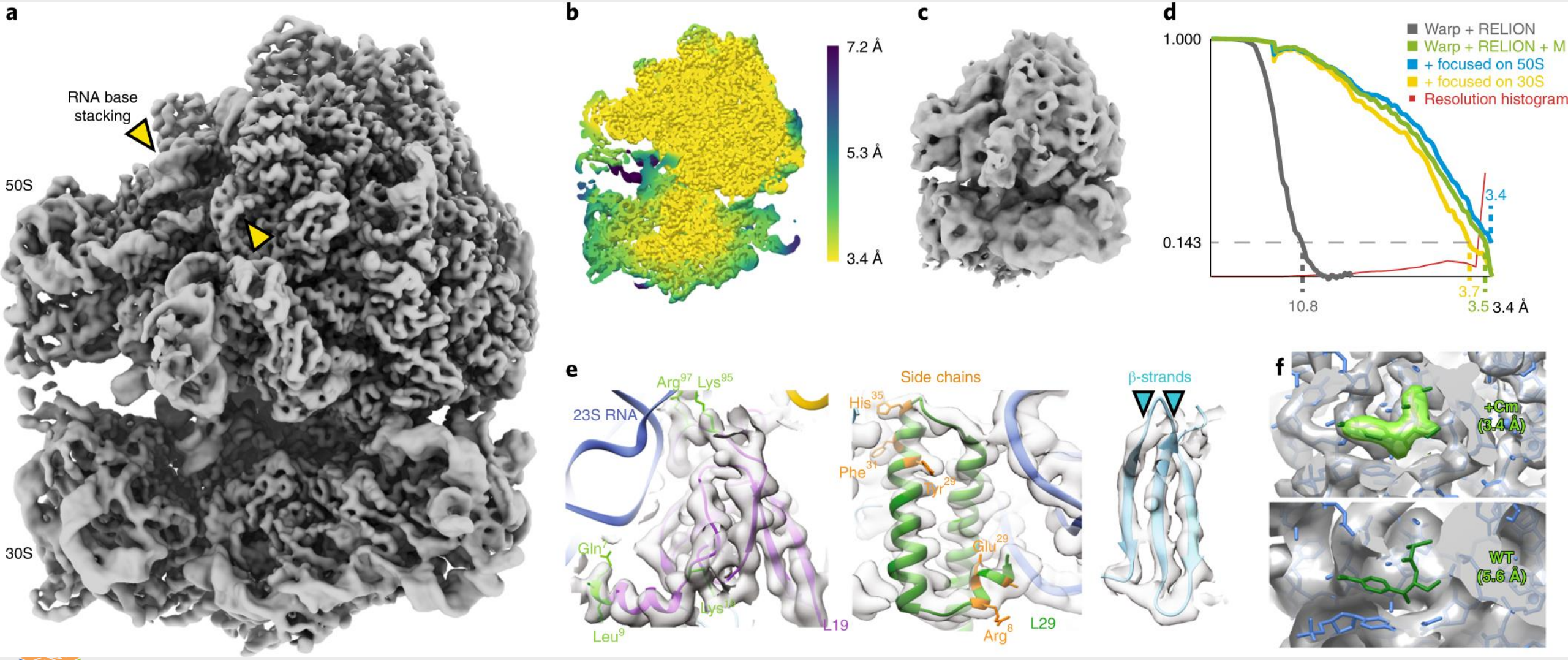
Tegunov et al., 2021

Warp/M Co-sub-tilt-series refinement: apoferritin



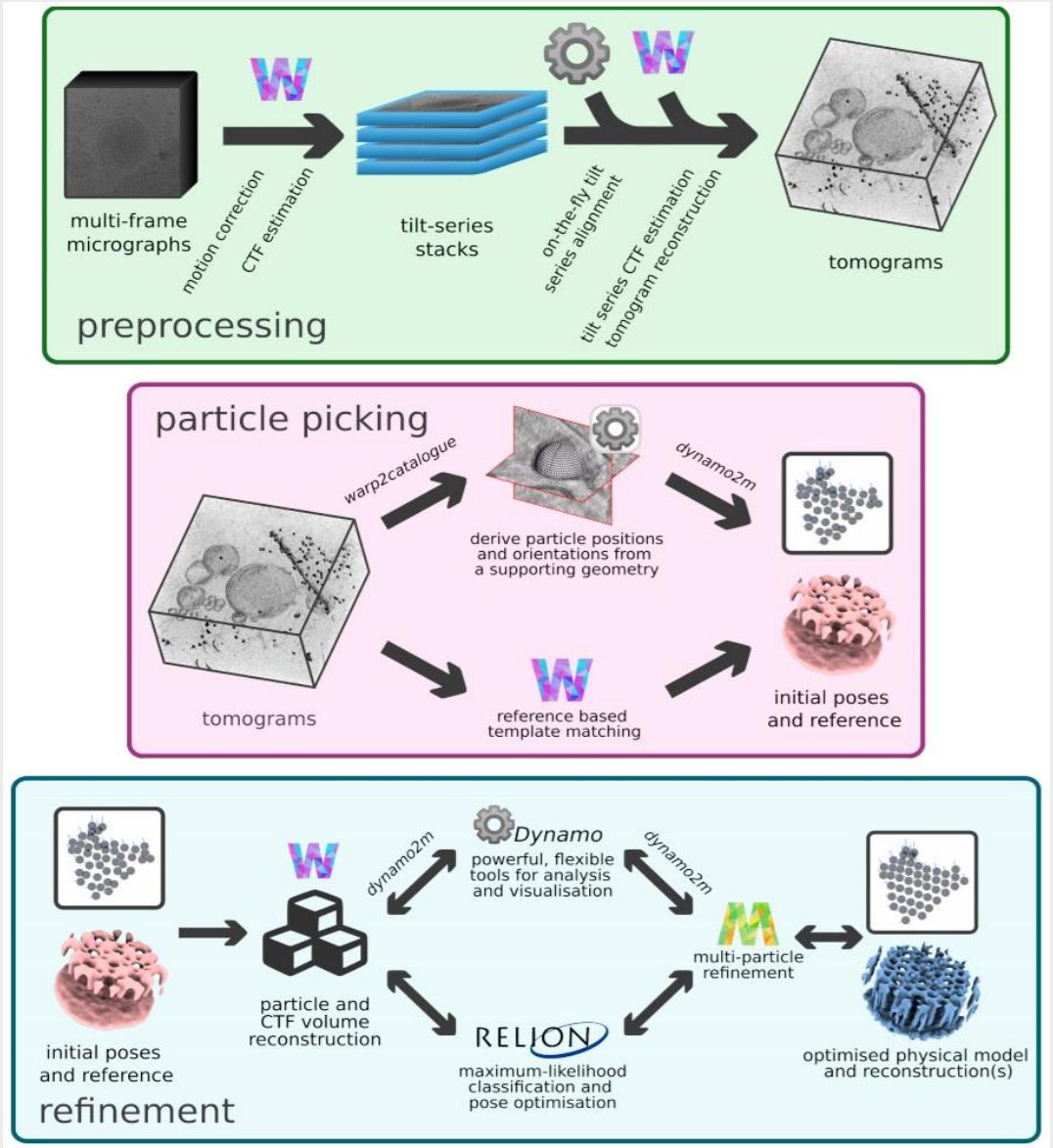
Tegunov et al., 2021

Warp/M Co-sub-tilt-series refinement: *In-situ* 70S ribosome



Tegunov et al., 2021

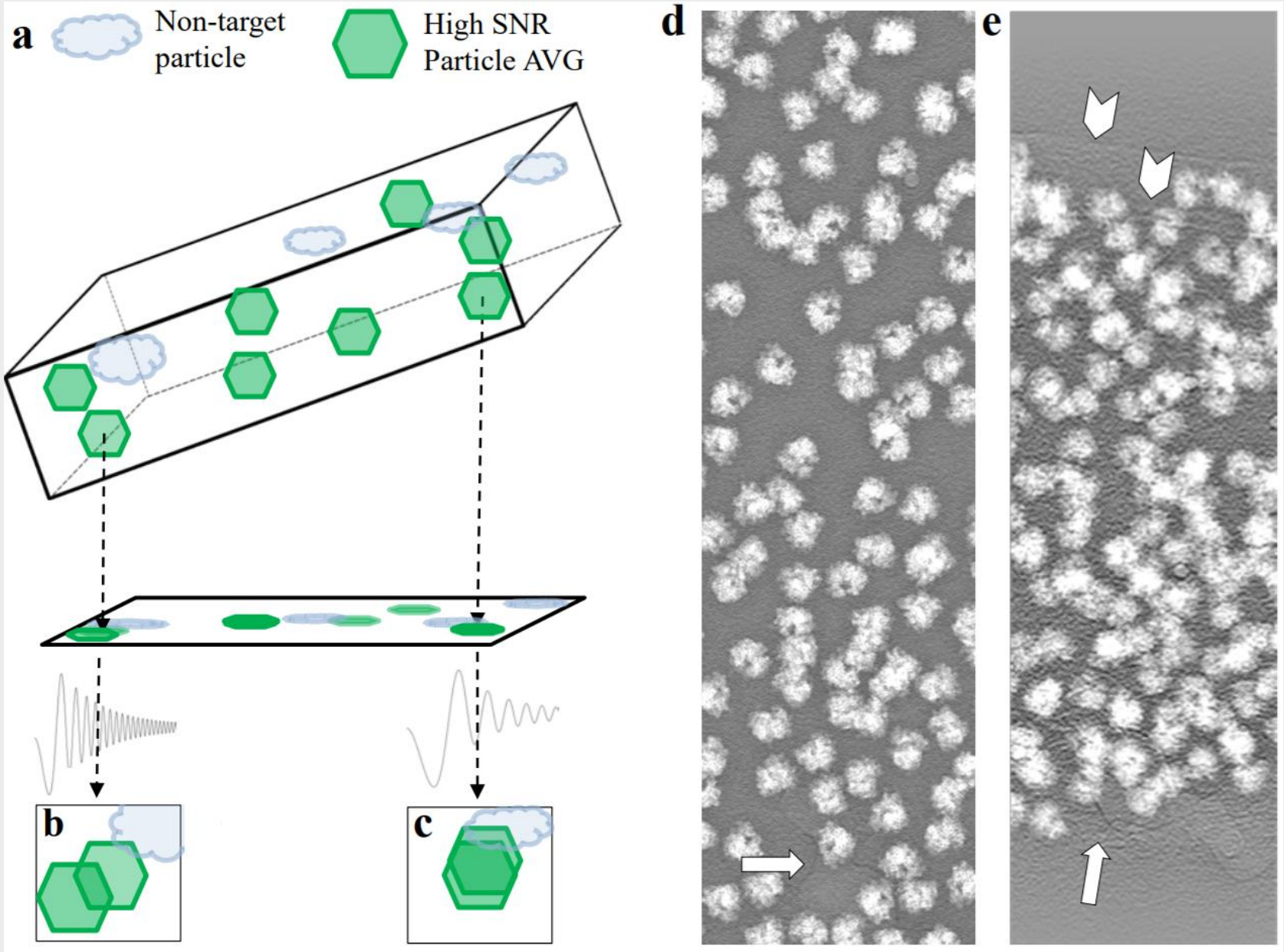
Warp/M Co-sub-tilt-series refinement: Dynamo-Warp/M-Relion workflow



teamtomo.org

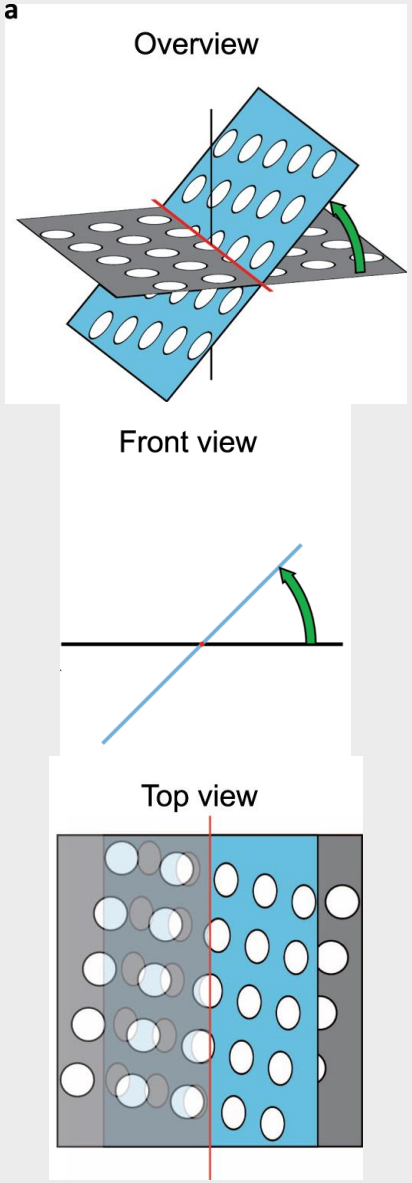
Burt et al., 2021

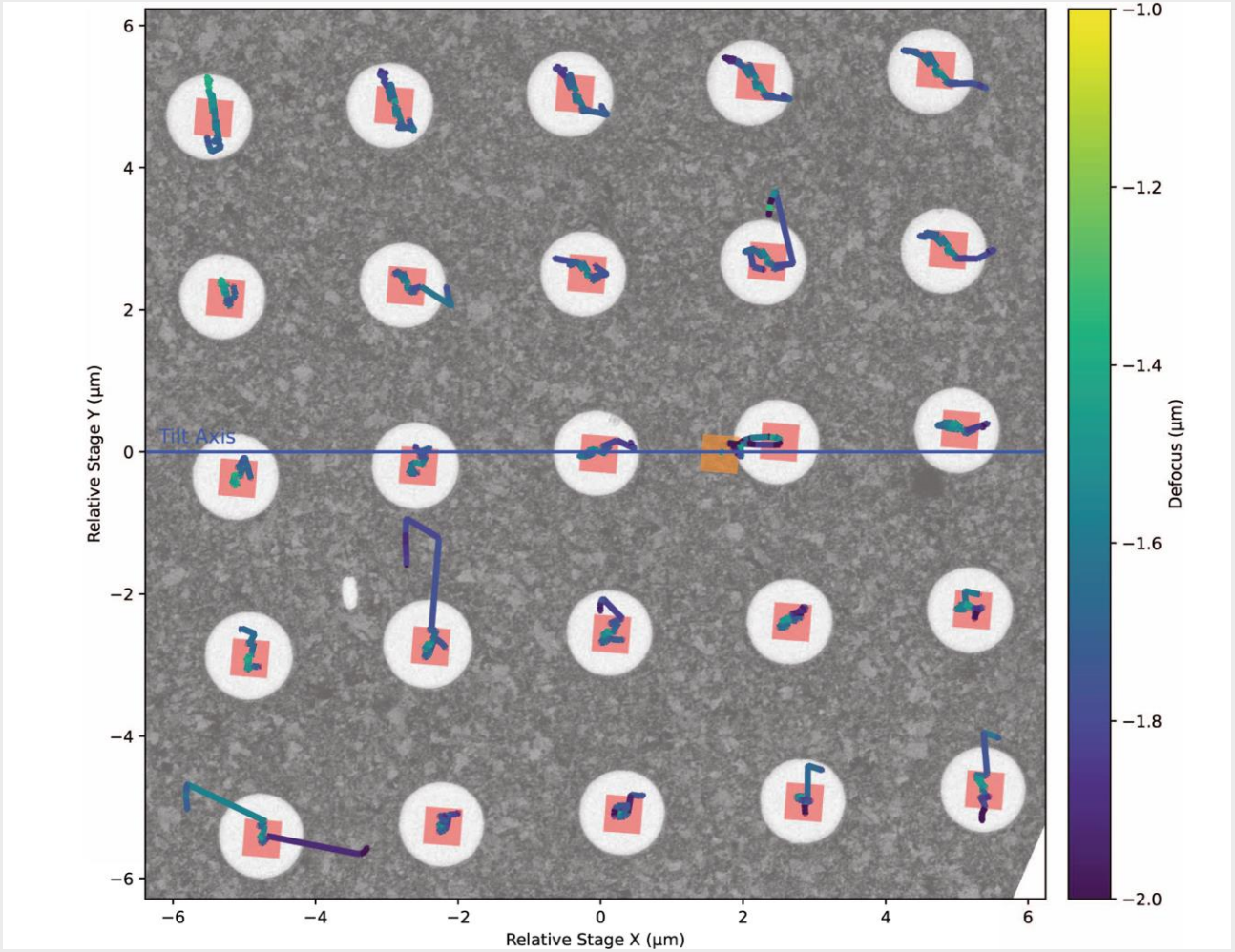
Refining tilt-series alignment by tracking just particles



Himes et al., 2017

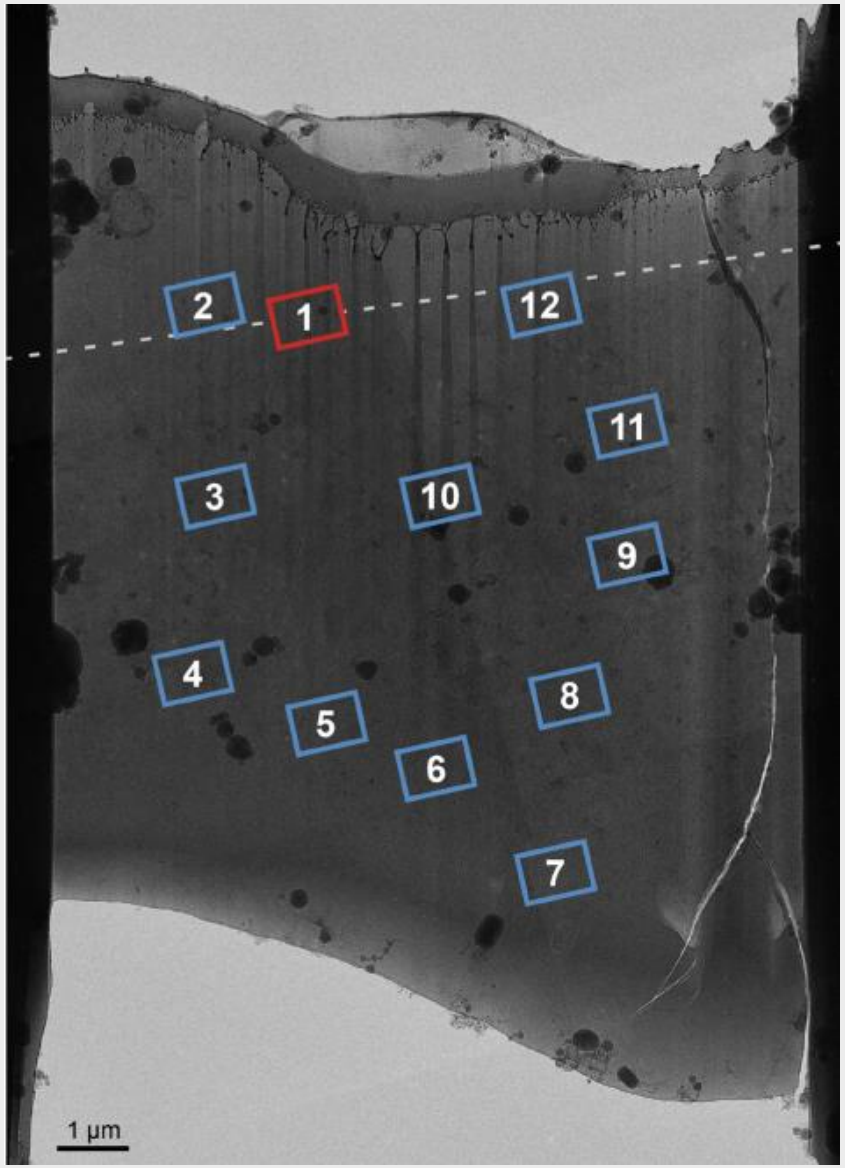
BISECT: Higher-throughput parallel acquisition





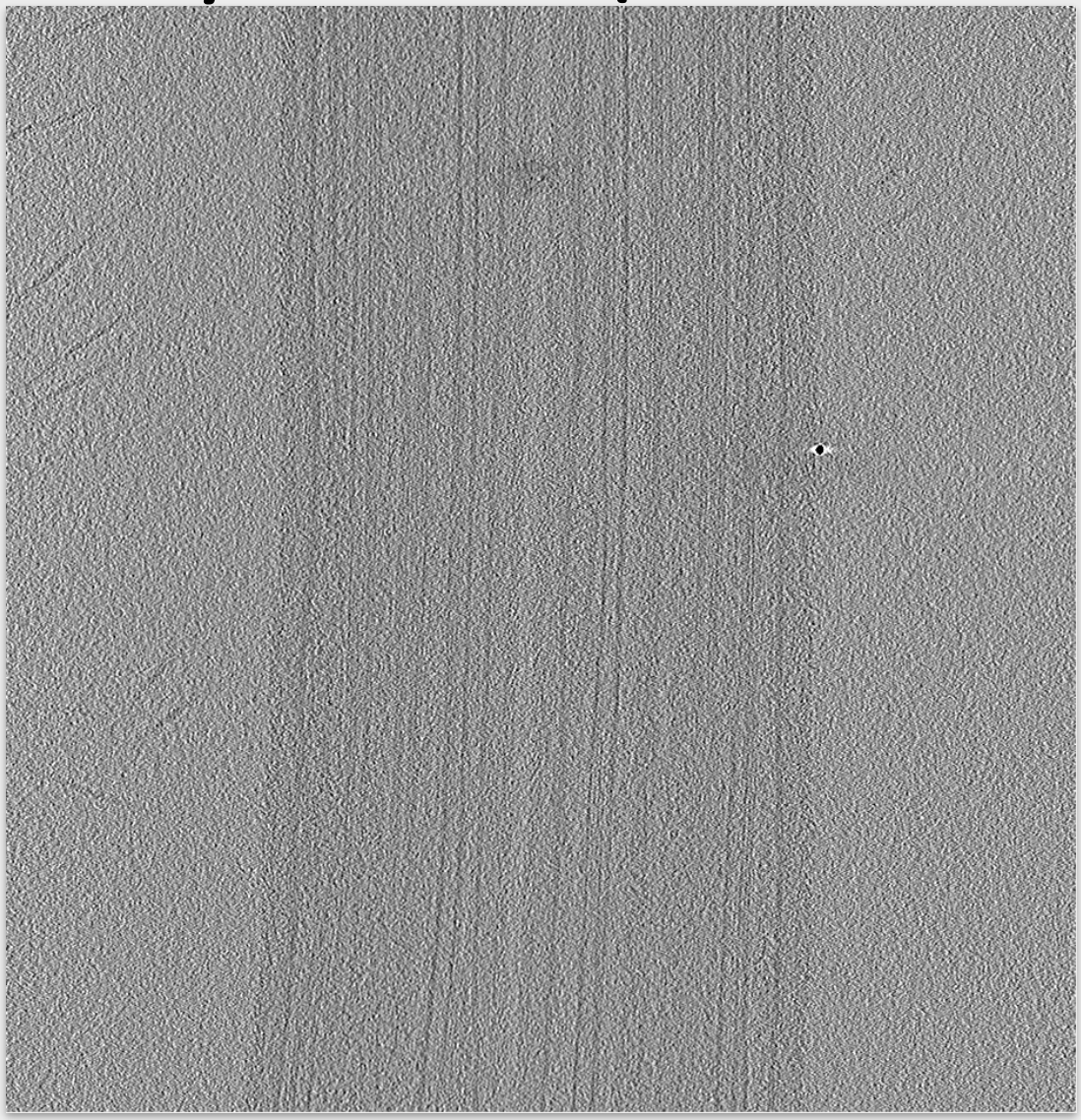
Bouvette et al., 2021

PACE: Even higher-throughput parallel acquisition

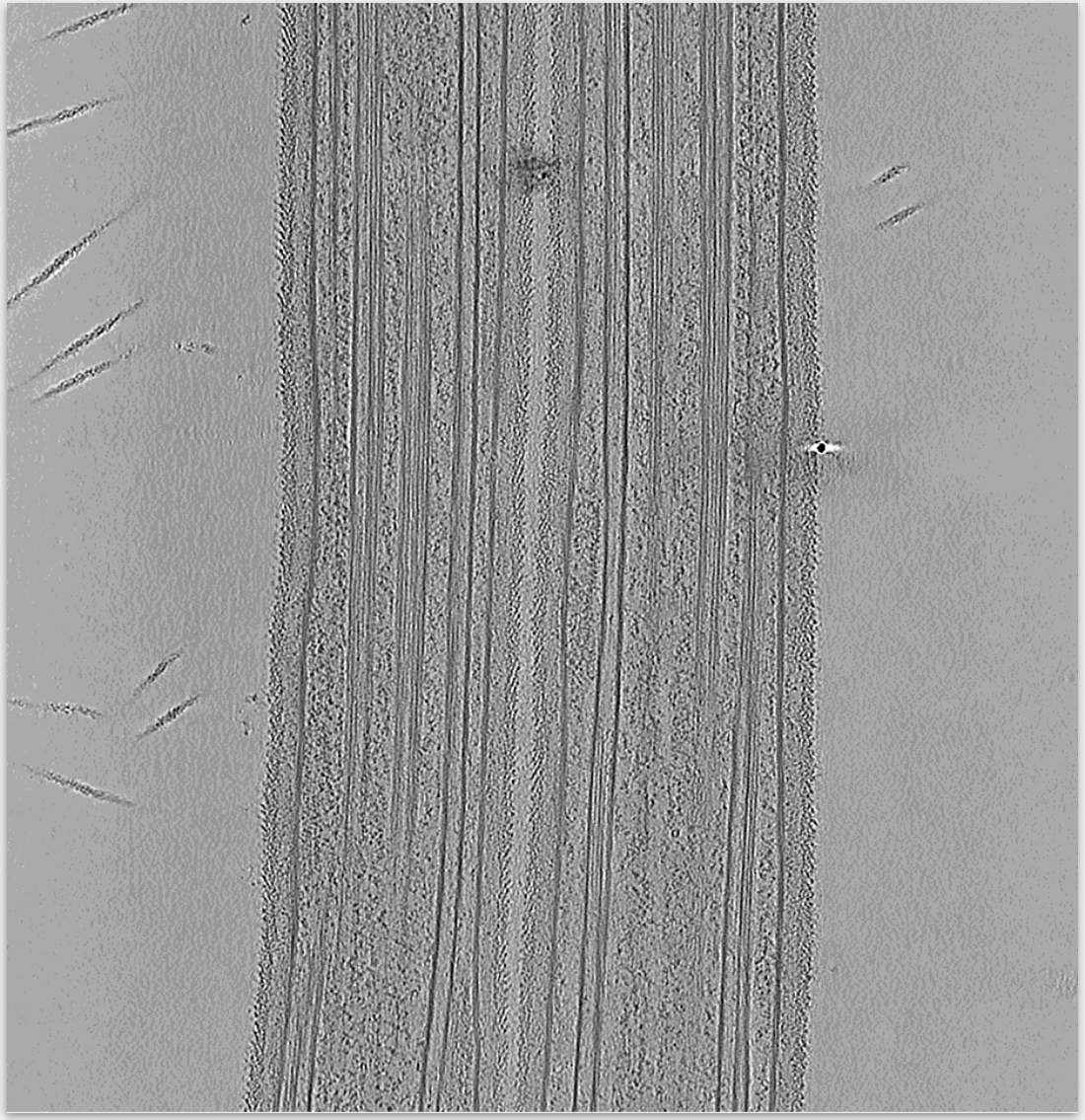


Eisenstein et al., 2022

Post-processing improvement - *Denoising* Cryo-CARE (3D Noise2Noise):



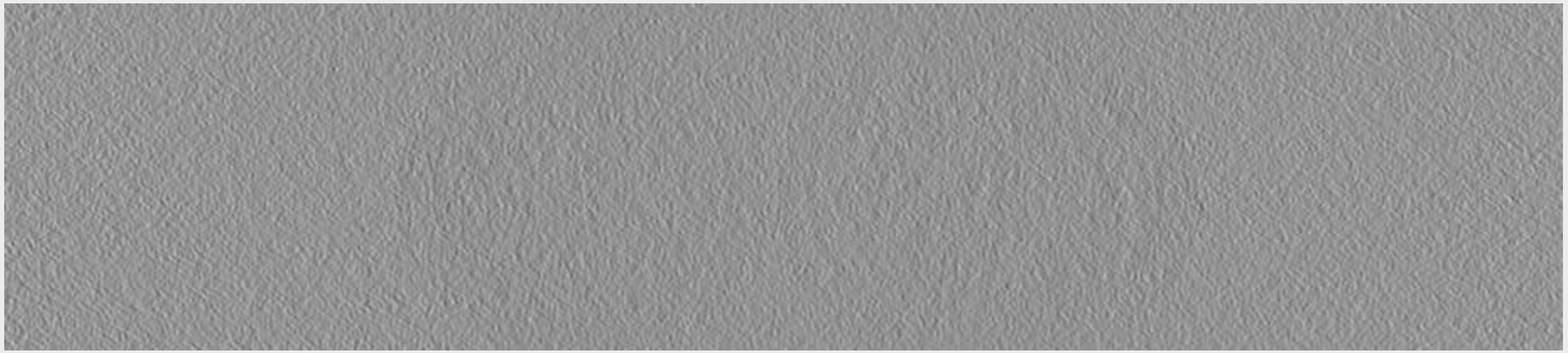
Before



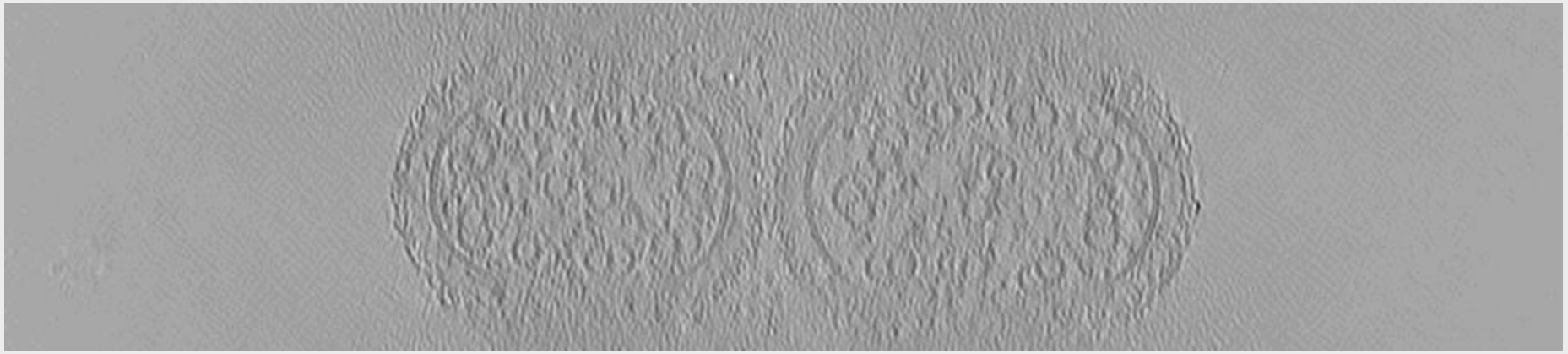
After!

Buchholz et al., 2019

Post-processing improvement - *Denoising* Cryo-CARE (3D Noise2Noise):



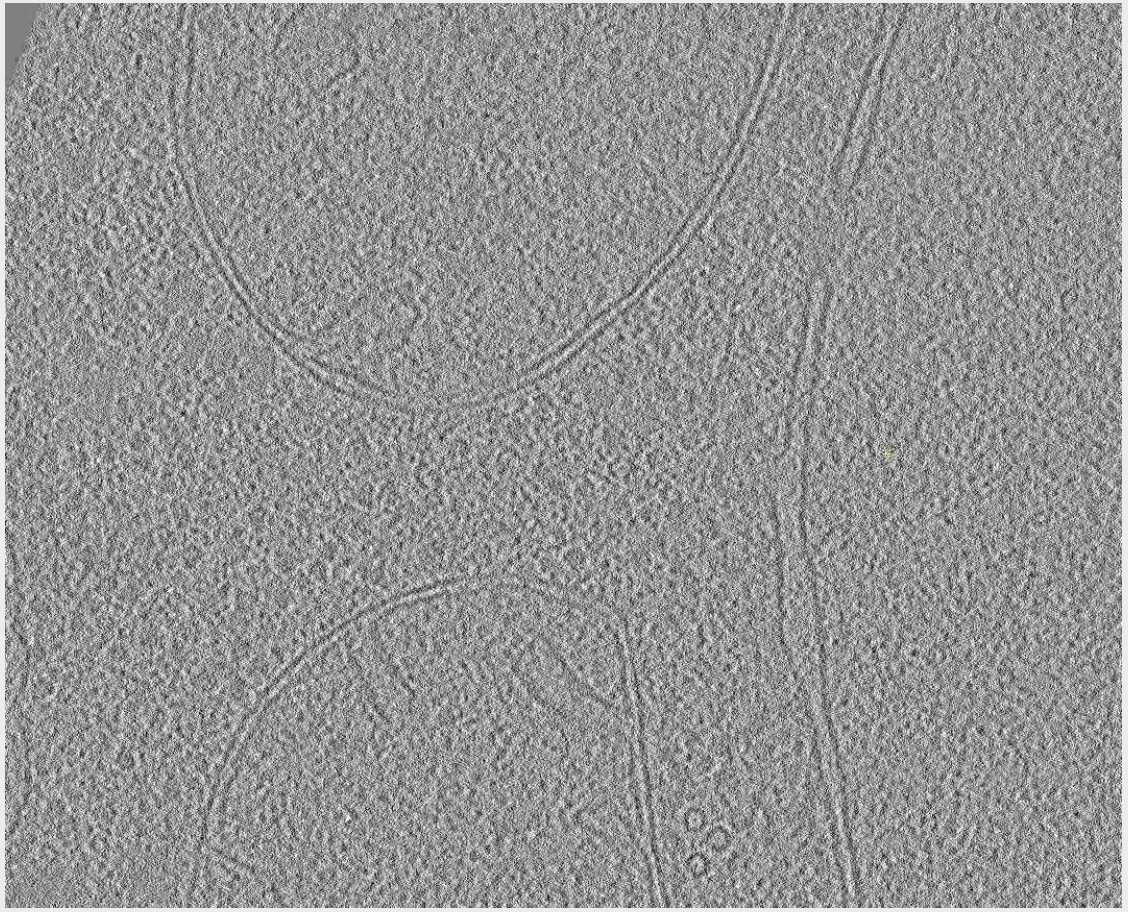
Before



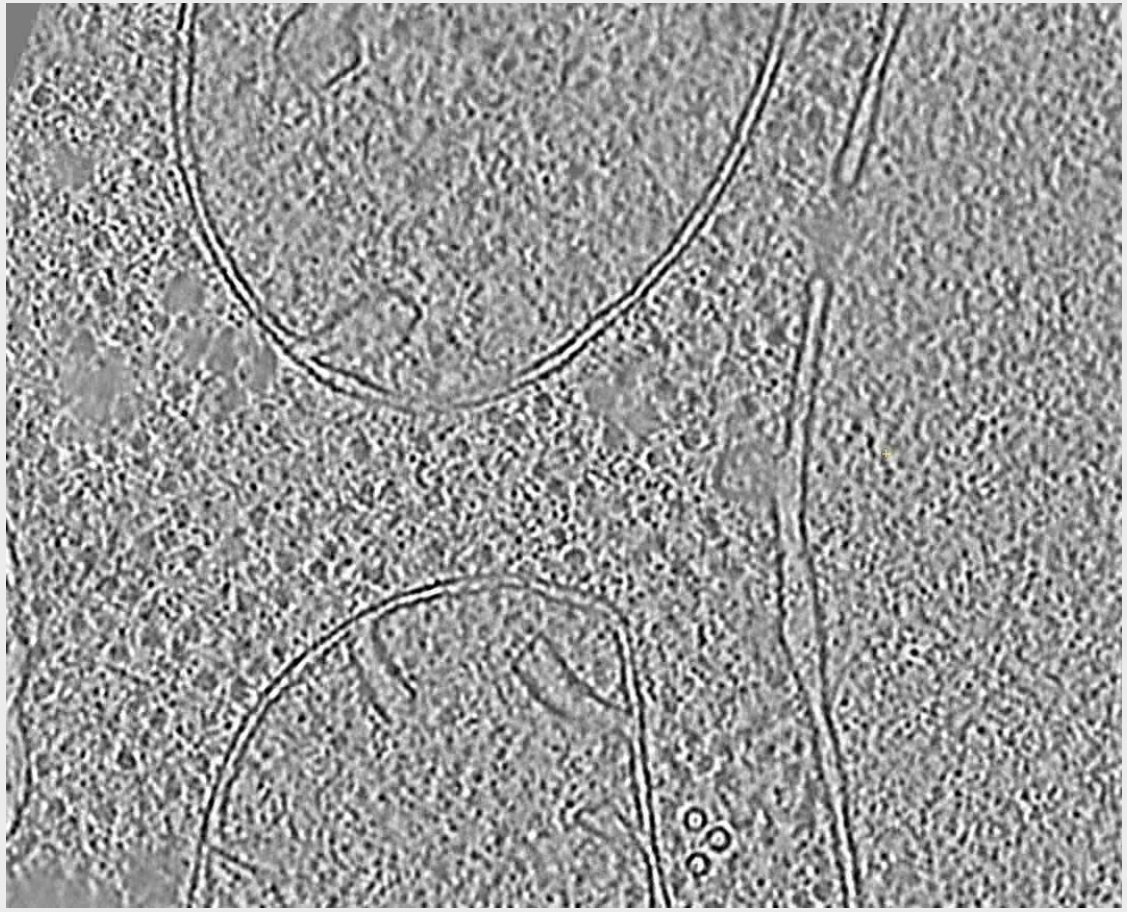
After!

Buchholz et al., 2019

Post-processing improvement - *Denoising* Topaz (3D Noise2Noise):



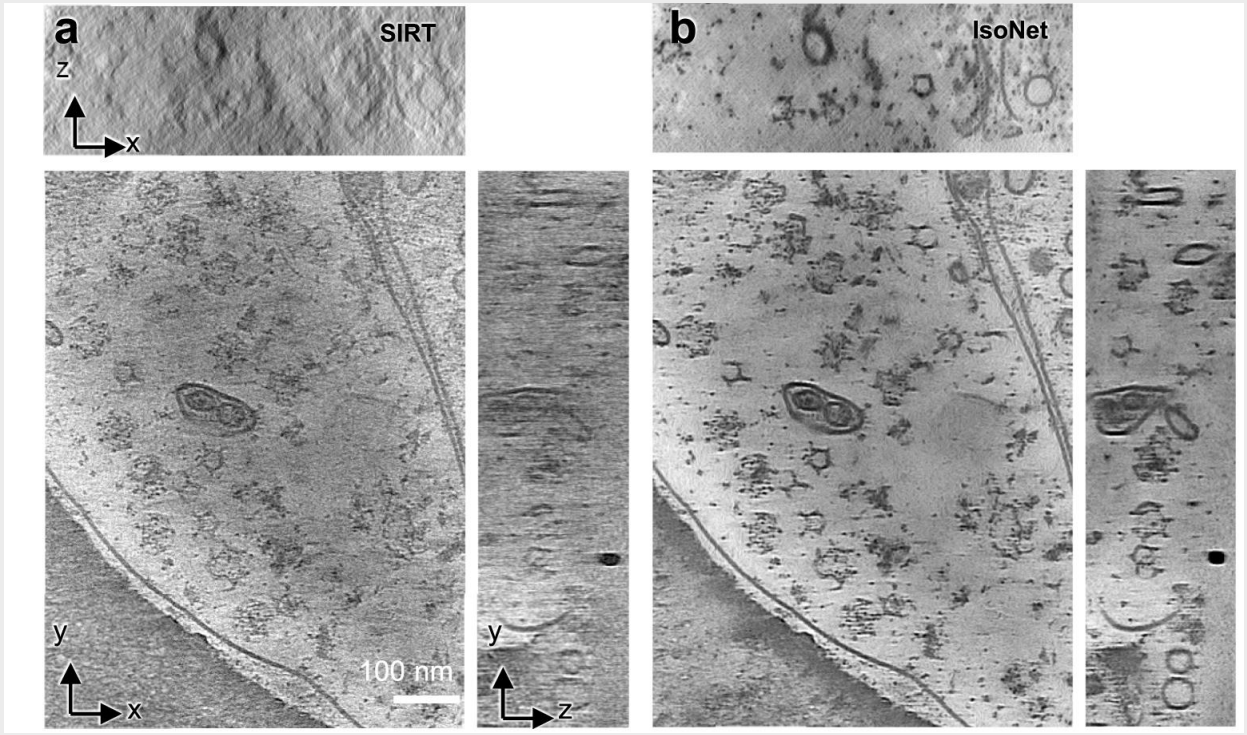
Original



Topaz denoised

Bepler et al., 2020

IsoNet: Missing wedge estimation by deep learning

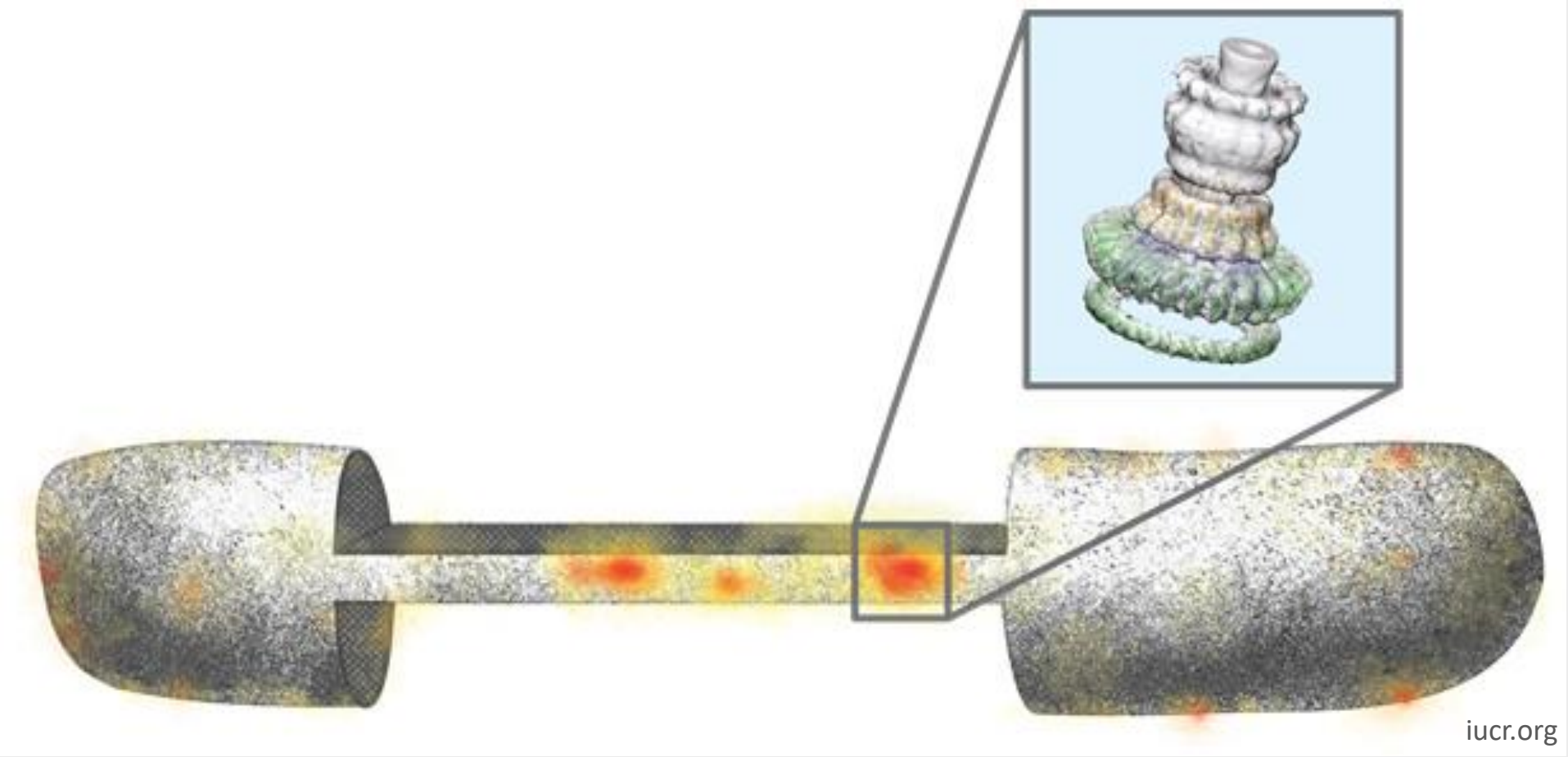


Before **After!**

Liu et al., 2022

Current/future directions in tomography

Future hardware improvements in the field: 3D cryo-CLEM

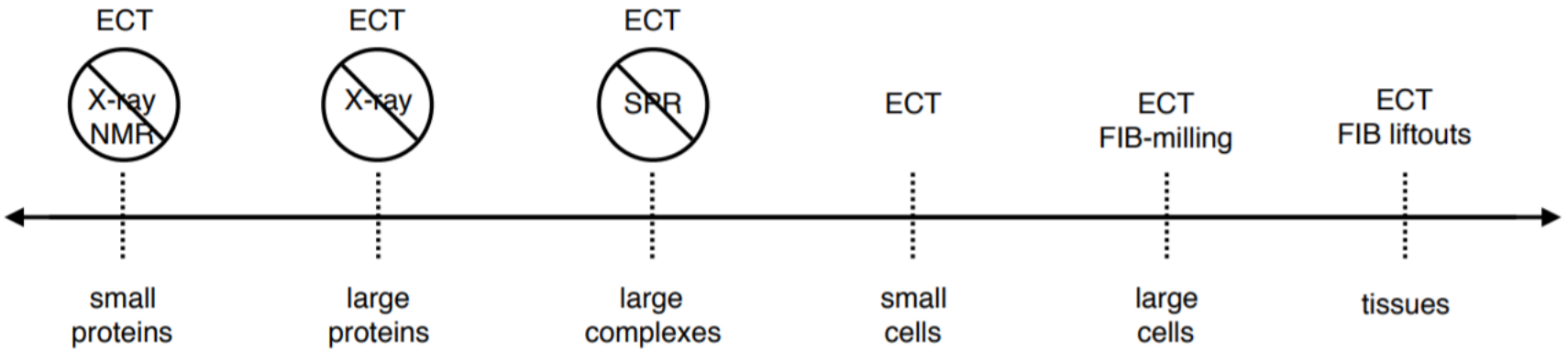


Hardware improvement – Rapid tilting

Nominal magnification	Pixel size (Å)	Exposure time (s)	Total frames	Total time per tilt-series (min)
33kx	4.32	126	5040 or less	9.7
53kx	2.74	50	2000 or less	7.6
81kx	1.78	20	800	6.7
130kx	1.09	12	480	5.0

MOSTLY *MOST*

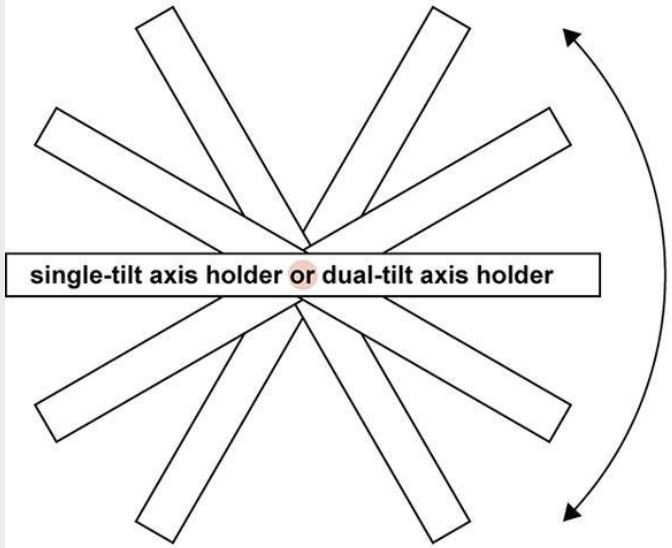
~~ALL~~ cryotomography, ~~ALL~~ the time



Chreifi et al., 2019

Hardware/software improvement
Pre-calibrated rapid tilting!

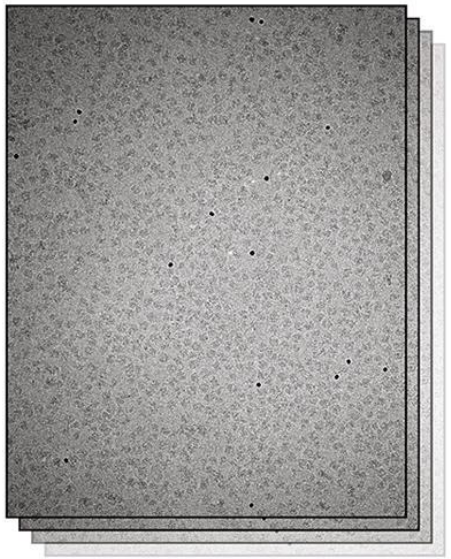
Fast-incremental single-exposure



Collection

< 5 min
per tilt series

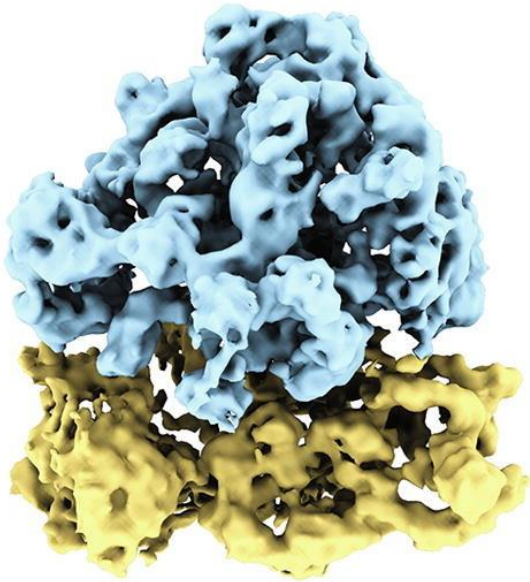
Tilt series movie



Processing

several days

Subtomogram average at subnanometer resolution



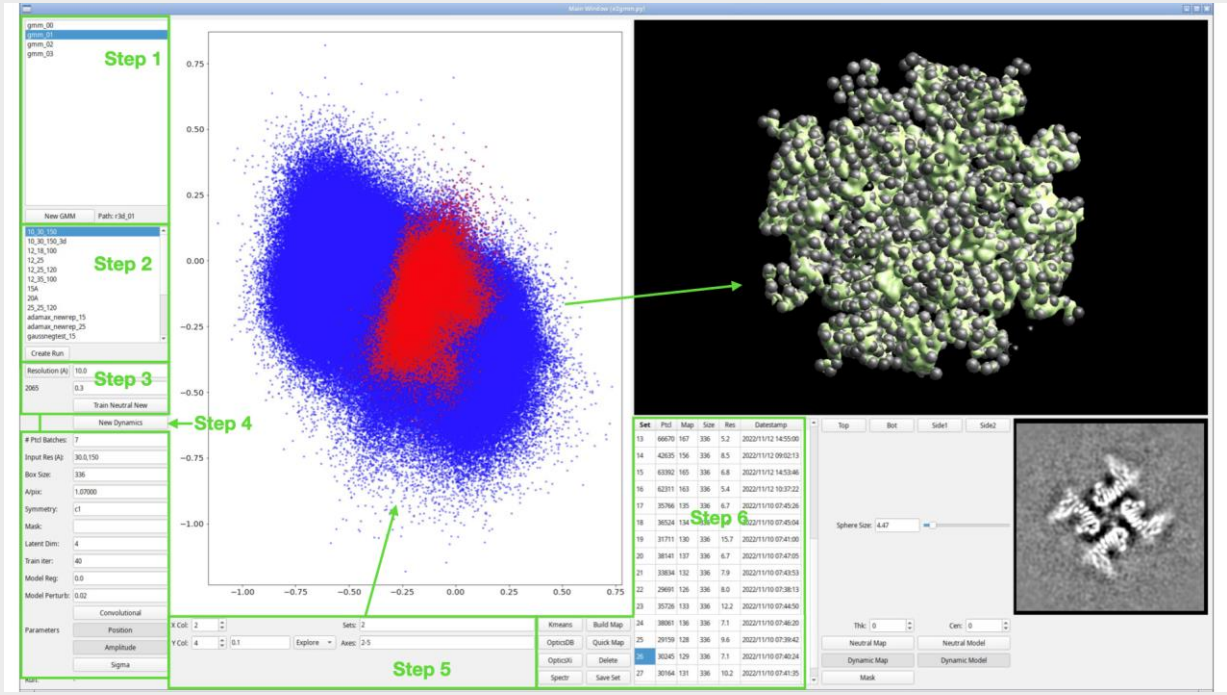
K3

x, y, z specimen shift compensation

Eisenstein et al., 2019

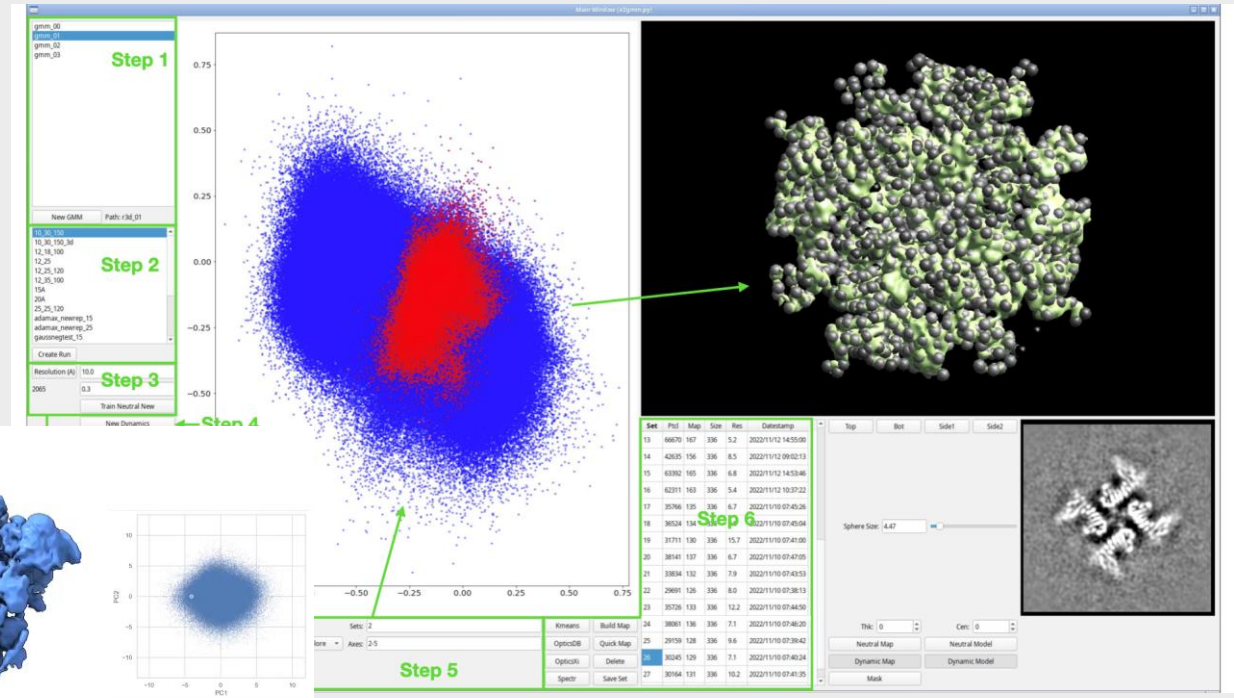
Flexibility analysis

- EMAN2 GMM:

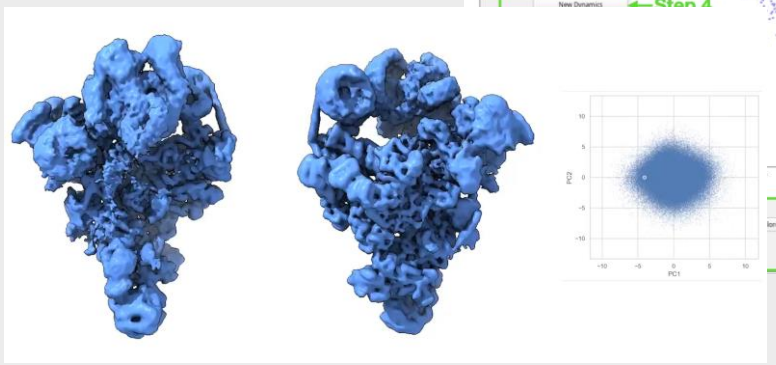


Flexibility analysis

- EMAN2 GMM:

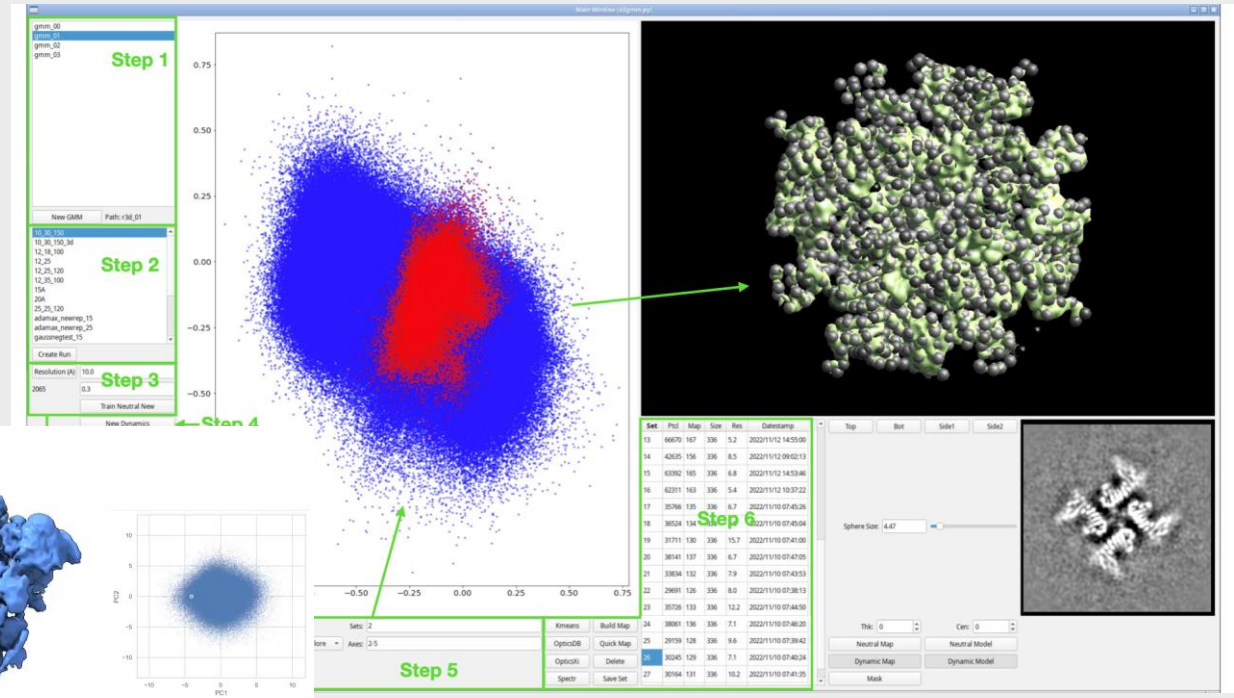


- TomoDRGN (soon?)

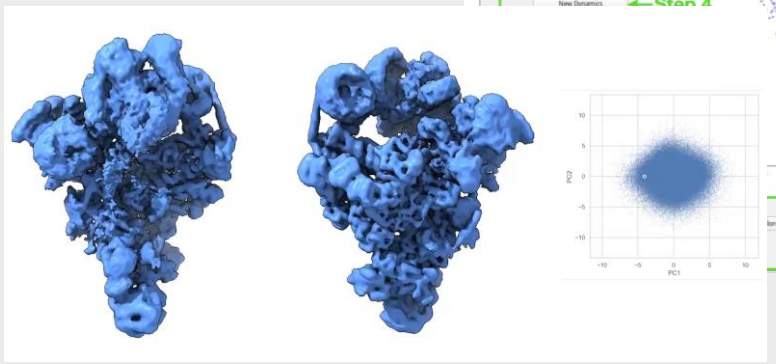


Flexibility analysis

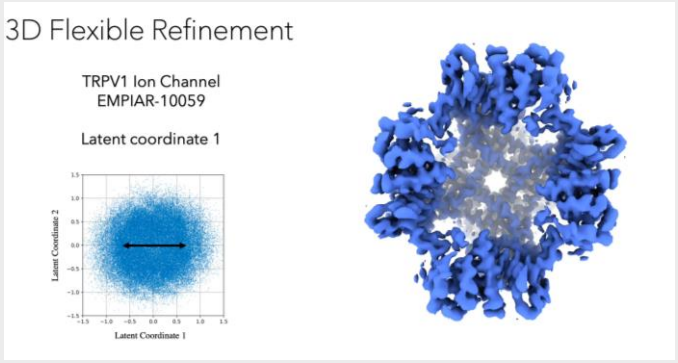
- EMAN2 GMM:

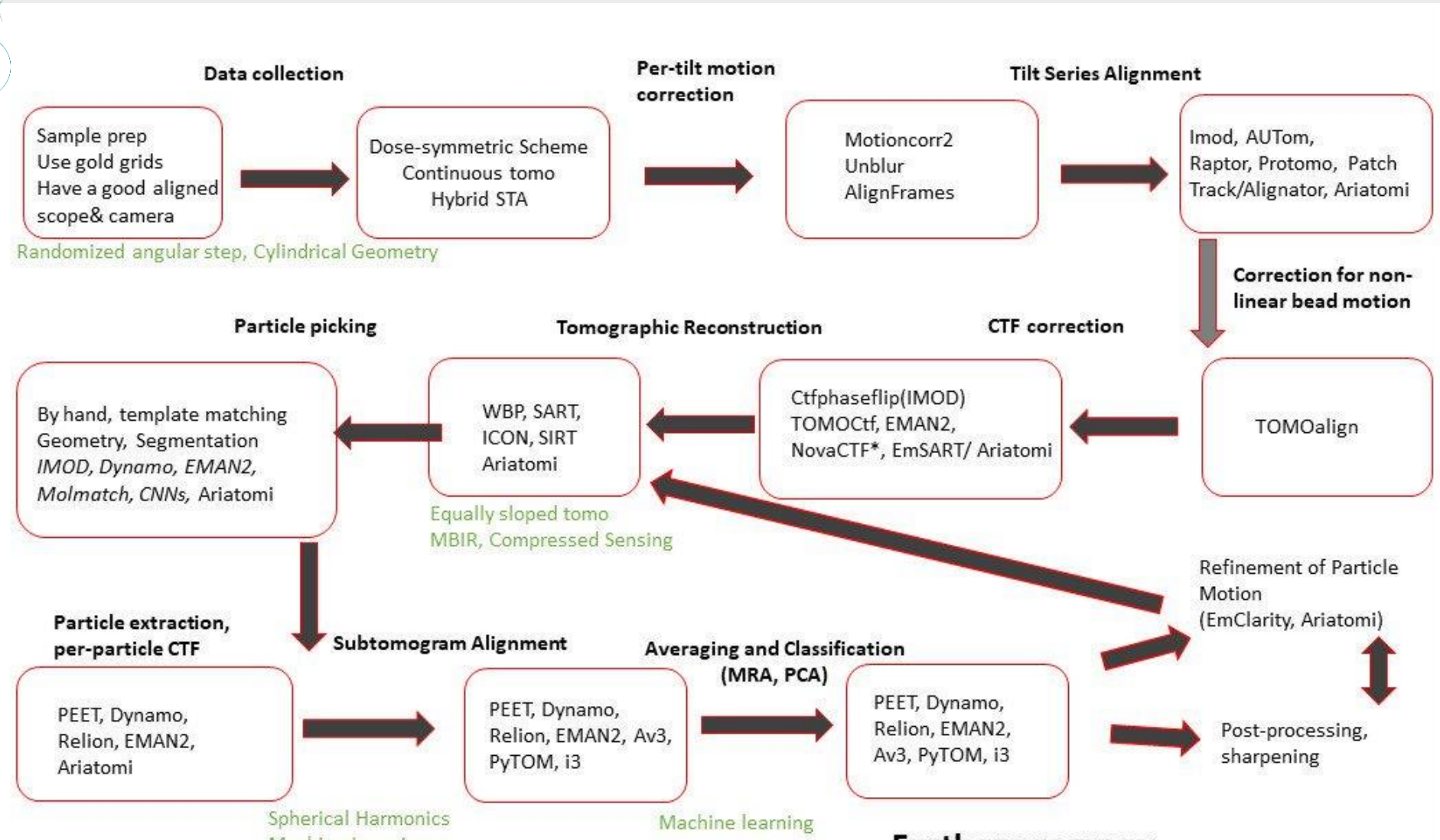


- TomoDRGN (soon?)



- 3DFlex?

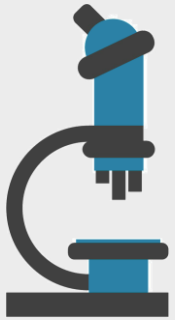




Leigh et al., 2019

Make sure you, your collaborators, and your PI have **reasonable expectations** at the beginning of tomo projects

Non-ribosome projects generally take **years**



Thank you!
Questions?