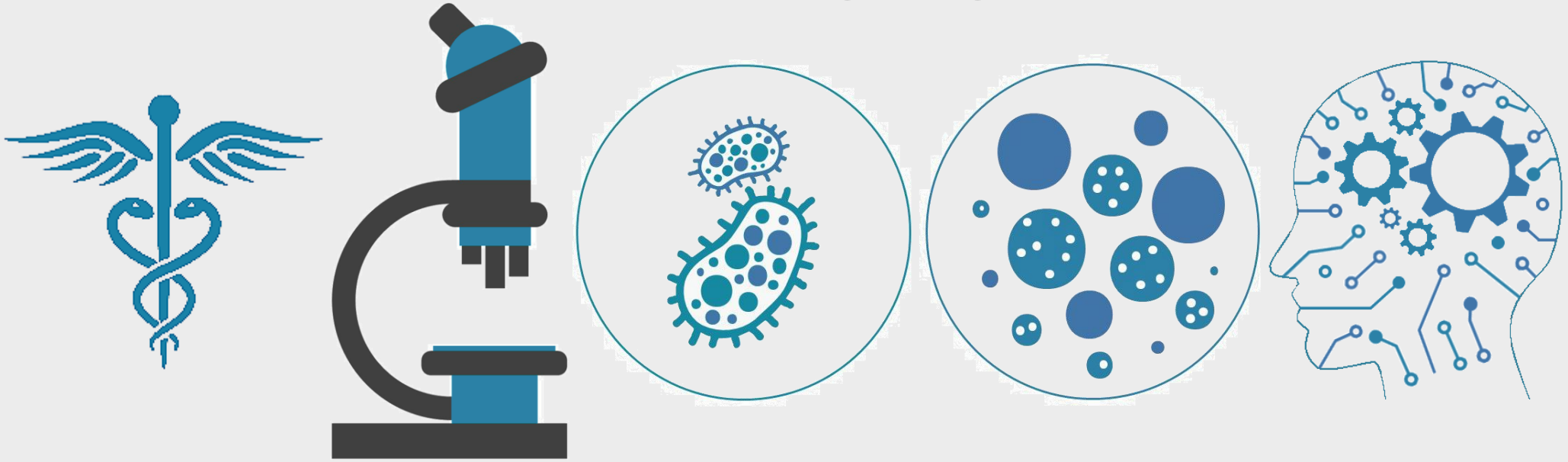
Cryo-Applications and sub-tomogram averaging



Tomography Short Course!

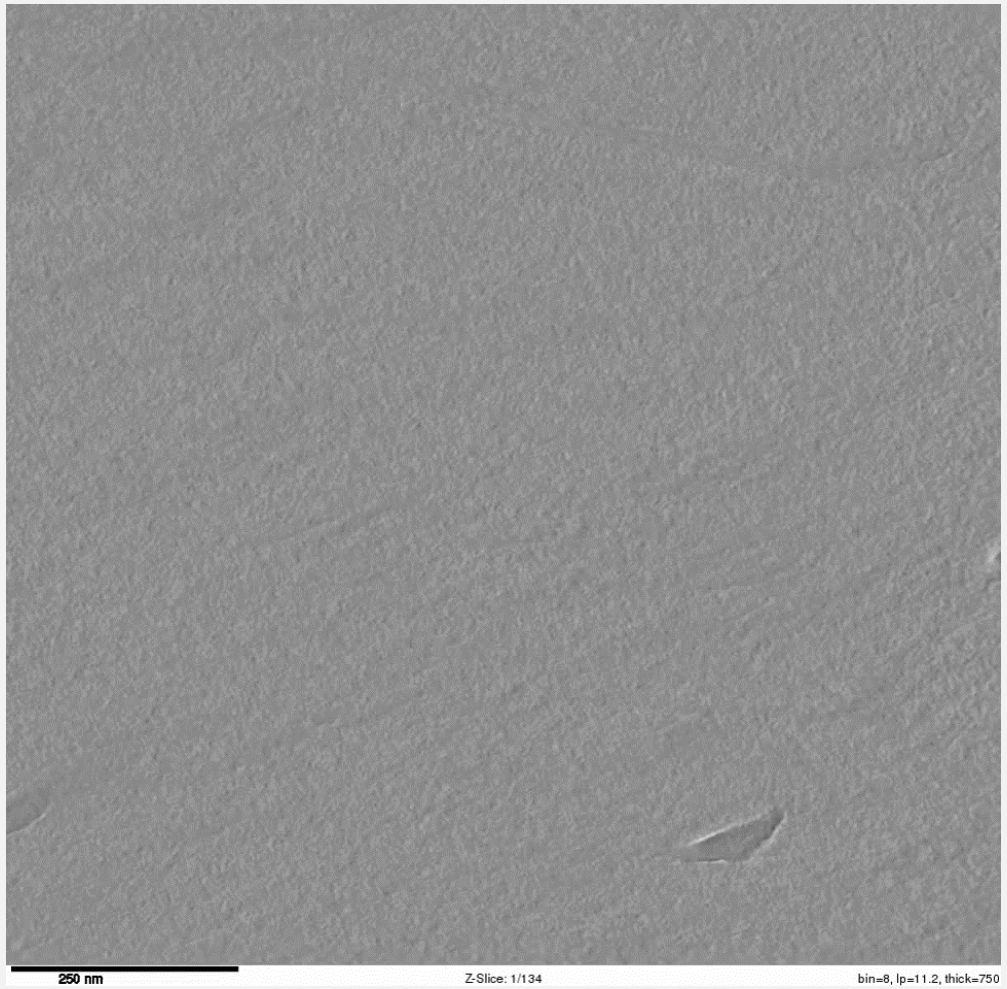
4-12-21

Alex Noble
anoble@nysbc.org

What is CryoET?

(cryo-electron tomography)

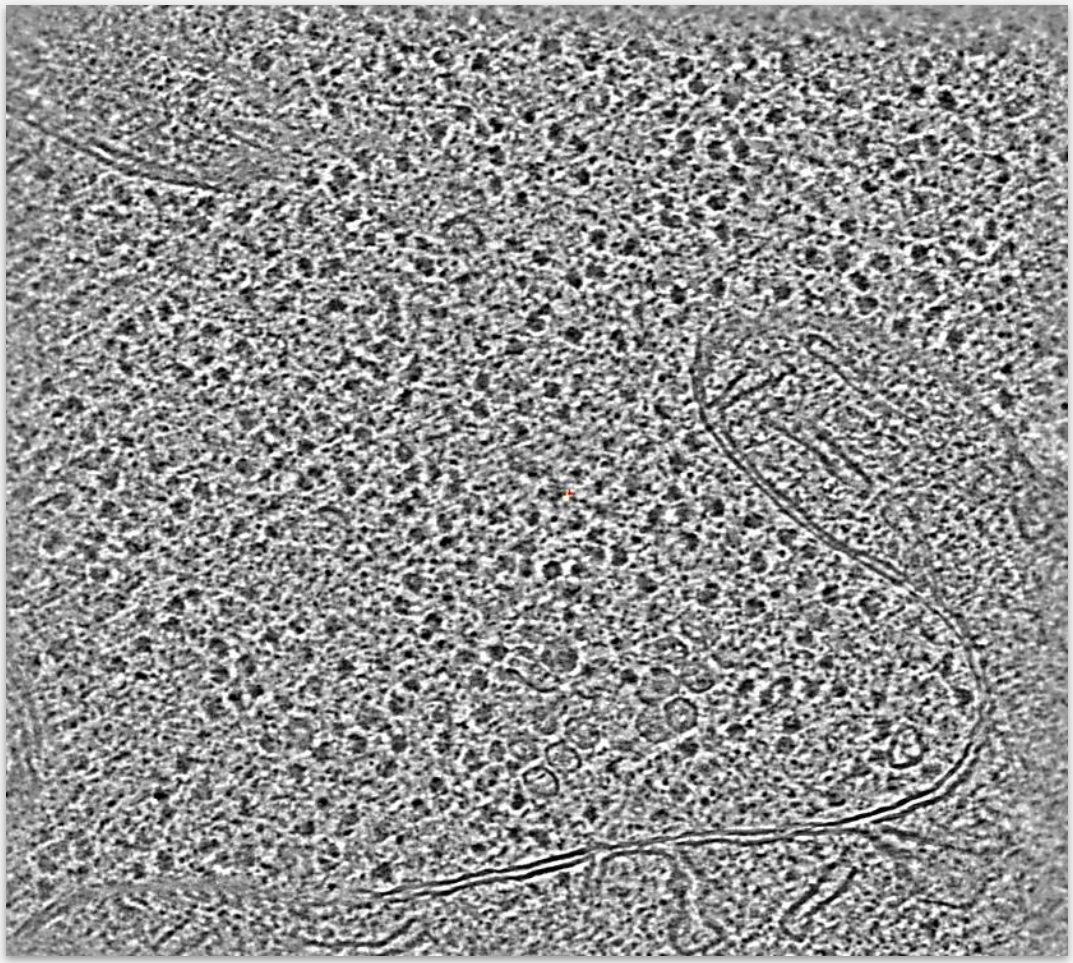
- Cells or complex reconstituted environments



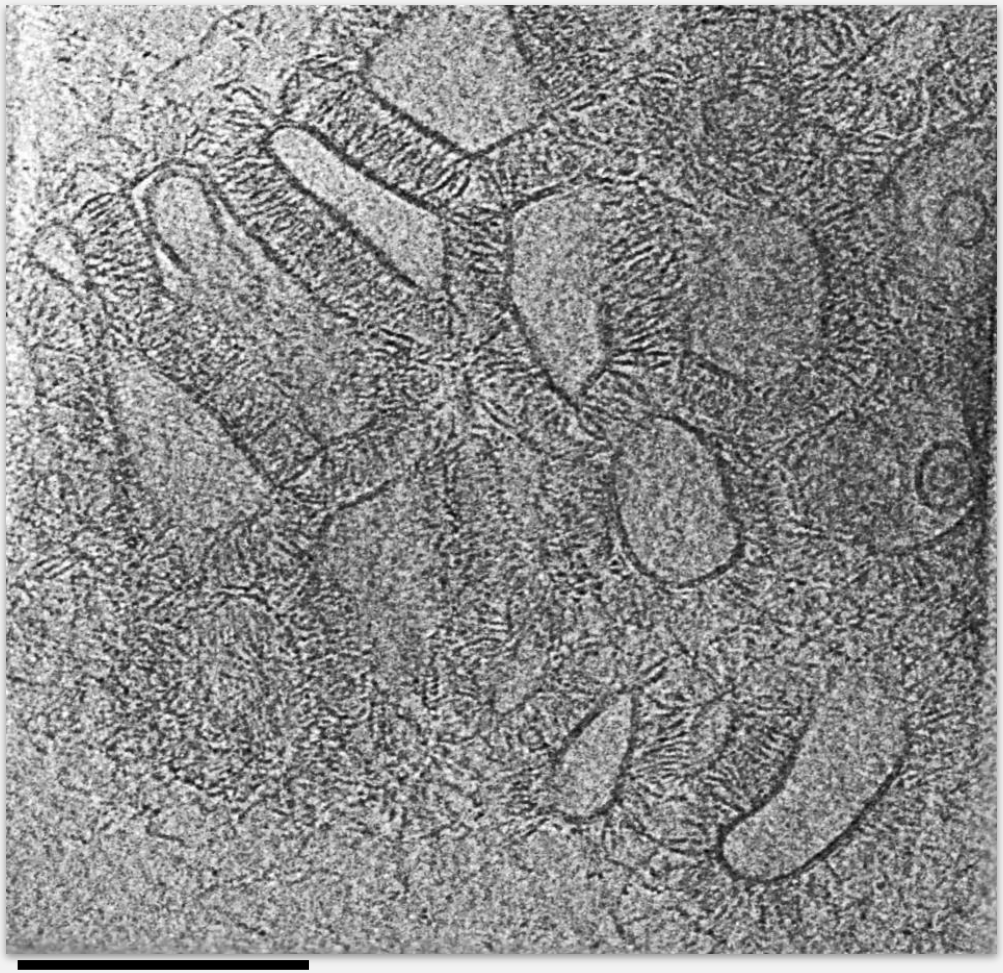
Tan ZY et al., bioRxiv 2021

Brasch J et al., Nature 2019

What is CryoET?
(cryo-electron tomography)

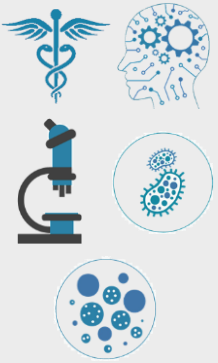


Tan ZY et al., bioRxiv 2021



Brasch J et al., Nature 2019

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



Overview – Why CryoET?

Why **cryo**?

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

Why **electrons**?

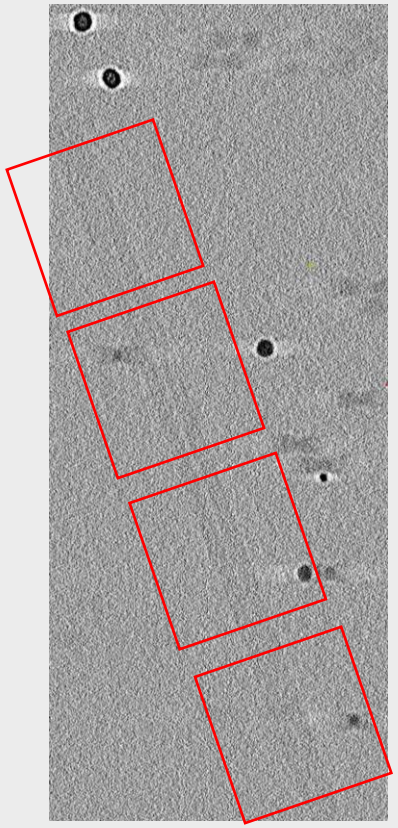
- **+**Small wavelengths (**high res**), **+**Can be focused, **–**Damage sample

Why **tomography**?

- Some combination of:
  - Sample is **unique**; e.g. cells,
  - Sample is too **heterogeneous** (structurally or morphologically); e.g. viruses with variable # of receptors, or viruses of different non-symmetric shapes,
  - **Domain-stoichiometry** and/or orientation is required,
  - **Sub-nanometer** information is usually **not** required, but may be possible.

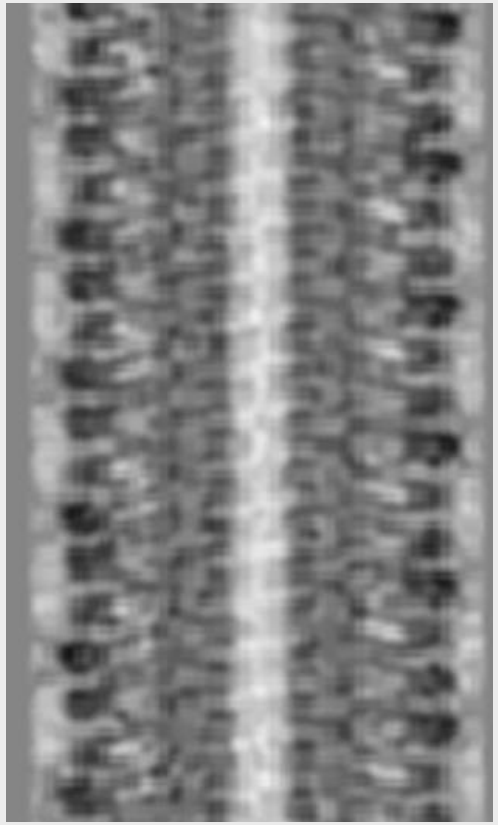
Overview – Why subtomogram averaging?

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



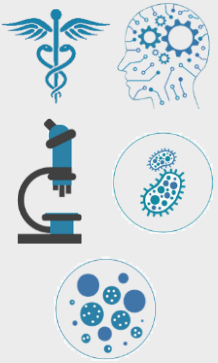
particles

align



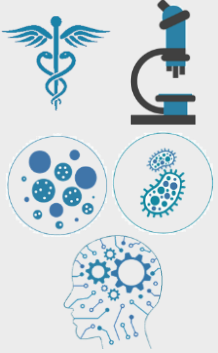
reconstruction

Courtesy of Misha Kudyashev



Overview

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

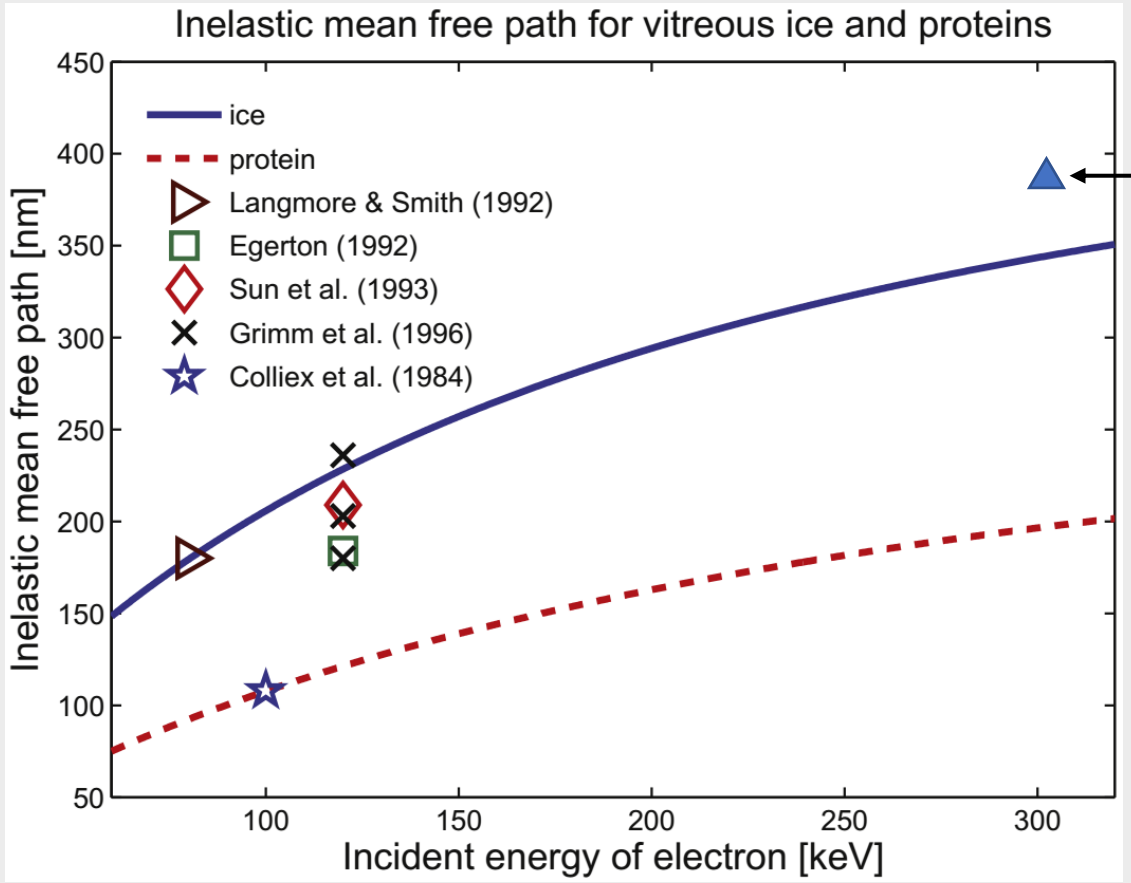


CryoET Limitations

Overview – Limitations

Limitation: Specimen/Ice **thickness**

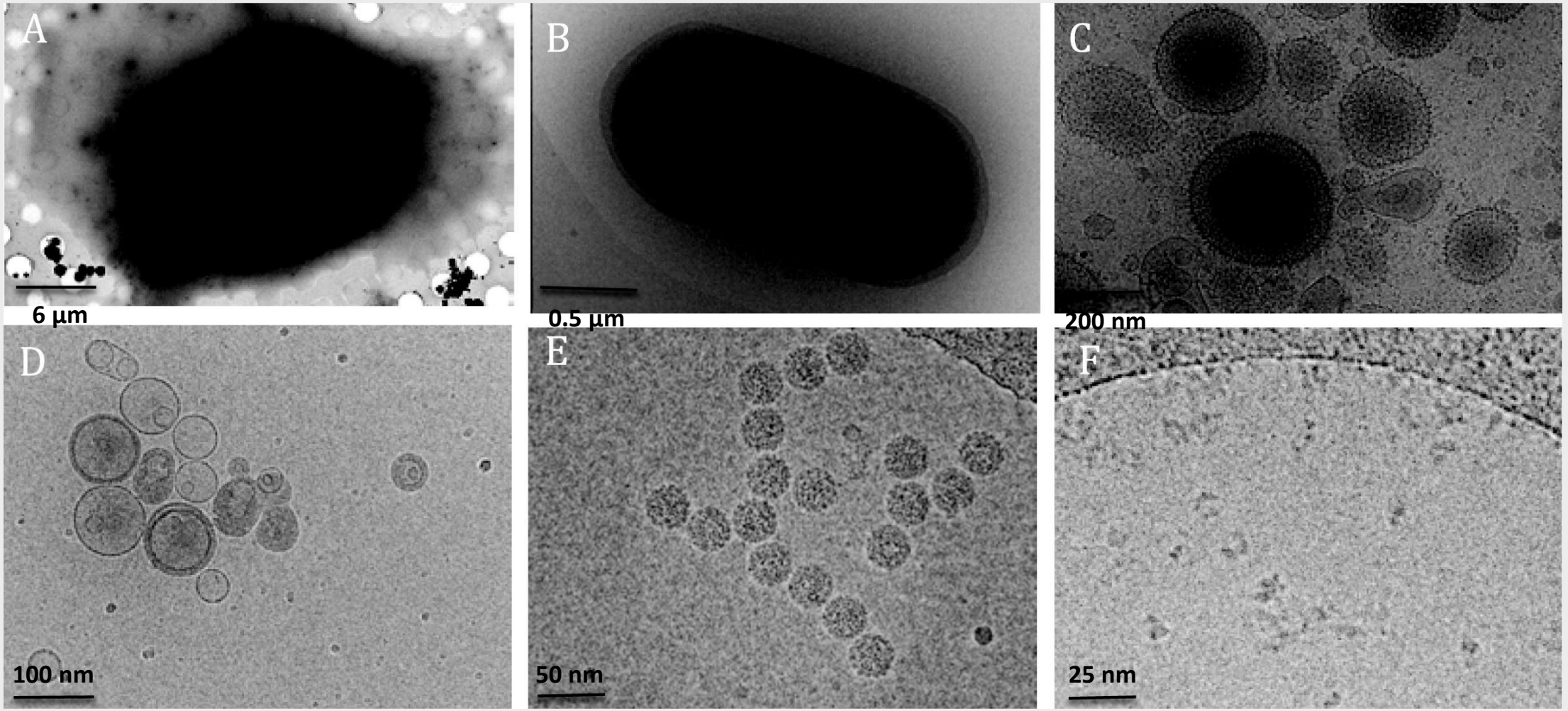
Vulović, 2013



William J. Rice, NYSBC, 2017
300 keV Krios

Overview – Limitations

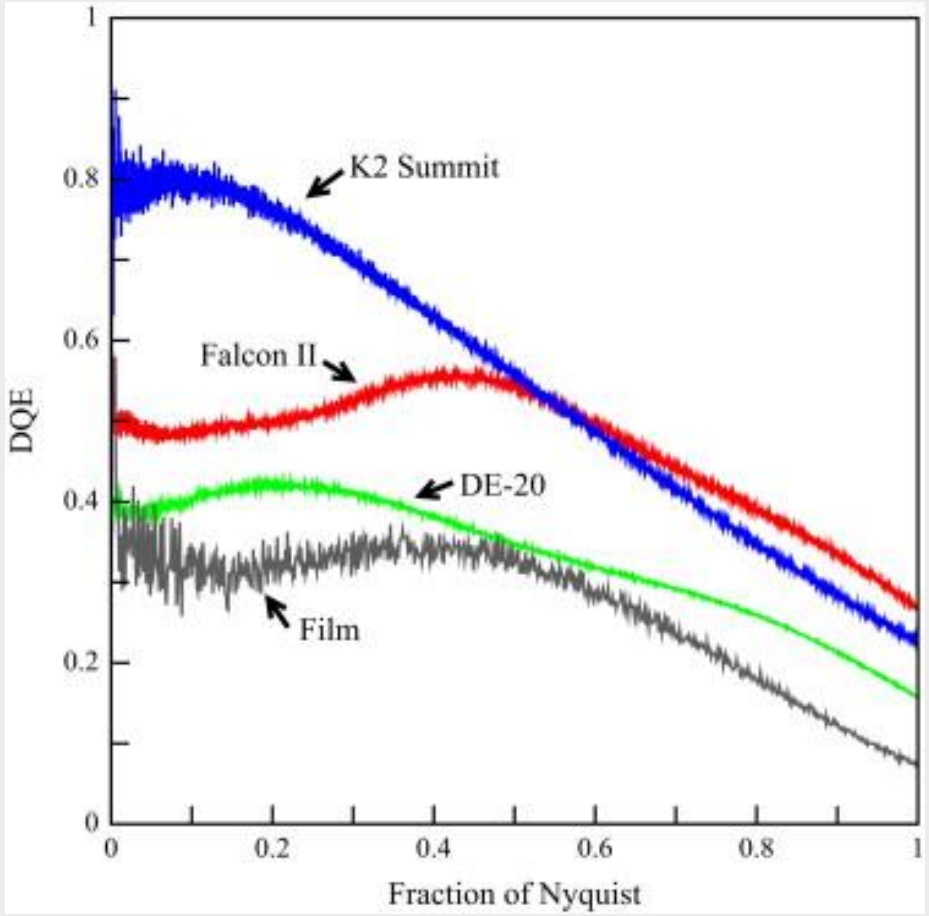
Limitation: Specimen/Ice **thickness**

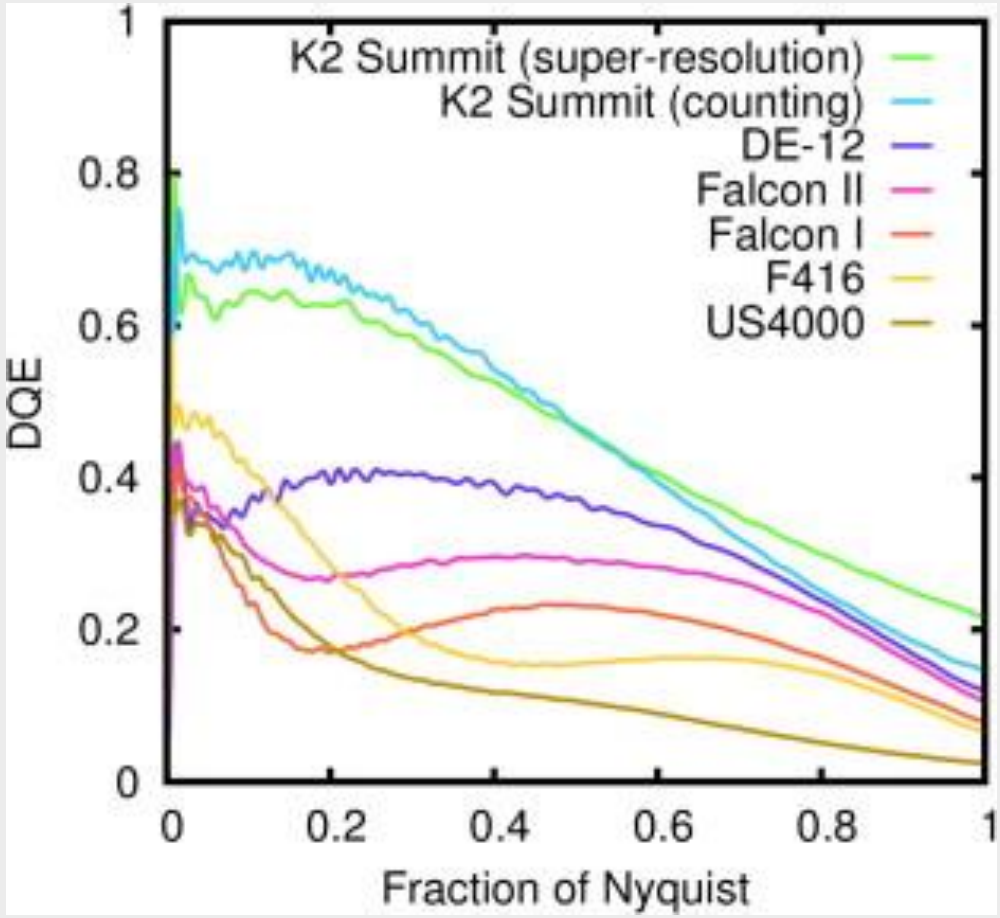


Thompson et. al., 2016

Overview – Limitations

Limitation: Camera fidelity



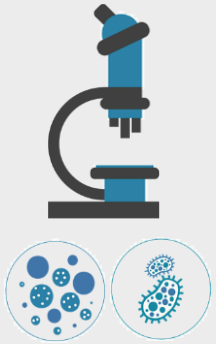


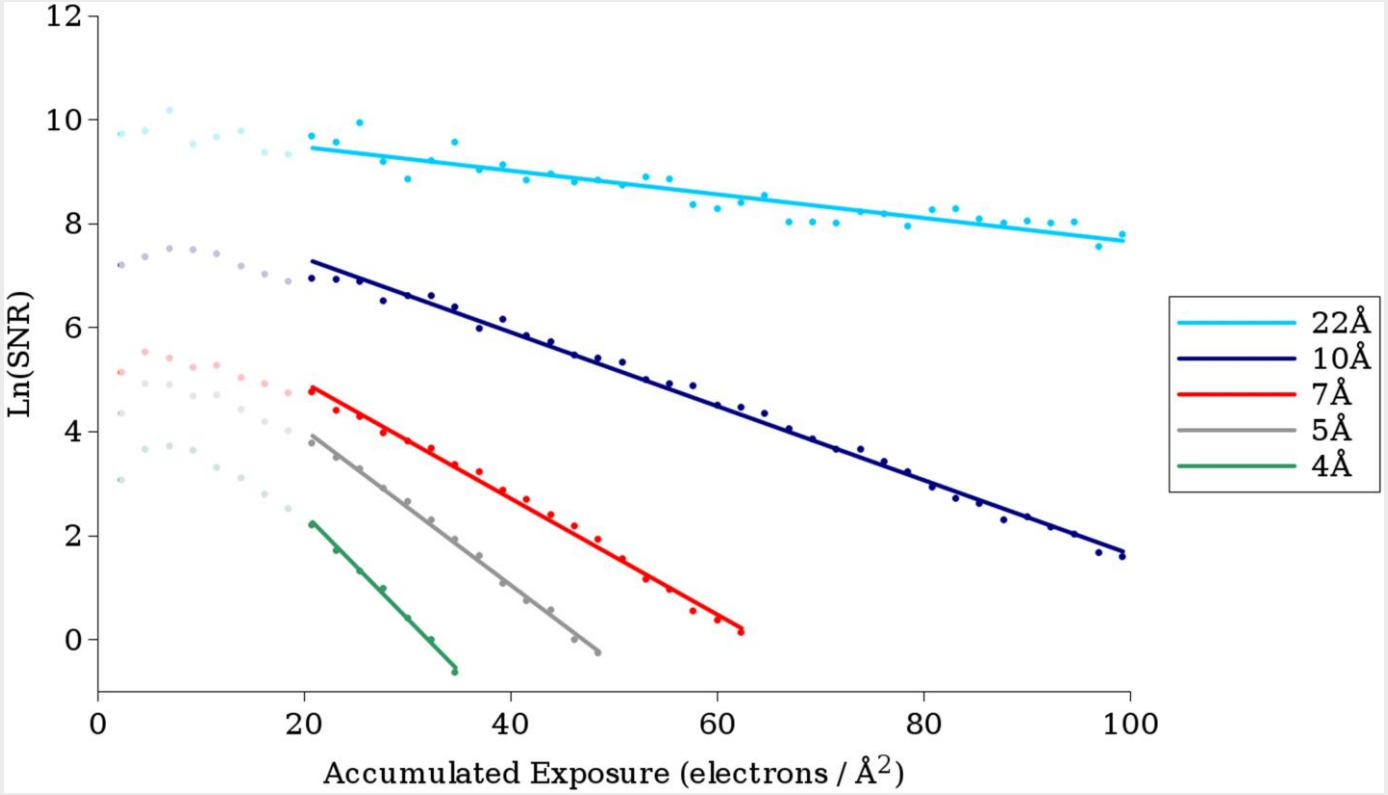
McMullin, 2014 &
Ruskin, 2013

Overview – Limitations

Limitation: **Electron damage** of the specimen

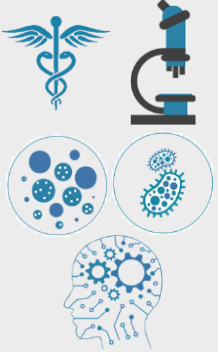
- **High resolution information is lost first**.



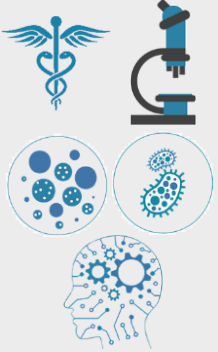


Grant & Grigorieff, 2015

Solution:
Remove damaged information from image frames



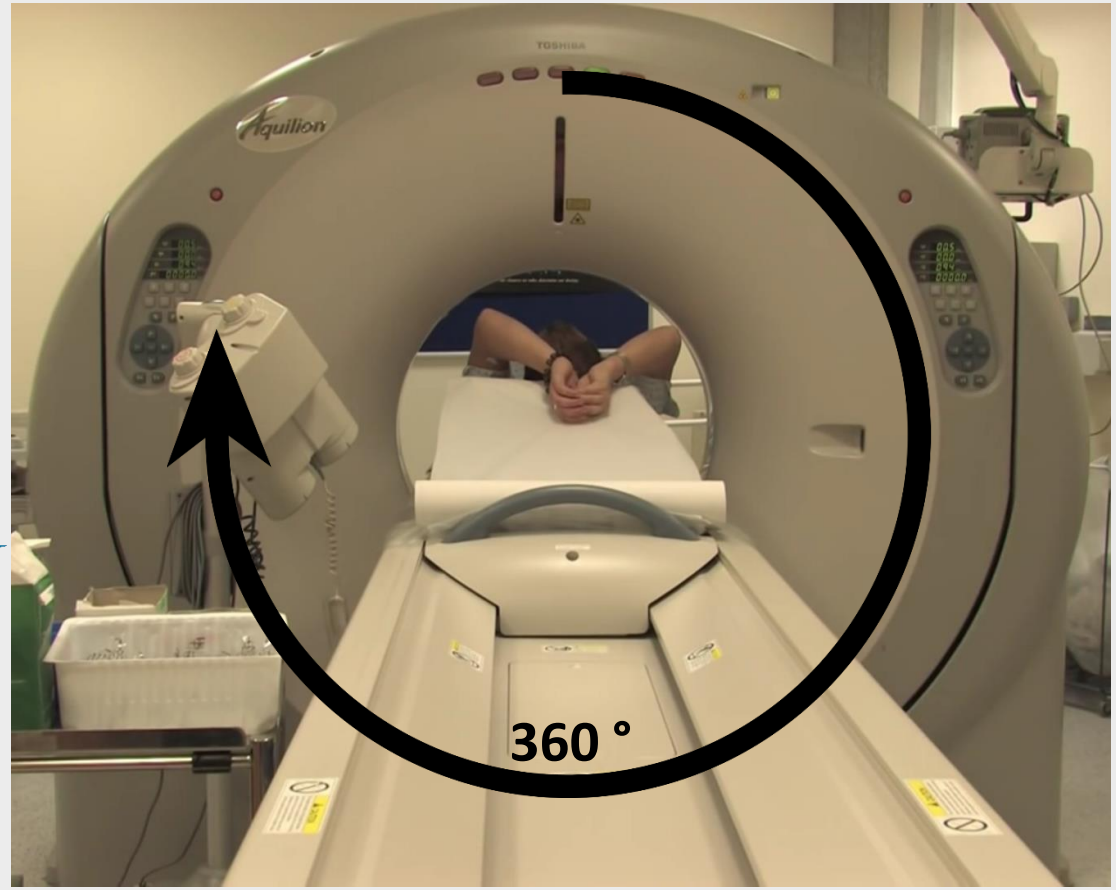
Tomography overview

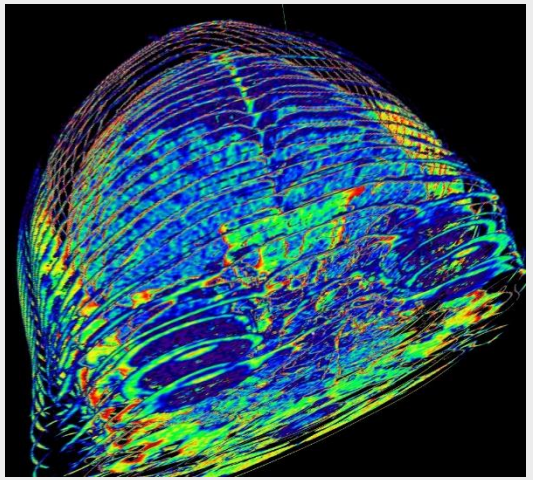


Tomography overview

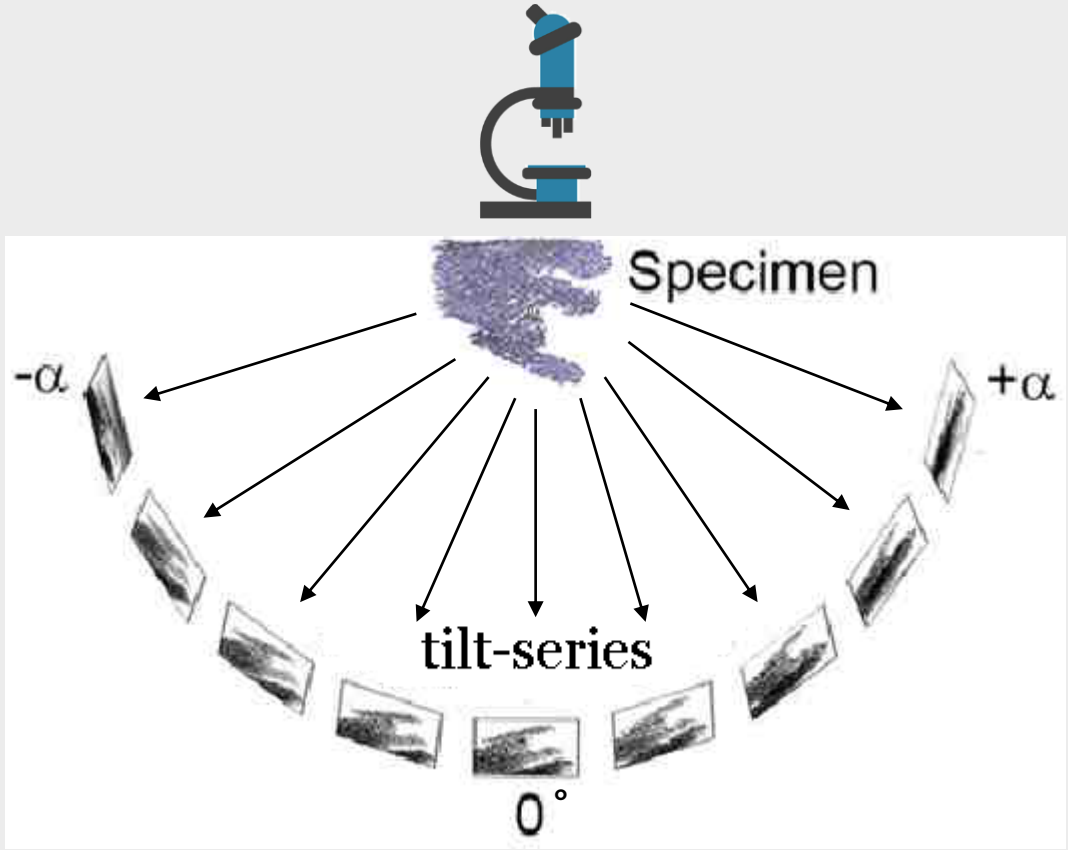
Tilt-series Collection

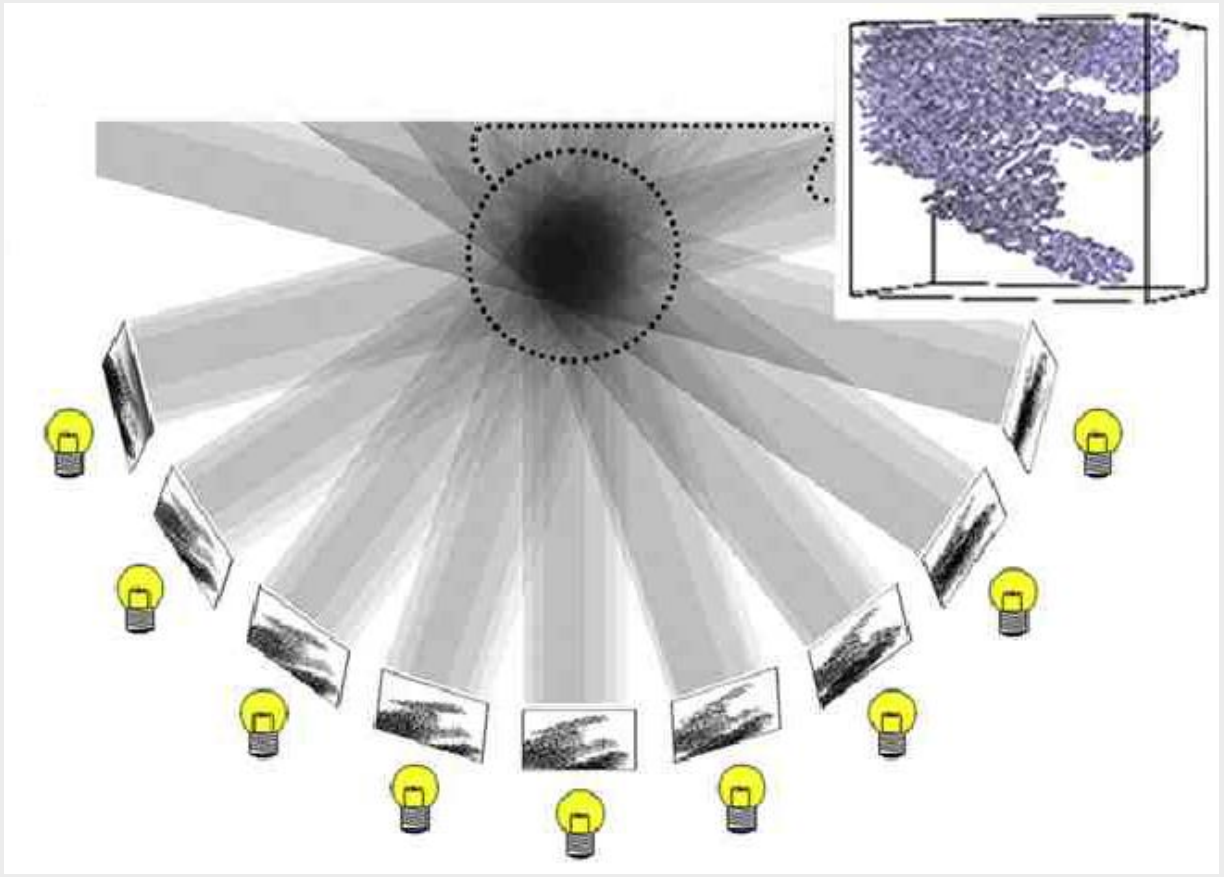
Tomography overview





ET/CryoET collection and processing overview

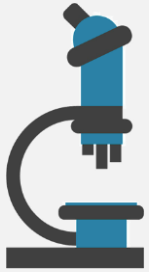


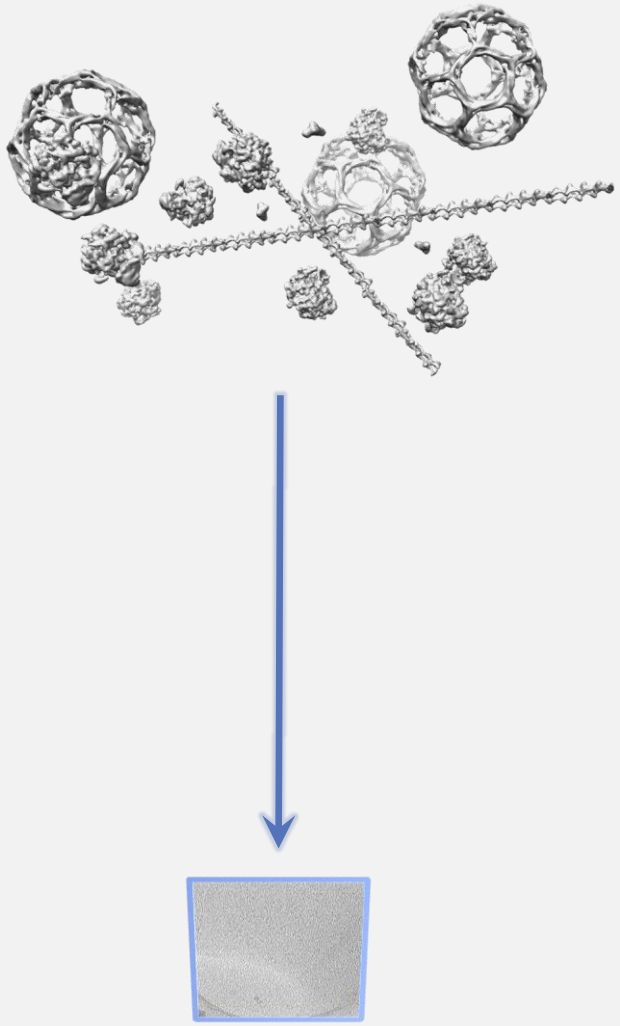


Collect → align → reconstruct

(UMET)

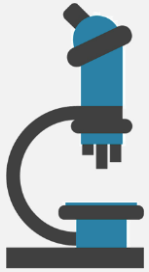
3D specimen movement during collection

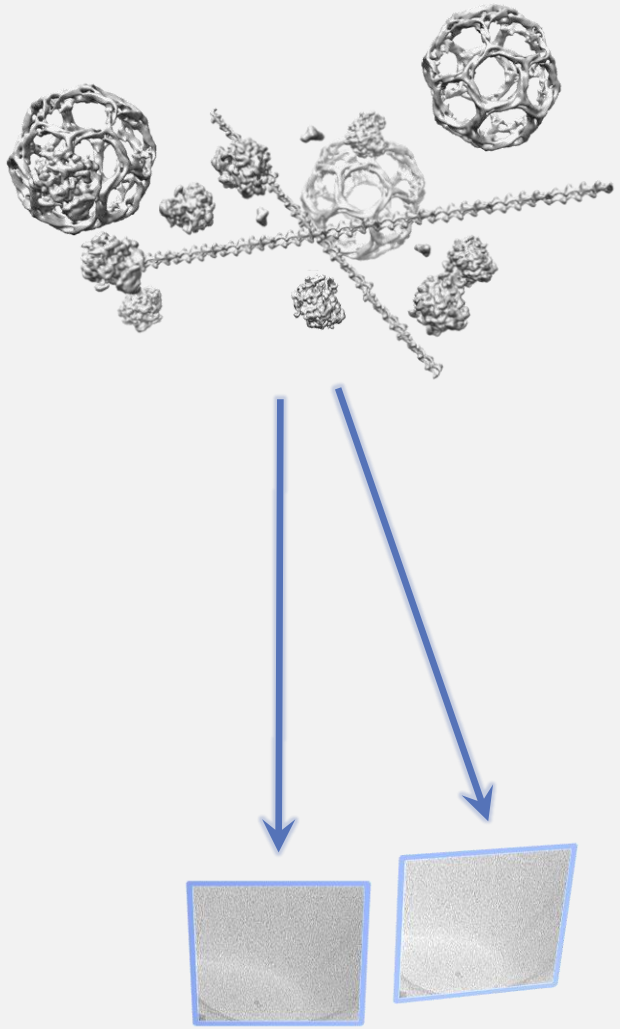




(movements are exaggerated)

3D specimen movement during collection





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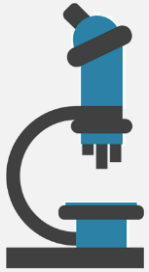
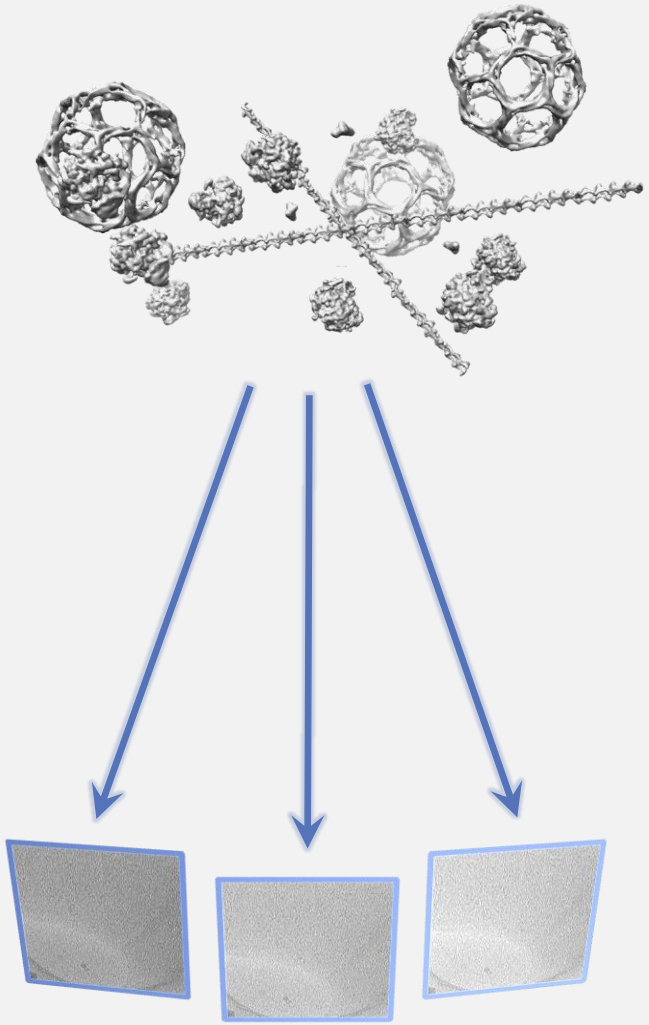
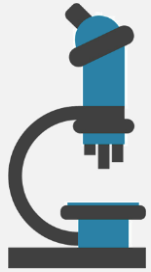
3D specimen movement during collection

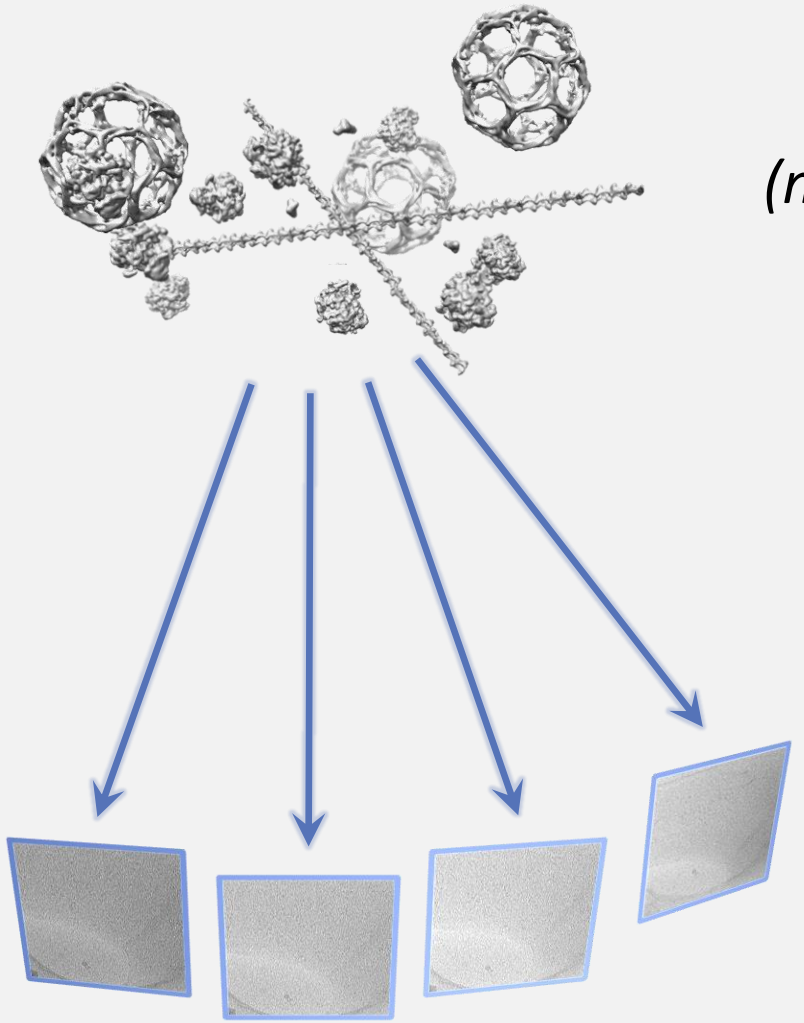




(movements are exaggerated)

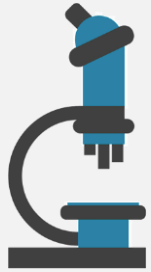
3D specimen movement during collection

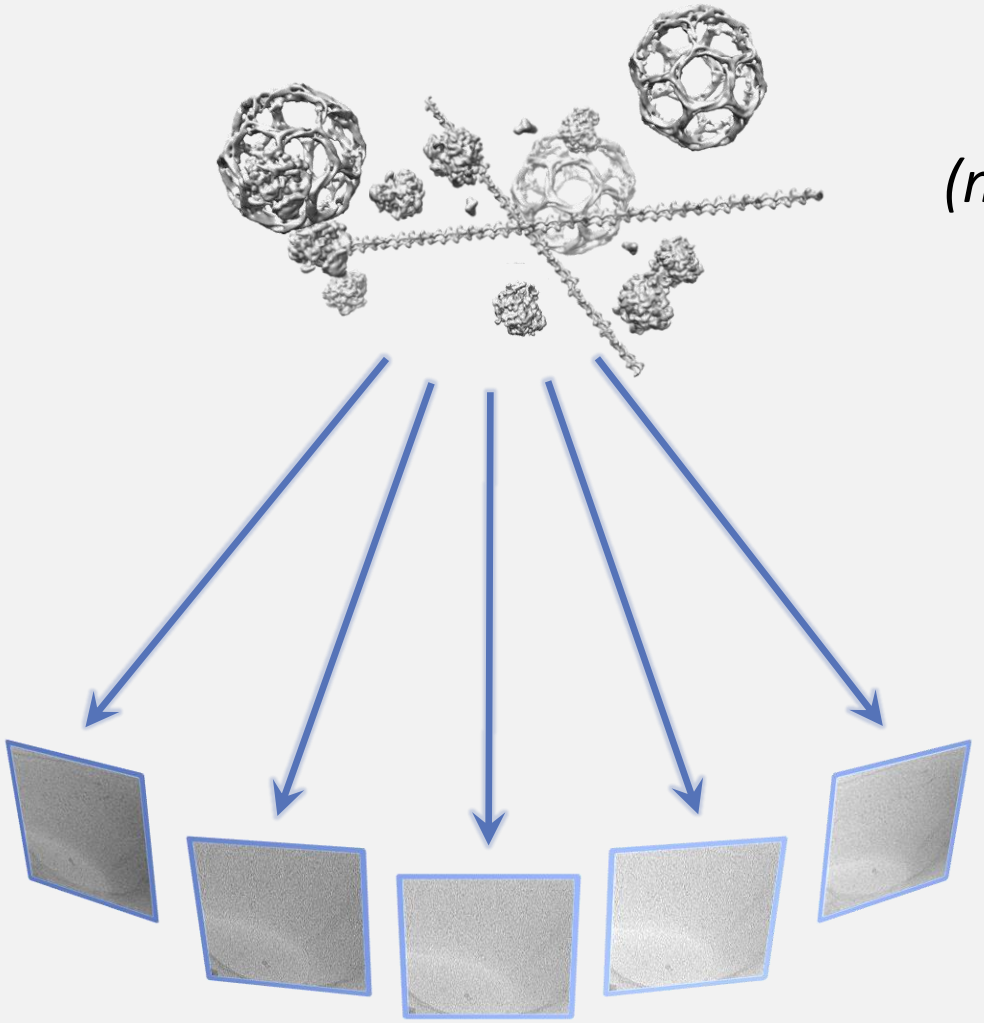




(movements are exaggerated)

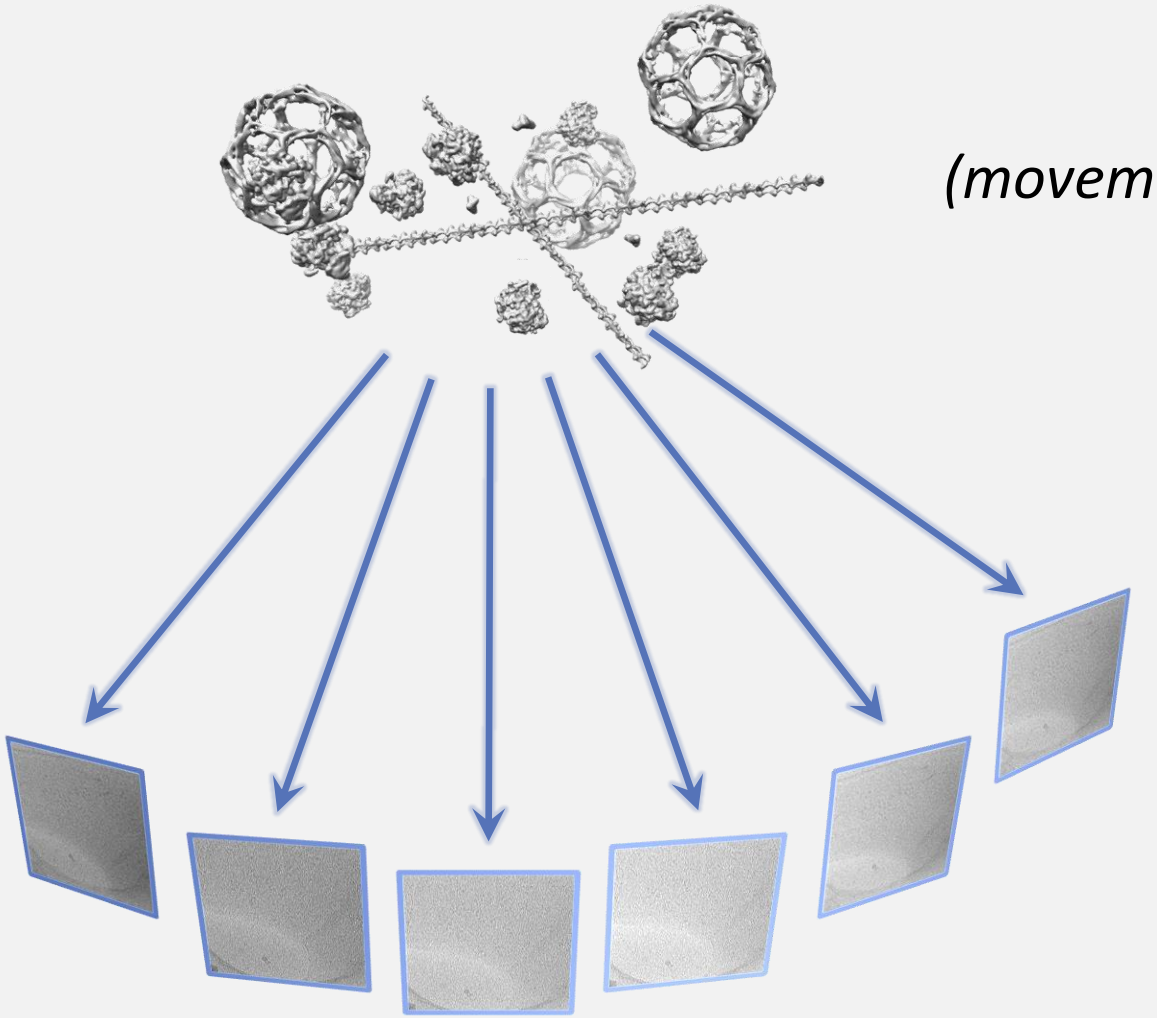
3D specimen movement during collection





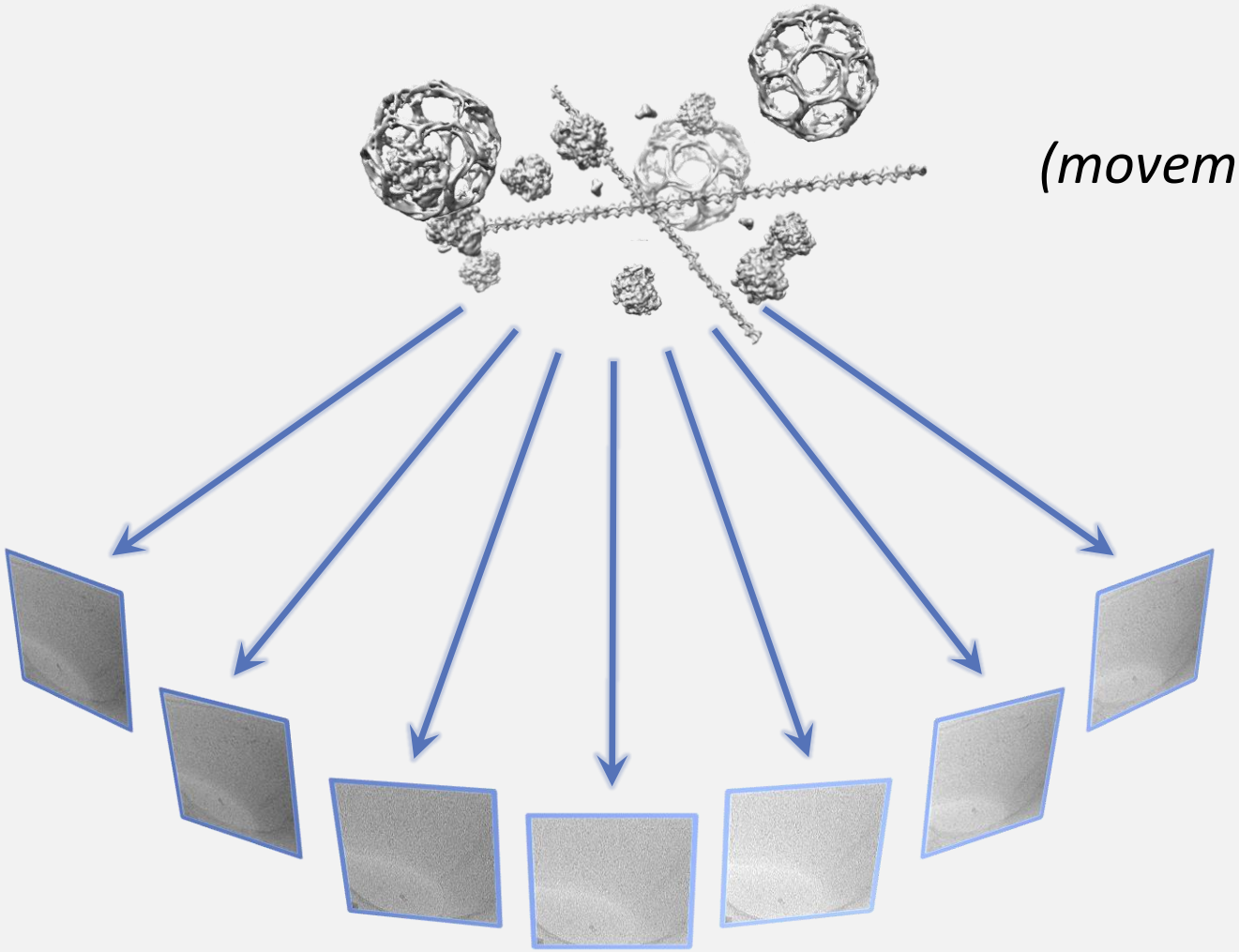
(movements are exaggerated)

3D specimen movement during collection



(movements are exaggerated)

3D specimen movement during collection



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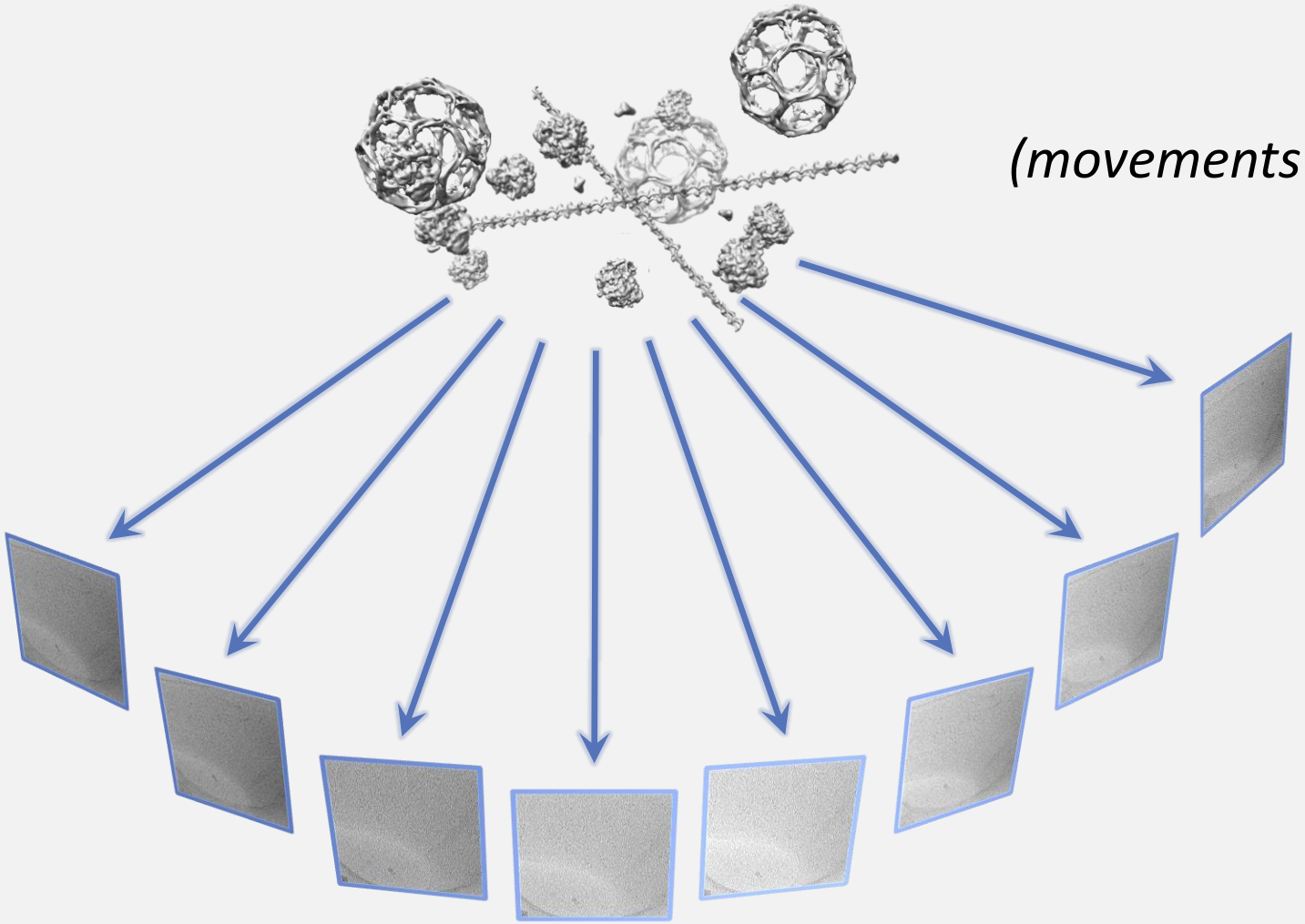
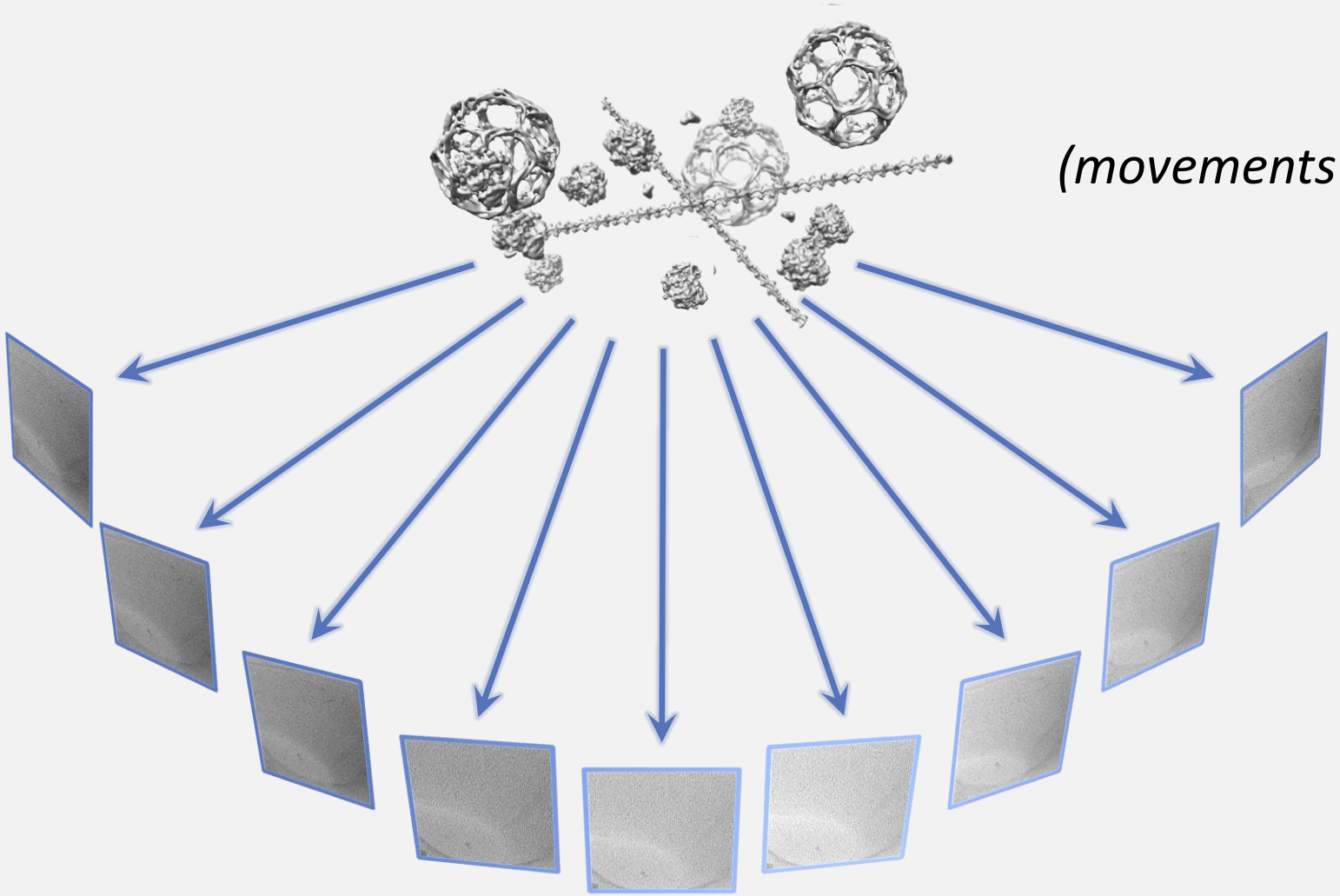
3D specimen movement during collection

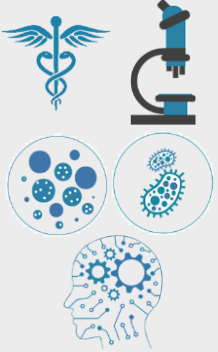


(movements are exaggerated)

3D specimen movement during collection

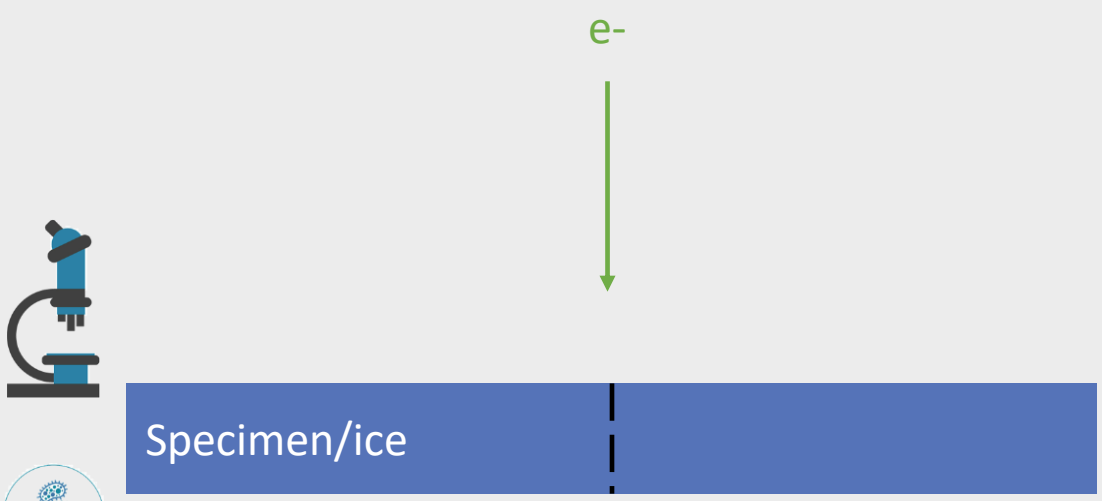


(movements are exaggerated)

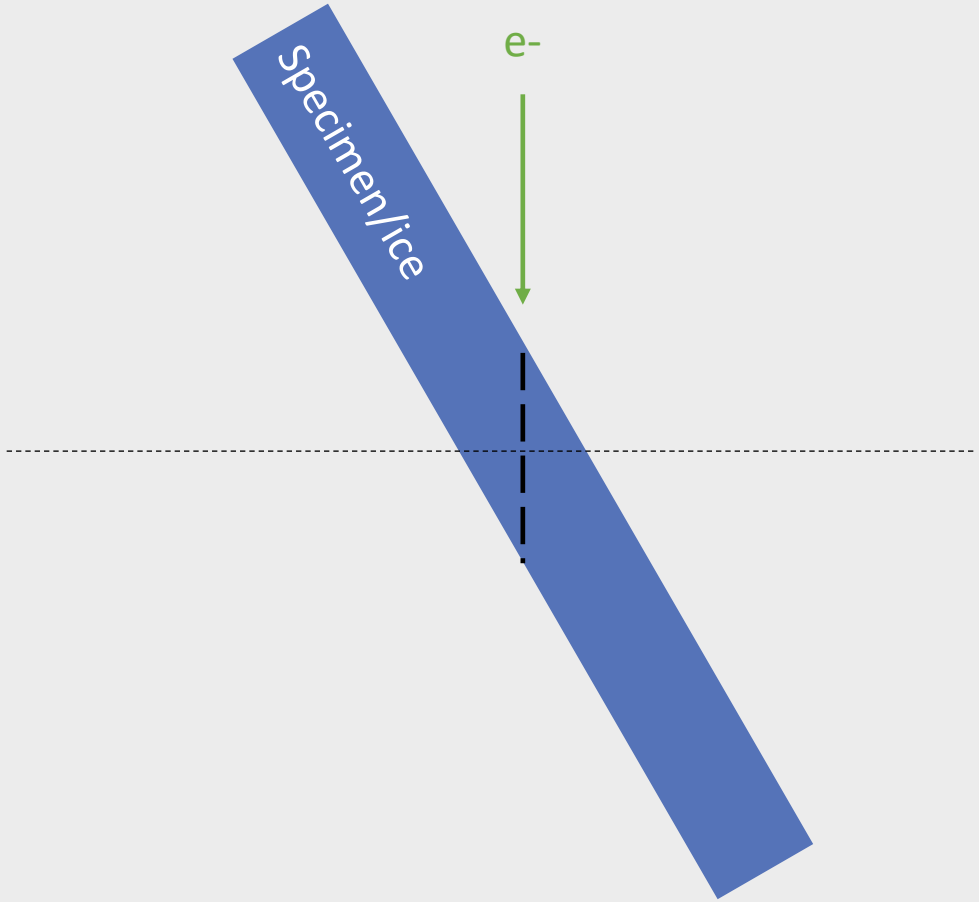


Some more CryoET Limitations

Grid tilting increases thickness

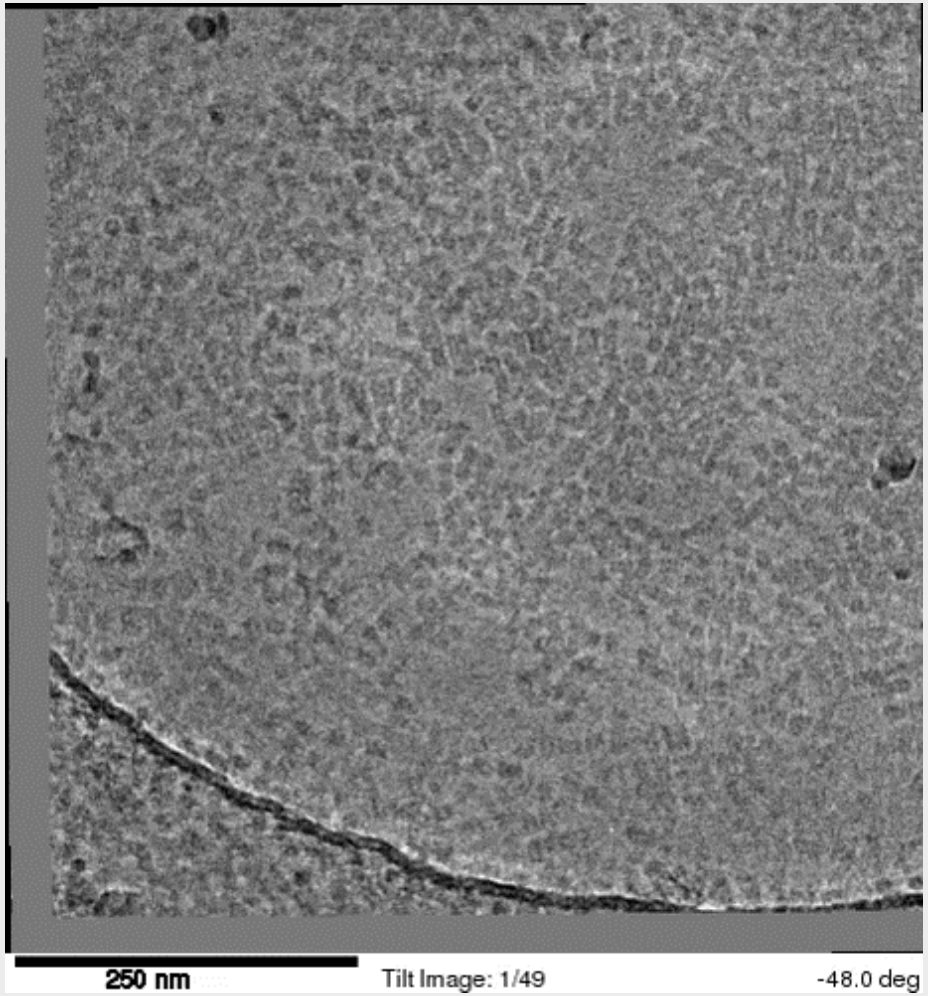


untilted grid



grid tilted 60° = 2x thickness

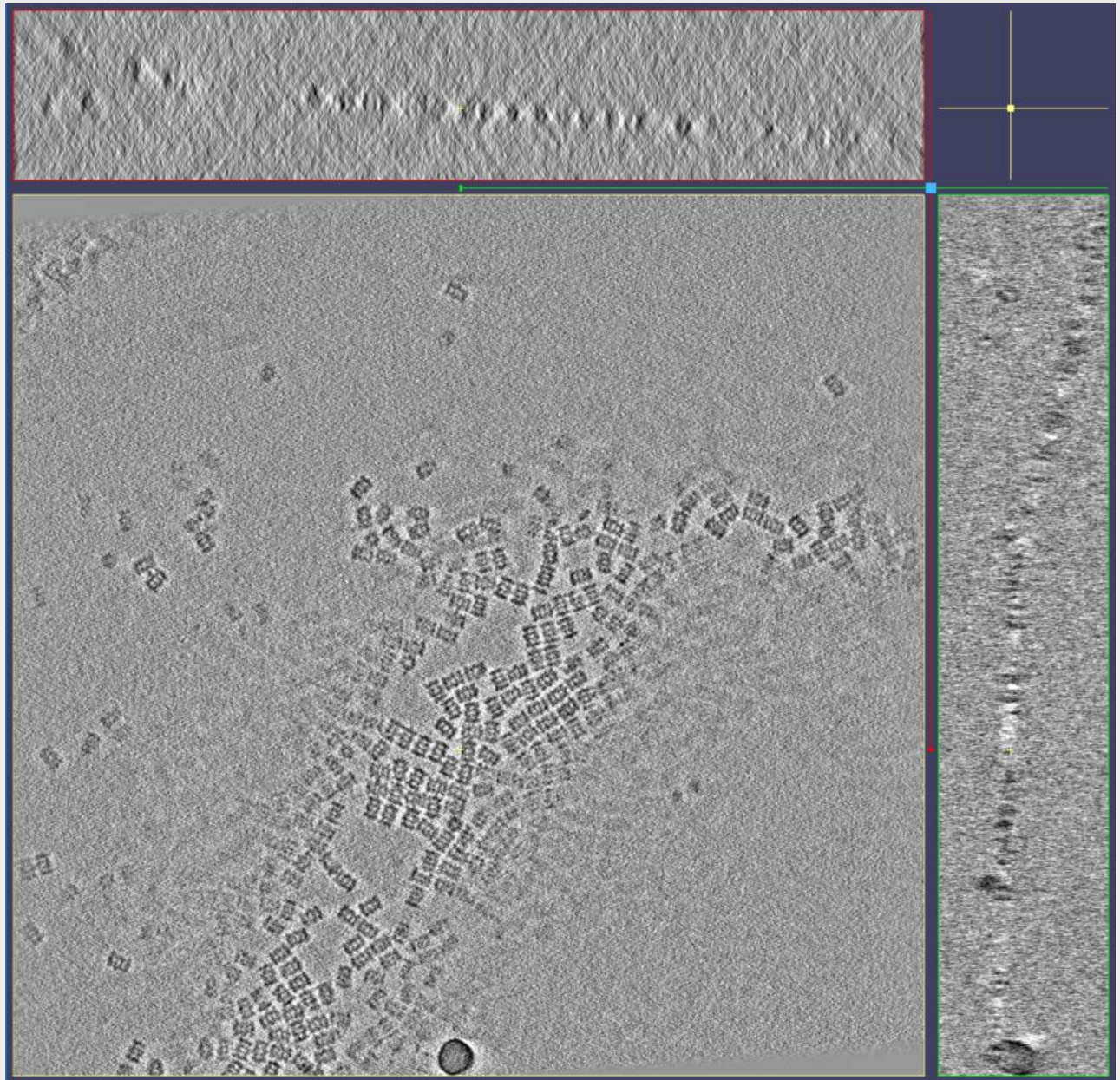
Grid tilting thickness increase limits tilting



Noble et al., eLife 2018

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

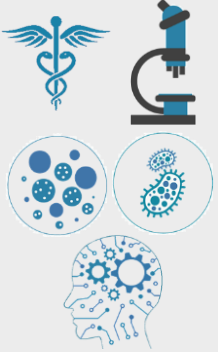
Grid tilting limit results in missing information



Phase plate tilt-series of T20S Proteasome.

Tilt axis is **vertical**

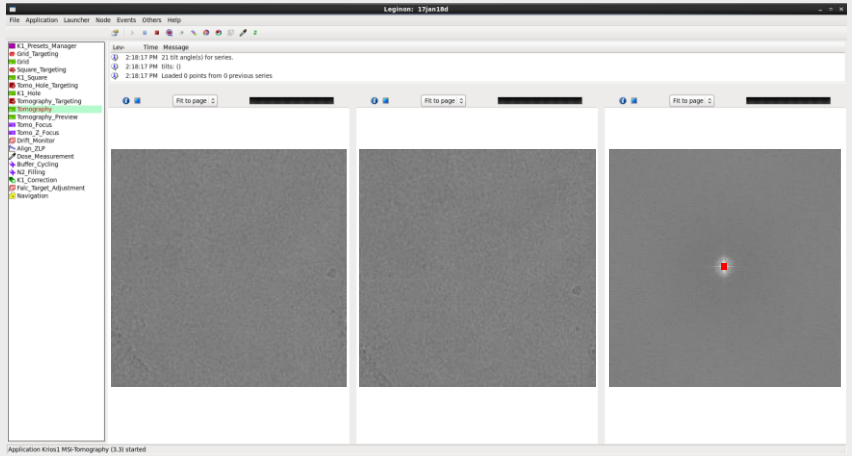
Noble et al., eLife 2018



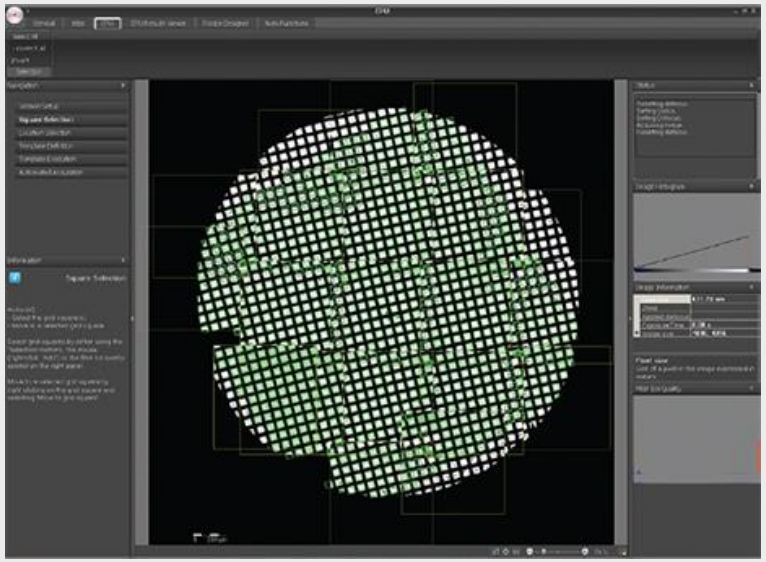
Tilt-series collection

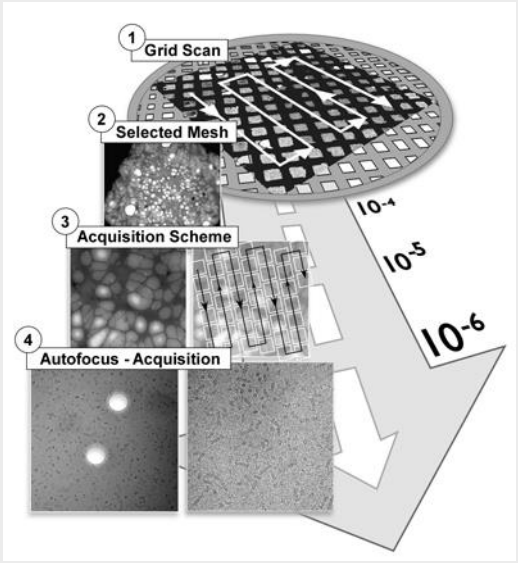
Tilt-series collection software

Leginon

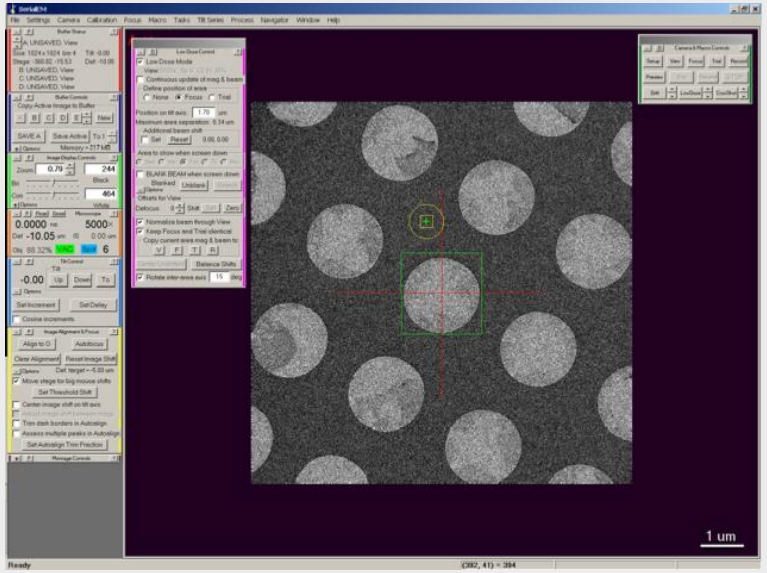


EPU

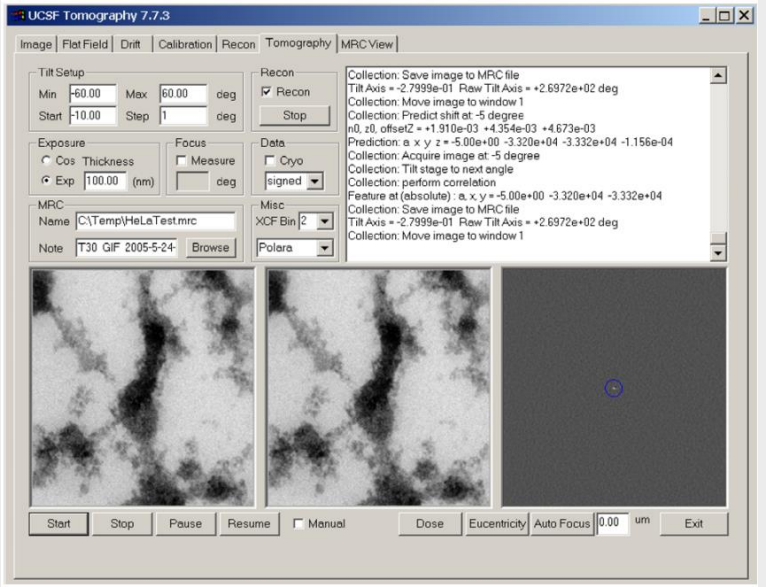




TOM Toolbox

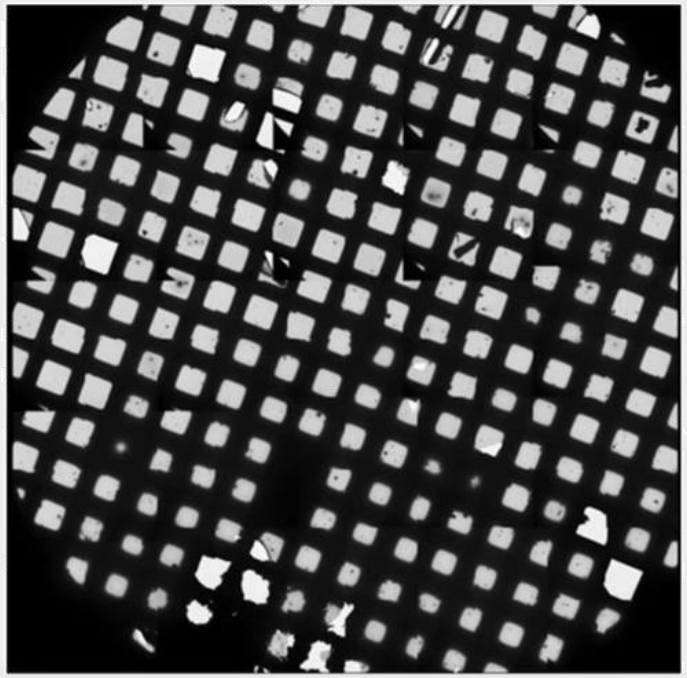
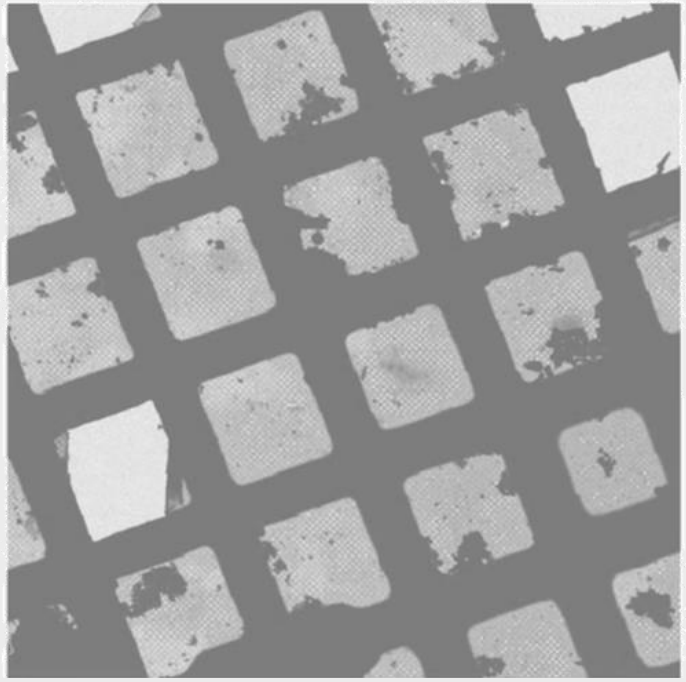
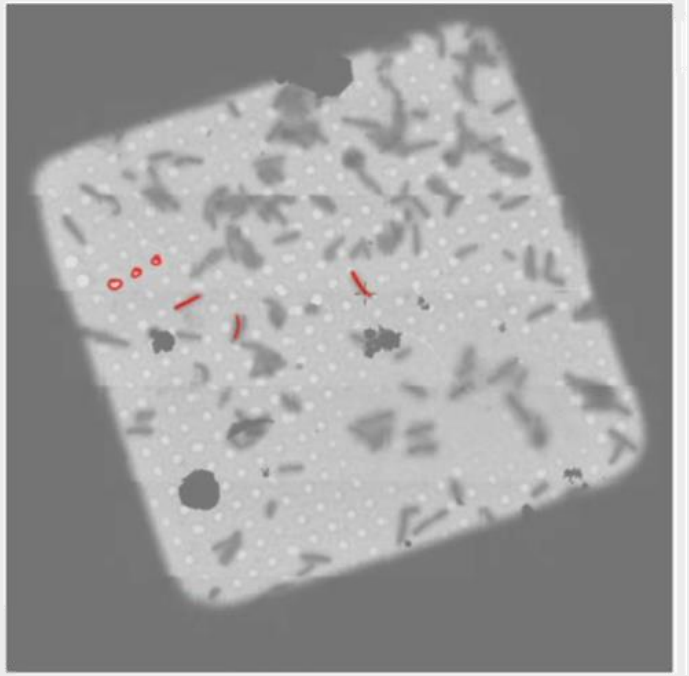


SerialEM



UCSF Tomography

Automated tilt-series collection

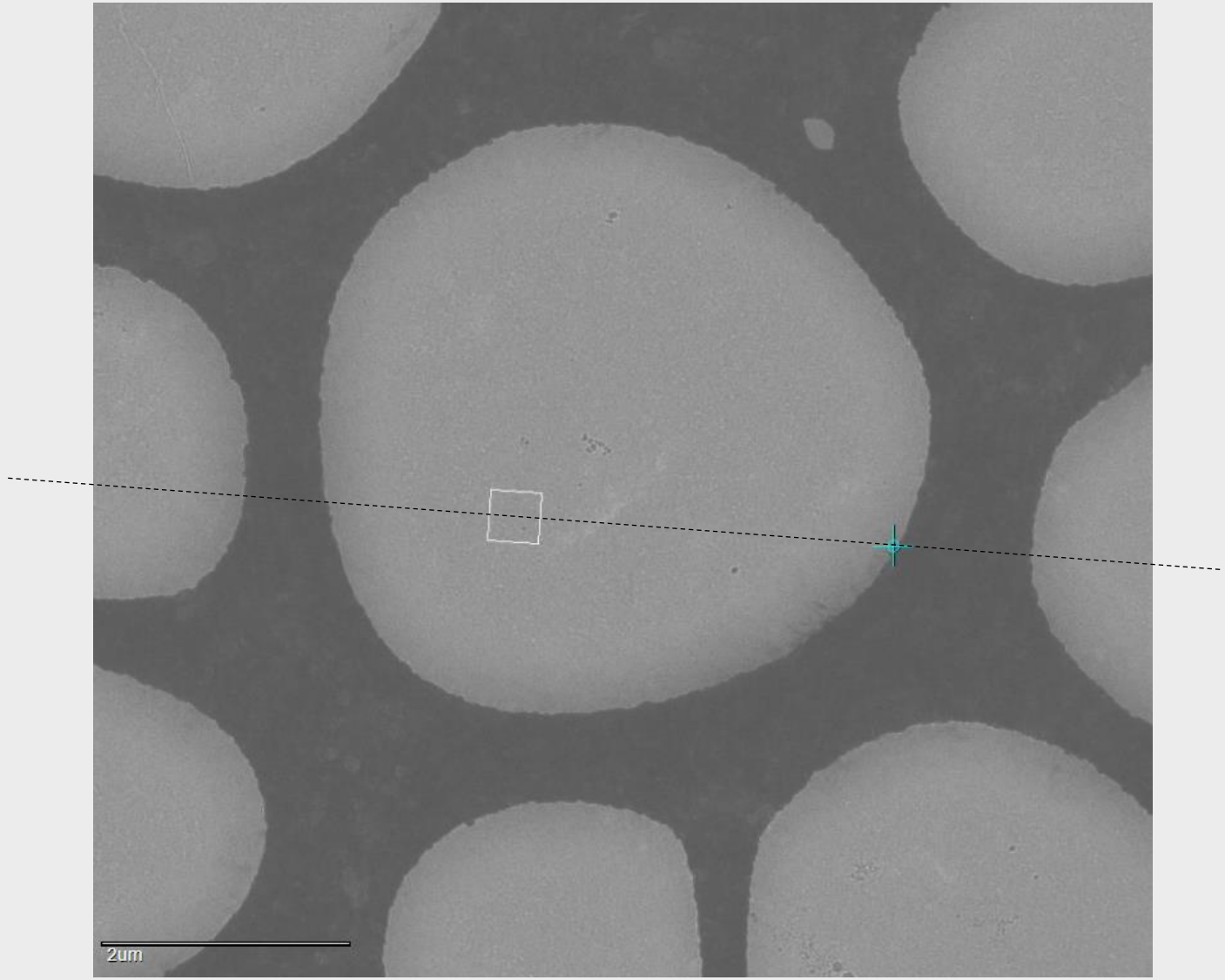
Automated tilt-series collection is currently **routine**

- 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!

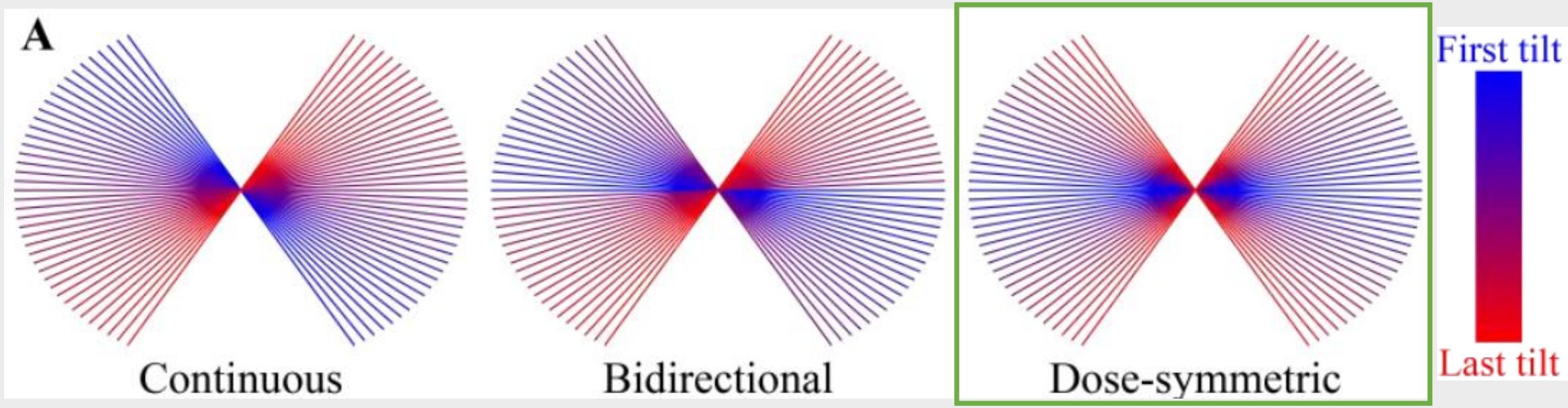
Automated tilt-series collection

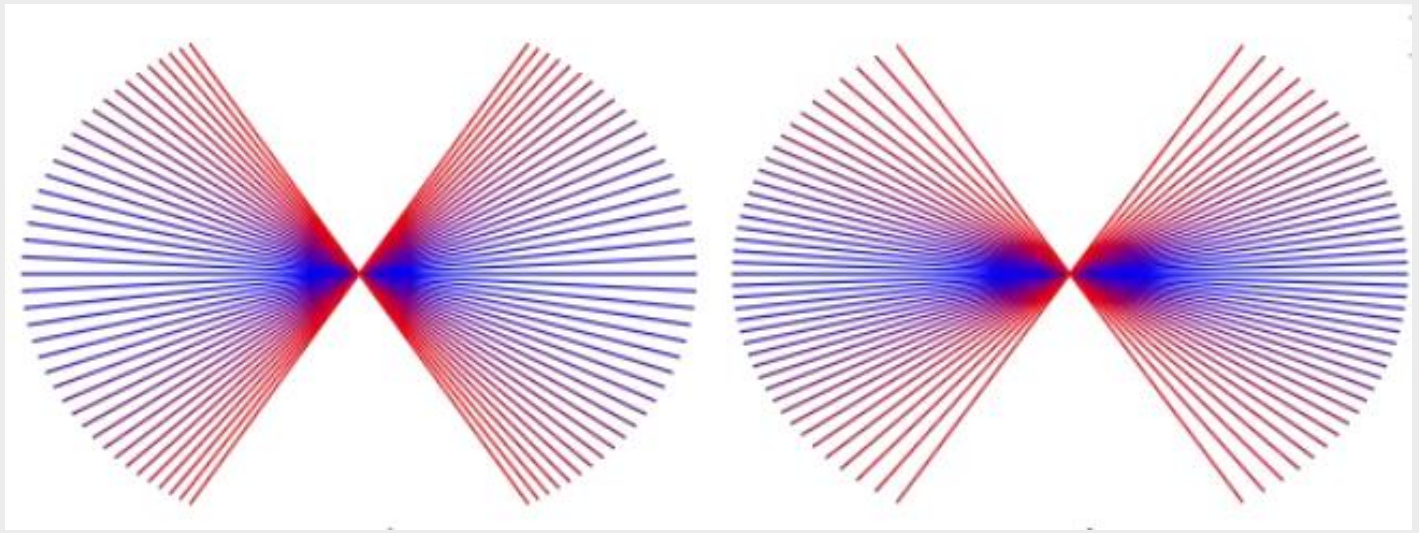
Focus on the tilt axis!

- 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**.



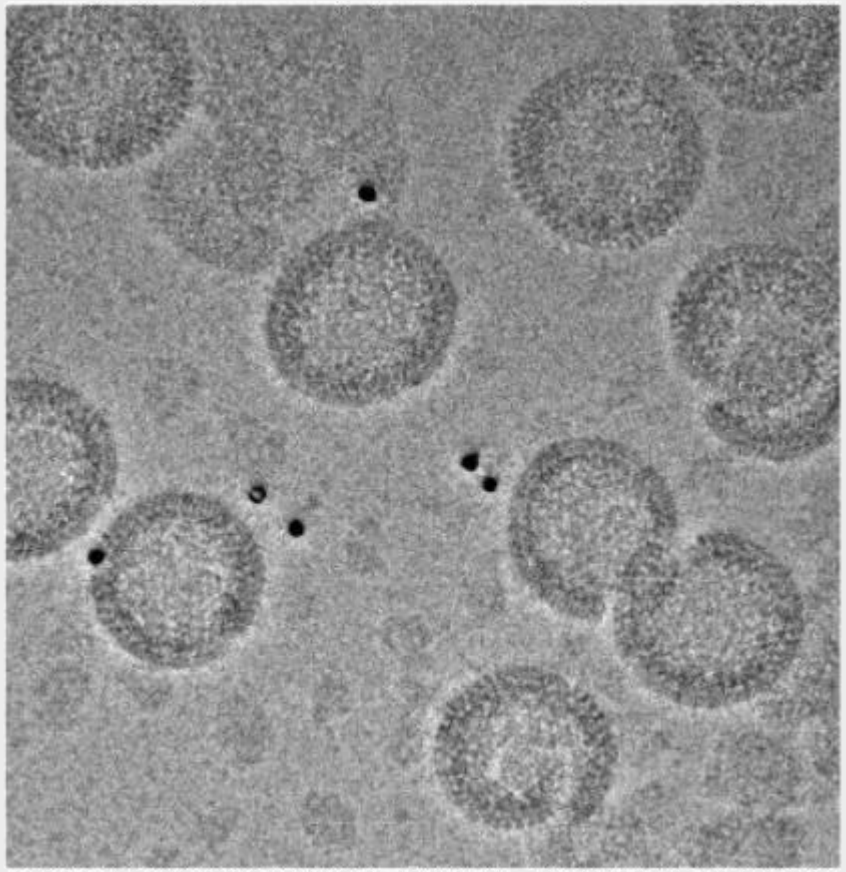
Some Collection Schemes

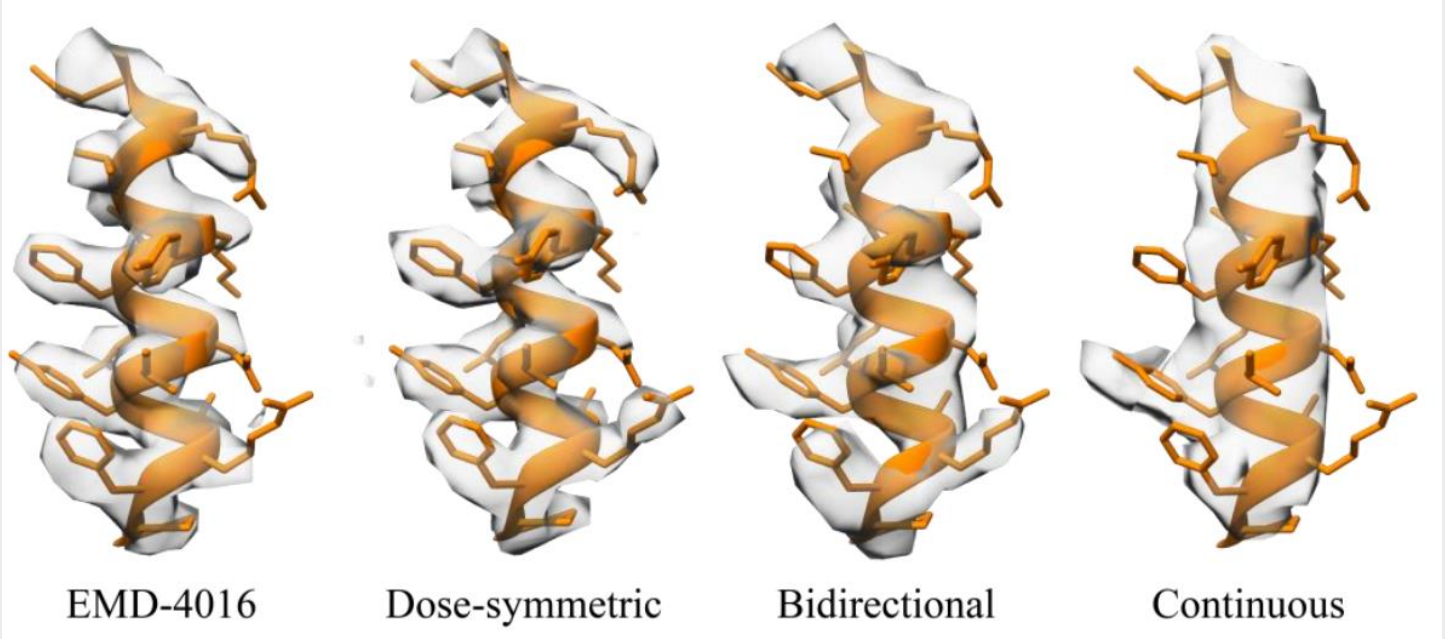




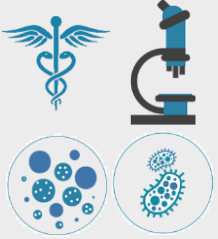
Turoňová et al., 2019 & 2020

Some Collection Schemes on an *Isotropic* Sample





Turoňová et al., 2019



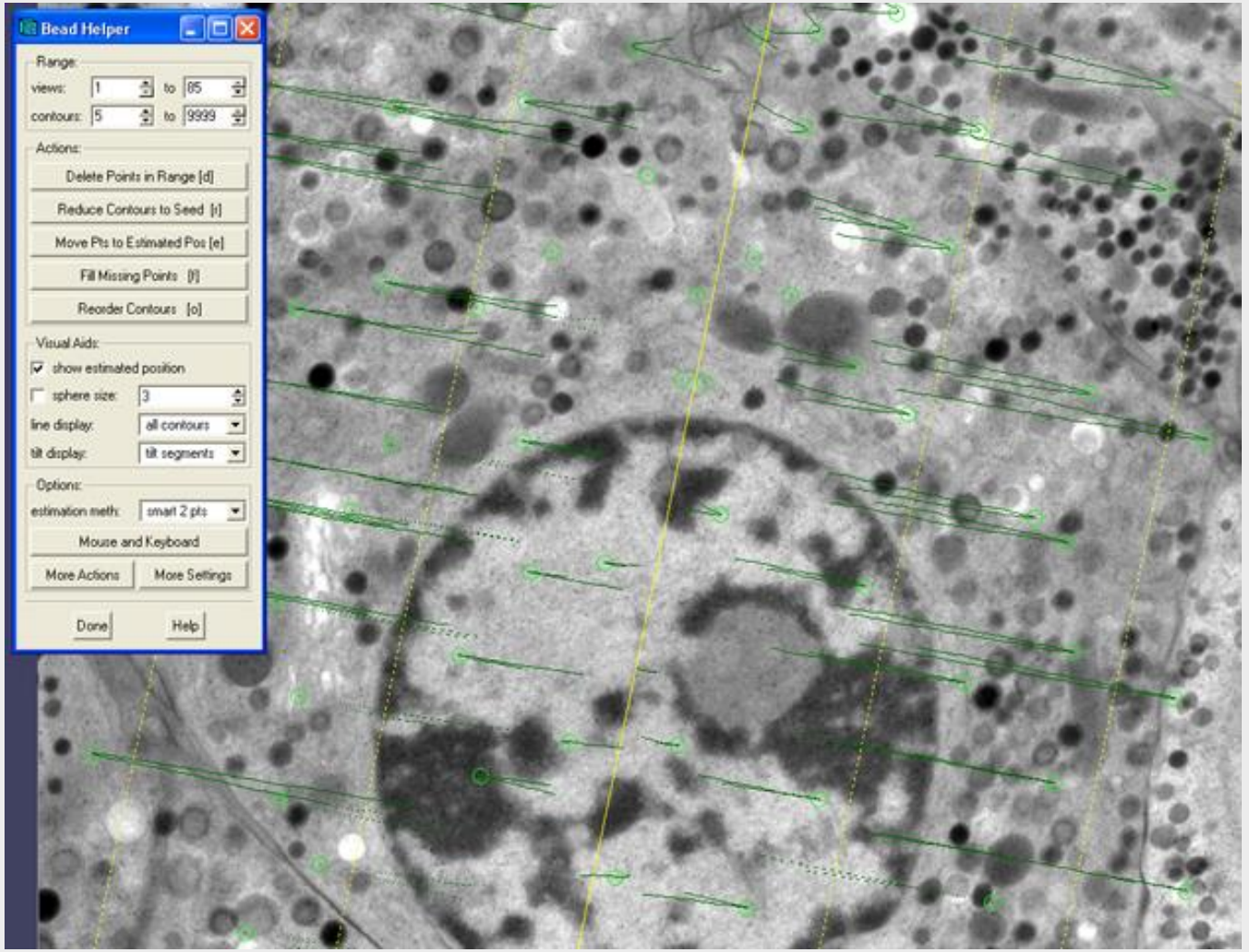
Tilt-series alignment

Tilt-series alignment

- **Software:**
  - 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

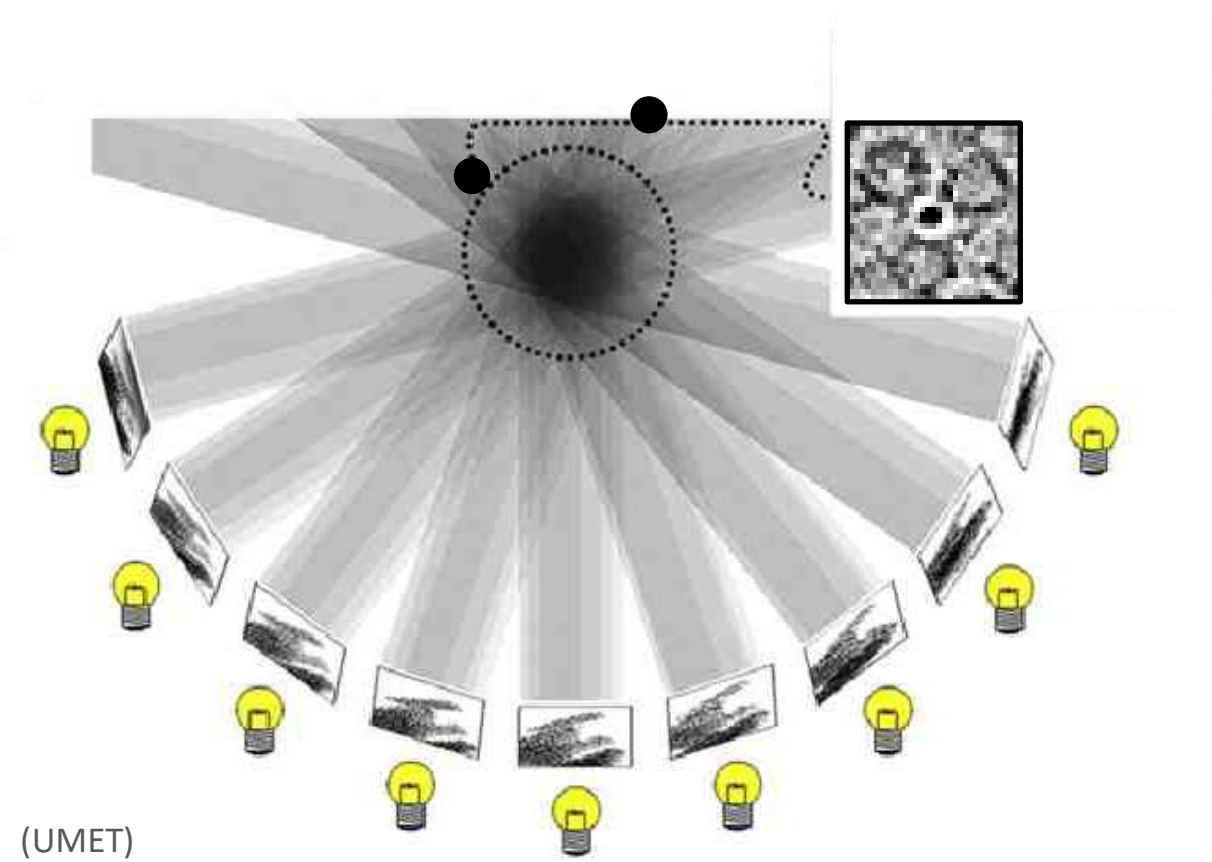
Fiducial-based tilt-series alignment

- 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

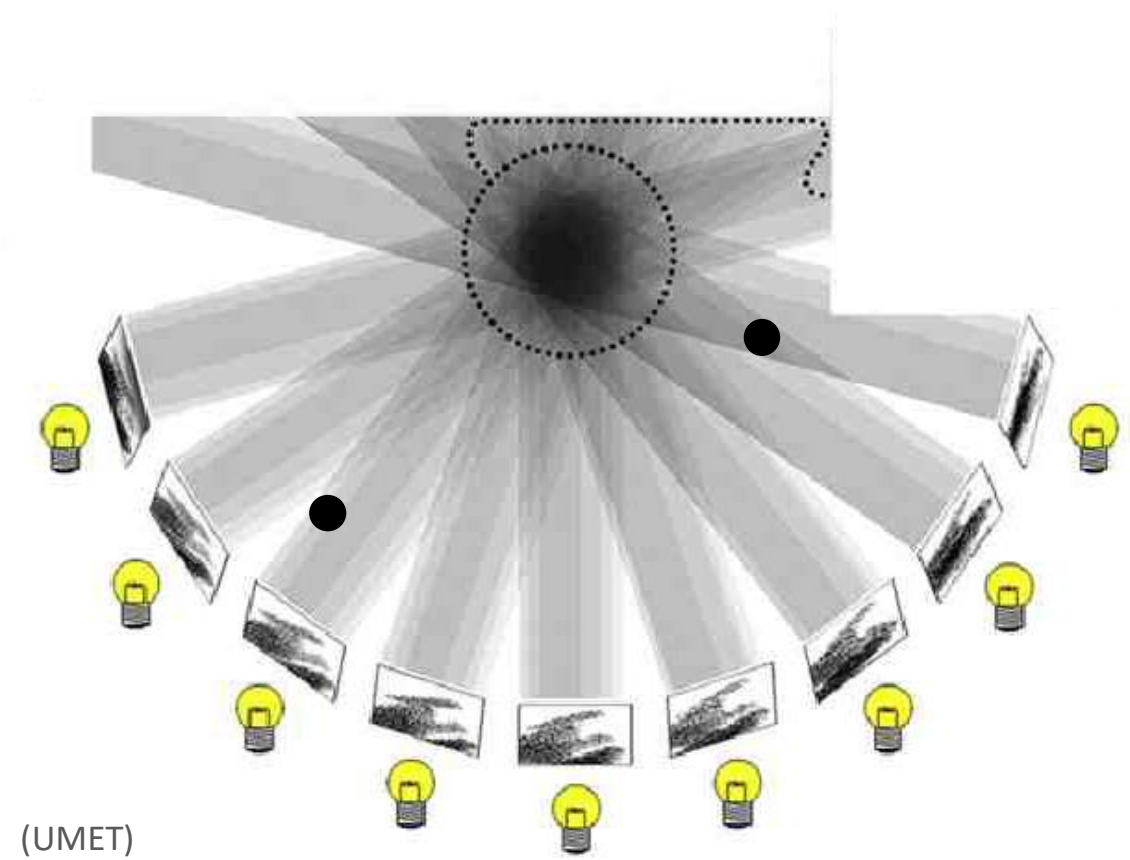


(UMET)

Nearby Fiducials Affect Signal and Contrast

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

Fiducial-based tilt-series alignment issues

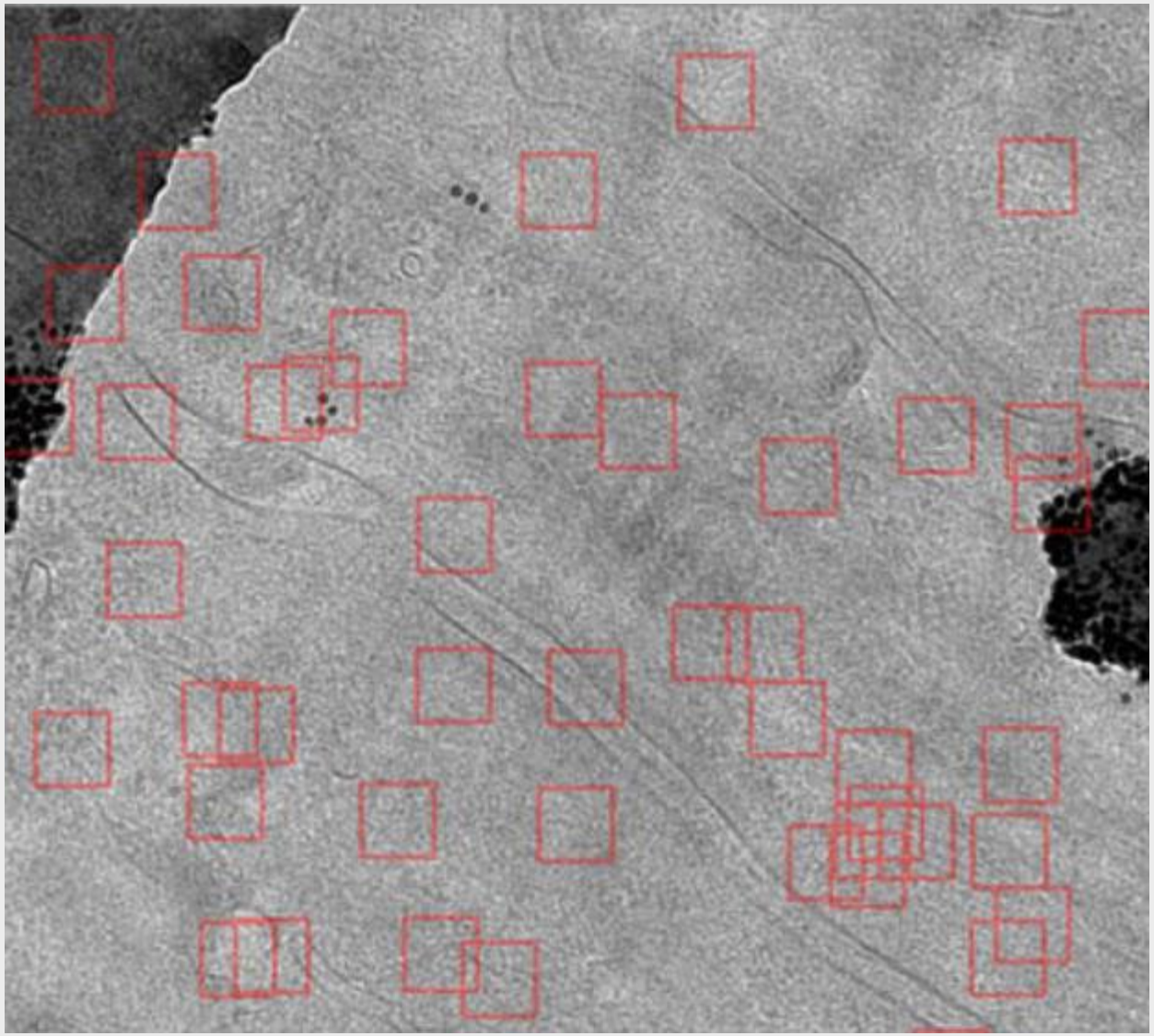


(UMET)

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

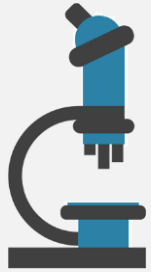


Castaño-Díez, 2010

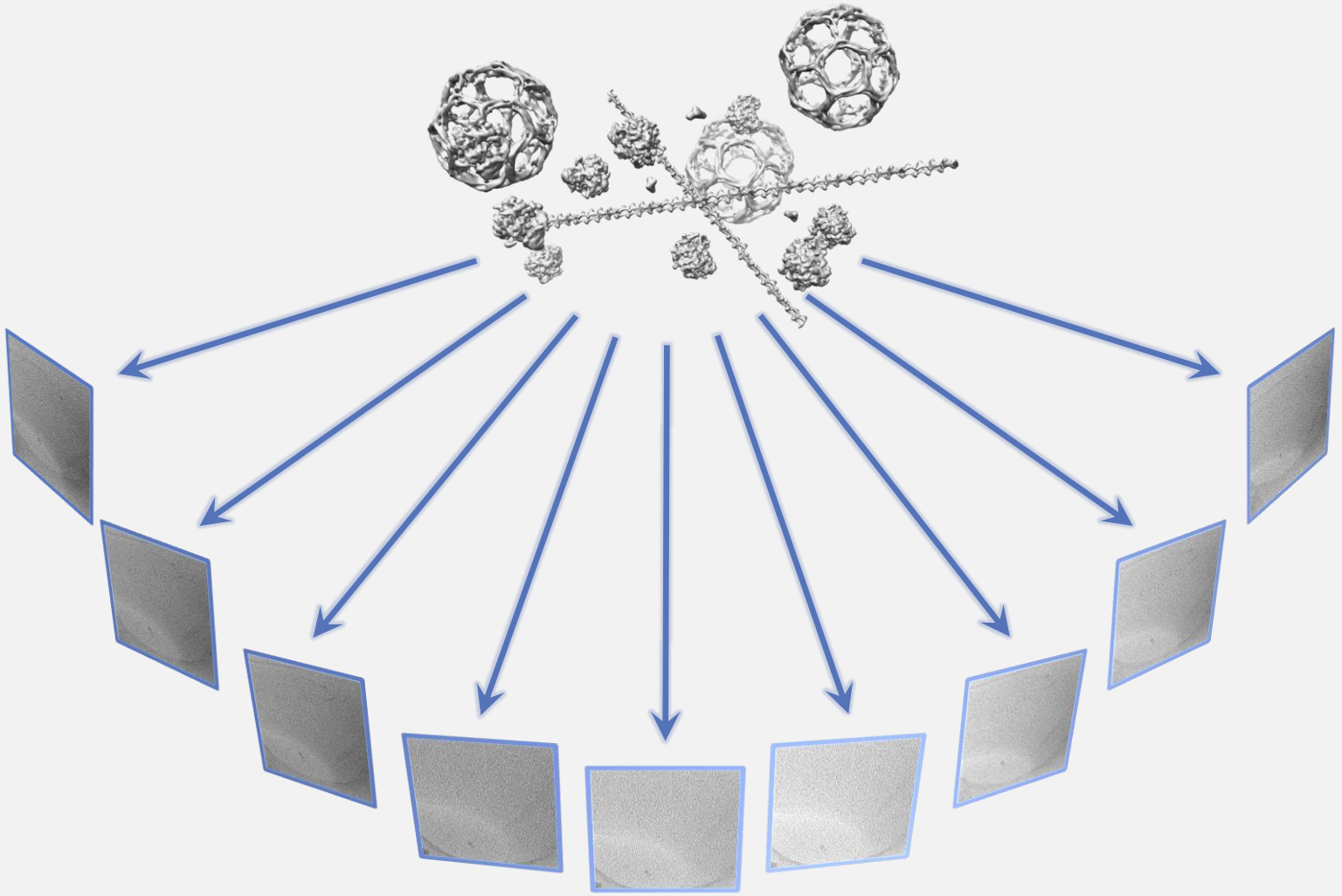
Identify featureful objects with contrast in all tilt images and track them.

- Semi-automated (IMOD, Alignator)

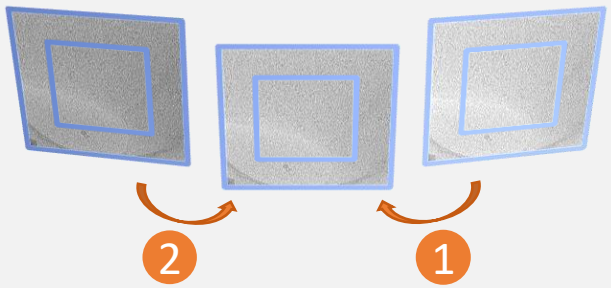
How does fiducial-less alignment in Protomo work?



Collect a tilt-series

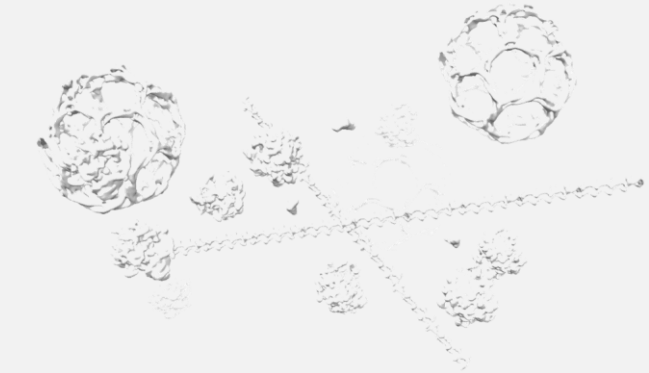


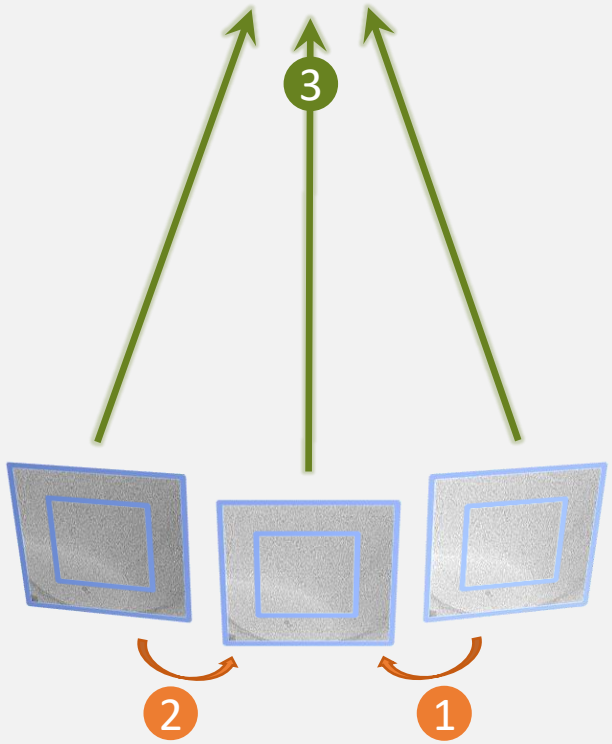
Protomo alignment



Nearest-neighbor correlation

Protomo alignment

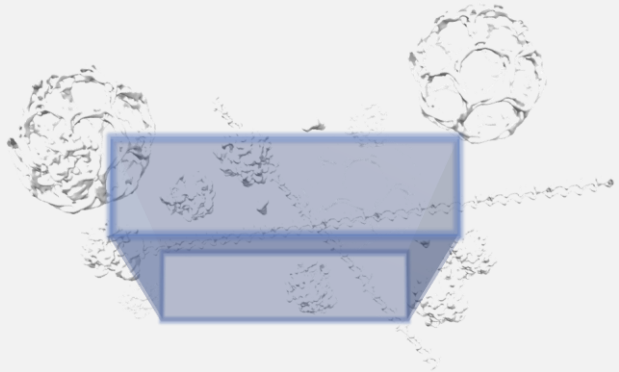


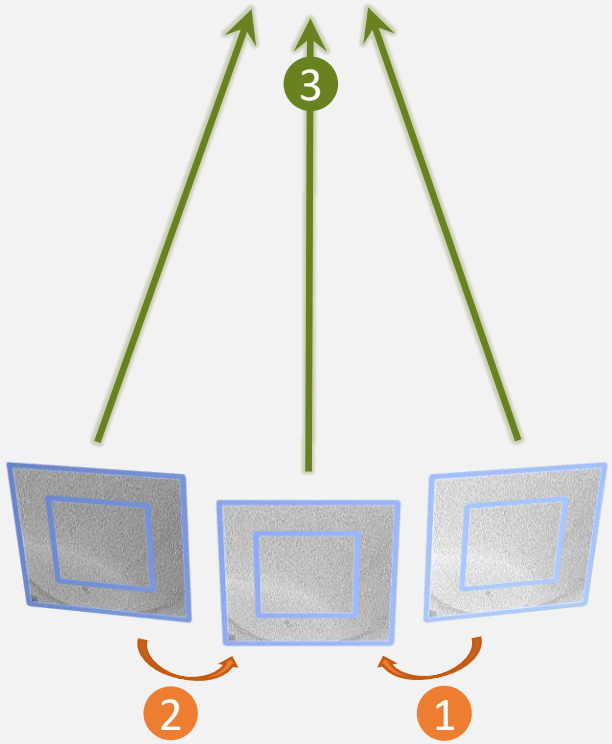


Nearest-neighbor correlation

Weighted back-projection

Protomo alignment





Nearest-neighbor correlation

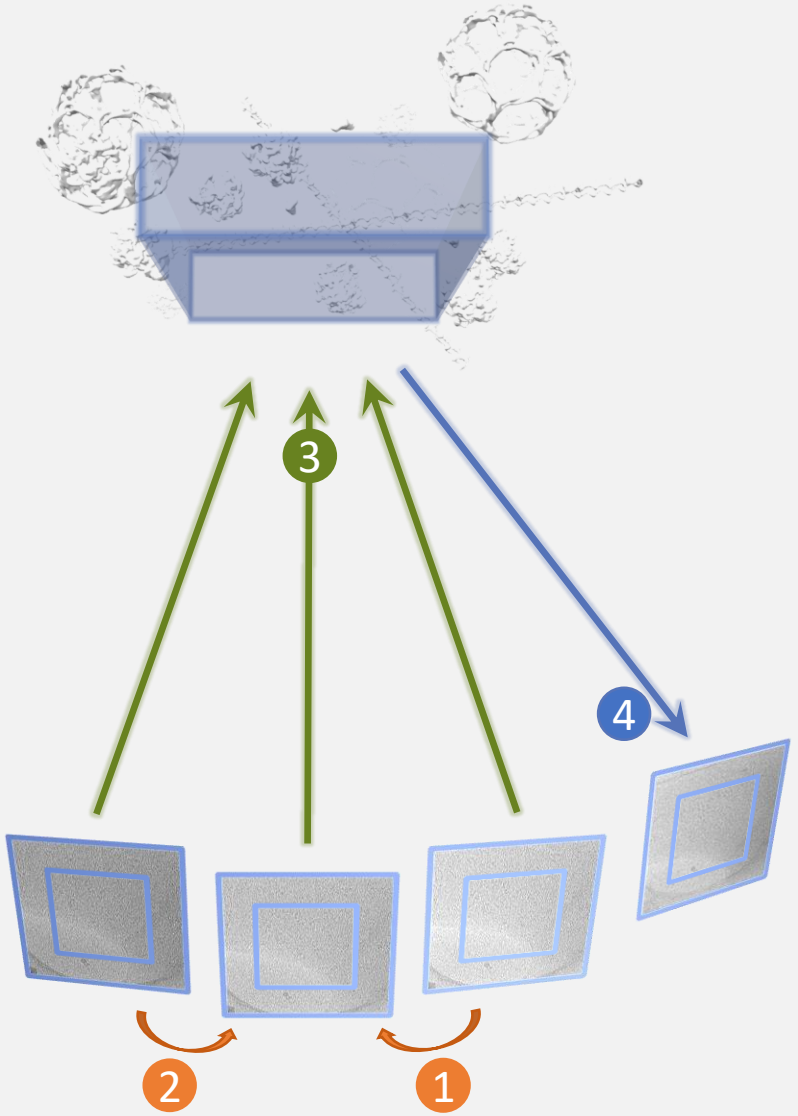
Weighted back-projection

alignment thickness = z

x y

Volume to be re-projected

Protomo alignment



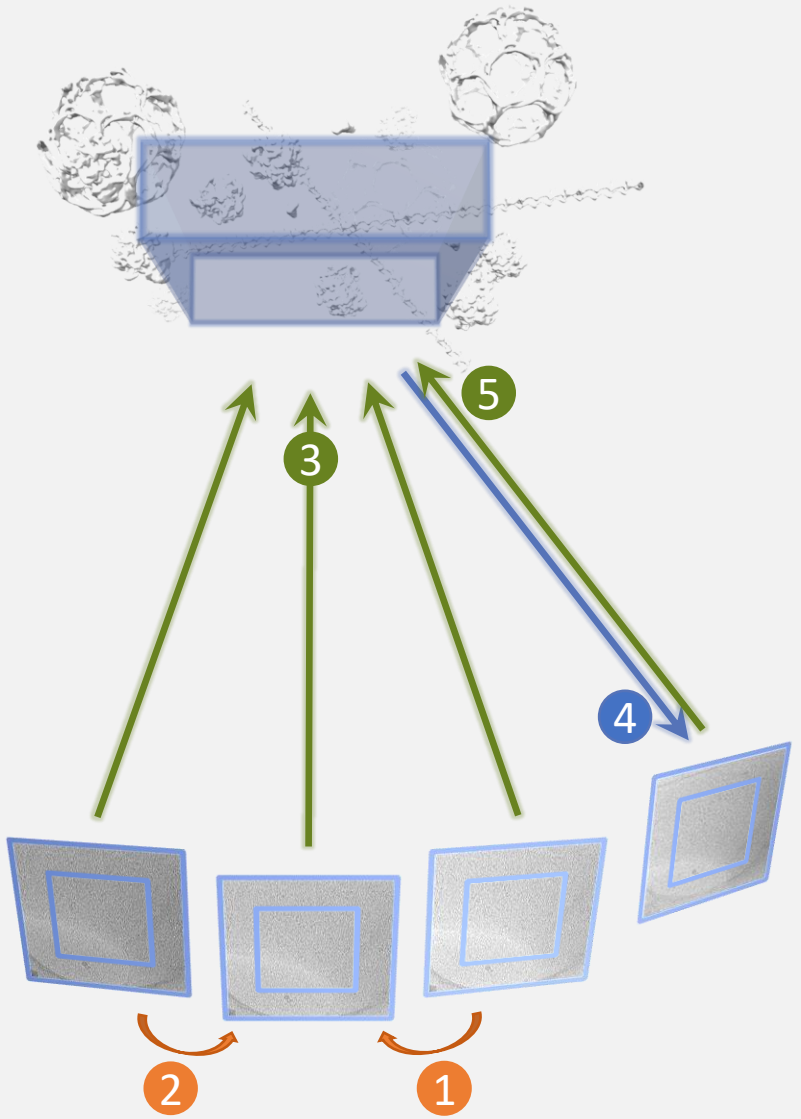
Nearest-neighbor correlation

Weighted back-projection

alignment thickness = z; x; y — Volume to be re-projected

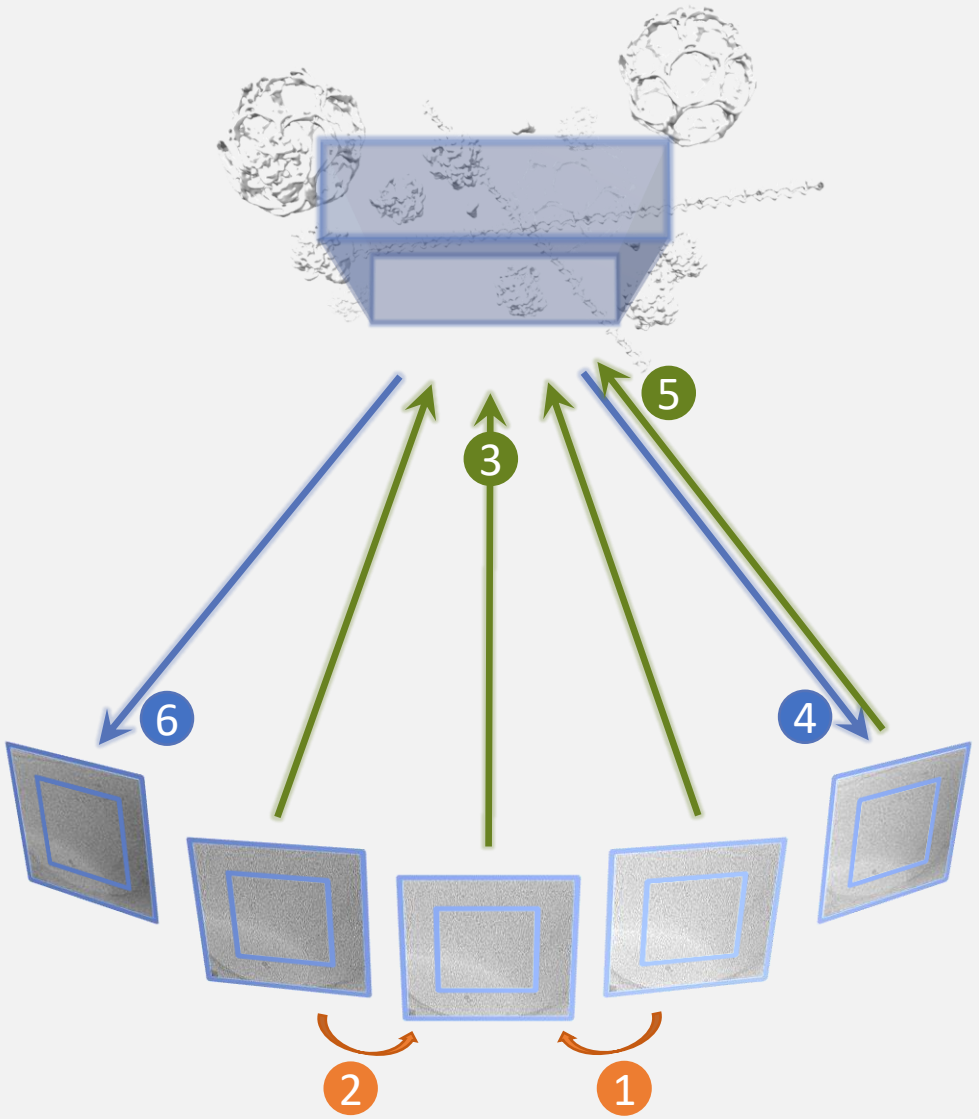
Re-projection ⟶ correlation

Protomo alignment



- Nearest-neighbor correlation
- Weighted back-projection
- Volume to be re-projected (alignment thickness = z; x, y)
- Re-projection ⟶ correlation

Protomo alignment



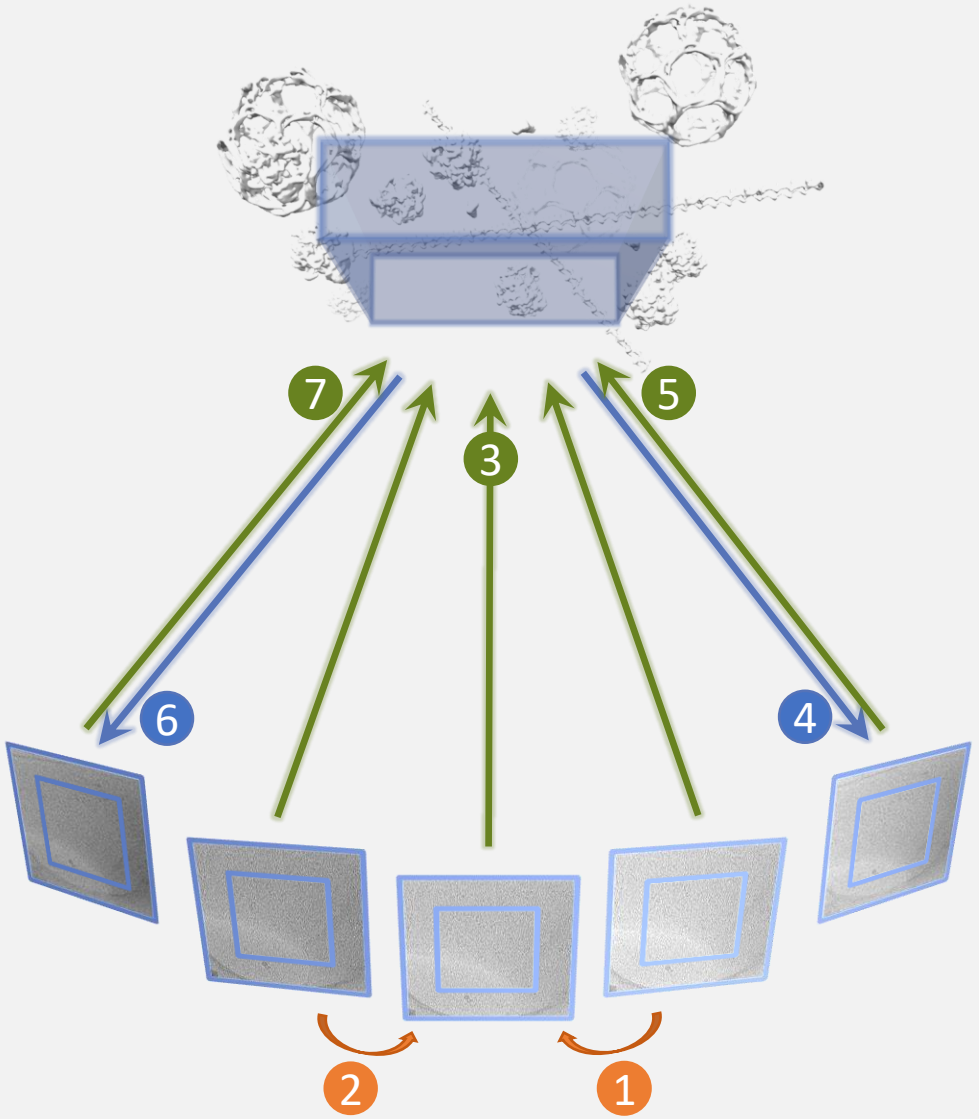
Nearest-neighbor correlation

Weighted back-projection

alignment thickness = z, x, y — Volume to be re-projected

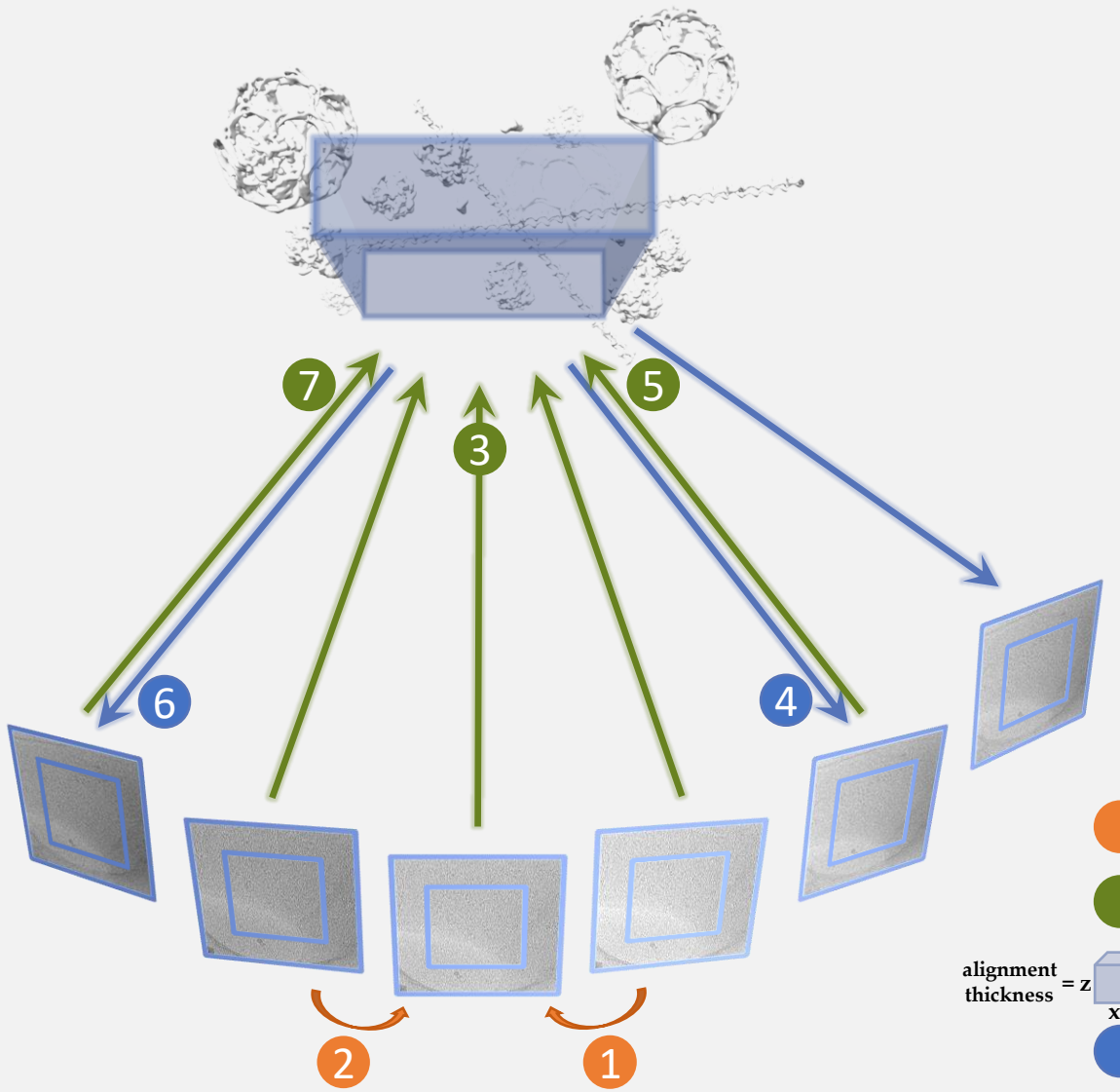
Re-projection ⟶ correlation

Protomo alignment



- Nearest-neighbor correlation
- Weighted back-projection
- Volume to be re-projected (alignment thickness = z; x, y)
- Re-projection ⟶ correlation

Protomo alignment



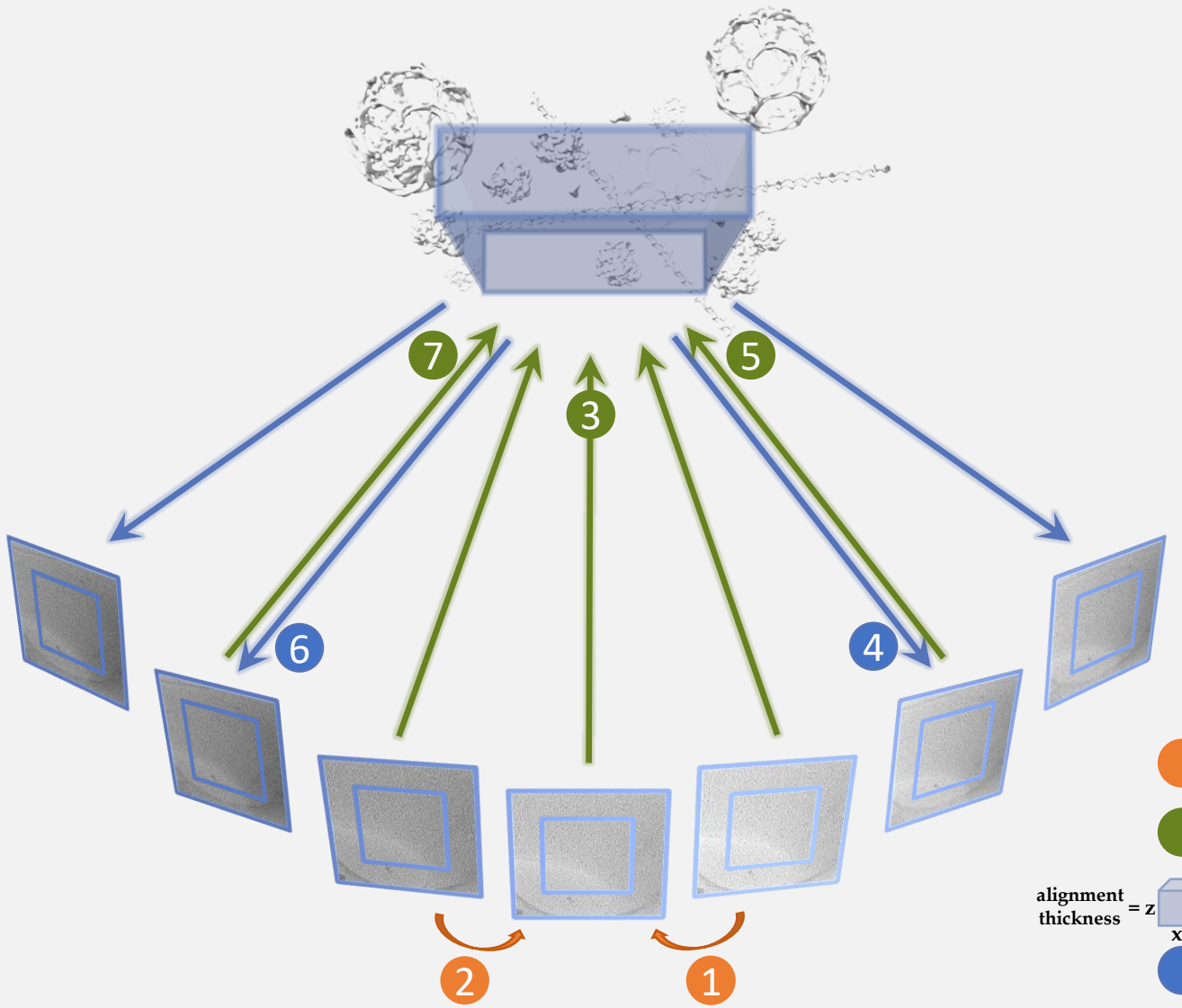
Nearest-neighbor correlation

Weighted back-projection

alignment thickness = z; x; y — Volume to be re-projected

Re-projection ⟶ correlation

Protomo alignment



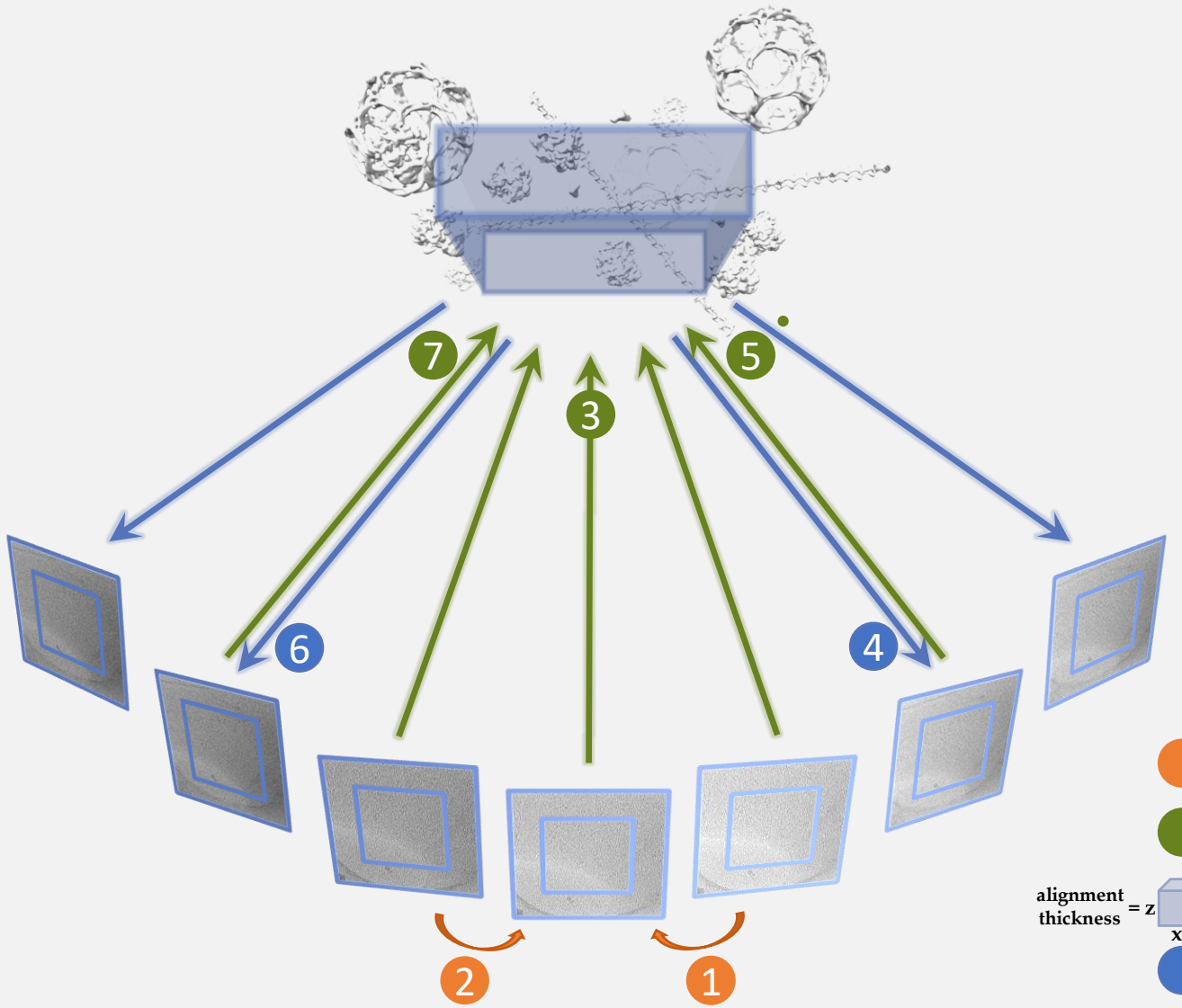
Nearest-neighbor correlation

Weighted back-projection

alignment thickness = z, x, y — Volume to be re-projected

Re-projection ⟶ correlation

Protomo alignment



Nearest-neighbor correlation

Weighted back-projection

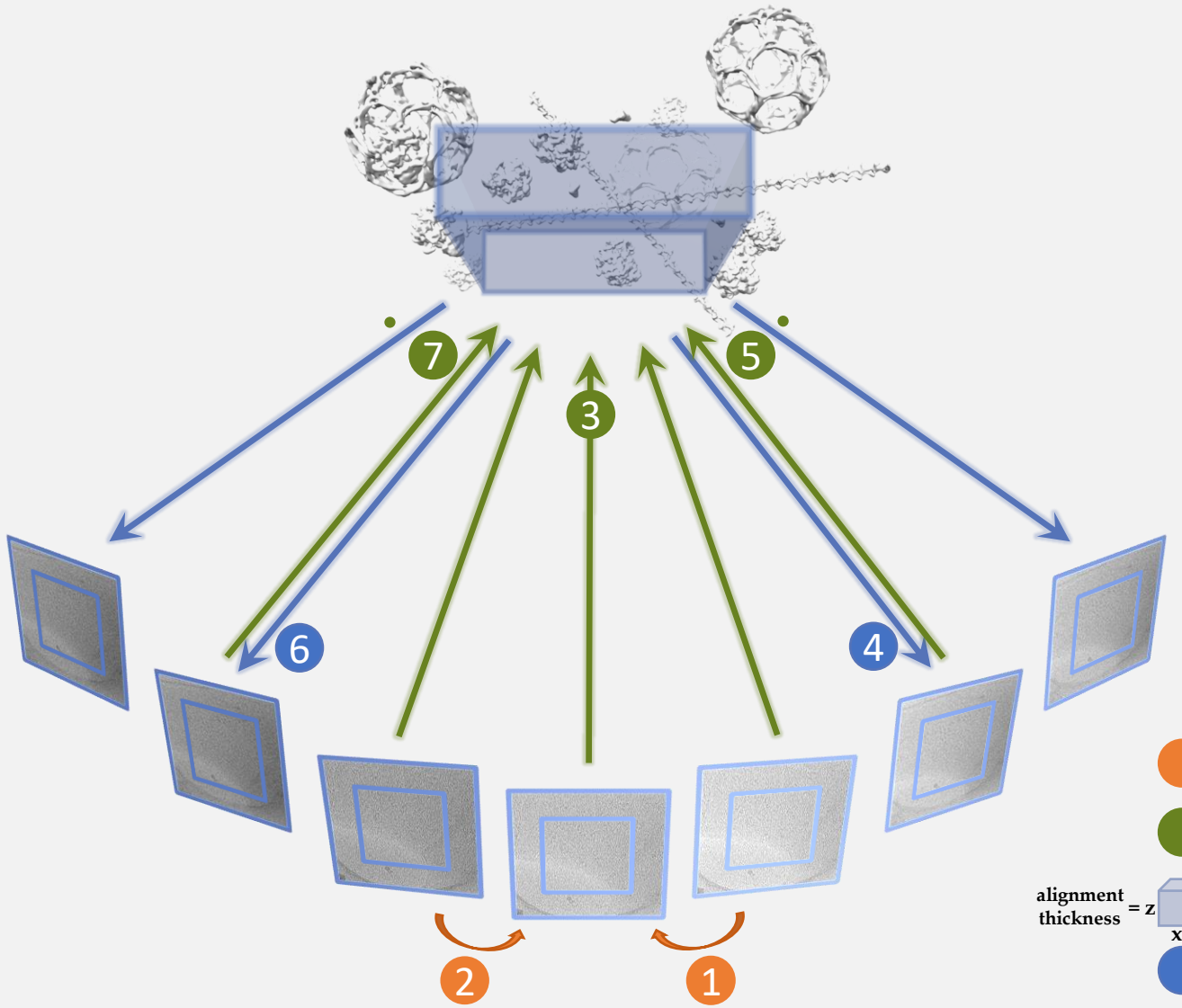
alignment thickness = z

x y

Volume to be re-projected

Re-projection ⟶ correlation

Protomo alignment



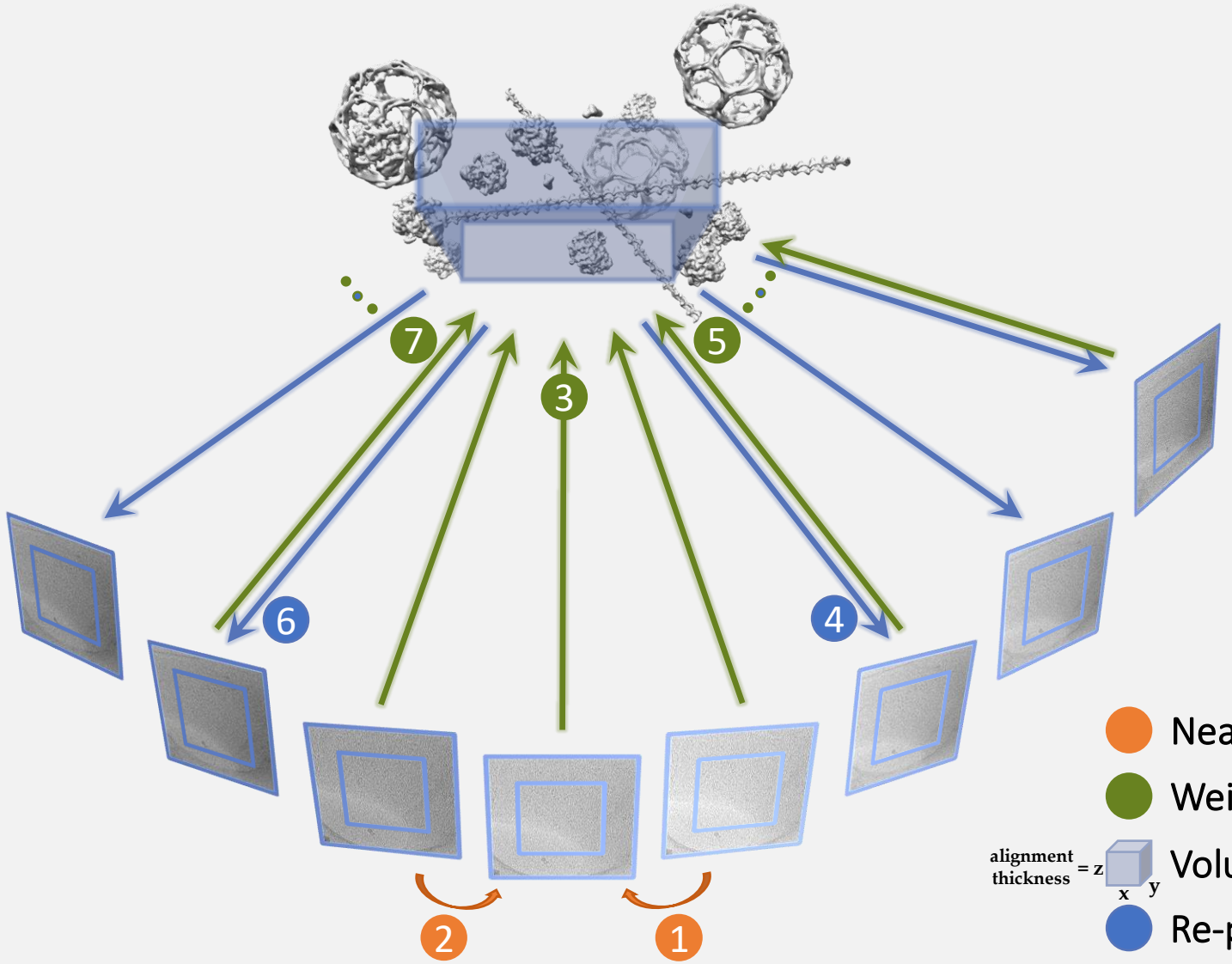
Nearest-neighbor correlation

Weighted back-projection

alignment thickness = z; x; y — Volume to be re-projected

Re-projection ⟶ correlation

Protomo alignment



Nearest-neighbor correlation

Weighted back-projection

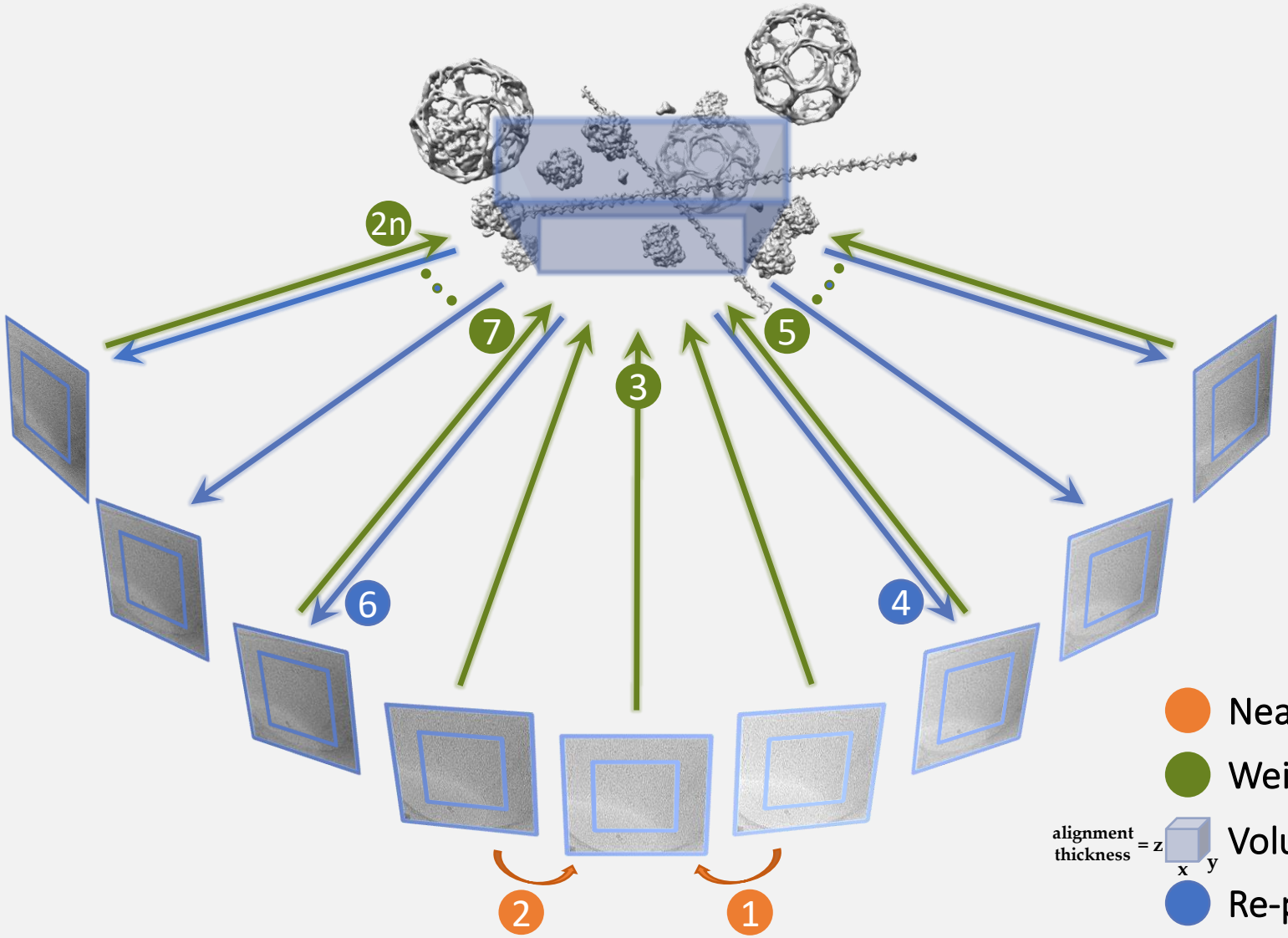
alignment thickness = z

x y

Volume to be re-projected

Re-projection ⟶ correlation

Protomo alignment



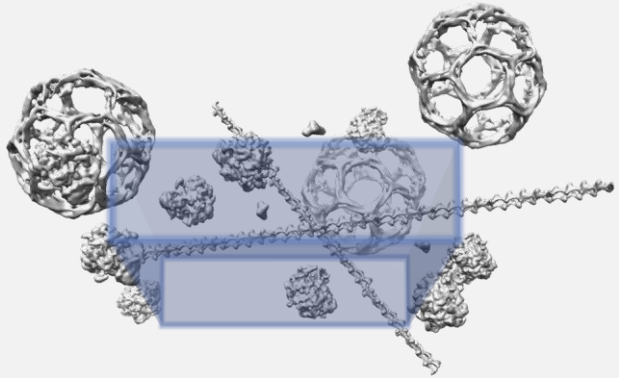
Nearest-neighbor correlation

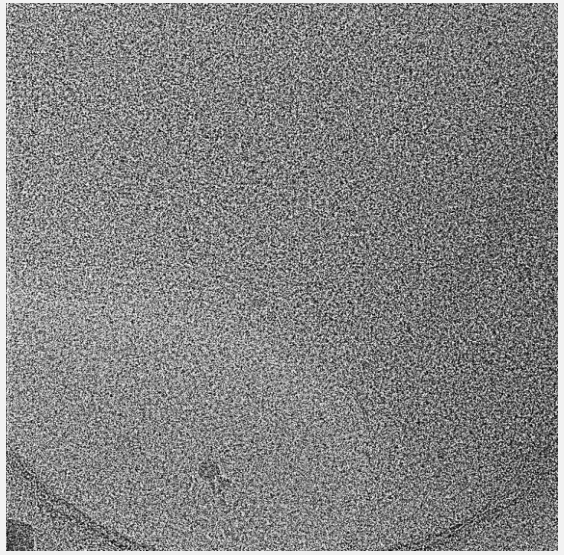
Weighted back-projection

alignment thickness = z; x; y — Volume to be re-projected

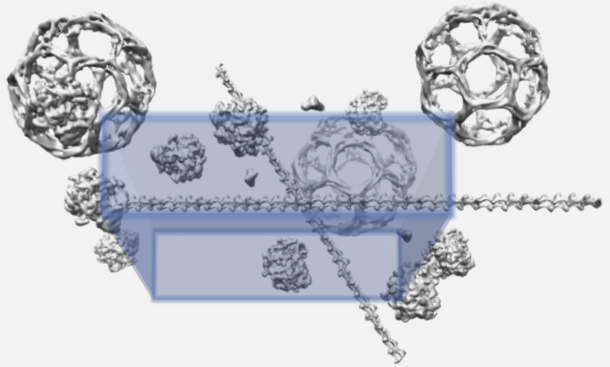
Re-projection ⟶ correlation

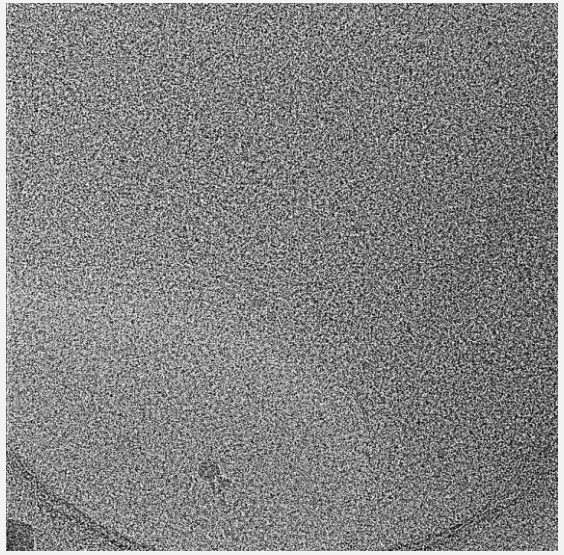
Protomo alignment





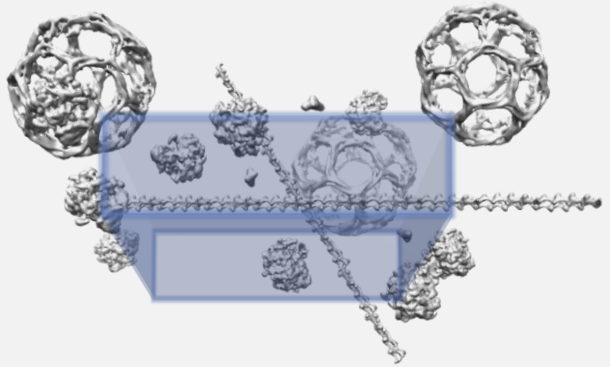
Protomo alignment

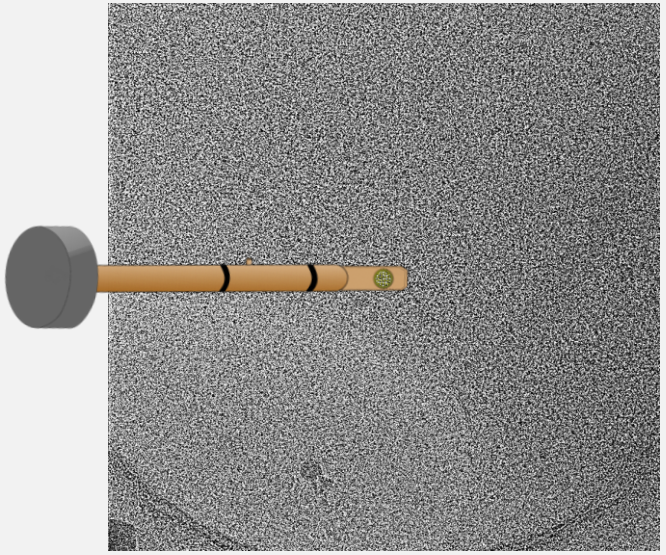




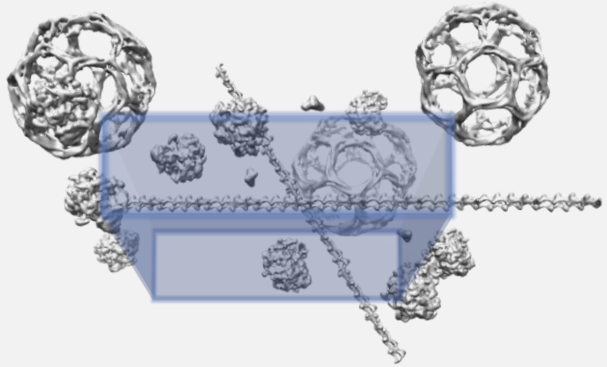
Refine orientations of objects

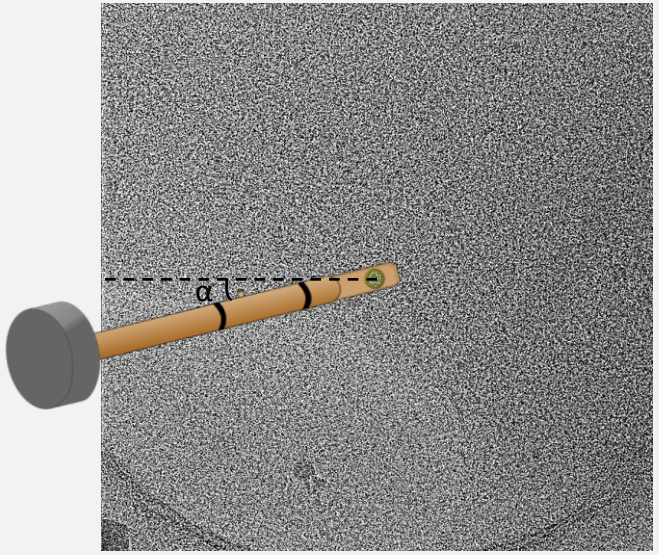
Protomo alignment





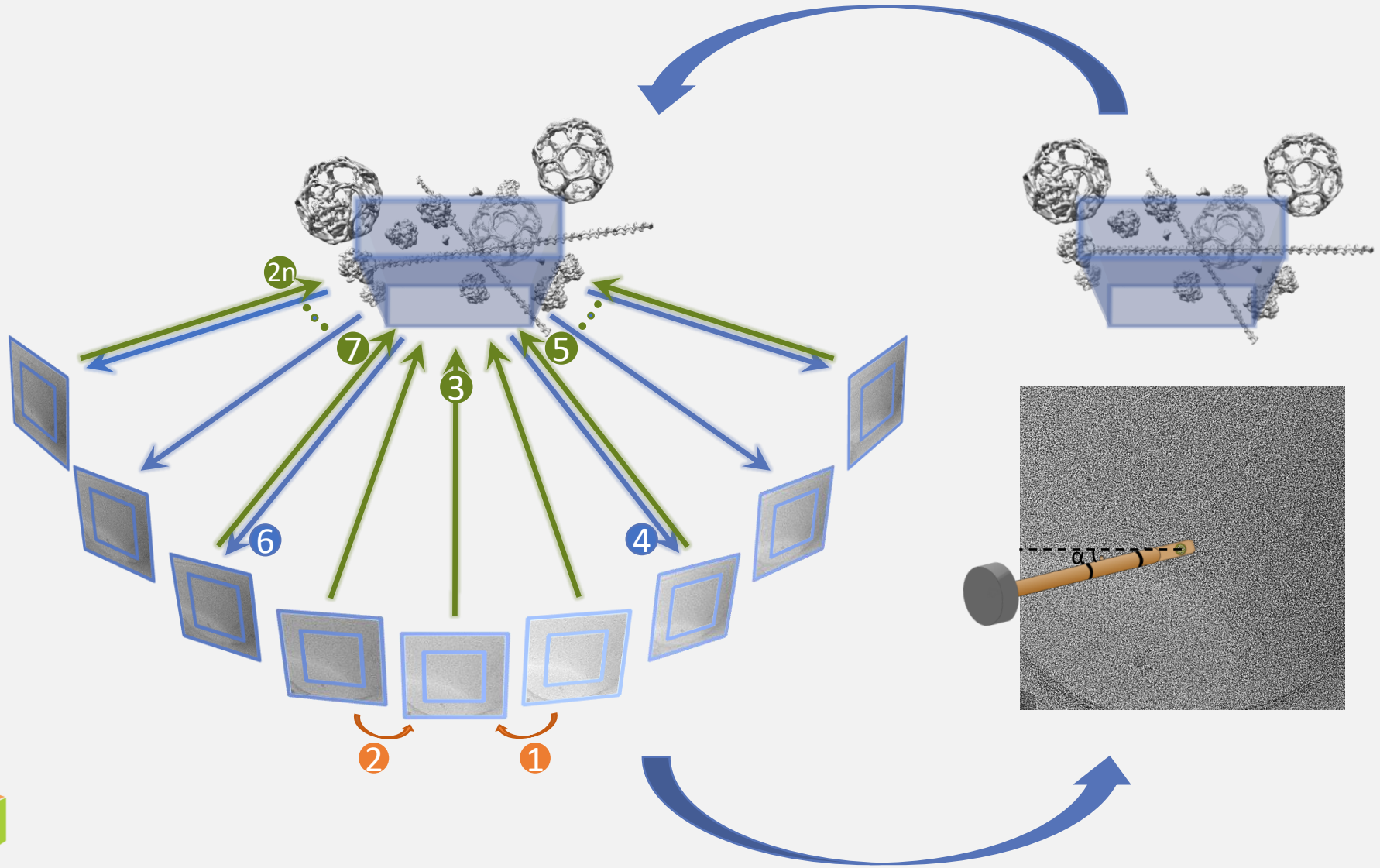
Protomo alignment





Refine tilt azimuth

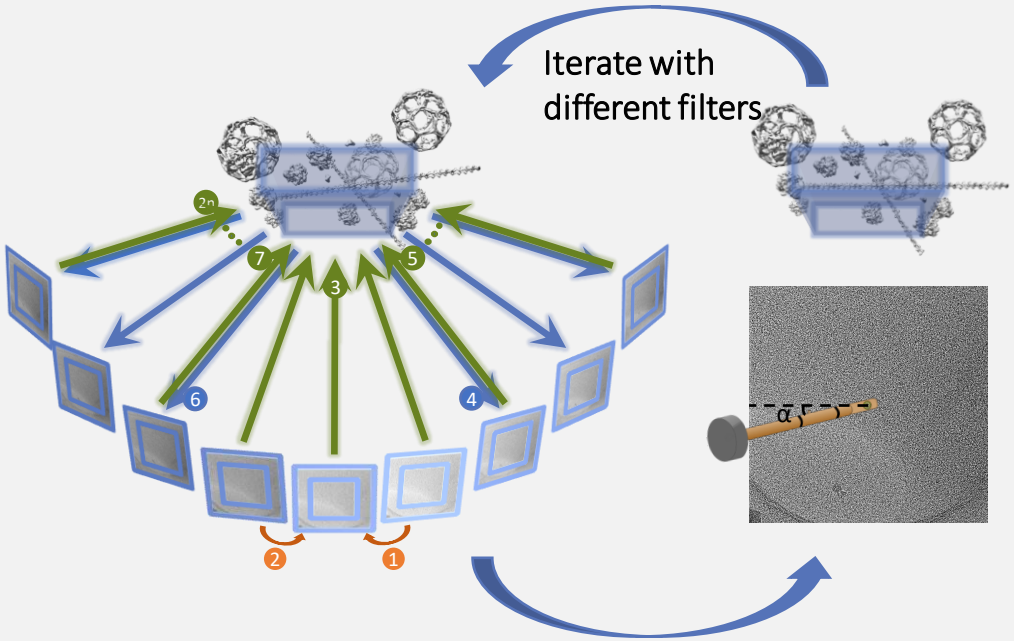
Appion-Protomo refinement



Iterate with different filters

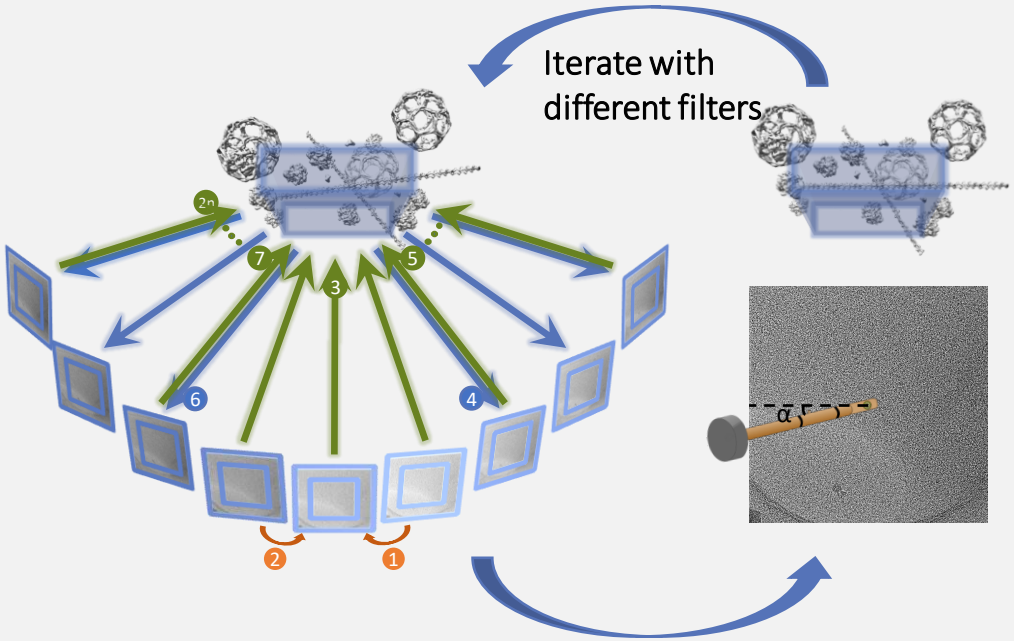
Appion-Protomo refinement

Why is this important?

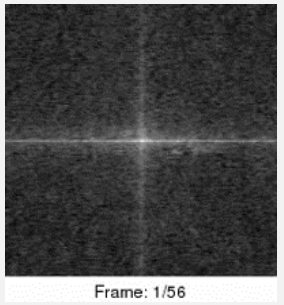


Appion-Protomo refinement

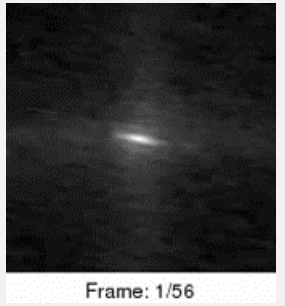
Why is this important?



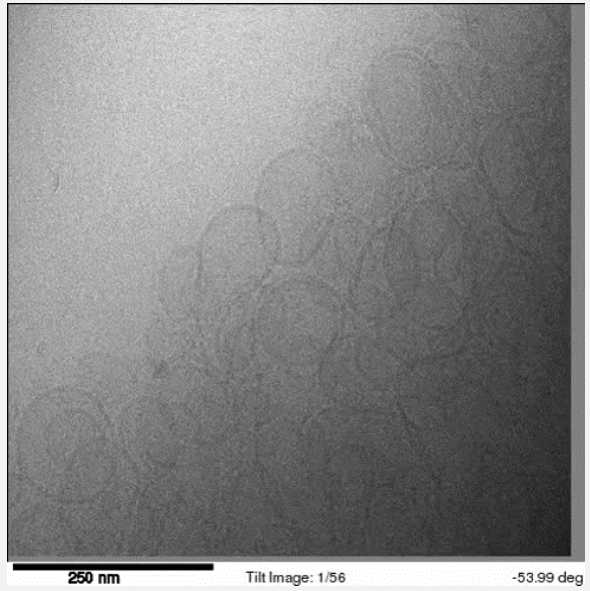
Nearest-neighbor alignment

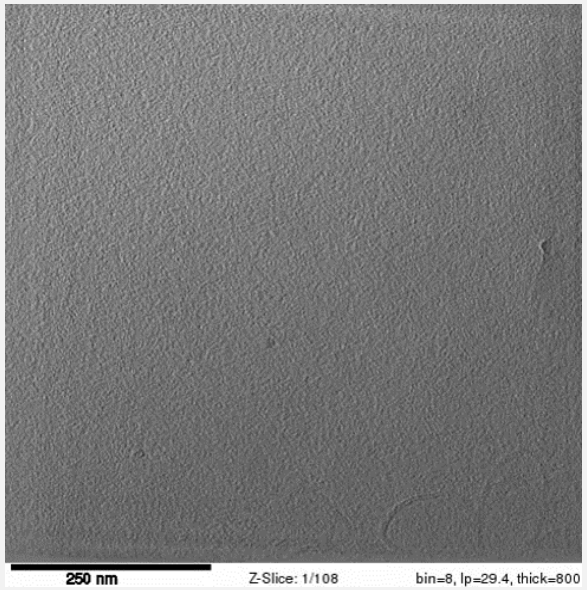


After refinement



After refinement



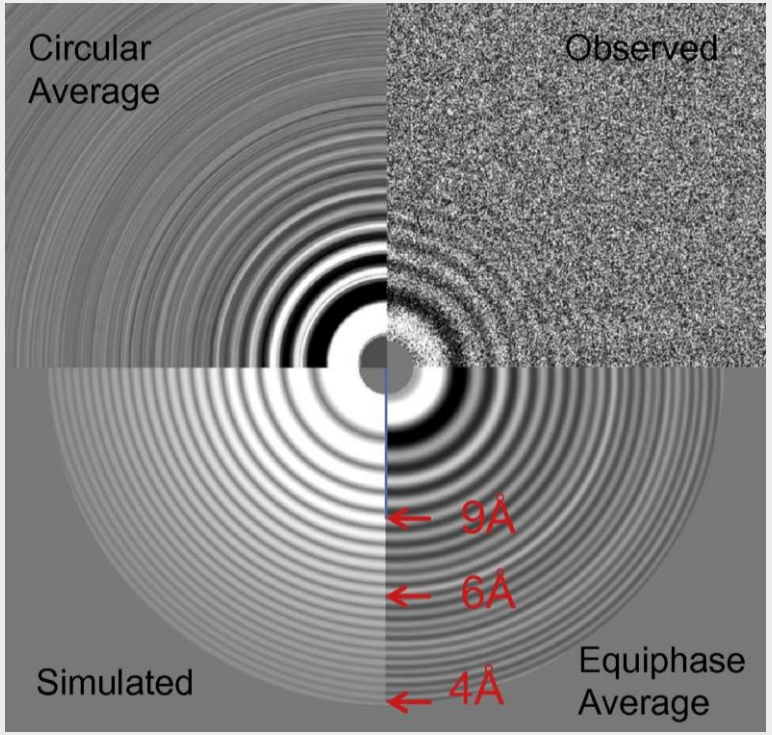


Defocus estimation

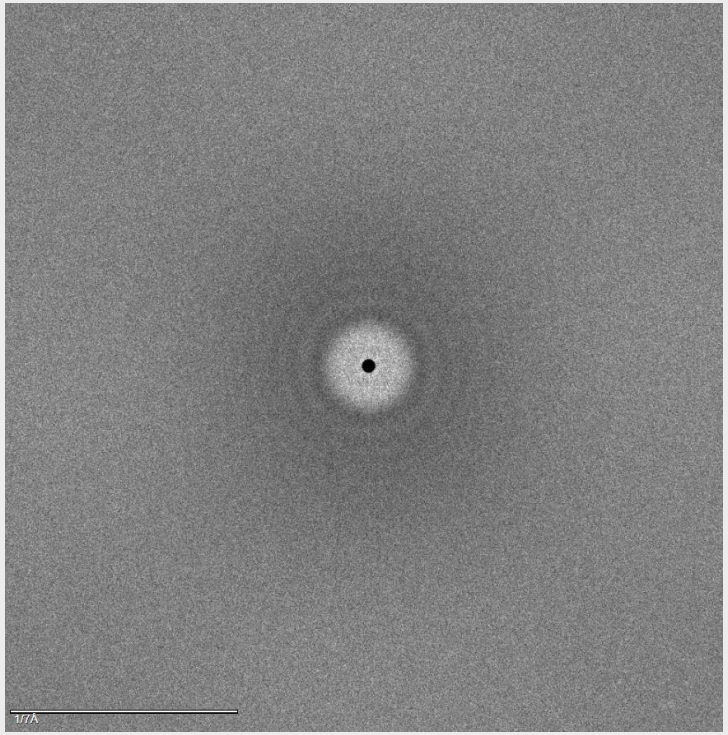
Goal: Find the **height of your objects** of interest to correct for microscope aberrations (CTF)

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

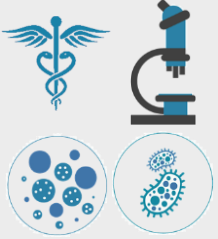
Zhang, 2016



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

Methods ordered approximately **worst-to-best** (depends on sample):

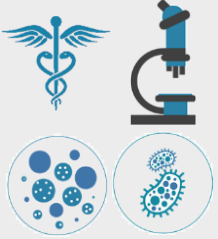
- **Per-image** defocus estimation accounting for tilts (CTFFIND4, GCTF, etc.)
- Per-tomogram post-hoc estimation by using **SPT FSC to locate the first CTF zero**
- **Image tiling** to estimate the **defocus of the untilted plane** (TomoCTF)

- Defocus estimation and **interpolation using two focus locations** on the tilt axis (Eibauer, 2012)
- Per-particle tilt image fine estimation and correction that accounts for the **3D location of each particle**
- Per-particle tilt image fine estimation and correction that takes into **account overlapping objects** in each tilt image of each particle and accounts for the 3D location of each particle – can use all particles in each tilt image to refine!

CTF correction methods

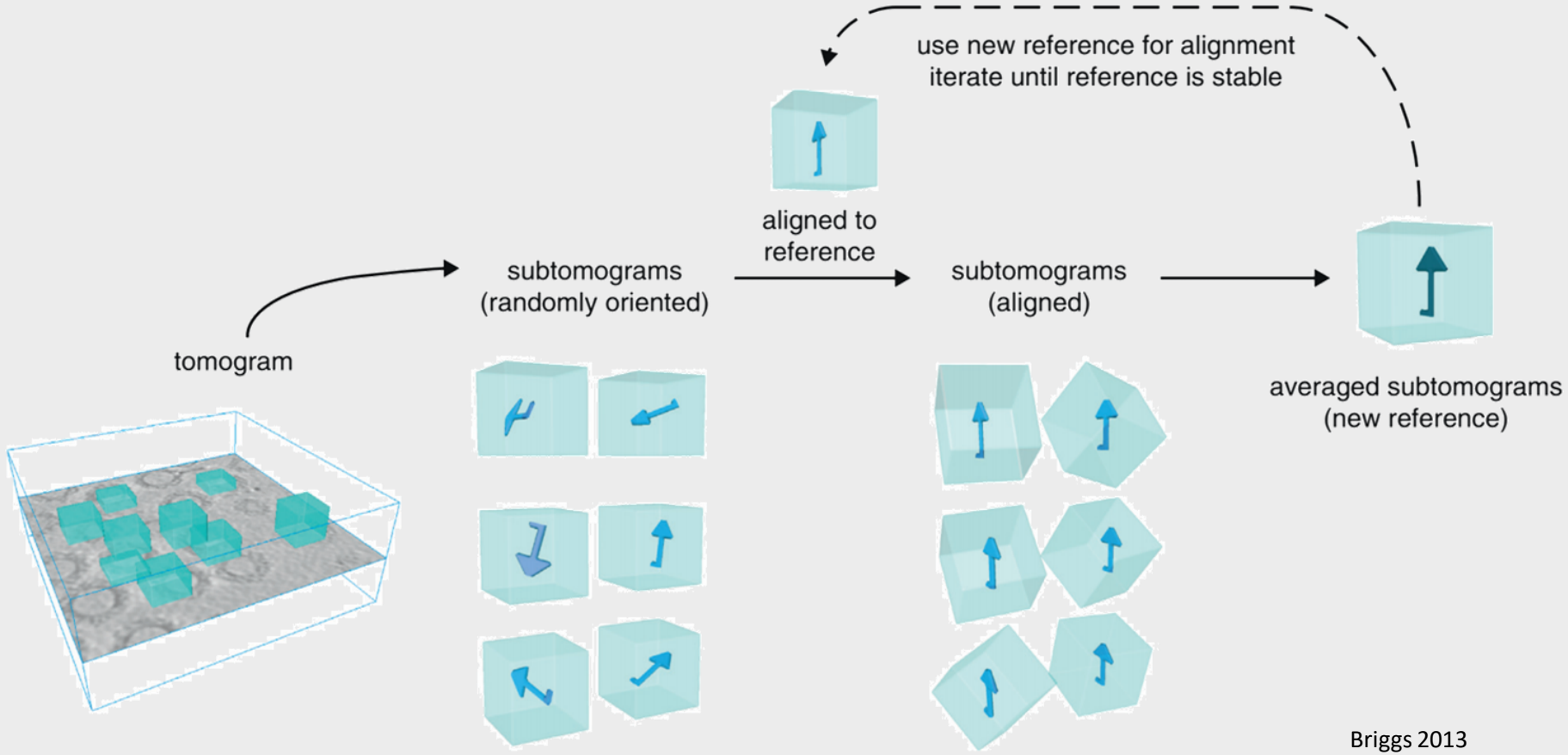
Methods ordered approximately **worst-to-best** (depends on sample):

- **Per-image** correction
- Strip-based correction with TomoCTF or IMOD ctfphaseflip
  - Flips phases and optionally corrects amplitudes (TomoCTF) on a strip-by-strip basis.
  - Error will depend on the amount of non-eucentricity
- 3D CTF model (Relion) takes into account x,y,z particle locations
- Per-particle/tiling CTF correction (EMAN2)
- During tomographic reconstruction (EmSART, NovaCTF)



Sub-tomogram processing

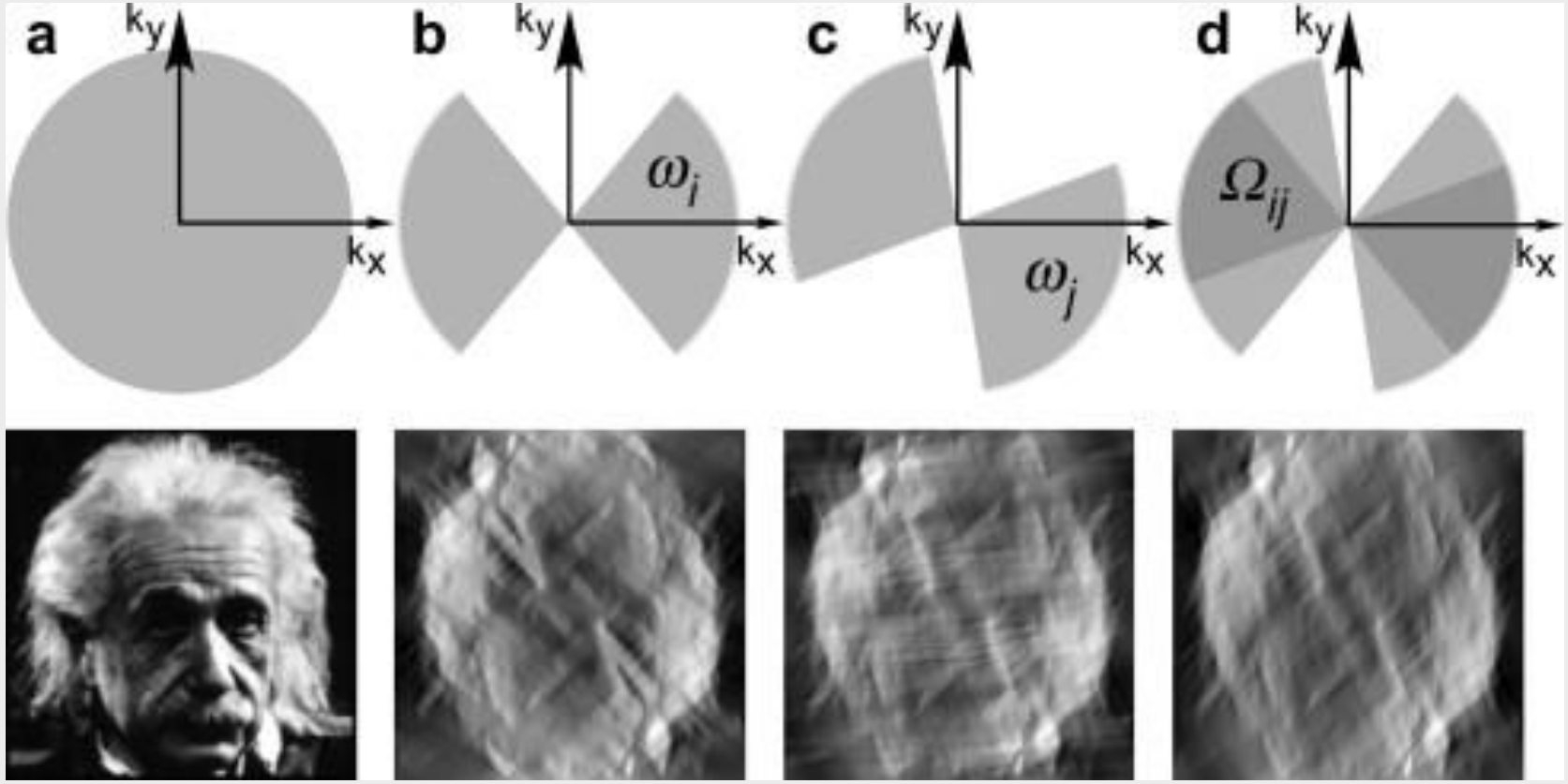
Sub-tomogram processing workflow



Briggs 2013

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

Must take into account subtomogram missing wedges



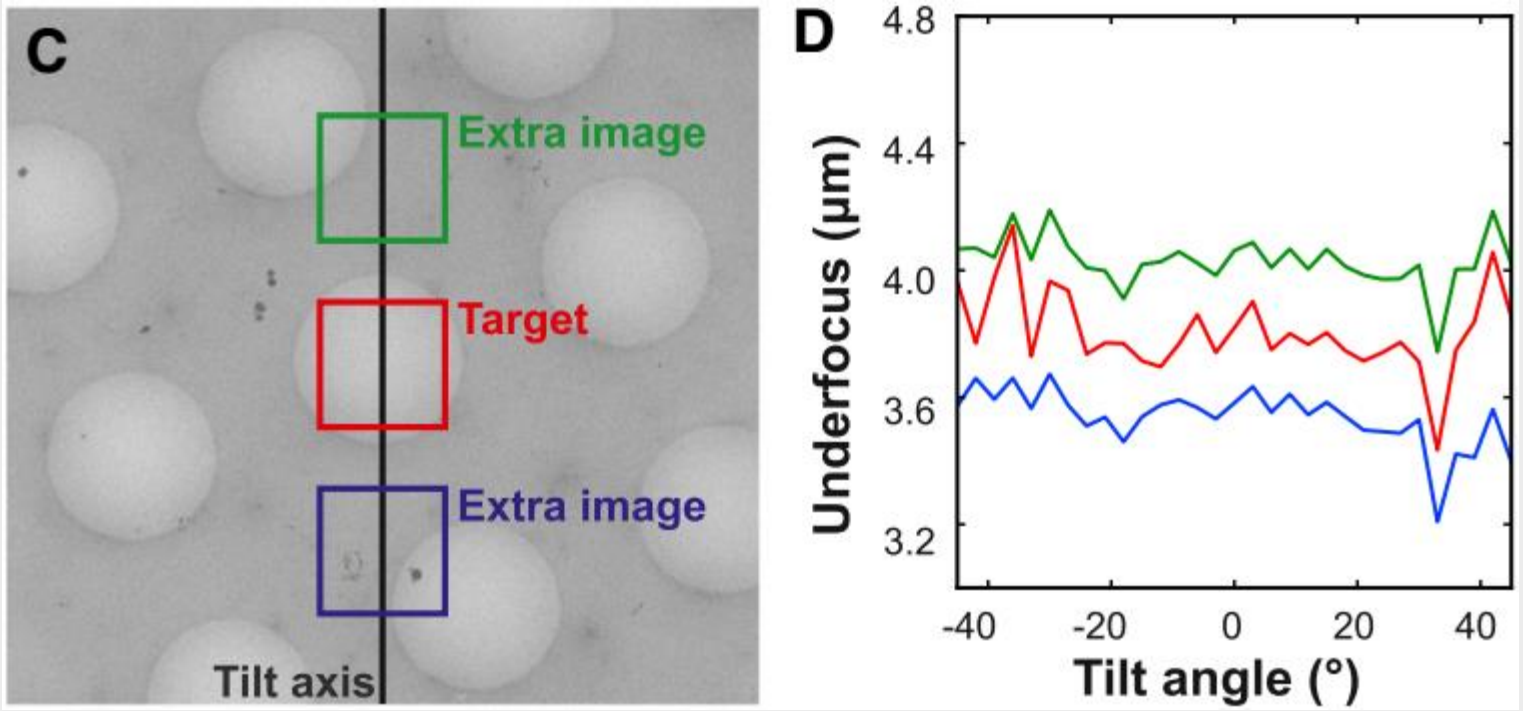
Forster et al, J. Struct. Biol, 2008

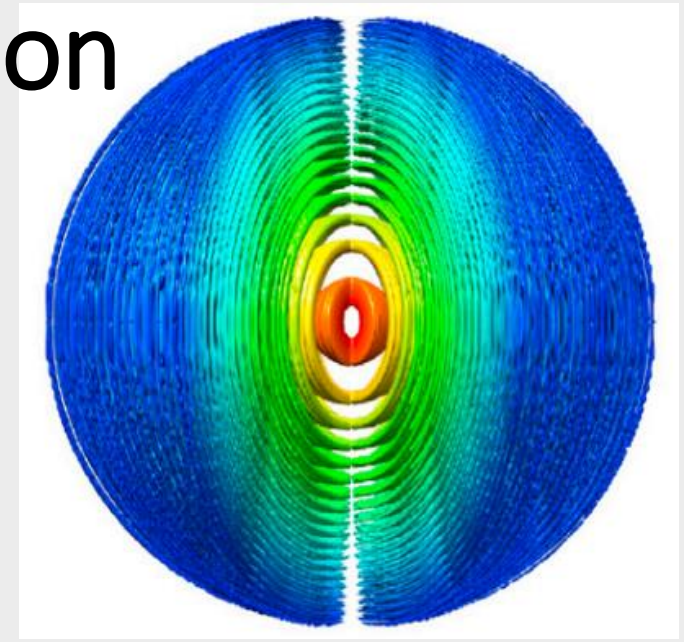
- Effectively align volume in common in Fourier space

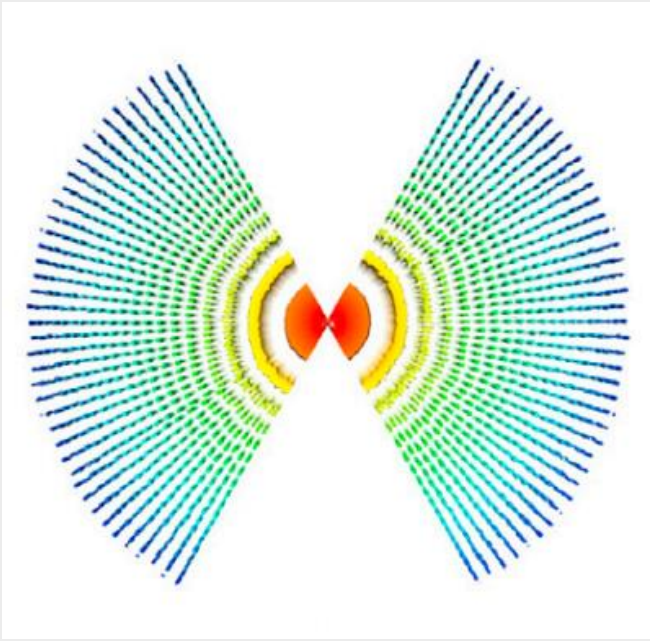
Sub-tomogram processing software

- Dynamo – GPU accelerated, tomogram database, extensive picking abilities
- Relion – 3D CTF model, Bayesian approach to alignment is used
- 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
- Warp

Sub-tomogram processing in Relion



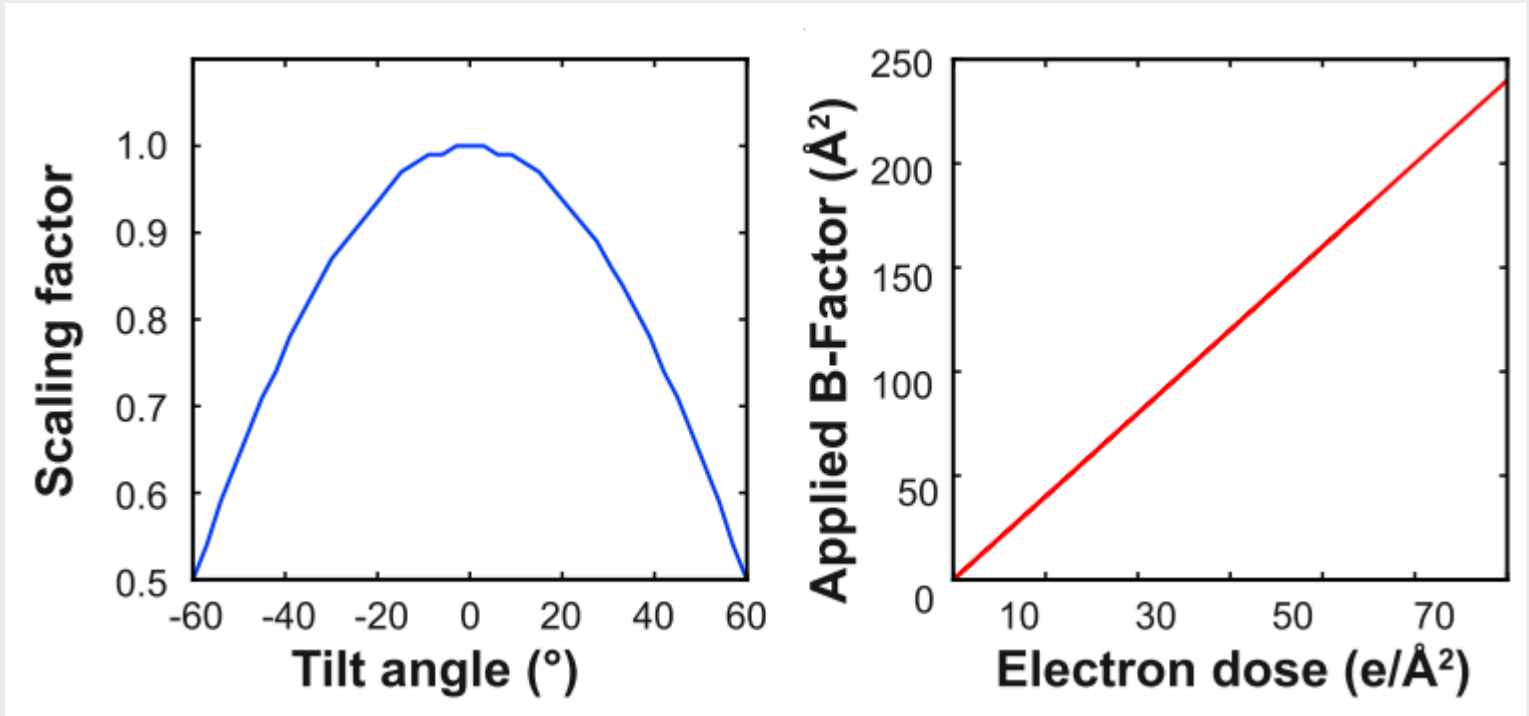


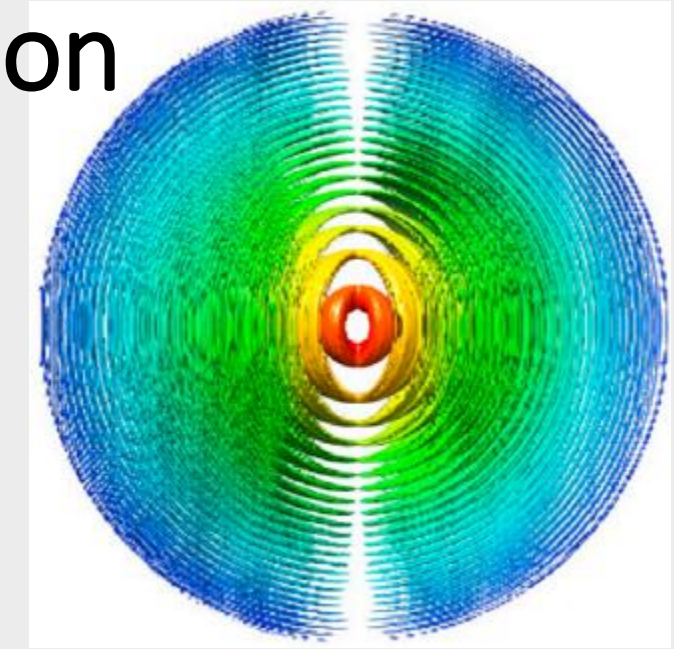


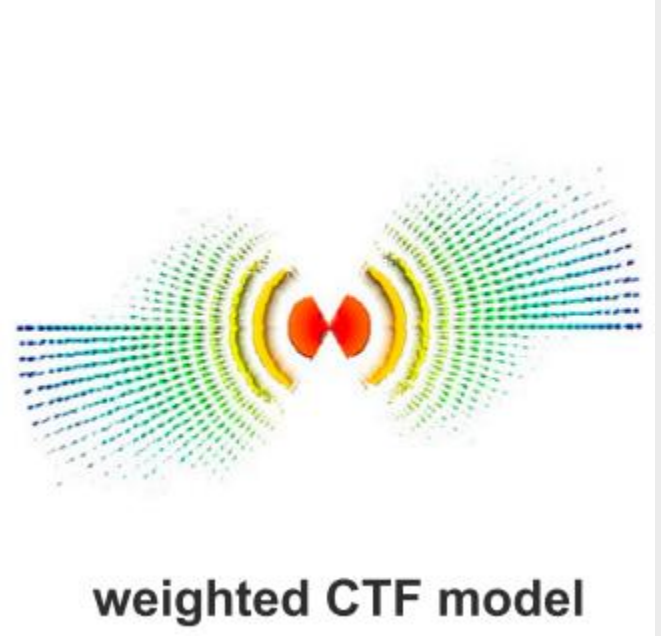
- Uses normal Relion workflow.
- Potential issues:
  - Extra images are likely not at the same focus as the Target
  - 3D FSC may eliminate properly interpolated values due to sampling

Bharat et. al., Structure 2015

Sub-tomogram processing in Relion

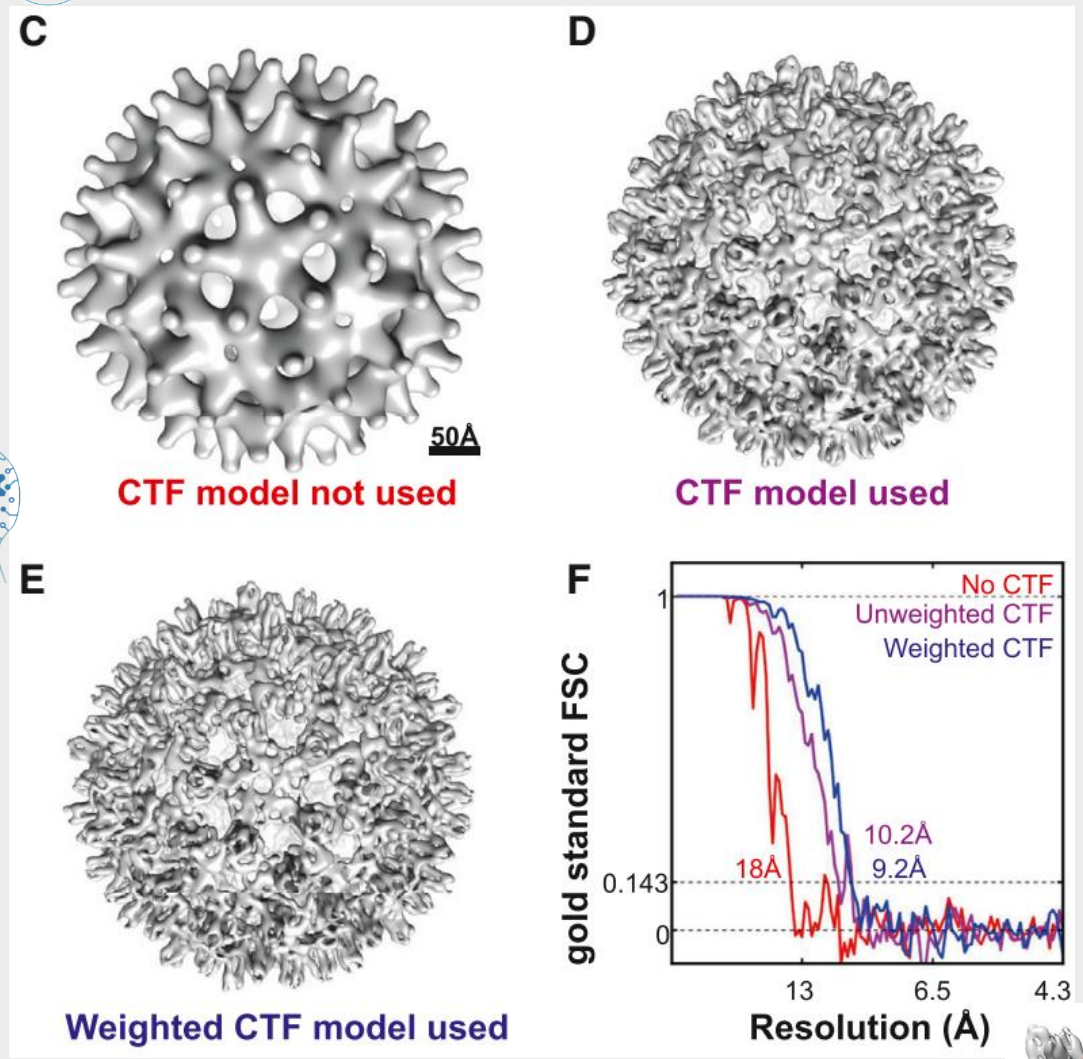


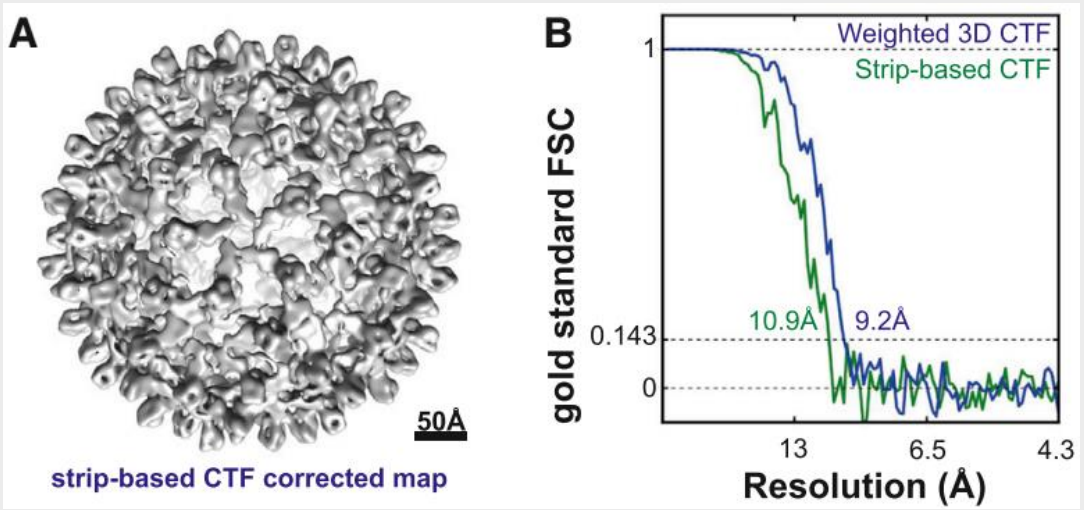


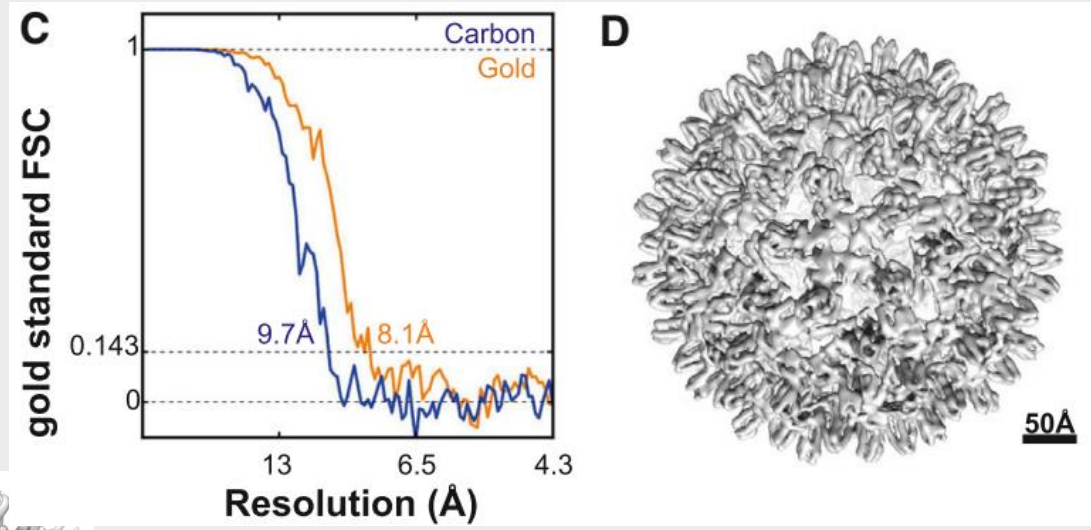


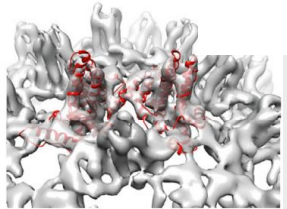
Bharat et. al., Structure 2015

Sub-tomogram processing in Relion





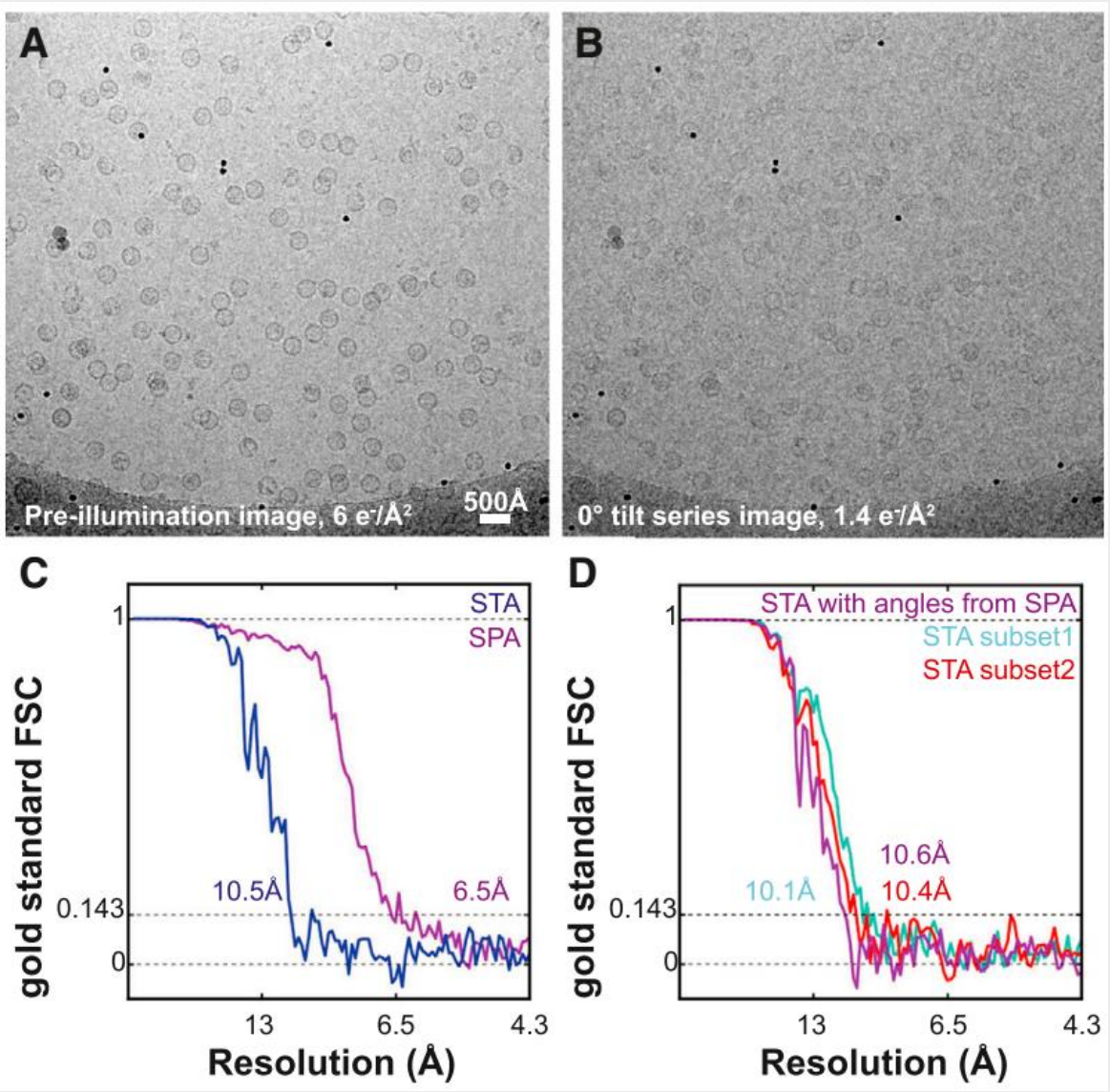




- Test case: Hepatitis B capsid

Bharat et. al., Structure 2015

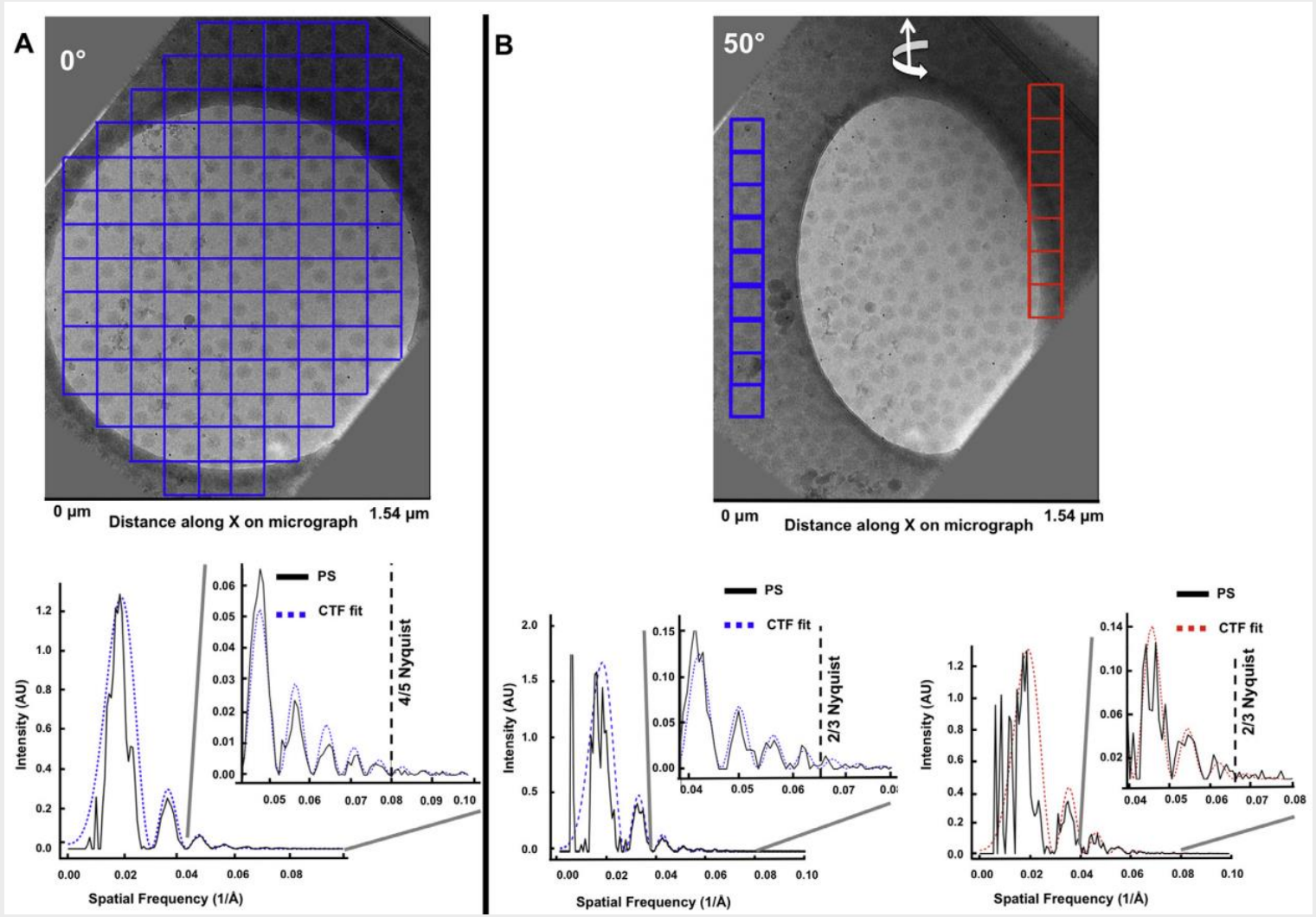
Sub-tomogram processing in Relion



- 6e-/A^2 pre-exposures prior to tilt-series collected were collected and analyzed with single particle

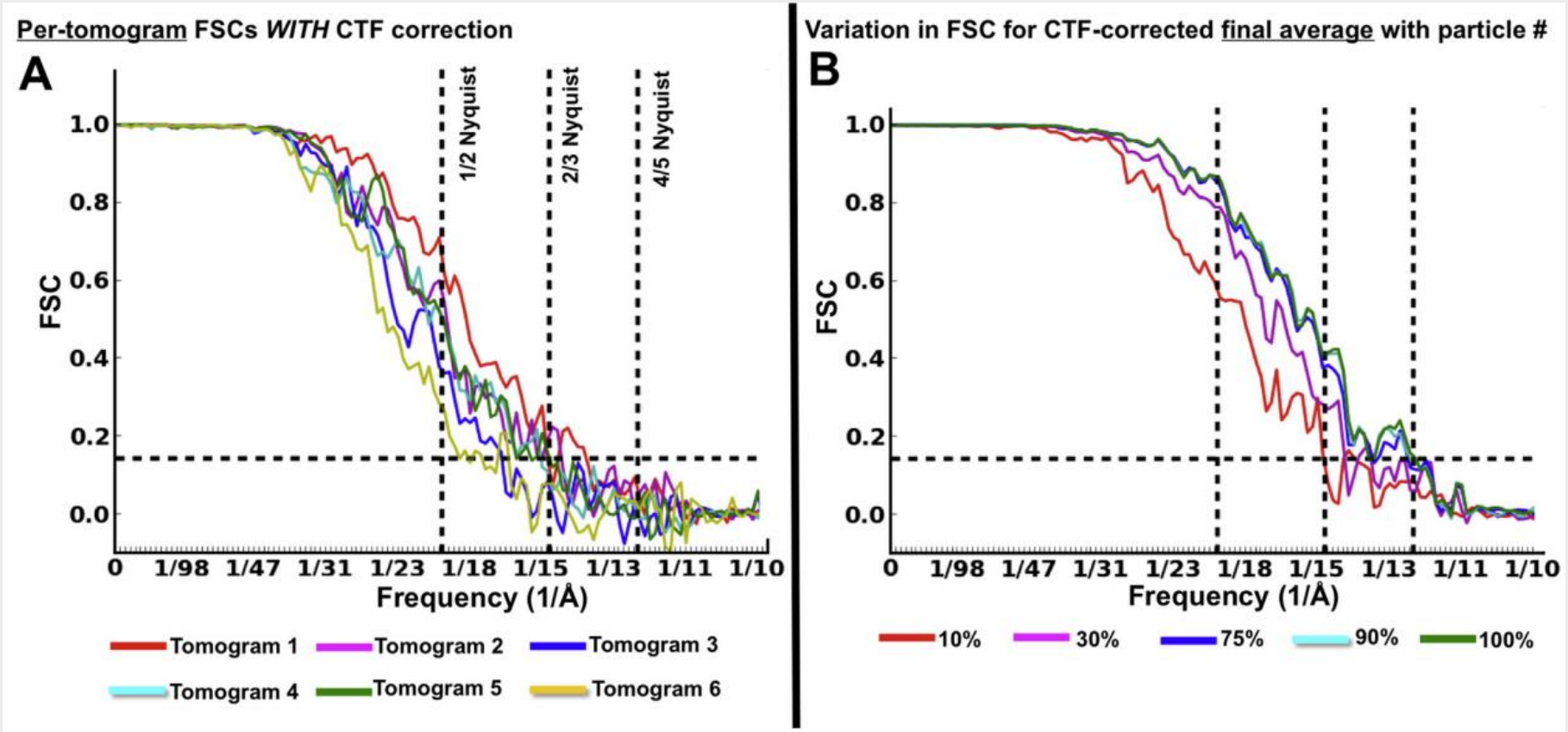
Bharat et. al., Structure 2015

Sub-tomogram processing in EMAN2



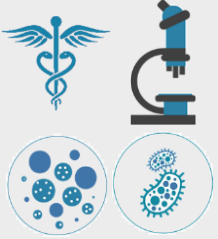
Galaz-Montoya, JSB 2016

Sub-tomogram processing in EMAN2



- Better than 2/3 Nyquist

Galaz-Montoya, JSB 2016

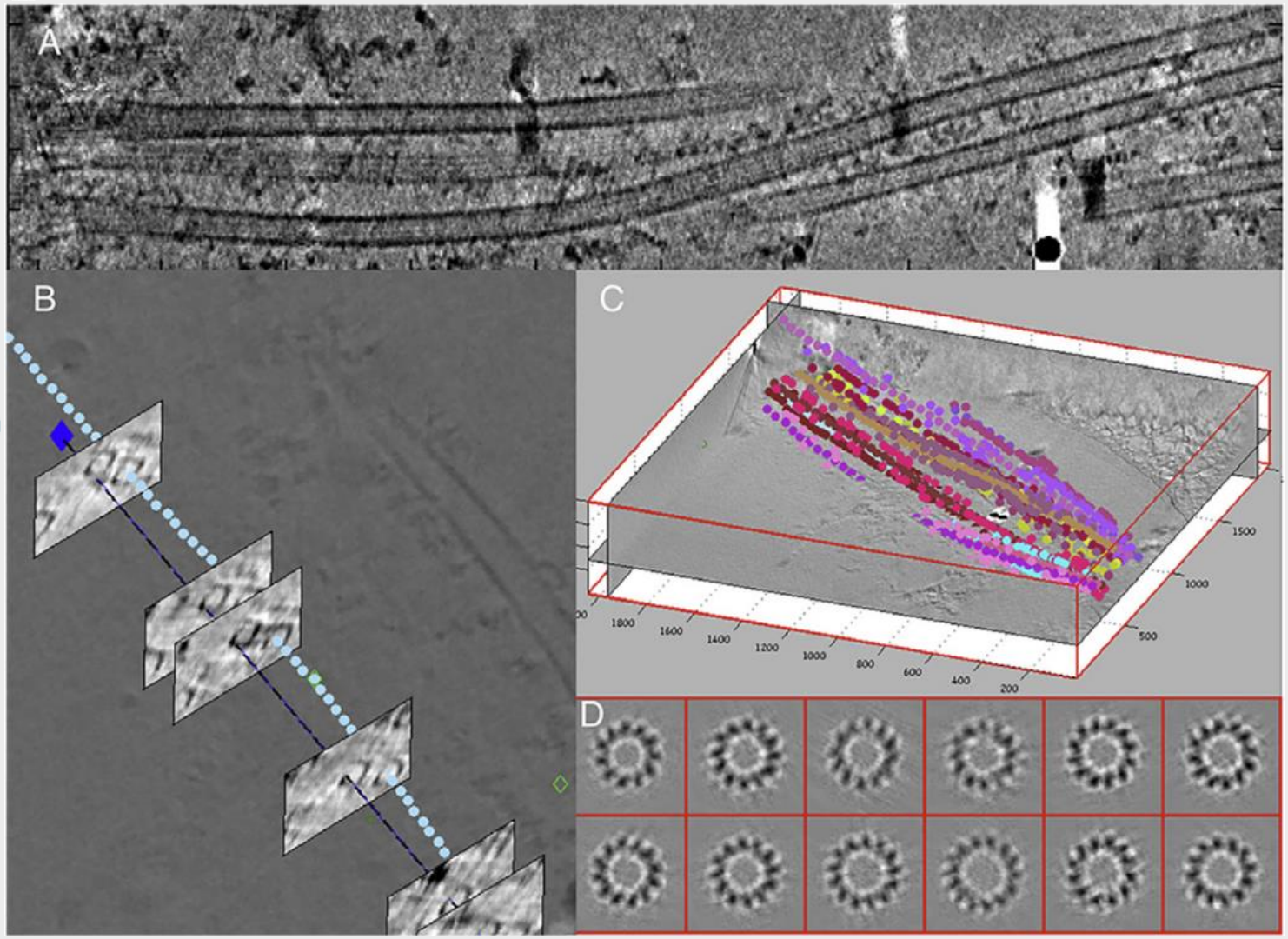


Tomogram annotation

Tomogram/sub-tomogram annotation and segmentation software

- Dynamo – Annotate membranes, tubes, helices, crystal structures, vesicles, etc.
- EMAN2 – Neural network segmentation
- Amira – Interactive segmentation and filtering suite
- UCSF Chimera w/ Segger - Interactive segmentation
- Template picking – MolMatch, Dynamo
- Various deep learning picking and segmentation softwares

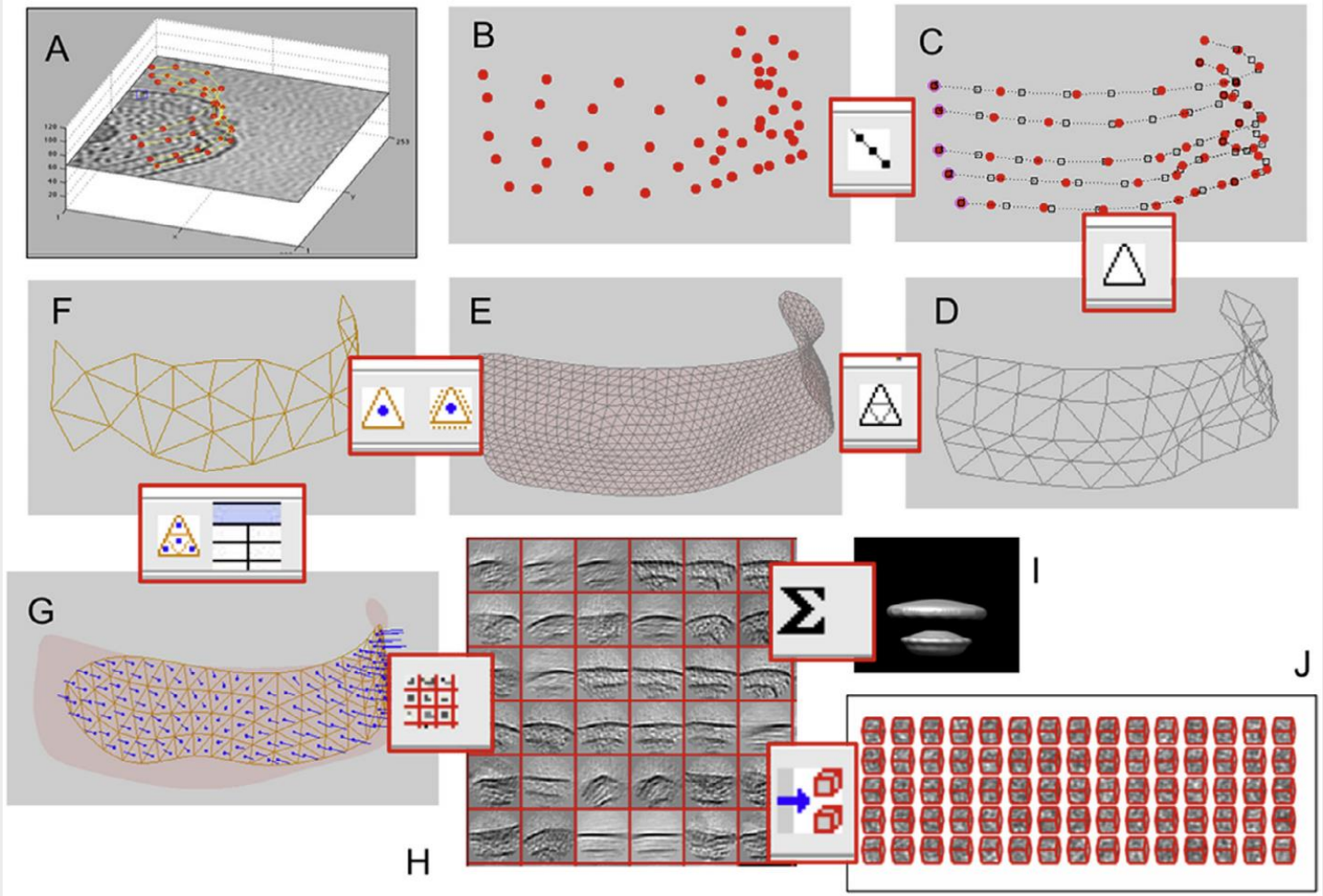
Sub-tomogram annotation processing in Dynamo



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

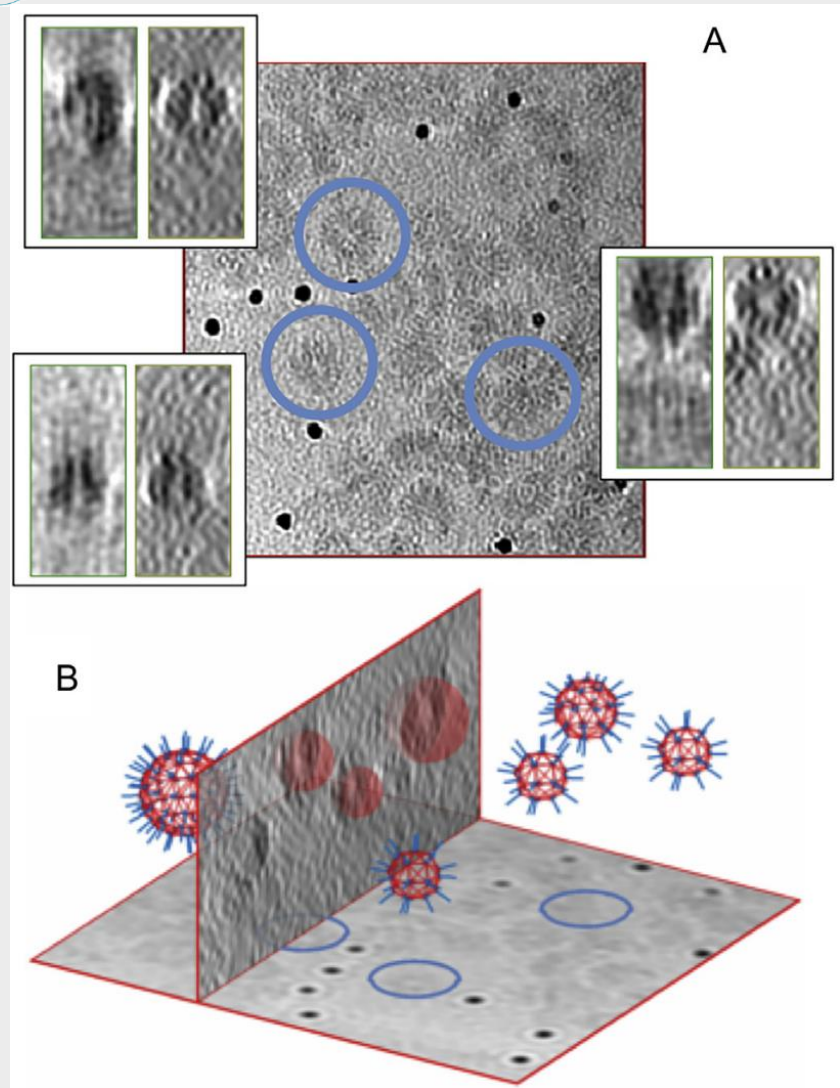
Castaño-Díez et. al., JSB 2012 & 2016

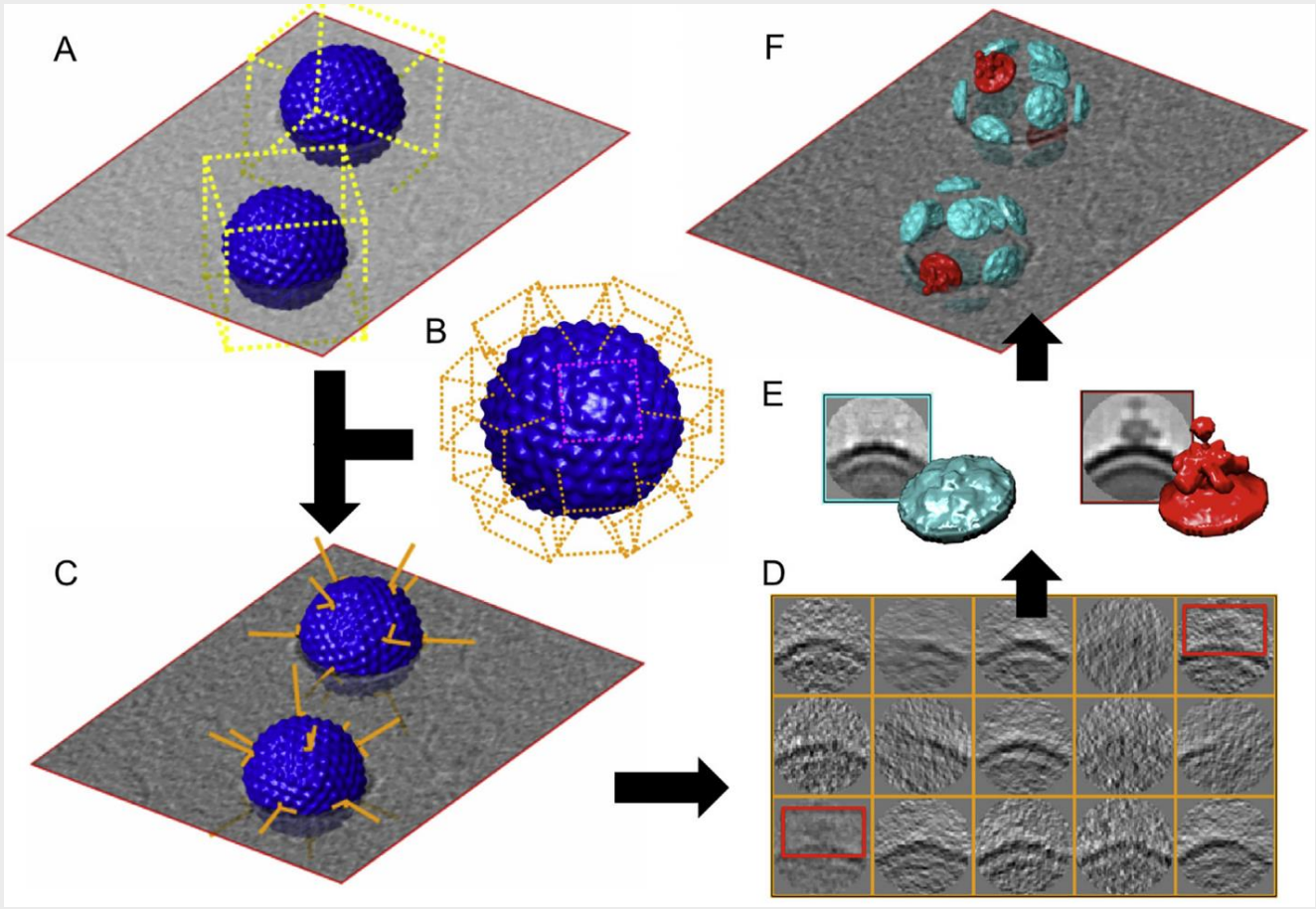
Sub-tomogram annotation processing in Dynamo



Castaño-Díez et. al., JSB 2012 & 2016

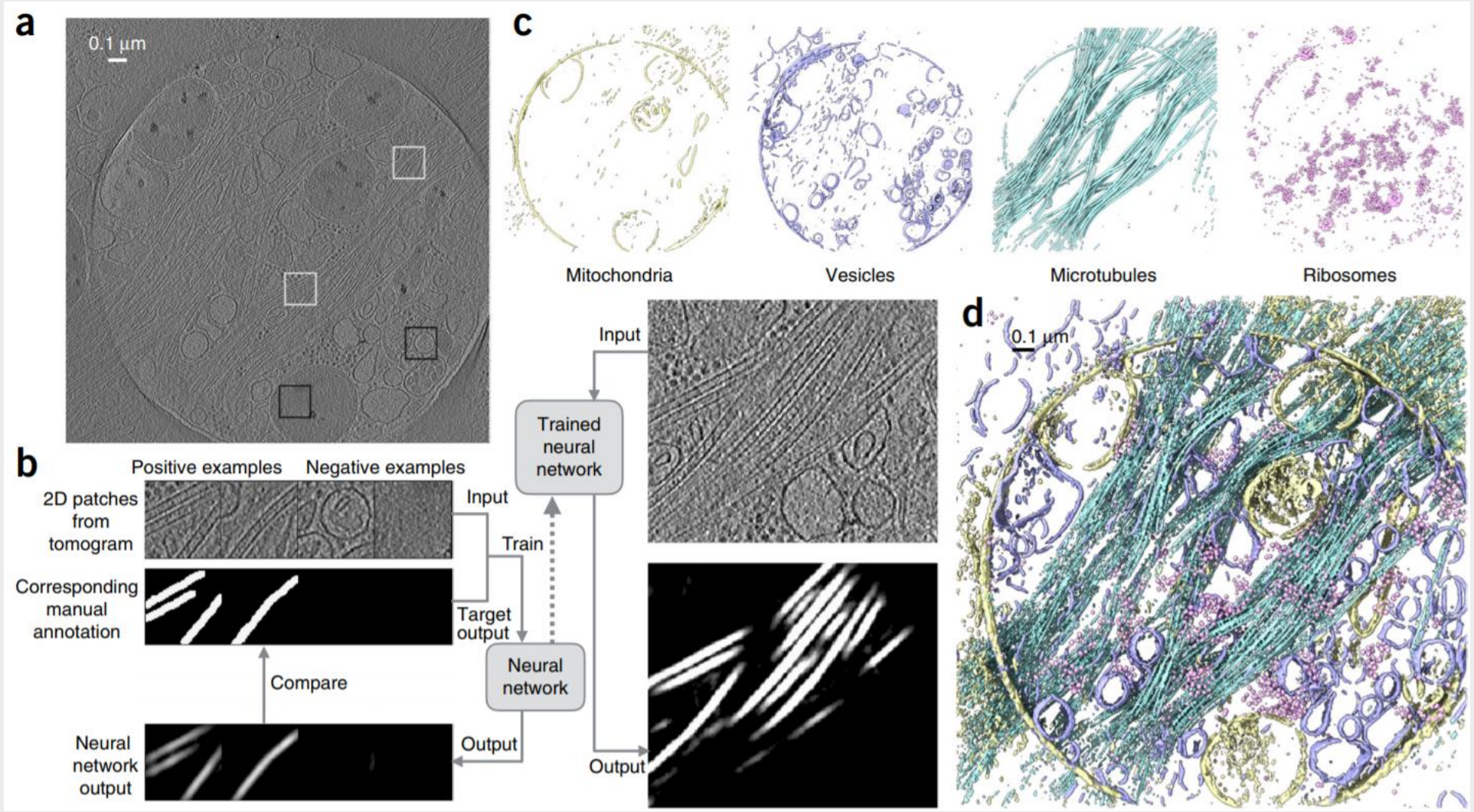
Sub-tomogram annotation processing in Dynamo





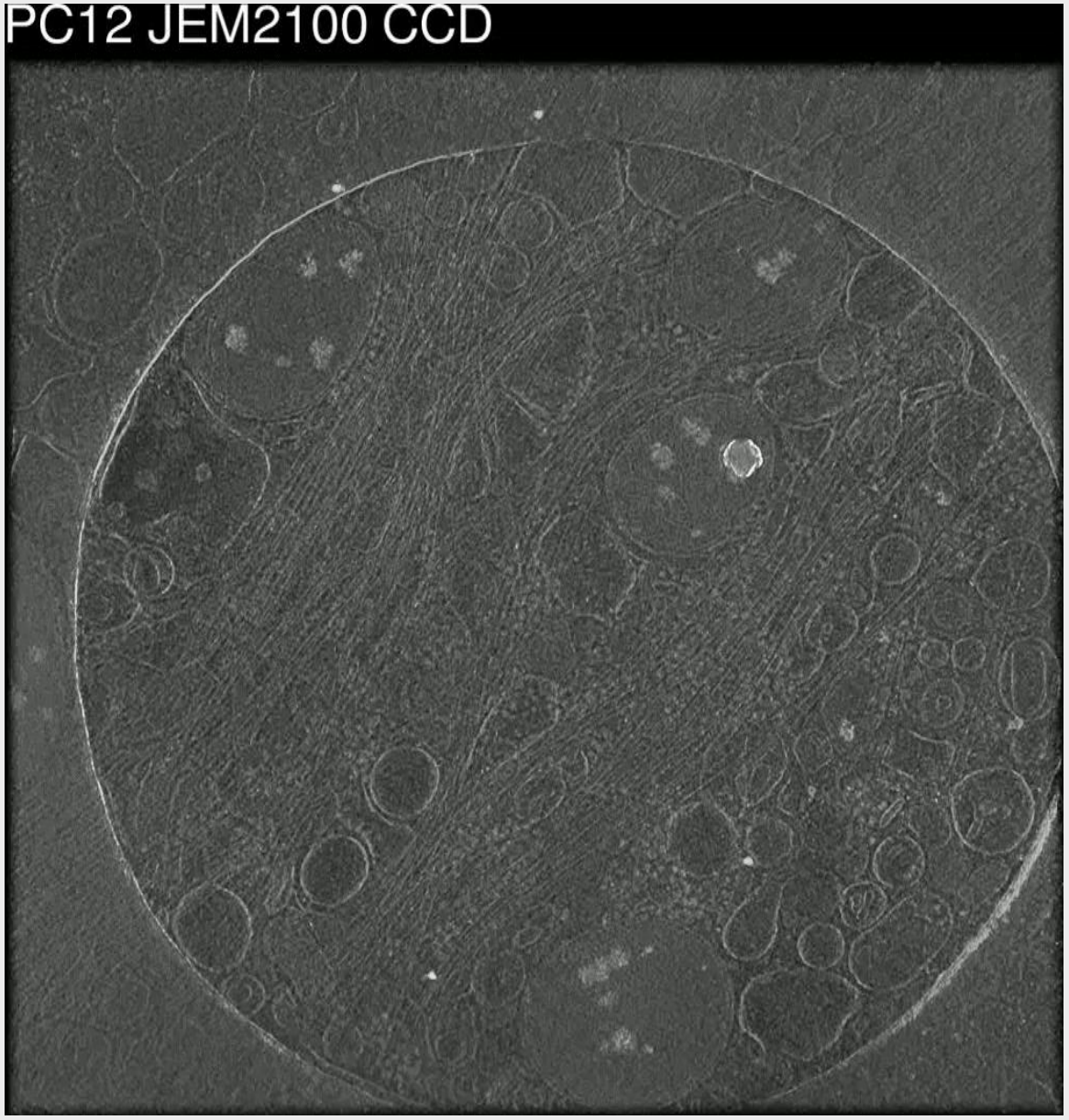
Castaño-Díez et. al., JSB 2012 & 2016

Sub-tomogram segmentation with CNNs in EMAN2



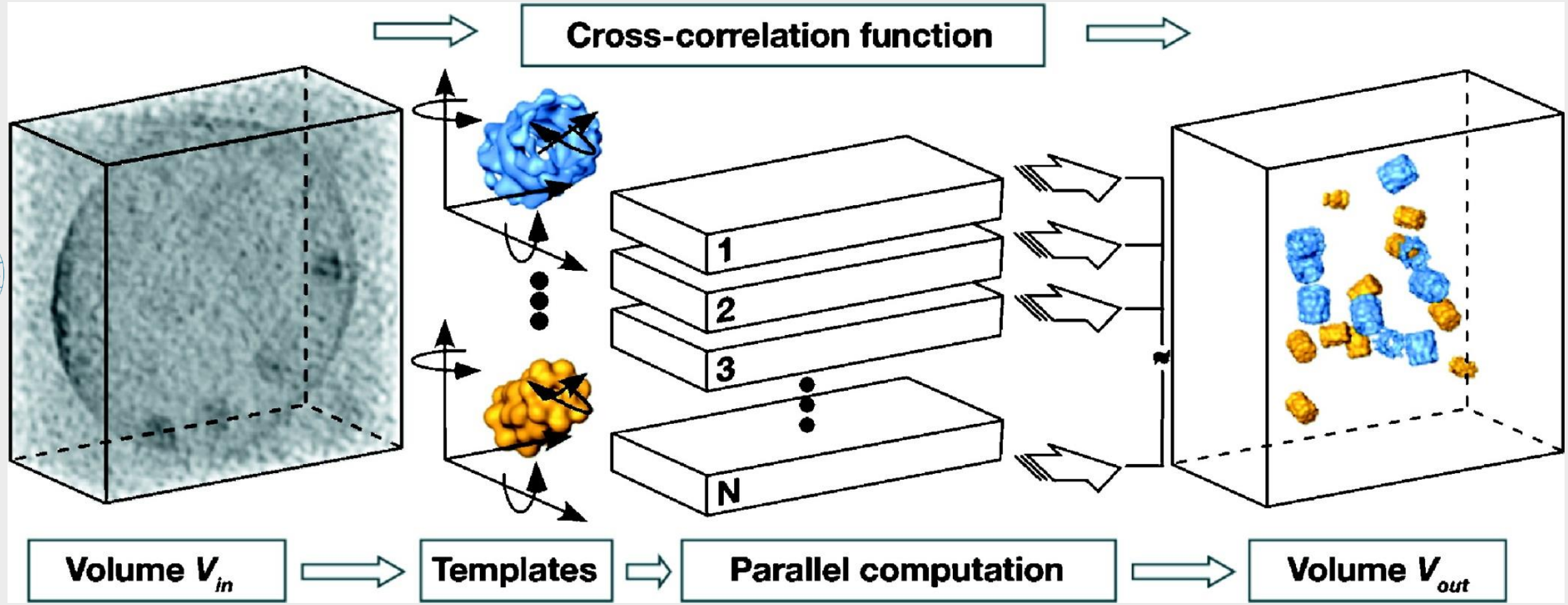
Chen et. al., Nat. Meth. 2017

Sub-tomogram segmentation with CNNs in EMAN2



Chen et. al., Nat. Meth. 2017

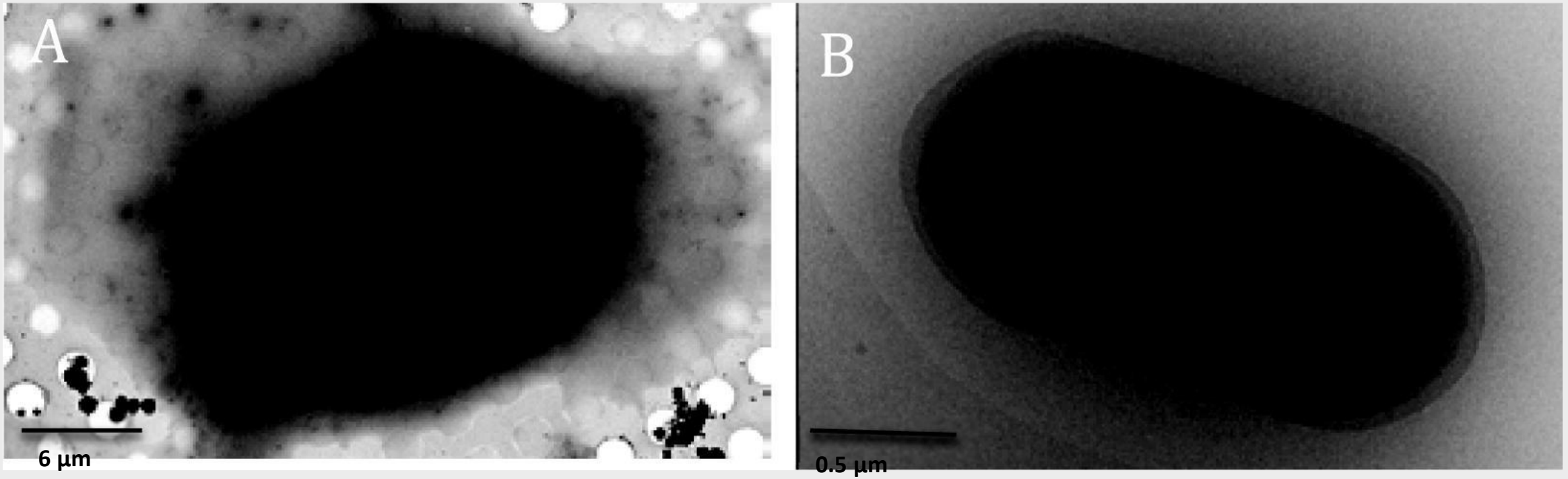
Template matching



Lucic et al, 2005, Annu. Rev. Biochem

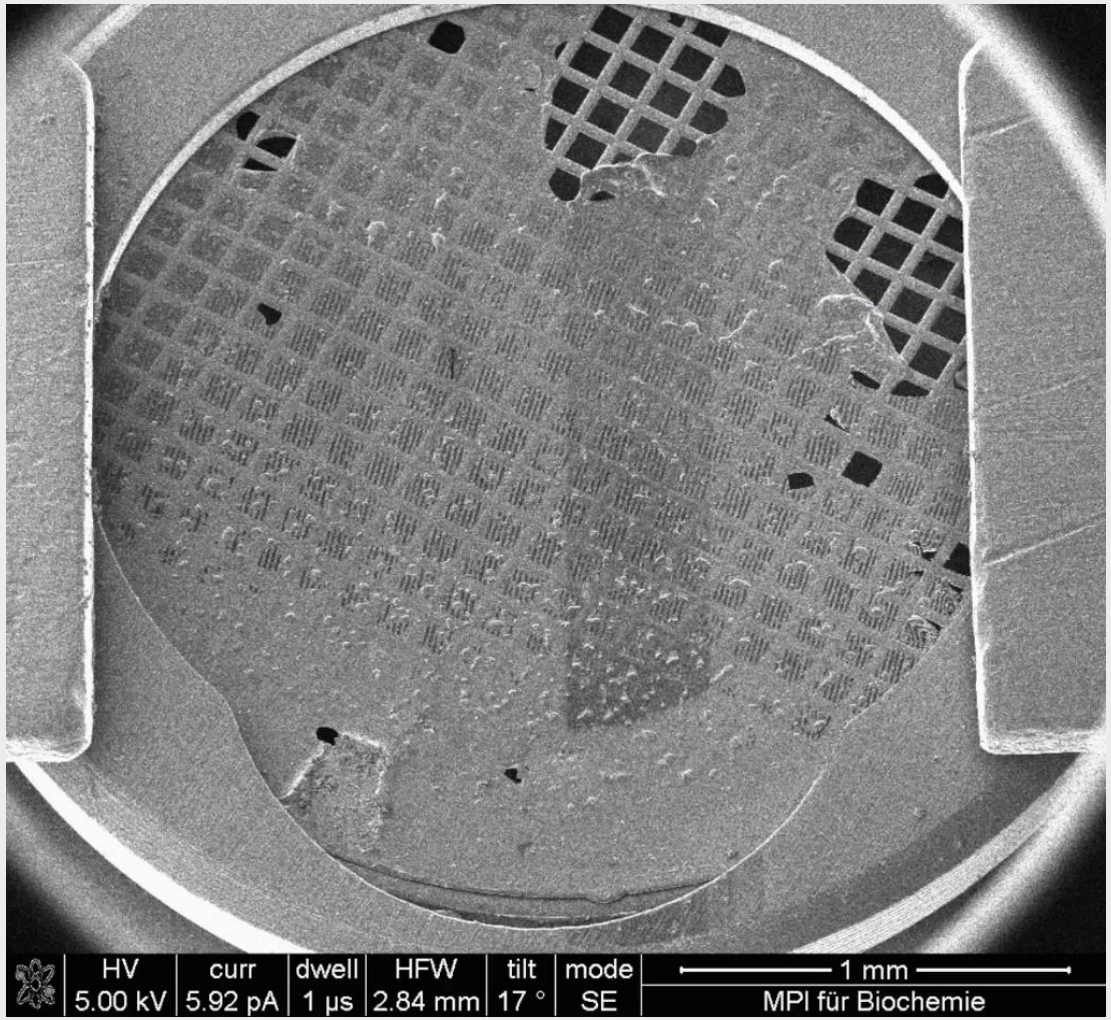
CryoET allows for glimpses into cells/tissues

CryoET allows for glimpses into cells/tissues



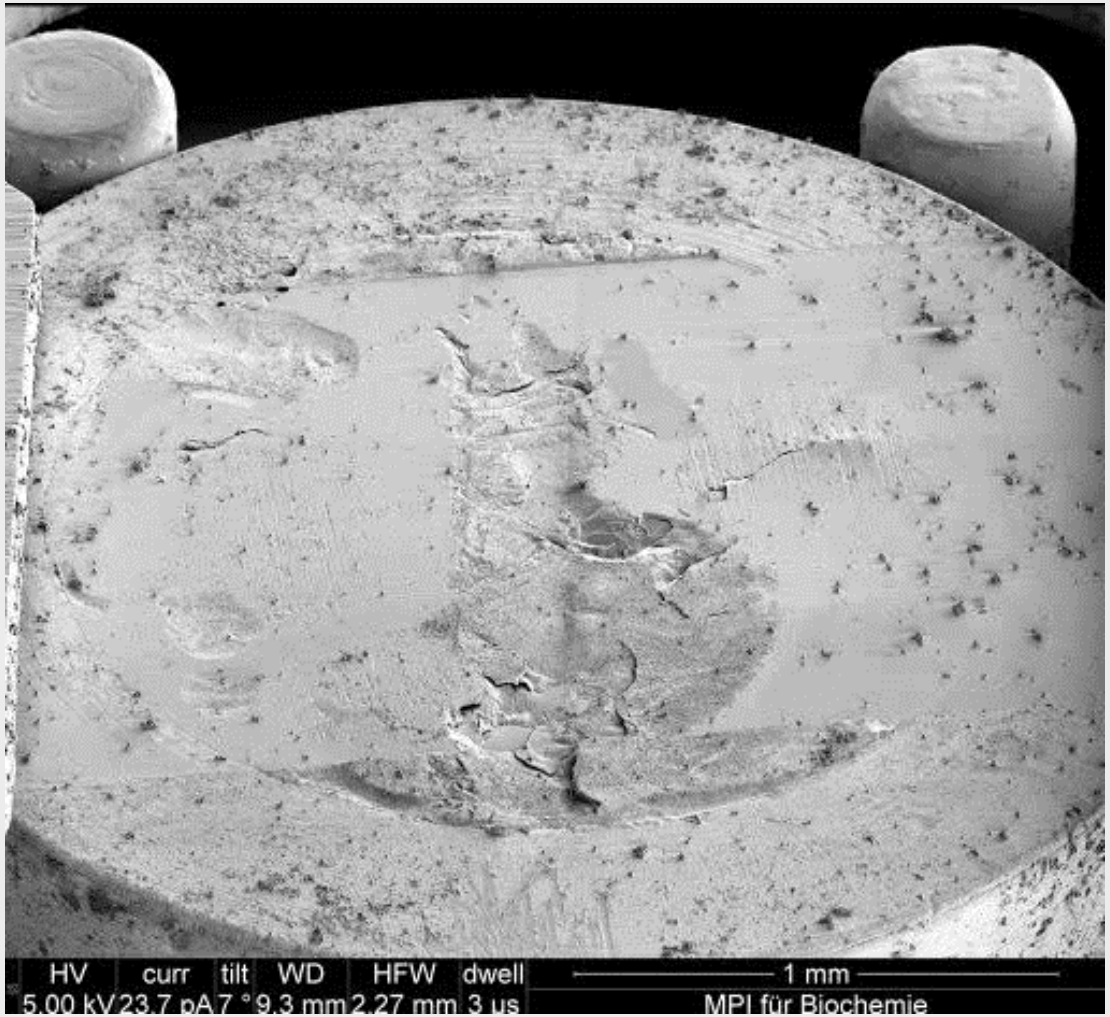
Thompson et. al., 2016

CryoET allows for glimpses into cells/tissues



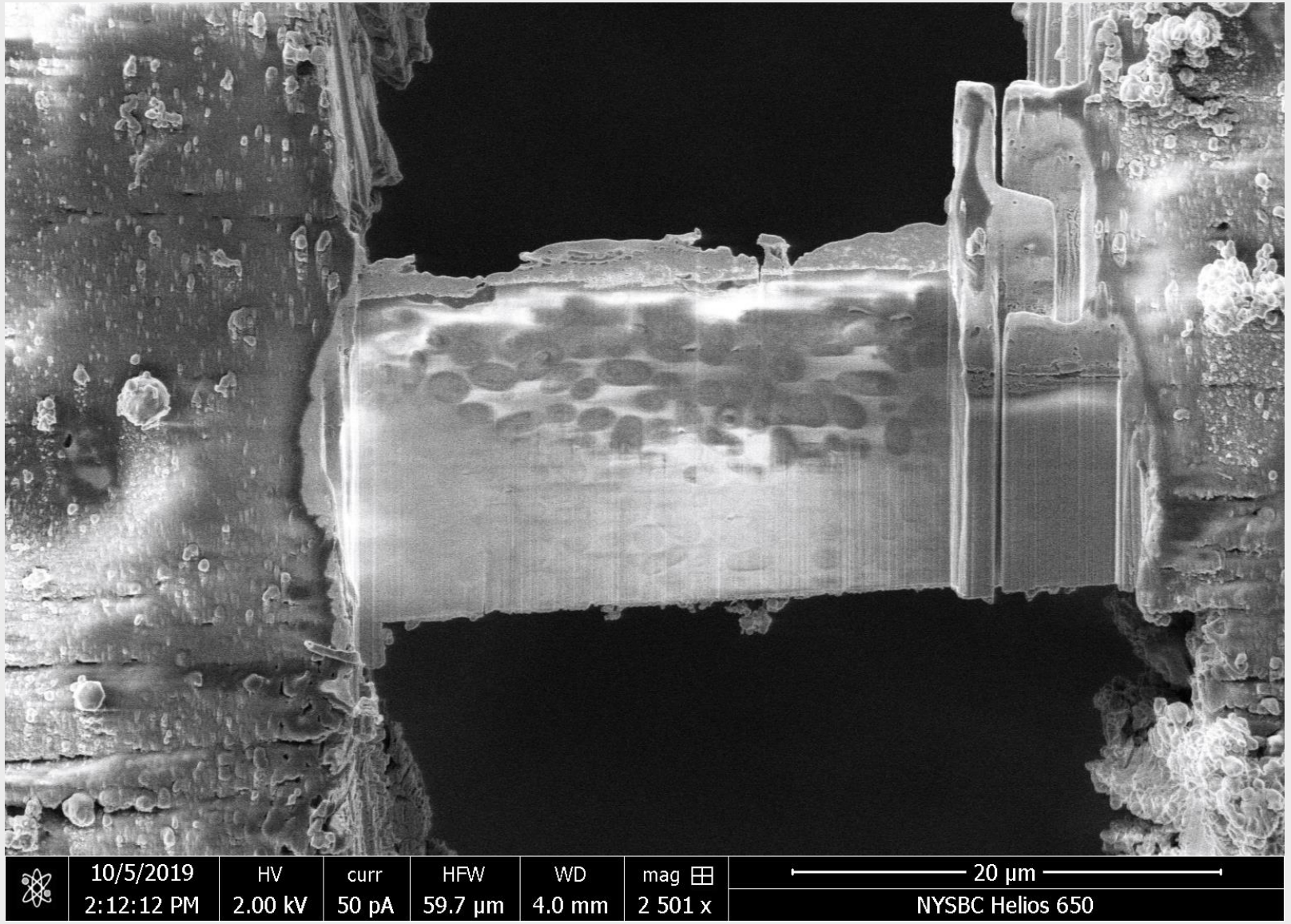
Baumeister et al., MPI

CryoET allows for glimpses into cells/tissues



Schaffer et al., Nat. Meth.

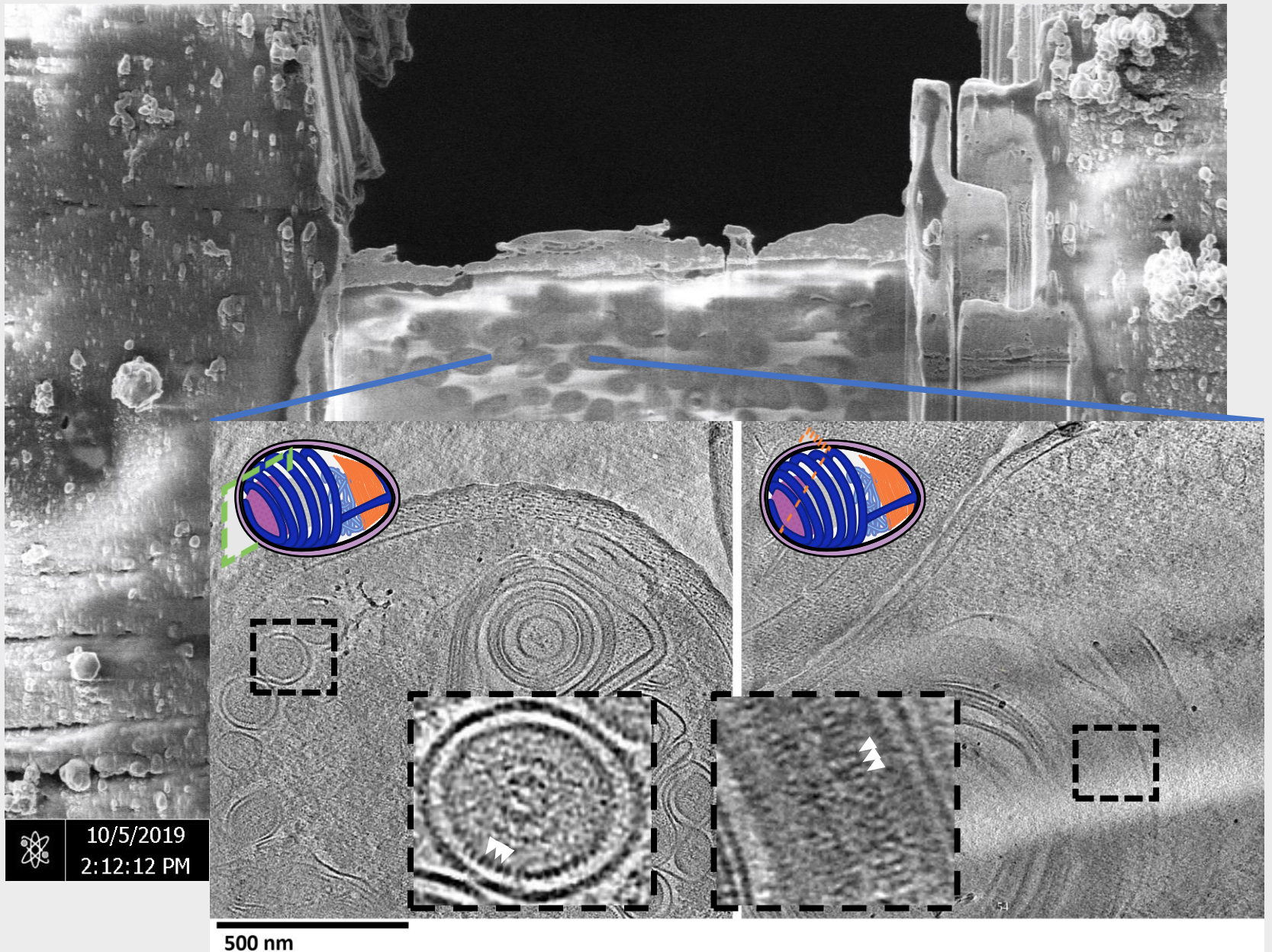
CryoET allows for glimpses into cells/tissues



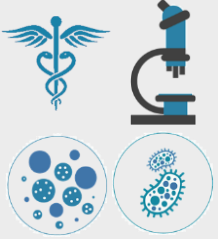
Waffle method

Kelley et al., 2020

CryoET allows for glimpses into cells/tissues

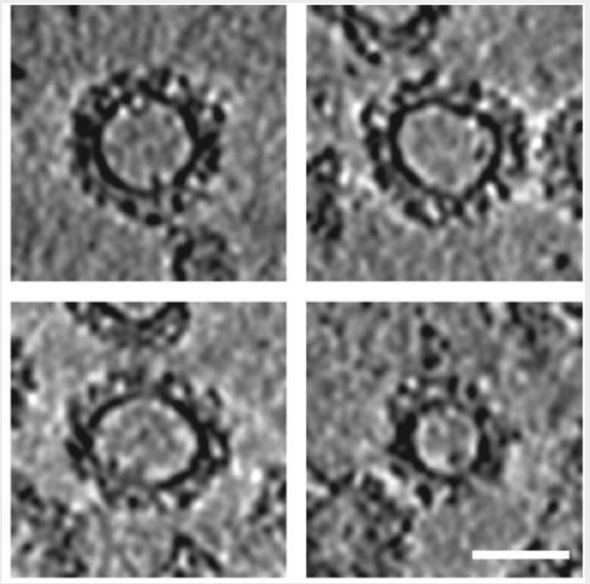


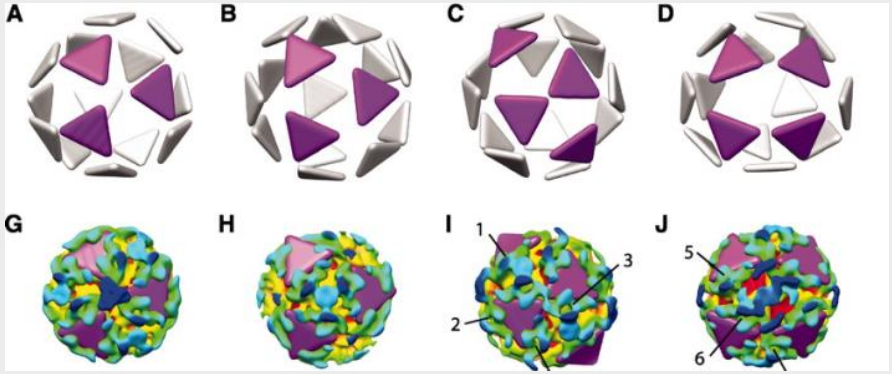
Kelley et al., 2020



Examples from the literature

Example: STA followed by placing averages to the tomograms

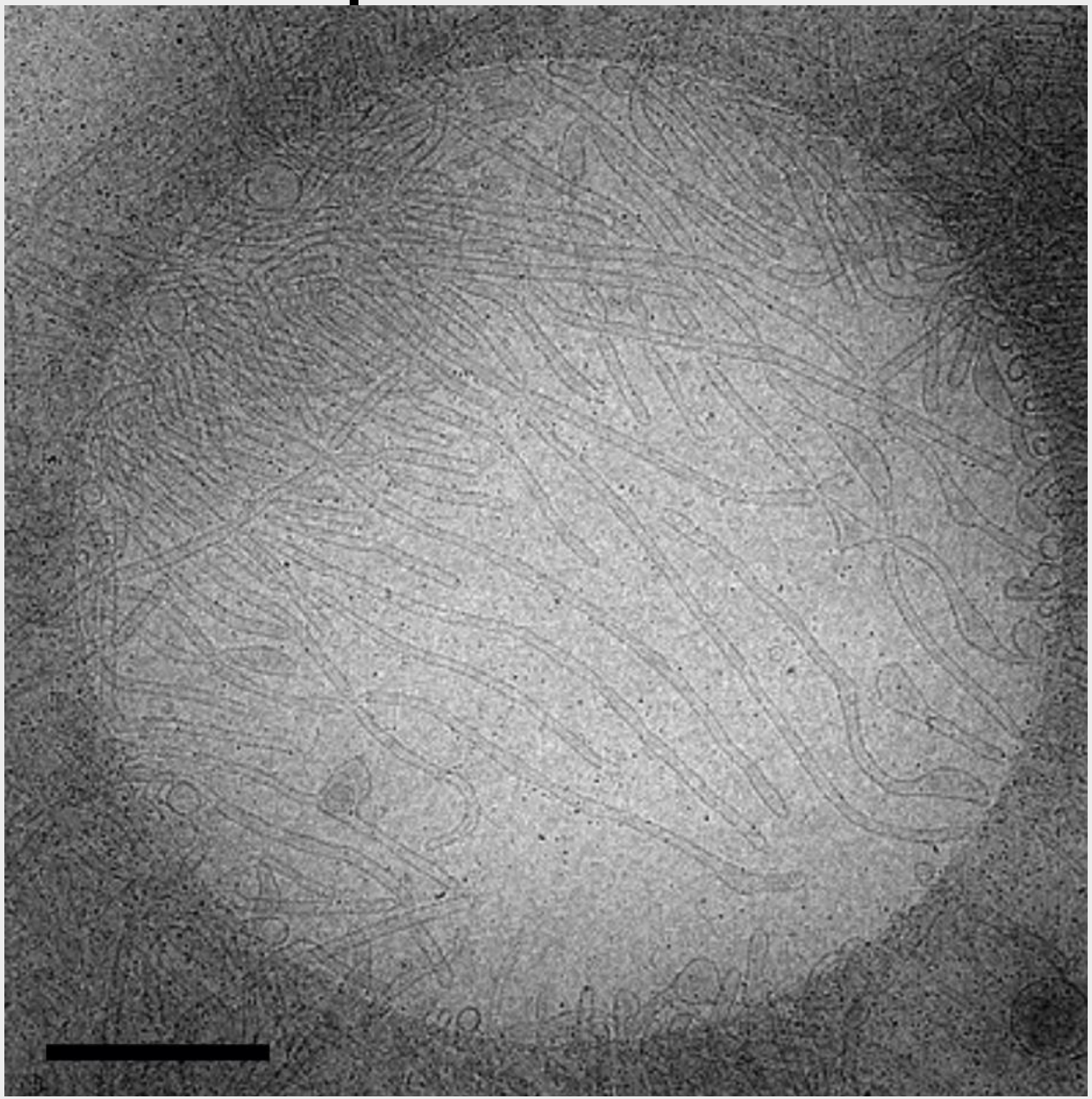


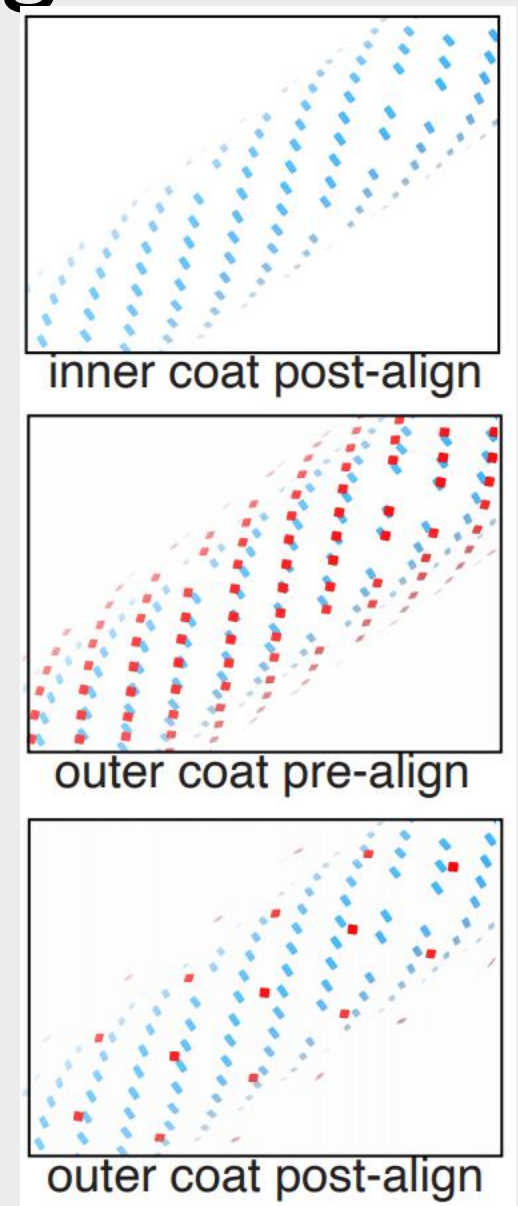


COP-I coated vesicles From: Faini et al, Science, 2012

Example: COPII proteins on budding GUVs

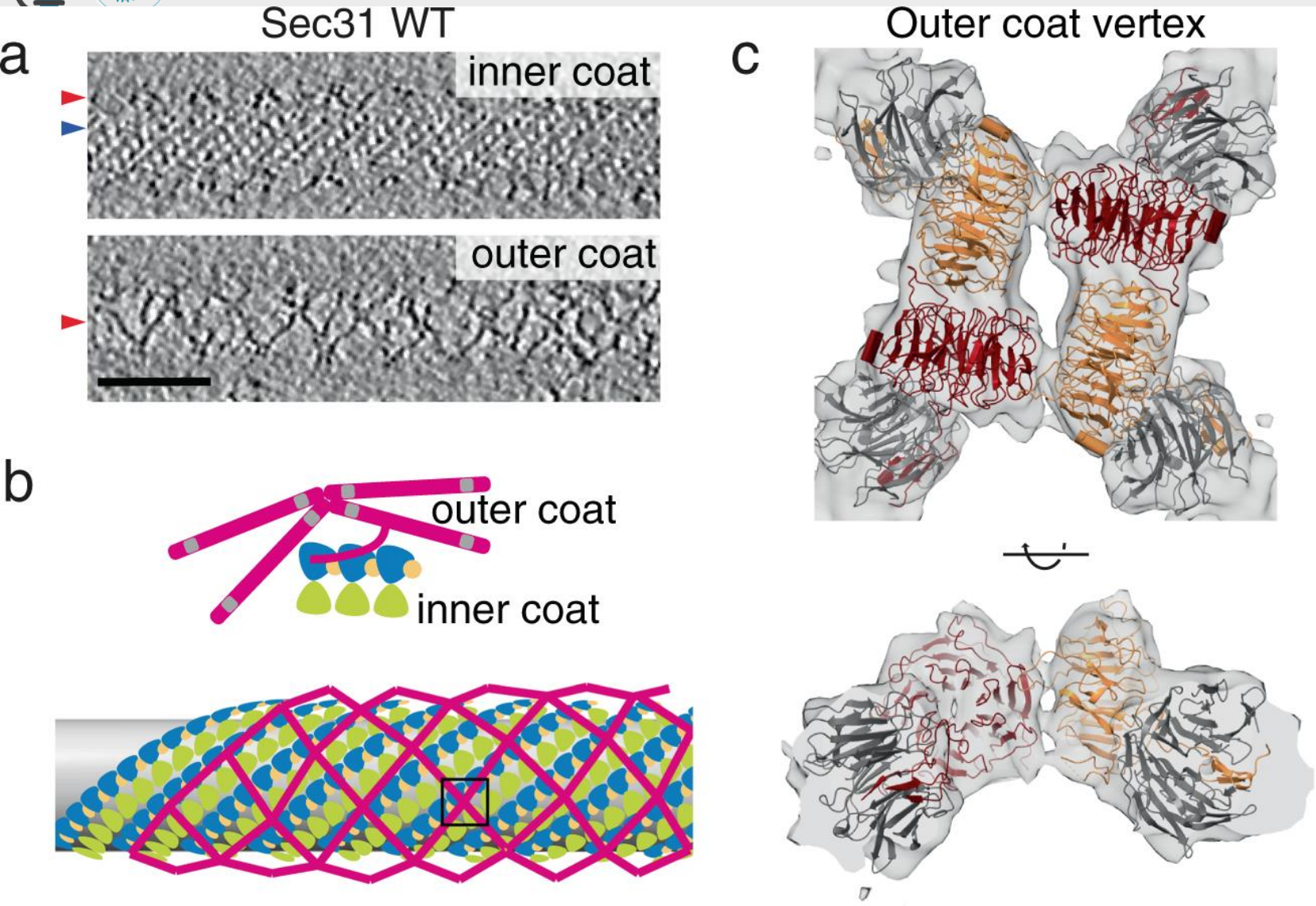
Over-picking to find repeating units





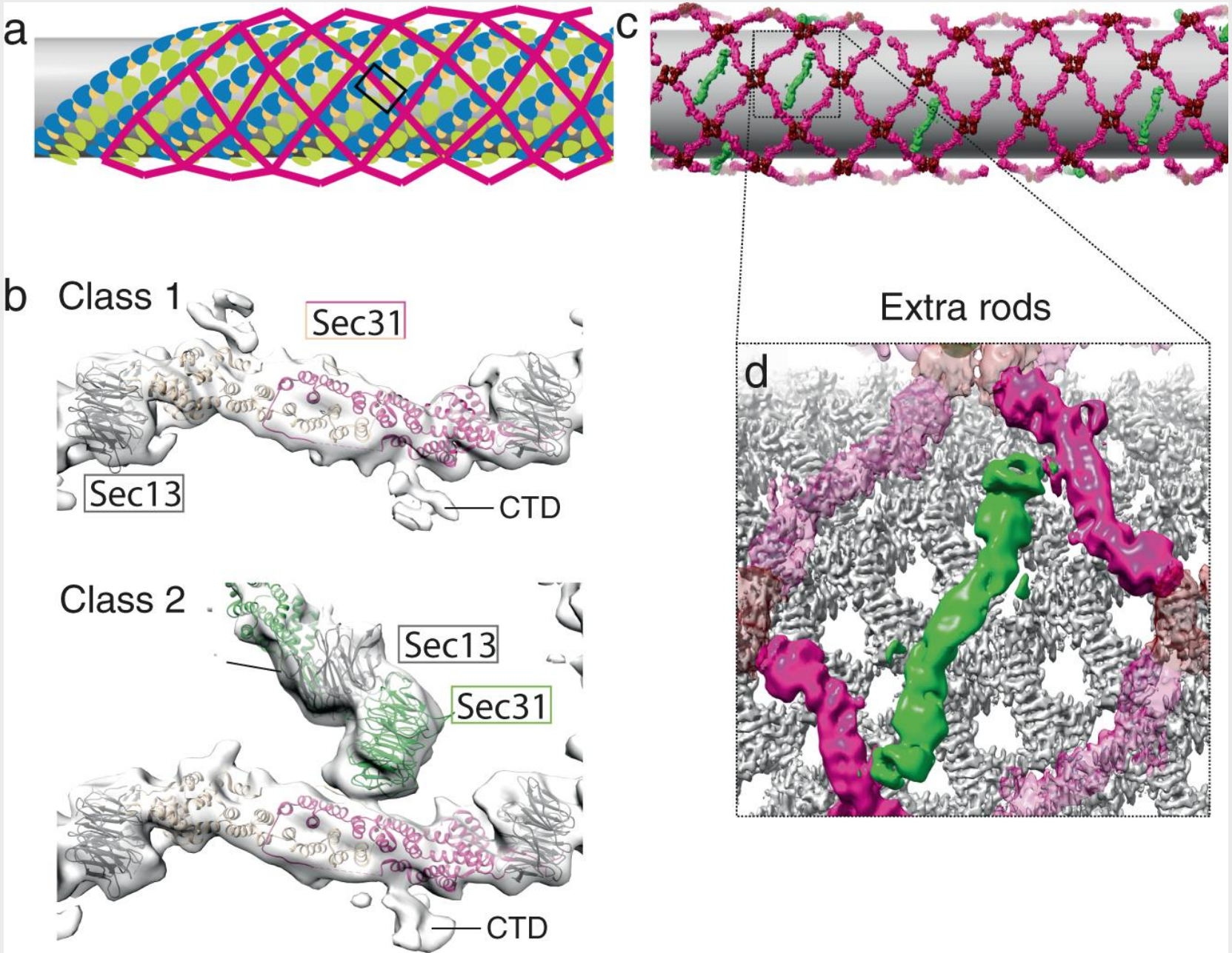
Hutchings et al, 2021

Example: COPII proteins on budding GUVs



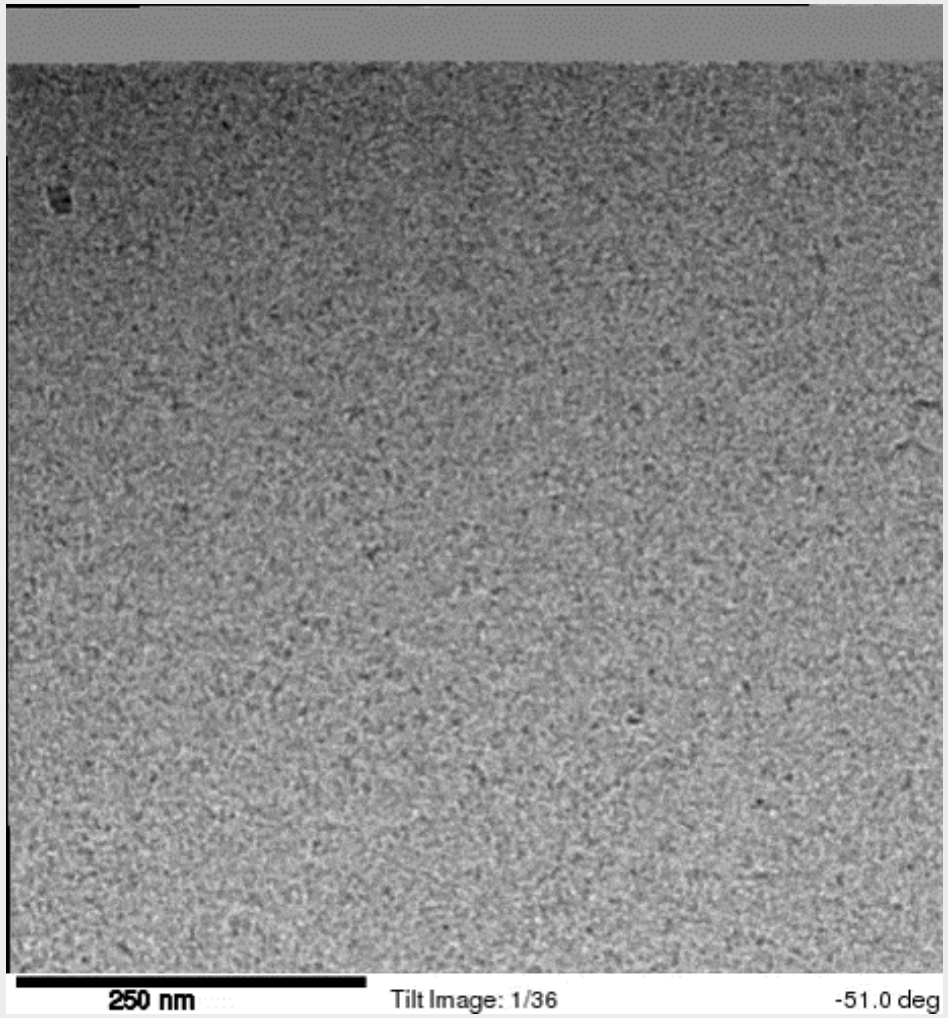
Hutchings et al, 2021

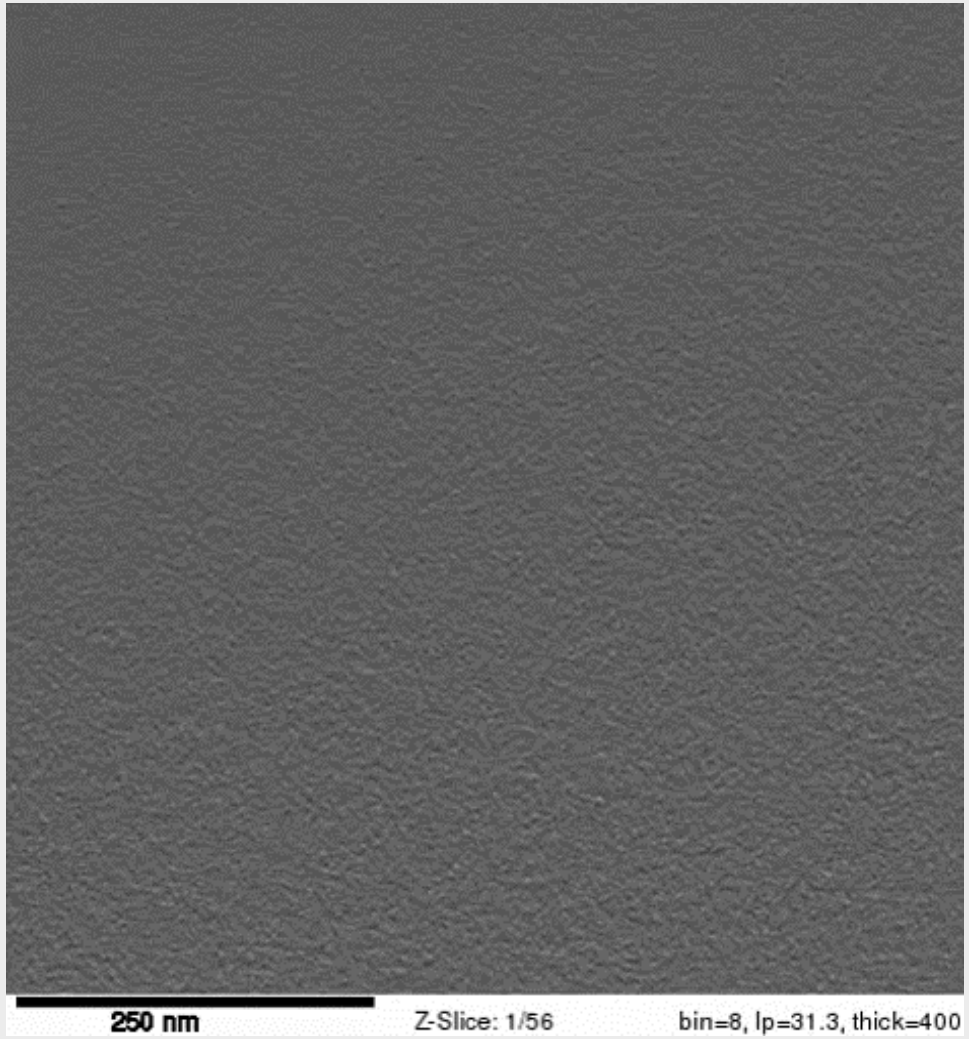
Example: COPII proteins on budding GUVs



Hutchings et al, 2021

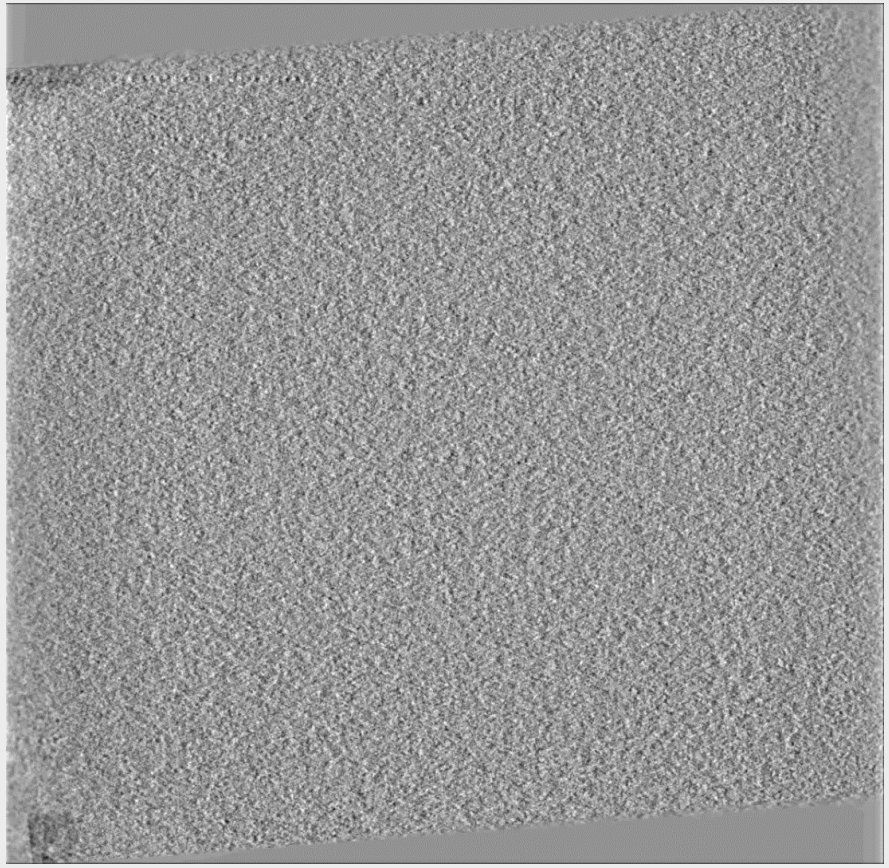
Example: HIV-1 trimer single particle





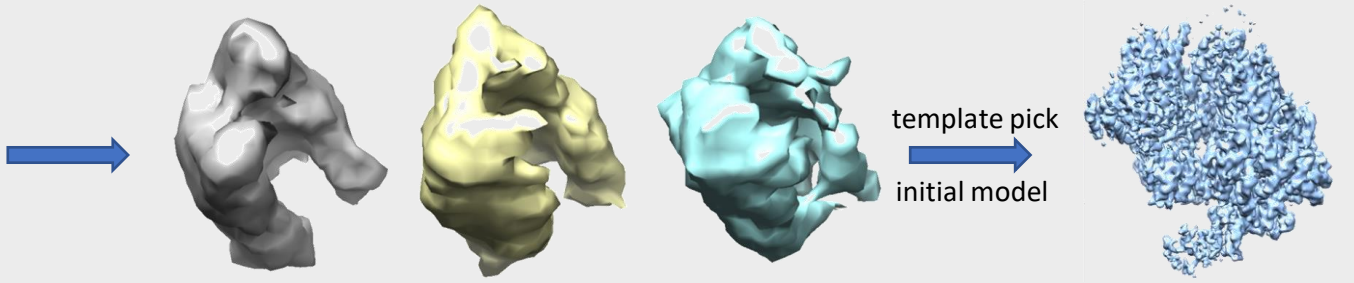
Priyamvada Acharya & Alex Noble
eLife, 2018

Example: Tomography for single particle initial model



250 nm

Z-slices through tomogram/ice



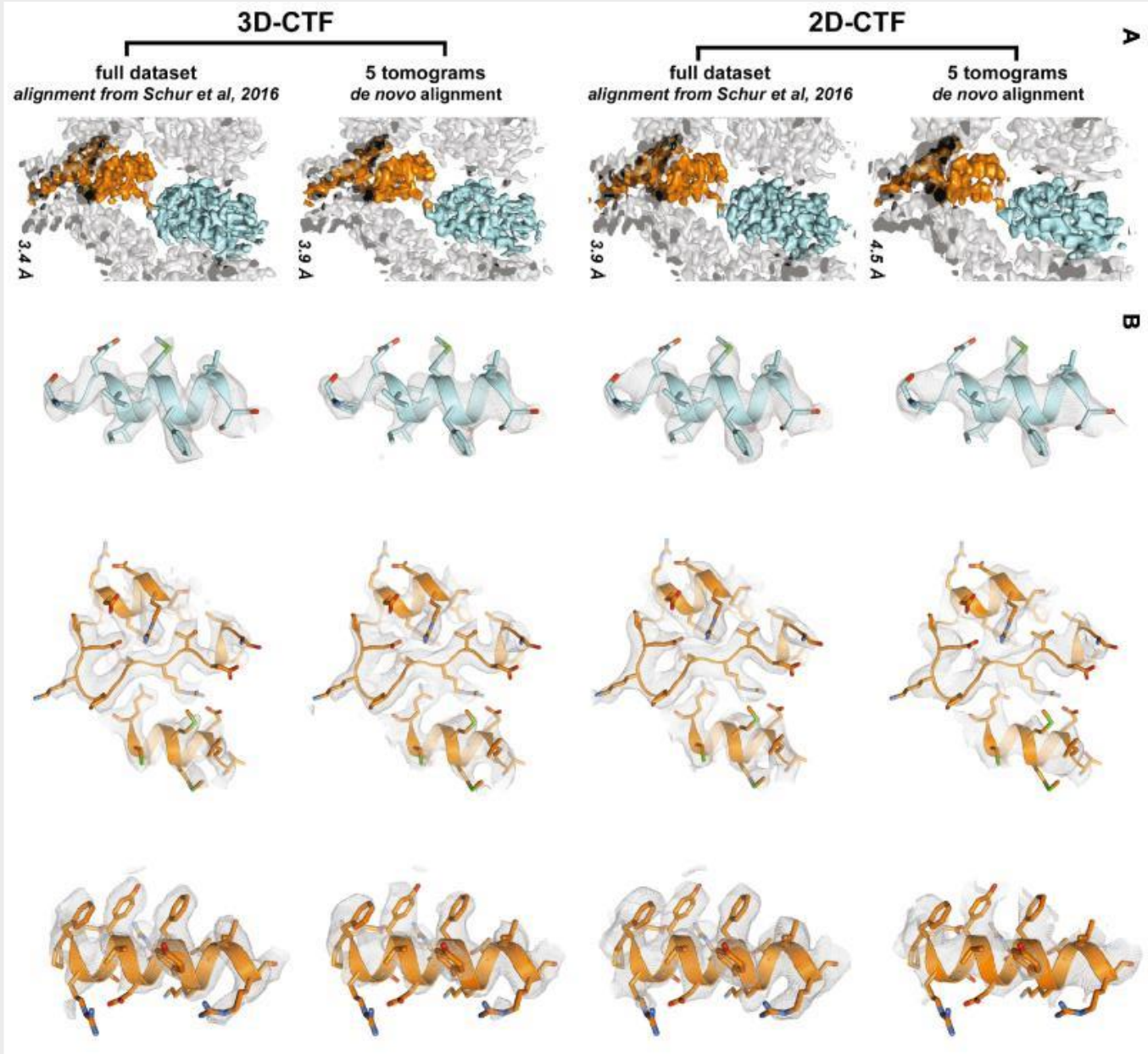
Structure now at 4 Å

- Classes used as templates for picking single particle micrographs
- Single particle now at 4 Å without anisotropy.

Jillian Chase
and Alex Noble
eLife, 2018 and 2019

Example: HIV-1 Capsid-SP1 at 3.9/3.4/3.2/3.0 Å

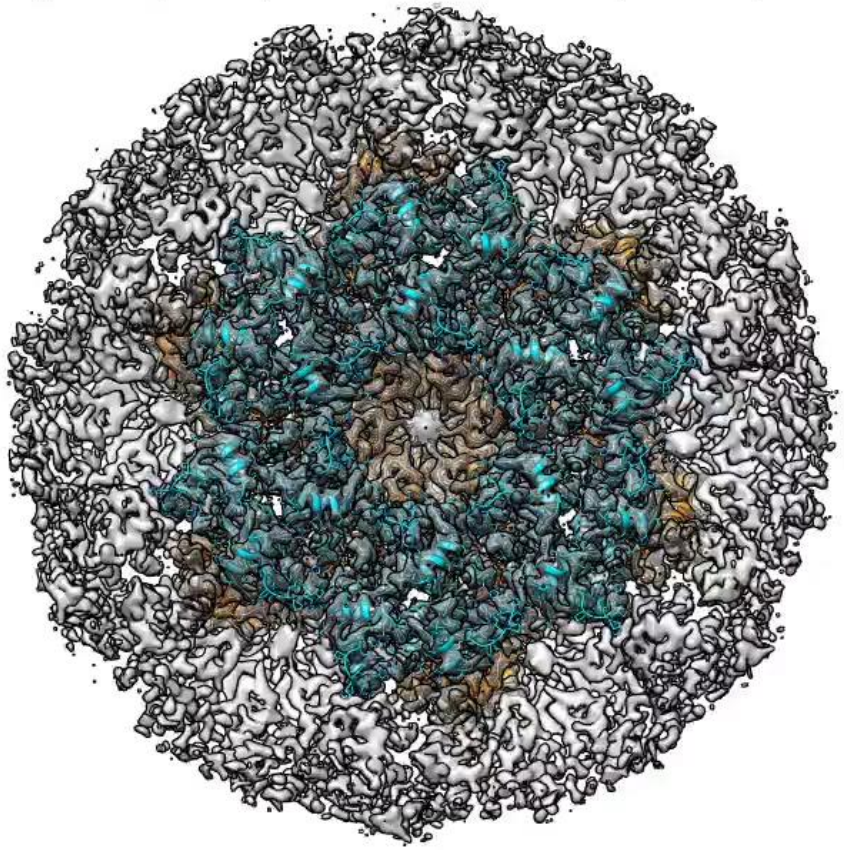
- Krios + Super-res K2 + Gatan Energy Filter
- Fiducial tilt-series alignment
- 1.5 – 5 micron defocus
- Strip-based CTF correction
- ~750,000 sub-particles used
- TOM, AV3, Dynamo, and in-house scripts were used
- NovaCTF 3D CTF pushed it to 3.4 Å
- emClarity pushed to 3.2 Å
- Warp/M to 3.0 Å



Schur, 2016
Turoňová, 2017

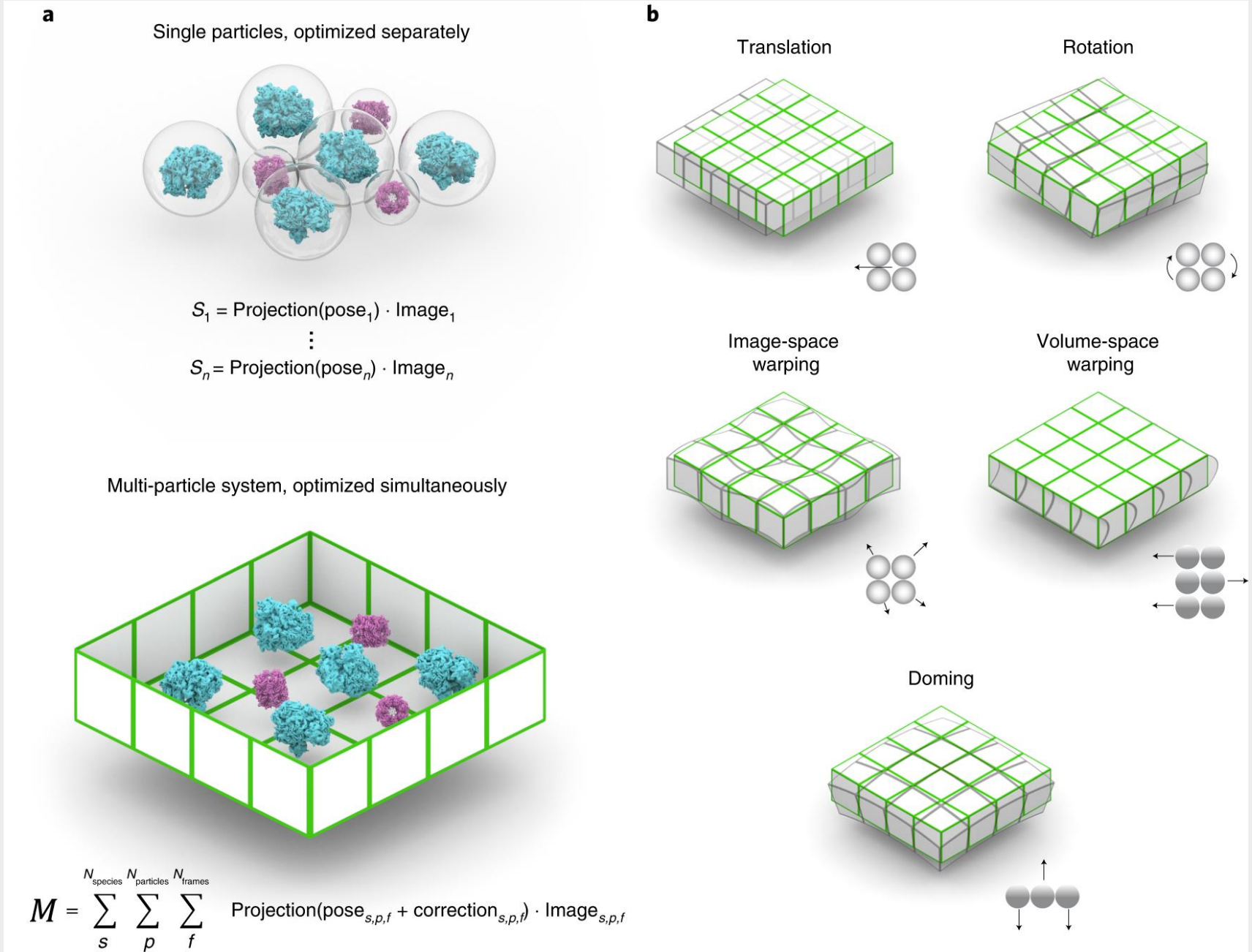
Example: HIV-1 Capsid-SP1 at 3.9 Å

An atomic model of HIV-1 capsid-SP1 reveals structures regulating assembly and maturation
Schur F.K.M, Obr M., Hagen W.J.H, Wan W., Jakobi A.J., Kirkpatrick J.M., Sachse C., Kräusslich H-G., Briggs J.A.G



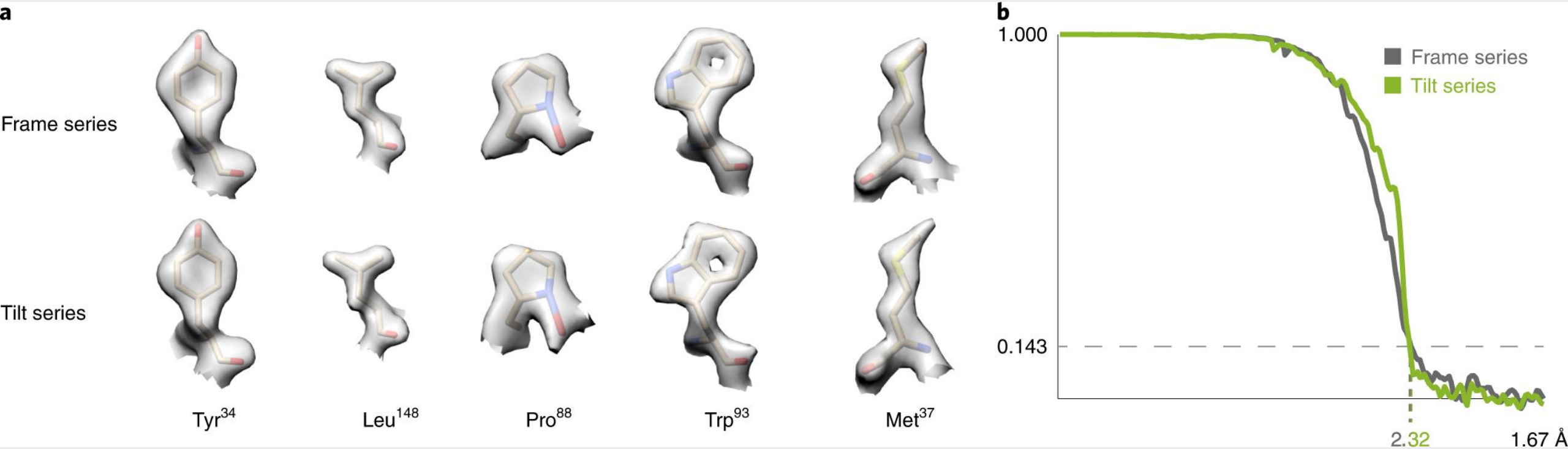
Schur et al., 2016

Warp/M Co-sub-tilt-series refinement



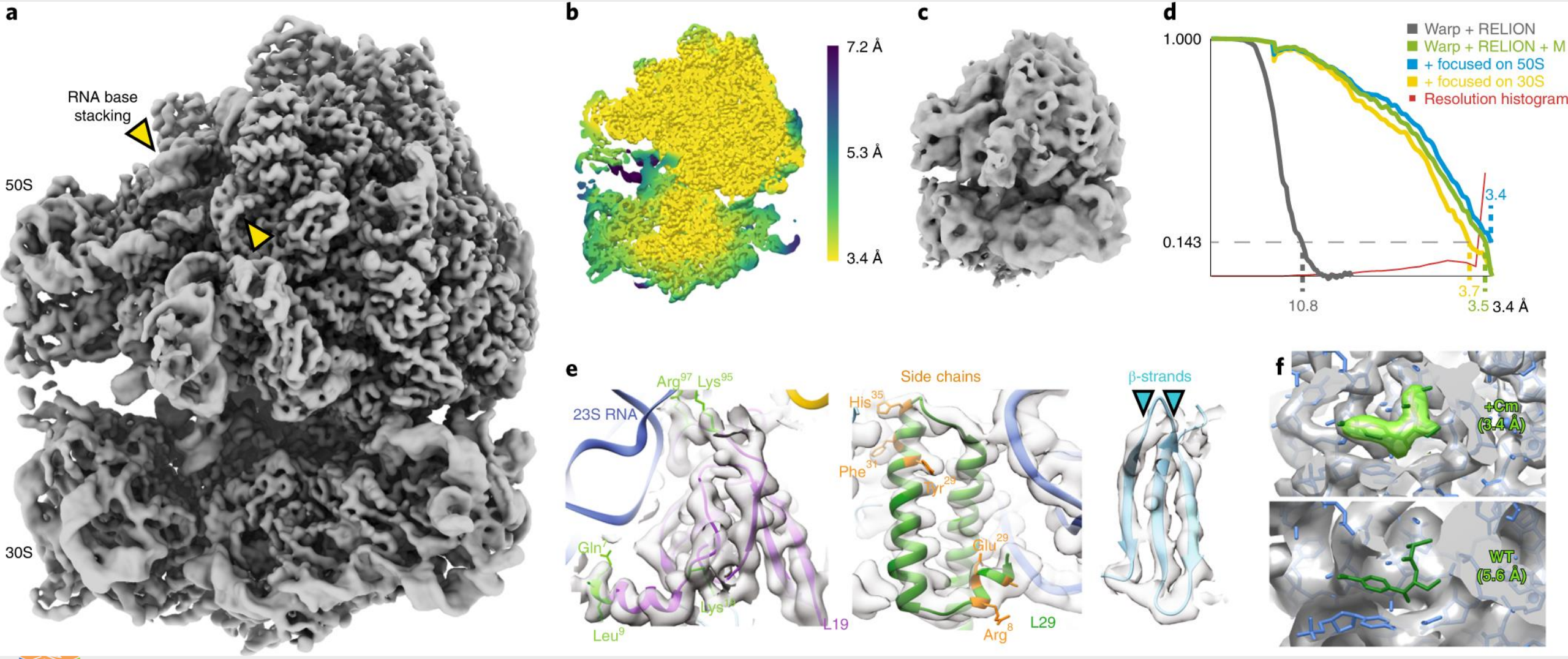
Tegunov et al., 2021

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



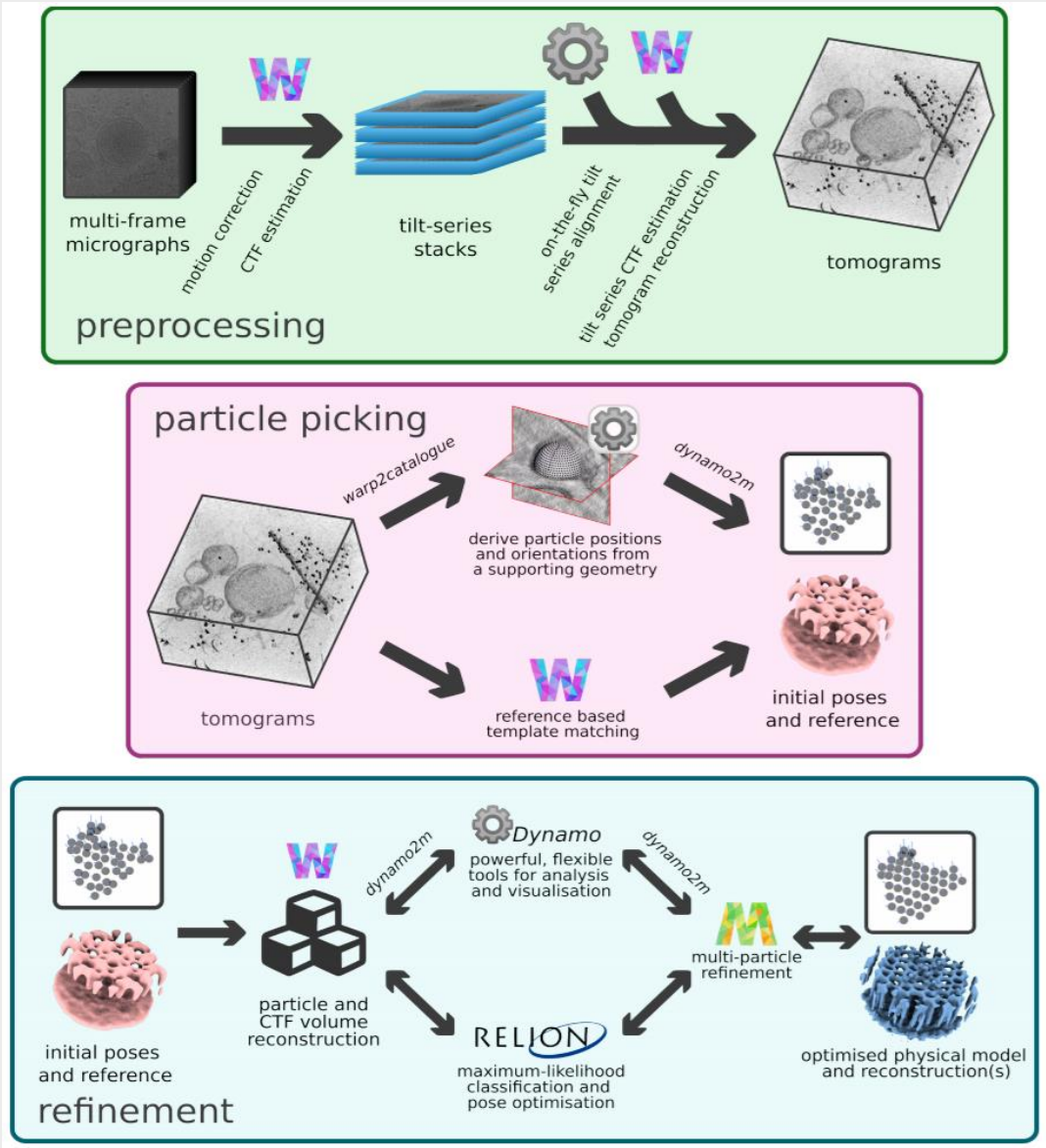
Tegunov et al., 2021

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



Tegunov et al., 2021

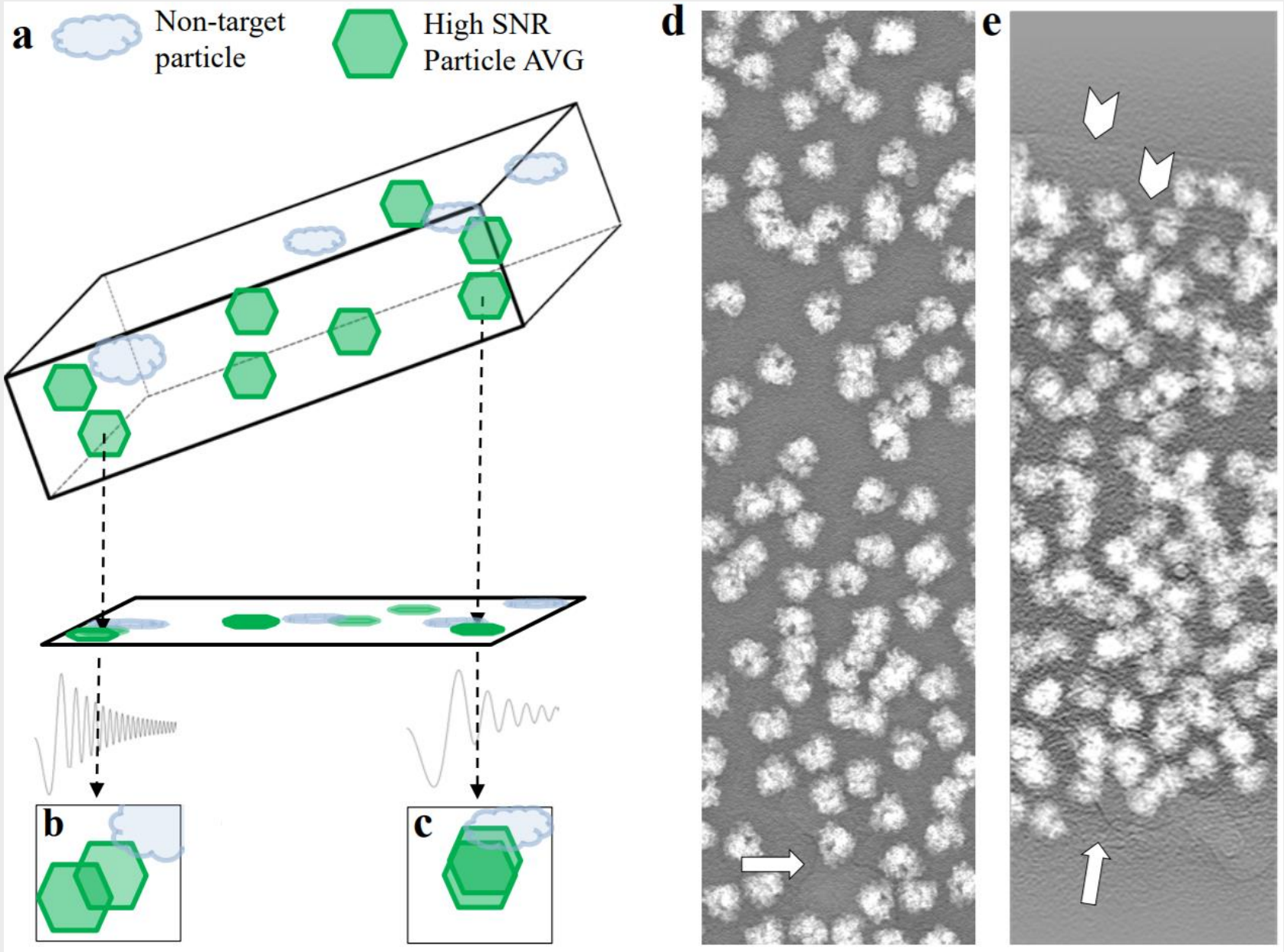
Warp/M Co-sub-tilt-series refinement: Dynamo-Warp/M-Relion workflow



teamtomo.org

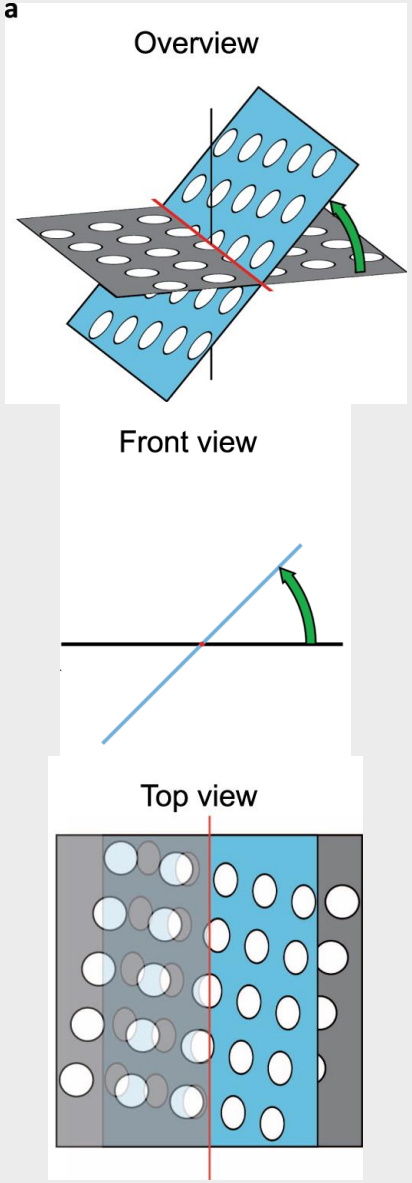
Burt et al., 2021

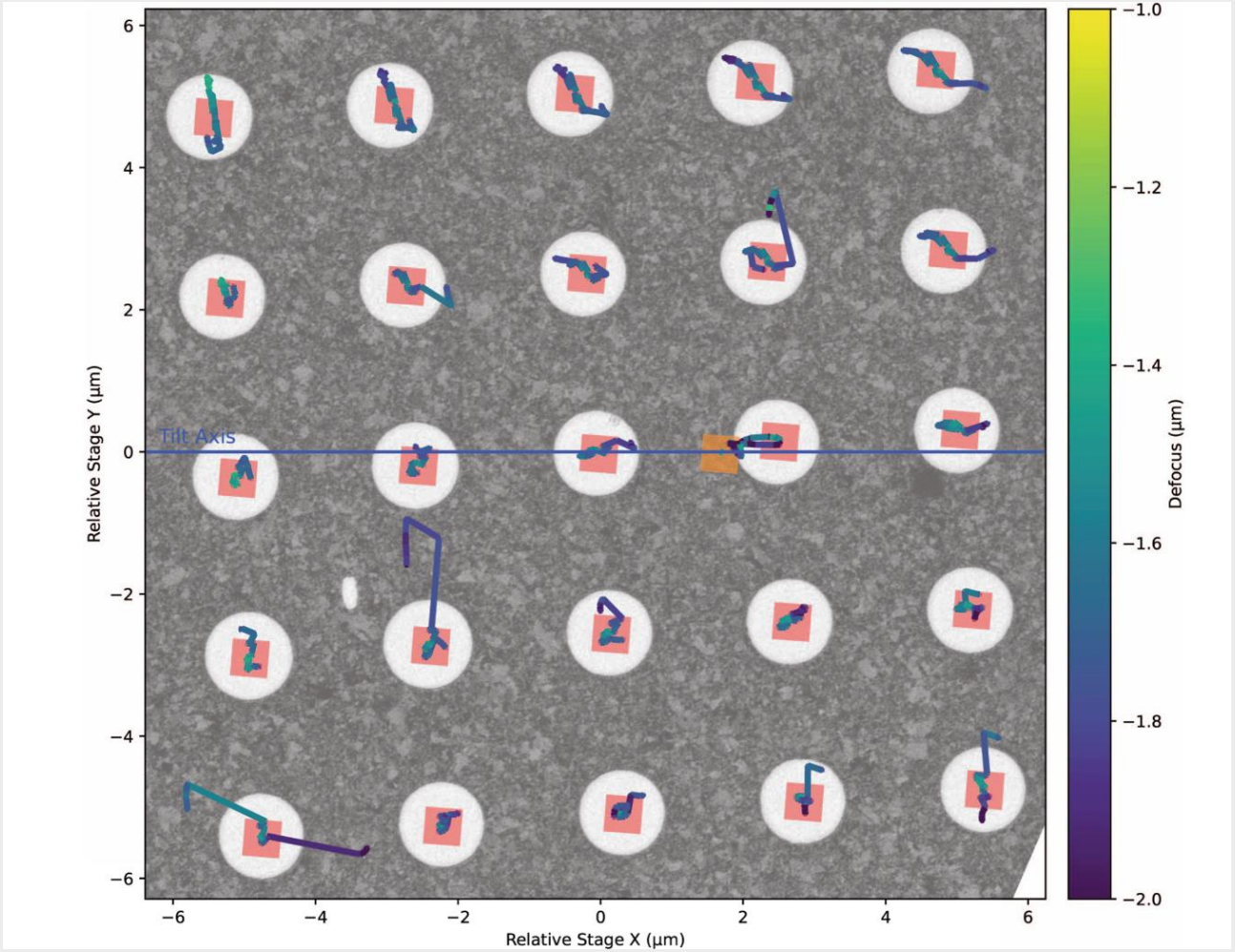
Refining tilt-series alignment by tracking just particles



Himes et al., 2017

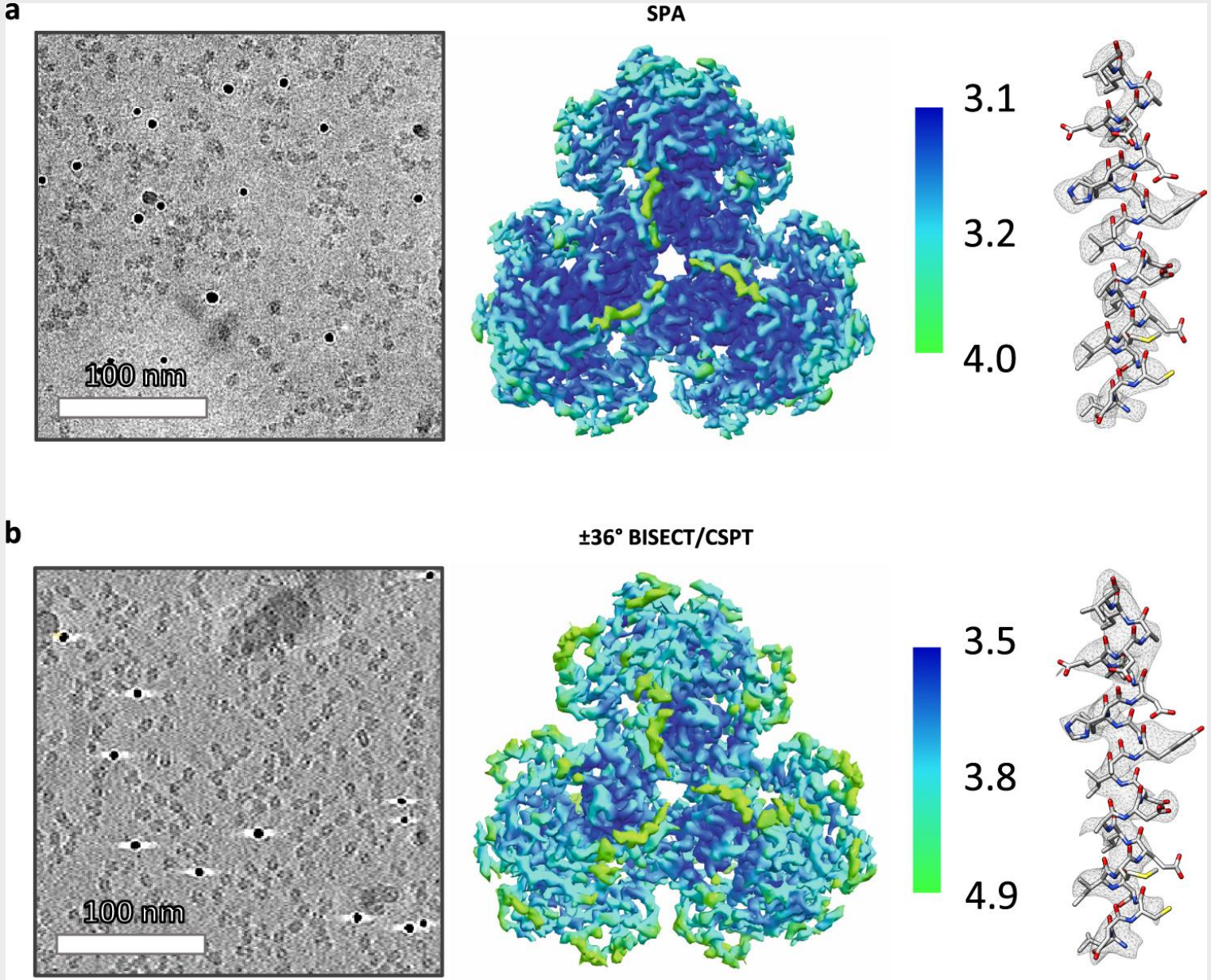
BISECT: Higher-throughput parallel acquisition





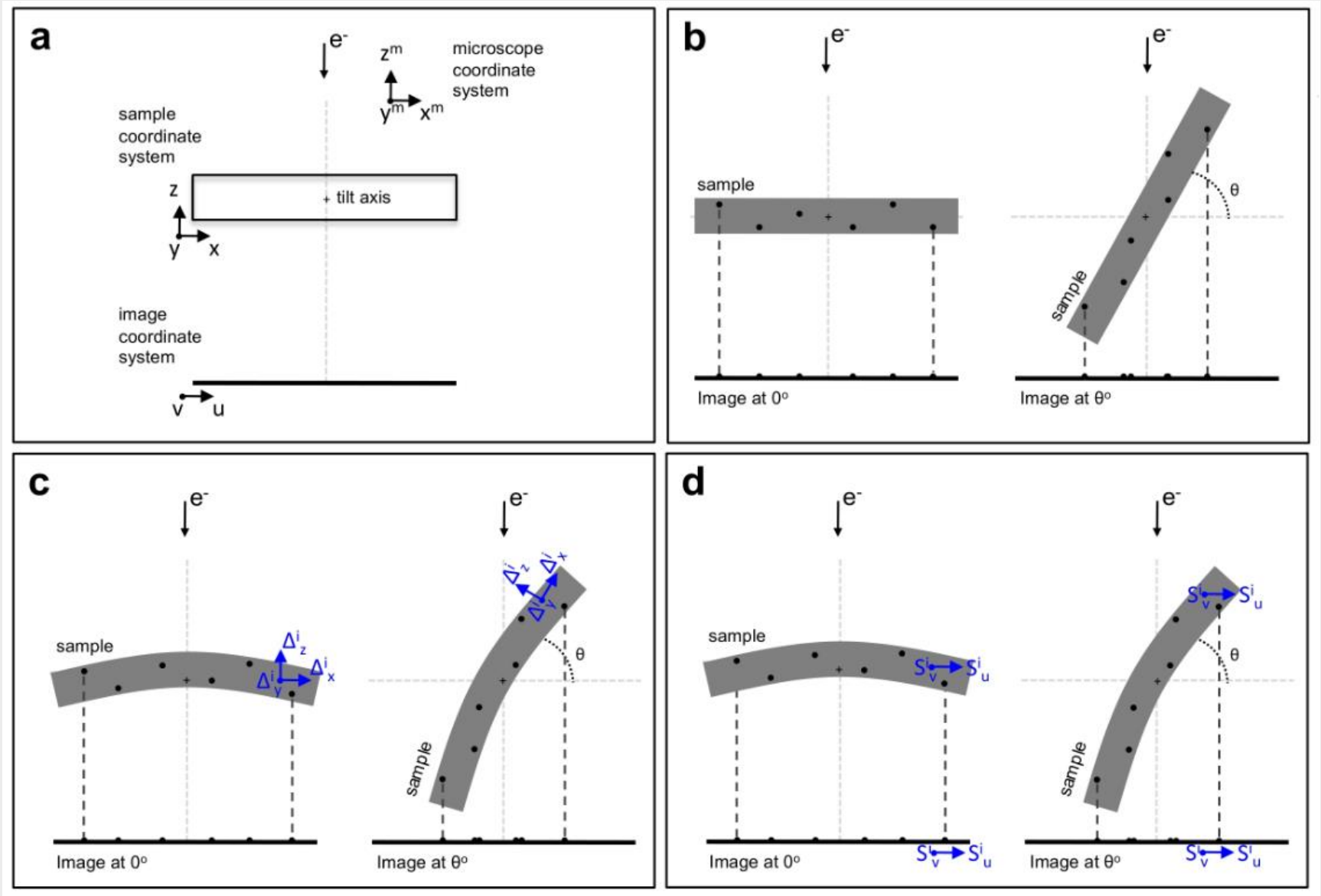
Bouvette et al., 2021

BISECT: Higher-throughput parallel acquisition



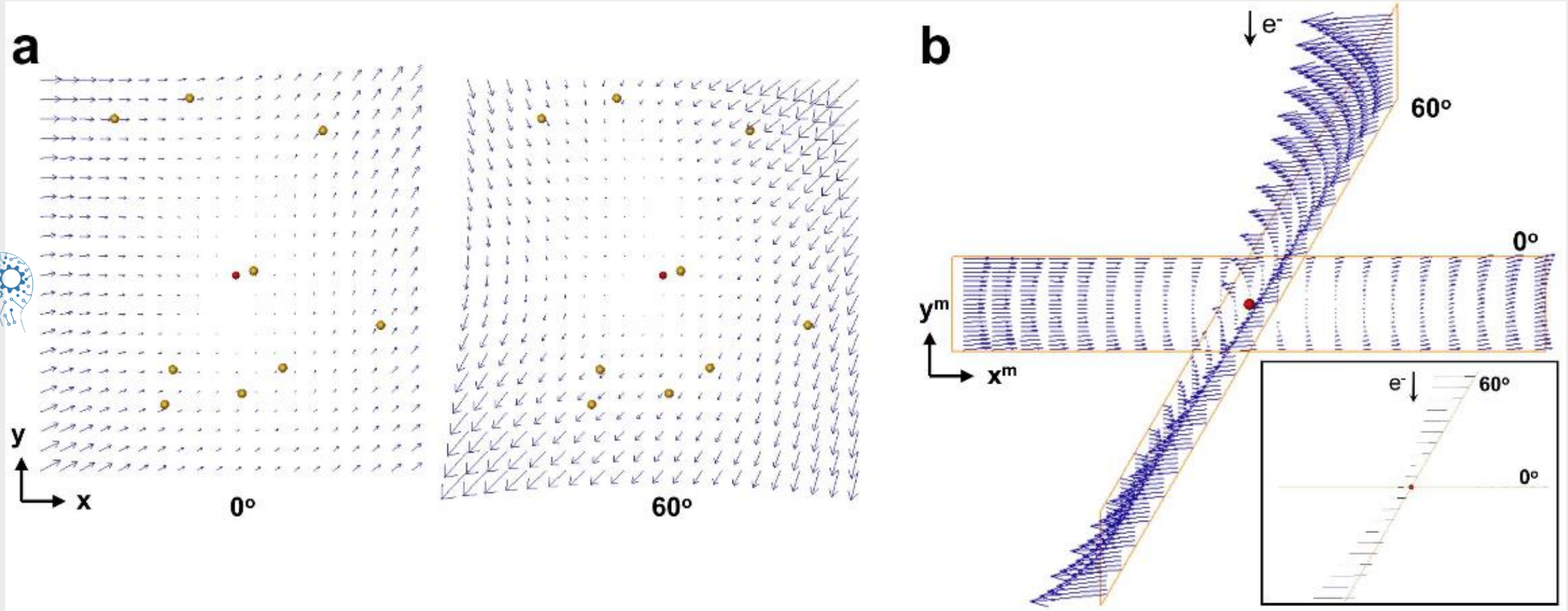
Bouvette et al., 2021

Refining tilt-series alignment by tracking beads in 3D

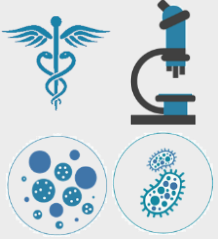


Fernandez et al., 2018

Refining tilt-series alignment by tracking beads in 3D



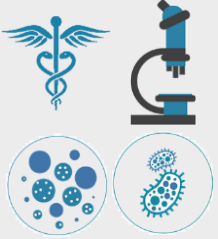
Fernandez et al., 2018



Processing/Resolution limits

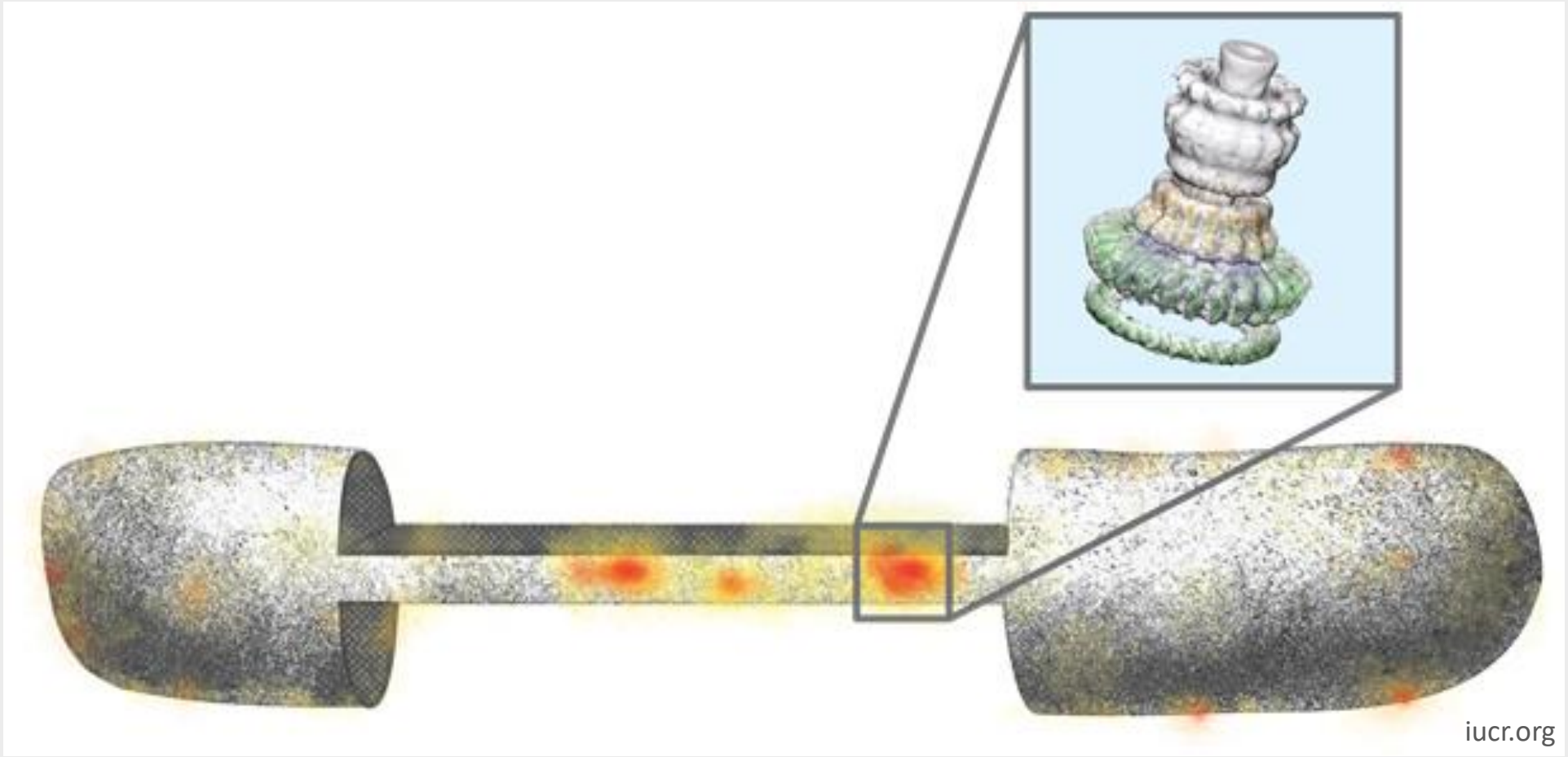
- Pixelsize (highest resolution = 2 x pixelsize = **Nyquist**)
- Isotropic motion (monitor your **drift** before full collection)

- Inherent specimen **flexibility**
- **Ice warping** in 3D during collection (doming)
- Beam-induced **motion of objects** of interest **in 3D** (particularly anisotropic)



Current/future directions in tomography

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

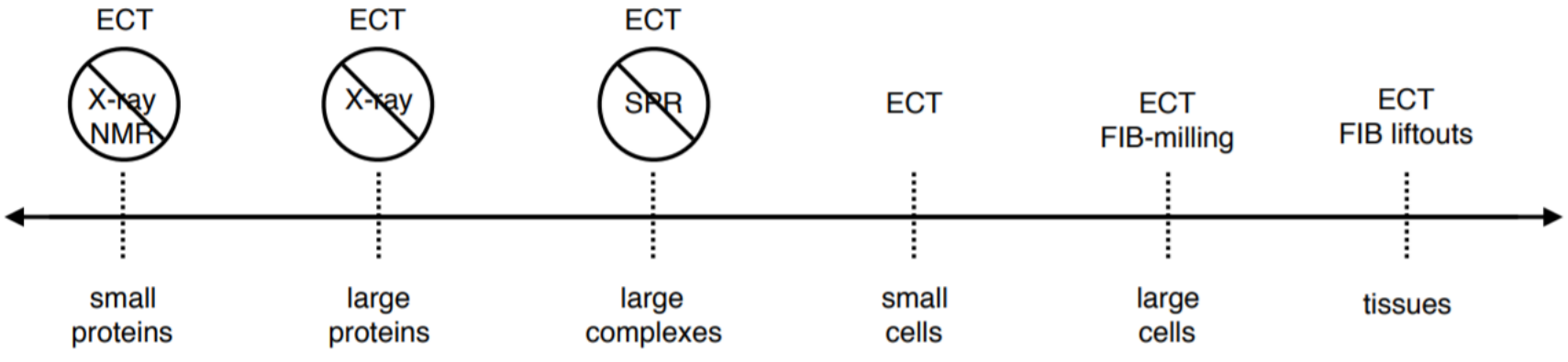


Hardware improvement – Rapid tilting

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*

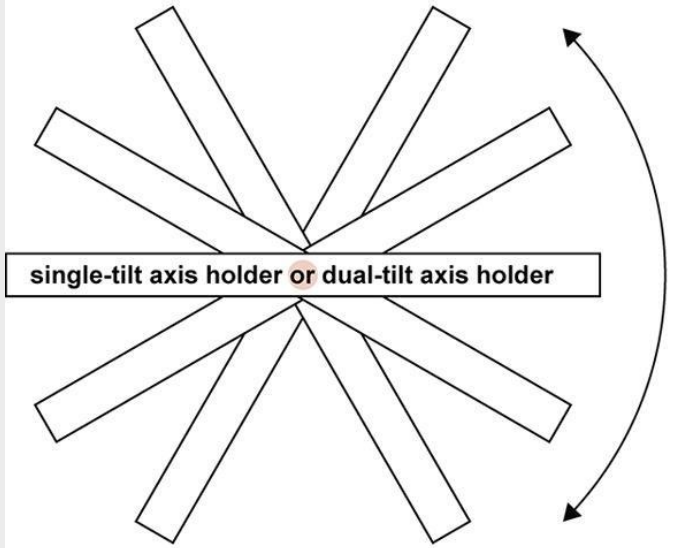
~~ALL~~ cryotomography, ~~ALL~~ the time



Chreifi et al., 2019

Hardware/software improvement
Pre-calibrated rapid tilting!

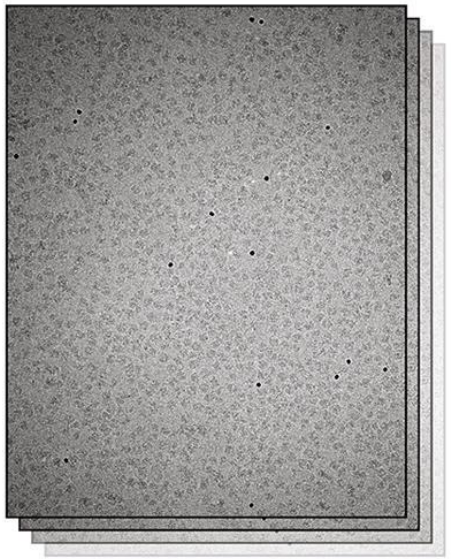
Fast-incremental single-exposure



Collection

< 5 min
per tilt series

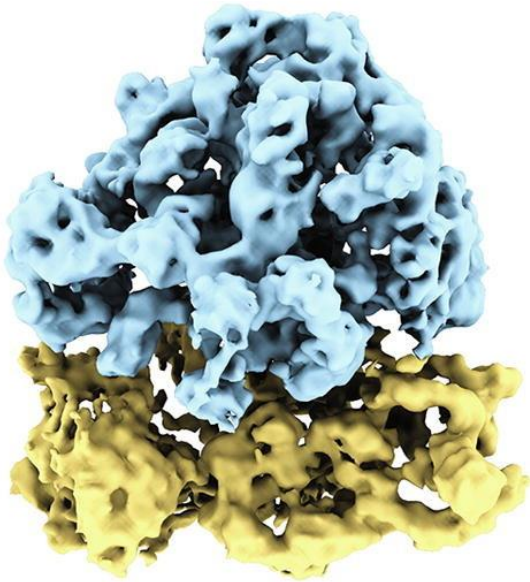
Tilt series movie



Processing

several days

Subtomogram average at subnanometer resolution

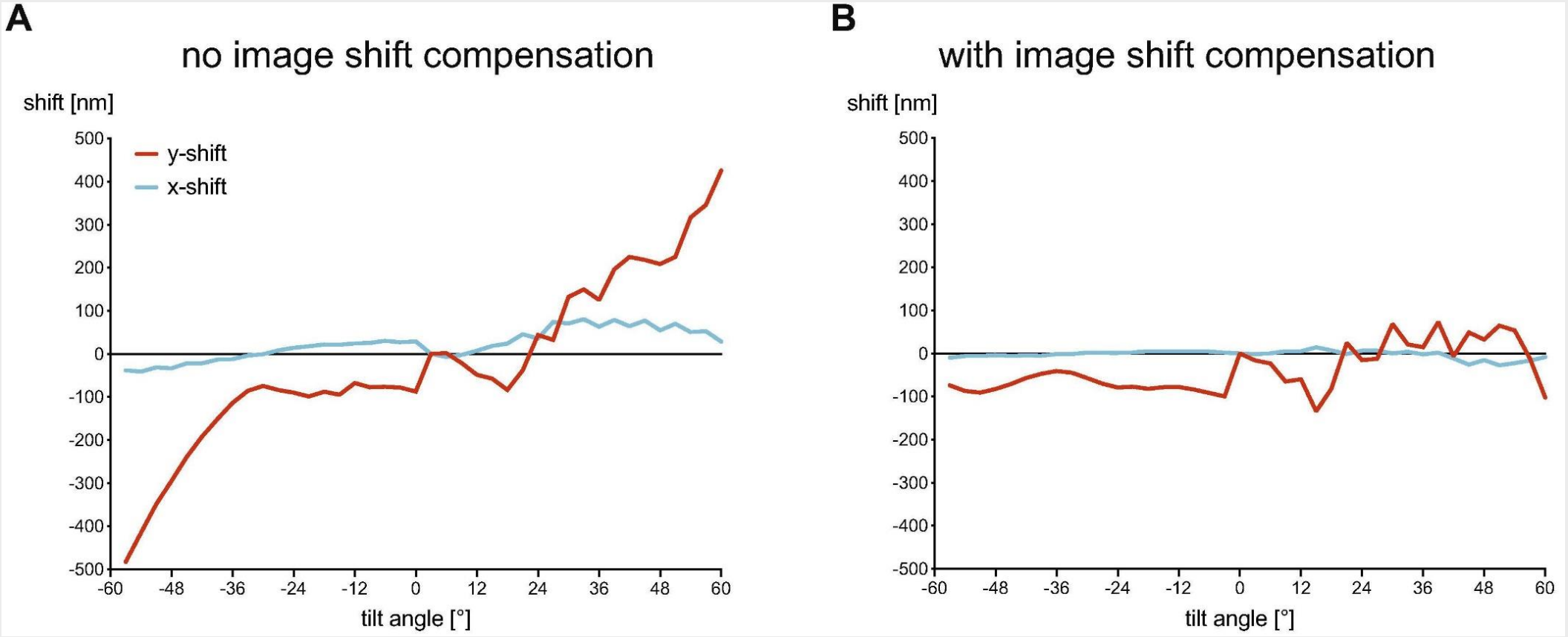


K3

x, y, z specimen shift compensation

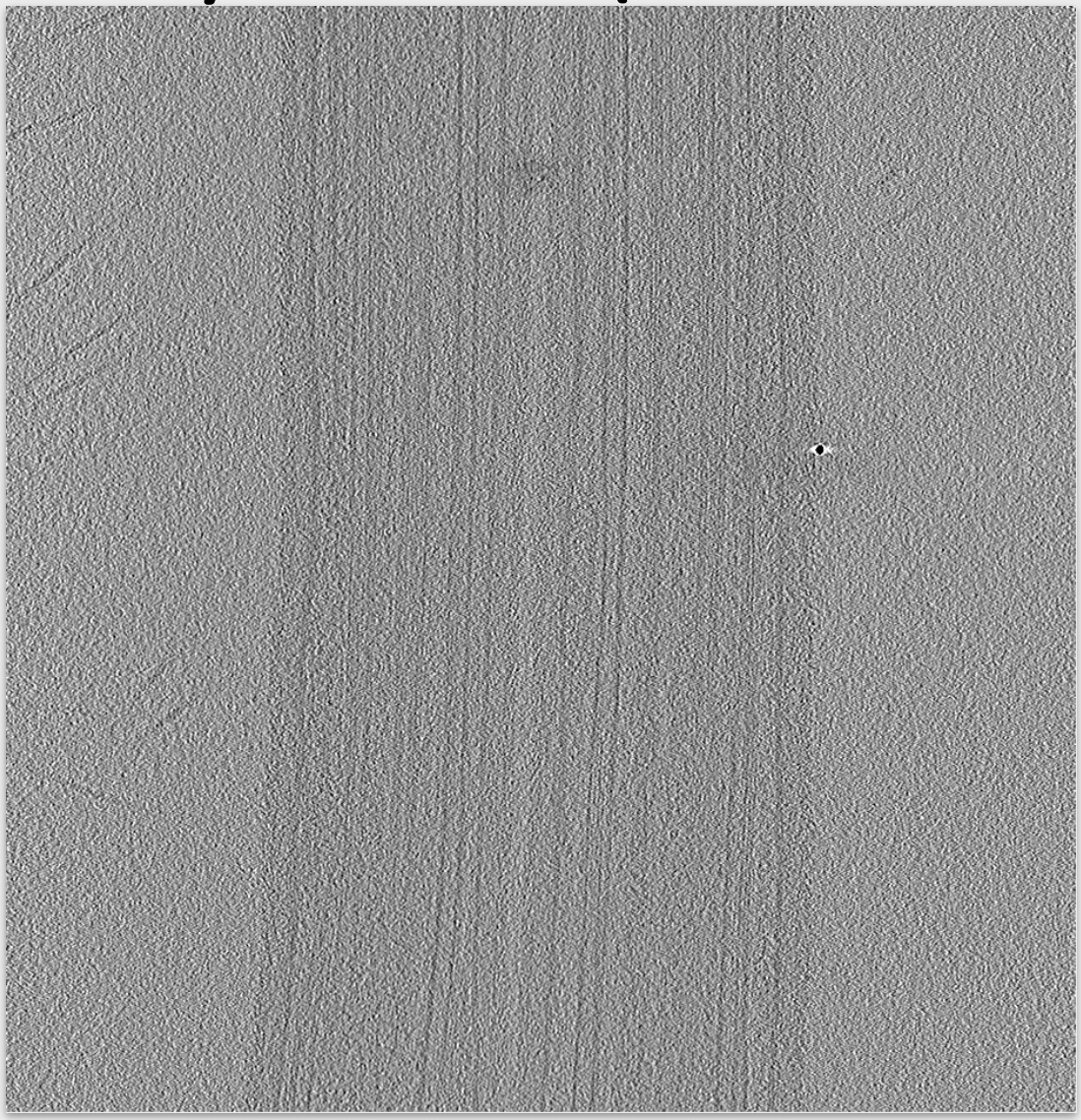
Eisenstein et al., 2019

Software improvements in the field
Pre-calibrated Rapid tilting

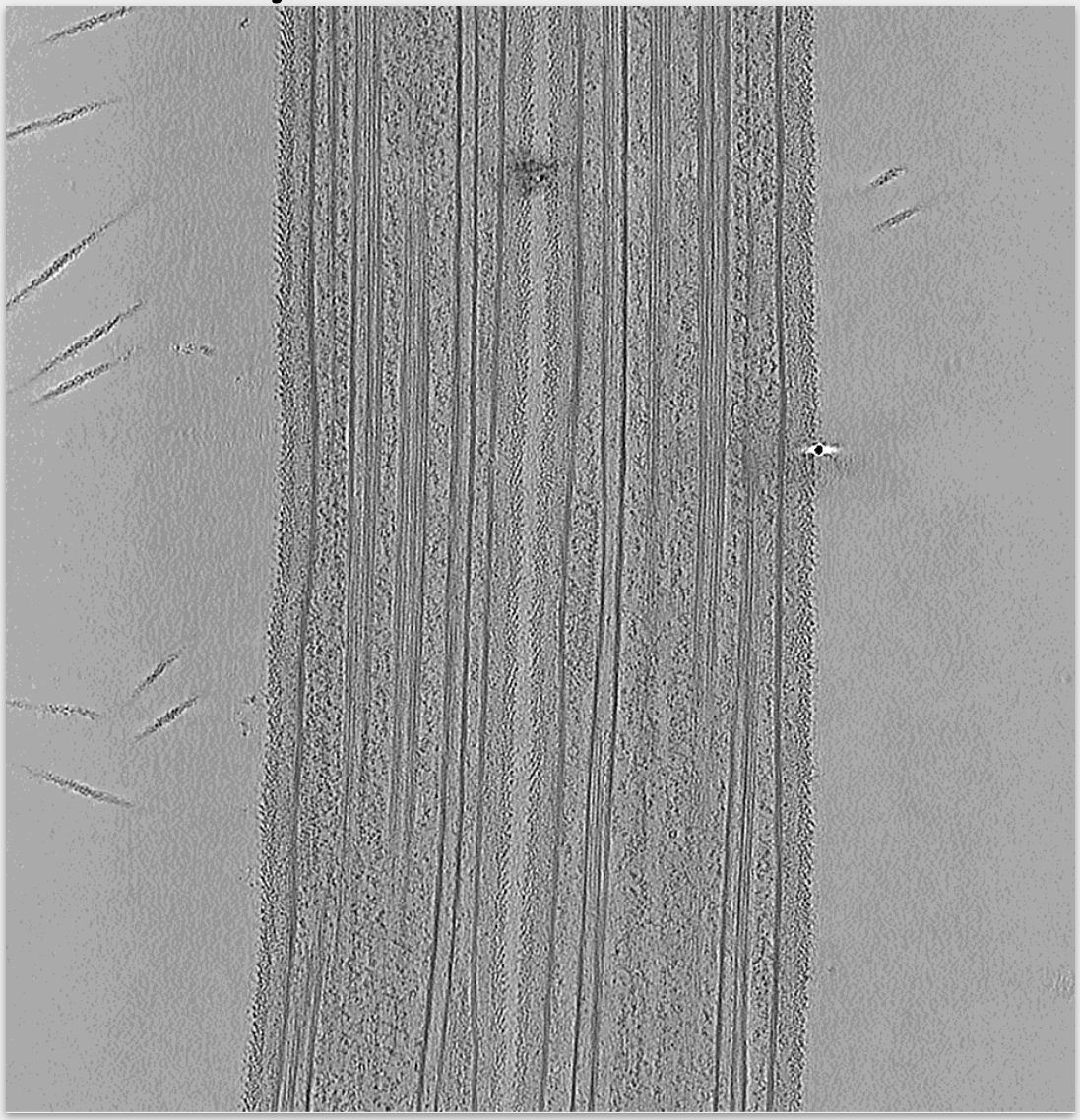


Eisenstein et al. 2019

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



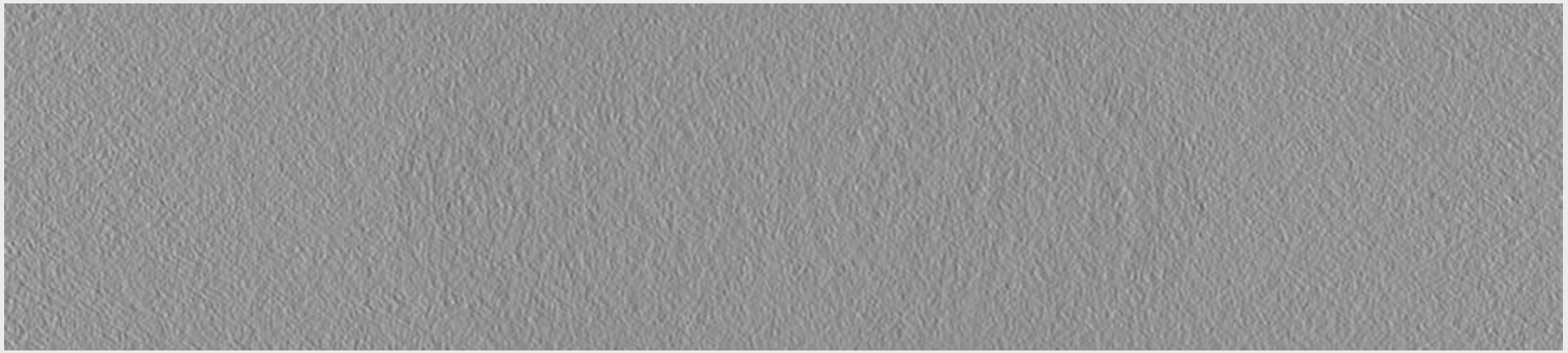
Before



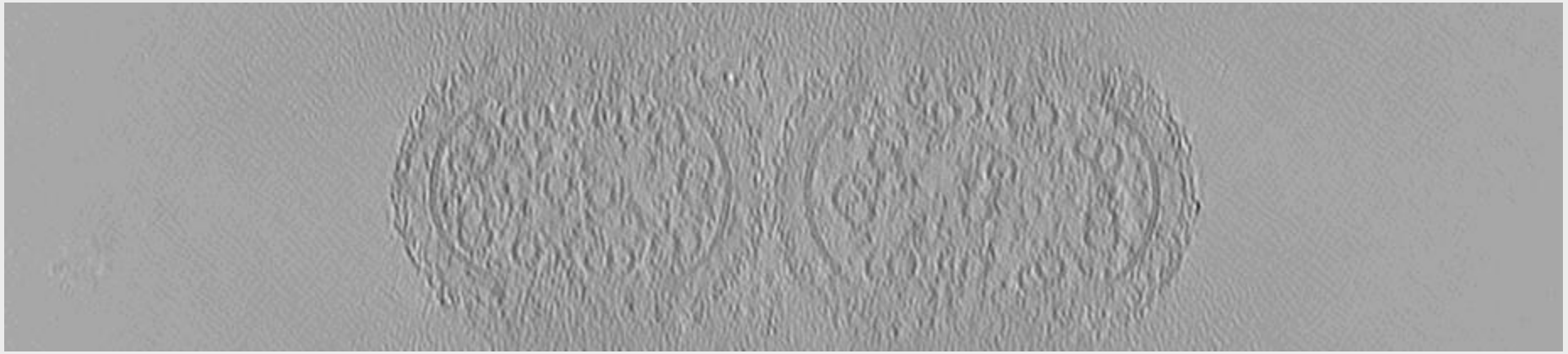
After!

Buchholz et al., 2019

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



Before

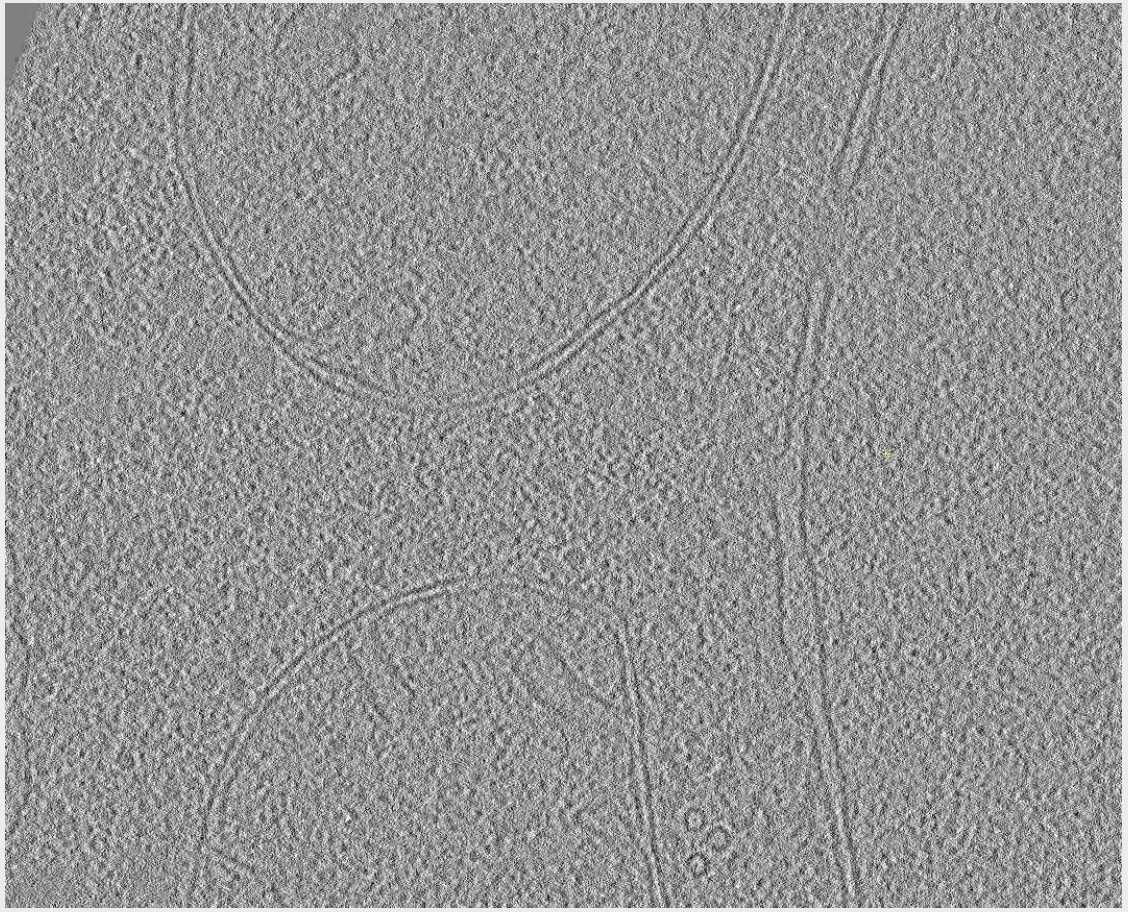


After!

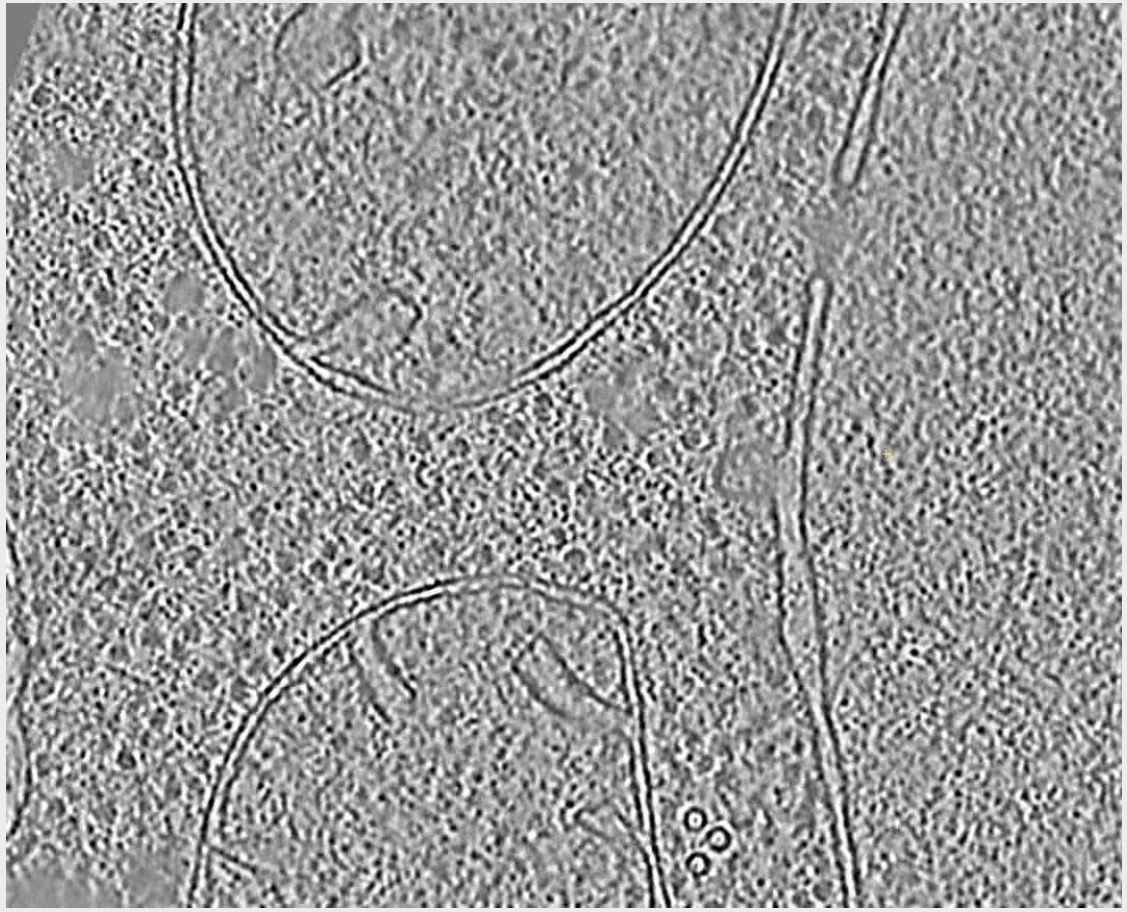
Buchholz et al., 2019

Post-processing improvement - *Denoising*

Topaz (3D Noise2Noise):

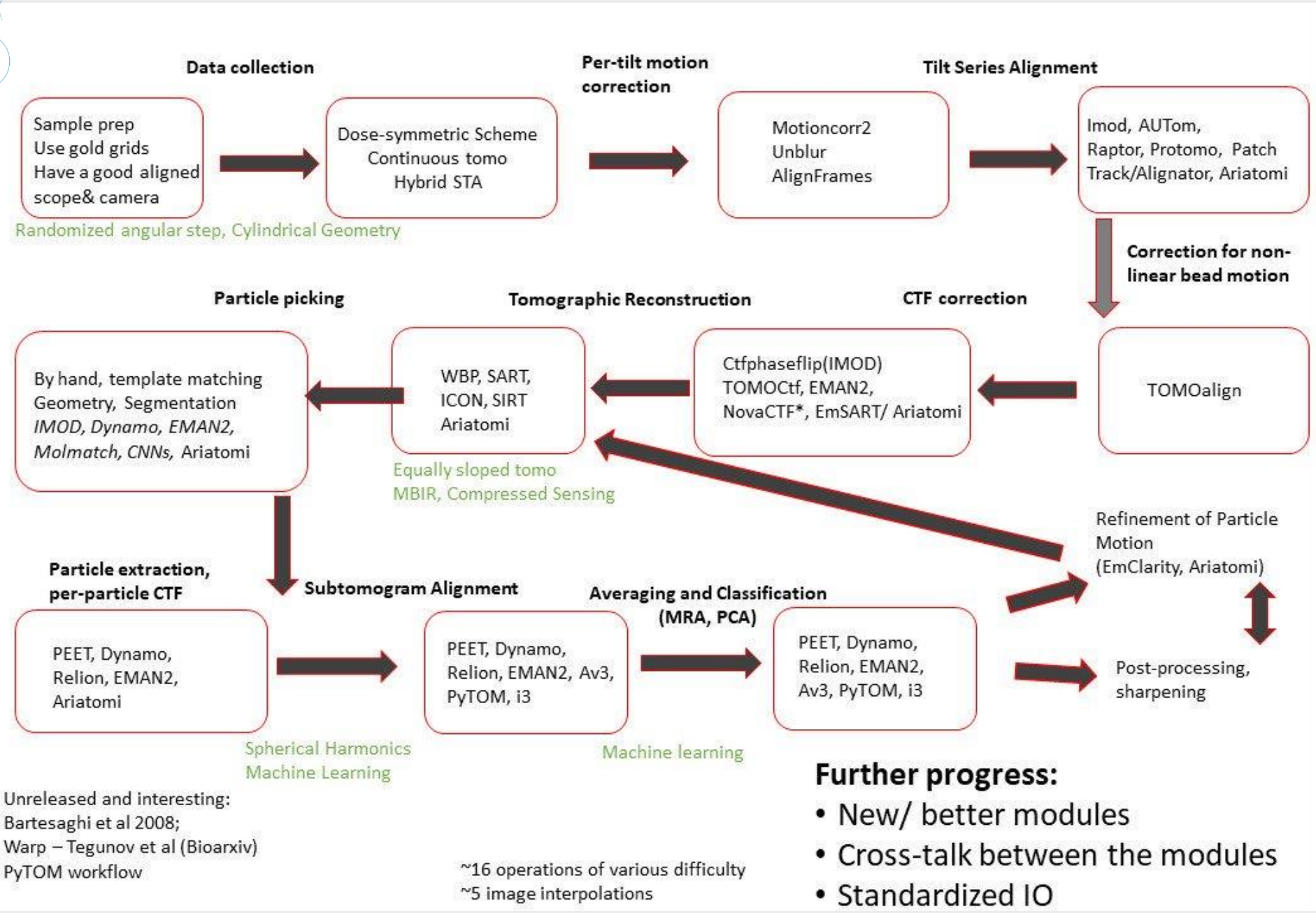


Original

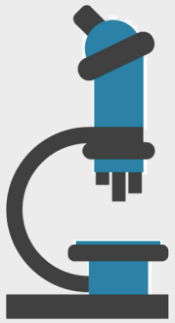


Topaz denoised

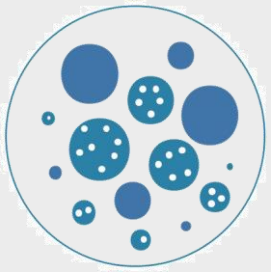
Bepler et al., 2020



Leigh et al., 2019



Thank you!
Questions?



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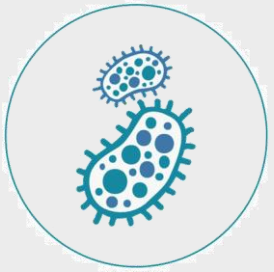

National Resource for Automated Molecular Microscopy
Simons Electron Microscopy Center
New York Structural Biology Center