Cryo Applications & Sub-tomogram Processing

SEMC Winter EM Course 2019

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Overview – Why CryoET?

Why **cryo**?

- Specimen preservation in native or near-native environments.

Why **electrons**?

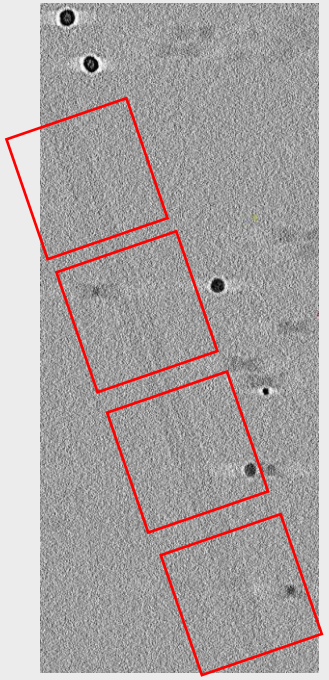
- **+**Small wavelengths (high res), **+**Can be focused, **–**Damage sample

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 may **not** be required, but may be possible.

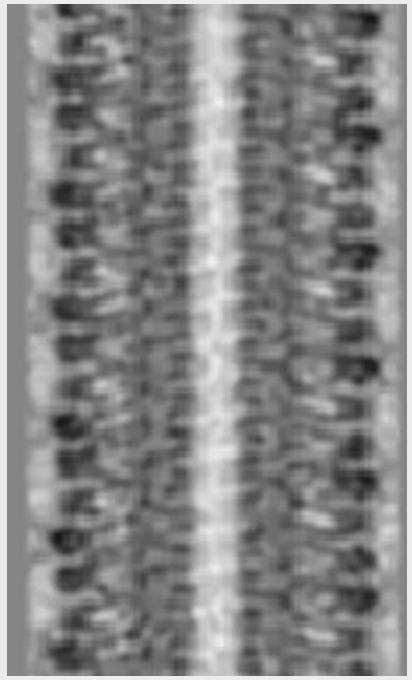
Overview – Why subtomogram averaging?

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



particles

align



reconstruction

Courtesy of
Misha Kudyashev

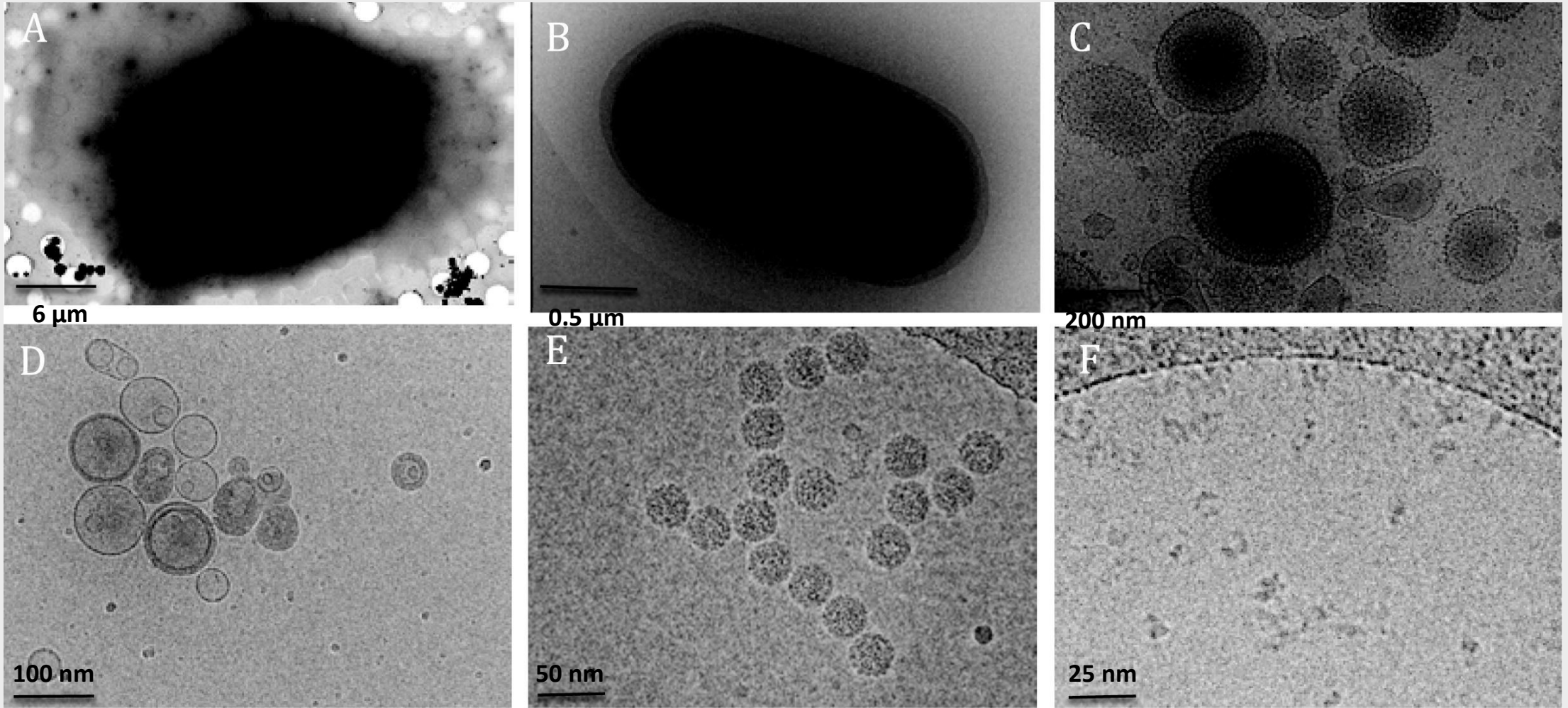
Overview

- 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

Overview – Limitations: Sample/Ice Thickness

Limitation: Specimen/Ice **thickness**

- At **300keV** in a TEM (e.g. Krios), electrons cannot penetrate more than **0.5-1 µm**



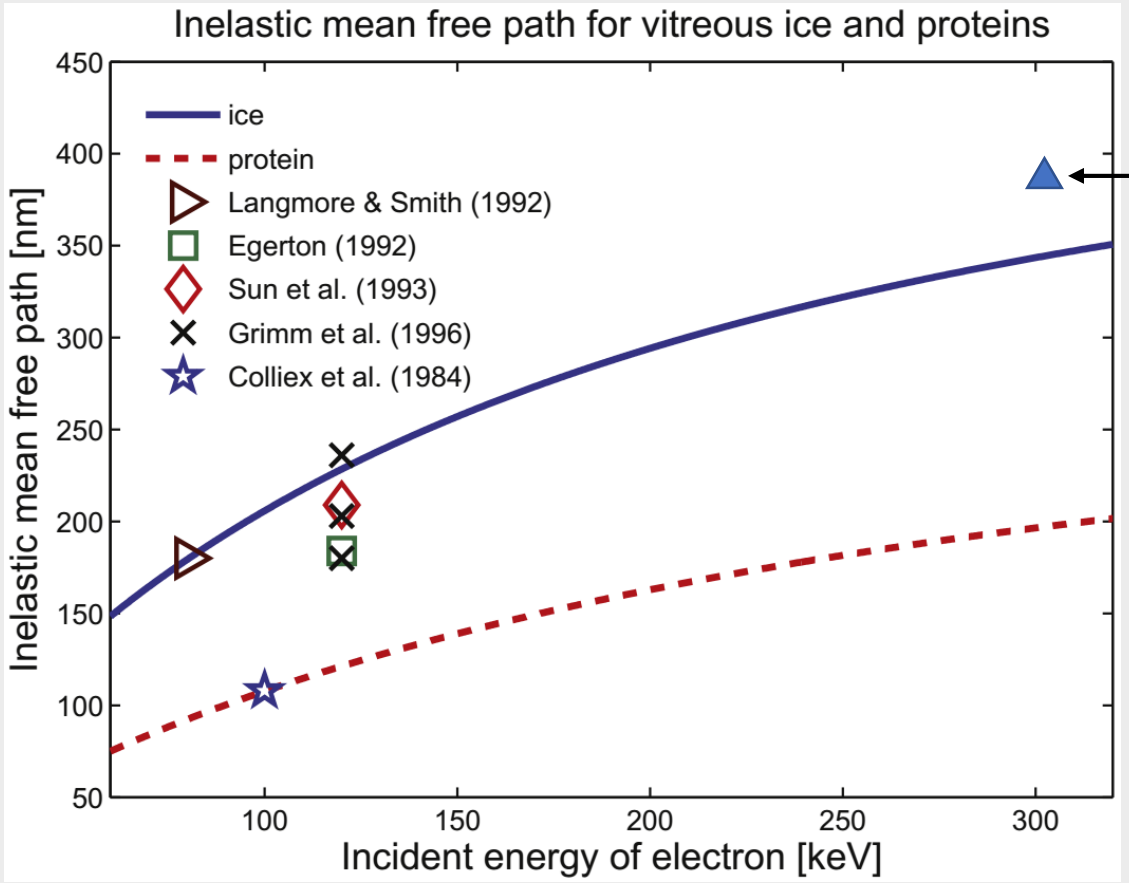
Thompson et. al., 2016

Overview – Limitations: Sample/Ice Thickness

Limitation: Specimen/Ice **thickness**

- At **300keV** in a TEM (e.g. Krios), electrons cannot penetrate more than **0.5-1 µm**

Vulović, 2013

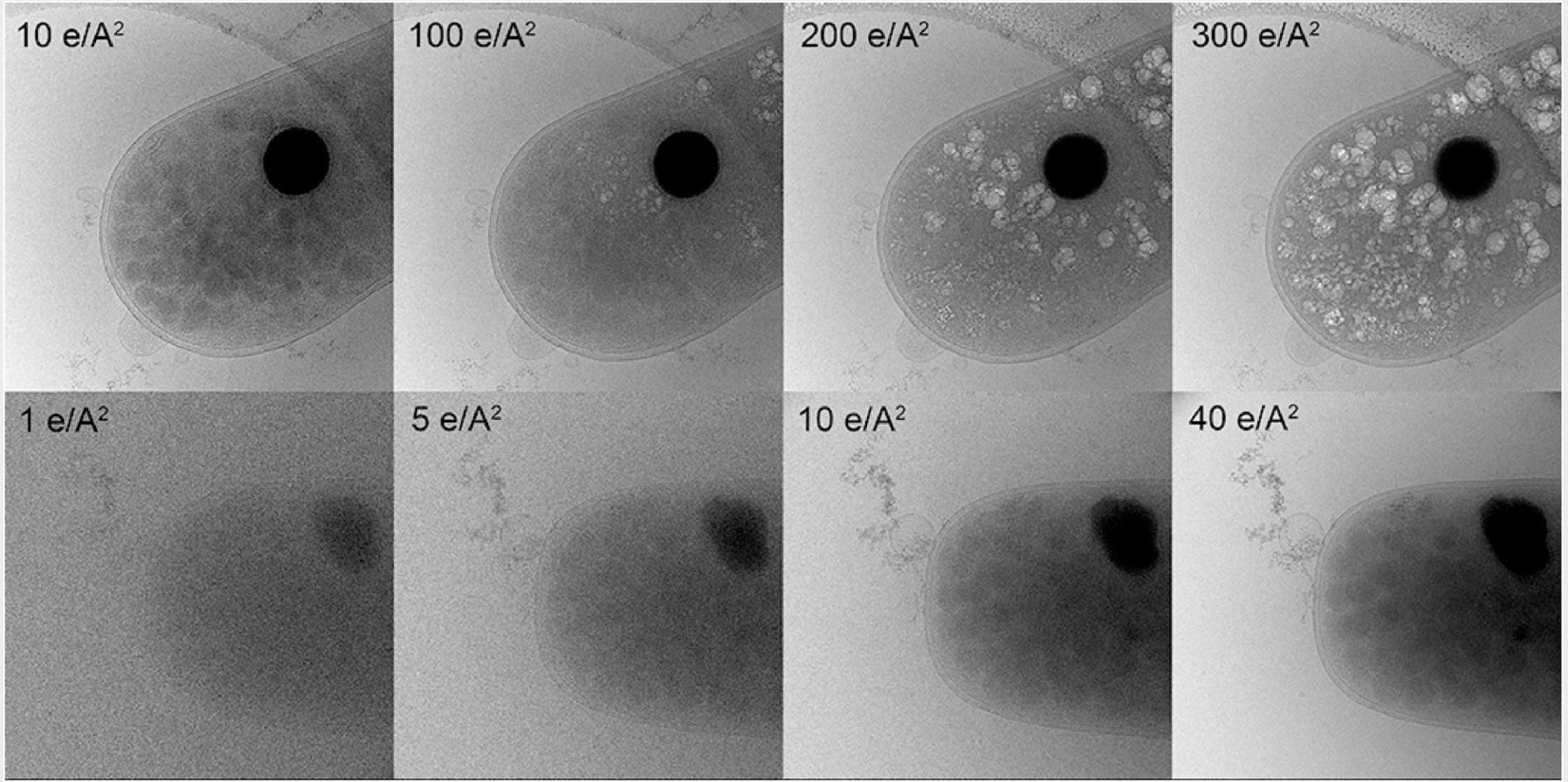


William J. Rice, NYSBC, 2017
300 keV Krios

Overview – Limitations: Electron Damage

Limitation: **Electron damage** of the specimen

- High voltage electrons damage biological specimen. This is **sample dependent**!



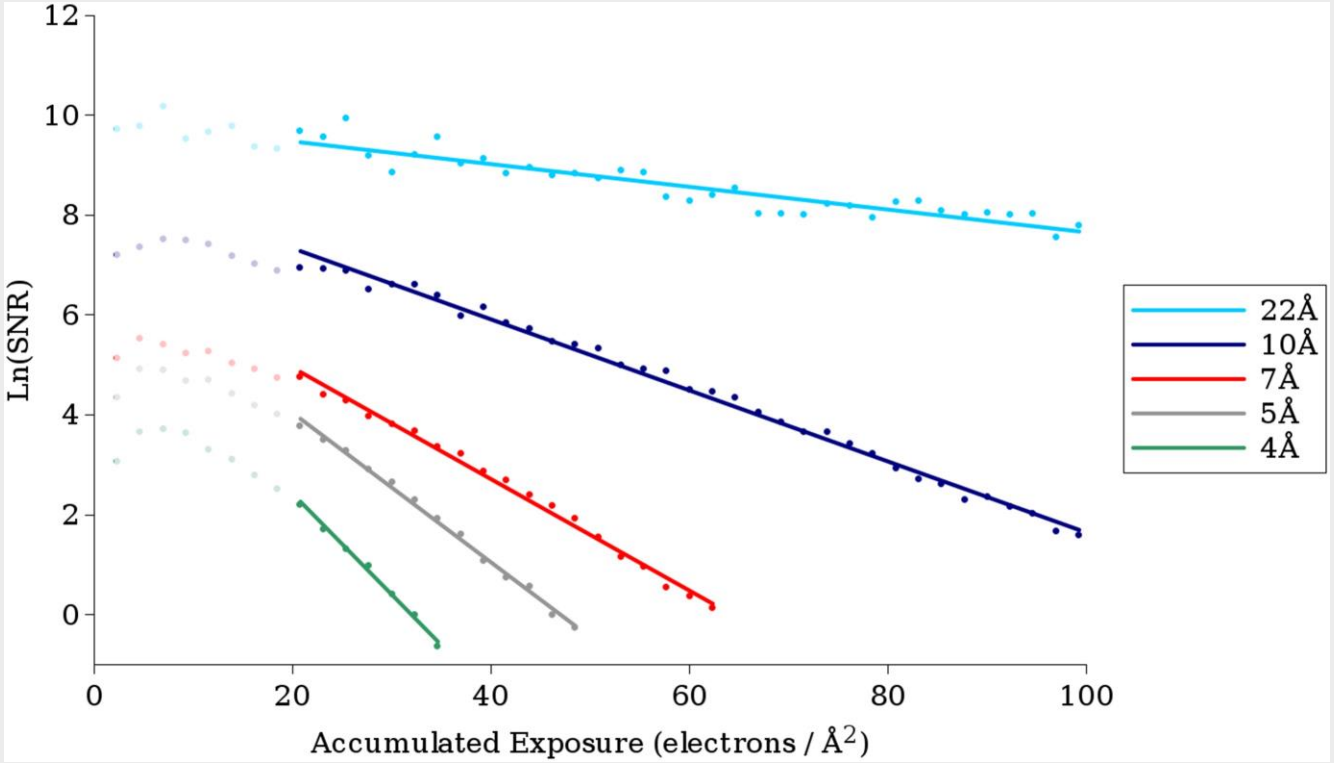
Koning, 2018

Overview – Limitations: Electron Damage

Limitation: **Electron damage** of the specimen

- High voltage electrons damage biological specimen.
  - **High resolution information is lost first** followed by lower resolution info.

Grant & Grigorieff, 2015



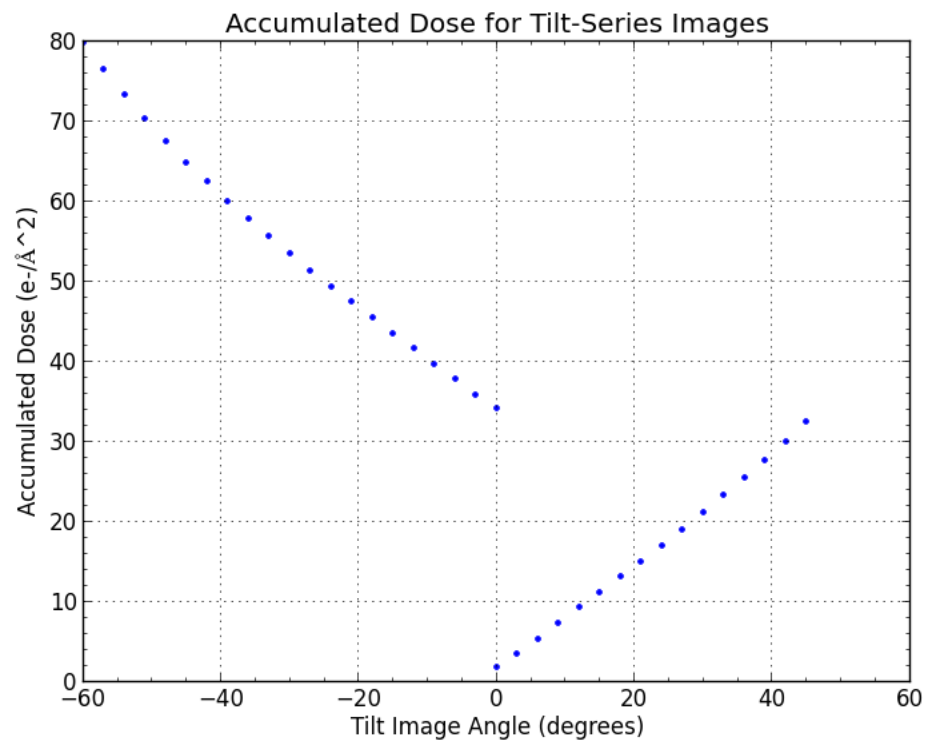
Solution:
Remove damaged information from image frames

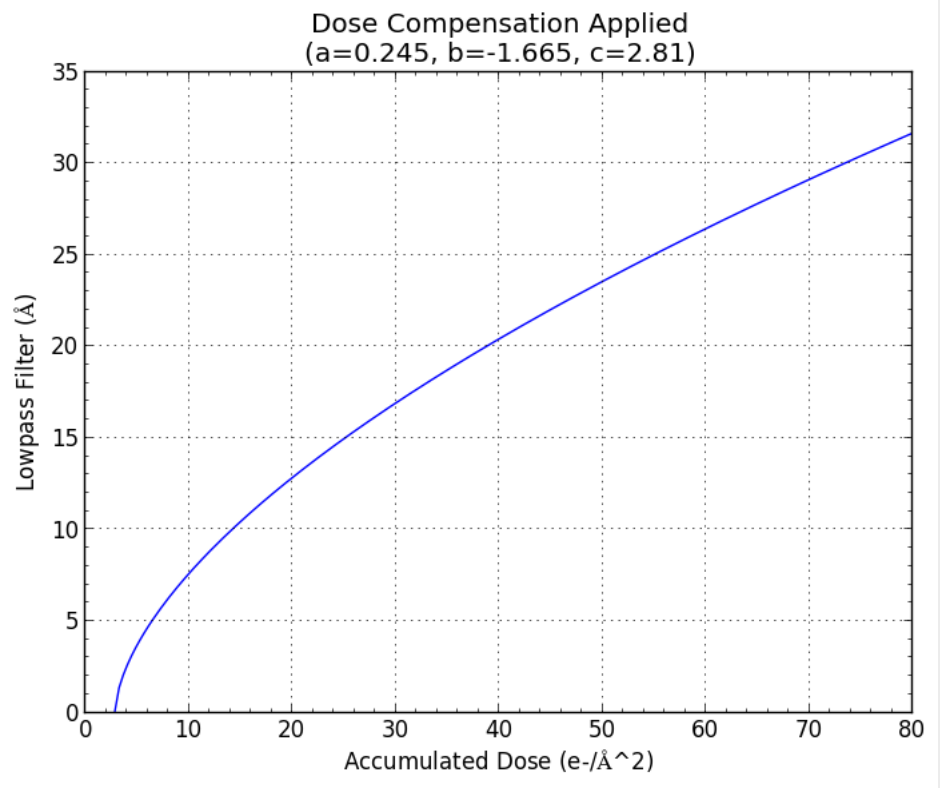
Overview – Limitations: Electron Damage

Limitation: **Electron damage** of the specimen

- **Solution:** Remove damaged information from image frames (single particle) or tilt images (tomography):

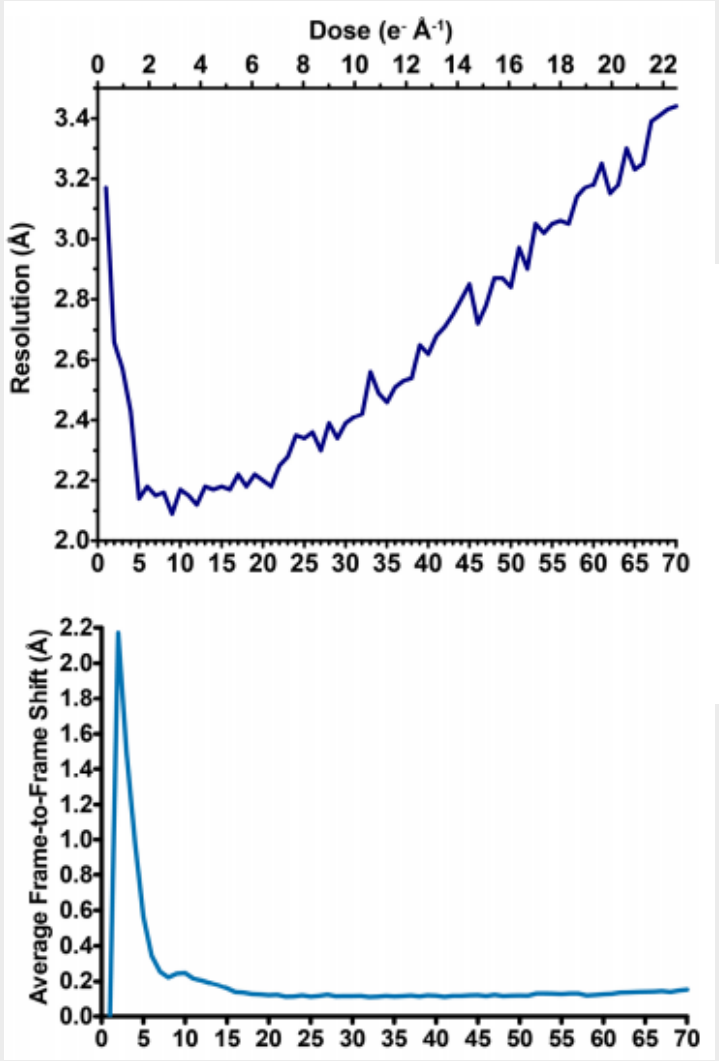
Noble & Stagg, 2015





Overview – Limitations: Electron Damage?

But be careful! There might be more information than you think:



Tan YZ et al., Nat. Comm. 2018

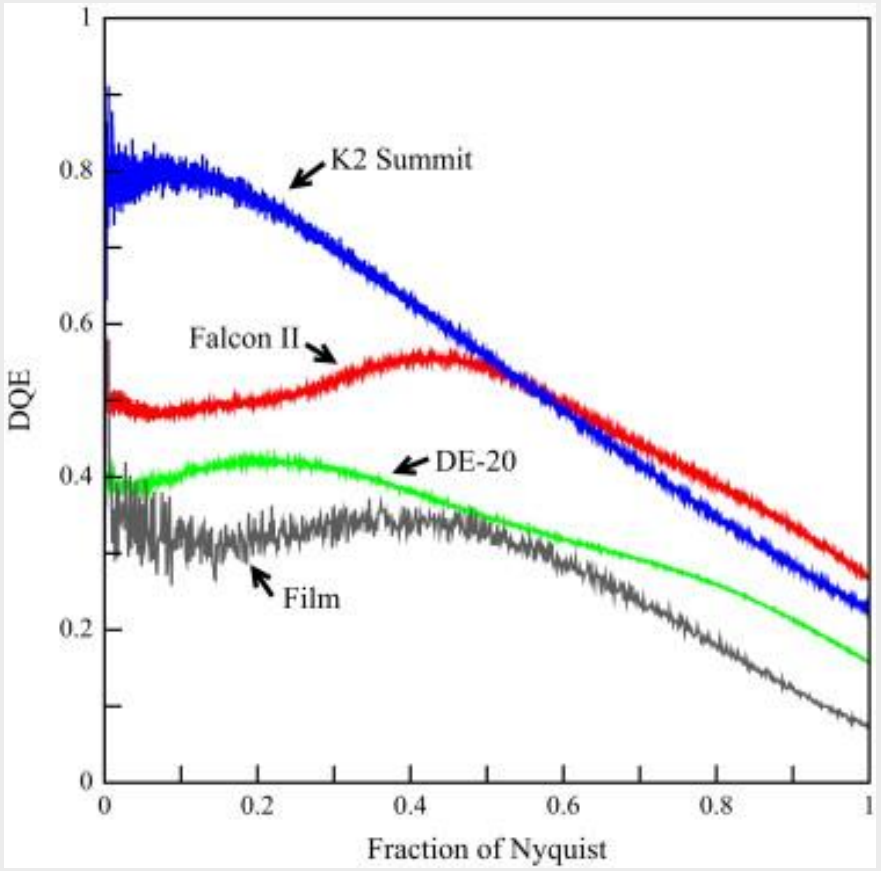
Supplementary Figure 3 | Resolution of individual frame reconstructions. (**a**) Using the best Euler angles and shifts, reconstructions were computed separately for each of the 70 frames. The resulting resolution shows two trends: the first 4 frames (3.17-2.43 Å) suffered from the initial effects of beam-induced motion; after frame 22, the resolution gradually worsens owing to the cumulative effects of radiation damage. (**b**) Frame-to-frame shifts in Ångstroms for all 70 frames are shown in blue. Frame-to-frame shifts were calculated using MotionCor2 global frame alignment mode.

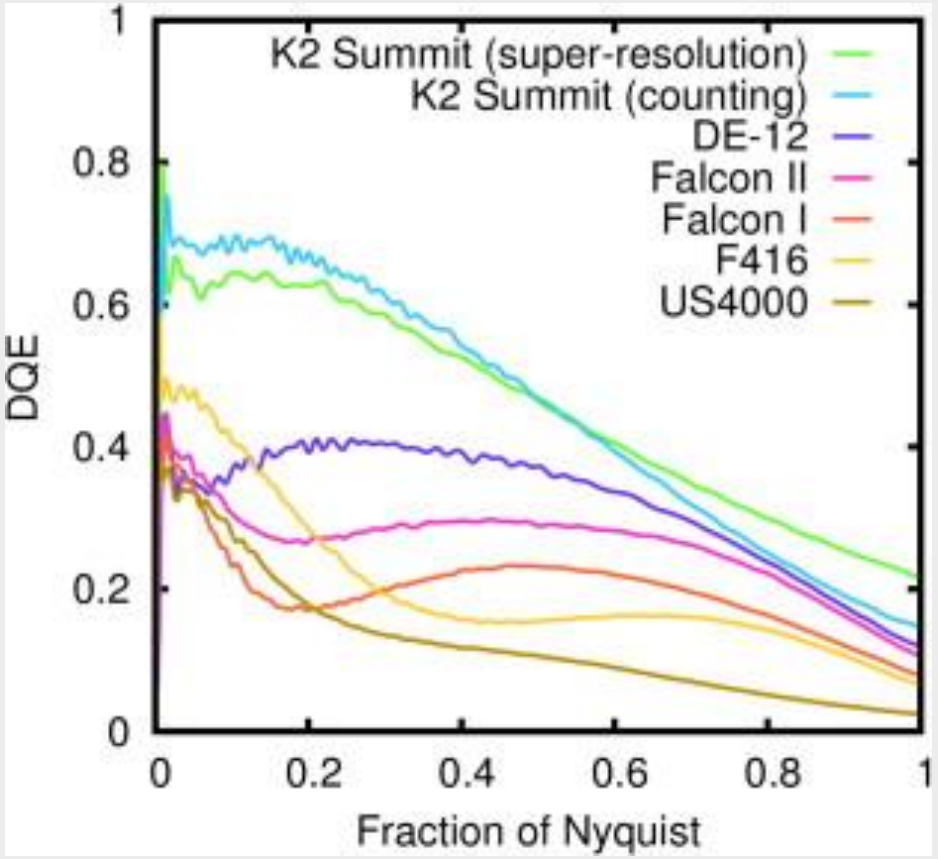
Overview – Limitations: Camera Fidelity

Limitation: Camera fidelity at **localizing electrons**

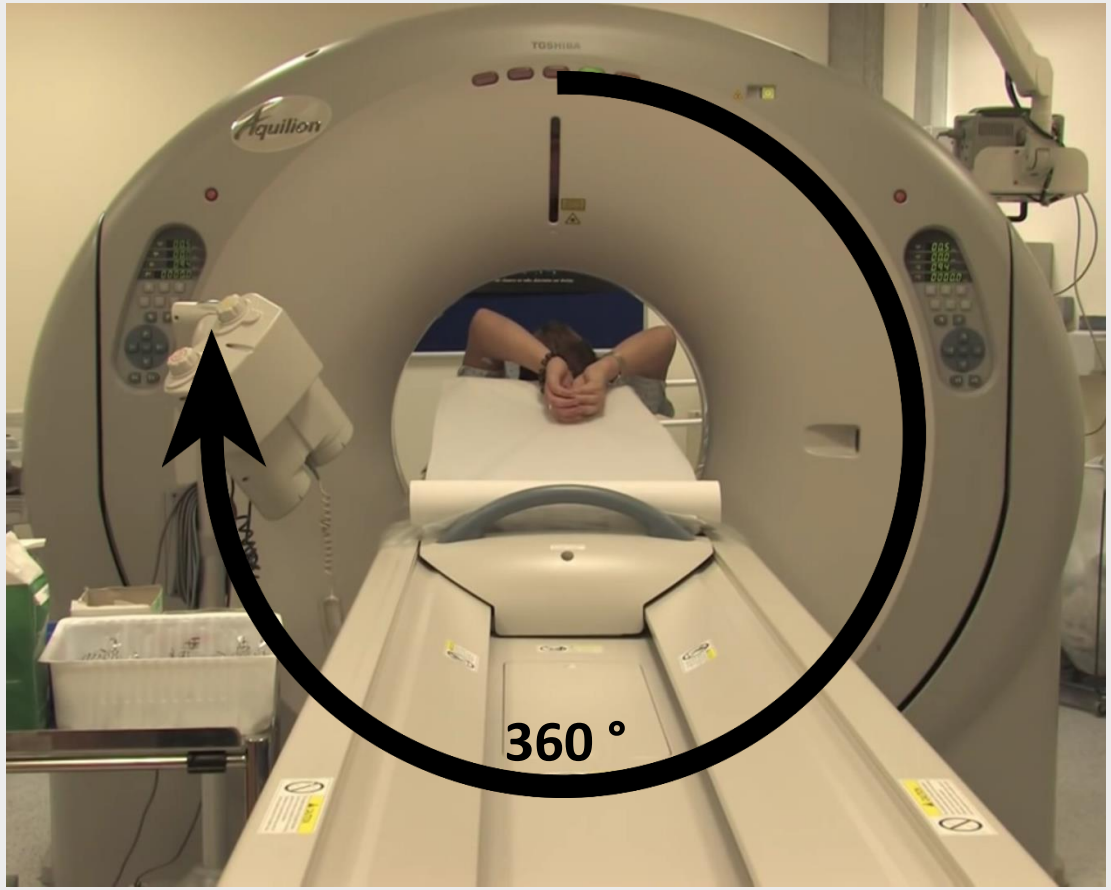
- Cameras do **not** transfer information **perfectly or equally across frequencies**.

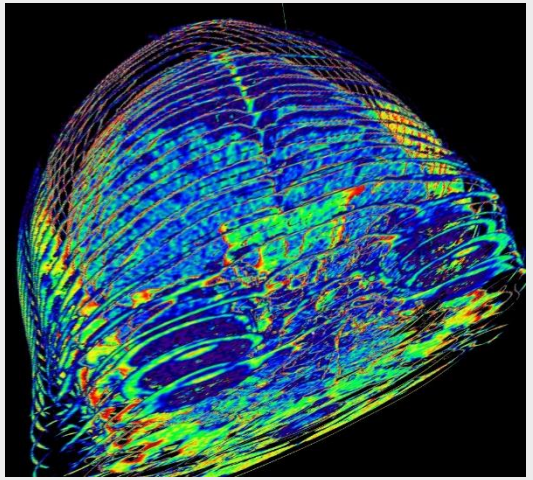
McMullin, 2014 & Ruskin, 2013

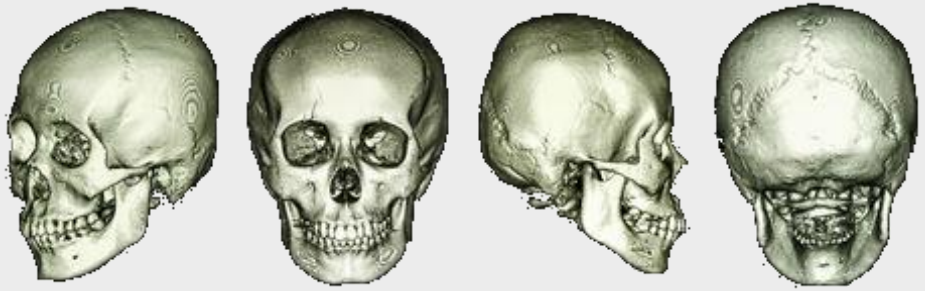




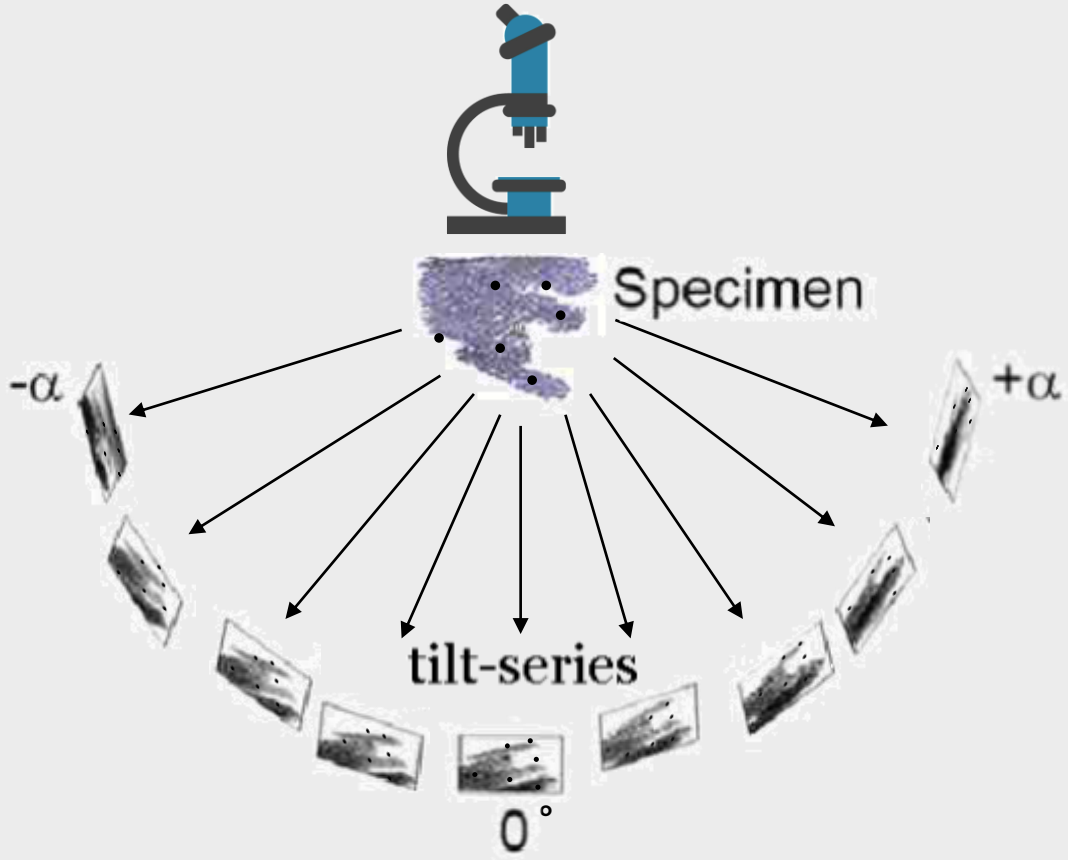
Tomography overview

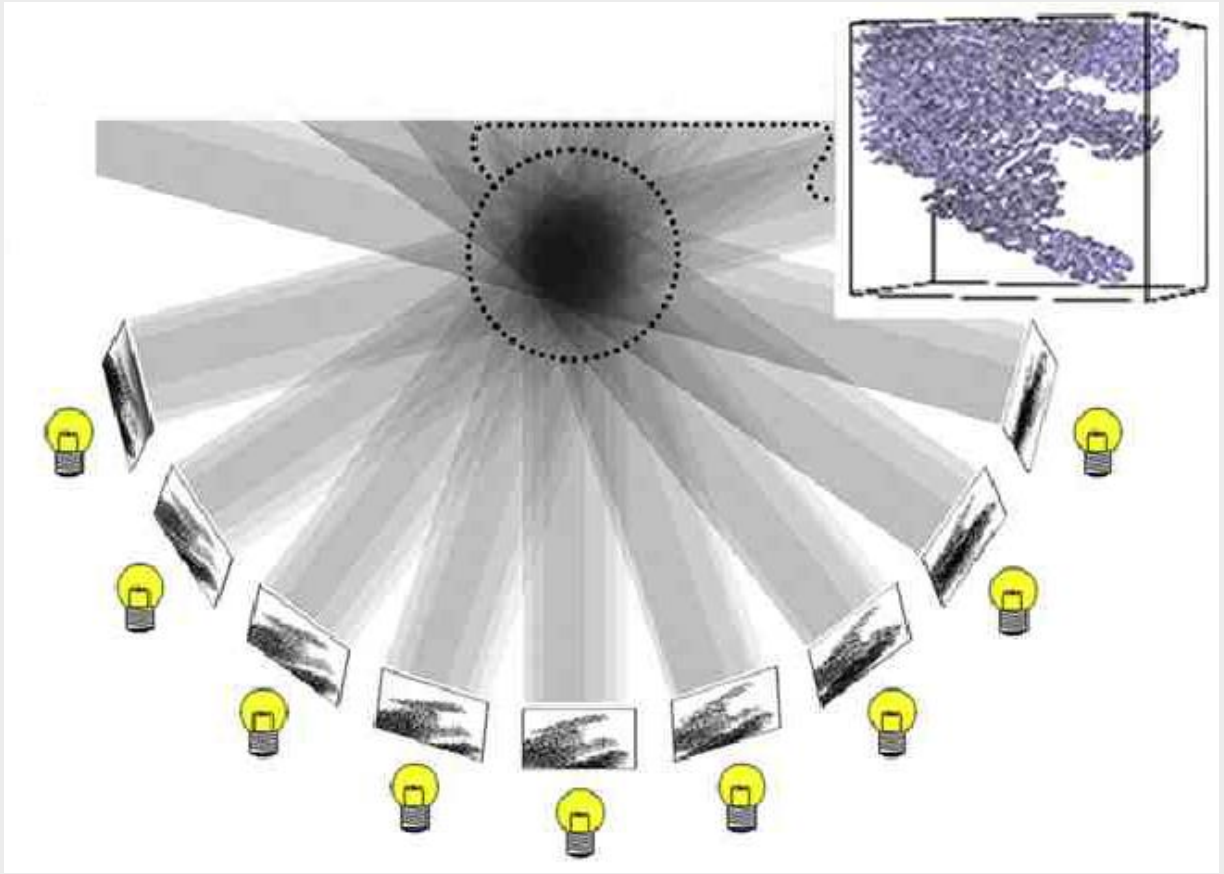






ET/CryoET collection and processing overview

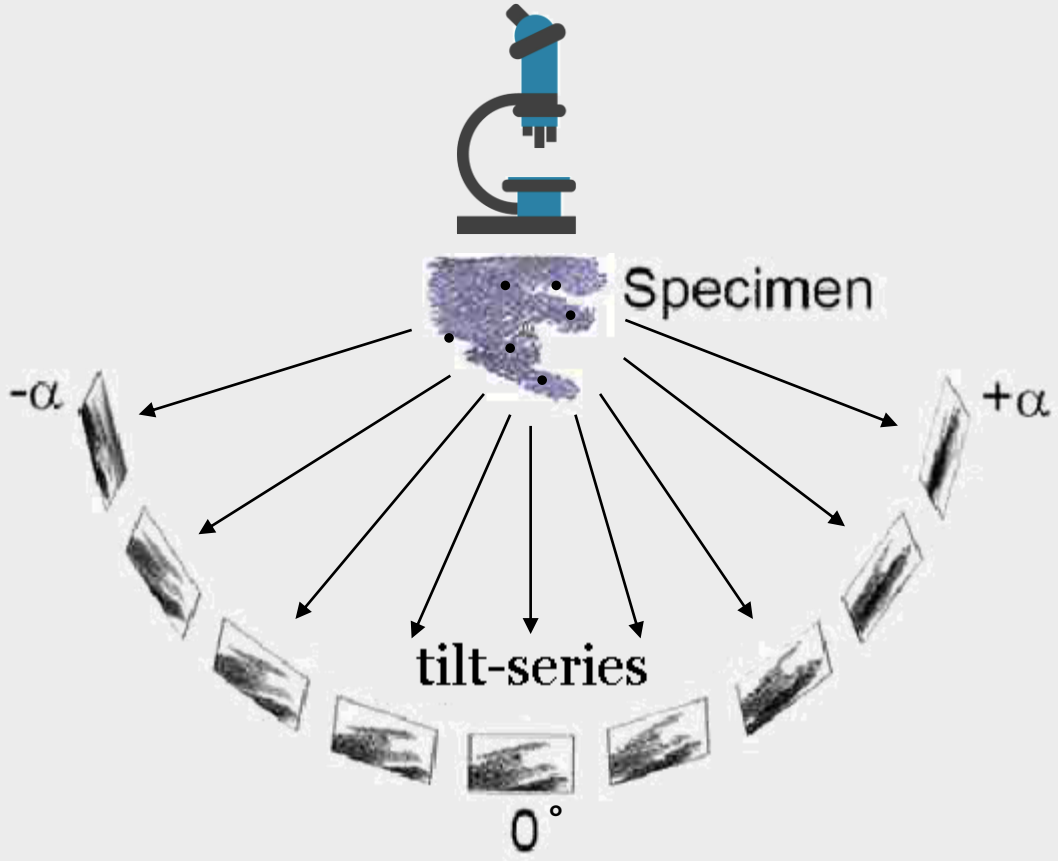


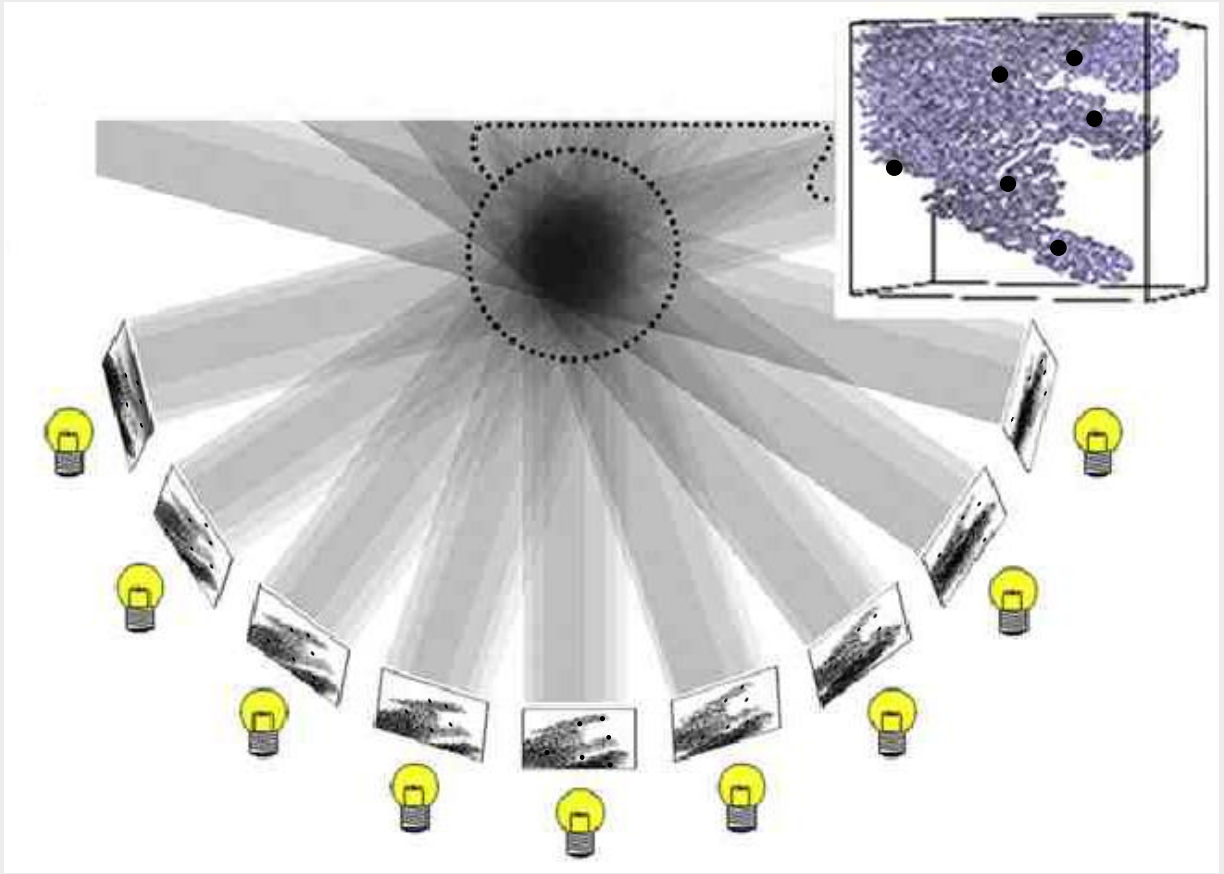


(UMET)

Collect → align → reconstruct

ET/CryoET collection and processing overview

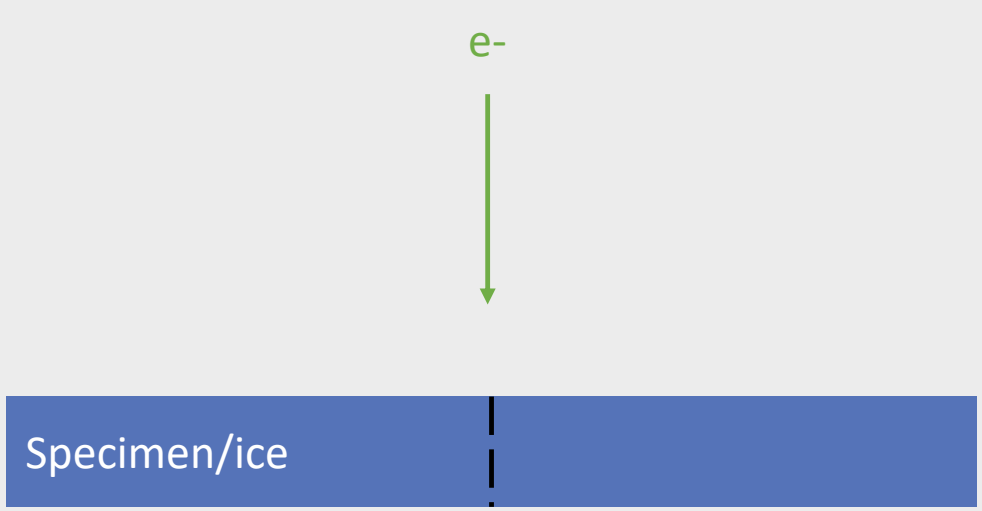




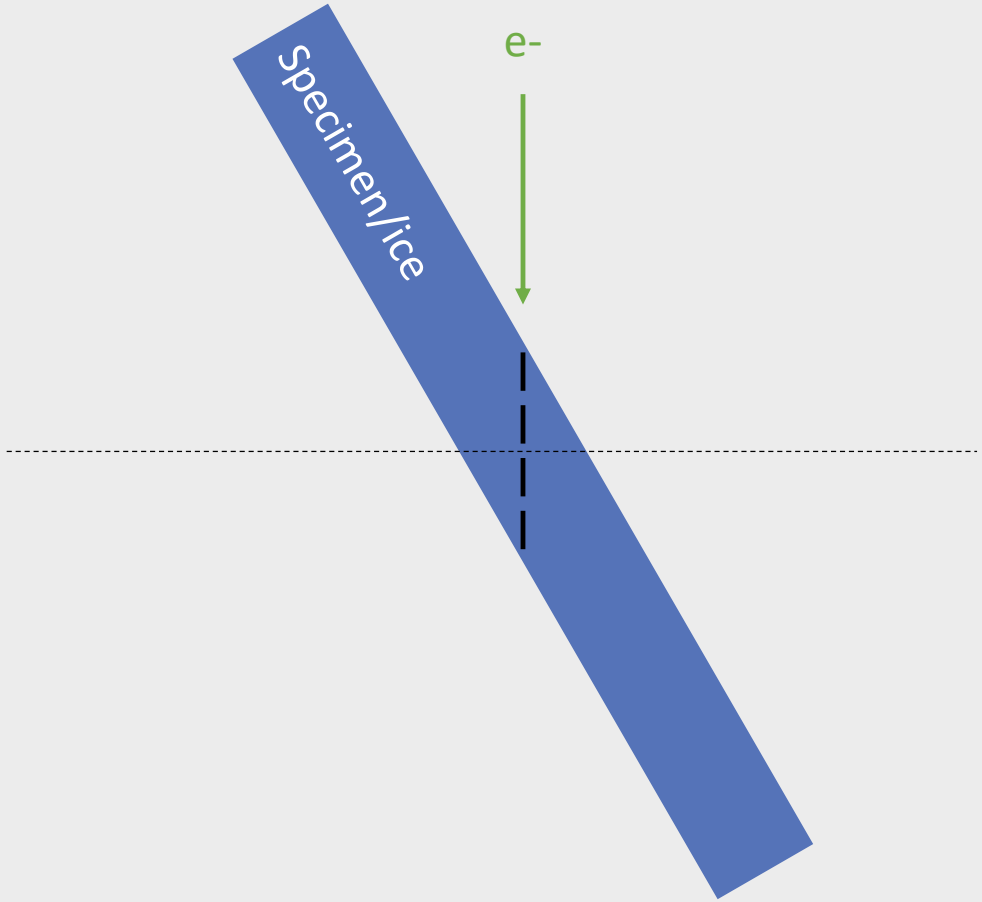
(UMET)

Collect → align → reconstruct

Grid tilting increases thickness



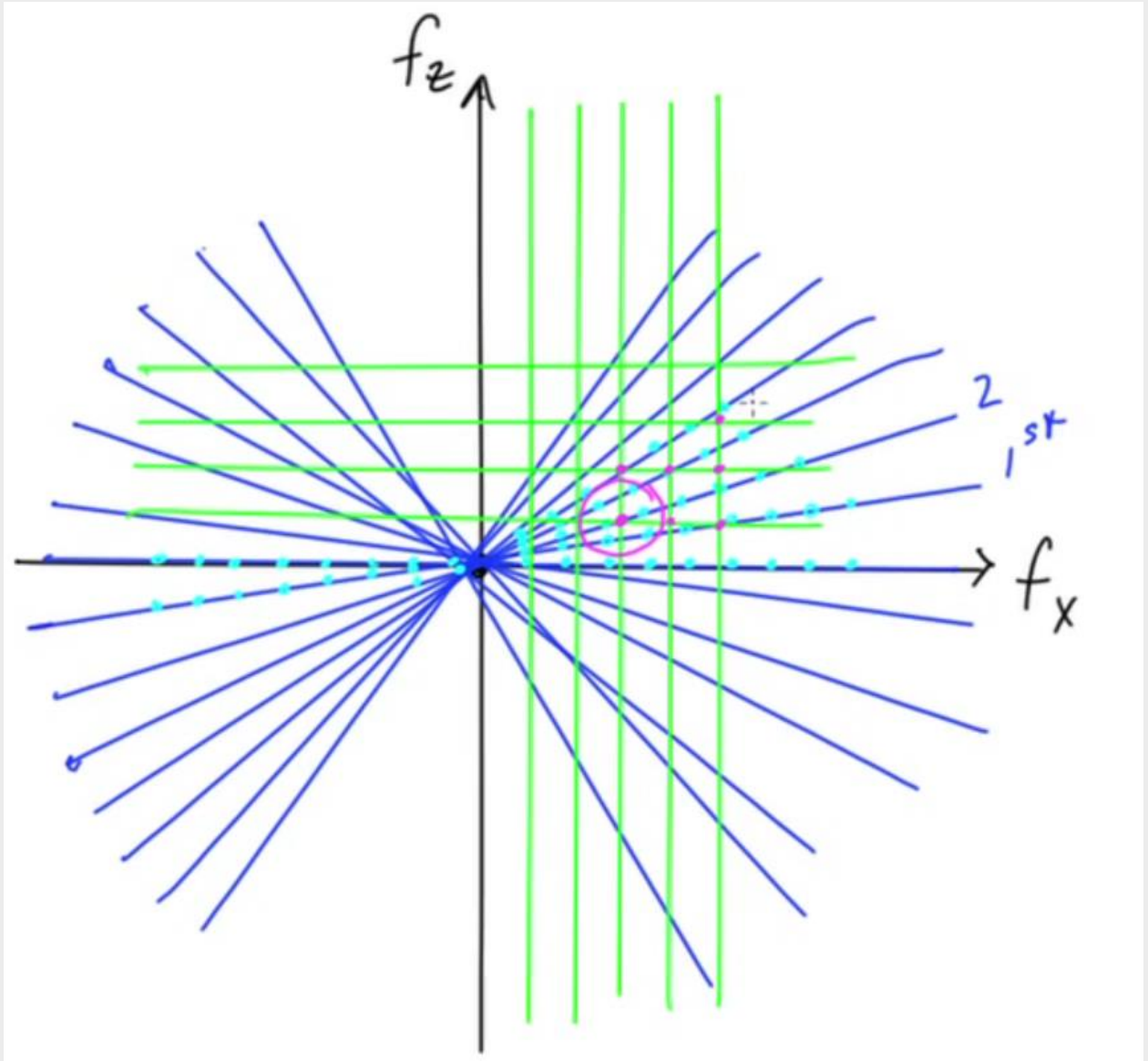
untilted grid



grid tilted 60° = 2x thickness

Reconstruction Implies Interpolation

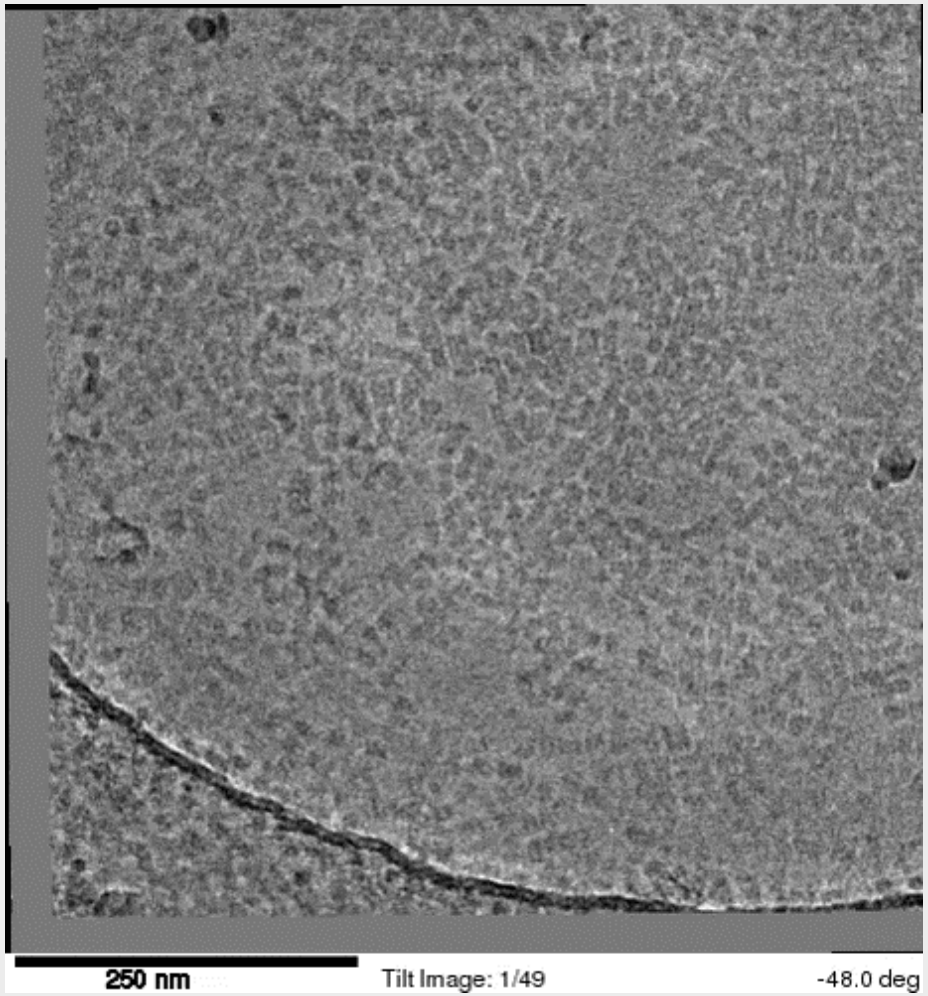
- Tomographic reconstruction on a 3D grid requires interpolation
- Larger tilt increment = more missing information at higher tilt angles



Grant Jensen, YouTube

Thickness increase from tilting limits tilting

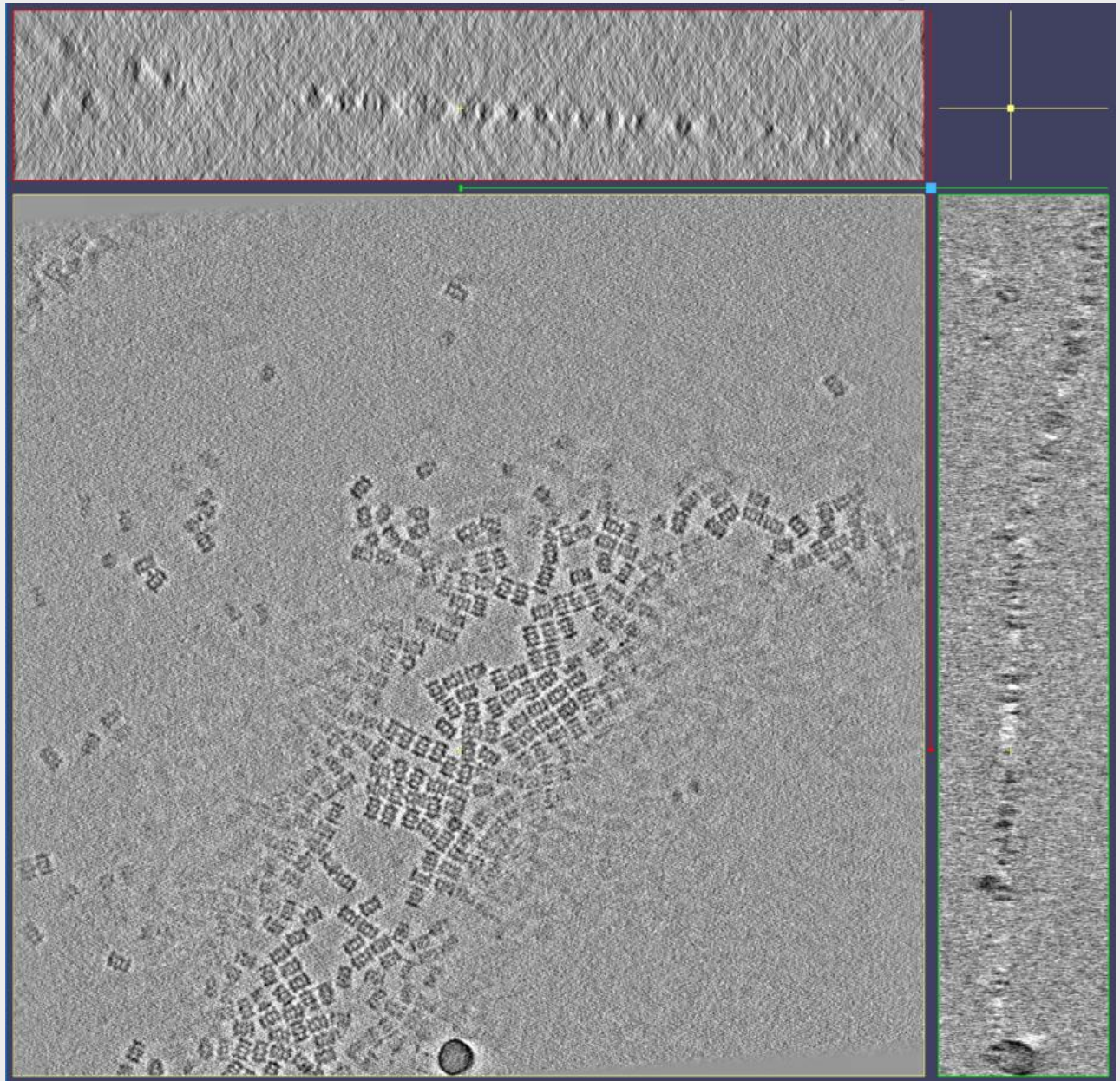
Noble, 2017



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

Grid tilting limit results in missing information

Noble, 2017



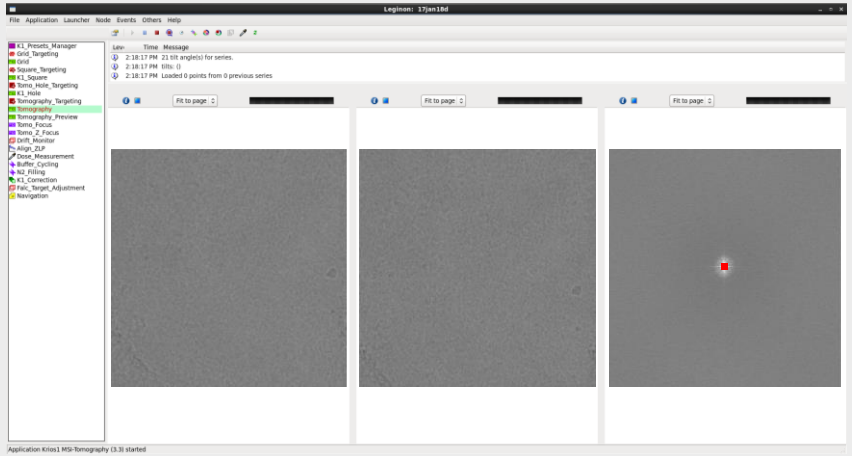
Phase plate tilt-series

of T20S Proteasome

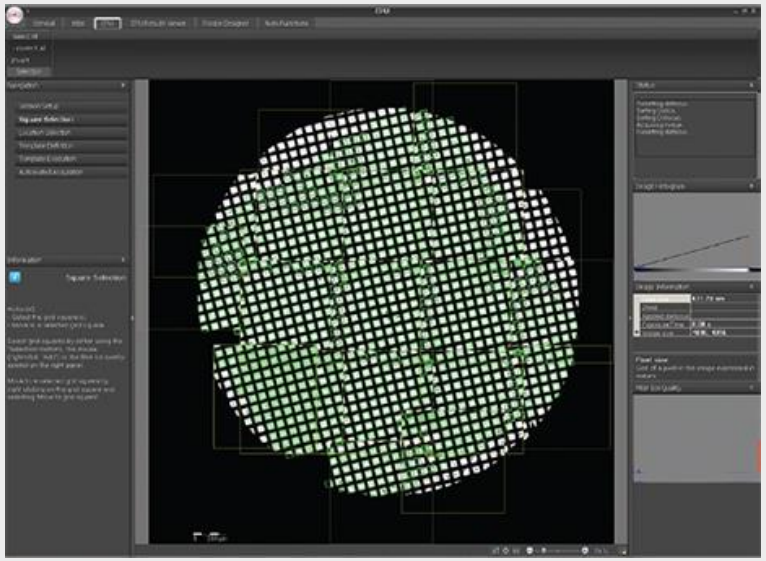
Tilt axis is **vertical**

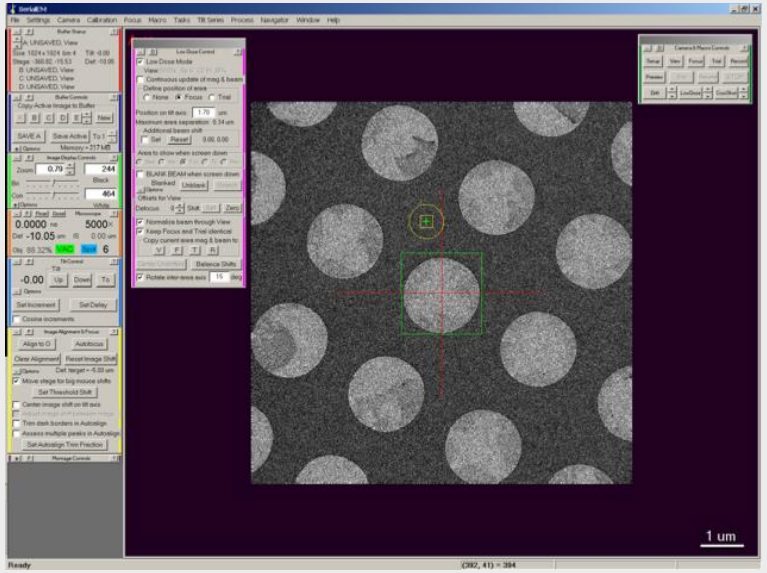
Tilt-series collection software

Leginon

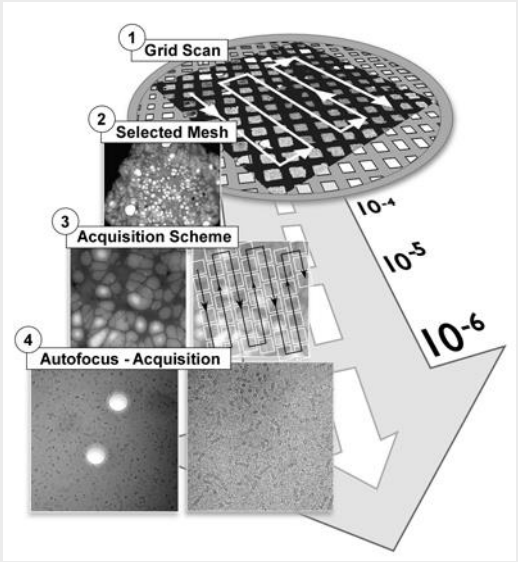


EPU

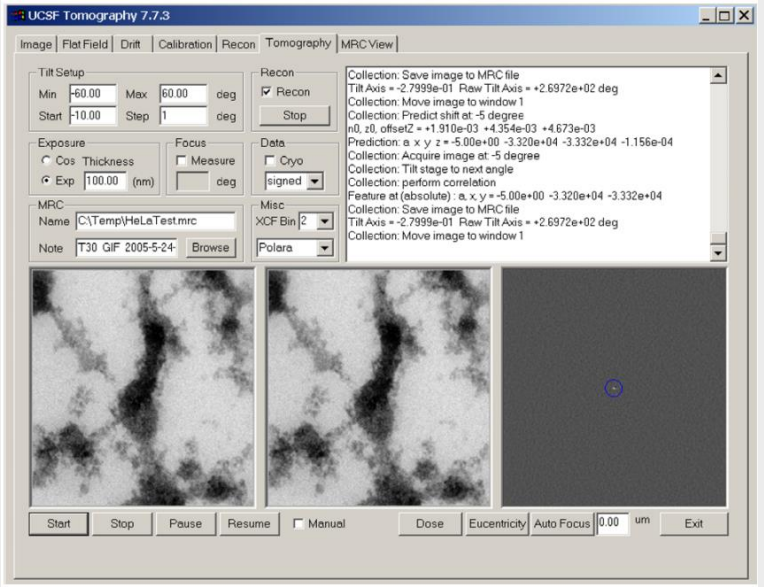




SerialEM



TOM Toolbox



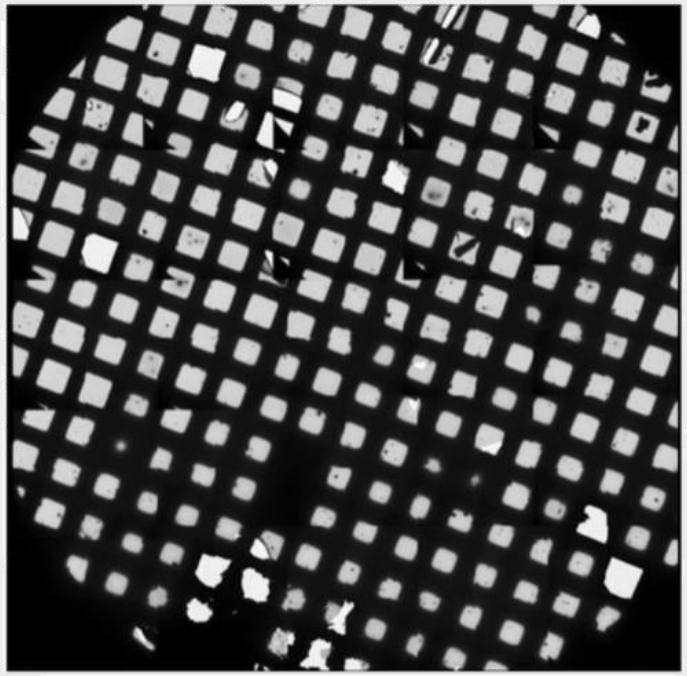
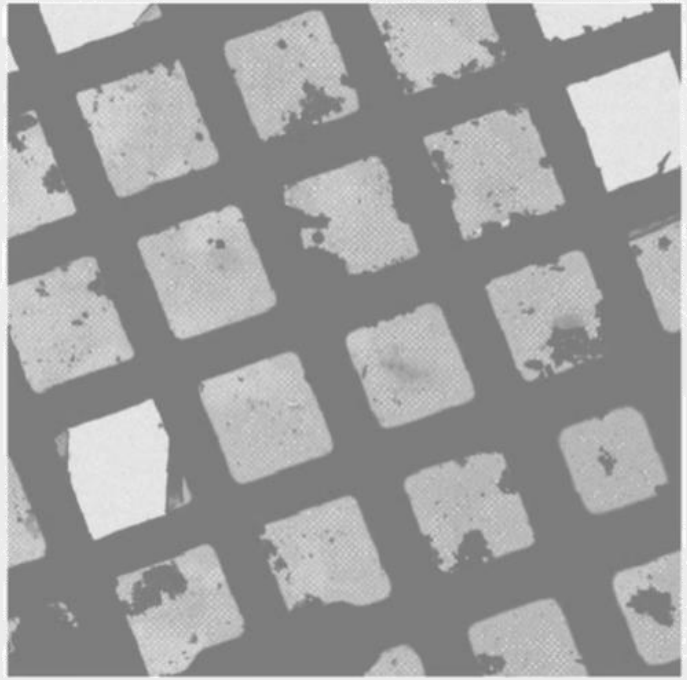
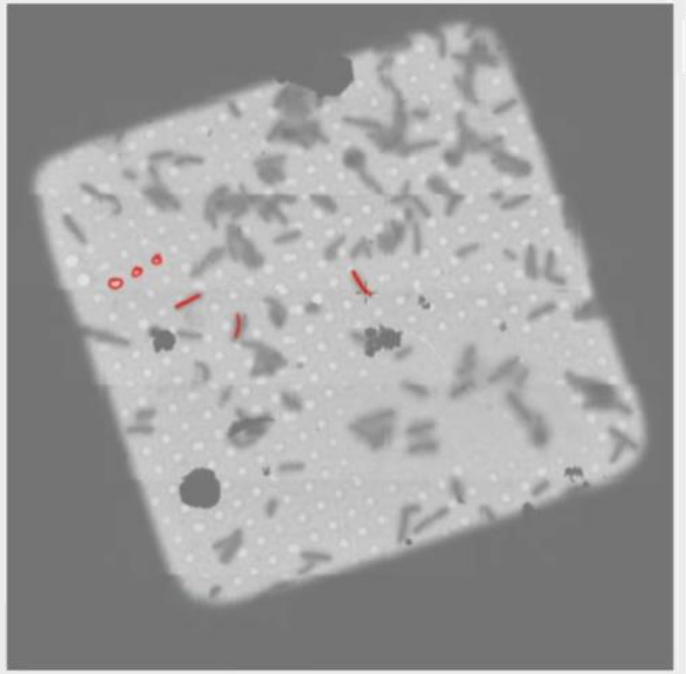
UCSF Tomography

Tilt-series tracking

- **Problem:** You **cannot trust the goniometer** to move where you tell it
- **Problem:** You **cannot use the area of interest to refine** your tracking because you will over-expose your sample
- **Problem:** You need to **refine x, y, and usually z to within 10-100 nm** for a high-mag tilt-series collection.

- **Solution 1: Predictive tracking** – Use previous tilt images, previous tilt-series, and possibly known goniometer instabilities.
- **Solution 2: Focus position method** – Identify one or two locations along the tilt axis the software will go to re-focus and re-track.

Automated tilt-series collection

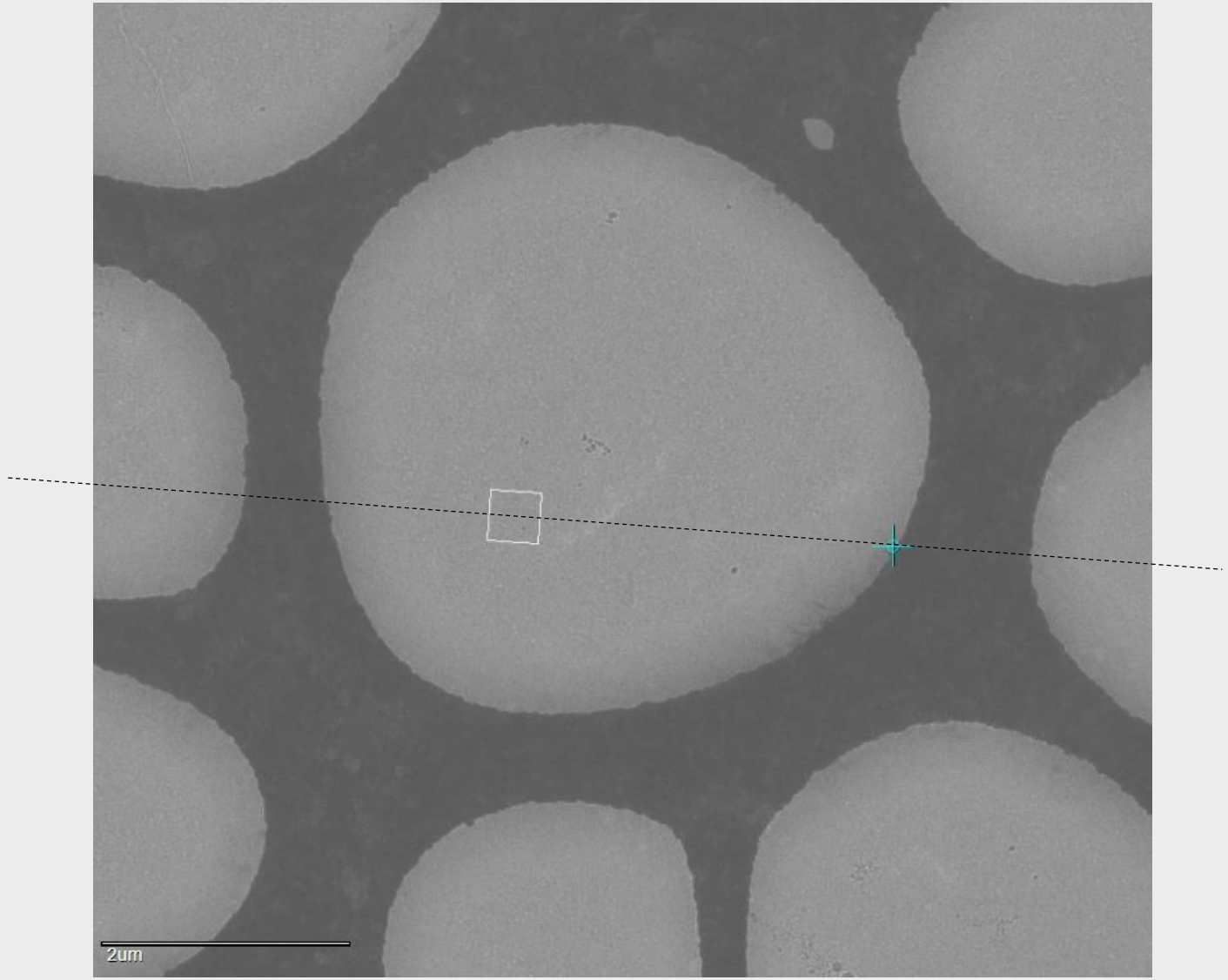
Automated tilt-series collection is currently **routine**

- 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**.



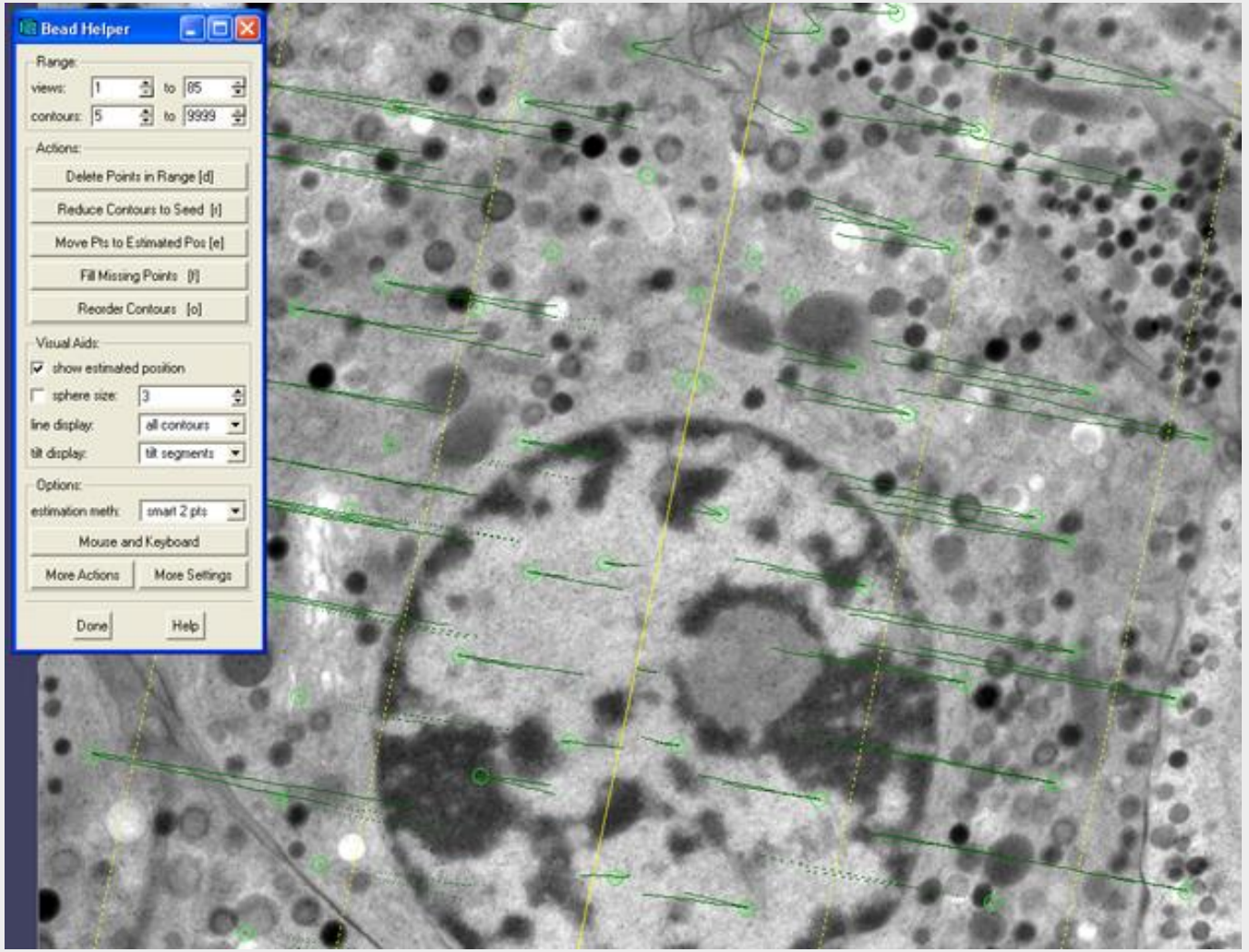
Tilt-series alignment

- **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
  - EMAN2 – Object tracking

- **Must refine** most or all of the following:
  - Tilt image shifts, rotations, and magnification changes (scaling)
  - 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



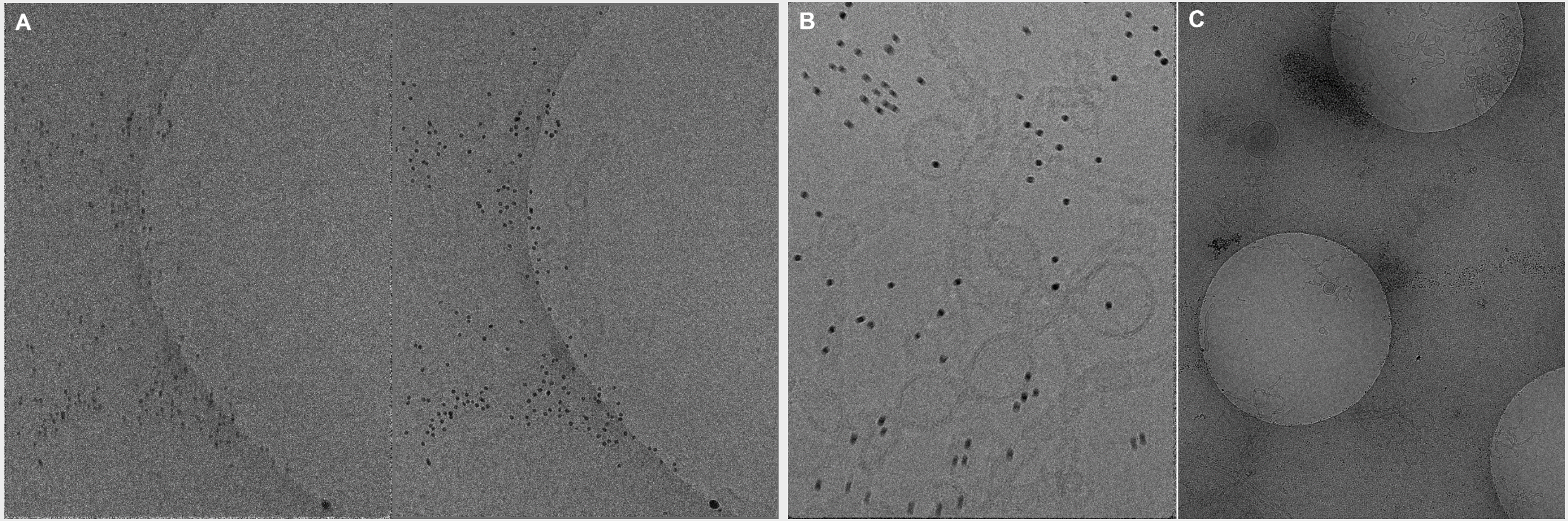
http://bio3d.colorado.edu

Fiducial-based tilt-series alignment issues

Fiducial Movement

Anisotropic Bead Motion

Bead Aggregation

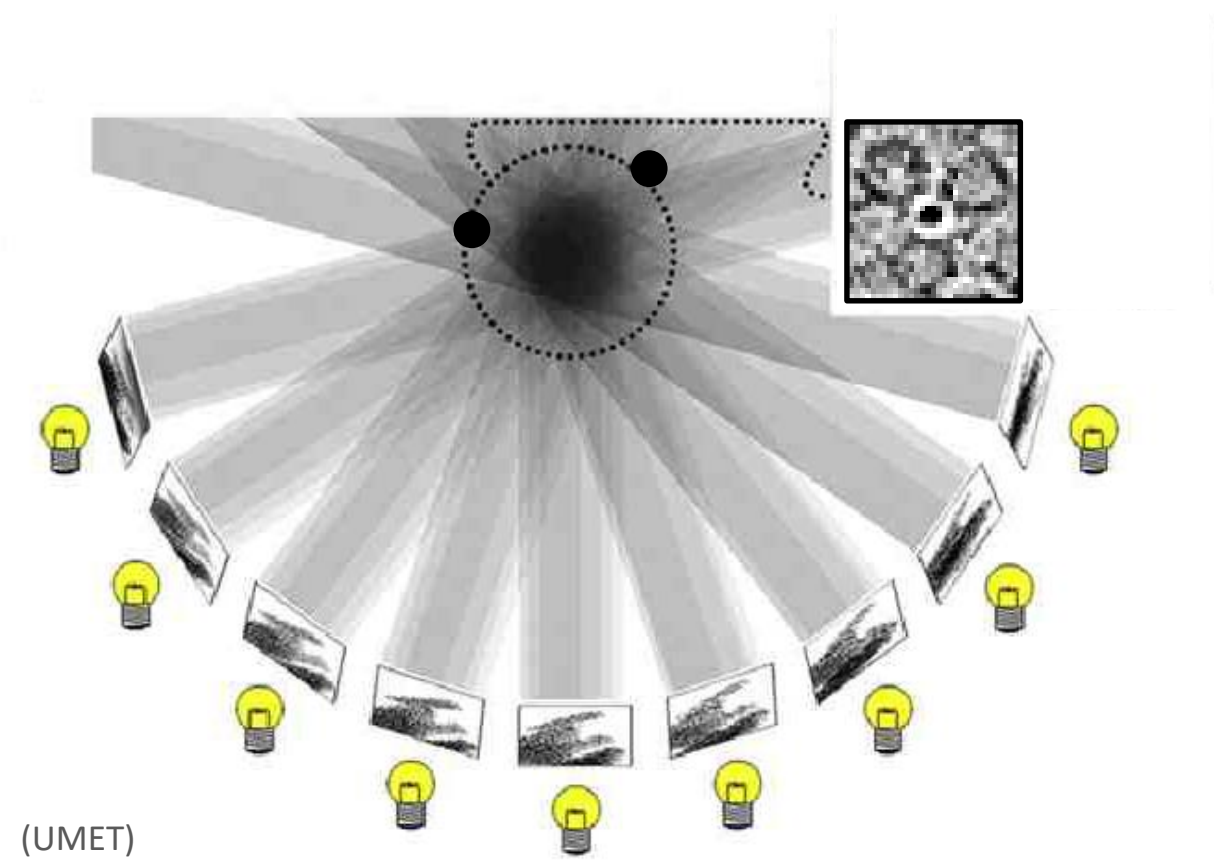


DE-20 @ 18kx; 51°, 2.34 $e^{-}/Å^2$ after a cumulative dose of 60 $e^{-}/Å^2$

DE-20; 57.5 $e^{-}/Å^2$, 0° exposure

Noble & Stagg, JSB 2015

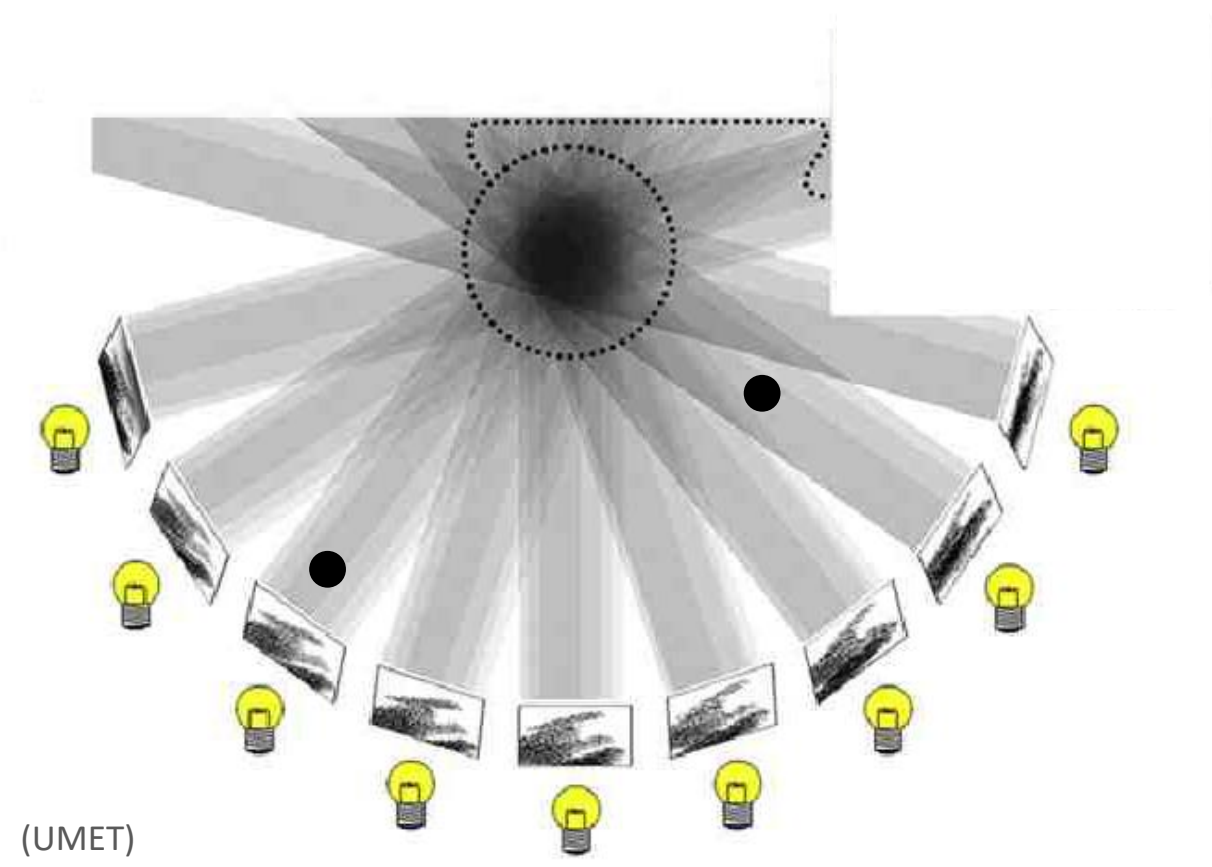
Fiducial-based tilt-series alignment issues


(UMET)

Nearby Fiducials Affect Signal and Contrast

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

Fiducial-based tilt-series alignment issues

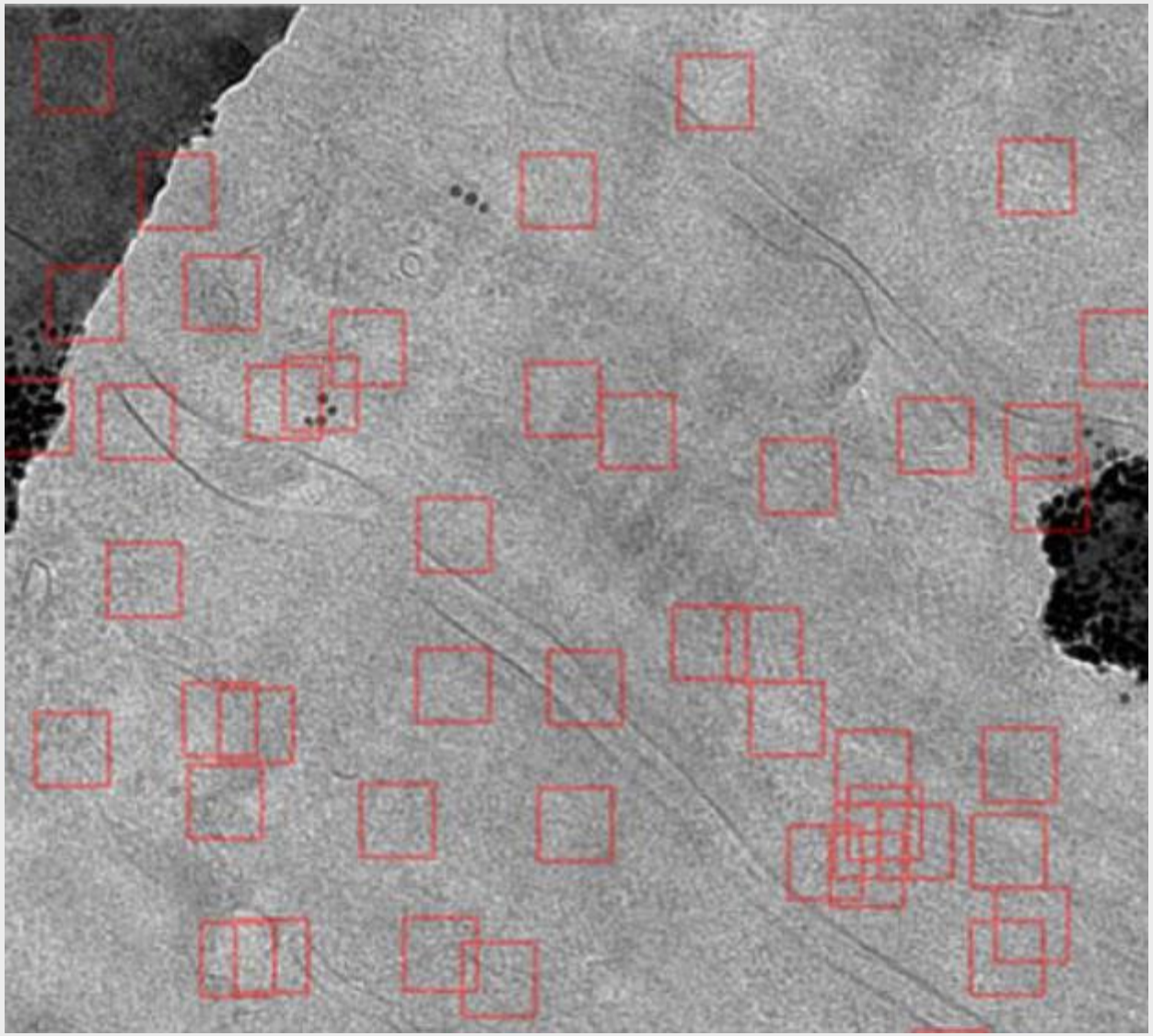


(UMET)

Fiducials are Present in Much of 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

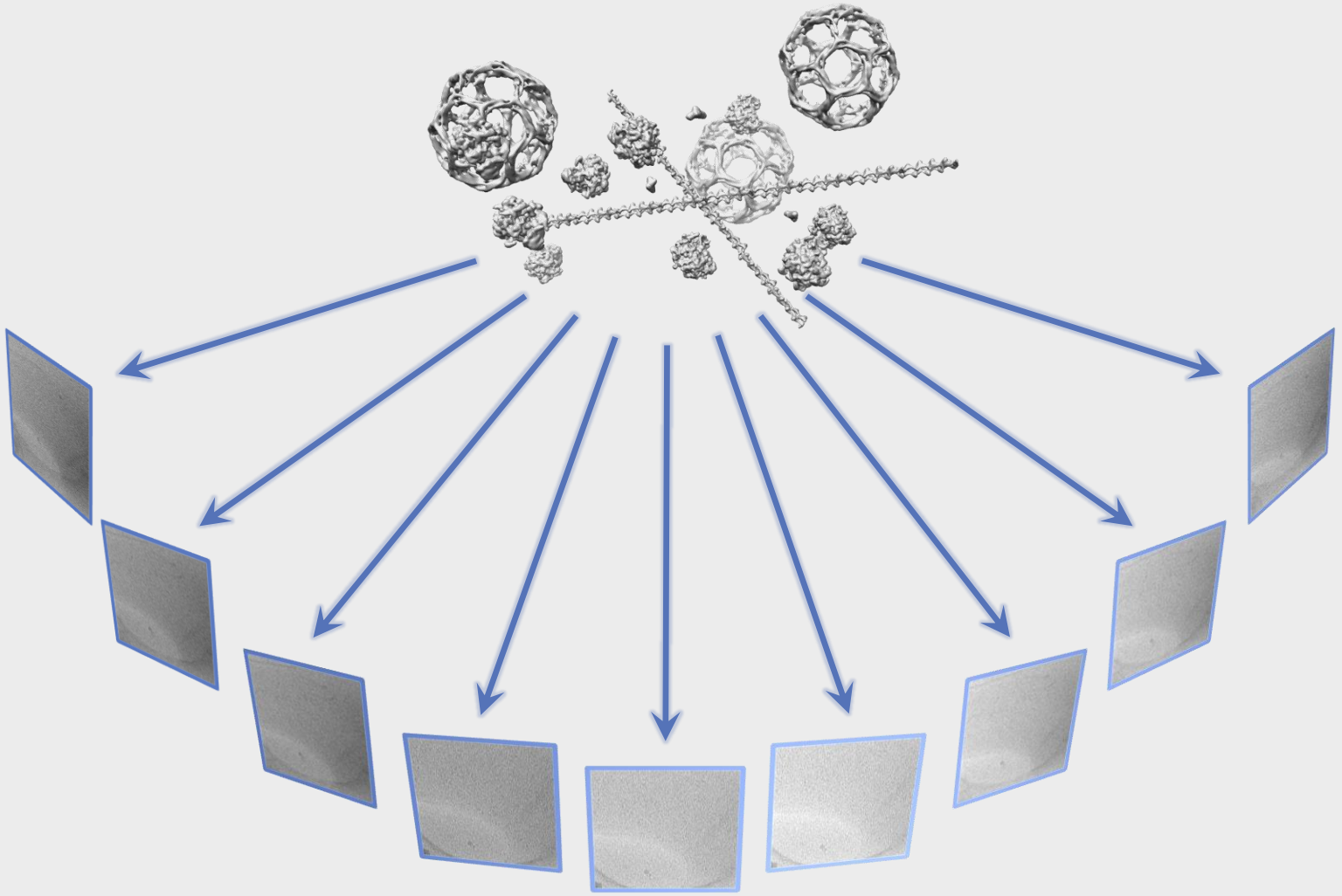


Castaño-Díez, 2010

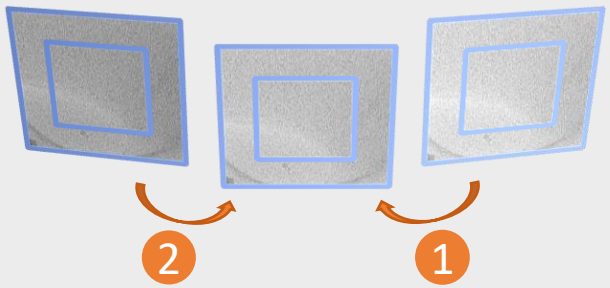
Identify featureful objects with contrast in all tilt images and track them.

- Semi-automated (IMOD, Alignator)

Collect a tilt-series

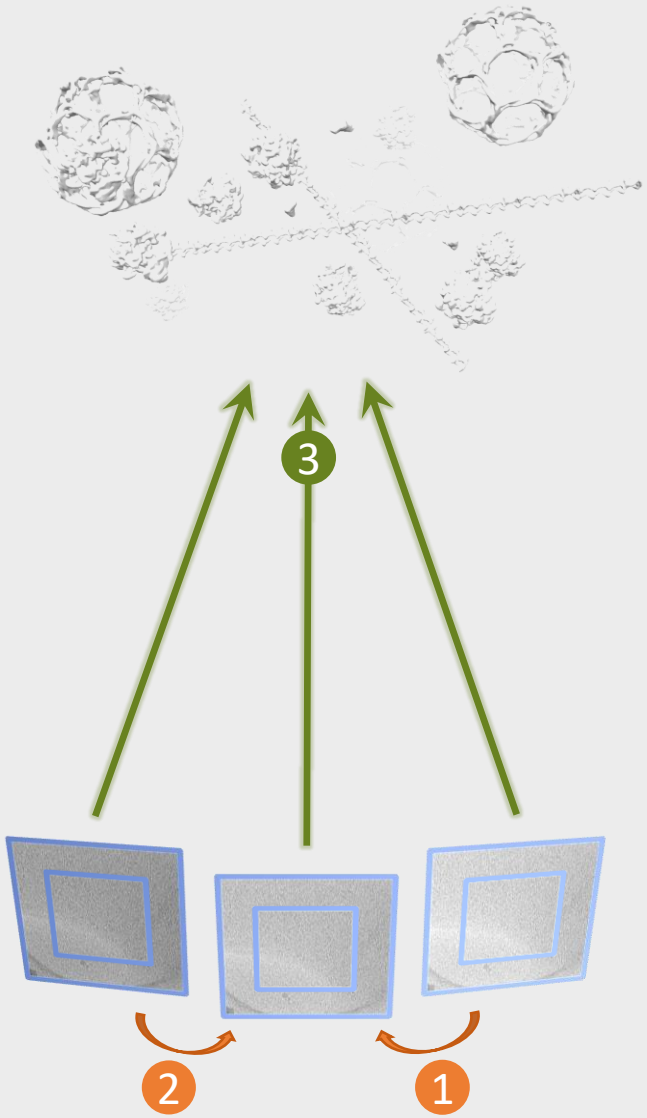


Protomo alignment



Nearest-neighbor correlation

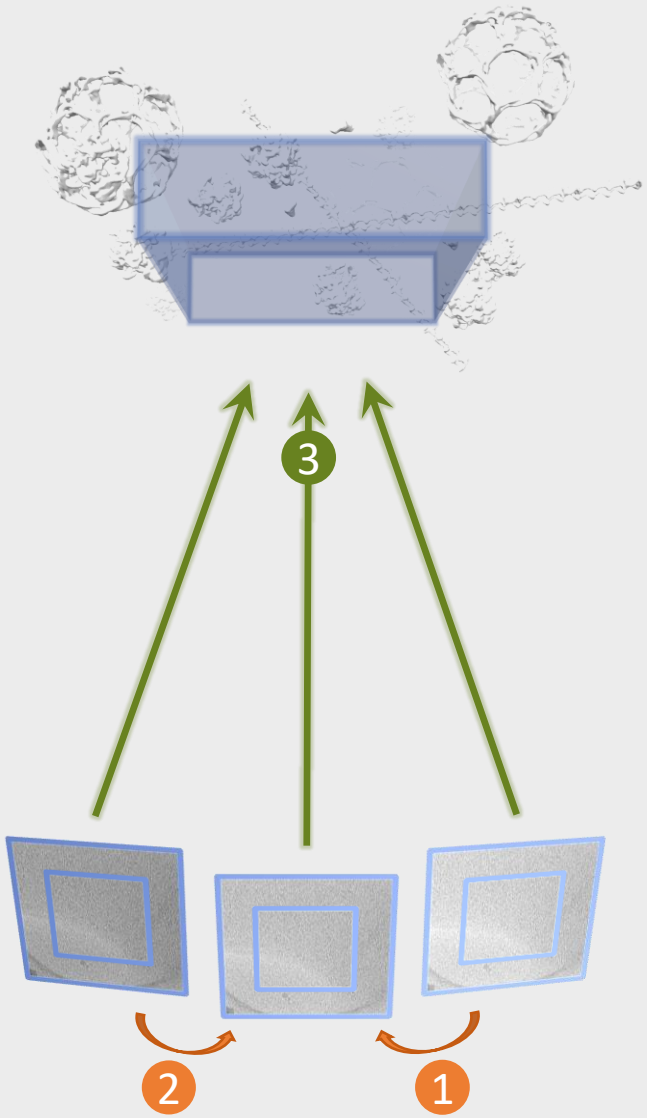
Protomo alignment



Nearest-neighbor correlation

Weighted back-projection

Protomo alignment



Nearest-neighbor correlation

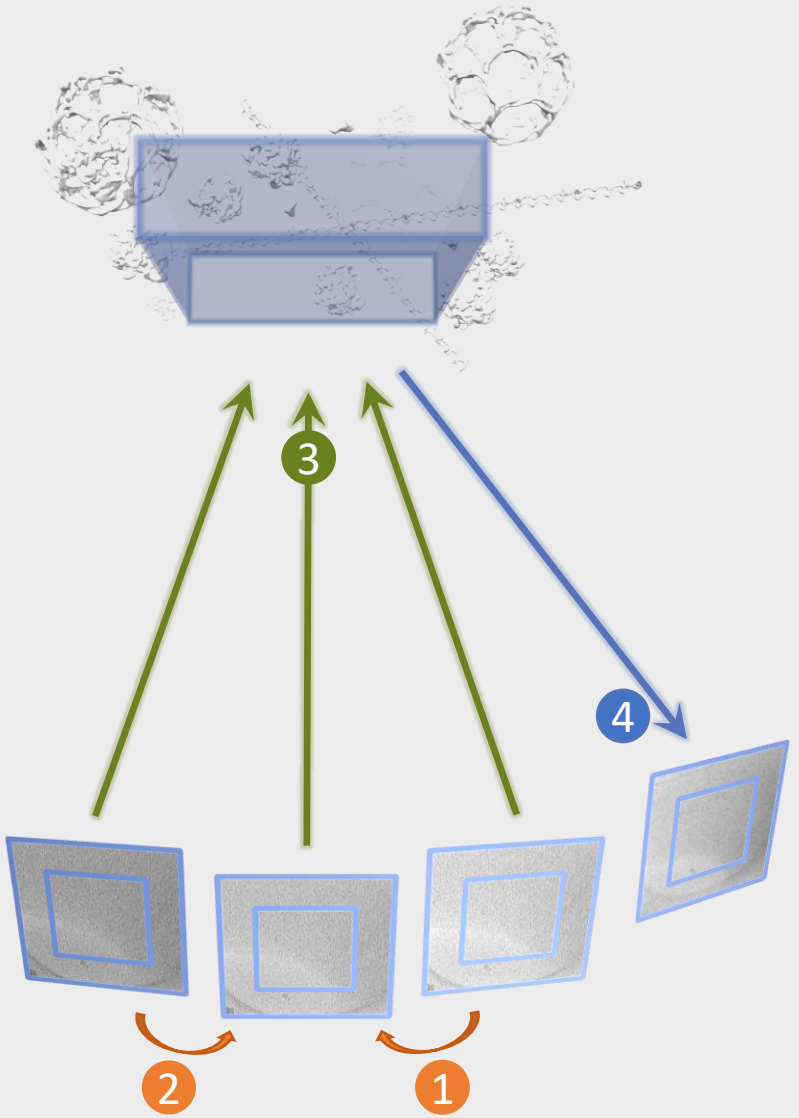
Weighted back-projection

alignment thickness = z

x y

Volume to be re-projected

Protomo alignment



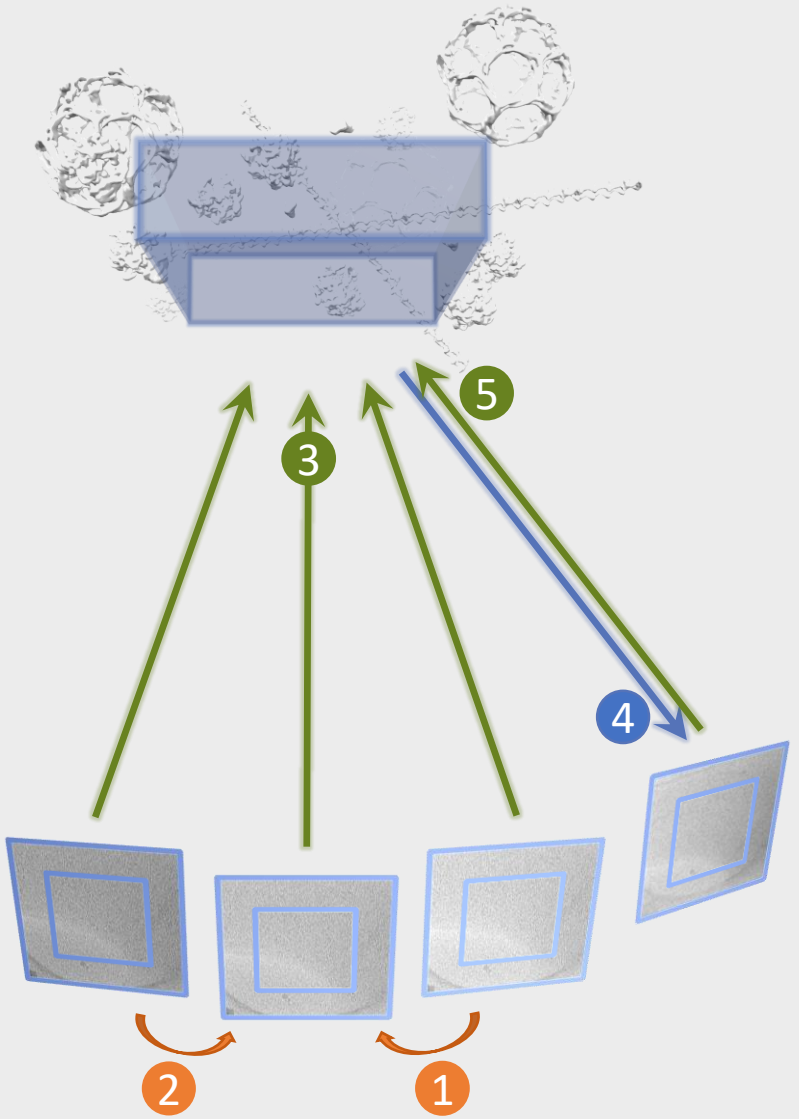
Nearest-neighbor correlation

Weighted back-projection

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

Re-projection ⟶ correlation

Protomo alignment



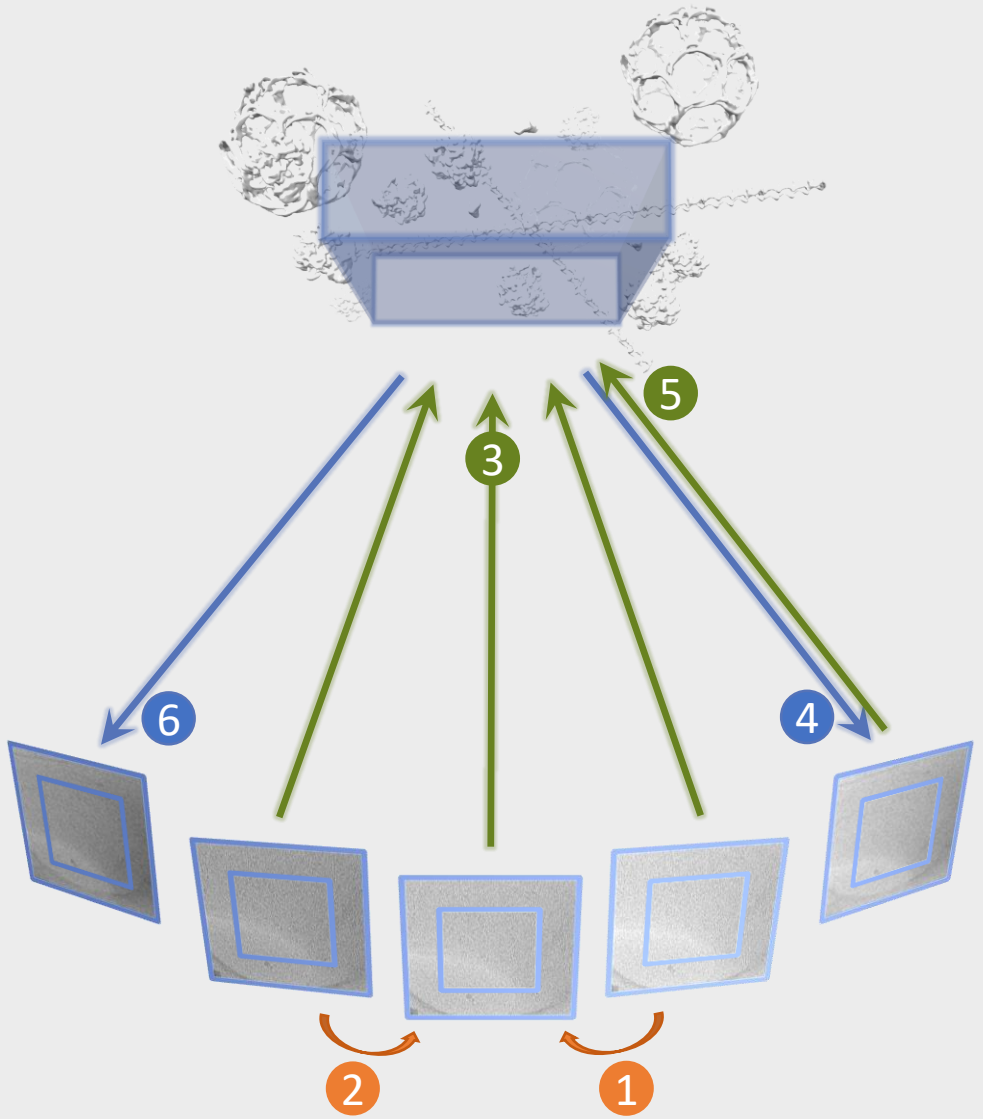
Nearest-neighbor correlation

Weighted back-projection

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

Re-projection ⟶ correlation

Protomo alignment



Nearest-neighbor correlation

Weighted back-projection

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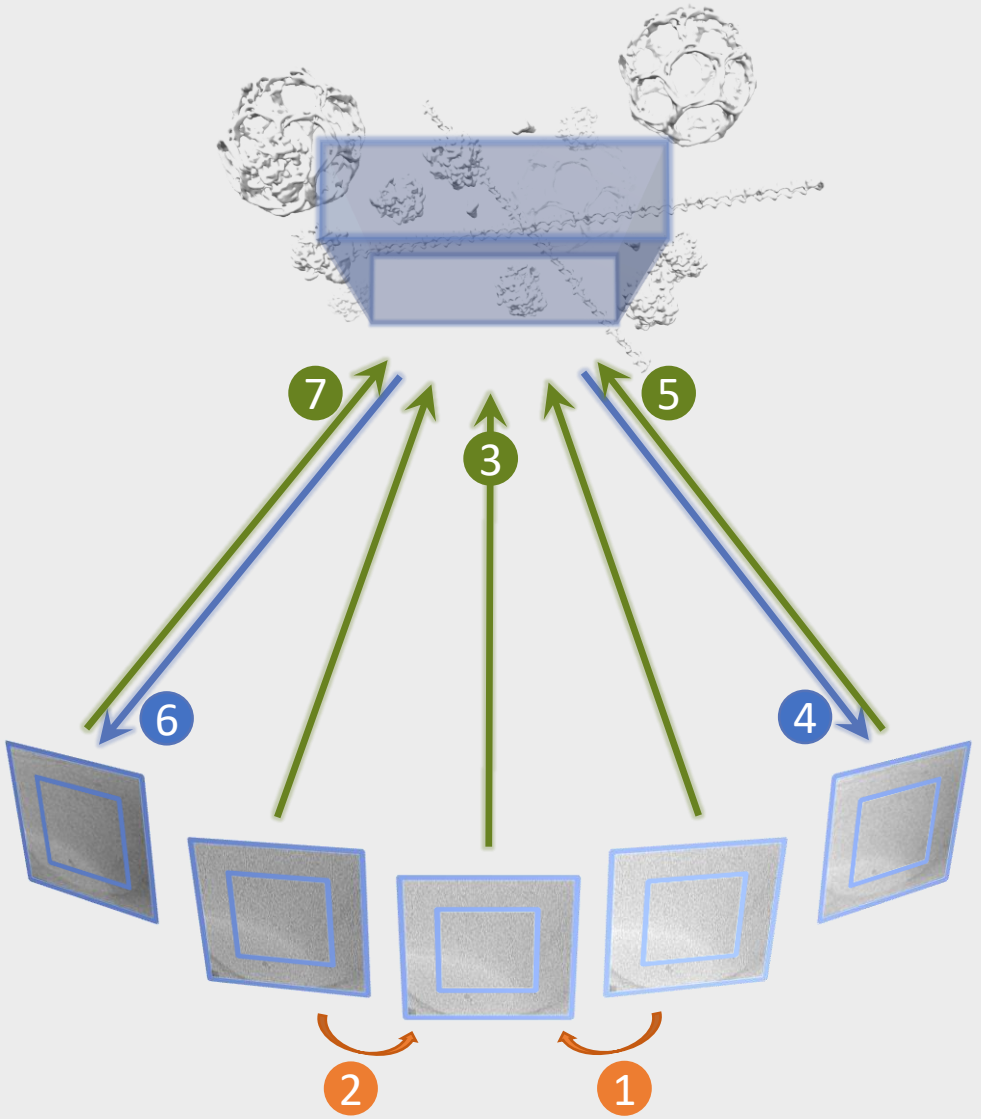
Re-projection ⟶ correlation

Protomo alignment



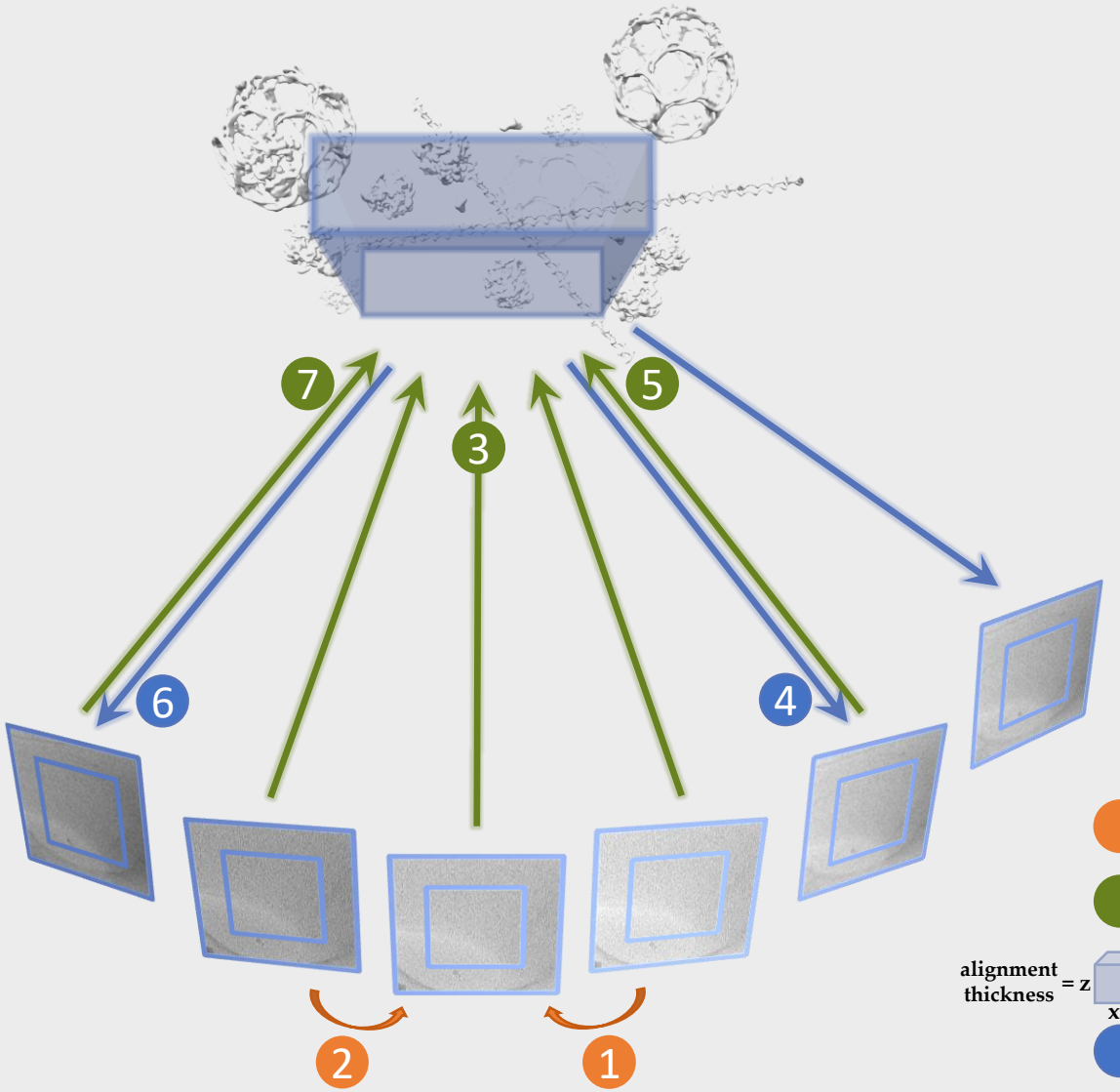
Nearest-neighbor correlation

Weighted back-projection

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

Re-projection ⟶ correlation

Protomo alignment



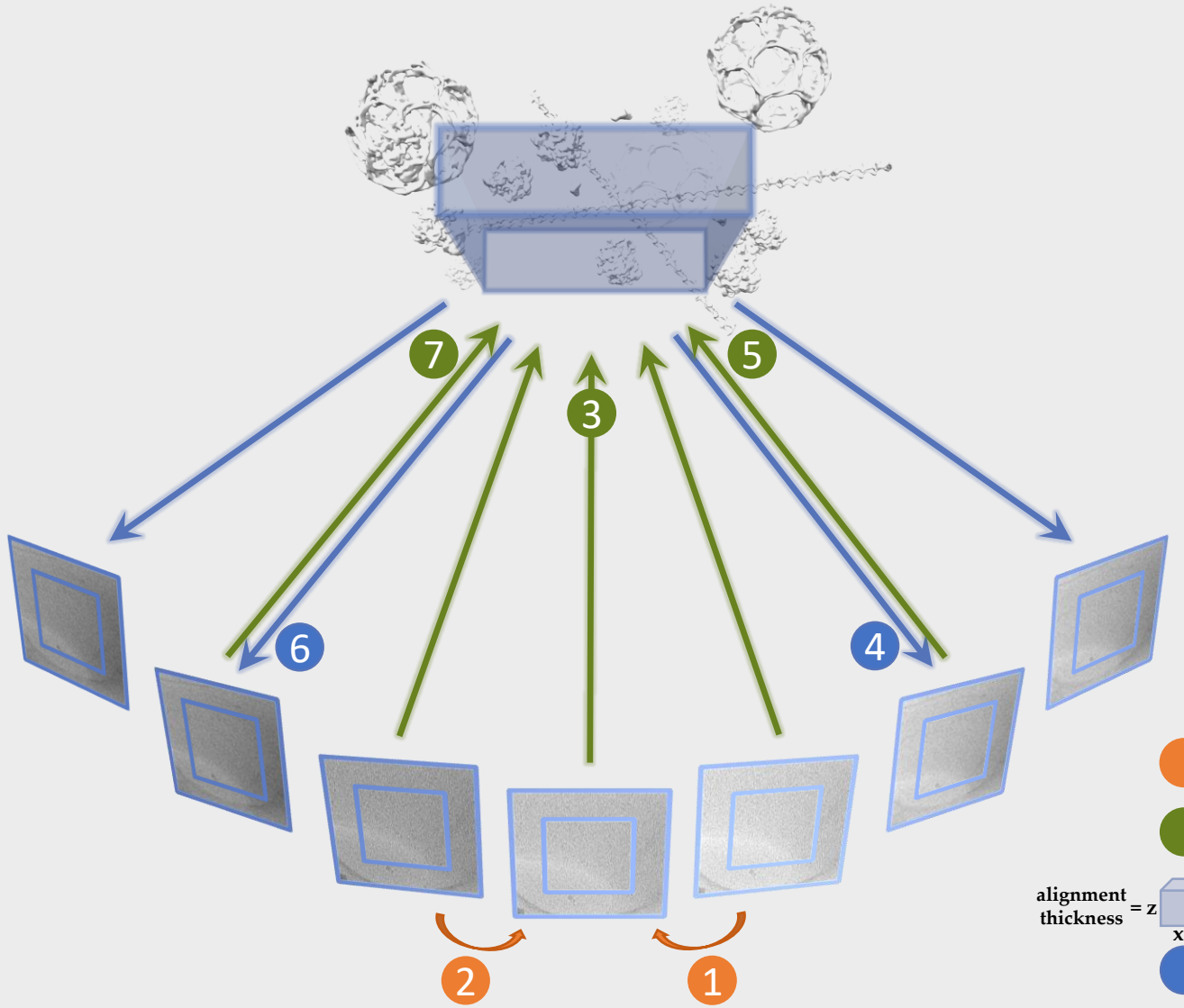
Nearest-neighbor correlation

Weighted back-projection

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

Re-projection ⟶ correlation

Protomo alignment



Nearest-neighbor correlation

Weighted back-projection

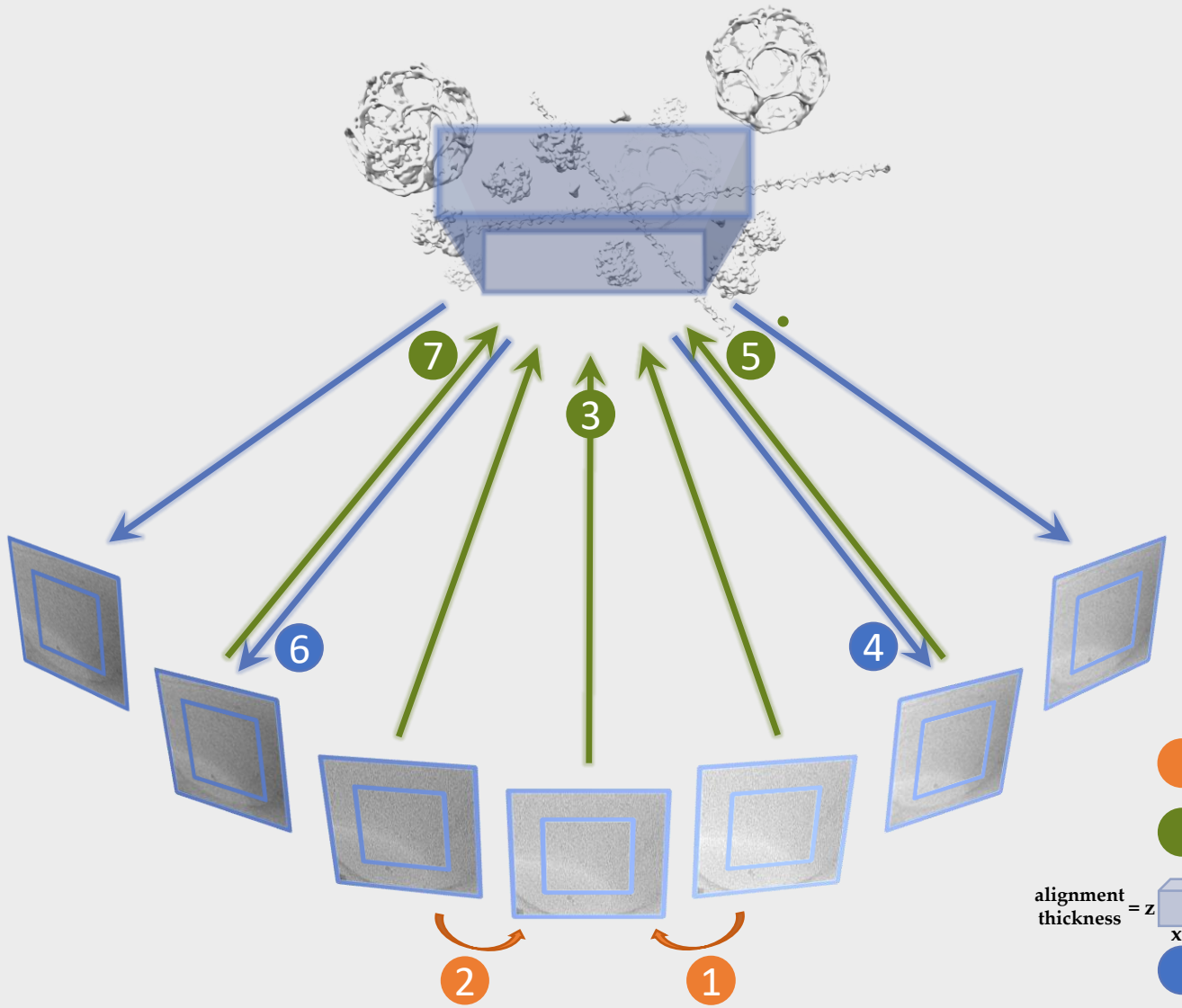
alignment thickness = z

x y

Volume to be re-projected

Re-projection ⟶ correlation

Protomo alignment



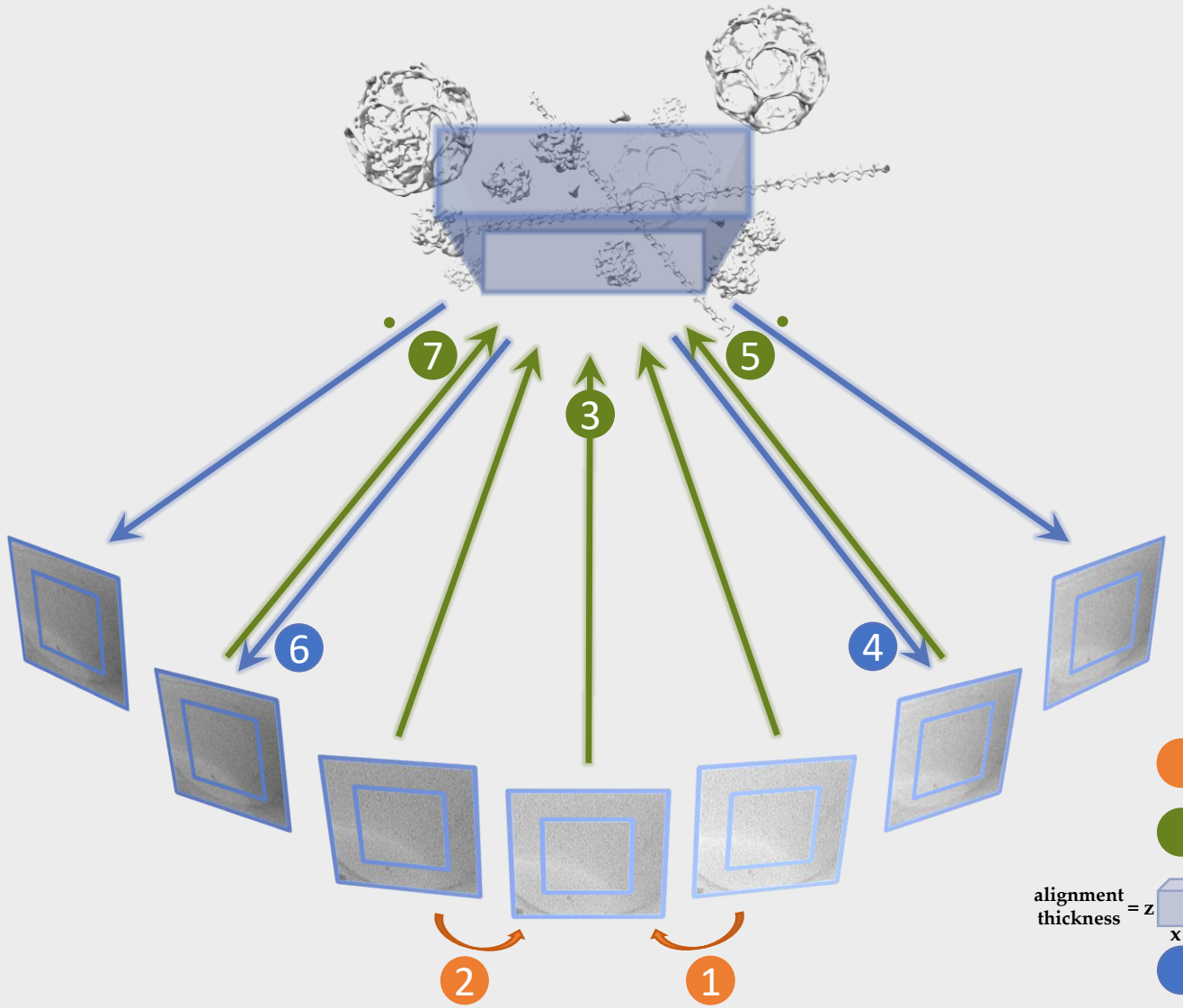
Nearest-neighbor correlation

Weighted back-projection

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

Re-projection ⟶ correlation

Protomo alignment



Nearest-neighbor correlation

Weighted back-projection

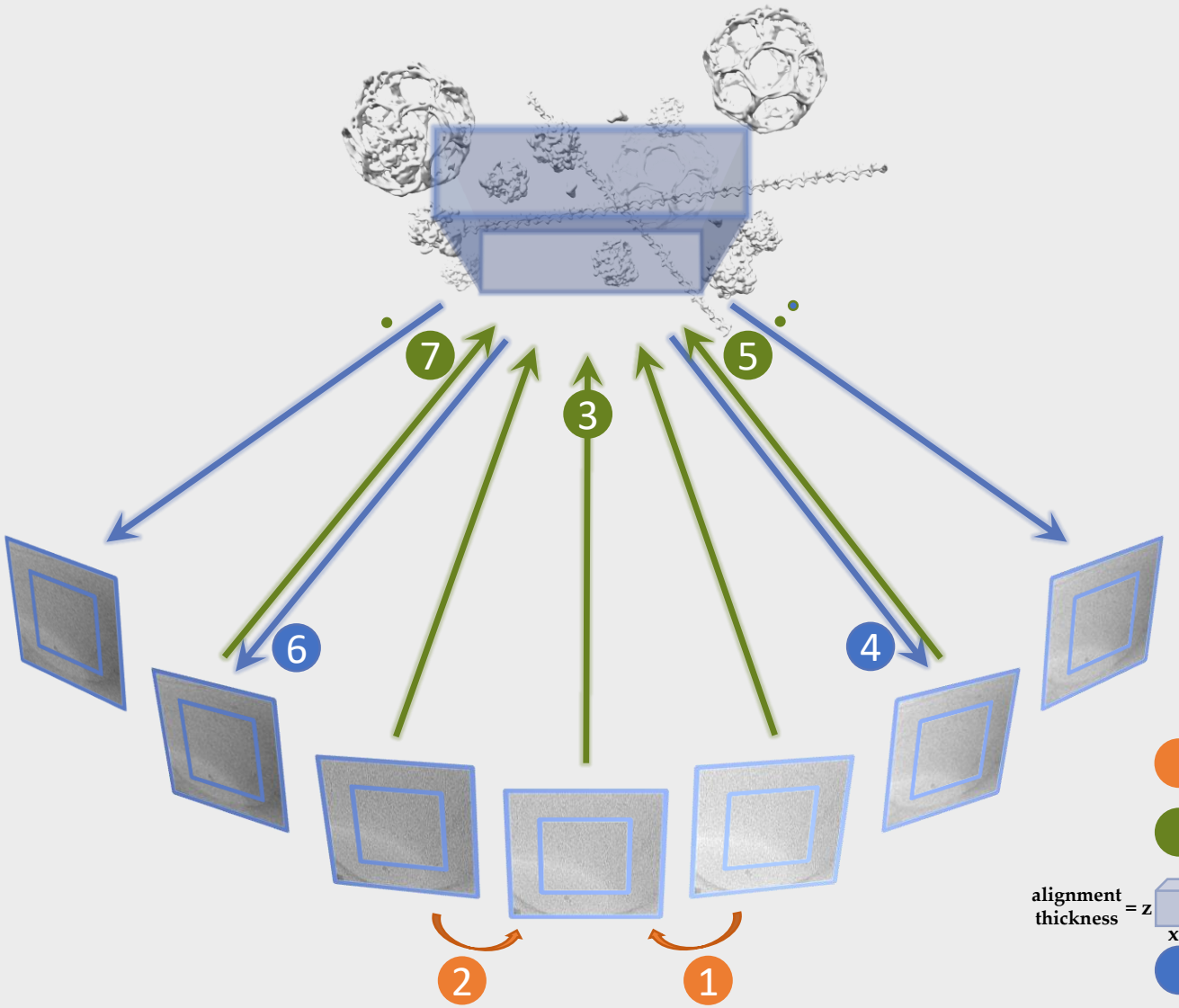
alignment thickness = z

x y

Volume to be re-projected

Re-projection ⟶ correlation

Protomo alignment



Nearest-neighbor correlation

Weighted back-projection

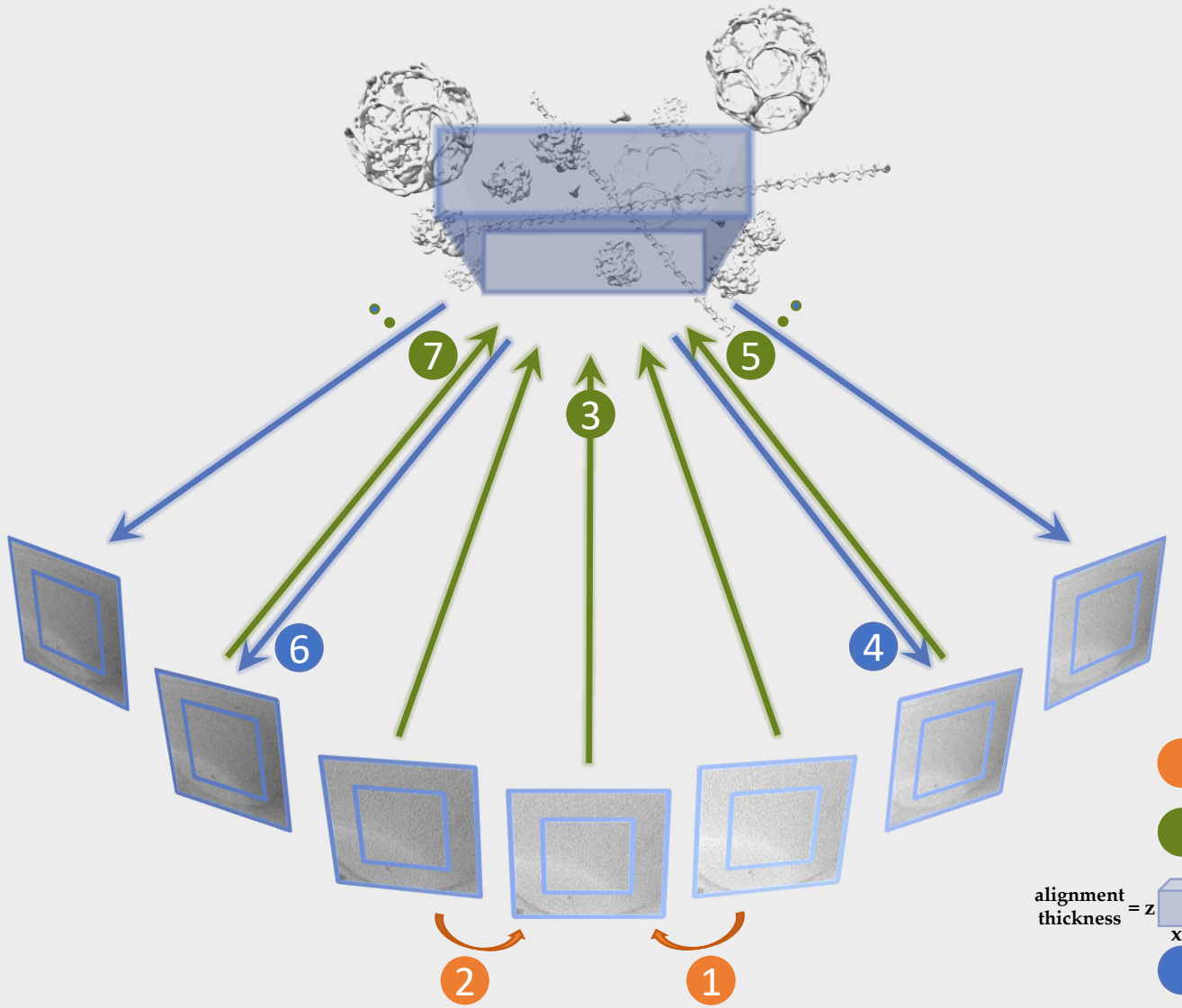
alignment thickness = z

x y

Volume to be re-projected

Re-projection ⟶ correlation

Protomo alignment



Nearest-neighbor correlation

Weighted back-projection

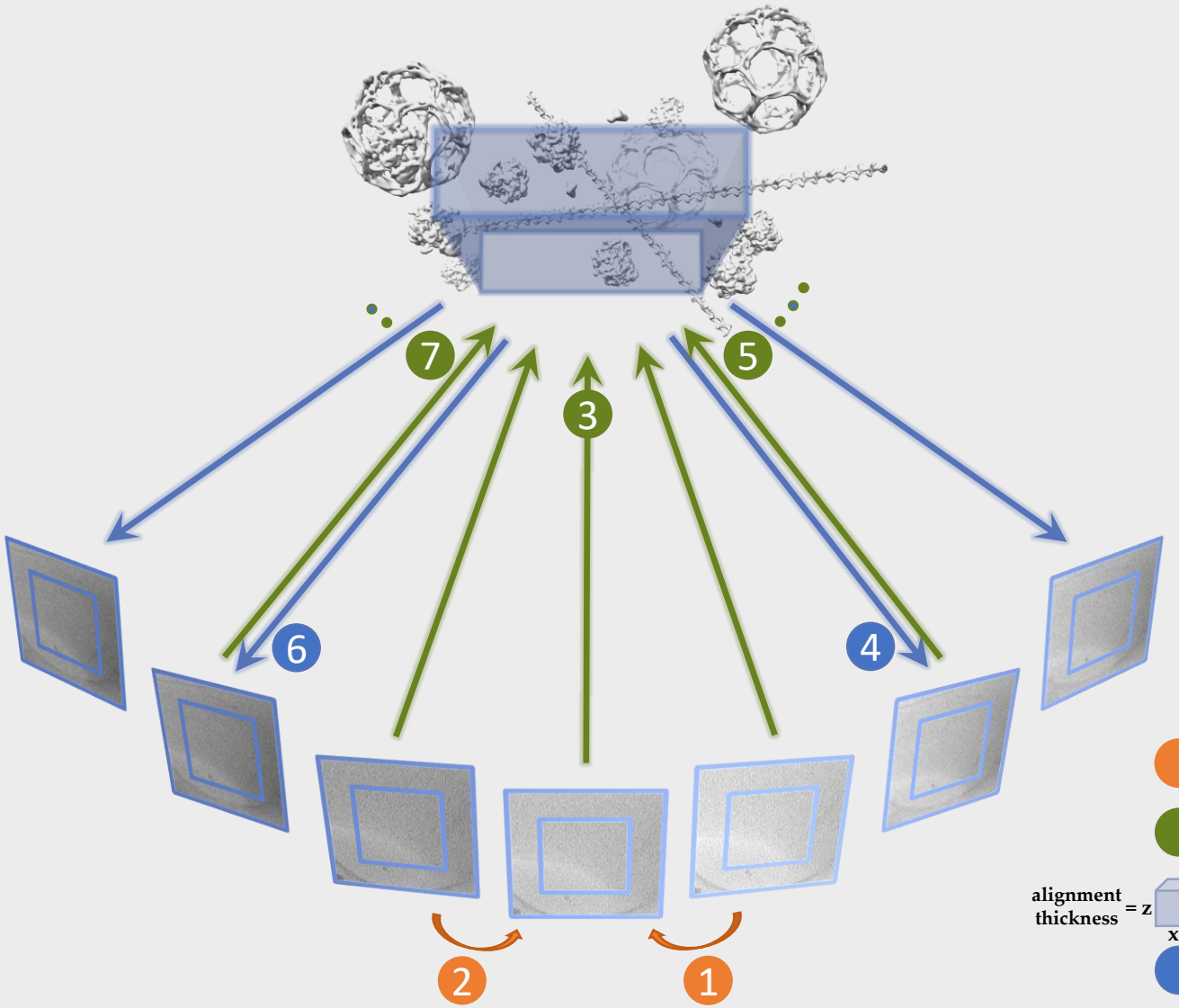
alignment thickness = z

x y

Volume to be re-projected

Re-projection ⟶ correlation

Protomo alignment



Nearest-neighbor correlation

Weighted back-projection

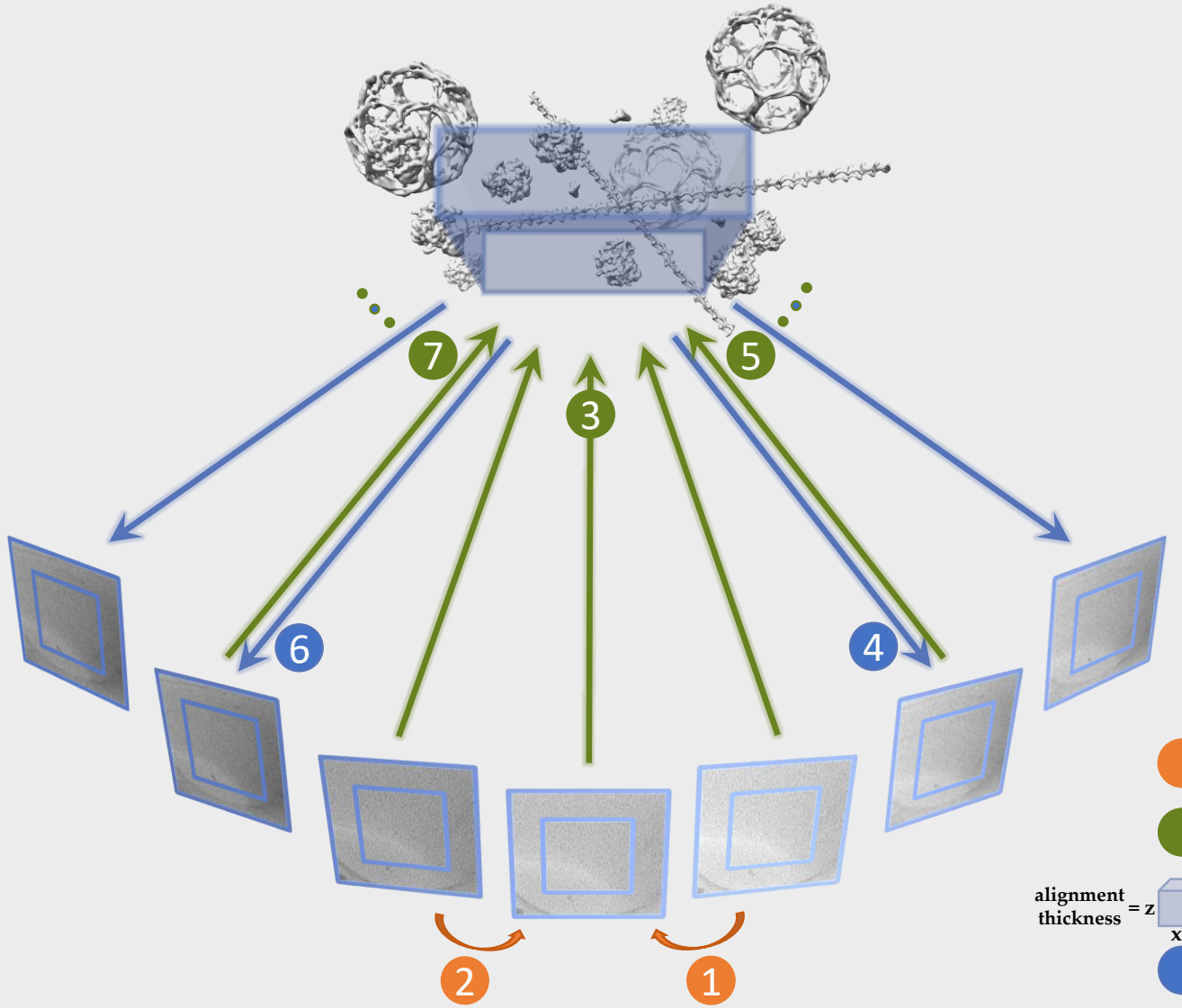
alignment thickness = z

x y

Volume to be re-projected

Re-projection ⟶ correlation

Protomo alignment



Nearest-neighbor correlation

Weighted back-projection

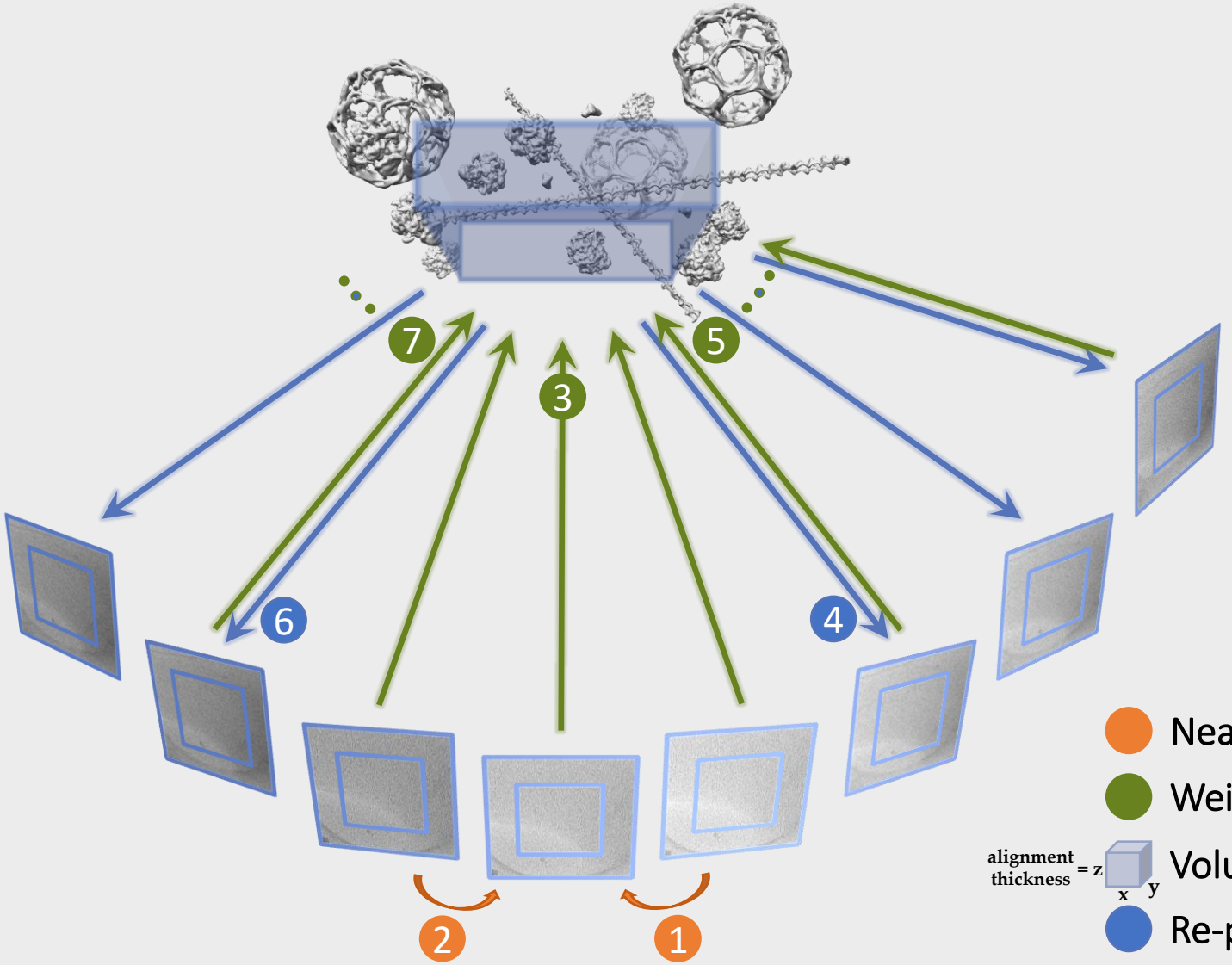
alignment thickness = z

x y

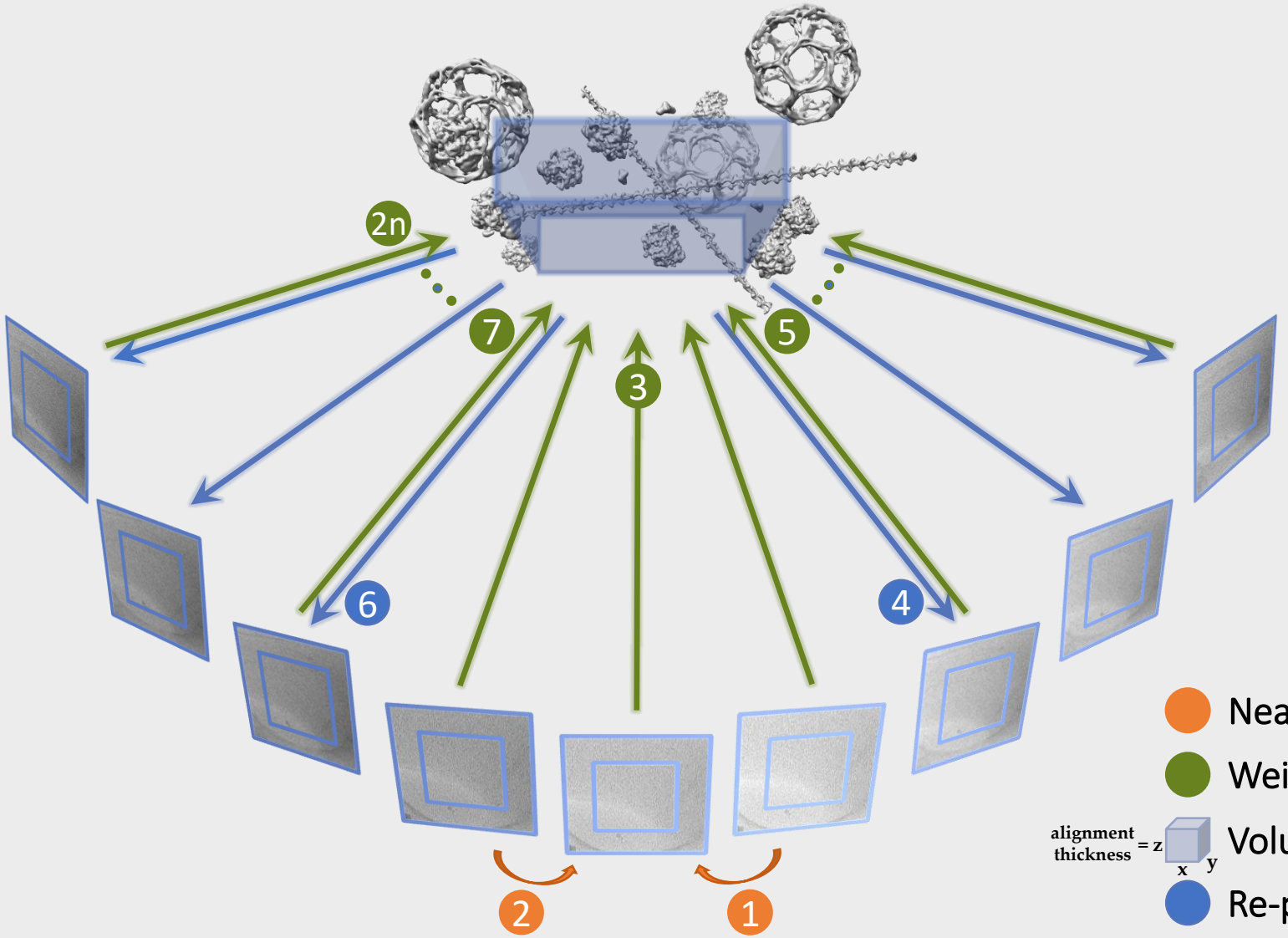
Volume to be re-projected

Re-projection ⟶ correlation

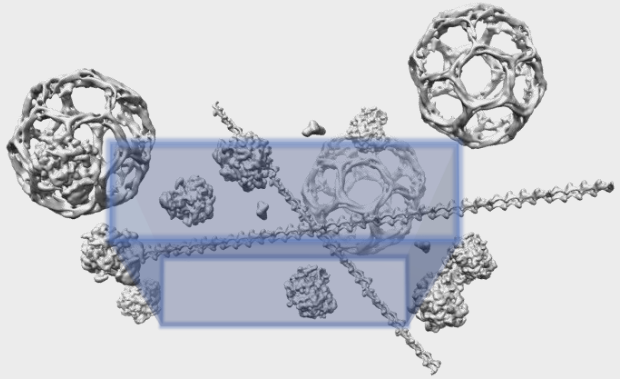
Protomo alignment

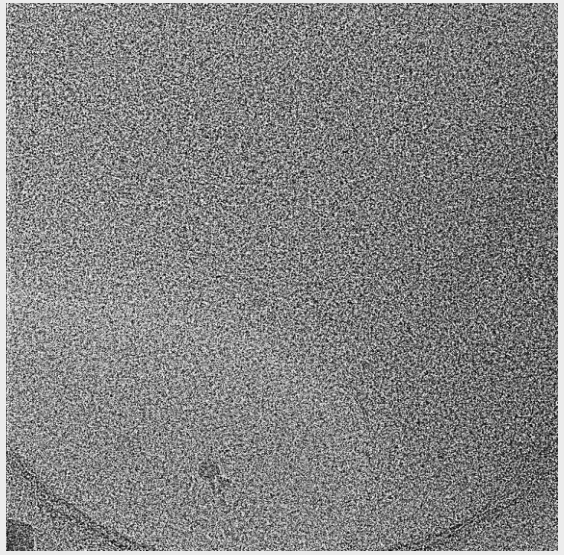


Protomo alignment

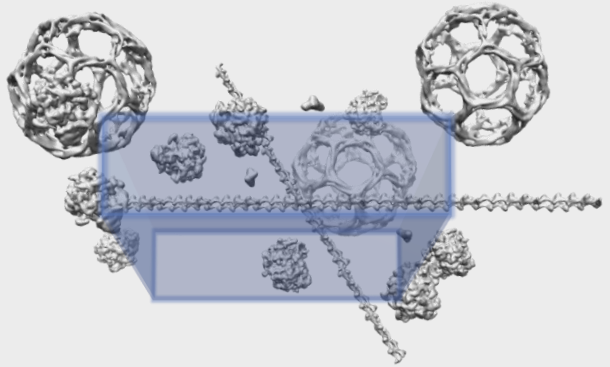


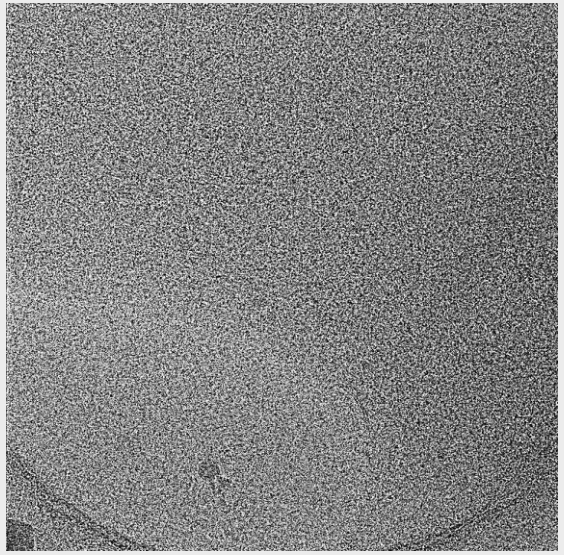
Protomo alignment





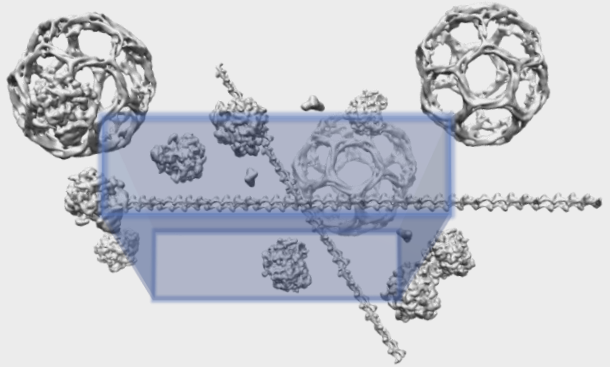
Protomo alignment

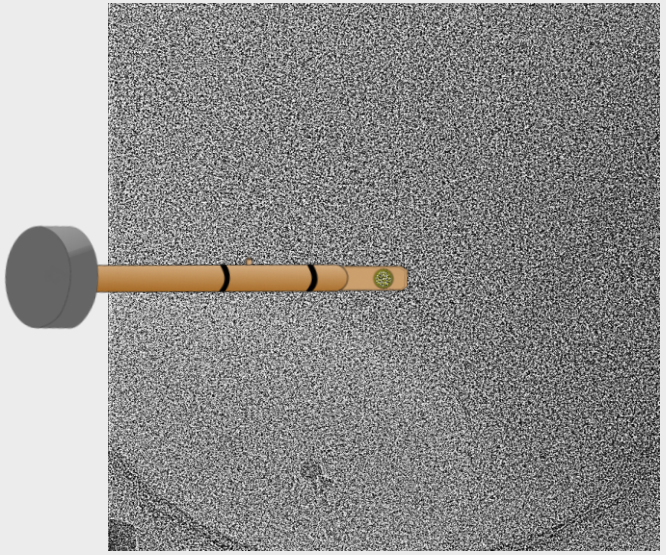




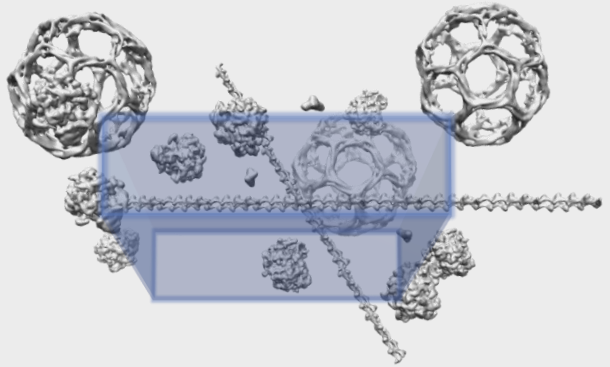
Refine orientations of objects

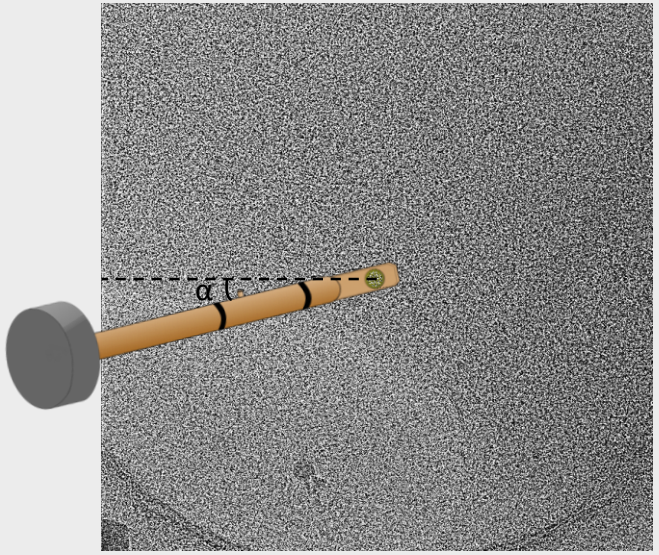
Protomo alignment





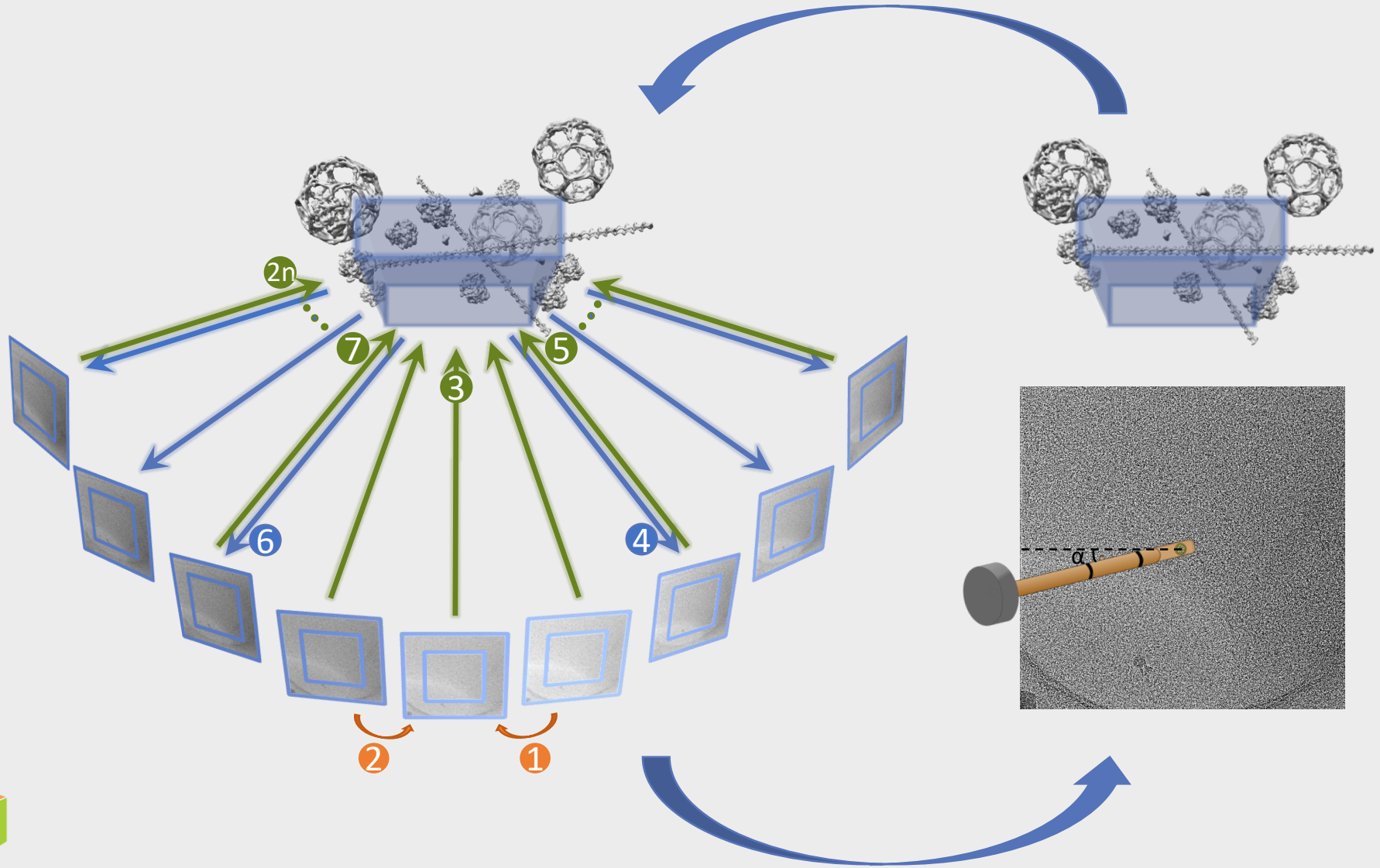
Protomo alignment





Refine tilt azimuth

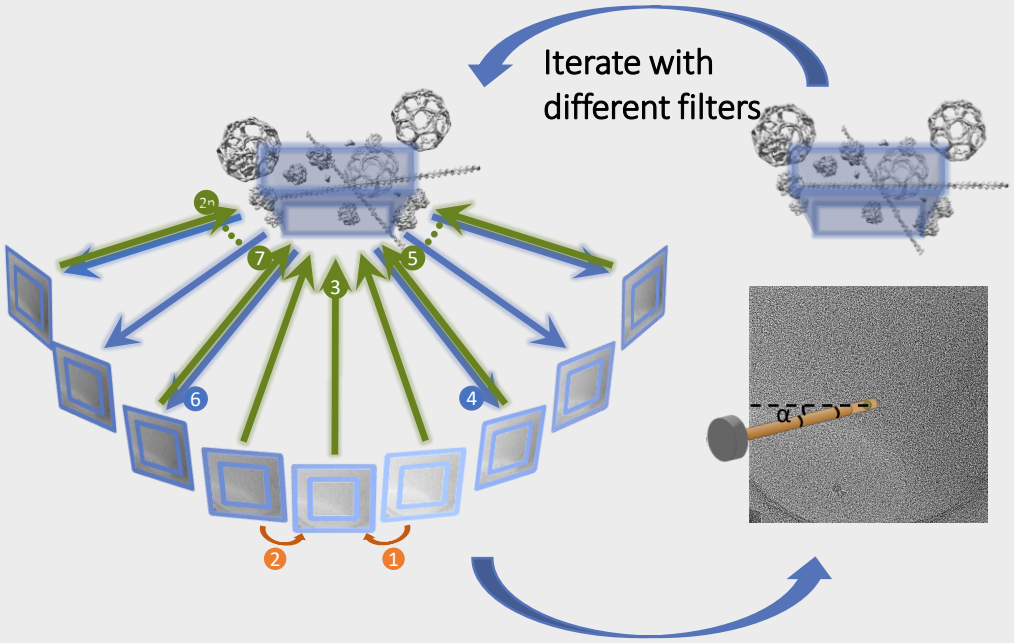
Appion-Protomo refinement



Iterate with different filters

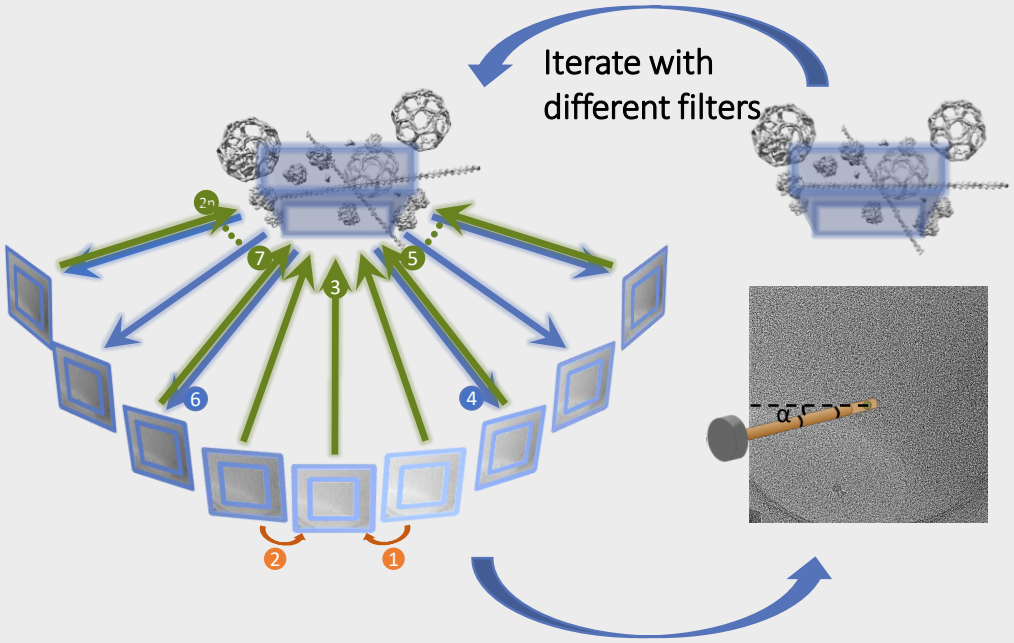
Appion-Protomo refinement

Why is this important?

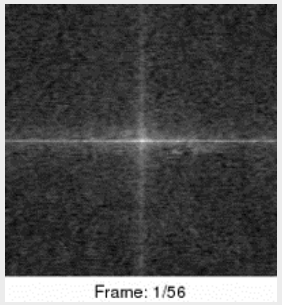


Appion-Protomo refinement

Why is this important?

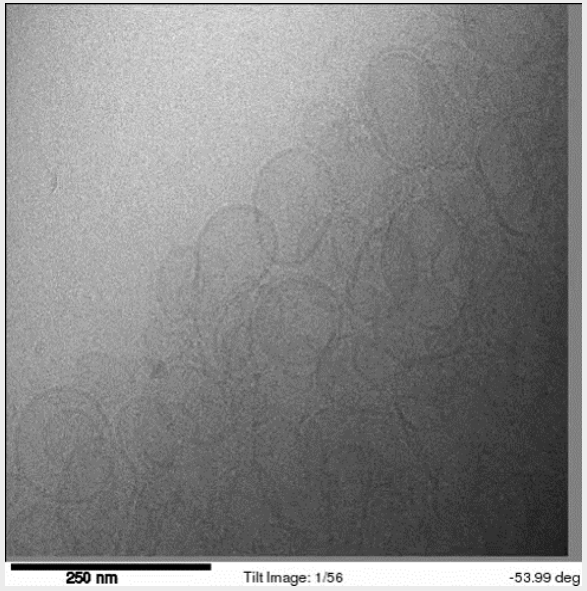


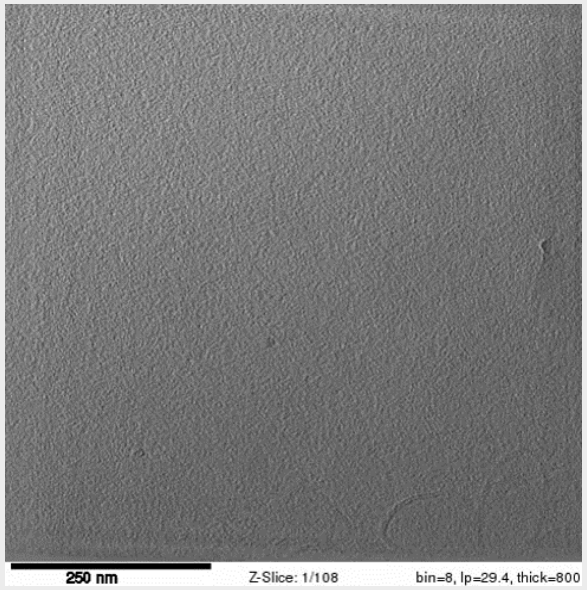
Nearest-neighbor alignment



After refinement

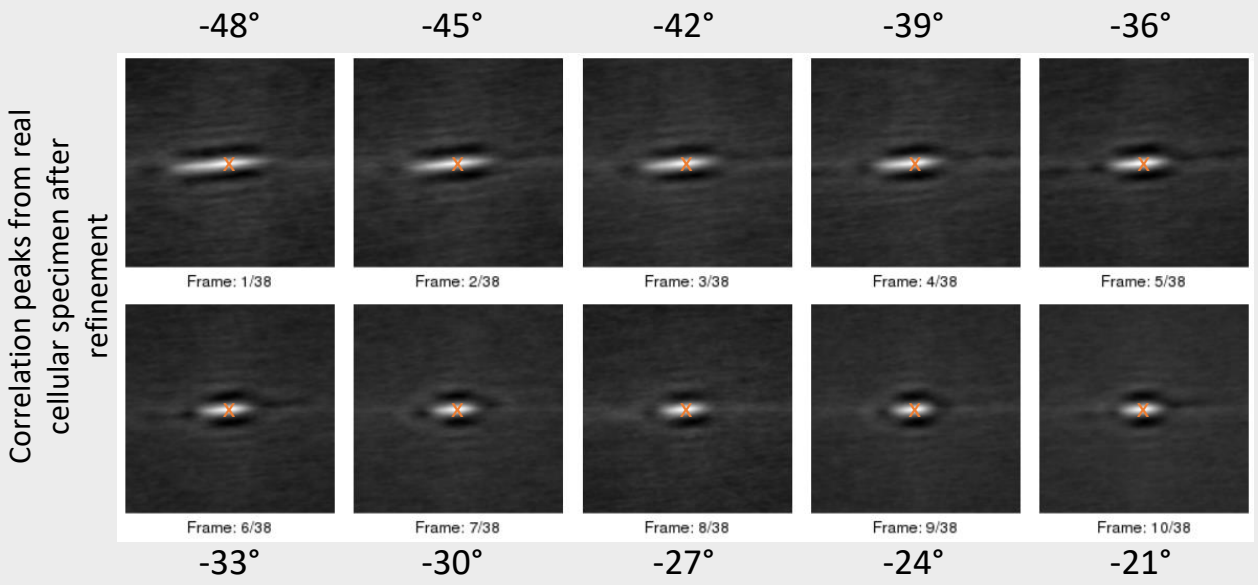
After refinement



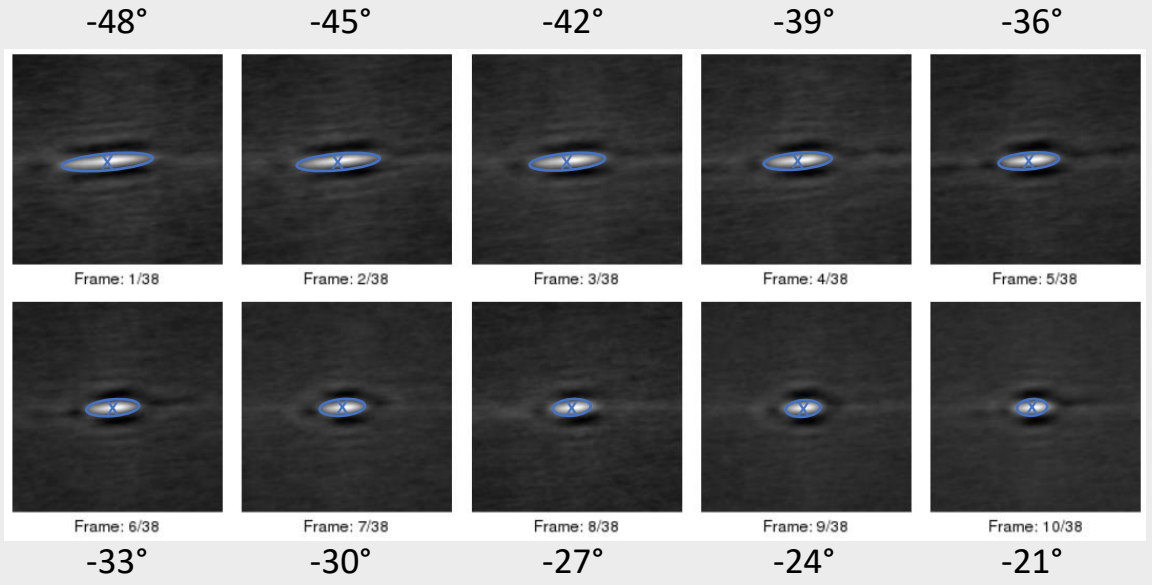


Protomo alignment of thick specimen has one potential issue

Systematic misalignment of images can stretch objects. *This is fixable!*

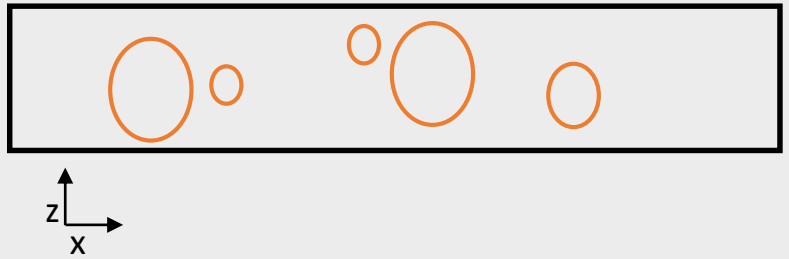


x = correlation peak using the brightest pixel

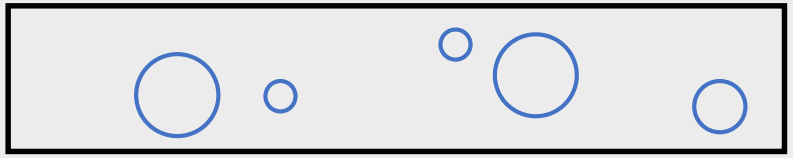


x = correlation peak using the center of the stretched peak

Side-view of incorrectly-aligned specimen



Side-view of correctly-aligned specimen

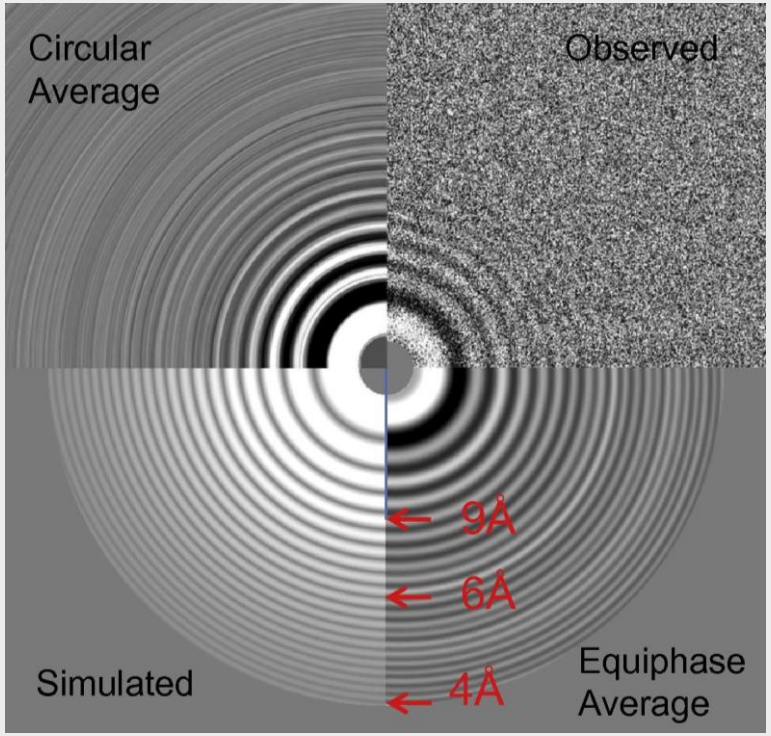


Defocus estimation

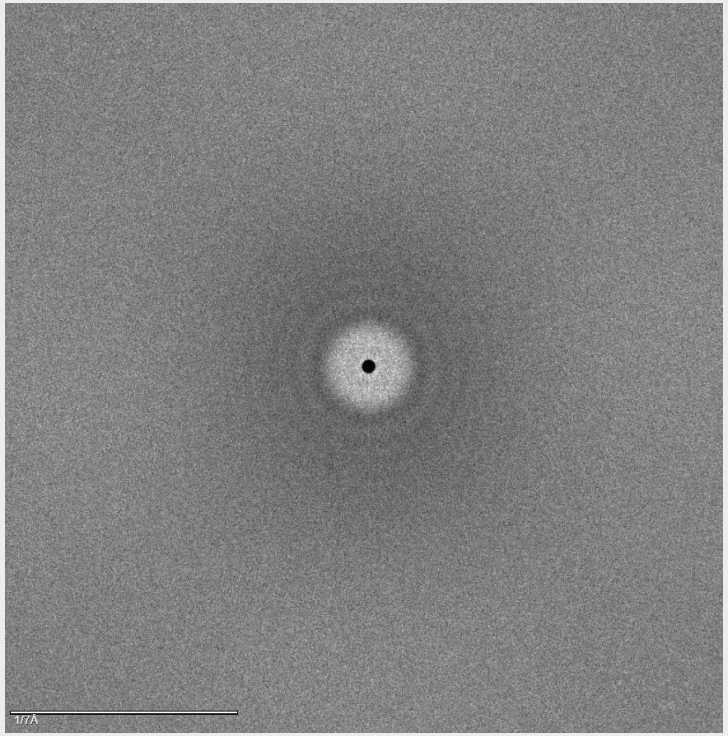
Goal: Find the **height of your objects** of interest to correct for microscope aberrations (CTF)

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

Zhang, 2016



High dose single particle image



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

Defocus estimation methods

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

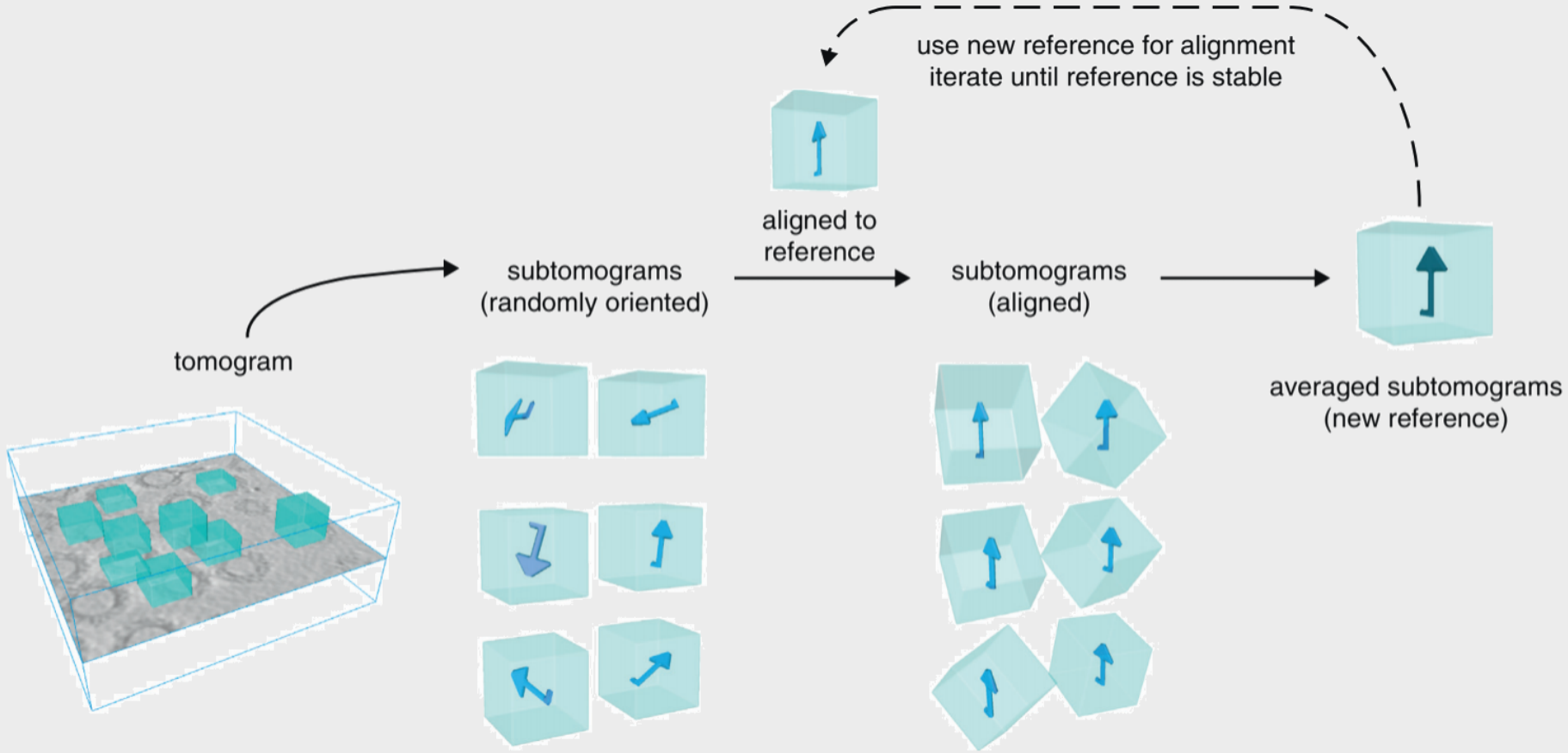
- **Per-image** defocus estimation accounting for tilts (CTFFIND4, GCTF, etc.)
- Per-tomogram post-hoc estimation by using **SPT FSC to locate the first CTF zero**
- **Image tiling** to estimate the **defocus of the untilted plane** (TomoCTF)
- Defocus estimation and **interpolation using two focus locations** 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)
- Per-particle tilt image refinement (emClarity)

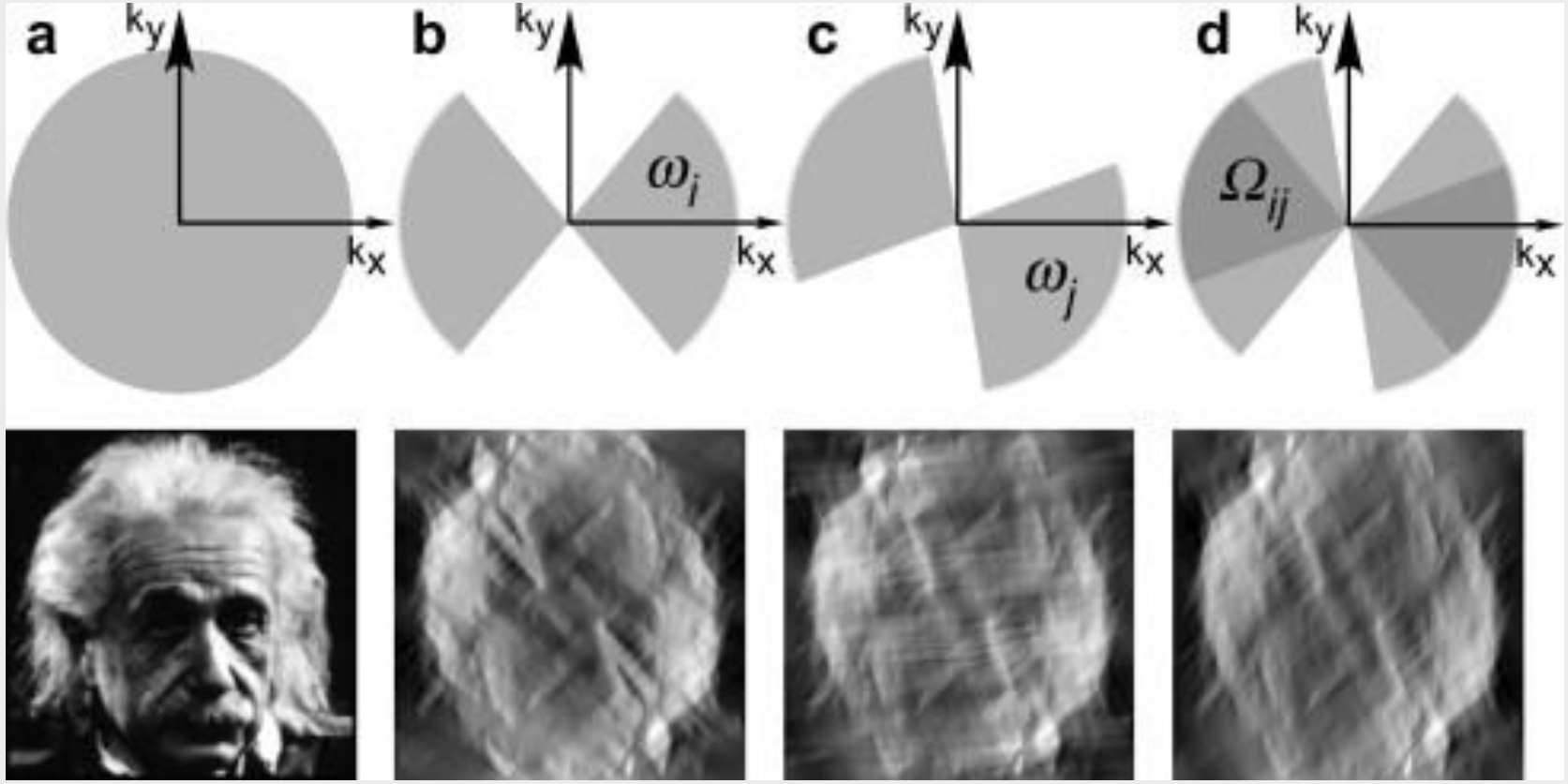
Sub-tomogram processing workflow



Briggs 2013

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

Must take into account subtomogram missing wedges



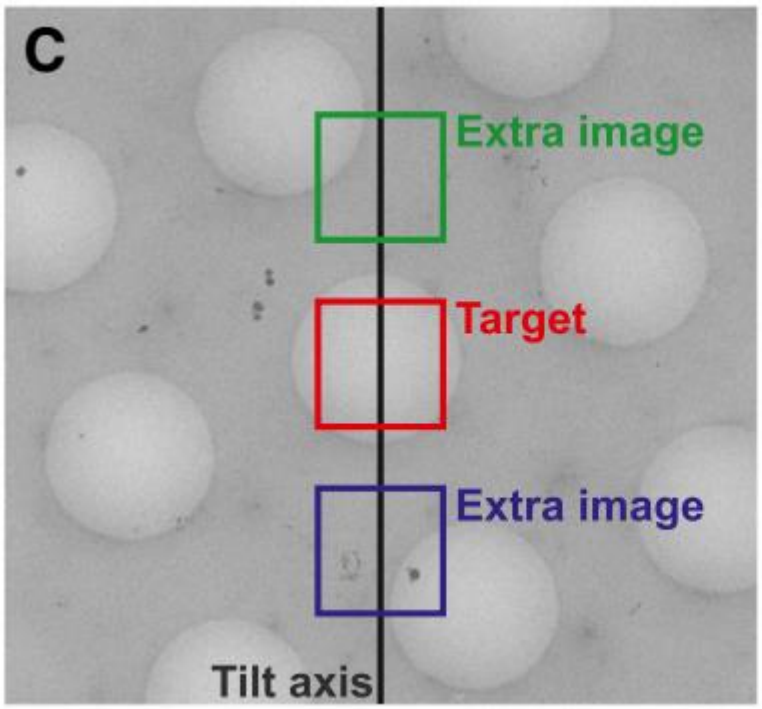
Forster et al, J. Struct. Biol, 2008

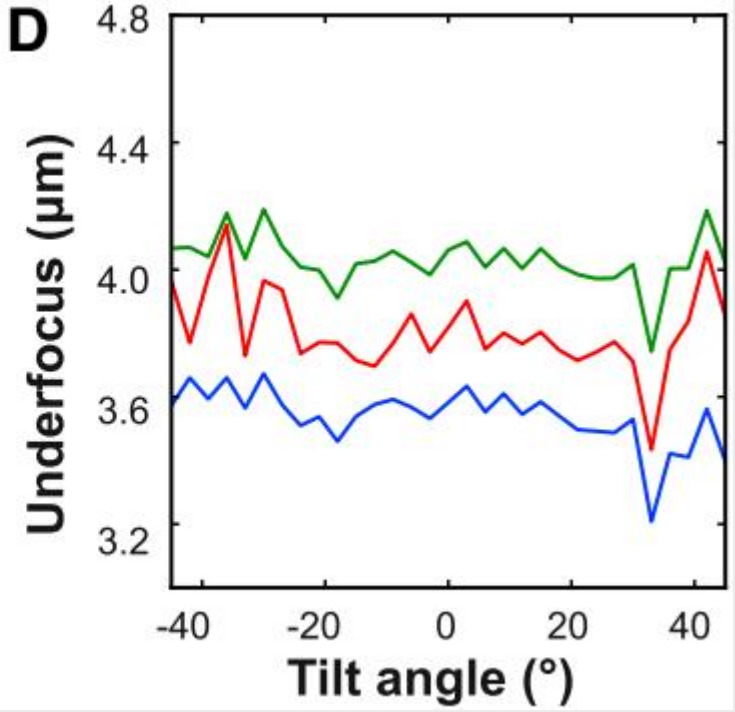
- Effectively align volume in common in Fourier space

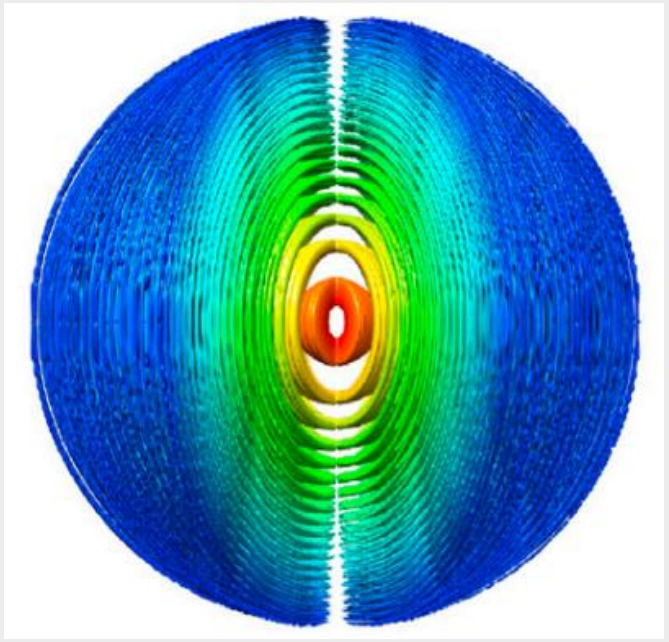
Sub-tomogram processing software

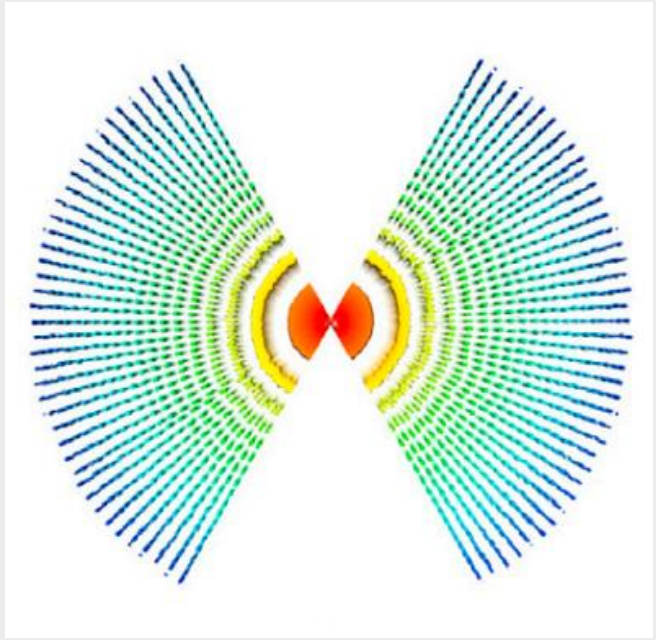
- Dynamo – GPU accelerated, tomogram database, extensive picking abilities
- Relion – 3D CTF model, Bayesian approach to alignment is used
- EMAN2 – Sub-tilt-series refinement and defocus estimation/correction
- emClarity – Sub-tilt-series refinement and defocus estimation/correction
- TYGRESS – Intended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

Sub-tomogram processing in Relion





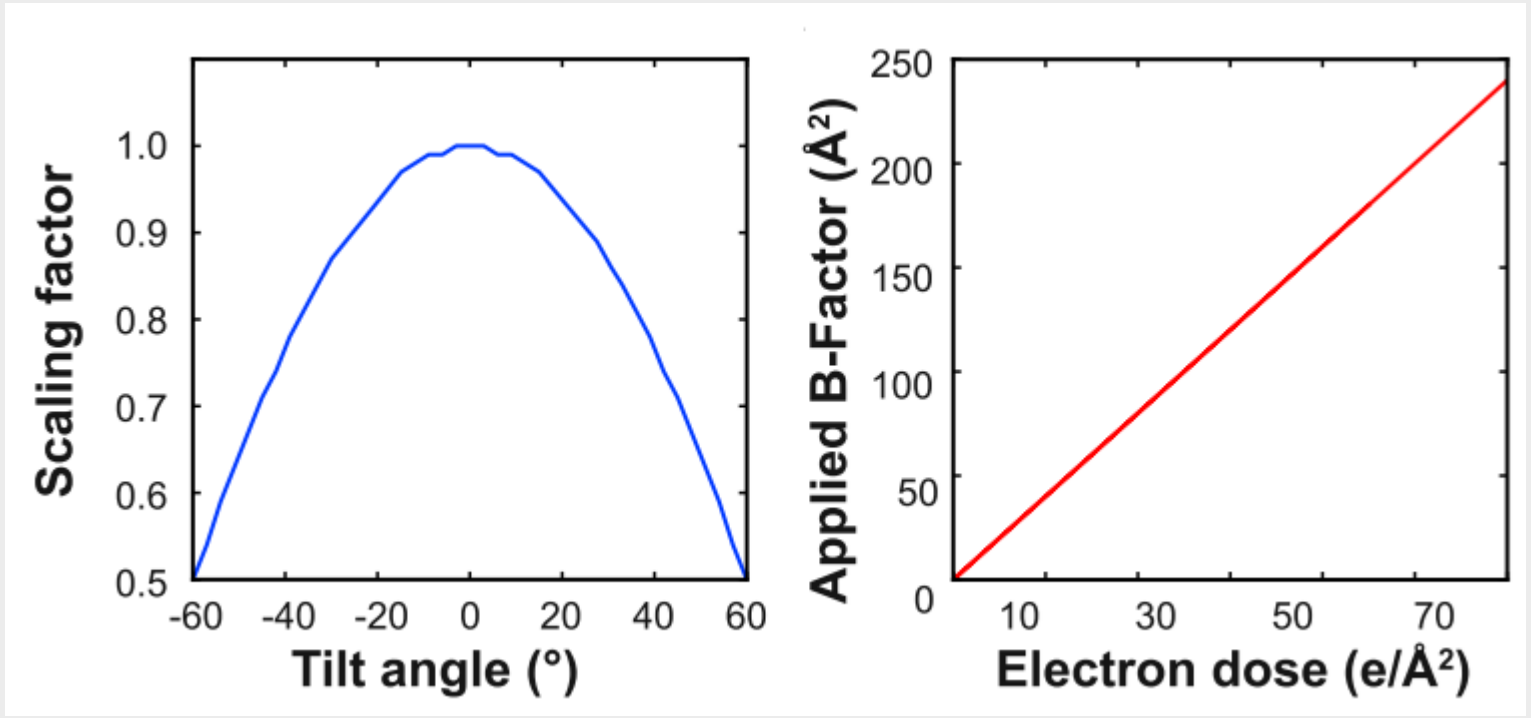


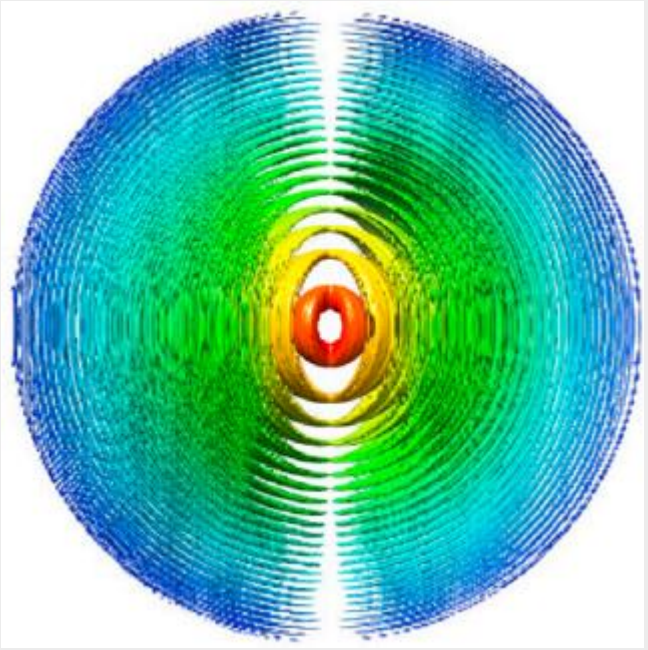


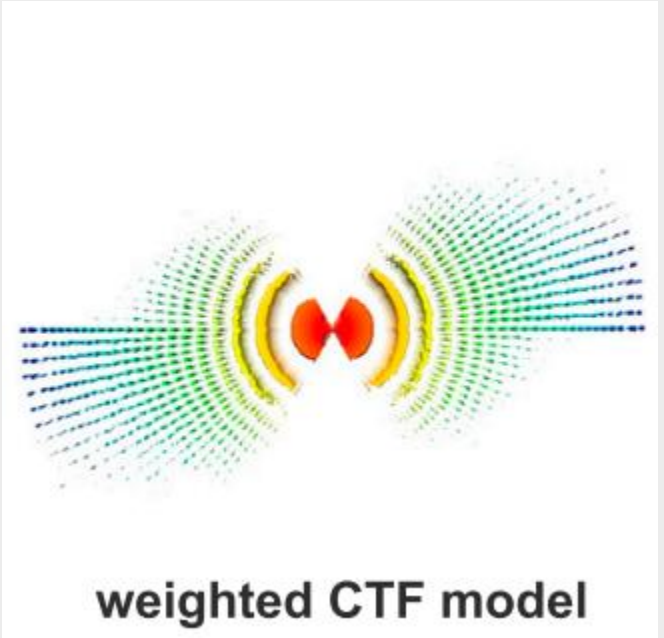
- Uses normal Relion workflow.
- Potential issues:
  - Extra images are likely not at the same focus as the Target
  - 3D FSC may eliminate properly interpolated values due to sampling

Bharat et. al., Structure 2015

Sub-tomogram processing in Relion

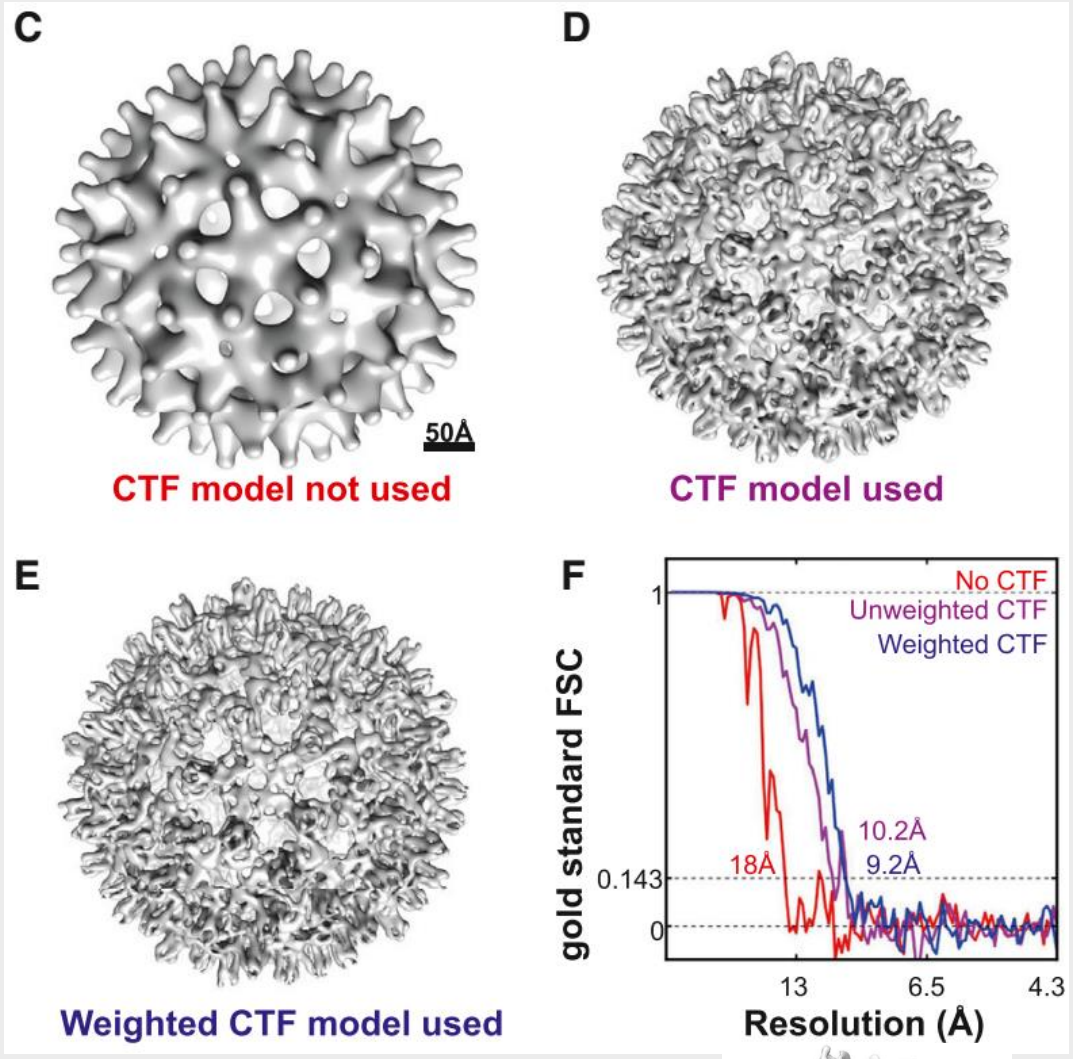


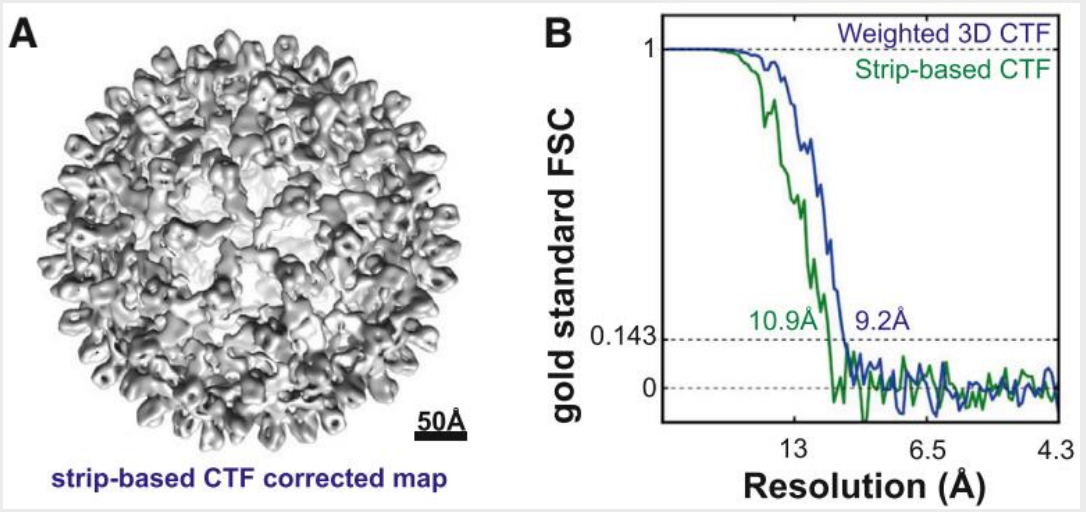


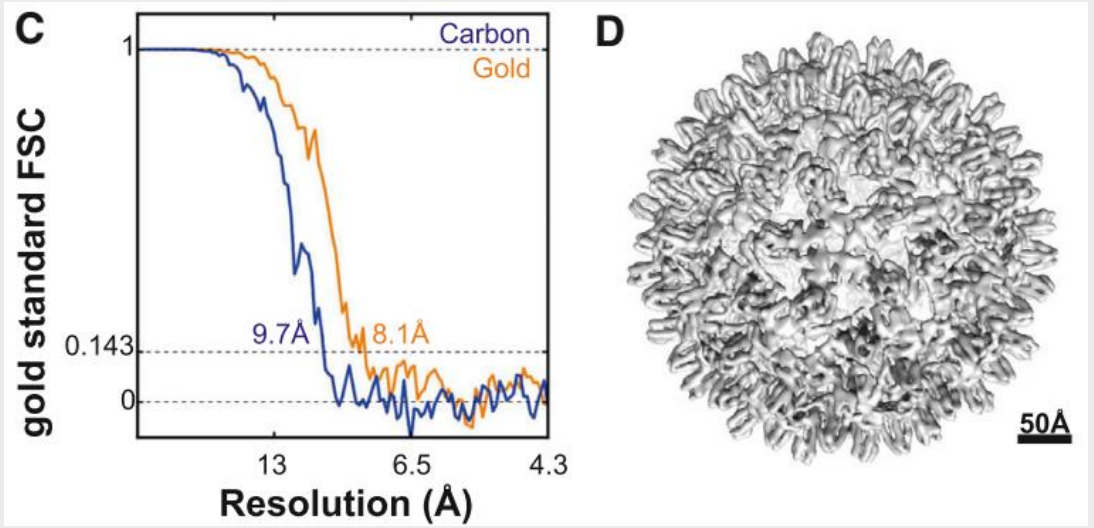


Bharat et. al., Structure 2015

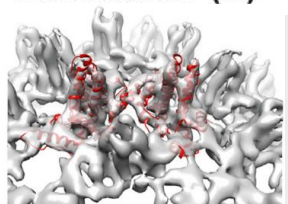
Sub-tomogram processing in Relion





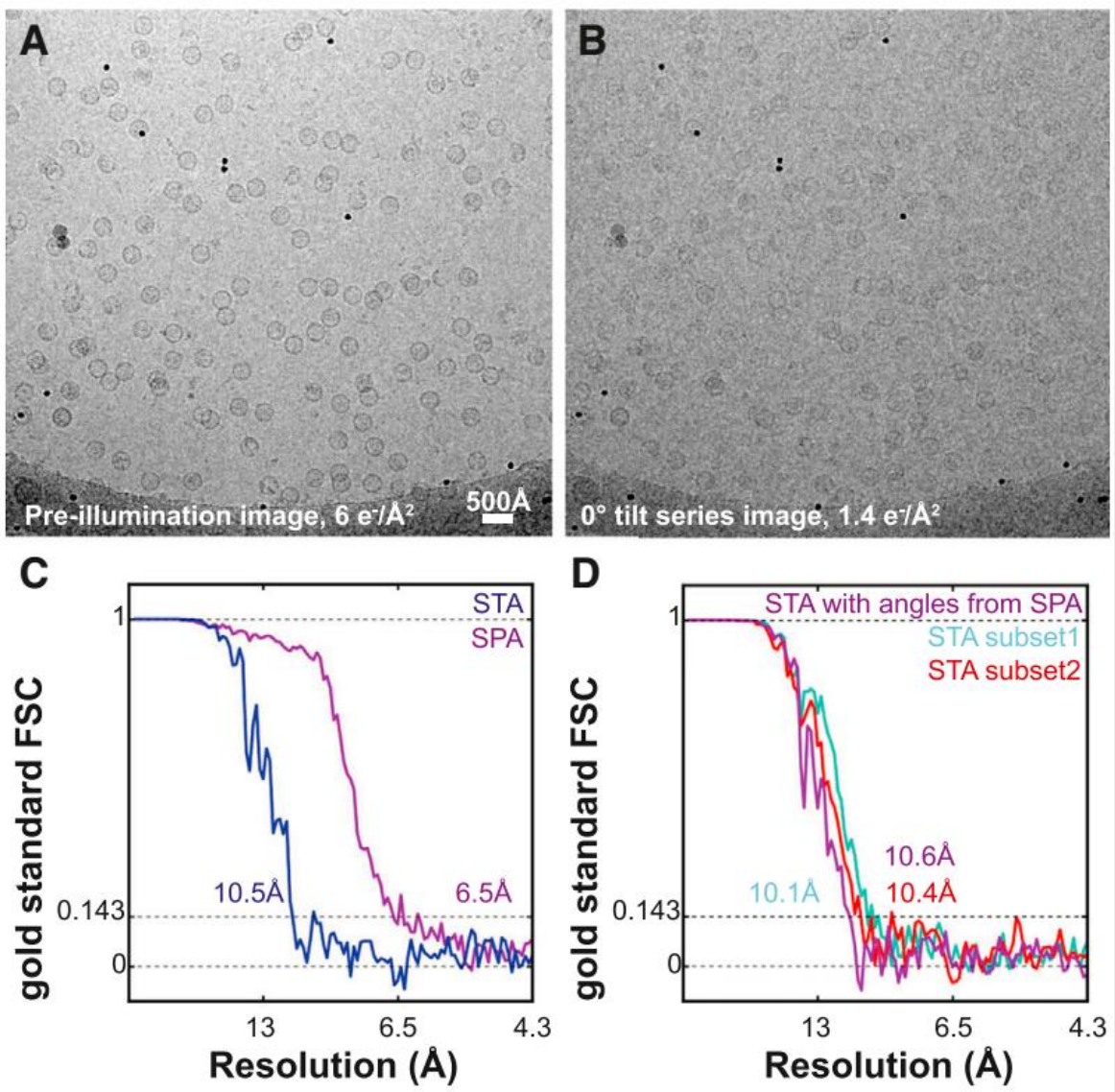


- Test case: Hepatitis B capsid



Bharat et. al., Structure 2015

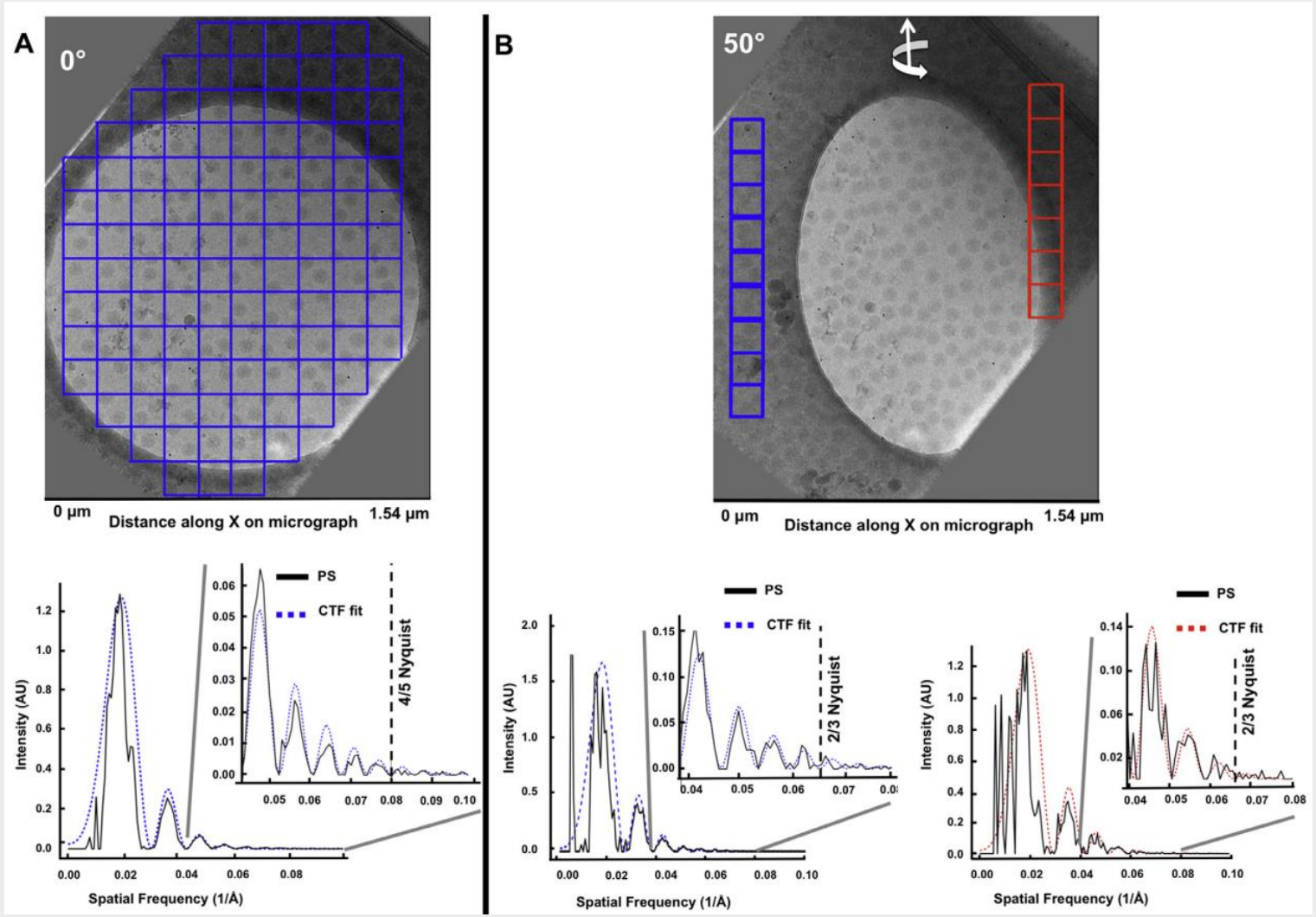
Sub-tomogram processing in Relion



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

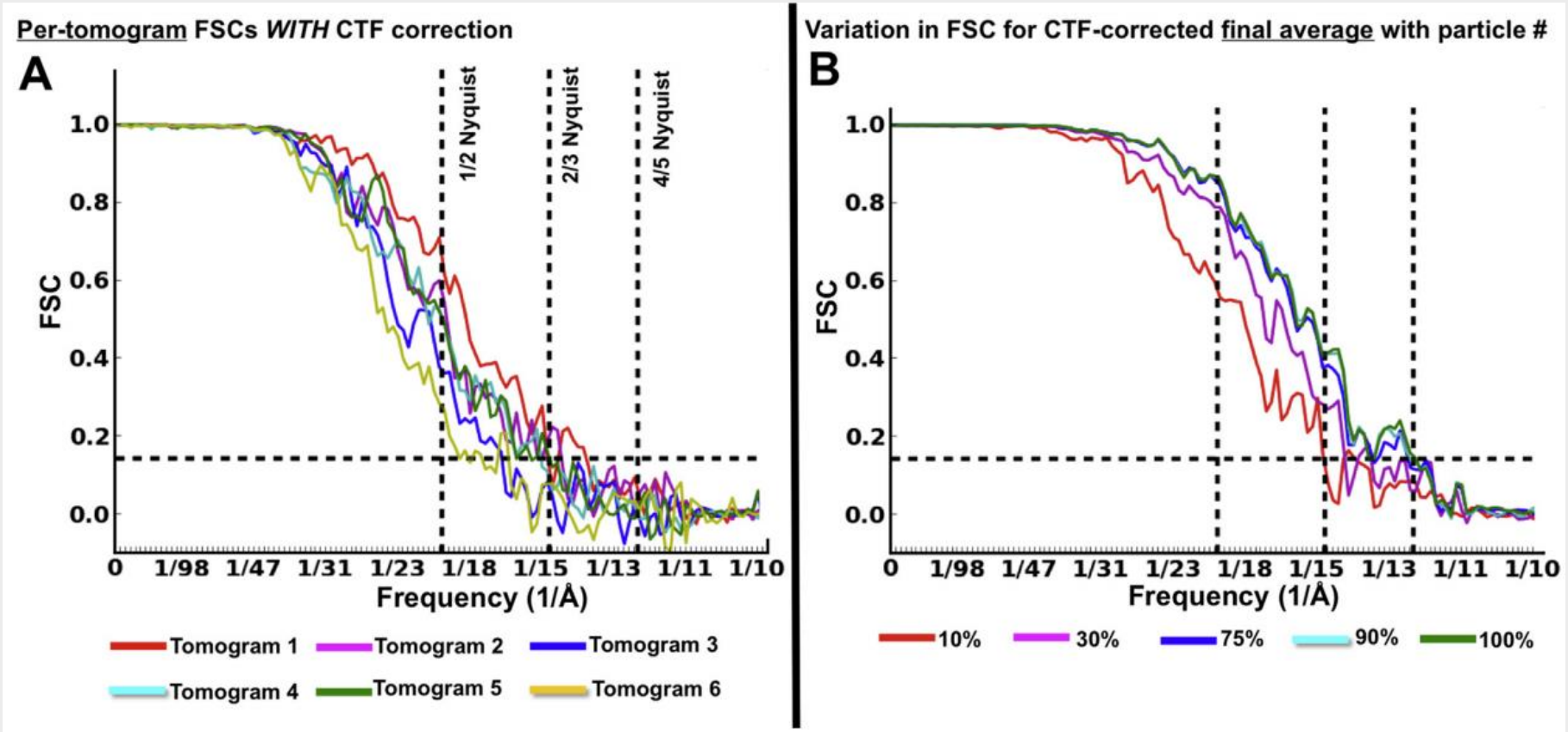
Bharat et. al., Structure 2015

Sub-tomogram processing in EMAN2



Galaz-Montoya, JSB 2016

Sub-tomogram processing in EMAN2



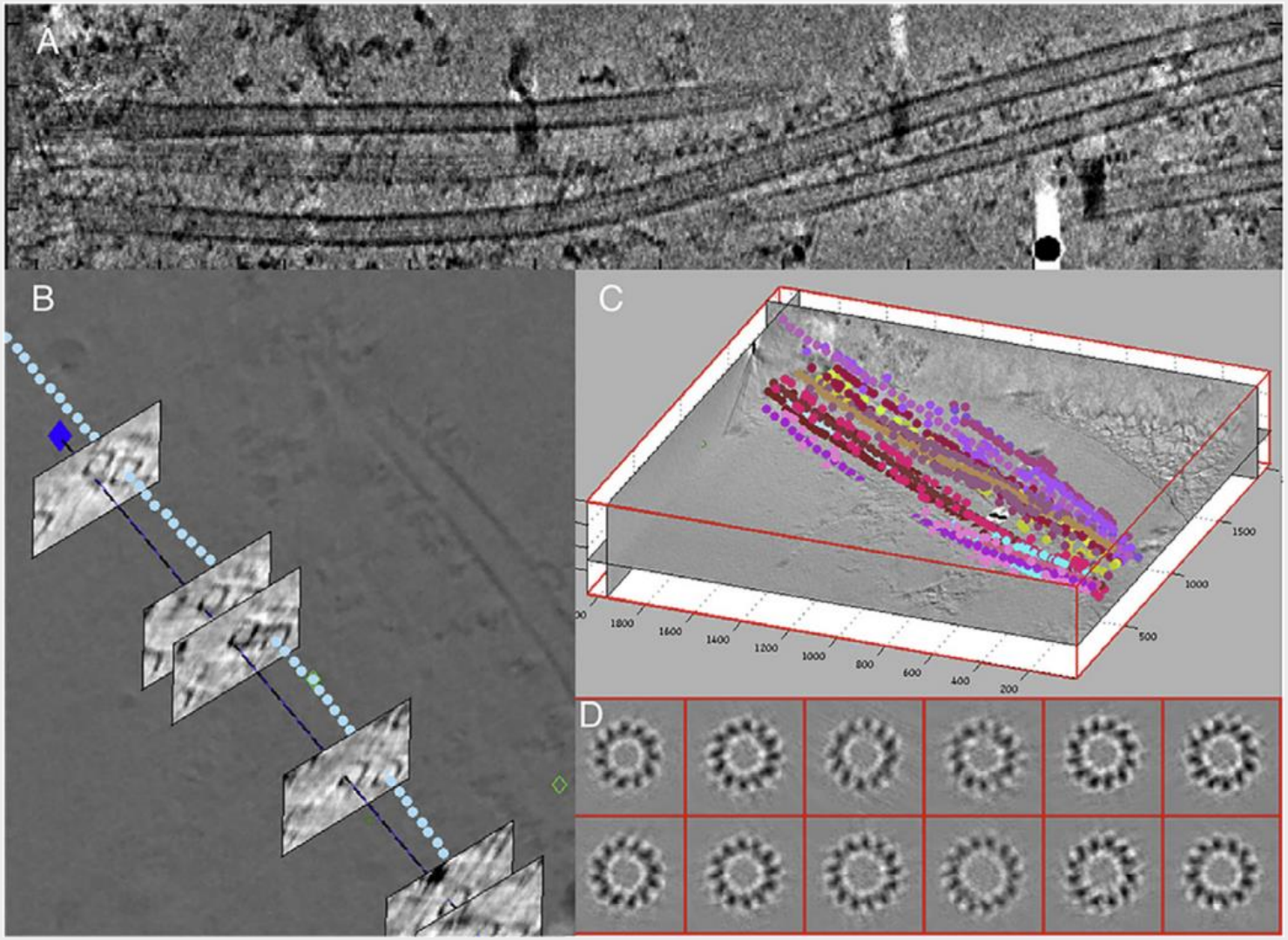
- Better than 2/3 Nyquist

Galaz-Montoya, JSB 2016

Tomogram/sub-tomogram annotation and segmentation software

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

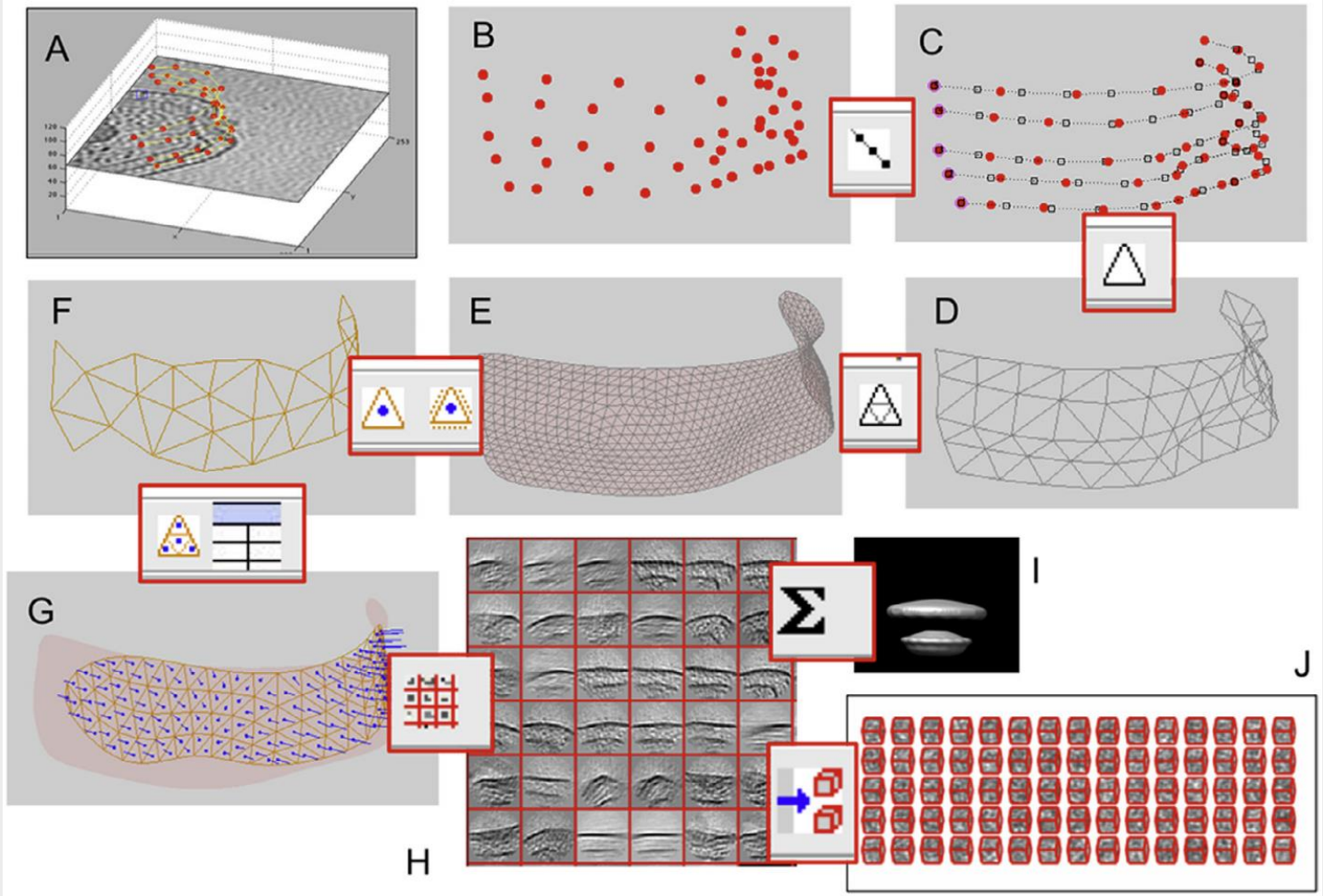
Sub-tomogram annotation processing in Dynamo



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

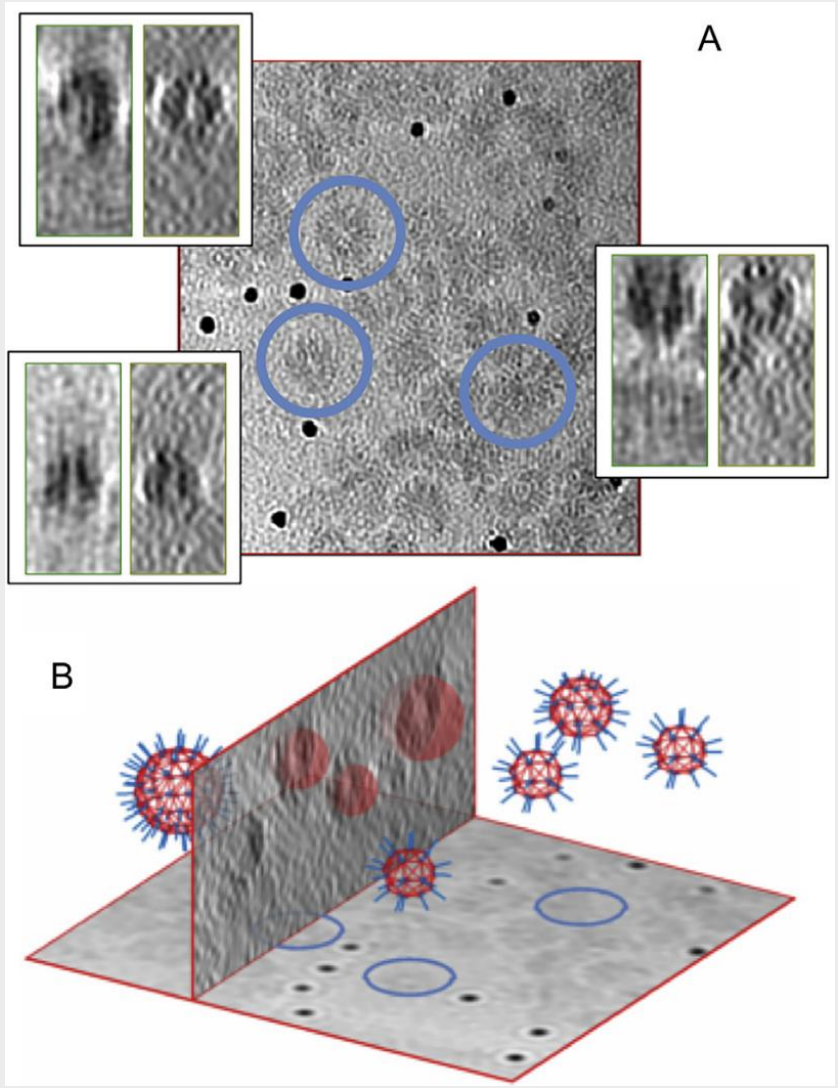
Castaño-Díez et. al., JSB 2012 & 2016

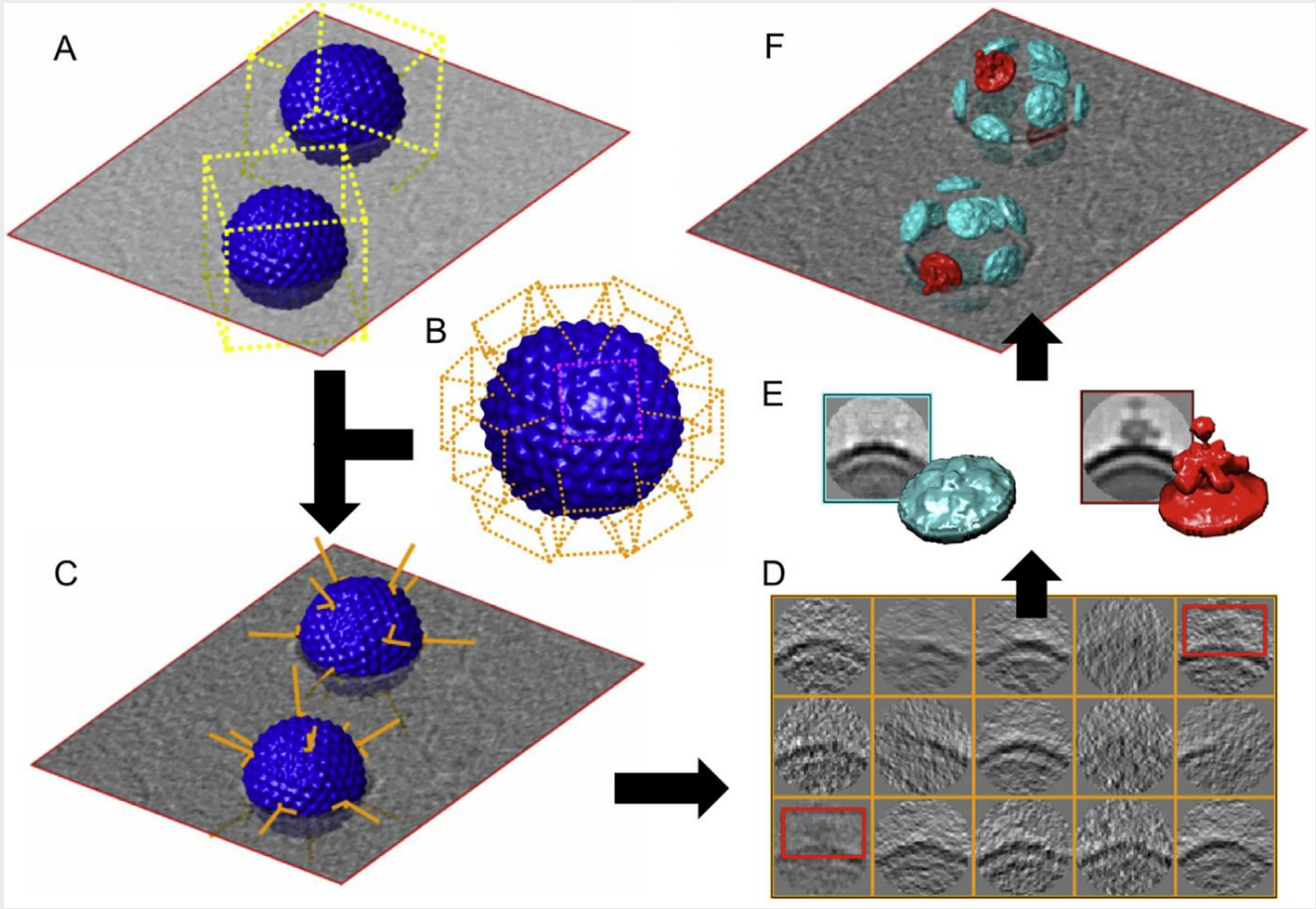
Sub-tomogram annotation processing in Dynamo



Castaño-Díez et. al., JSB 2012 & 2016

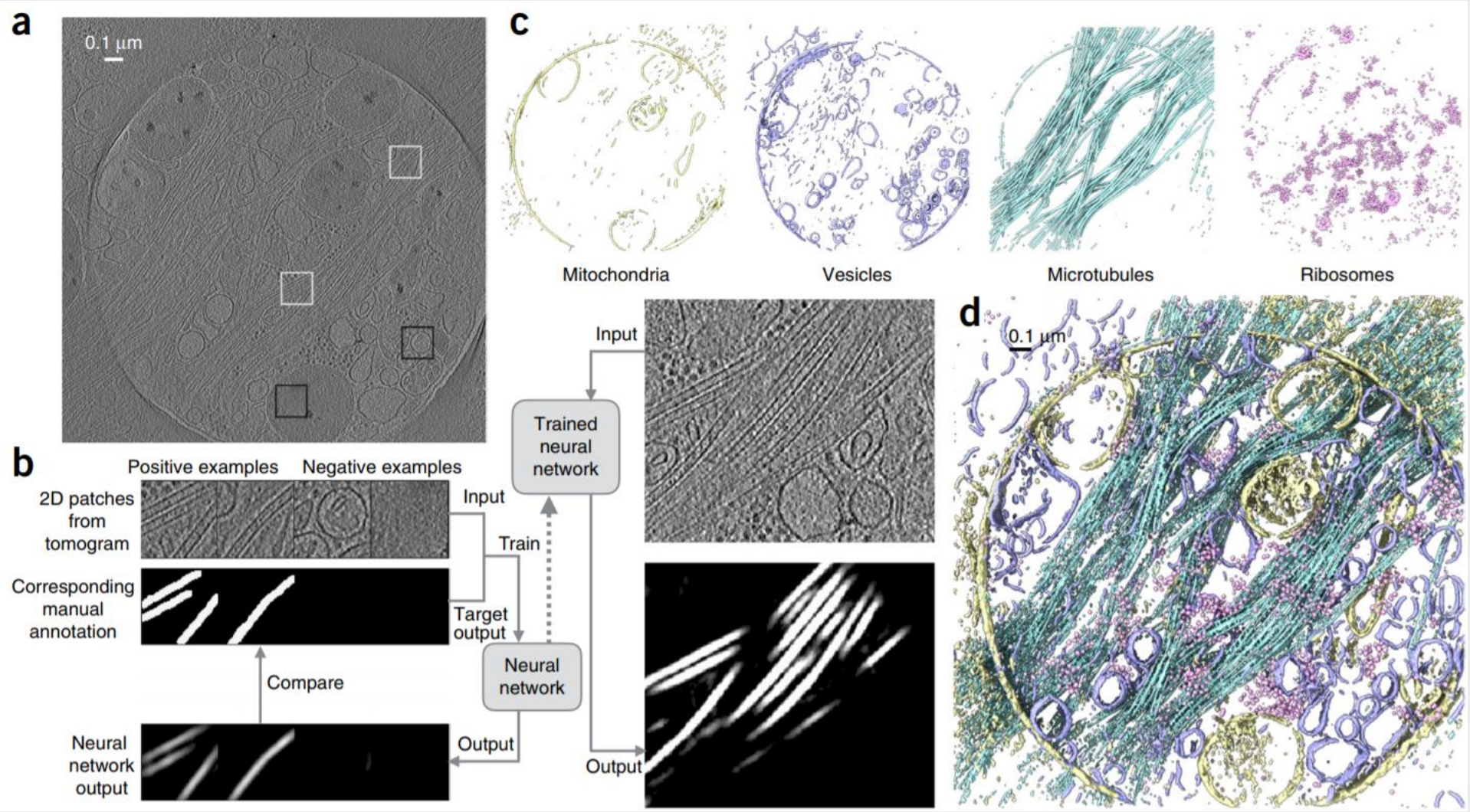
Sub-tomogram annotation processing in Dynamo





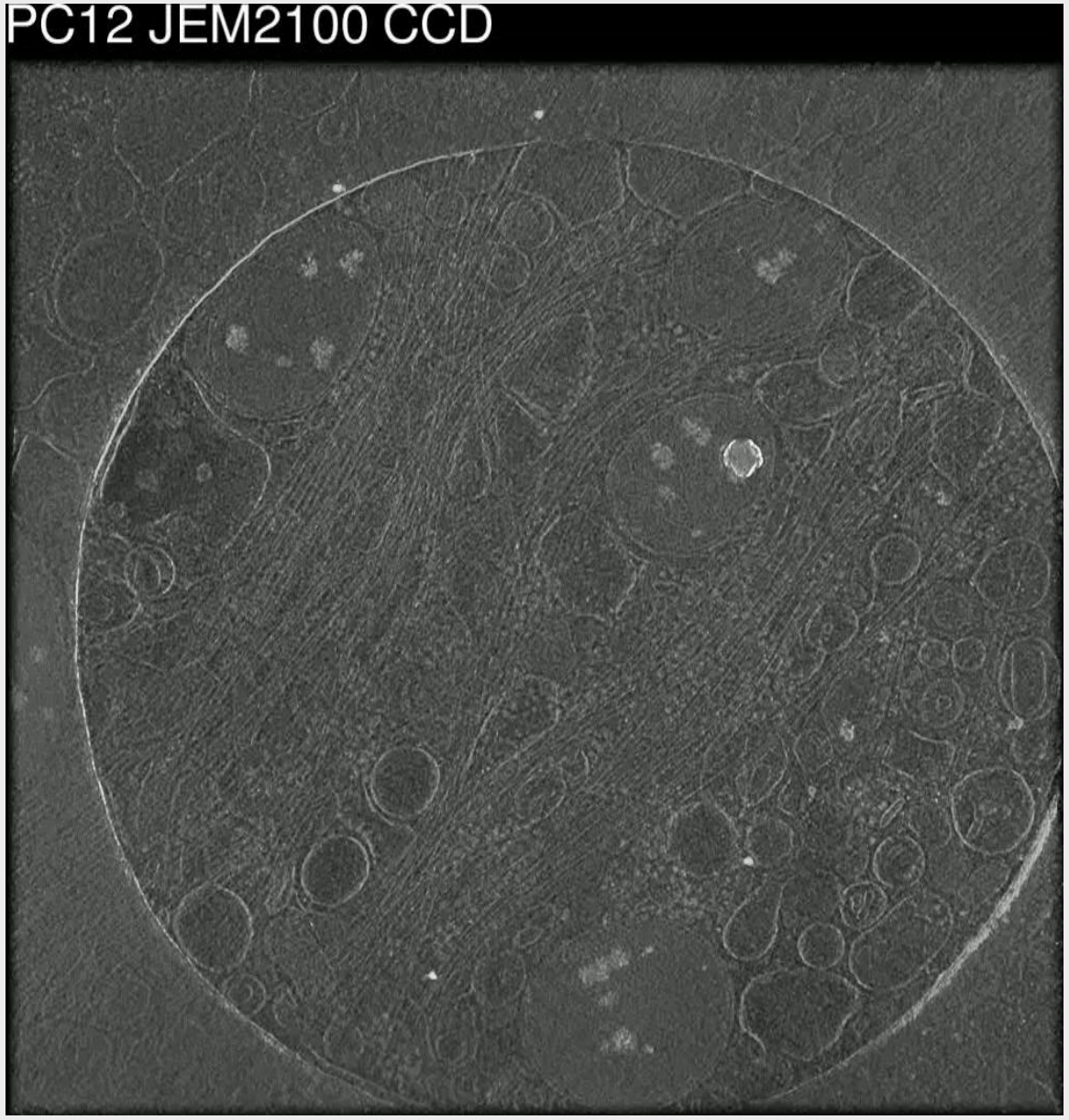
Castaño-Díez et. al., JSB 2012 & 2016

Sub-tomogram segmentation with CNNs in EMAN2



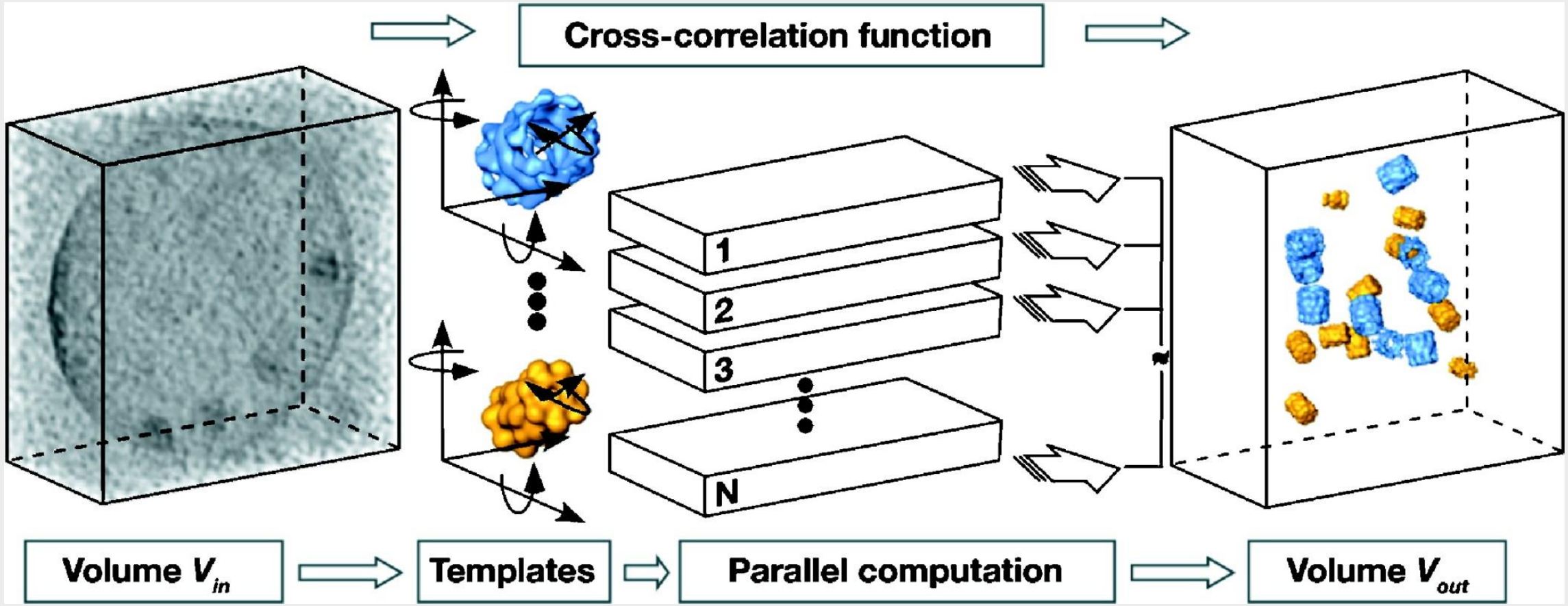
Chen et. al., Nat. Meth. 2017

Sub-tomogram segmentation with CNNs in EMAN2



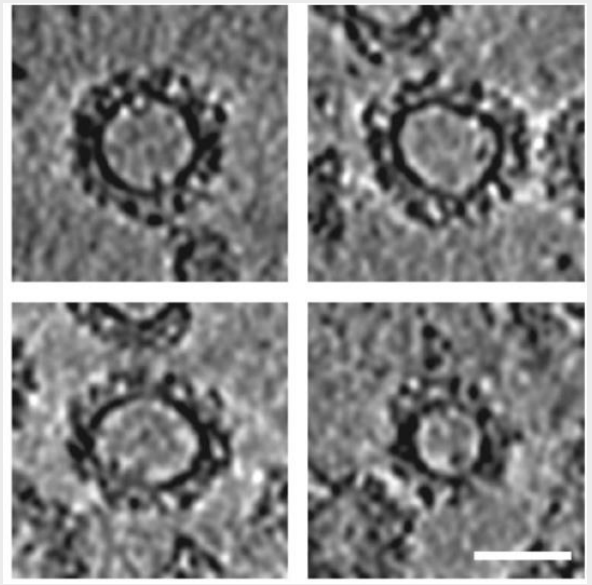
Chen et. al., Nat. Meth. 2017

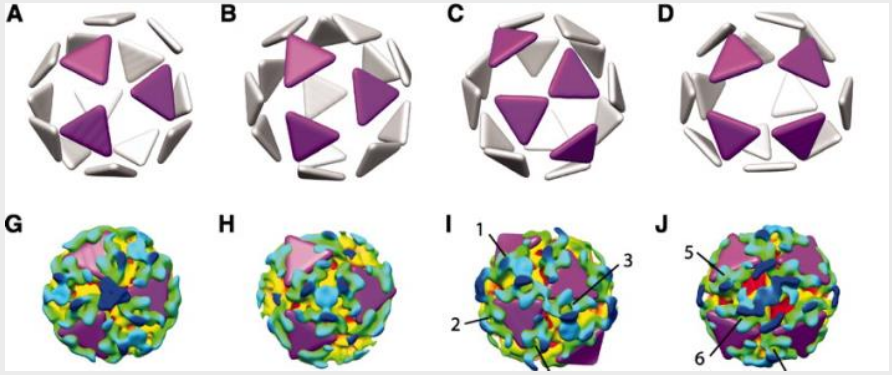
Template matching



Lucic et al, 2005, Annu. Rev. Biochem

Example: STA followed by placing averages to the tomograms

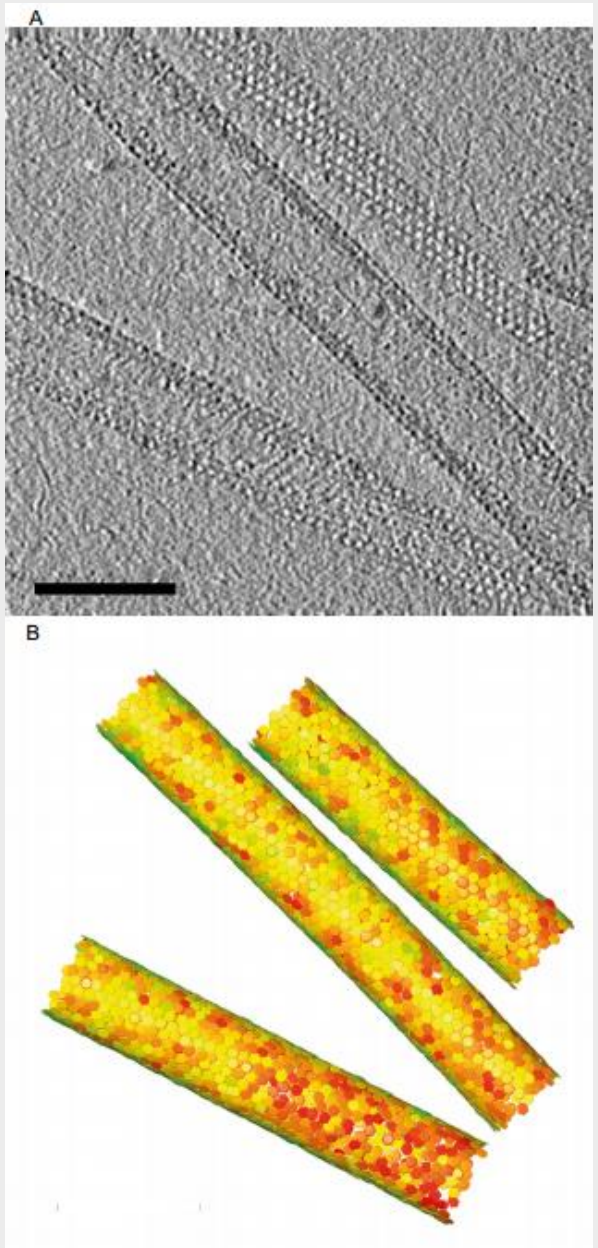


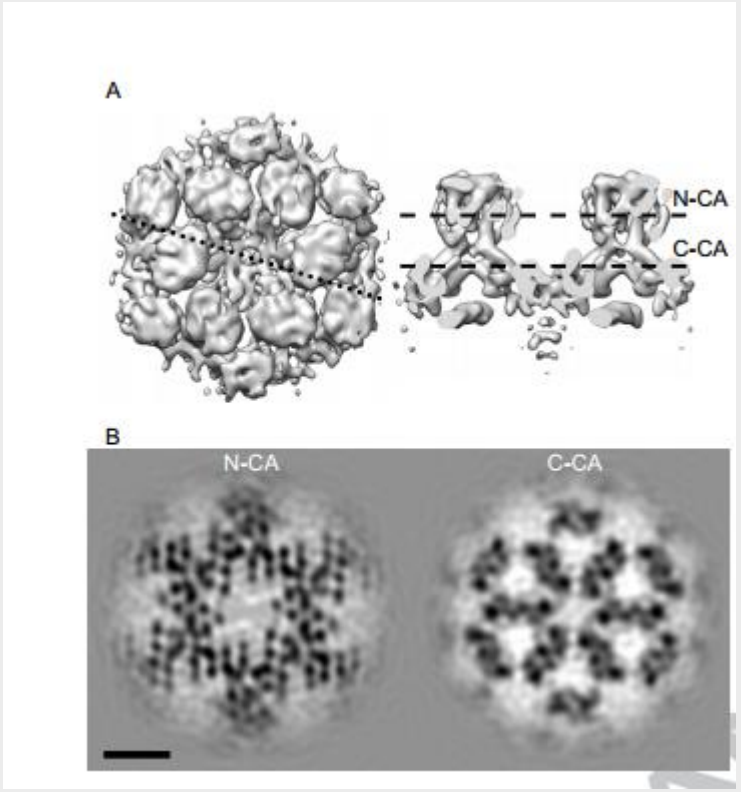


COP-I coated vesicles From: Faini et al, Science, 2012

Example: Mason-Pfizer monkey virus Gag protein

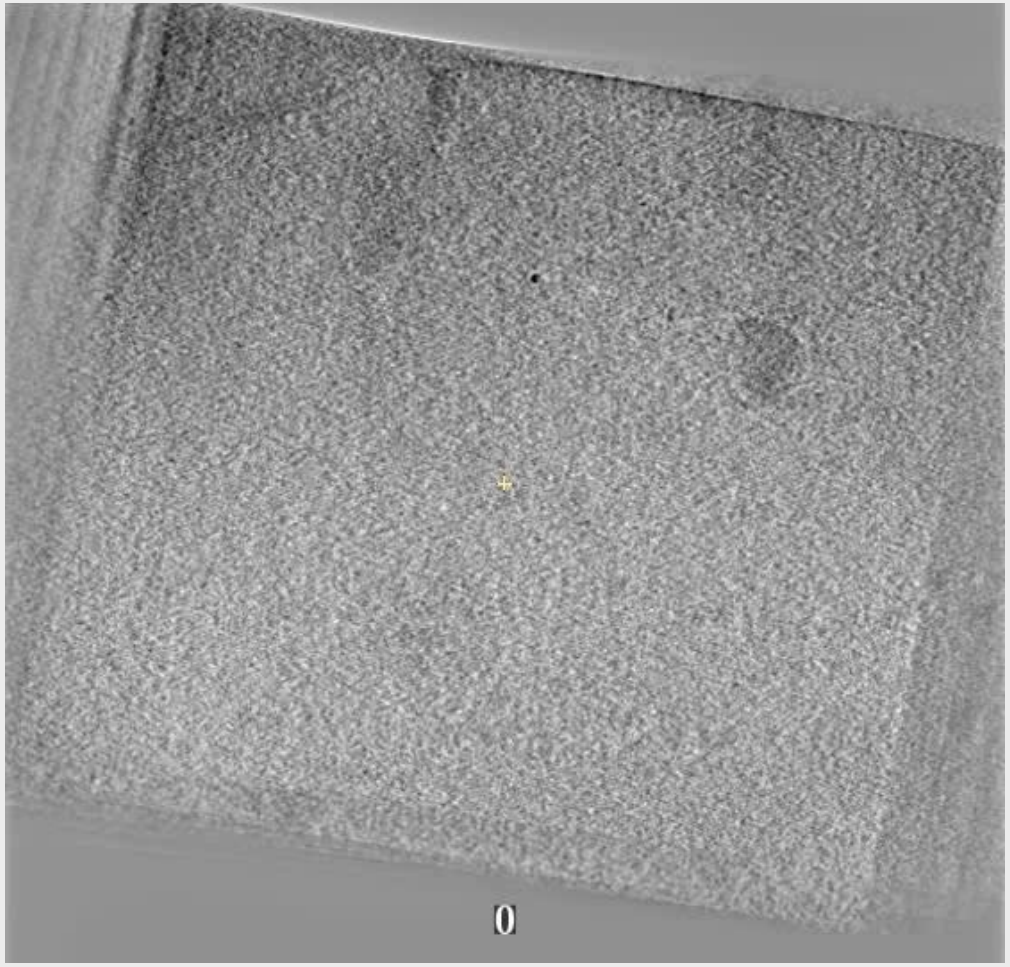
Over-picking to find repeating units



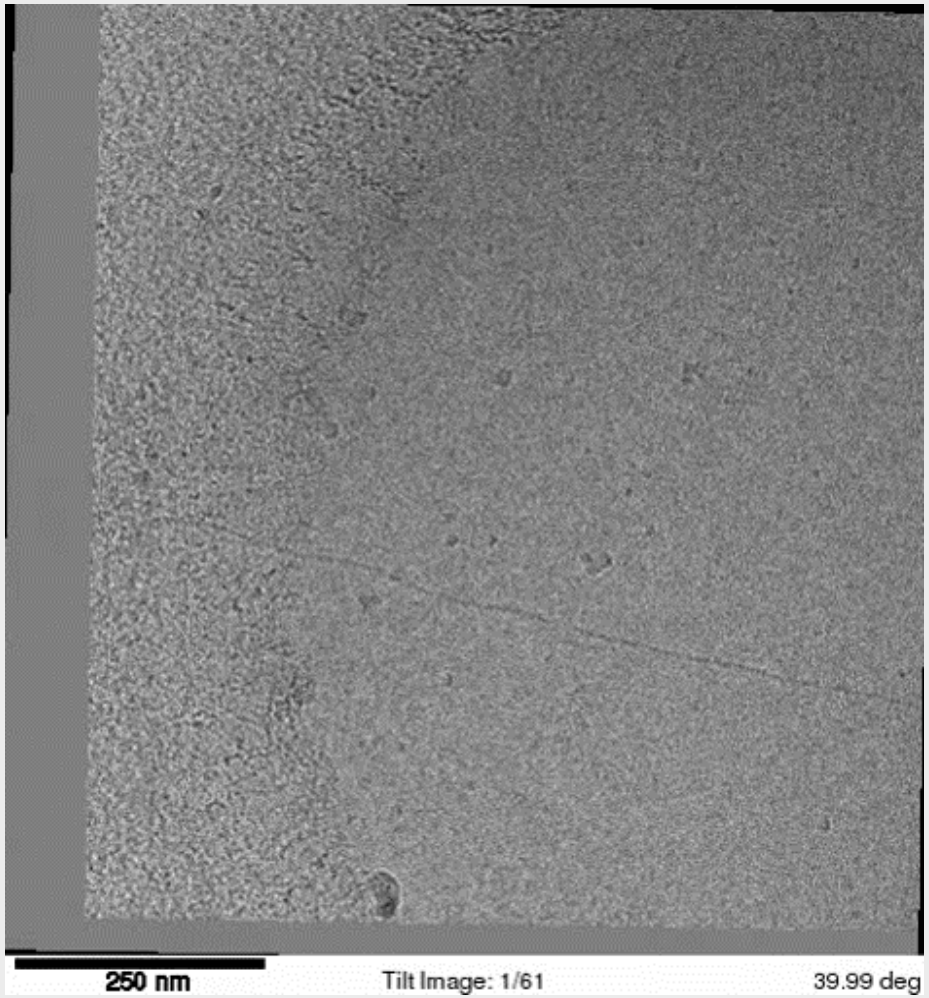


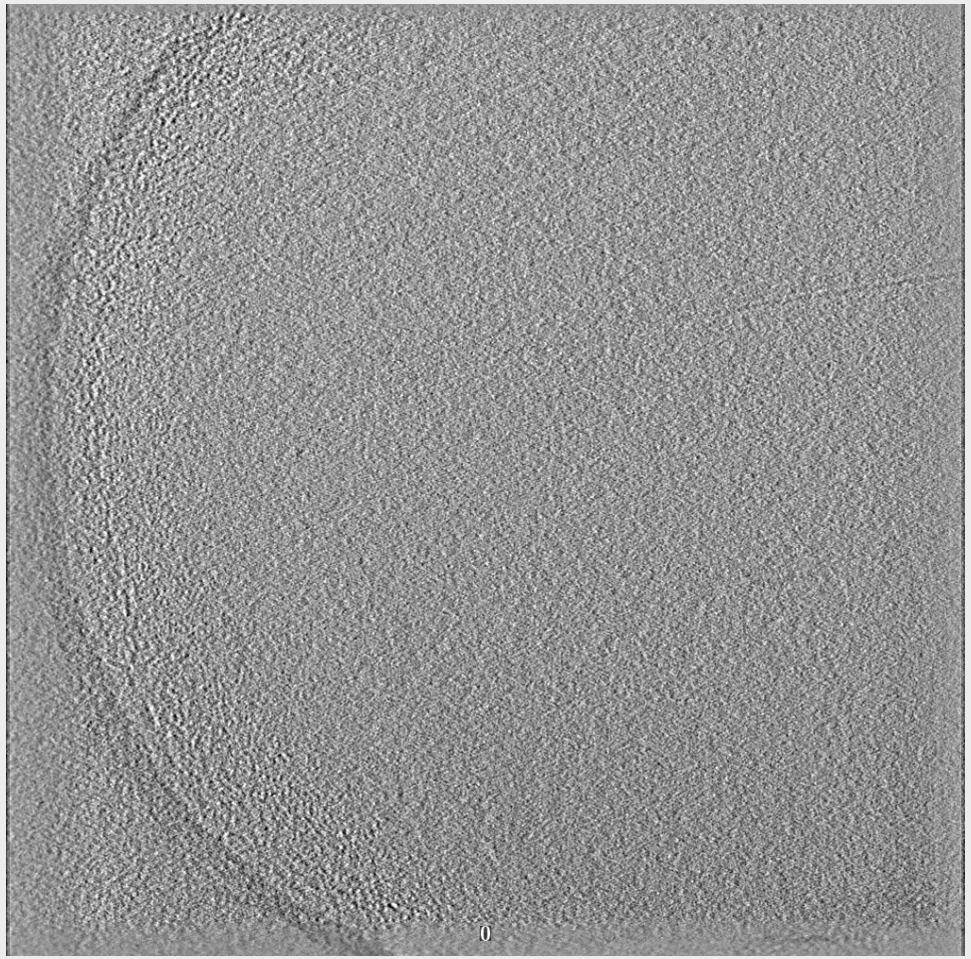
Schur et al, J. Struct Biol, 2013
Schur et al, Nature , 2015

Example: Liposomes and VLPs



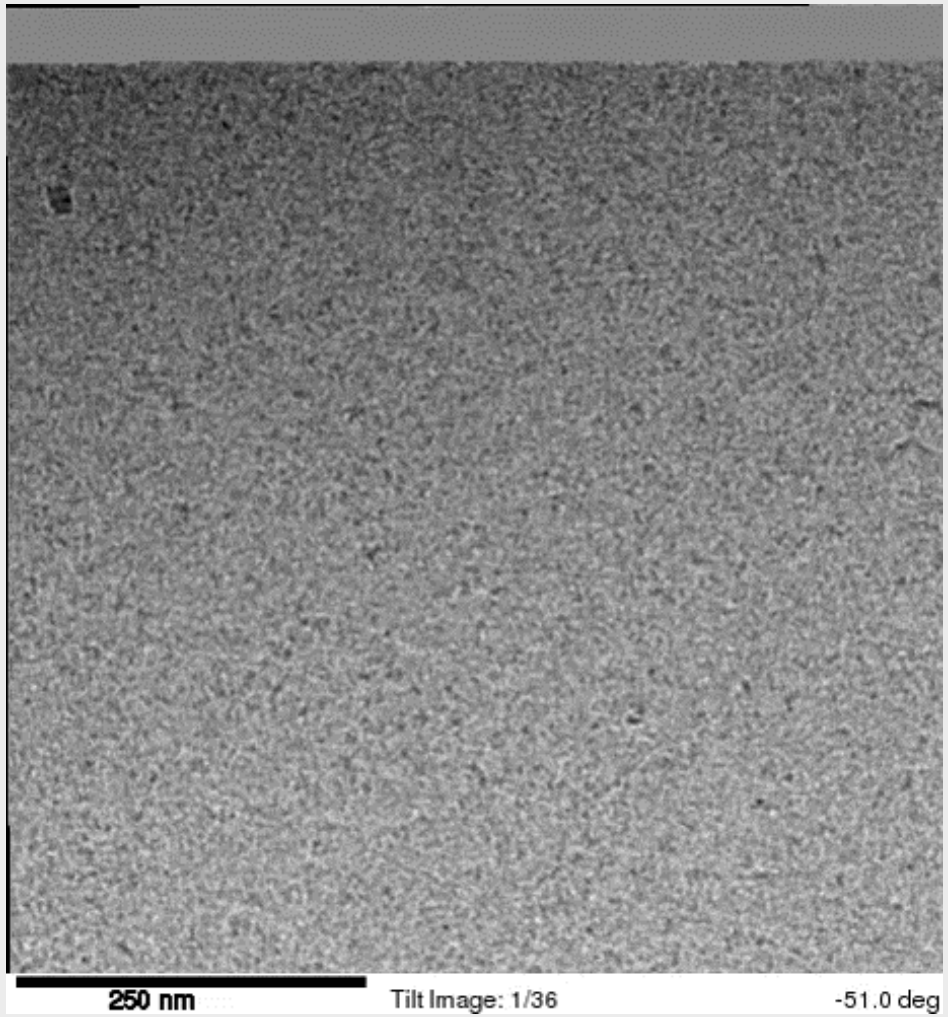
Example: Actin Filaments

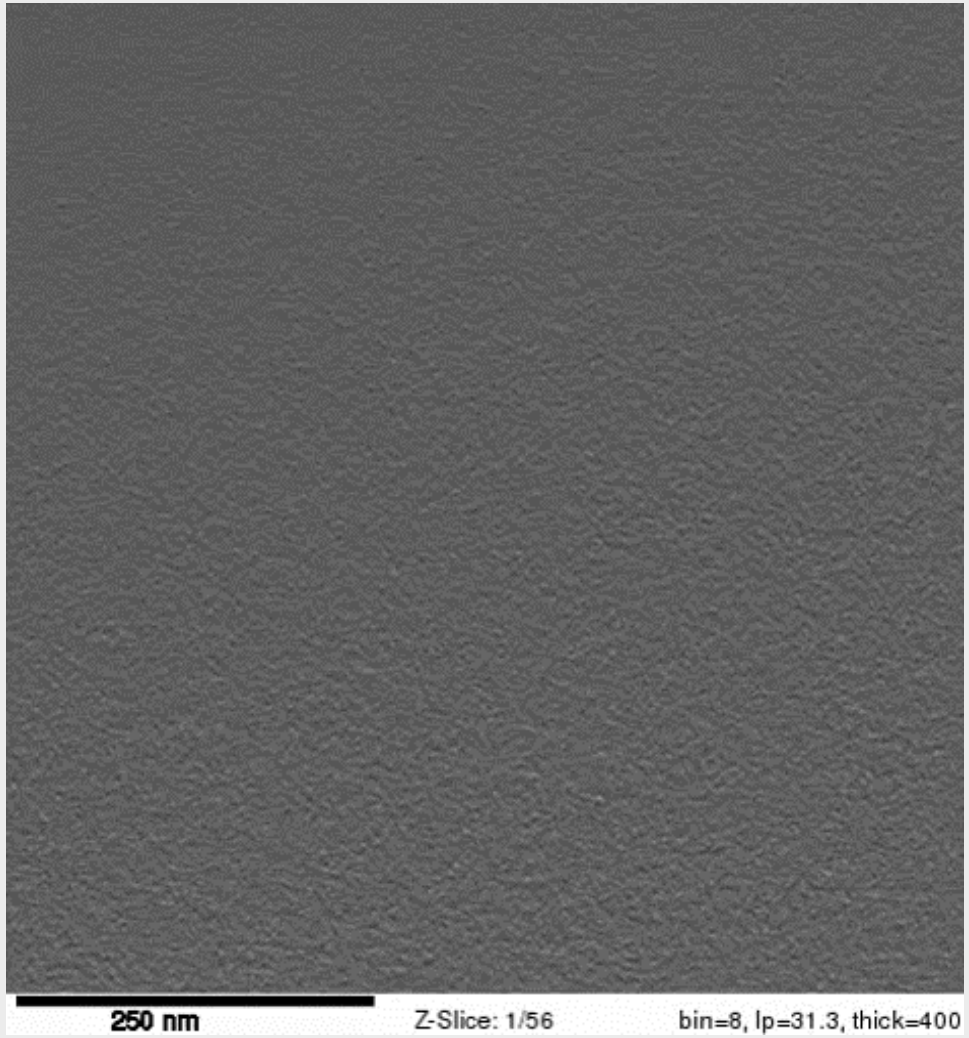




Pinar Gural, Greg Alushin, Alex Noble
unpublished

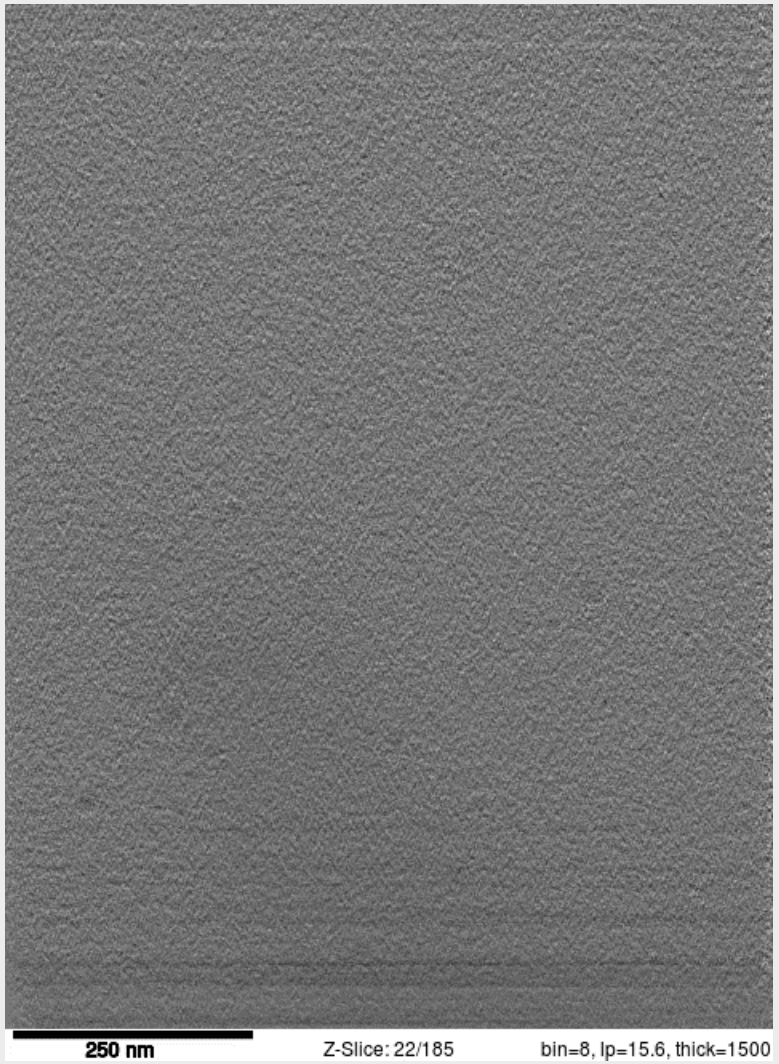
HIV-1 trimer single particle





Priyamvada Acharya & Alex Noble
eLife, 2018

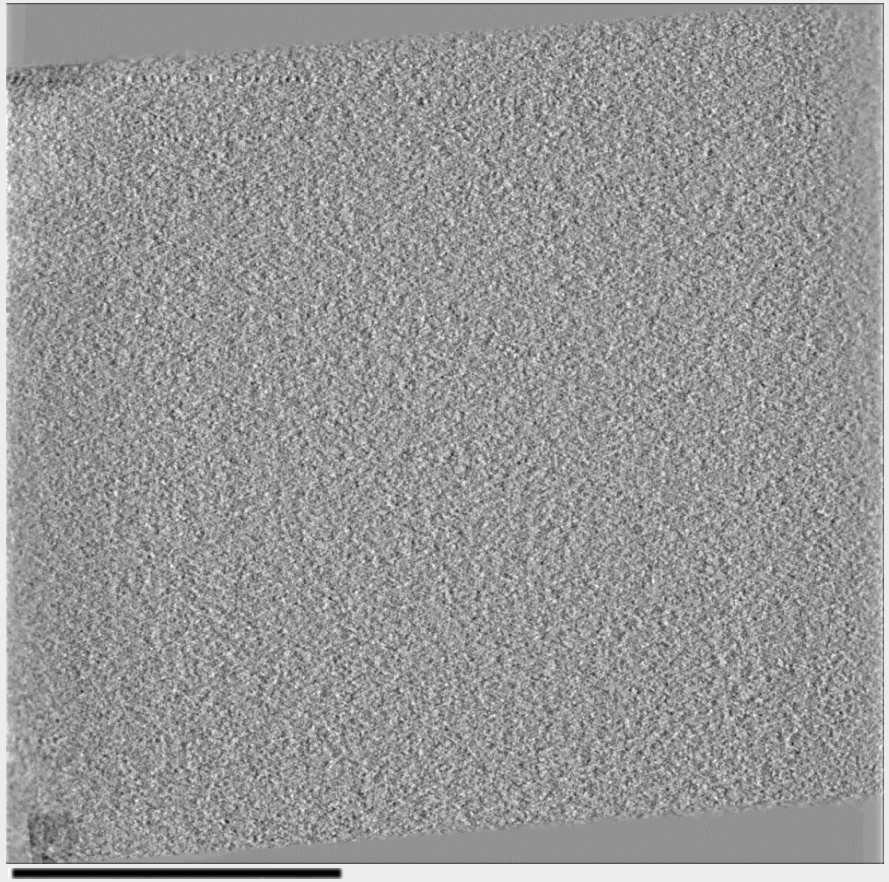
Example: Exotically Shaped Samples



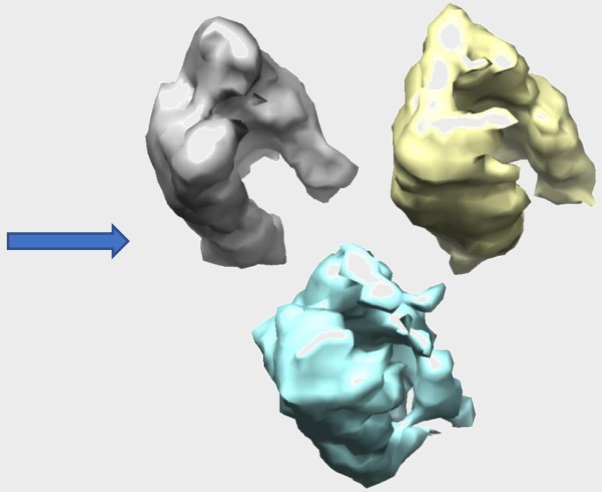
ZmNDPK1: GTP complex

Mykhailo Kopylov & Beth Stroupe
unpublished

Example: Tomography for single particle initial model

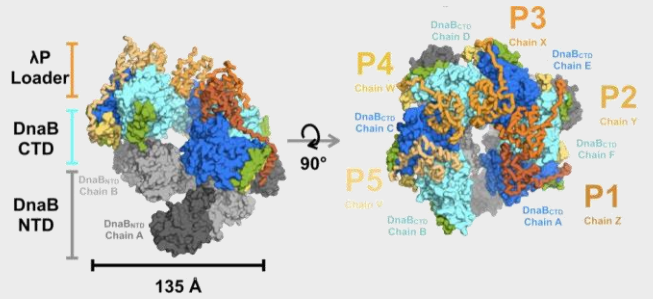


Z-slices through tomogram/ice



template pick

initial model

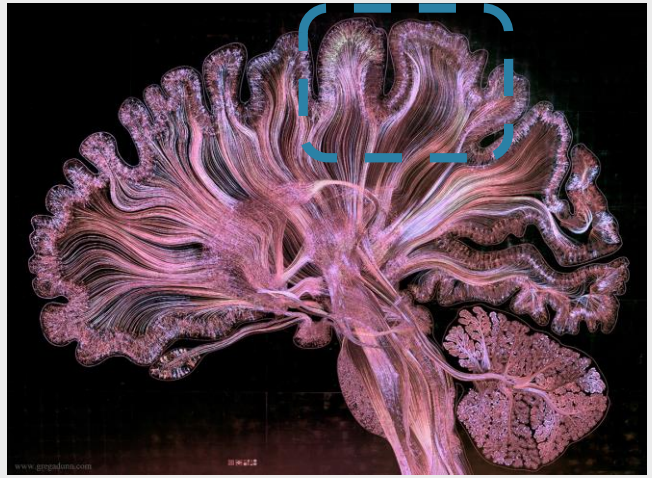


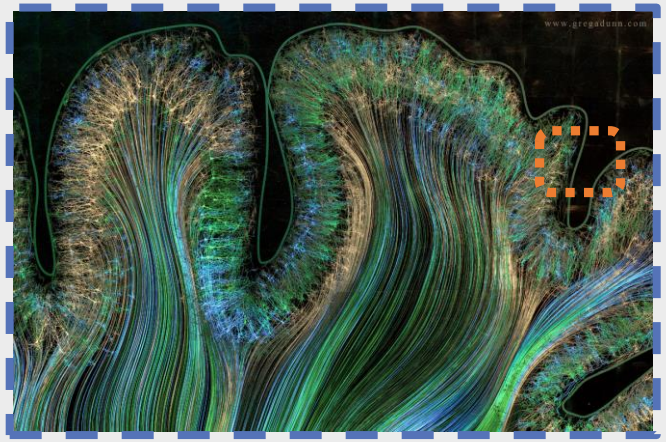
4 Å structure!

- 5 tomograms were collected
- ~1,000 particles picked, aligned, and classified
- Classes used as templates for picking single particle micrographs
- Single particle now at 4 angstroms without anisotropy.

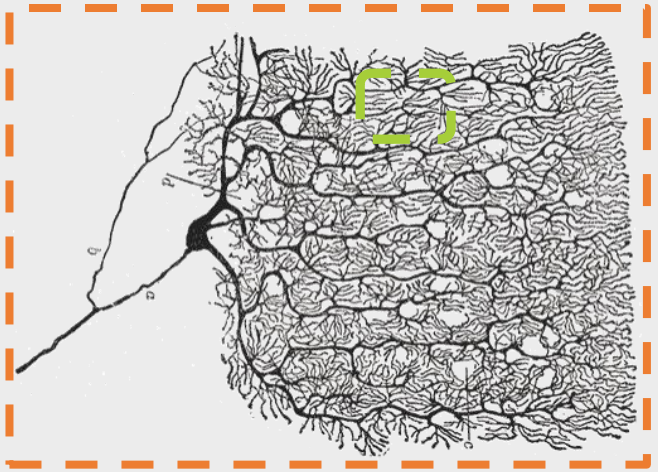
Jillian Chase
and Alex Noble
eLife, 2018 (tomography) and eLife, 2018 (biology)

Example: Tomography of clustered protocadherins on membranes





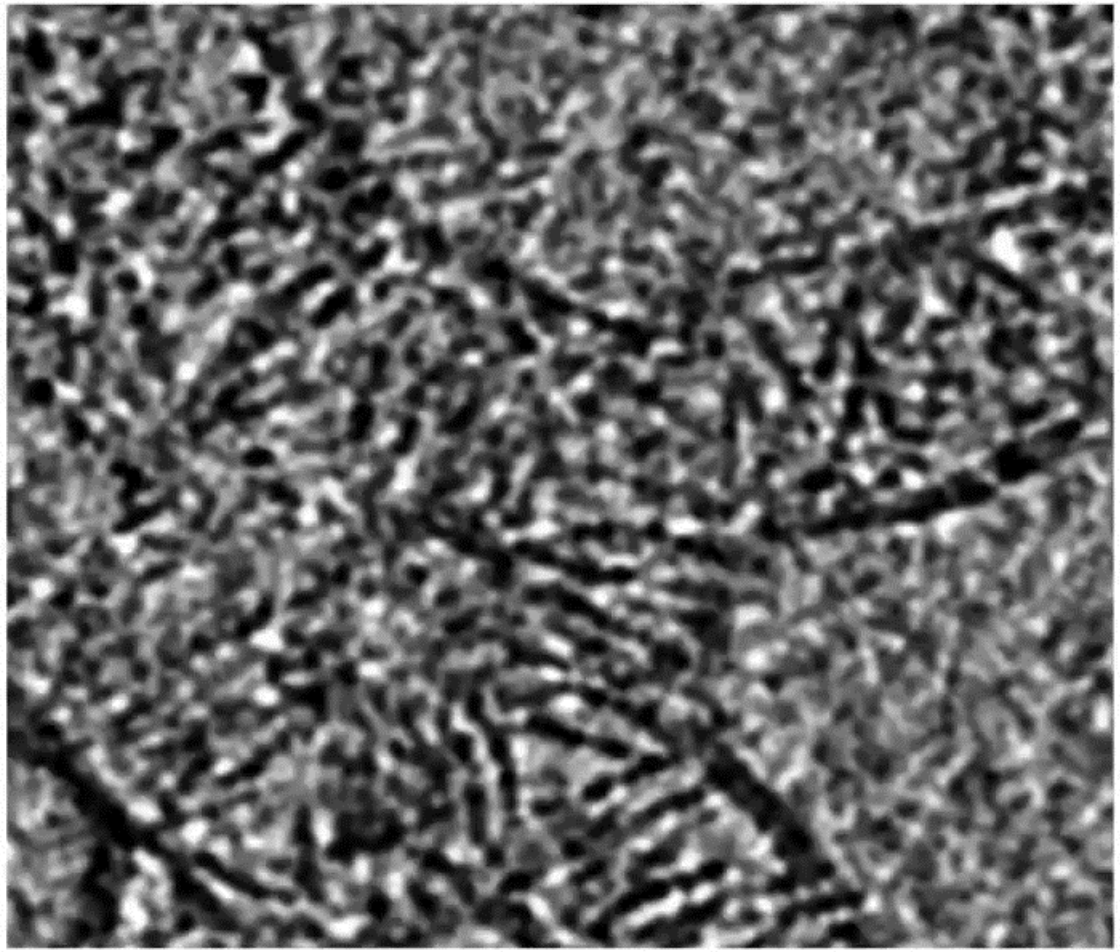
Single neuron self-avoidance

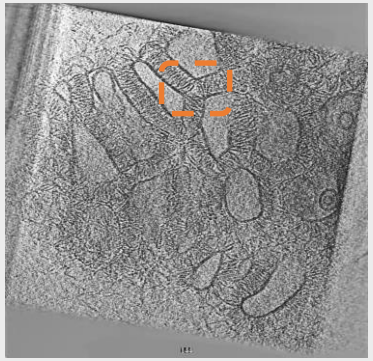


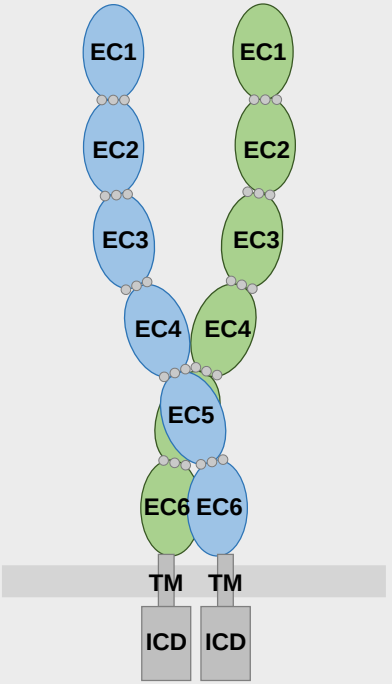
Purkinje cell, Ramon y Cajal, ~1890

Brasch, Goodman, Noble et al., Nature, 2019

Example: Tomography of clustered protocadherins on membranes

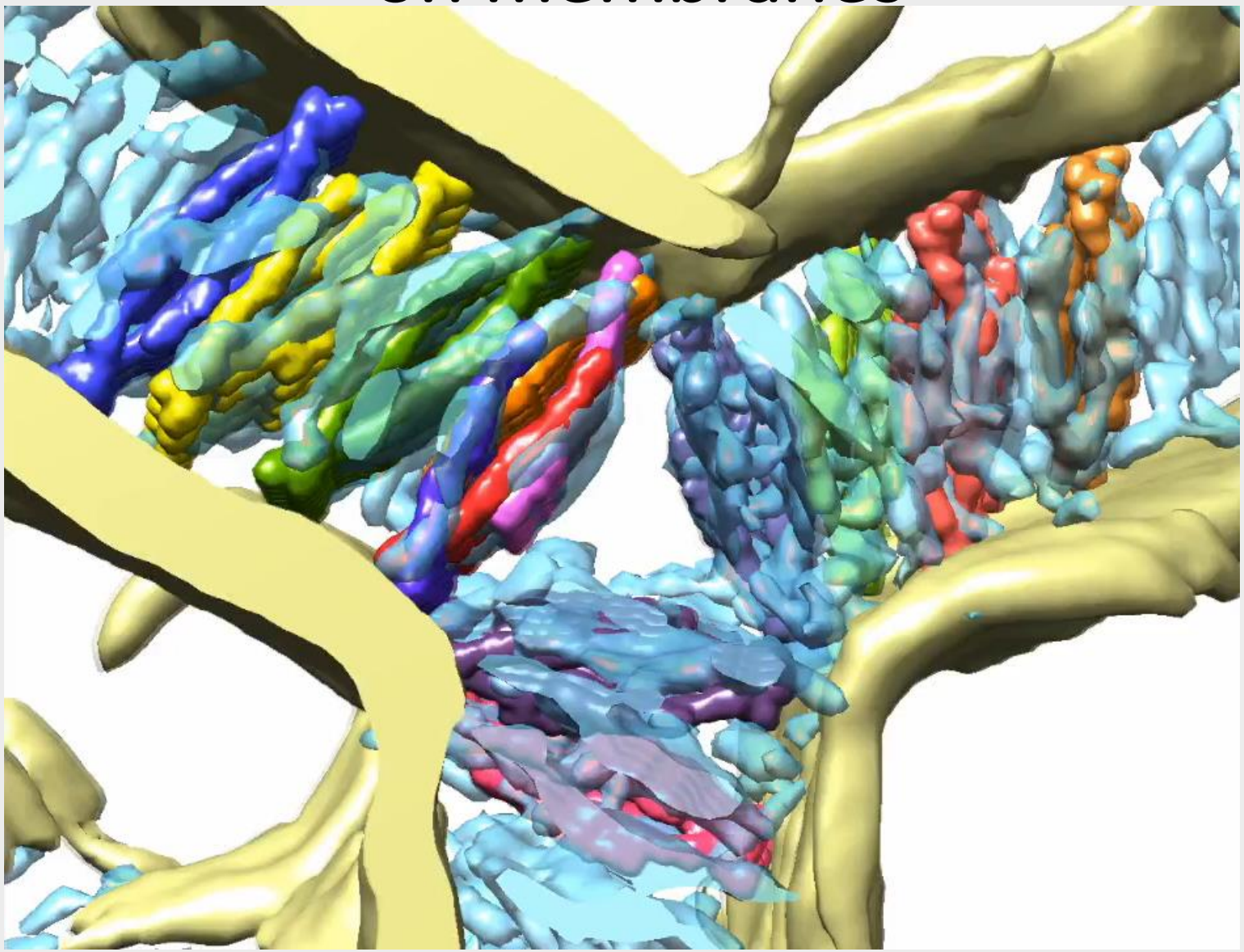


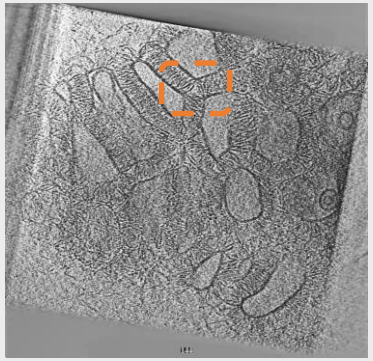


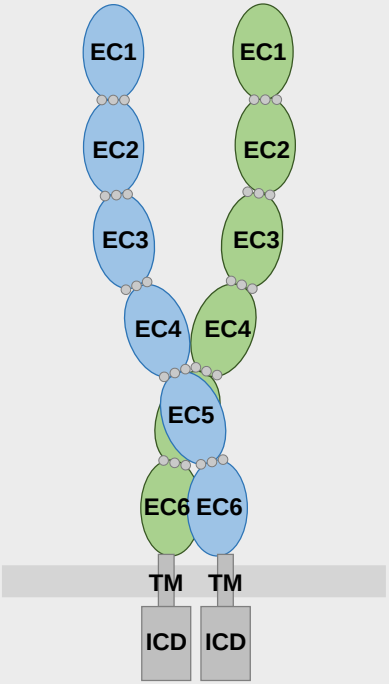


Brasch, Goodman, Noble et al., Nature, 2019

Example: Tomography of clustered protocadherins on membranes

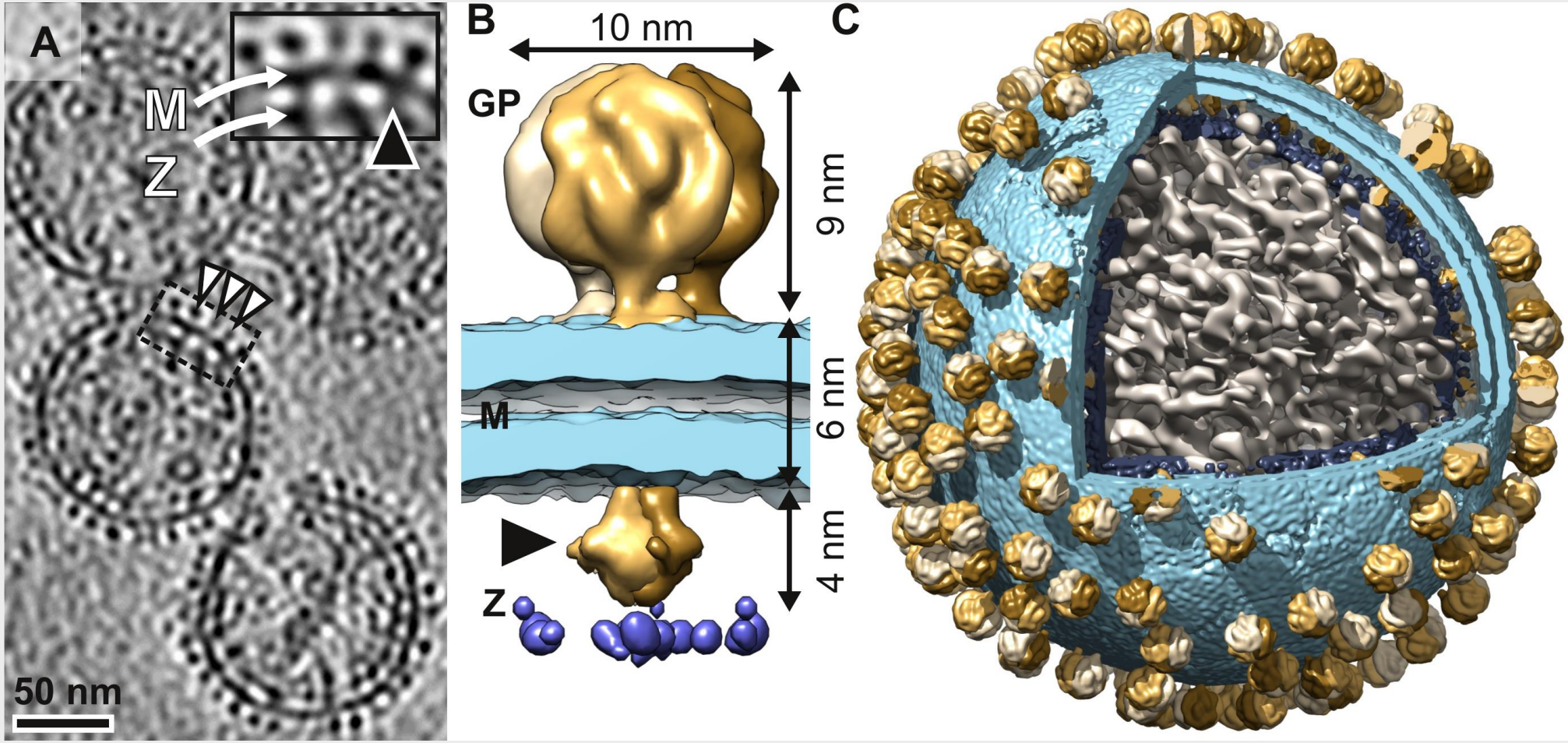






Brasch, Goodman, Noble et al., Nature, 2019

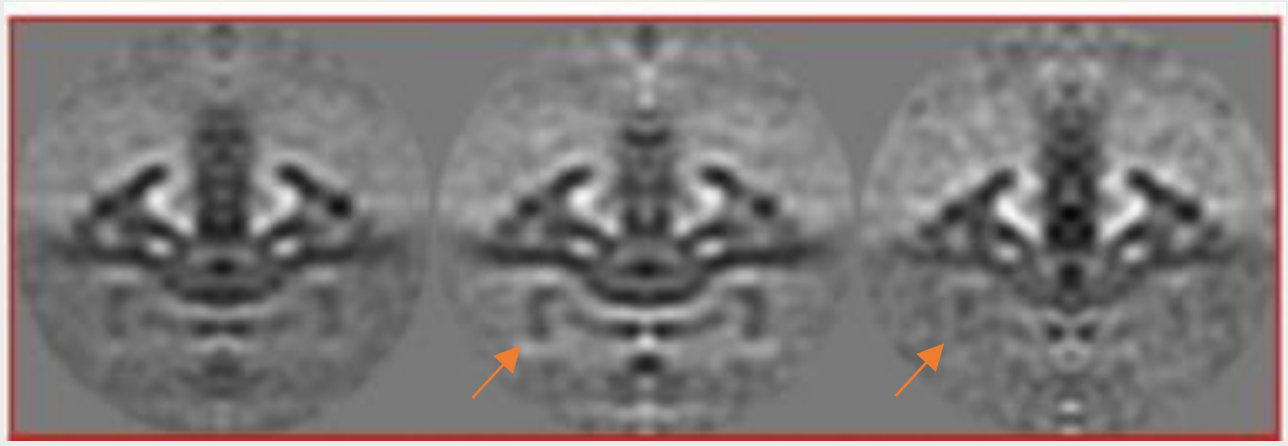
Example: Lassa virus glycoprotein spike



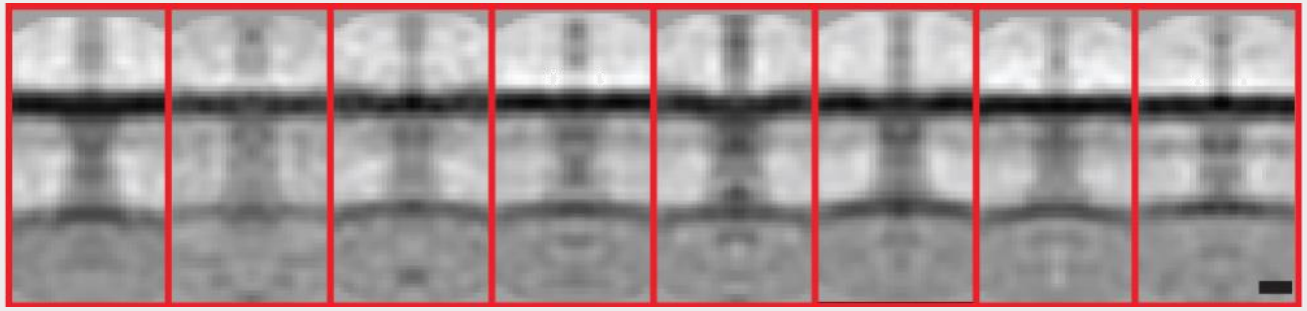
- **Heterogeneous shape** makes single particle difficult/impossible
- Sub-tomogram processing on spiked allows for **13.6 Å spike structure**
- Can **re-map spikes** onto all particles in the tomogram

Li et. al., PLOS 2016

Example: Bacterial flagella motor and type III secretion injectisome



Kudryashev et al, JSB, 2010 and
Castaño-Díez, et al, JSB, 2012

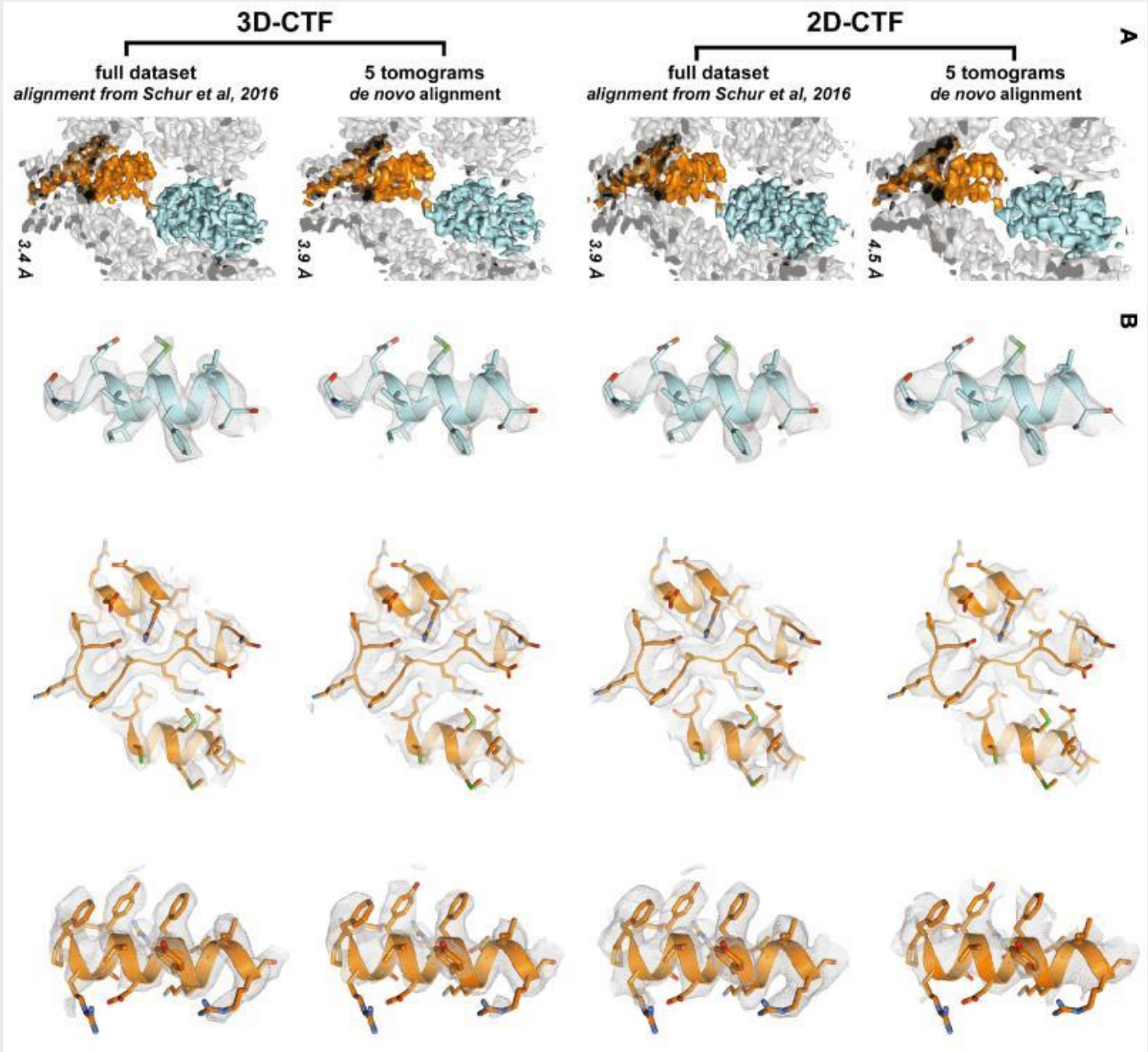


Kudryashev et al, eLife, 2013

- Conformational states studied *in situ*
- Presence and absence of C-ring
- Elongation of injectisome

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

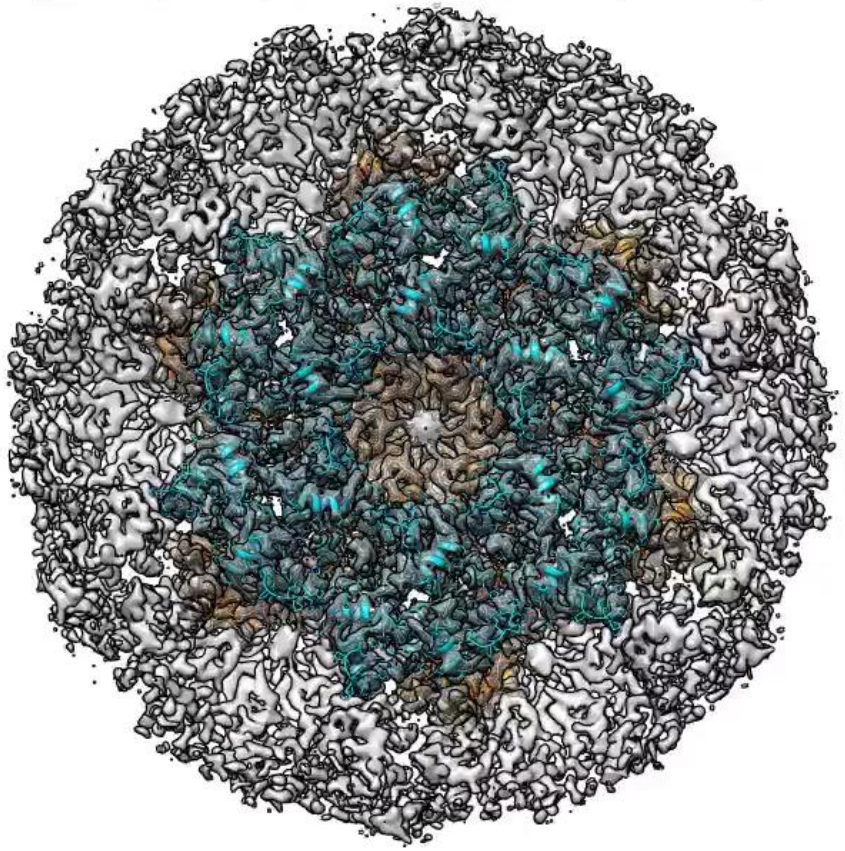
- Krios + Super-res K2 + Gatan Energy Filter
- Fiducial tilt-series alignment
- 1.5 – 5 micron defocus
- Strip-based CTF correction
- ~750,000 sub-particles used
- TOM, AV3, Dynamo, and in-house scripts were used
- NovaCTF 3D CTF pushed it to 3.4 Å
- emClarity pushed to 3.2 Å
- Warp pushed it to the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



Schur, 2016
Turoňová, 2017

Example: HIV-1 Capsid-SP1 at 3.9/3.4/3.2 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, 2016

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

Sample		HIV-1 ΔMACANCSP2 VLPs	HIV-1 ΔMACANCSP2 VLPs + 100 µg/ml Bevirimat	Immature HIV-1 (D25A) virus
Acquisition settings	**Microscope**	FEI Titan Krios	FEI Titan Krios	FEI Titan Krios
	Voltage (keV)	300	300	300
	Detector	Gatan Quantum K2	Gatan Quantum K2	Gatan Quantum K2
	Energy-filter	Yes	Yes	Yes
	Slit width (eV)	20	20	20
	Super-resolution mode	Yes	Yes	Yes
	Å/pixel	1.35	1.35	1.35
	Defocus range (microns)	-1.5 to -4.5	-1.5 to -5.0	-1.5 to 5.0
	Defocus step (microns)	0.25	0.25	0.25
	Acquisition scheme	-60/60°, 3°, Serial EM	-60/60°, 3°, Serial EM	-60/60°, 3°, Serial EM
	Total Dose (electrons/Å^2)	~90 - 270	~120 - 145	~120-221
	Dose rate (electrons/Å^2/sec)	~3 - 8	~3 - 3.8	~1.5 – 5.5
	Frame number	6 – 10	8 – 10	10 – 12
	Tomogram number	93	43	74
Processing settings	**VLPs/Viruses**	285	383	484
	Asymmetric units Set A	265,506	386,040	301,302
	Asymmetric units Set B	263,910	386,598	301,920
	Final resolution (0.143 FSC) in Å	4.5	3.9	4.2

Schur et. al., Science 2016

Nanometer SPT studies (~1 year old)

Sample	Sample type	Instrumentation	Tilt-series alignment method	Defocus range (μm)	CTF correction method	Number of asymmetric units	Reported resolution (Å), Nyquist fraction	Citation
HIV-1 capsid-SP1	VLP spikes	Titan Krios, K2 Summit @ 8kx8k, GIF	fiducial	1.5 – 5	Strip-based/3D CTF	~750,000	3.9, 0.35 3.4	(Schur et al., 2016) (Turoňová, 2017)
Rous-Sarcoma Virus Gag particles	Isolated viruses	Titan Krios, 2k CCD, GIF	fiducial	1.5 – 5	Strip-based	50,000	7.7, 0.27	(Schur et al., 2015)
Hepatitis B capsid	Isolated viruses	Titan Krios, K2 Summit, GIF	fiducial	3.2 – 5.6	Per-particle 3D CTF model	68,000	8.1, 0.53	(Bharat et al., 2015)
M-PMV CANC Gag dimer	Lattice-decorated tubes	Titan Krios, 2k CCD, GIF	fiducial	1.5 – 3.3	Tile-based	121,000	8.3, 0.49	(Schur et al., 2013)
GroEL	Isolated particles	Titan Krios, 4k CCD	fiducial	2 – 3	Per-particle, each projection	10,000	8.4, 0.41	(Bartesaghi et al., 2012)*
HIV-1	Isolated viruses	Titan Krios, 2k CCD, GIF	fiducial	1.2 – 4	Strip-based	195,000	8.8, 0.46	(Schur et al., 2014)
Sec61 protein-conducting channel	Isolated vesicles	Titan Krios, K2 Summit, GIF	fiducial	3 – 4	Strip-based	17,600	9, 0.58	(Pfeffer et al., 2015)
M-PMV Gag-derived protein	Isolated viruses	Titan Krios, 2k CCD, GIF	fiducial	1.4 – 4.5	Strip-based	77,500	9.7, 0.41	(Schur et al., 2014)
HIV-1	Isolated viruses	Titan Krios, Falcon II	fiducial	2 – 5.5	Strip-based	63,000	10.9, 0.42	(Schur et al., 2014)
Histidine Kinase CheA	Latticed proteins	Tecnai Polara, 4k CCD	correlation	5 – 8	Strip-based	4,000	11.3, 0.53	(Cassidy et al., 2015)
Mouse Serotonin Receptor	Isolated viruses	Titan Krios, K2 Summit	fiducial	2.5 – 4	Strip-based	65,000	12, 0.28	(Kudryashev et al., 2016)
VEEV	Isolated viruses	JEM3200FSC, DE-20, GIF	fiducial	4 – 8	Per-particle sub-tilt-series	21,000	13, 0.77	(Galaz-Montoya et al., 2016)

Processing/Resolution limits

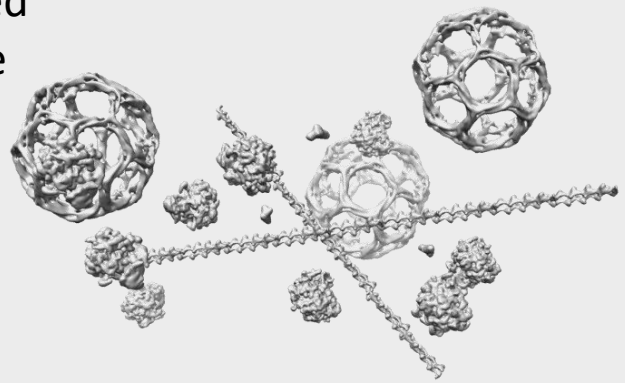
Already discussed: Sample thickness, camera accuracy, and specimen damage

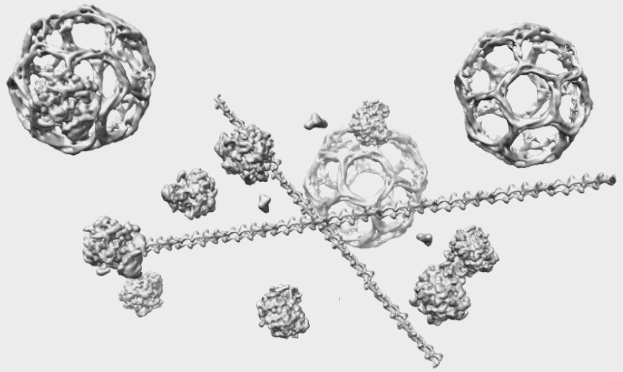
- Pixelsize (highest resolution = 2 x pixelsize = **Nyquist**)
- Isotropic motion (monitor your **drift** before full collection)
- Inherent specimen **flexibility**
- **Ice warping** in 3D during collection (doming)
- Beam-induced **motion of objects** of interest **in 3D** (particularly anisotropic)

Problem: Objects and ice move non-uniformly!

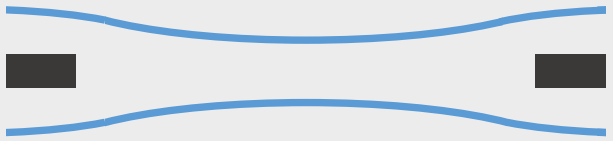
Beam-induced motion type

Particle



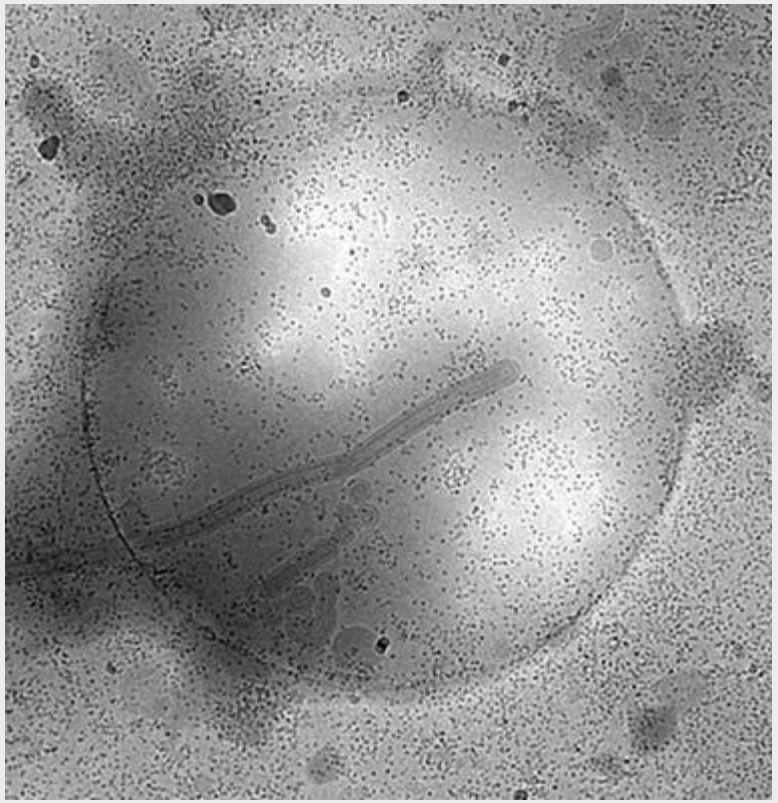


Ice



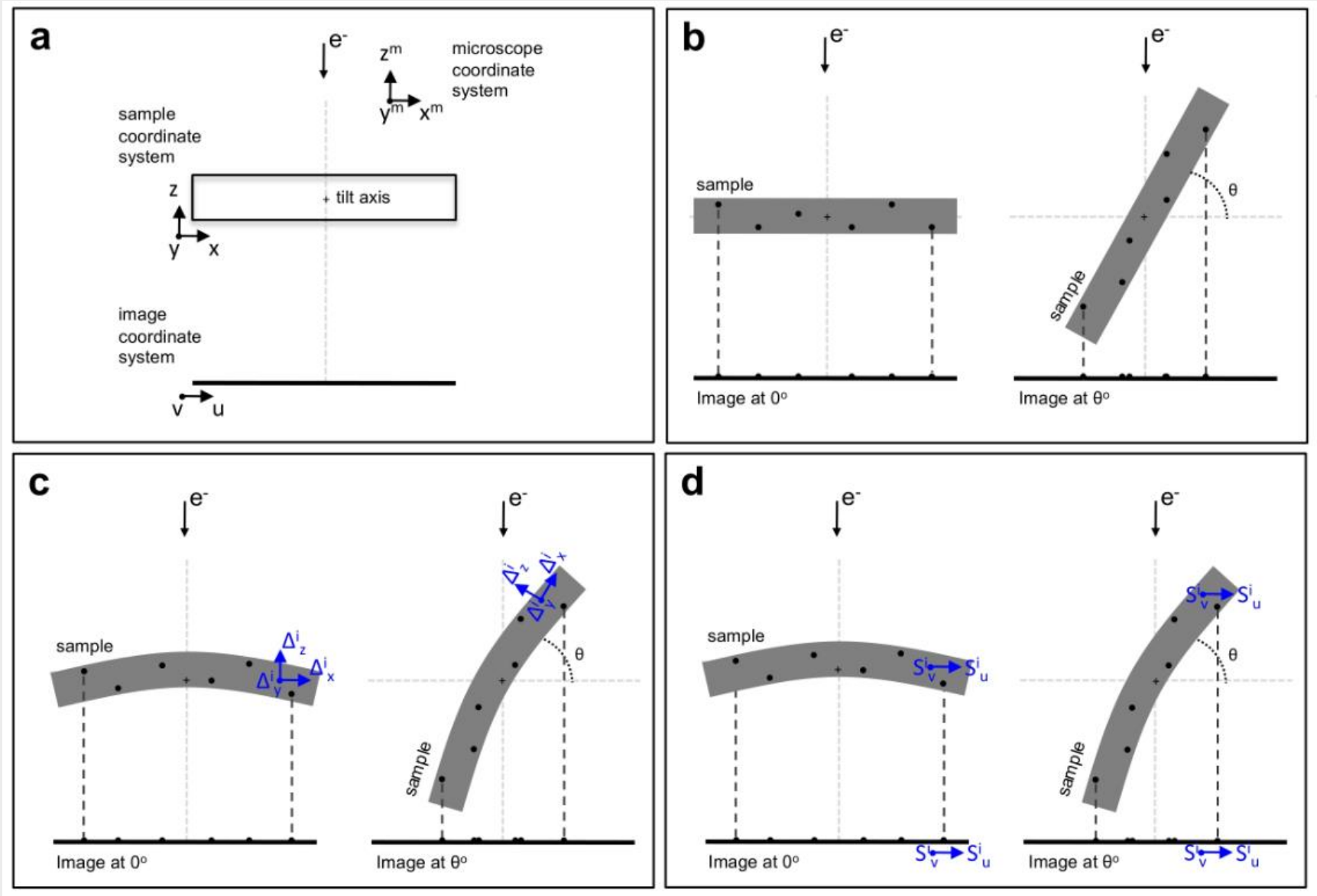
0 e-/A^2

20 e-/A^2



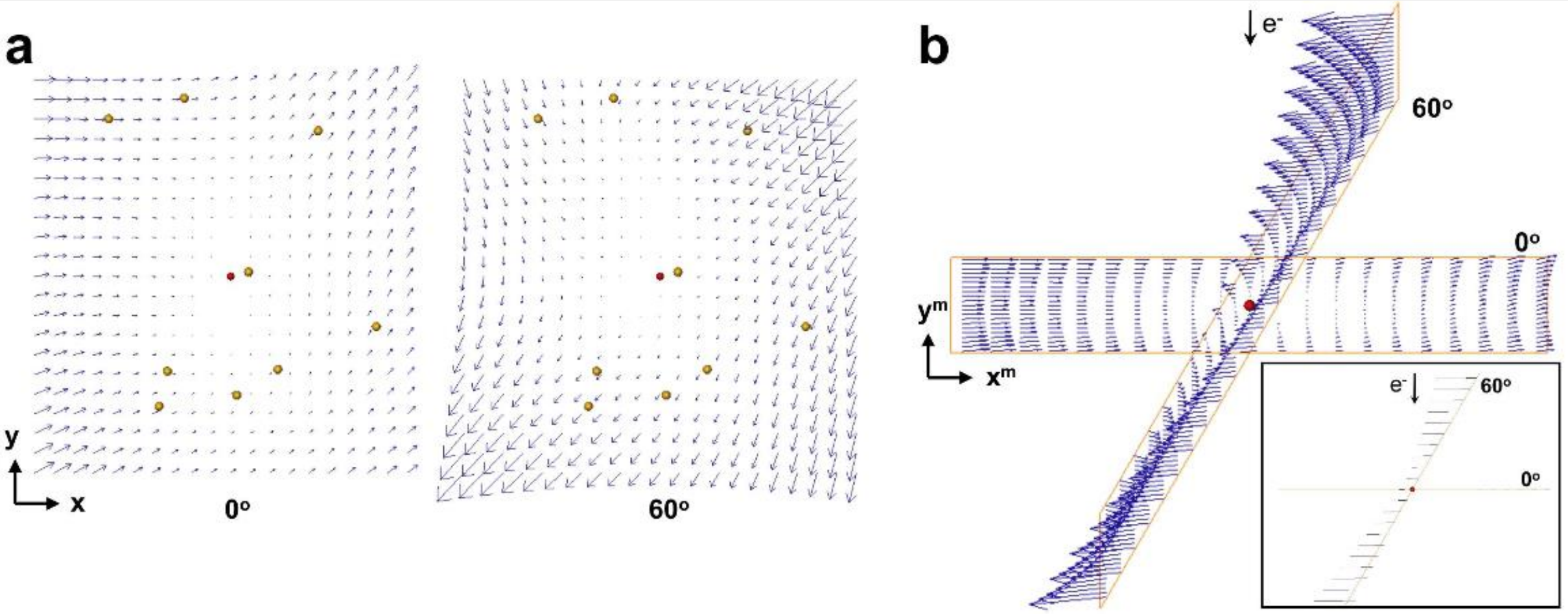
0 e-/A^2 – 20 e-/A^2 Wim Hagen, The esteemed journal of **Twitter**

Refining tilt-series alignment by tracking beads in 3D



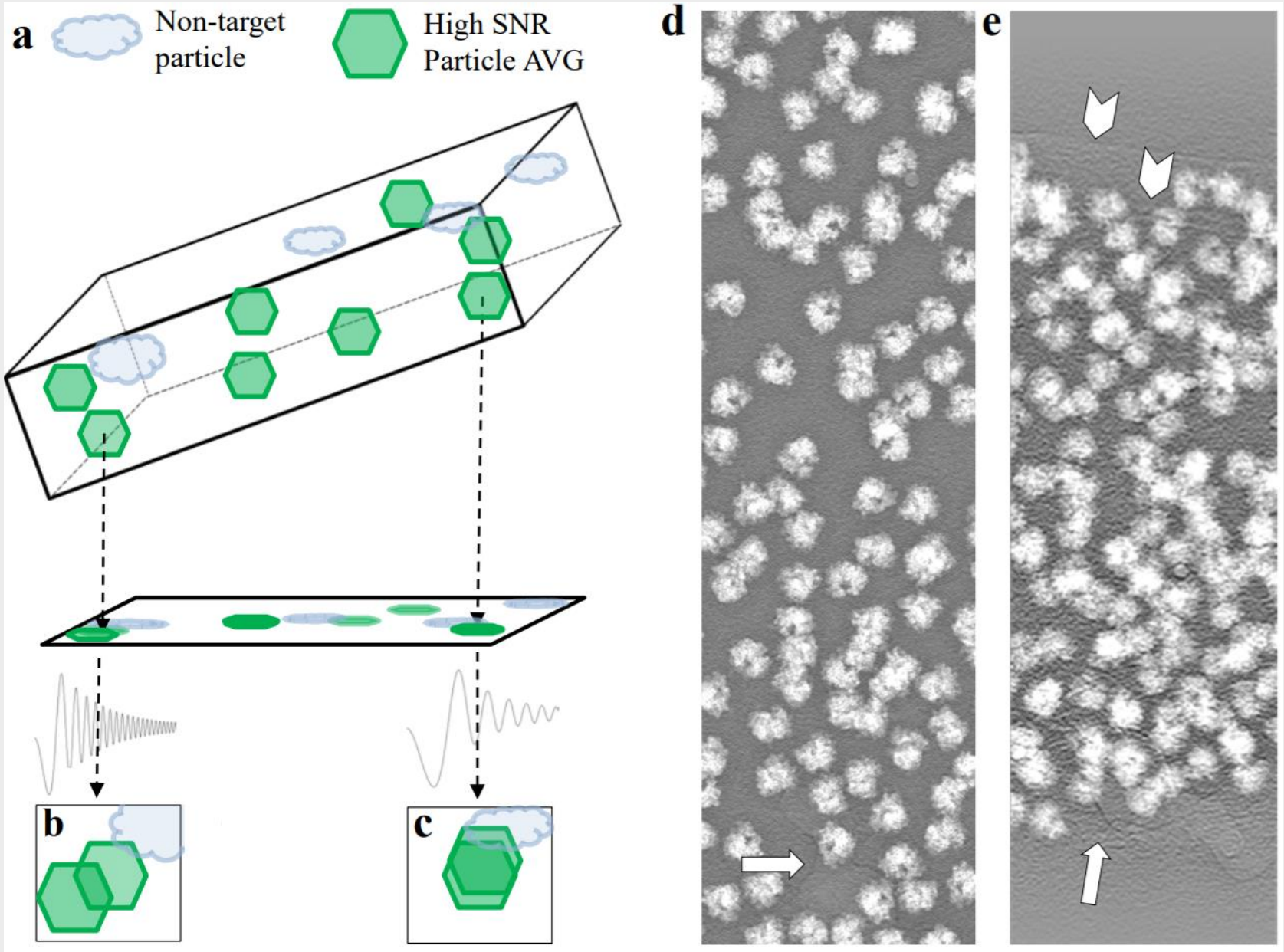
Fernandez, 2018

Refining tilt-series alignment by tracking beads in 3D



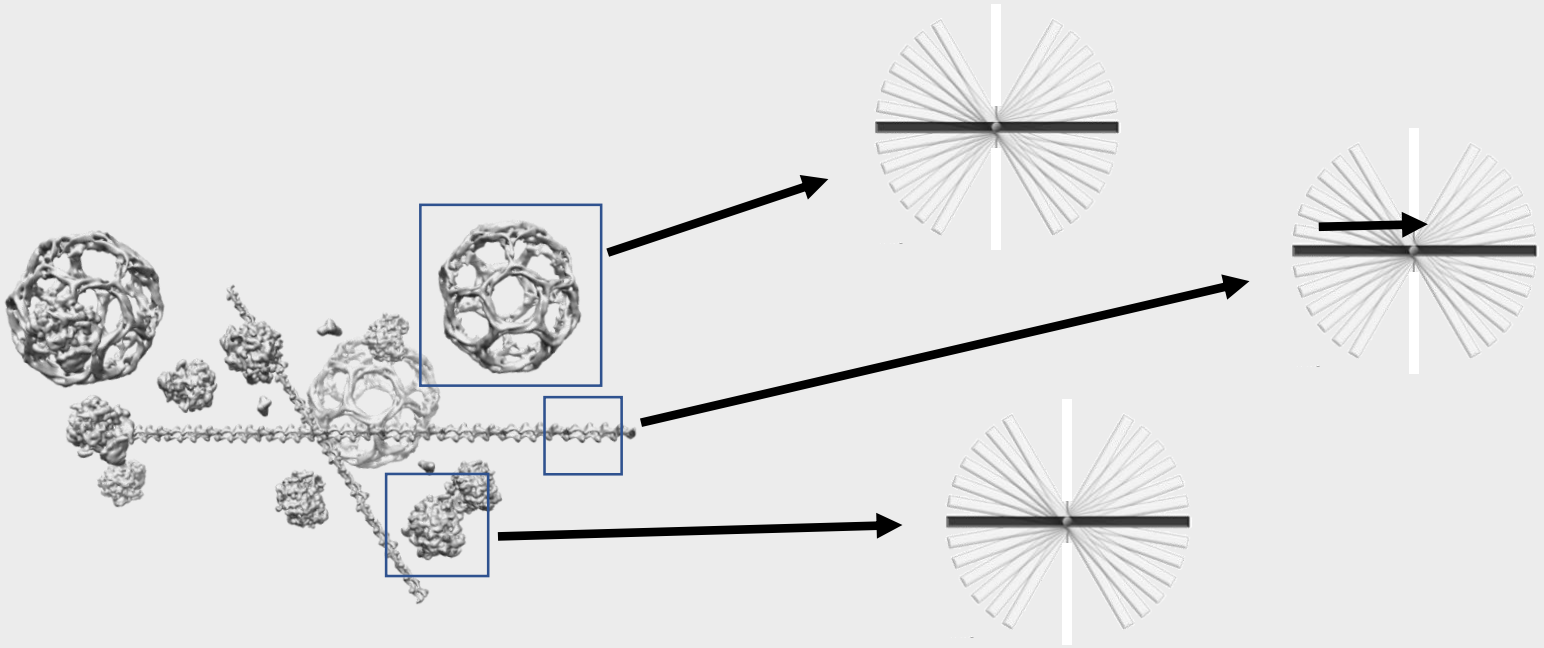
Fernandez, 2018

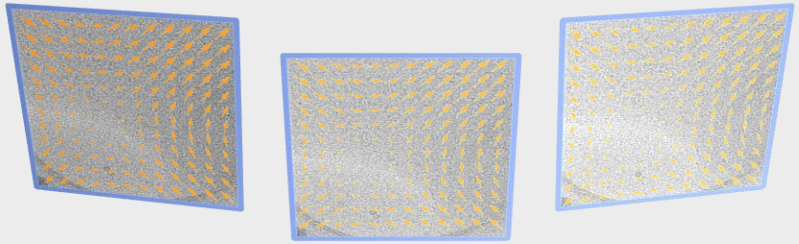
Refining tilt-series alignment by tracking just particles



Himes, 2017

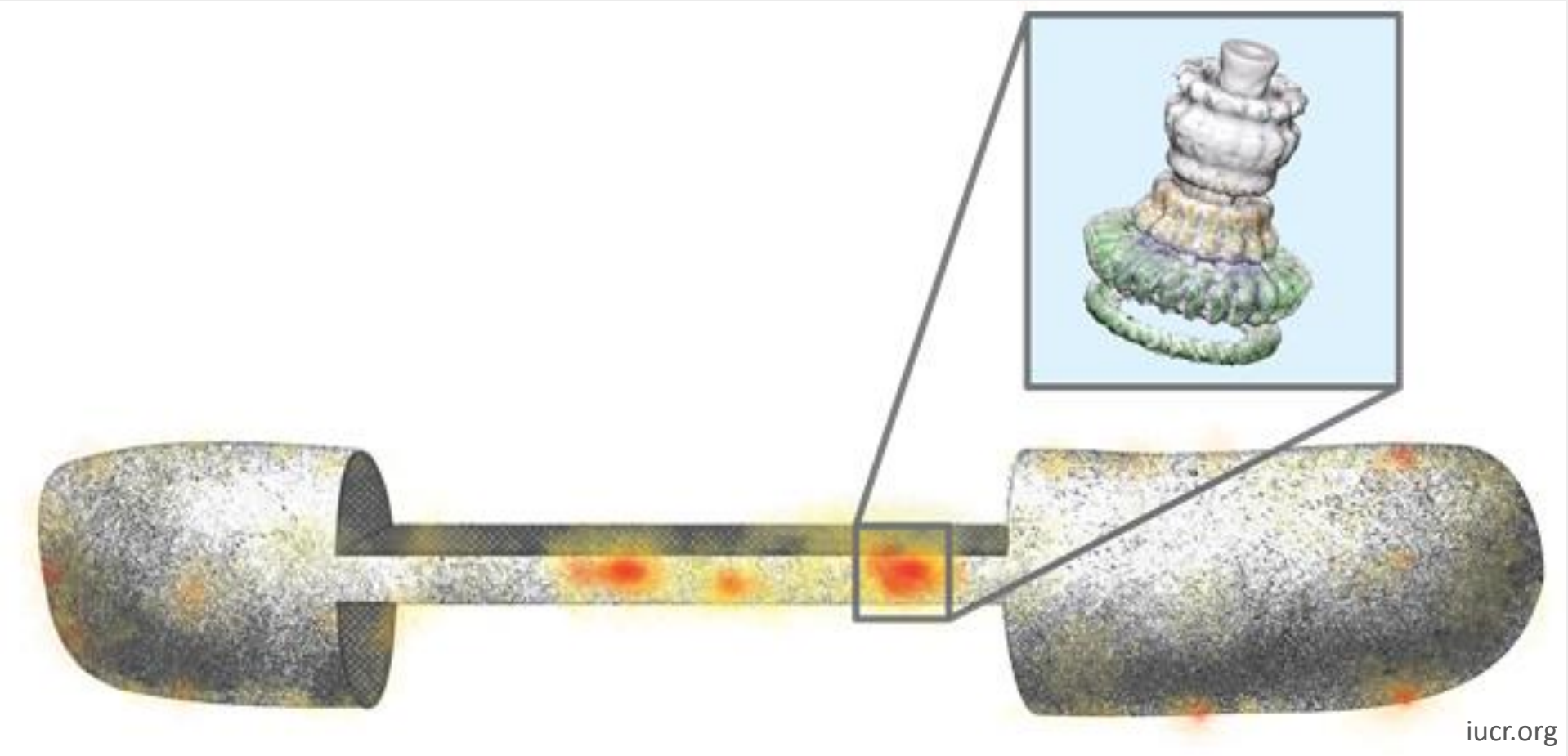
Refining tilt-series alignment by tracking just particles



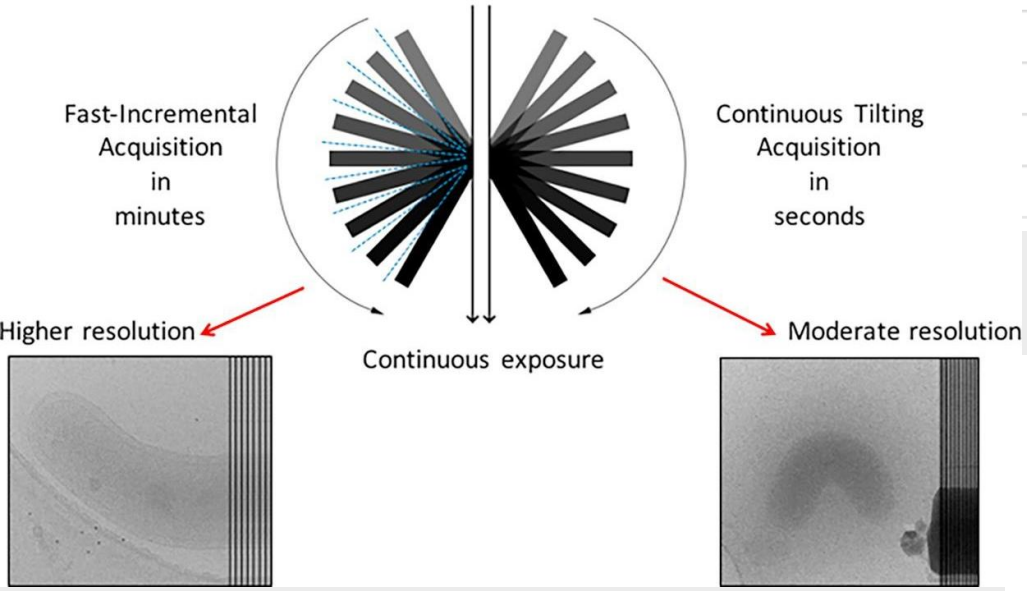


Interpolate **vector field** from particle refinements to **refine all objects**

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



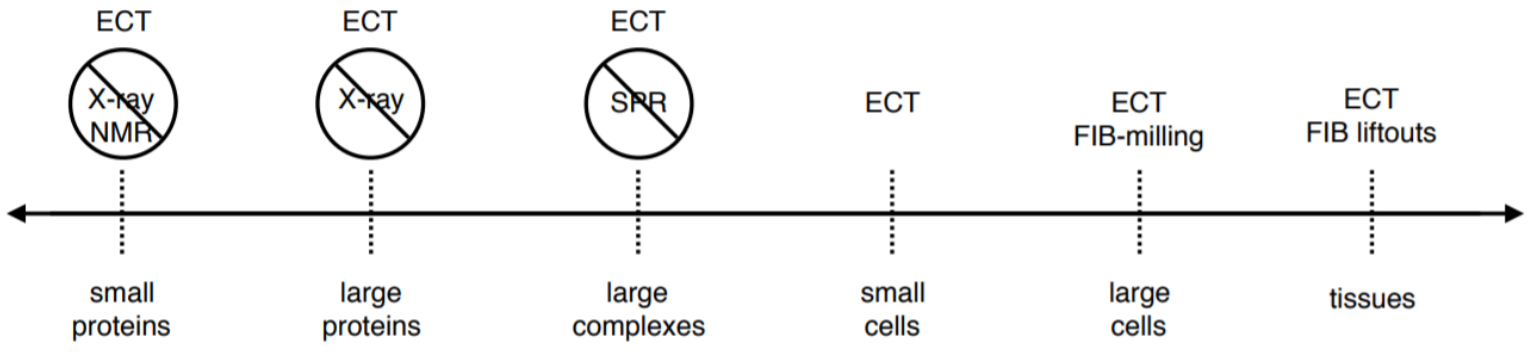
Future hardware improvements in the field – Rapid tilting



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

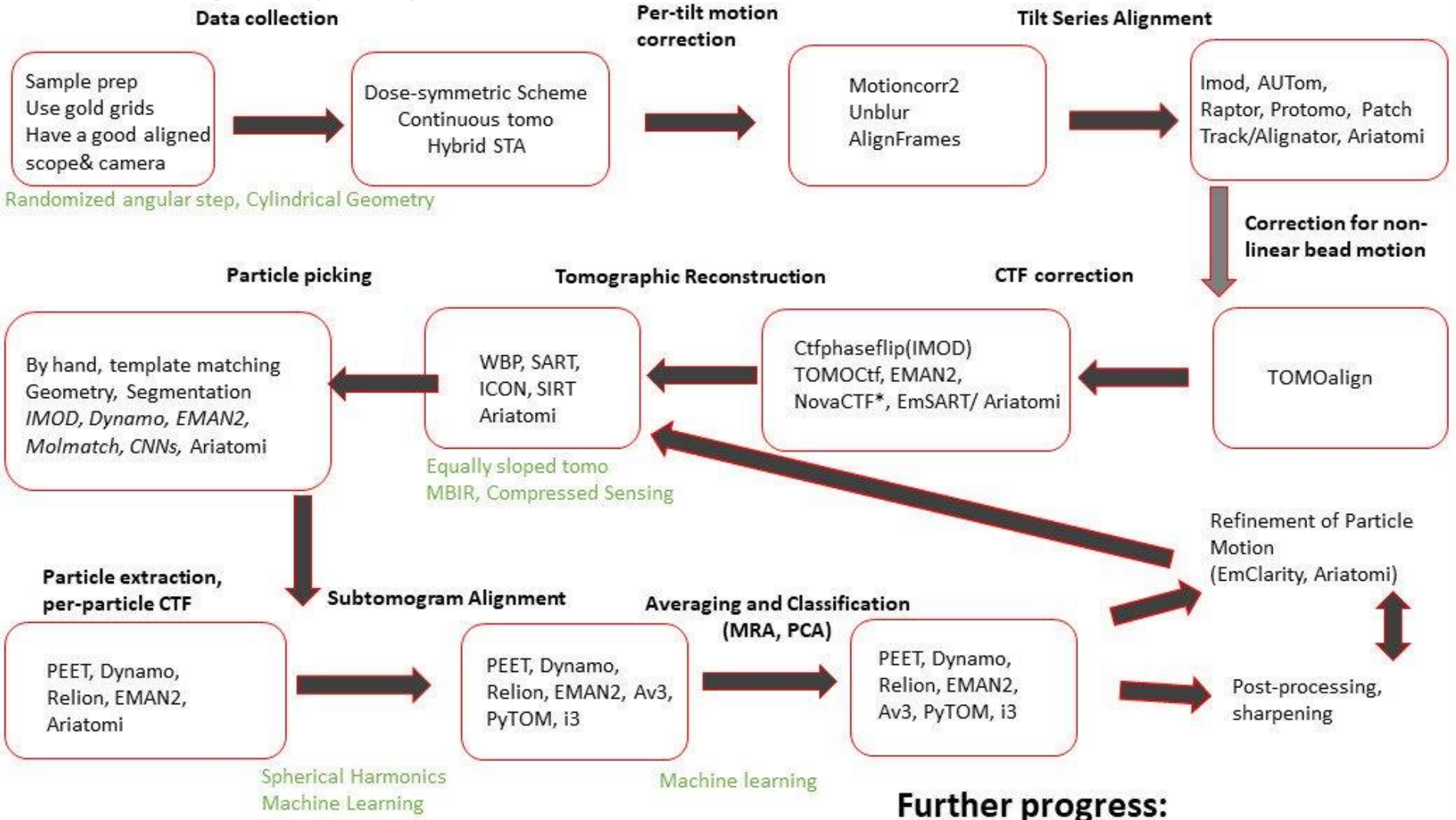
Most of

ALL cryotomography, ~~ALL~~ the time



Chreifi, JSB, 2019

Tomography workflows



Do it some early time

Dose Weighting
Motioncorr2, Unblur, Relion, etc

Throw away bad projections

Per tilt CTF determination
GCTF, CTFFind
Ctfplotter, TOMOCtf
EmClarity

Anisotropic mag correction
IMOD, Unblur, Motioncorr2, Ariatomi

Unreleased and interesting:
Bartesaghi et al 2008;
Warp – Tegunov et al (Bioarxiv)
PyTOM workflow

~16 operations of various difficulty
~5 image interpolations

Further progress:
- New/ better modules
- Cross-talk between the modules
- Standardized IO

Misha Kudryashev and Alex Noble

Thank you!
Questions?

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