Cryo Applications & Sub-tomogram Processing

SEMC Winter EM Course 2018

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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 is probably **not** required.

Overview

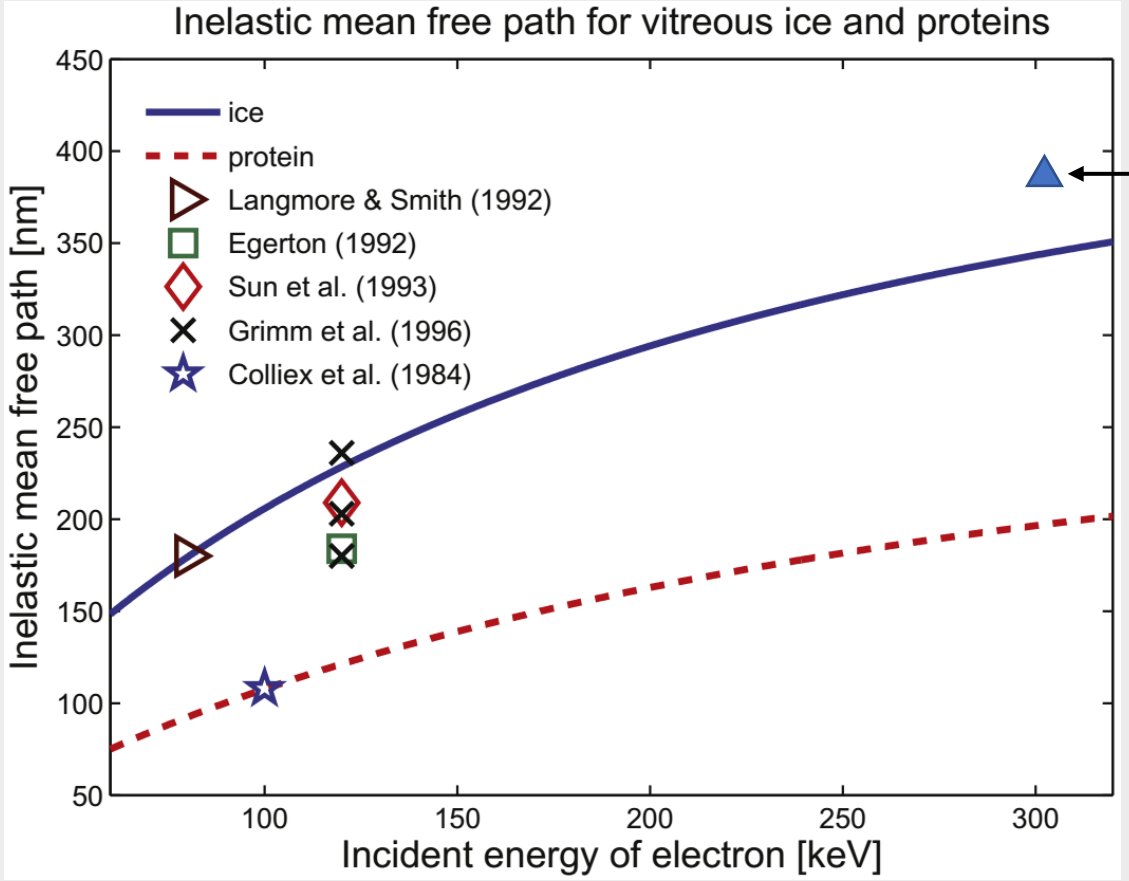
- CryoET limitations
- Tilt-series collection
- Tilt-series alignment
- Defocus estimation and CTF correction
- Sub-tomogram localization
- Sub-tomogram alignment and averaging
- Examples
- Processing limitations
- Future directions and improvements

Overview – Limitations

Primary 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

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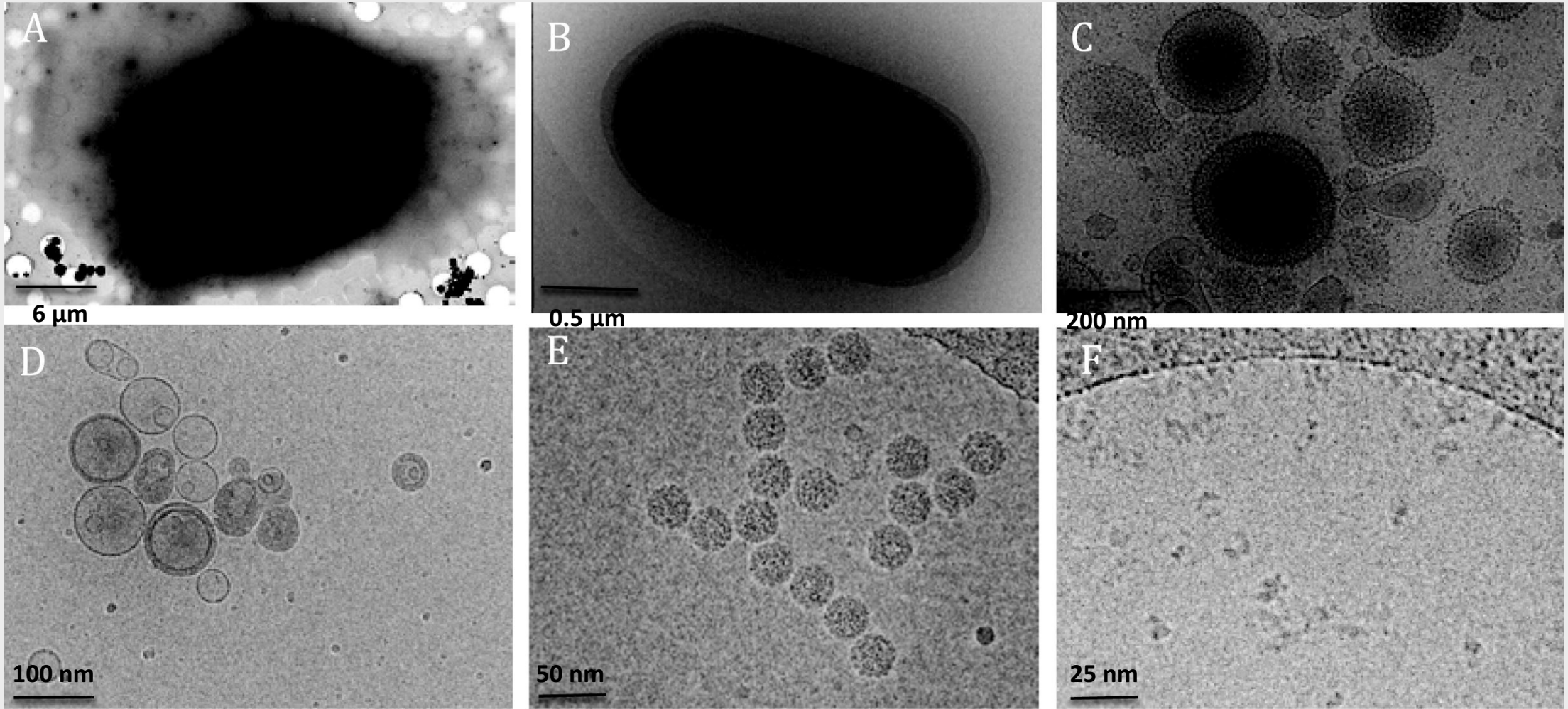


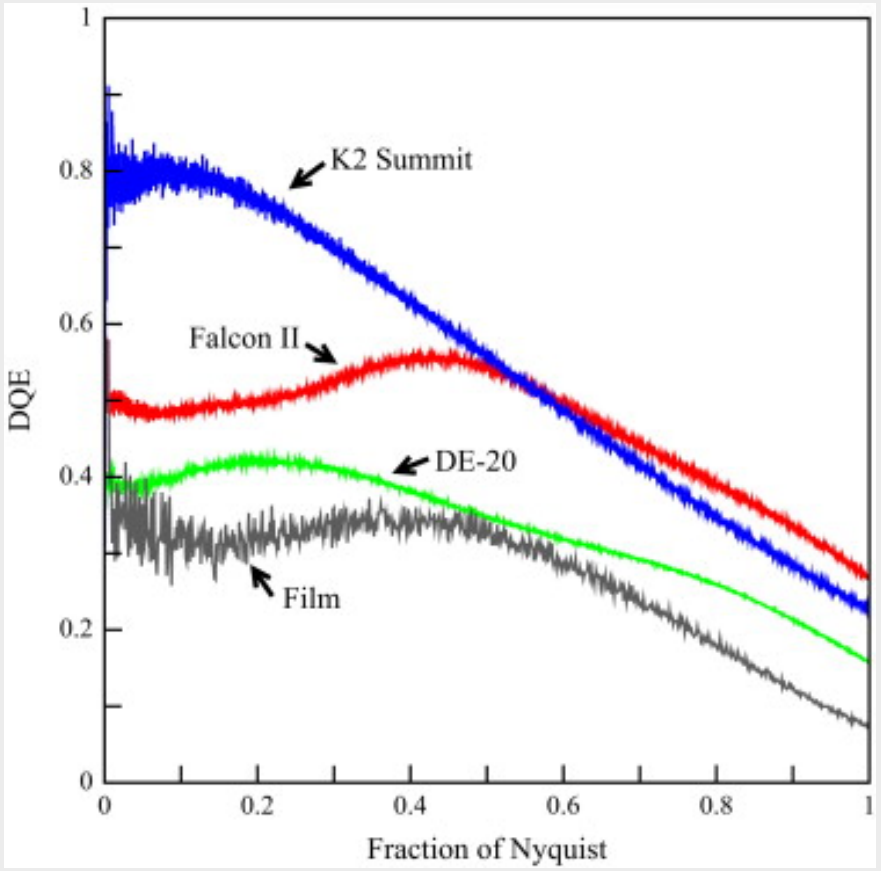
Thompson et. al., 2016

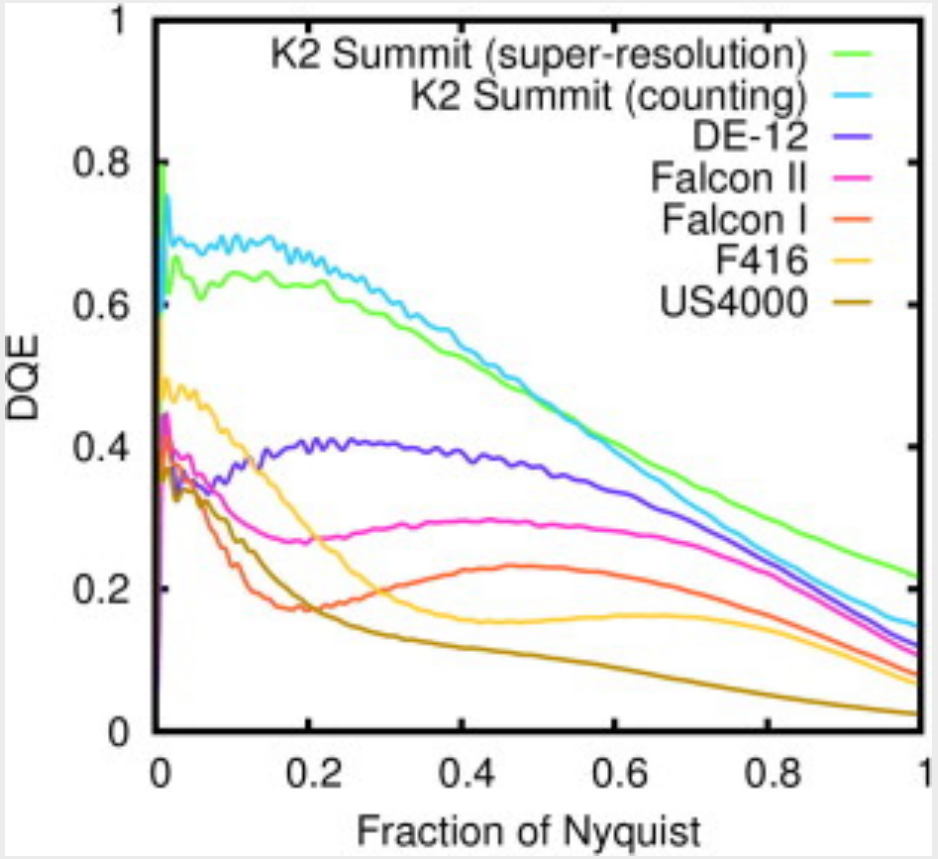
Overview – Limitations

Limitation: Camera fidelity at **localizing electrons**

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

McMullin, 2014 & Ruskin, 2013



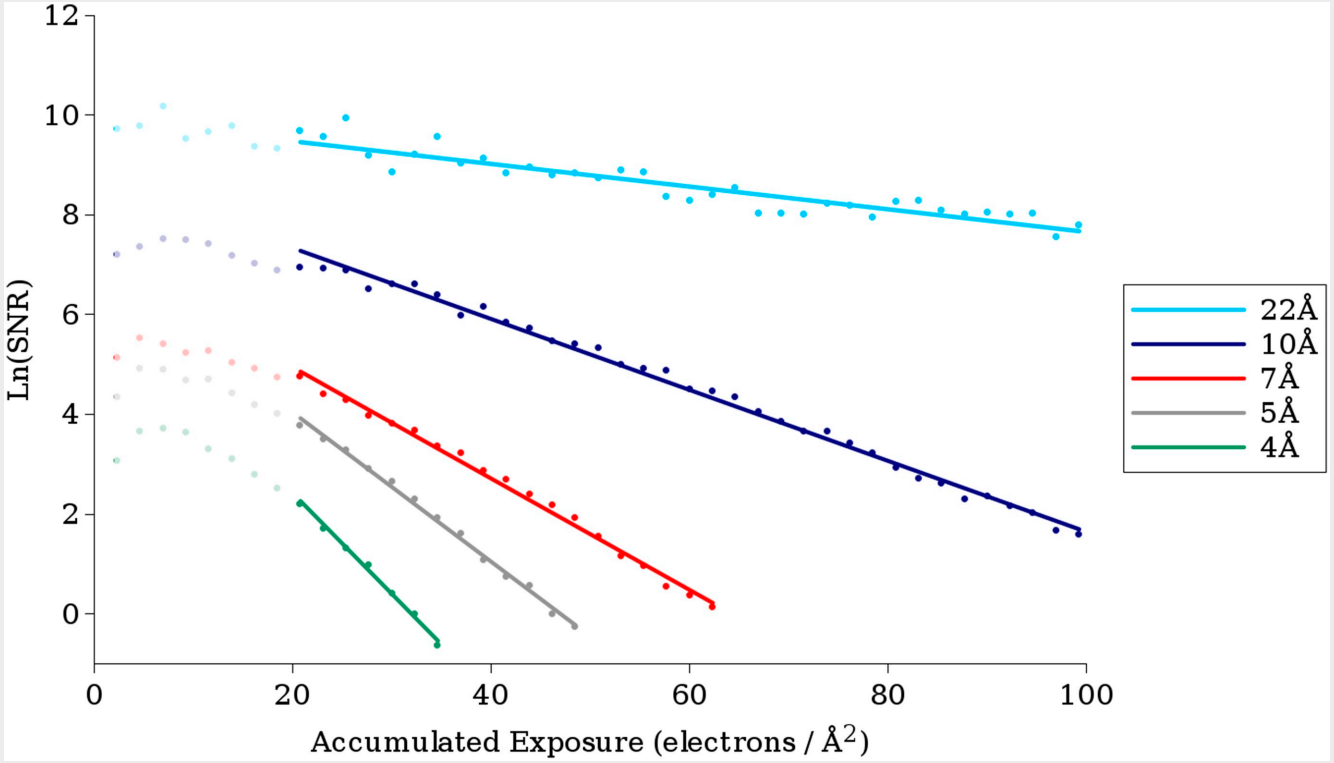


Overview – Limitations

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



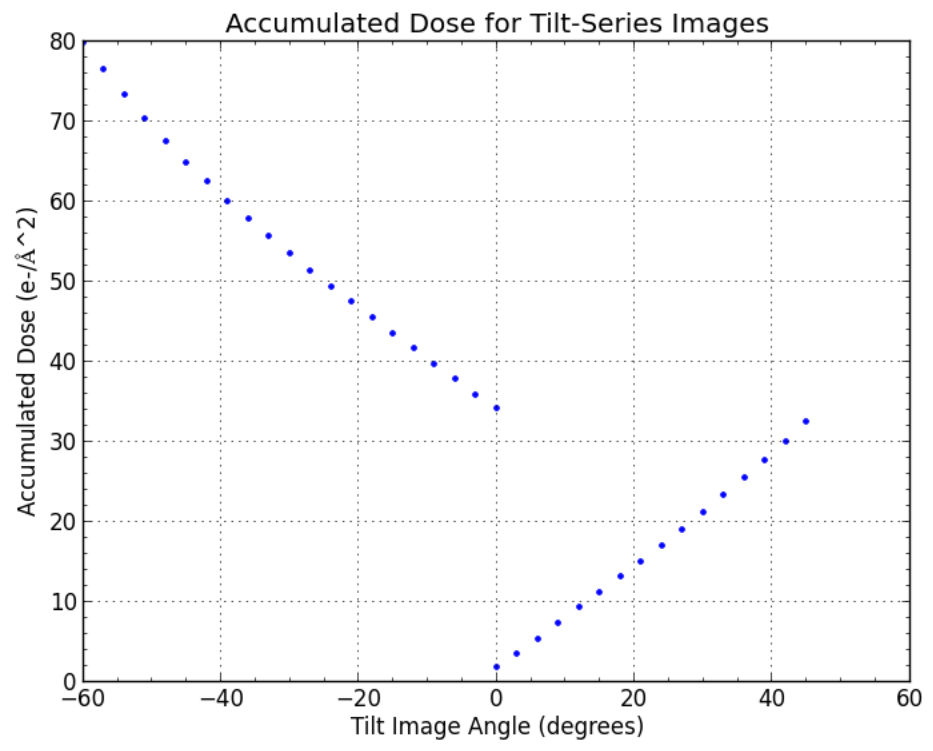
Solution:
Remove damaged information from image frames

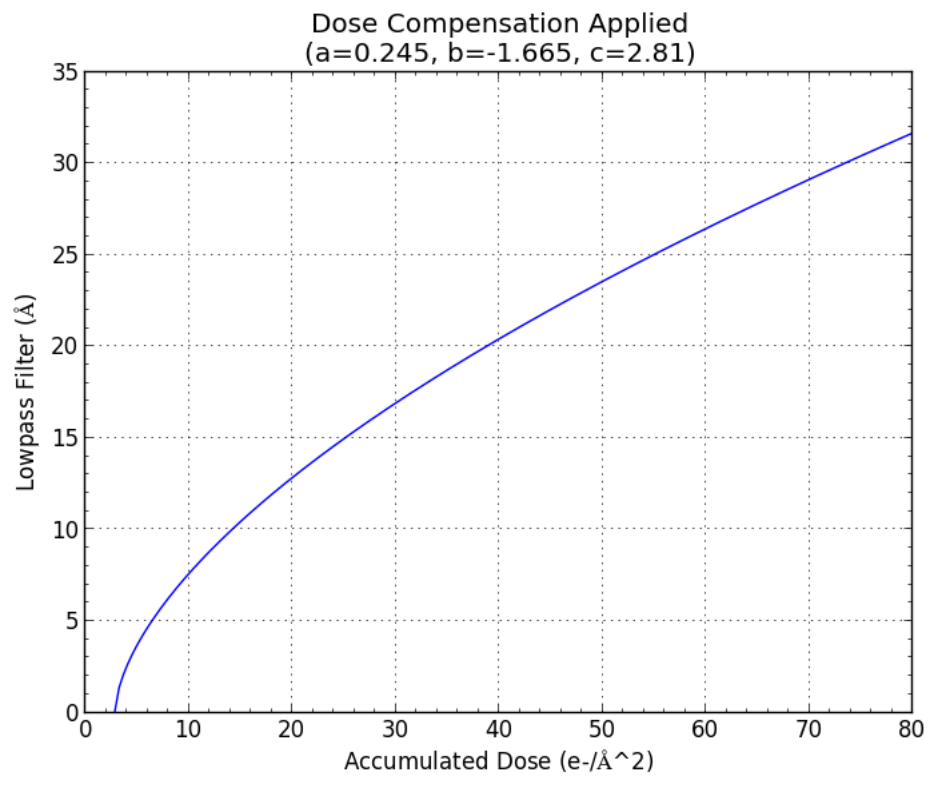
Overview – Limitations

Limitation: **Electron damage** of the specimen

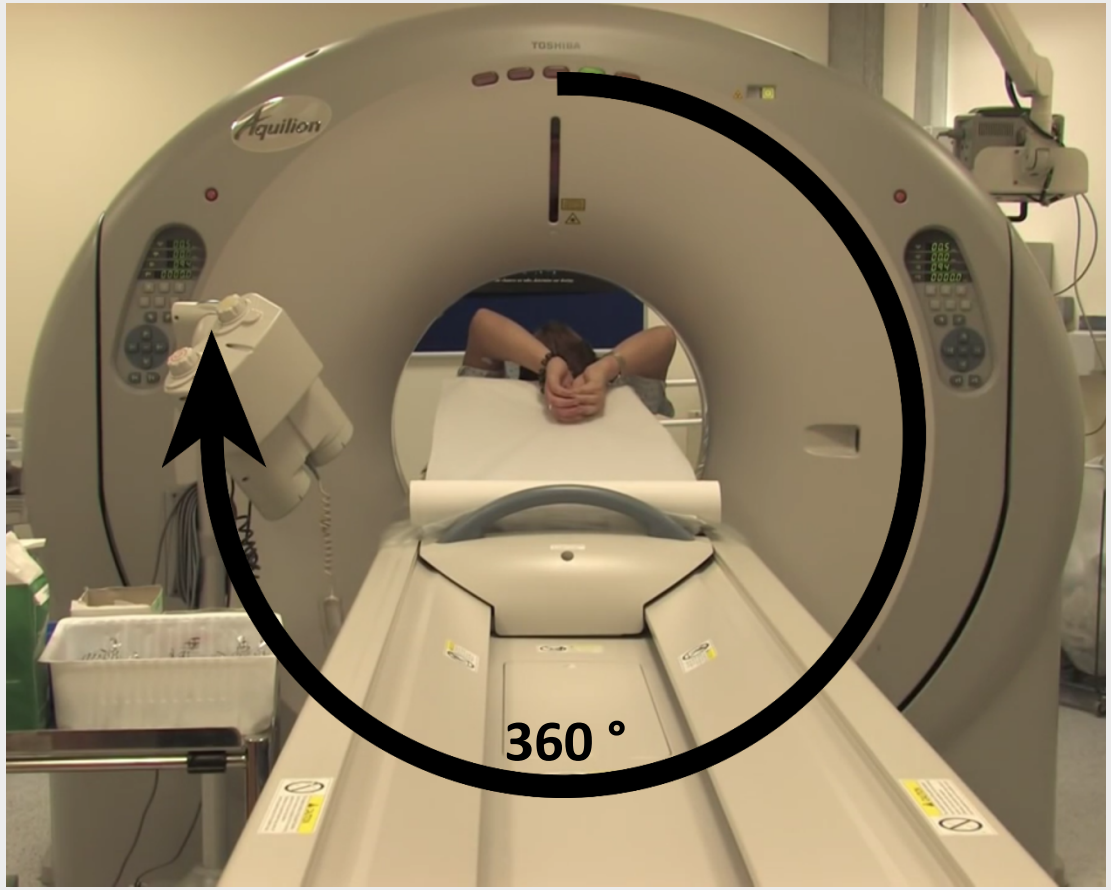
- **Solution:** Remove damaged information from image frames (single particle) or tilt images (tomography):

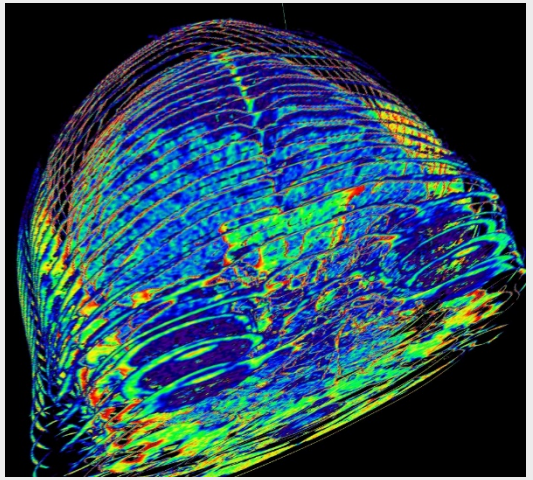
Noble & Stagg, 2015

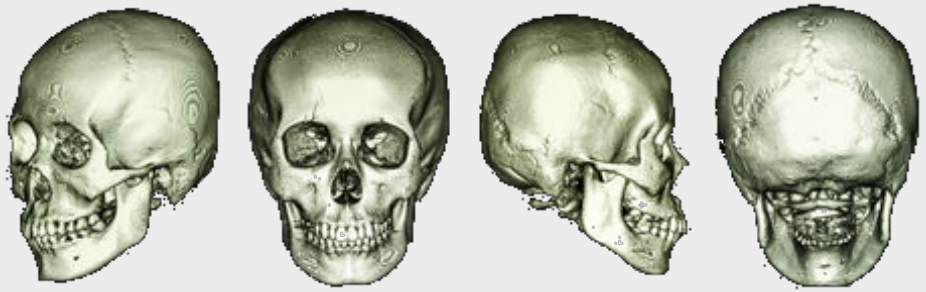




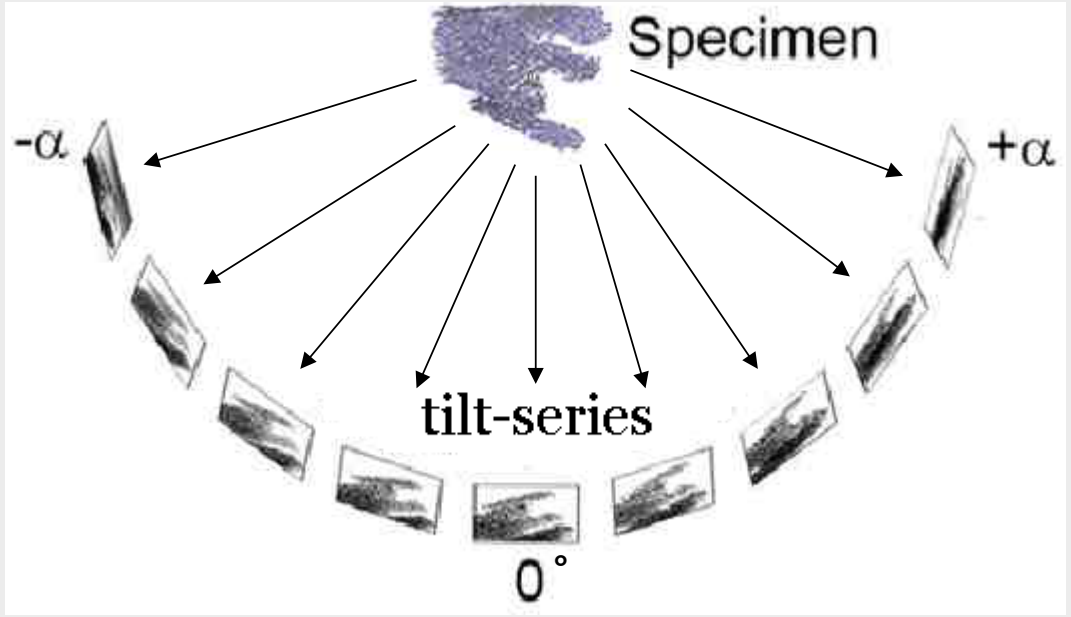
Tomography overview

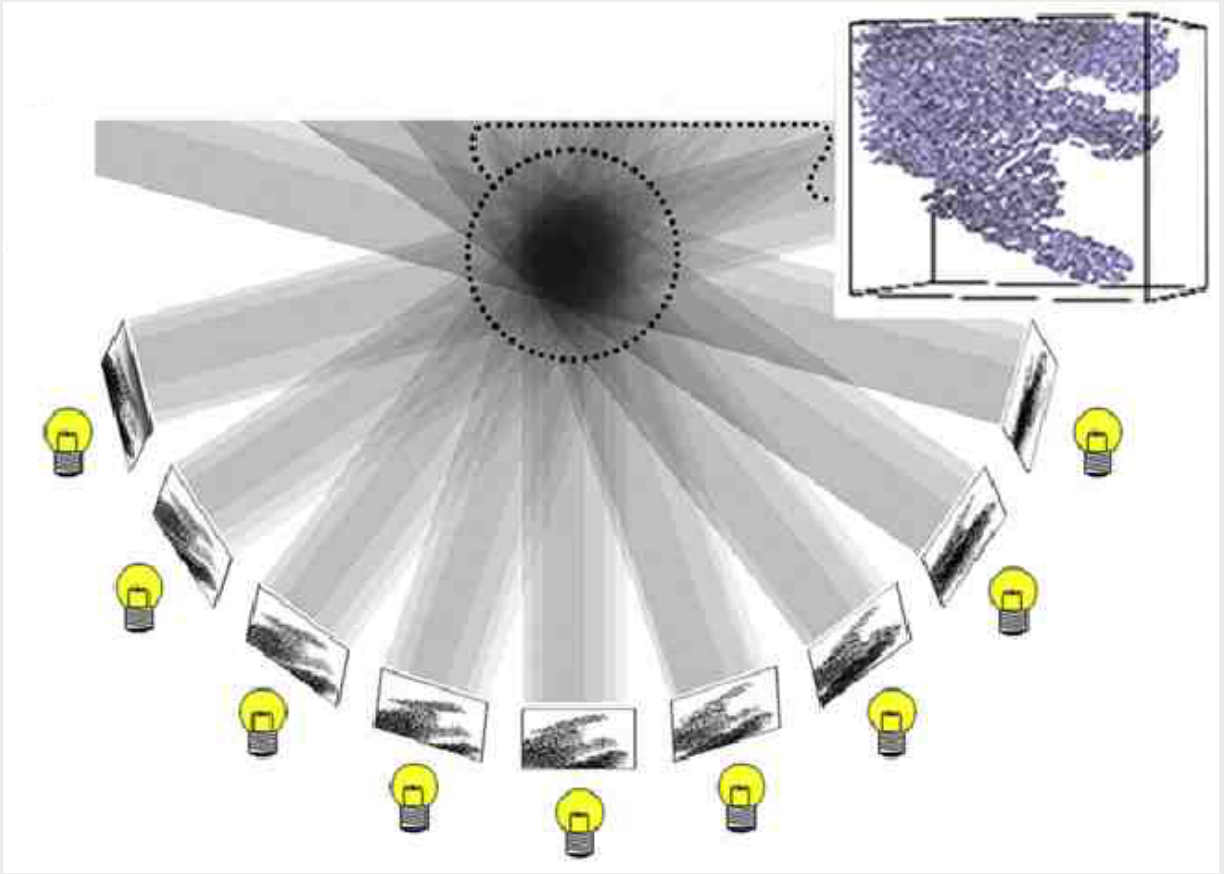






ET/CryoET collection and processing overview



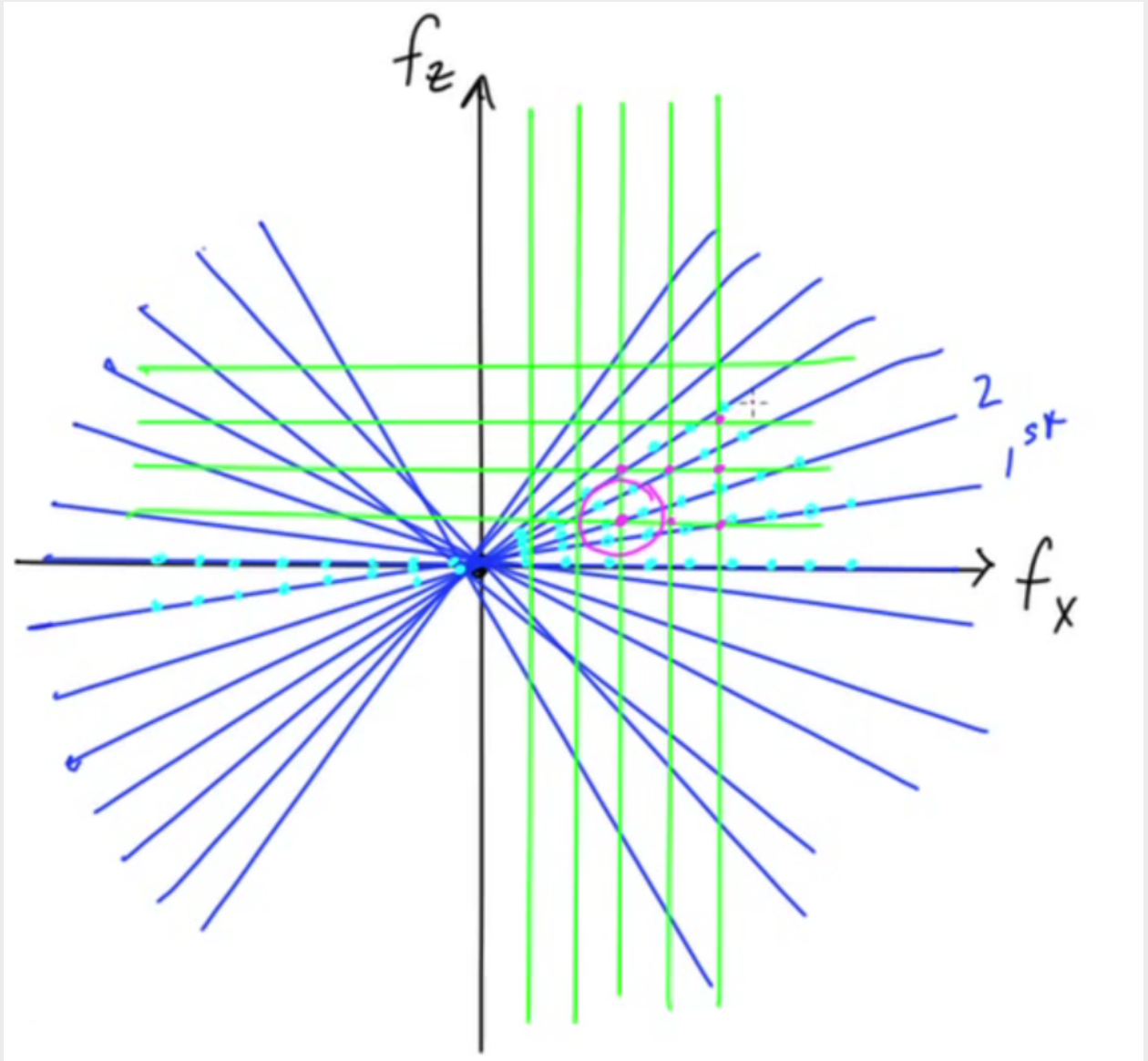


(UMET)

Collect → align → reconstruct

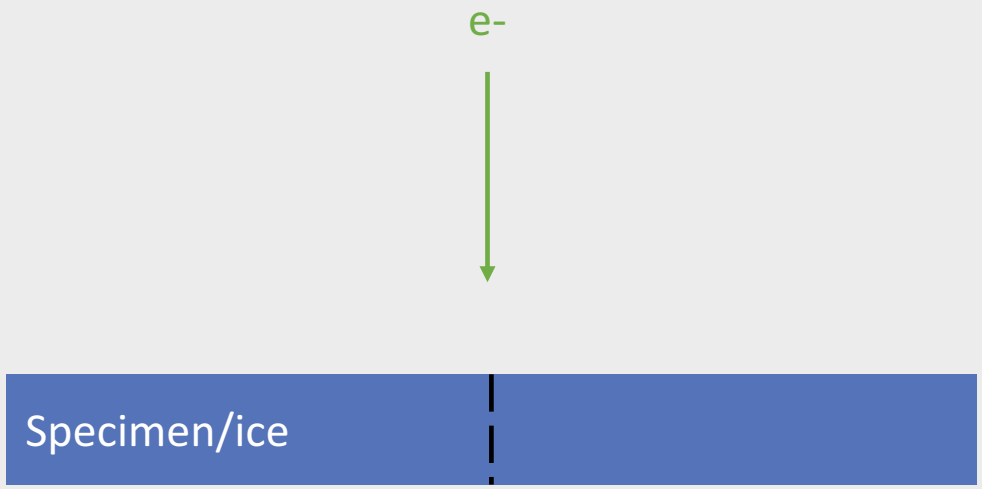
Reconstruction Implies Interpolation

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

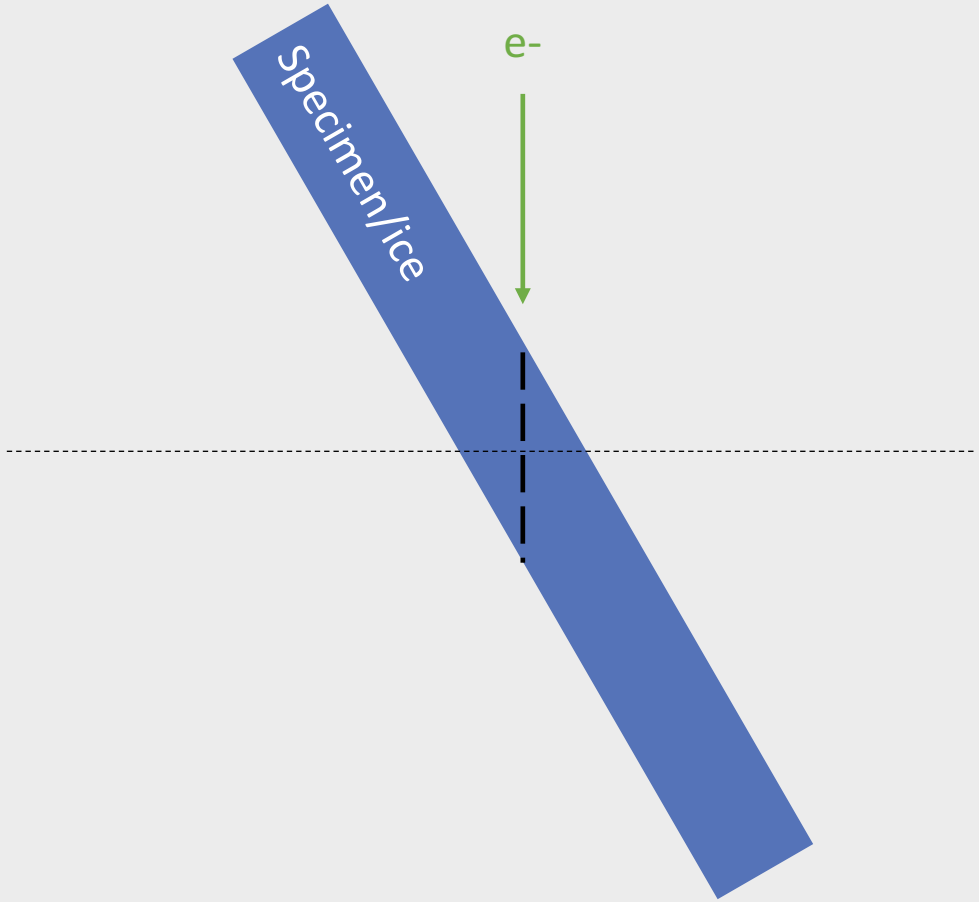


Grant Jensen, YouTube

Grid tilting increases thickness



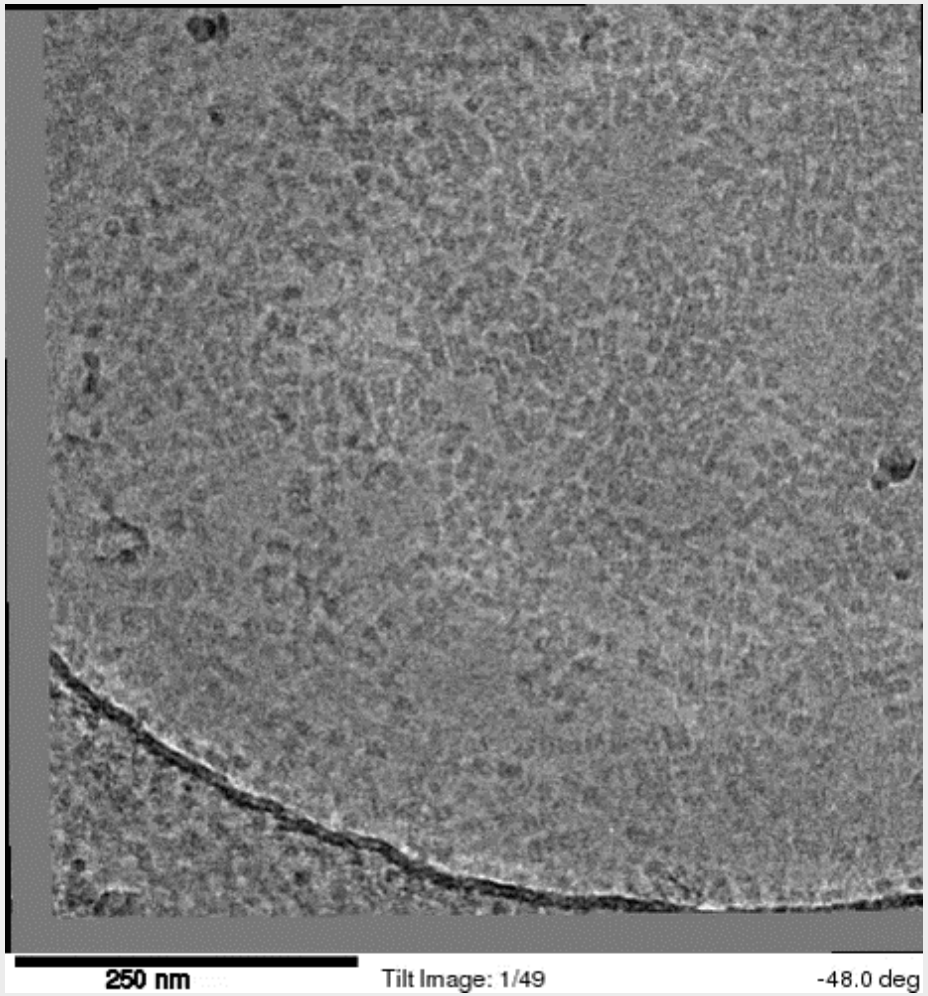
untilted grid



grid tilted 60° = 2x thickness

Grid tilting thickness increase limits tilting

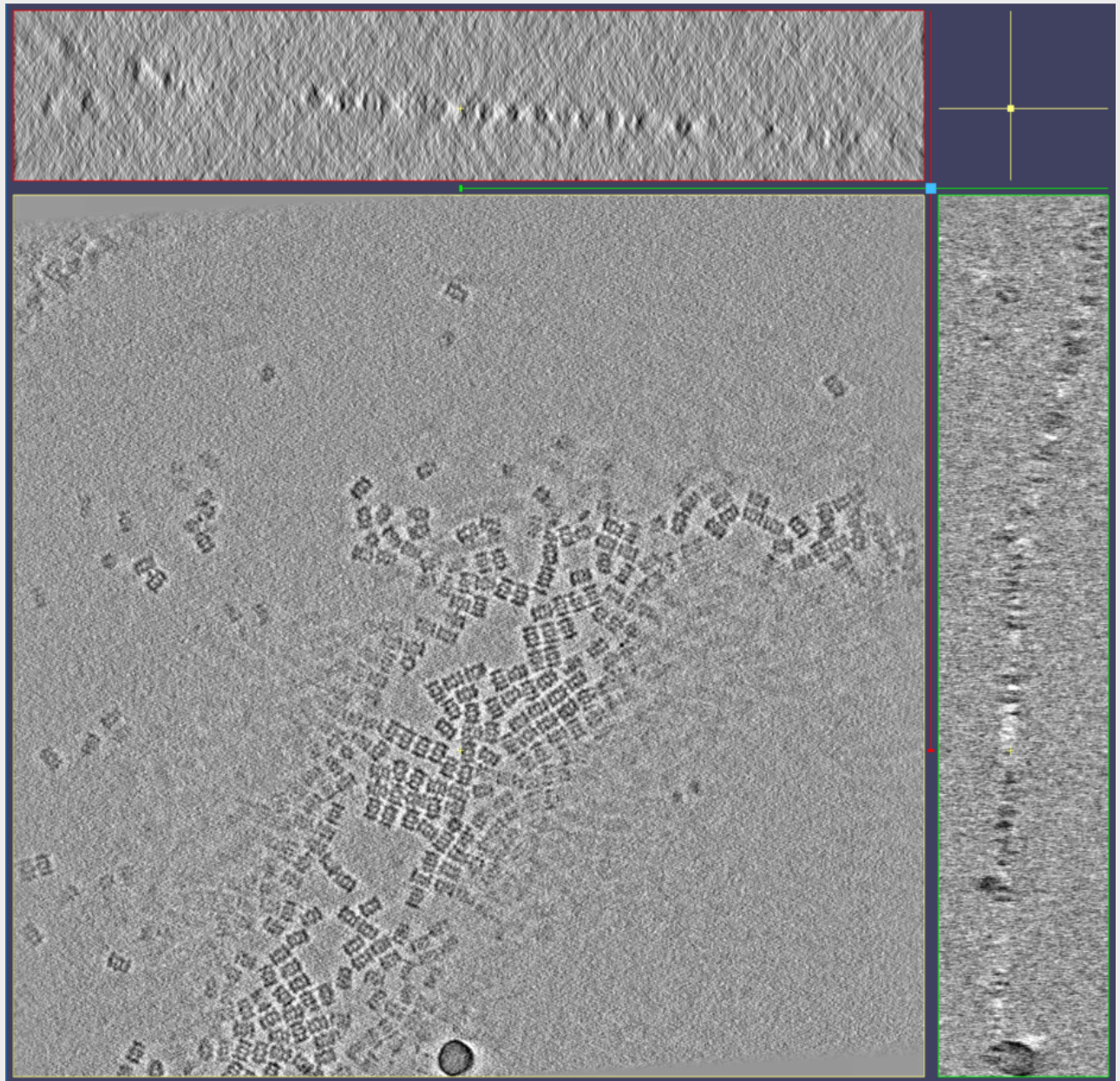
Noble, 2017



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

Grid tilting limit results in missing information

Noble, 2017



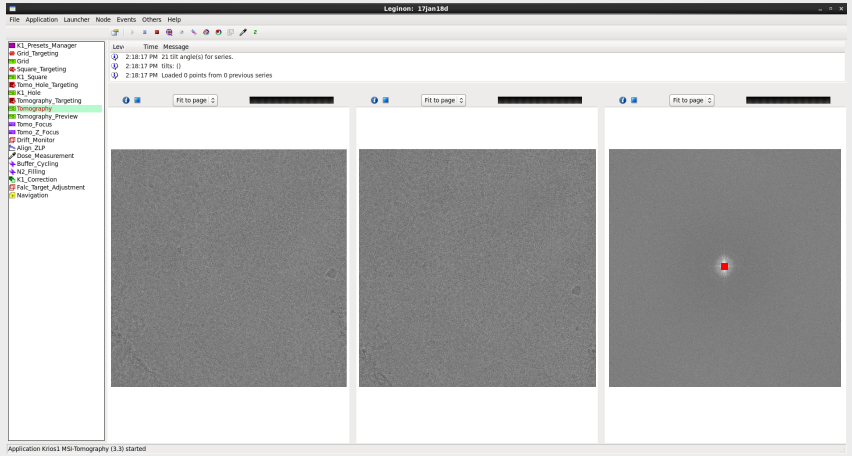
Phase plate tilt-series

of T20S Proteasome

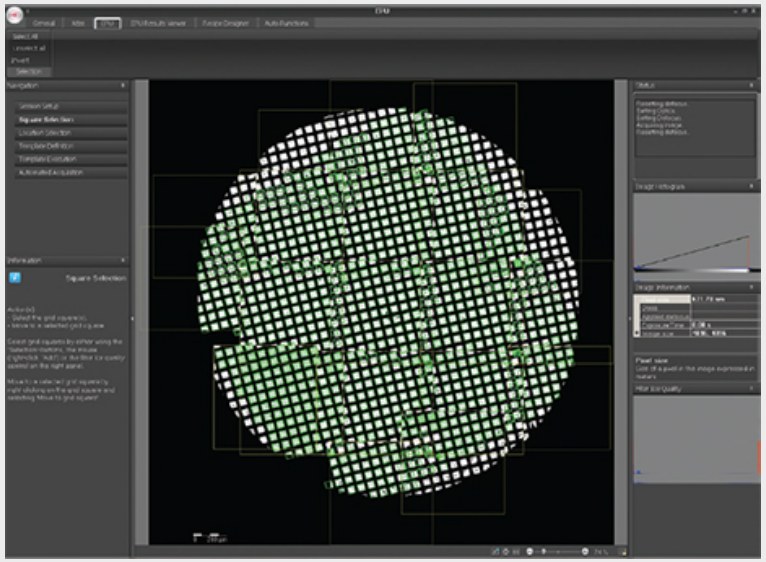
Tilt axis is **vertical**

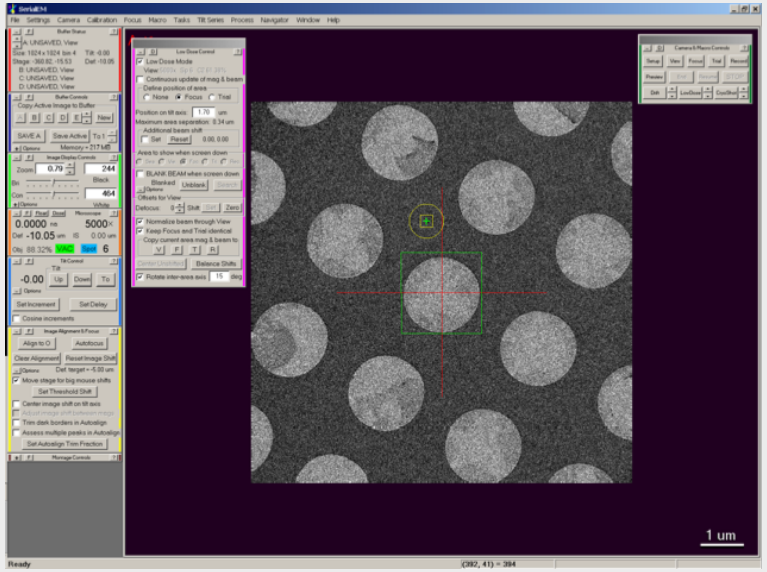
Tilt-series collection software

Leginon

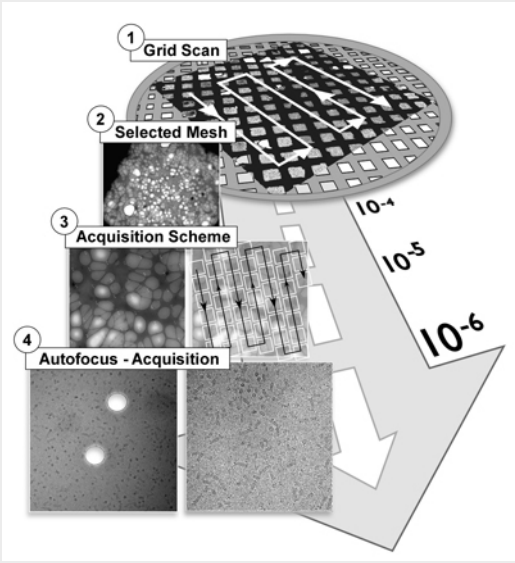


EPU

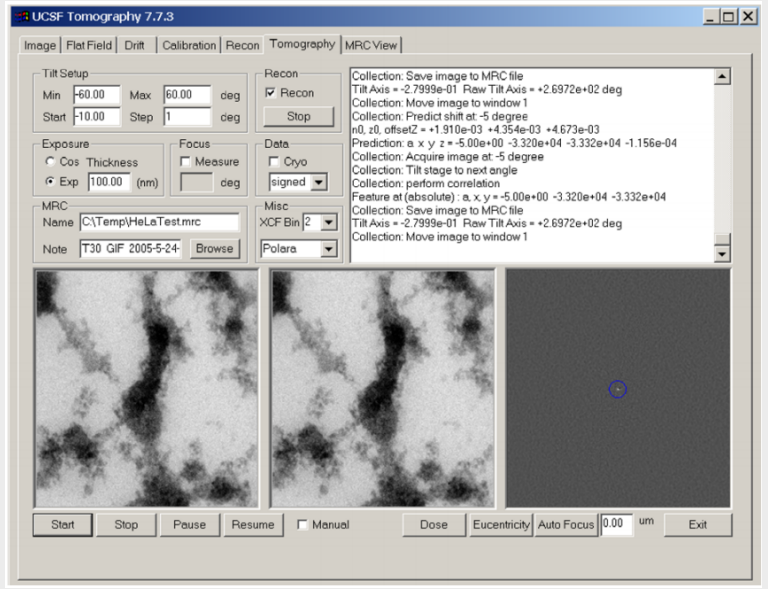




SerialEM



TOM Toolbox



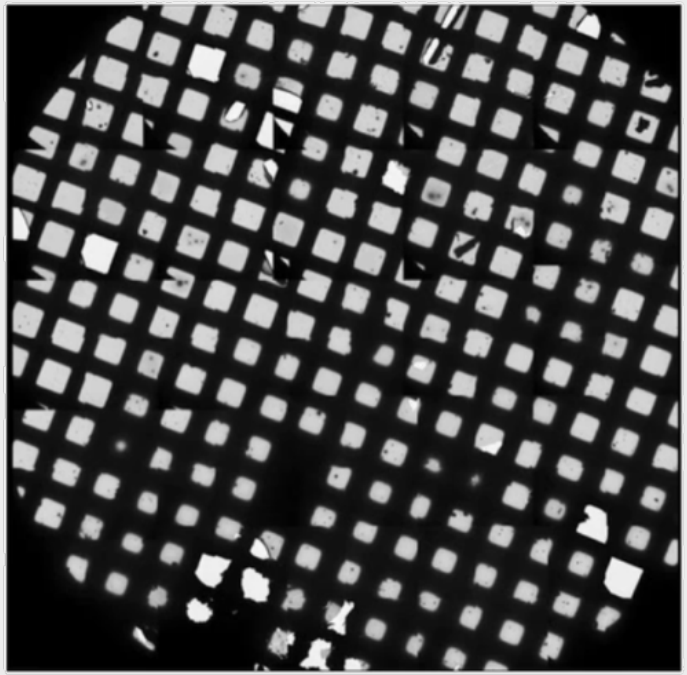
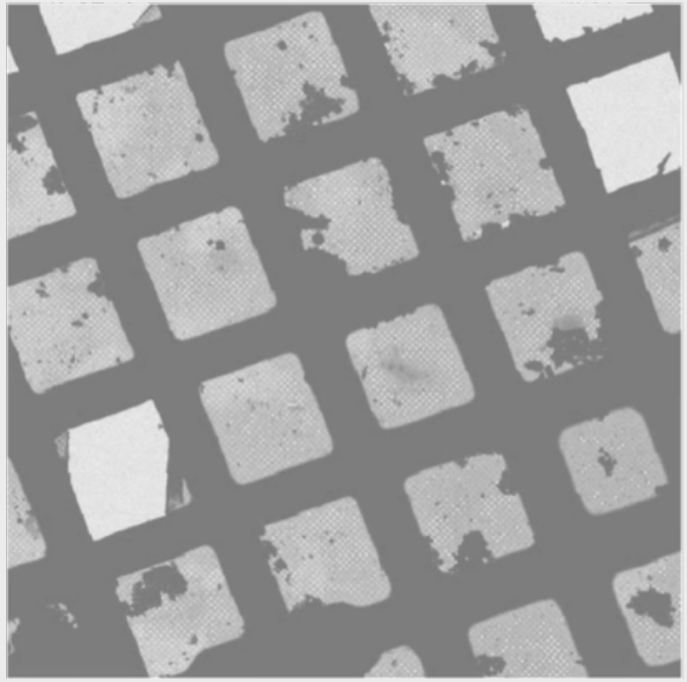
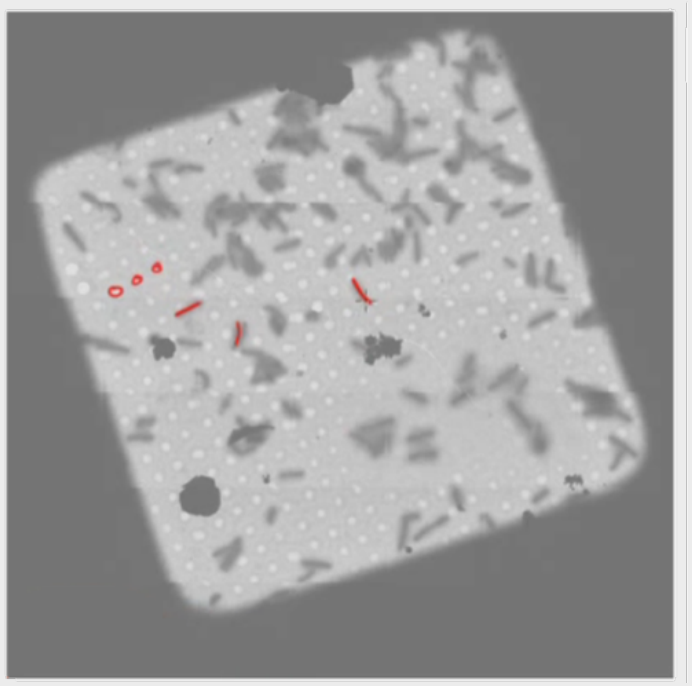
UCSF Tomography

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 the software will go to to re-focus and re-track.

Automated tilt-series collection

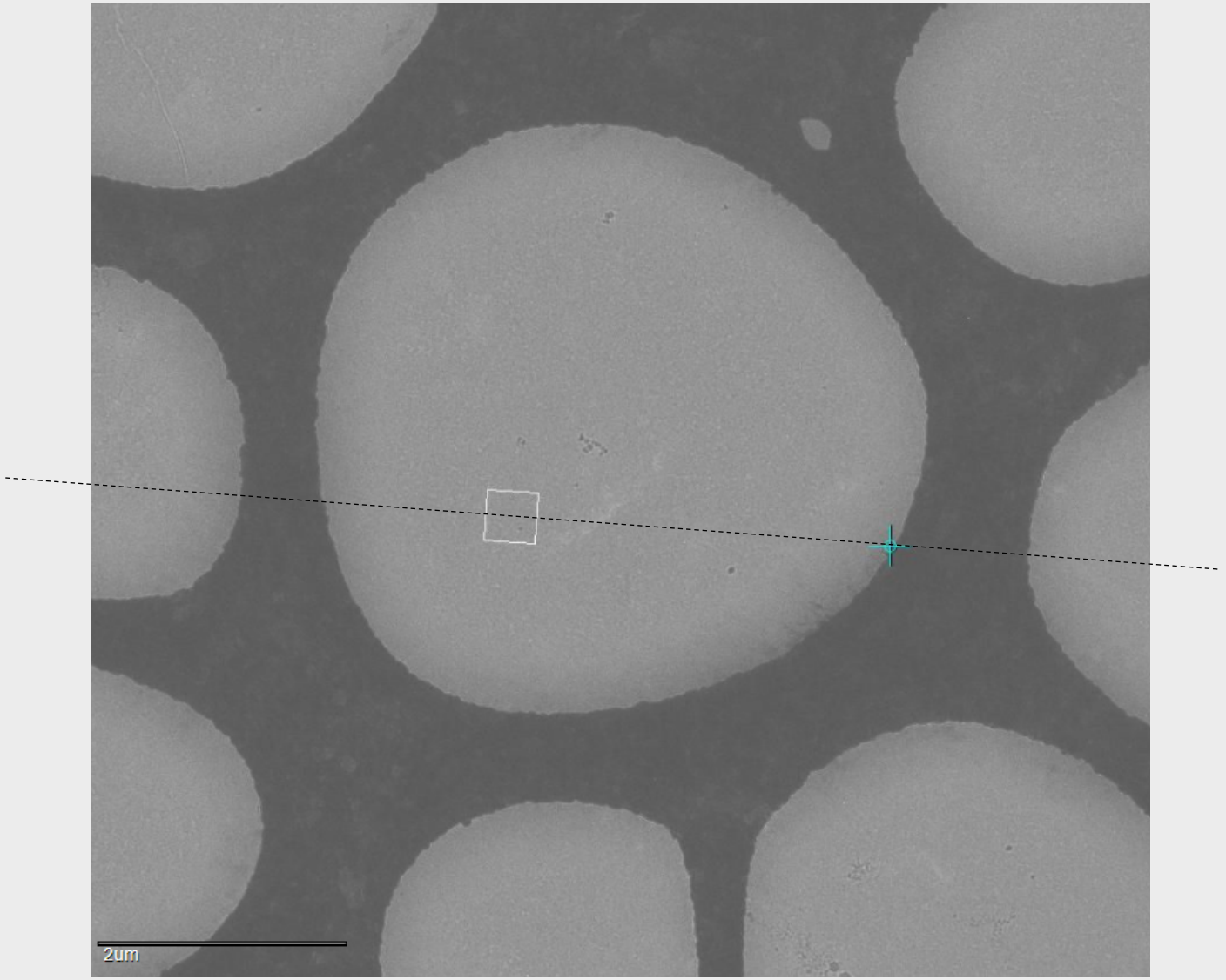
Automated tilt-series collection is currently **routine**

- 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**.



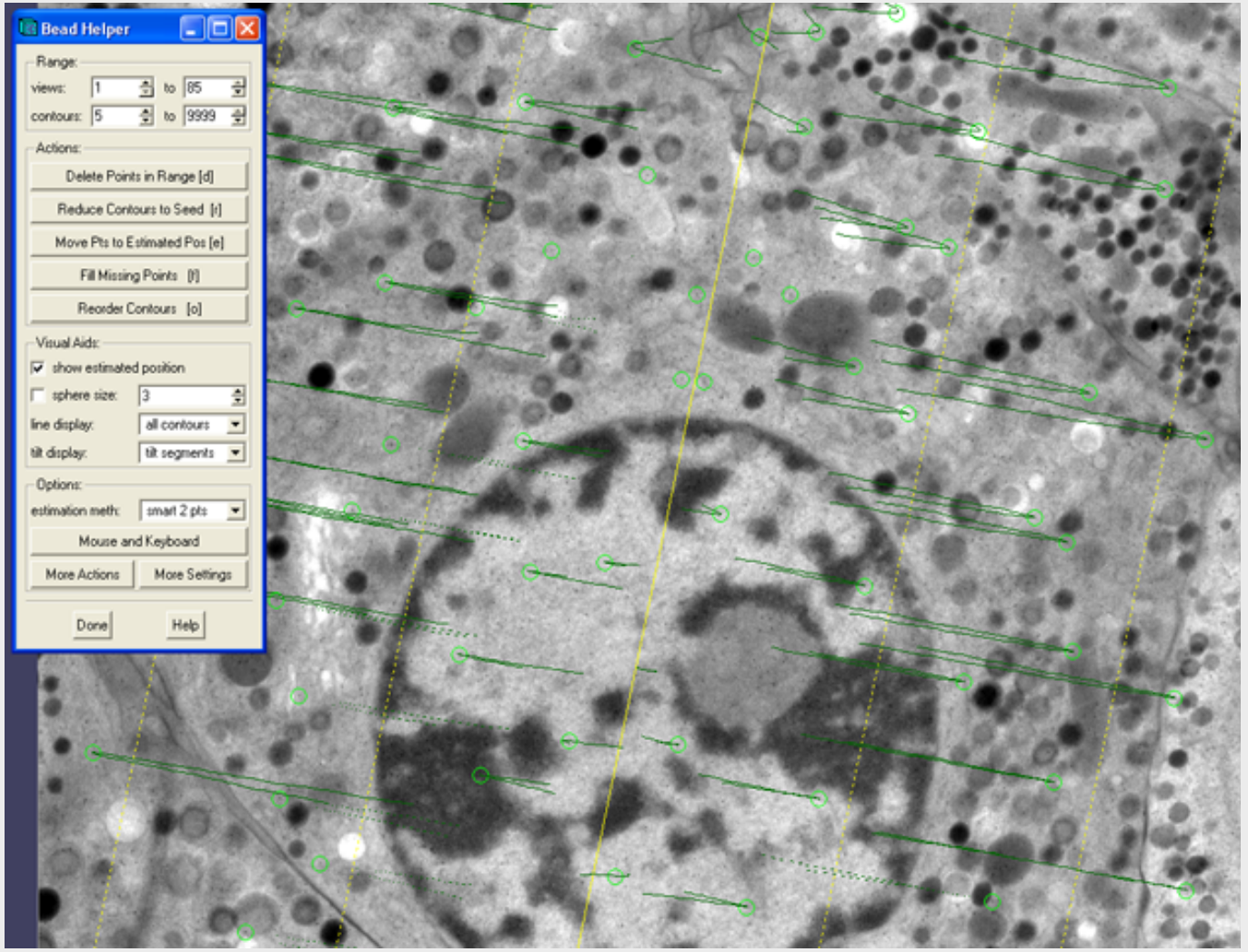
Tilt-series alignment

- **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

- **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) or automated (markerauto, IMOD) processing



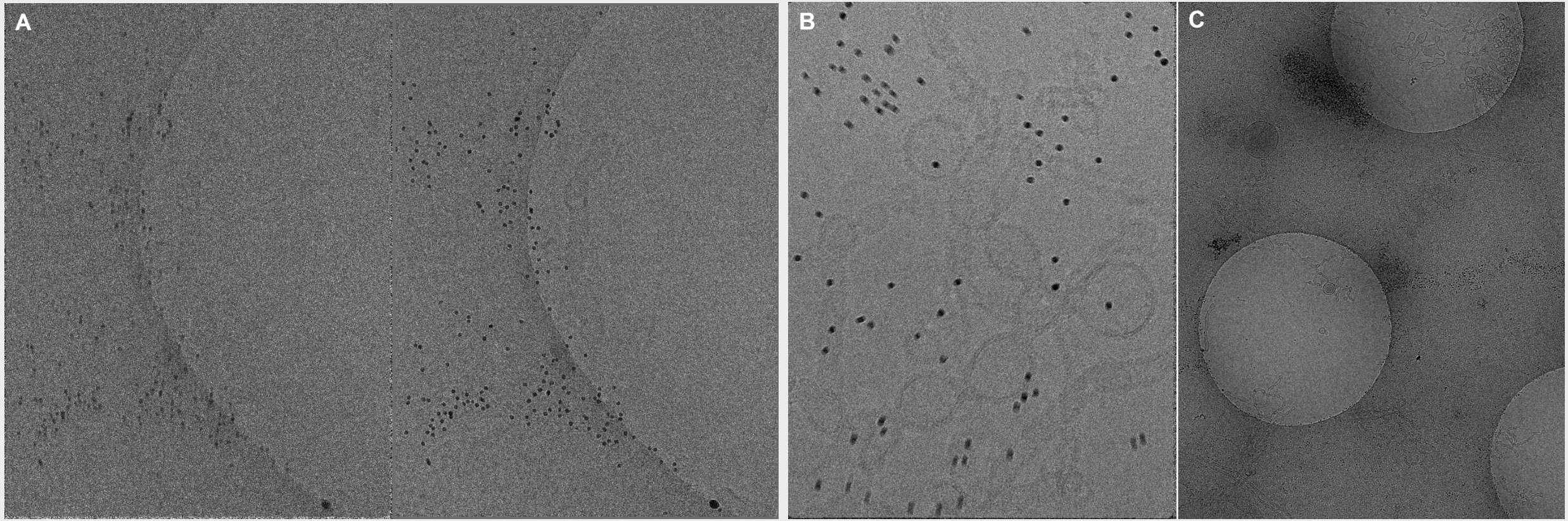
http://bio3d.colorado.edu

Fiducial-based tilt-series alignment issues

Fiducial Movement

Anisotropic Bead Motion

Bead Aggregation

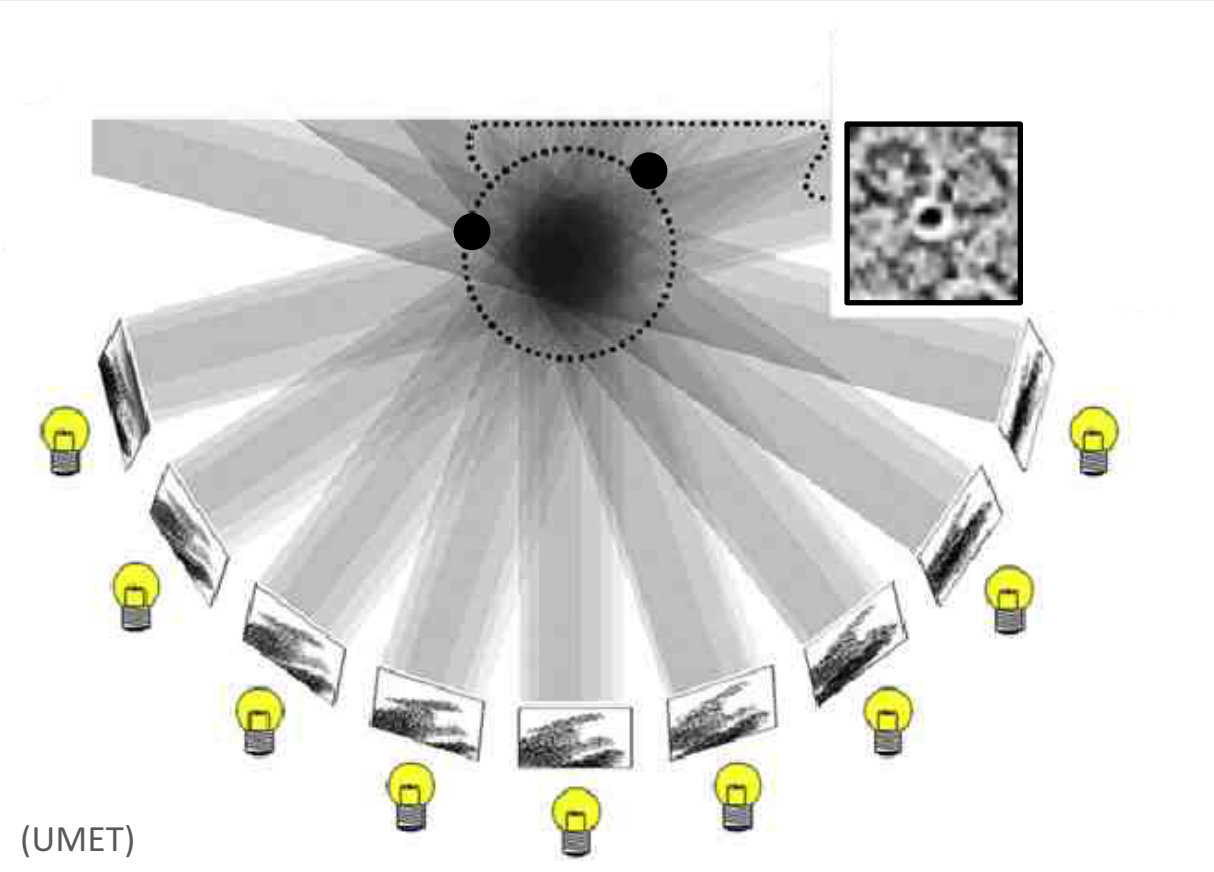


DE-20 @ 18kx; 51°, 2.34 $e^{-}/Å^2$ after a cumulative dose of 60 $e^{-}/Å^2$

DE-20; 57.5 $e^{-}/Å^2$, 0° exposure

Noble & Stagg, JSB 2015

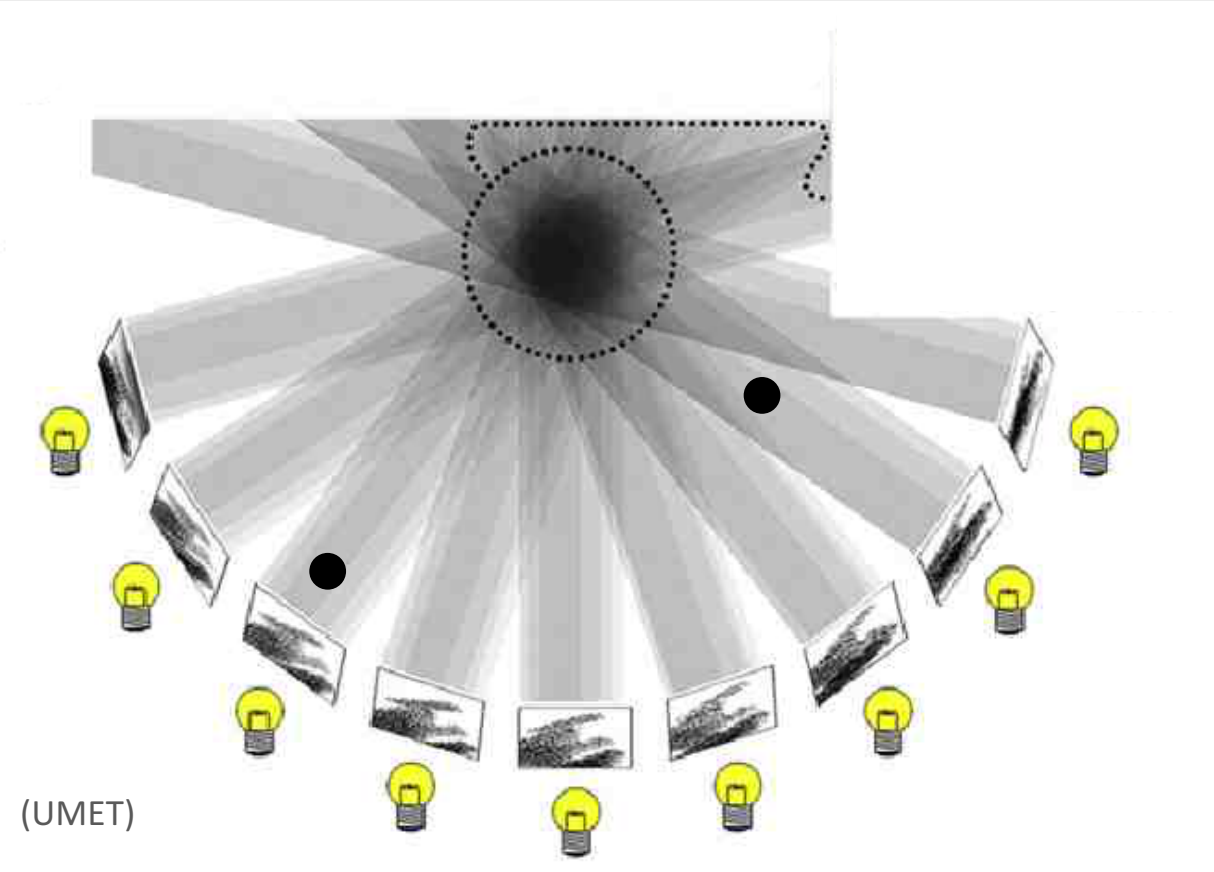
Fiducial-based tilt-series alignment issues


(UMET)

Nearby Fiducials Affect Signal and Contrast

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

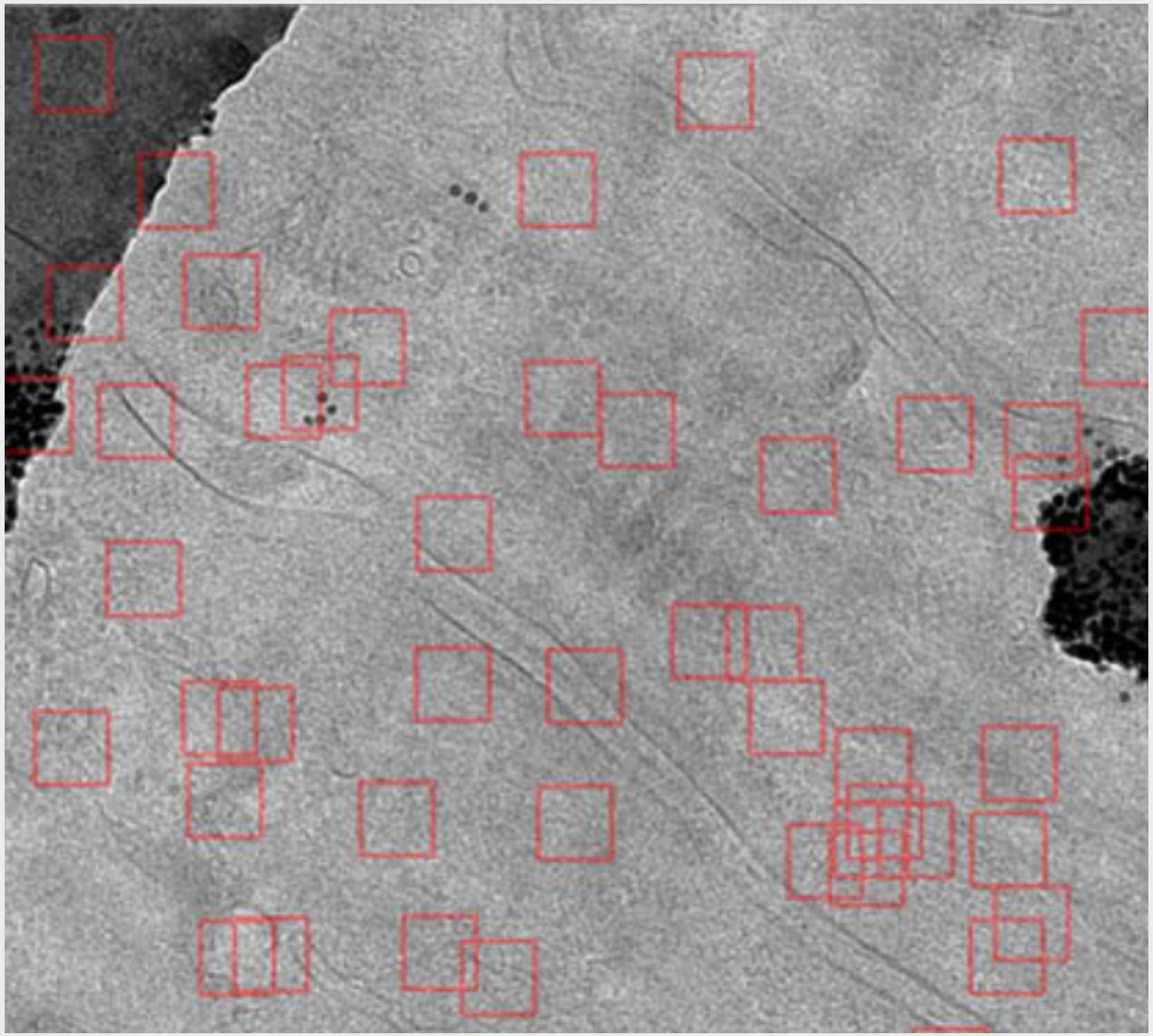
Fiducial-based tilt-series alignment issues


(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

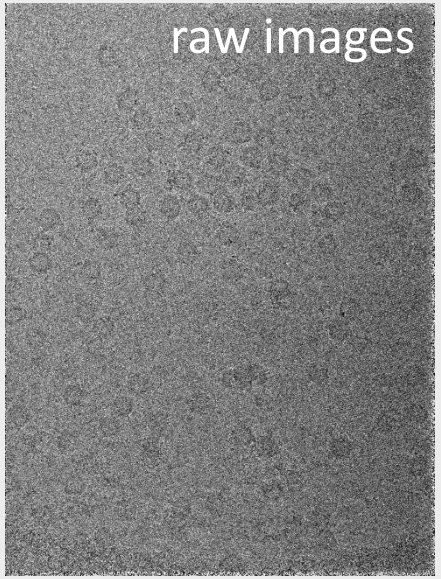


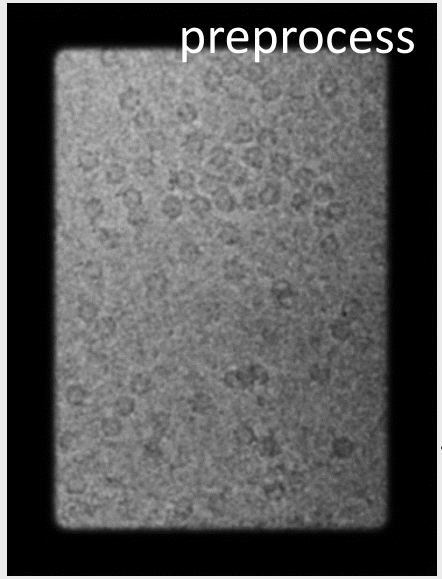
Castaño-Díez, 2010

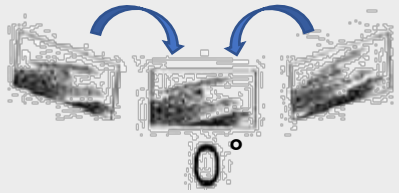
Identify featureful objects with contrast in all tilt images and track them.

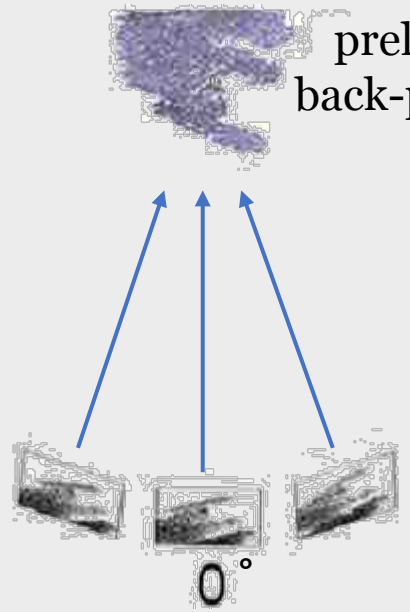
- Semi-automated (IMOD, Alignator)

Fiducial-less tilt-series alignment (Protomo)

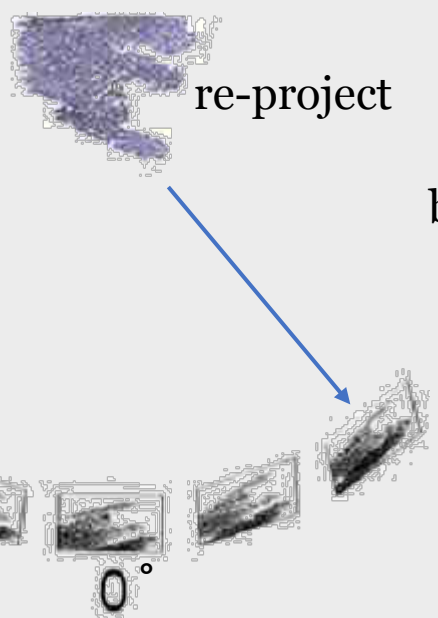


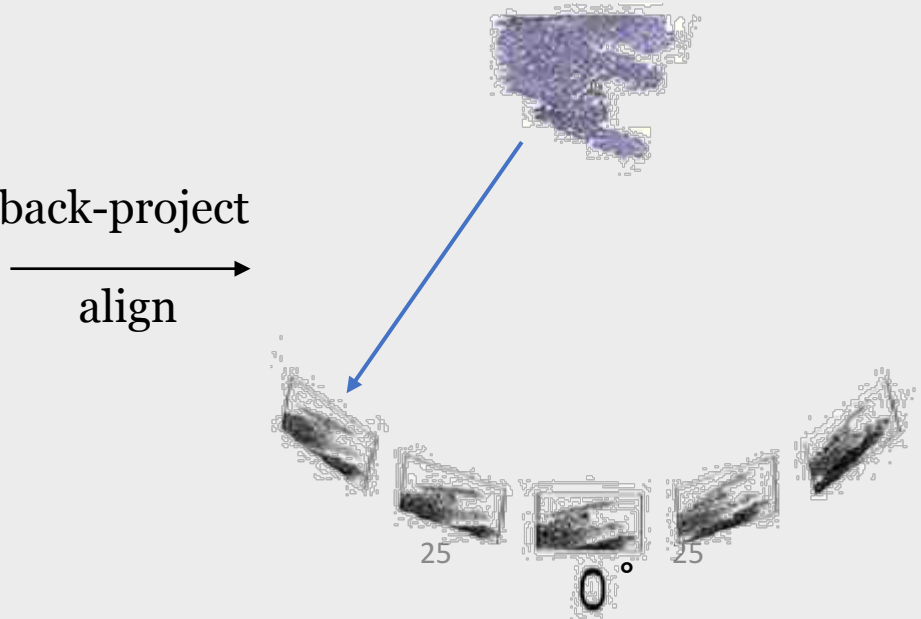


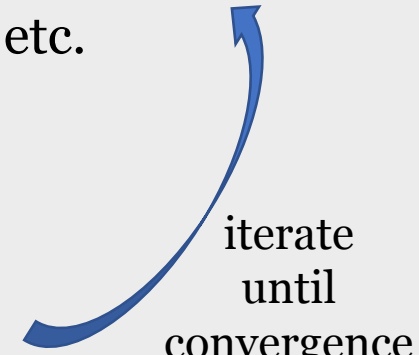




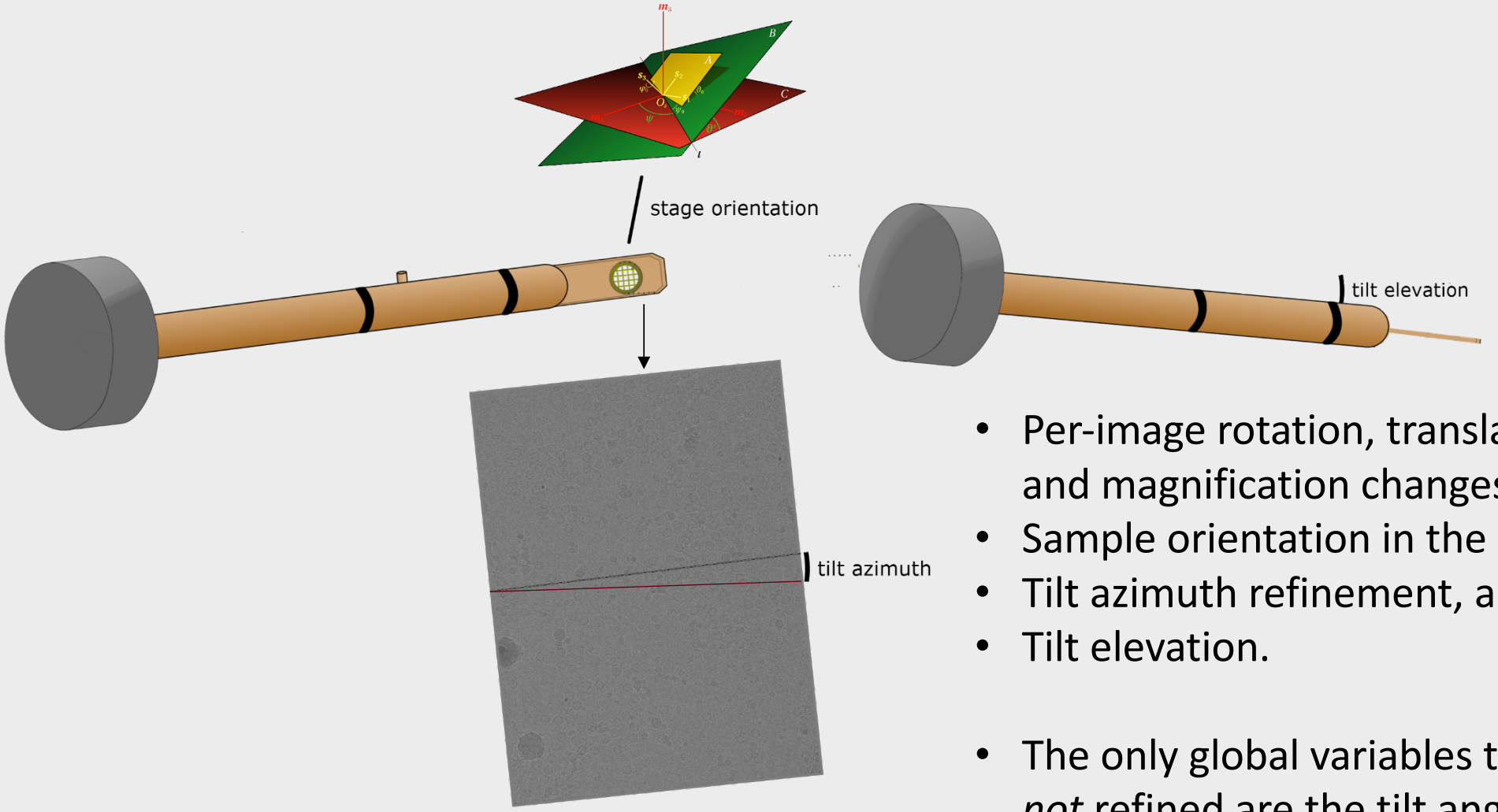
- Re-projection matching
- Correlation
- Common lines







Fiducial-less tilt-series alignment (Protomo)

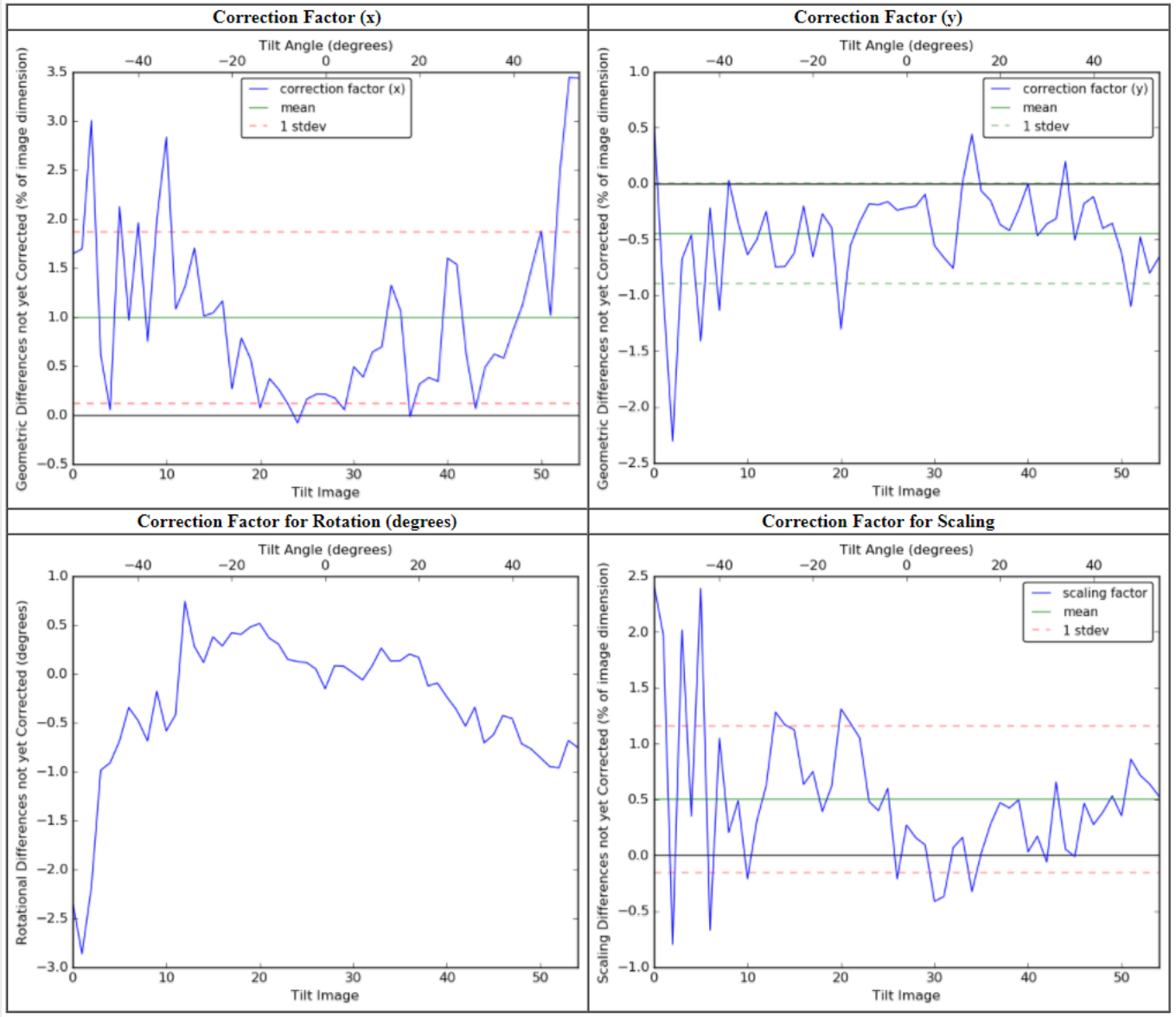


- Per-image rotation, translation, and magnification changes,
- Sample orientation in the ice,
- Tilt azimuth refinement, and
- Tilt elevation.

- The only global variables that are *not* refined are the tilt angles.

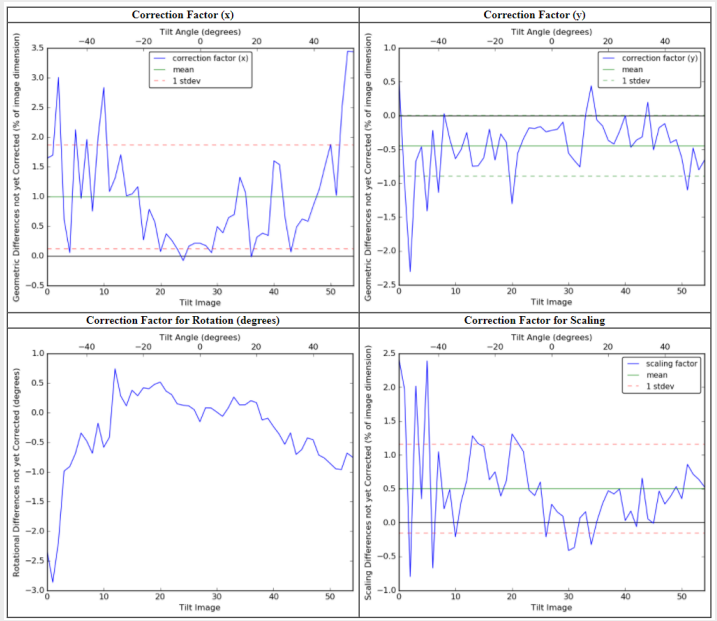
Winkler & Taylor, JSB 2006

Protomo measures alignment errors for each image

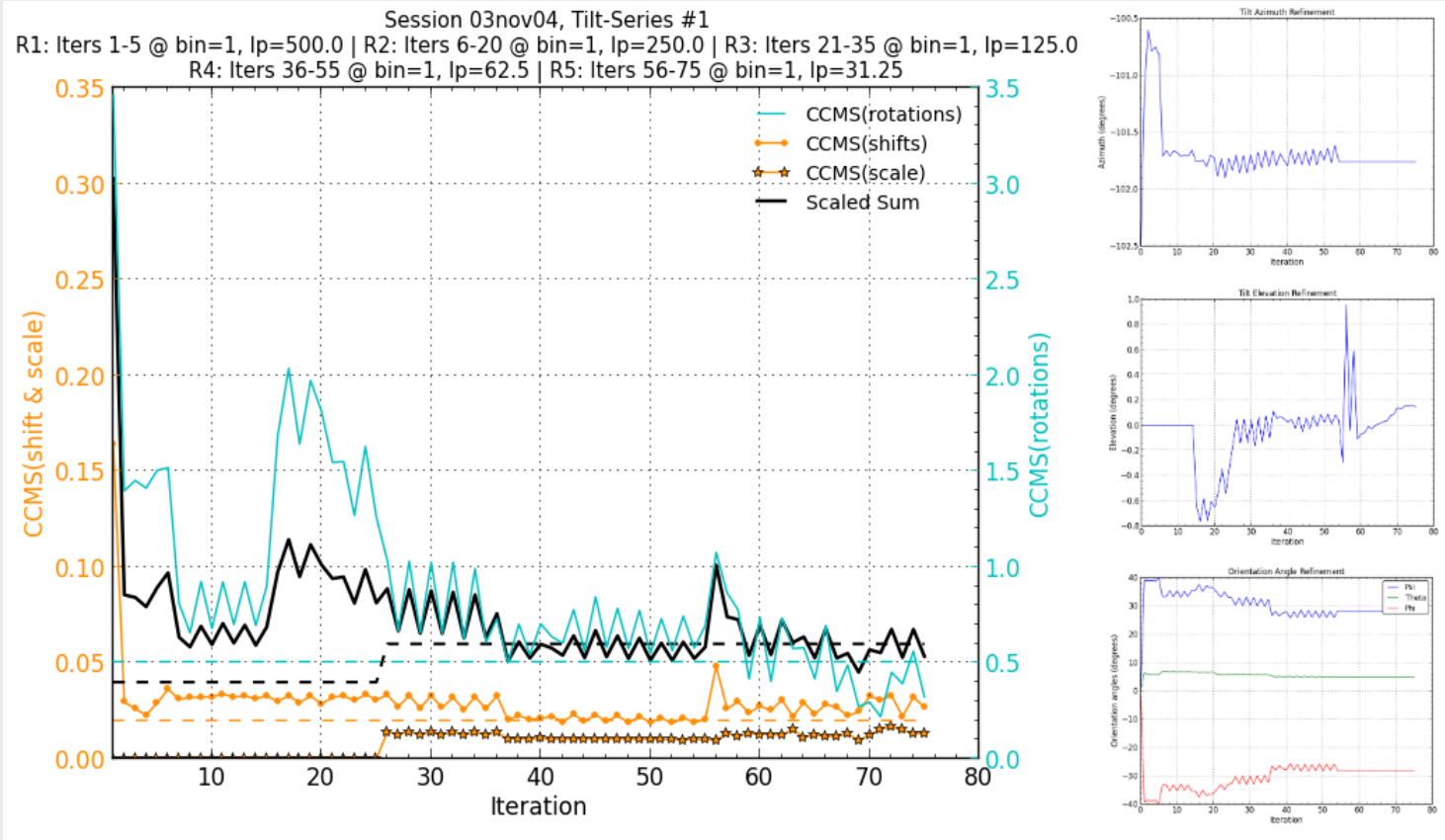


- Alignment is performed by **matrix diagonalization**,
- Residual **off-diagonal** are recorded as **errors**
  - Per image errors in translation, rotation, and scaling.

Appion-Protomo automated Protomo



Per-Iteration



All Iterations

Tilt & Sample Geometry

Goal becomes:

1) Minimize all image transformation errors,
2) Stabilize tilt and sample geometry.

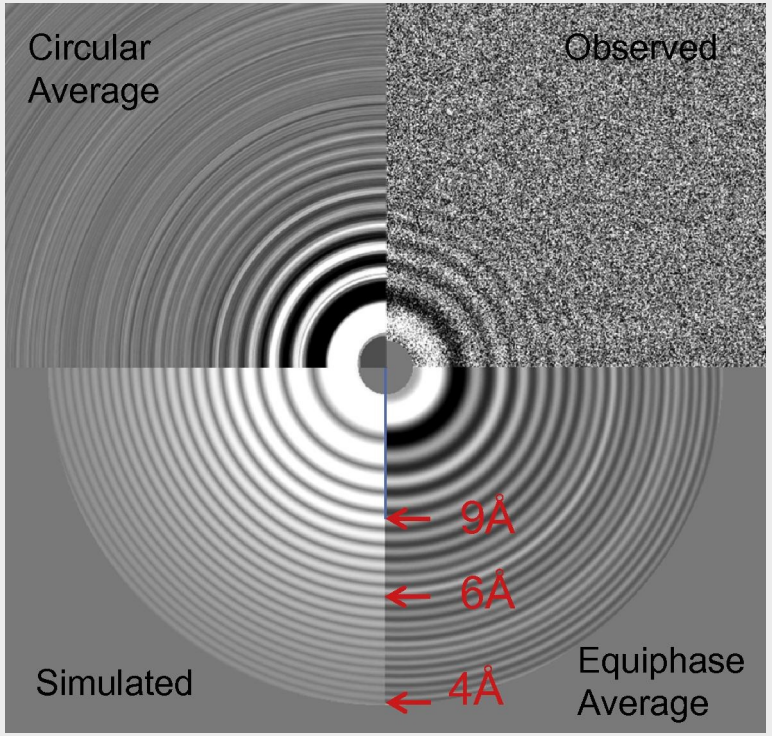
https://github.com/nysbc/appion-protomo

Defocus estimation

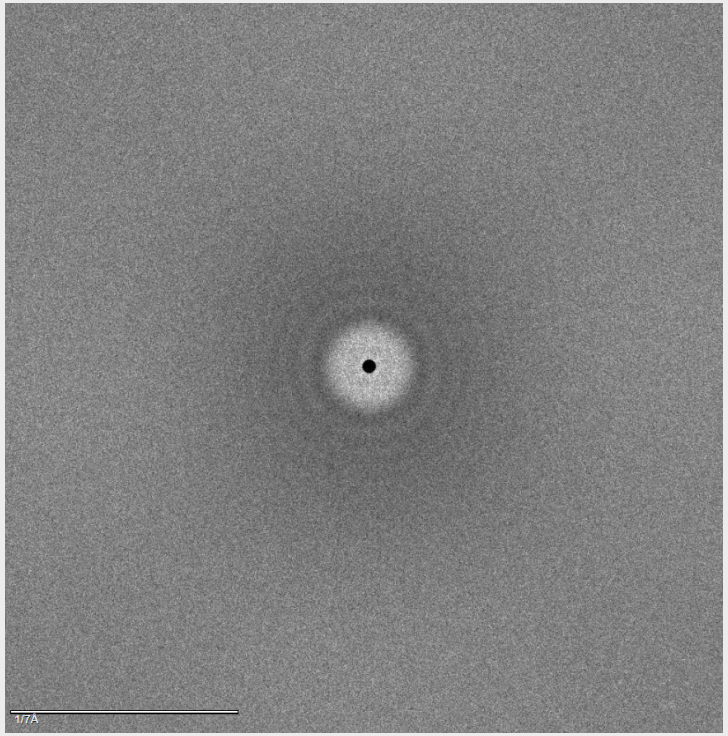
Goal: Find the **height of your objects** of interest to correct for microscope aberrations (CTF)

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):

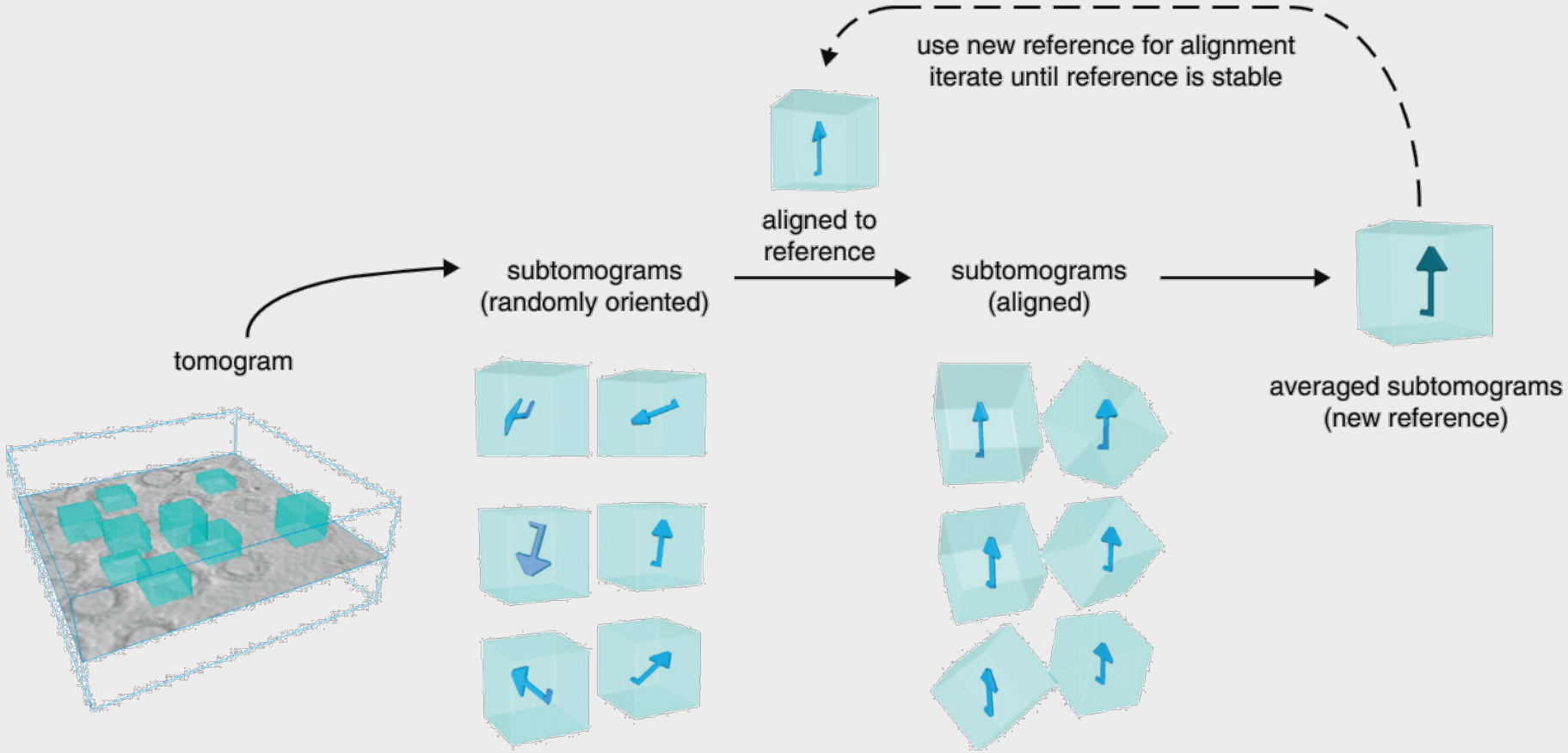
- **Per-image** defocus estimation accounting for tilts (CTFFIND4, GCTF, etc.)
- Per-tomogram post-hoc estimation by using **SPT FSC to locate the first CTF zero**
- **Image tiling** to estimate the **defocus of the untilted plane** (TomoCTF)
- Defocus estimation and **interpolation using two focus locations**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

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 workflow



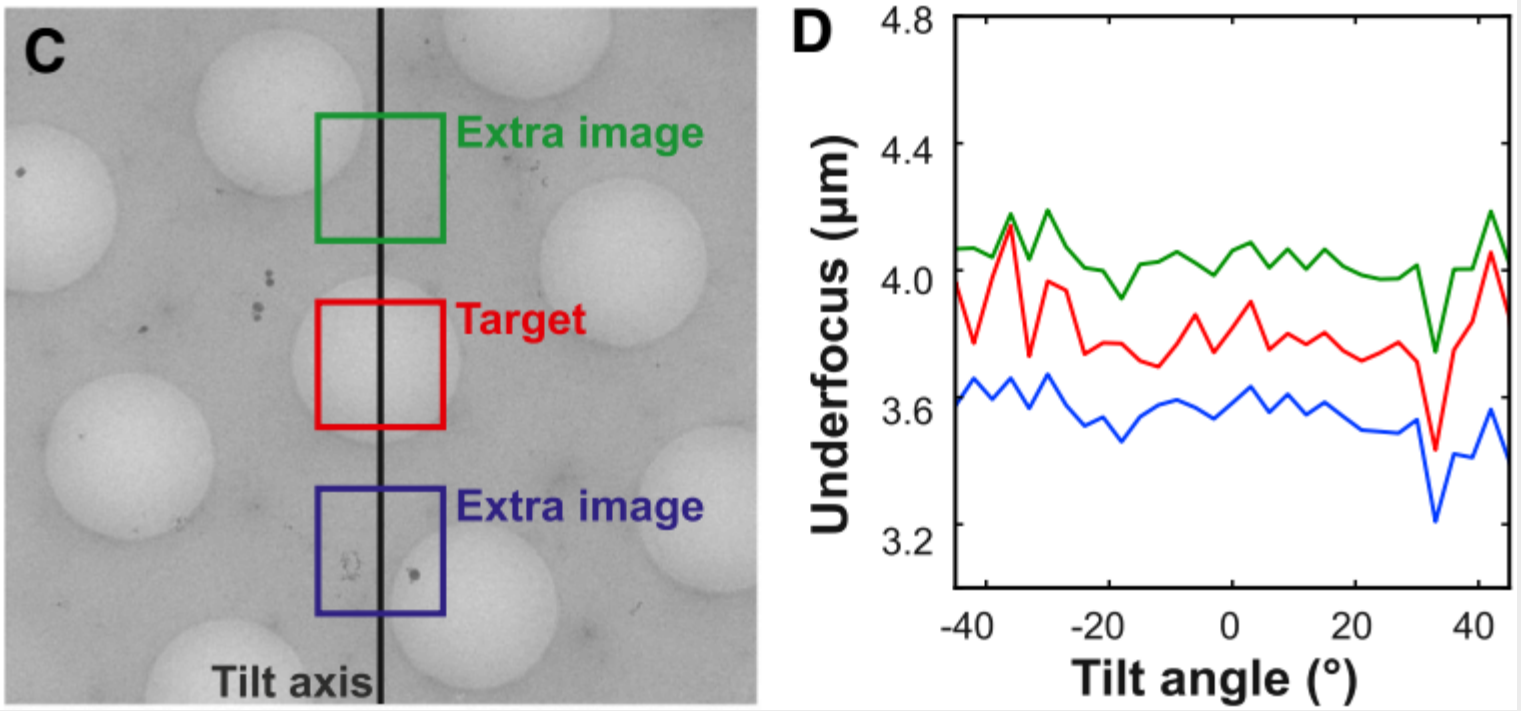
Briggs 2013

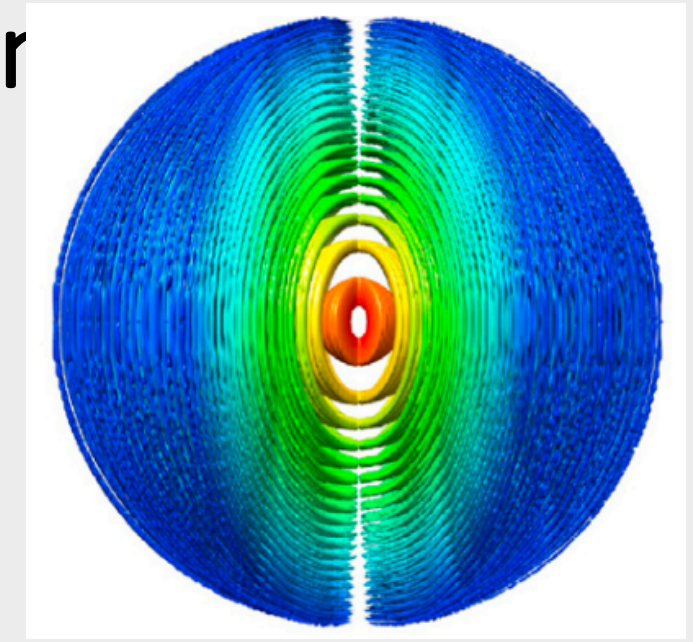
- **Missing wedge** must be taken into account for each sub-tomogram

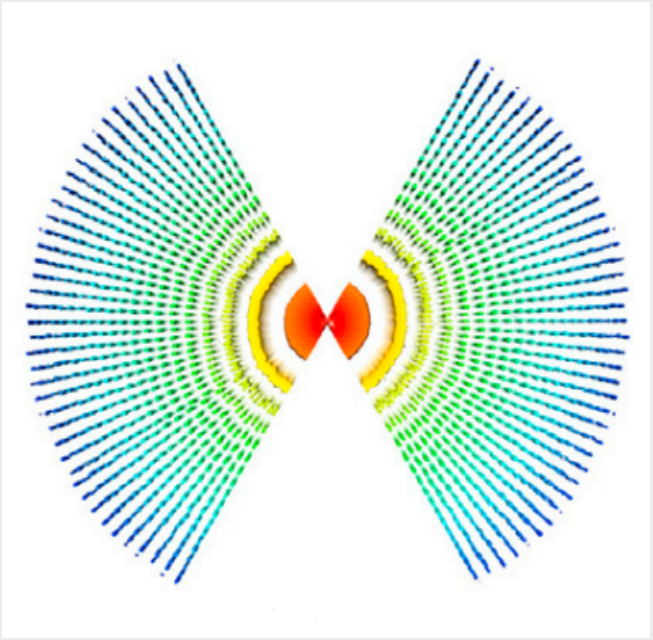
Sub-tomogram processing software

- 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
- PyTom
- PEET
- Jsubtomo
- TOM & AV3
- XMIPP

Sub-tomogram processing in Relion



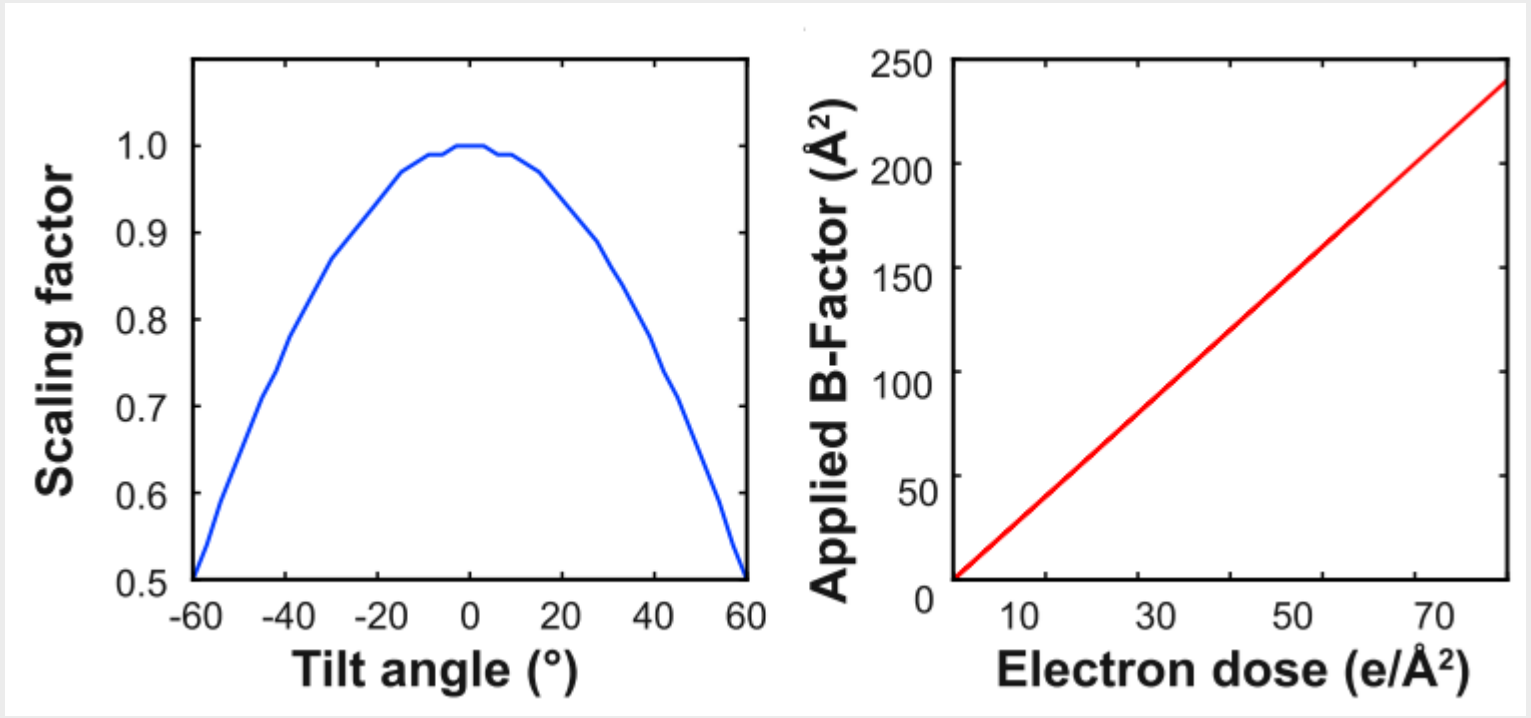


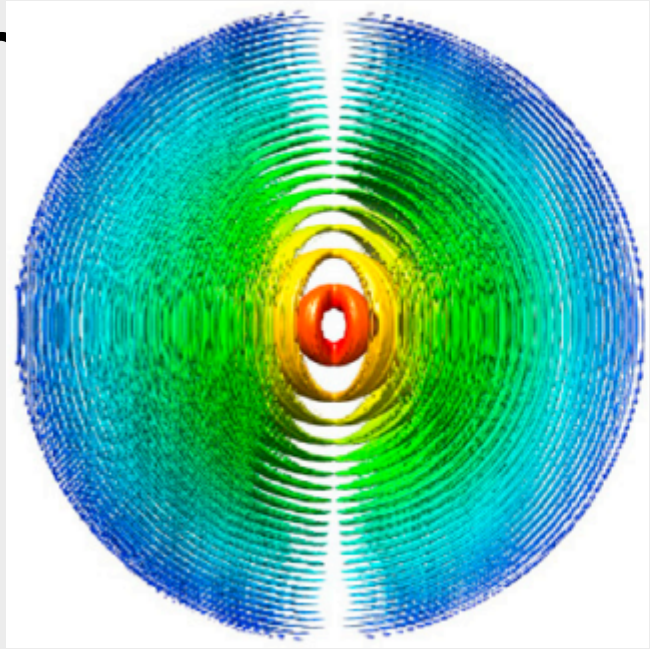


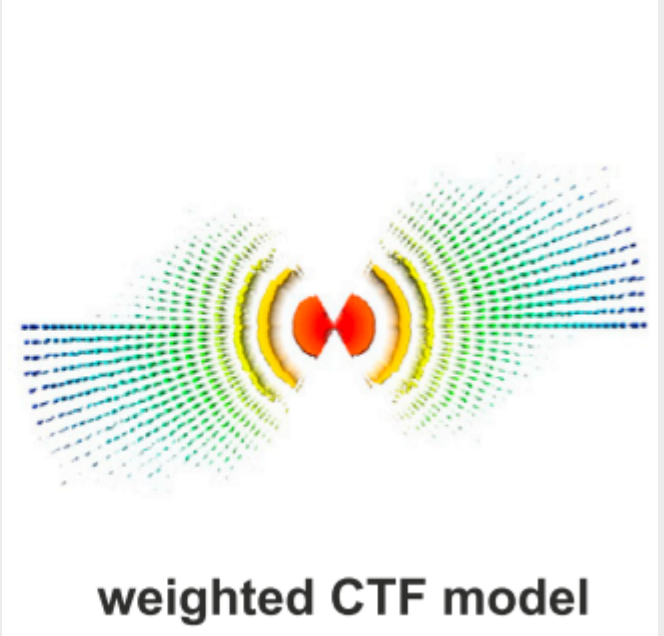
- Uses normal Relion workflow.
- Potential issues:
  - Extra images are likely not at the same focus as the Target
  - 3D FSC may eliminate properly interpolated values due to sampling

Bharat et. al., Structure 2015

Sub-tomogram processing in Relion

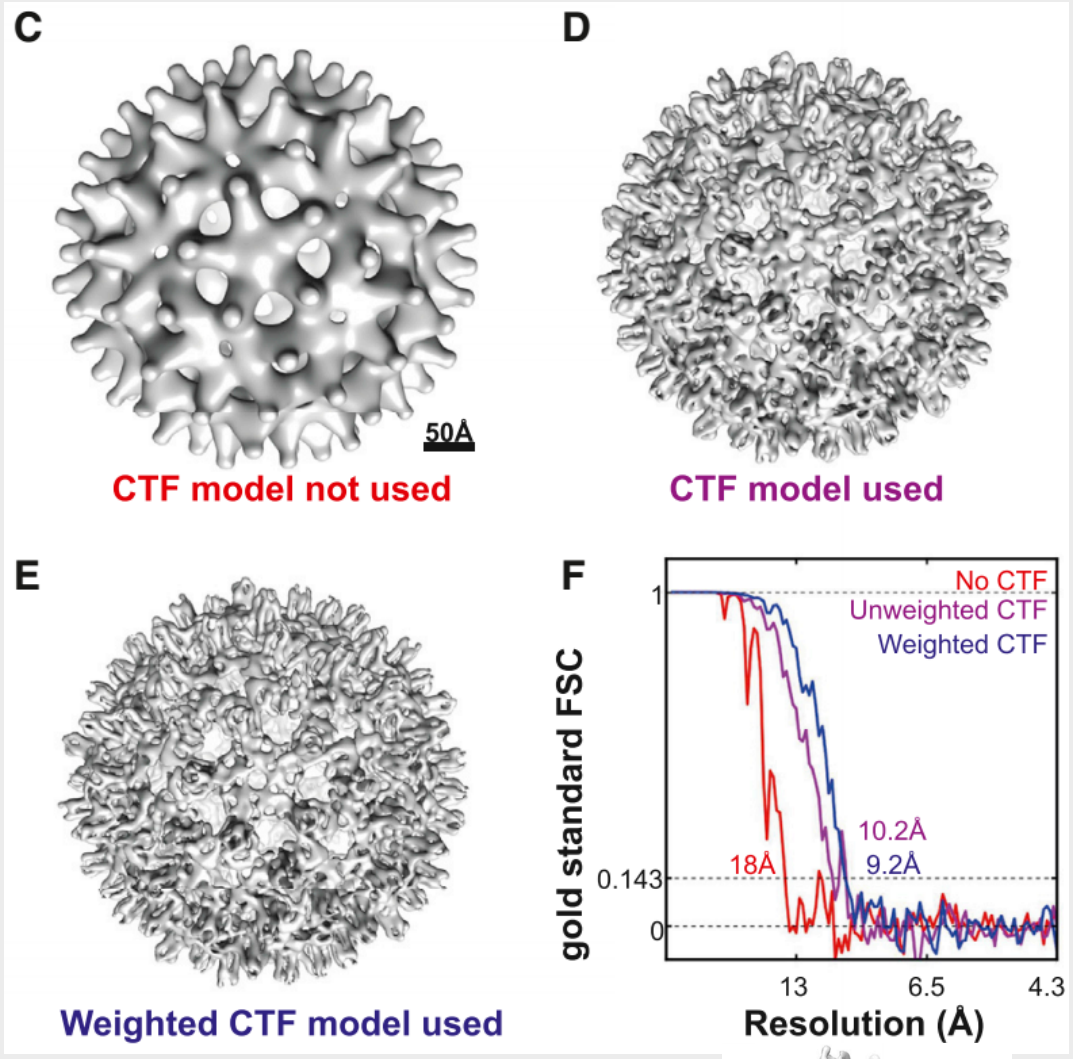


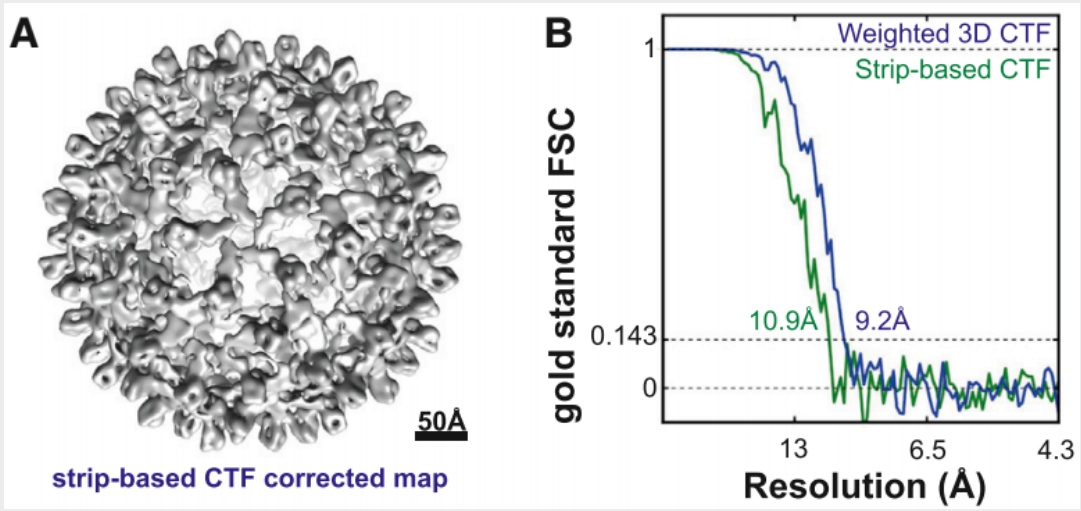


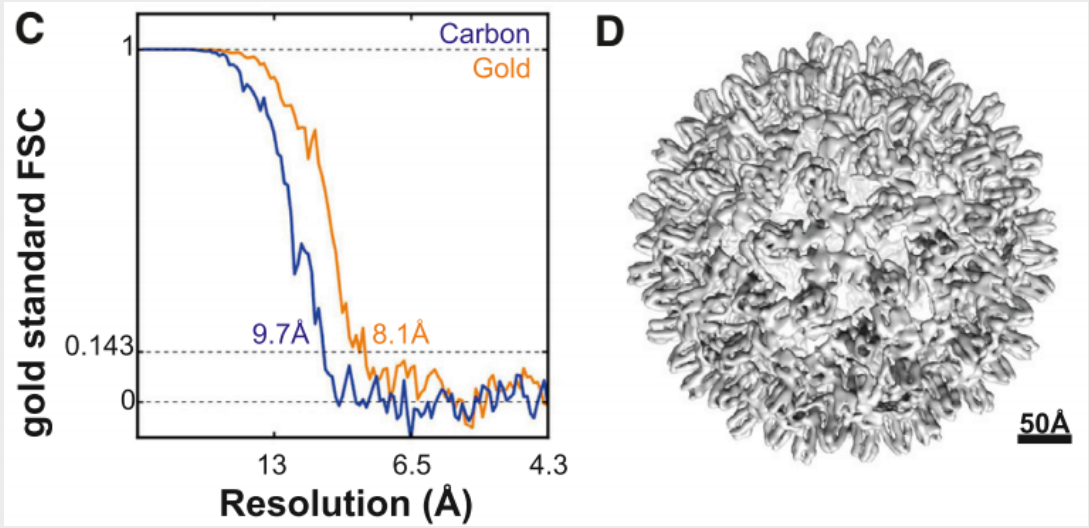


Bharat et. al., Structure 2015

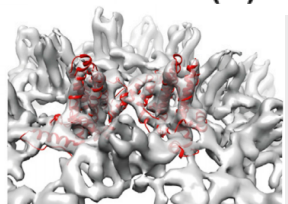
Sub-tomogram processing in Relion





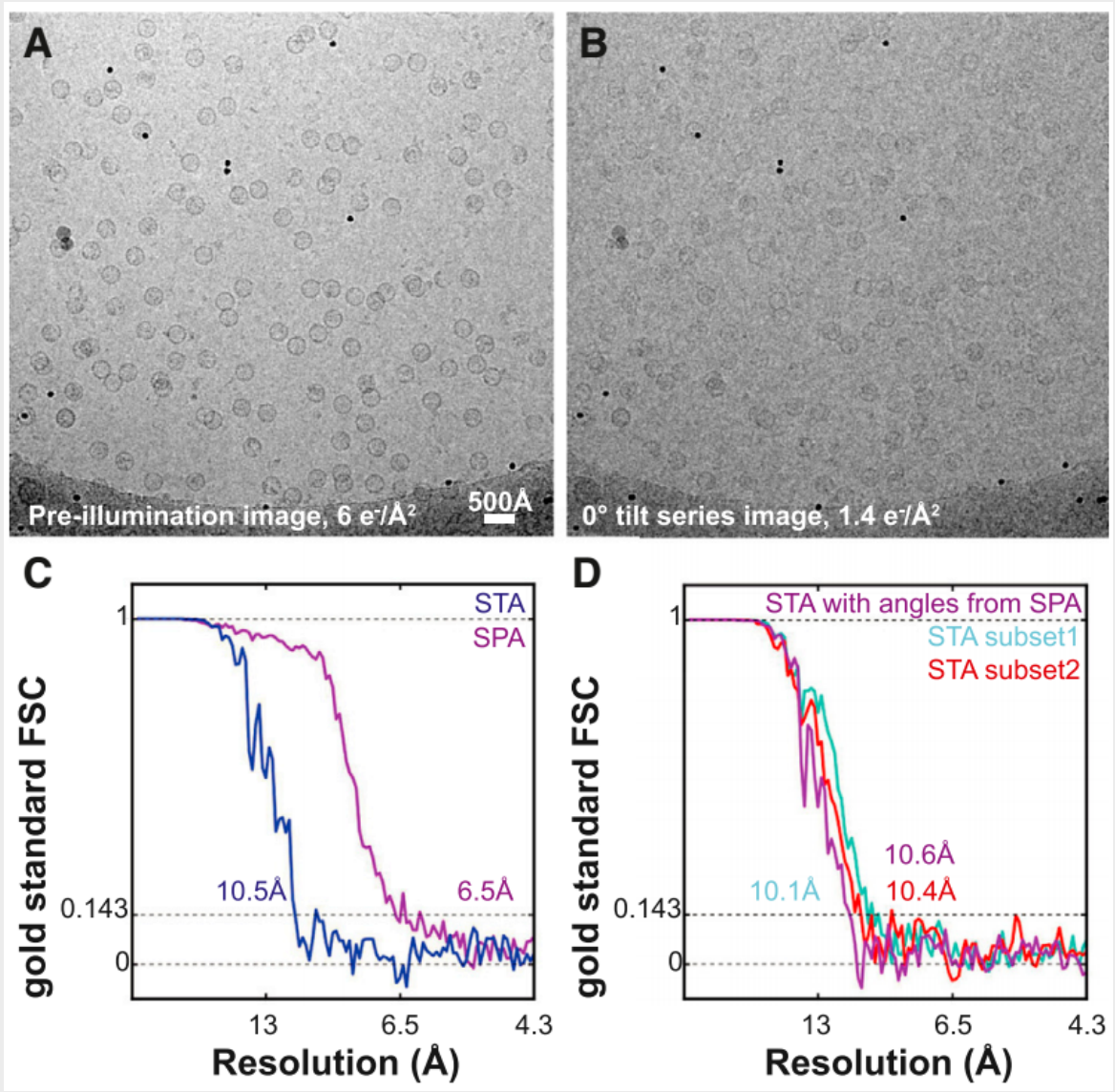


- Test case: Hepatitis B capsid



Bharat et. al., Structure 2015

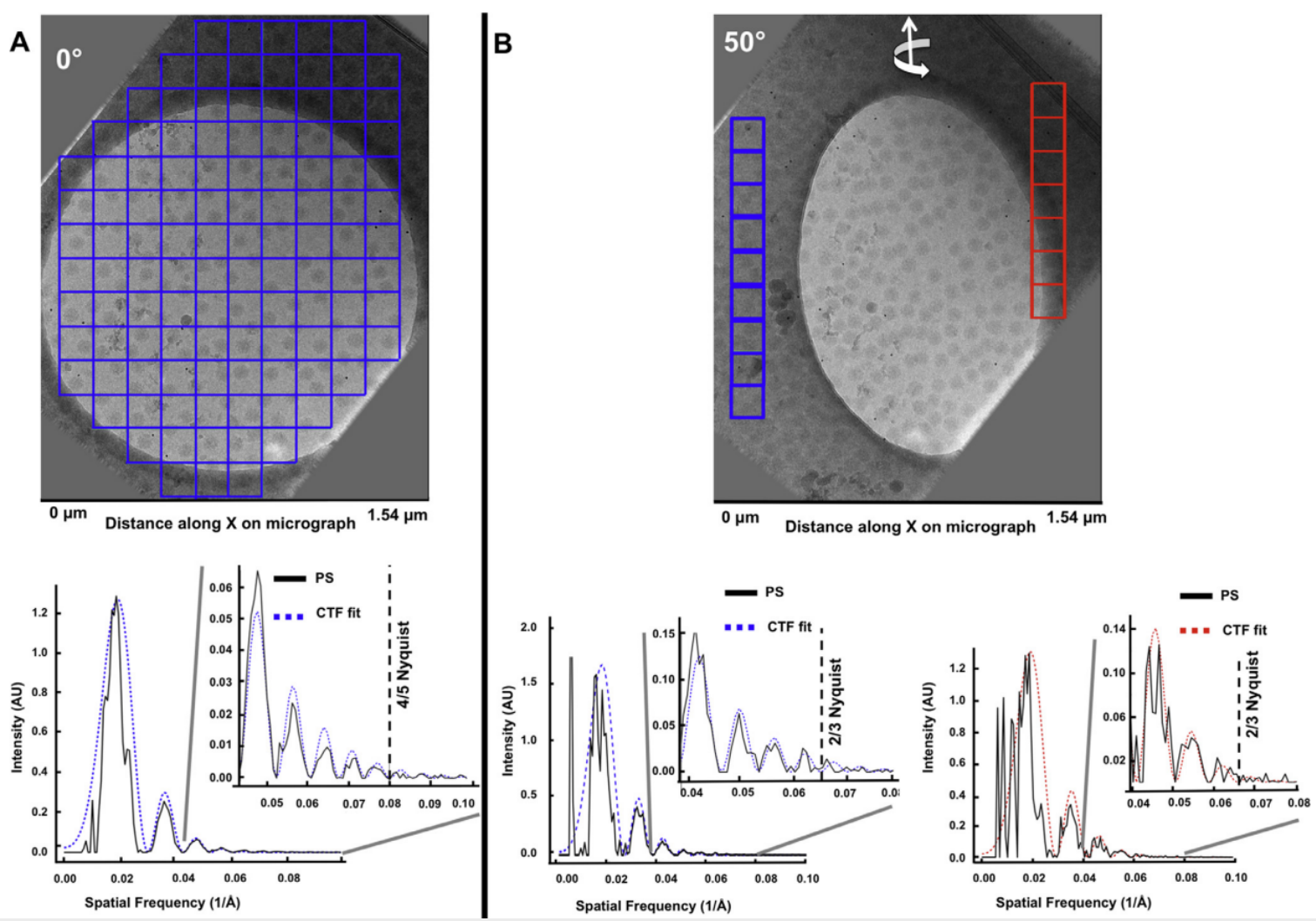
Sub-tomogram processing in Relion



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

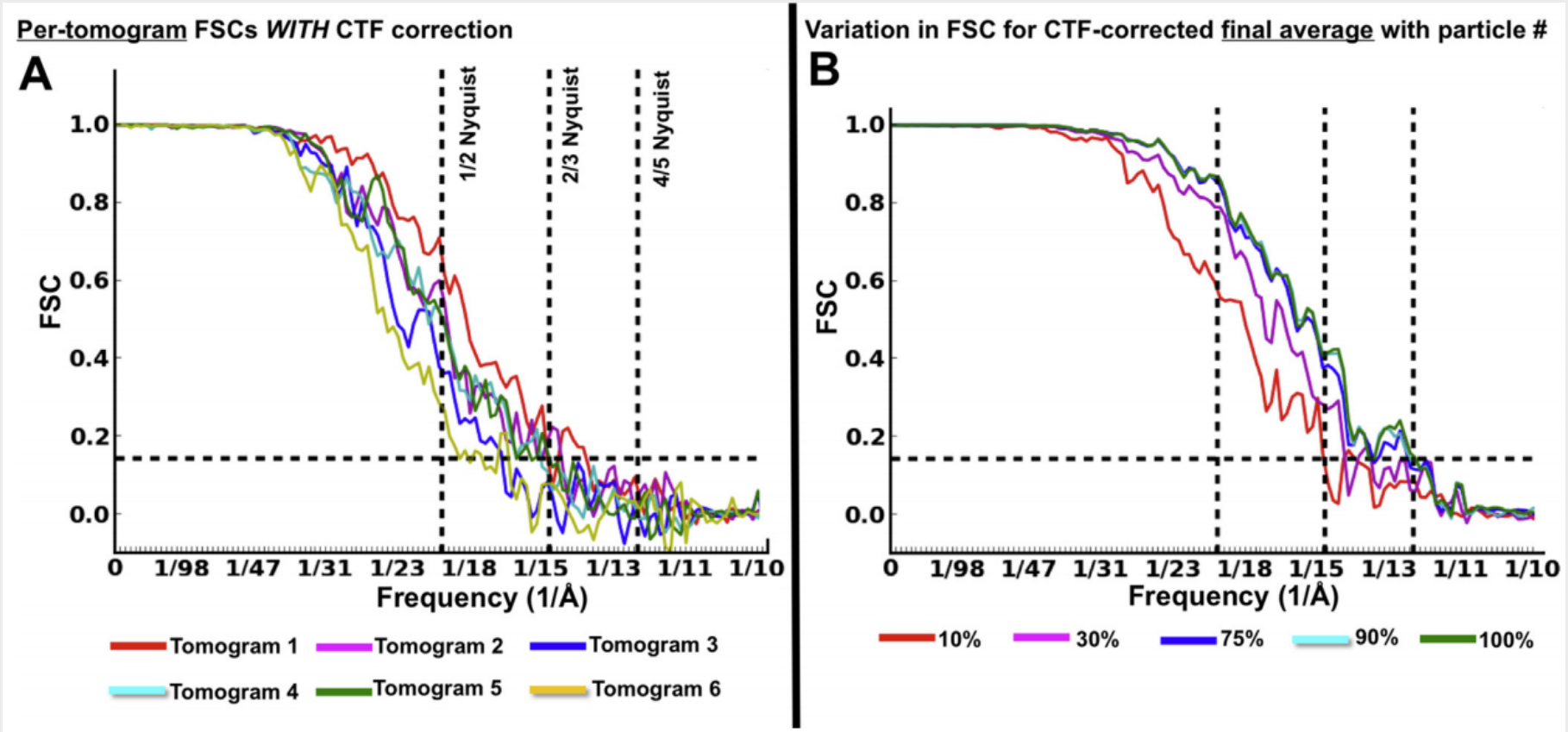
Bharat et. al., Structure 2015

Sub-tomogram processing in EMAN2



Galaz-Montoya, JSB 2016

Sub-tomogram processing in EMAN2



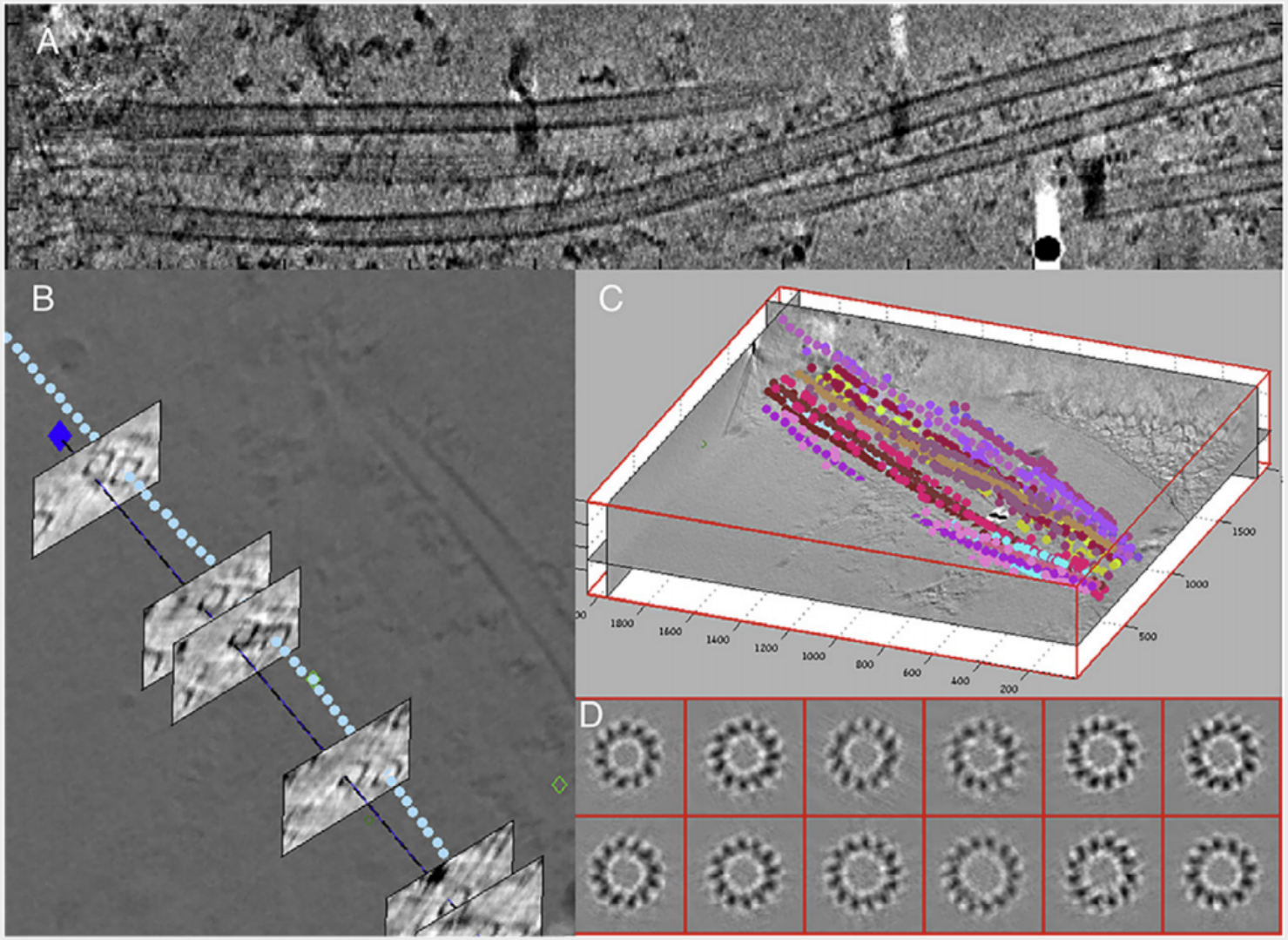
- Better than 2/3 Nyquist

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

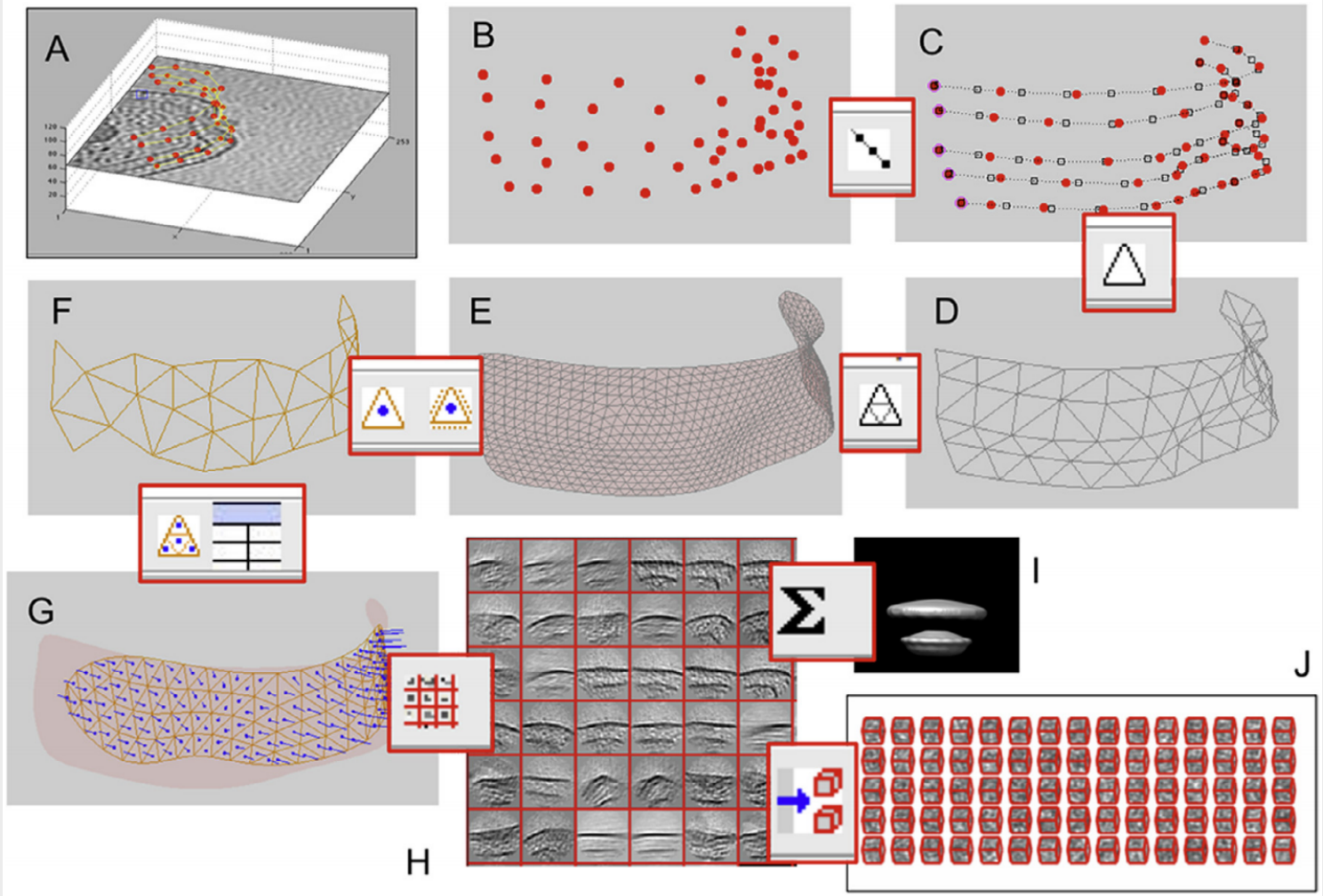
Sub-tomogram annotation processing in Dynamo



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

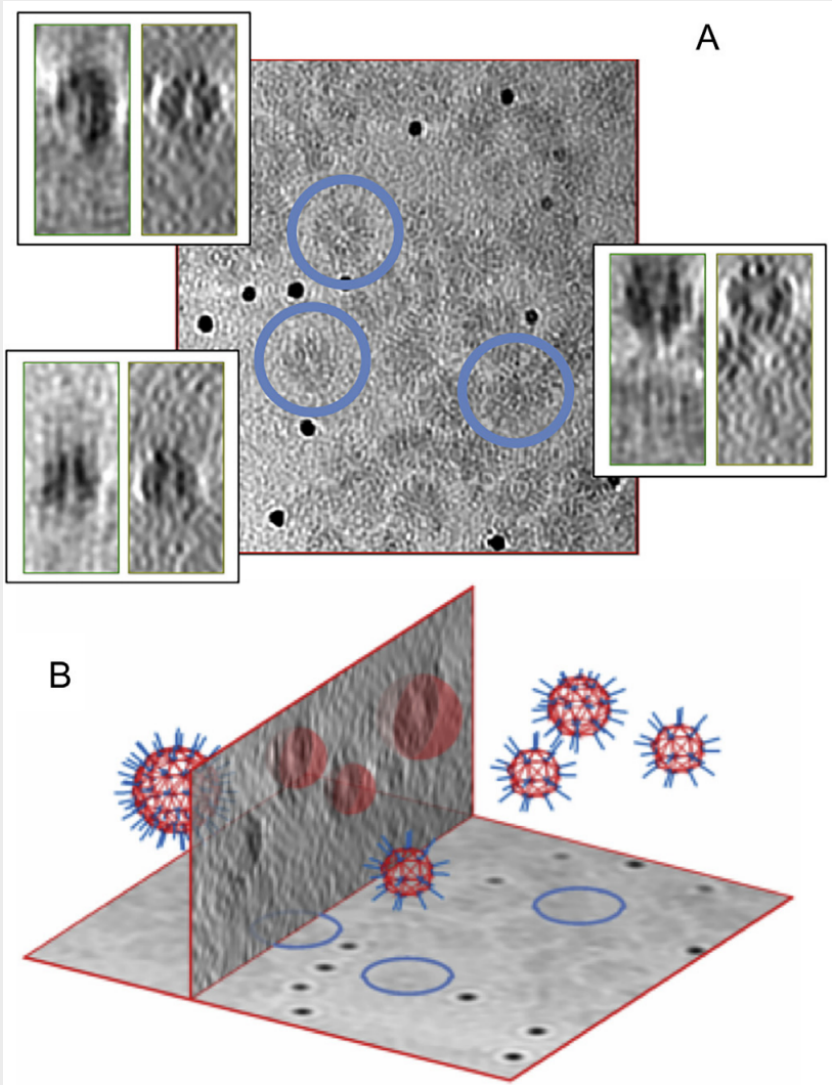
Castaño-Díez et. al., JSB 2012 & 2016

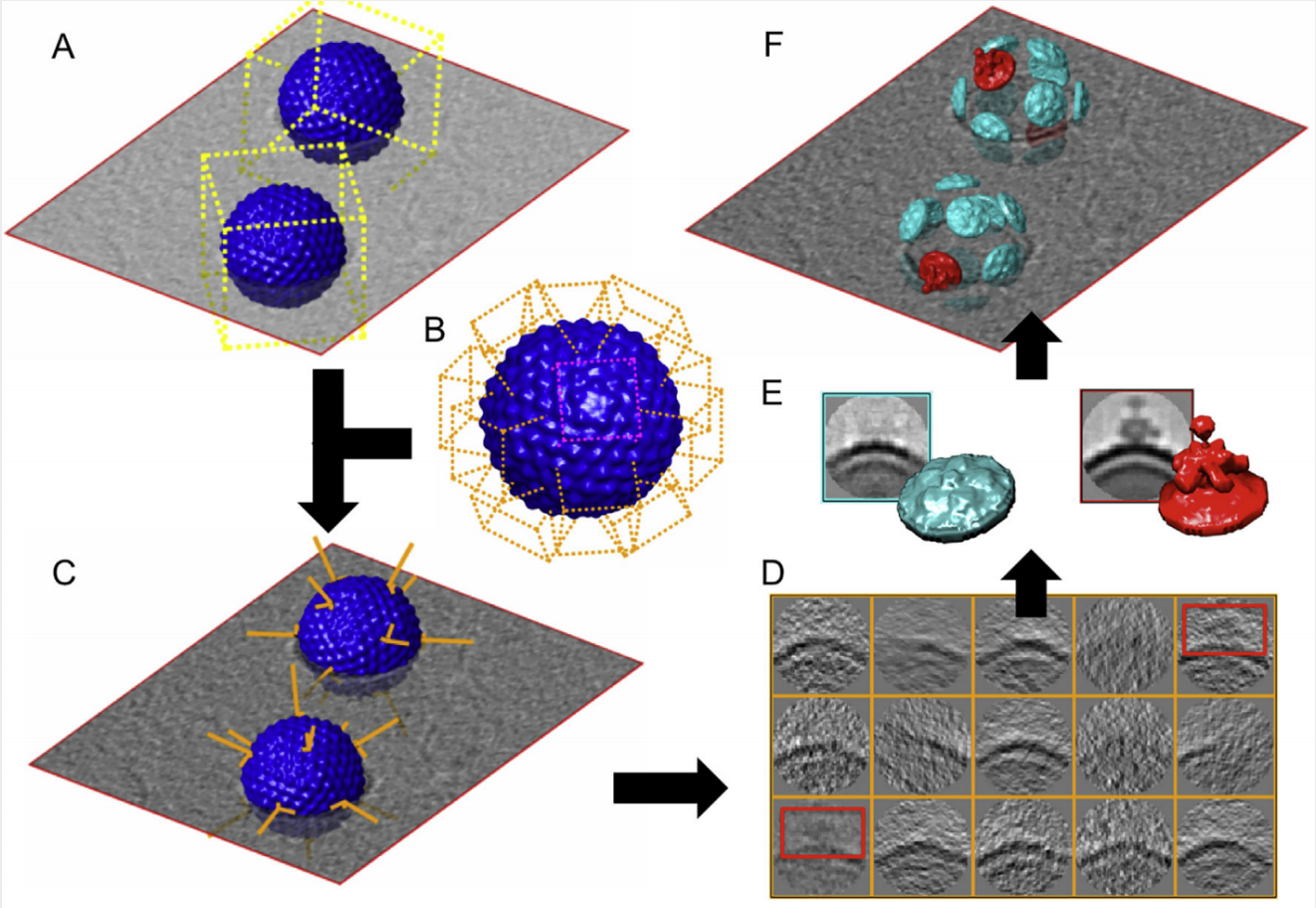
Sub-tomogram annotation processing in Dynamo



Castaño-Díez et. al., JSB 2012 & 2016

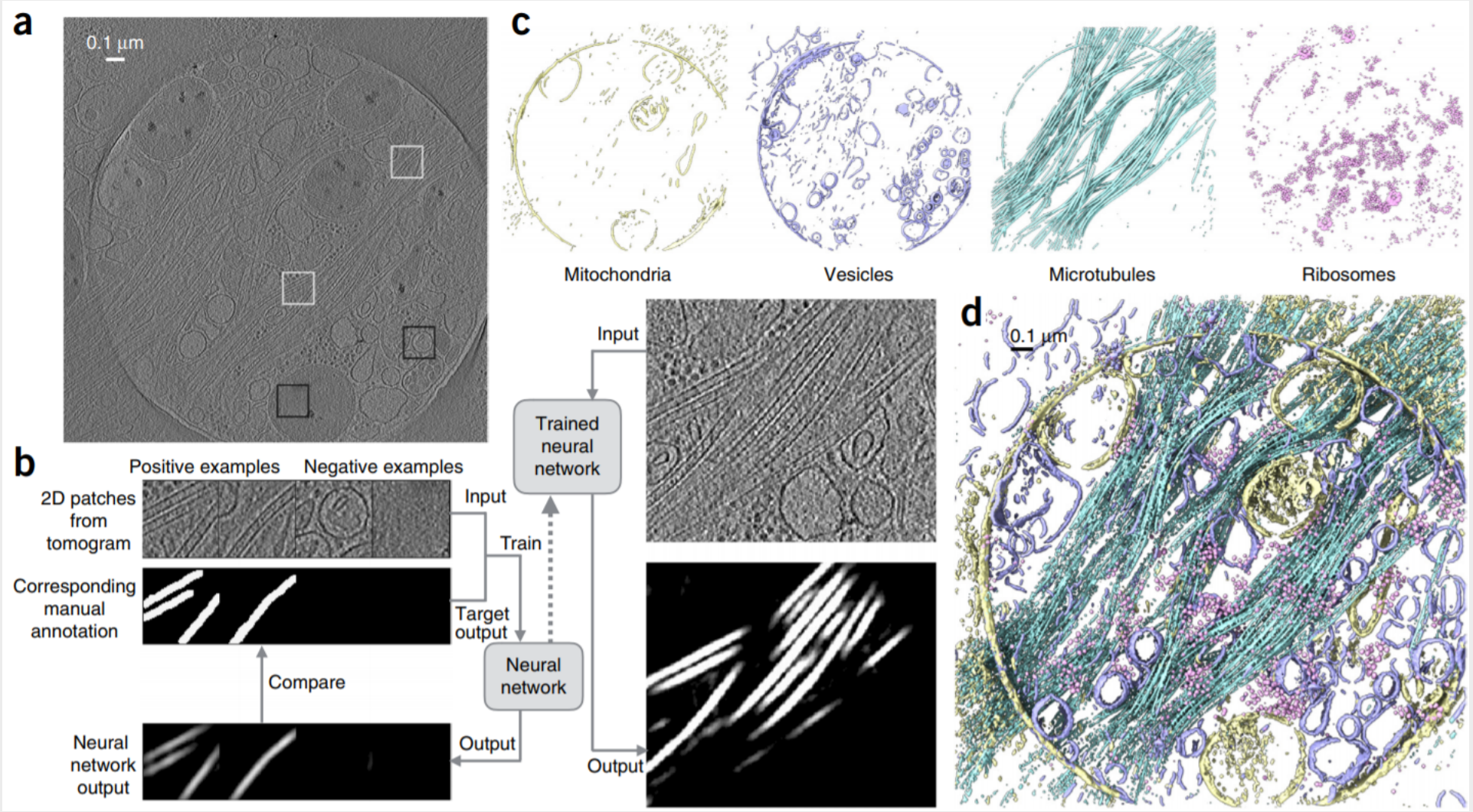
Sub-tomogram annotation processing in Dynamo





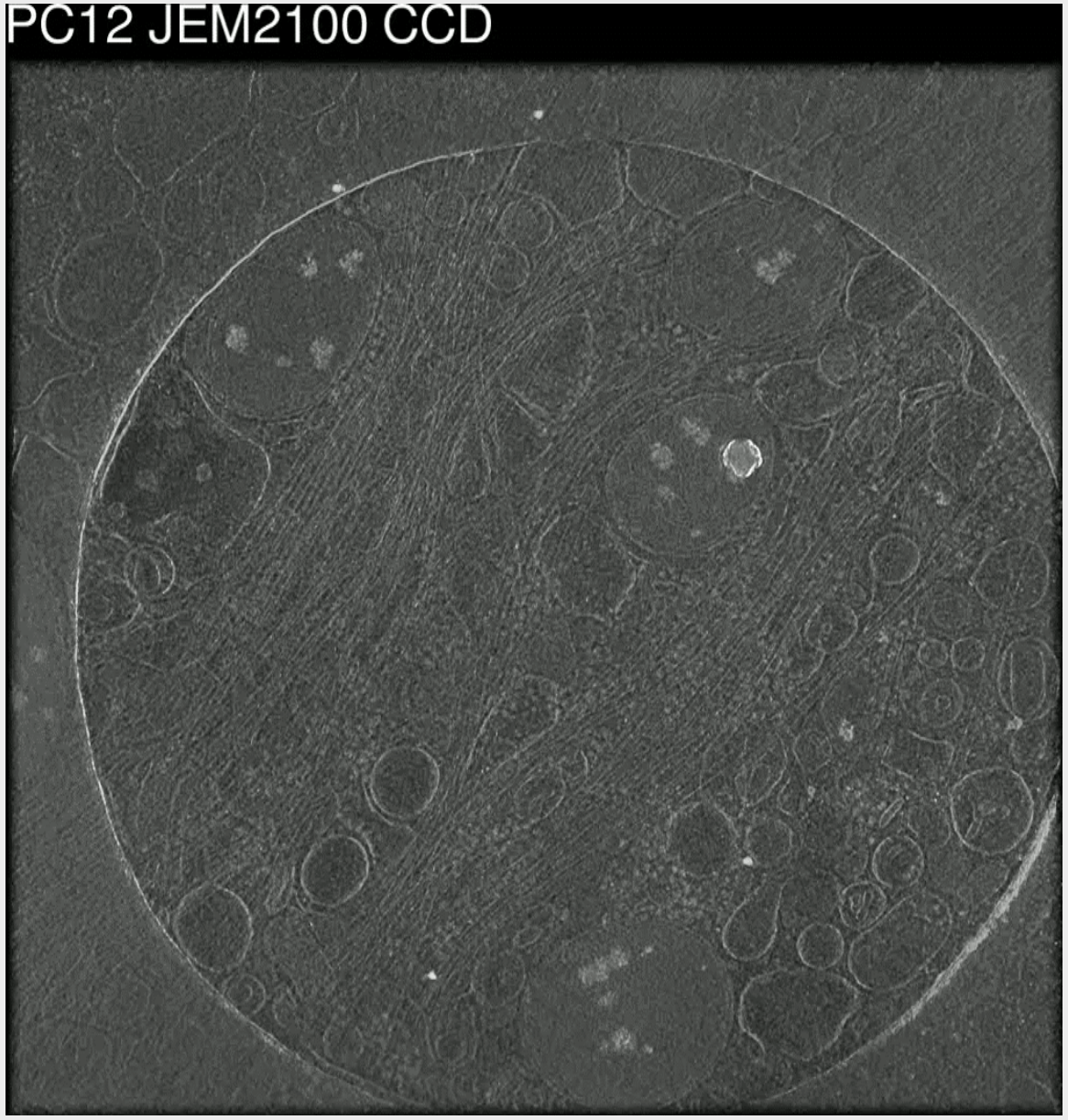
Castaño-Díez et. al., JSB 2012 & 2016

Sub-tomogram segmentation processing in EMAN2



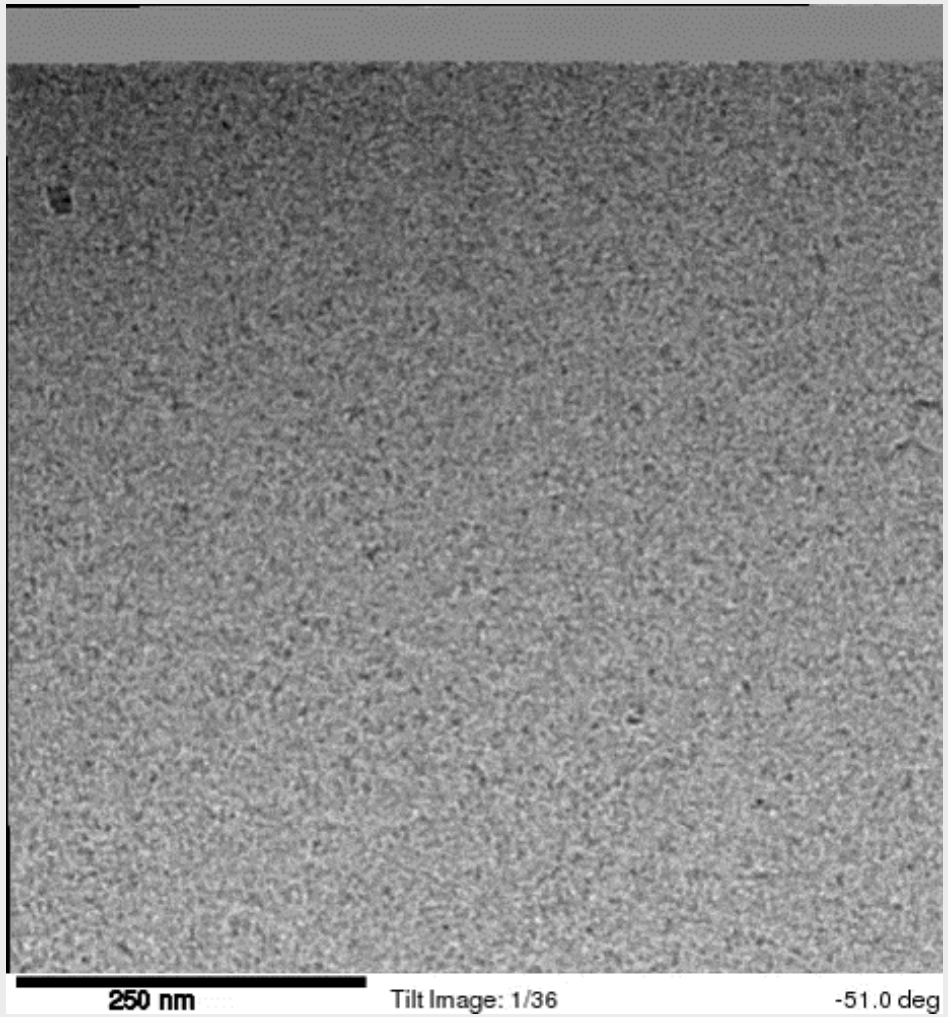
Chen et. al., Nat. Meth. 2017

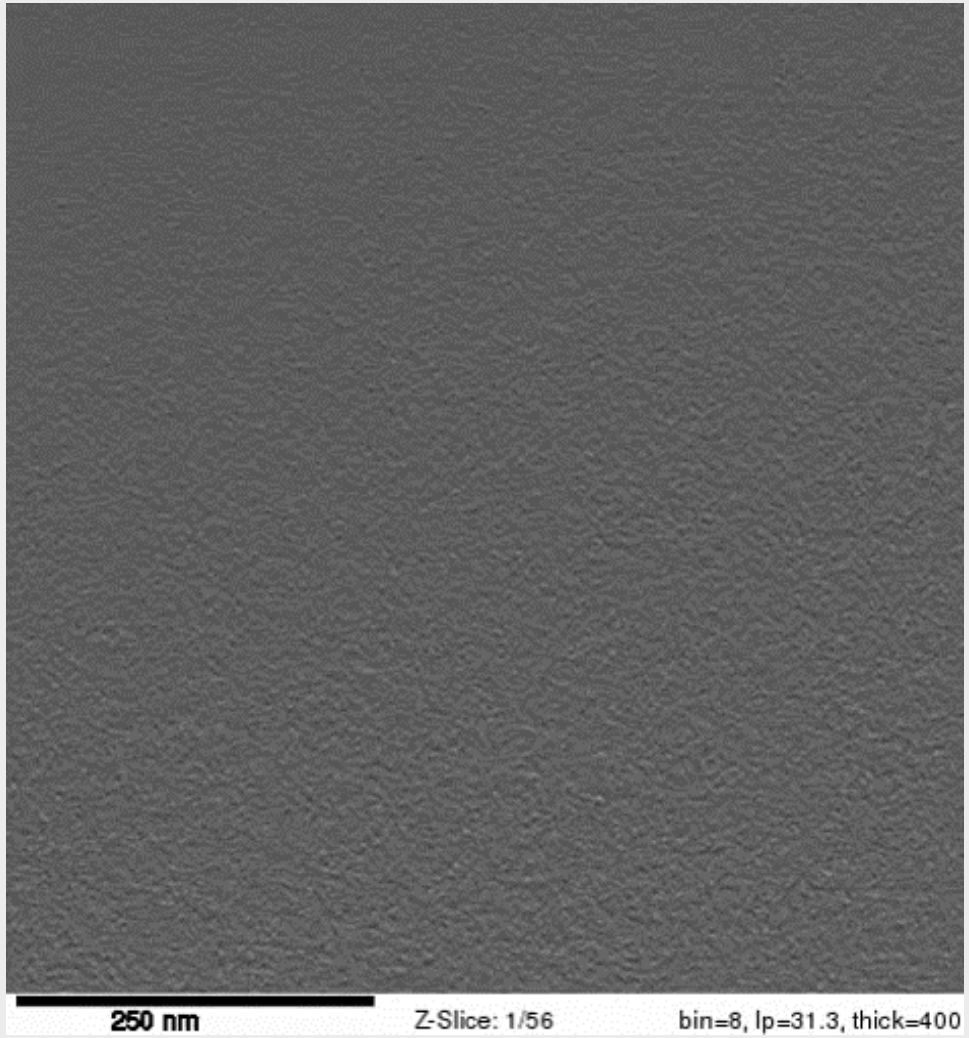
Sub-tomogram segmentation processing in EMAN2



Chen et. al., Nat. Meth. 2017

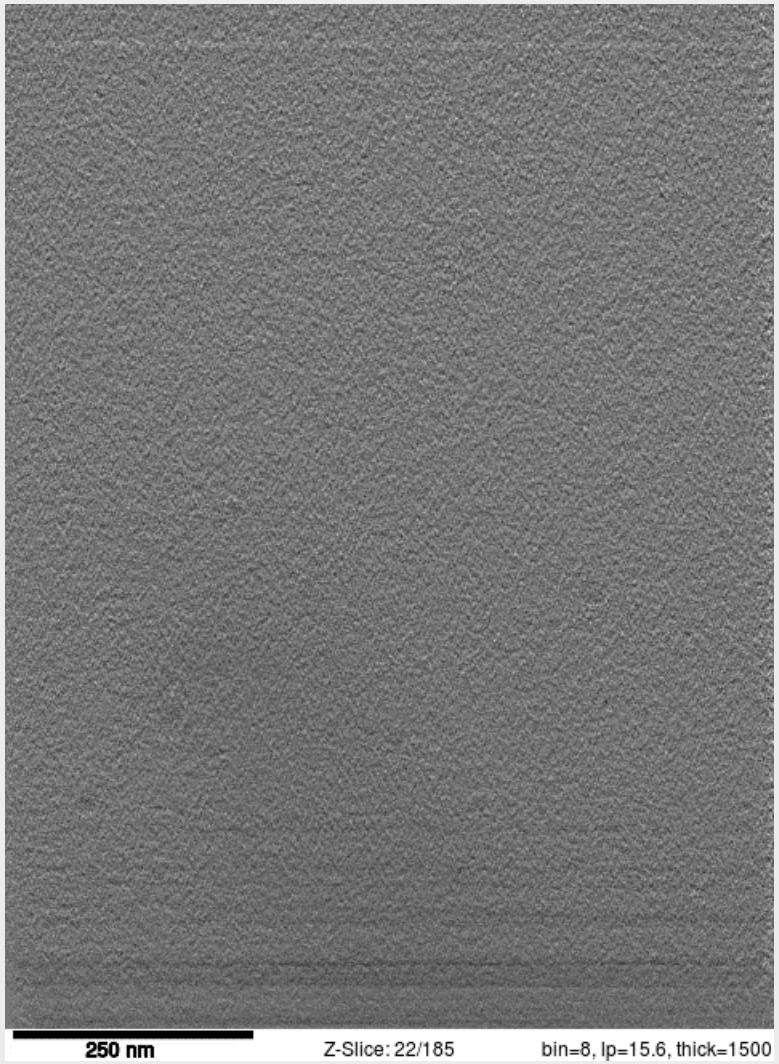
HIV-1 trimer single particle





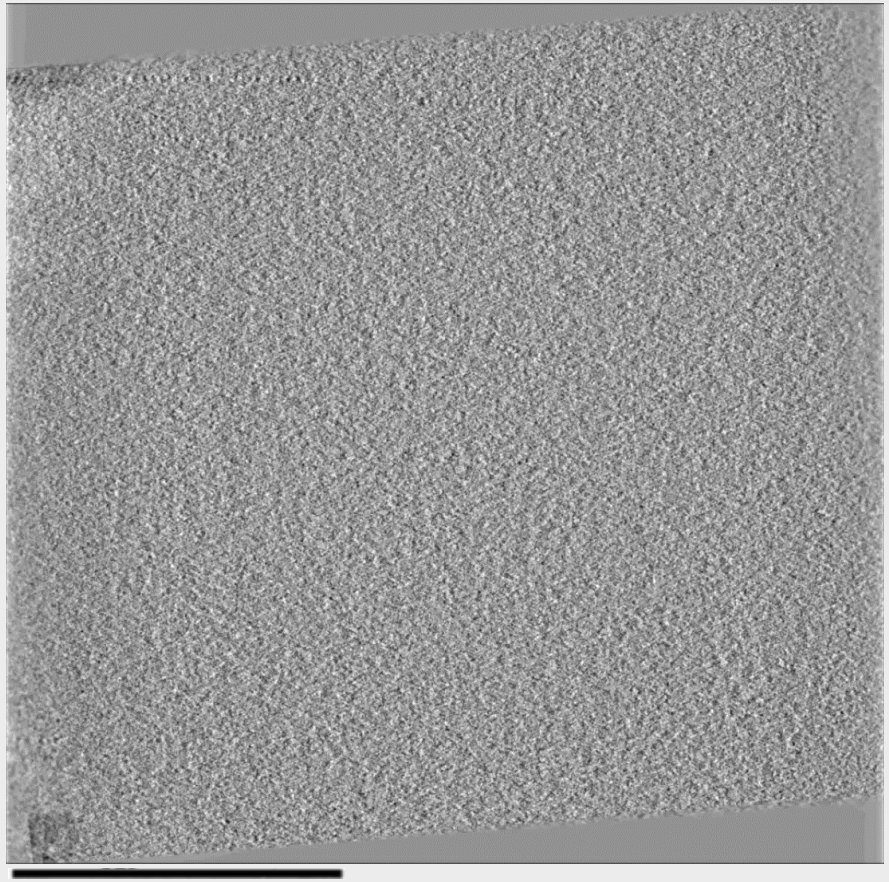
Priyamvada Acharya & Alex Noble

Example: Exotically Shaped Samples

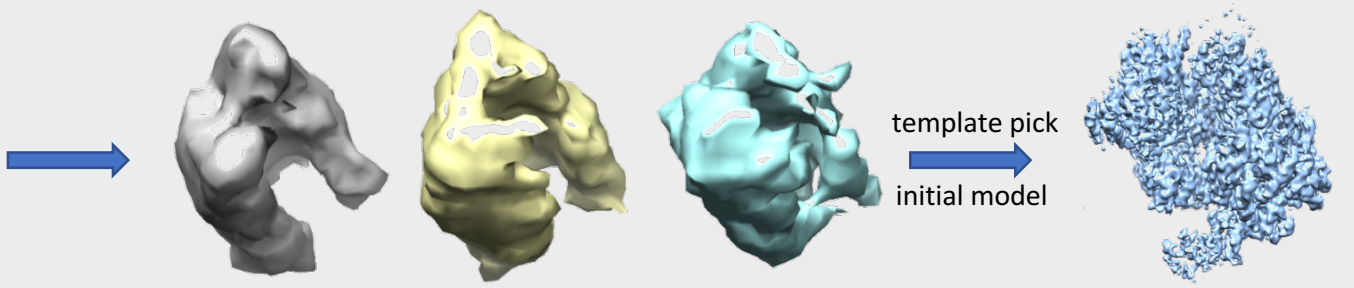


ZmNDPK1: GTP complex

Example: Tomography for single particle initial model



Z-slices through tomogram/ice

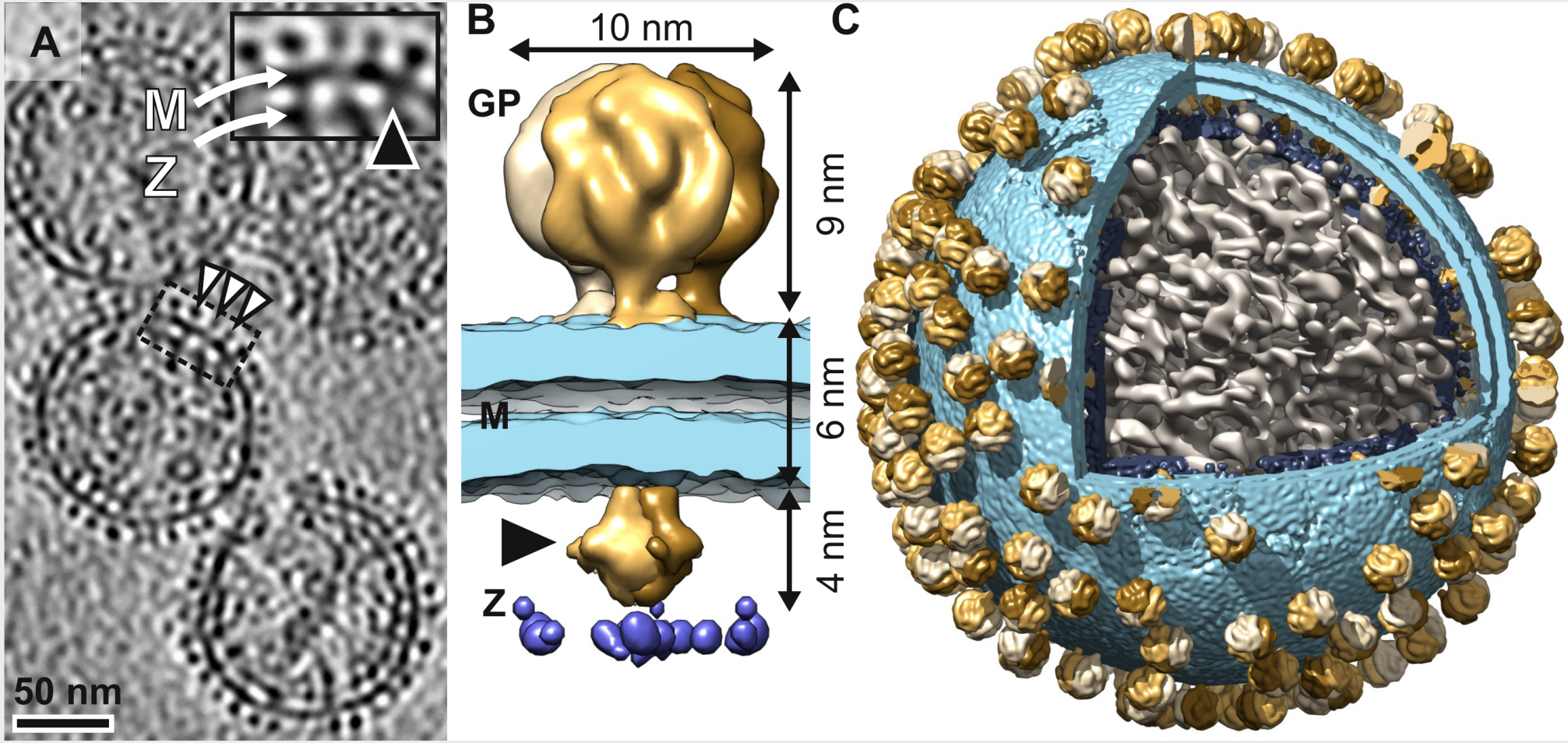


Structure now at 4 Å!

- 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

Example: Lassa virus glycoprotein spike

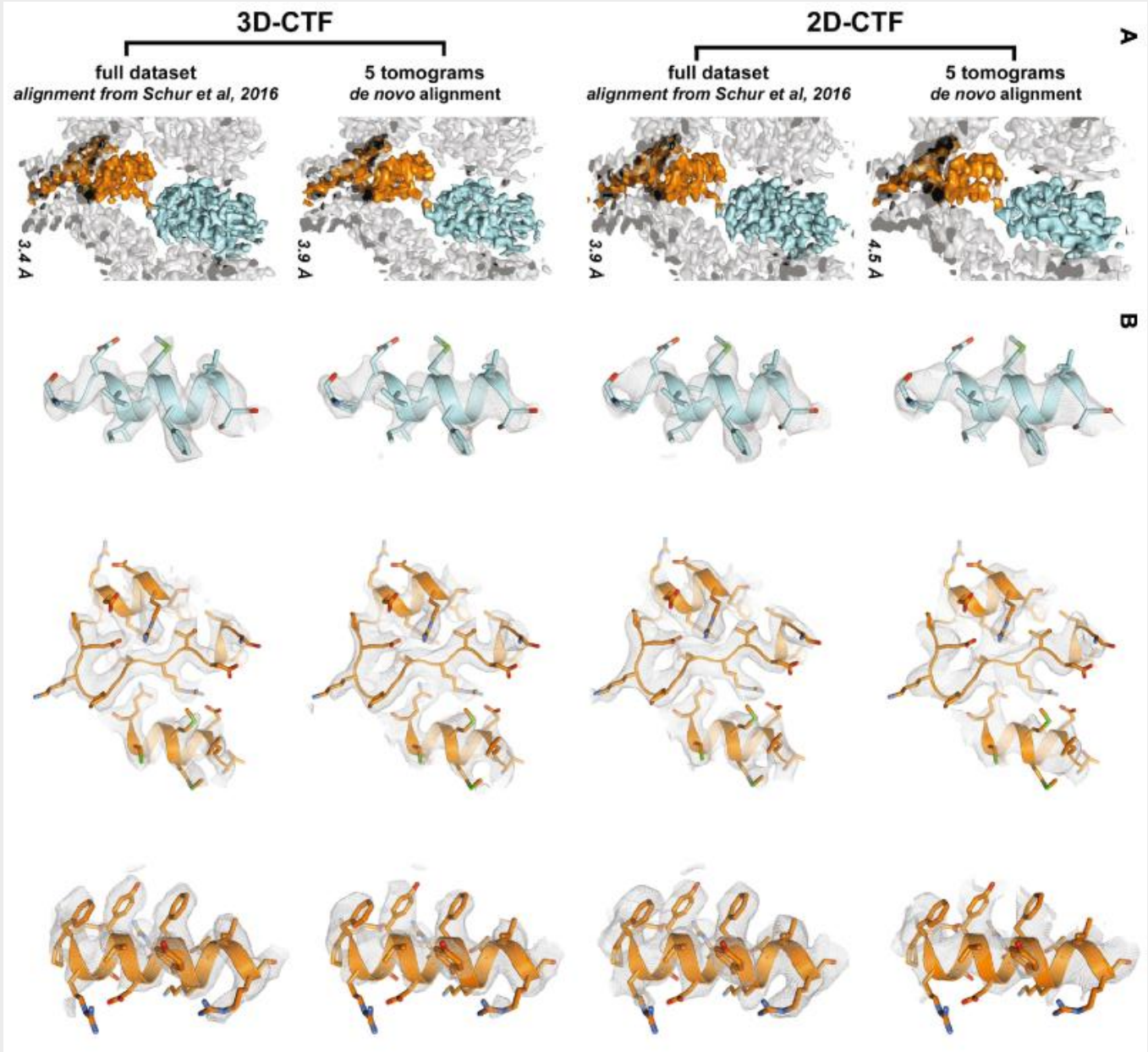


- **Heterogeneous shape** makes single particle difficult/impossible
- Sub-tomogram processing on spiked allows for **13.6 Å spike structure**
- Can **re-map spikes** onto all particles in the tomogram

Li et. al., PLOS 2016

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

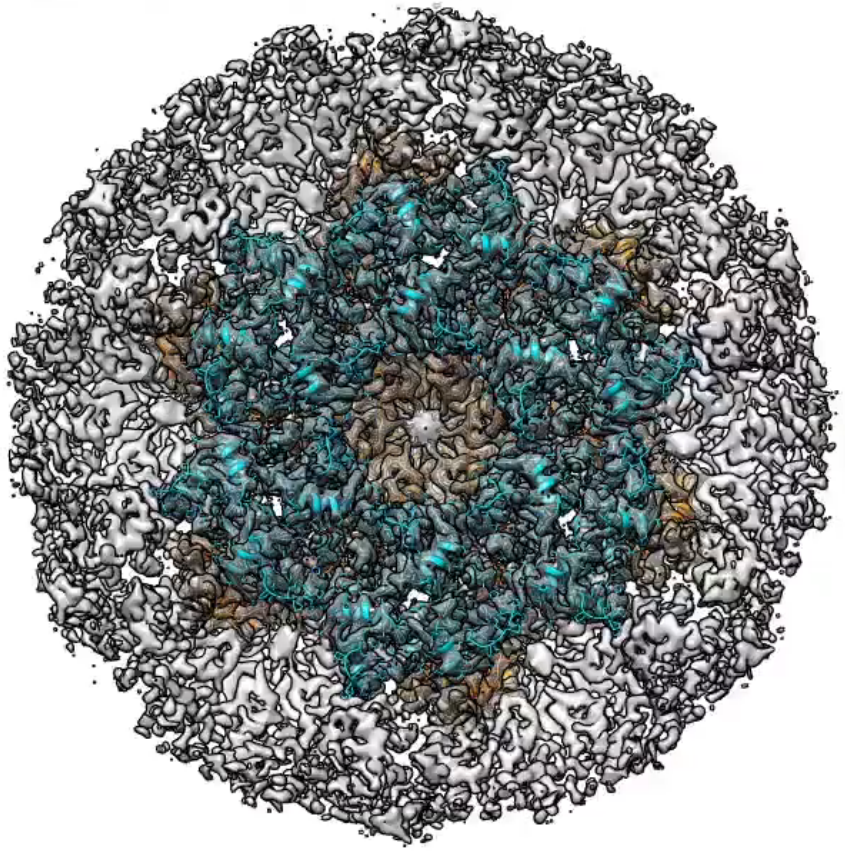
- Krios + Super-res K2 + Gatan Energy Filter
- Fiducial tilt-series alignment
- 1.5 – 5 micron defocus
- Strip-based CTF correction
- ~750,000 sub-particles used
- TOM, AV3, Dynamo, and in-house scripts were used
- NovaCTF 3D CTF pushed it to 3.4 Å



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, 2016

Example: HIV-1 Capsid-SP1 at 3.9 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)

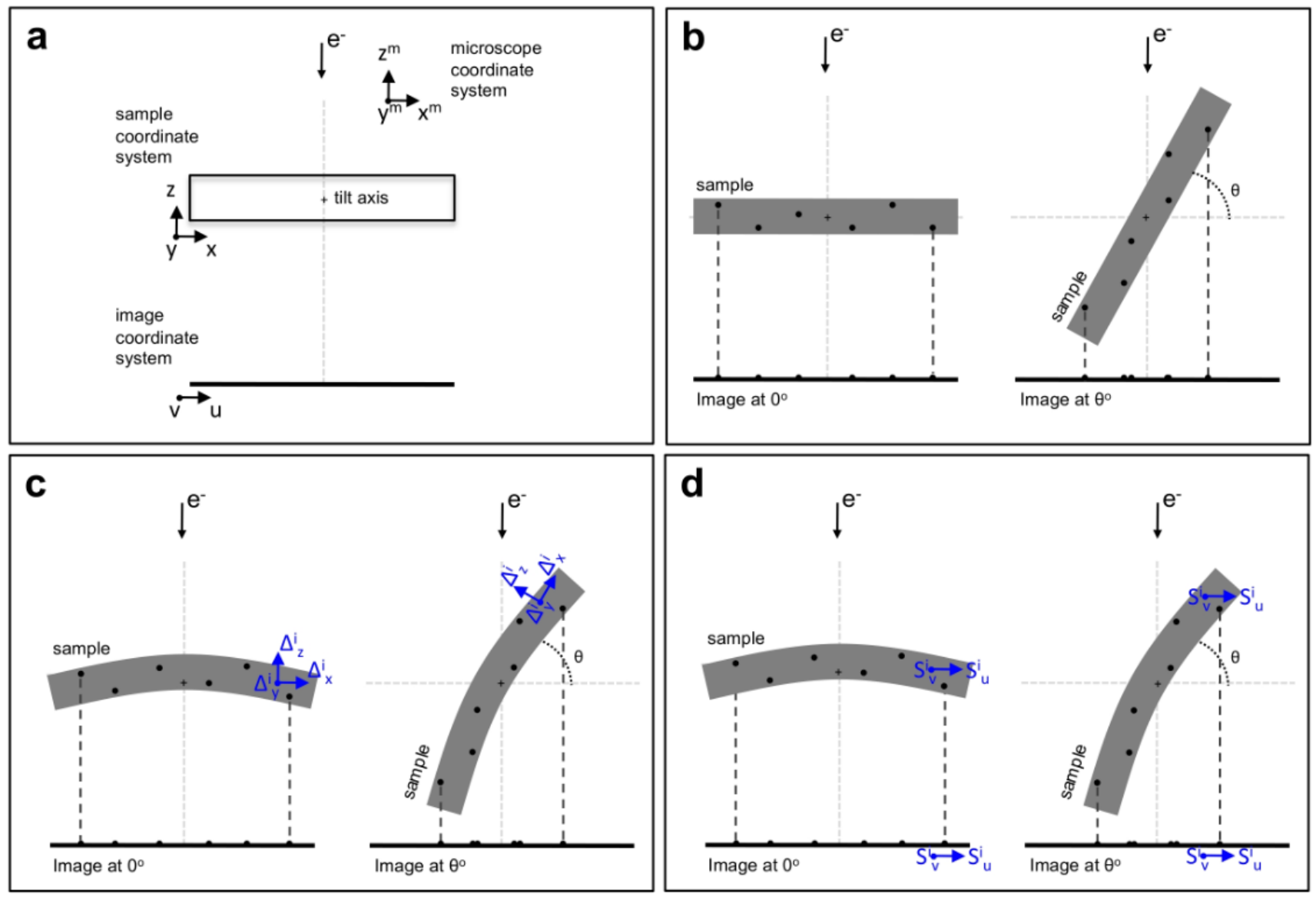
Sample	Sample type	Instrumentation	Tilt-series alignment method	Defocus range (µm)	CTF correction method	Number of asymmetric units	Reported resolution (Å), Nyquist fraction	Citation
HIV-1 capsid-SP1	VLP spikes	Titan Krios, K2 Summit @ 8kx8k, GIF	fiducial	1.5 – 5	Strip-based/3D CTF	~750,000	3.9, 0.35 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

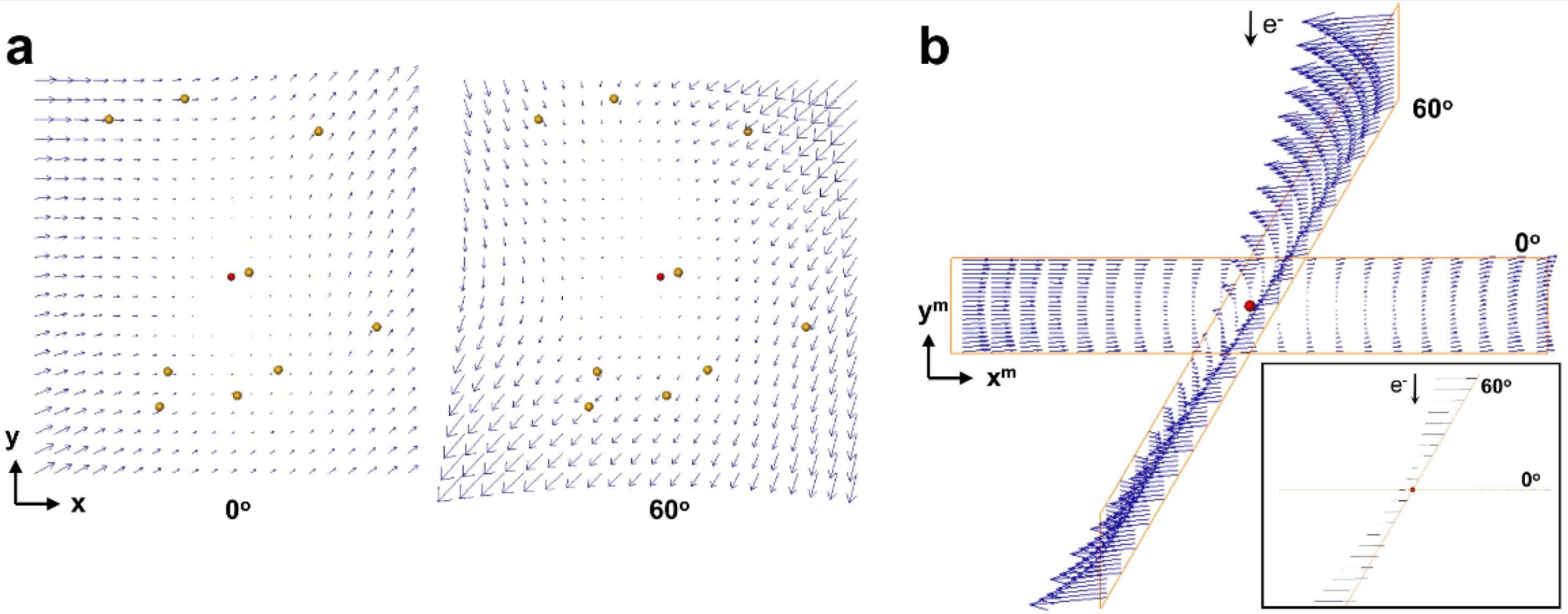
- Pixelsize (highest resolution = 2 x pixelsize = **Nyquist**)
- Isotropic motion (monitor your **drift** before full collection)
- Inherent specimen **flexibility**
- **Ice warping** in 3D during collection
- Beam-induced **motion of objects** of interest **in 3D** (particularly anisotropic)

Refining tilt-series alignment by tracking beads in 3D



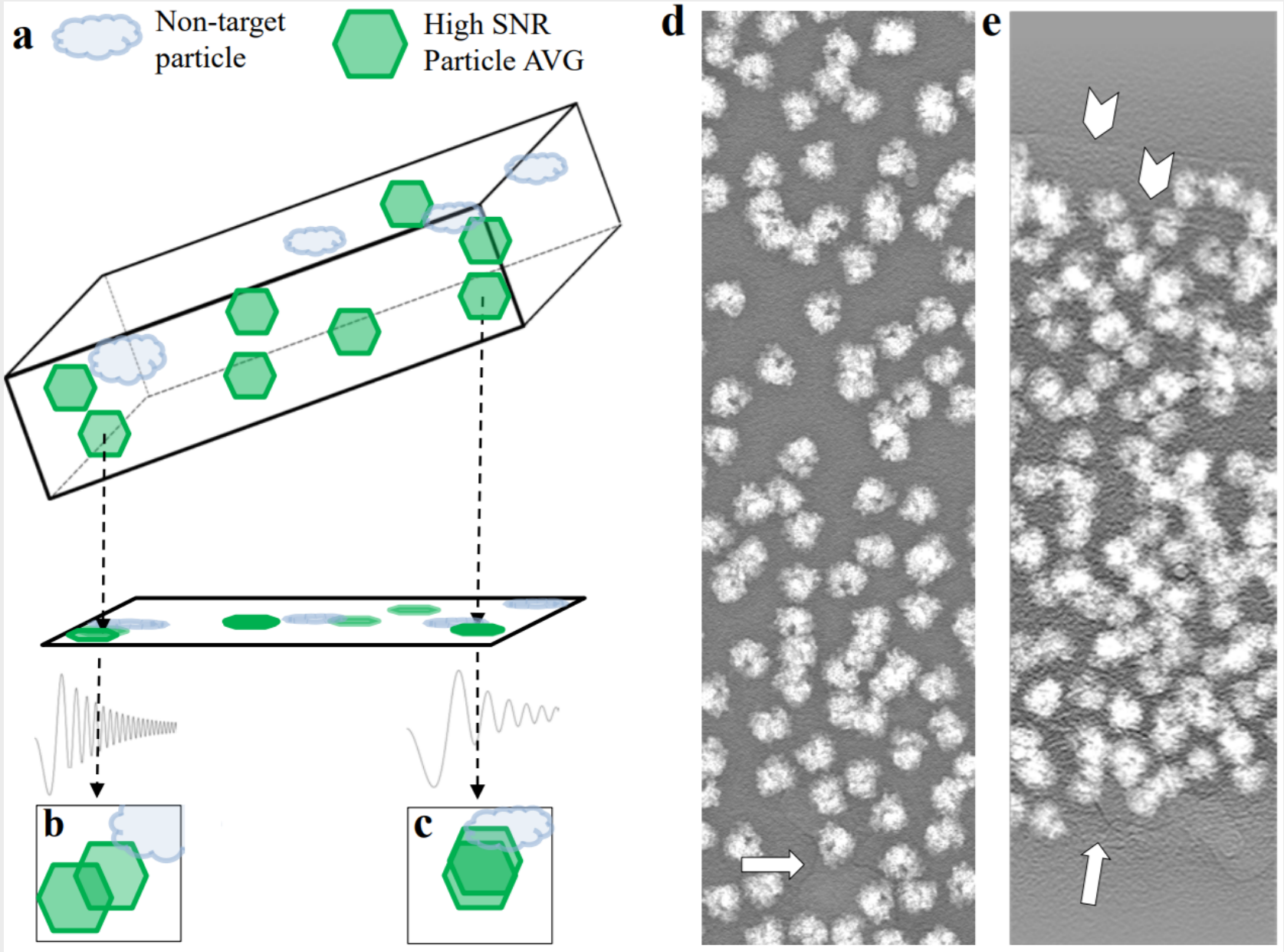
Fernandez, 2018

Refining tilt-series alignment by tracking beads in 3D



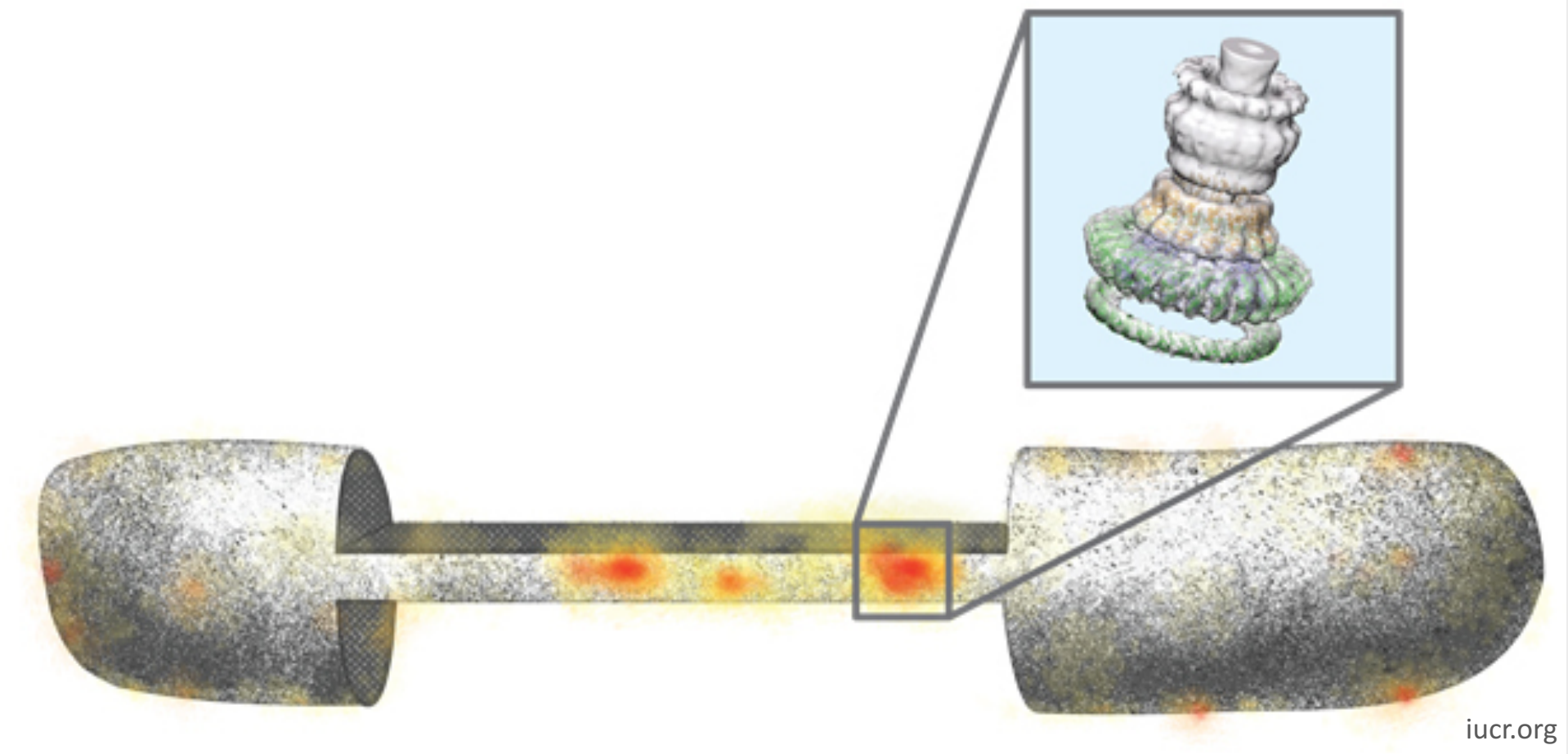
Fernandez, 2018

Refining tilt-series alignment by tracking just particles



Himes, 2017

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



Thank you!
Questions?

Appion
Tomography
Workshop
March 29

Alex Noble
anoble@nysbc.org

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