CryoET & Sub-tomogram Processing

Winter EM Course 2017

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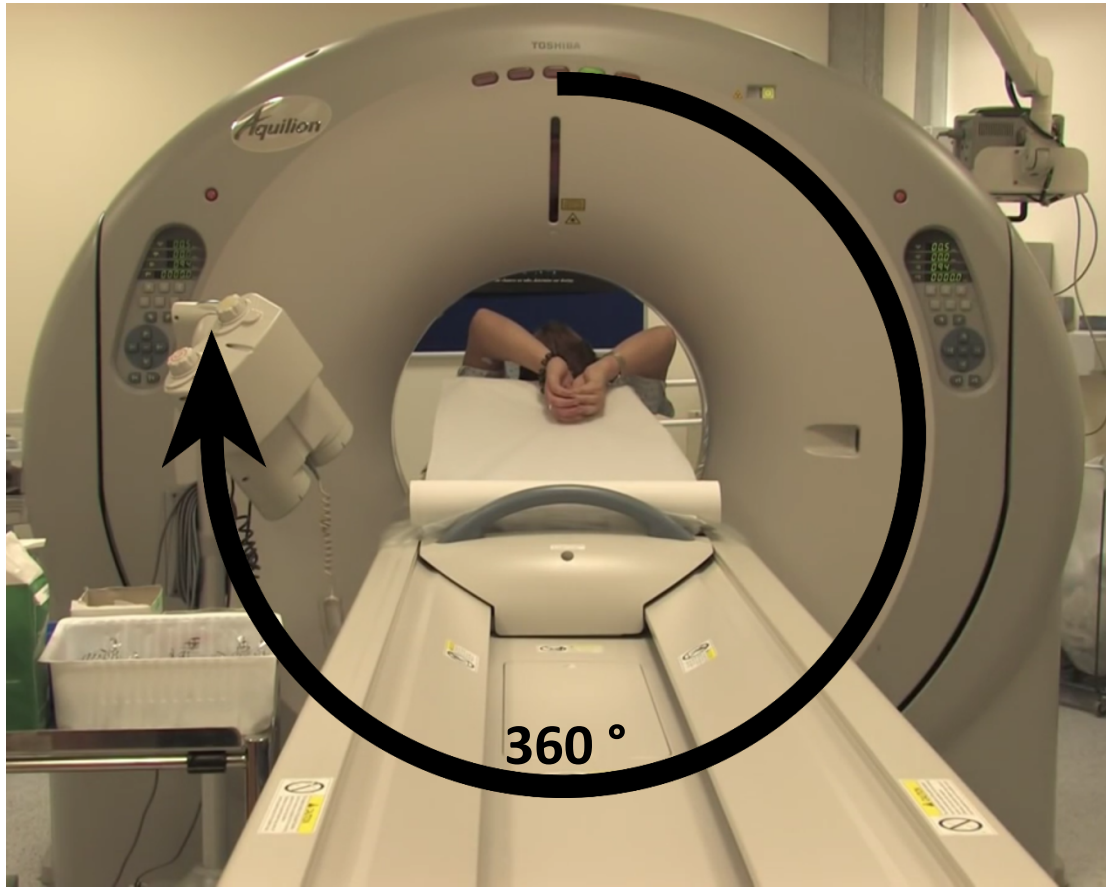
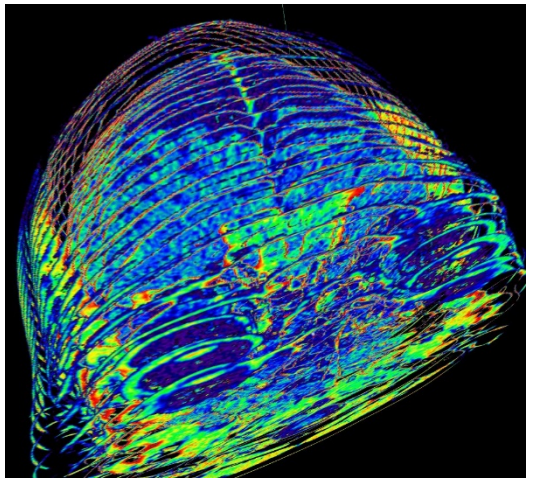
Overview

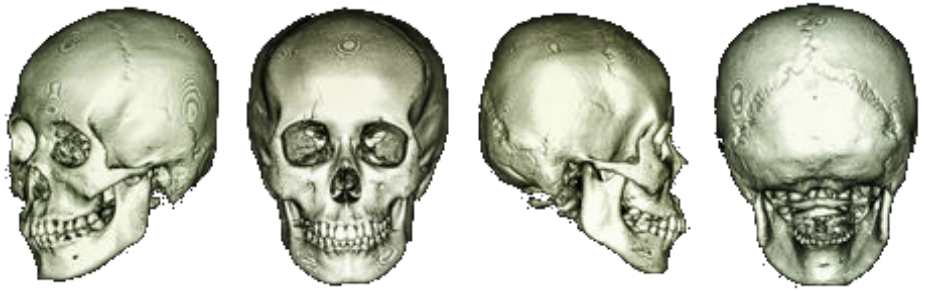
Why CryoET? Commonly used to observe in-situ structures and objects with structural and/or morphological heterogeneity that is difficult or impossible for single particle cryoEM.

- Tilt-series collection
- Tilt-series alignment
- Defocus estimation & CTF correction
- Dose compensation
- Sub-tomogram localization
- Sub-tomogram alignment and averaging
- Numerous examples
- Future directions and improvements

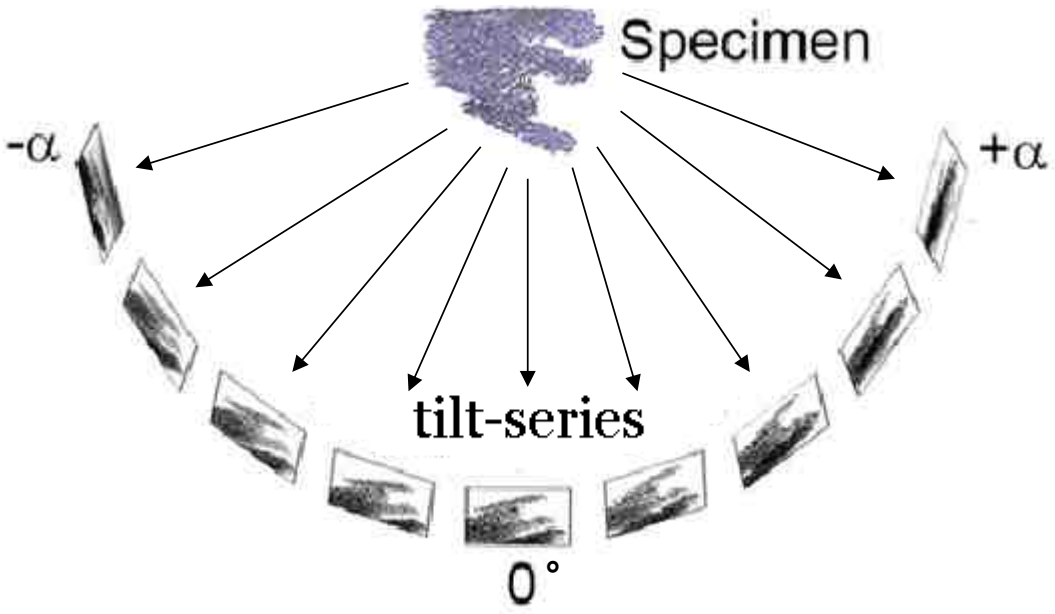
Tomography Overview – Consider How a CT Scan Works

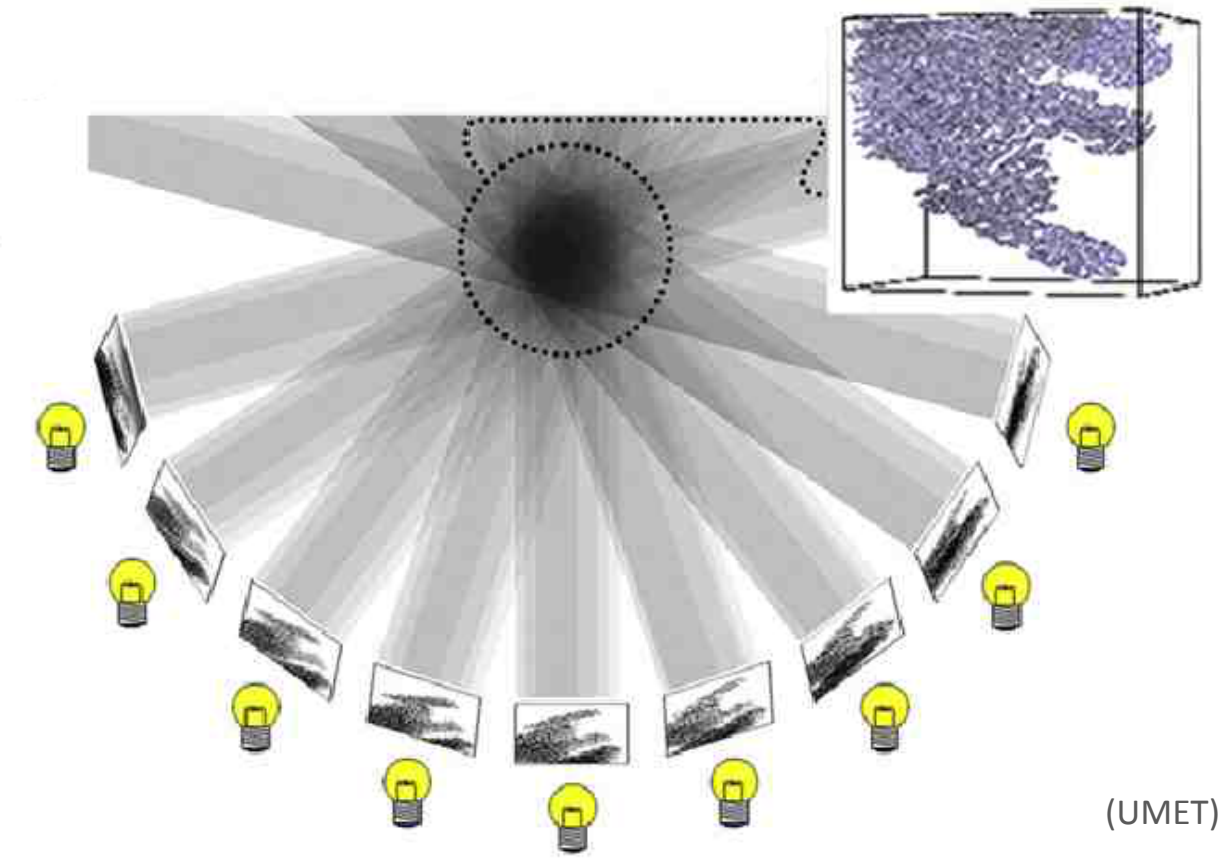






In ET Accuracy is Primarily Limited by Electron Dose, Angular Increment, and Tilt-Series Alignment



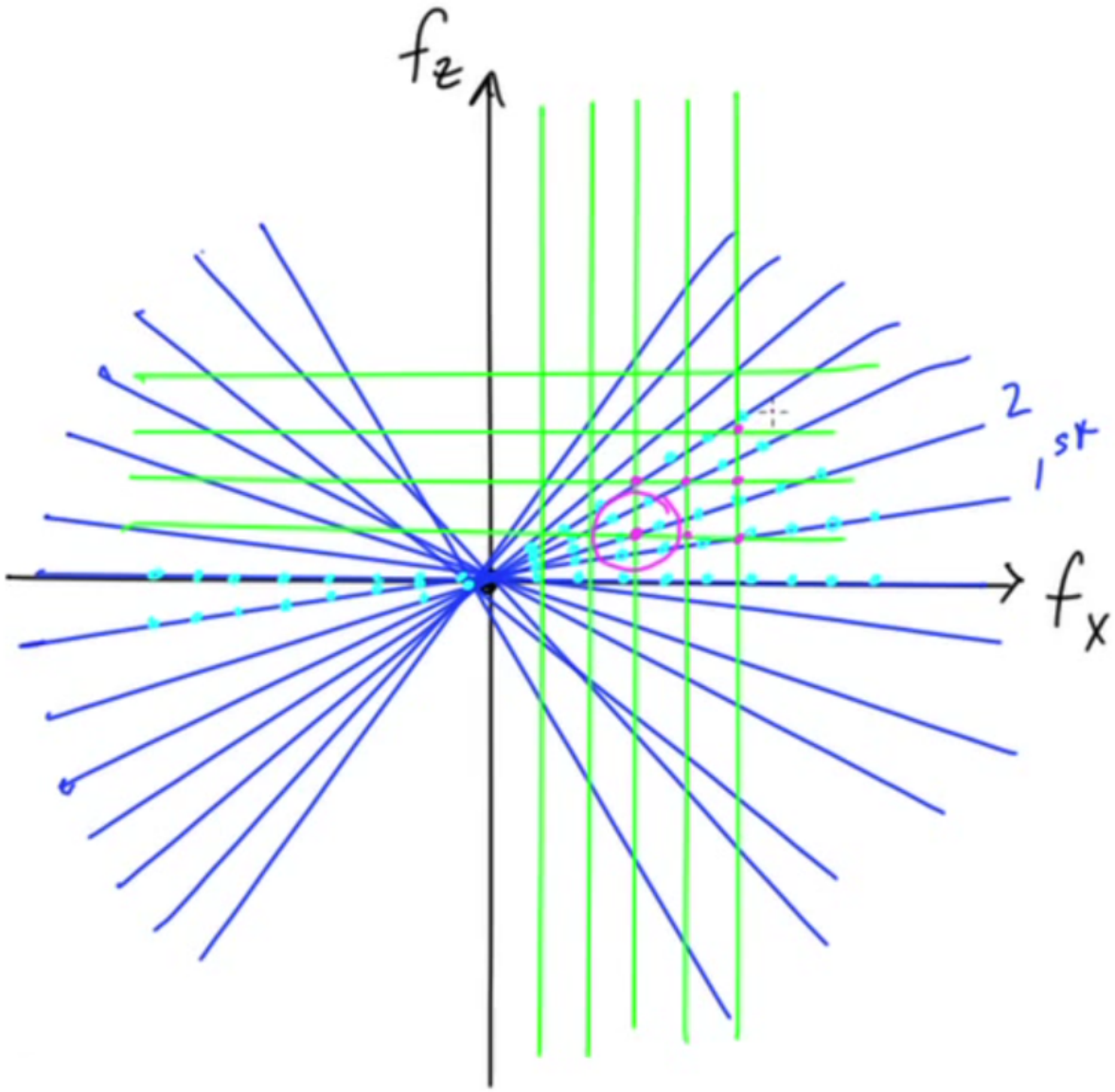


(UMET)

Collect → align → reconstruct

Reconstruction Implies Interpolation

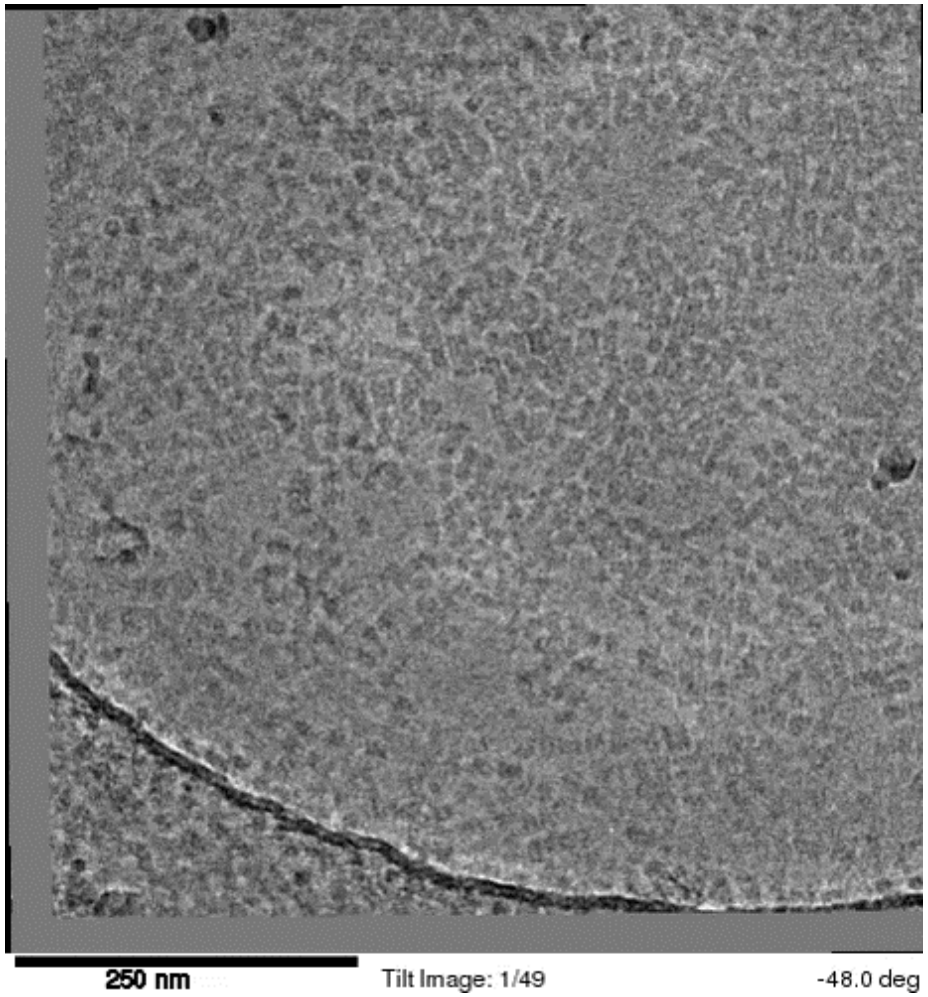
- Tomographic reconstruction in Fourier space always requires interpolation
- Larger tilt increment = more missing information at higher tilt angles



Grant Jensen, YouTube

Missing Wedge Effects

- Phase plate tilt-series of T20S Proteasome aligned with Appion-Protomo
- Tilt axis is horizontal



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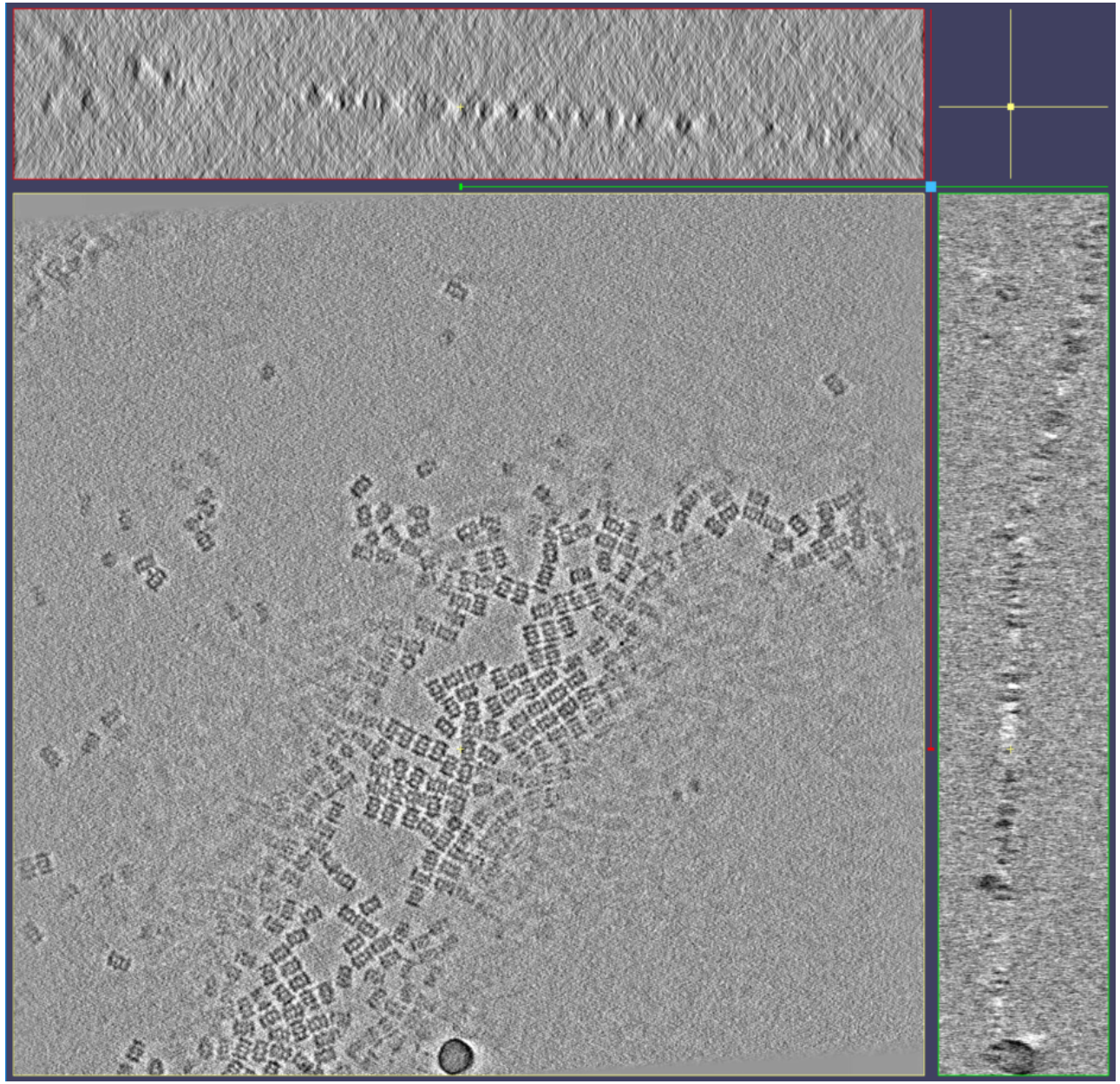
Anchi Cheng, Radostin Danev, Alex Noble

Missing Wedge Effects

- Phase plate tomogram of T20S Proteasome aligned with Appion-Protomo, reconstructed with Tomo3D
- Tilt axis is vertical



(rotated 90 degrees)

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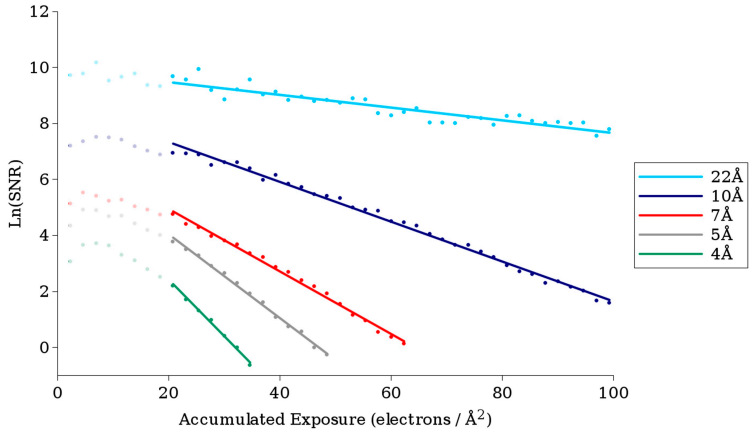
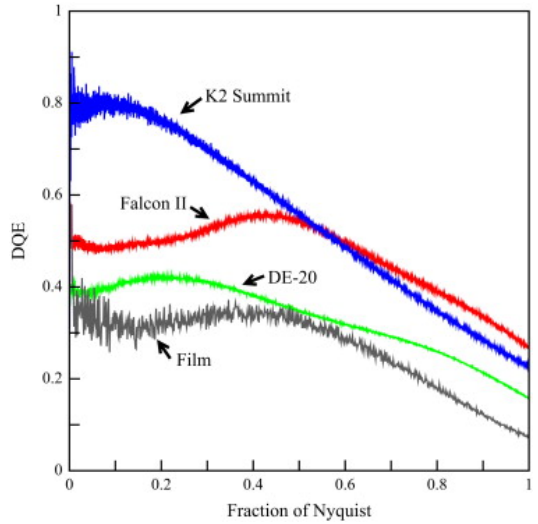
Fundamental Resolution Limits in CryoEM

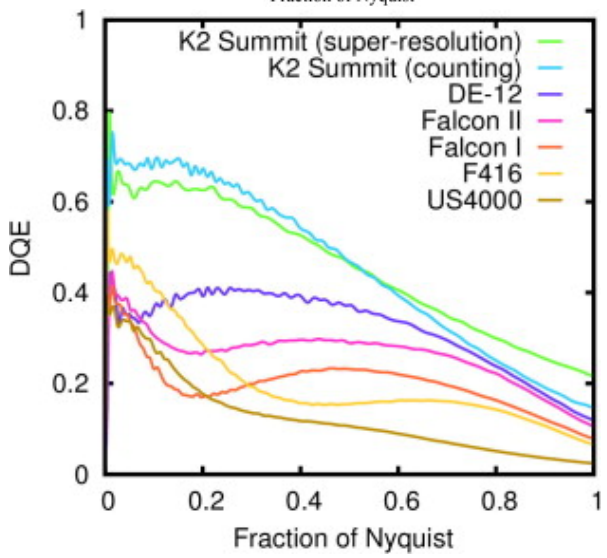
Dose on Specimen



Grant & Grigorieff, 2015

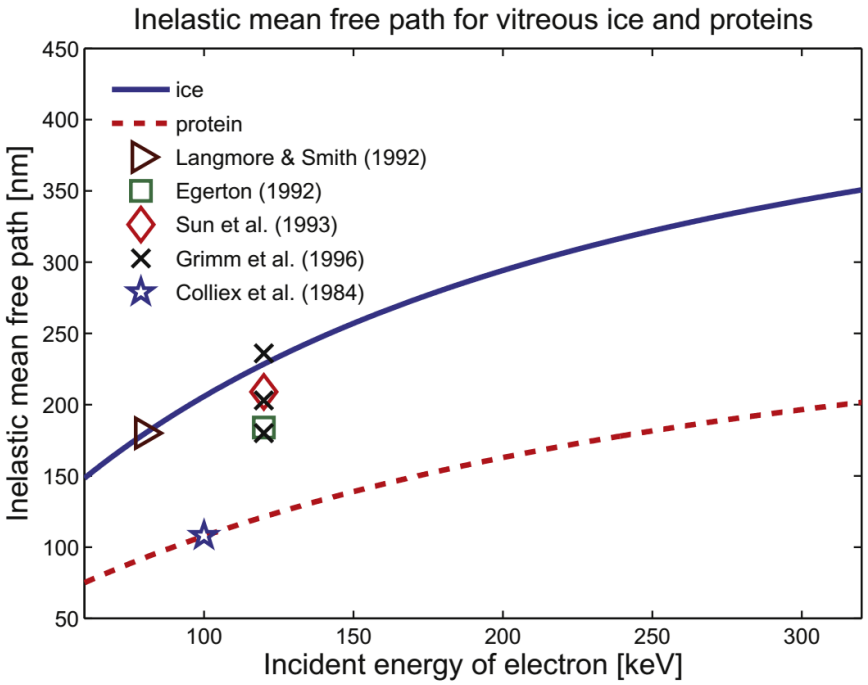
DQE





McMullin, 2014 & Ruskin, 2013

Ice Thickness



Vulović, 2013

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Tilt-Series Collection is a Game of Trade-Offs

- Resolution is always limited by the accumulated dose on the sample.
- For a given amount of total dose, commonly between 50 and 200 e-/A^2, a certain number of tilt images are possible for a given tilt increment
  - For example, with a total dose of 100 e-/A^2 one may wish to spread the electrons out across 100 tilt images from -50 degrees to 50 degrees at 1 degree increments; ie. [-50:50:1]
  - For example, with a total dose of 50 e-/A^2 one may choose to collect at a coarse increment of 3 degrees over
  - Minimum tilt increment is determined by possible dose per image and accuracy of goniometer
  - Maximum practical tilt increment, in my experience, is 5 degrees
- Microscope setup (DD? EF? PP?), magnification choice (ie. pixelsize), and defocus range will all determine the feasibility of reliable automated collection and alignment of tilt-series and sub-tomograms.
- This all depends on the intended post-processing of the sample and on the resolution desired

Tilt-Series Collection is a Game of Trade-Offs

Examples of possible collection setups:

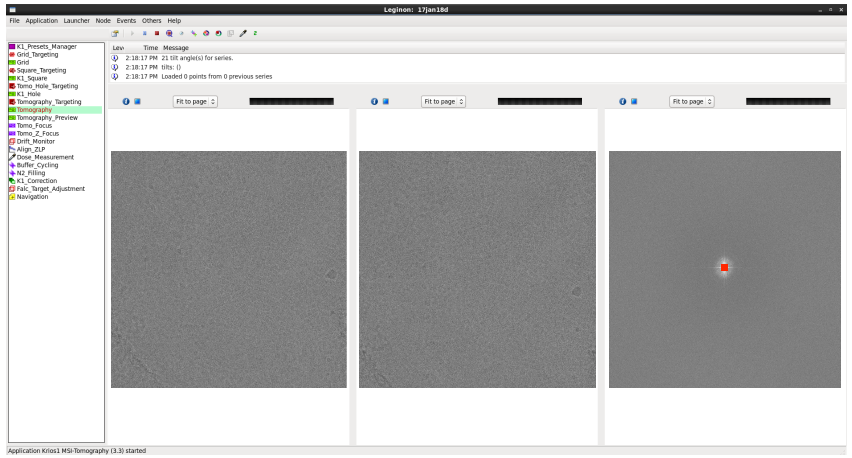
- For a **SPT project** where particles exhibit **low heterogeneity**, it may be appropriate to use a somewhat **coarse tilt increment** of ~3 degrees and a **low tilt range** of perhaps +-45 degrees so that a **higher dose and lower defocus** of 1 – 2 µm may be used.
- For a tilt-series collection of a **cell** with the intention of **viewing and segmenting** large objects clearly, a **finer tilt increment** of ~1 degree and **higher tilt range** of +-65 degrees may be used with a **moderate dose and higher defocus** of around 5 µm. Consider using the Saxton tilt scheme to increase

In practice, alternating tilt-series collection currently tracks less accurately than bi-directional or uni-directional collection.

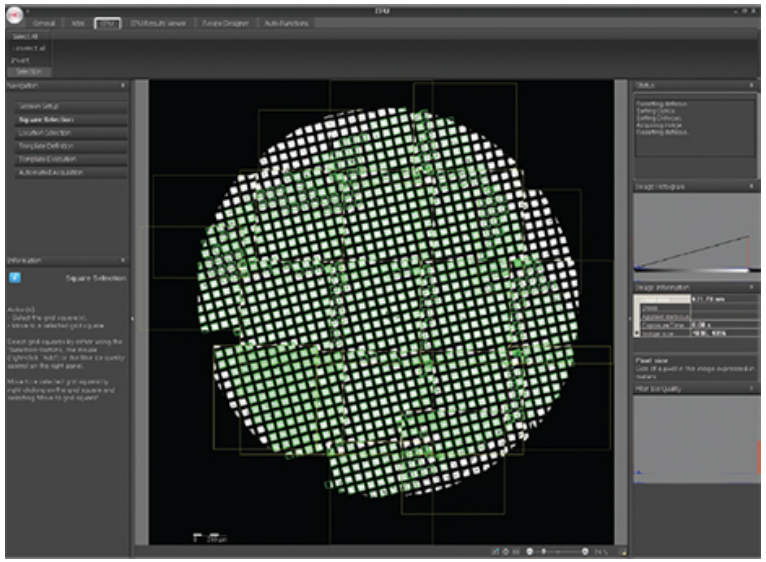
For optimal resolution in a single tomogram, one would collect with an alternating Saxton scheme.

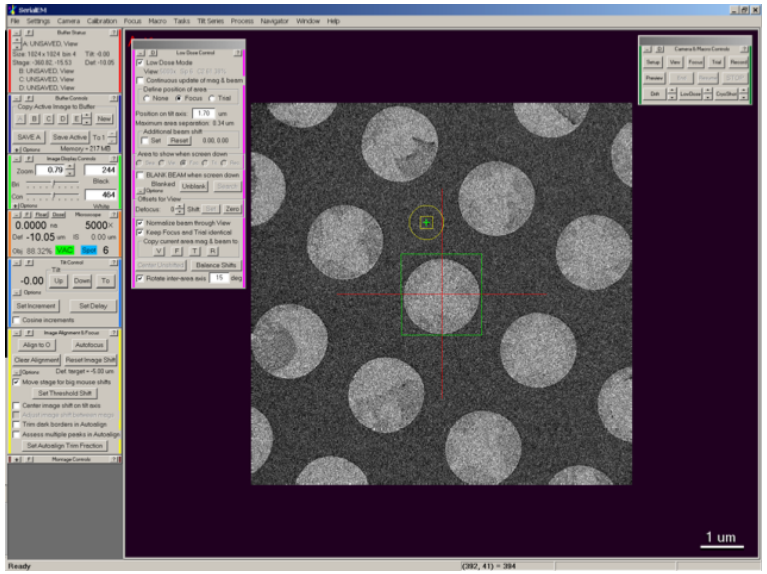
Tilt-Series Collection Software

Leginon

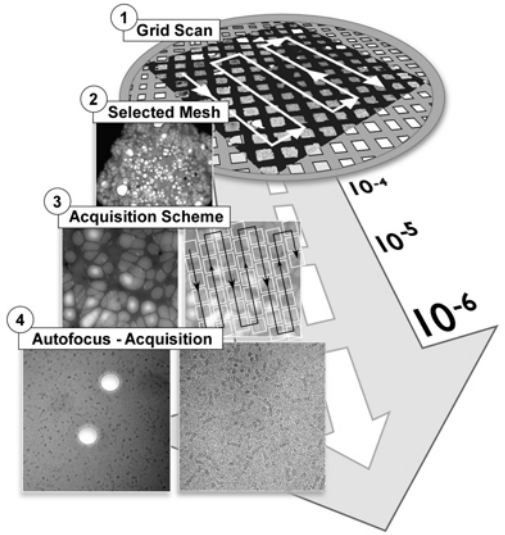


EPU

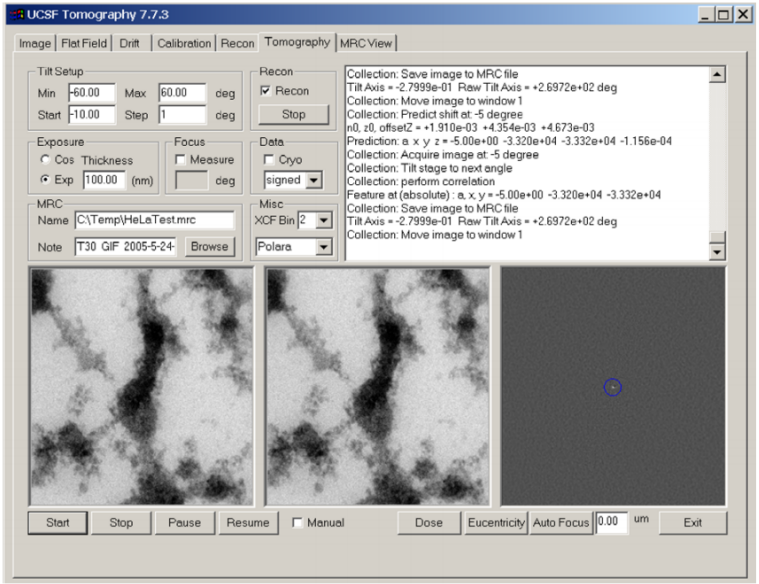




SerialEM



TOM Toolbox



UCSF Tomography

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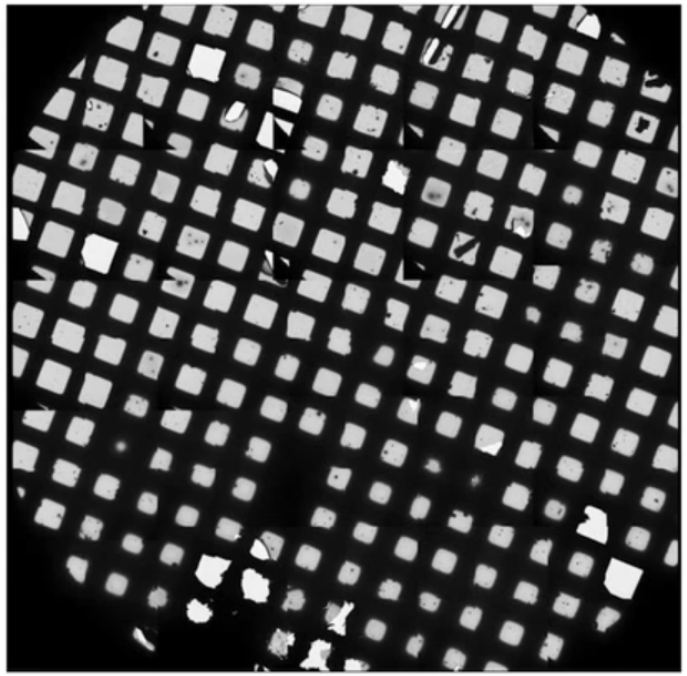
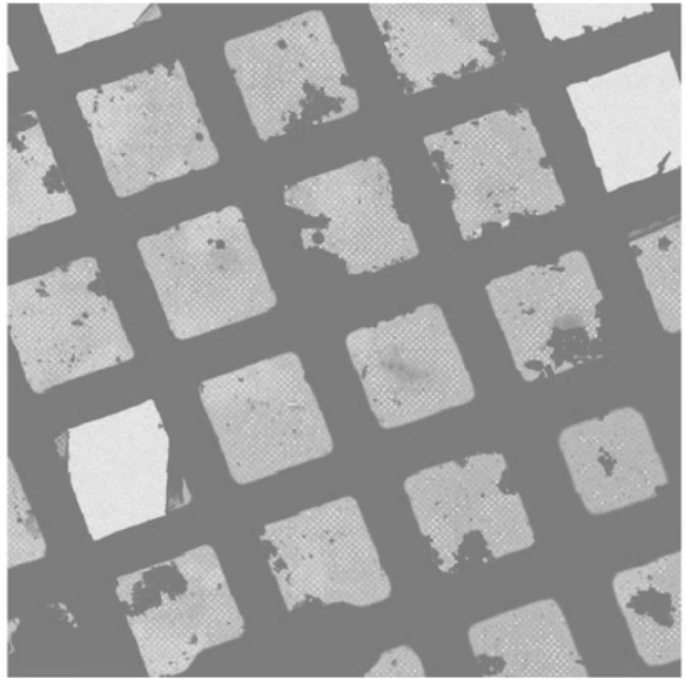
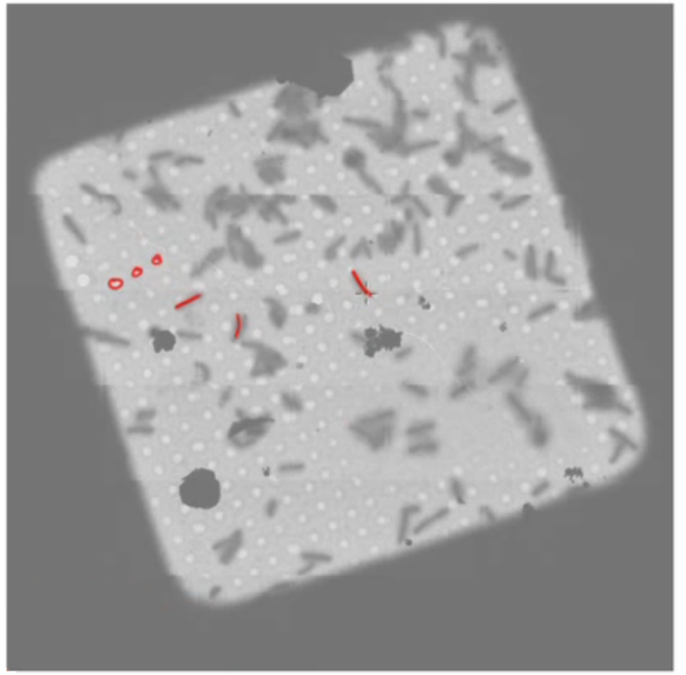
Tilt-Series Collection Tracking

In order to not apply unnecessary dose to the sample, tilt images must be located a-priori, called “tracking”

- Predictive tracking – Previous tilt images, previous tilt-series, and possibly information regarding known goniometer instabilities may be used
- Focus position method – Identify two locations along the tilt axis that the microscope software will use to focus, then interpolate to the exposure location

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!

Tilt-series Alignment Software

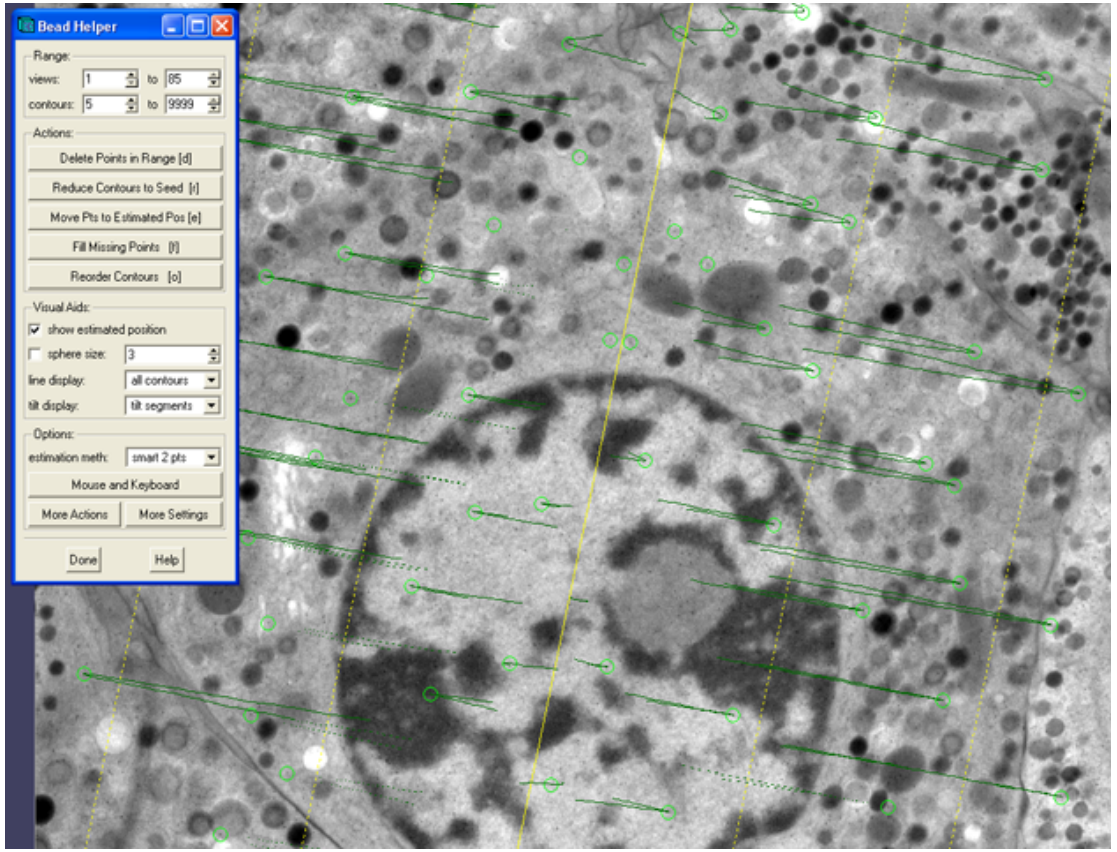
- Etomo in IMOD – fiducial-based alignment
- Protomo – fiducial-less alignment
- Alignator – Patch tracking, GPU-accelerated
- ATOM – fiducial-less alignment

Each 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 processing



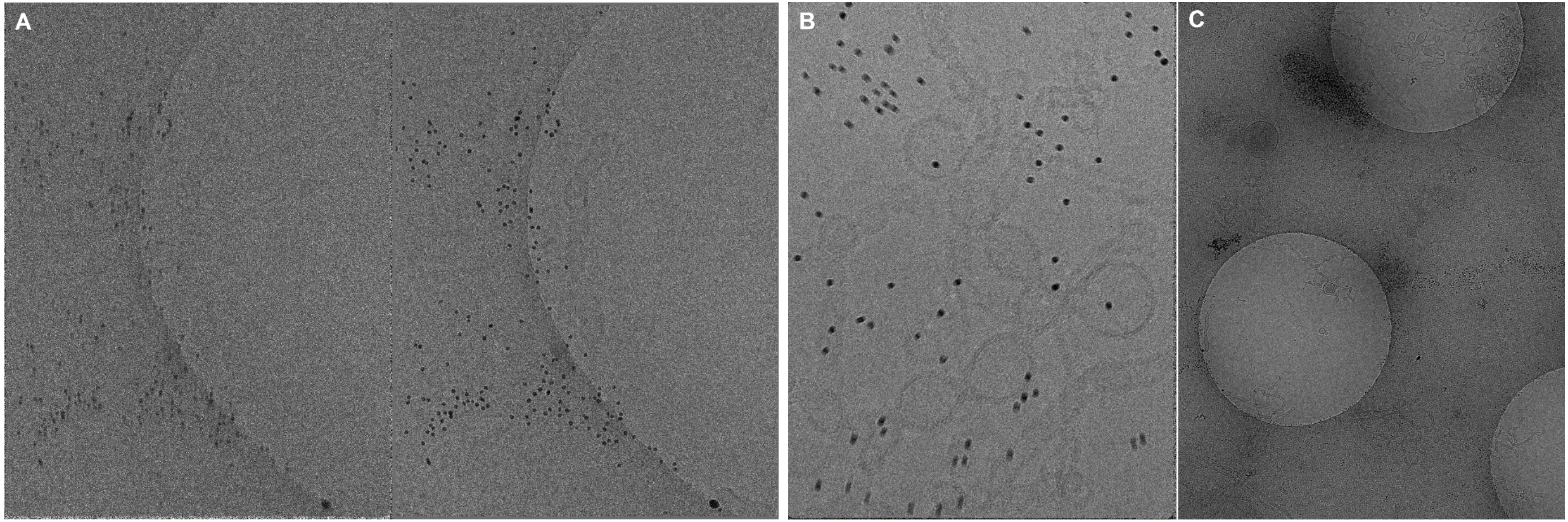
http://bio3d.colorado.edu

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

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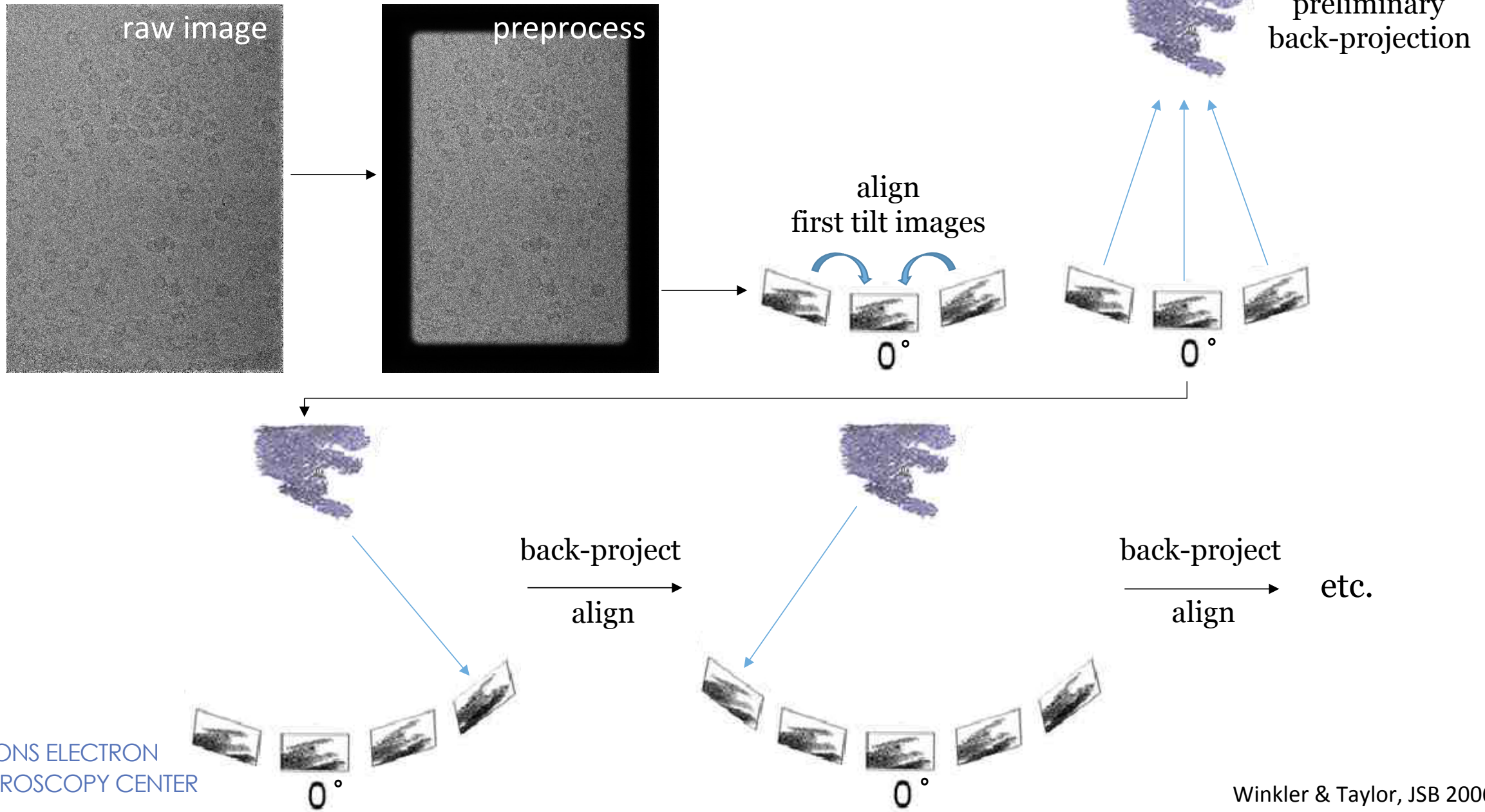
Noble & Stagg, JSB 2015

Protomo Correlation-based Alignment



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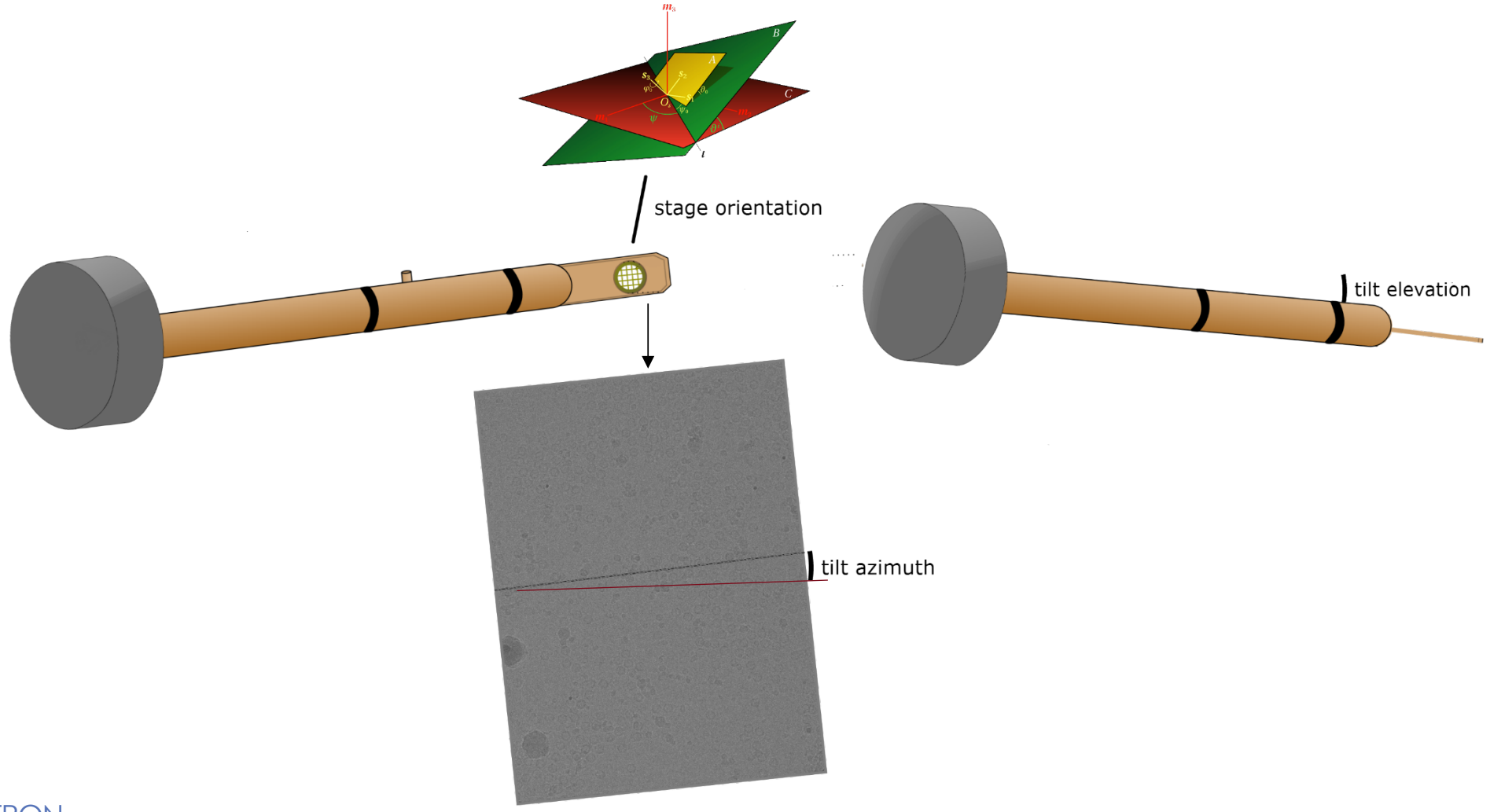
Winkler & Taylor, JSB 2006

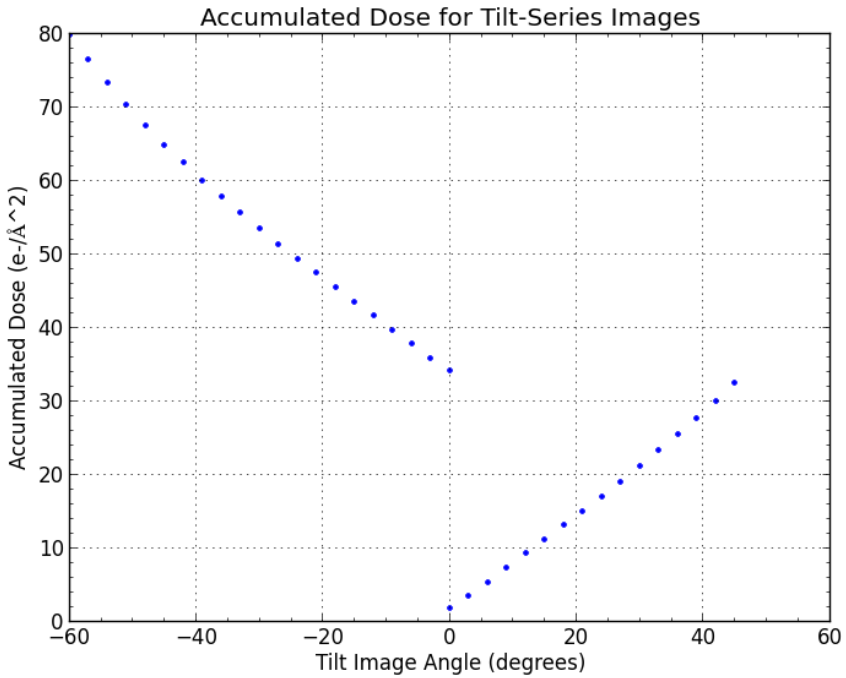
Protomo Accounts for the Full Geometry of Sample on Grid

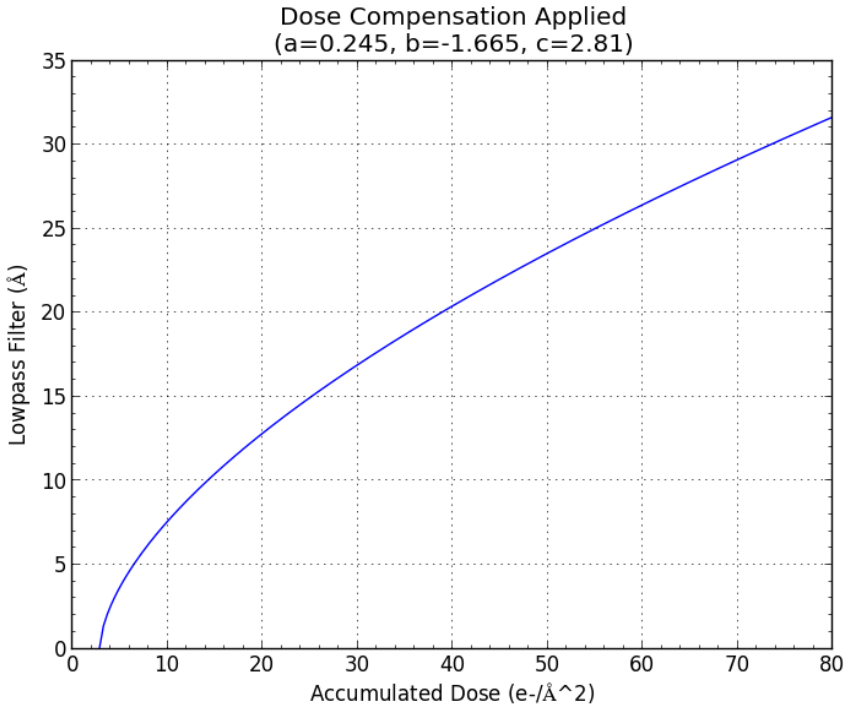


Winkler & Taylor, JSB 2006

Per-Image Dose Compensation in Appion-Protomo

- Uses accumulated dose
- Based on Grant & Grigorieff, 2015





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Noble & Stagg, JSB 2015

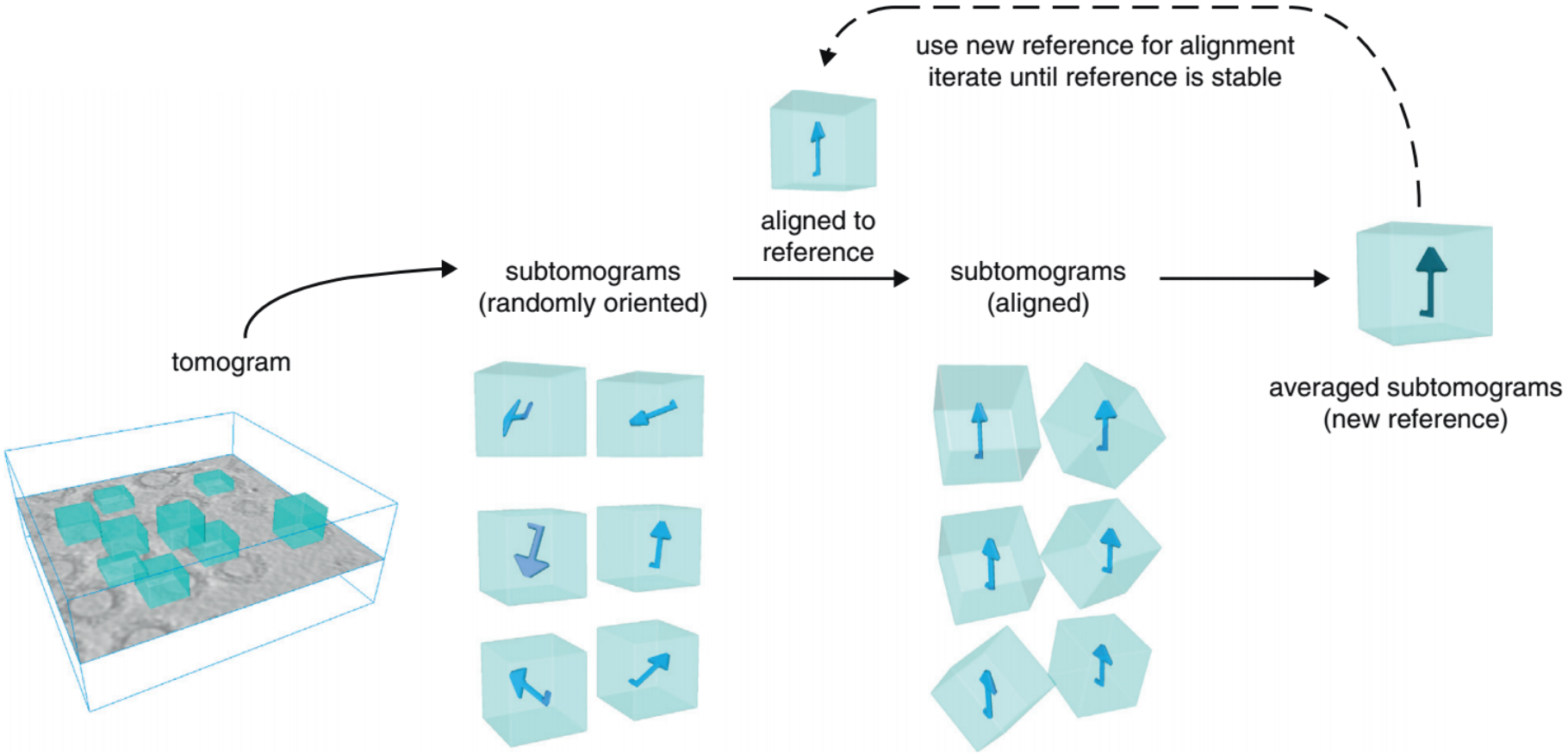
Defocus Estimation Methods

- Per-image defocus estimation accounting for tilts
  - CTFFIND4
  - GCTF
- Defocus estimation and interpolation using two focus locations on the tilt axis (Eibauer, 2012)
- EMAN2 – Per-particle image defocus estimation
- TomoCTF – Image tiling to estimate the defocus of the untilted plane
- Post-hoc defocus estimation by using a SPT FSC to locate the first CTF zero

CTF Correction Methods

- Full image correction – limited by the height difference from the mean at tilts
- Strip-based correction with IMOD's ctfphaseflip or TomoCTF
  - Flips the phases and optionally amplifies amplitudes (TomoCTF) on a strip-by-strip basis parallel to the tilt axis
  - Requires an accurate goniometer and sample at eucentric height
- 3D CTF model – Implemented in Relion
- Per-particle image correction – Implemented in EMAN2
- During tomographic reconstruction

Sub-tomogram Processing Workflow



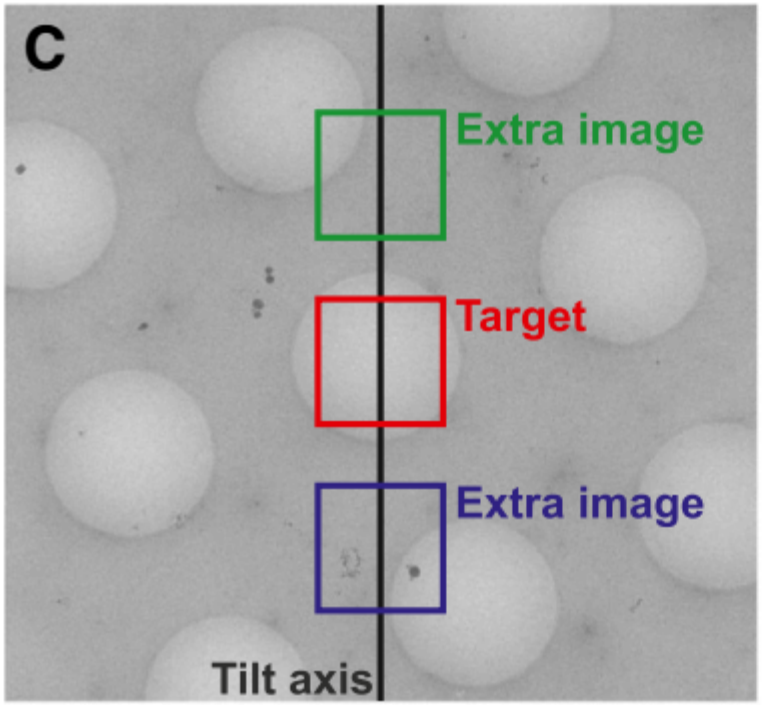
Current Opinion in Structural Biology, Briggs 2013

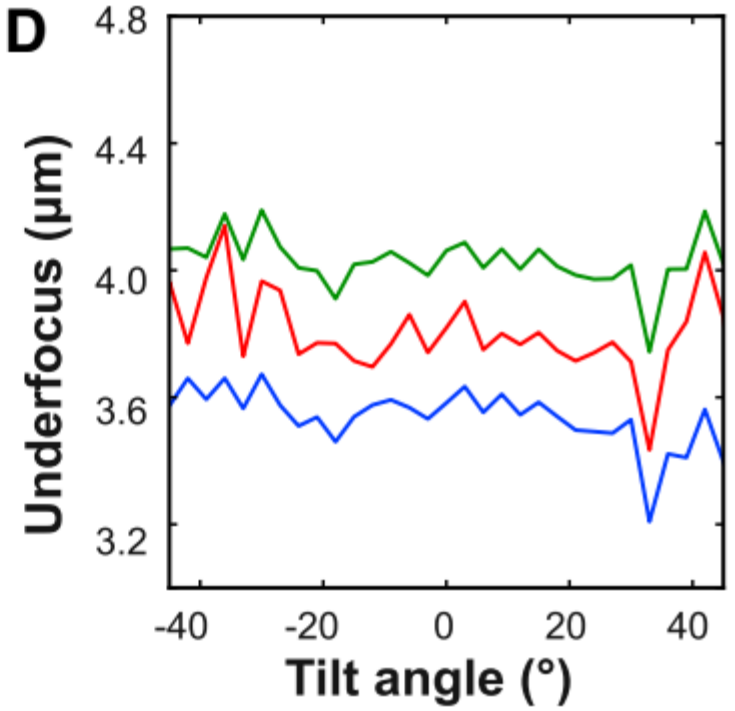
- Missing wedge must be taken into account for each sub-tomogram

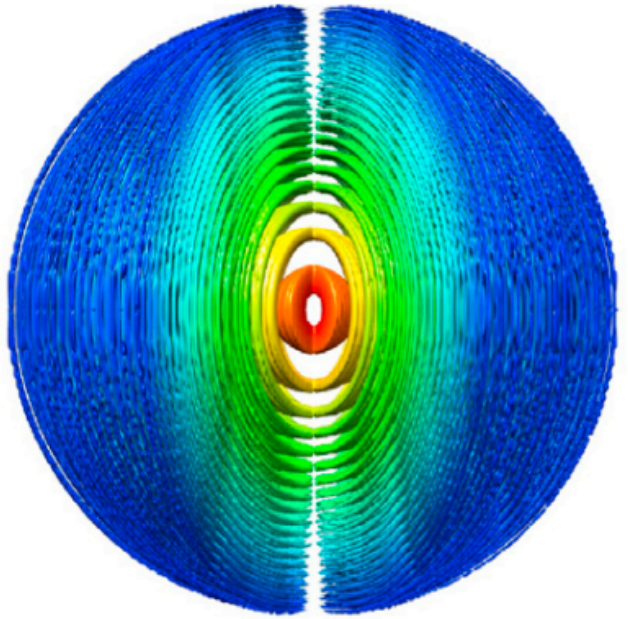
Sub-tomogram Processing Software

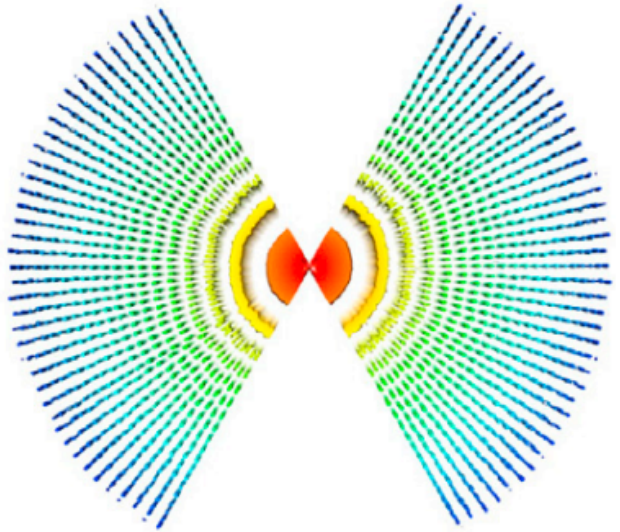
- Dynamo – GPU accelerated, tomogram database, extensive picking abilities
- Relion – 3D CTF model, Bayesian approach to alignment is used
- EMAN2 – Sub-tilt-series refinement and defocus estimation
- PyTom
- PEET
- Jsubtomo
- TOM & AV3
- XMIPP

Relion SPT









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

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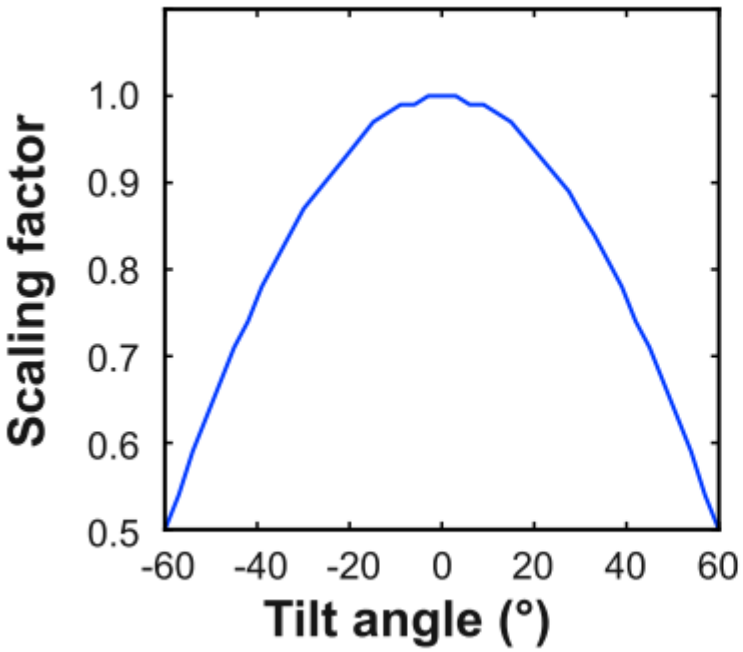
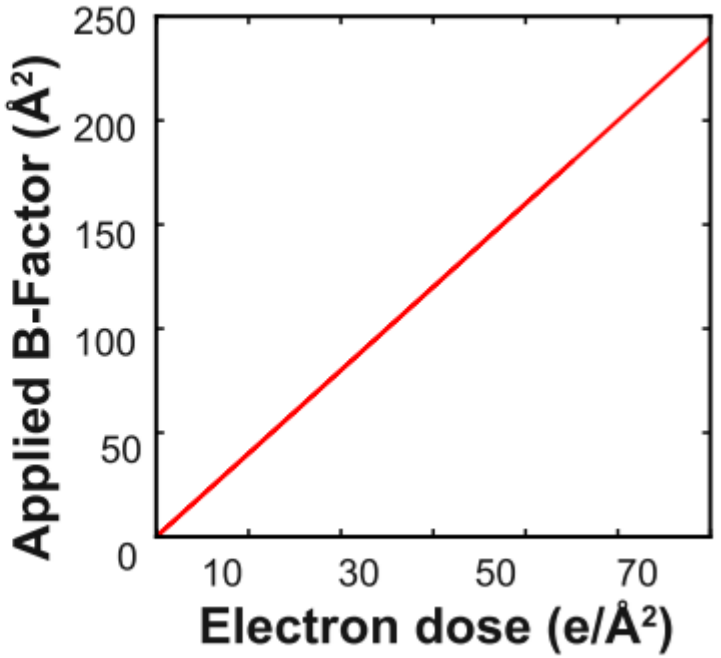
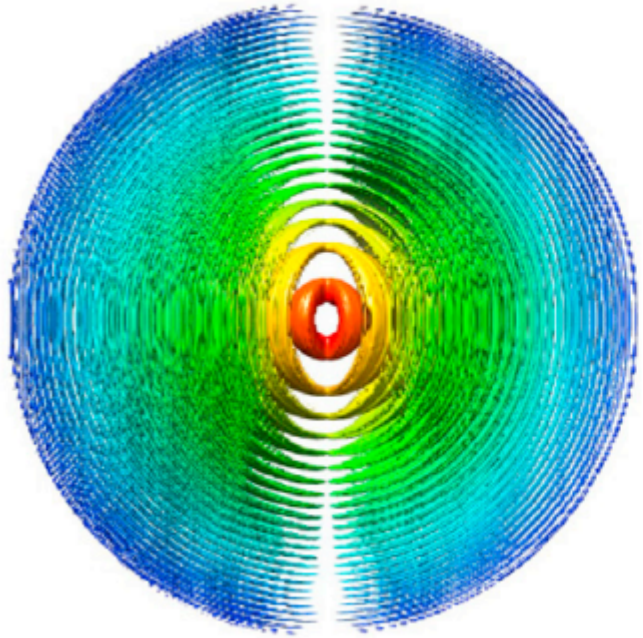
Bharat et. al., Structure 2015

Relion SPT







weighted CTF model

Bharat et. al., Structure 2015

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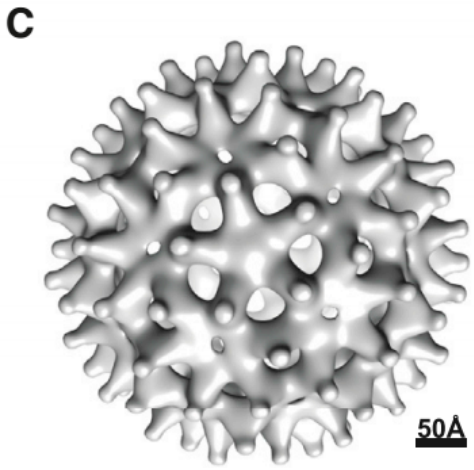
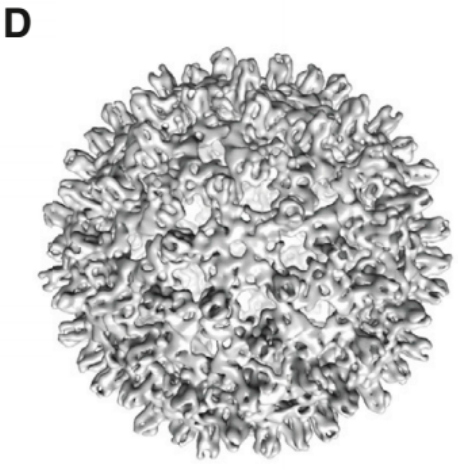
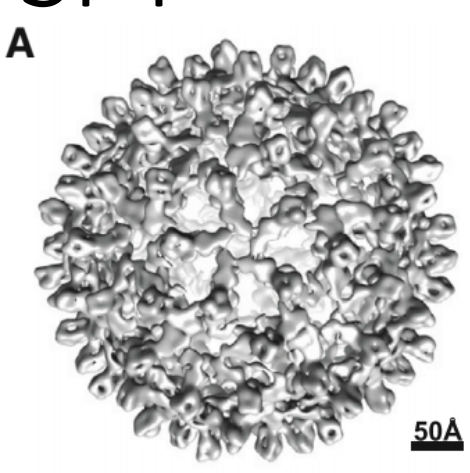
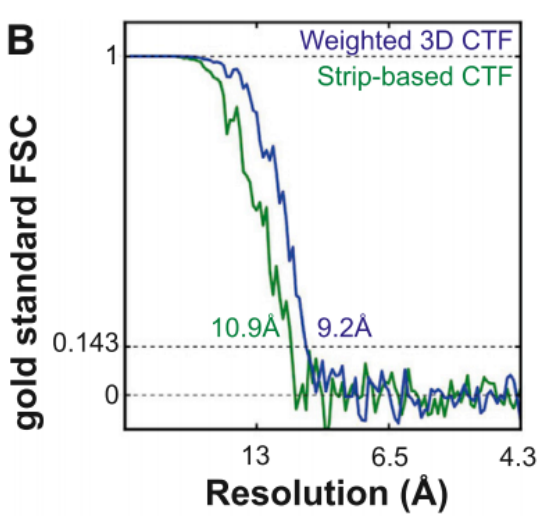
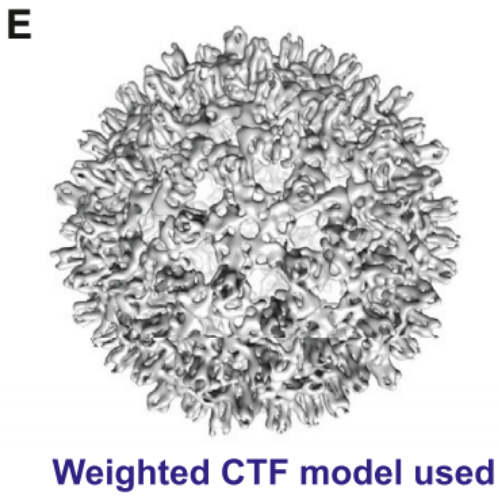
Relion SPT

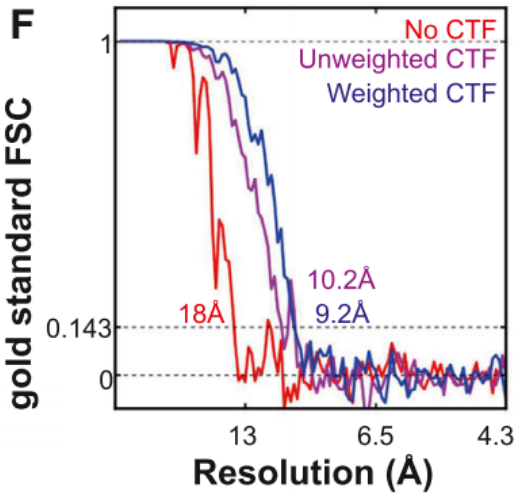


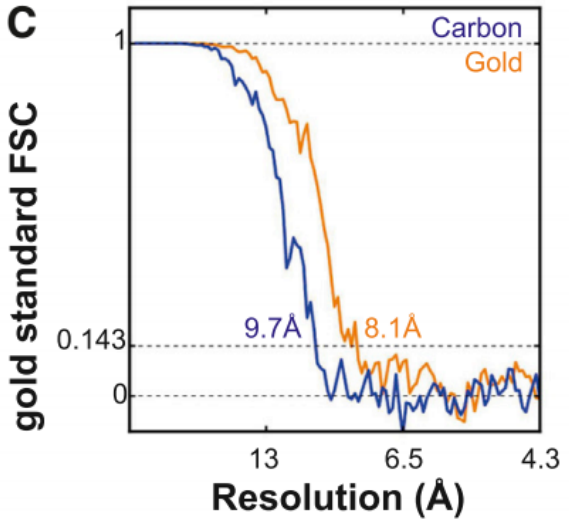


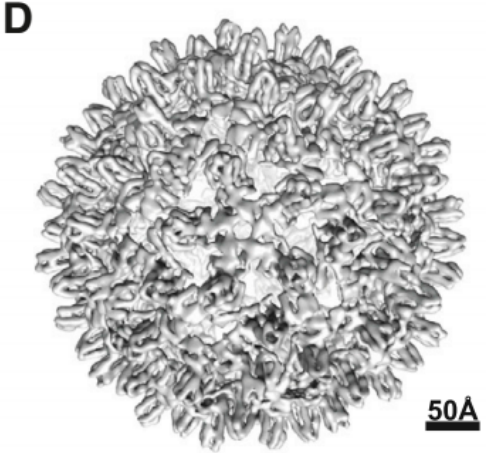




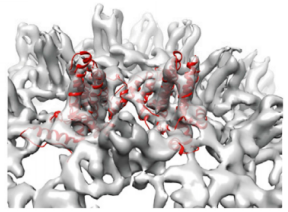








- Test case: Hepatitis B capsid



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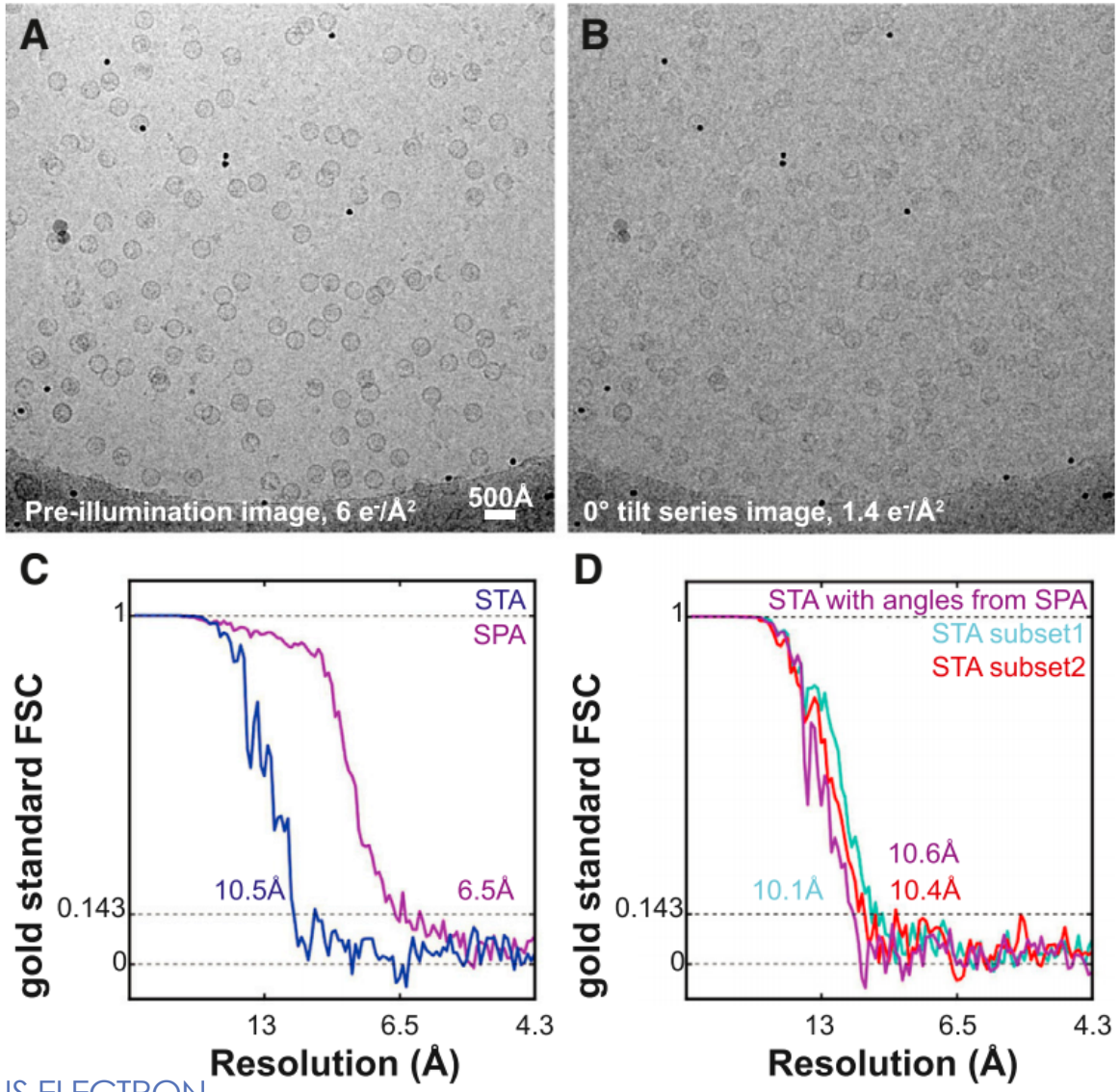
Bharat et. al., Structure 2015

Relion SPT – Comparison to Single Particle



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

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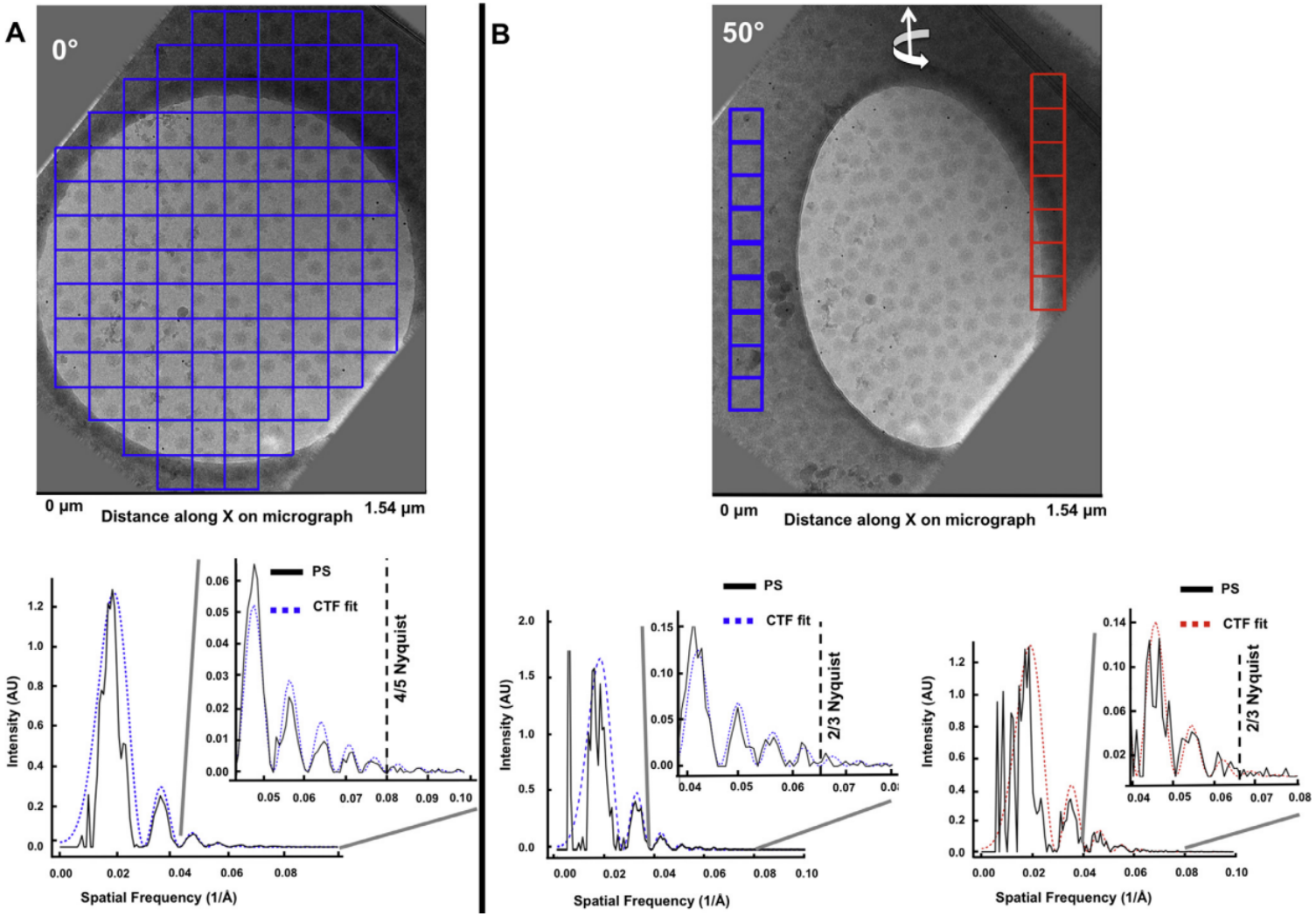
Bharat et. al., Structure 2015

EMAN2 SPT Tiled Defocus Estimation



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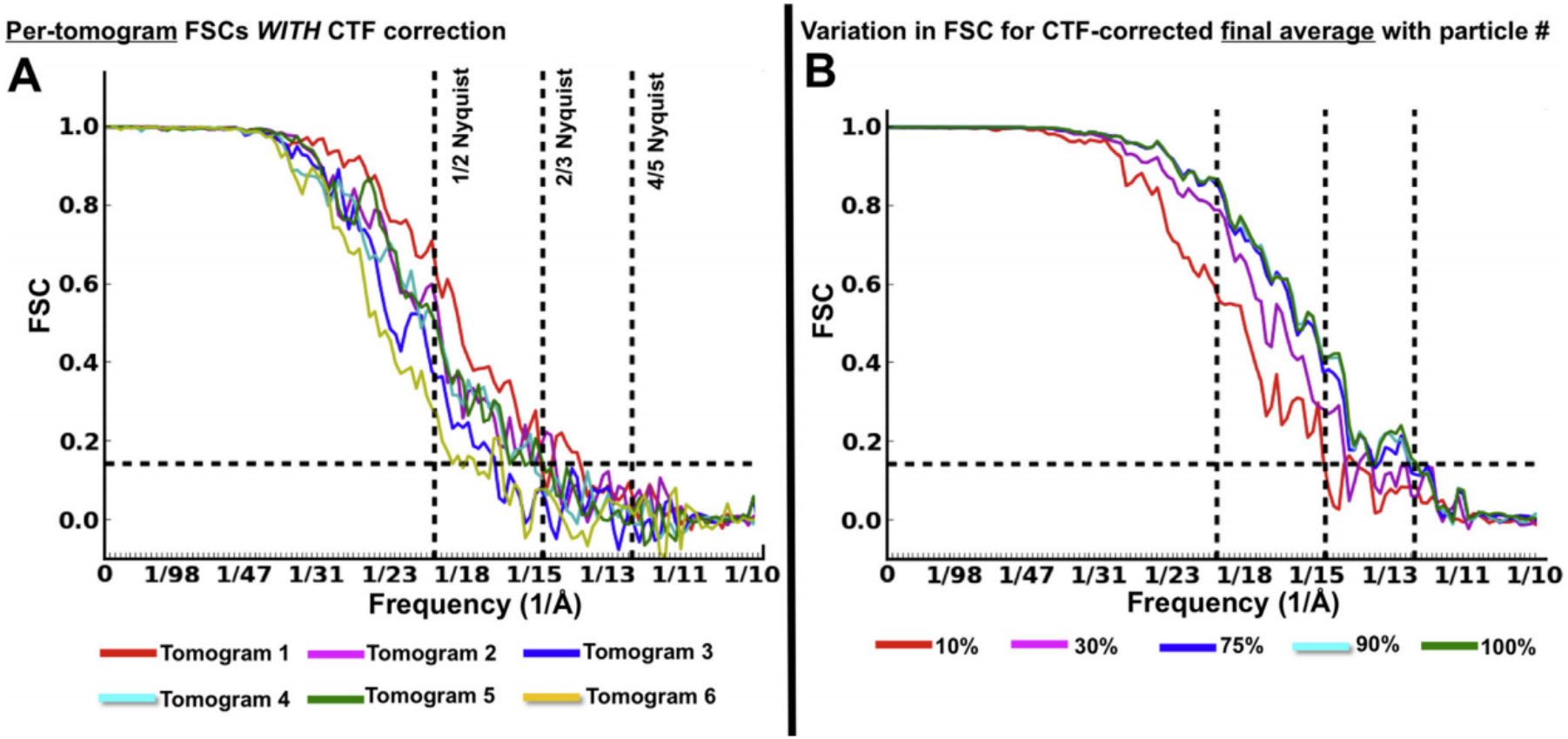
Galaz-Montoya, JSB 2016

EMAN2 SPT Tiled Defocus Estimation



- Better than 2/3 Nyquist

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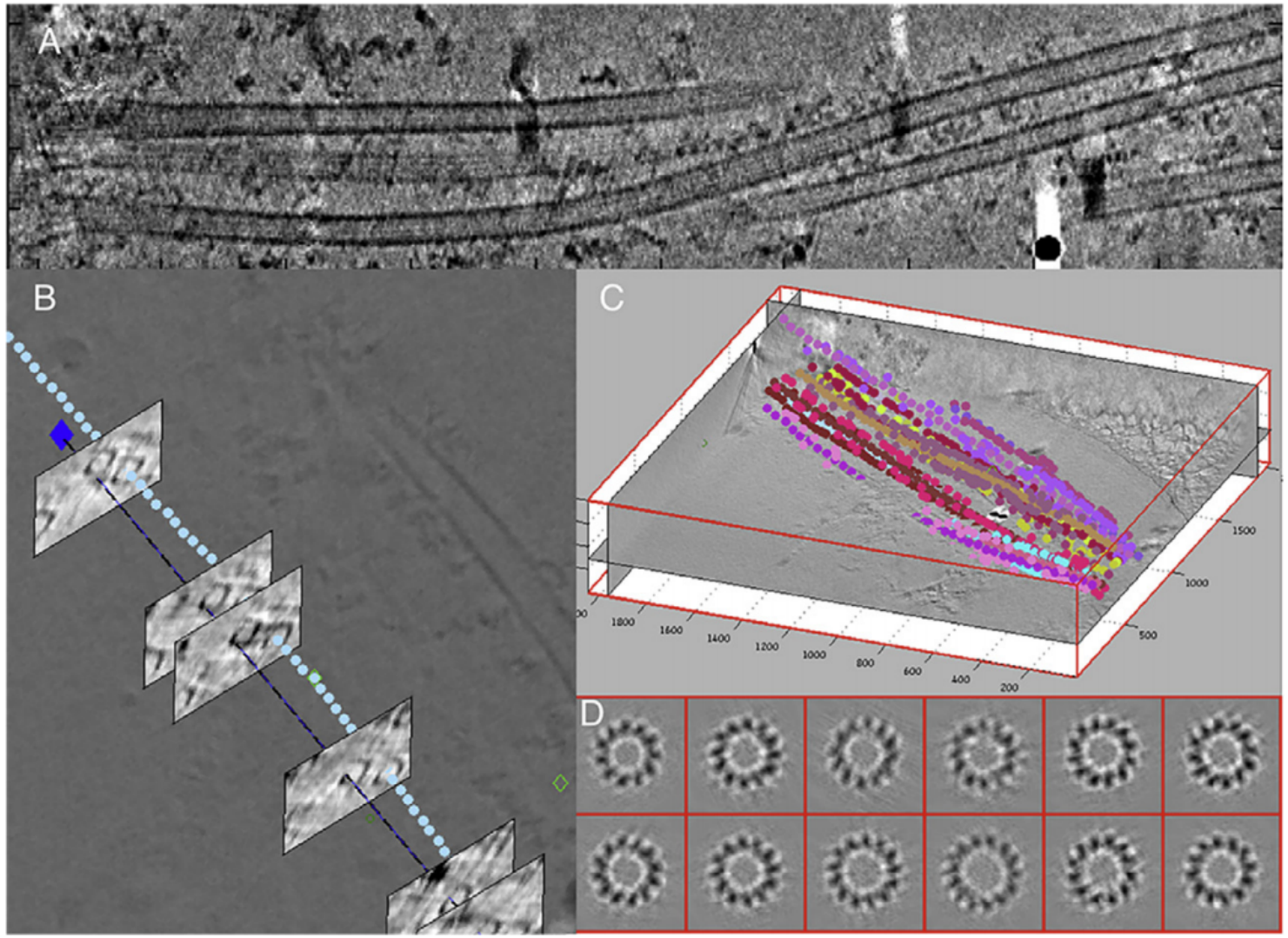
Galaz-Montoya, JSB 2016

Annotation and Segmentation Software

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

Dynamo Filament Picking



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

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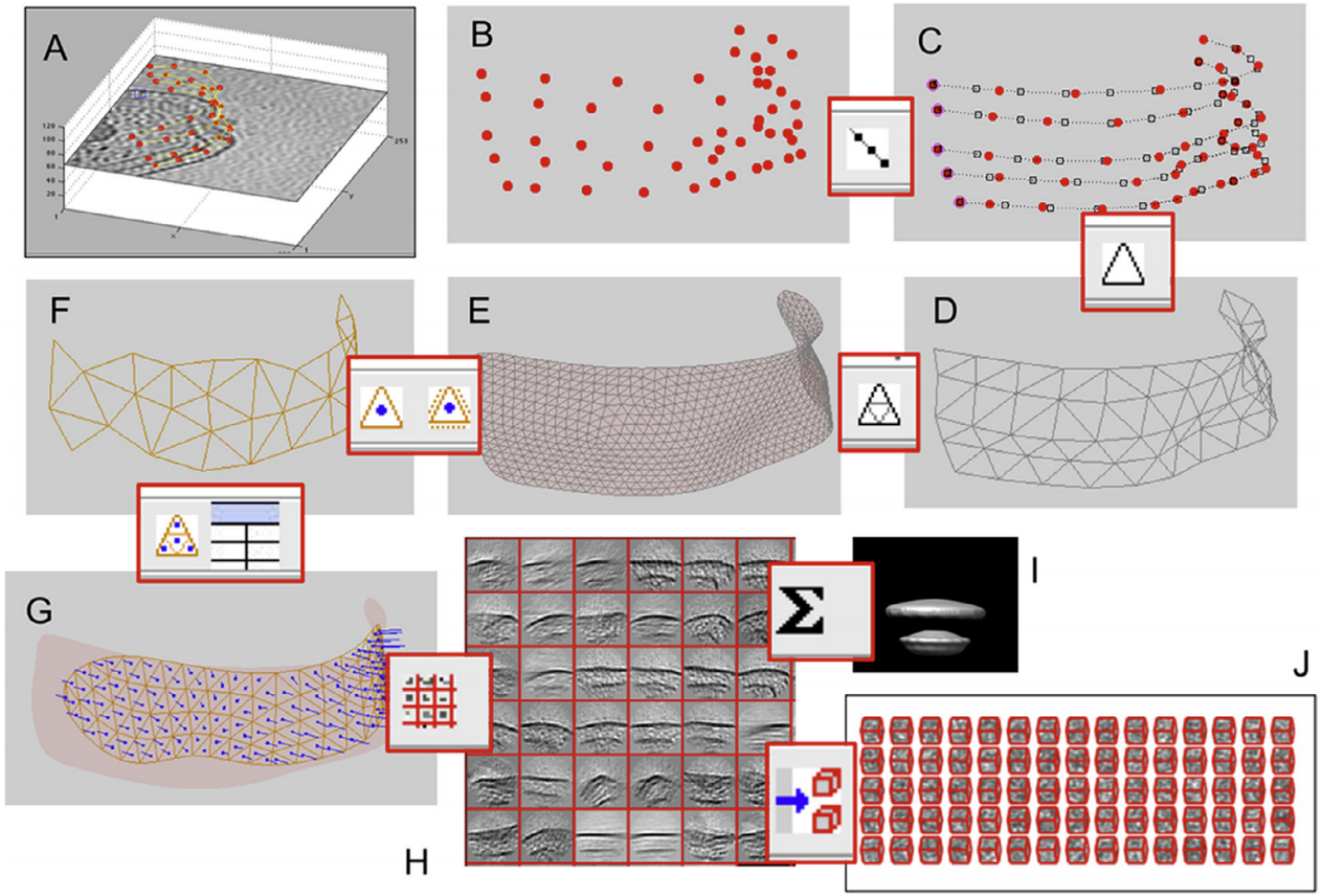
Castaño-Díez et. al., JSB 2012 & 2016

Dynamo Membrane Picking



- a

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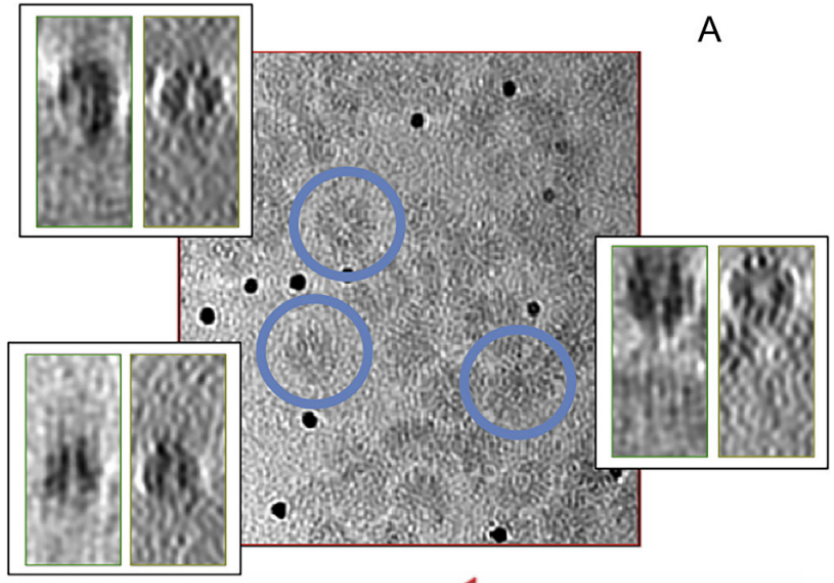
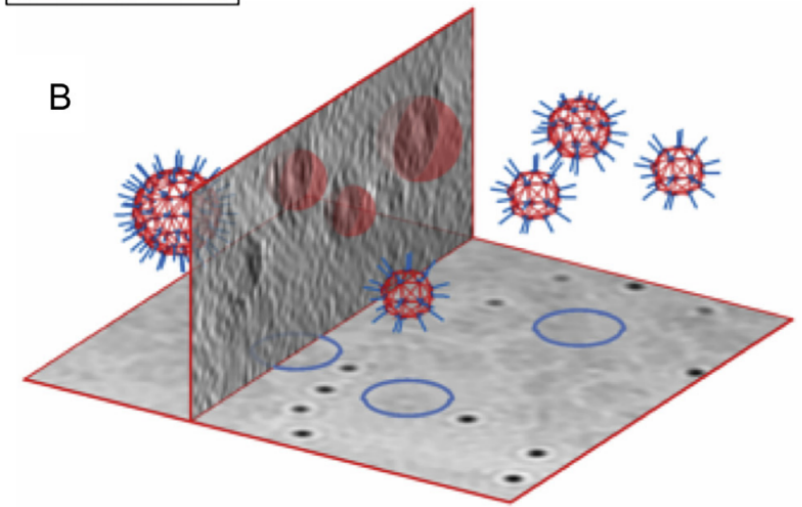
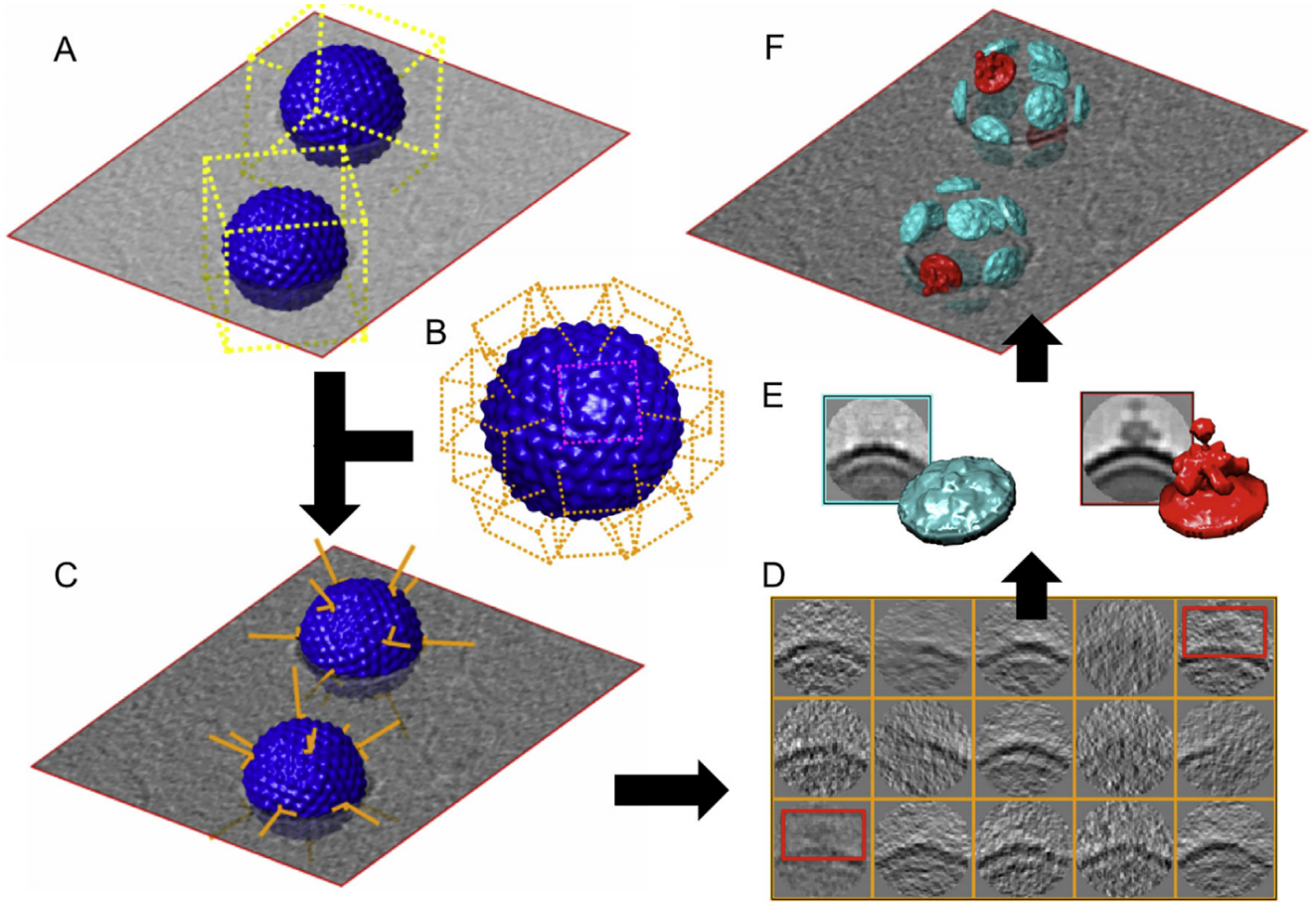
Castaño-Díez et. al., JSB 2012 & 2016

Dynamo Vesicle and Sub-Particle Picking







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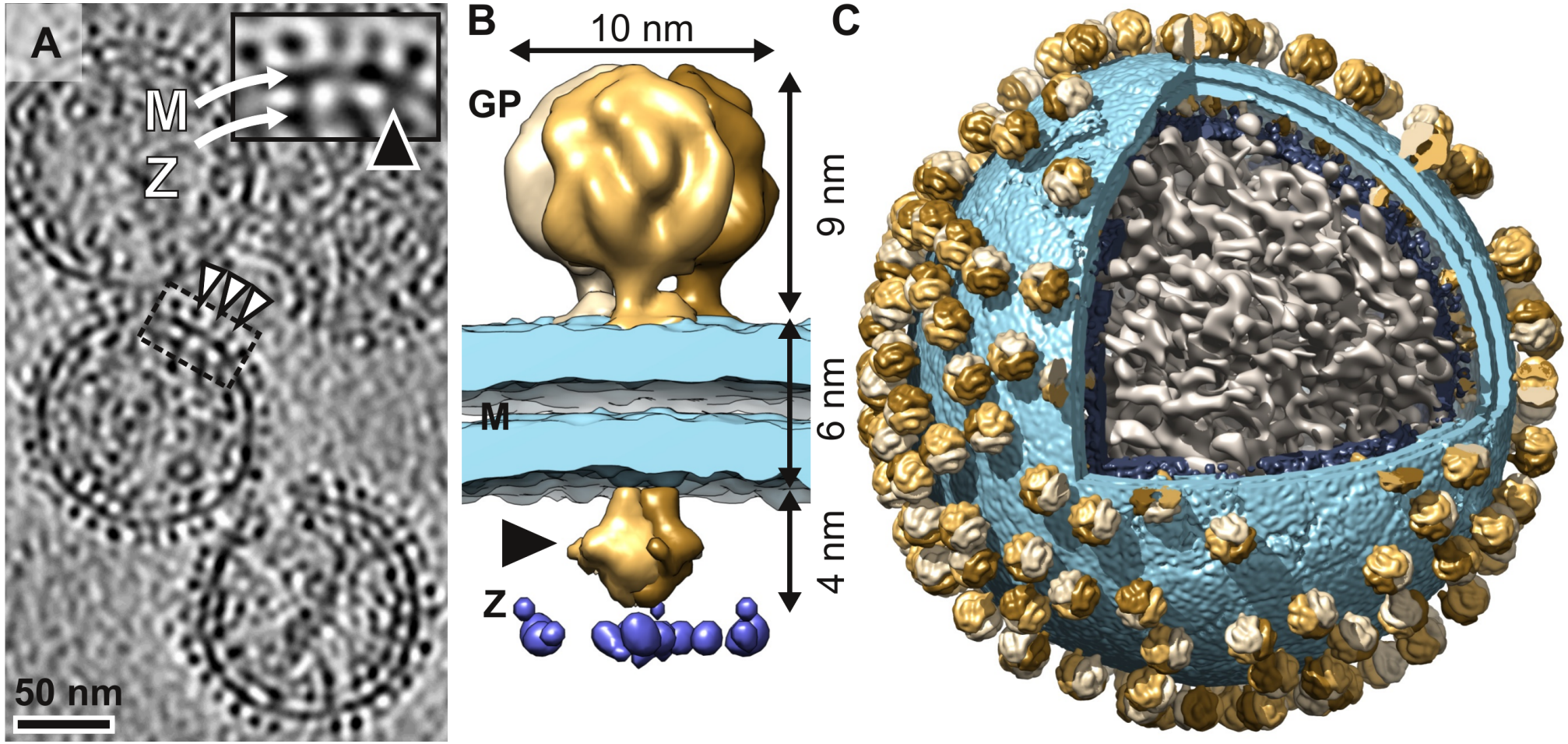
Sub-Tomogram Processing Examples

(break)

Lassa Virus Glycoprotein Spike



- Spheroidal enveloped viruses of various sizes with irregularly spaced spikes were picked with a Dynamo membrane model

Li et. al., PLOS 2016

Lassa Virus Glycoprotein Spike

	LASV	VLP pH 7.3	VLP pH 5.2	VLP+LAMP1 pH 5.5	VLP pH 3.0
Data acquisition					
Magnification	37,037	37,037	37,037	37,037	37,037
Tilt series	27	31	16	30	10
Tilt range (°)	-45–45	-45–45	-45–45	-45–45	-45–45
Interval (°)	5	5	5	5	5
Frames per tilt	8	8	8	8	8
Dose (e^{-}/Å^2)	~60	~60	~60	~60	~60
Defocus (um)[a]	2.3–3.9	1.7–3.7	2.8–6.7	1.4–4.6	3.0–3.9
Data processing					
Virions/VLPs	48	112	22	113	23
Seeds [b]	n/a	n/a	9,406	n/a	7,821
Sub-tomograms	6,496	2,764	2,578	8,527	1,454
Box size (pixels)	128	128	128	128	128
Pixel size (Å)	2.7	2.7	2.7	2.7	2.7
Resolution (Å) [c]	13.6	13.9	16.4	14.8	16.7

[a]Positive defocus denotes underfocus.

[b]Number of 'seeds' created on the virion/VLP surfaces. If n/a is indicated, manual picking was used instead of seeds.

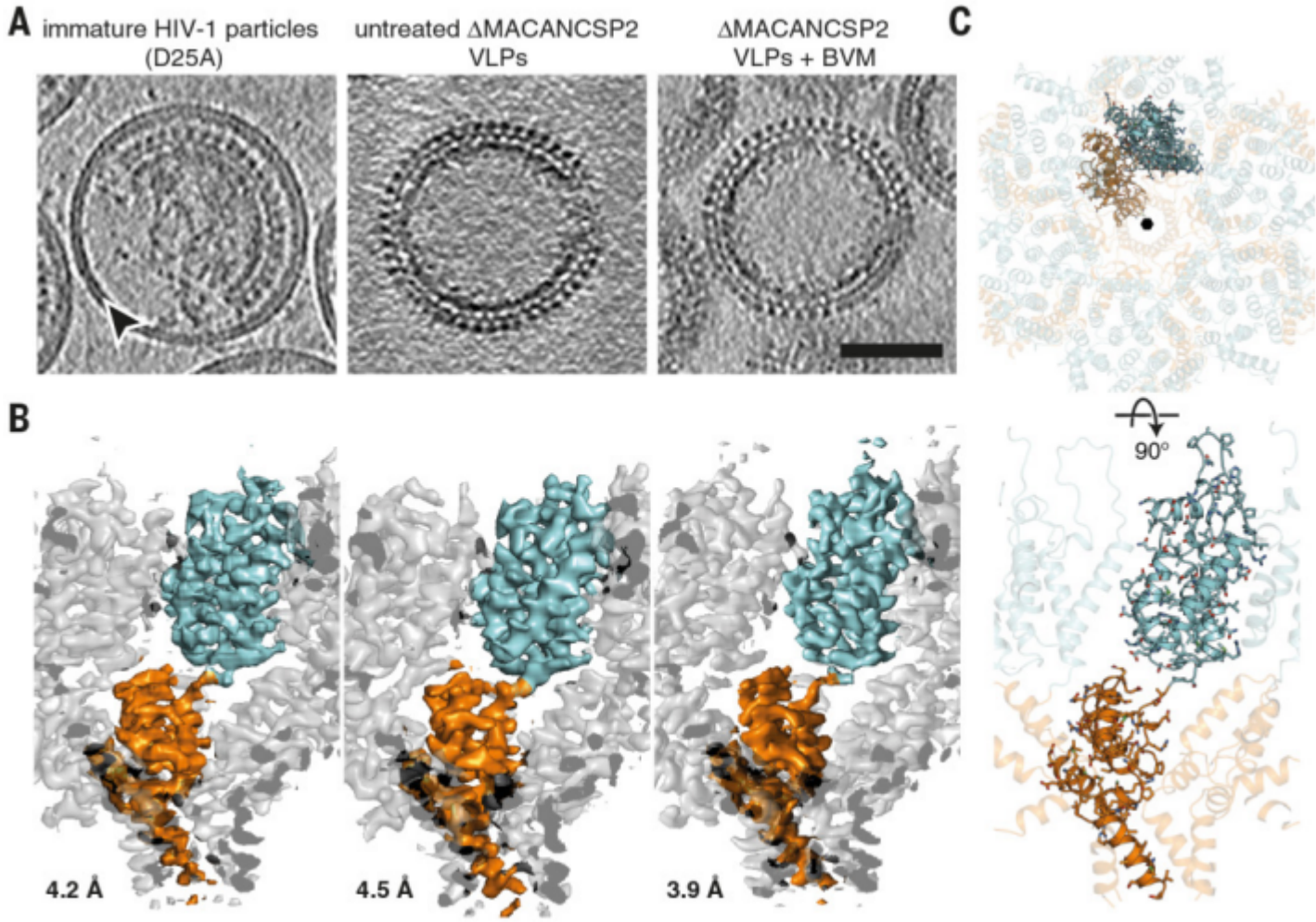
[c]Resolution (Fourier shell correlation = 0.5).

doi:10.1371/journal.ppat.1005418.t001

Li et. al., PLOS 2016

HIV-1 Capsid-SP1 at 3.9 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



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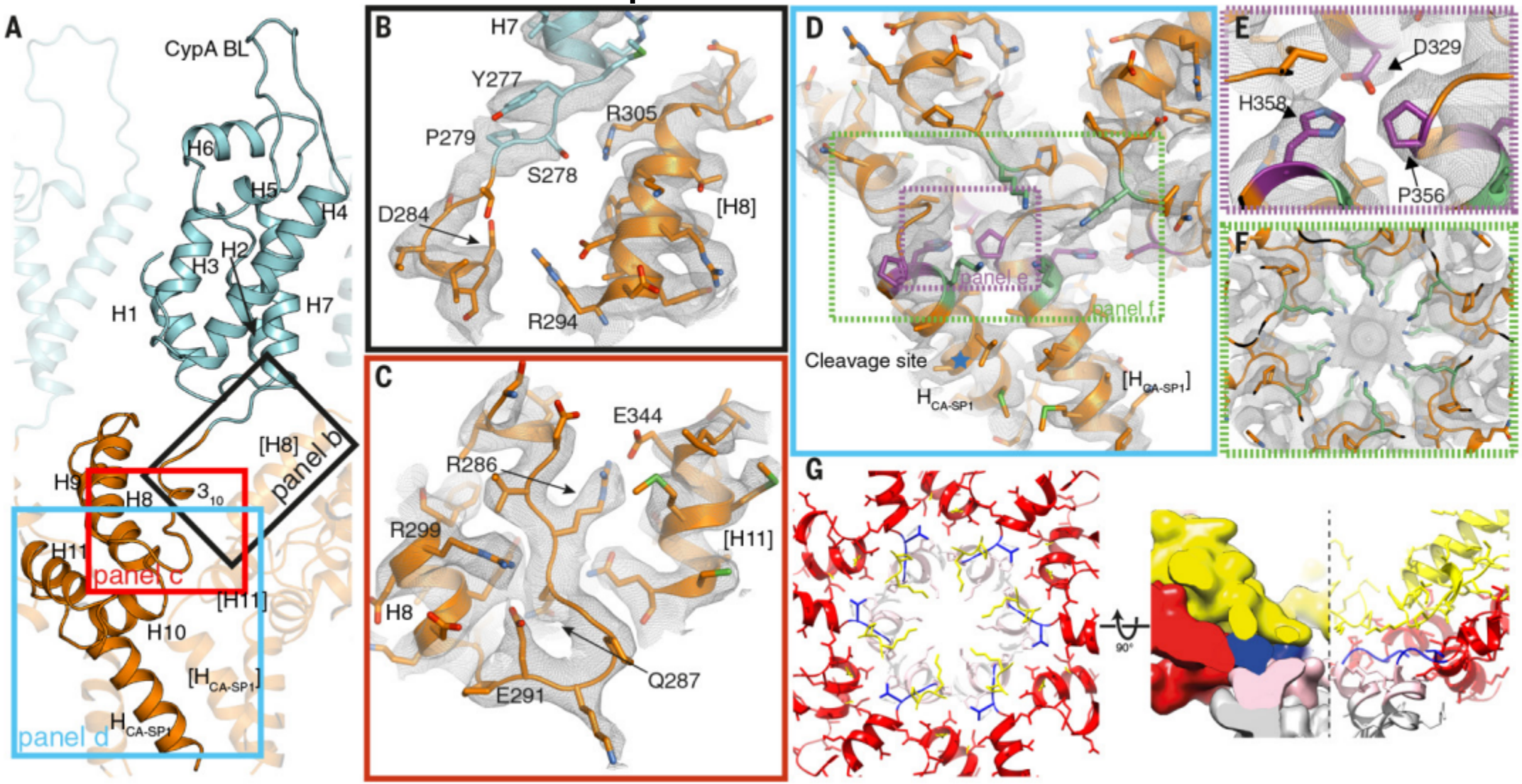
Schur et. al., Science 2016

HIV-1 Capsid-SP1 at 3.9 Å



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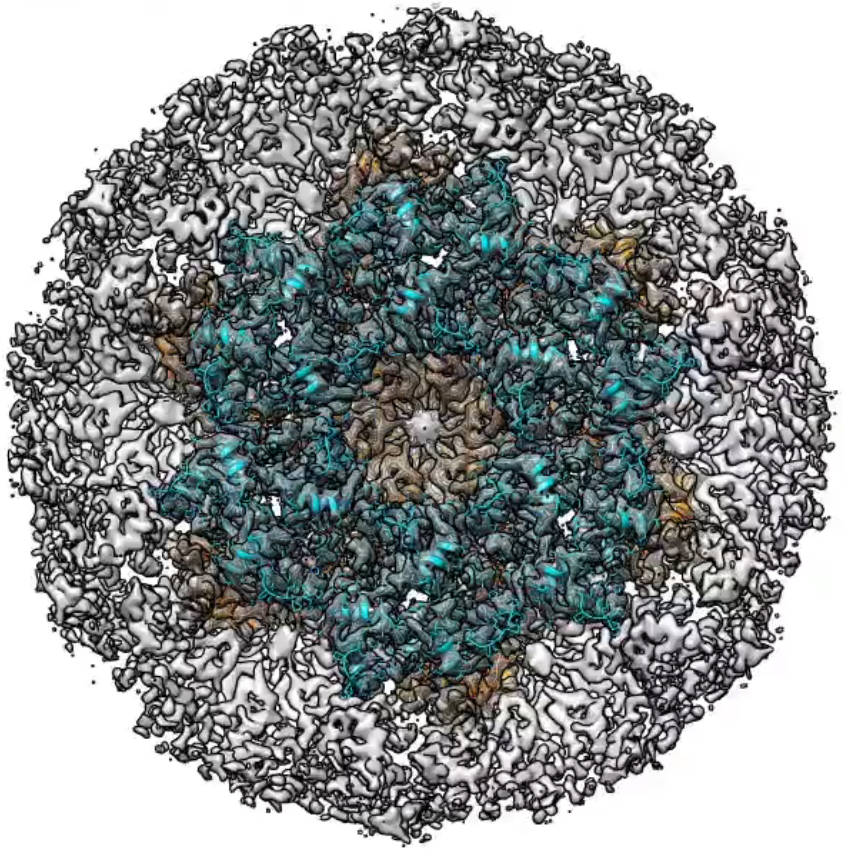
Schur et. al., Science 2016

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



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Schur et. al., Science 2016

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

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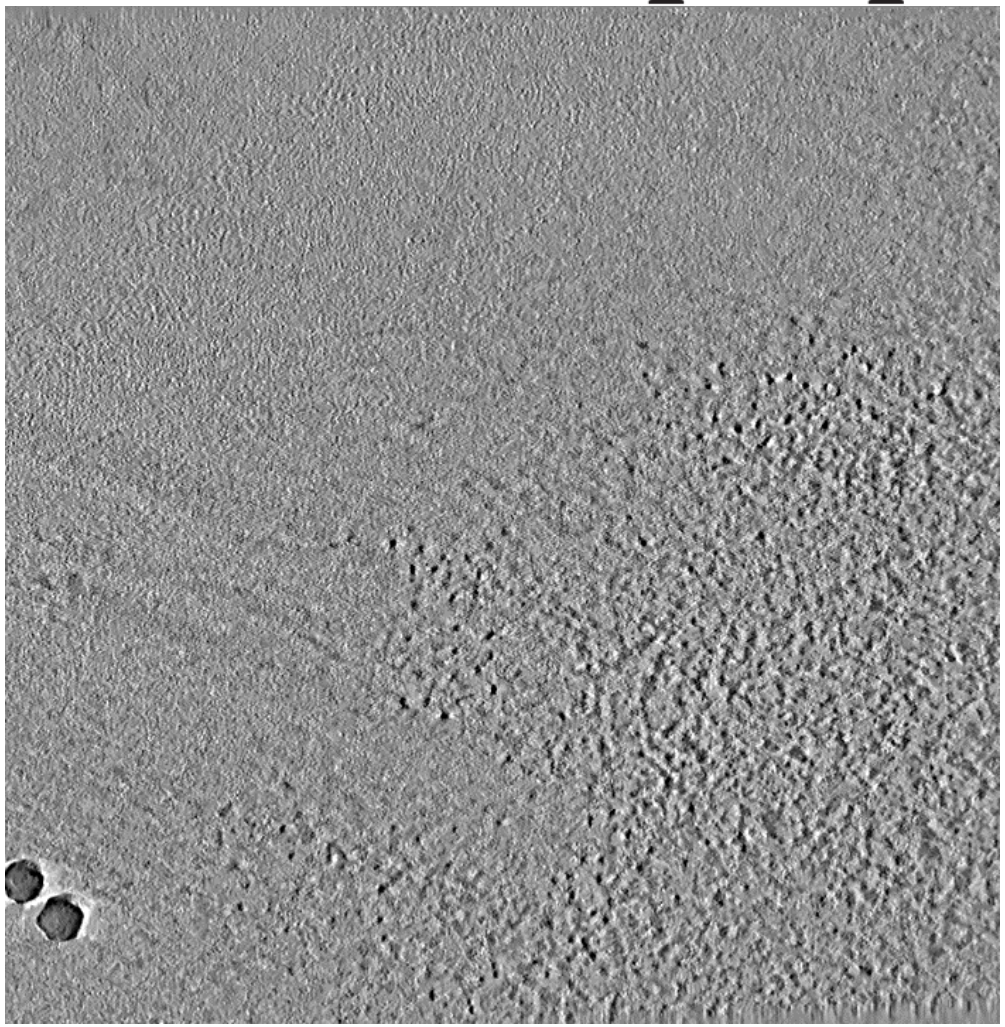
Schur et. al., Science 2016

CELL NUCLEUS

SCIENCE 26 FEBRUARY 2016 • VOL 351 ISSUE 6276

Visualizing the molecular sociology at the HeLa cell nuclear periphery



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Mahamid et. al., Science 2016

Hardware Setup

- Cryo-FLM: FEI CorrSight w/ cryo stage and spinning disk confocal
  - 405/488/561/640 nm
- Correlation w/ Zeiss EC Plan-Neofluar
- EM: Krios @ 300kV, K2, VPP, Quantum energy filter
- Gold Quantifoil R2/2 200 mesh
  - 20-25nm carbon coat on film side
  - Sterilized with UV radiation
  - > cell culture

Mahamid et. al., Science 2016

Biology Setup

- HeLa cells grown on grids
  - Expressing beta-tubulin tagged with GFP
  - Expressing histone2B:mCherry
- Blotted w/ Virtobot Mark IV
  - Added 2 micron green fluorescent beads
  - Blotforce 10
  - 10s blot
  - 2s before plunging
  - Blotted on back side – other side has Teflon pad
- Plunged into liquid ethane/propane mix
- Sputter-coated ~5nm platinum layer to avoid charging

Mahamid et. al., Science 2016

Tomography Setup

- SerialEM
- [-30:50] then [-30:-70] @ 2 degree increments
- Pixelsize: 4.21 Å
- VPP
  - slightly defocused (~0.5microns) to guarantee no overfocus
  - 60 e-/Å^2
- Defocus imaging
  - 6 microns defocus
  - 100 e-/Å^2

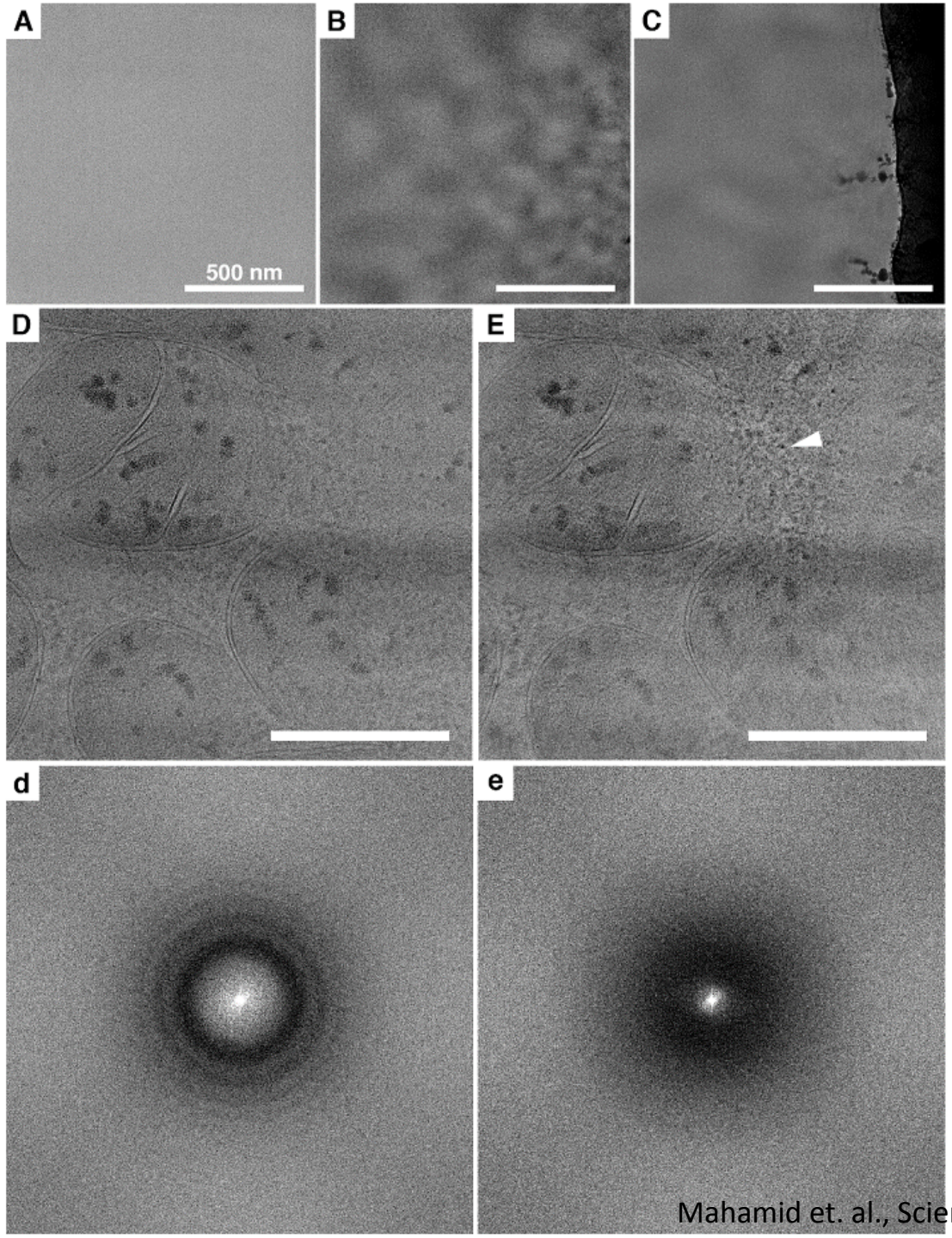
> Collected 10 tomograms from 6 cells

- Aligned w/ IMOD patch tracking, segmented w/ Amira, sub-tomogram processing w/ PyTom

Mahamid et. al., Science 2016

Charging without Platinum Coat

- A: Empty hole w/ VPP – uniform background
- B&C: Edge of non-coated lamella shows distortion
- D&d: No VPP @ 4micron def 10e-/A^2
  - Distortion
- E&e: VPP @0.5 micron def
  - Distortion and no increase in contrast



Mahamid et. al., Science 2016

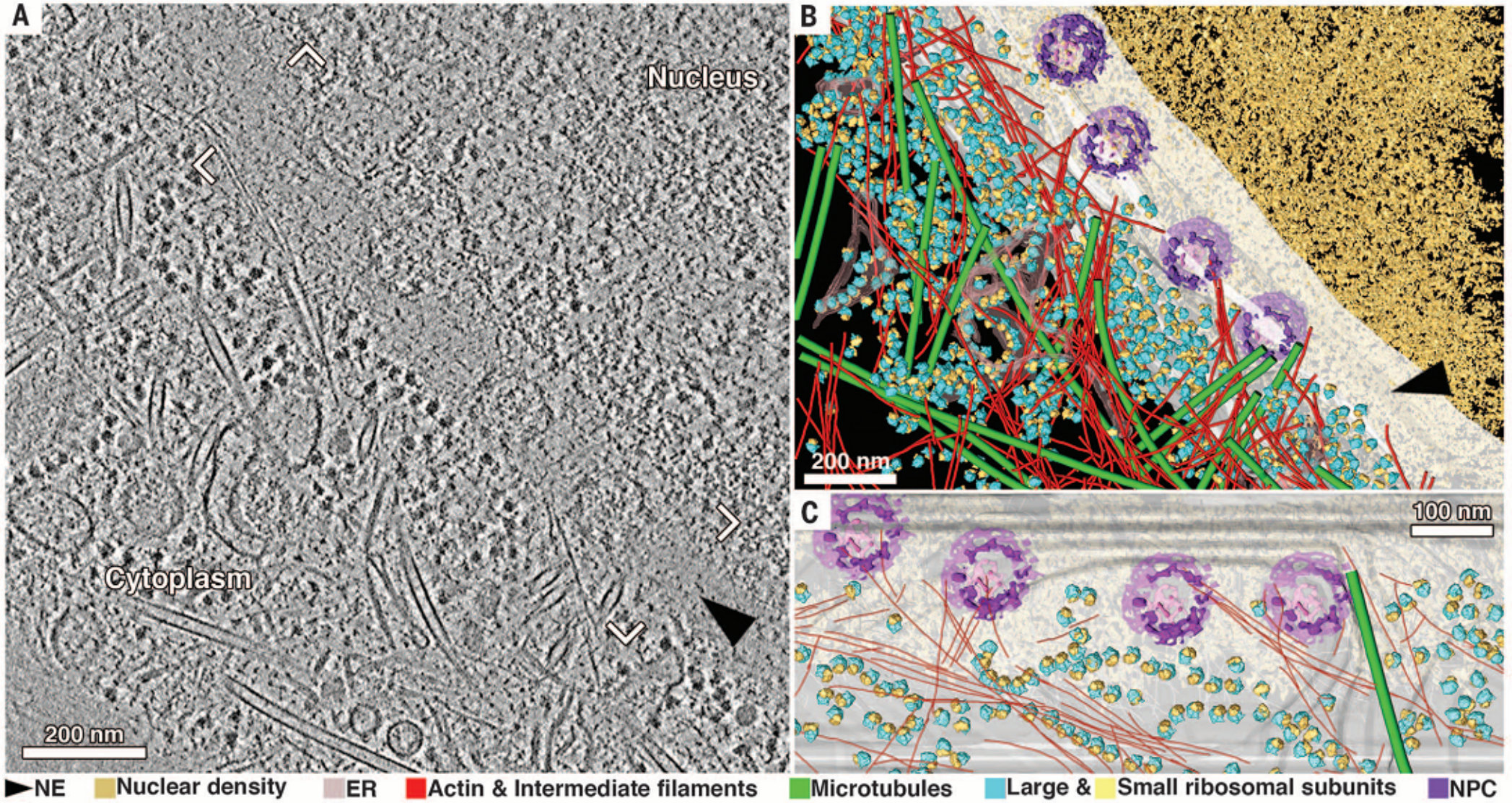
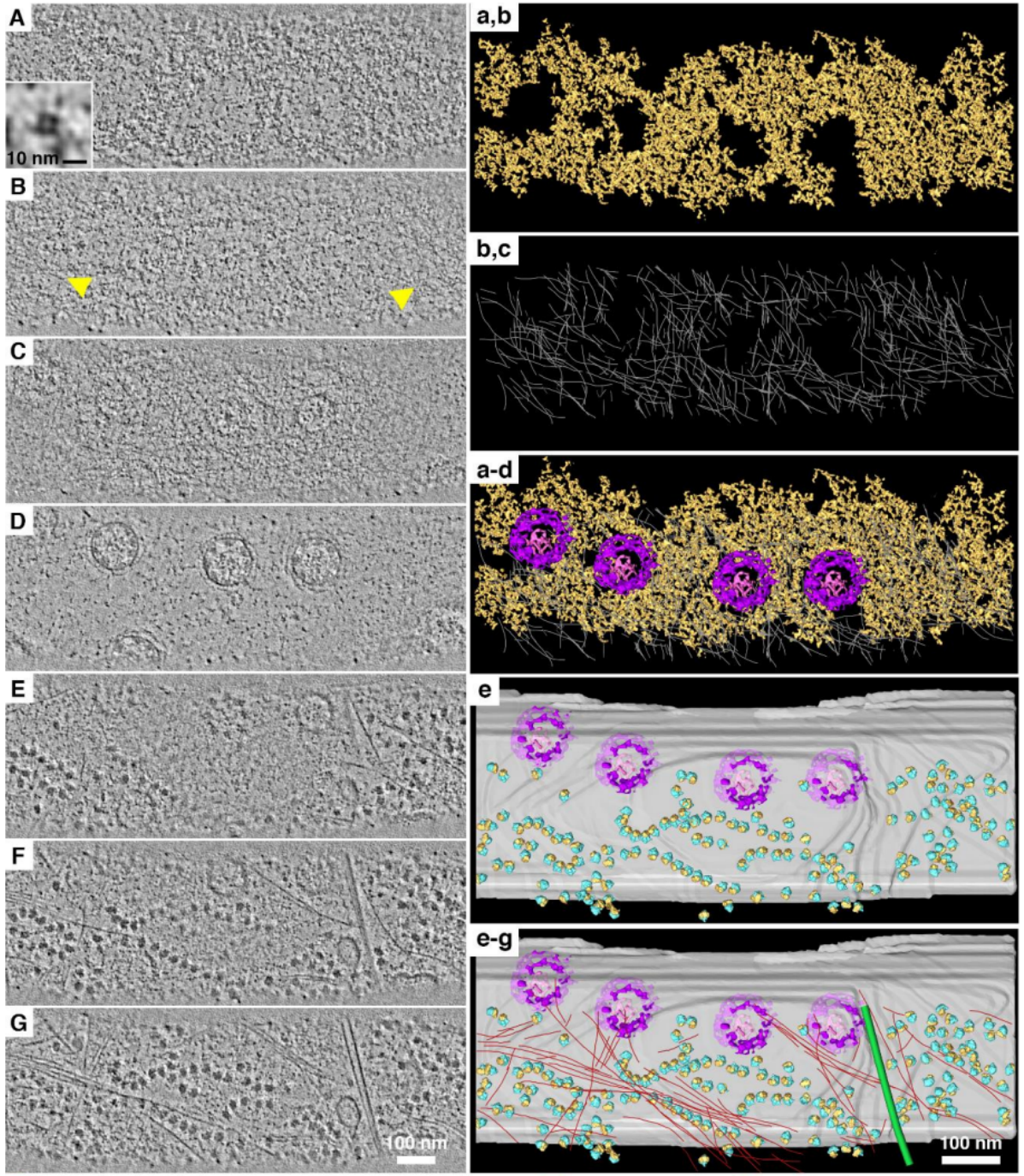


Fig. 1. The nuclear periphery of a HeLa cell revealed by cryo-ET. (**A**) Tomographic slice with 8.4-nm thickness of an interphase HeLa cell thinned by cryo-FIB. (**B**) Annotated view of the tomographic data. Color labels are defined for each structure in (B) and (C). (**C**) Cross-section view of the segmentation in the vicinity of the nuclear envelope [frame in (A)]. NE: nuclear envelope; ER: endoplasmic reticulum; NPC: nuclear pore complex.

Mahamid et. al., Science 2016



Nucleosome chains segmented by intensity thresholding

Lamina fibers segmented based on template matching

Nuclear pore complexes segmented manually

Nuclear envelope segmented based on position and texture

Ribosomes segmented based on template matching

Actin segmented based on template matching

SIMONS ELECTRON MICROSCOPY

Mahamid et. al., Science 2016

Nanometer SPT Studies

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	~750,000	3.9, 0.35	(Schur et al., 2016)
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)

Possible Future Improvements

As exemplified by the highest resolution SPT study by far:

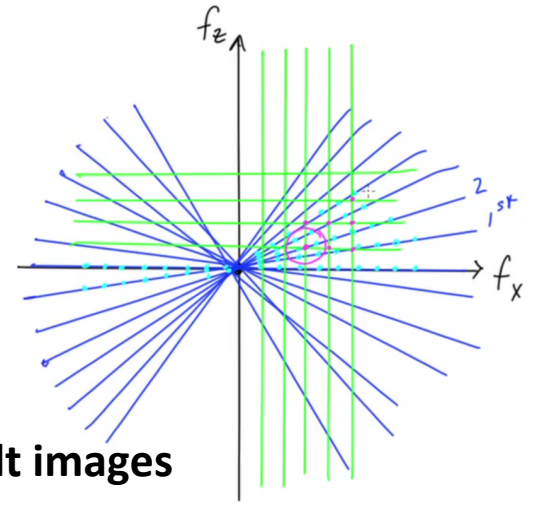
- 3.9 Å with 750,000 subunits by Schur et al., 2016)

and by the dual SPT and single particle study shown in the recent Relion SPT paper:

- 6.5 Å with single particle analysis
- 10.5 Å with sub-tomogram analysis (same exposure areas)

SPT techniques today are not producing resolutions expected given the quality of data being studied. **Why?**

Possible Future Improvements

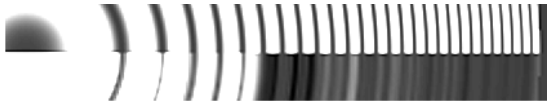


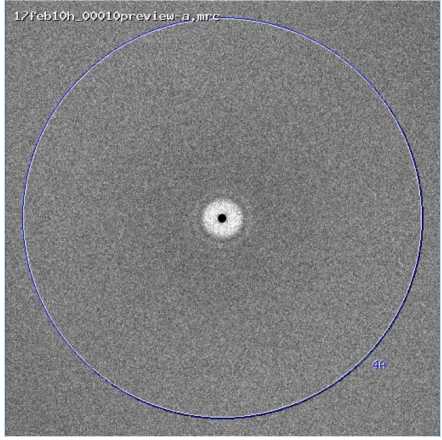
Grant Jensen, YouTube

Interpolation during reconstruction

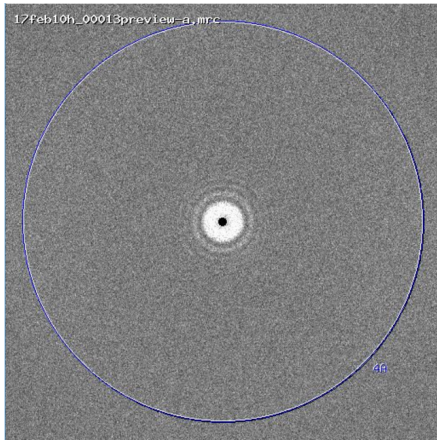
- Possible solution: After subvolume alignment, **trace-back objects to their original tilt images** and perform **constrained single particle alignment** (see Bartesaghi et al., Structure 2012)
    - Issue: **overlapping objects** need to be dealt with, possibly by subtracting unwanted re-projected densities from the images before single particle processing (see Ilca et al., Nat. Comm., 2015)
- Collect **more continuous tilt-series** to properly fill in Fourier space
    - Issue: Low dose = low SNR, which makes tracking and fiducial-less alignment less accurate
- Experiment more with more **constrained Fourier masks** rather than using solid missing wedges.
    - Relion and Dynamo (and possibly other software) allow for custom Fourier masks

Possible Future Improvements





No EF

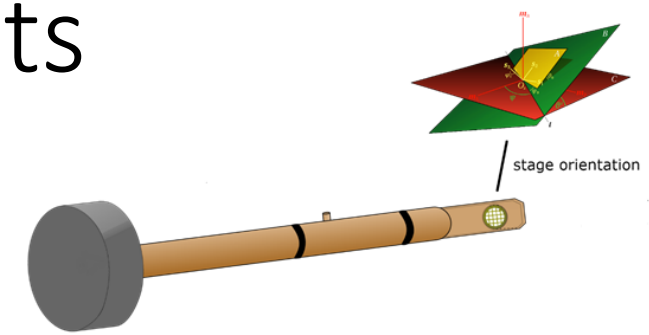


15eV EF

Inaccurate defocus estimation

- **Low** per-tilt image **SNR** makes estimating per-image defocus highly error-prone
- **Tiled defocus estimation** depends heavily on perfect eucentric height, accurate tilt axis estimation, and goniometer stability
- **Movement of objects** in the beam direction **at every tilt angle** may anisotropically move, rotate, and thus blur objects
- Possible solutions: Better detectors, use of energy filters and phase plates to boost overall SNR, include sub-volume height when correcting for defocus (various software does this already), test full-frame alignment versus patch-tracking frame alignment

Possible Future Improvements



Errors in tilt-series alignment

- Alignment errors include: Shifts, rotations, scaling factors, and tilt axis refinement.
- **If gold beads move** relative to the sample in fiducial alignment, then this imposes tilt-series alignment error of at least that overall error and decreases the accuracy of the estimated tilt axis, shifts, and rotations
  - Possible solution: Don't align with fiducials
- For **fiducial-less alignment**, if the **SNR is too low**, then the per-tilt image alignment error will be on the order of the resolvability of the respective correlation peak
  - Possible solution: Collect at a higher dose per tilt image

Thank you!
Questions?