Lecture 8

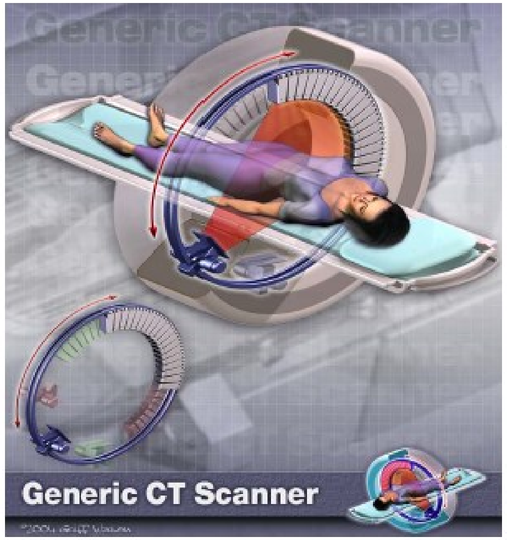
3DEM methods

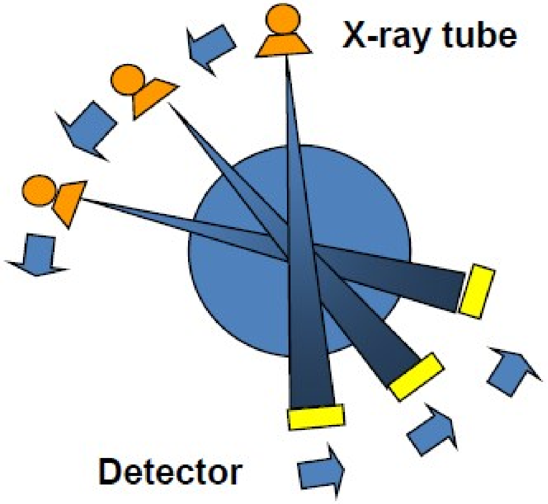
Electron tomography

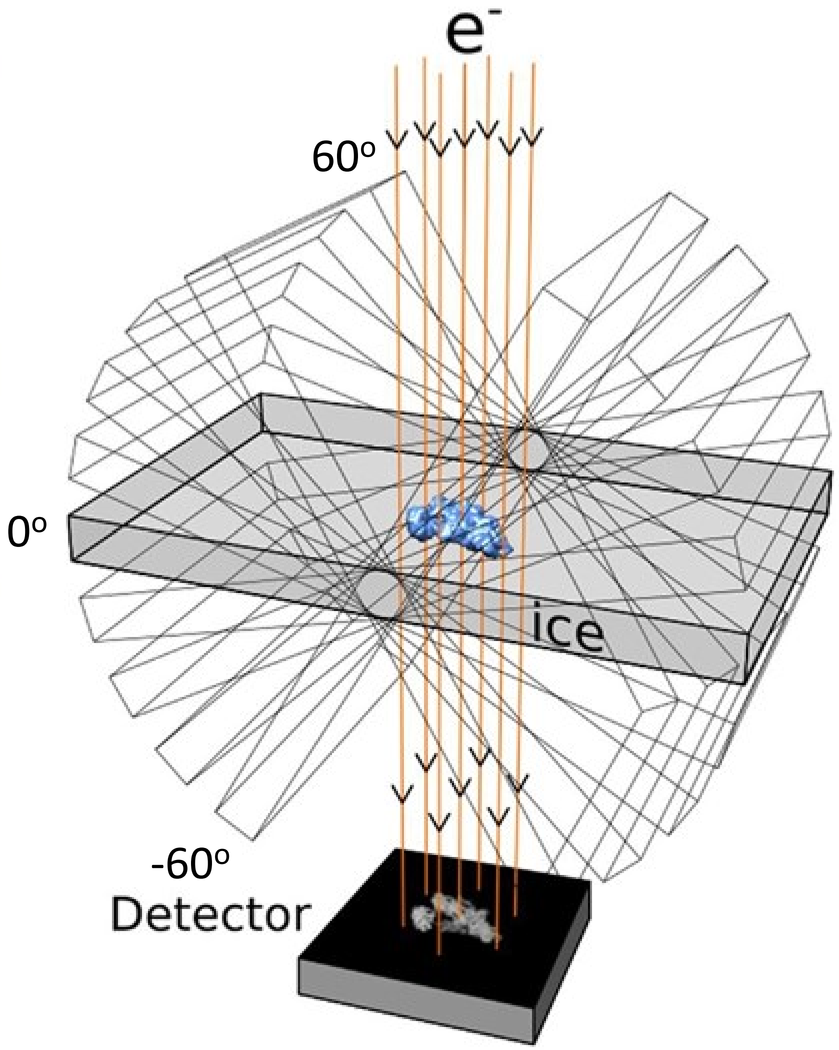
Tibor Füzik

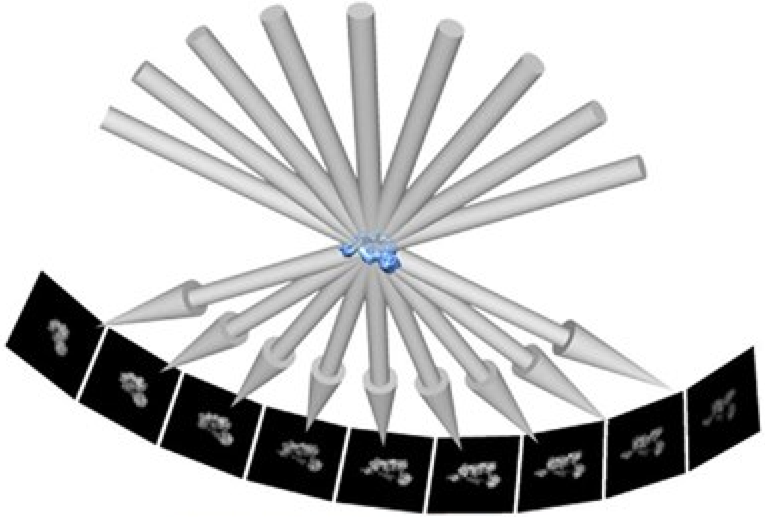
Electron tomography

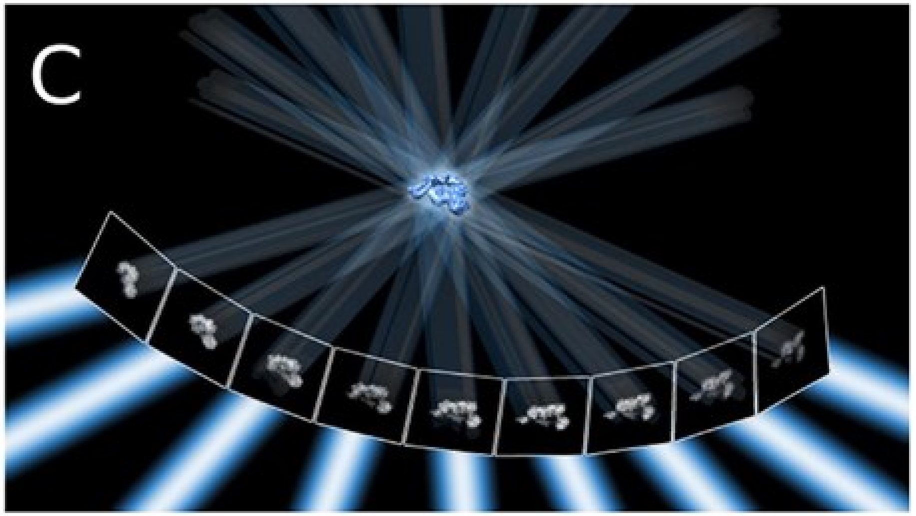
Computer Tomography







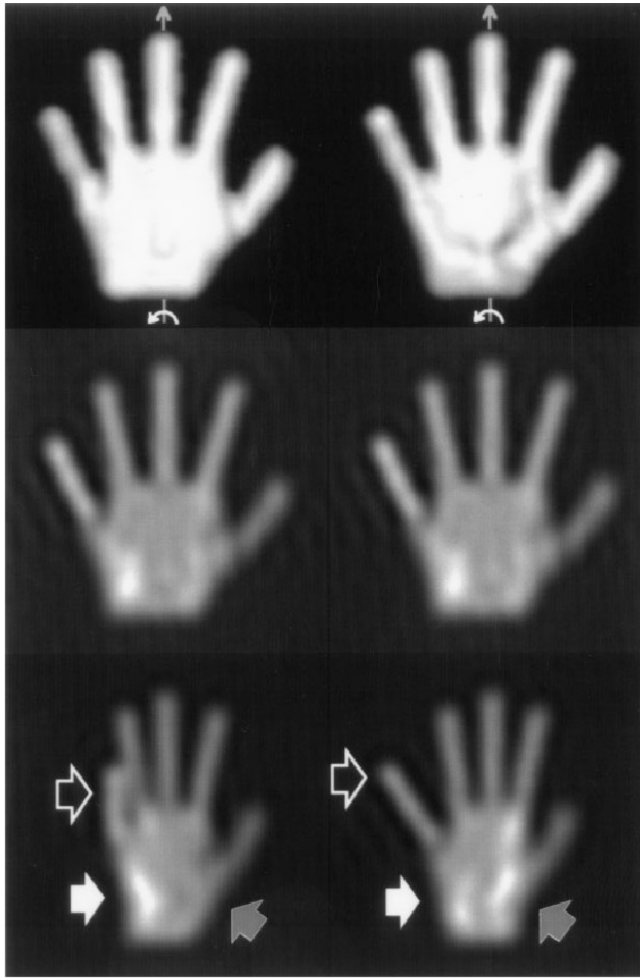




Koning et al. 2018 https://doi.org/10.1016/j.aanat.2018.02.004

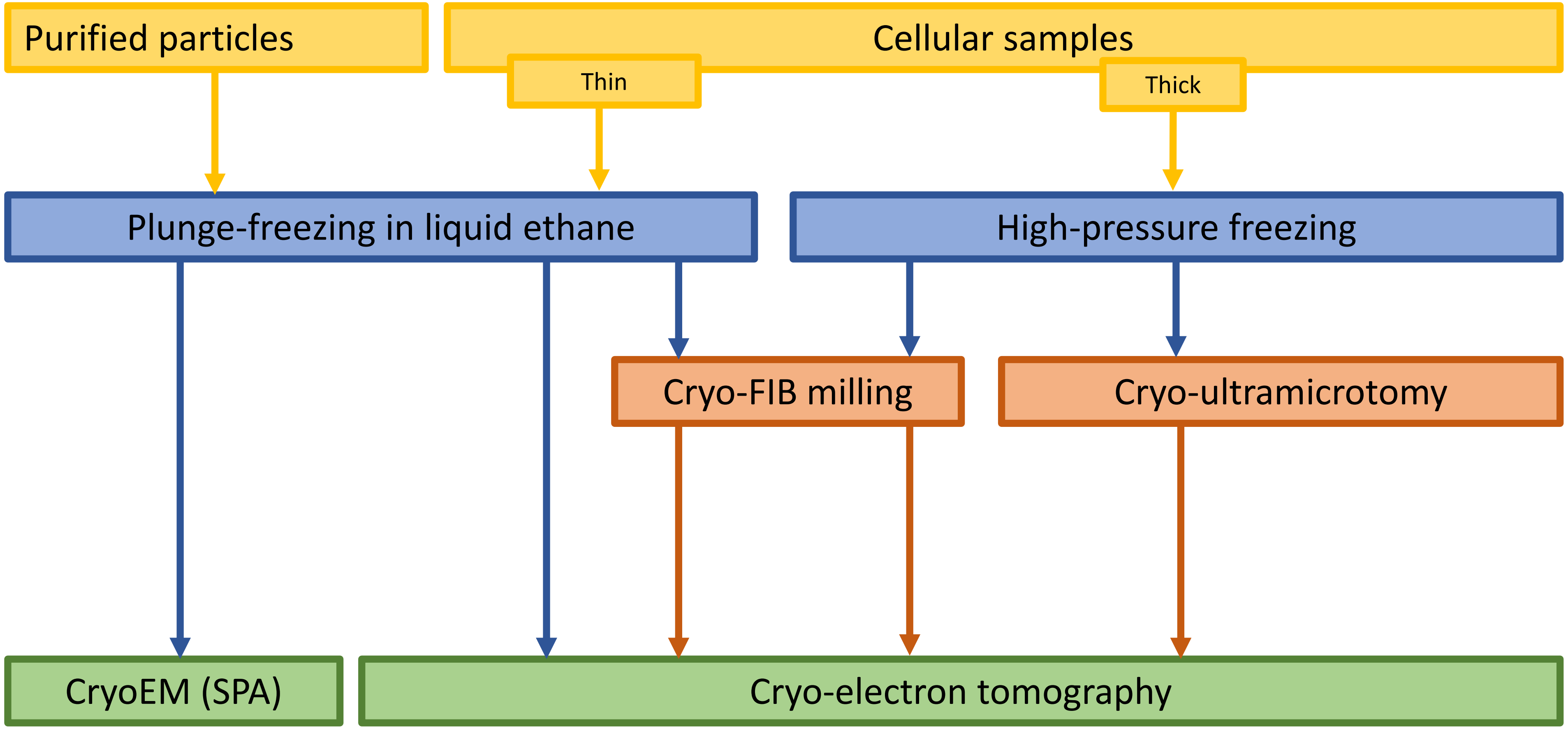
Tomography vs Single particle analysis

- **Single particle analysis**
  - Taking single projection image of the sample
  - Exposing the acquisition area only once (“high” SNR)
  - Assuming that the object of interest is in random uniform orientations
  - Cannot make 3D reconstruction form a single 2D projection
  - Not suitable for samples where 3D information from single acquisition area is needed (cells, non-uniformly organized structures)
  - Sample needs to be electron transparent
- **Tomography analysis**
  - Taking multiple projection images of the same area under different tilts
  - Exposing the acquisition area multiple times (low dose, low SNR)
  - Tilt angles can be assigned to the projection images
  - From series of tilts, we can reconstruct a 3D volume
  - Suitable for studying large samples, macromolecules *in situ,* poorly organized structures
  - No ambiguity in handedness determination
  - Sample needs to be electron transparent

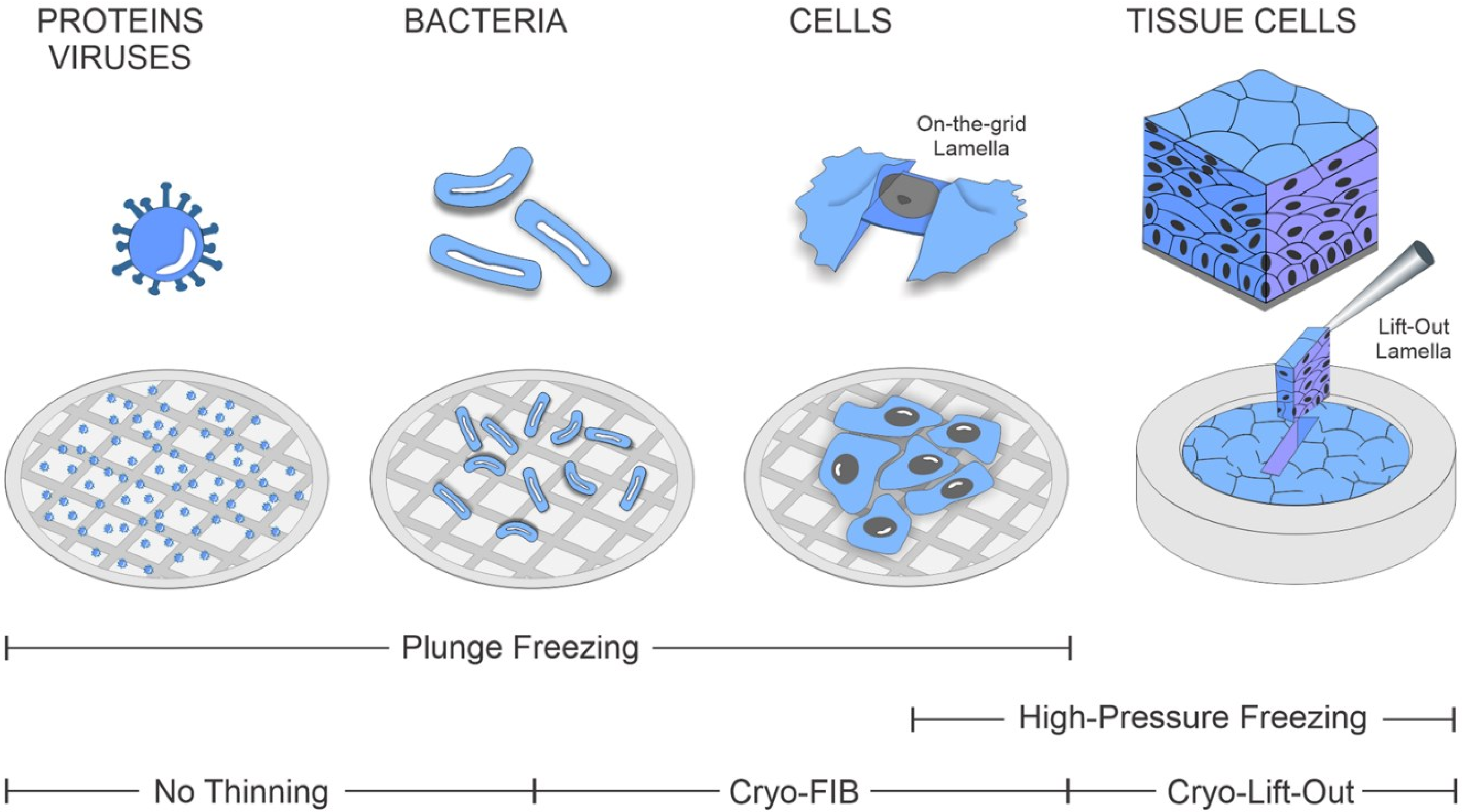


Belnap et al., 1997 https://doi.org/10.1006/JSBI.1997.3896

Sample preparation for tomography



Sample preparation for tomography

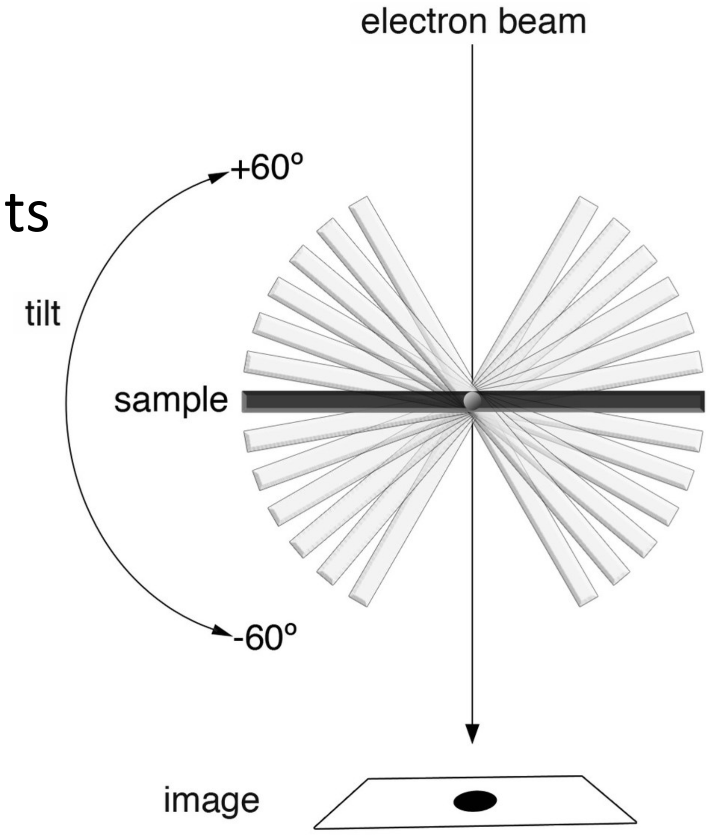


Klumpe et al. 2022

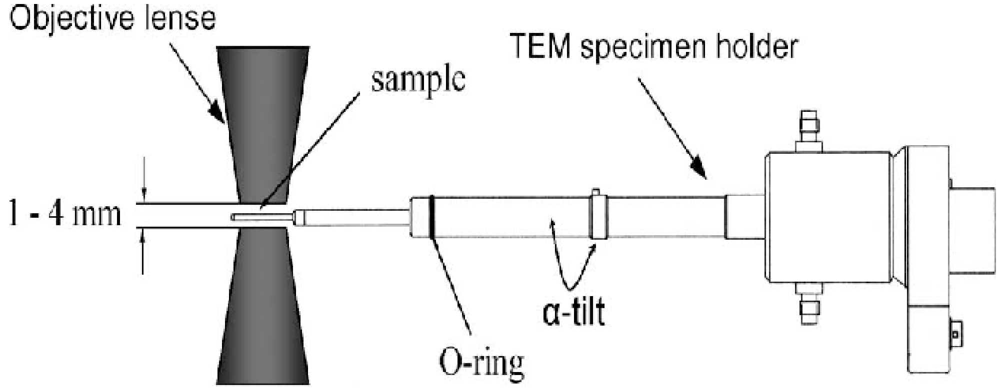
https://doi.org/10.1017/S1551929521001528

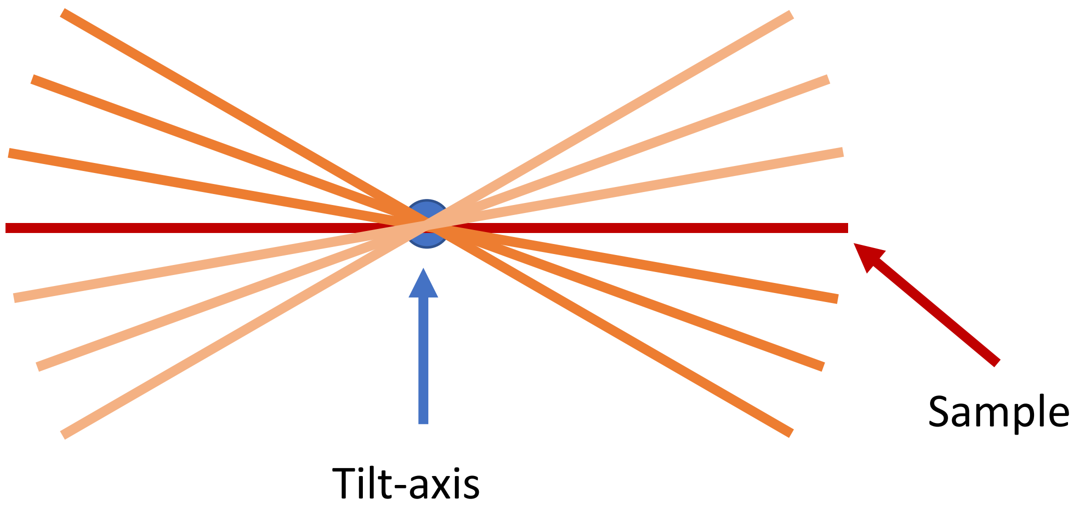
Acquisition

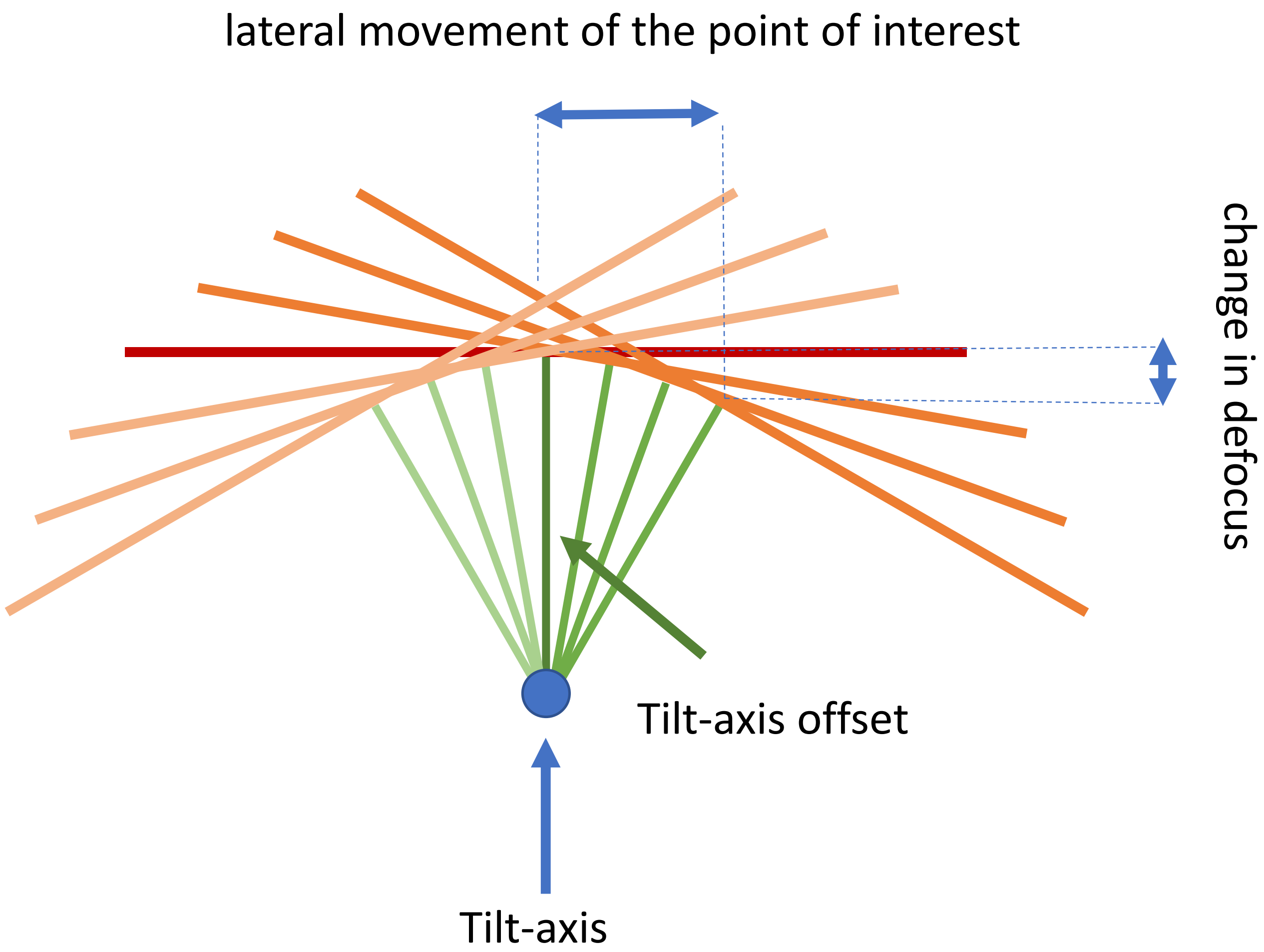
- Acquisition of tilt-series
  - Single acquisition area exposed multiple times
- Radiation damage
  - Electron dose per tilt ~2-3 e-/$Å^2$
  - Total electron dose = number of tilts * dose per tilt (100-150 e-/$Å^2$)
  - Dose symmetric tilt scheme
- Inclusion of fiducials in the sample
  - Small (5-10 nm) gold beads allowing precise tracking of the tilts
- Defocus during tilt-series
  - Usually kept constant
- Proper alignment of tilt axis
  - Set of eucentric height (center of rotation)
  - Corrections



Correct eucentric height – no tilt axis offset







Zero tilt-axis offset

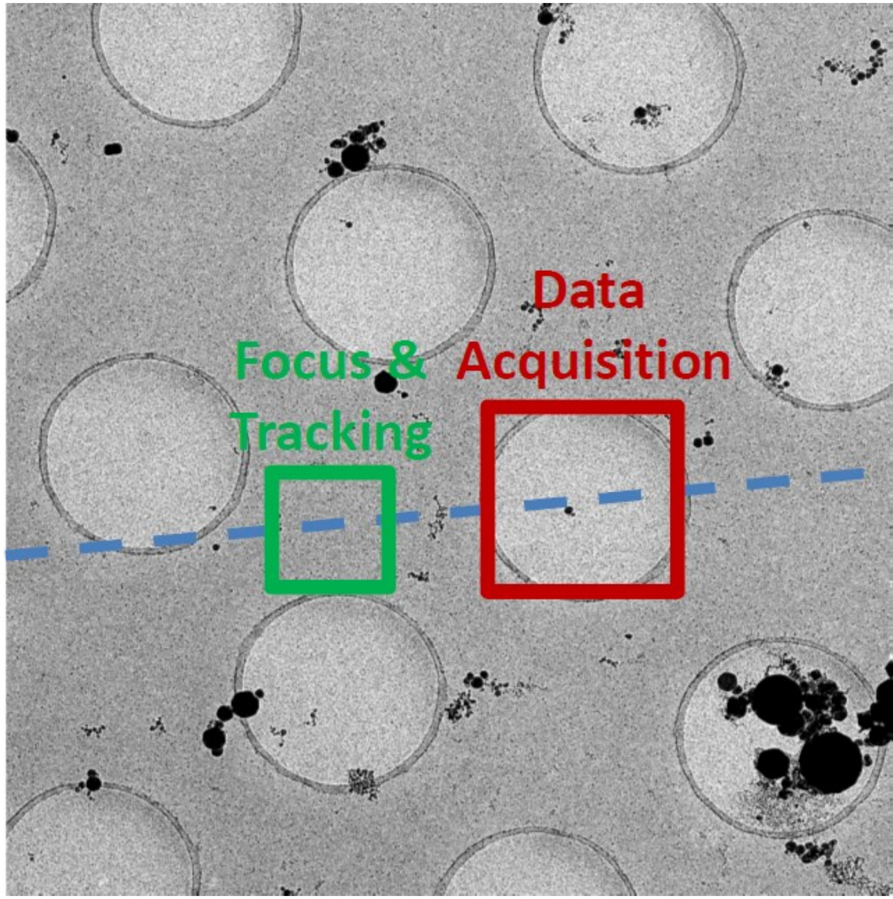
Non-zero tilt-axis offset

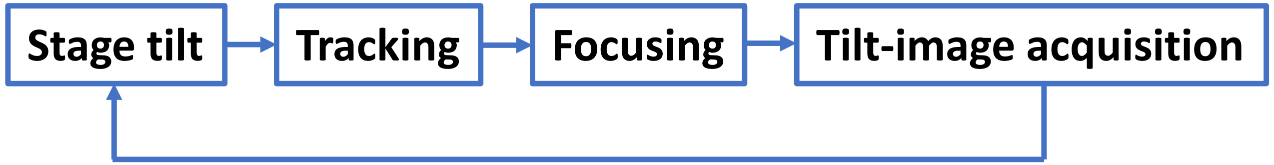
Nafari et al. 2008

http://dx.doi.org/10.1109/JMEMS.2007.912714

Compensation for tilt-axis offset

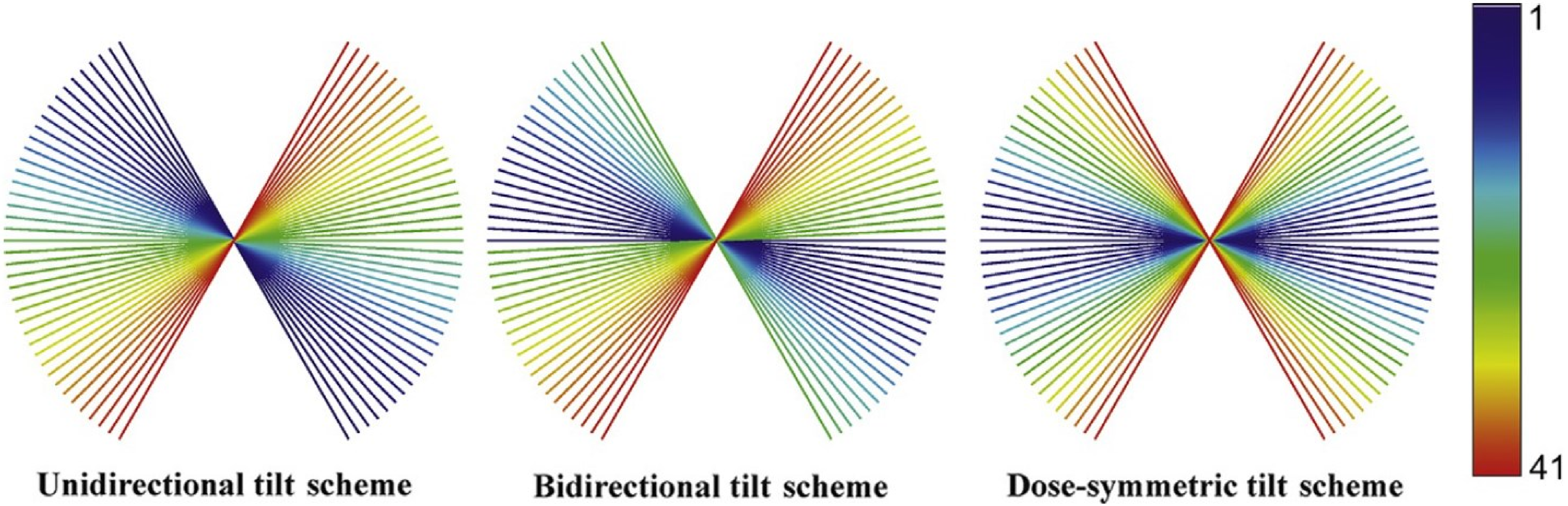
- After each tilt change a “tracking area” is imaged and by cross correlation with the previous image correction to image shift is done
- After each tilt change autofocus is done on focusing area
- Tracking and focusing area **must** lay on the tilt-axis





Tilt schemes

- Order in which the tilts are collected



From most positive
to most negative tilt
60^{o} -> -60^{o}

From zero to most positive
From zero to most negative tilt
0^{o} -> 60^{o} ; 0^{o} -> -60^{o}

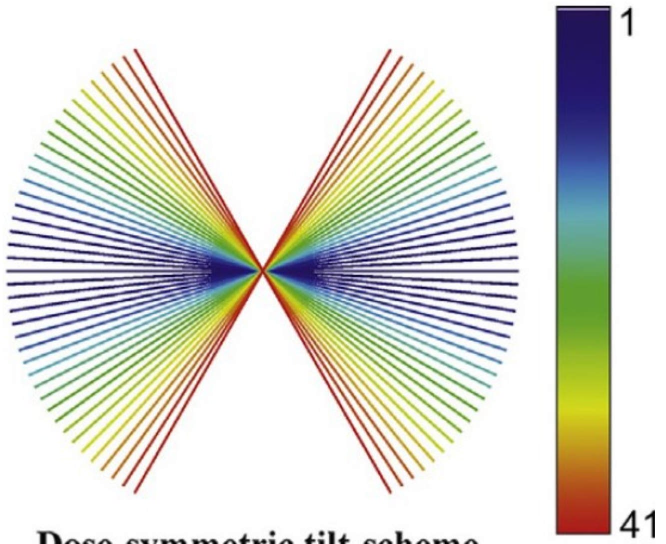
Positive, positive, negative, negative,
positive, positive, negative, negative,
0, 3; -3, -6; 6, 9; -9, -12; 12, 15; -15….

Hagen et al., 2017

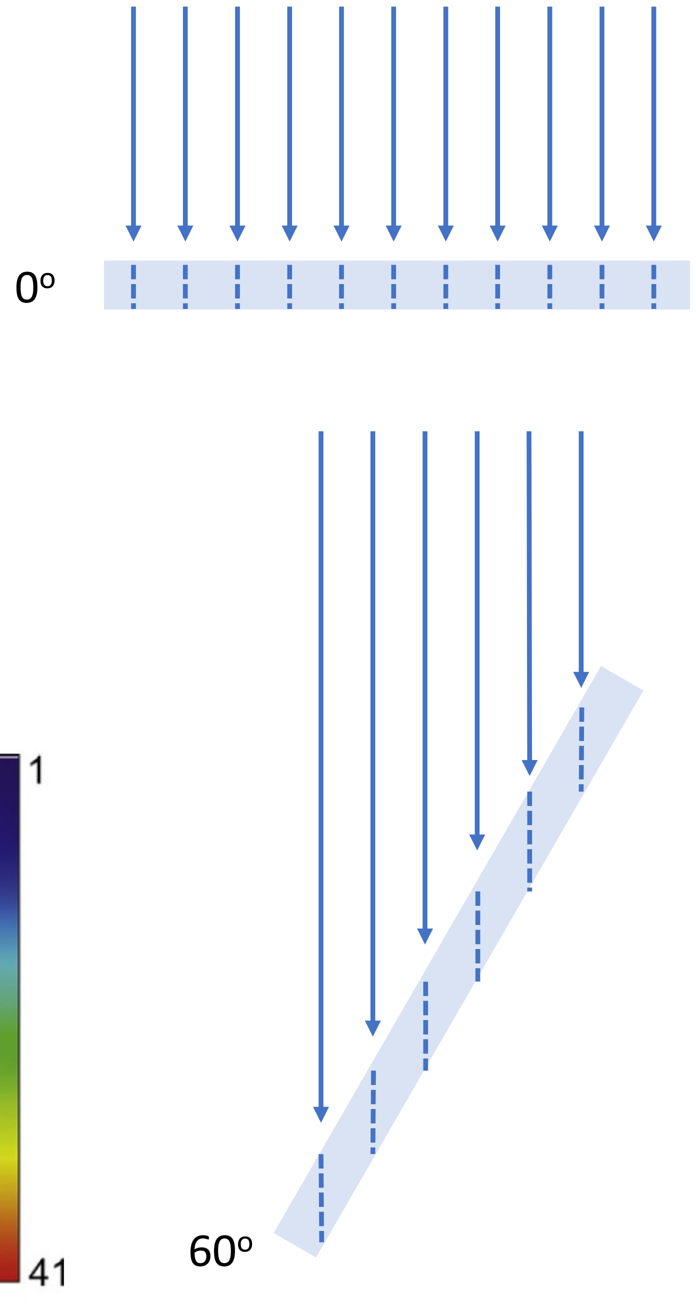
https://doi.org/10.1016/j.jsb.2016.06.007

Dose symmetric tilt-scheme

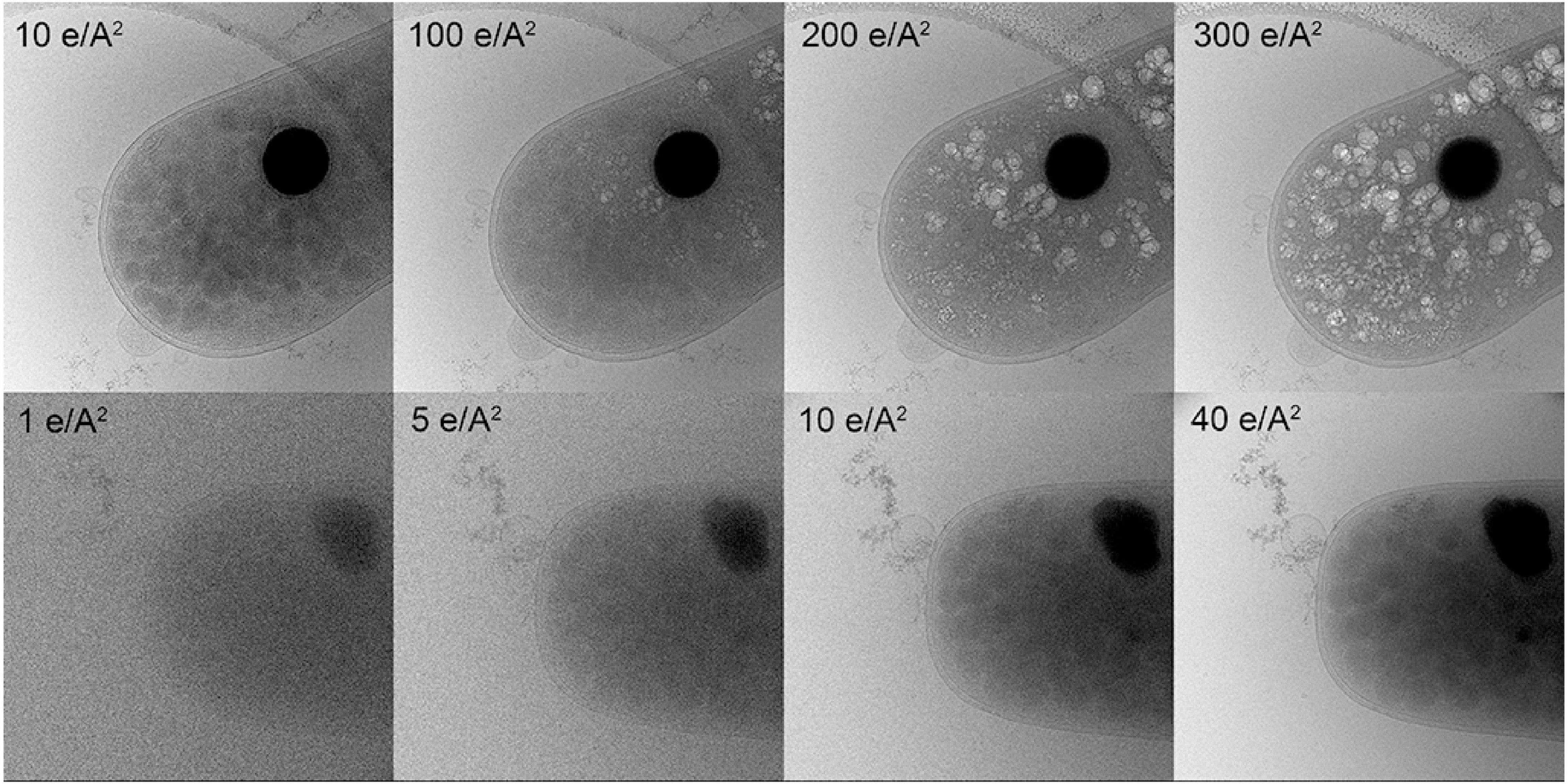
- First the small angle tilts are collected
  - Sample thickness is minimal => most transparent part
  - High contrast
  - Lower radiation damage
  - Contain the most useful high freq. information
- Last the high angle tilts are collected
  - Tilt-induced grow in sample thickness => decreased transparency
  - Lower contrast
  - Higher radiation damage



Dose-symmetric tilt scheme



Radiation damage of cellular samples

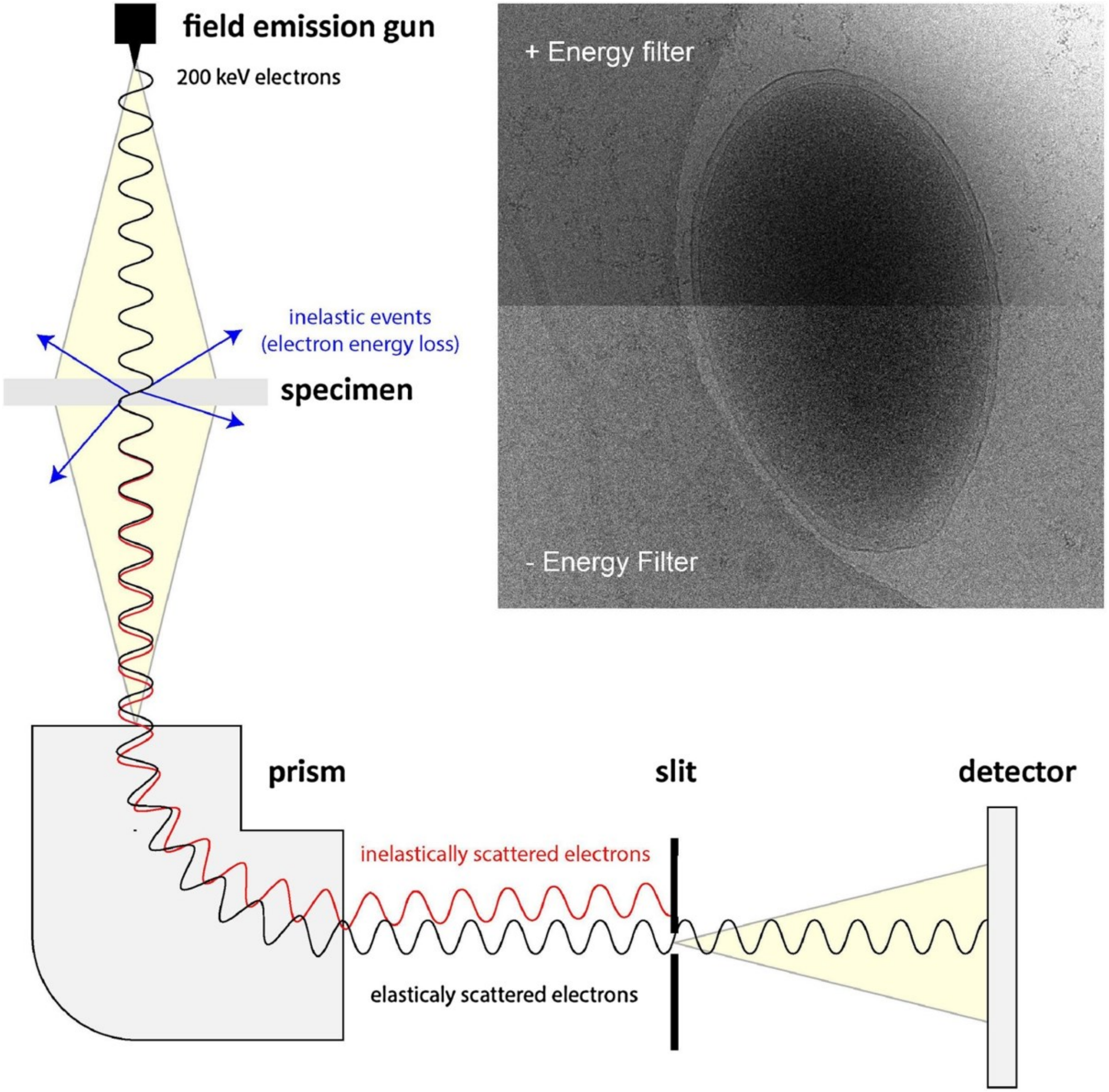


Koning et al. 2018 https://doi.org/10.1016/j.aanat.2018.02.004

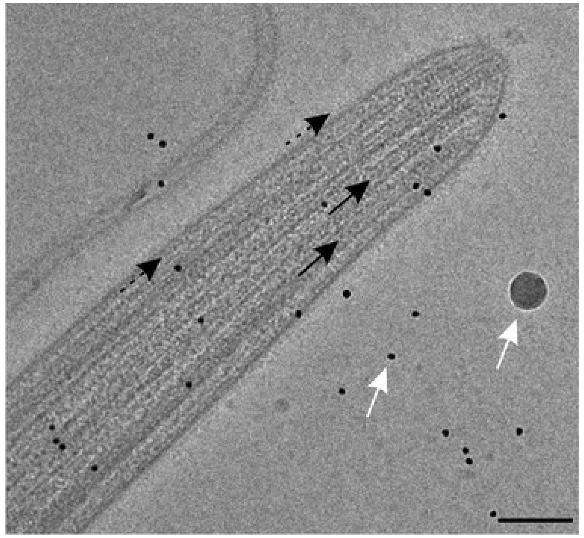
Acquisition setup

- Stage setup
  - Choice of angular increment / max tilt angle (e.g. +-60^o, 3^o step)
  - Single axis/dual axis
- Camera setup
  - Counting mode (ideally CDS mode on K3)
  - Short exposure times (per tilt dose 2-3 e^-/A^2 => on K3 ~0.5 sec)
  - Fractionation into few dose fractions (~4 fractions; <1e-/A^2/fraction)
- Energy filter setup
  - High-tilts => thick sample (more inelastically scattered electrons)
  - Zero loss mode – slit set to 10-20 eV
  - Increased contrast
- Phase-plate setup
  - Low dose low contrast => compensated by high defocus
  - Volta phase plate – comparable contrast at lower defocus (by applying phase shift on CTF)
- Combining SPA and tomography
  - Zero tilt acquired at higher dose (10-20 e-/A^2) – serves as micrograph for SPA
  - Other tilts acquired at standard dose

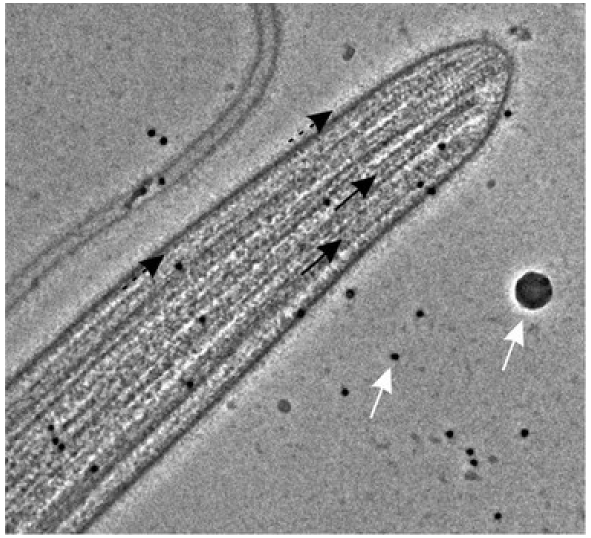
Energy filter, Volta phase plate (VPP)



Koning et al. 2018 https://doi.org/10.1016/j.aanat.2018.02.004



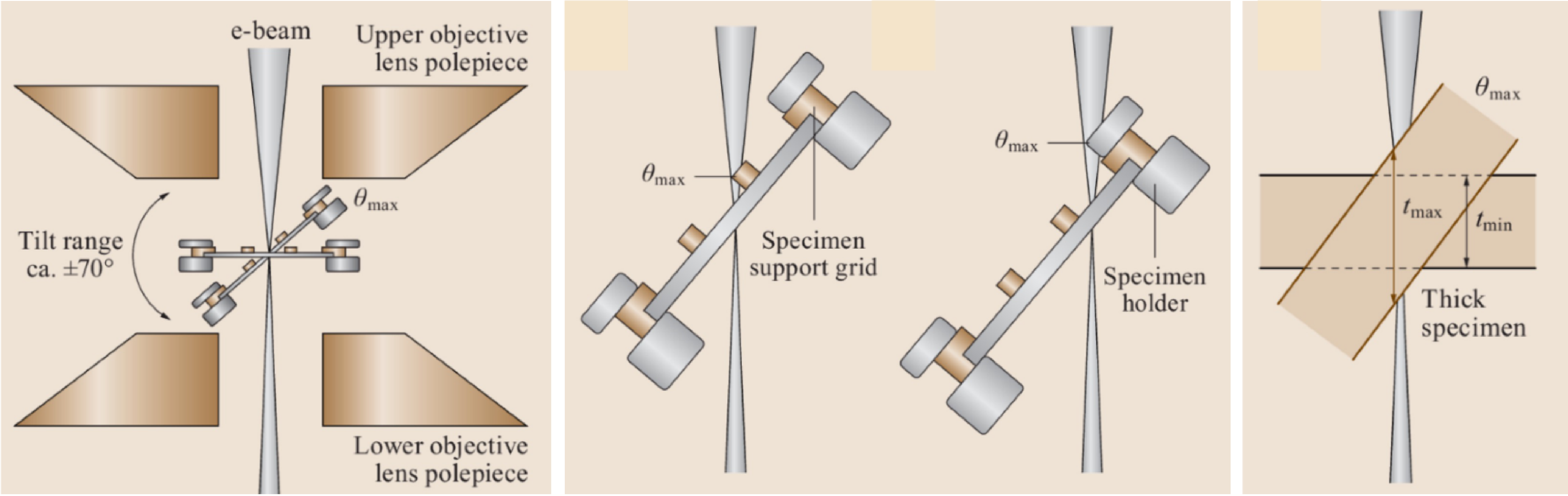
-5µm defocus, without VPP



In focus, width VPP

Danev et al. 2014 https://doi.org/10.1073/pnas.1418377111

Tilt range limitation

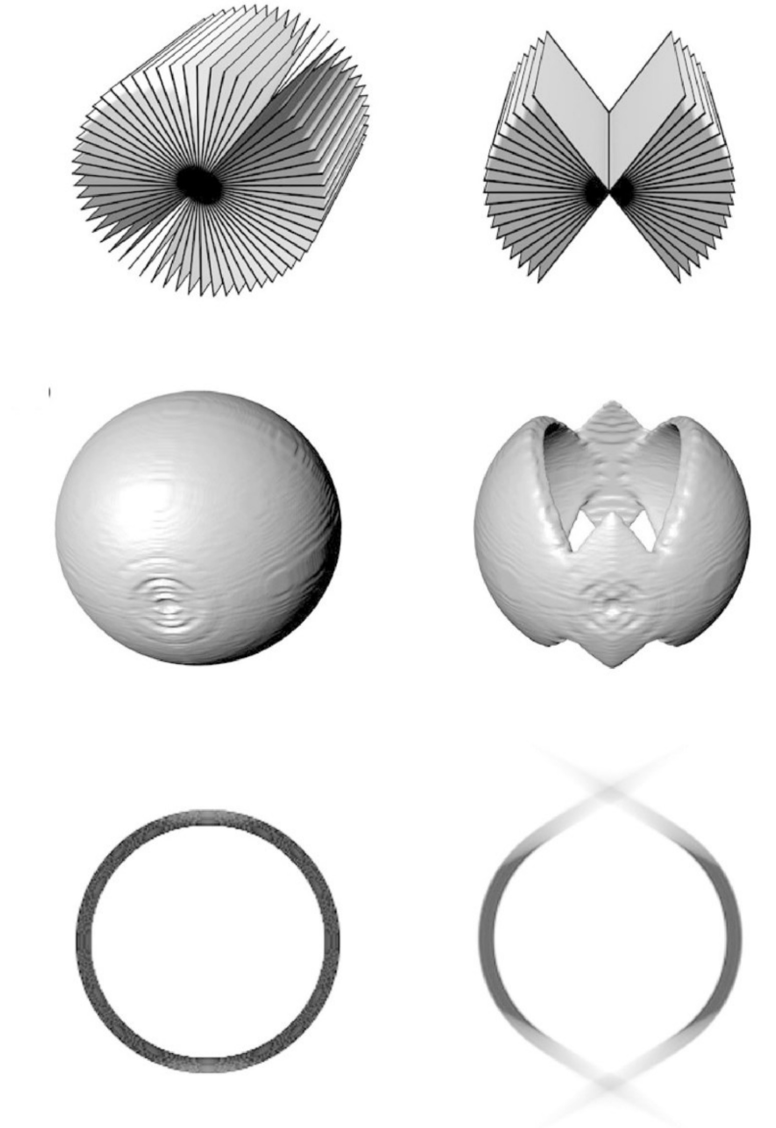


High tilt polepieces

Tomography holders

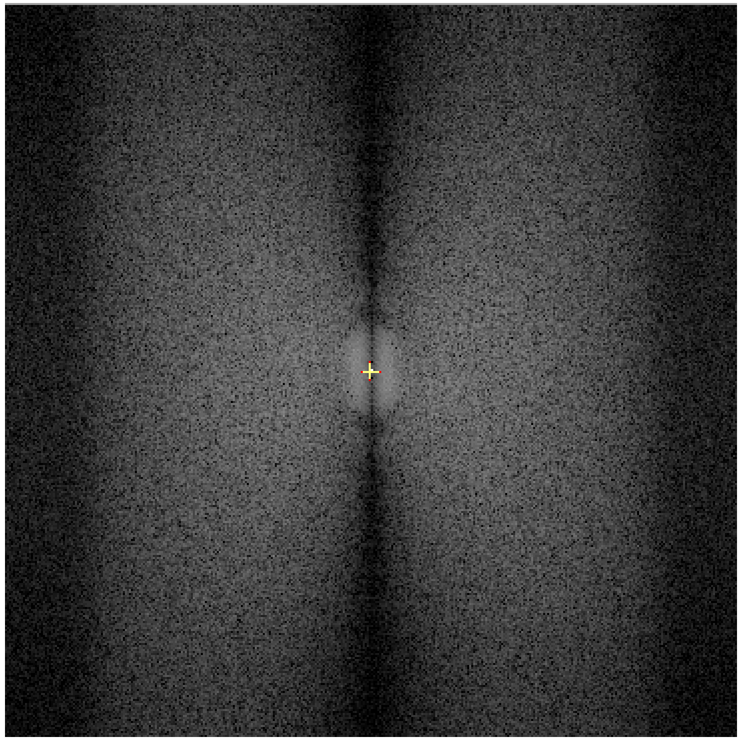
Leary & Midgley (2019) Electron tomography in material sciences, Springer Handbook of microscopy, pp 1279-1329

Missing wedge

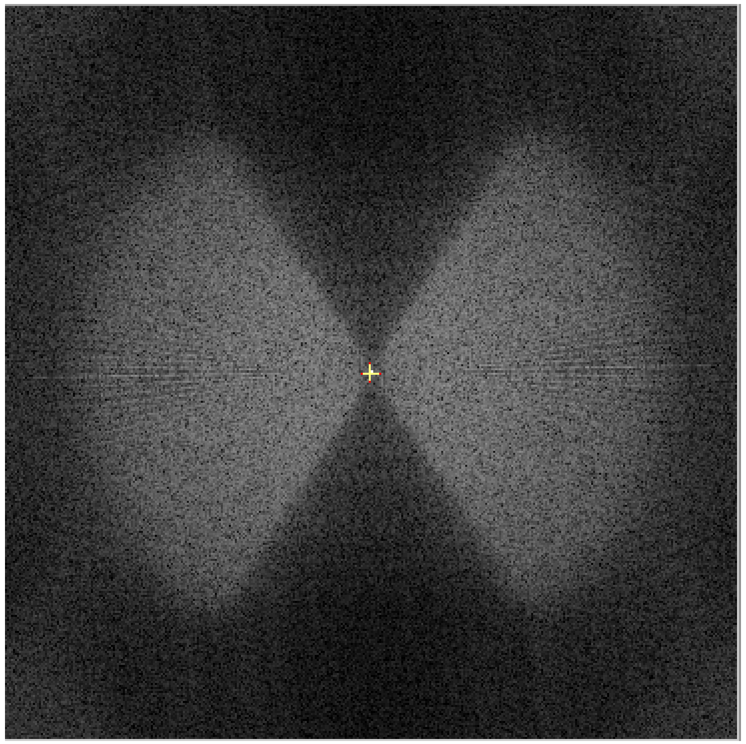


Ideal (+- 90^o) Real (+- 60^o)

- Missing wedge – missing in Fourier space (therefore affects all the point in real-space)
- The information is missing there is no possibility to add it or recover it (it was not recorded at all)



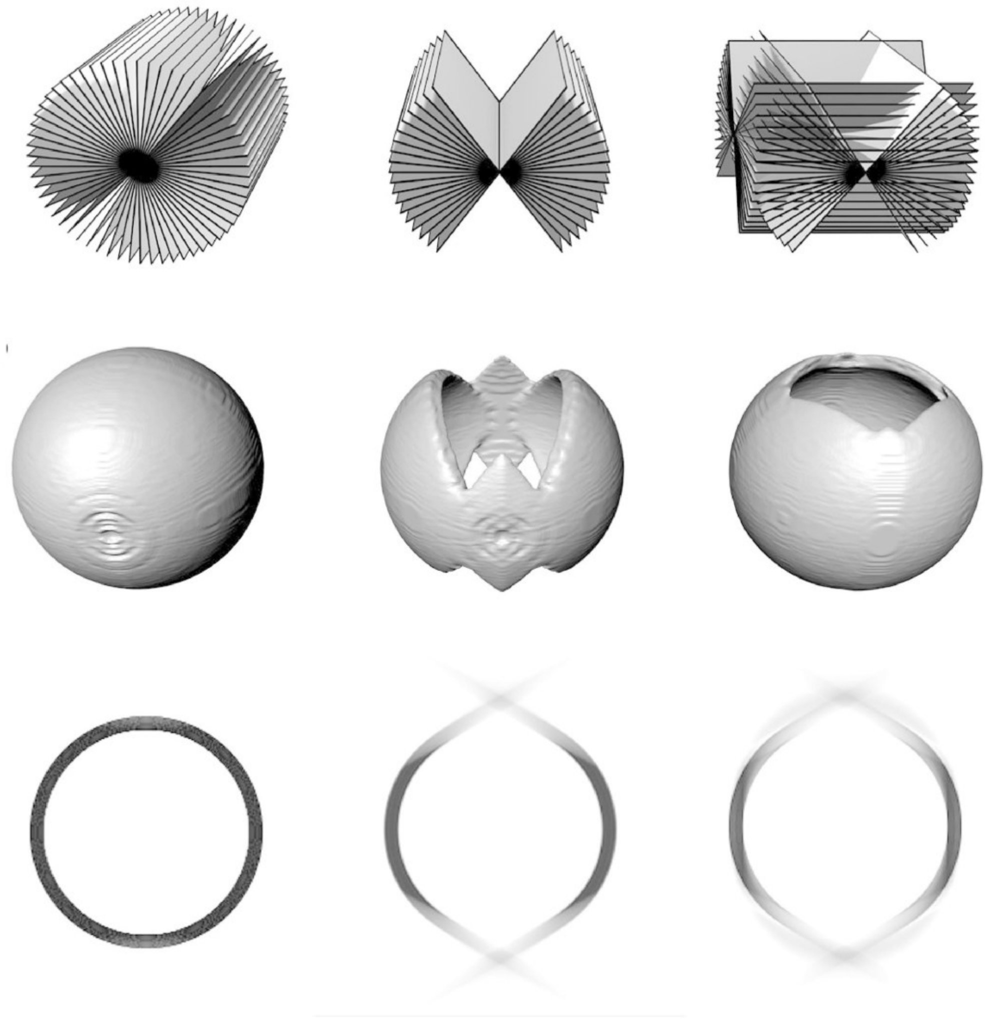
FFT XY plane of a tomogram slice



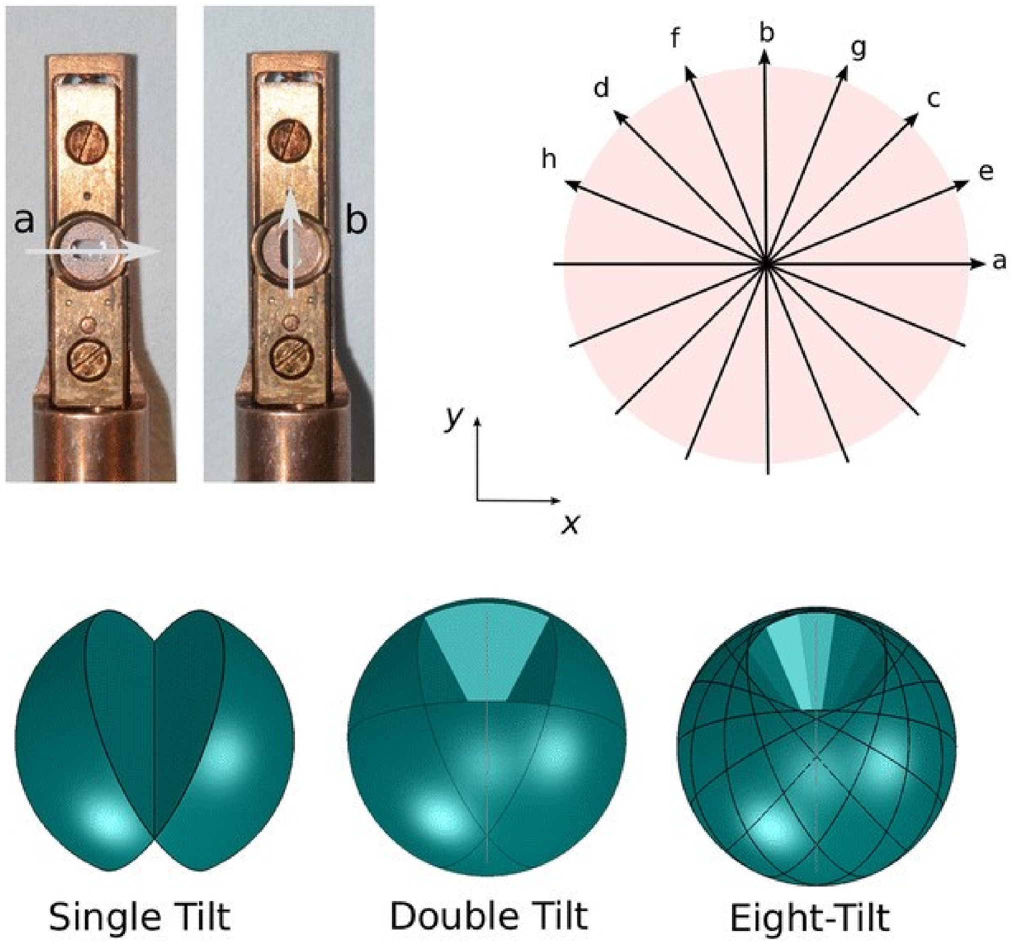
FFT XZ plane of a tomogram slice

Koning et al. 2018 https://doi.org/10.1016/j.aanat.2018.02.004

Dual (multi) tilt tomography



- Dual-axis sample holders



Koning et al. 2018 https://doi.org/10.1016/j.aanat.2018.02.004

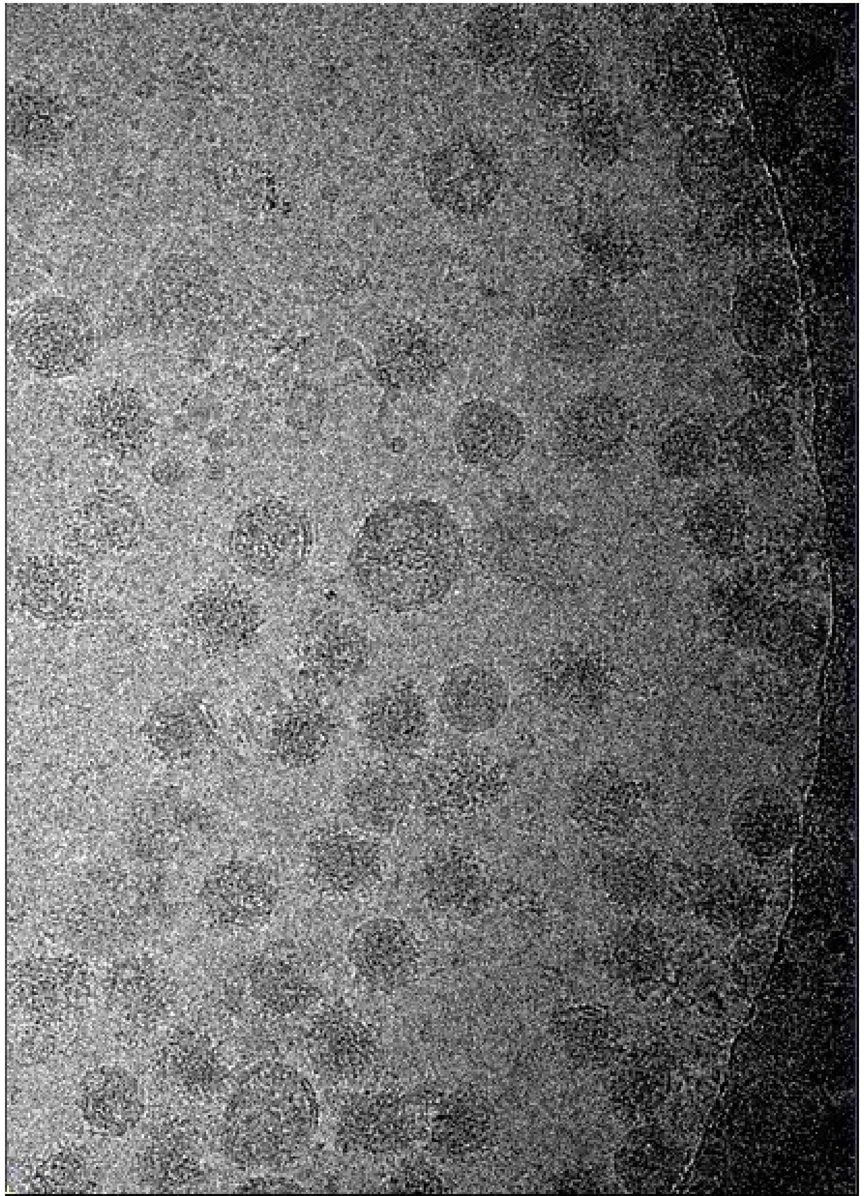
Phan et al. 2016 DOI: 10.1186/s40679-016-0021-2

Tomography - Data processing

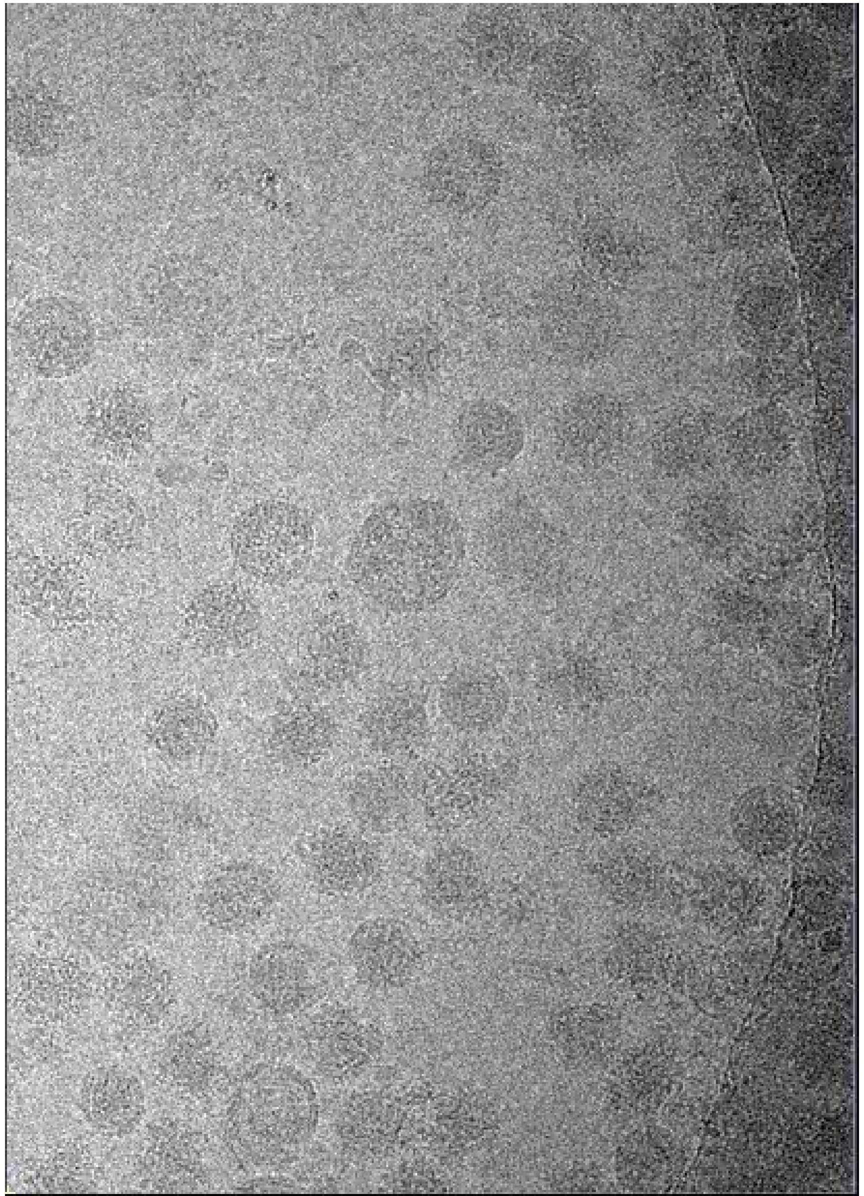
- Motion correction of raw movie data of single tilts
  - Possibility of dose weighting of single tilts
- Alignment of tilt series
  - Coarse alignment – cross correlation between neighboring tilts
  - Fine alignment – fiducial model that describe the tilting transformations
- Aligned tilt series postprocessing
  - Fiducial removal, CTF correction
- Reconstruction of the tomogram
  - Back-projection algorithms
- Tomogram filtering
- Segmentation of tomogram
- Sub-tomogram averaging
  - Refining a high-resolution structure from the subparts of the tomogram

Tilt alignment – Coarse align

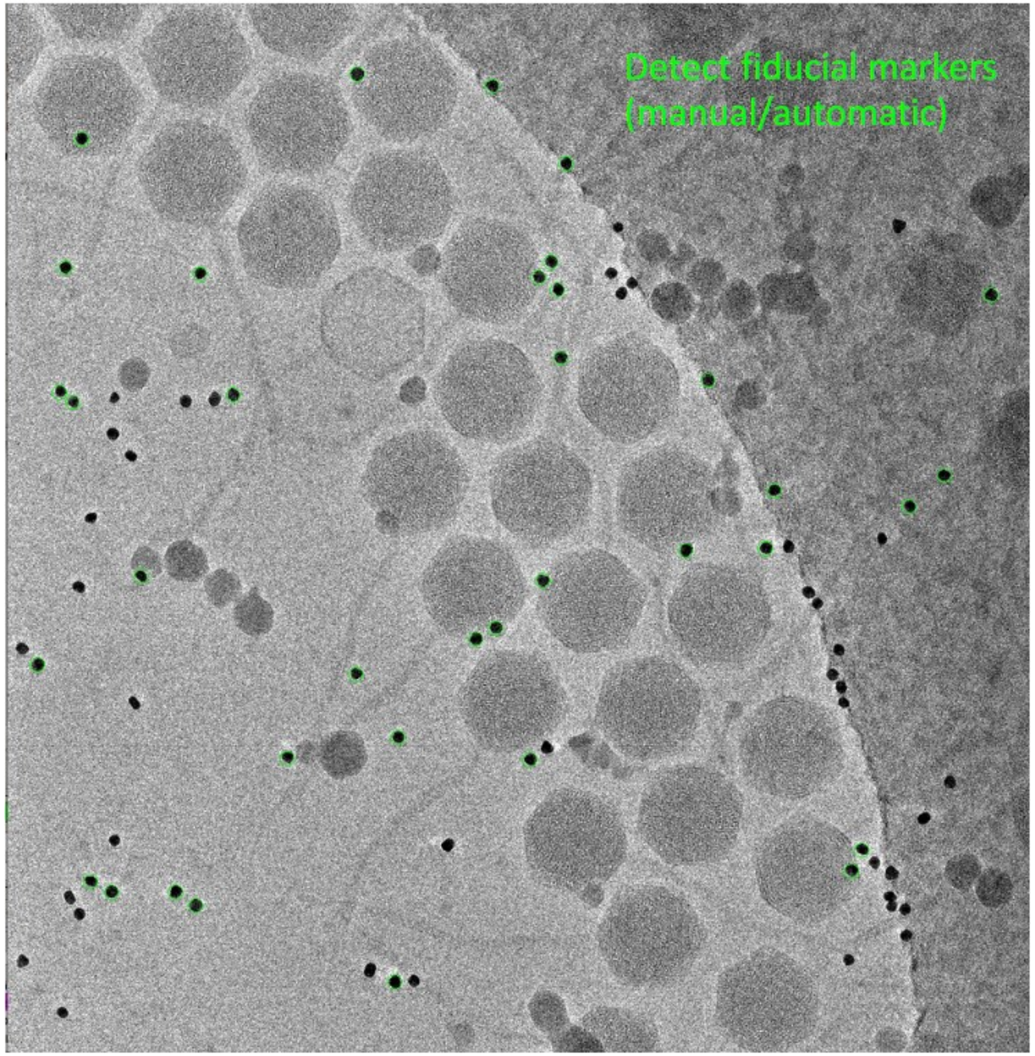
After acquisition

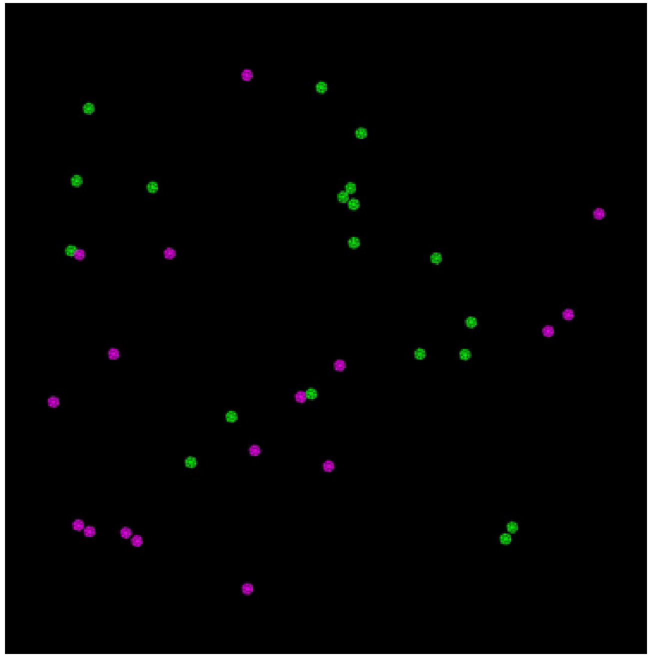


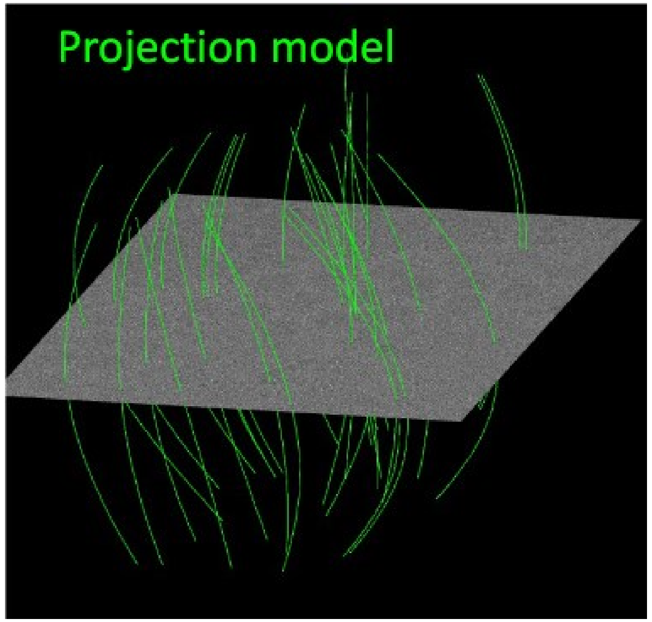
After coarse alignment



Fiducial based fine alignment



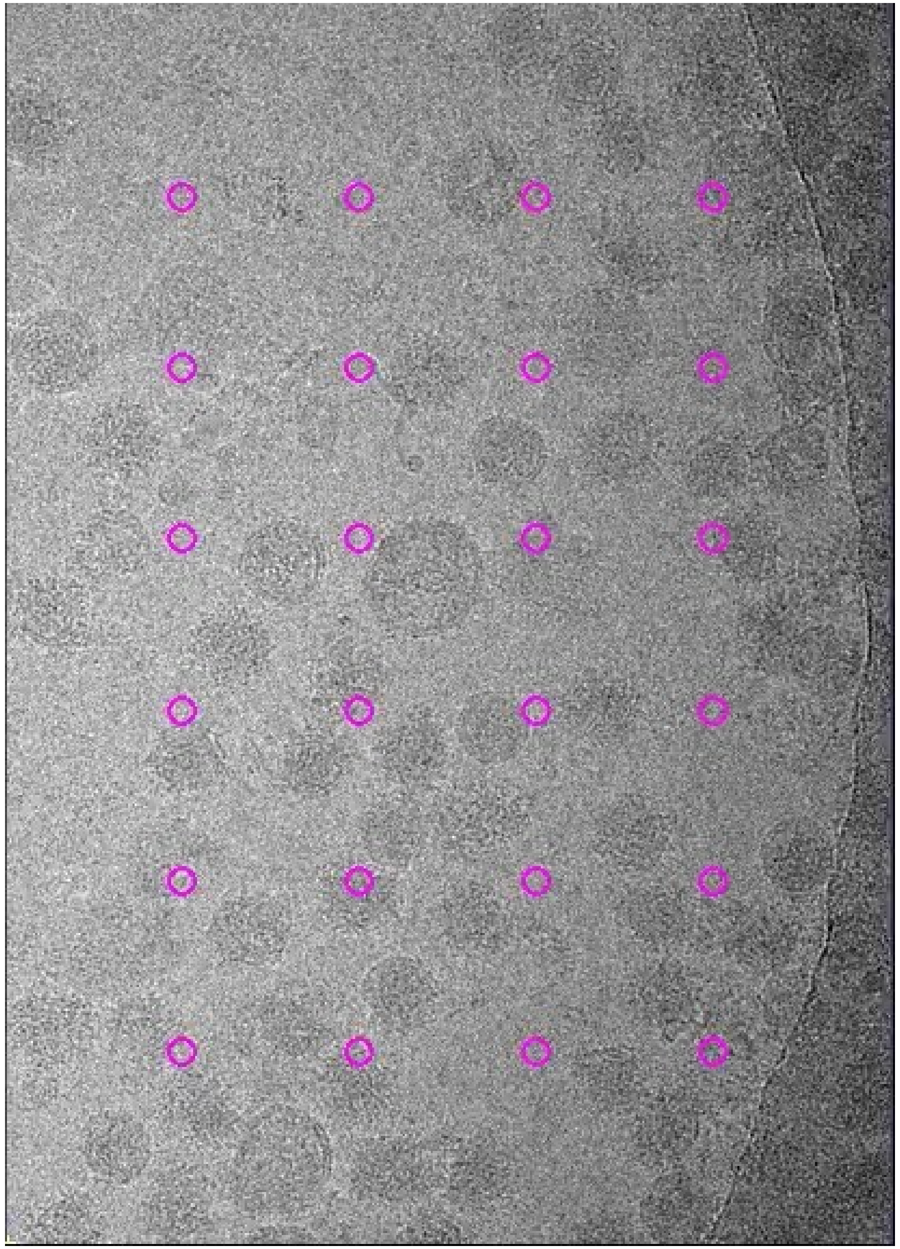




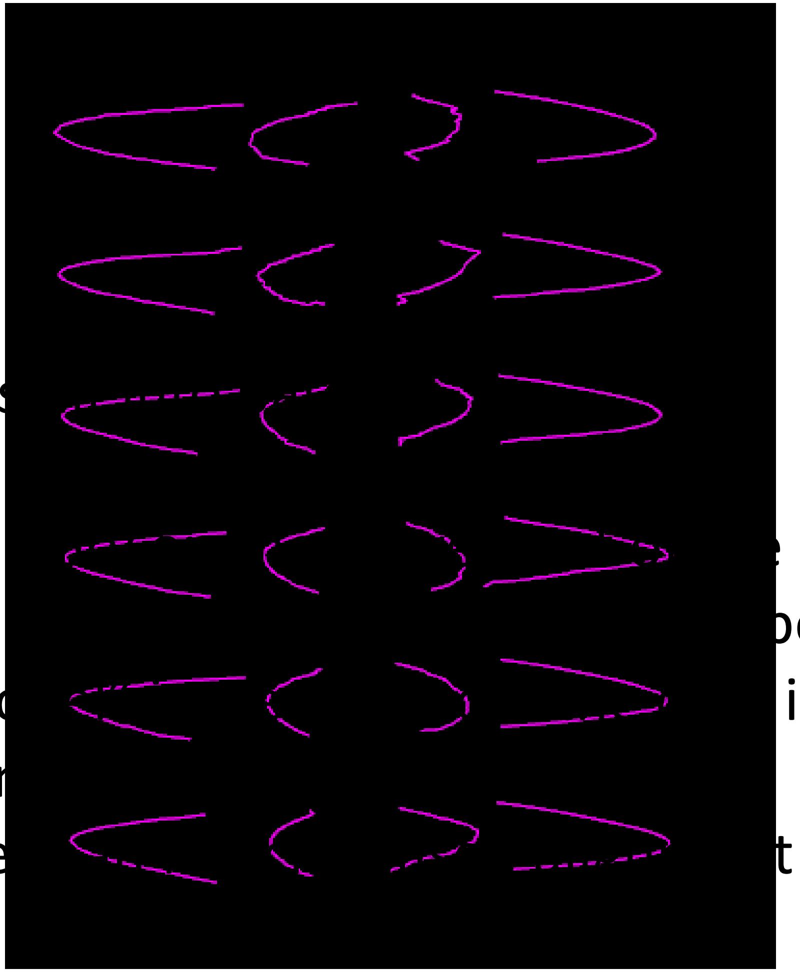
- Need fiducials
- Finding the same fiducial on all of the tilts and fitting a model on their trajectory
- Allows subpixel precision alignment of tilt series

https://www.renafobis.fr/...../leforestier-tomography-renafobis_2021.pdf

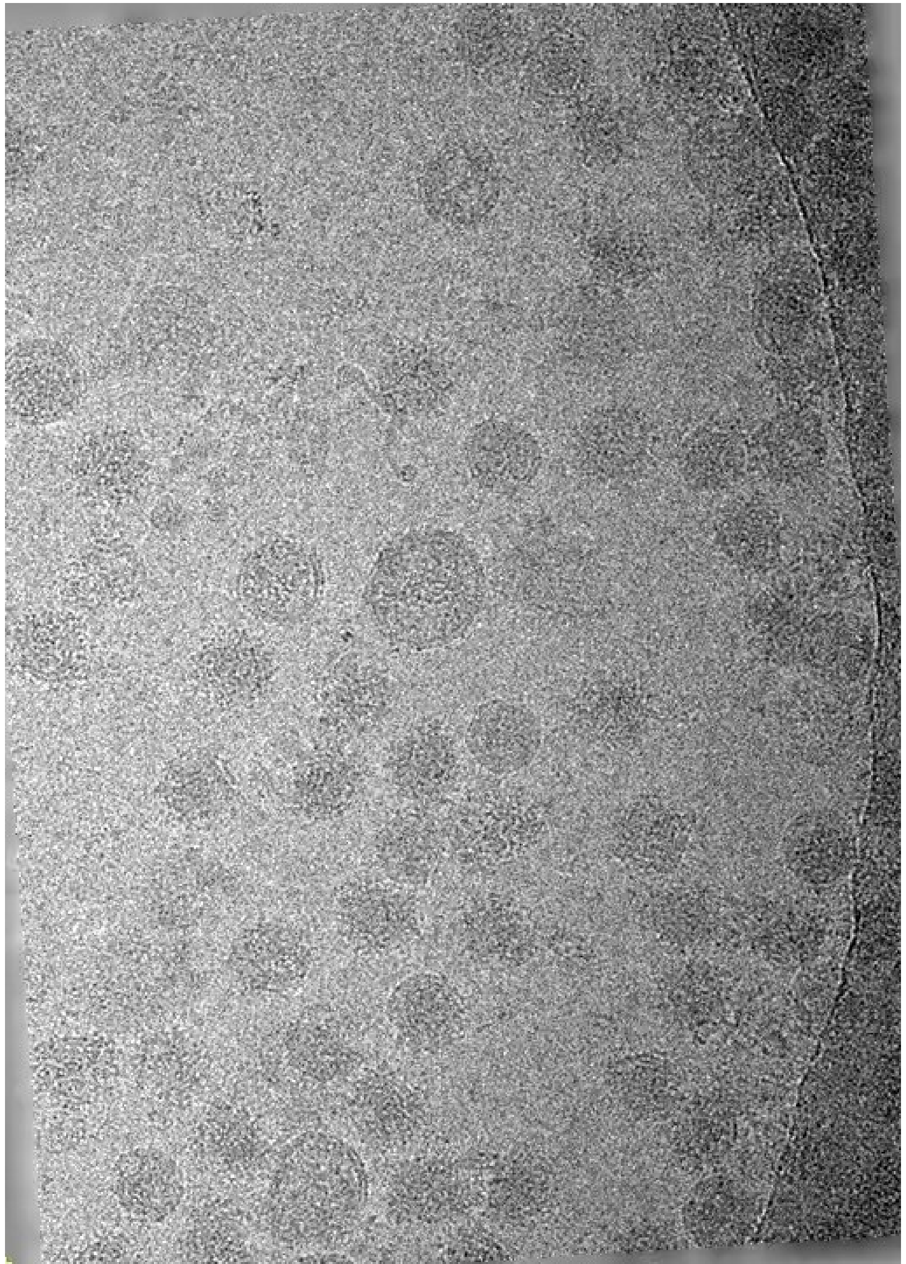
Patch tracking – fine alignment



- Tilt images s
- Correlating
- Fitting traje e patches
- Every patch be traceable
- “Fiducial mo itself without any additior
- Less precise t models

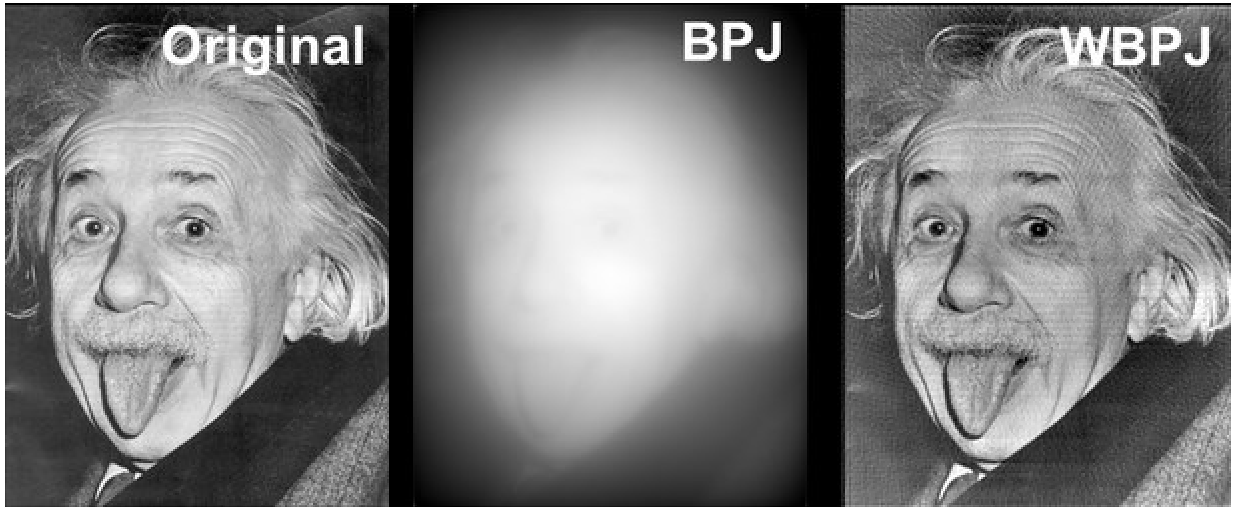


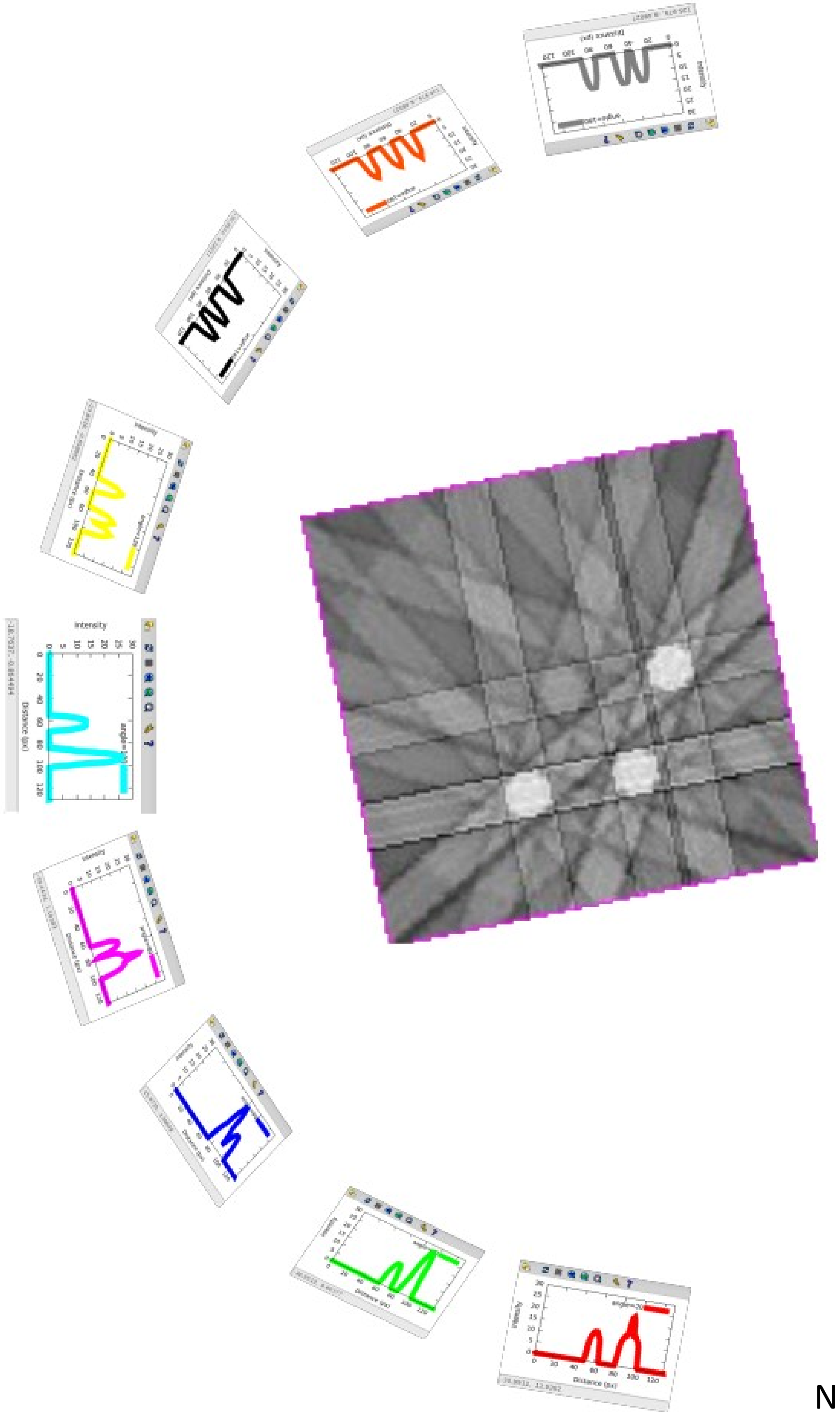
Fine alignment using the fiducial model



Reconstruction

- Back-projection
  - Low frequencies are over-represented
- Weighted back-projection
  - Low frequencies are down weighted
- SIRT
  - Simultaneous Iterative Reconstruction Technique



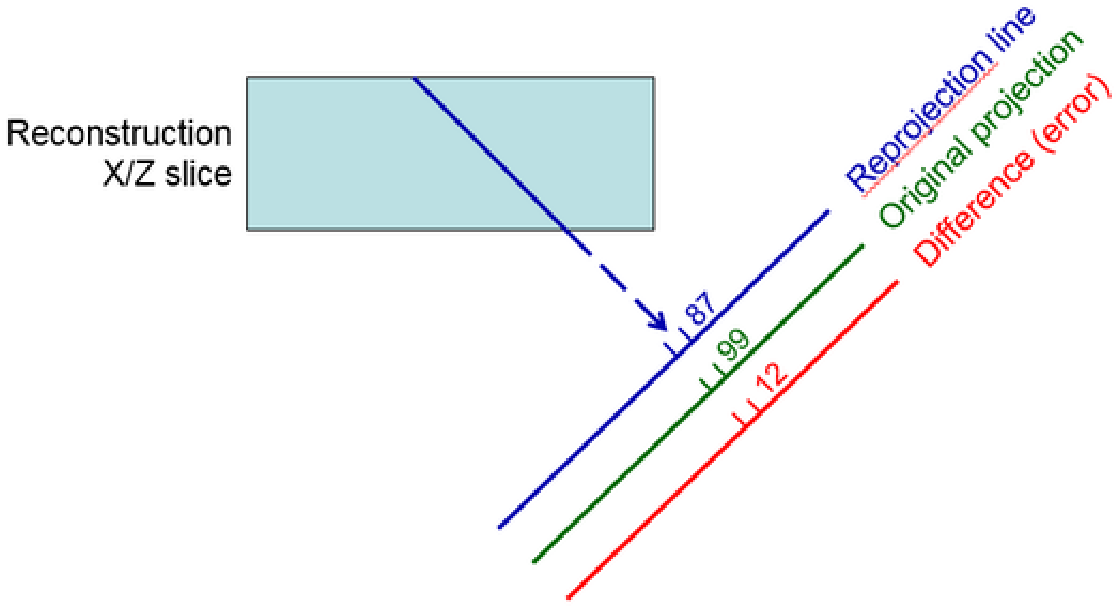


https://www.int.kit.edu/1731.php

Nováček J.

SIRT (Simultaneous Iterative Reconstruction Technique)

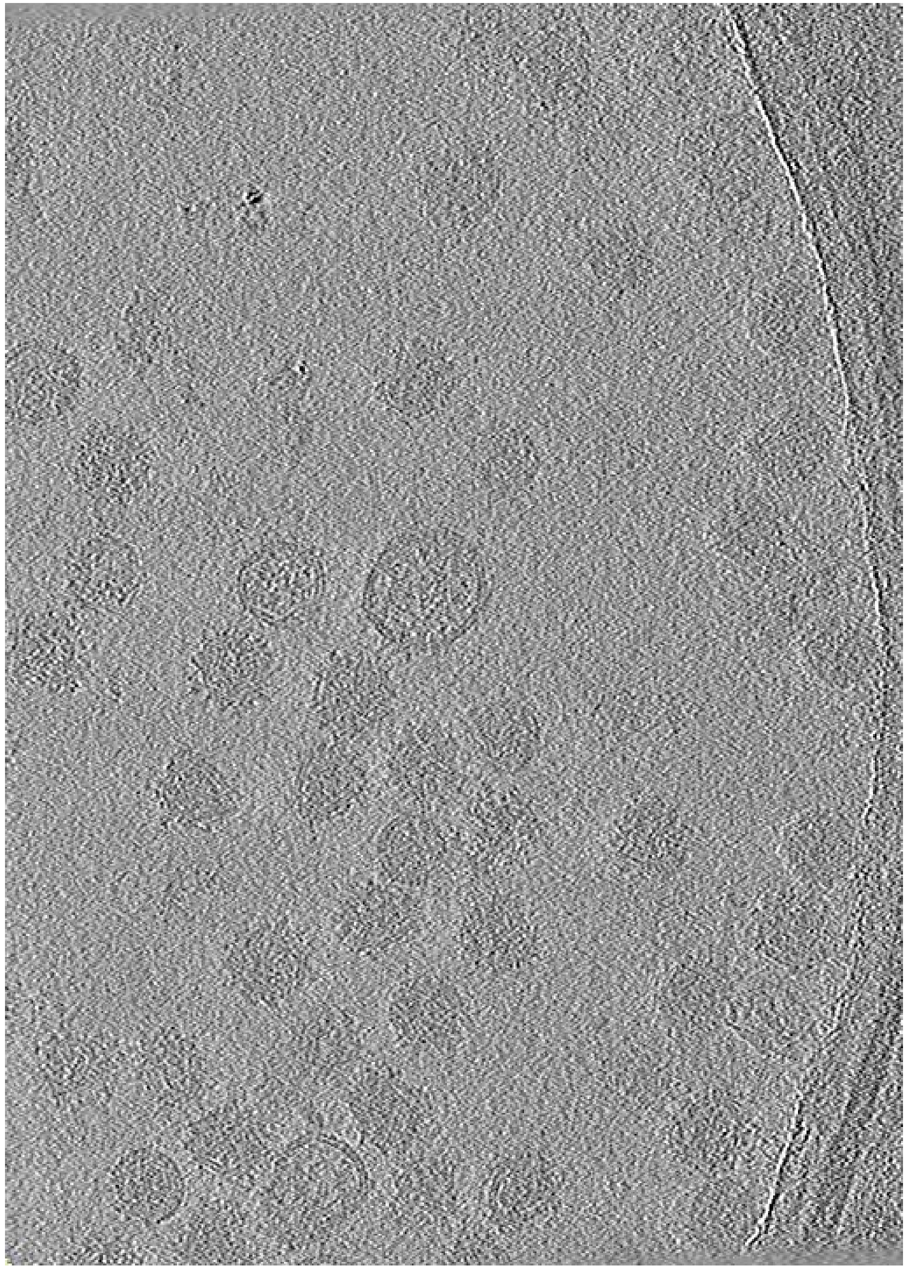
- Start with a tomogram reconstruction from the backprojection of the tilts
- Reprojecting from the tomogram in the original tilt orientations
- Taking the difference between the original projection data and this reprojection at each pixel (this difference represents the amount of error in the current estimate)
- Adding the error to the tomogram by a back-projection operation

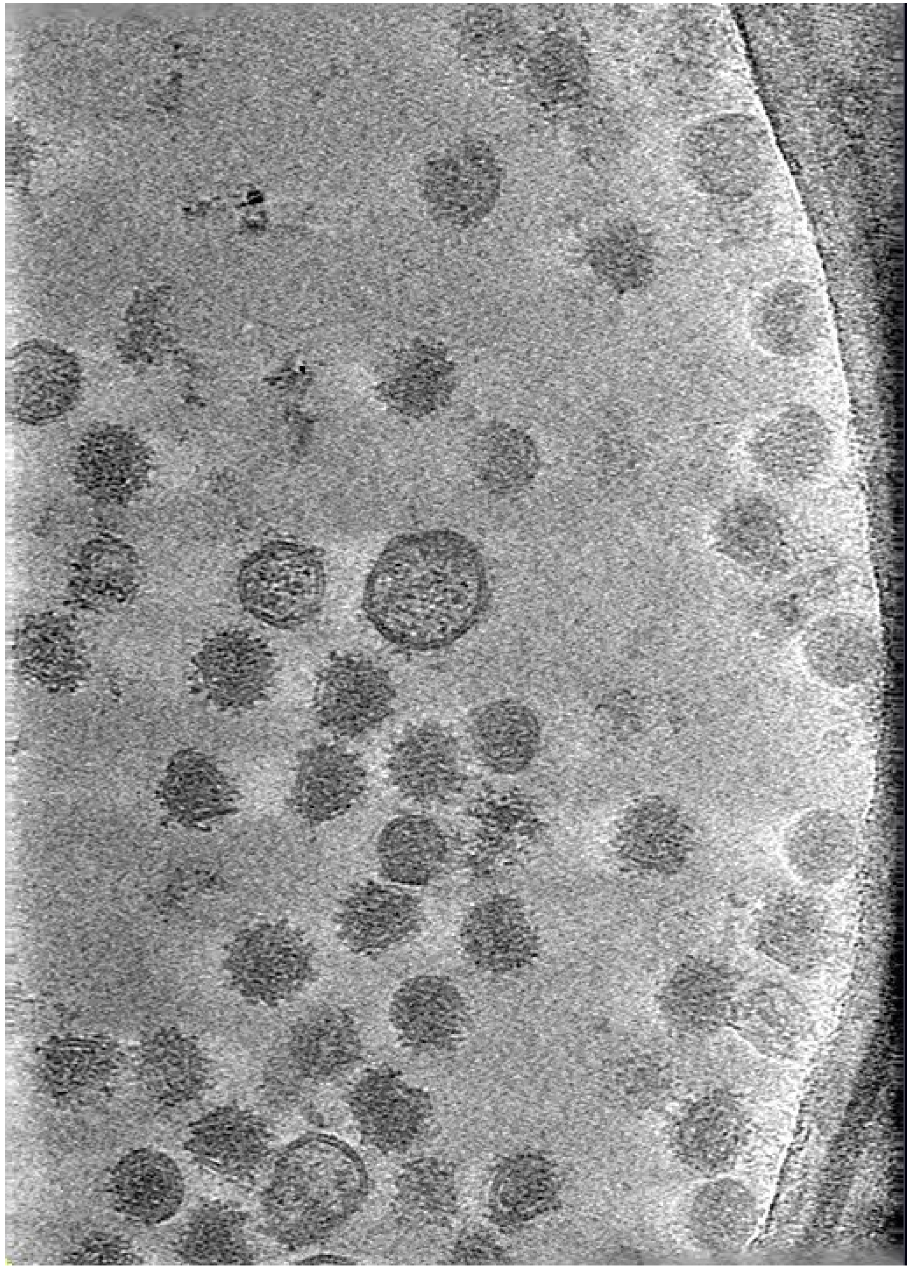


https://bio3d.colorado.edu/imod/doc/SIRTexample.html

Reconstruction

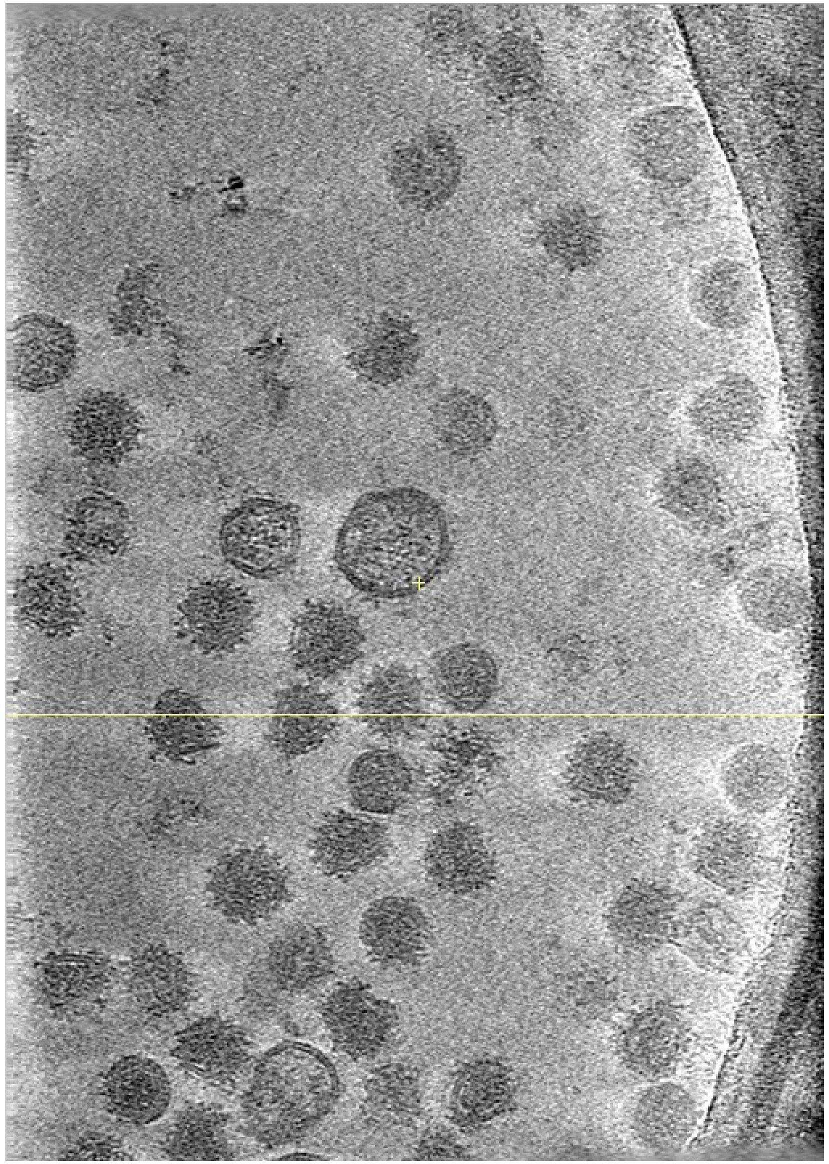
Weighted Back-projection



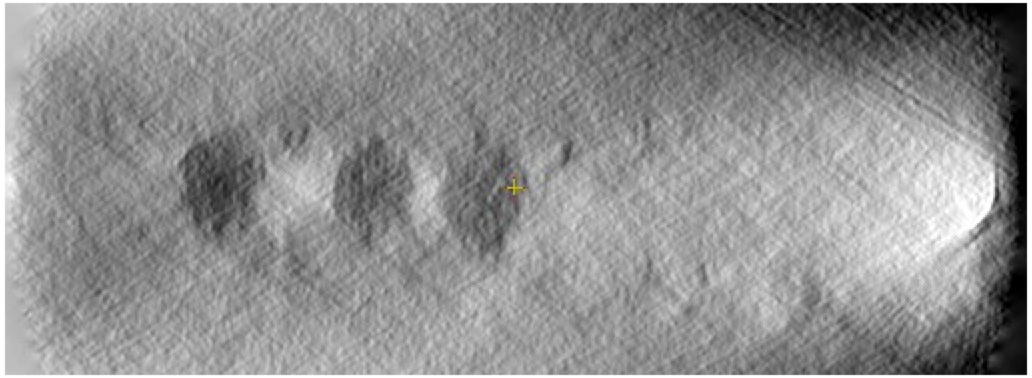


SIRT

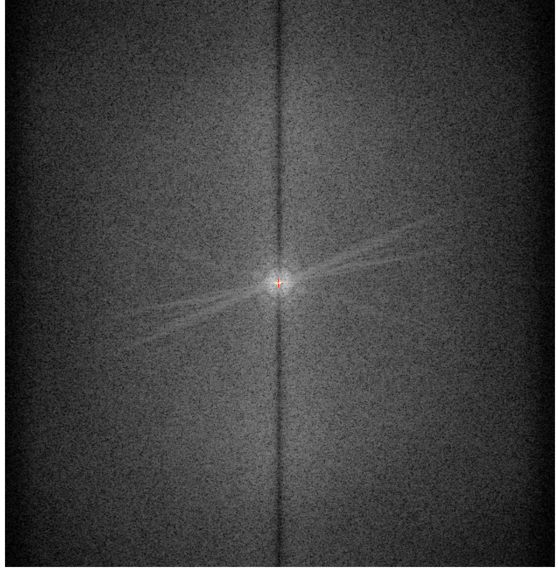
Missing wedge



XY Plane

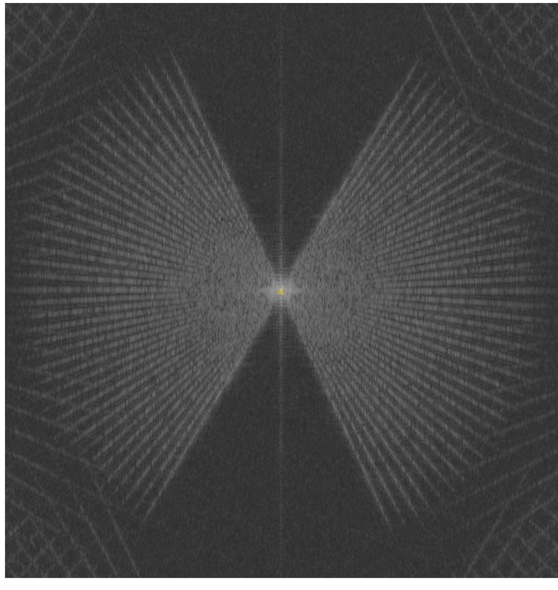


XZ Plane

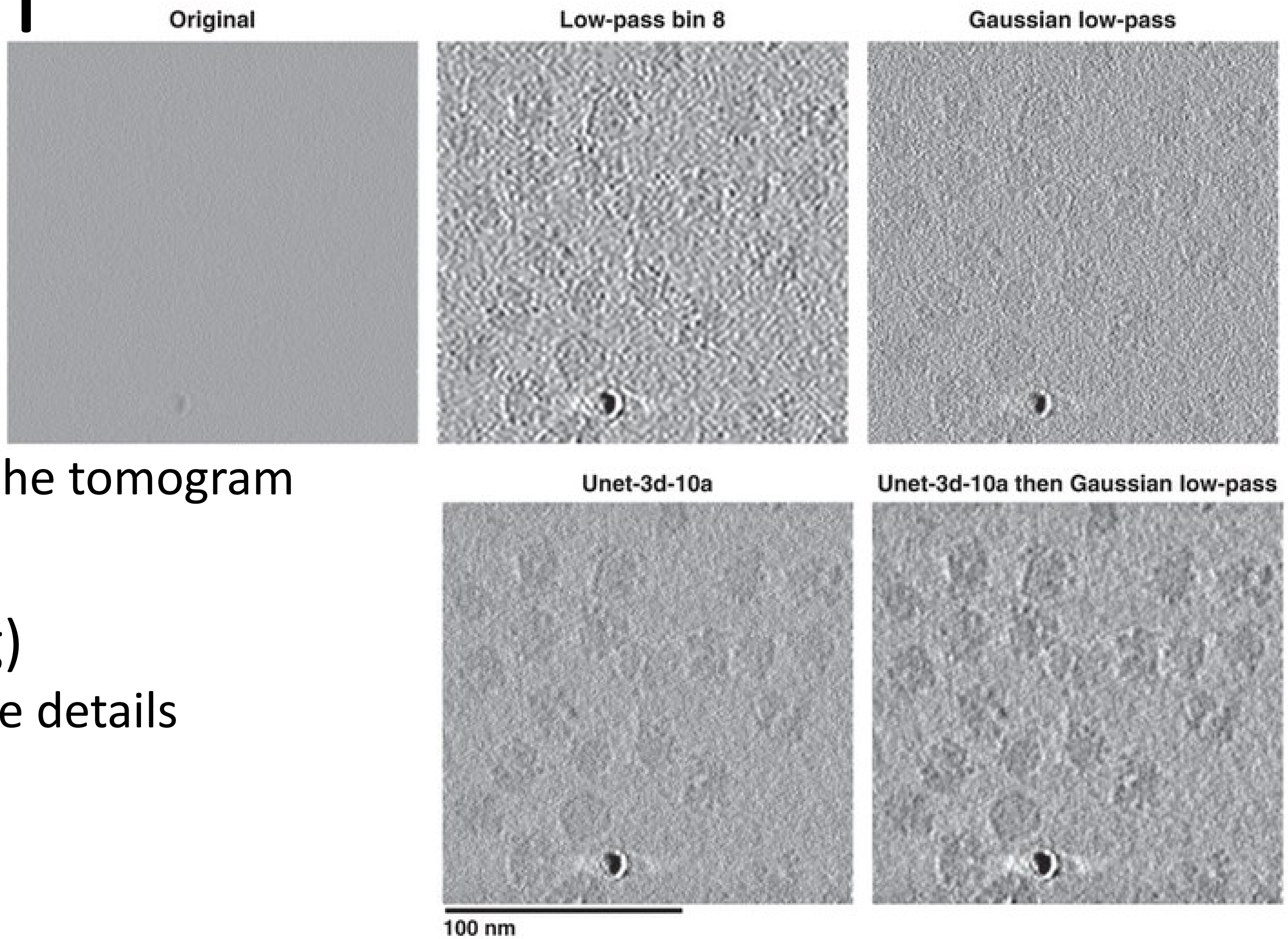


FTT XY Plane

FFT XZ Plane



Tomogram interpretation



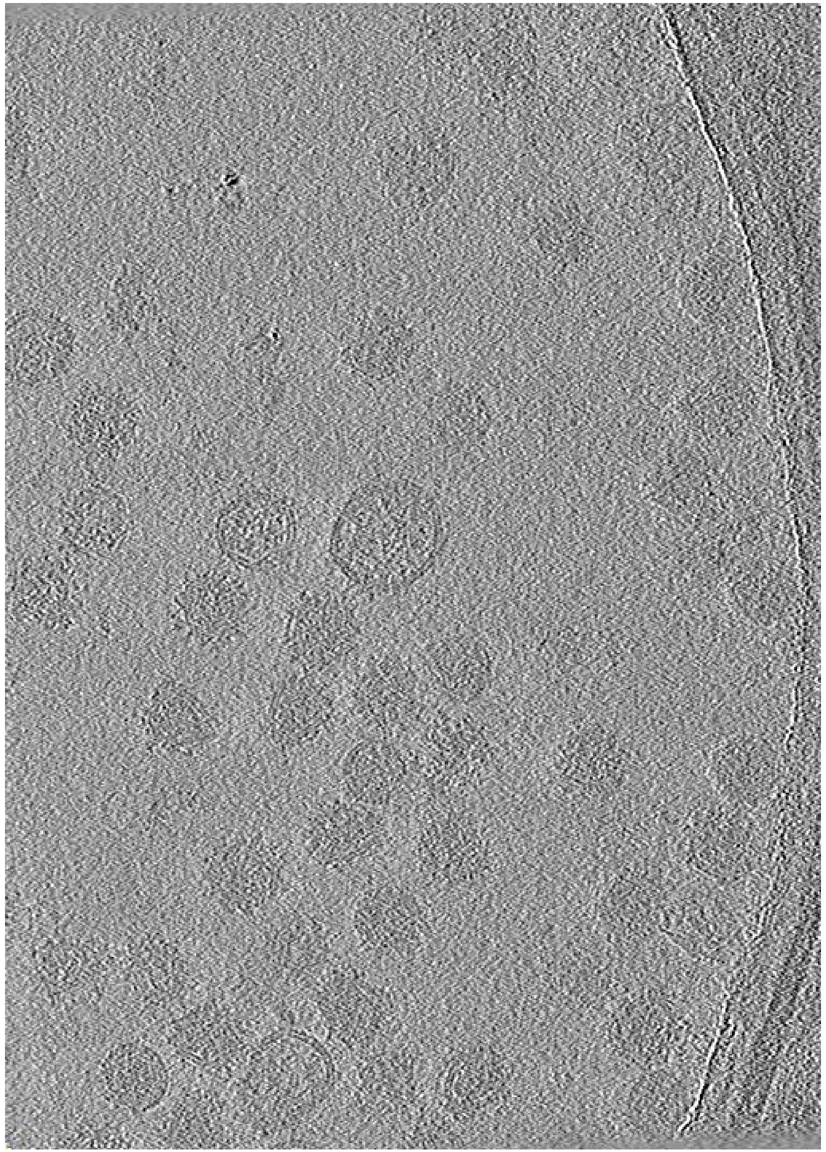
- Visual inspection
  - Annotation of features
- Segmentation
  - Annotating/extracting segments from the tomogram
- Sub-tomogram averaging
- Filtering – postprocessing (denoising)
  - Lowpass filters – increase contrast loose details
  - High pass filters – edge detection
  - Median filters
  - NAD – Nonlinear Anisotropic diffusion
    - Need to be tuned for balance between contrast and preserved details
  - Neural network based denoising
    - Noise model trained on data

Bepler et al., 2020

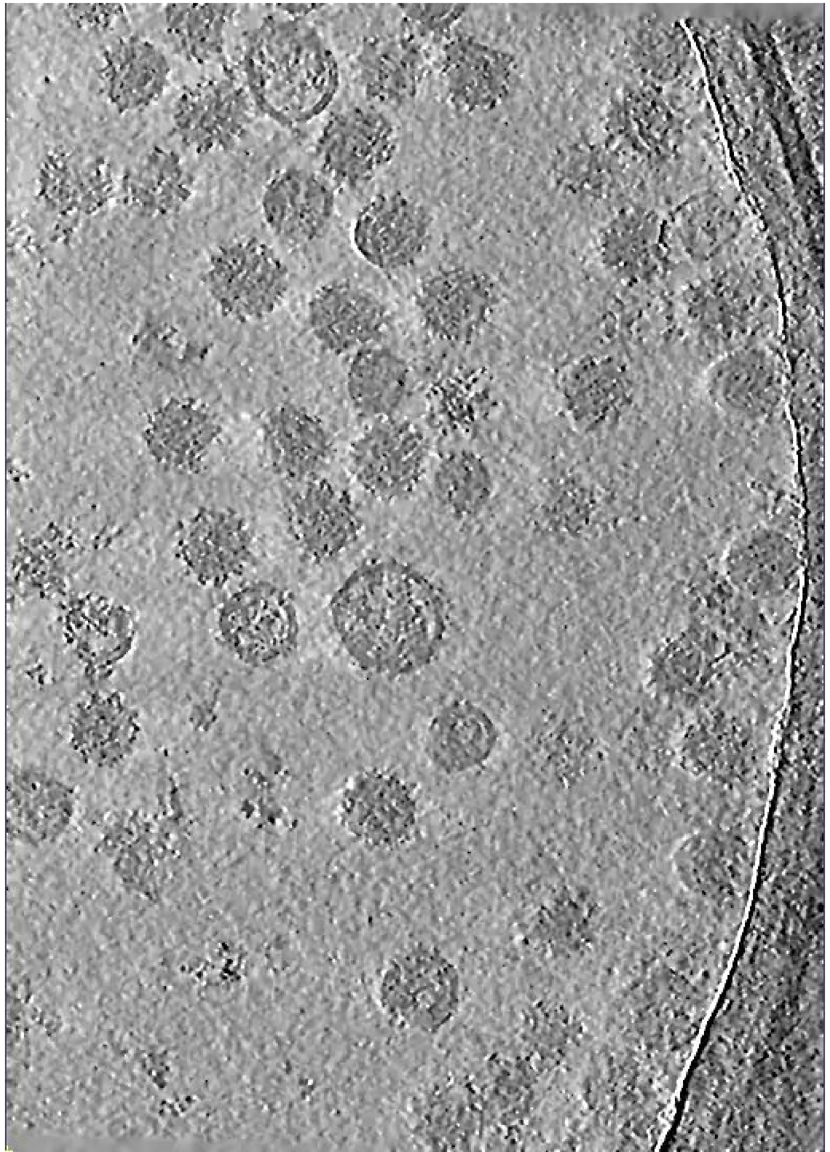
https://doi.org/10.1038/s41467-020-18952-1

NAD – nonlinear anisotropic difusion

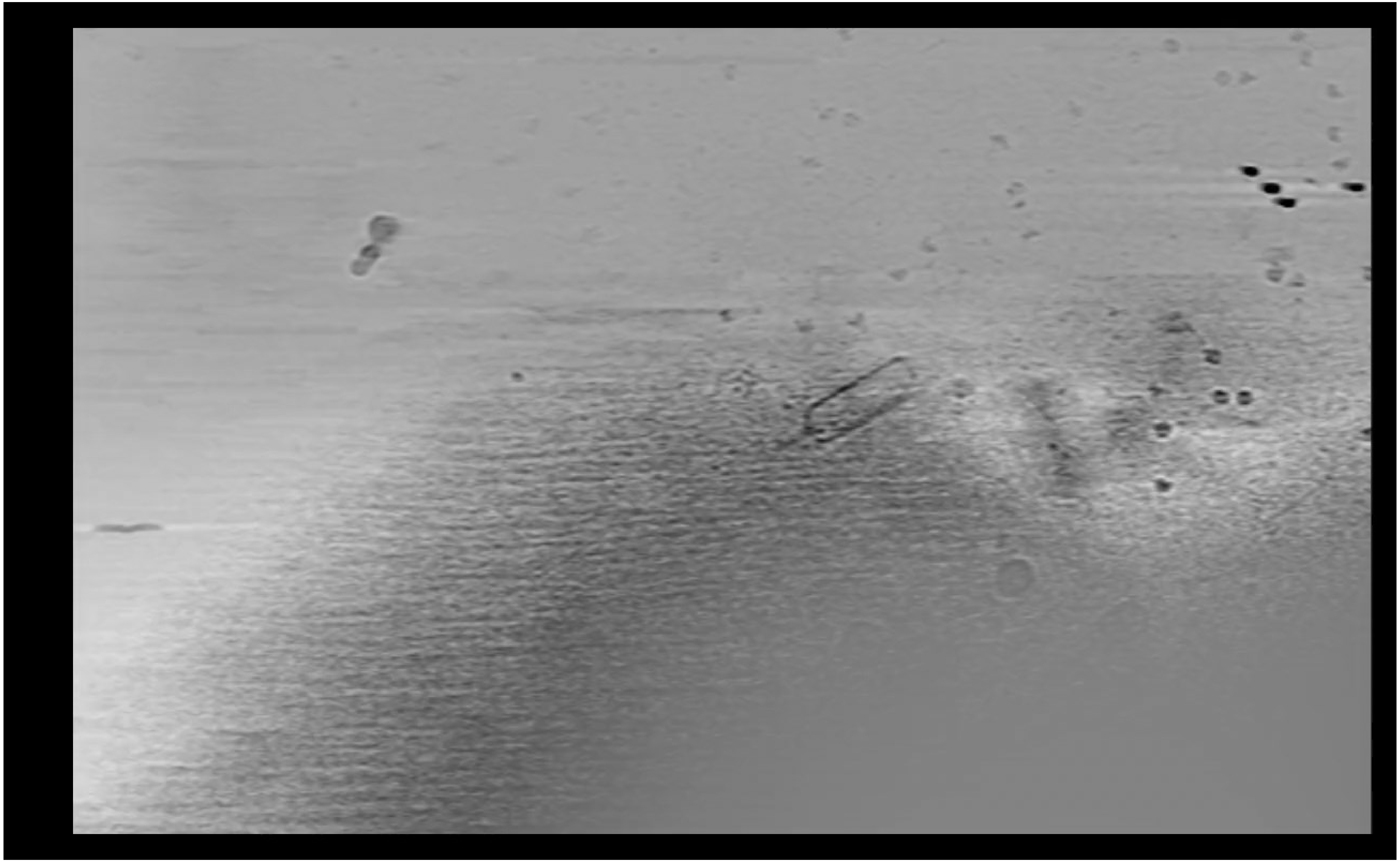
Before



After



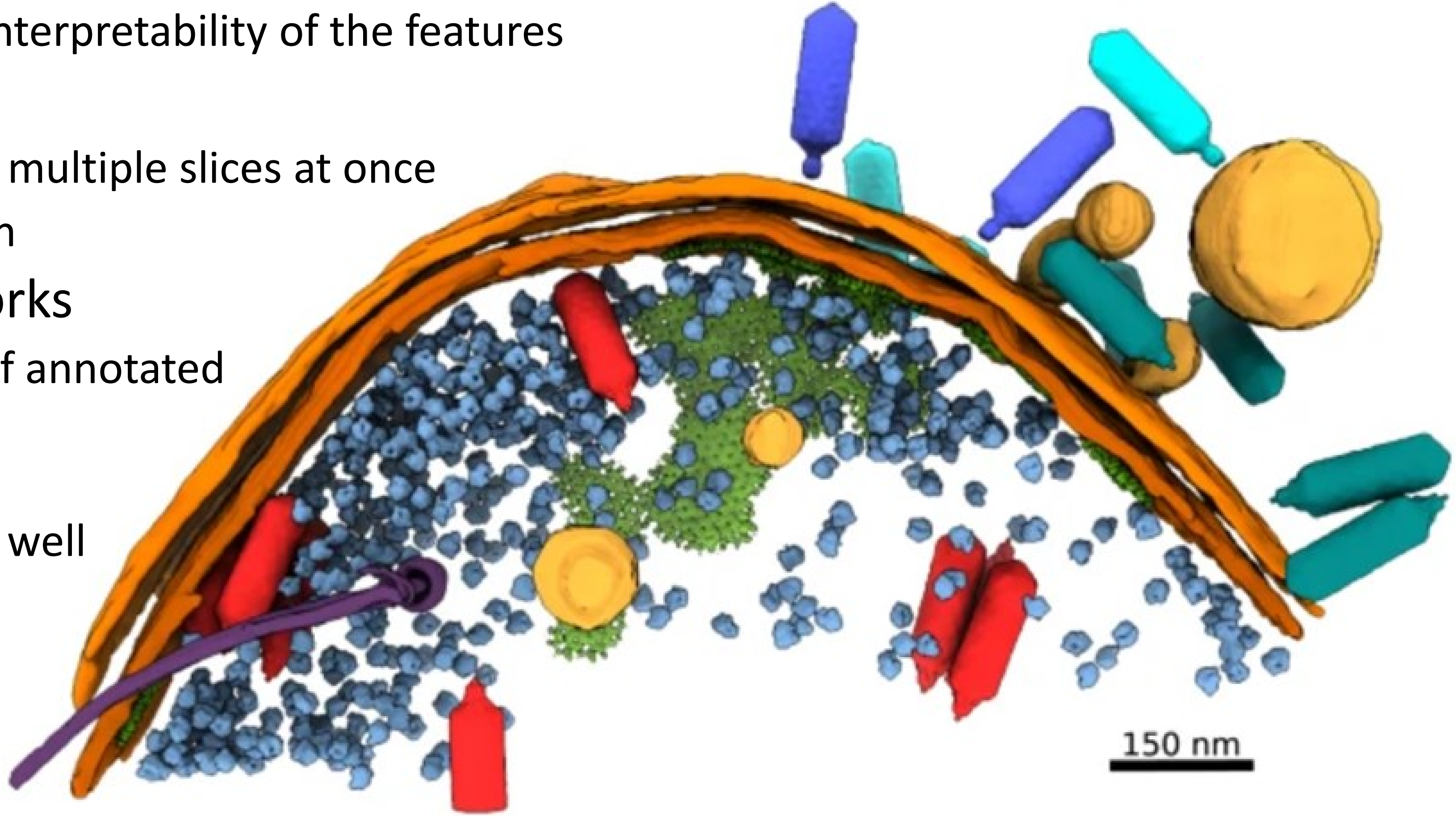
Segmentation



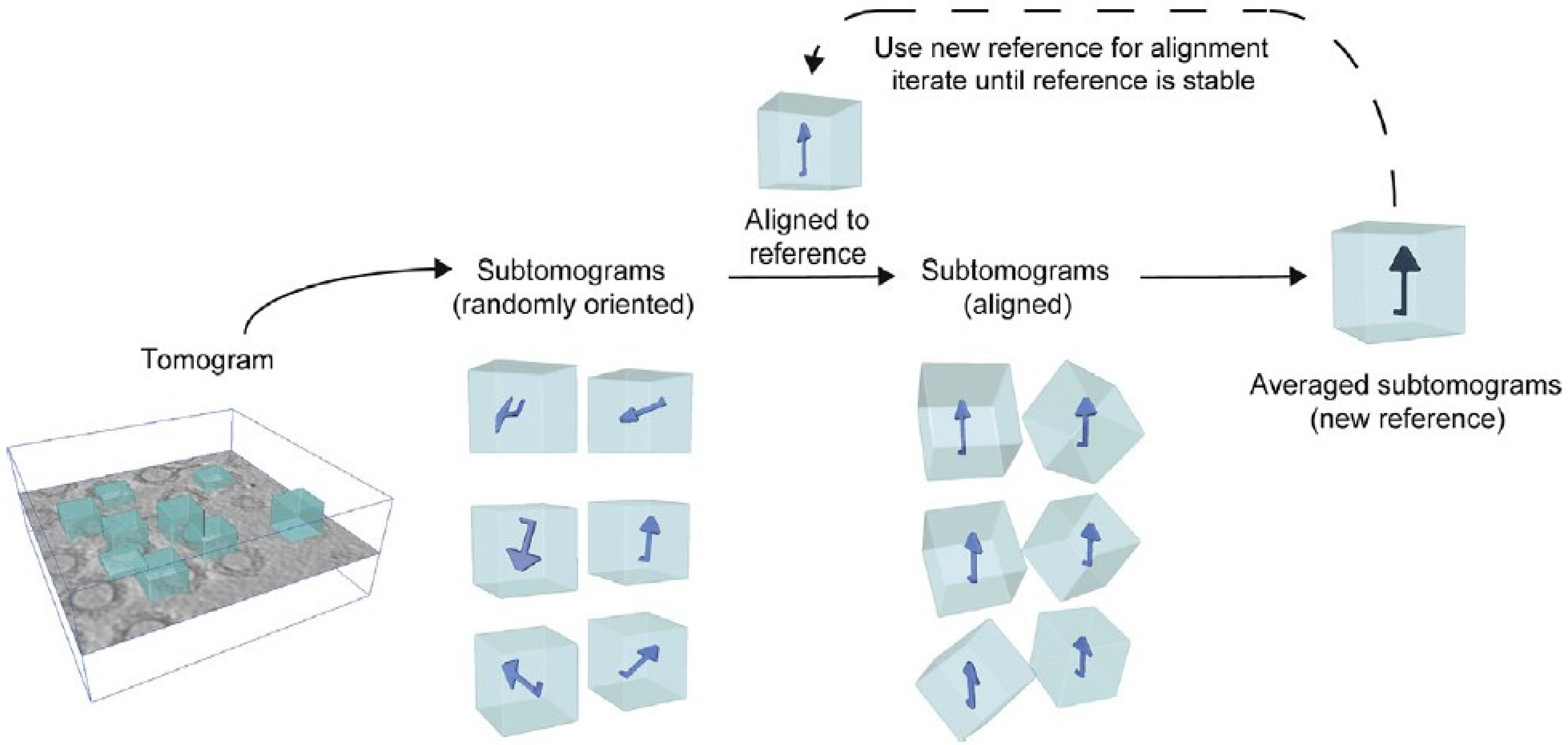
Šiborová, M. et al. 2022

Segmentation

- Annotating parts of the tomogram by connecting continuous segments
  - Manual
    - Drawing
    - Thresholding
    - “Missing wedge” limits the interpretability of the features
  - Semiautomatic
    - Simultaneous annotation on multiple slices at once
    - Correlation based annotation
  - Convolutional neural networks
    - Training CNN on small part of annotated tomogram
      - Long training process
    - General models not working well



Sub-tomogram averaging



- Increasing signal to noise ratio
- Increasing resolution
- By averaging uniformly orientationally distributed sub-tomograms we fill the missing wedge in the Fourier space

Wan & Briggs 2016

http://dx.doi.org/10.1016/bs.mie.2016.04.014

Tilt-dose weighting

- High angle tilts heavily radiation damaged
  - Not containing high resolution information
- Essential for high resolution sub-tomogram averaging
- Dose weighting during movie motion correction process
- Implemented in many sub-tomogram averaging software
  - Beware not to double dose weight the data

$$q(k, N) = e^{-\frac{N}{2N_e(k)}}$$

Spatial frequency (k)

exposure-dependent amplitude attenuation (q)

accumulated exposure of the frame (N)

$$\widetilde{F}^W(\mathbf{k}) = \frac{\sum_{i=1}^{n} q(k, N_i) F_i(\mathbf{k})}{\sqrt{\sum_{i=1}^{n} q(k, N_i)^2}}$$

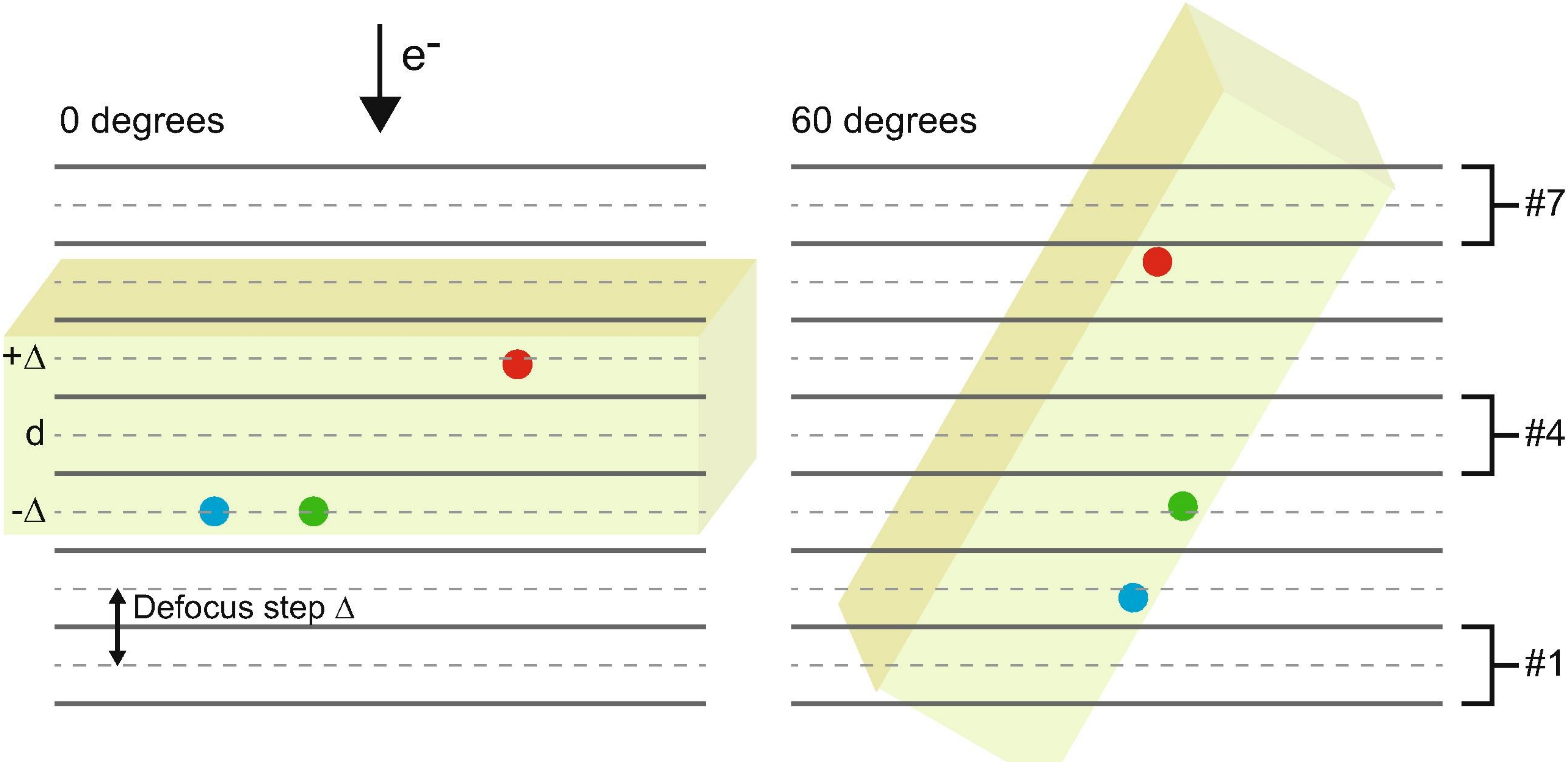
Dose-weighted Fourier transform of Image ($\widetilde{F}^W$)

i-th fraction / i-th tilt (i)

From lecture 7

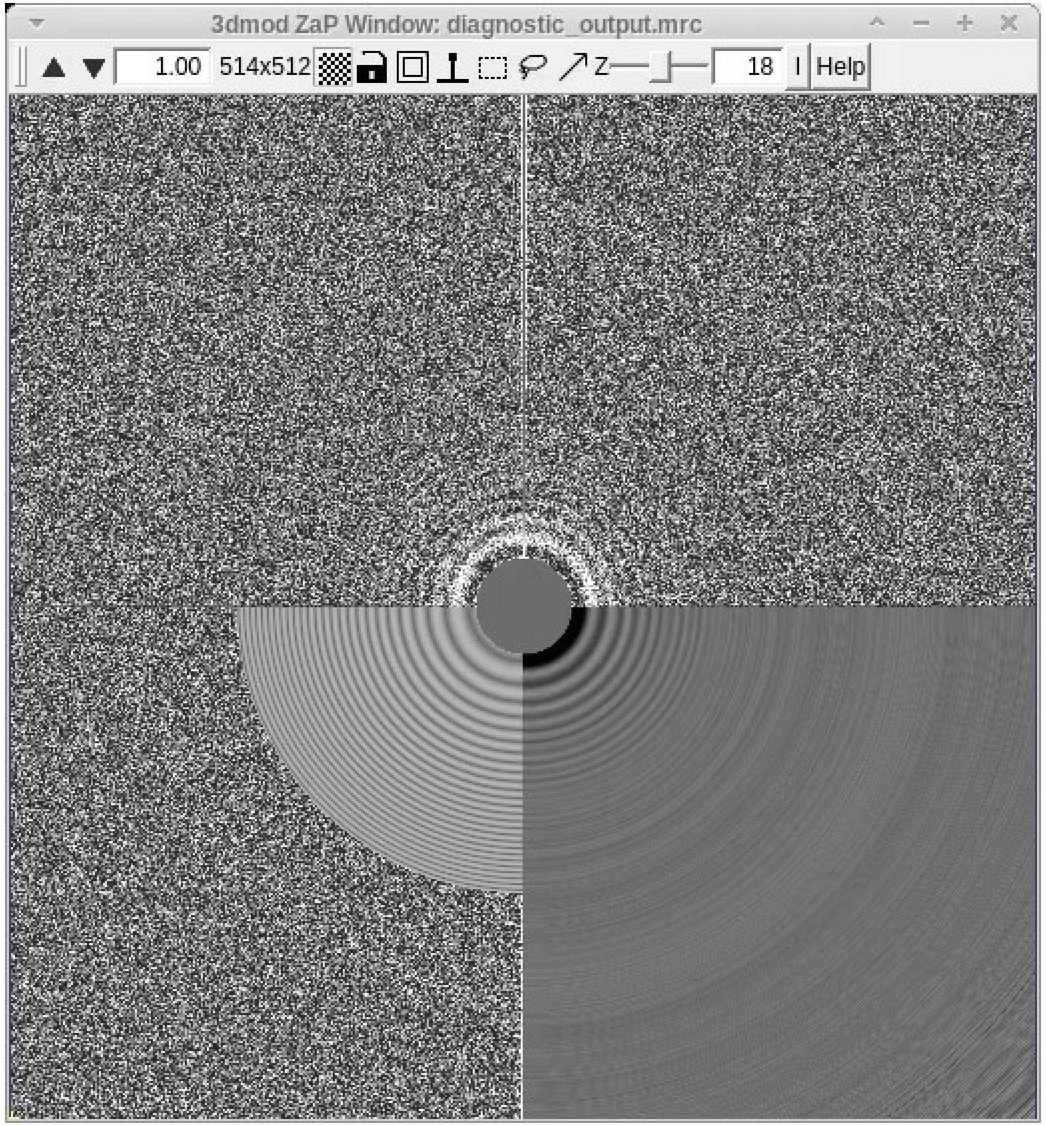
Grant & Grigorieff 2015
https://doi.org/10.7554/eLife.06980.001

CTF estimation – defocus gradient

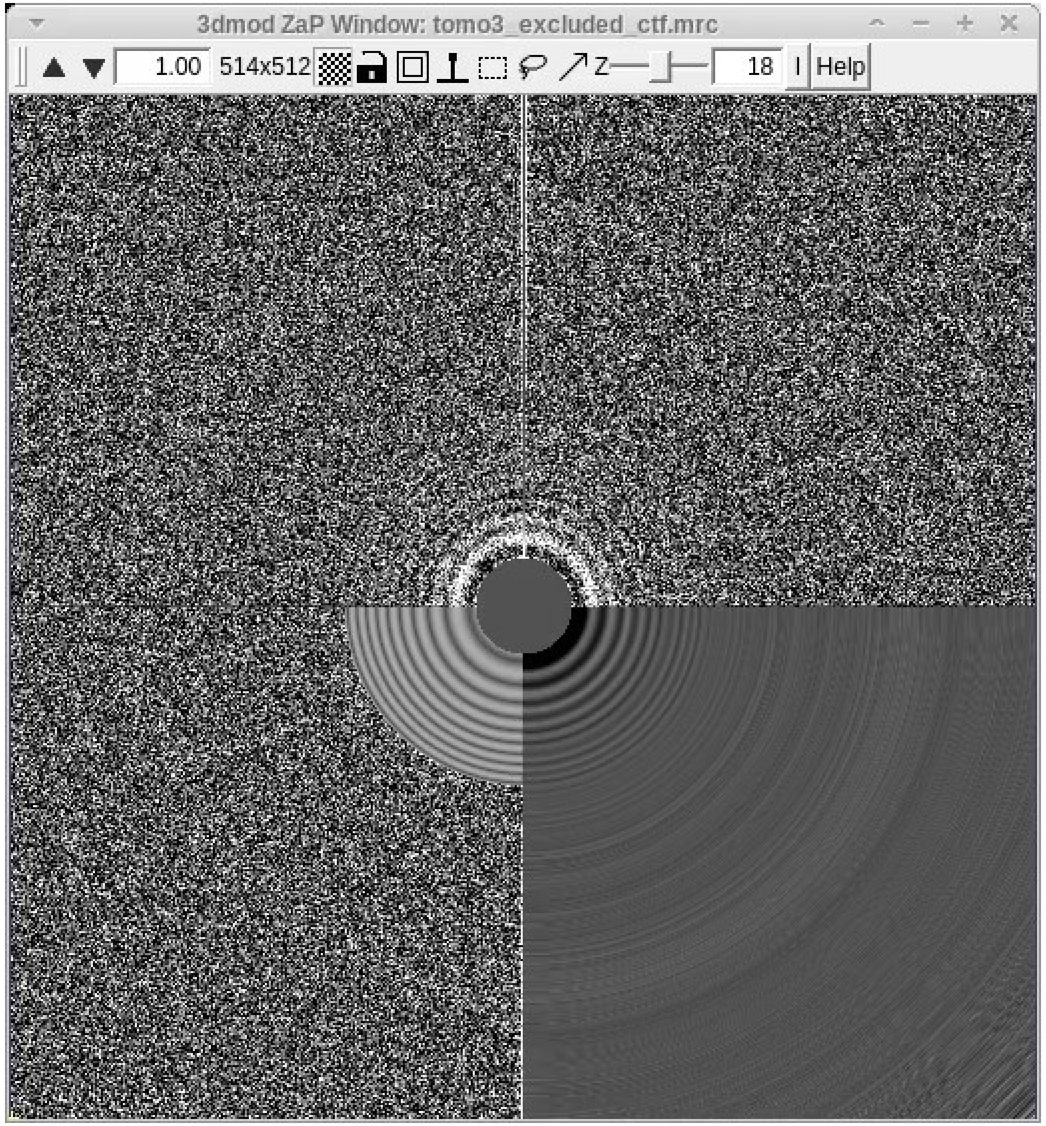


CTF Estimation

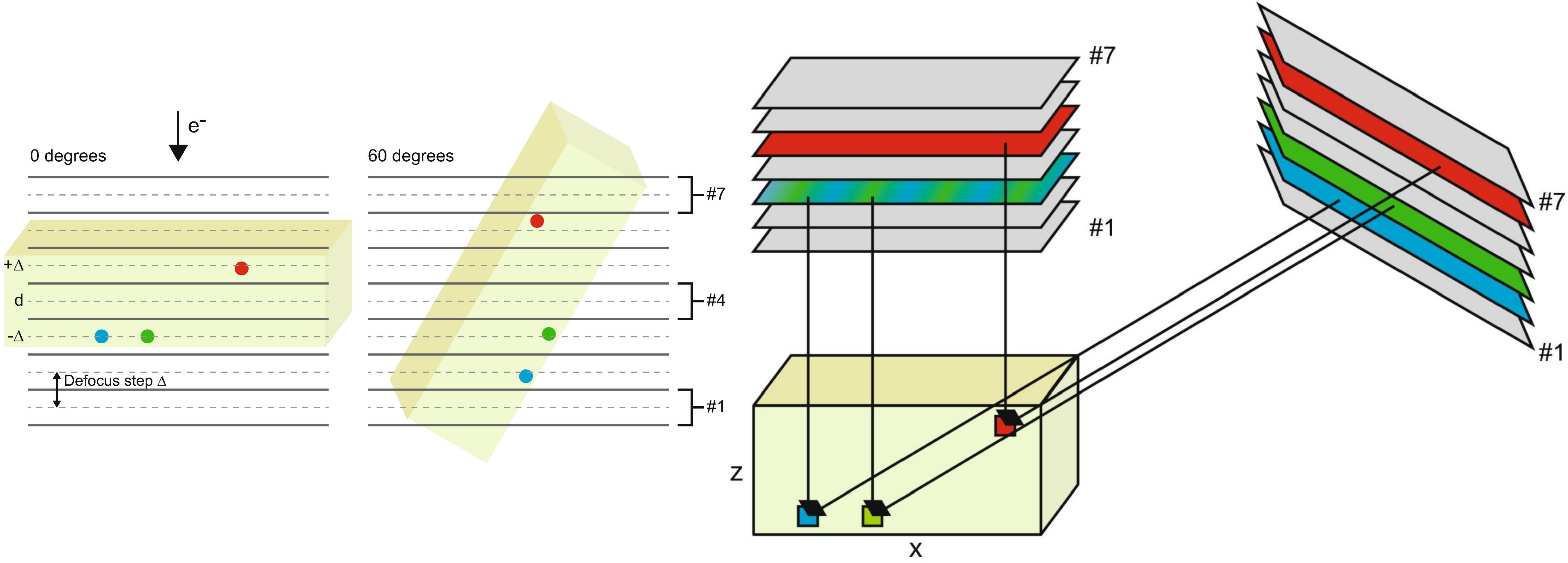
Unconstrained CTF fit



Constrained search range, constraining resolution
Gradually depending on the tilt angle



CTF correction during tomogram reconstruction



Turonova et al., 2017

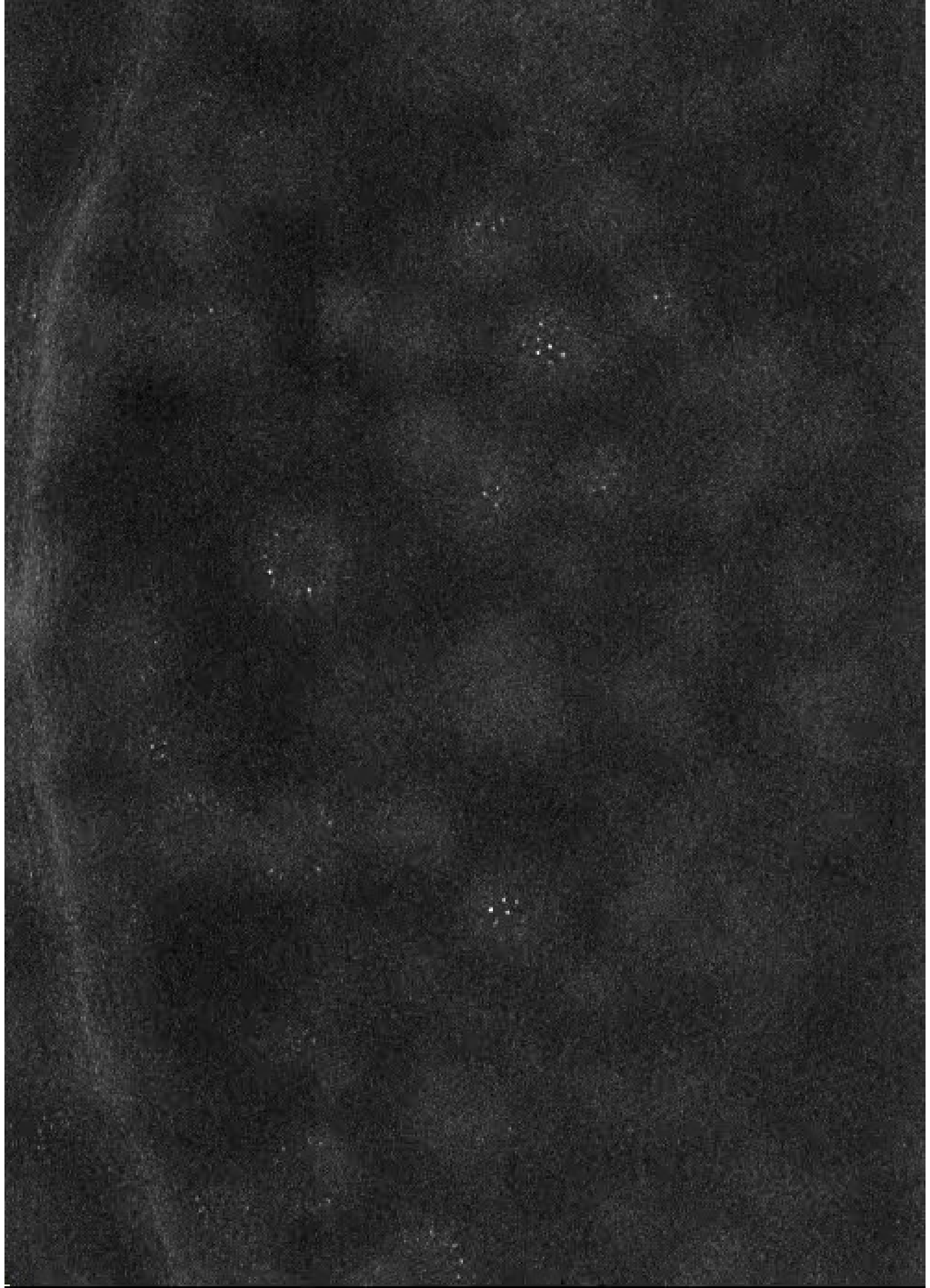
https://doi.org/10.1016/j.jsb.2017.07.007

Template matching

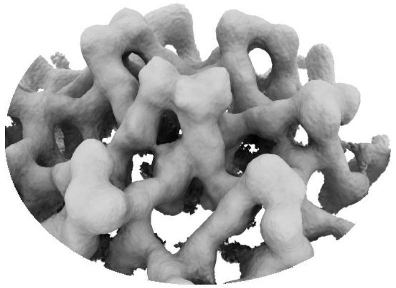
- Hard to locate particles in 3D tomograms with “missing wedge”
- Manual picking
  - When no template is available
  - Manual curation of template matching results
- Template matching
  - Need template for matching
  - Looking for a 3D volume in a 3D volume
  - Cross-correlation methods
  - Orientational search of the template
  - Computationally expensive
  - Produce not only coordinates (X, Y, Z) but also the best fitting orientation (ϕ, θ ,ψ)
  - Many false positive matches

3D correlation map

template



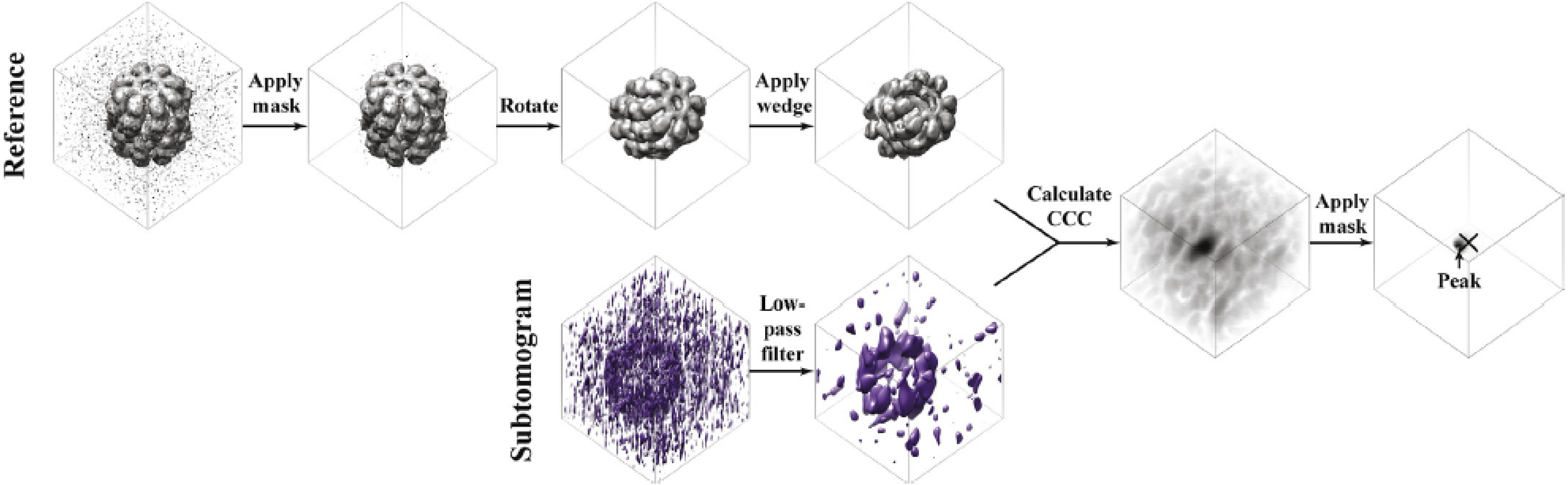
Template Matching



Sub-tomogram Averaging process

- Iterative process as SPA refinement
- Initial model can be generated from the data (need to be lowpass filtered)
- Computationally expensive
  - Aligning 3D volume on 3D volume
  - 3 Euler angles, 3 shifts
- Cross correlation/Maximum Likelihood methods
  - Need to compensate for the “Missing wedge”
- Speeding up calculations by gradual unbinning of the sub-tomograms
- Coarse search on heavily binned particles
- Fine (local) search on unbinned particles

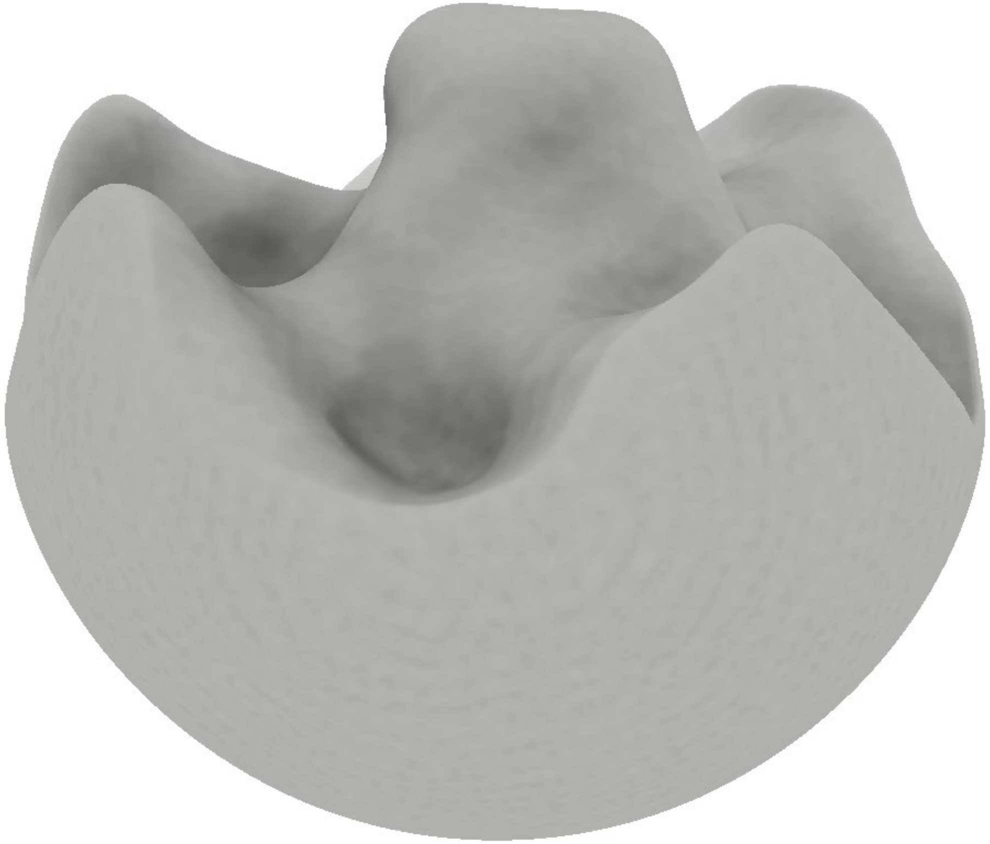
Sub-tomogram Averaging process

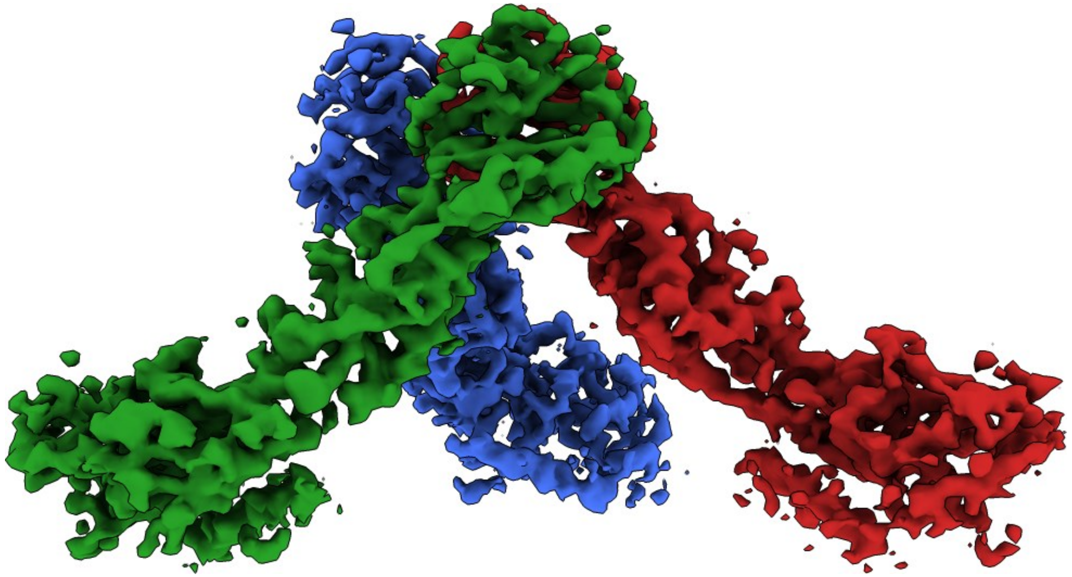


Wan & Briggs 2016

http://dx.doi.org/10.1016/bs.mie.2016.04.014

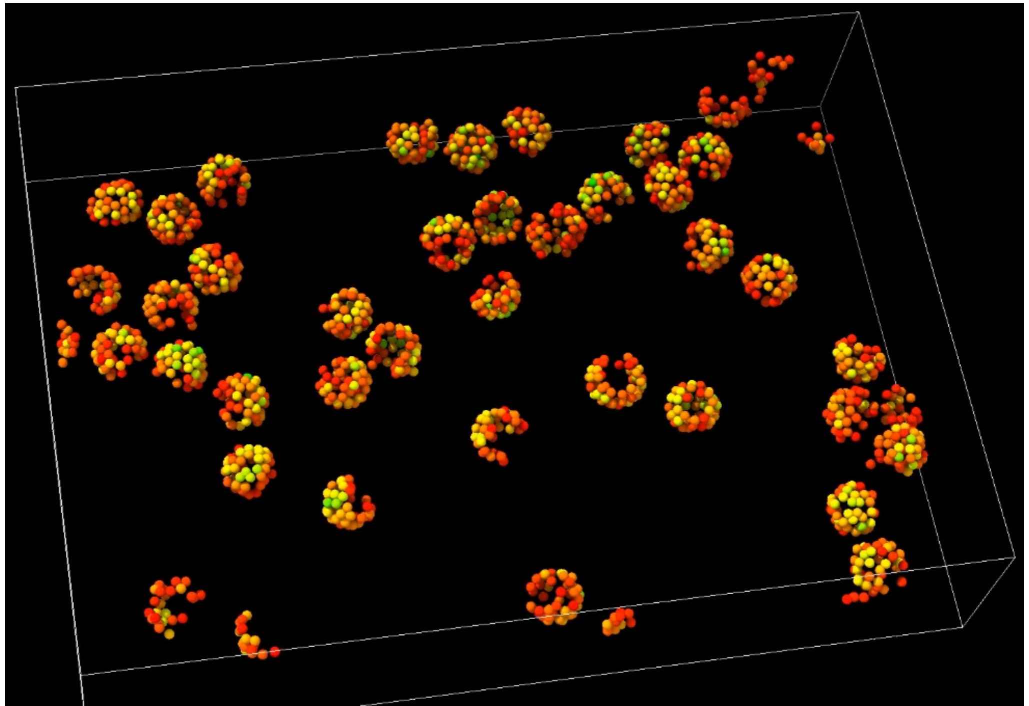
Sub-tomogram averaging – gradual refinement



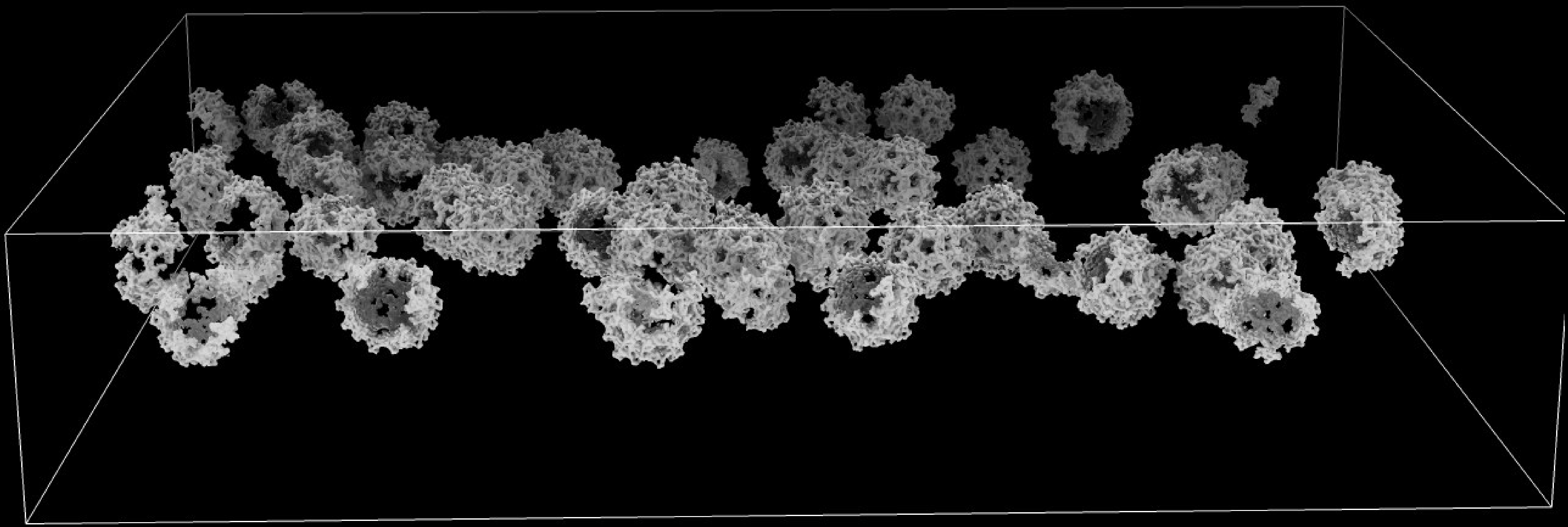


Placeback of averaged sub-tomograms

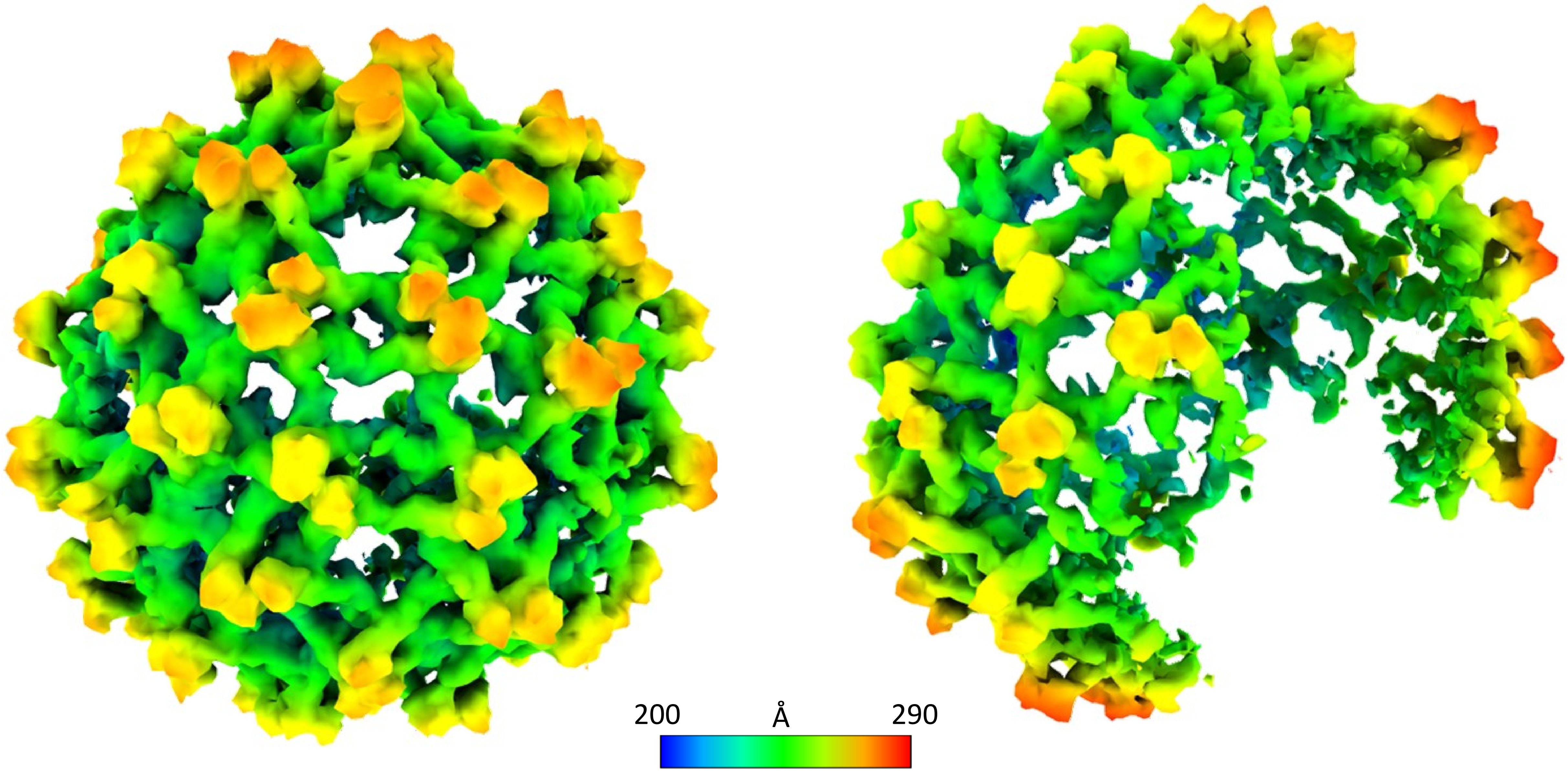
- Positions of the sub-tomograms int the tomogram is known
- After averaging the sub-tomograms have refined orientations and shifts
- Placing back the refined volume into the original tomogram
- Describes better the data in tomogram than segmentation



Placeback of averaged sub-tomograms



Selected placeback particles



Conclusion

- Electron tomography
  - Advantages / disadvantages
  - Sample preparation
  - Data acquisition (limitations)
  - Data processing
- Electron tomography future
  - Sub-tomogram averaging *in situ*
  - Speeding up data acquisition
  - Automated data processing
  - Combination of tomography and SPA
  - Correlative microscopy (combination of fluorescent and electron microscopy)

Thanks for your attention!

#teamtomo