Image selection, Initial Model generation and Movie processing

Vinothkumar K.R.

MRC Laboratory of Molecular Biology

MRC | Laboratory of Molecular Biology

Overview

- Image selection
- *Ab-initio* 3d reconstruction procedures
- Movie processing

What is a good image?

The ideal image:

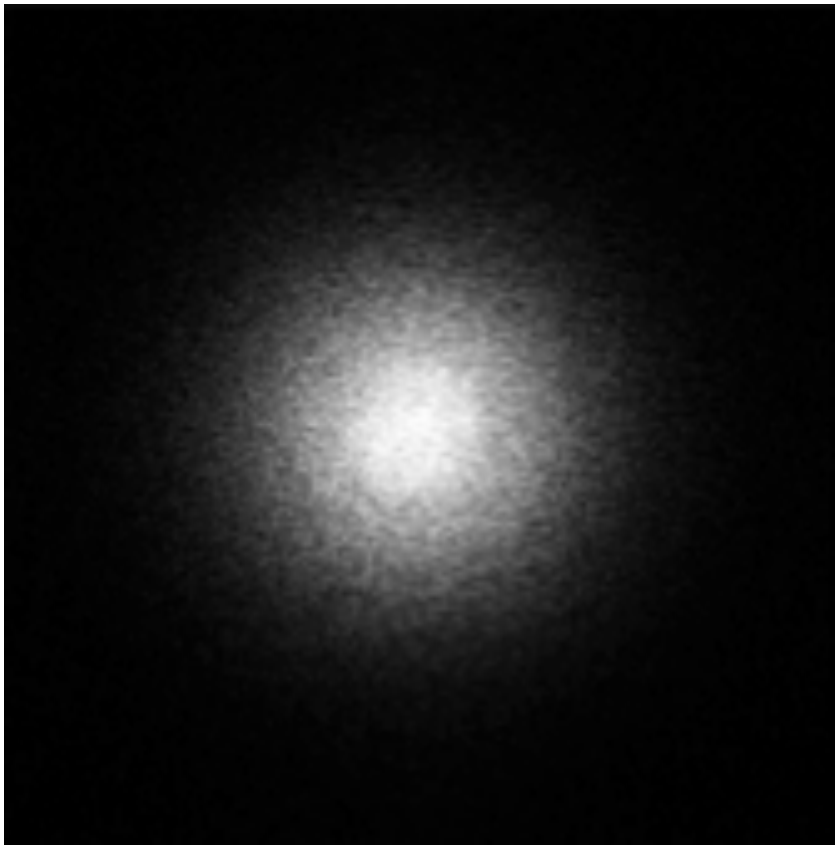
- high signal/noise ratio
- mono-disperse homogeneous particles evenly spaced
- random particle orientation
- power spectrum showing strong and isotropic Thon rings to the Nyquist limit

The real image:

- noisy images
- different levels of heterogeneity
- different levels of orientation bias
- low dose and weak specimen scattering attenuate the recovery of CTF modulation

Image selection

- And if I don’t see nice Thon rings?



- And if I don’t see nice Thon rings?

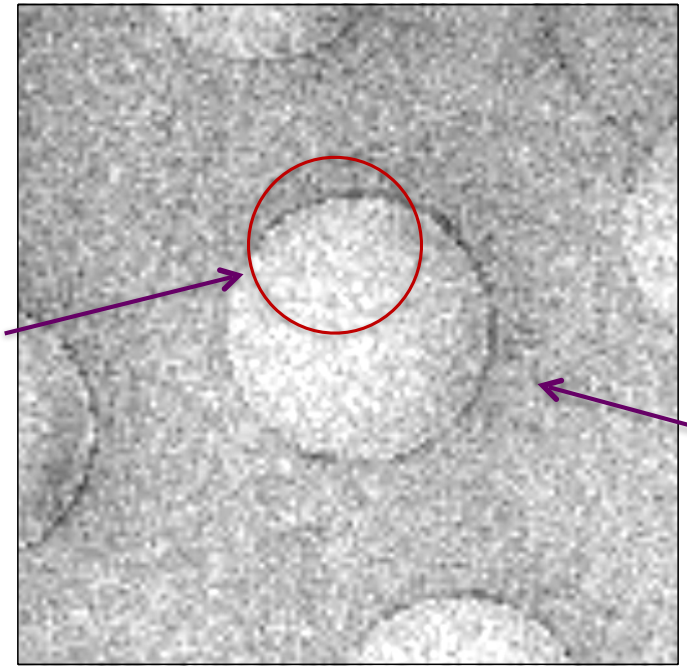
  - was the microscope working optimally and properly aligned?

Confirm the performance of the microscope at the time of data collection:

If you are imaging specimens that are weak electron scatterers (i.e. imaging smaller proteins in unsupported ice) this is the best way to have some confidence in the recovery of high resolution information

Image selection

- And if I don’t see nice Thon rings?



Imaging at the edges of holes including some of the supporting carbon is a way to increase electron scattering required for the visualisation of the Thon rings in the image power spectrum

Image selection

- And if I don’t see nice Thon rings?

Covering the holey film with a continuous layer of thin carbon will significantly increase the strength of the Thon rings in the power spectrum of the recorded images allowing a much easier evaluation.

This will somewhat increase the image background - but this is overcome by the advantages

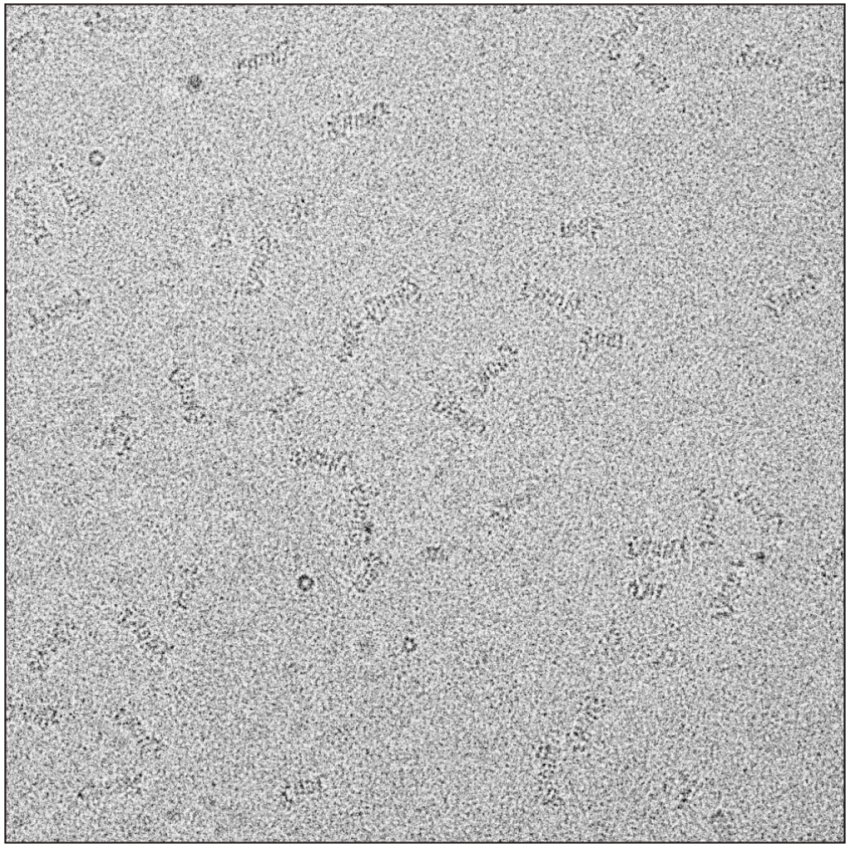
- it will also be suitable for samples with lower protein concentration - (~0.1-0.2 mg/ml protein is sufficient)

Image selection

- what to look for -

- Good distribution of target particles

optimise number of molecular images per frame, but avoid contact and/or overlap



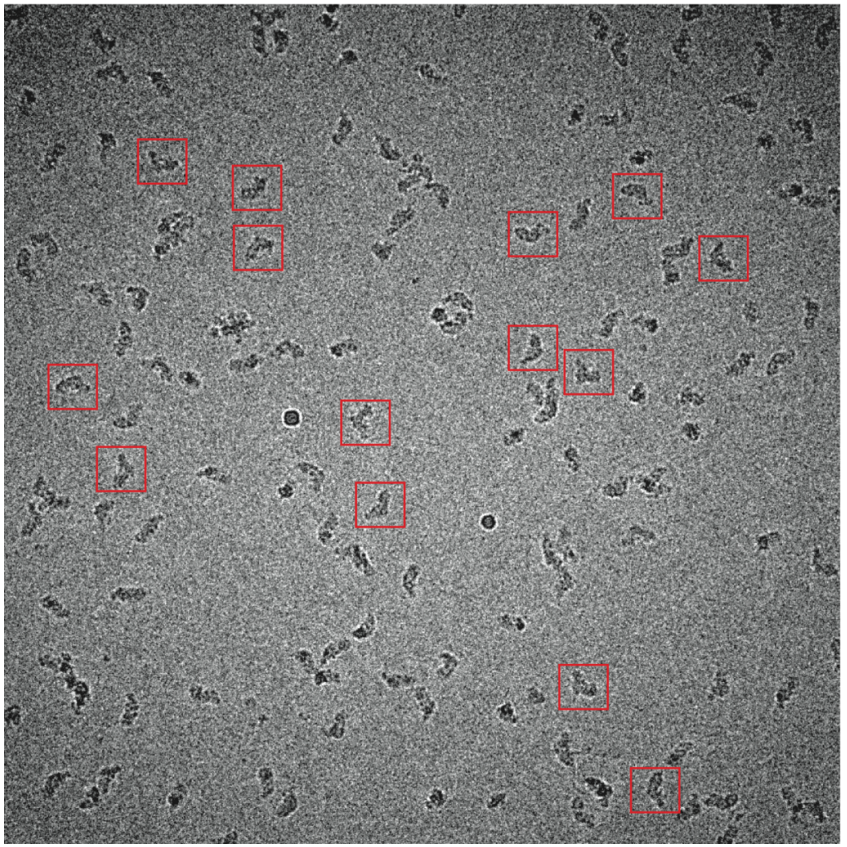


Image selection

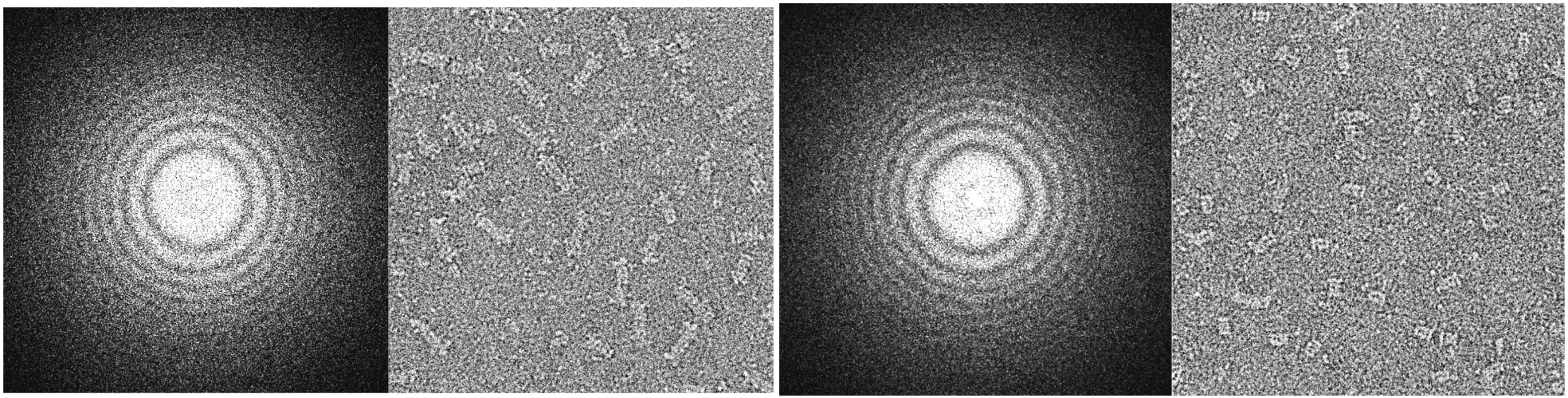
- what to look for -

- Optimal ice thickness:

as thin as possible

- but beware of possible exclusion of target particles when ice is too thin -

ice too thin



different regions of the same grid

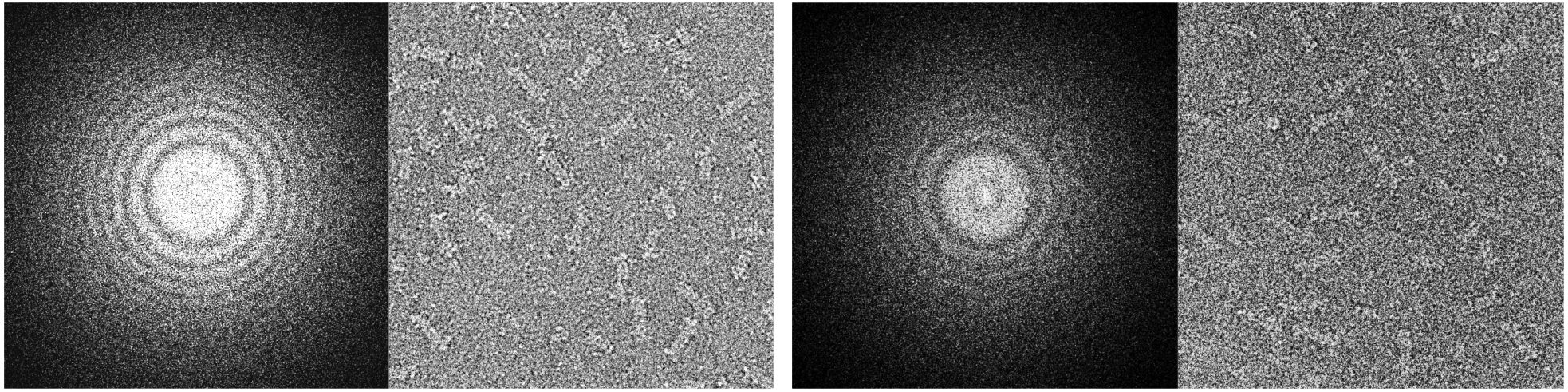
Image selection

- what to look for -

- Optimal ice thickness:

- even if molecular images can be identified in regions of thicker ice, the loss of high resolution information cannot be recovered during processing -

ice too thick



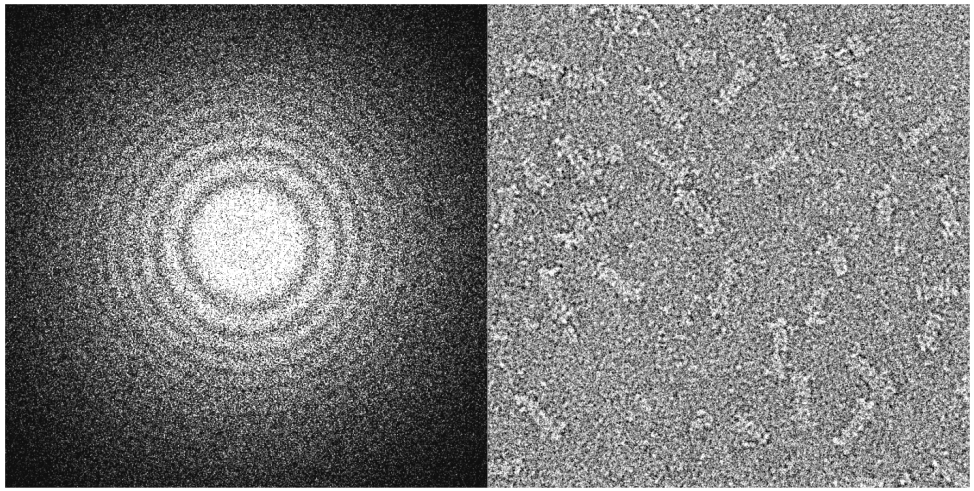
different regions of the same grid

Image selection

- what to look for -

- stability during exposure

  - minimise beam-induced motion/charging



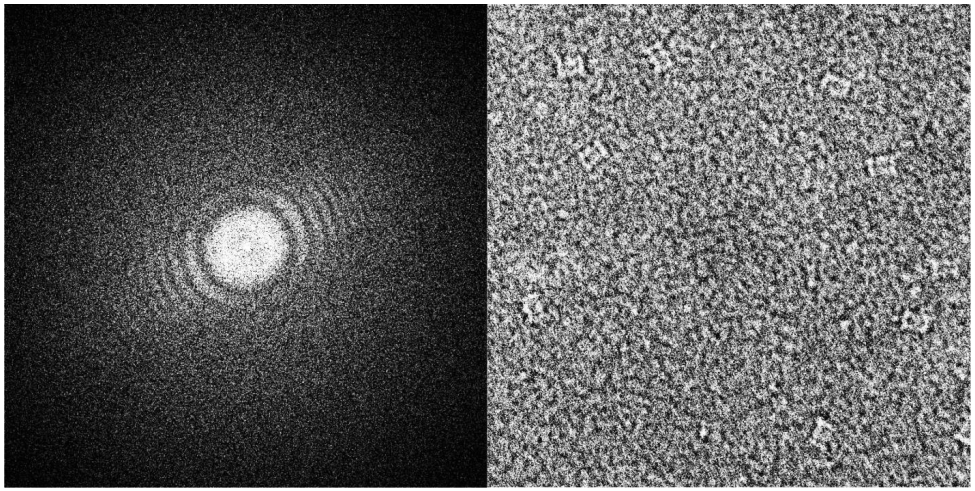
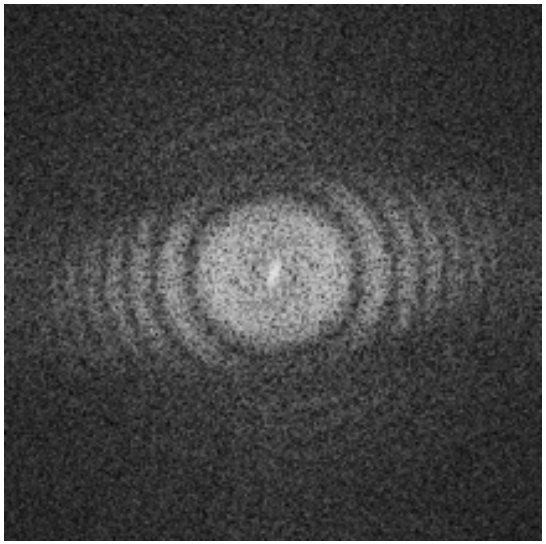


Image selection

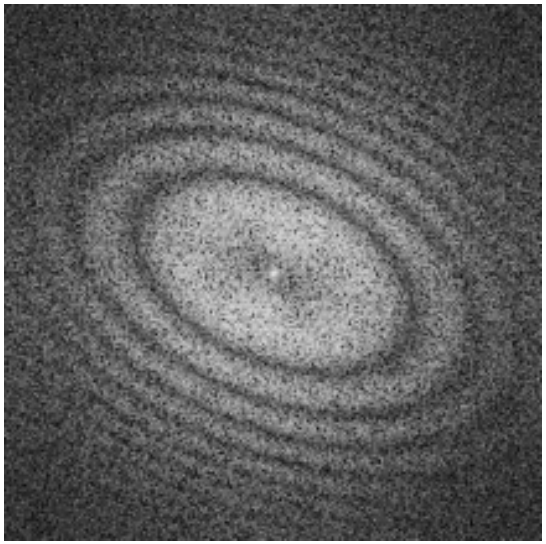
- what to look for -

- stability during exposure

- minimal drift/astigmatism



drift



astigmatism

••• minimise everything that may reduce the image contrast and/or the recovery of Thon rings on the image power spectra

Charging on specimen

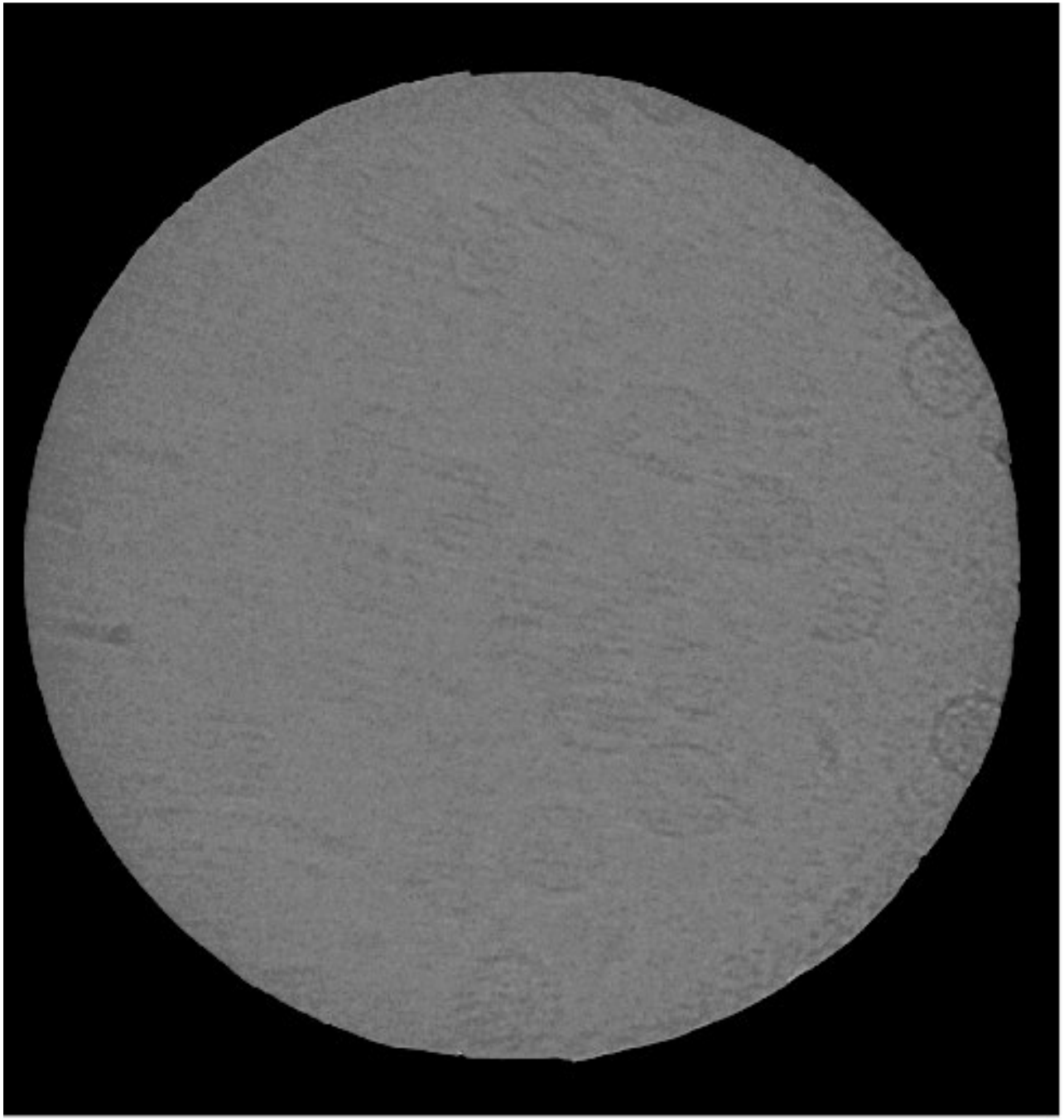
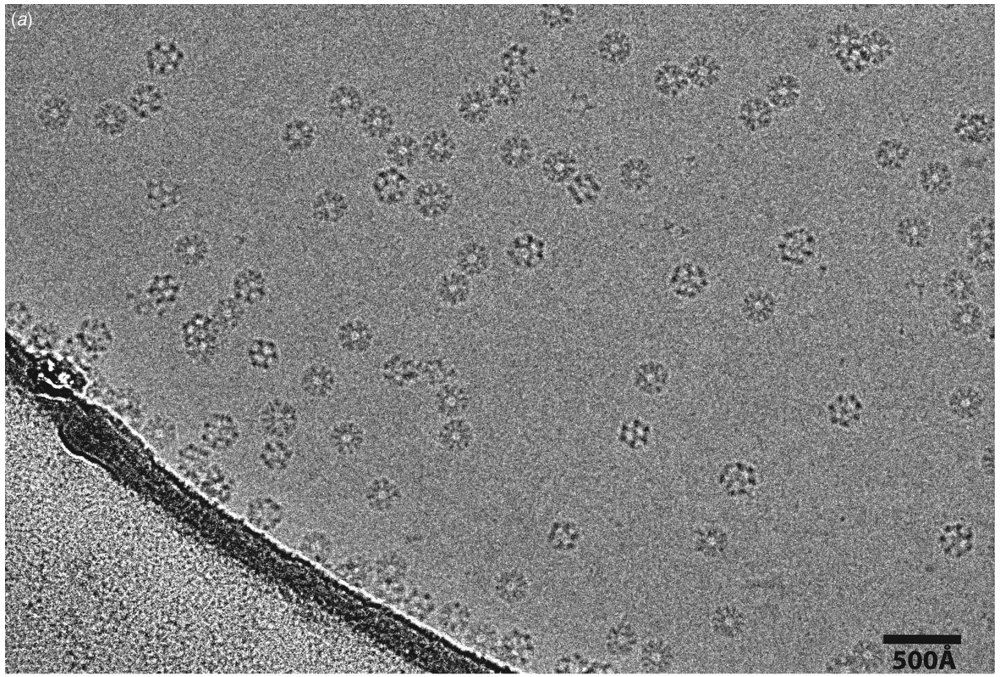


Image selection

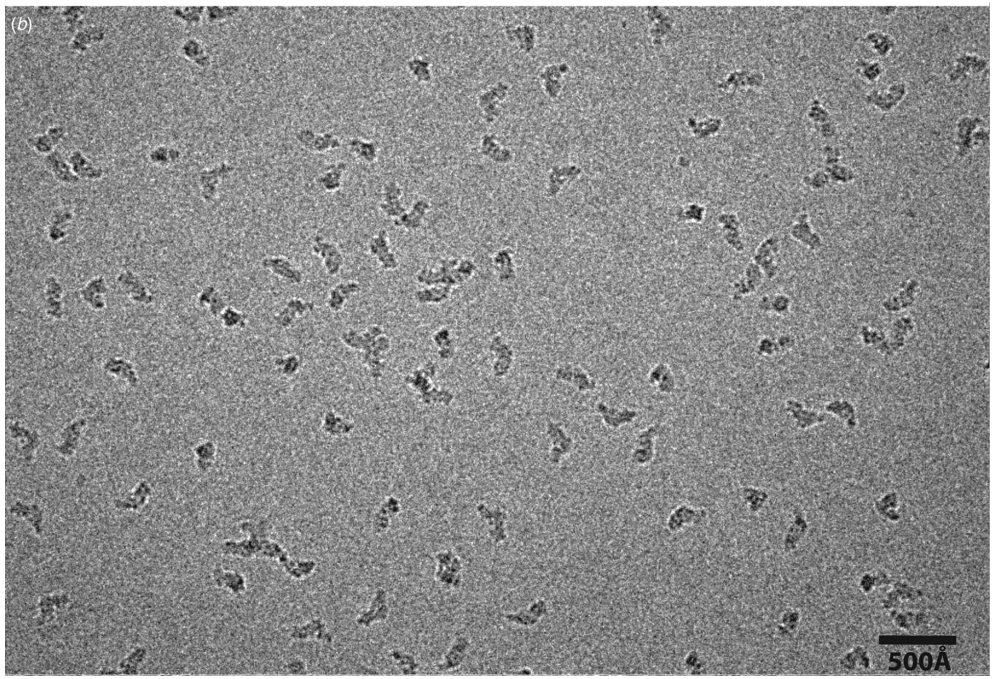
Select for analysis images where you can clearly identify your particles and where you are confident high resolution information is preserved

- It can be more effective to do image selection at the time of data collection:
  - if you only record in good areas, it saves time going over and excluding all the bad frames afterwards and if you only save the good images the amount of data you have to handle is much reduced
- If you take time to optimise your grids and if you have a grid where you know you have areas that reliably yield good images then you may chose to do automated data collection

Gallery of Micrographs of macromolecules

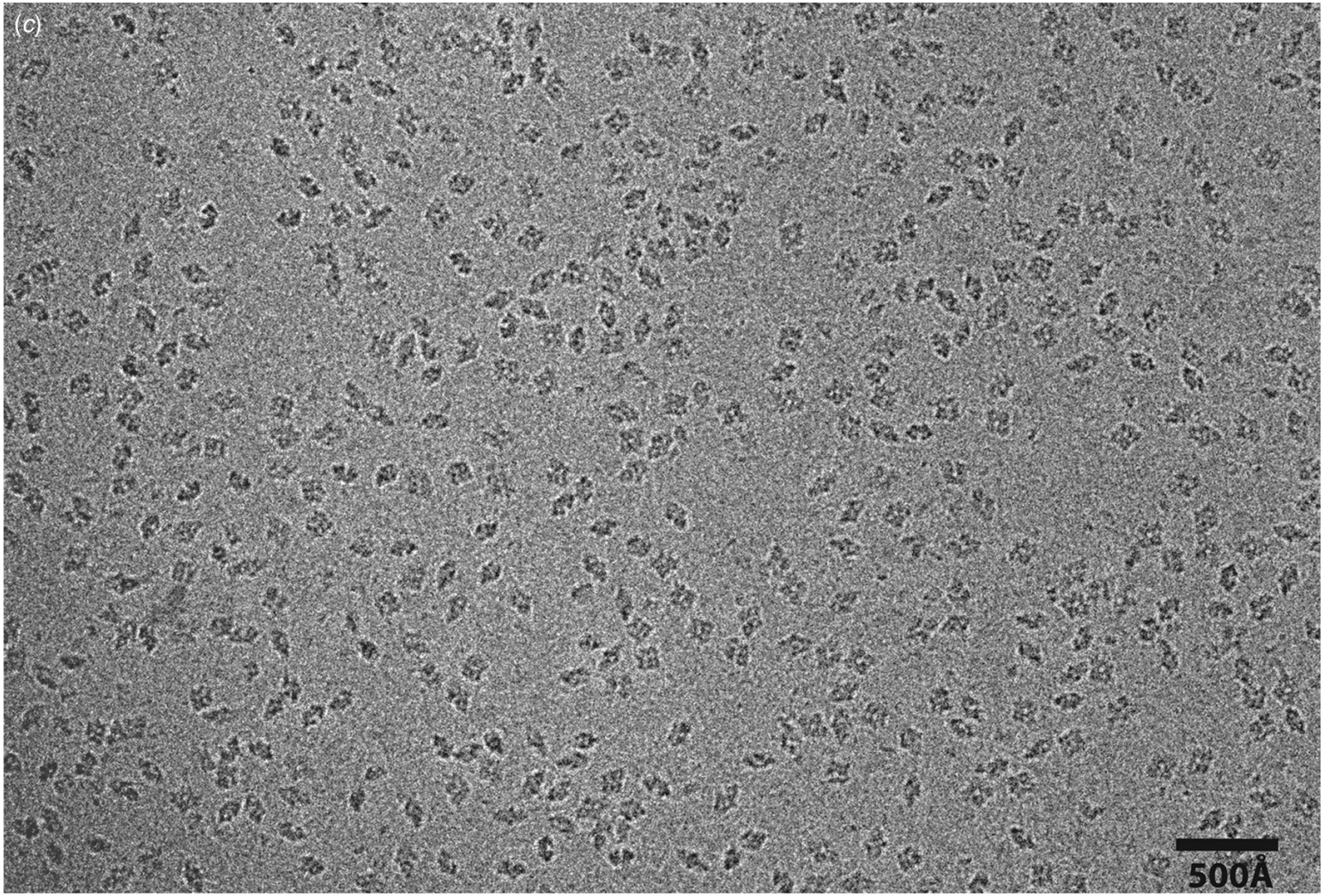


Pyruvate dehydrogenase, 1600 kDa



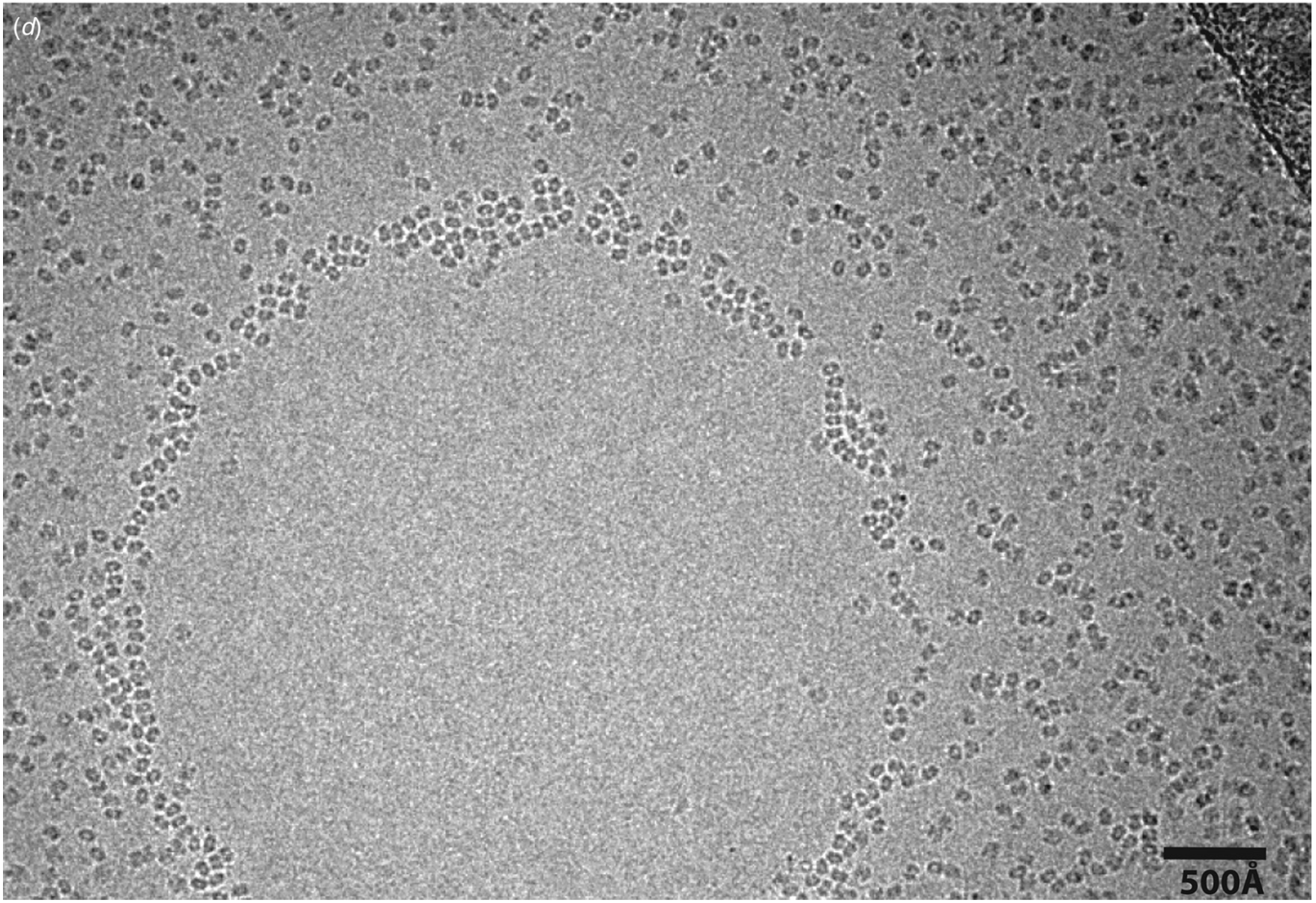
bovine Complex I, 900 kDa

Gallery of Micrographs of macromolecules



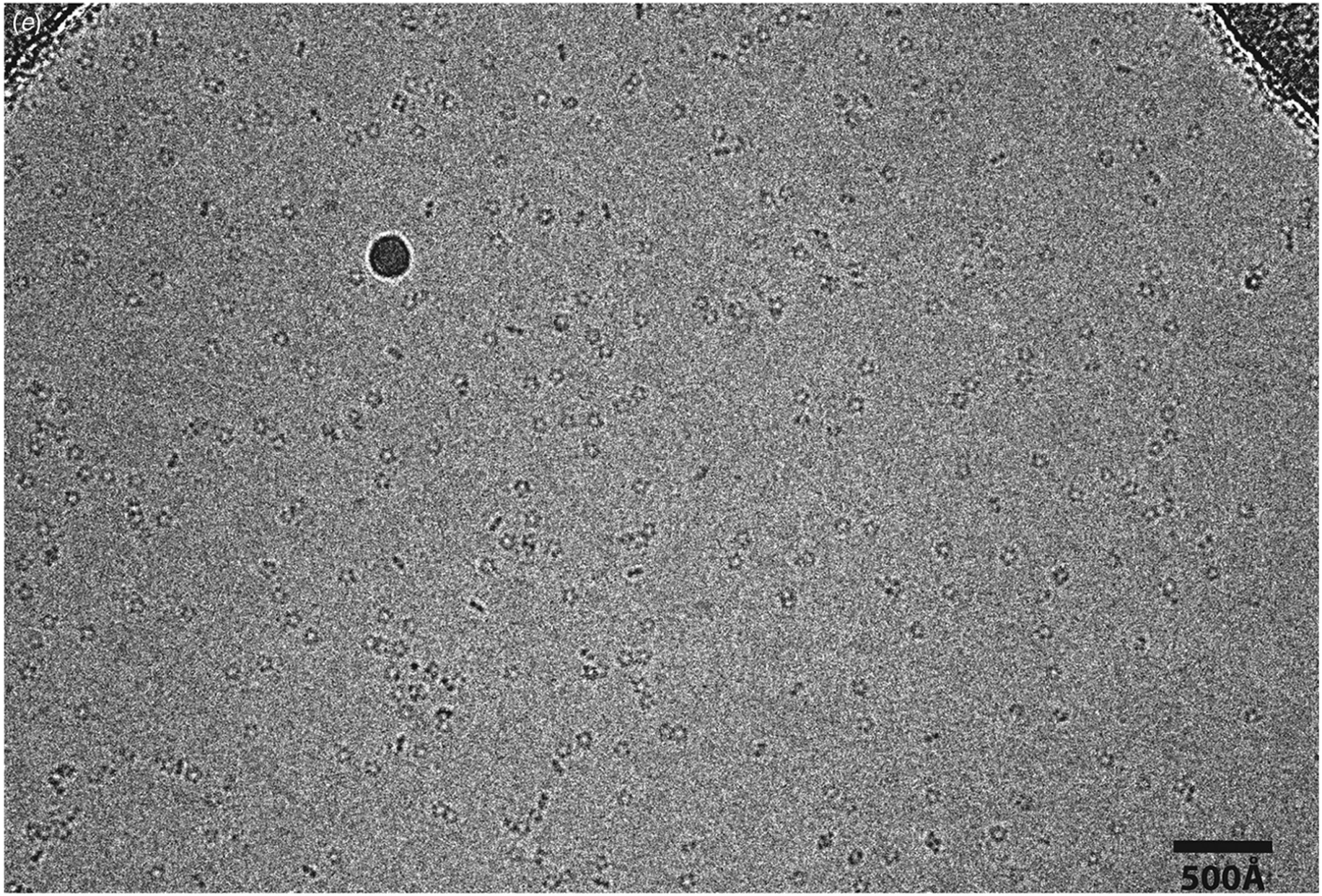
Beta galactosidase, 440 kDa

Gallery of Micrographs of macromolecules



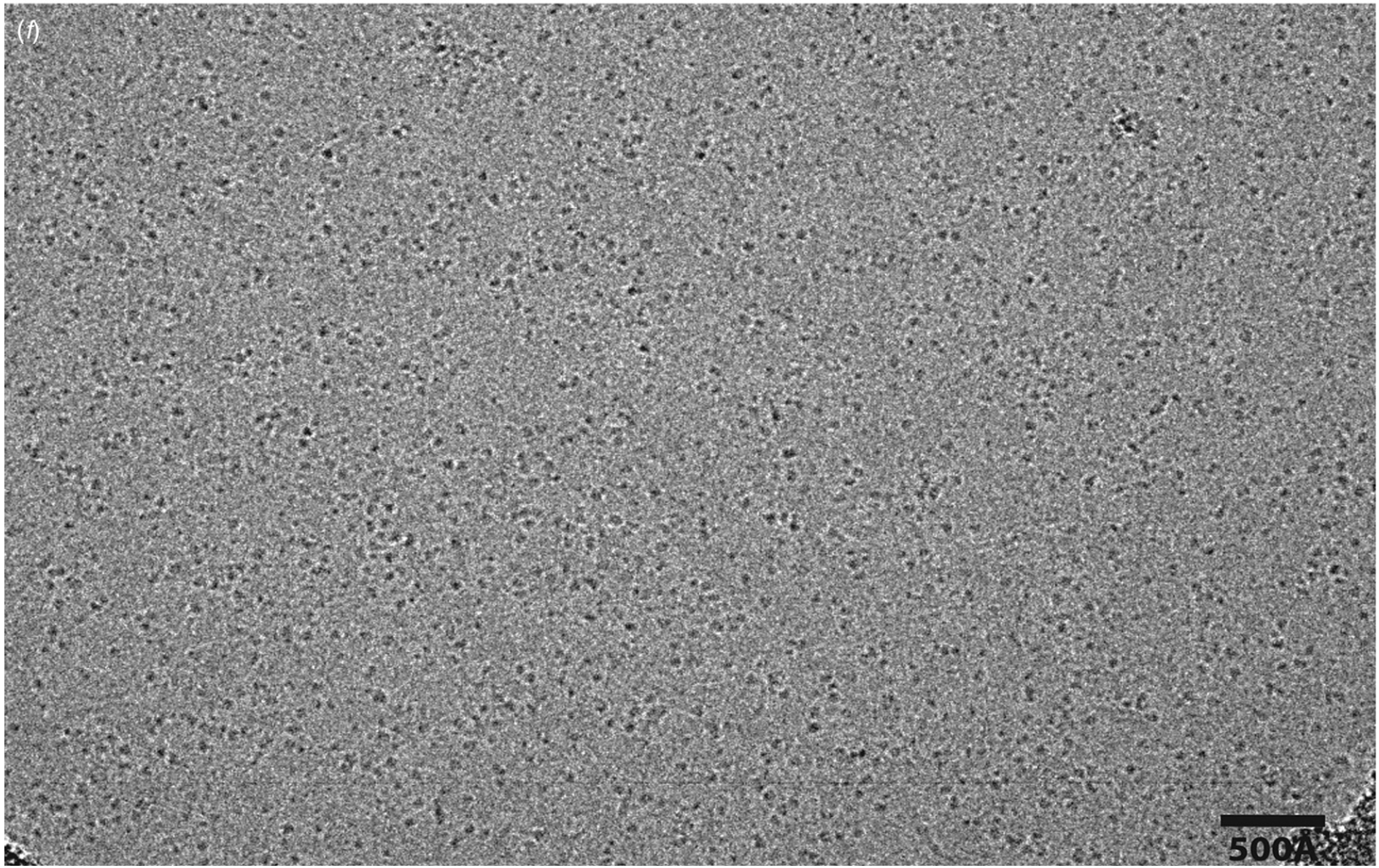
Catalase, 236 kDa

Gallery of Micrographs of macromolecules



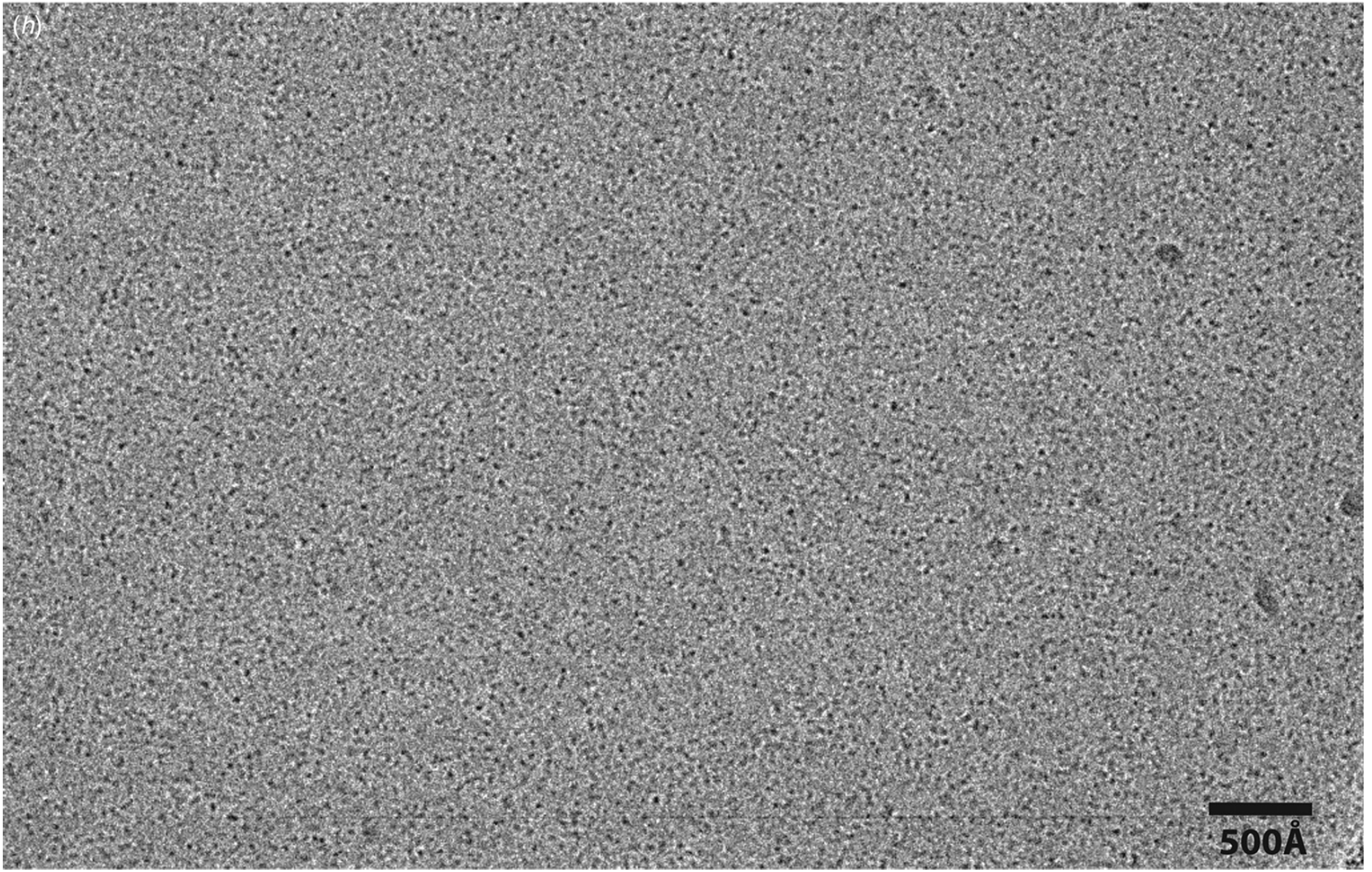
C-reactive protein, 125 kDa

Gallery of Micrographs of macromolecules



Haemoglobin, 64 kDa

Gallery of Micrographs of macromolecules



Lysozyme, 14 kDa

Number of particles in projection/μm^2 in 800 Å thick ice film (separation)

Concentration

M.W.	10mg/ml	2mg/ml	0.5mg/ml	0.1mg/ml	20μg/ml
10 kD	48000 (45Å)	10000 (100Å)	2500 (200Å)	500 (450 Å)	100 (1000 Å)
50 kD	10000 (100Å)	2000 (220Å)	500 (400Å)	100 (1000Å)	20 (0.2μm)
250kD	2000 (220Å)	400 (500 Å)	100 (1000 Å)	20 (0.2μm)	4 (0.5μm)
1 MD	500 (400Å)	100 (1000Å)	25 (0.2μm)	5 (0.4μm)	1 (1μm)
5 MD	100 (1000Å)	20 (0.2μm)	5 (0.4μm)	1 (1μm)	0.2 (2.2μm)
25 MD	20 (0.2μm)	4 (0.5μm)	1 (1μm)	0.2 (2.2μm)	0.04 (5μm)

Initial Model generation

Ab initio 3D reconstruction

- Standard 3D single particle analysis consists of iterative image alignment and angular assignment with respect to a 3D map (typically that obtained in the previous iteration)

  - But where does this all start? Do we need a model at all?

  - Which initial reference 3d map should we use for the first iteration?

  - is one map sufficient or multiple maps required?

Ab initio 3D reconstruction

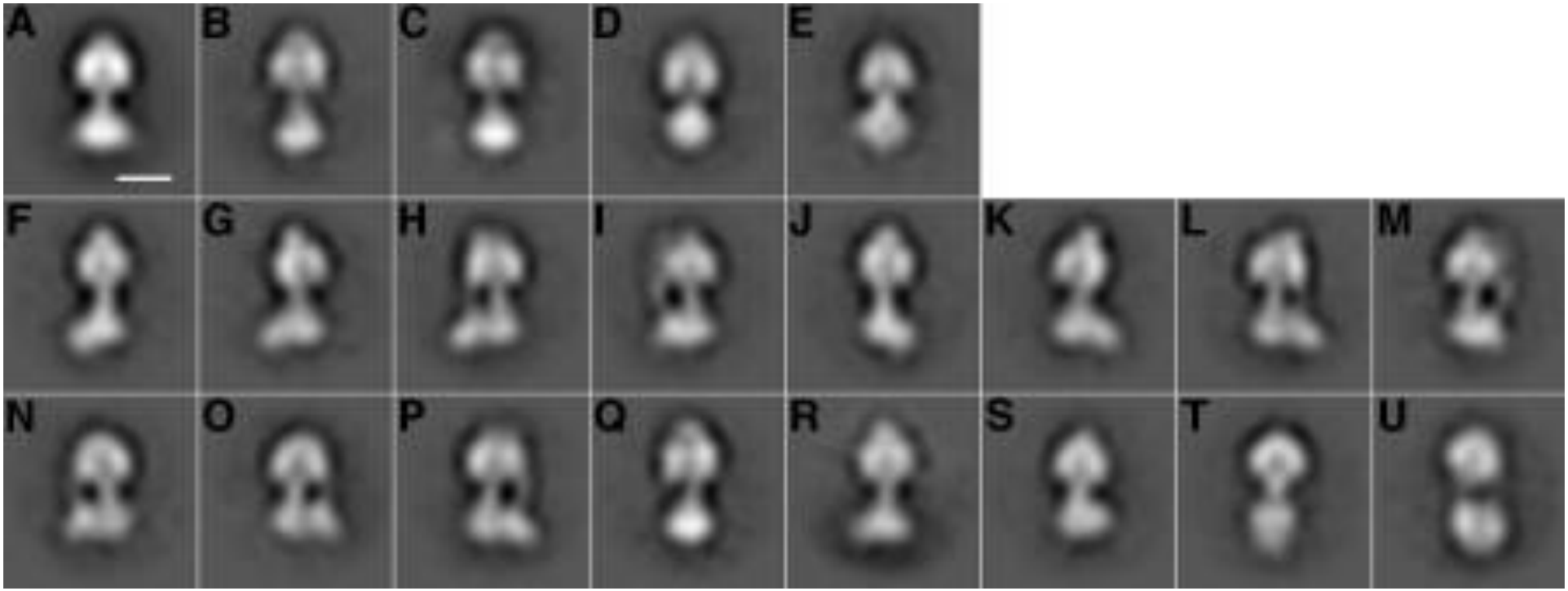
Possible sources of a starting model

1. You may be lucky enough to already have an accurate 3D structure closely related to your sample (eg the ribosome)

2. Otherwise you will need to calculate a first 3D map from your (2D) molecular images or other means

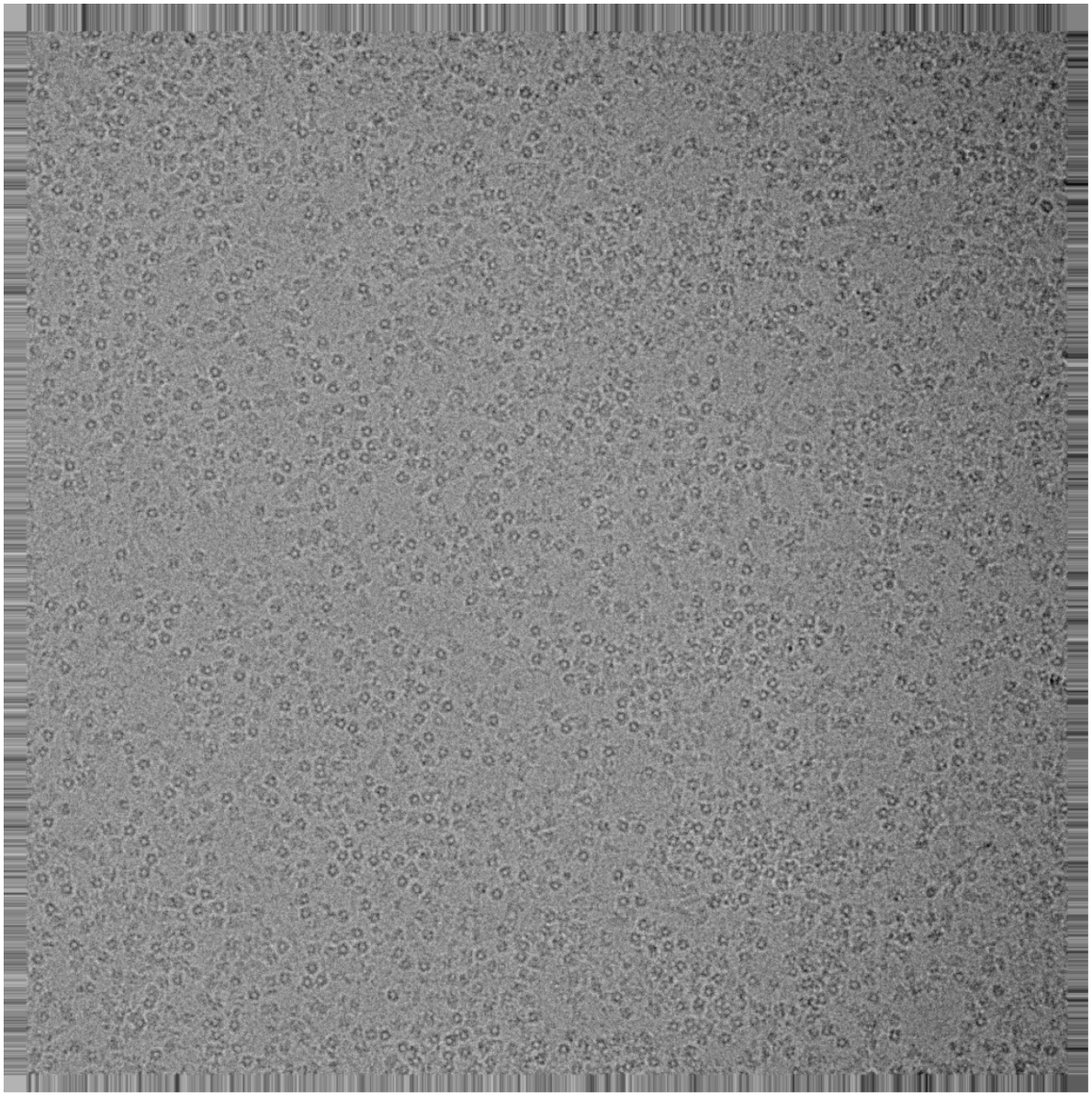
3. It is possible to use a PDB model from X-ray but needs to be correctly filtered to eliminate model bias

2D classes averages - useful tool to access your data

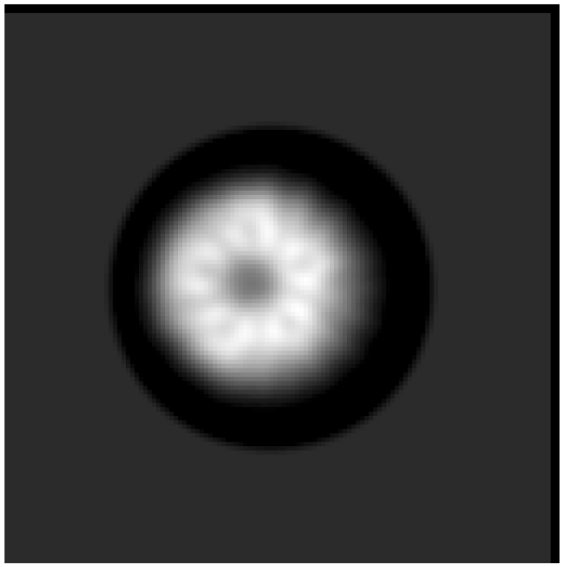


Rubinstein et al 2012

AChR - molecule with preferred orientation

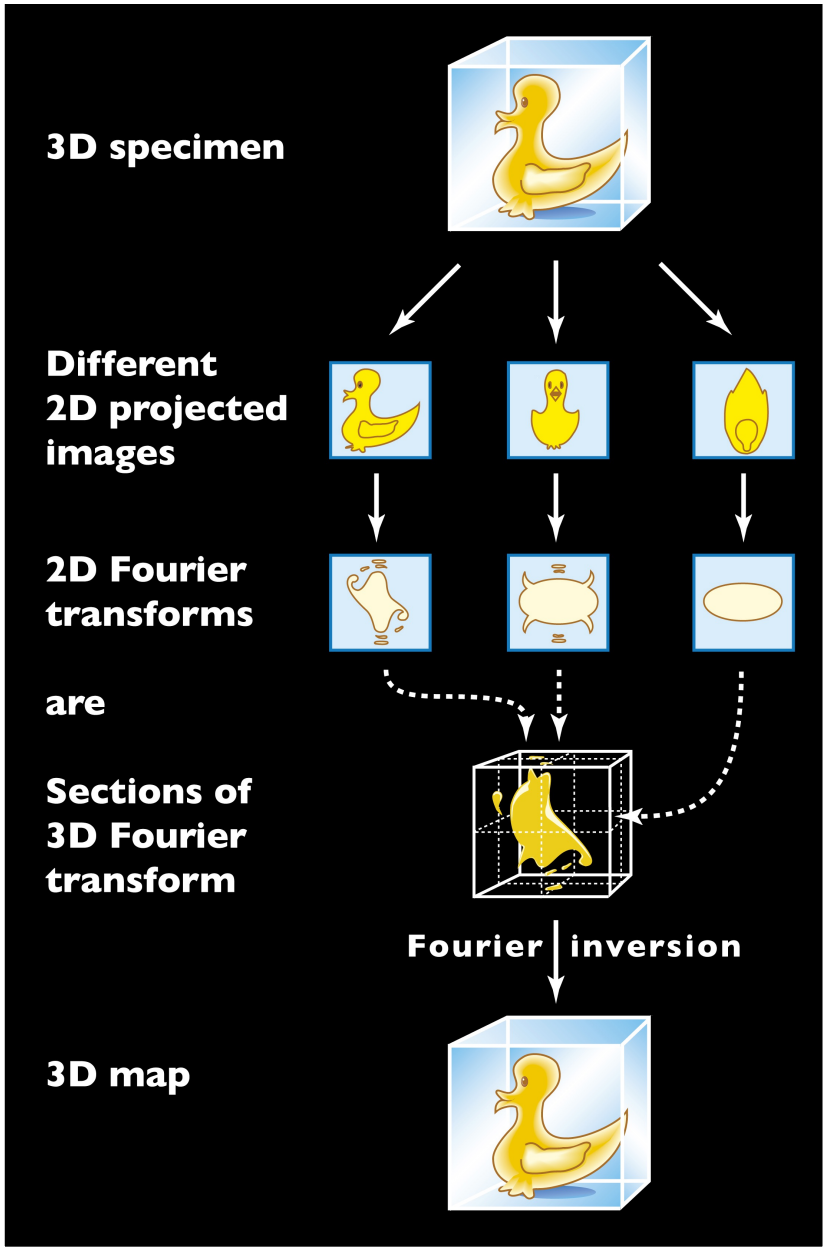


Micrograph



Class average

Ab initio 3D reconstruction



The actual reconstruction algorithms are based on the projection/section relation for object and transform

The challenge is to determine the angular relation of the 2D projected images to the 3D specimen

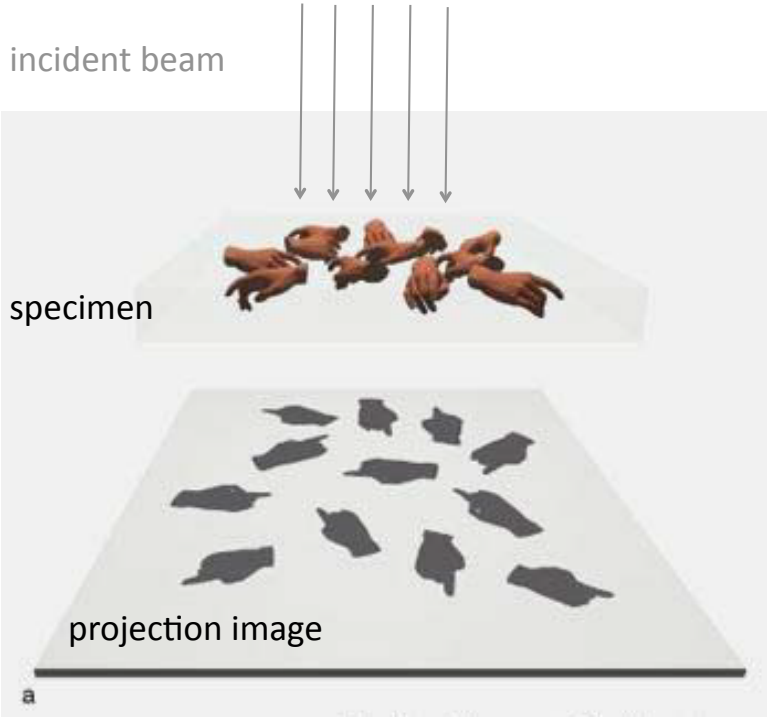
Ab initio 3D reconstruction

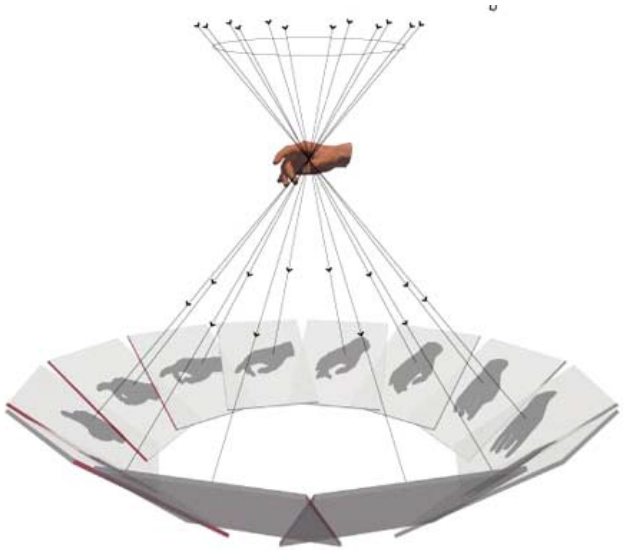
Caution: you can always get a 3D volume from your data

Methods - initial model generation

- Random conical tilt
- Orthogonal tilt (a variant of Random conical tilt)
- Tomography
- Common lines - fourier space
  - real space
- PRIME - Probabilistic Initial 3D model generation

Random conical tilt



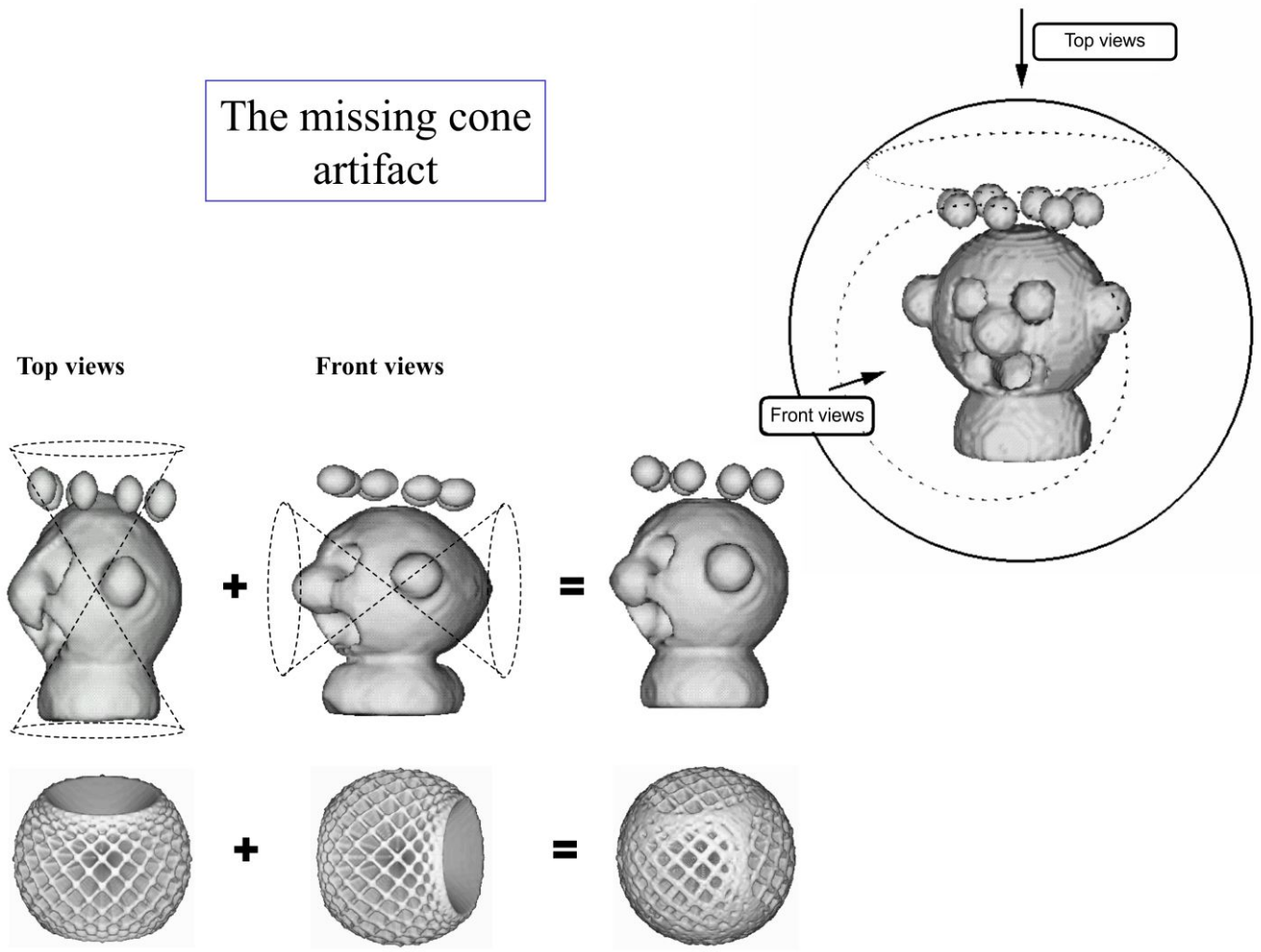


- Projections of particles related only by in-plane rotations are identical, but if the specimen is tilted the projections will correspond to different views of the specimen.
- Two images are recorded at first a defined tilt angle followed by another at 0 tilt.
- In-plane rotation angles between different projections can de determined by the alignment of the un-tilted specimen projections , which combined with the known tilt angle, defines the relative orientation of the corresponding tilt projections

Radermacher M. (1988) . *Electron Microsc. Tech. 9, 359*

Random conical tilt

- A 3D volume can be calculated from the particles in the tilted image



Slide from Nicolas Boisset

A typical procedure in Random conical tilt (RCT) image processing

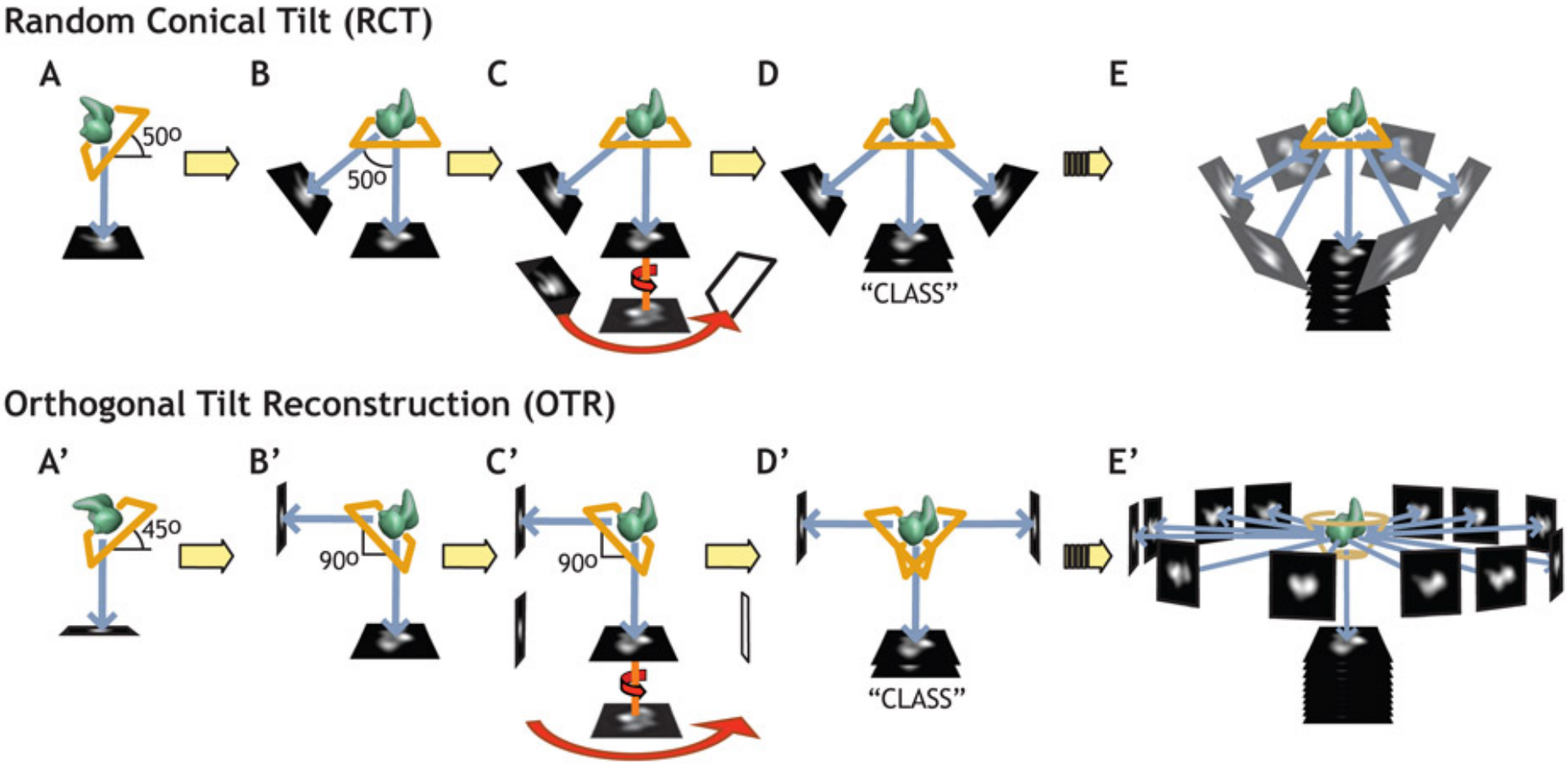
1. Pick tilt pairs - particle coordinates, TiltAxis in both images, tilt angle
2. Particles are boxed out, rotated such that the direction of the tilt axis coincides with the Y-axis in each image and contrast normalised
3. Classification (2D) of untilted particles
4. Centering of tilted particles, by aligning with the corresponding centered untilted particles
5. 3D reconstruction using only centered tilted particles (SHX, SHY=0)

   PHI = in-plane rotation (from 3)
   THETA = Tilt angle (from 1)
   PSI = 0 [due to rotation in (2)]

Random conical tilt

- Works better with samples with biased orientations on the grid
- Technically very demanding
- It is the only ab-initio reconstruction procedure that may give the correct handedness of the calculated structure

Orthogonal tilt

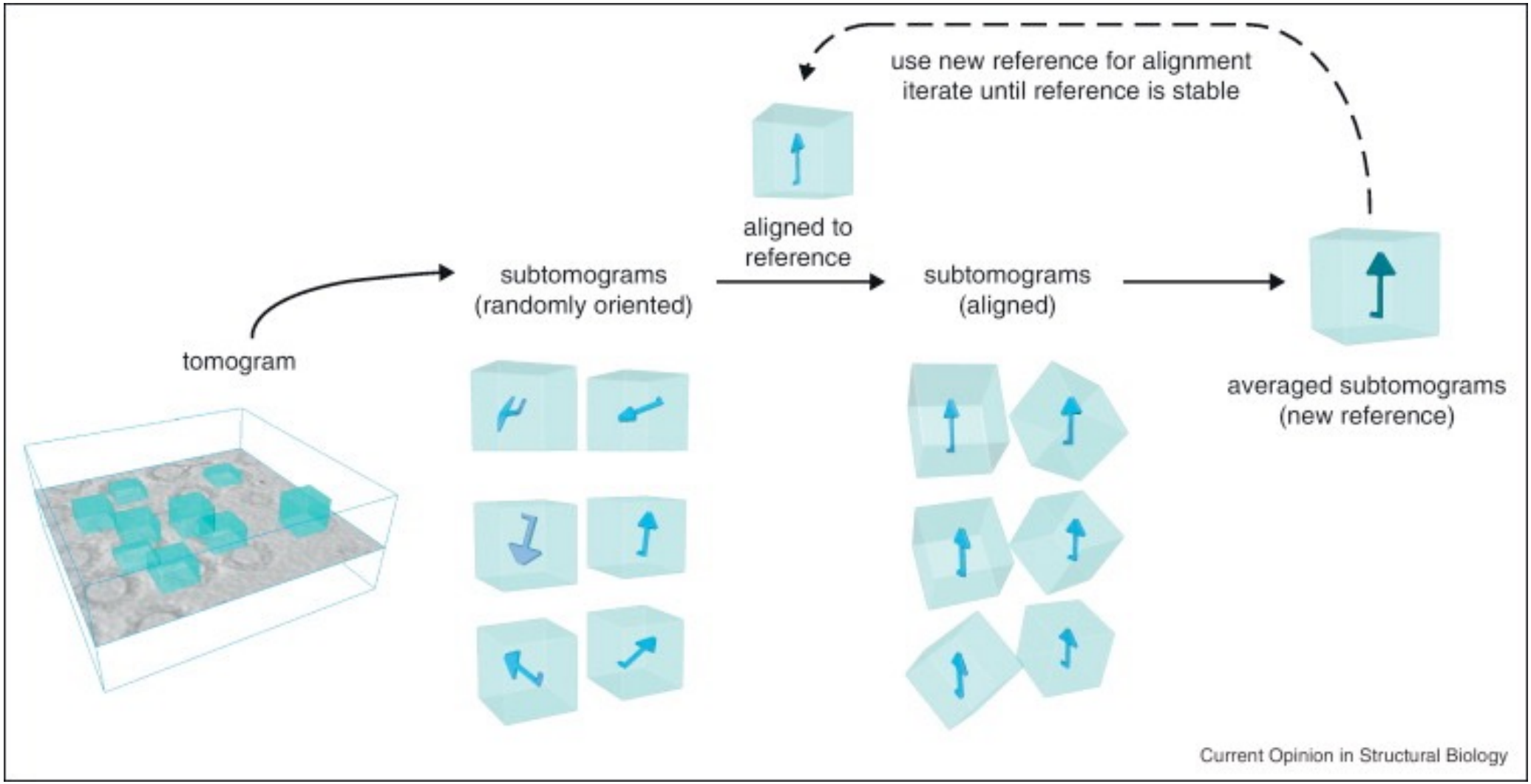


Leschziner A, 2010

Orthogonal tilt

- needs samples with all possible orientations on the grid
- Technically very demanding
- In OTR (orthogonal tilt reconstruction), the images used for alignment and classification comes from one of the tilted image.

Sub-tomogram averaging

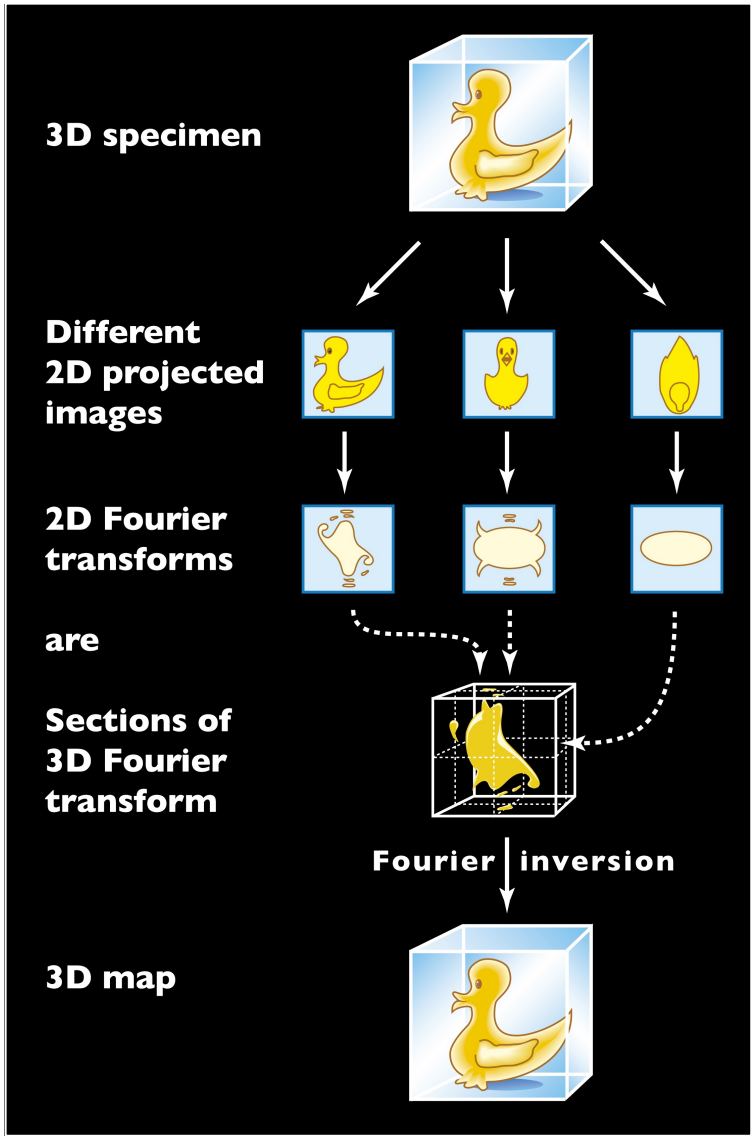


3D averaging of repeating features in tomograms.

Briggs, Curr Op Struct Biol, 2013

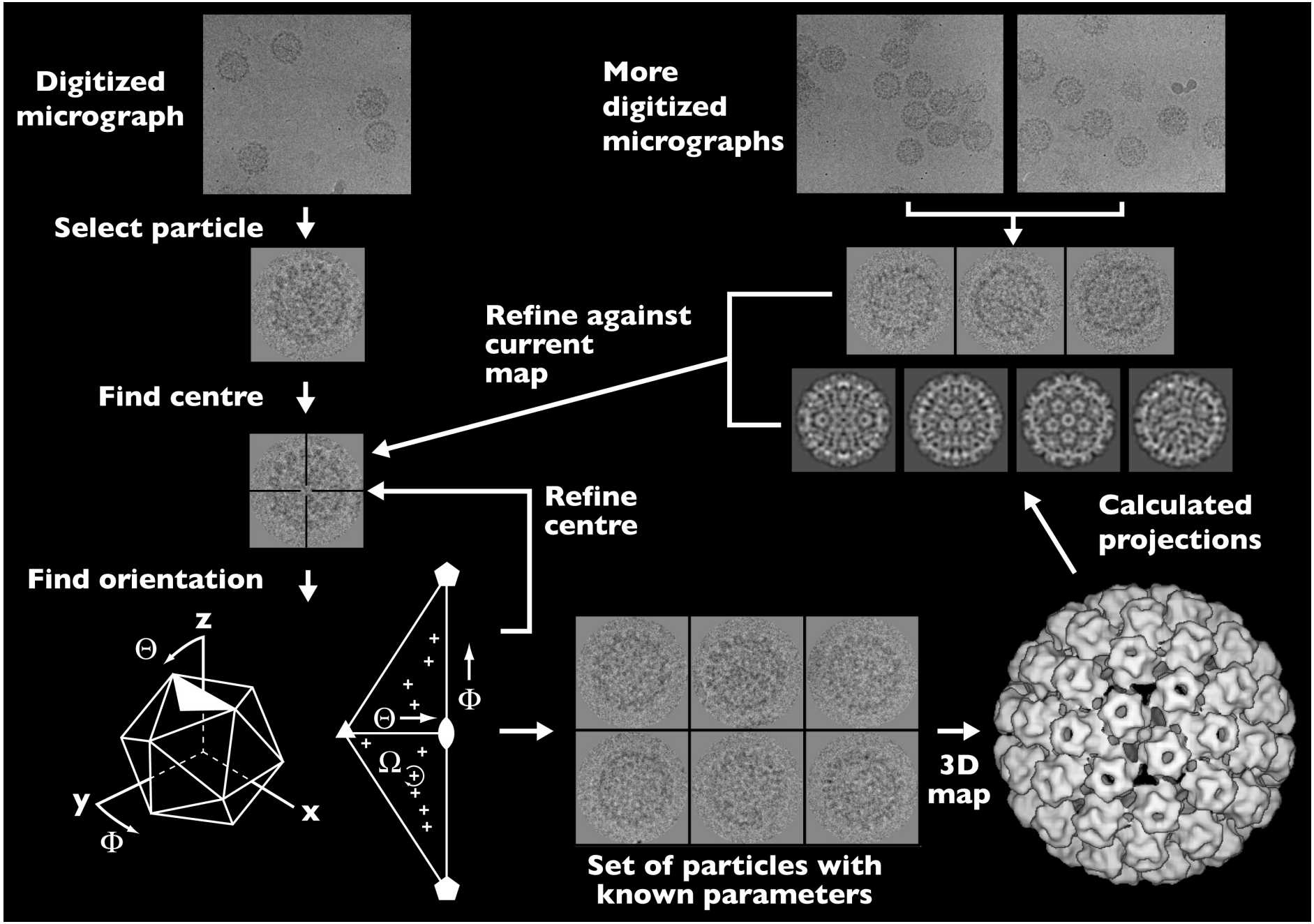
Ab initio 3D reconstruction - common lines

Projection slice theorem



The orientation of a particle can be determined by the existence of a set of pairs of 'common lines' in the 2D transform of any view of a symmetrical particle - Crowther 1971

Common line - fourier space

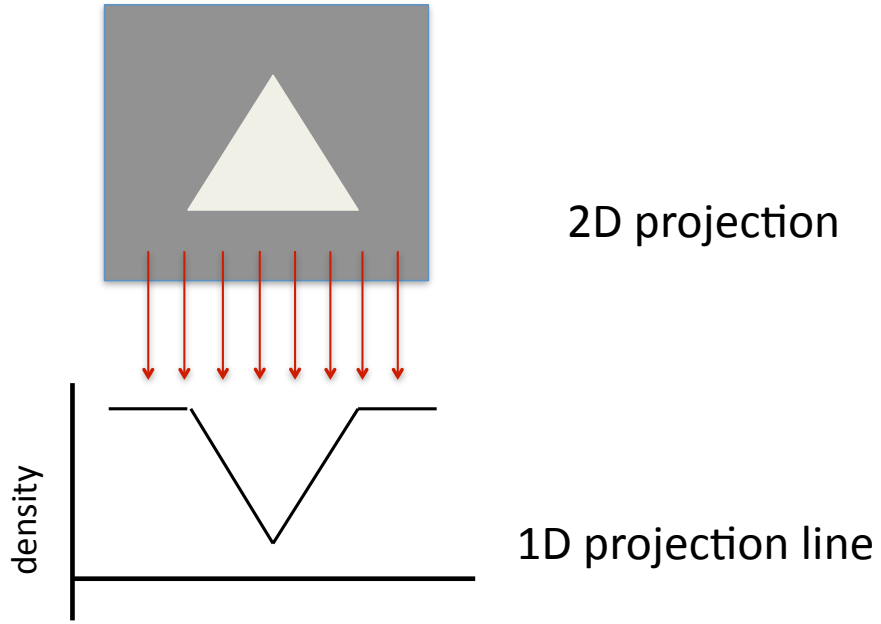


Crowther 1971

Common line - real space approach

Based on the “common-line projection” theorem:

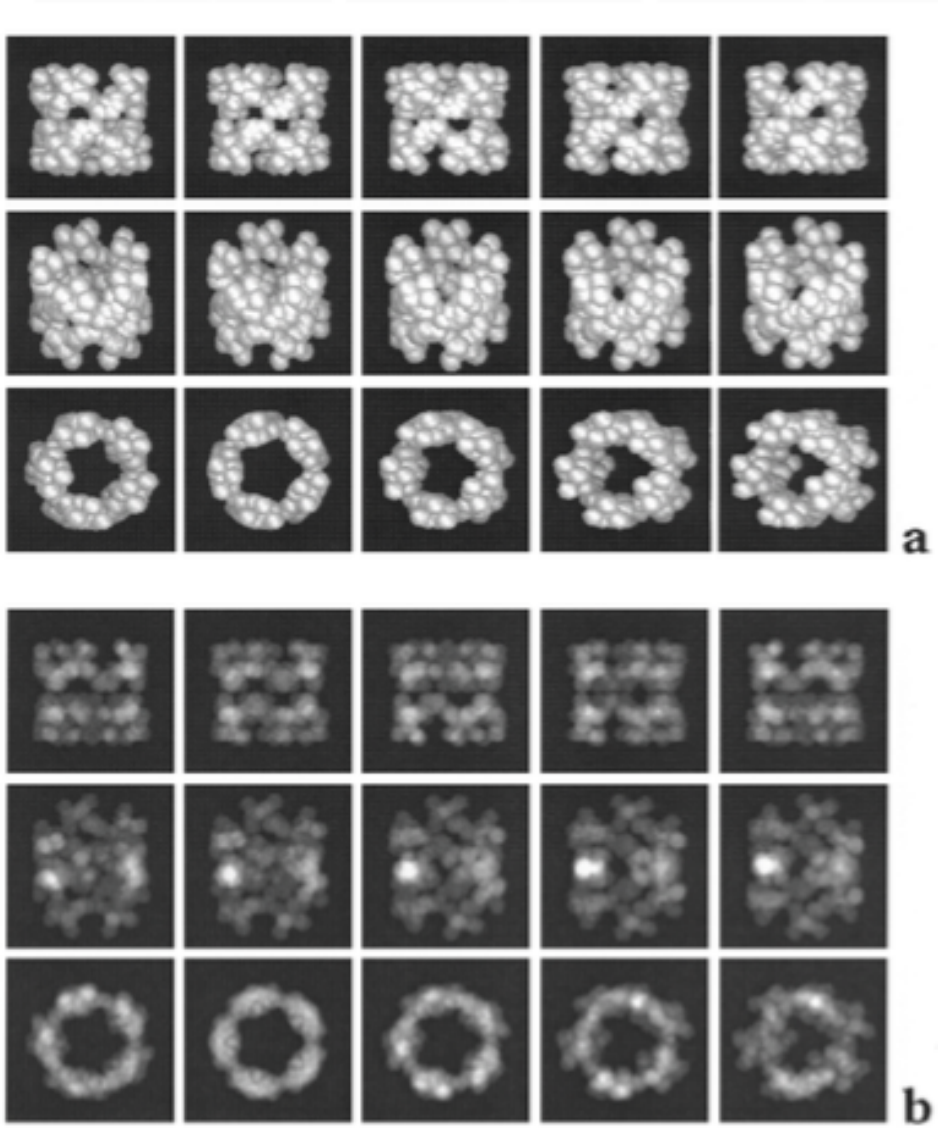
Any two 2D projections of a 3D object have at least one common 1D line projection



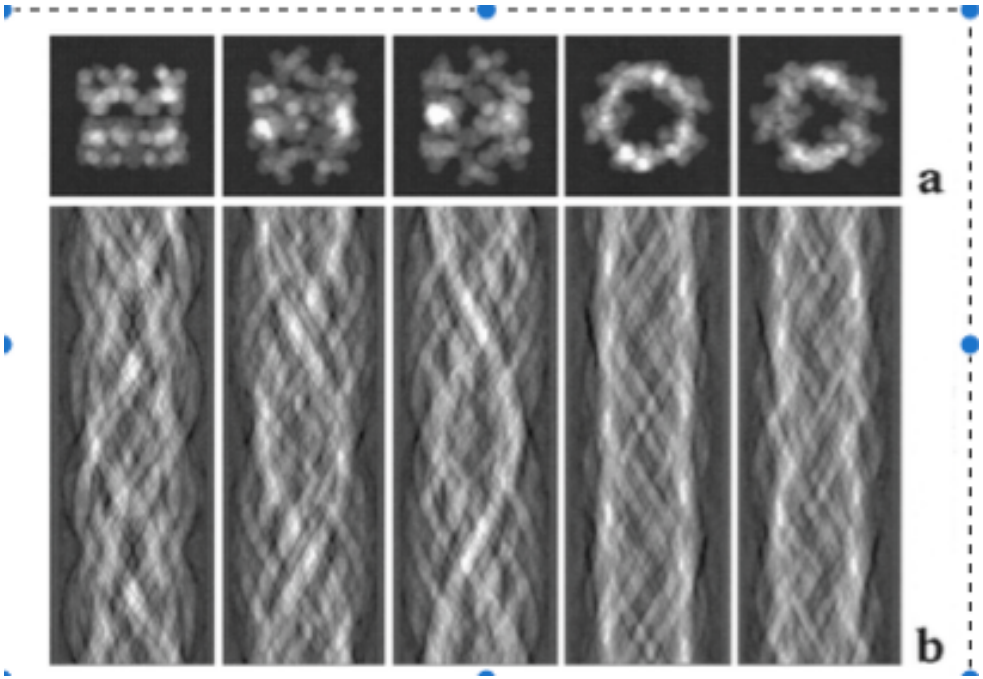
Sinogram/Radon transform: compilation of 1D projection lines of a 2D projection around a 360^{O} rotation

van Heel et al. (1987)

Angular reconstitution



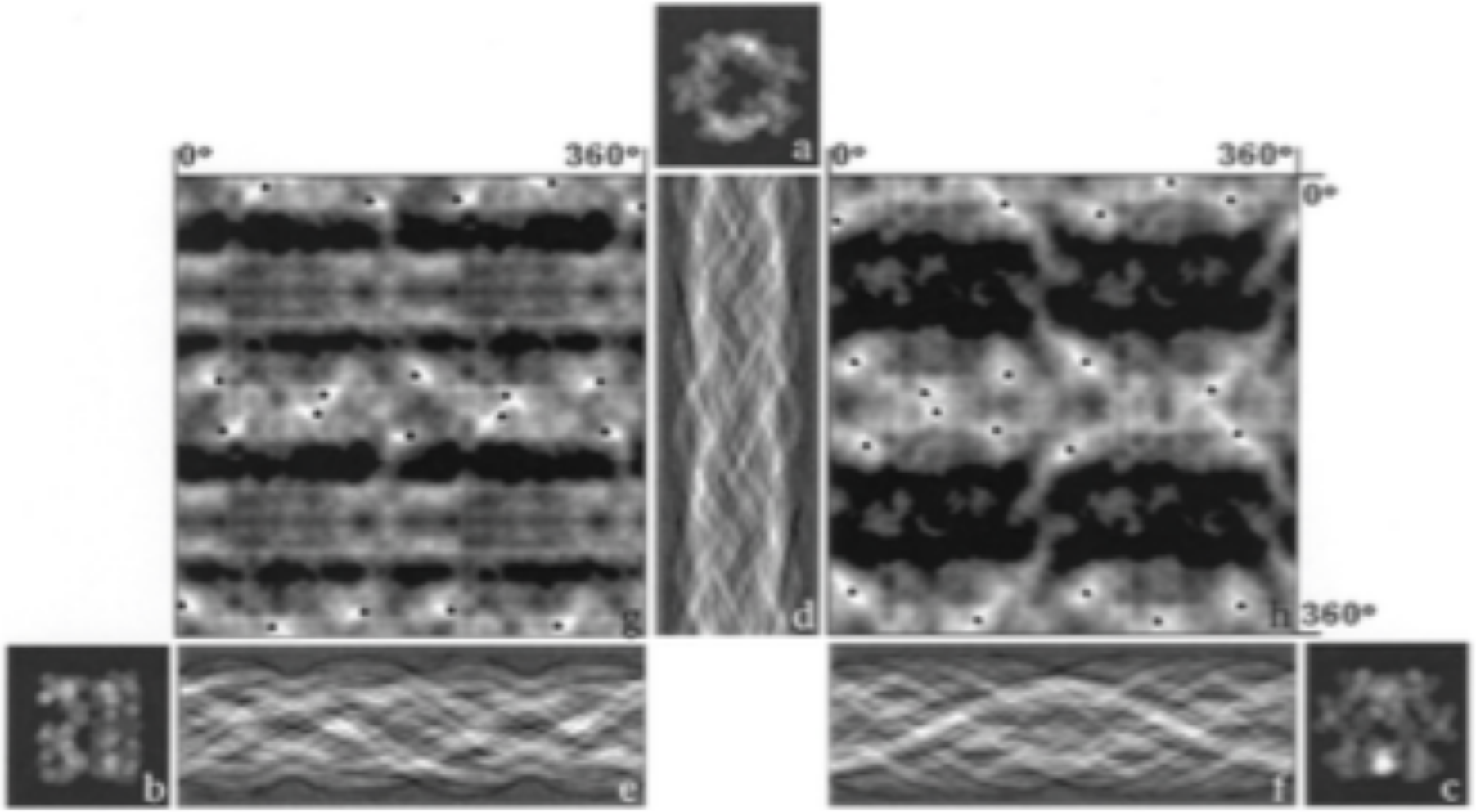
D5 symmetry



1D projection image - Sinogram

van Heel et al. (1997)

Angular reconstitution



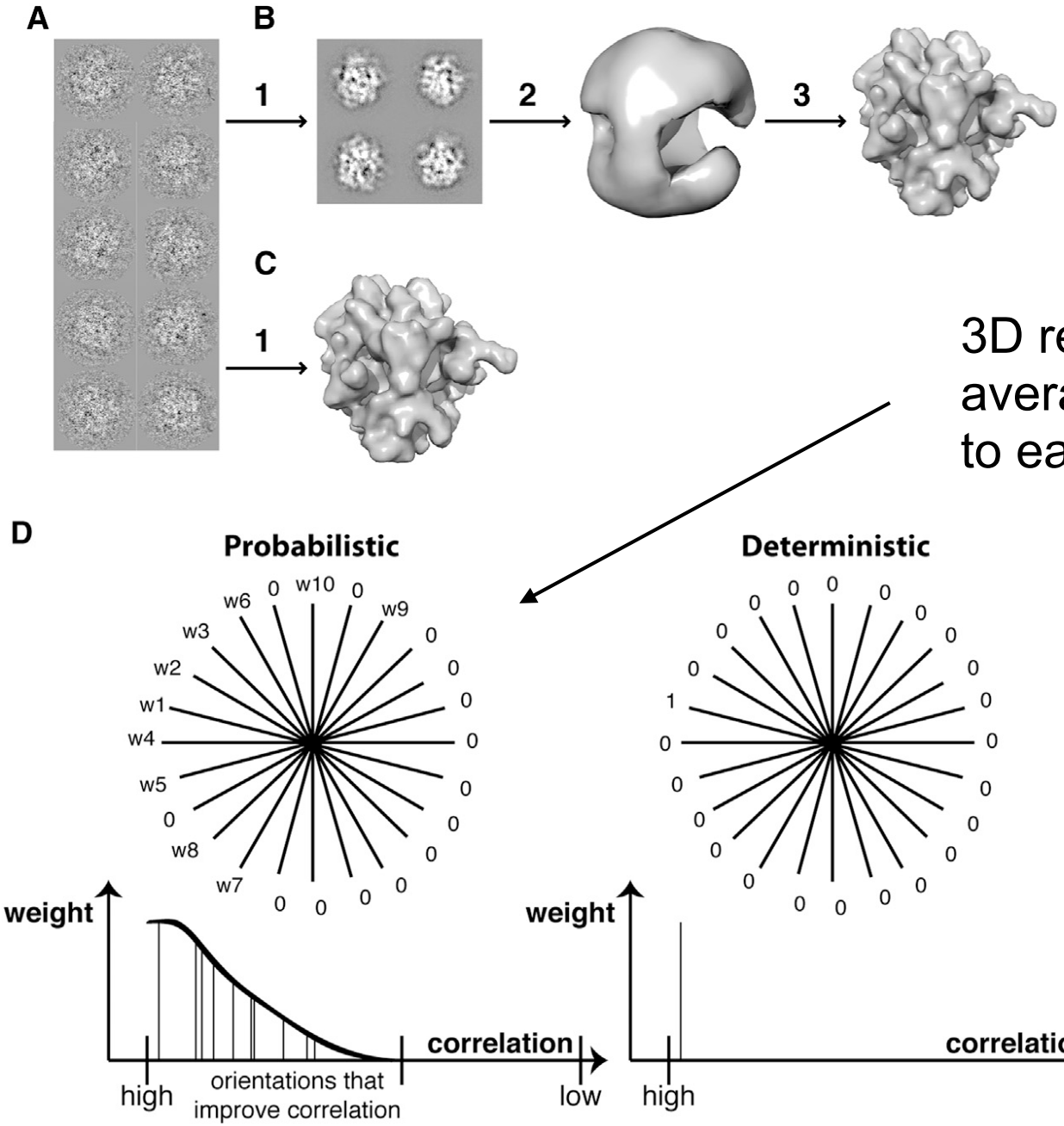
sinogram correlation function

van Heel et al. (1997), van Heel 1987

Common line - real space approach

- Requires high signal to noise ratio (good class averages)
- It requires a relatively large number of good classes representing distinct orientations of the specimen
- It requires the 2D classes to be on the same origin (not always achieved during the 2D classification)

PRIME
(Probabilistic Initial 3D model generation)



3D reconstruction is a weighted average of the images assigned to each orientation

Elmlund et al. (2013)

PRIME
(Probabilistic Initial 3D model generation)

$$S = (\{\psi_1, \theta_1, \varphi_1\}_1, \ldots, \{\psi_n, \theta_n, \varphi_n\}_n) \in \Omega$$

all possible orientations and all possible combinations of orientations for the *n* images using stochastic hill climbing

$$\lambda_i = \frac{\int_1^{n_{pix}} dj \left(X_j^{(i)} Y_j^{(k)} \right)}{\sqrt{\int_1^{n_{pix}} dj \left(X_j^{(i)} \right)^2 \int_1^{n_{pix}} dj \left(Y_j^{(k)} \right)^2}},$$

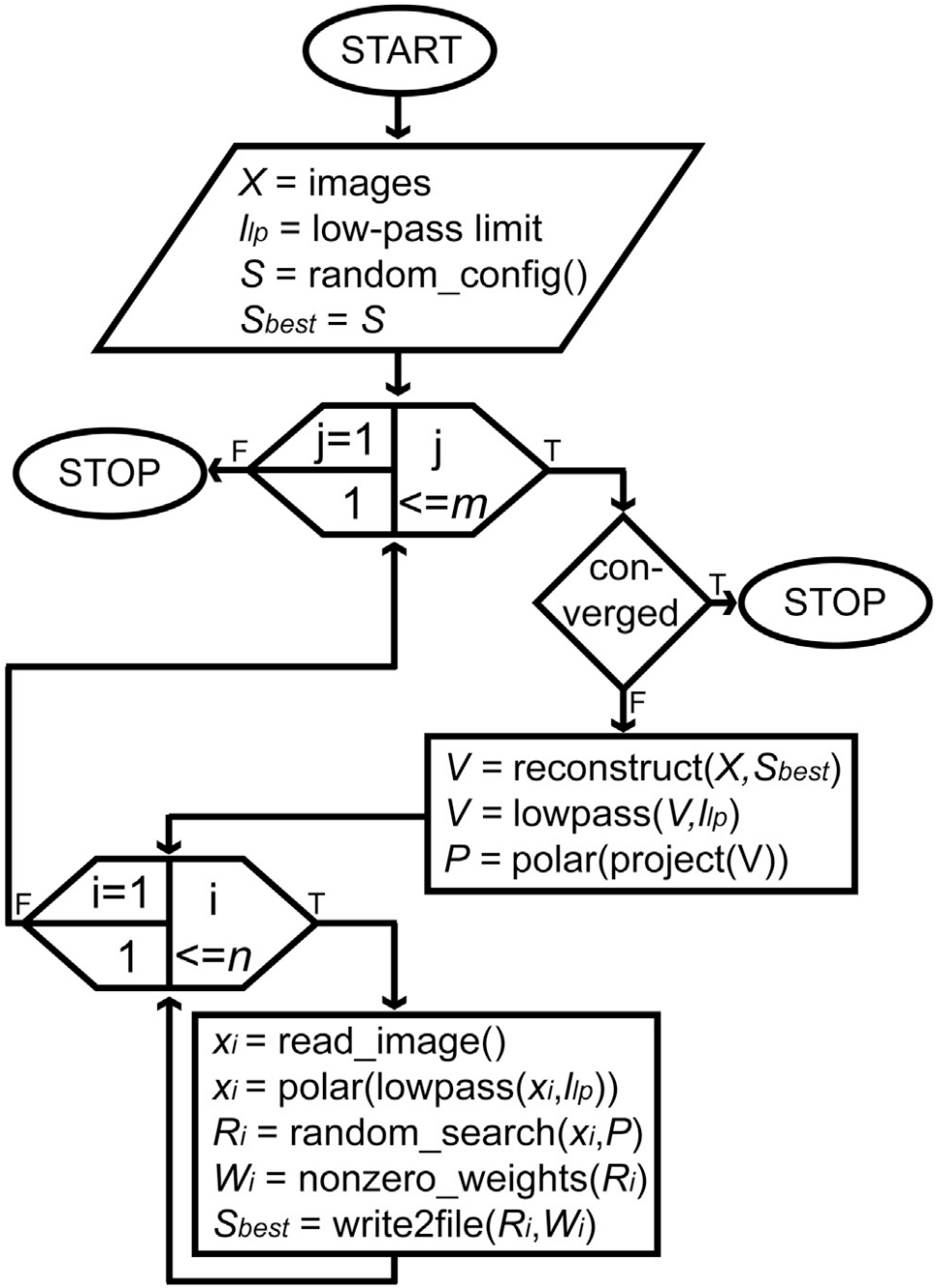
Individual score - correlation between the image (i) and projection of the reconstruction (k)

$$g = \int_1^n di \int_1^p dj \int_0^{2\pi} d\theta \lambda_{ij}^{\theta} w_{ij}^{\theta},$$

global score - weighted average of individual score (shown only for one rotation variable)

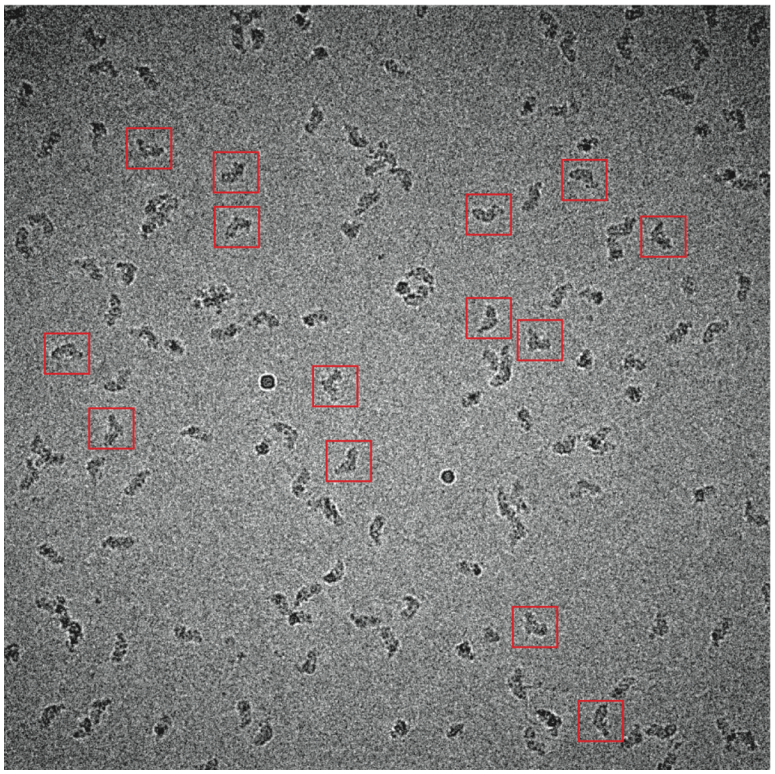
Elmlund et al. (2013)

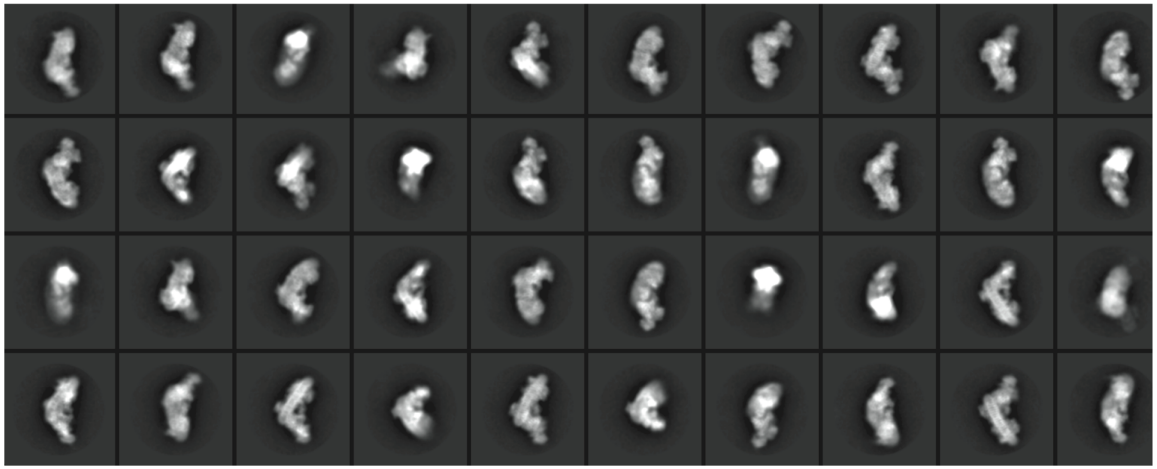
PRIME

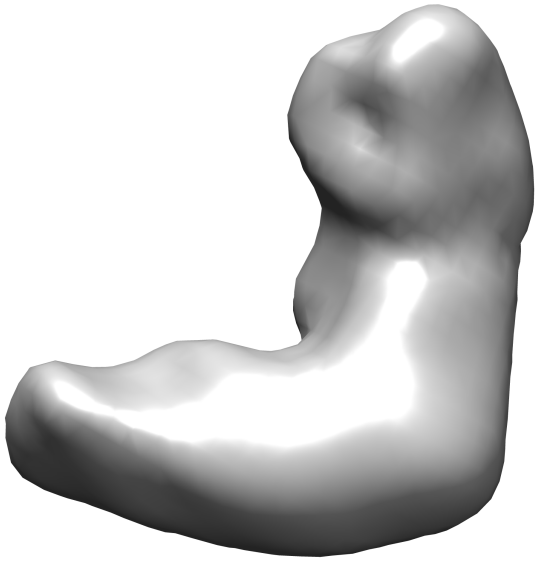


Elmlund et al. (2013)

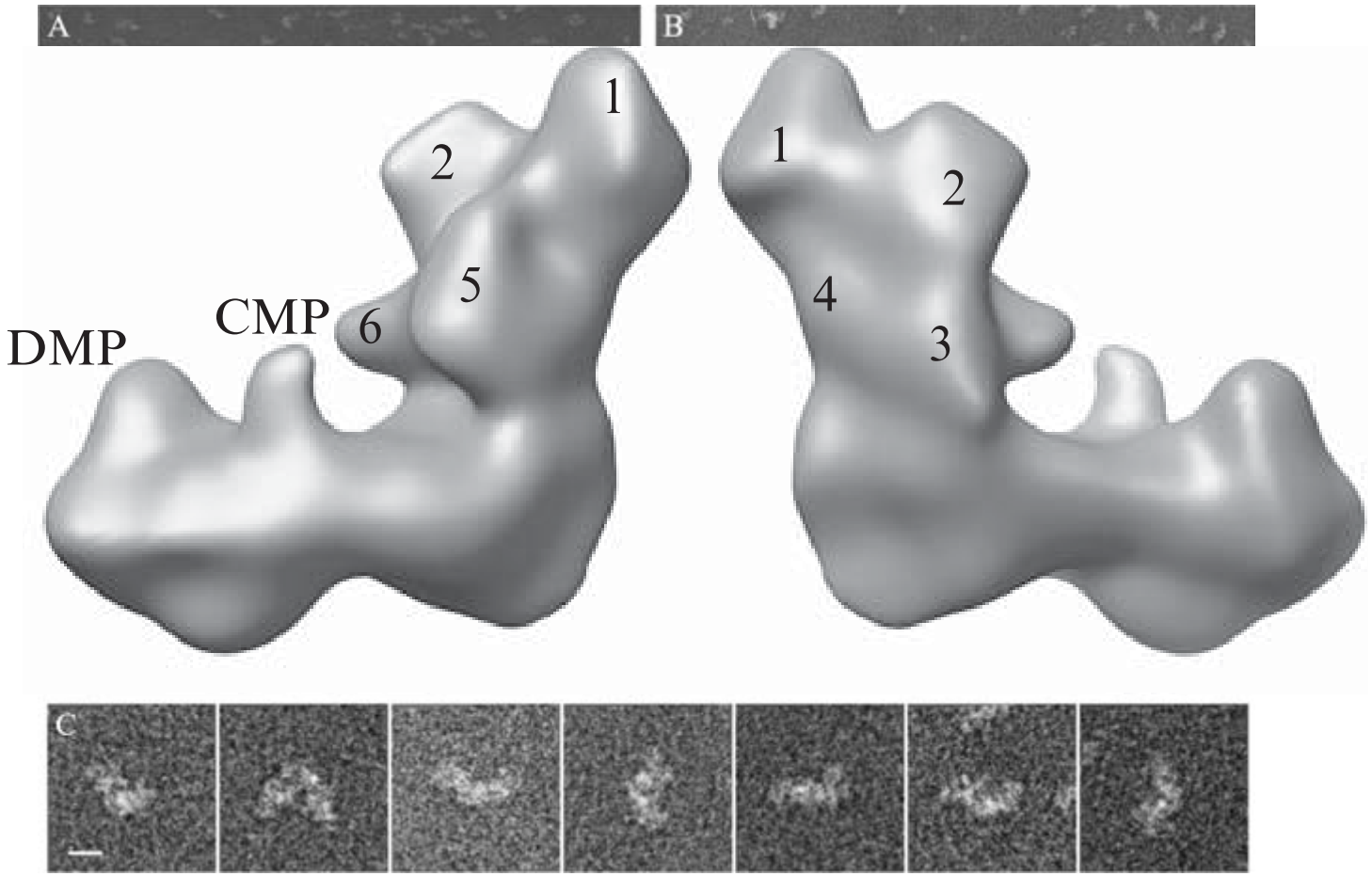
Complex I - initial model generated with EMAN2





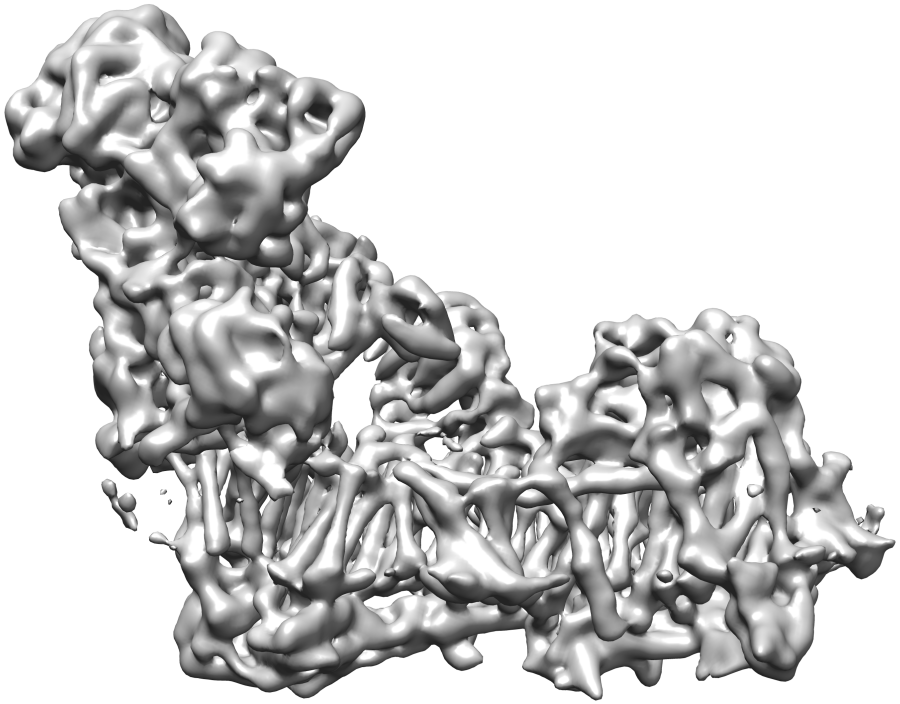


Complex I RCT

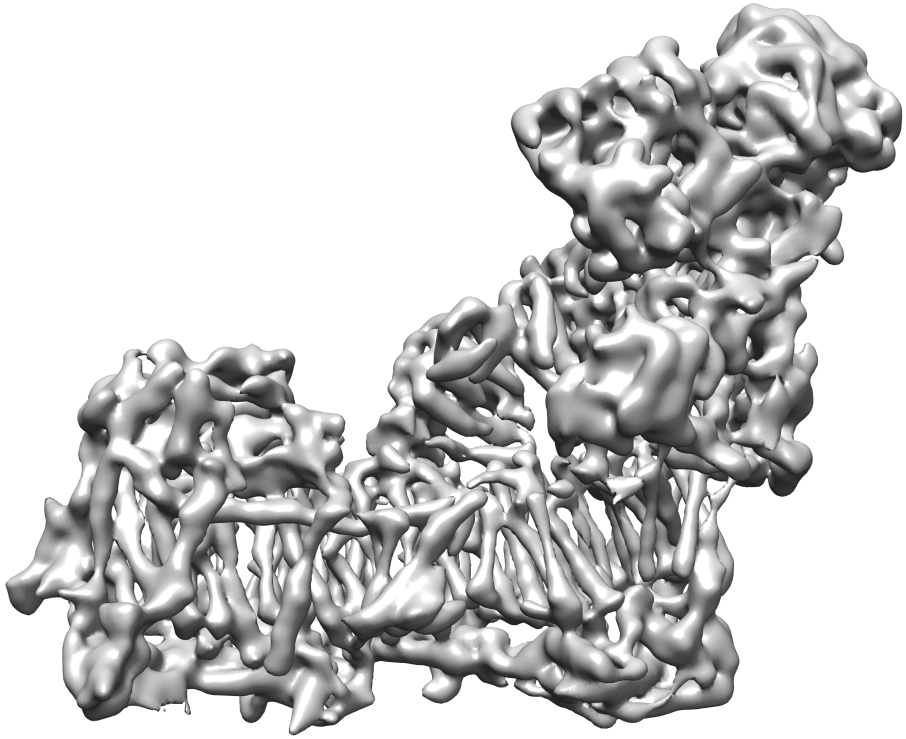


Radermacher, M Methods in Enzymology 2009

Complex I - map handedness

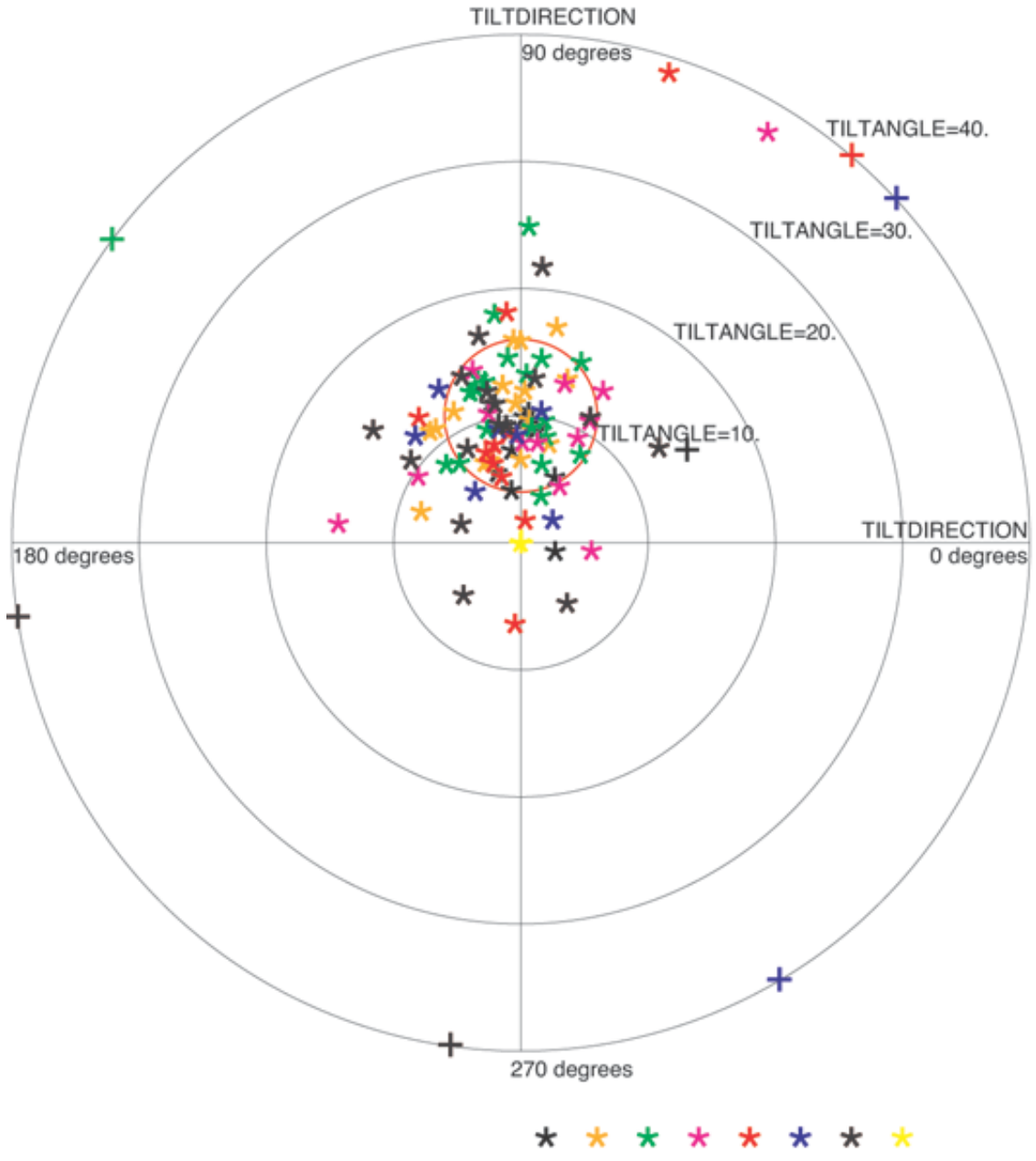


Wrong hand

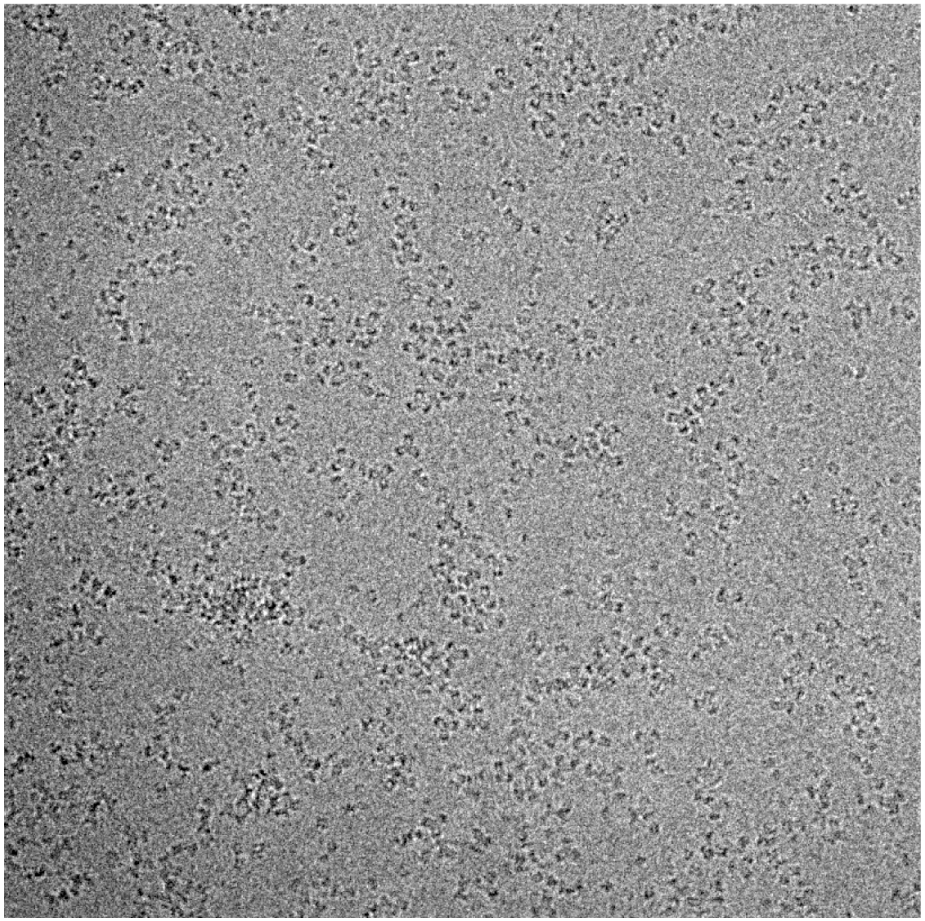


Right hand

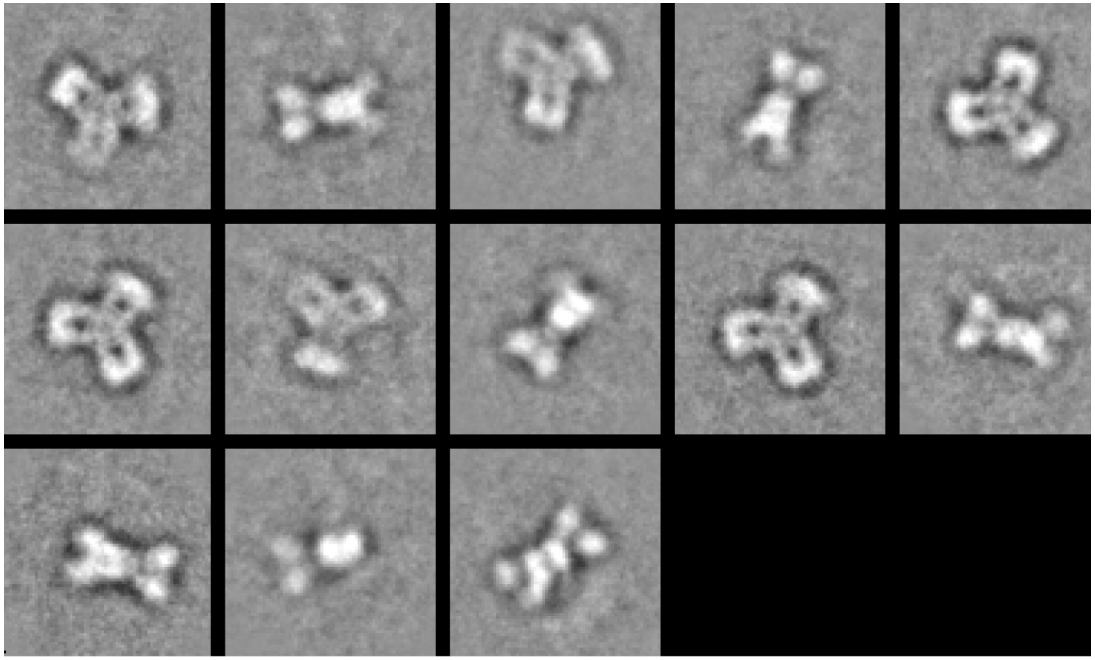
Tilt pair validation of Complex I



PaaZ - a bifunctional enzyme

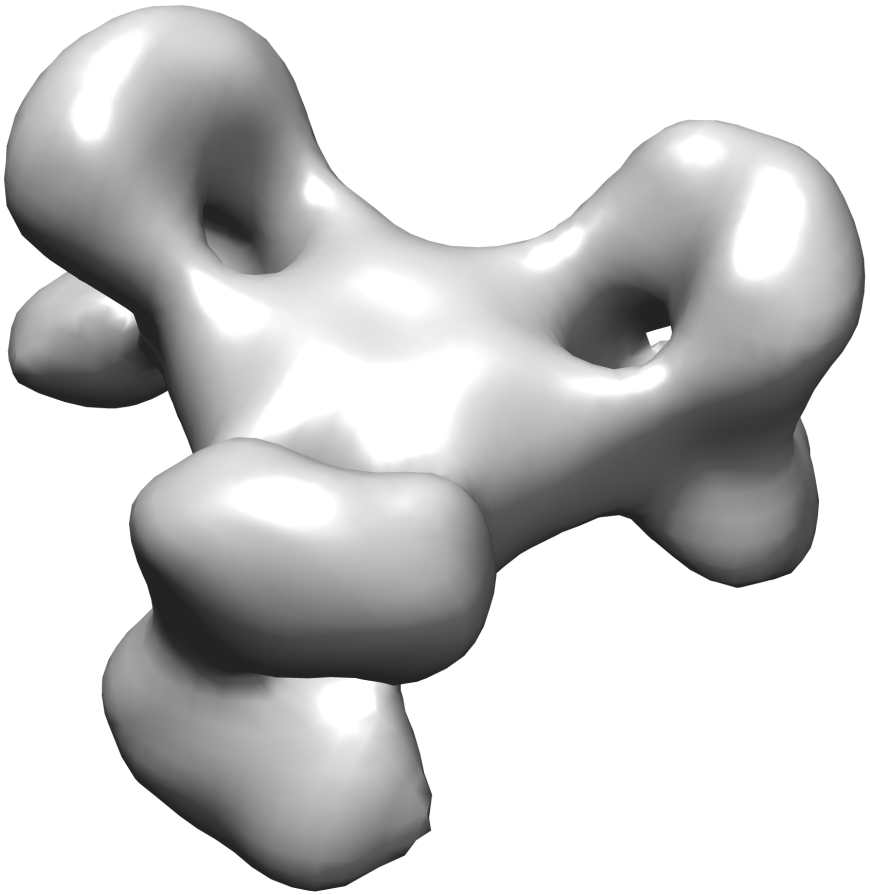


Micrograph

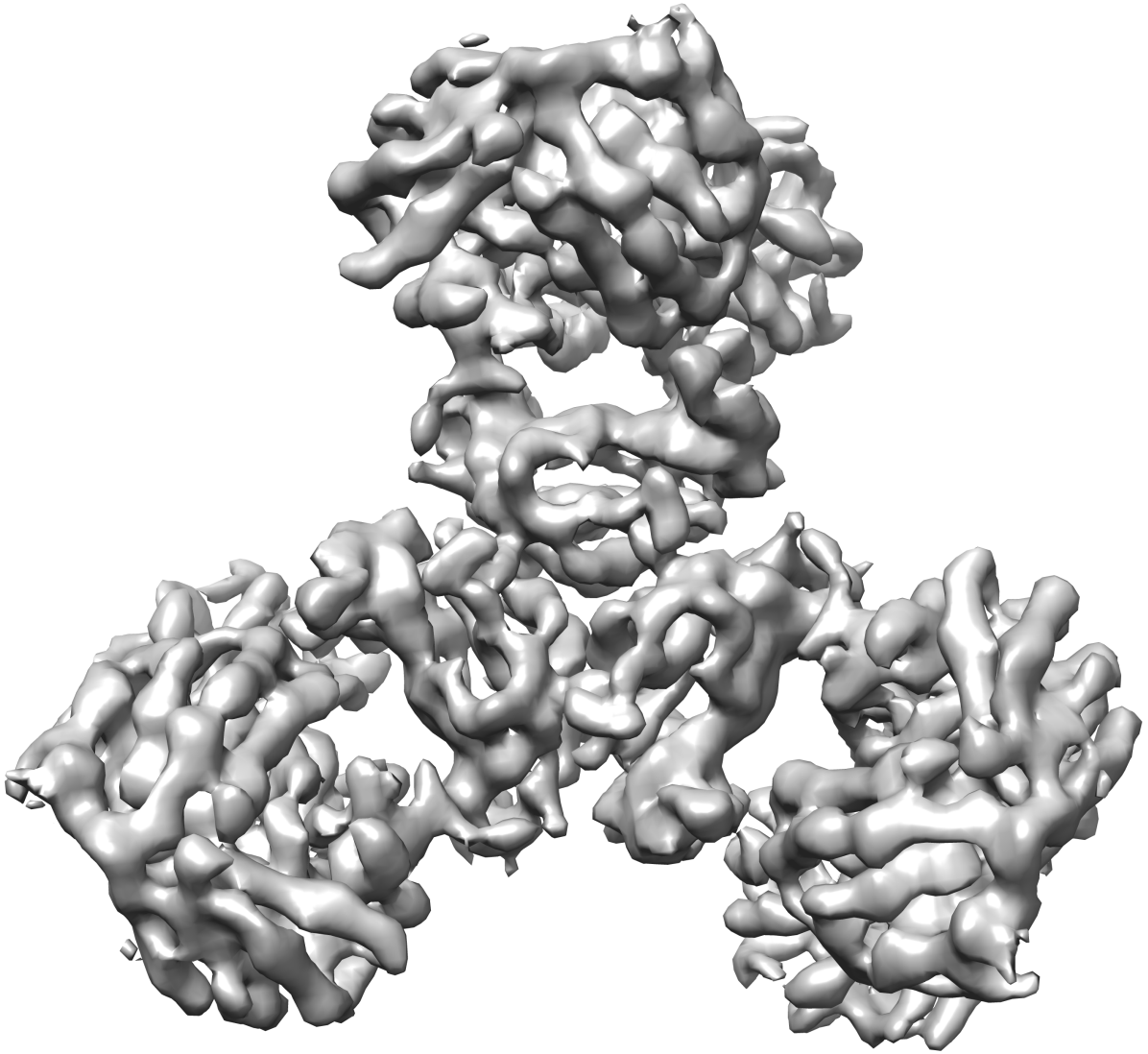


Class averages

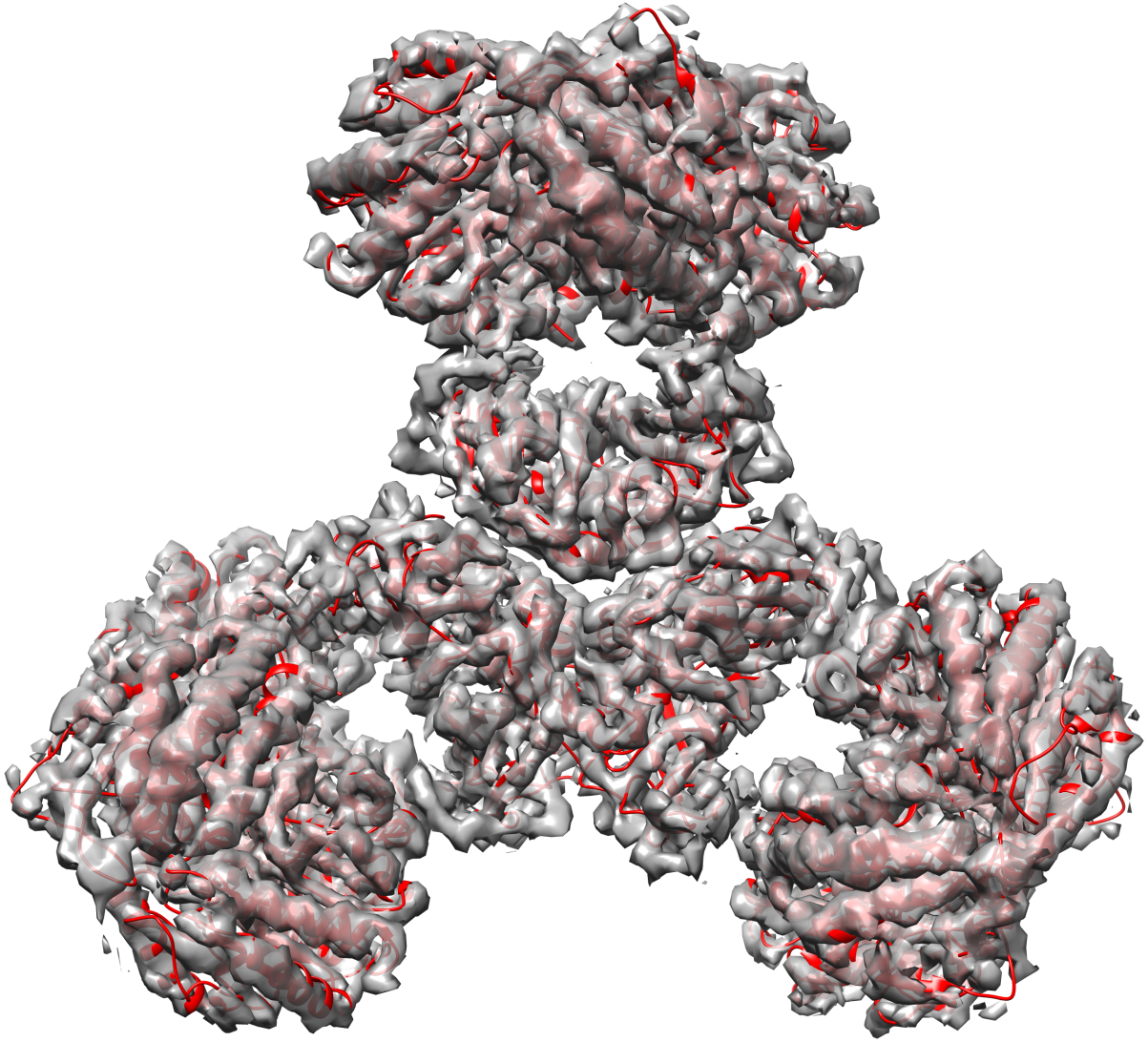
PaaZ - initial model



PaaZ - map after refinement



PaaZ - map with model



None of these methods are perfect – they don't get it right all the time

The ab initio 3D reconstruction of low symmetry small molecular weight specimen is in particular challenging

Make use of any prior information (known biochemical information/ crystal structure) and validation methods

At all stages be very critical of your results!!

Further reading

Cong, Y and Ludtke, S.J. (2010) Single particle analysis at high resolution . *Meth. Enzymology*, 482, 211-235

Crowther, .A., DeRosier, D.J. and Klug,A. (1970) The reconstruction of a three dimensional structure from projections and its application to electron microscopy. *Proc. R. Soc. Lond*, 317, 319-340

Elmlund, H. , Elmlund, D. and Bengi, S. (2013)PRIME: Probabilistic initial 3D model generation for single-particle cryo-electron microscopy. *Structure,* 21, 1299-1306

Frank, J. (2006) Three-dimensional electron microscopy of macromolecular assemblies. Oxford University Press

Radermacher, M. (1988) The three-dimensional reconstruction of single particles from random and non-random tilt series. *J. Electron Microsc. Tech.* 9, 359-394

van Heel, M. (1987) Angular reconstitution: a posteriori assignment of projection directions for 3D reconstructions. *Ultramicroscopy,* 21, 114-126

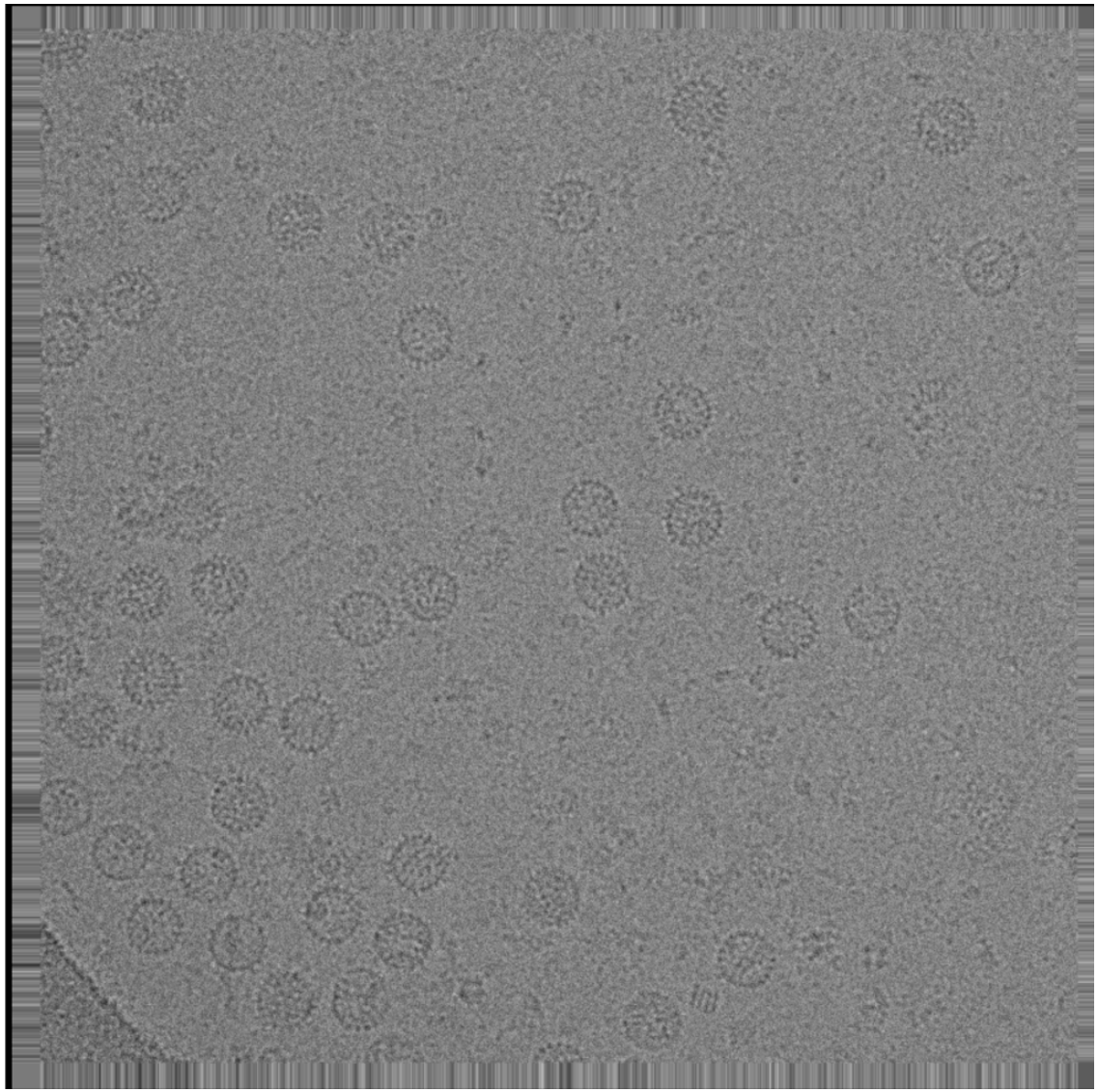
Movies, motion-correction and higher-resolution maps

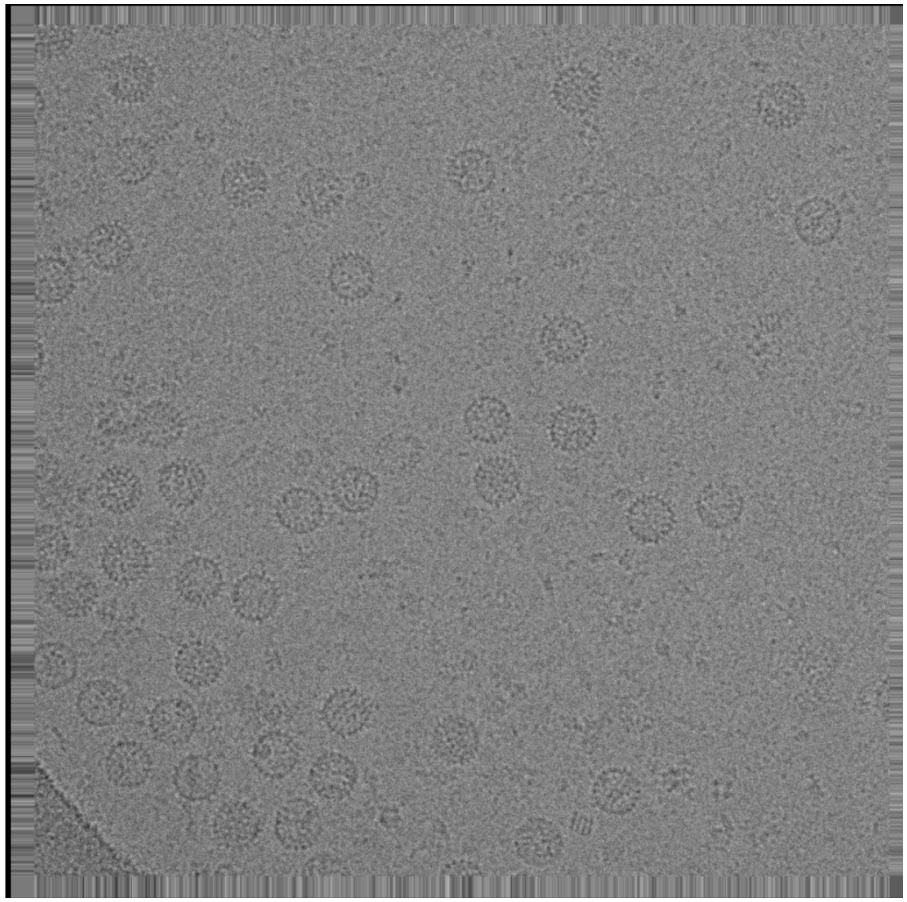
Imaging of HepatitisB viral core with a Falcon detector

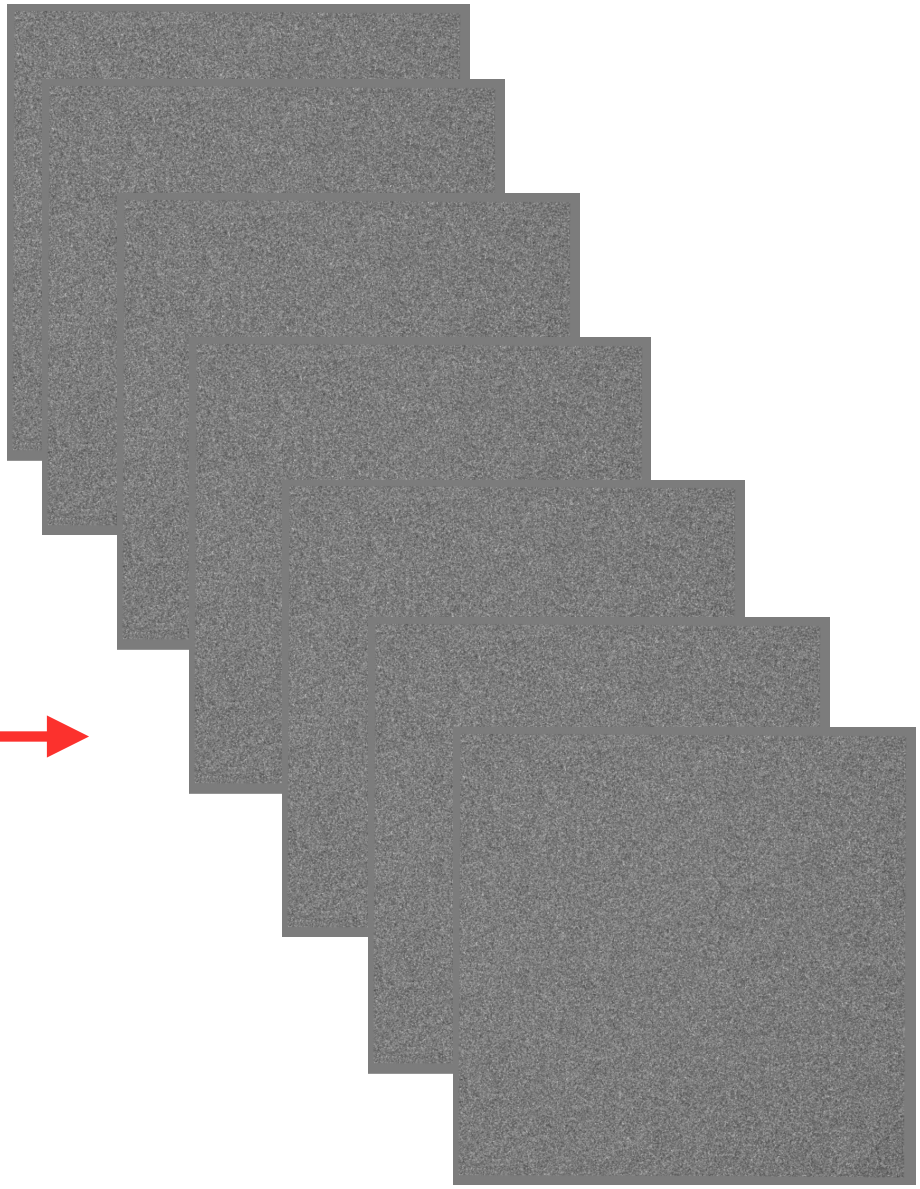
HepB core - ~4 MDa

Falcon - CMOS detector

Microscope	Polara
Magnification	104,000
Dose	~25 $e^-/Å^2$
No. of frames	33
Exposure	2S



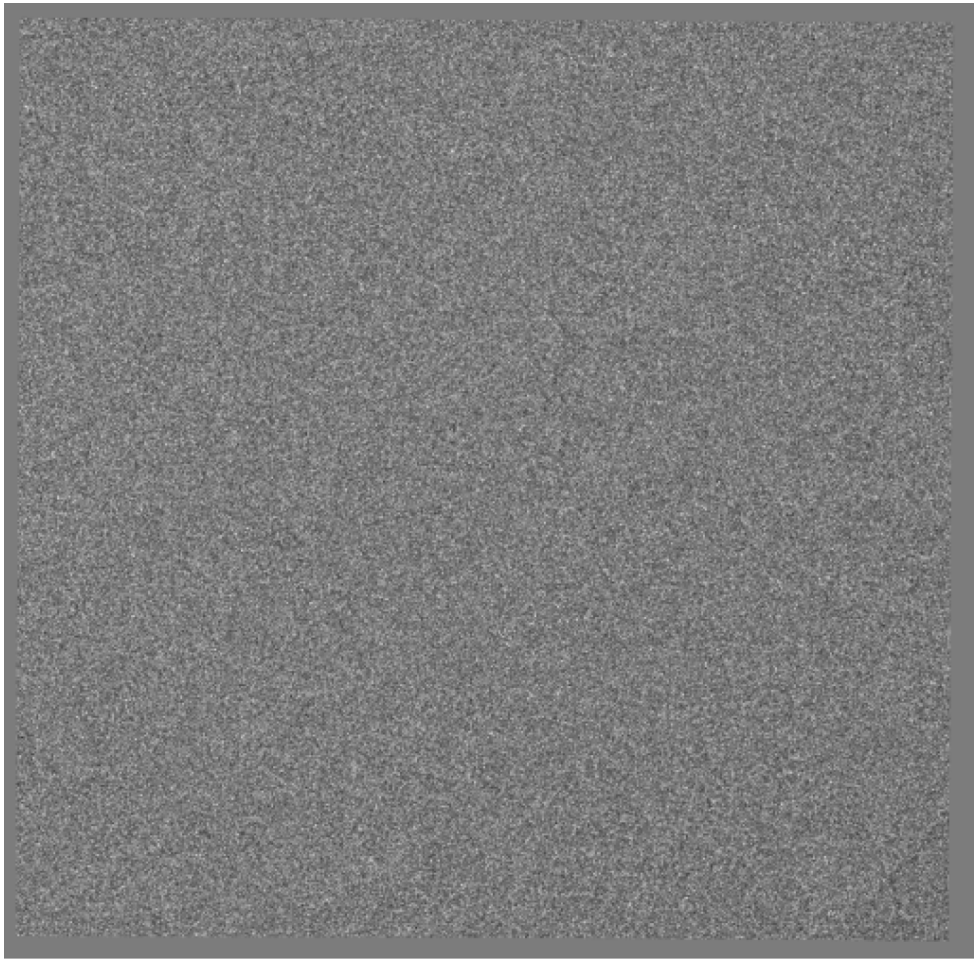




Summed image

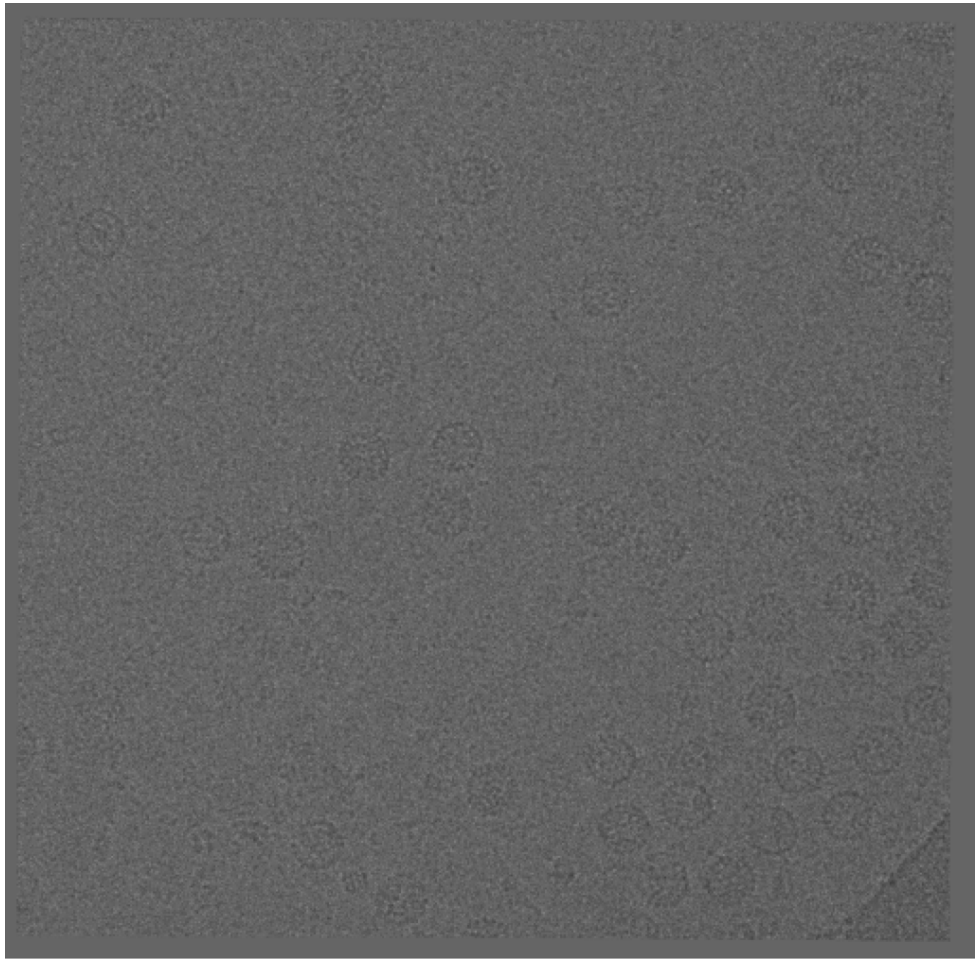
Movies - single frames

Single frame



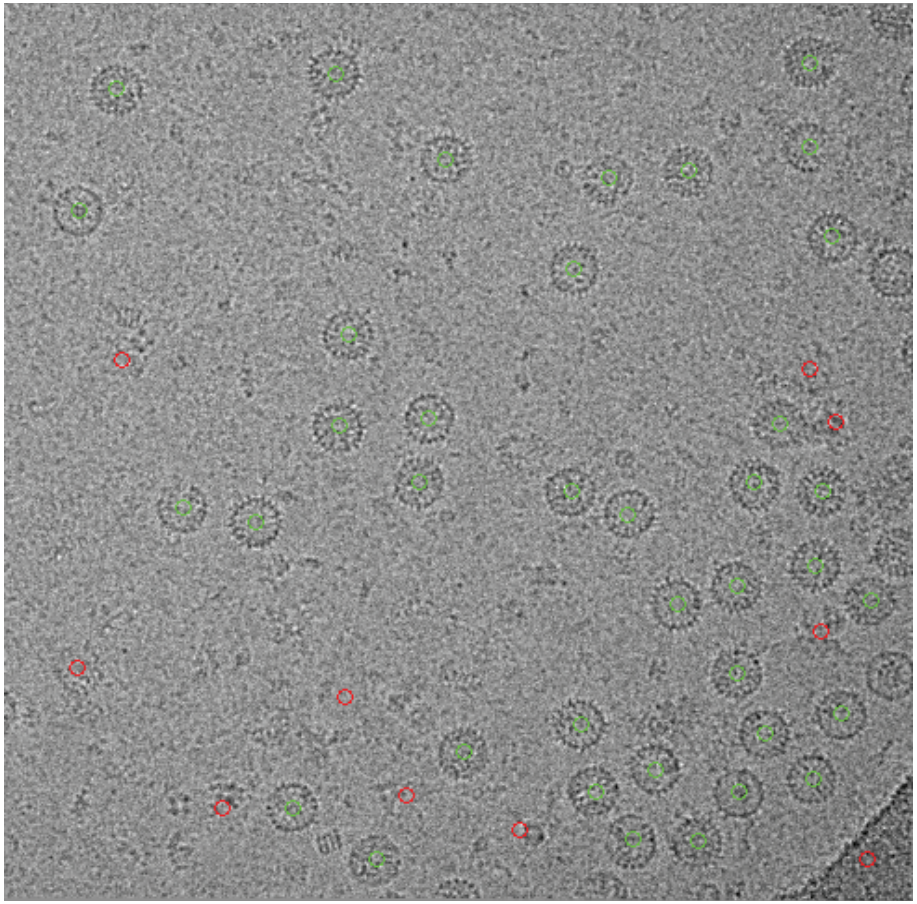
0.6 e^-/pixel

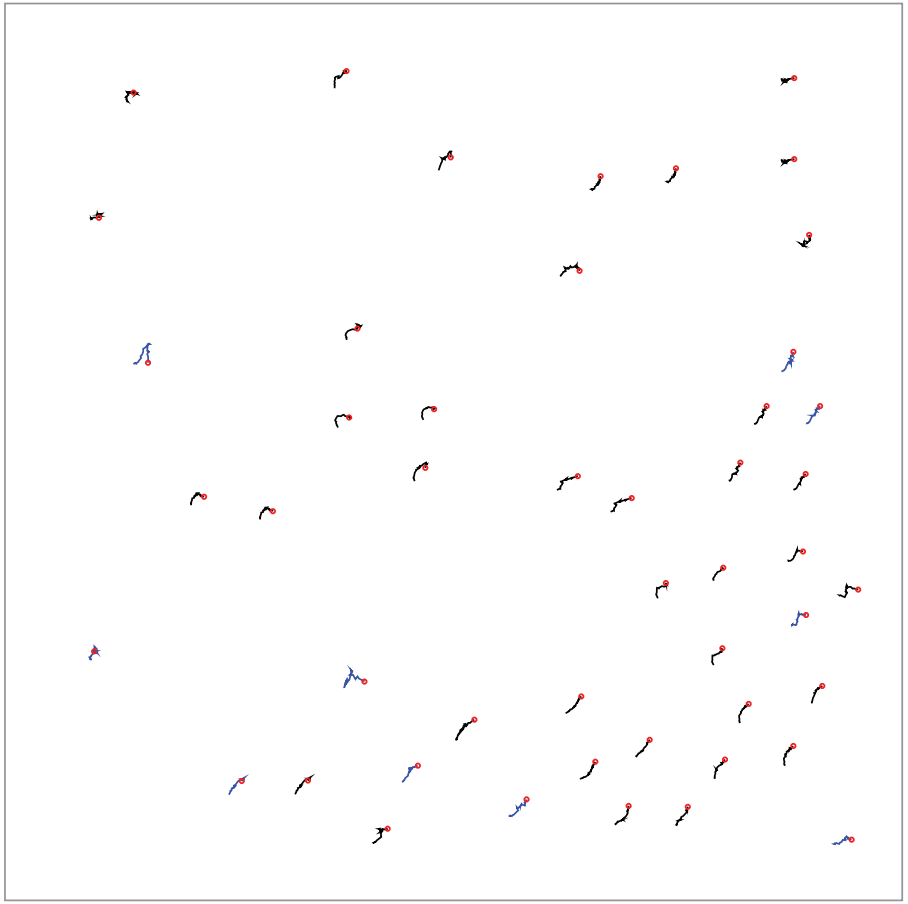
Average of 9 frames



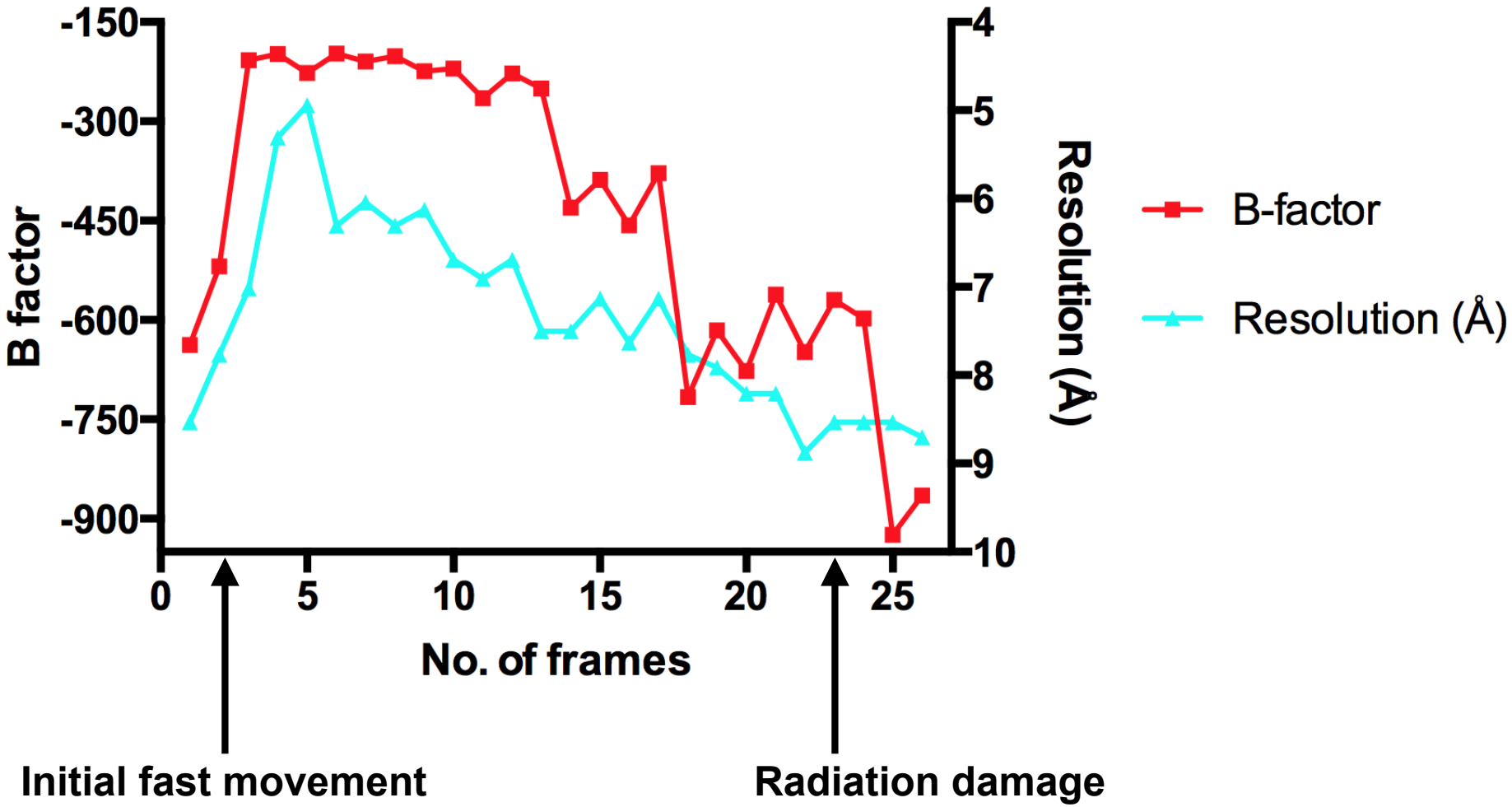
5.4 e^-/pixel

Beam-induced movement of single particles

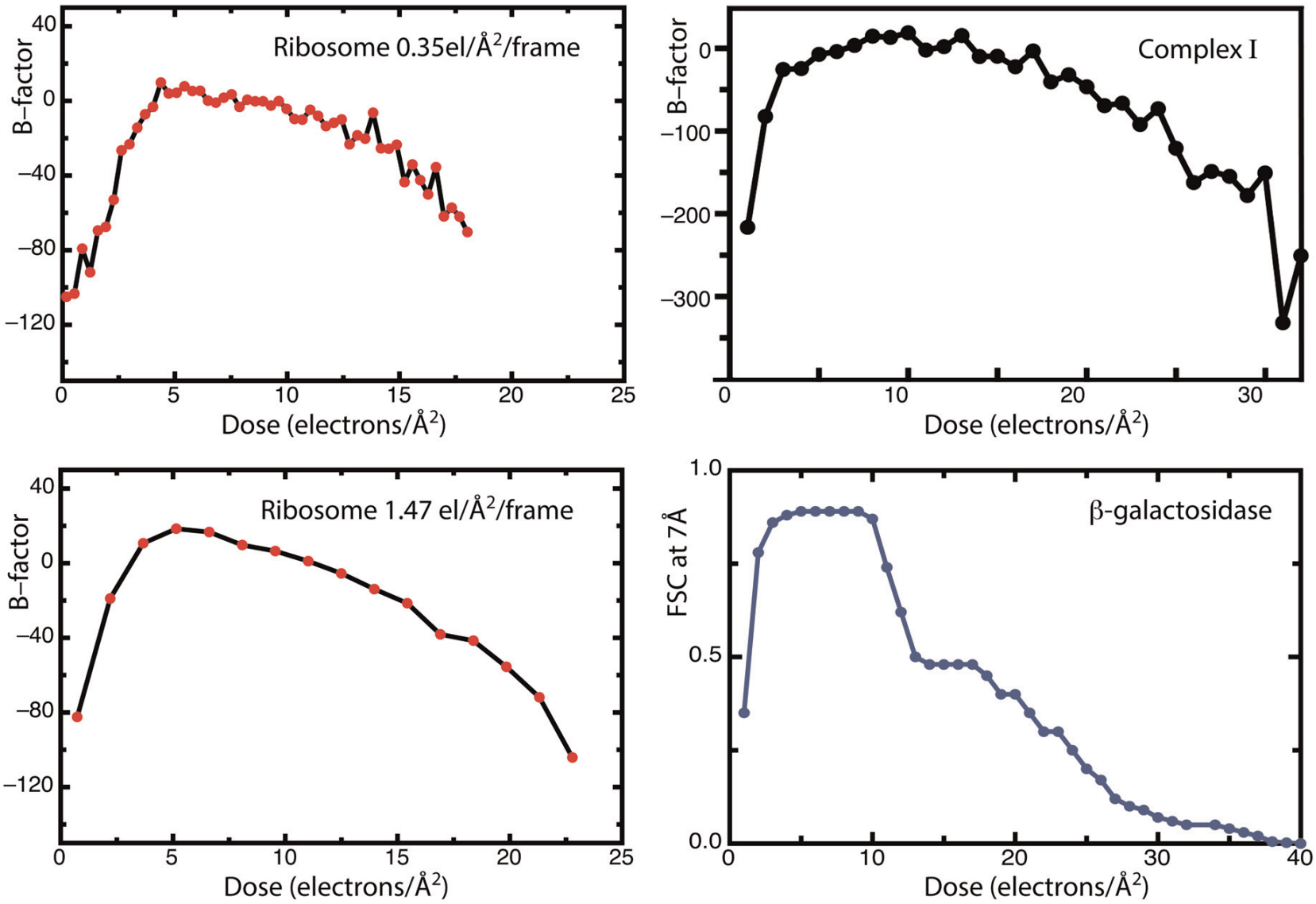




Beam-induced damage in the first few electrons

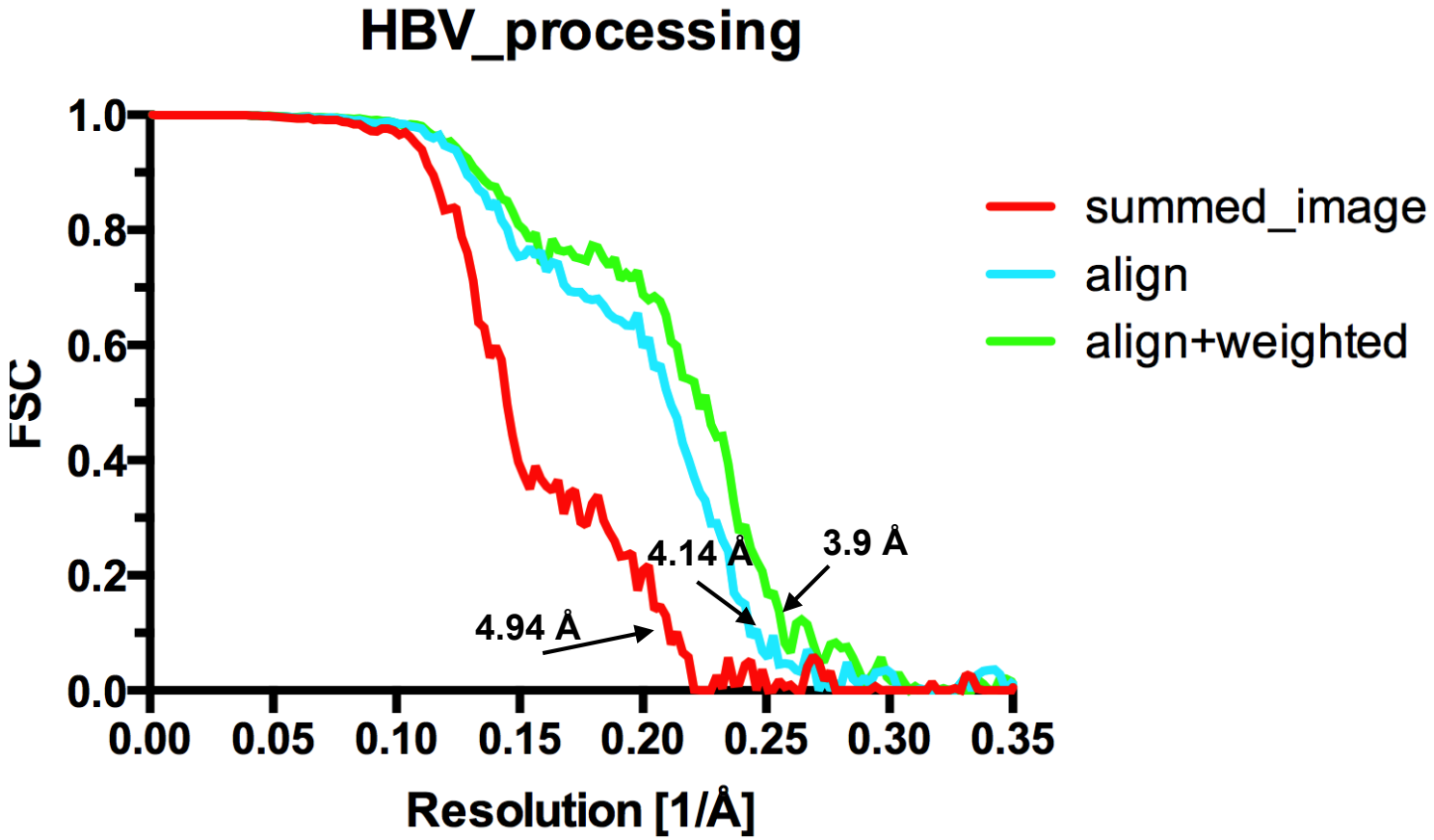


Different data sets - same pattern



Vinothkumar & Henderson 2016; Henderson 2015

Progressive increase in resolution of HepB viral core (<1000 particles)



Vinothkumar et al., 2013; Scheres et al., 2014

Further reading

Cheng, Y et al., A primer to single-particle cryo electron microscopy. **Cell,** 2015, **438,** p1.

Bai et al., How cryo-EM is revolutionising structural biology. **TIBS,** 2015, **40,** p49.

Vinothkumar, K.R., Membrane protein structures without crystals, by single particle electron cryomicroscopy **COSB,** 2015, **33,** p103

Henderson, R., The potential and limitations of neutrons, electrons, and X-rays for atomic resolution microscopy of unstained biological molecules **QRB**, 1995, **28,** p171.

van Heel et al., Single-particle electron cryo-microscopy: towards atomic resolution **QRB**, 2000, **33,** p307.