



Laboratory for BioMolecular Structure

2022 Virtual Cryo-EM Course

Day 2, Wednesday June 15

EM IMAGE FORMATION AND SINGLE PARTICLE RECONSTRUCTION

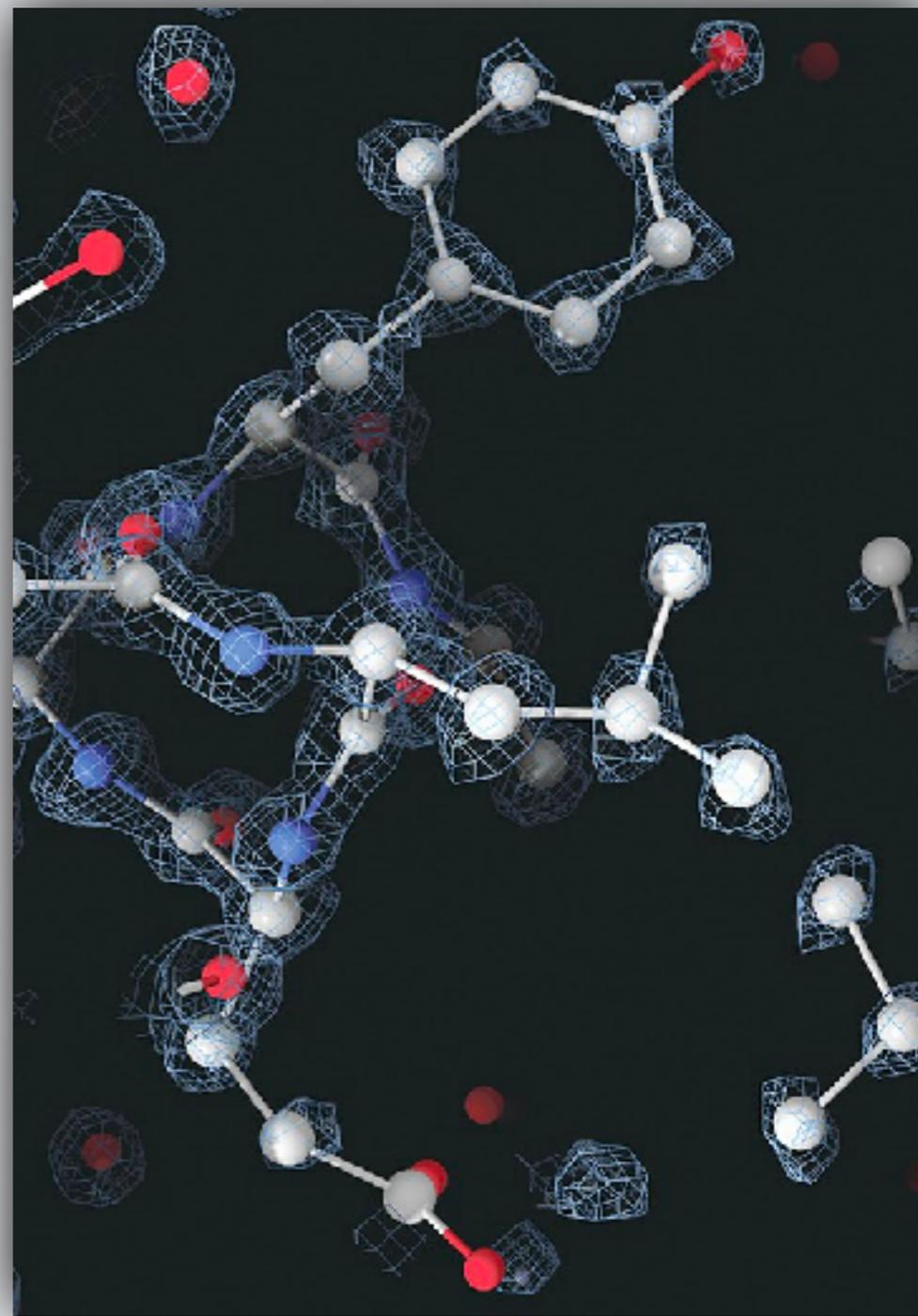
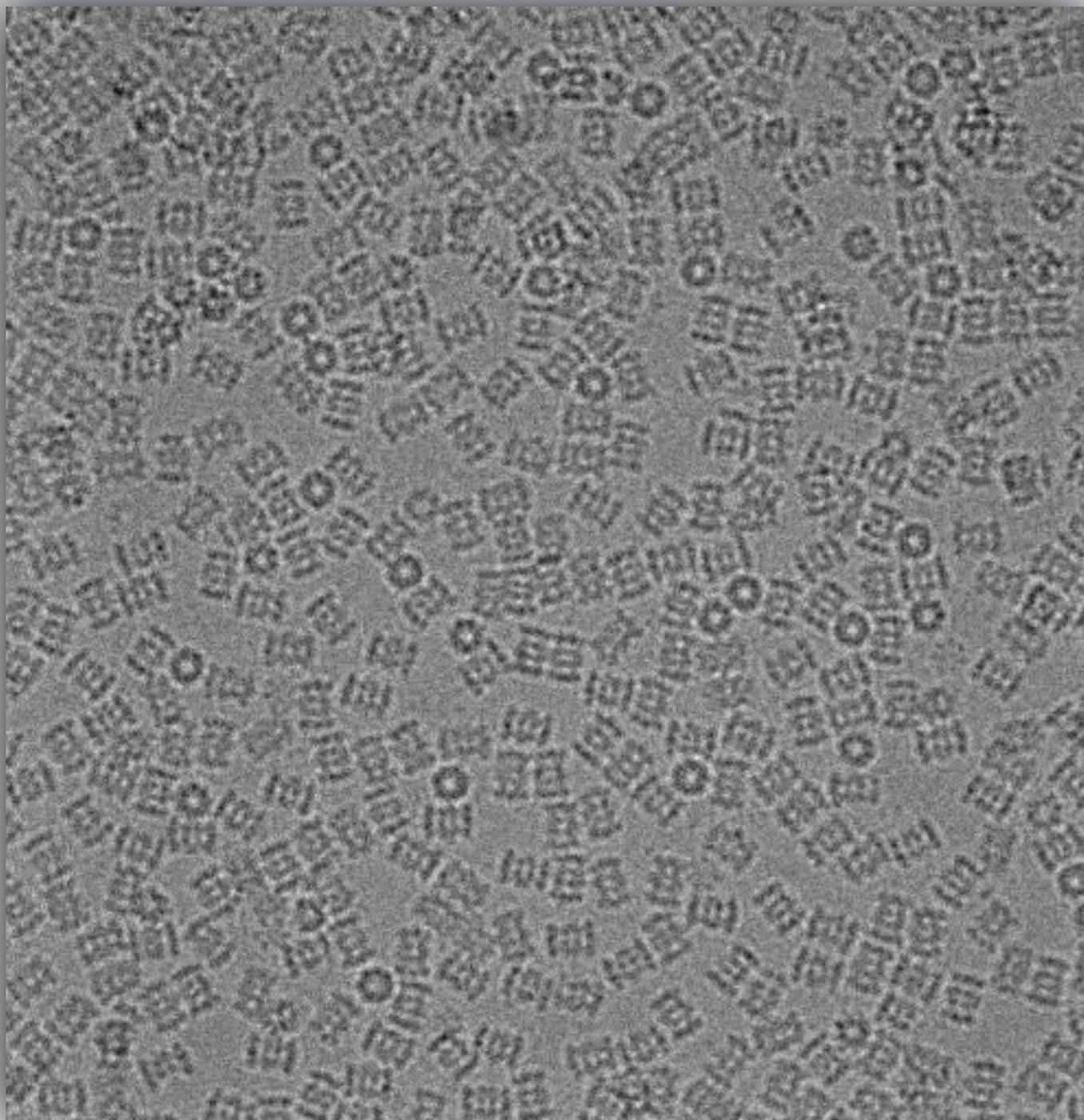
Gabe Lander

Dept. Integrative Struct. & Comp Bio



Scripps Research

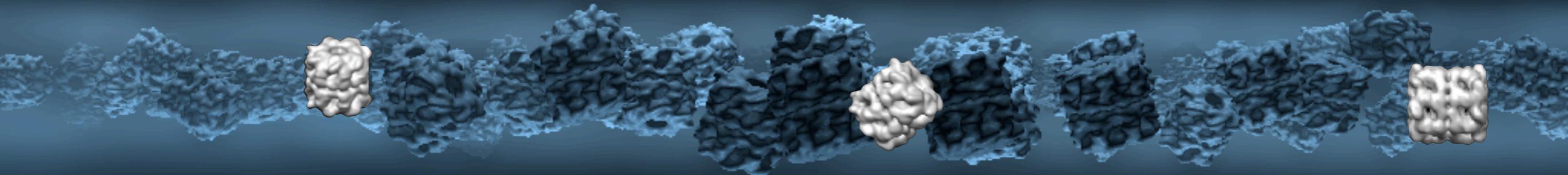
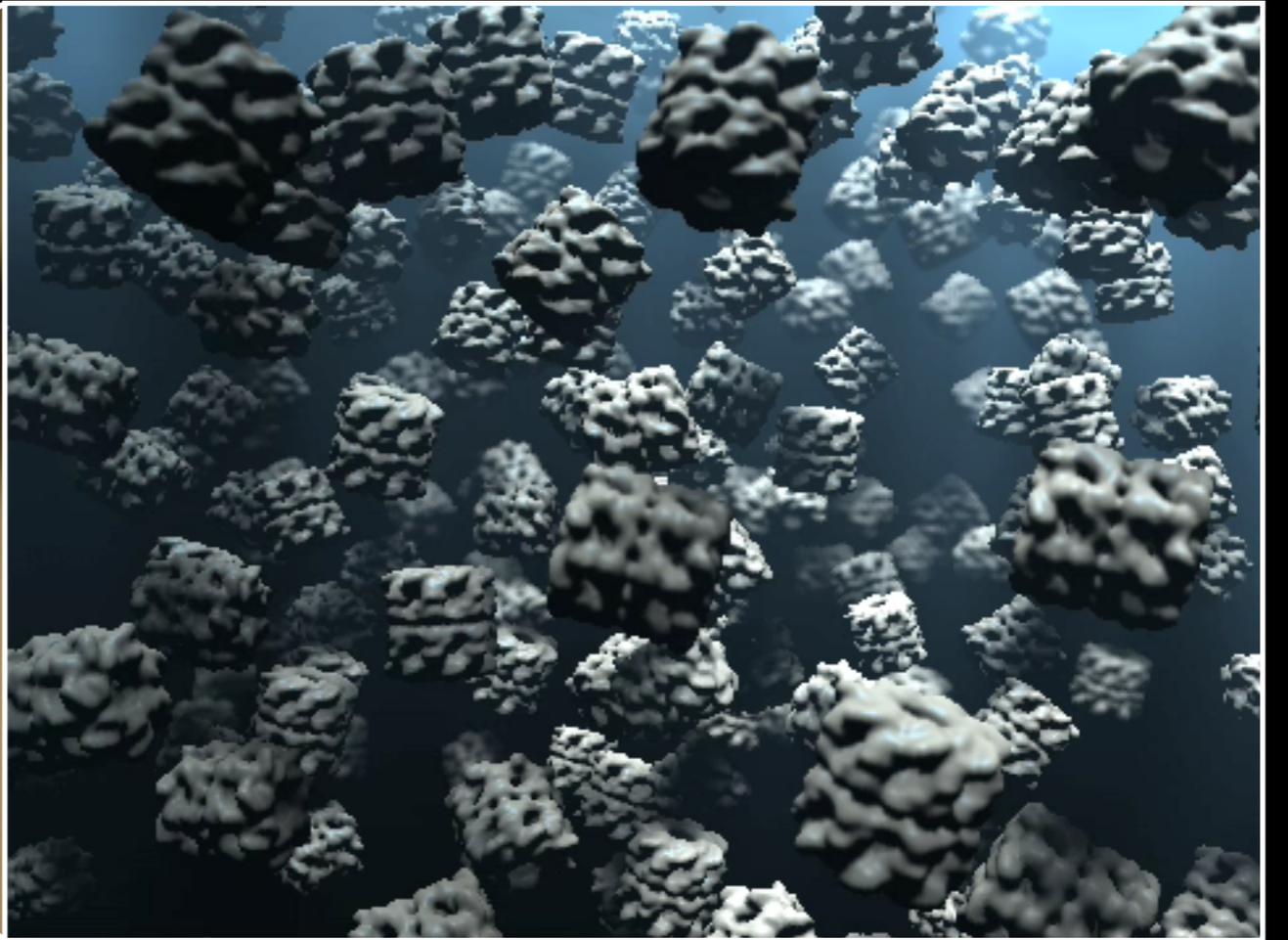
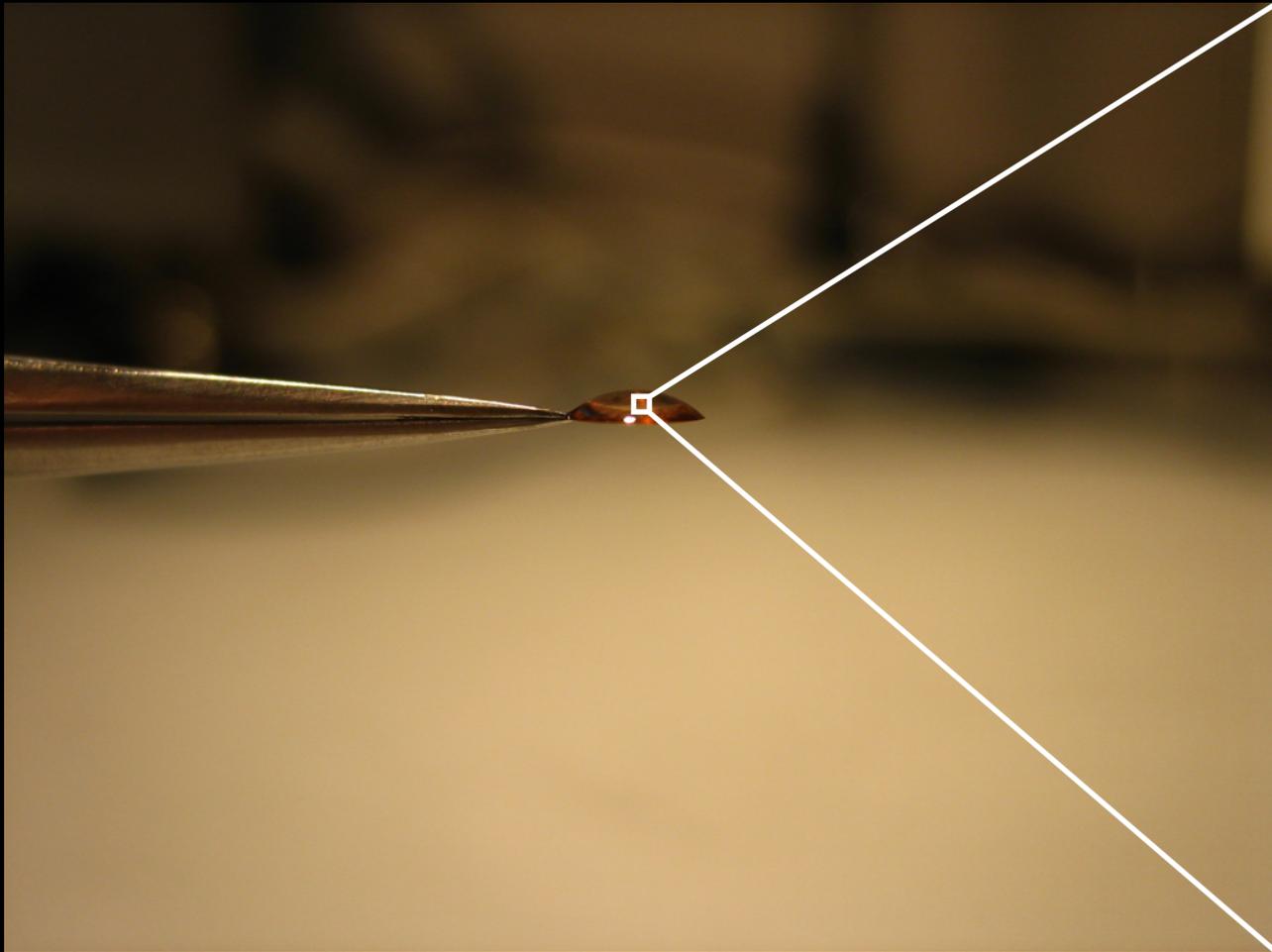
TODAY'S GOAL:

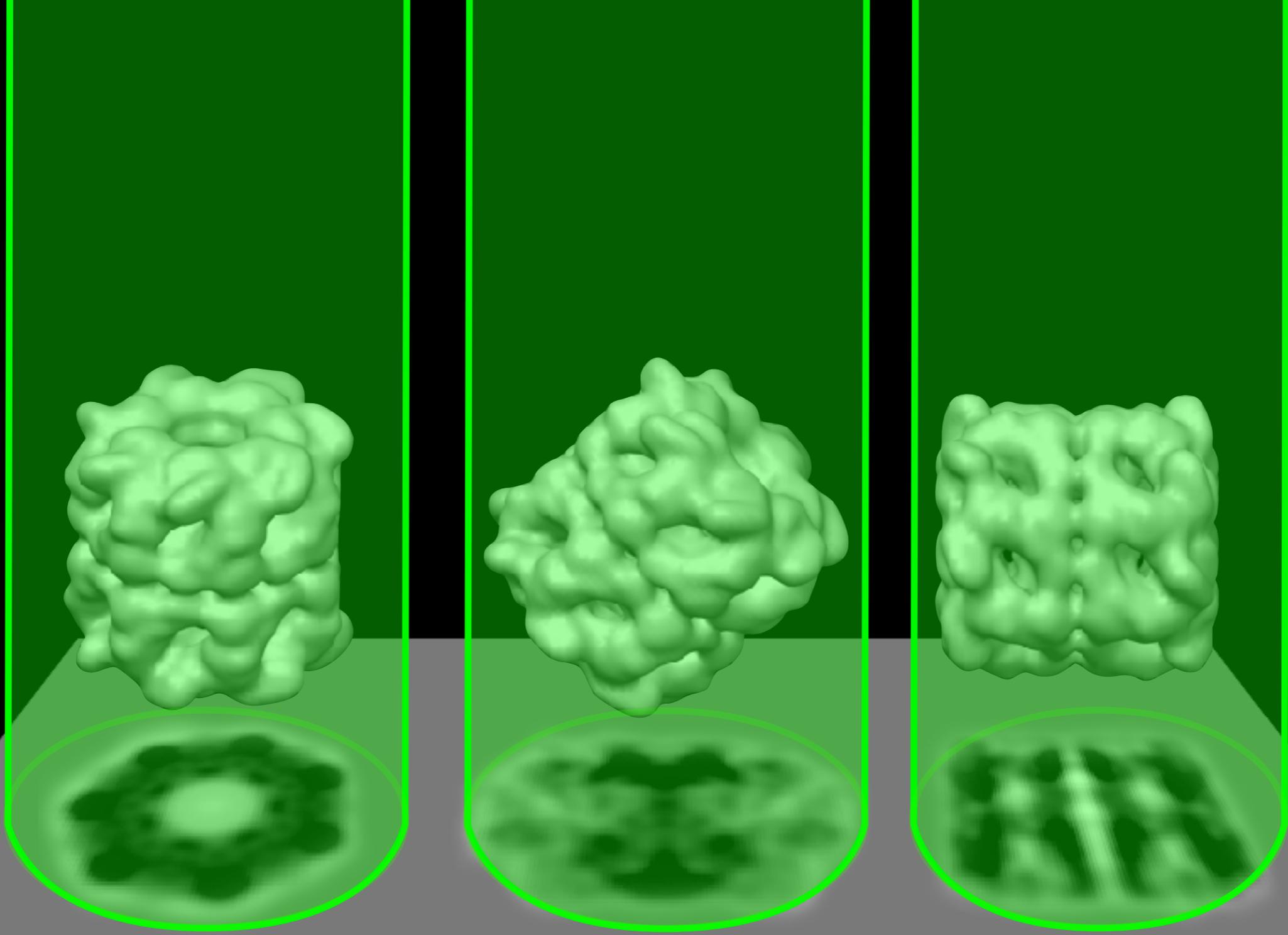


Nakane *et al.* 2020

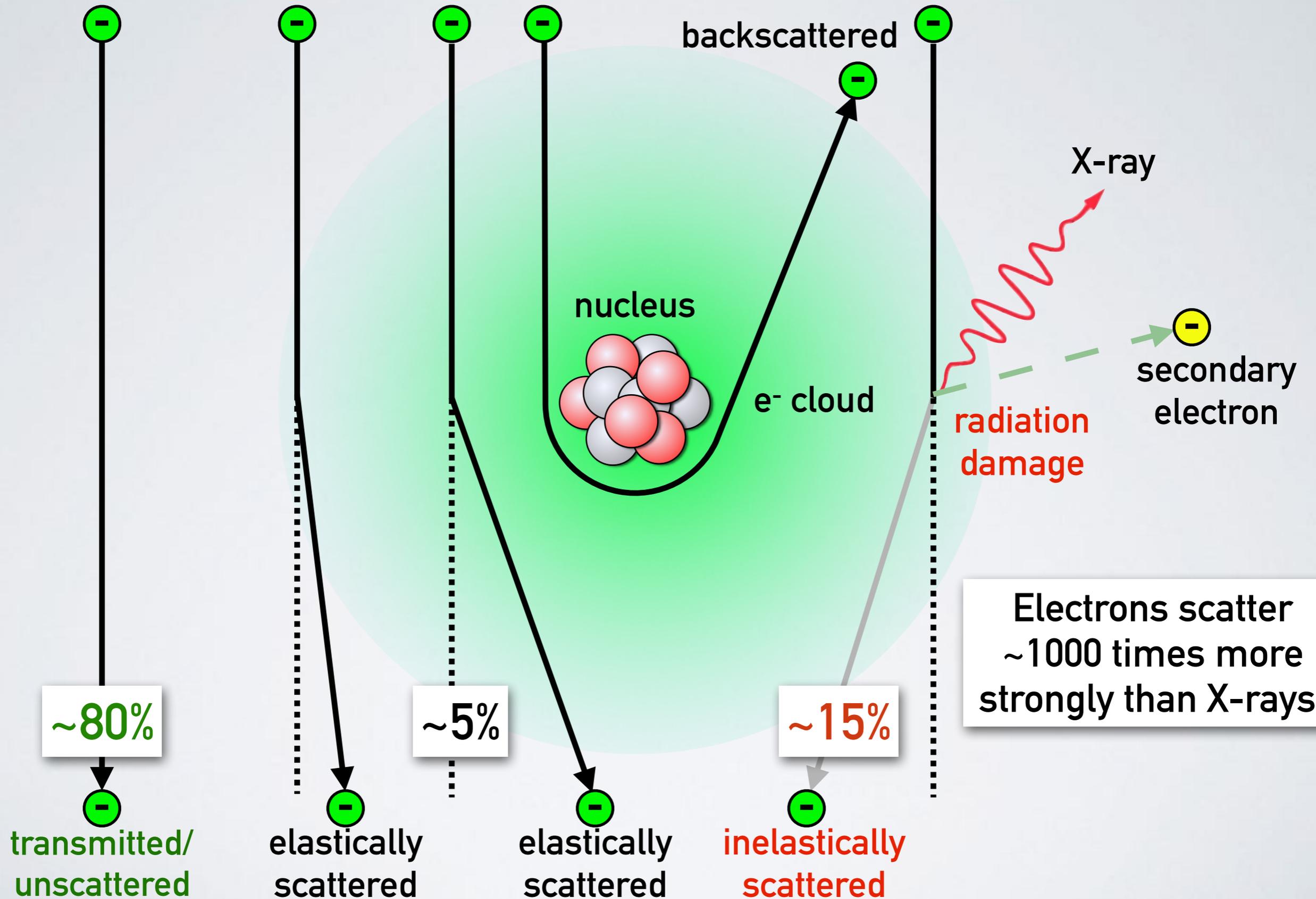
Yip *et al.* 2020

image from Herzik 2020



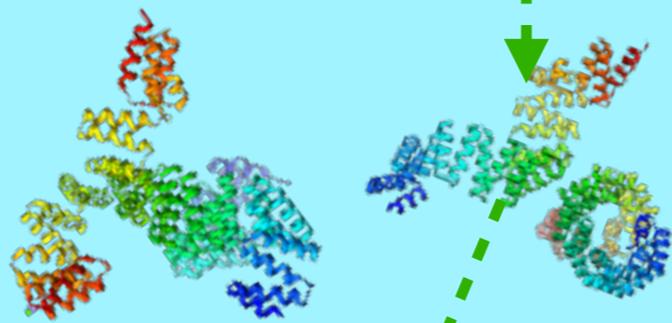


Electron Scattering

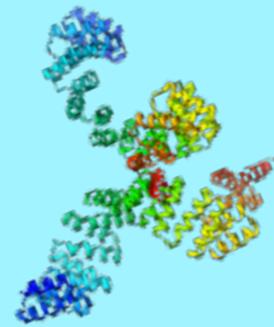


We need contrast! (Discriminate scattered from unscattered)

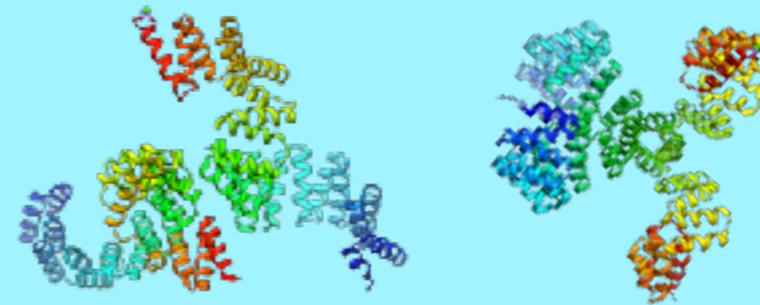
water = 1 g/cm³
protein = 1.4g/cm³



scattered



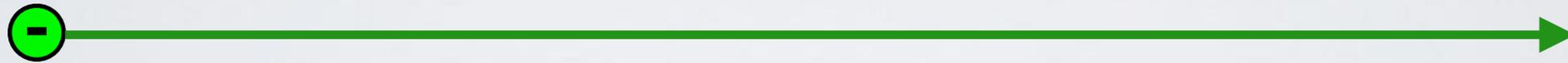
unscattered



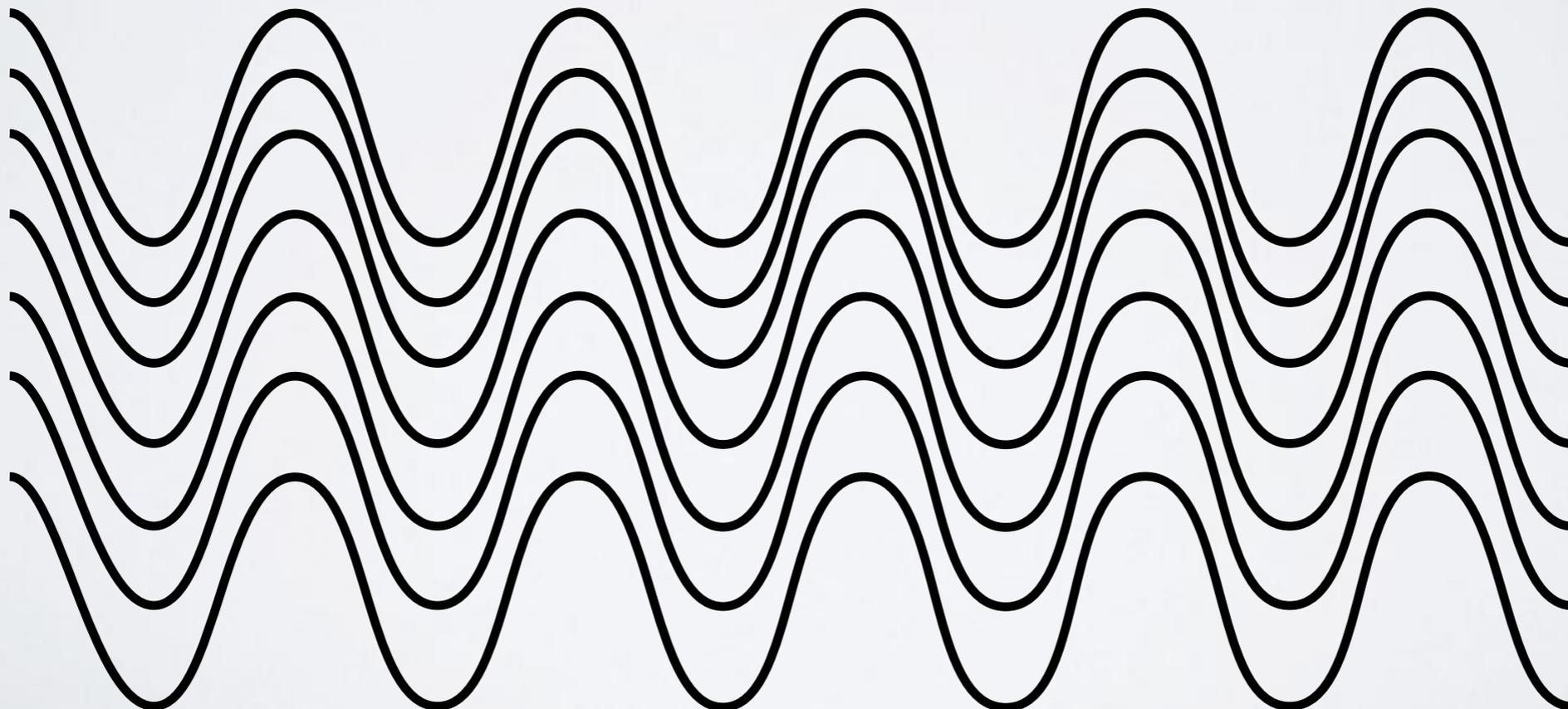
Phase Contrast

wave-particle duality of electrons
give rise to two representations:

ray diagrams (e- as particles)



wave diagrams (e- as waves)



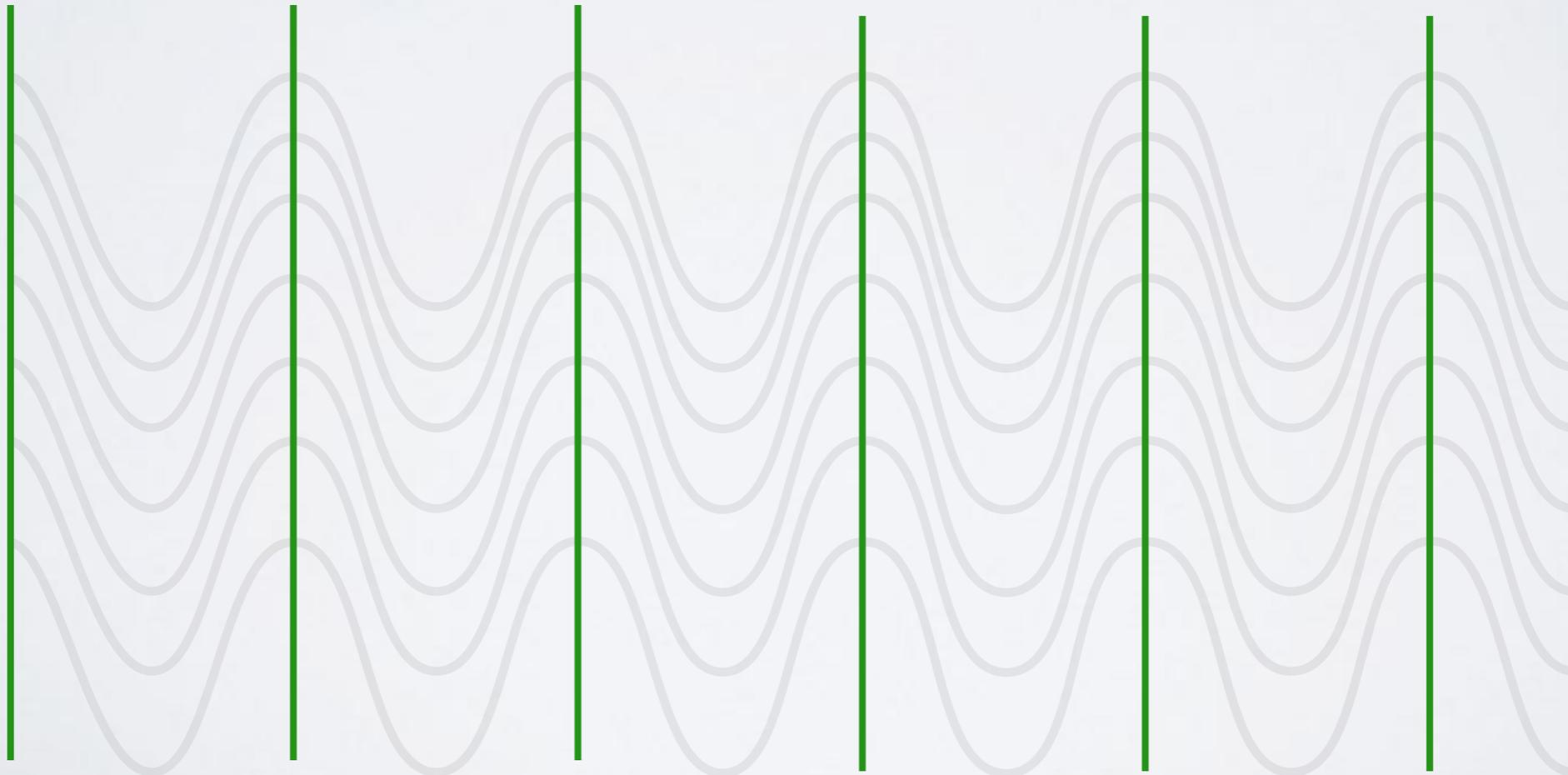
Phase Contrast

wave-particle duality of electrons
give rise to two representations:

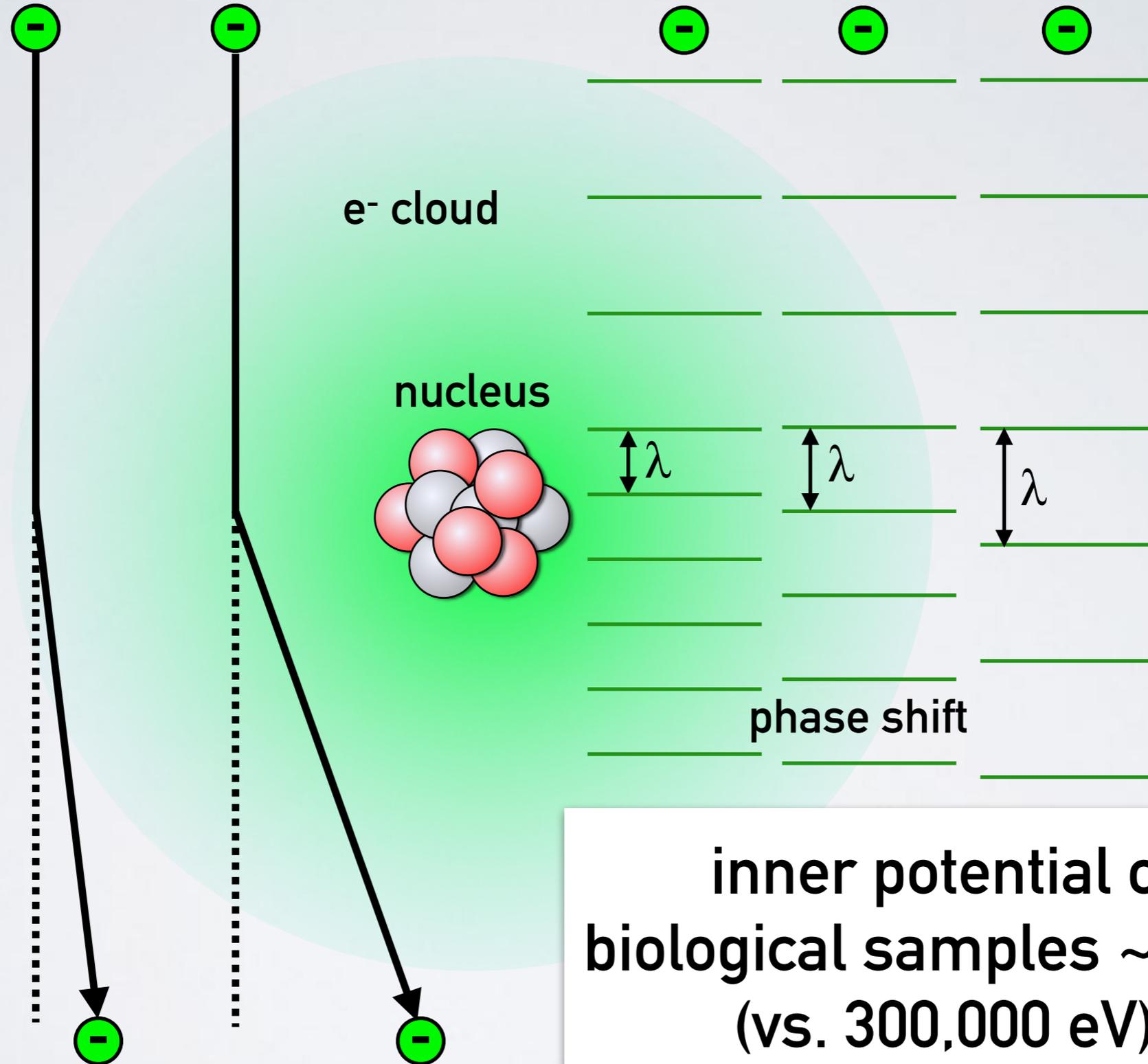
ray diagrams (e- as particles)



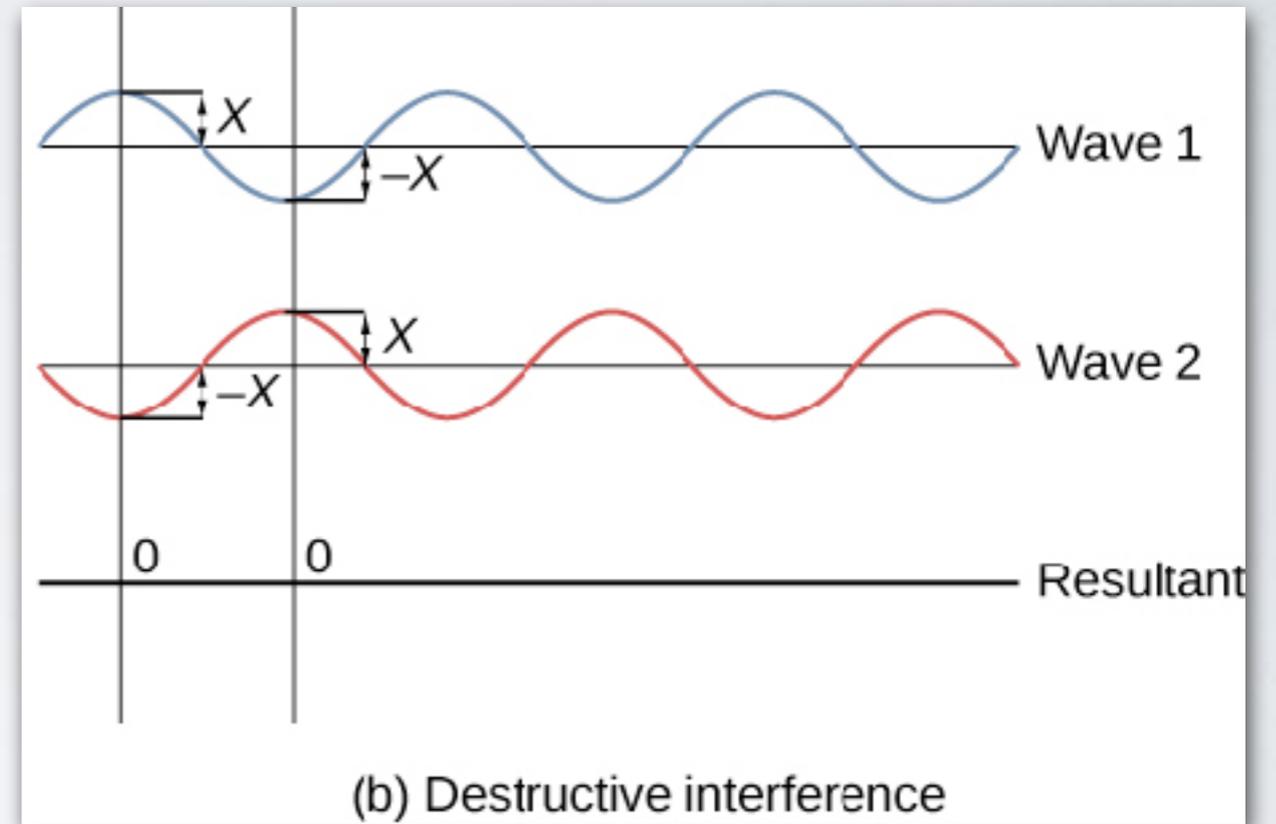
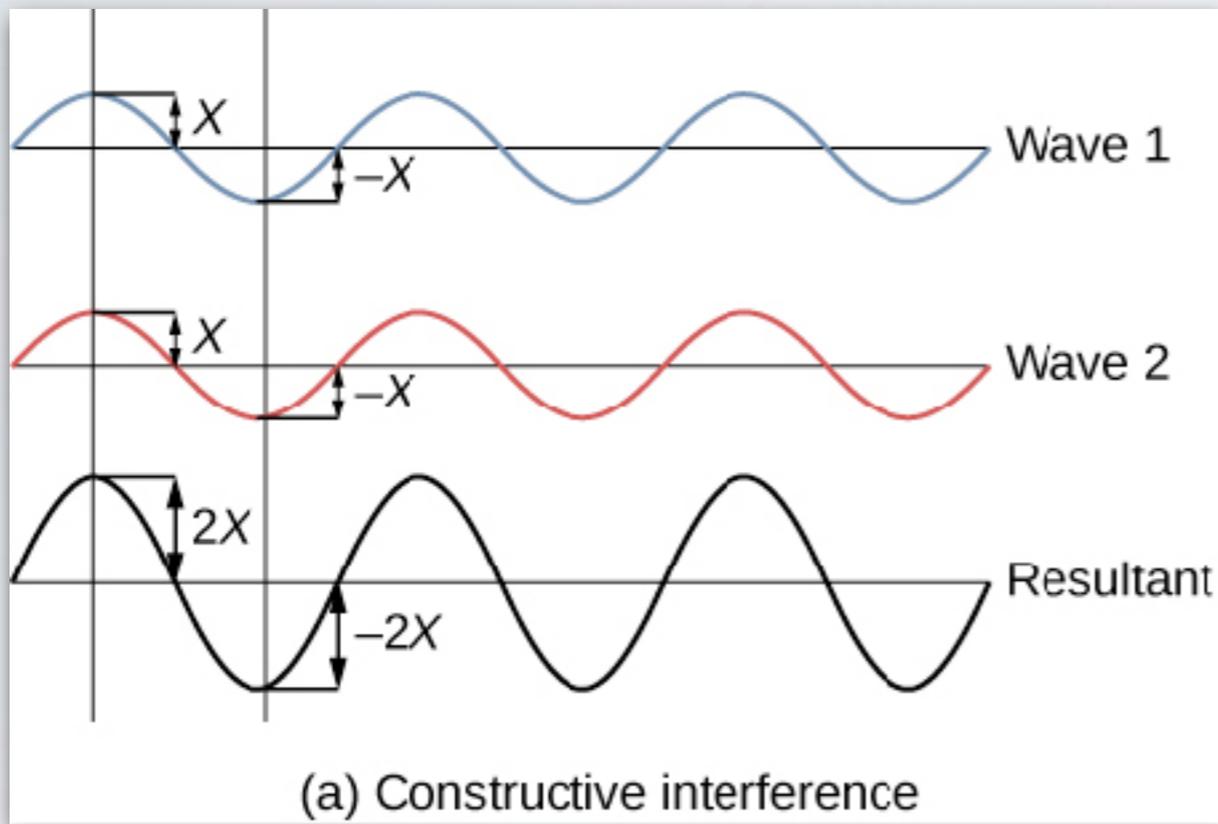
wave diagrams (e- as waves)



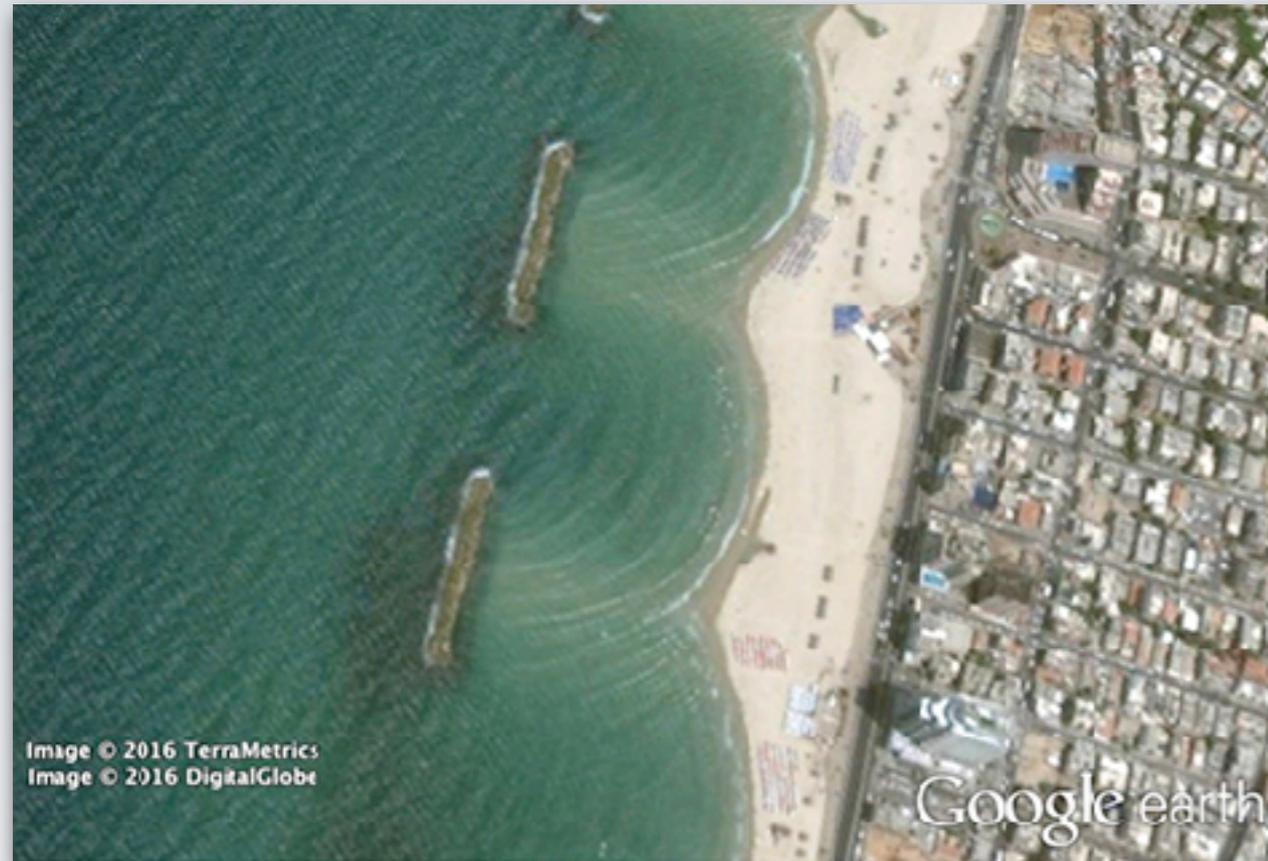
Biological samples are weak phase objects



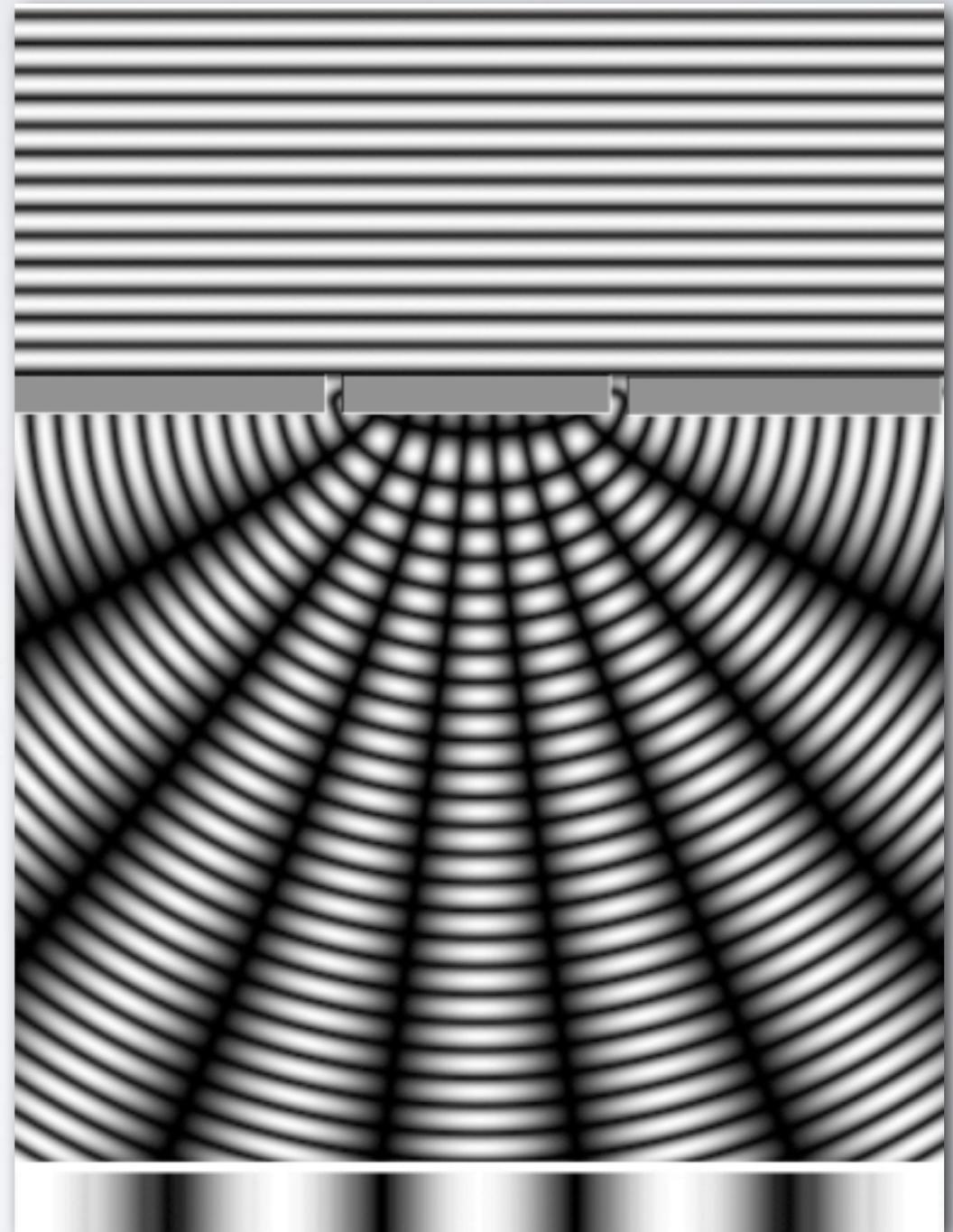
Wave interference



Wave interference



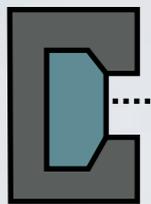
affects amplitudes!



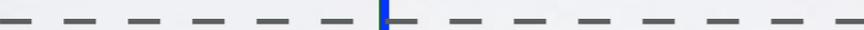
sample



EM
lens

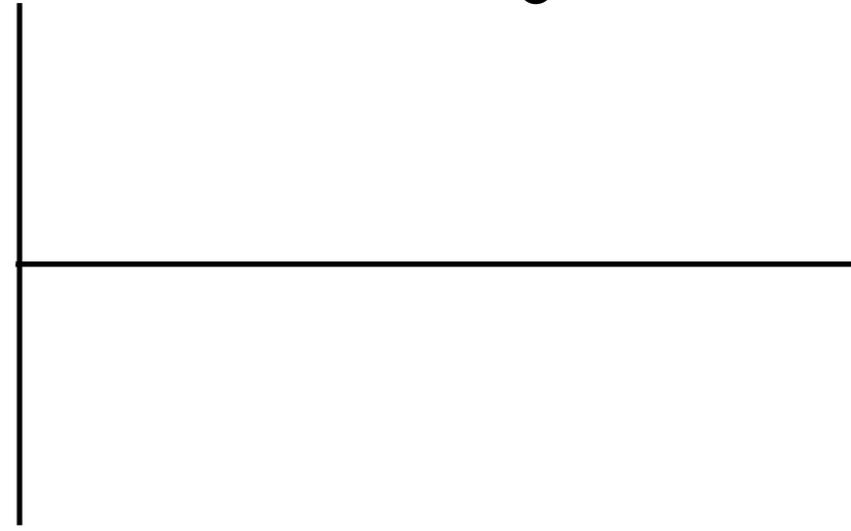


back focal plane

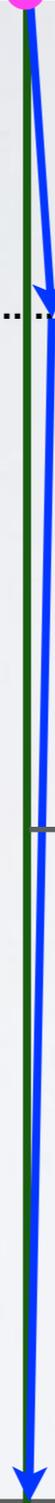
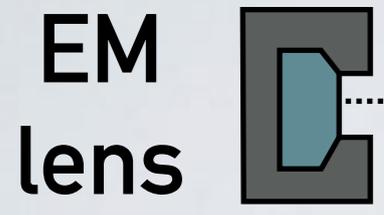


Contrast transferred
to image

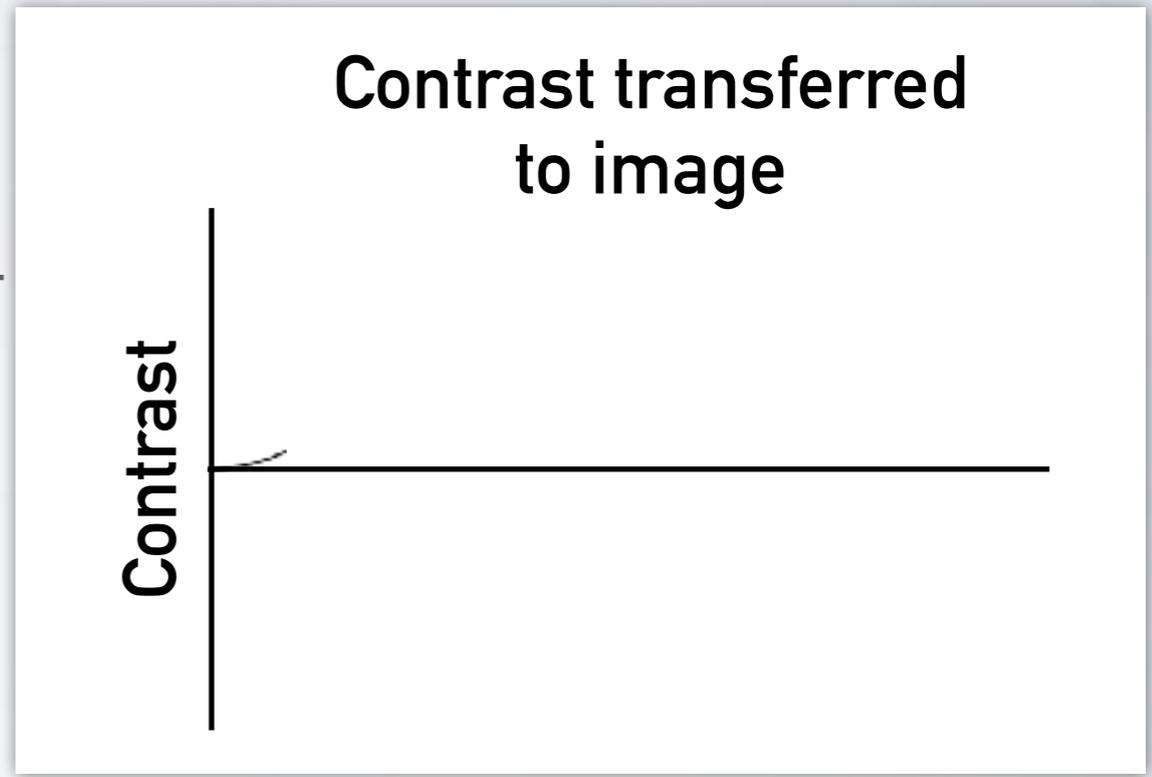
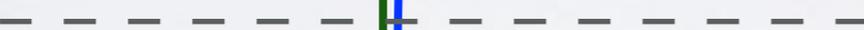
Contrast



sample



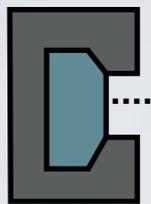
back focal plane



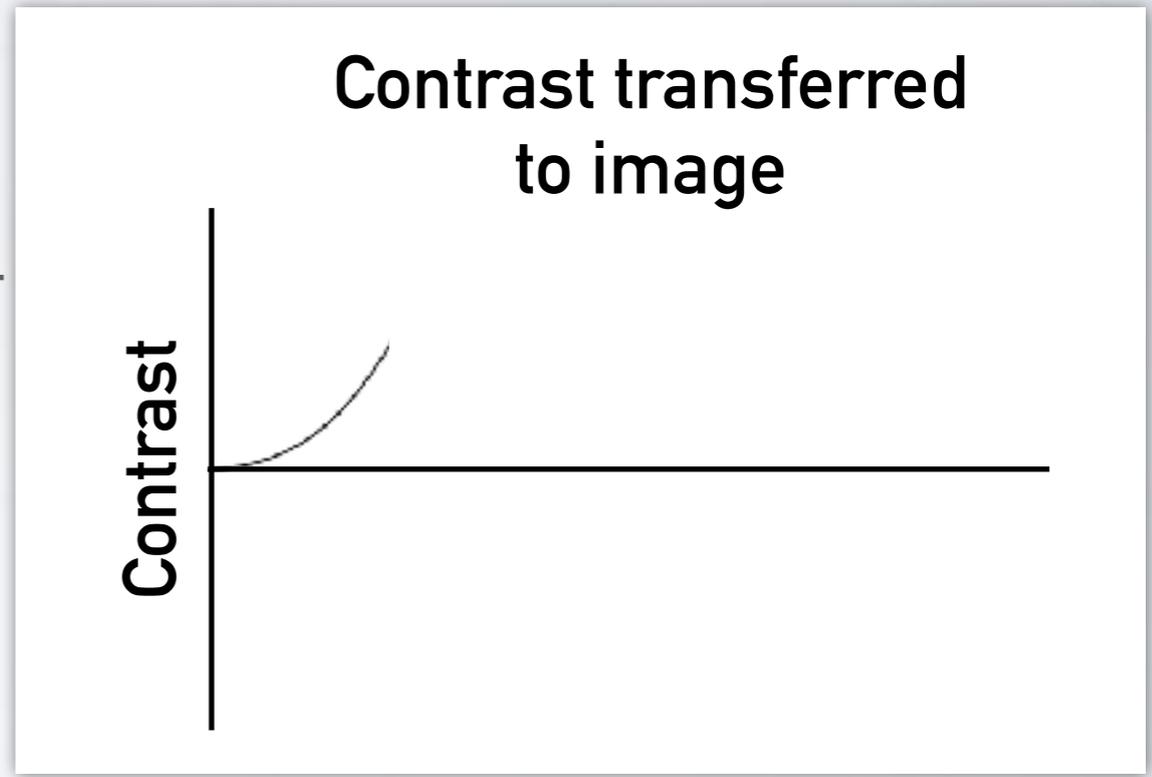
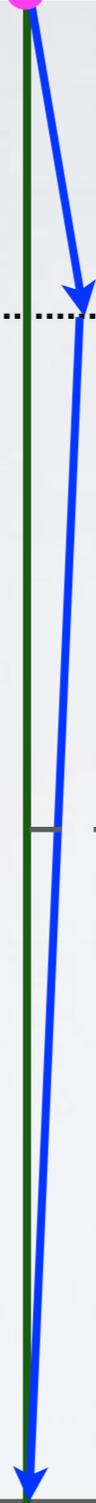
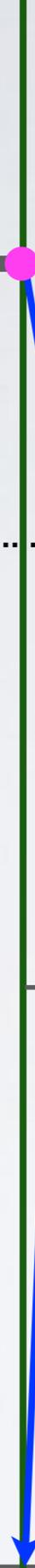
sample



EM
lens



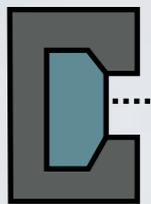
back focal plane



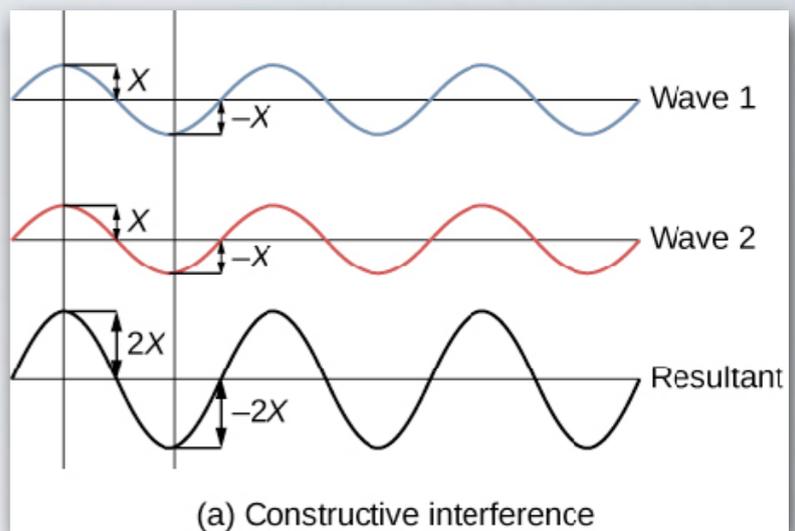
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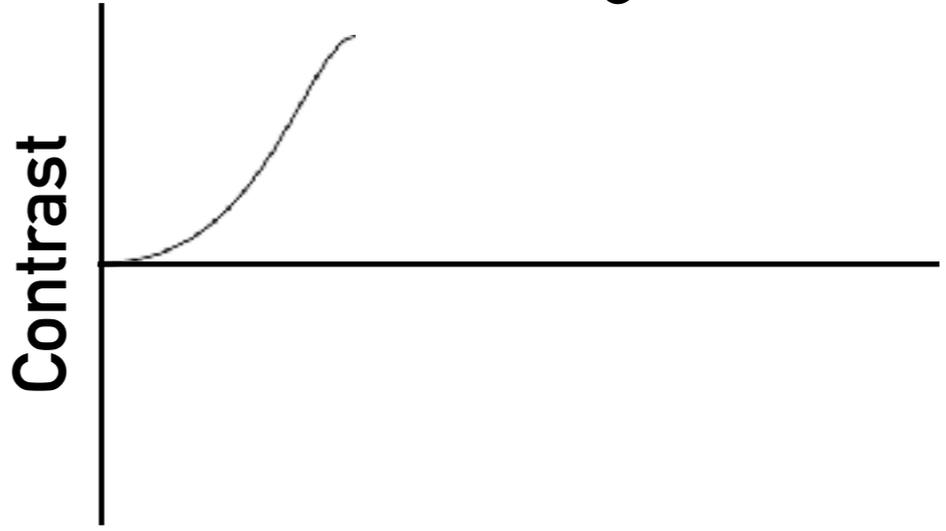
EM lens



back focal plane



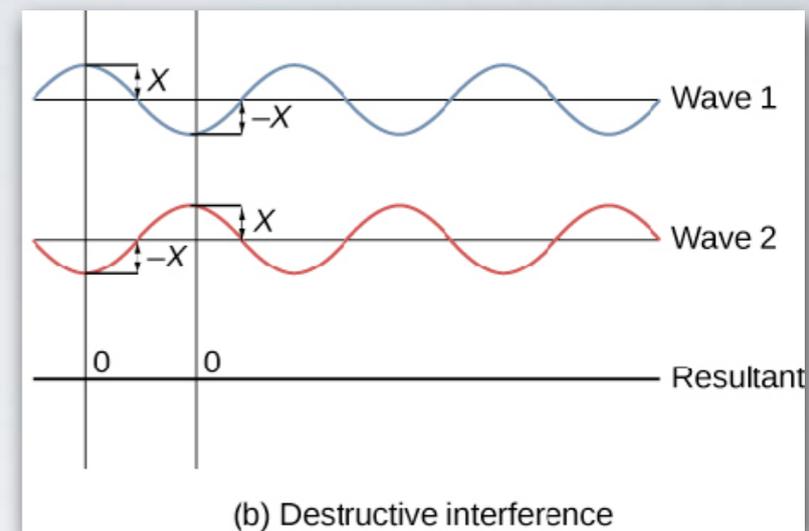
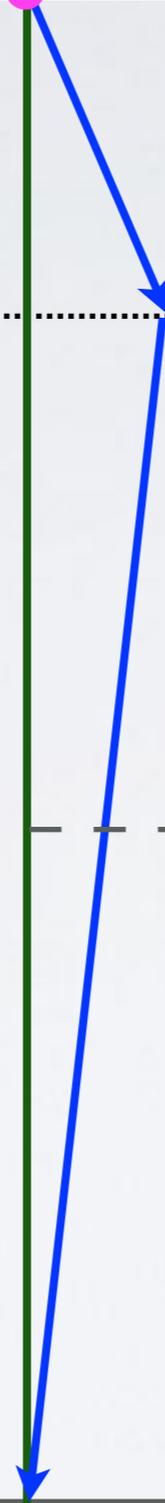
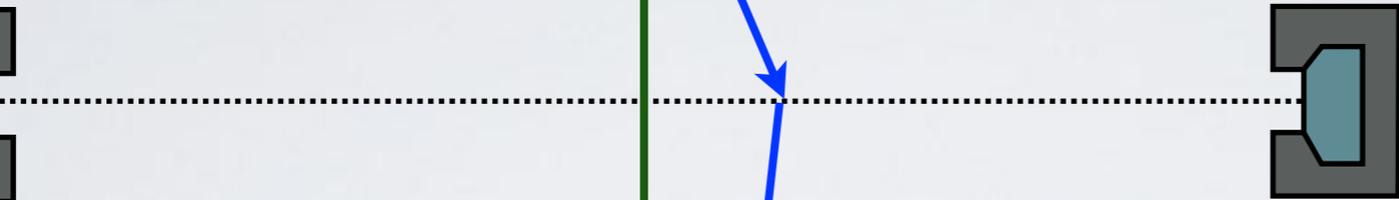
Contrast transferred to image



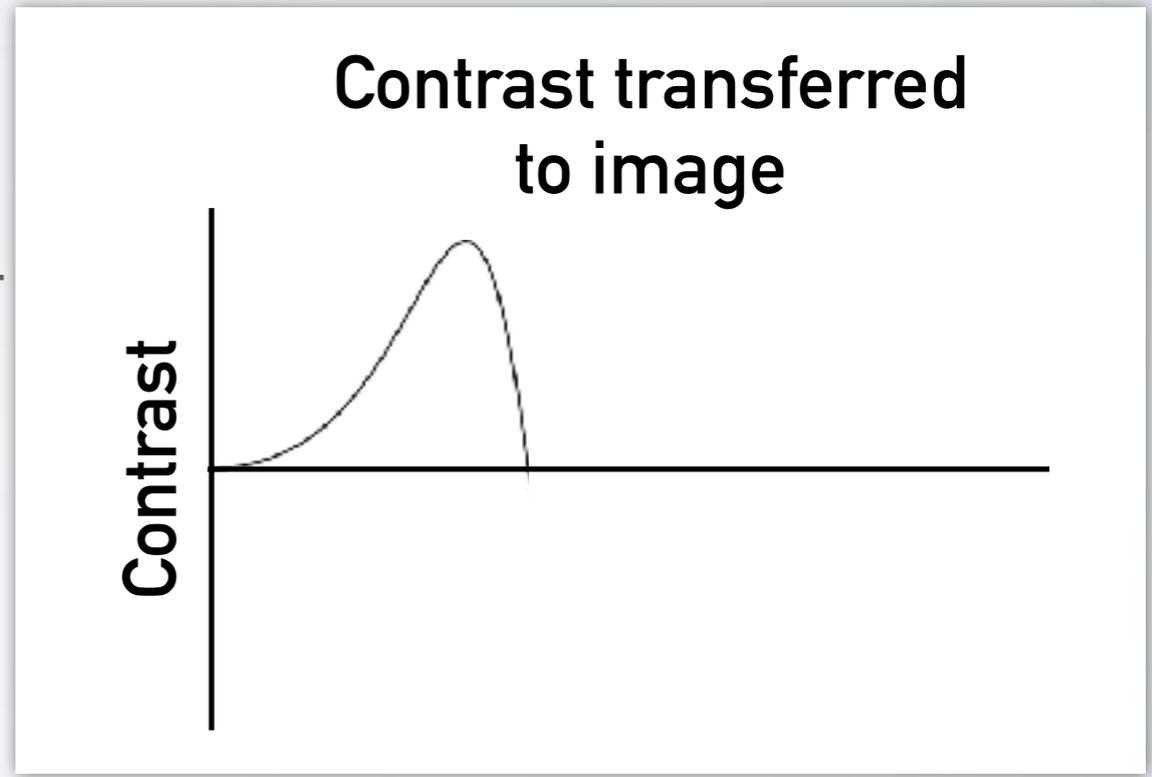
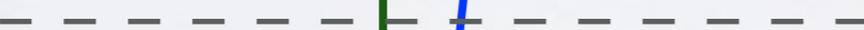
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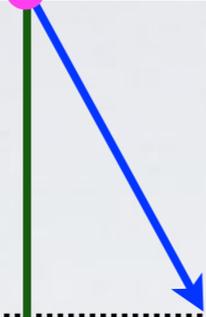
EM lens



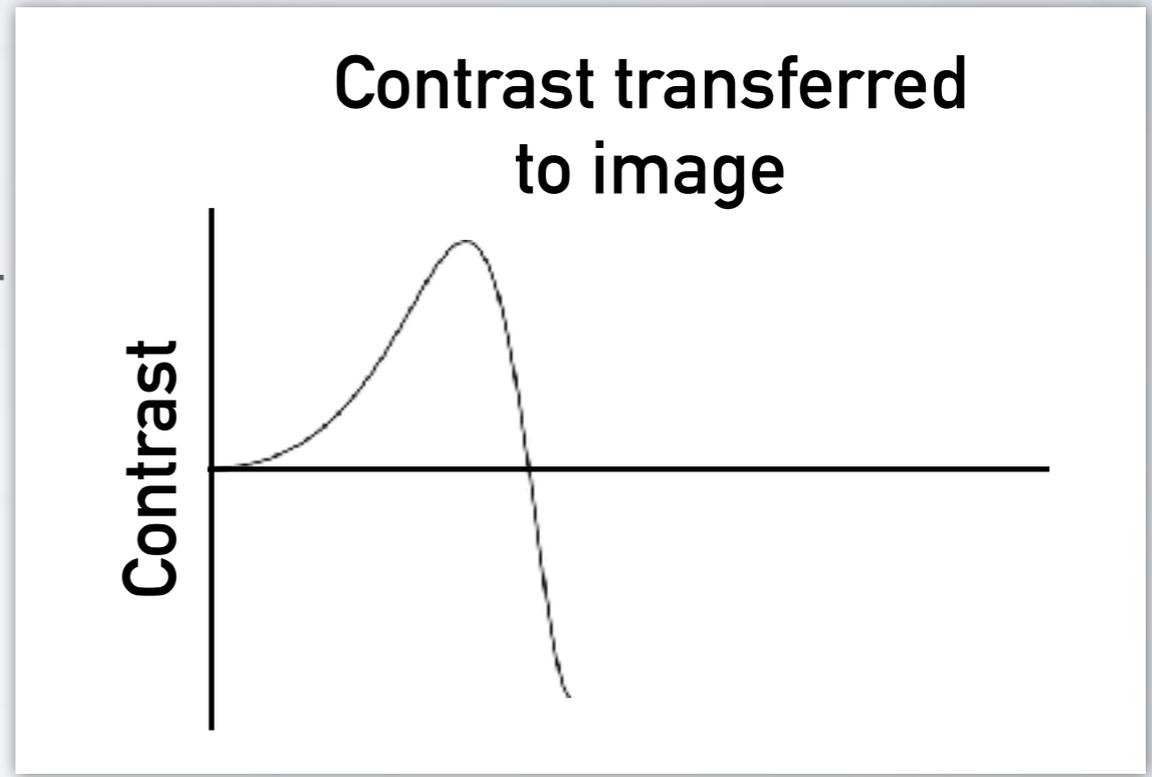
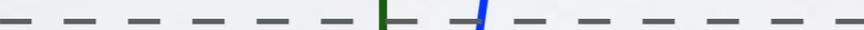
back focal plane



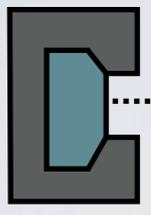
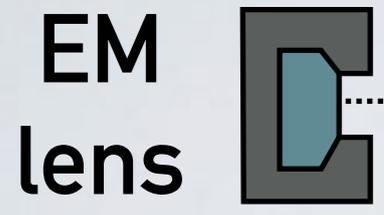
sample



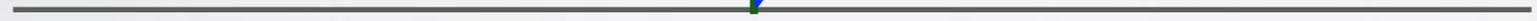
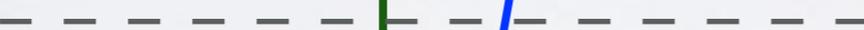
back focal plane



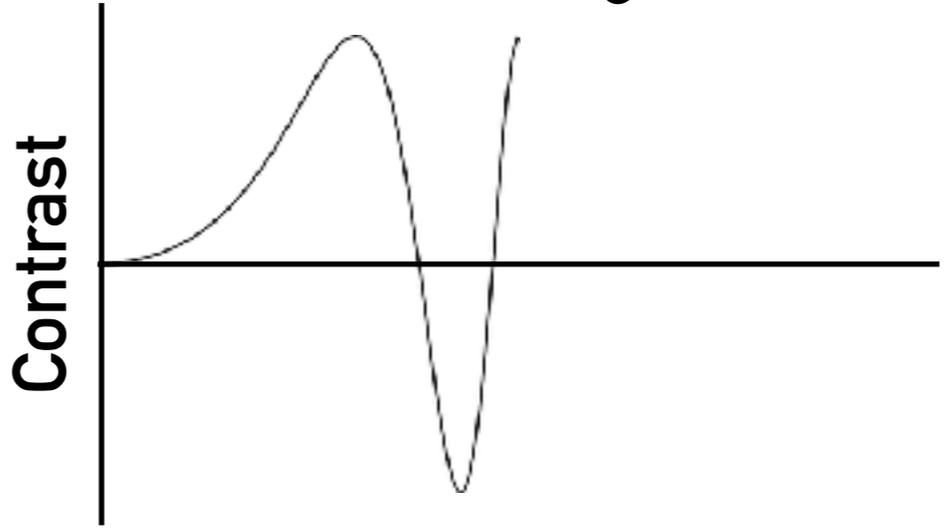
sample



back focal plane



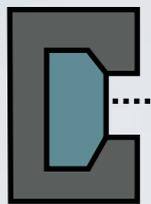
Contrast transferred to image



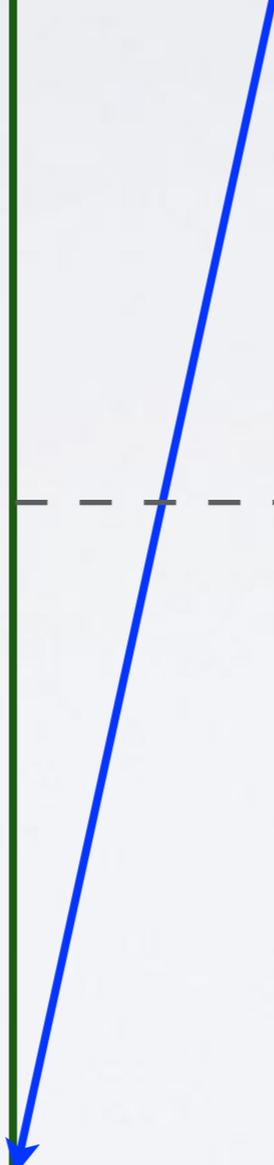
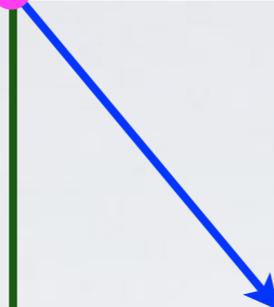
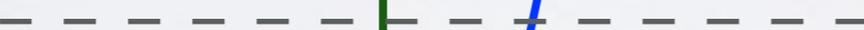
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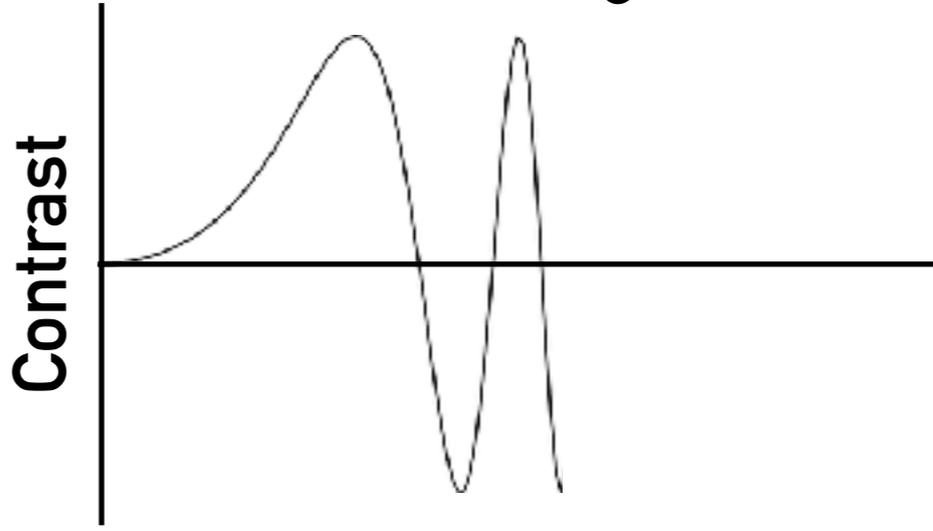
EM lens



back focal plane



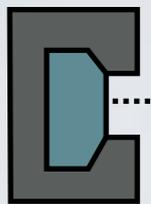
Contrast transferred to image



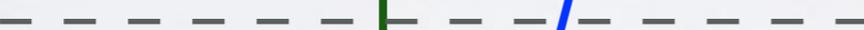
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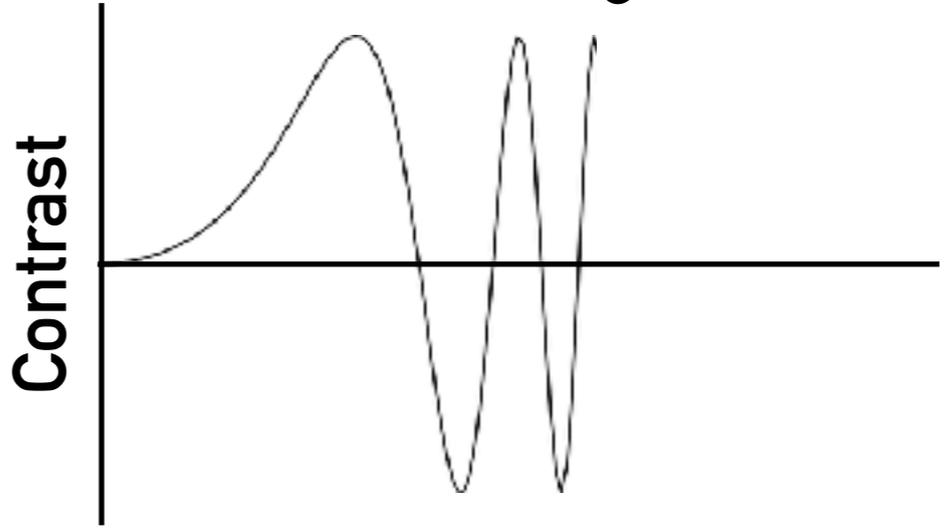
EM
lens



back focal plane



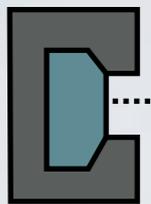
Contrast transferred
to image



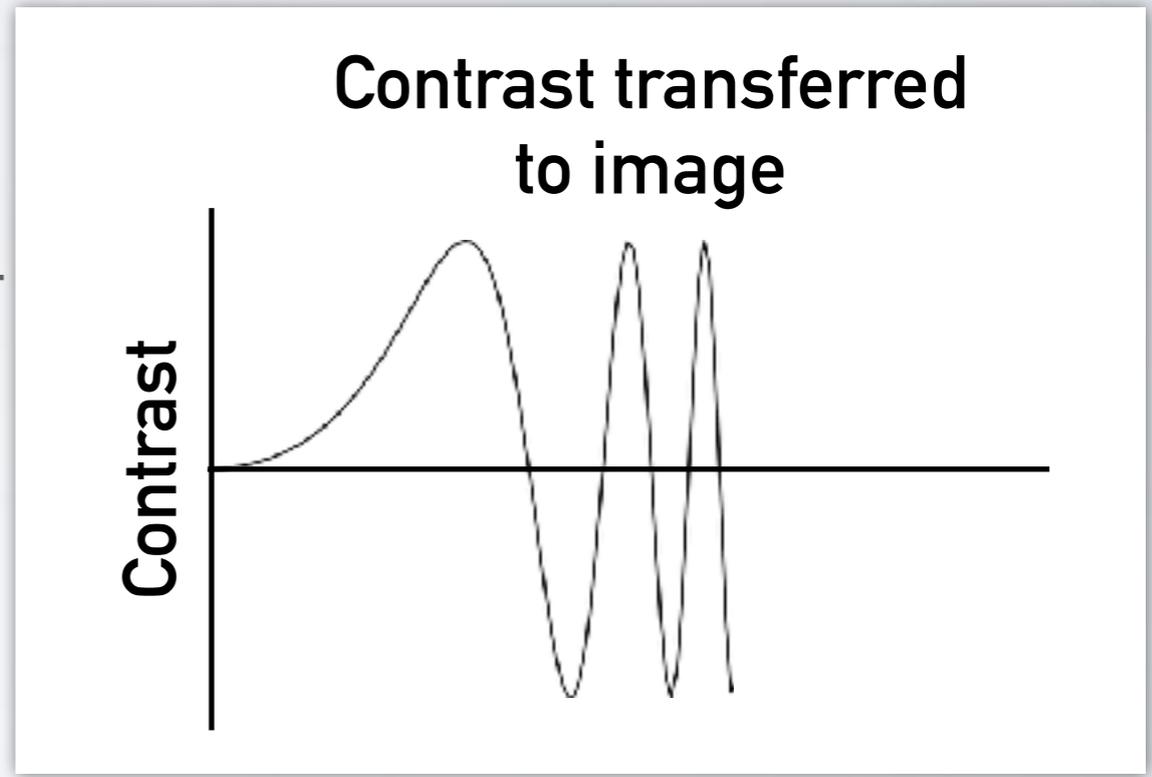
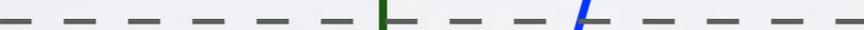
sample



EM
lens



back focal plane



sample



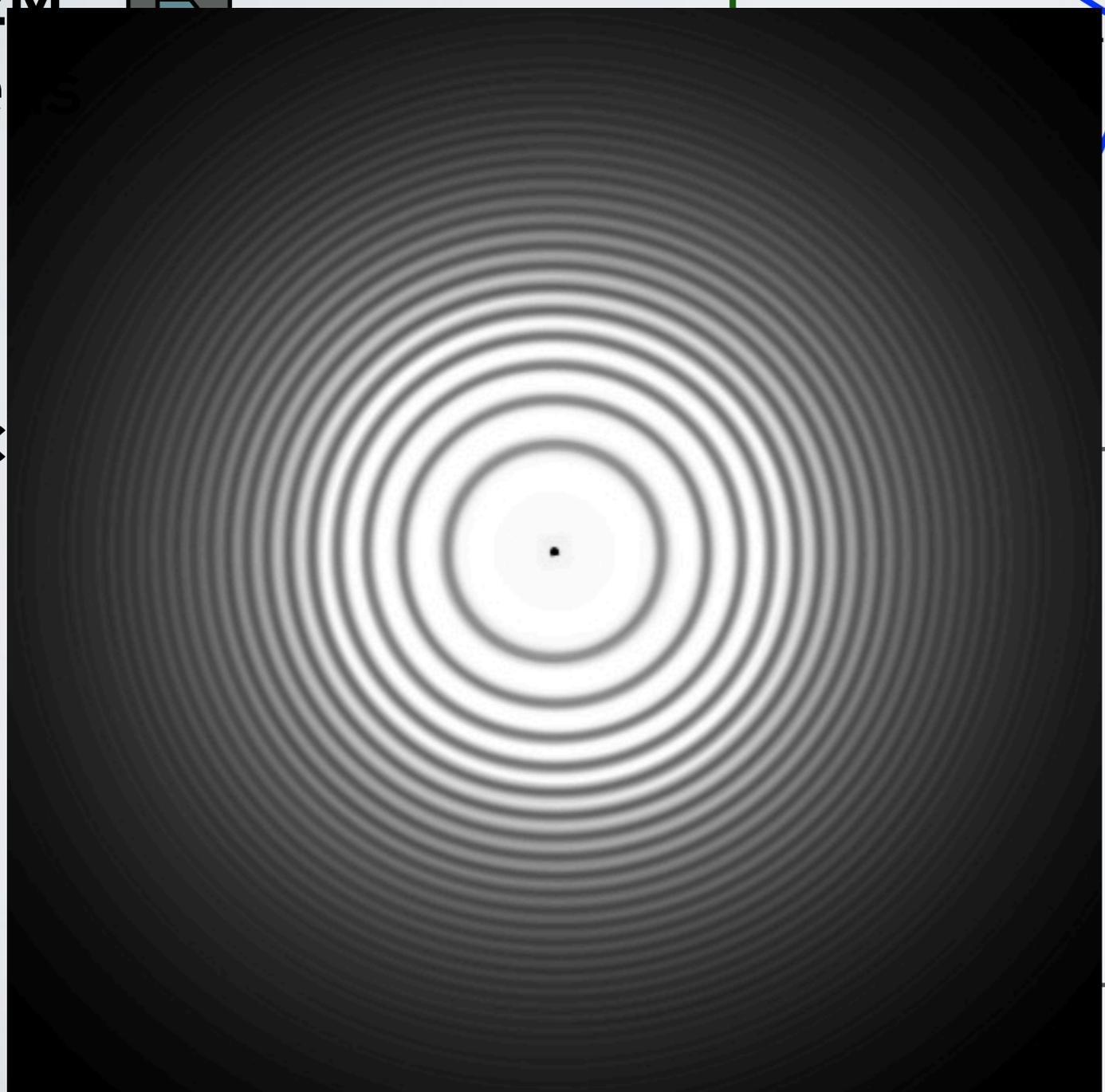
EM



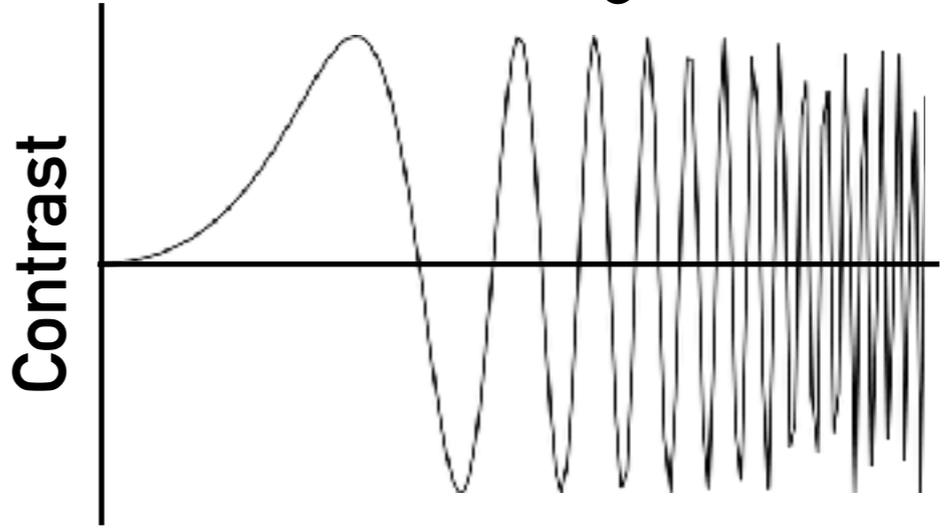
le



bac



Contrast transferred to image



The Contrast Transfer Function

$$\text{CTF}(\vec{s}) = \sqrt{1 - A^2} \cdot \sin(\gamma(\vec{s})) + A \cdot \cos(\gamma(\vec{s}))$$

$$\gamma(\vec{s}) = -\frac{\pi}{2} C_s \lambda^3 s^4 + \pi \lambda z(\theta) s^2$$

s = spatial frequency

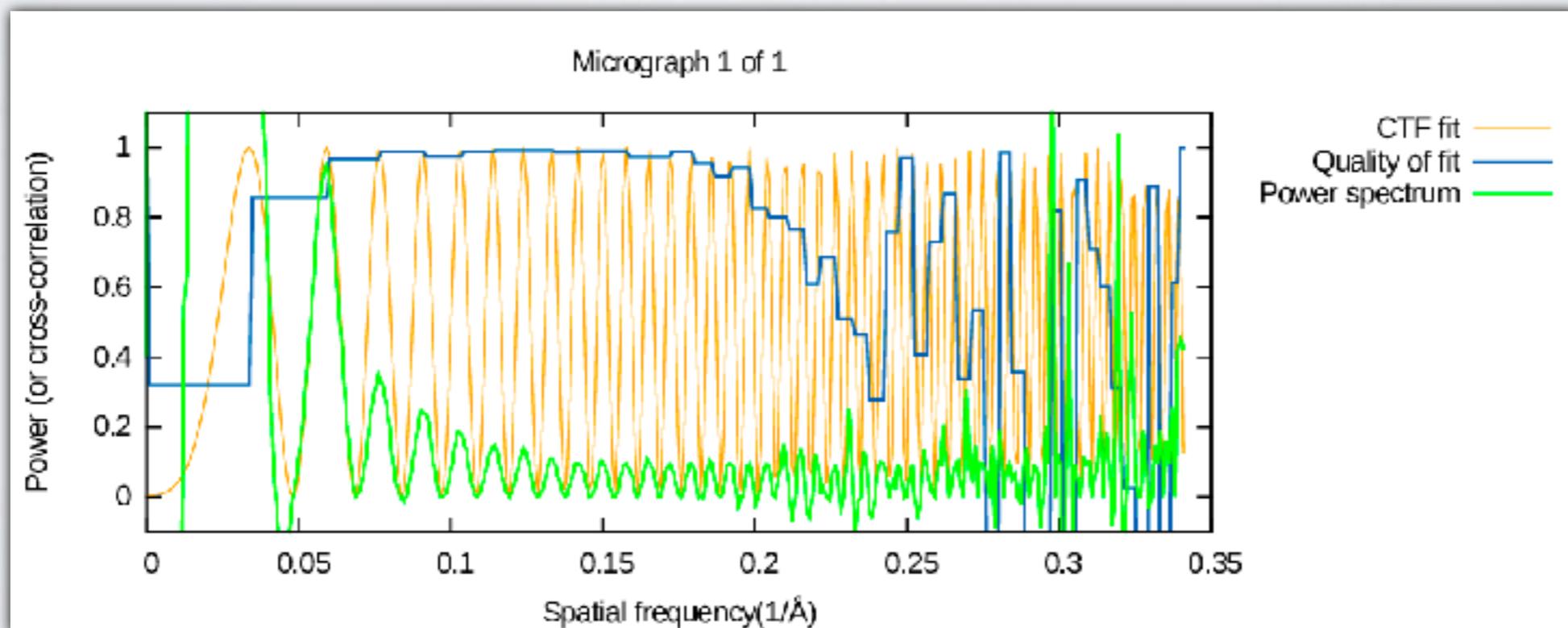
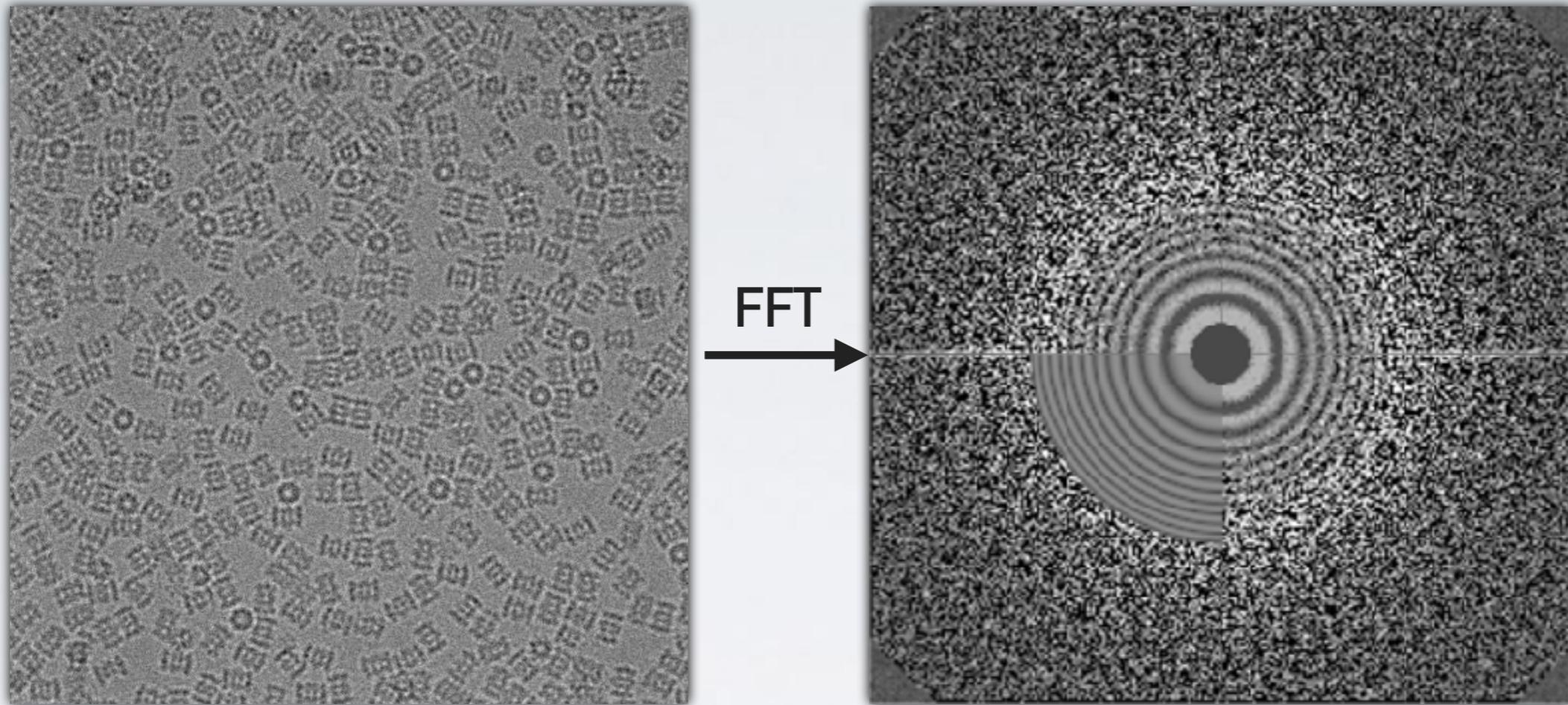
A = amplitude contrast

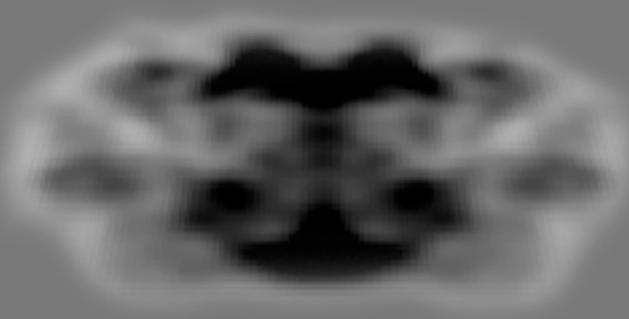
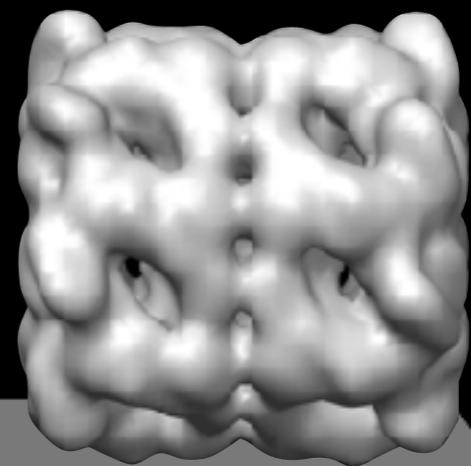
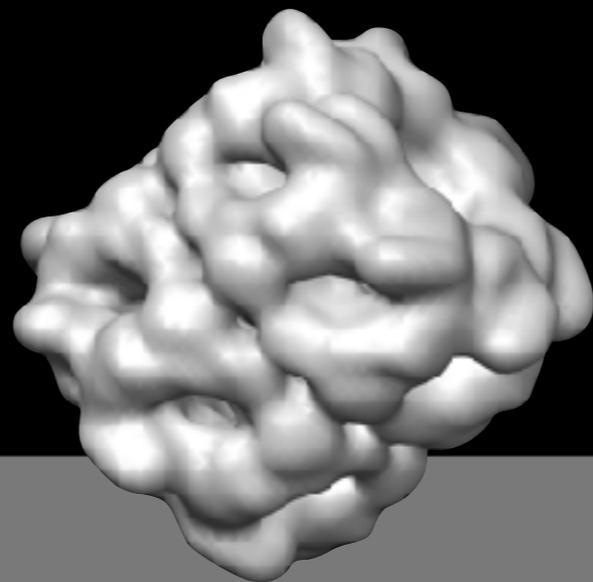
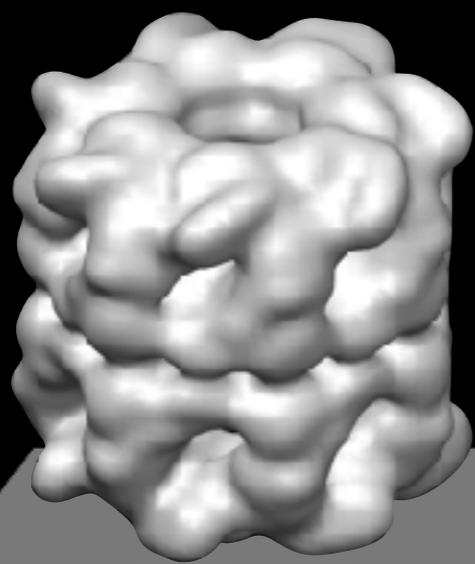
C_s = spherical aberration

λ = wavelength of electrons

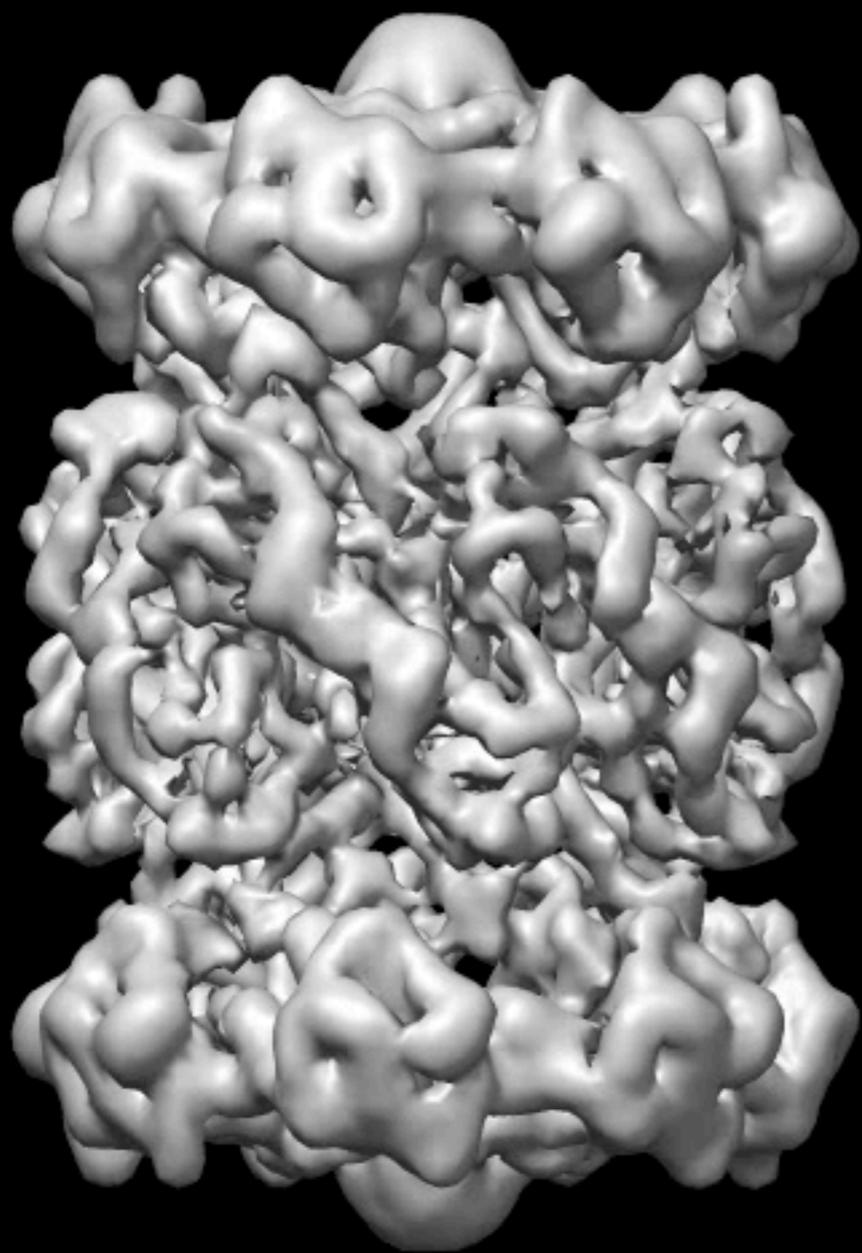
$z(\theta)$ = defocus

Estimating the CTF

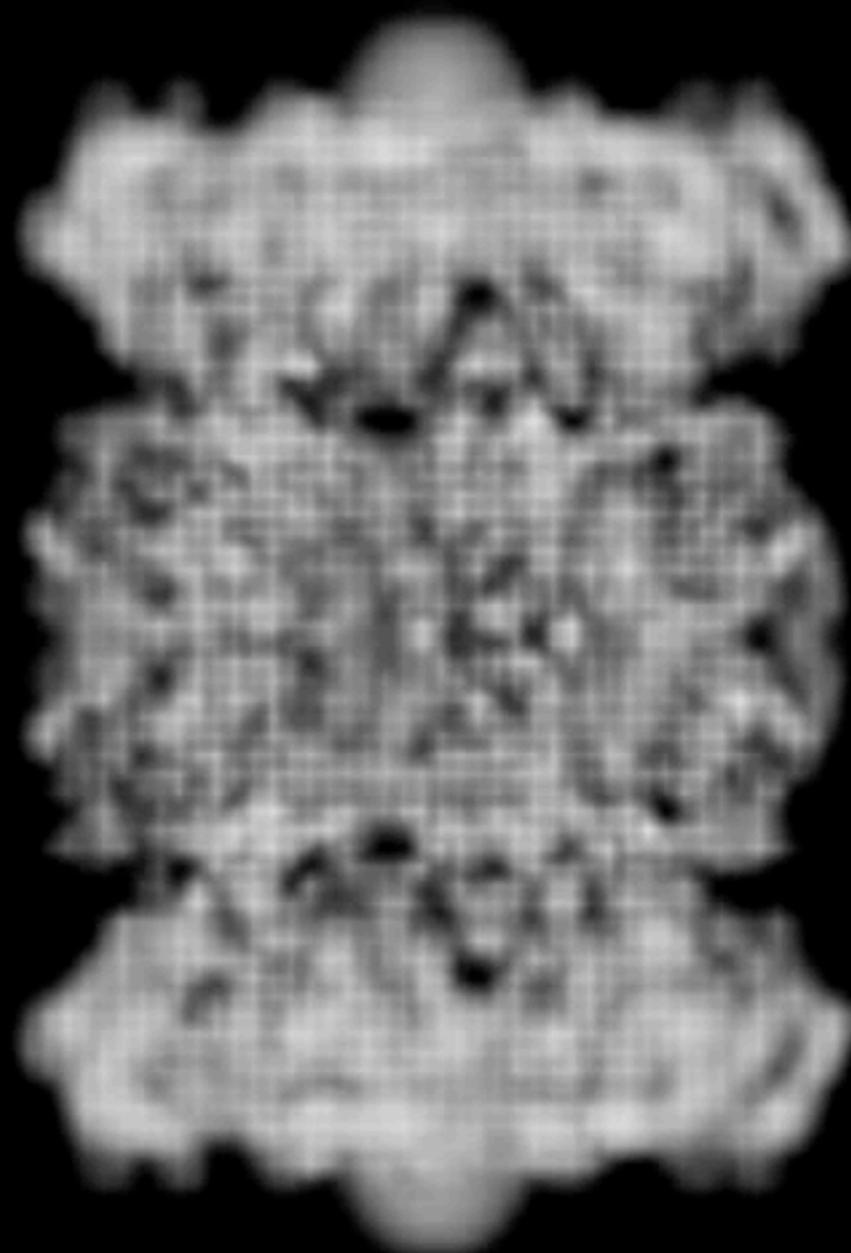




3D object

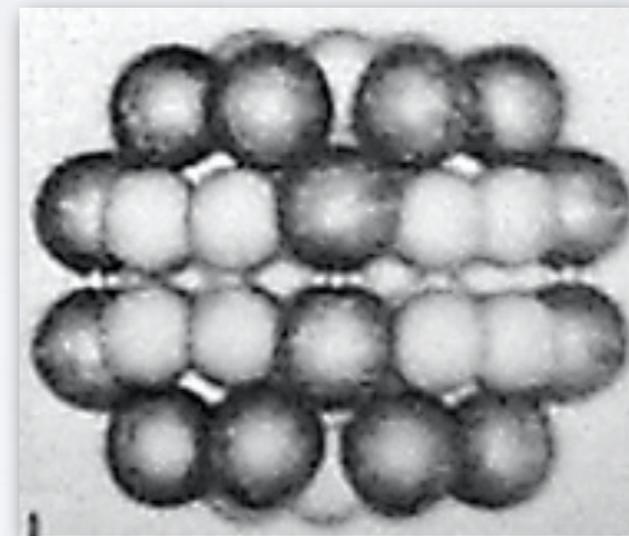
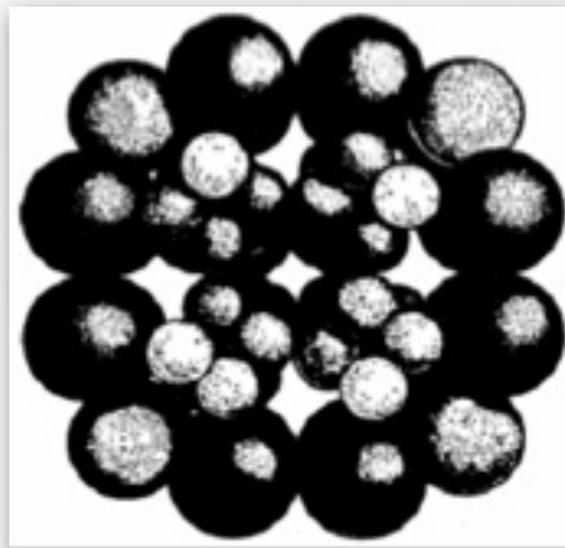
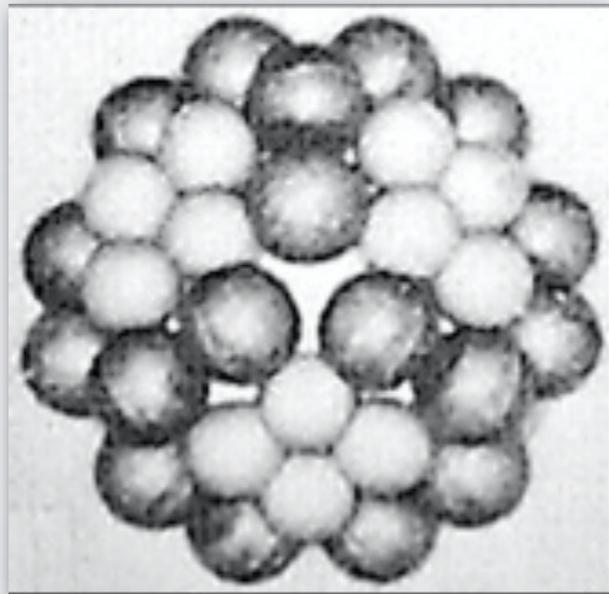
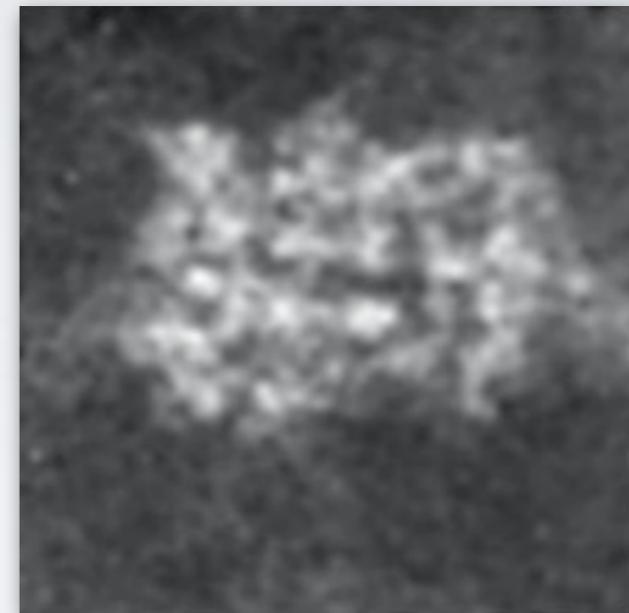
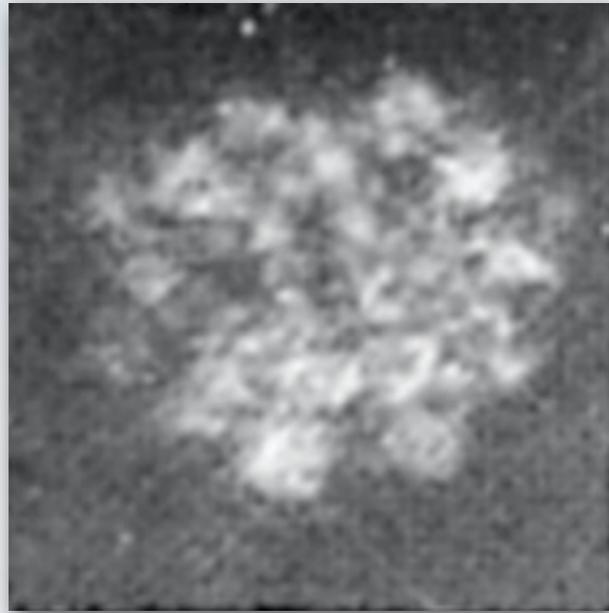


2D projection



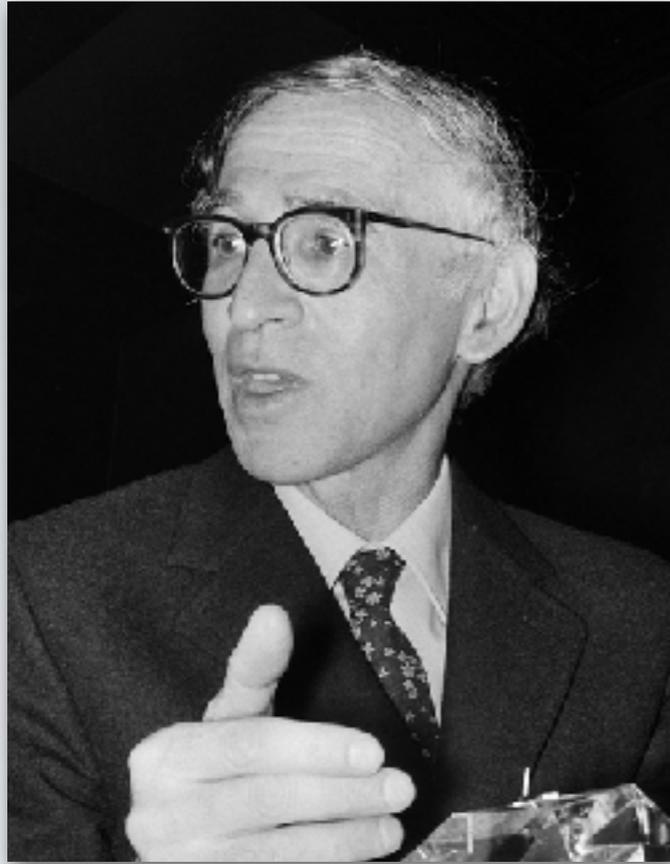
“Image analysis” of biological specimens circa 1965

pyruvate dehydrogenase



Reed & Cox, The Enzymes, 1970

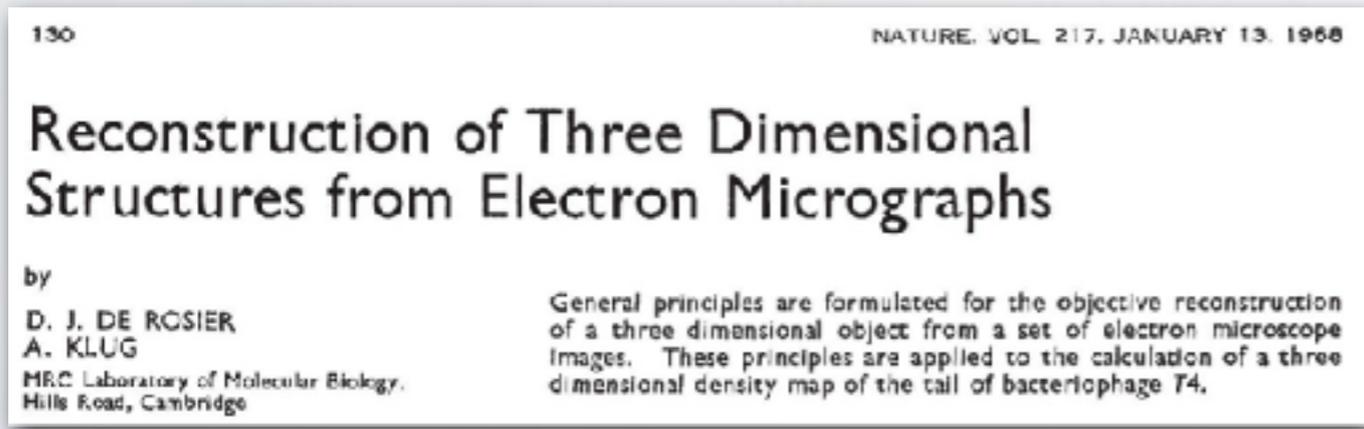
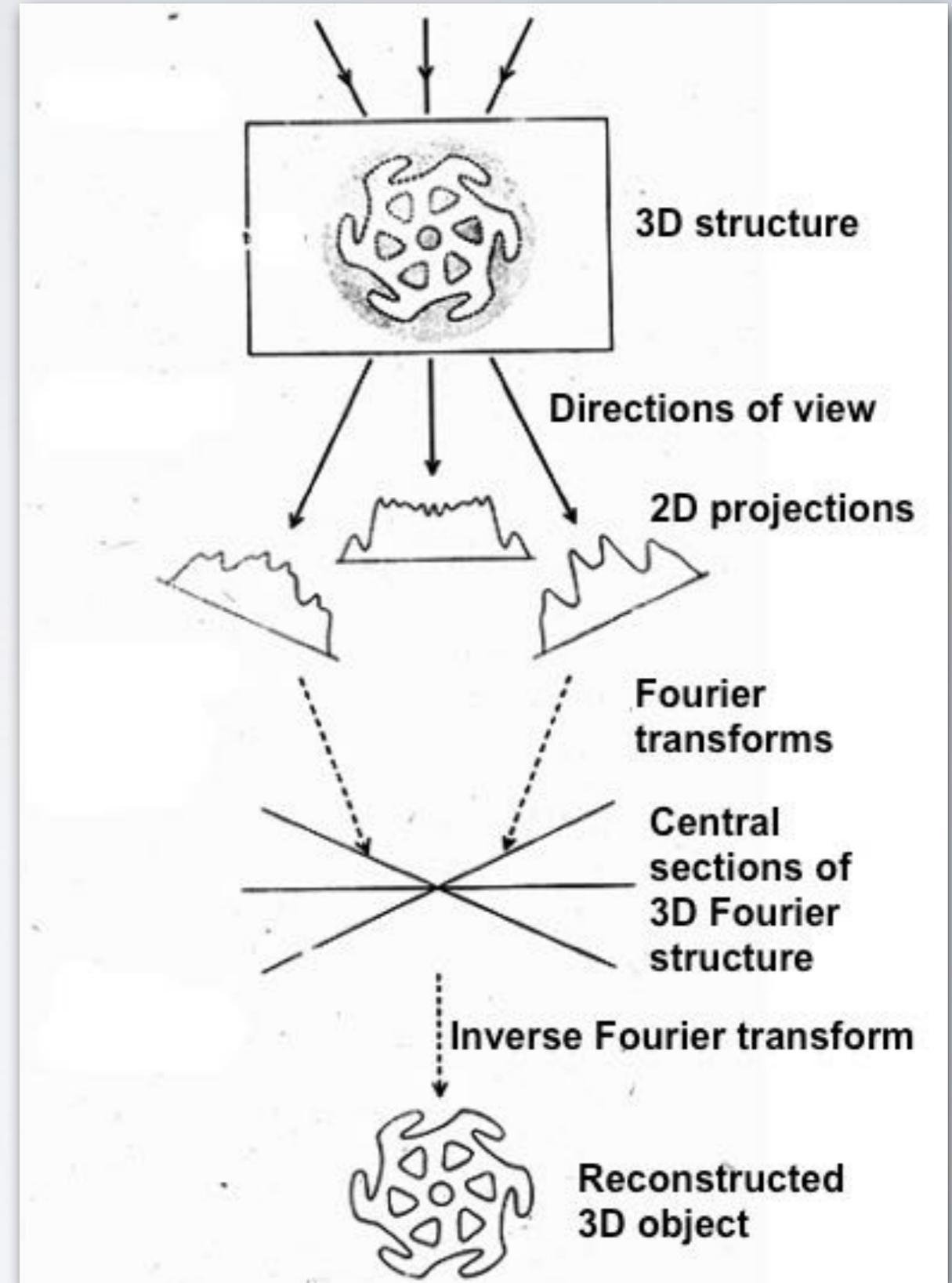
Introduction of 3D Reconstruction



Aaron Klug

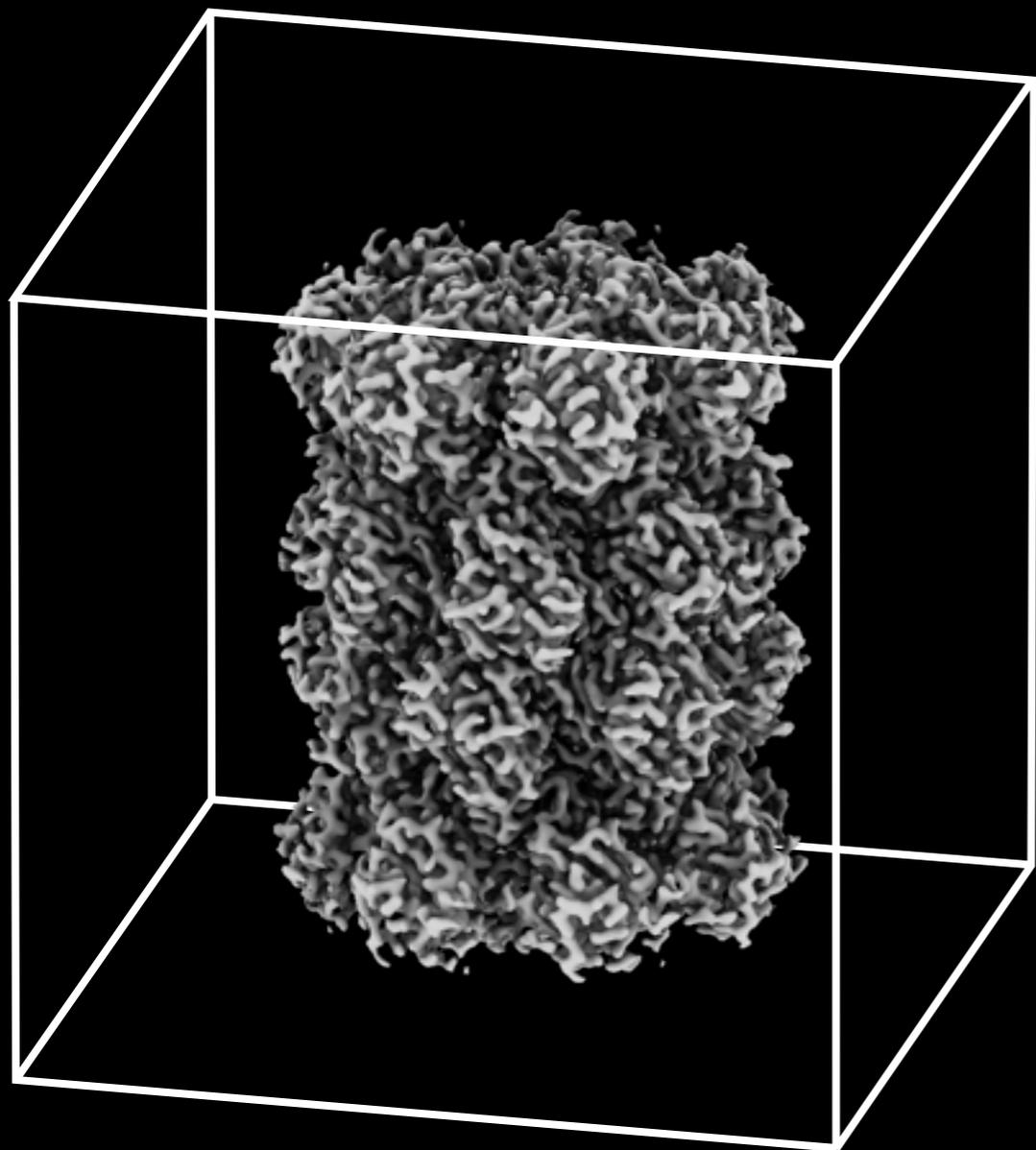


David DeRosier

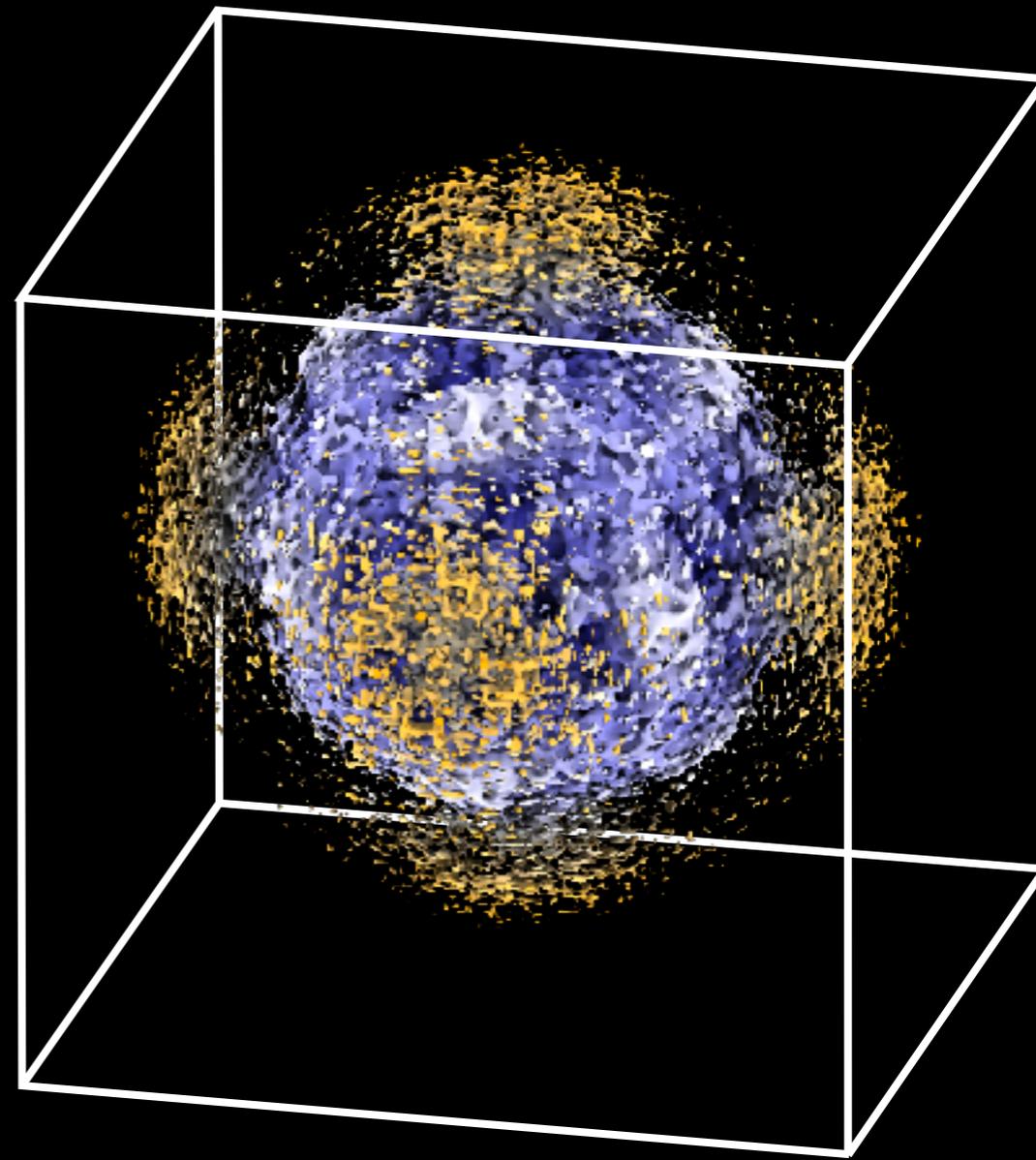


DeRosier & Klug, Nature 1968

Real Space Structure



Fourier Structure



FT

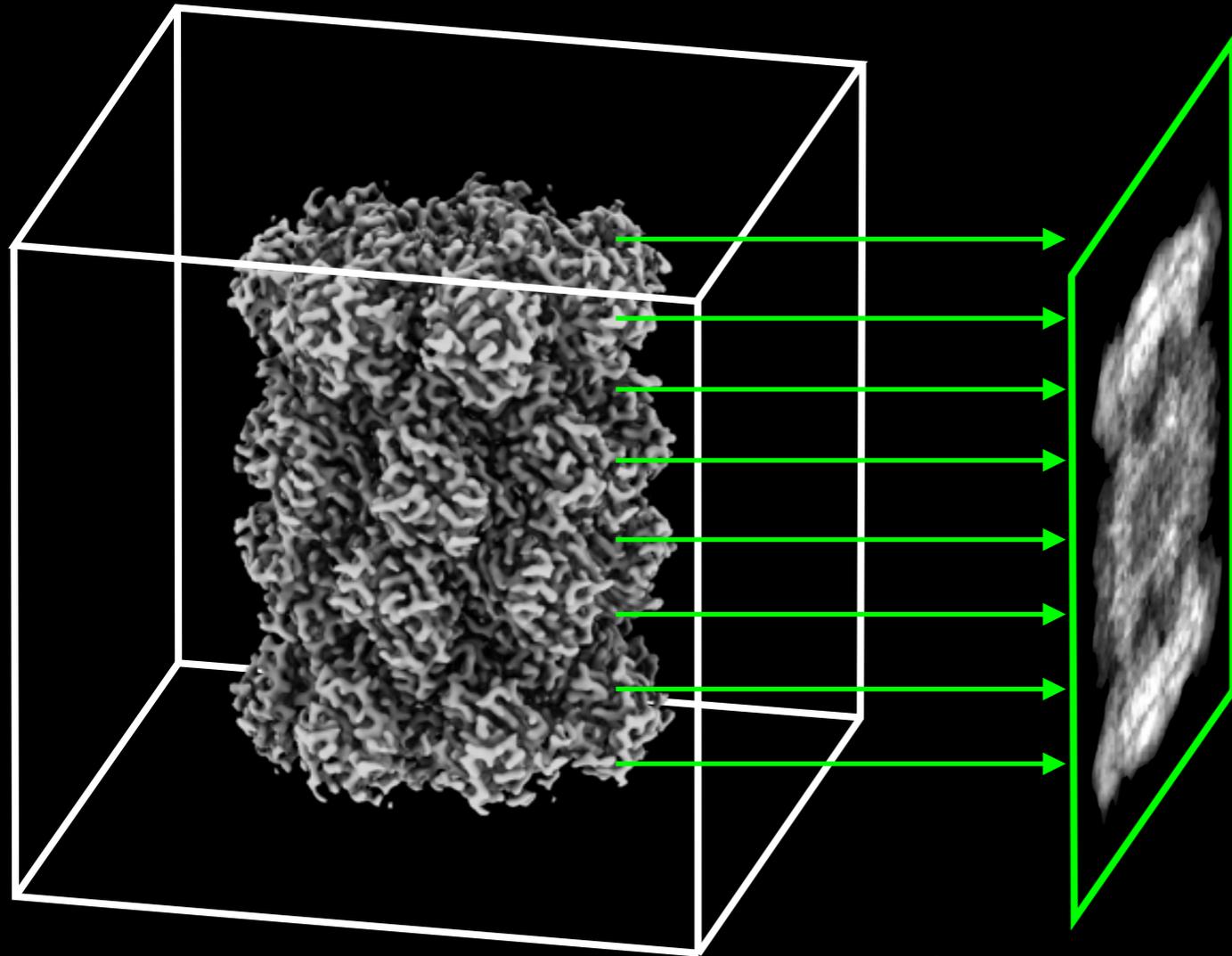


inverse



FT

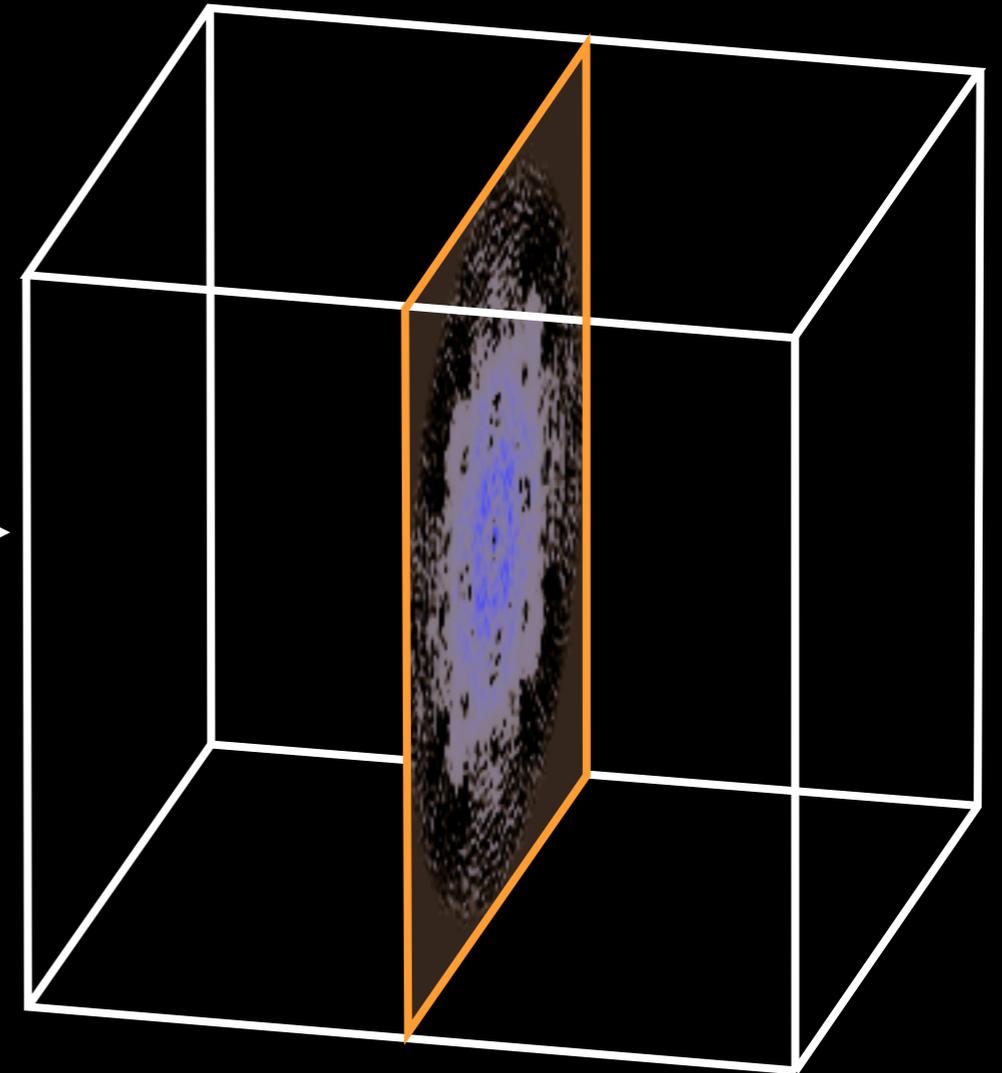
Real Space Structure



2D Projection

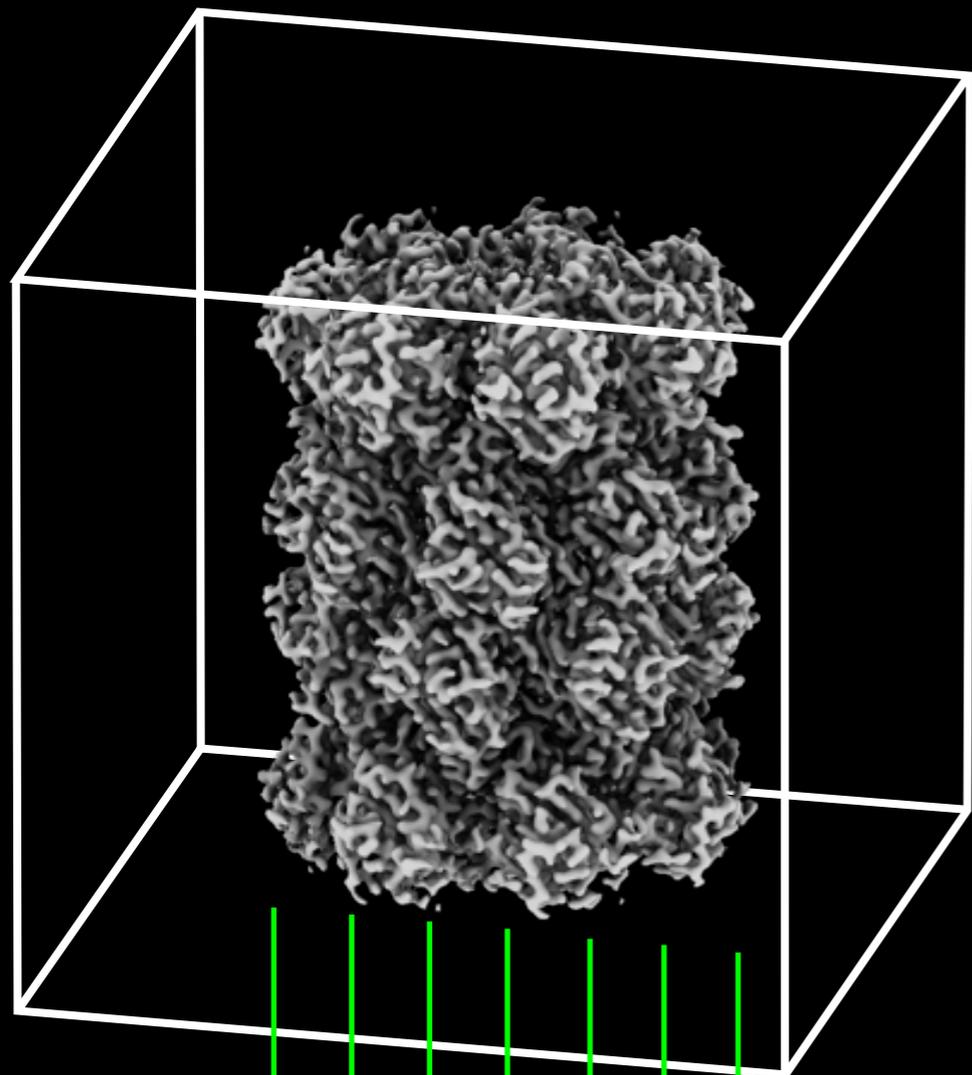
FT
↓

Fourier Structure

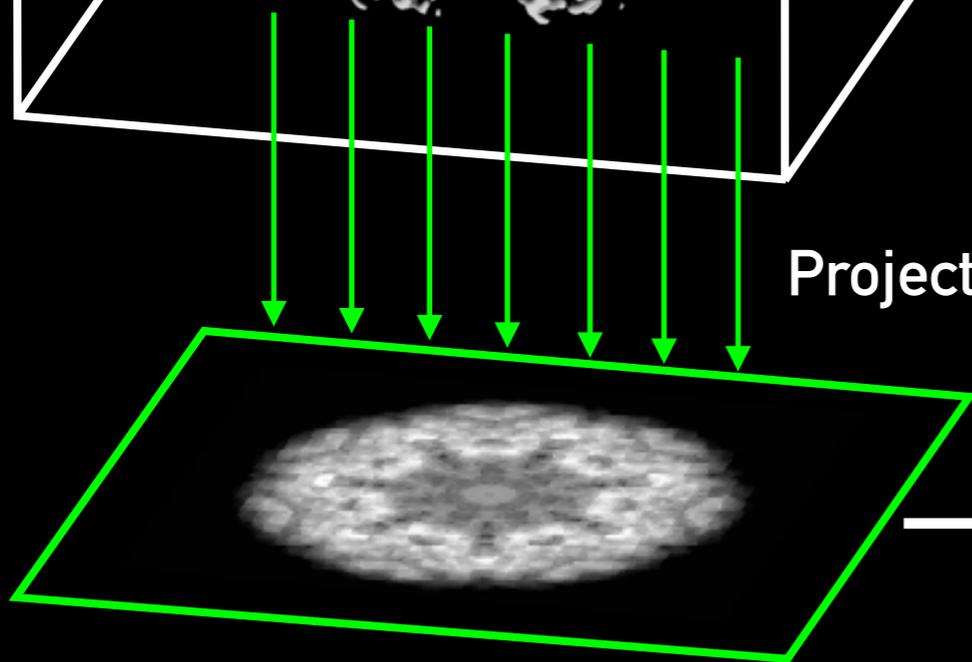


Central Slice of 3D
Fourier Structure

Real Space

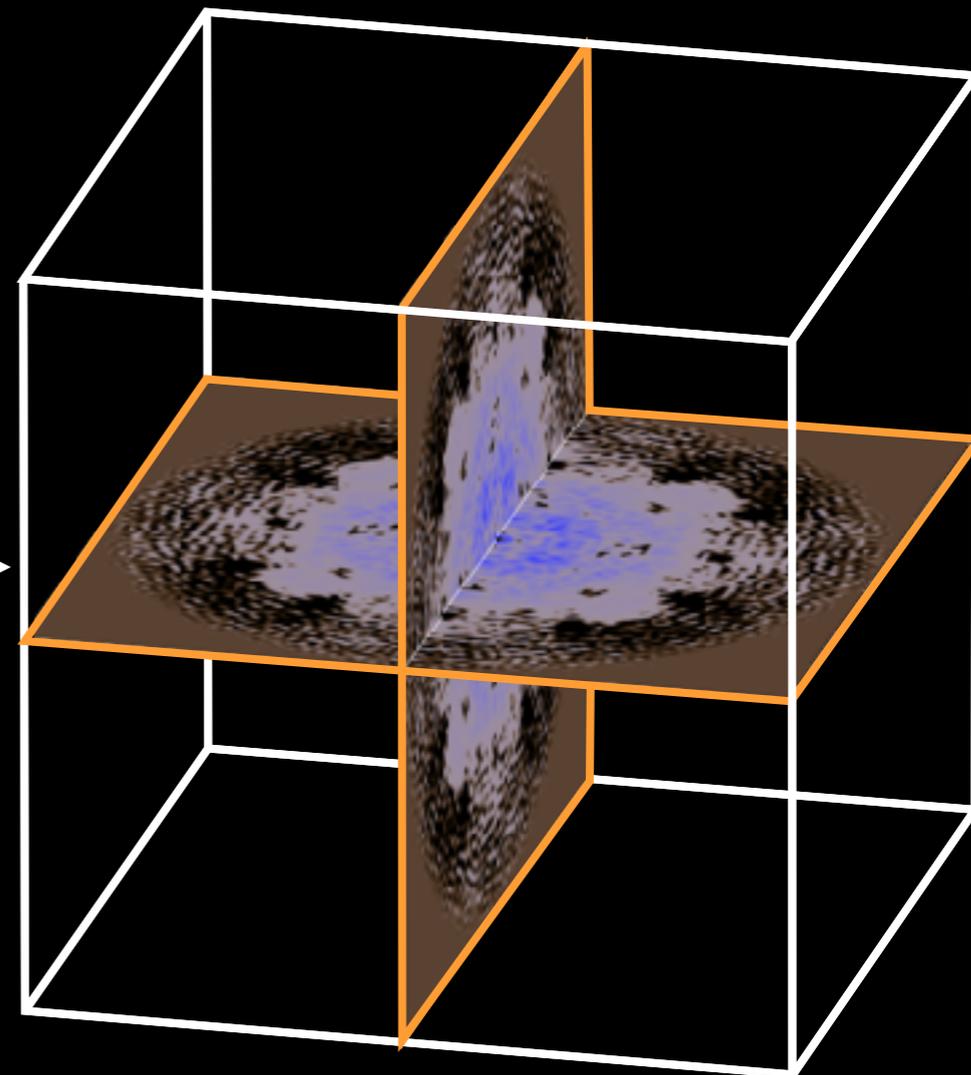


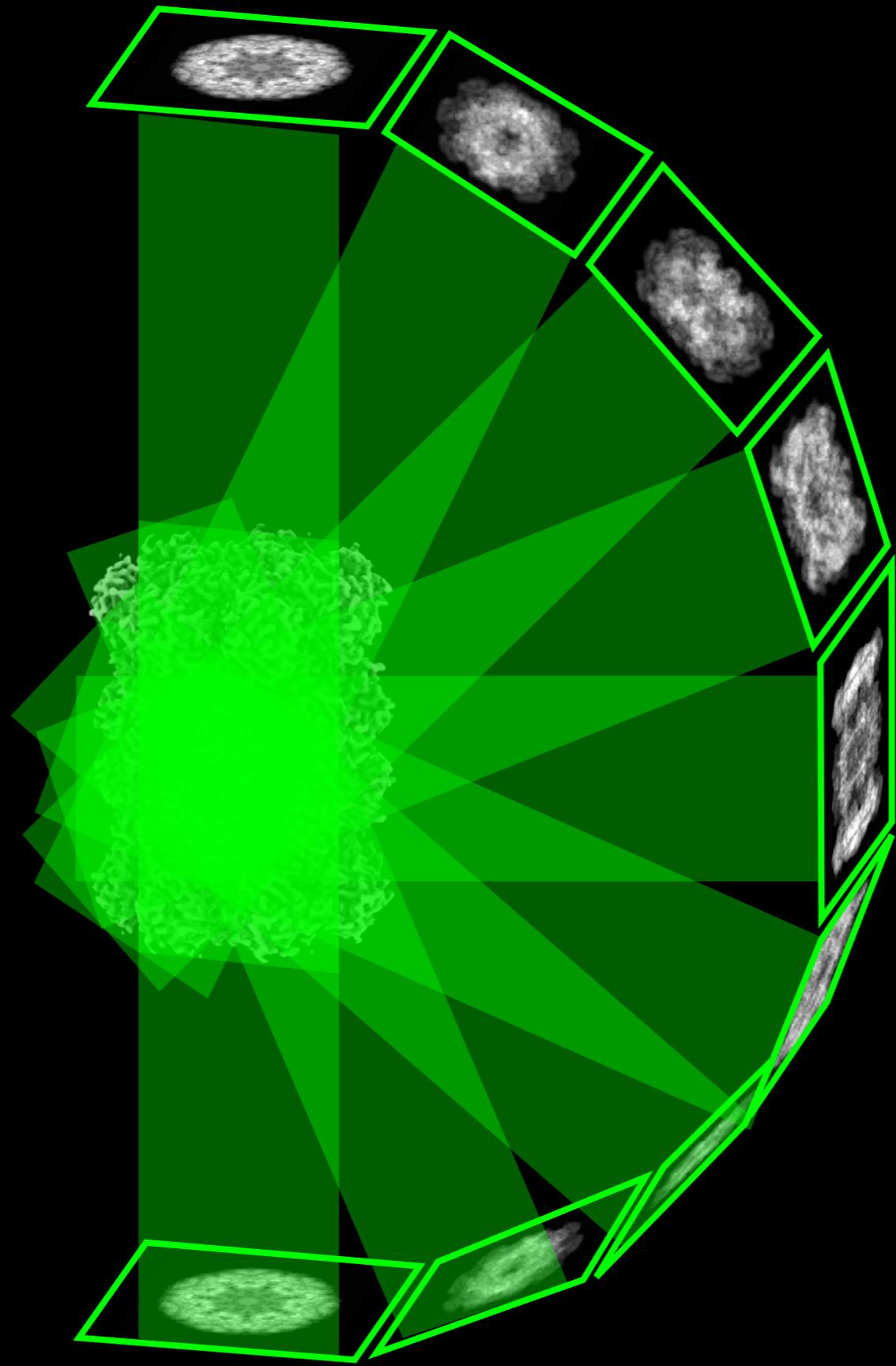
Projection



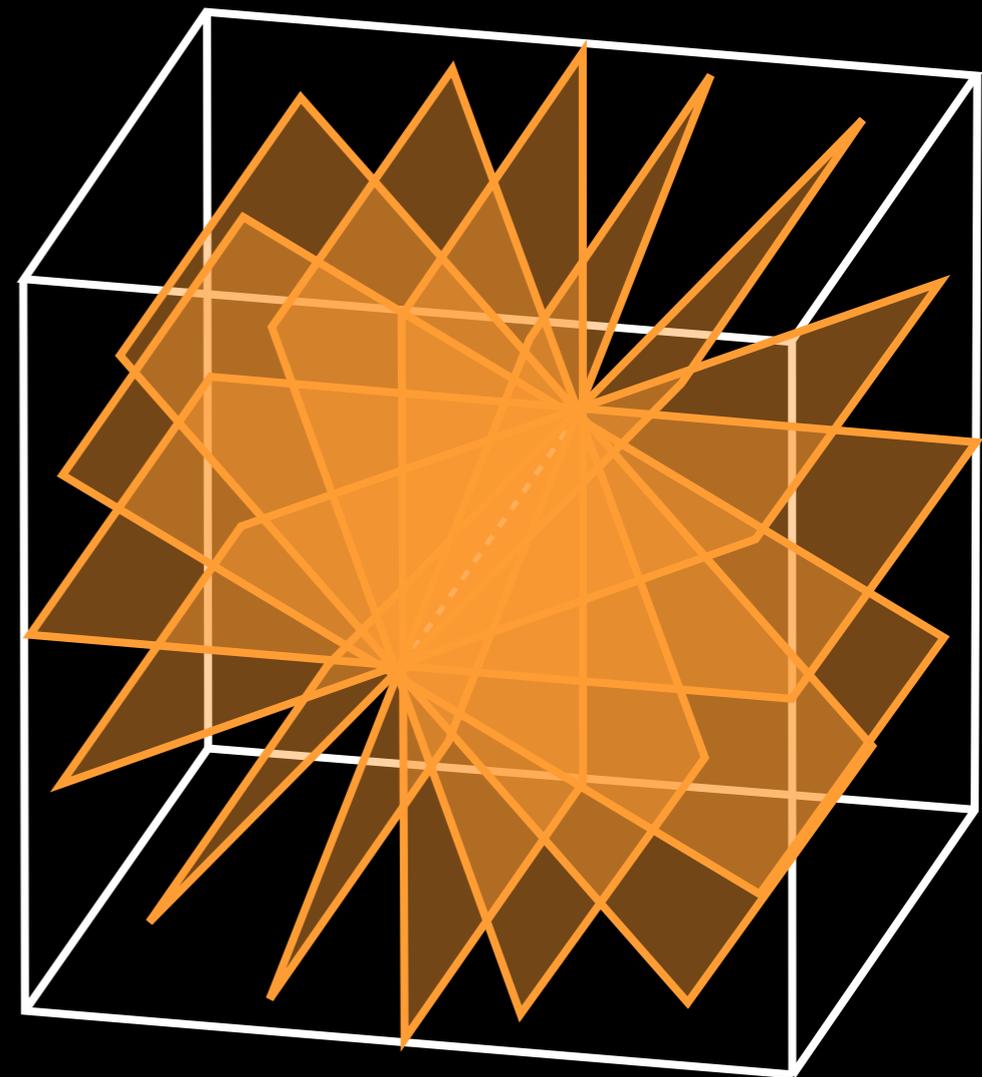
FT

Fourier Structure

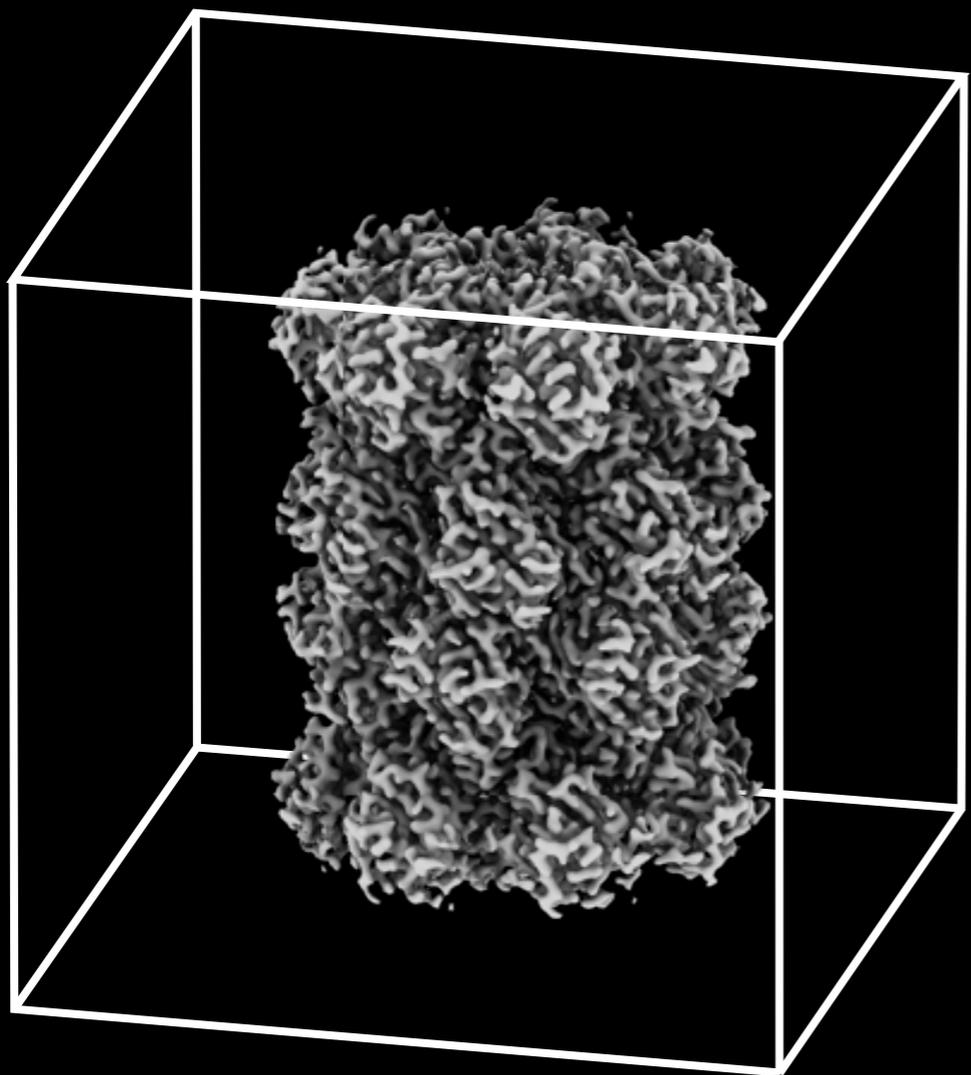




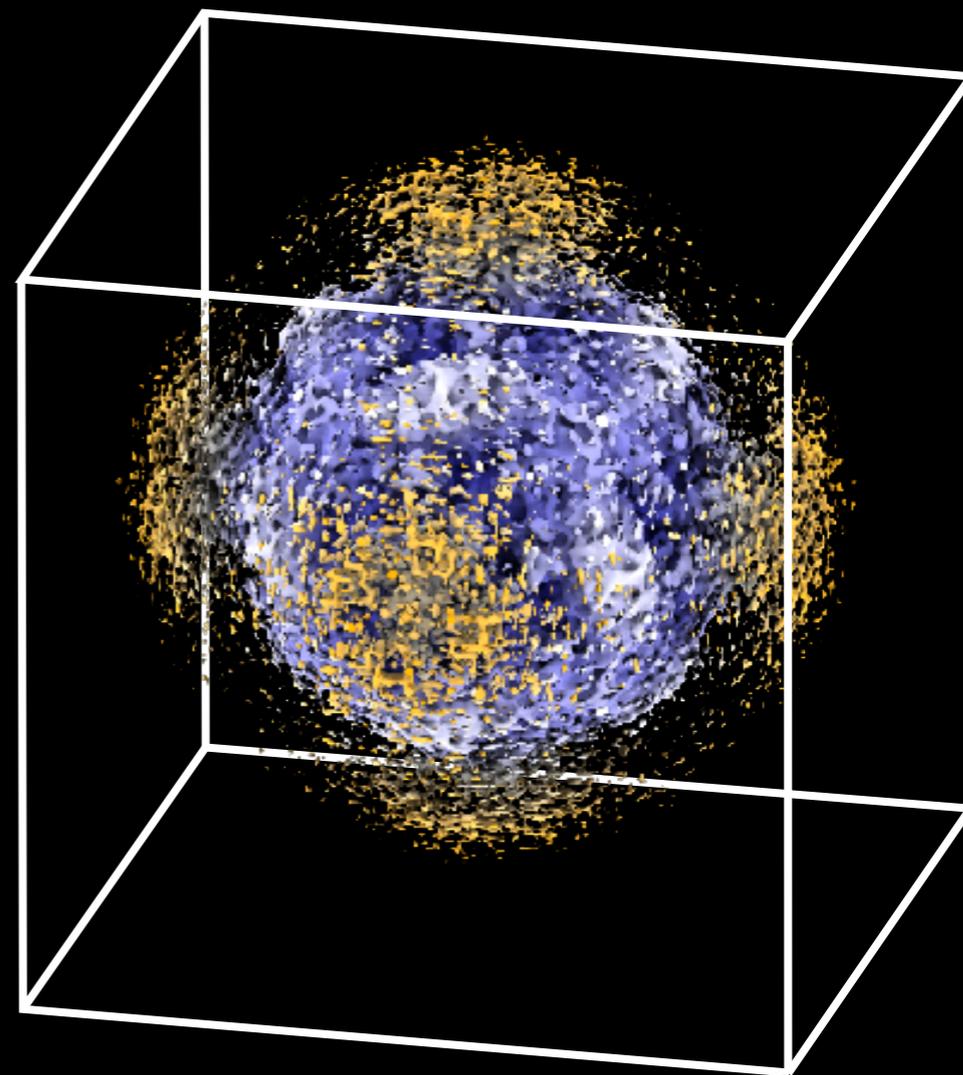
Fourier Structure



Real Space Structure

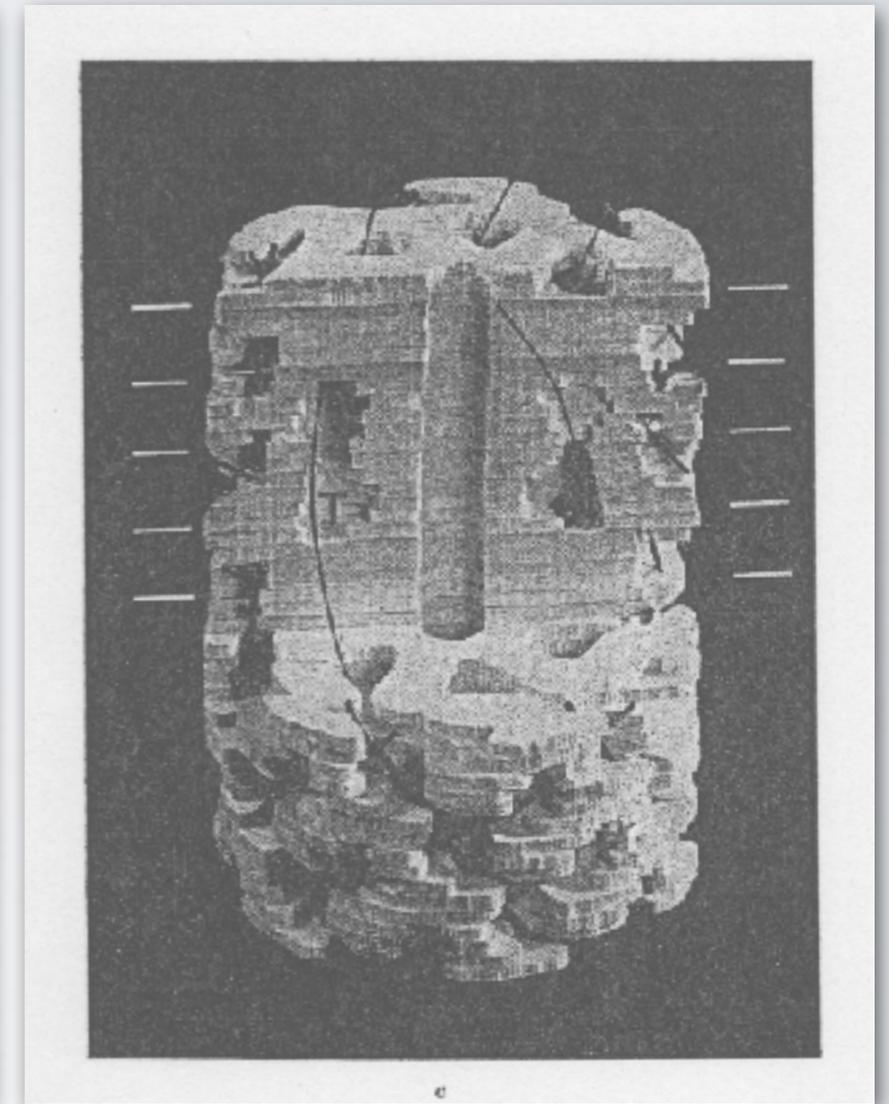
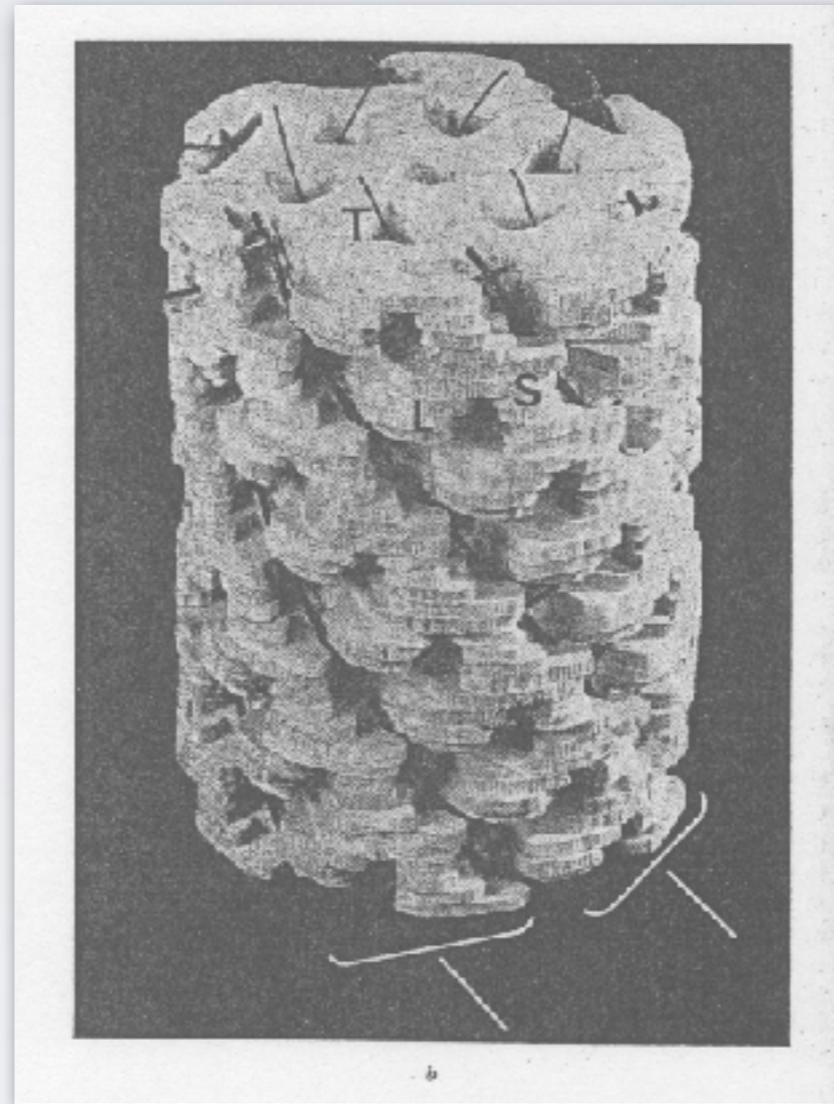
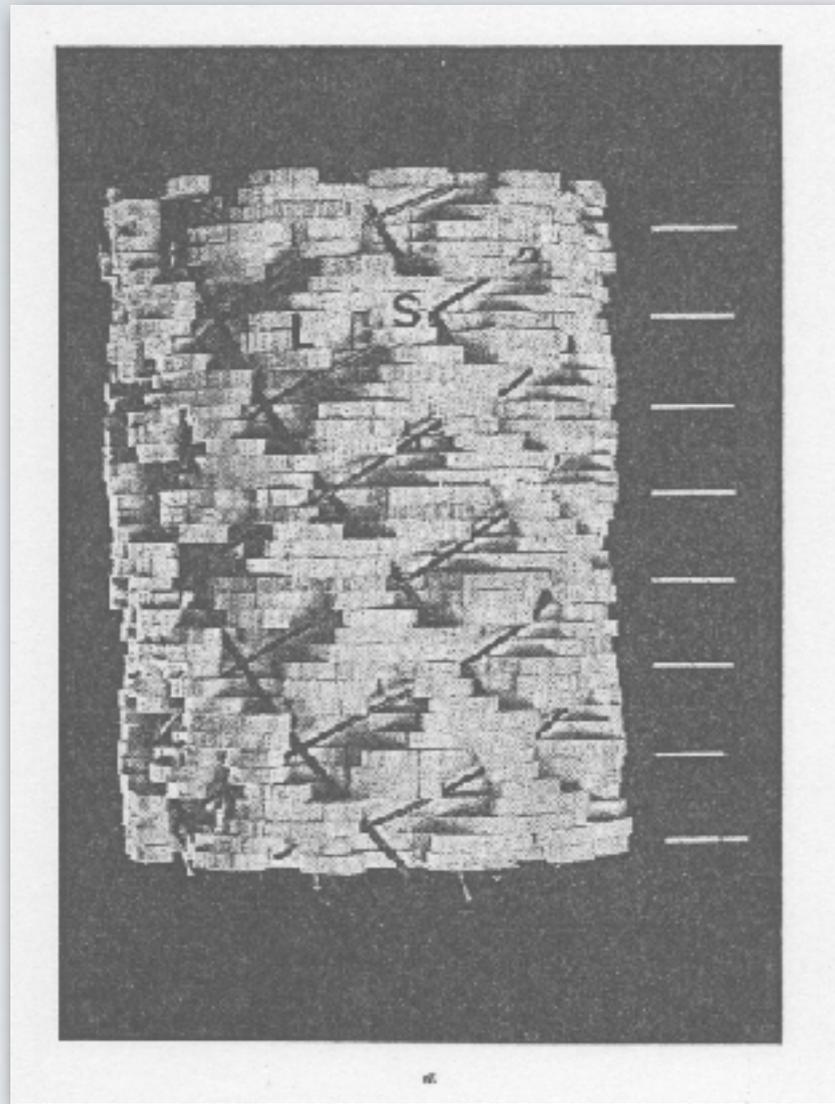


Fourier Structure



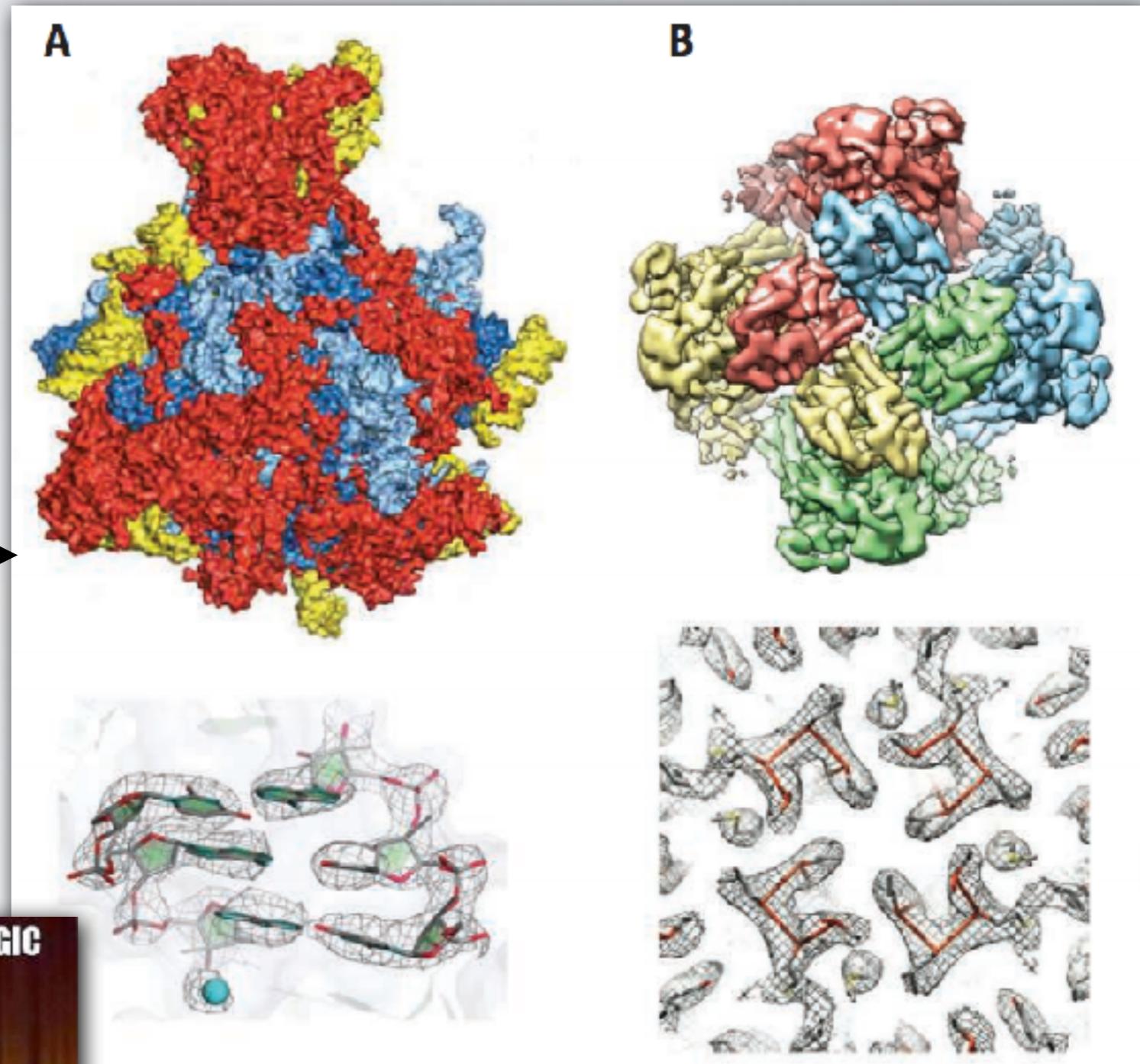
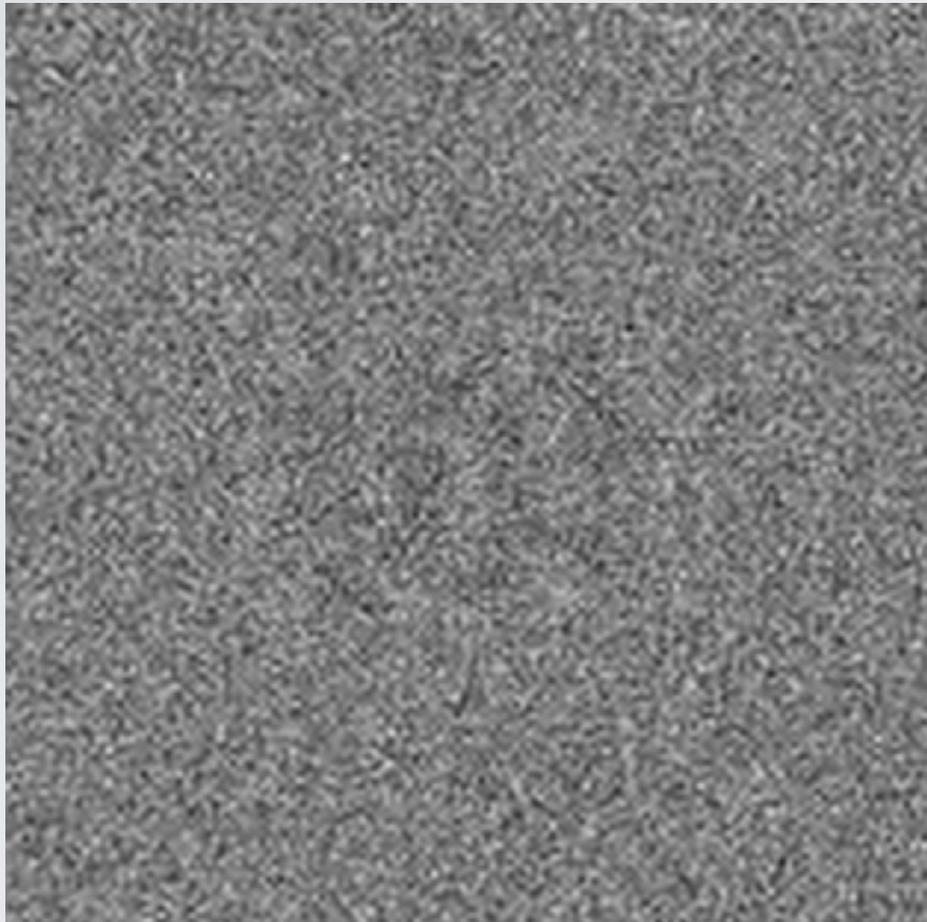
←
inverse
FT

First 3D Reconstruction - T4 phage tail



~2014: Attainable Resolutions dramatically improved

Very noisy 2D projection images that are radiation damaged



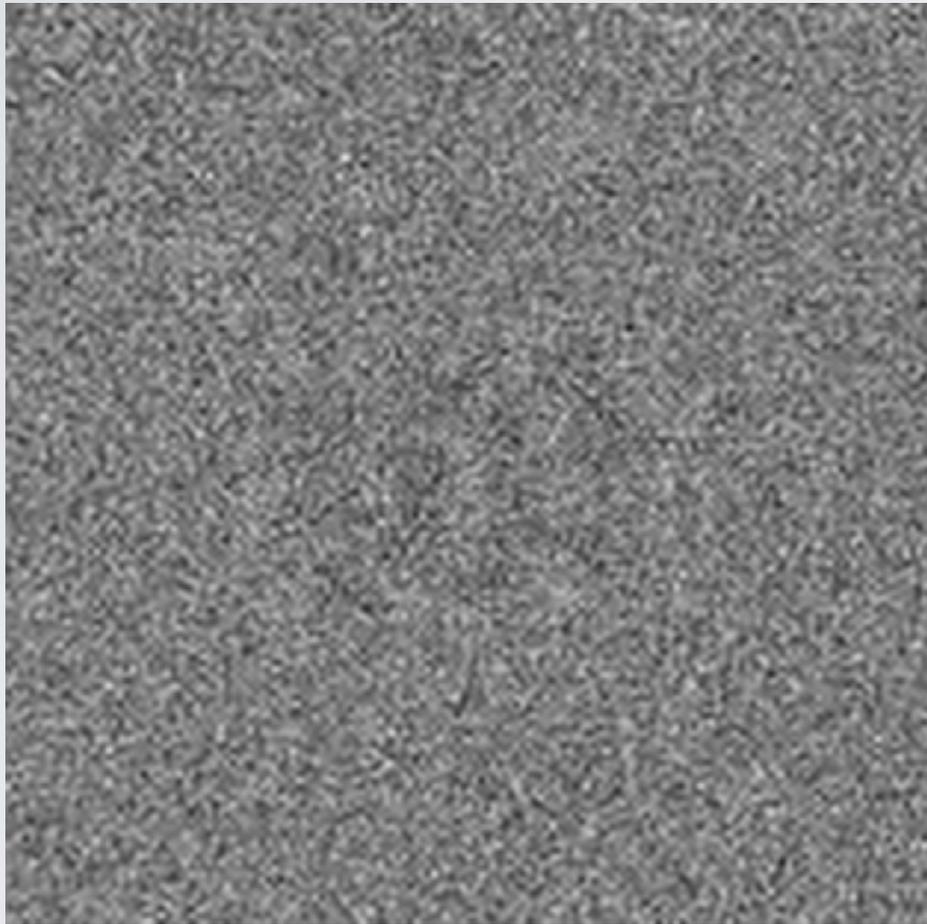
How is this possible?



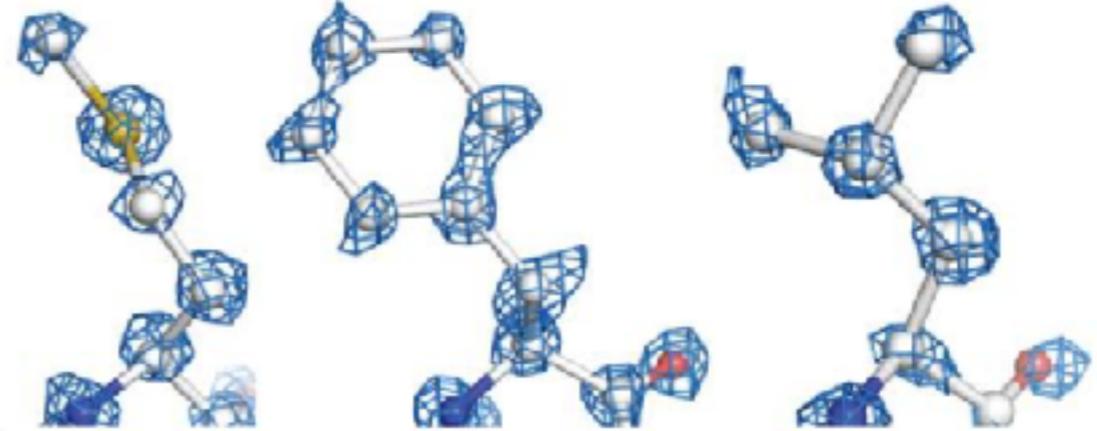
Kühlbrandt, Science 2014

2020: Attainable Resolutions dramatically improved AGAIN

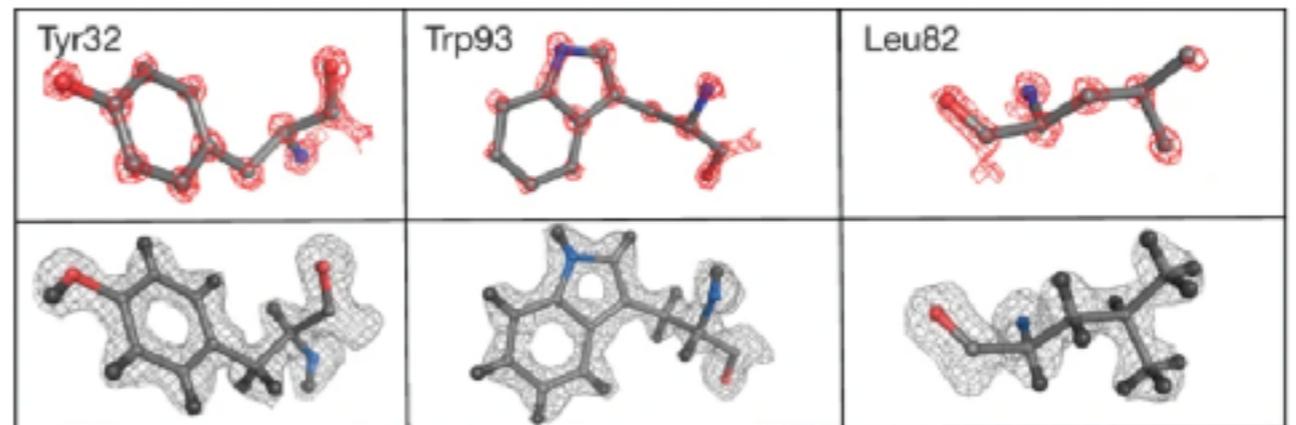
Very noisy 2D projection images that are radiation damaged



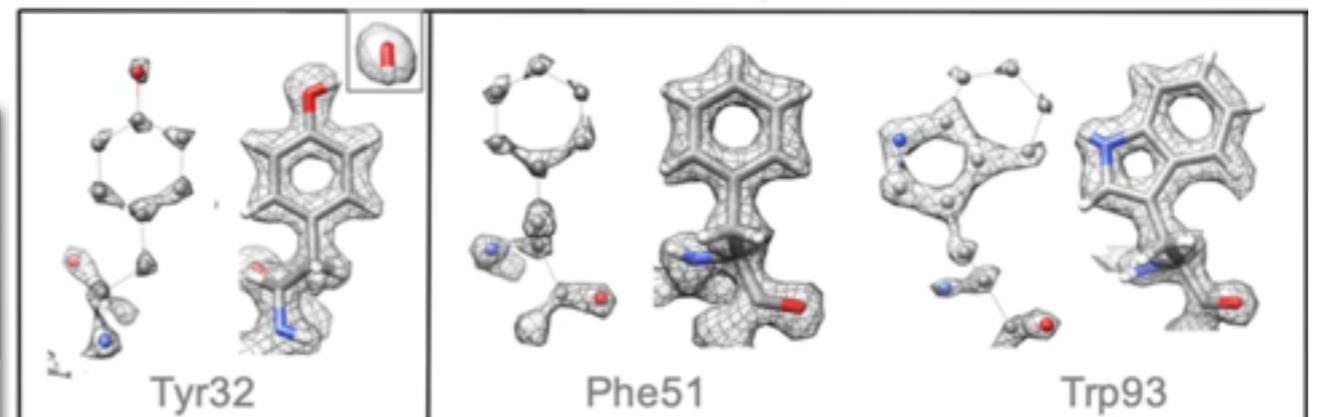
Atomic resolution structures



Nakane et al., Nature 2020

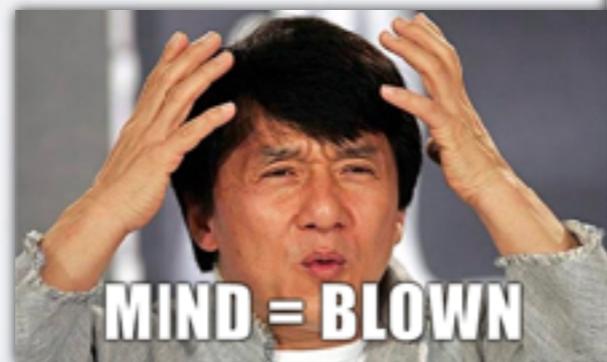


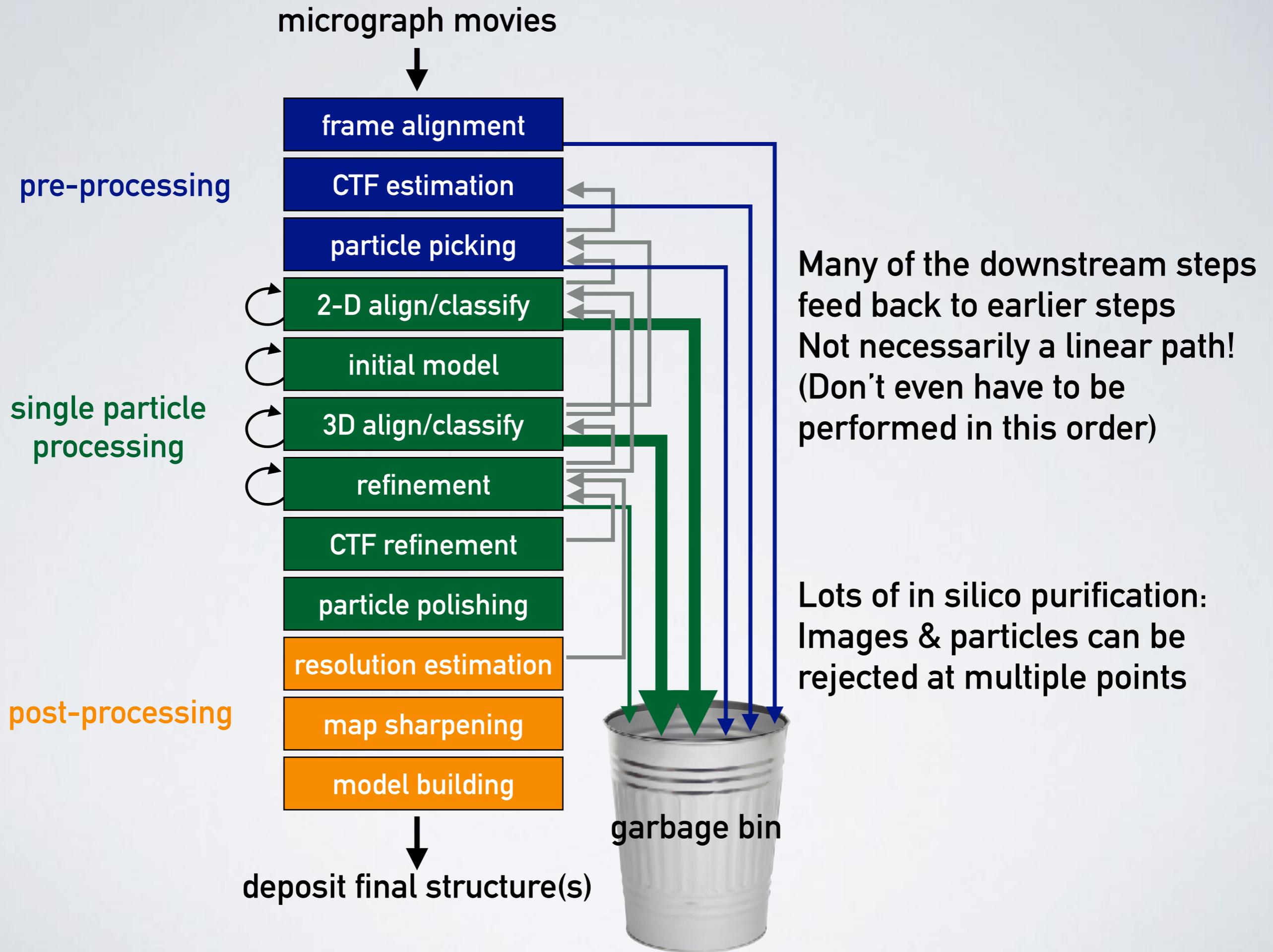
Yip et al., Nature 2020



Zhang et al., Cell Research 2020

How is this possible?!!





micrograph movies



frame alignment

pre-processing

CTF estimation

particle picking

2-D align/classify

single particle processing

initial model

3D align/classify

refinement

CTF refinement

particle polishing

resolution estimation

post-processing

map sharpening

model building



deposit final structure(s)



garbage bin

Many of the downstream steps feed back to earlier steps
Not necessarily a linear path!
(Don't even have to be performed in this order)

Lots of in silico purification:
Images & particles can be rejected at multiple points

Reconstructions need lots of particle data

frame alignment

CTF estimation

particle picking

2-D align/classify

initial model

3D align/classify

refinement

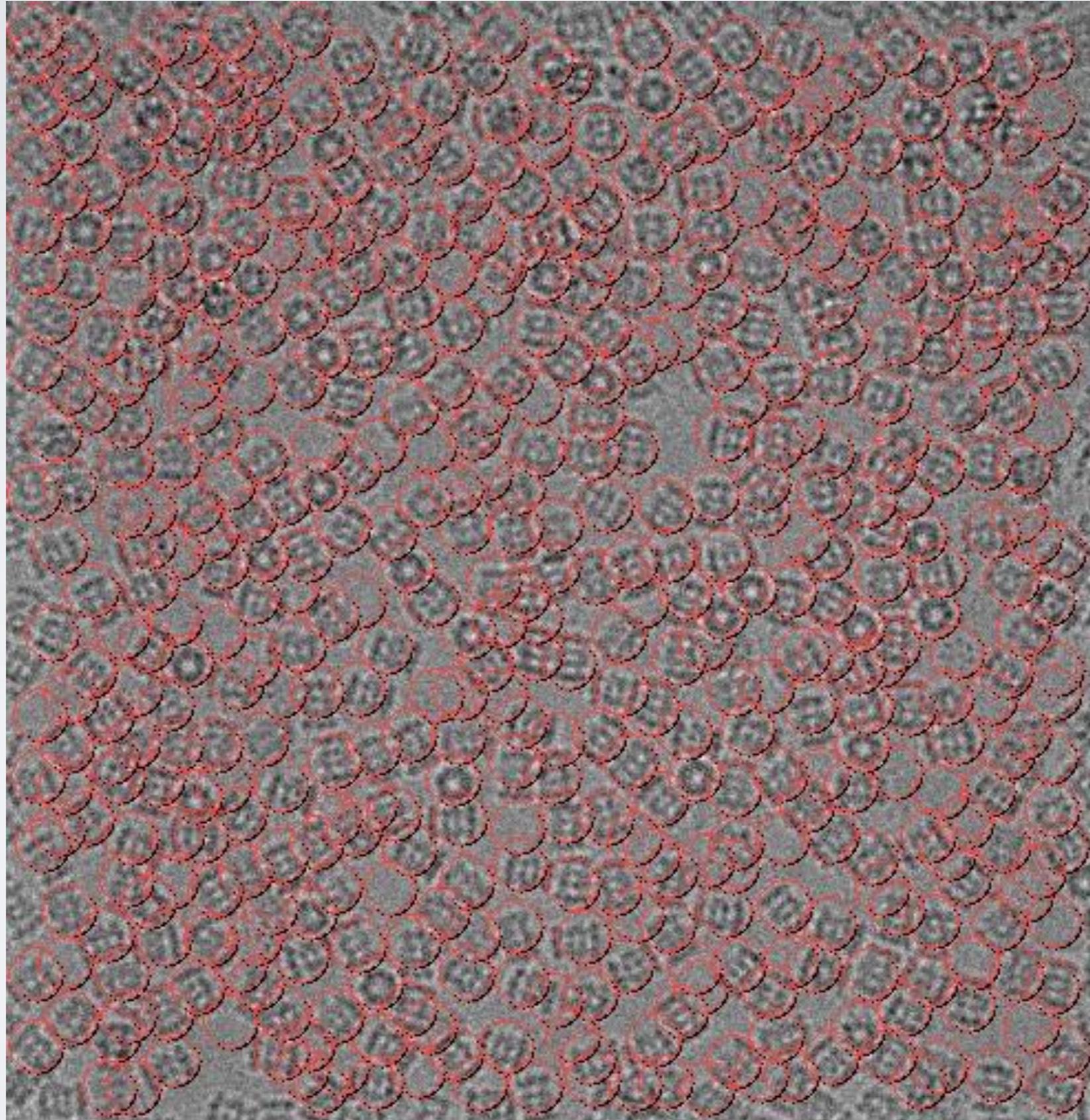
CTF refinement

particle polishing

resolution estimation

map sharpening

model building



Particle Extraction

frame alignment

CTF estimation

particle picking

2-D align/classify

initial model

3D align/classify

refinement

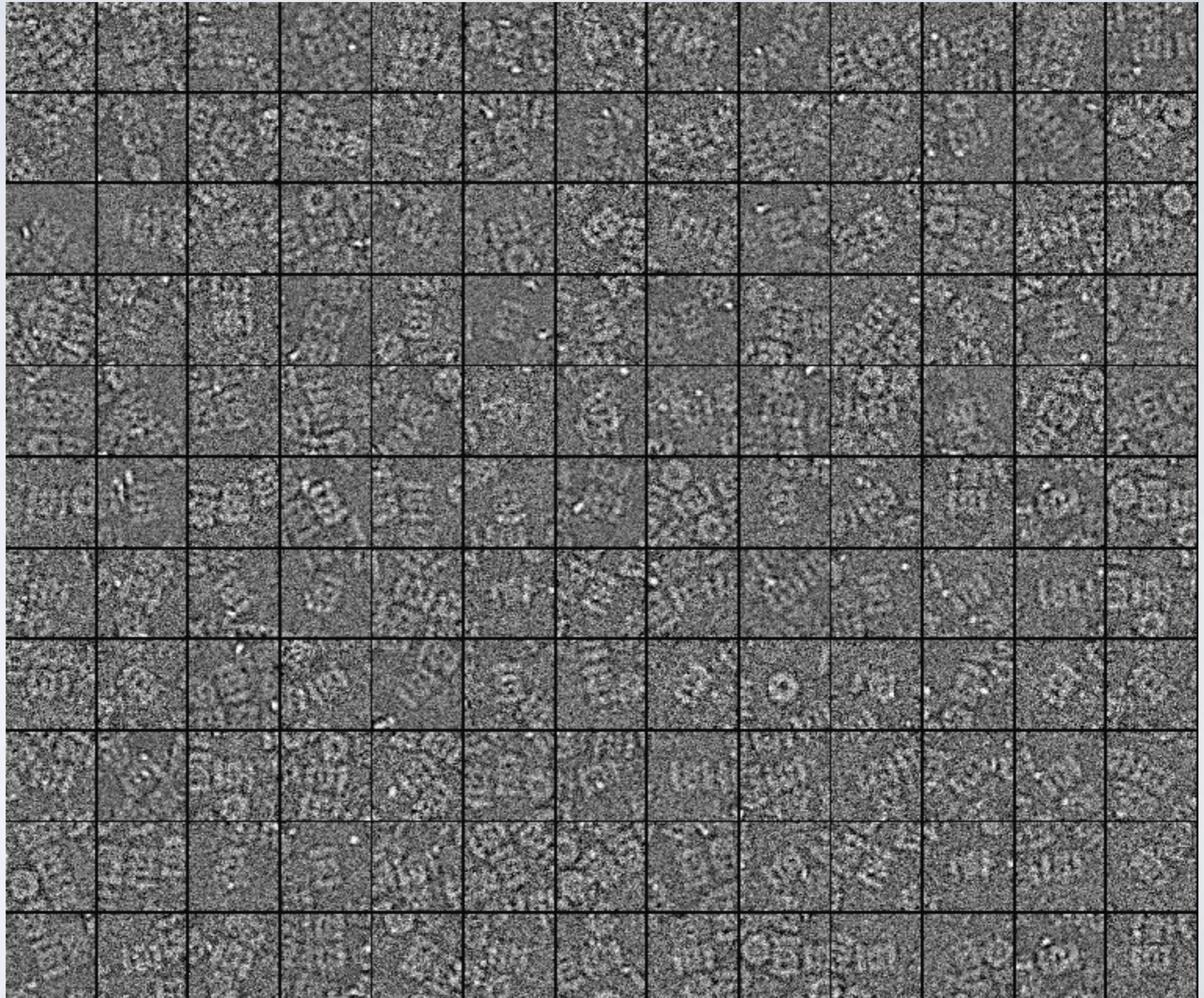
CTF refinement

particle polishing

resolution estimation

map sharpening

model building



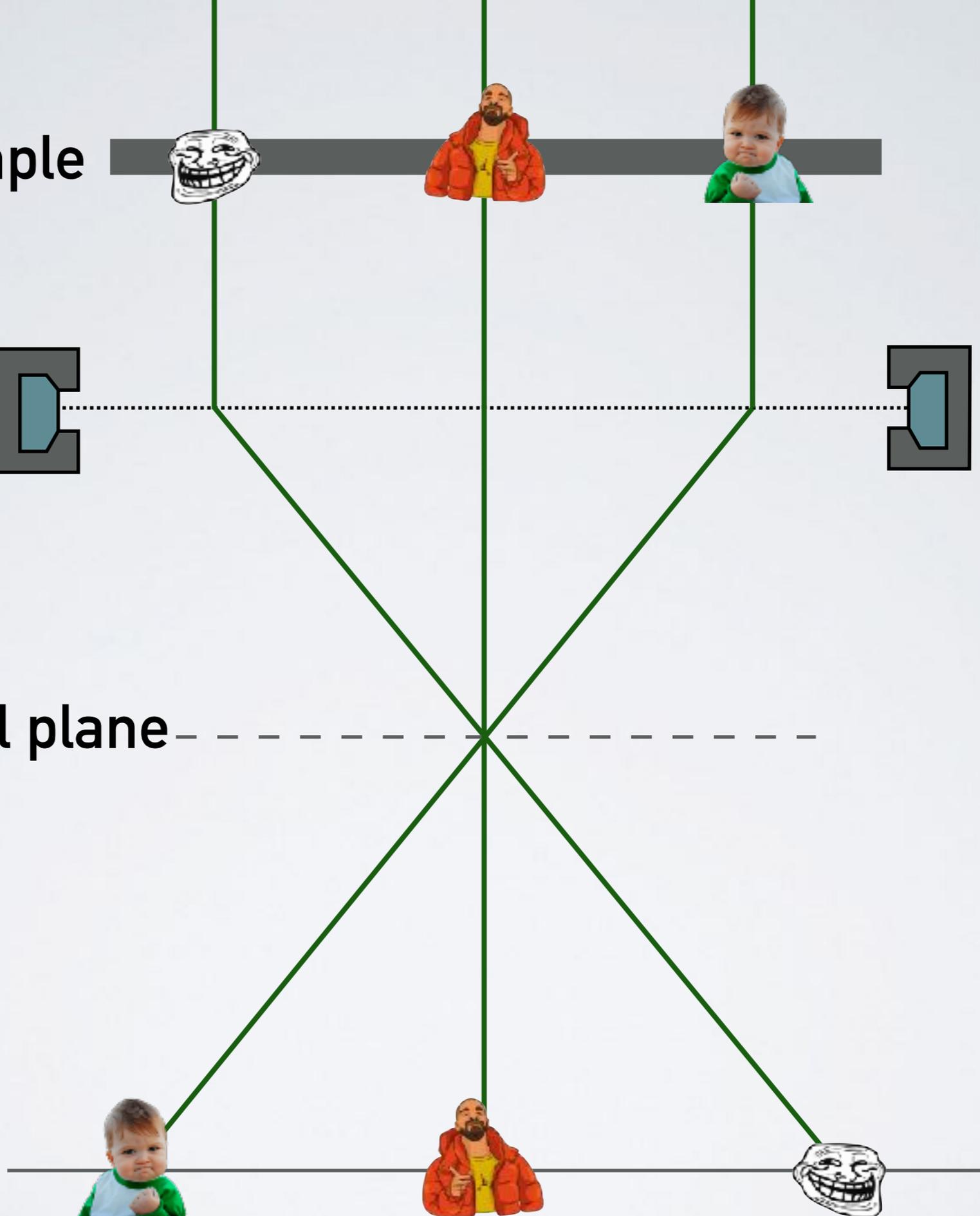
sample

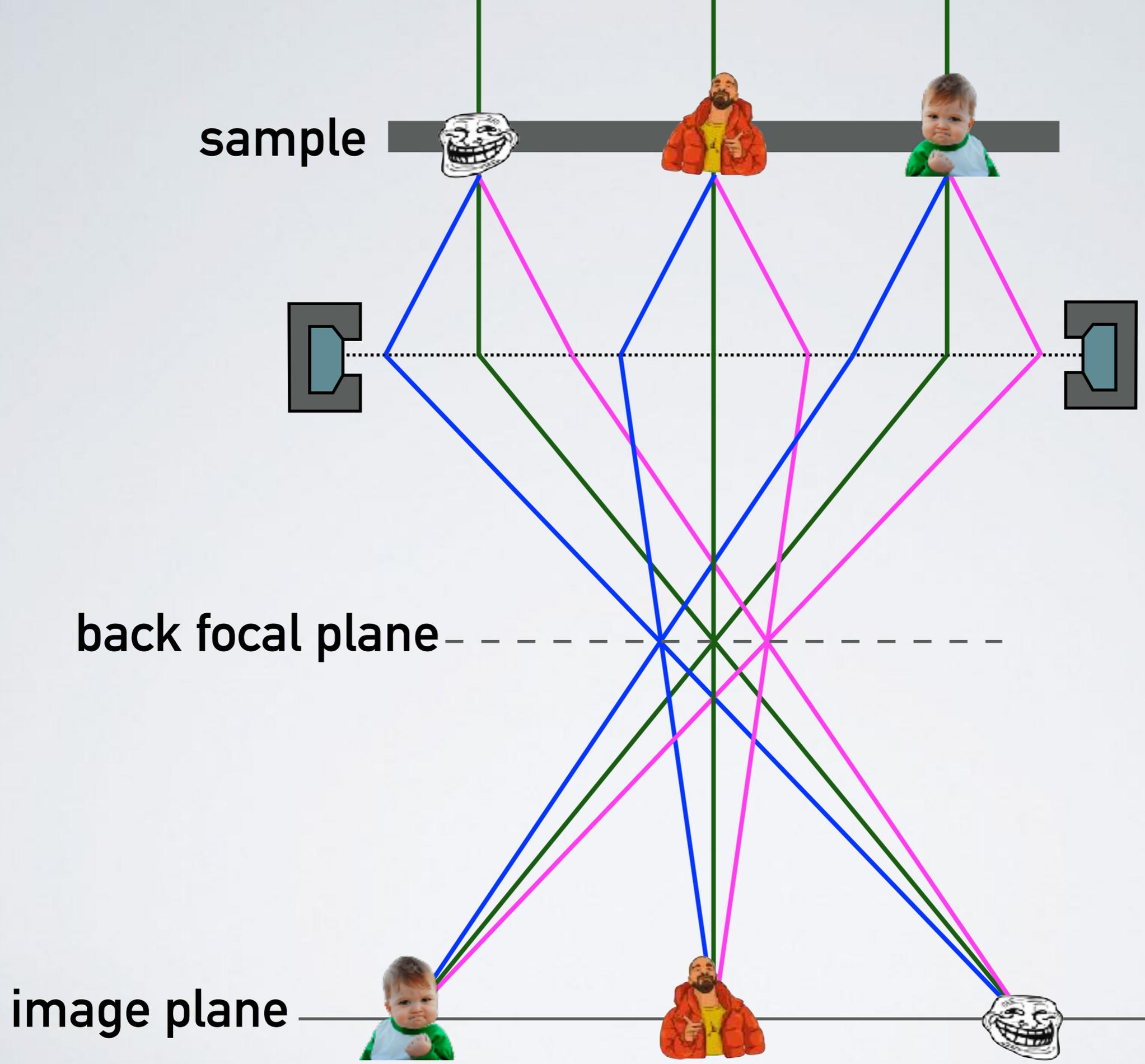


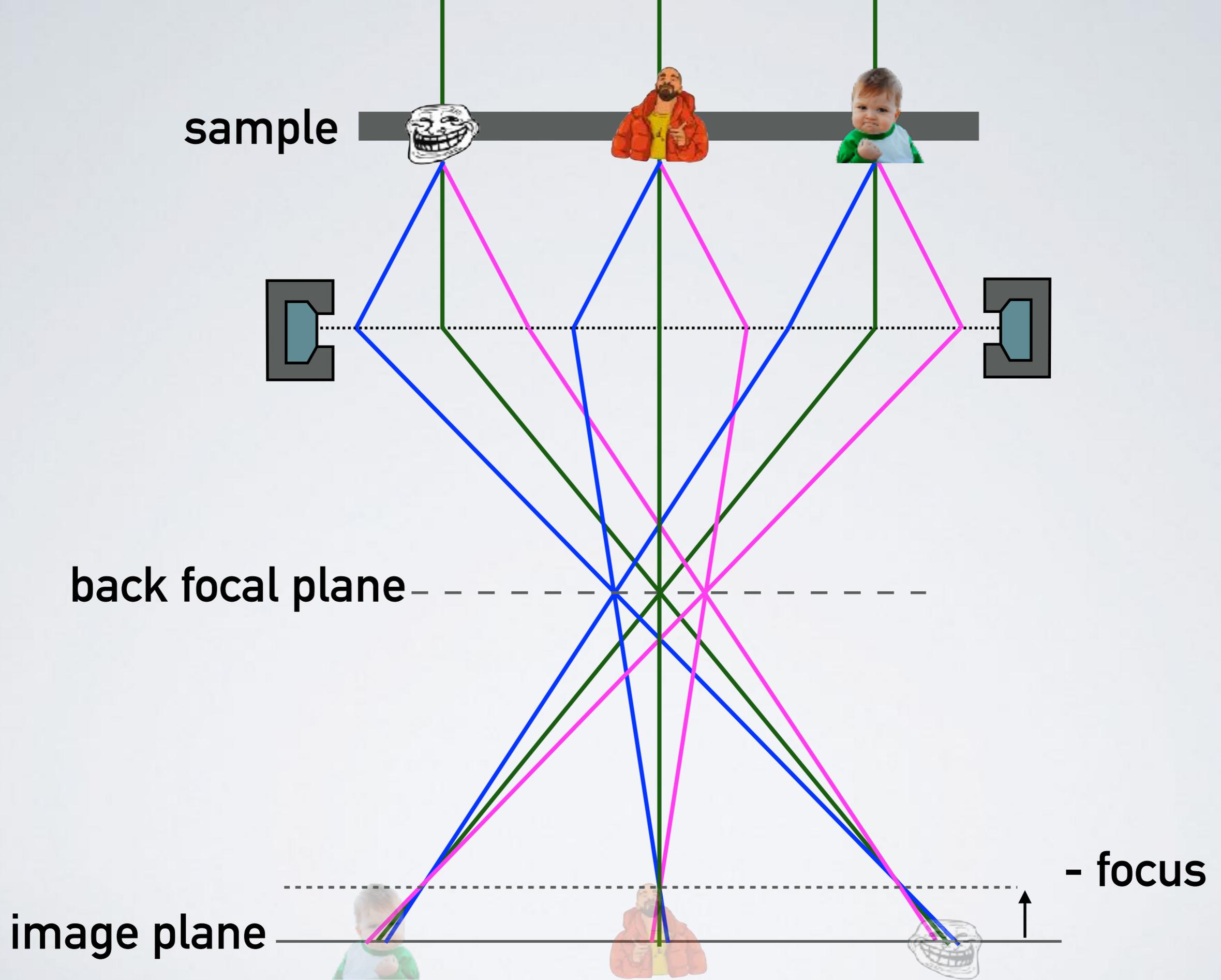
back focal plane

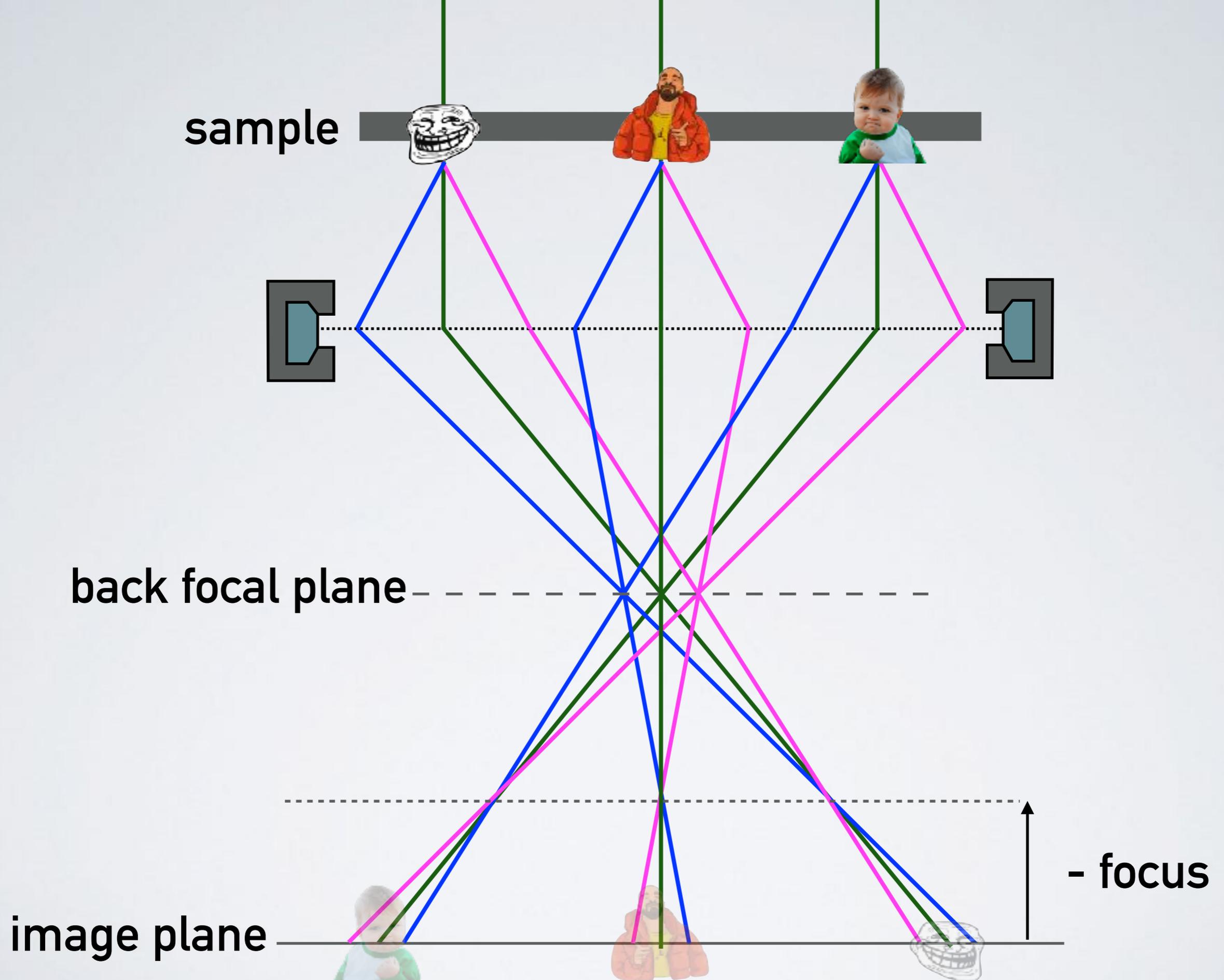


image plane







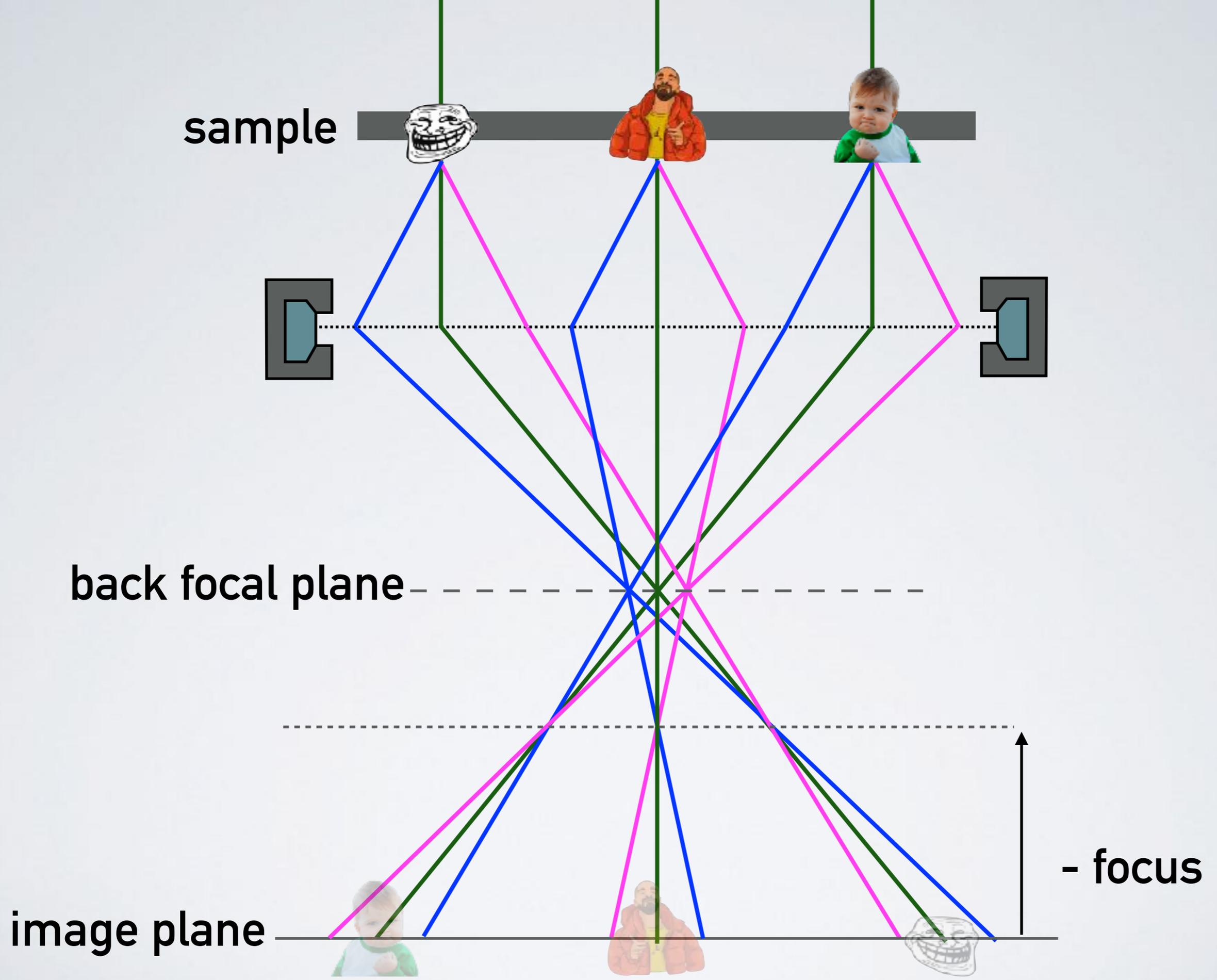


sample

back focal plane

image plane

- focus



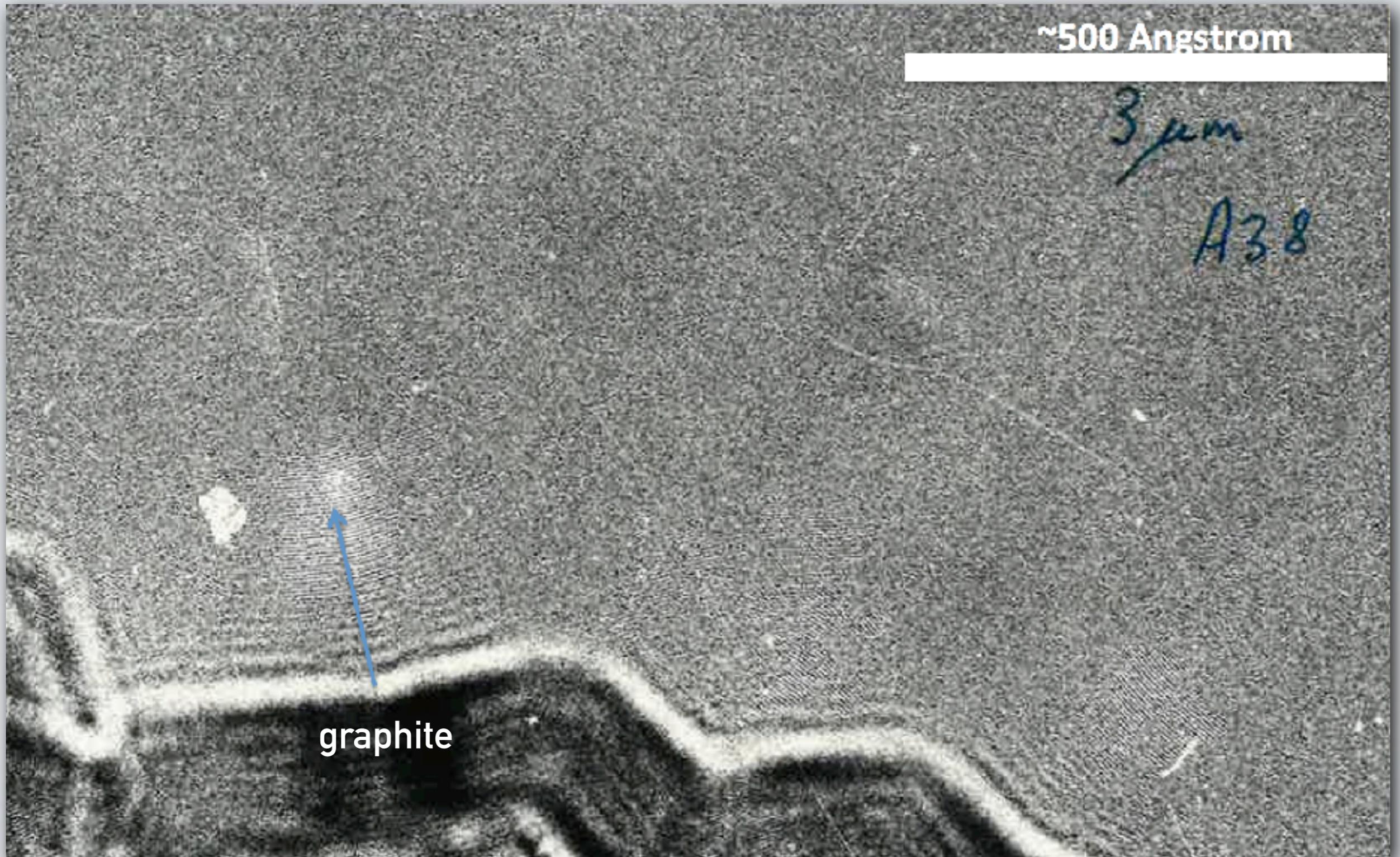
sample

back focal plane

image plane

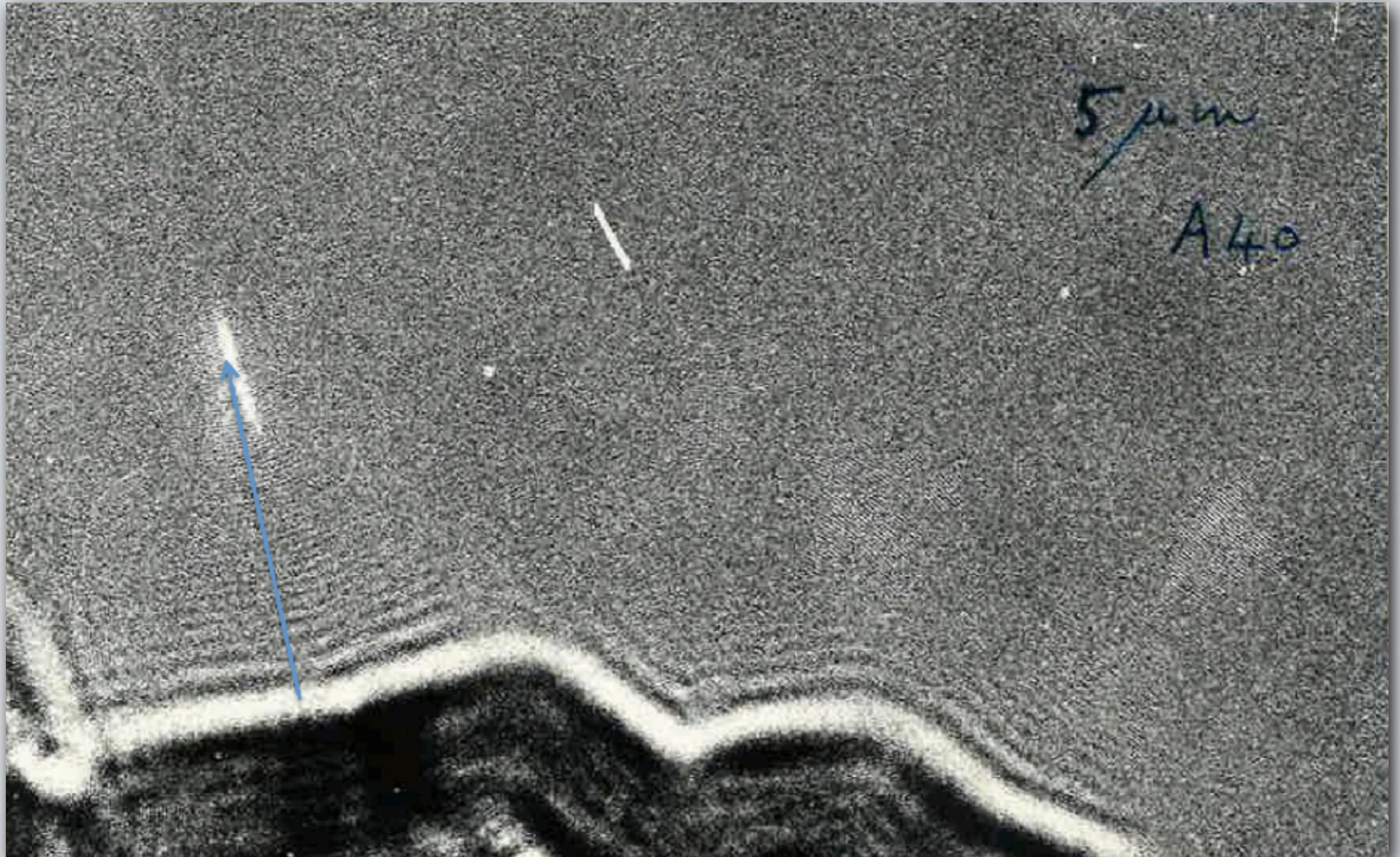
- focus

Signal Delocalization



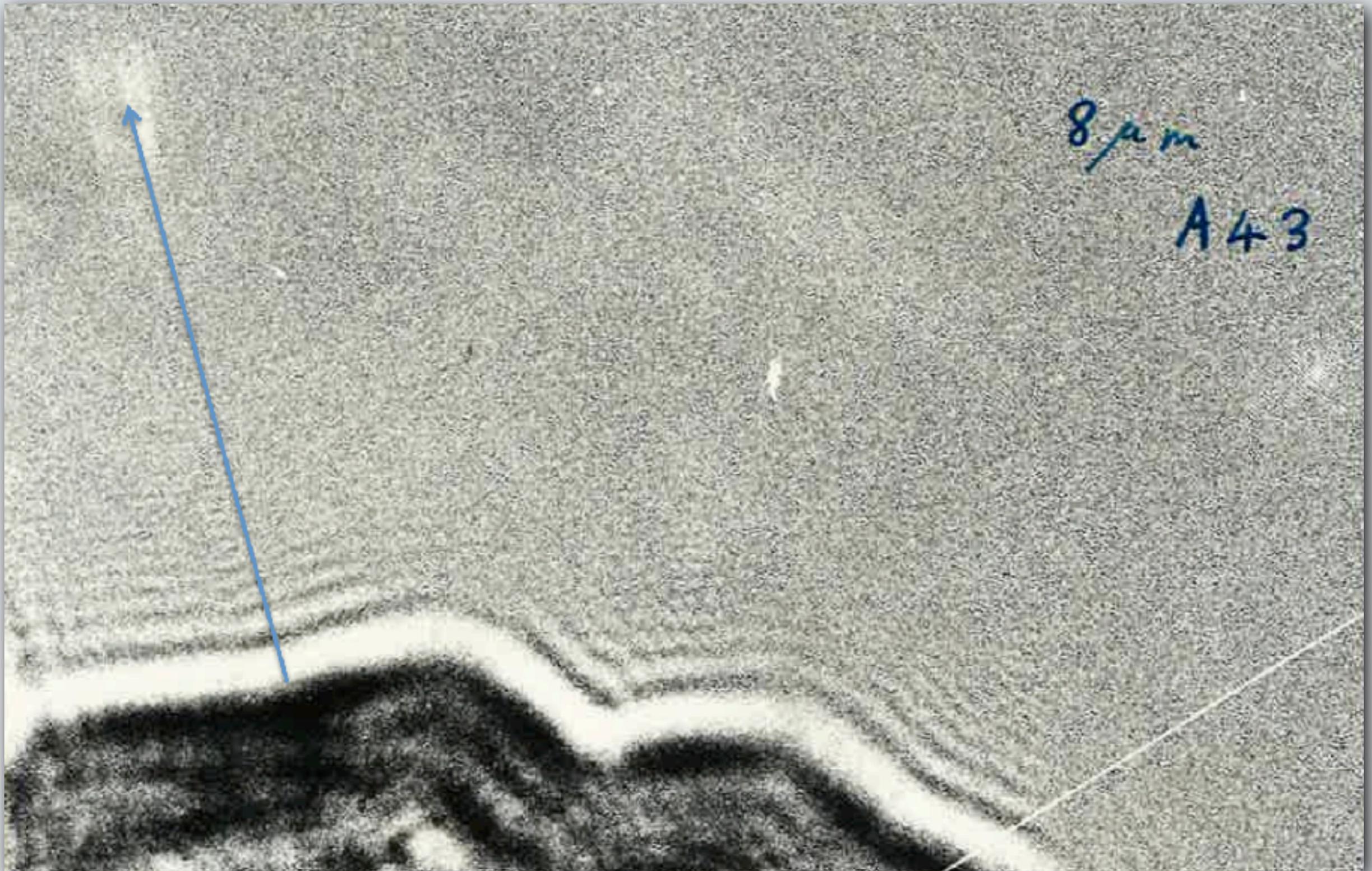
Images courtesy Richard Henderson

Signal Delocalization



Images courtesy Richard Henderson

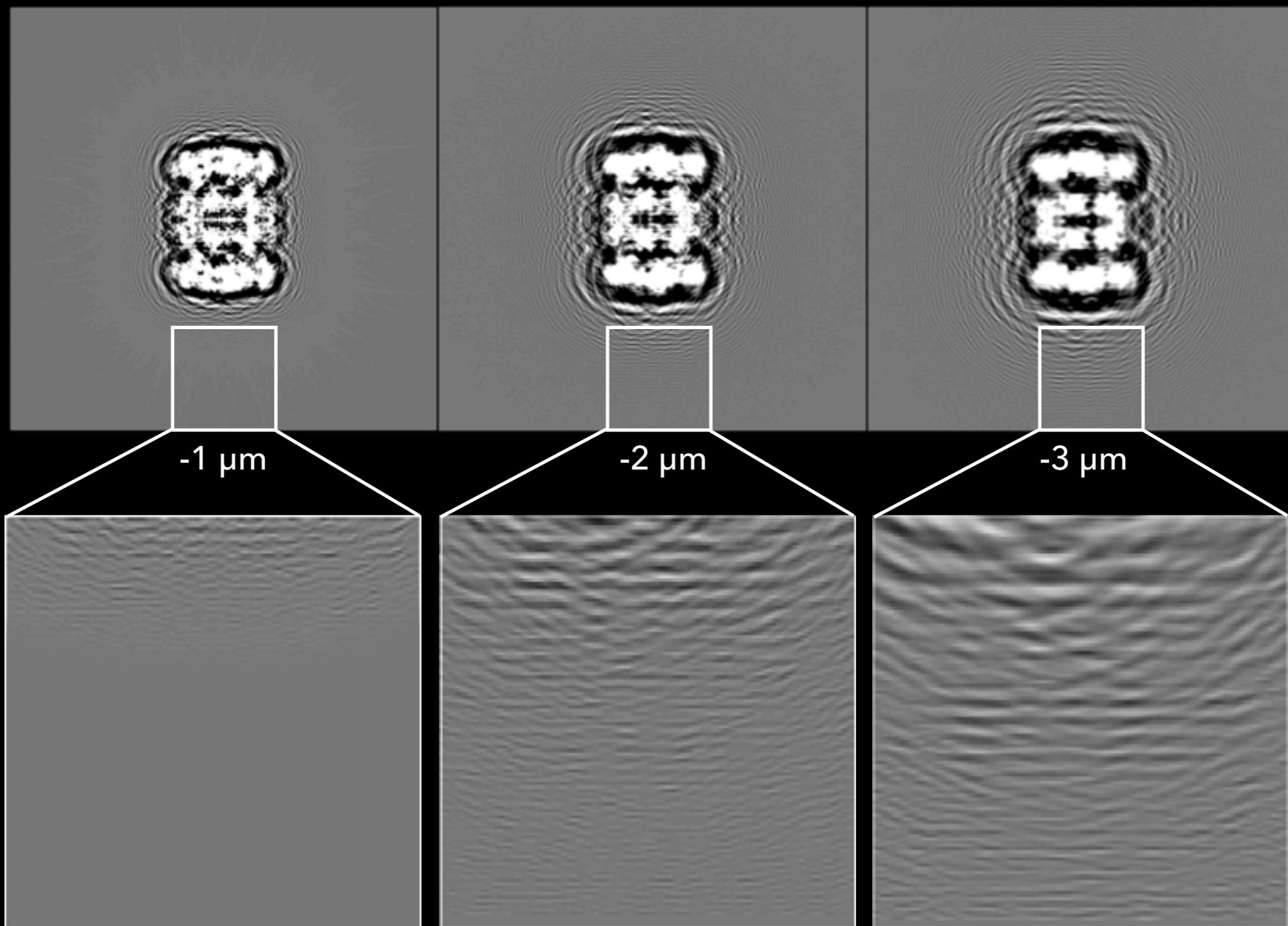
Signal Delocalization



Images courtesy Richard Henderson

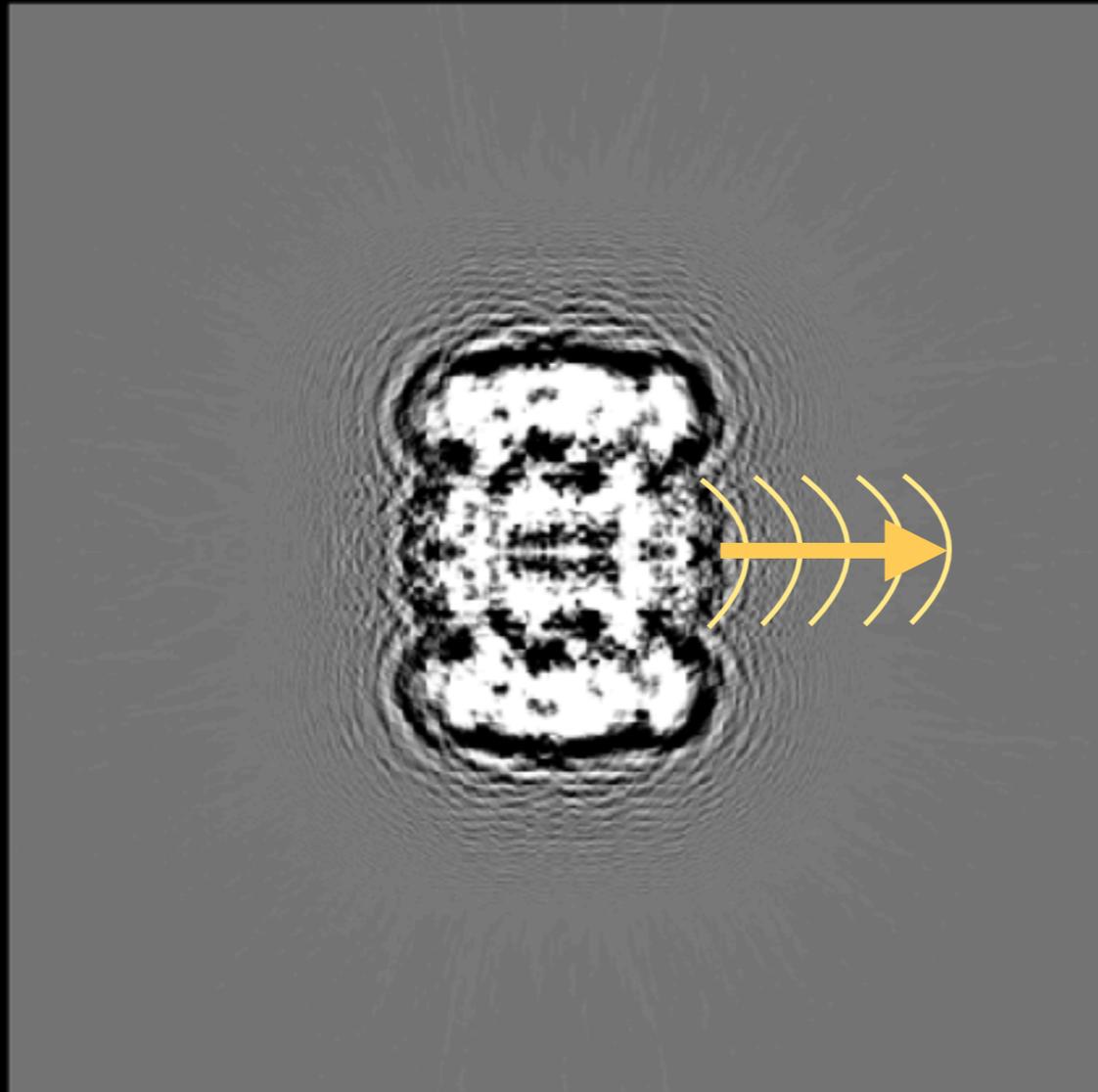
Signal delocalization and box size

Simulated 300 keV, $0.91 \text{ \AA}/\text{pixel}$, 512 box size



Signal delocalization and box size

Simulated 300 keV, 0.91 Å/pixel, 512 box size

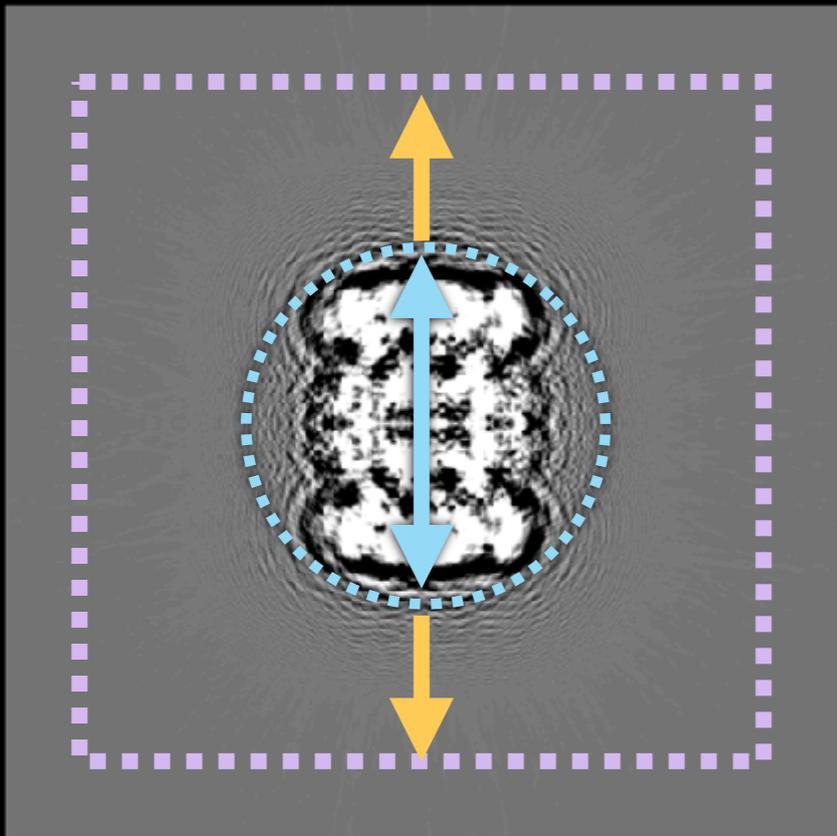


How far signal corresponding to a given resolution will be displaced in a defocused image can be determined:

$$\frac{\lambda \cdot defocus}{resolution}$$

Signal delocalization and box size

Simulated 300 keV, 0.91 Å/pixel, 512 box size



$$\text{Box size} = D + 2 \left(\frac{\lambda F_{max}}{r} \right)$$

D = particle diameter

λ = electron wavelength

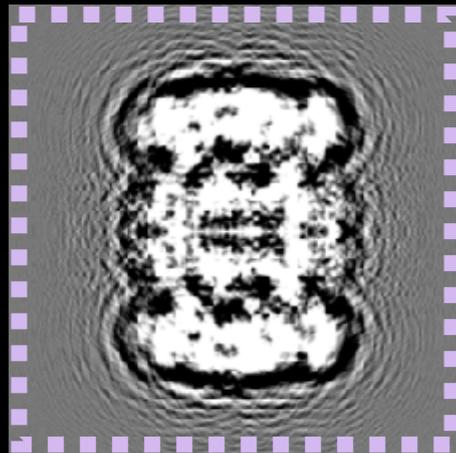
F_{max} = maximum defocus

r = resolution

To keep 3 Å information with max defocus of 1.5 μm: 396 pixel box size

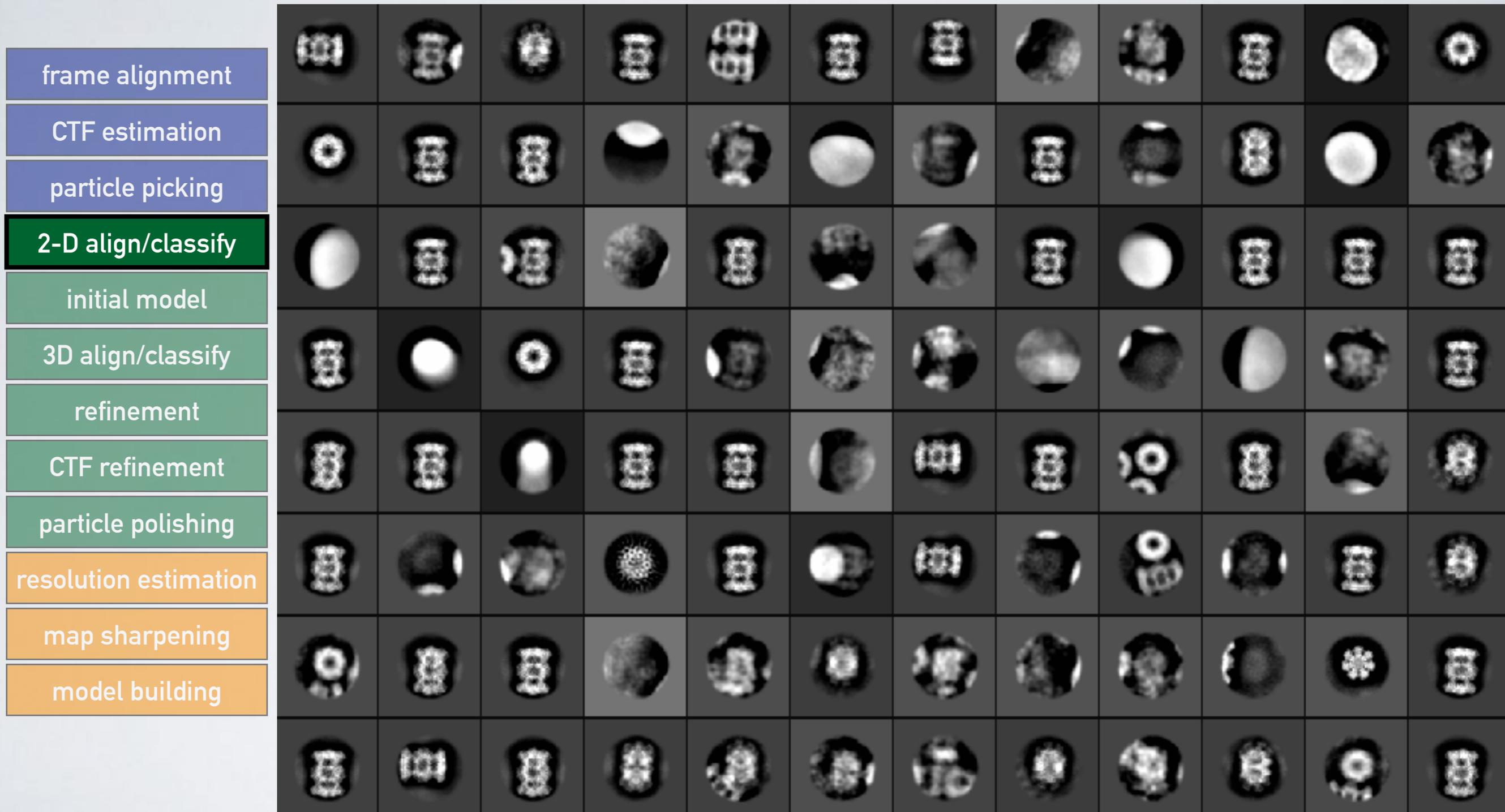
To keep 2 Å information with max defocus of 3.0 μm: 693 pixel box size

Signal delocalization and box size



For preliminary image processing, (2D classification, initial model generation, 3D classification) don't need a large box

Use 2D classification to assess quality of particles



Use 2D classification to assess quality of particles

frame alignment

CTF estimation

particle picking

2-D align/classify

initial model

3D align/classify

refinement

CTF refinement

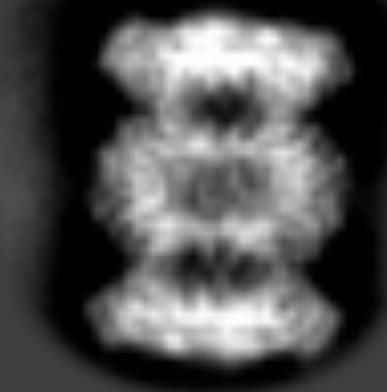
particle polishing

resolution estimation

map sharpening

model building

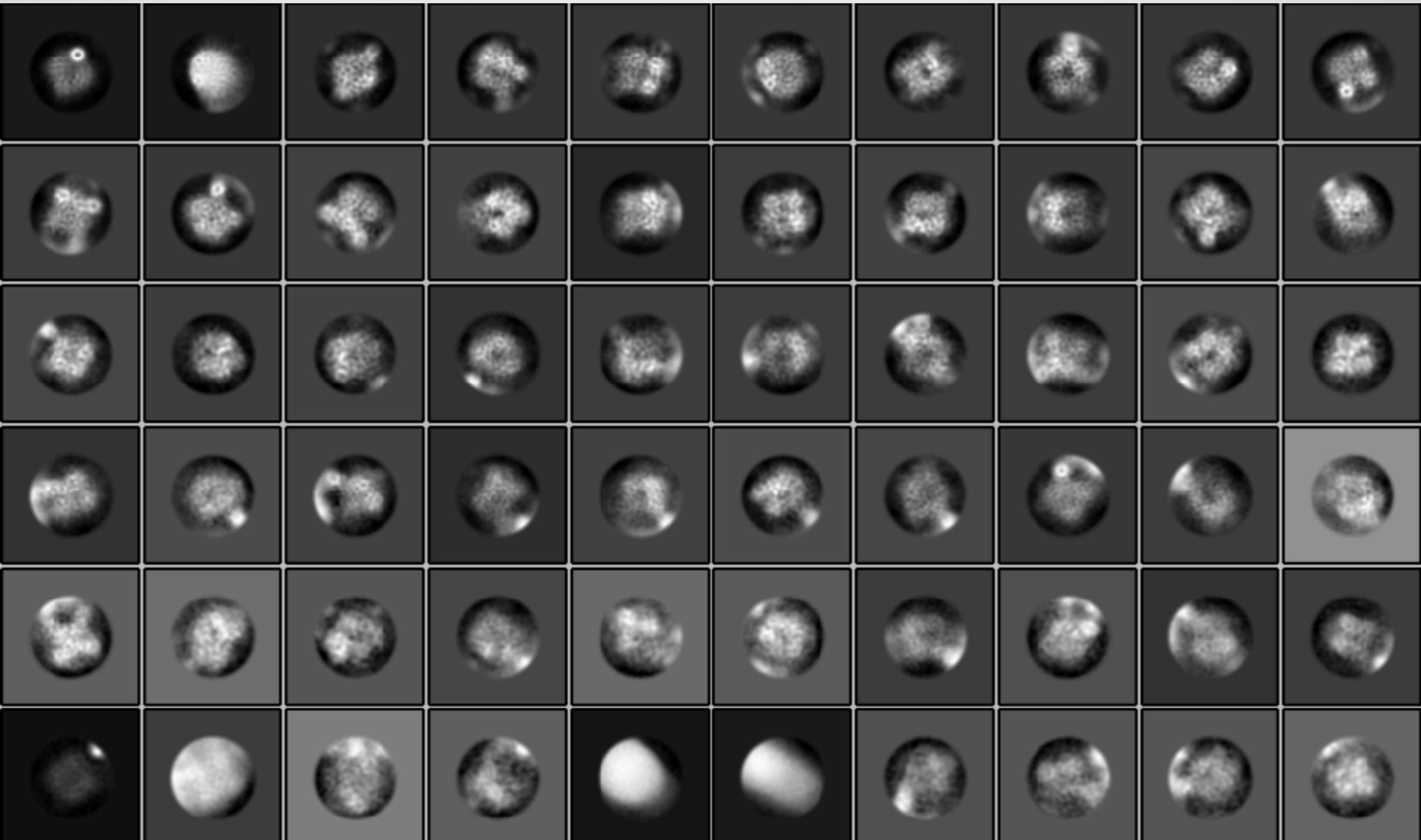
KEEP



THROW AWAY

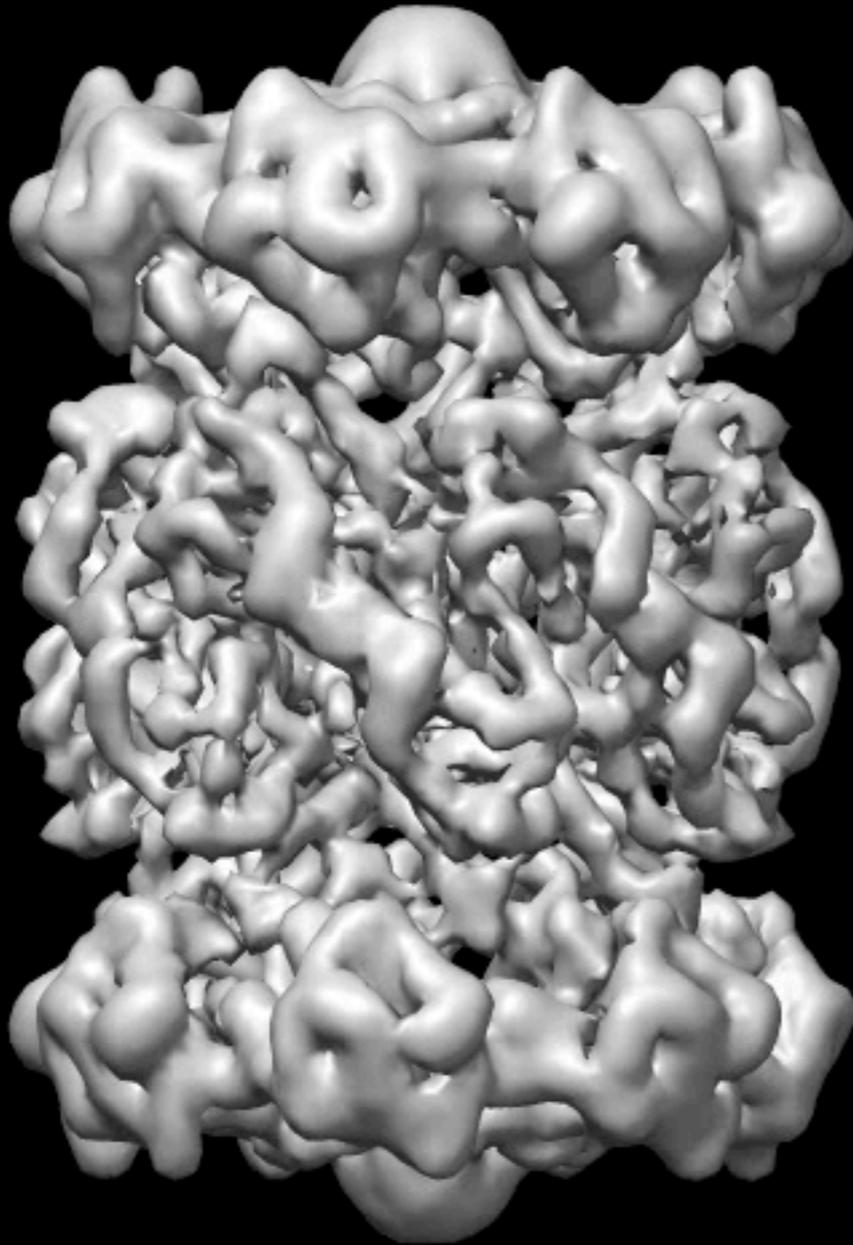


Don't assume you will be able to "process" your way to a high resolution structure!

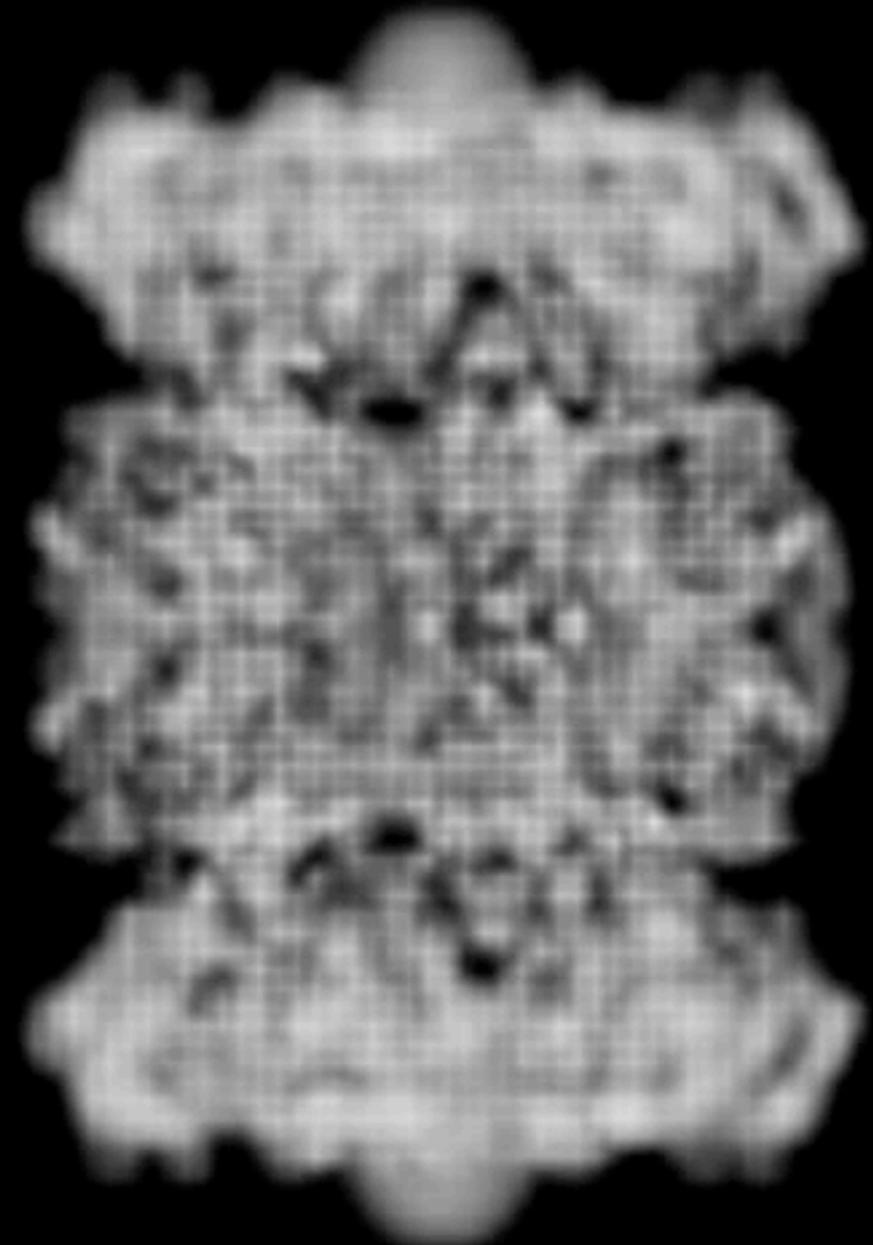


How do we define particle orientations?

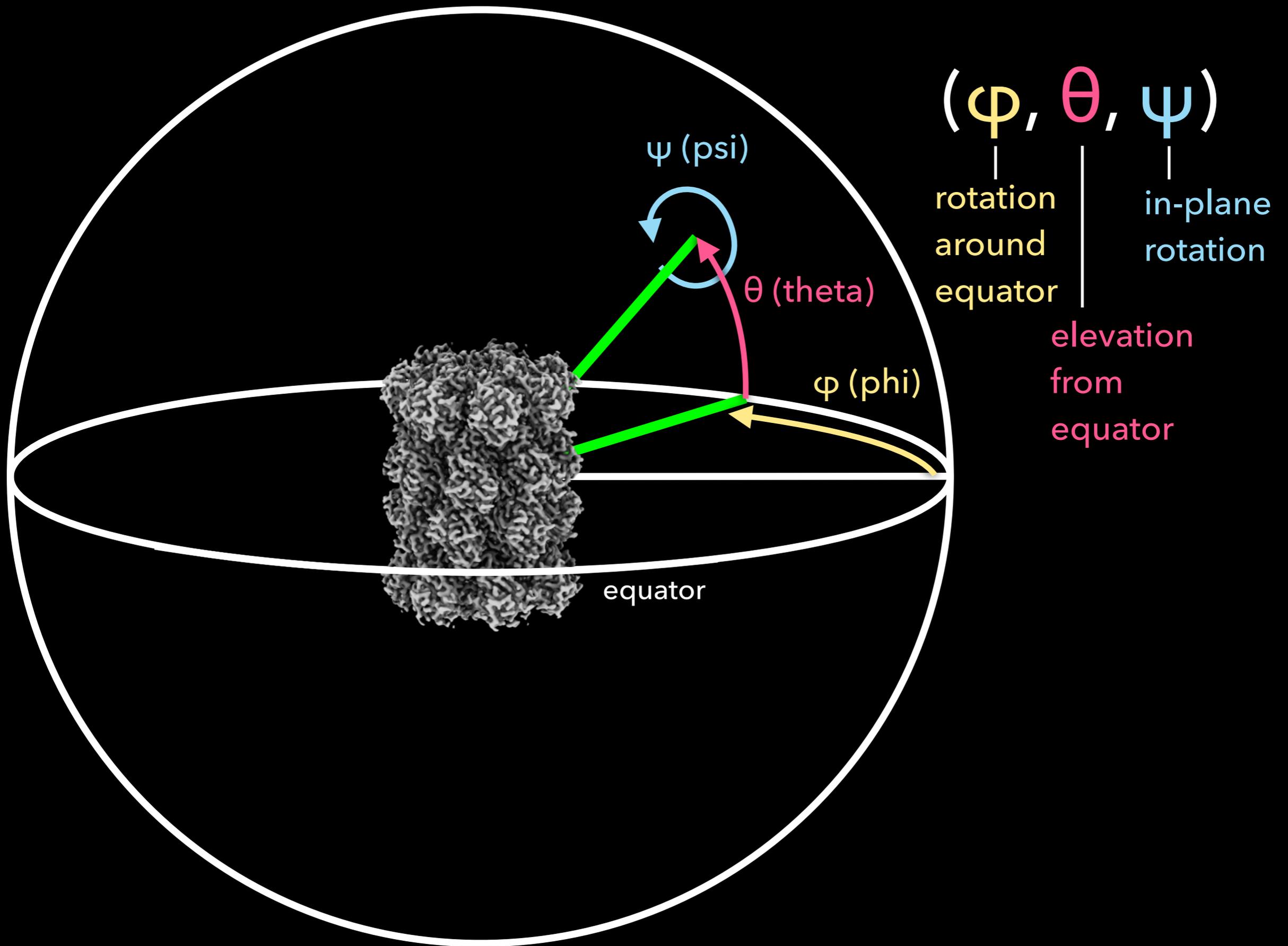
3D object



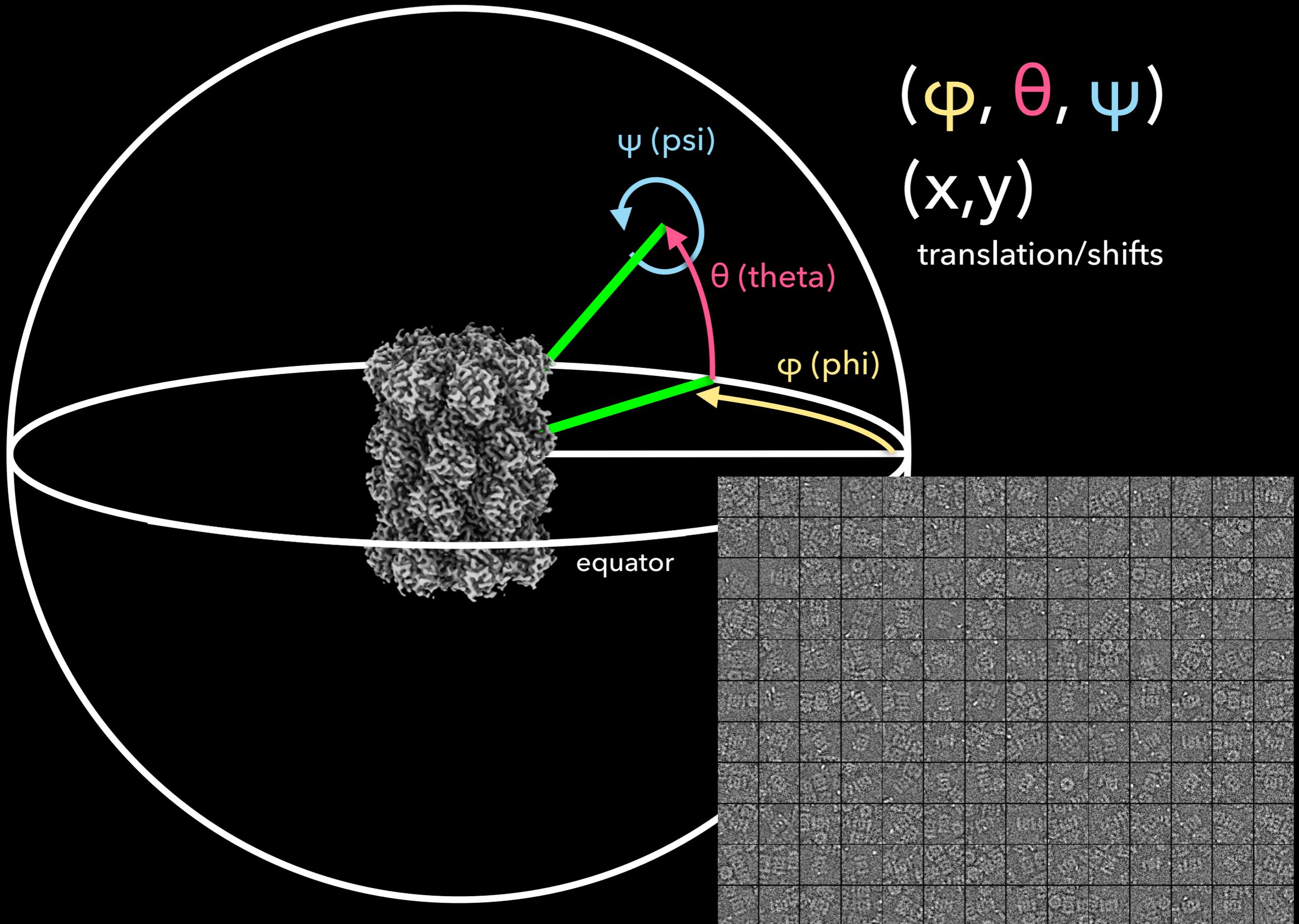
2D projection



Defining particle orientation



Defining particle orientation



Defining particle orientation

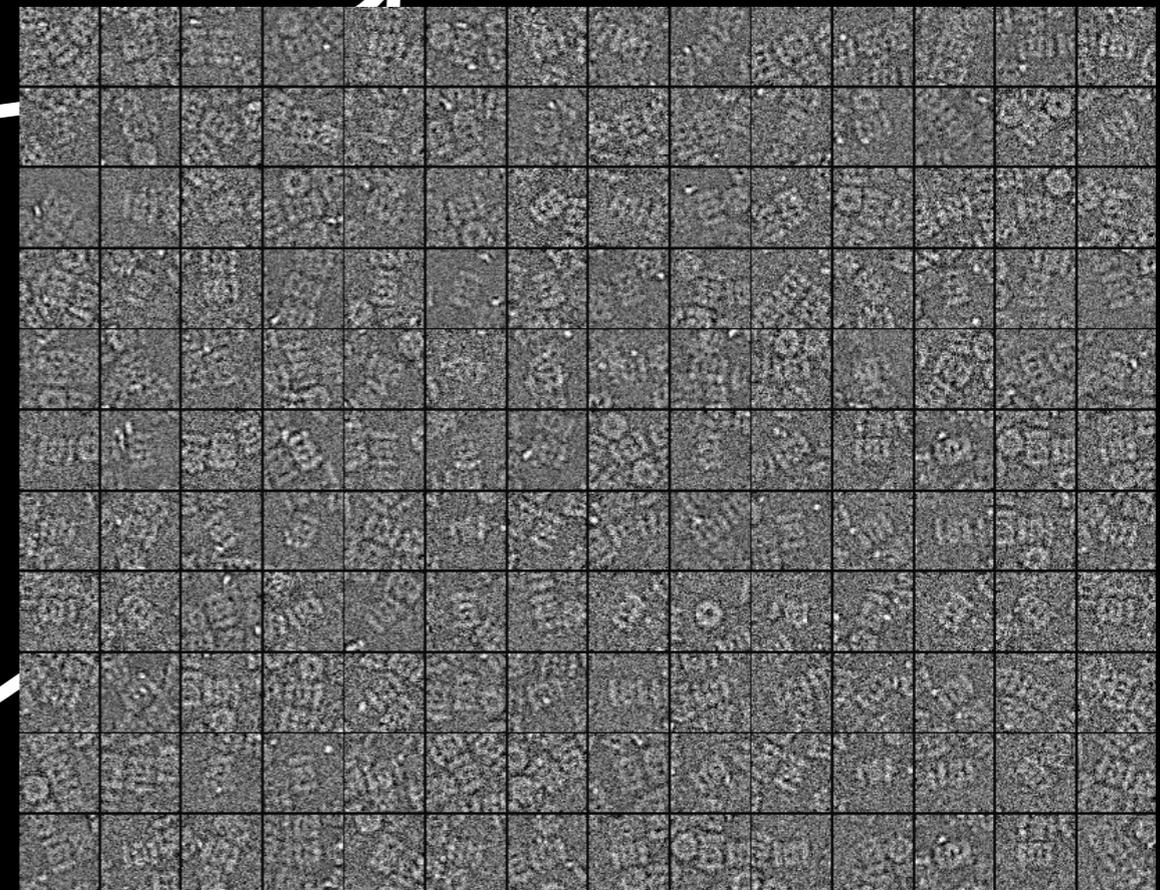
(φ, θ, ψ)

(x, y)

Where does
our reference
model come
from?



equator



The mathematical challenge of single particle cryo-EM: Solving 3D structures from 2D images



Generating an initial model for particle alignment

frame alignment

CTF estimation

particle picking

2-D align/classify

initial model

3D align/classify

refinement

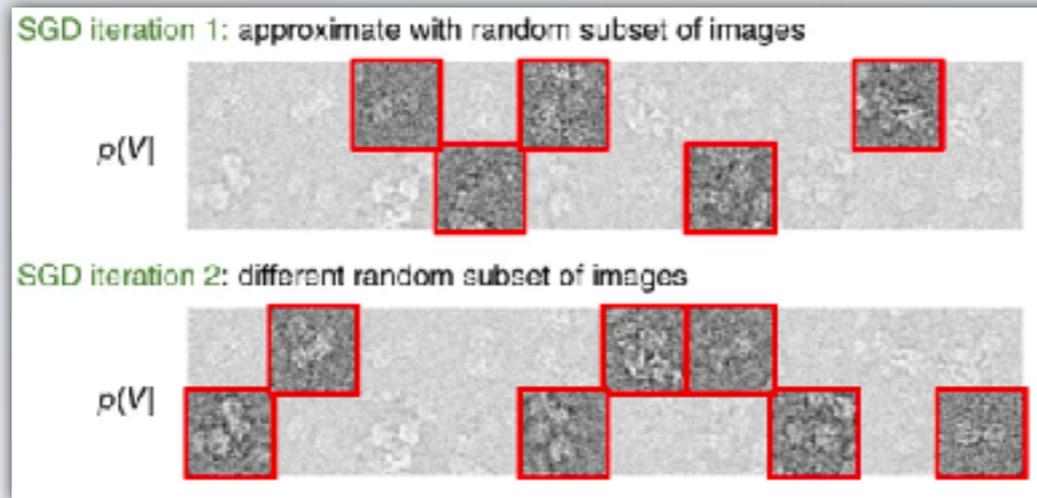
CTF refinement

particle polishing

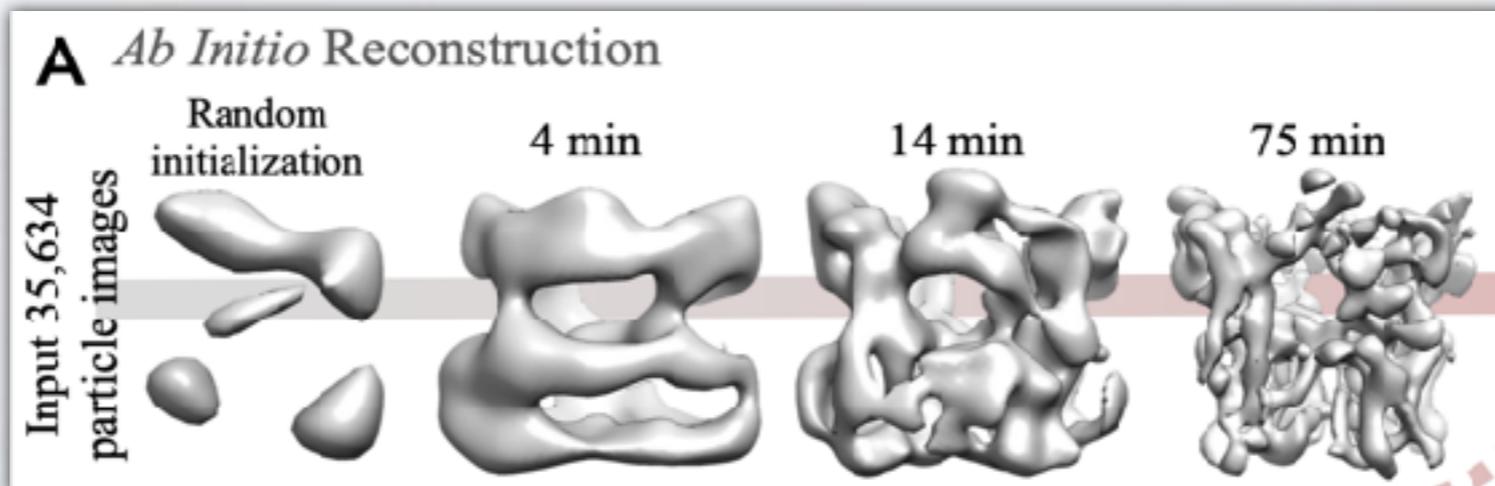
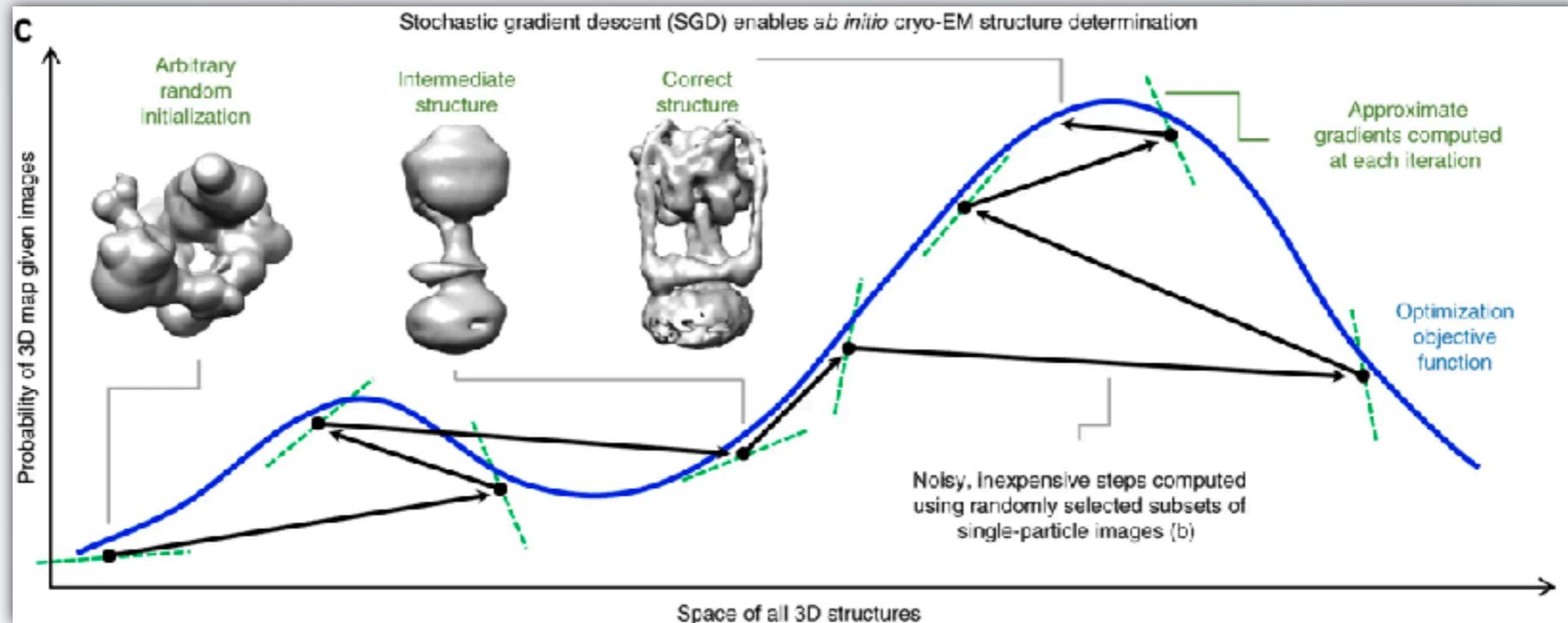
resolution estimation

map sharpening

model building



ab initio model
generation using
Stochastic Hill Climbing
(Gradient Descent)



Punjani et al.,
Nat Methods (2017)

Generating an initial model for particle alignment

Make sure that your model makes sense
& is consistent with 2D averages!

frame alignment

CTF estimation

particle picking

2-D align/classify

initial model

3D align/classify

refinement

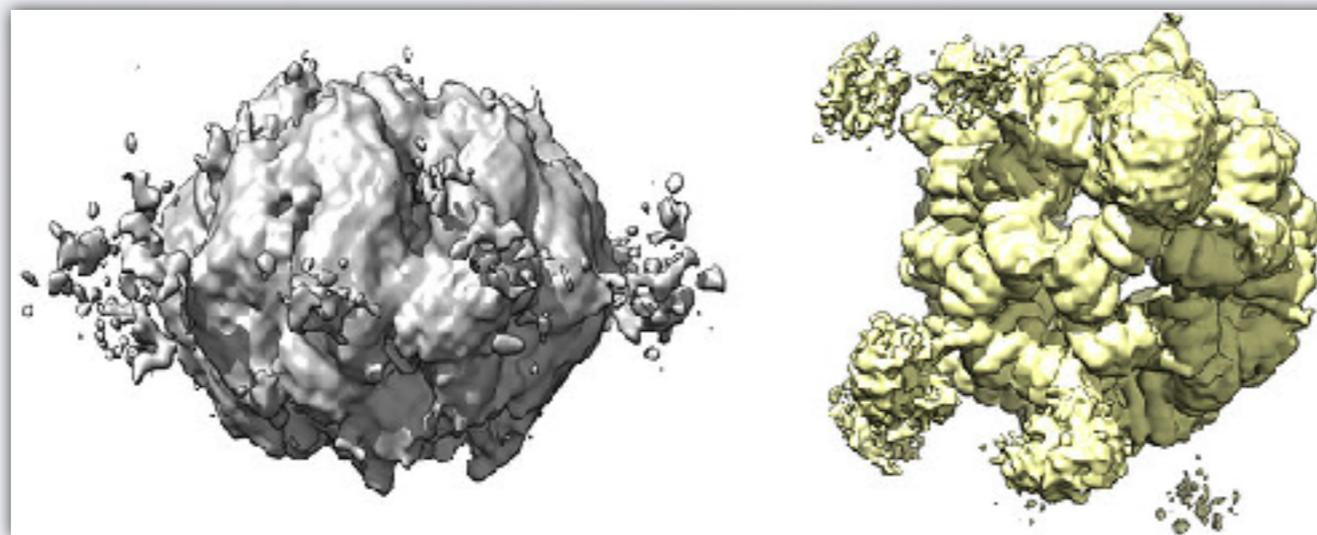
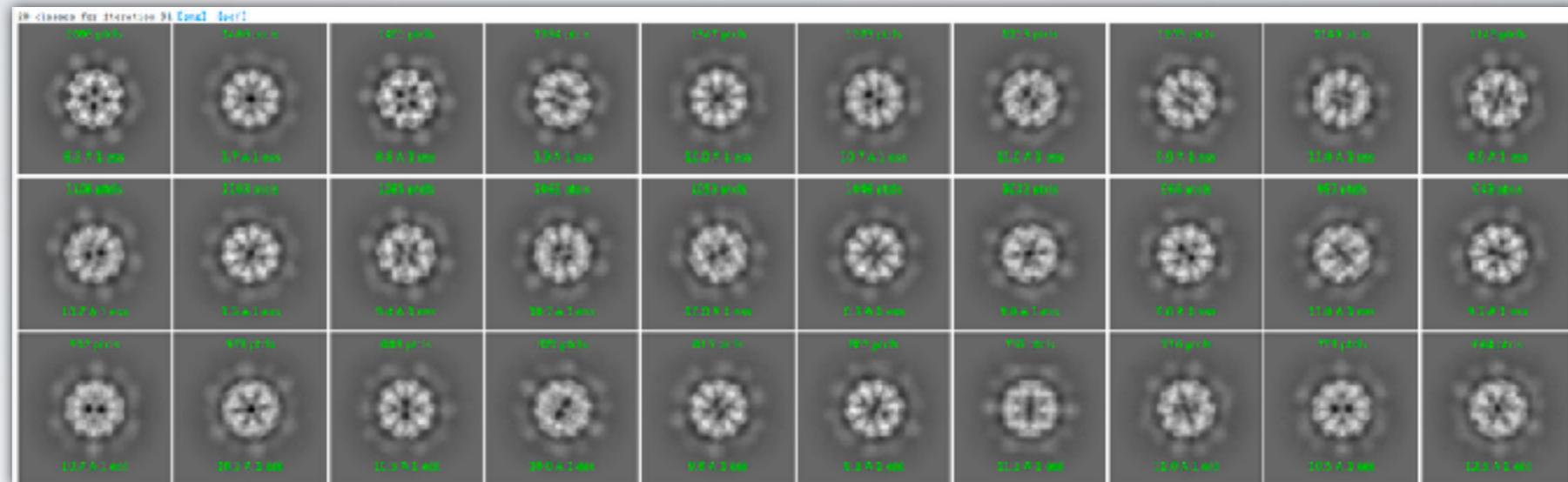
CTF refinement

particle polishing

resolution estimation

map sharpening

model building



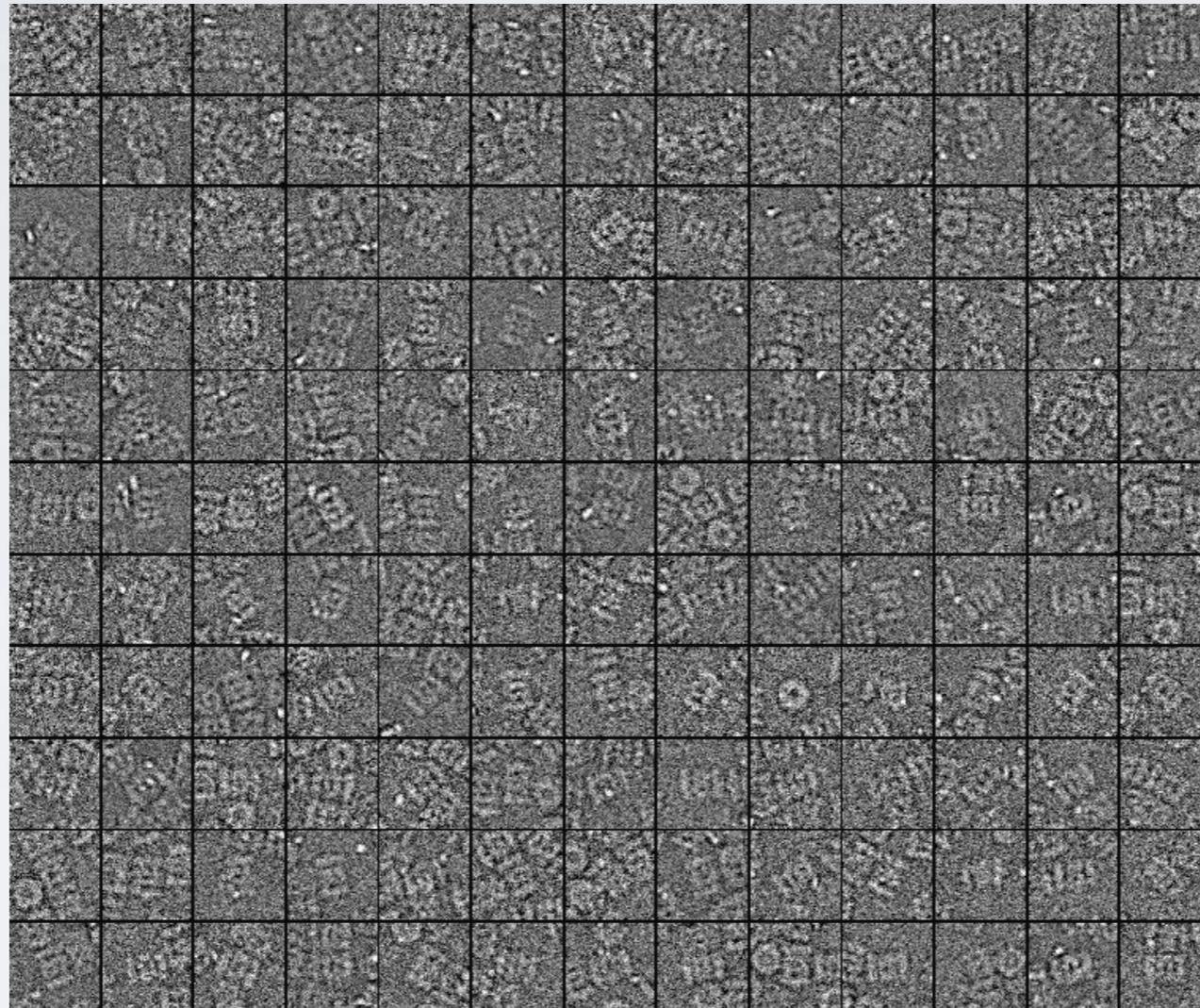
cryoSPARC

RELION

Stressosome
(expecting icosahedral
symmetry)

Improving the resolution of initial model

particles



initial model



frame alignment

CTF estimation

particle picking

2-D align/classify

initial model

3D align/classify

refinement

CTF refinement

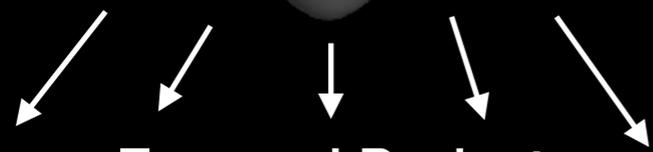
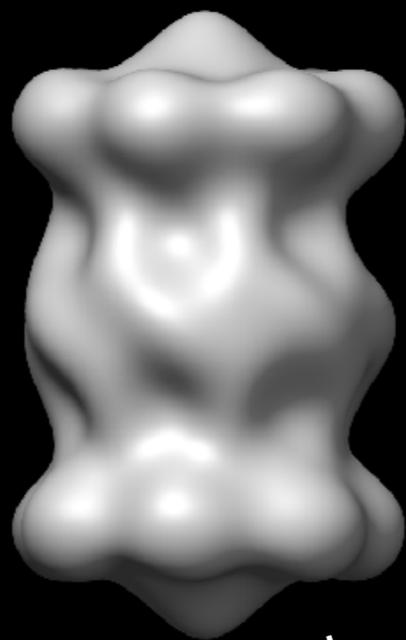
particle polishing

resolution estimation

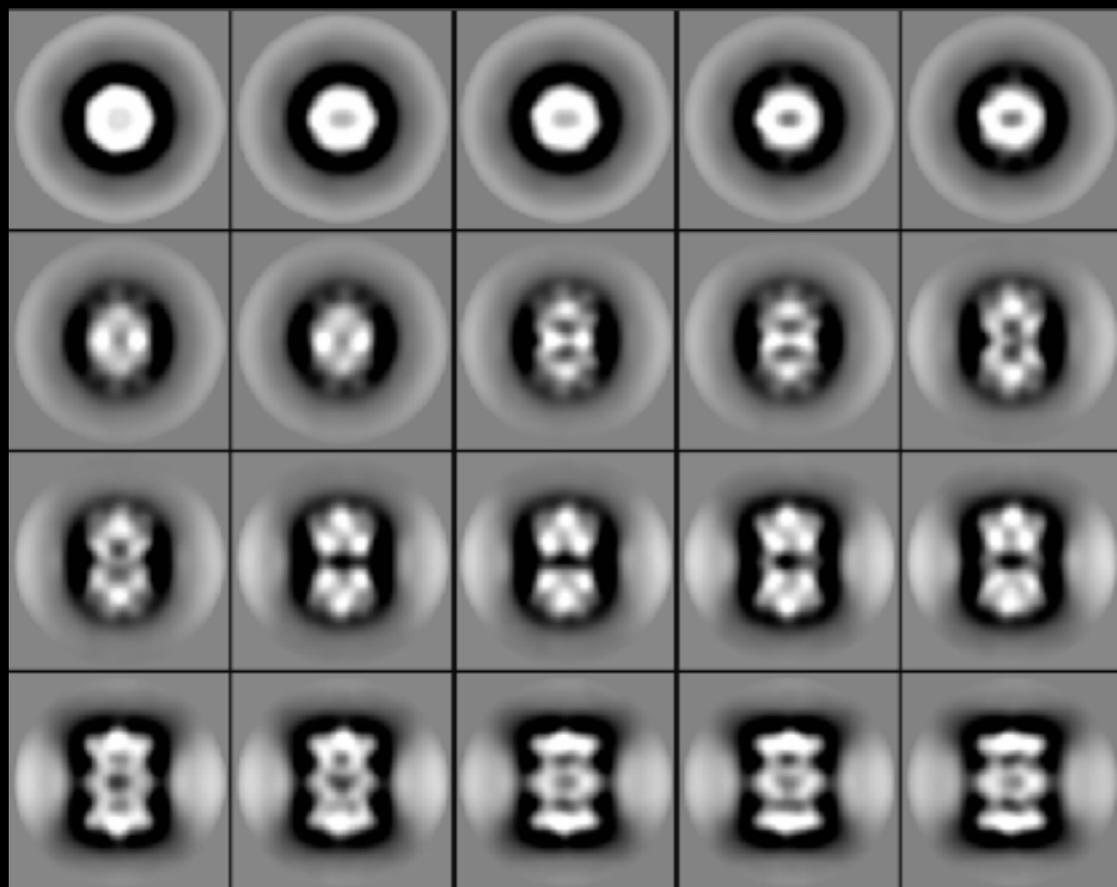
map sharpening

model building

3D
Model



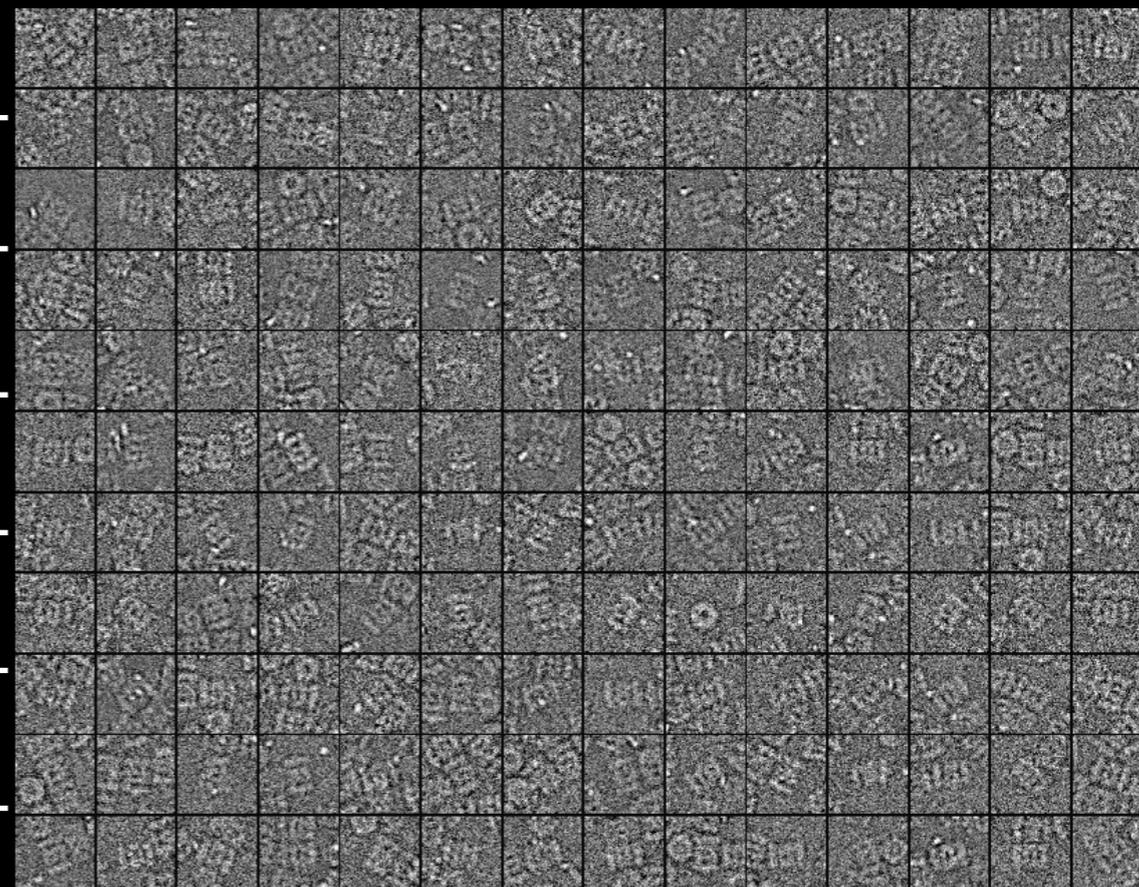
Forward Project



Projection
Matching

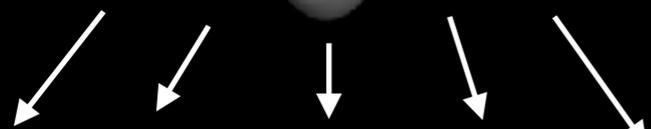


Back Project

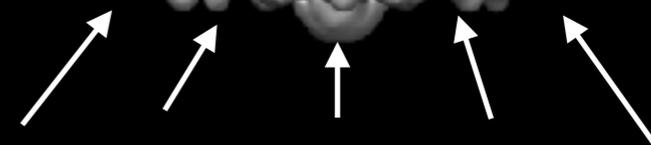
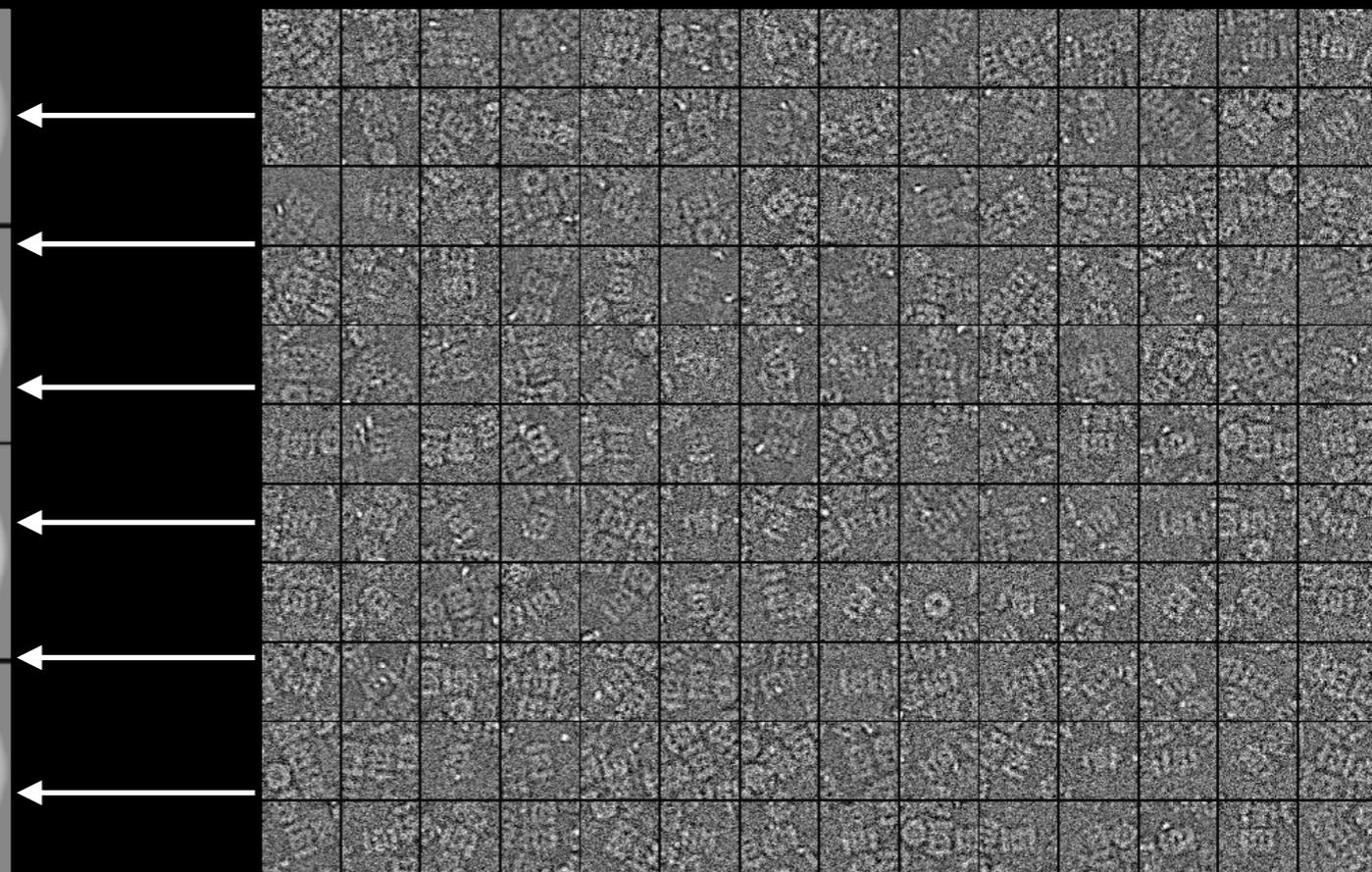
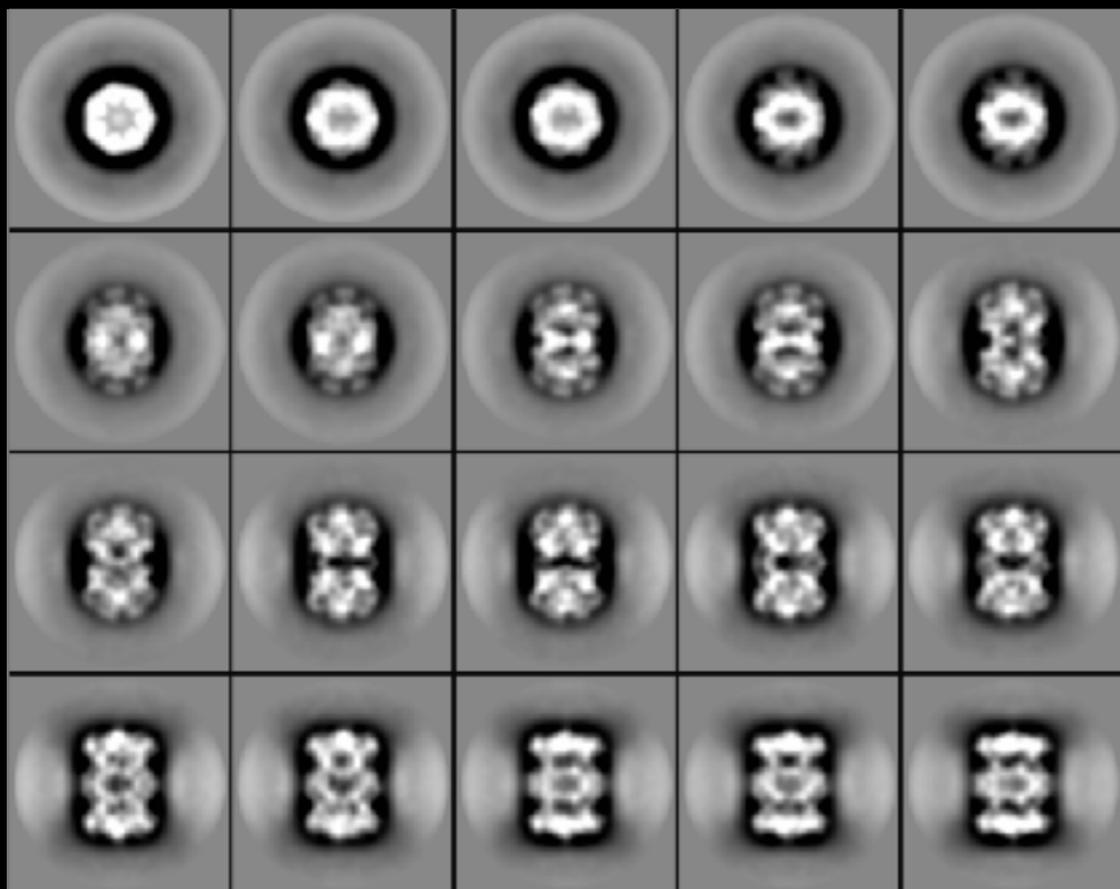


CryoEM Dataset

3D
Model



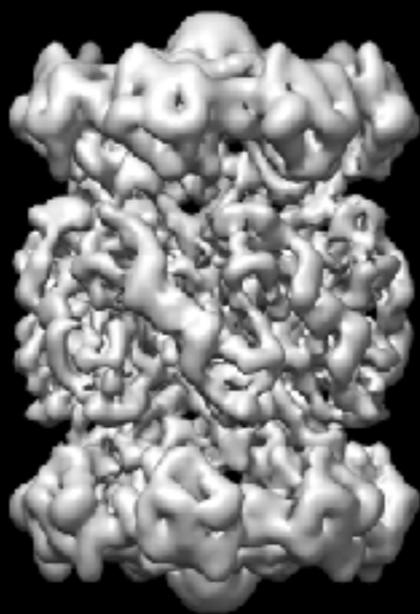
Forward Project



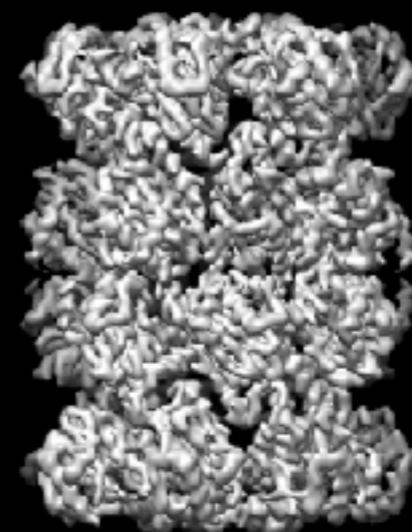
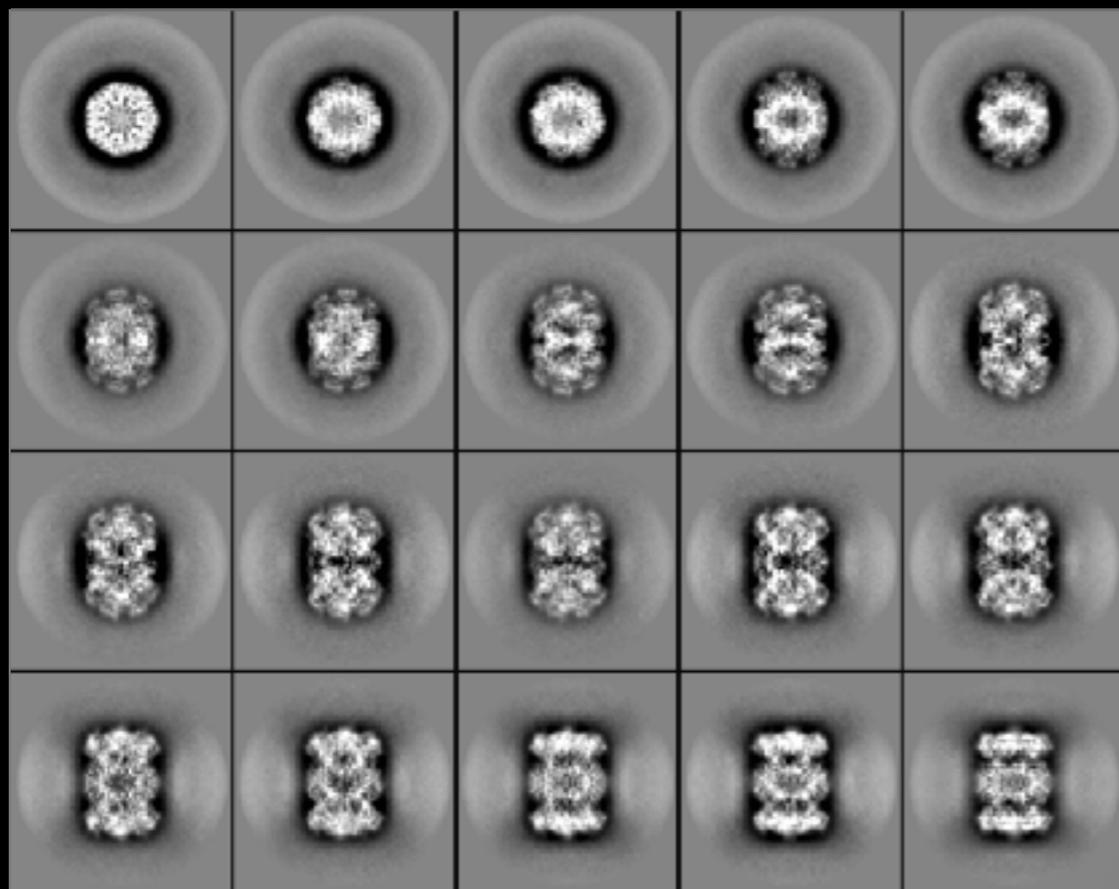
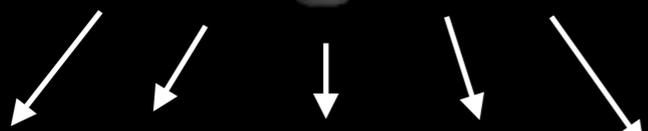
Back Project

Projection
Matching

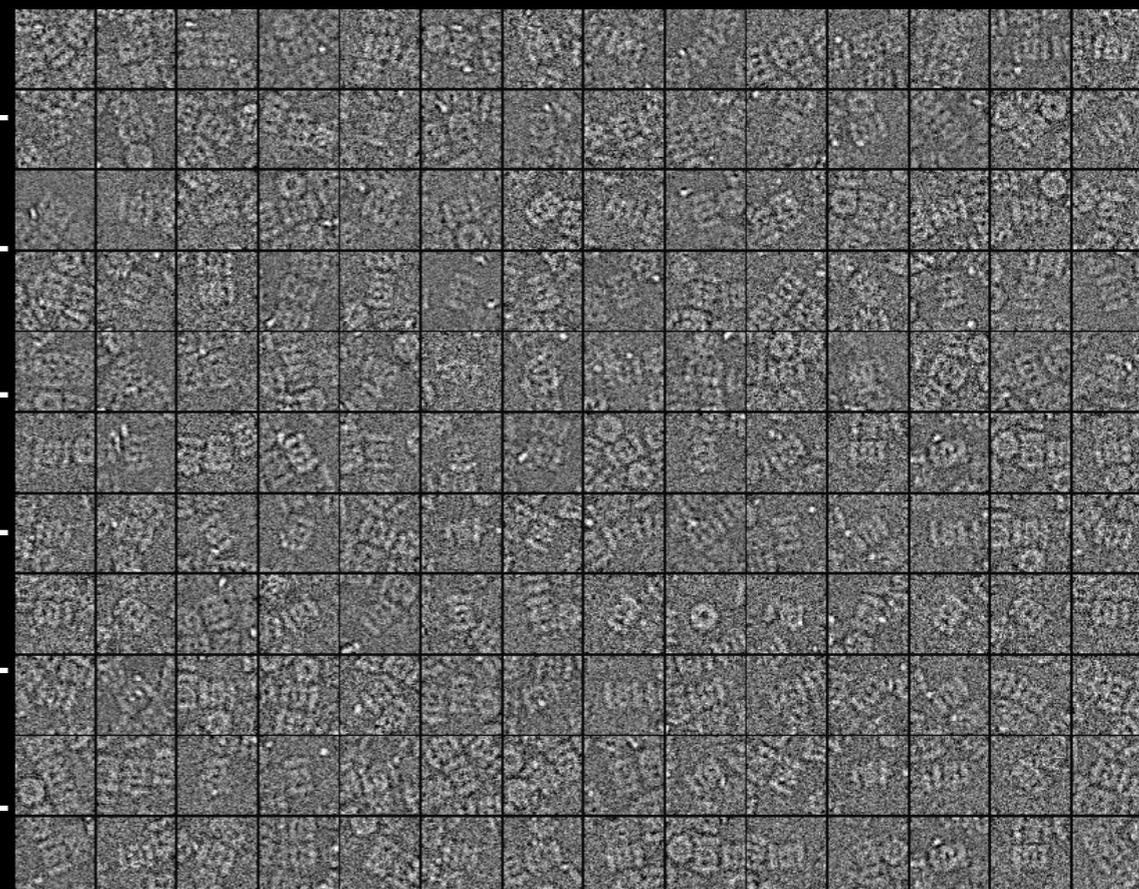
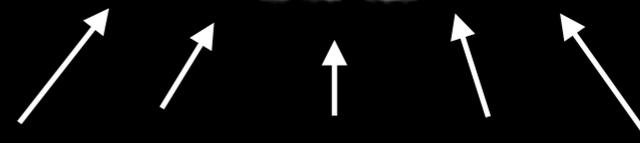
3D
Model



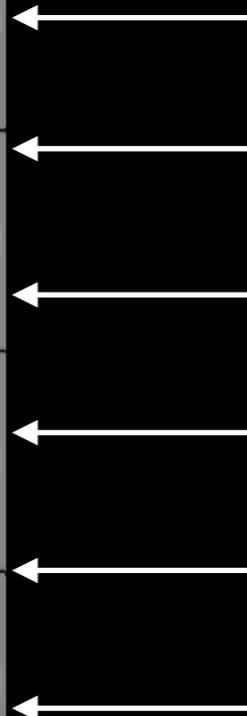
Forward Project



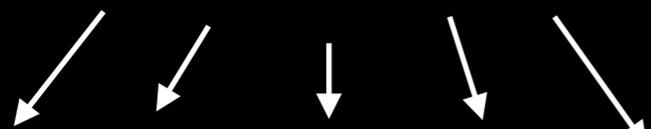
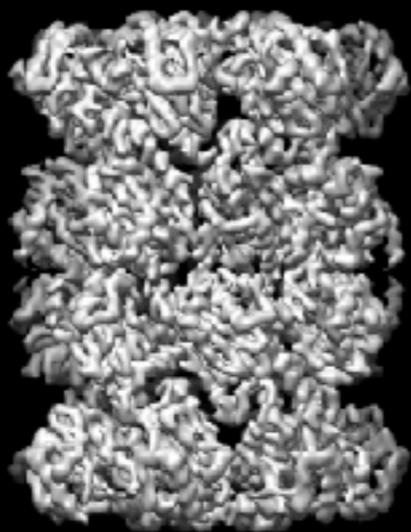
Back Project



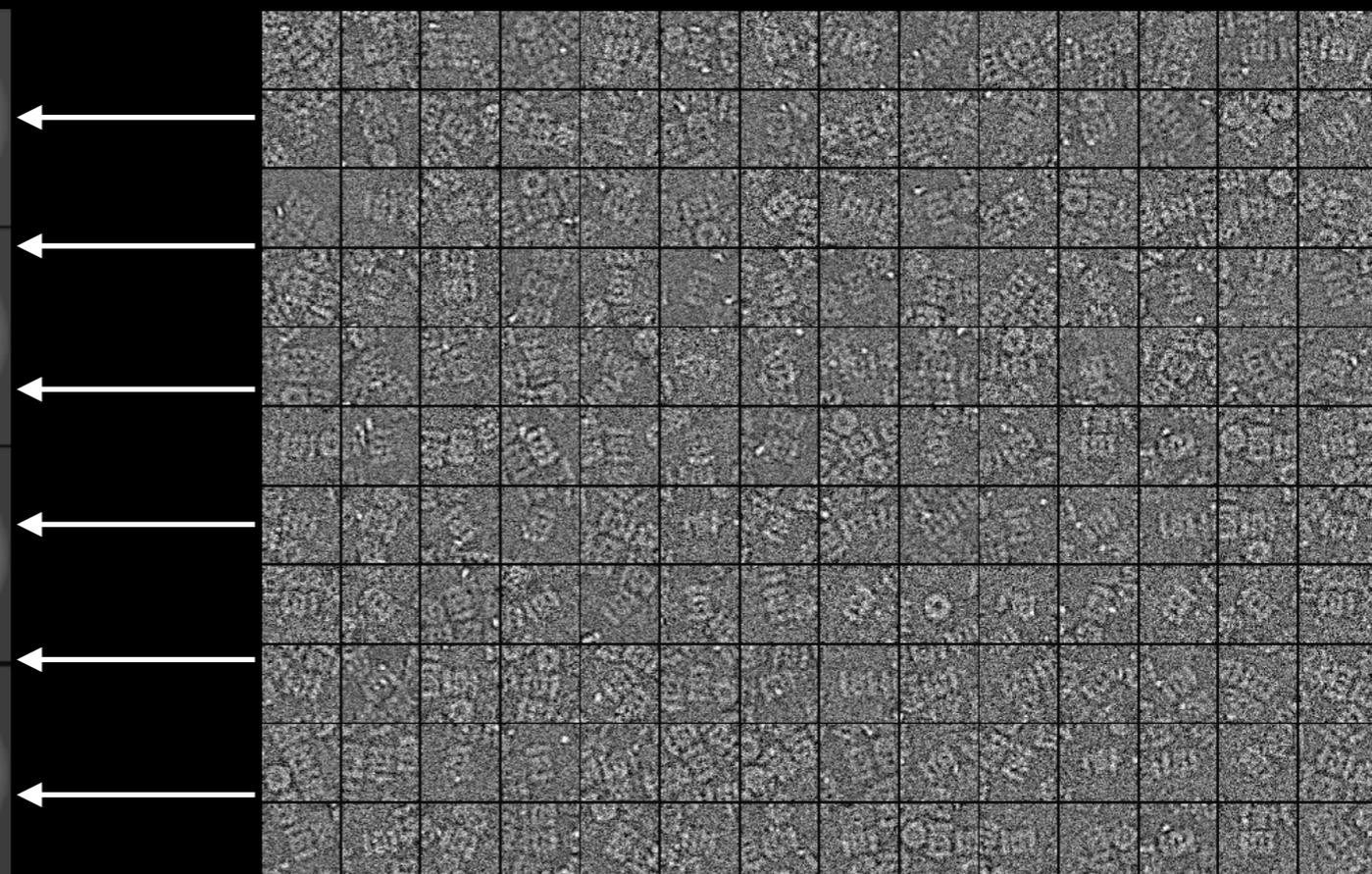
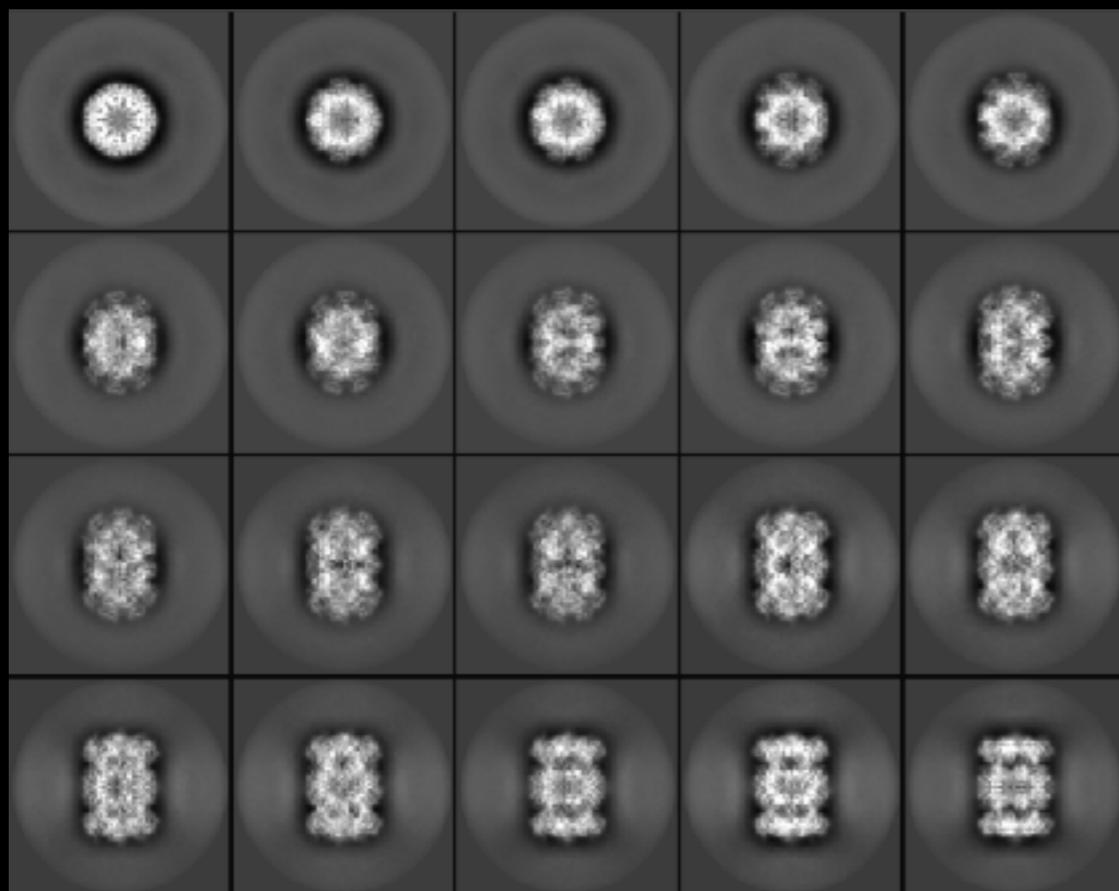
Projection
Matching



3D
Model



Forward Project



Back Project

Projection
Matching

frame alignment

CTF estimation

particle picking

2-D align/classify

initial model

3D align/classify

refinement

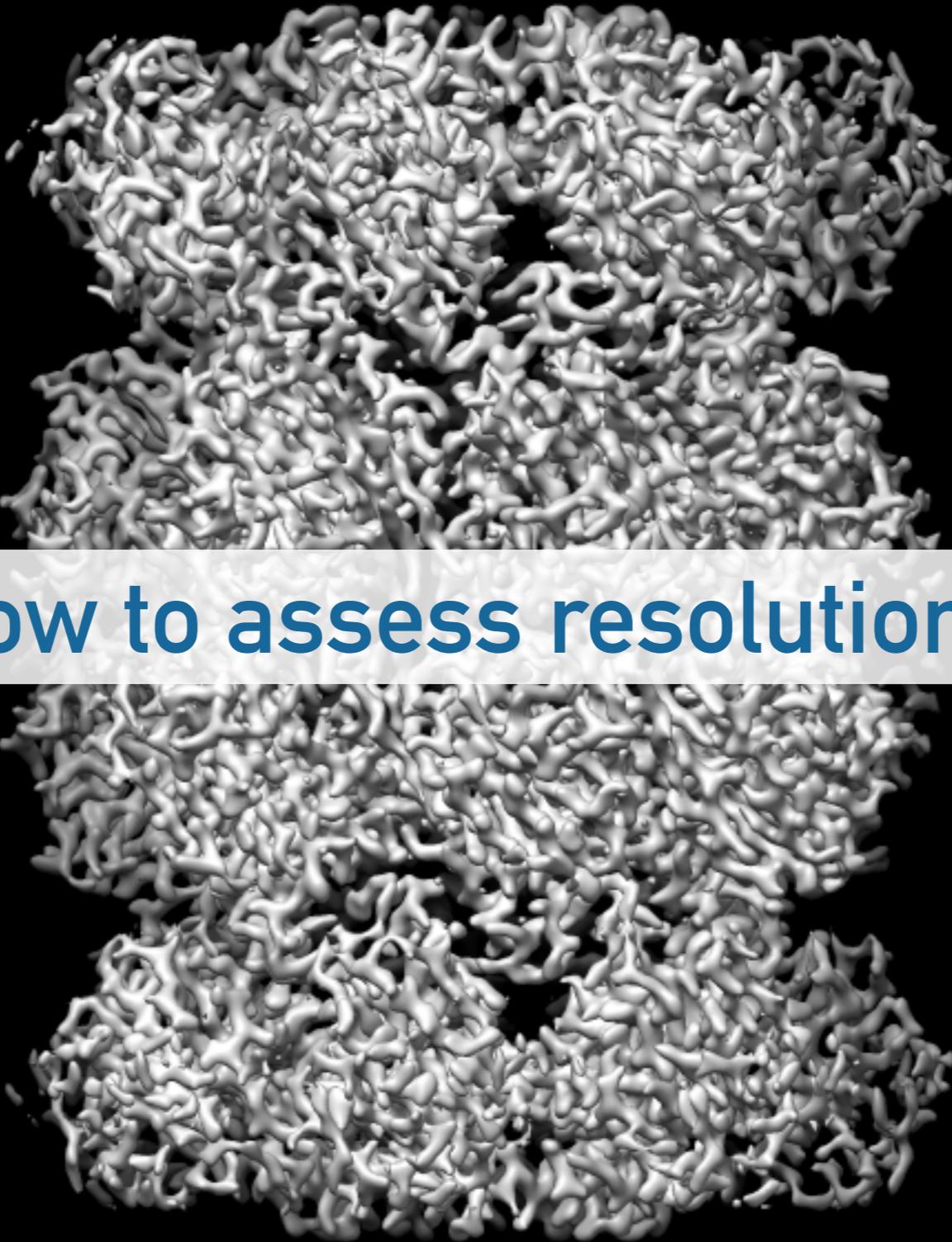
CTF refinement

particle polishing

resolution estimation

map sharpening

model building



How to assess resolution?

Fourier Shell Correlation

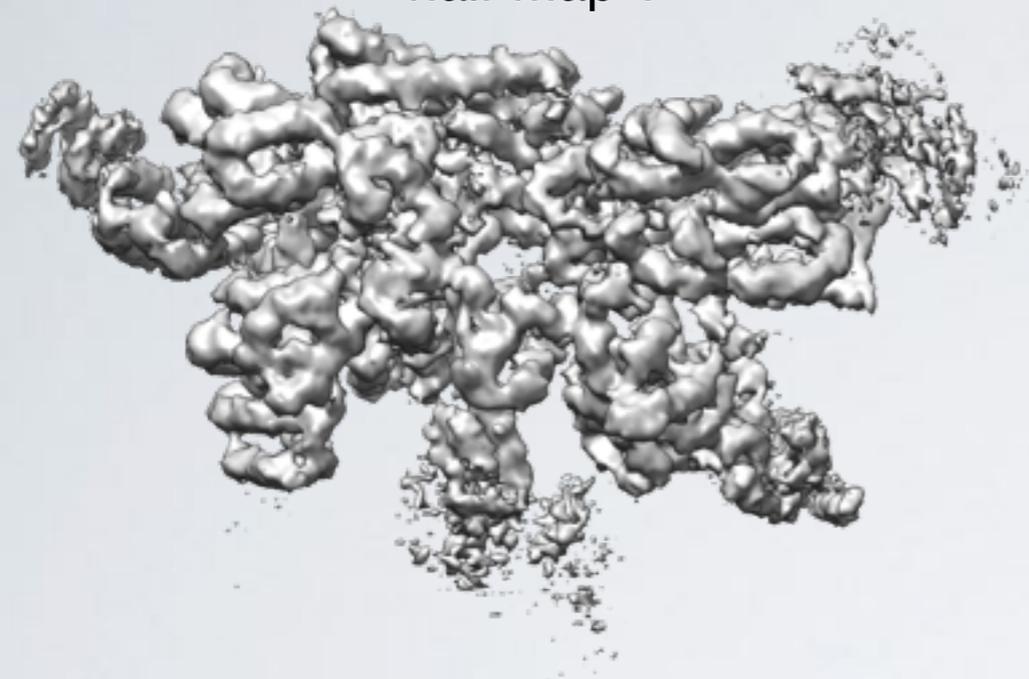


**combine all data
at the end for final
reconstruction**

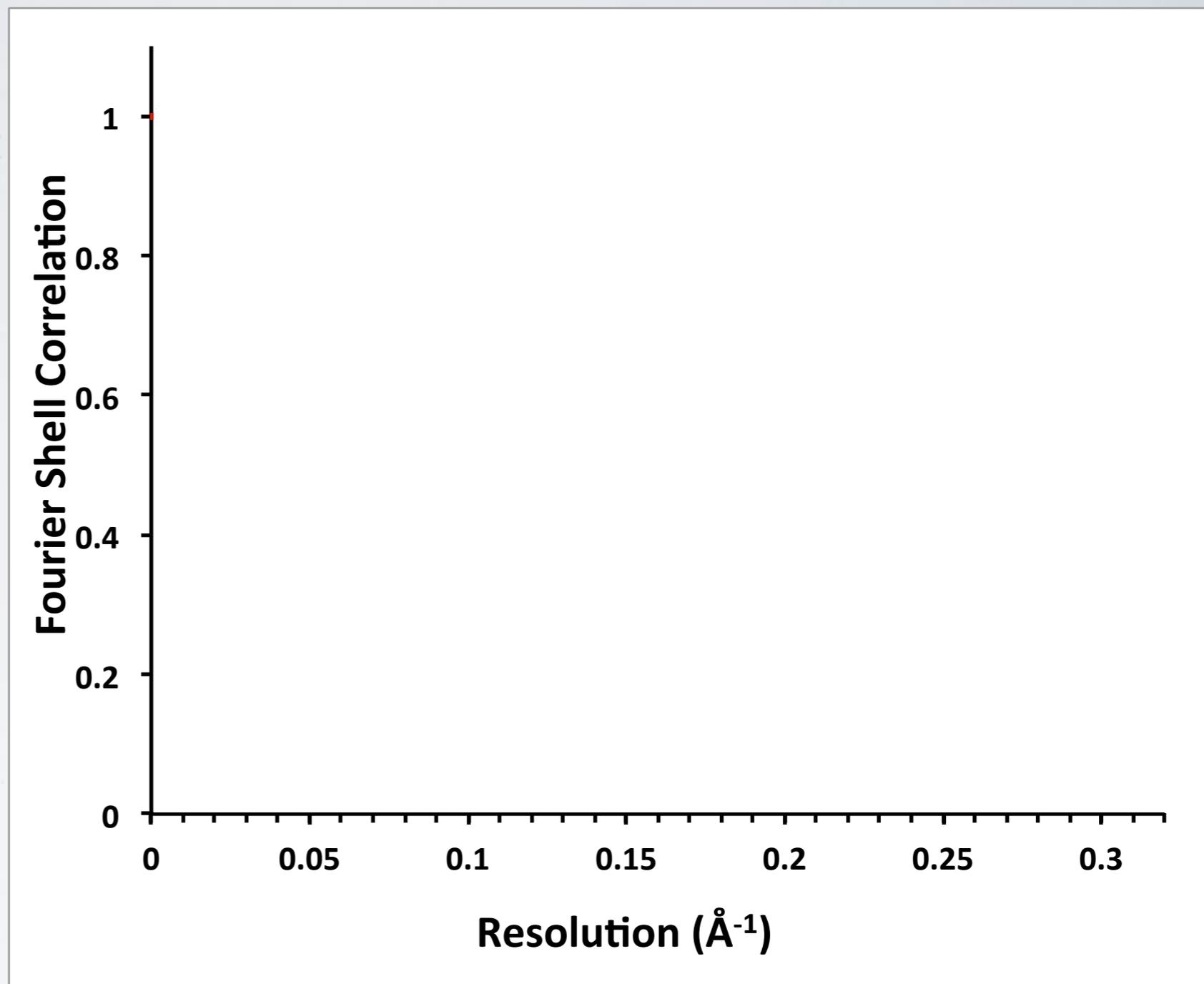
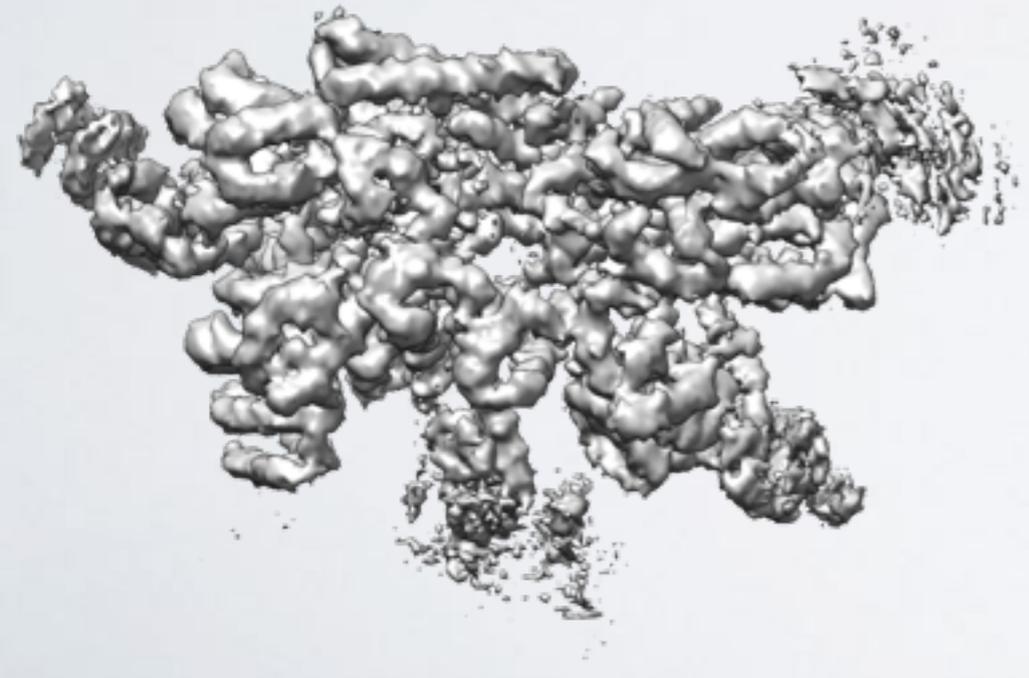
“Gold Standard” FSC

Fourier Shell Correlation

half-map 1



half-map 2

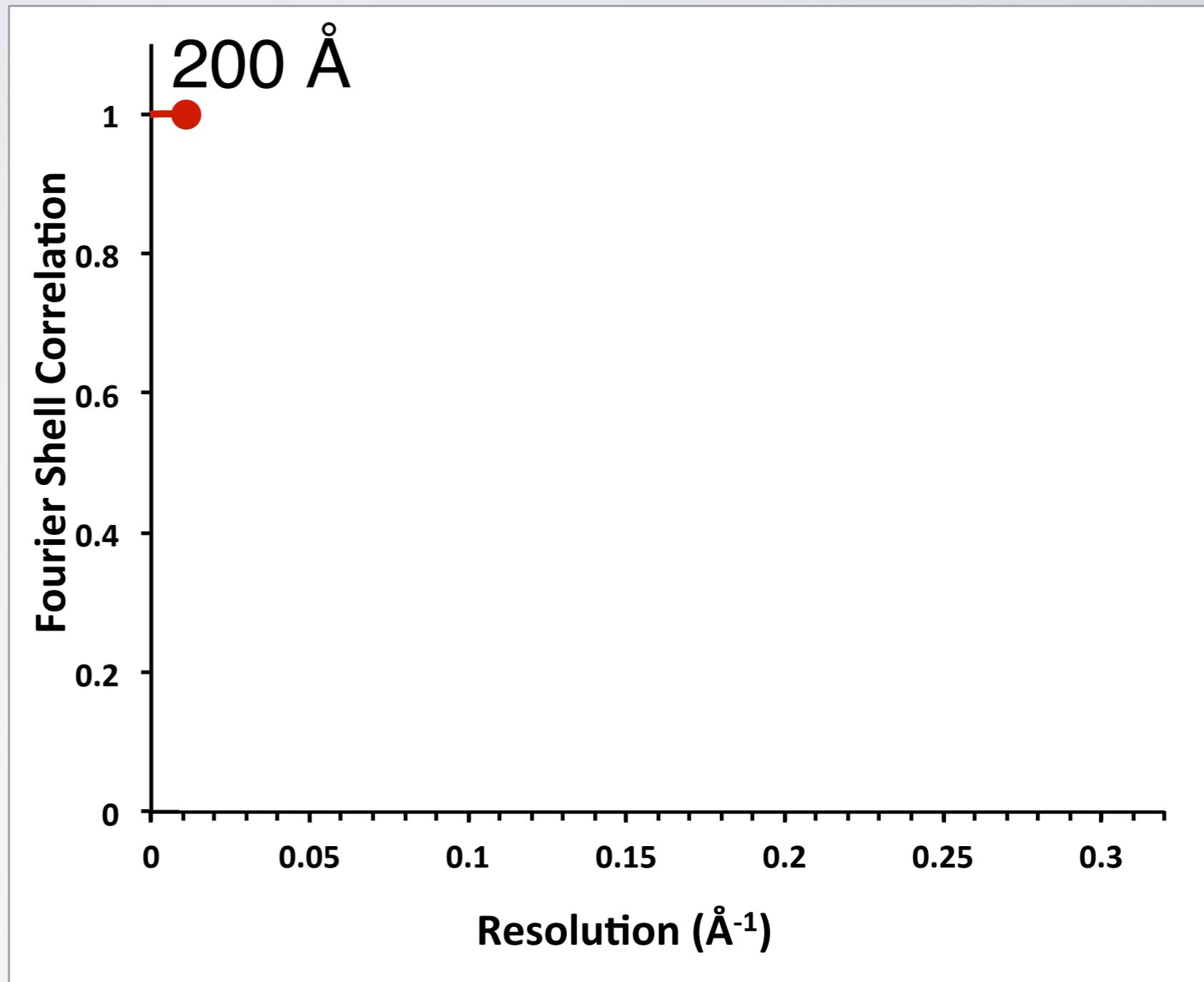
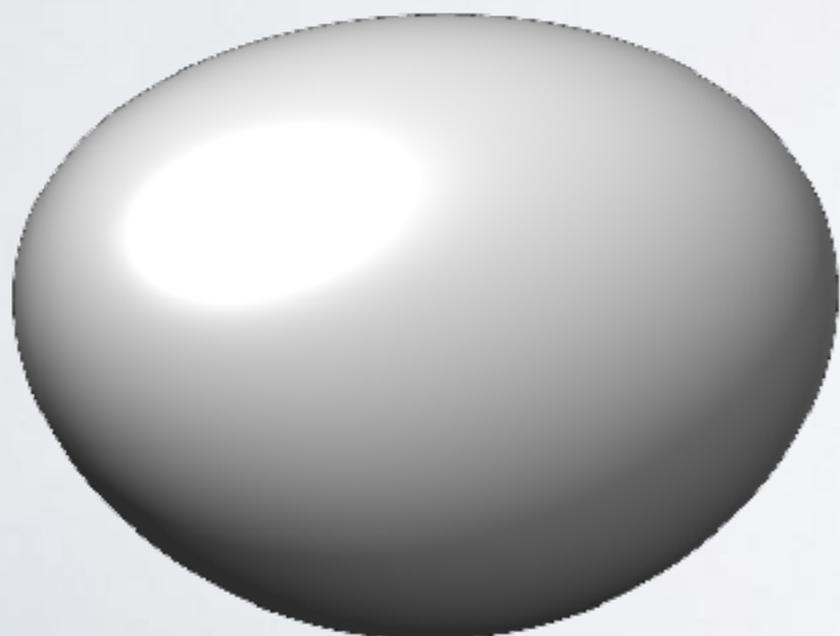


Fourier Shell Correlation

half-map 1

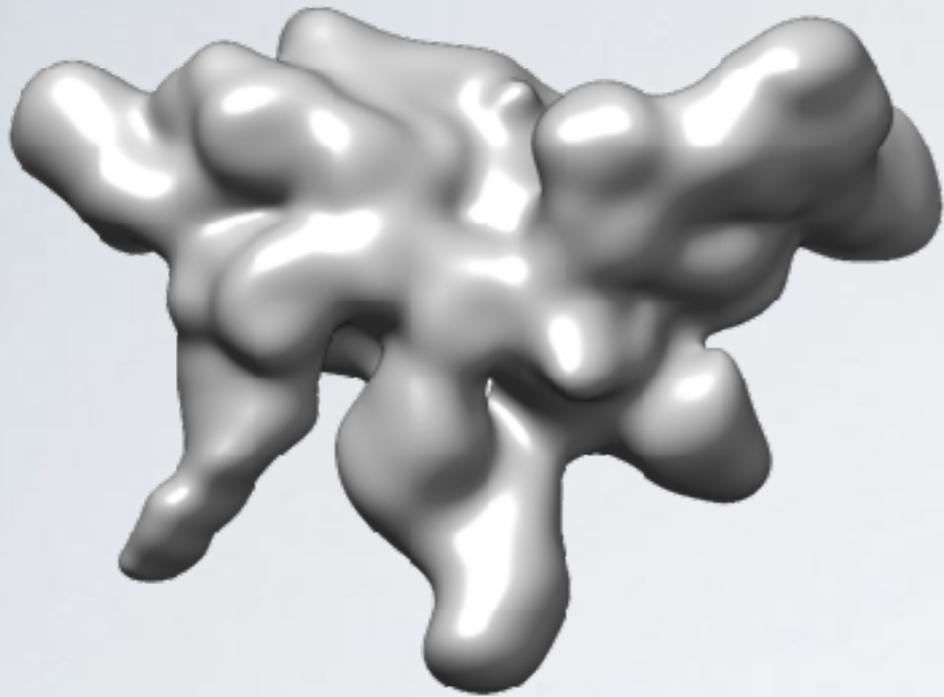


half-map 2

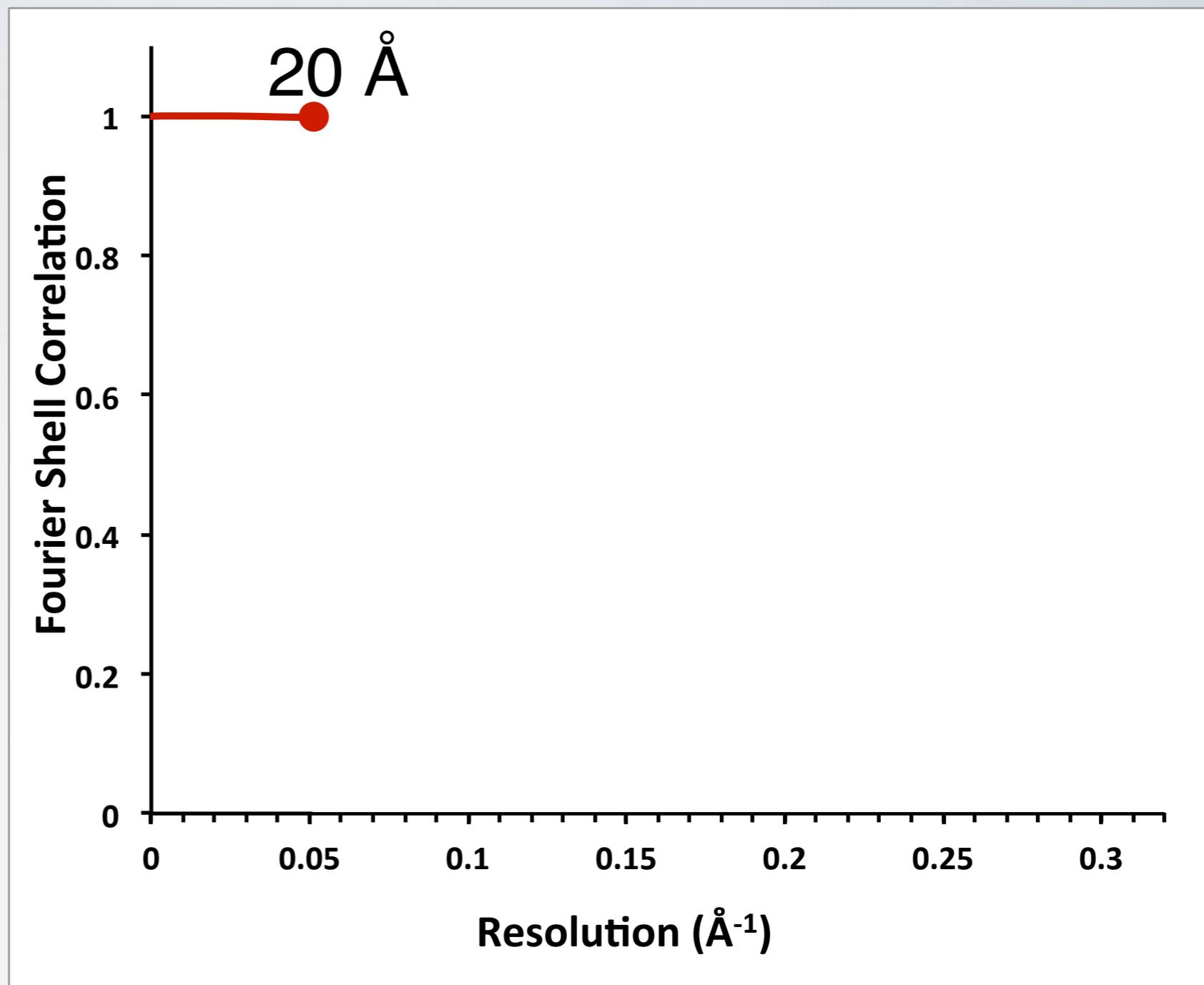
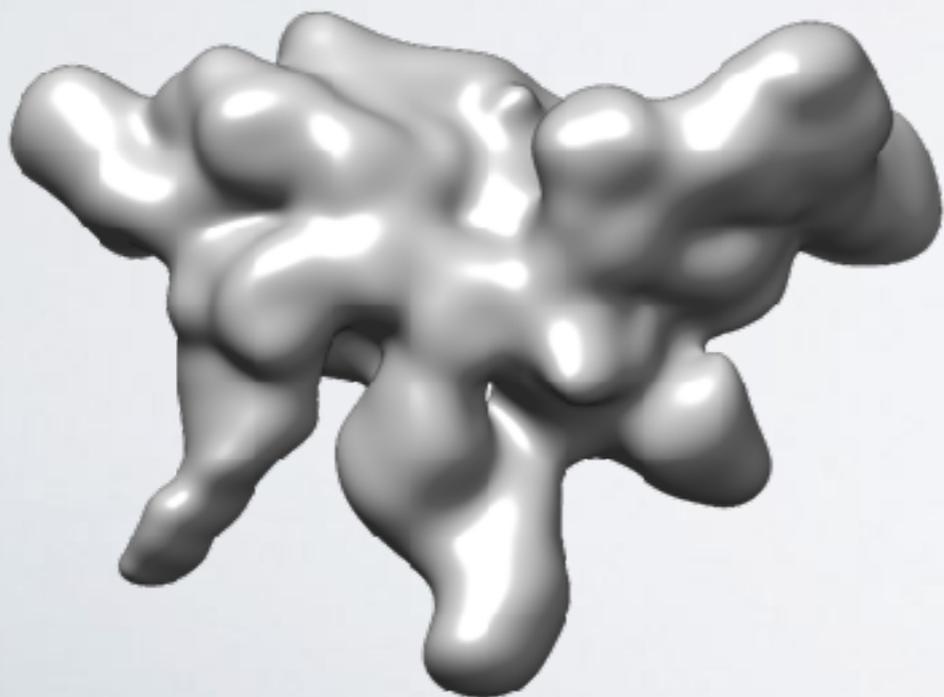


Fourier Shell Correlation

half-map 1

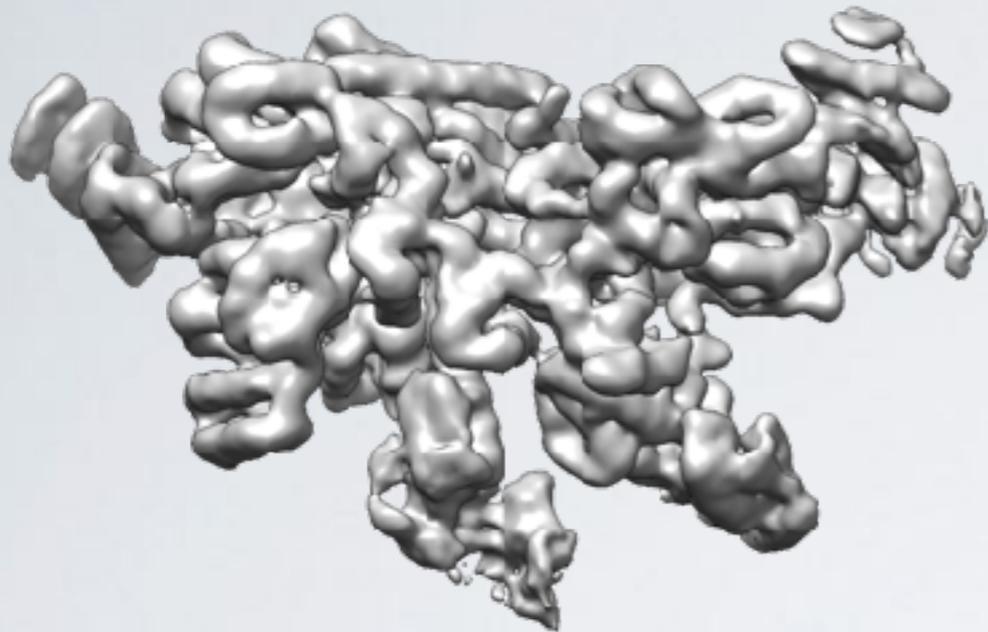


half-map 2

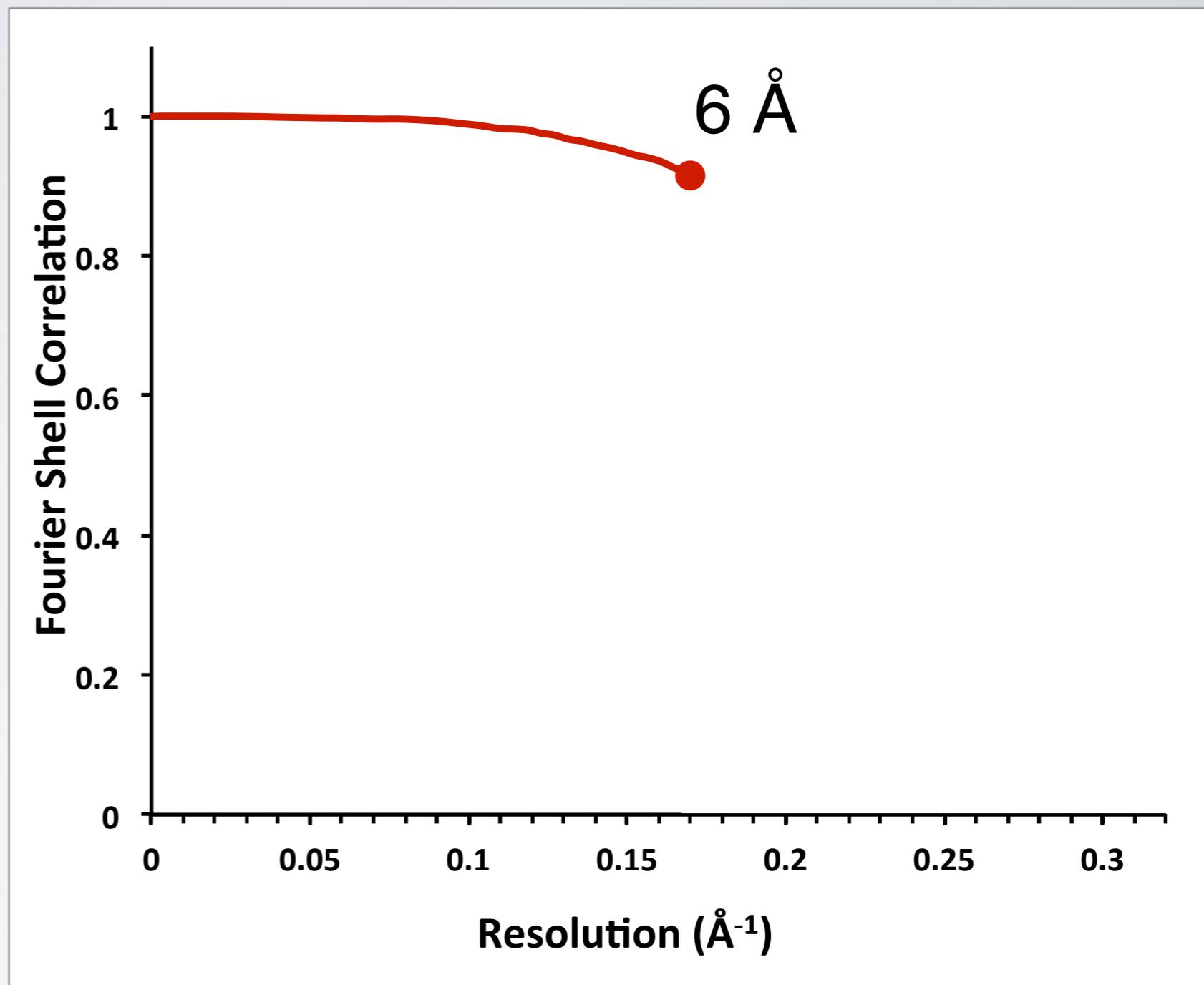
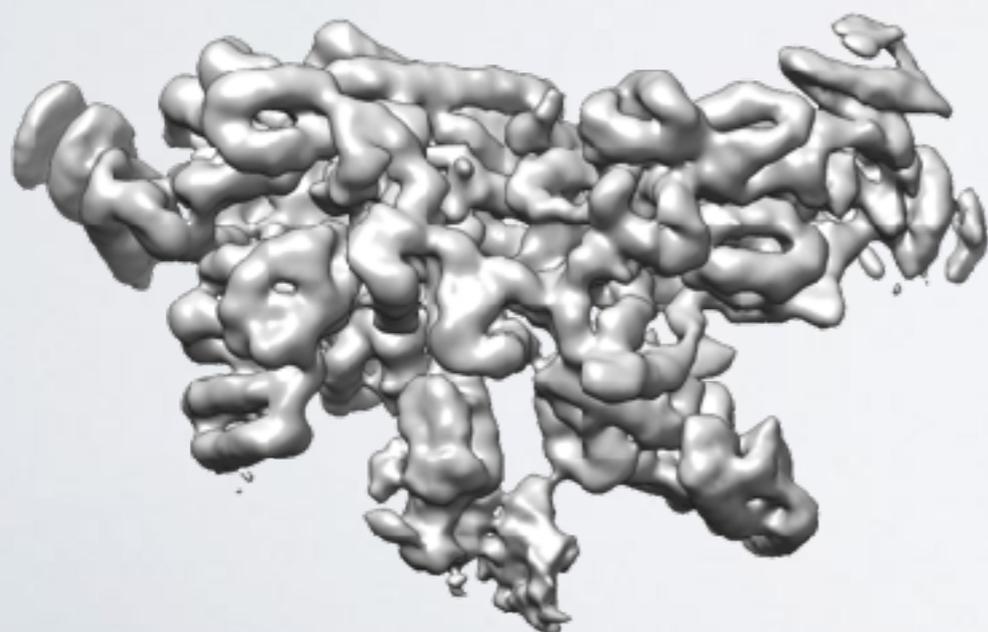


Fourier Shell Correlation

half-map 1

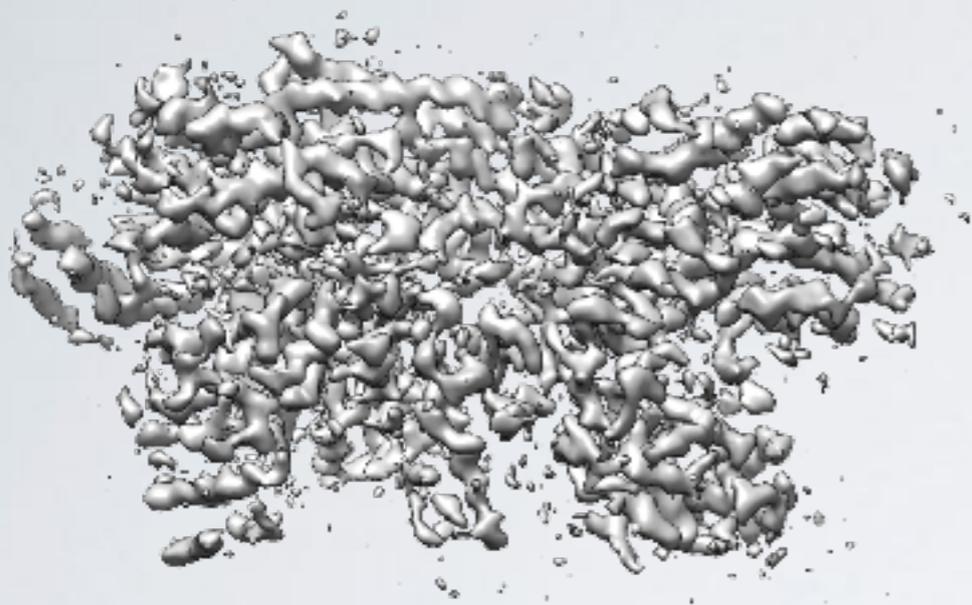


half-map 2

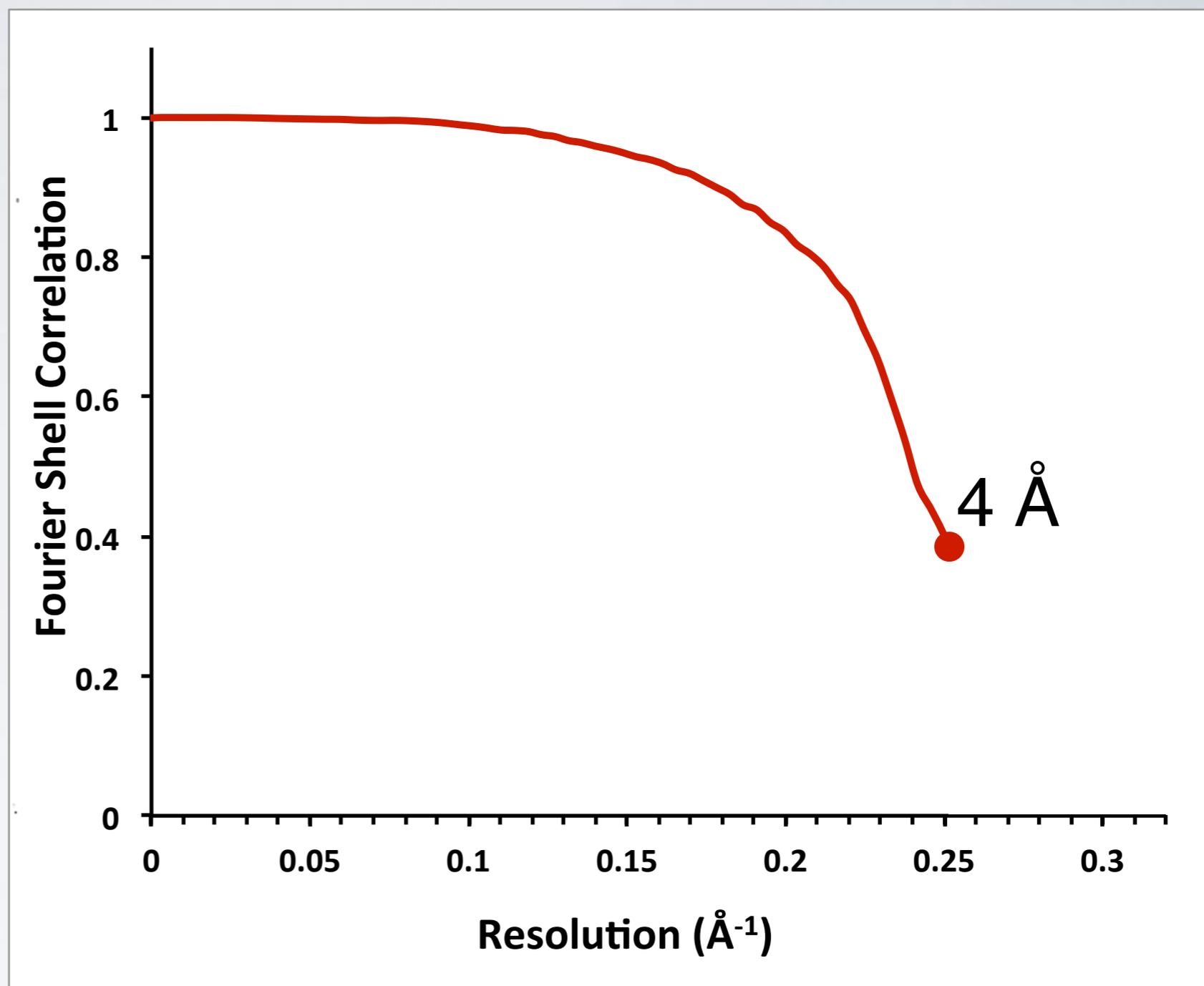
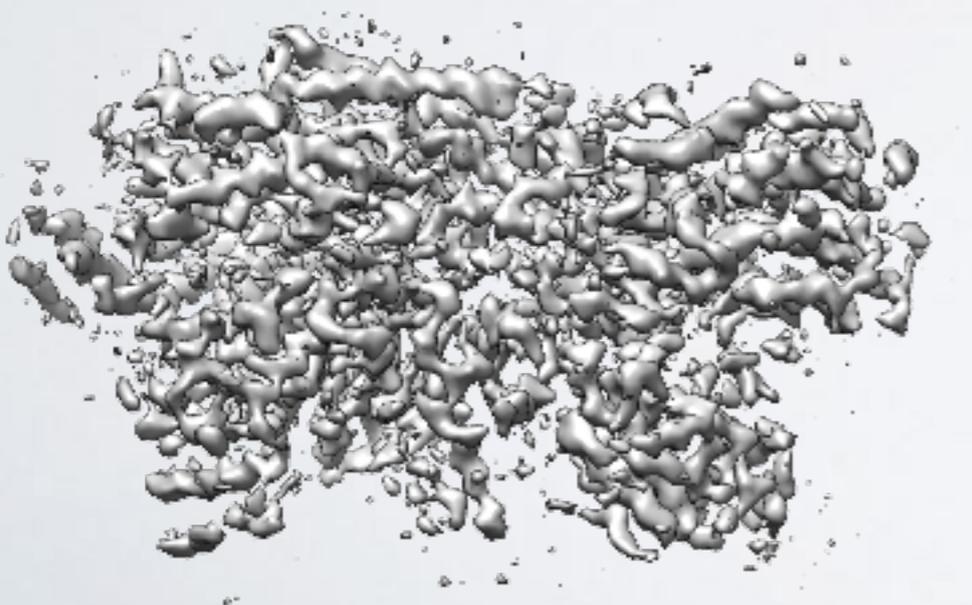


Fourier Shell Correlation

half-map 1



half-map 2

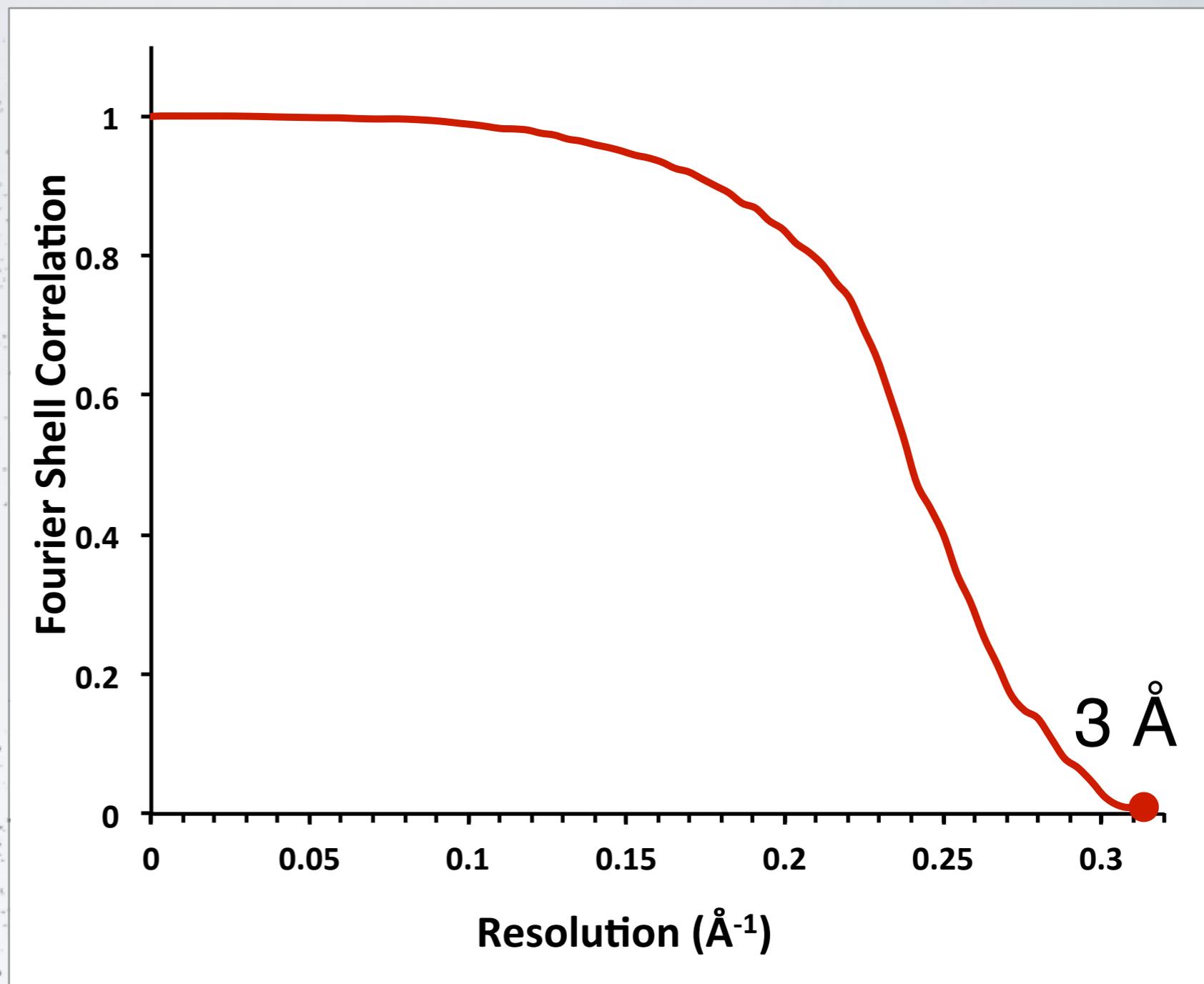


Fourier Shell Correlation

half-map 1

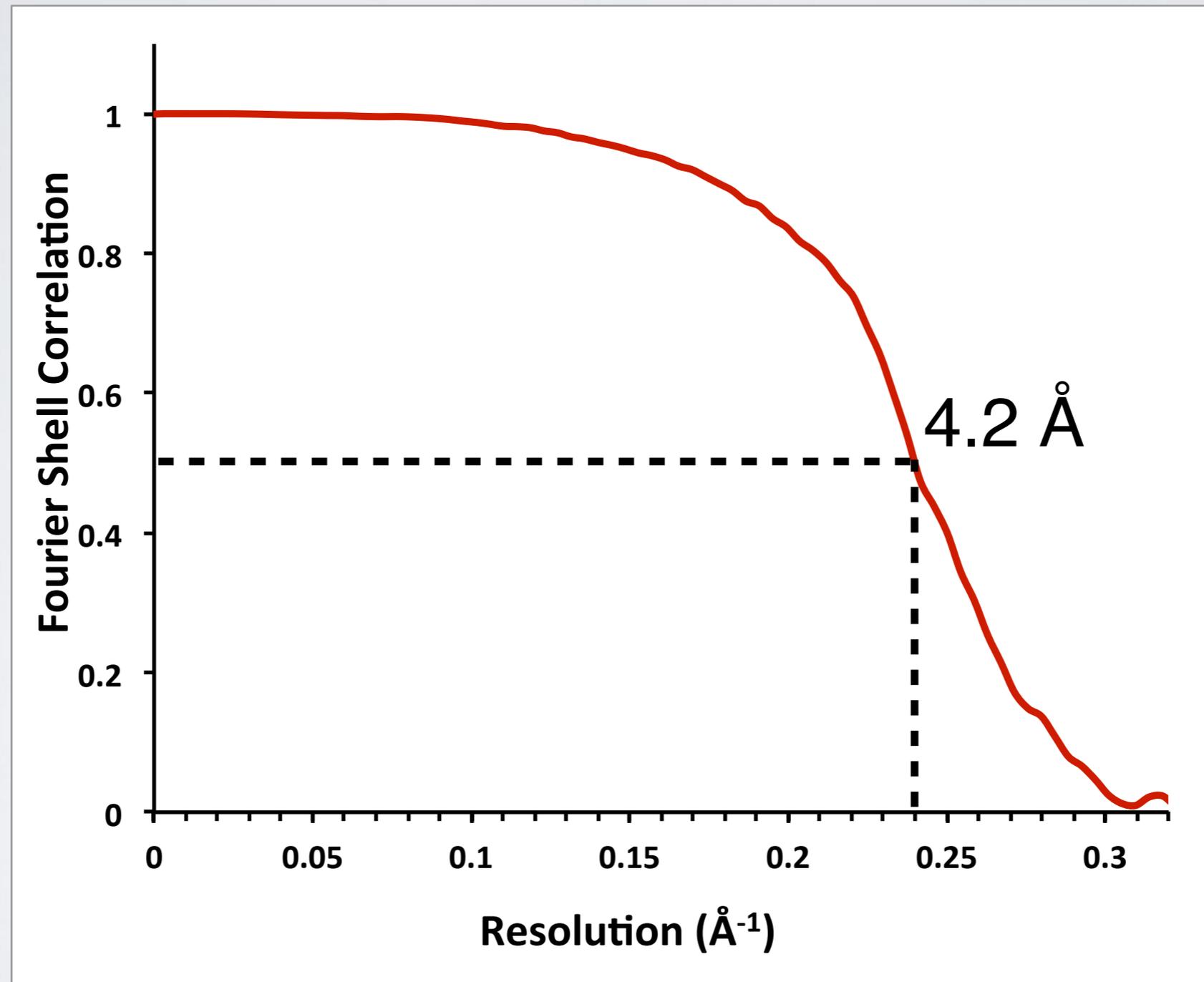


half-map 2



Fourier Shell Correlation

How to assign a single value from this curve?



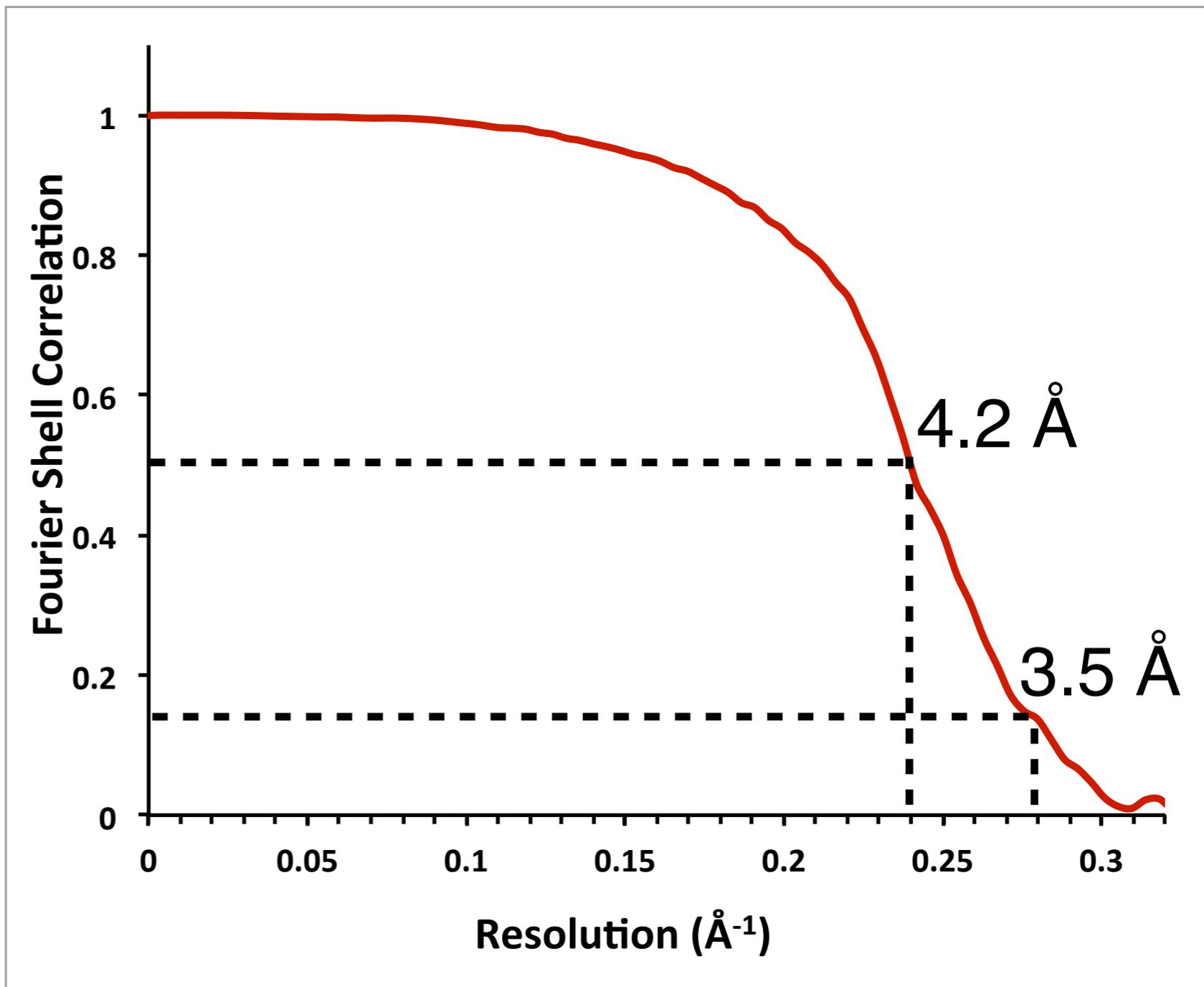
Fourier Shell Correlation

$$FoM = \sqrt{\frac{2 \cdot FSC}{1 + FSC}}$$

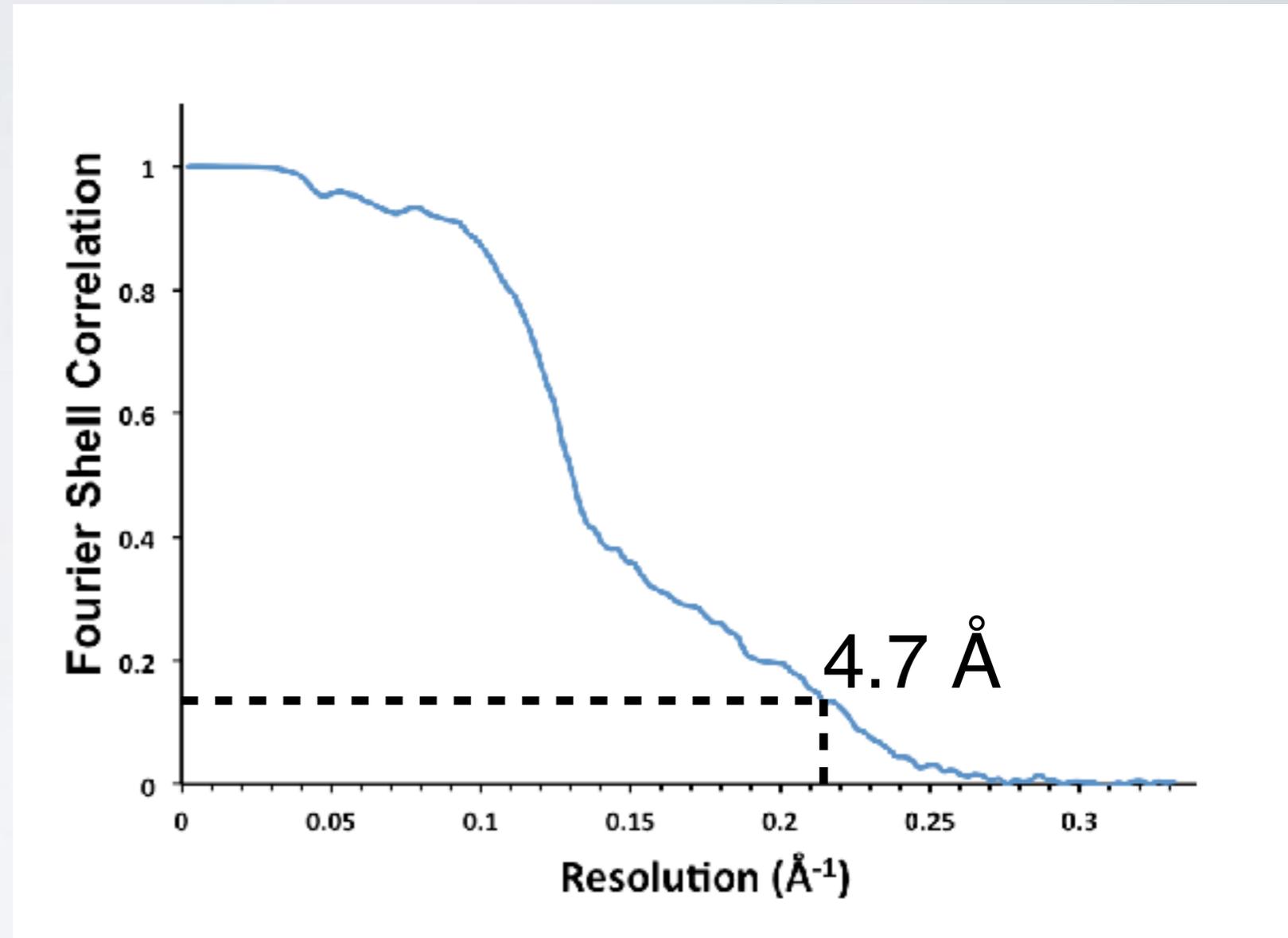
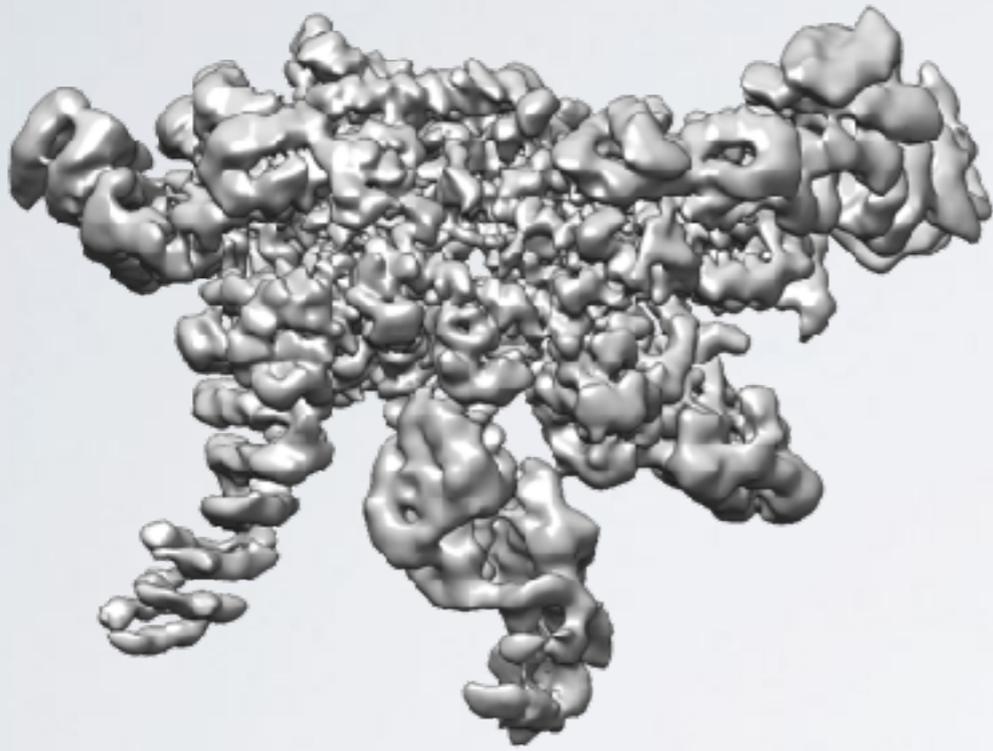
at FSC = 0.5
FoM is 0.816

at FSC = 0.143
FoM is 0.5

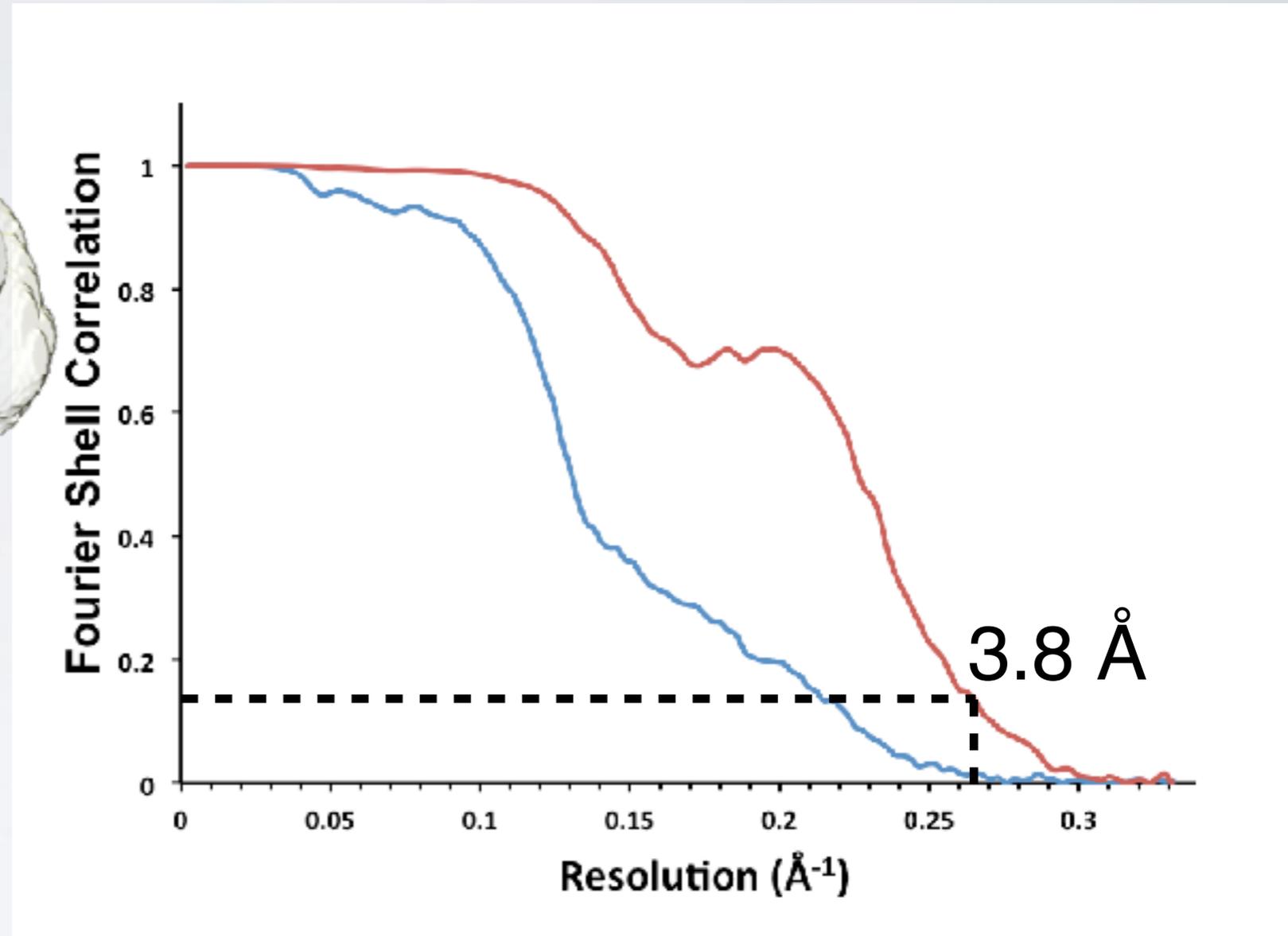
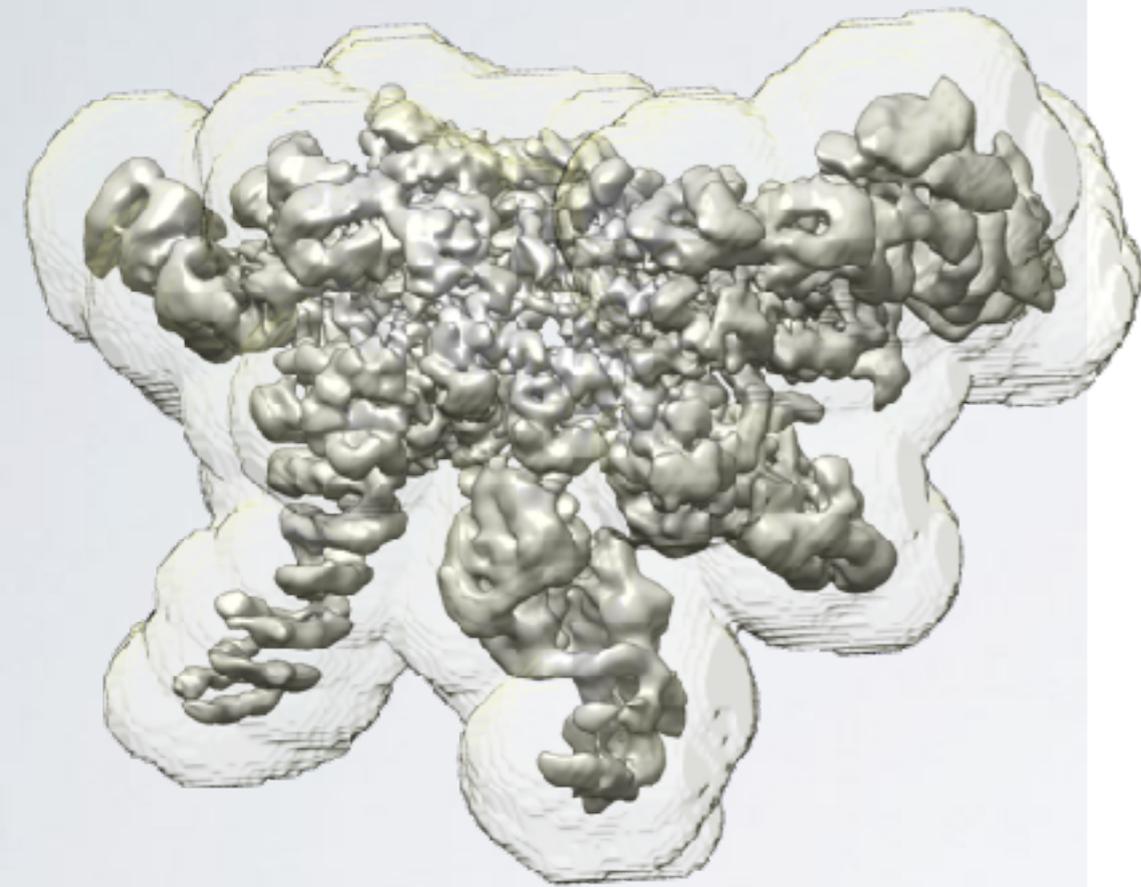
Rosenthal and Henderson JMB (2003)



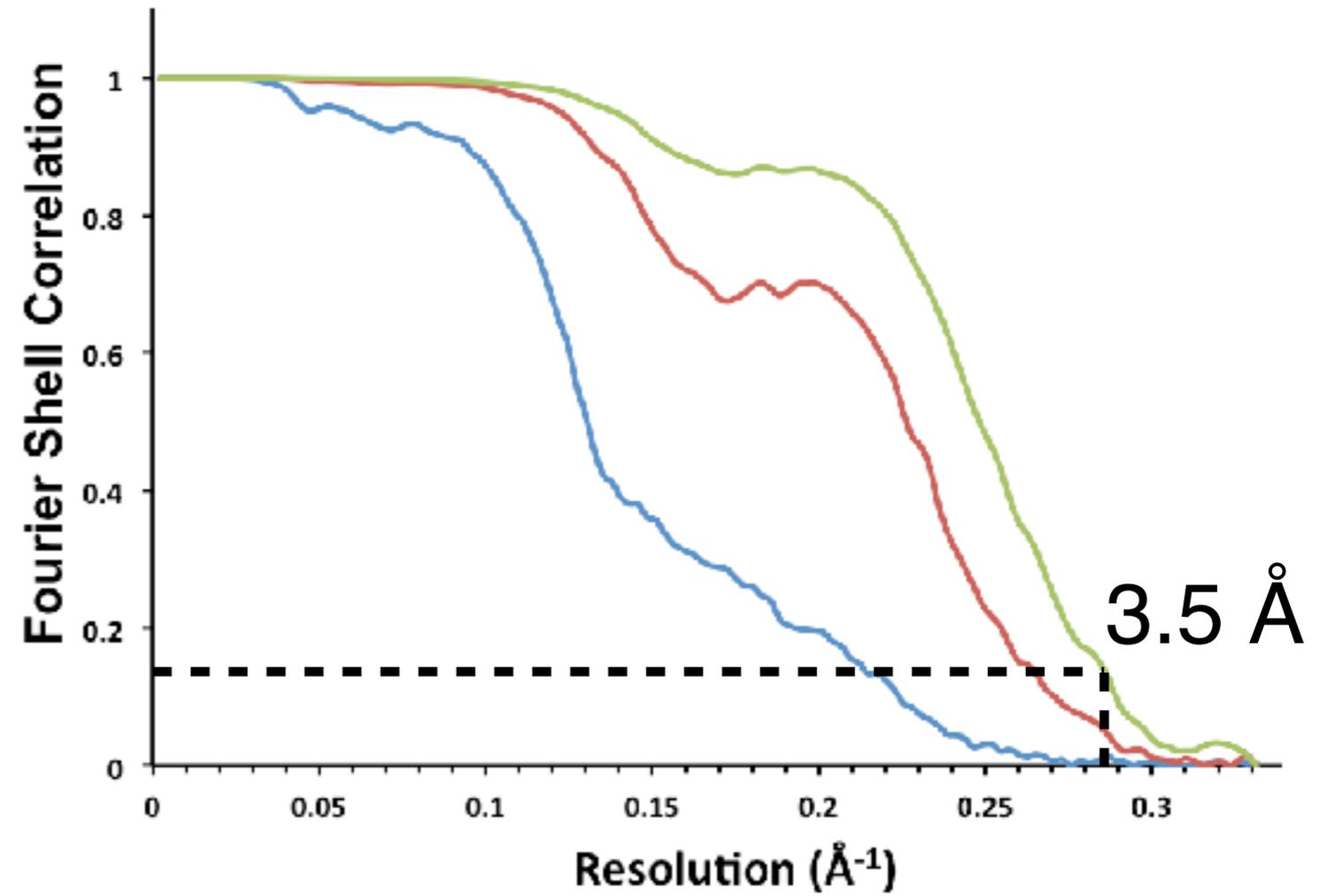
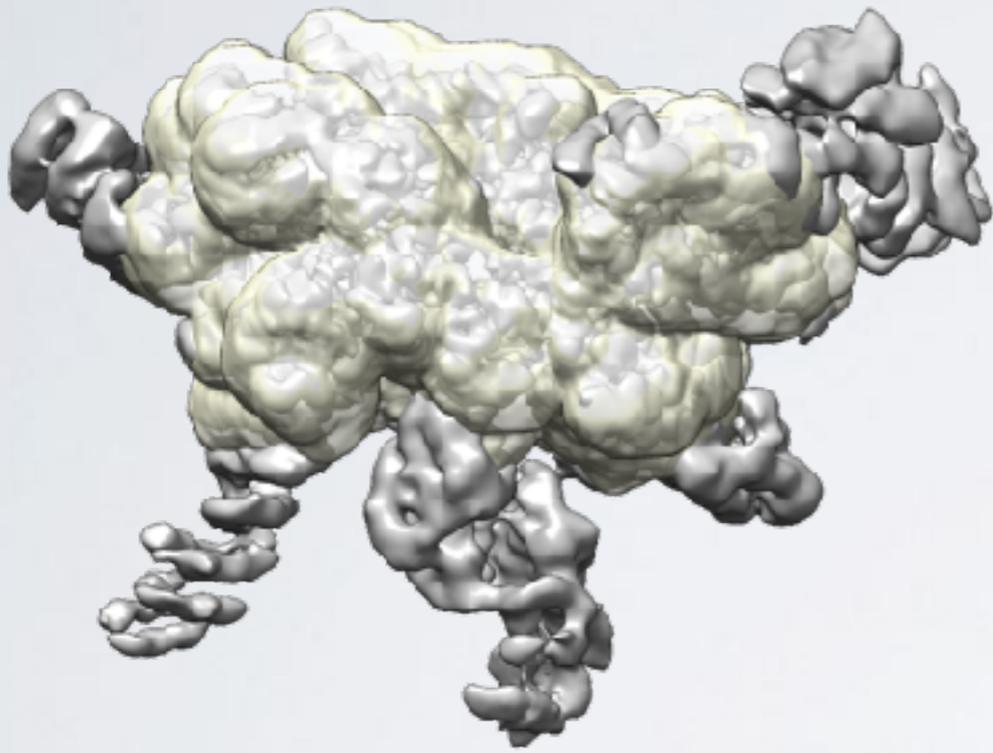
Fourier Shell Correlation - 3D Masking



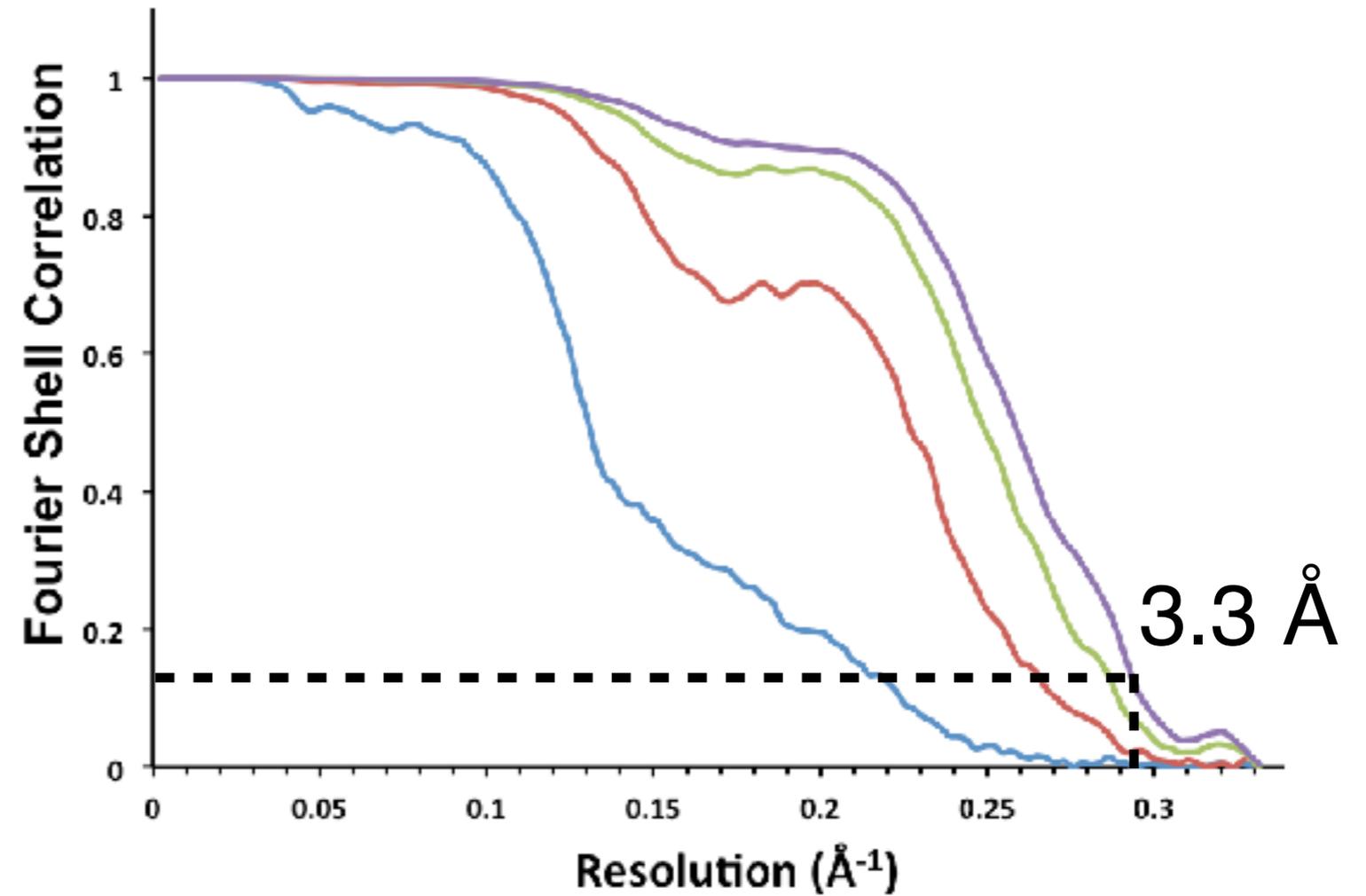
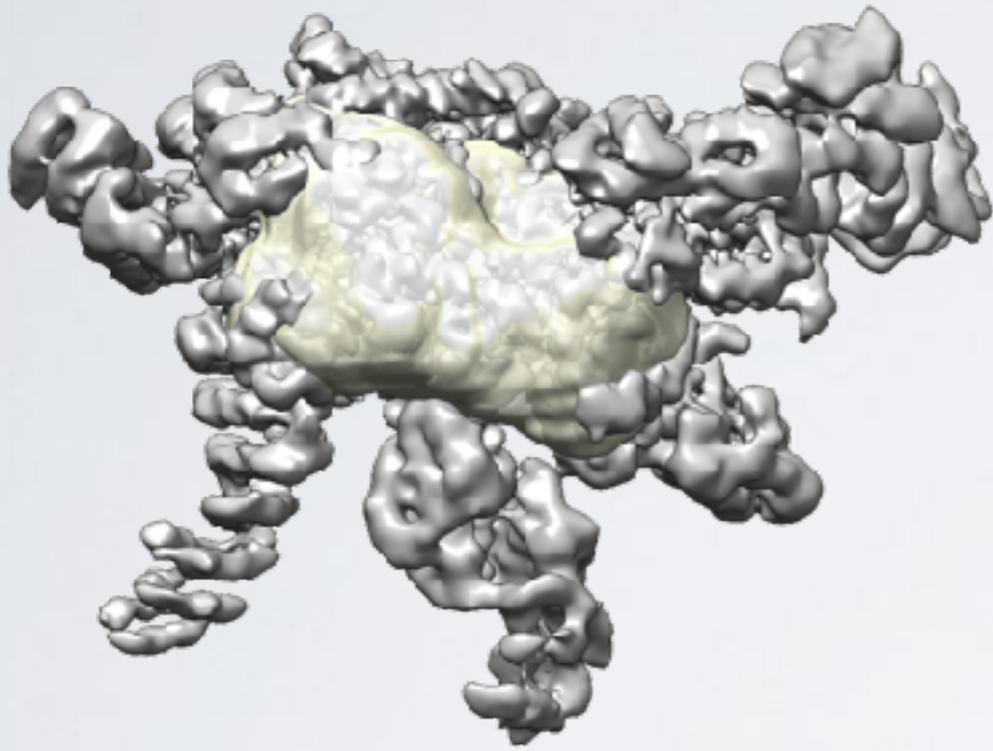
Fourier Shell Correlation - 3D Masking



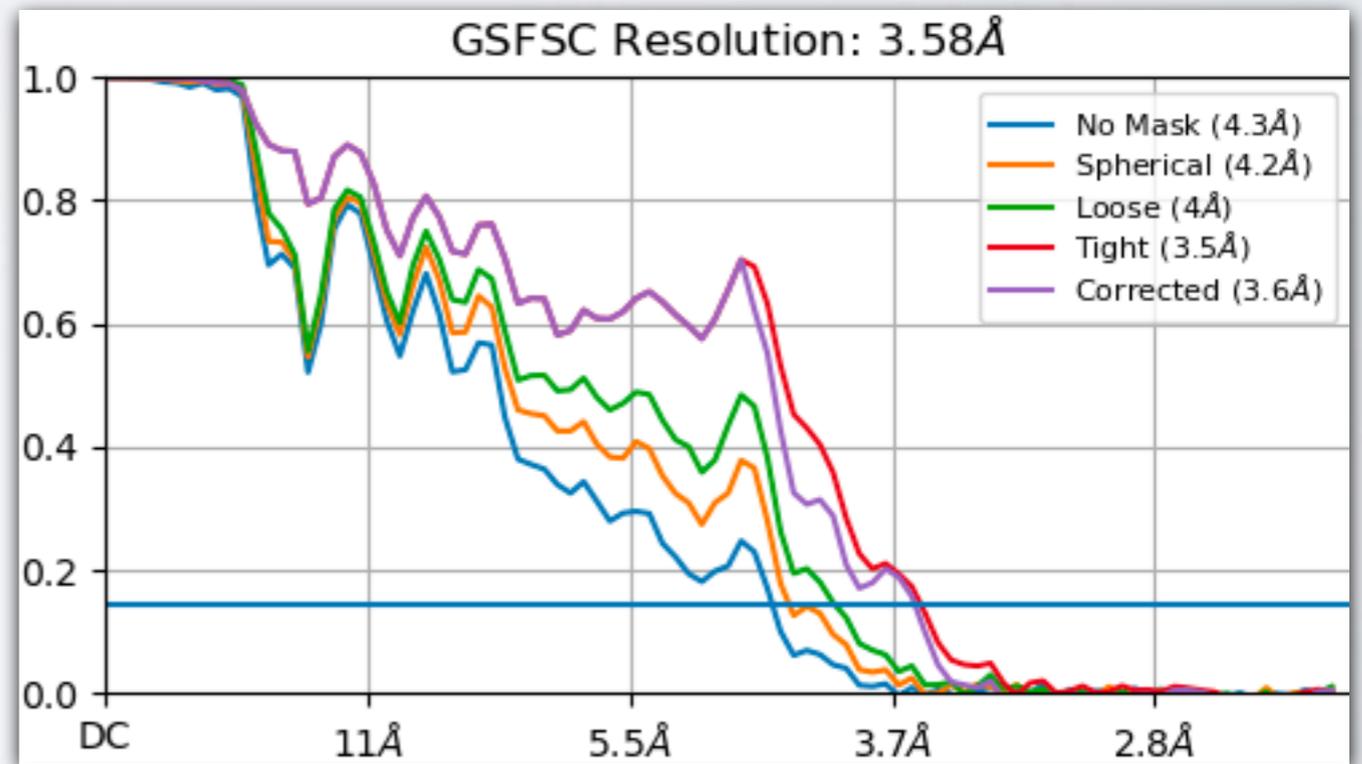
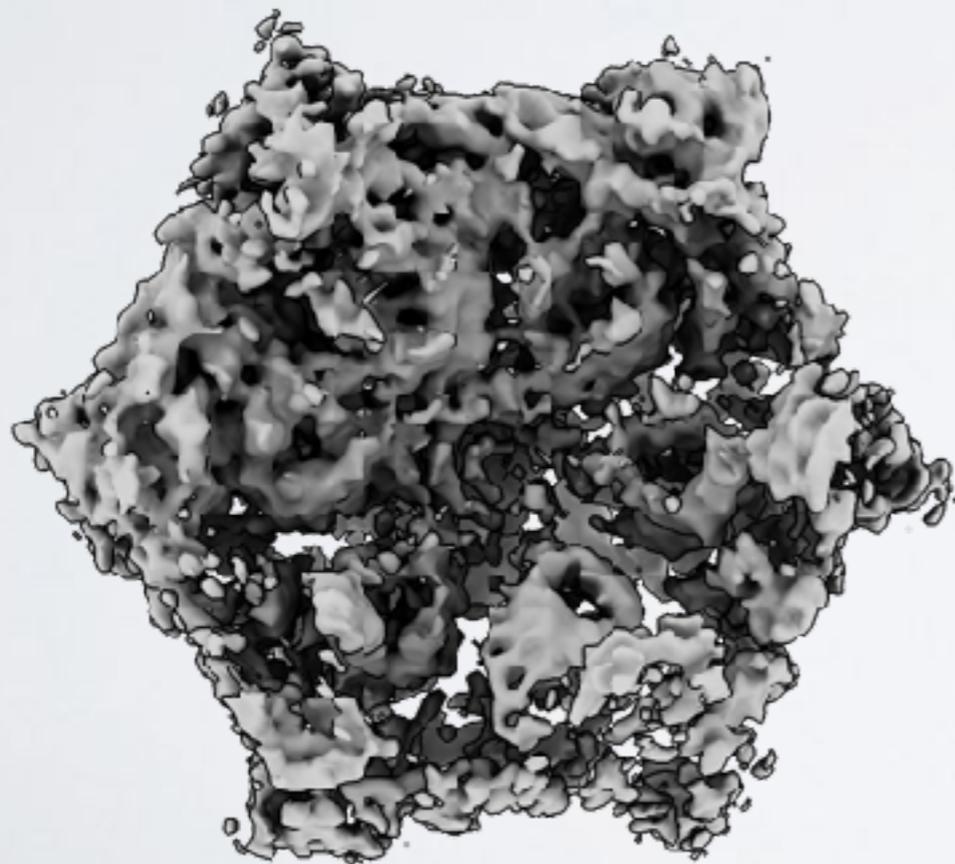
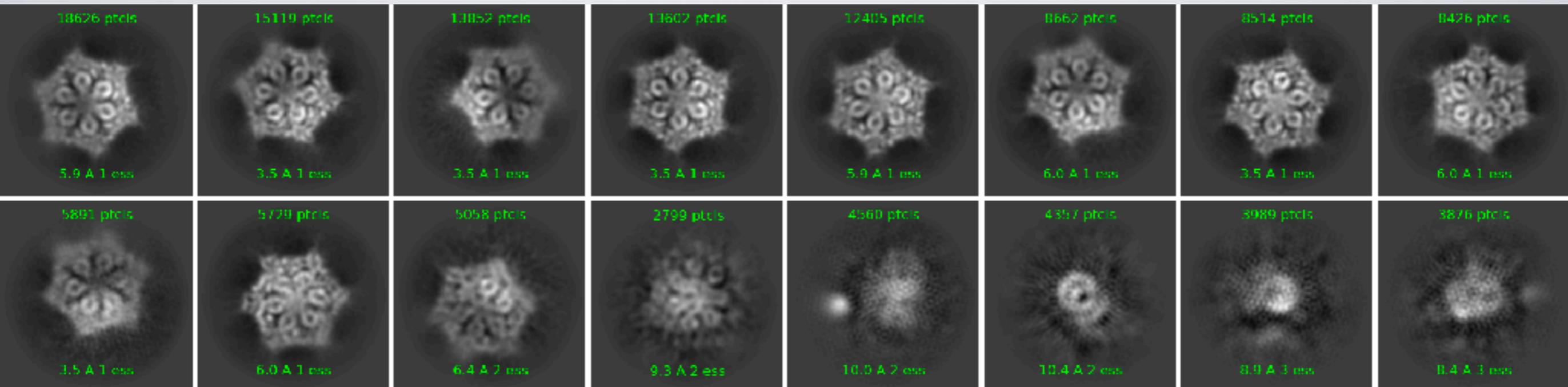
Fourier Shell Correlation - 3D Masking



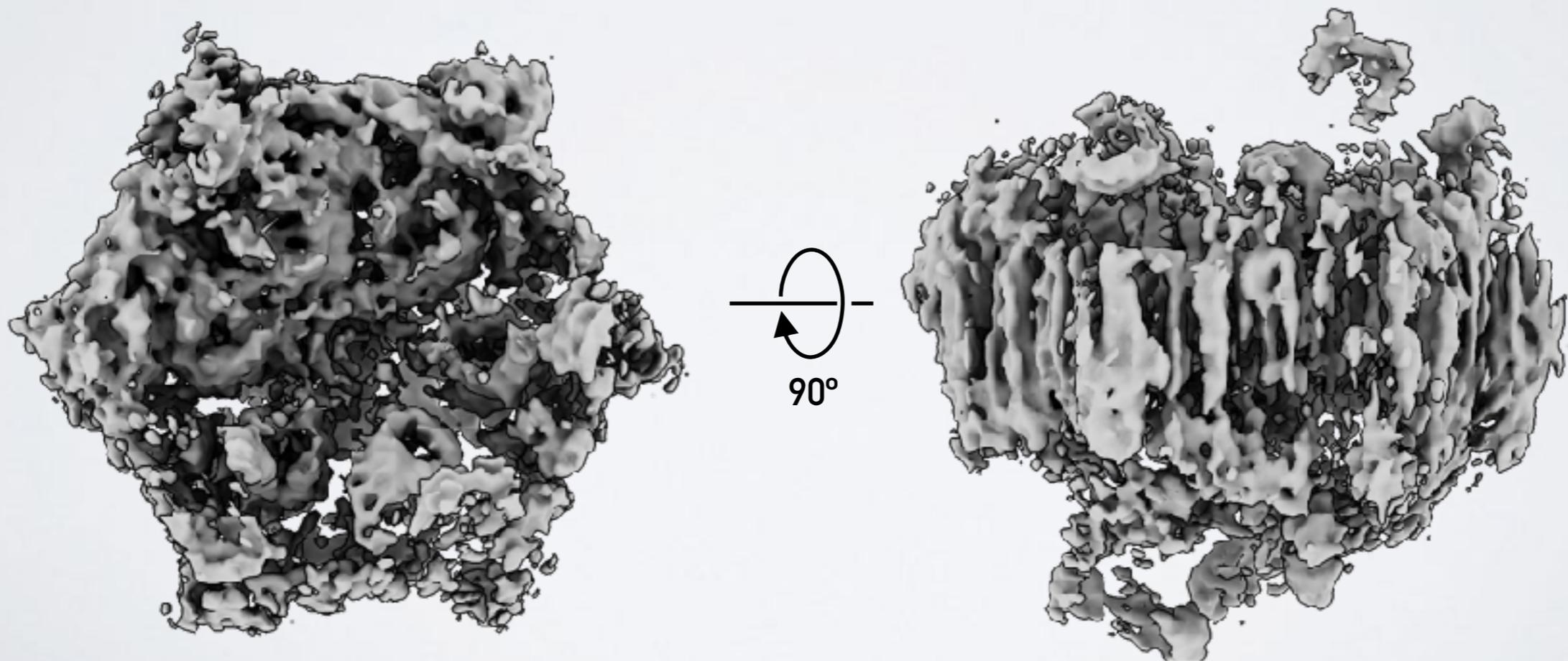
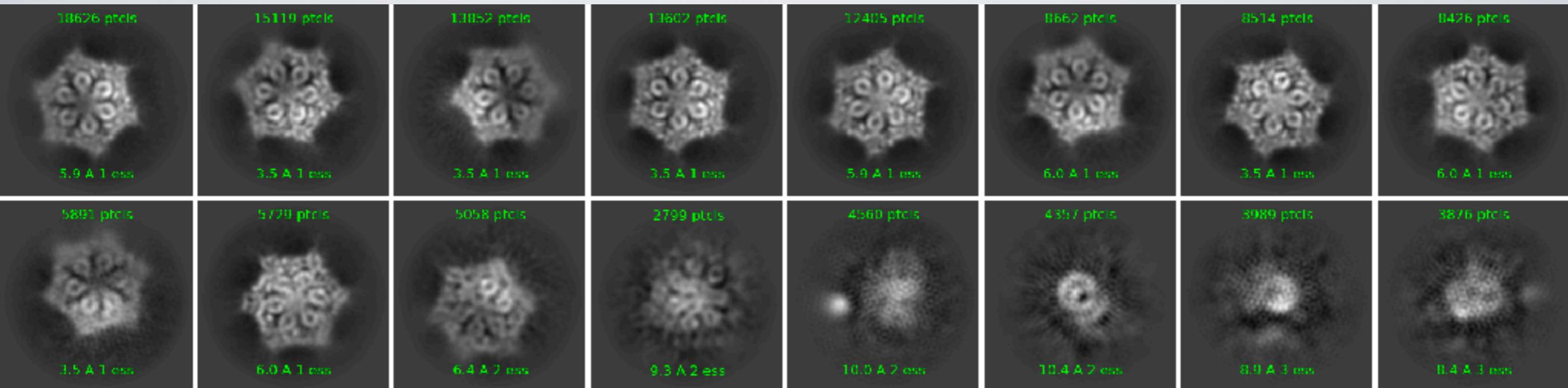
Fourier Shell Correlation - 3D Masking



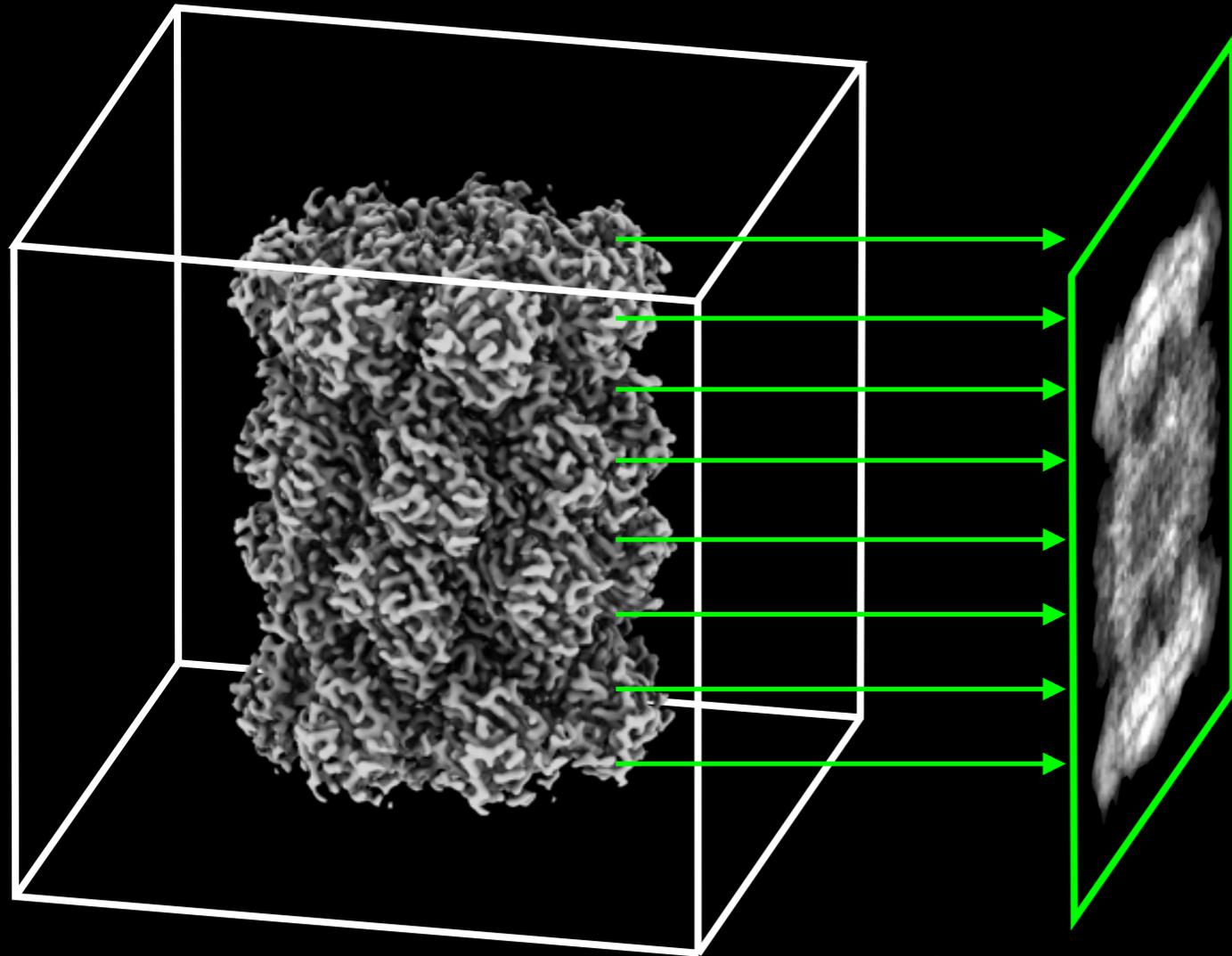
Reported Resolution: 3.6 Å



Reported Resolution: 3.6 Å



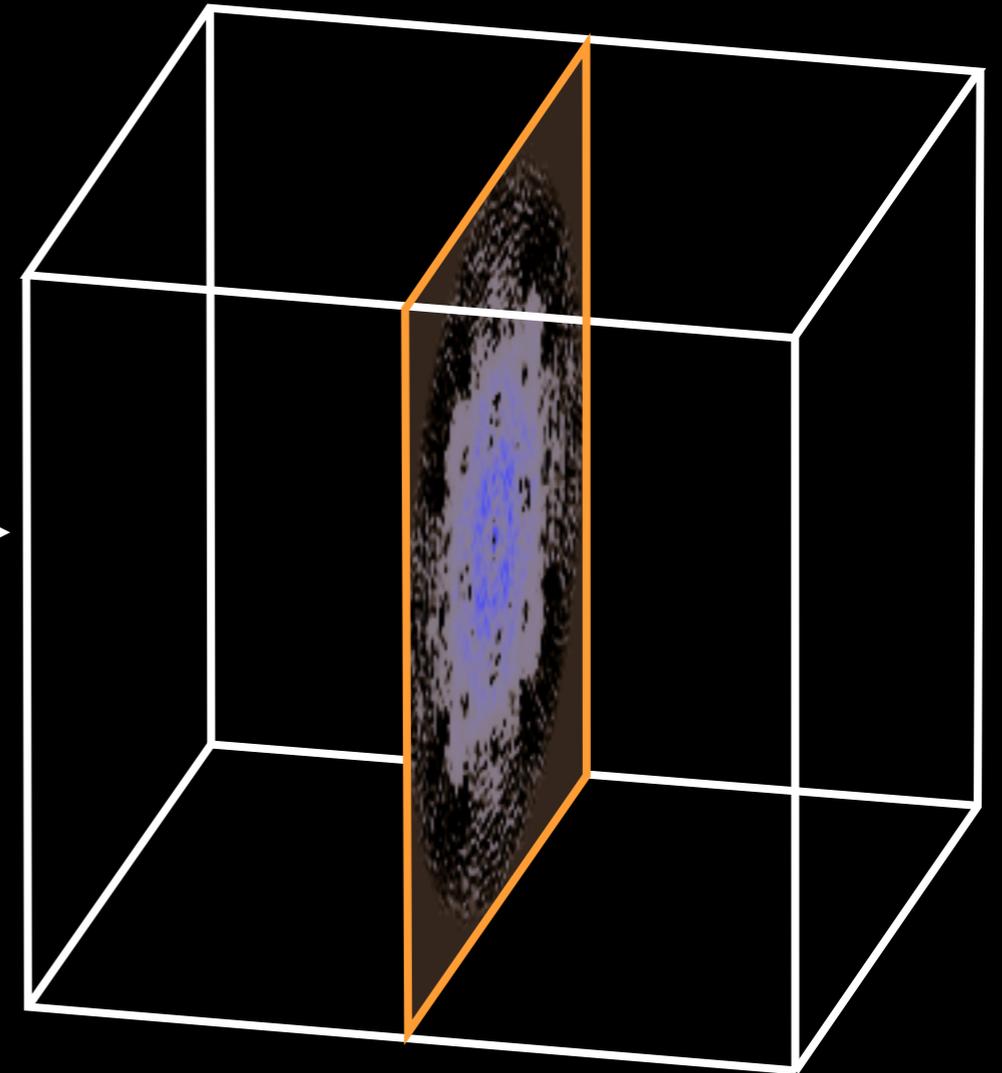
Real Space Structure



2D Projection

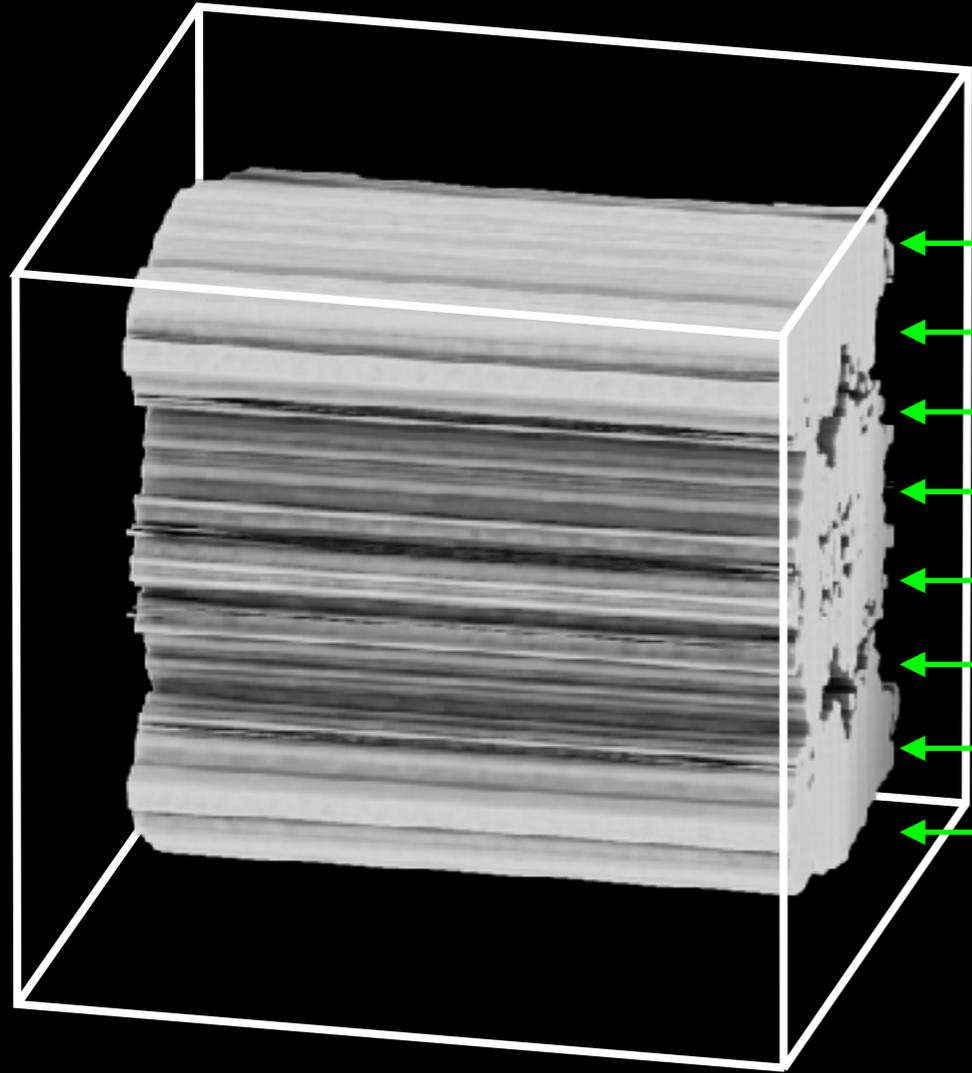
FT
↓

Fourier Structure

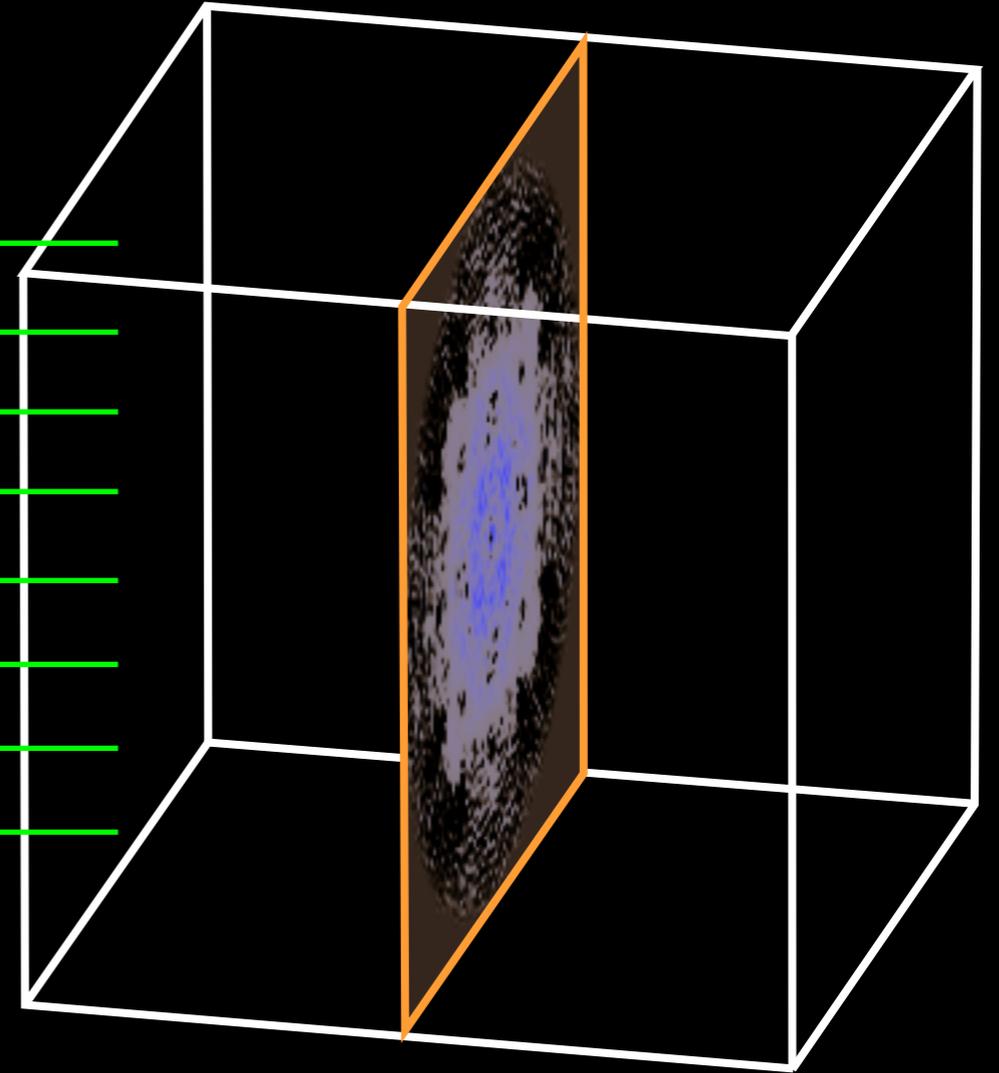


Central Slice of 3D
Fourier Structure

Real Space Structure

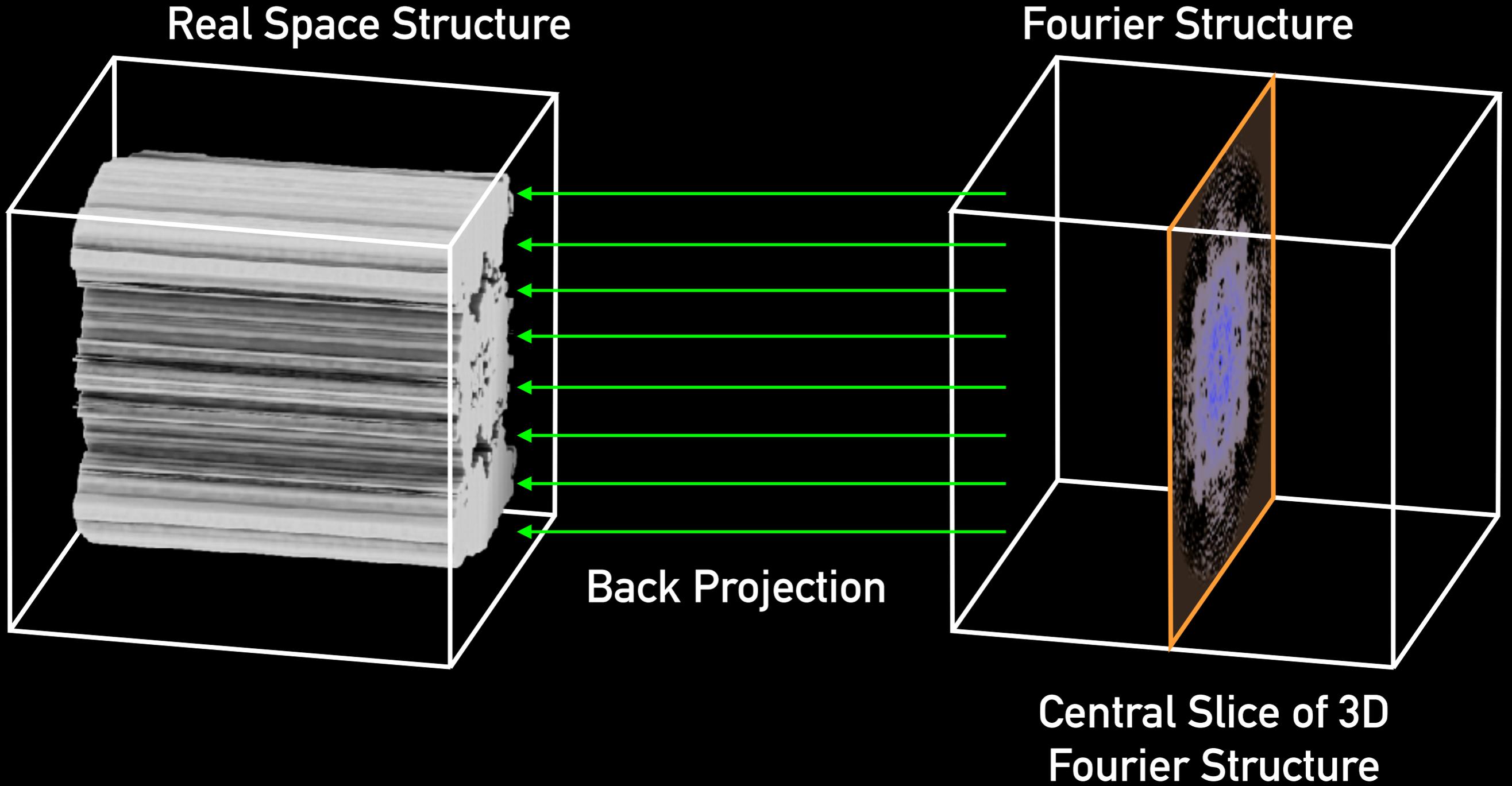


Fourier Structure

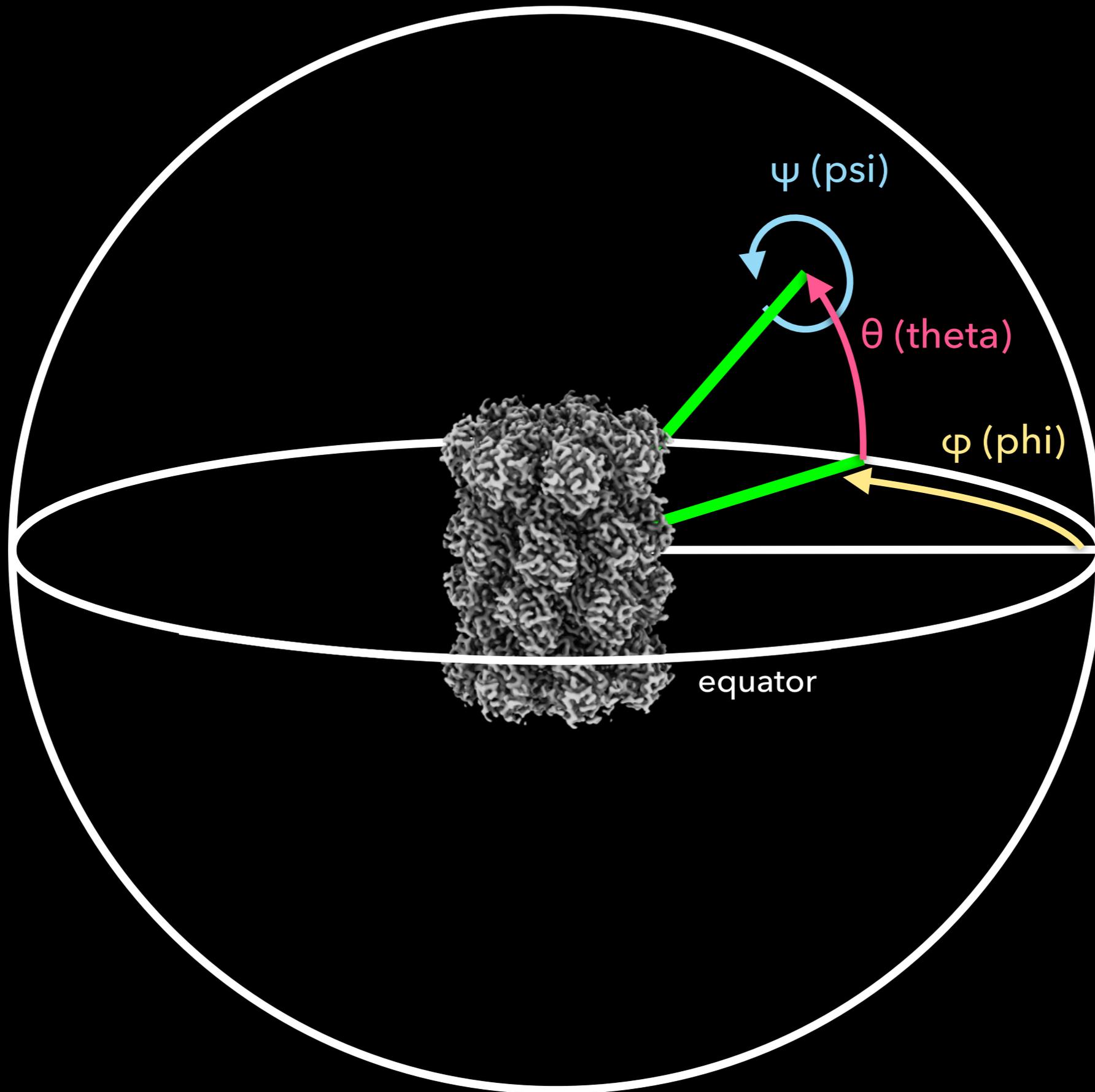


Back Projection

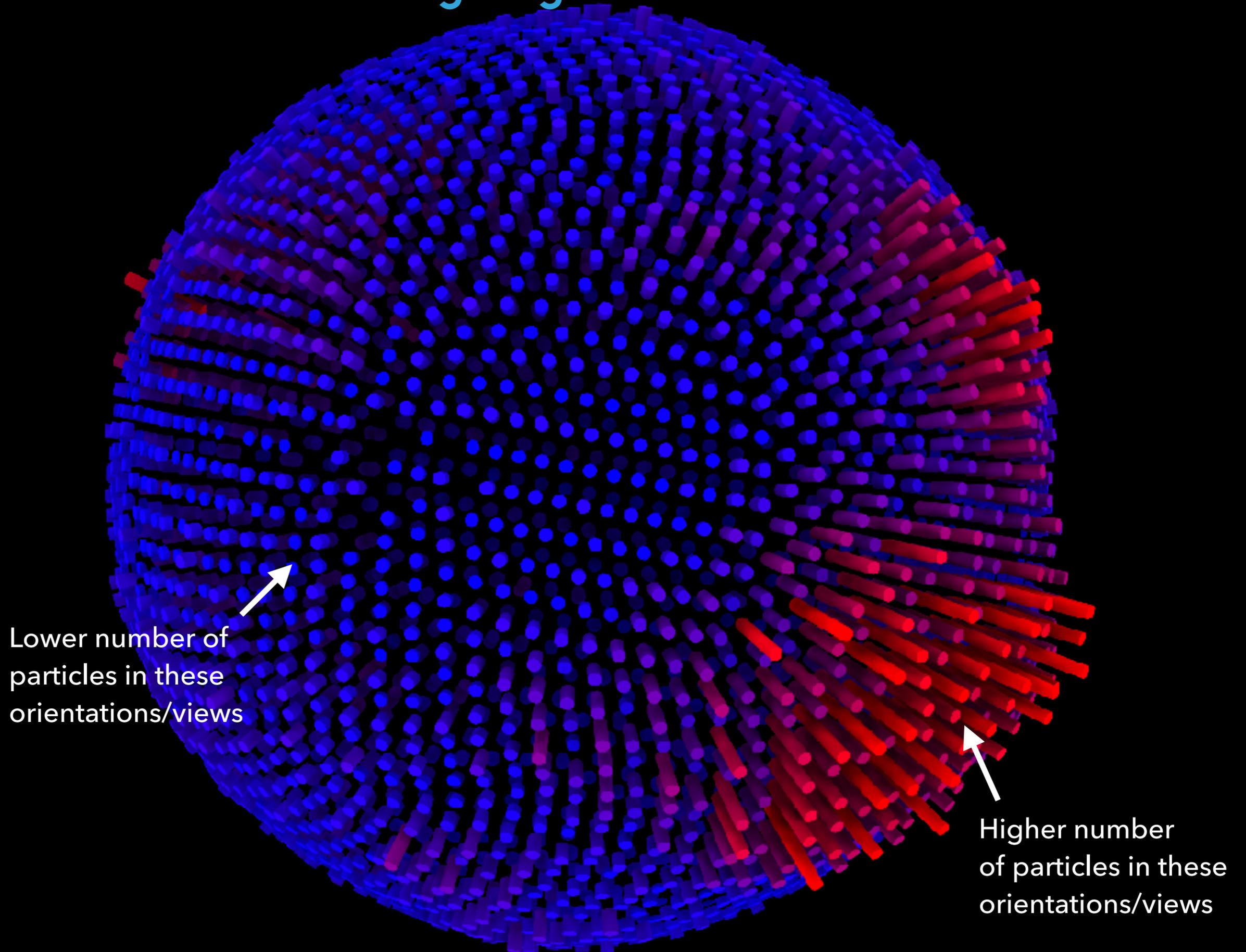
**Central Slice of 3D
Fourier Structure**



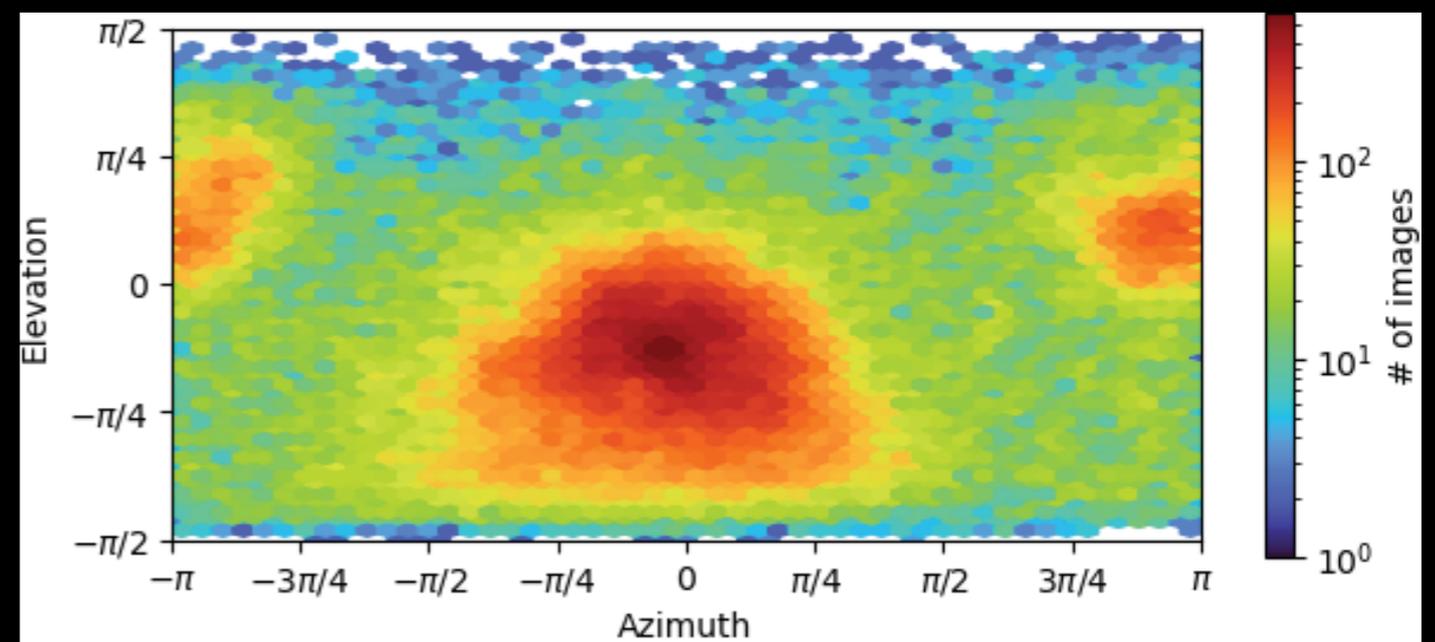
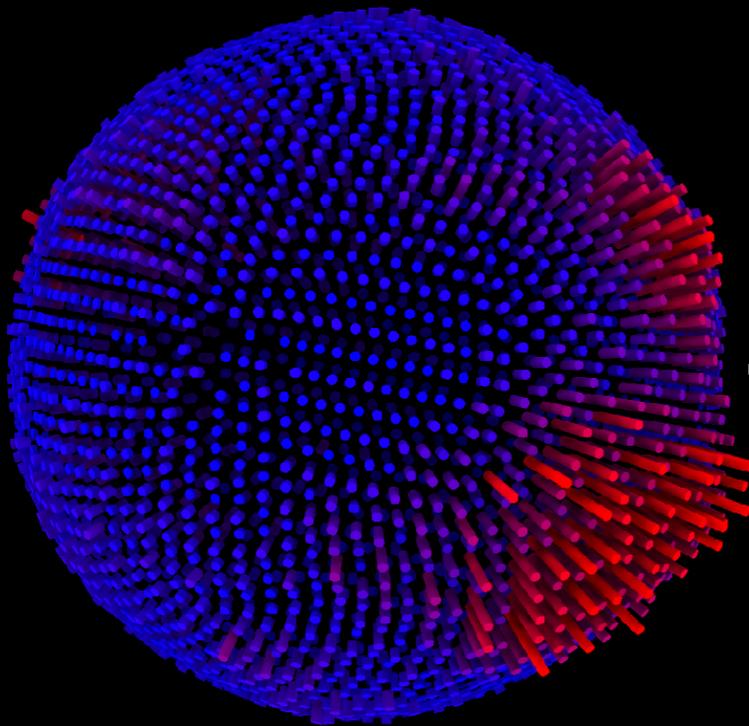
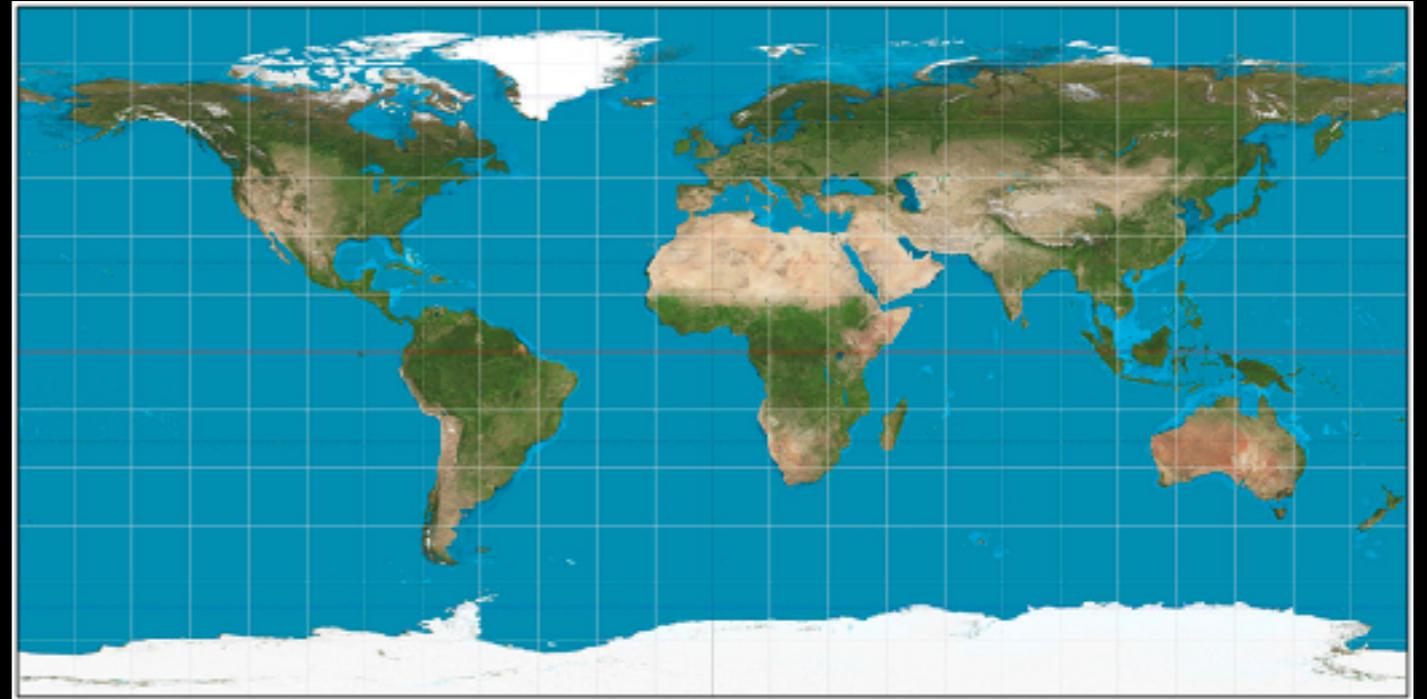
Examining angular distribution



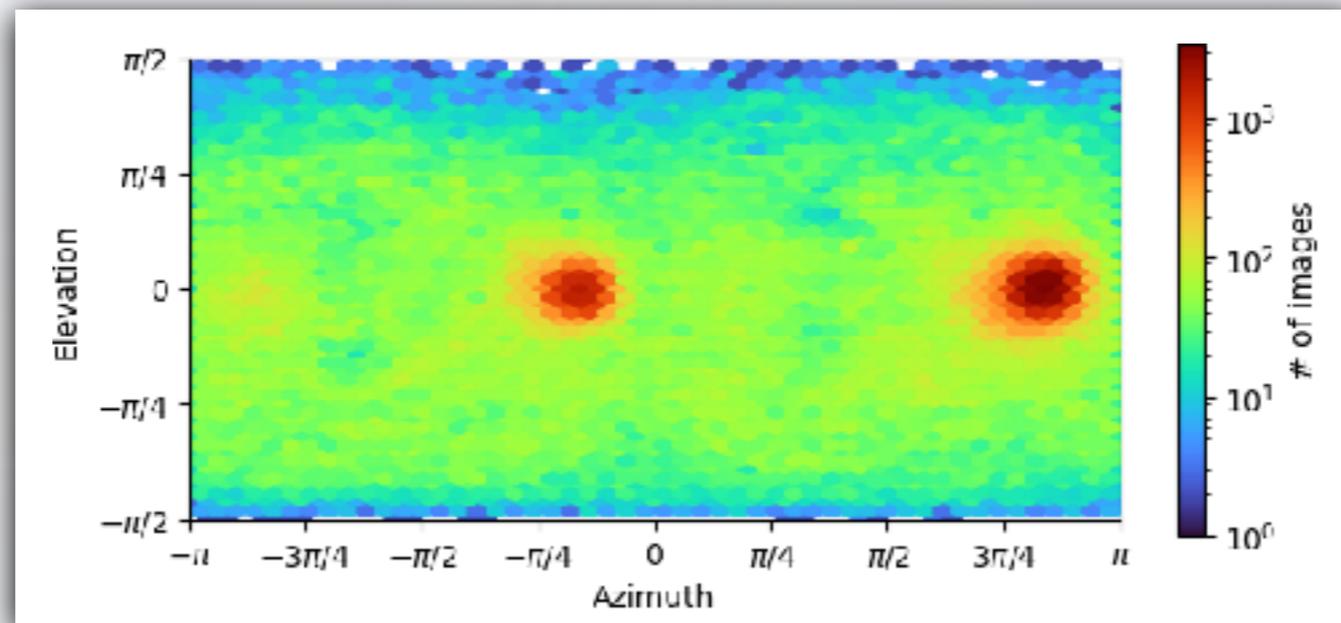
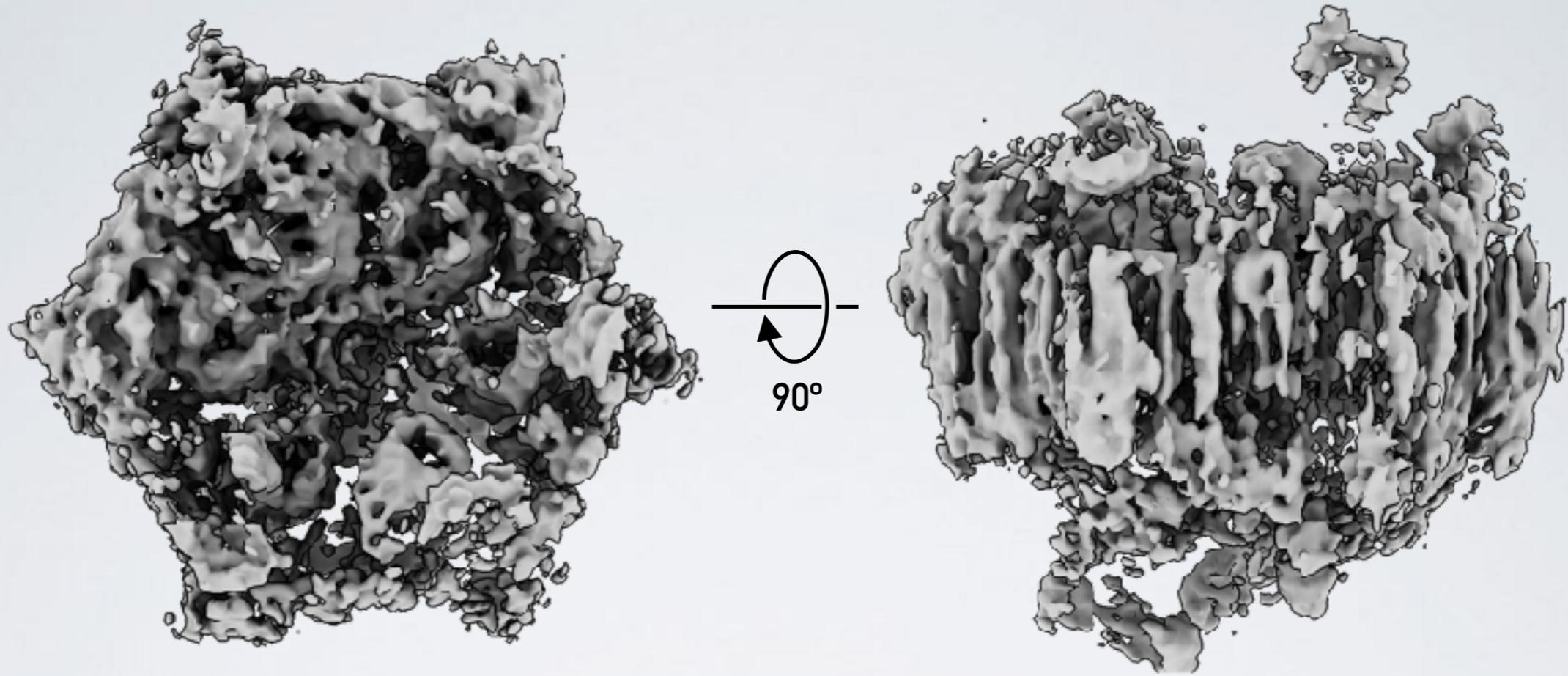
Examining angular distribution



Examining angular distribution



Reported Resolution: 3.6 Å



severe preferred orientation

Reporting Resolution Anisotropy

Published: 03 July 2017

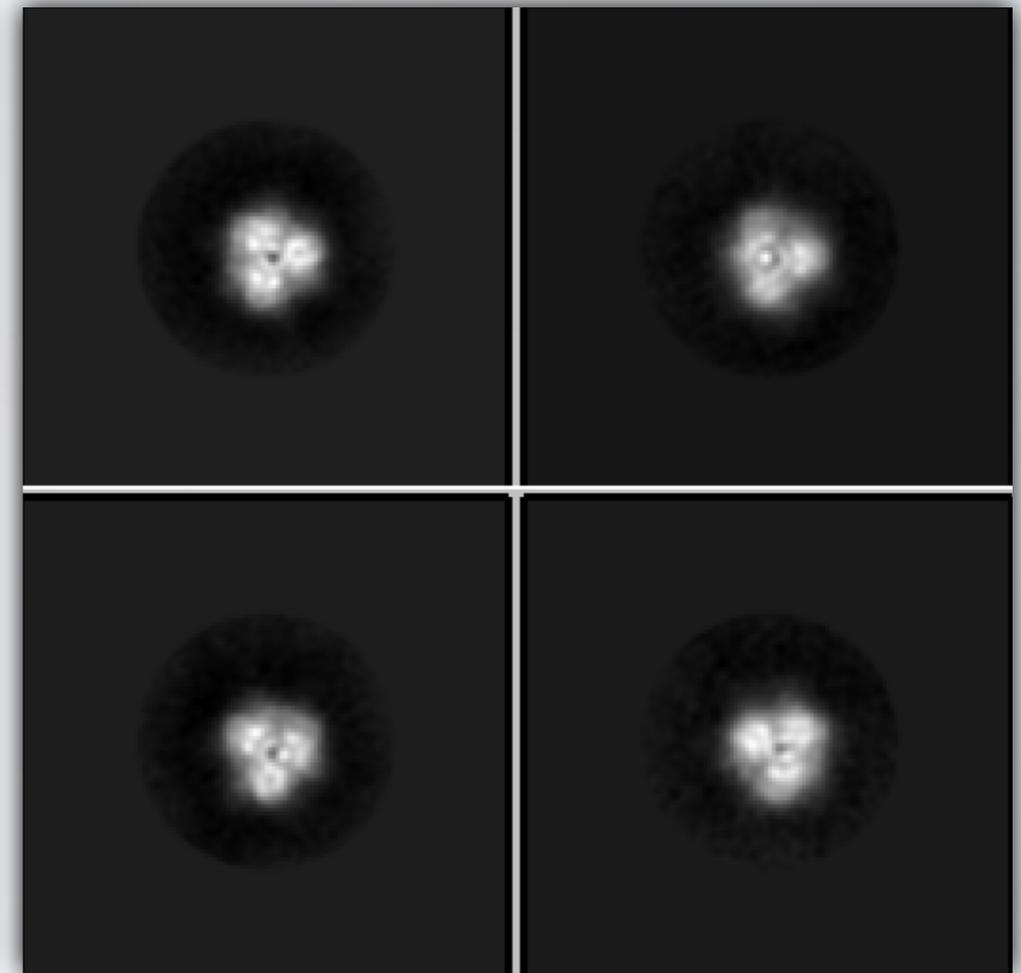
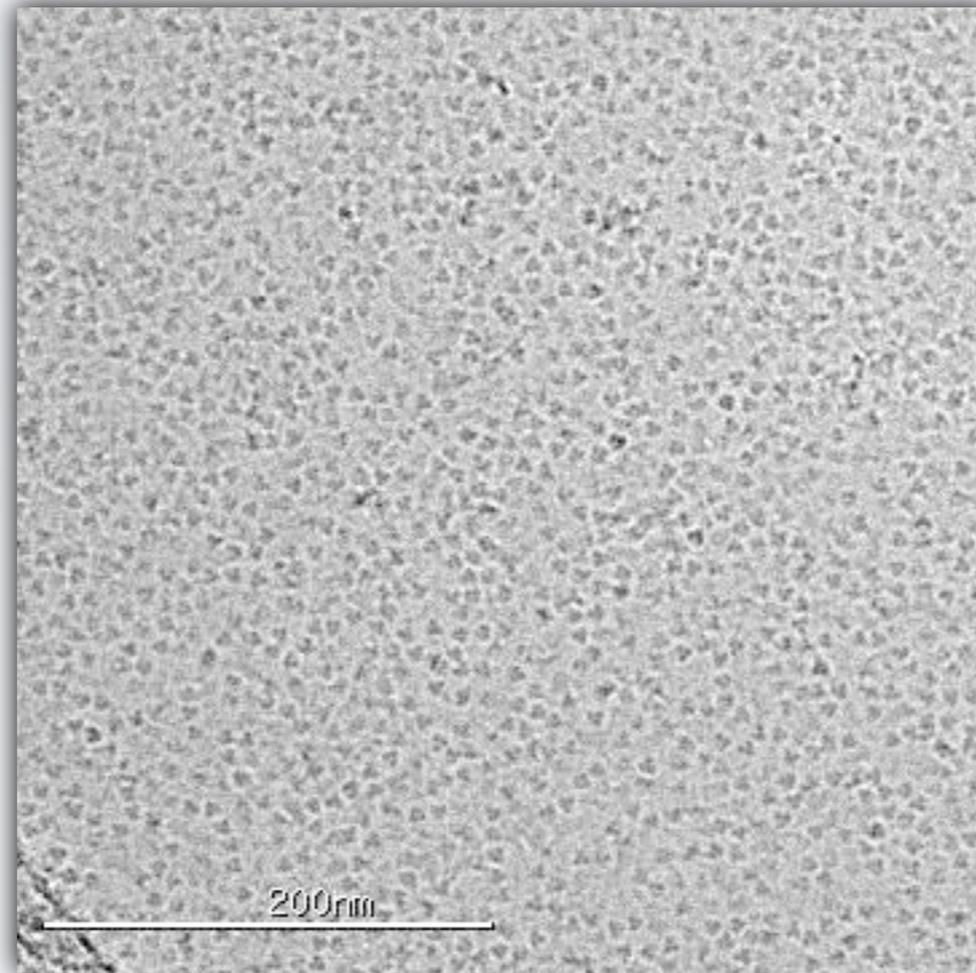
Addressing preferred specimen orientation in single-particle cryo-EM through tilting

Yong Zi Tan, Philip R Baldwin, Joseph H Davis, James R Williamson, Clinton S Potter, Bridget Carragher & Dmitry Lyumkis 

Nature Methods **14**, 793–796 (2017) | [Cite this article](#)

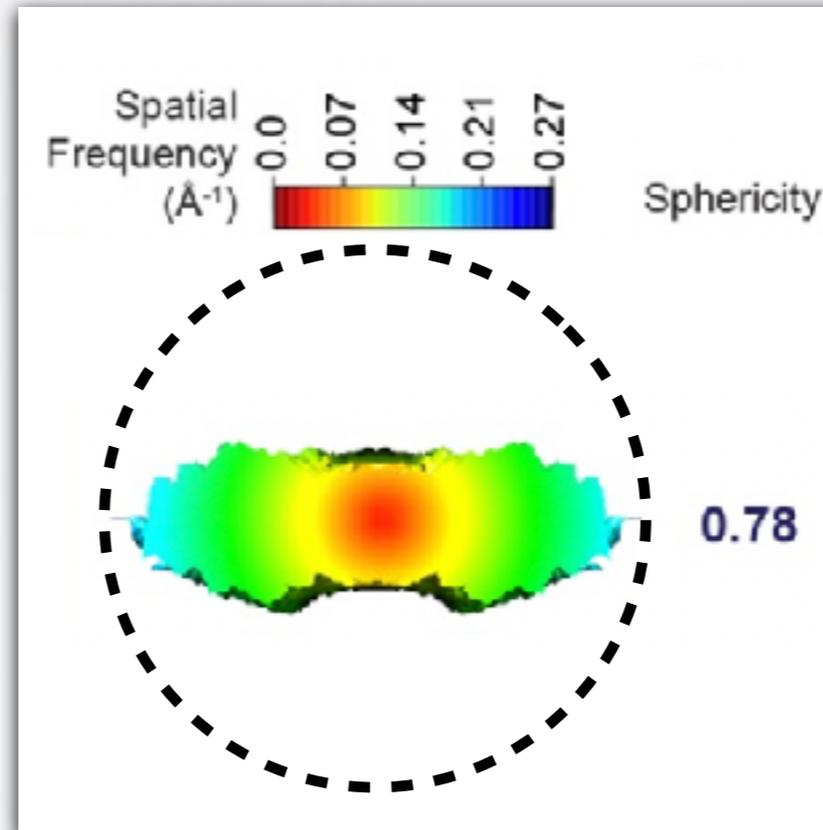
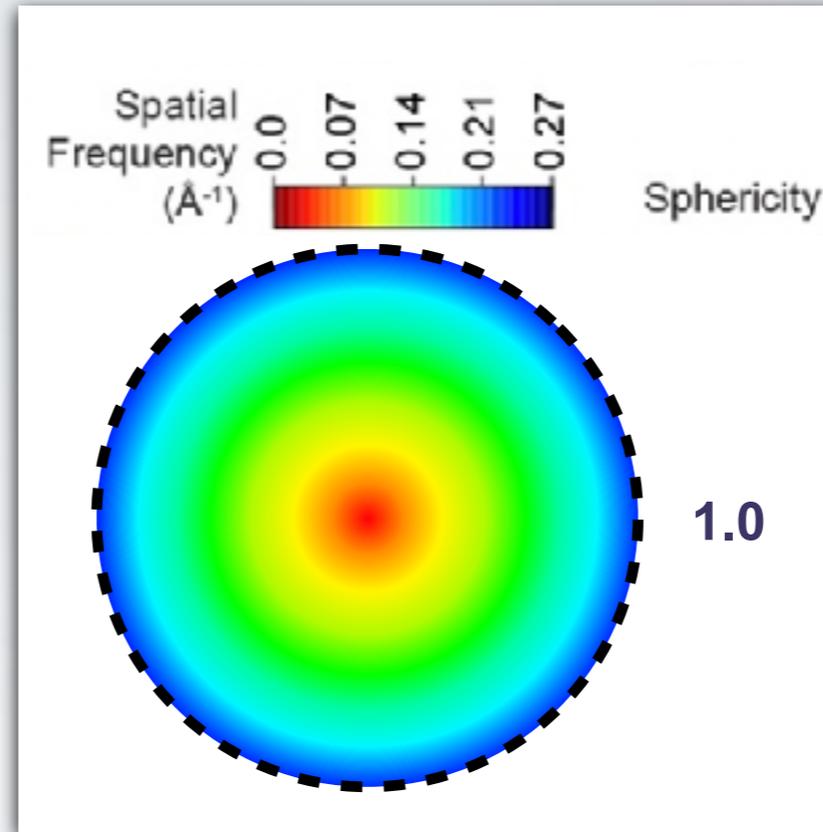
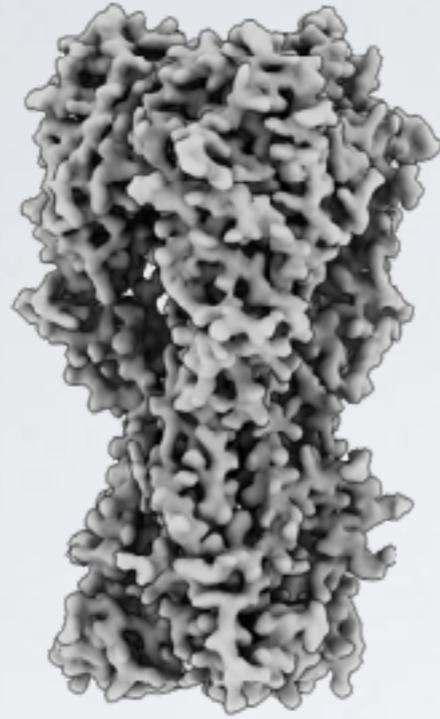


Hemagglutinin (150kD)

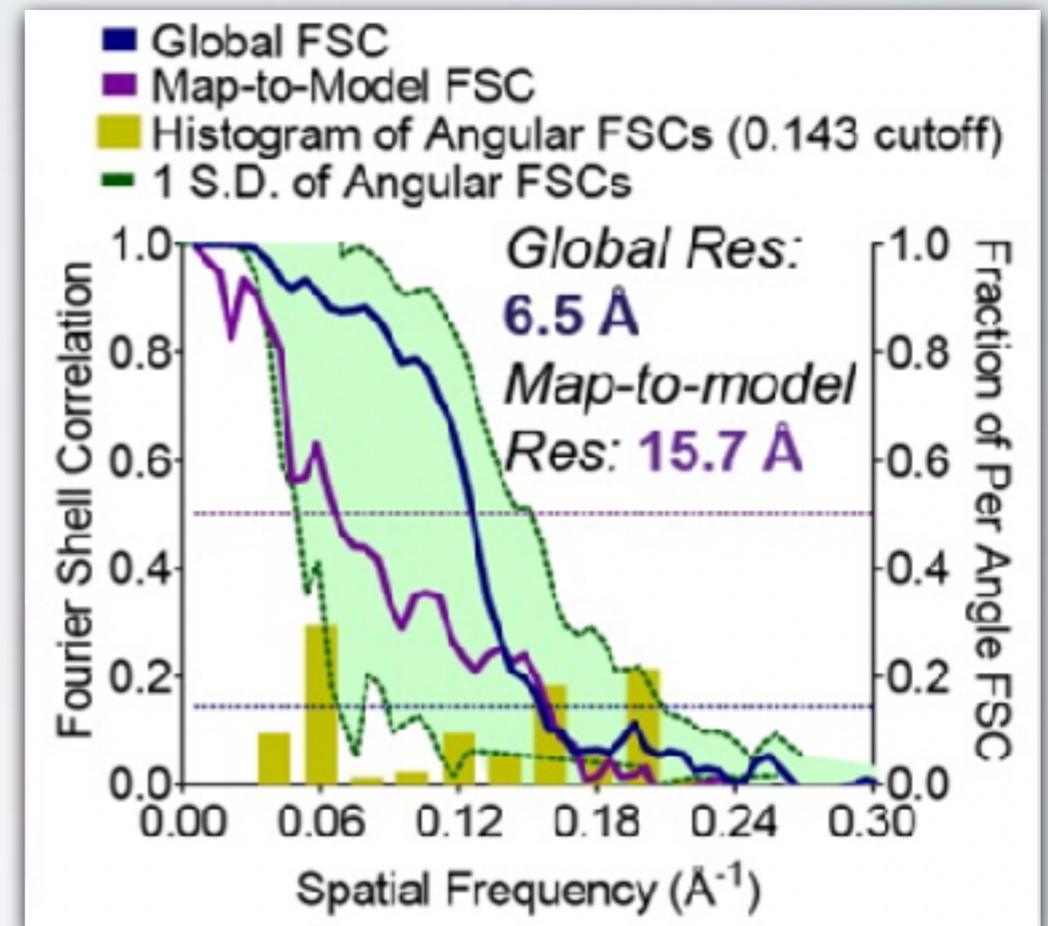
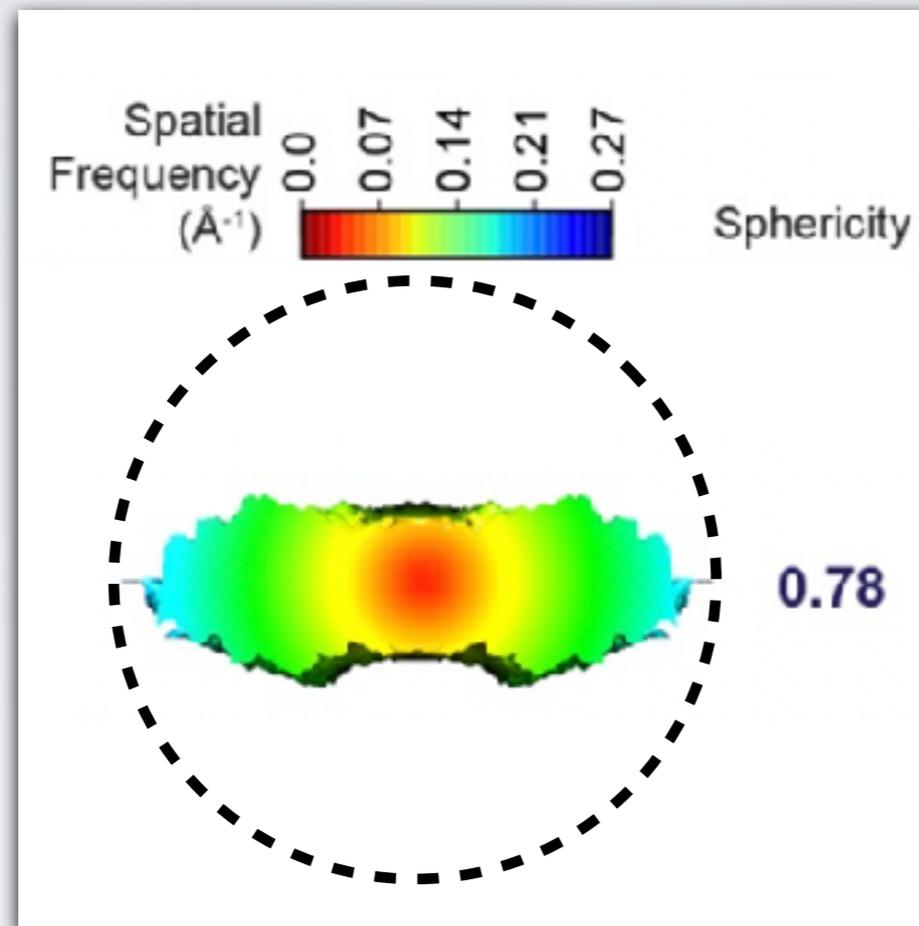
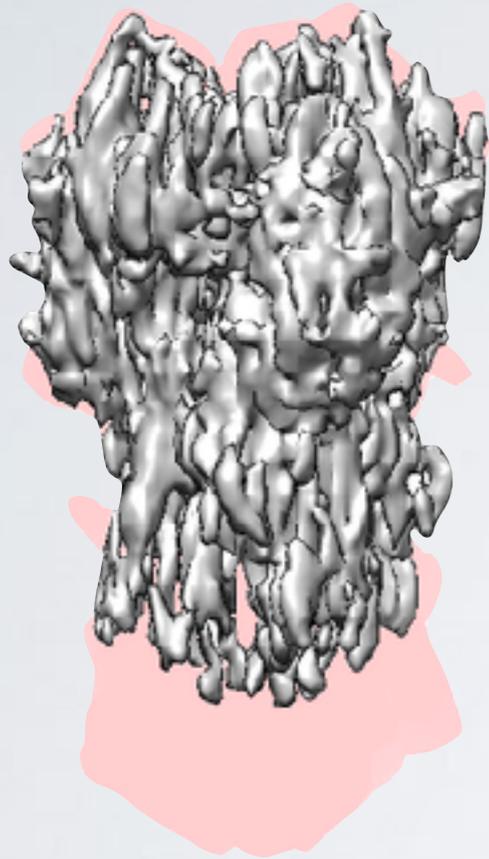


2D averages show preferred orientation
(images courtesy Bridget Carragher)

Reporting Resolution Anisotropy



Reporting Resolution Anisotropy



Remote 3DFSC Processing Server

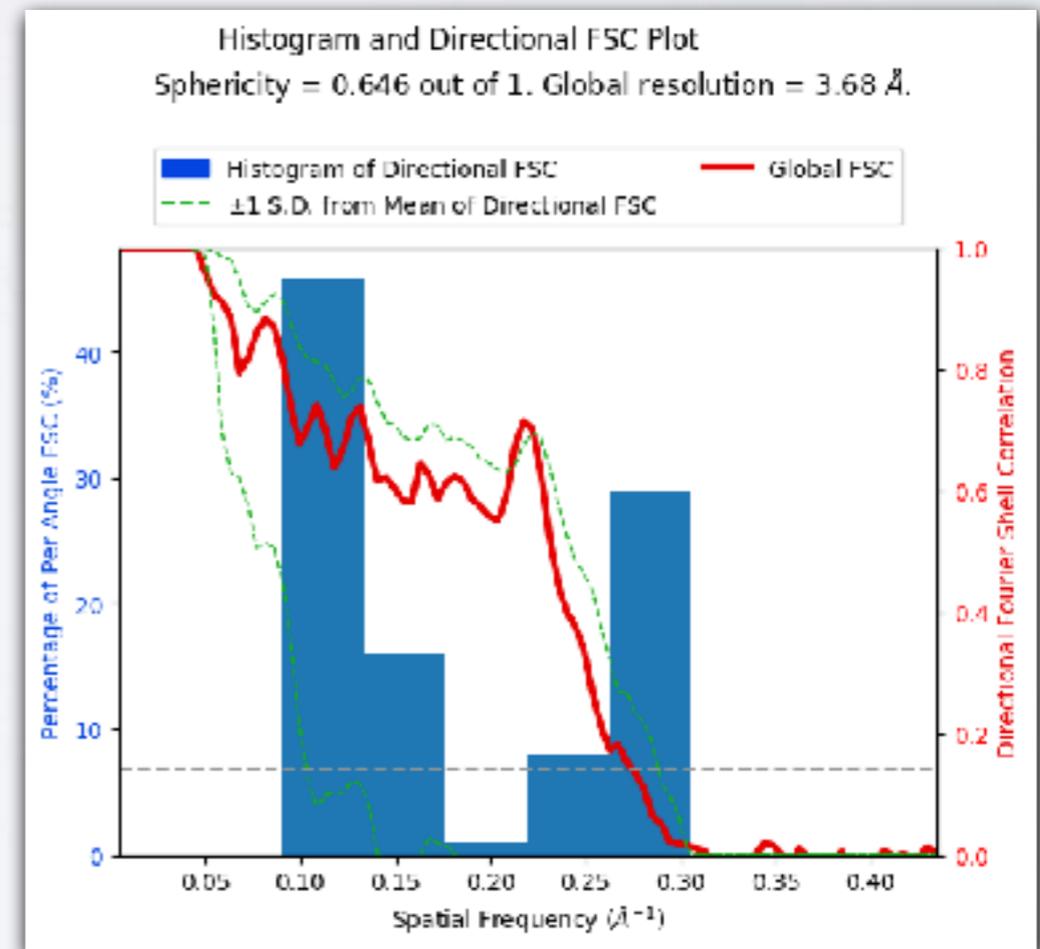
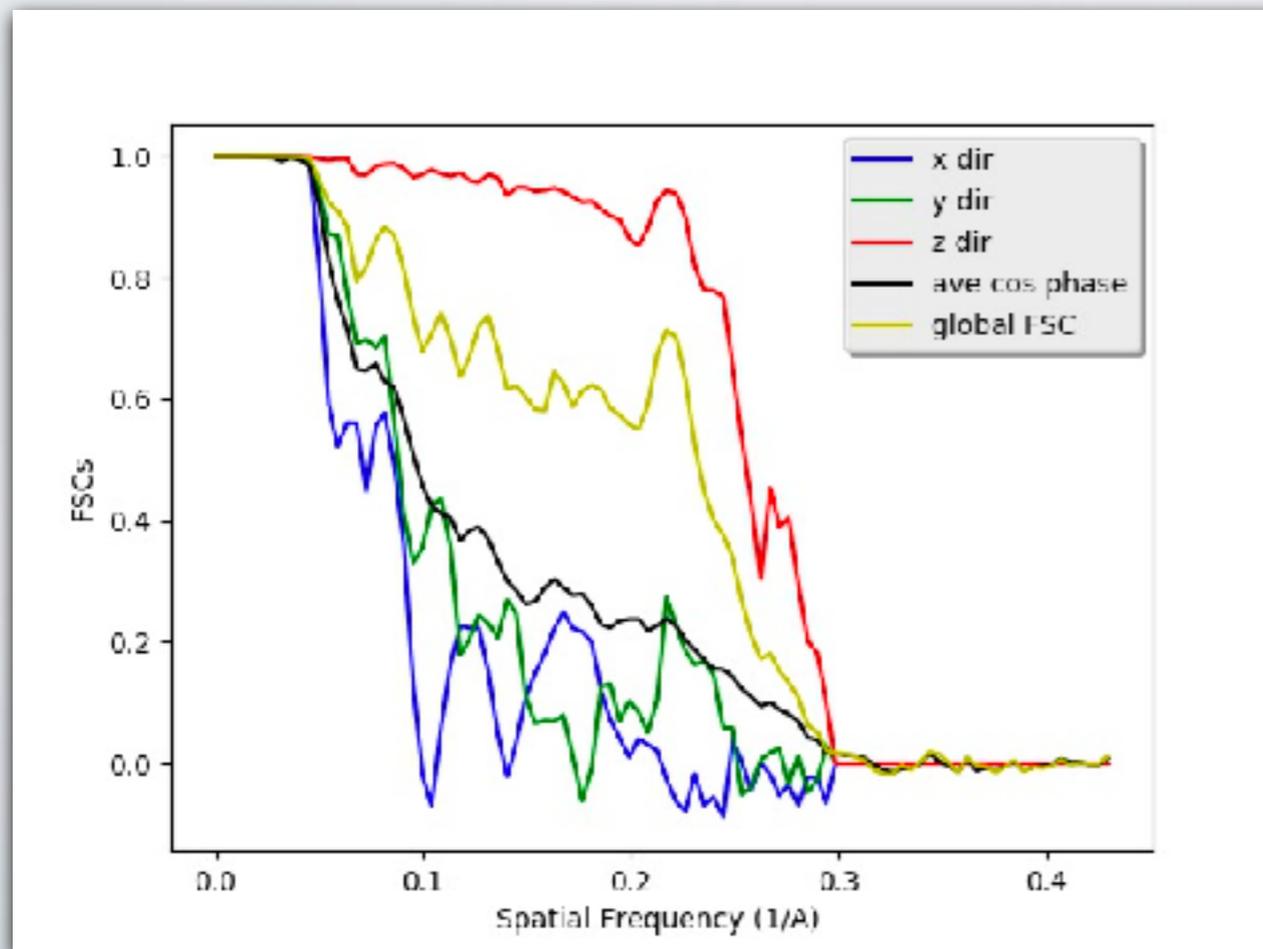
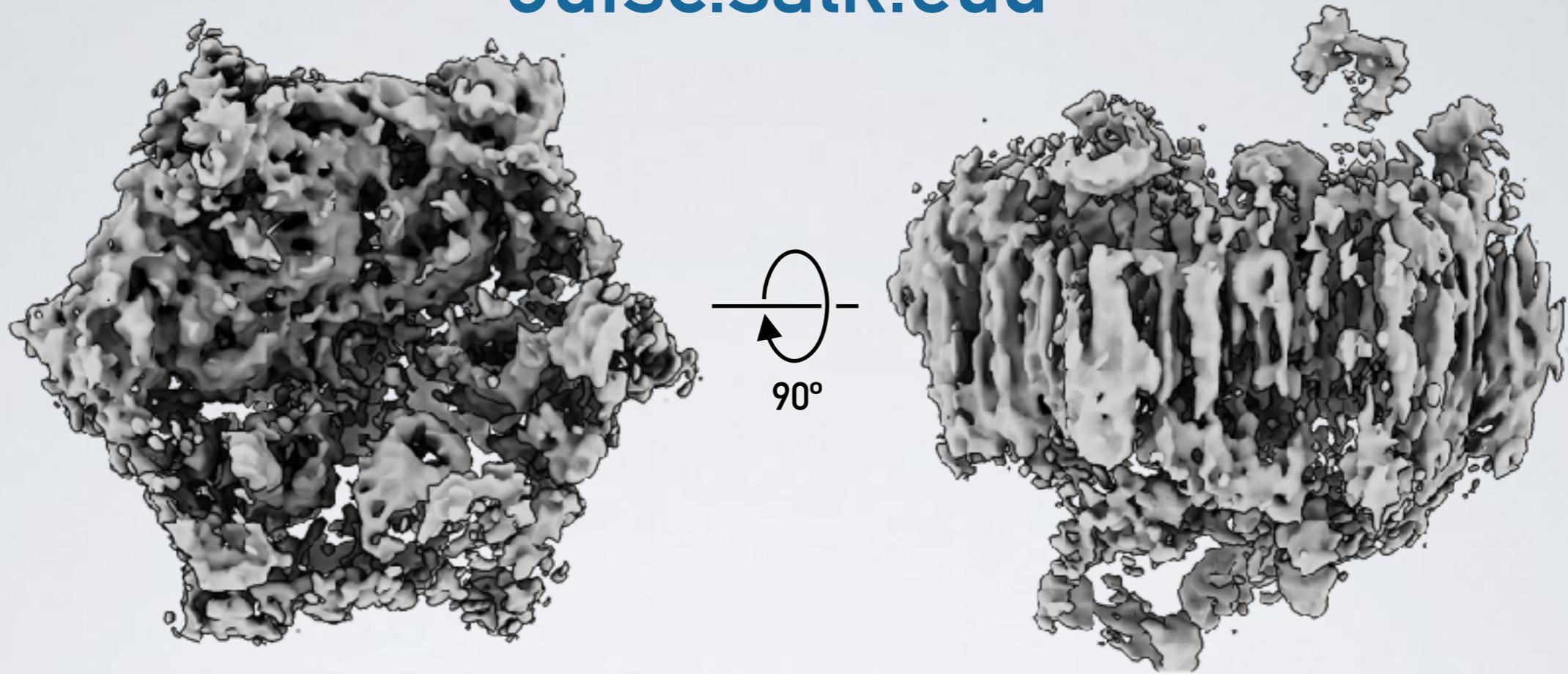
This is an application for remotely processing the 3D Fourier shell correlation of cryoEM maps.

Instructions:

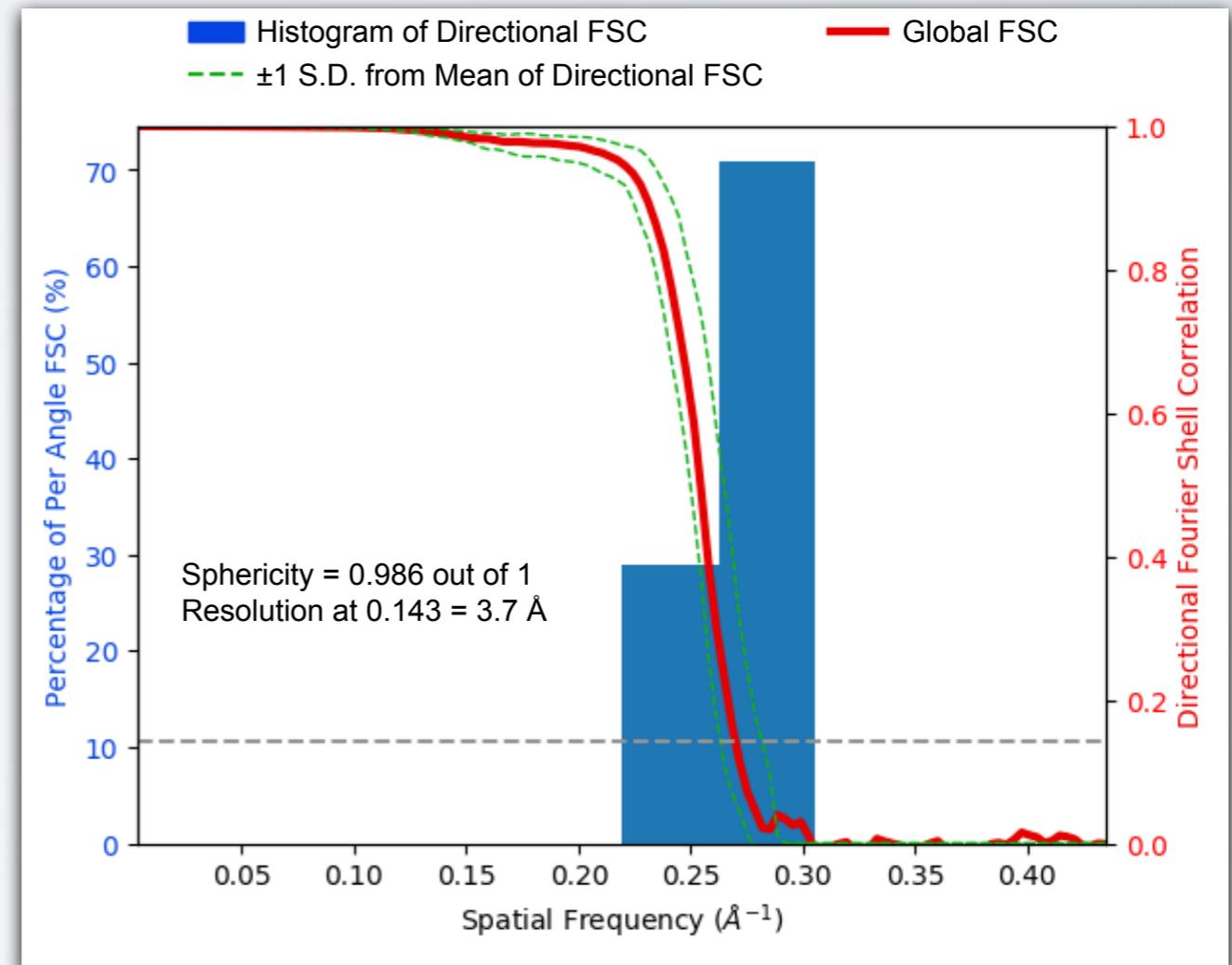
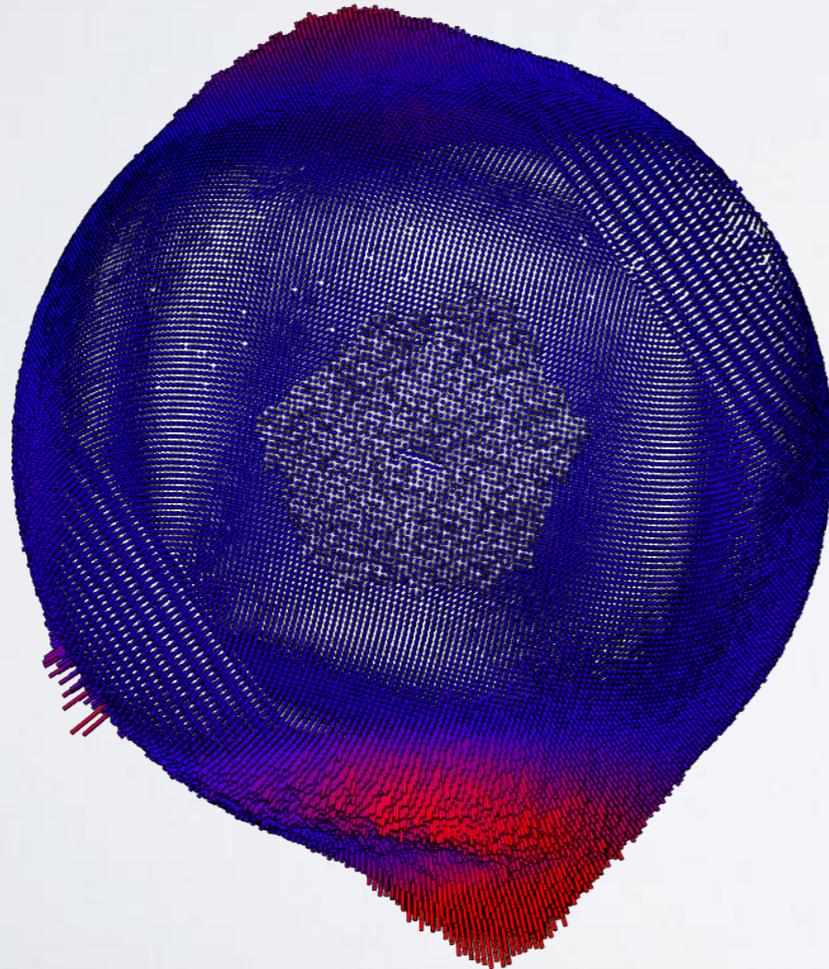
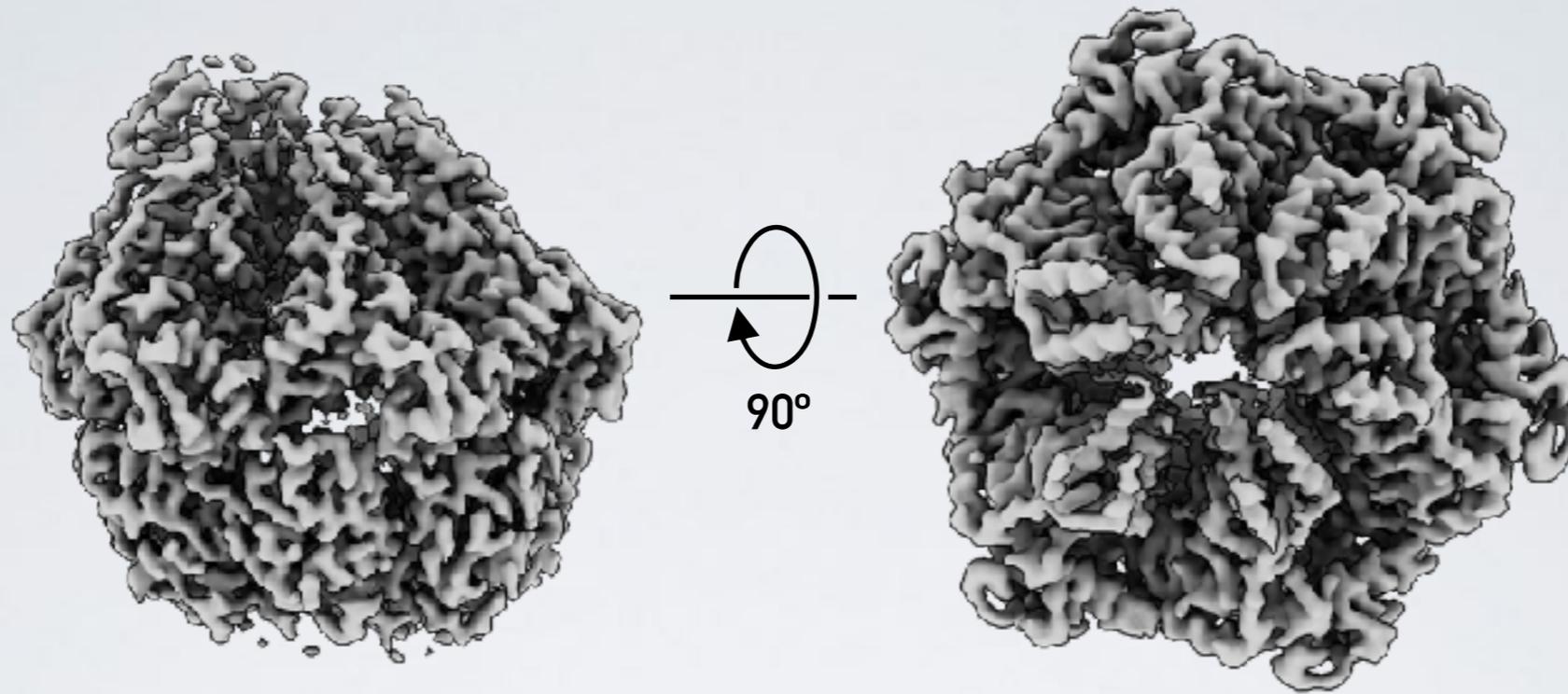
- 1) Click "Register" on the navigation bar and follow the instructions to create an account.
- 2) Navigate to the processing form via the "Submit job" link.
- 3) Enter your email address and other required parameters in the form. You must upload a job name, two half maps (.mrc format), a full map (also .mrc format), and an appropriate pixel size. Click "Submit job".
- 4) You should receive an email to confirm your processing job. If you do not receive an email, please check your spam folders.
- 5) When your job is complete, you will receive another email with a link to view the results.



3dfsc.salk.edu



3dfsc.salk.edu



Reporting Resolution Anisotropy

Measuring the effects of particle orientation to improve the efficiency of electron cryomicroscopy

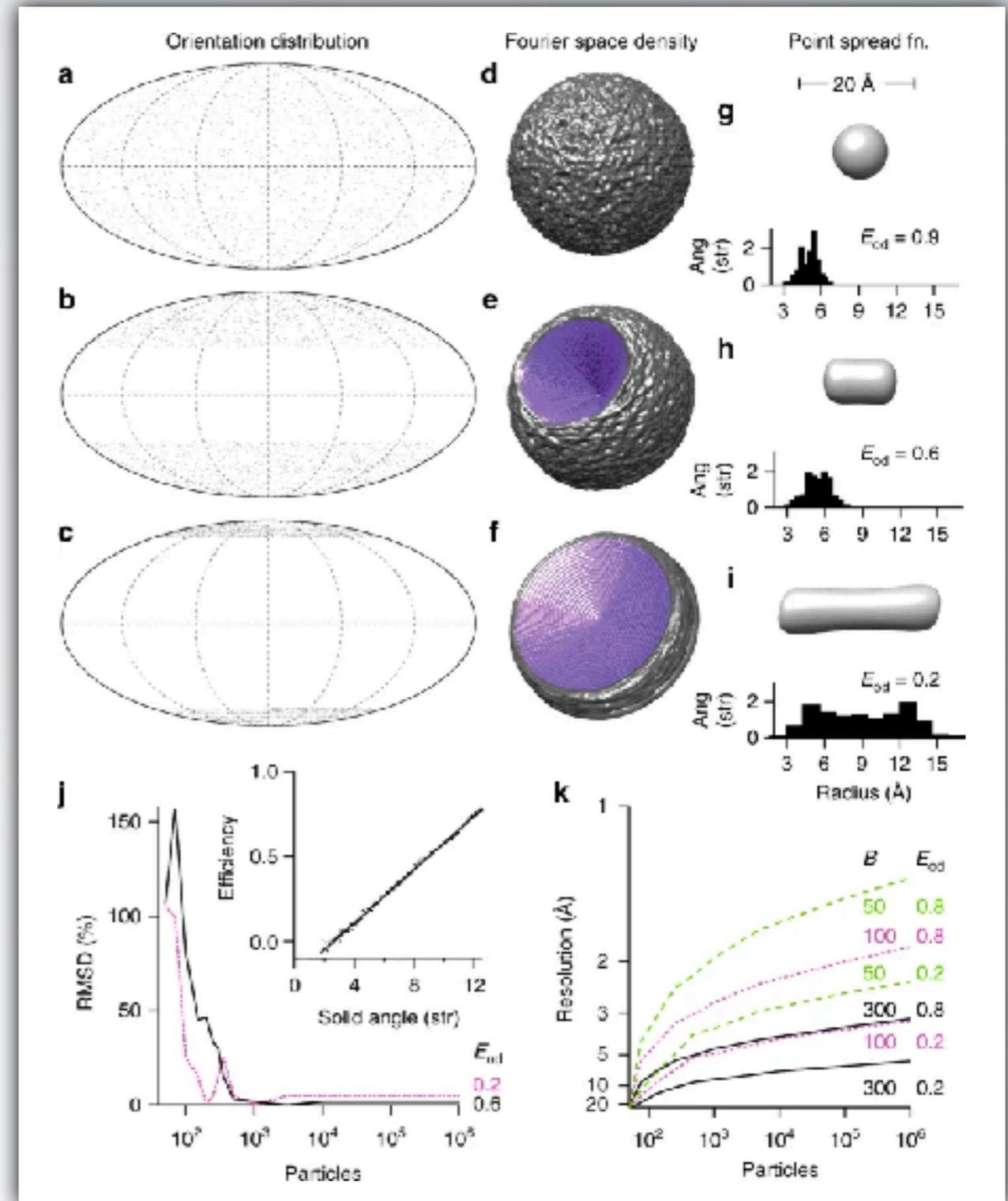
Katerina Naydenova & Christopher J. Russo

Nature Communications 8, Article number: 629 (2017) | [Cite this article](#)

Defines uniformity of resolution by characterizing the point spread function of the map (E_{od})

Download cryoEF:

<https://www.mrc-lmb.cam.ac.uk/crusso/cryoEF/>



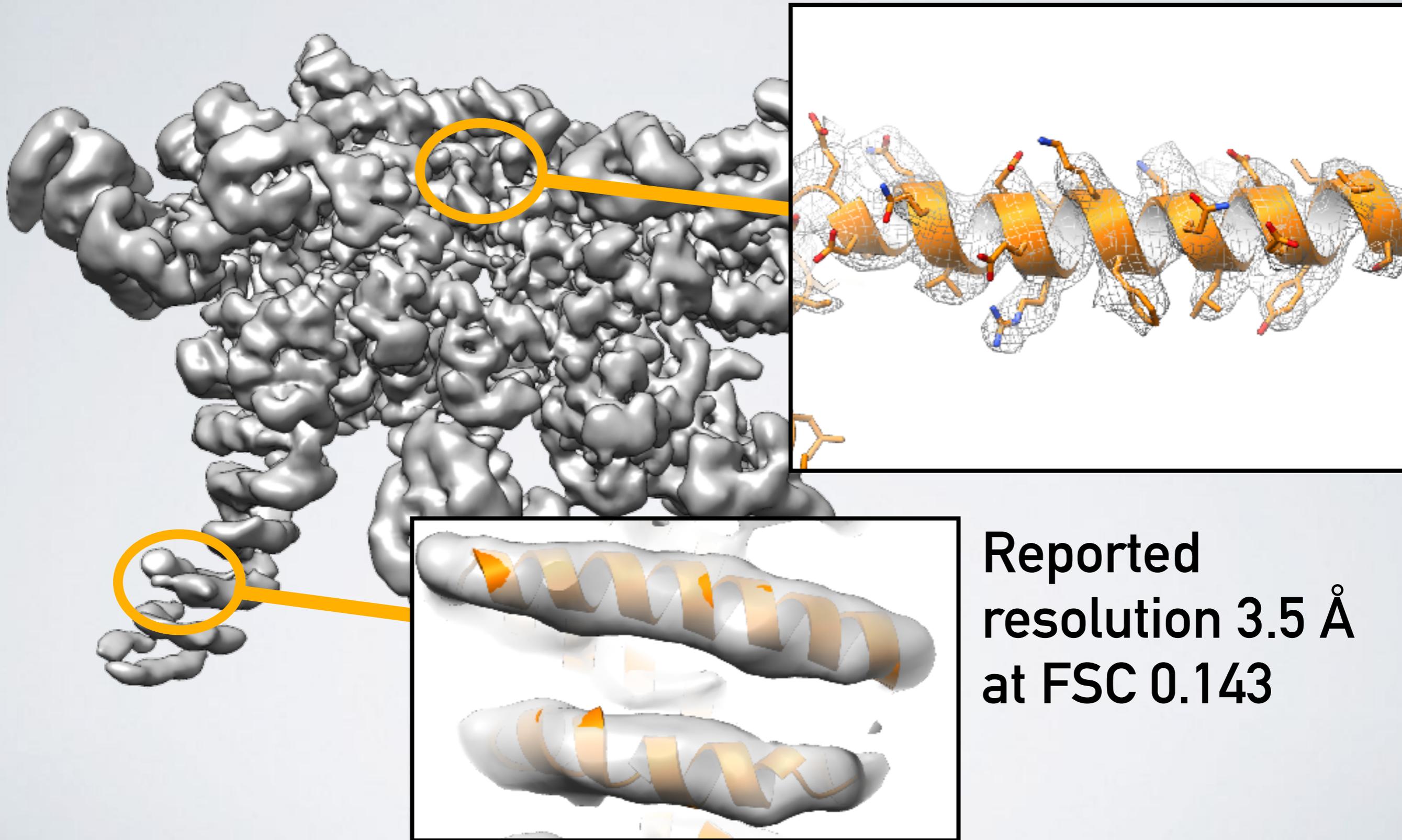
How to report resolution?

Abstract The 26S proteasome is responsible for the selective, ATP-dependent degradation of polyubiquitinated cellular proteins. Removal of ubiquitin chains from targeted substrates at the proteasome is a prerequisite for substrate processing and is accomplished by Rpn11, a deubiquitinase within the 'lid' sub-complex. Prior to the lid's incorporation into the proteasome, Rpn11 deubiquitinase activity is inhibited to prevent unwarranted deubiquitination of polyubiquitinated proteins. Here we present the atomic model of the isolated lid sub-complex, as determined by cryo-electron microscopy at 3.5 Å resolution, revealing how Rpn11 is inhibited through its interaction with a neighboring lid subunit, Rpn5. Through mutagenesis of specific residues, we describe the network of interactions that are required to stabilize this inhibited state. These results provide significant insight into the intricate mechanisms of proteasome assembly, outlining the substantial conformational rearrangements that occur during incorporation of the lid into the 26S holoenzyme, which ultimately activates the deubiquitinase for substrate degradation.

DOI: [10.7554/eLife.13027.001](https://doi.org/10.7554/eLife.13027.001)

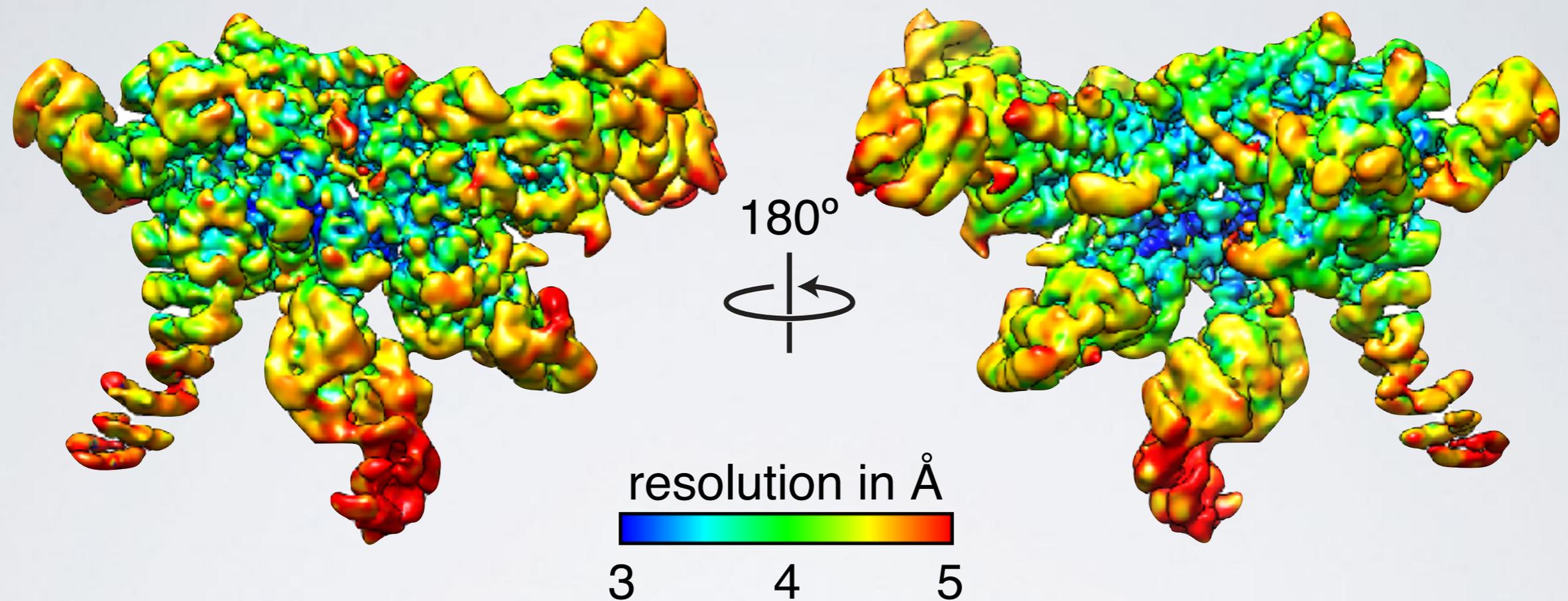
Dambacher, Herzik, Worden et al. eLife 2016

Resolution is rarely consistent across a reconstruction



**Reported
resolution 3.5 Å
at FSC 0.143**

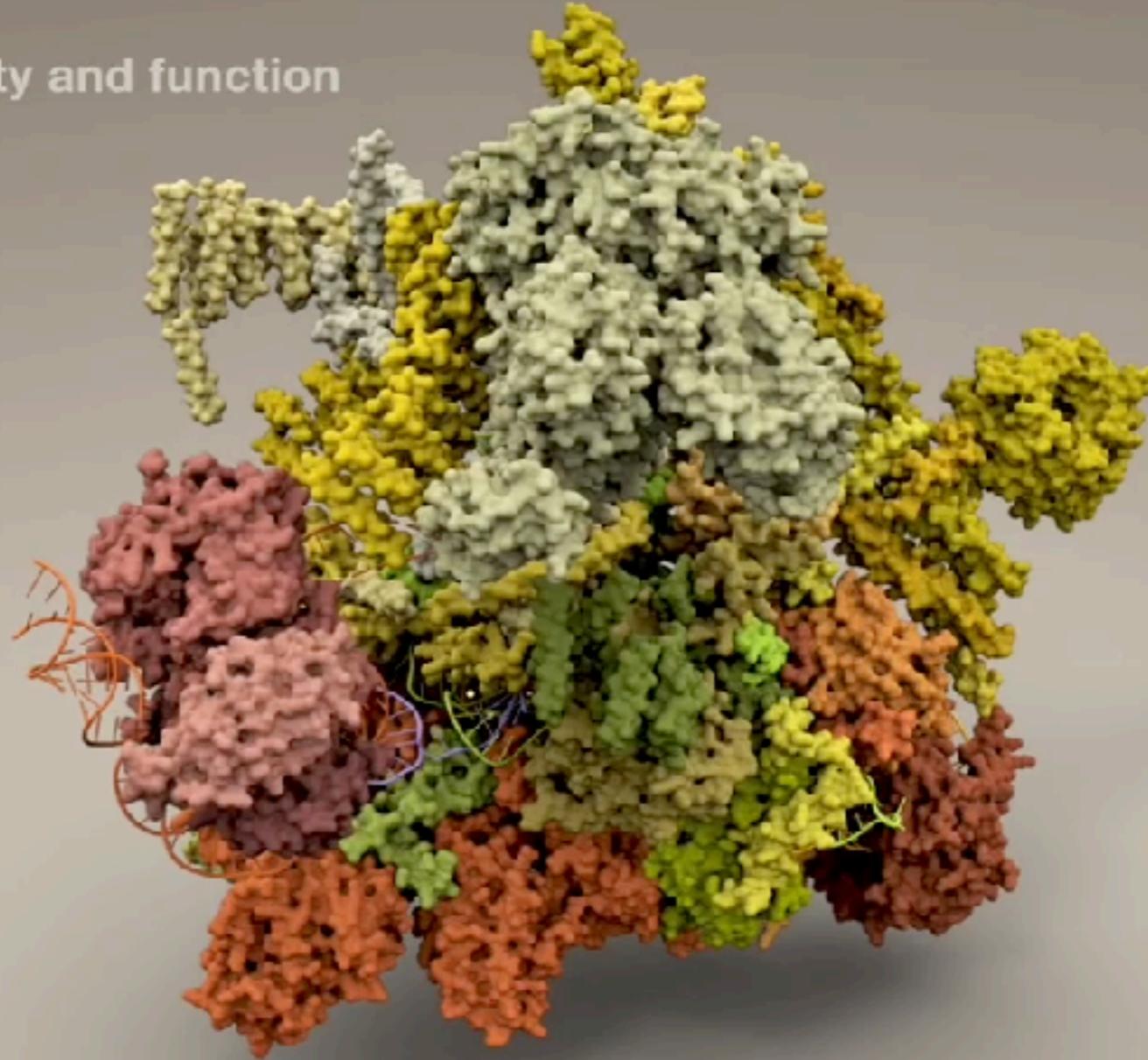
Estimating local resolution



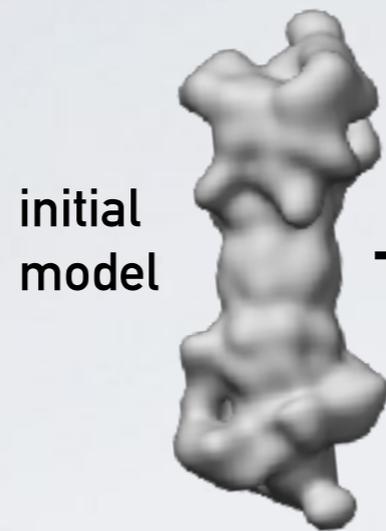
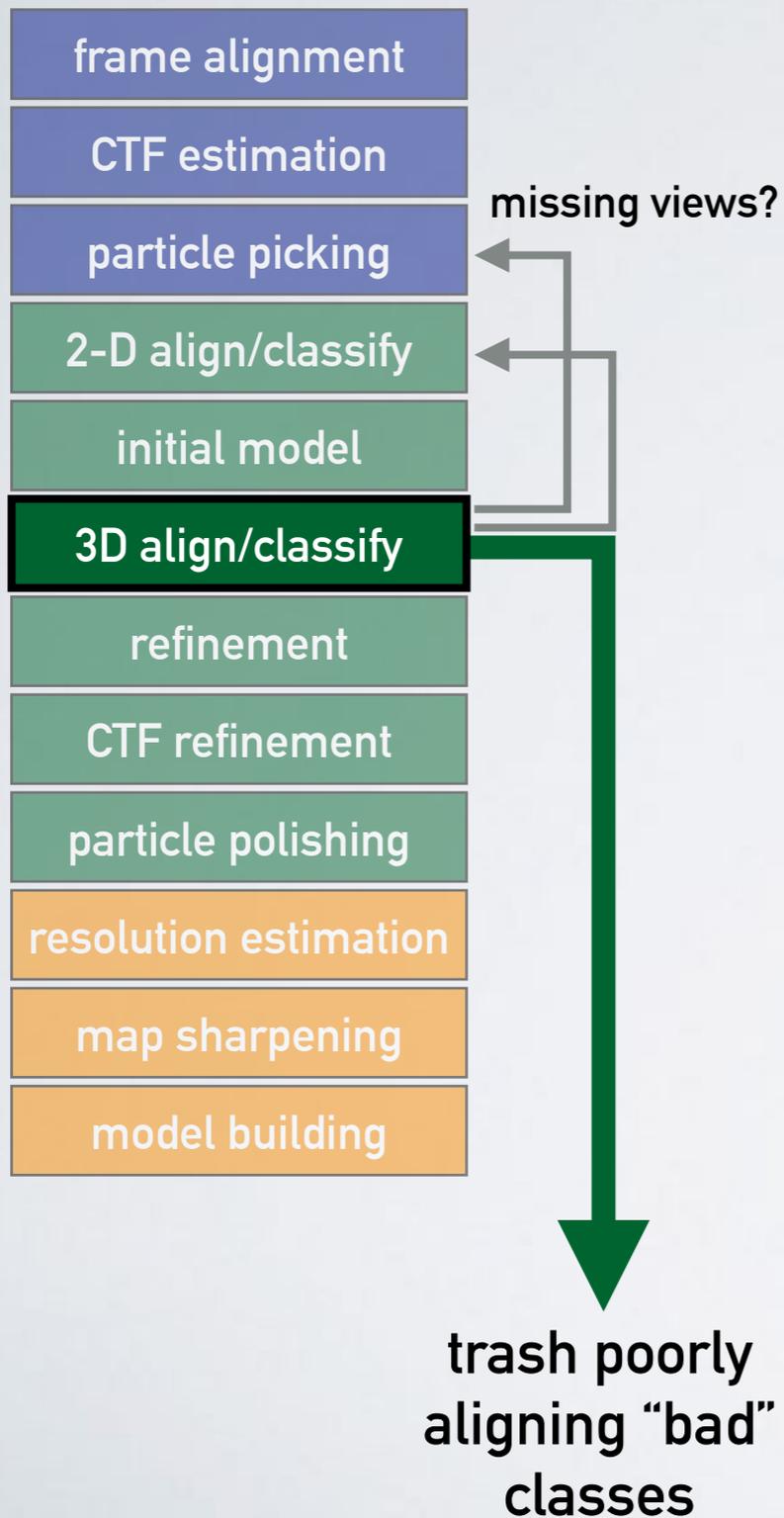
- Resmap - compares power of Fourier components
- Bsoft - calculates windowed FSCs
- RELION - calculates windowed FSCs
- Sparx - calculates local variance from 2D images
- CryoSPARC - calculates windowed FSCs

How do we deal with conformational & compositional variability?

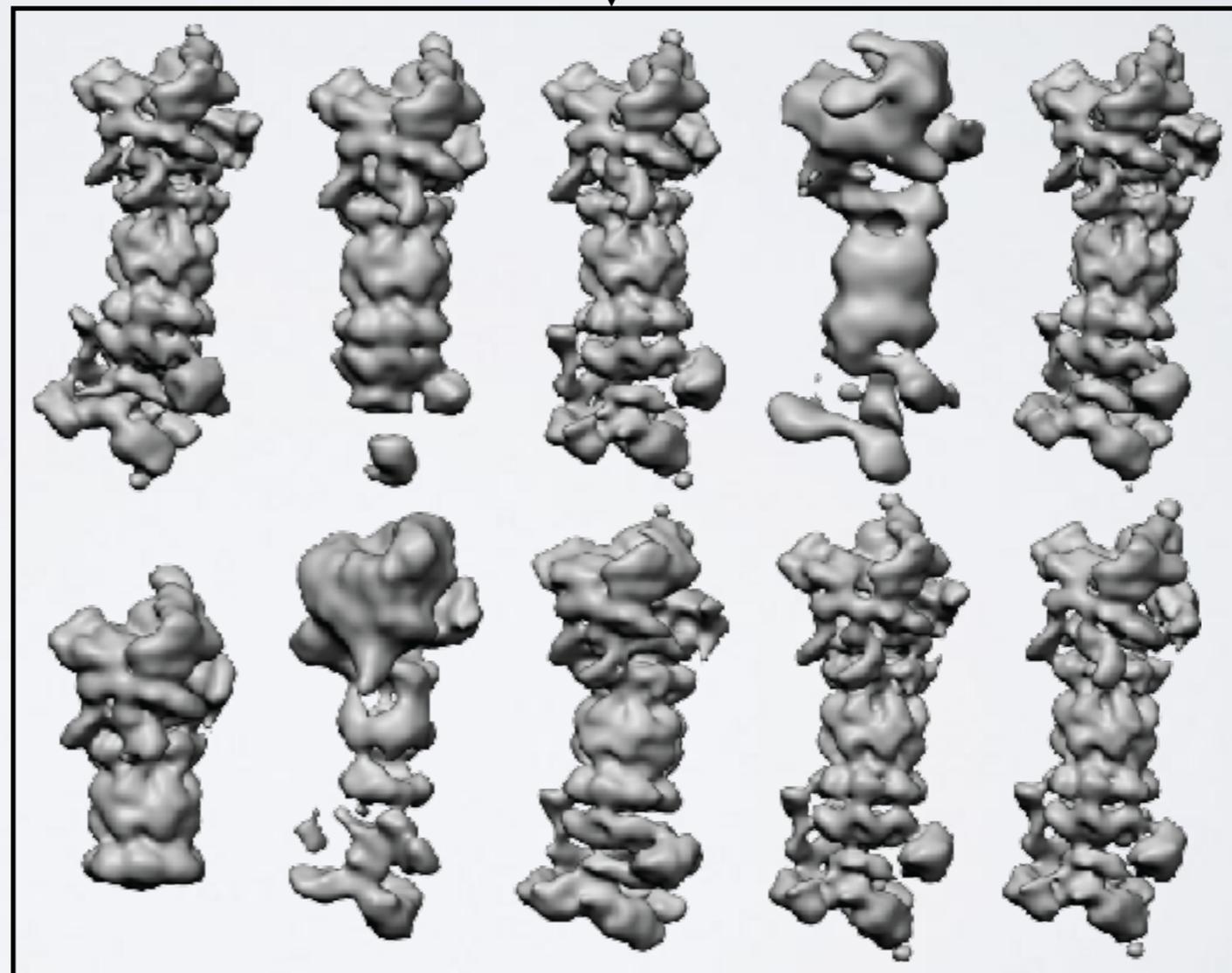
Extended structure,
conformational flexibility and function



3D Classification



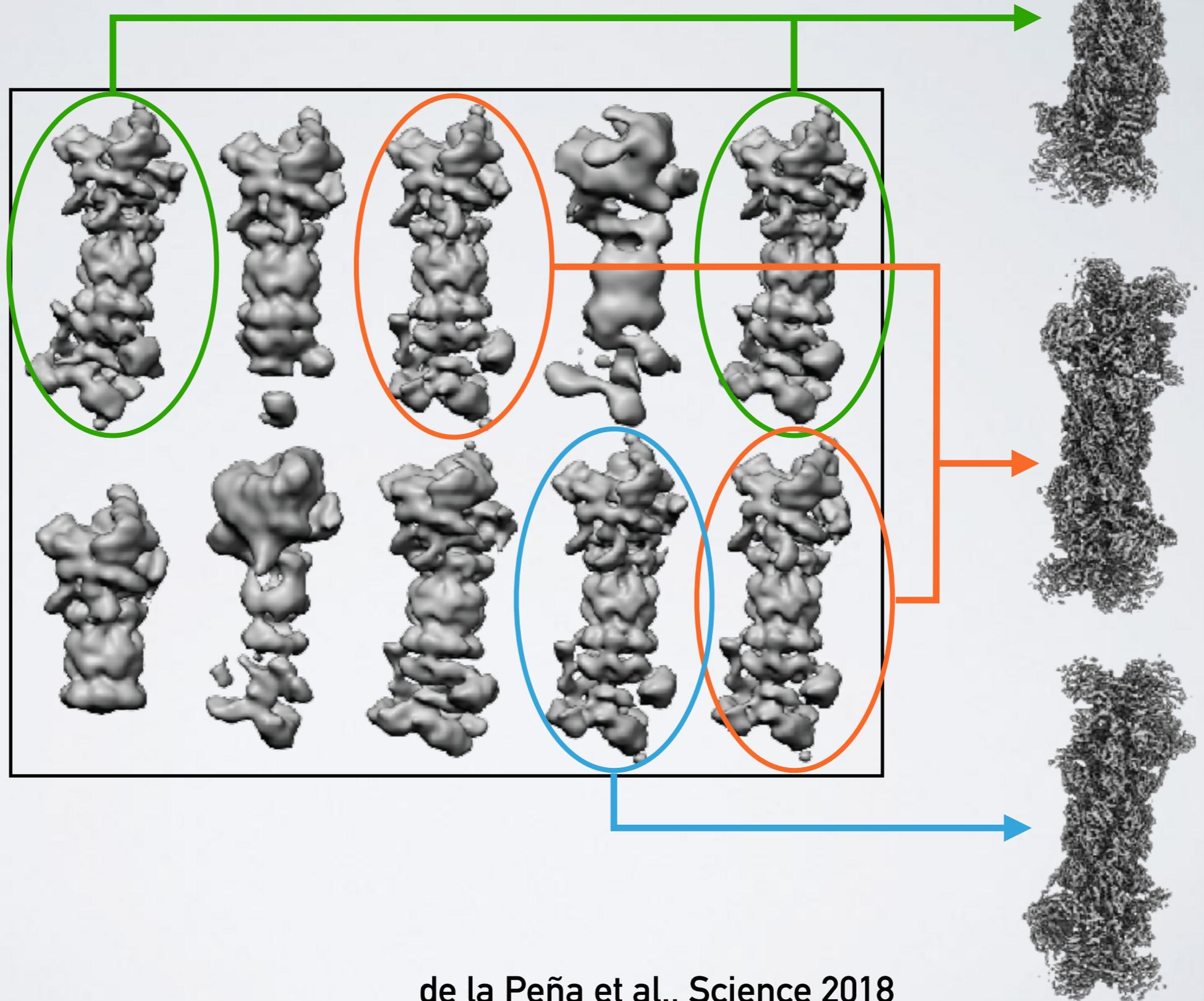
Assess conformational and compositional heterogeneity, and identify junk



de la Peña et al., Science 2018

3D Refinement

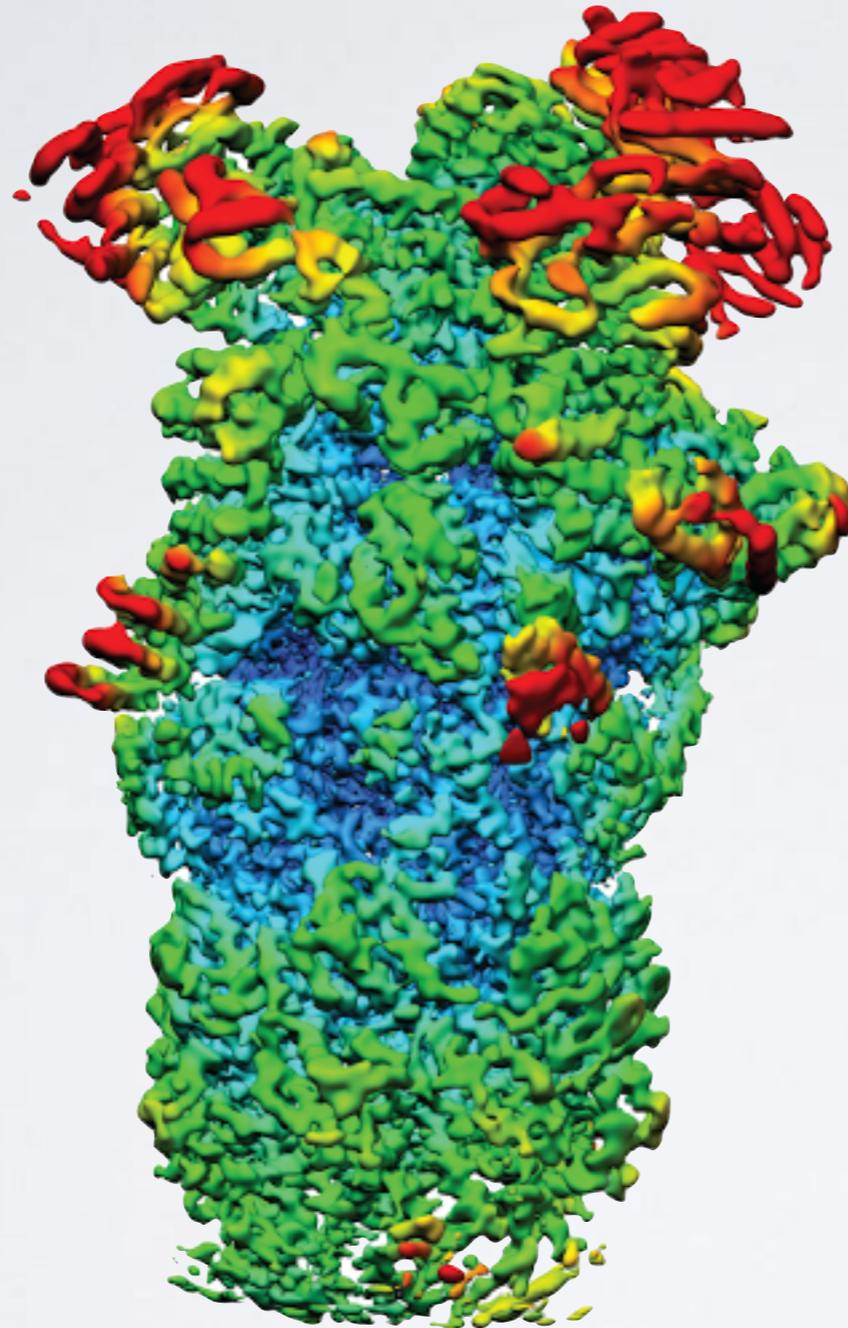
frame alignment
CTF estimation
particle picking
2-D align/classify
initial model
3D align/classify
refinement
CTF refinement
particle polishing
resolution estimation
map sharpening
model building



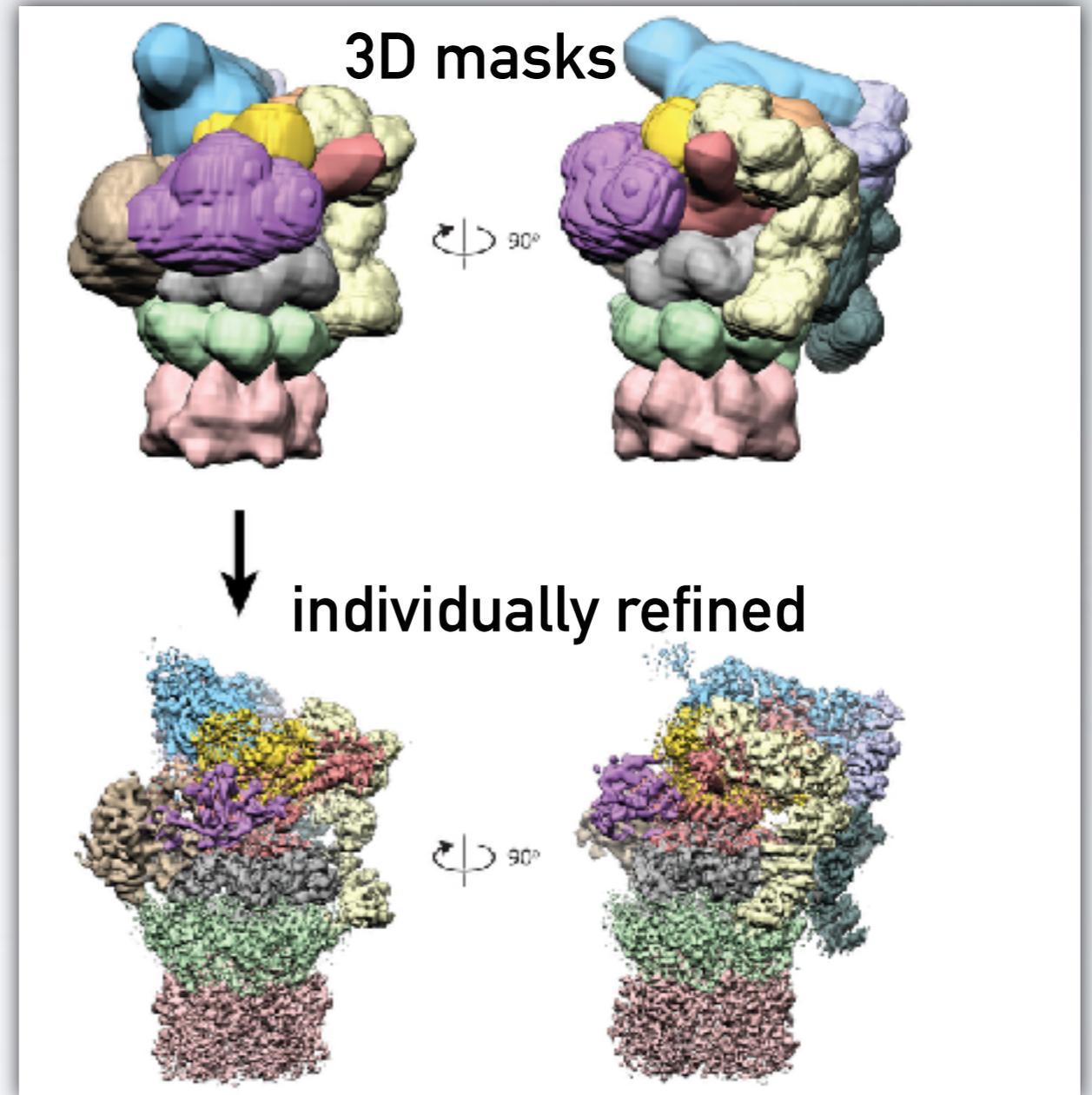
de la Peña et al., Science 2018

Mask-and-align / Focused refinement

- frame alignment
- CTF estimation
- particle picking
- 2-D align/classify
- initial model
- 3D align/classify
- refinement**
- CTF refinement
- particle polishing
- resolution estimation
- map sharpening
- model building

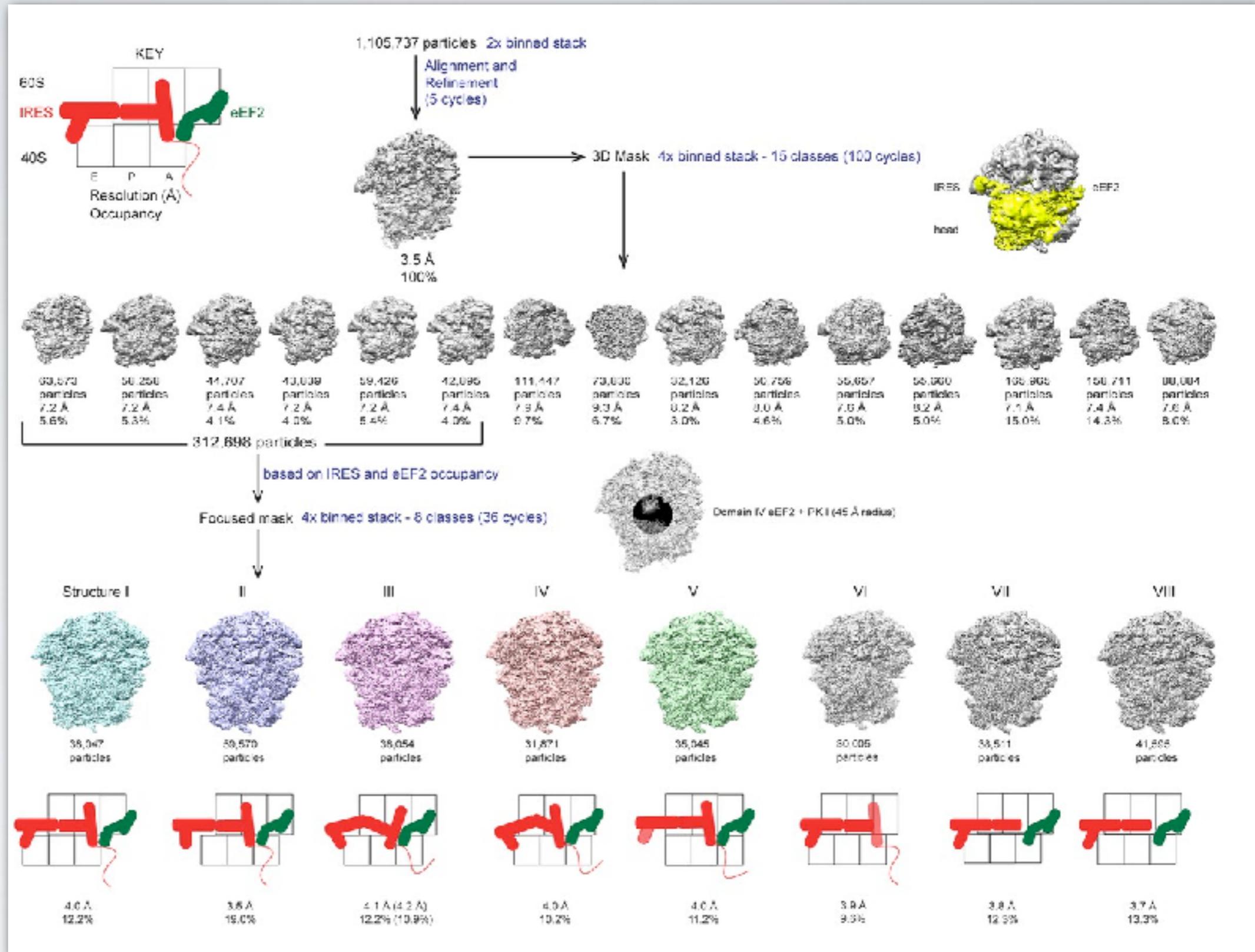


local resolution map



Mask-and-classify / Focused classification

- frame alignment
- CTF estimation
- particle picking
- 2-D align/classify
- initial model
- 3D align/classify**
- refinement
- CTF refinement
- particle polishing
- resolution estimation
- map sharpening
- model building



Abeyrathne et al. eLife 2016

Mask-and-classify with signal subtraction

frame alignment

CTF estimation

particle picking

2-D align/classify

initial model

3D align/classify

refinement

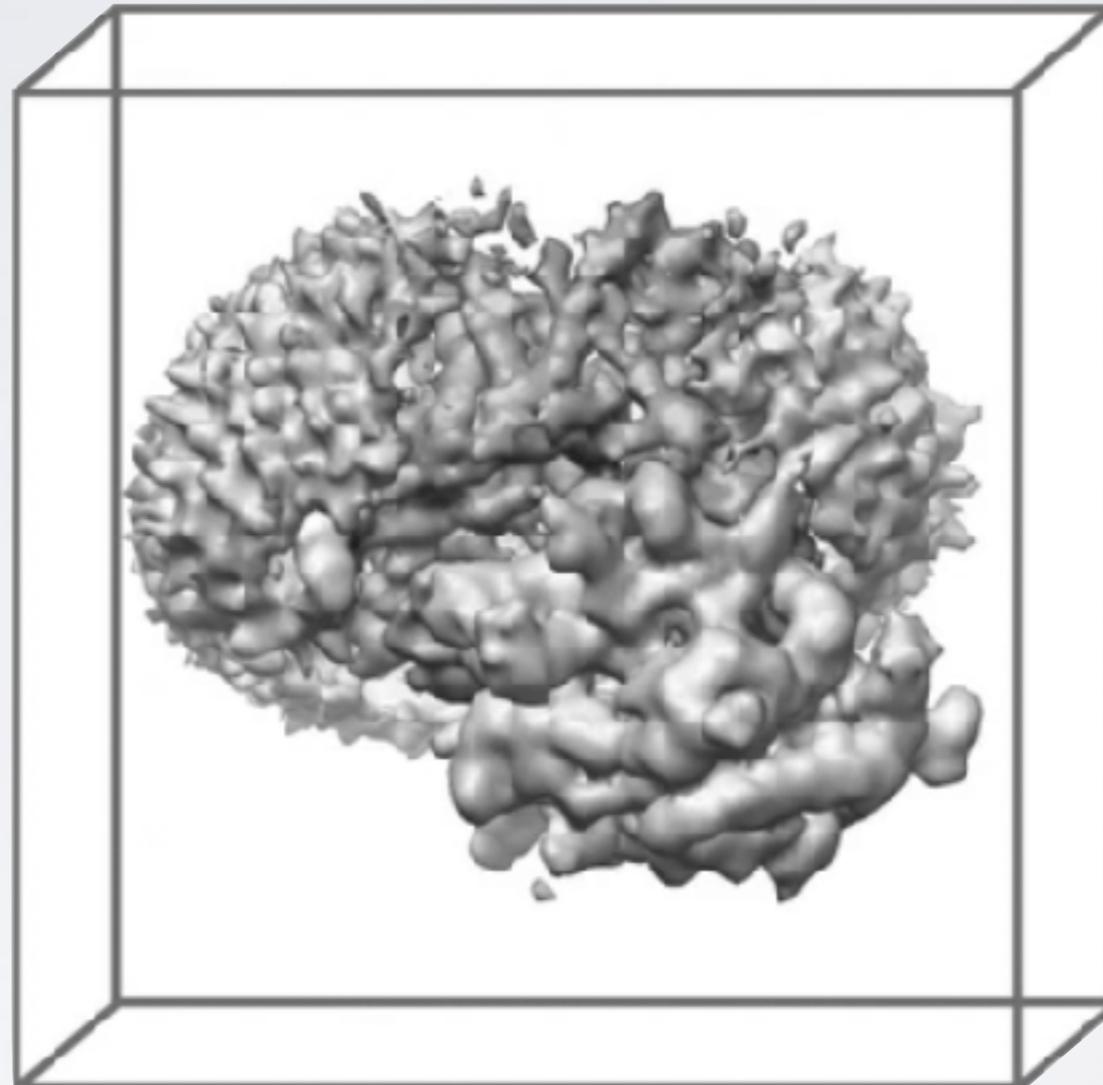
CTF refinement

particle polishing

resolution estimation

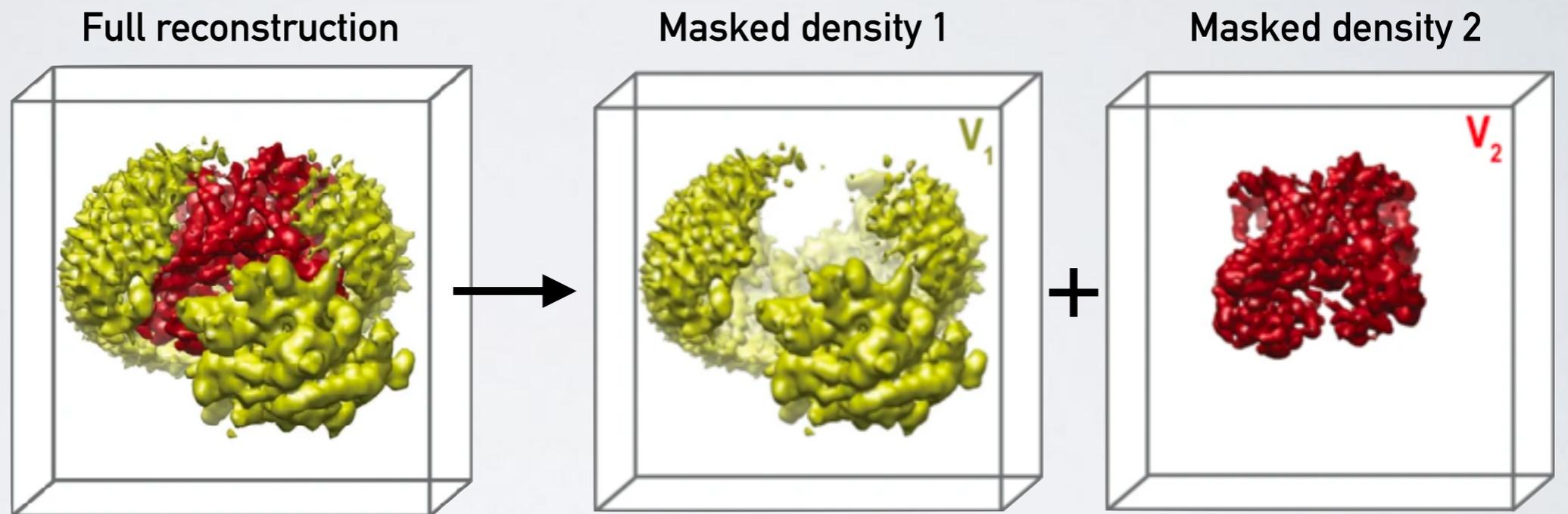
map sharpening

model building



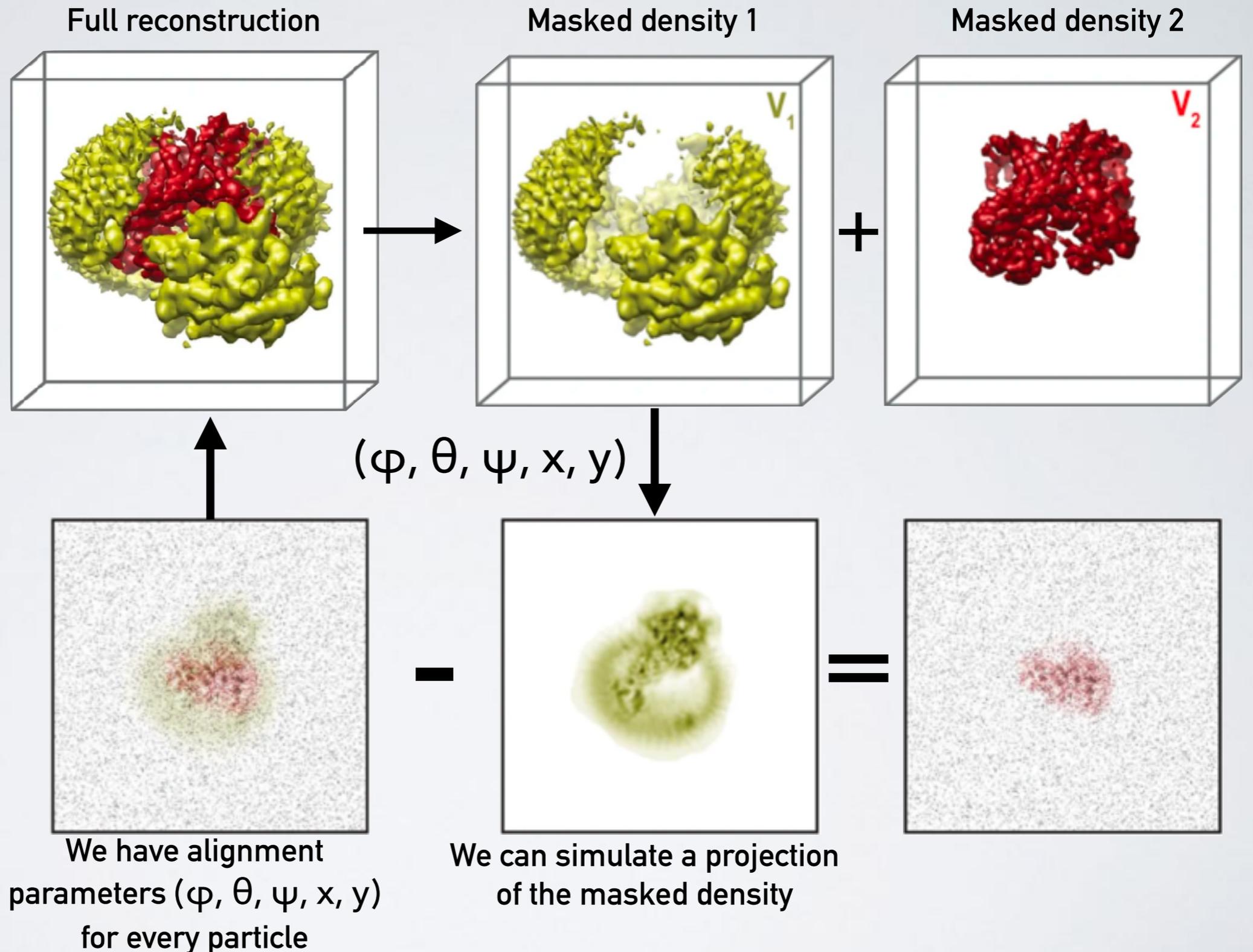
Mask-and-classify with signal subtraction

- frame alignment
- CTF estimation
- particle picking
- 2-D align/classify
- initial model
- 3D align/classify**
- refinement
- CTF refinement
- particle polishing
- resolution estimation
- map sharpening
- model building



Mask-and-classify with signal subtraction

- frame alignment
- CTF estimation
- particle picking
- 2-D align/classify
- initial model
- 3D align/classify**
- refinement
- CTF refinement
- particle polishing
- resolution estimation
- map sharpening
- model building



Mask-and-classify with signal subtraction

frame alignment

CTF estimation

particle picking

2-D align/classify

initial model

3D align/classify

refinement

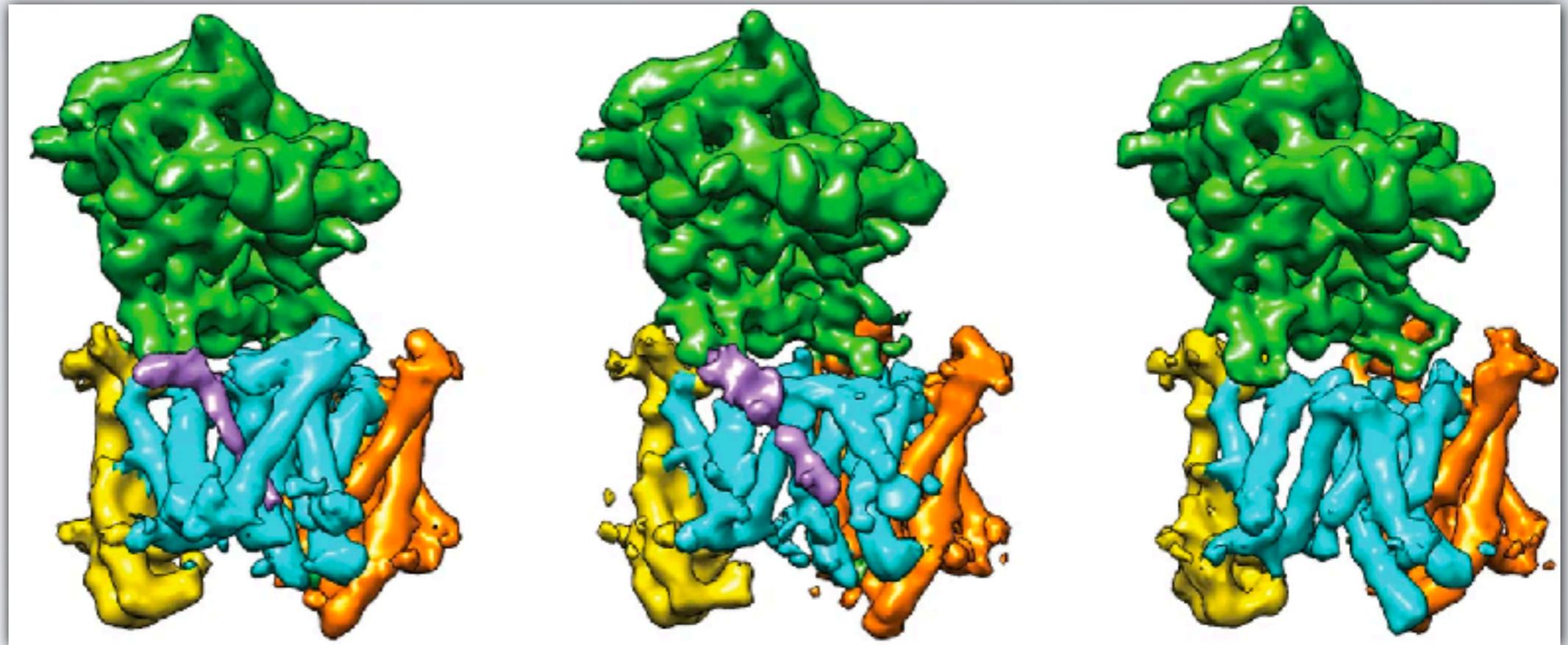
CTF refinement

particle polishing

resolution estimation

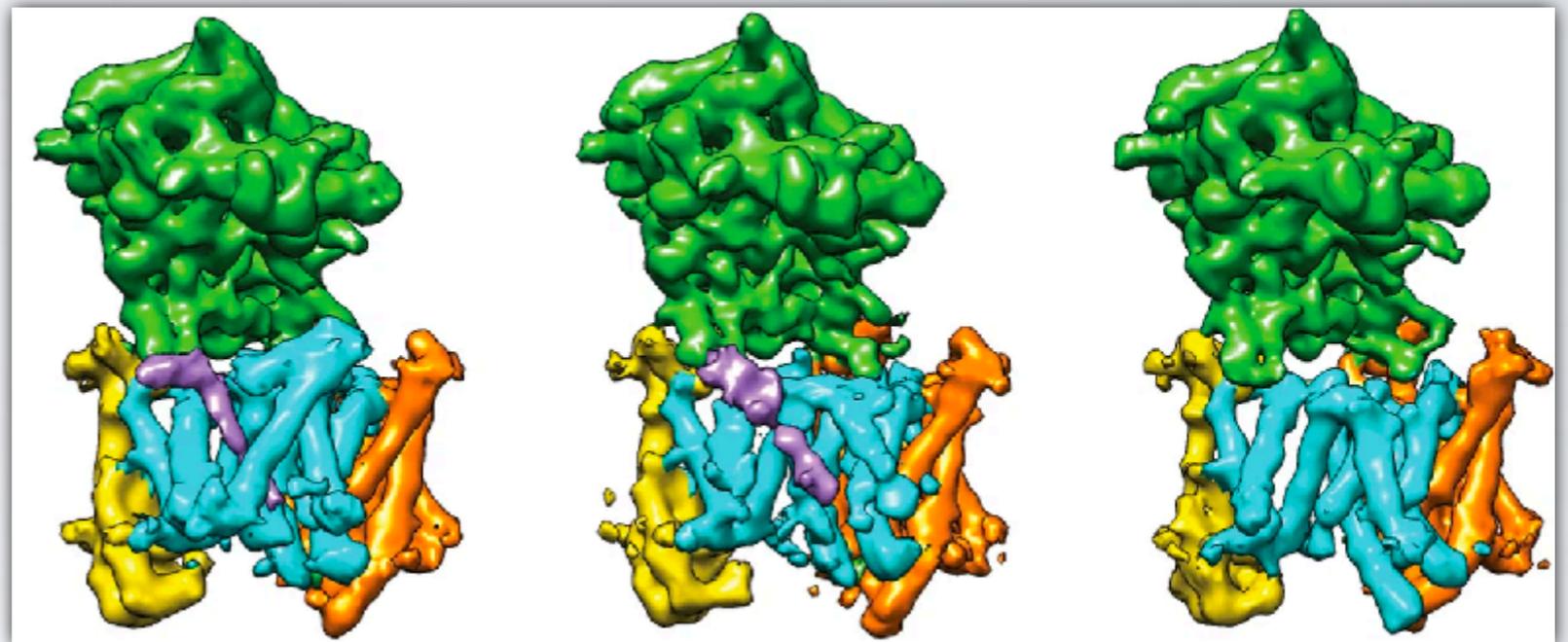
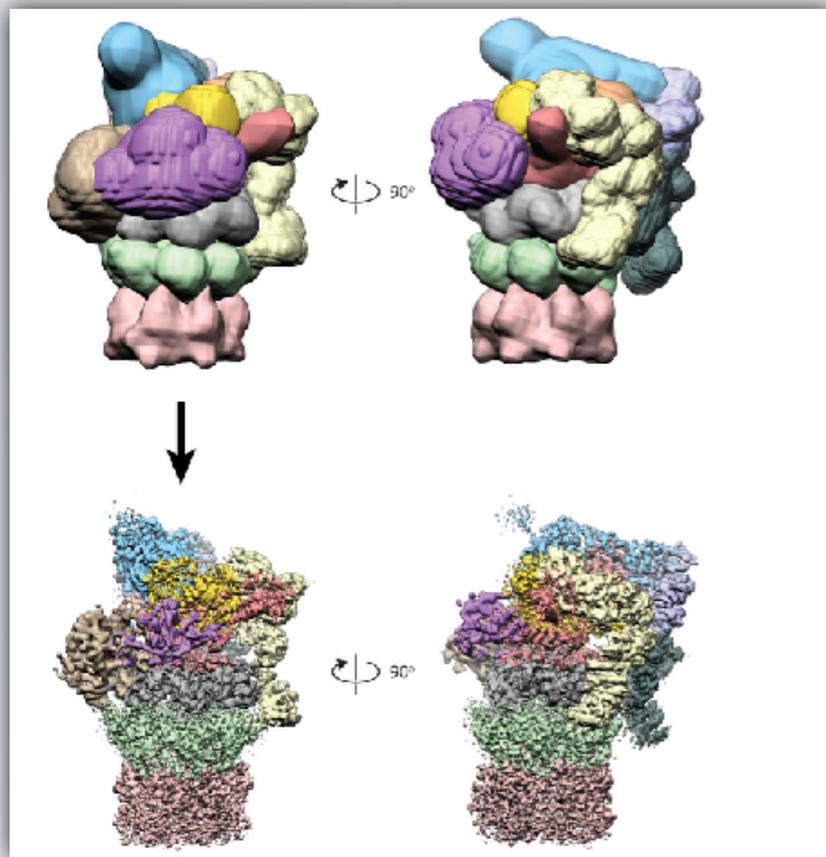
map sharpening

model building



**enabled classification of 30 kDa
density into distinct states**

Provide representations of discrete conformations



Can we represent the conformational landscape?

Methodologies for more complete descriptions of conformational variability

frame alignment

CTF estimation

particle picking

2-D align/classify

initial model

3D landscape characterization

CTF refinement

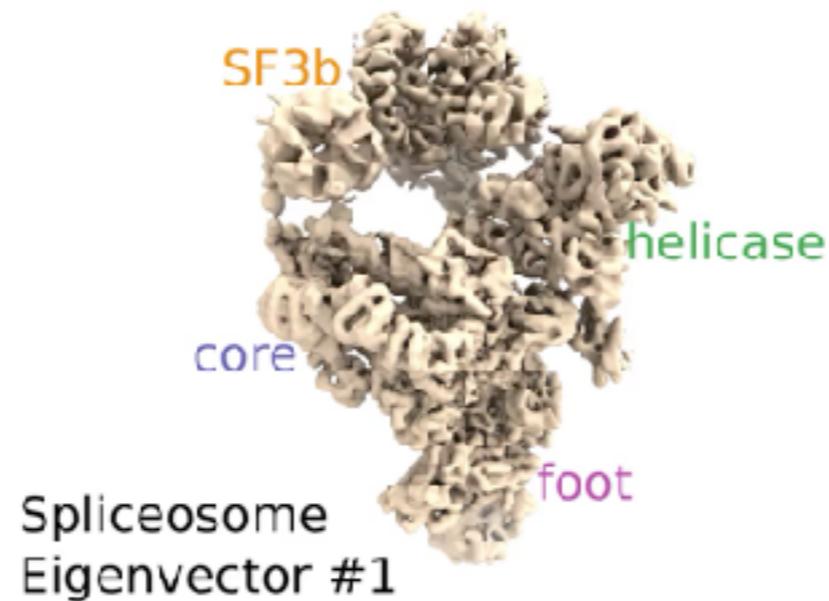
particle polishing

resolution estimation

map sharpening

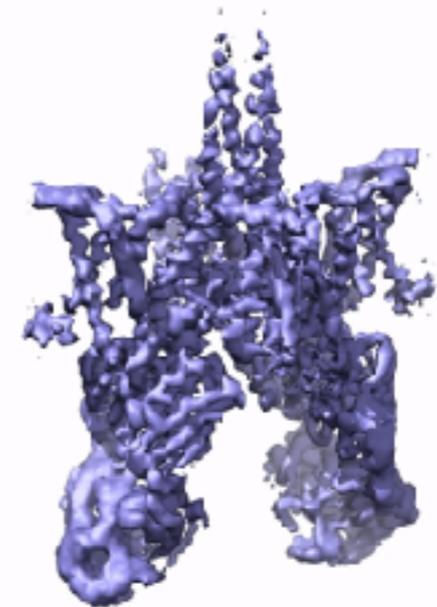
model building

Multi-body refinement (RELION)



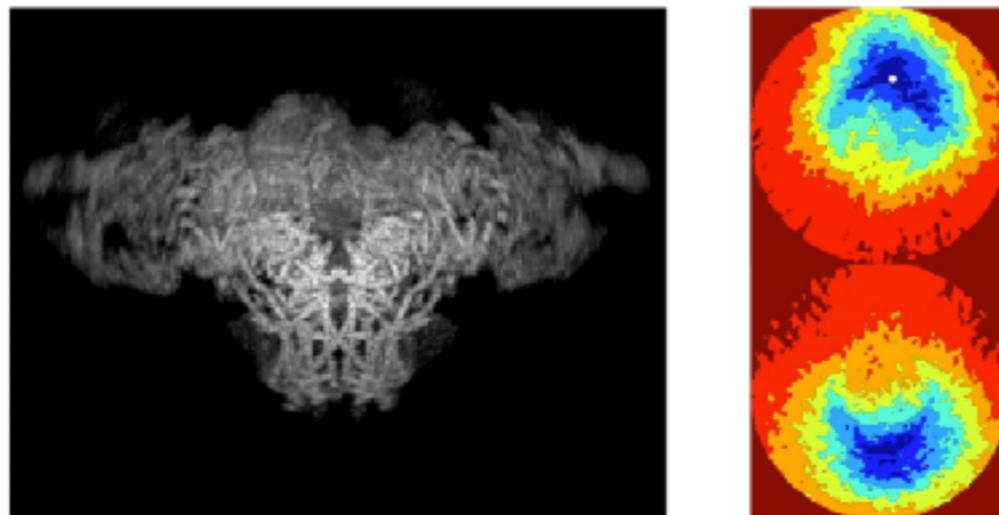
Nakane et al. eLife 2018

Variability Analysis (CryoSPARC)



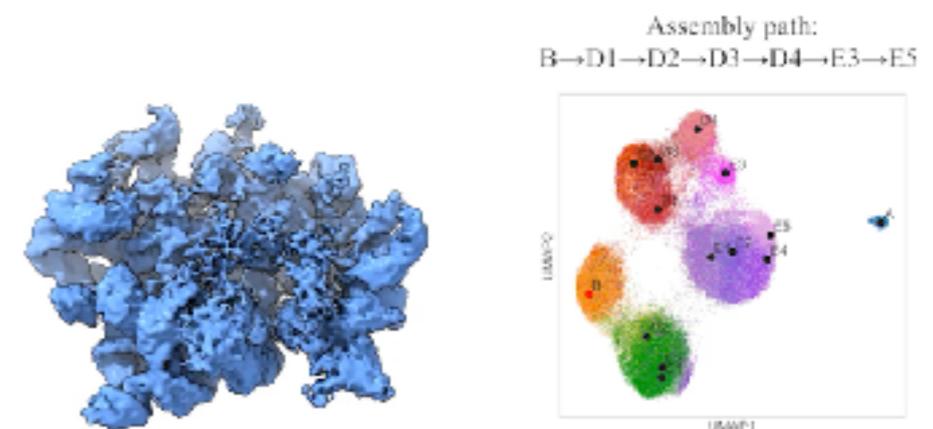
Punjani & Fleet JSB 2021

ManifoldEM



Dashti et al. Nat Comm 2020

CryoDRGN



Zhong et al. Nat Methods 2021

glander@scripps.edu

 [@LanderLab](https://twitter.com/LanderLab)