

Physics of Life

PHYS-468

Electron Microscopy in Life Sciences

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3D imaging techniques with beams

Technique	Beam	Sample size	Technical Resolution
SPECT (single photon emission)	Gamma emission	Human	2 mm
PET (positron emission)	Pair of gamma emission	Human	1 mm
CT (computed tomography)	X-ray	Human	0.5 mm
Ultrasound	Sound	tissue	0.3 mm
MRI (magnetic resonance imaging)	Magnetic field	human	0.1 mm
OCT (optical coherence tomography)	Infrared light	2 mm	10 μm
Synchotron X-ray tomography	X-ray	2 mm	100 nm
Synchotron X-ray ptychography	X-ray	2 mm	30 nm
Helium microscopy	He ²⁺	100 nm	1 nm
Electron microscopy: SBF-SEM	Electron (3kV)	1 mm	3 nm
Electron microscopy: FIB-SEM	Electron (3kV)	30 μm	2 nm
Electron microscopy: TEM	Electron (300kV)	500 nm	0.1 nm

BRAIN IMAGING



X-RAY



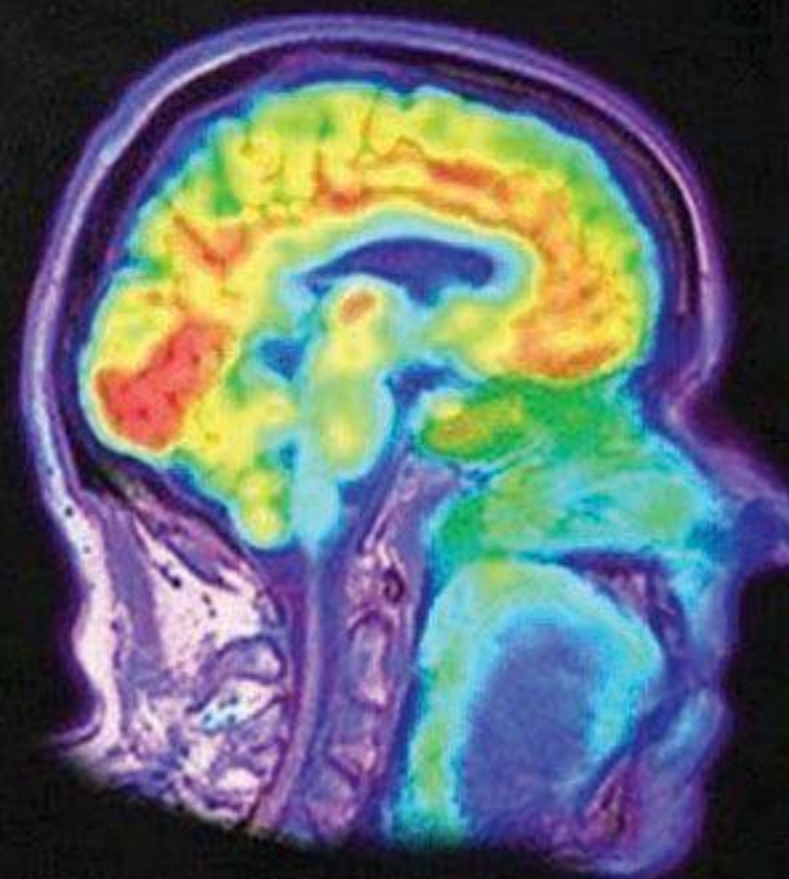
MRA



MRI



CT



PET SCAN

X-Ray shows bone/skull only, it does not show the brain. Best used to detect if there are bone fractures. It produces only a 2D projection of the object.

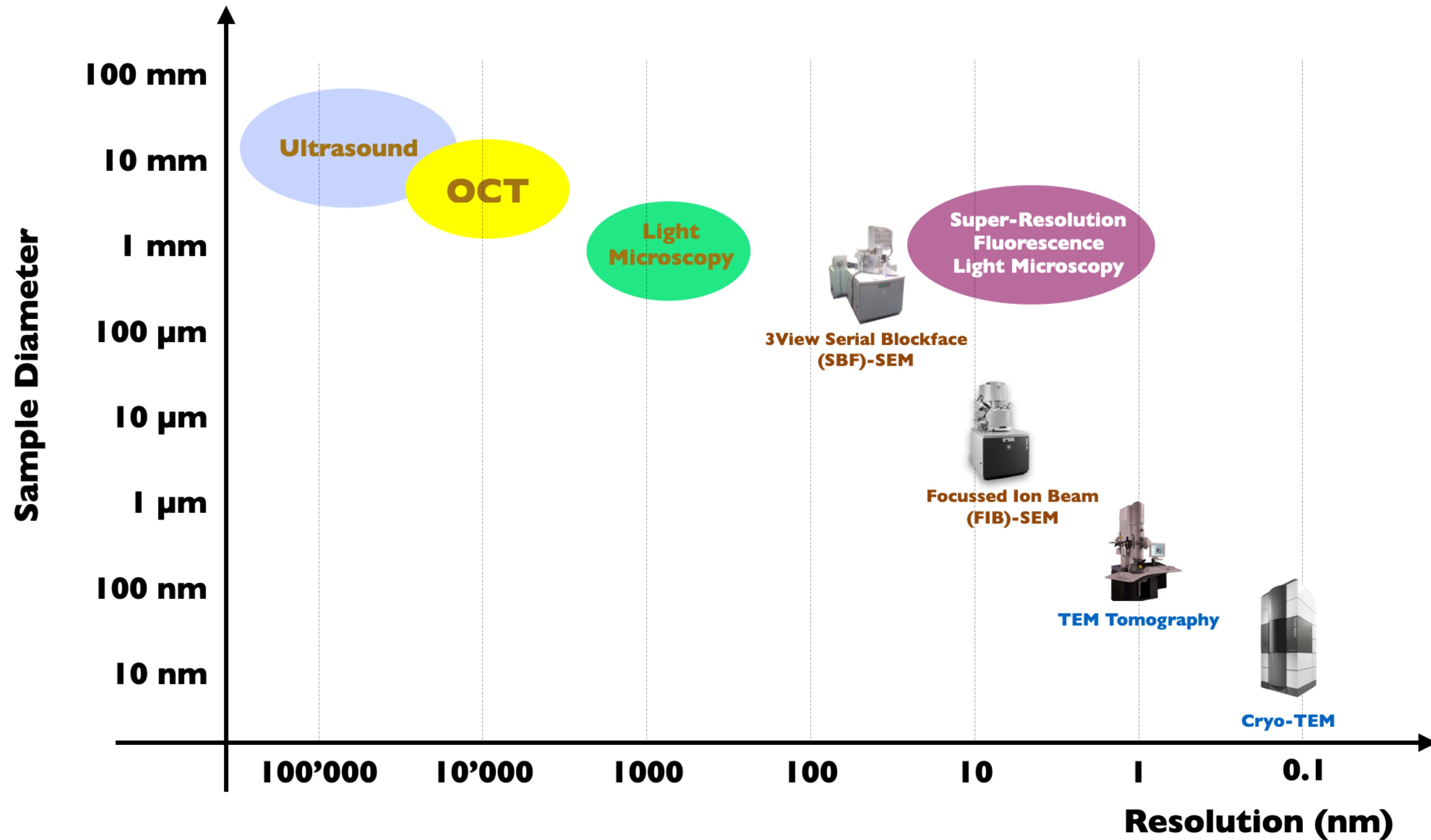
MRI scanners utilize strong magnetic fields, magnetic field gradients, and radio waves to produce images of the internal organs in the body. It provides a 3D map.

MRA, a specialised form of MRI, focuses a contrast material in the blood stream to visualize specifically the body's blood vessels.

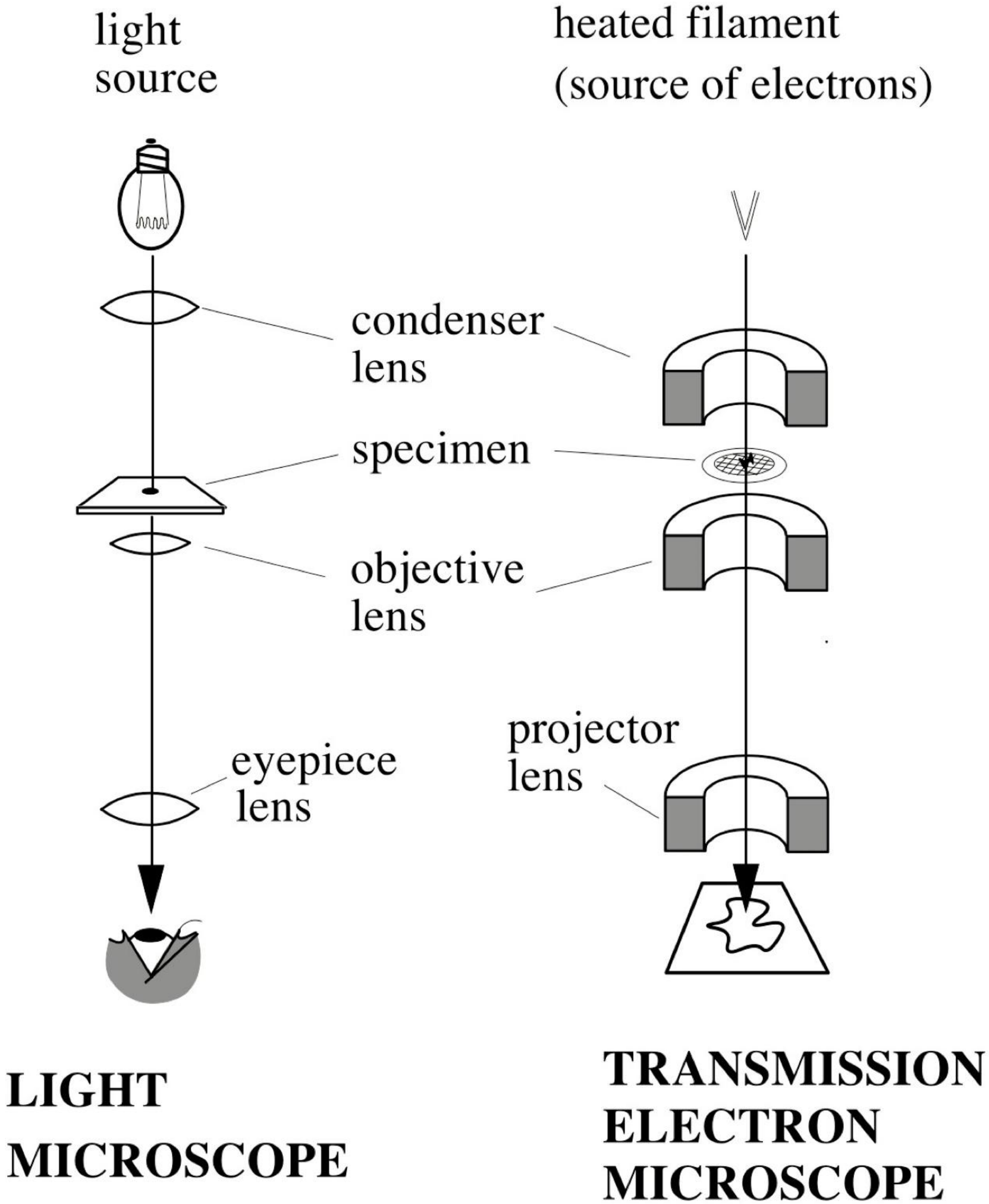
CT scans are a series of X-ray images transformed into 3D map of the brain, from which cross-sectional images can be produced.

PET scans involve the use of a radioactive tracer that binds to glucose in the bloodstream. As the brain primarily relies on glucose for fuel, the tracer accumulates in regions with high brain activity. It provides a 3D map.

No single instrument can cover all scales



The electron beam



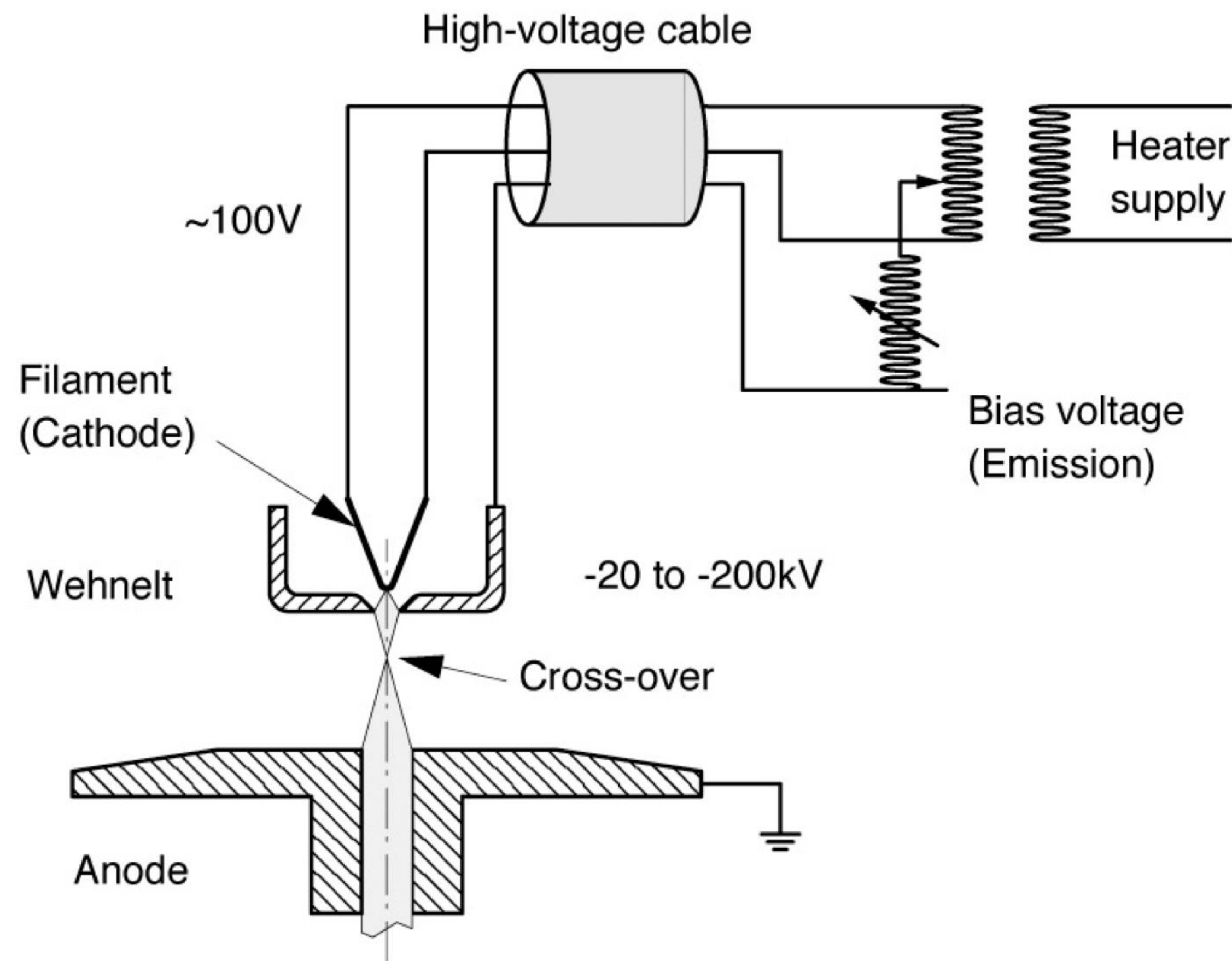
Type	Energy	Wavelength
Infrared	0.1 – 1 eV	10-1 μm
Visible	1-3 eV	1-0.3 μm
Ultraviolett	3-500 eV	0.3-0.01 μm
X-rays, γ	≥ 5000 eV	≤ 2 Å
Electrons	1 keV	0.4 Å
	10 keV	0.12 Å
	100 keV	0.037 Å
	1000 keV	0.009 Å

$$E = h \cdot \nu$$

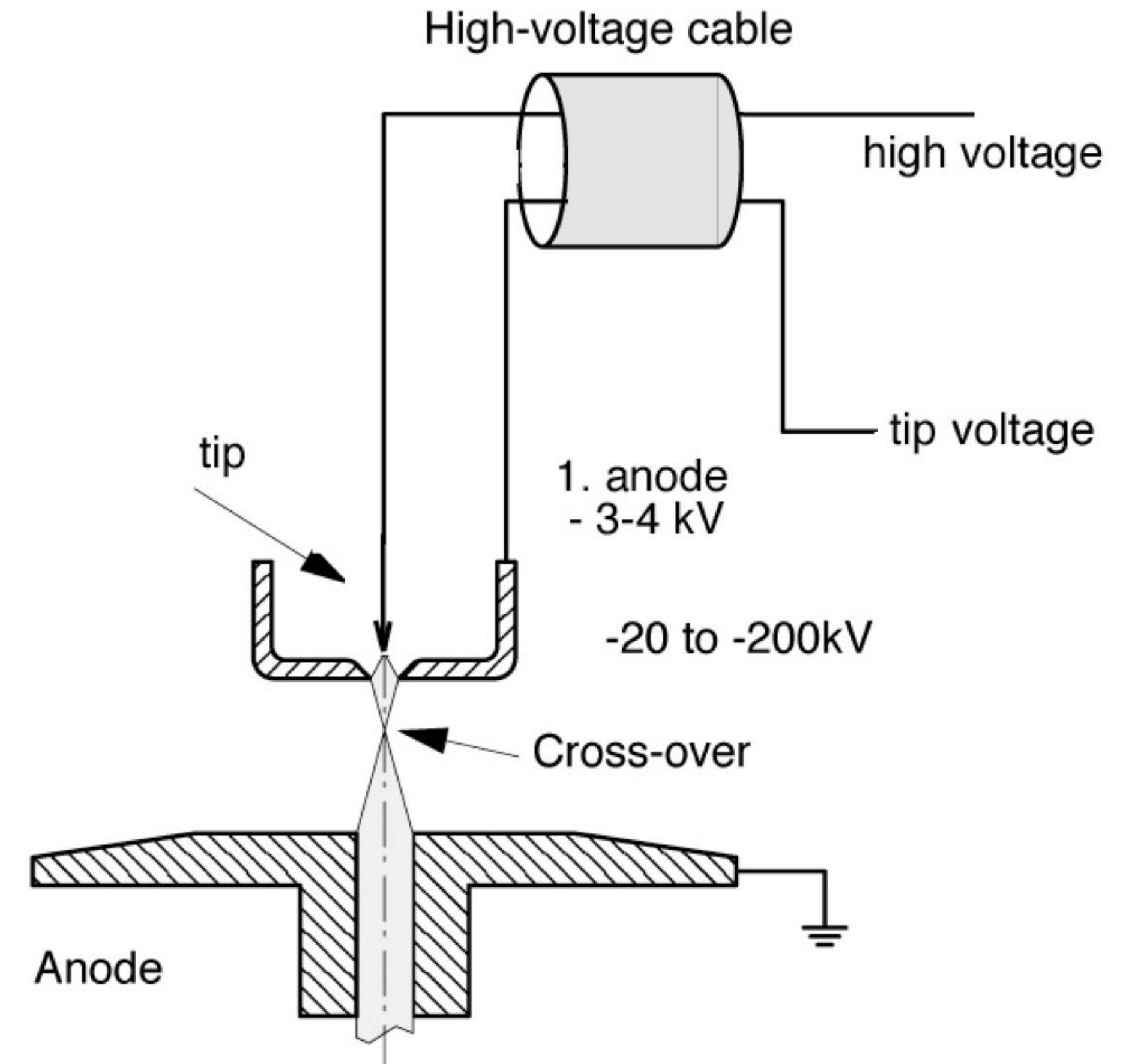
$$\lambda = \frac{c}{\nu}$$

$$d = \frac{\lambda}{2NA}$$

Electron Sources

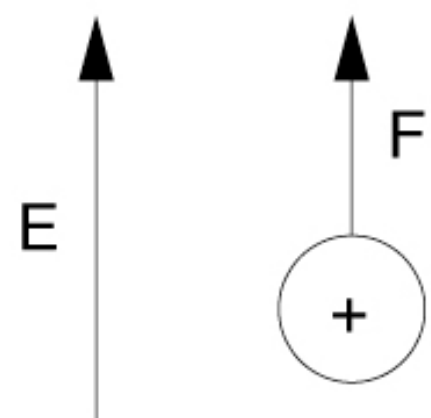
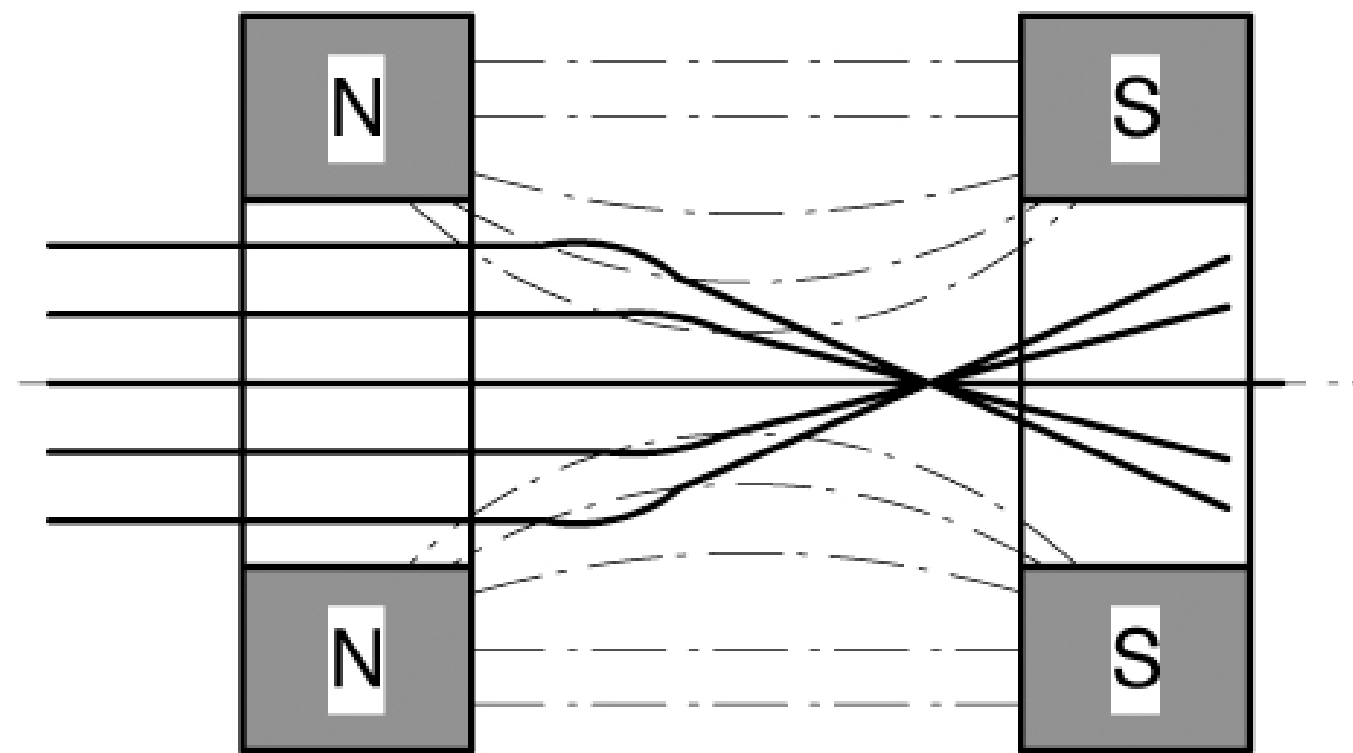


Heated filament



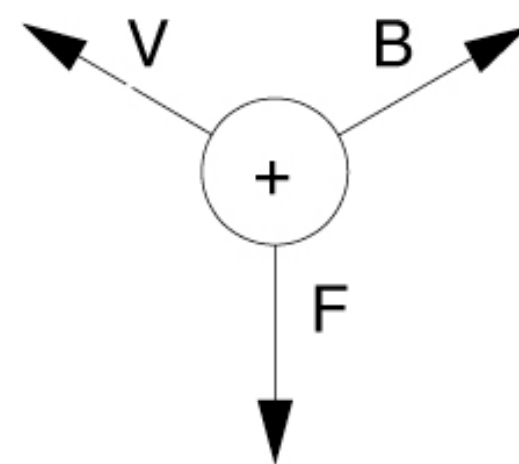
Field emission gun

Electrons are focused by magnetic fields



$$\vec{F} = q \cdot \vec{E}$$

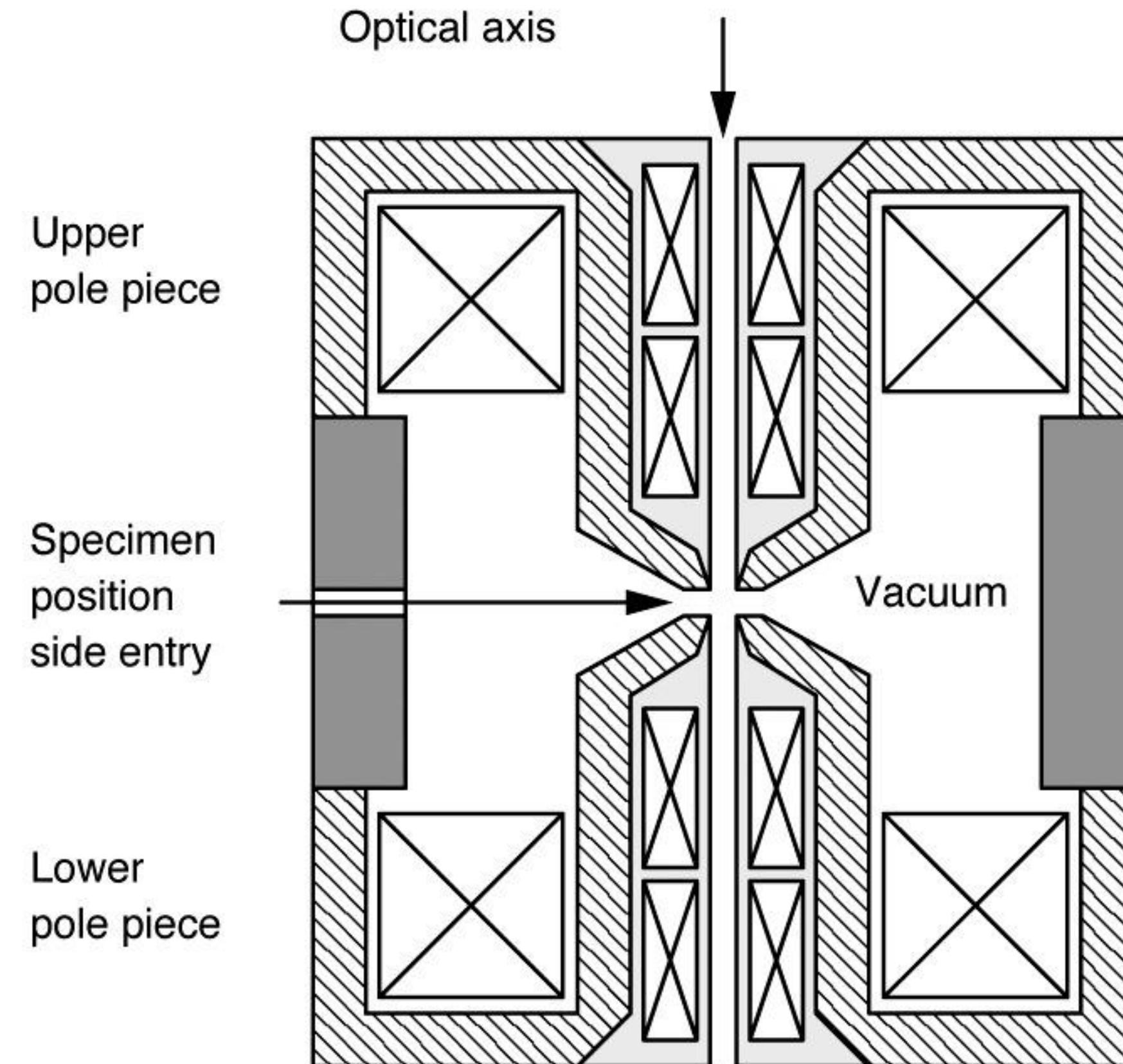
Electrostatic Force



$$\vec{F} = q \cdot \vec{v} \times \vec{B}$$

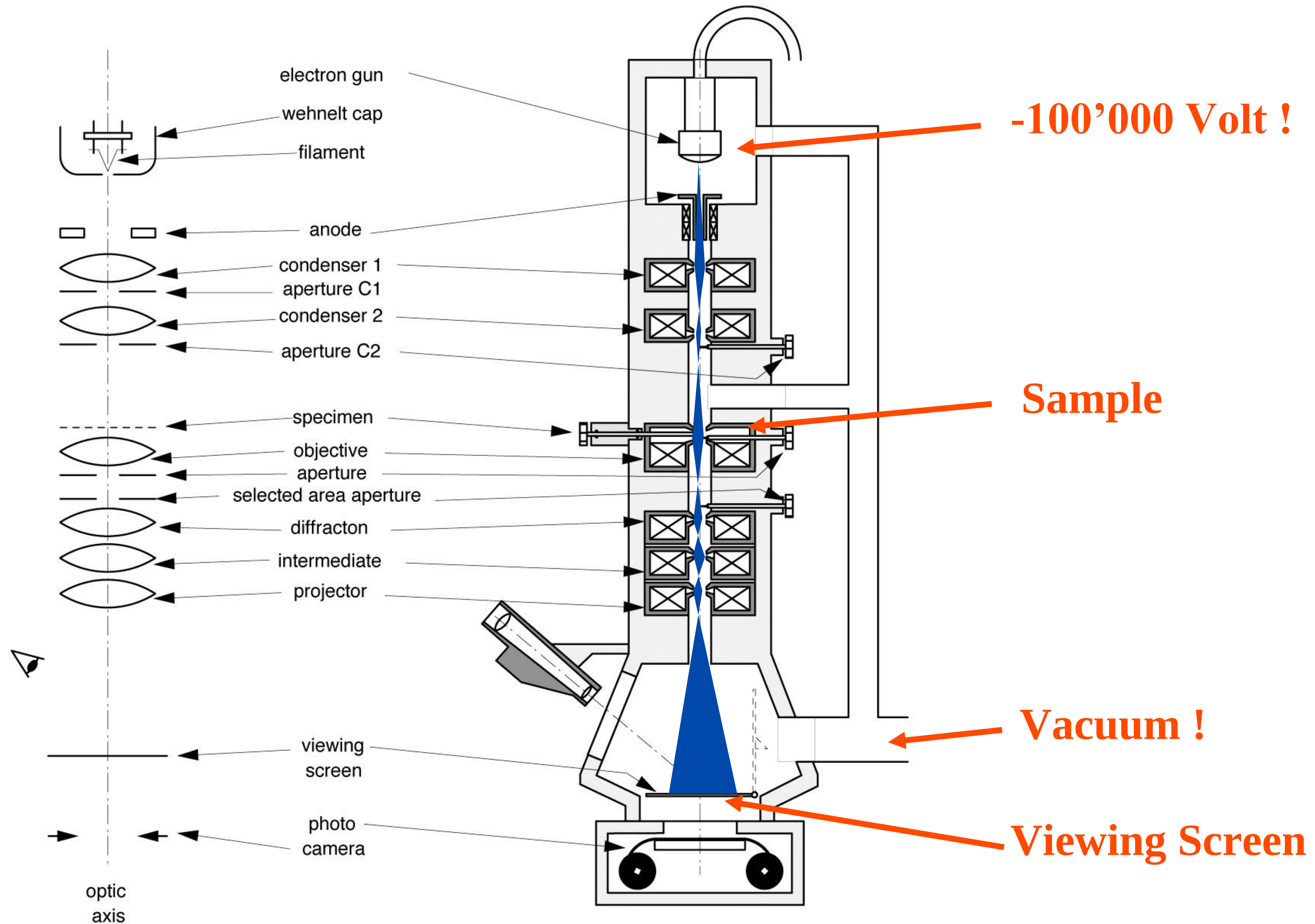
Lorentz Force

(Left-hand rule for
negative electrons)



The magnetic electron lens

The Transmission Electron Microscope



3D imaging techniques with beams

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Transmission Electron Microscopes

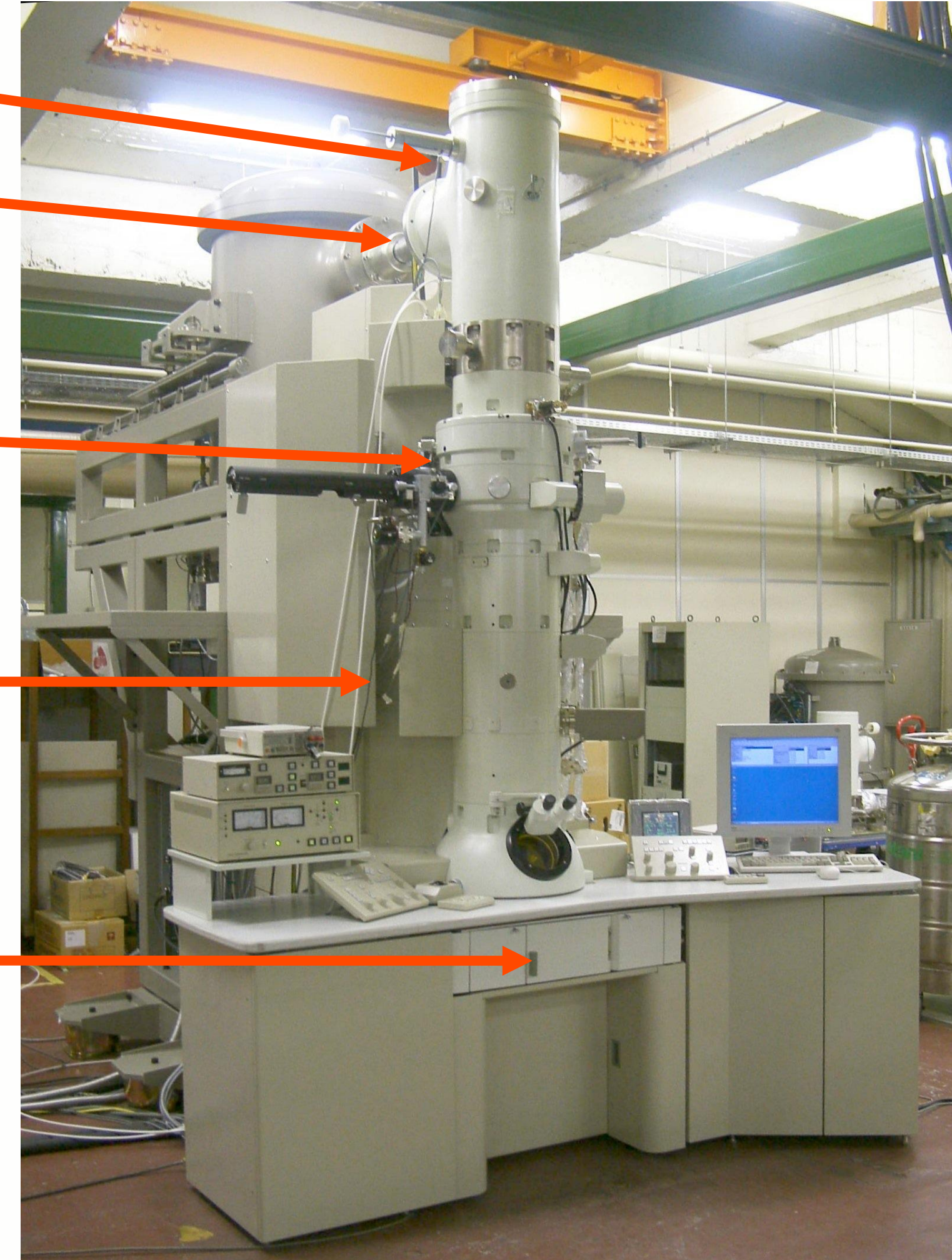
FEG

300 kV

Helium Cold Stage

Energy Filter

4k CCD Camera



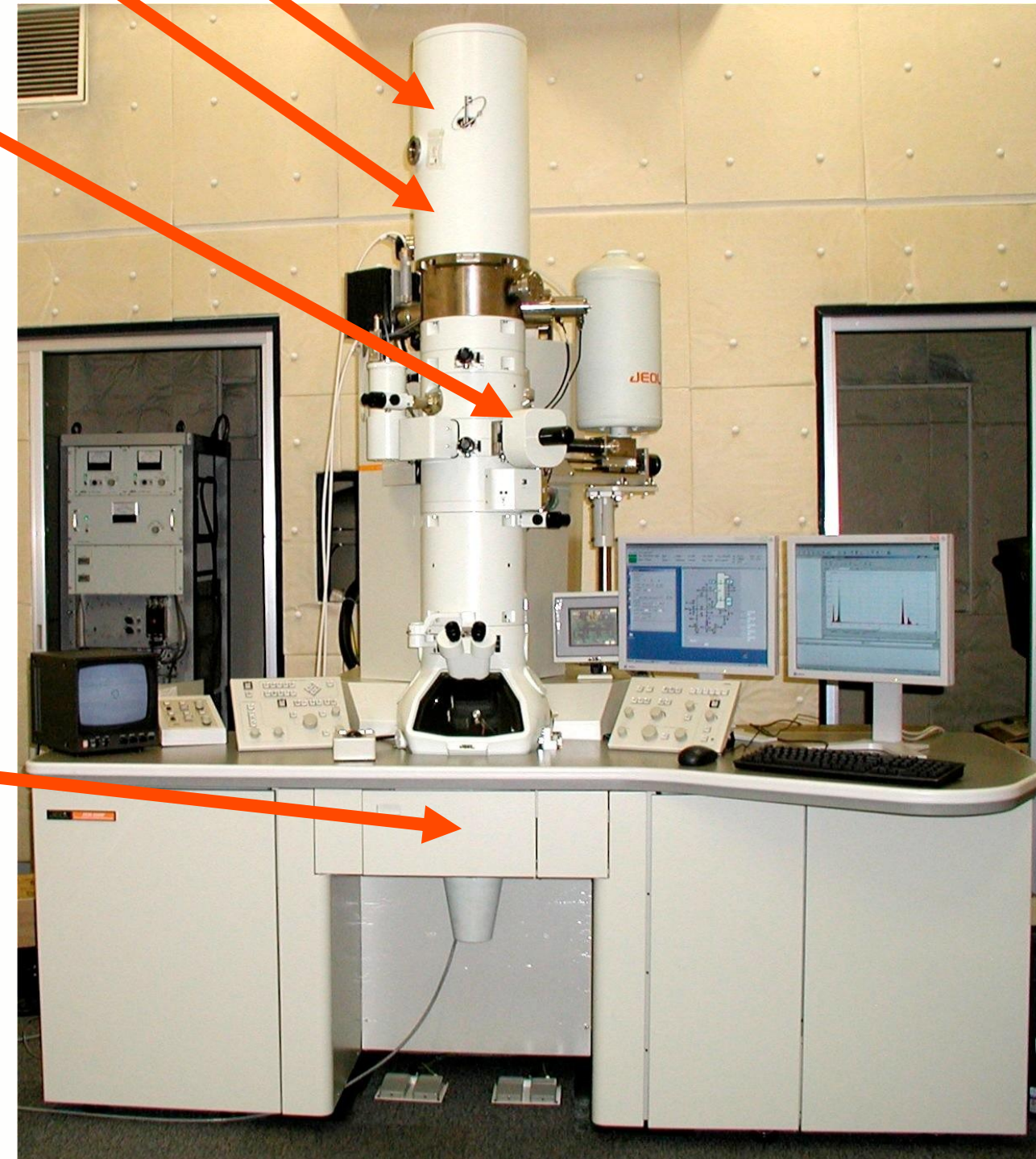
Transmission Electron Microscopes

FEG

200 kV

LN2 Cold Stage

4k CCD Camera

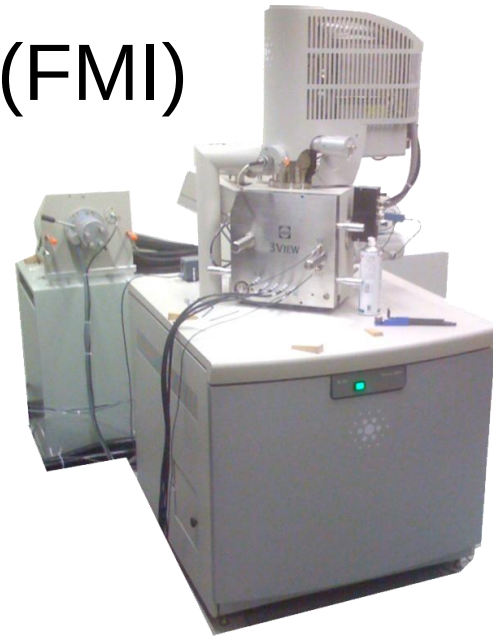


Electron Microscopes

FEI Quanta200/3View

Serial Block Face SEM

(FMI)



Zeiss Merlin/3View

Serial Block Face SEM

(FMI)



(SEM)

FEI Versa3D

Dual-Beam FIB-SEM



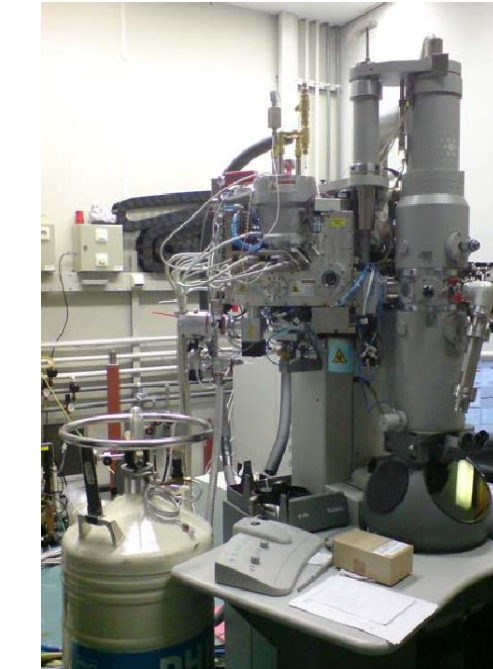
Philips CM100

100 kV, CCD
negative stain EM, sections



FEI T12

120kV, F416 CMOS
Cryo-EM screening



(TEM)

FEI Titan Krios

GIF / K2 Summit

300kV, GIF
Cryo-EM and Cryo-ET



FEI Polara

300kV, K2 Summit
Cryo-EM and Cryo-ET



FEI Talos

200kV, Ceta 16M CMOS
Cryo-EM and STEM



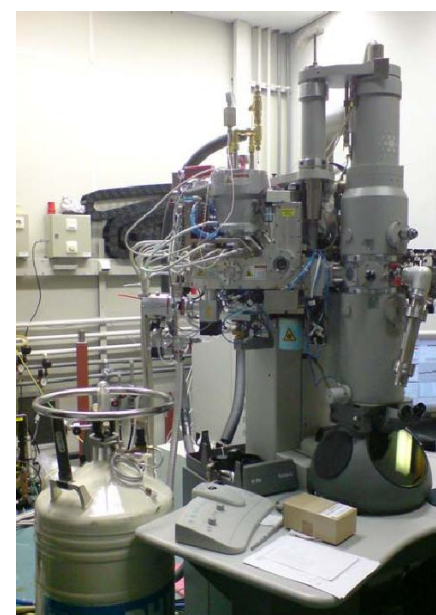
Philips CM200F

200kV, F416 CMOS
Cryo-EM



FEI T12

120kV, F416 CMOS
Cryo-EM screening



Scanning Electron Microscopy (SEM)



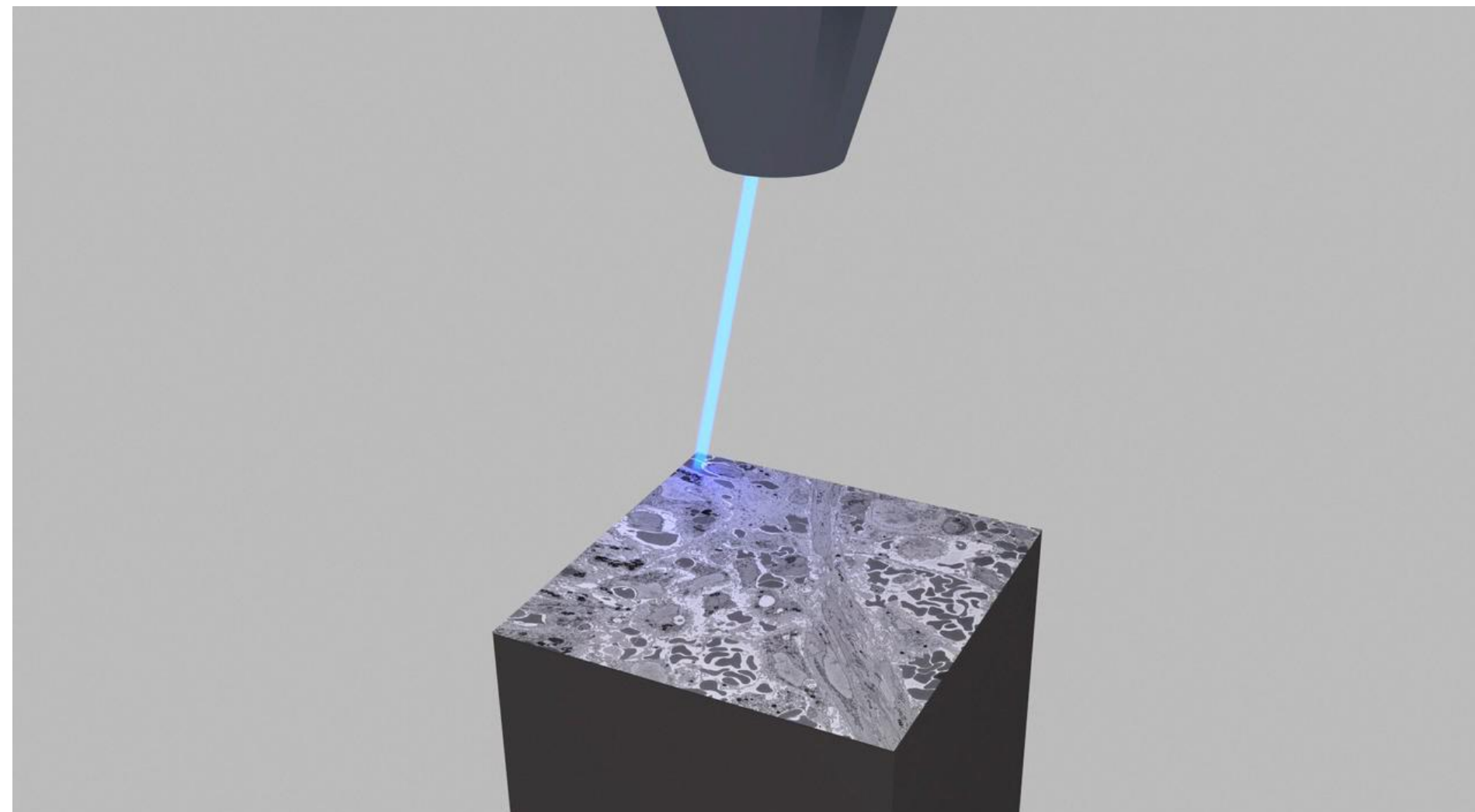
Transmission Electron Microscopy (TEM)



Scanning Electron Microscopy (SEM)



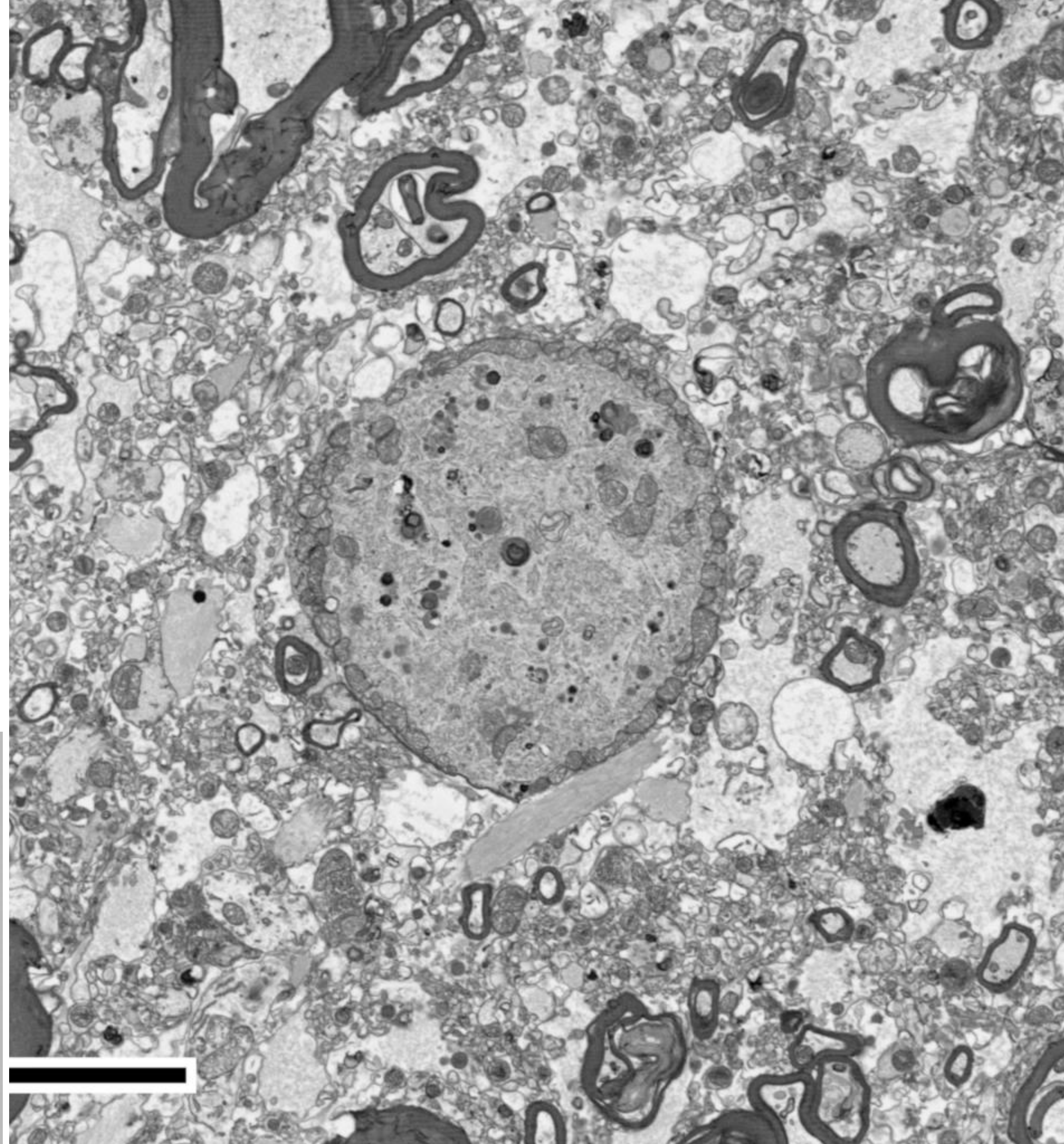
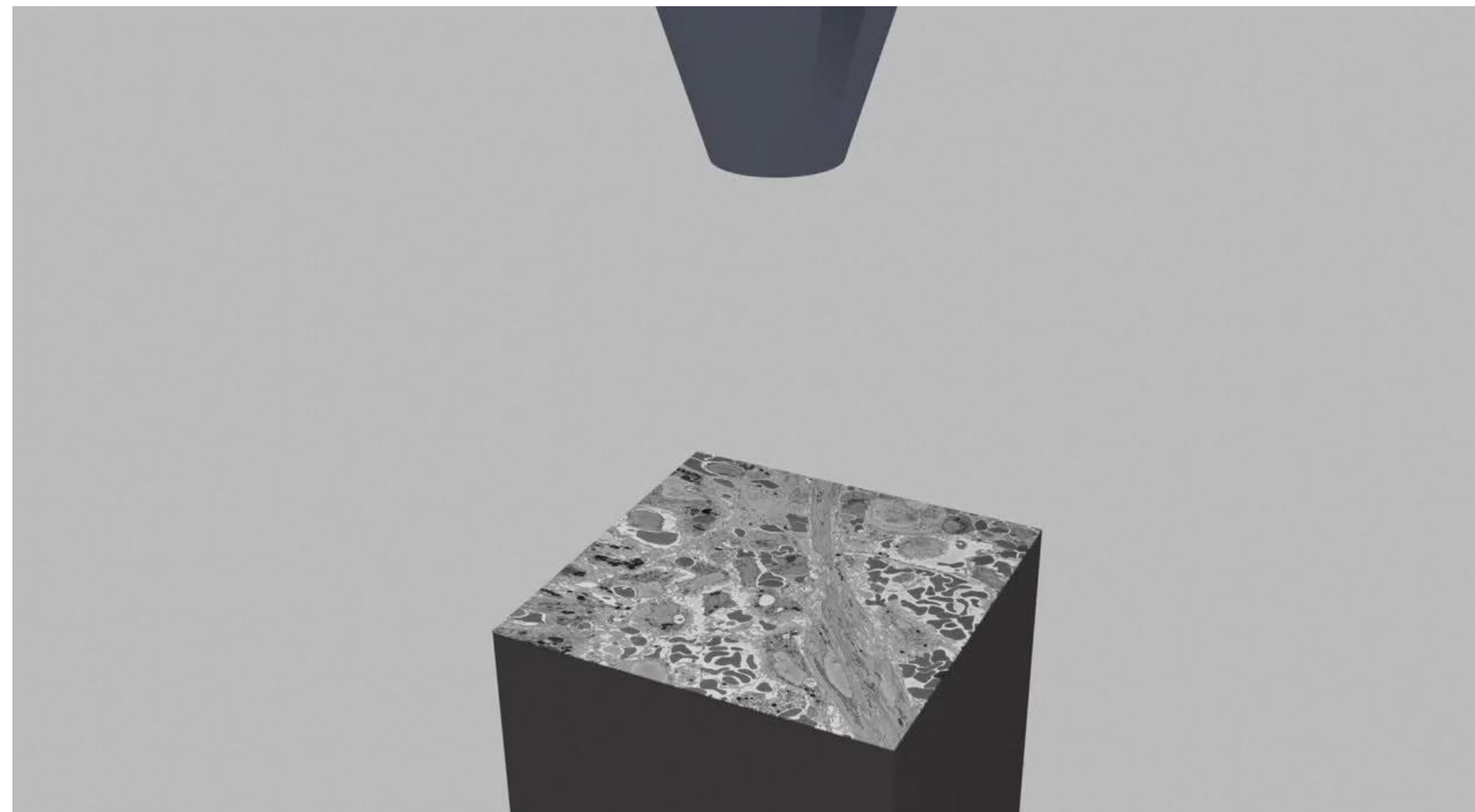
Transmission Electron Microscopy (TEM)



Scanning Electron Microscopy (SEM)

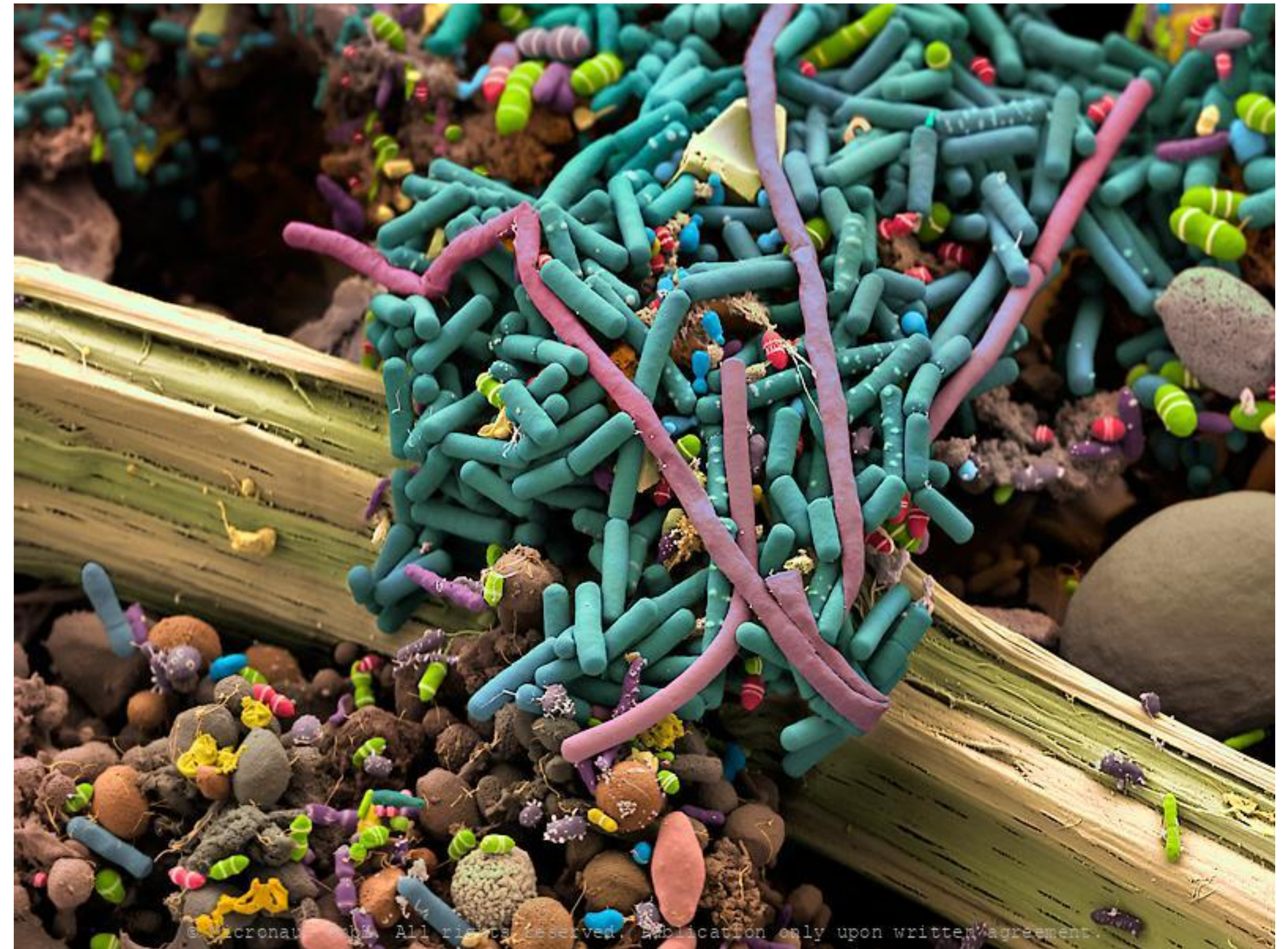
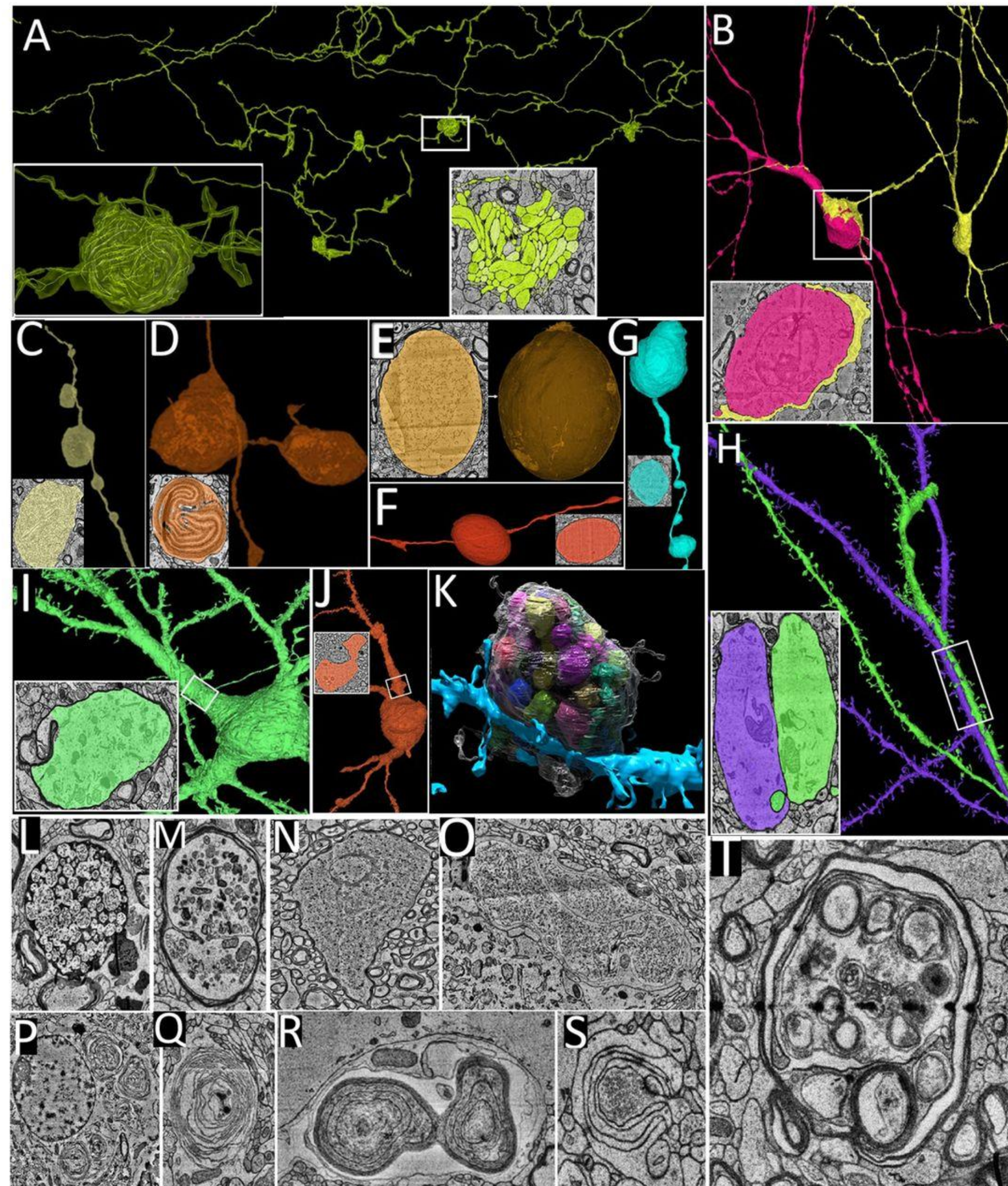


Serial Block-Face Scanning Electron Microscopy (SBF-SEM)



SBF-SEM vs SEM

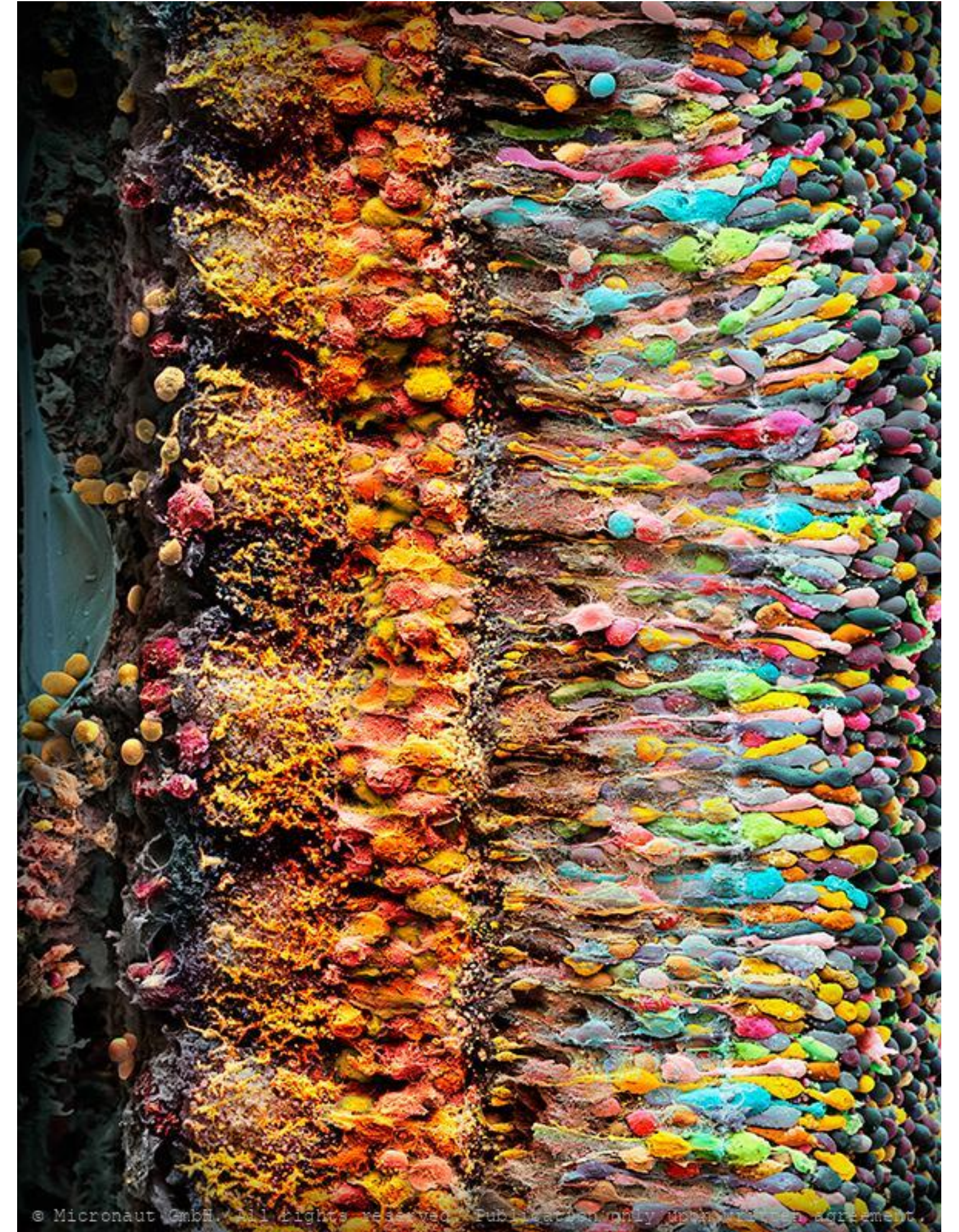
(SEM colors are from Photoshop)



<https://www.micronaut.ch>

SEM images

(false colors are from Photoshop)

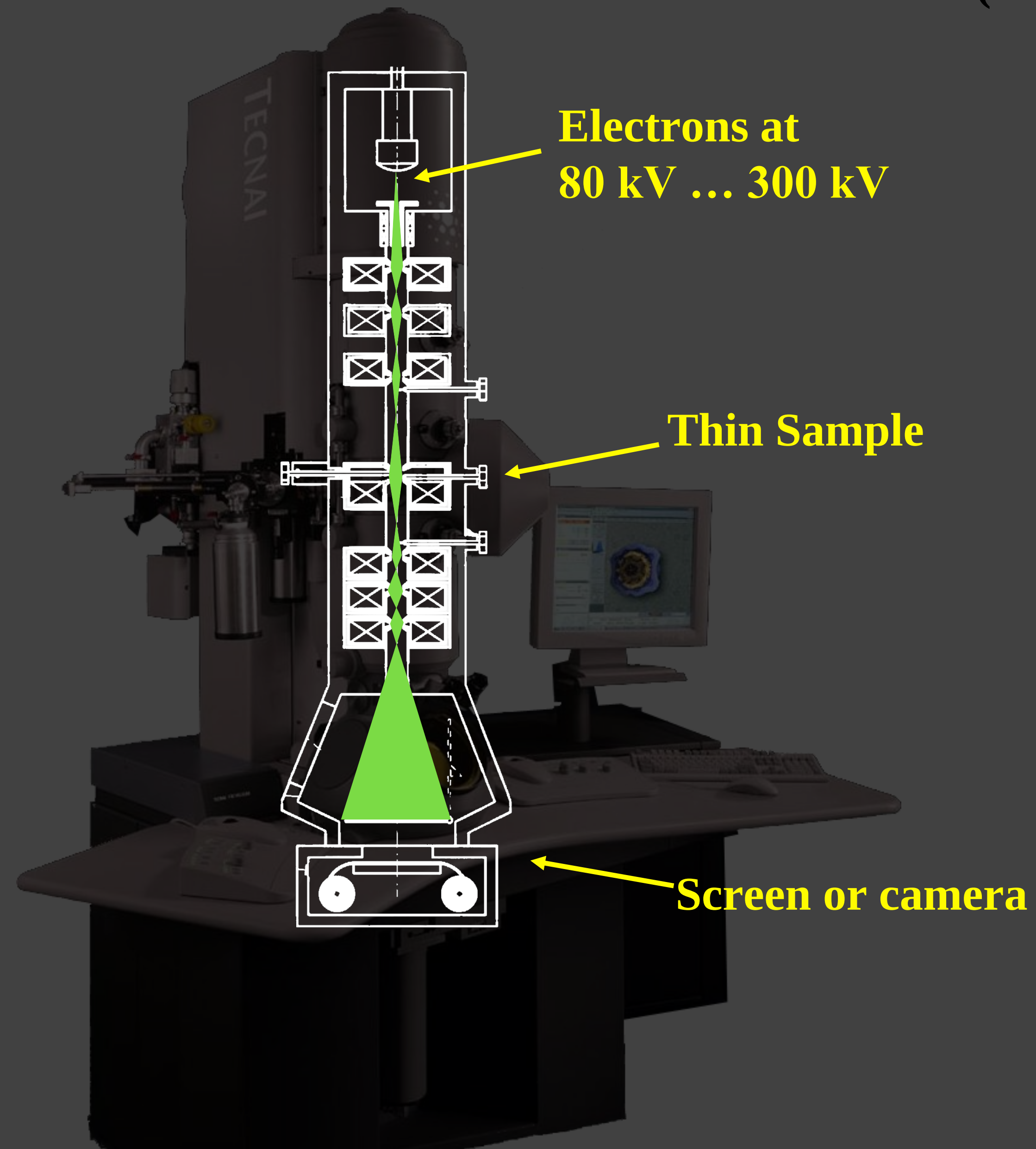


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Transmission Electron Microscopy (TEM)



Transmission Electron Microscopy (TEM)

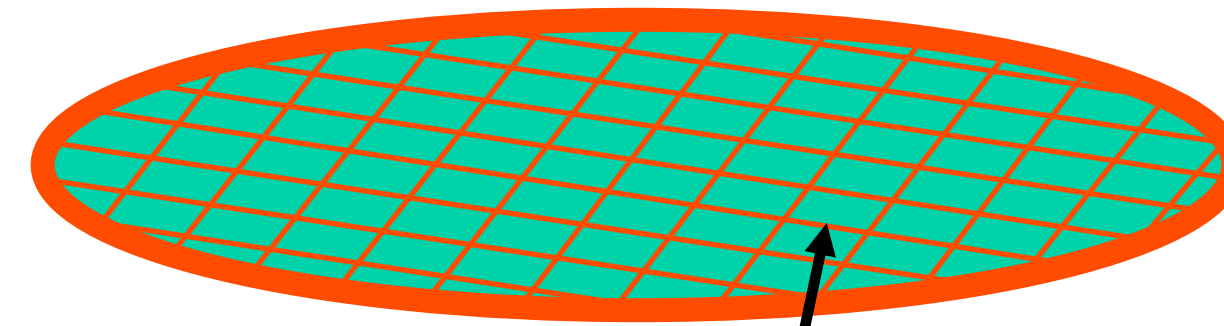
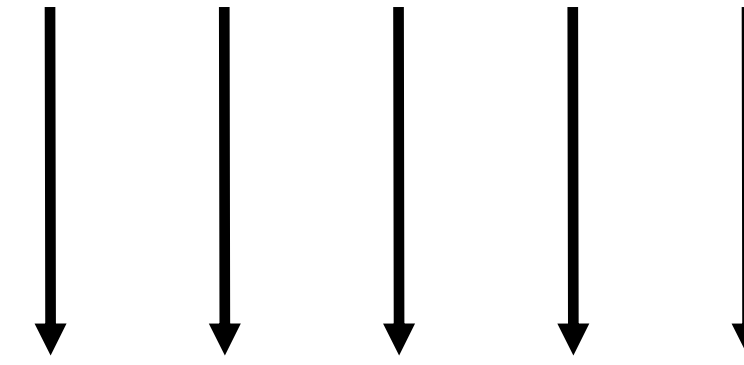


Transmission Electron Microscopy



Specimen Preparation

e^-



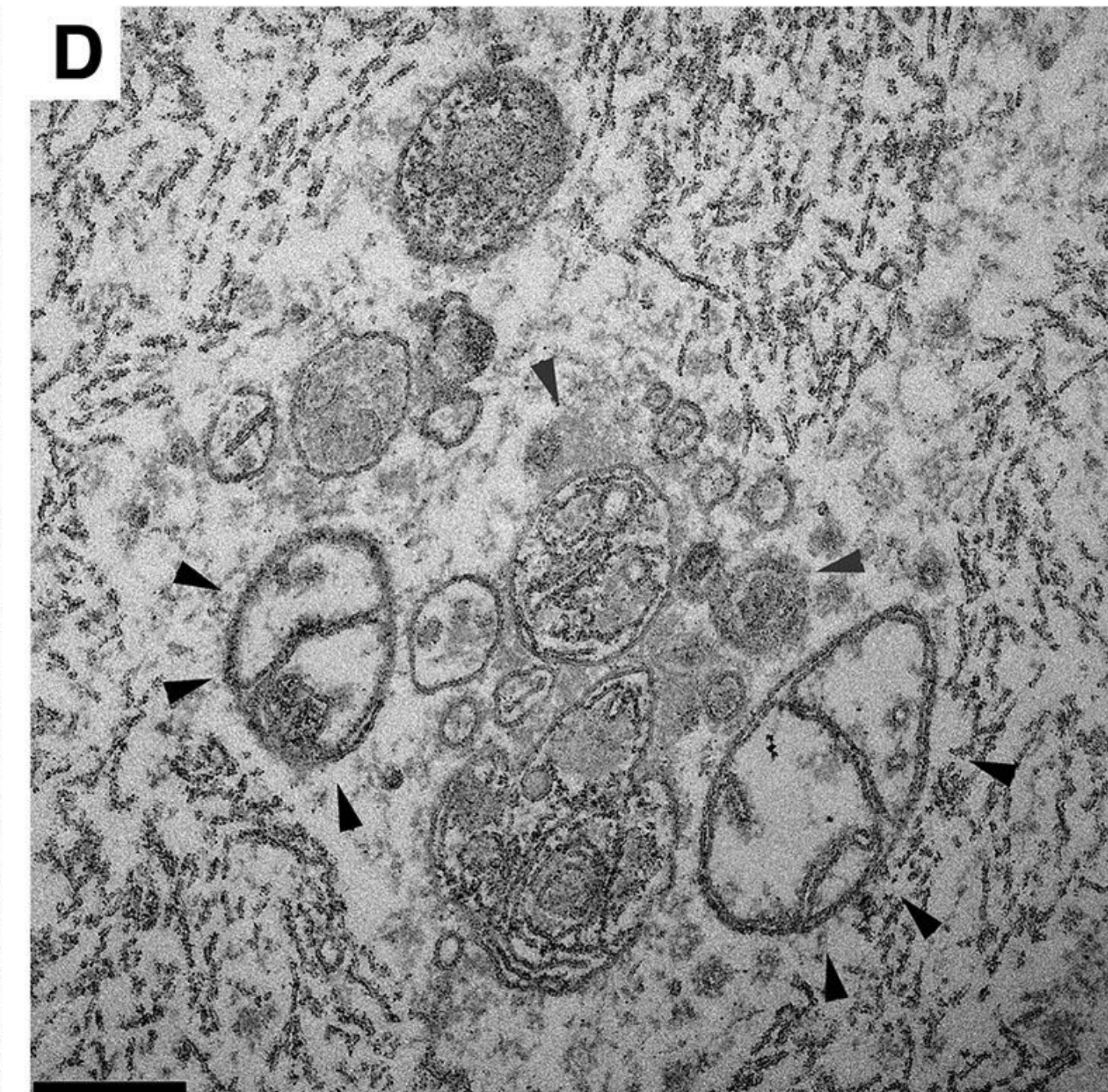
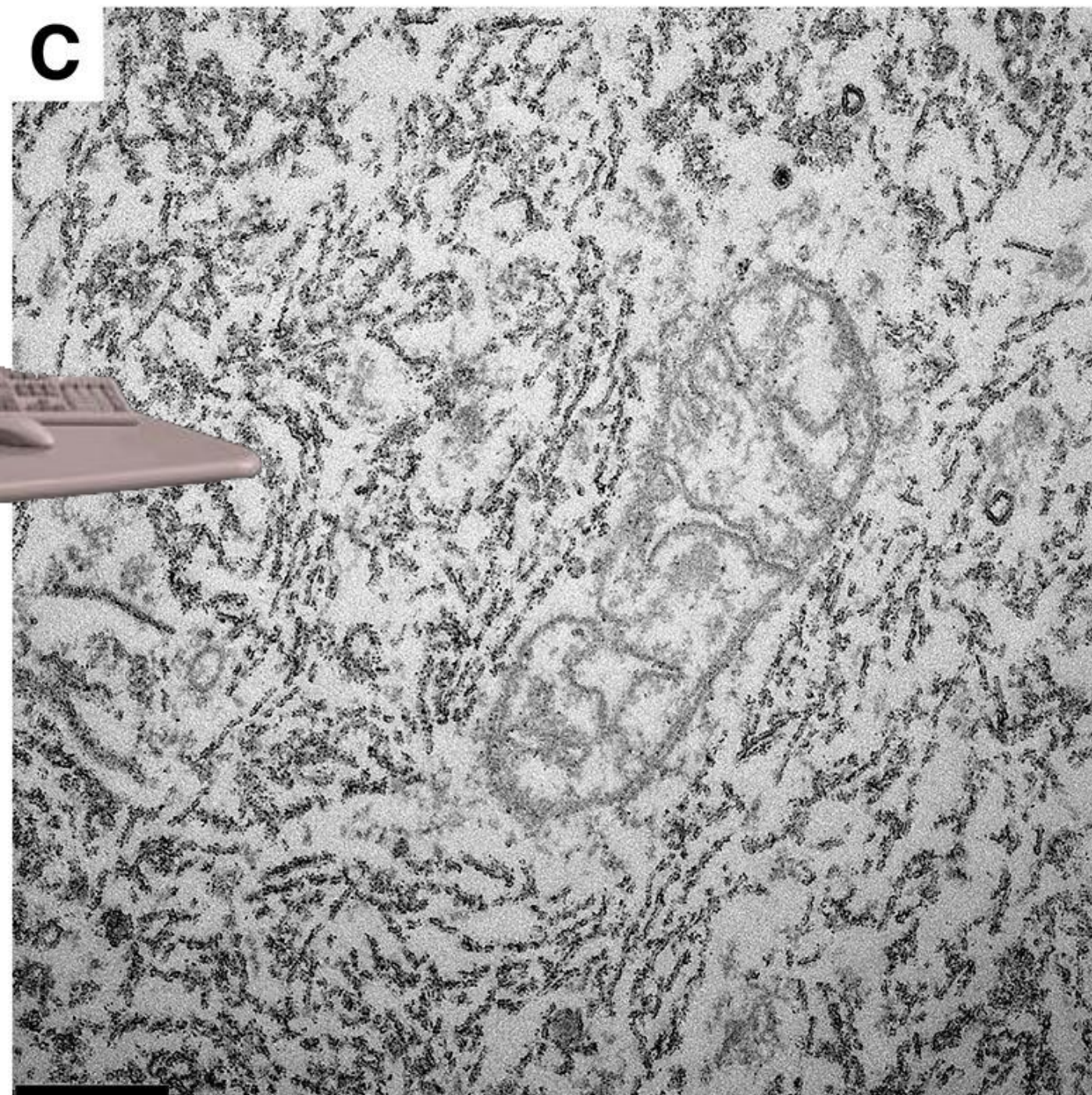
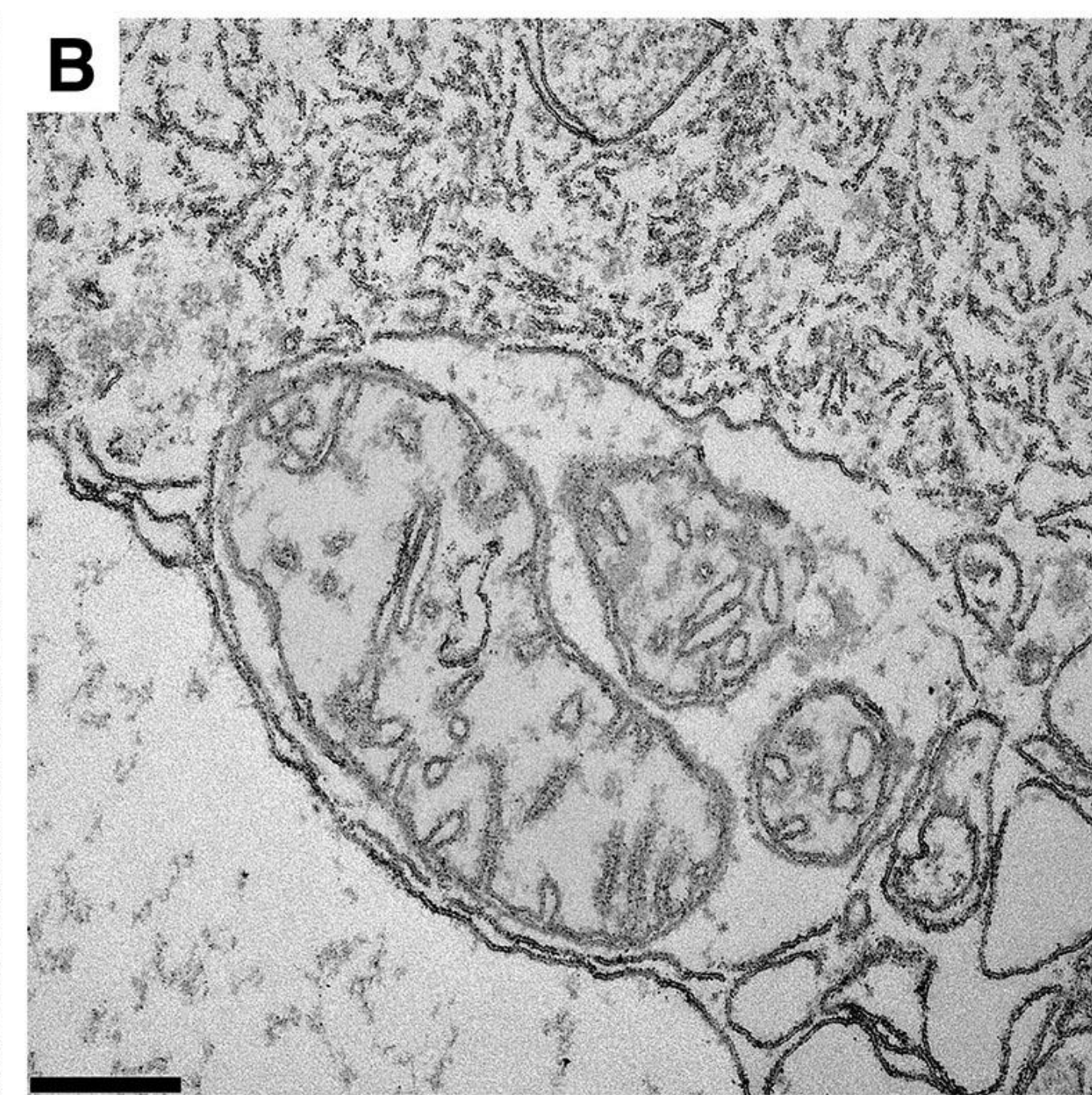
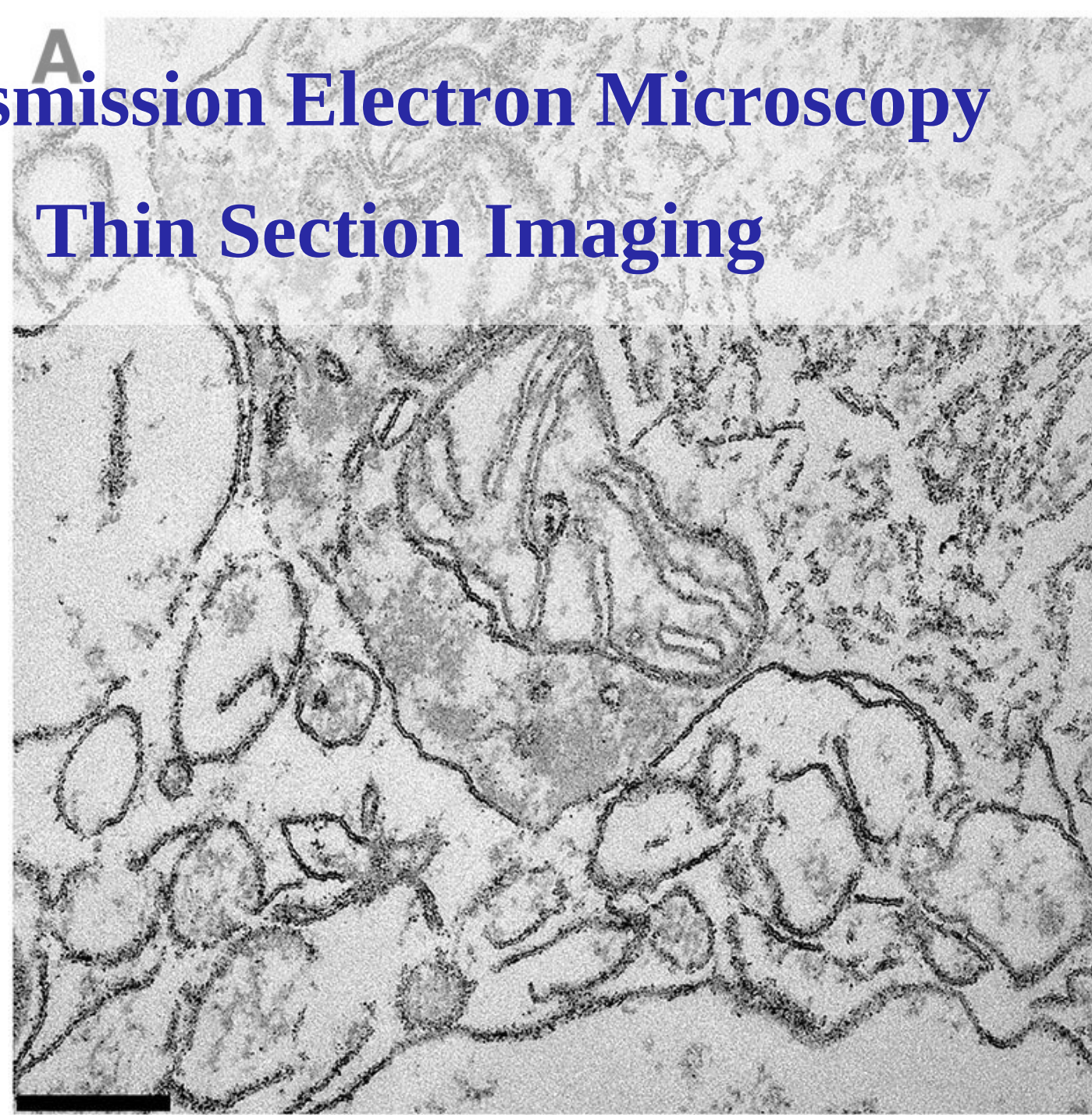
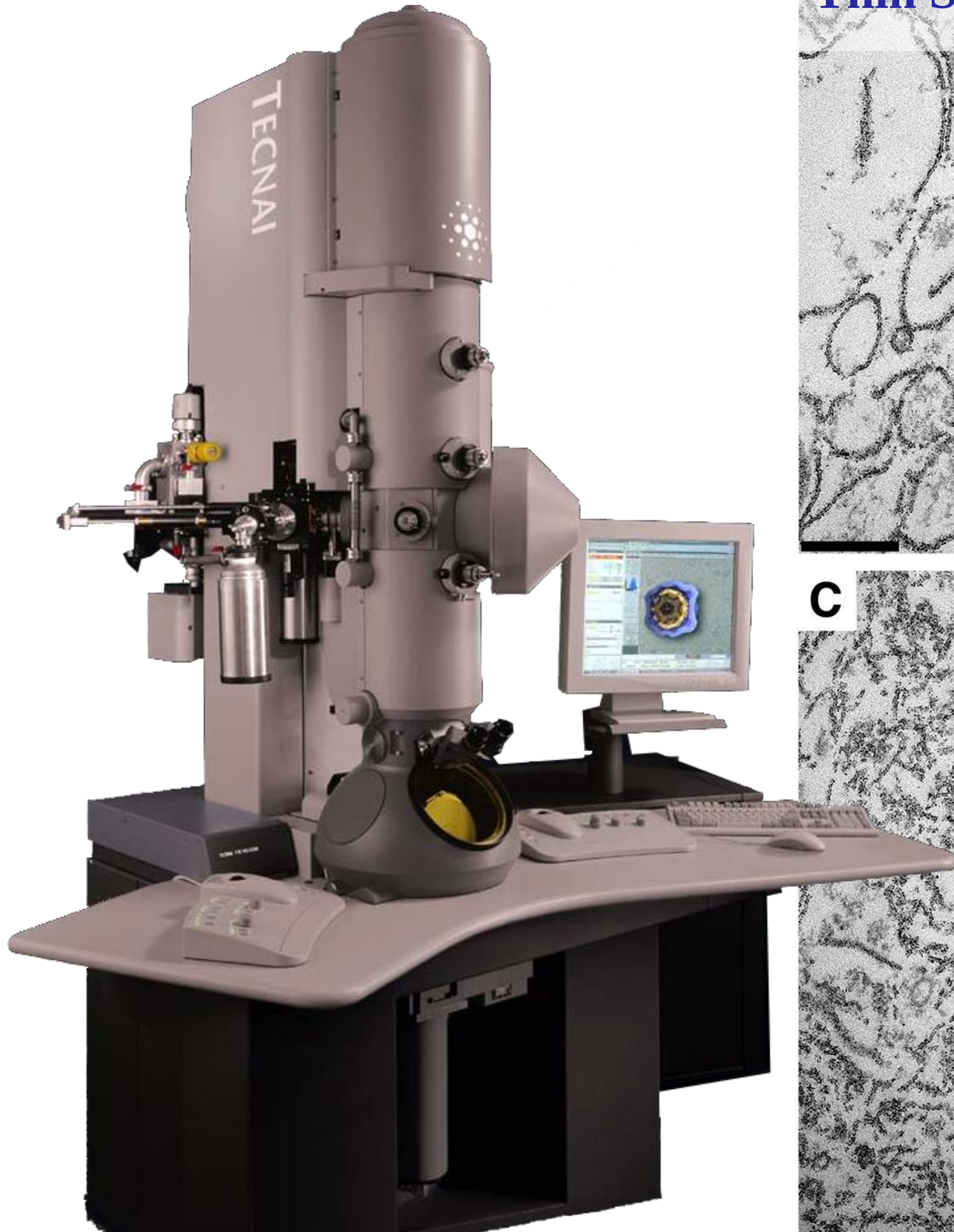
Microscopy Grid

Carbon-Film

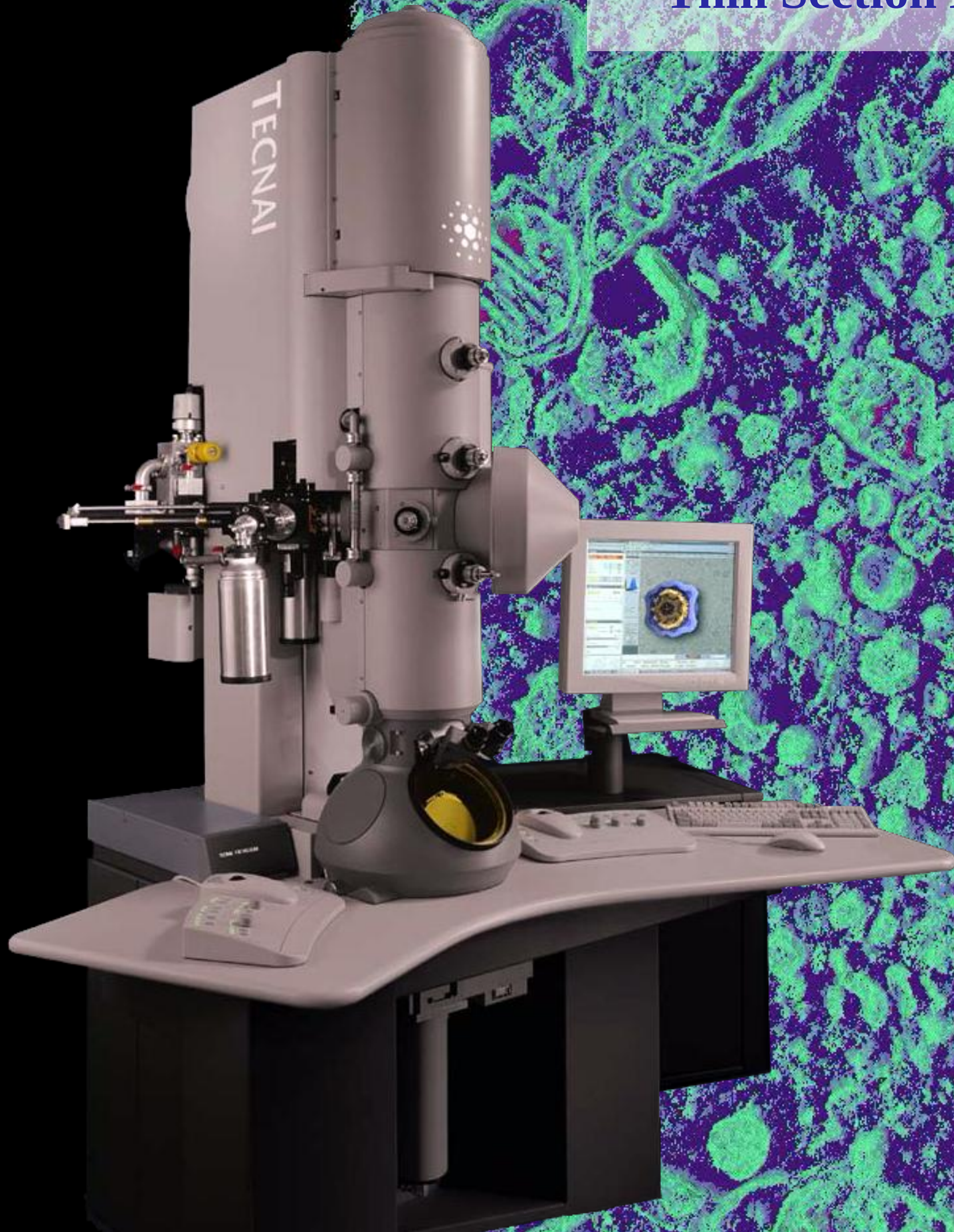
A

Transmission Electron Microscopy

Thin Section Imaging



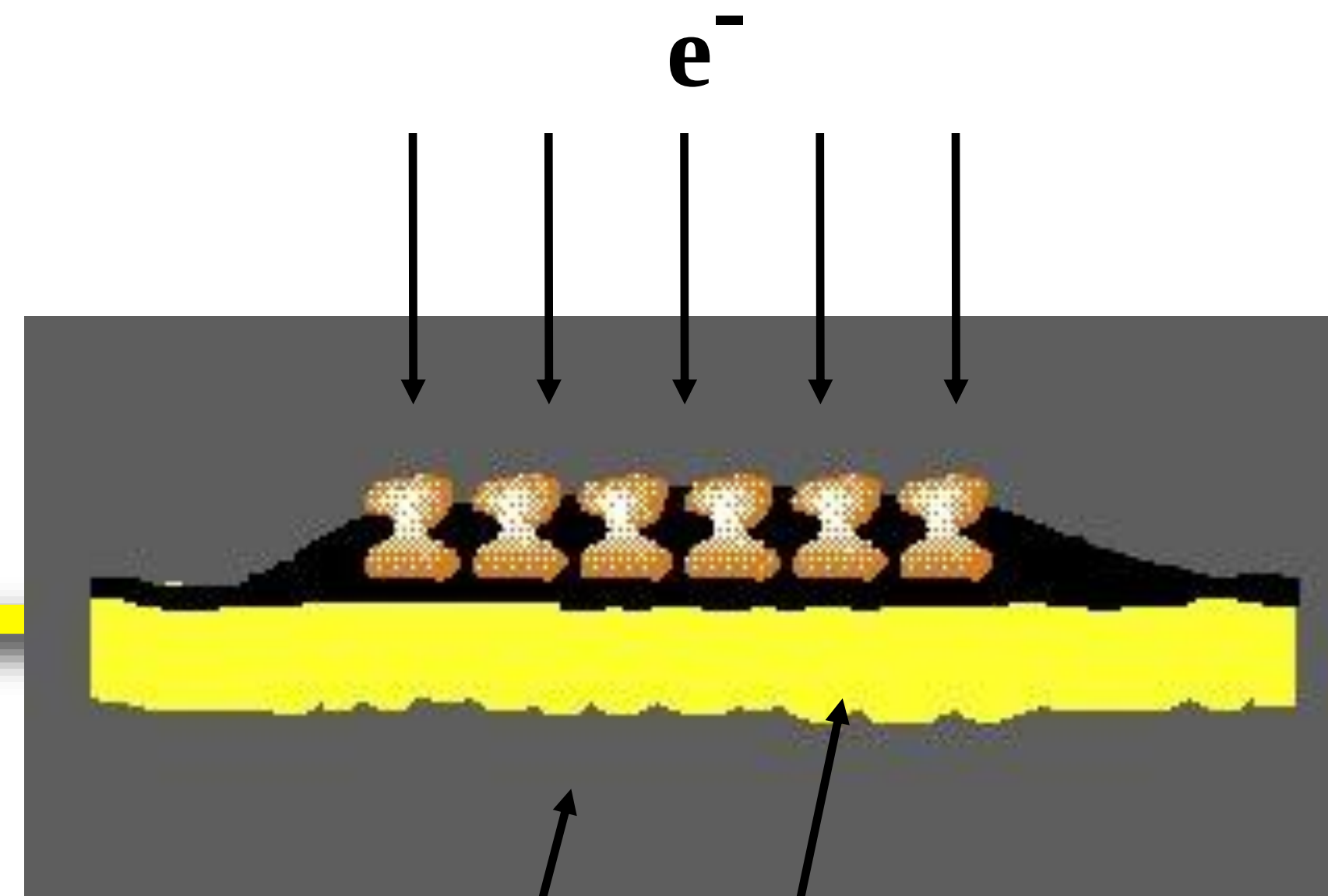
Transmission Electron Microscopy
Thin Section Electron Tomography



Transmission Electron Microscopy

Negative Stain TEM

Specimen Preparation



Microscopy Grid

Carbon-Film

Transmission Electron Microscopy

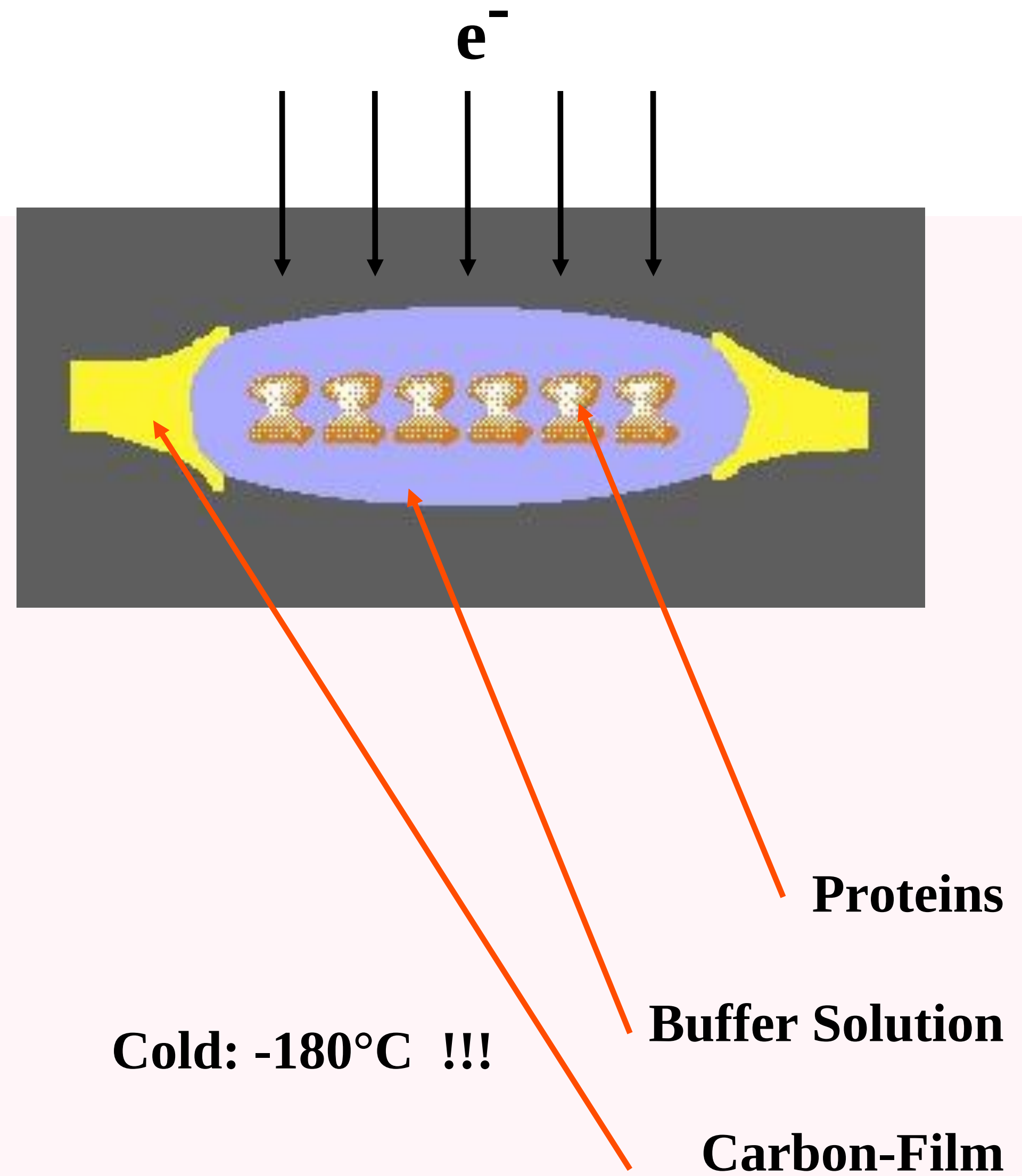
Negative Stain TEM



Transmission Electron Microscopy

Cryo-EM

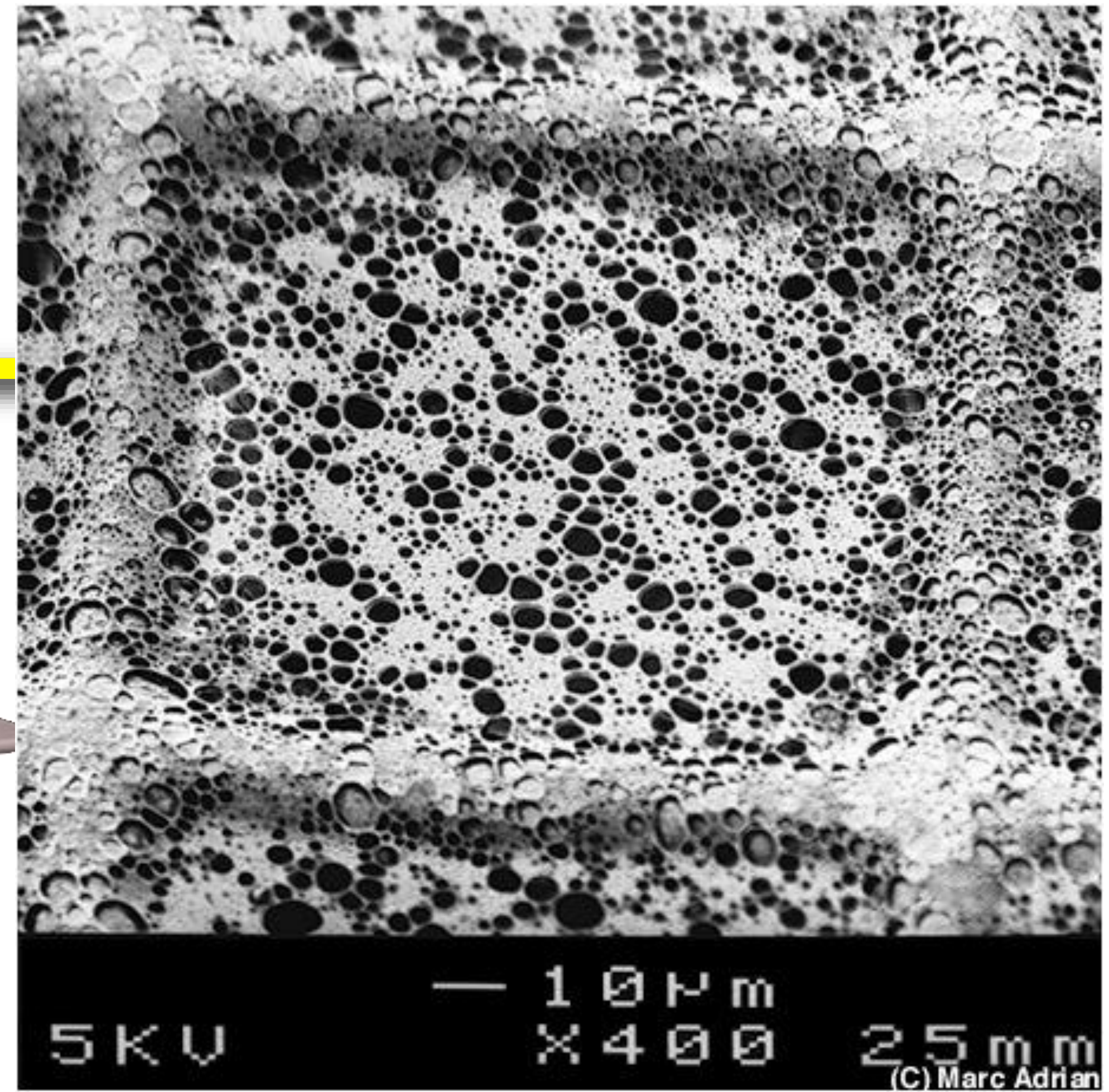
Specimen Preparation



Transmission Electron Microscopy

Cryo-EM

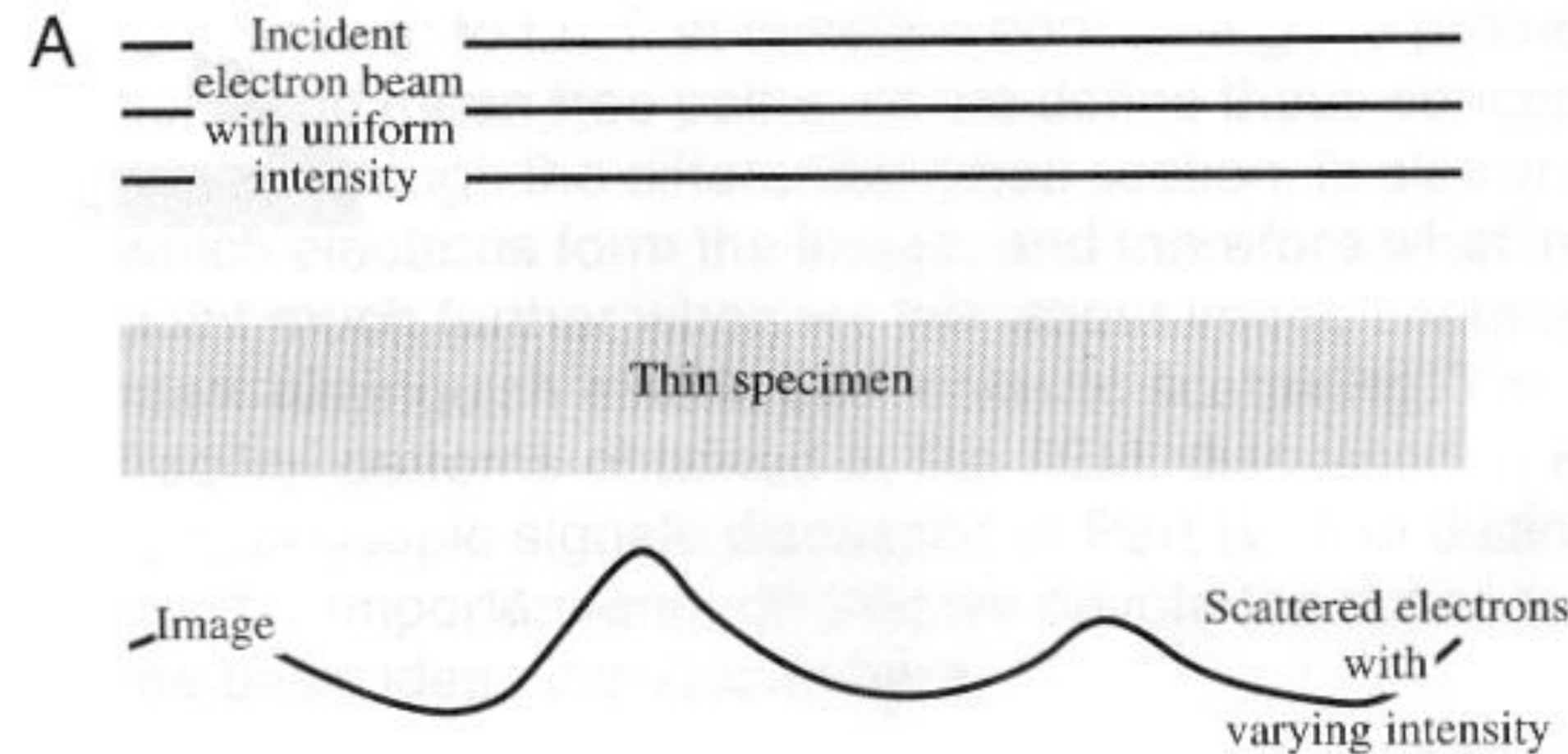
Holey Carbon Film



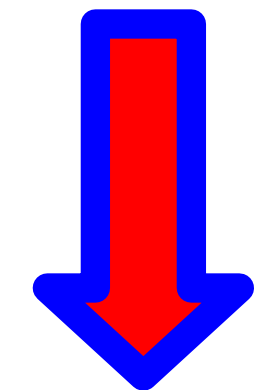
Electron beam / sample interaction

What is an electron beam?

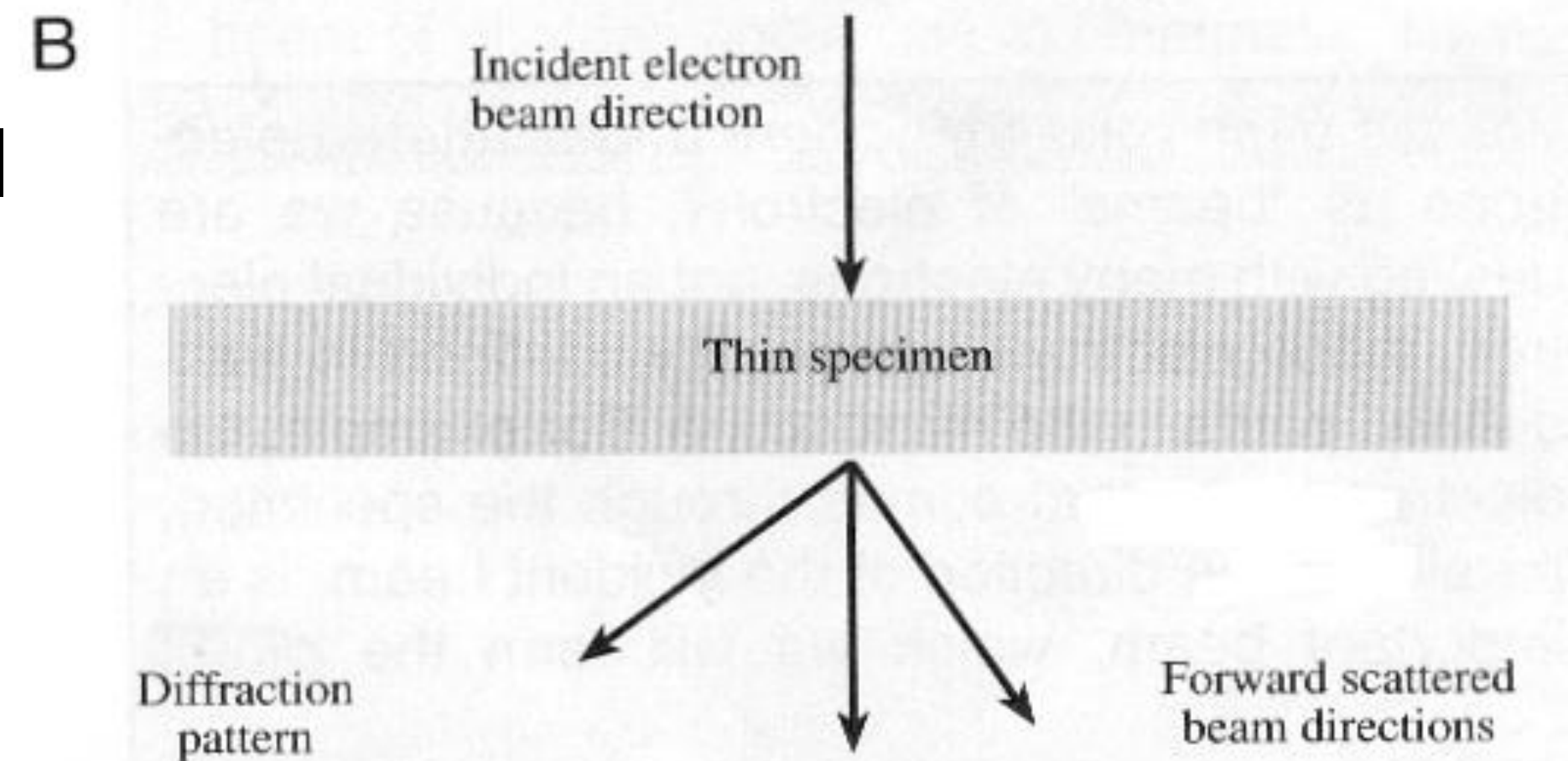
Wave Model



Both angular and spatial distribution to scattering

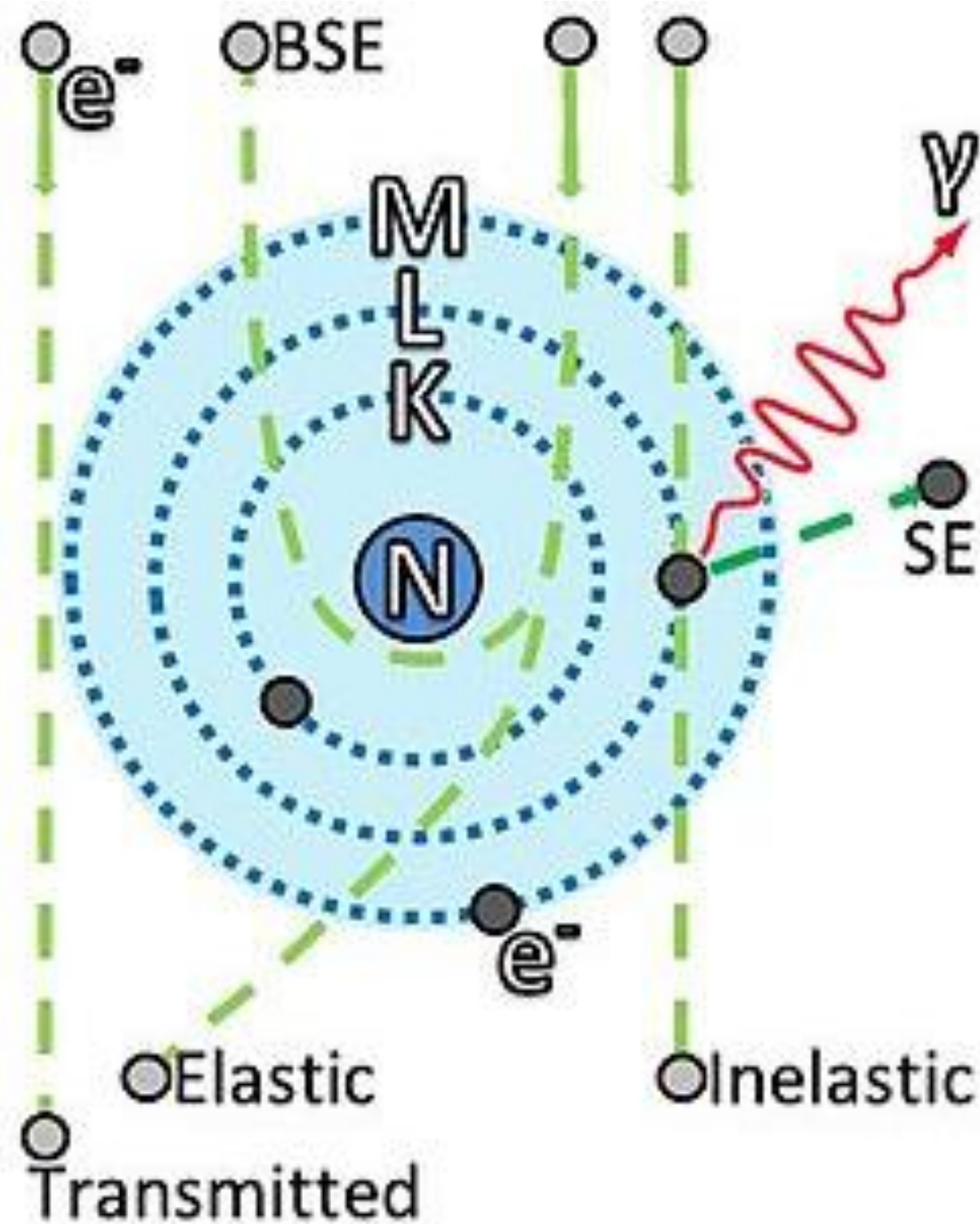


Particle Model



Diffraction patterns and images

Interaction Electron / Specimen Atom



- Beam Electron
- Atomic Shell Electron
- Electron Cloud
- Beam Electron Path
- Secondary Electron Path
- Characteristic X-Ray

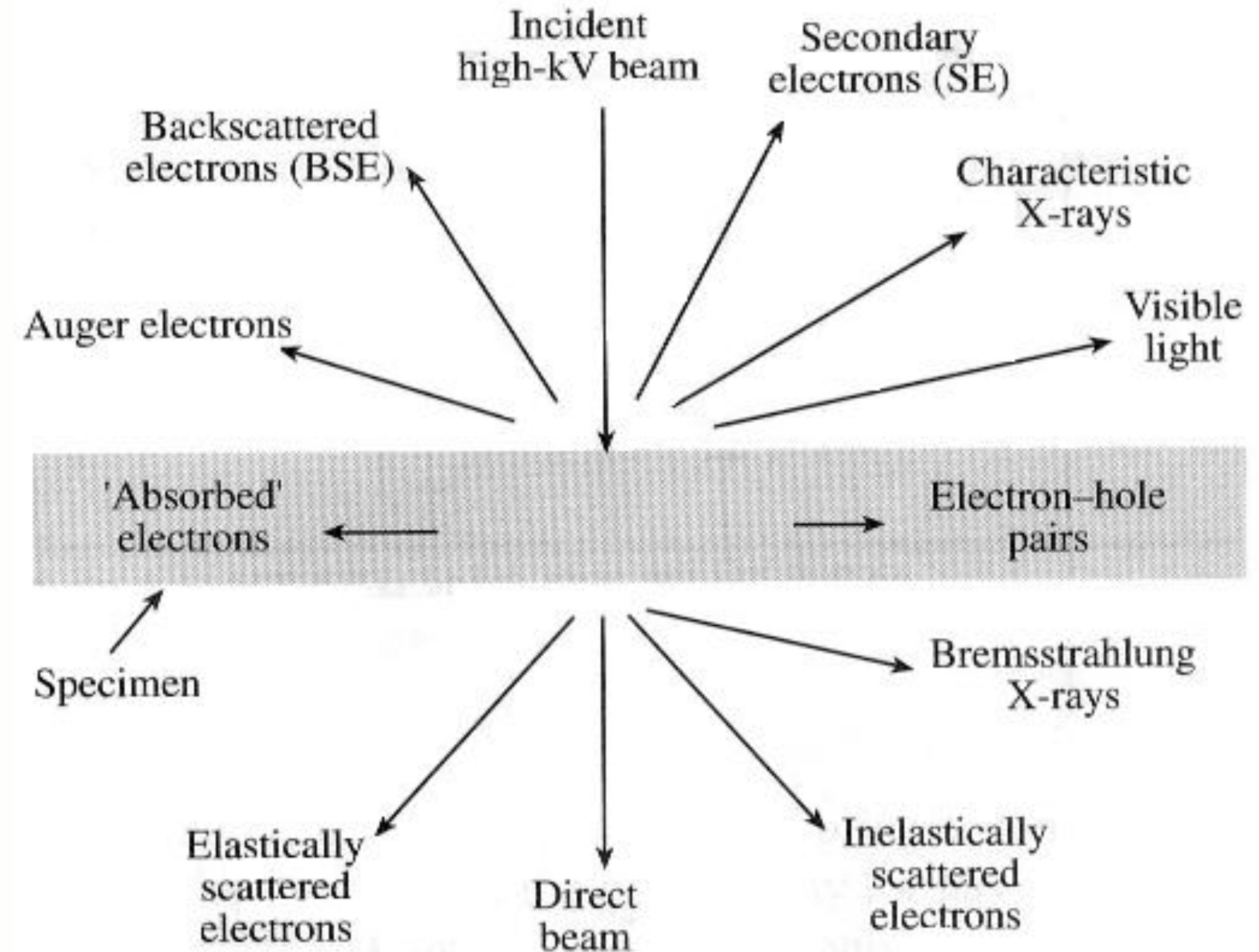
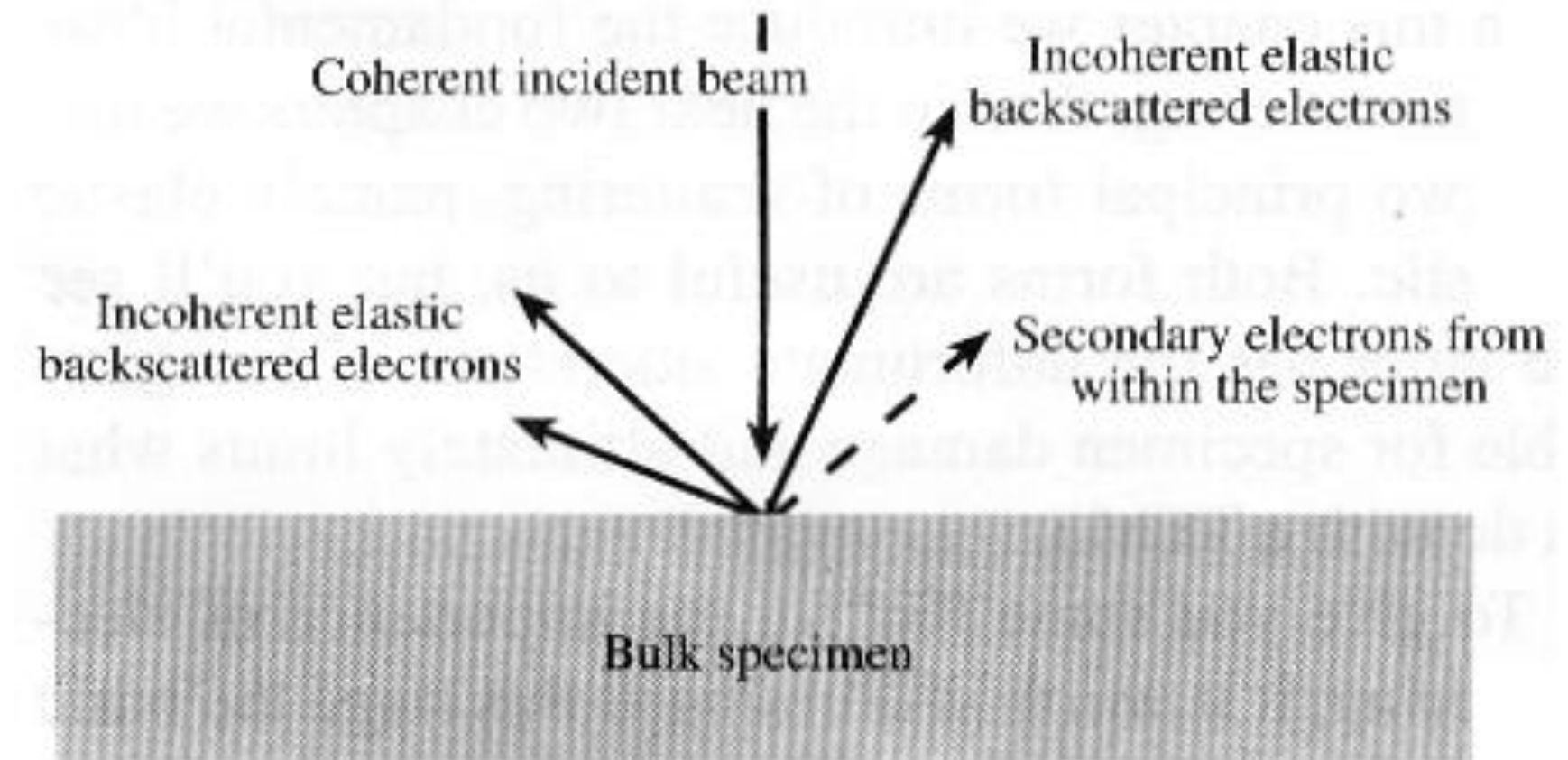
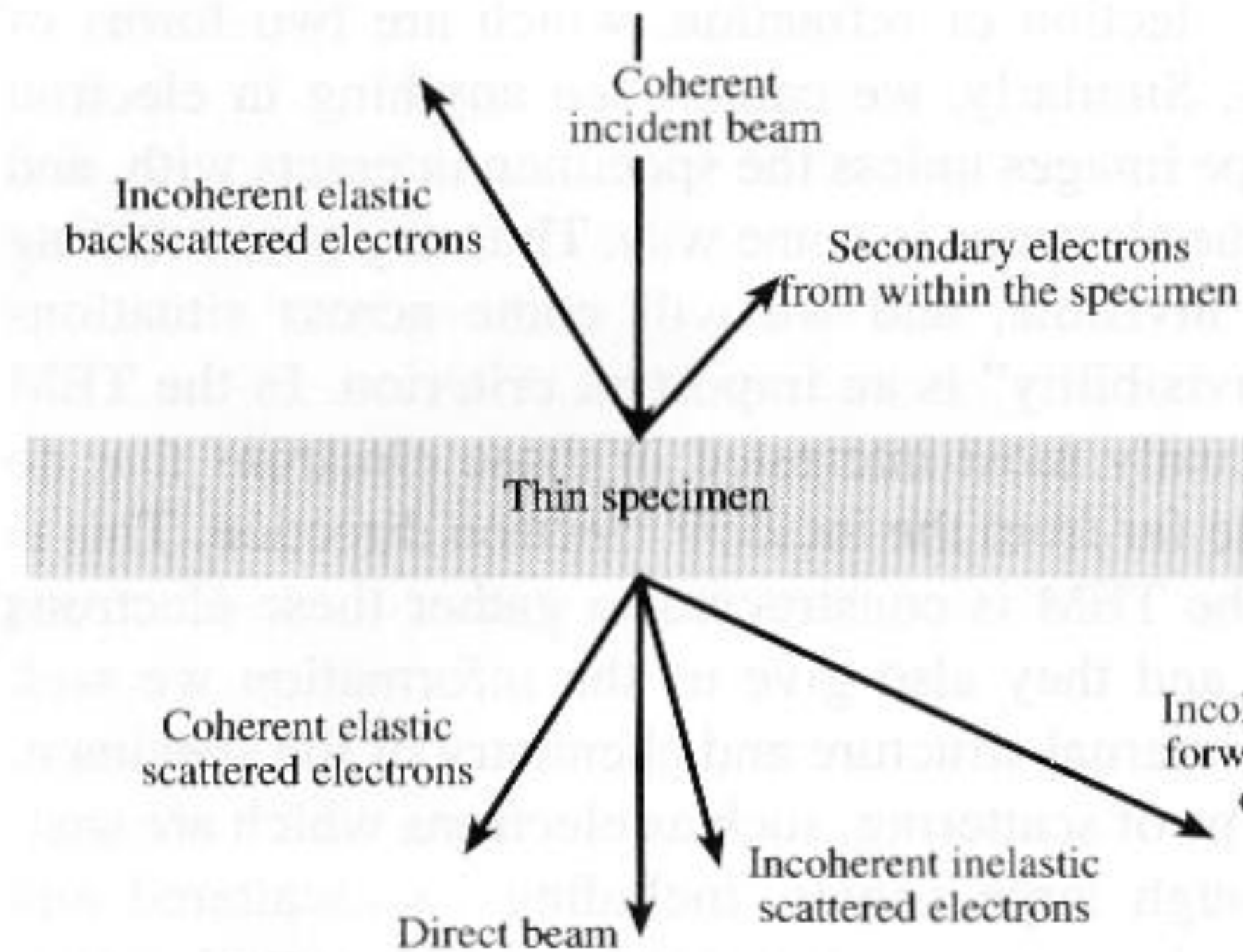


Figure 1.3. Signals generated when a high-energy beam of electrons interacts with a thin specimen. Most of these signals can be detected in different types of TEM. The directions shown for each signal do not always represent the physical direction of the signal but indicate, in a relative manner, where the signal is strongest or where it is detected.

Scattering Terminology



	Coherent (same phase)	Incoherent (phase is random)
Elastic (same energy)		
Inelastic (lost energy)		

Interaction Cross-Section

If I throw a ball at a glass window of 1 m² area,
there may be a chance of one in ten that the
window will break and nine in ten that the ball will just bounce.

In physics language this means that for this ball,
the window has a
disintegration cross-section of 0.1 square meters,
and an elastic cross-section of 0.9 square meters.

For an electron in a transmission electron microscope (TEM),
the typical interaction cross sections for a sample atom is
 $\sim 10^{-22} - 10^{-26} \text{ m}^2$

Scattering of proteins

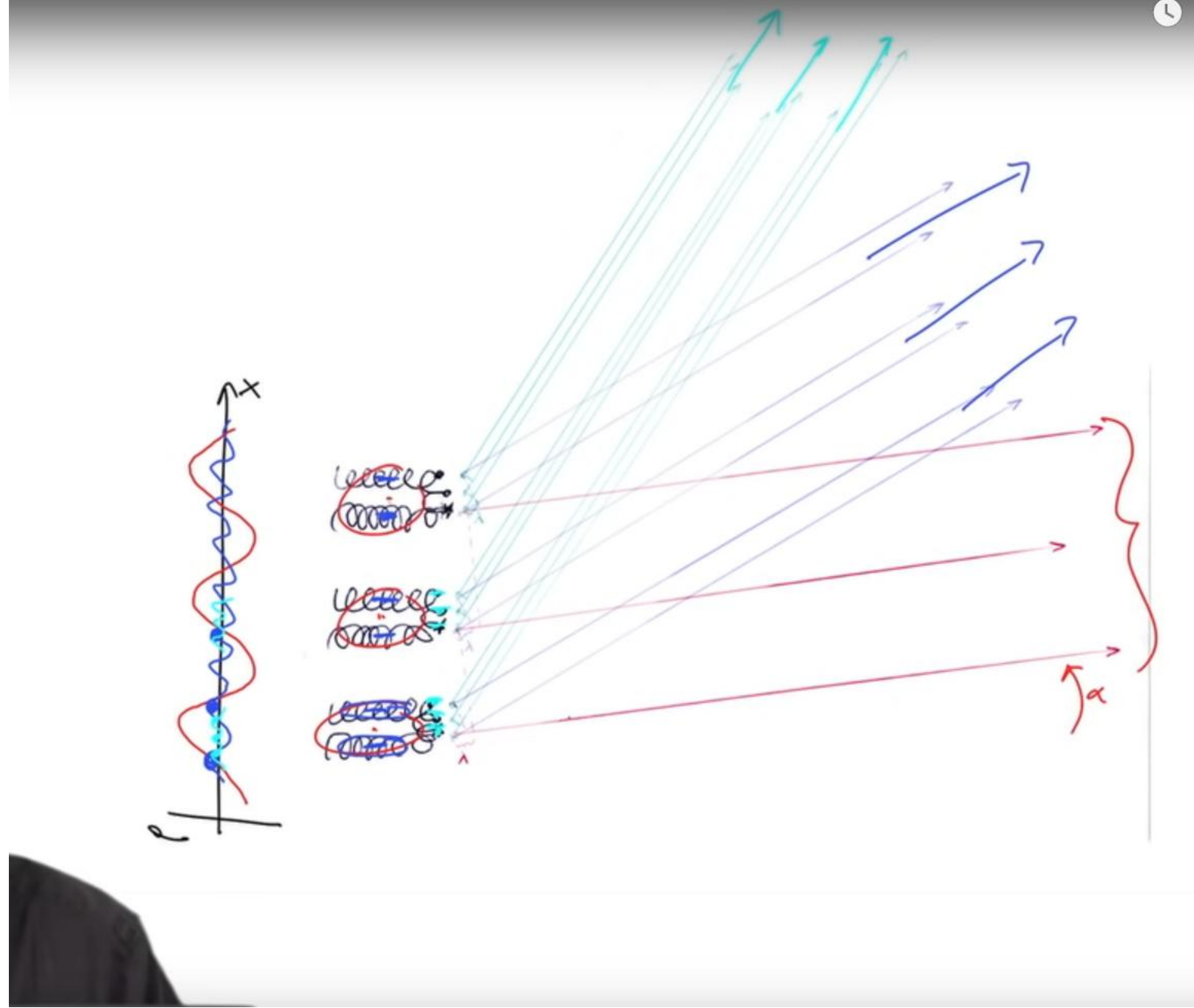


Image formation

<https://cryo-em-course.caltech.edu/overview>

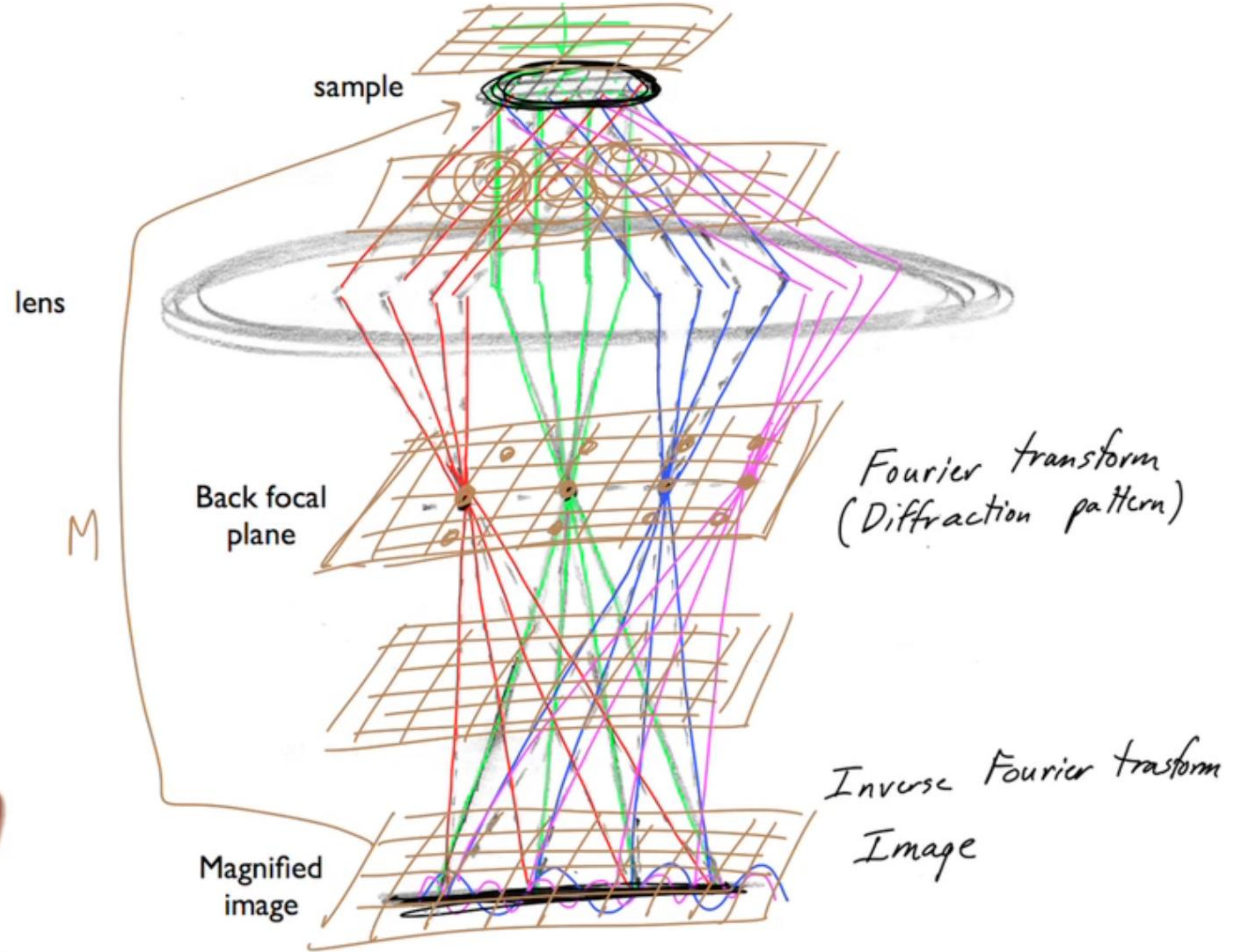
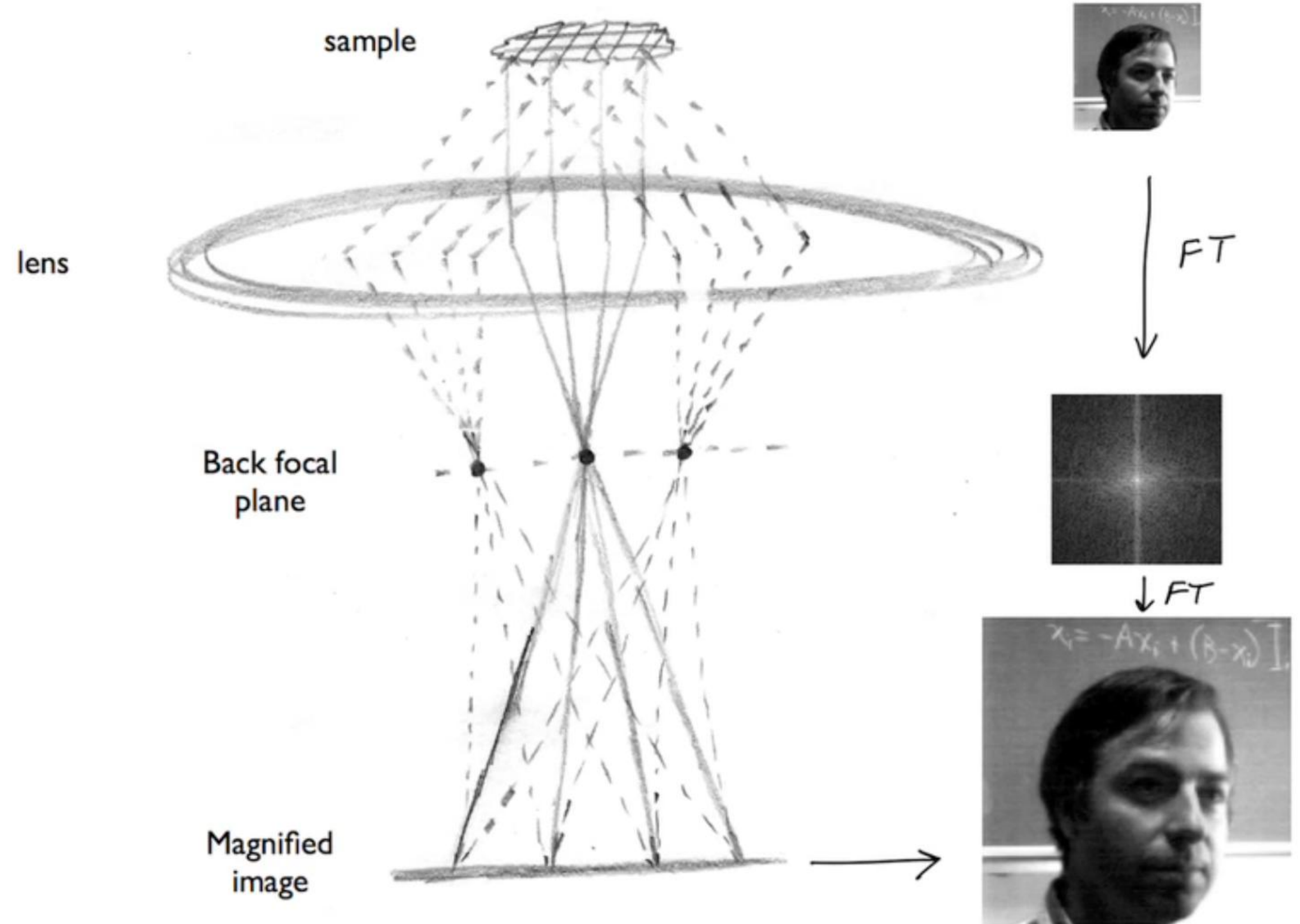
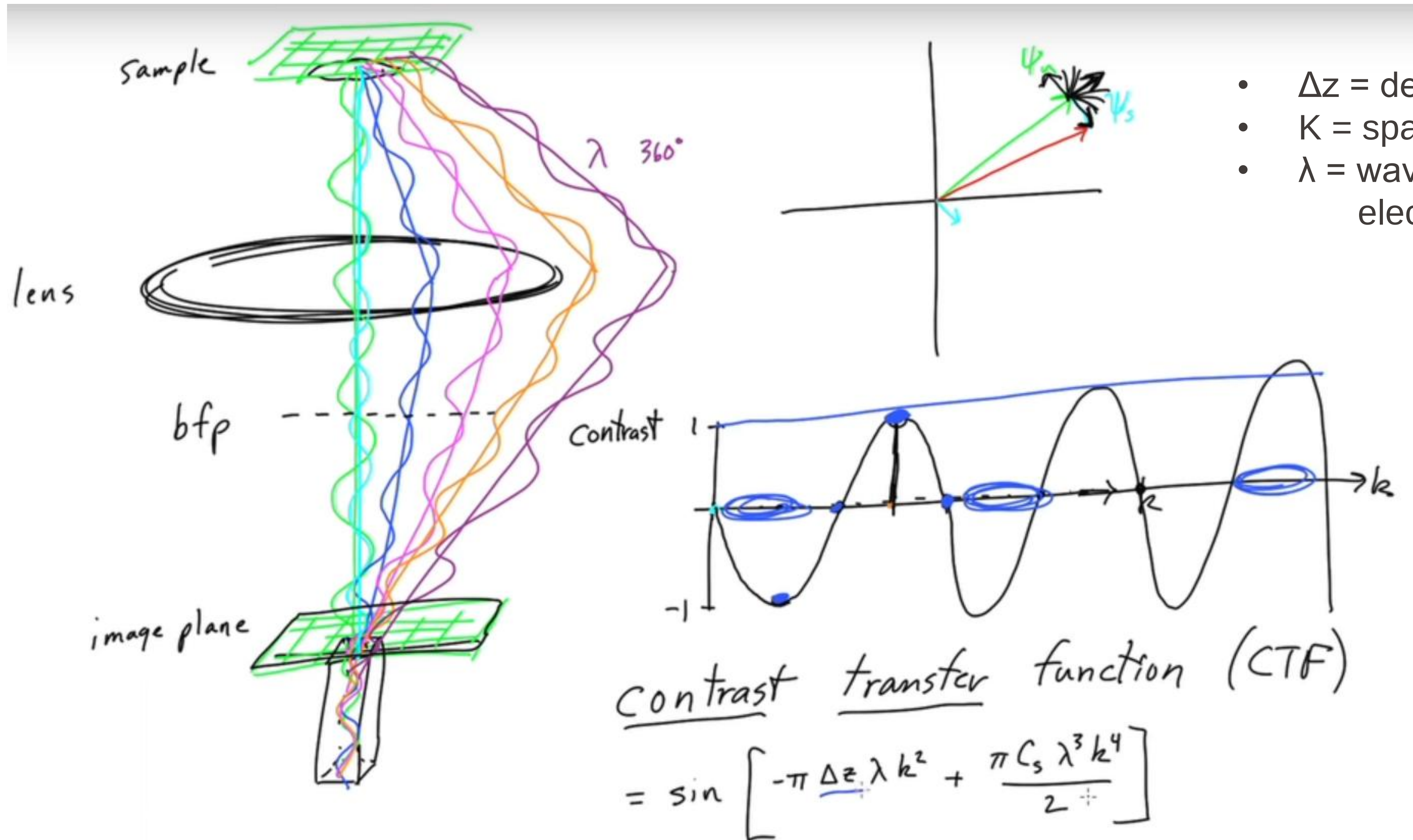


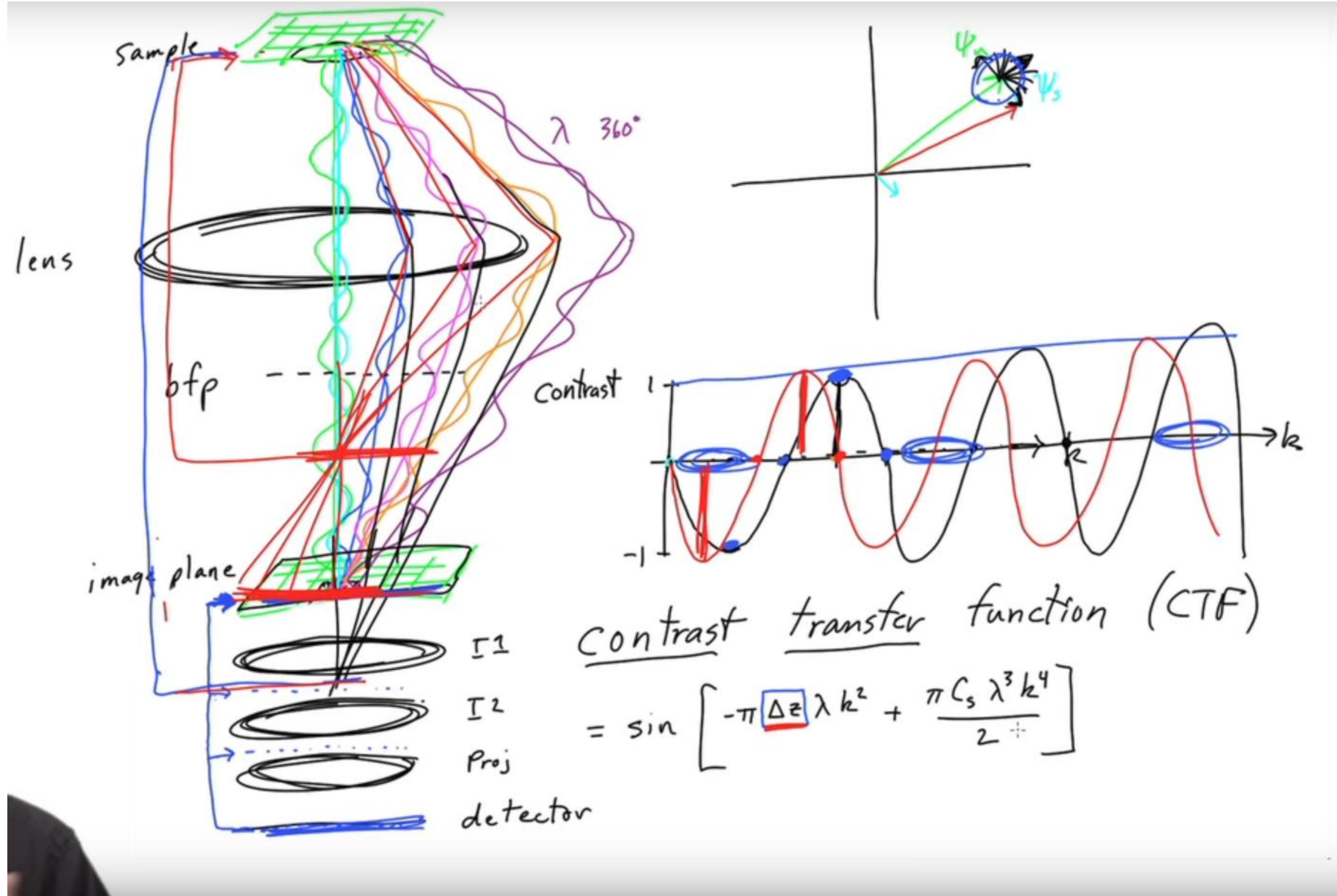
Image formation





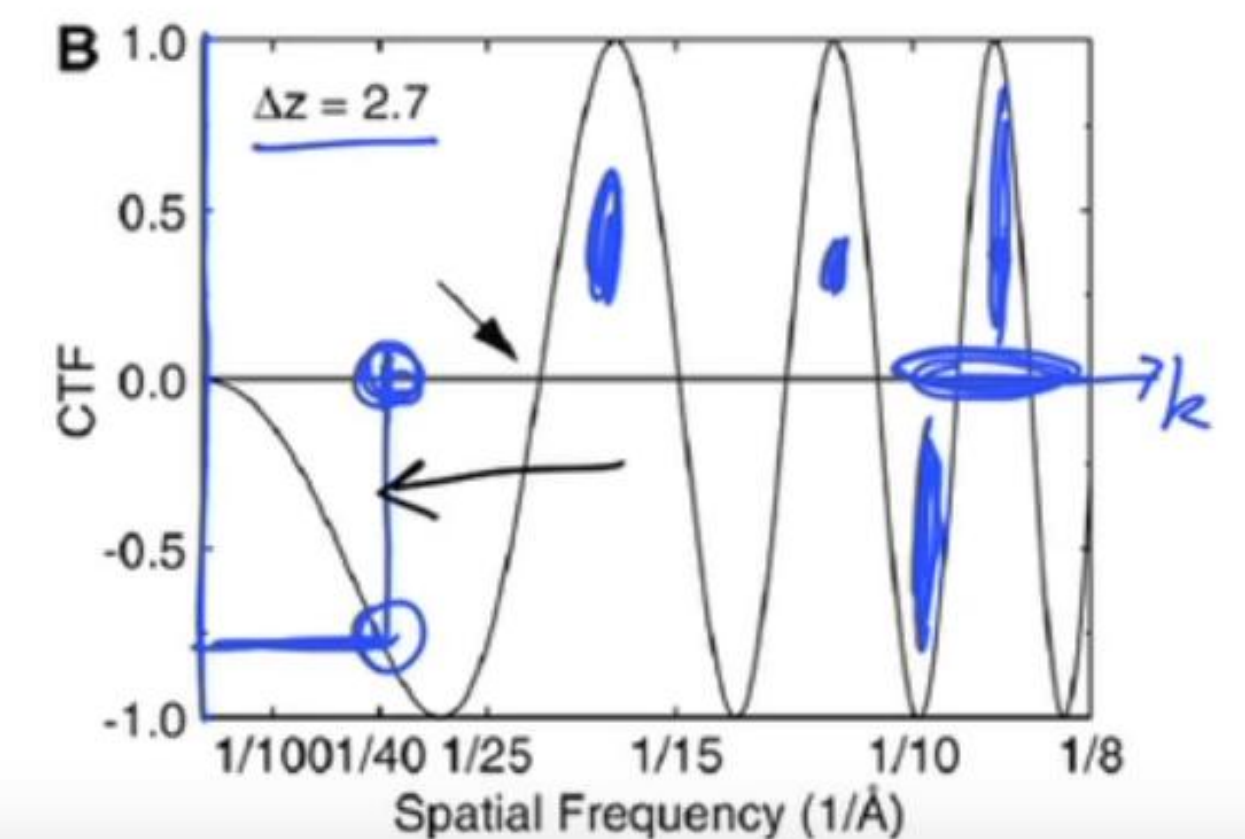
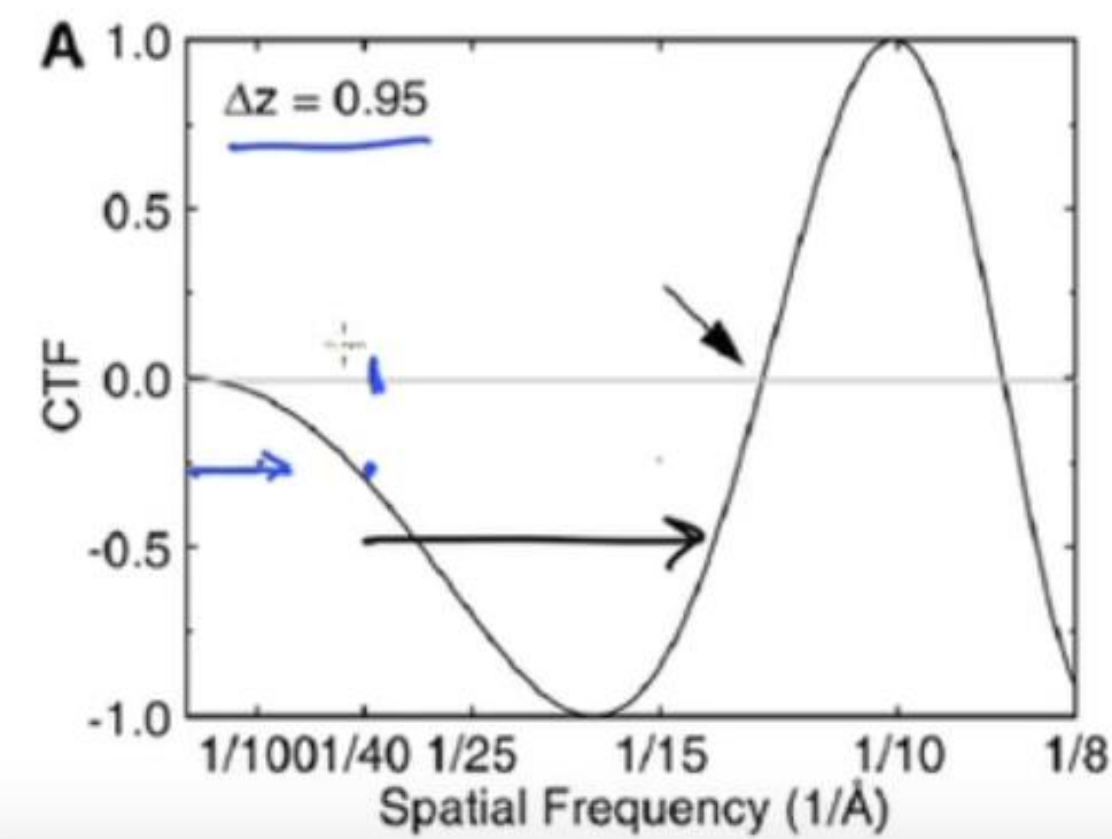
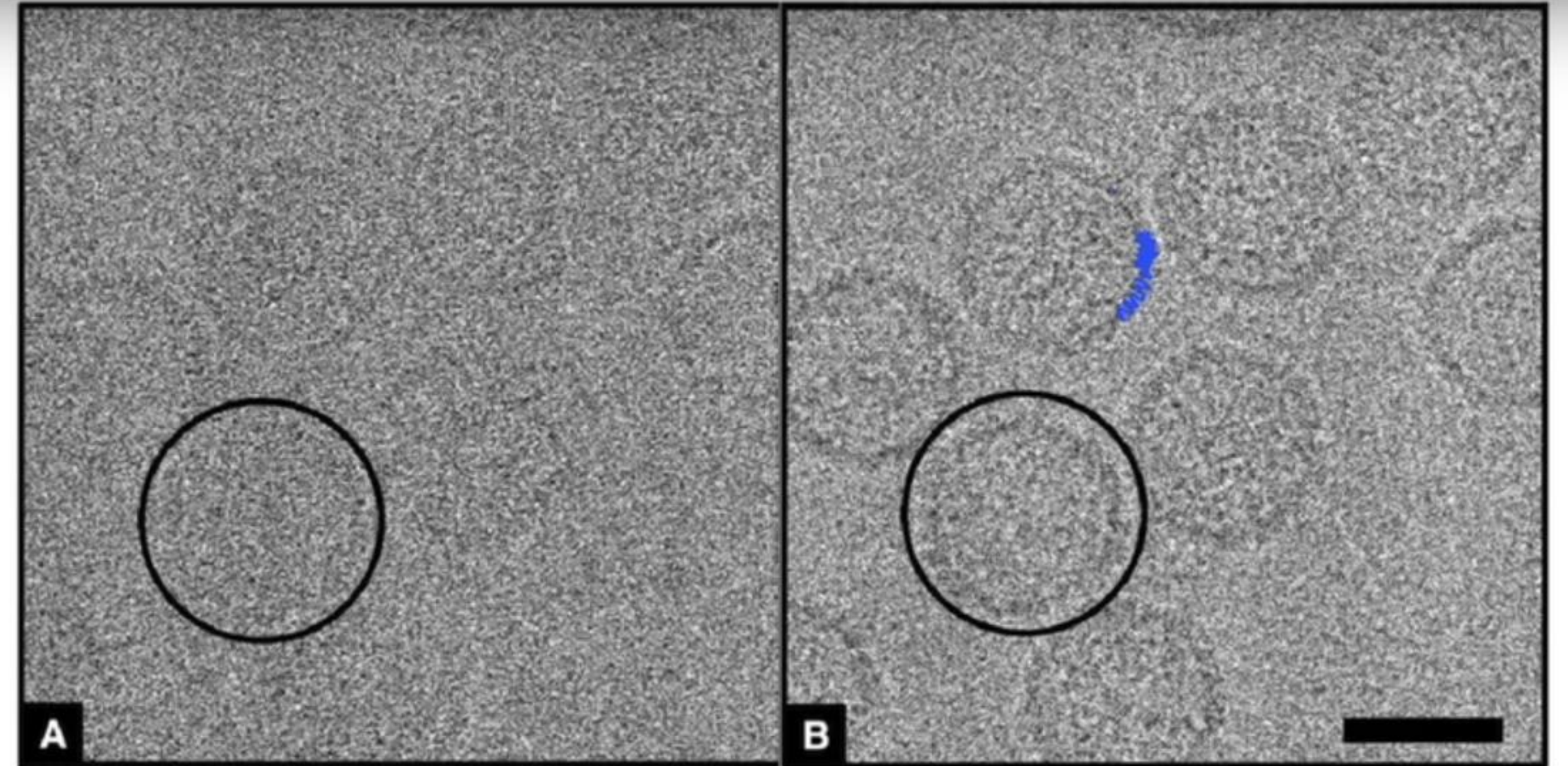
Defocusing

<https://cryo-em-course.caltech.edu/overview>



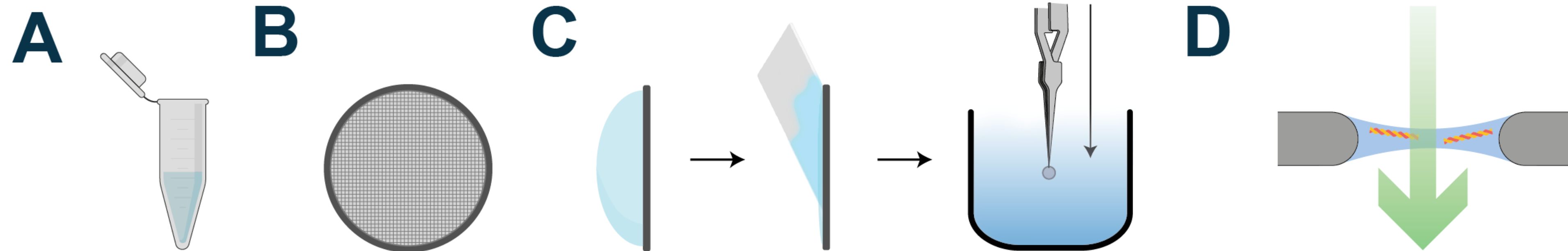
Defocusing and CTF

Part 3: Defocus and Its Effects - G. Jensen



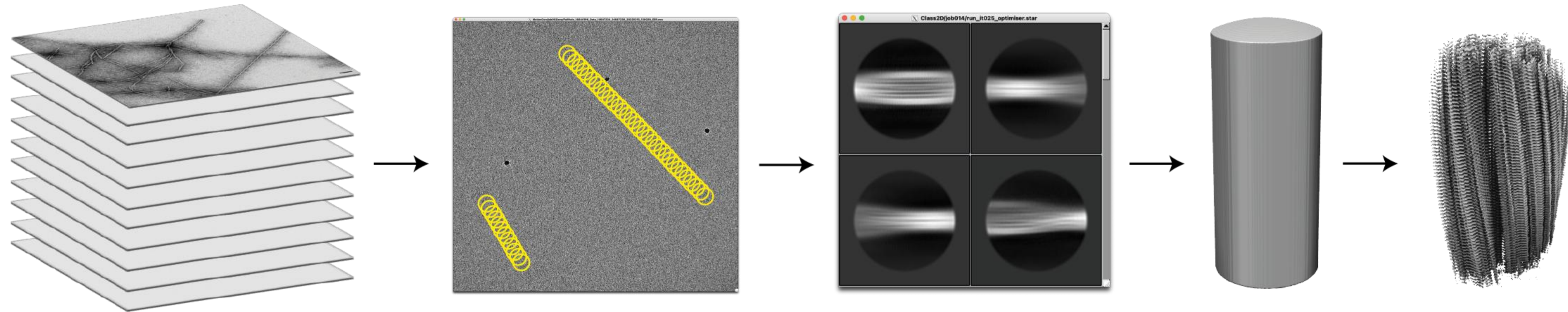
Cryo-Electron Microscopy

- Pure sample imaging
- «Close-to» native state
- No need for heavy metals
- Low contrast but high resolution



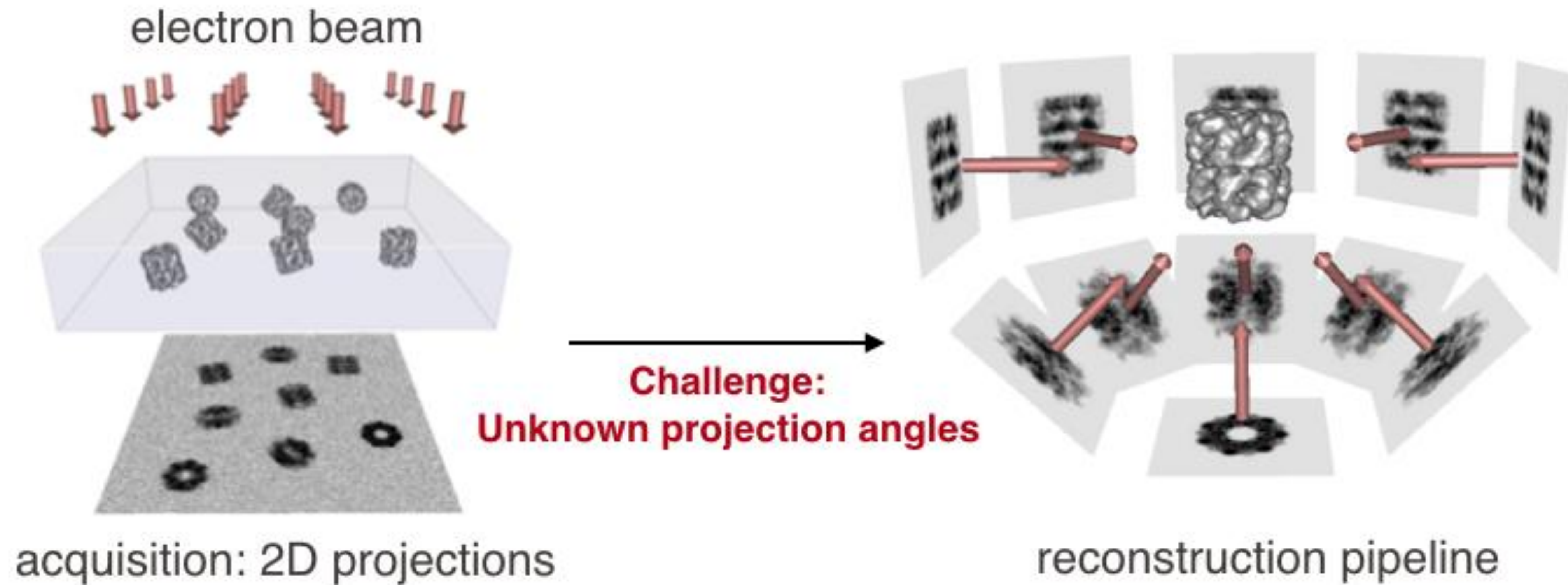
- Homogenous sample (purified)
- Small sample aliquots (3 μ l) on a copper/gold grid
- Blot excess liquid ($\approx 99\%$)
- Rapid plunge freezing (in liquid ethane @ -180°C)
 - Sample in a close to native state frozen in vitreous ice
 - Acquisition of projection images

Cryo-EM: principle workflow

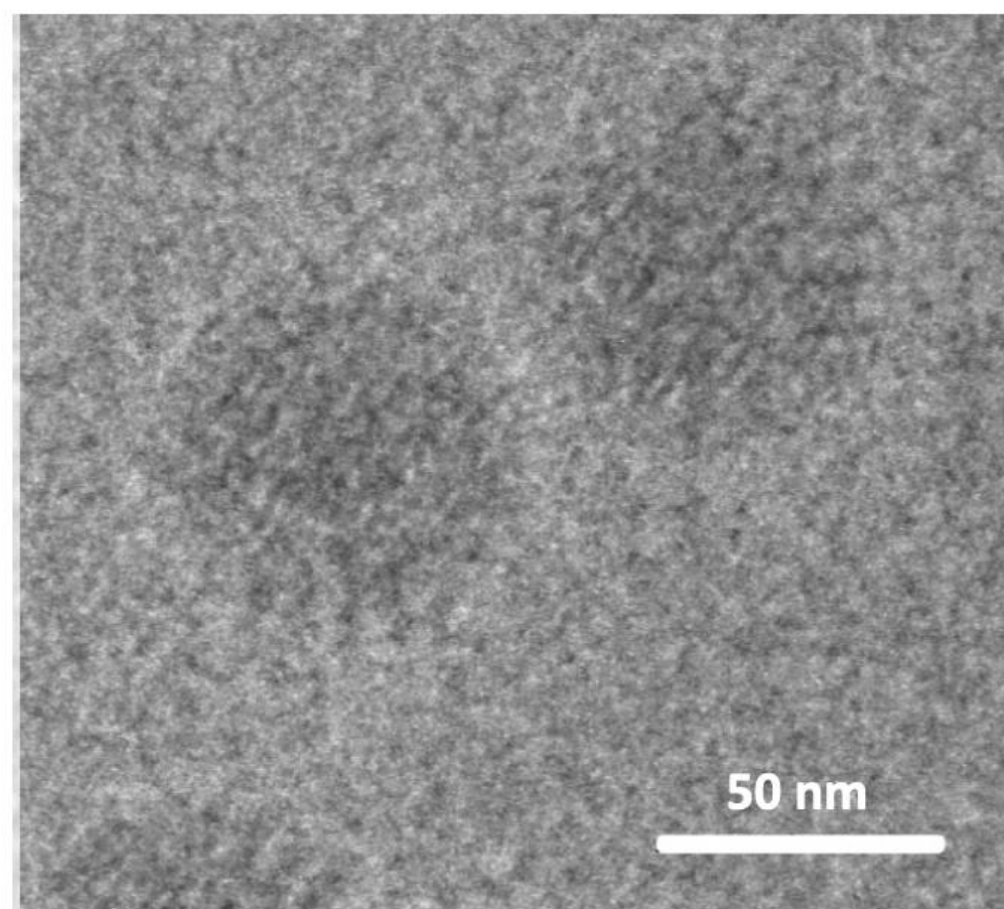


- Large datasets of your sample in random orientation
- Processing / Correction: particle motion, damage, and CTF estimation
- Extraction of individual particles
- Signal averaging for 2D classes
- Backprojection into a 3D volume, alignment
- Iterative process to obtain a final map -> model building

Cryo-EM: principle workflow

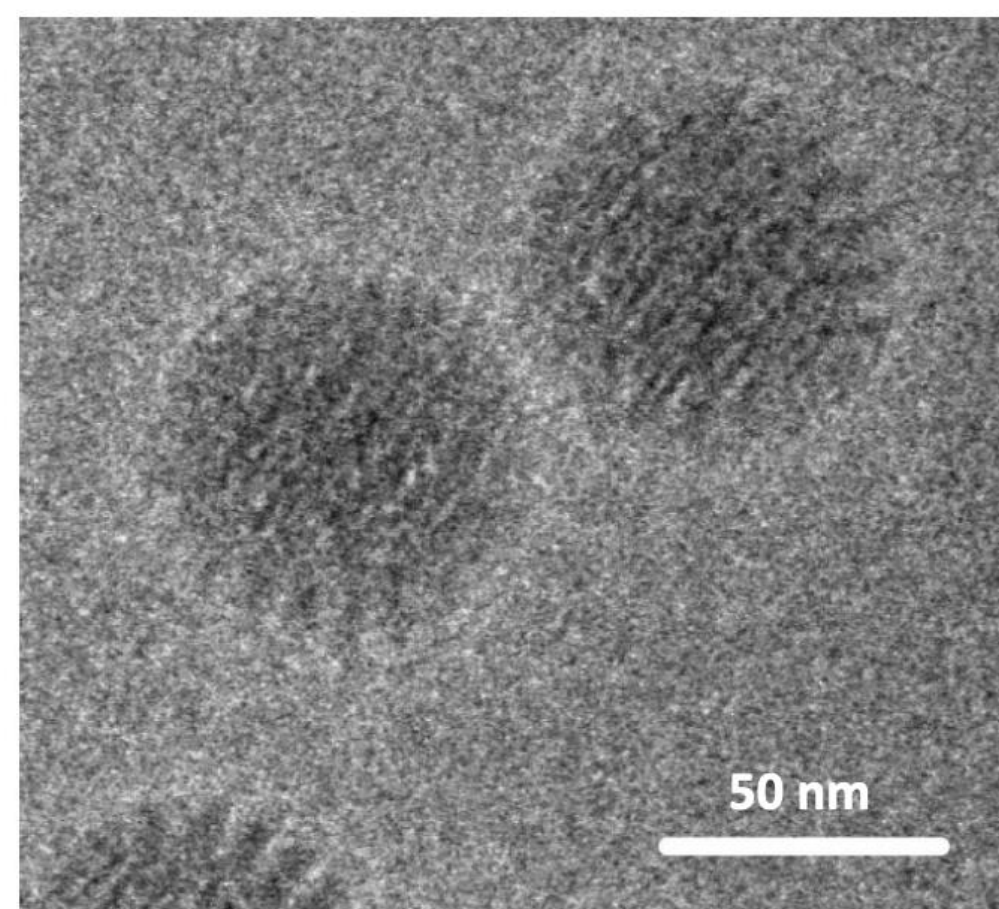


until 2012
Photographed on a
CCD camera or on film

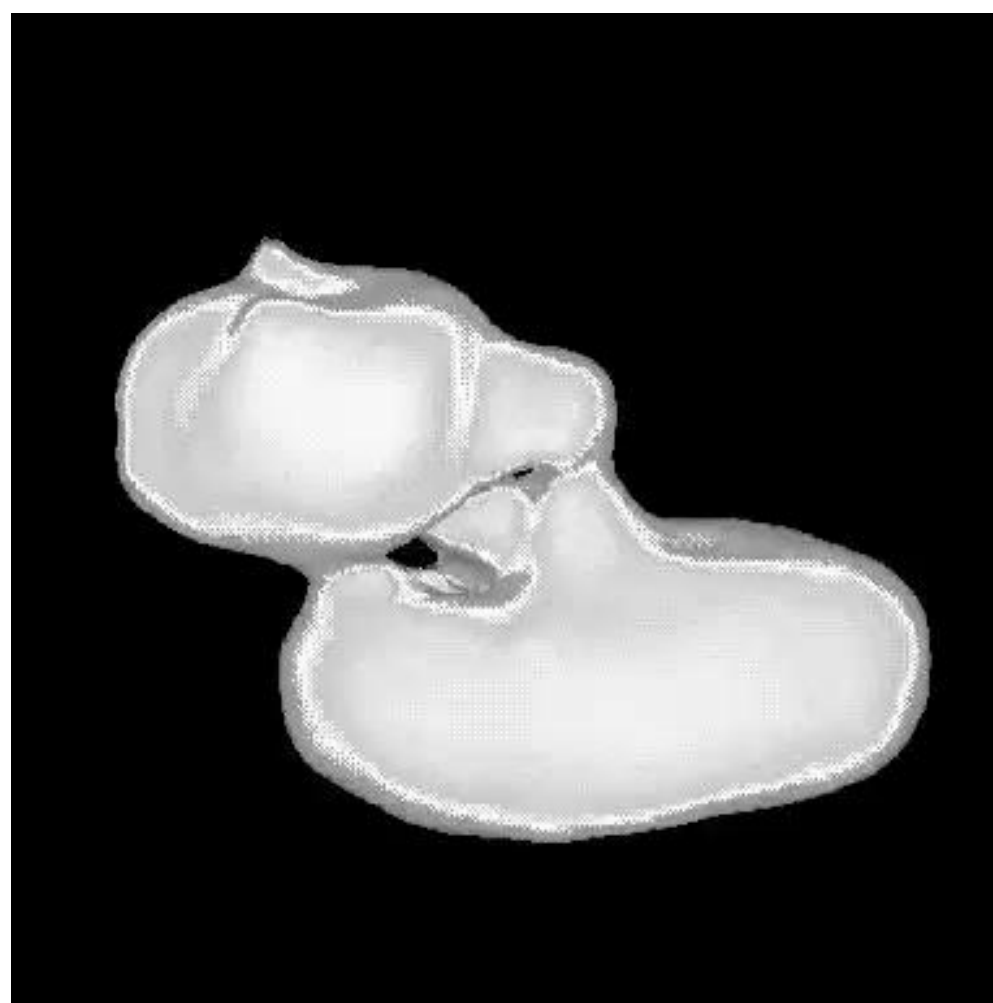
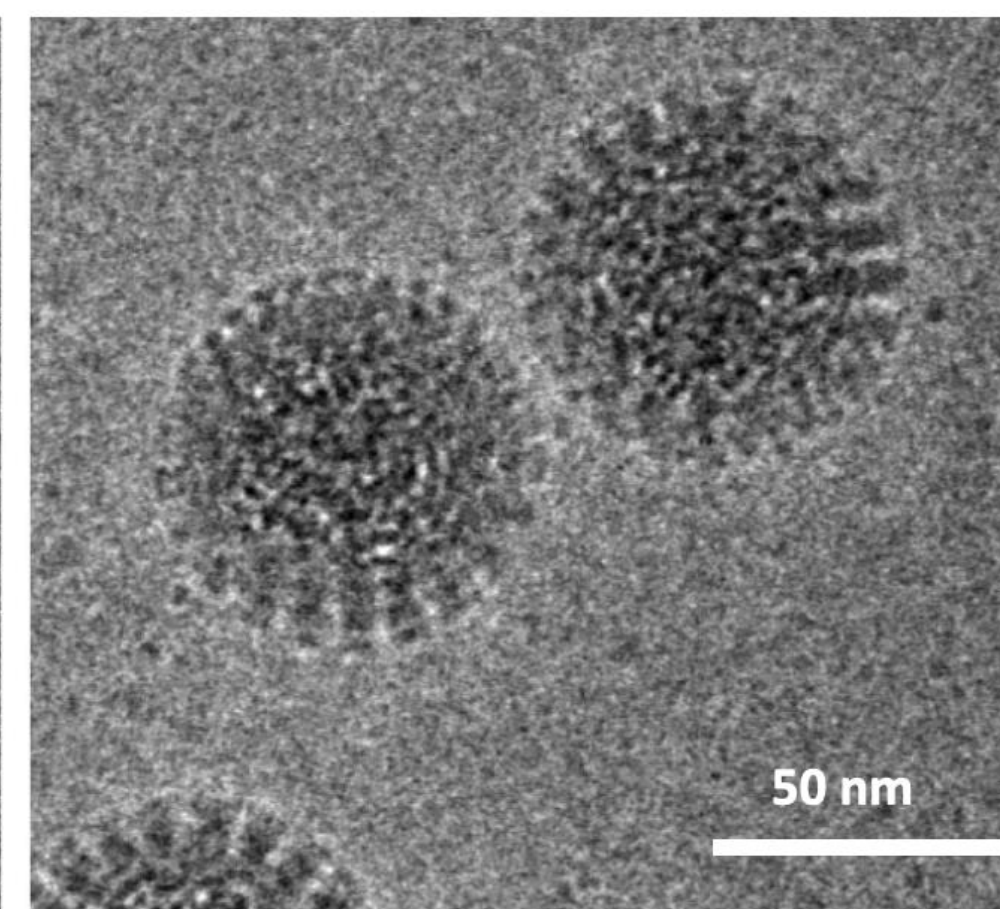


Rotavirus, mockup images adapted from Grigorieff, *eLife*, 2013;2:e00573.

2013
Photographed on a
direct electron detector



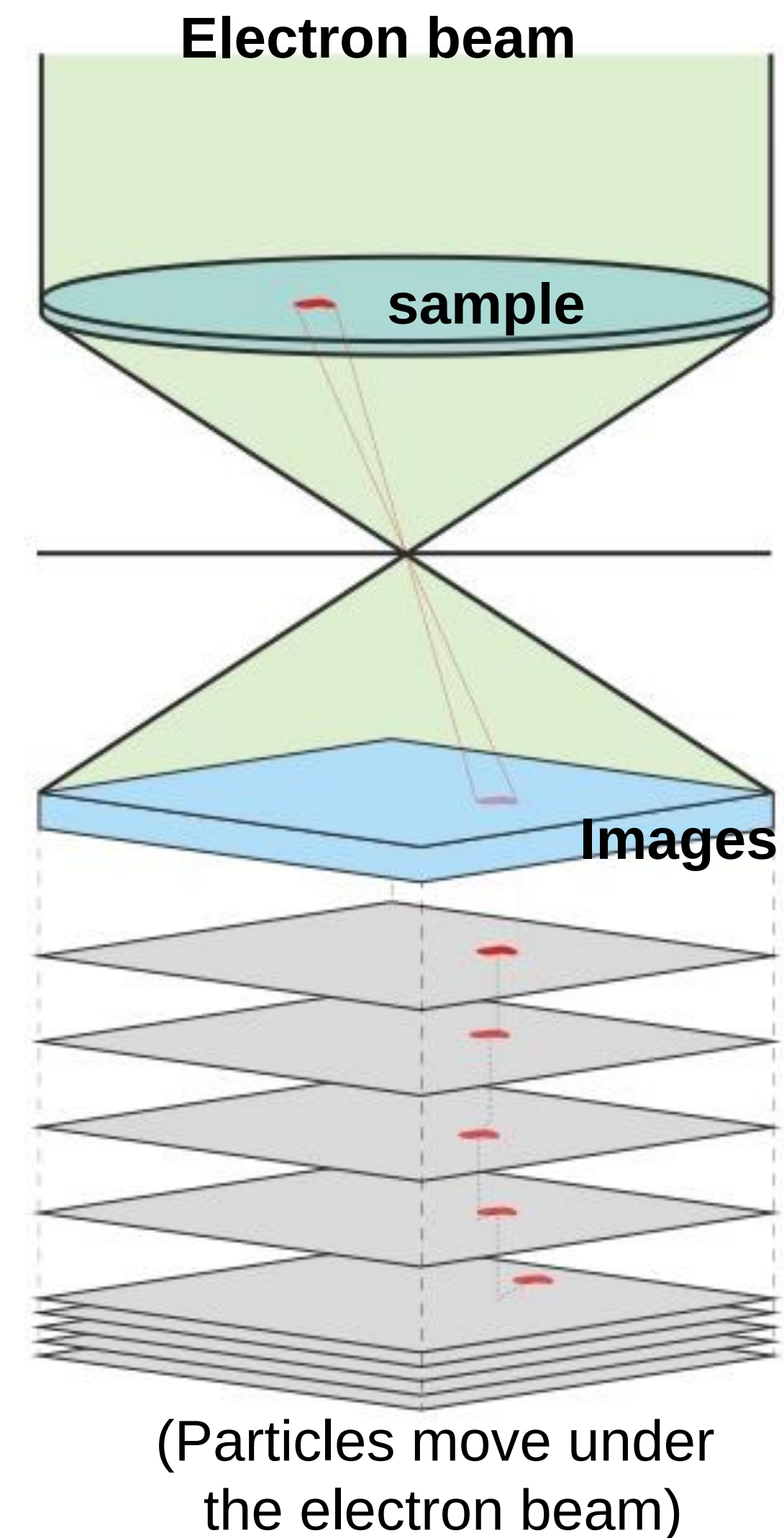
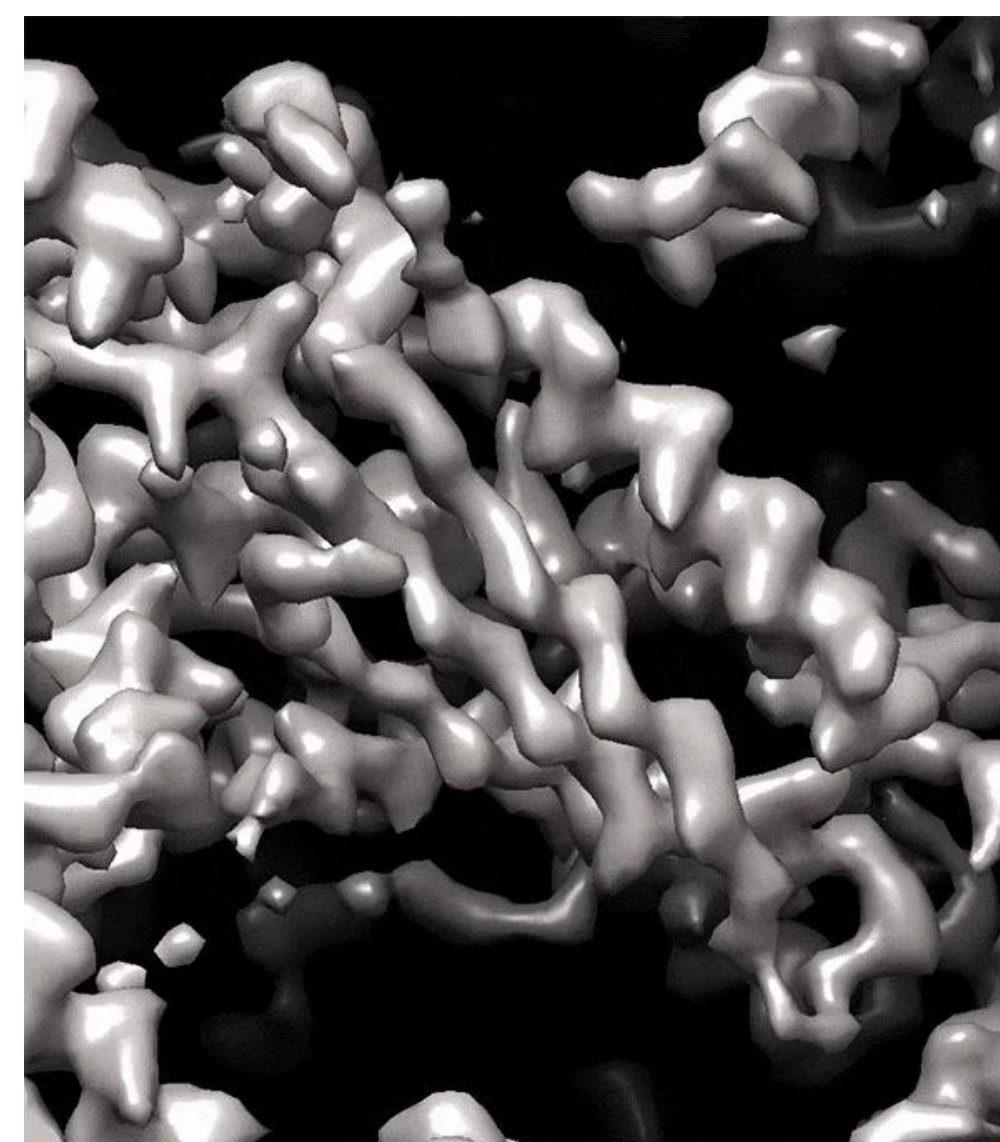
since 2014
Filmed on a
direct electron detector



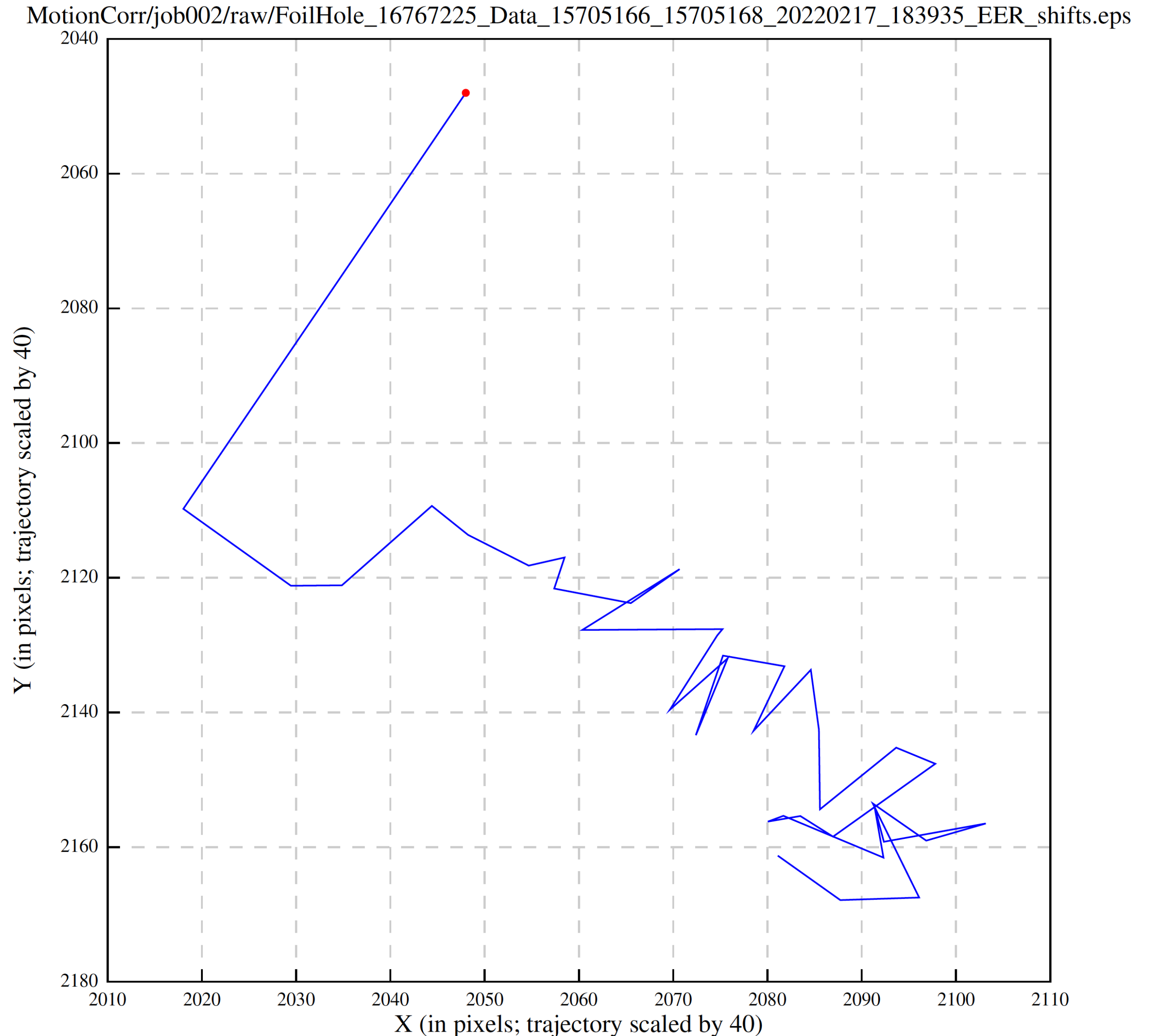
«Blobology»



To chemistry



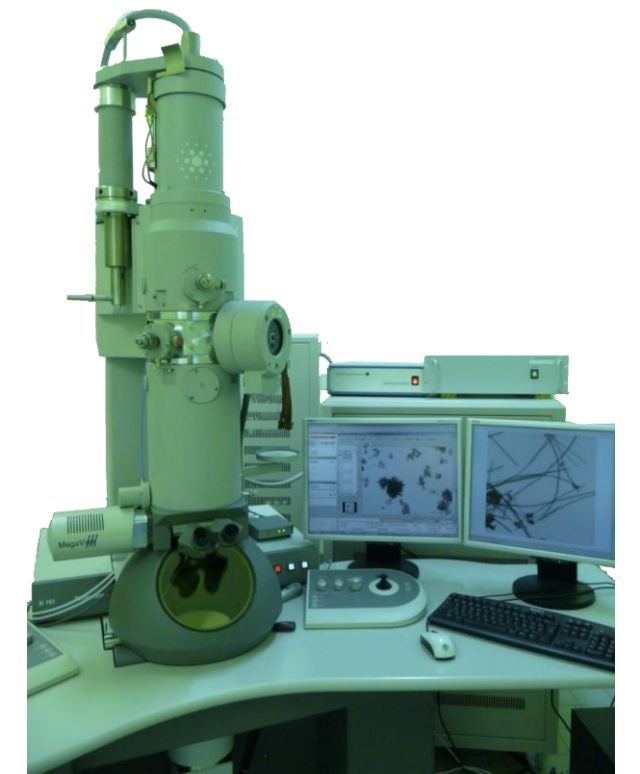
- Acquisition of movies instead of image.
 - .tiff, .mrc, .eer
- Allows for corrections induced by the electron beam
- Allows for deletion of frames (radiation damage)
- Results in sharper images and better signal to noise ratio



Cryo-EM: workflow of a project

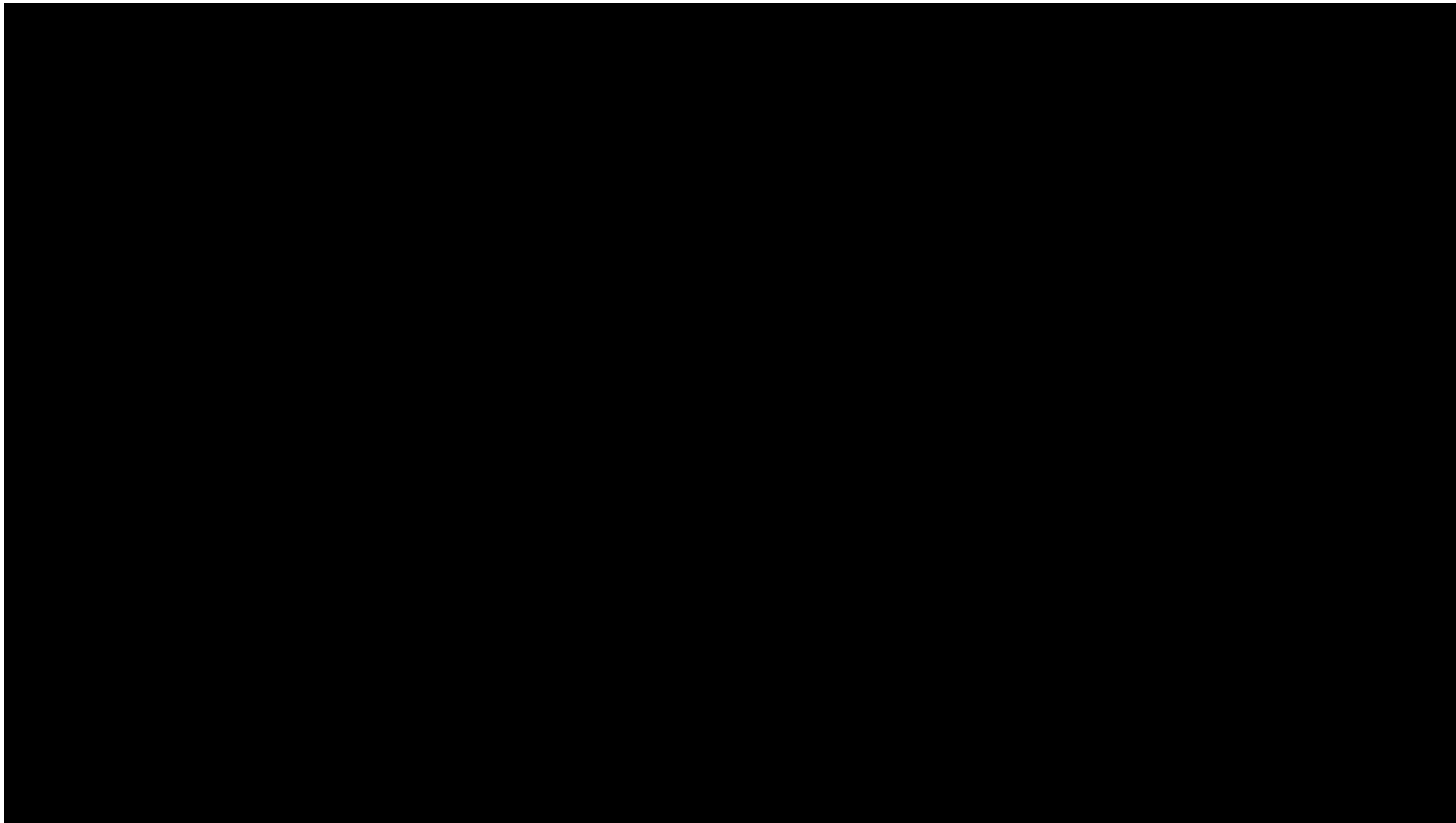
- Stage 1: Is my sample suitable (pure, homogenous, stable)?
 - Preparation of negatively stained grid
 - TEM imaging @120kV
 - Computer analysis for sample homogeneity
- Stage 2: Can my sample be frozen well?
 - Preparation of frozen grids
 - Cryo-EM imaging @ 200kV
 - Computer analysis for sample quality control
- Stage 3: Get the high-resolution structure
 - Identification of the optimal, frozen grids
 - Cryo-EM imaging @300kV
 - Computer analysis for 3D structure reconstruction

Mostly done in facilities



<https://cryoem101.org/>

Cryo-EM: the grid



Cryo-EM: freezing



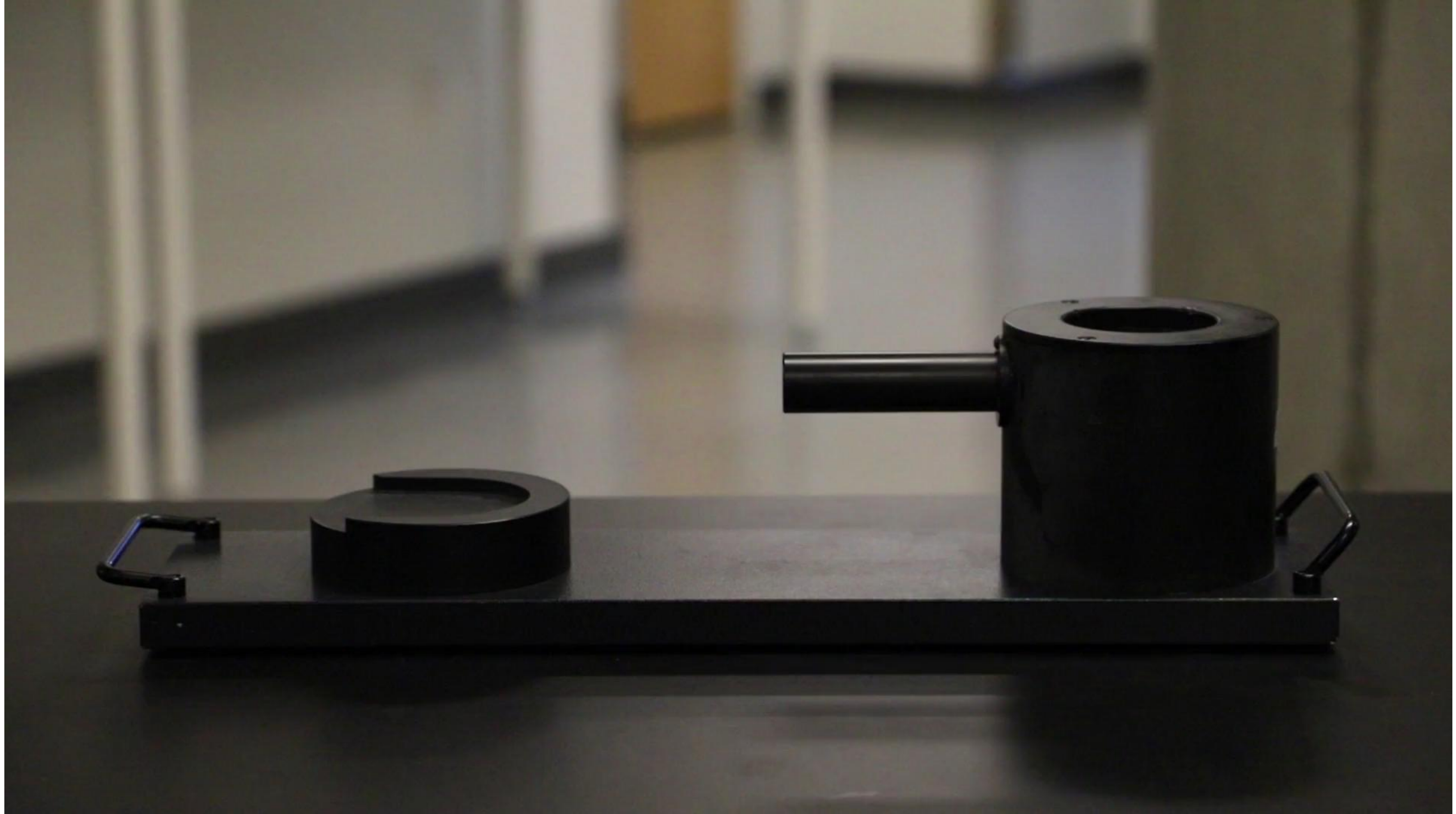
Transmission Electron Microscopy

Cryo-EM



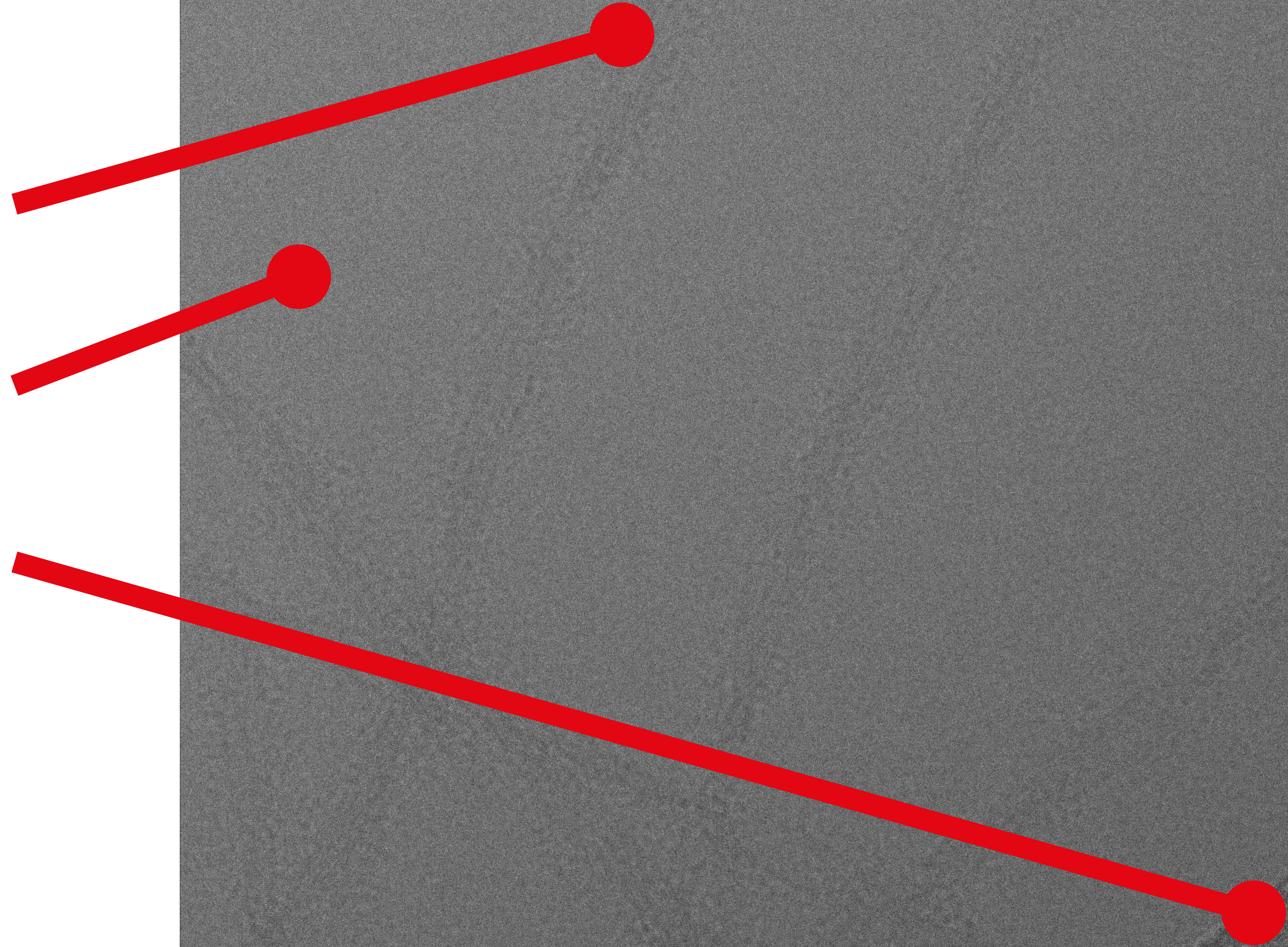
3 μ l

Cryo-EM: loading & imaging



Typical image

- Protein in solution
- Buffer / vitreous
- Carbon edge





**Cryo
TEM**

**Continous
C-film**

-180°C



da Vinci

100 %



90 %



80 %



70 %



AFM

TEM neg.stain

60 %



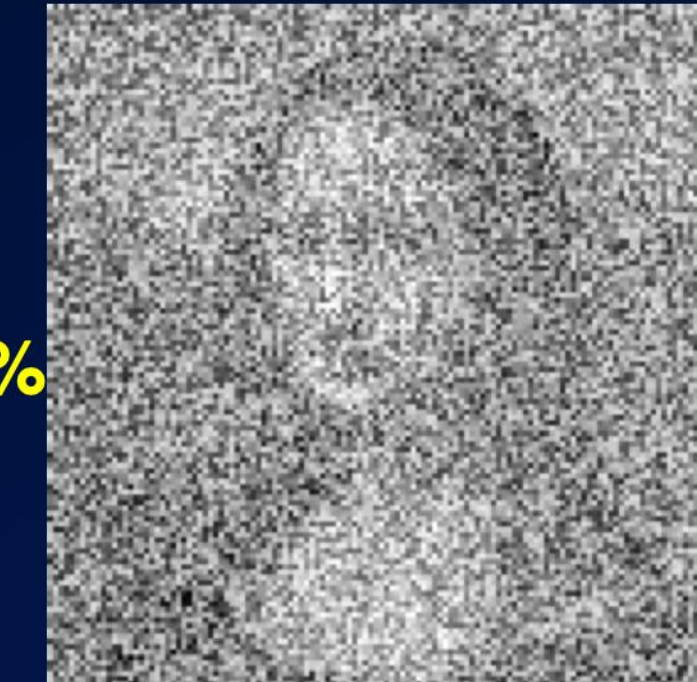
50 %



40 %

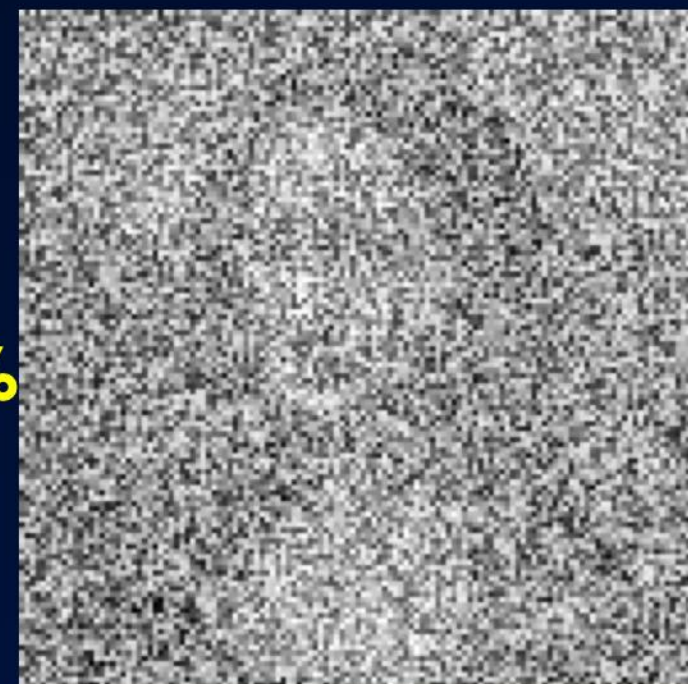


30 %

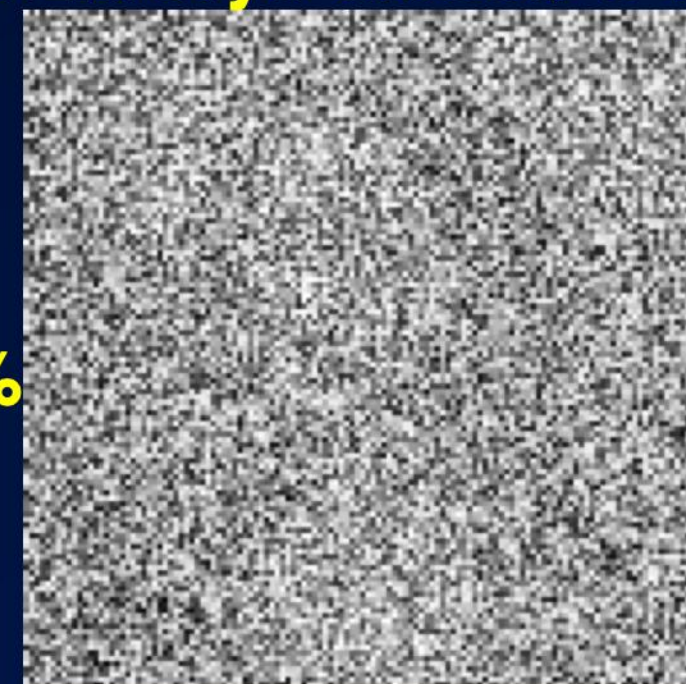


TEM cryo closed C-film

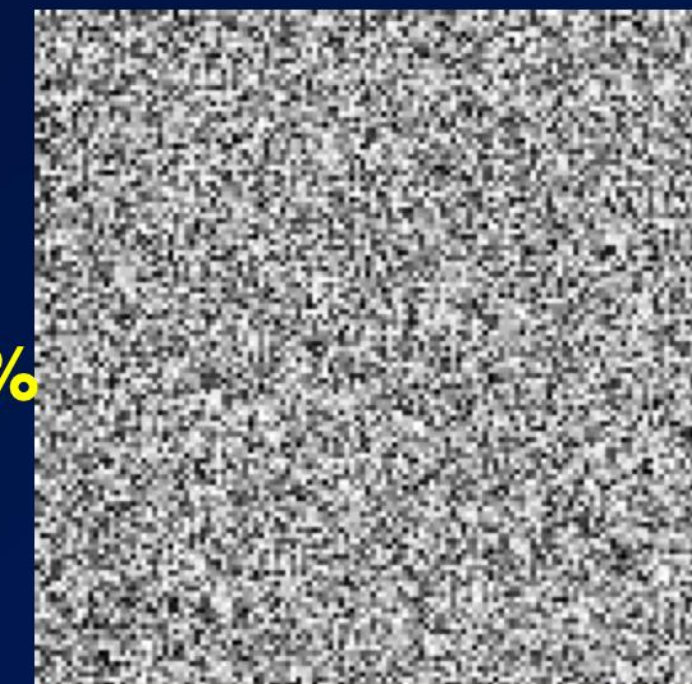
20 %

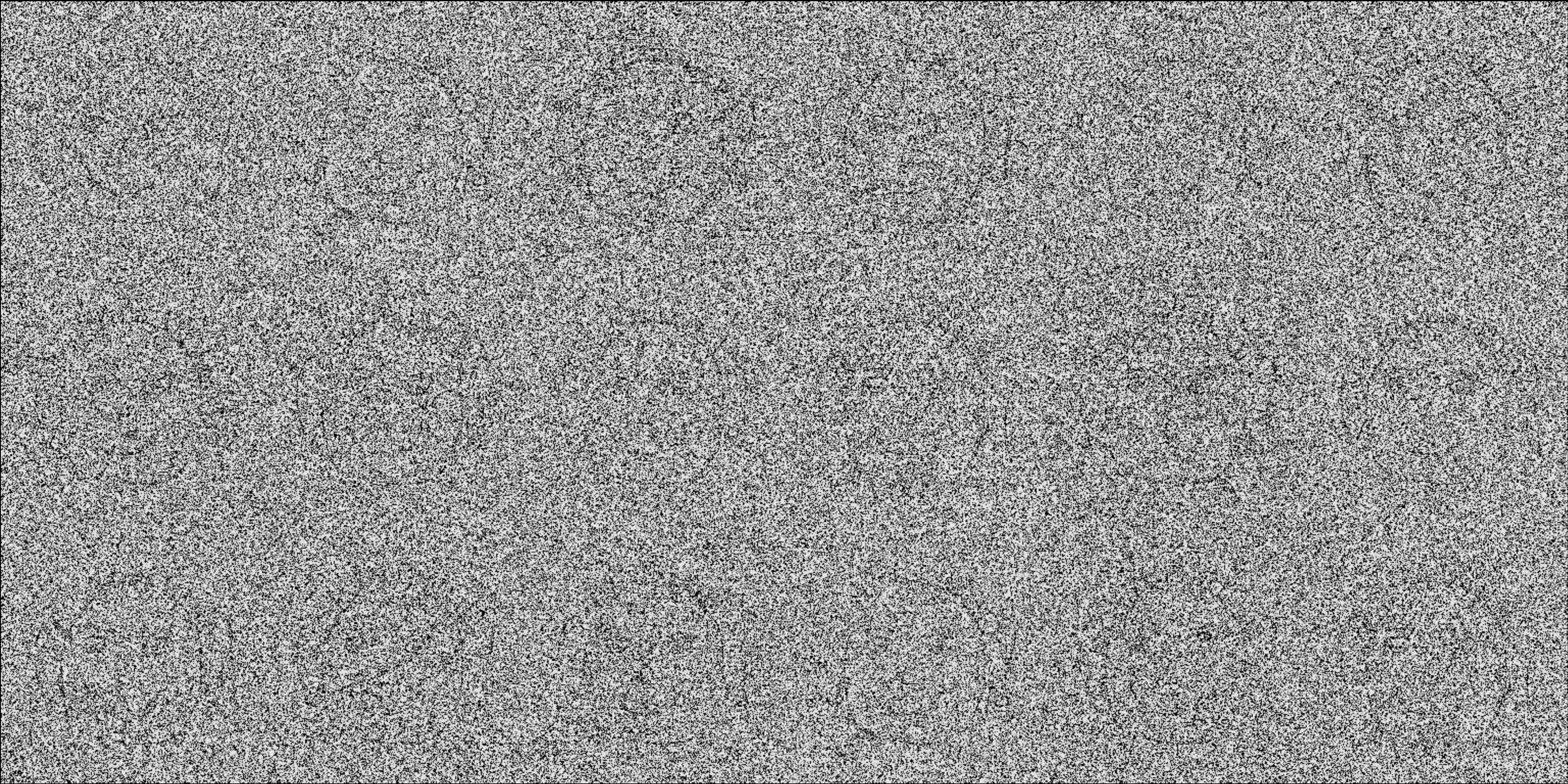


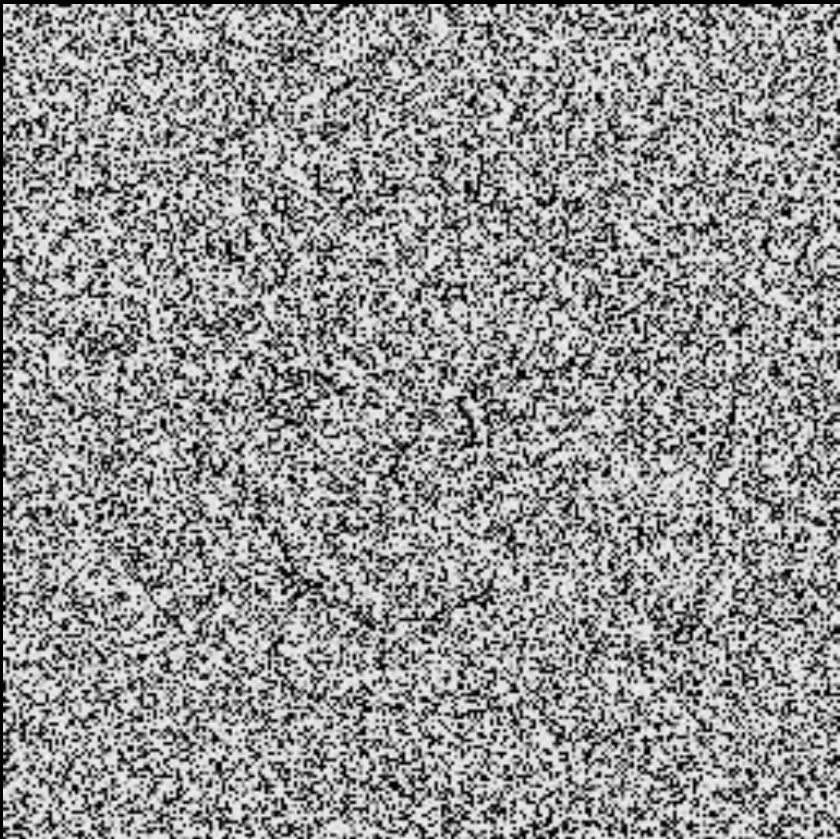
10 %



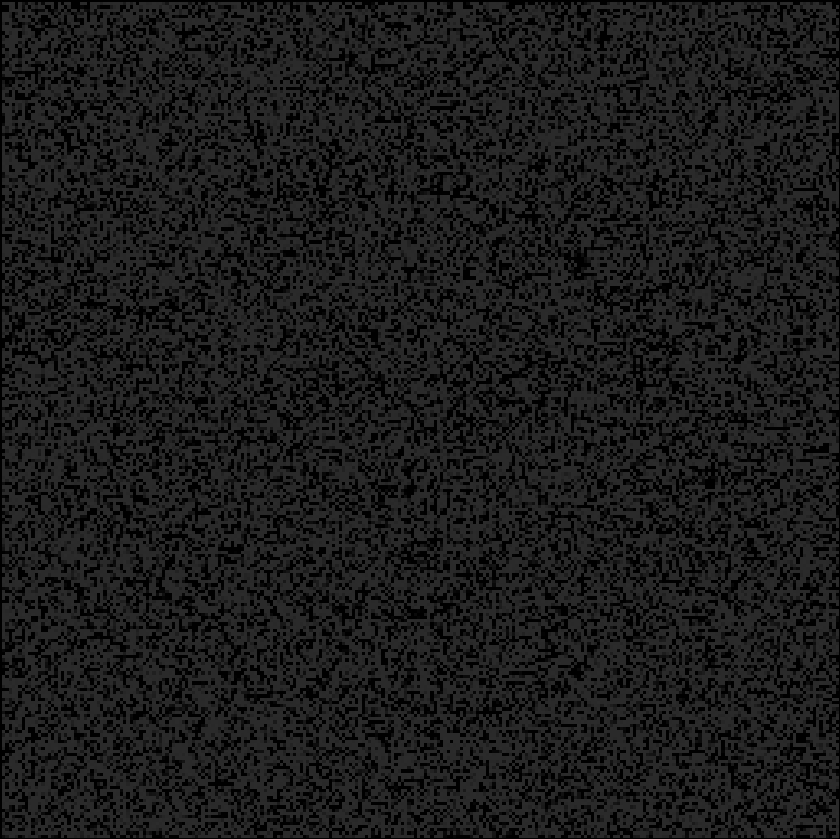
0 %





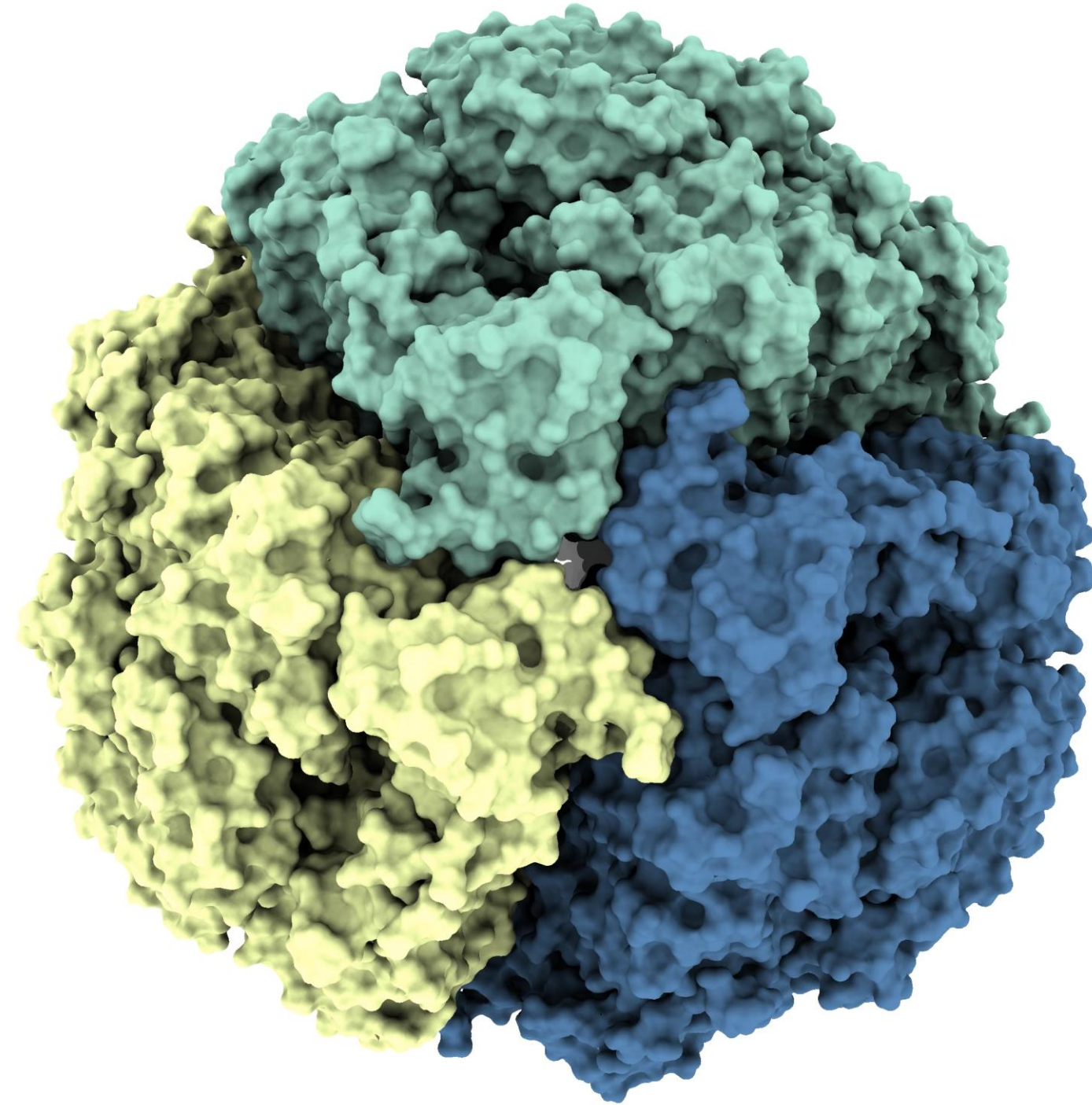


One particle

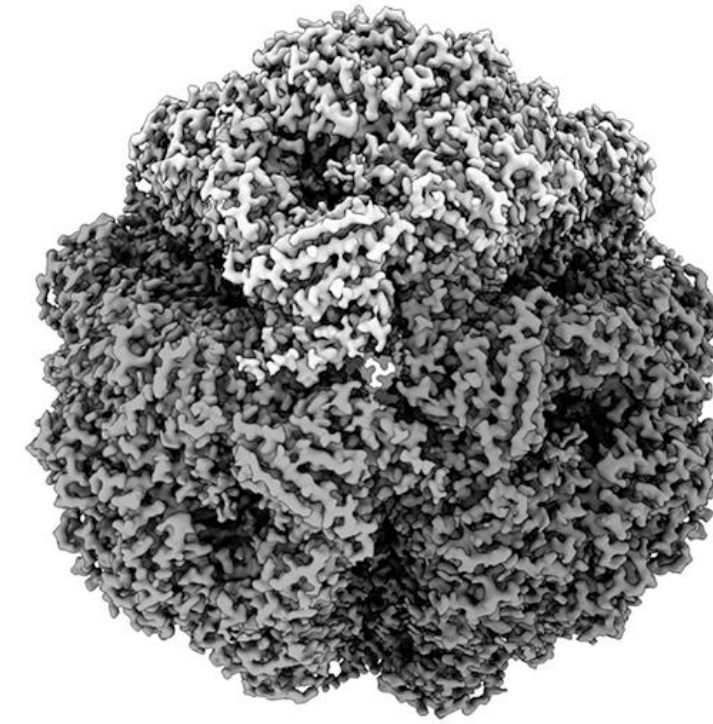


Average

Imaging: Reducing noise

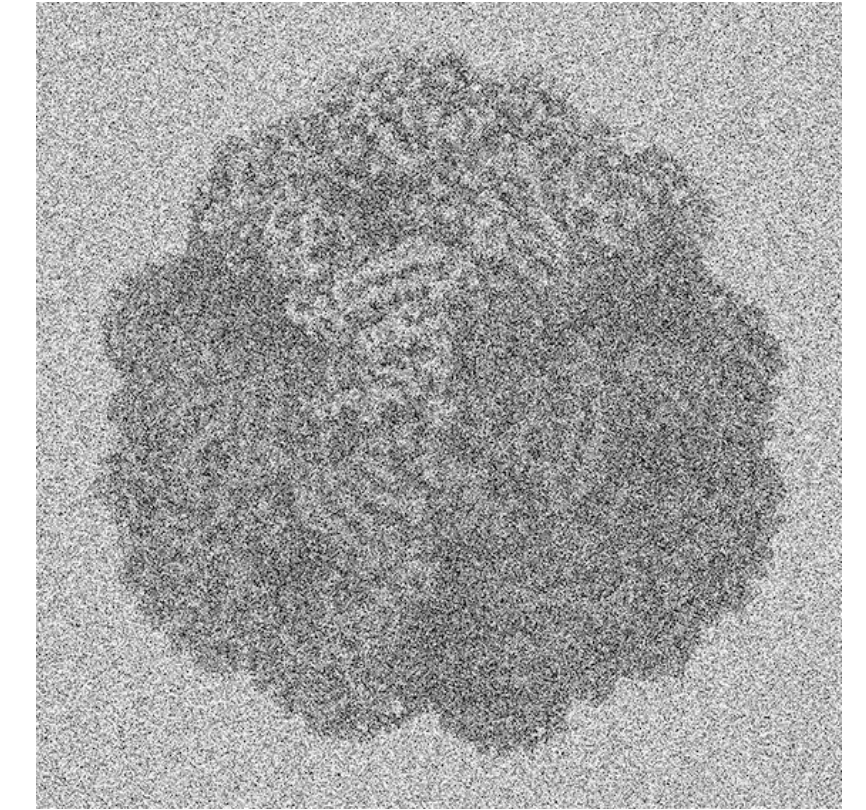


“true” projection

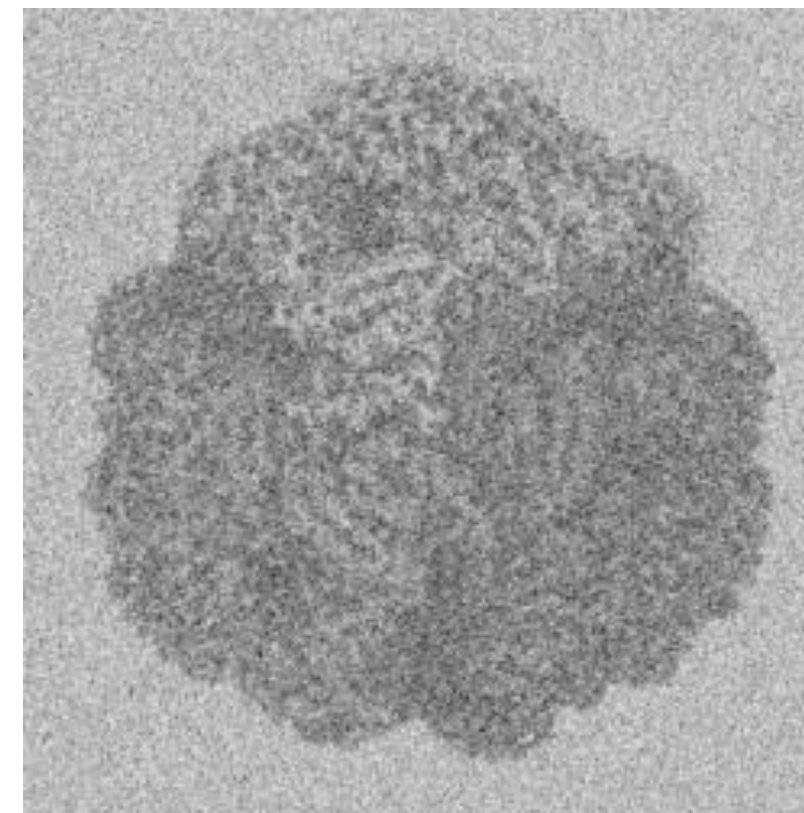


noise

acquired projection

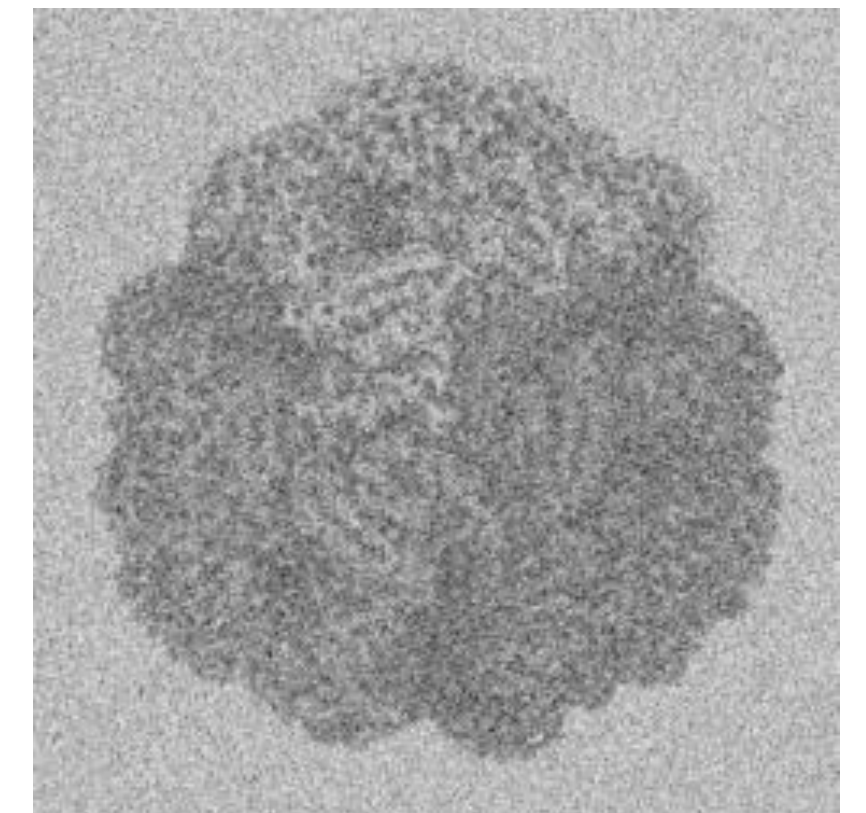


multiple acquired projection



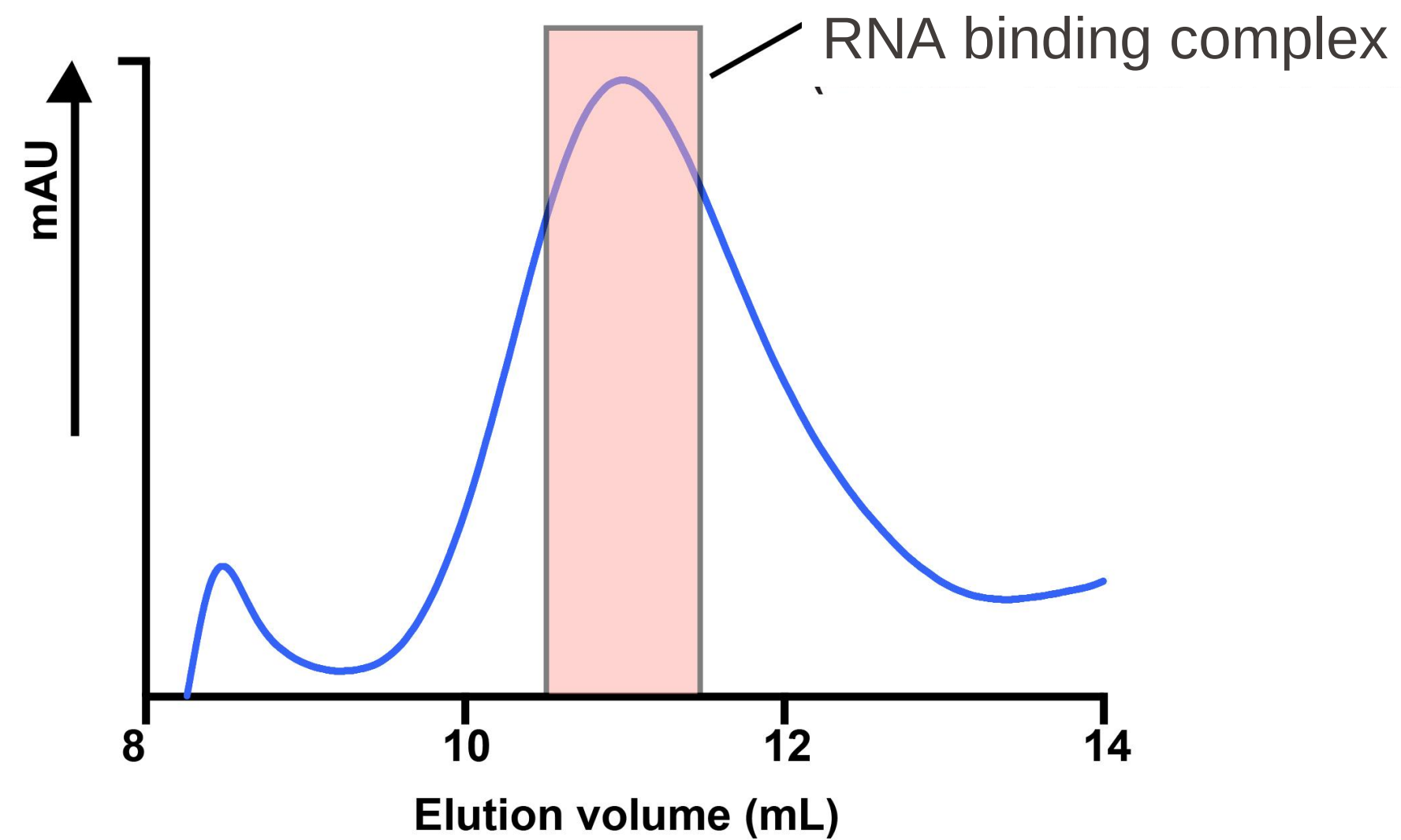
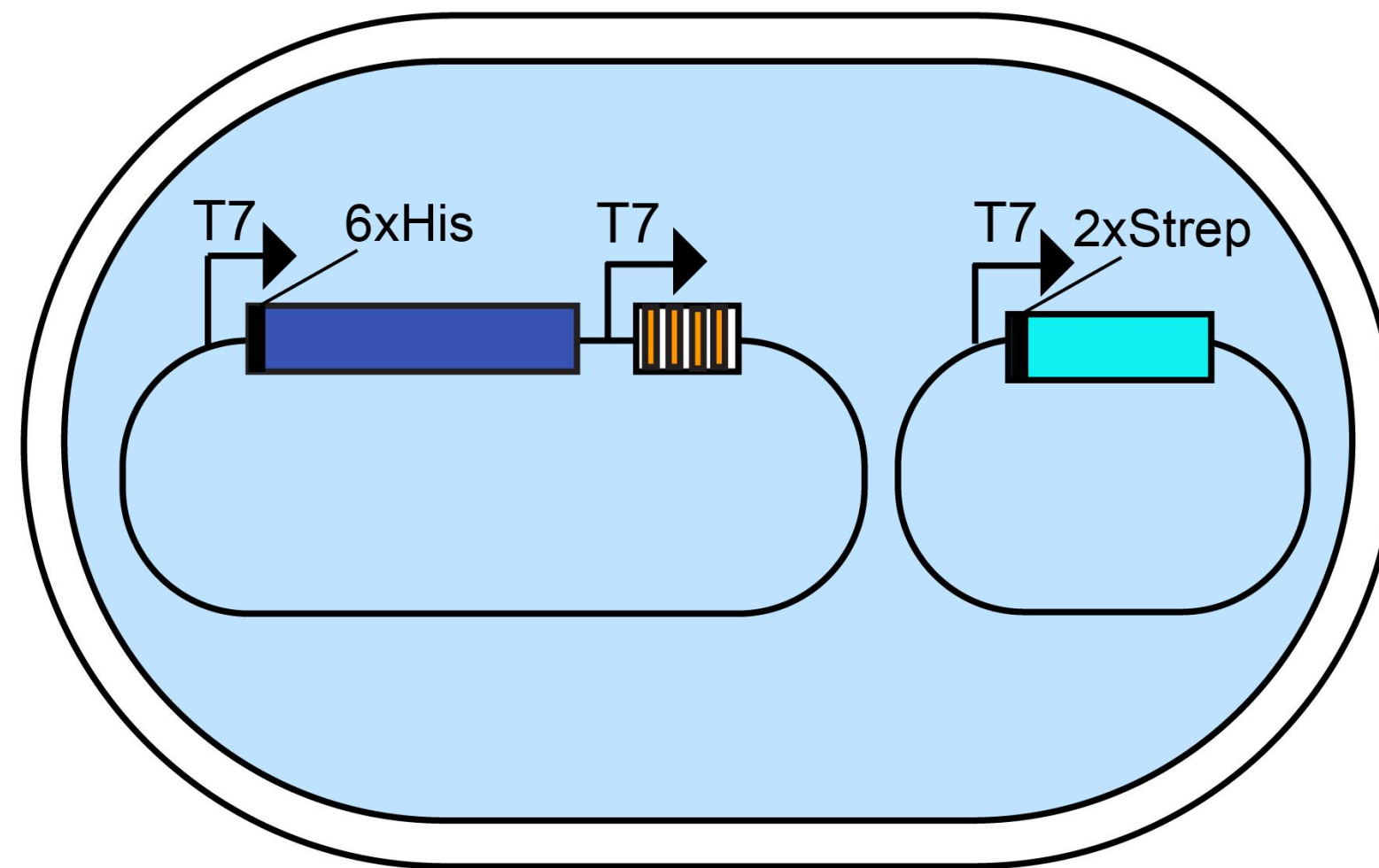
averaging

reconstructed projection

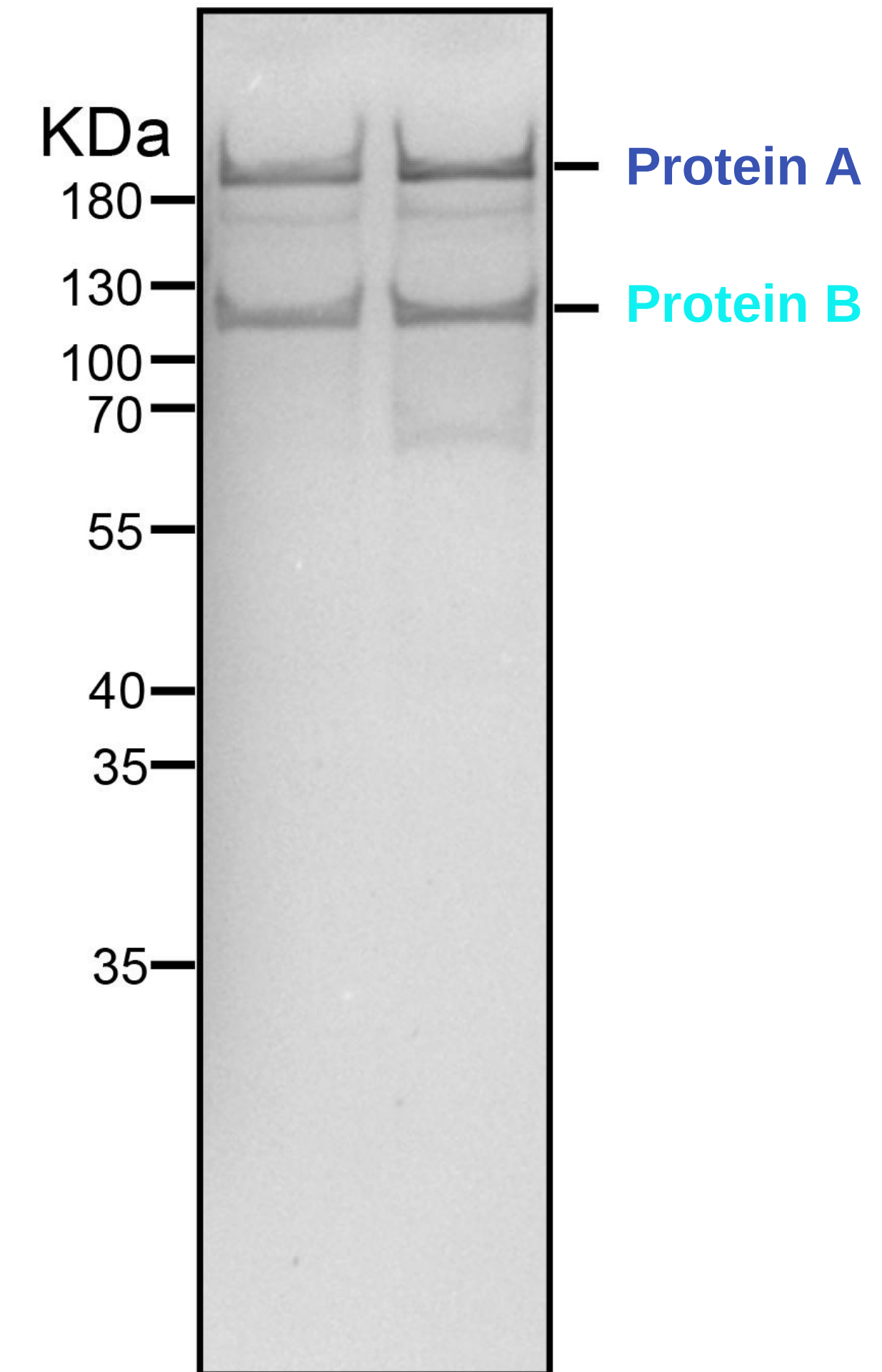


Example: A RNA binding complex

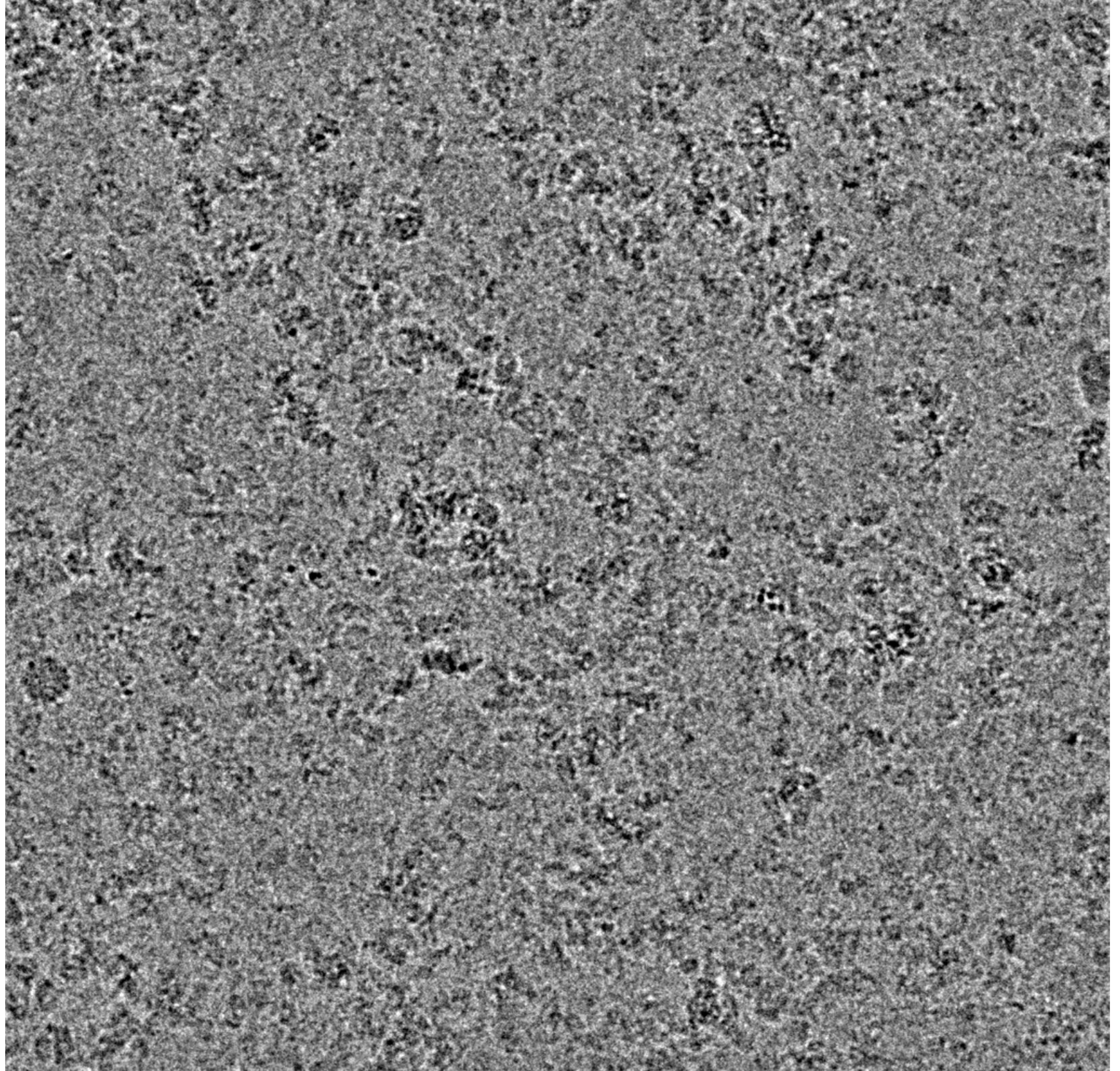
E. coli BL21(DE3)



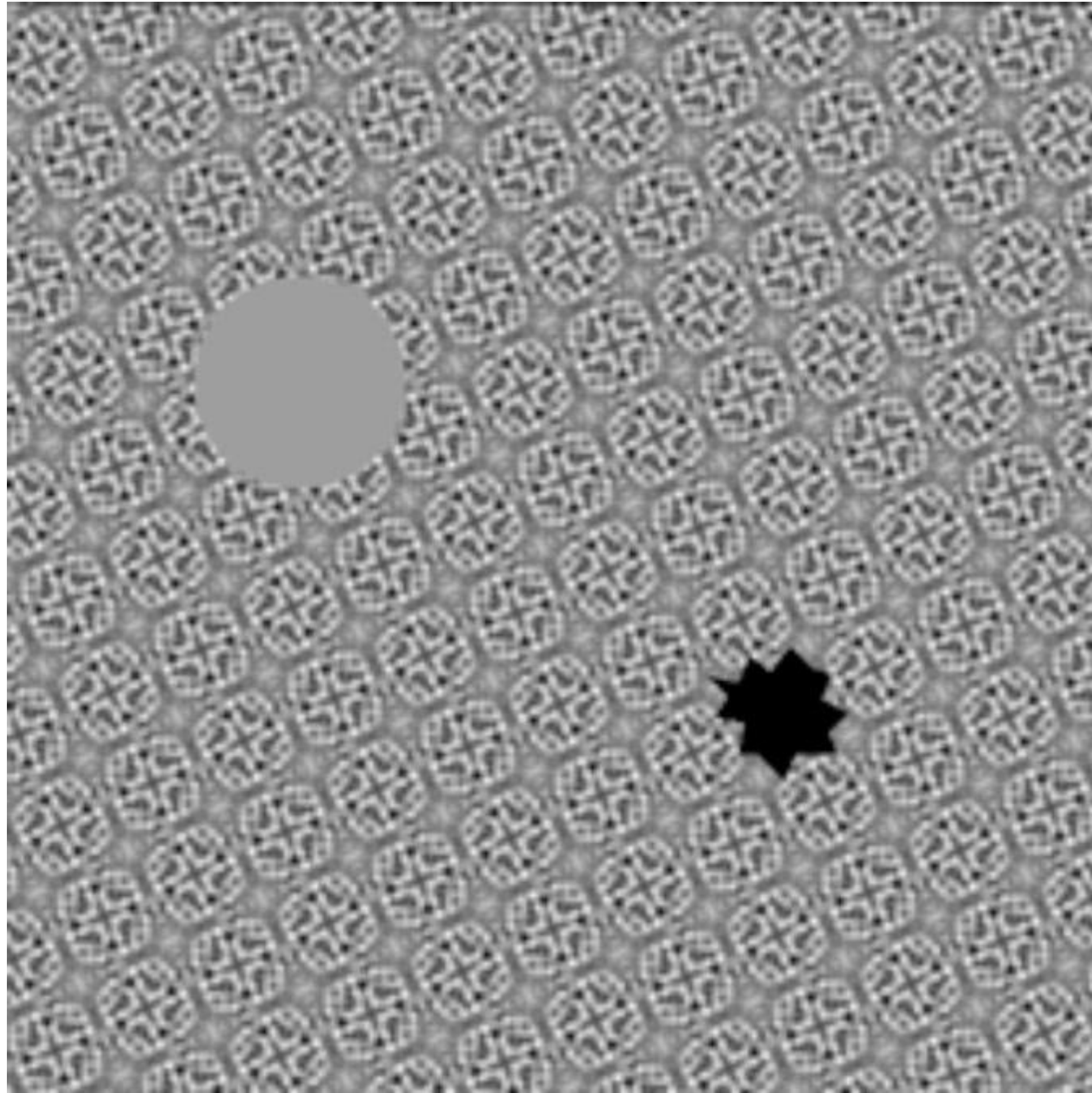
SDS-PAGE



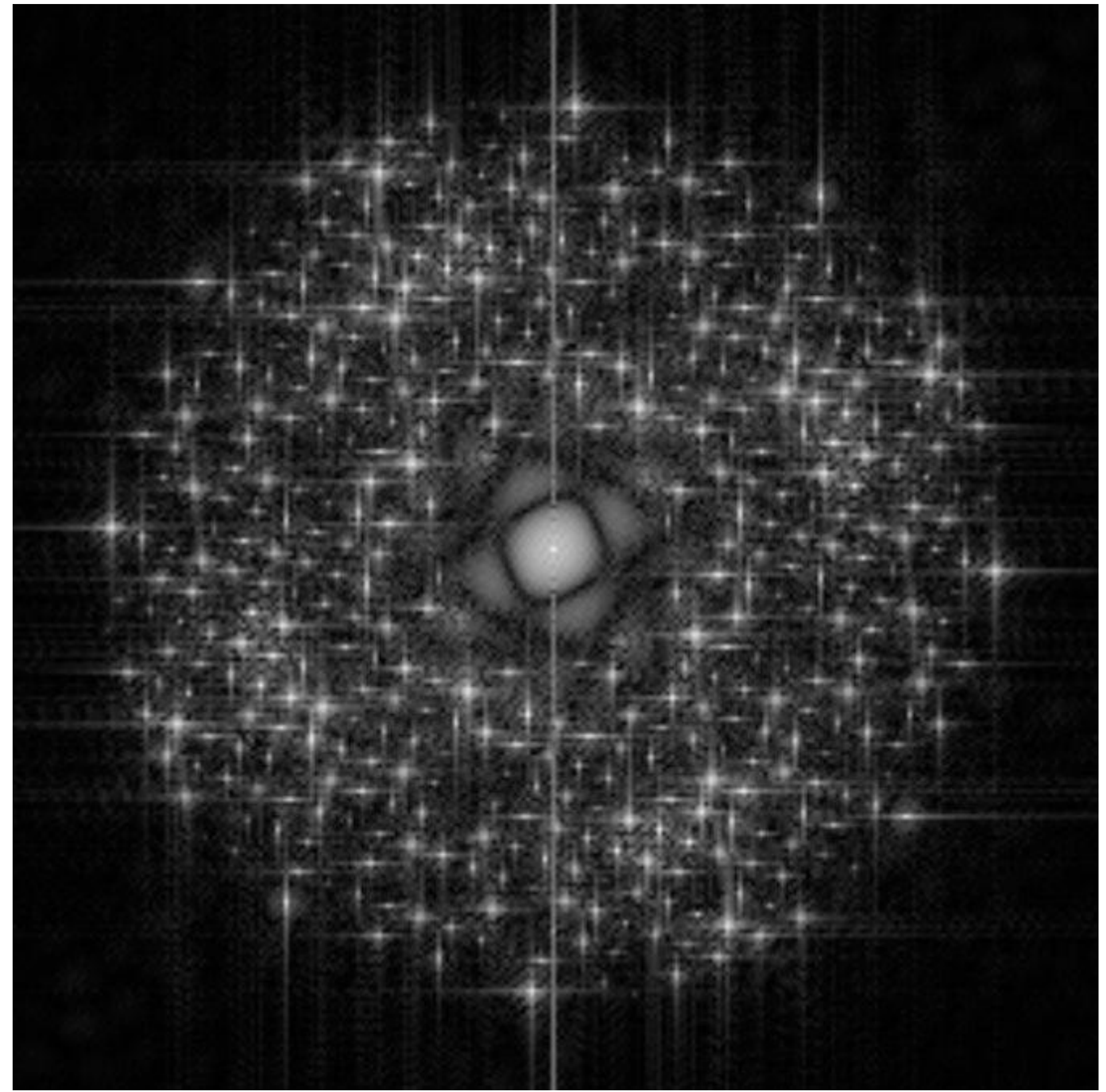
A RNA binding complex



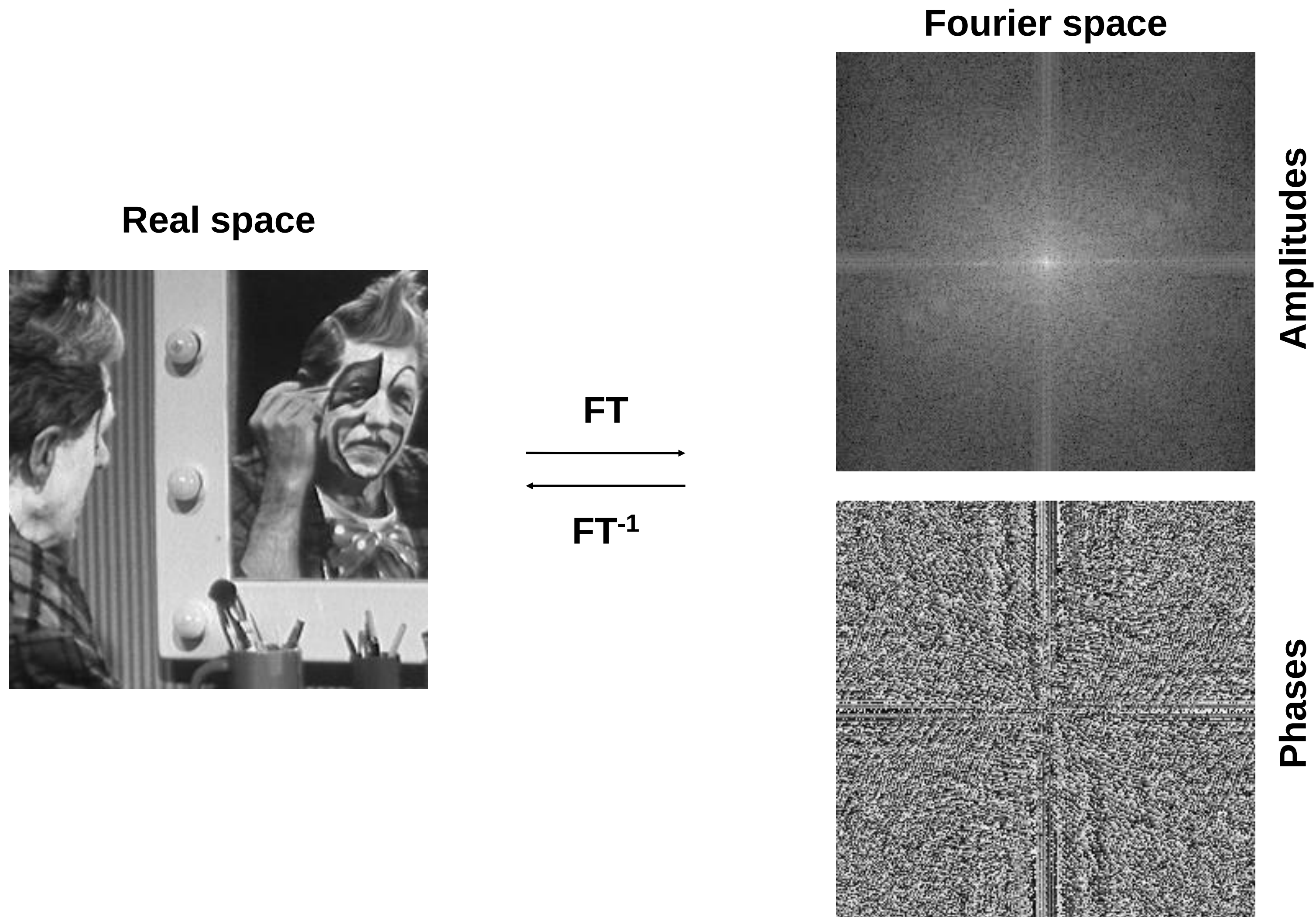
Real Space



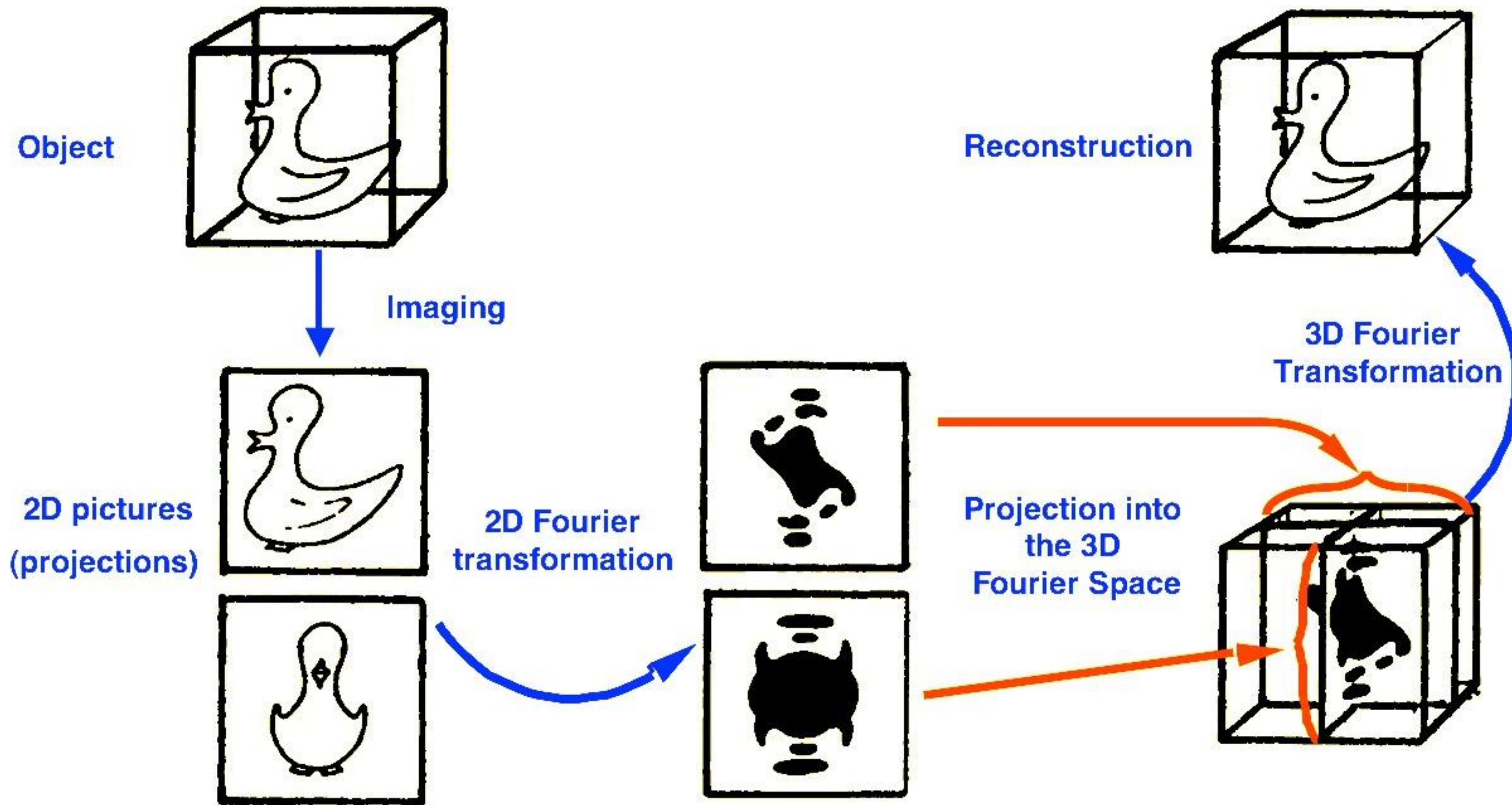
Fourier Transform



Real-space image $\Leftrightarrow$ Fourier Transform



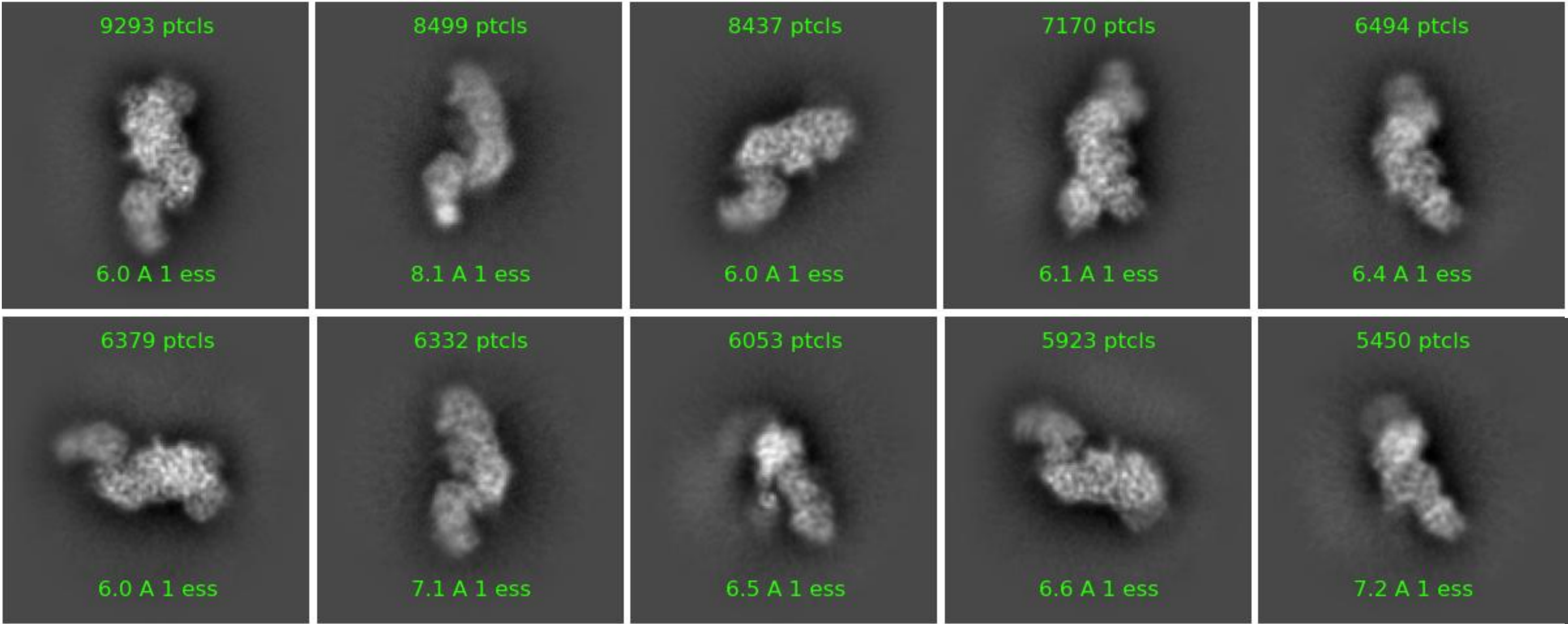
The Radon projection theorem for 3D reconstructions



Lake, J. (1971). In "Optical Transforms", (H. Lipson, Ed.), Academic Press, London.

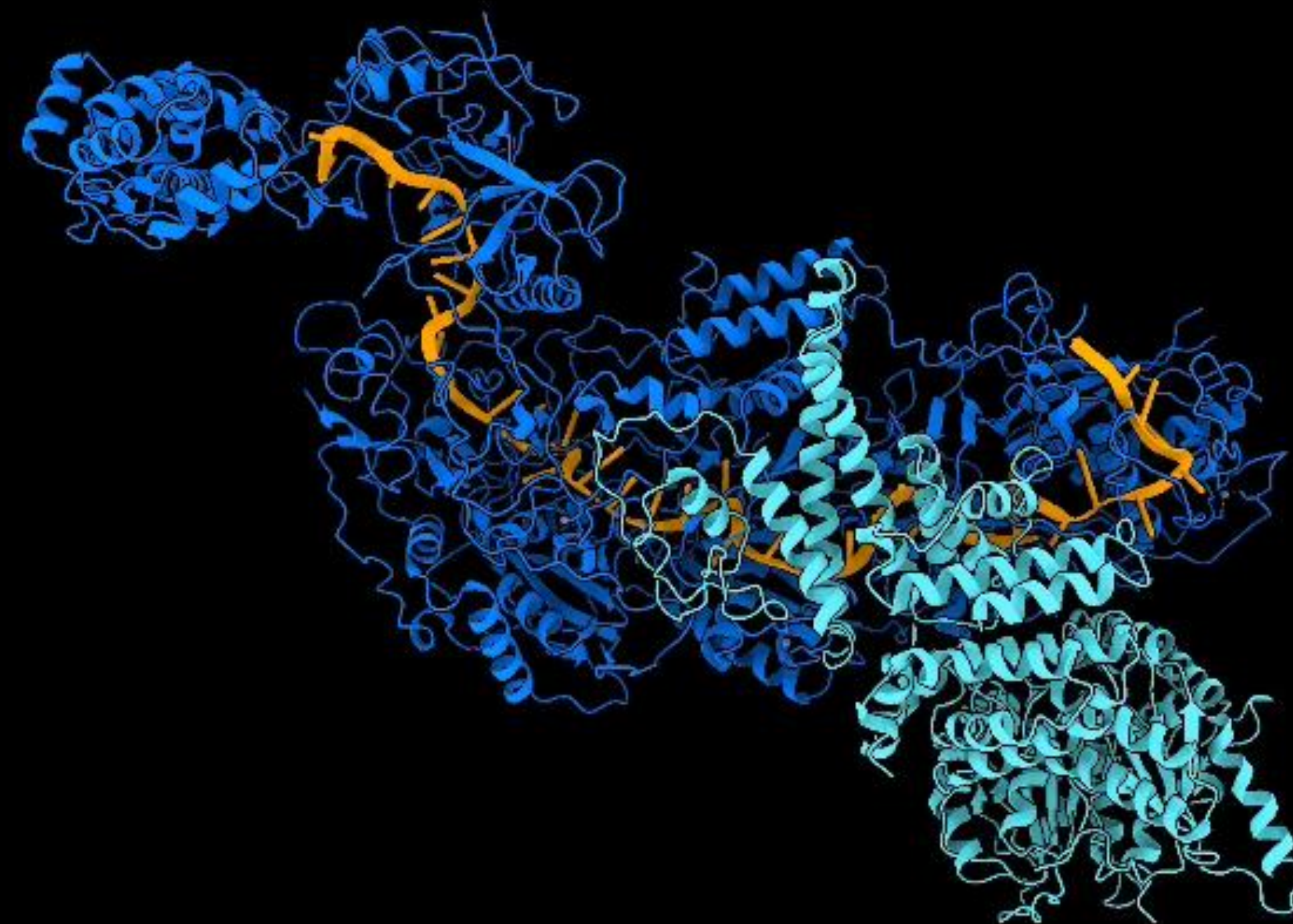
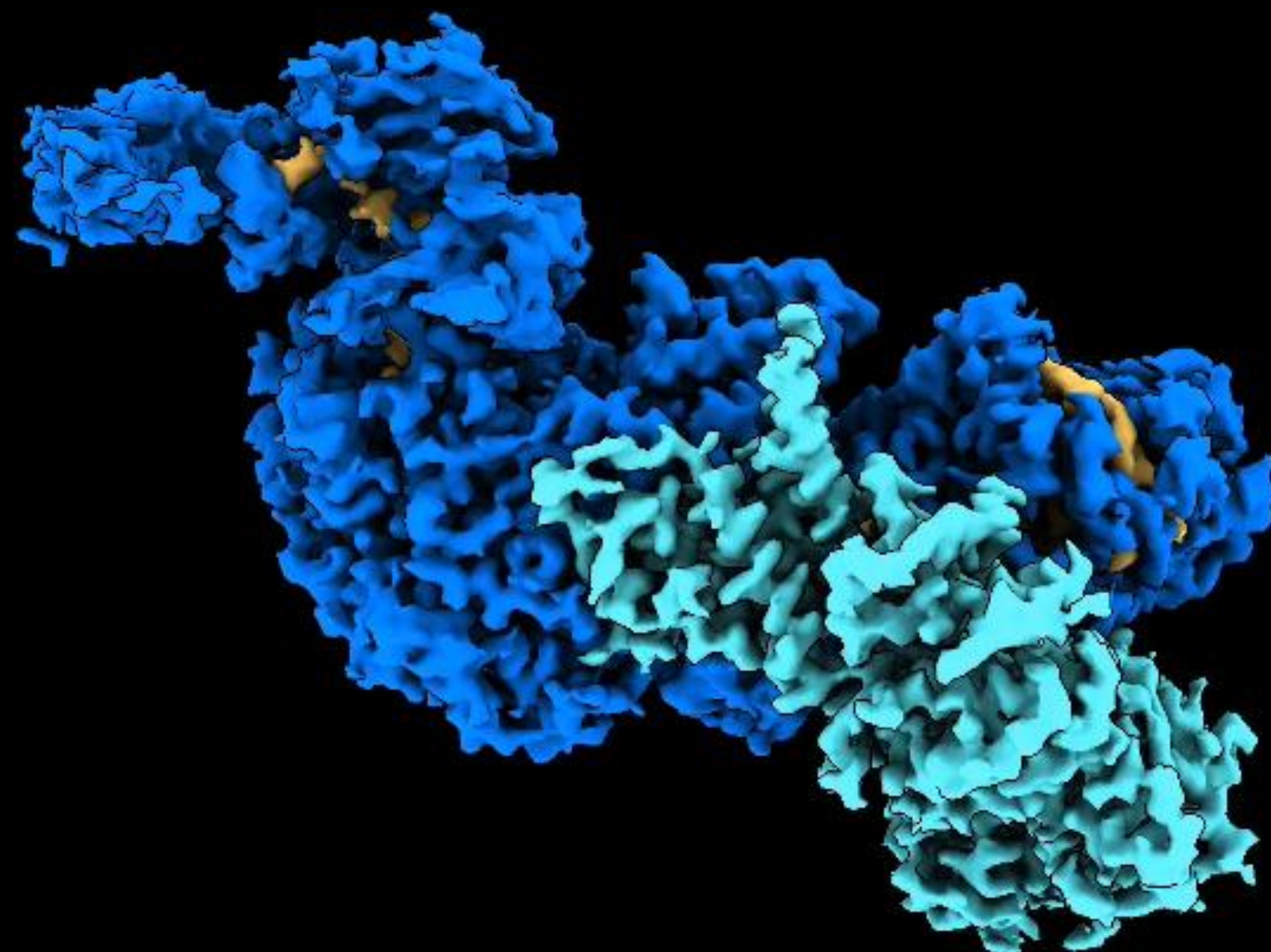
Fourier-Space Method

A RNA binding complex

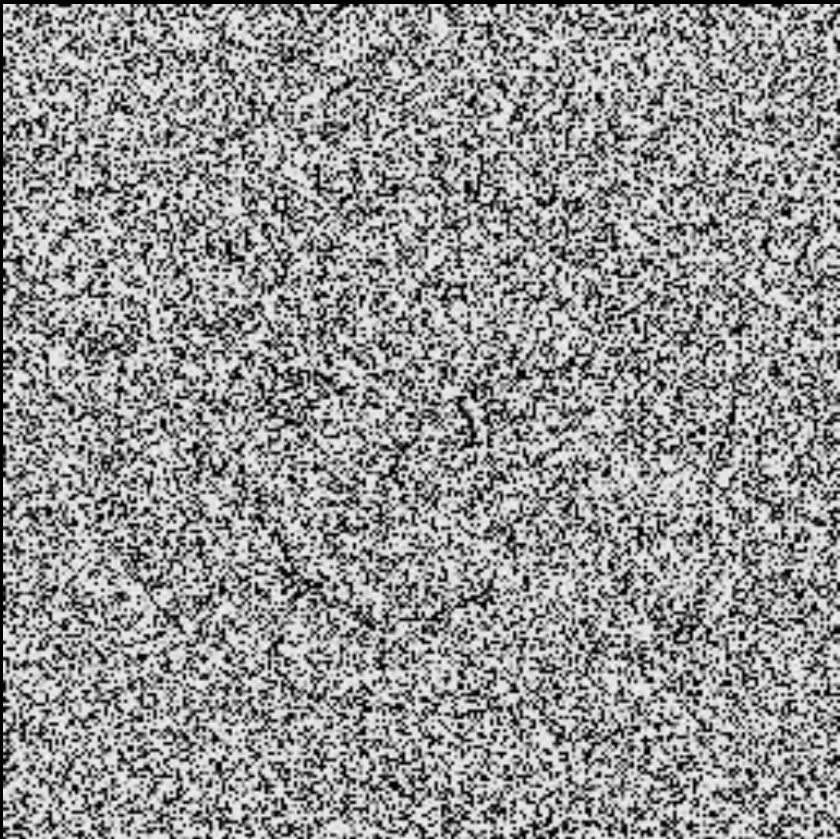


A RNA binding complex

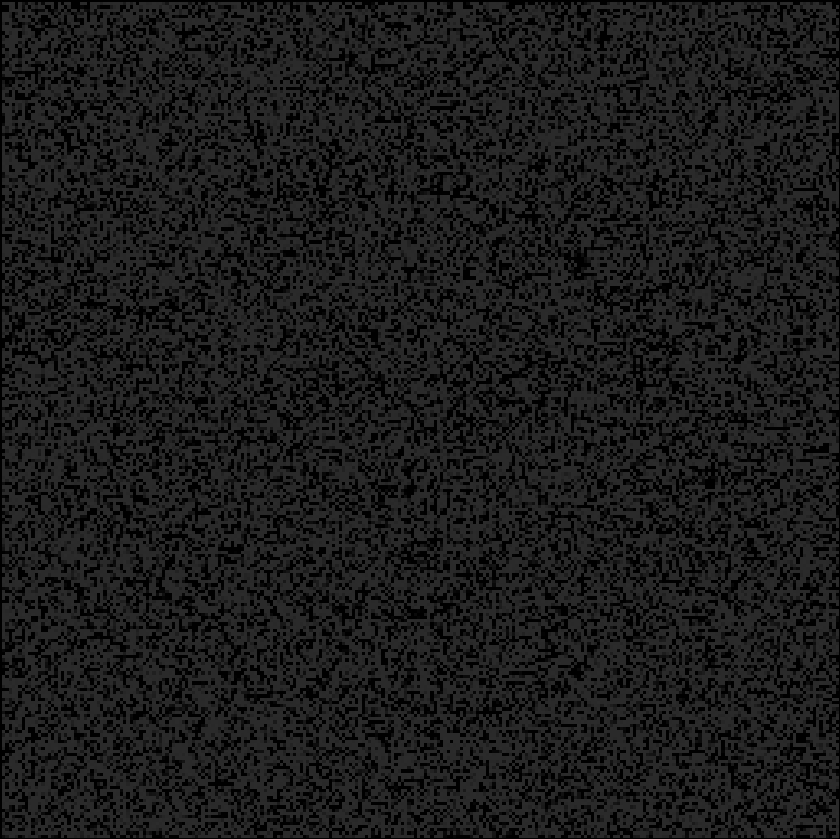
Structure of *D. magnum* Craspase (Cas7-11-TPR-CHAT)



Cas7-11
TRP-CHAT
gRNA/CrRNA



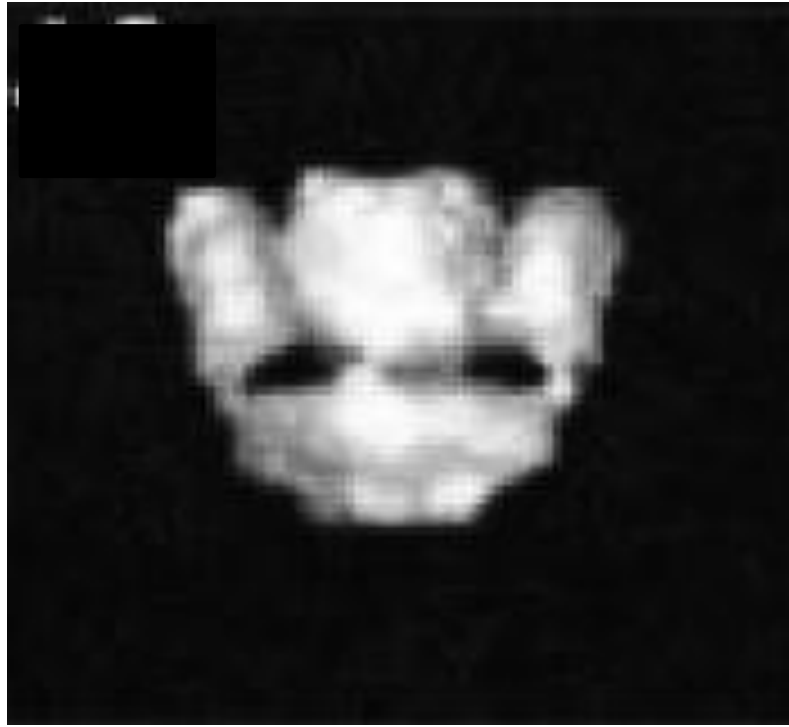
One particle



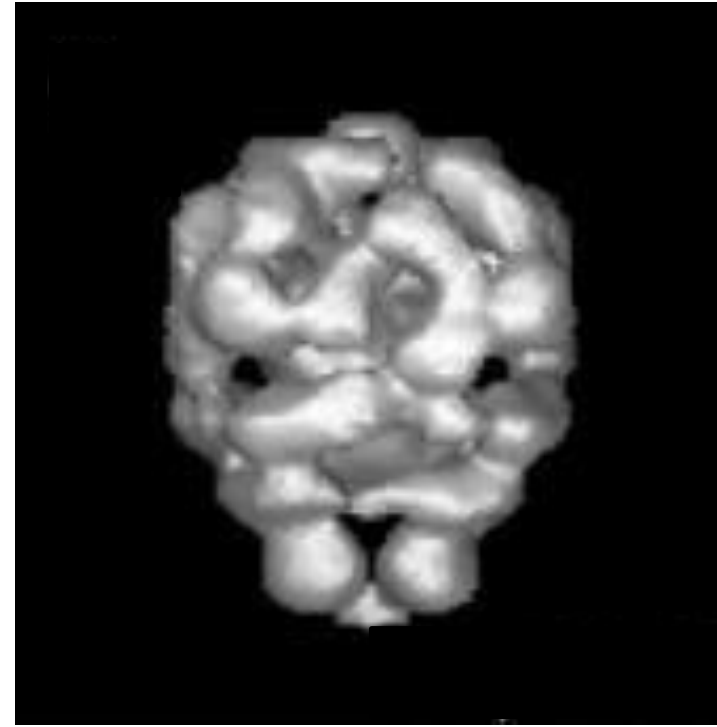
Average

Single Particle Cryo-EM: The “Einstein from Noise” Problem

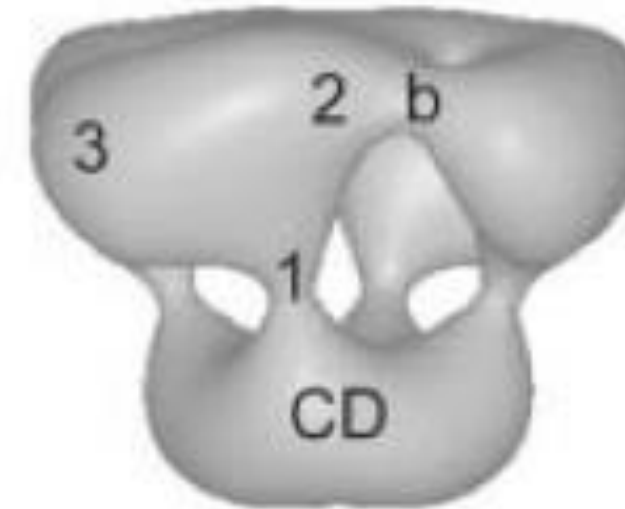
da Fonseca, 2003
Morris, 2010
neg. stain, 30Å



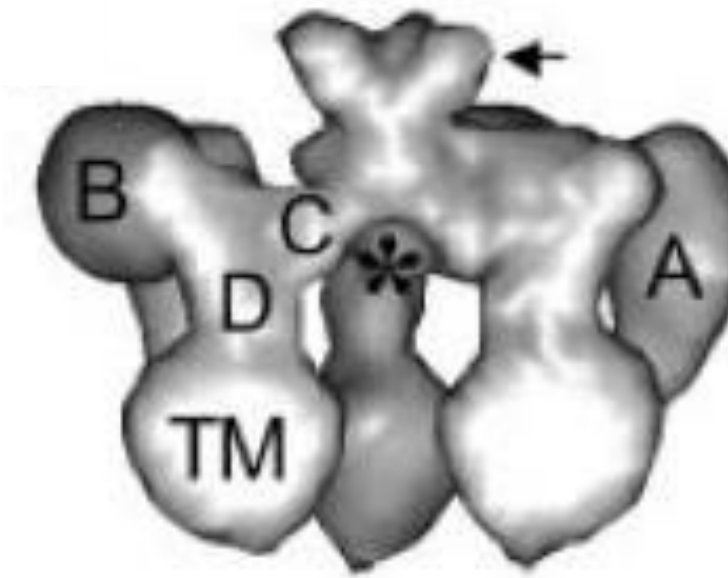
Sato, 2004
cryoEM 15Å



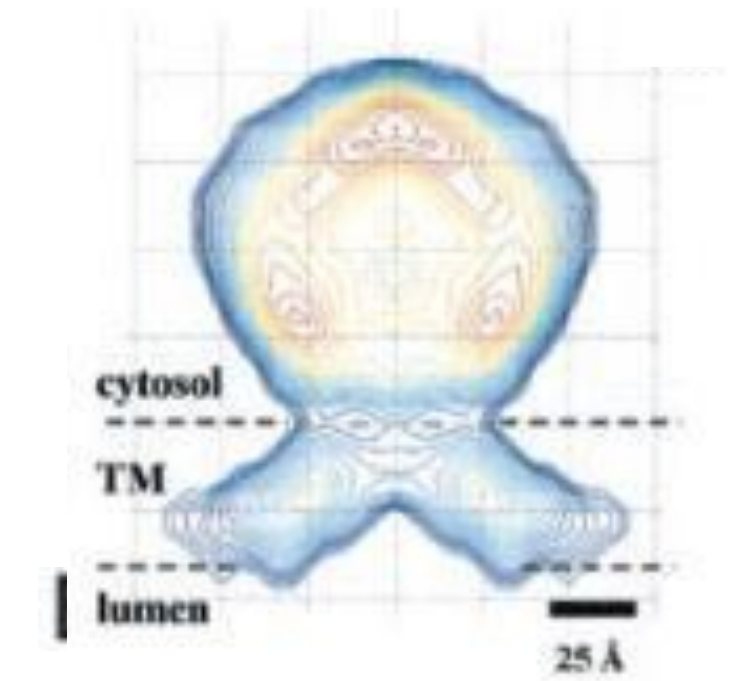
Hamada, 2003
neg. stain 34Å



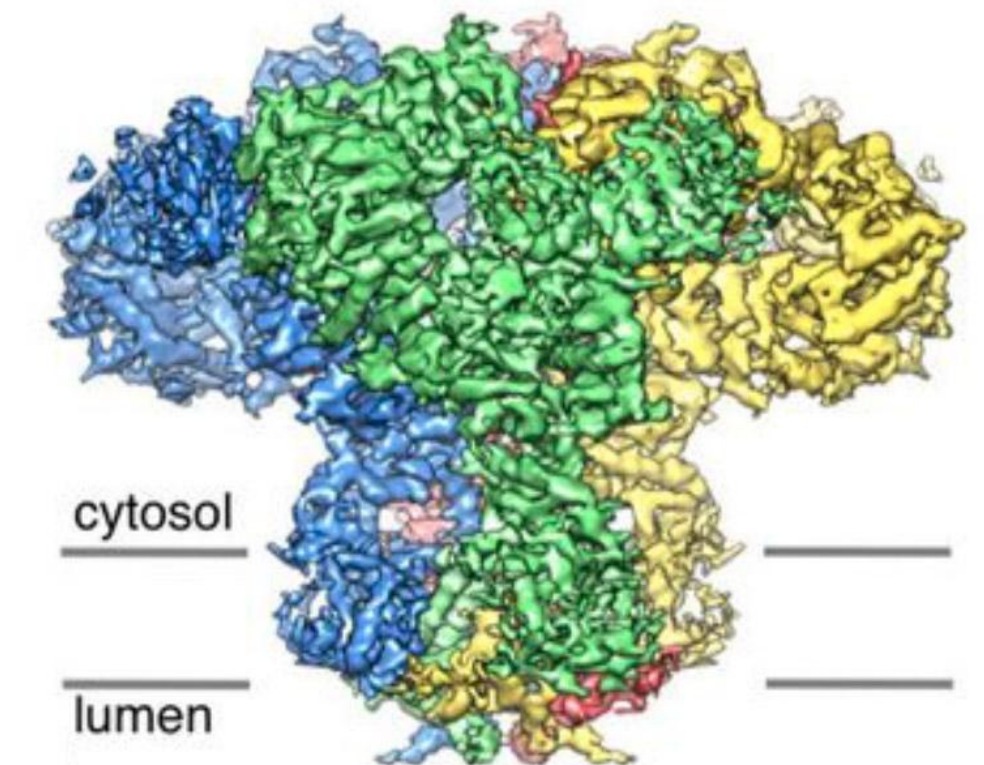
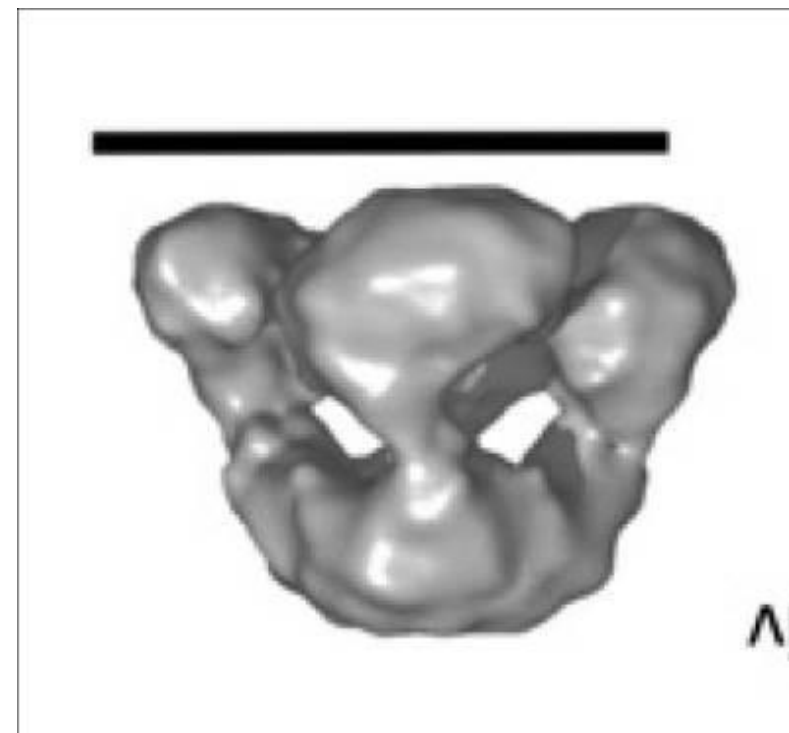
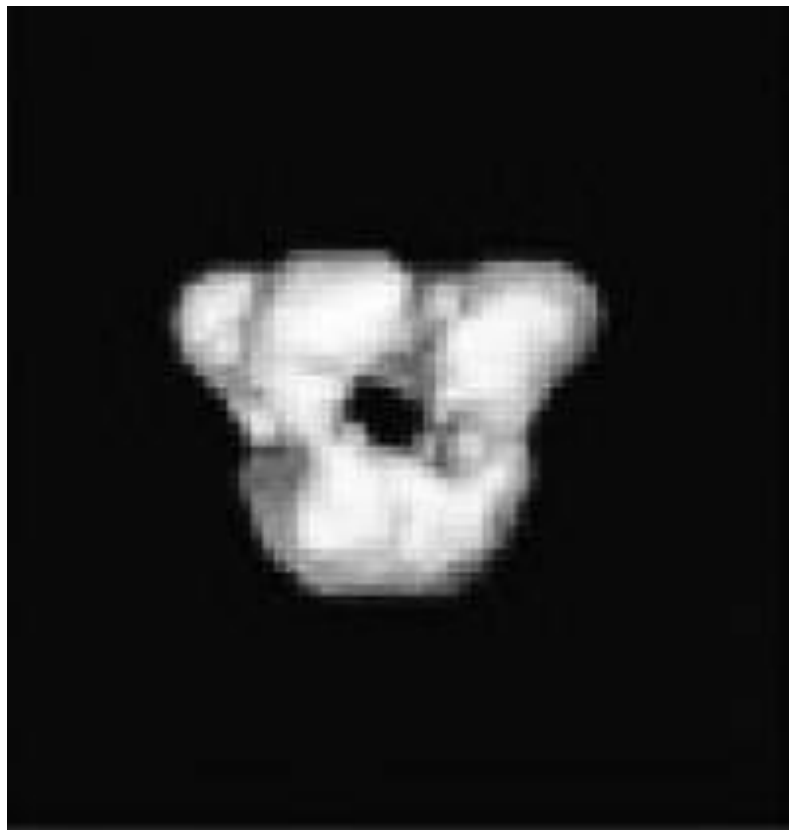
Serysheva, 2003
cryoEM, 30Å



Sigworth, 2002
cryoEM, 24Å

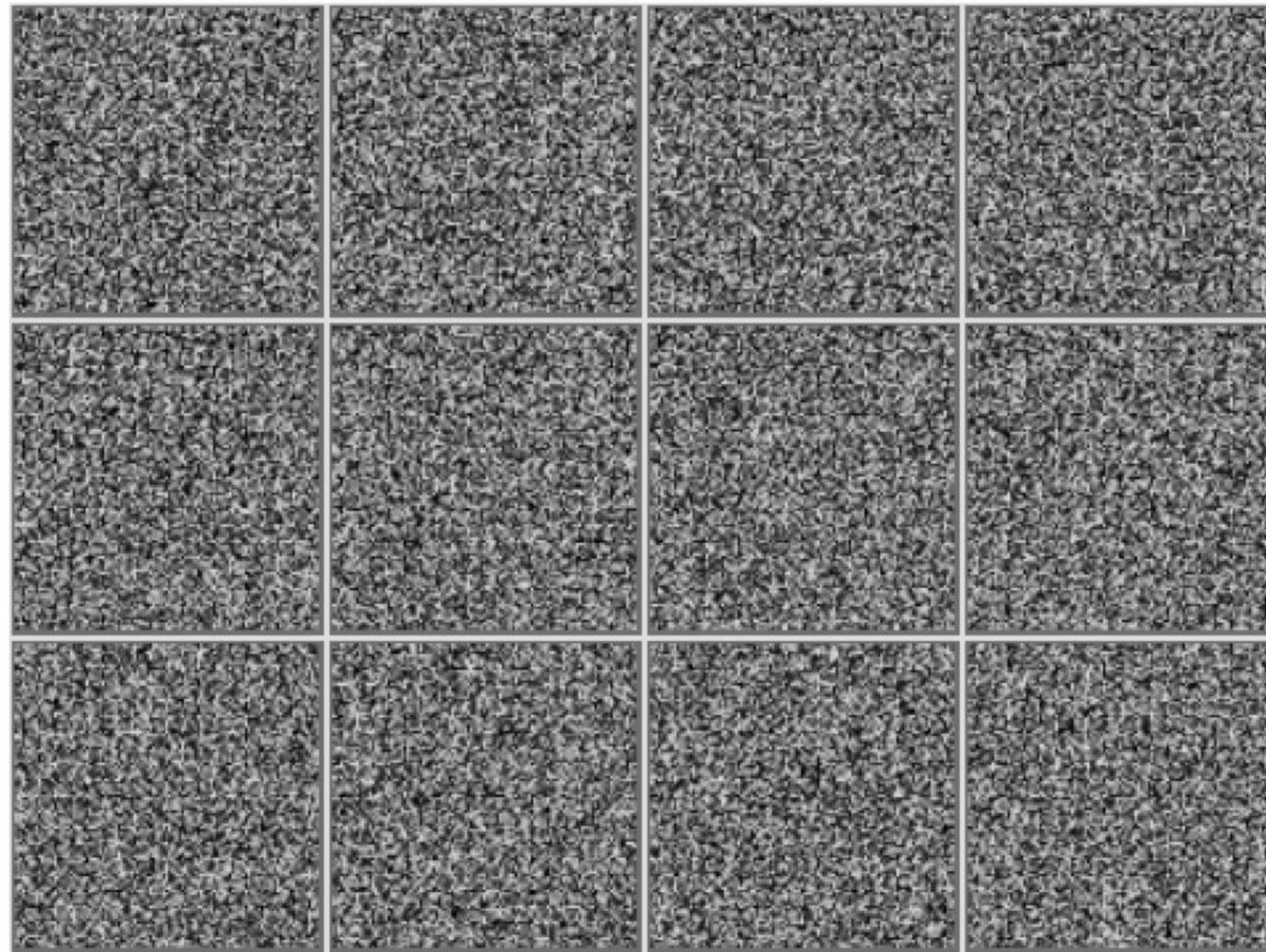


IP3 receptor, 1.3 MDa

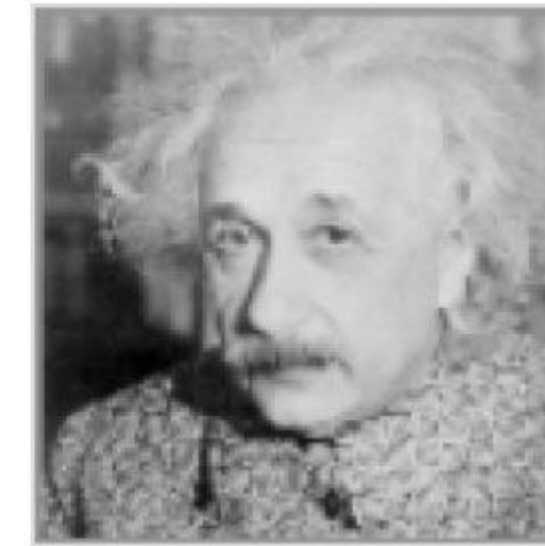


Fan et al., Nature 2015 (4.7Å)

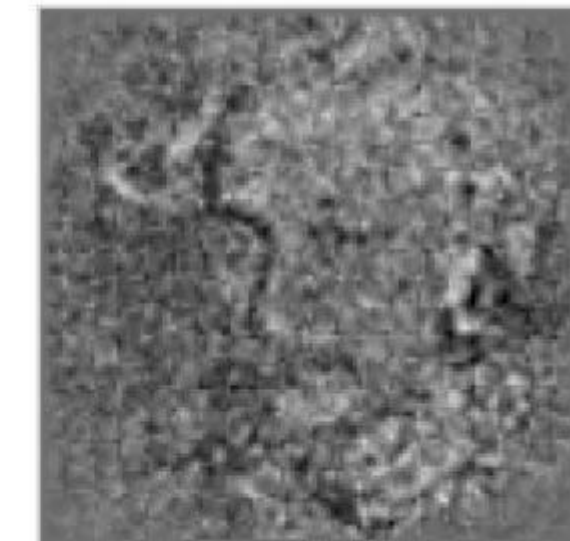
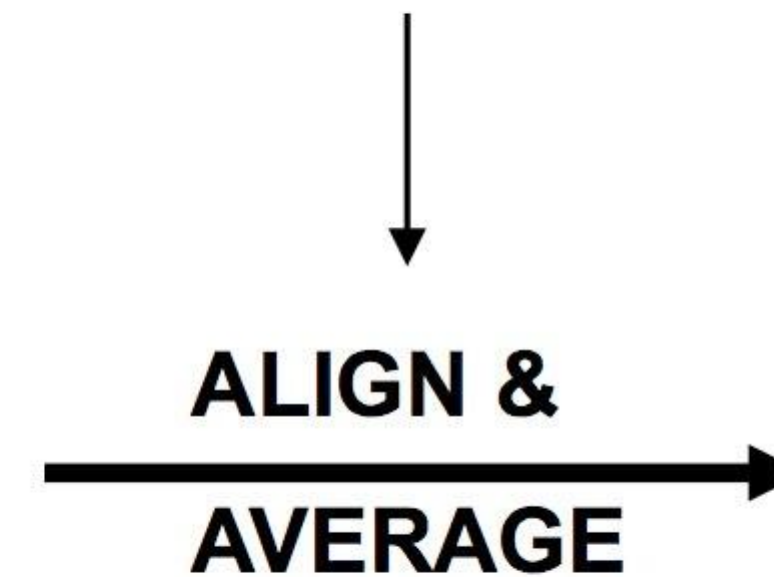
Reference Bias: A dangerous phenomenon.



Random noise



Reference

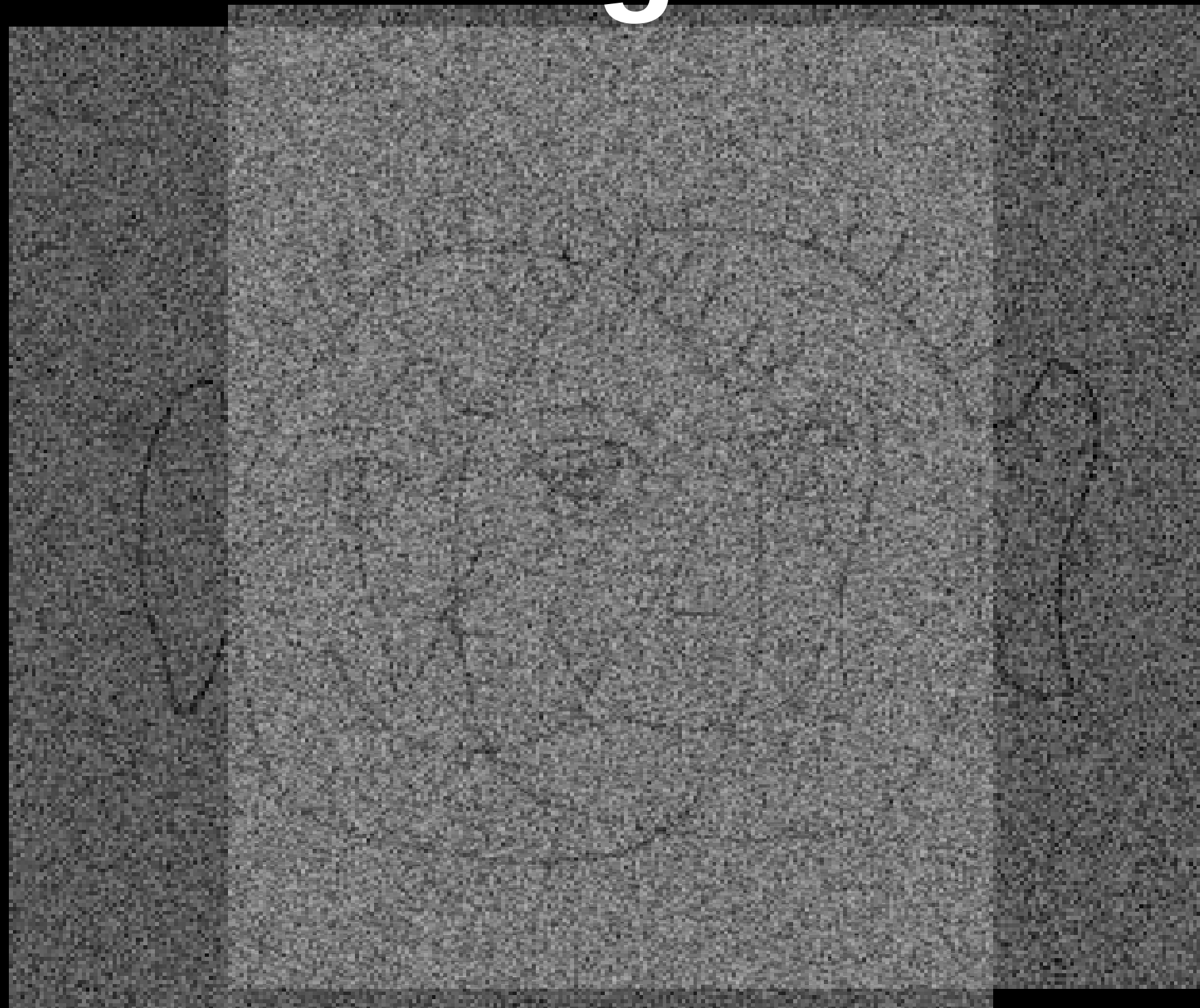


Average

Particle Selection or in Particle Alignment can reproduce the reference feature, even if

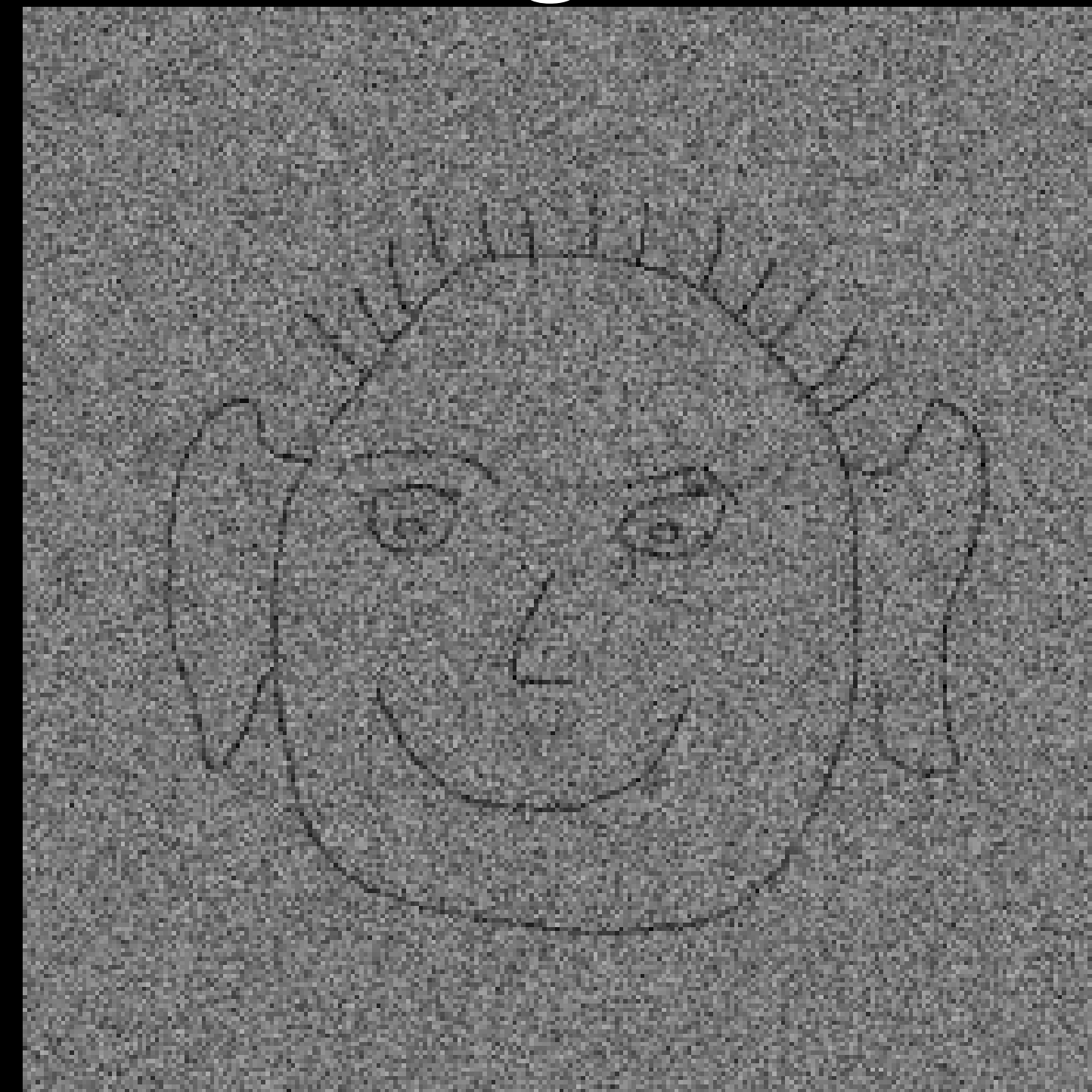
REFERENCE BIAS

**Wrongly
Aligned**



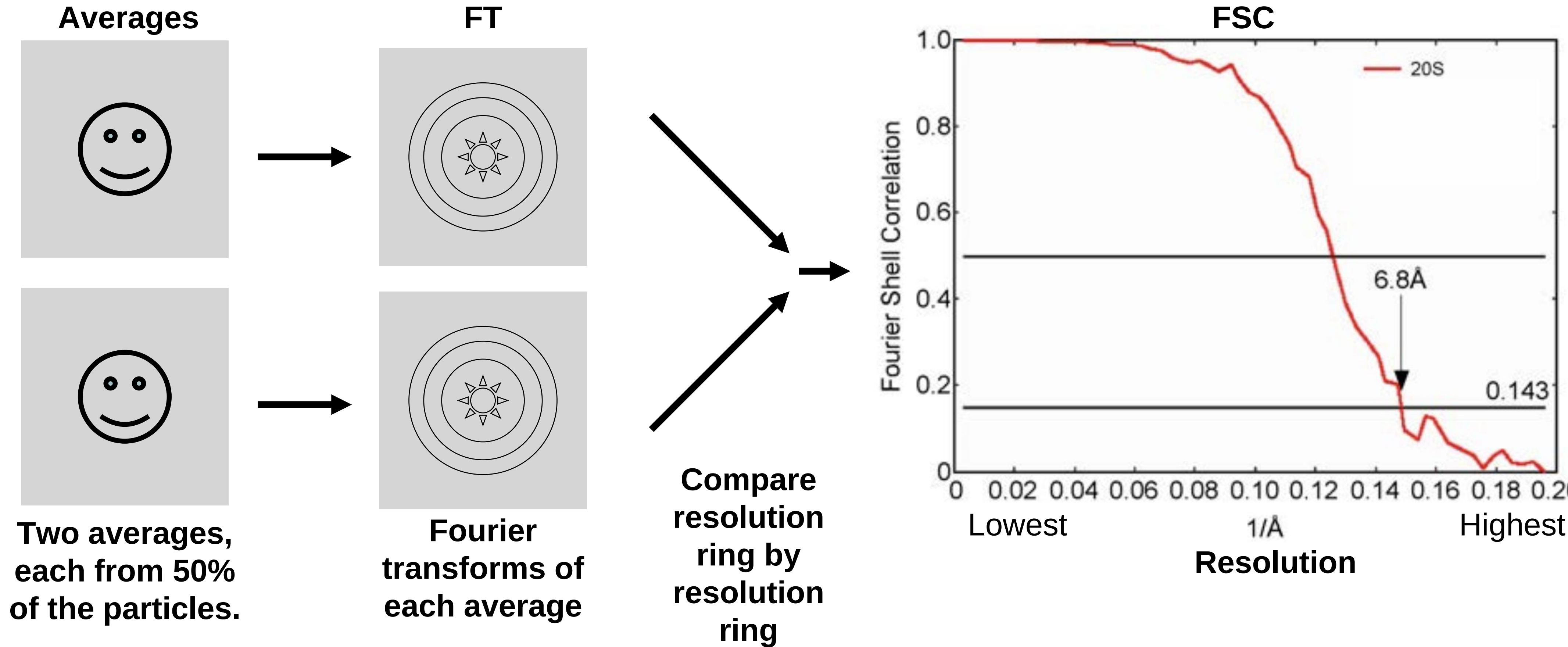
Three eyes

**Correctly
Aligned**

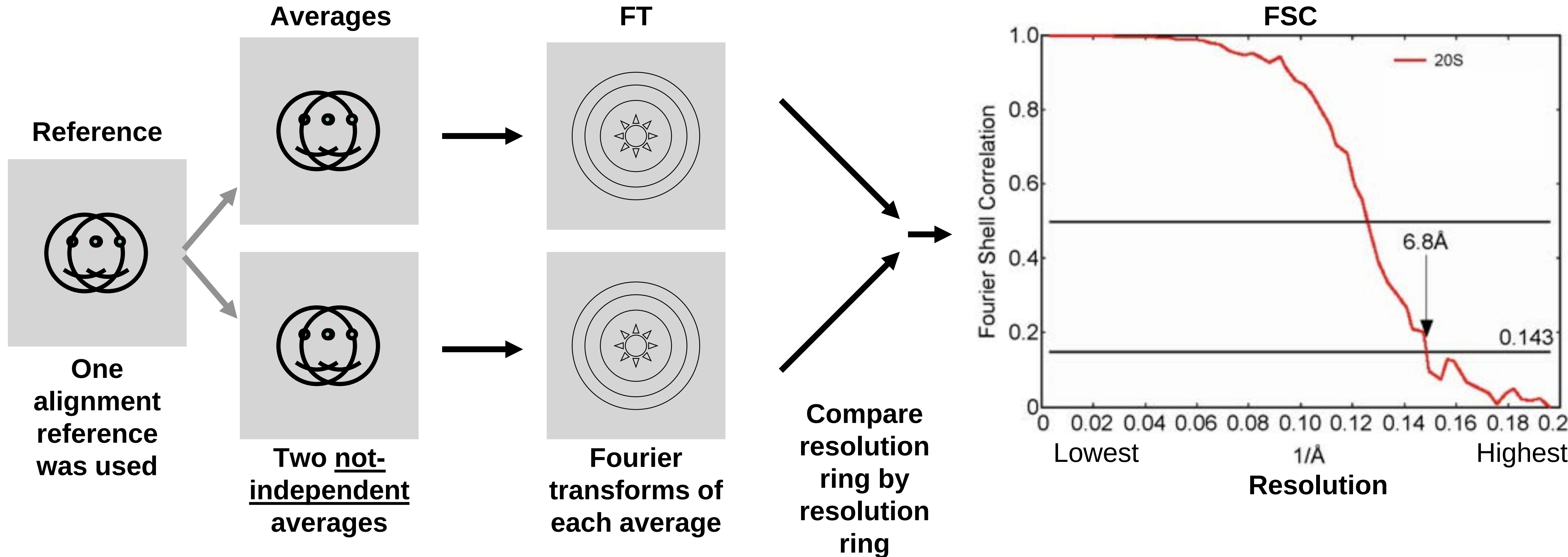


Two eyes

Resolution: Fourier ring correlation



Reference Bias Problem leads to apparently high resolution



So-called “Gold Standard” Method

Work with two half-sets of the particles fully separated from A-Z.
(You will need twice as many particles)

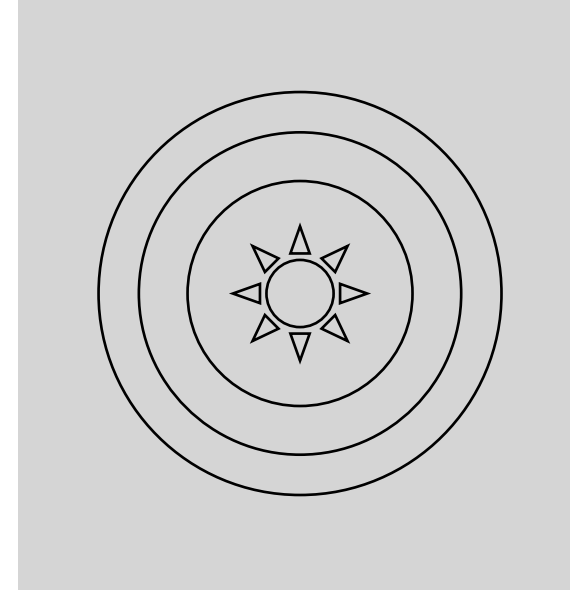
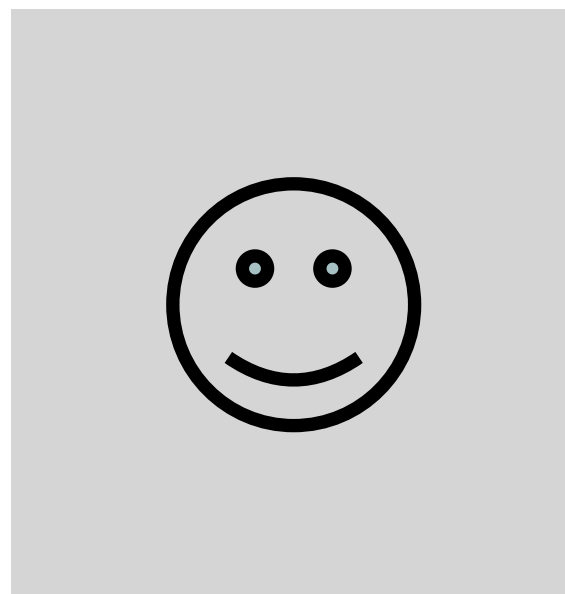
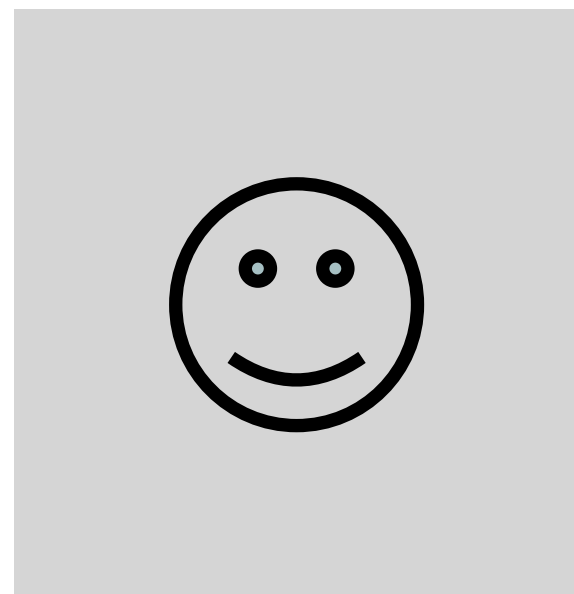
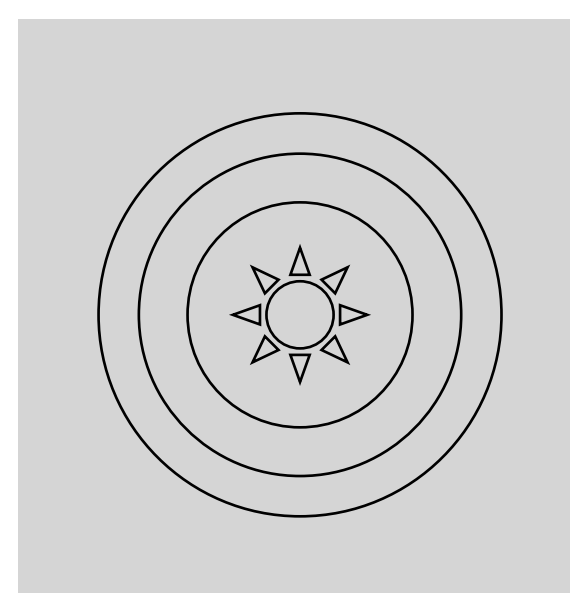
References



Averages



FT

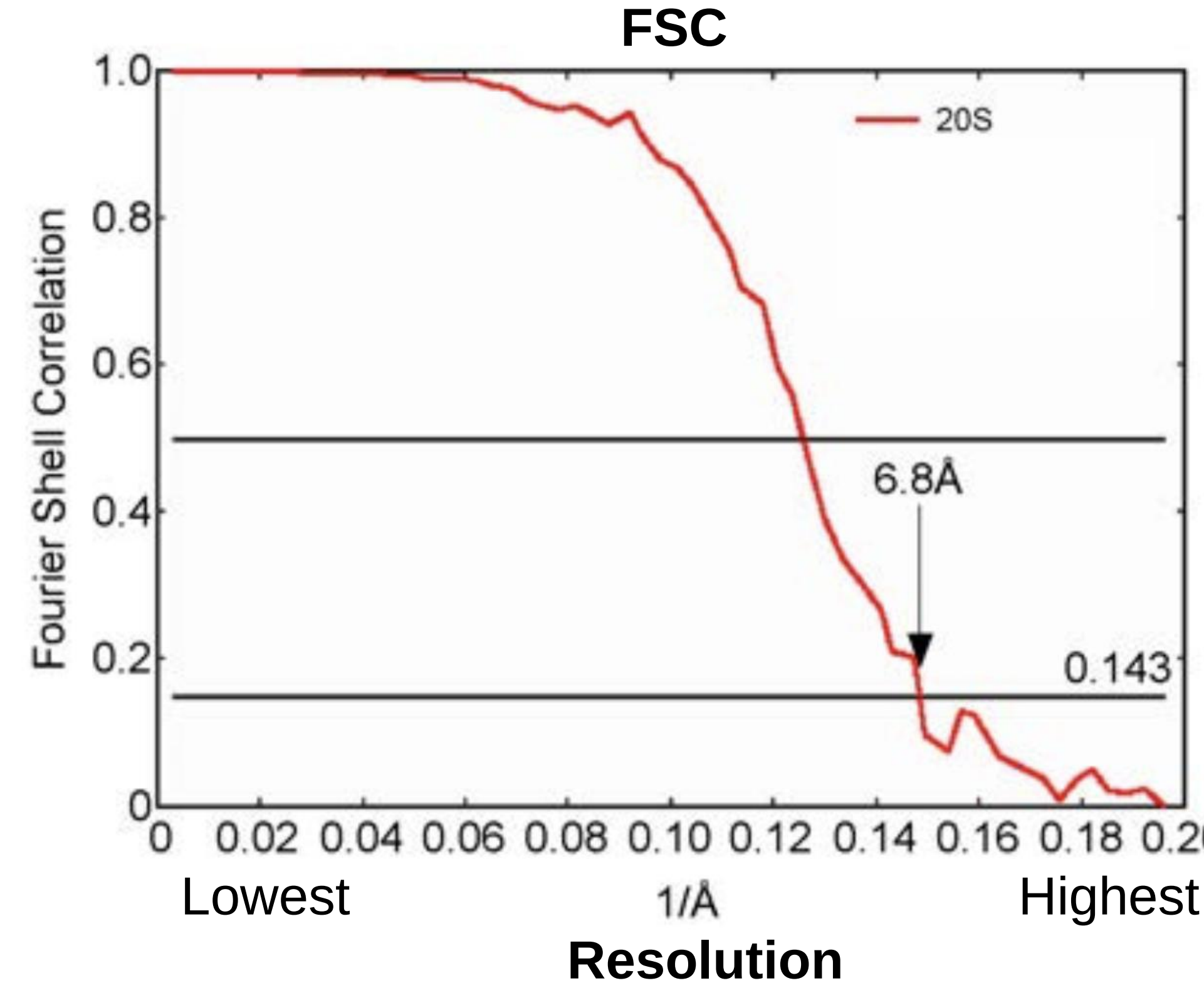


Two fully
separate
alignment
references
were used

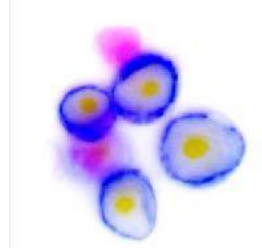
Two fully
independent
averages

Fourier
transforms of
each average

Compare
resolution
ring by
resolution
ring



Single Particle Cryo-EM



Expression



Purification



Sample Preparation



Imaging

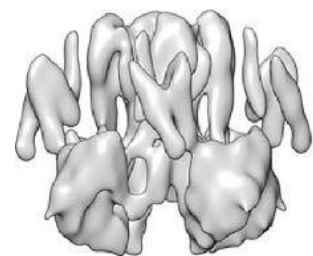
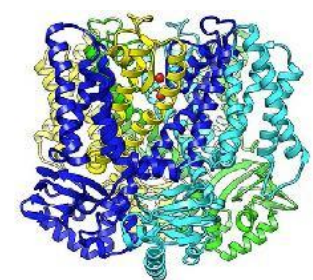


Image Processing



Model Building

- **Stage 1: Is my sample suitable (pure, homogeneous, stable)?**
 - Preparation of negatively stained grids
 - TEM imaging @ 120kV
 - Computer analysis for sample homogeneity control
- **Stage 2: Can my sample be frozen well?**
 - Preparation of frozen grids
 - Cryo-EM imaging @ 200kV
 - Computer analysis for sample quality control
- **Stage 3: Get the high-resolution structure**
 - Identification of the optimal, frozen grid
 - Cryo-EM imaging @ 300kV
 - Computer analysis for 3D structure reconstruction



Single Particle Cryo-EM



Expression



Purification



Sample
Preparation



Imaging

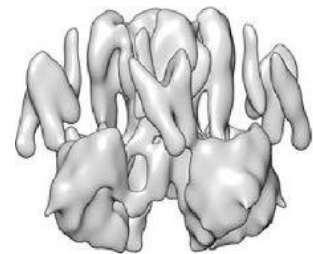
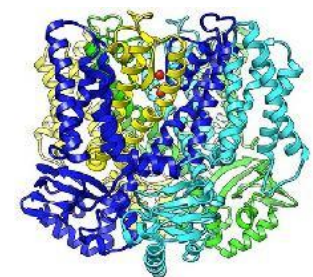
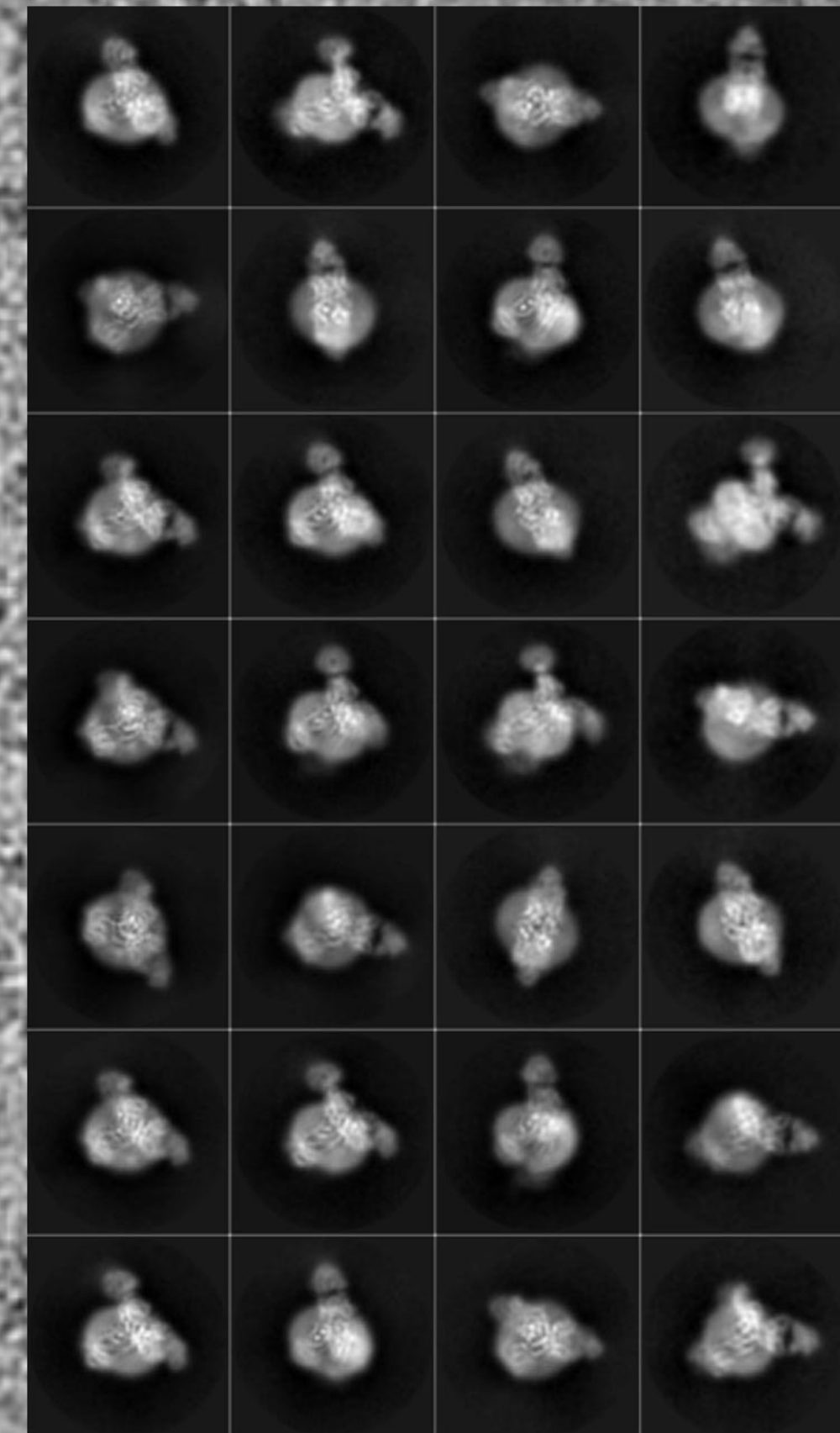


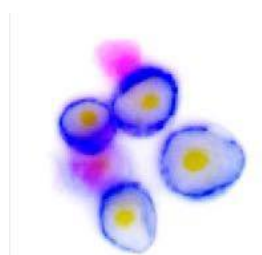
Image Processing



Model Building



Cryo-EM of the human ABC transporter ABCG2



Expression



Purification



Sample
Preparation



Imaging

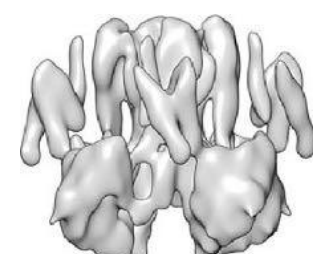
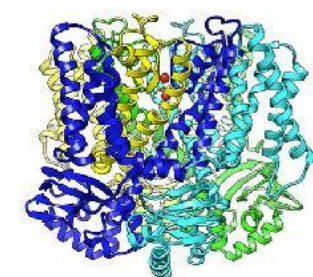
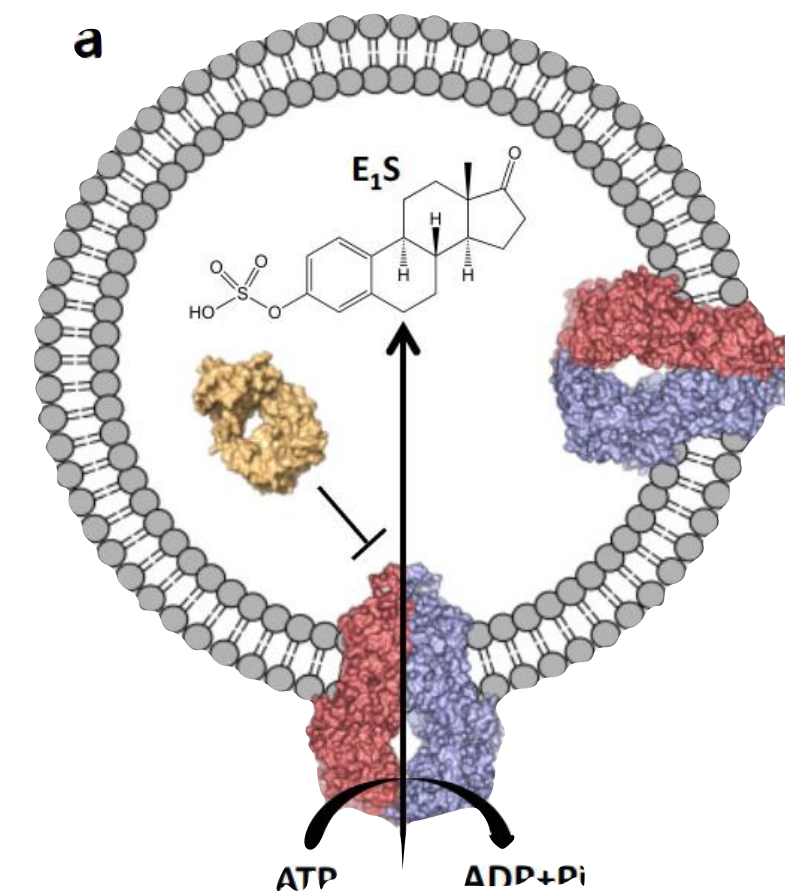
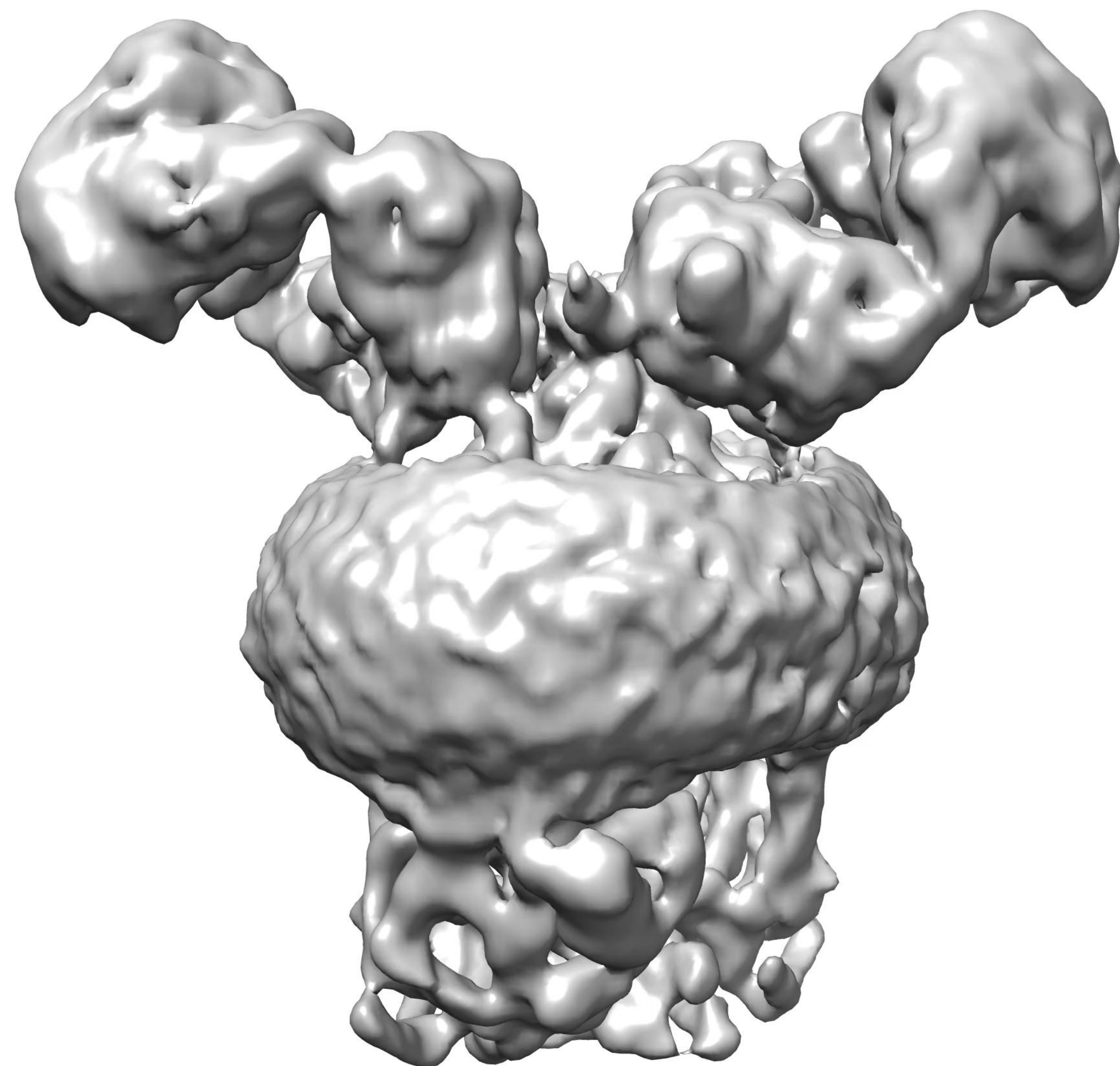


Image Processing



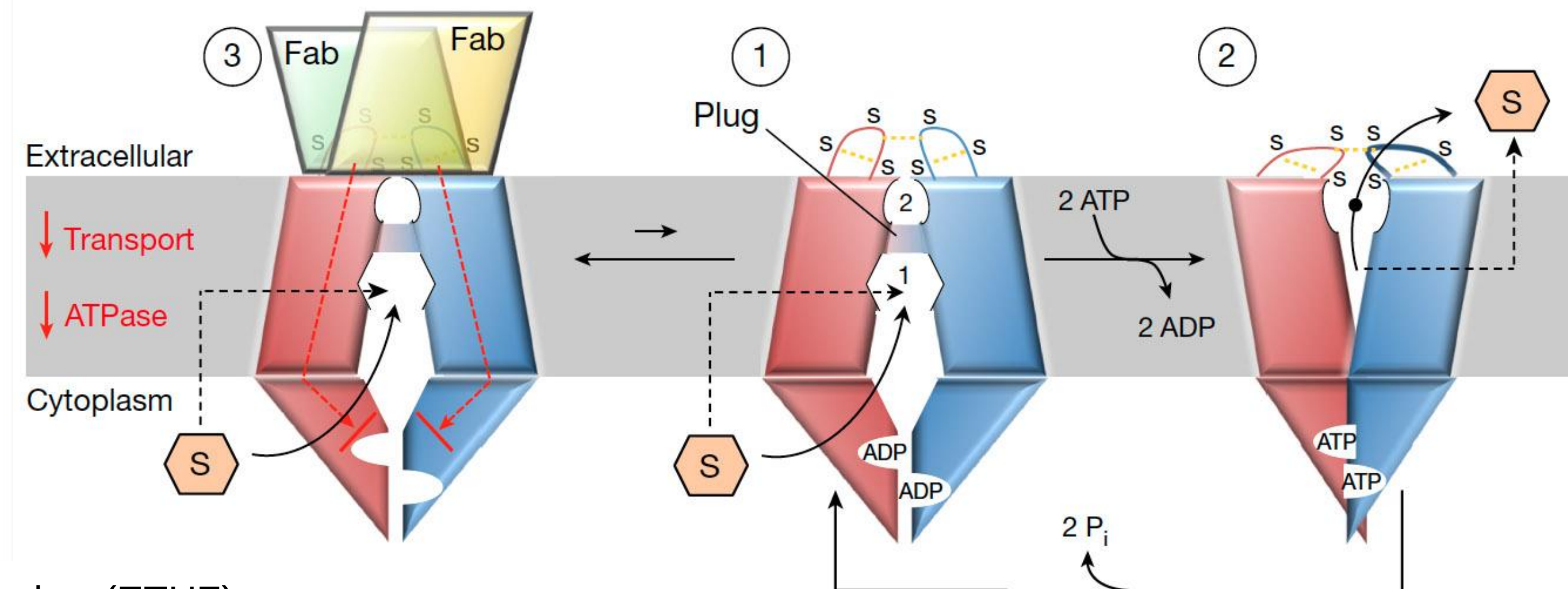
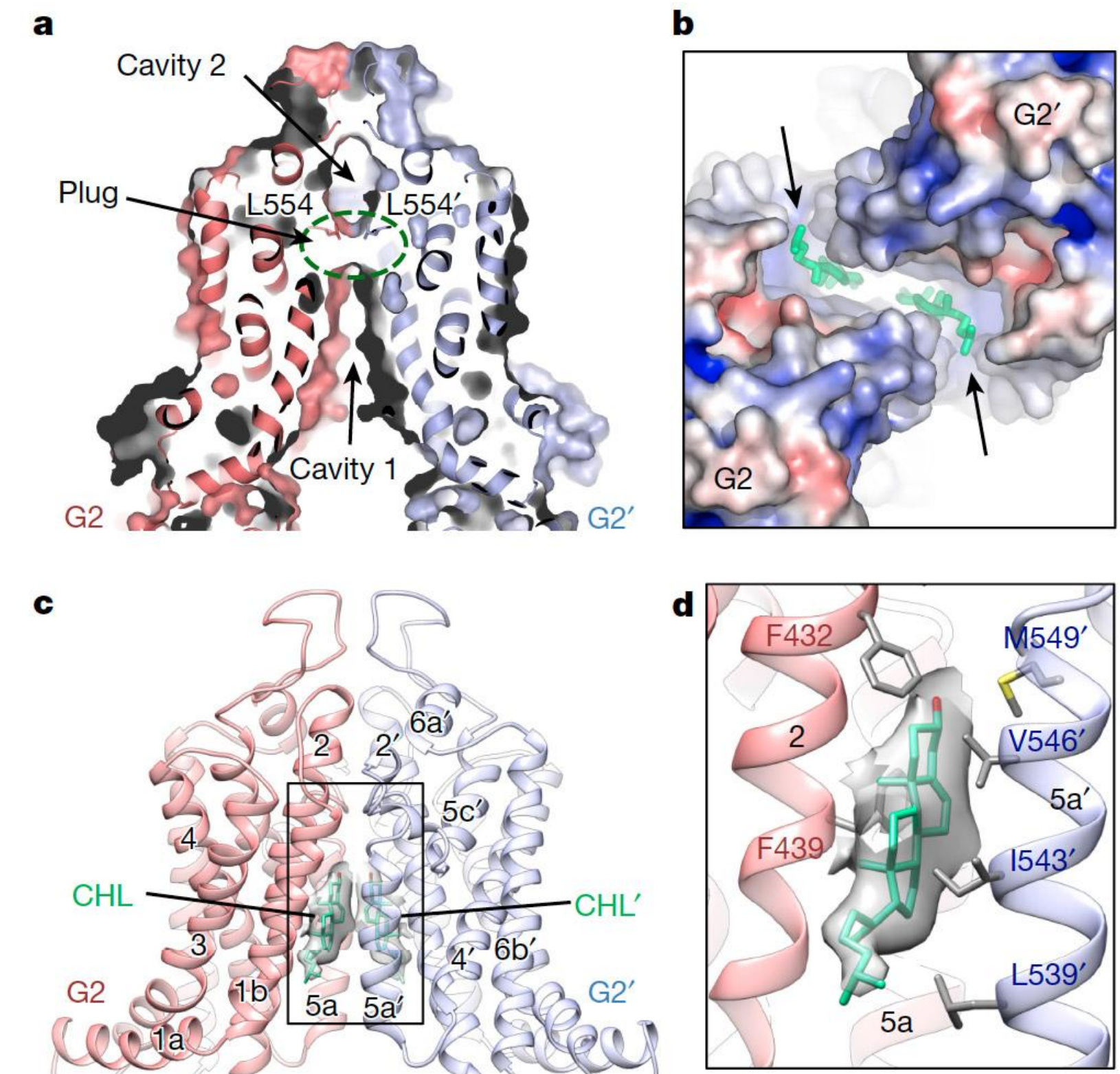
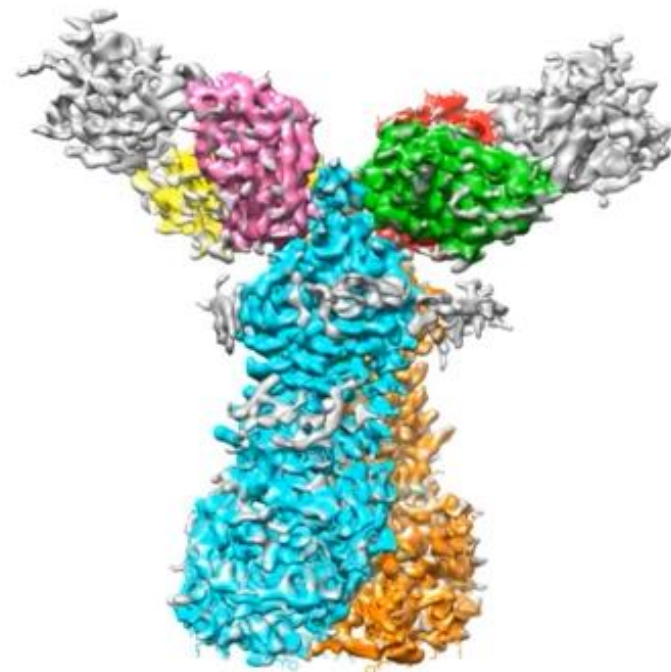
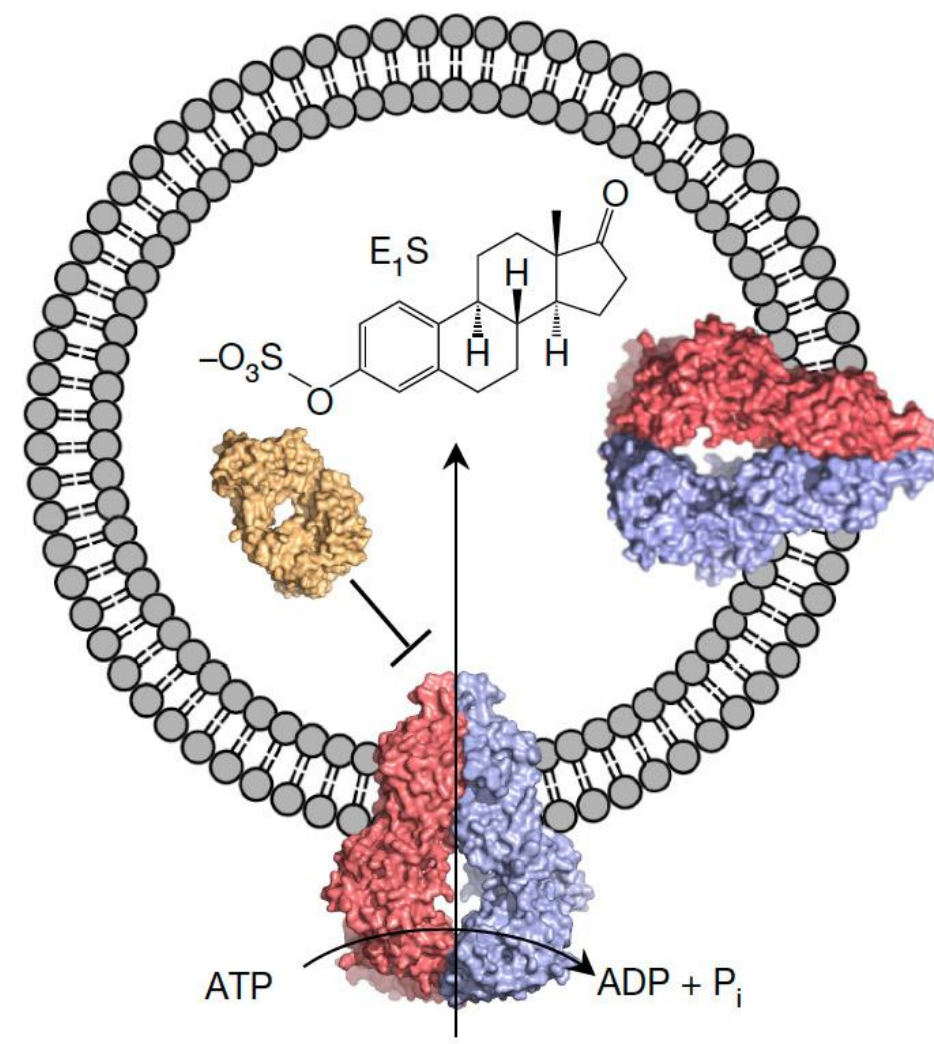
Model Building



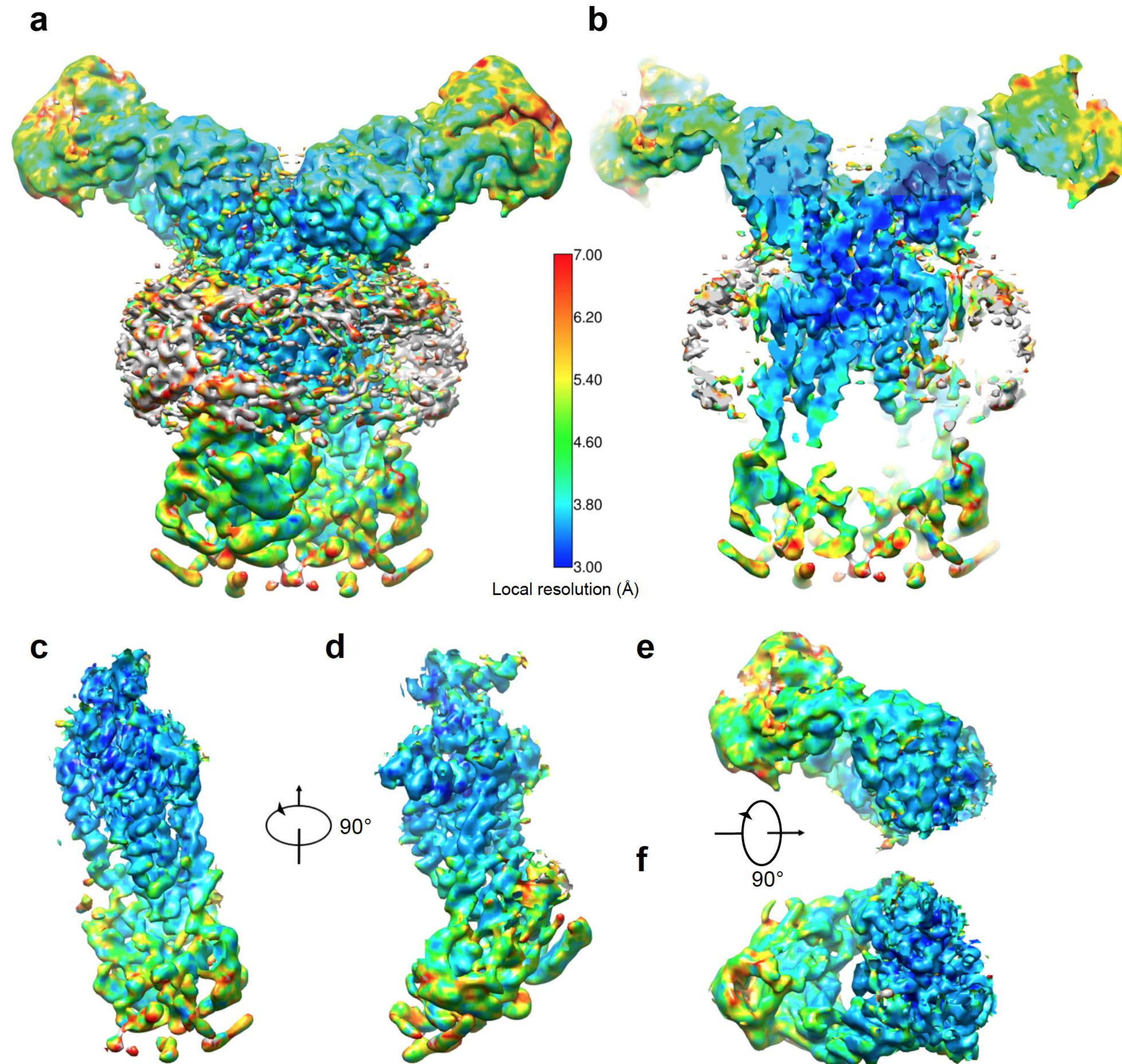
Membrane protein
in a lipid nanodisk.
Data collection:
3 days on C-CINA Titan,
1702 movies,
232'608 particles
97'612 particles in final map.
Final resolution 3.8Å

Collaboration with Kaspar Locher, ETHZ

Cryo-EM Structure of a human ABC Transporter



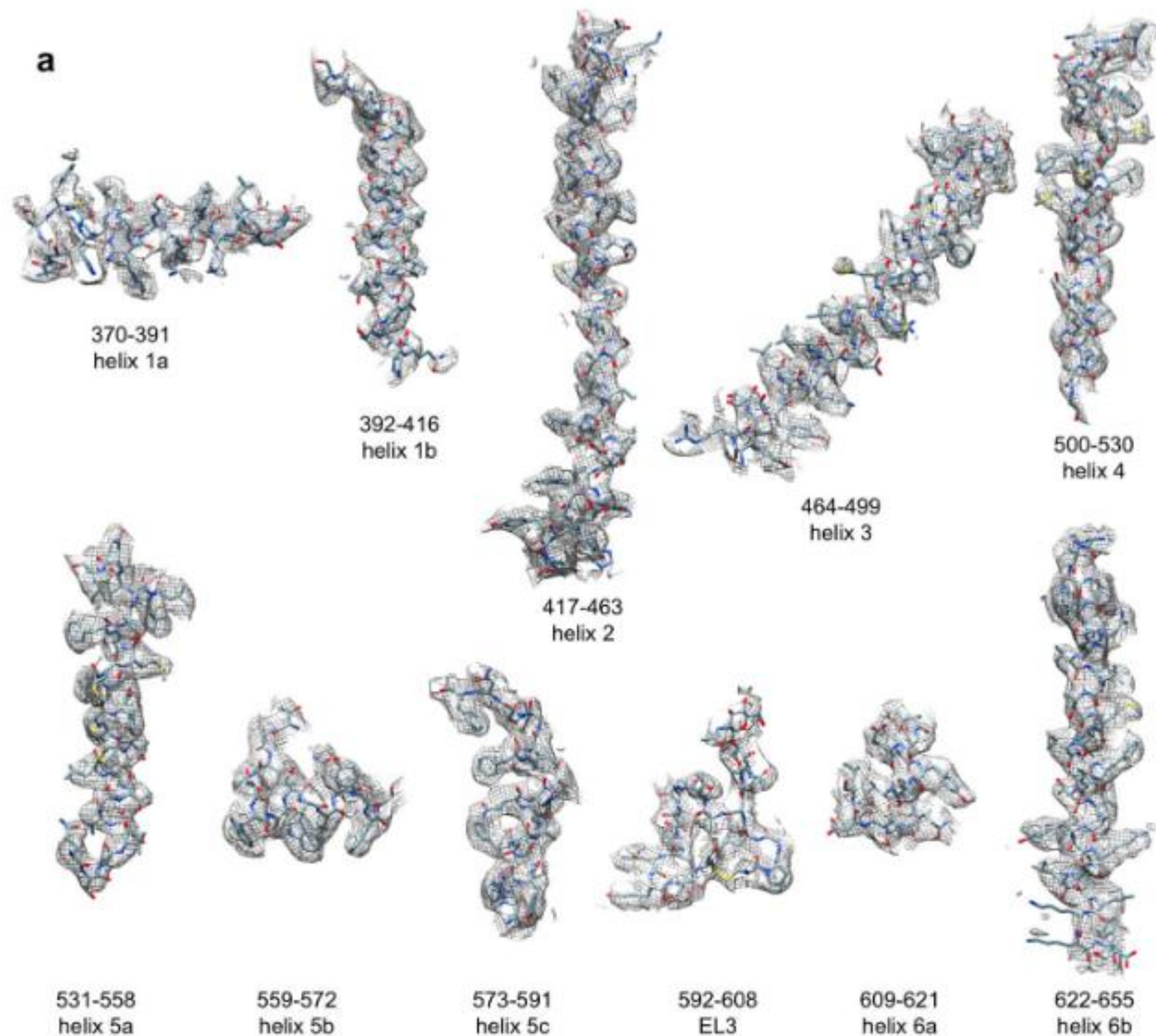
Cryo-EM of the human ABC transporter ABCG2



This plot shows the “local resolution” of the final 3D map, which ranges from 3.0 to 7.0 Å, on average 3.8Å.

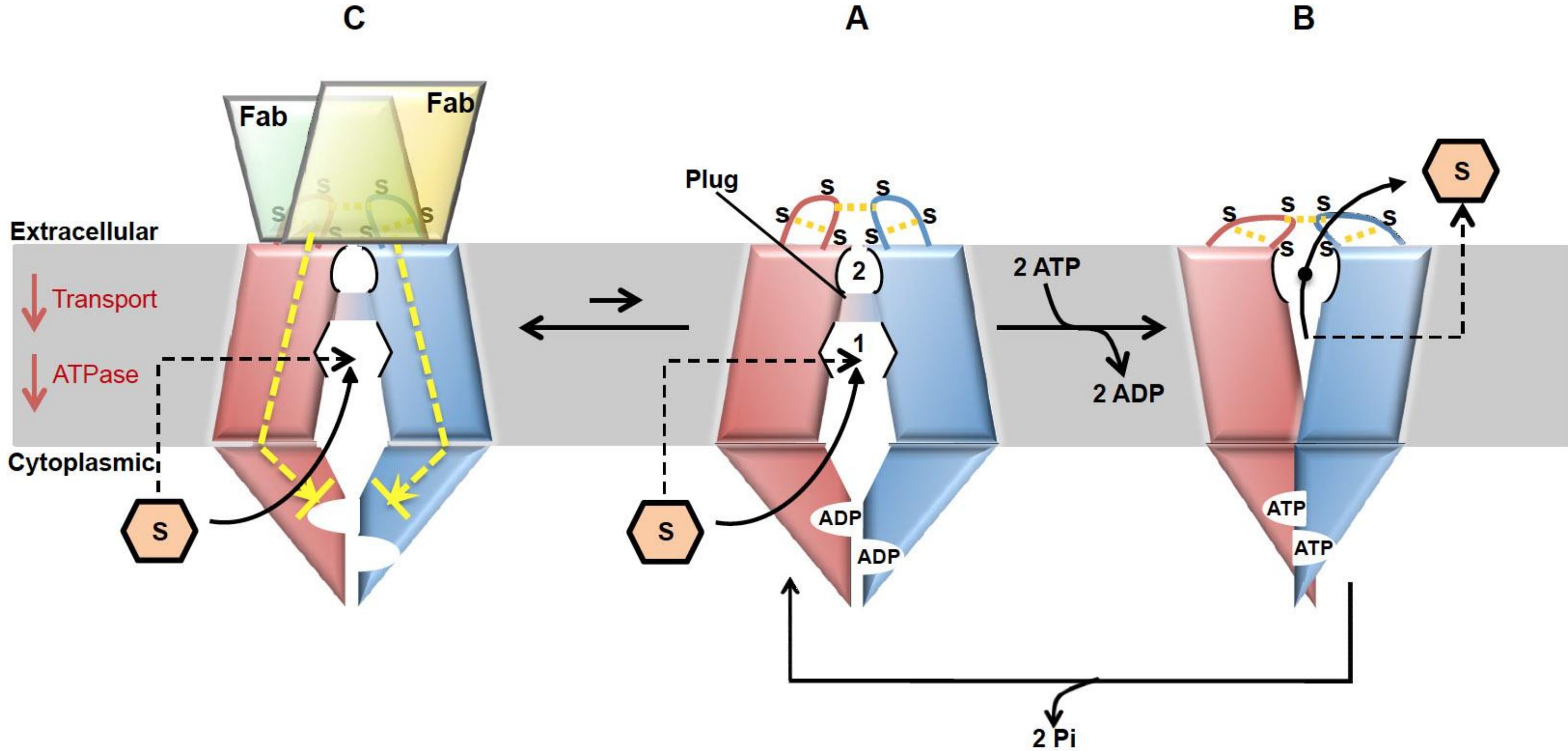
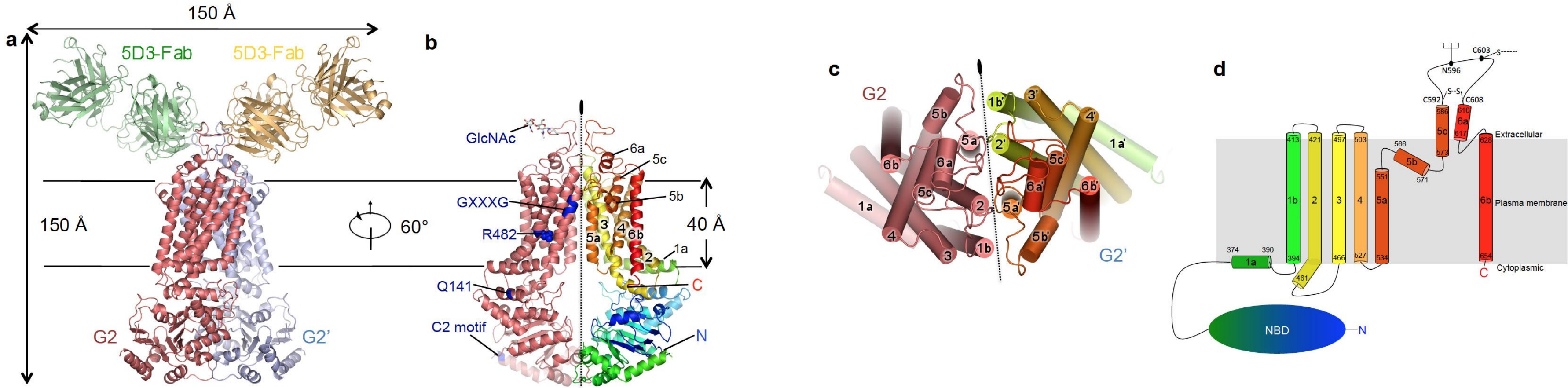
Structure of the human multidrug transporter ABCG2

Nicholas M. I. Taylor^{1*}, Ioannis Manolaridis^{2*}, Scott M. Jackson^{2*}, Julia Kowal², Henning Stahlberg¹ & Kaspar P. Locher²

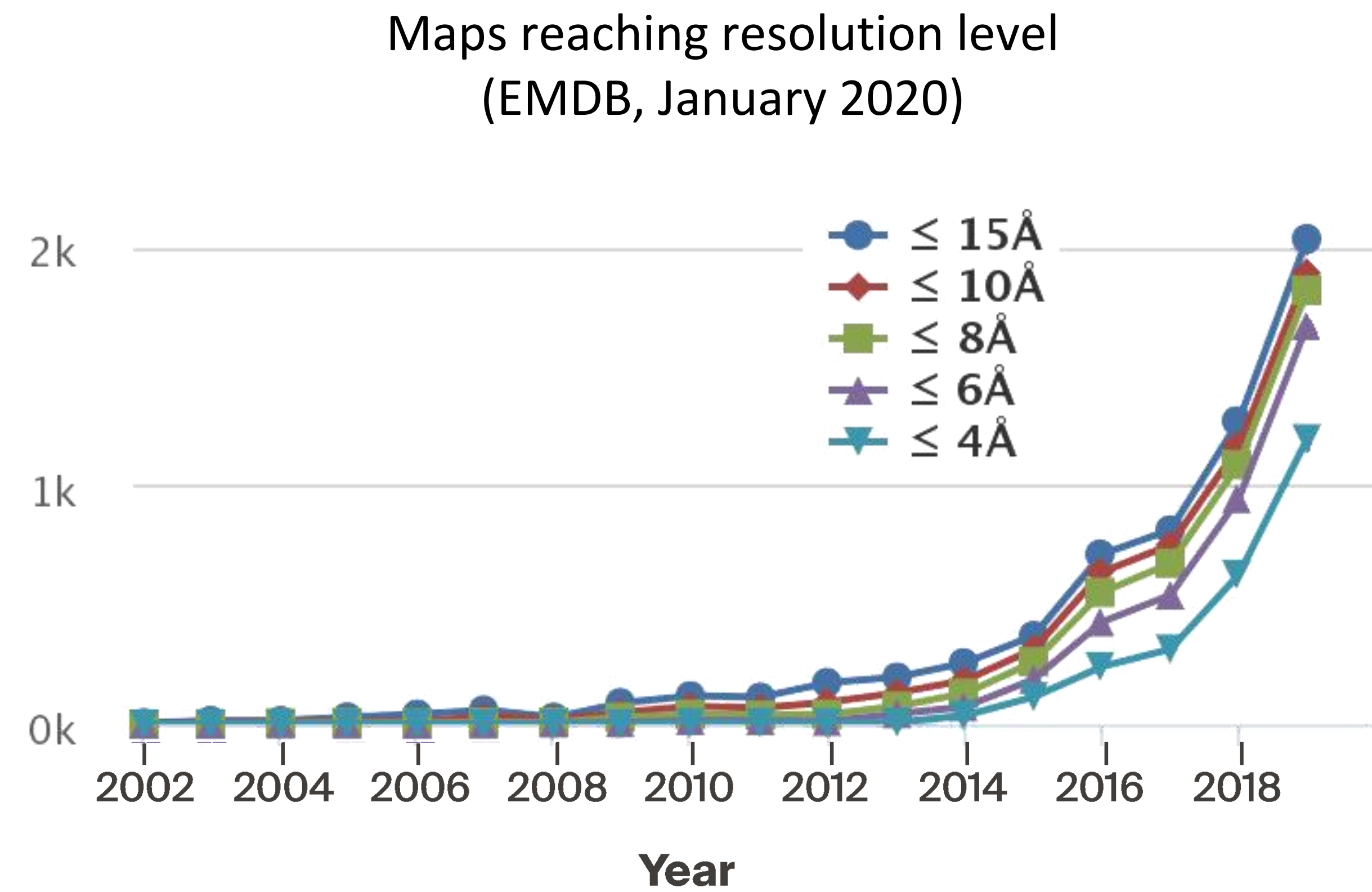
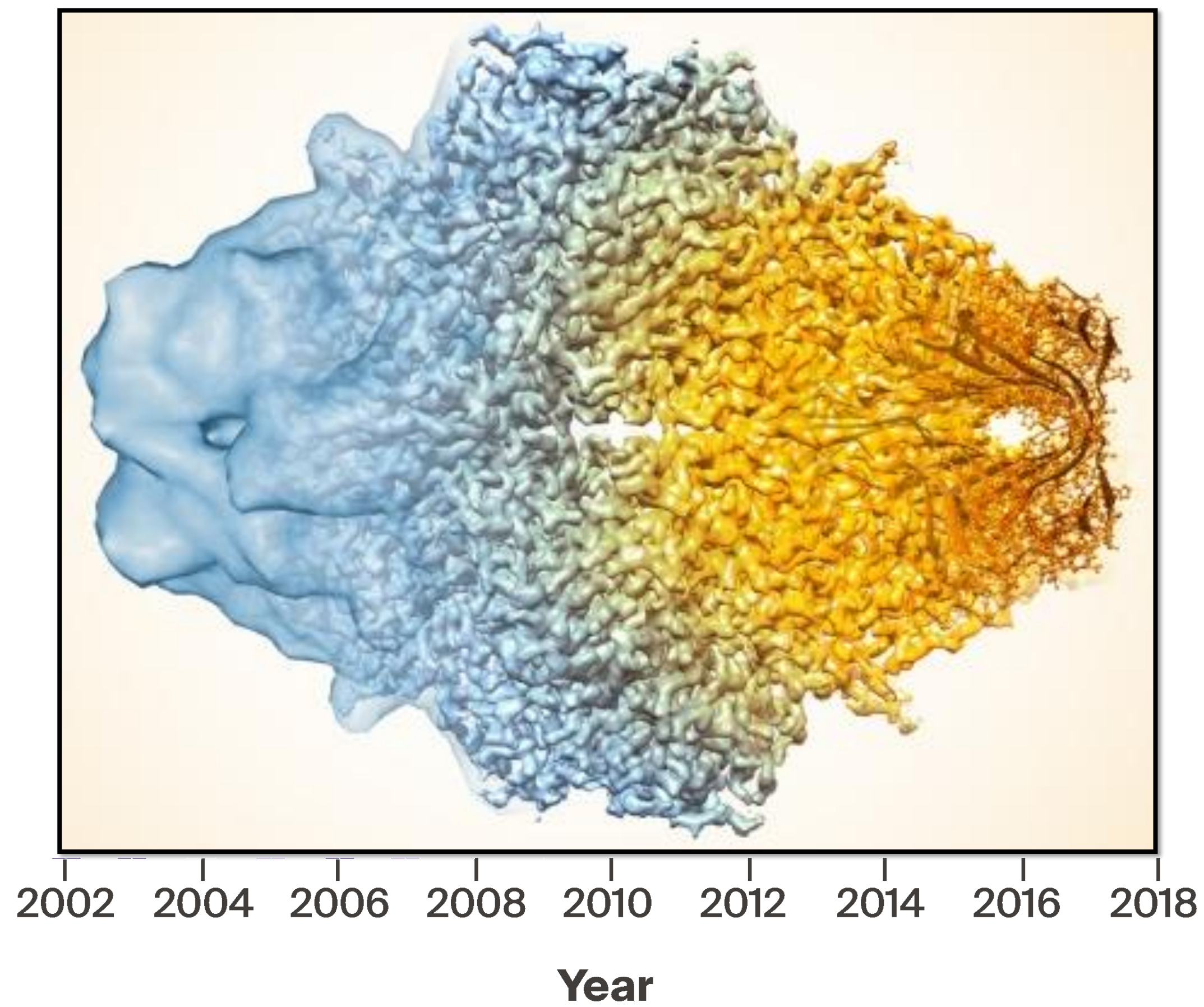


Membrane protein
in a lipid nanodisk.
Data collection:
3 days on C-CINA Titan,
1702 movies,
232'608 particles
97'612 particles in final map.
Final resolution 3.8Å

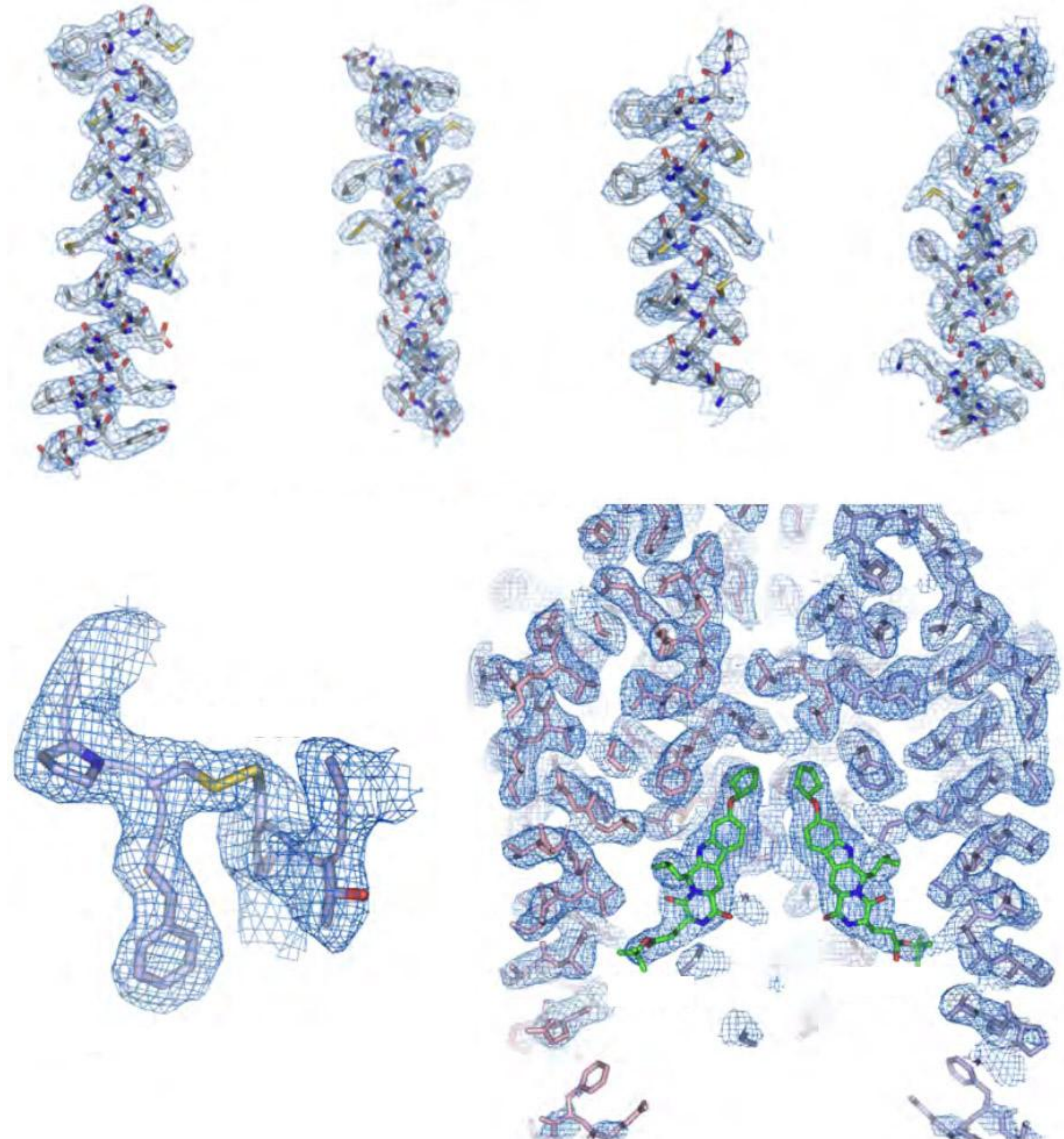
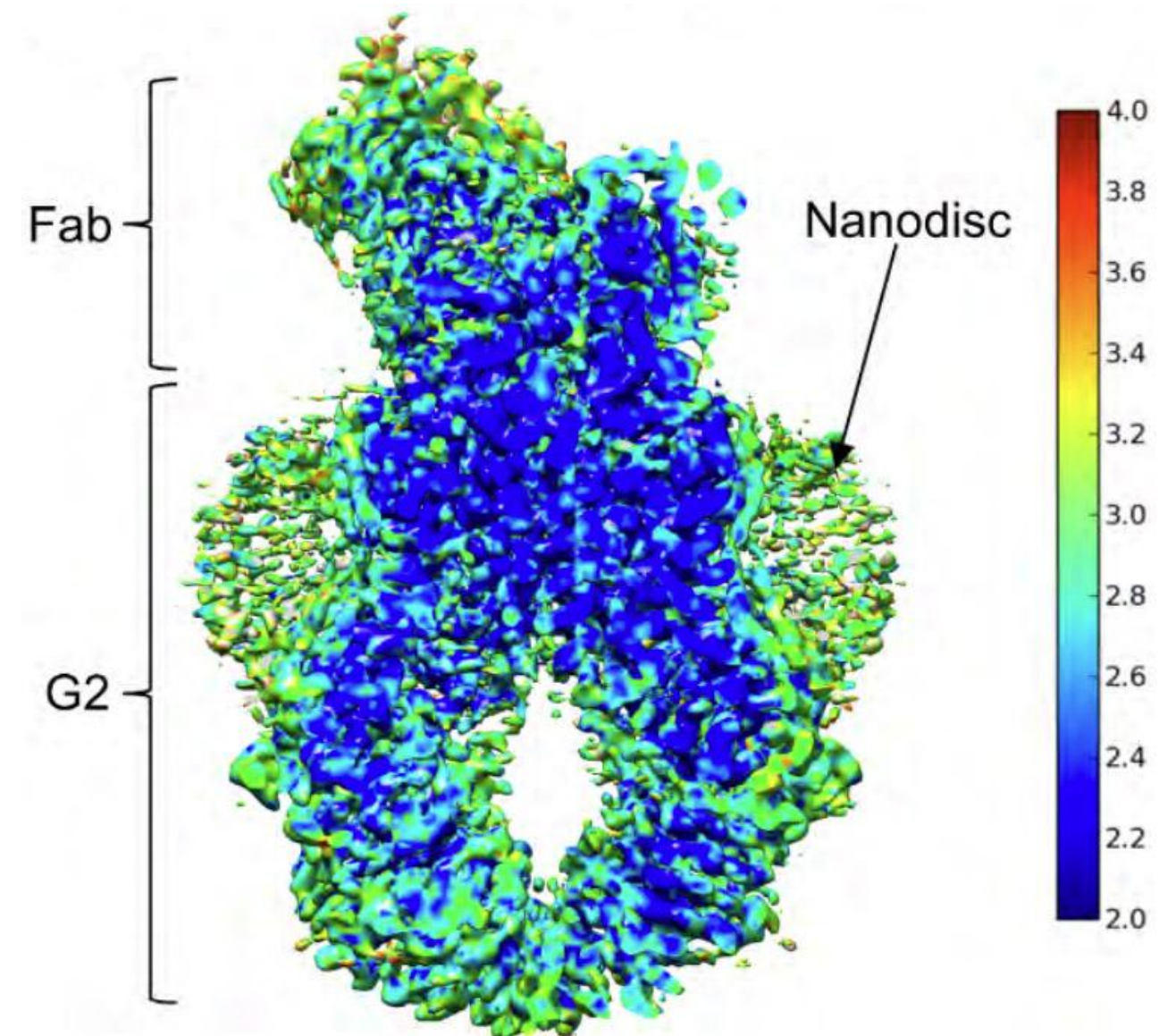
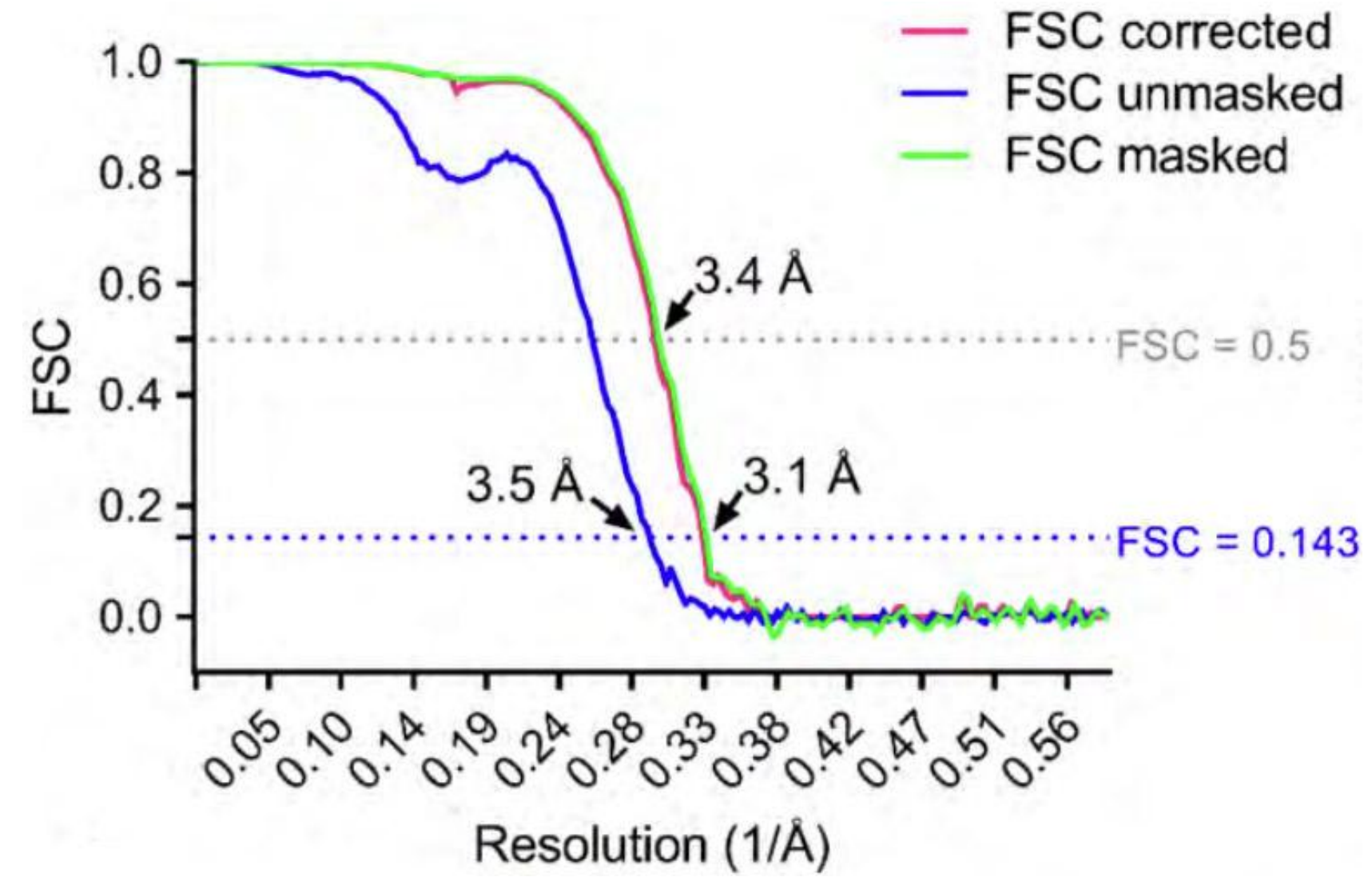
Cryo-EM of the human ABC transporter ABCG2



The Resolution Revolution in Cryo-EM

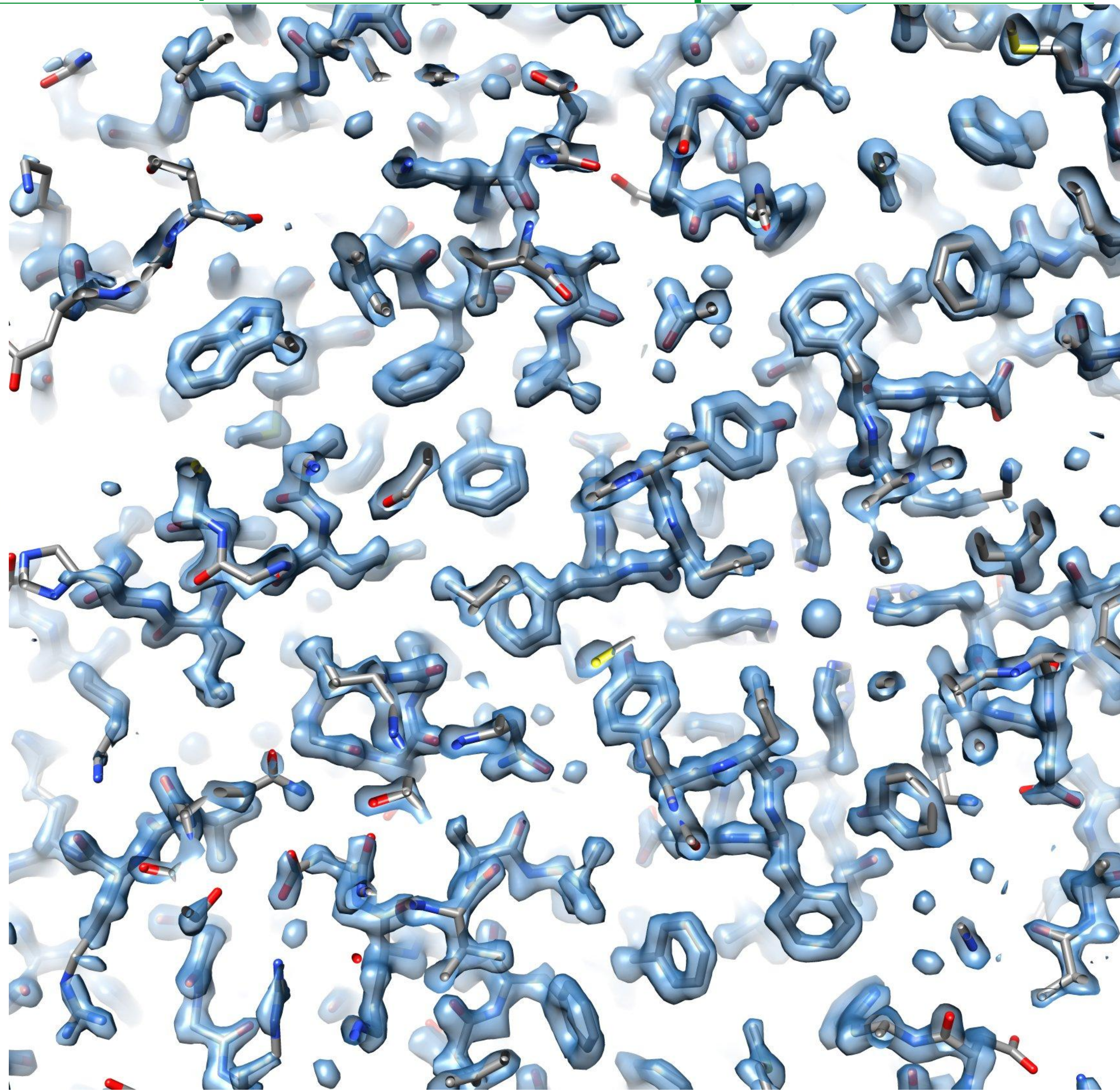
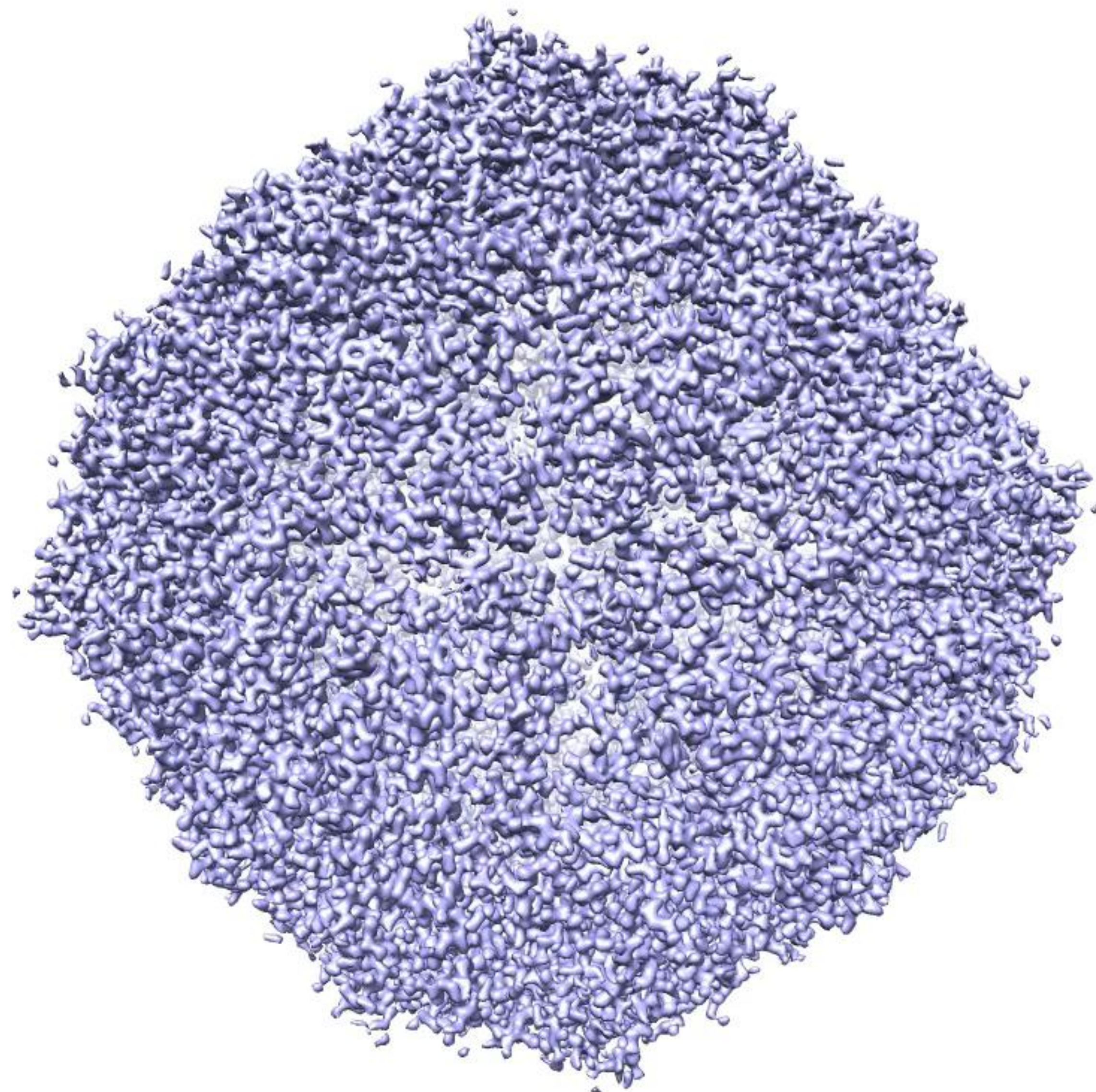


Cryo-EM Resolution



Second-Highest resolution: JEOL CryoARM 300, Namba lab Osaka: Apoferritin at 1.54Å

Apoferritin, mouse heavy chain, Fth1

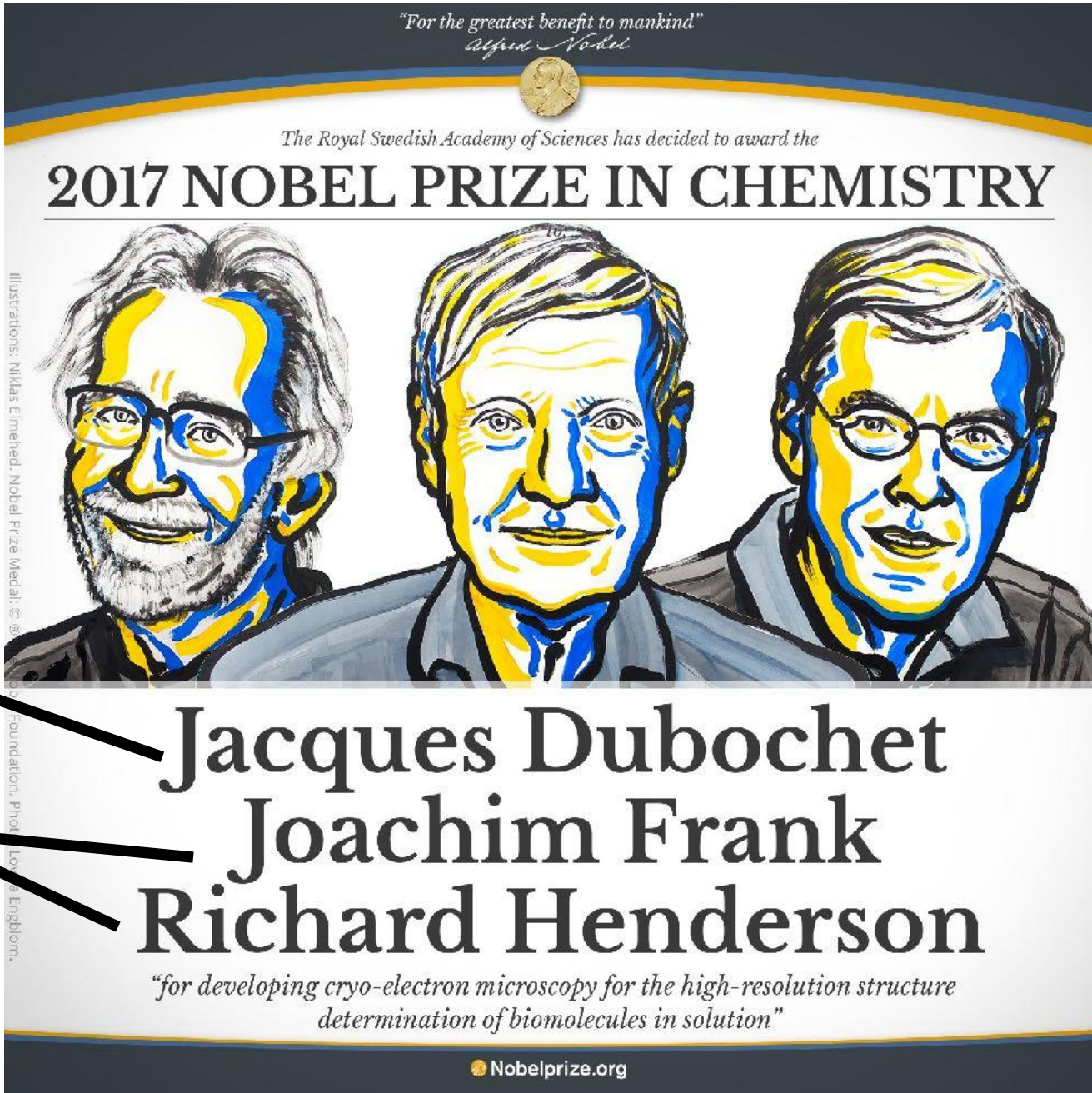


Radostin Danev, Tokyo University

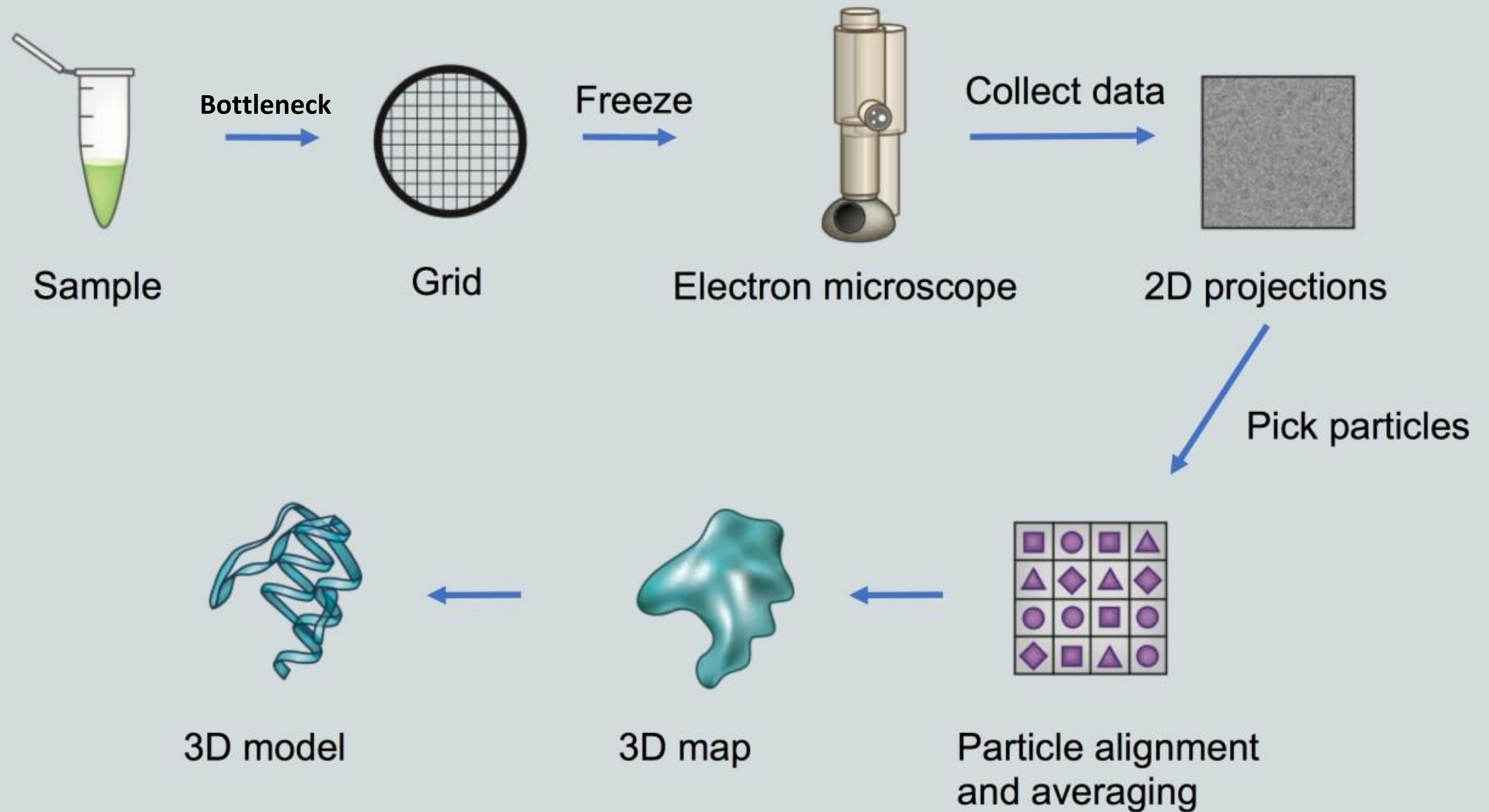
Titan Krios, Falcon III EC camera: 1.62 Å

Cryo-EM as of today

<i>What</i>	<i>Time</i>	<i>Resource</i>
Expression, Purification	years	Pure, homogeneous, stable
Cryo-EM grid optimization	3 months	200kV Cryo-EM
Cryo-EM imaging	1 week	300kV high-end Cryo-EM
Image processing	1 month	500 CPUs or 10 GPUs



Workflows in Single Particle Cryo-EM



Frontiers in Single Particle Cryo-EM

Resolution:

- 3.0 Å resolution is routine => Backbone, larger side chains
- 2.0 Å resolution is difficult => ligands, inhibitors, ions, drug design
- < 1.0 Å resolution is possible with the Titan => Limitation by sample and software

Sample Size:

- > 150 kDa is ok
- 100 kDa is difficult
- 45 kDa is current world record (only with phase plate)

Sample Homogeneity:

- < 10 discrete conformations ok
- Continuous variations are difficult to process.

Speed:

Today: 1 day on 300kV microscope, 1 month on computer
Desired: 30 min on microscope, processing in real time.

Sample Quantity:

- > 3 µl per cryo-EM grid => mostly lost in filter paper
- 3 nl per cryo-EM grid => needed for imaging

Cryo-EM application: Multi-Scale Microscopy

Single Particle Cryo-Electron Microscopy

**Atomic structures of
proteins and protein complexes**

Atomistic function of enzymes

Method Development:

- Automation
- Sample preparation
- Acceleration

Related:

- Protein production
- Biophysics
- XRD, NMR, MS, AFM
- Modeling

Tissue Cryo-Electron Tomography

**Cellular and multi-cellular
structure**

Biological organization, structure

Method Development:

- cryo-FIB-SEM lamella
- 3D reconstruction
- 3D segmentation

Related:

- FIB-SEM Slice and View

Correlative Light and Electron Microscopy

**Cellular and multi-cellular
organization**

Biological function

Method Development:

- Label technology
- Coordinate transform
- Super-resolution LM

Related:

- Correlated FIB-SEM

Technique	Beam	Sample size	Technical Resolution
SPECT (single photon emission)	Gamma emission	Human	2 mm
PET (positron emission)	Pair of gamma emission	Human	1 mm
CT (computed tomography)	X-ray	Human	0.5 mm
Ultrasound	Sound	tissue	0.3 mm
MRI (magnetic resonance imaging)	Magnetic field	human	0.1 mm
OCT (optical coherence tomography)	Infrared light	2 mm	10 μm
Synchotron X-ray tomography	X-ray	2 mm	100 nm
Synchotron X-ray ptychography	X-ray	2 mm	30 nm
Helium microscopy	He ²⁺	100 nm	1 nm
Electron microscopy: SBF-SEM	Electron (3kV)	1 mm	3 nm
Electron microscopy: FIB-SEM	Electron (3kV)	30 μm	2 nm
Electron microscopy: TEM	Electron (300kV)	500 nm	0.1 nm