Physics of Life

PHYS-468

Electron Microscopy in Life Sciences

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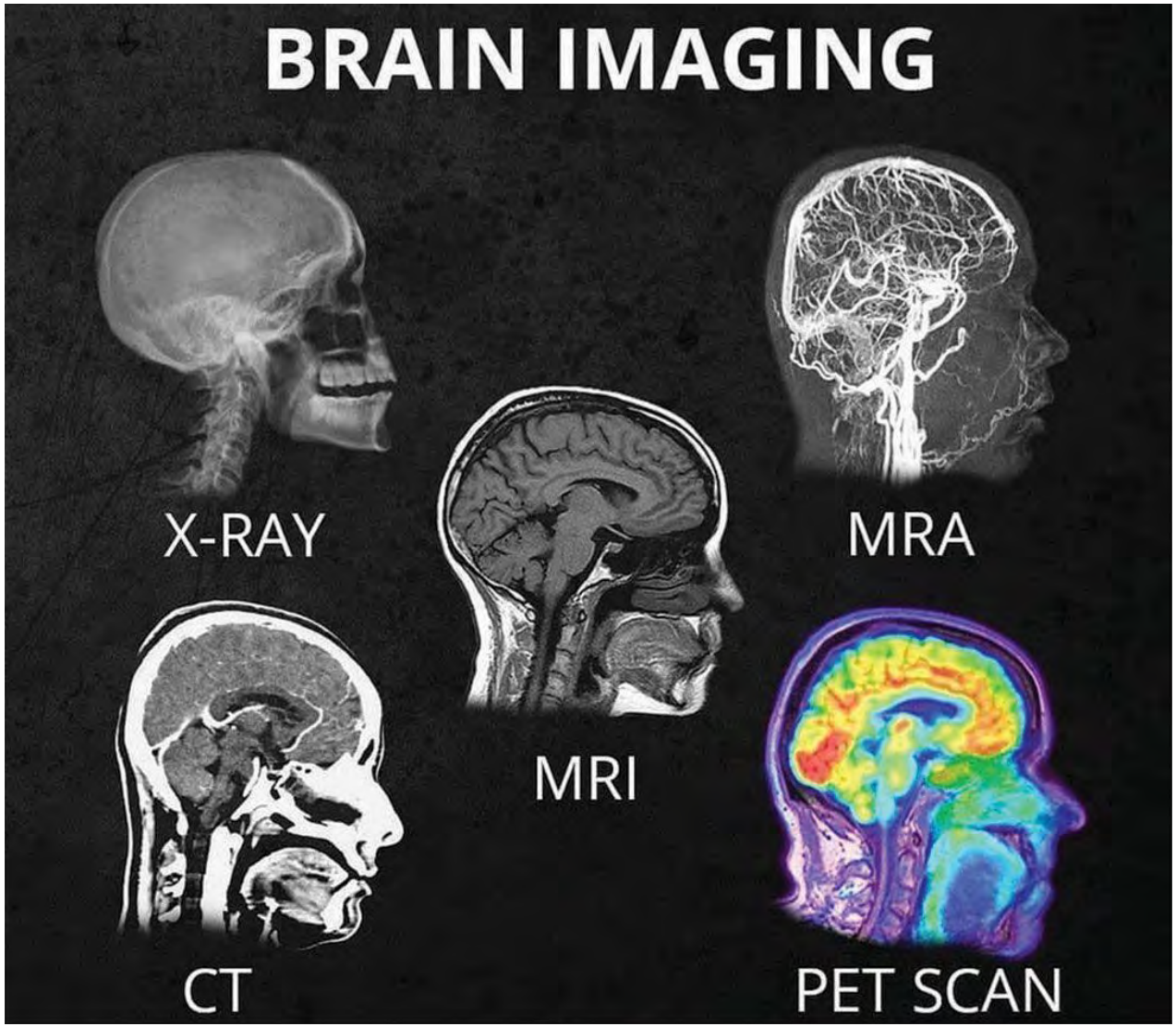
3D imaging techniques with beams

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

3D imaging techniques with beams



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.

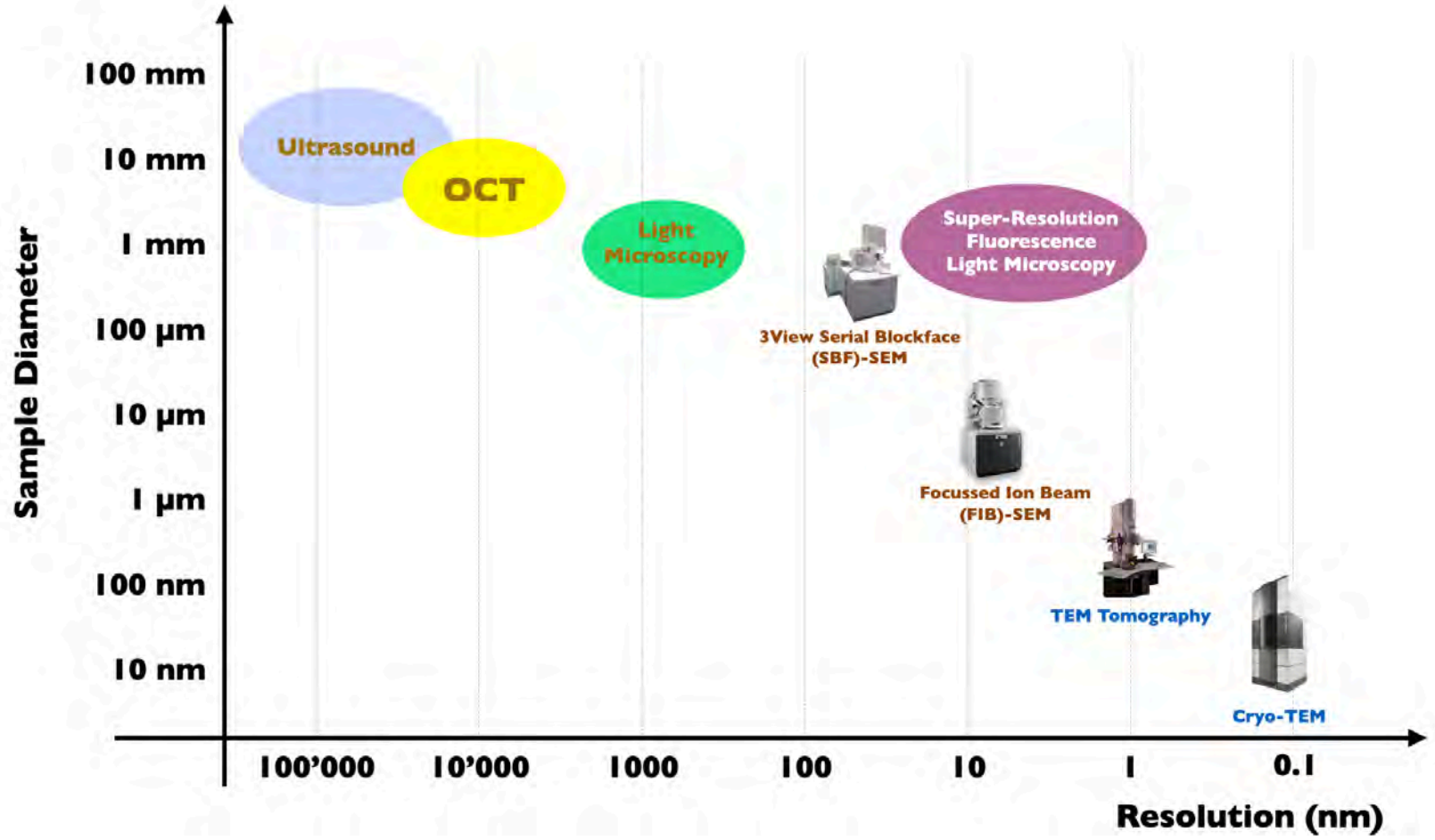
MRI scanners utilize strong magnetic fields, magnetic field gradients, and radio waves to produce images of the internal organs in the body. It provides a 3D map.

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

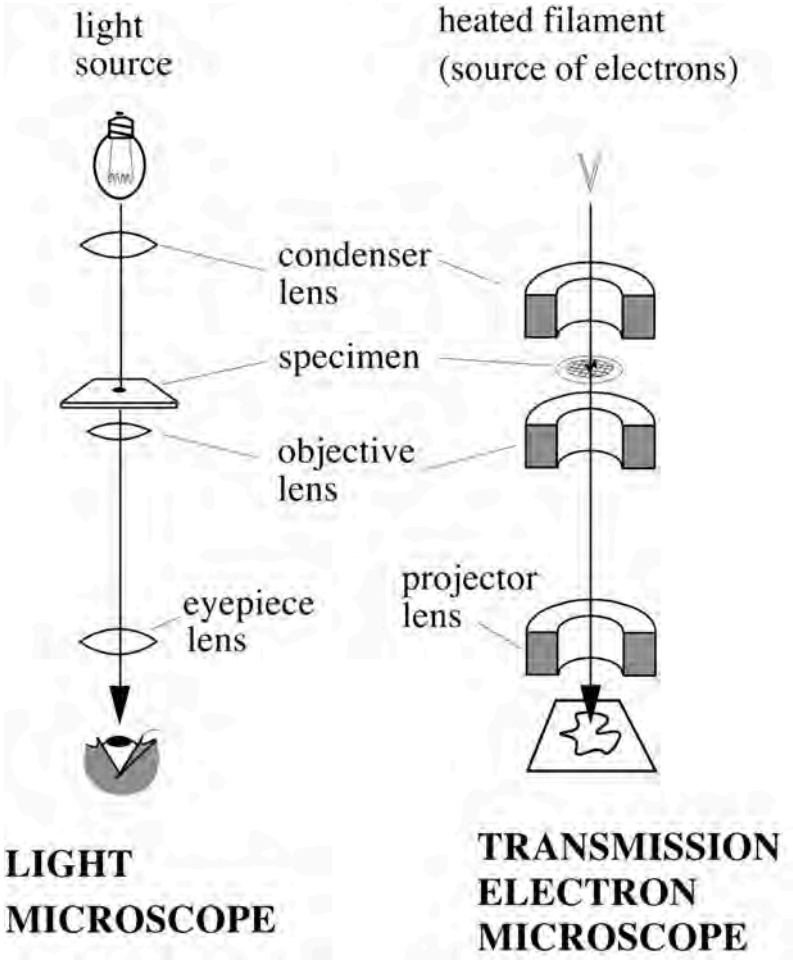


History of TEM

Ernst Ruska was awarded the Nobel prize 1986 for his construction of the first transmission electron microscope, together with Gerd Binning and Heinrich Rohrer for their invention of the scanning tunnelling microscope. As students, **Ernst Ruska** and **B. v. Borries** were in 1928 members of the group of **M. Knoll**, who was a group leader in the laboratory for high-tension technology and electric devices of Prof. **A. Matthias** at the Technische Hochschule (TH) Berlin, which later became the Technische Universität (TU) after the second world war. Ernst Ruska worked on a focusing coil for cathode beams. After his diploma thesis in the year 1930, he was unemployed, but he was allowed to continue his work unpaid. In April 1931 he managed to construct a magnifying device with 16 times magnification, using an electron beam. He and Knoll discussed at that time, if it would be possible to construct a high resolving microscope with an electron beam and the "lens" of Ruska. They imagined electrons as infinitely small particles, which would not limit the resolution. Only later they considered the possibility of a limitation to the resolution due to the "matter-wavelength" of an electron according to the definition of **Louis de Broglie** 1924/25. On July 4, 1931, Knoll presented their results at the public Cranz-colloquium in the TH Berlin. Their first publication using the word "electron microscope" was submitted on the 10th of September 1931.

The first patent application, however, was submitted on May 30, 1931, by Prof. **Reinhold Rüdenberg**, who was at that time head of the scientific department of the Siemens-Schuckert-Werke in Berlin. Rüdenberg had been informed about the advances of Ernst Ruska by his assistant Dr. **Max Steenbeck**, who had visited the laboratory of Knoll and Ruska. This application resulted in three patents (DBP 895 635, 889 660 and 906 737), that were accepted only in the years 1953 and 1954. No participation of any kind in the development or the construction of an electron microscope by Rüdenberg has ever been documented.

The electron beam



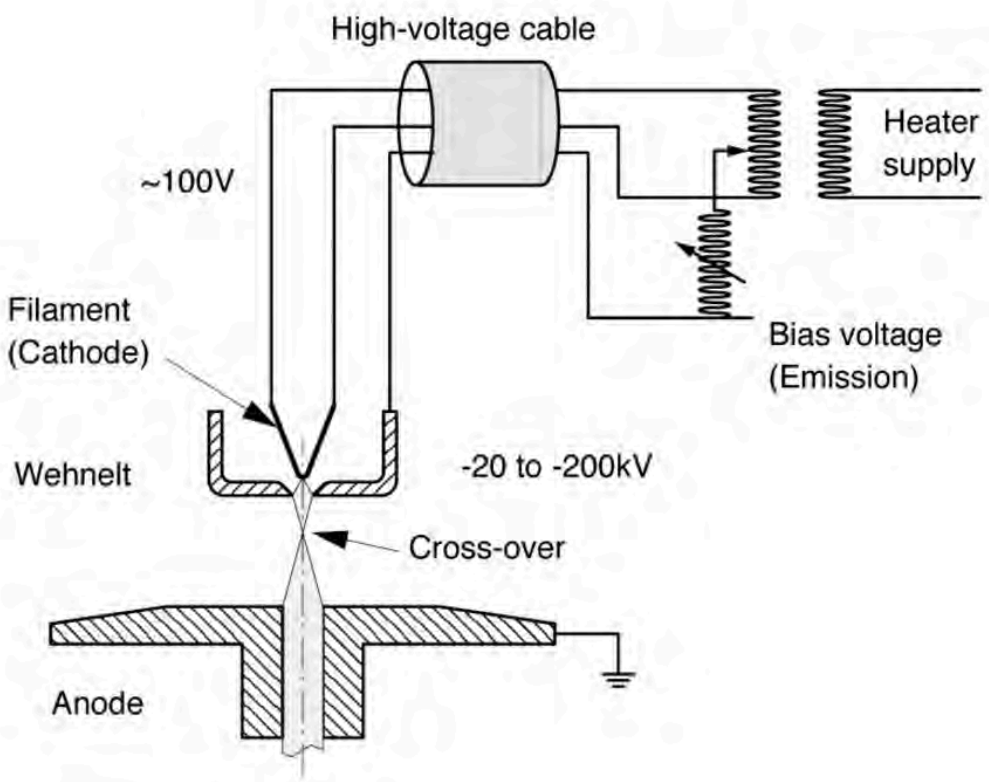
Type	Energy	Wavelength
Infrared	0.1 – 1 eV	10-1 μm
Visible	1-3 eV	1-0.3 μm
Ultraviolett	3-500 eV	0.3-0.01 μm
X-rays, ɣ	≥ 5000 eV	≤ 2 Å
Electrons	1 keV	0.4 Å
	10 keV	0.12 Å
	100 keV	0.037 Å
	1000 keV	0.009 Å

$$E = h \bullet v \qquad \lambda = \frac{c}{v}$$

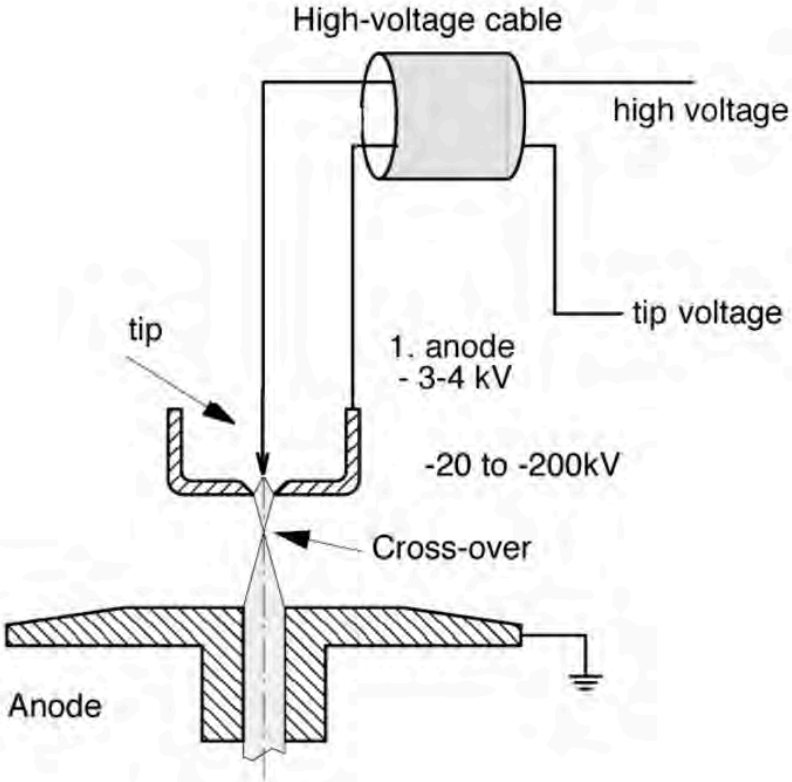
$$d = \frac{\lambda}{2NA}$$

1Å = 0.1 nm

Electron Sources

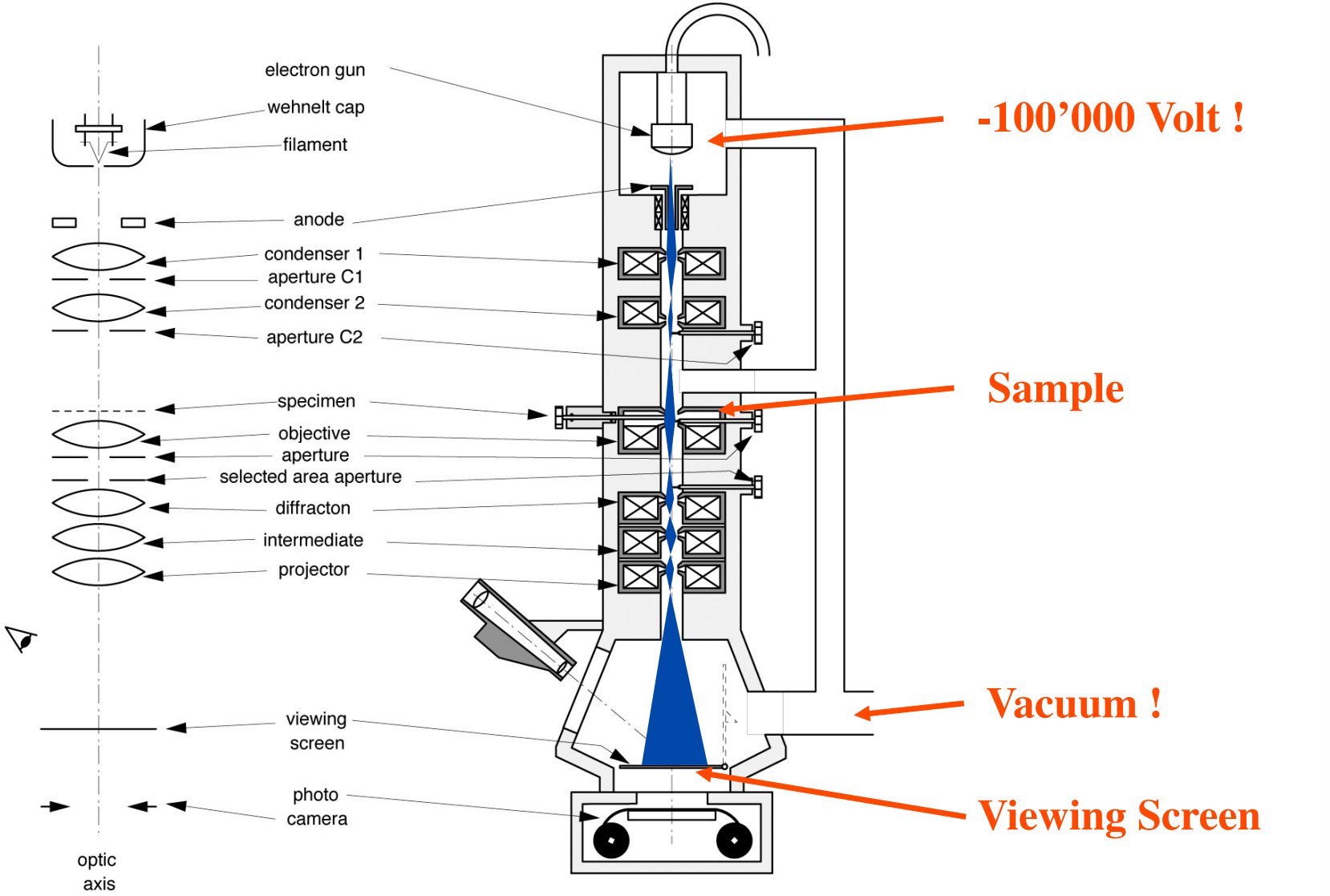


Heated Filament



Field Emission Gun (FEG)

The Transmission Electron Microscope



Transmission Electron Microscopes

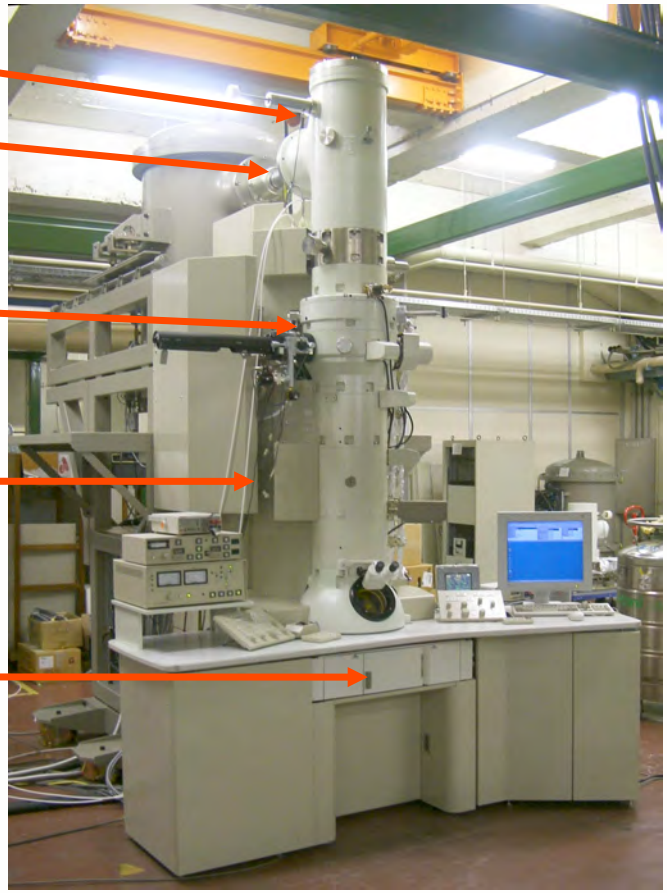
FEG

300 kV

Helium Cold Stage

Energy Filter

4k CCD Camera



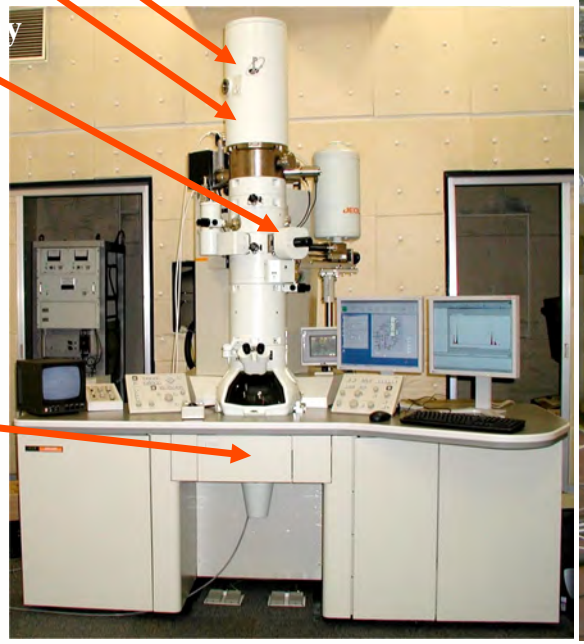
Transmission Electron Microscopes

FEG

200 kV

LN2 Cold Stage

4k CCD Camera



C-CINA

Electron Microscopes

FEI Quanta200/3View

Serial Block Face SEM

(FMI)

Zeiss Merlin/3View

Serial Block Face SEM

(FMI)

FEI Versa3D

Dual-Beam FIB-SEM

(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

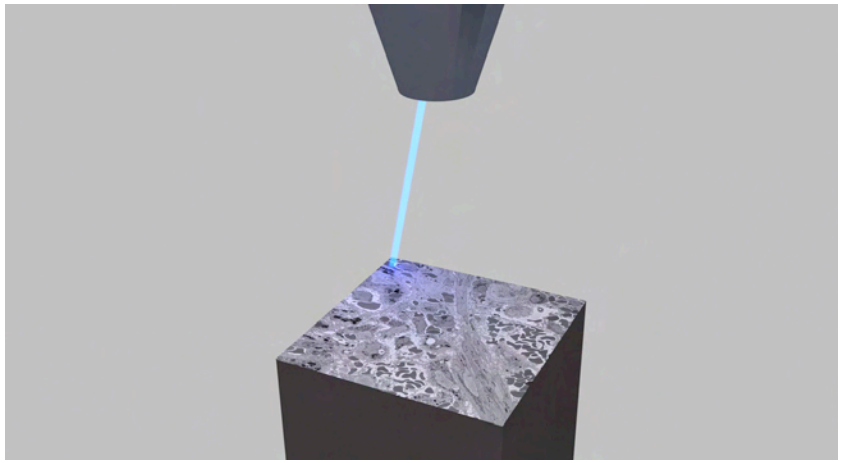
120kV, F416 CMOS

Cryo-EM screening

Scanning Electron Microscopy (SEM)

Transmission Electron Microscopy (TEM)

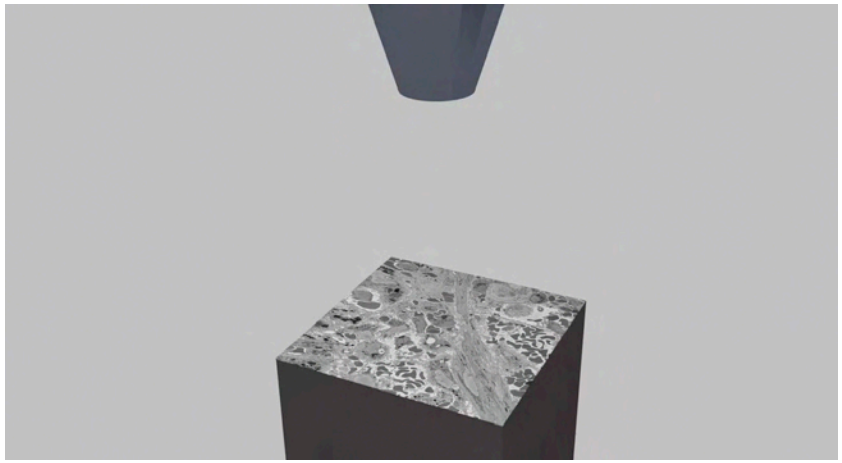
Scanning Electron Microscopy (SEM)

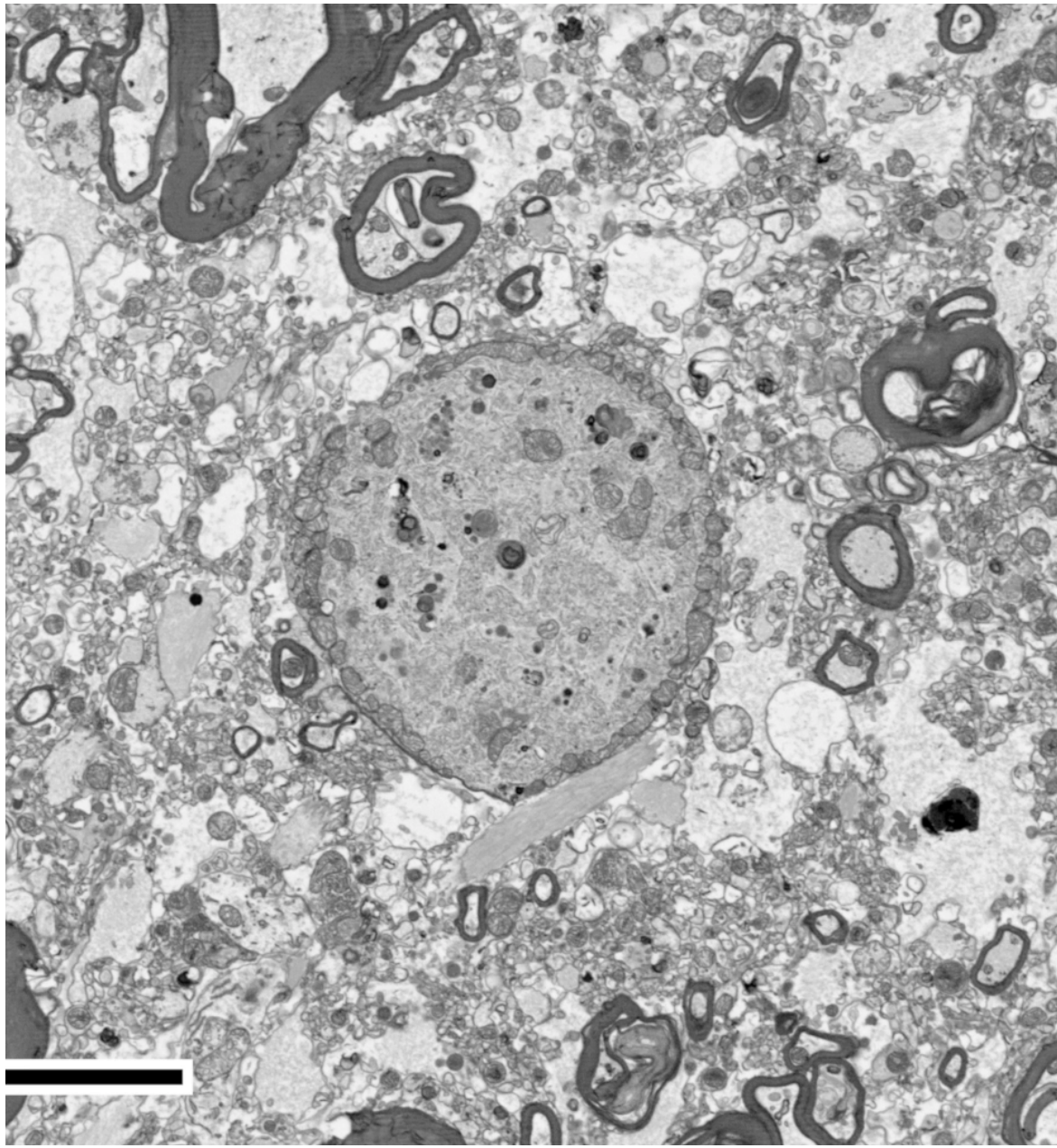


Transmission Electron Microscopy (TEM)

Scanning Electron Microscopy (SEM)

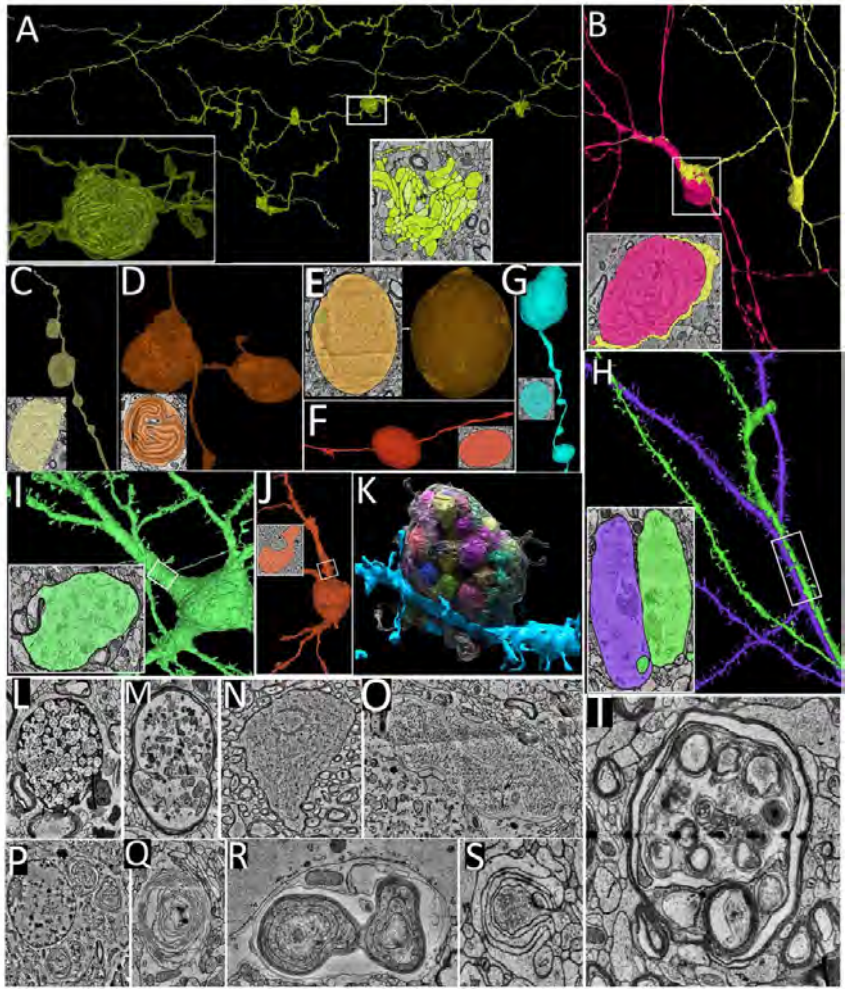
Serial Block-Face Scanning Electron Microscopy (SBF-SEM)

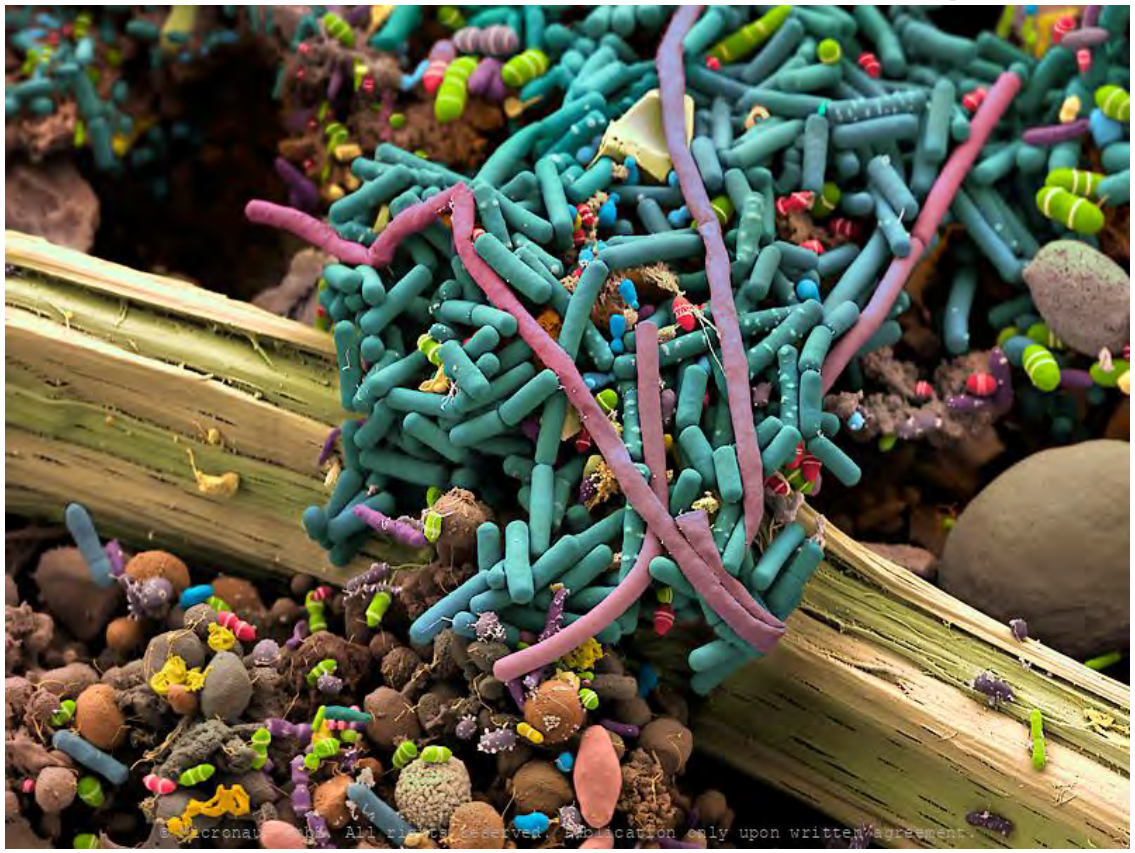




SBF-SEM vs SEM

(SEM colors are from Photoshop)





https://www.micronaut.ch

Alexander Shapson-Coe et al., A connectomic study of a petascale fragment of human cerebral cortex, bioRxiv, 2021

SEM images

(false colors are from Photoshop)

https://www.micronaut.ch

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Technique	Beam	Sample size	Technical Resolution
SPECT (single photon emission)	Gamma emission	Human	2 mm
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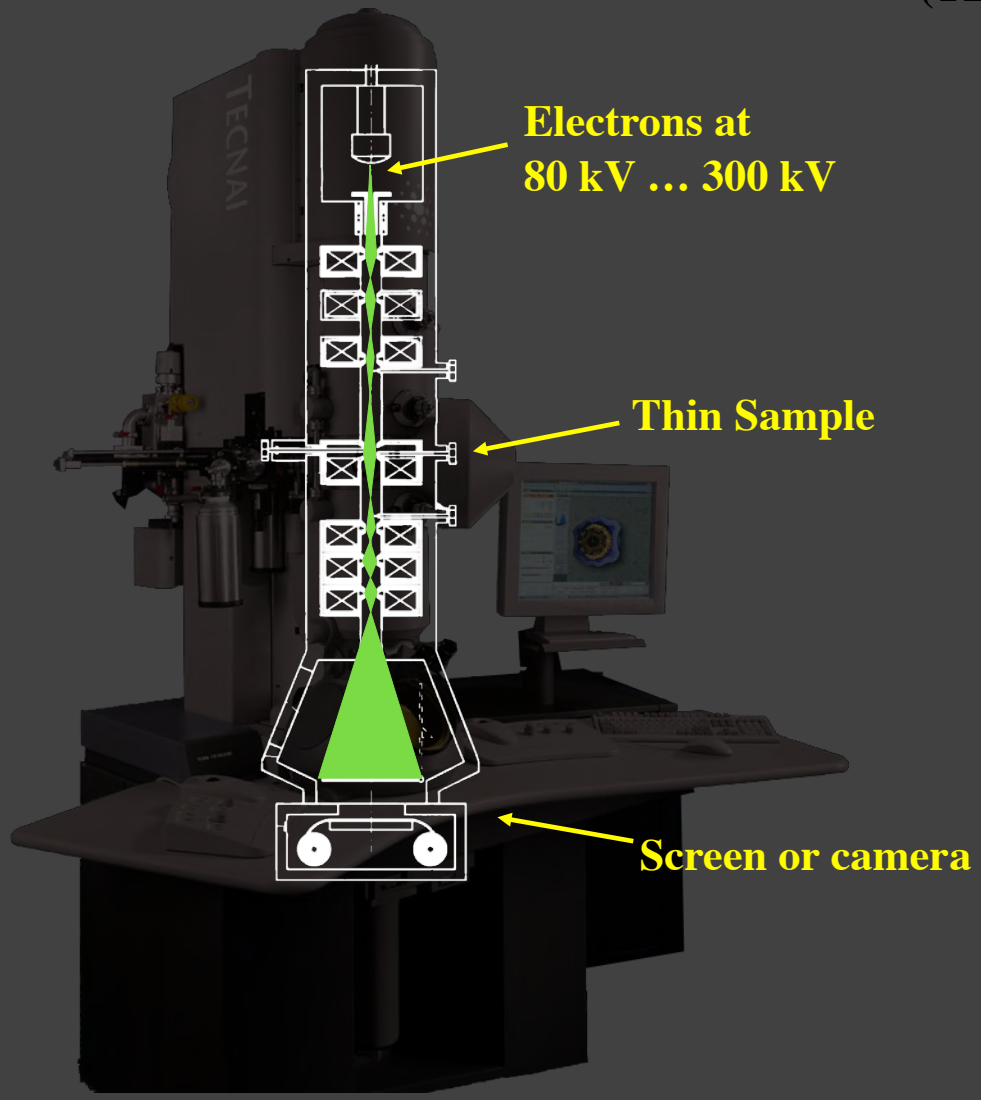
Synchotron X-ray ptychography	X-ray	2 mm	30 nm
Helium microscopy	He^{2+}	100 nm	1 nm
Electron microscopy: SBF-SEM	**Electron (3kV)**	**1 mm**	**3 nm**
Electron microscopy: FIB-SEM	Electron (3kV)	30 µm	2 nm
Electron microscopy: TEM	**Electron (300kV)**	**500 nm**	**0.1 nm**

Transmission Electron Microscopy (TEM)

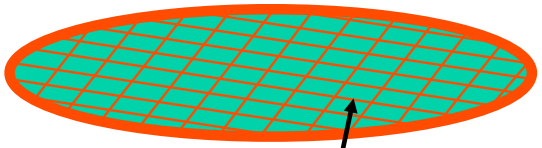
Transmission Electron Microscopy (TEM)



Transmission Electron Microscopy

Specimen Preparation

e^-

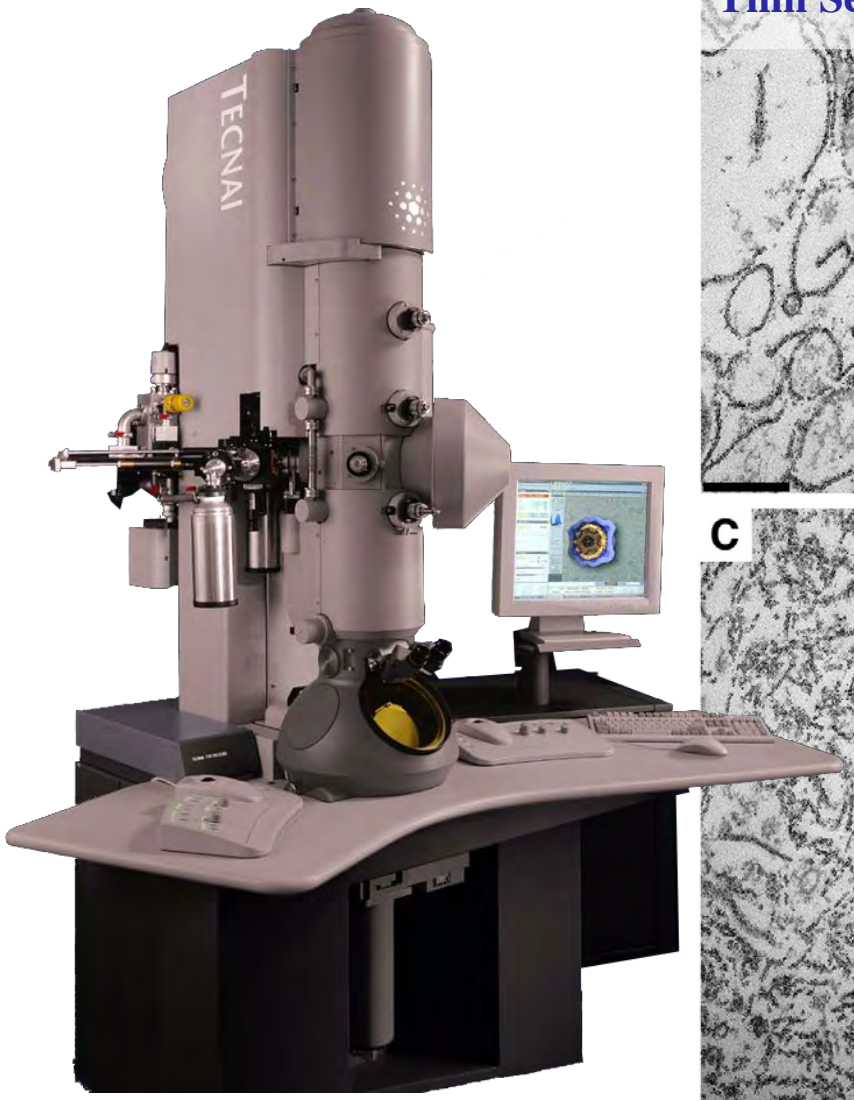


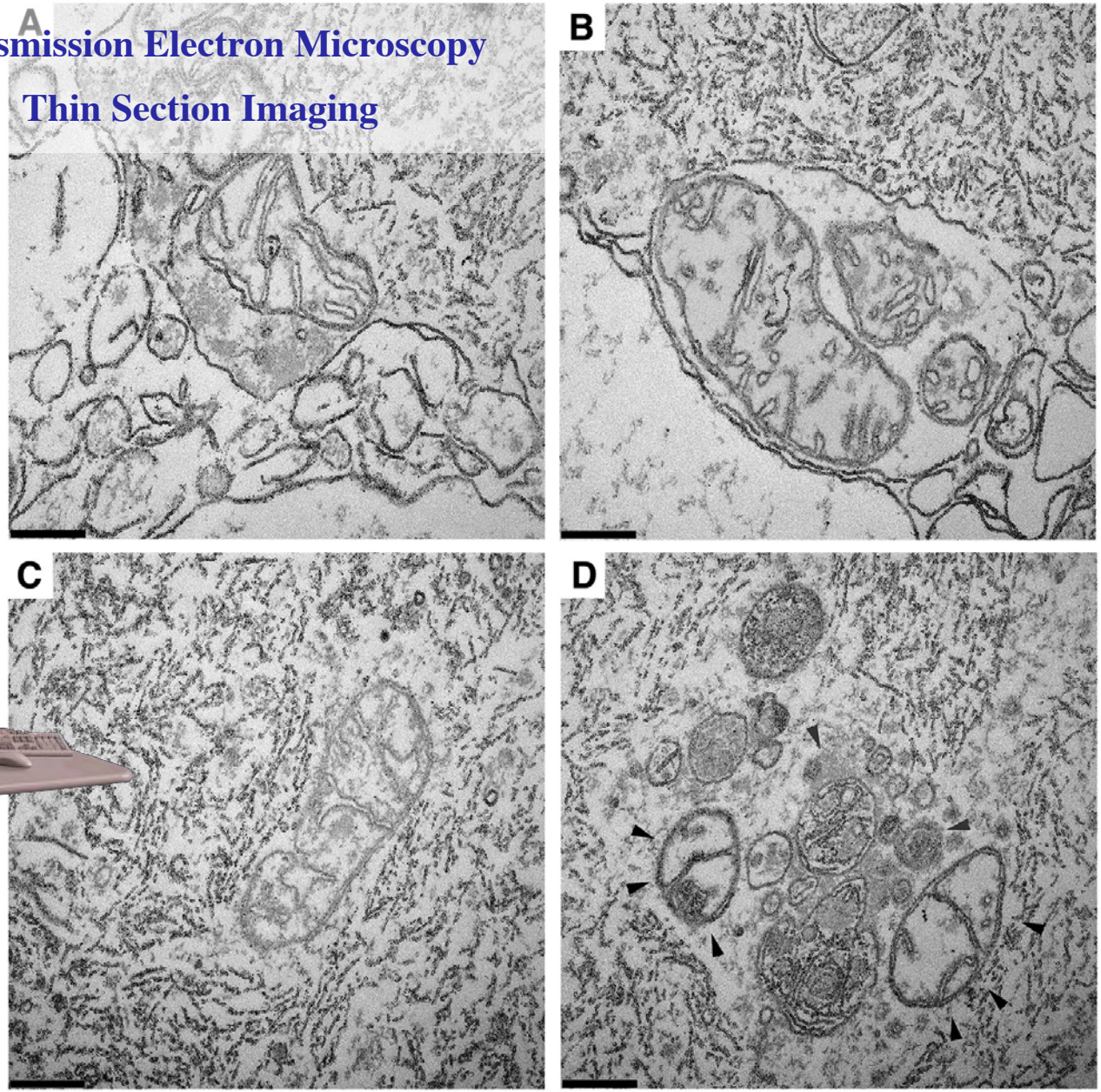
Microscopy Grid

Carbon-Film

Transmission Electron Microscopy

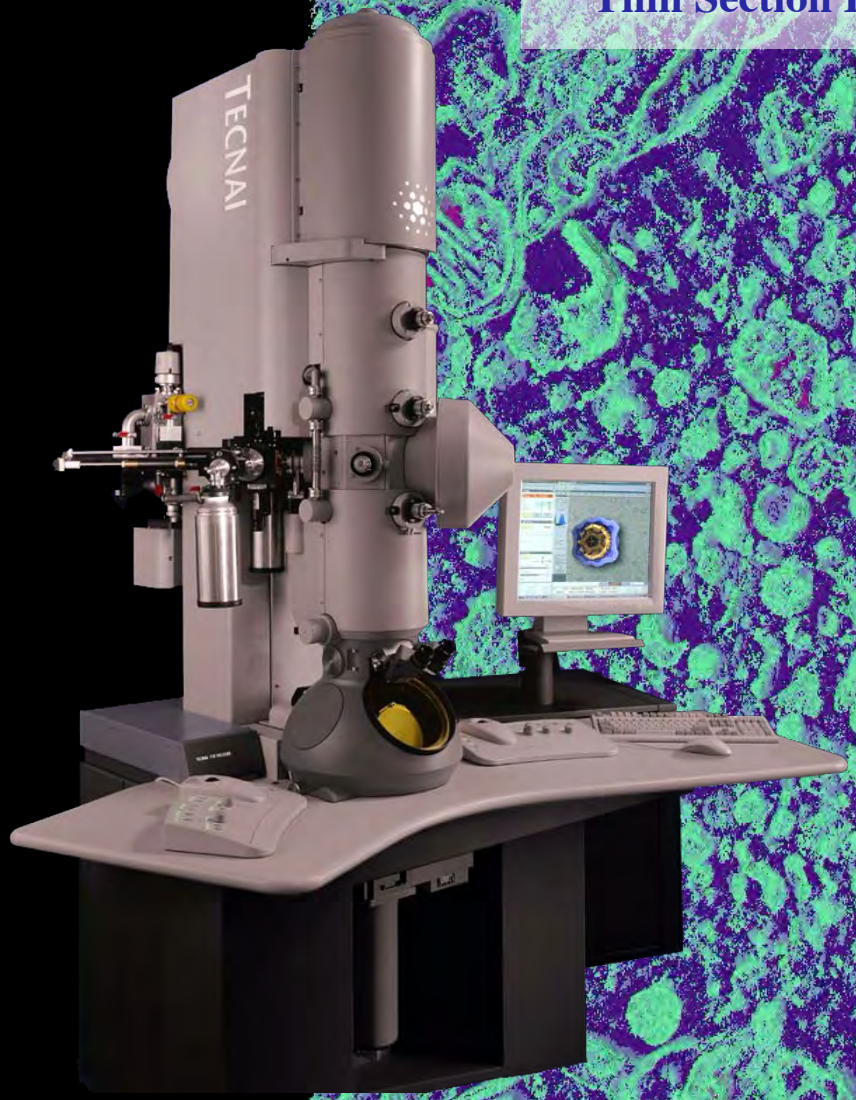
Thin Section Imaging





Transmission Electron Microscopy

Thin Section Electron Tomography

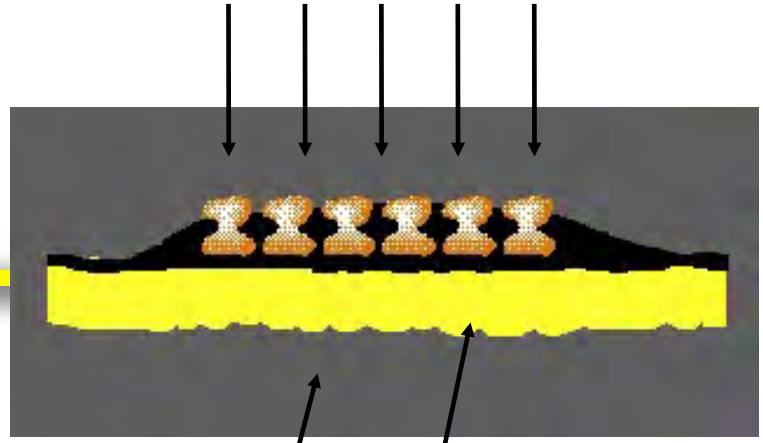


Transmission Electron Microscopy

Negative Stain TEM

Specimen Preparation

e^-



Microscopy Grid

Carbon-Film

Transmission Electron Microscopy

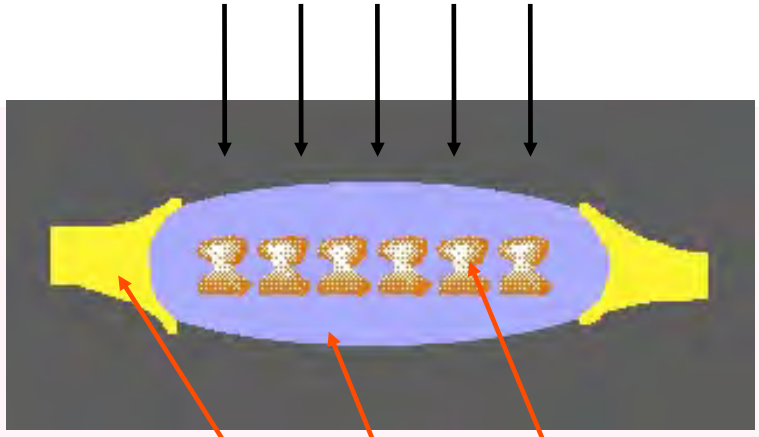
Negative Stain TEM

Transmission Electron Microscopy

Cryo-EM

Specimen Preparation

e^-



Proteins

Buffer Solution

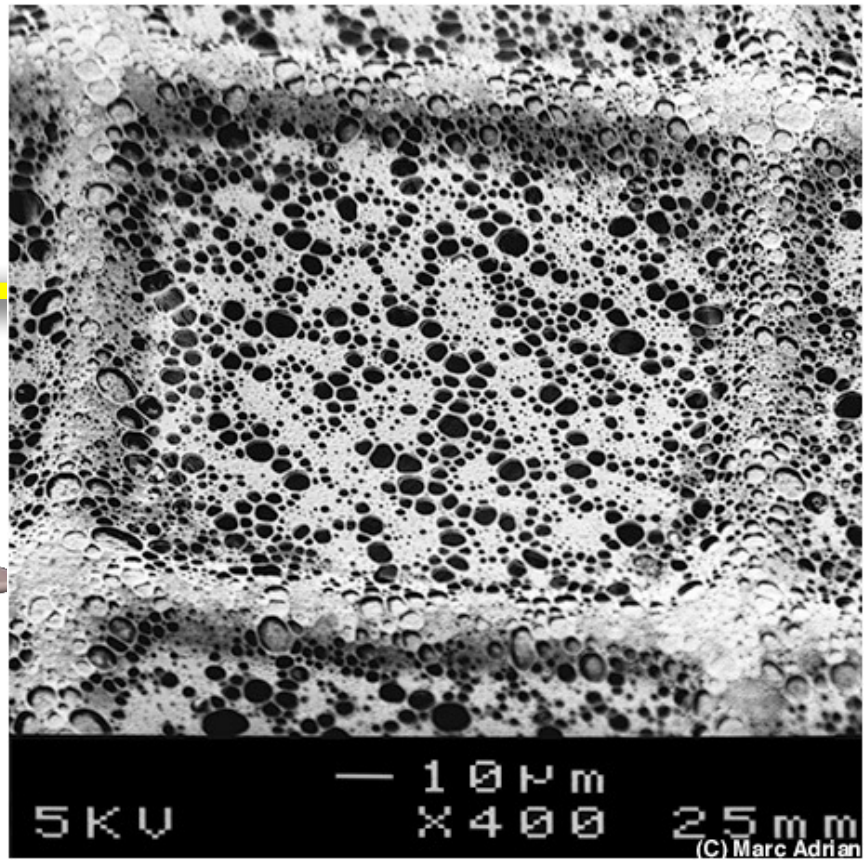
Cold: -180°C !!!

Carbon-Film

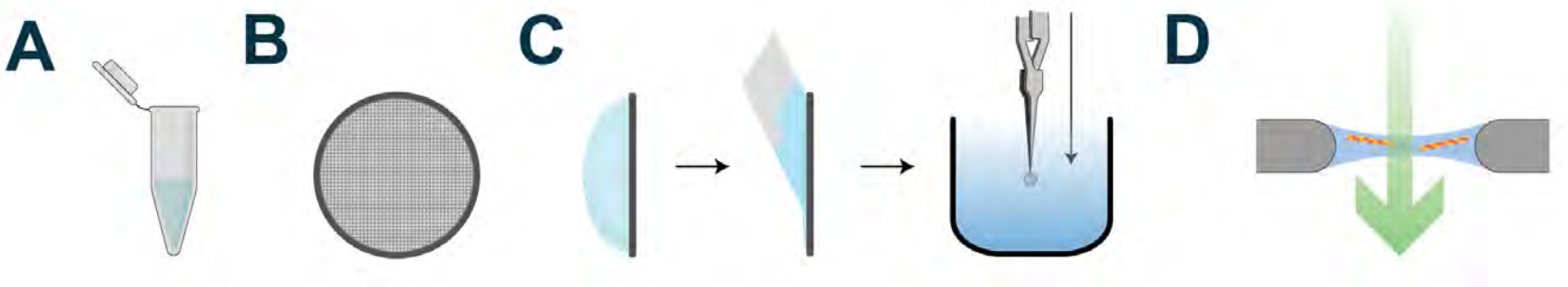
Transmission Electron Microscopy

Cryo-EM

Holey Carbon Film



Cryo-EM: principle workflow



- Homogenous sample (purified)
- Small sample aliquots (3µl) on a copper/gold grid
- Blot excess liquid (≈ 99%)
- Rapid plunge freezing (in liquid ethane @ -180°C)

➢Sample in a close to native state frozen in vitreous ice

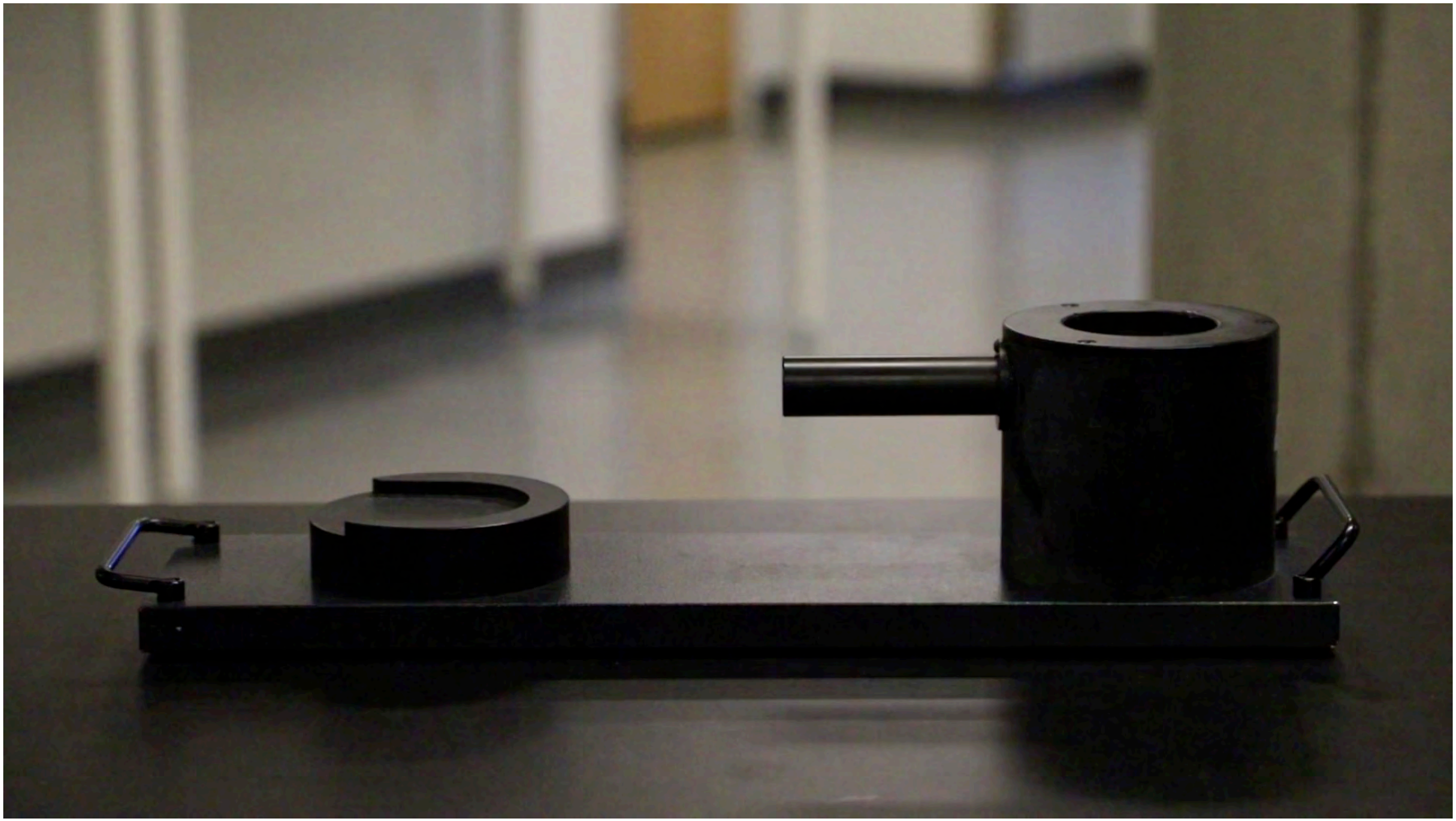
➢Acquisition of projection images

https://cryoem101.org/


Transmission Electron Microscopy
Cryo-EM
3 µl

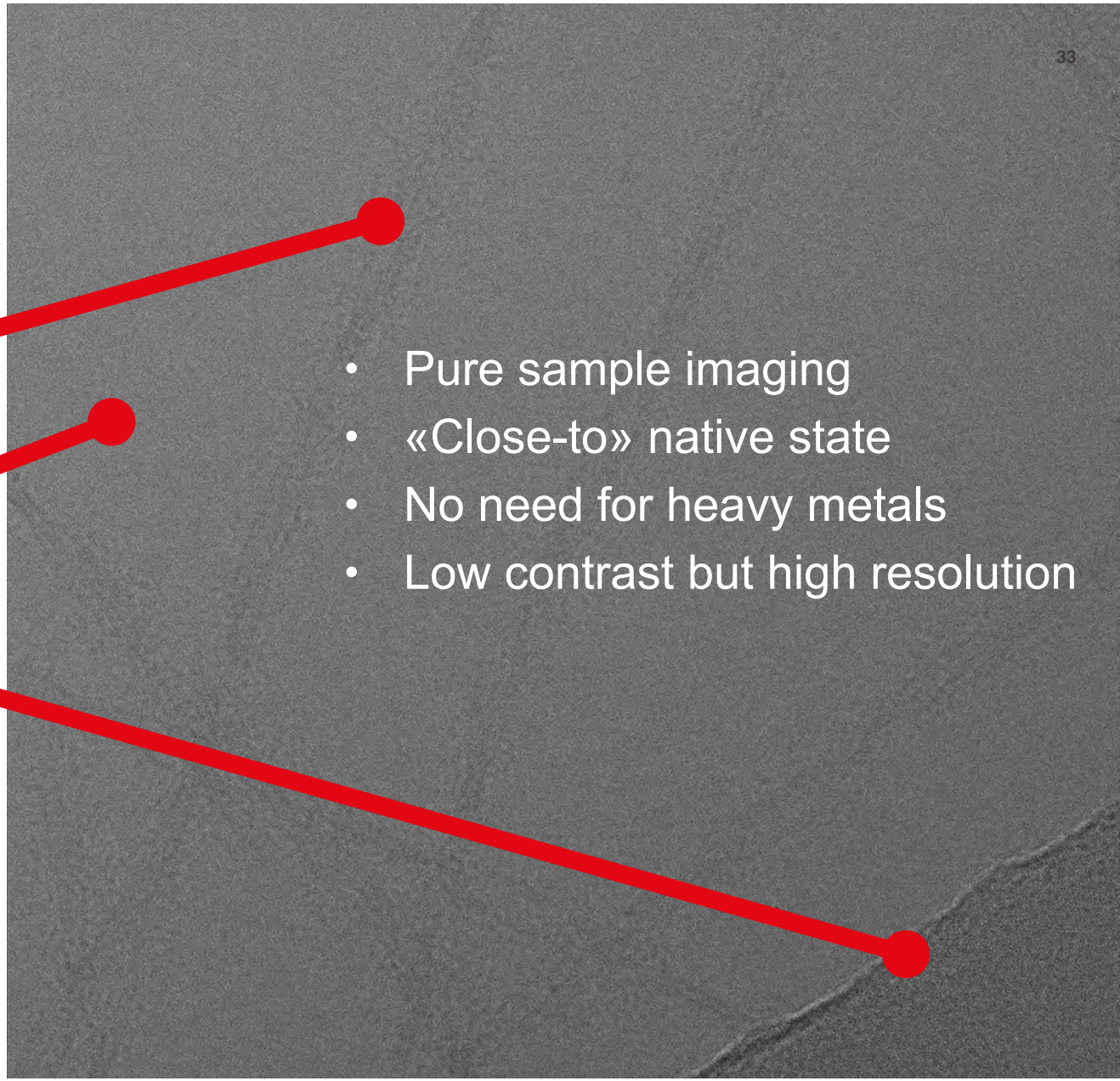
Cryo-EM: freezing

Cryo-EM: loading & imaging

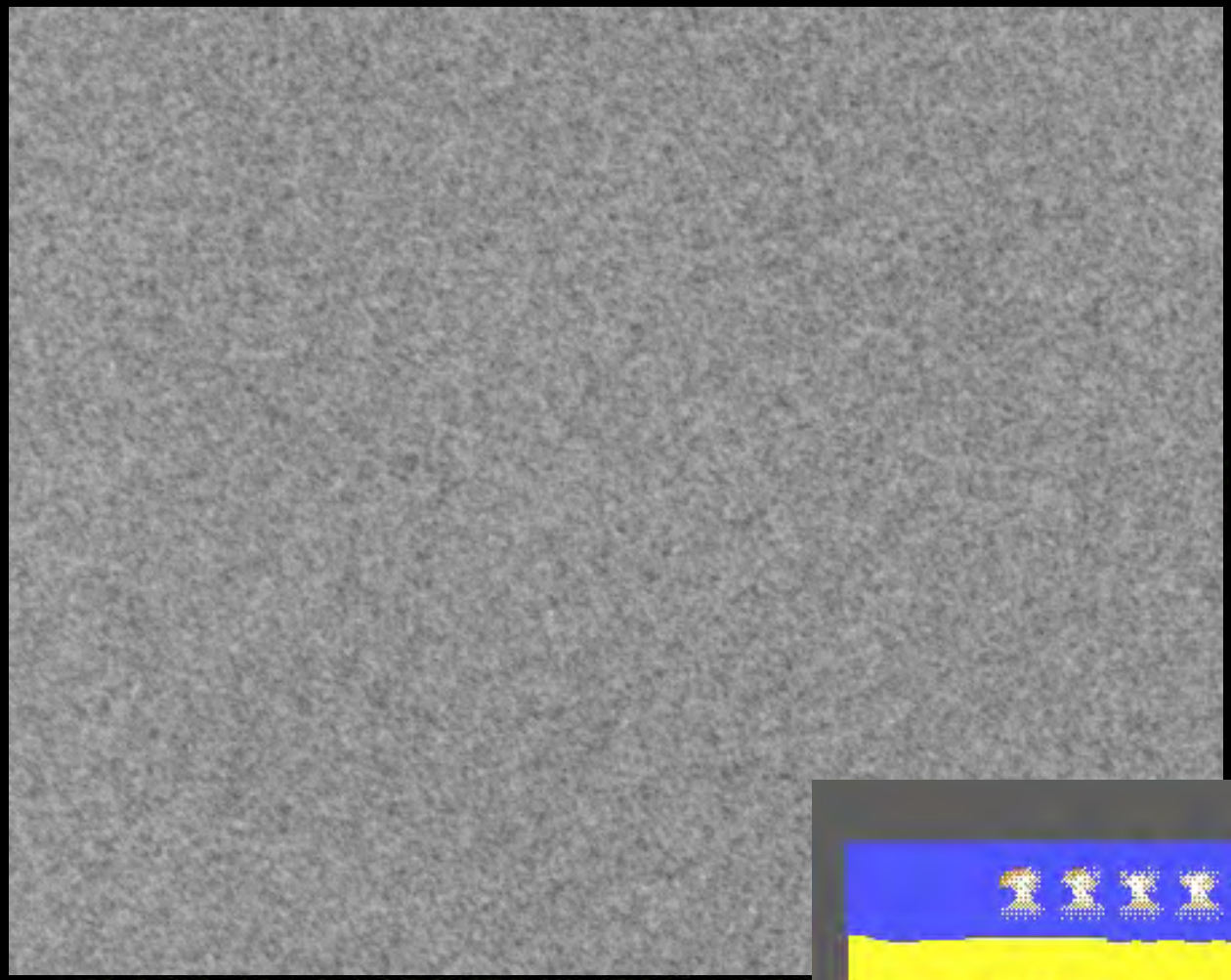


Cryo-electron microscopy

- Protein in solution
- Buffer / vitreous
- Carbon edge



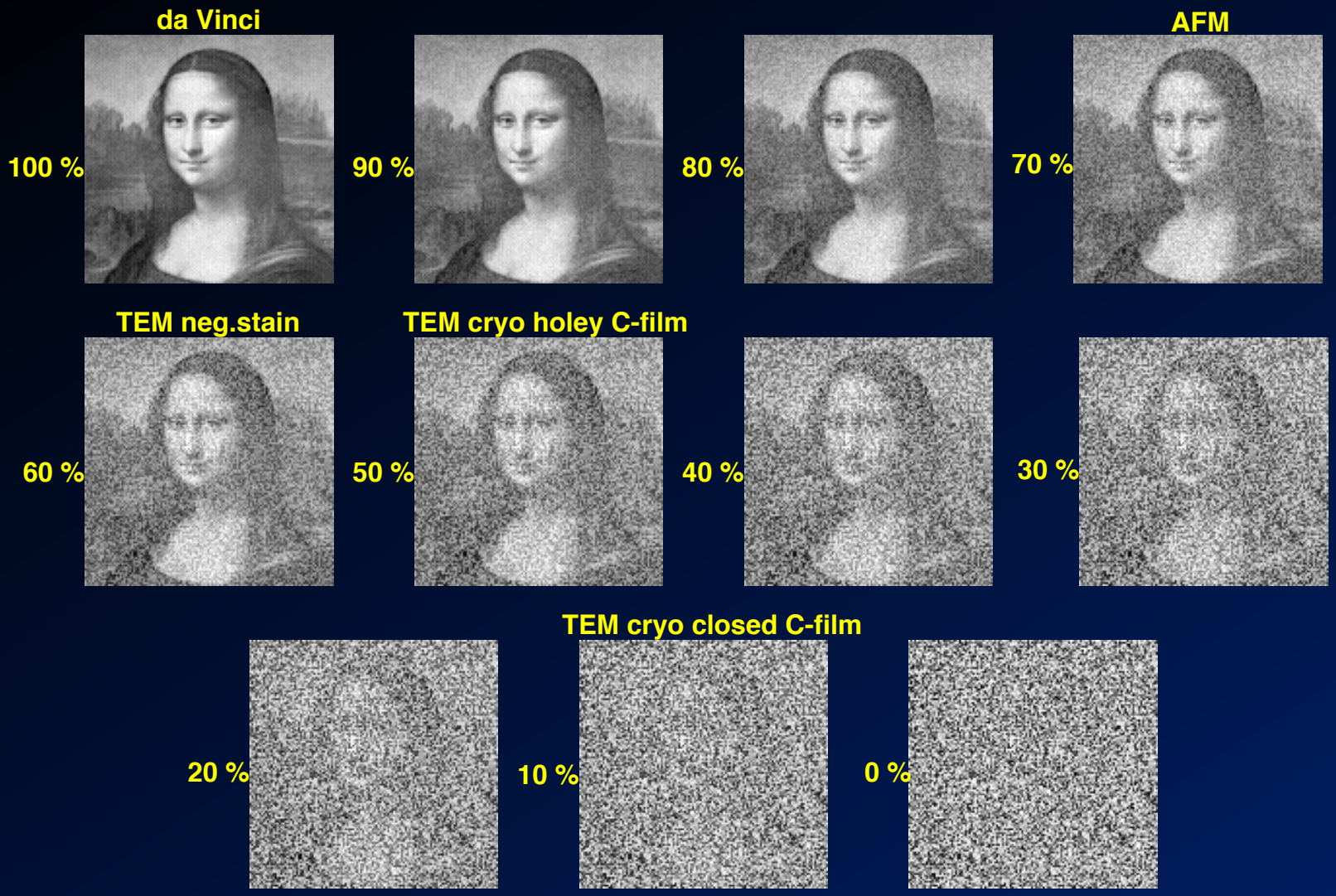
- Pure sample imaging
- «Close-to» native state
- No need for heavy metals
- Low contrast but high resolution



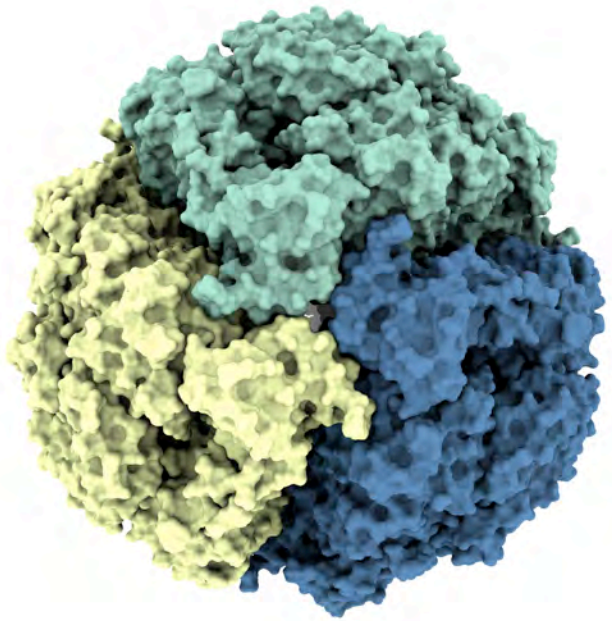
Cryo
TEM

Continous
C-film

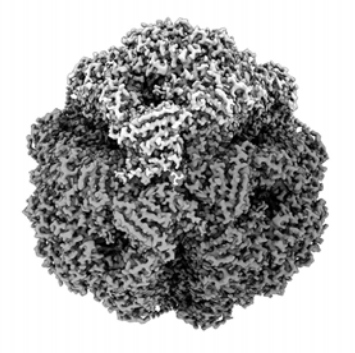
-180°C


da Vinci
AFM
100 %
90 %
80 %
70 %
TEM neg.stain
TEM cryo holey C-film
60 %
50 %
40 %
30 %
TEM cryo closed C-film
20 %
10 %
0 %

Imaging: Reducing noise

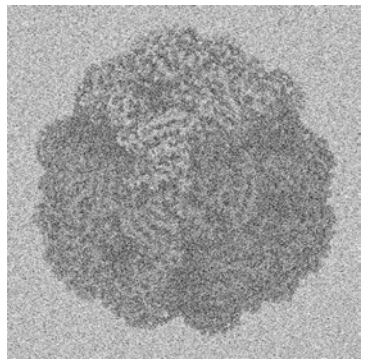


"true" projection

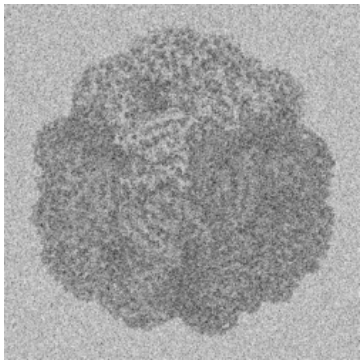


noise →

acquired projection

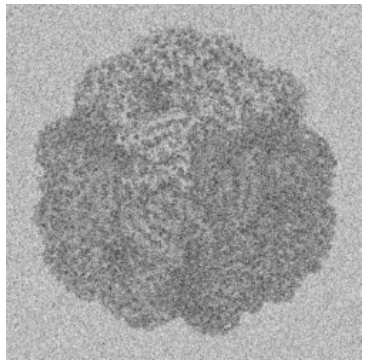


multiple acquired projection

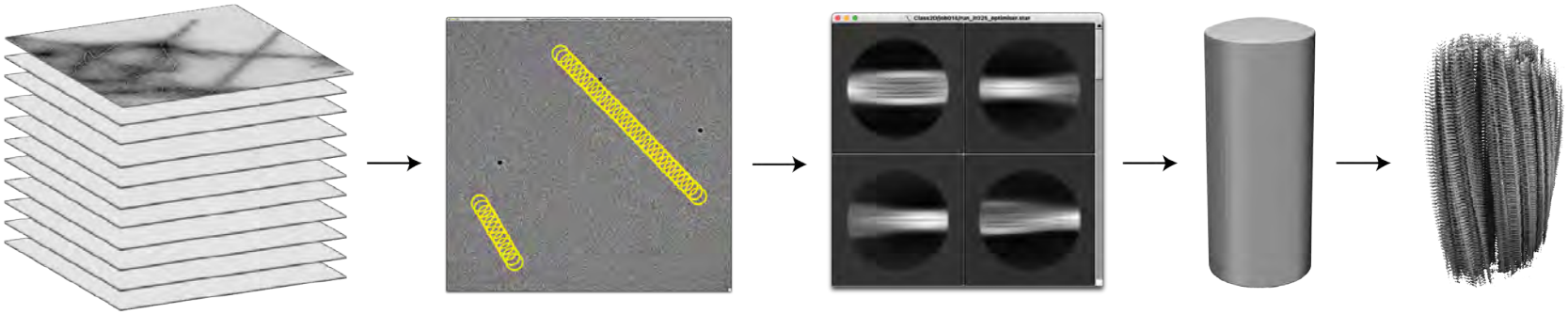


averaging →

reconstructed projection

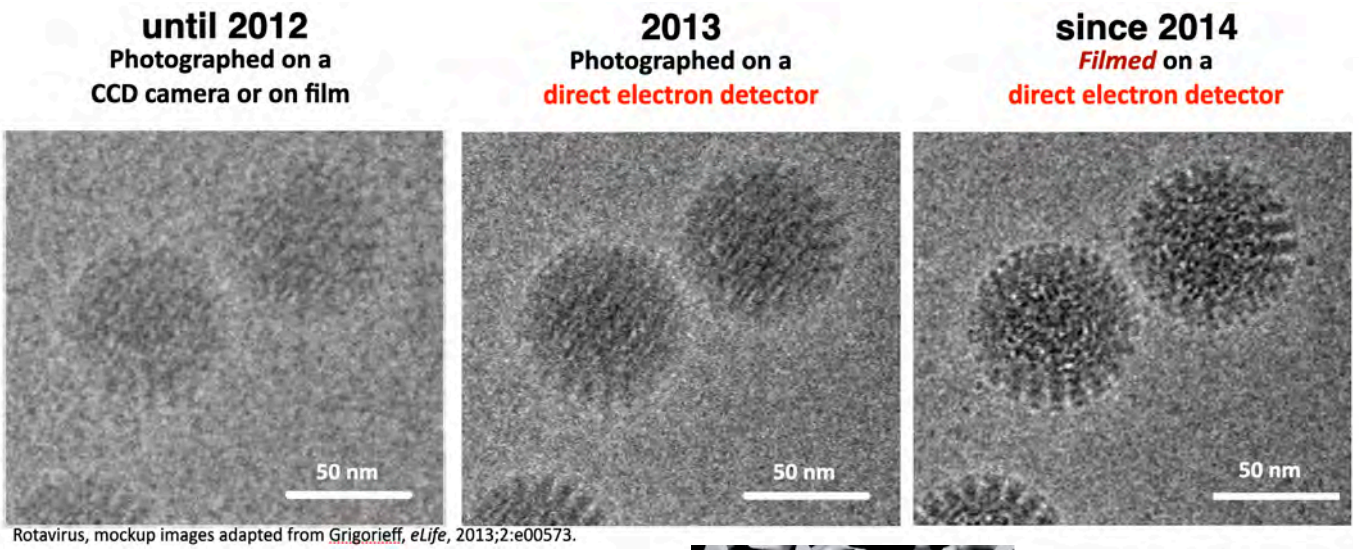


Cryo-EM: principle workflow

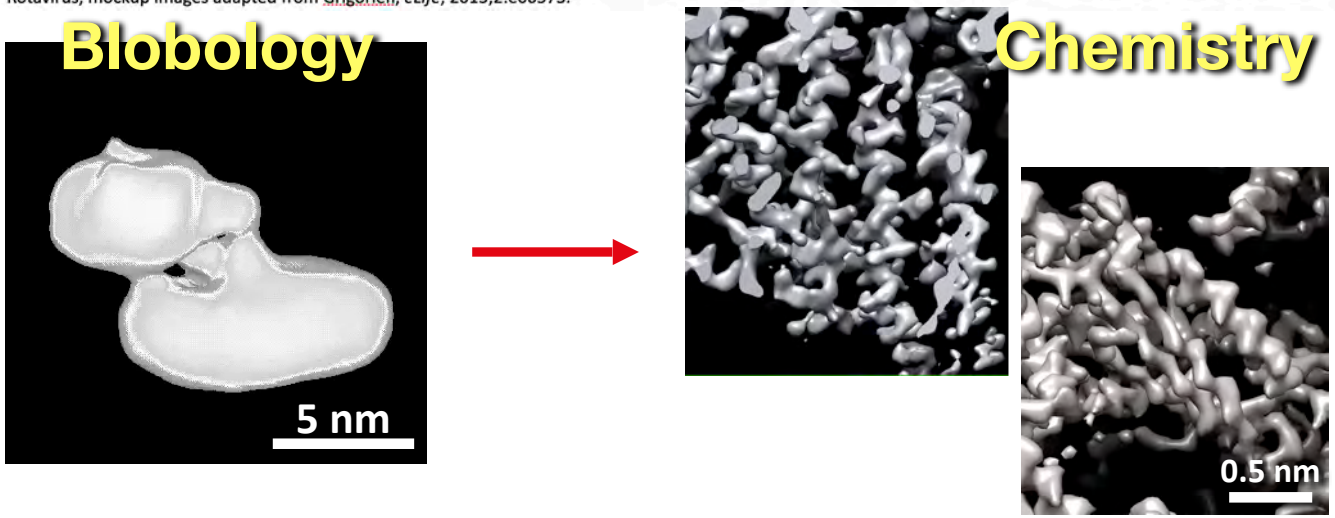


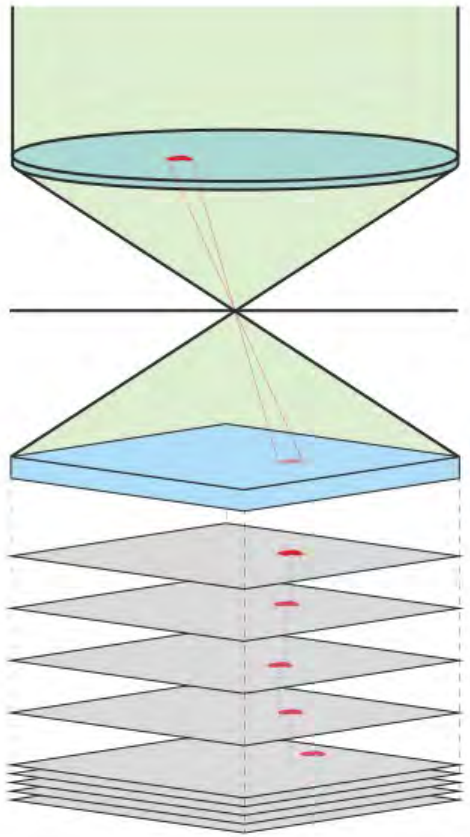
- Large datasets of your sample in random orientation
- Processing / Correction: particle motion, damage, and CTF estimation
- Extraction of individual particles
- Signal averaging for 2D classes
- Backprojection into a 3D volume, alignment
- Iterative process to obtain a final map -> model building

Cryo-EM: resolution revolution



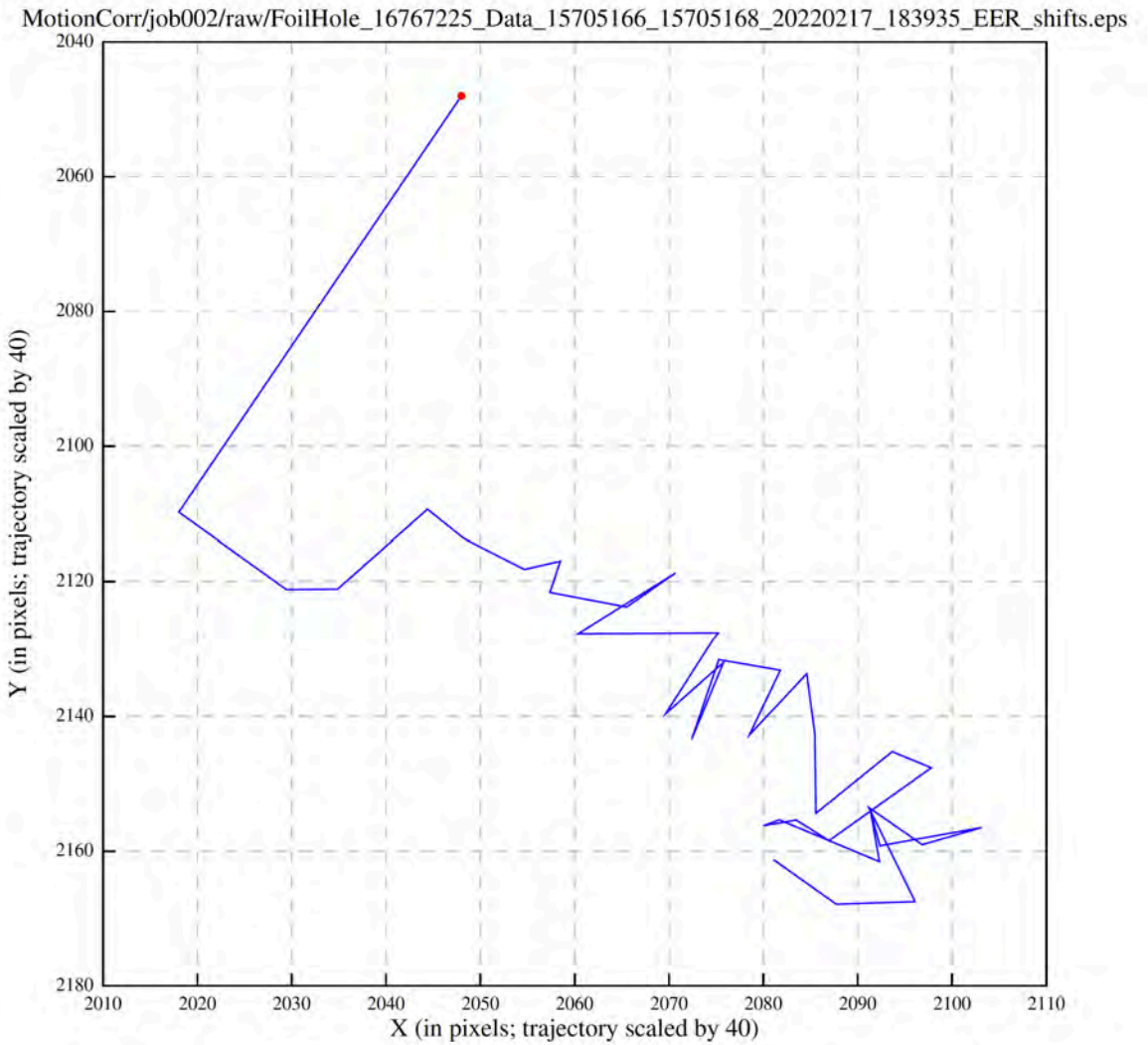
Rotavirus, mockup images adapted from Grigorieff, *eLife*, 2013;2:e00573.





Cryo-EM: resolution revolution

- 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

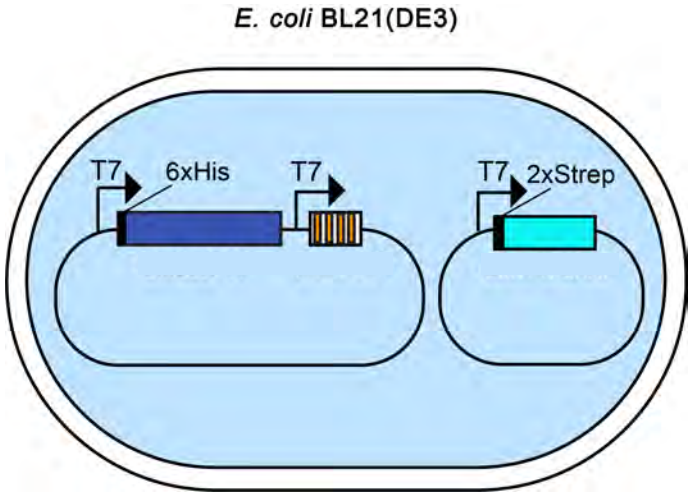
- Stage 1: Is my sample suitable (pure, homogenous, stable)?
  - Preparation of negatively stained grid
  - TEM imaging @120kV
  - Computer analysis for sample homogeneity

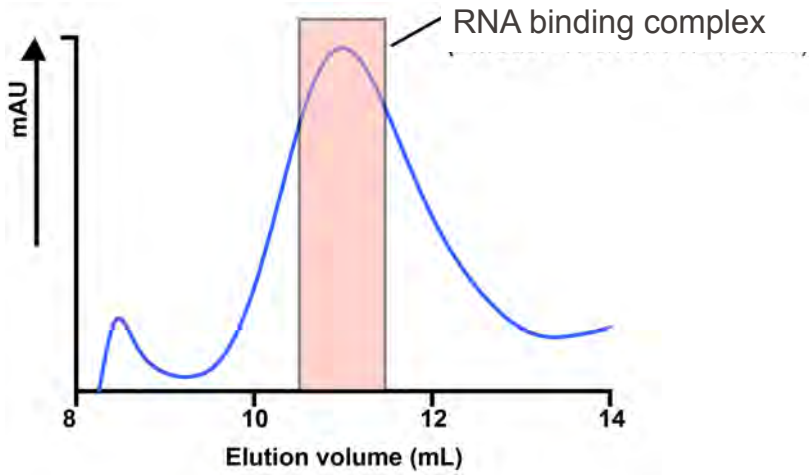
- Stage 2: Can my sample be frozen well?
  - Preparation of frozen grids
  - Cryo-EM imaging @ 200kV
  - Computer analysis for sample quality control

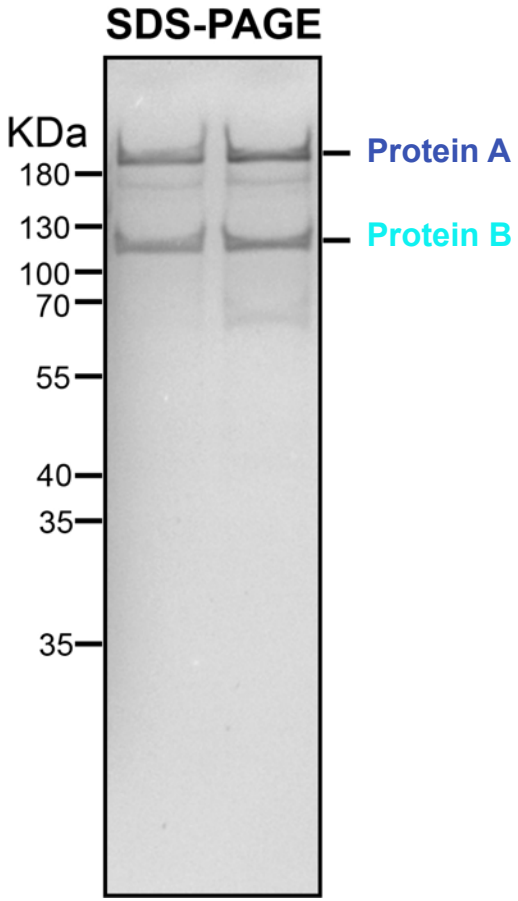
- Stage 3: Get the high-resolution structure
  - Identification of the optimal, frozen grids
  - Cryo-EM imaging @300kV
  - Computer analysis for 3D structure reconstruction

Mostly done in facilities

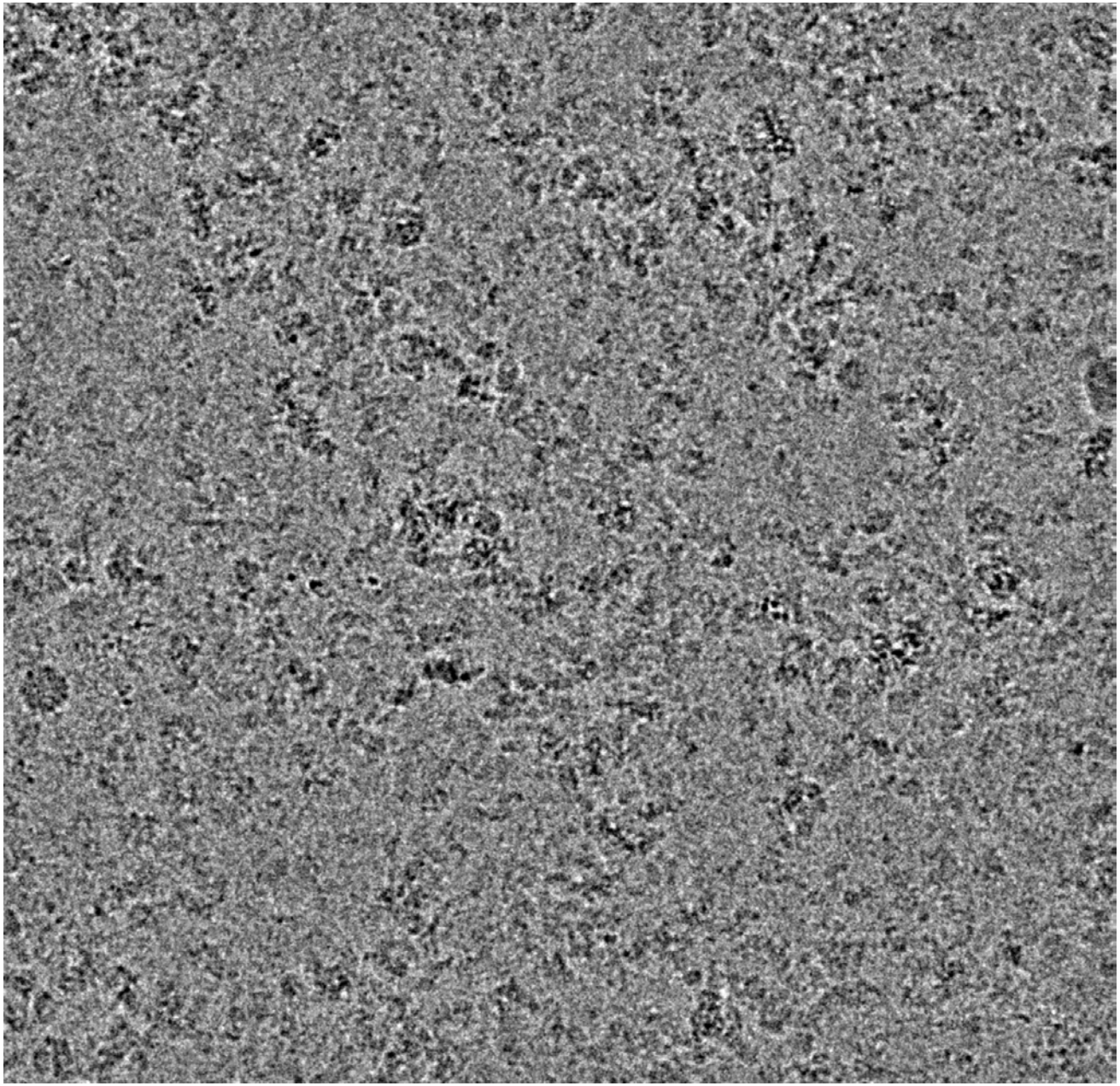
Example: A RNA binding complex





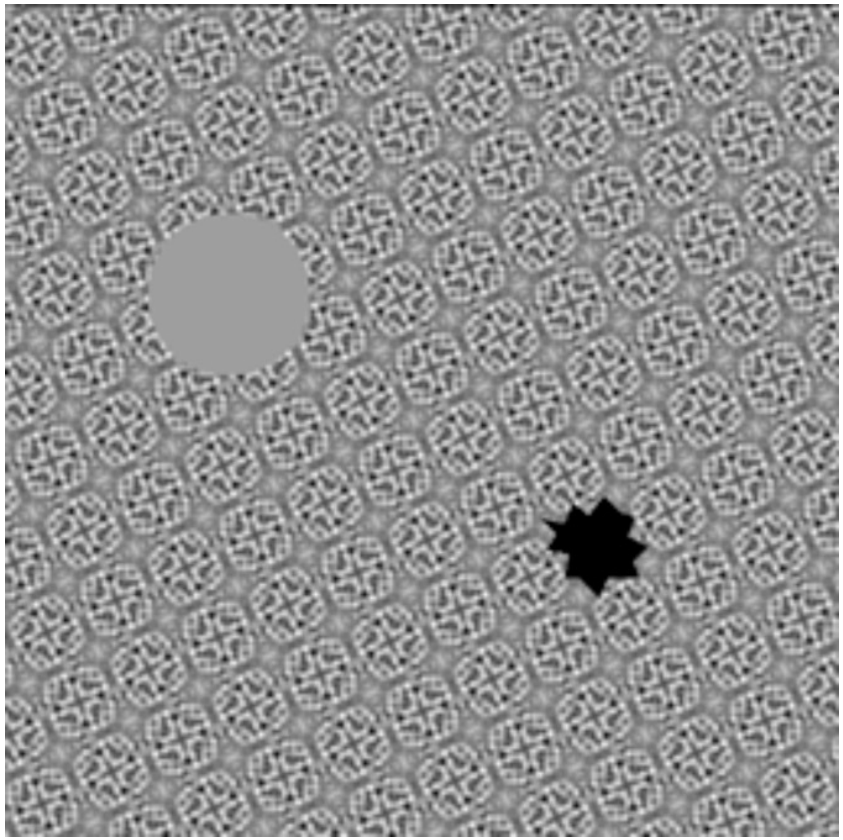


A RNA binding complex

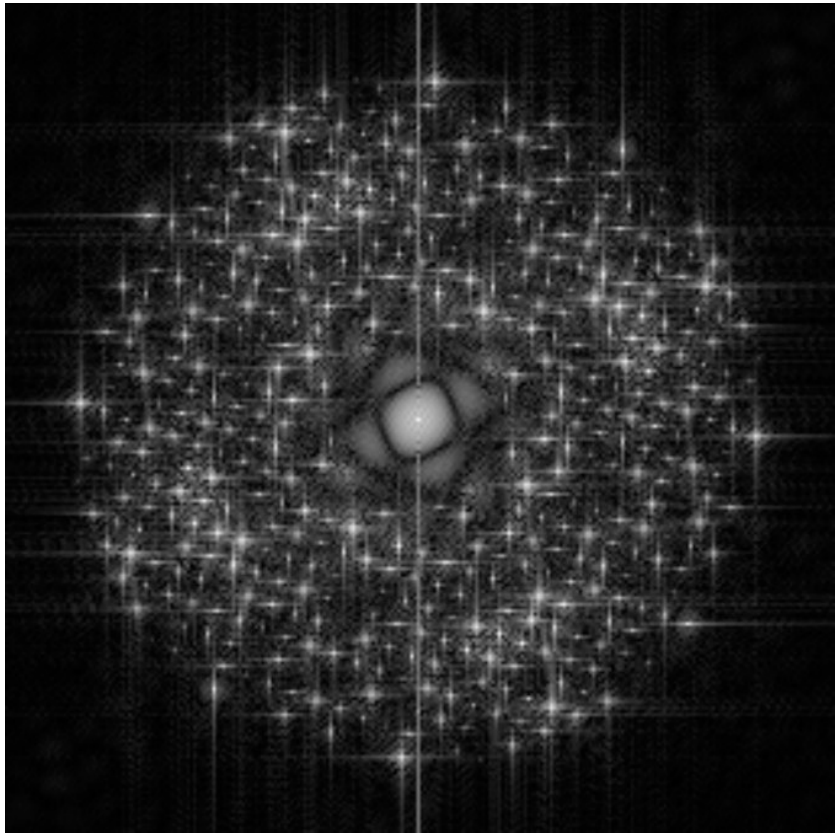


Unpublished work, 2022, Babatunde Edukpe Ekundayo, LBEM EPFL

Real Space



Fourier Transform



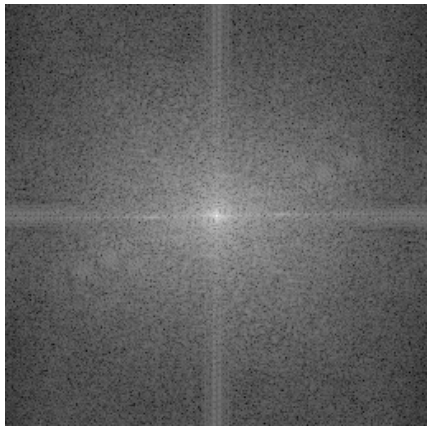
Real-space image <=> Fourier Transform

Real space

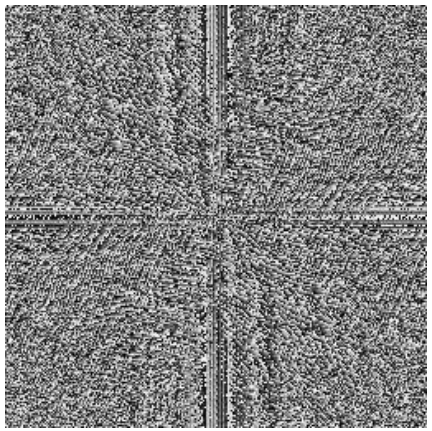
FT

FT^{-1}

Fourier space

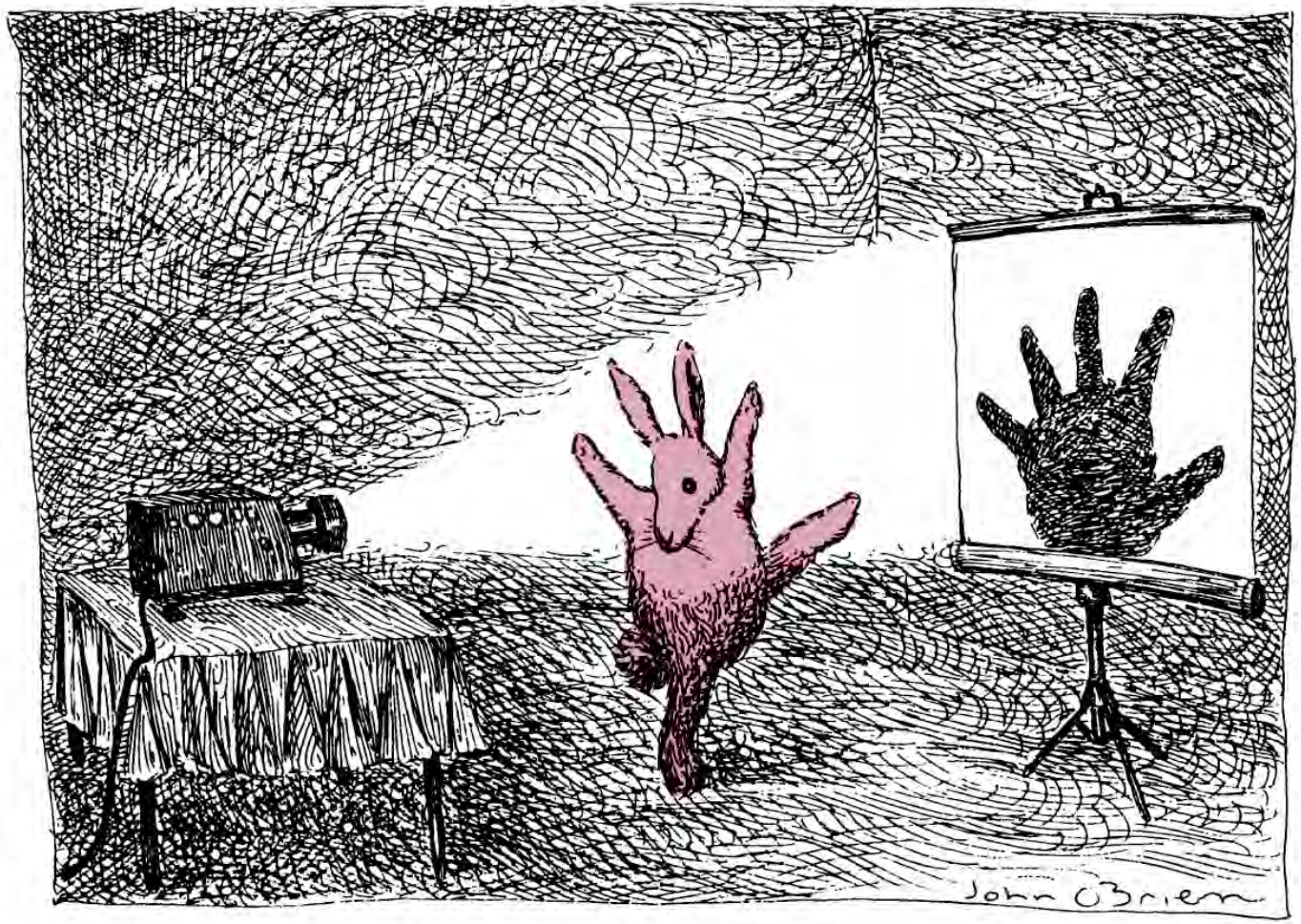


Amplitudes



Phases

2D -> 3D

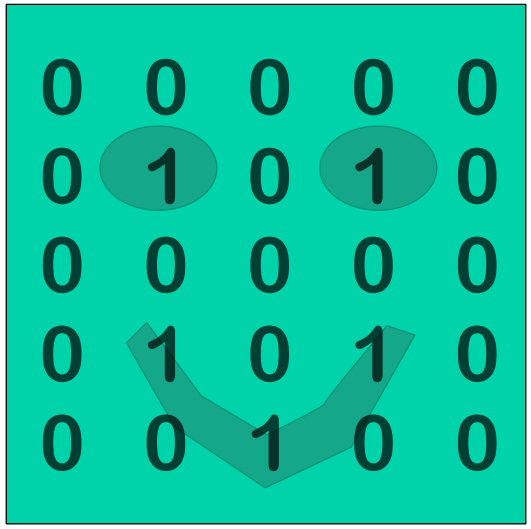


Drawing by John O'Brien; © 1991 The New Yorker Magazine

Projection

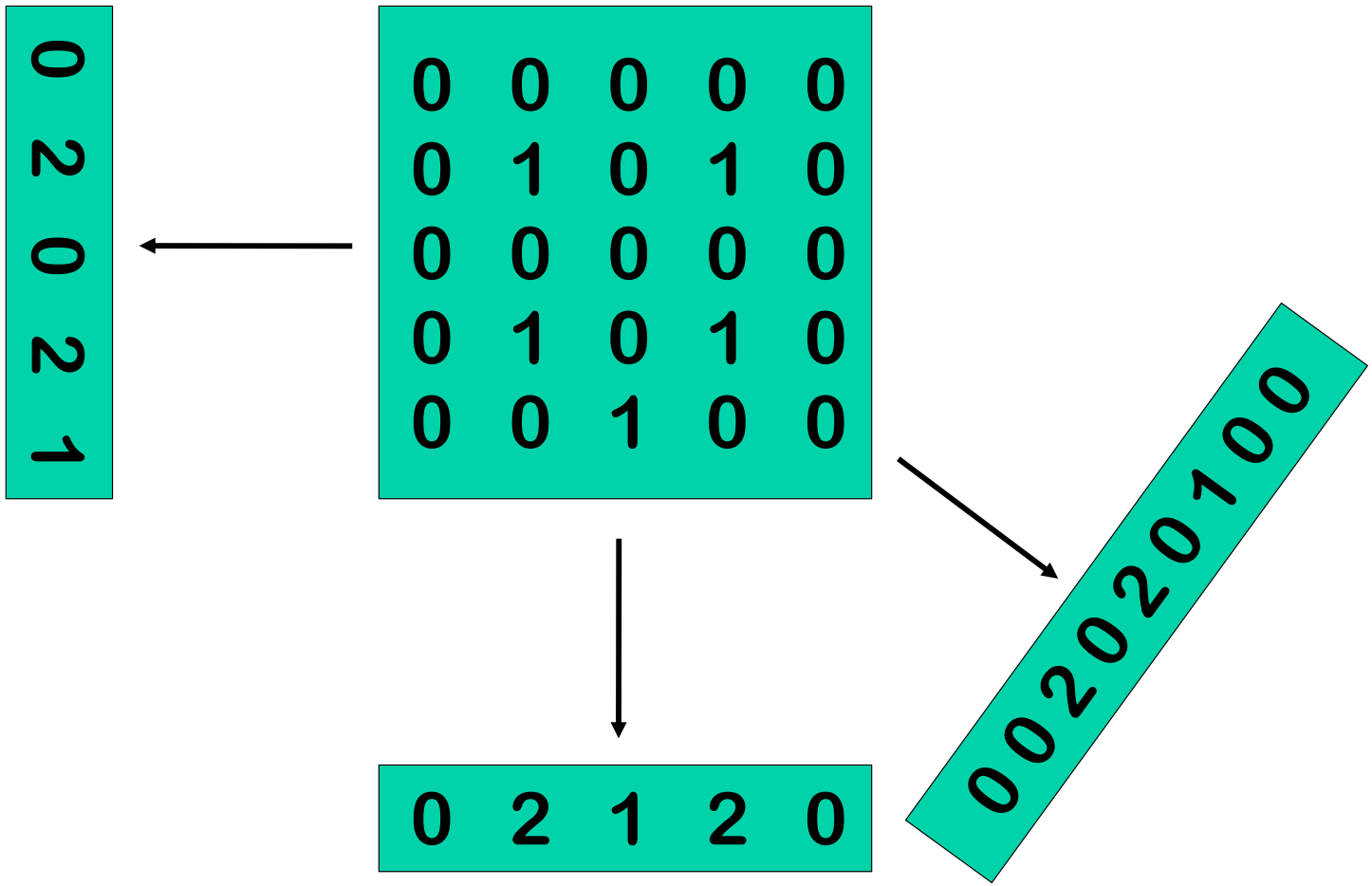
Real-Space Method

Projection



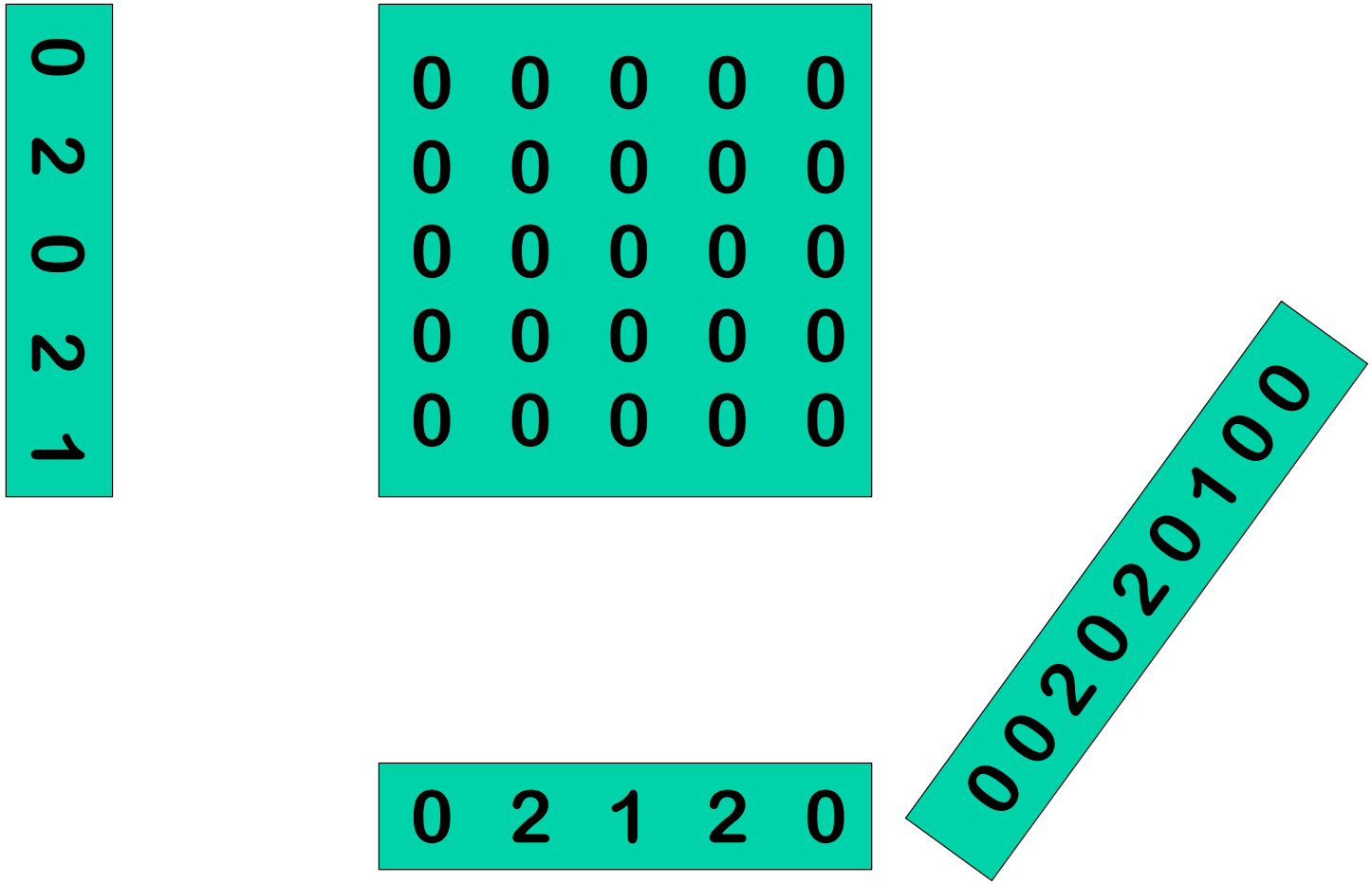
Real-Space Method

Projection



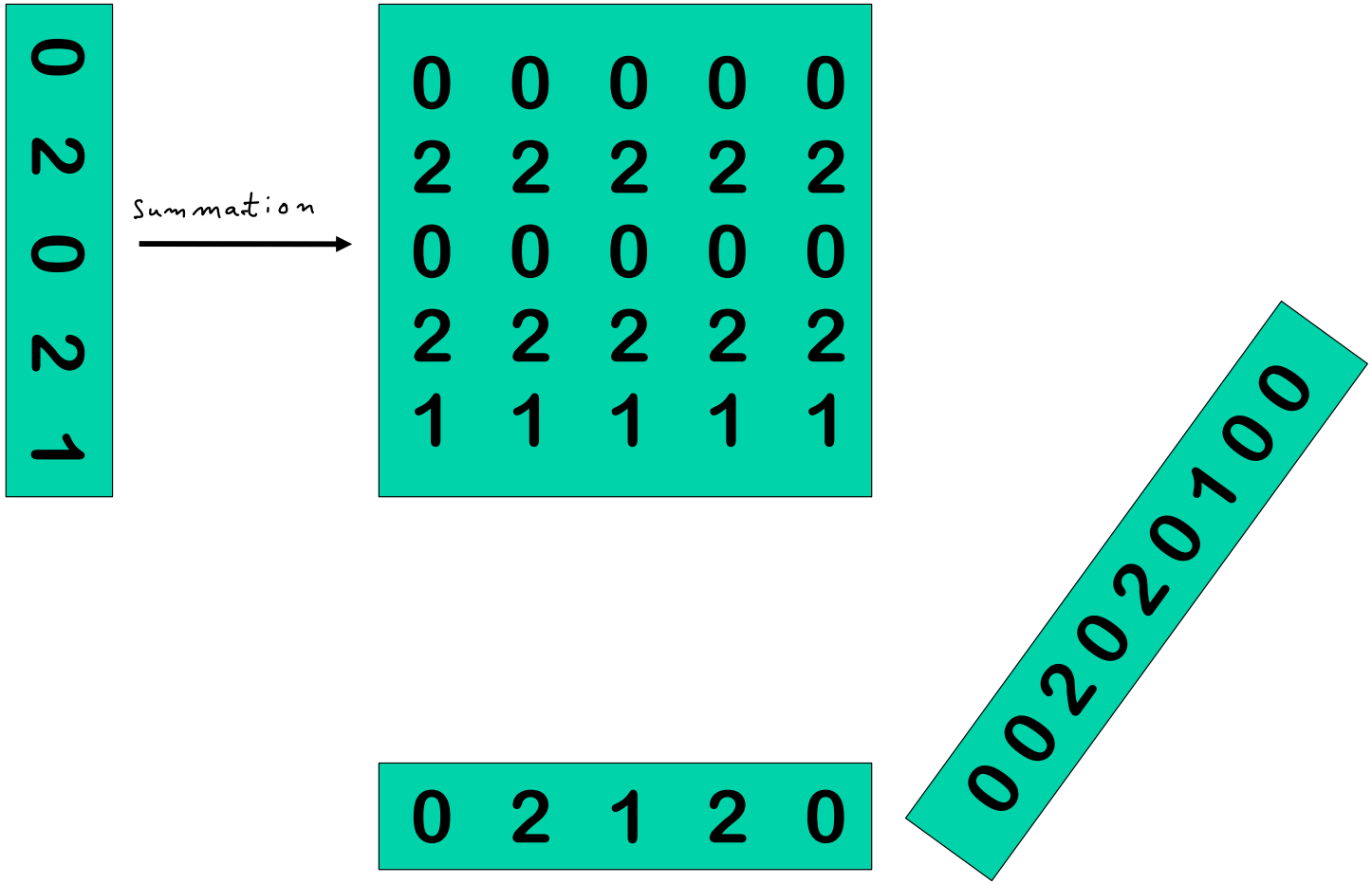
Real-Space Method

Backprojection



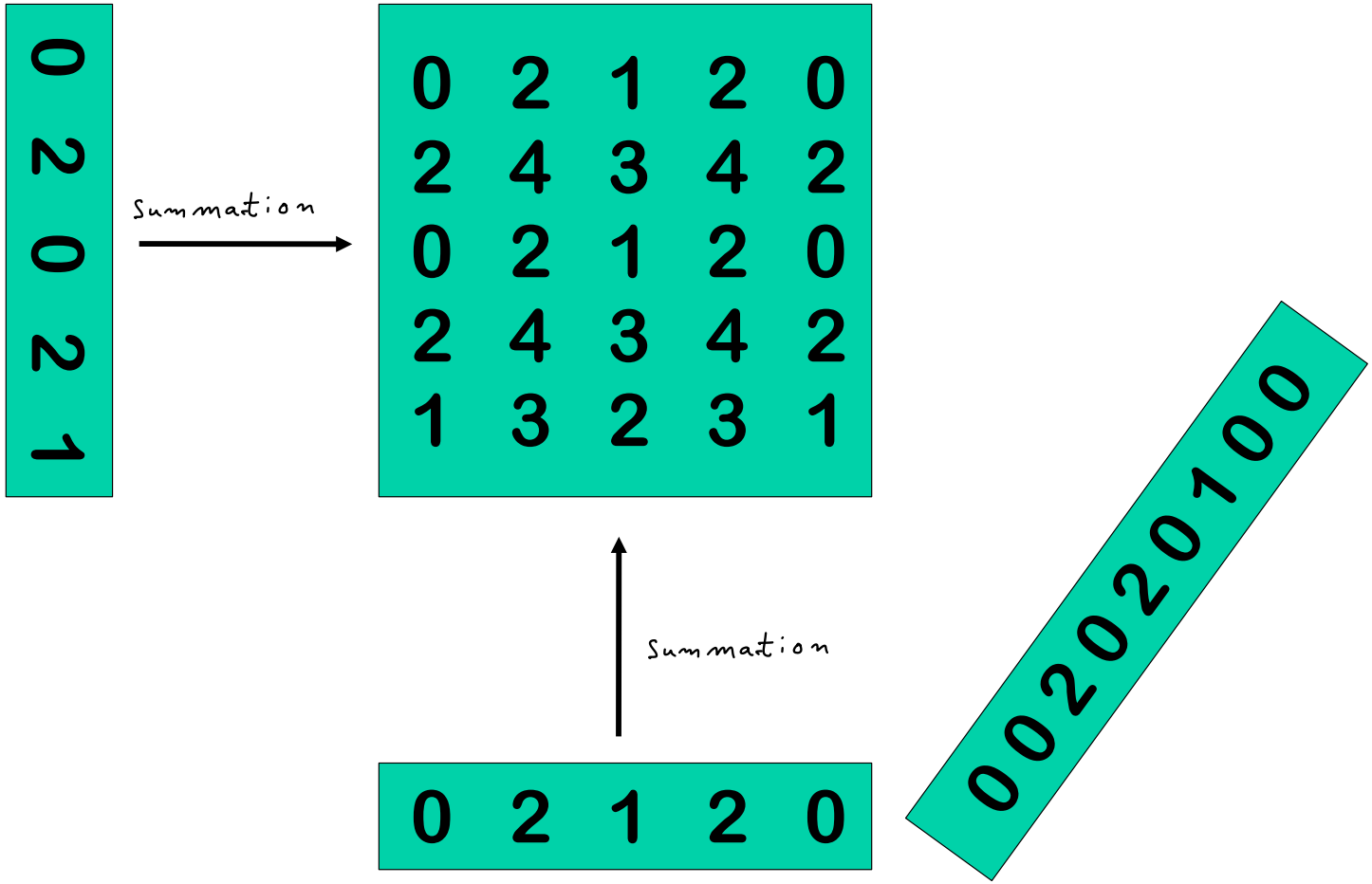
Real-Space Method

Backprojection



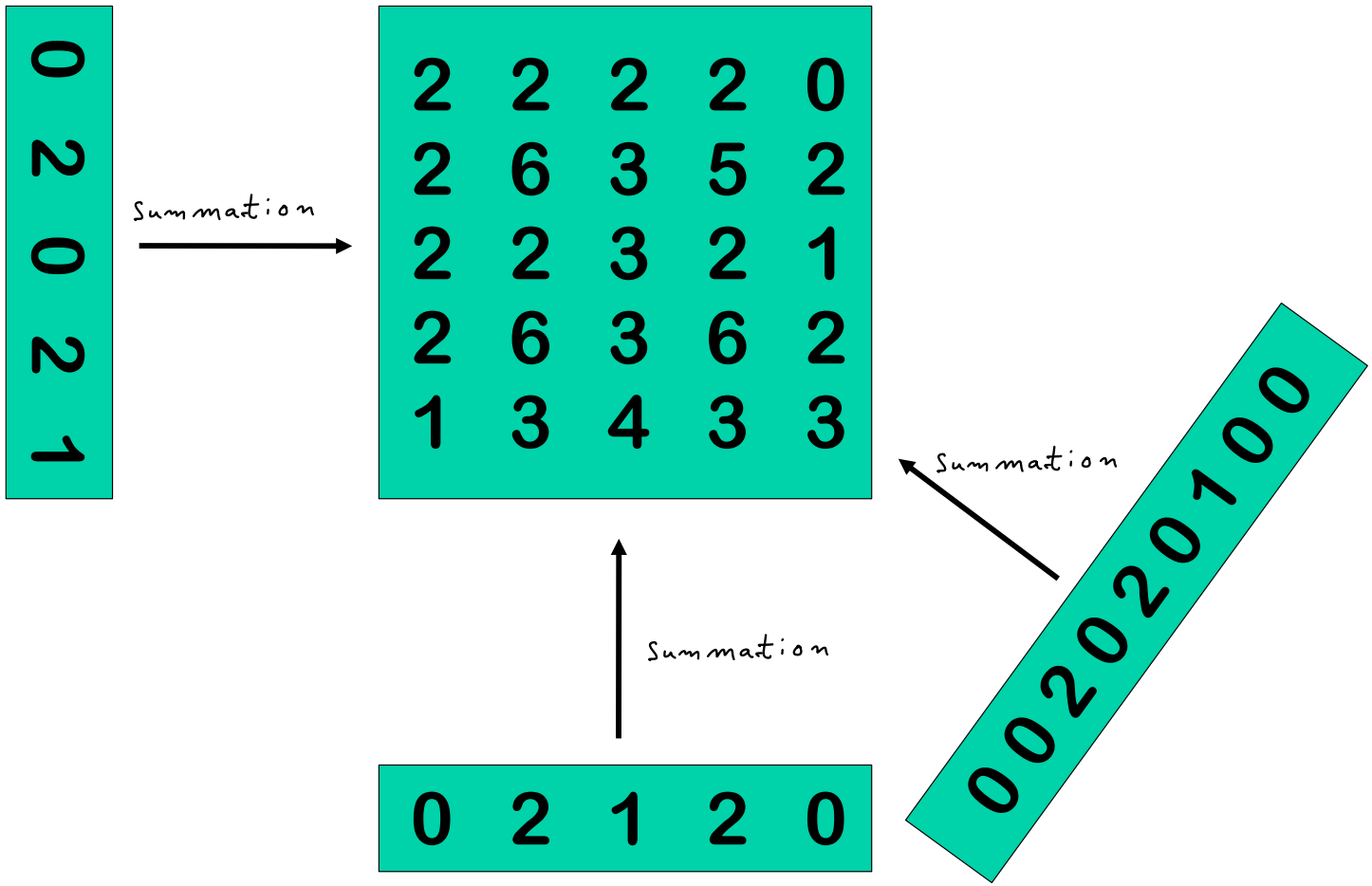
Real-Space Method

Backprojection



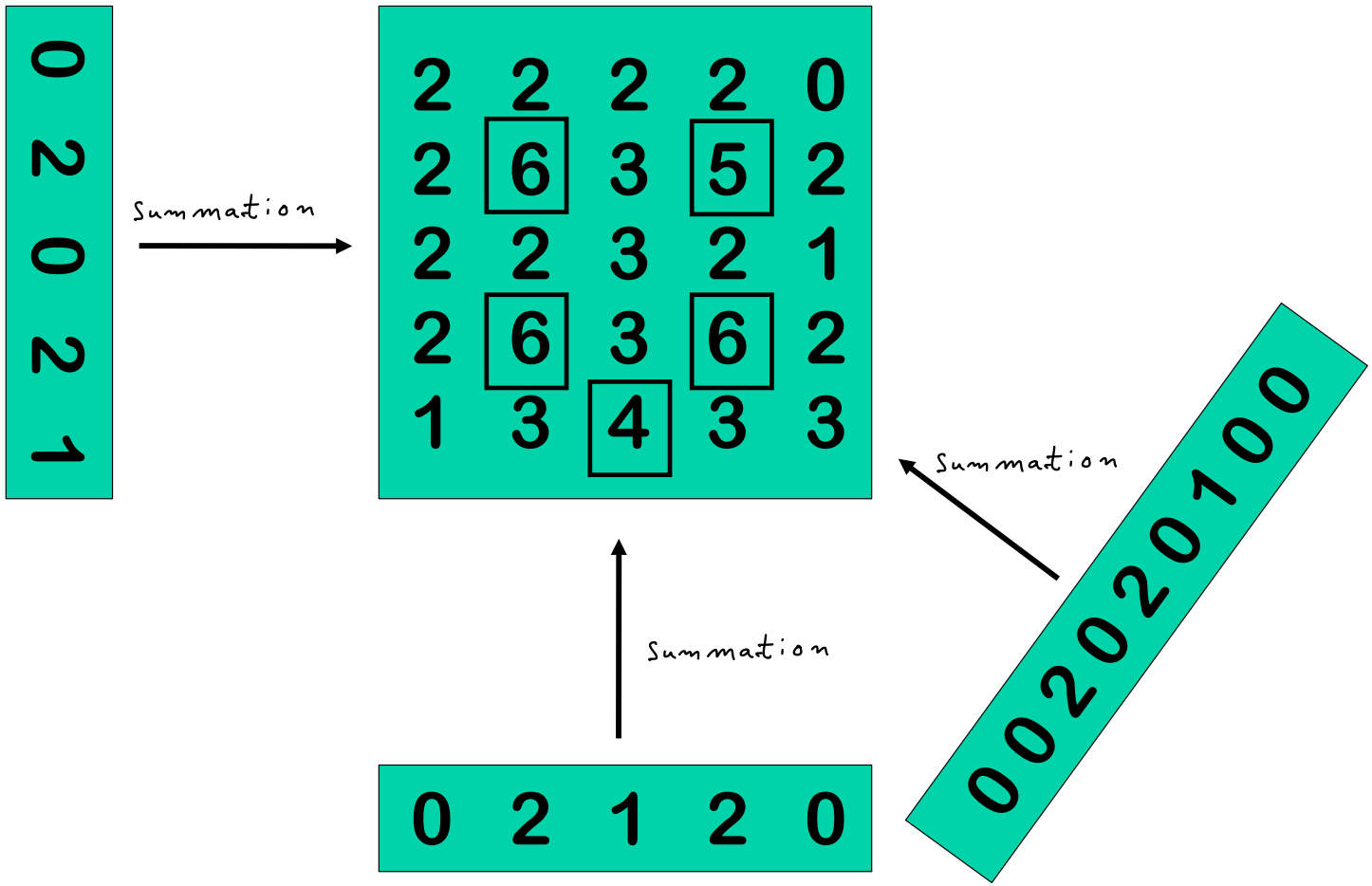
Real-Space Method

Backprojection



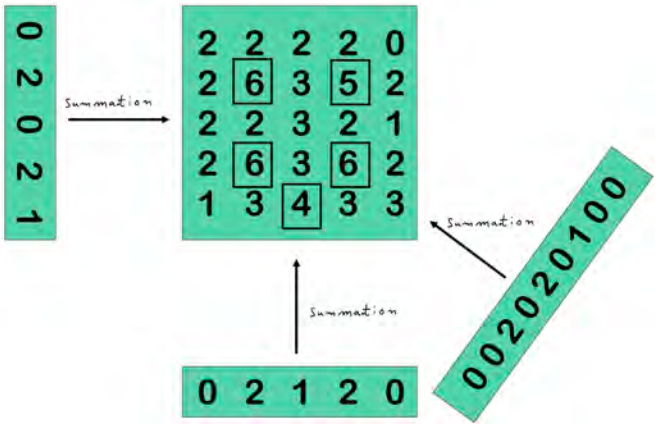
Real-Space Method

Backprojection



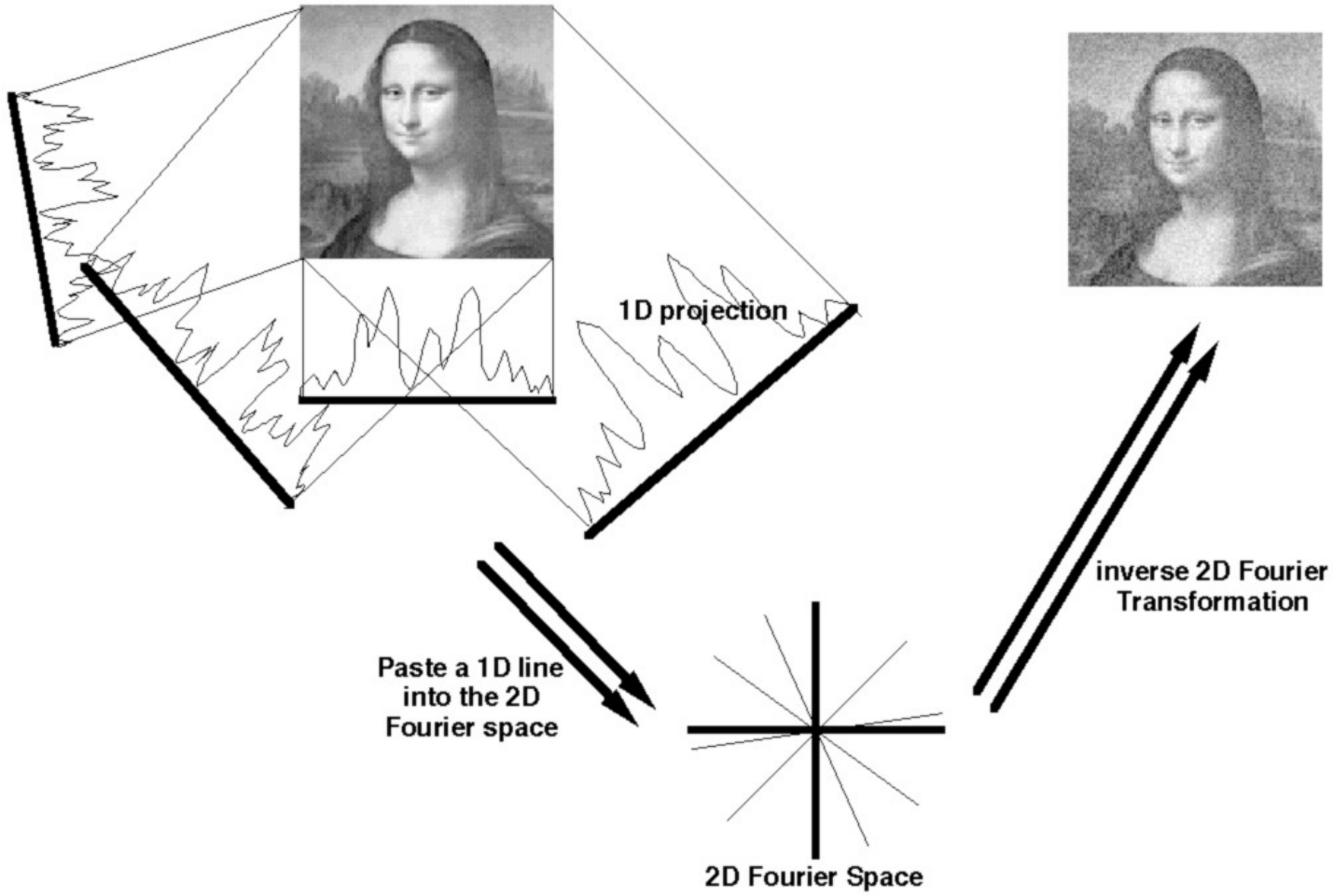
Real-Space Method

Shaping 3D structures out of snow



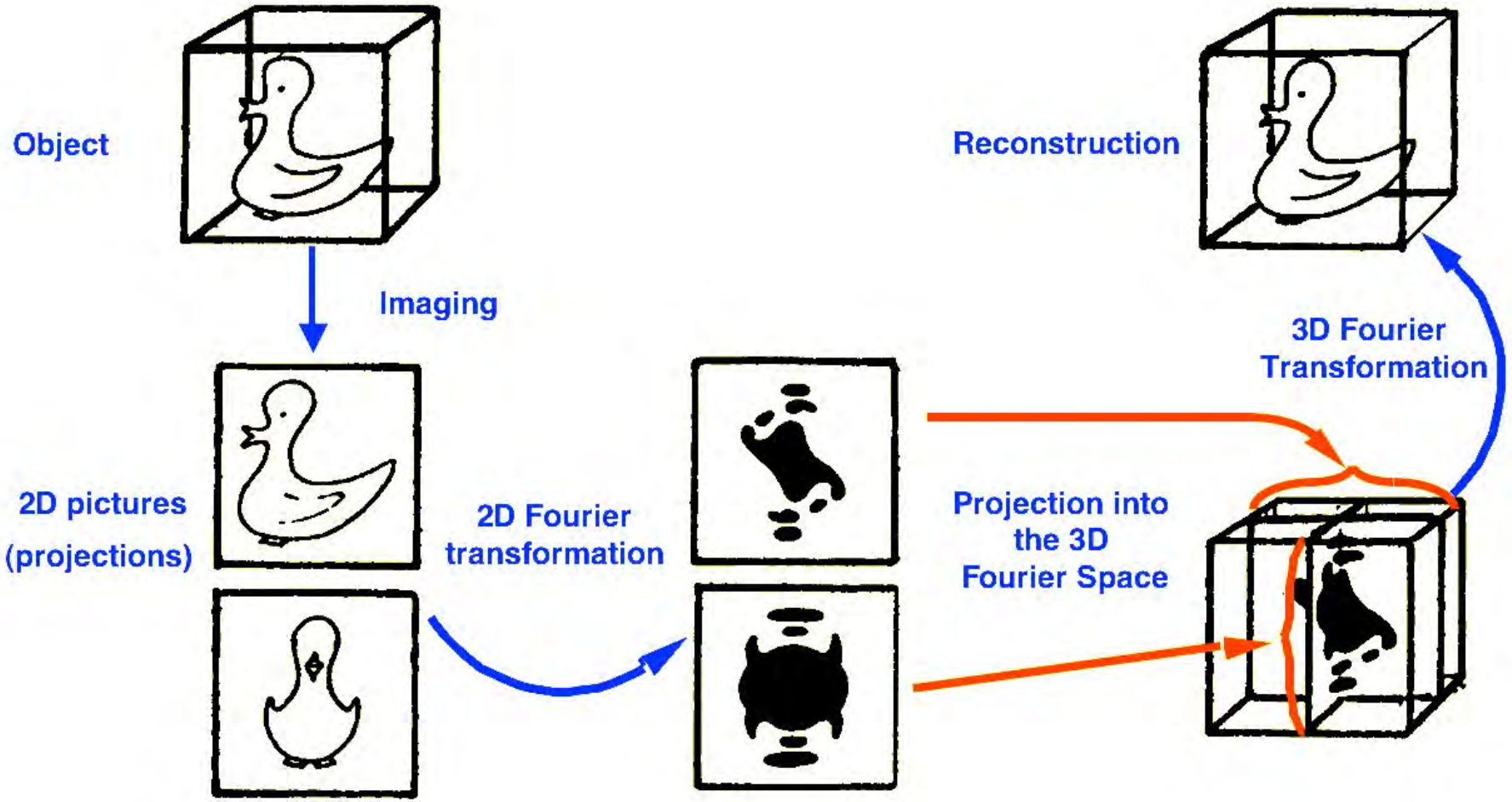
Real-Space Method

The Radon projection theorem for 3D reconstructions



Fourier-Space Method

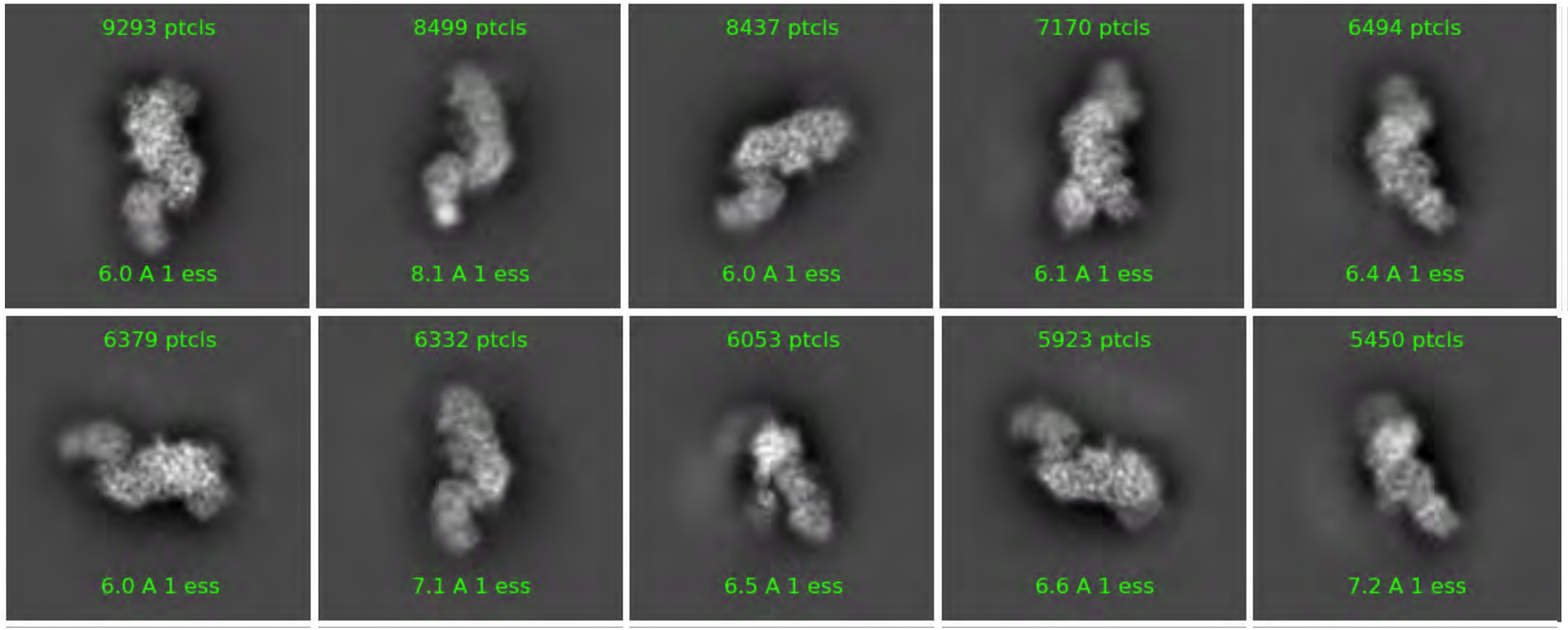
The Radon projection theorem for 3D reconstructions



Fourier-Space Method

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

A RNA binding complex



Cryo-EM: principle workflow

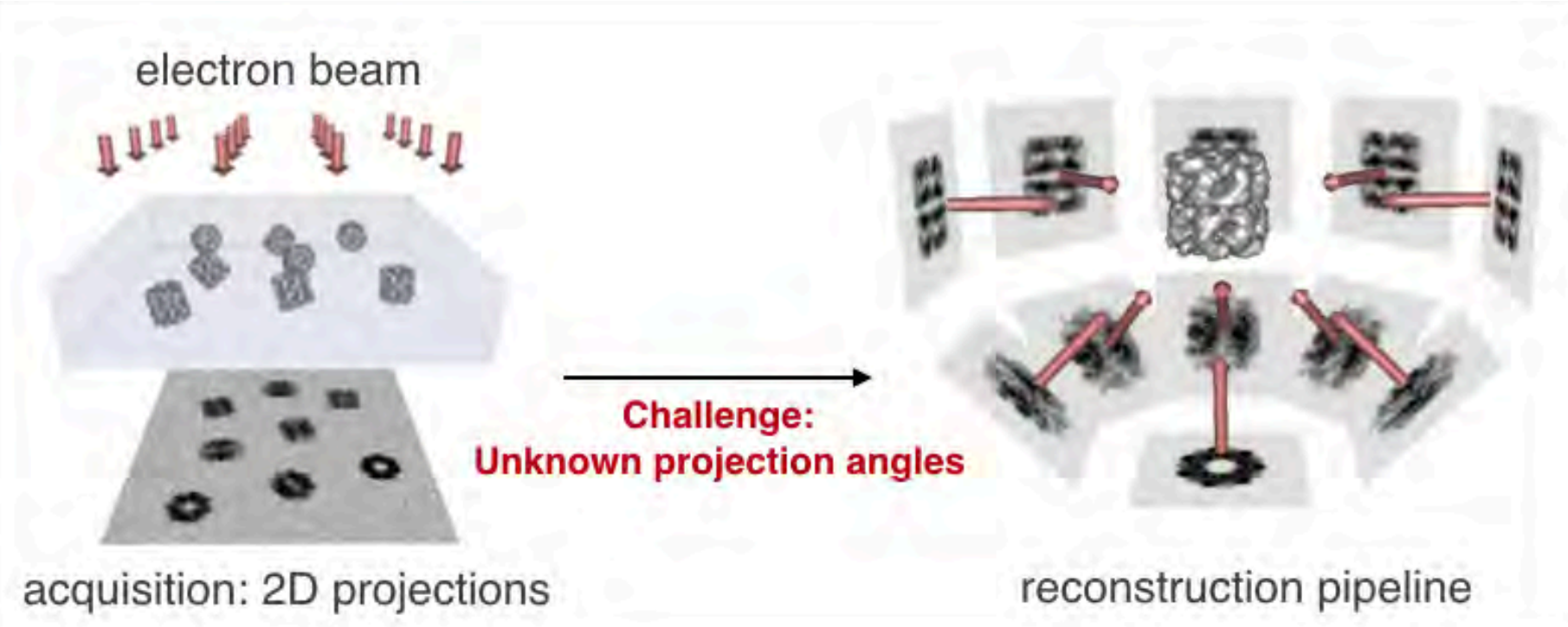
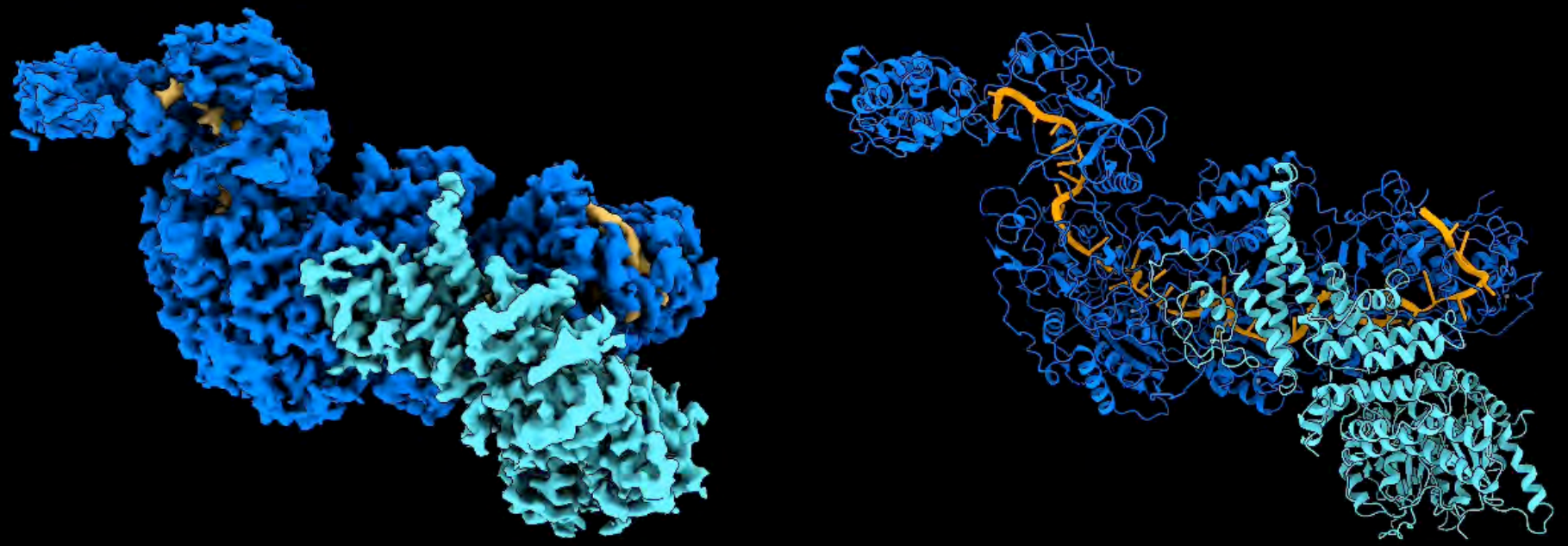


Illustration from Greg Pintilie, Ph.D. (MIT)

A RNA binding complex

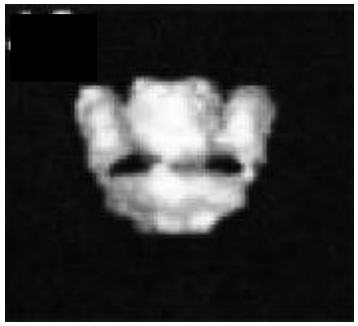
Structure of *D. magnum* Craspase (Cas7-11-TPR-CHAT)

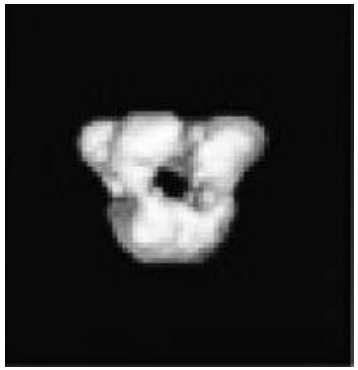


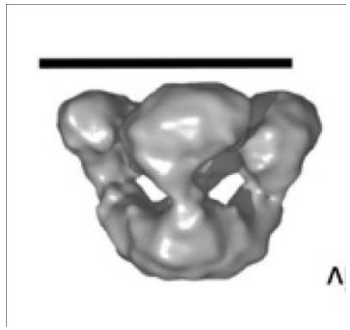
Single Particle Cryo-EM:
The “Einstein from Noise” Problem

IP3 receptor, 1.3 MDa

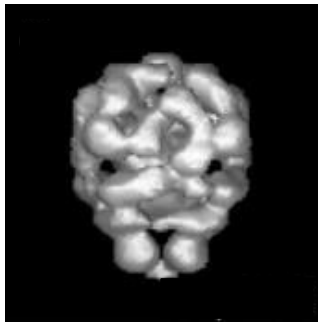
da Fonseca, 2003
Morris, 2010
neg. stain, 30Å



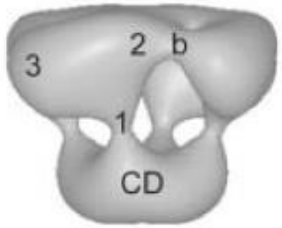




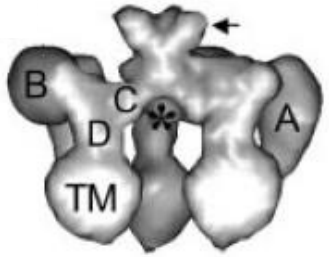
Sato, 2004
cryoEM 15Å



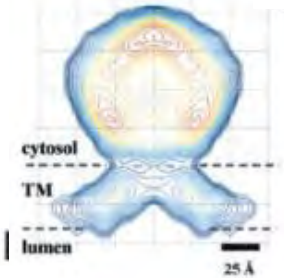
Hamada, 2003
neg. stain 34Å

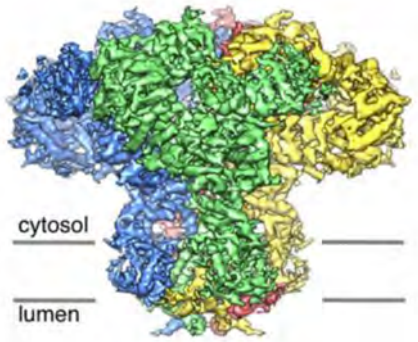


Serysheva, 2003
cryoEM, 30Å



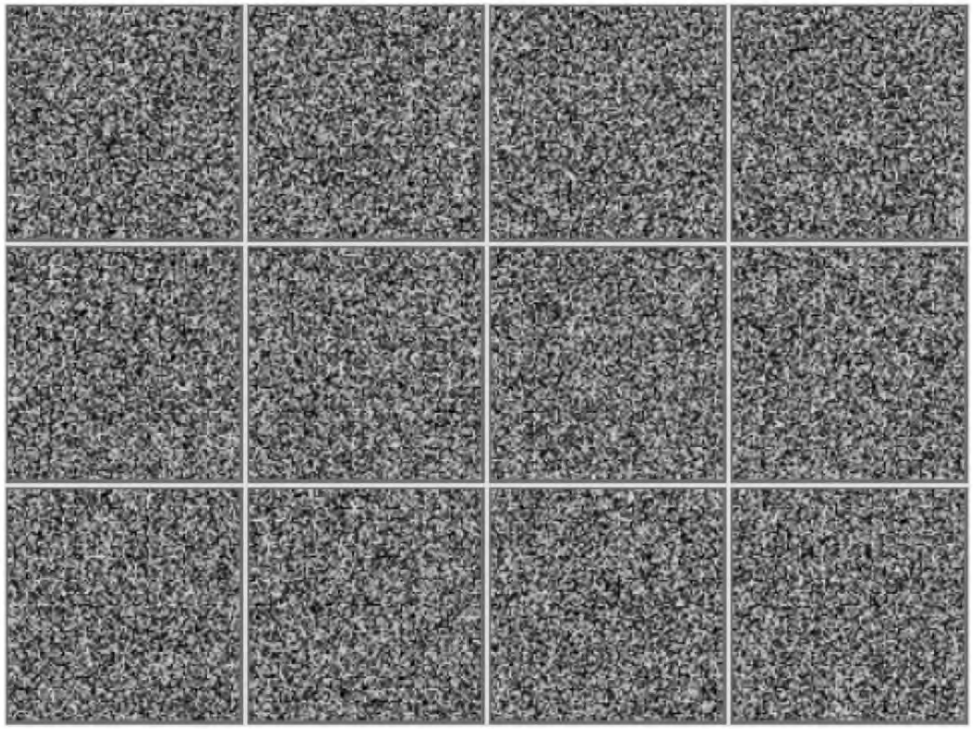
Sigworth, 2002
cryoEM, 24Å





Fan et al., Nature 2015 (4.7Å)

Reference Bias: A dangerous phenomenon.

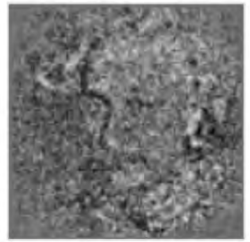


Random noise

Reference

ALIGN &

AVERAGE

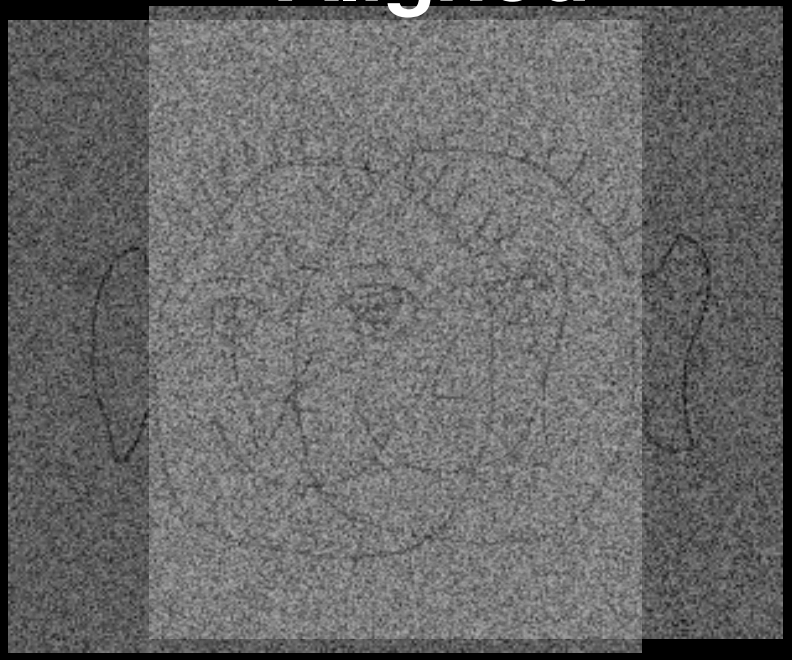


Average

Attention: Reference Bias in Particle Selection or in Particle Alignment can reproduce the reference feature, even if it was not present in the dataset.

REFERENCE BIAS

Wrongly Aligned

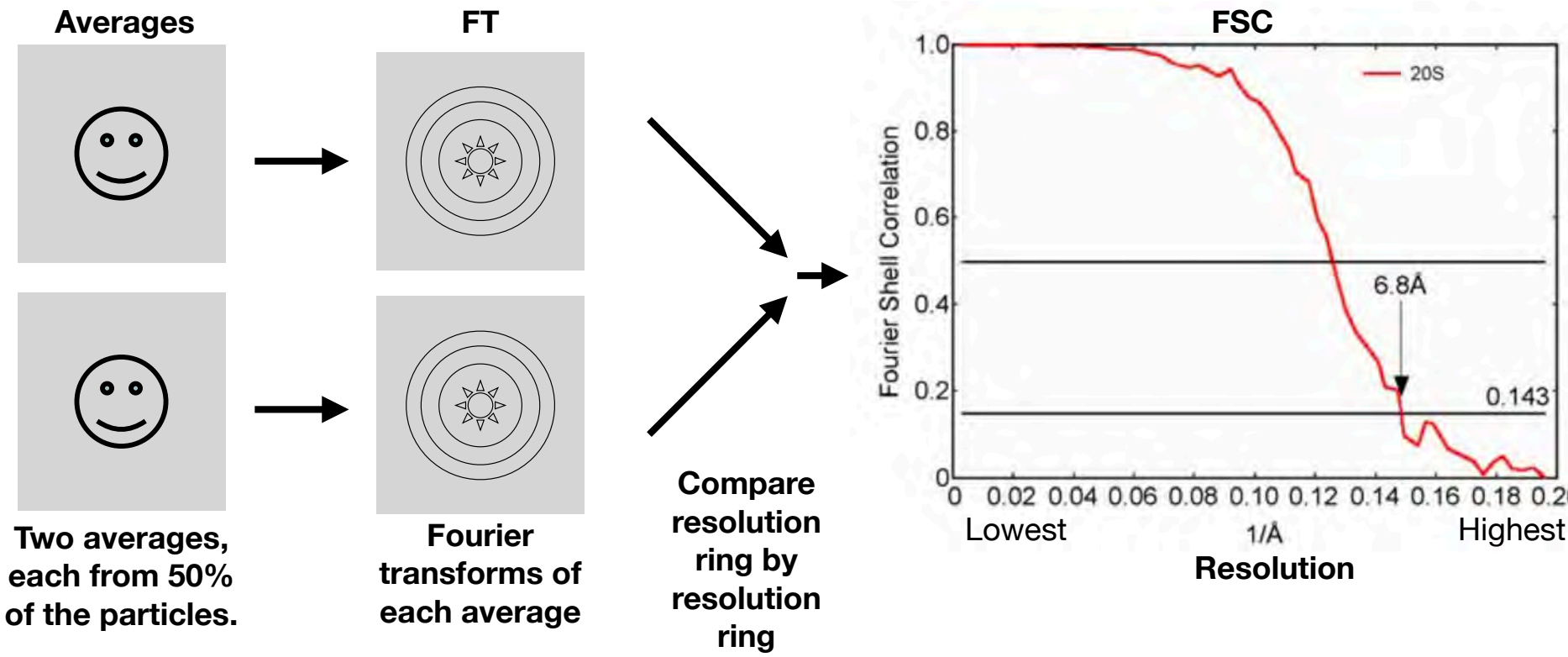


Three eyes

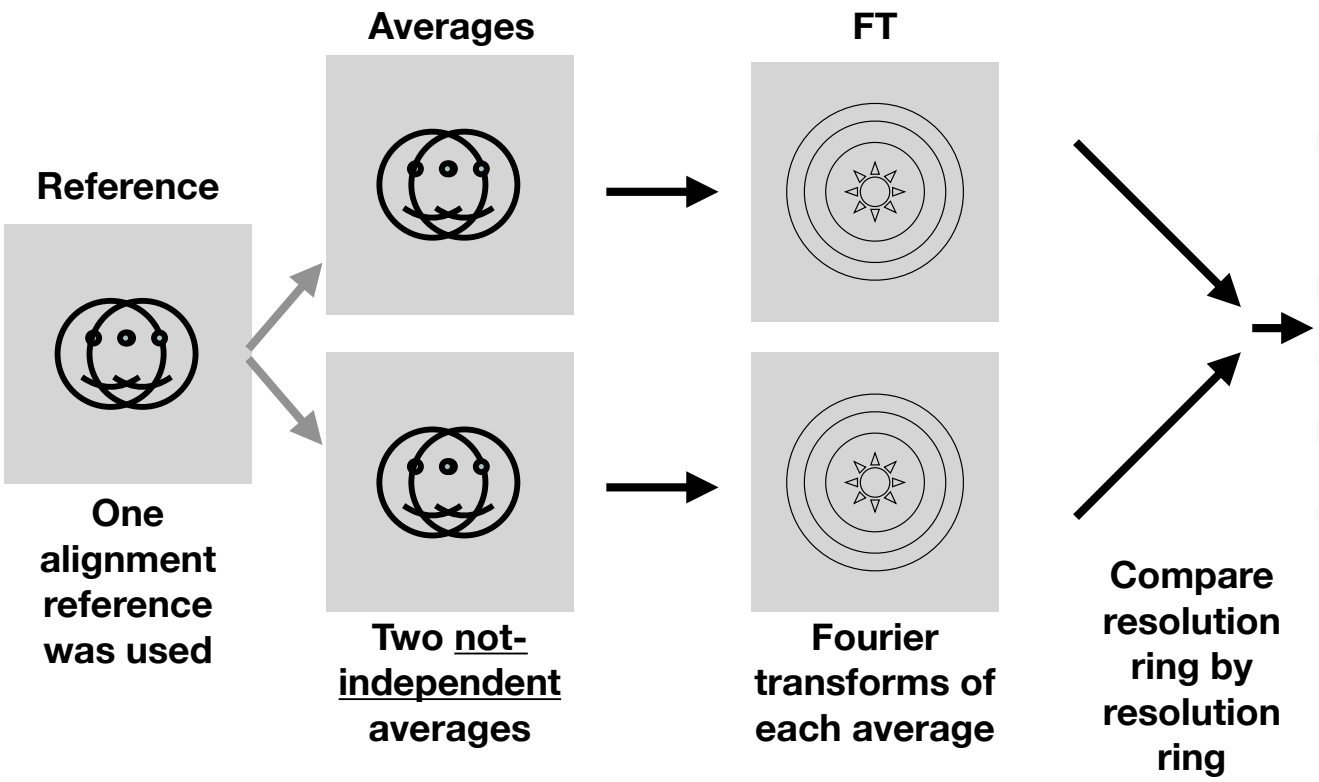
Correctly Aligned

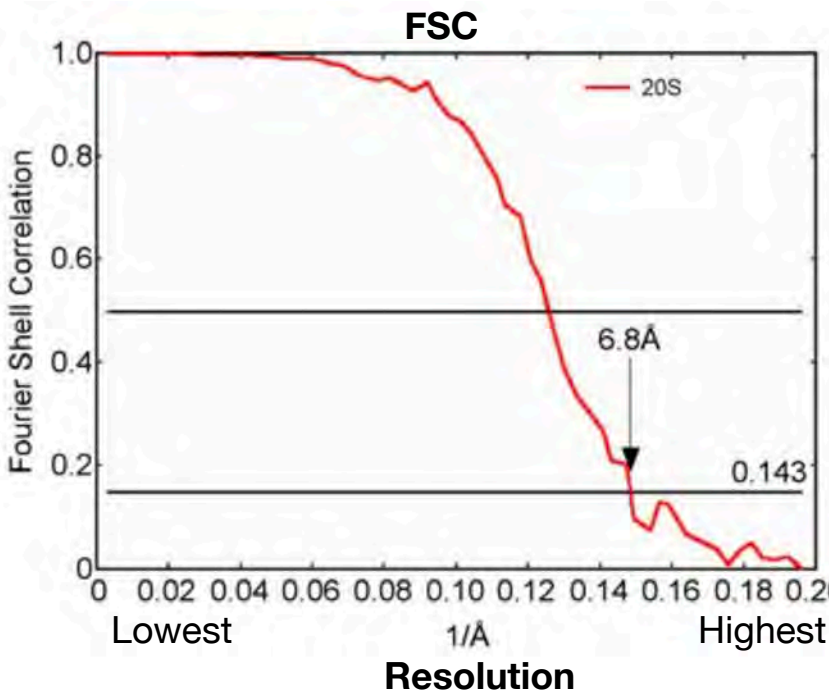
Two eyes

Resolution: Fourier ring correlation



Reference Bias Problem leads to apparently high resolution

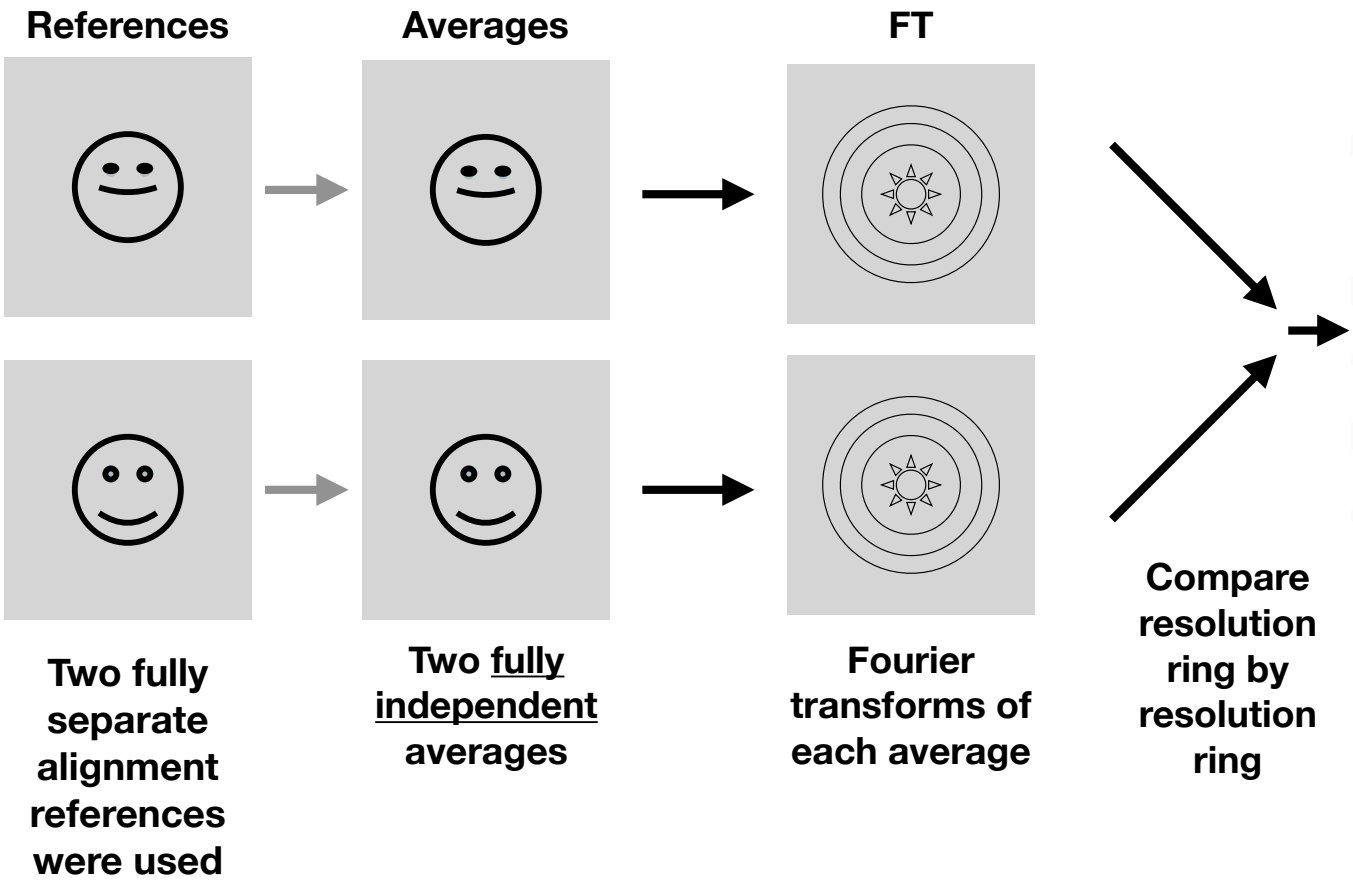


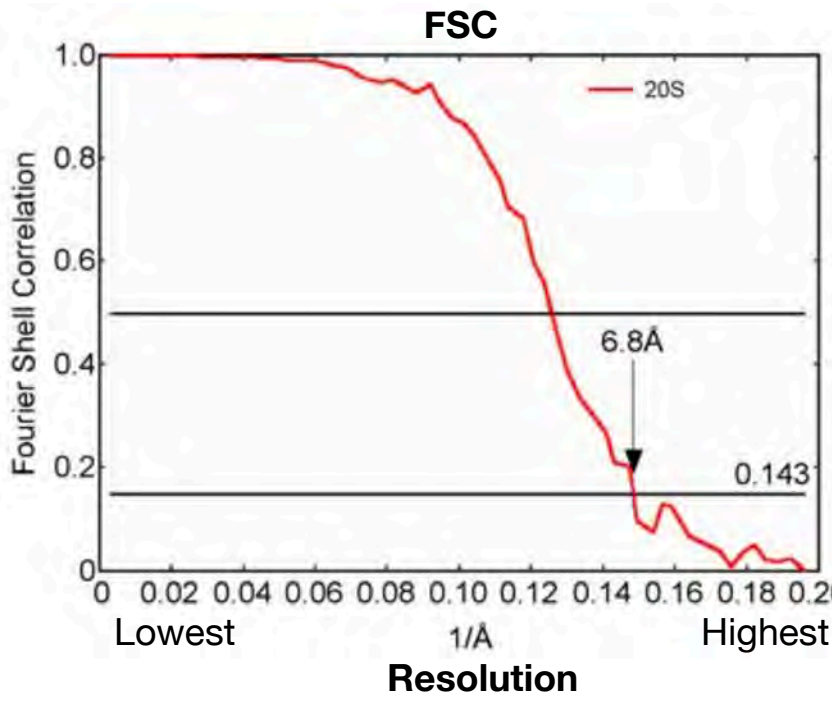


So-called “Gold Standard” Method

Work with two half-sets of the particles fully separated from A-Z.

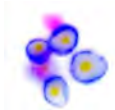
(You will need twice as many particles)





CryoSPARC, RELION

Single Particle Cryo-EM


Expression


Purification


Sample Preparation


Imaging

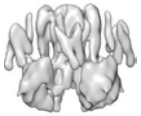
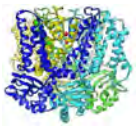


Image Processing


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

Expression

Purification

Sample Preparation

Imaging

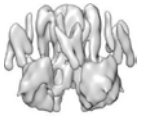
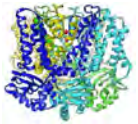
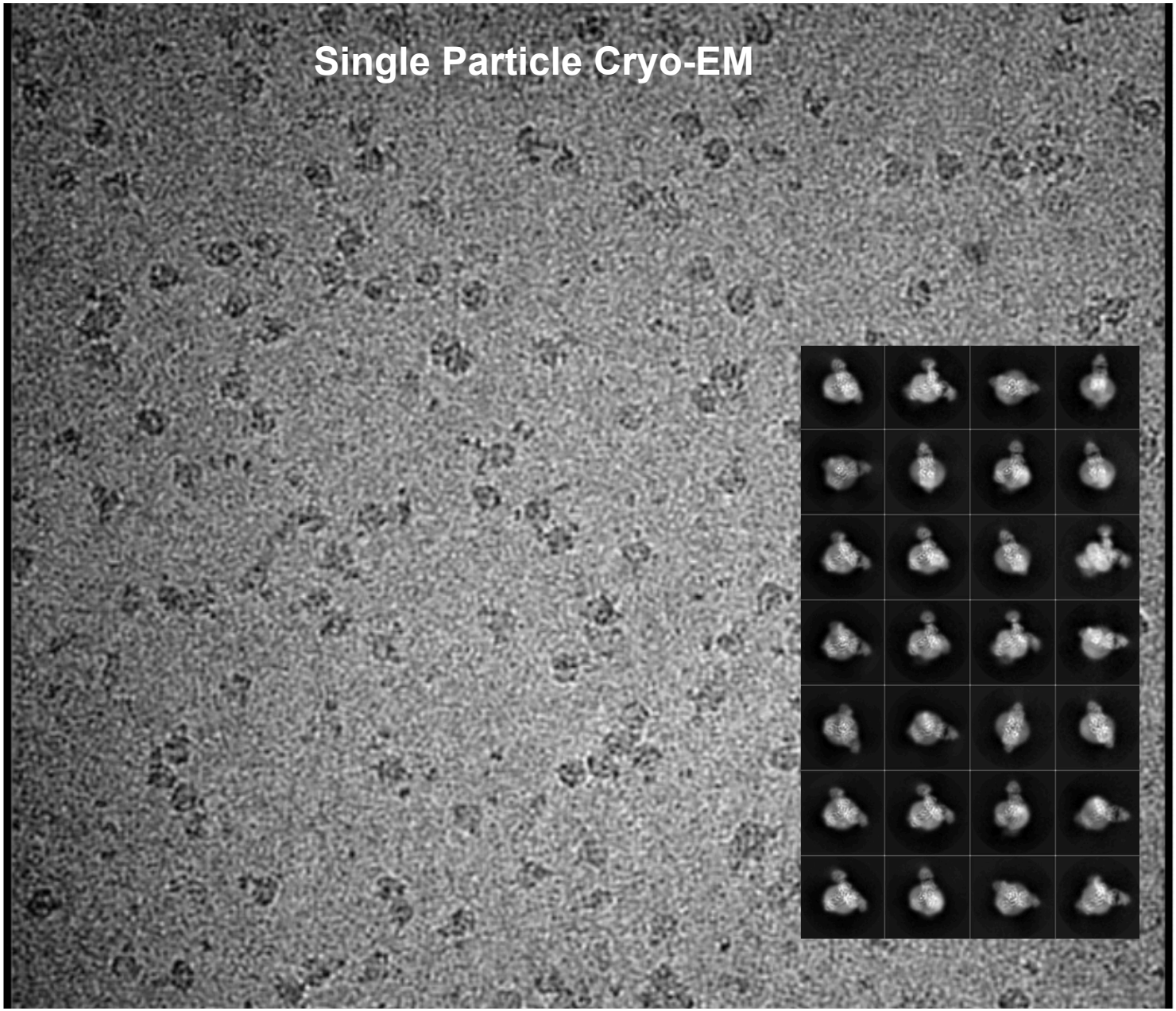


Image Processing



Model Building



Cryo-EM of the human ABC transporter ABCG2


Expression


Purification


Sample Preparation


Imaging

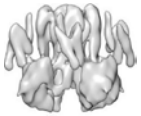
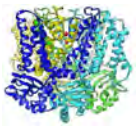
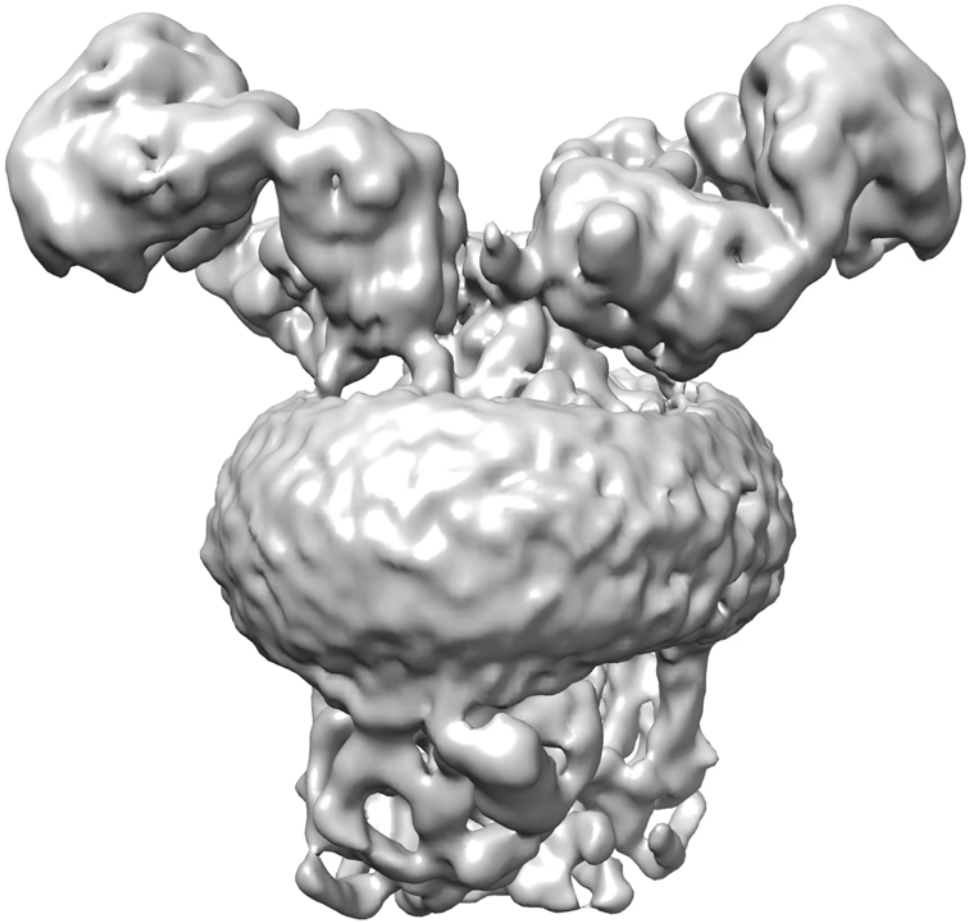
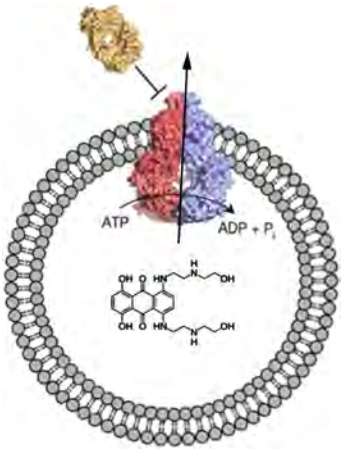


Image Processing


Model Building



Collaboration with Kaspar Locher, ETHZ

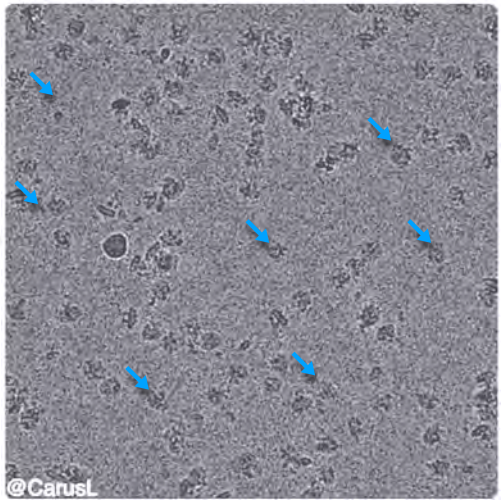


Membrane protein
in a lipid nanodisk.
Data collection:
3 days on C-CINA Titan,
1702 movies,
232’608 particles
97’612 particles in final map.
Final resolution 3.8Å

Cryo-Electron Microscopy

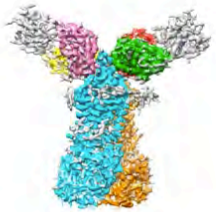
Frozen sample goes into microscope

10’000 Images (> 10 TB raw data)

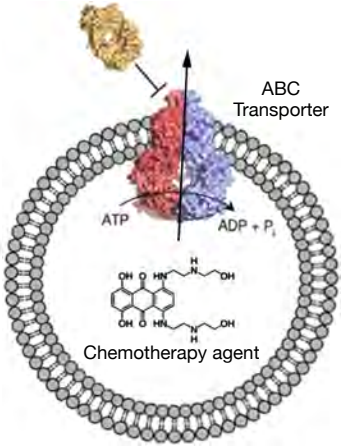


(1’000’000 particle images are needed)

Computer 3D Reconstruction



Atomic Model

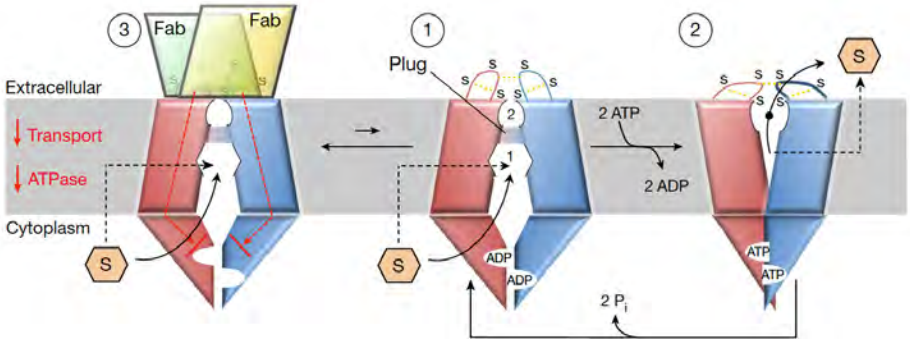


Understanding the mechanism of chemotherapy resistance

Cancer cells defend themselves from mitoxantrone chemotherapy with the protein ABCG2.

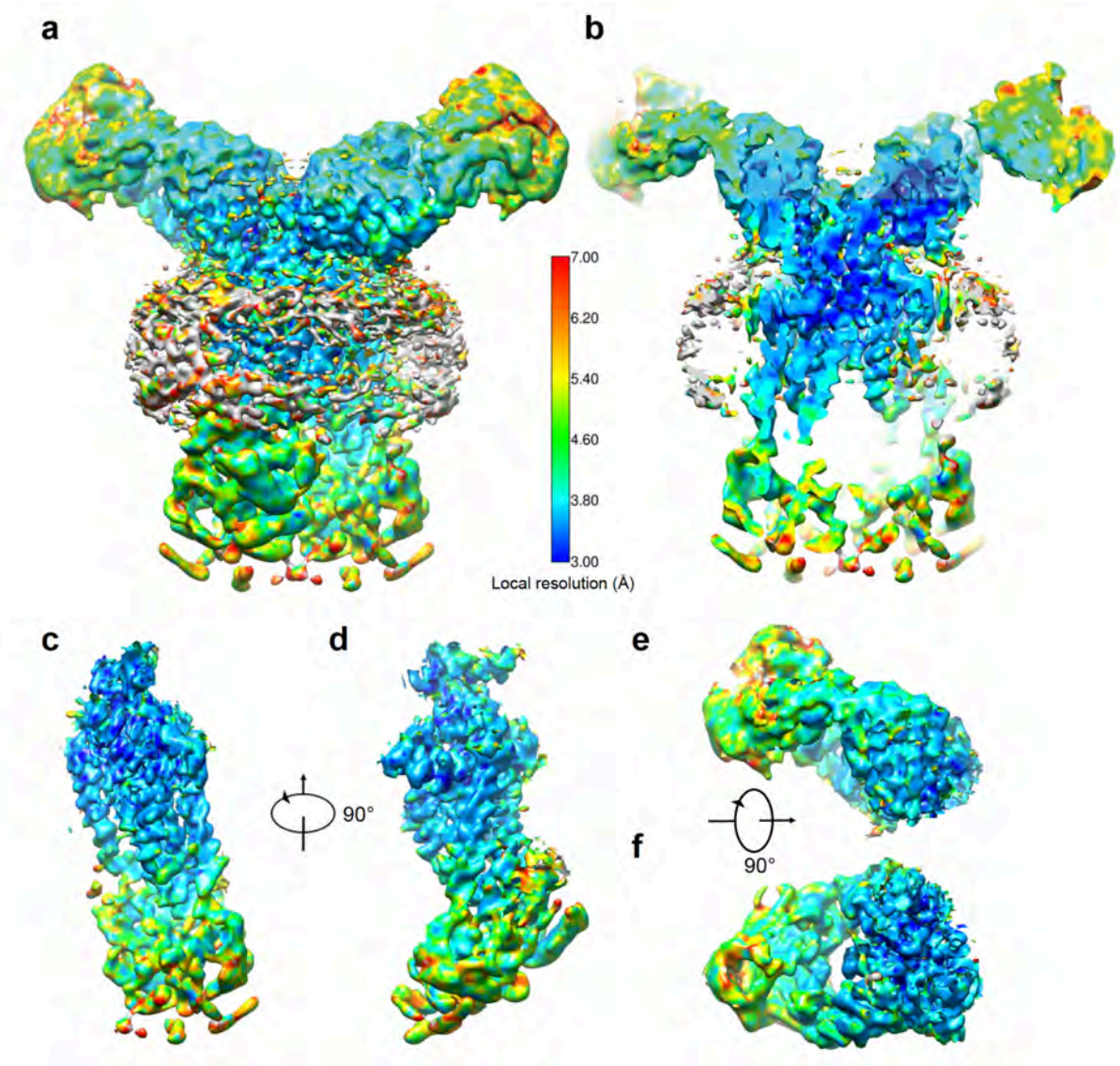
Blocking ABCG2 with a small *Fab* during chemotherapy kills the tumor.

<u>Understanding and fighting cancer resistance.</u>



Taylor et al., Nature (2017). With Kaspar Locher lab, ETHZ

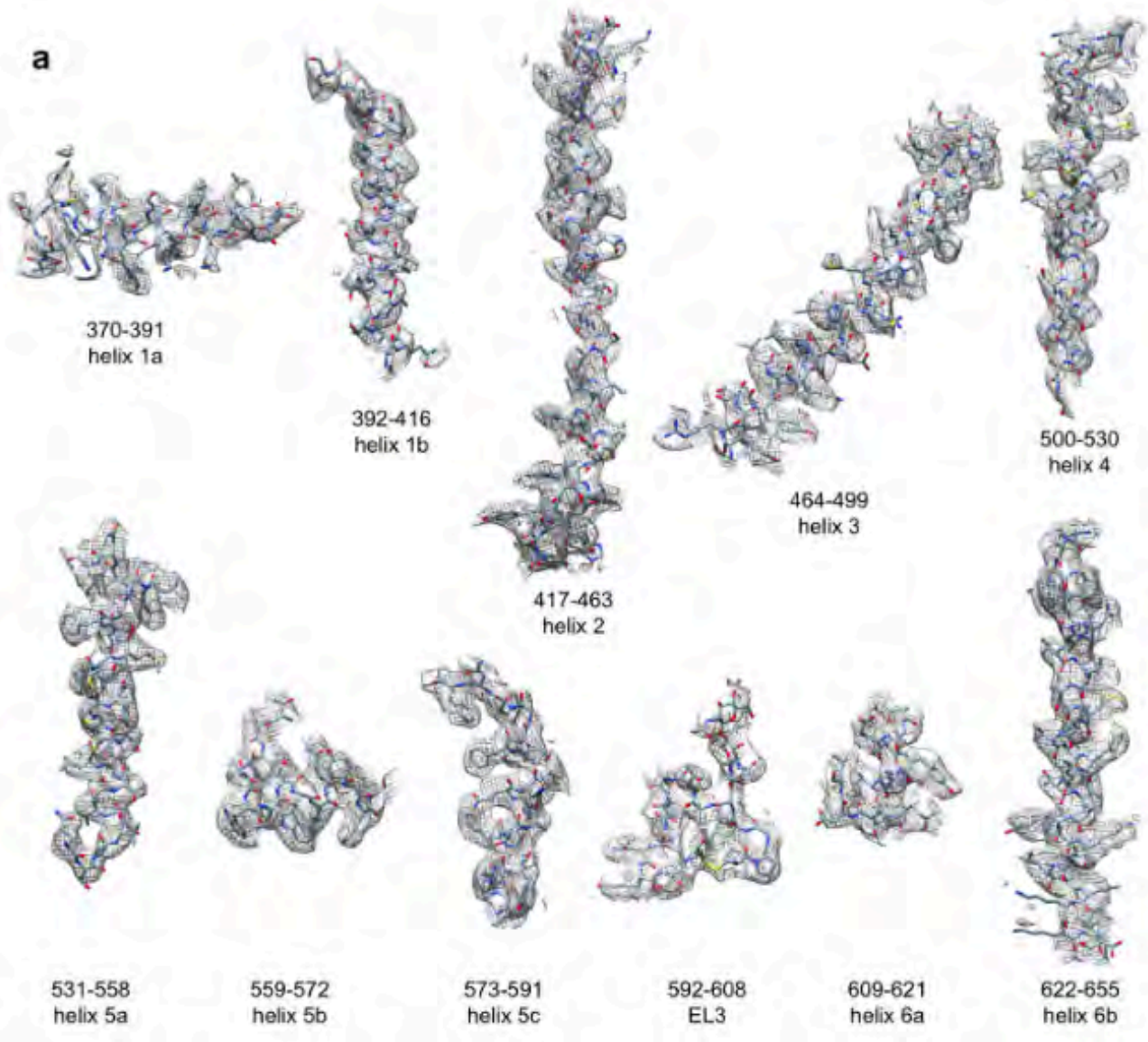
Cryo-EM of the human ABC transporter ABCG2



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

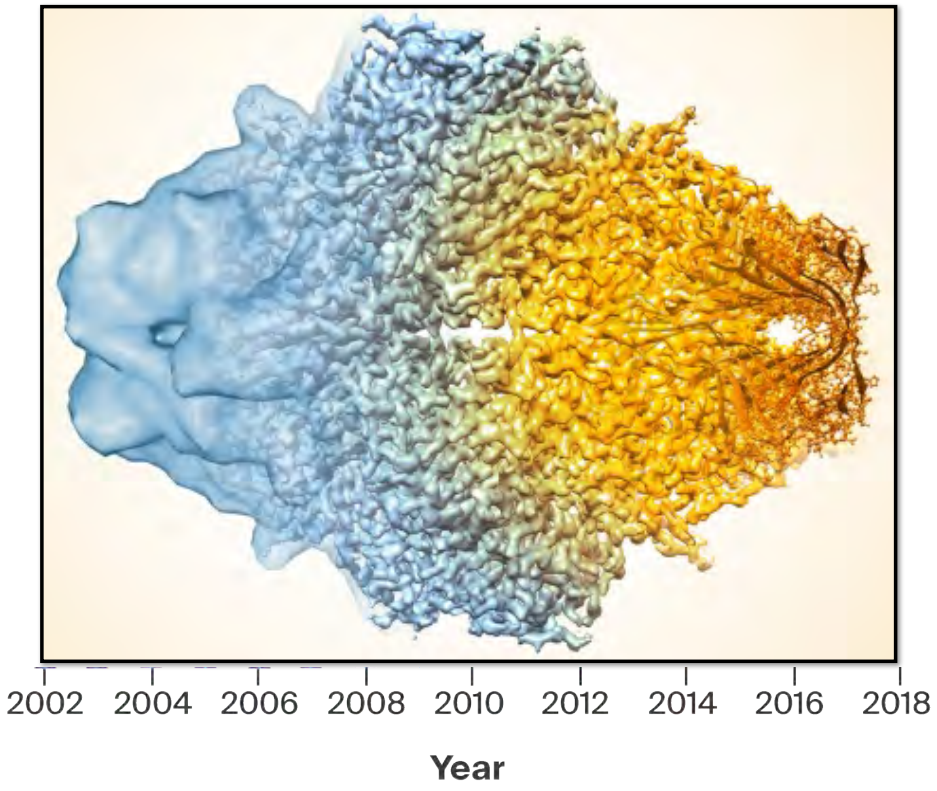
Nicholas M. I. Taylor[1]*, Ioannis Manolaridis[2]*, Scott M. Jackson[2]*, Julia Kowal[2], Henning Stahlberg[1] & Kaspar P. Locher[2]

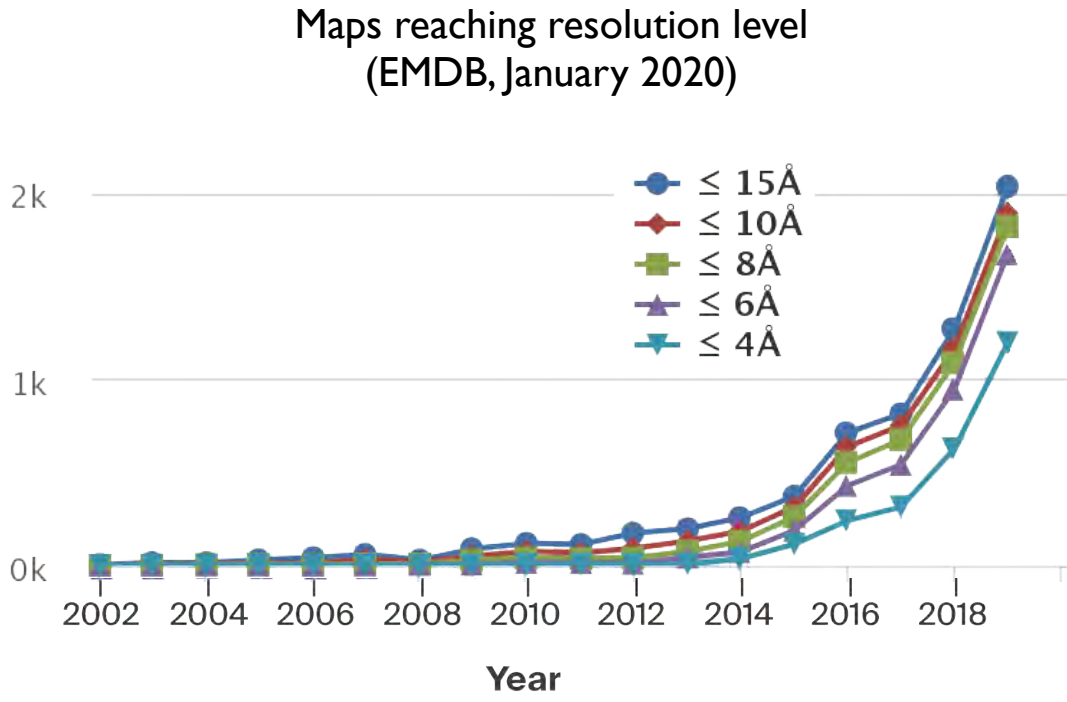


Membrane protein
in a lipid nanodisk.
Data collection:
3 days on C-CINA Titan,
1702 movies,
232’608 particles
97’612 particles in final map.
Final resolution 3.8Å

Nicholas Taylor et al., Nature (2017), with Kaspar Locher

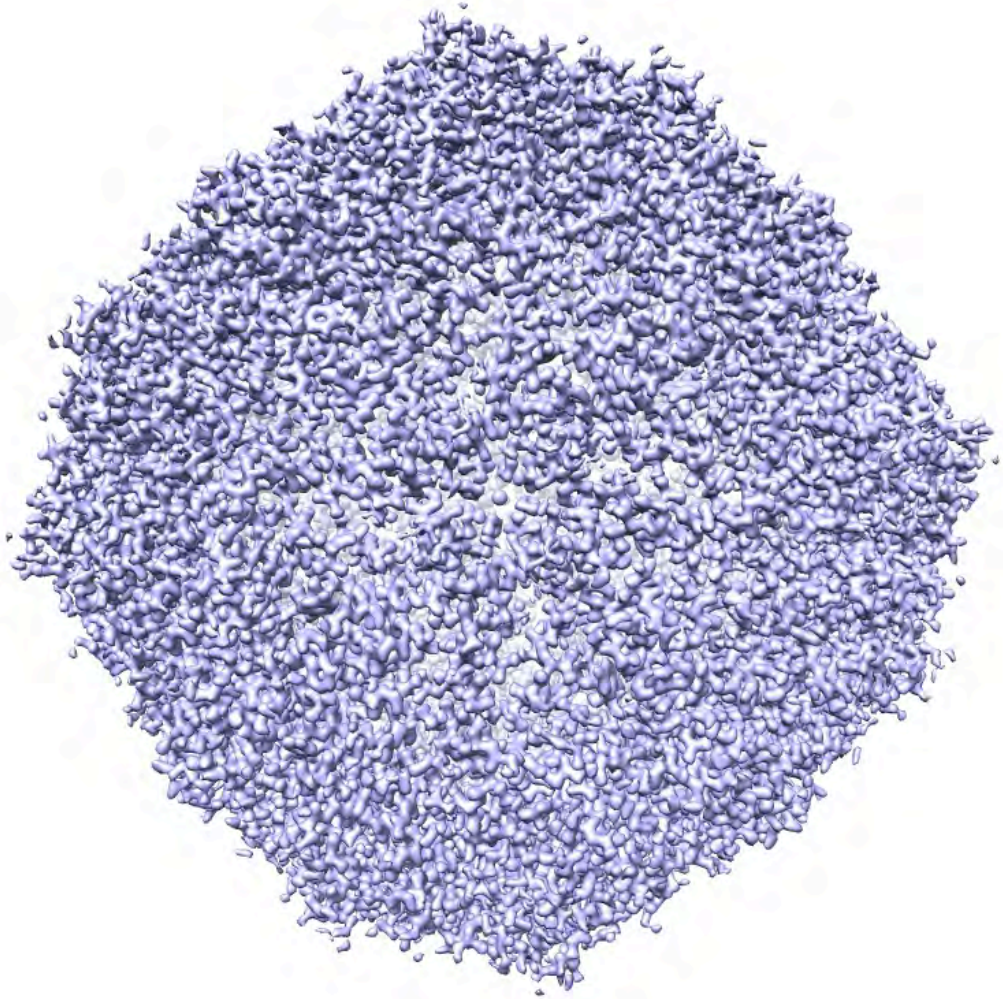
The Resolution Revolution in Cryo-EM

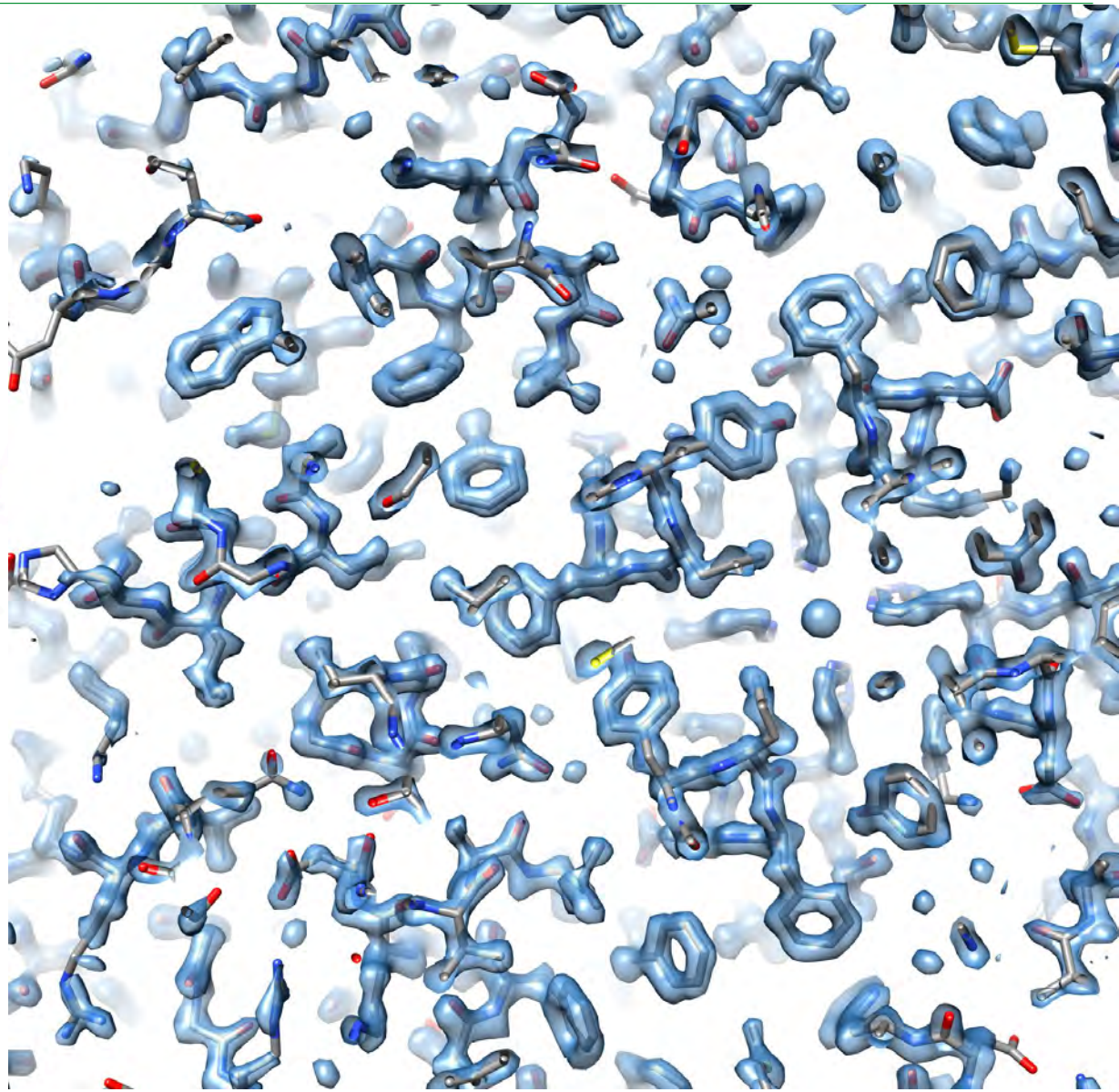




An ideal test protein: Apoferritin

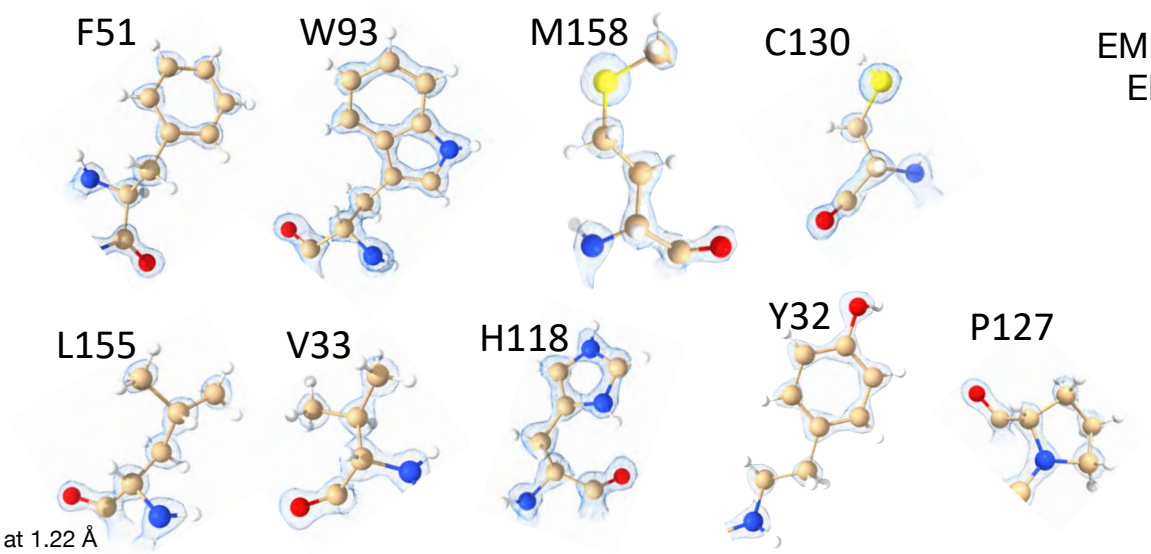
Apoferritin, mouse heavy chain, Fth1





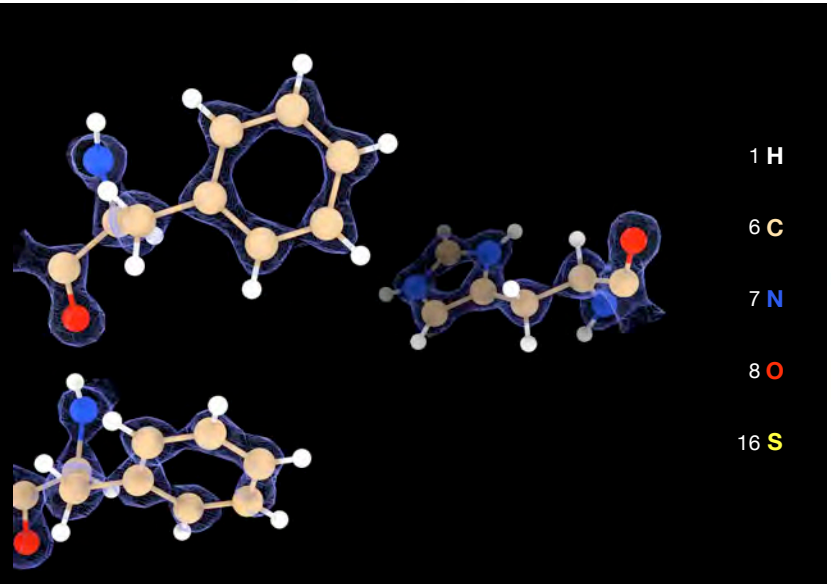
Radostin Danev, Tokyo University
Titan Krios, Falcon III EC camera: 1.62 Å

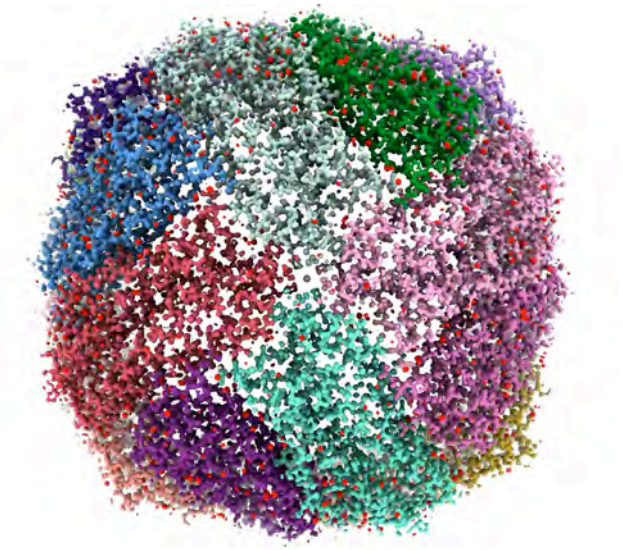
Dubochet Center for Imaging Lausanne: Apoferritin @ 1.09 (March 2024)



EMPIAR: 11866
EMDB: 19436
PDB: 8RQB

Here shown at 1.22 Å





Alex Myasnikov
Technical Director

Christel Genoud
Executive Director

Bertrand Beckert

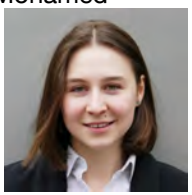
Emiko Uchikawa

Inay Mohamed

Sergey Nazarov

Mireille Fasmeyer

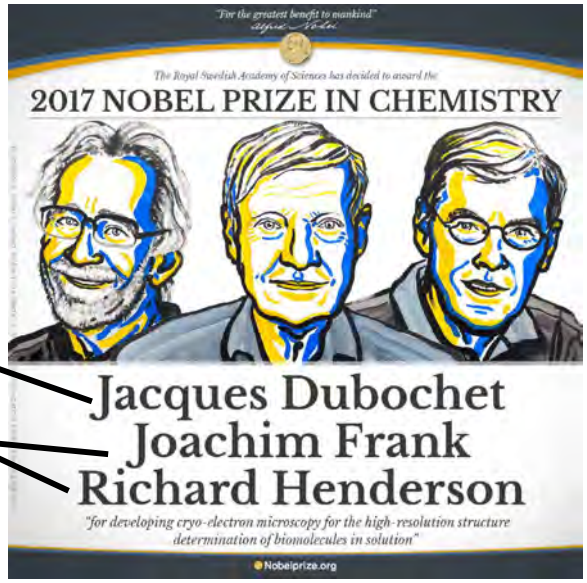
Florent Wenger



Sofya Laskina

Cryo-EM as of today

What	*Time*	*Resource*
Expression, Purification	years	Pure, homogeneous, stable
Cryo-EM grid optimization	2 weeks	200kV Cryo-EM
Cryo-EM imaging	2 days	300kV high-end Cryo-EM
Image processing	1 month	500 CPUs or 10 GPUs



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:

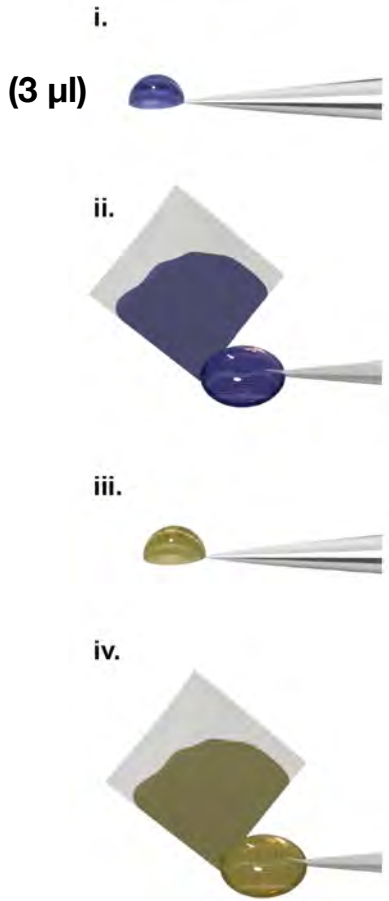
> 3 µl per cryo-EM grid	=> mostly lost in filter paper
3 nl per cryo-EM grid	=> needed for imaging

Classical EM Grid Preparation: 99.99% of the sample goes to filter paper.

Sample Quantity:

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

Negative stain:



Cryo:

Protein Purification

Expression

(Liters)

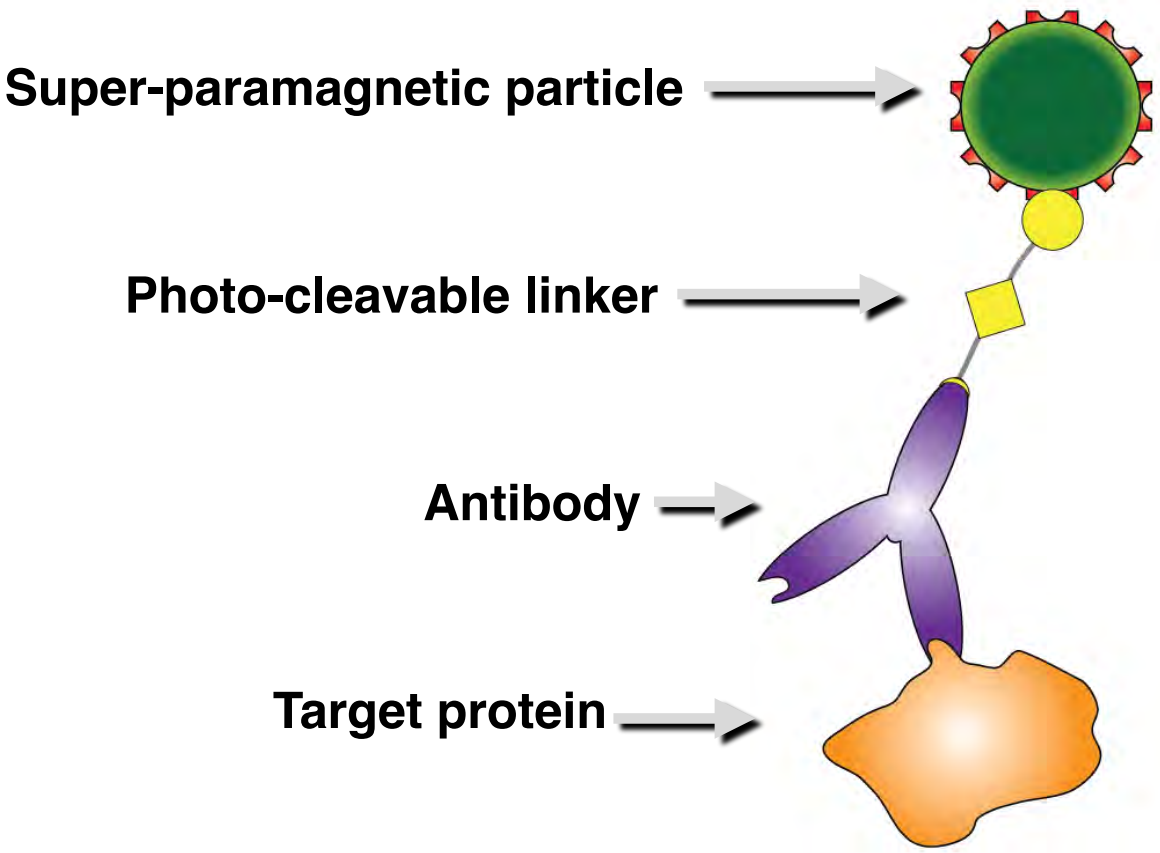
Purification

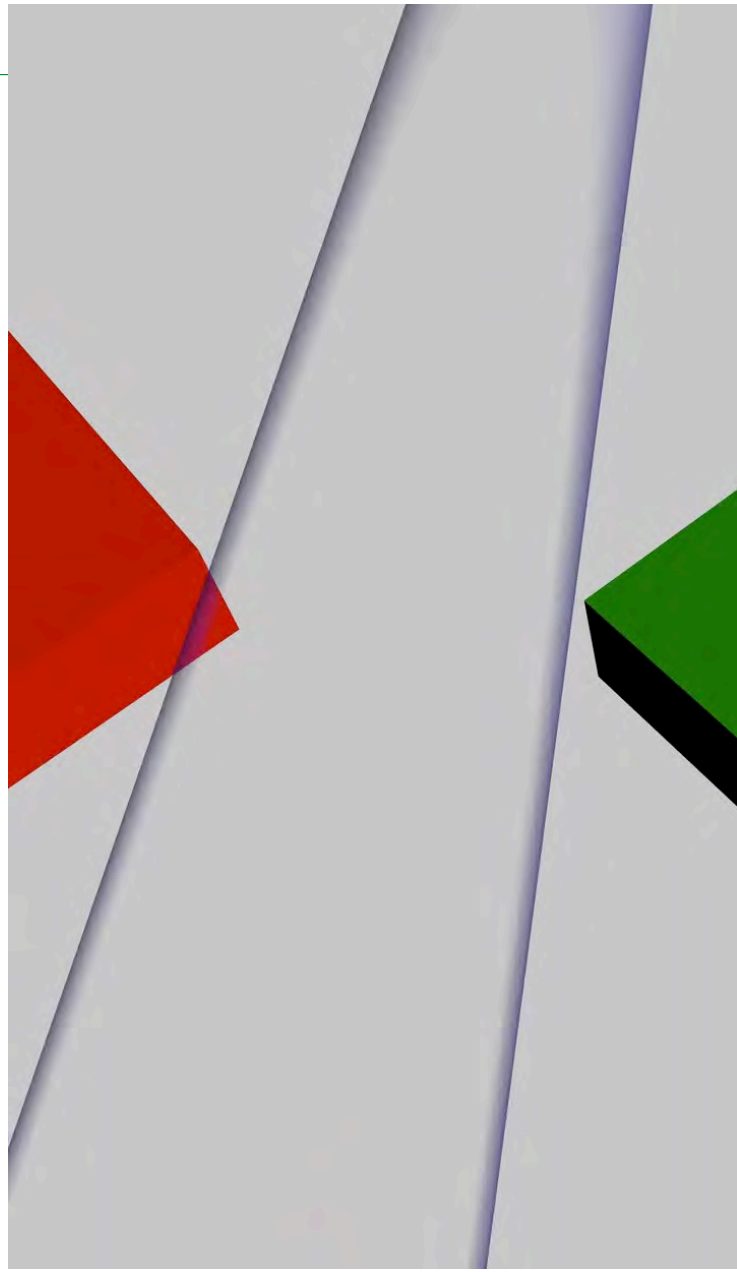
(hours)

Cryo-EM Grid Freezing

(3 µl)

Microfluidic Protein Purification, using Magnetic Beads

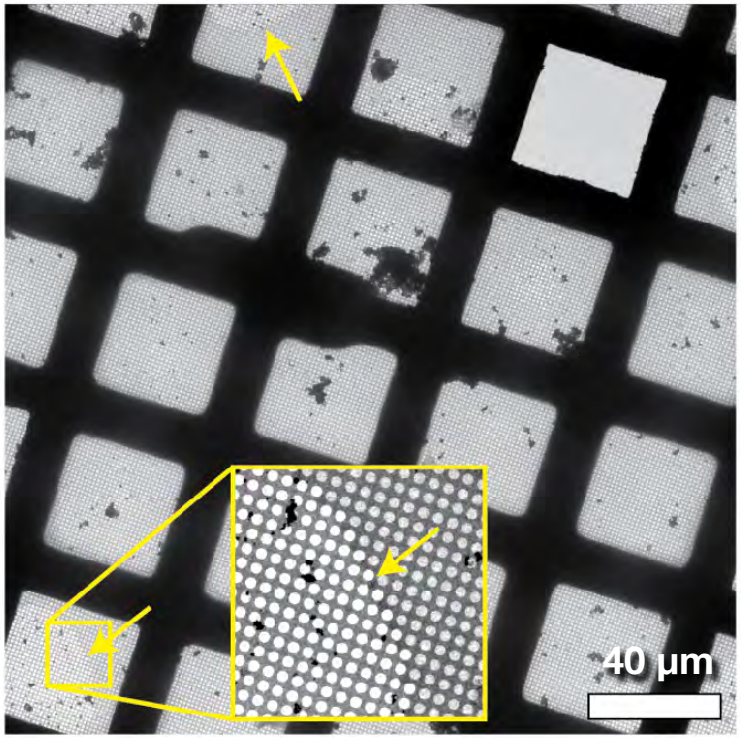


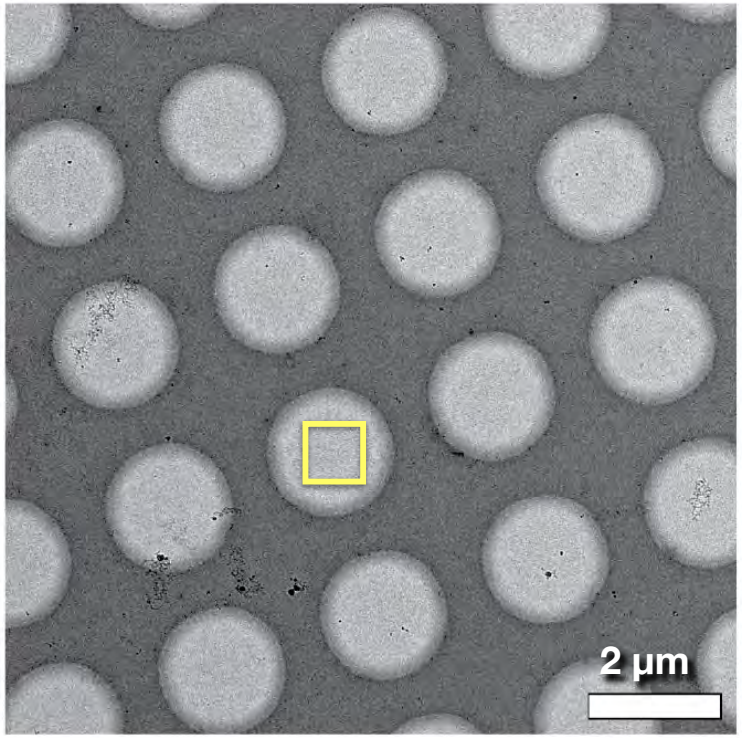


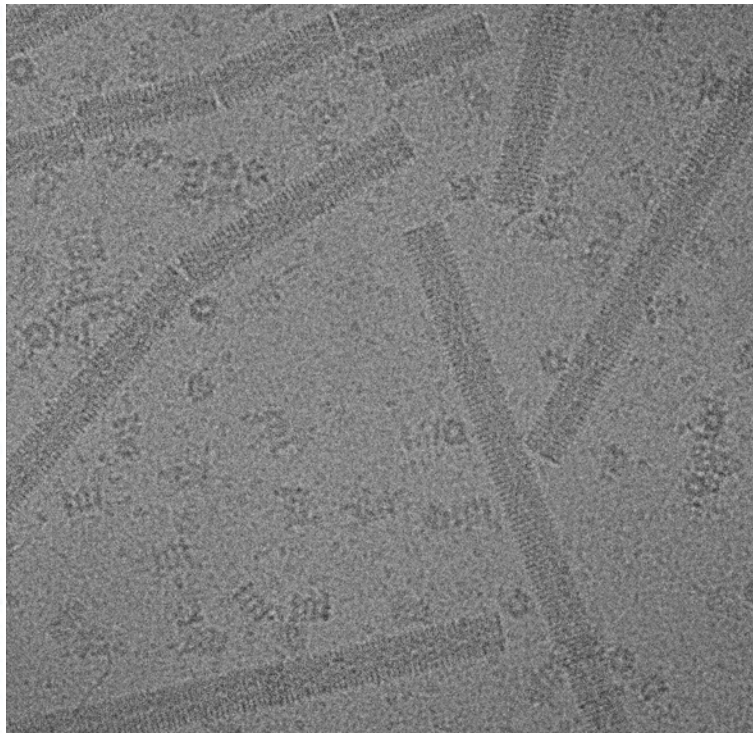
D. Giss, et al., Anal. Chem. 86(10), 4680–4687 (2014).

Microfluidic Protein Purification, using Magnetic Beads

Microfluidic Cryo-EM Grids



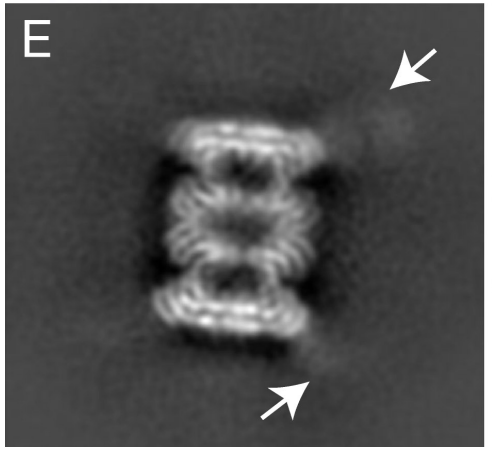


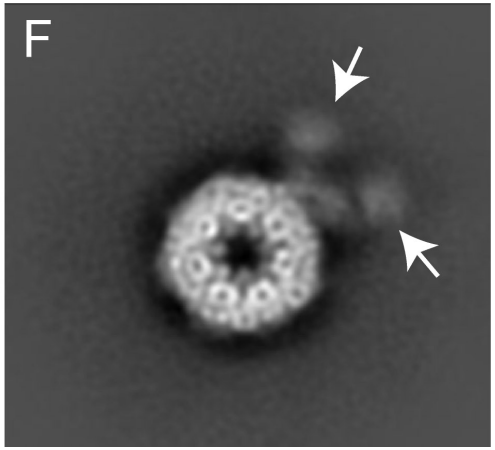


Thomas Braun et al.

Human 20S Proteasome,
purified from 0.75 µL of HeLa cytosol

class averages





Proteasome

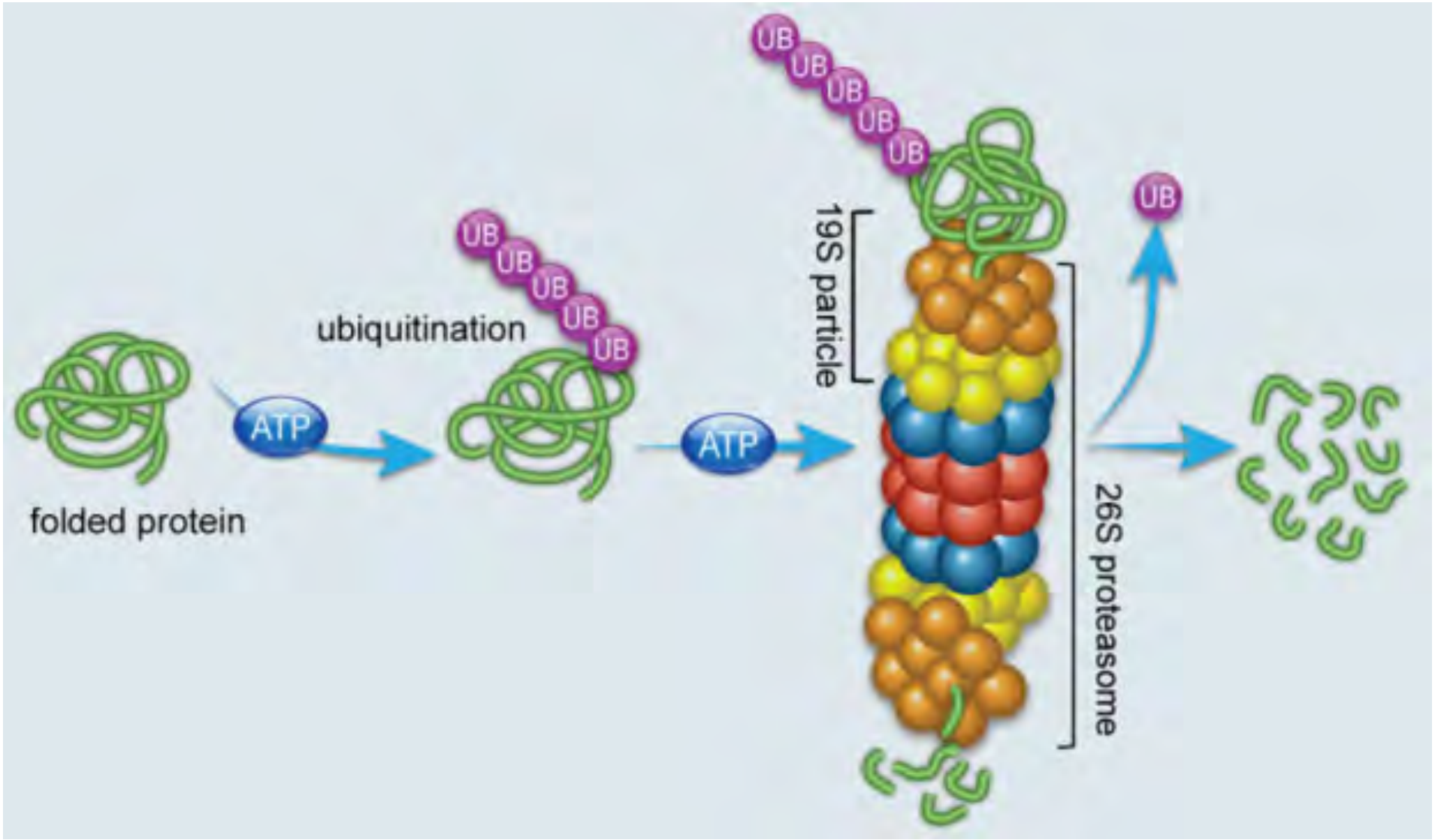
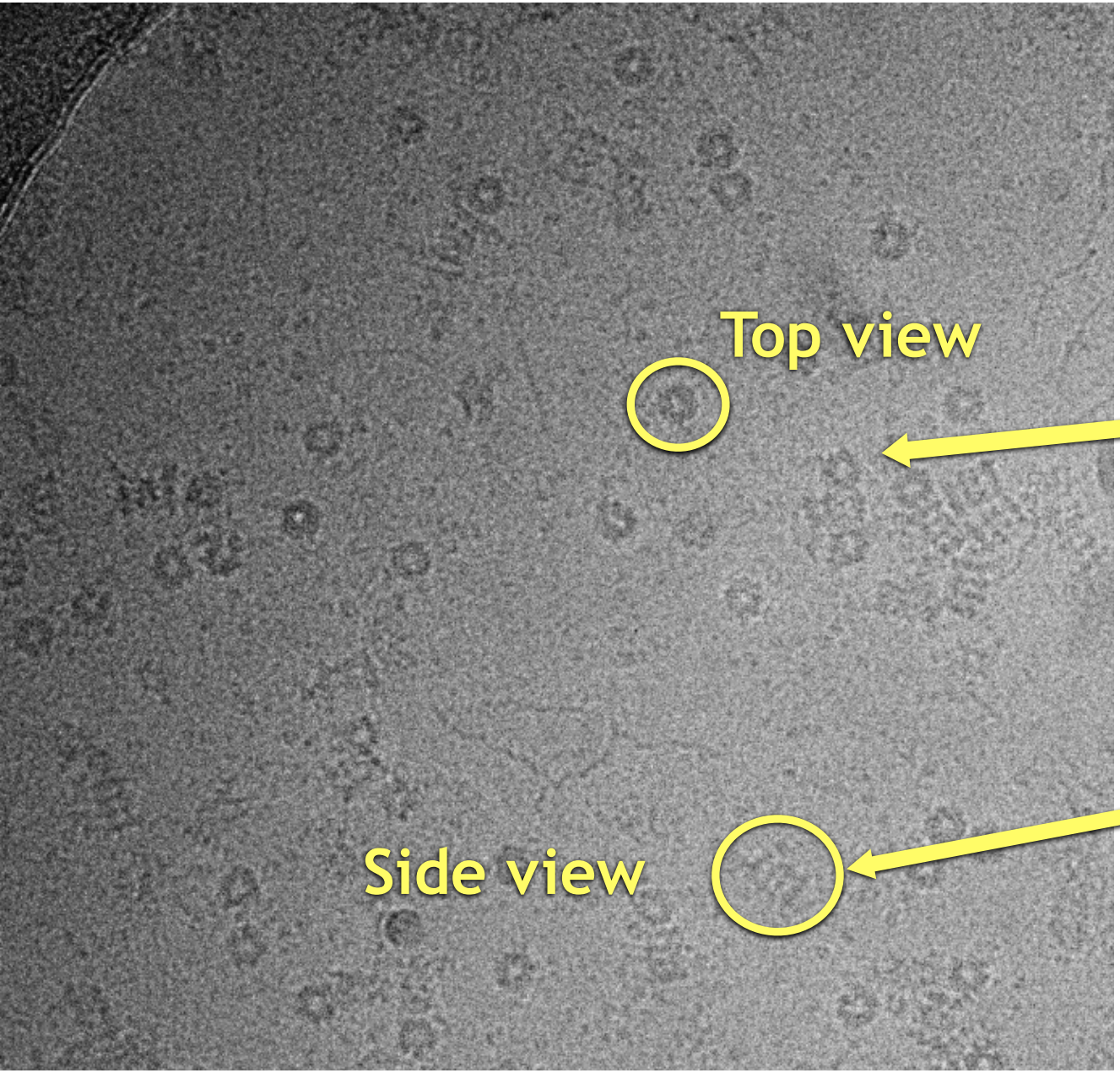
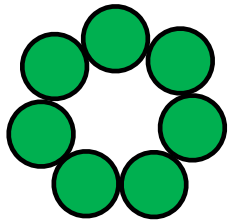


Illustration from R&D Systems, Inc.



Top view



Side view

165k magnification
→ 0.832 A/pix

20 eV sit width for energy filter
(Gatan Quantum-LS)

1.2-2.2 um defocus

35 frames

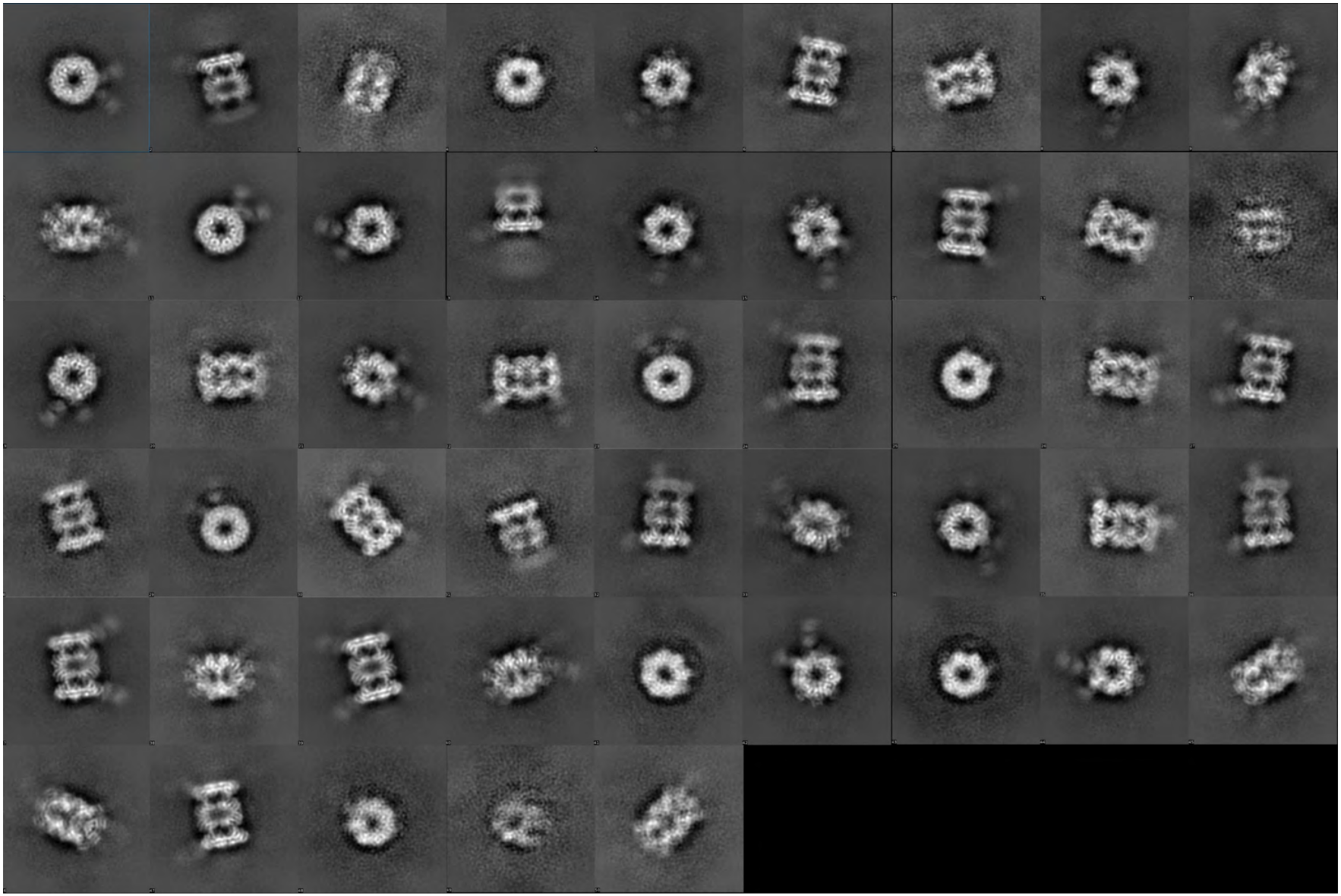
7 s exposure

70 e/A2 total dose

MotionCor2

CTFFIND4

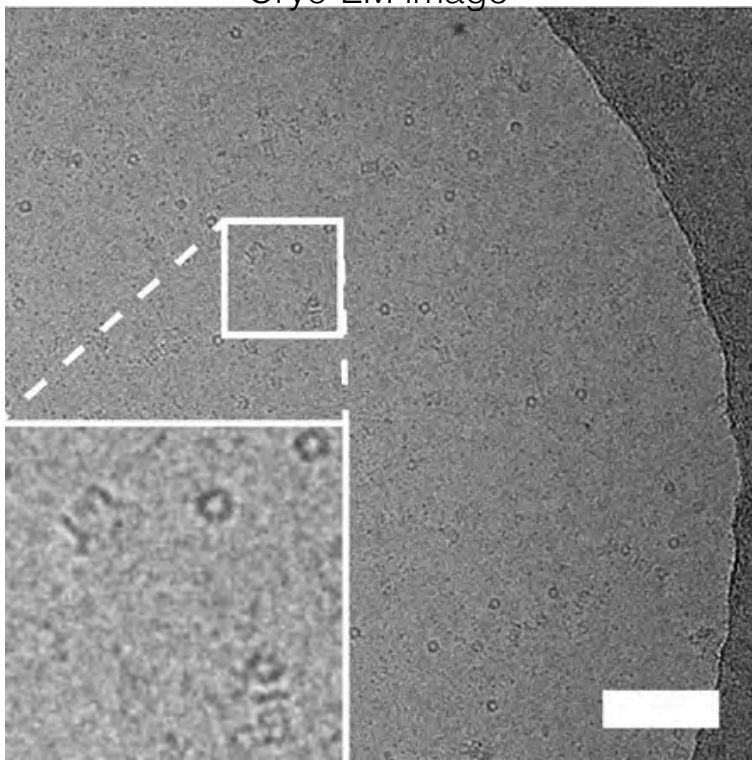
Class averages



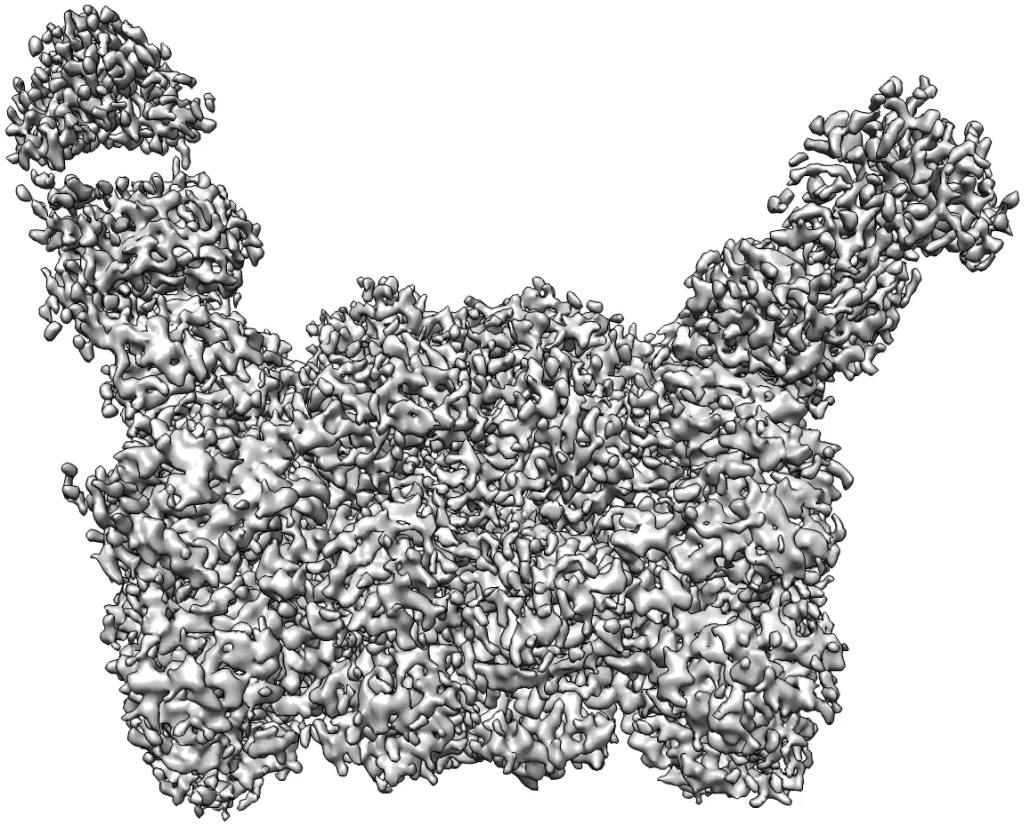
Human 20S Proteasome, purified from 0.75 µL of HeLa cytosol

Purified with Fab fragments on magnetic beads, out of the cytosol of ~10'000 cells.

Cryo-EM image



3D reconstruction at 3.7Å



Thomas Braun et al.

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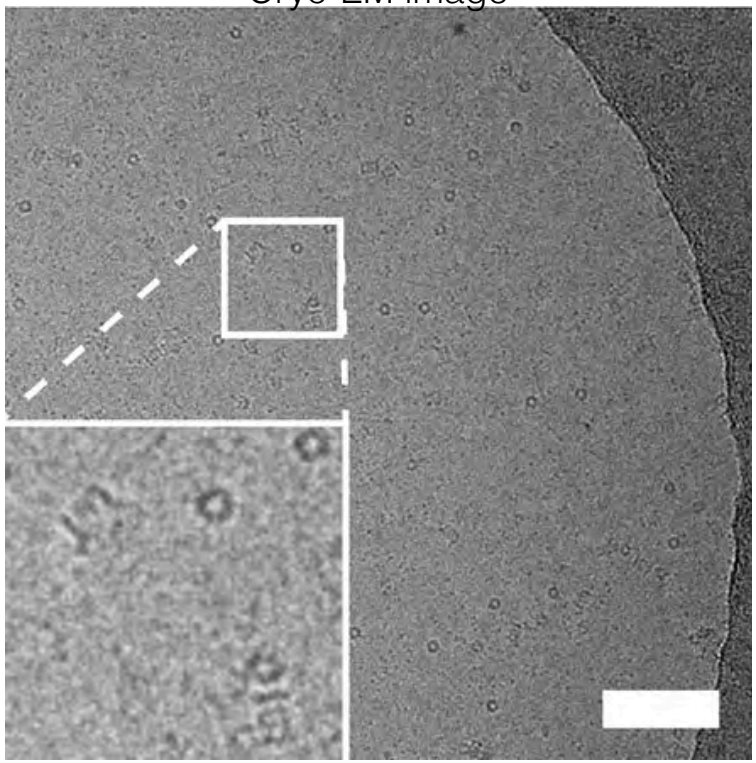
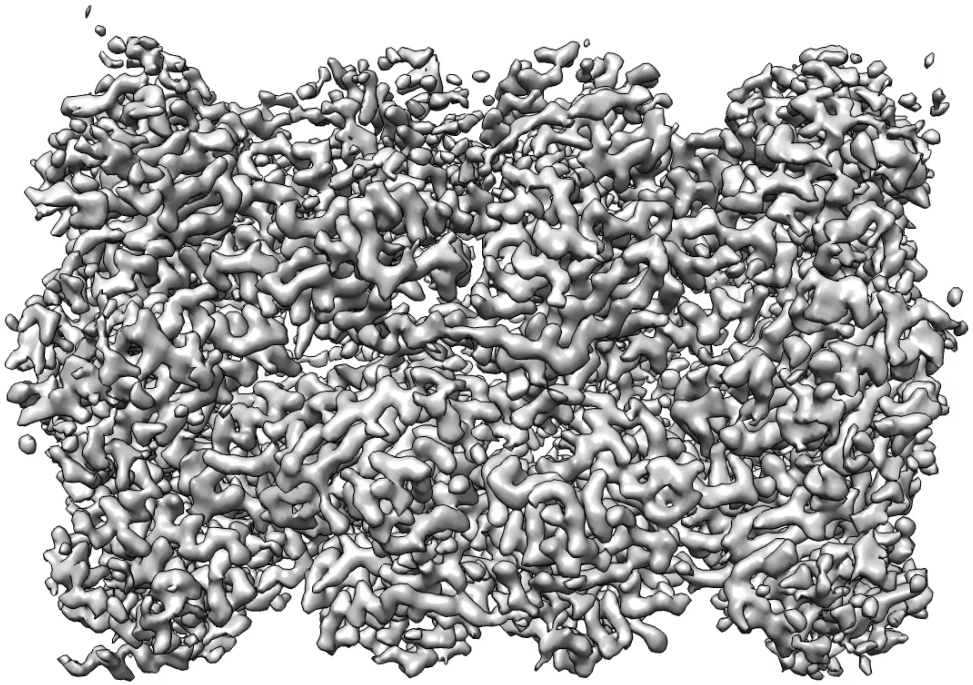


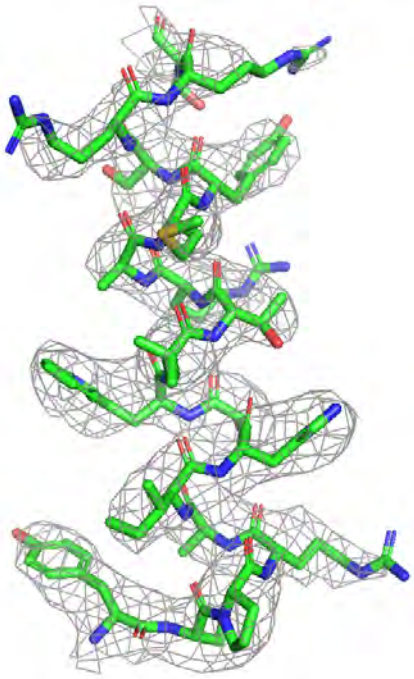
3D reconstruction at 3.6Å



Thomas Braun et al.

Model Building

Alpha Helix



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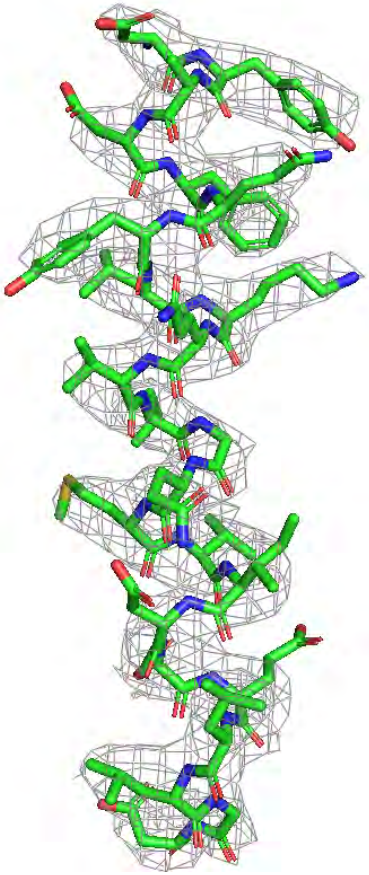
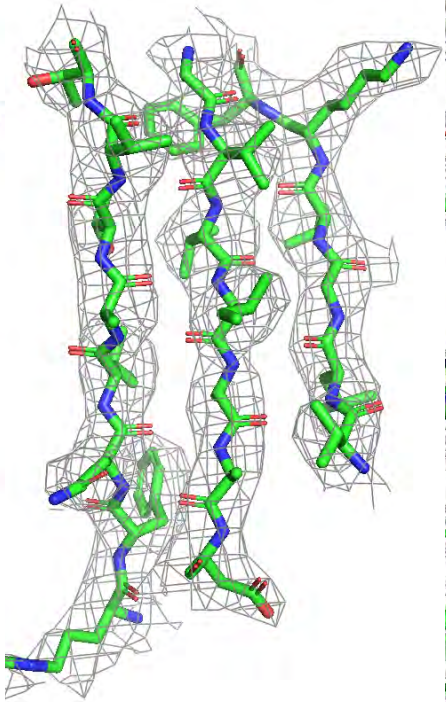
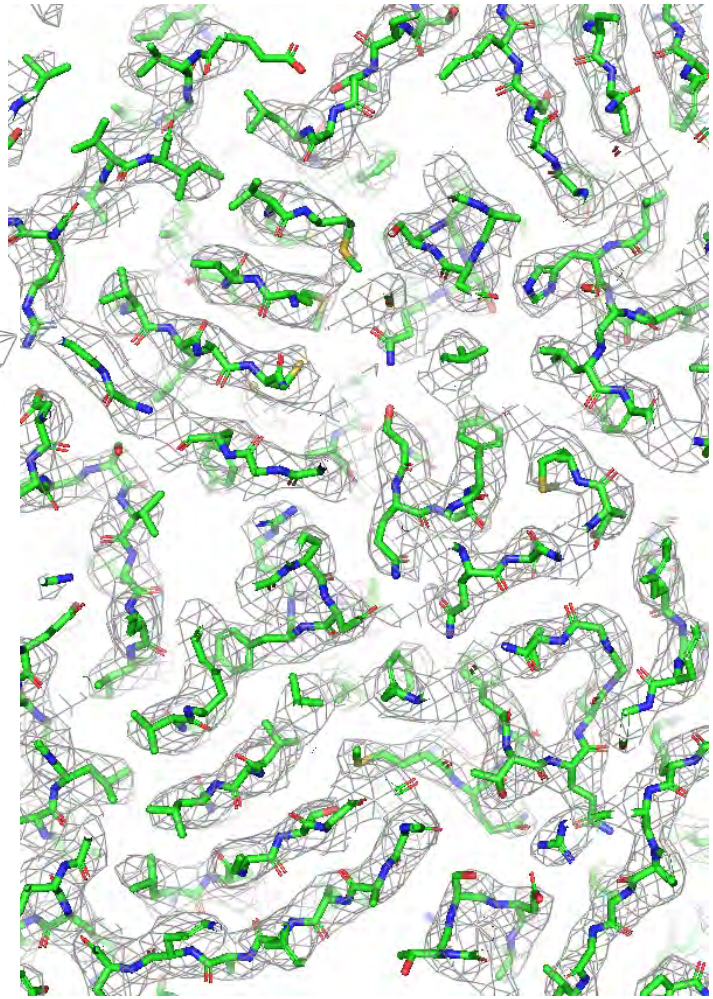
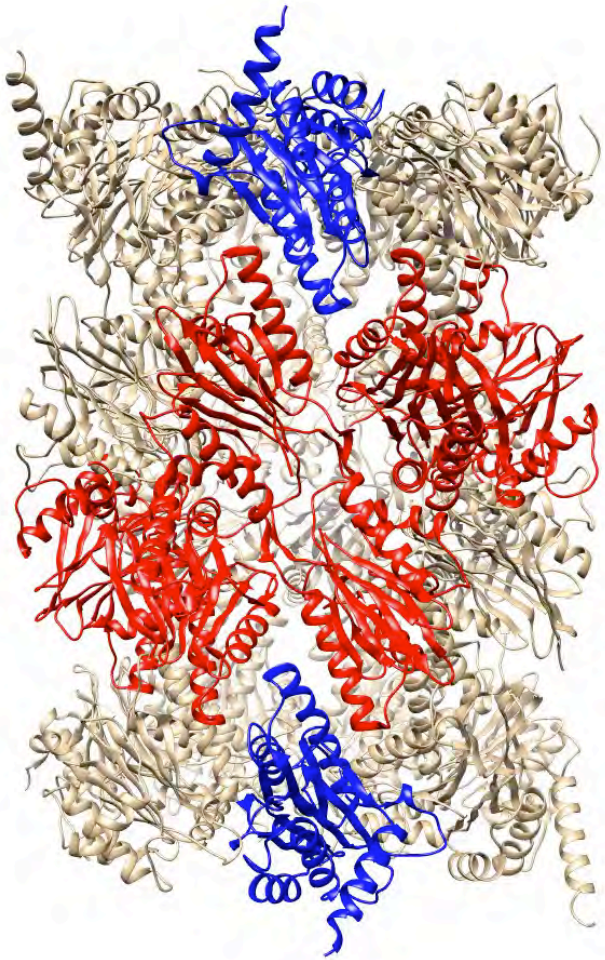


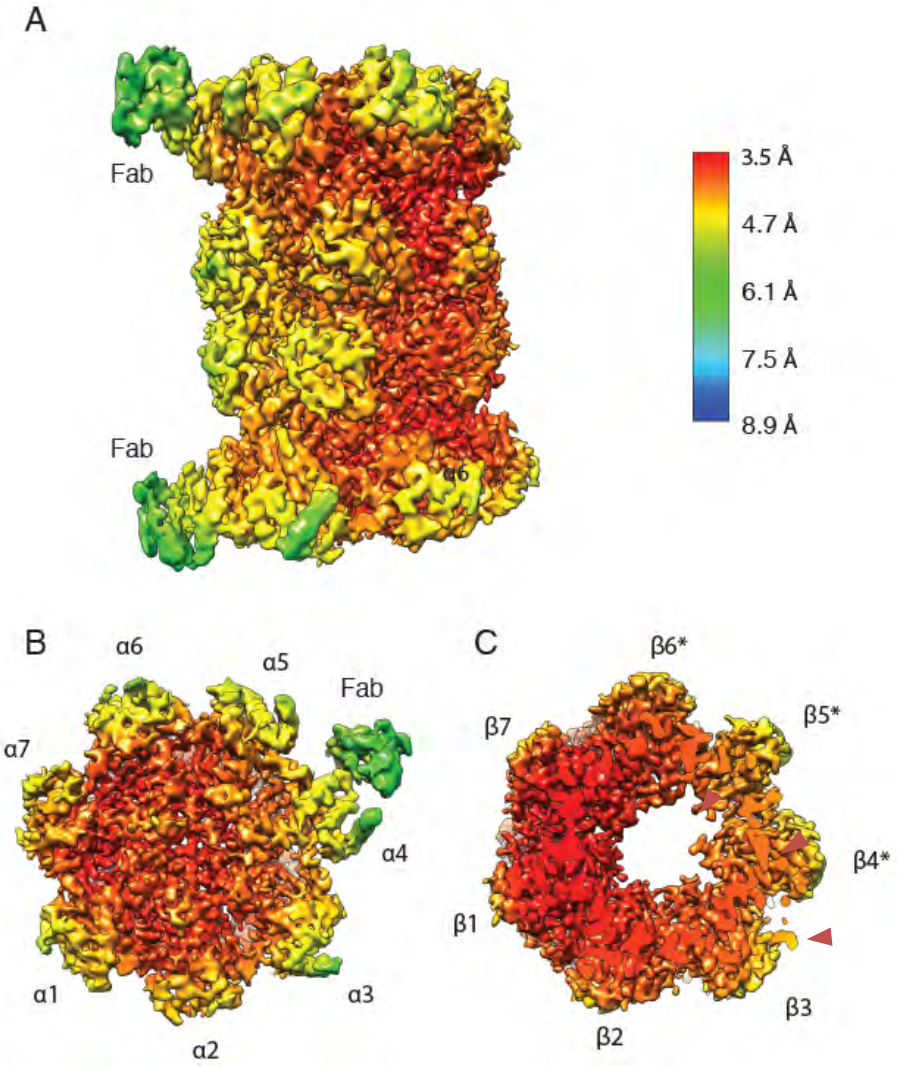
Beta Sheet





Model Building, Local Resolution





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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