There is no better way to understand how macromolecules function in a cell than to have a visual image of their parts and how they interact ...

– *Kensal E. Van Holde*

电镜研究方法与技术

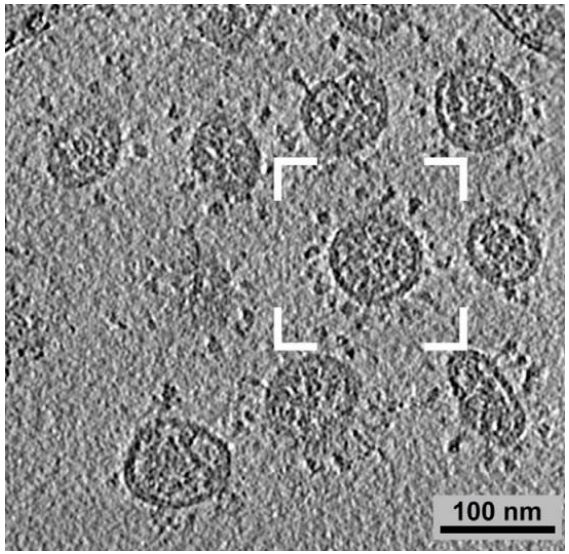
中科院神经科学研究所

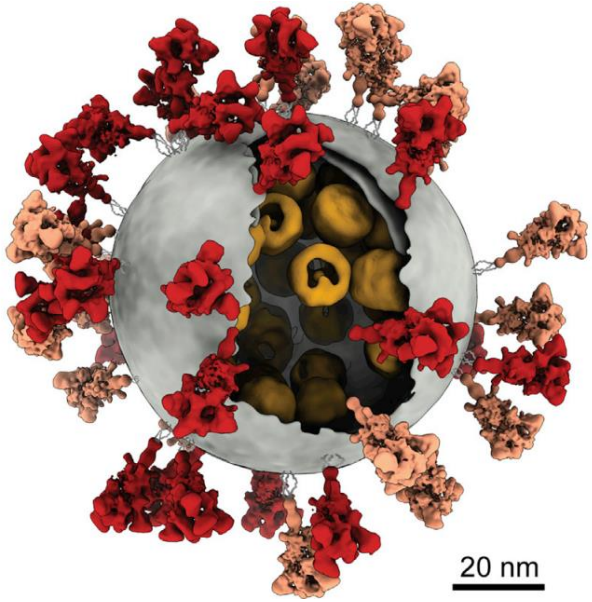
突触蛋白的结构与功能研究组

竺淑佳

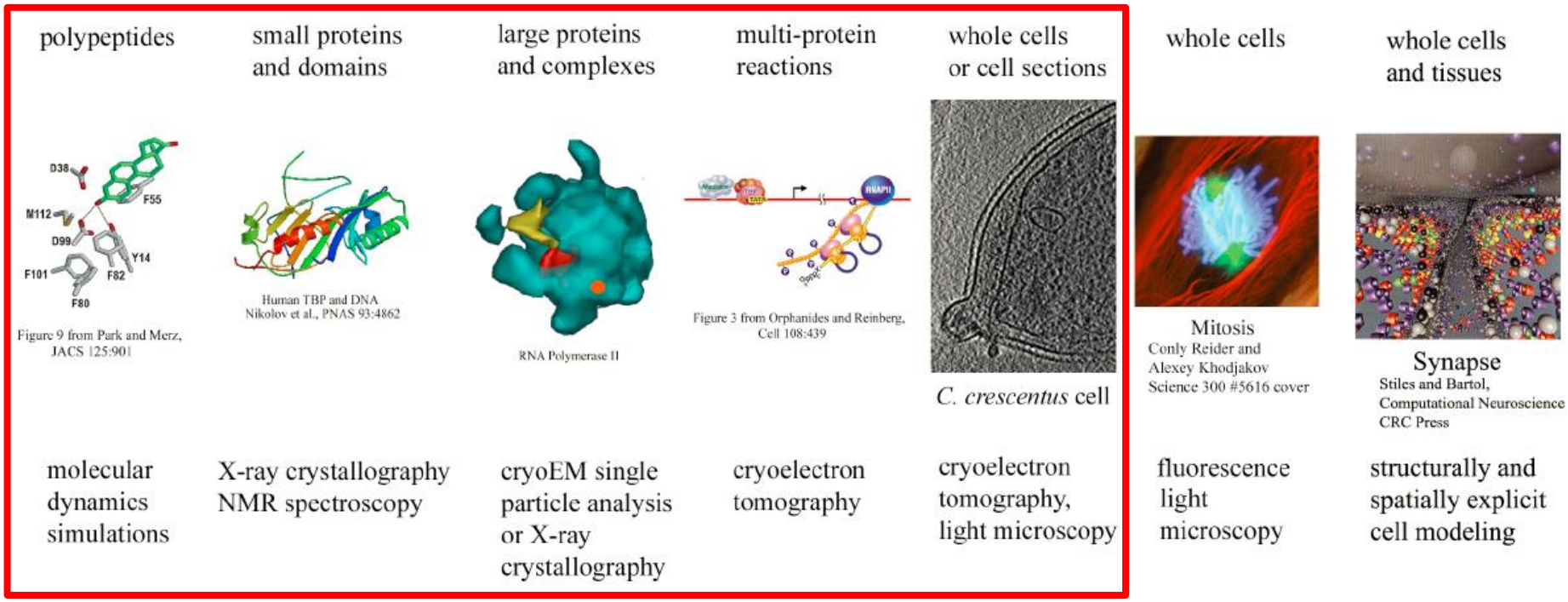
shujiazhu@ion.ac.cn

2020-11-12





- 前 言
- 电镜(扫描和透射)构造及基本原理
- 基本操作步骤
- 单颗粒冷冻电镜（Single-Particle Analysis，SPA)
- 冷冻断层扫描（Cryo Electron Tomography，Cryo-ET）



2017年诺贝尔化学奖与冷冻电镜

2017 Chemistry Laureates. Ill: N. Elmehed.
© Nobel Media 2017

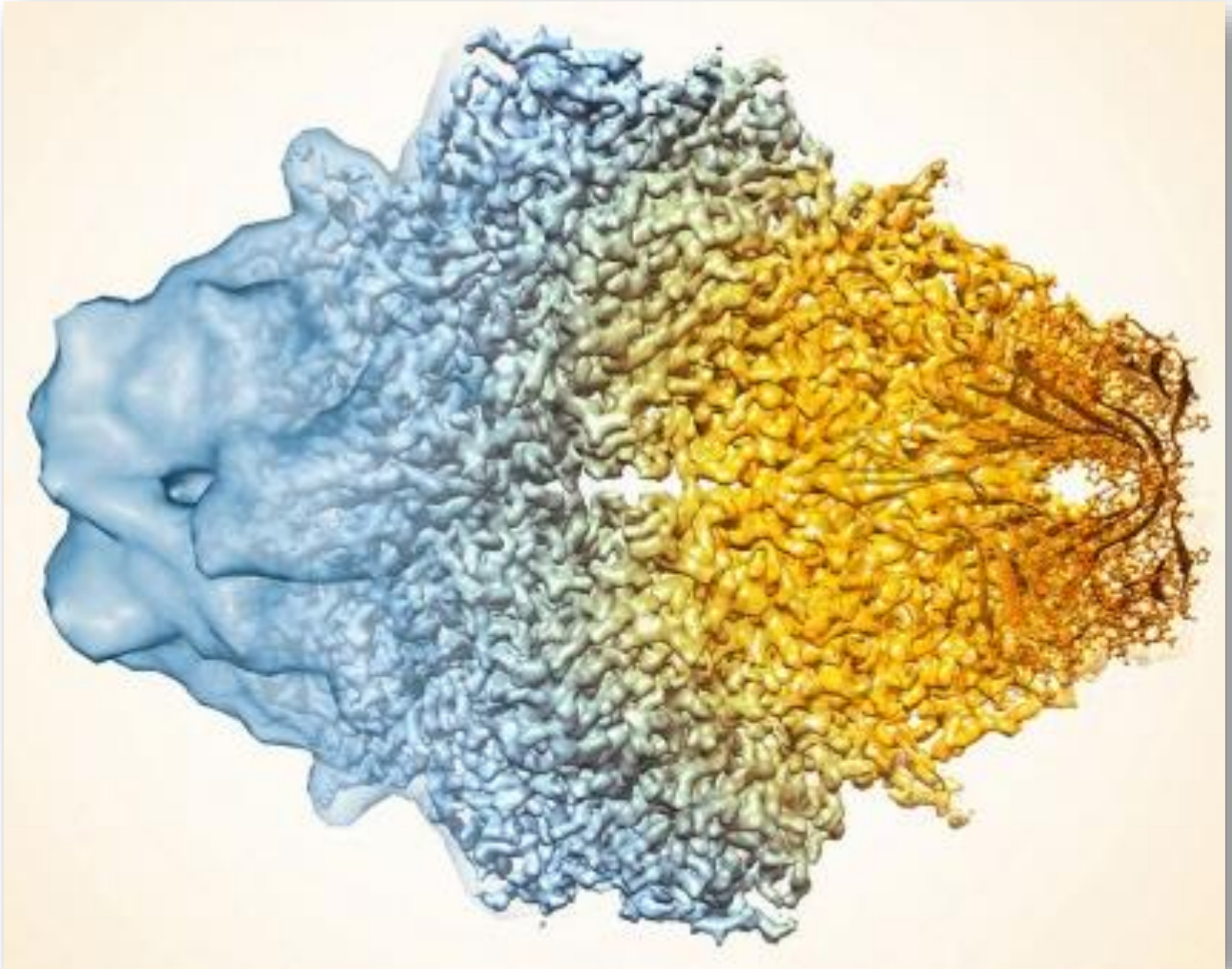
2017 Nobel Prize in Chemistry

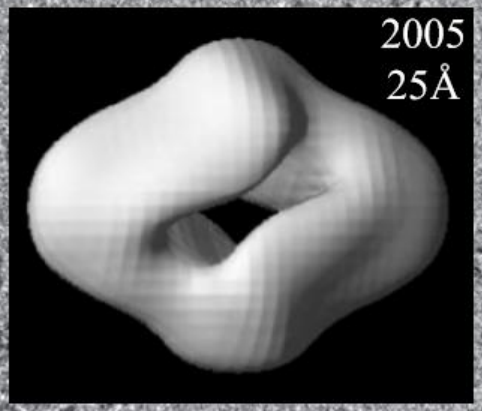
The Nobel Prize in Chemistry 2017 was awarded to Jacques Dubochet, Joachim Frank and Richard Henderson

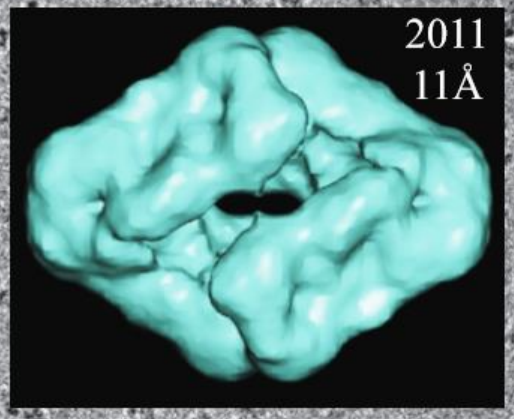
1. Richard Henderson改进了传统电子显微镜，取得了原子级分辨率的图像；
2. Joachim Frank开发了图像合成算法，能将电子显微镜模糊的二维图像合成清晰的三维图像；
3. Jacques Dubochet发明了迅速将液体水冷冻成玻璃态以使生物分子保持自然形态的技术。

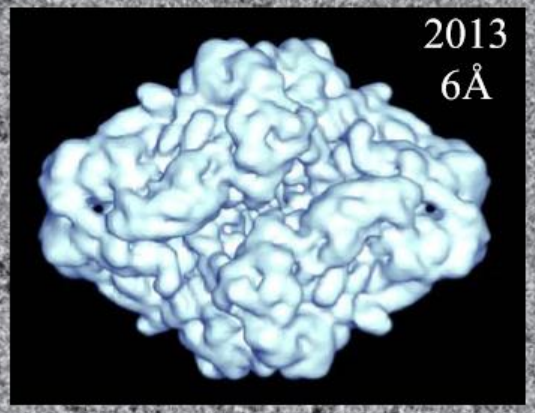
For developing cryo-EM for the high-resolution structure determination of biomolecules in solution

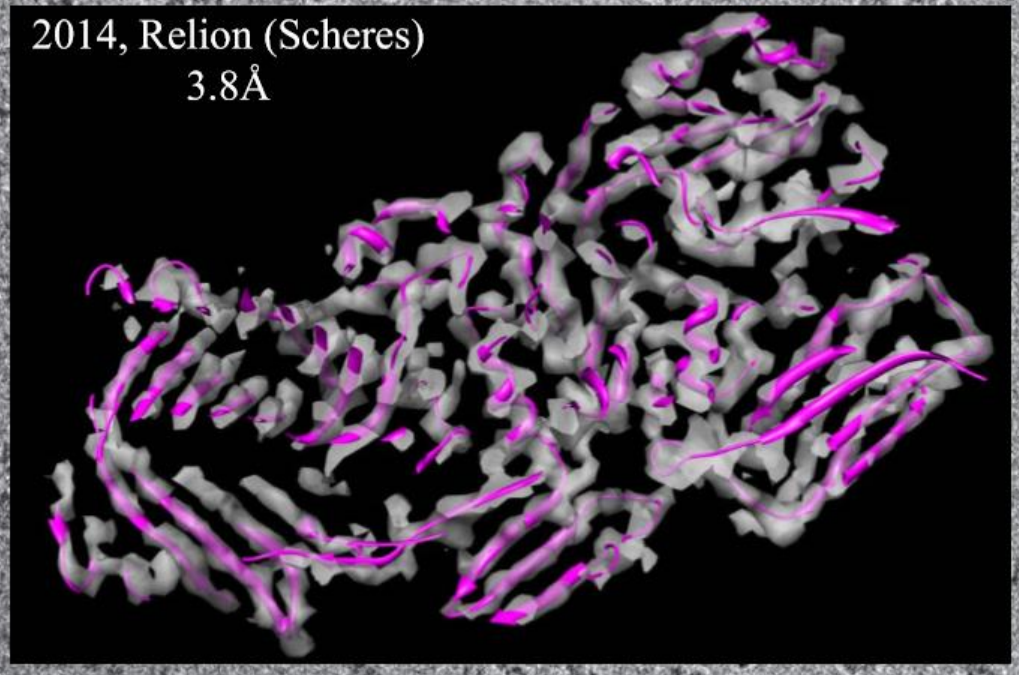
From blobology to atomic resolution



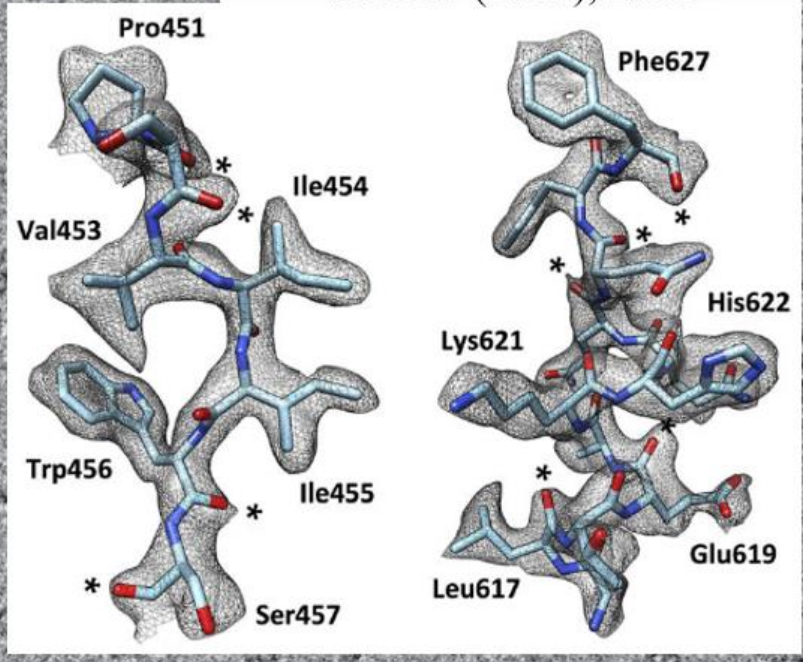








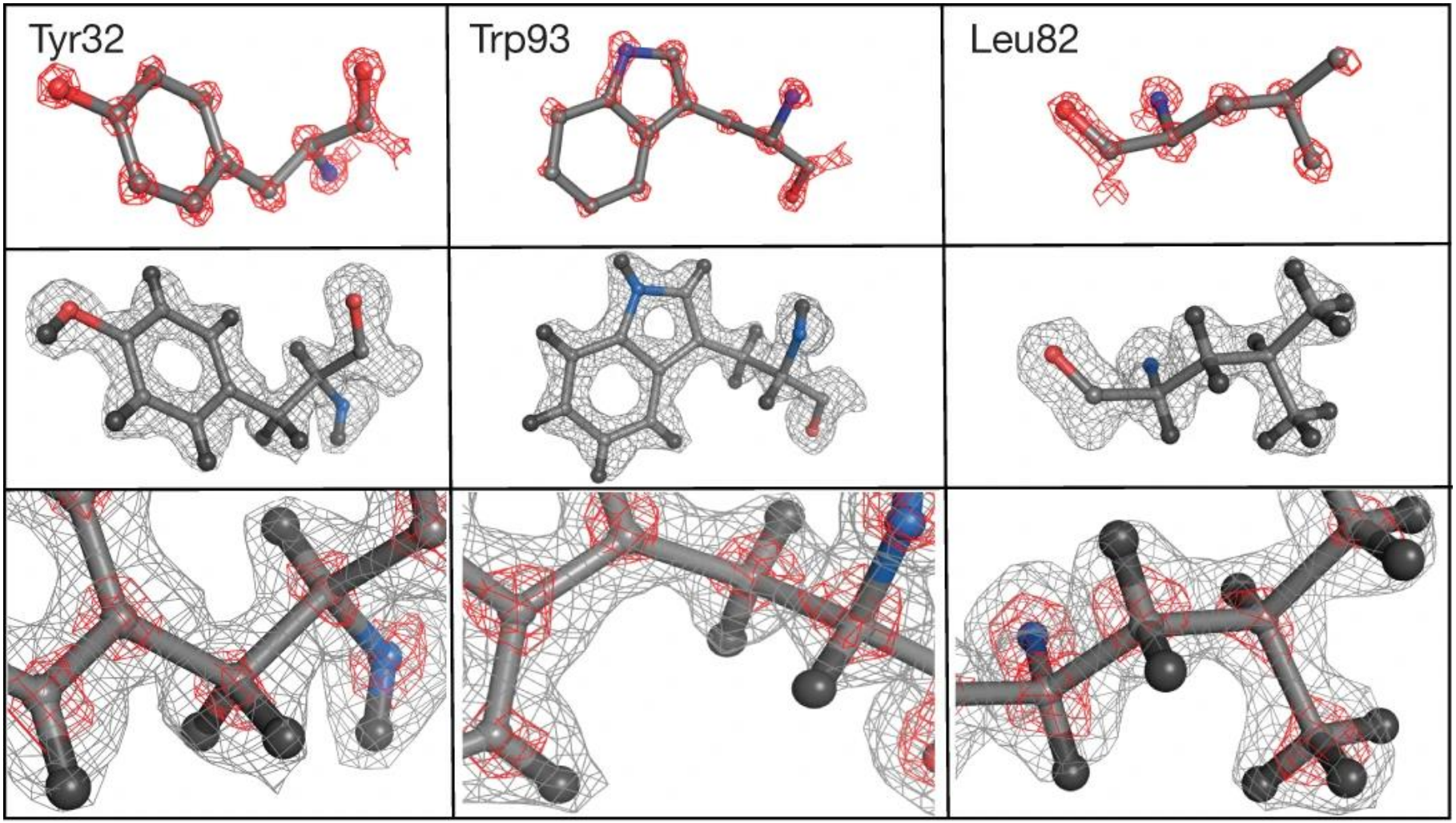
Bartesaghi et al & Subramaniam
Science (2015), 2.2Å



Nature. 2020 Nov;587(7832):157-161. doi: 10.1038/s41586-020-2833-4. Epub 2020 Oct 21.

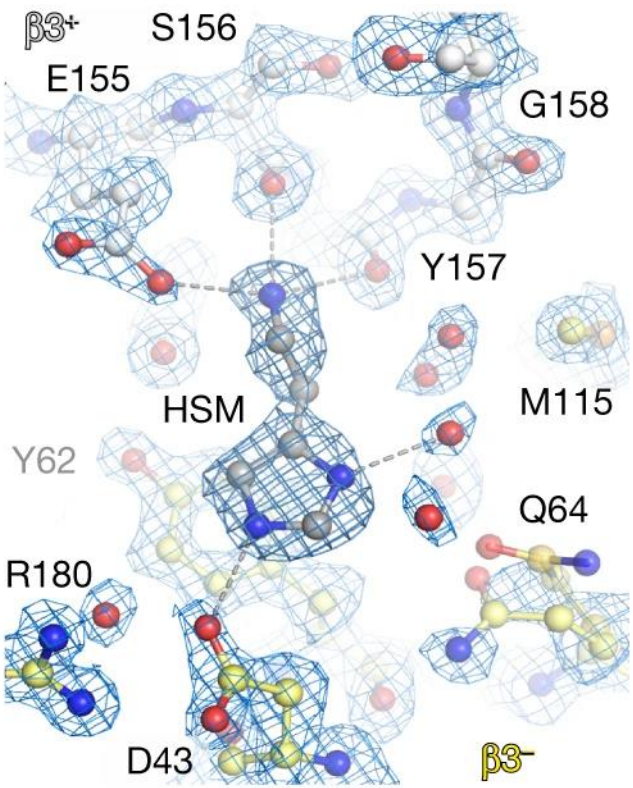
Atomic-resolution protein structure determination by cryo-EM

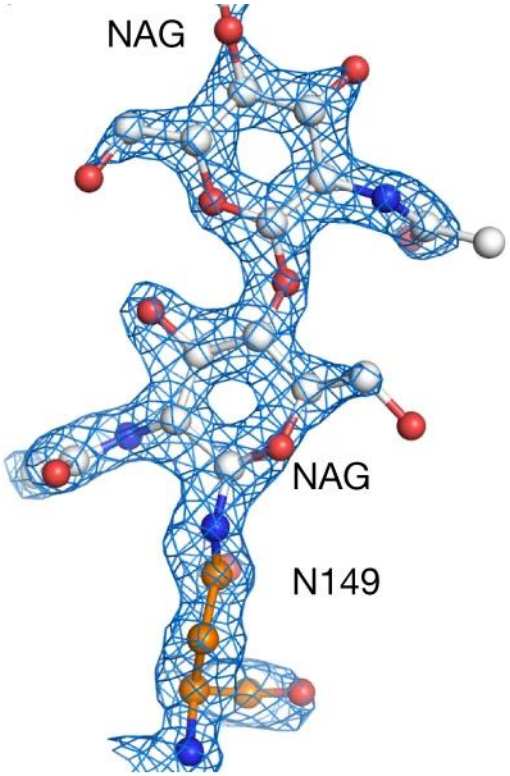
Ka Man Yip [1], Niels Fischer [1], Elham Paknia [1], Ashwin Chari [1], Holger Stark [2]



Article | Published: 21 October 2020

Single-particle cryo-EM at atomic resolution





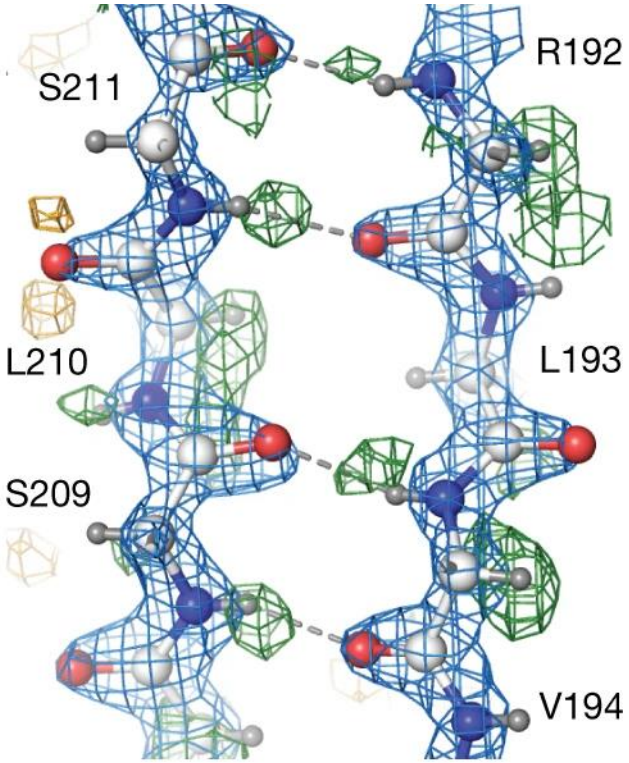
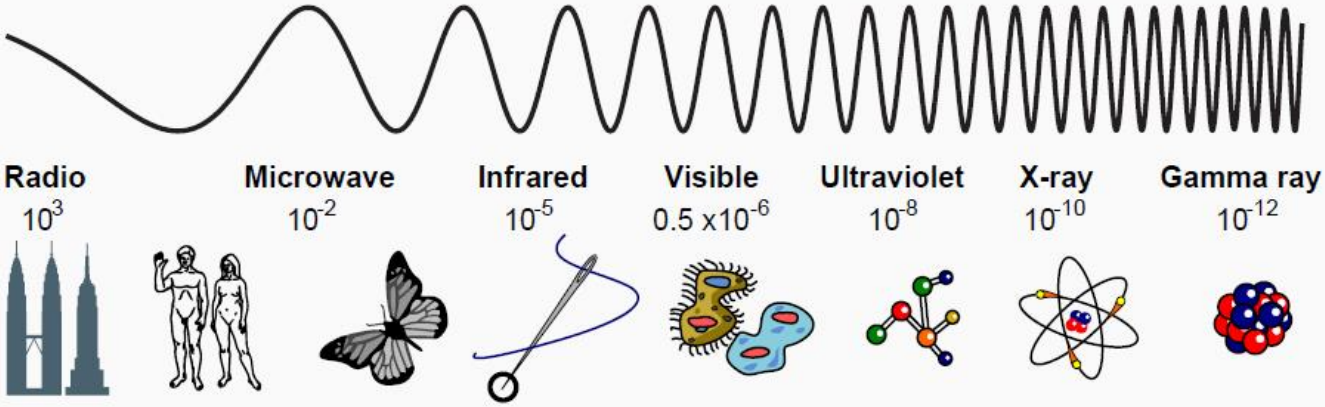


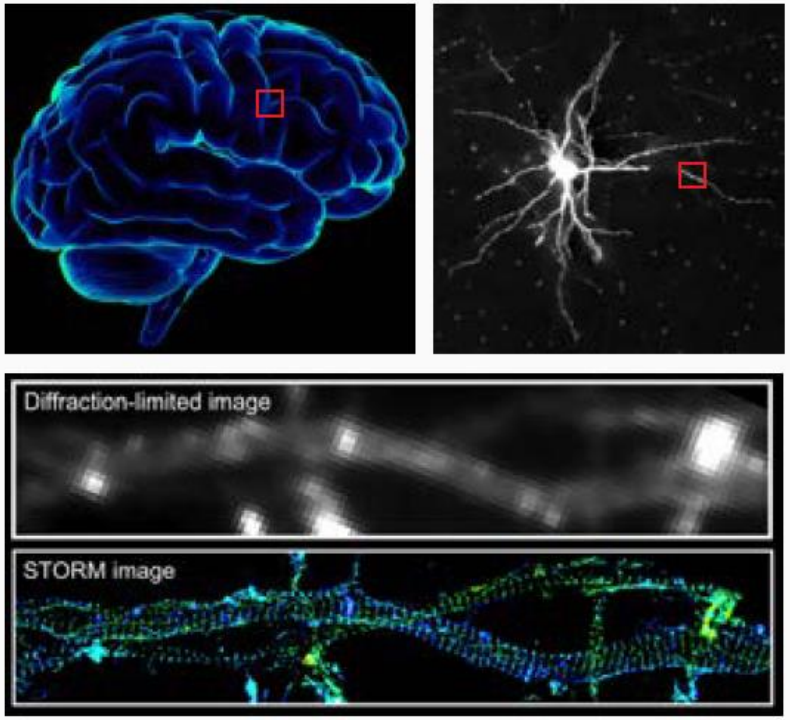
Fig. 2: $GABA_AR$ reconstructions.

Wavelength and resolution

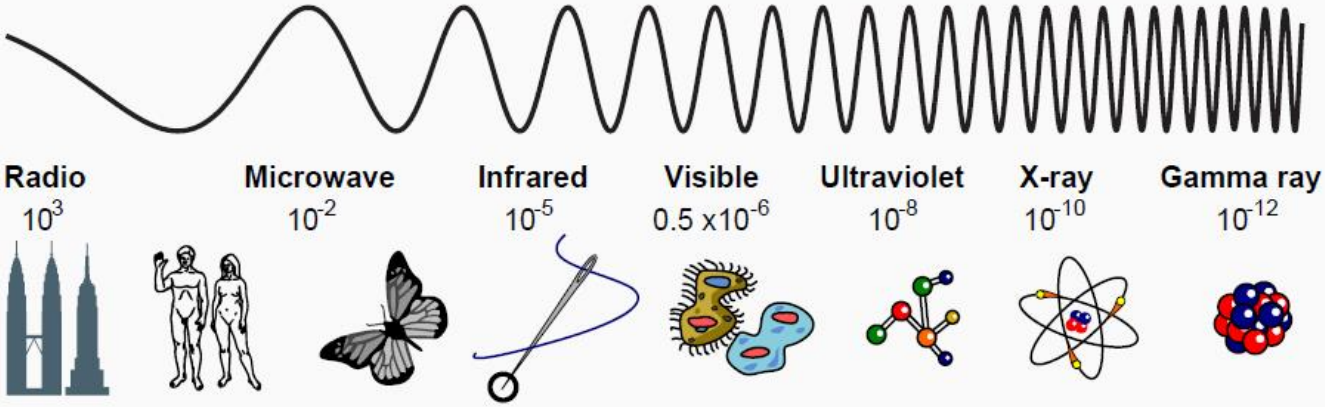


Visible light

- easy to get
- easily focused
- eye wonderful detector
- not very damaging
- long wavelength ($\sim$ 400–700 nm)
- super-resolution microscopy
- Nobel Prize 2014

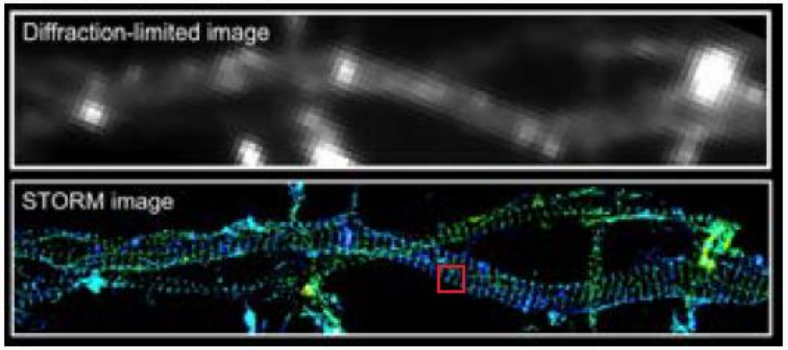


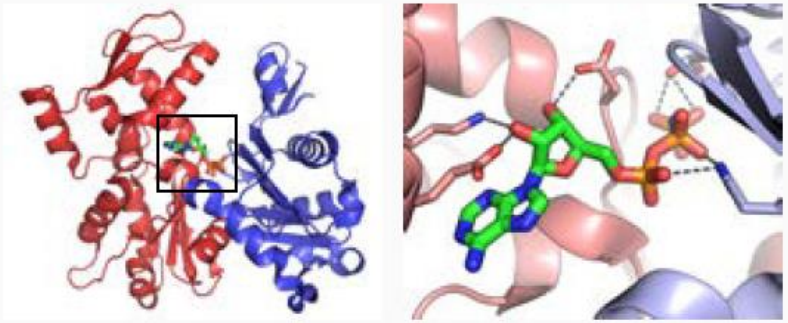
Wavelength and resolution



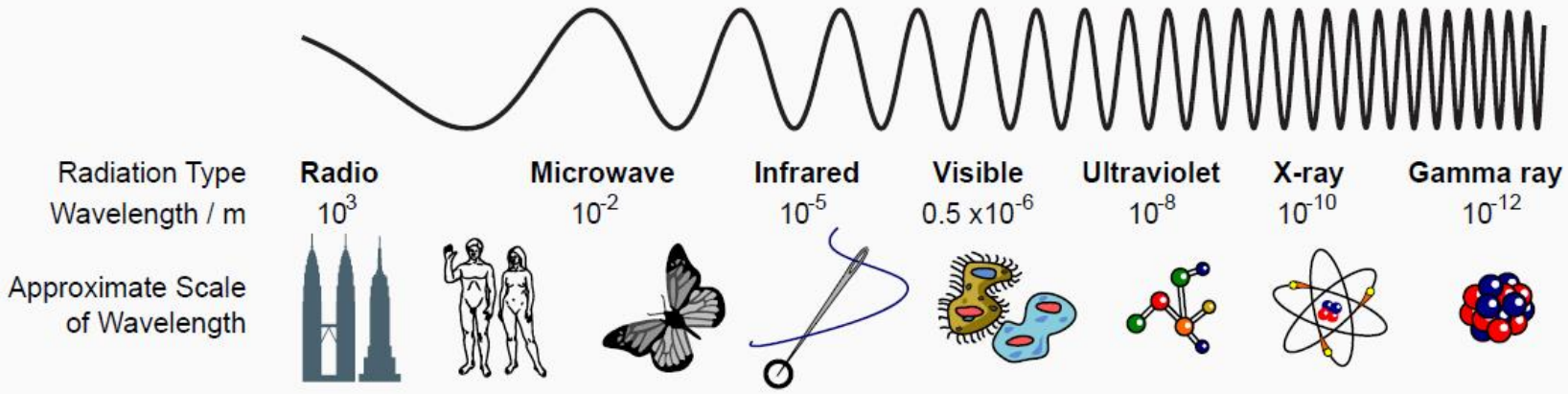
X-ray

- small wavelength: ∼0.8–2.3 Å
- atomic resolution
- good penetration
- hard to focus
- damage samples
- requires crystals



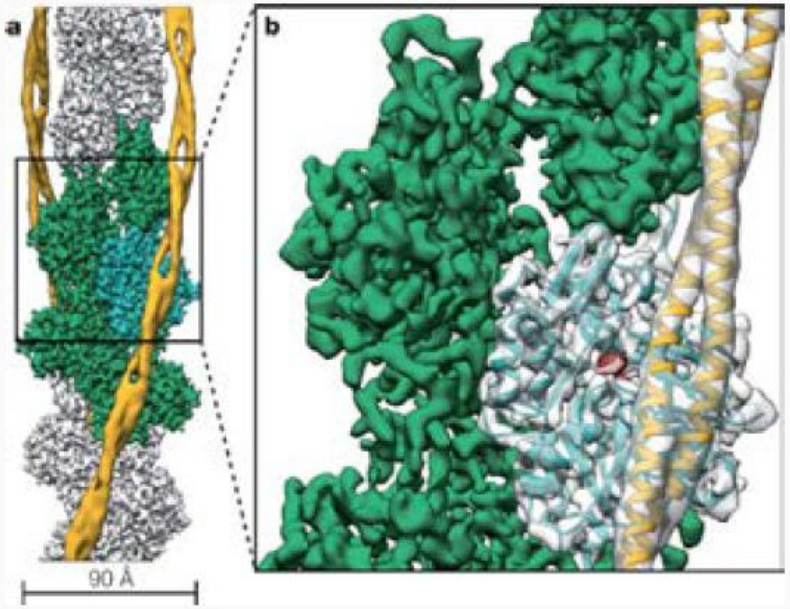


Wavelength and resolution



Electron

- small wavelength (pm)
- do not require crystals!
- can be focused
- poor penetration
- damage samples



Why electrons?

•The **electron** is a subatomic particle, with a negative elementary electric charge.

	Advantages	Disadvantages
Visible light	Not very damaging Easily focused Eye wonderful detector	Long wavelengths (~400 nm)
X-rays	Small wavelength (Angstroms) Good penetration	Hard to focus Damage sample
Electrons	Small wavelength (pm) Can be focused	Damage sample Poor penetration
Neutrons	Low sample damage Small wavelength (pm)	Hard to produce in controlled ways Hard to focus

Wave-particle duality of electron

It all started with the De Broglie's hypothesis:

$$\lambda = \frac{h}{p}$$

λ is wavelength, h is Planck's constant, and p is momentum.

The original motivation of building an electron microscope came from the shorter wavelength of the electron.

p=mv

加速每个电子（电子的电荷为-e）所做的功（eU）就是电子获得的全部动能，即 eU=1/2.m.v2

Electron wavelength

Applying the principle of energy conservation to an electron (-*e*) traveled in voltage E_0:

$$eE_0 = \frac{h^2}{2m\lambda^2}$$

$$\lambda = \frac{h}{\sqrt{2meE_0}}$$

E_0 = acceleration voltage

λ = wavelength

m = electron mass

e = electron charge

h = Planck's constant

Electron wavelength

Take the relativity into consideration, the wave length is:

$$\lambda = \frac{h}{\sqrt{2m_0 e E_r}} \qquad E_r = E_0 + \left(\frac{e}{2m_0 c^2}\right) E_0^2$$

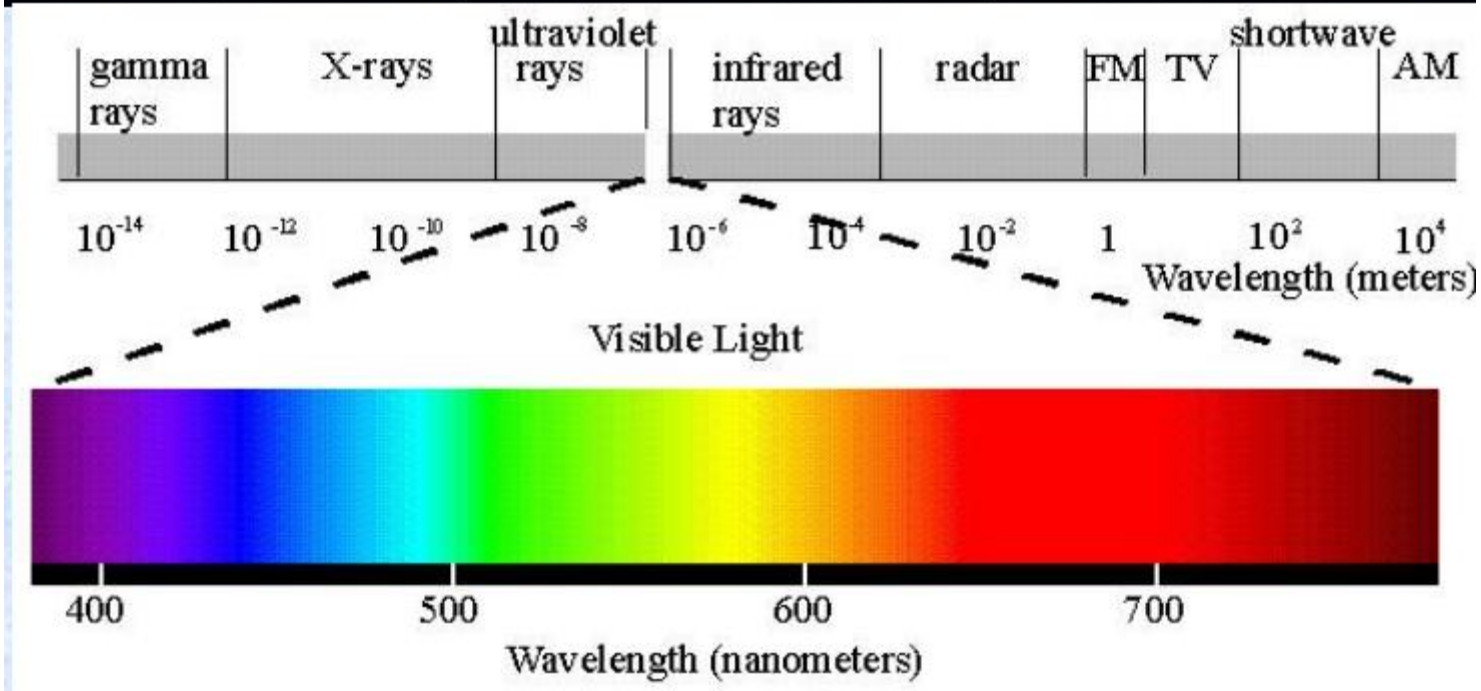
$$\lambda = \frac{1.22639}{\sqrt{E_0 + 0.97845 \times 10^{-6} E_0^2}}$$

120kV λ=0.033Å; 200kV λ=0.025Å; 300kV λ=0.020Å;

Note that these wavelength is considerably shorter than that used in X-ray crystallography, which is ~Å.

不同加速电压下的电子波长和速度

U/kV	λ/nm	v/(10^{11}mm·s^{-1})
40	0.006 01	1.121 6
60	0.004 37	1.338
80	0.004 18	1.506
100	0.003 70	1.644
200	0.002 51	2.079
500	0.001 42	2.587
1 000	0.000 87	2.822

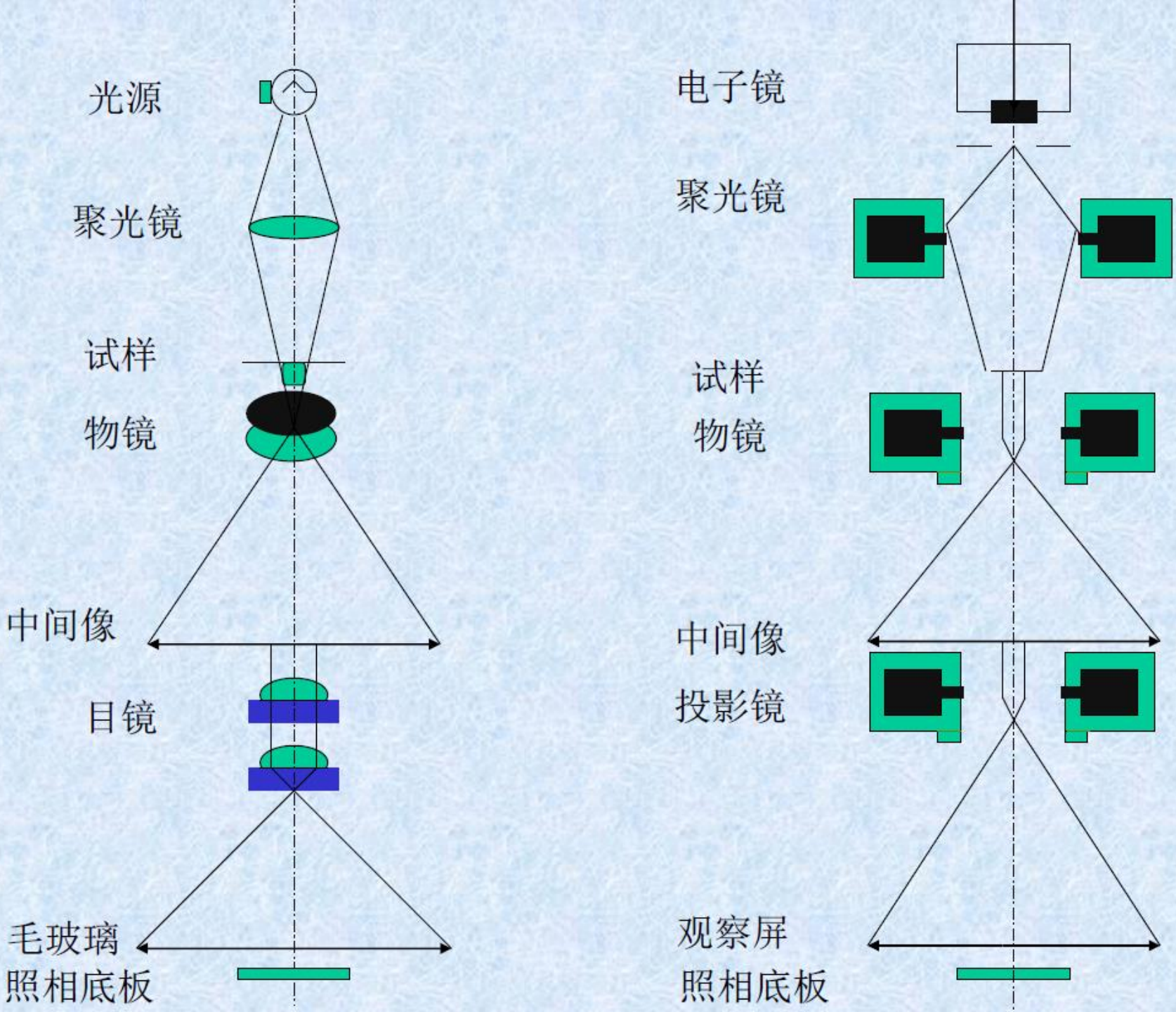


Life Sciences TEM Portfolio

120 kV	200 kV		300 kV	
High degree of automation, class leading optics and data quality	Flexibility at high performance, automation and ease-of-use	Full Automation for SPA and Tomo building on the Talos platform	Maximum flexibility and versatility on best possible platform	The ultimate fully automated high end cryo-TEM for SPA and Tomo
				
Talos L120C	Talos	Talos Arctica	Titan Halo	Titan Krios

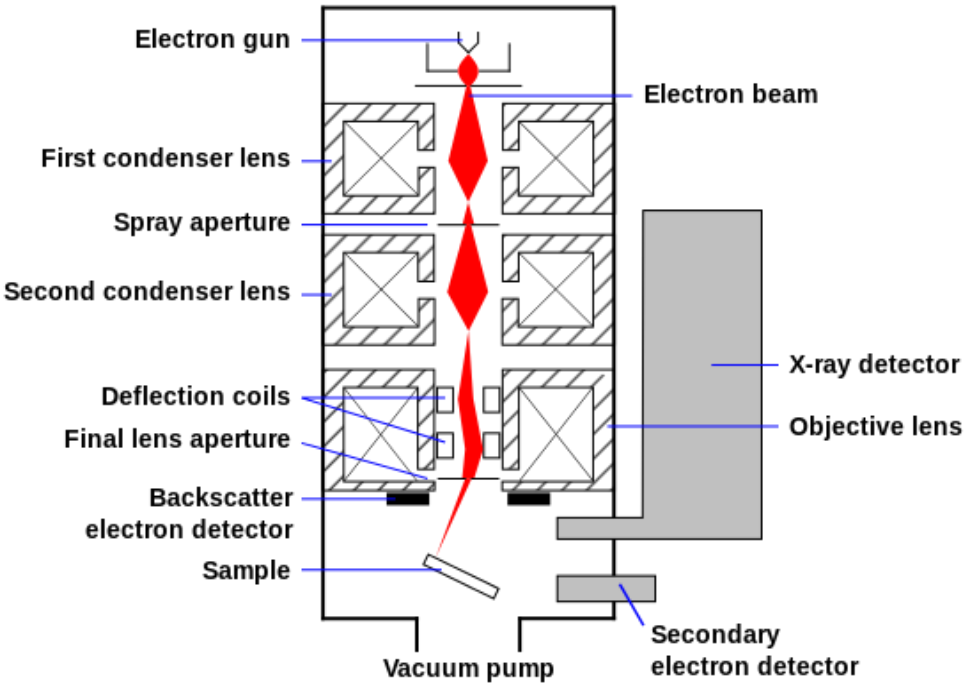
电镜与光镜的比较

显微镜	分辨本领	光源	透镜	真空	成像原理
LM	200nm	可见光（400-700）	玻璃透镜	不要求真空	利用样品对光的吸收形成明暗反差和颜色变化
	100nm	紫外光（约200nm)	玻璃透镜	不要求真空	
TEM	0.1nm	电子束（0.01-0.9nm）	电磁透镜	要求真空	利用样品对电子的散射和透射形成明暗反差

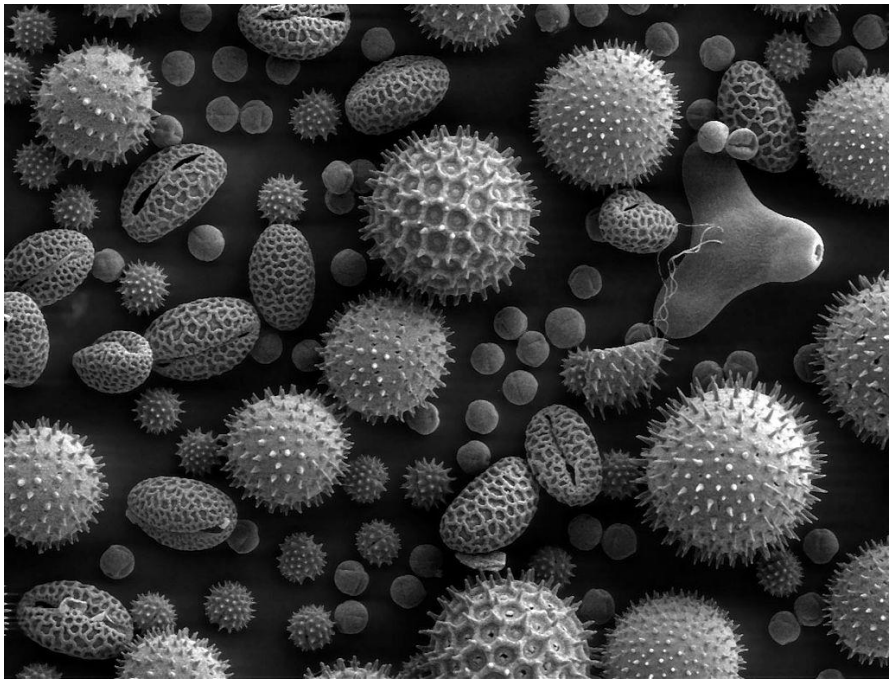

光源
聚光镜
试样
物镜
中间像
目镜
毛玻璃
照相底板
电子镜
聚光镜
试样
物镜
中间像
投影镜
观察屏
照相底板

- Scanning Electron Microscope (SEM)
- Transmission electron microscope (TEM)
- Scanning transmission electron microscope (STEM)

Scanning Electron Microscope (SEM)

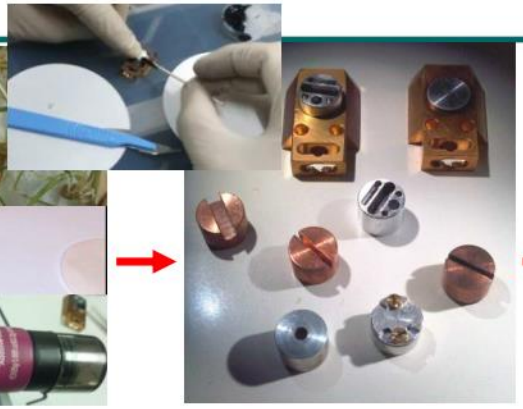


A **scanning electron microscope** (**SEM**) is a type of electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about the sample's surface topography and composition.

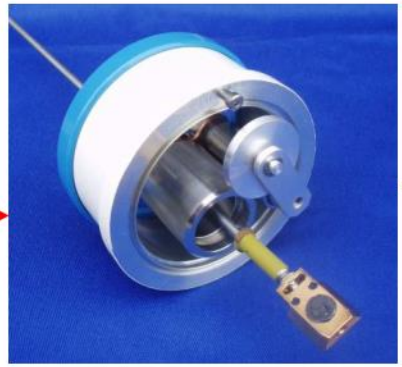


Cryo –SEM 制样过程

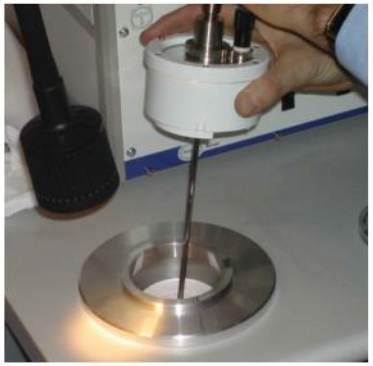
含水（液体）样品取样



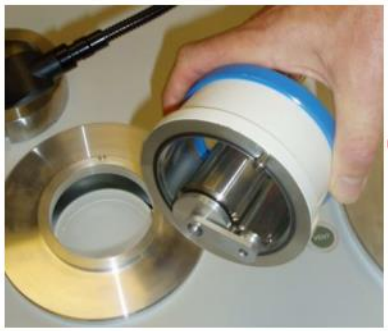
装载(各种样品座)



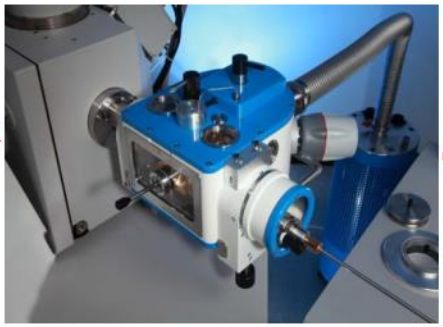
样品装载到传输装置



样品预冷(插入液氮泥)

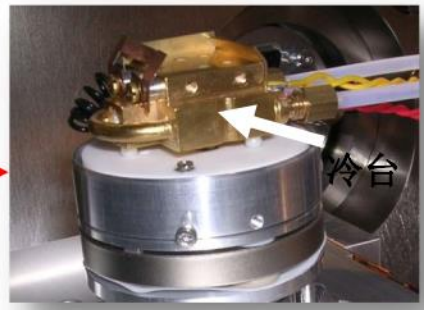


真空下转移



与制备腔室气锁对接

断裂、升华刻蚀和镀膜

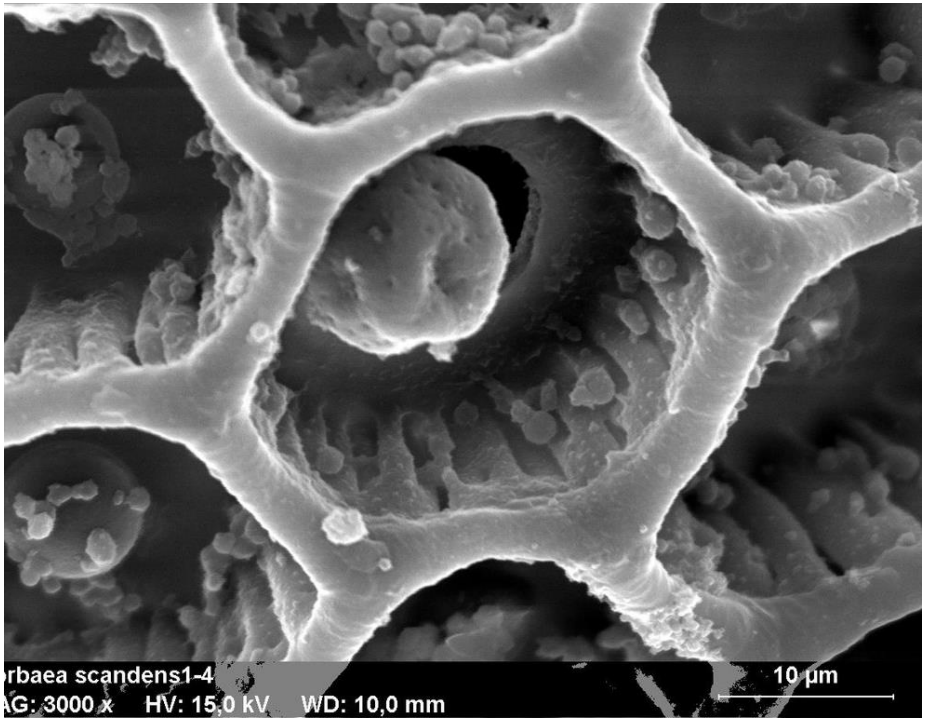


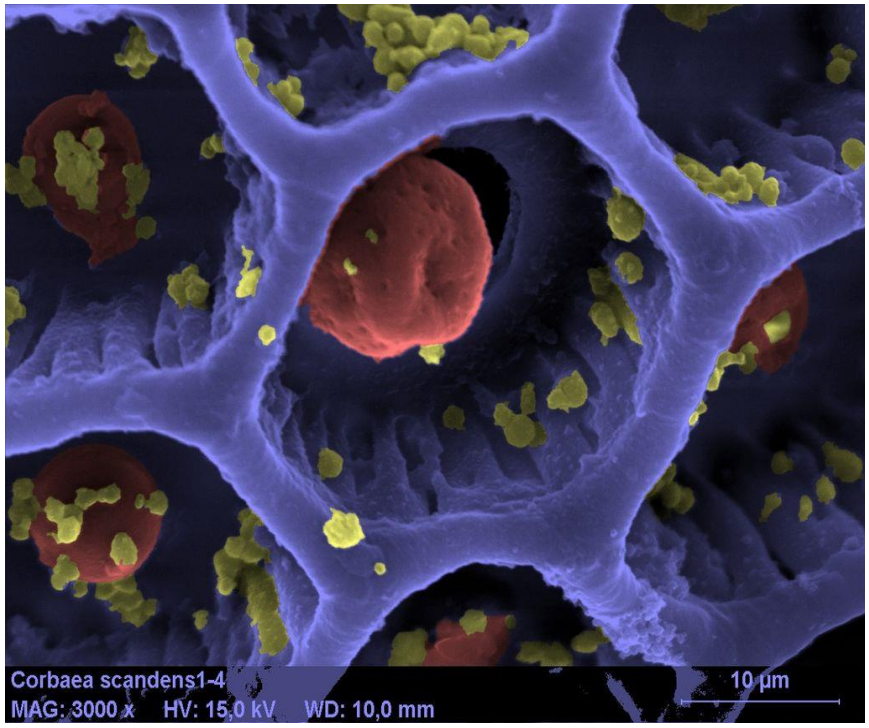
传输至电镜腔室
(SEM观察和拍照)

整个CryoSEM操作过程均在“低温冷冻”和“真空状态”两个前提下进行

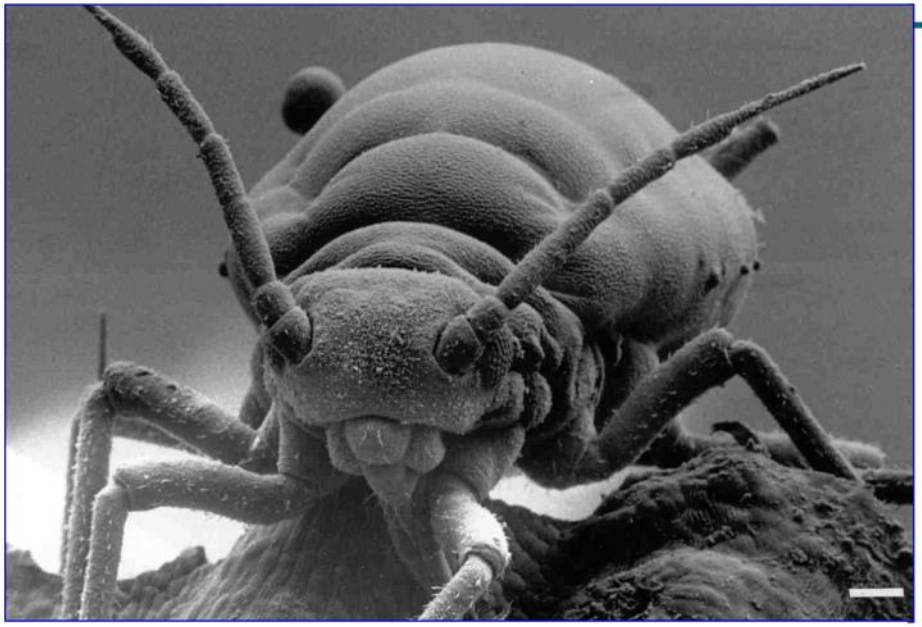
通过低温断裂(通常为-140°C)可从样品得到更多额外信息；断裂后可进行升华刻蚀(通常为-90°C)以显示样品更多内在信息。

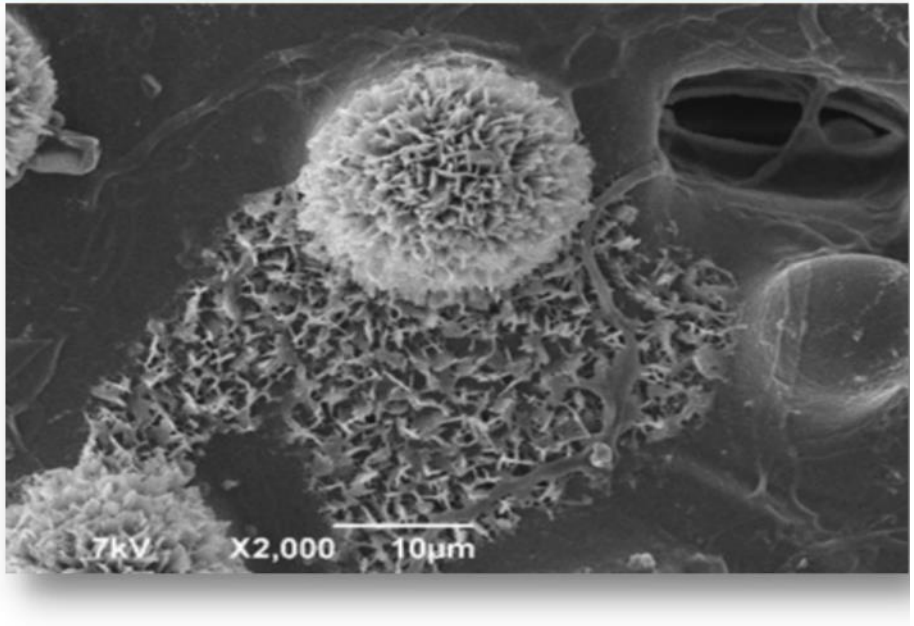
关于电镜图像的“颜色”


rbaea scandens1-4
AG: 3000 x HV: 15,0 kV WD: 10,0 mm
10 µm


Corbaea scandens1-4
MAG: 3000 x HV: 15,0 kV WD: 10,0 mm
10 µm

Cryo -SEM

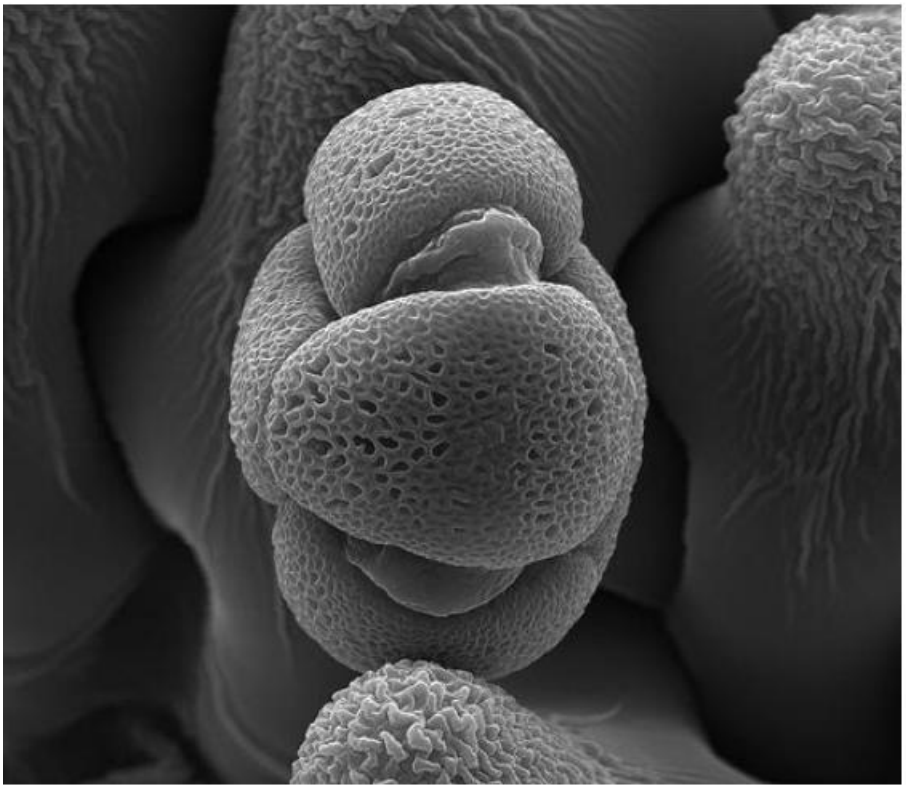




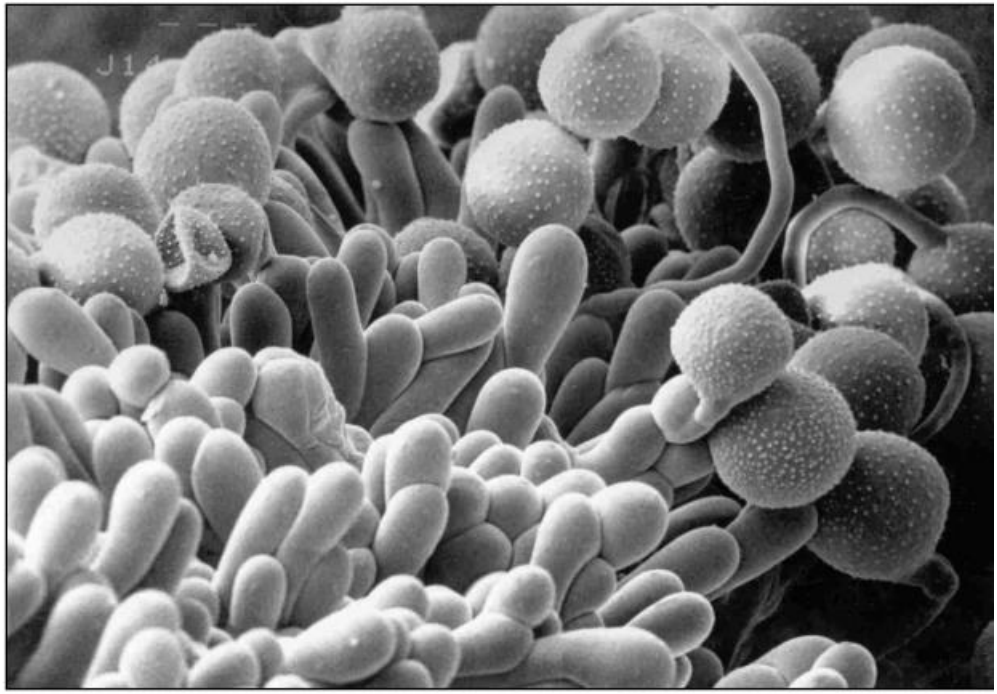
- 无皱缩变形及机械损伤
- 常规处理样品表面蜡质通过溶剂和液态CO2被移去，而CryoSEM样品蜡质被保留

左图：CryoSEM，蚜虫及其表面蜡质　　右图：植物表面蜡质纹饰

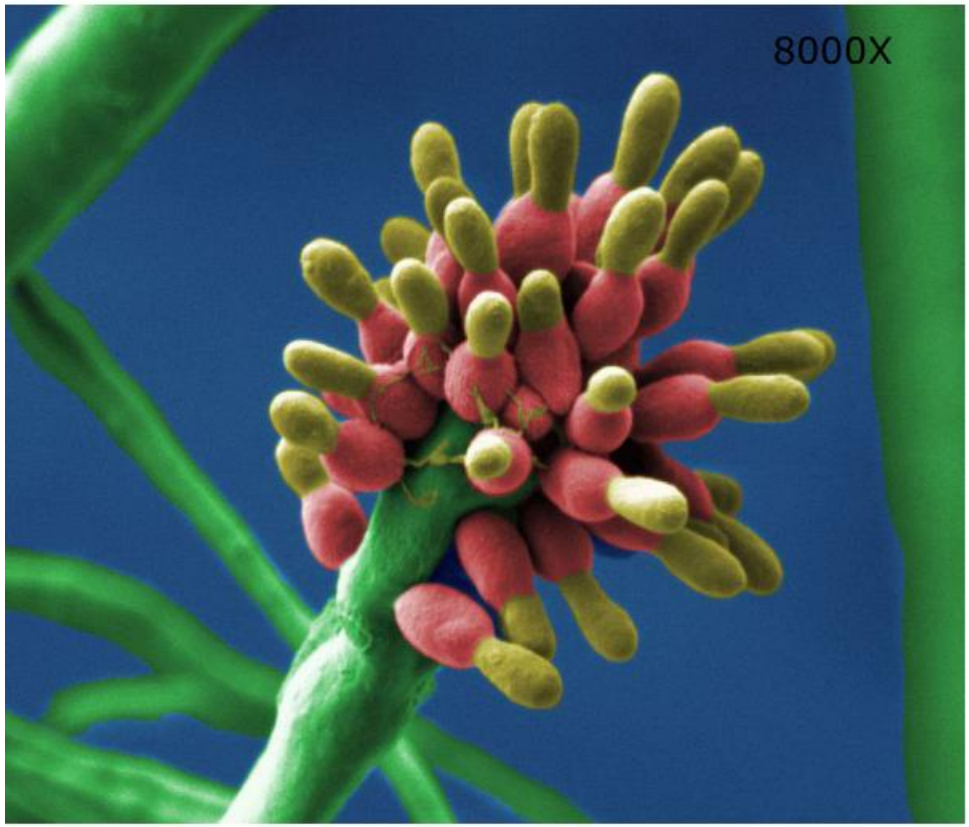
不失真观察样品(不变形_形状结构真实、可溶材料被保留_成分真实)

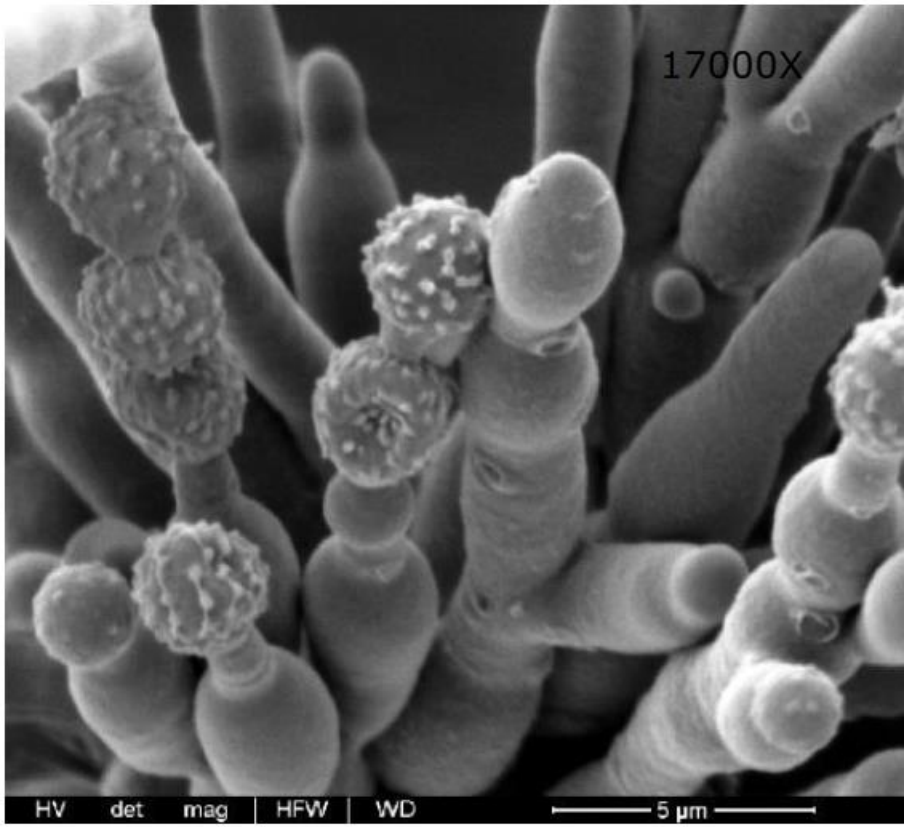


Mother hugging a baby? Actually a pollen grain on the petal of Mazus.
通泉草花粉粒



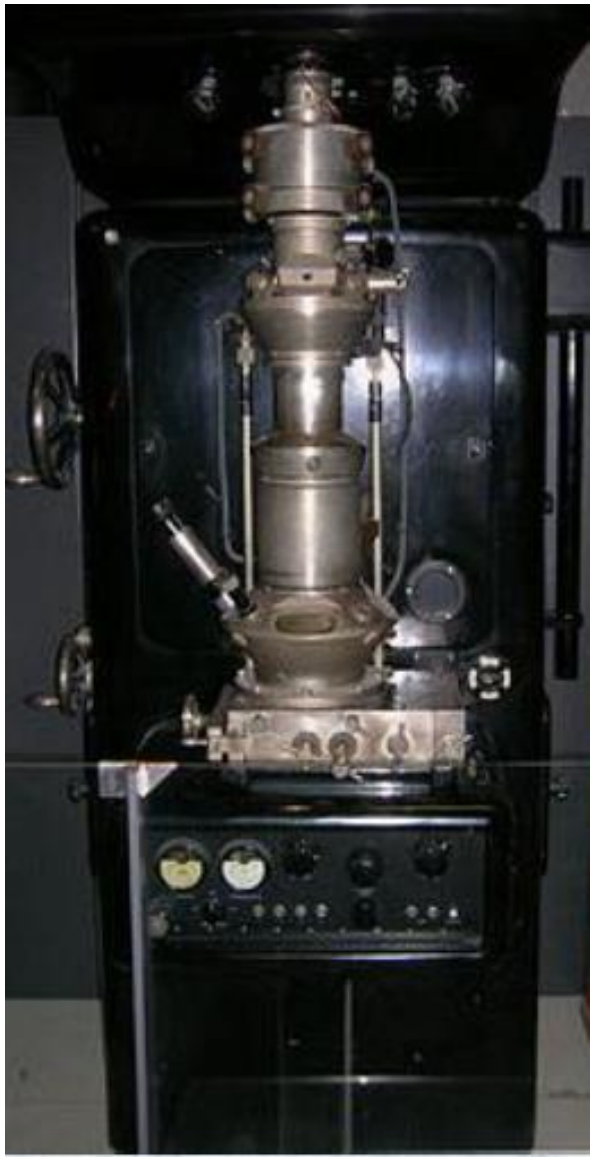
仙人掌花粉



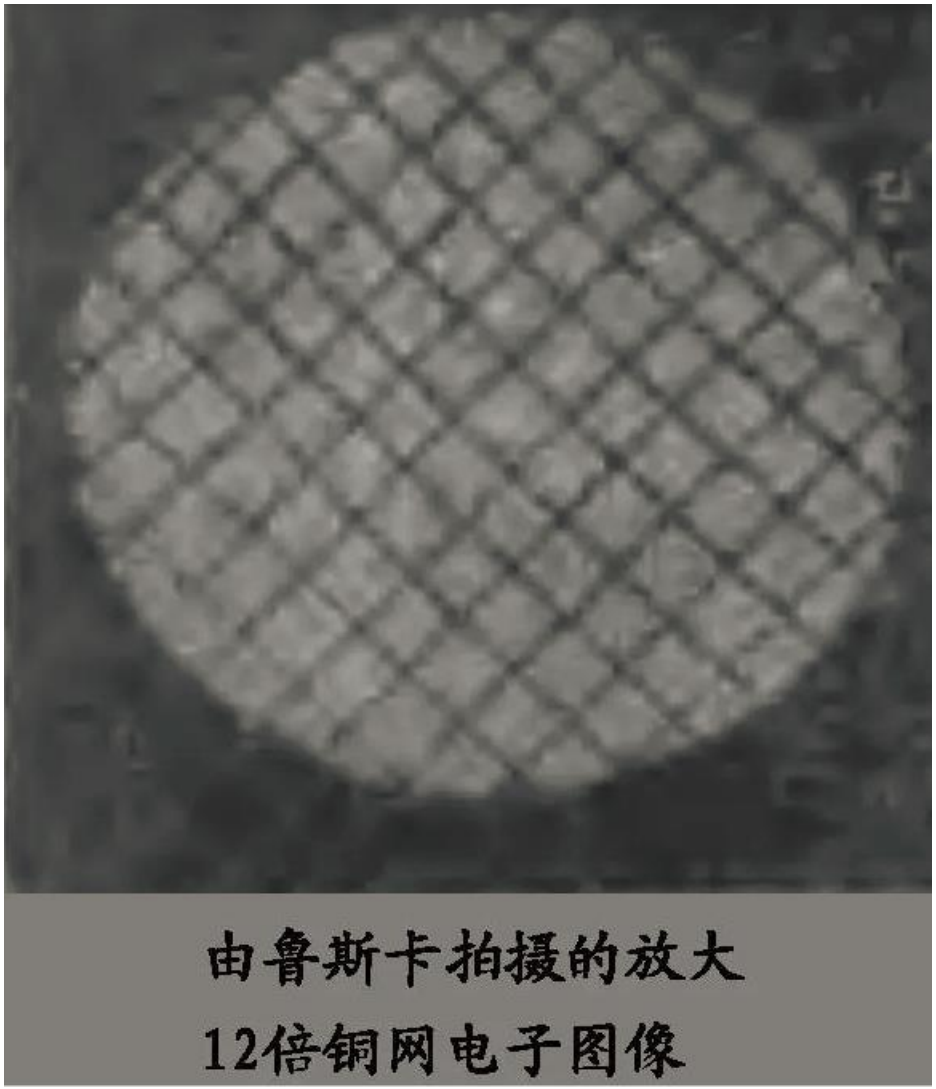


与植物根系互惠共生的**菌根真菌**

透射电镜的基本构造

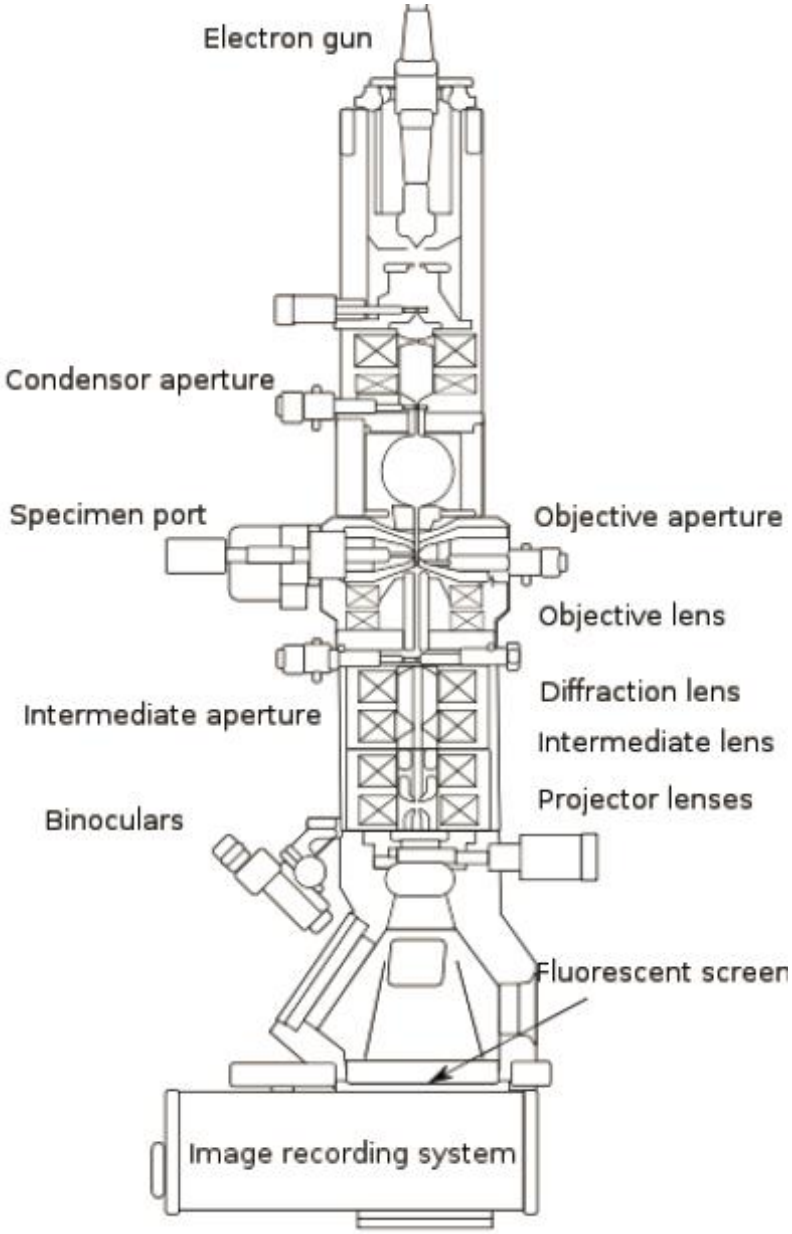


Electron microscope constructed by Ernst Ruska in 1933



由鲁斯卡拍摄的放大
12倍铜网电子图像

Instrumentation of EM



Gun

Wehnelt cylinder

Condenser lens system

"gun" deflectors

Condenser lenses

Condenser stigmators

Condenser aperture

Objective lens system

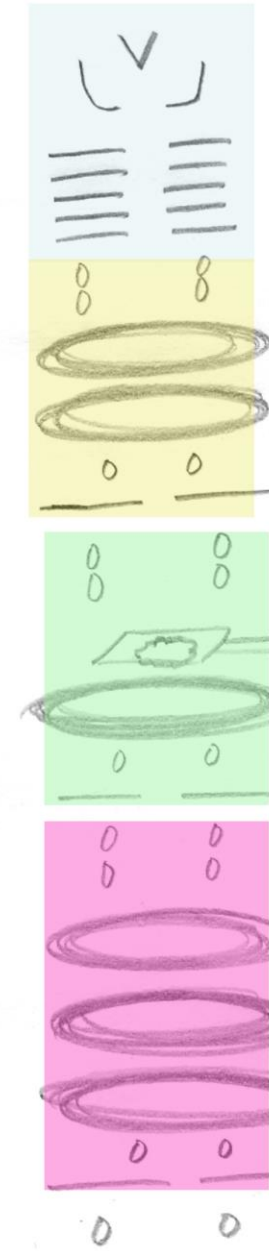
"image" deflectors

Projector lens system

"intermediate" lenses

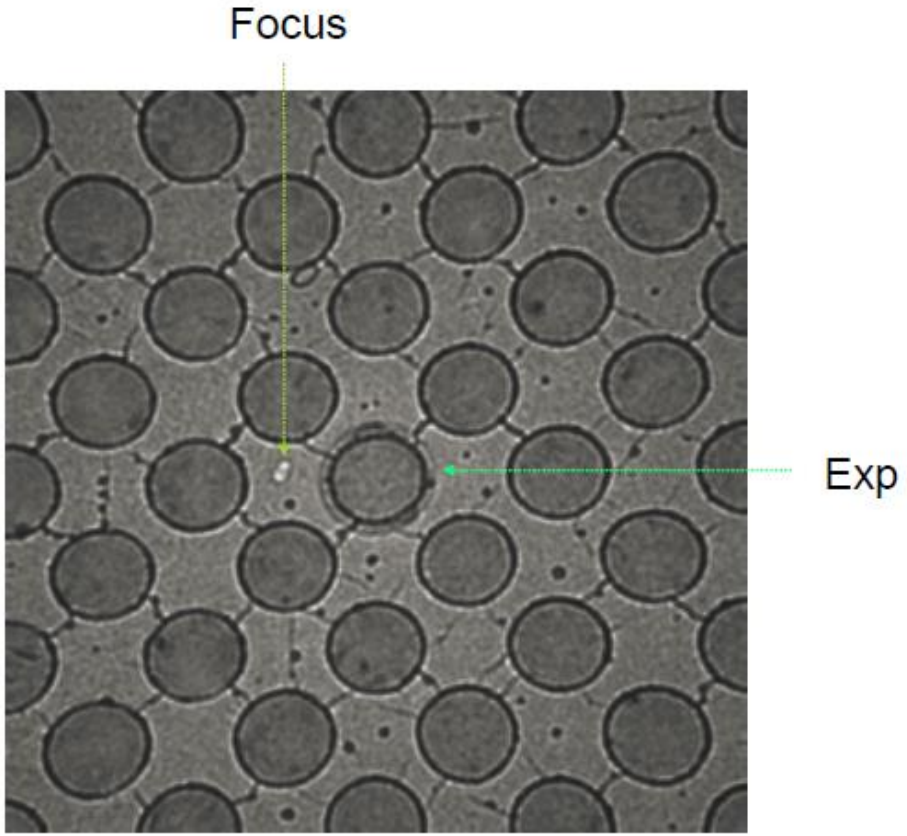
"diffraction" stigmator

"selected area" aperture



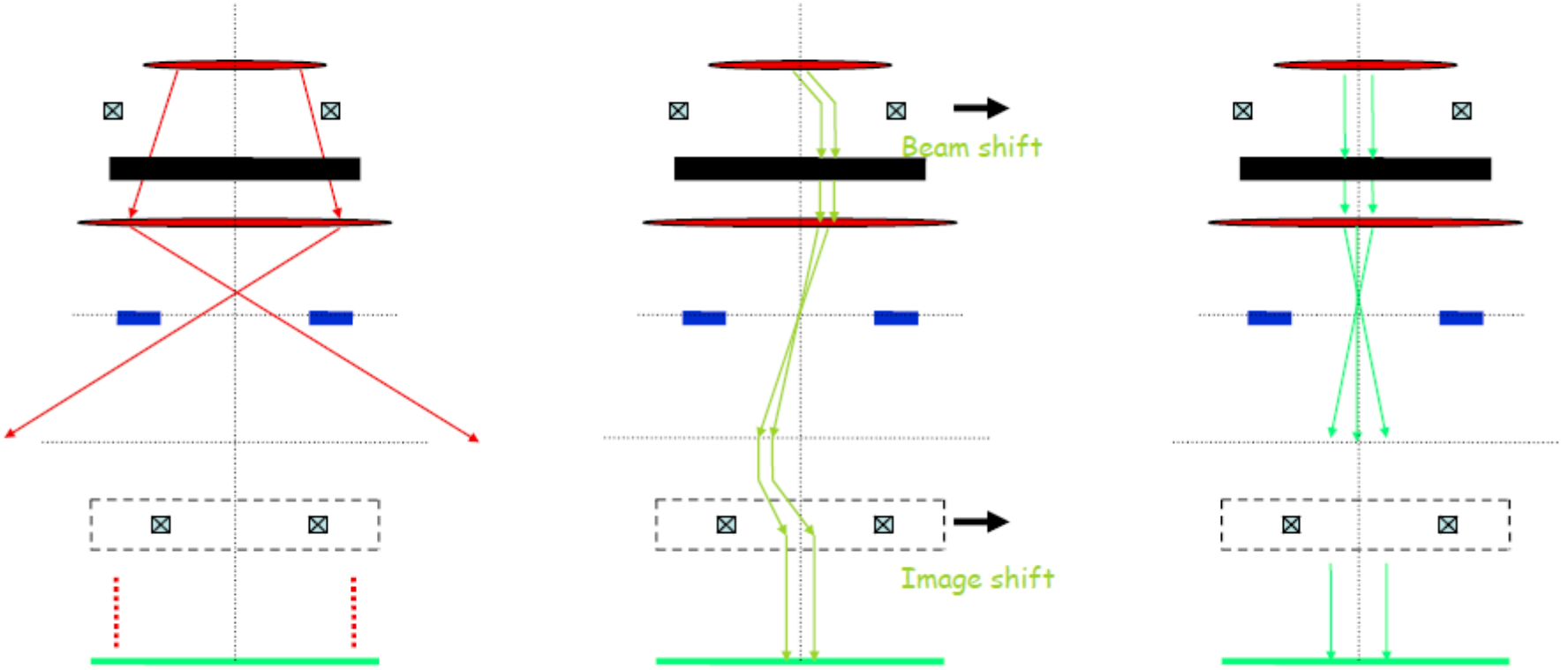
Three different modes in low dose

* Search: lowest possible beam intensity;
* Focus: off-exposure area, high magnification;
* Exposure: desired magnification and beam intensity;



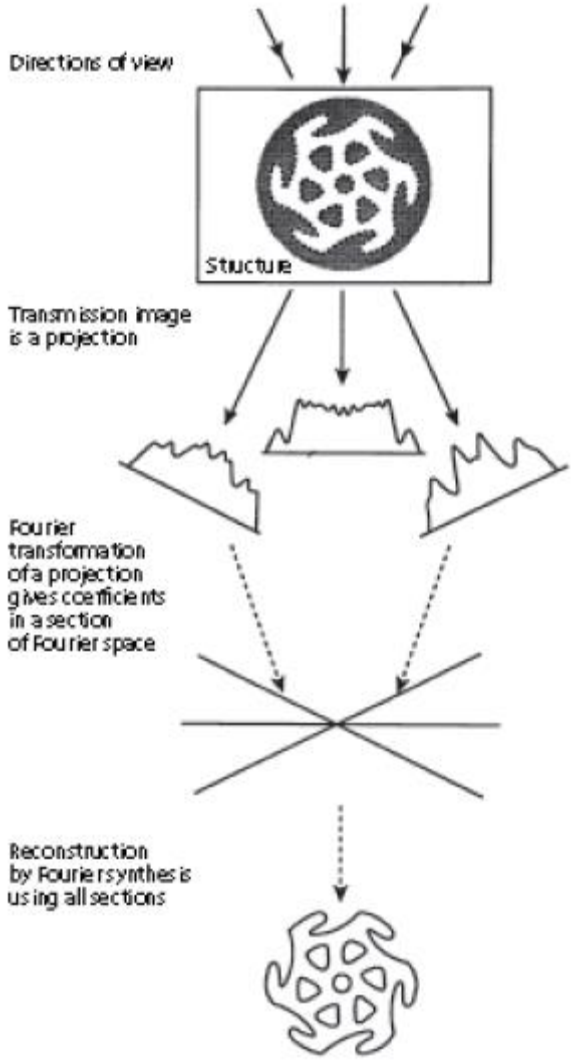
* SEARCH: extremely low-dose, ~$10^{-3}e^{-}/Å^2/sec$;
* FOCUS: high magnification, away from the imaging area;
* Exposure: 10 ~ 30 $e^{-}/Å^2$ dose rate to record image;

Electron optics of Low-Dose imaging



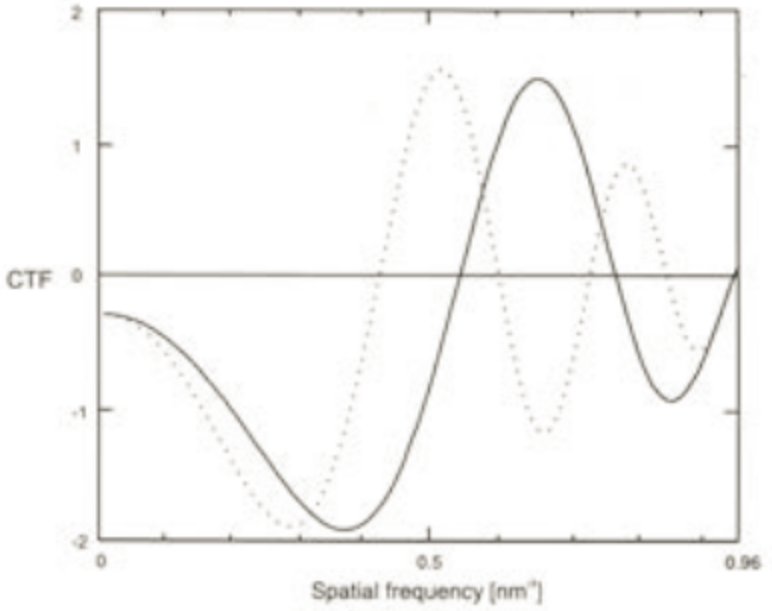
SEARCH FOCUS EXPOSURE

From Two-Dimensional Projections to Three-Dimensional Reconstructions



Contrast Transfer Function (CTF)

$$CTF = \sin(2\pi\chi k)$$

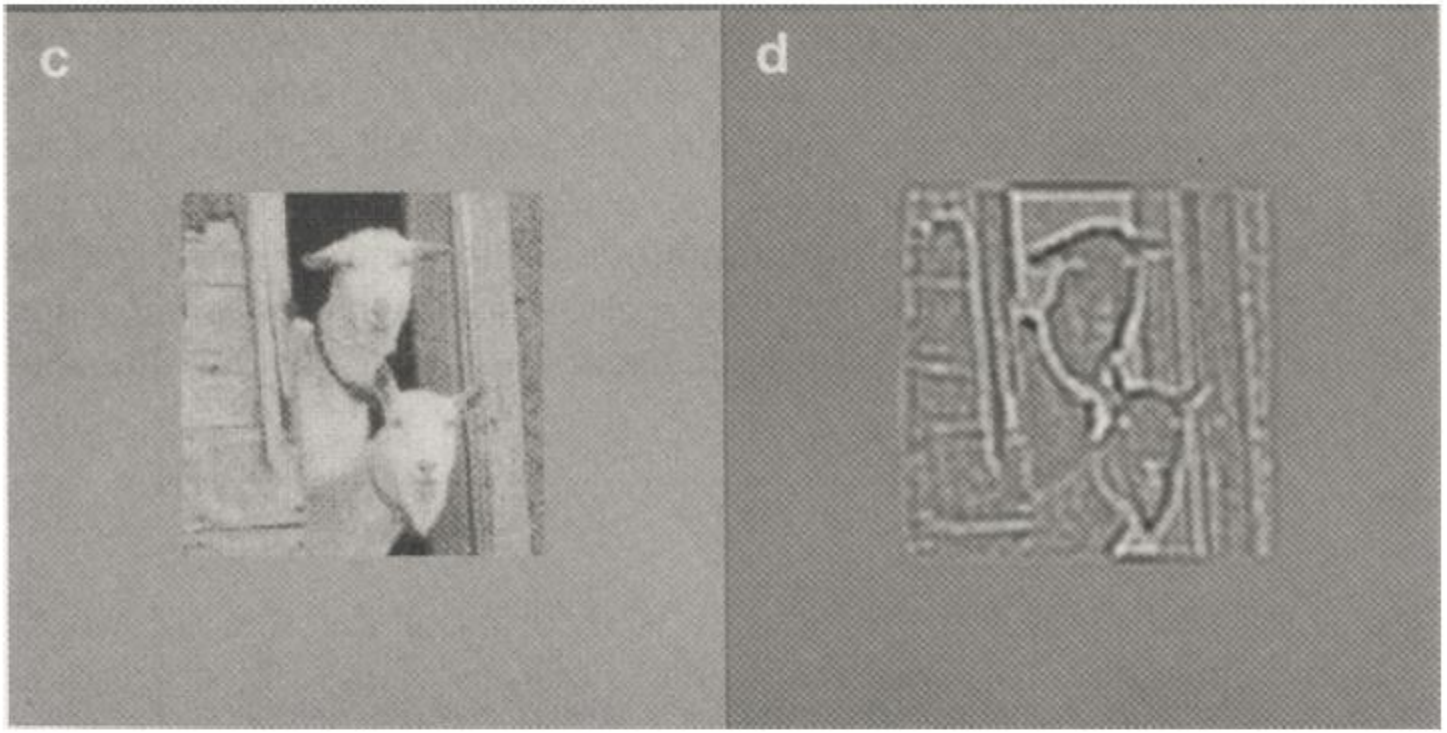


The intensity of a recorded image is directly related to the projection of specimen (good!) but modified by the FT of CTF (bad!).

$$I_i(\vec{r}) = \psi_i(\vec{r})\psi_i^*$$

$$= 1 + 2\Phi(-\vec{r}) \otimes J_0(\vec{r}) \otimes F^{-1}\left(CTF\right) \qquad (12)$$

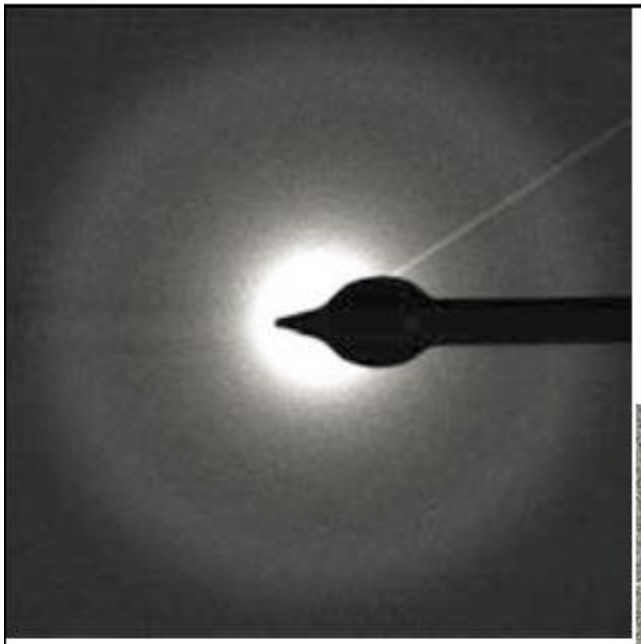
What is this CTF thing anyway and why do I care?



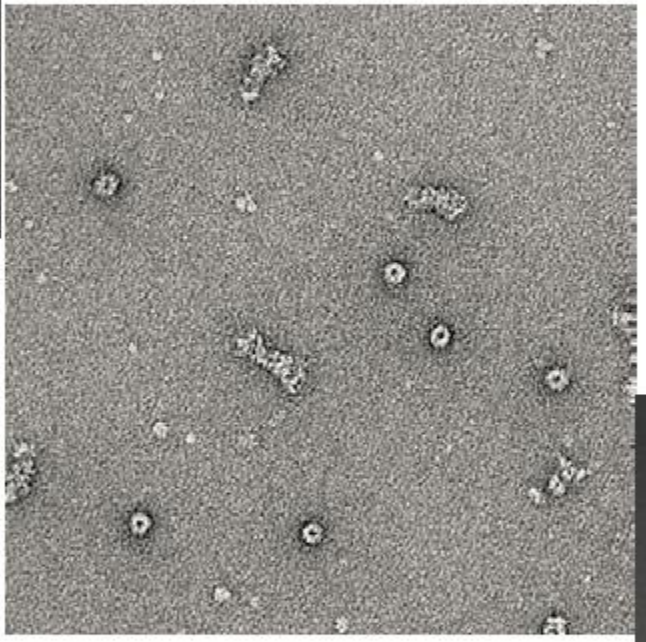
Distortions of CTF to the image are:
1) Contrast reverve of large area; 2) diminished contrast in large area; 3) edge enhancement and 4) appearance of fringes along the borders.

From Joachim Frank

Diffraction, image and power spectra

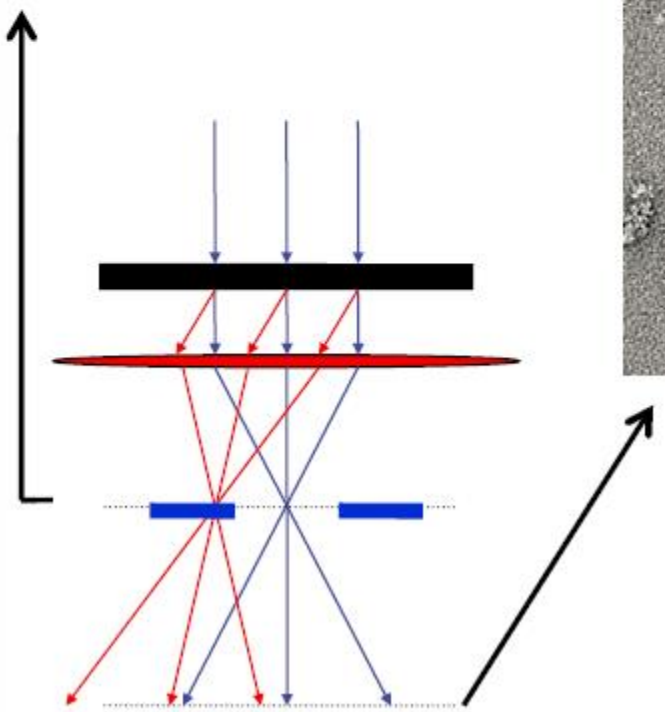


diffraction

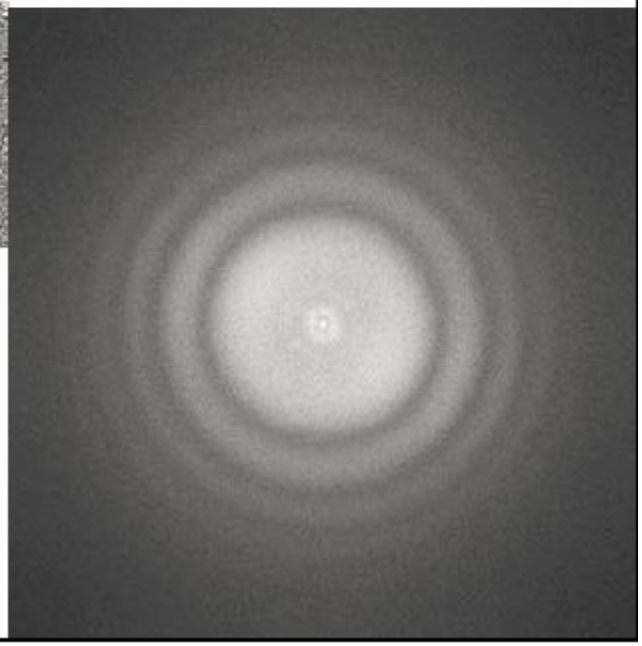


iamge

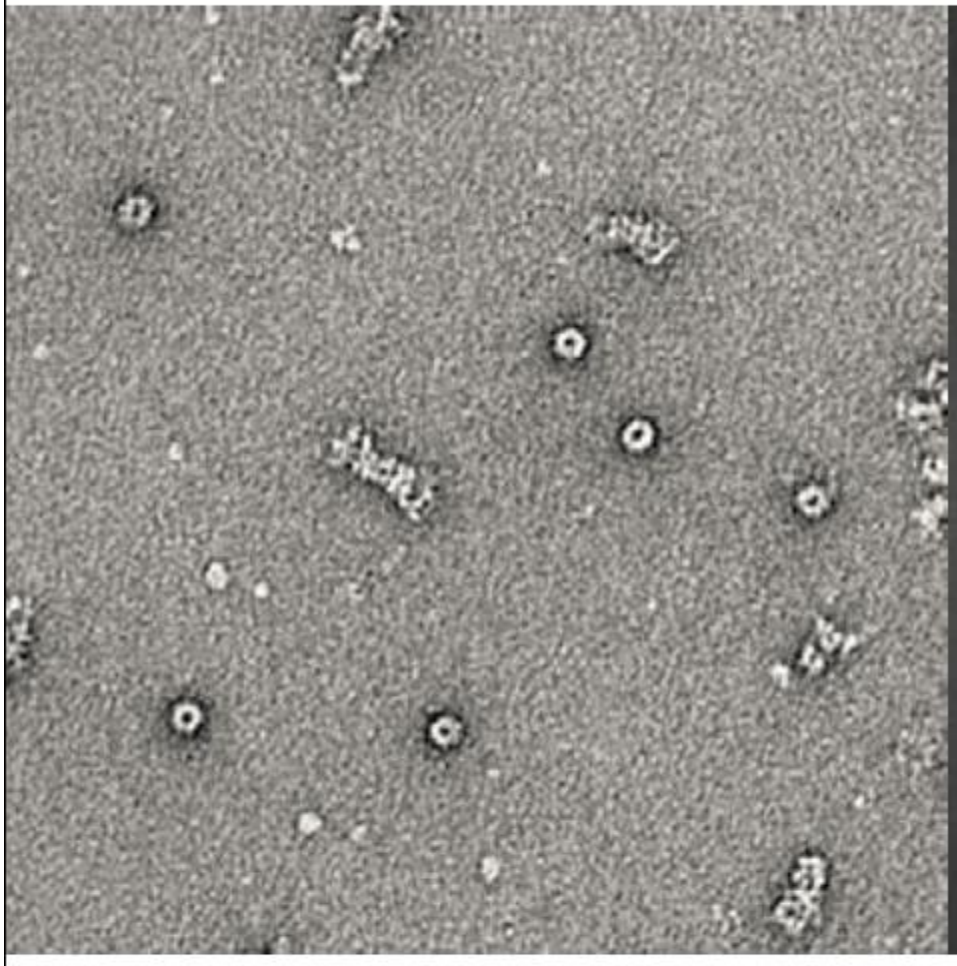
Fourier power spectrum

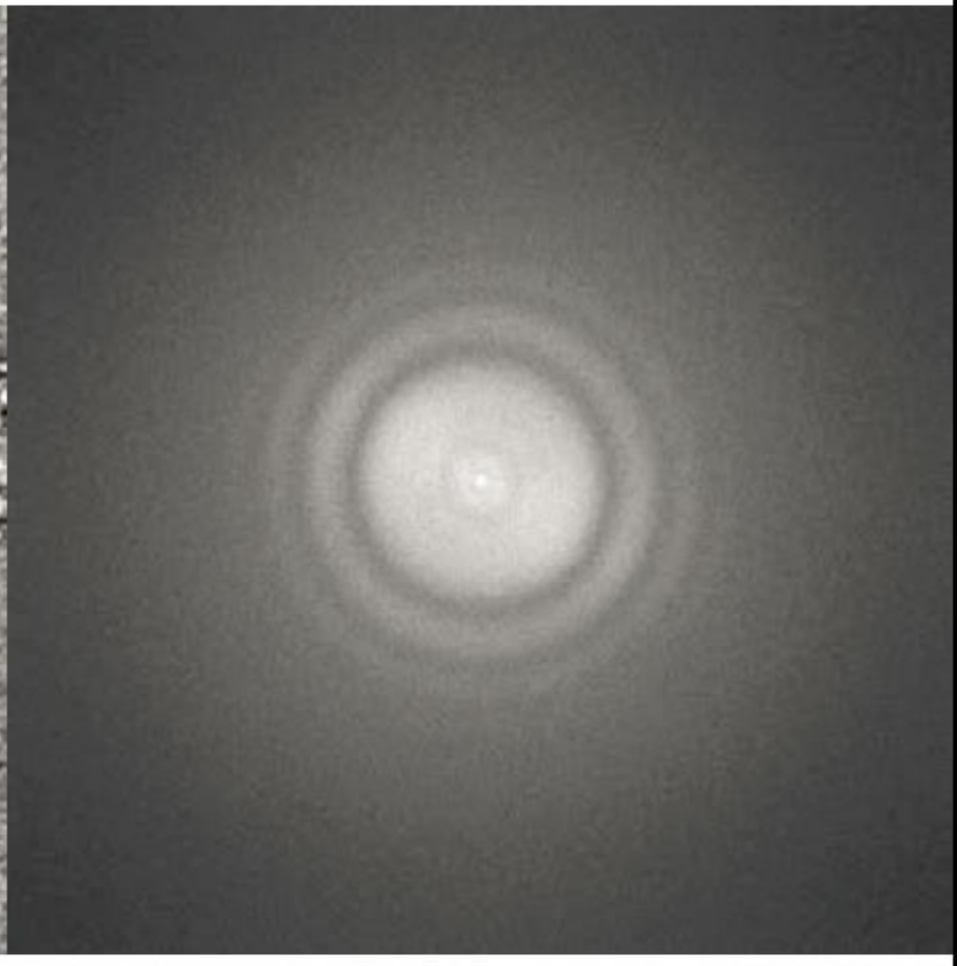


FT

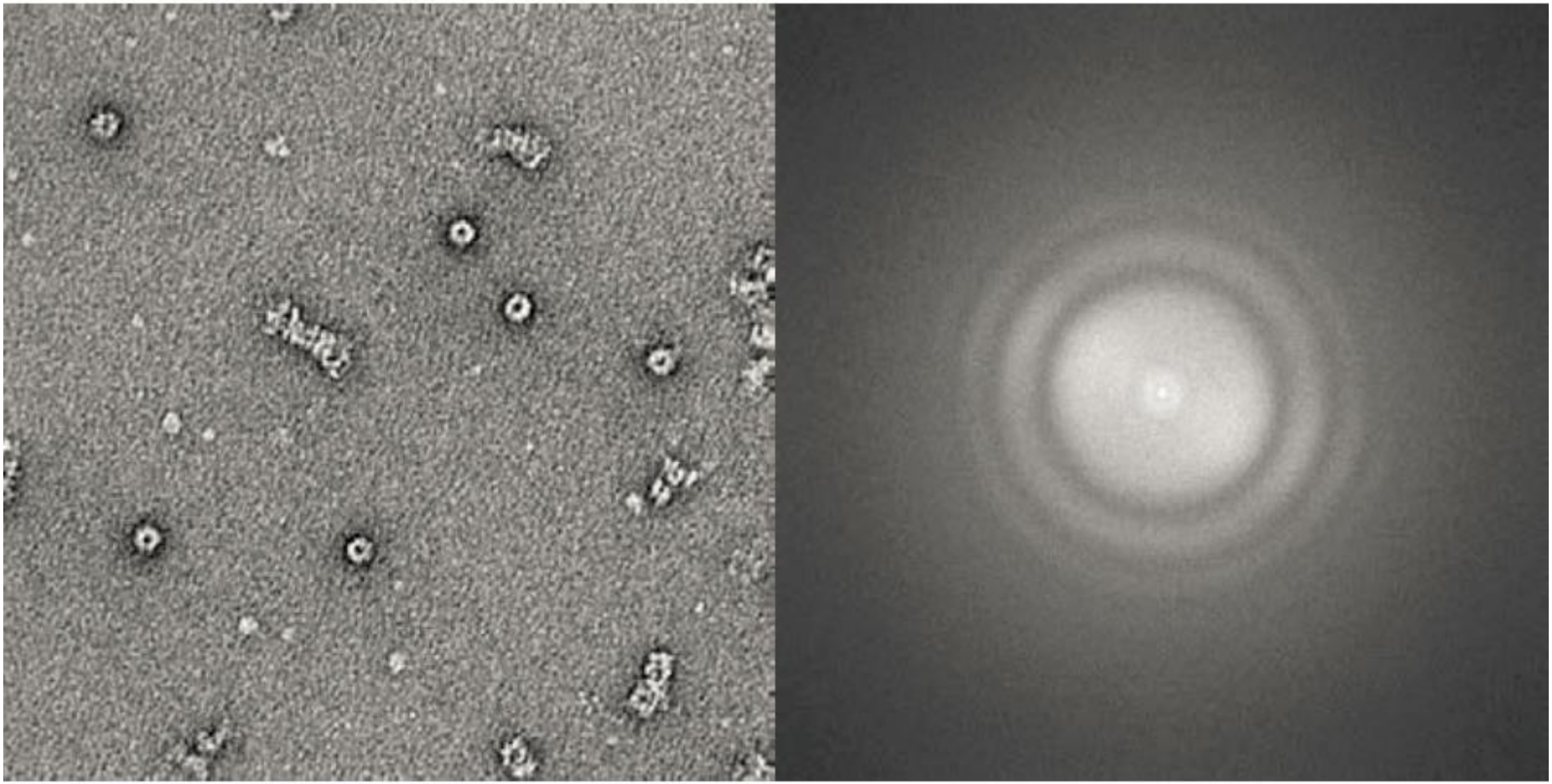


Defocus -2μm

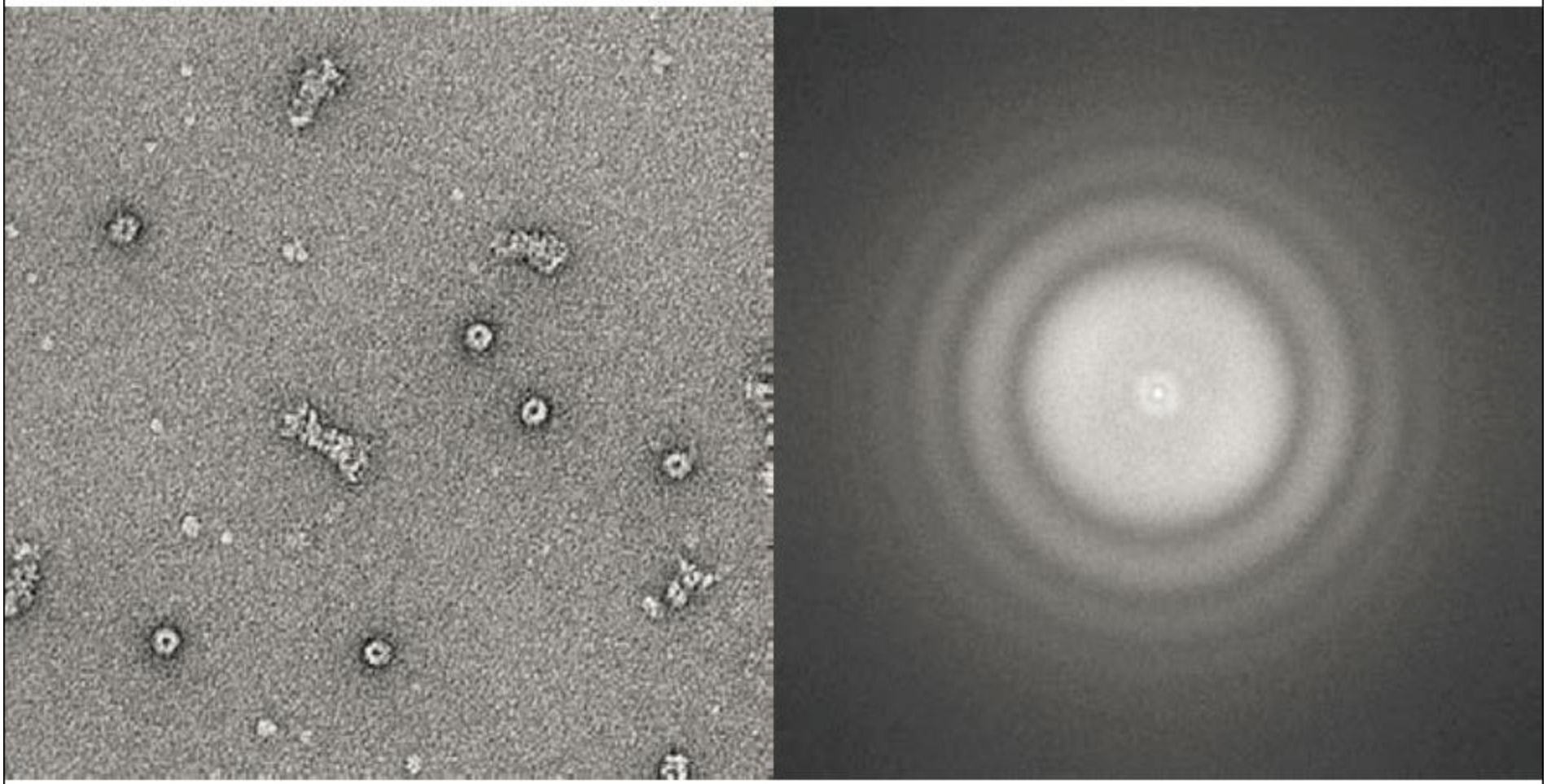




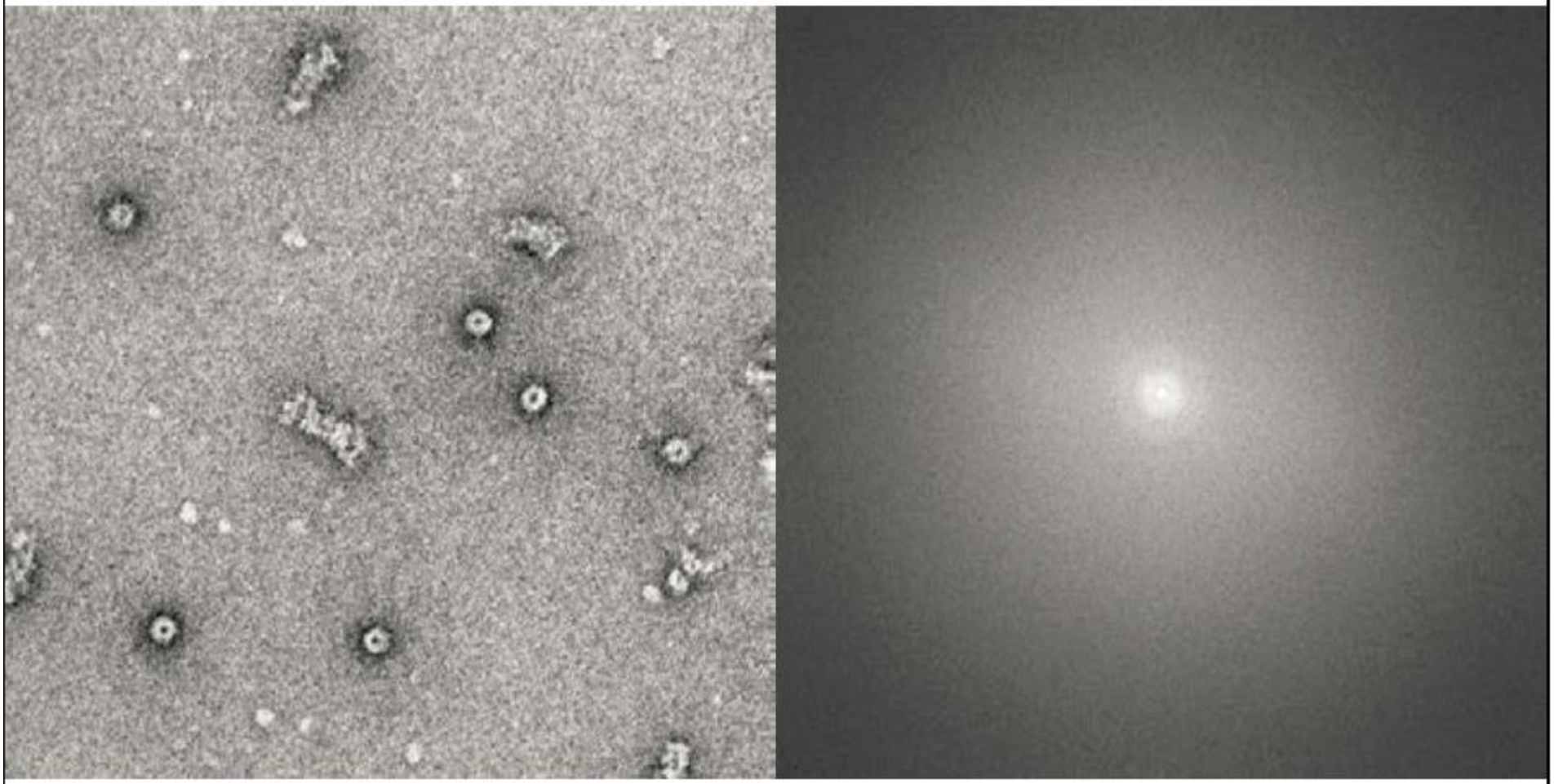
Defocus -1.5μm



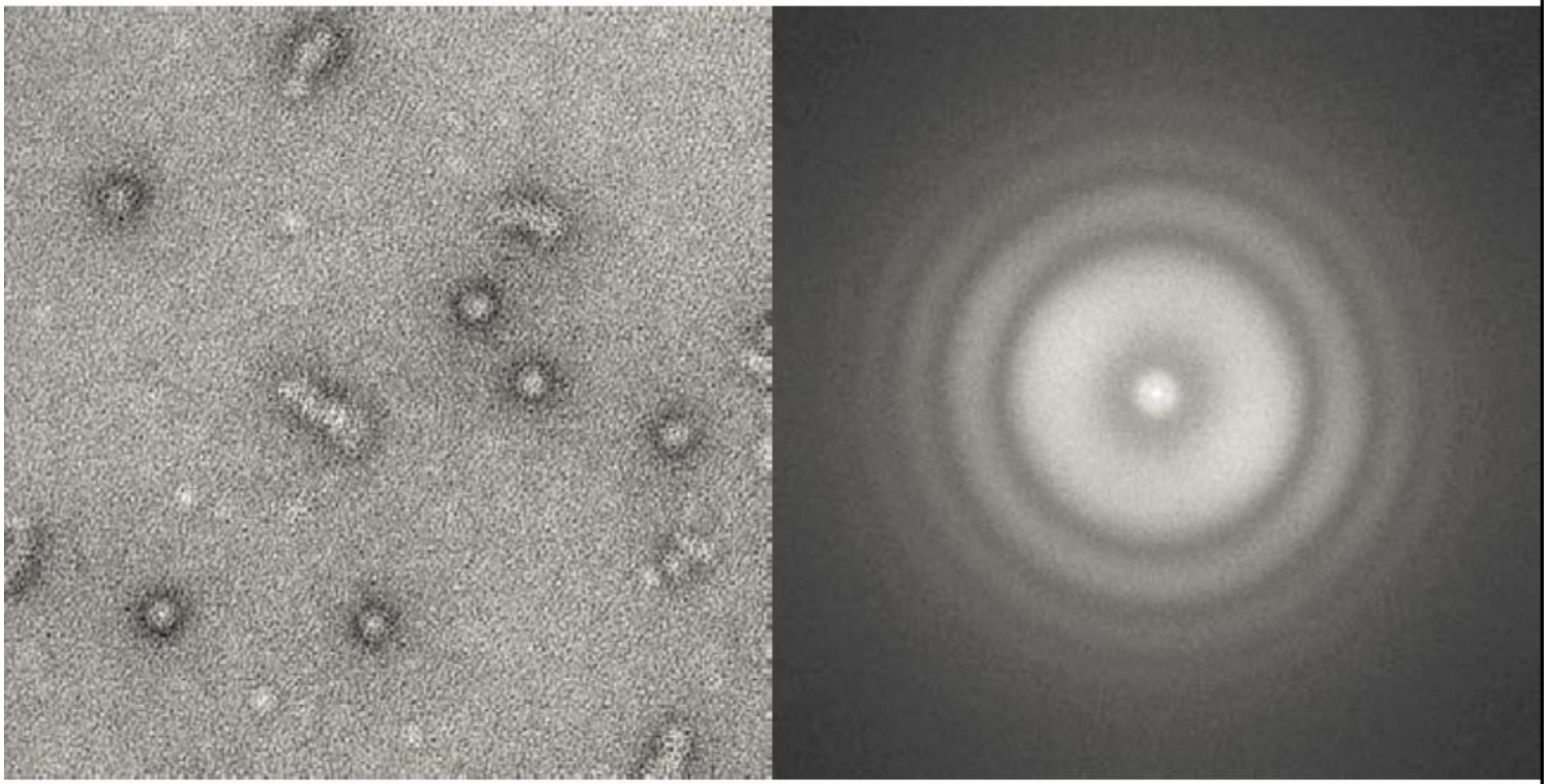
Defocus -1μm



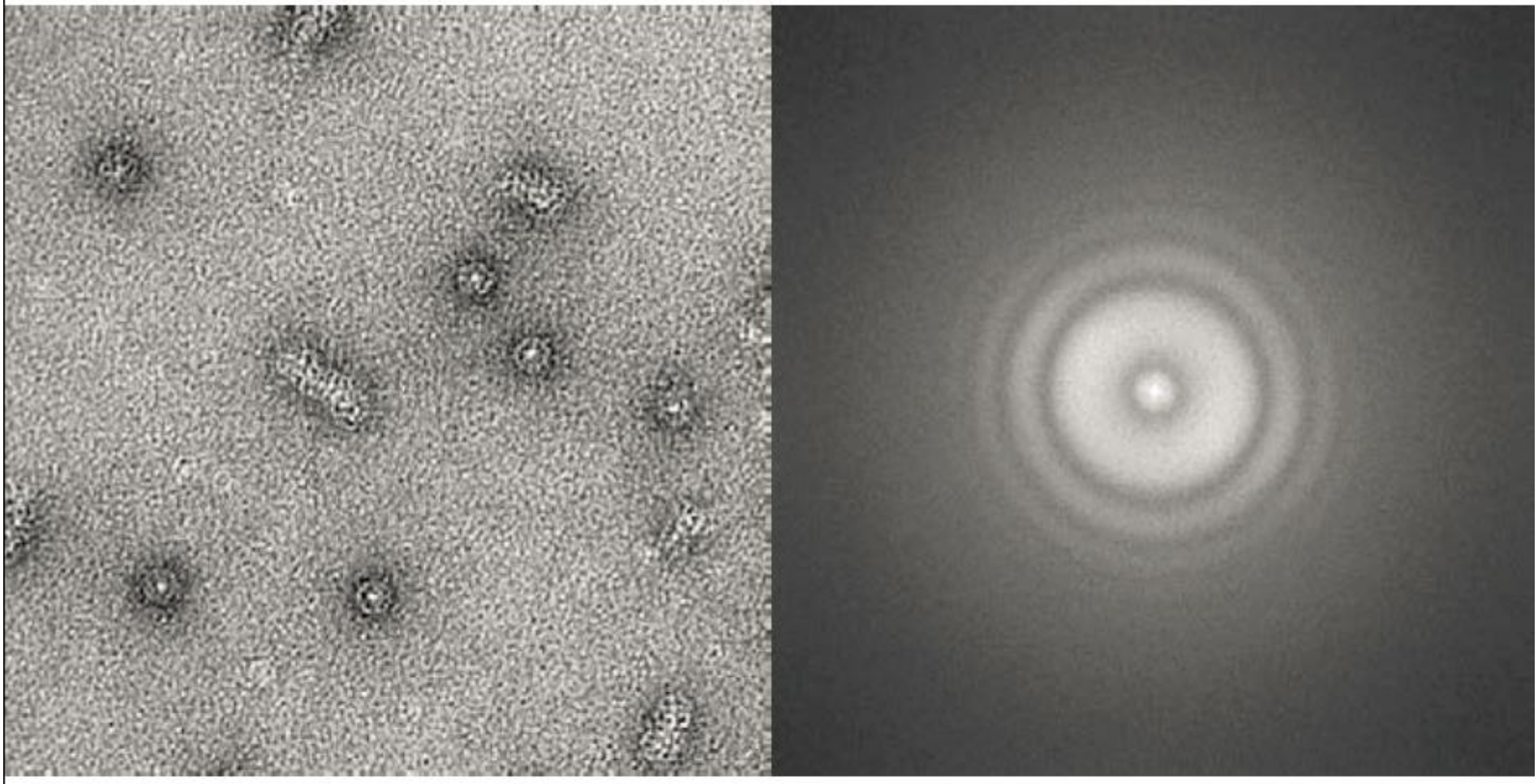
Defocus ~0μm



Defocus +1μm

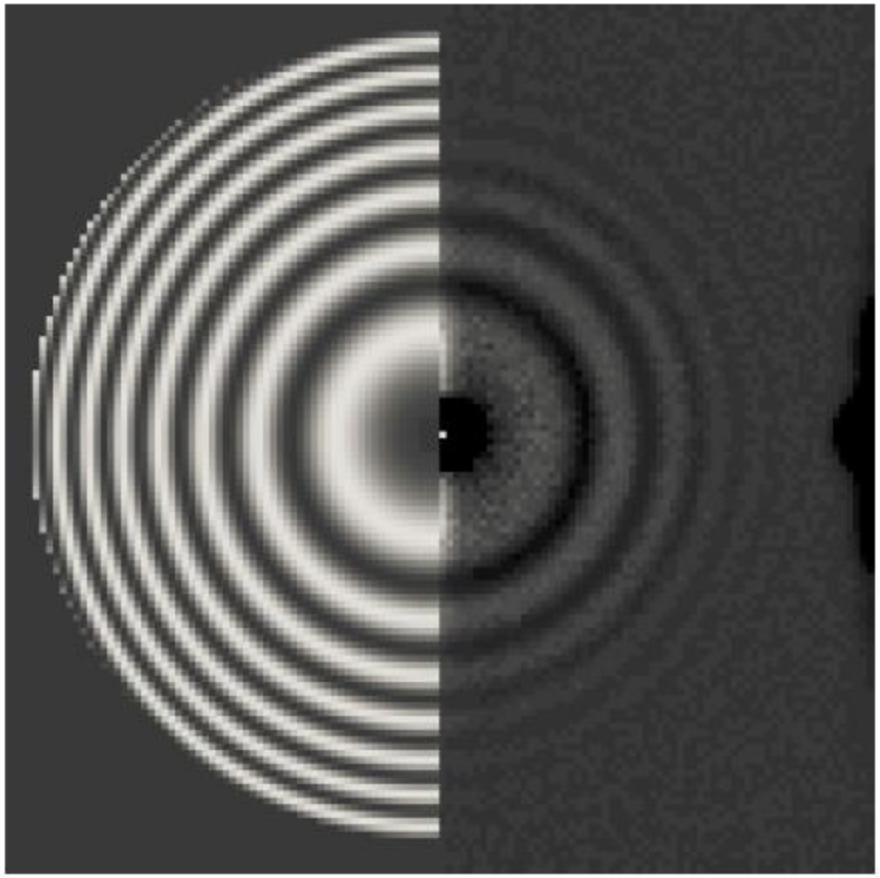


Defocus +2μm



Determine CTF

Image power spectrum



Model

Experiment

E = 120 kV, Δf = 21000 Å, C_s = 2 mm, A = 0.15

Maximum Likelihood approach

The iterative refinement procedure based on cross correlation is equivalent to a least square optimization procedure.

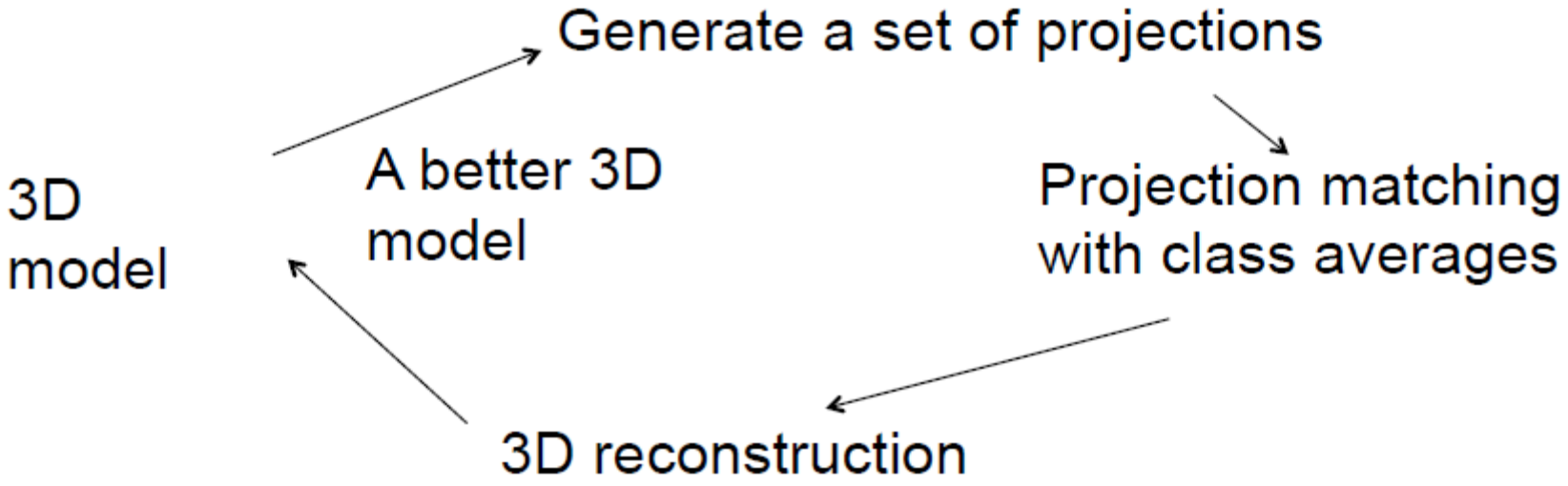
* Maximum Likelihood approach:
Given a set of images X, we would like to maximize is the probability $P(\Theta|X)$ that this model Θ is the correct one.

Sigworth, et al. “An introduction to maximum-likelihood methods in cryo-EM” Method in Enzymology, Cryo-EM, part C.

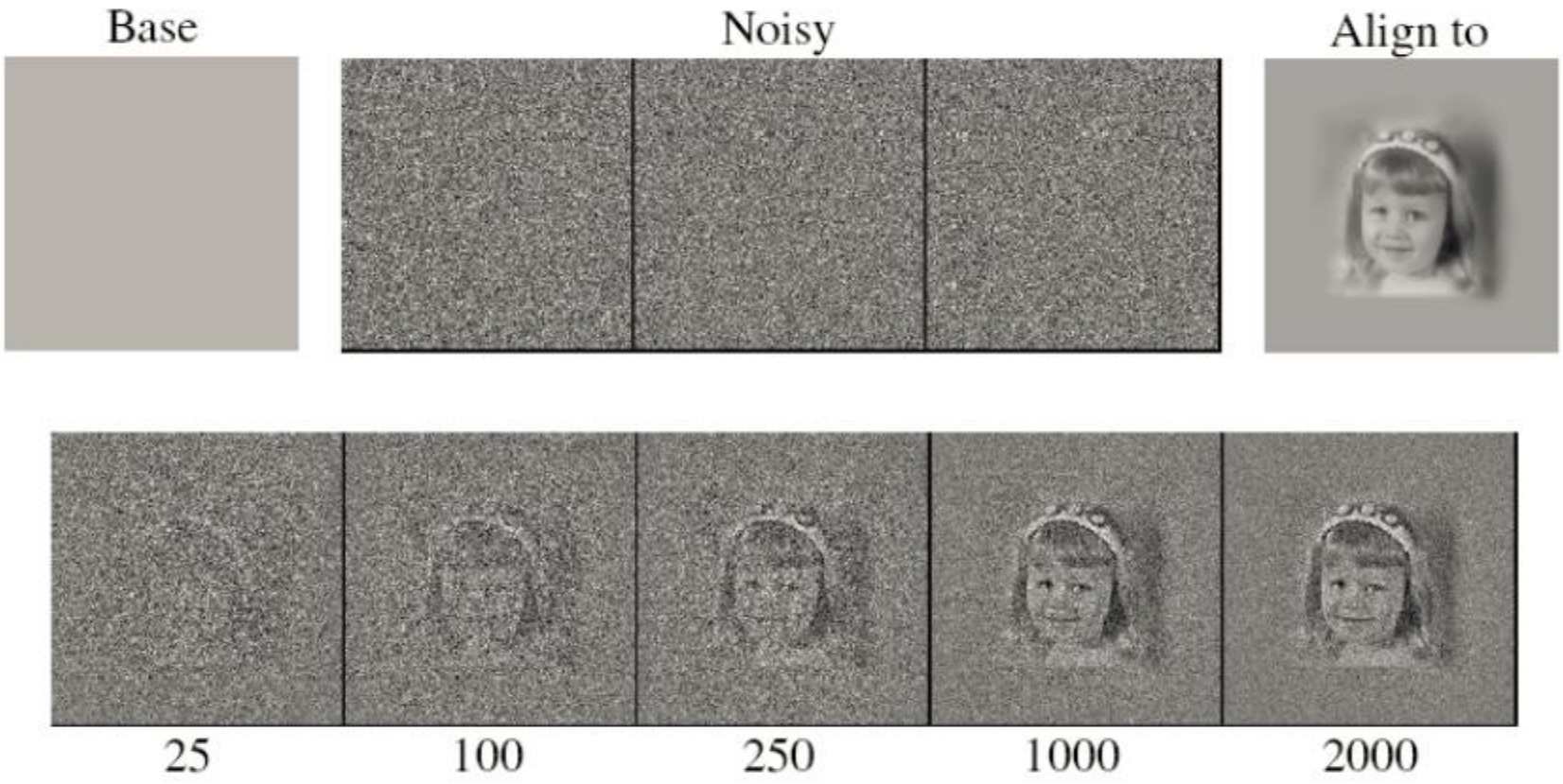
Maximum likelihood algorithm is now implemented in a number of programs, including RELION, XIMP, FREALING, etc.

Iterative refinement procedure

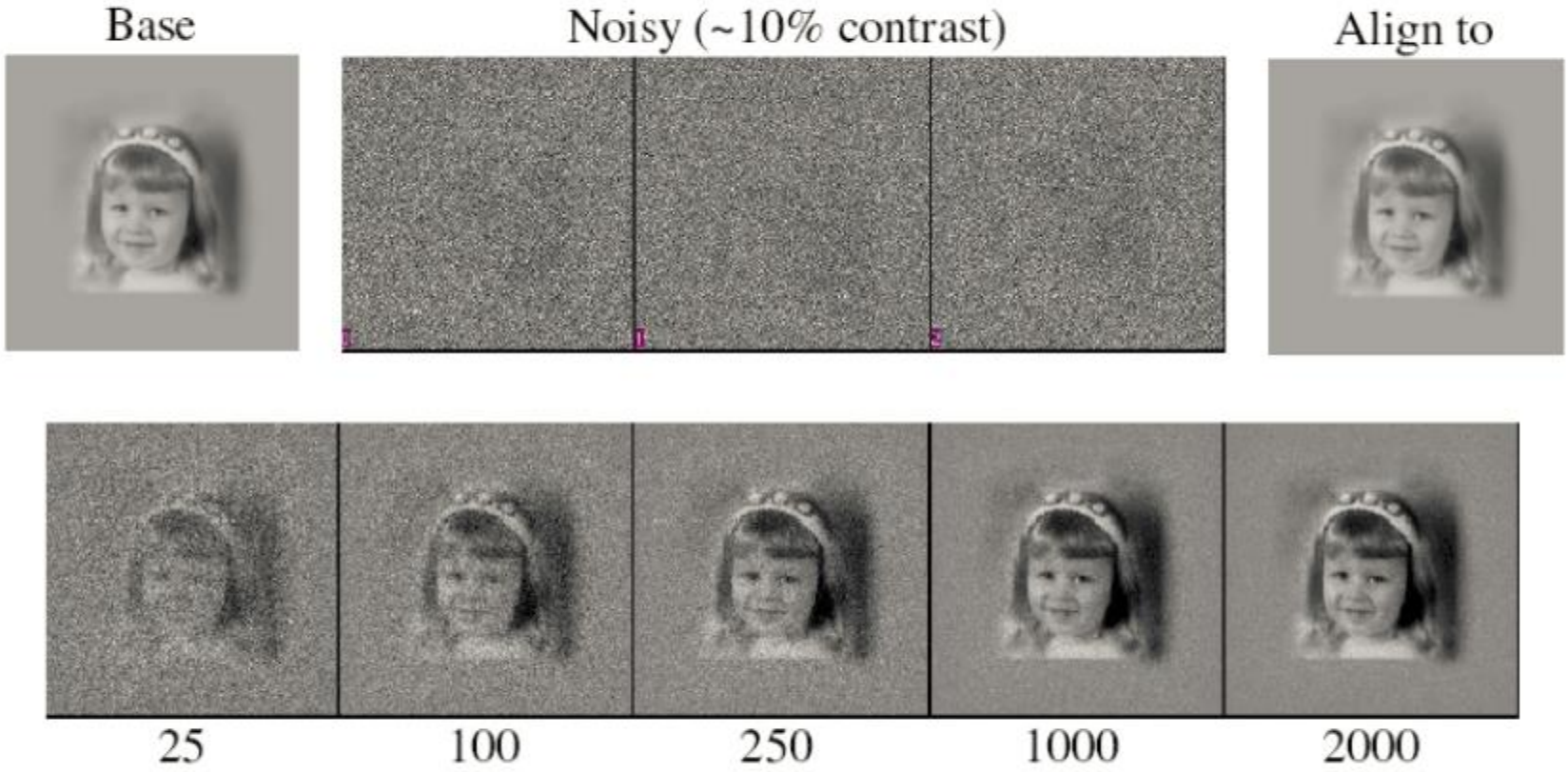
Iterative refinement procedure, using reference model based projection matching:



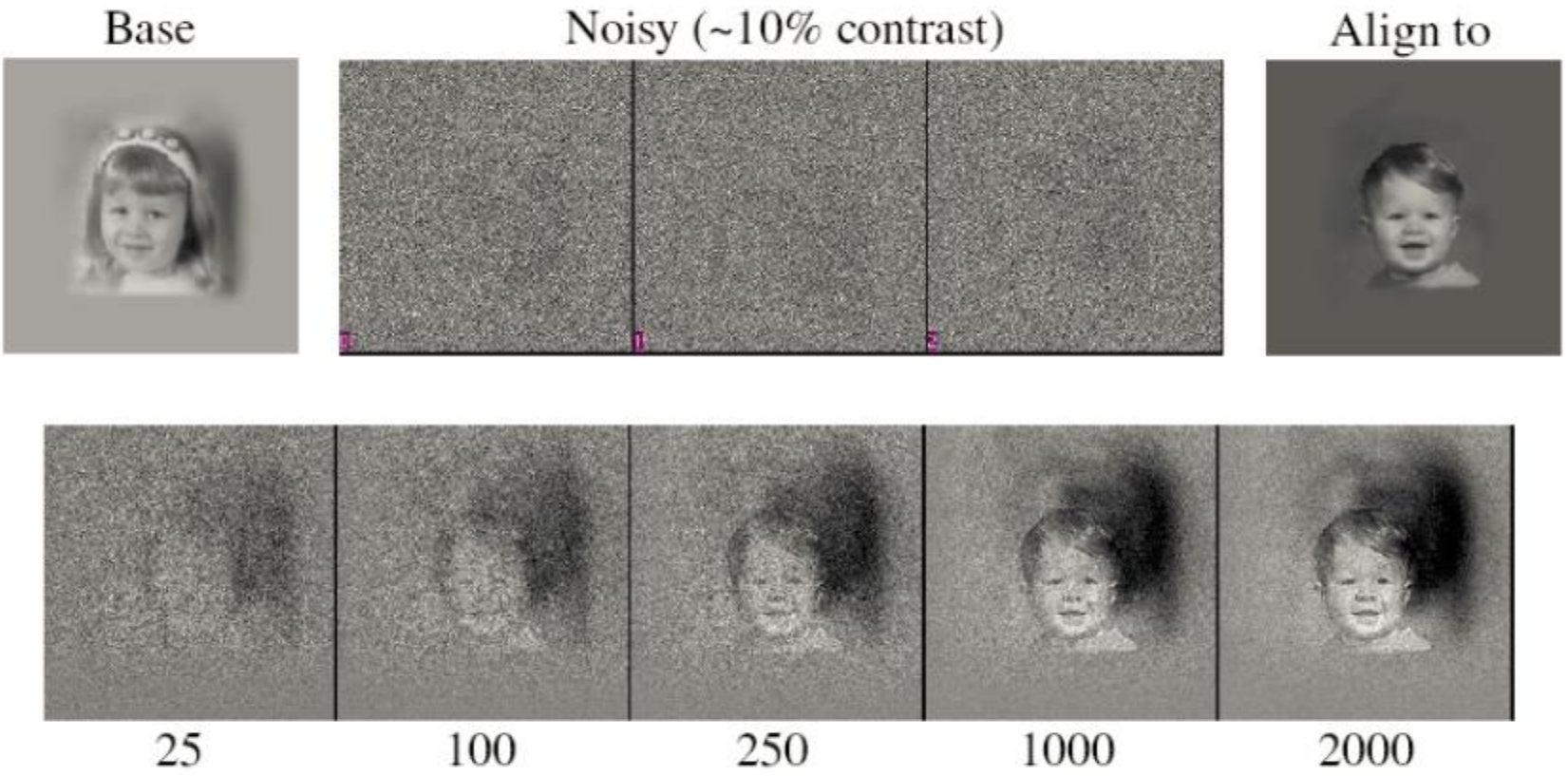
Caveat: Model Bias



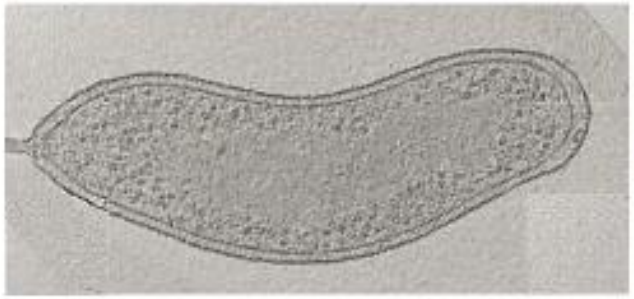
Caveat: Model Bias



Caveat: Model Bias

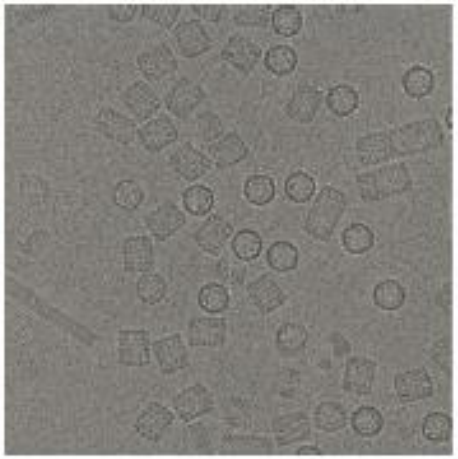


Basic approaches in cryo-EM



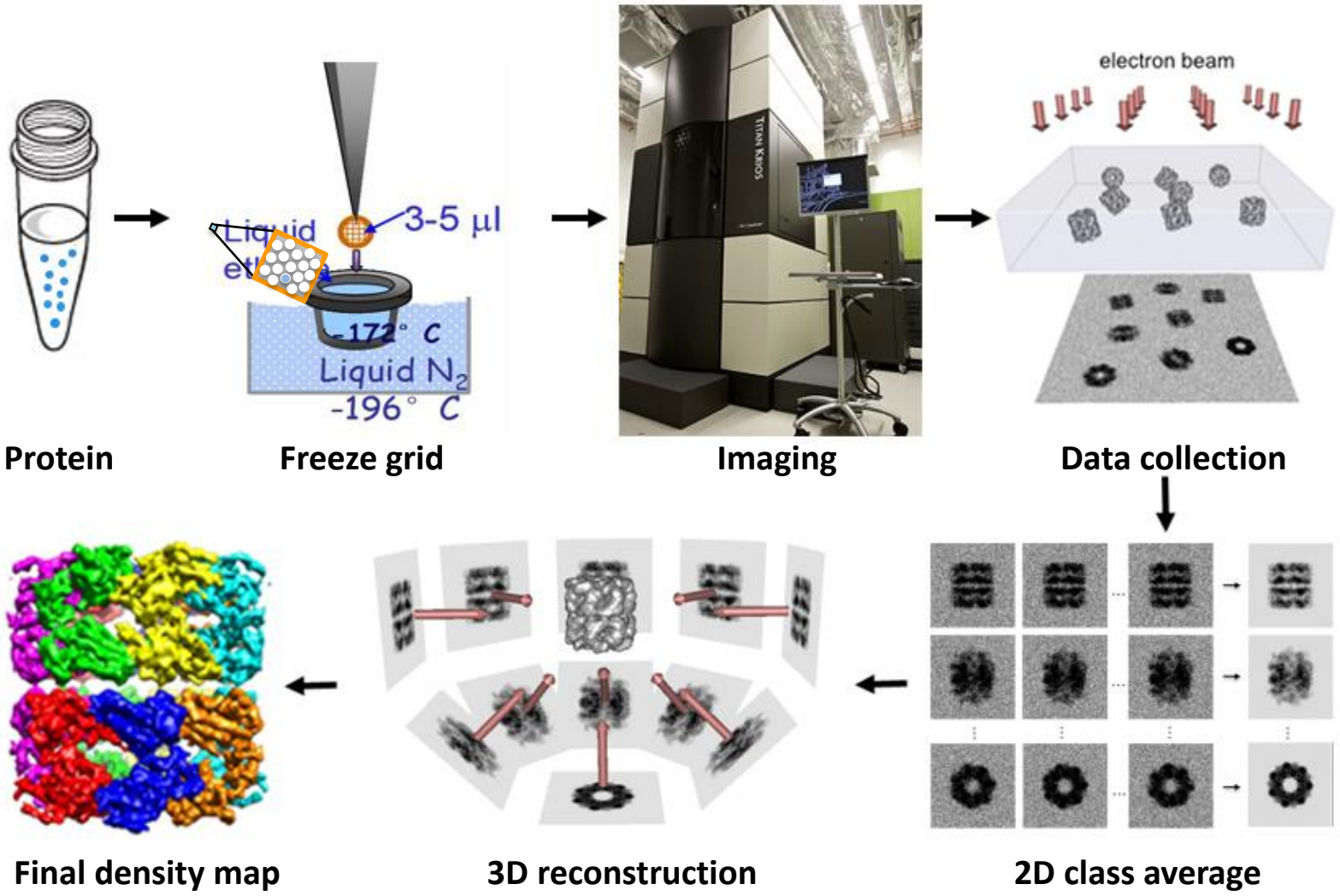
Tomography

Single particle analysis

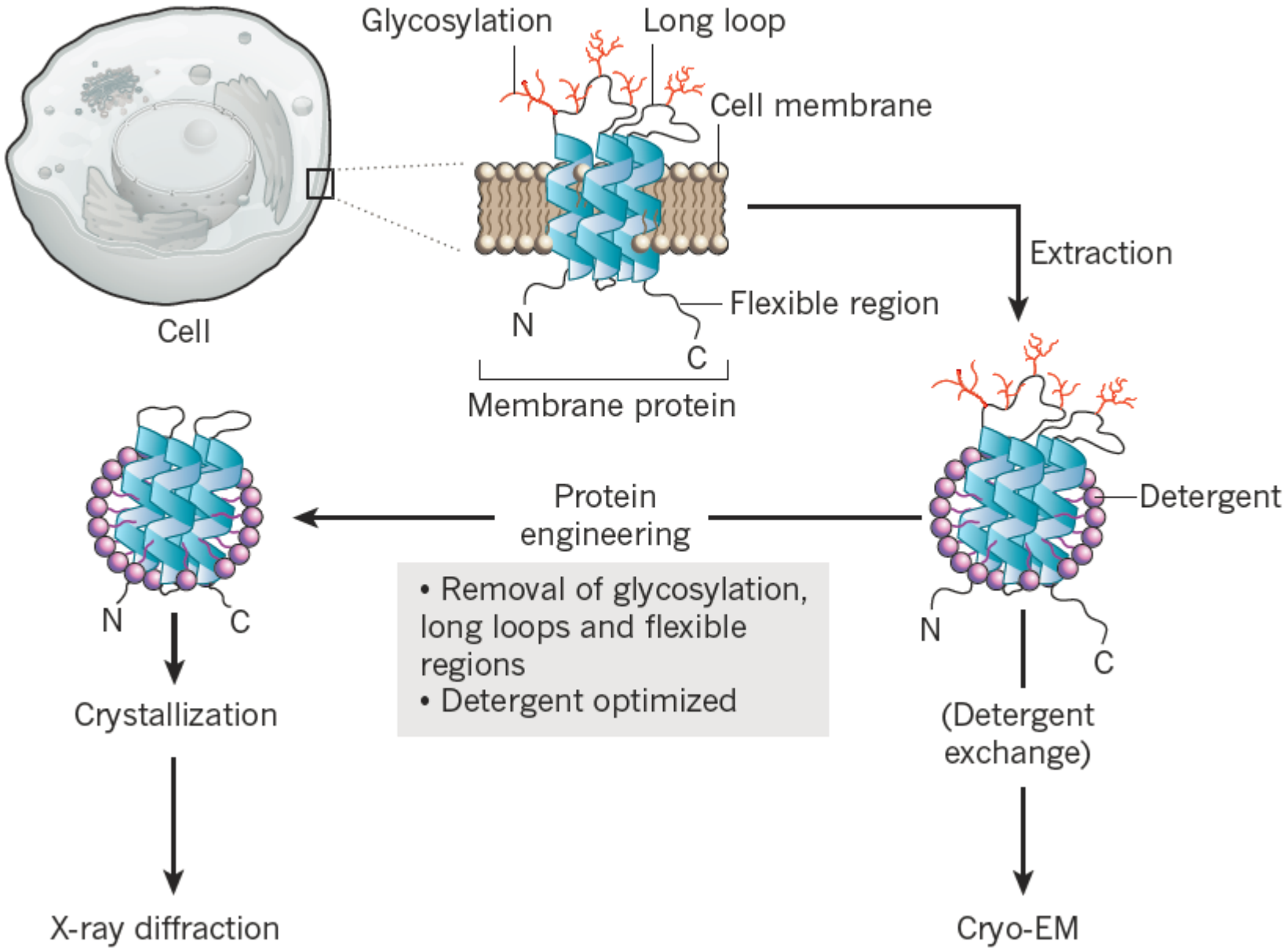


2D crystallography

单颗粒冷冻电镜（Single-Particle Analysis，SPA)

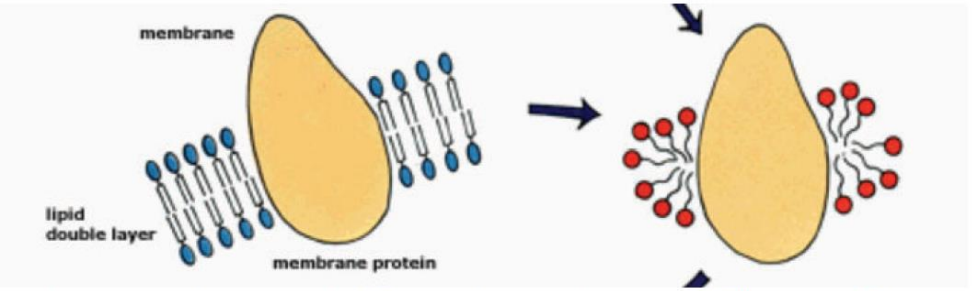


a **Extraction of membrane proteins**



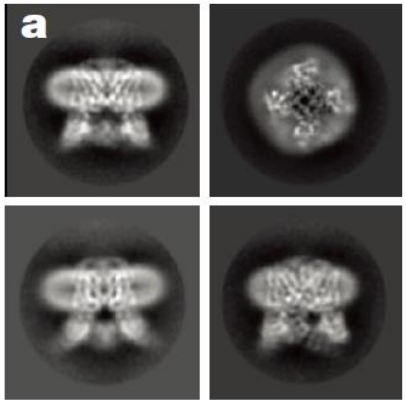
How to stabilize membrane proteins?

1. Detergent solubilization

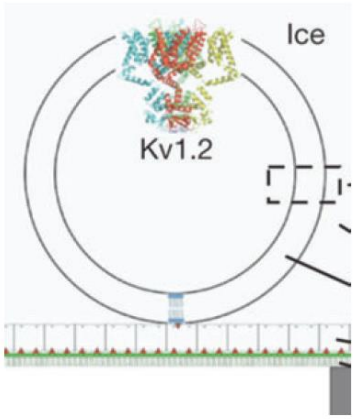


http://www.nobelprize.org/nobel_prizes/chemistry/laureates/1988/illpres/crystals.html

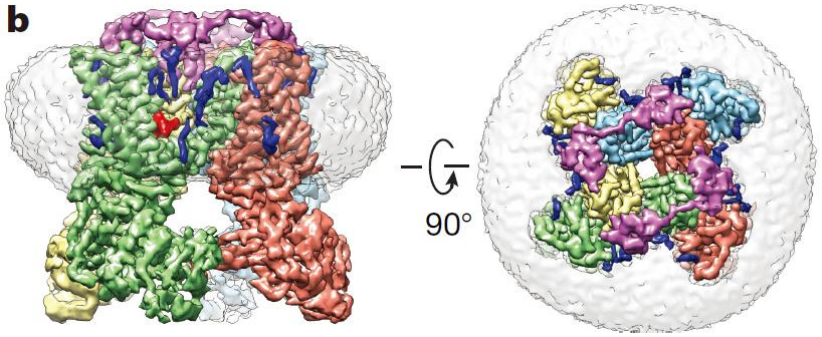
2. Nanodisc



3. Embed in liposomes

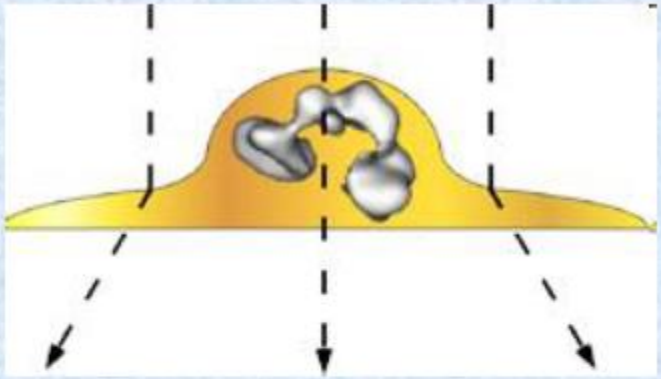


Wang and Sigworth, Nature 2009



Gao et al., Nature 2016

Negative Stain



A sample deposited on a carbon coated grid and surrounded by stain (light gray shading) interacting with an electron beam (arrows).

Phosphotungstate
uranyl actetate/formate
molybdate (ammonium)

Benefits:

Very high contrast..

Radiation damage causes less change in the stain area.

The sample is easy to prepare.

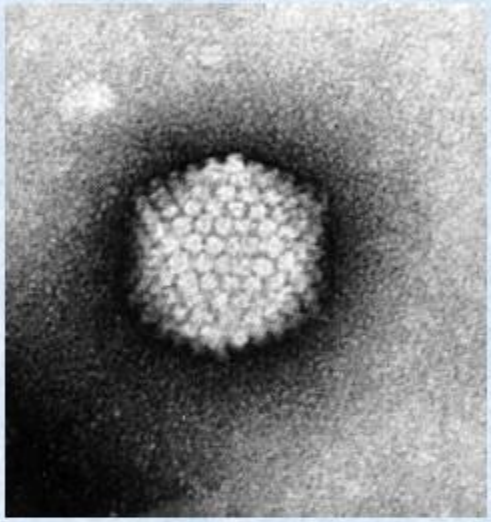
Drawbacks:

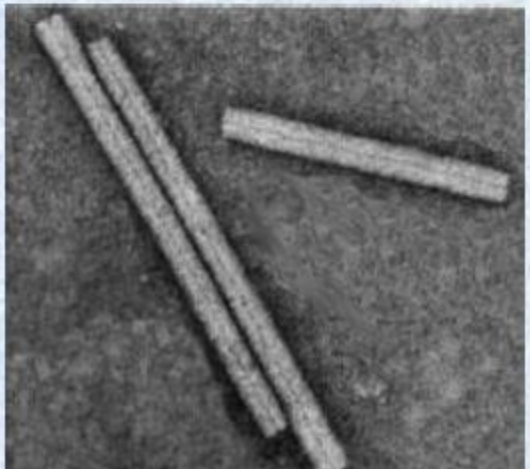
The particle is distorted during the staining process.

Artifacts can arise if the stain is uneven.

The resolution is limited to approximately 20Å.

Negative staining



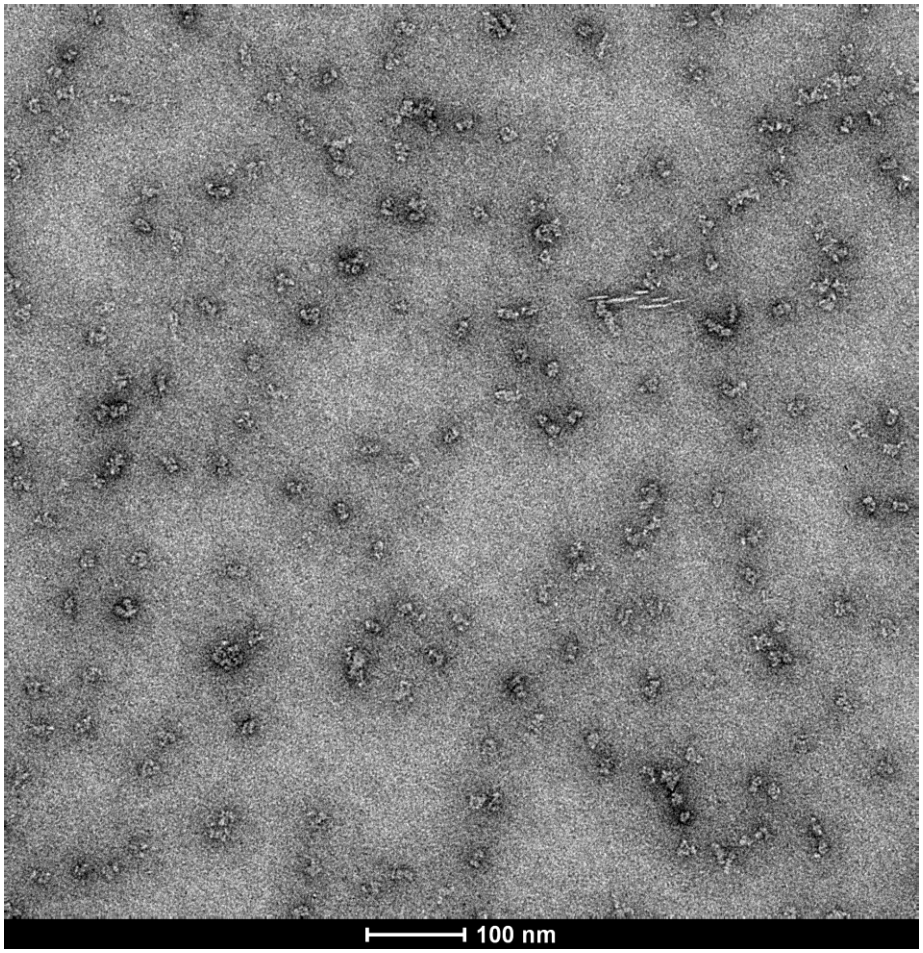


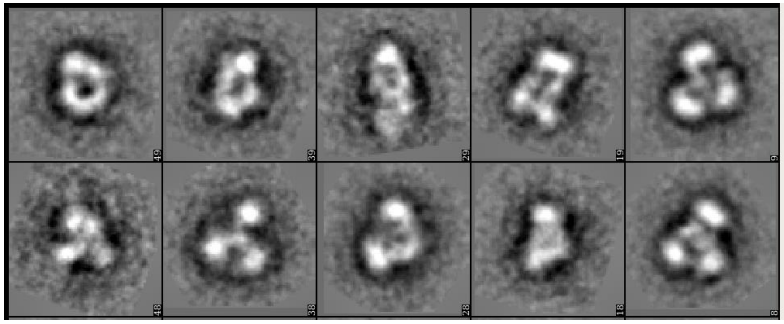
Cryo-EM vs. Negative stain EM

- Negative stain
  - Easy to prepare
  - Good contrast
  - Preservation
  - Sample distortion
  - Resolution limited to about 20 angstroms

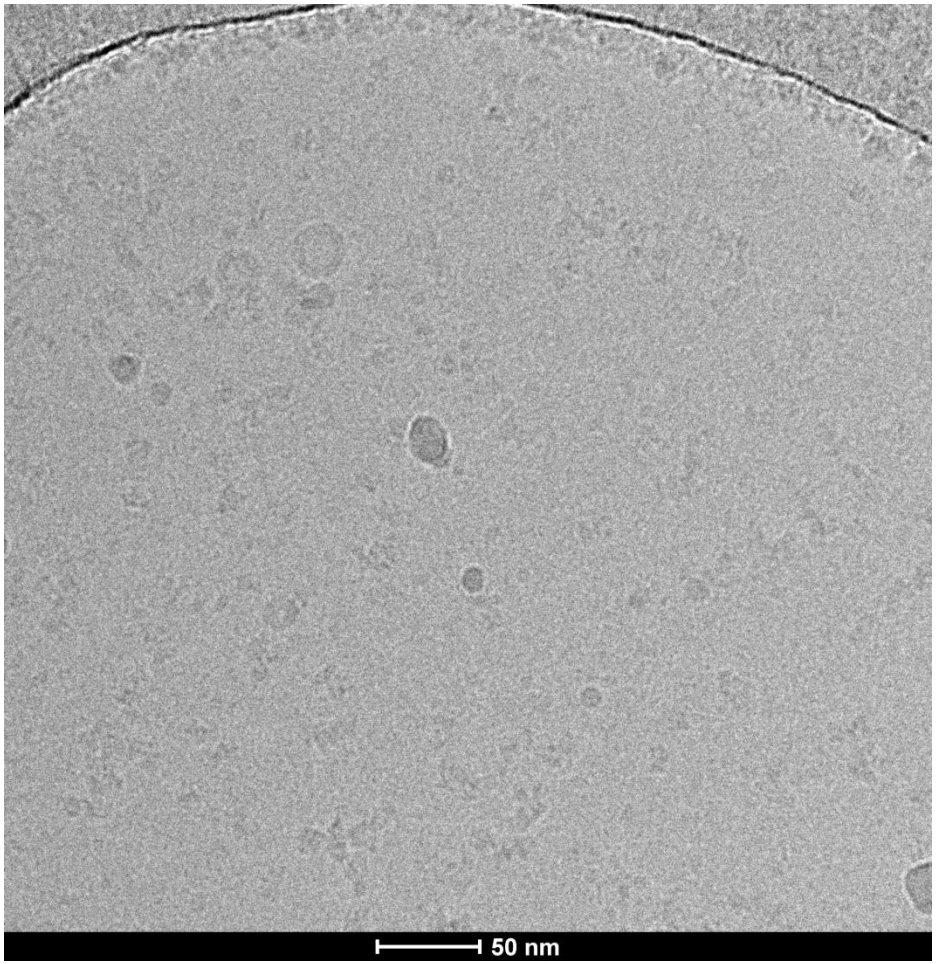
- Cryo
  - Difficult sample prep
  - Low contrast
  - Best preservation and therefore resolution

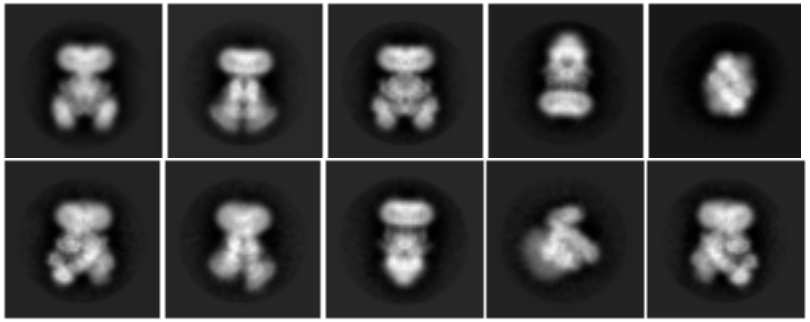
Negative staining





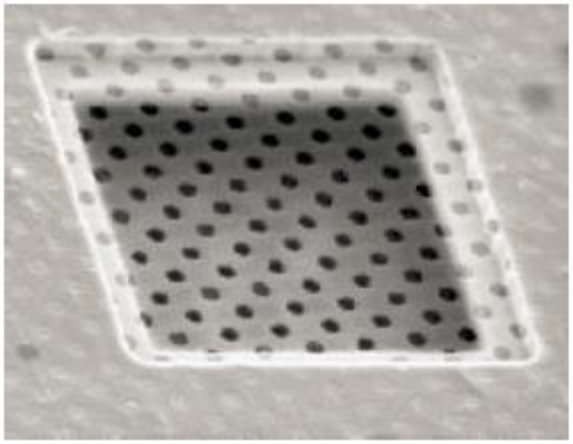
Cryo-EM



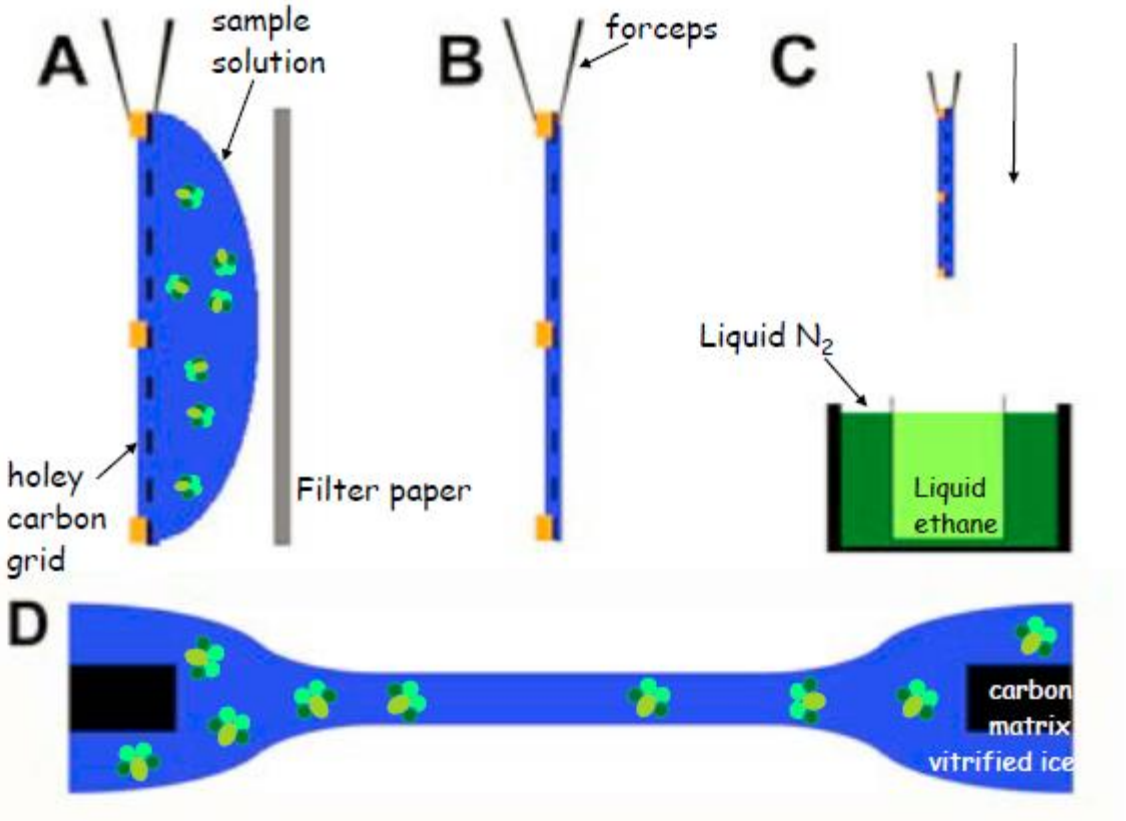


Frozen hydrated specimen preparation

Adrian M, Dubochet J, Lepault J & McDowall AW (1984) Cryo-electron microscopy of viruses. *Nature* **308**, 32-36.

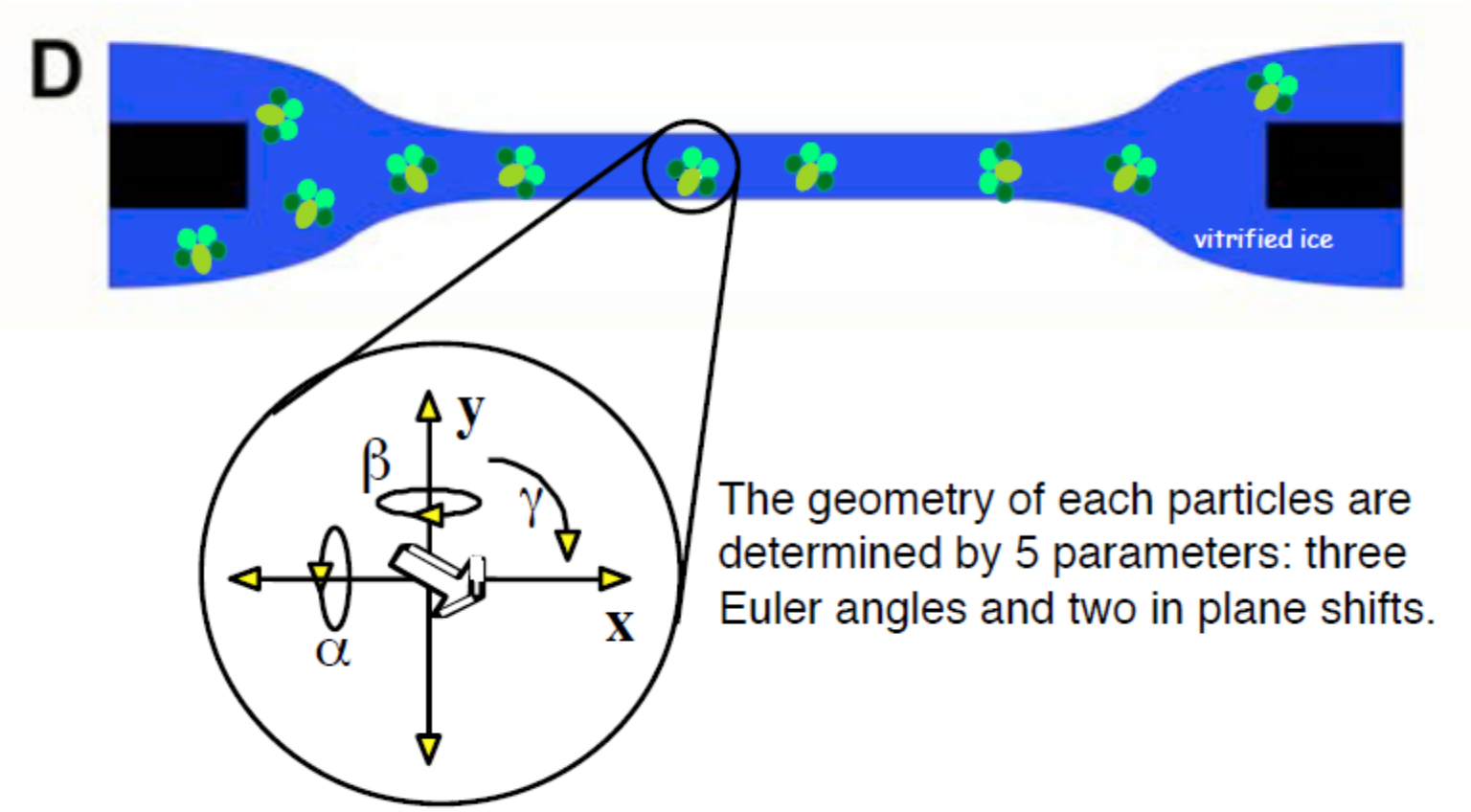


Quantifoil grid



Plunge freezing

Protein molecules embedded in vitrified ice as single particles



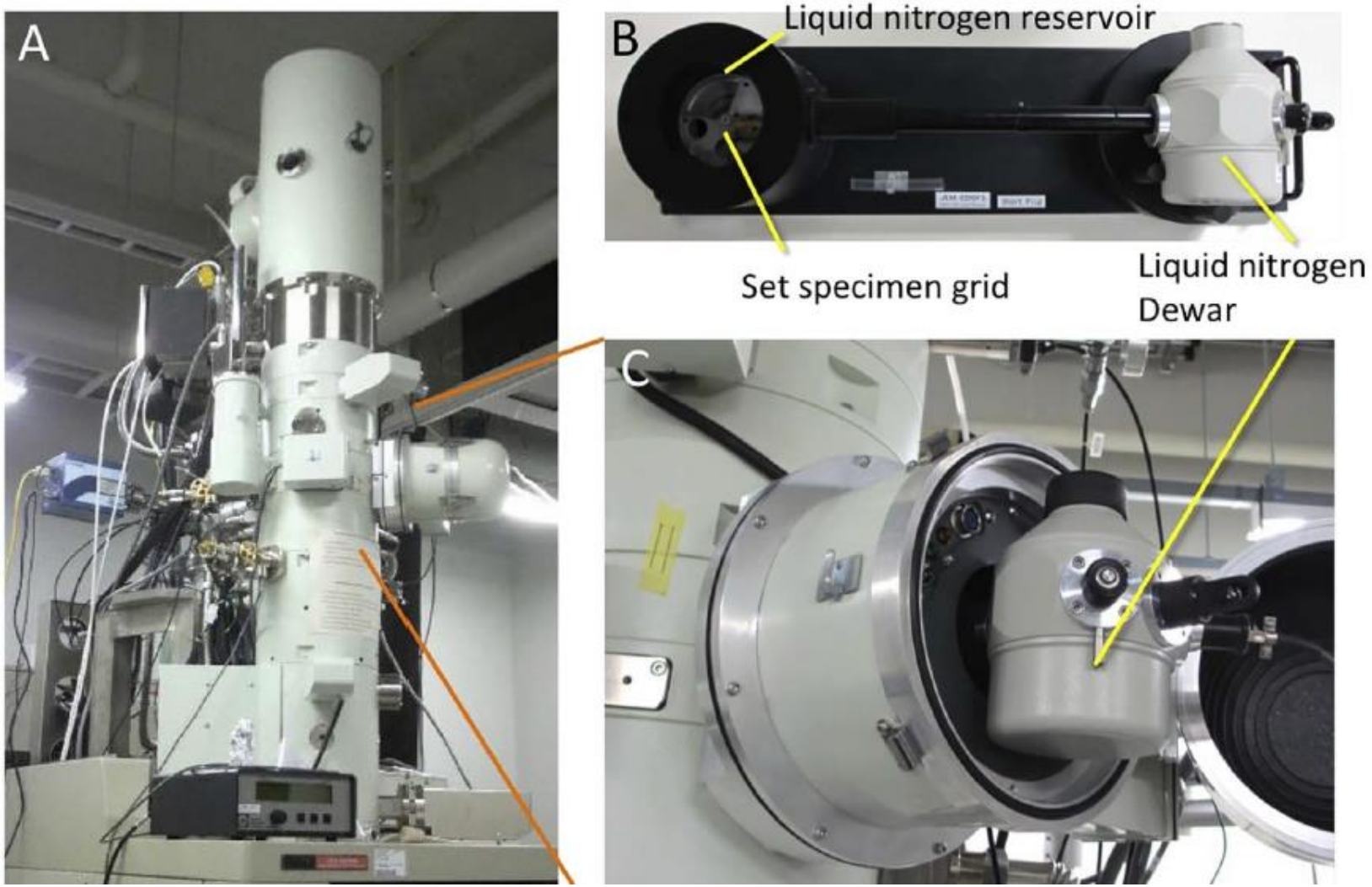
The geometry of each particles are determined by 5 parameters: three Euler angles and two in plane shifts.

Single particles are randomly oriented in vitreous ice

Equipment for cryo-electron microscopy

Cryo specimen holder

- cryo plunger for rapid freezing;
- cryo-holder and cryo-transfer station;



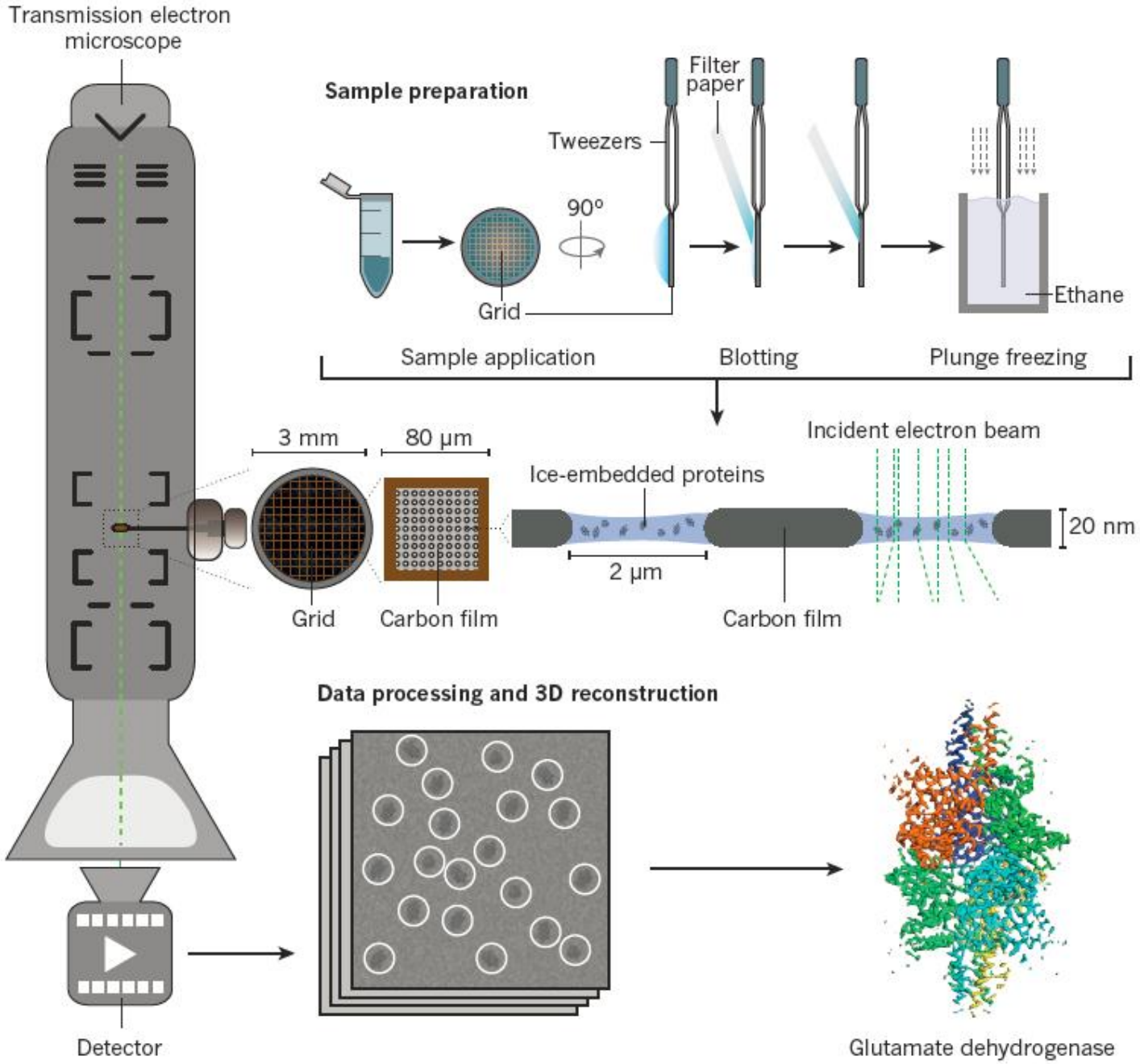

Transmission electron microscope
Sample preparation
Filter paper
Tweezers
90°
Grid
Ethane
Sample application
Blotting
Plunge freezing
3 mm
80 µm
Incident electron beam
Ice-embedded proteins
20 nm
2 µm
Grid
Carbon film
Carbon film
Data processing and 3D reconstruction
Detector
Glutamate dehydrogenase

Image recording

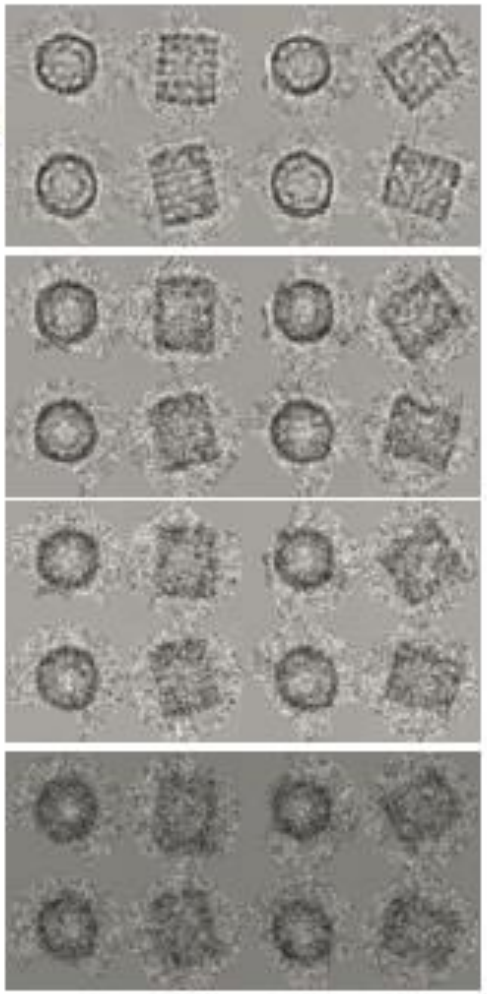
For native samples, the ultimate resolution limit is radiation damage

10 or 20 e^-/A^2

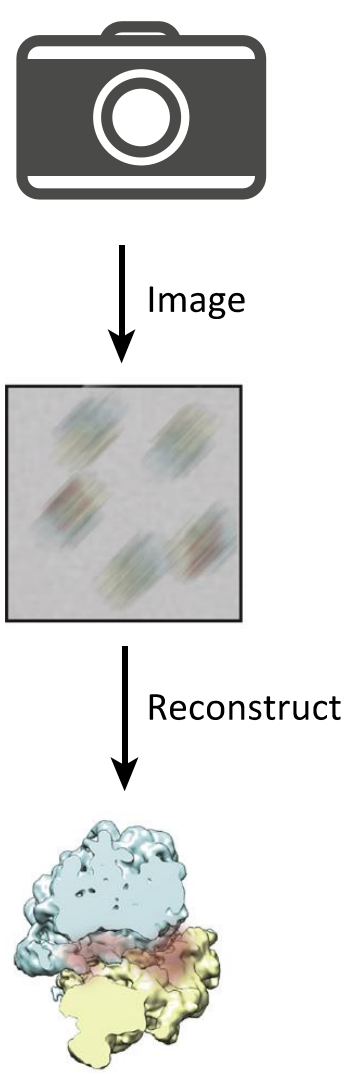
120 e^-/A^2

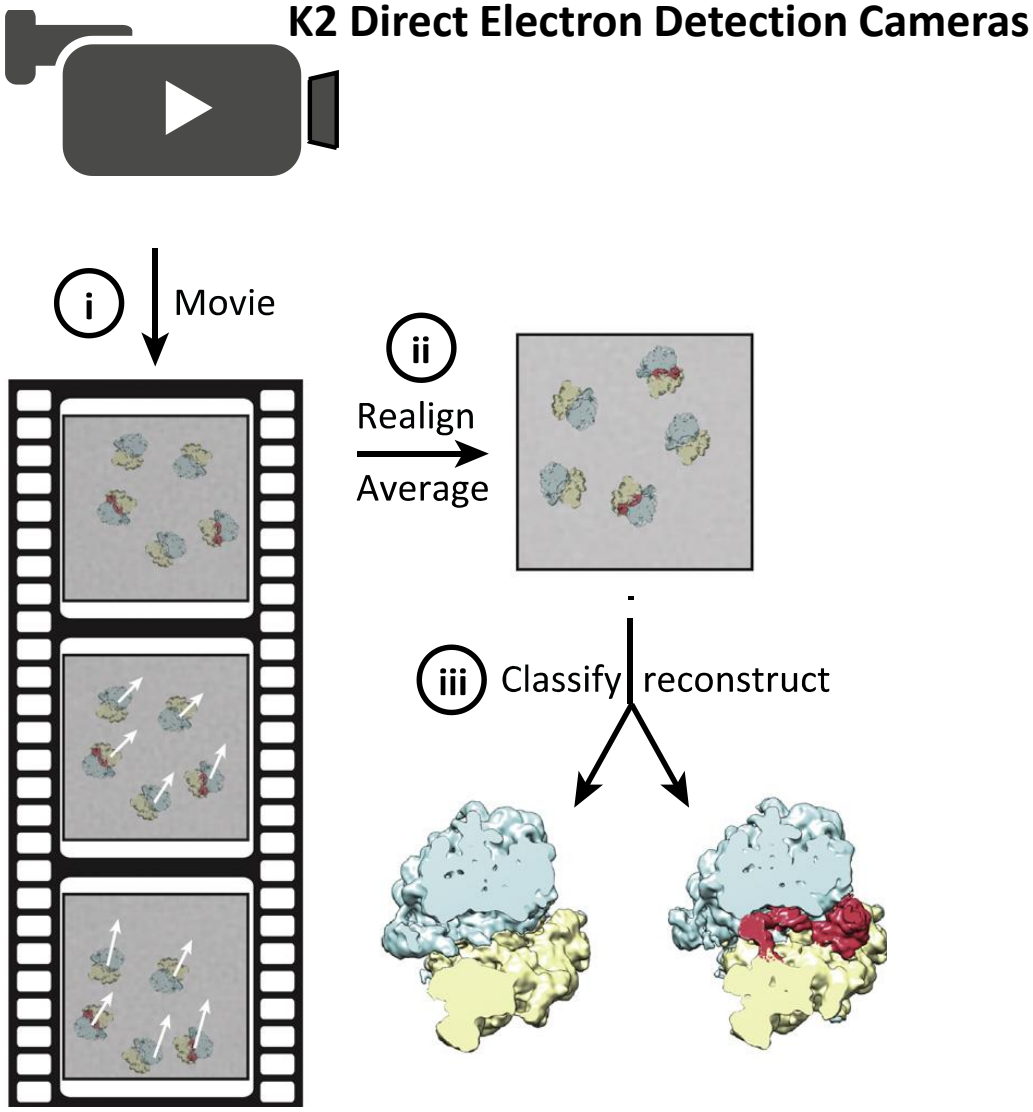
200 e^-/A^2

350 e^-/A^2

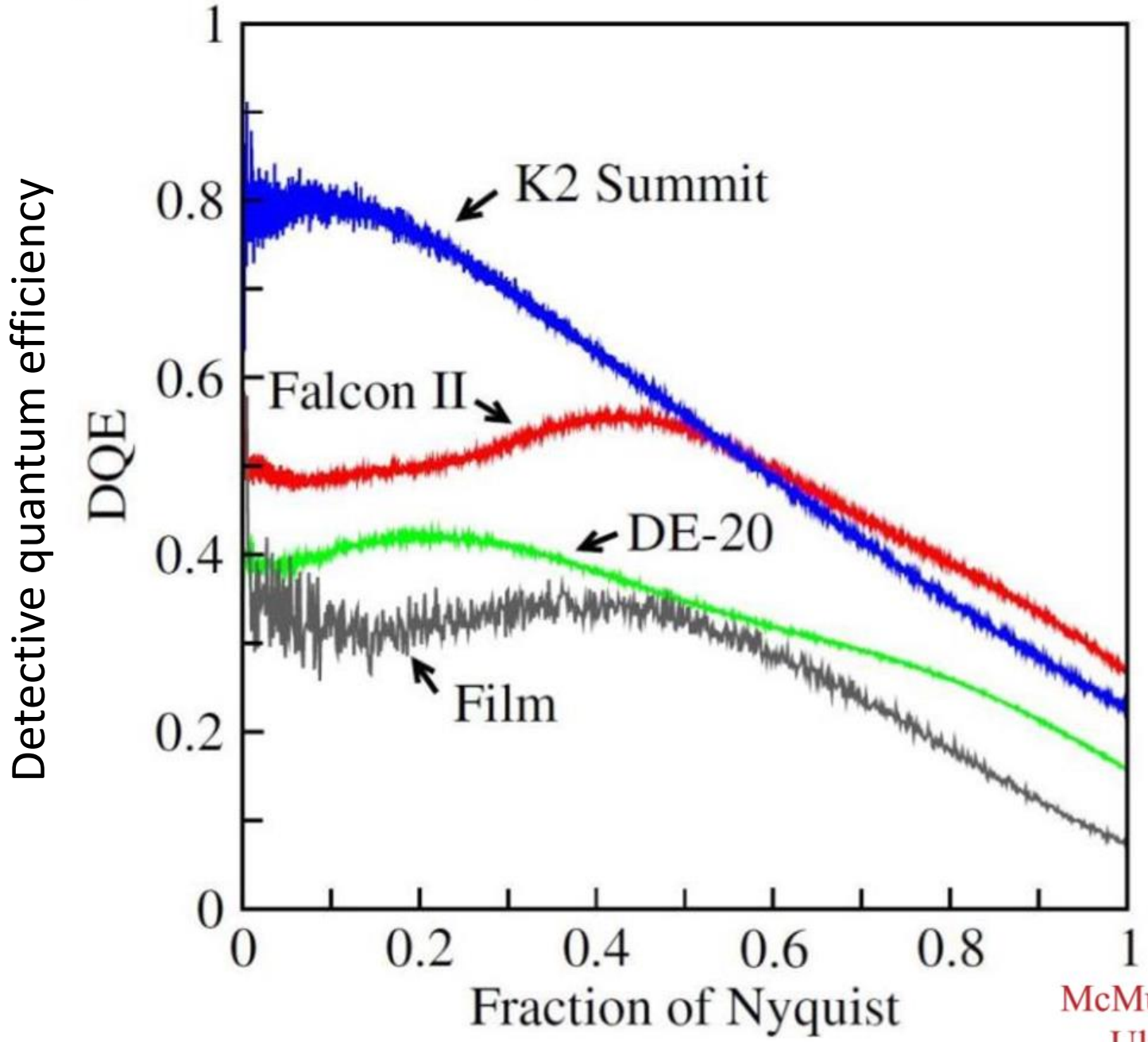


Direct electron detector





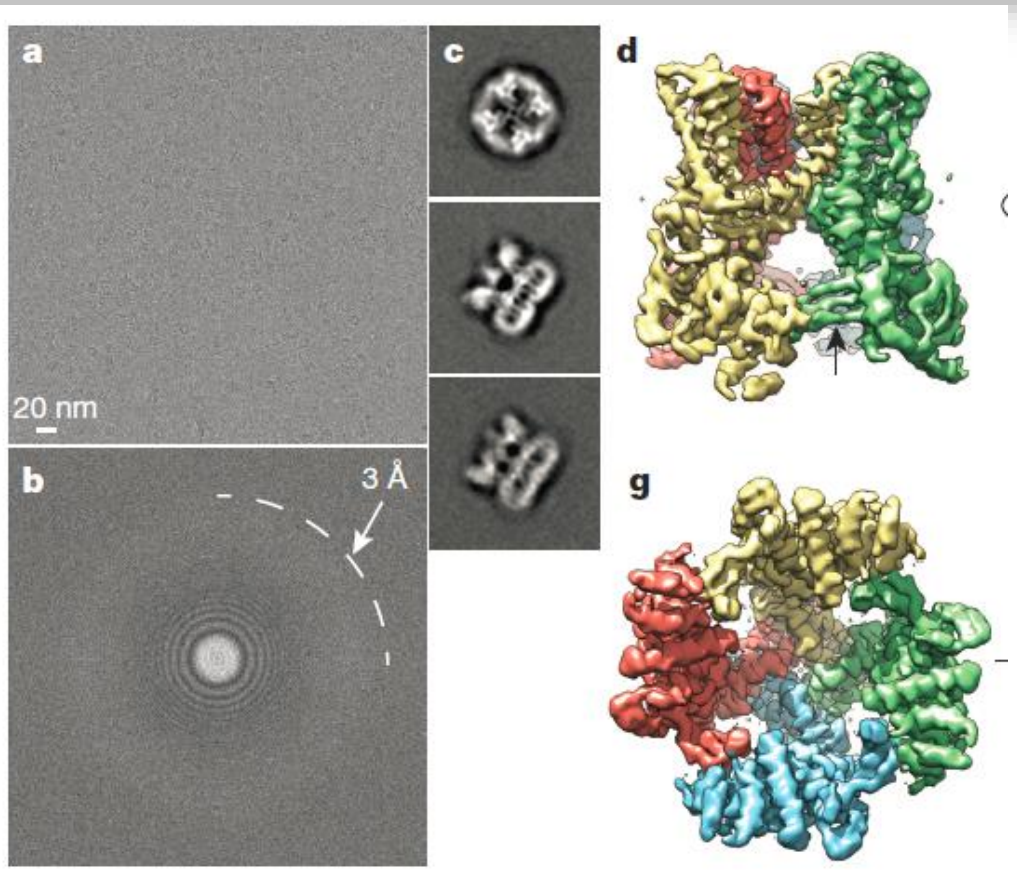
Comparison of 300keV DQE of direct electron detectors versus film



McMullan et al (2014)
Ultramicroscopy

The Resolution Revolution

Yifan Cheng, UCSF Professor,
HHMI Investigator



ARTICLE

doi:10.1038/nature12822

Structure of the TRPV1 ion channel determined by electron cryo-microscopy

Maofu Liao[1]*, Erhu Cao[2]*, David Julius[2] & Yifan Cheng[1]

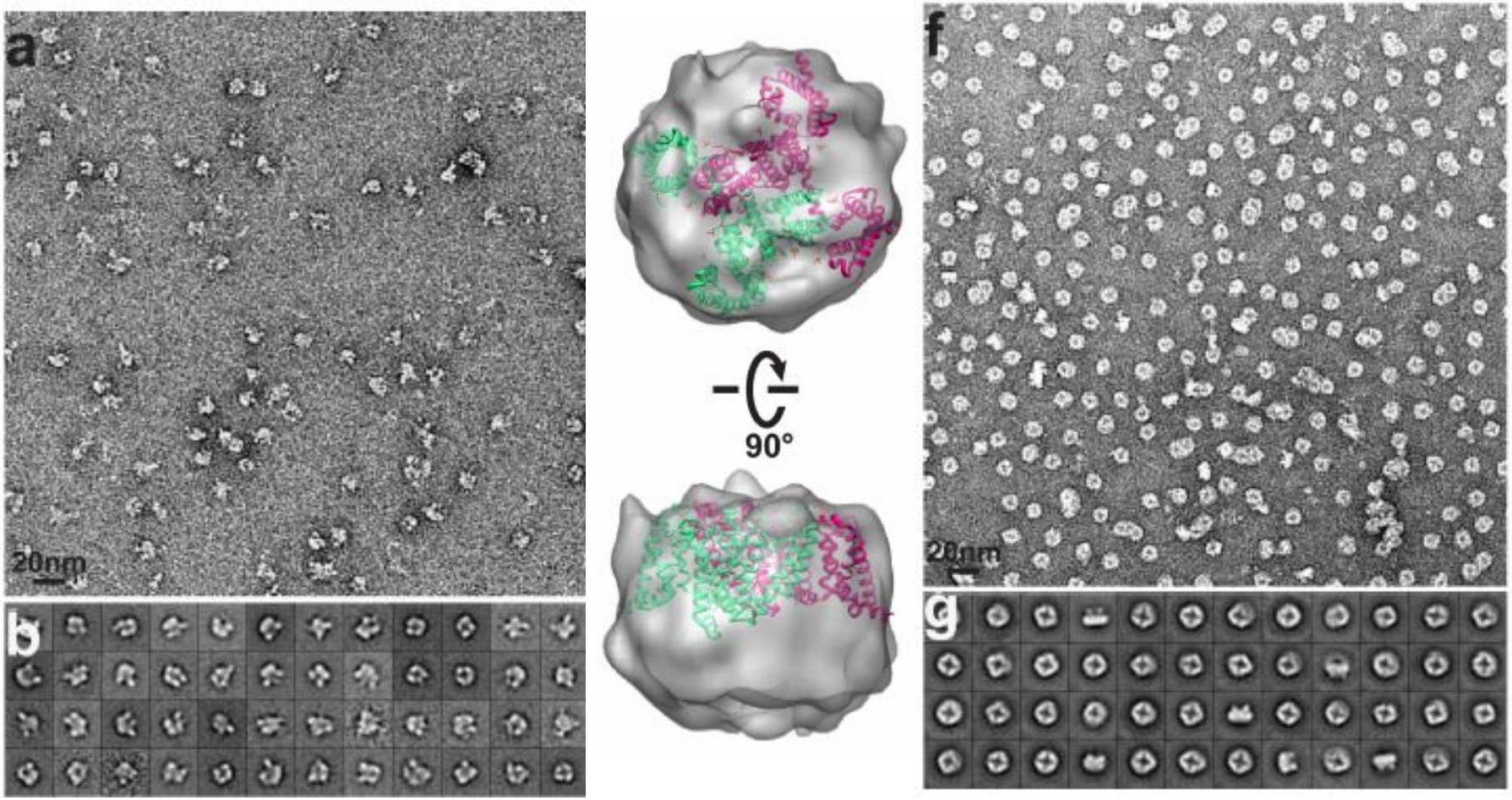
ARTICLE

doi:10.1038/nature12823

TRPV1 structures in distinct conformations reveal activation mechanisms

Erhu Cao[1]*, Maofu Liao[2]*, Yifan Cheng[2] & David Julius[1]

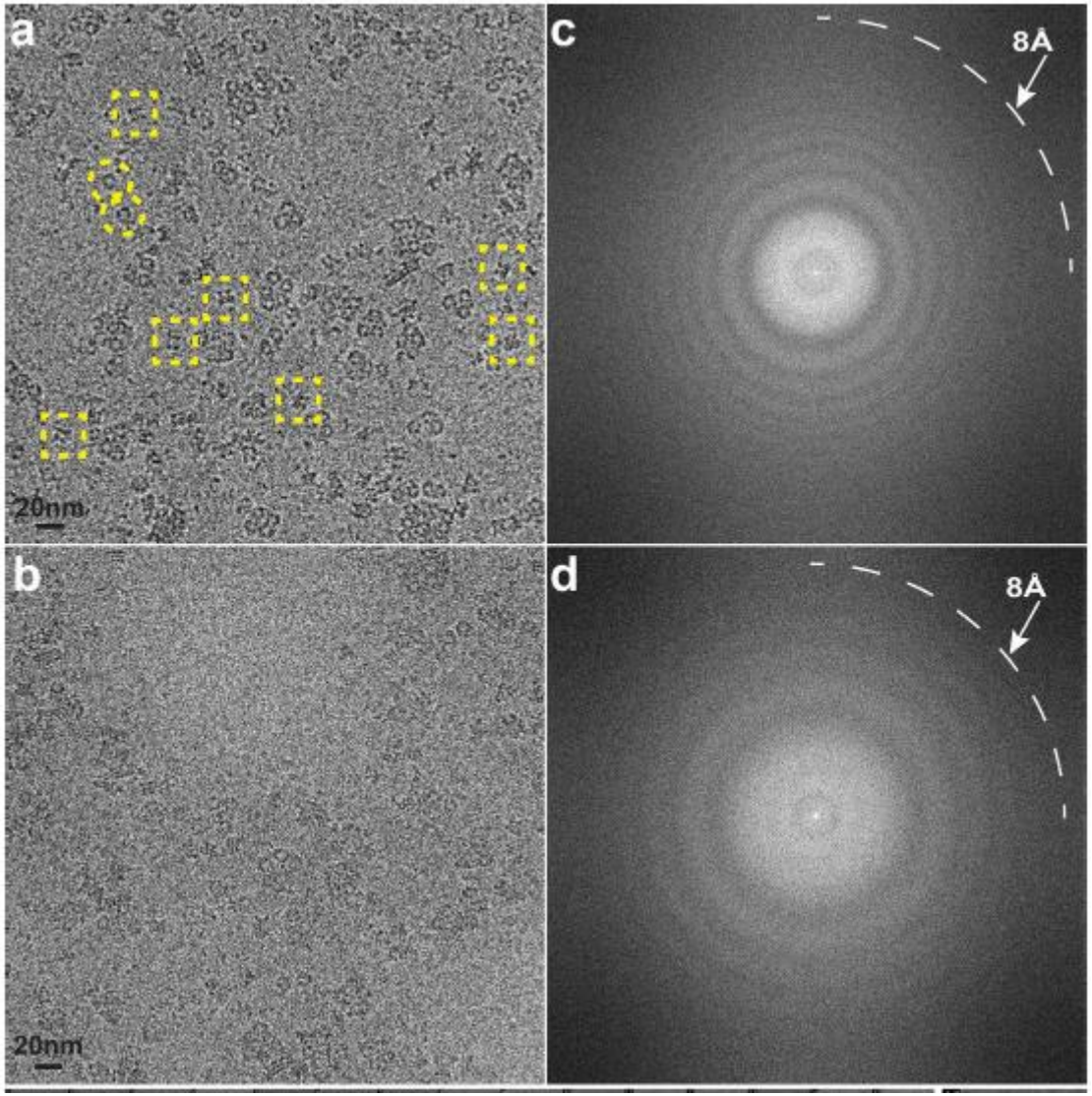
Negative-stain EM of TRPV1

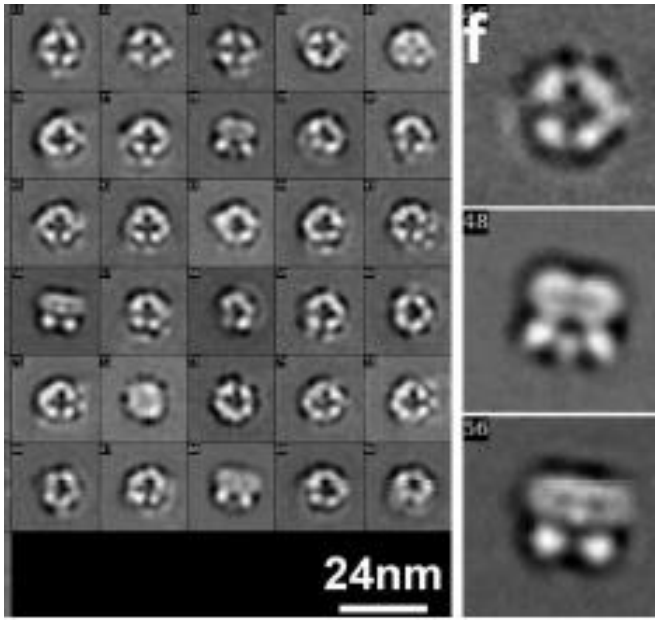


Negatively stained TRPV1 inDDM

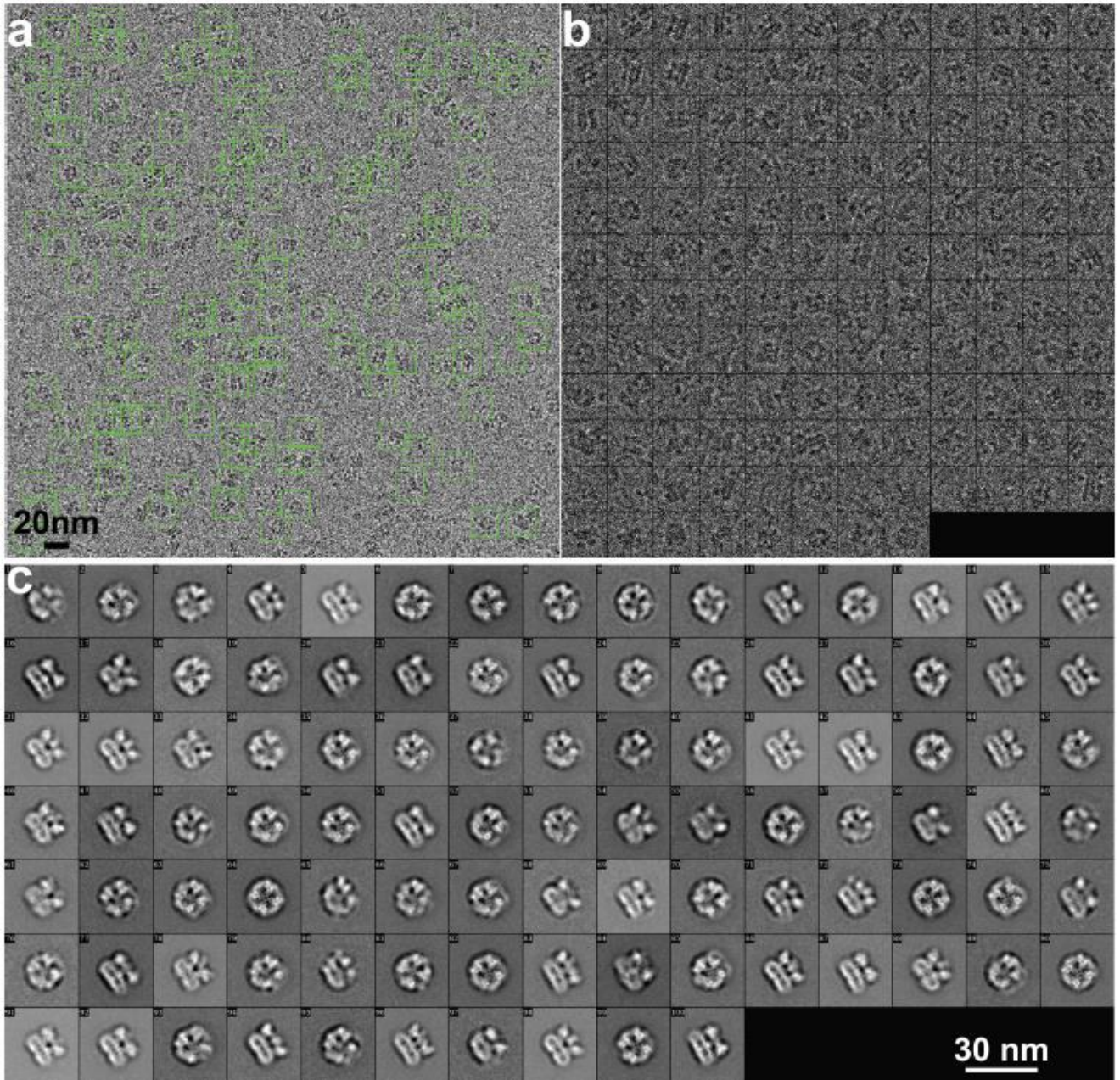
in amphipols

Cryo-EM of TRPV1 using Tecnai TF20 microscope and TemF816 8kX8k CMOS camera

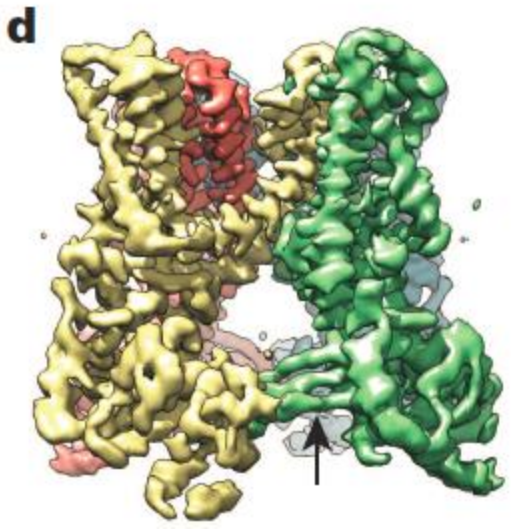


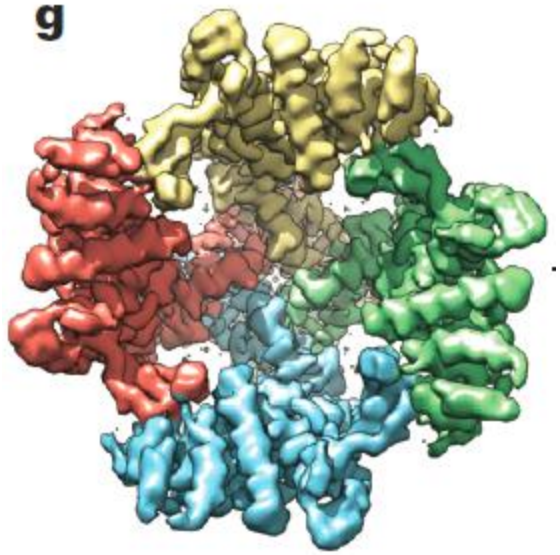


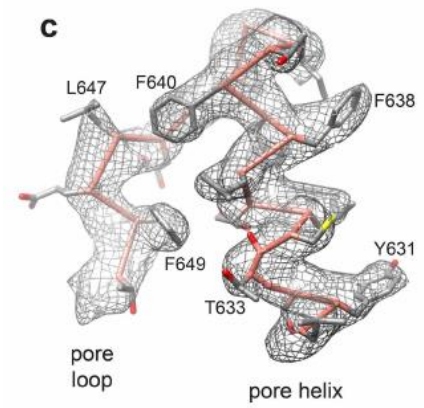
Picking and 2D classification of TRPV1 Cryo-EM particles collected on Polora TF30 microscope

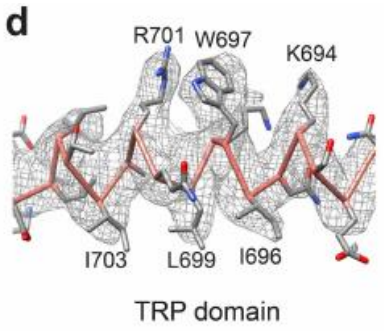


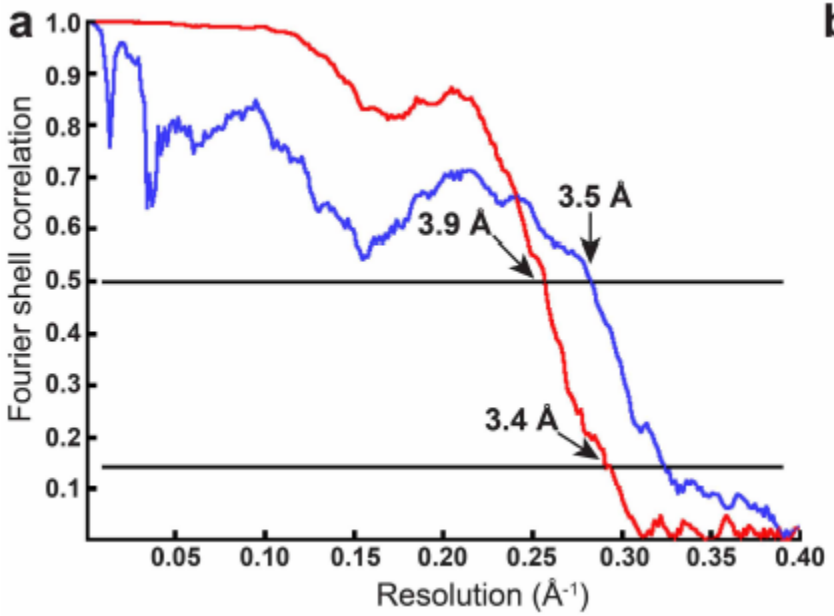
3D reconstruction of TRPV1 calculated from TF30 data.

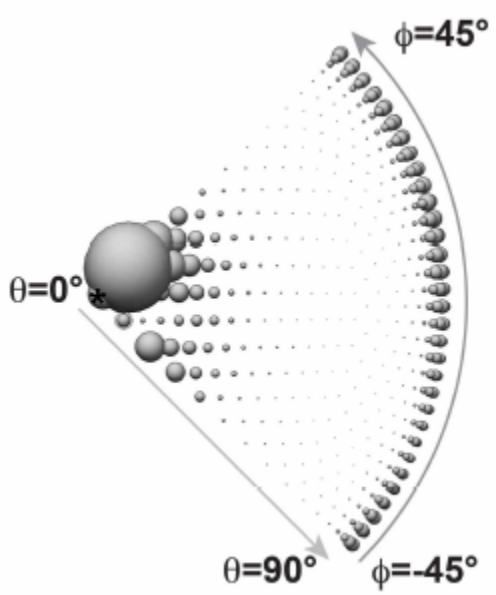










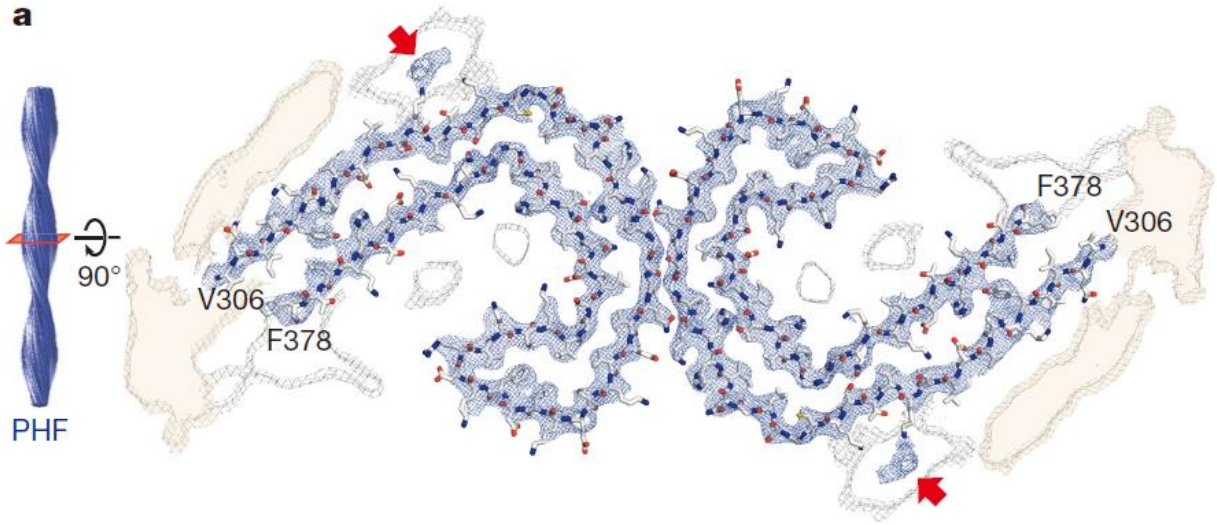


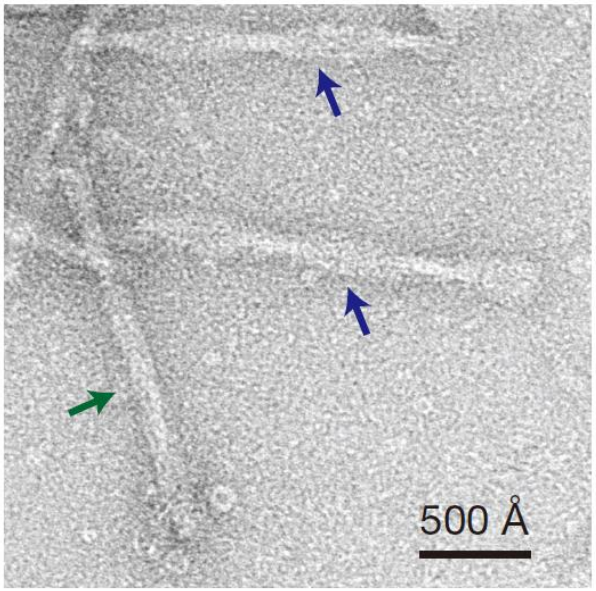
ARTICLE

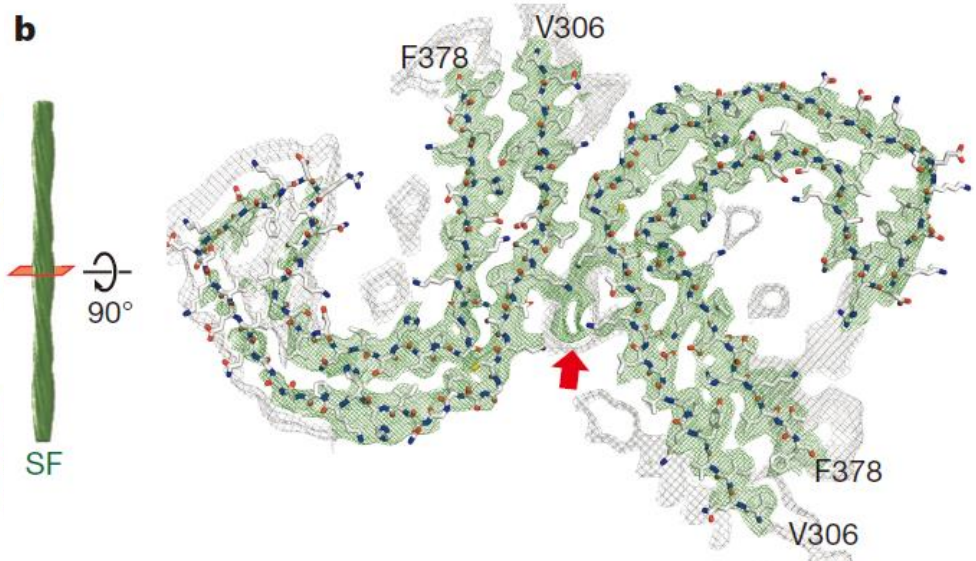
doi:10.1038/nature23002

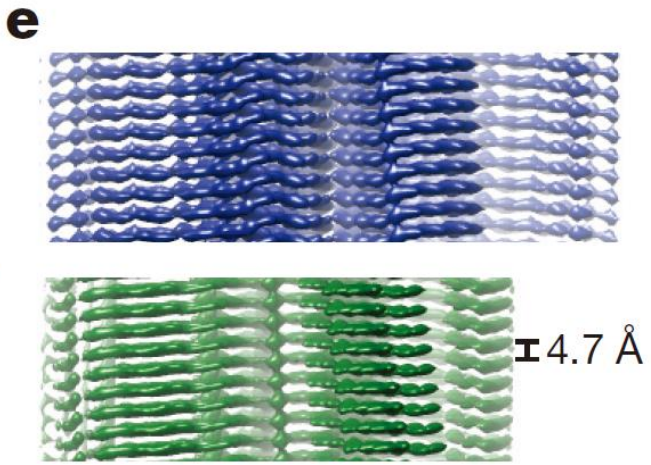
Cryo–EM structures of tau filaments from Alzheimer's disease

Anthony W. P. Fitzpatrick[1], Benjamin Falcon[1], Shaoda He[1], Alexey G. Murzin[1], Garib Murshudov[1], Holly J. Garringer[2], R. Anthony Crowther[1], Bernardino Ghetti[2], Michel Goedert[1]§ & Sjors H. W. Scheres[1]§









Single particle analysis

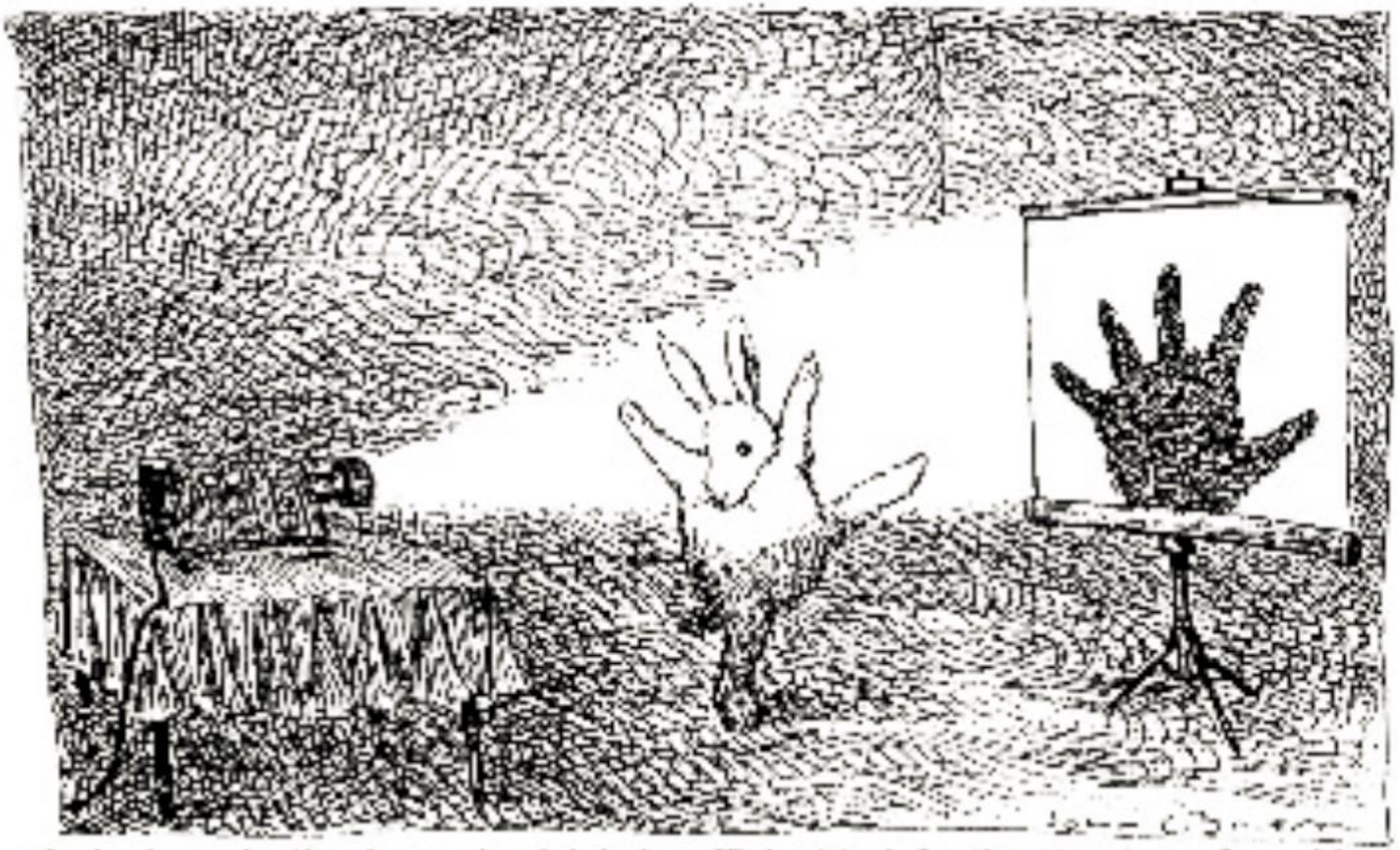
Advantages of single particle analysis

- Does not require crystals
- Samples can be partially inhomogeneous
- Physiological conditions possible
- Requires small amount of sample
- Rapid - many steps automated
- May eventually even be possible in vivo

Limitations

- Radiation damage
- Precision of image alignment
- Numbers of particles averaged
- Conformational heterogeneity
- Orientational preferences

An image is the projection of a 3D object

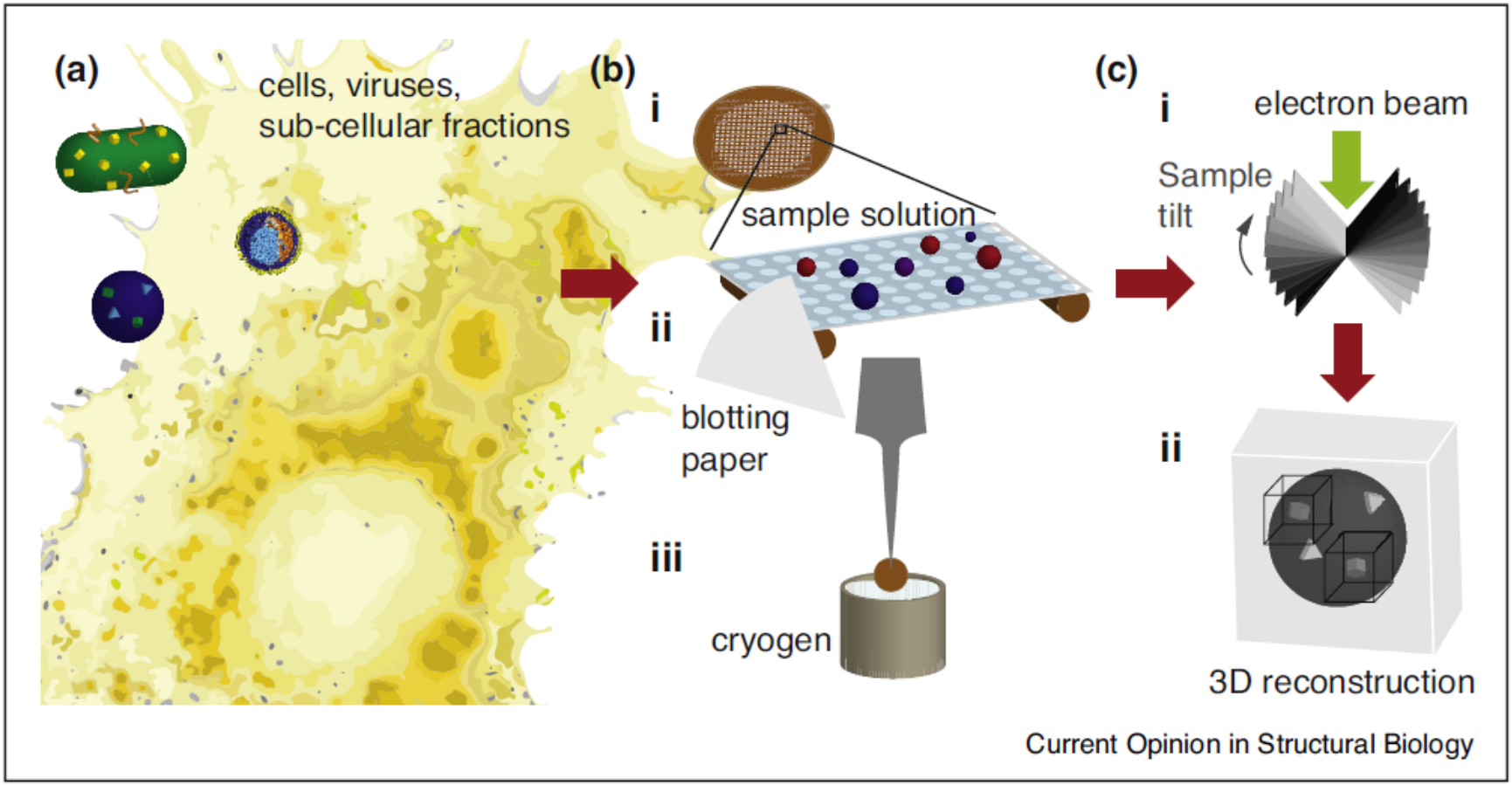


A single projection image is plainly insufficient to infer the structure of an object.

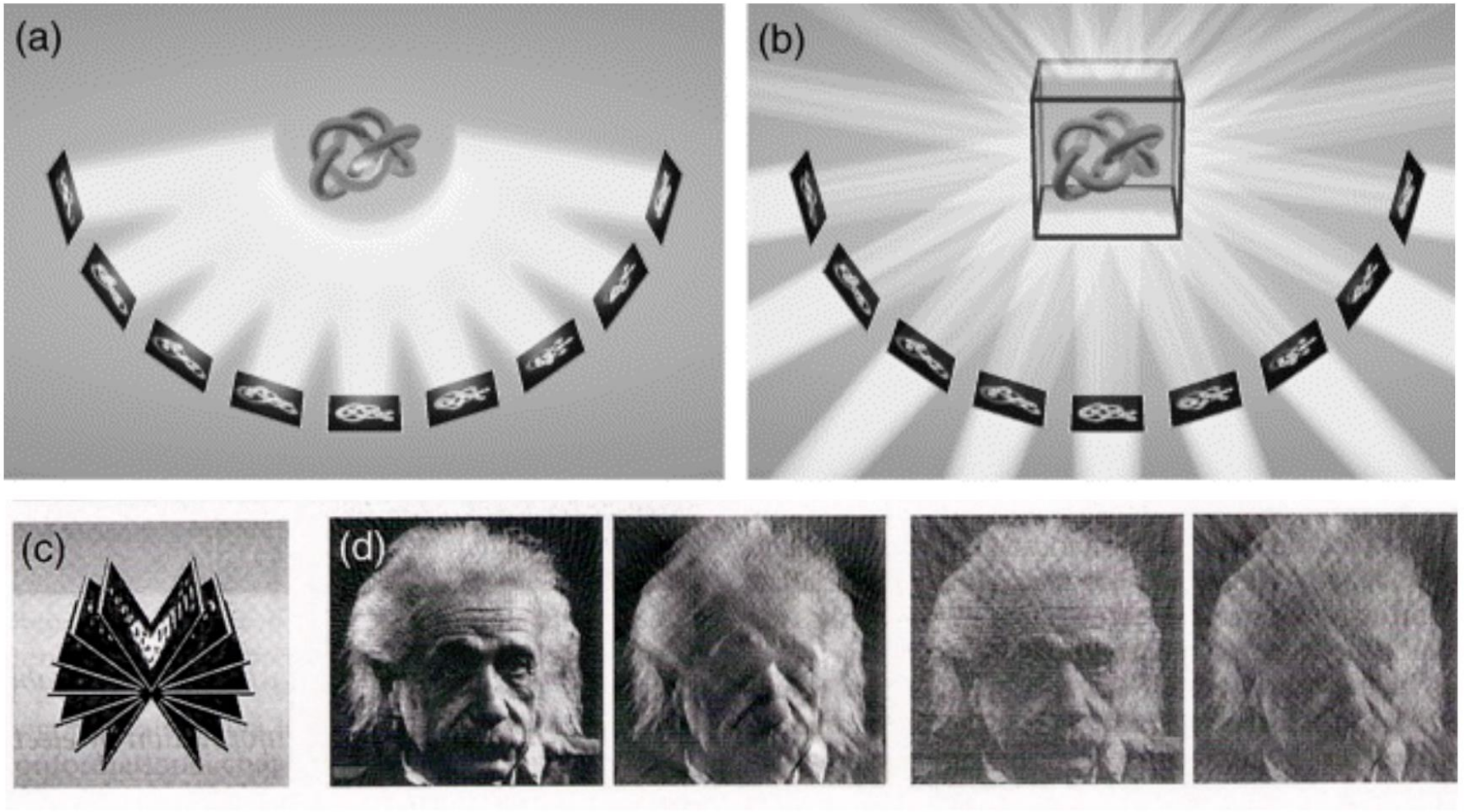
John O'Brien; © 1991 The New Yorker Magazine

Cryo-electron tomography (cryo-ET)

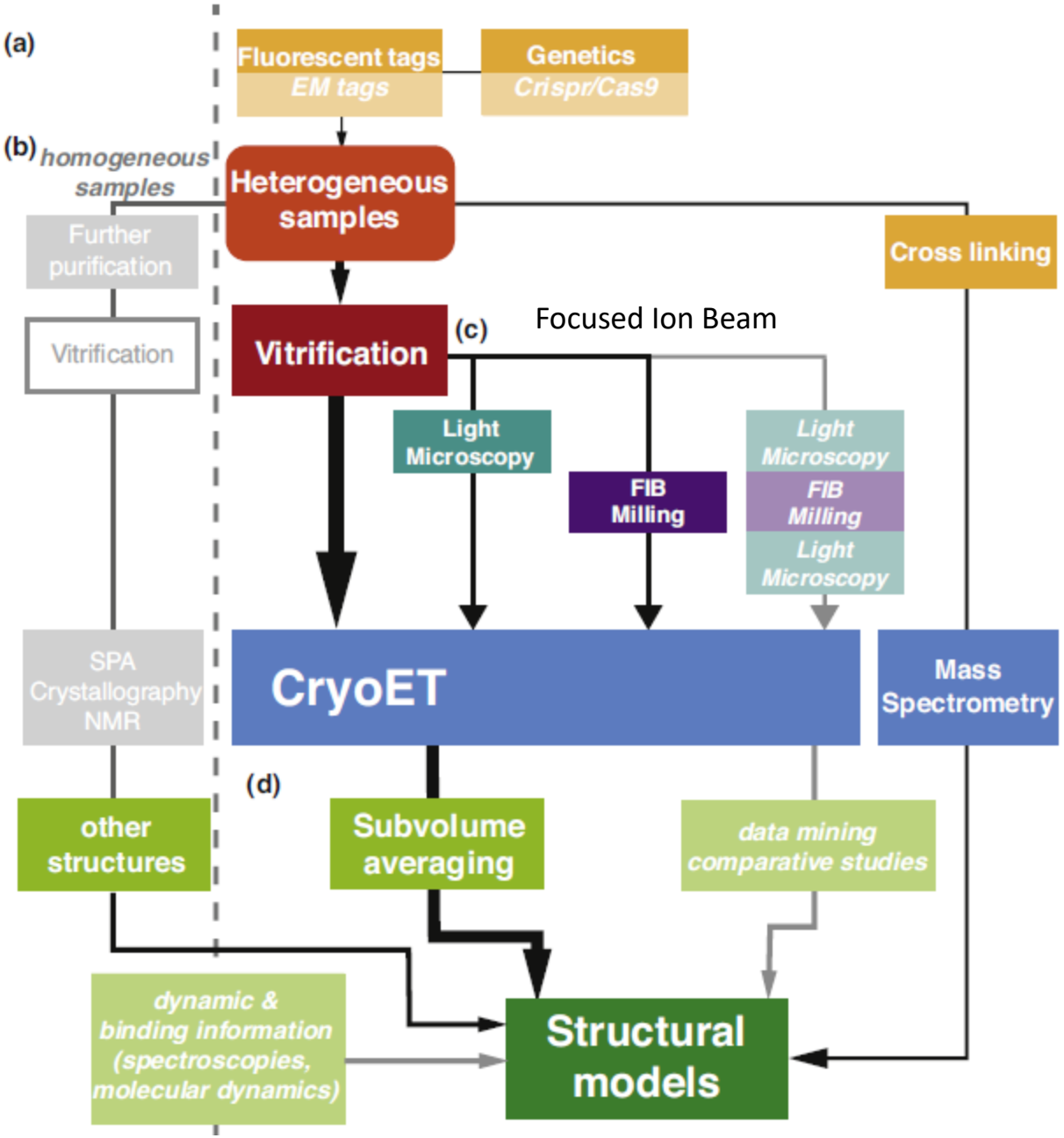
is a three-dimensional imaging technique that makes it possible to analyse the structure of complex and dynamic biological assemblies in their native conditions.



Effects of tilt increment and missing wedge

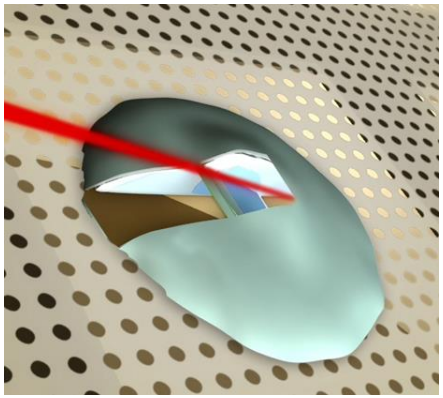


Baumeister et al., *Trends in Cell Biology* **9**:81

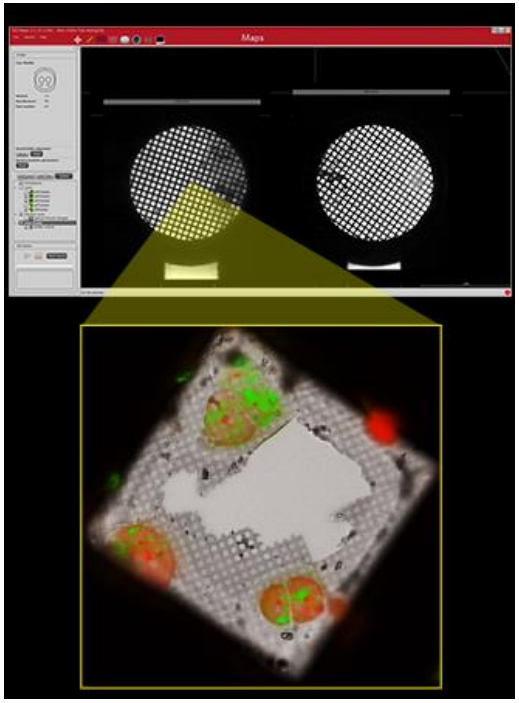

(a)
Fluorescent tags
EM tags
Genetics
Crispr/Cas9
(b)
homogeneous samples
Heterogeneous samples
Further purification
Cross linking
Vitrification
(c)
Focused Ion Beam
Vitrification
Light Microscopy
FIB Milling
Light Microscopy
FIB Milling
Light Microscopy
SPA
Crystallography
NMR
CryoET
Mass Spectrometry
(d)
other structures
Subvolume averaging
data mining
comparative studies
dynamic & binding information (spectroscopies, molecular dynamics)
Structural models

Cryo-Tomography Workflow

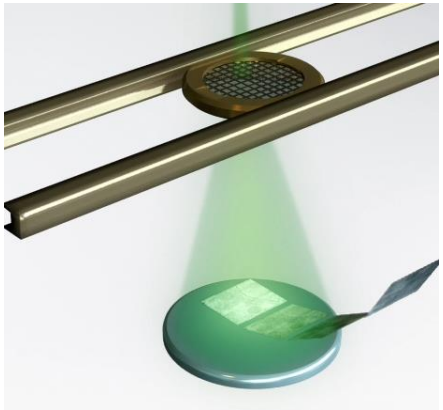
Step one: Sample Preparation



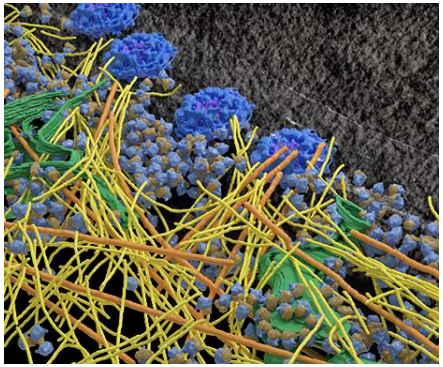
Step three: Cryo-FIB Milling



Step two: Cryo-LM

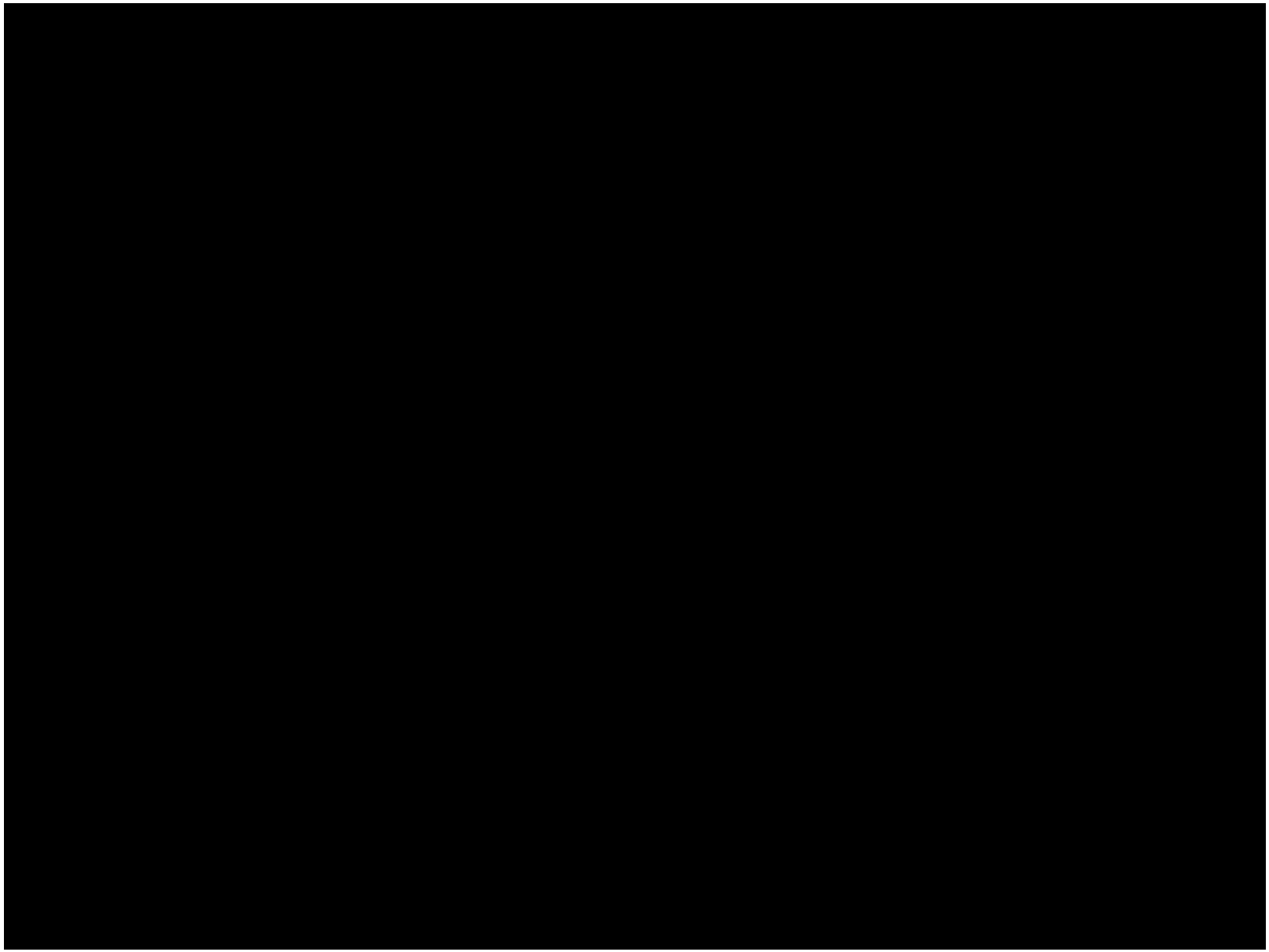


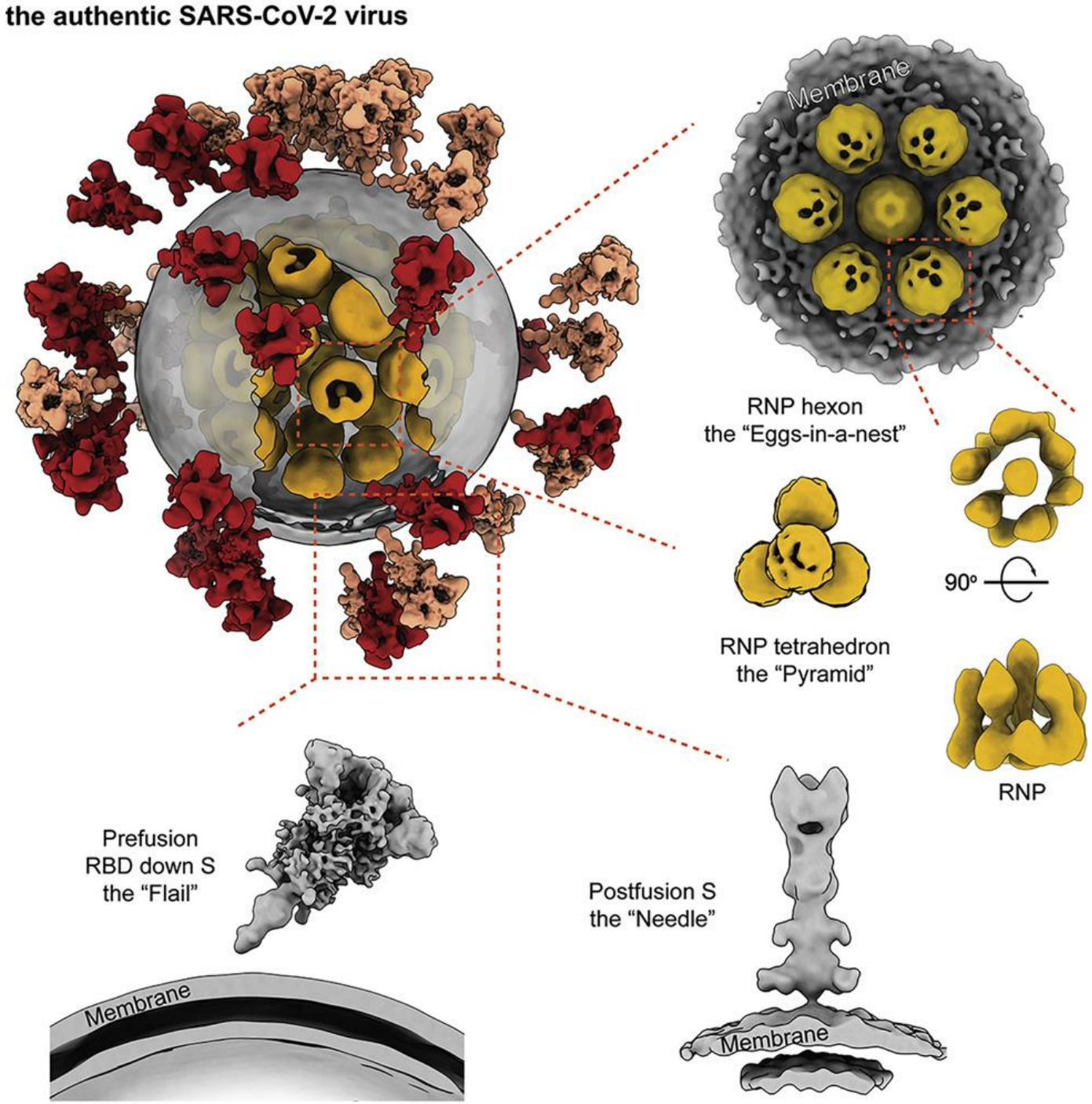
Step four: Cryo-TEM Tomography



Step five: Reconstruction & Visualization

Cryo-focused Ion Beam （Cryo-FIB）Sample Preparation
低温聚焦离子束方法制备样本

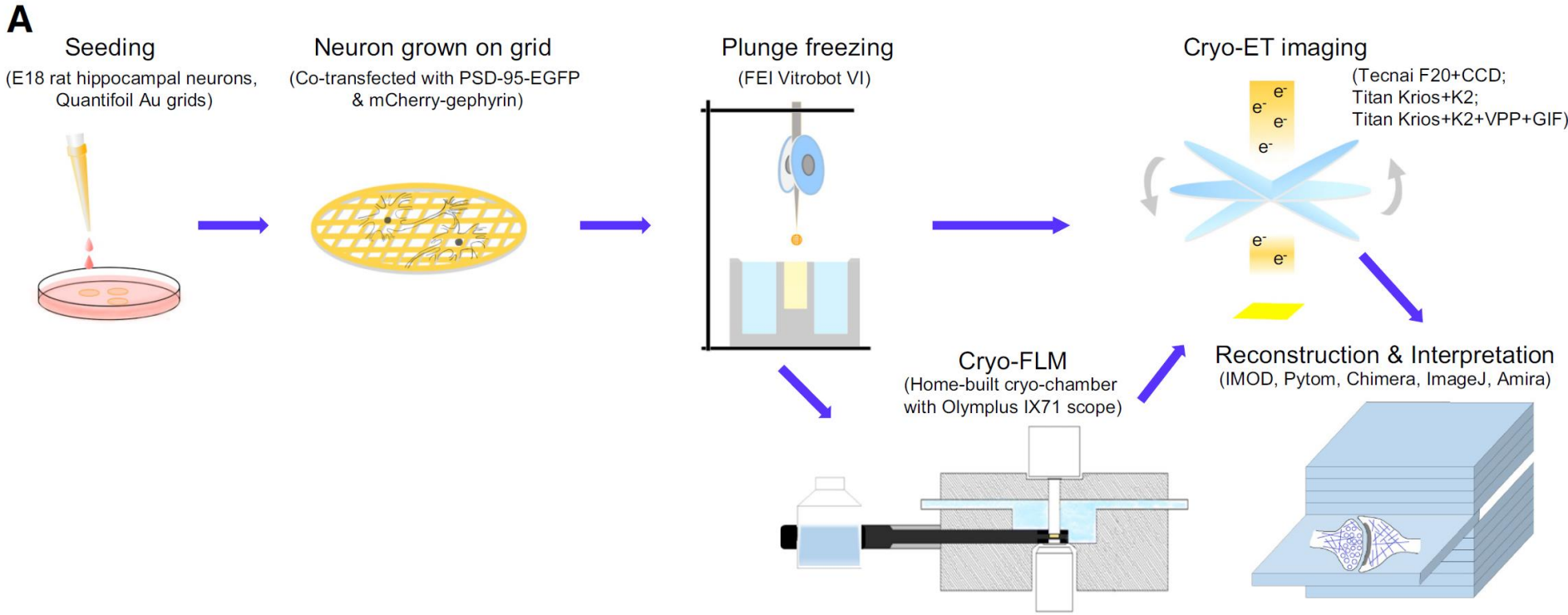




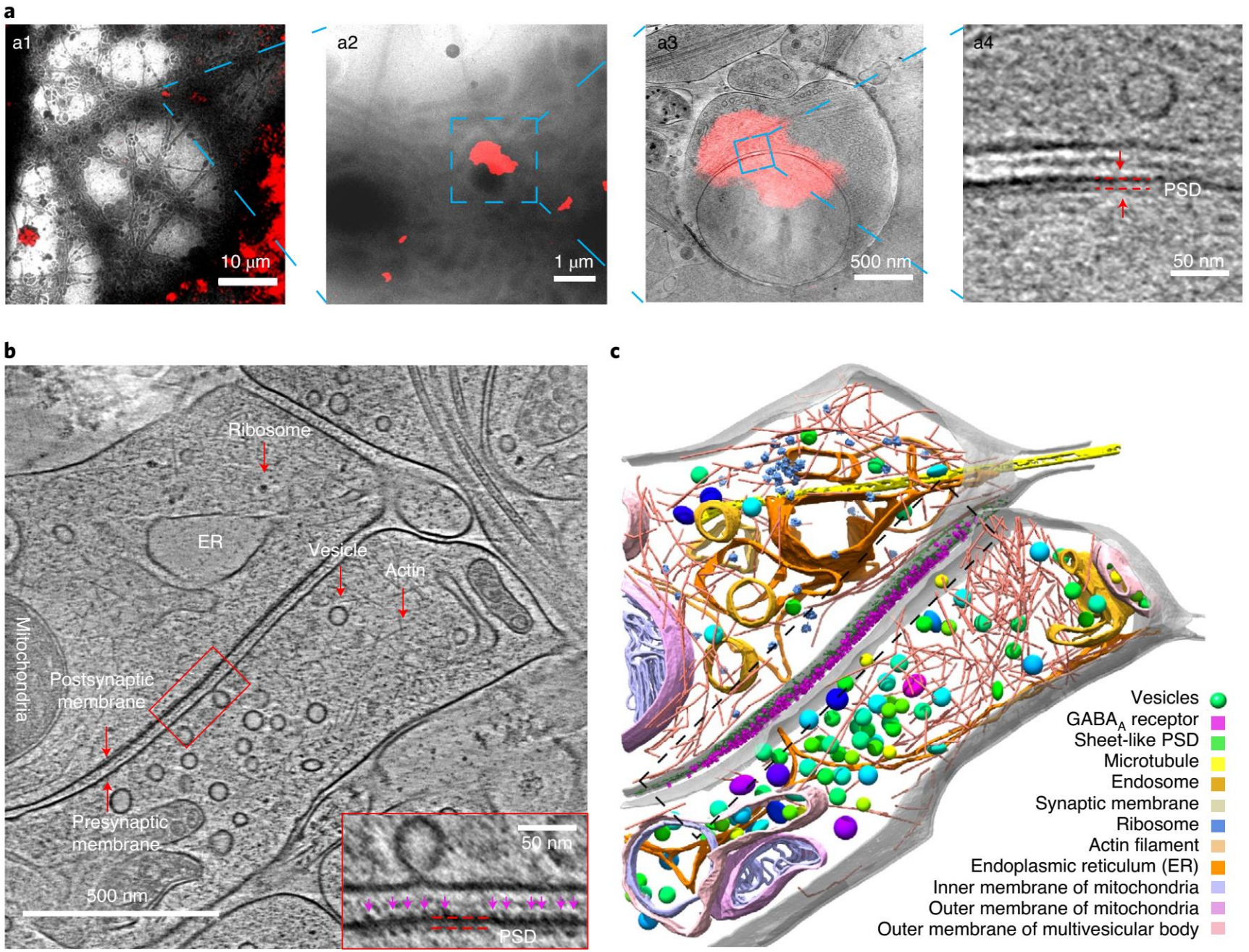
Yao et al., 2020. Cell

Workflow of cryo-ET/cryo-CLEM

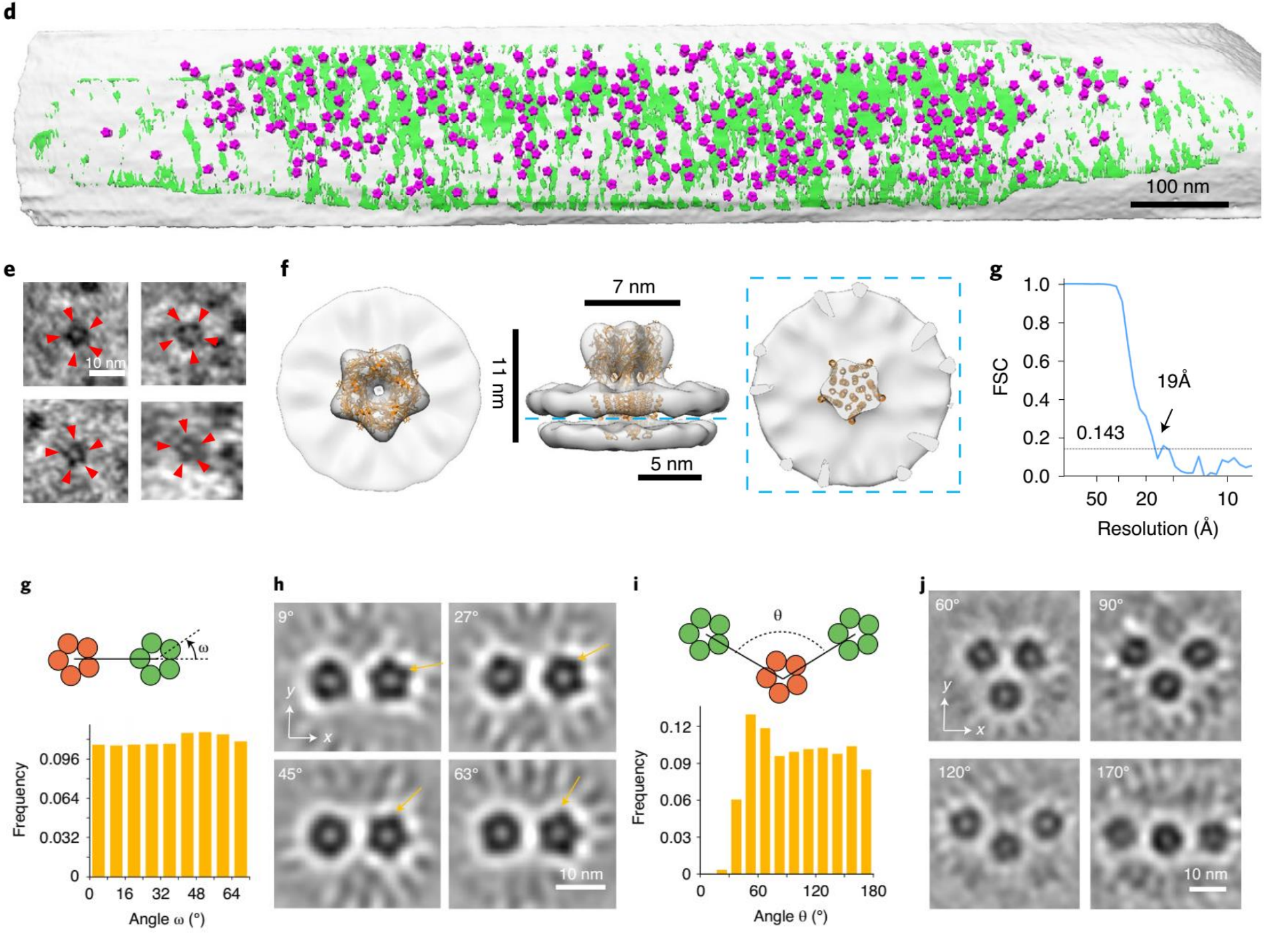
(Correlative Light and Electron Microscopy)

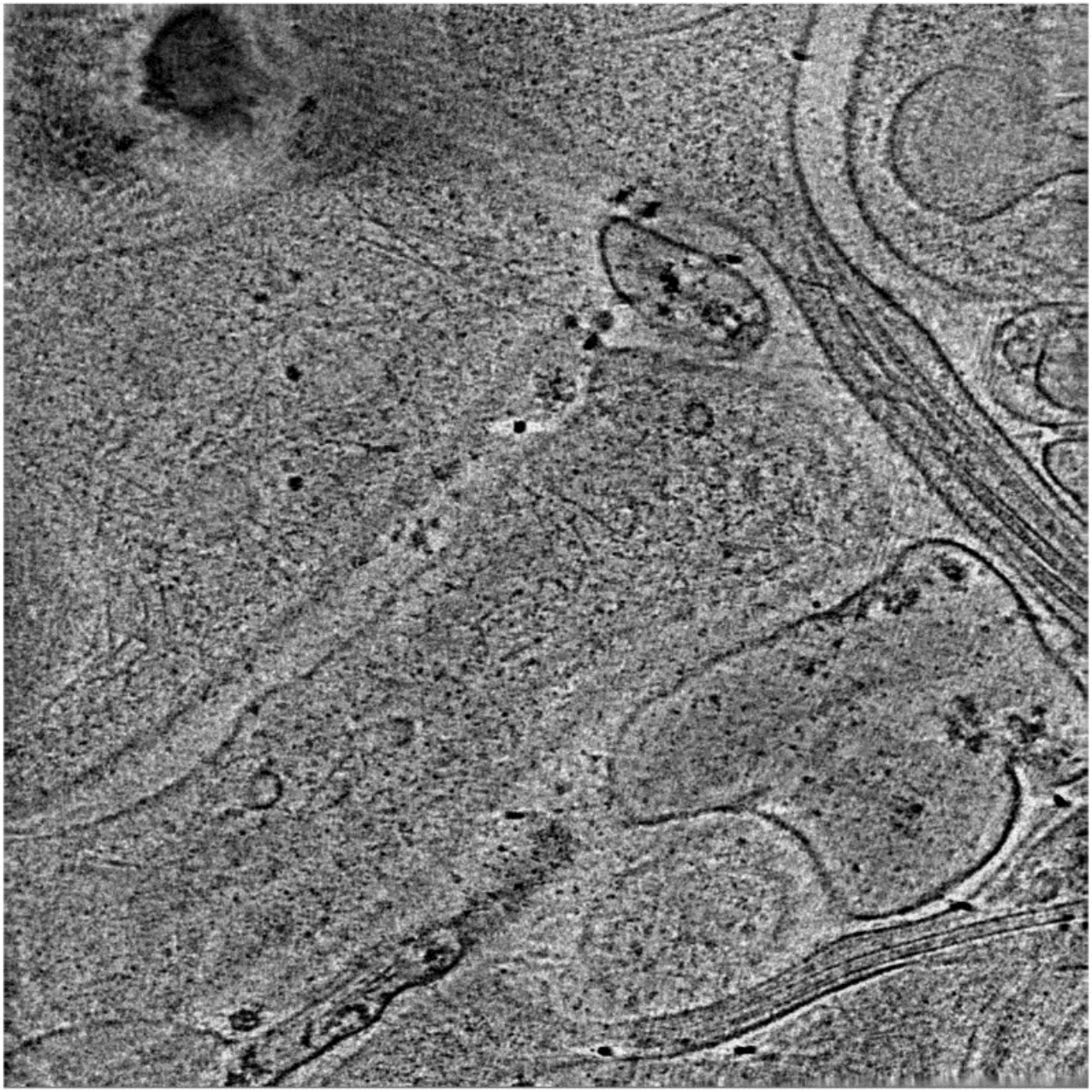


Tao et al., 2018. J Neurosci.



Liu et al., 2020. Nat Neurosci.


d
100 nm
e
10 nm
f
7 nm
11 nm
5 nm
g
1.0
0.8
0.6
0.4
0.2
0.0
FSC
19Å
0.143
50
20
10
Resolution (Å)
g
ω
Frequency
0.096
0.064
0.032
0
0
16
32
48
64
Angle ω (°)
h
9°
27°
45°
63°
y
x
10 nm
i
θ
Frequency
0.12
0.09
0.06
0.03
0
0
60
120
180
Angle θ (°)
j
60°
90°
120°
170°
y
x
10 nm



Caltech Getting Started in Cryo-EM

Welcome Course Overview Outline Lecture Videos Instructor Links

WELCOME TO THE COURSE

Before diving into the lecture videos, start by watching the trailer and reading the course overview and outline.

We hope you enjoy learning about cryo-electron microscopy (cryo-EM)!

http://cryo-em-course.caltech.edu/cryoem_welcome

Contact: shujiazhu@ion.ac.cn