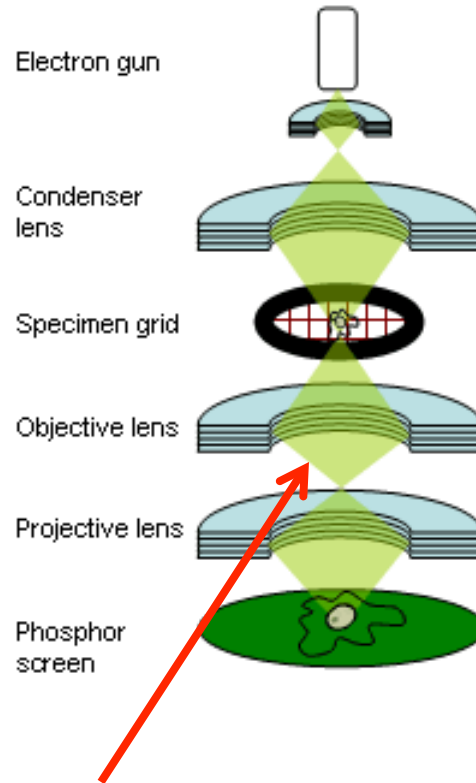
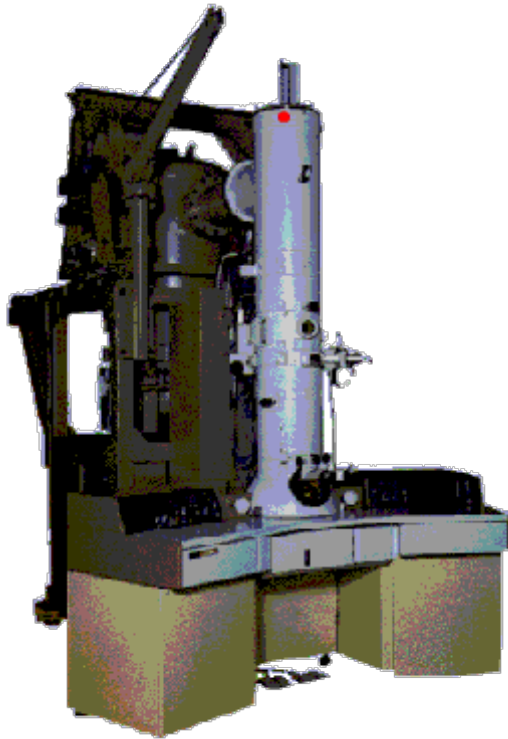


Cryo-Electron Microscopy

Pavel Plevka

Transmission Electron Microscope



Vacuum!

Electron source:

Thermal emission from heated cathode

Focussing:

Electro-magnetic Lenses

Detection:

Phosphor screen or CCD camera (former times: negative)

Pro & Con of cryo-EM

Pro

- Short wavelength => high resolution
- Strong interaction with materials => good contrast
- Electromagnetic lenses => standard optics (in contrast to X-ray crystallography)
- High intensity is easy to produce
- Inner structure of biomolecules is accessible

Con

- High vacuum requires special treatment of sample
- Sample has to be thin to avoid 100% absorption
- Electron beam damages biological samples => short measurements
=> low contrast of biomolecules

Microscope Optics

In order to see an “object” which is too small to be seen by our eyes, one needs to magnify the image. An example of magnifying an image by a lens is illustrated in Fig. 2.1:

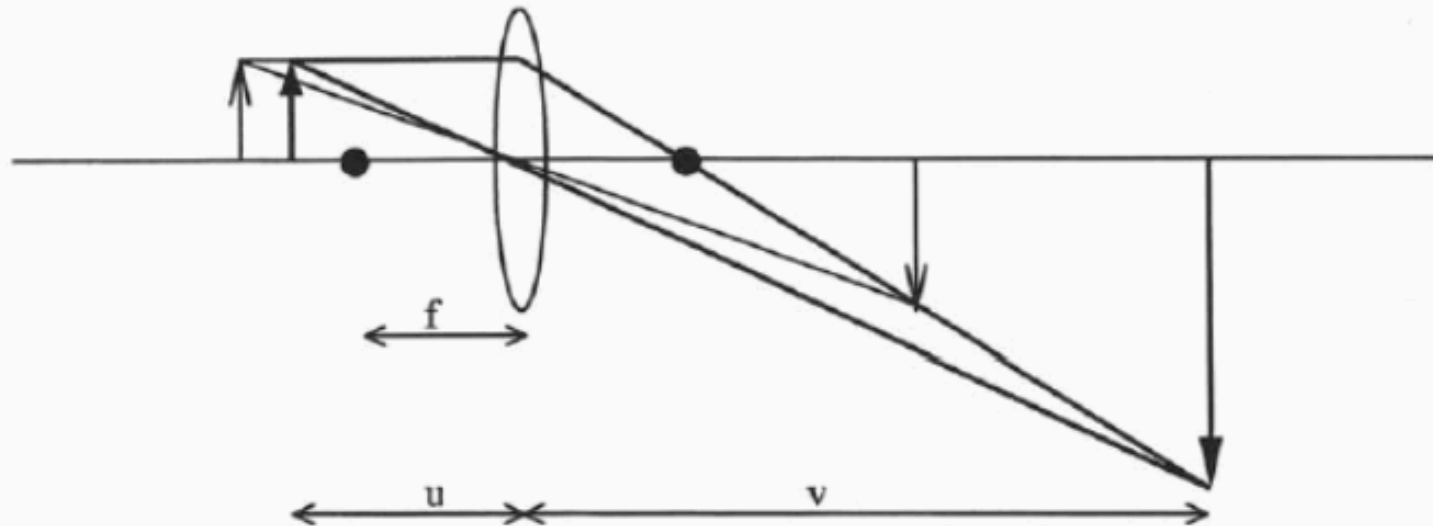


Figure 2.1 Ray diagram illustrating the formation of images by a lens. f = focal length, u = the distance between the object and the lens, v = the distance between the image and the lens.

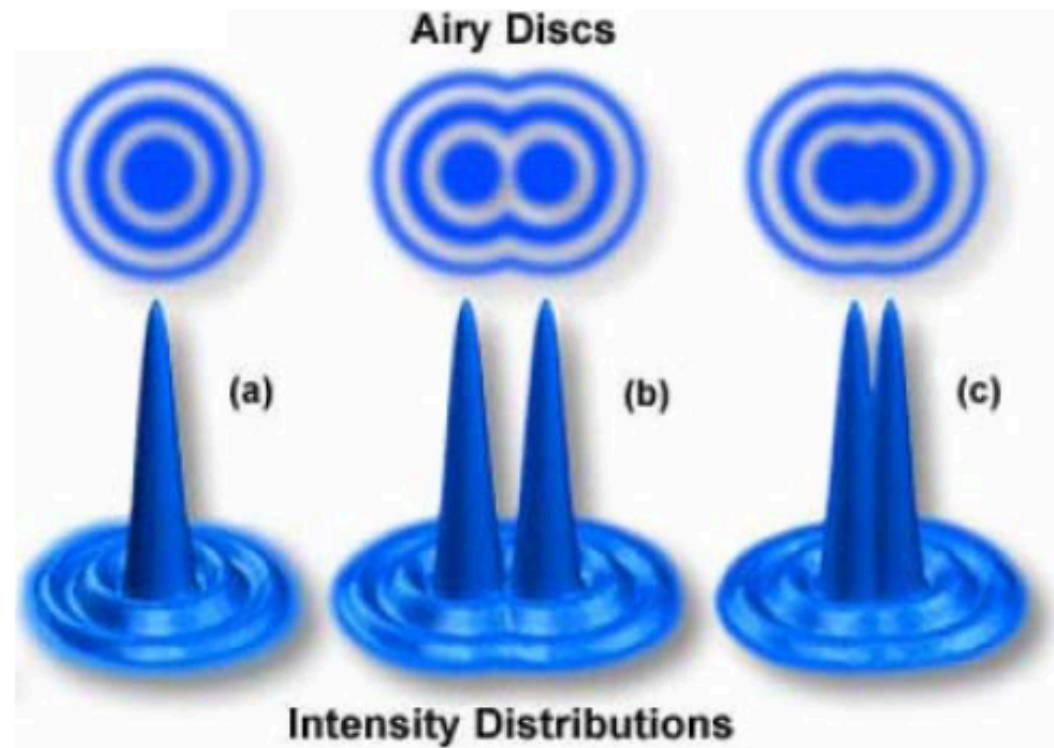
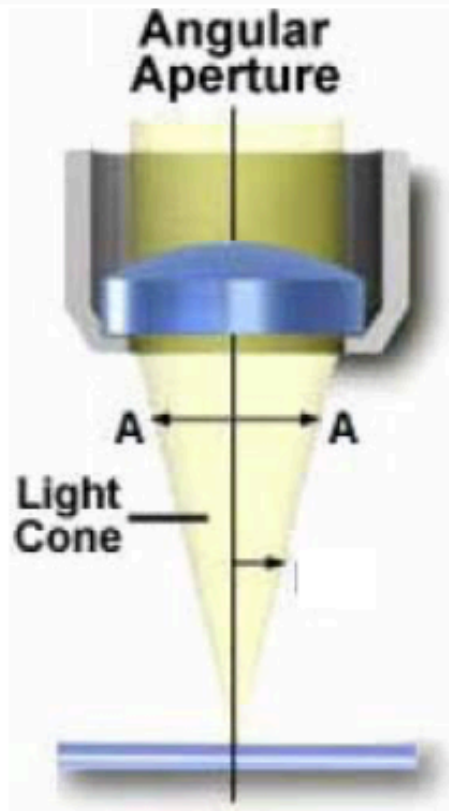
The relation between the “object distance” u , the “image distance” v and the focal length f is:

$$\frac{1}{f} = \frac{1}{u} + \frac{1}{v} \quad (2-1)$$

The closer the object distance u is to the focal length f , the larger the image is magnified.

Optical Resolution

An image cannot be endlessly enlarged due to the limit of the resolution. “Resolution” is the closest distance between two points on the object which can be clearly seen through the microscope to be separate entities.



The central maximum of the Airy patterns is often referred to as an Airy disk, which is defined as the region enclosed by the first minimum of the Airy pattern and contains 84 percent of the luminous energy.

Rayleigh Criterion

Lord Rayleigh proposed a criterion to define the resolution - Rayleigh resolution: when the intensity maximum of the Airy disc from one point coincides with the first minimum of the Airy disc from the second point, then the two points can be just resolved. The Rayleigh resolution can be derived from diffraction theory to be:

$$r = \frac{d}{2} = \frac{0.61\lambda}{\mu \sin \alpha} \quad (2-2)$$

where λ is the wavelength of the light and μ is the refractive index of the medium between the object and objective lens. α is the semi-angle above which the light is stopped by the aperture, see in Fig. 2.3.

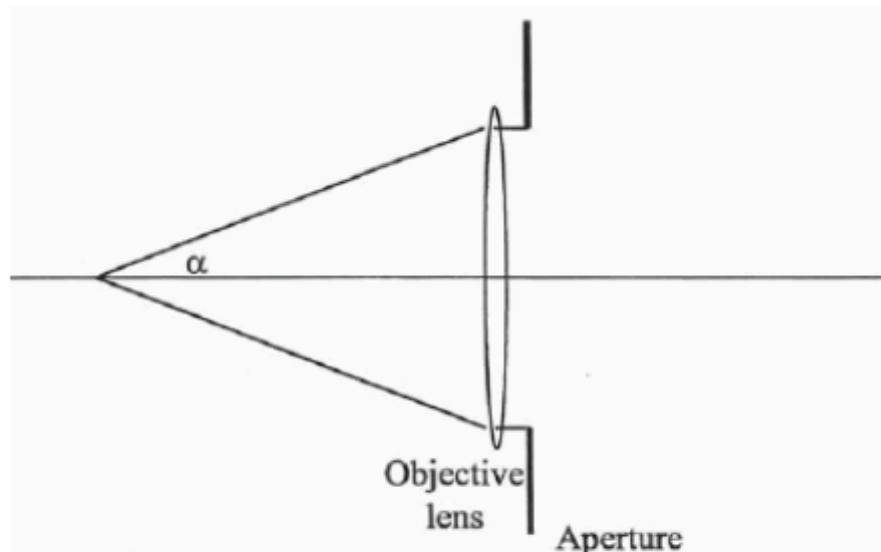
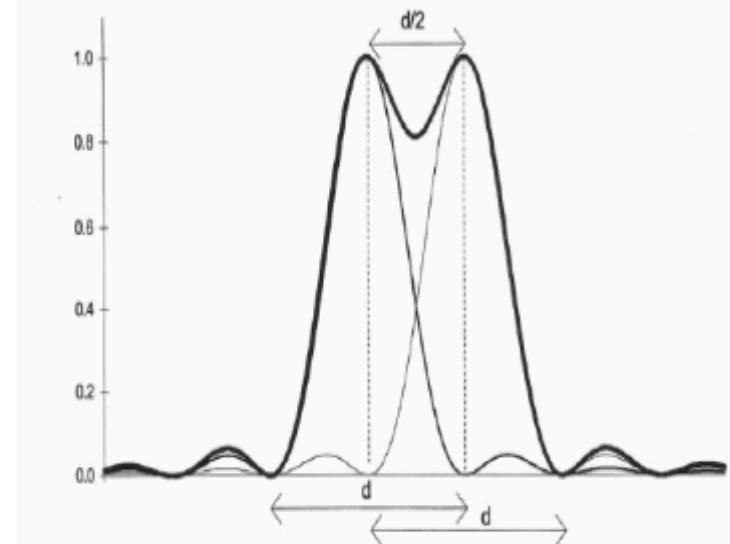
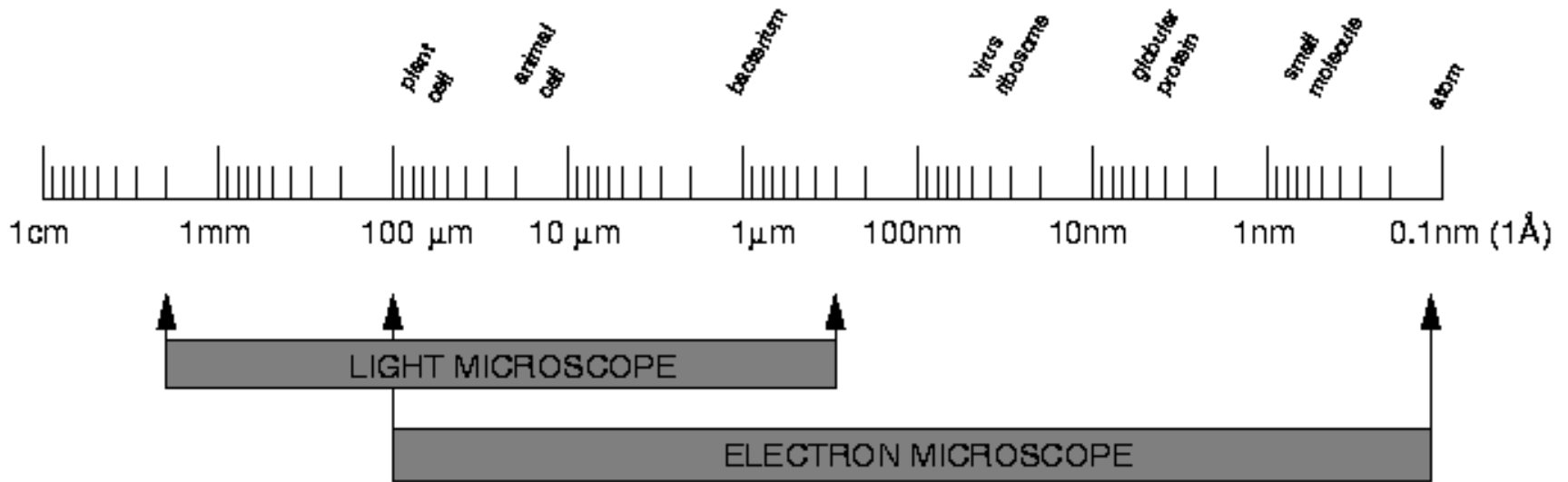


Figure 2.3 The definition of the semi-angle, α .



Why electrons?



Visible Light:

$$\lambda = 400 - 600 \text{ nm}$$

Electrons:

$$\lambda = 0.002 - 0.004 \text{ nm}$$

Remember: wave-particle dualism

de Broglie: $\lambda = h / m \cdot v$

$$E = \frac{1}{2} \cdot m \cdot v^2$$

Electrons are Both Particles and Waves

Electrons, like other particles, can be considered both as particles and waves. The wavelength of an electron depends on its velocity, v :

$$\lambda = \frac{h}{mv} \quad (2-3)$$

When an electron is accelerated through a potential difference U (volt), its wavelength can be calculated as follows:

$$\lambda = \frac{h}{(2emU + \frac{e^2 U^2}{c^2})^{\frac{1}{2}}} = \left(\frac{1.5}{(U + 10^{-6} U^2)} \right)^{\frac{1}{2}} \text{ nm} \quad (2-4)$$

where h is Planck's constant, c is the speed of light, e and m are the mass and charge of the electron. Typical values of the electron wavelengths used in a TEM are:

U (kV)	100	200	300	400	500	1000
λ (Å)	0.0370	0.0251	0.0197	0.0164	0.0142	0.0087

The wavelength depends on the accelerating voltage used. The higher the accelerating voltage, the shorter the wavelength.

Sample Preparation - Staining

⇒ To increase contrast: heavy atoms interact with electrons stronger than biomolecules (C, N, O, S, P)

- **Positive Staining**

treat sample with solution of salt like uranyl acetate, lead citrate, osmium tetroxide – object is black on light background

- **Negative Staining**

place sample on dried film of heavy metal salt – object is light spot on black background

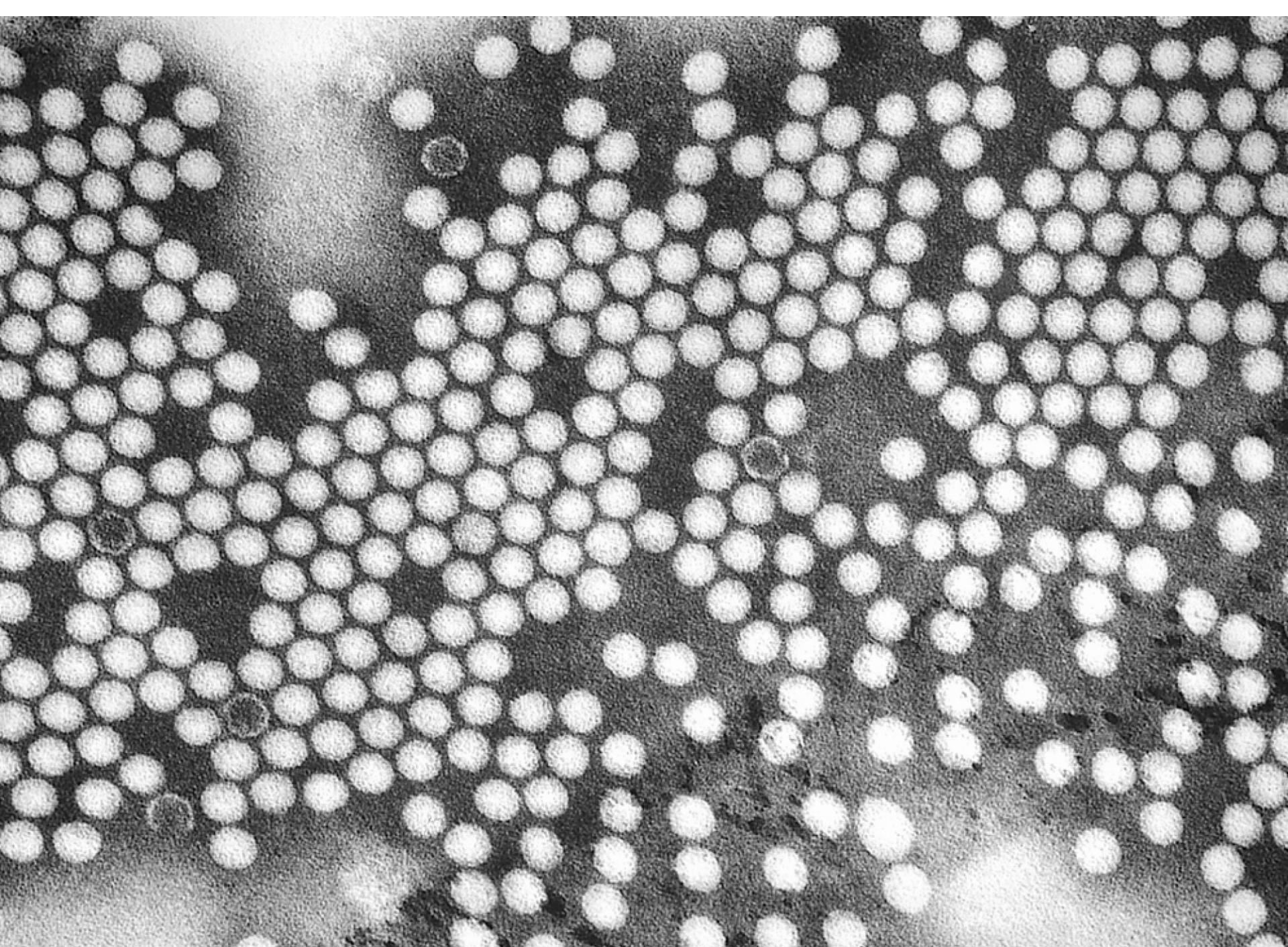
- **Shadowing**

spray thin layer of heavy metal on sample to produce a shadow

Disadvantage:

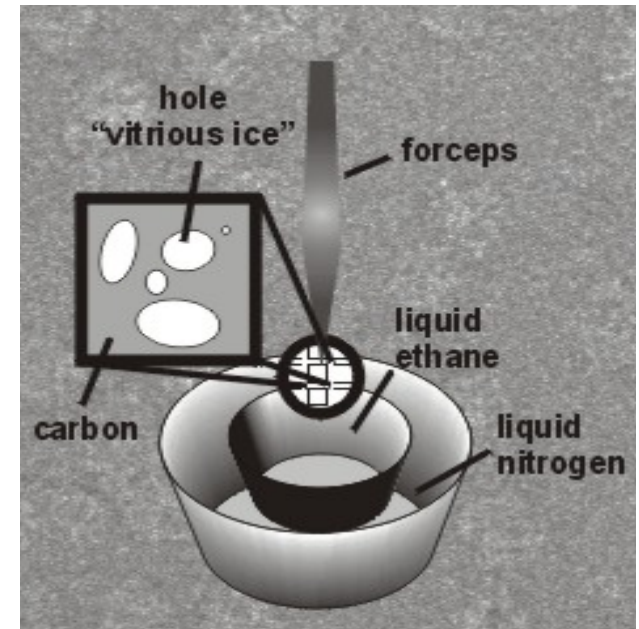
Size of stain reduces resolution to about 20-30 Å





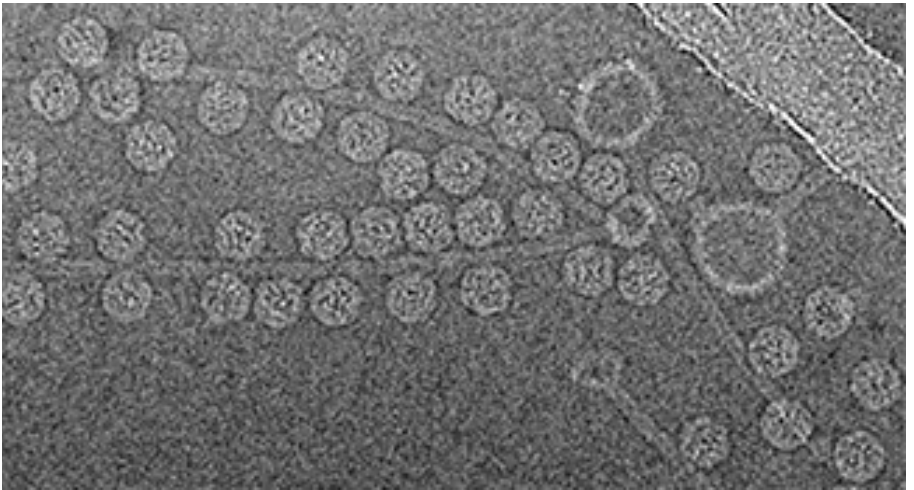
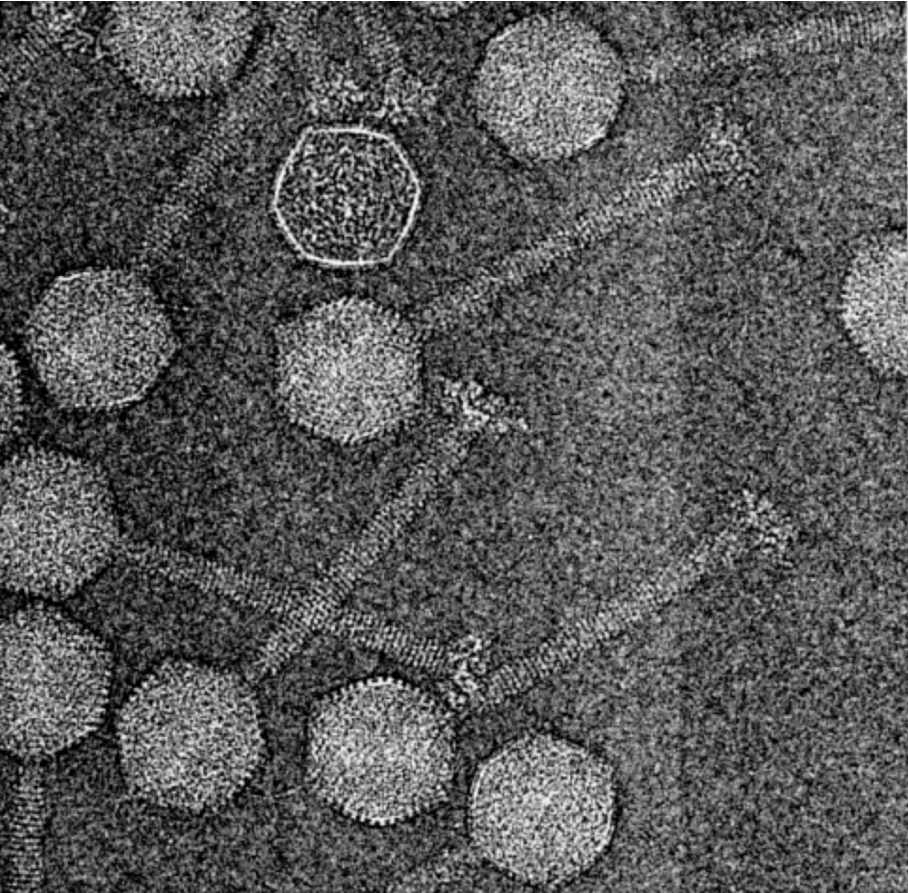
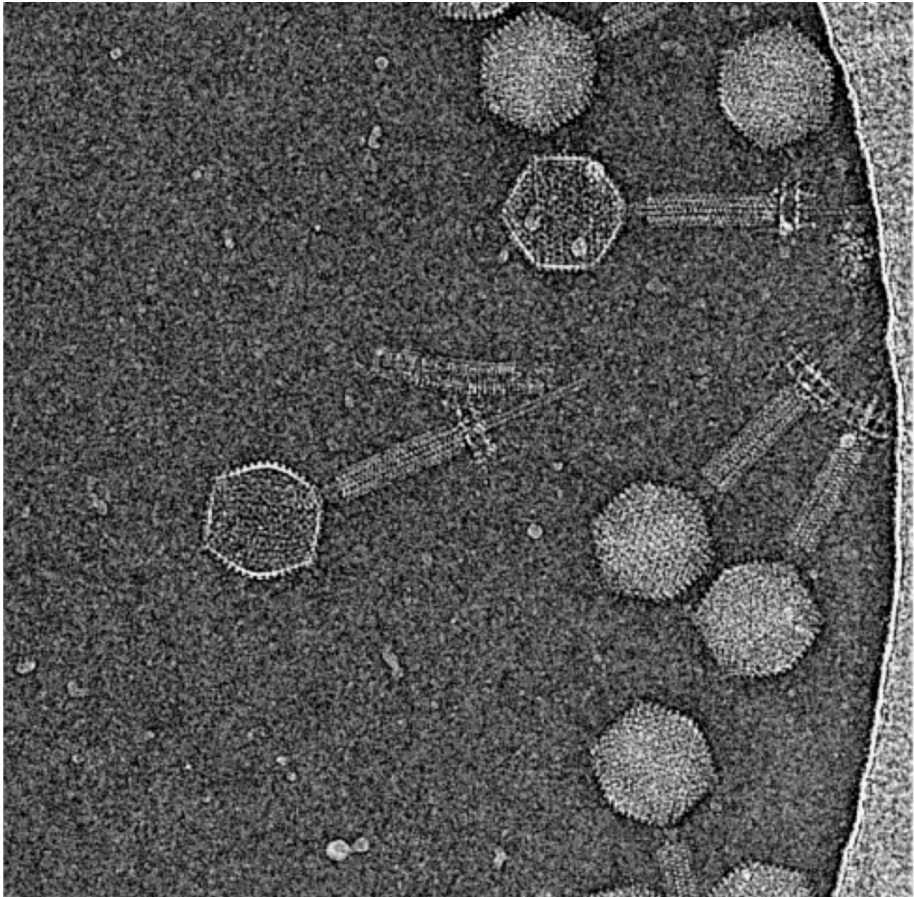
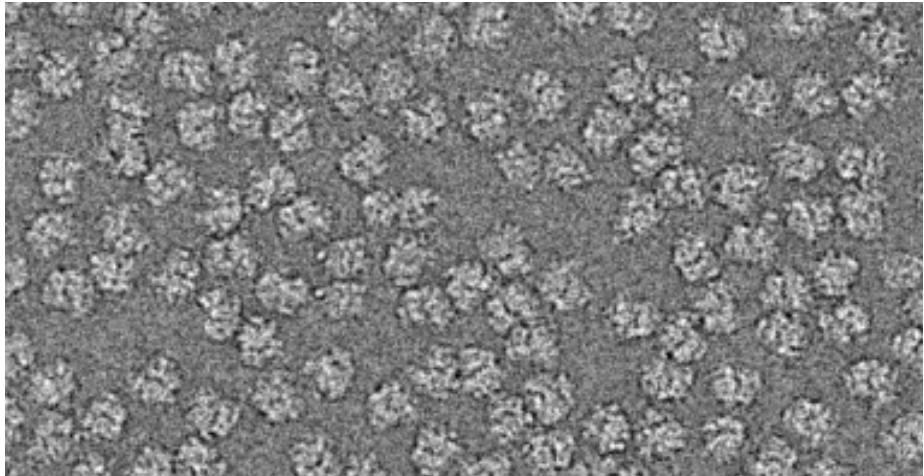
Alternative: Cryo-EM

- to avoid harsh staining which may change the structure of your sample
- stabilization of sample by rapid freezing of sample in liquid ethane to form vitreous ice
- into electron microscope at low temperatures to keep sample stable in hydrated state in vacuum
- thickness of ice layer as small as possible!



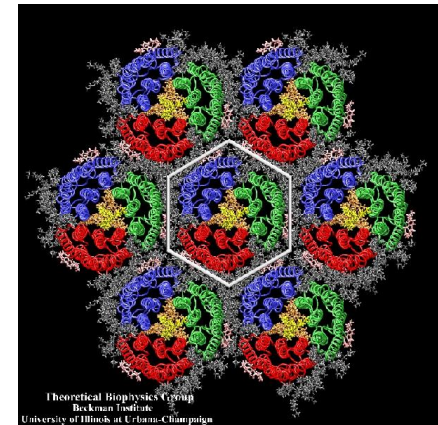
Advantage:

- sample structure unchanged
- inner structure of molecule is accessible

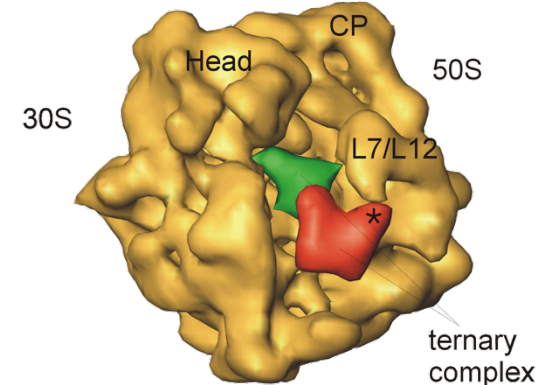


Types of Samples

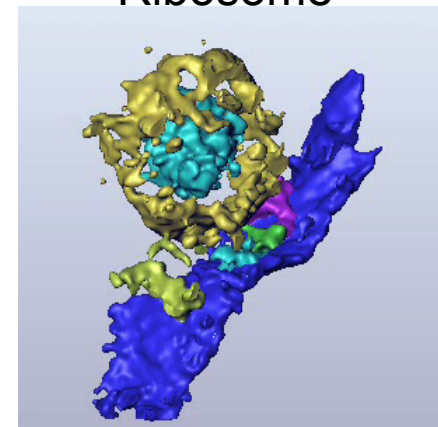
- Periodic arrangement
=> 2D electron crystallography
small or membrane proteins < 200 kDa
resolution up to 2.5 Å
- Random arrangement
=> single particle technique
macromolecular complexes > 200 kDa
up to atomic resolution
- Large Organelles (Golgi, ER), whole cells
=> tomography
resolution > 40 Å



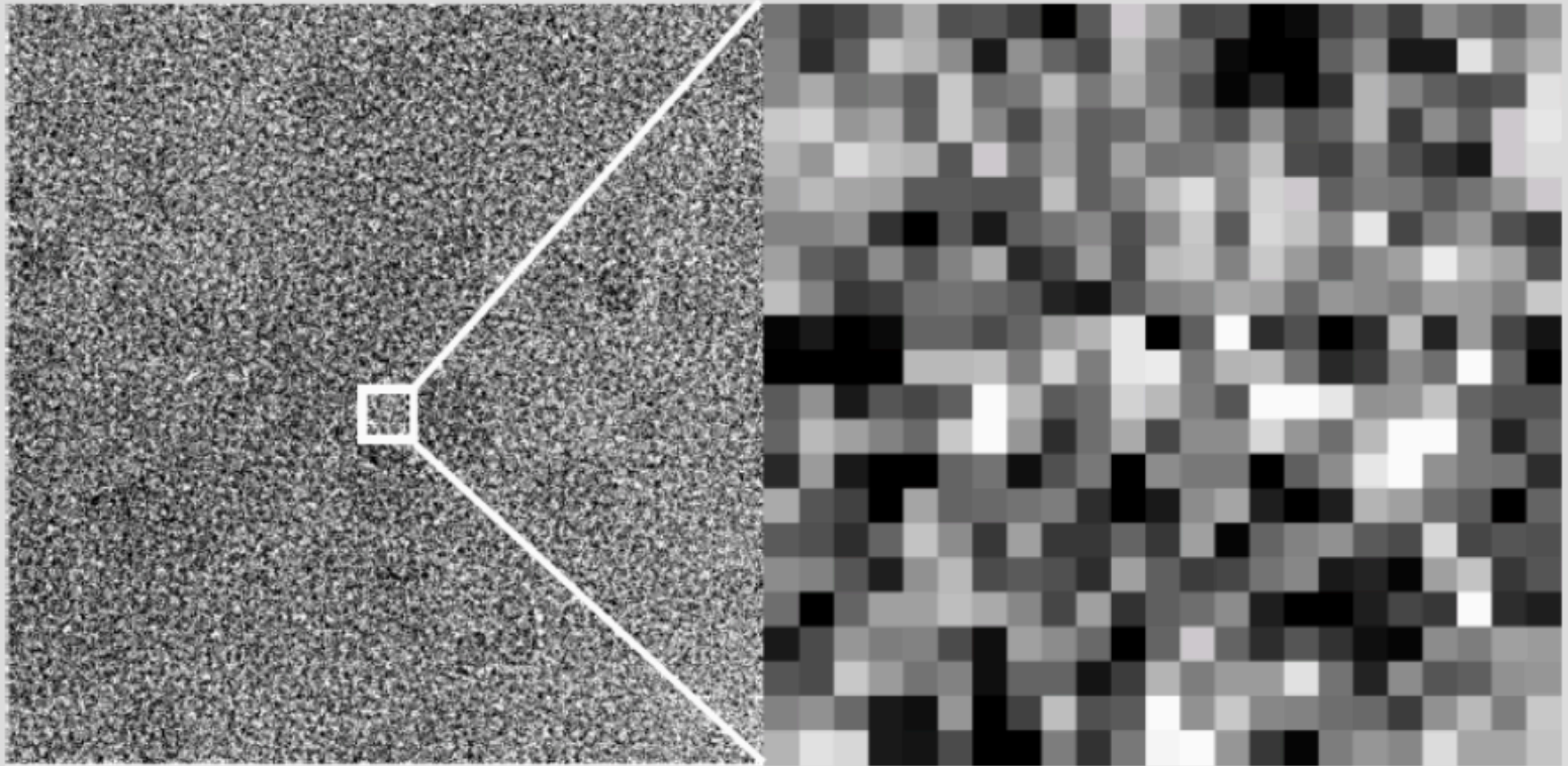
Bacteriorhodopsin



Ribosome

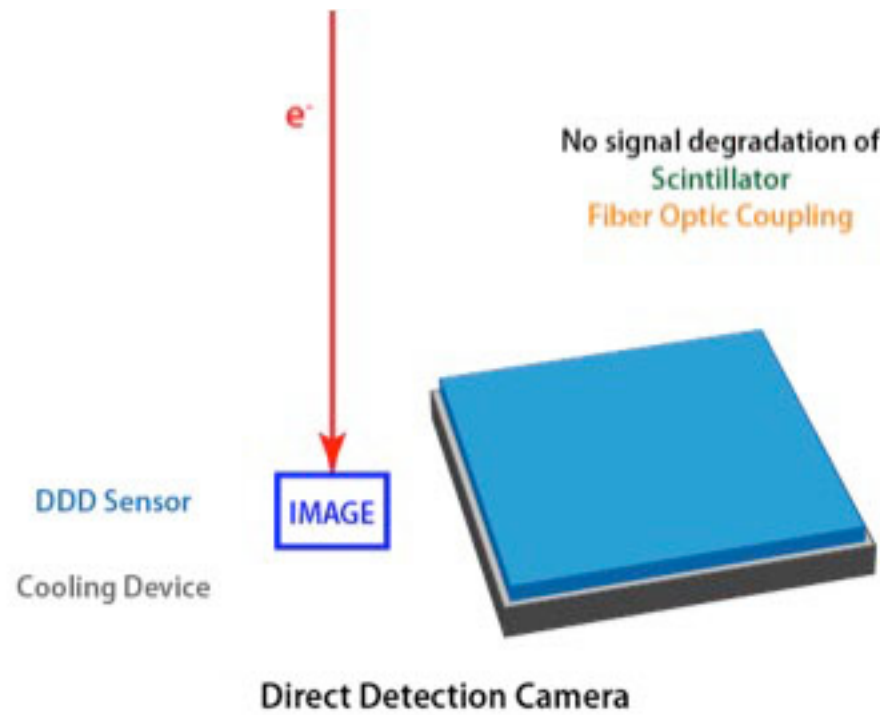
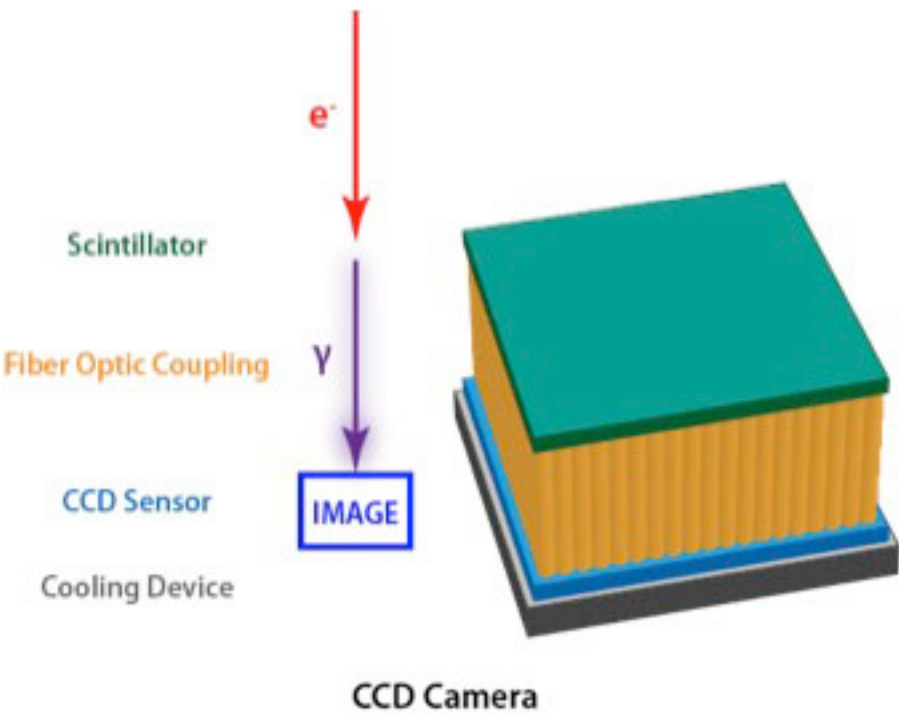


Digitization of Recorded Image

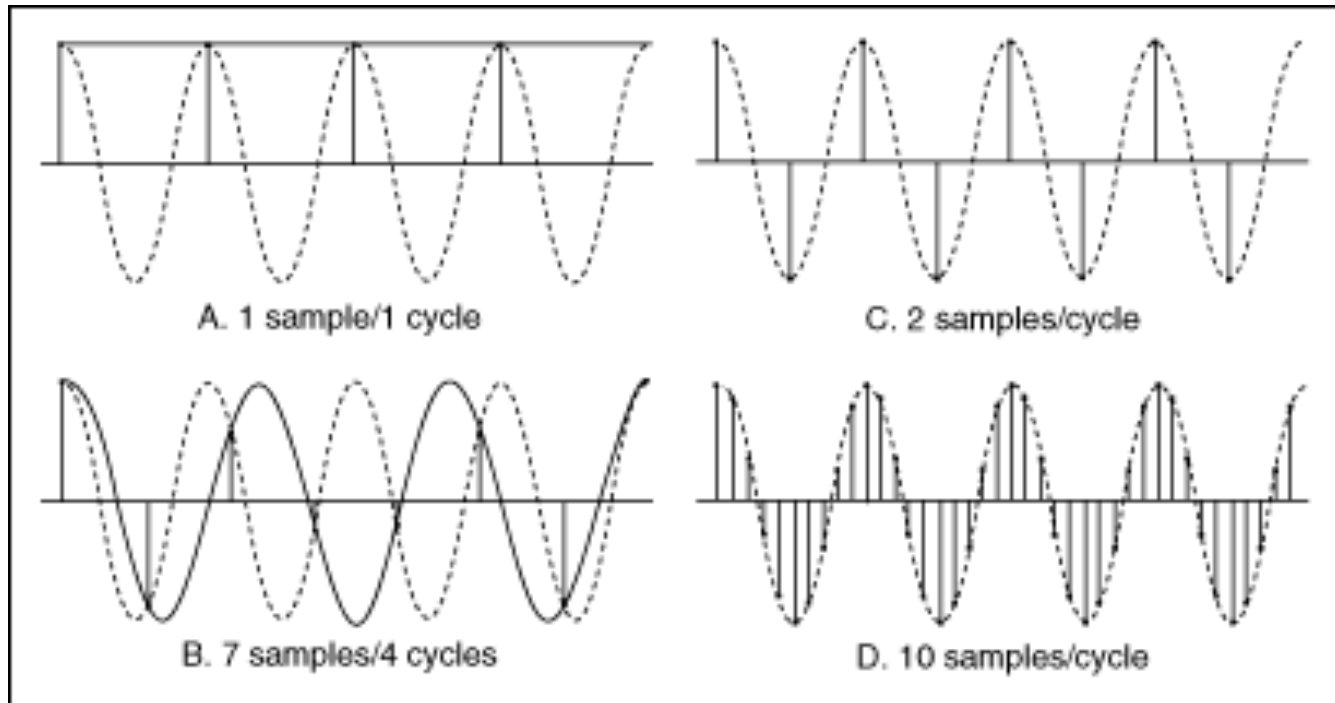


image

magnified sample of image showing
how the image is formed by a grid of
 $10 \times 10 \mu\text{m}$ pixel



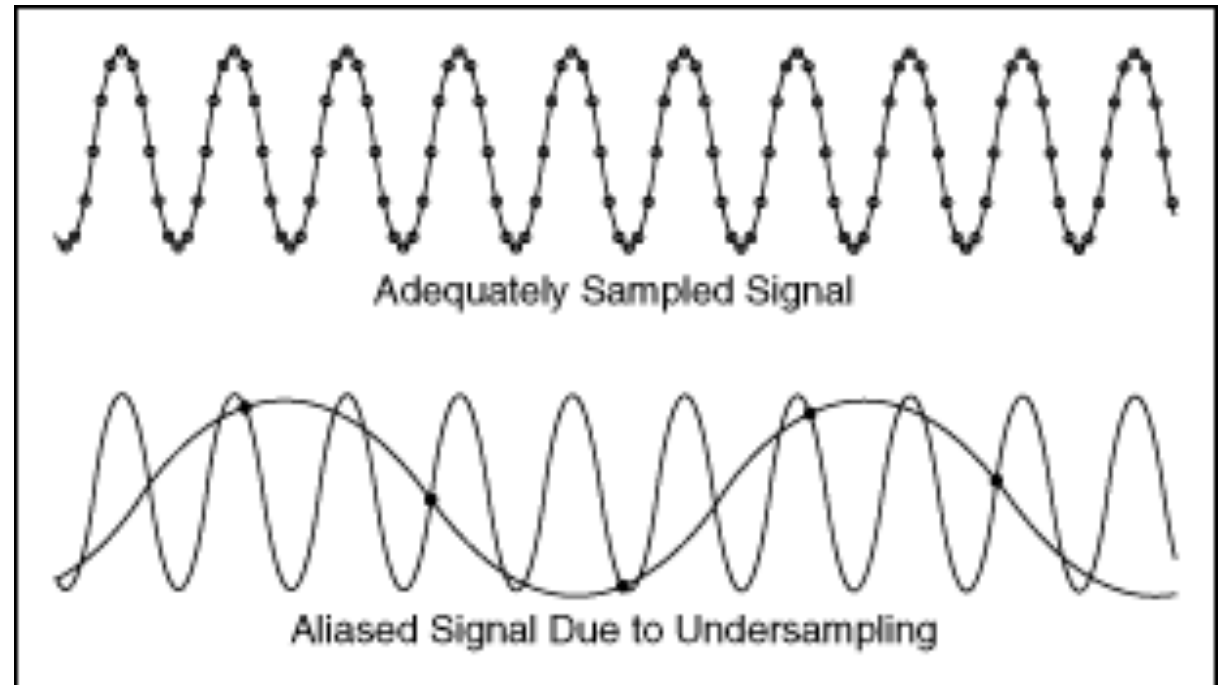
Whittaker-Shannon sampling theorem





For a given sampling frequency, the maximum frequency you can accurately represent without aliasing is the Nyquist frequency. The Nyquist frequency equals one-half the sampling frequency, as shown by the following equation.

$$f_N = \frac{f_s}{2}$$

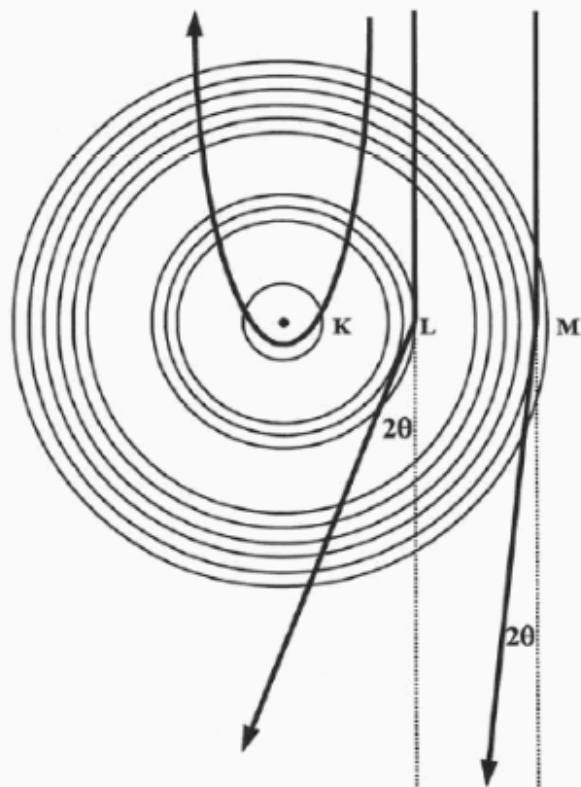


Electrons See the Electrostatic Potential

Electrons are negatively charged particles and they interact with atoms via Coulomb forces. The interactions are between the incident electrons and

- Valence electrons - low angle scattering
- Inner shell electrons - medium angle scattering
- The nucleus - high angle scattering

Incident electrons



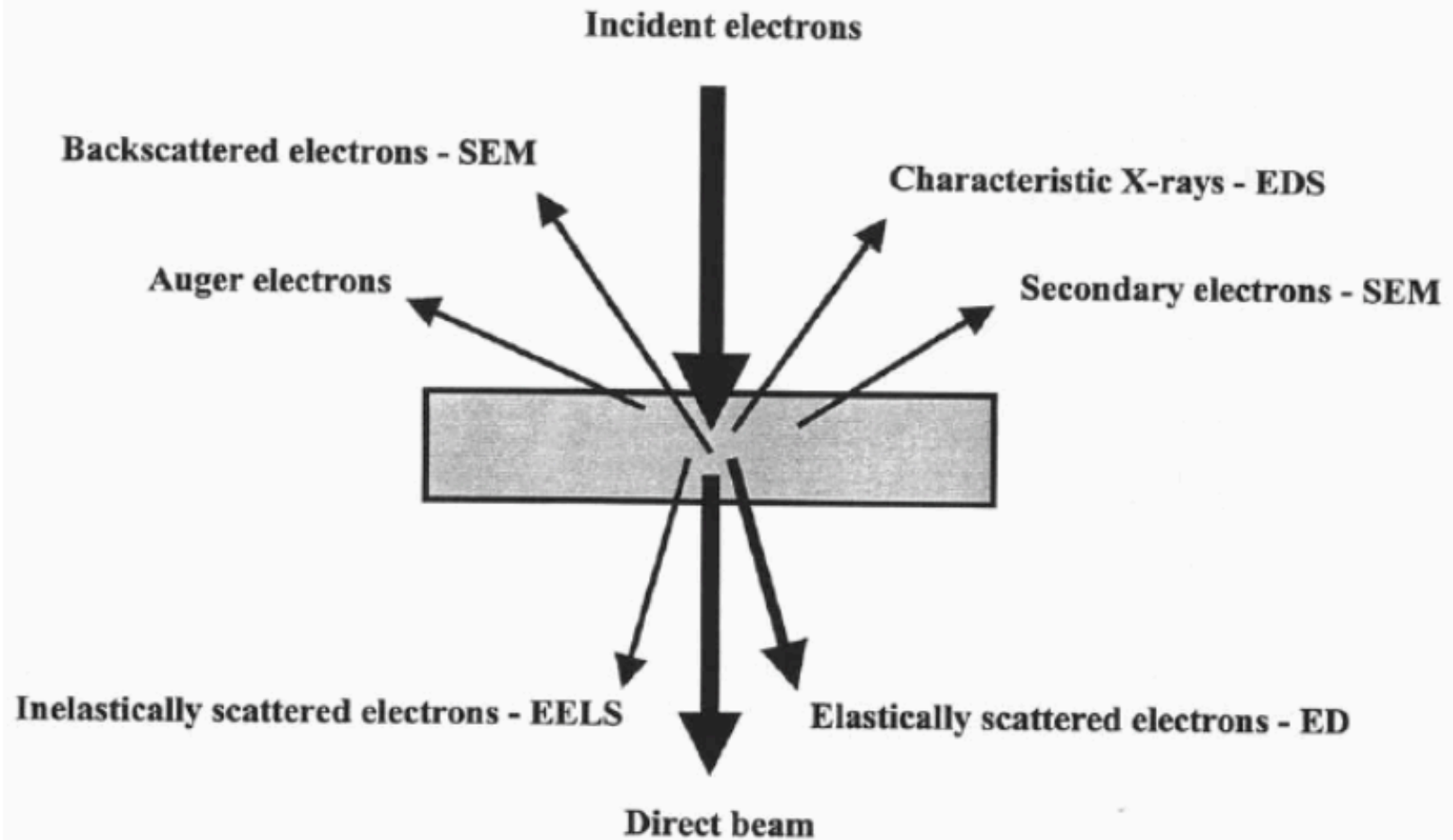
Shell	Sub-shell		No. e ⁻
	KLM	spdf	
K	K	1s	2
L	L1	2s	2
	L2	2p	2
	L3	2p, 2p	4
M	M1	3s	2
	M2	3p	2
	M3	3p, 3p	4
	M4	3d, 3d	4
	M5	3d, 3d, 3d	6

Electrons interact with both the electrons and nuclei, while X-rays interact only with electrons and neutrons only with nuclei. Thus electrons “see” the electrostatic potential, X-rays the electron density and neutrons the nuclei in a specimen.

Electrons are scattered much more strongly than X-rays or neutrons:

Because of the strong interaction between electrons and matter, the sample has to be very thin in the TEM to allow electrons to pass through. The maximum thickness is less than 500 Å for materials containing heavy elements and more for those containing light elements such as organic molecules.

Scattering and Energy Loss

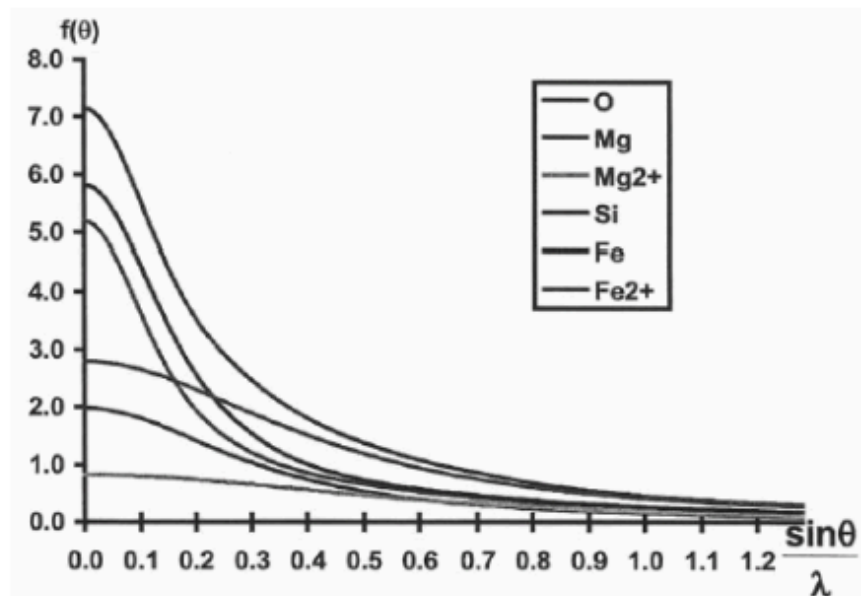


Atomic Scattering Factors for Electrons

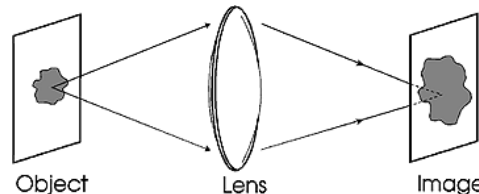
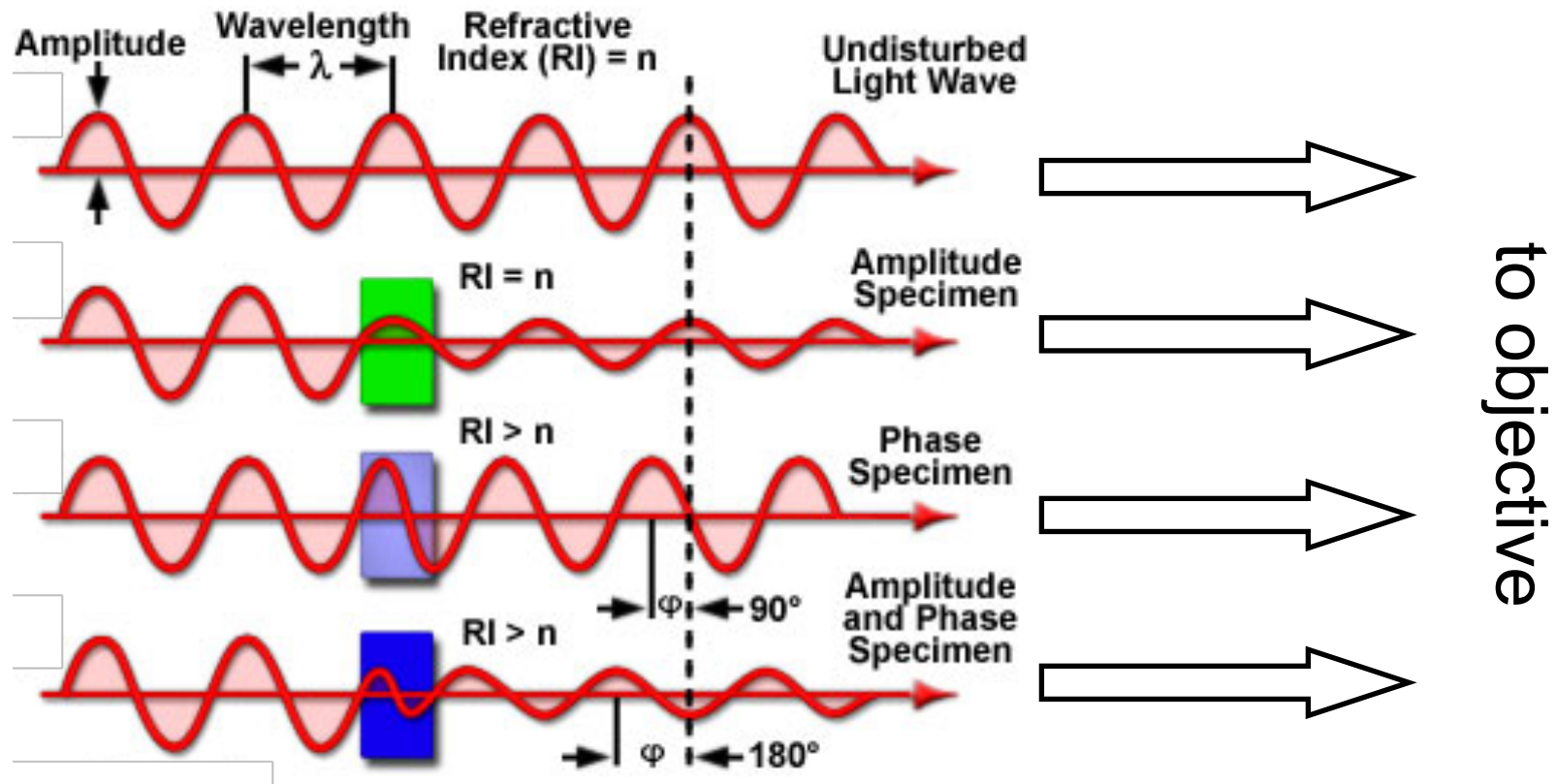
The atomic scattering factors for electrons $f(\theta)$ represent the probabilities that an electron is scattered into a specific direction (2θ with respect to the incident beam) when it passes near an isolated atom. They can be derived from the atomic scattering factors for X-rays $f_x(\theta)$ by the Mott-Bethe formula:

$$f(\theta) = 0.023934 \left(\frac{\lambda}{\sin \theta} \right)^2 (Z - f_x(\theta)) \quad (3-1)$$

where $f_x(\theta)$ is calculated from the Schrödinger wave equation. $f(\theta)$ for neutral and some of the ionized atoms are listed in the International tables for Crystallography, Volume C.

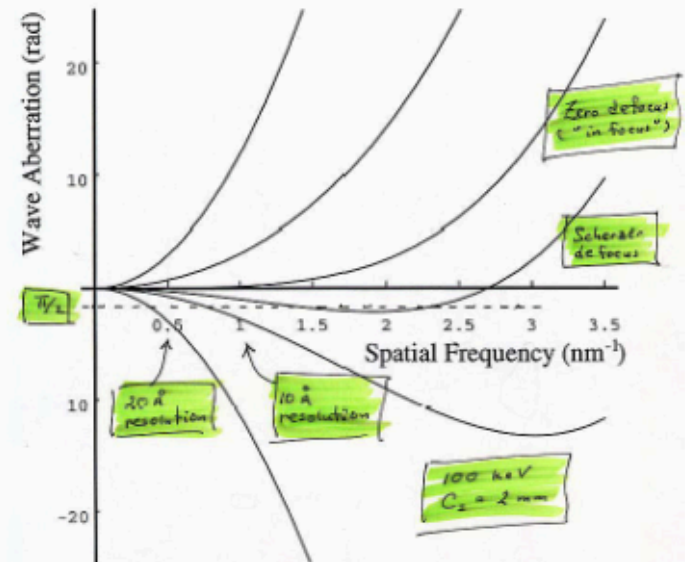
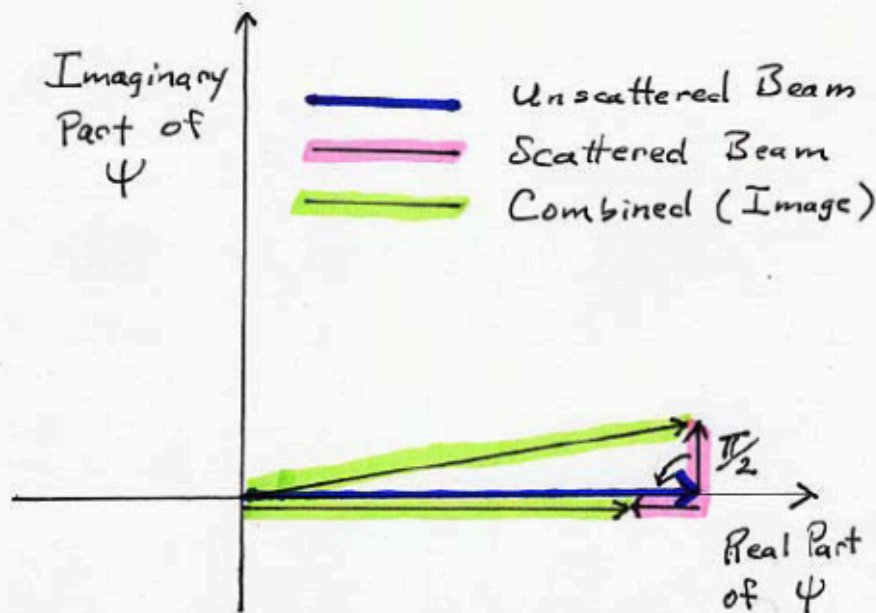


Macromolecules in water / vitreous ice are phase objects



Phase Objects Require an Additional Phase Shift to be Seen

- THE SCATTERED BEAM GIVES NO CONTRAST FOR A PHASE OBJECT BECAUSE IT IS $\pi/2$ OUT OF PHASE WITH THE UNSCATTERED BEAM
- APPLYING AN ADDITIONAL $\pi/2$ PHASE SHIFT CAN THUS PRODUCE CONSIDERABLE CONTRAST



DEFOCUS AND SPHERICAL ABERRATION IMPOSE A PHASE SHIFT

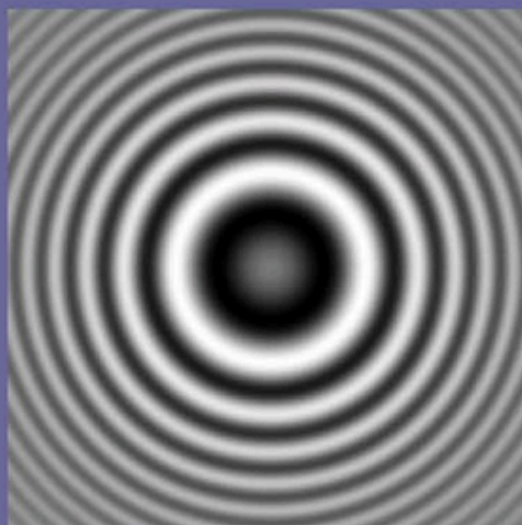
$$\gamma(s) = 2\pi [C_s/4 \lambda^3 s^4 - \Delta Z/2 \lambda s^2]$$

RESOLUTION-ZONES OF HIGH CONTRAST CAN BE “TUNED” BY ADJUSTING THE DEFOCUS



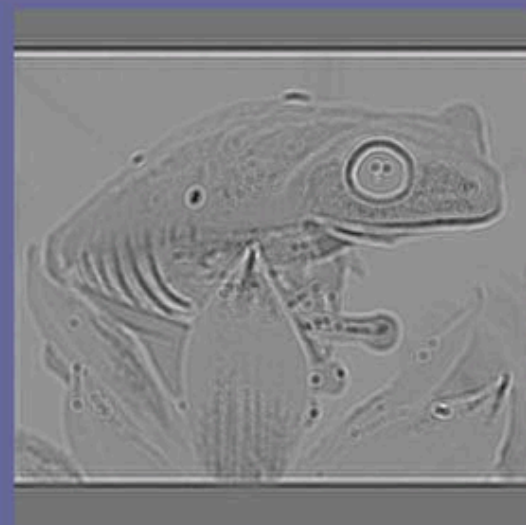
original object

\times

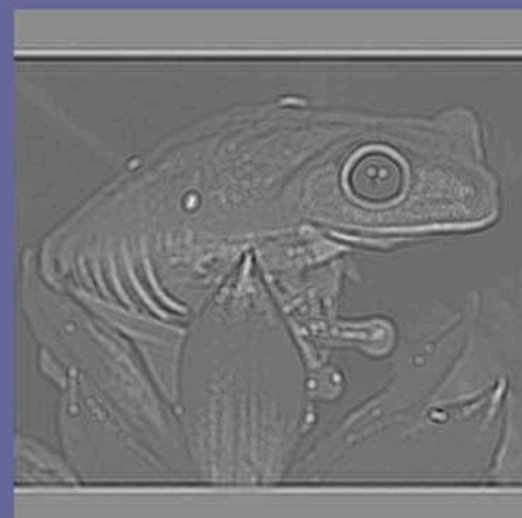
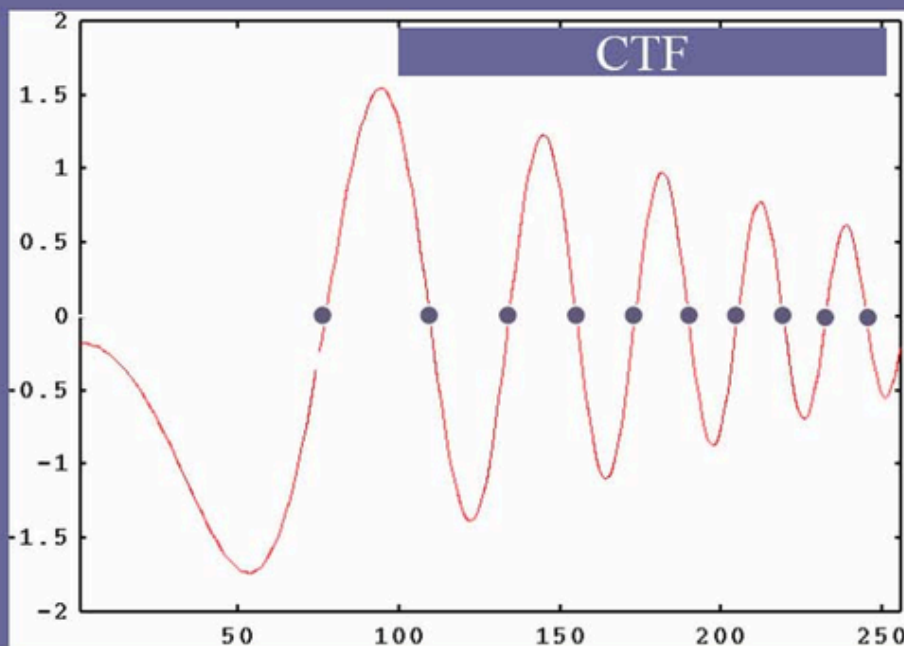


CTF for $\Delta z = 2.500 \mu\text{m}$

$=$



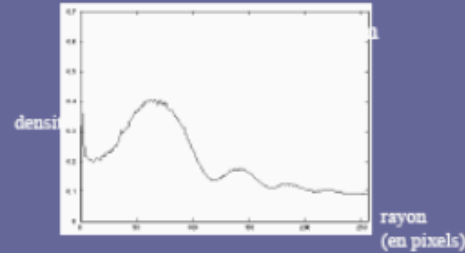
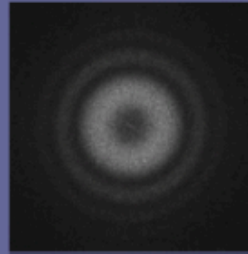
cryo-EM image



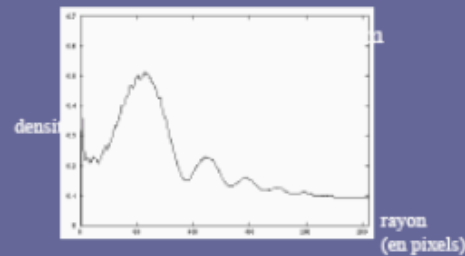
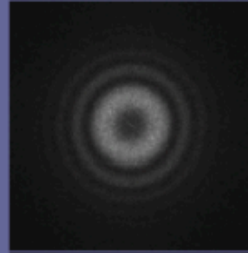
cryo-EM image,
contrast-inverted

Gallery of Power Spectra at Different Defocus

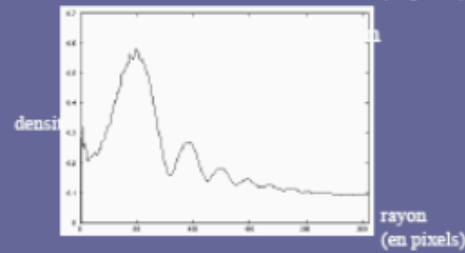
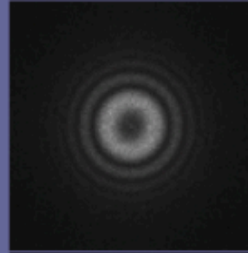
A



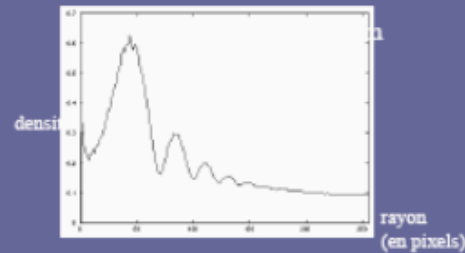
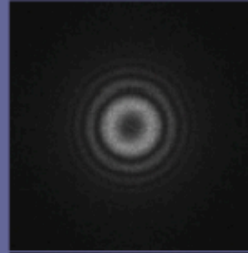
B



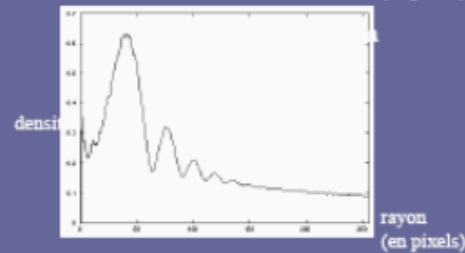
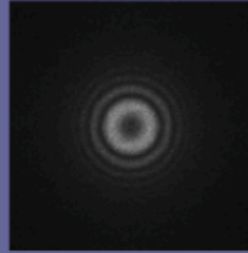
C



D



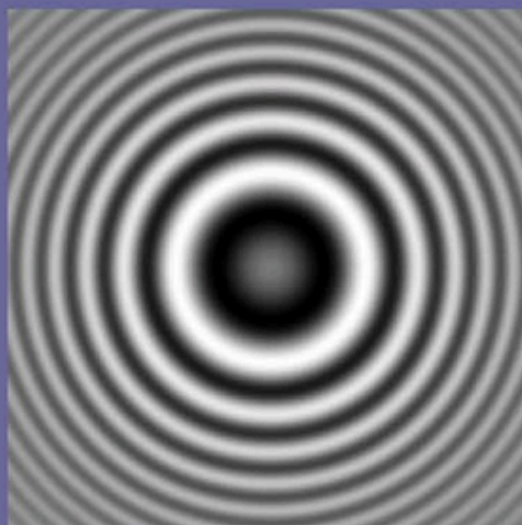
E





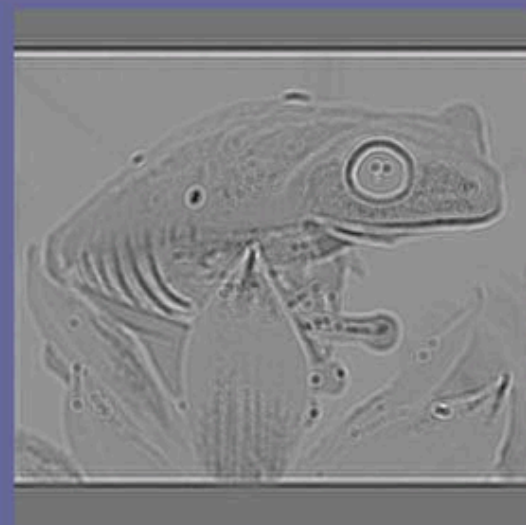
original object

\times

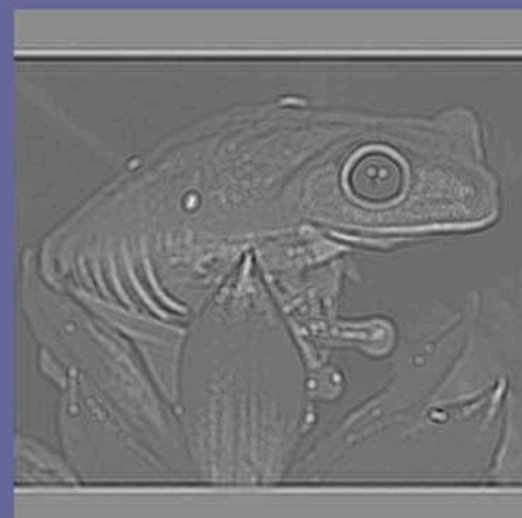
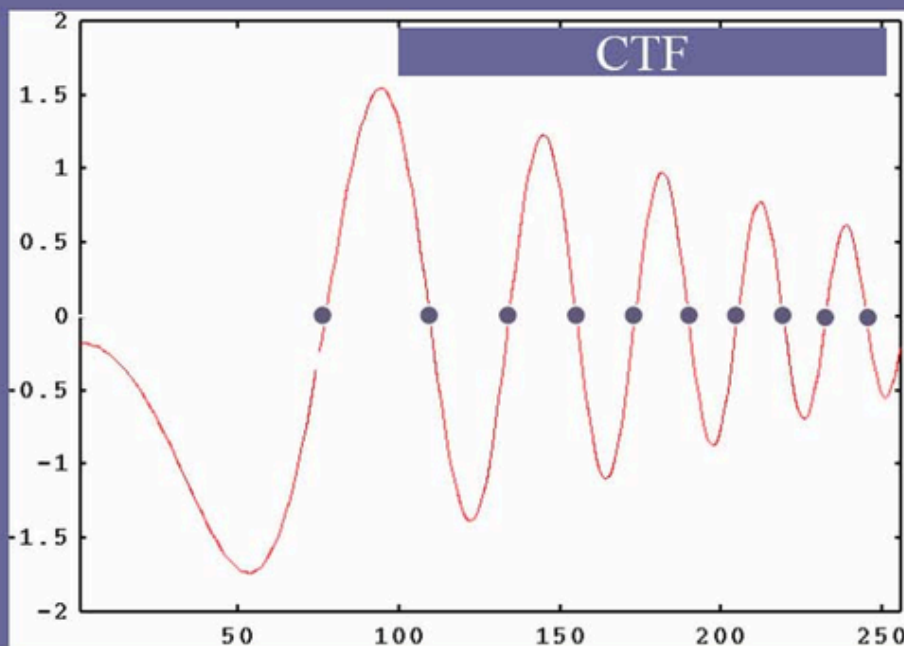


CTF for $\Delta z = 2.500 \mu\text{m}$

$=$

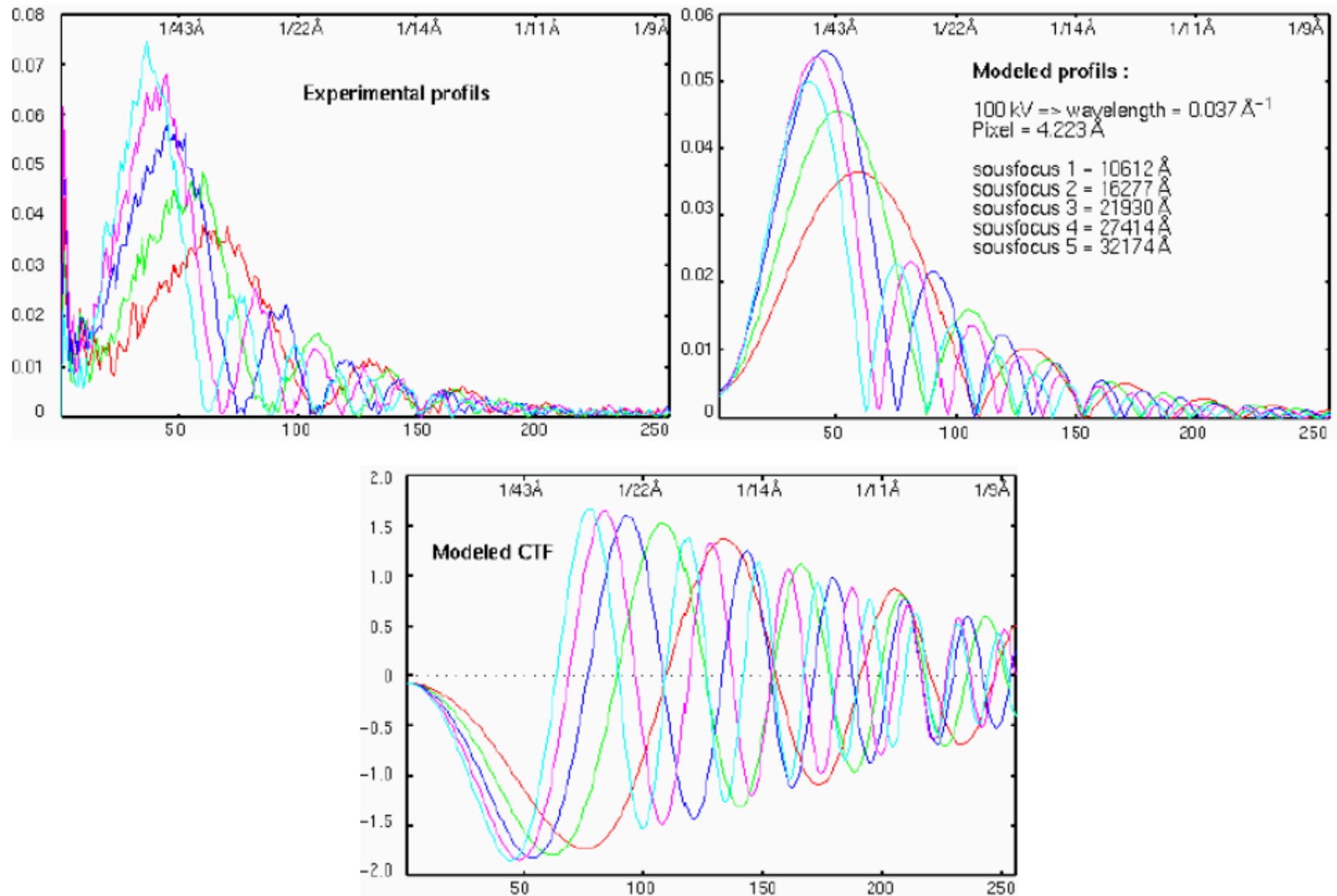


cryo-EM image

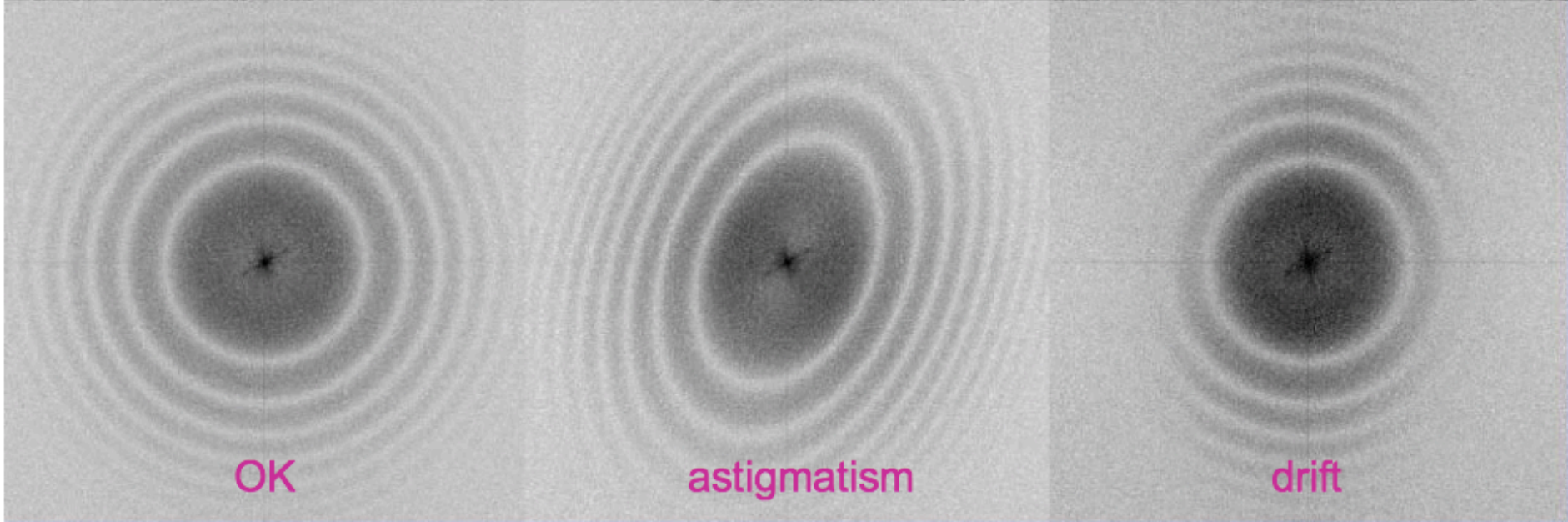
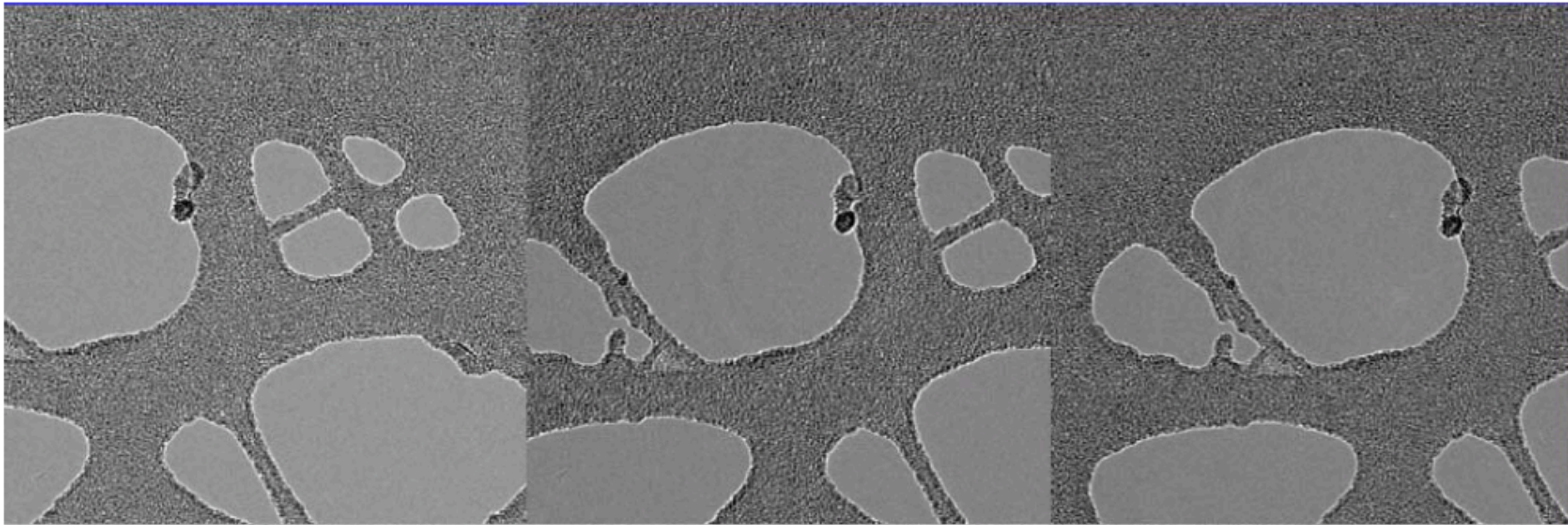


cryo-EM image,
contrast-inverted

Multiple Defocus Groups



Can You Tell the Difference?

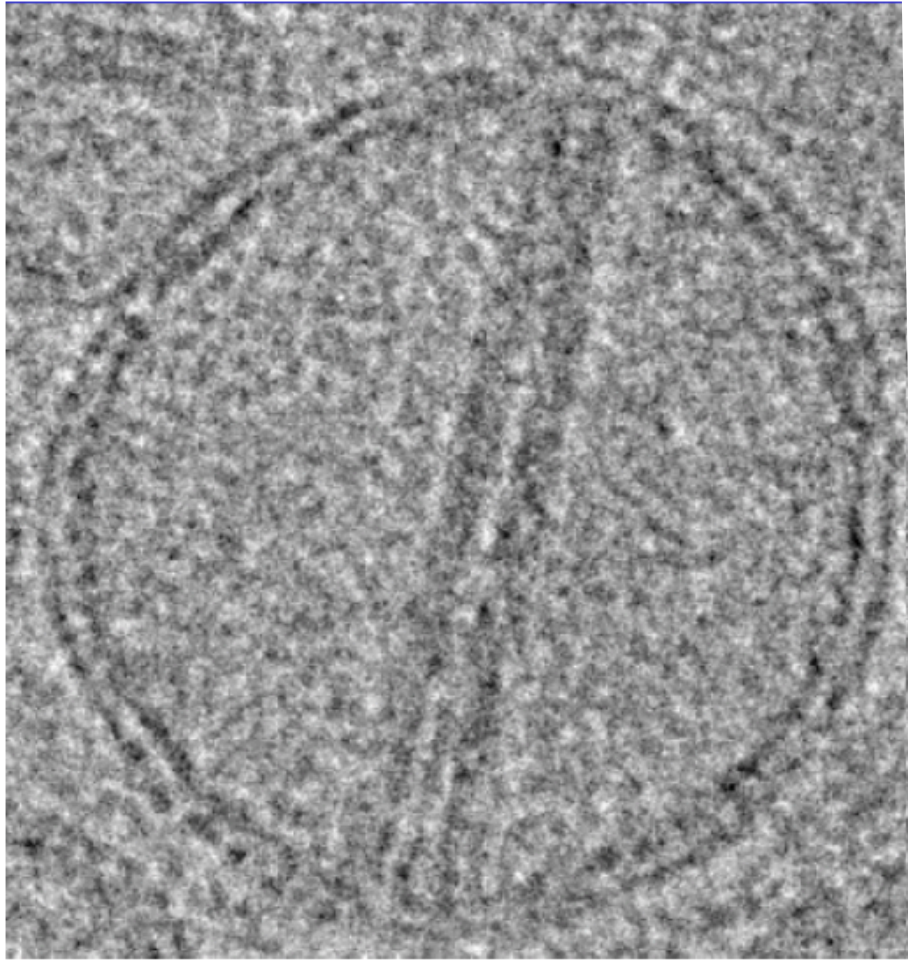


OK

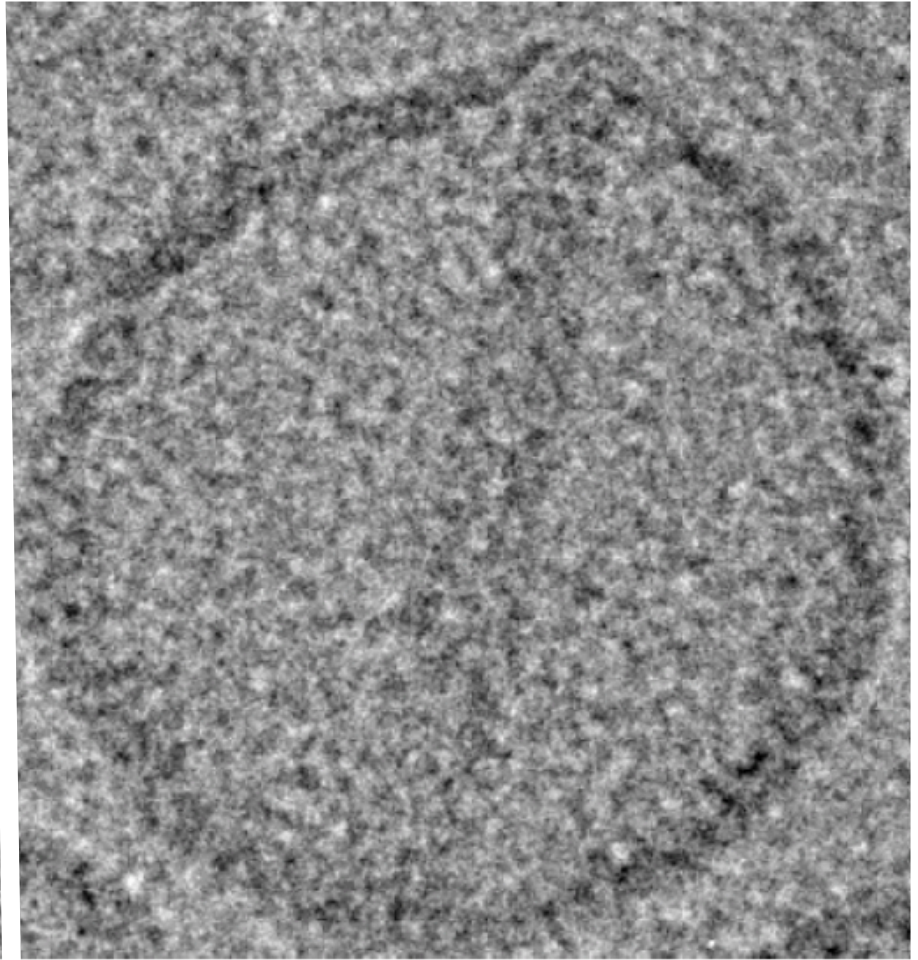
astigmatism

drift

Destructive Power of Electrons

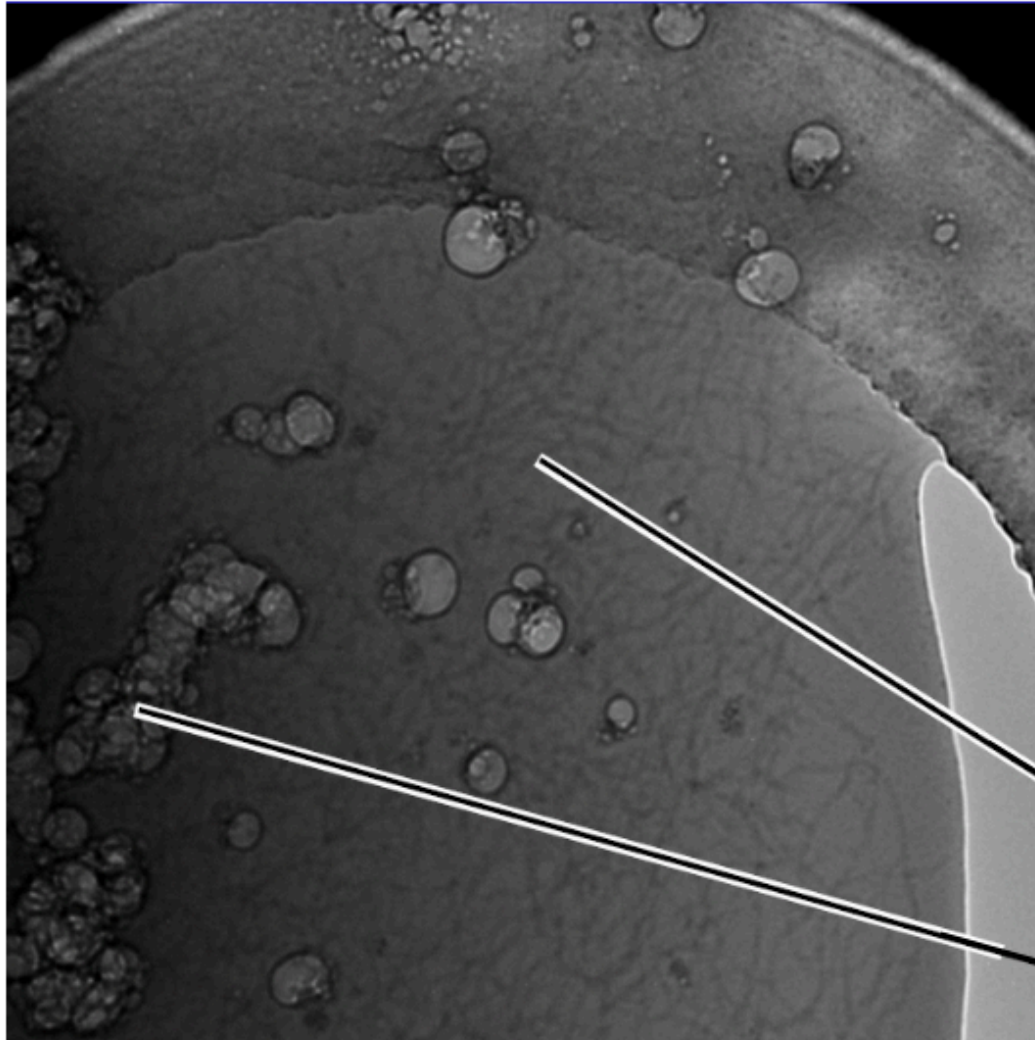


after 0.2 sec



1 sec exposure

Bubbling: A Sign of Radiation Damage



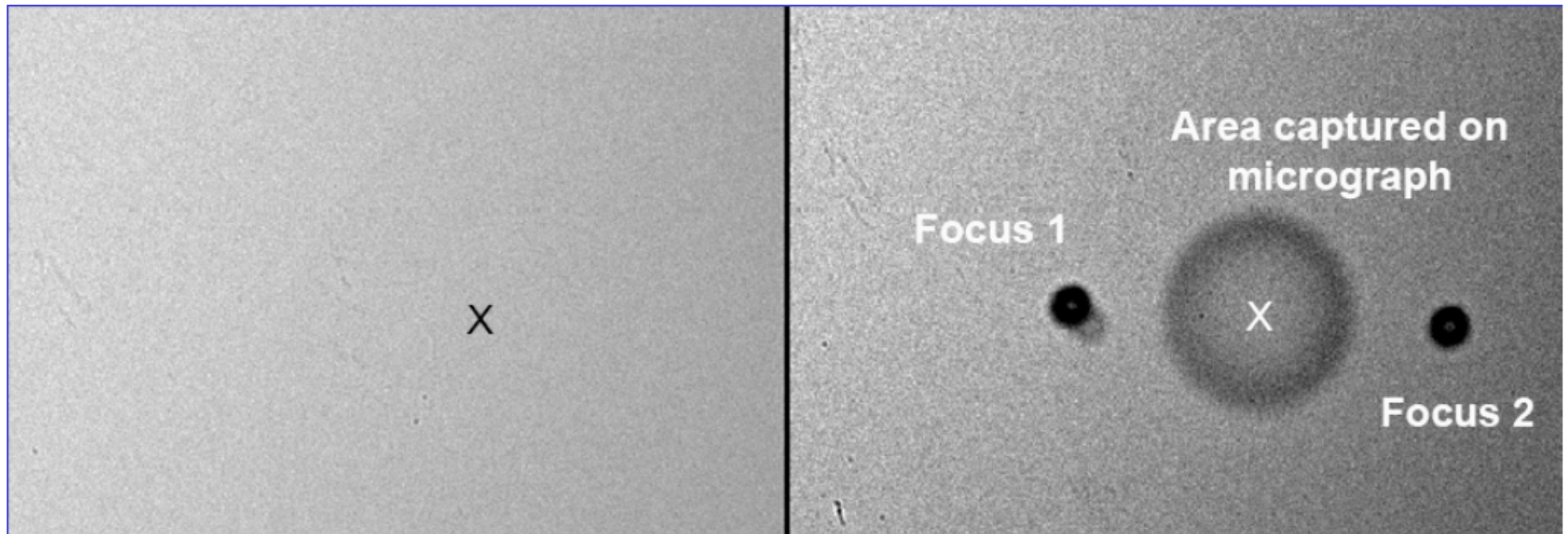
A sample of unstained amyloid materials after a few seconds of illumination with an electron beam.

While some fibers can still be detected, “bubbling” within the field of view indicates total destruction of the sample

amyloid fibers

bubbles

Low-Dose Microscopy



Appearance of trehalose dried down on a carbon film (left). The sugar allows to demonstrate how “low-dose” microscopy is done (right). Let X be the area of interest (for instance a crystal or virus/single particle). Prior to taking a picture some parameters such as “defocus” and “astigmatism” need to be adjusted. To avoid destruction of the specimen, any adjustments are made on small areas (Focus 1 and 2) located adjacent to the area that will be photographed. In the example, the trehalose burned as it was exposed at high magnification (220kx, Focus 1 and 2). Similarly, by exposing the area to be captured for about 30 seconds at 52,000 fold magnification.

Resolution Limits Due to Shot Noise

- ALBERT ROSE DETERMINED A QUANTITATIVE RELATIONSHIP BETWEEN FEATURE SIZE AND VISUAL DETECTABILITY:

$$d C > 5 / (N)^{1/2}$$

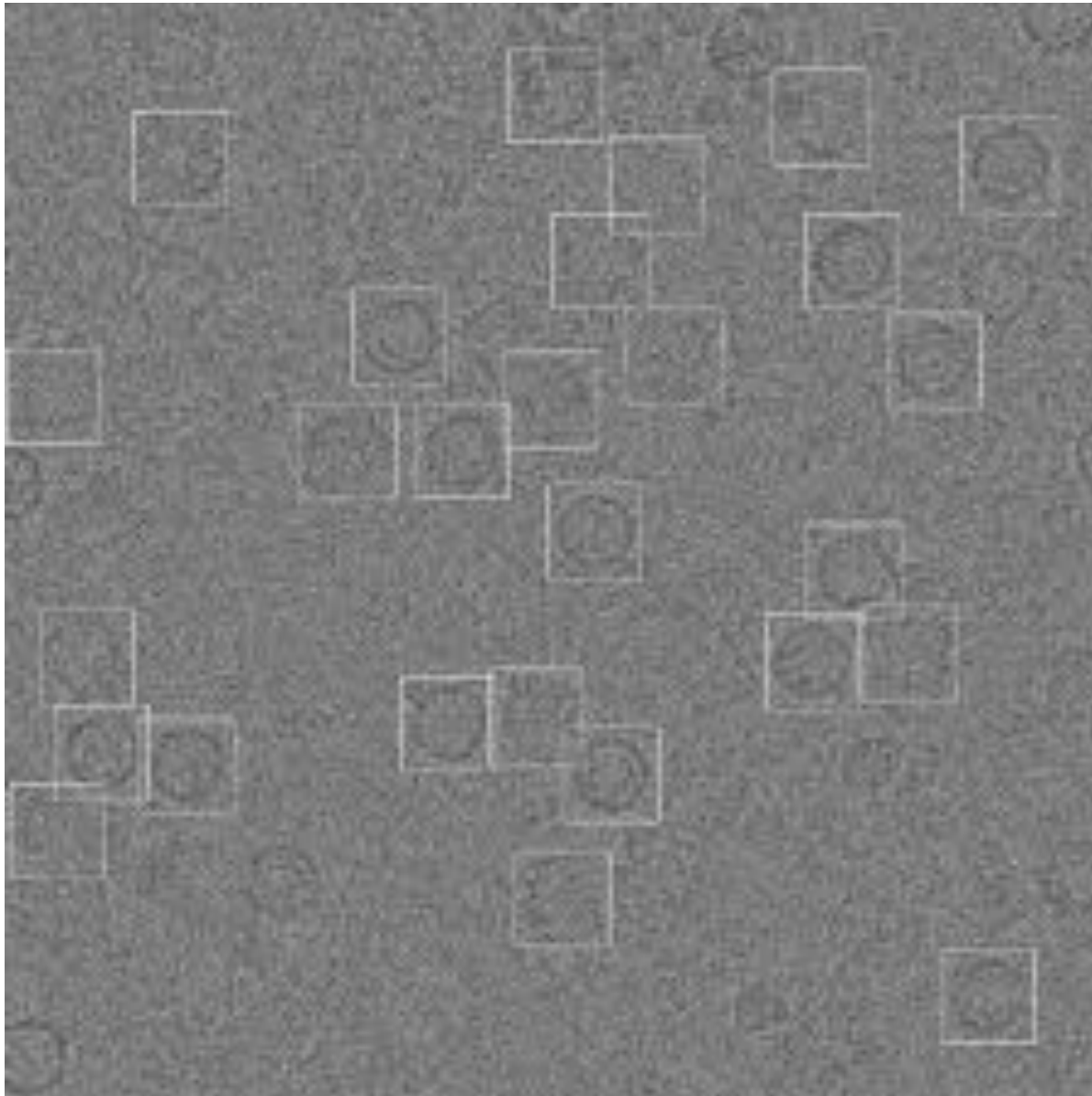
WHERE “N” IS THE NUMBER OF QUANTA PER UNIT AREA

- FEATURES SMALLER THAN 25 λ MAY NOT BE DETECTABLE FOR EXPOSURES AS LOW AS 25 e/A²
- THE ONLY WAY TO OVERCOME THIS LIMITATION IS TO AVERAGE INDEPENDENT IMAGES OF IDENTICAL OBJECTS

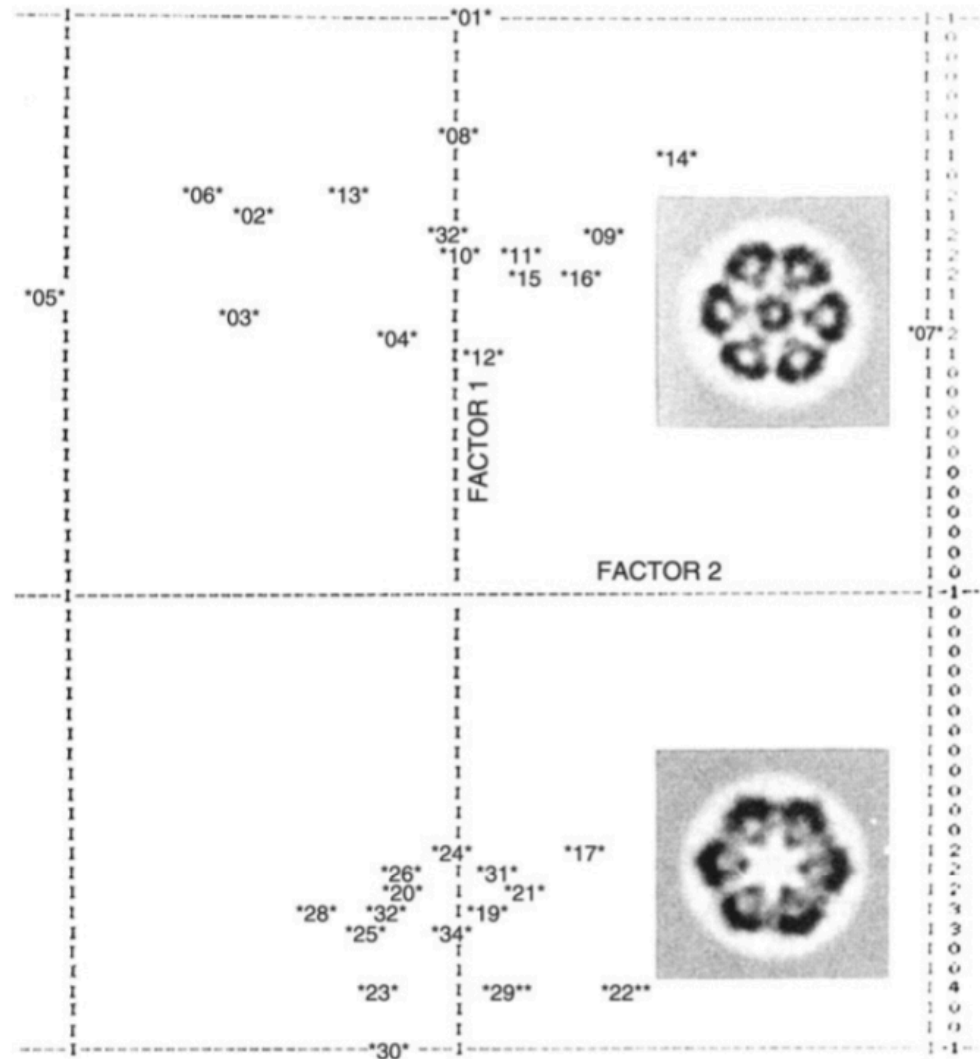
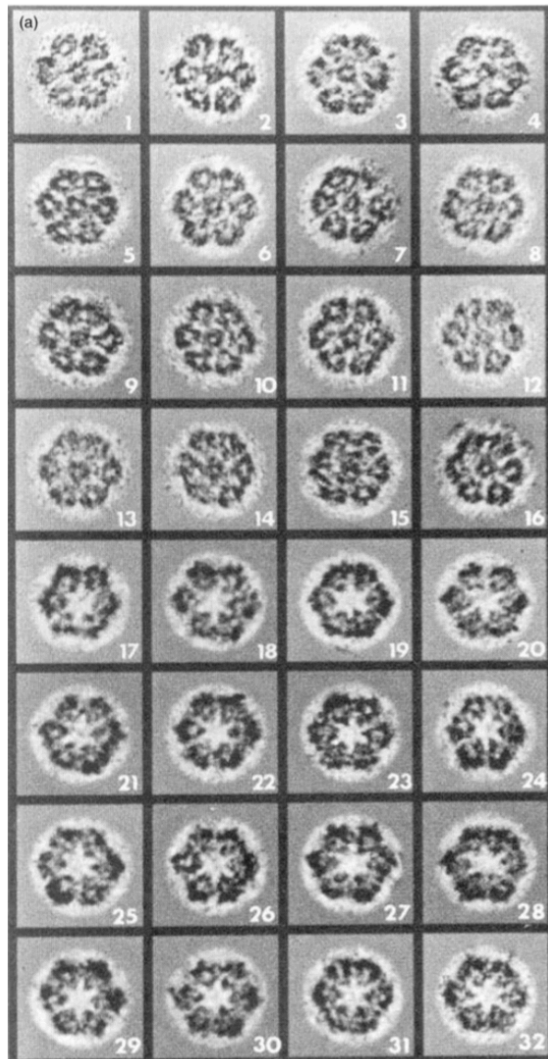


Picture	Number of photons	High-light brightness, foot-lamberts
a	3×10^3	10^{-6}
b	1.2×10^4	4×10^{-6}
c	9.3×10^4	3×10^{-5}
d	7.6×10^5	2.5×10^{-4}
e	3.6×10^6	1.2×10^{-3}
f	2.8×10^7	9.5×10^{-3}

Signal to noise ratio



Classification and averaging (principal component analysis)



Overview of steps

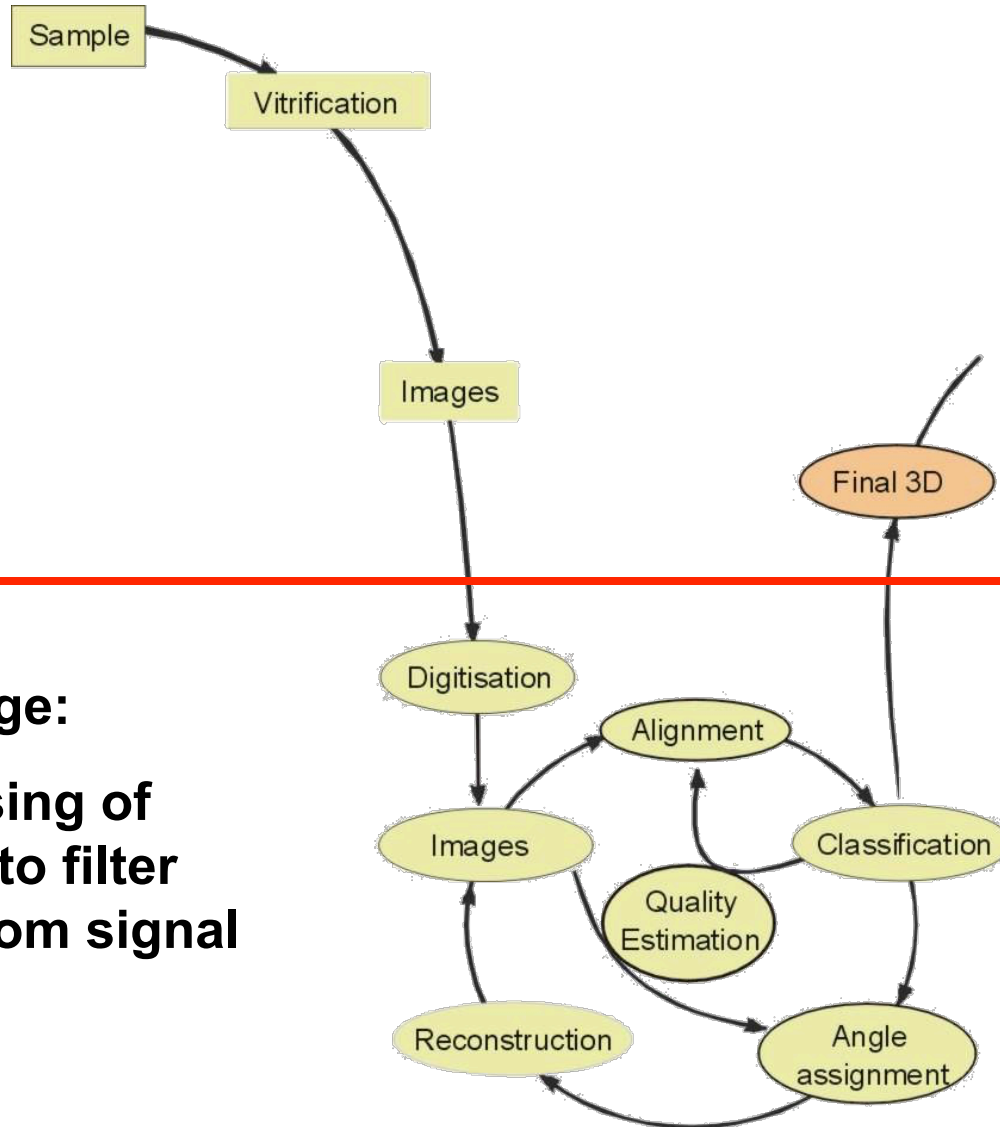
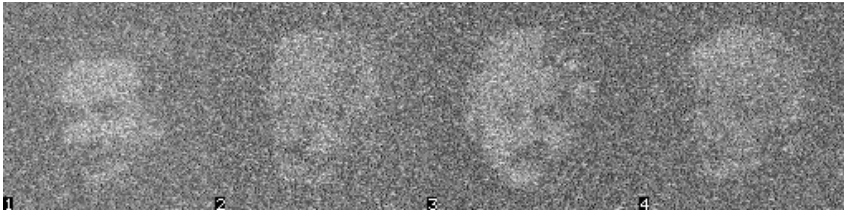
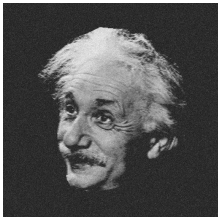


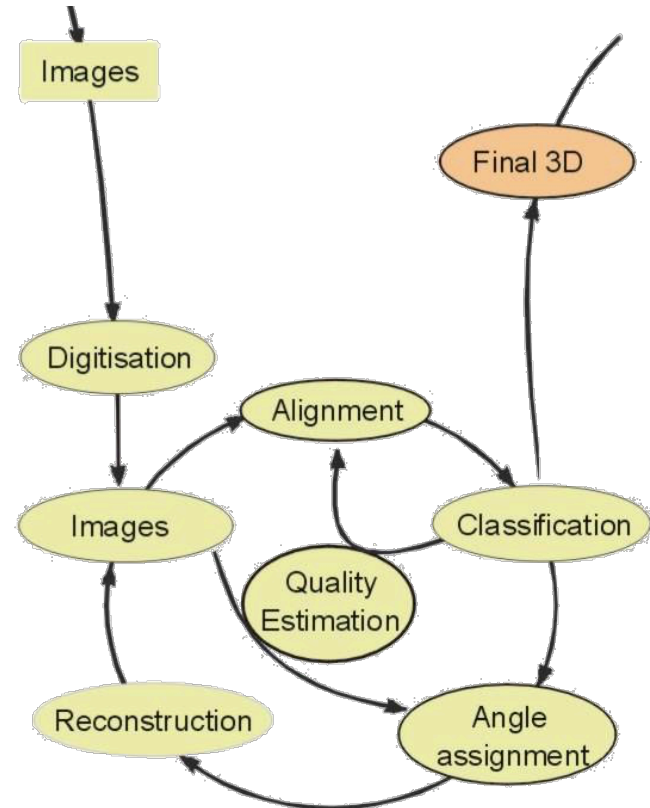
Image Processing



Improve signal to noise by
Overlaying many pictures



Problem:
objects are arranged in
different orientations



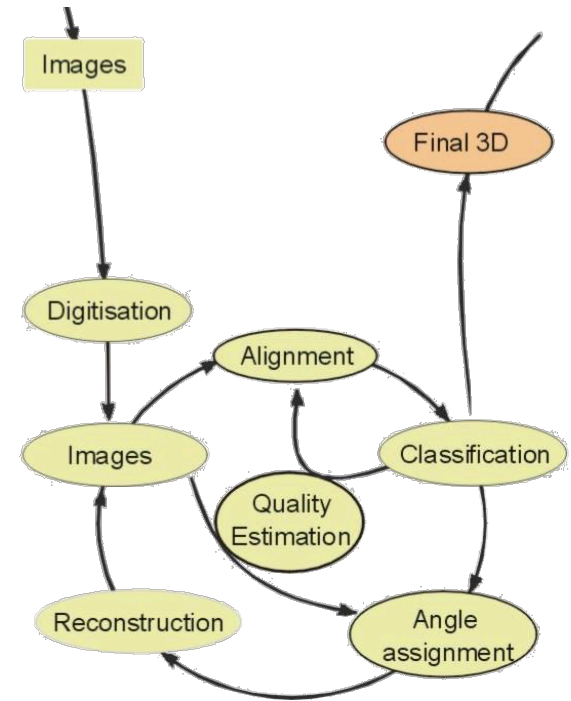
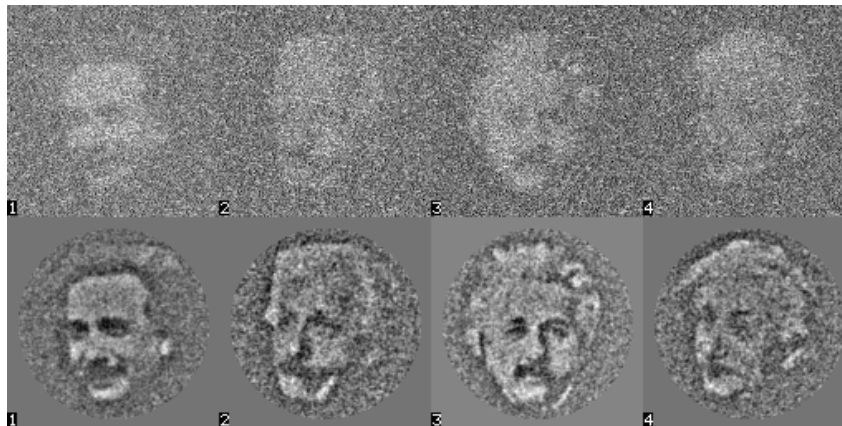
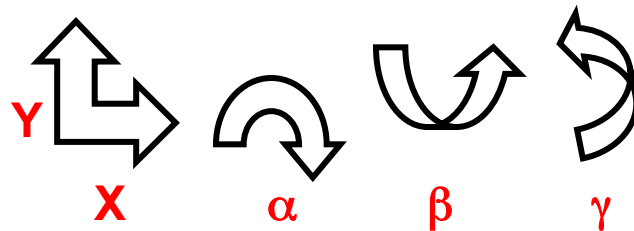
Alignment & Classification

Rough alignment of pictures (up to 100,000)

Correspondence analysis groups images using statistical methods

Challenging process requiring at least 5 parameters

Images with same orientation will be averaged (class averages)

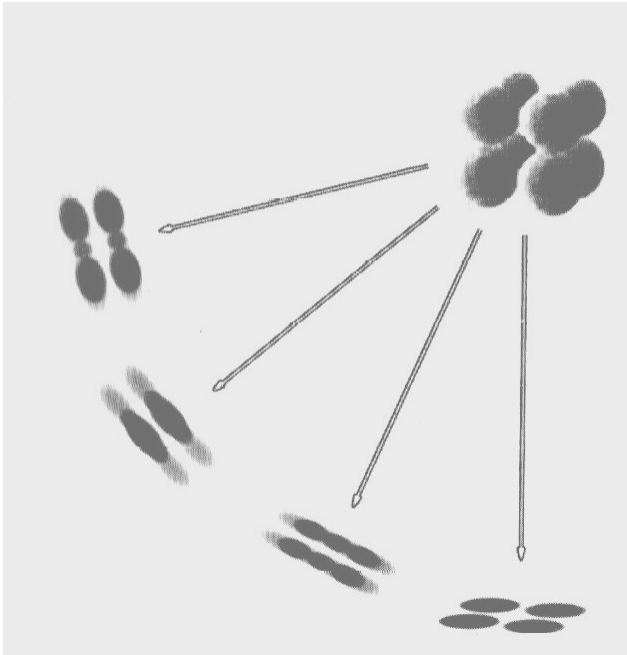


Raw Images

Class Averages

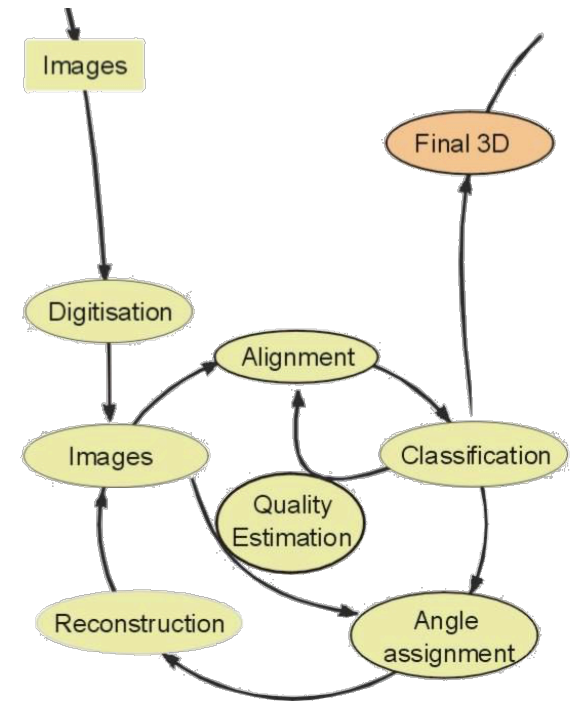
3D Reconstruction

When the angles between the different classes are known (estimated), a 3D model can be calculated.



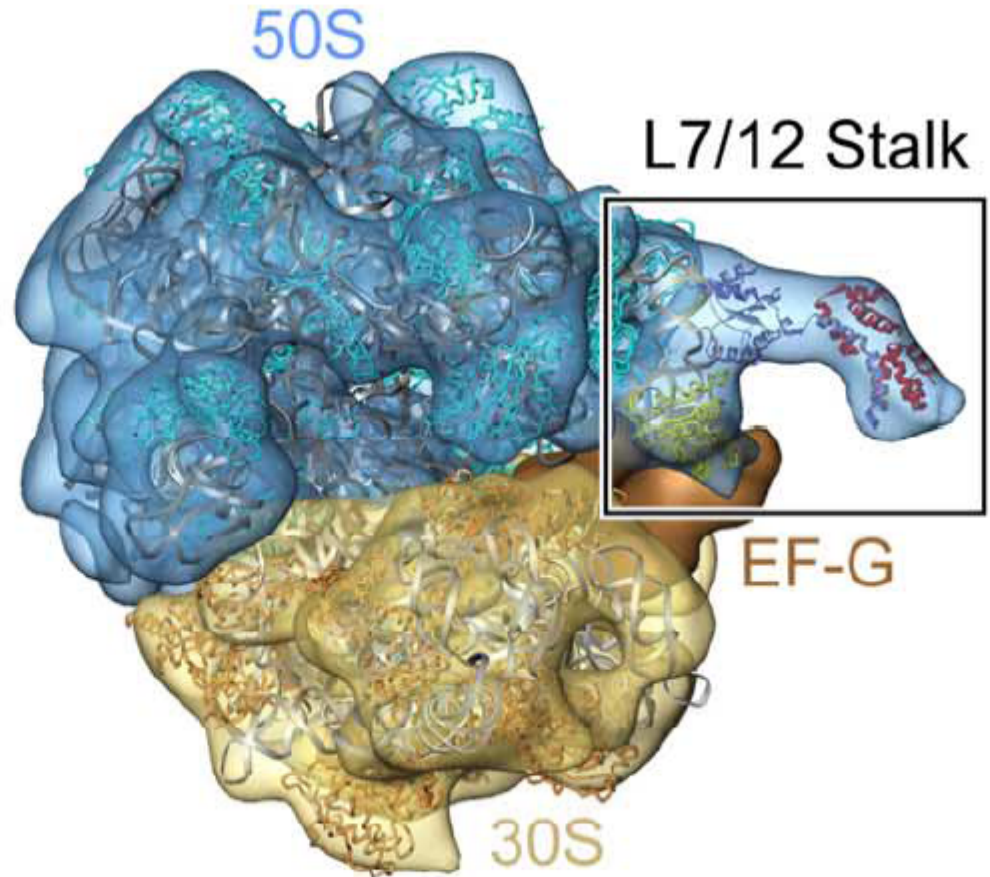
Iterative Process:

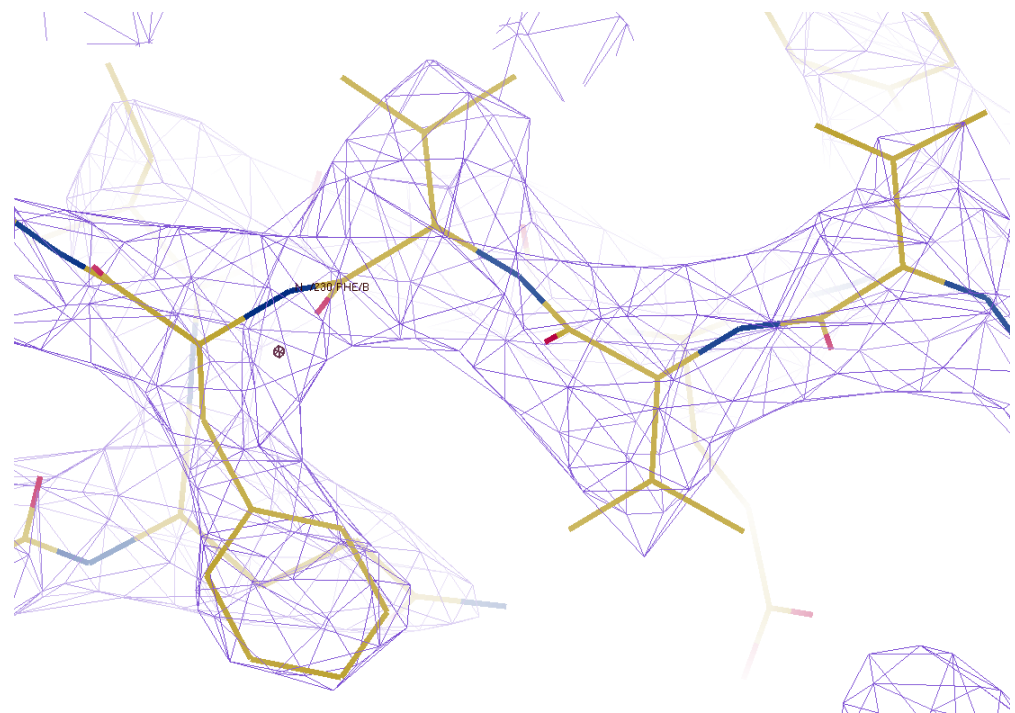
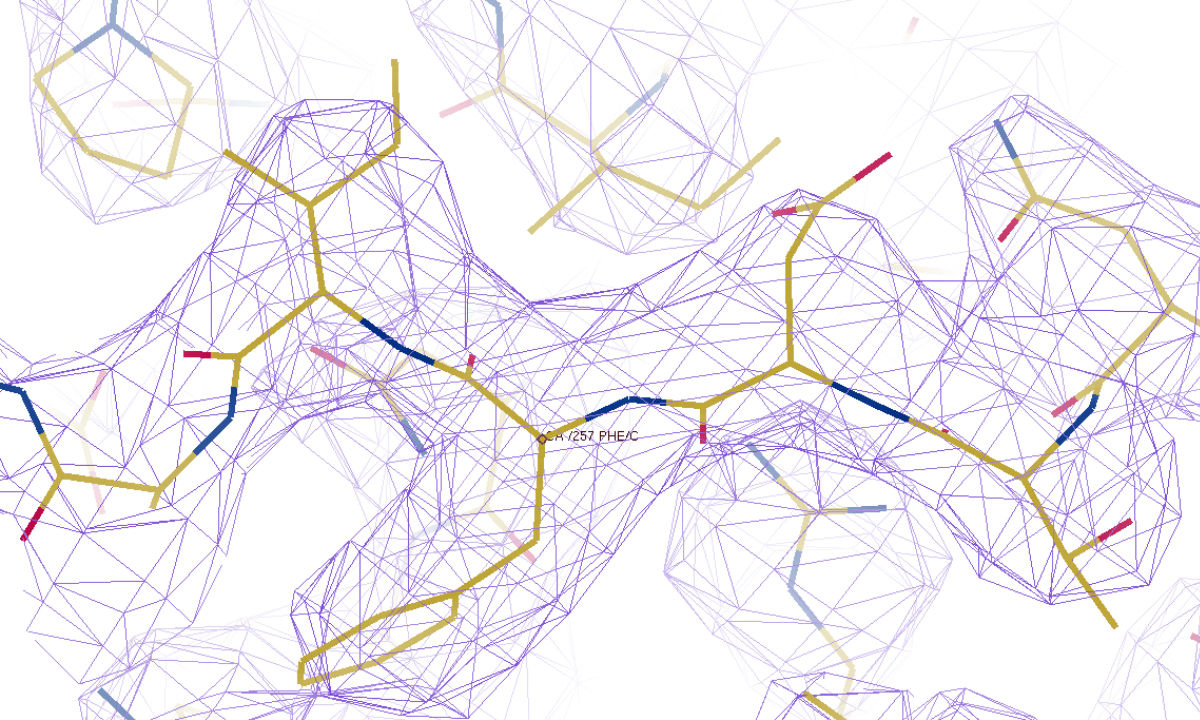
3D model is used to generate 2D images which are fed into statistical analysis of images (alignment and classification).

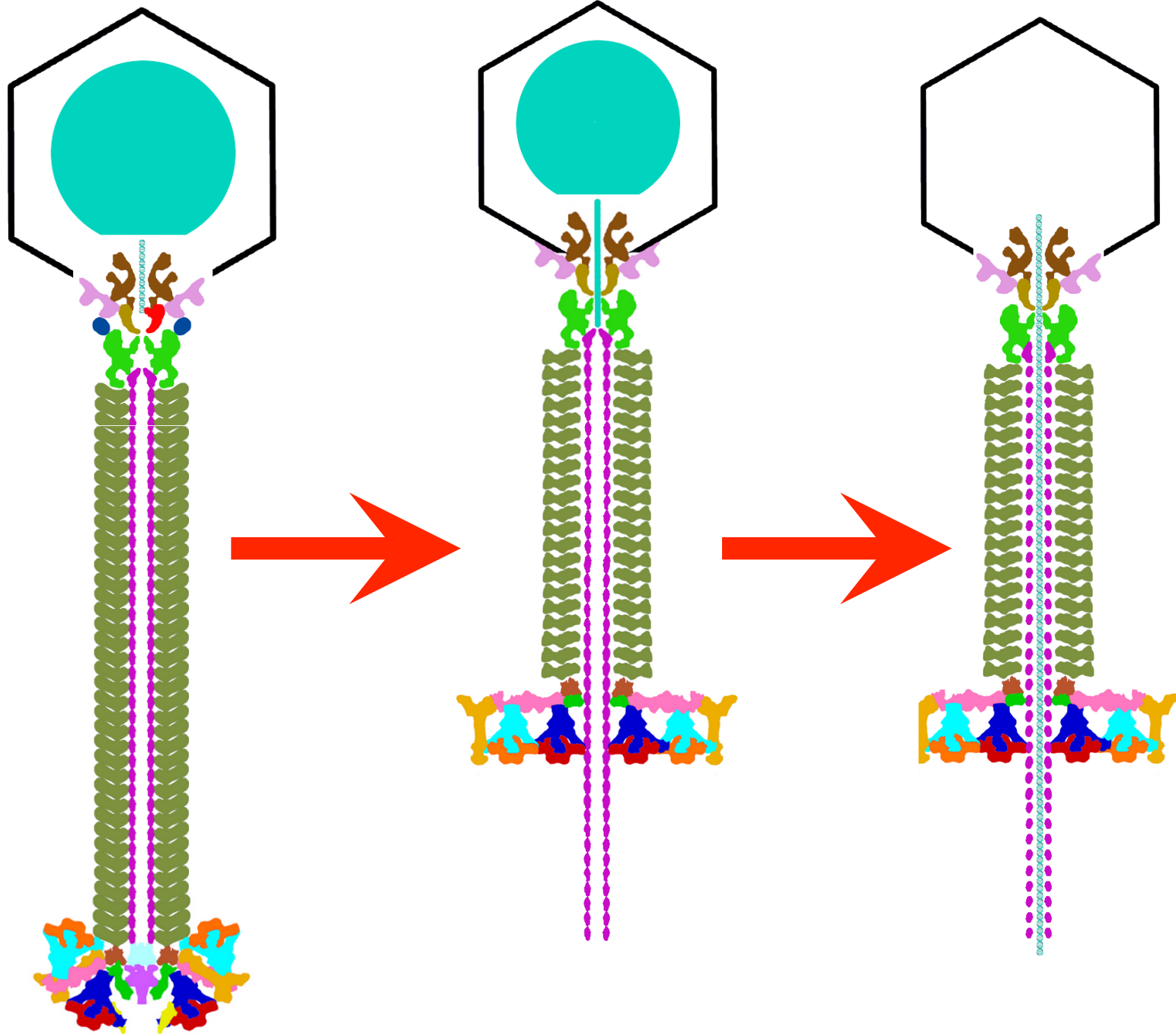


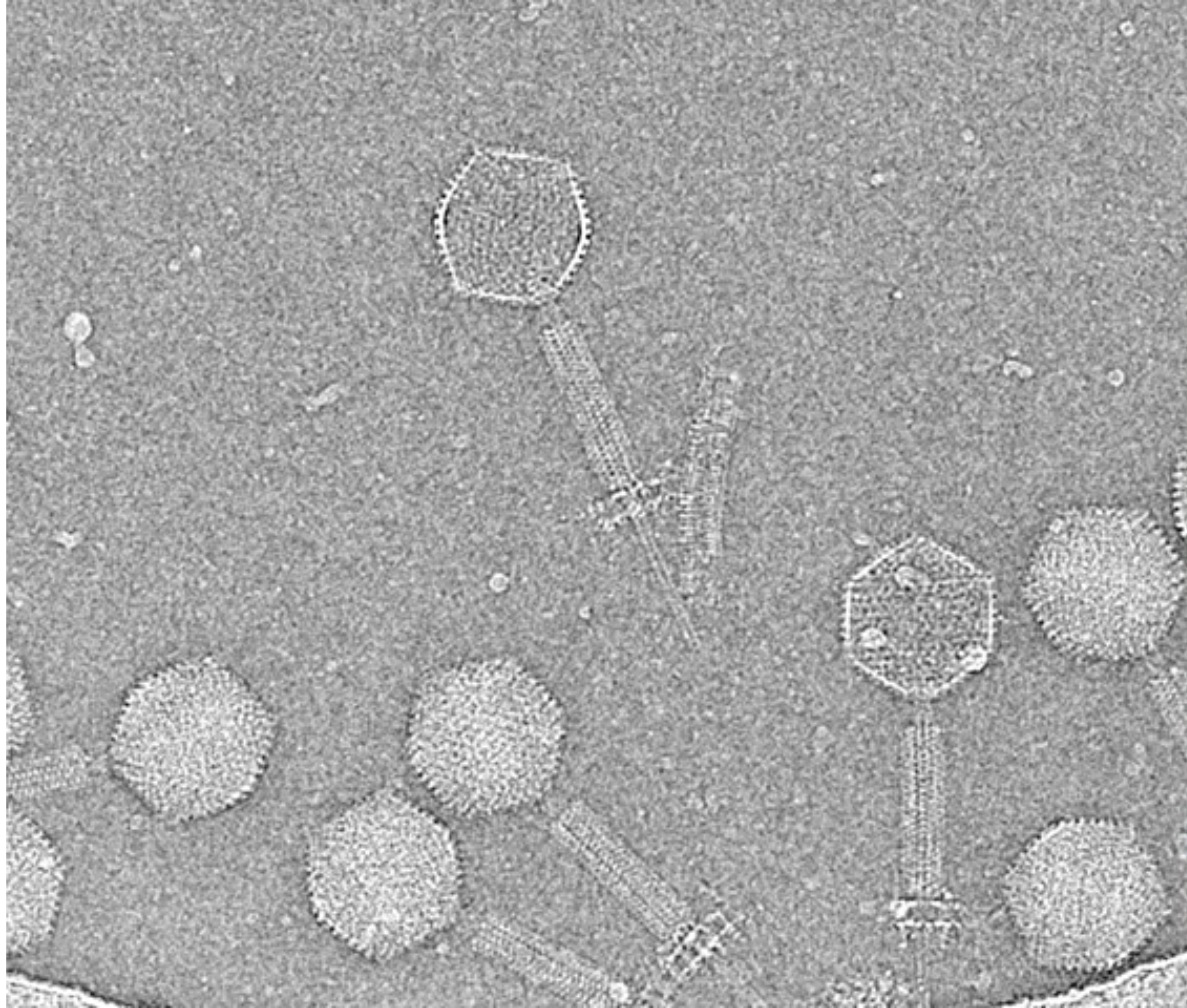
And then?

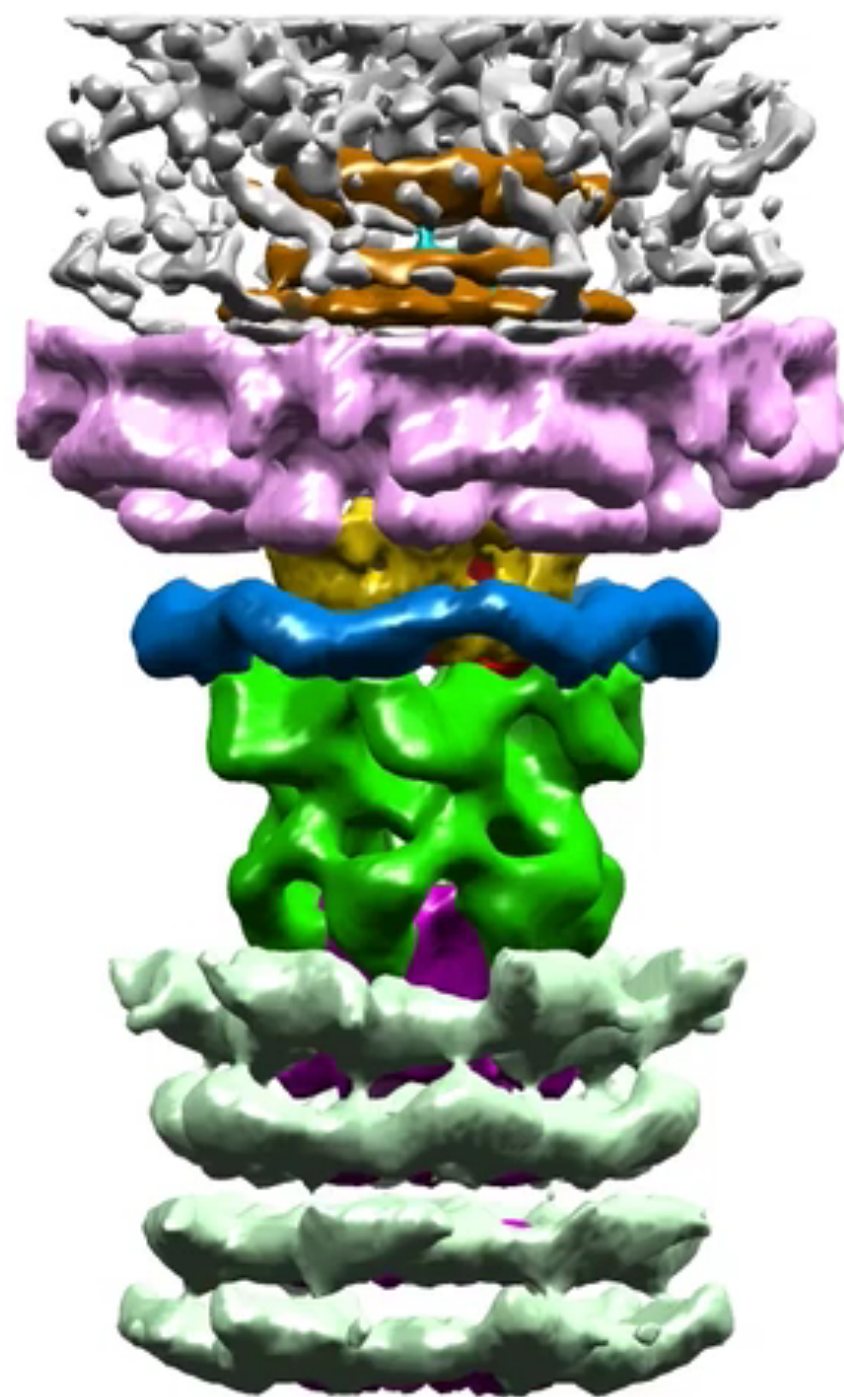
Try to interpret 3D map,
e.g. try to fit known
crystal structures into
electron density map

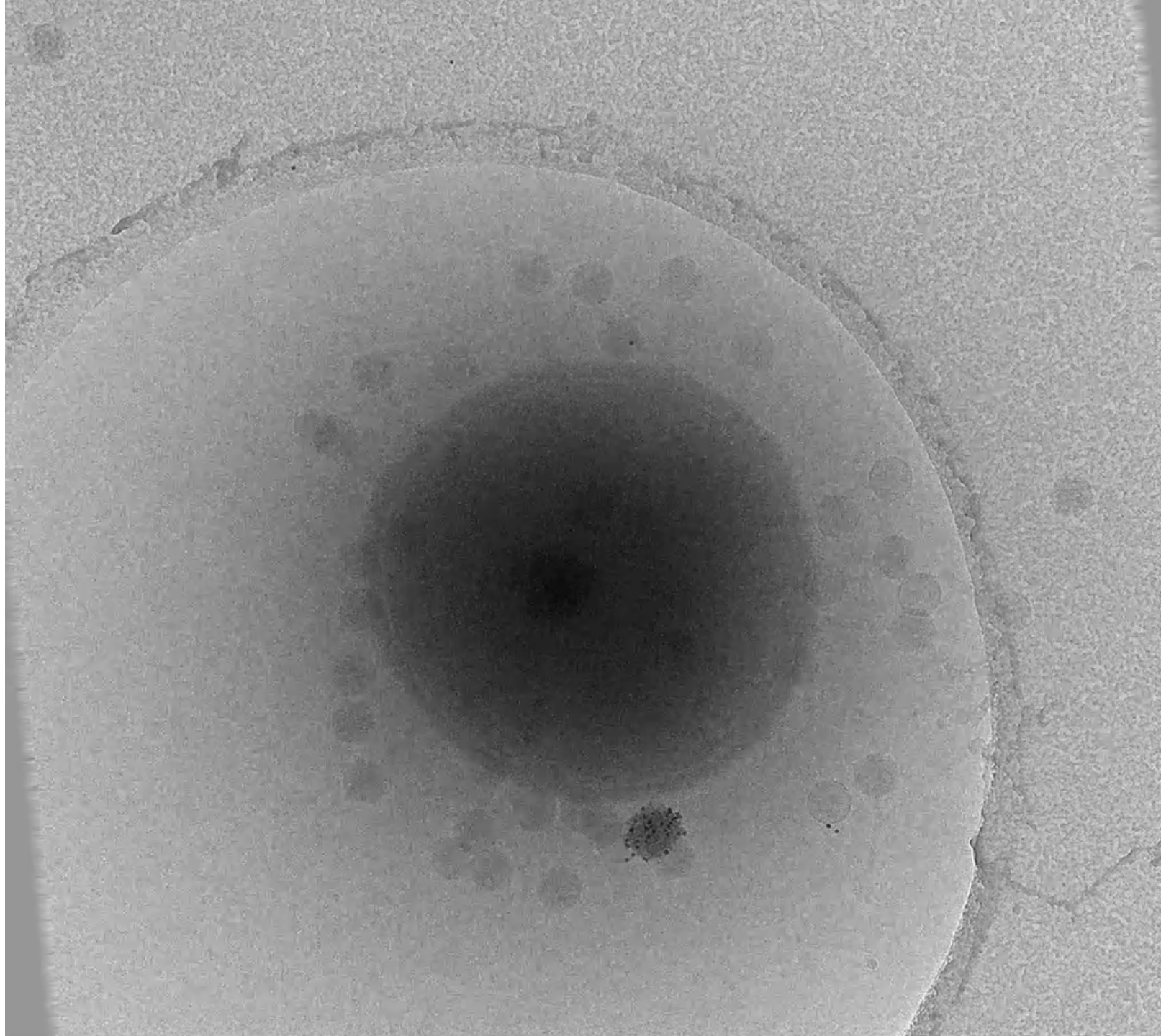




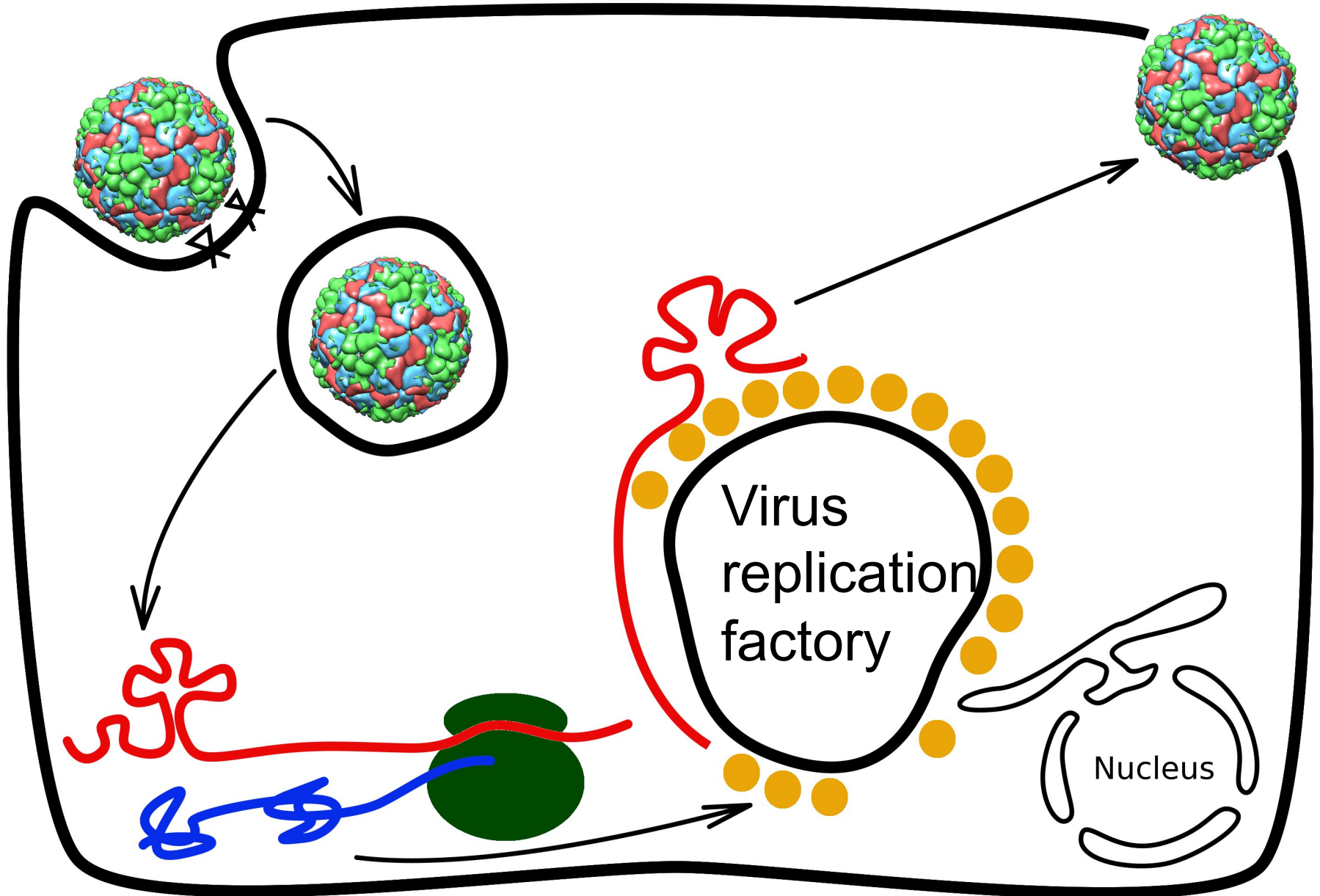




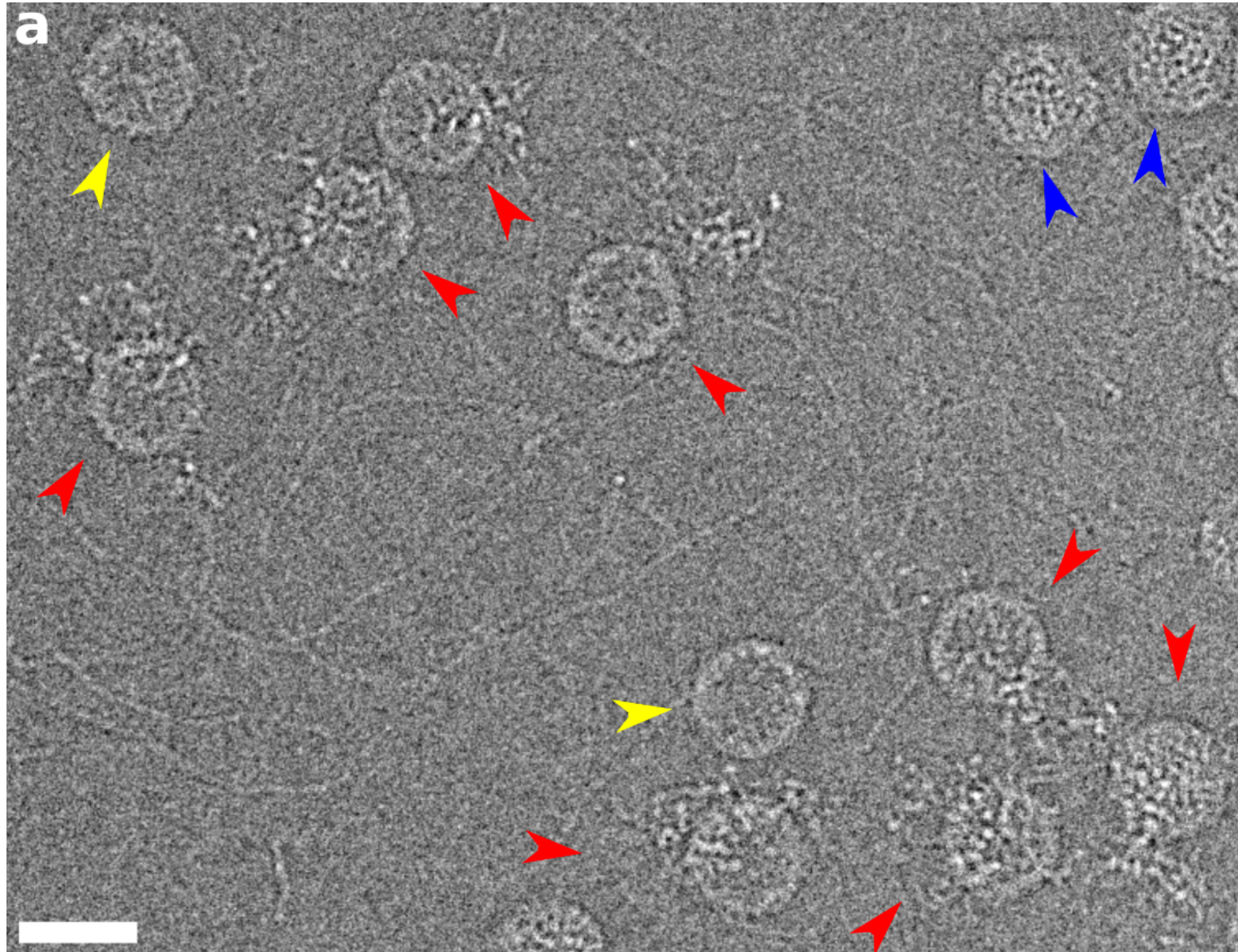




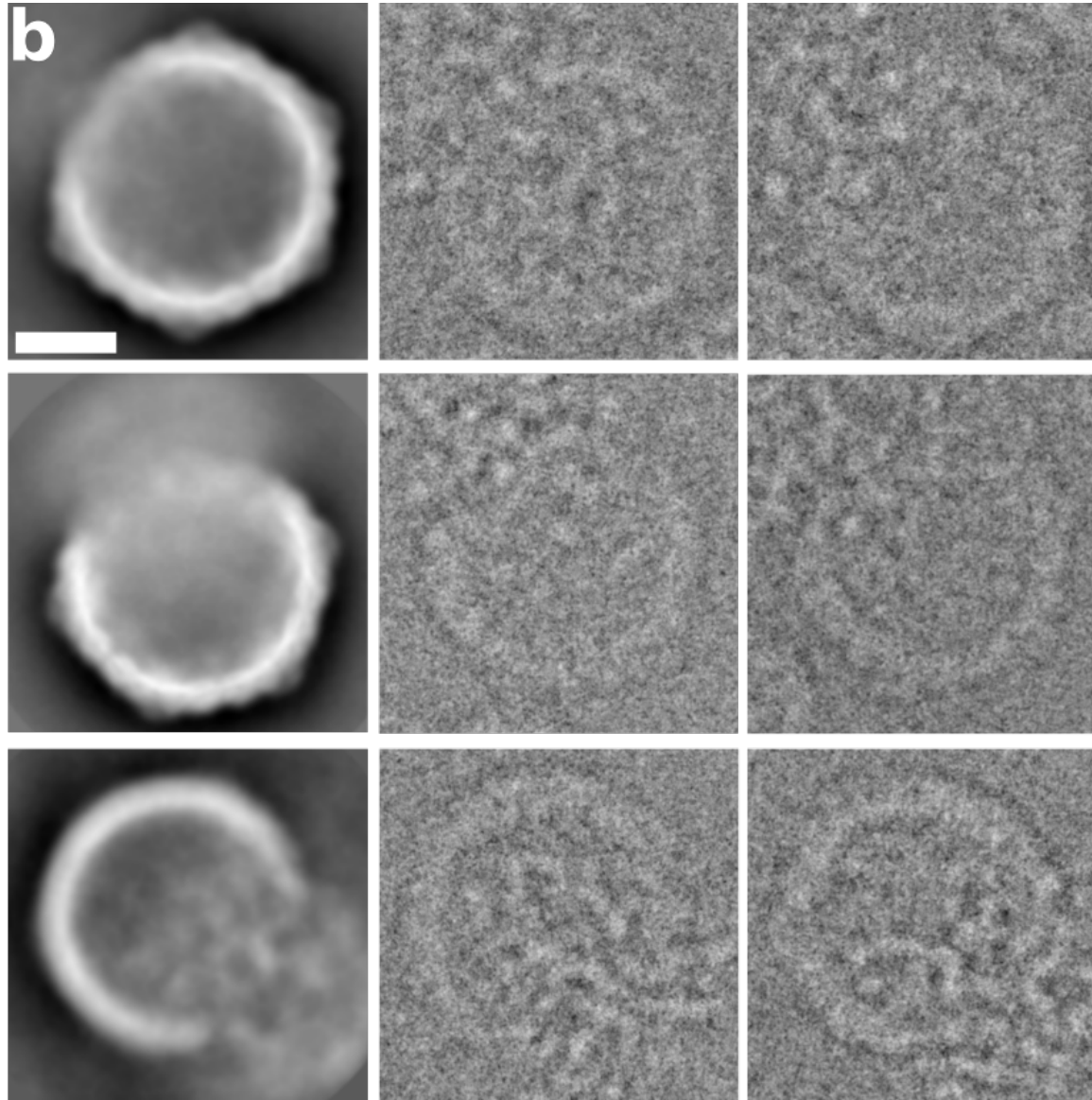
Enterovirus replication cycle



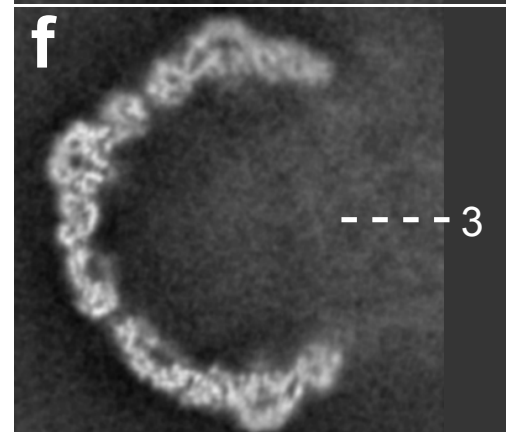
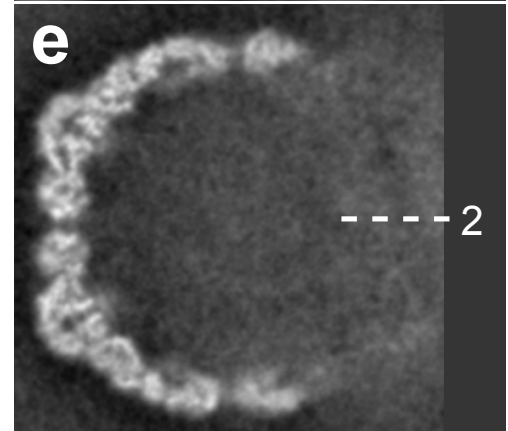
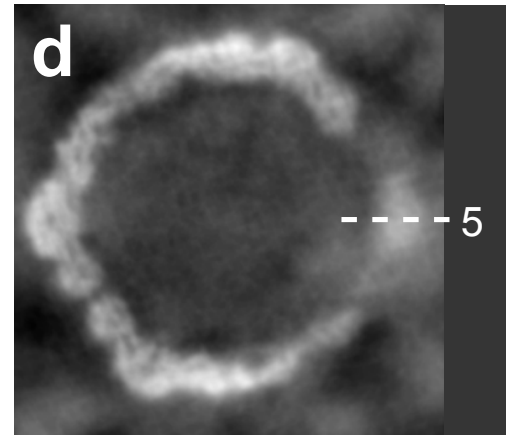
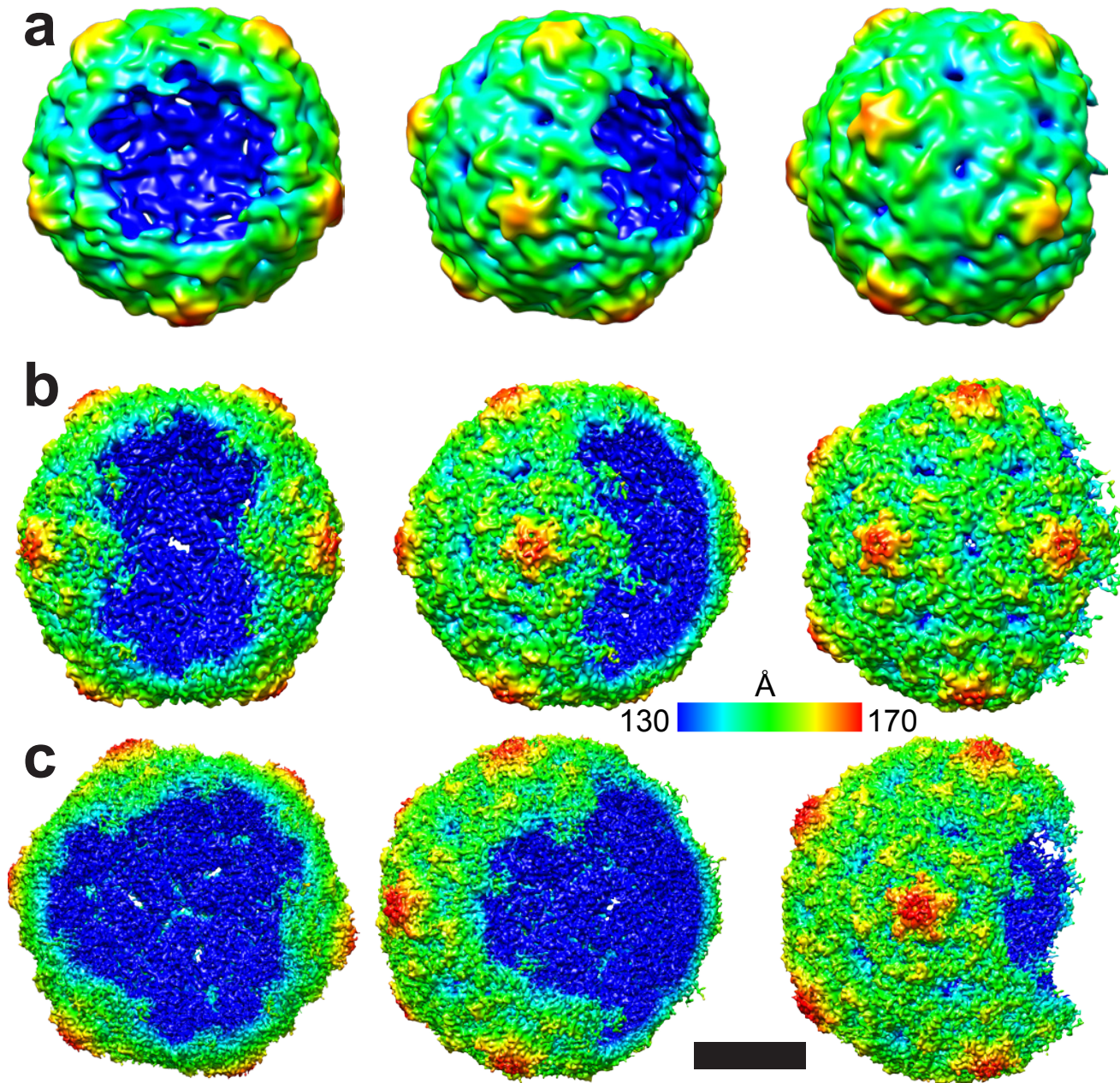
Acidic pH induced genome release of echovirus 18



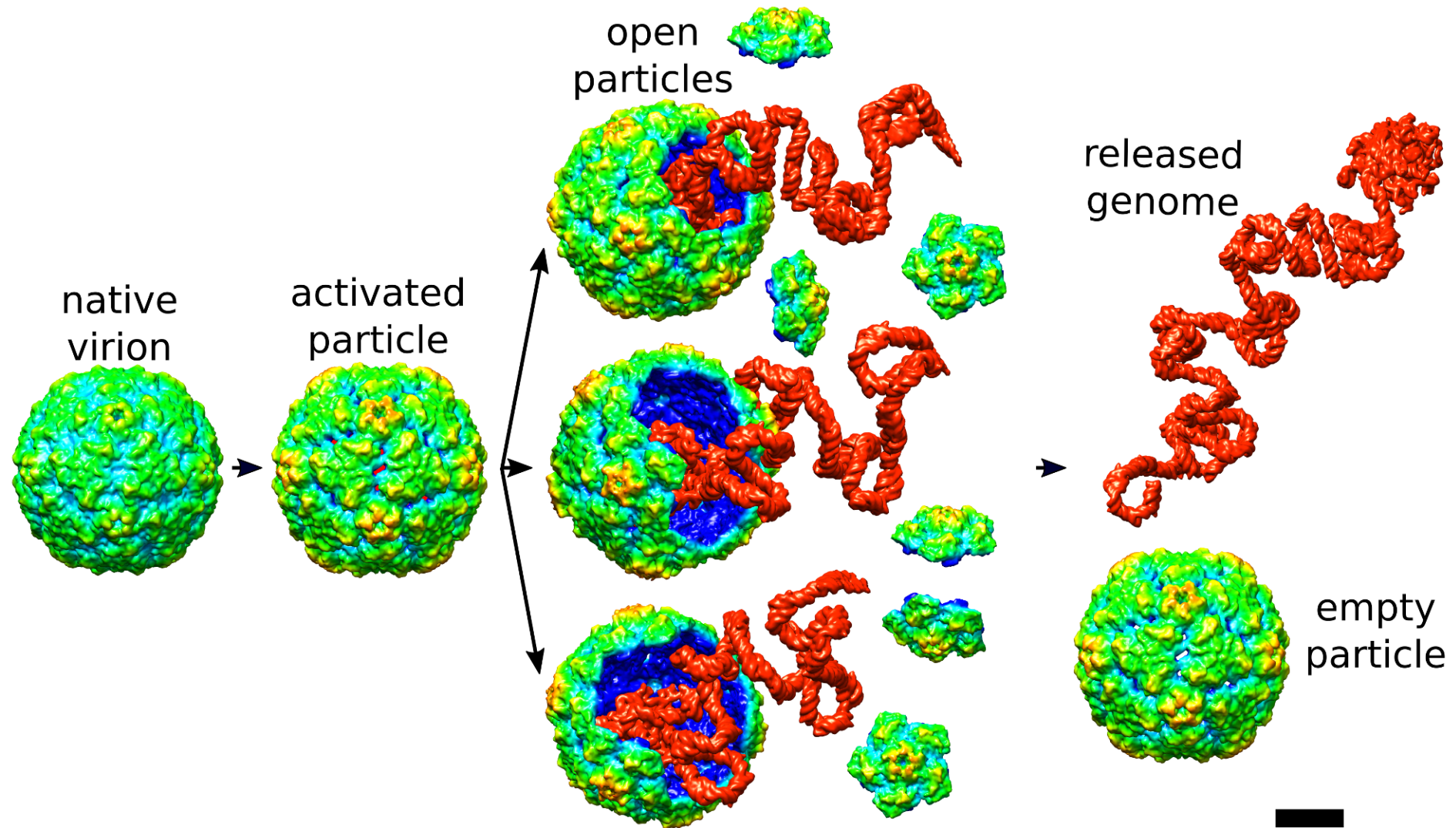
Reference-free two-dimensional class averages of genome-releasing particles



Asymmetric structures of open particles



Model of enterovirus genome release



1. Ktere z nize uvedených zření a částic interaguje nejsilněji s biologickým materiálem?

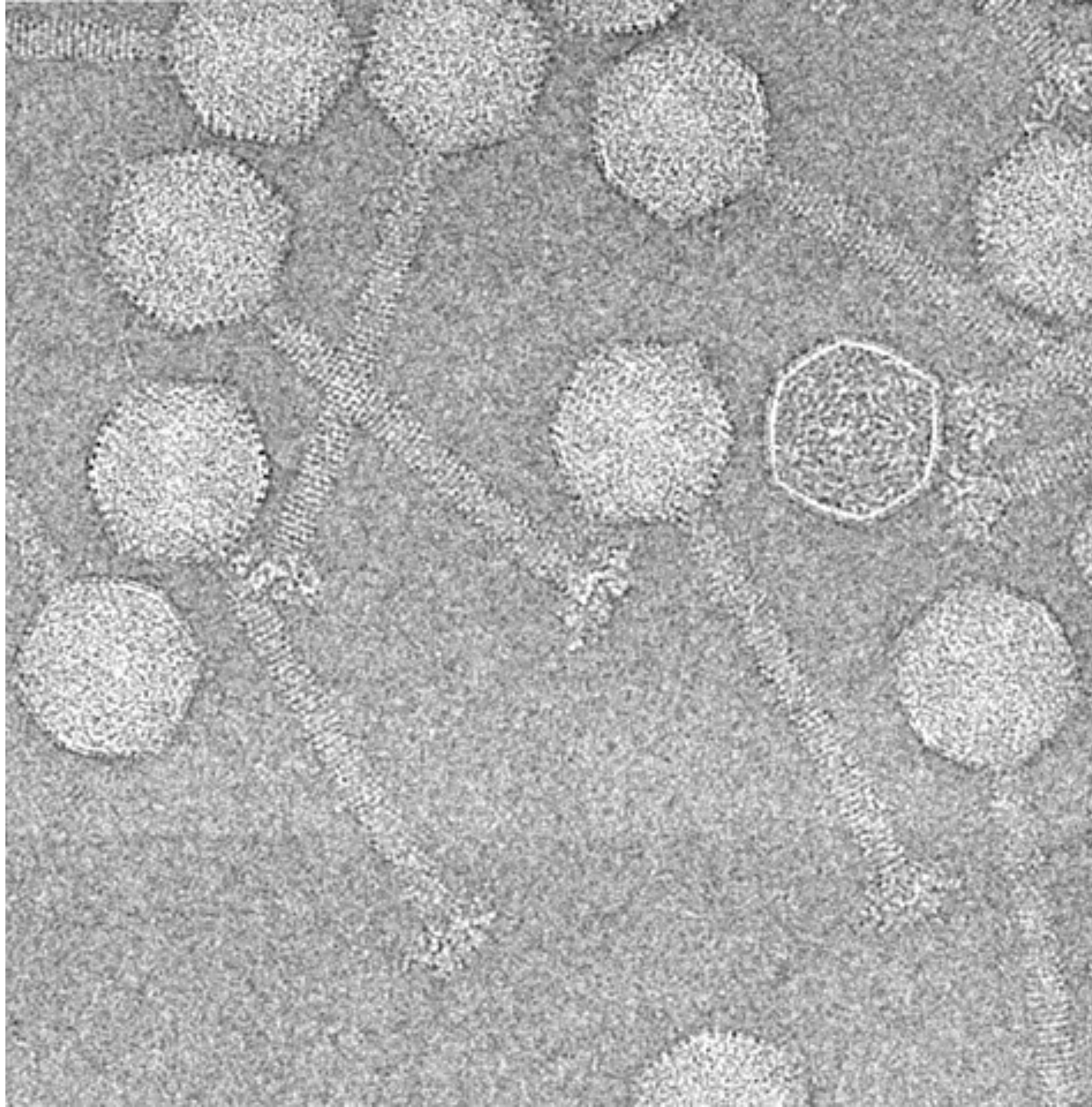
- a) elektrony
- b) viditelné světlo
- c) paprsky X

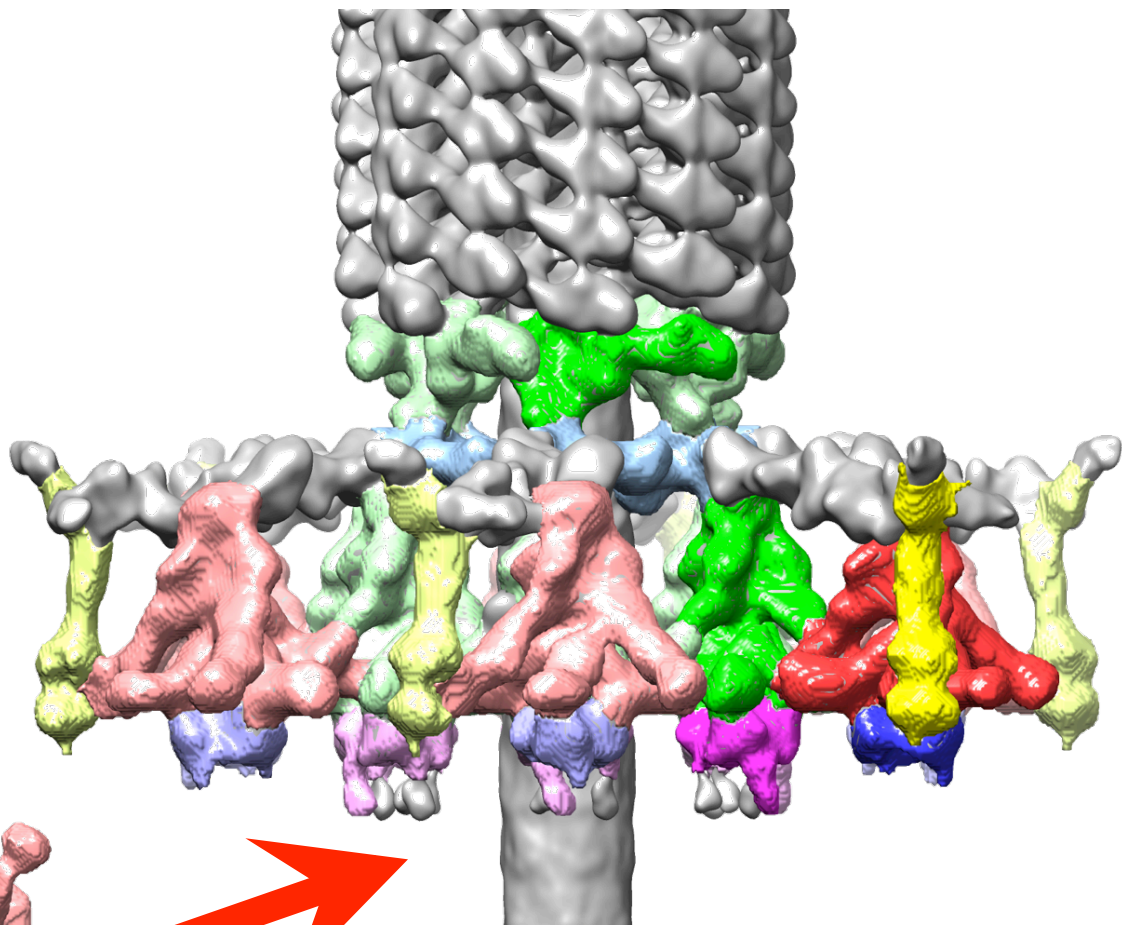
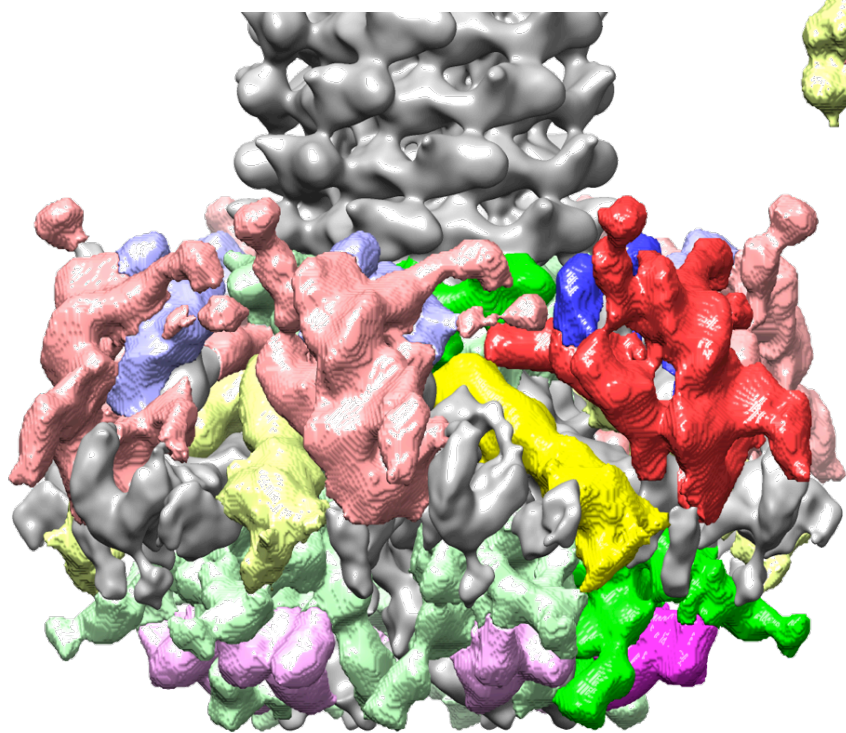
2. Jaké je v současnosti nejvyšší rozlišení dosažené při studiu makromolekul pomocí kryo-elektronové mikroskopie?

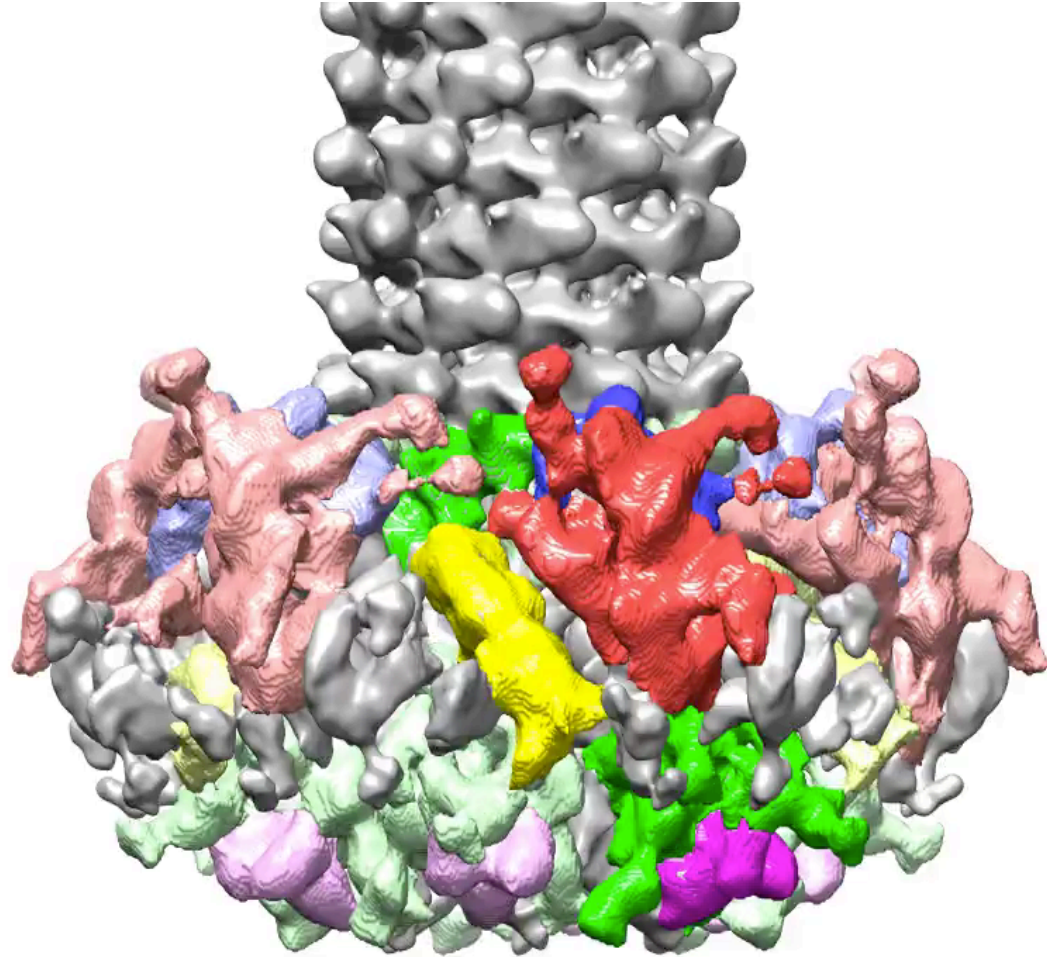
- a) 1.8 Å
- b) 0.23 μm
- c) 0.5 nm

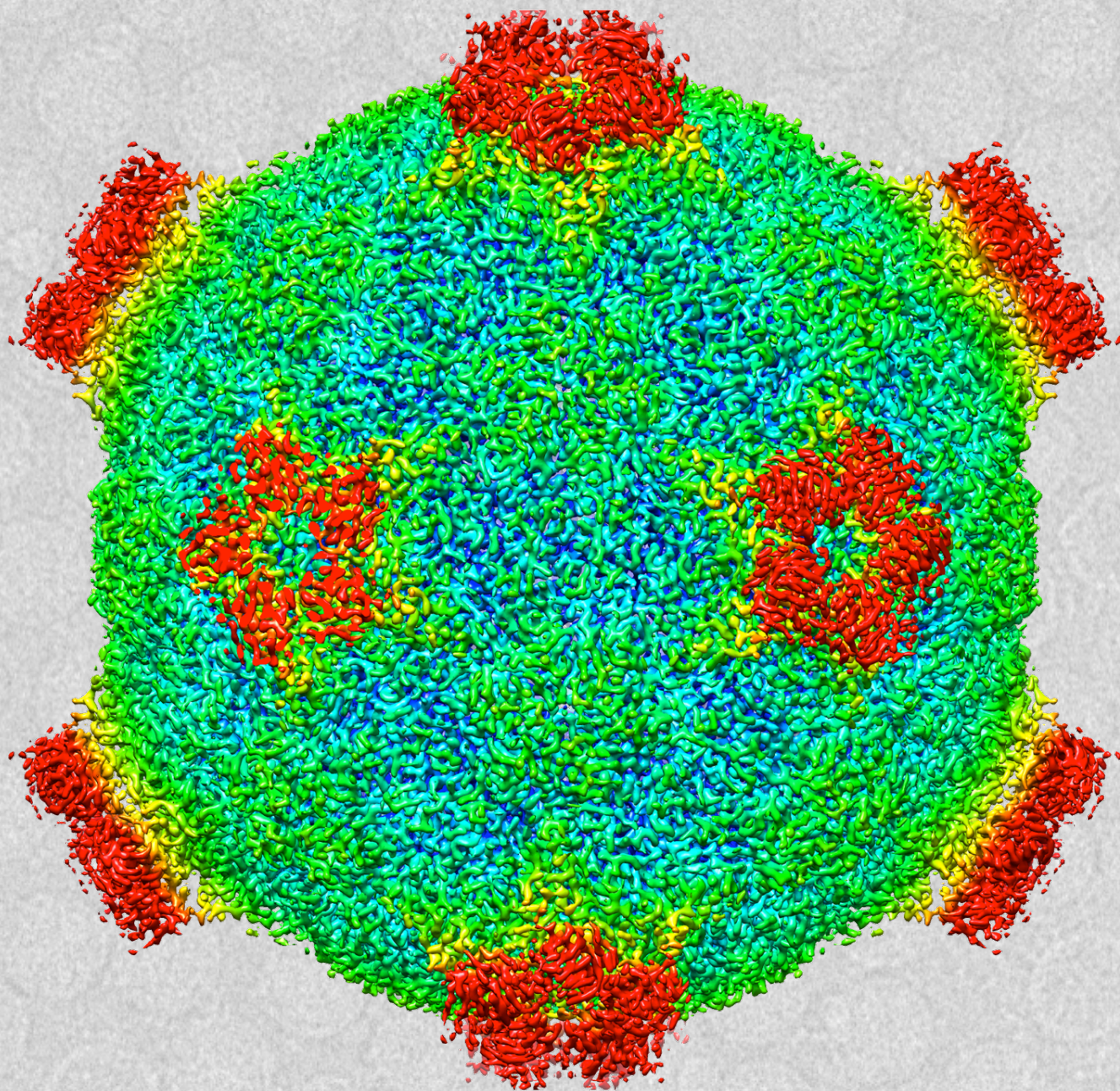
3. Jaké je nejvyšší možné rozlišení obrazku, který má velikost pixelu 1.1 Å?

- a) 1.1 Å
- b) 2.2 Å
- c) 3.3 Å

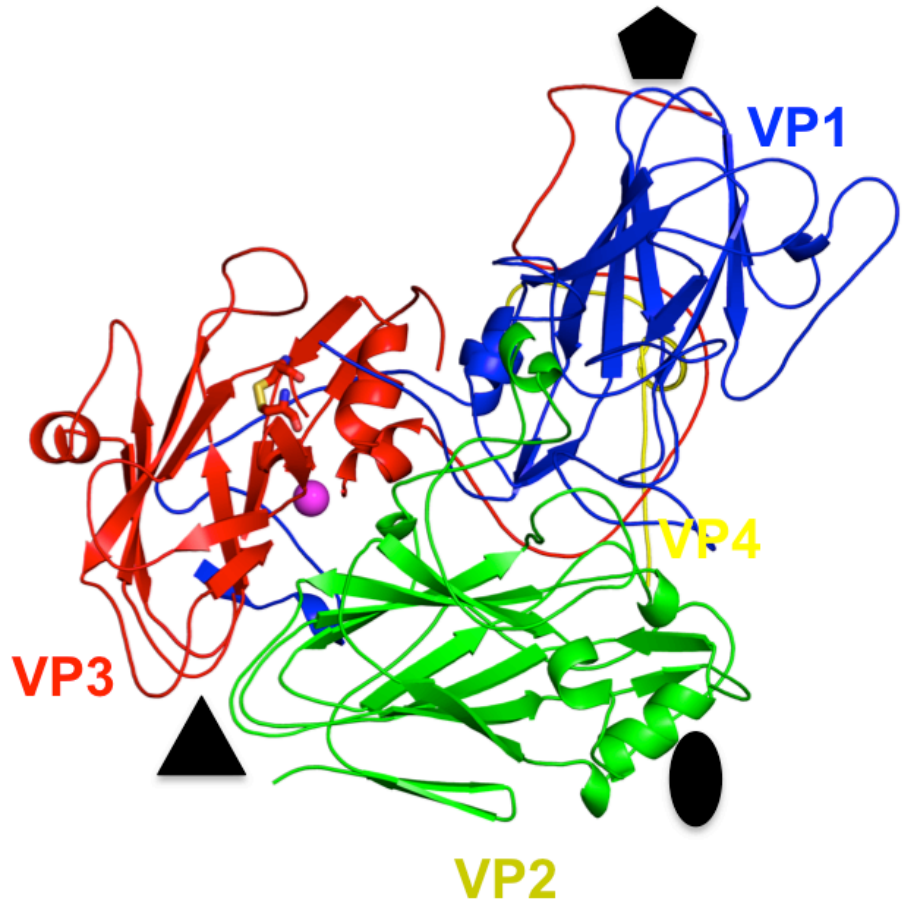
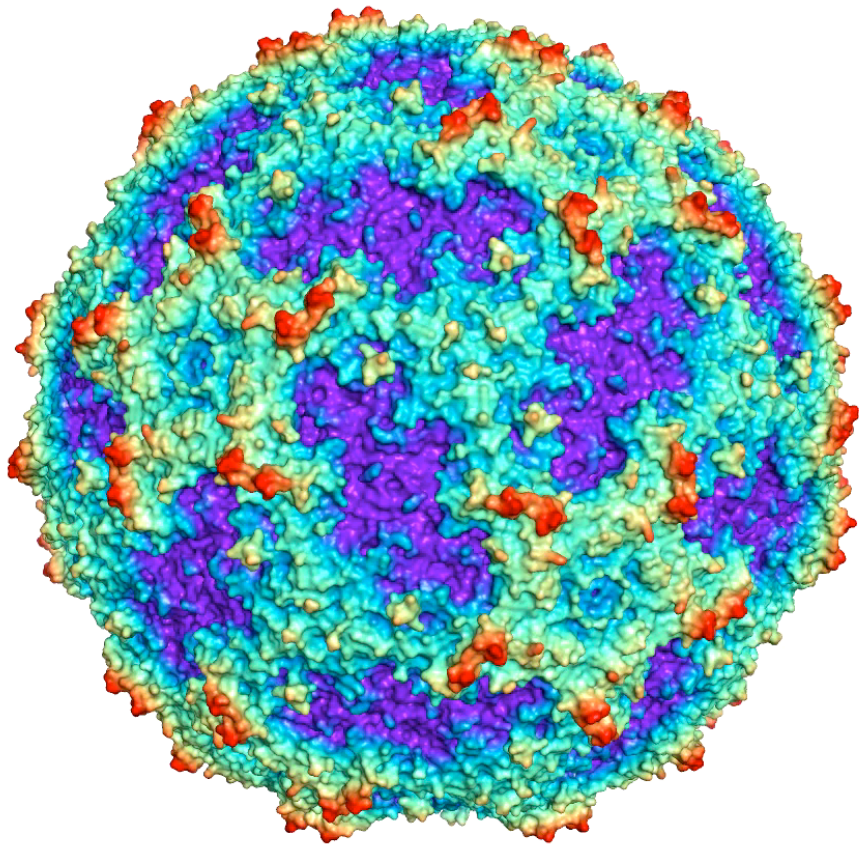




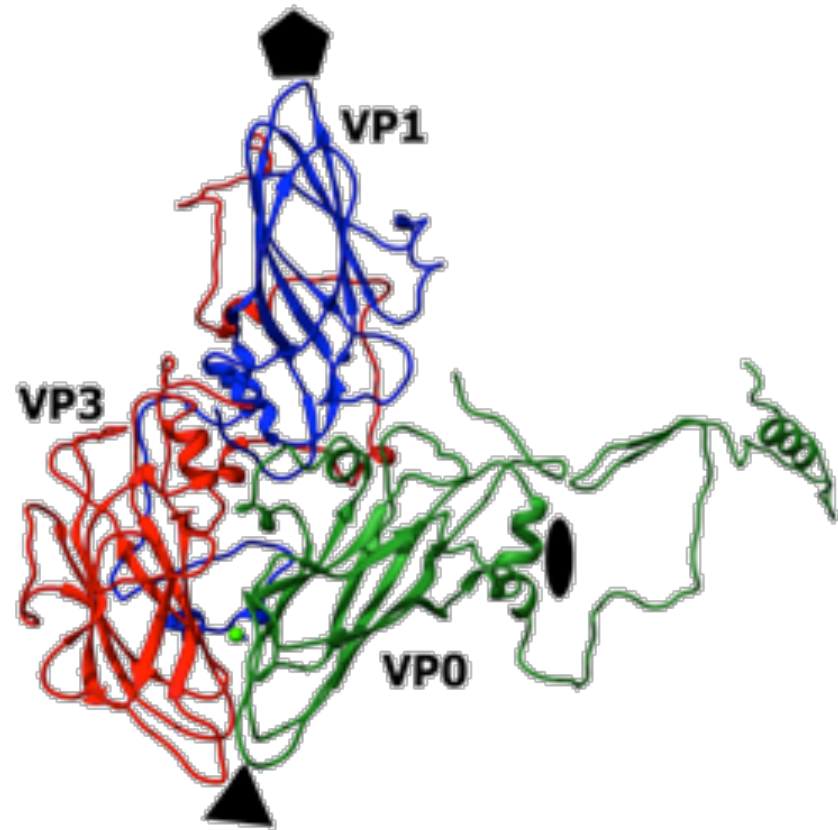
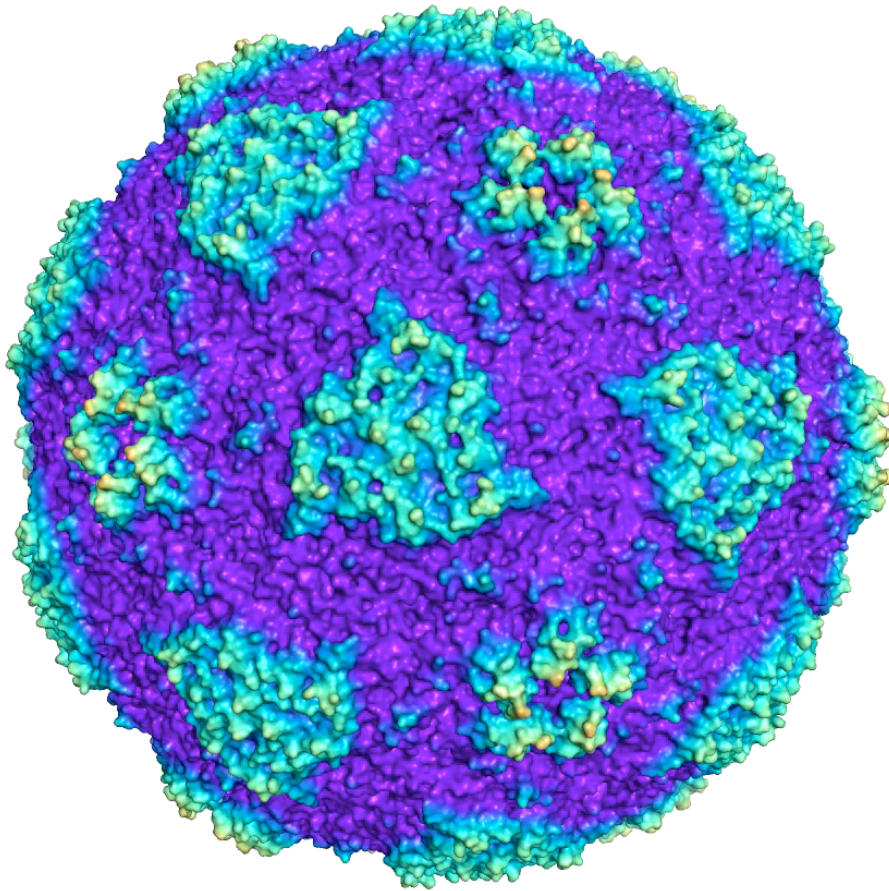


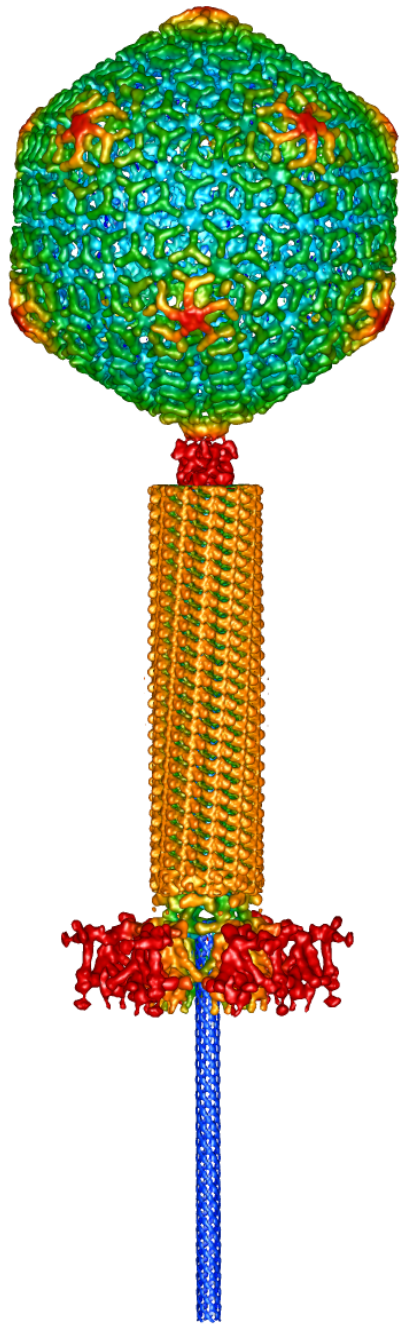
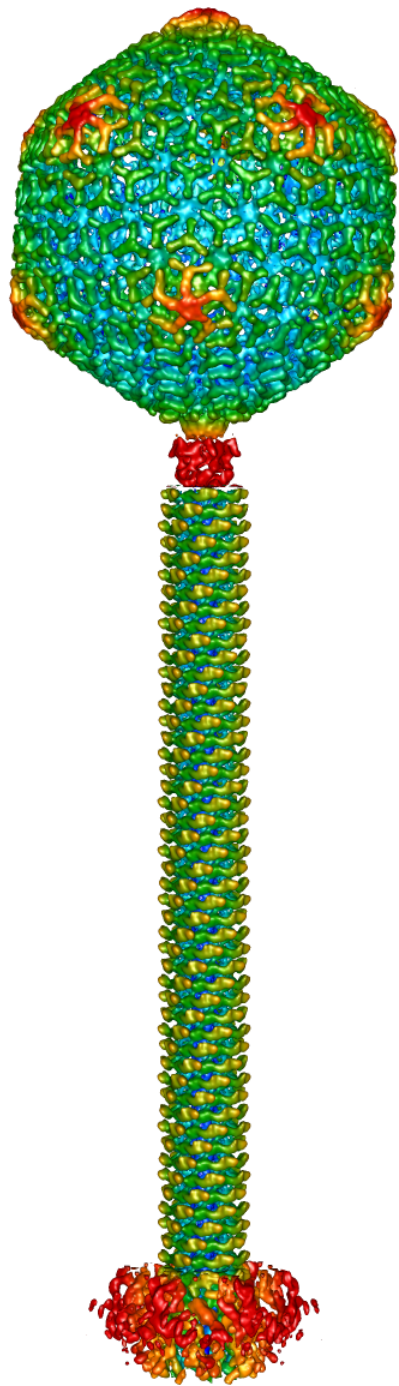


Human coronavirus Saffold virus 3 (2.5Å resolution)

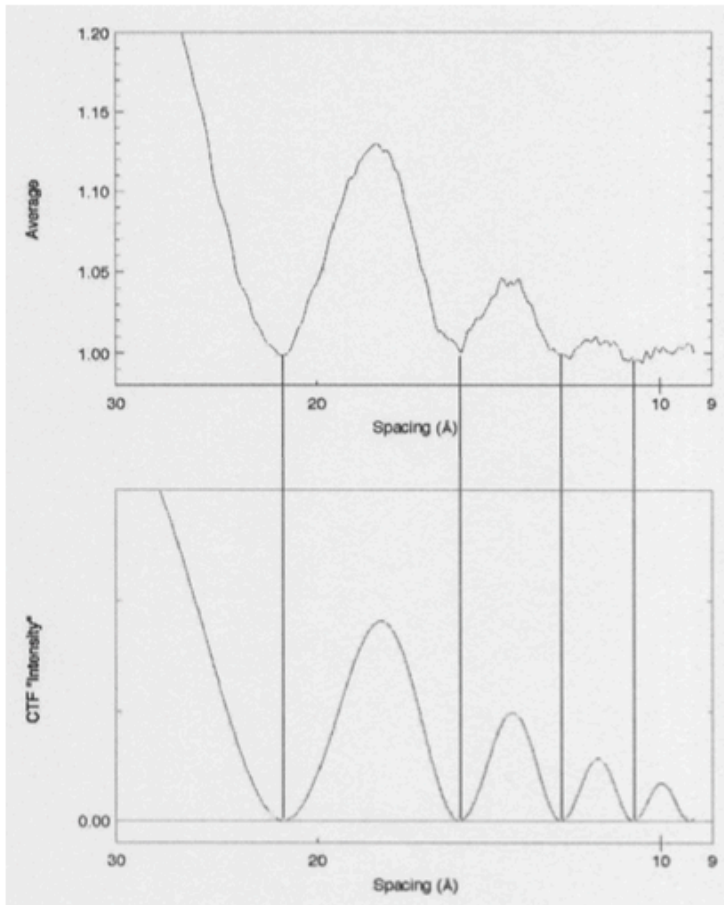


Human Parechovirus 1 @ 3.1Å





Example



CTF measured from images (see previous page):
Thon rings clearly identifiable.

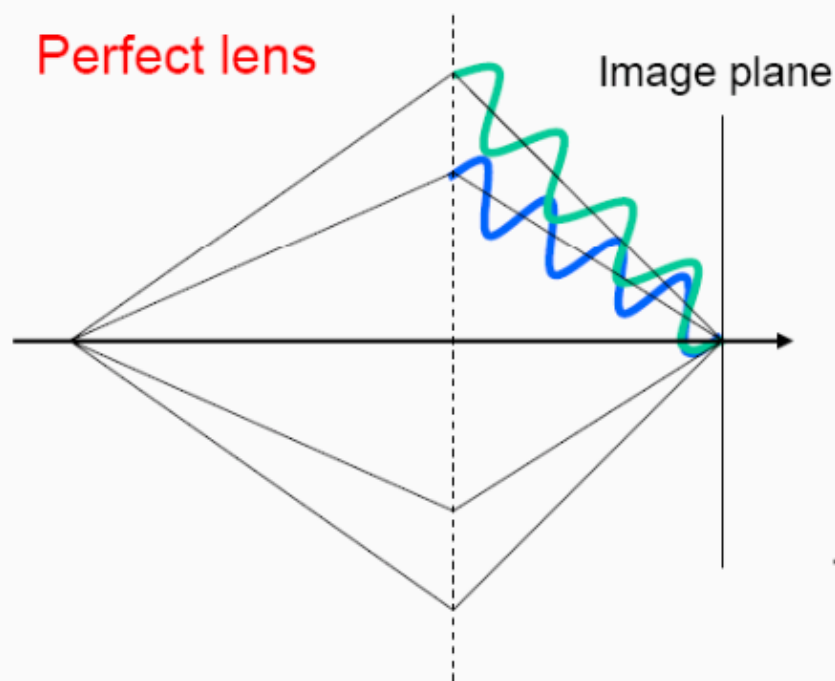
CTF estimated

The microscope settings (accelerating voltage, defocus) and observed image decay were used to calculate this theoretical squared CTF “Intensity” curve.

Use this information to flip phases and to amplify high frequencies (careful). This is a partial correction because information at zeros of CTF is lost.

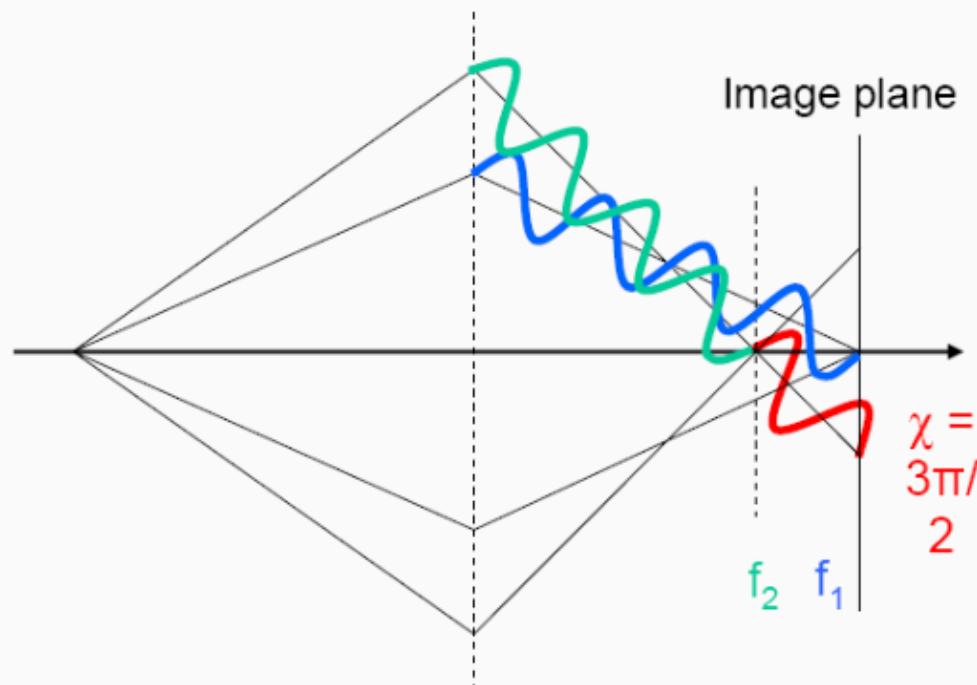
Spherical Aberration C_s

Perfect lens



© Werner Kühlbrandt

Lens with spherical aberration



Spherical aberration causes a phase shift which depends on the scattering angle and Δf

- Typical defect of electron lens, contributes to phase shift
- Modern lens designs seek to compensate this
- Normally C_s not modifiable, fine-tuning of CTF is done by adjusting defocus ΔZ

The Phase Contrast Function

- $T(x,y) = \exp[i \phi(x,y)]$
 $\sim 1 + i \phi(x,y)$

WHERE $\phi(x,y)$ IS
 PROPORTIONAL TO THE
 COULOMB-POTENTIAL
 "DENSITY" OF THE OBJECT

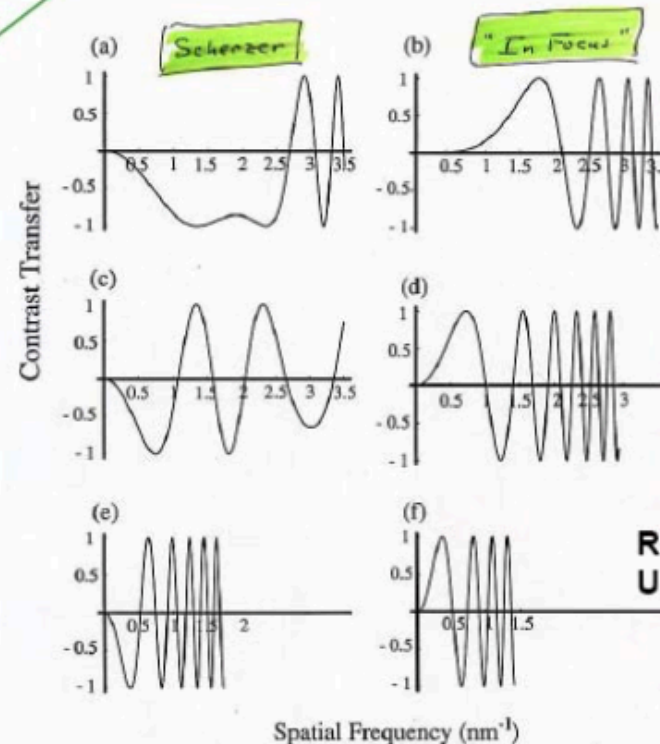
- WHEN THIS LINEAR
 APPROXIMATION IS VALID,
 THE FOURIER TRANSFORM OF
 THE IMAGE INTENSITY IS
 PROPORTIONAL TO

$\sin \gamma(s) \{FT [\text{object}]\}$

- $\sin \gamma(s)$ OSCILLATES
 BETWEEN +/- 1.0
- $\sin \gamma(s)$ IS KNOWN AS THE
 PHASE CONTRAST TRANSFER
 FUNCTION (CTF)



Downing & Jap
 PhoE porin image
 (unpublished)



RMG,
 Unpublished