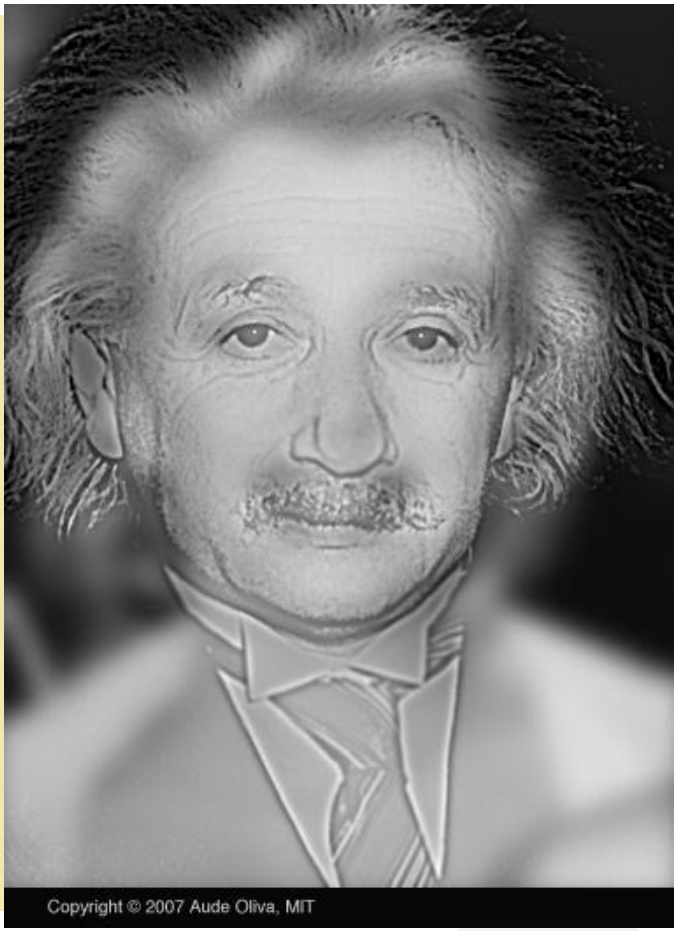
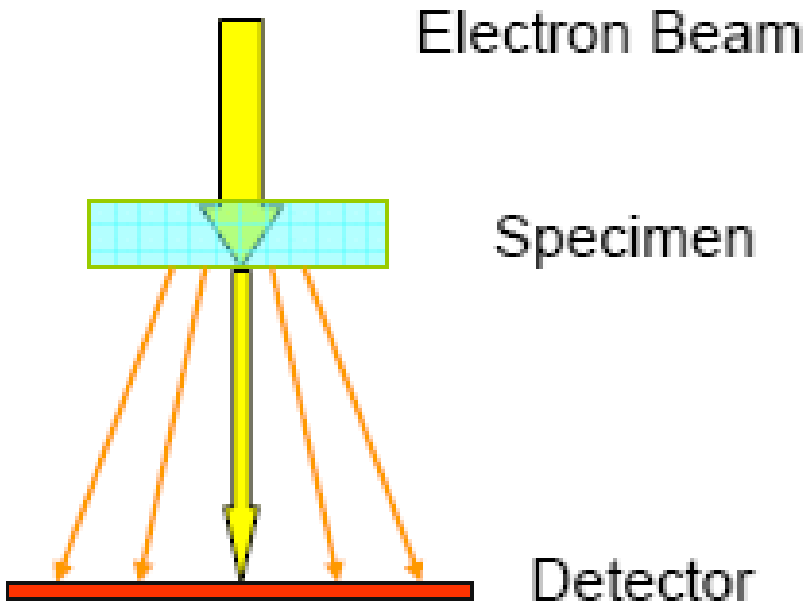
What can we see with a transmission electron microscope?



o.lambert@cbmn.u-bordeaux.fr

Transmission electron microscope



electron

1897 J.J. Thomson **Electron discovery** m= 9.1083.10-^{31}Kg

e= -1.60206.10^{-19}C

1924 Louis de Broglie **Duality wave length-particle**

A particle with a mass m and a speed v is characterized by a wave length λ:

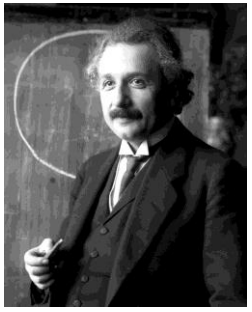
λ = h/(m.v)

A electron submitted to electric field with a tension V **Ec=1/2 mv^2= e.V**

λ=h /(2e.m.V)$^{1/2}$≈ 12,25/ √V

For high tension, v> c 3 10^8 m/s

Albert Einstein (relativistic effects on electron)

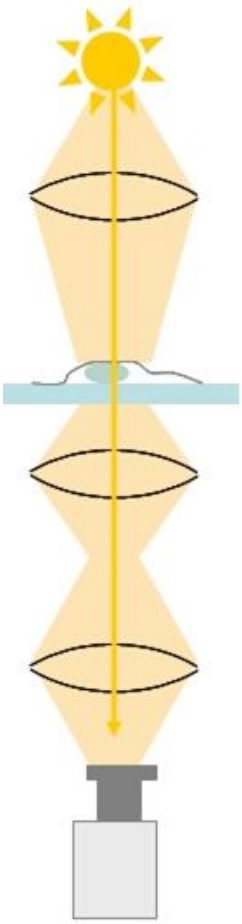


Correction for the mass $m_1=m_0/(1-v^2/c^2)^{1/2}$

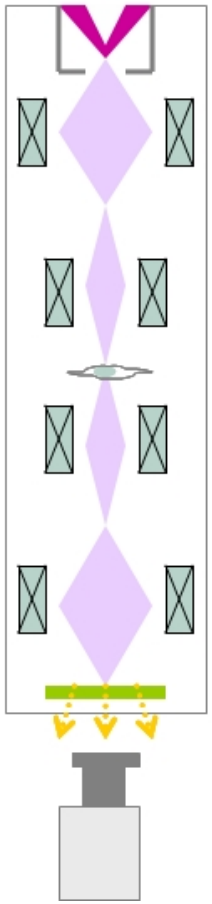
$$\lambda = \frac{1.23}{\sqrt{V + 10^{-6}V^2}}\,nm$$

Optical vs electron Microscope

OM



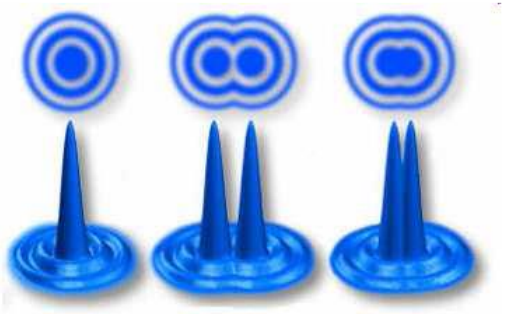
TEM



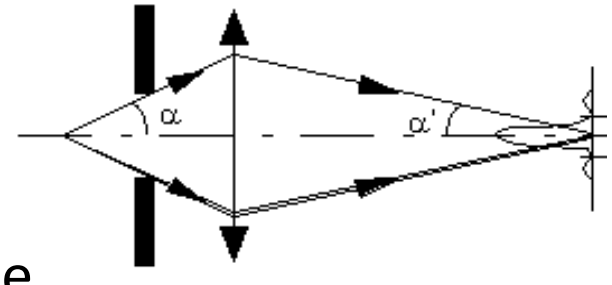
	Photon	Electron
Mass	0	$9x10^{-31}$ kg
Charge	0	$-1.6x10^{-19}$ C

Resolution of an optical microscope

- **Resolution: the smallest distance below which two points won't be seen as separate**
- Rayleigh/Abbe criteria **$d = 0{,}61.\lambda/n.\sin\alpha$**



  - **λ** : wavelength of the emission source
  - **n** : refraction index (air, oil)
  - **α** : opening angles of rays entering the objective lens (numerical aperture of the objective)



- **Resolution ≈ ½ λ**

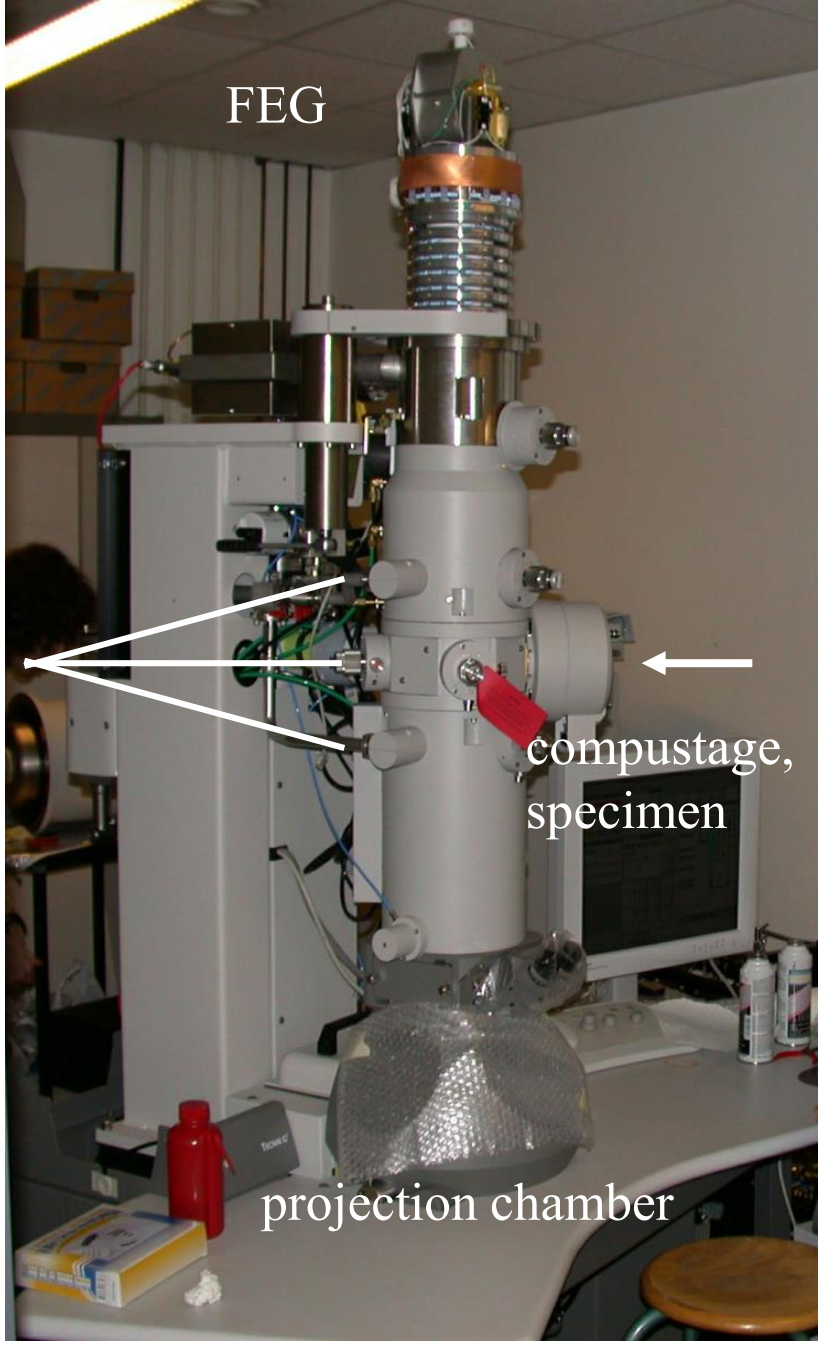
Resolution of an electron microscope

Relation Acceleration voltage - wavelength

V	λ (nm)	Theoretical resolution (nm)
10,000	0.01223	0.00611
50,000	0.00536	0.00268
100,000	0.00370	0.00185
1,000,000	0.00086	0.00043

80-300 kV

A transmission electron microscope (TEM)



source

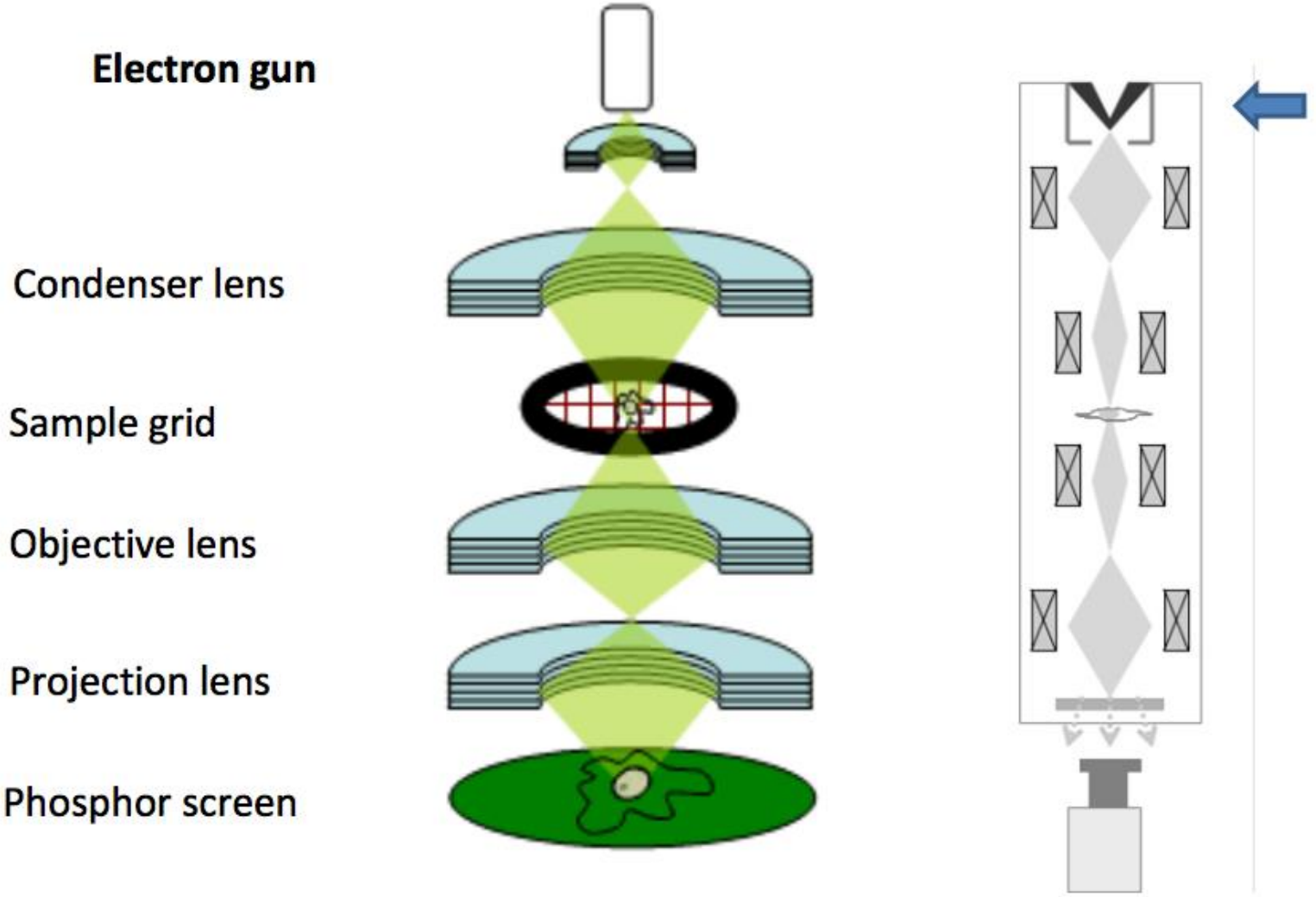
sample

image acquisition

- vacuum: ~10^{-6} Pa
- potentially high electron dose
- potentially high resolution
  ($\lambda \approx 0.025$Å at 200kV)
  i.e.resolution <u>not limited by the wavelength</u>

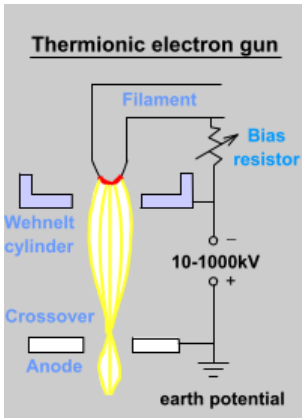
<u>Field emission gun (FEG) electron microscope (Tecnai20, IGBMC)</u>

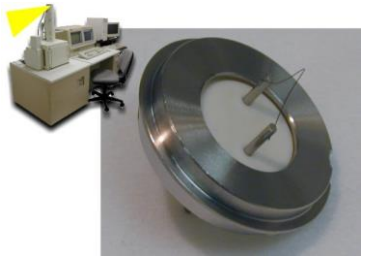
Composition of a TEM

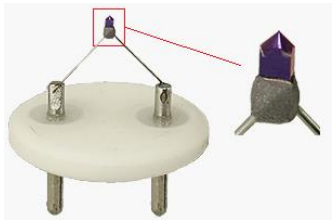


Electron source

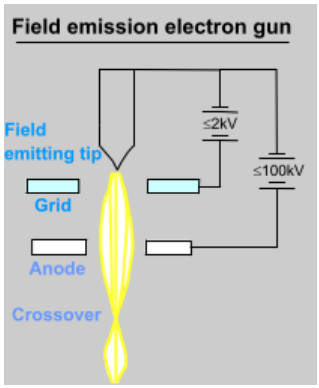
- Purpose: generation of electrons that can be accelerated by high tension to obtain the illuminating electron beam



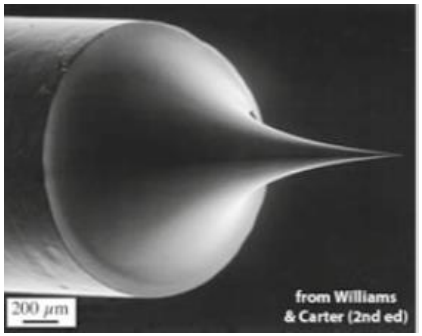




Thermionic gun: W or LaB6
Electrons come out when the emitter is heated



Field emission gun



Central development for high resolution EM !

Composition of a TEM

Electron gun

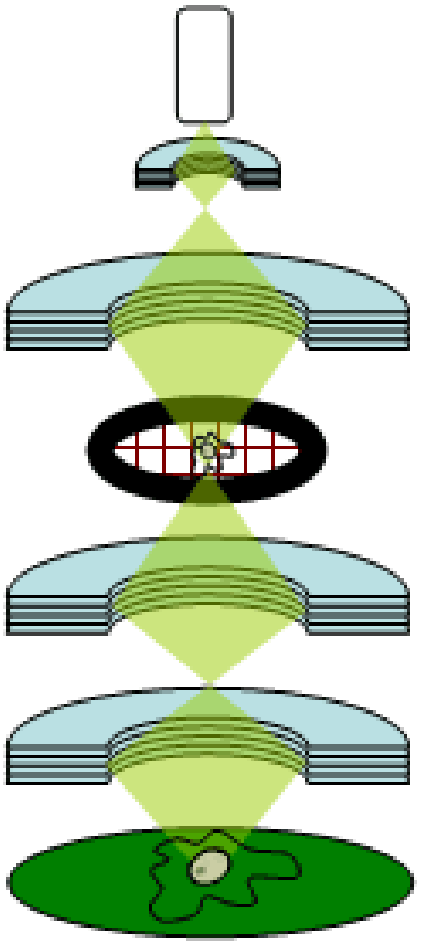
Condenser lens

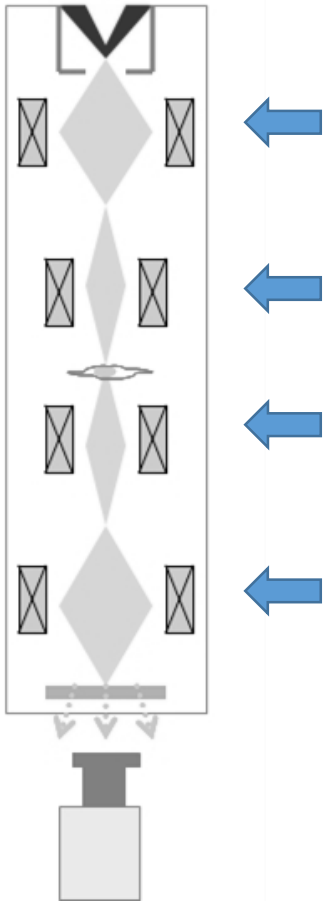
Sample grid

Objective lens

Projection lens

Phosphor screen

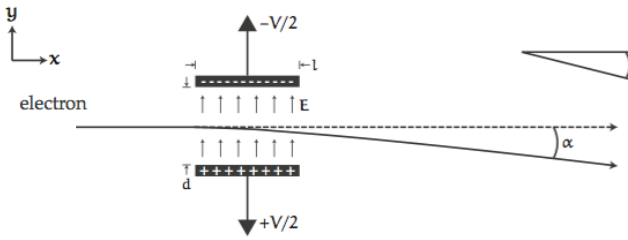


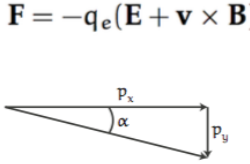


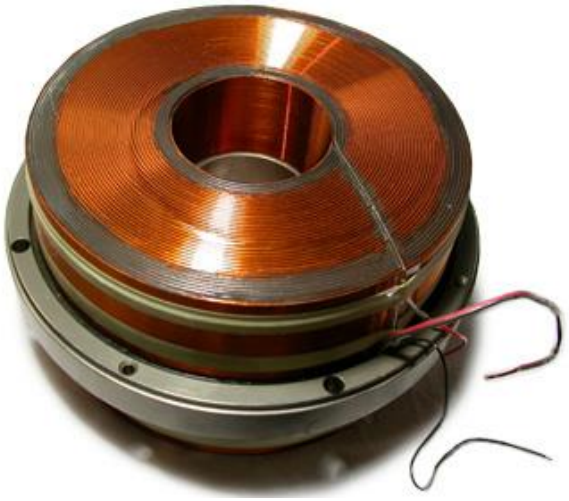
Electromagnetic lens

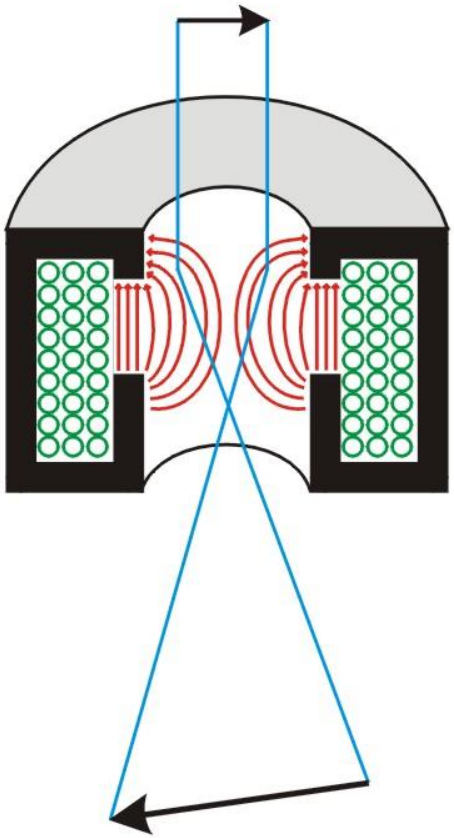
The Lorentz force

$$\mathbf{F} = -q_e(\mathbf{E} + \mathbf{v} \times \mathbf{B})$$









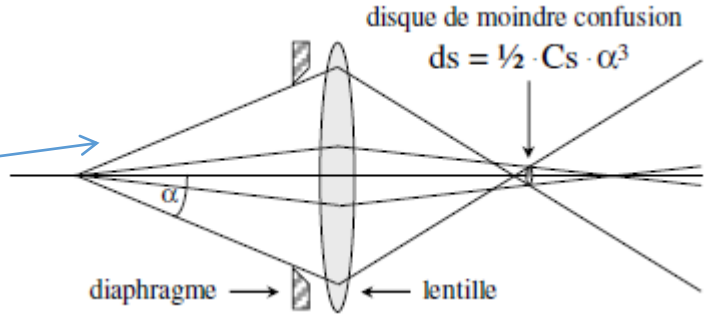
$$f = KV_r/(N \cdot I)^2$$

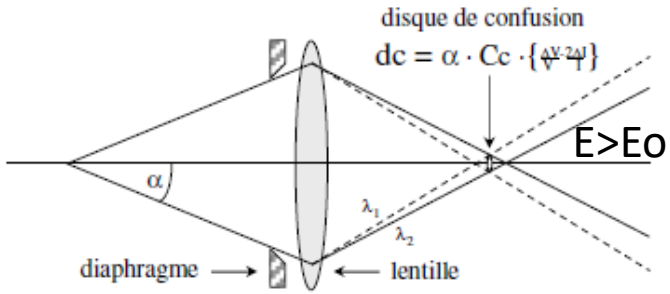
where f = the focal length of the lens
K = a constant
V_r = the accelerating voltage, relativistically corrected
$N \cdot I$ = the number of ampere turns in the excitation coils

drawbacks

Lens:

- Spherical aberration
- Chromatic aberration
- Astigmatism





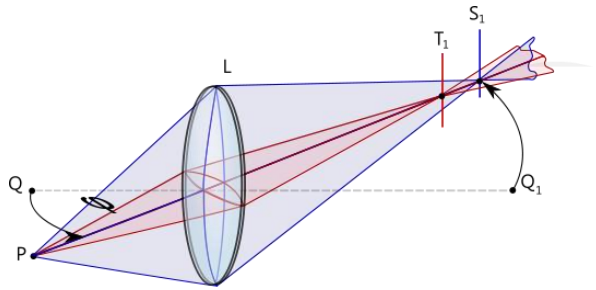
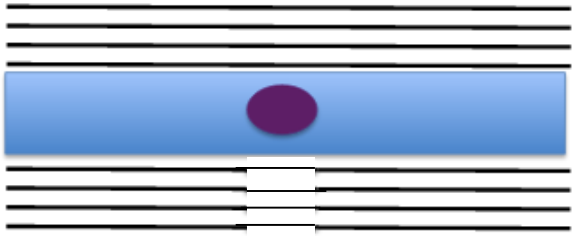


Image formation

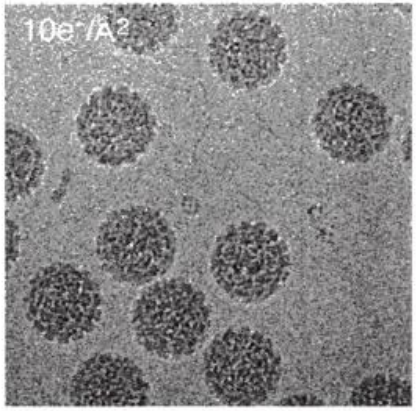
Electrons « seen as » particles:



✓The transmitted electrons are used to produce an image

✓In thicker region, more scattering, less direct beam: appear darker.

✓Provides AMPLITUDE contrast



Courtesy of Helene Malet

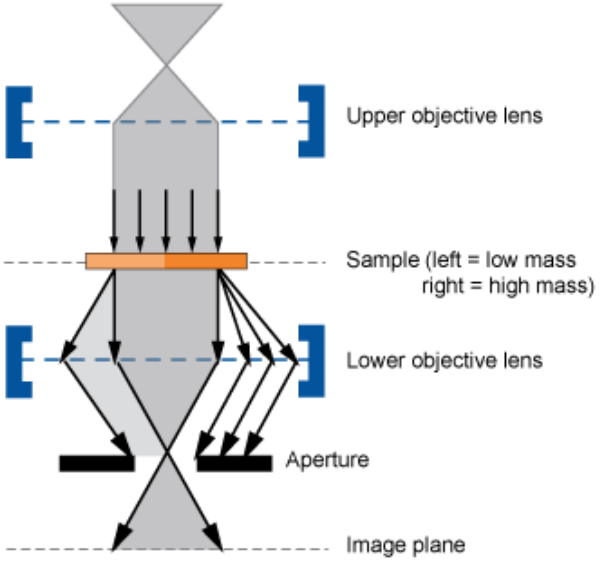
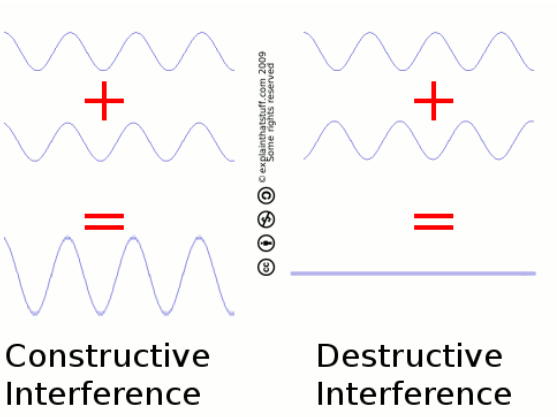


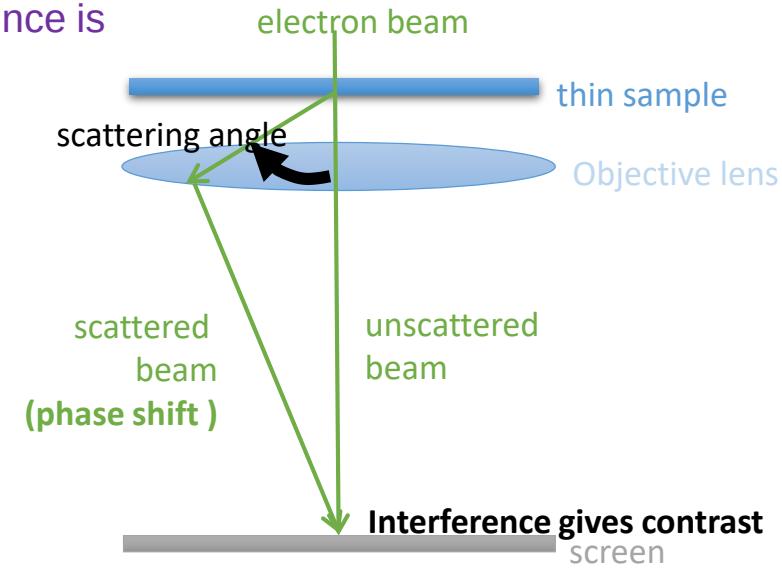
Image formation

Electrons « seen as » wave:

✓Scattered and non scattered electron don't have the same phase. Their interference is producing difference in intensity.

➔Provides PHASE contrast



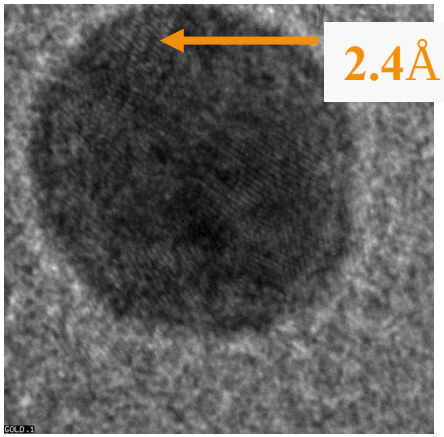


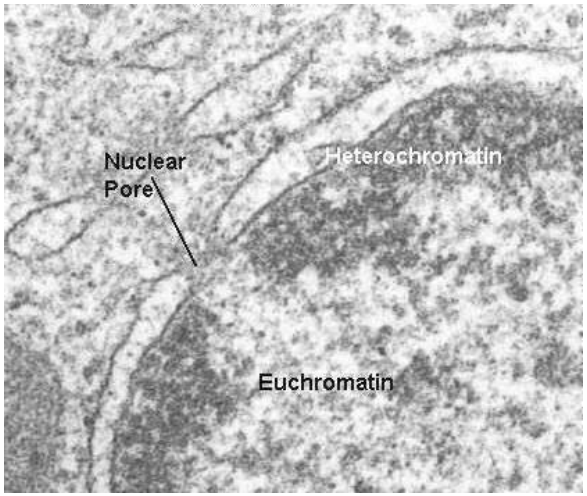
Diversity of samples

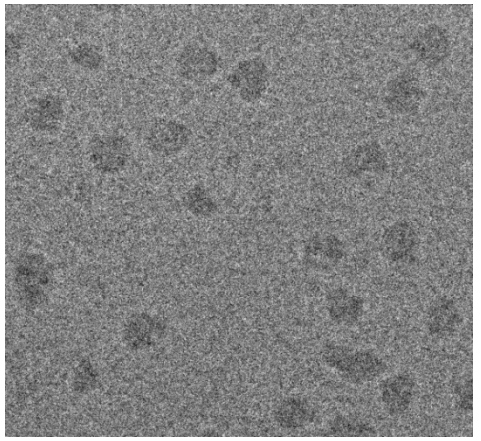
A) material sciences

B) cellular biology
(cell sections)

C) molecular biology
(extracted, purified
single molecules)



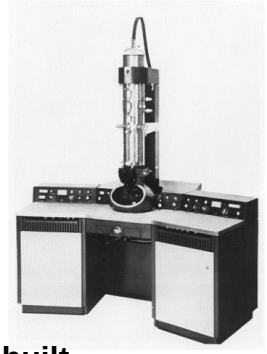




Visual inspection of sample quality

Direct visualization, and more… **3D reconstruction!**

What can be seen depends also on 1) imaging system, 2) sample preparation 3)image processing



***Ernst Ruska* (1906-1988) built the first TEM in 1931 (mag 17.4) (Nobel prize 1986)**

De Rosier and Klug
3D reconstruction
1968

Unwin and Henderson.
3D structure bacteriorhodpsin
1975

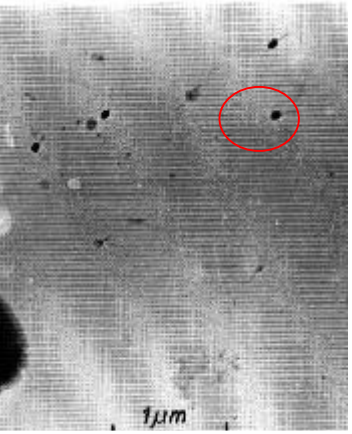
1931 1970 1990 2000 2008

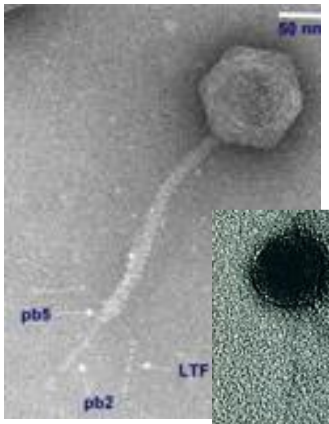
Bacteriophages. (H Ruska, 1941-1942)

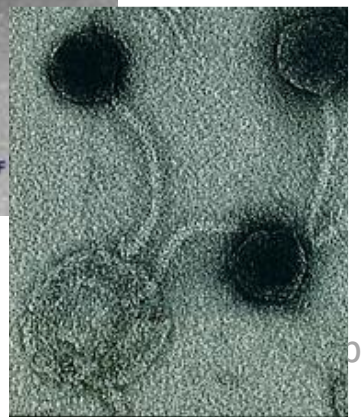
Negative staining

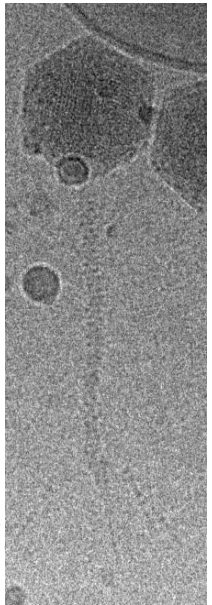
1982
Vitrification Dubochet *et al.*

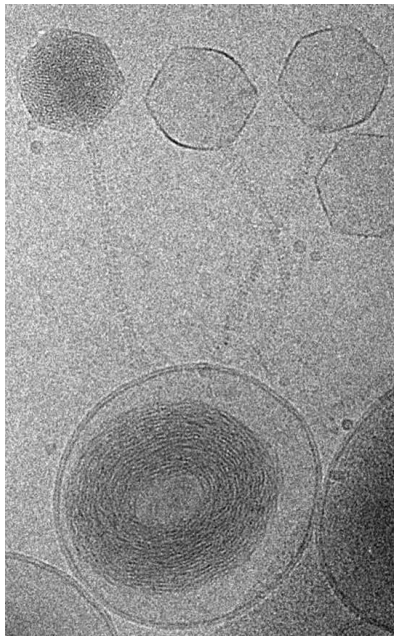
cryoTEM

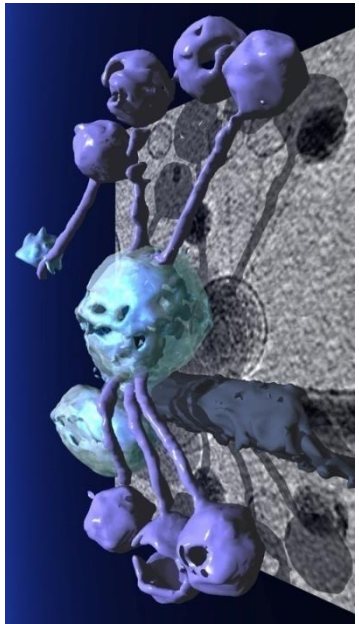






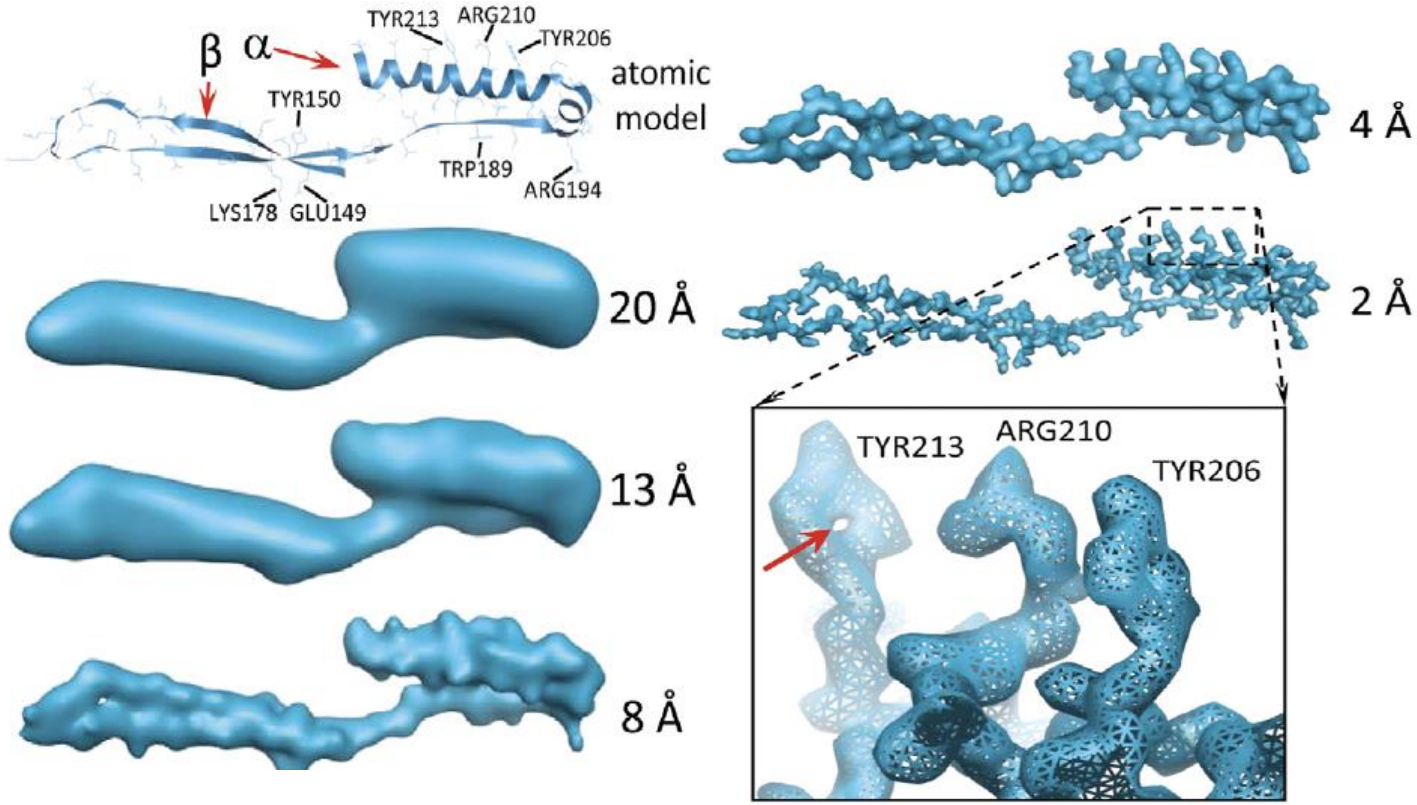






Resolution: what do we see ?

Secondary structure elements at different resolutions. The segment is extracted from the atomic model of BacteriophageHK97 capsid protein



Zhou , 2008

Wikoff , Science 2000

Breaking Cryo-EM Resolution Barriers to solve the 3D structure with an electron microscope

Science AAAS

BIOCHEMISTRY

The Resolution Revolution

Werner Kühlbrandt

Advances in detector technology and image processing are yielding high-resolution electron cryo-microscopy structures of biomolecules.

Review

CellPress

How cryo-EM is revolutionizing structural biology

Xiao-chen Bai, Greg McMullan, and Sjors H.W Scheres

MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge, CB2 0QH, UK

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Archive > Volume 525 > Issue 7568 > News Feature > Article

NATURE | NEWS FEATURE

The revolution will not be crystallized: a new method sweeps through structural biology

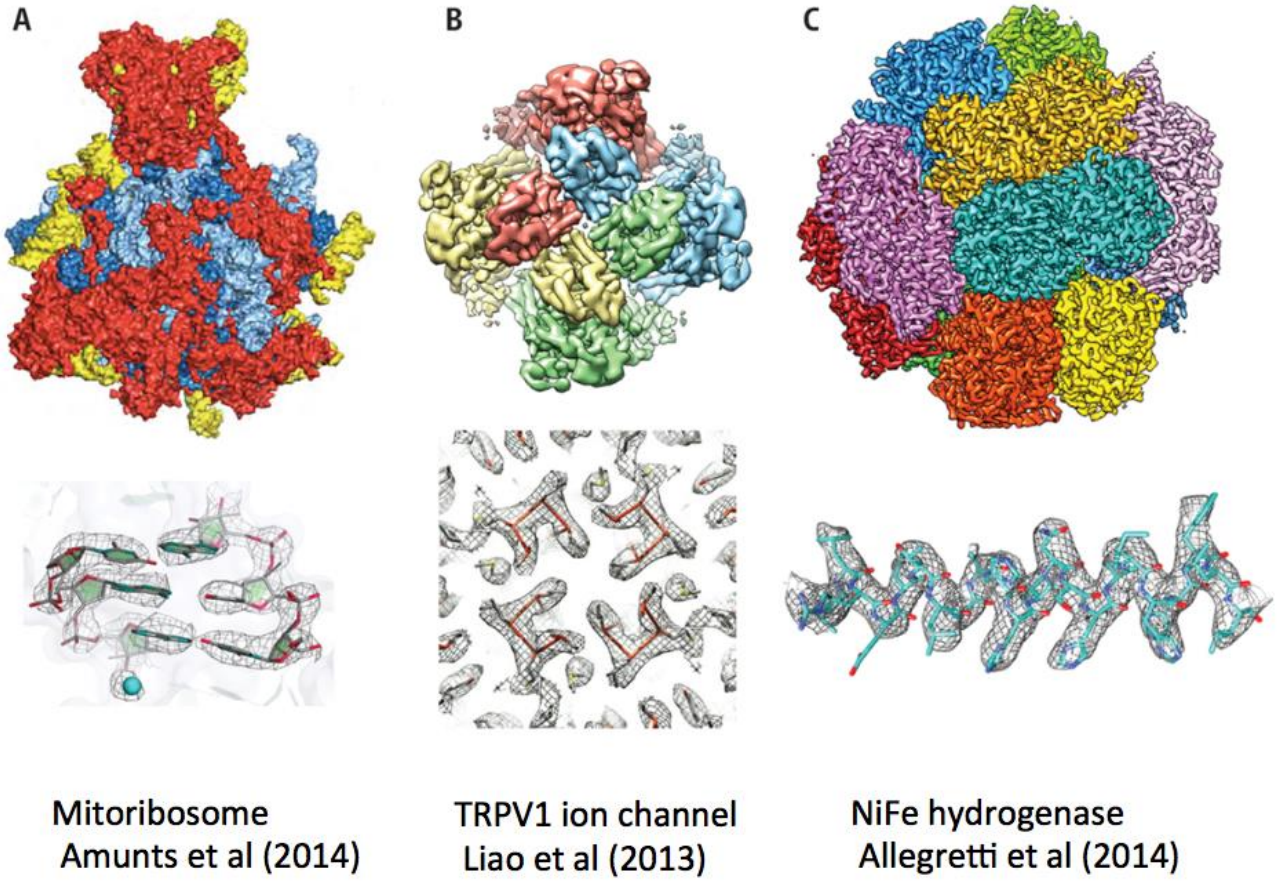
Move over X-ray crystallography. Cryo-electron microscopy is kicking up a storm by revealing the hidden machinery of the cell.

Method of the Year 2015

The end of 'blob-ology': single-particle cryo-electron microscopy (cryo-EM) is now being used to solve macromolecular structures at high resolution.

The resolution revolution

2013/2014 kick-off

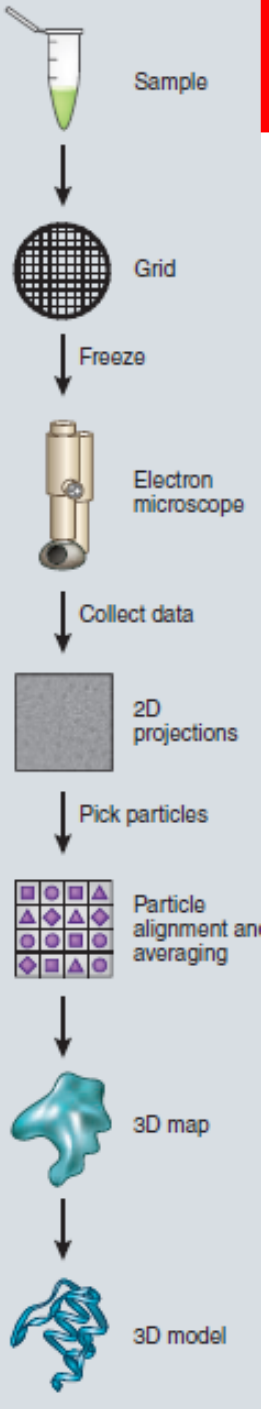


Mitoribosome
Amunts et al (2014)

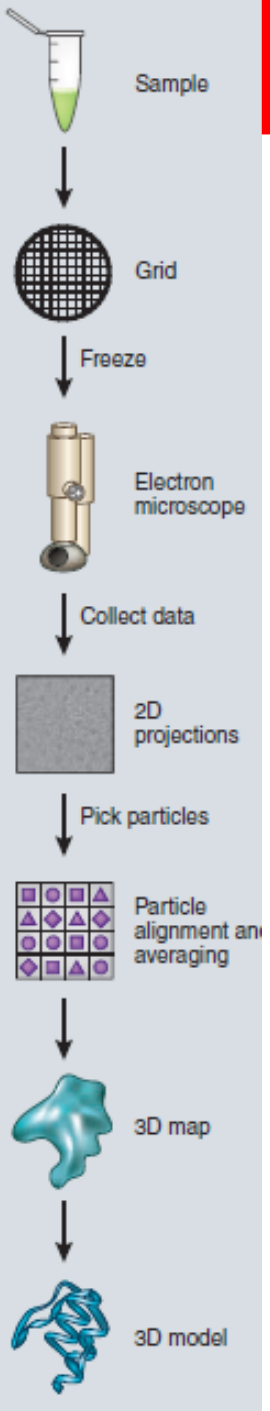
TRPV1 ion channel
Liao et al (2013)

NiFe hydrogenase
Allegretti et al (2014)

how to solve a macromolecular structure using single-particle cryo-electron microscopy



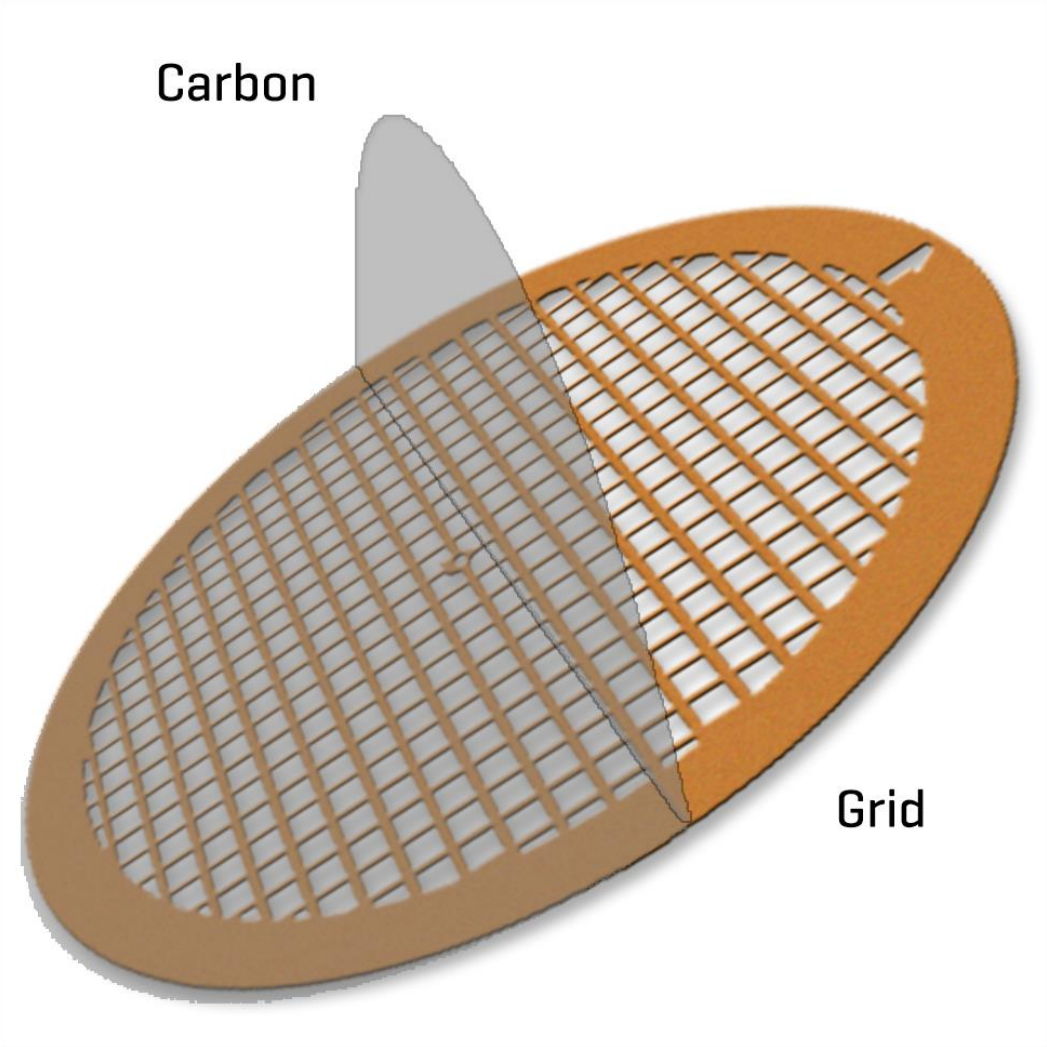
how to solve a macromolecular structure using single-particle cryo-electron microscopy



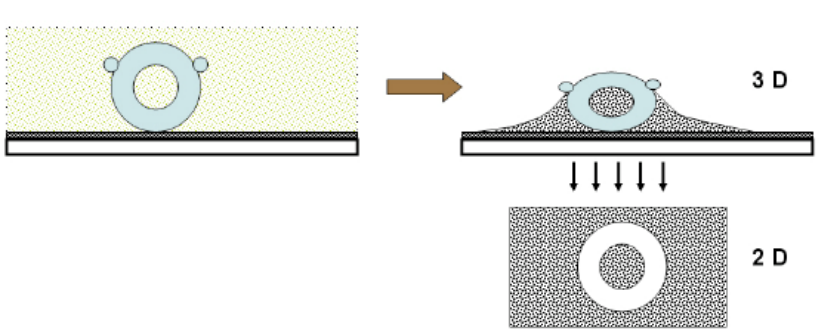
Protein preparation: Purity, homogeneity Stability, biochemical activity

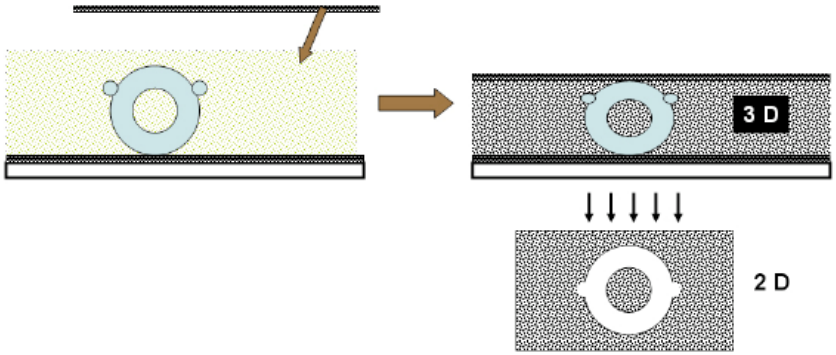
Negative Staining

Support : grid coated with thin amorphous carbon



Negative Staining :Principle



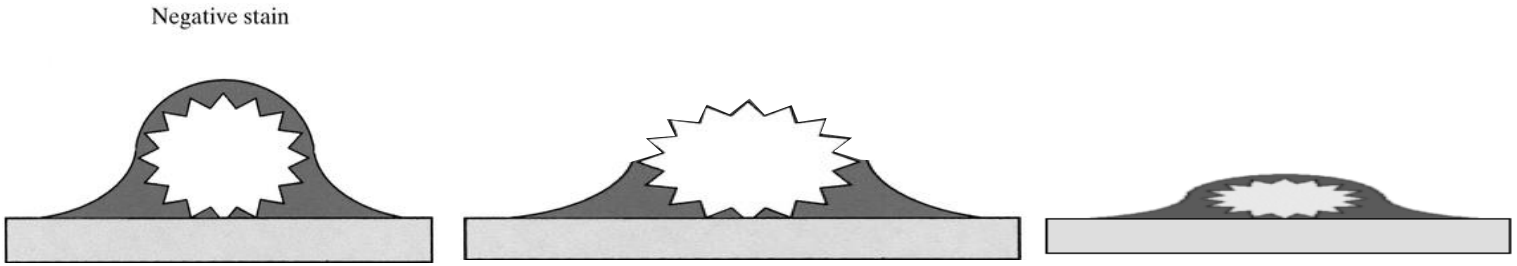


Advantages :

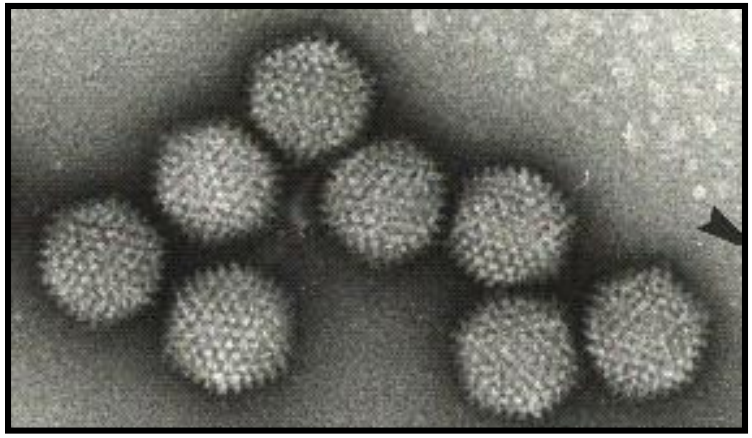
Fast
Small amount of protein (concentration 0.01-0.1mg/ml, few μl)
Small proteins are visible
High contrast

Drawbacks :

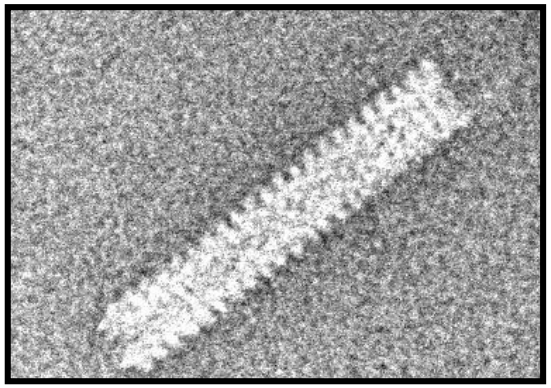
Low resolution (15 Å)
Flattening and drying
Footprint of the sample in the heavy atom
Artefacts due to the stain



Negative Staining : Examples of macromolecular assemblies

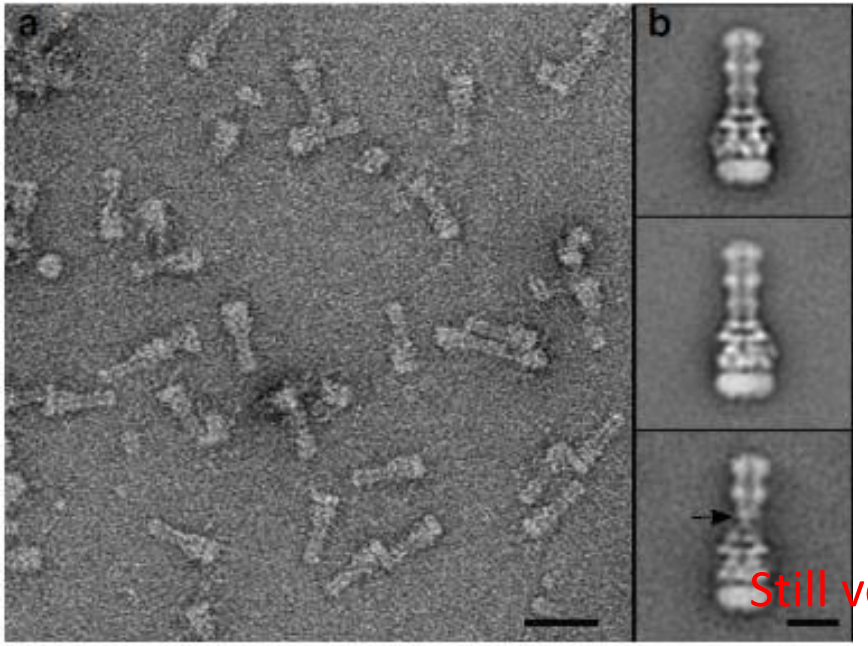


Adenovirus
(100 nm)
Courtesy of G Schoehn

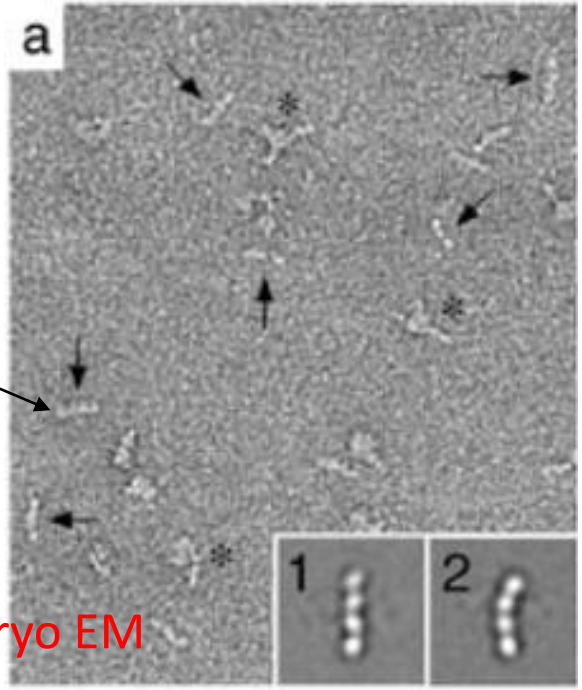


Measles nucleocapsid Pitch 5 nm
Courtesy of G Schoehn

Tripartite efflux system (TSS1-like)
Daury et al 2016, Nature comm



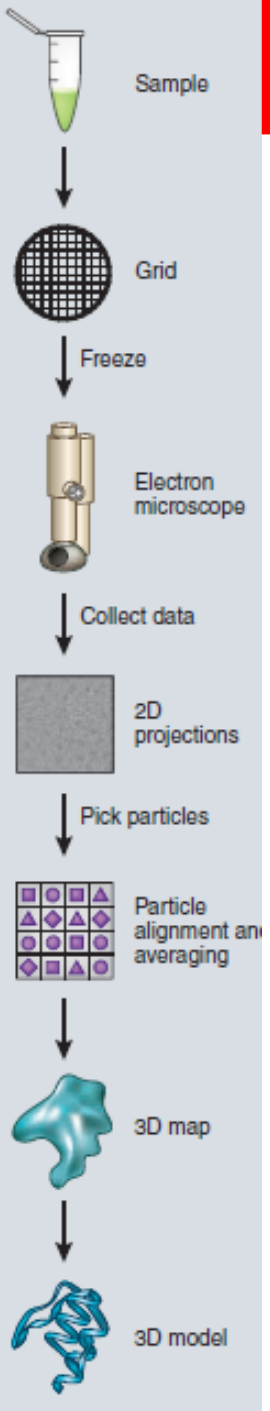
Integrin α5β1 in complex with fibronectin.
Takagi J,*EMBO J* 2003



Fn7-10 alone,
40 kDa

Still very usefull prior to cryo EM

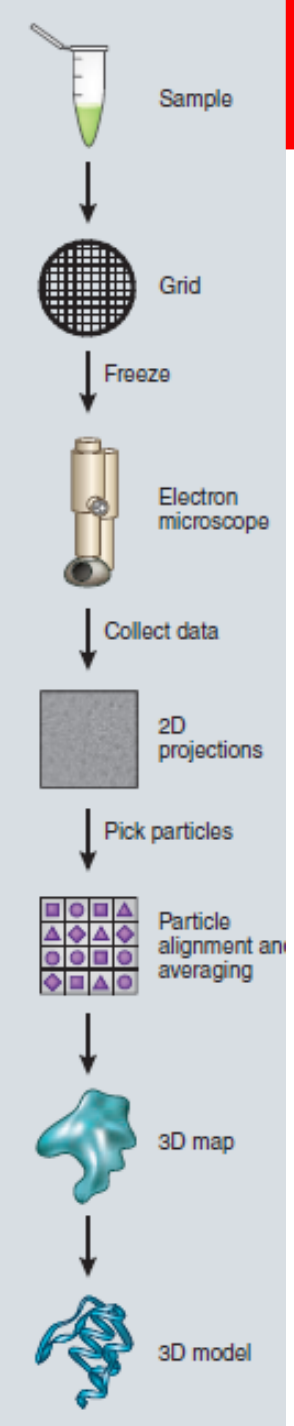
how to solve a macromolecular structure using single-particle cryo-electron microscopy



Protein preparation: Purity, homogeneity Stability, biochemical activity

1982 CryoEM preparation

how to solve a macromolecular structure using single-particle cryo-electron microscopy



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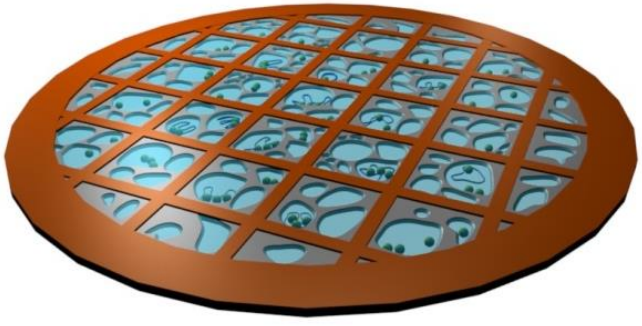
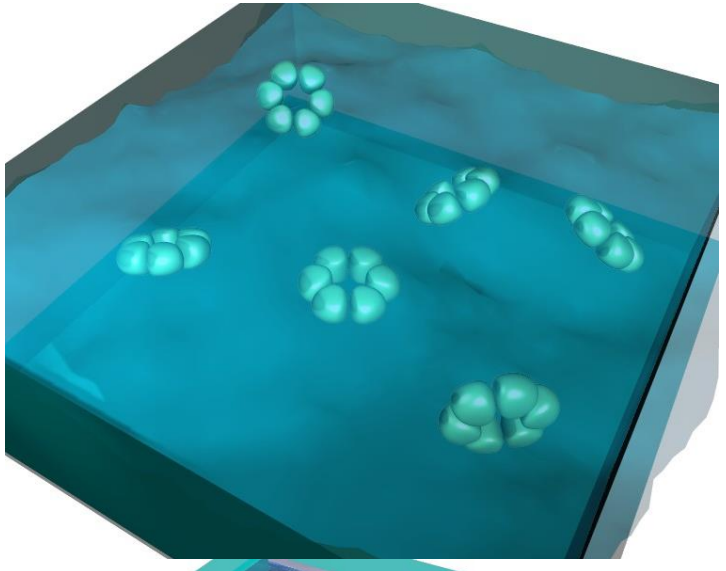
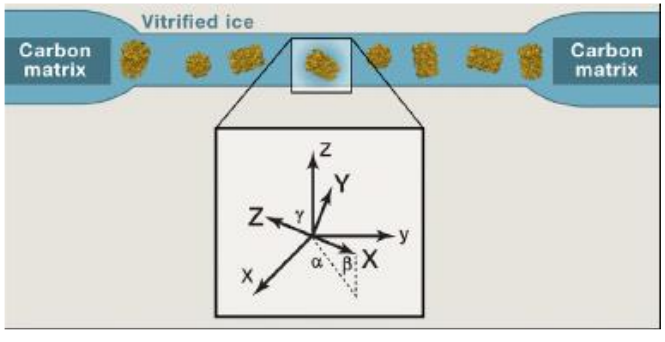
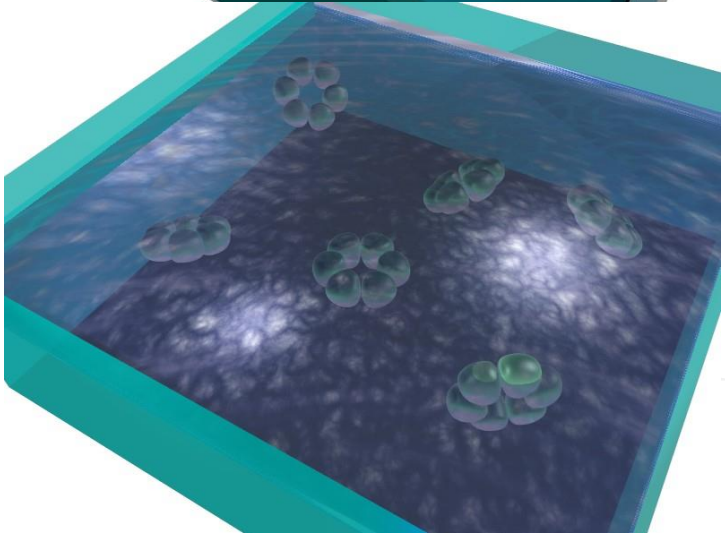




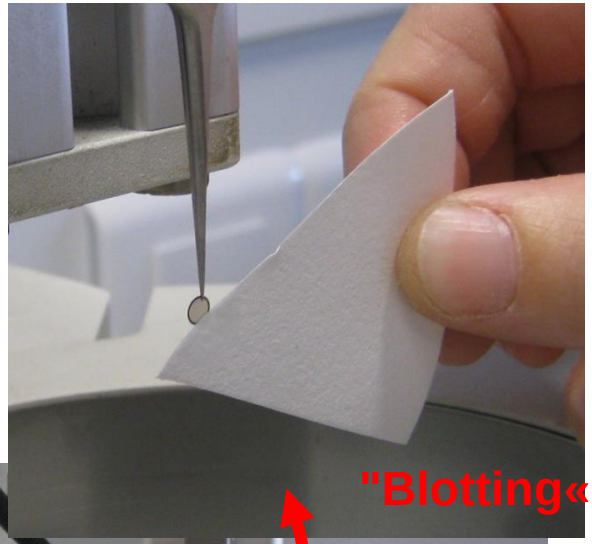




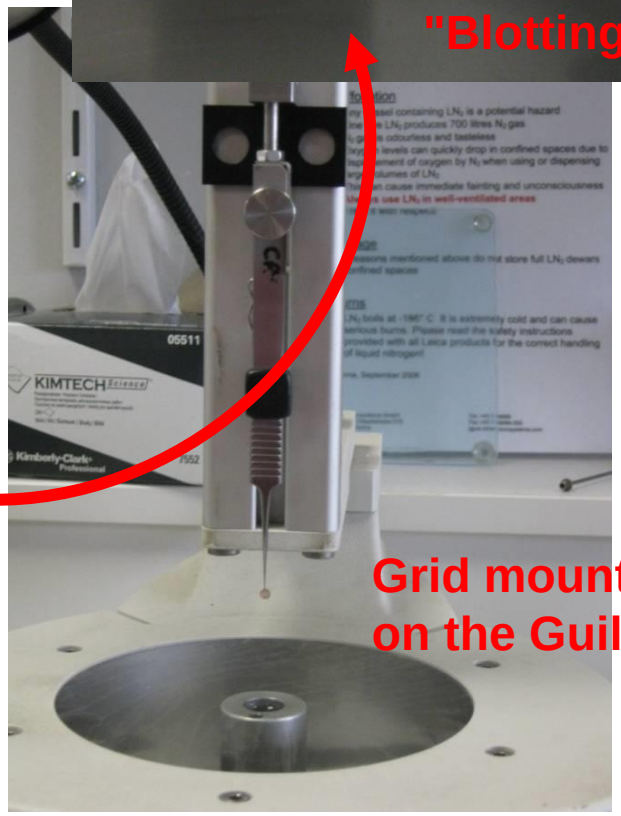
Liquid Ethane
-180°C

filling

5 µl of sample

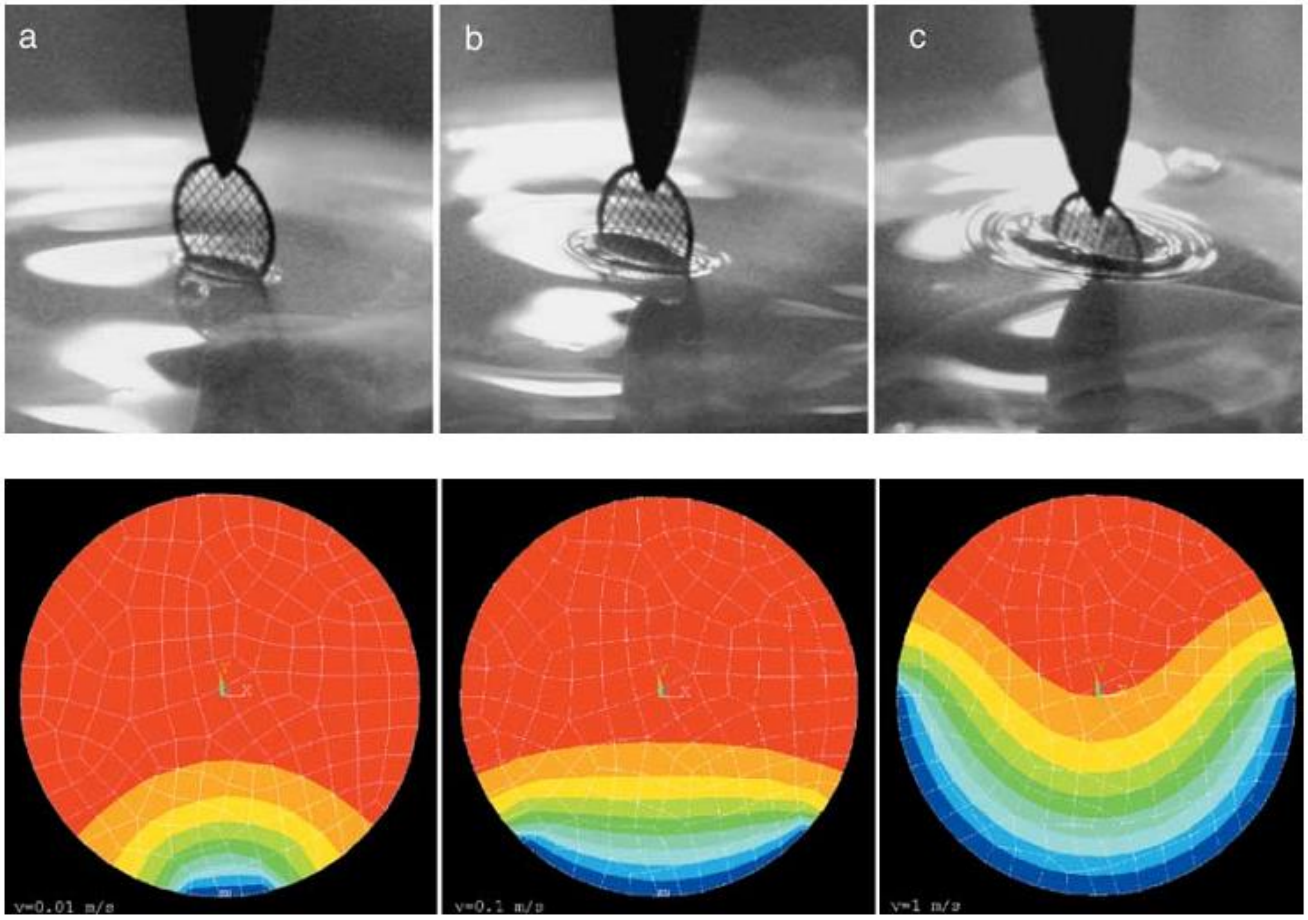


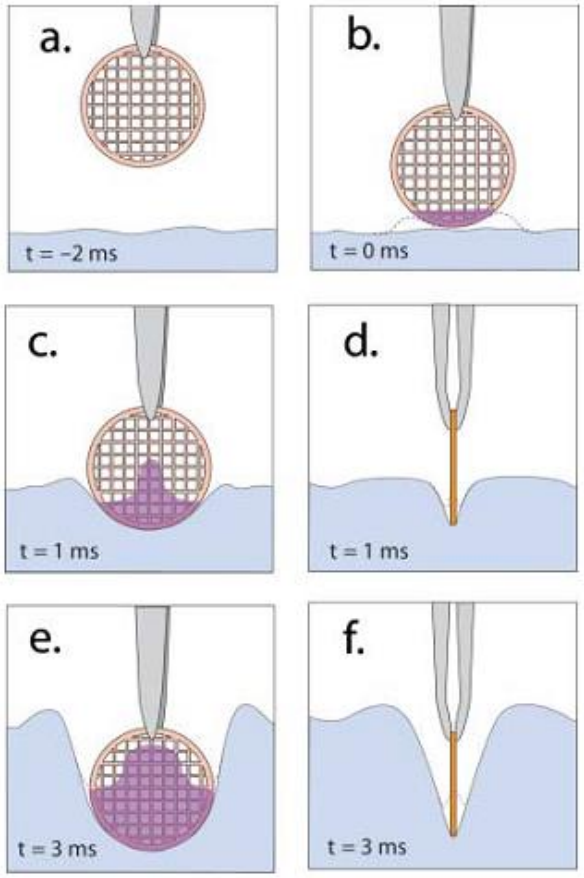
"Blotting« step



Grid mounted
on the Guillotine

The importance of cryo-approaches in cryo-EM





Kasas et al. & Adrian,
Journal of Microscopy, 2003

Advantages of flash-freezing:

- → vitrified water (amorphous ice)
- → specimen conservation (frozen-hydrated)
- → very weak ice sublimation in the vacuum of the microscope
- → fixation of particle orientations

The importance of cryo-approaches

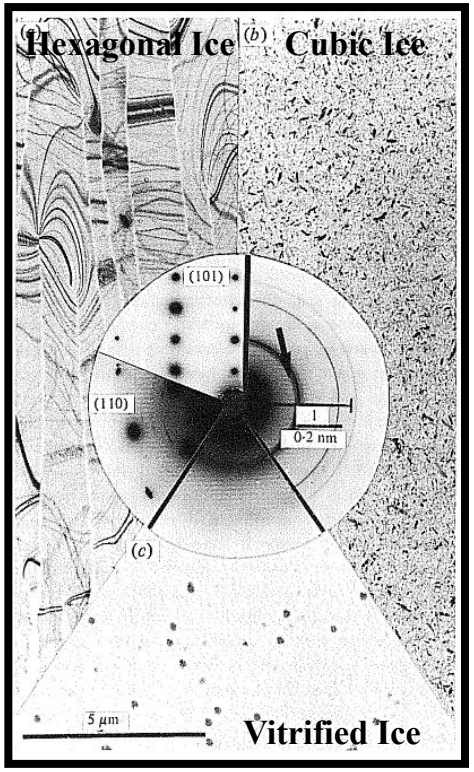
Vitreous ice:

forms by flash-cooling, is metastable and converts to crystalline ice modifications:

- cubic ice, forms when vitreous ice is warmed up above -135°C → keep samples below ~-135°C

- hexagonal ice, forms when water is (relatively slowly) cooled down at atmospheric pressure

(is typical source of contamination in cryo-EM)

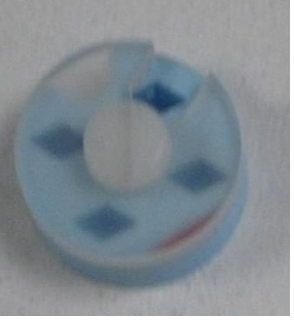


Dubochet *et al.*, 1988

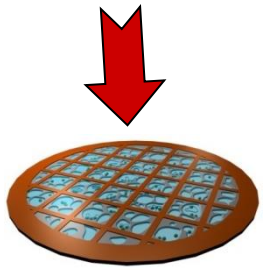
cooling rate required to obtain vitreous ice: ~10^4 K / s

Boiling and melting points of liquid ethane: -88.7 °C / -183.3 °C,
Boiling and melting points of Nitrogen: -196 °C / -210 °C,

Transfer into cryoTEM

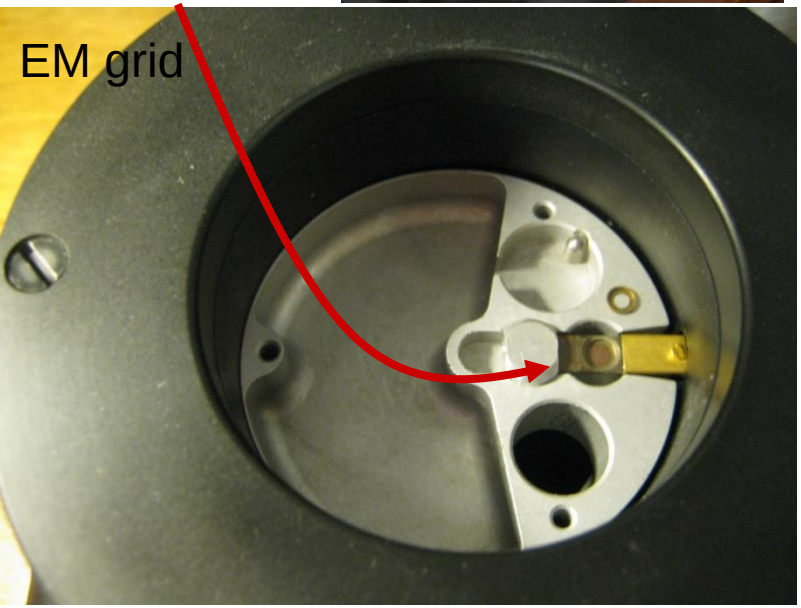


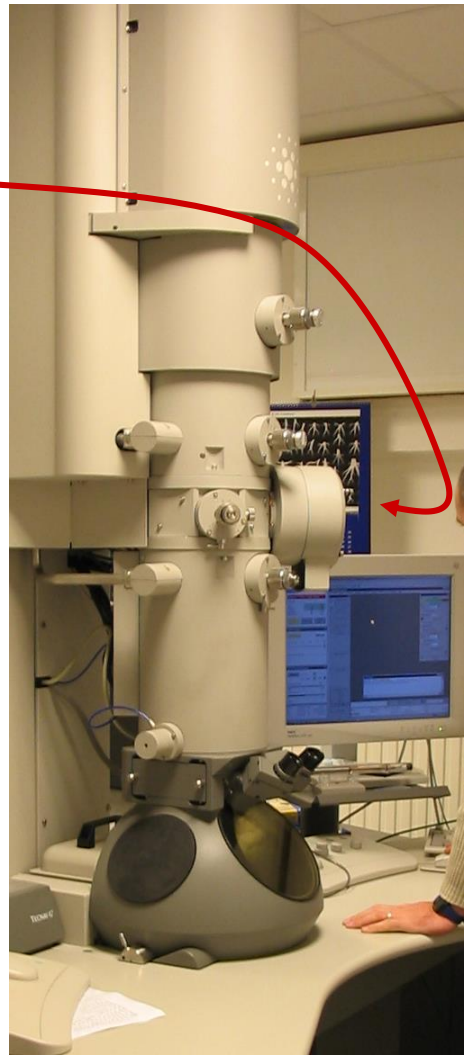
specimen



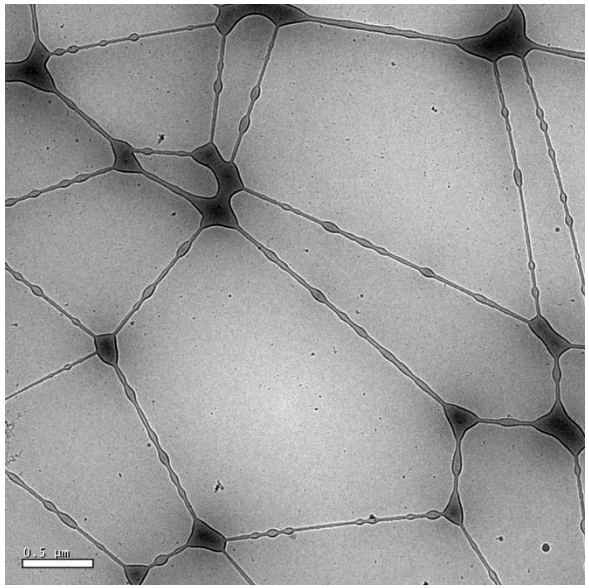
Cryo-holder

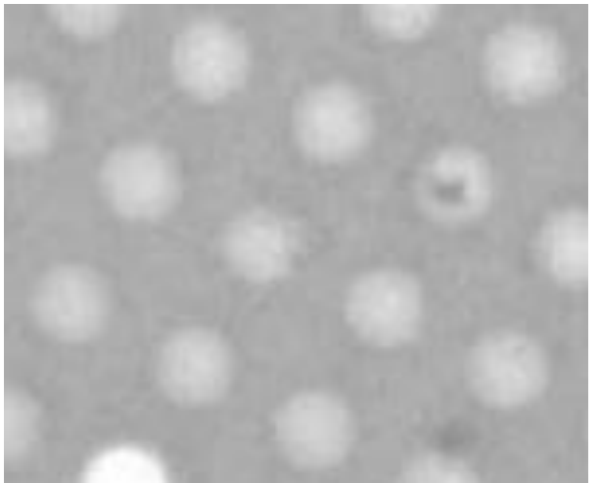
EM grid



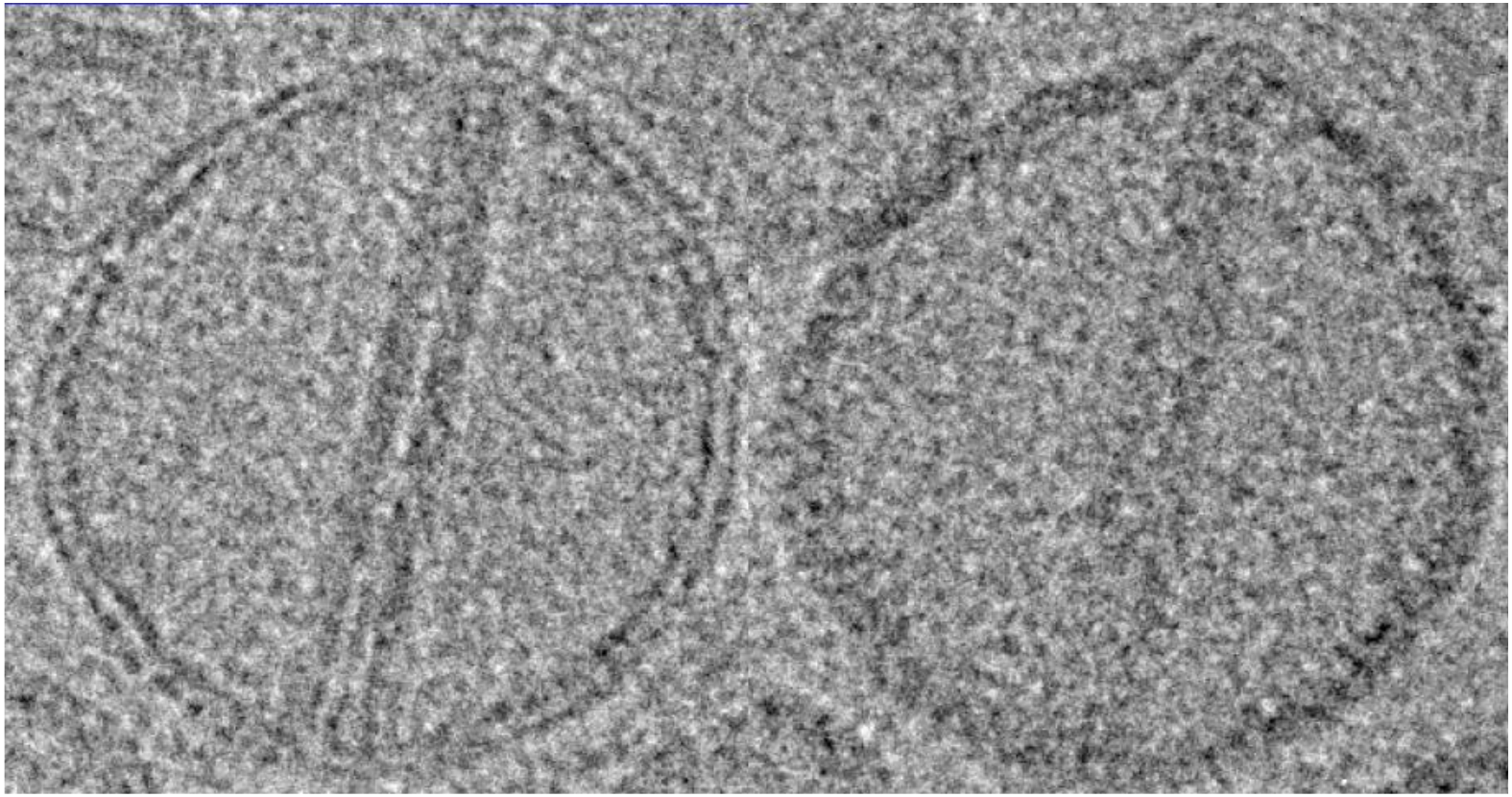


observation





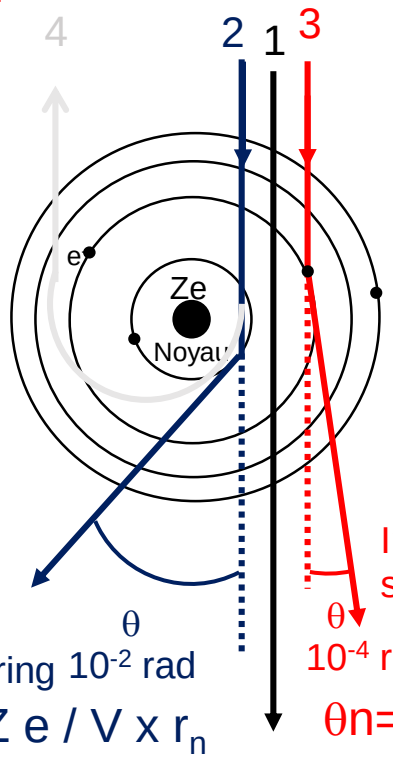
Destructive Power of Electrons



after 0.2 sec

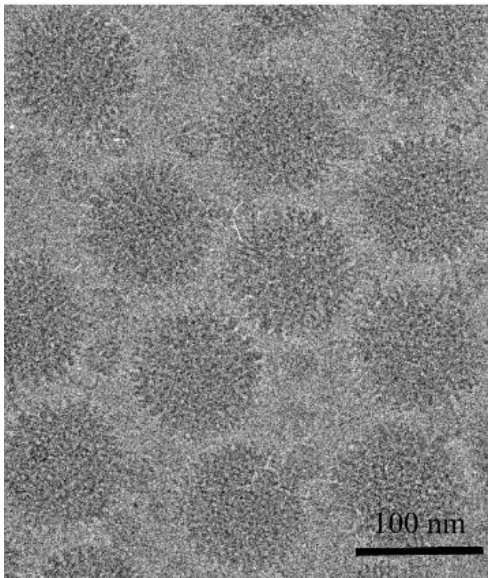
1 sec exposure

Low contrast image



Radiation damage

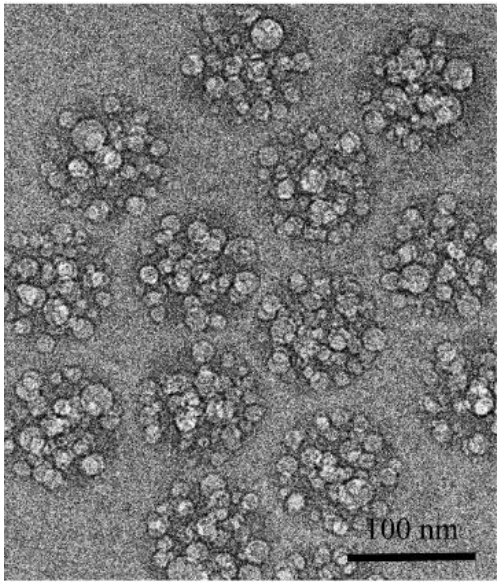
Noise



plus de 95%

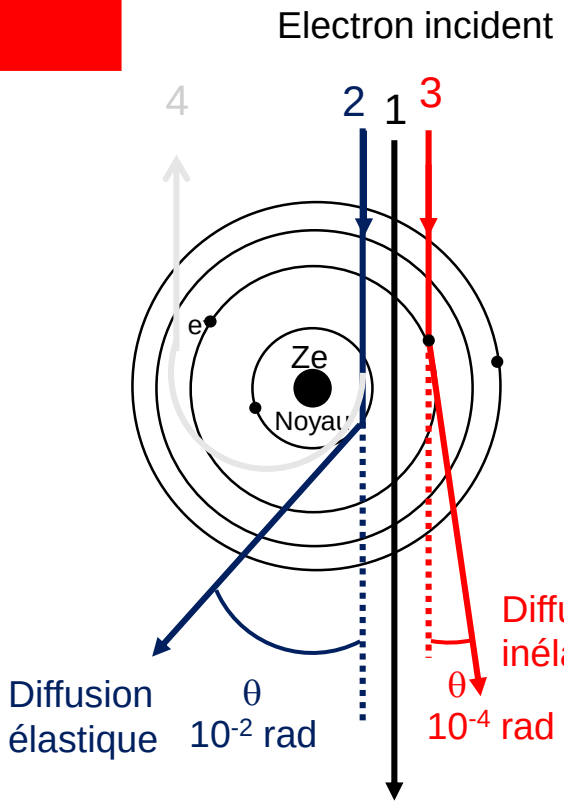
Noisy image

Low electron dose



Courtesy
Guy Schoehn

Low contrast image



10-20eV

Noise

Radiation damage

Noisy image

Only elastically scattered electrons contribute to the theoretical image intensity

Inelastically scattered electrons produced an unwanted background

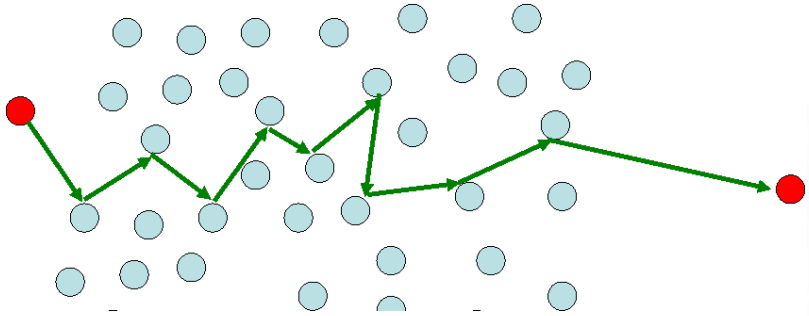
Signal Contrast

De=Z/20 Di

Sample thickness and inelastic scattering

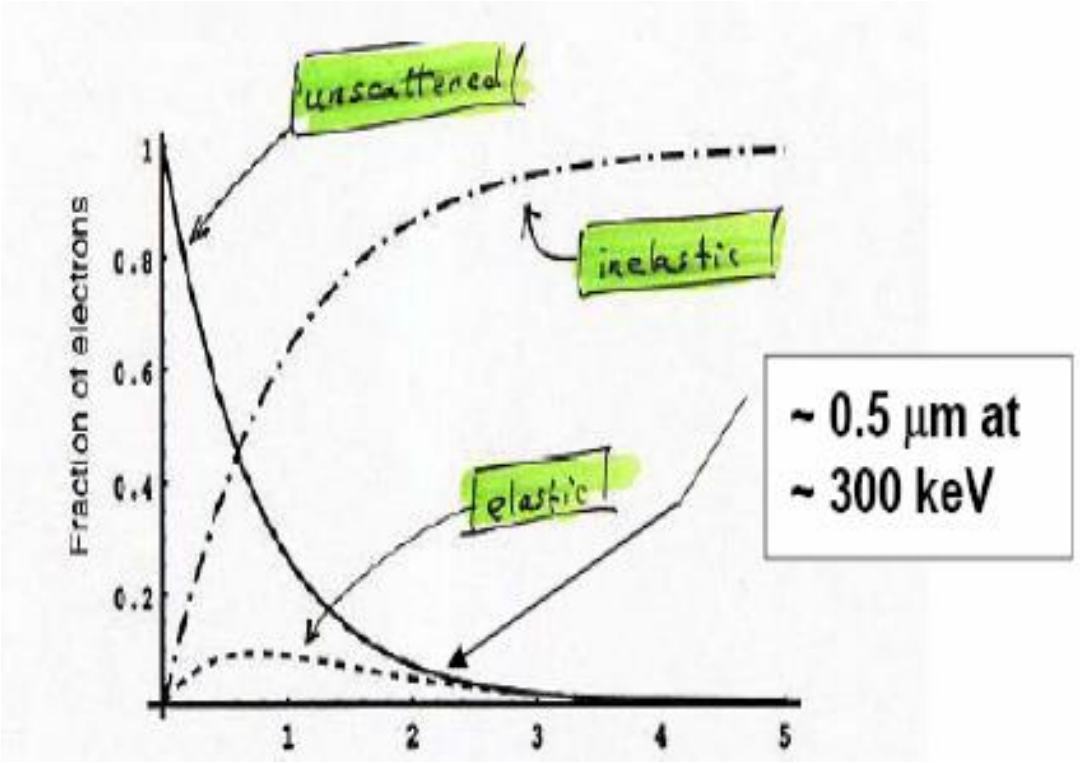
Mean free path

Distance of two successive elastic interactions



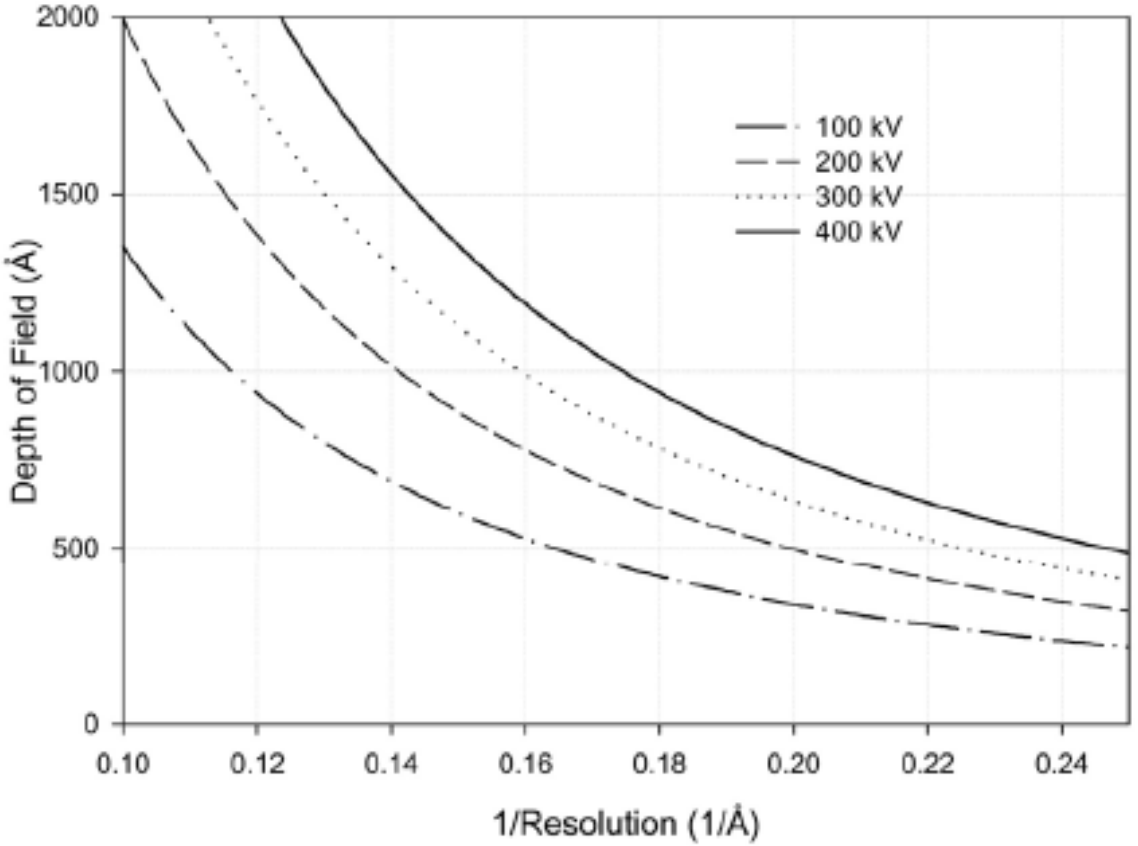
Λ = 500 Å for carbon, beyond multiple diffusion

When the specimen becomes too thick essentially all of the electrons will have been inelastically scattered



Mean free path

The Allowable Thickness is Also Resolution and Energy Dependent

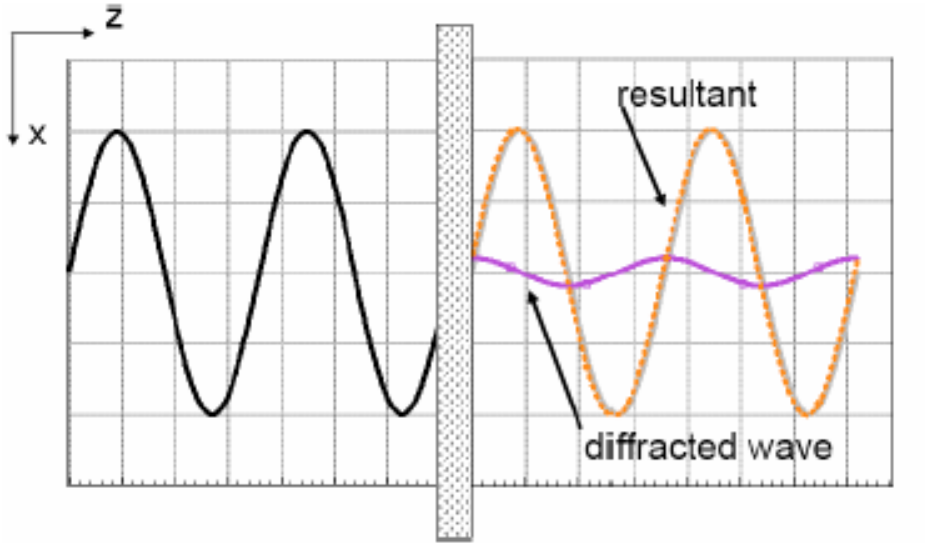


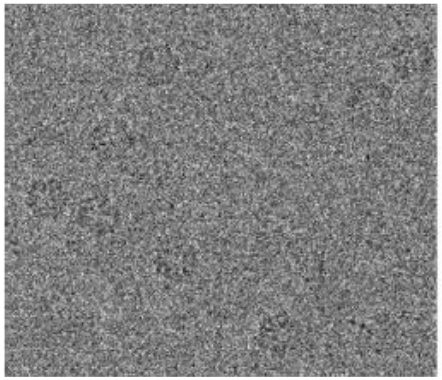
Necessary Assumptions:

- Image is a true 2-D projection of the 3-D object with the same focus throughout
- Only elastically scattered electrons form the image

Biological samples are phase objects

In cryo EM thin specimens do not absorb electrons, instead most of the electrons pass through the sample. The resultant wavefront emerges with almost the same amplitude, but has suffered a small phase shift proportional to the projection of the Coulomb potential. This can be reconstructed vectorially by interfering the undiffracted beam with a diffracted beam of low intensity that is shifted by ~90° ($\pi/2$) with respect to the undiffracted beam.

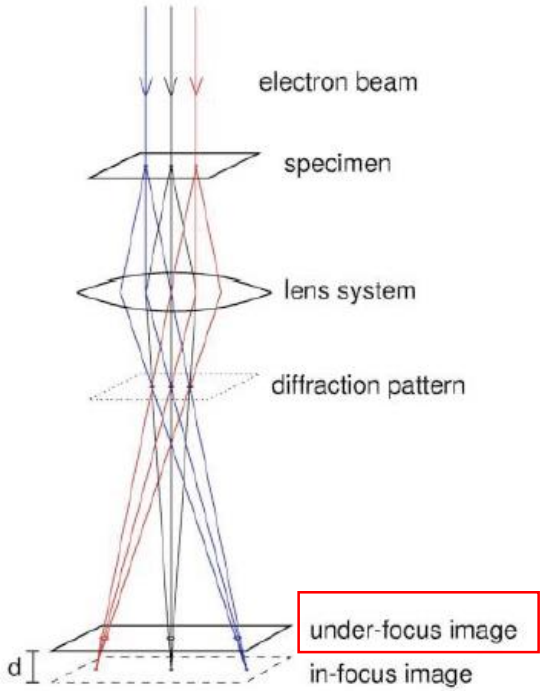




This is BAD news for imaging because in order to record a signal we need differences in amplitude…

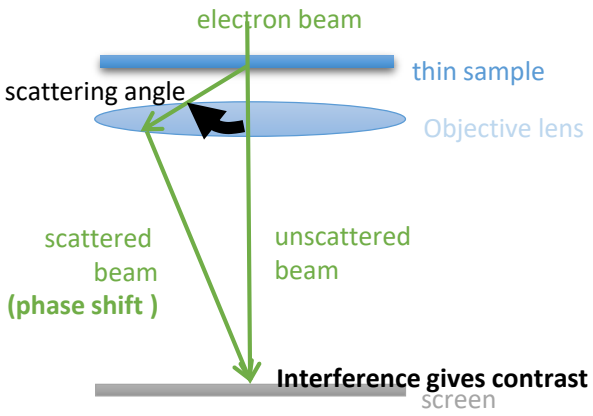
NO CONTRAST IMAGE

Image recorded in underfocus



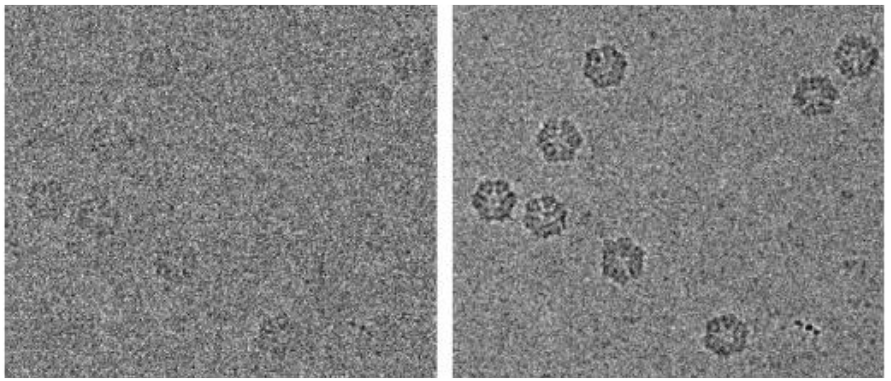
Volkmann & Hanein, 2002

We need to **underfocus ΔF** the image to add an additional shift that will produce considerable contrast



Close to **focus**

-2 µm defocus

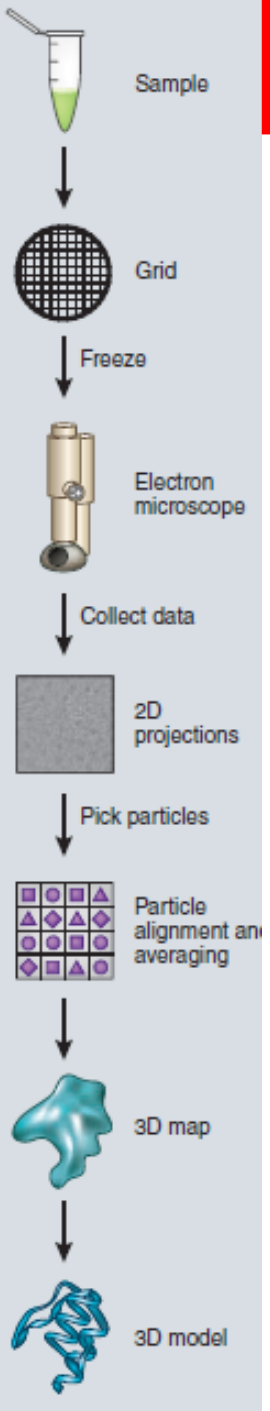


Nejadasl, 2011

➔ The signal in EM suffers from multiple aberrations (lenses, defocus) :
Contrast Transfer Function

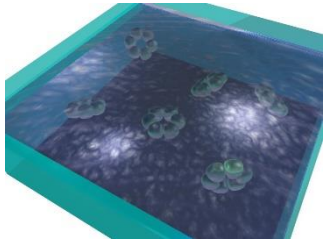
$$\gamma(X) = 2\pi\lambda\left(\Delta F\frac{X^2}{2} - C_s\lambda^3\frac{X^4}{4}\right)$$

how to solve a macromolecular structure using single-particle cryo-electron microscopy



Protein preparation: Purity, homogeneity Stability, biochemical activity

1982 CryoEM preparation

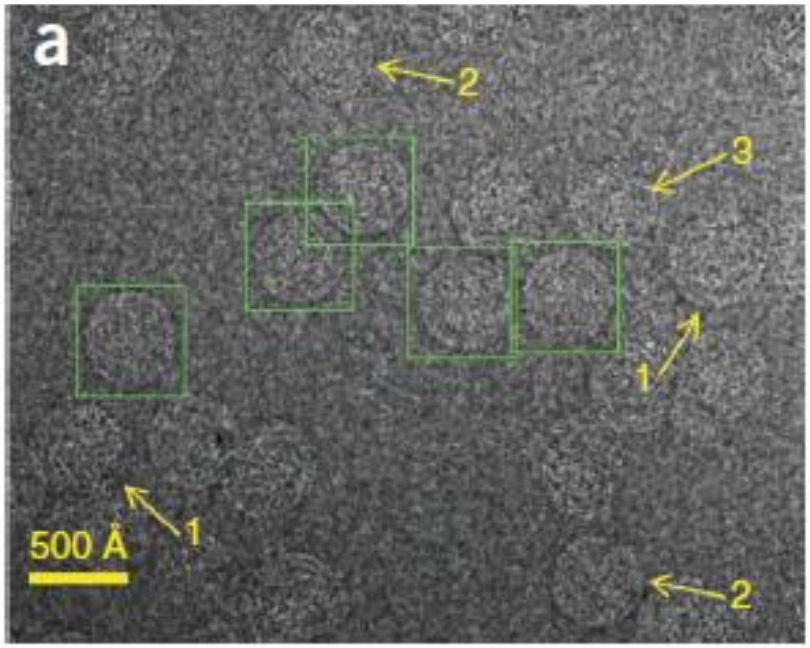


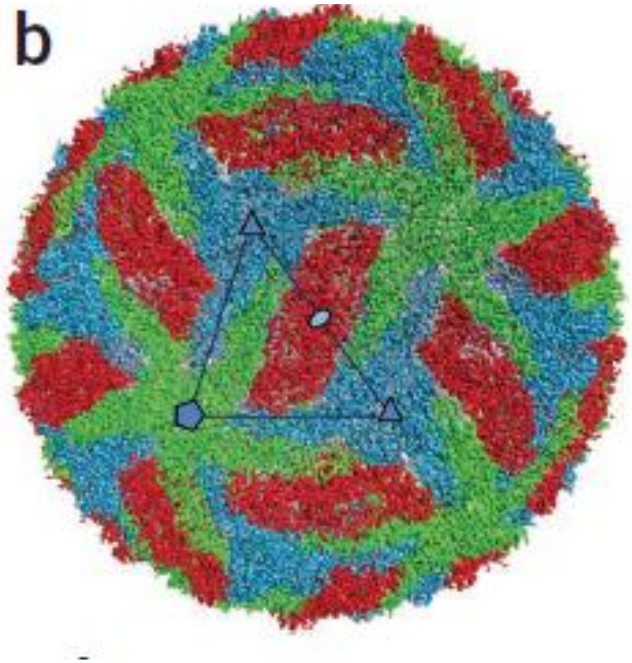
2008 300 kV FEG, Parallel illumination, Automated collection very stable stage, autoloader

Cryo-EM structure of the mature dengue virus at 3.5-Å resolution

Nature Struct Mol Biol, 2013

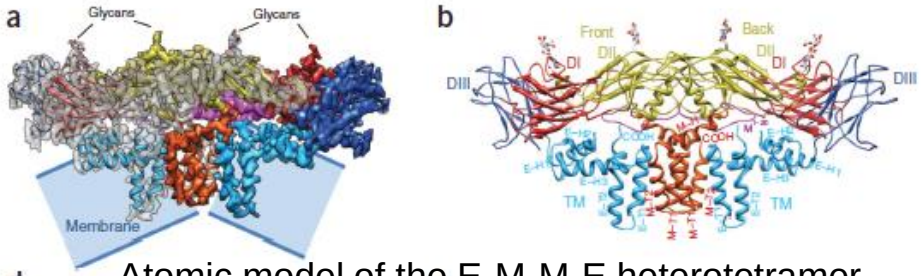
Xiaokang Zhang[1–5,9], Peng Ge[1–3,9], Xuekui Yu[1–3], Jennifer M Brannan[3,8], Guoqiang Bi[4,5], Qinfen Zhang[6], Stan Schein[2,7] & Z Hong Zhou[1–5]





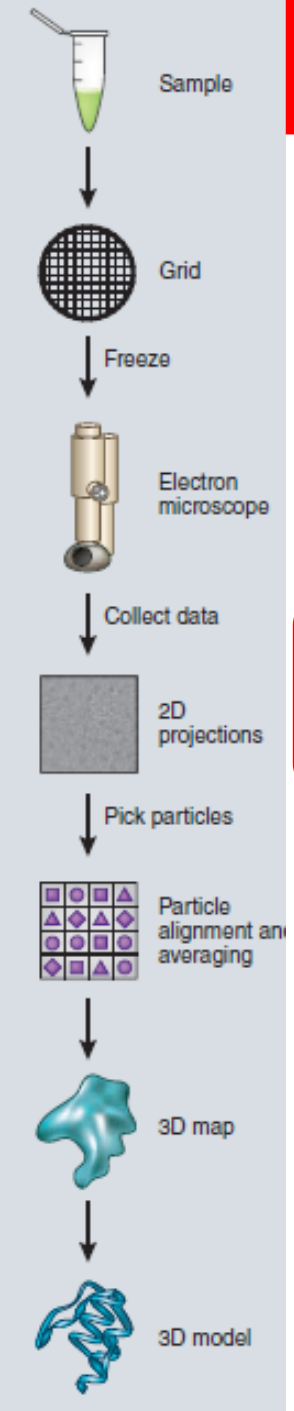
FEI Titan Krios cryo–electron microscope operated at 300 kV.

These images were recorded on **Films** and were digitized in Nikon CoolScan **scanners** (pixel size 1.104 Å/pixel). Approximately **32,569** particles were selected from the **1,103 films**



Atomic model of the E-M-M-E heterotetramer

how to solve a macromolecular structure using single-particle cryo-electron microscopy



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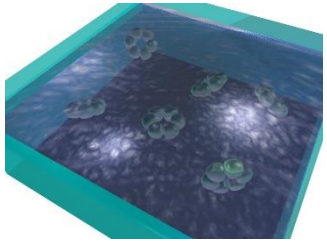
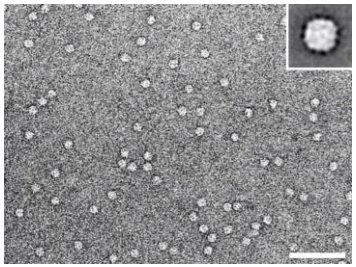


2008 300 kV FEG, Parallel illumination, Automated collection very stable stage

2012 New cameras : Direct Electron Detectors high sensitive very fast acquisition

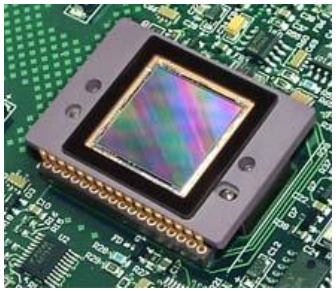
Conversion electron into images

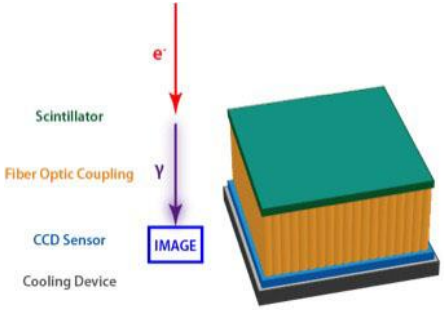
Film sensitive to electrons



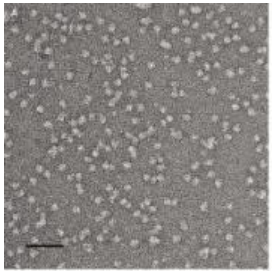
Pixel size = 8 µm
1 image / s

CCD



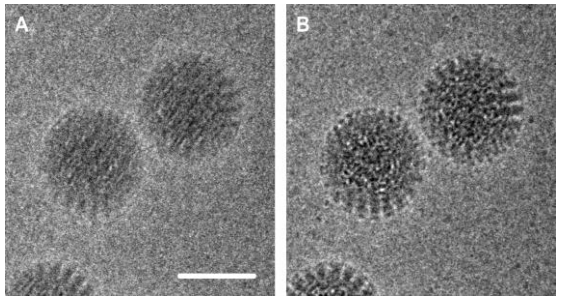


CCD Camera



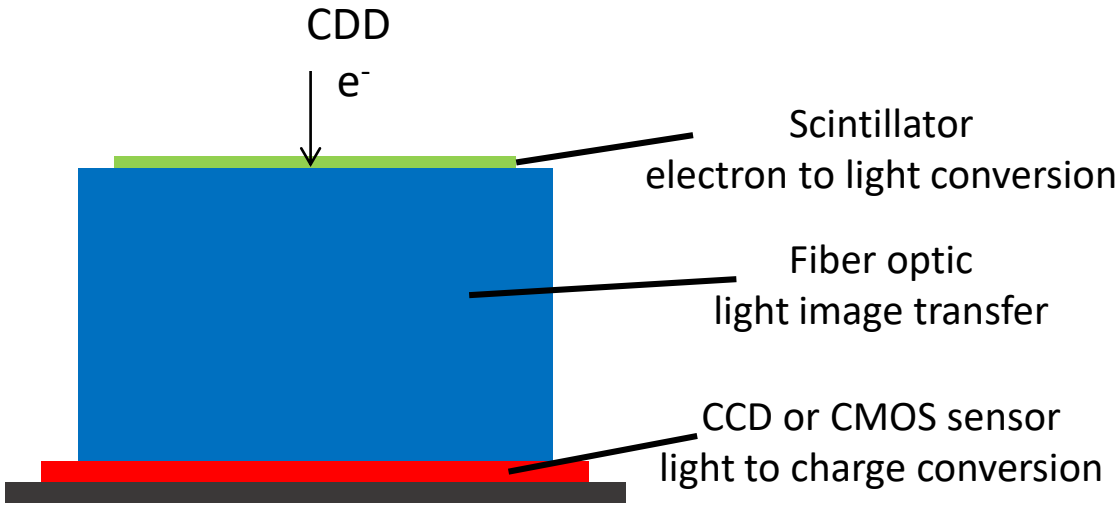
14 µm 1 image / s

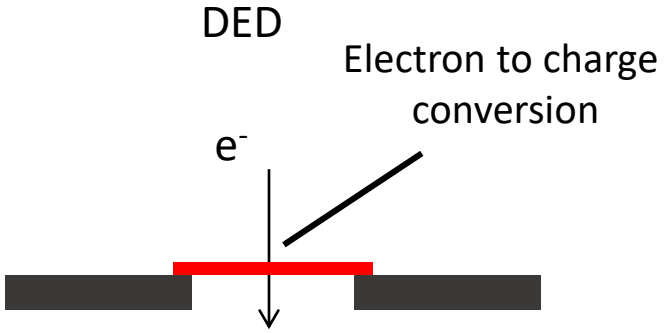
Direct Electron Detector CMOS

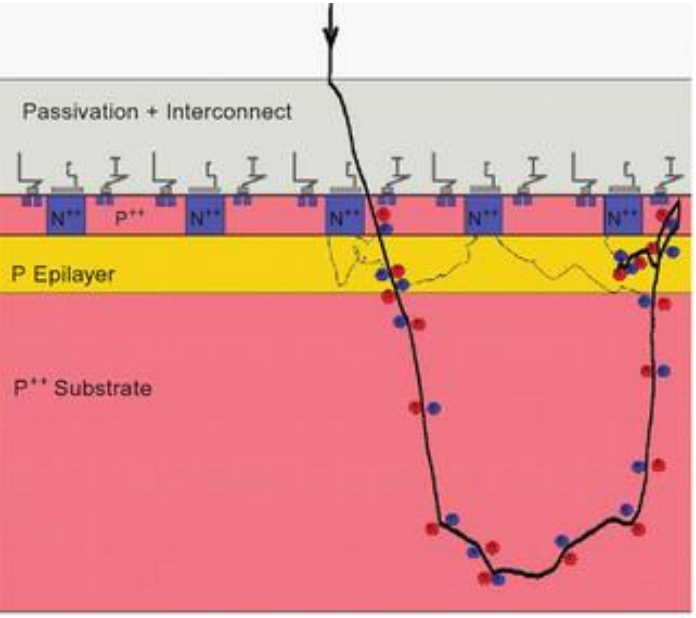


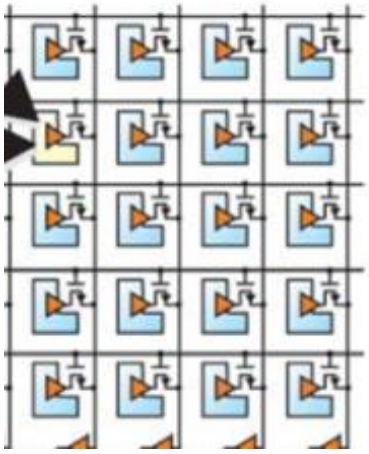
5 µm
20 images / s

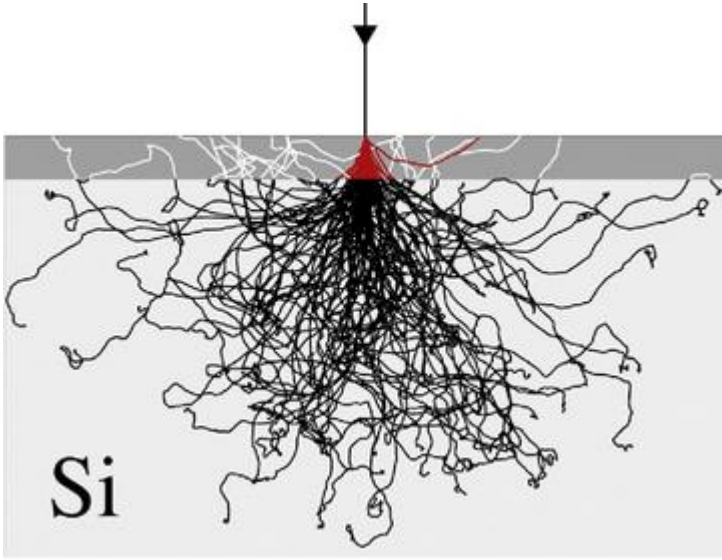
Advantage of Direct Electron Detector vs CCD camera







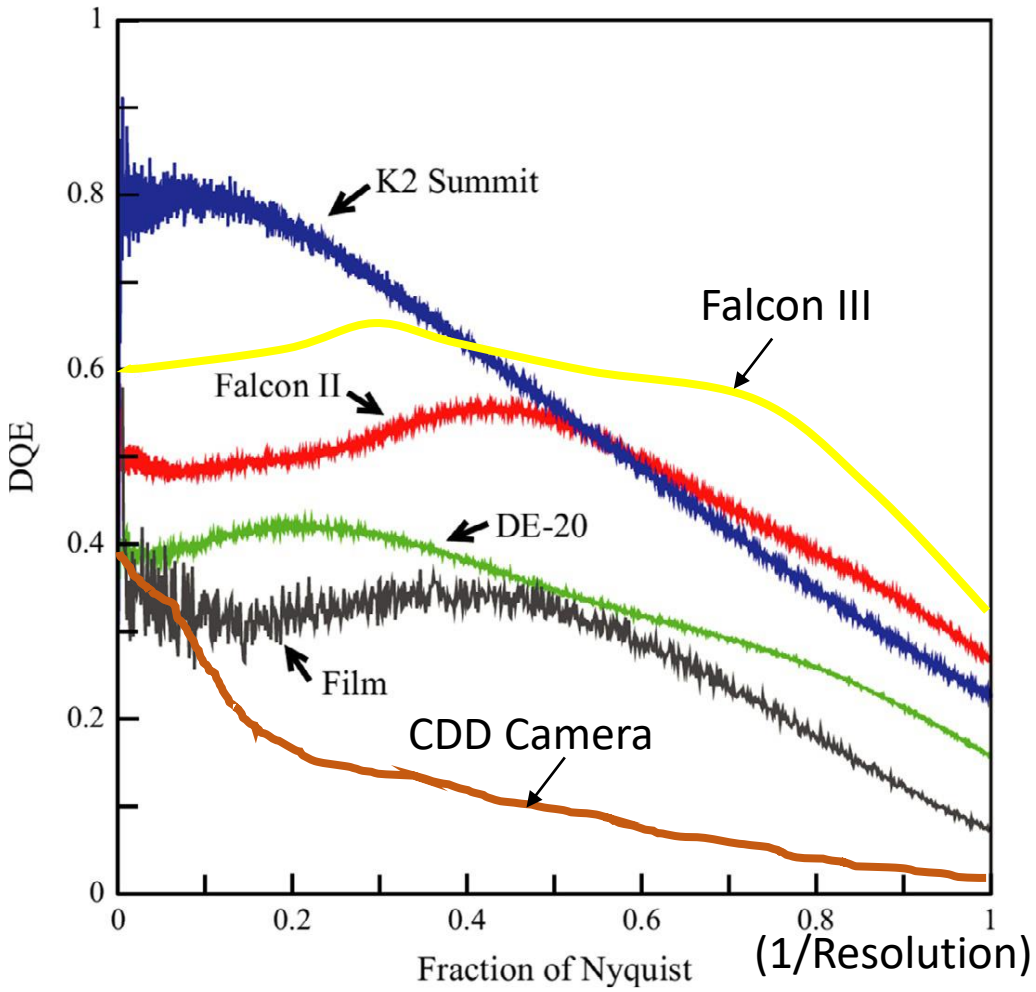




1 electron creates ~80 electron-hole pairs per micron (Faruqi, 2013)

Reduction of electronic noise
Reduction of electron scattering in the sensor

Detective quantum efficiency ≈ Sensitivity
% incident electron converted in signal



(1/Resolution)

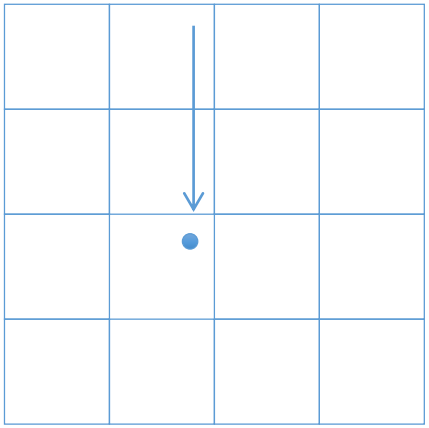
$$DQE = (S/N)^2_{out}/(S/N)^2_{in}$$

Higher DQE for DED
K2 summit (Gatan)
Falcon II and III (FEI)
DE-20 (Direct electron)

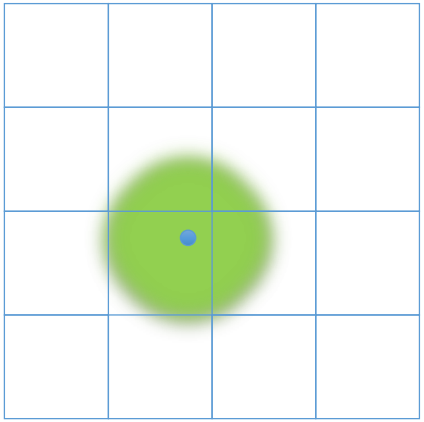
McMullan et al, 2014, and 2016

Counting mode

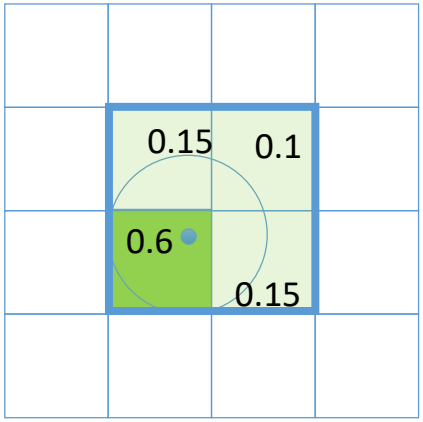
1. Electron enters detector



2. Signal is scattered

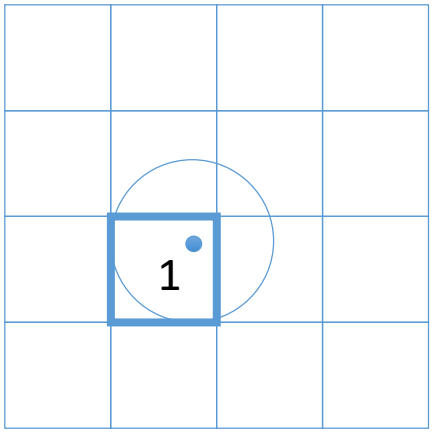


3. Charge collects in each pixel



Integration mode

4. Events reduced to the highest charge pixels

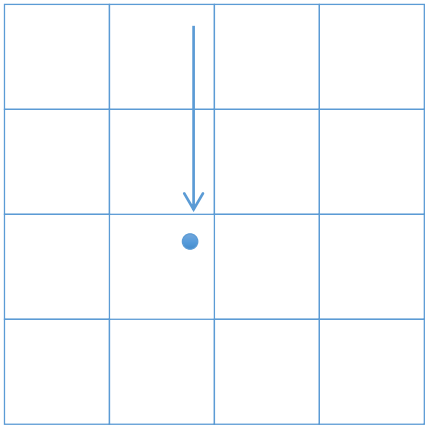


Counting mode

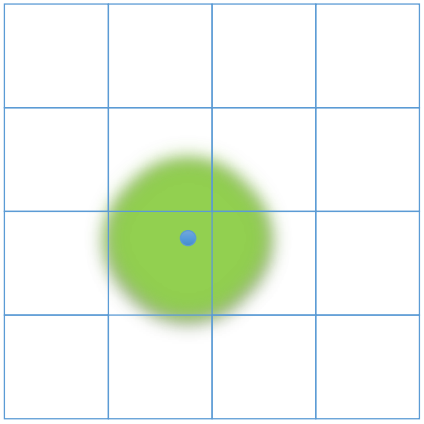
Improved DQE

Super resolution mode

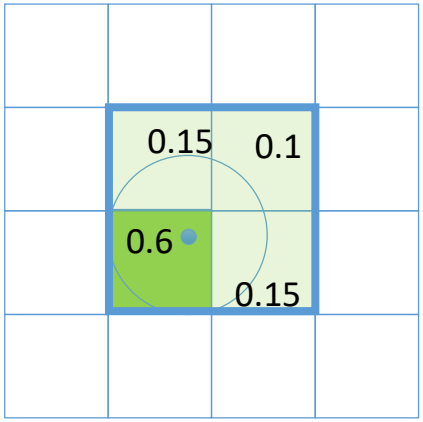
1. Electron enters detector



2. Signal is scattered

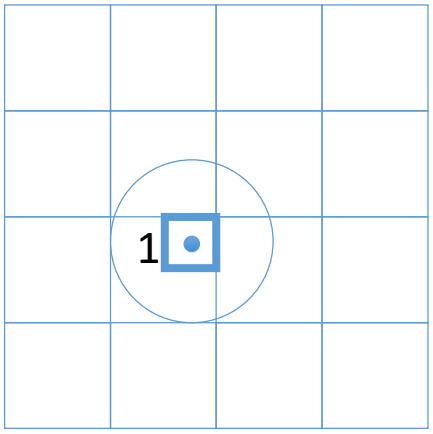


3. Charge collects in each pixel



Integration mode

4b. Events localized with sub-pixel accuracy

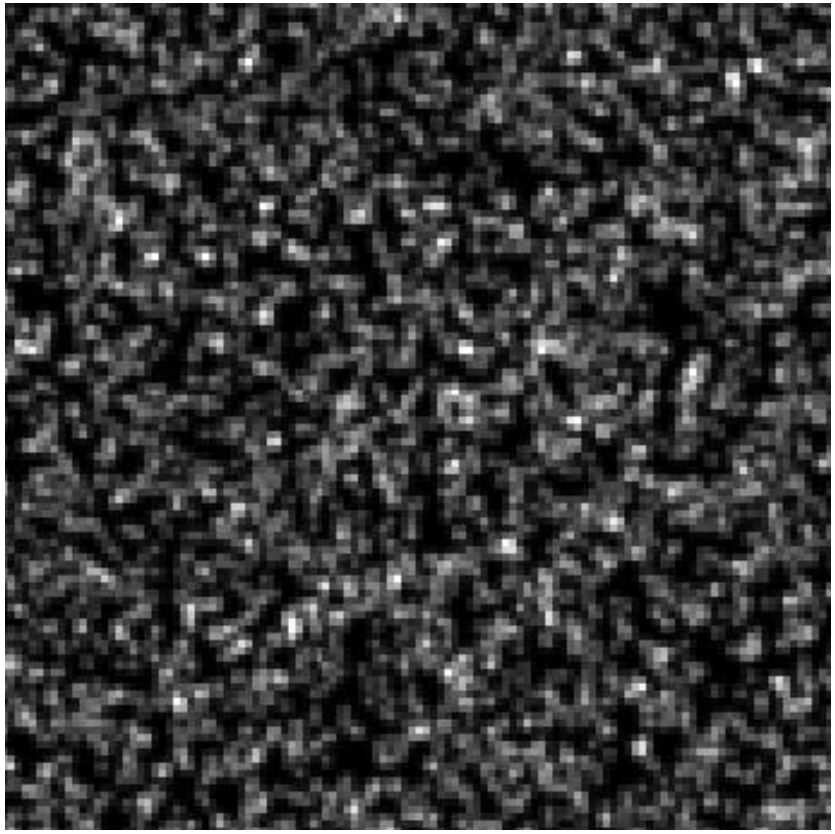


Super-Resolution counting mode

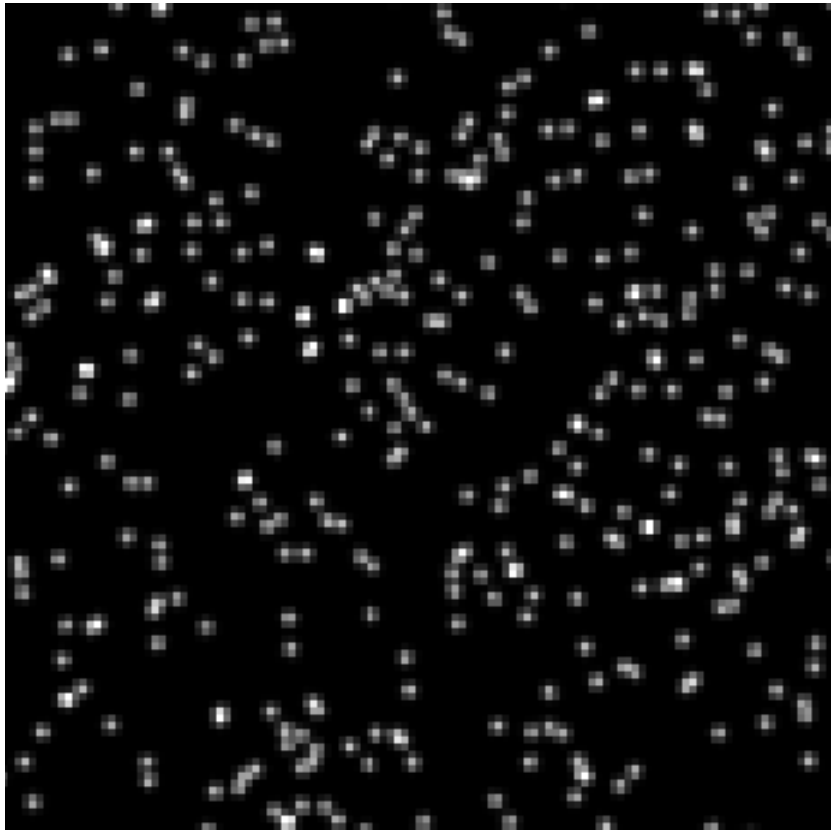
Improved DQE **7680 x 7424** pixels

Counting requires speed

Typical dose rate of 10 e^-/pix/s.



40 frames per second: events overlap and cannot be resolved.

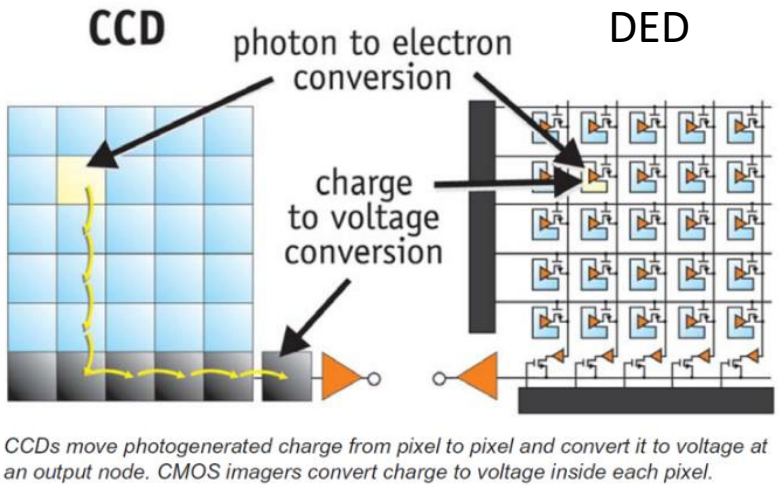


400 frames per second: events are resolved.

It takes 400 fps to resolve electrons at a dose rate of 10 e^-/pix/s.

Movie acquisition

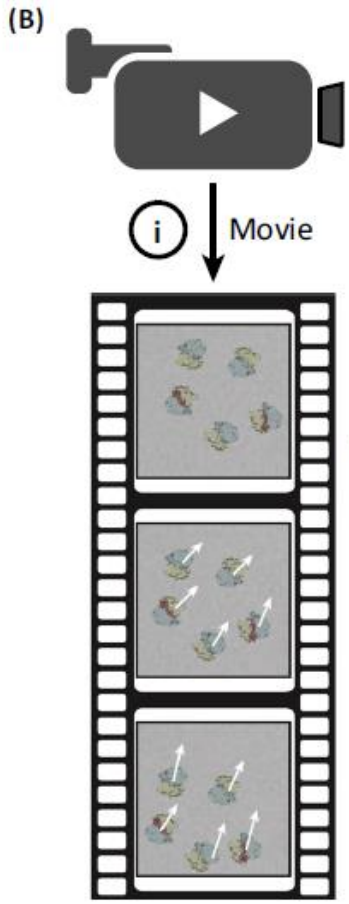
CMOS characteristic Rolling shutter : high speed read out
400 frames per second (for K2 camera)



Film/CDD

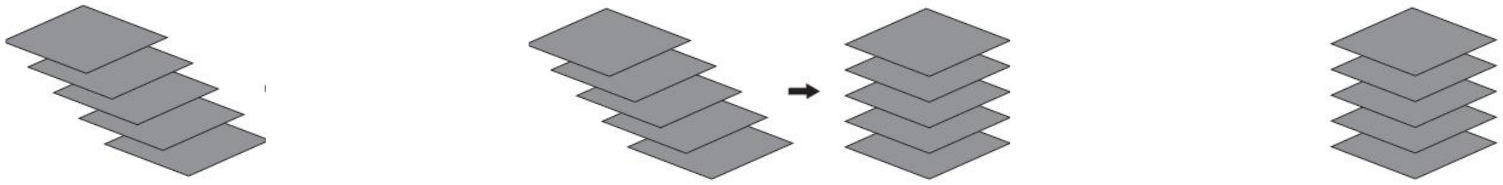
40 e/A2 in 1 image/ 1second

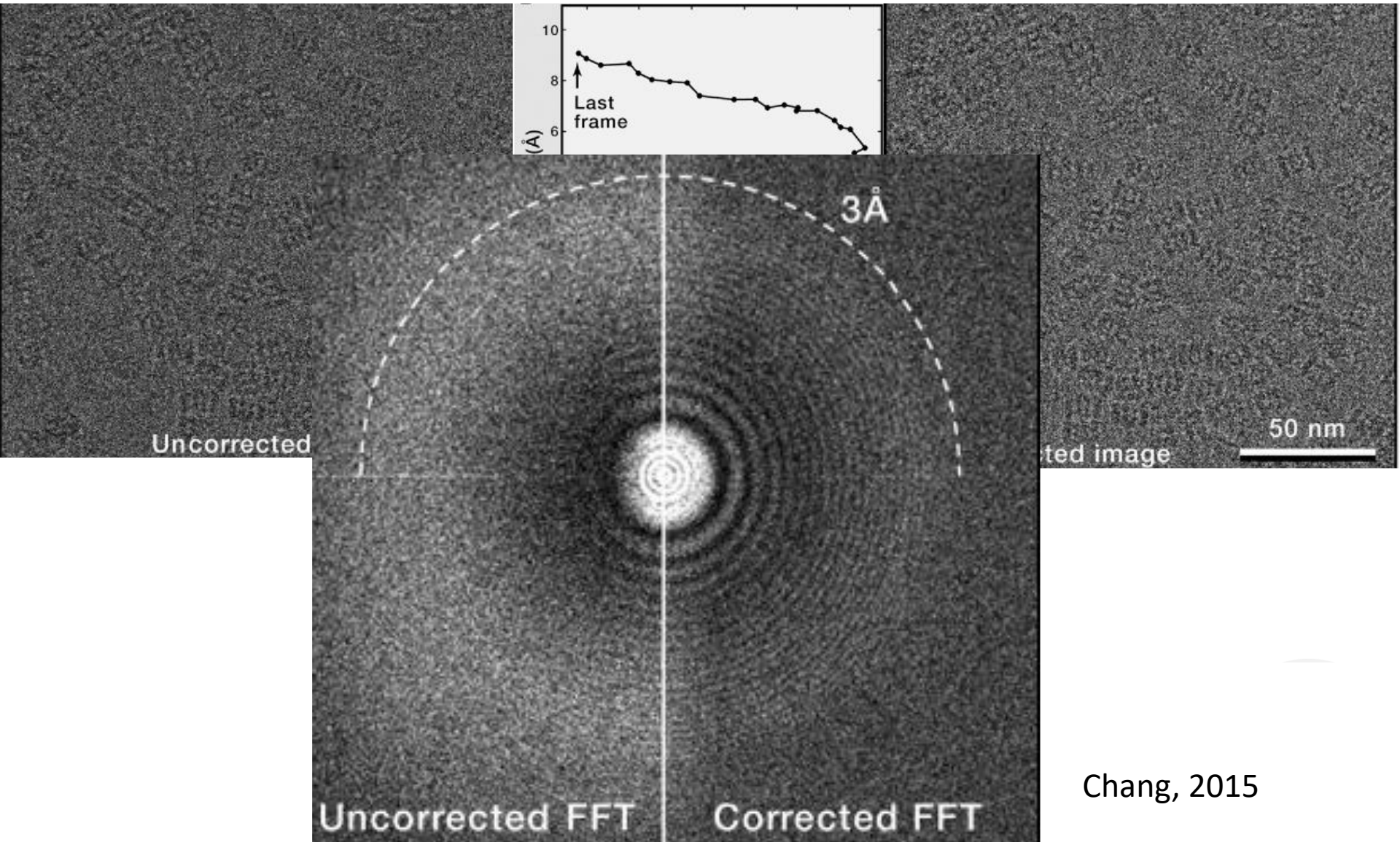
DED



Typically a movie of 40 frames
1 e/A2 /0.4 s
40 e/A2
Total 16 second
Dose fractioning

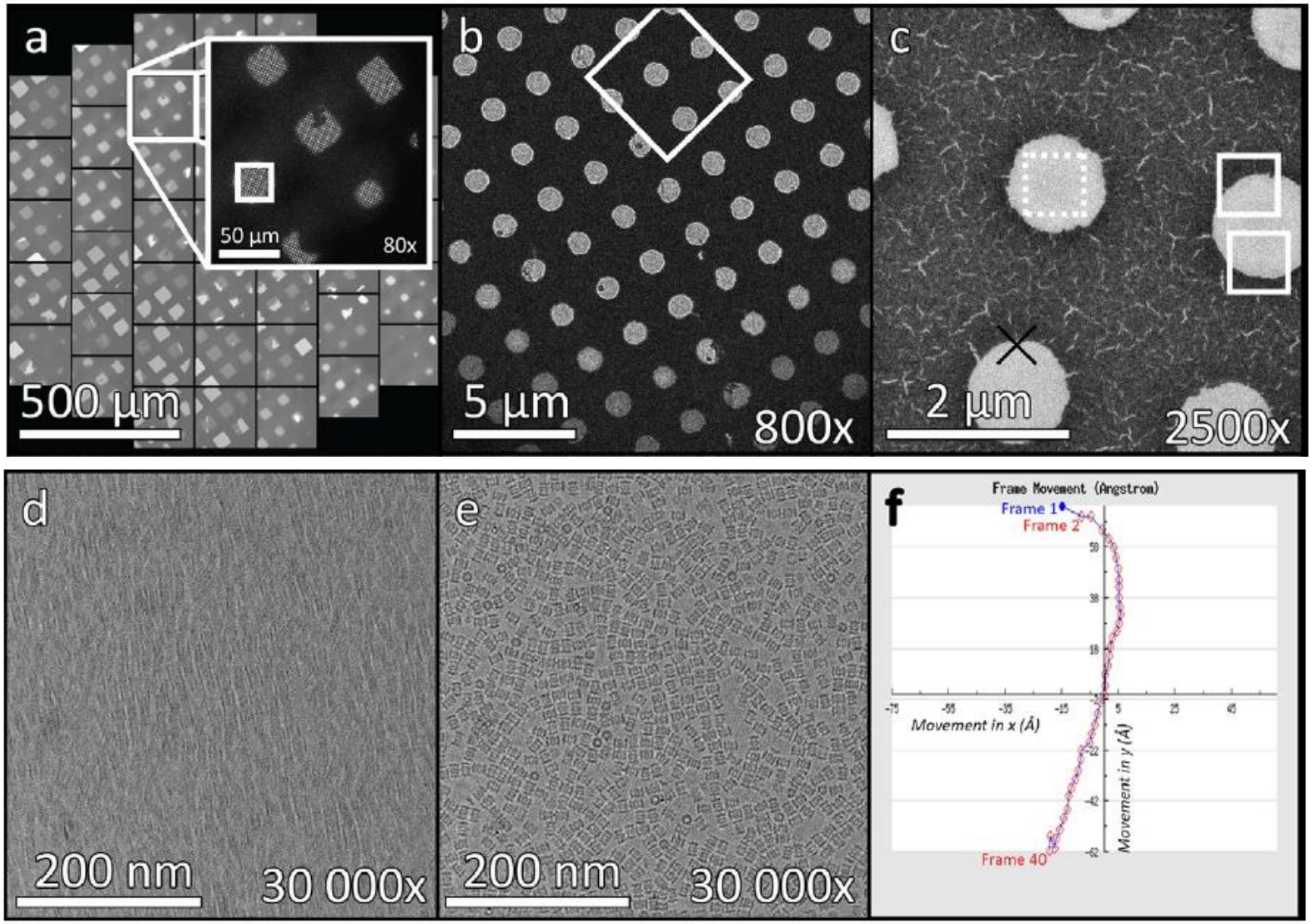
Advantage of Movie acquisition





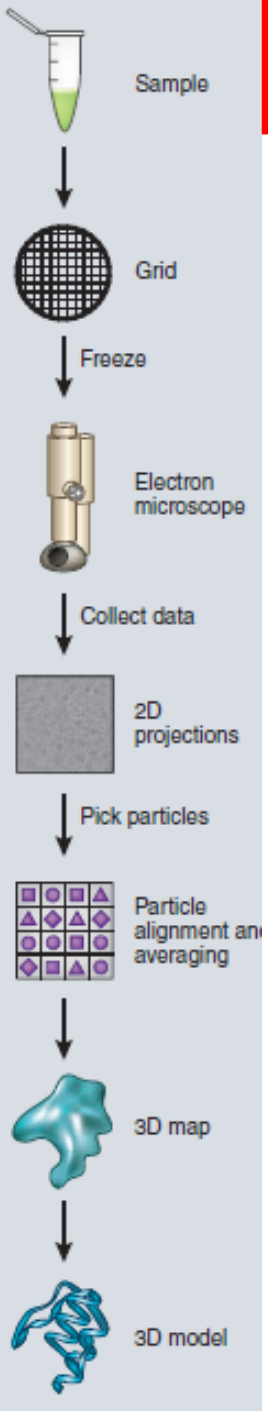
Chang, 2015

Automated CryoEM Data Collection Using Direct Detectors



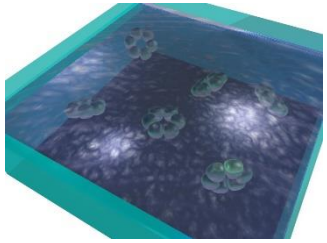
Chang, 2015

how to solve a macromolecular structure using single-particle cryo-electron microscopy



Protein preparation: Purity, homogeneity Stability, biochemical activity

1982 CryoEM preparation



2008 300 kV FEG, Parallel illumination, Automated collection very stable stage

2012 New cameras : Direct Electron Detectors
high sensitive
very fast acquisition

2012- New software configured for CPU clusters and GPU

Computational processing have been accelerated for

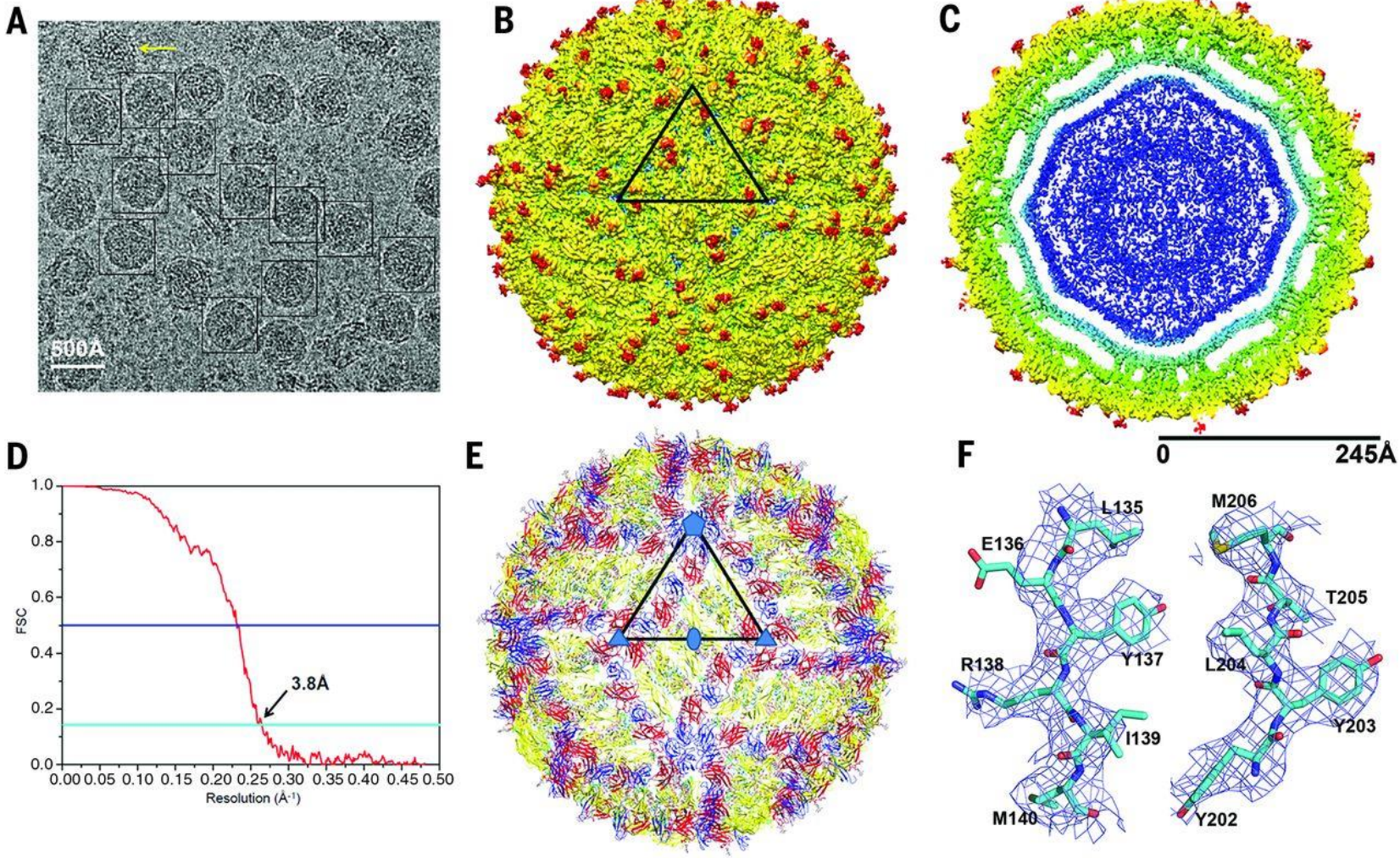
- particle autopicking,
- 2D- and 3D-classification,
- 3D refinement **on homogeneous data set**

Relion, Eman,…

STRUCTURAL BIOLOGY

The 3.8 Å resolution cryo-EM structure of Zika virus

Devika Sirohi et al. Science 2016;352:467-470

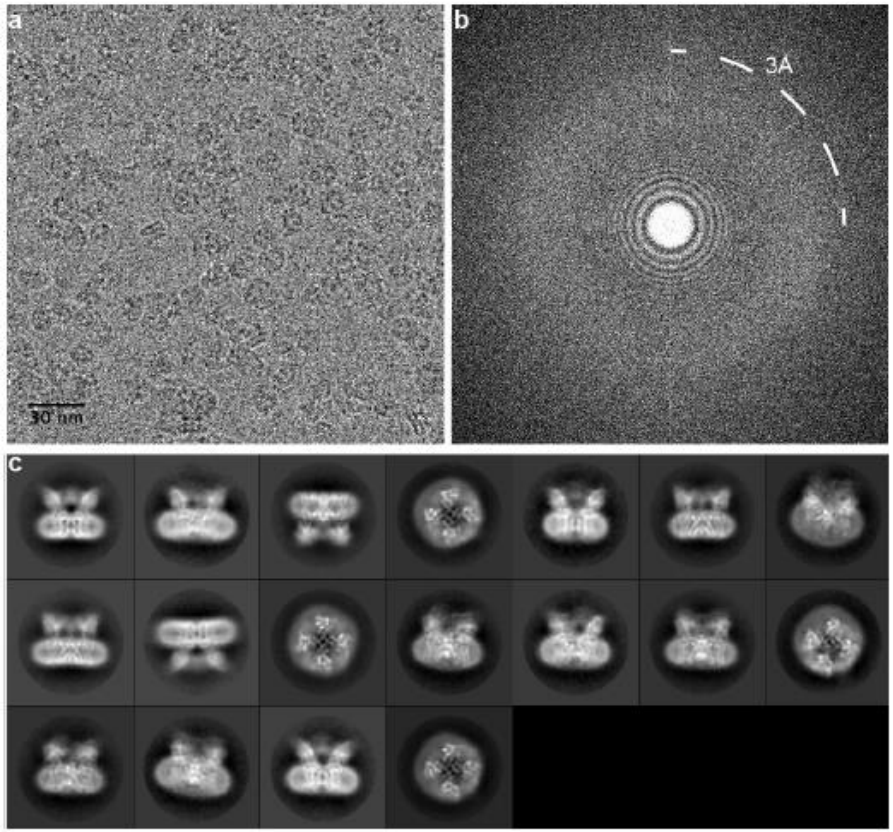


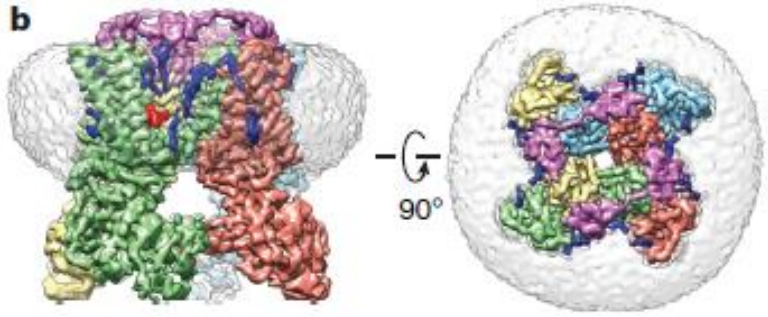
Krios, K2 pixel size of 1.04 Å 70 frames, Exp 14 s (2 electrons $Å^{-2}$ s^{-1}.)
2974 images 64,518 particles; 2D classification 20,151 particles
3D classification, **11,842 particles** at 3.8 Å resolution

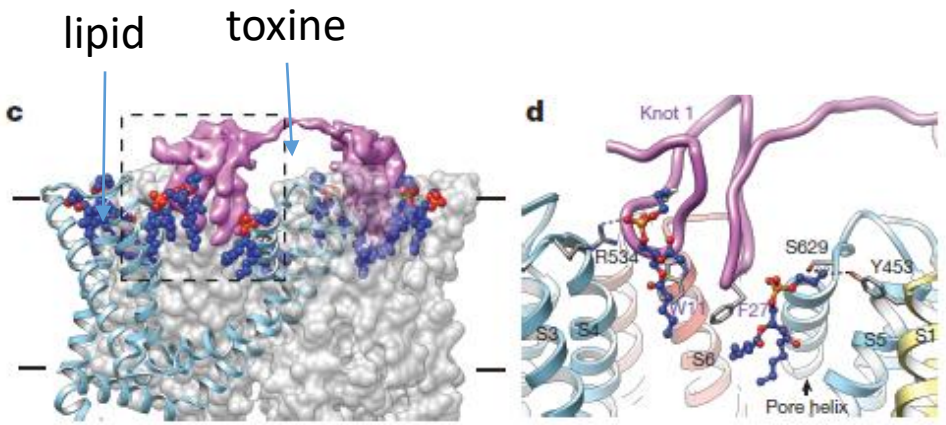
Dengue virus FEI Titan Krios
1,103 films (pixel size 1.104 Å/pixel)
32,569 particles for 3.5 Å resolution

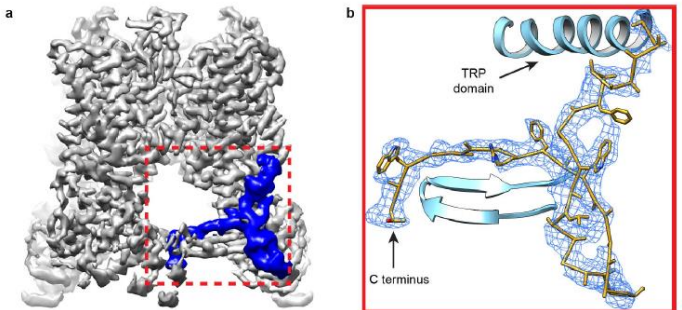
TRPV1 channel (transient receptor potential in nanodiscs Gao et al, Nature 2016

Polara, K2, 3A, ~30,000 particles from 1000 images



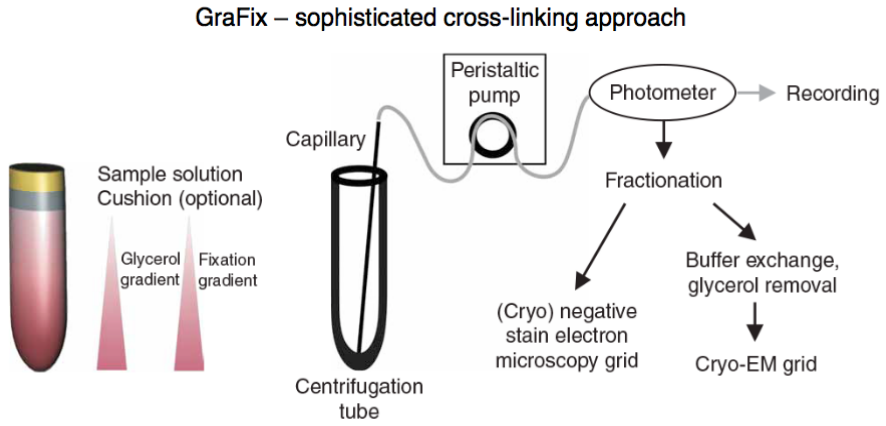


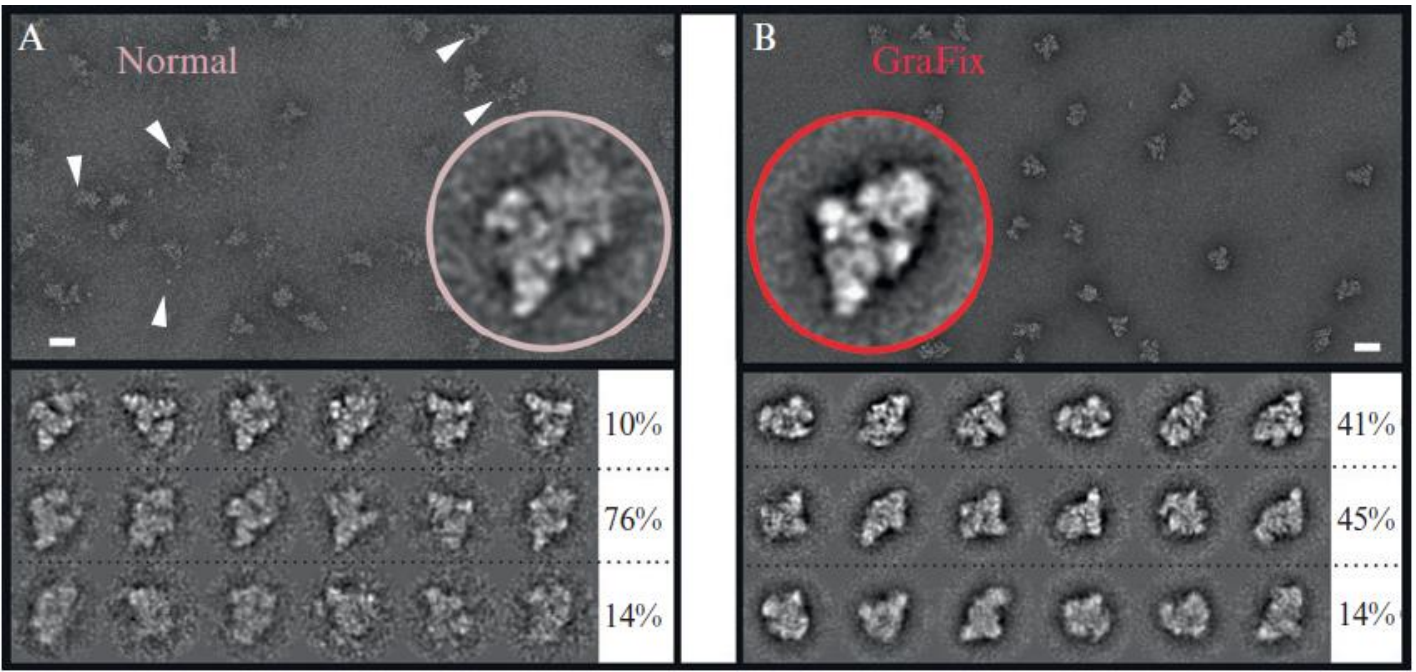




Improve the sample "stability"

Mild chemical fixation improves the stability of complex of protein (once deposited on the EM grid)



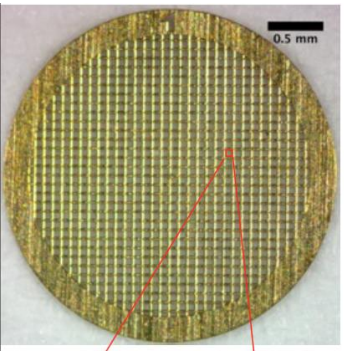


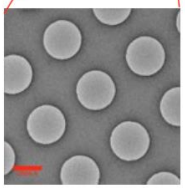
Kastner et al., (2008)
Nat. Methods 5:53-55.

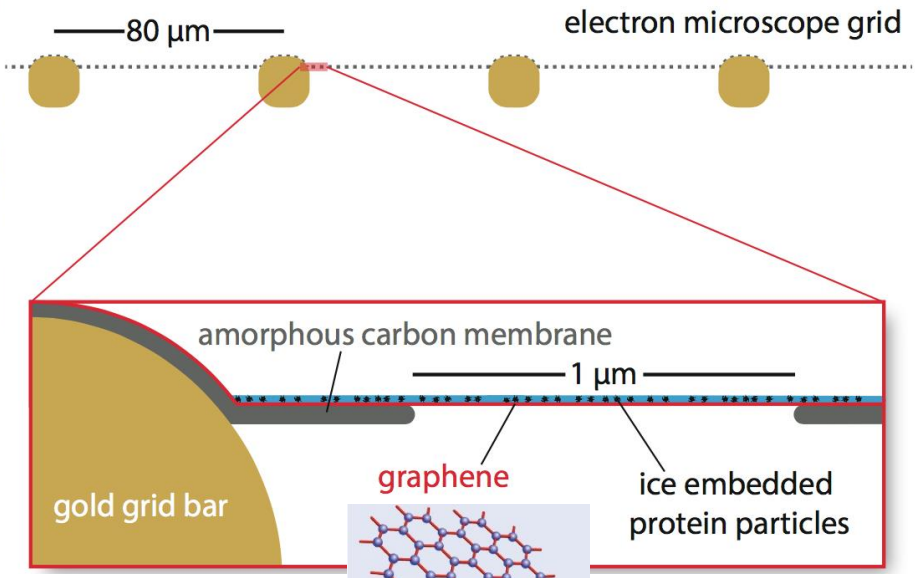
Spliceosomes
in uranyl formate

Improve the sample distribution

Use an extra graphene support

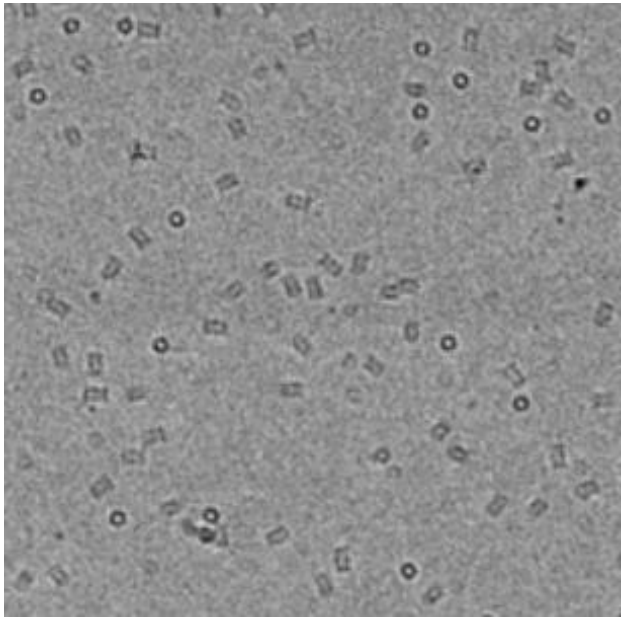




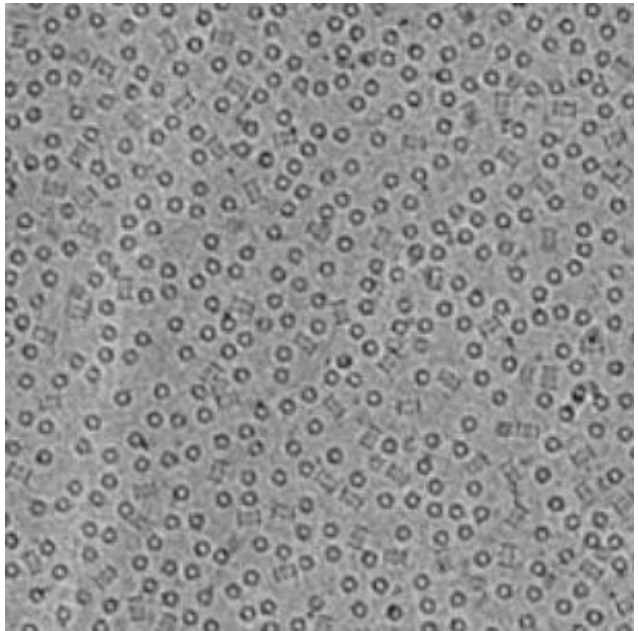


Russo & Passmore
Nature Methods 2014

Graphene modified with
low energy hydrogen plasma

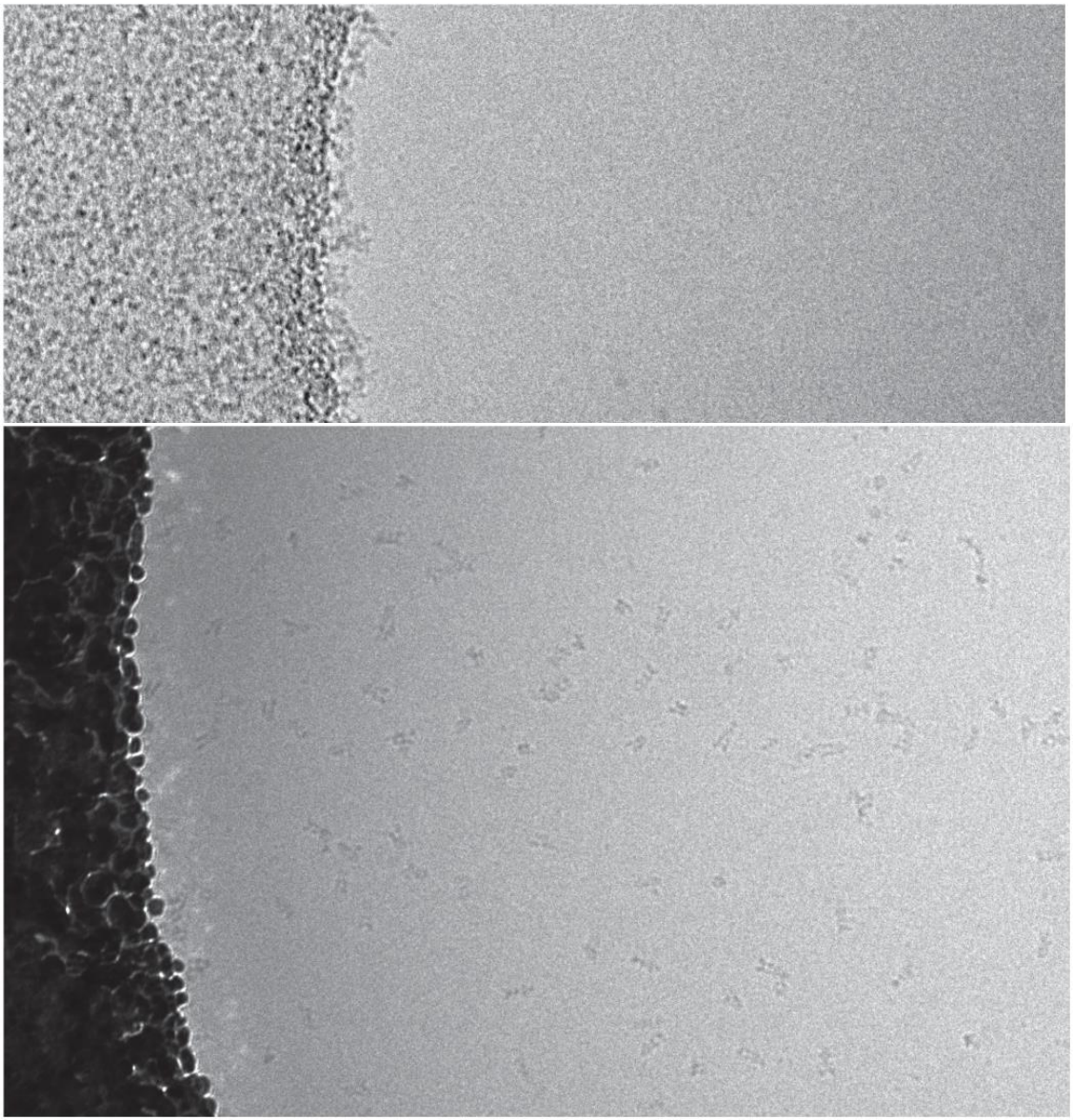


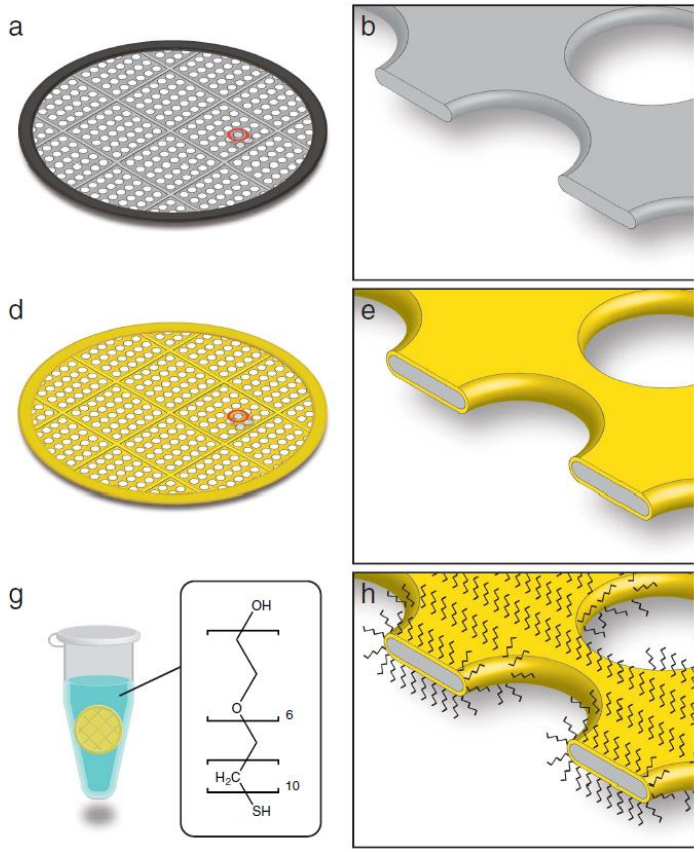
No Graphene



Improve the sample distribution

Use self assembled monolayers on gold grid



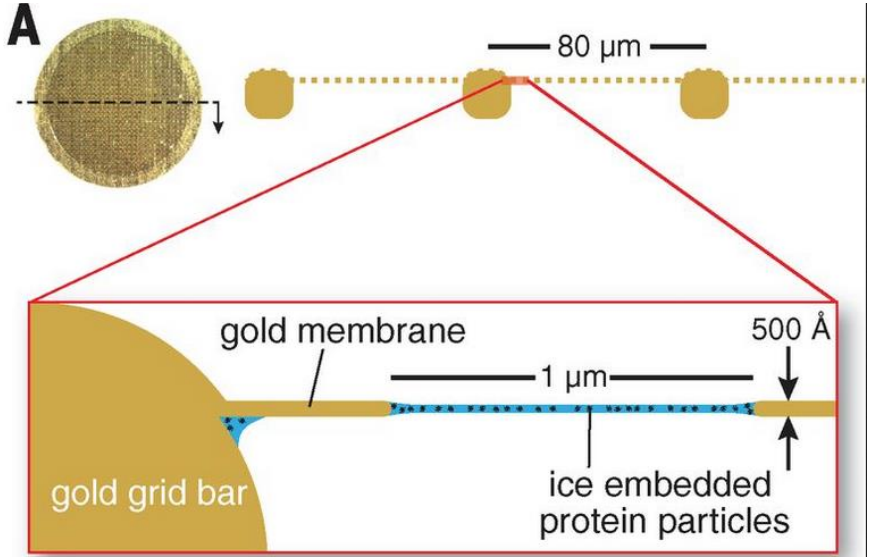


Meyerson Sci rep, 2014
Glutamate receptor

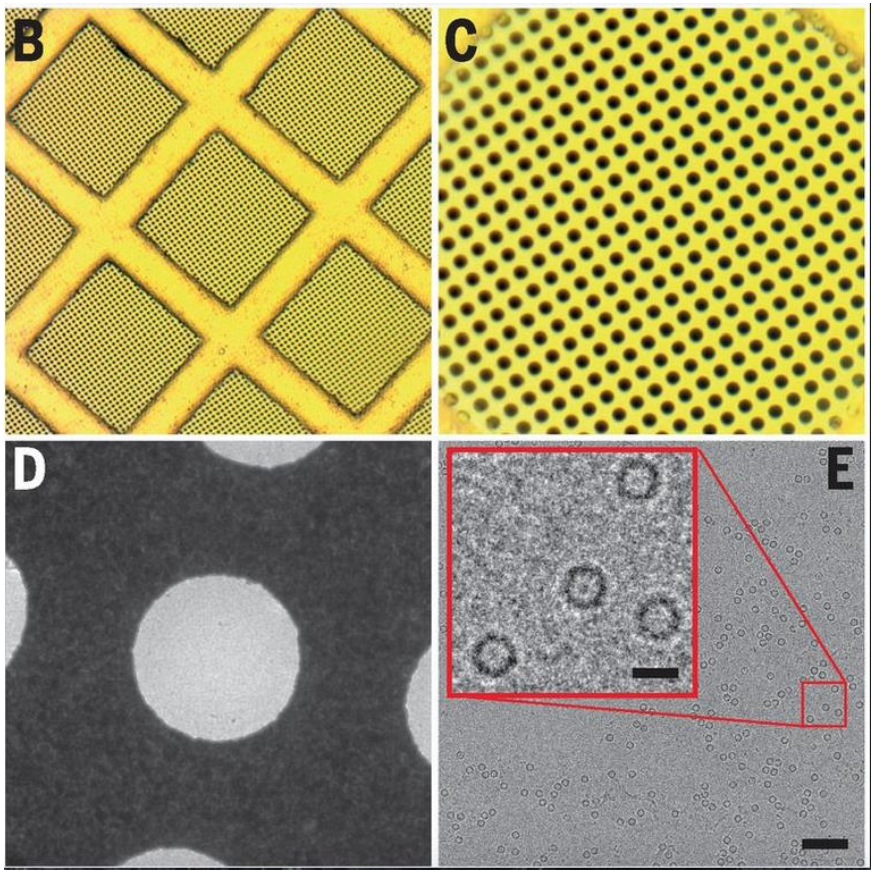
Improve the stability of the substate under electron beam

Use a gold grid

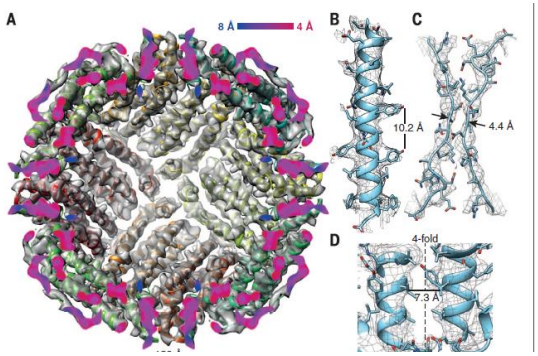
a gold specimen support
nearly eliminates substrate motion during irradiation

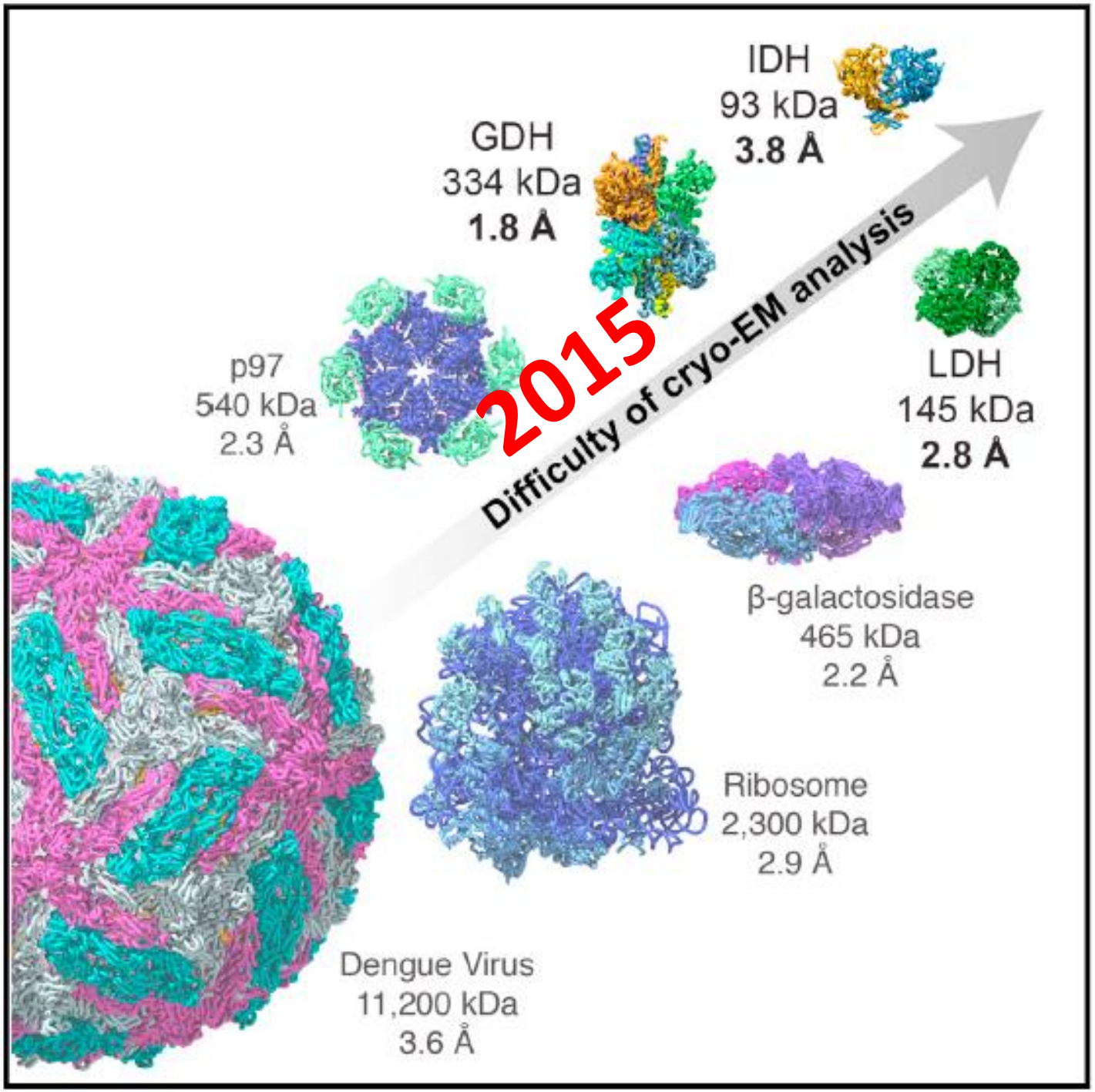


Russo & Passmore Science 2014



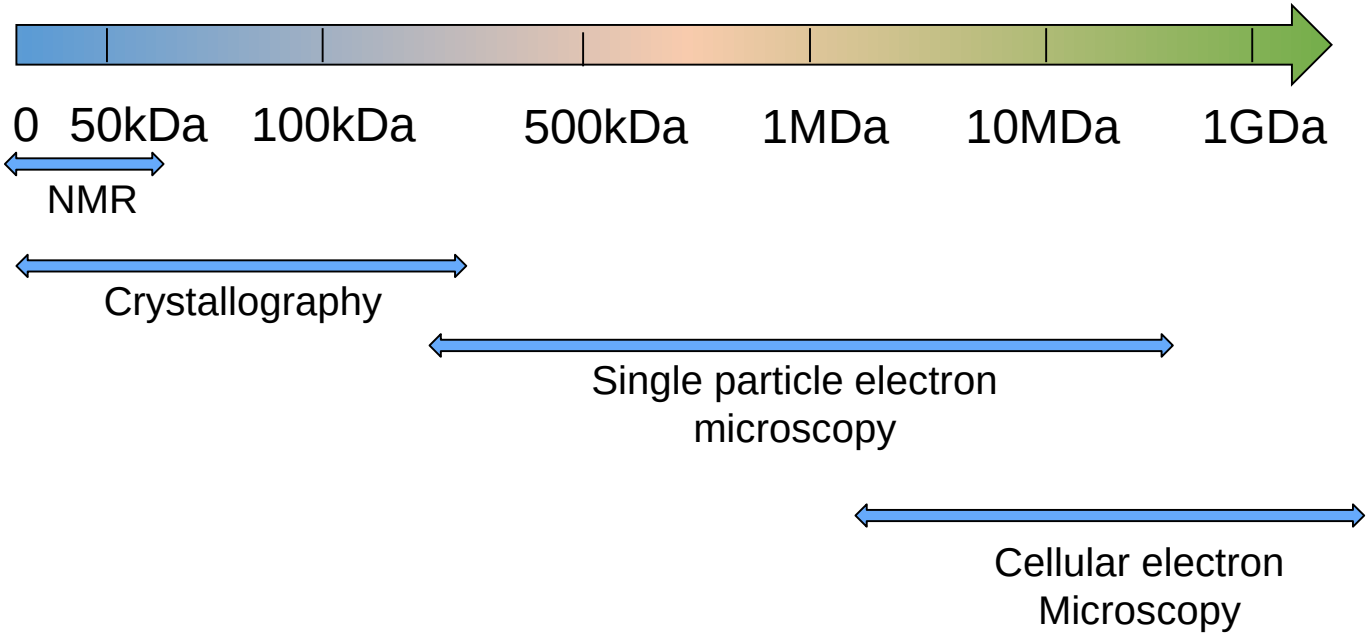
Compared with commercial am-C supports with nearly identical geometry, there was a 40-fold reduction movement. Apoferritin, 483 images , resolution 4.7 A.



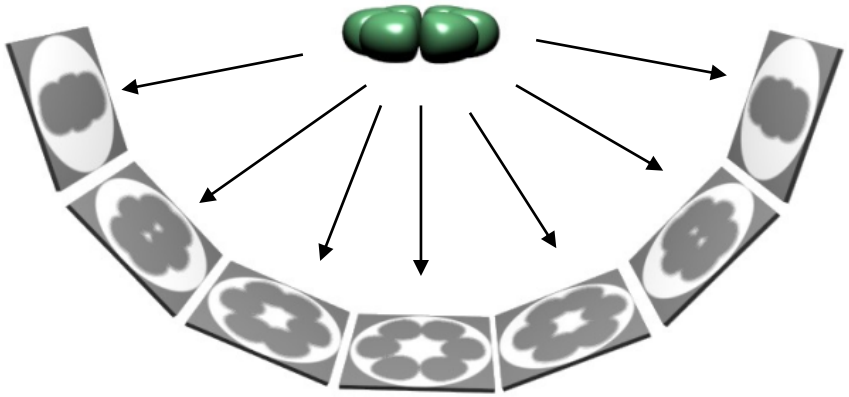

IDH
93 kDa
3.8 Å
GDH
334 kDa
1.8 Å
2015
Difficulty of cryo-EM analysis
p97
540 kDa
2.3 Å
LDH
145 kDa
2.8 Å
β-galactosidase
465 kDa
2.2 Å
Ribosome
2,300 kDa
2.9 Å
Dengue Virus
11,200 kDa
3.6 Å

Merk, 2016

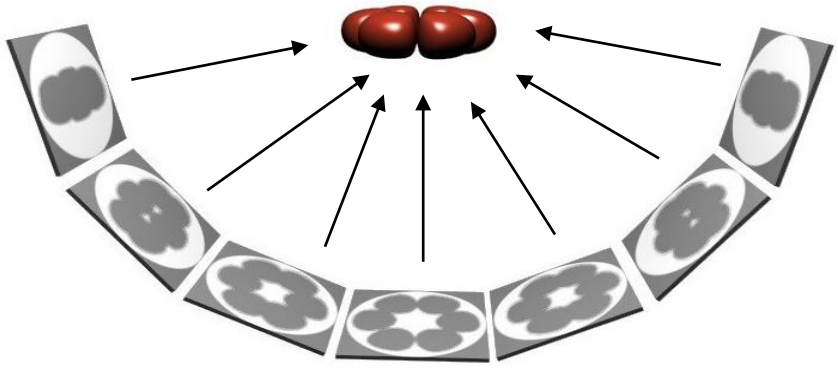
Electron microscopy allows to visualize a wide range of particle sizes



Cryo electron tomography: Sample which is not suitable for single particle analysis

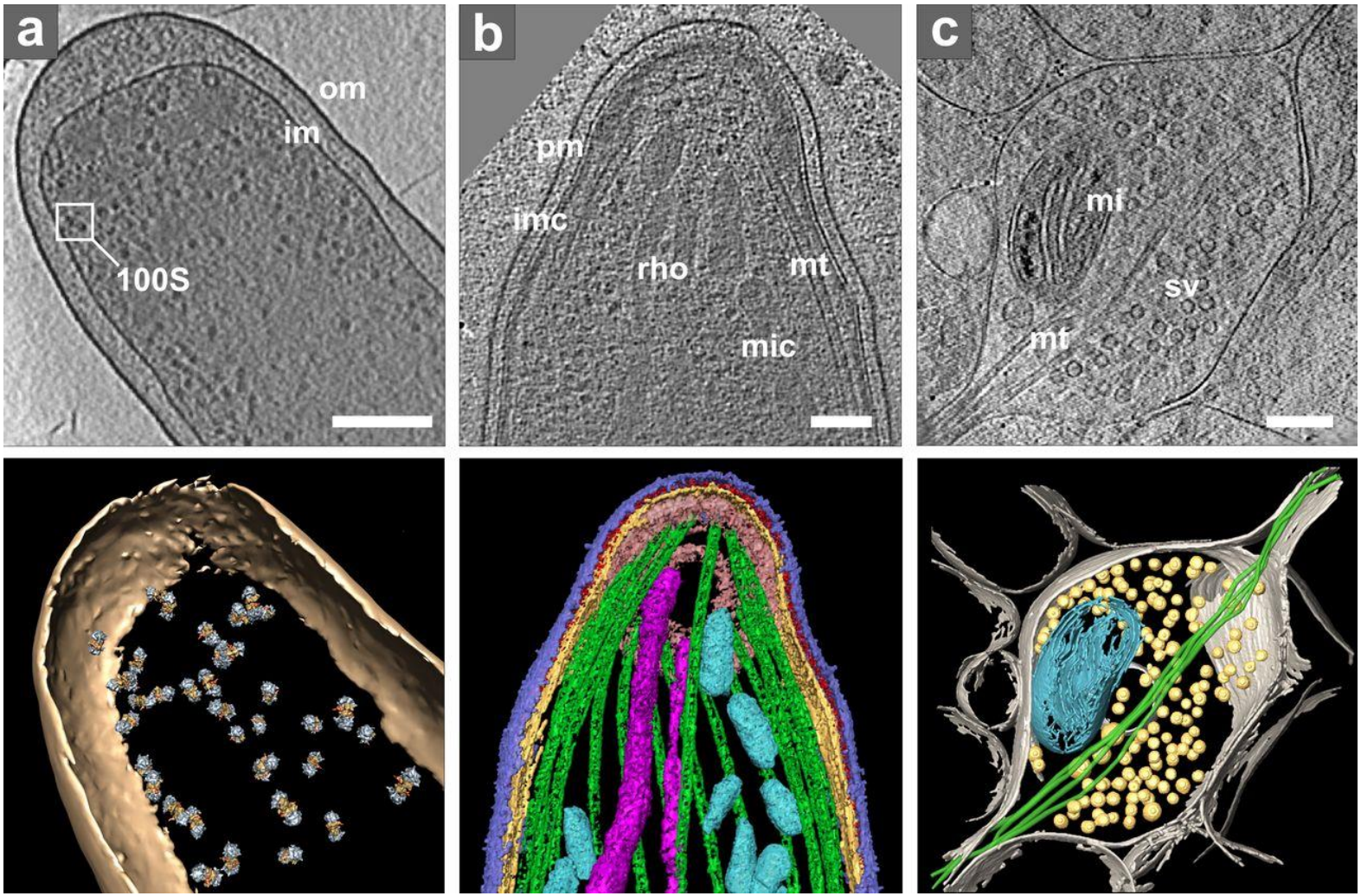


Cryo-electron tomography



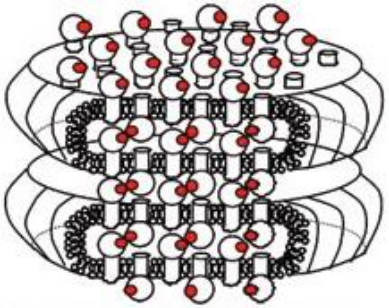
For review see
Beck M, **Baumeister,** Trends Cell Biol. 2016

Cryo electron tomography of whole cell or sections

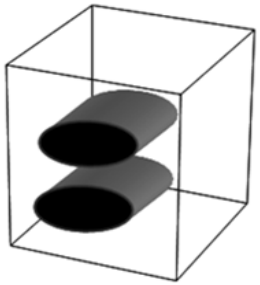


Lučić et al, 2013

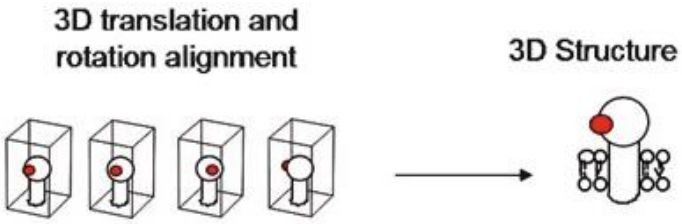
Cryo electron tomography: Sample which is not suitable for single particle analysis

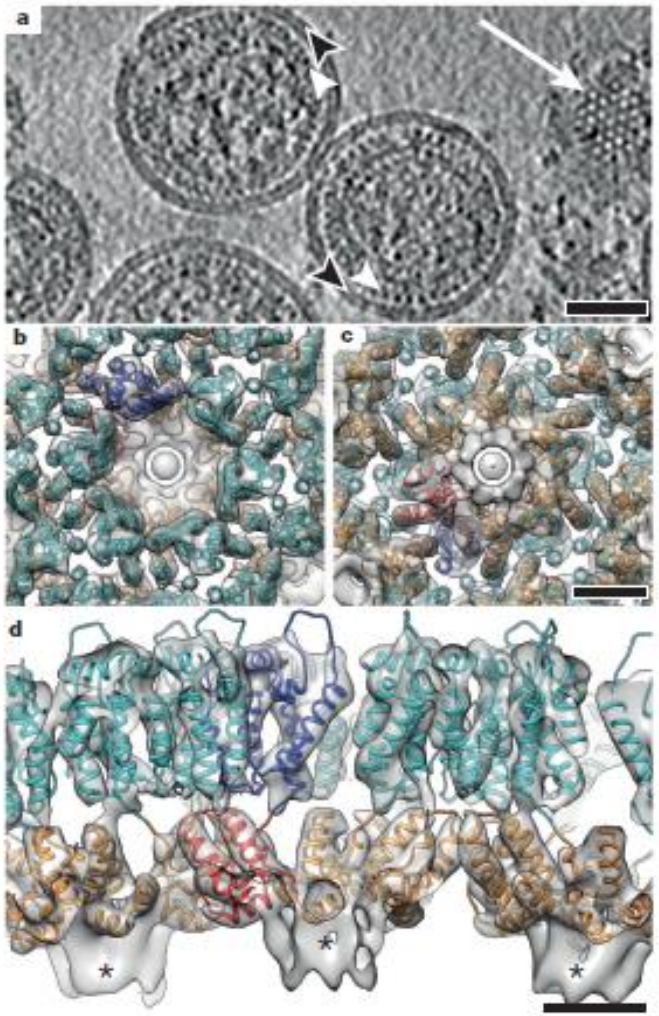


3D tomogram reconstruction

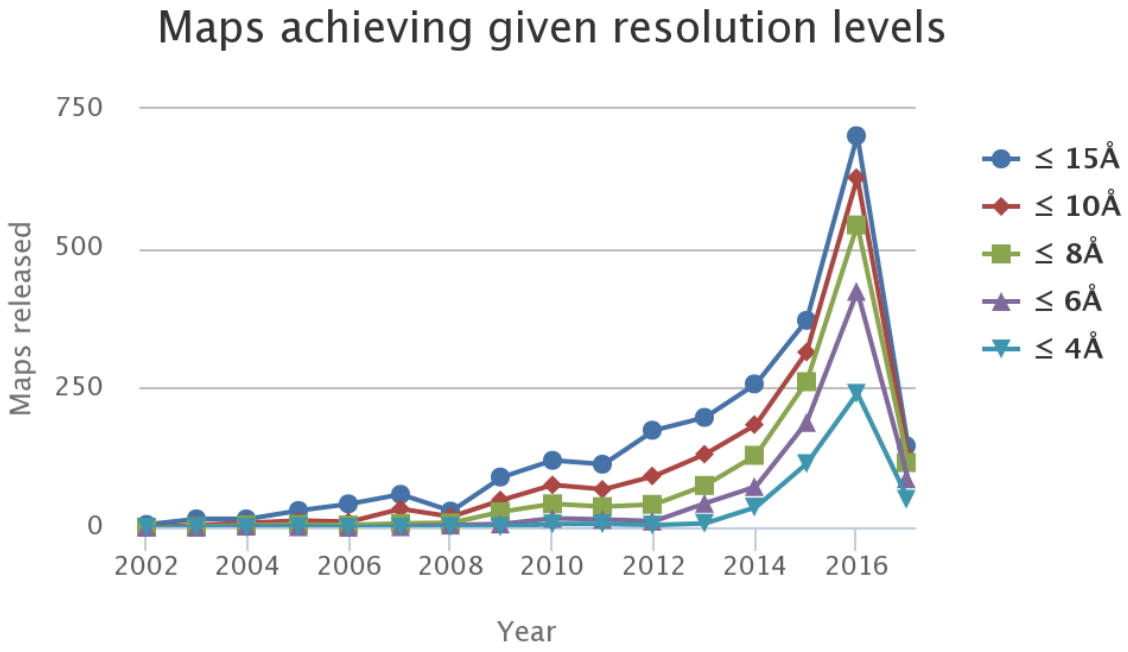


SUBTOMOGRAM AVERAGING



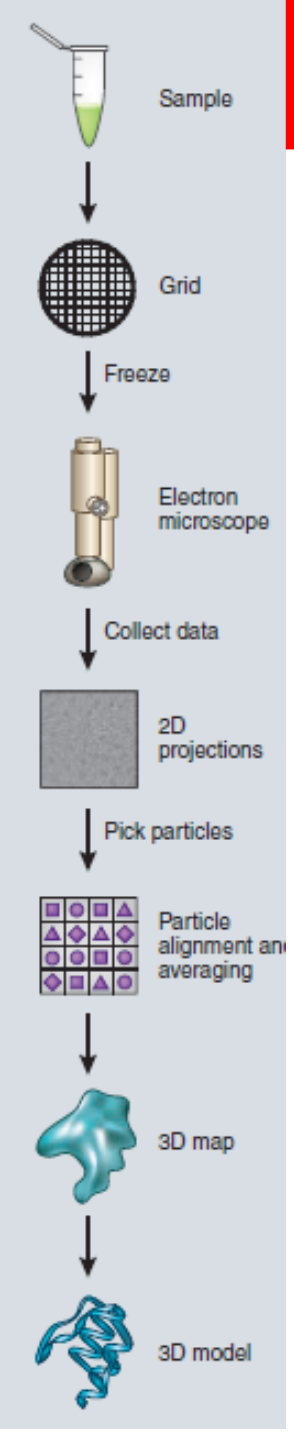


Structure of the immature HIV-1 capsid in intact virus particles at 8.8 Å resolution
Schur et al., Nature 2015



EM Databank Statistics

(March 2017)



how to solve a macromolecular structure using single-particle cryo-electron microscopy

Image Analysis

Bruno Klaholz
Hélène Malet