BIO-315
Structural Biology

Introduction to Electron Microscopy
- Lecture 1 -

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Global Health Institute

EPFL

12-03-25

Slides adapted from:
- Gabriel Lander, TSRI
- Andrew Ward, TSRI
- Matteo Dal Peraro, EPFL

What are the goals for this module?

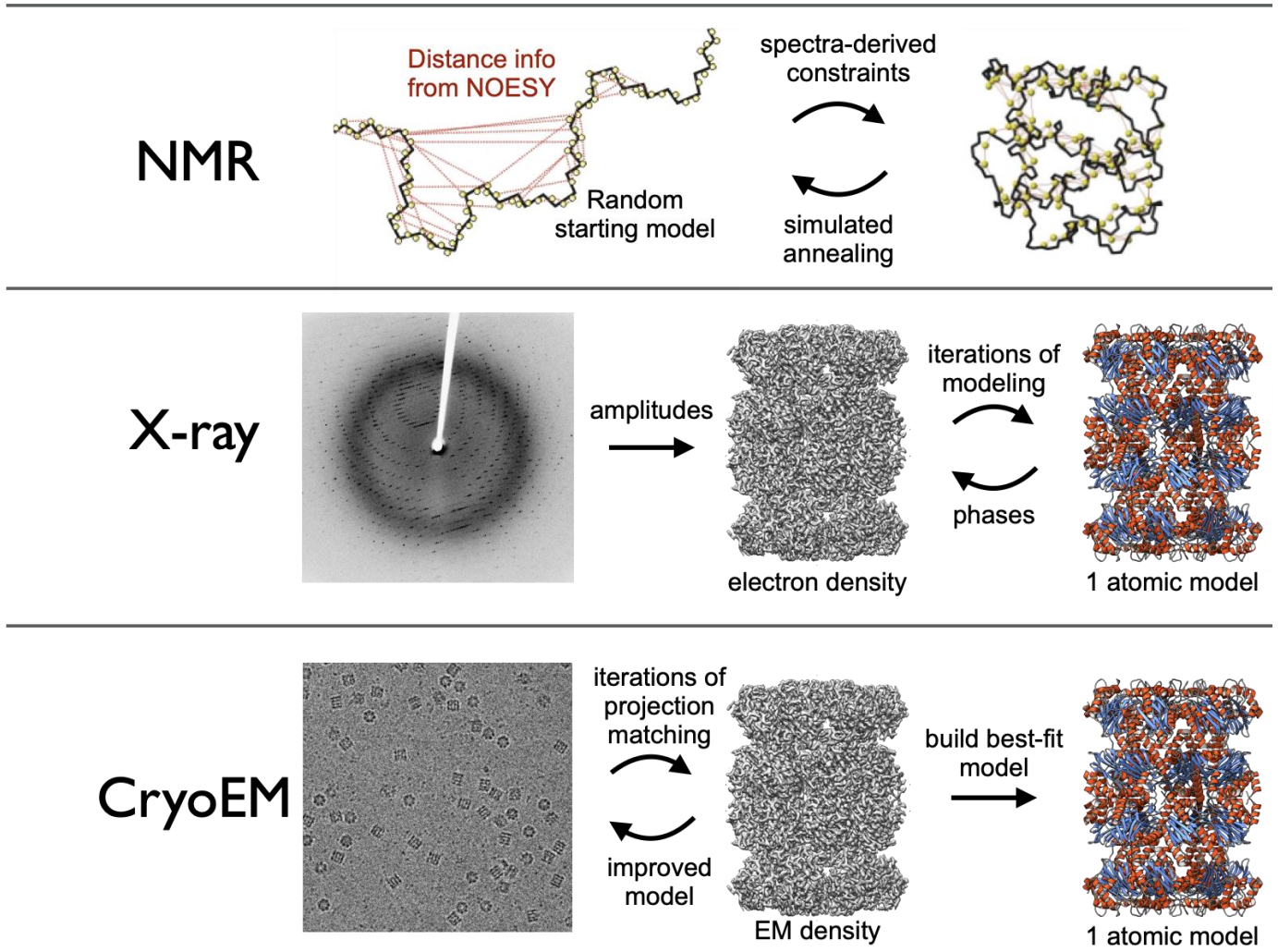
- To learn about the essential electron microscope components and their purpose.
- To learn about the theory of sample preparation for electron microscopy imaging and acquire hands-on practical experience
- To learn about image generation in transmission electron microscopy
- To cover the theory behind image processing and reconstruction of 3D maps
- To acquire hands-on experience in data processing using cryoSPARC software package
- To learn the theory of model reconstruction based on cryoEM maps
- To get an overview of the available EM infrastructure and research efforts on EPFL campus

Plan of action

- 12/03/25 – Lecture 1 (DIA004)
  - Introduction to EM, history, microscope design, image acquisition,
- 13/03/25 – Practical Section 1 (CIME)
  - Grid preparation and imaging, microscope alignment, visit to DCI
- 19/03/25 – Lecture 2 (DIA004)
  - Image generation, processing and model reconstruction, essential curves and plots
- 20/03/25 – Practical Section 2 (DCI)
  - Visit to Dubochet Center for Imaging in Lausanne
- 26/03/25 – Lecture 3 (DIA004)
  - Map interpretation, model building, comparison to other methods, new concepts
- 27/03/25 – Practical Section 3 (DIA004)
  - Image processing and model reconstruction

People assisting with the course:
- Verena Rukes
- Edoardo Cavani
- Parth Bibekar

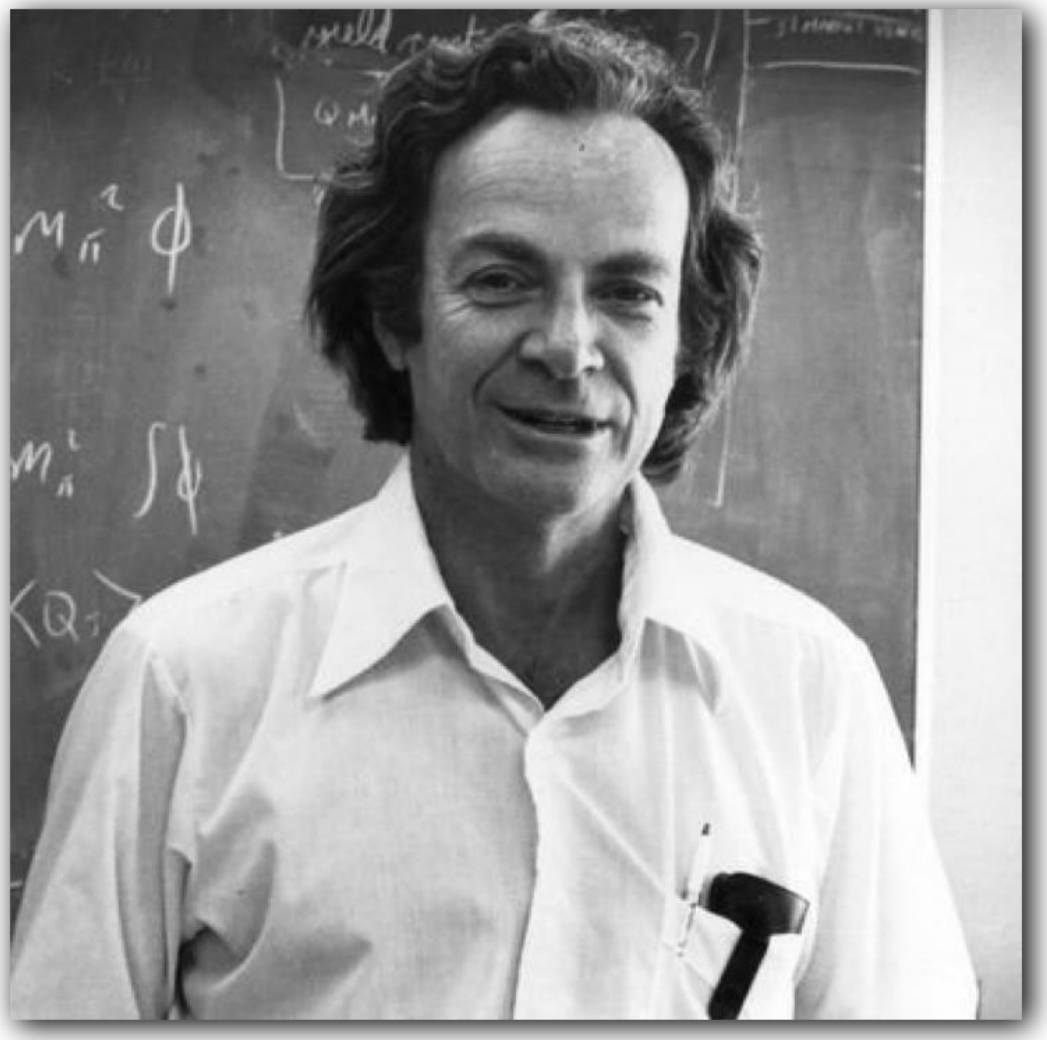
Methods for determining biomolecule structures



How to visualize biomolecules?

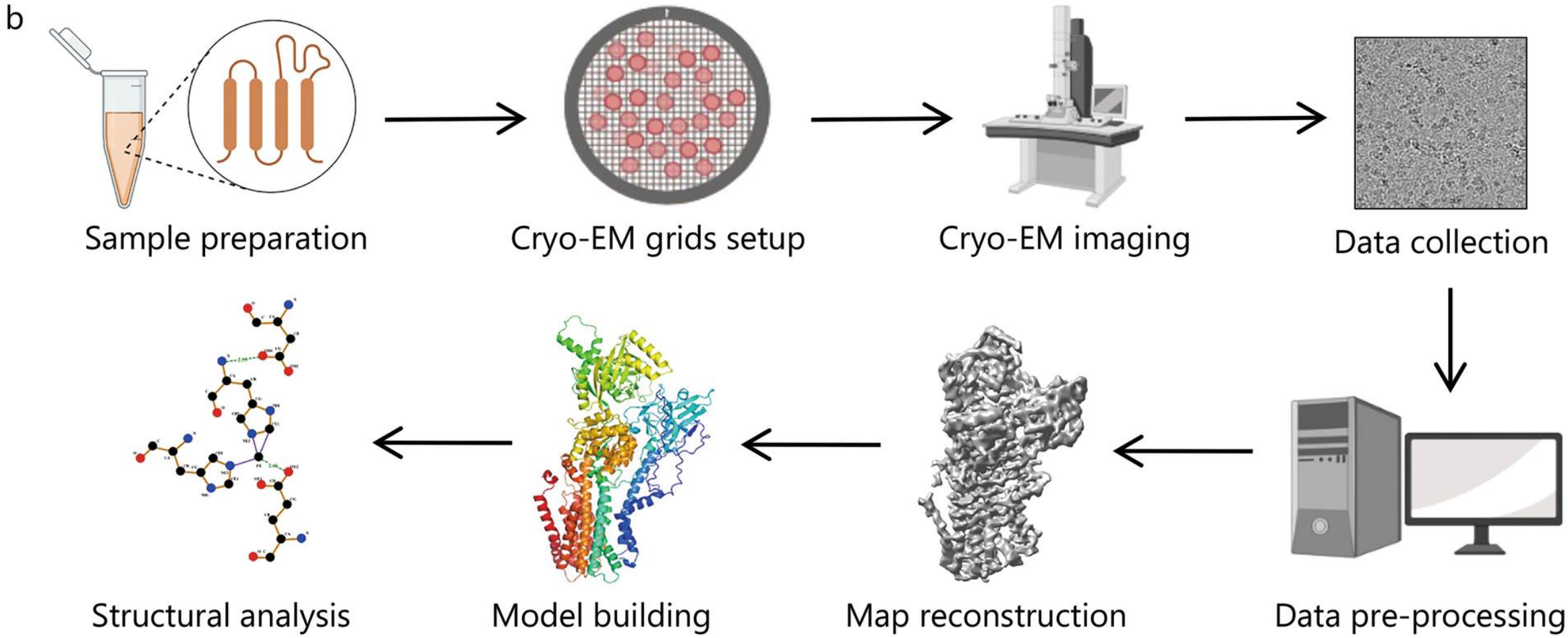
"It is very easy to answer many of these fundamental biological questions;
you just look at the thing!... Make the microscope one hundred times more powerful, and many problems of biology would be made very much easier.
I exaggerate, of course, but the biologists would surely be very thankful
to you ….."

Richard P. Feynman



"There's plenty of room at the bottom"
lecture given at the APS in 1959

Electron Microscopy for Structure Determination



Why electrons?

Abbé's law on diffraction limited optical systems

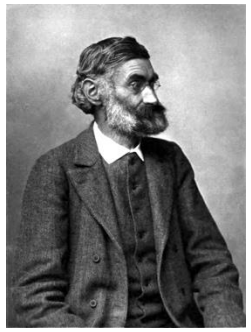
- The wavelength of electromagnetic radiation needs to be at a similar or lower order of magnitude as the features that are visualized

Resolution limit of a microscope

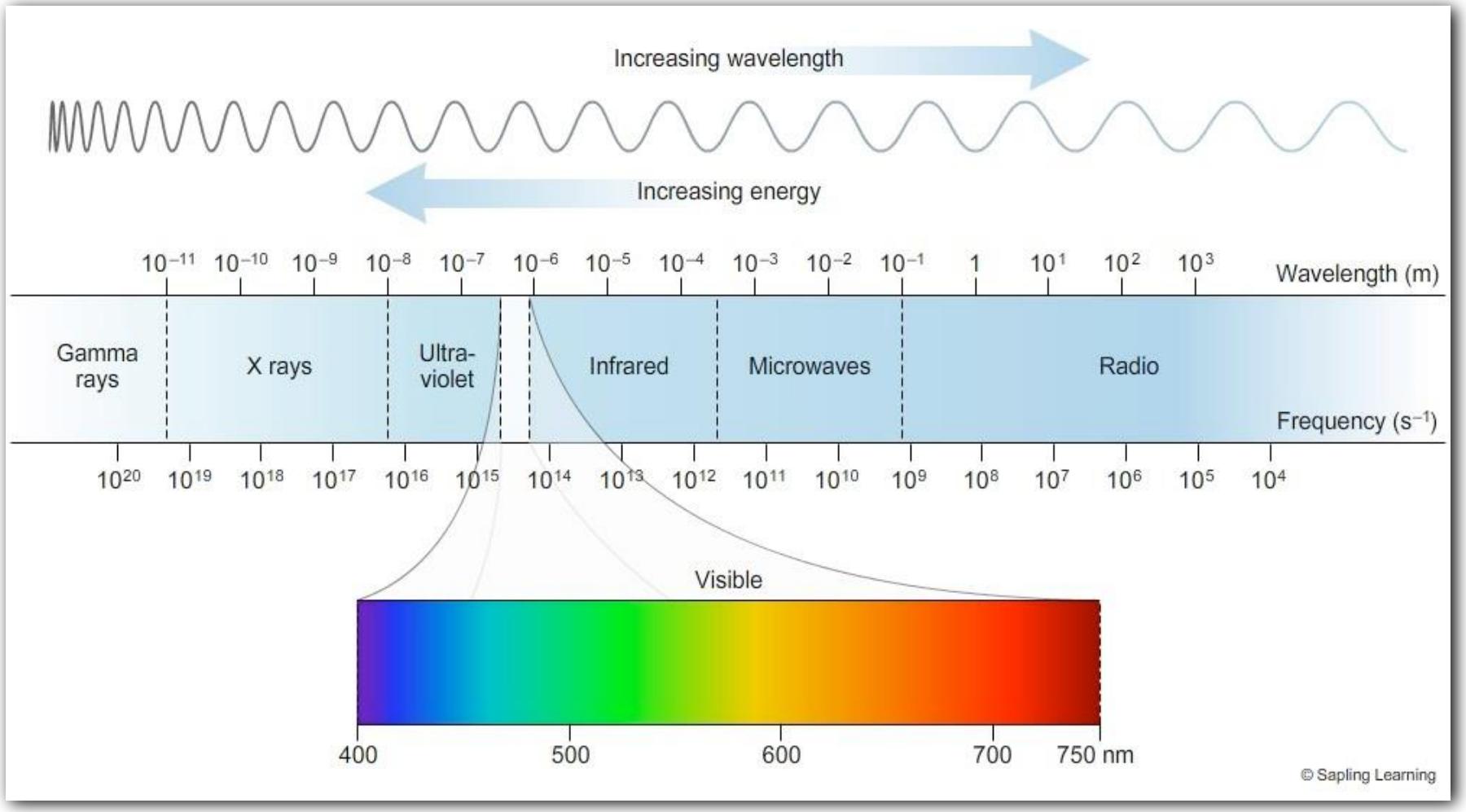
Light wavelength

$$d = \frac{\lambda}{2n \sin\theta} = \frac{\lambda}{2\mathrm{NA}}$$

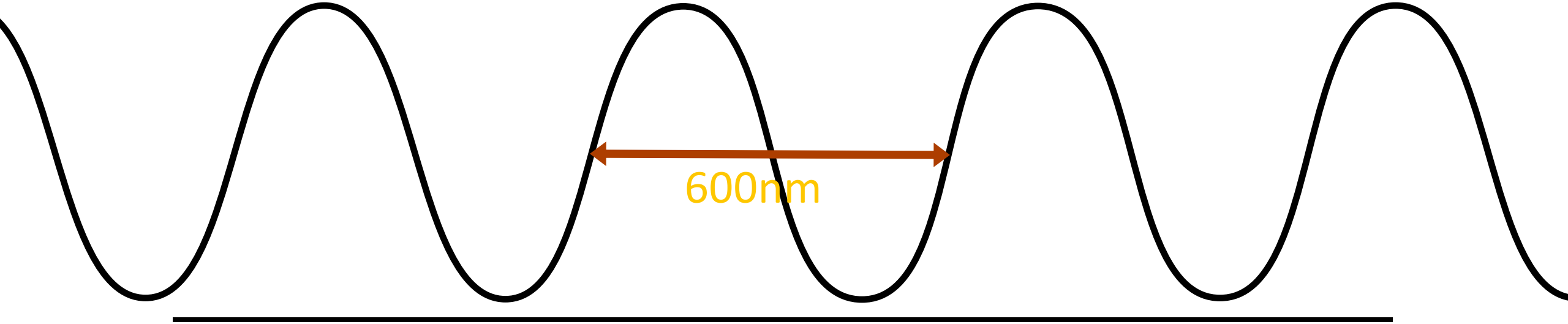
Numerical Aperture



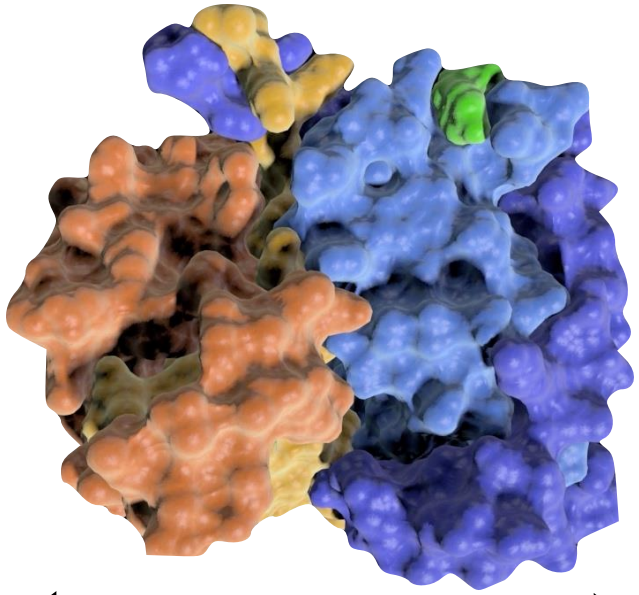
Ernst Abbe
(1840-1905)



Visible light is too “coarse” for visualization of biomolecules



Wavelength of visible light is 400-700nm



X-rays for microscopy applications

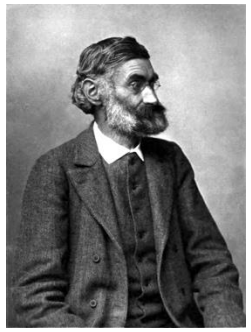
- The wavelength of X-rays is sufficiently fine to image biomolecules and visualize molecular details of their assembly

Resolution limit of a microscope

Light wavelength

$$d = \frac{\lambda}{2n \sin\theta} = \frac{\lambda}{2\mathrm{NA}}$$

Numerical Aperture



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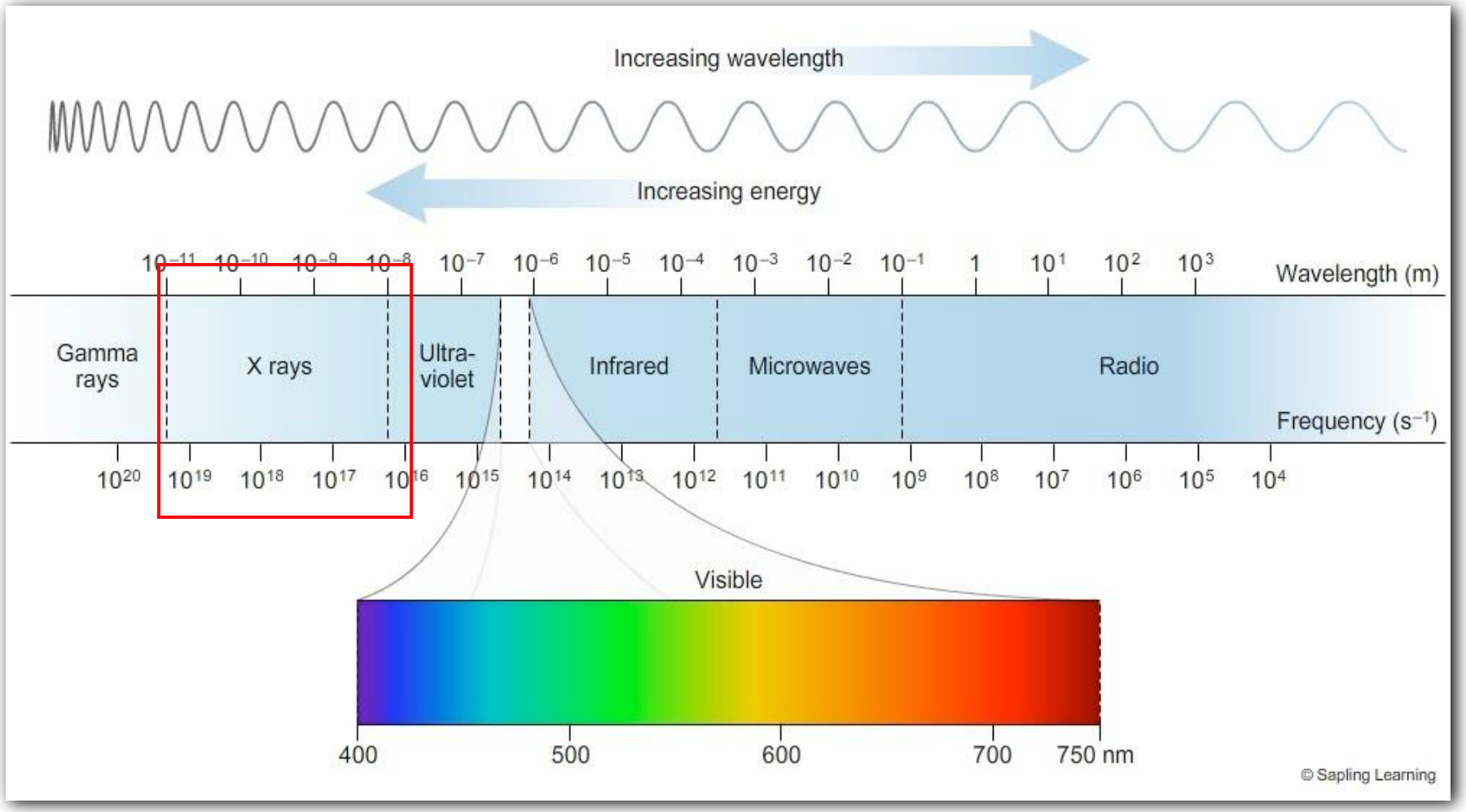
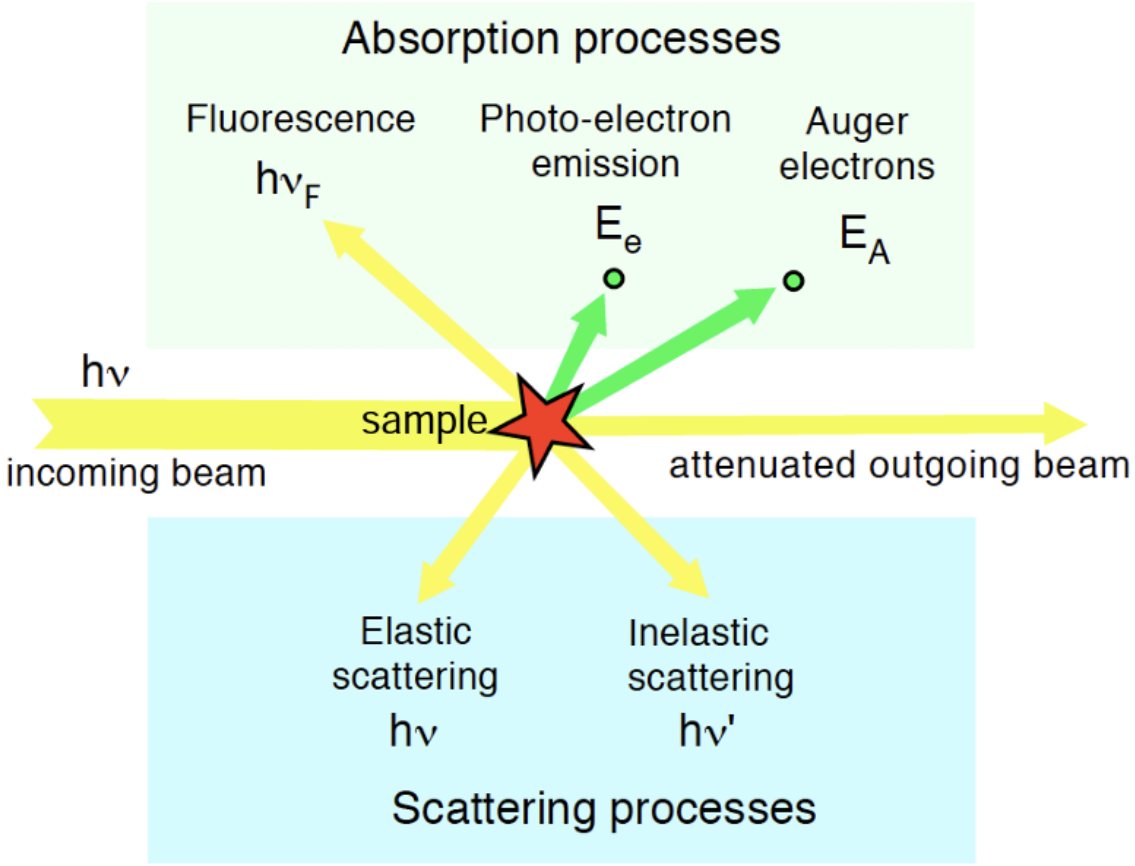
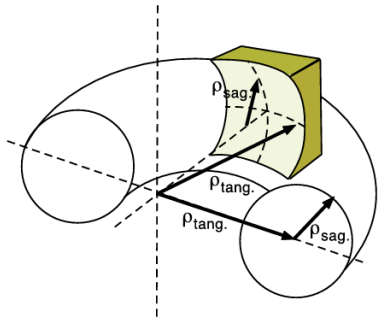


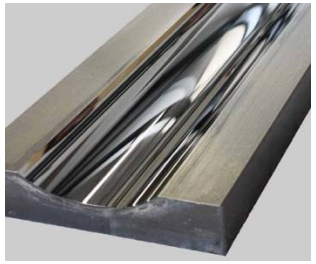
X-rays for microscopy applications

- X-rays can interact with matter in different ways but are relatively poorly scattered

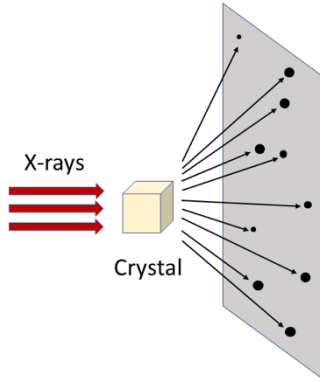


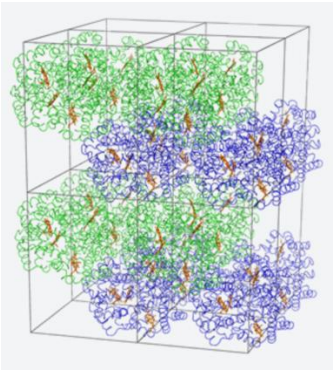
- Difficult to produce good lenses/mirrors





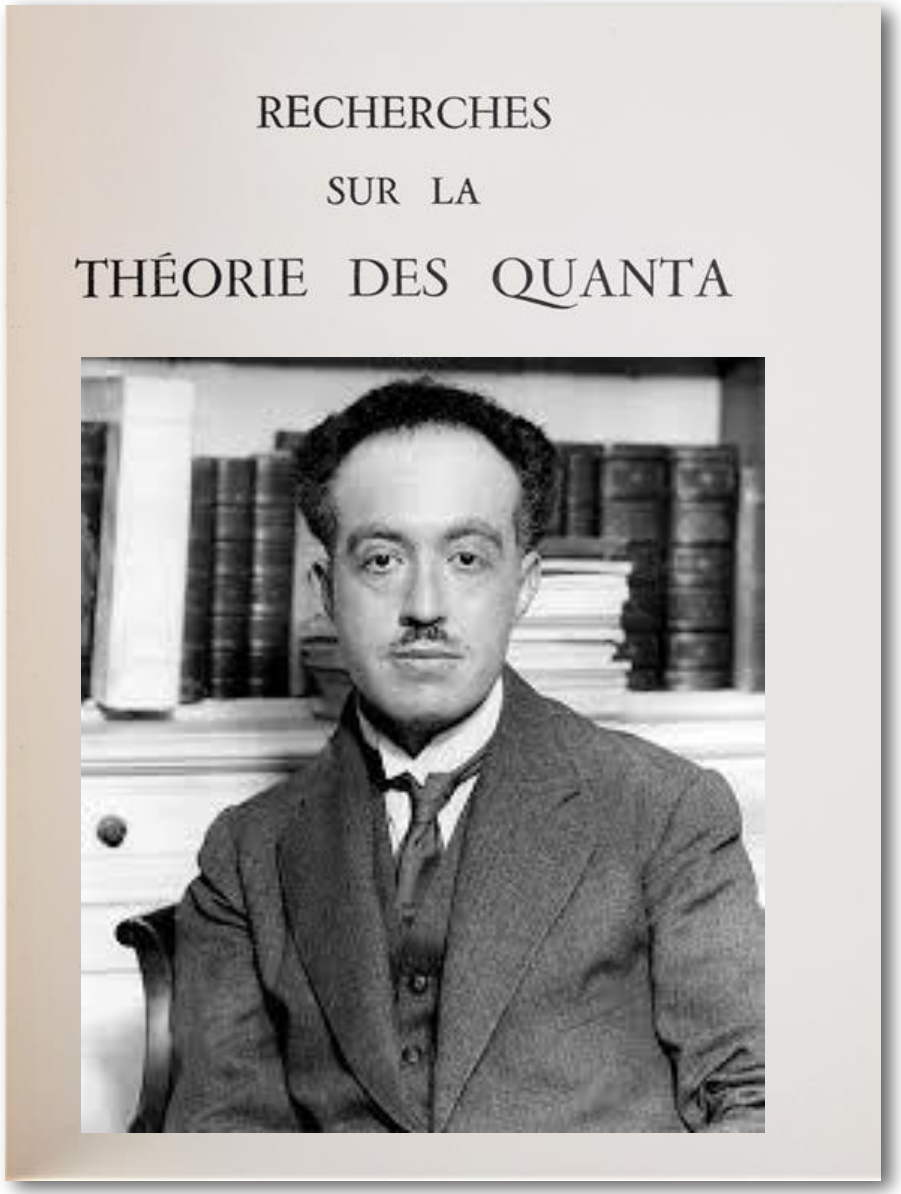
- Poor image contrast in a microscope





- Protein crystals help amplify the scattering signal

Accelerated electrons as an alternative for X-rays



1924 thesis of Louis de Broglie

Combined the equations of special relativity & quantum theory to suggest that electrons could be thought of as waves as well as particles.

et la fréquence ν des ondes s'exprime par :

$$\nu = \frac{1}{T} = \frac{\nu_0}{\sqrt{1-\beta^2}} = \frac{m_0 c^2}{h\sqrt{1-\beta^2}}$$

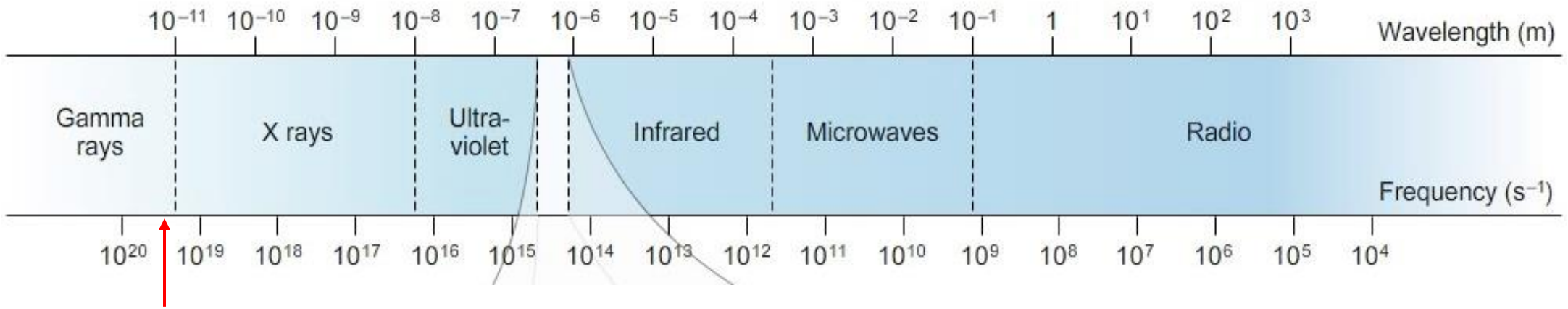
$$\lambda = \frac{h}{\gamma m_0 v} = \frac{h}{m_0 v}\sqrt{1-\frac{v^2}{c^2}}$$

$$f = \frac{\gamma m_0 c^2}{h} = \frac{m_0 c^2}{h} \Big/ \sqrt{1-\frac{v^2}{c^2}}$$

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

Accelerated electrons as an alternative for X-rays

- Electrons with energy of **~100keV** have a wavelength of **3.88pm**



- Typical voltage in a microscope is 100-300kV

- 100X shorter **λ** than typical X-rays in a synchrotron

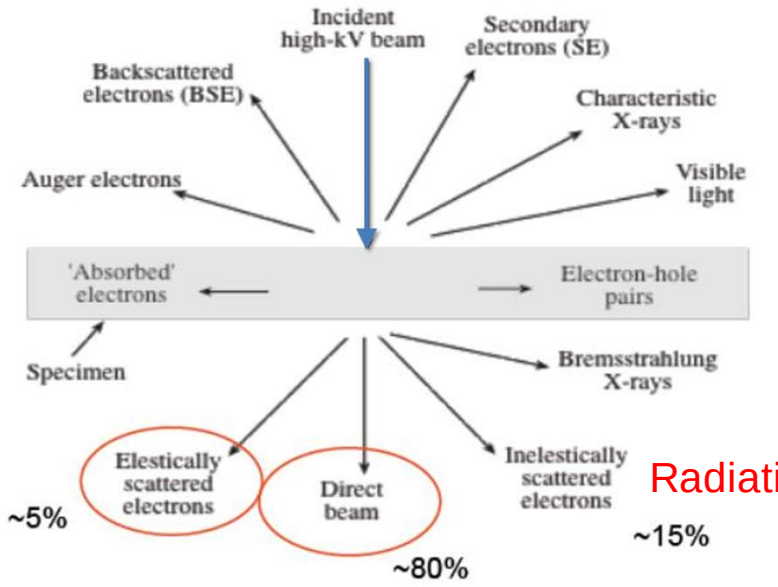
$$\lambda = \frac{6.626\times10^{-34}}{\sqrt{2V\times1.6\times10^{-19}\times9.11\times10^{-31}}}$$

$$\Rightarrow \lambda = \frac{12.27\times10^{-10}}{\sqrt{V}}\text{meter}$$

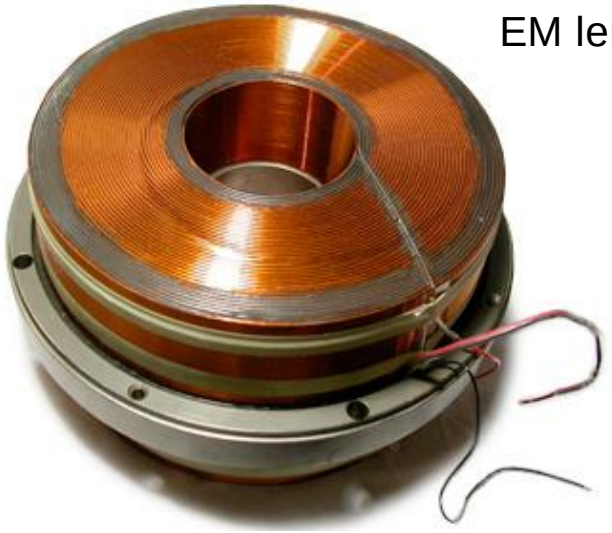
$$\text{(or)}\ \lambda = \frac{12.27}{\sqrt{V}}\text{Å}$$

What are the advantages of electrons?

- The main benefit comes from the negative charge
- Compared to X-rays, elastic scattering of electrons is ~10^3-10^6 times stronger
- Electrons are easily accelerated using potential difference
- Electron beam can be readily collimated with electromagnetic lenses
- Readily detected by films and cameras



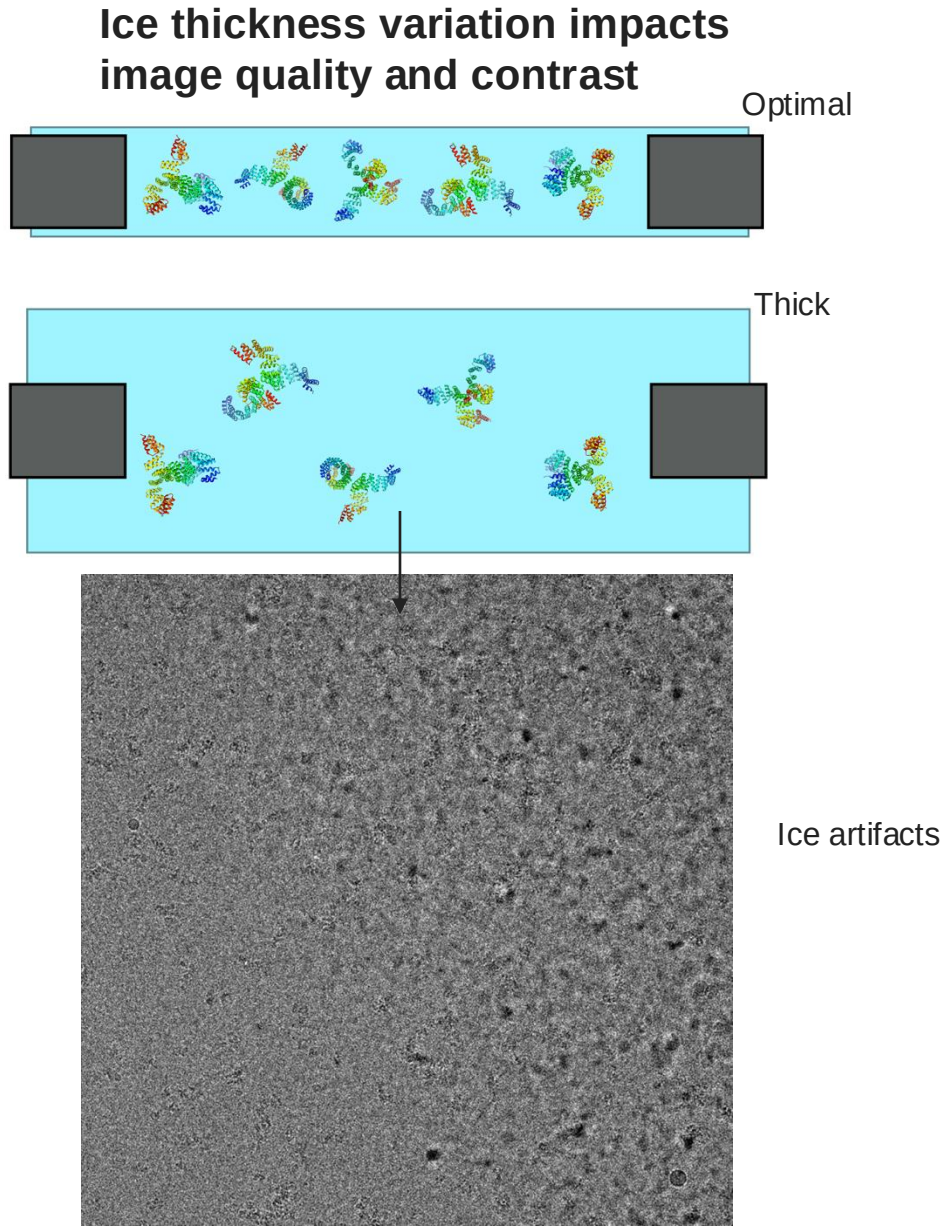
Radiation damage



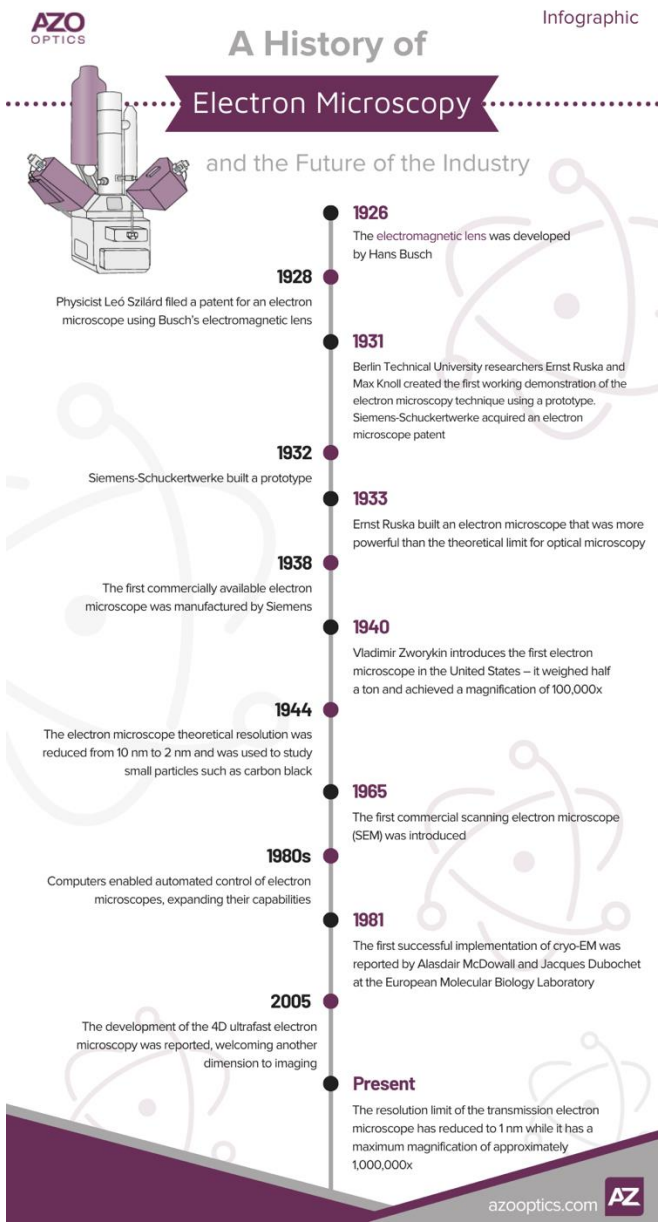
EM lens

What are the disadvantages of electrons?

- Need high vacuum within the microscope to reduce interactions with air molecules
- Samples need to be applied as a very thin (~10-100nm) layer to achieve sufficient contrast
- Radiation damage in the sample caused by inelastic scattering of electrons (i.e., collisions with biological material leading to energy transfer)
- Insufficient contrast when imaging small proteins (lower molecular weight limit for EM is ~50kDa)

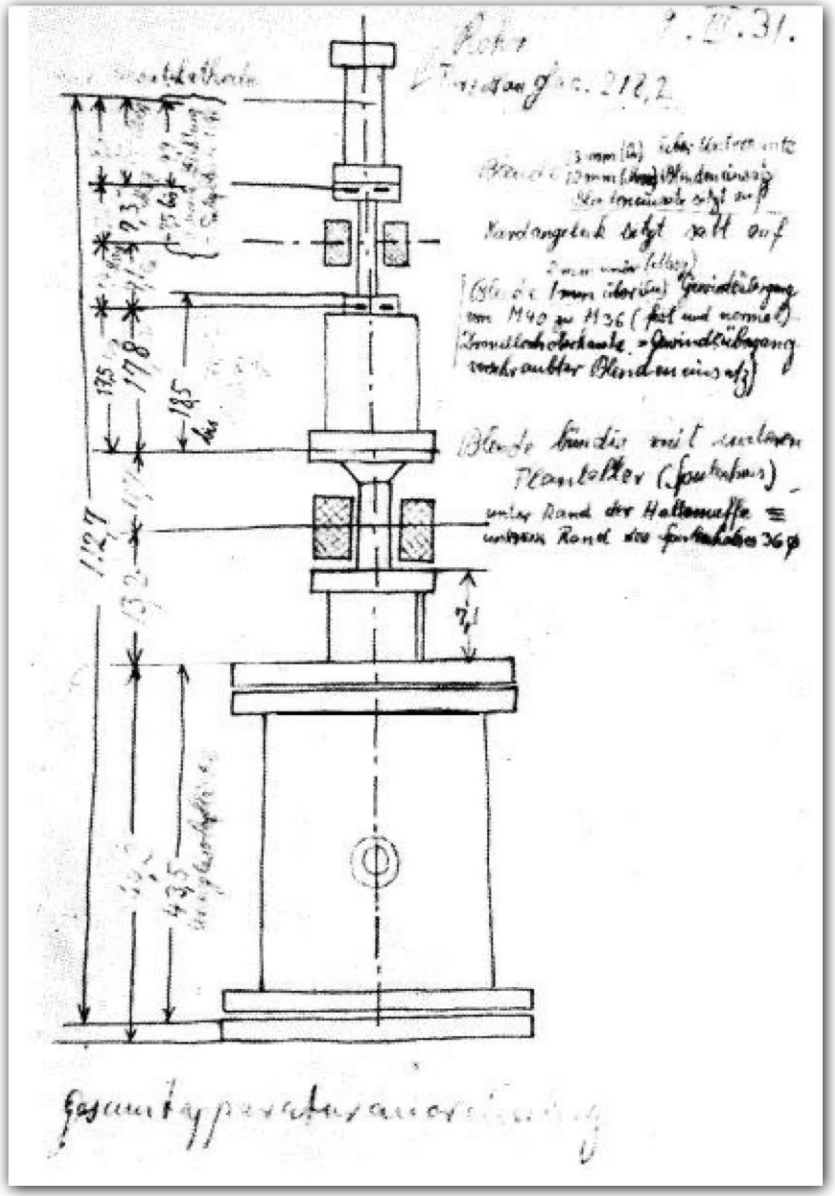


Brief History of EM



Adapted from: www.azooptics.com

The First Transmission Electron Microscope



• Ernst Ruska and Max Knoll building the first transmission electron microscope in **1931**

The First Transmission Electron Microscope

• Ernst Ruska and Max Knoll building the first transmission electron microscope in **1931**

The Nobel Prize in Physics 1986

Ernst Ruska
Prize share: 1/2

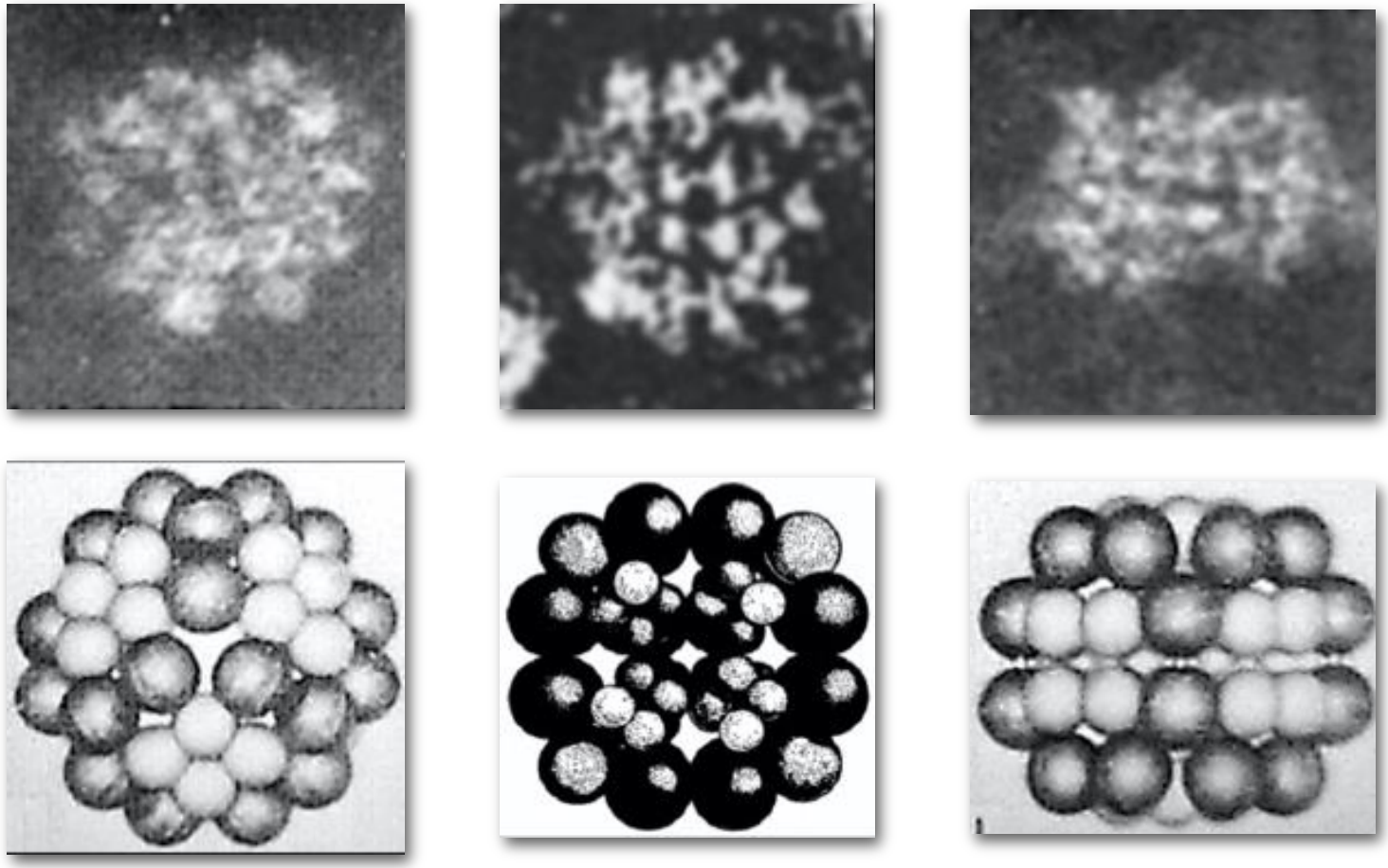
Gerd Binnig
Prize share: 1/4

Heinrich Rohrer
Prize share: 1/4

The Nobel Prize in Physics 1986 was divided, one half awarded to Ernst Ruska *"for his fundamental work in electron optics, and for the design of the first electron microscope"*, the other half jointly to Gerd Binnig and Heinrich Rohrer *"for their design of the scanning tunneling microscope"*.

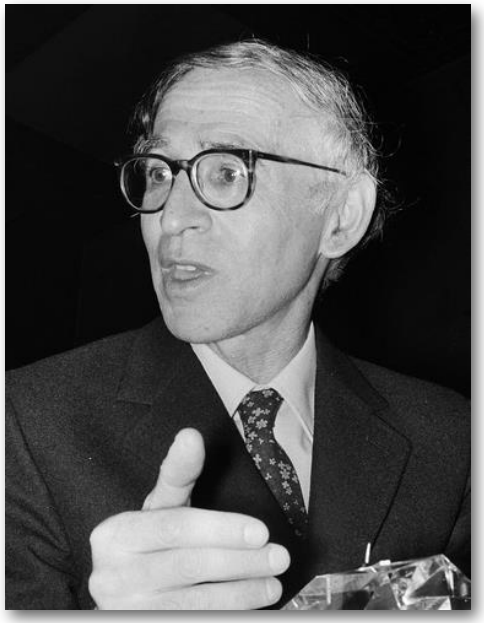
“Image analysis” of biological specimens circa 1965

- In late 60s and early 70s researchers demonstrated that EM can be used for imaging of biological specimens
- Imaging was performed using photographic film
- Recreating 3D structures using pebles or similar objects representing protein subunits for interpretation



Reed & Cox, The Enzymes, 1970

Introduction of 3D Reconstruction



Aaron Klug David DeRosier

DeRosier & Klug. “Reconstruction of three-dimensional structures from electron micrographs” Nature 1968

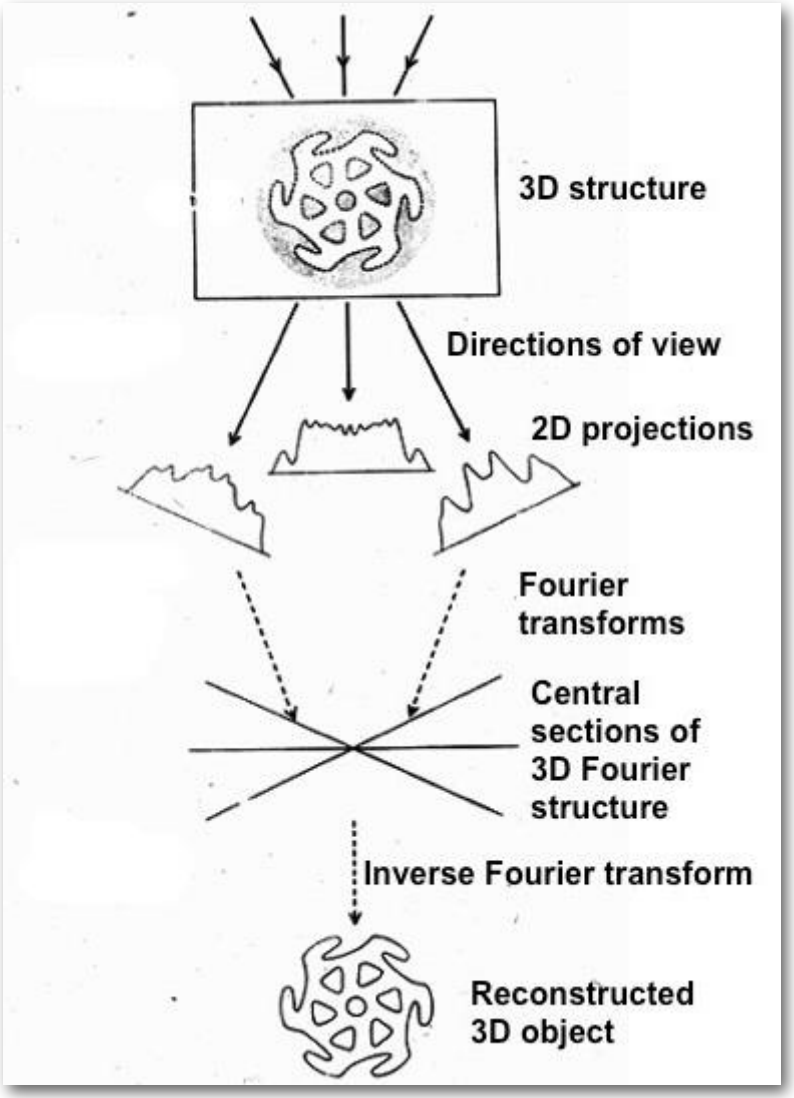
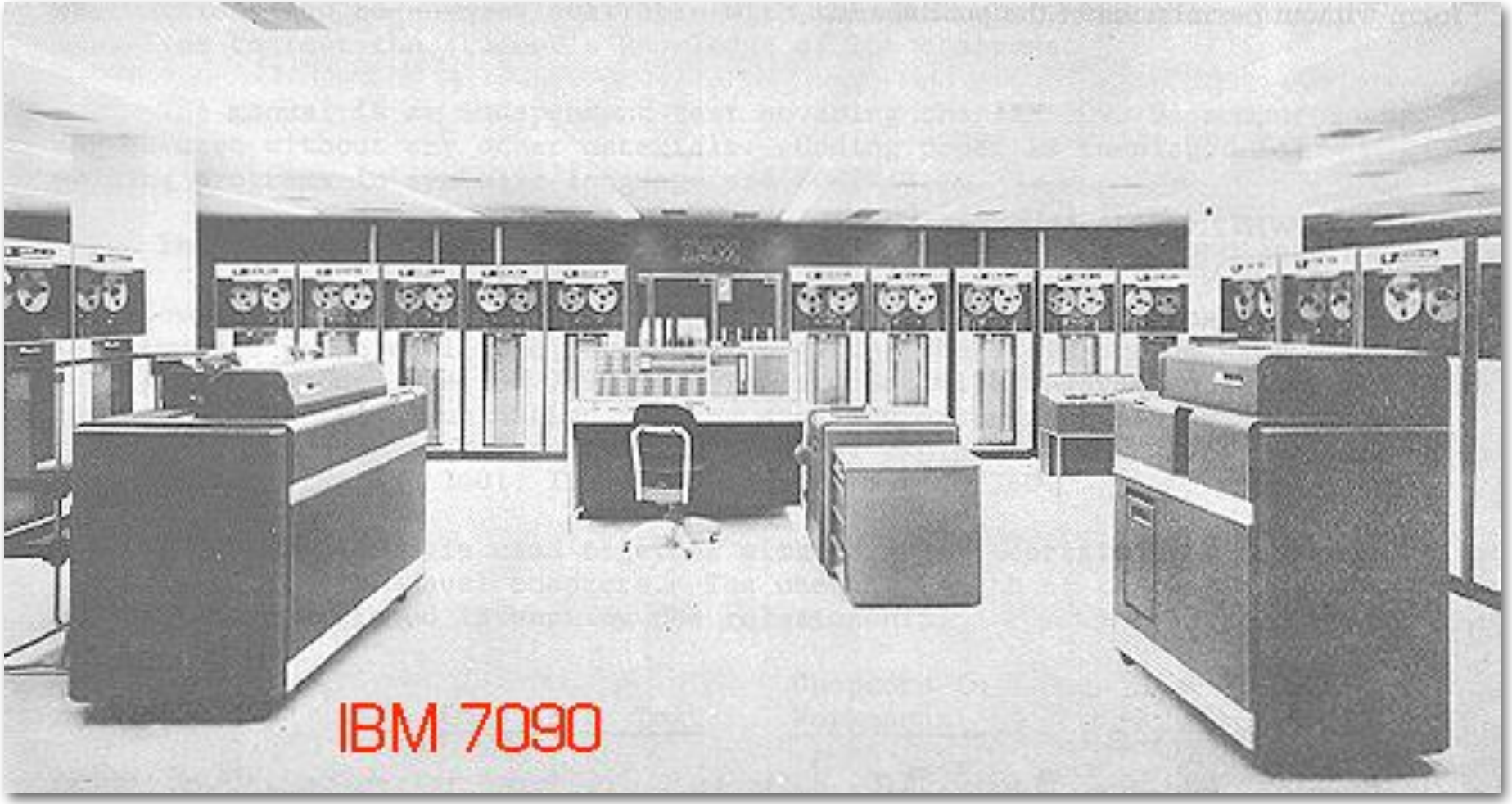


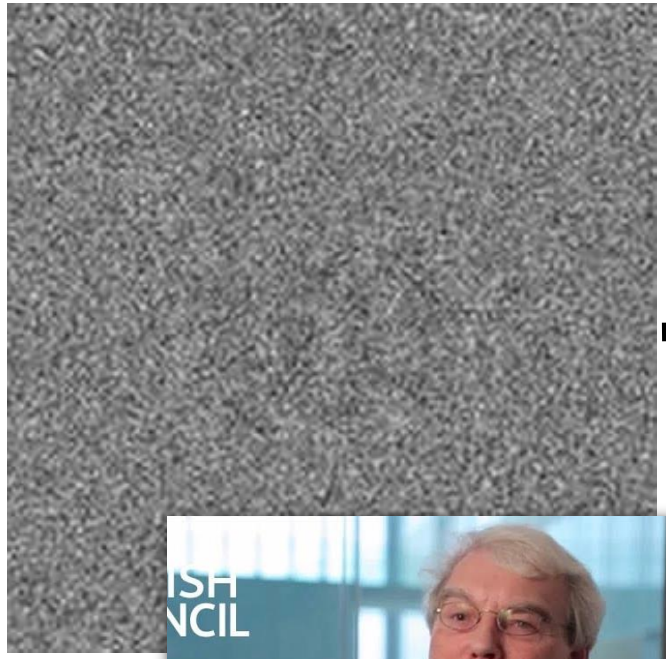
Image processing was performed on COMPUTERS!

1959 - IBM 7090:
32kb of memory, 200k adds/sec, 40k mult/sec, 33k div/sec

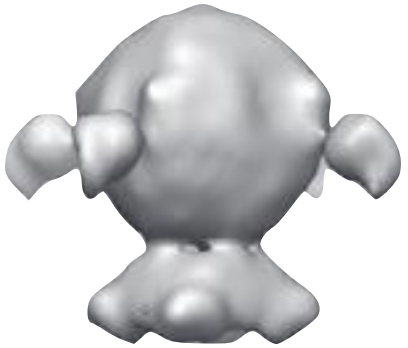


Single Particle Cryo-EM “Blobology” (1970 to 2010)

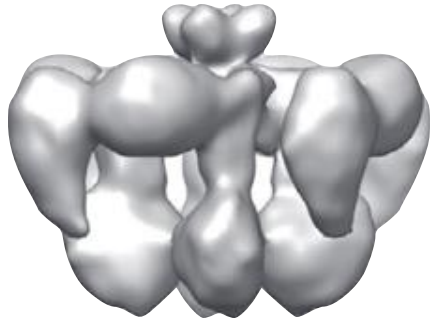
Very noisy 2D projection images that are radiation damaged



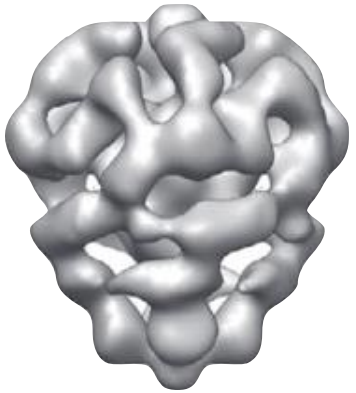
Inositol 1,4,5-trisphosphate receptor (IP3R1)



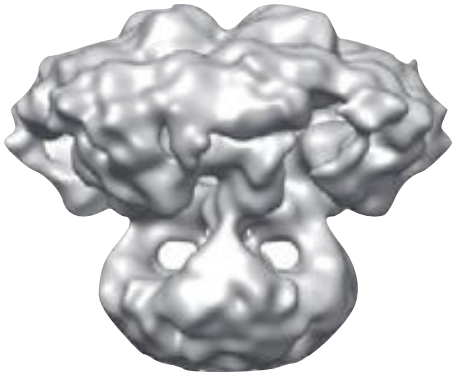
Jiang et al. EMBO J 2002



Serysheva et al. JBC 2003



Sato et al. JMB 2004



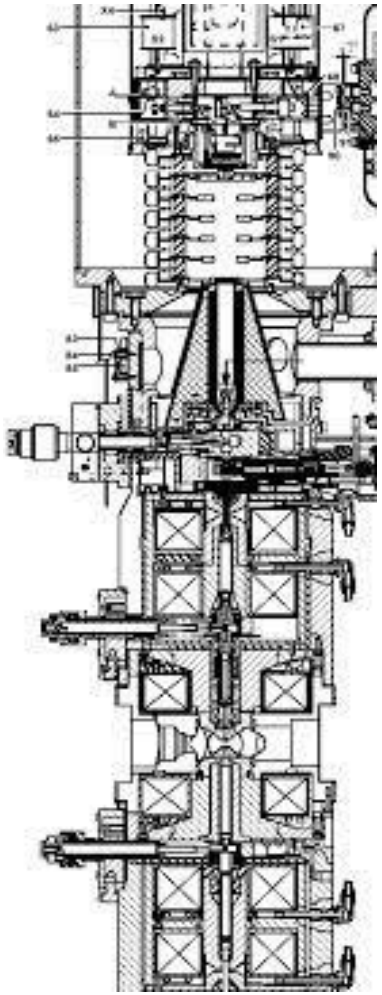
Ludtke et al. Structure 2011

“We know that at least 3 of them are wrong”

Electron microscopy after 2010

• Modern TEM

• Schematic of a modern TEM



• Direct electron detectors

• Computational resources

• Software:

- Acquisition (Leginon, EPU, SerialEM)
- Image pre-processing (MotionCorr, CTFFind)
- Processing packages (Relion, CryoSPARC)

Figure sources: ThermoFisher, Gatan, NVIDIA webpages

“Resolution Revolution” and “Democratization” of EM

Improved resolution of EM data

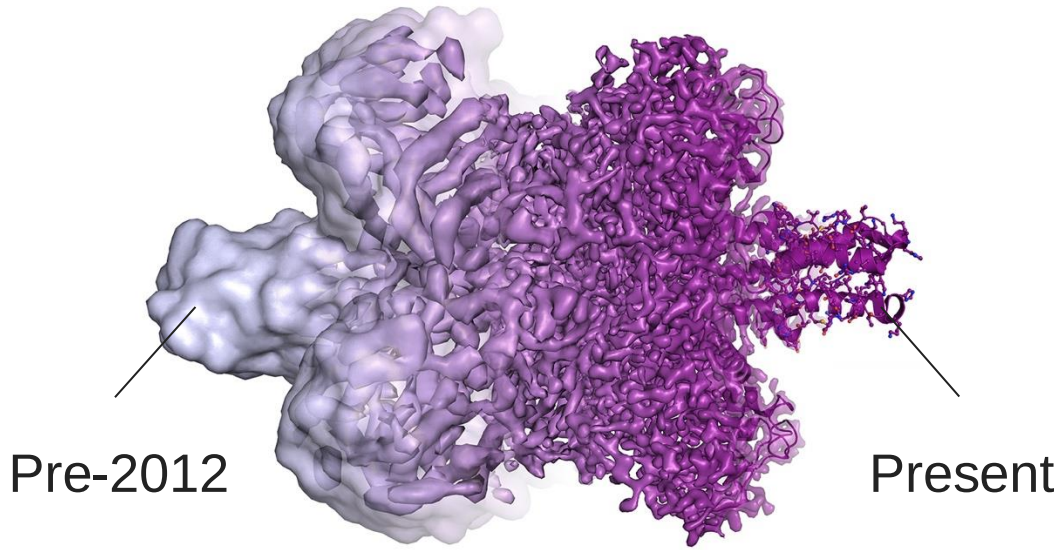
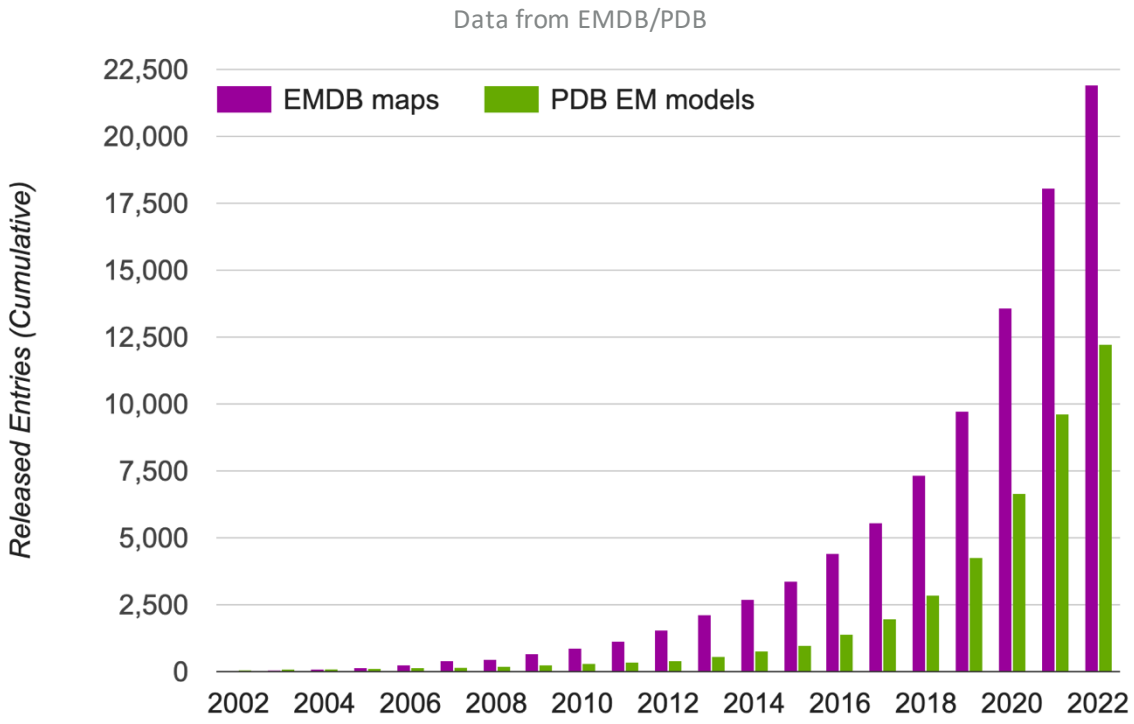


Illustration by Martin Högbom; The Royal Swedish Academy of Science

EM map/model depositions



- High-resolution maps became readily attainable starting ~2012 as a result of technological breakthroughs in the field

“Resolution Revolution” and “Democratization” of EM

Improved resolution of EM data

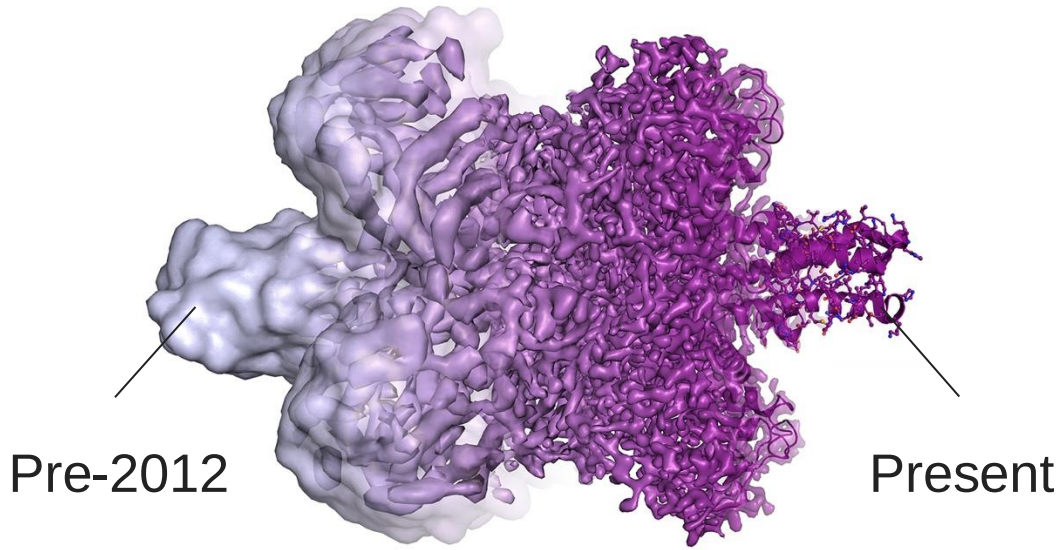


Illustration by Martin Högbom; The Royal Swedish Academy of Science

Nobel Prize in Chemistry, 2017

“for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution”

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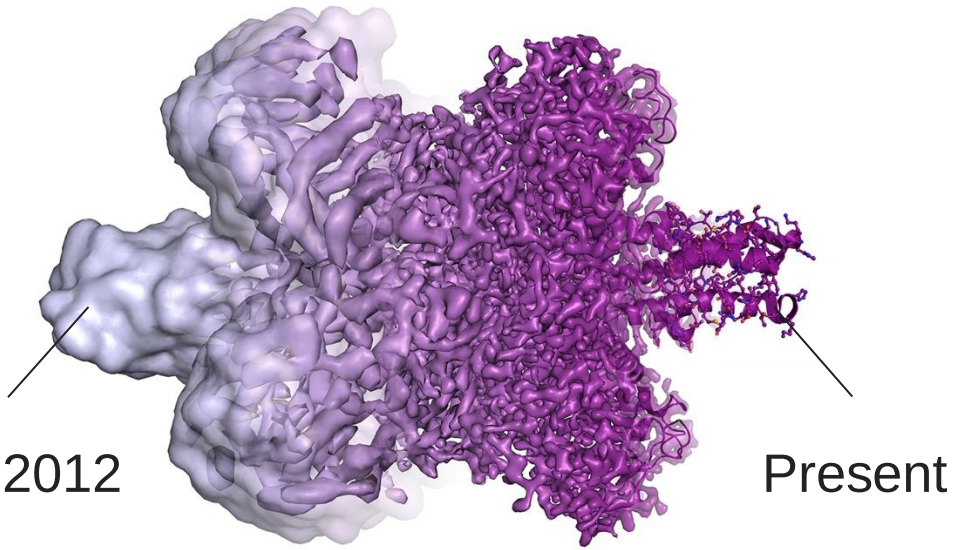
“Resolution Revolution” and “Democratization” of EM

Improved resolution of EM data



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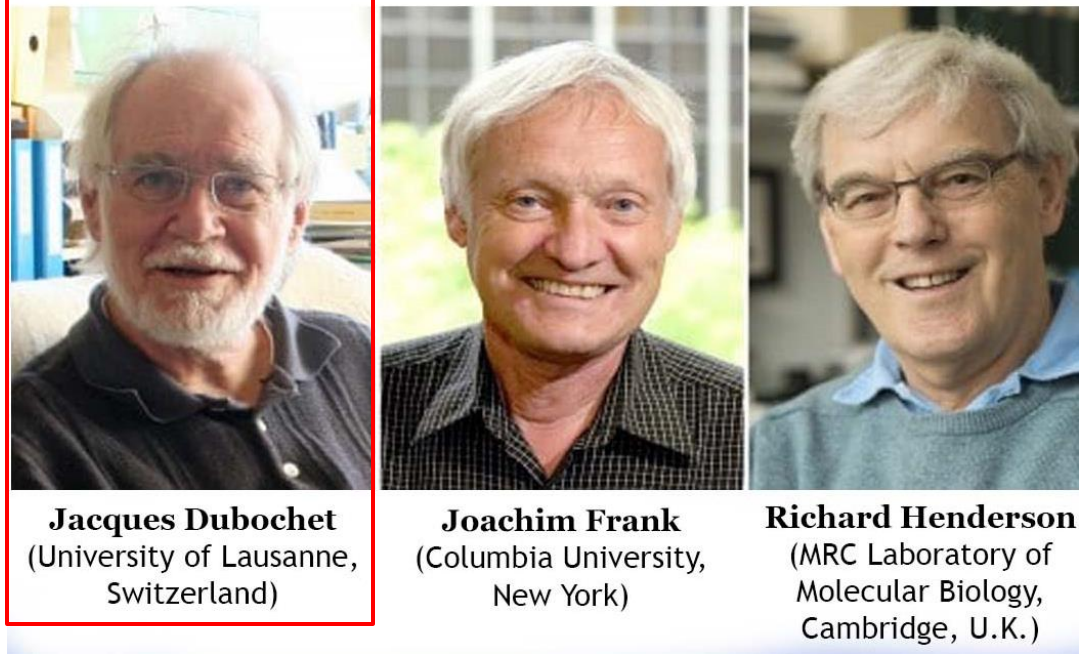
Nobel Prize in Chemistry, 2017



“for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution”

- High-resolution maps became readily attainable starting ~2012 as a result of technological breakthroughs in the field

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Instruments at the DCI Lausanne

Titan Krios (300kV), E-CFEG, SelectrisX, Falcon4

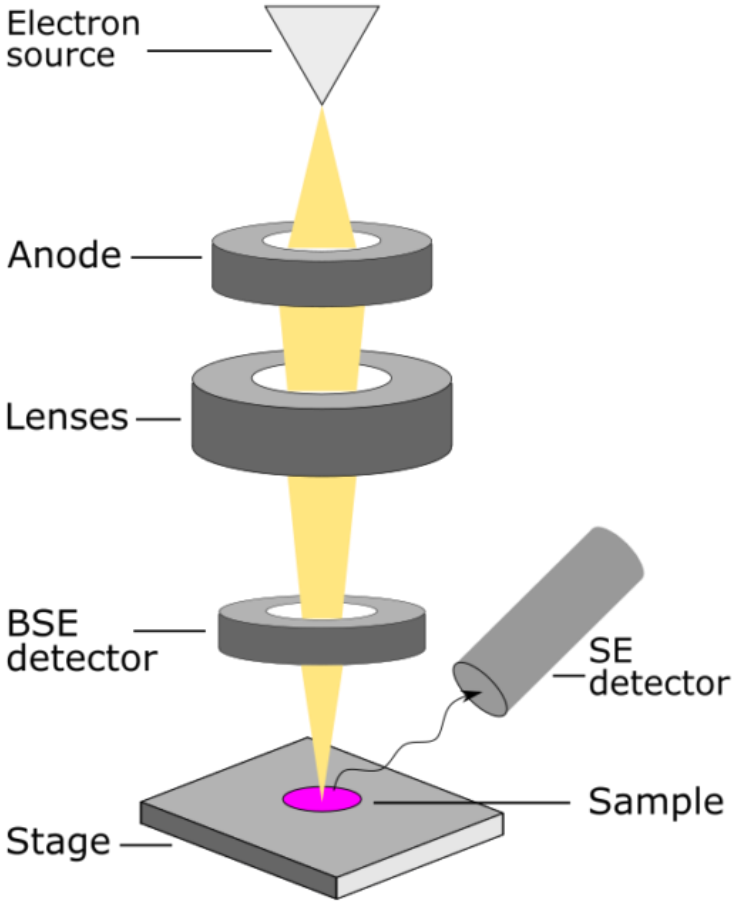
Titan Krios (300kV), E-CFEG, Falcon4

Glacios (200kV), X-FEG, Falcon4

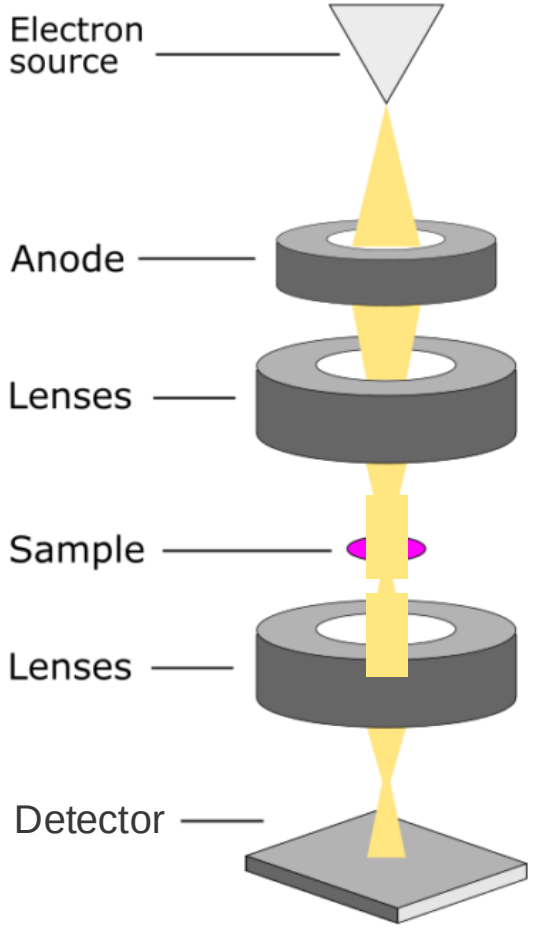
Transmission electron microscope (TEM)

Different types of electron microscopes

Scanning EM



Transmission EM

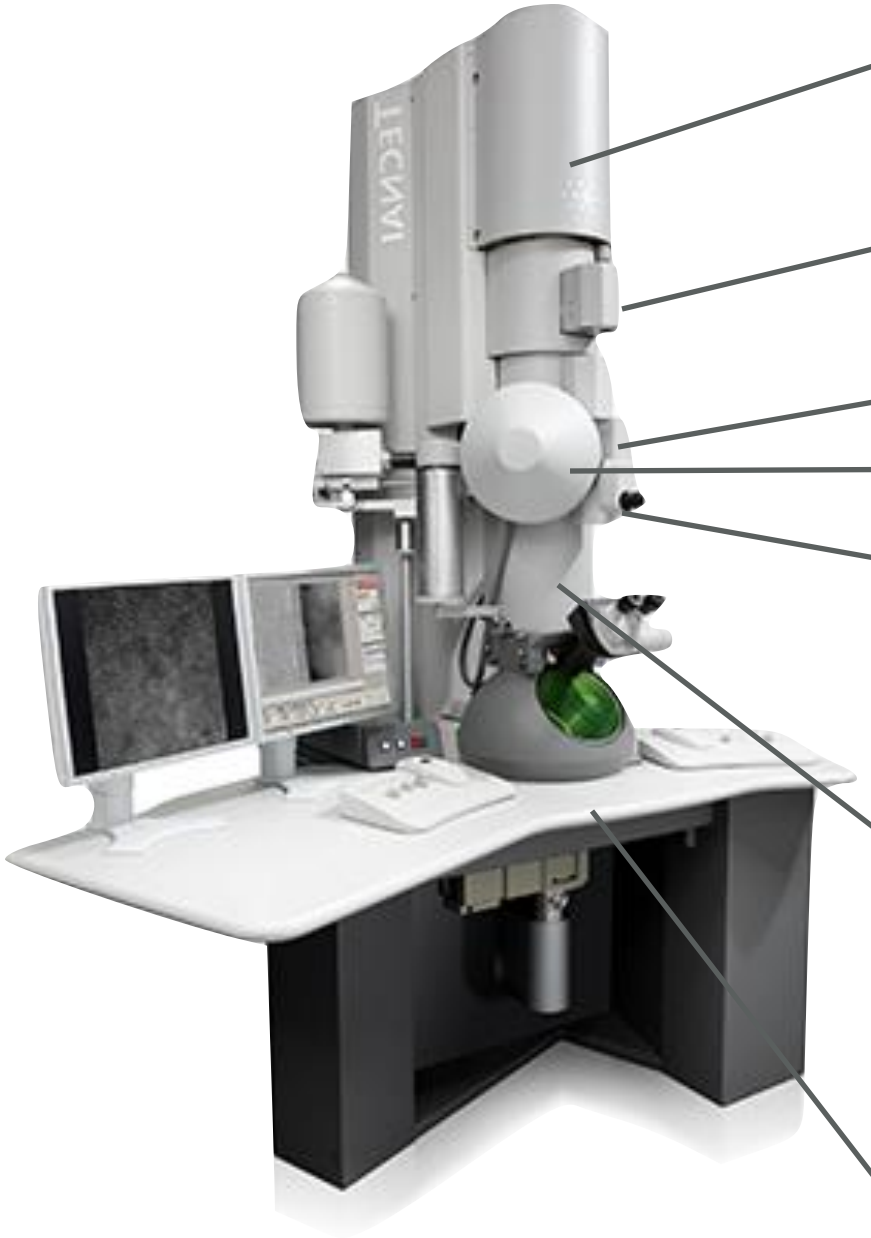


- SEM detects electrons diffracted at high angles (low probability events)
- SEM images give insight into a sample's topography and elemental composition but only at low resolution (~nm)
- SEM can be used to image thick and thin samples (e.g., whole cells)

- TEM detects non-diffracted and weakly diffracted electrons
- TEM samples need to be very thin (<200nm) to allow electrons to pass through (e.g., proteins, viruses, thin cell sections)
- TEM allows to image (certain) biological samples at atomic resolution (~Å)

- Other examples of imaging methods: Scanning Transmission EM, Correlated Light and EM, Cathodoluminescence EM…

Microscope components



Electron Source

Condenser Lens
(collecting the beam)

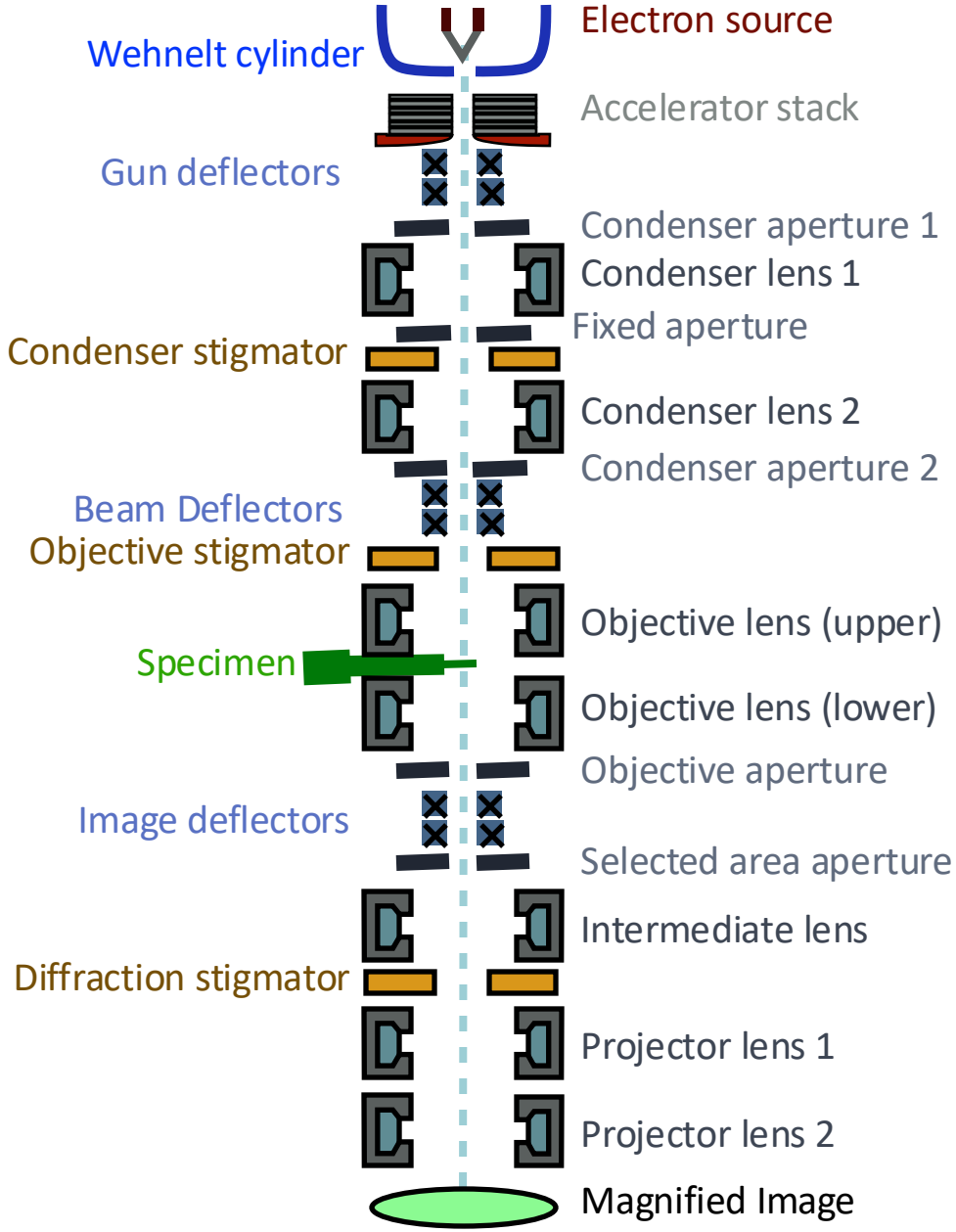
Specimen

Objective Lens
(focusing)

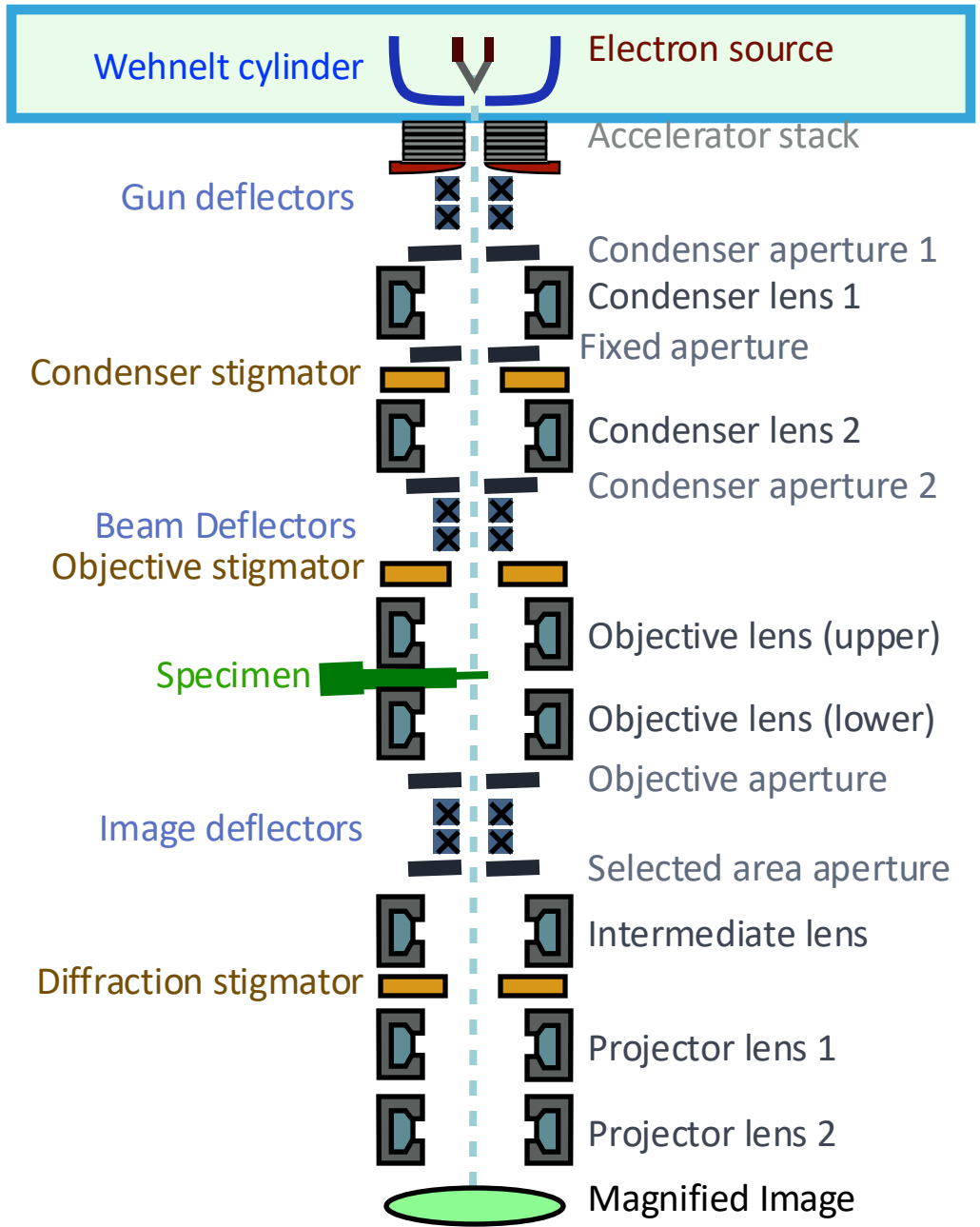
Projector Lens
(producing magnification)

Magnified Image

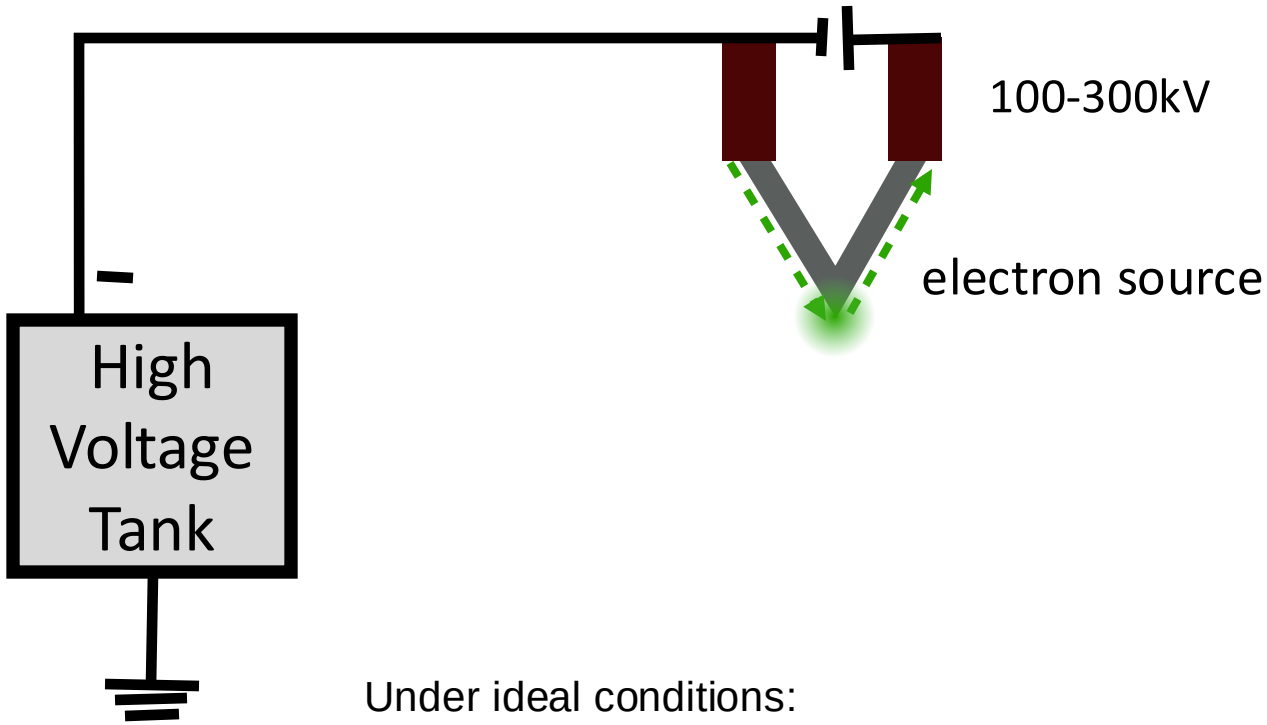
Microscope components



Generating electrons



We want every electron to have exactly the same wavelength and be emitted in the same direction.

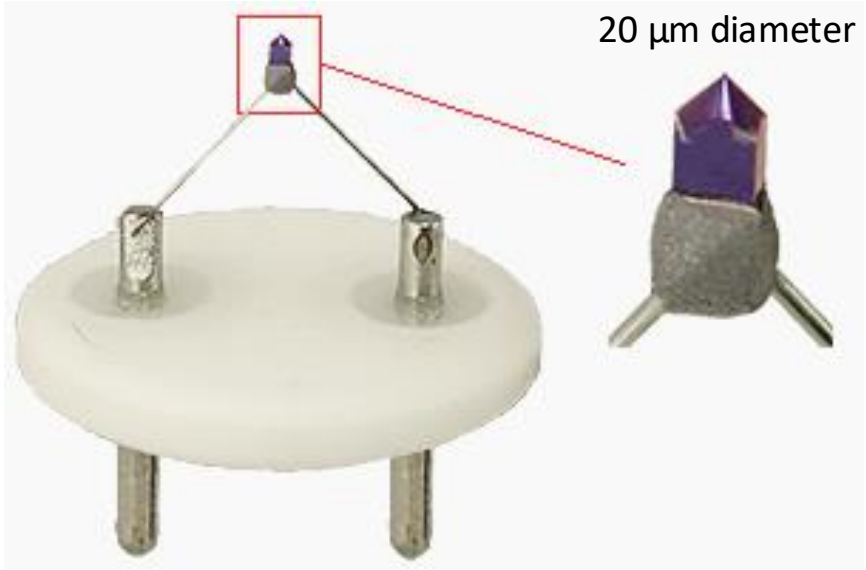


Under ideal conditions:

- A point source of electrons (1 atom)
- Energy distribution = 0 (no variation in eV)

Electron Sources – Thermal Filaments vs FEG

Thermal Filament

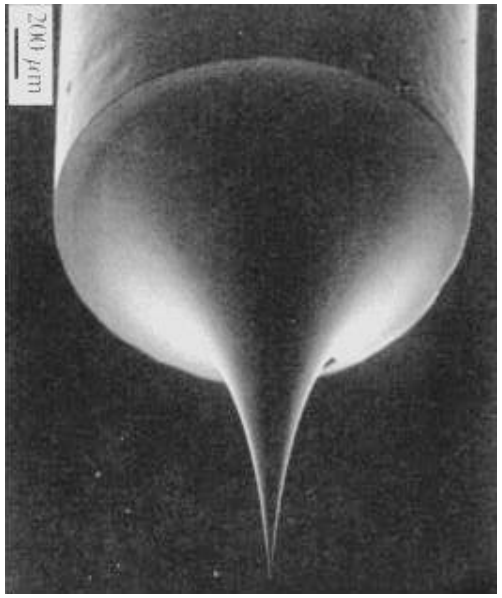


LaB_6

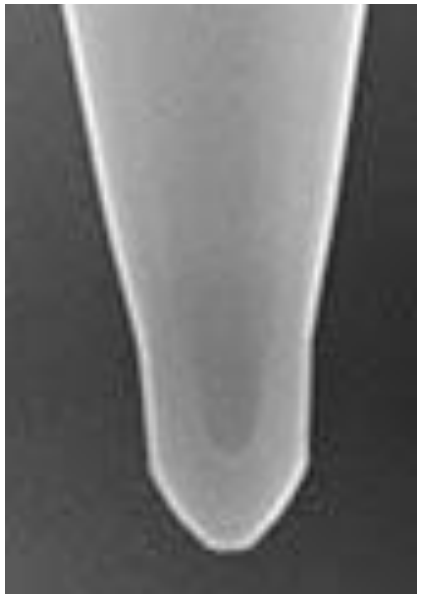
Lanthanum Hexaboride (LaB_6)
temperature: 1900 K
energy distr = 1.5 eV

Electron generated by thermal emission

Field Emission Gun (FEG)



Tungsten crystal
temperature: 1800 K
energy distribution = 1.0 eV

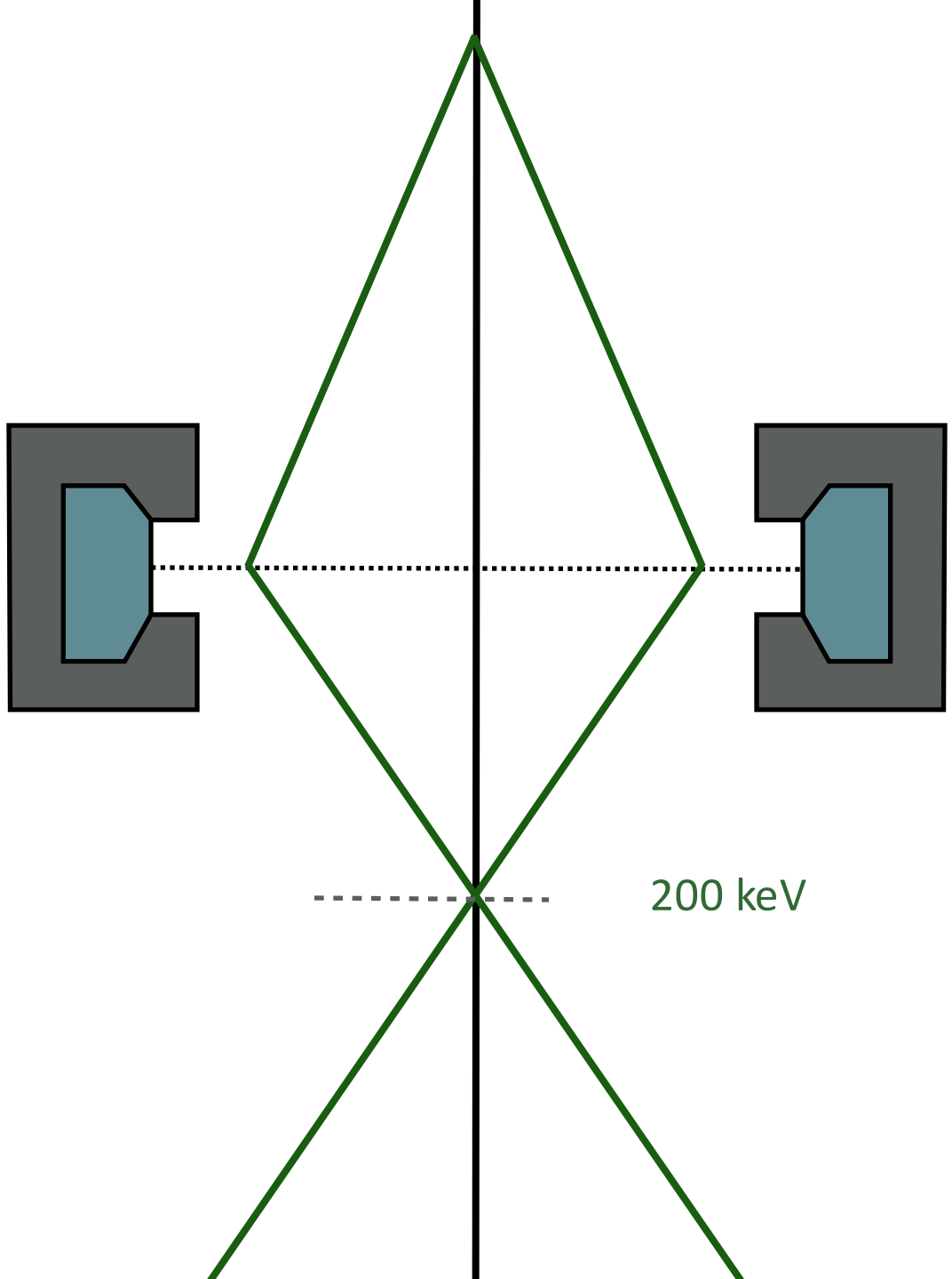


“Cold” FEG
temperature: 300 K
energy distribution = 0.25 eV

Field electron emission (negative potential applied for e^- extraction)

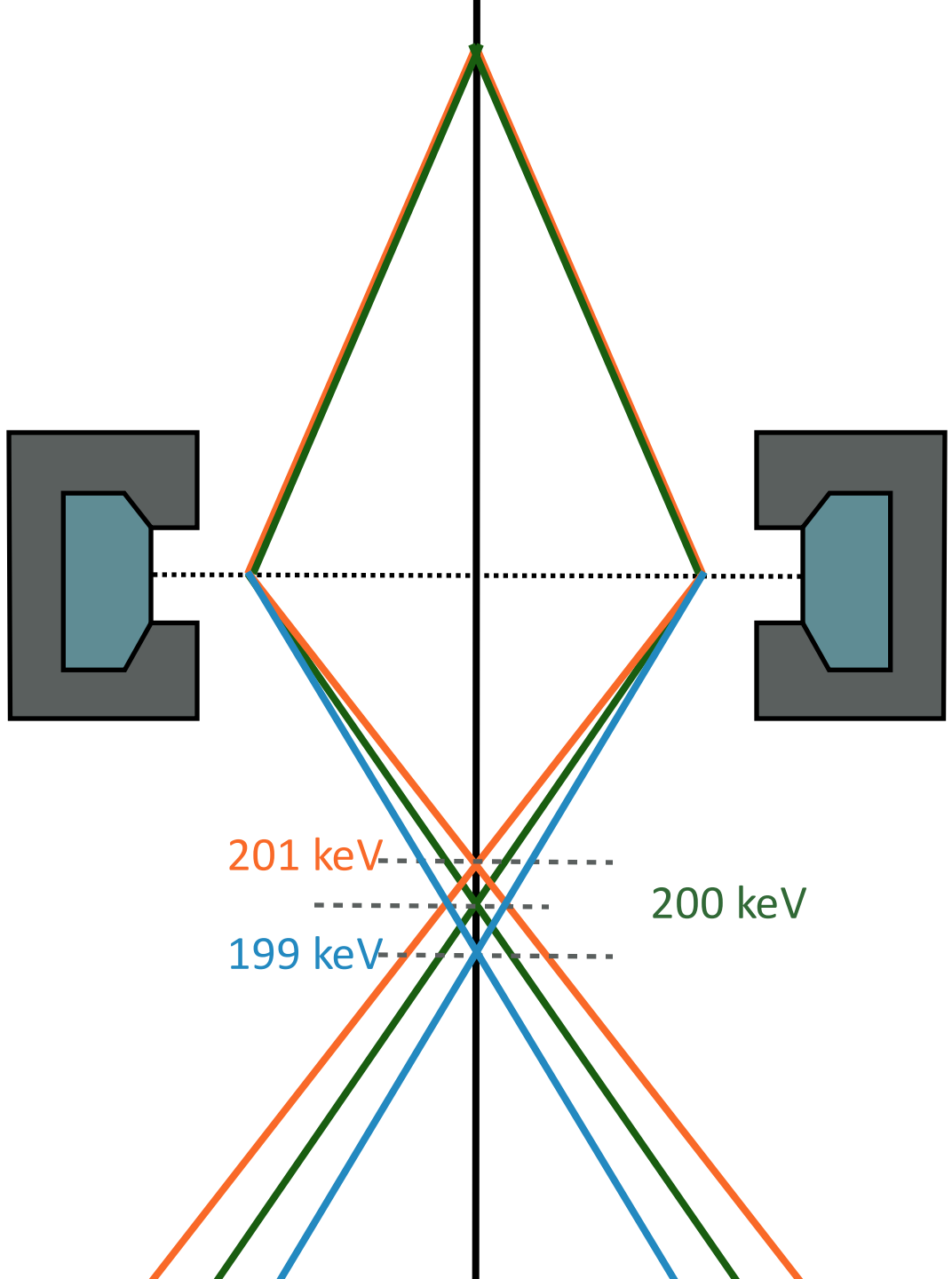
Ideal electron beam

All electrons of the same energy are focused to a single point



Variable electron energies

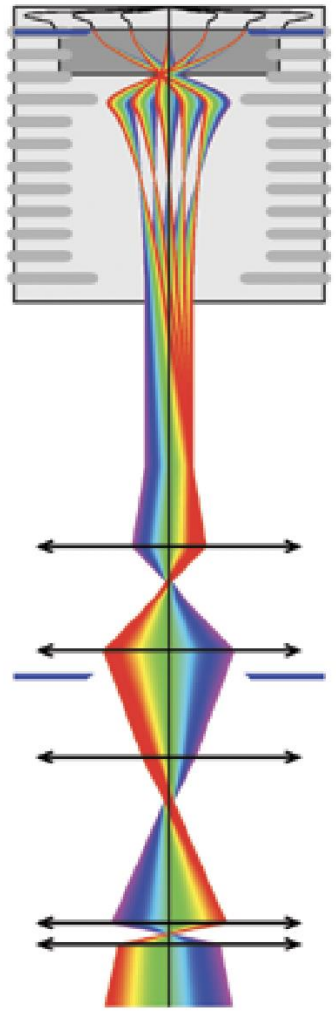
Electrons of different energies are focused differently by the EM lens



Variable electron energies

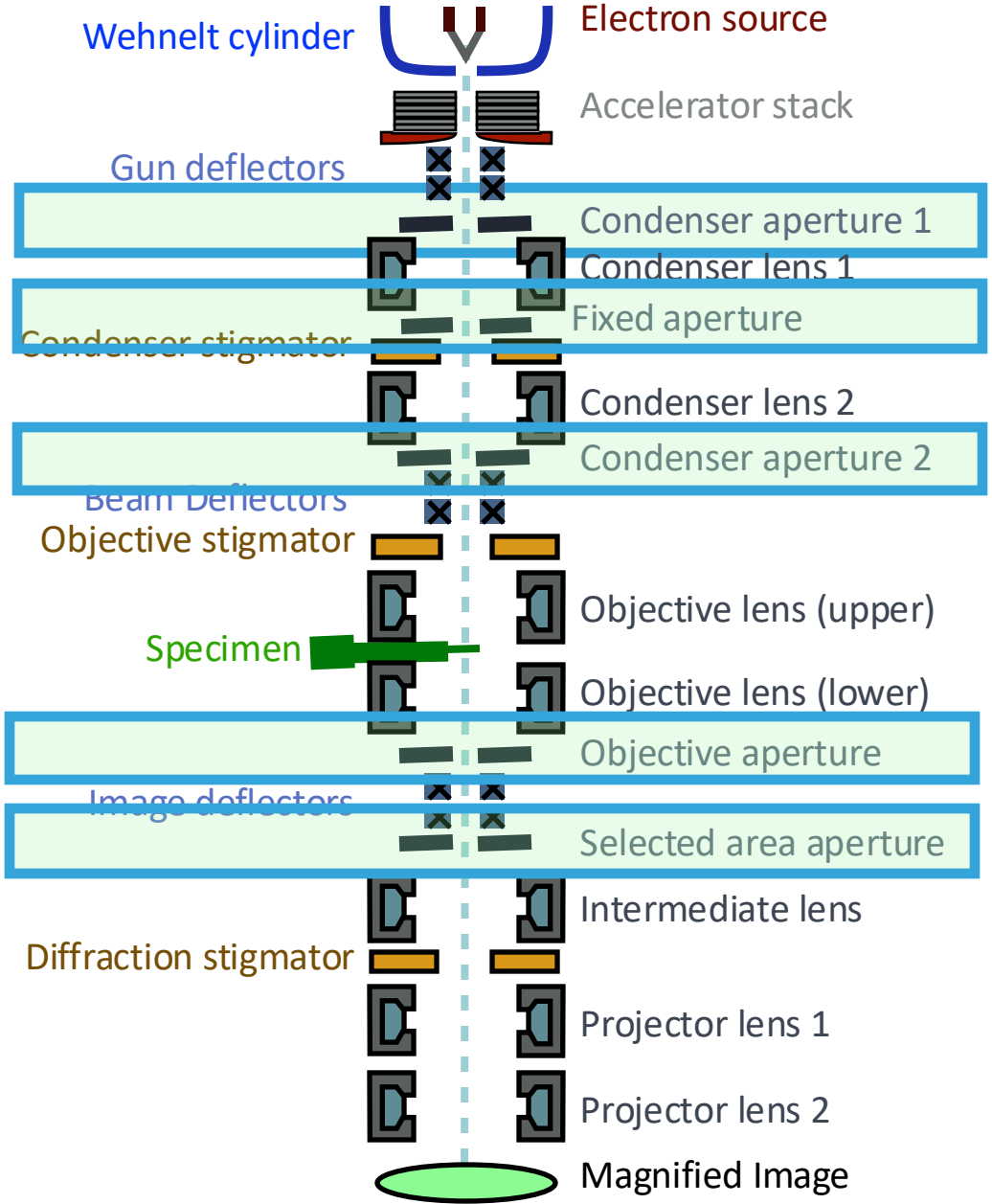
This leads to a phenomenon known as **chromatic aberration**

In optical microscopy **wavelength = color**



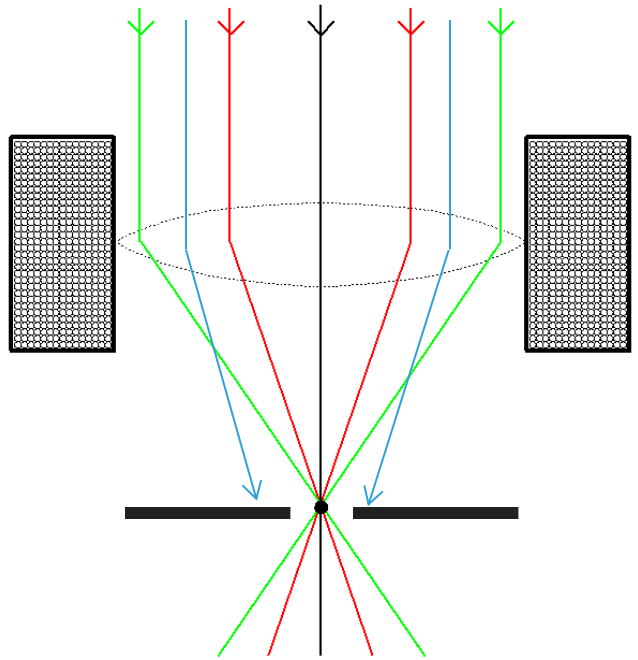
Chromatic aberration

Apertures



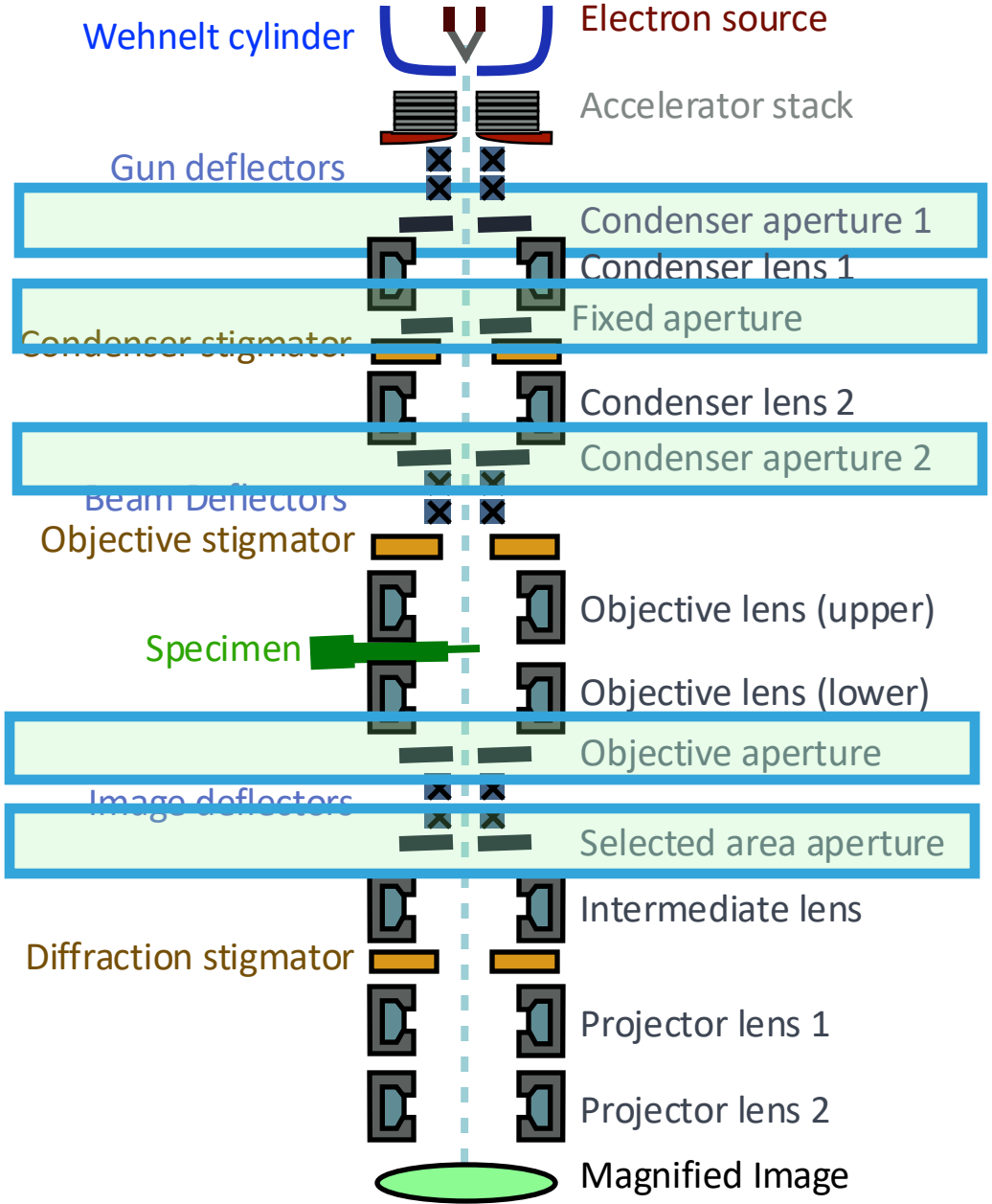
• Apertures are pinholes (20µm – 1500µm in diameter) that help remove electrons far from the optical axis (e.g., due to differing energy)

• This is helpful for (i) controlling beam intensity, (ii) reducing noise caused by inelastic scattering and (iii) correcting chromatic aberration.



Eliminating poorly focused electrons (blue)

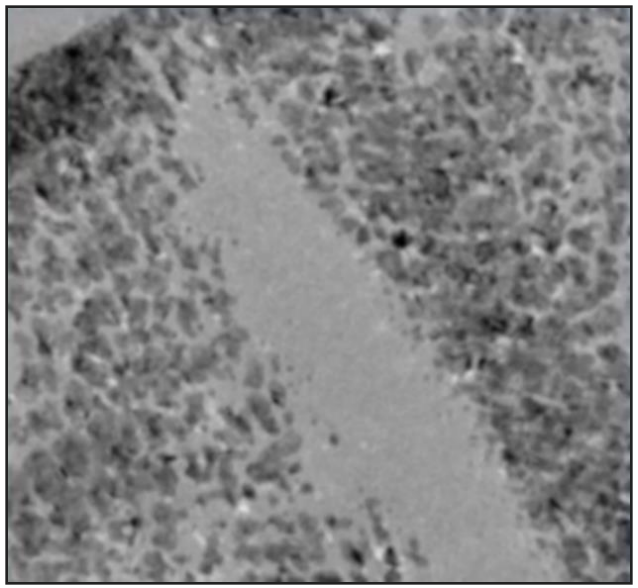
Apertures



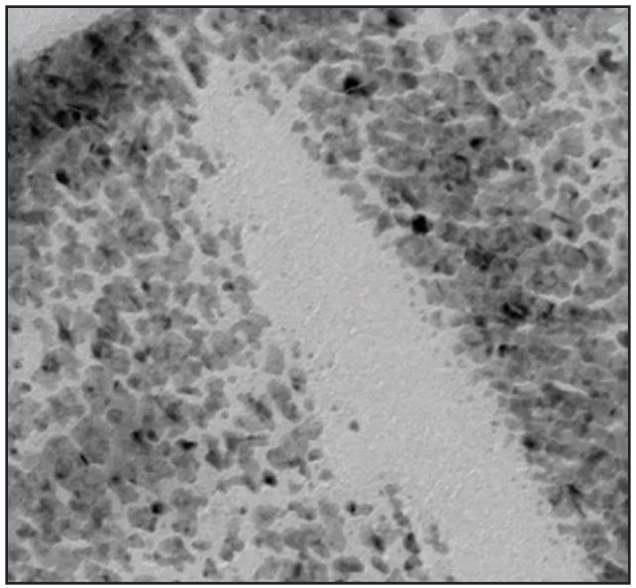
- Apertures are pinholes (20µm – 1500µm in diameter) that help remove electrons far from the optical axis (e.g., due to differing energy)

- This is helpful for (i) controlling beam intensity, (ii) reducing noise caused by inelastic scattering and (iii) correcting aberrations.

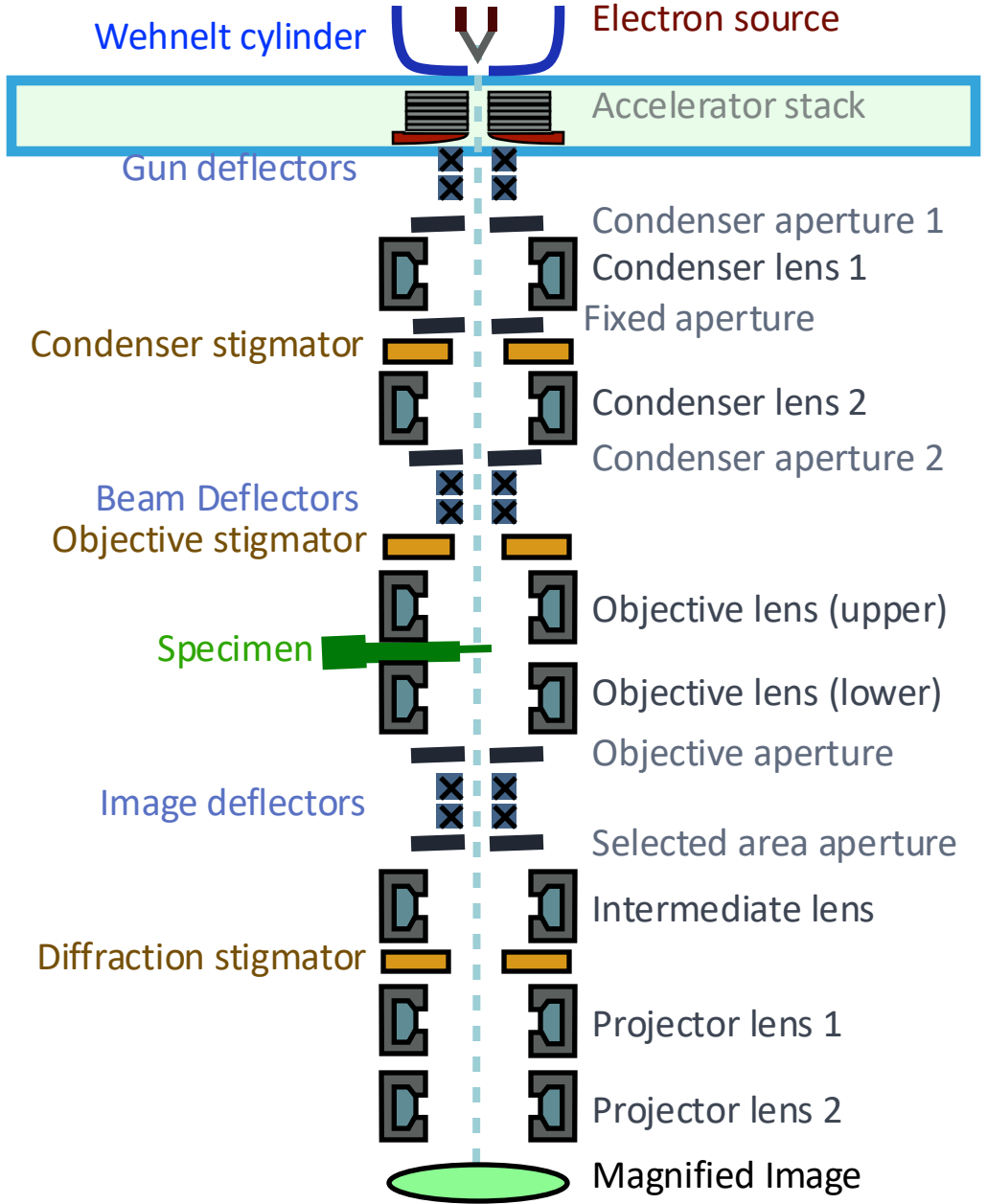
No Objective Aperture



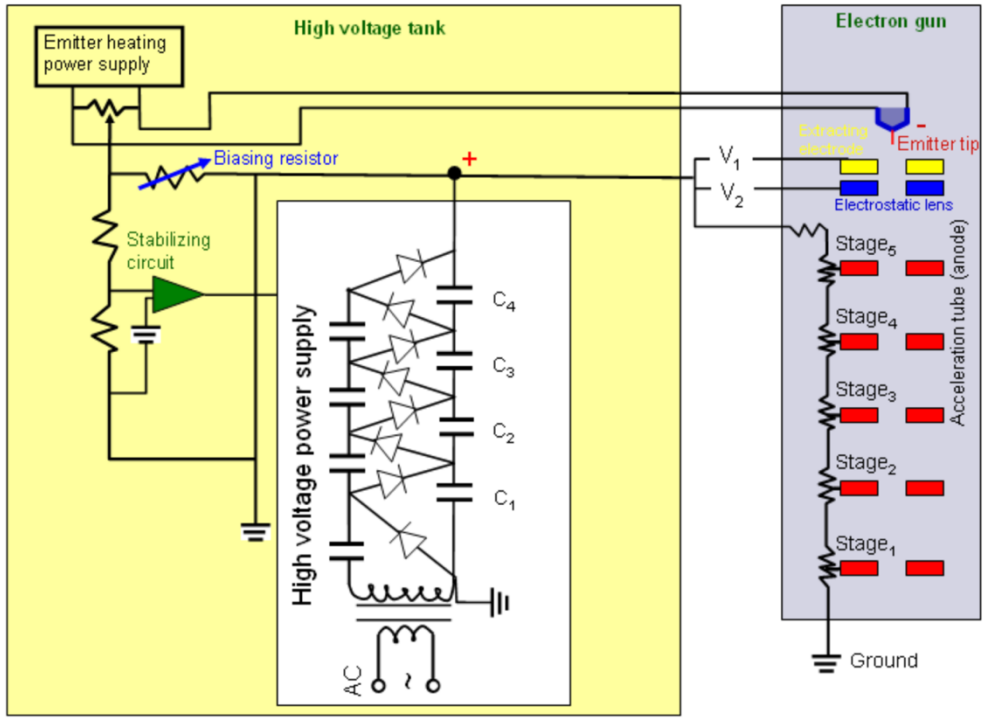
With Objective Aperture



High tension (voltage) generator



- The accelerator stack is based on **Cockcroft–Walton** voltage generator (Nobel Prize 1951)
- Originally designed by **Heinrich Greinacher**, a Swiss physicist

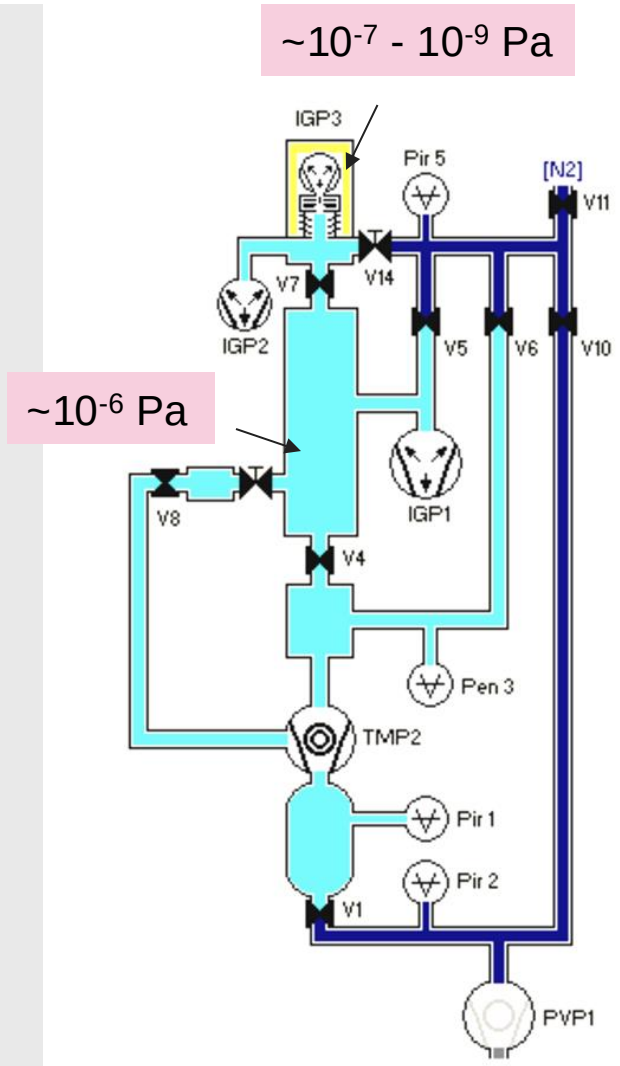


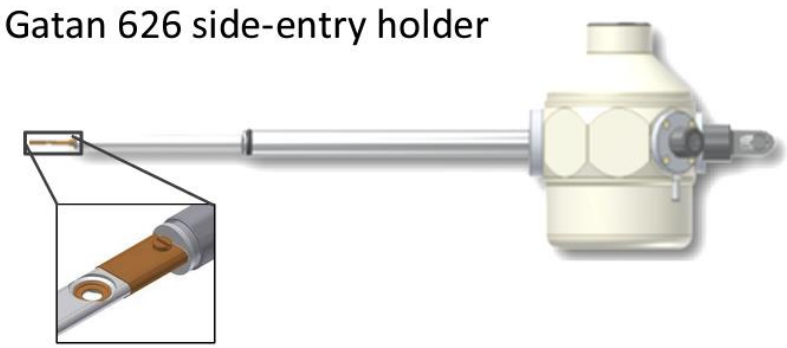
- Accelerating voltage is typically 100-300kv
- High stability of the generator (<1ppm) is necessary to achieve atomic resolutions

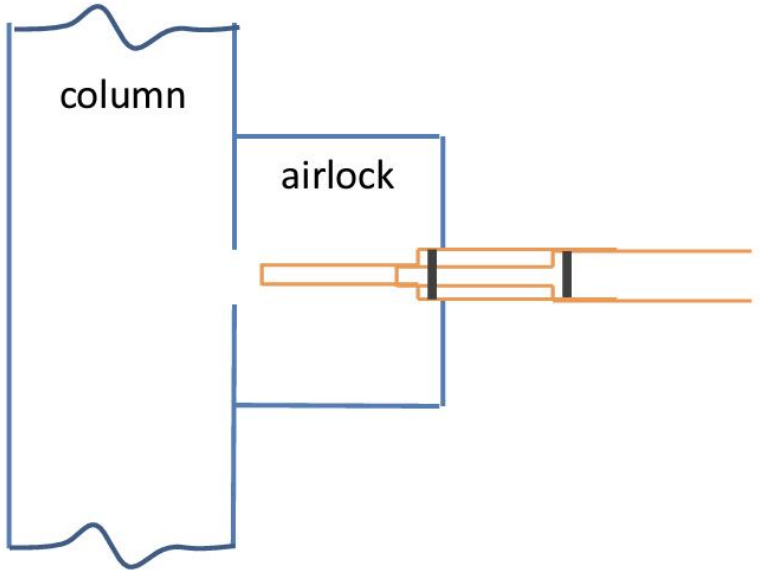
Vacuum system and sample insertion

- Electrons interact with air, thereby the column needs to be kept at high vacuum

- Different types of vacuum pumps:
  - Rotary pump
  - Oil diffusion pump
  - Turbomolecular pumps
  - Ion-getter pumps

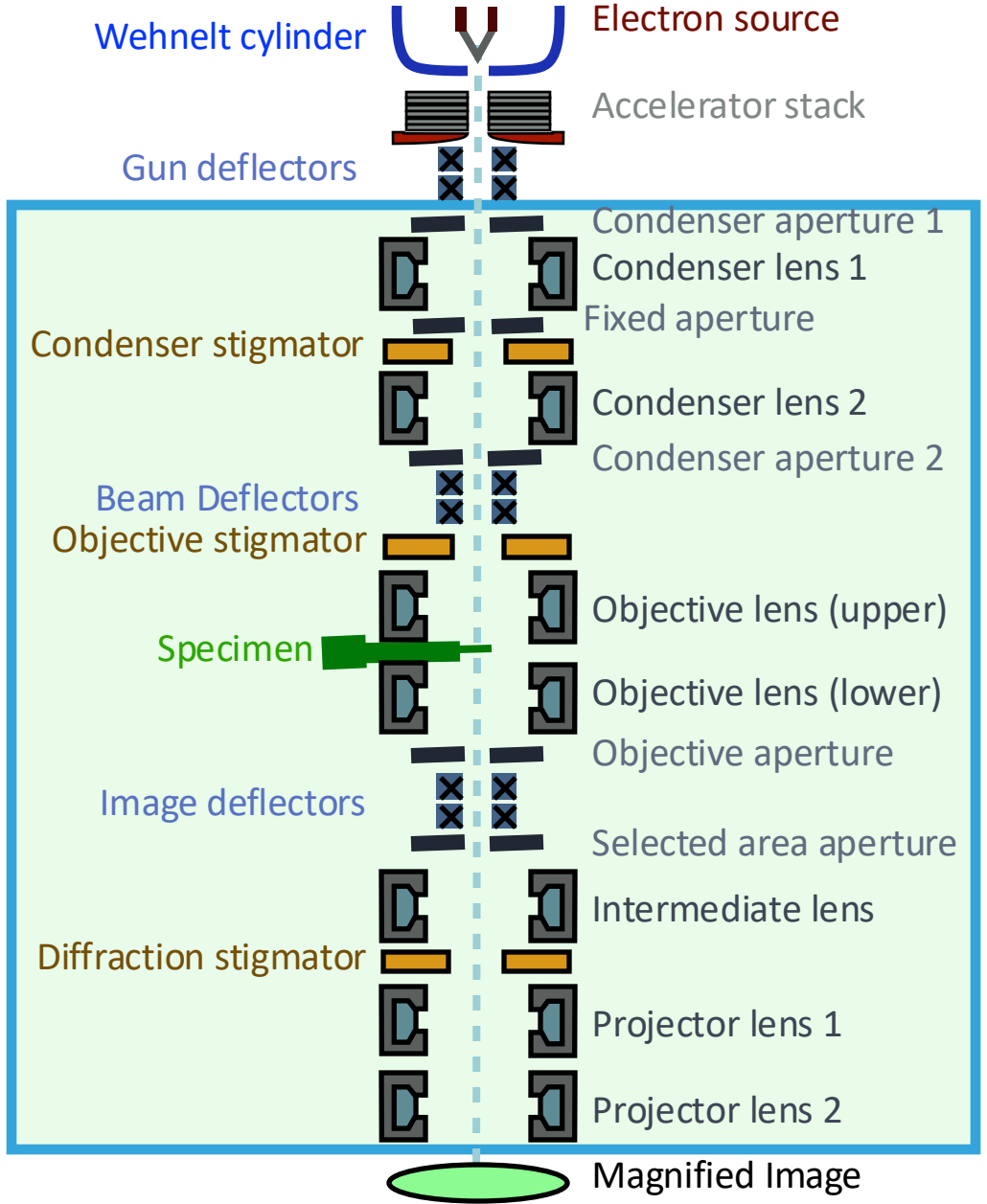






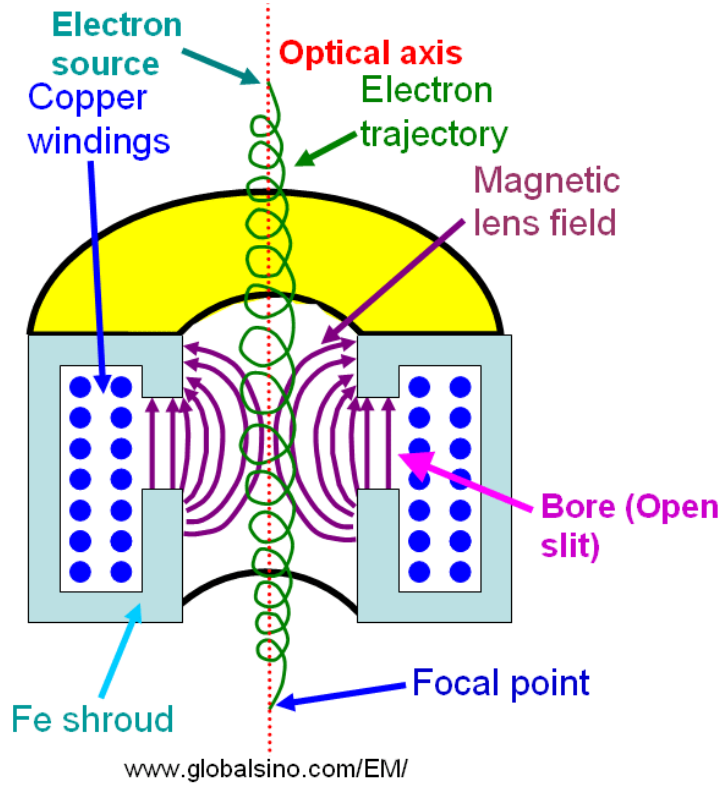
Fun fact: Even at high vacuum (~10^{-10} Pa) 30k molecules per cm^3 will still be present.

Electromagnetic lens system



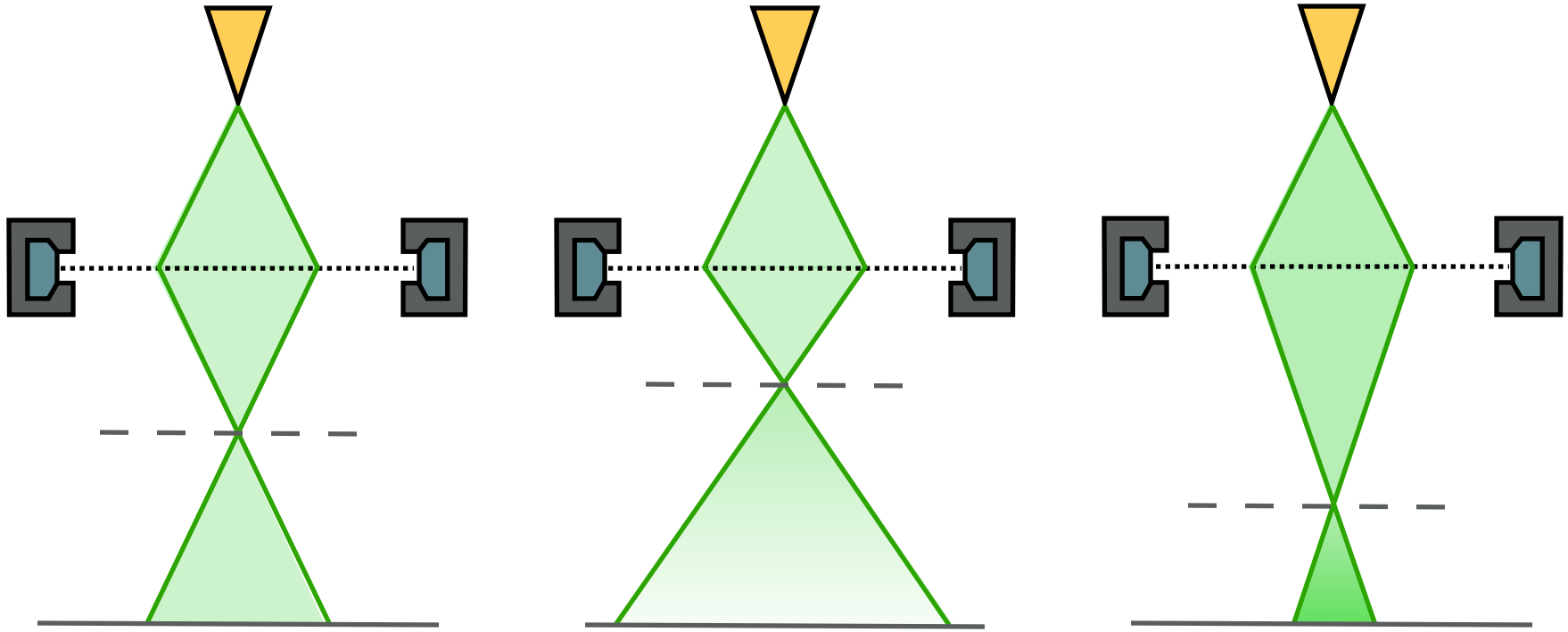
- Electromagnetic lenses change strength as excitation current changes.

- The magnetic field of the lens gives electrons a spiral path.



- The further the electrons are from the optical axis, the stronger the focusing effect.

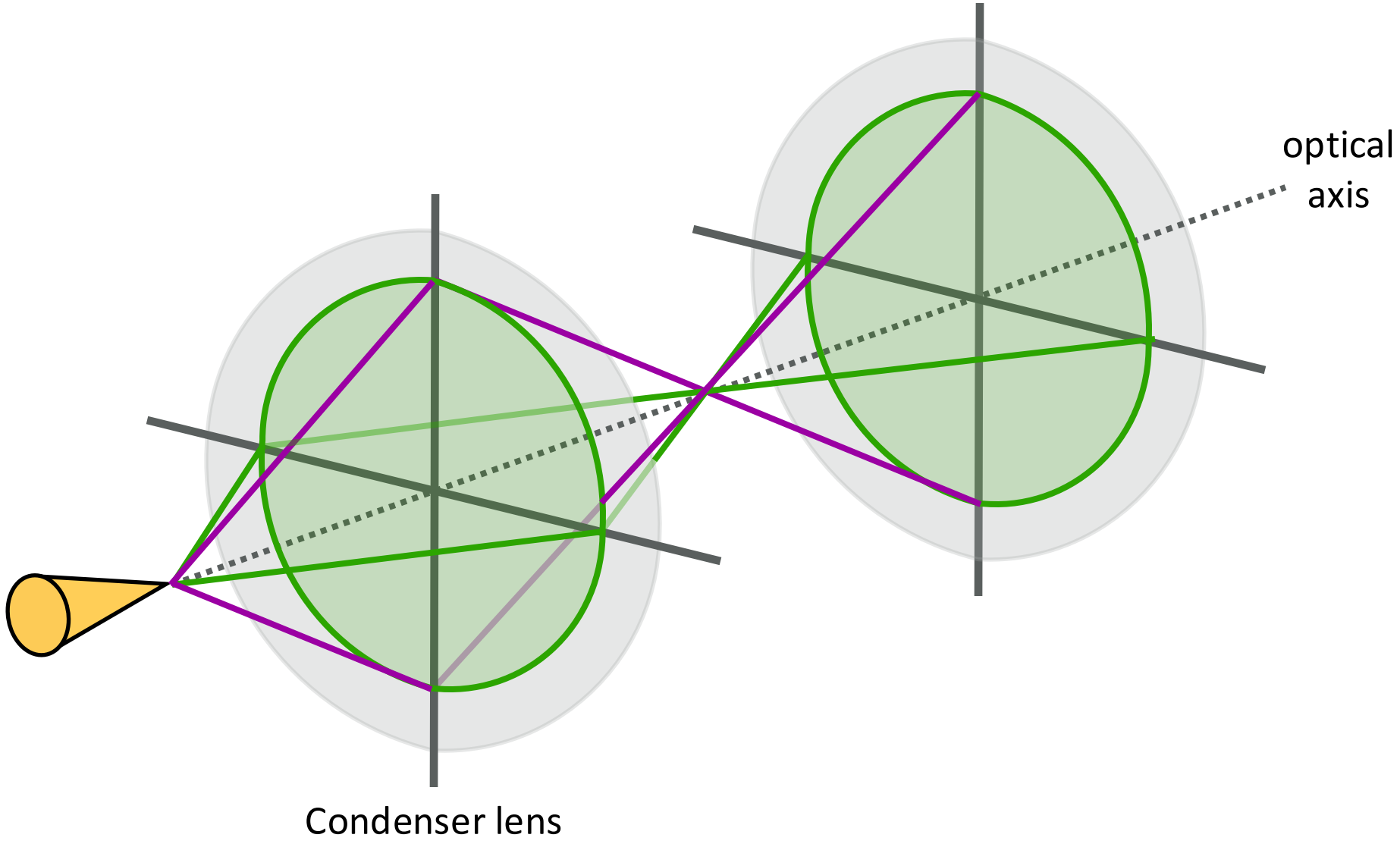
Lenses allow to control beam intensity and magnification



higher current,
bigger spot size

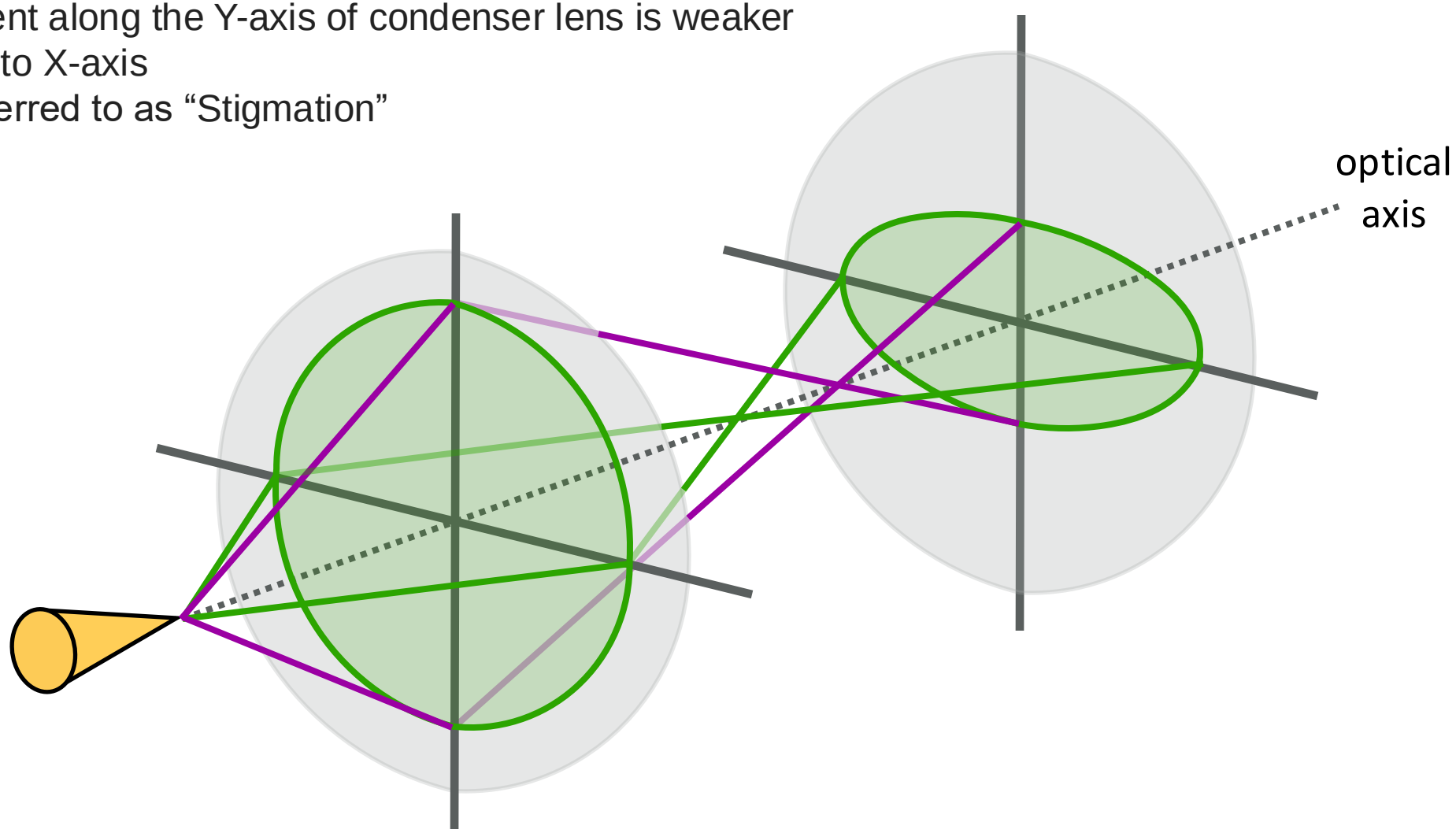
lower current,
smaller spot size

In a perfect lens the currents are the same in X- and Y-directions

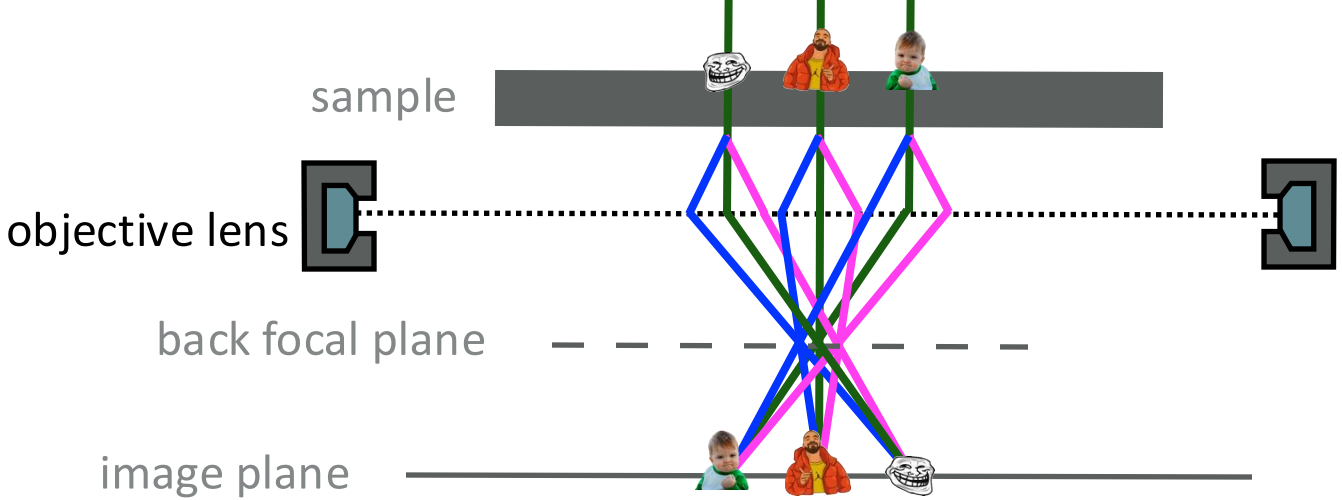


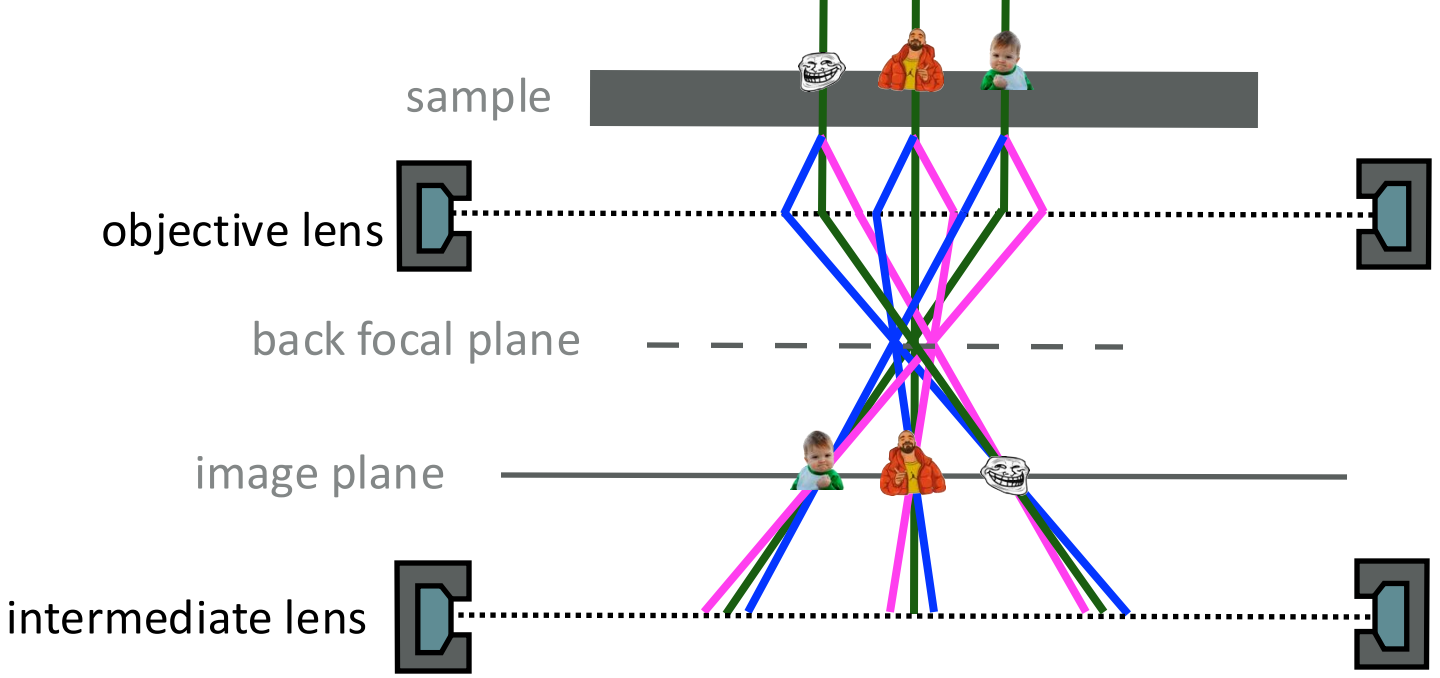
Astigmatism: Aberration caused by uneven lens current

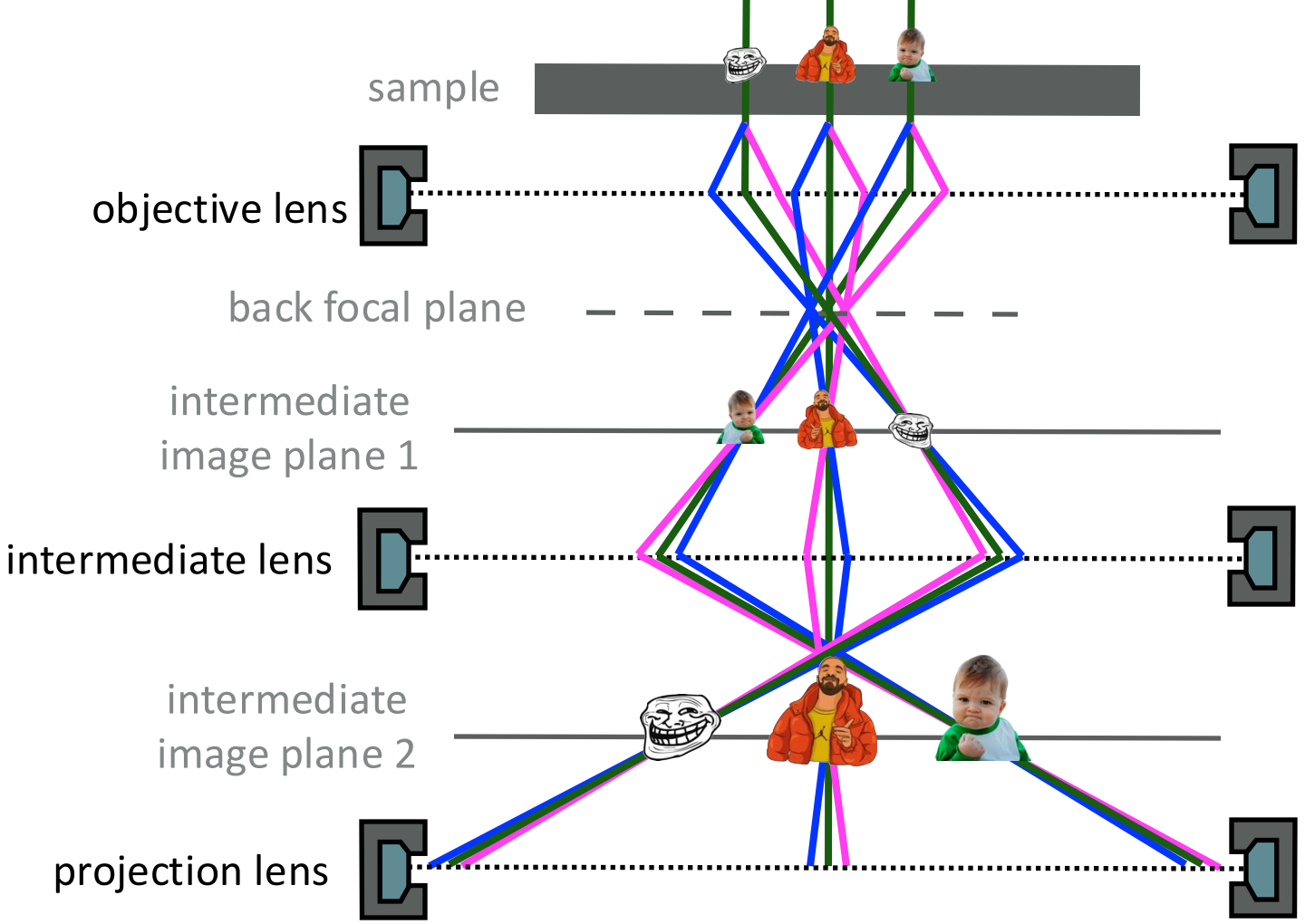
- The current along the Y-axis of condenser lens is weaker compared to X-axis
- Often referred to as “Stigmation”

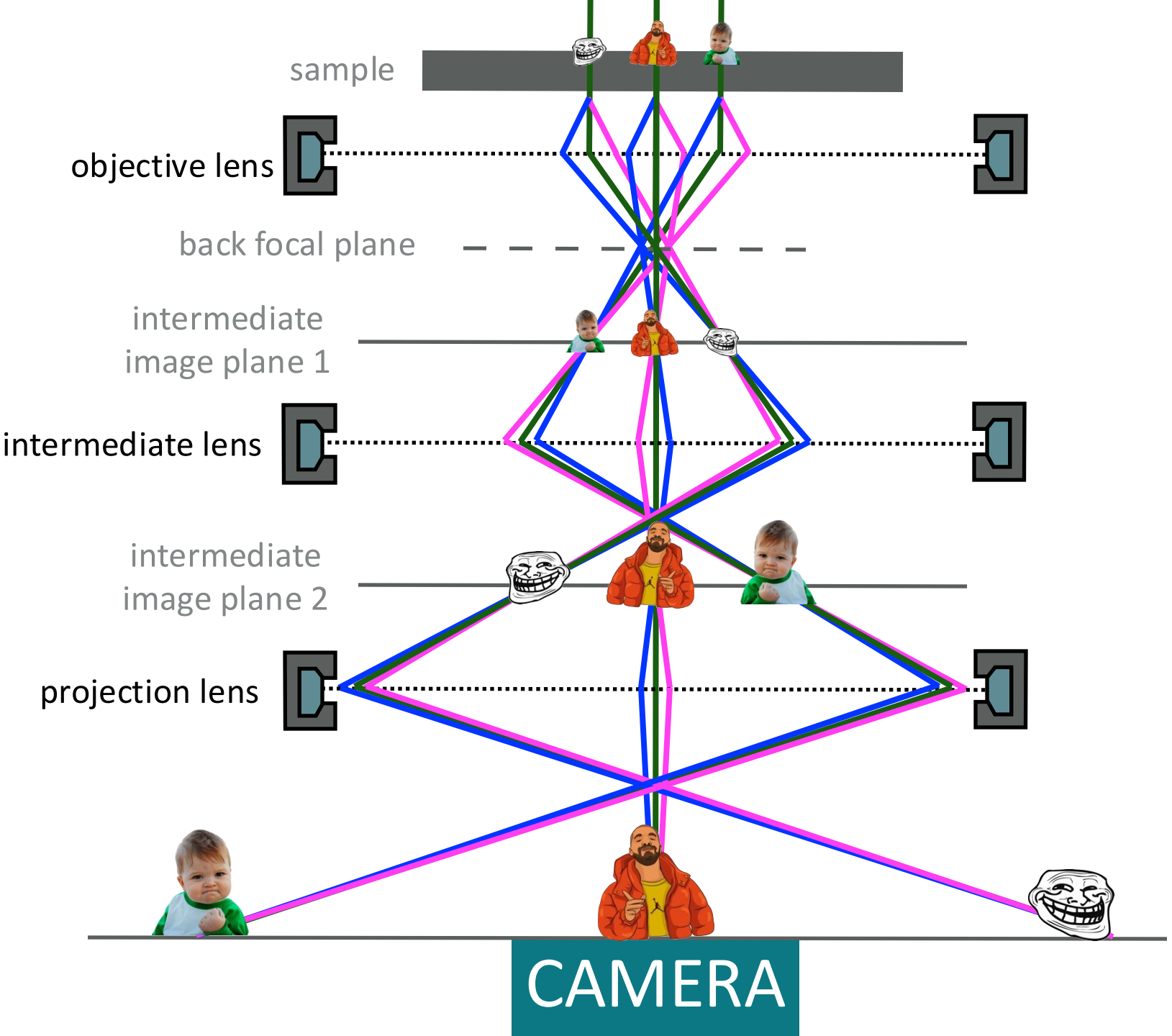


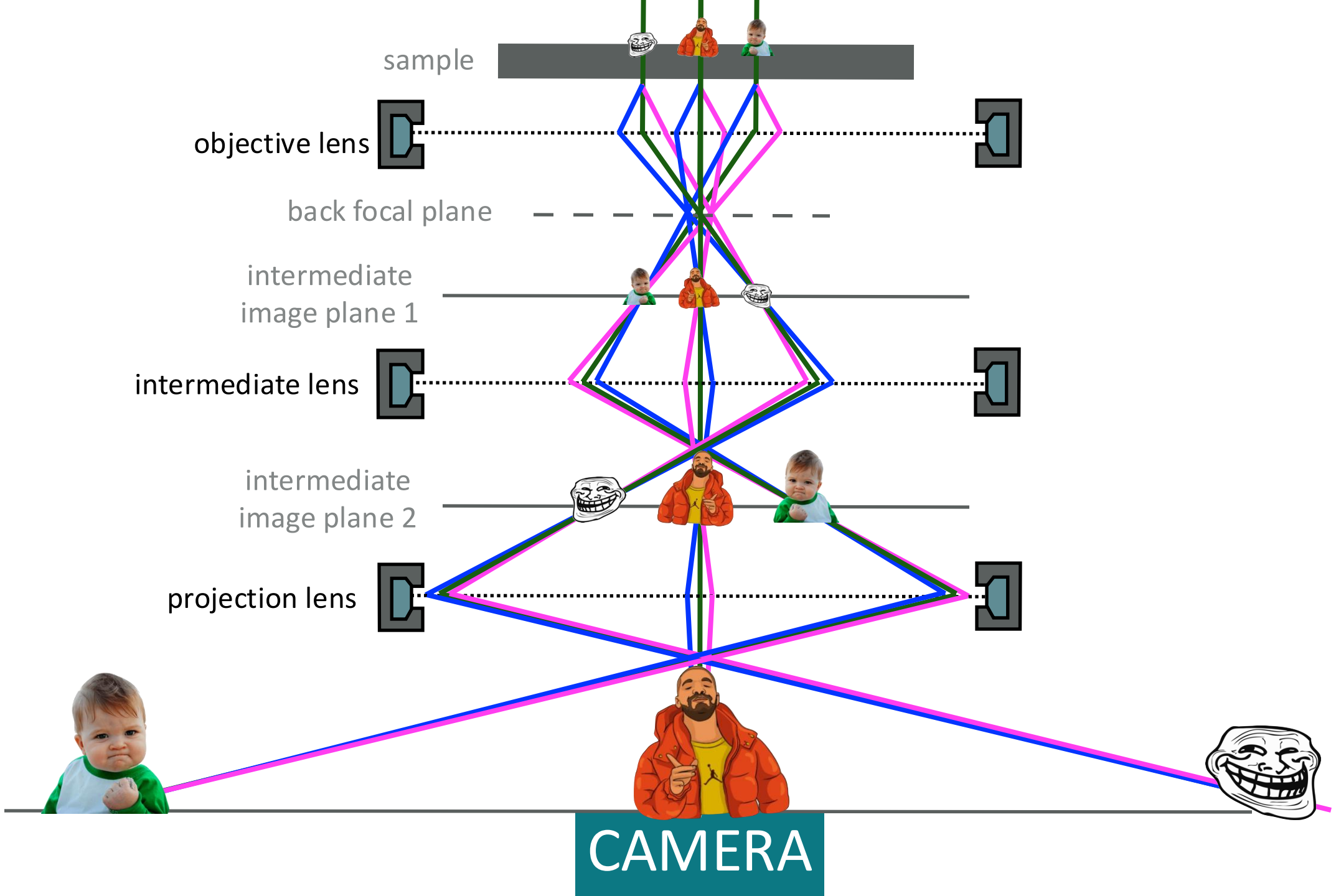
- Corrected by adjusting **stigmator lenses**

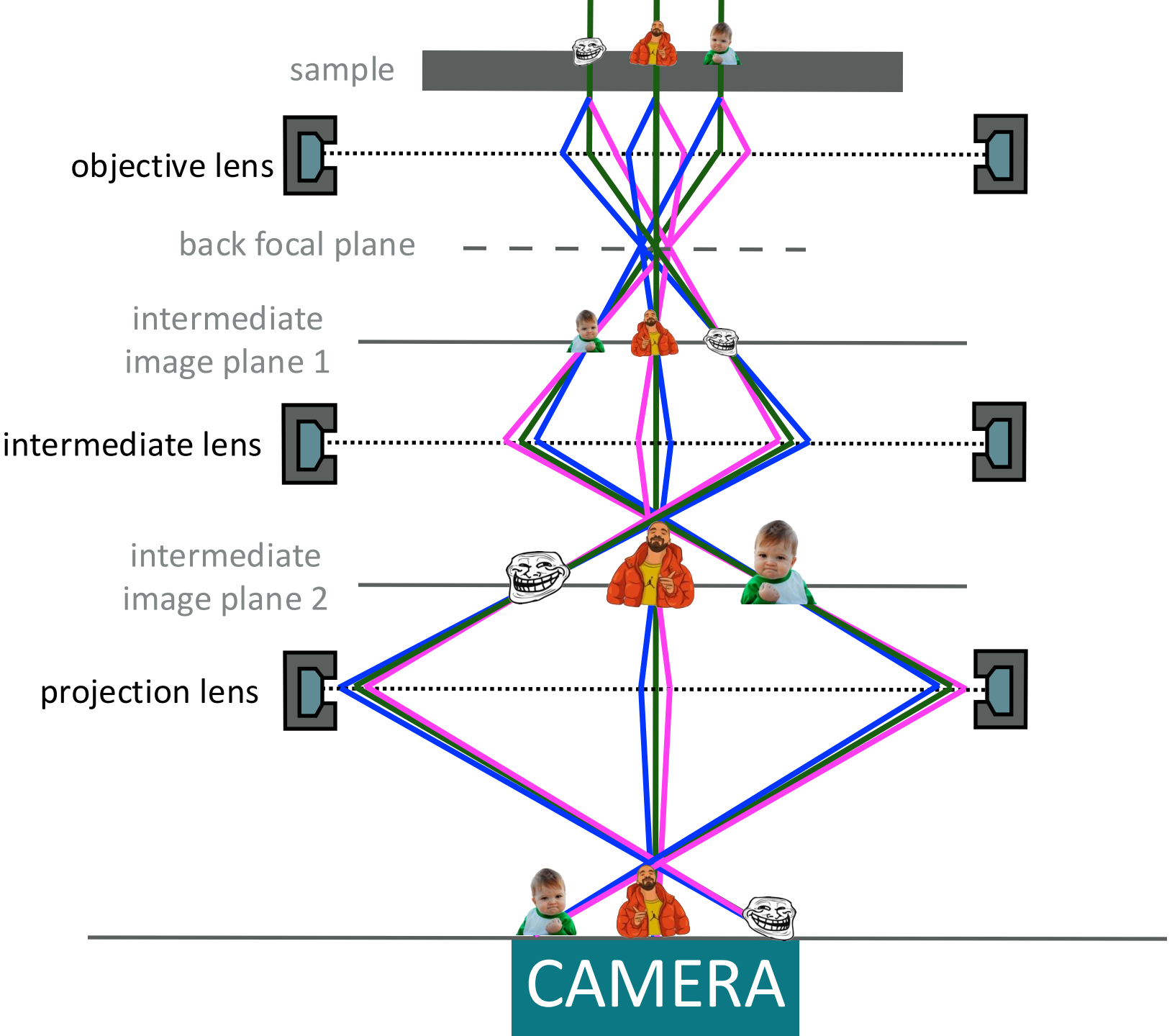

sample
objective lens
back focal plane
image plane


sample
objective lens
back focal plane
image plane
intermediate lens

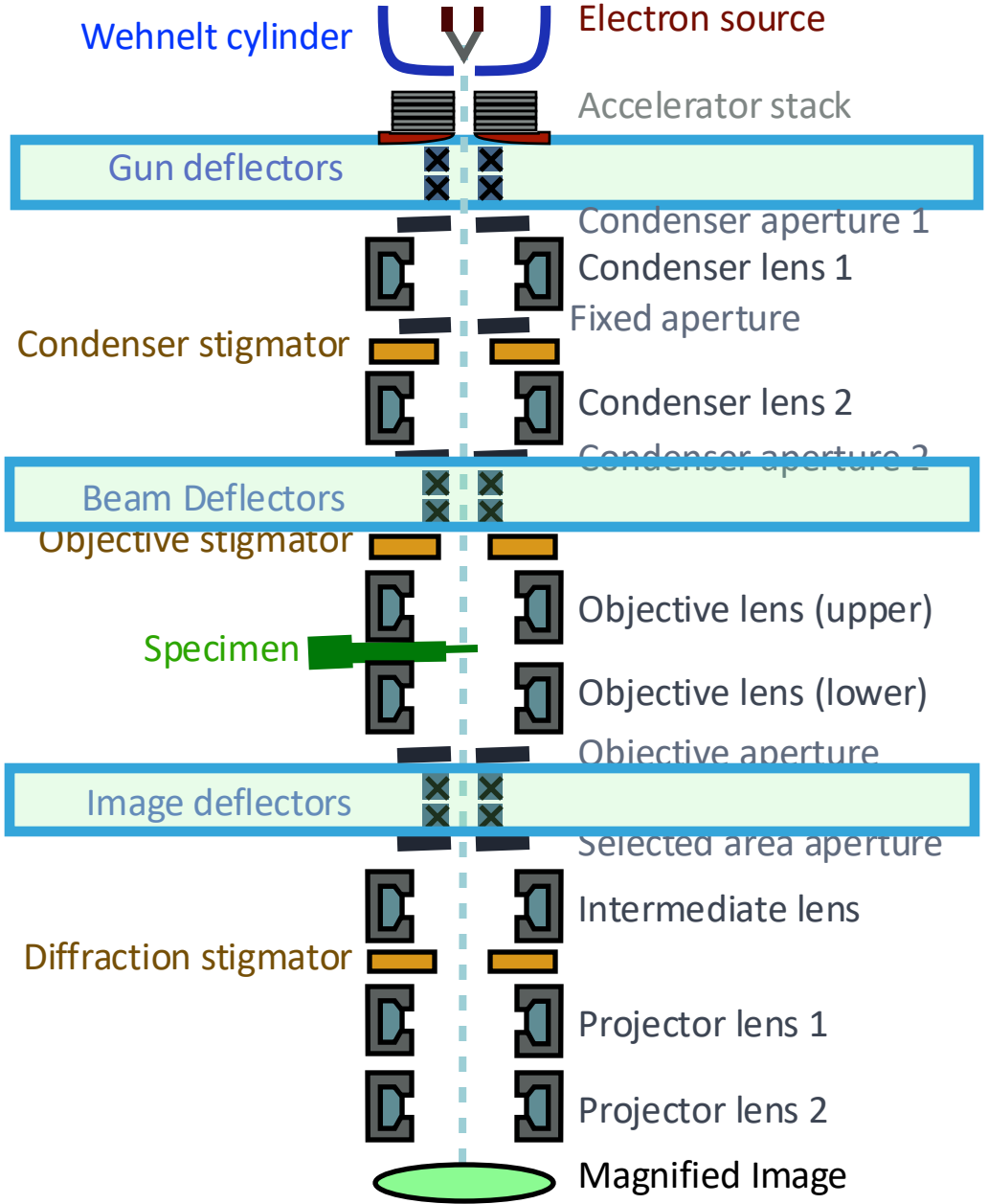

sample
objective lens
back focal plane
intermediate
image plane 1
intermediate lens
intermediate
image plane 2
projection lens


sample
objective lens
back focal plane
intermediate
image plane 1
intermediate lens
intermediate
image plane 2
projection lens
CAMERA

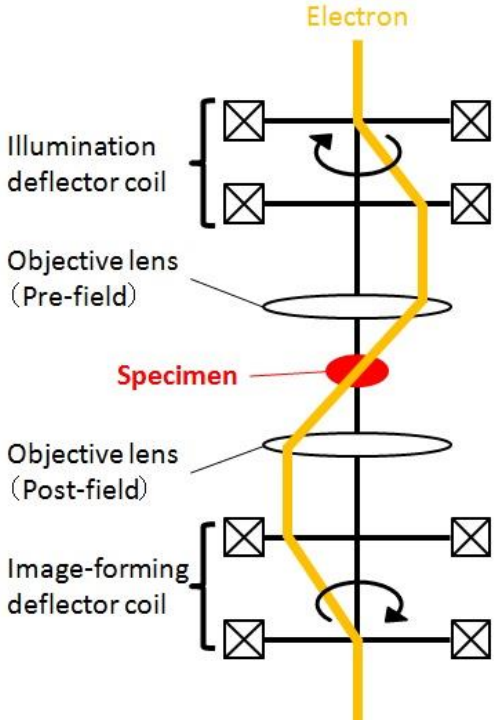

sample
objective lens
back focal plane
intermediate
image plane 1
intermediate lens
intermediate
image plane 2
projection lens
CAMERA


sample
objective lens
back focal plane
intermediate
image plane 1
intermediate lens
intermediate
image plane 2
projection lens
CAMERA

Deflector lenses

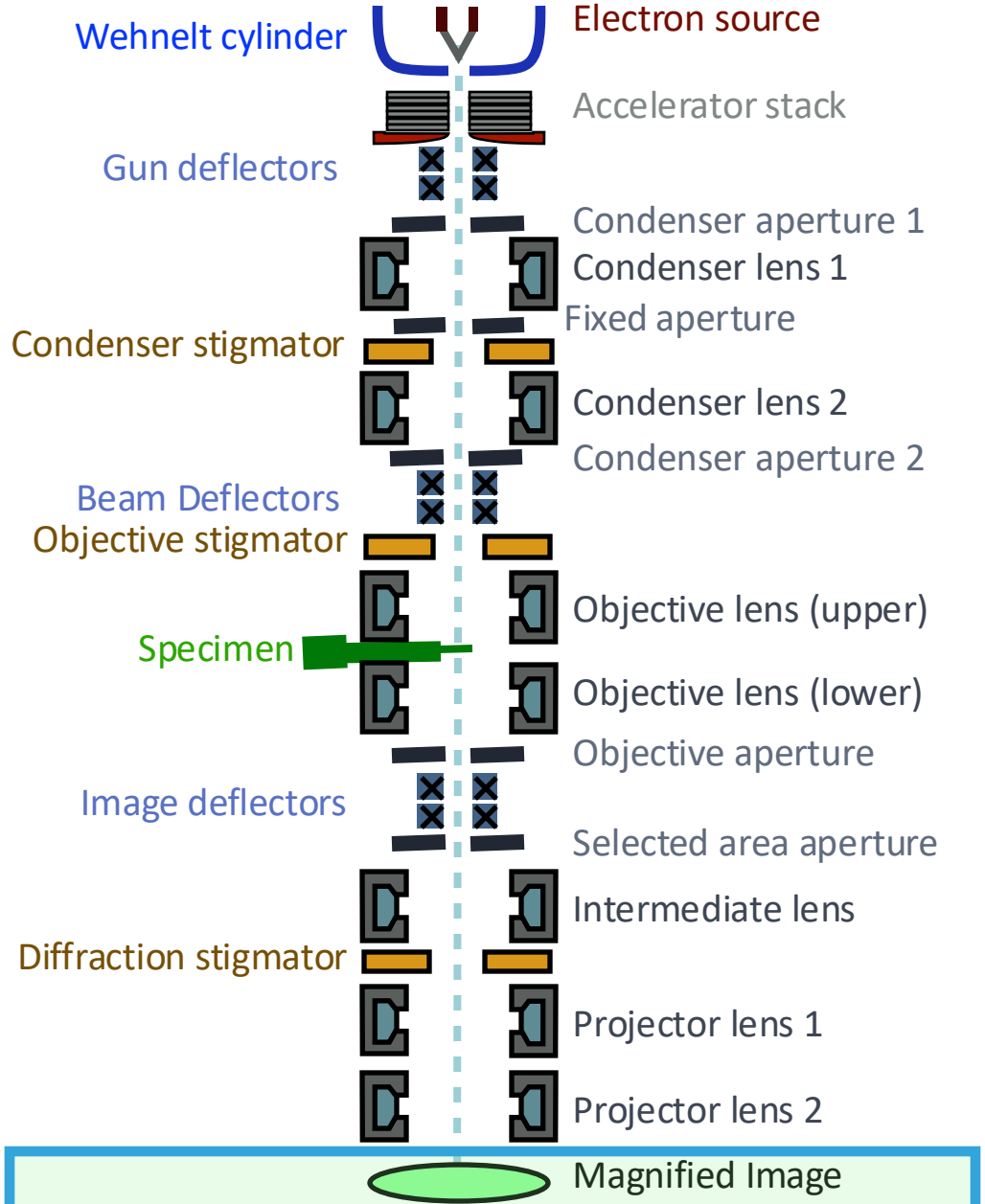


- Deflector lenses create field orthogonal to electron path
- The main purpose is to correct beam position (shift) or angle (tilt) relative to the main optical axis of the instrument



- Deflector lenses are critical for focusing during imaging

Detectors – The biggest contributors to the “Resolution Revolution"

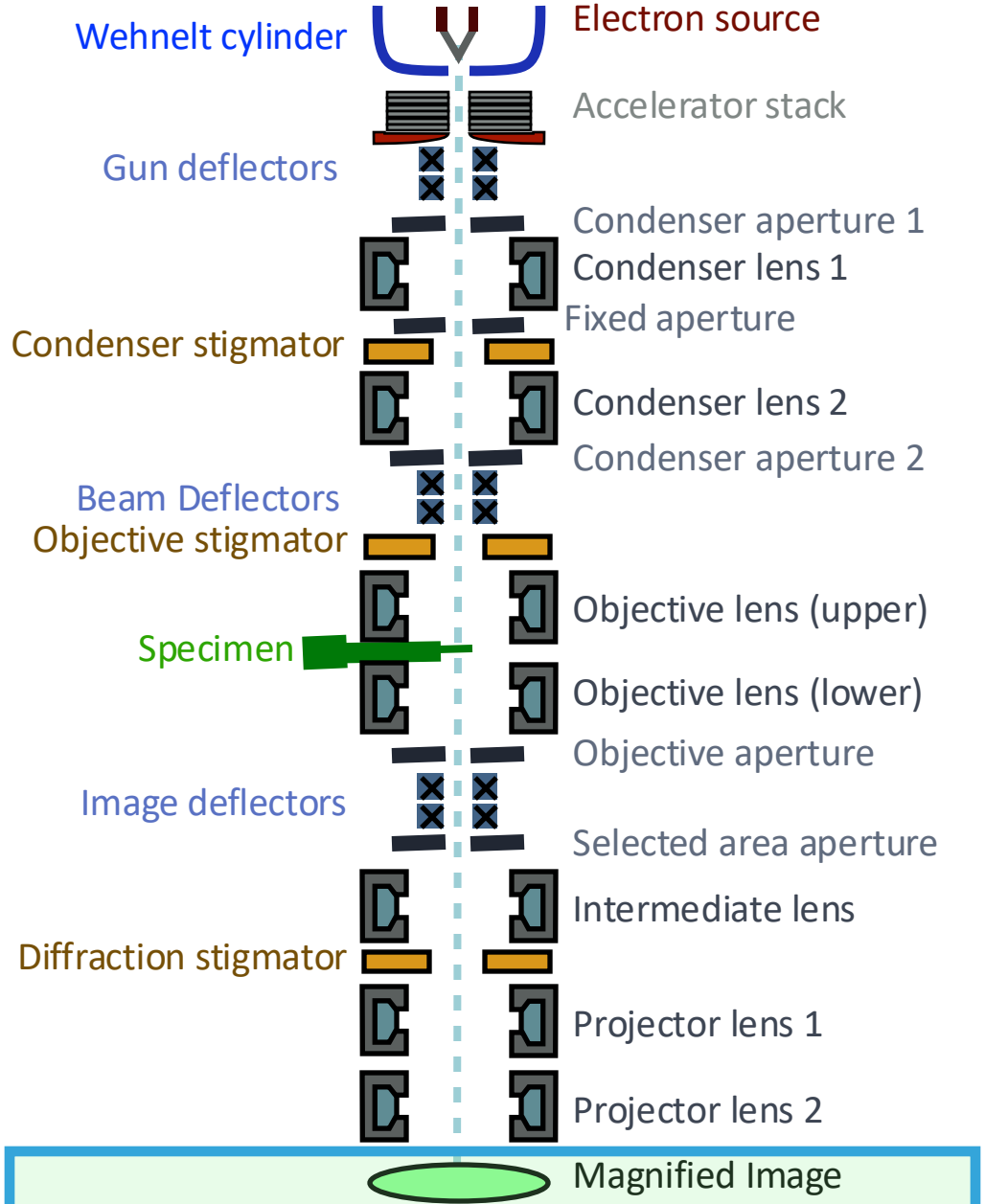


- The first detectors were based on imaging films

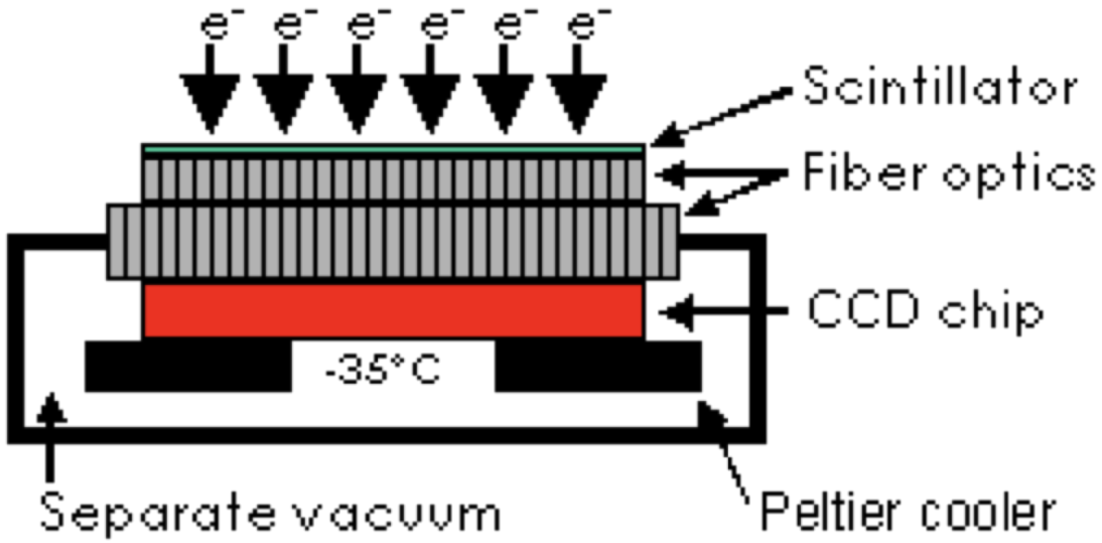
- Used until recently

- Good resolution, difficult to automate, slow to process and environmentally unfriendly

Detectors – The biggest contributors to the “Resolution Revolution"

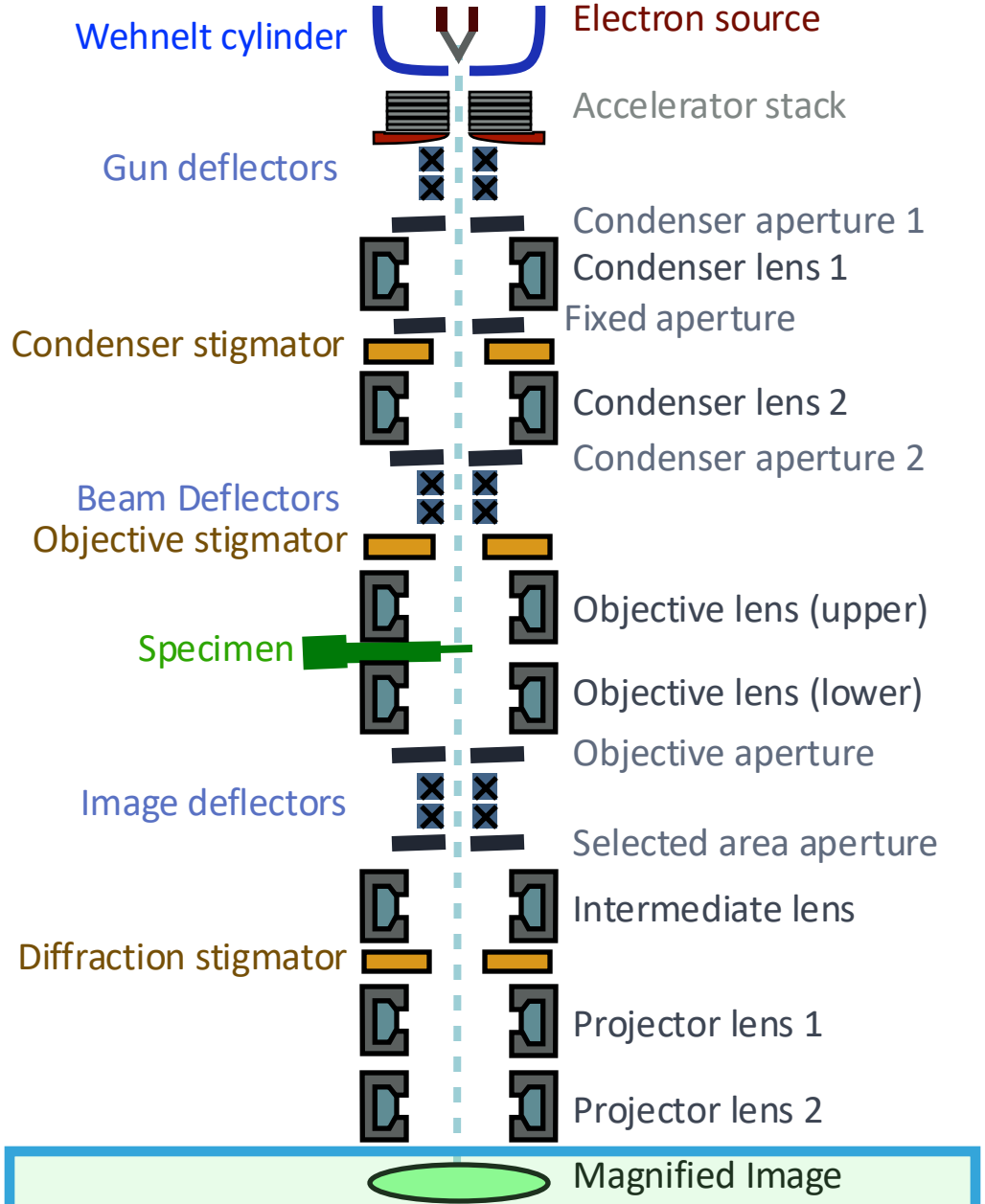


• Then came the era of CCD cameras (since 1980s)



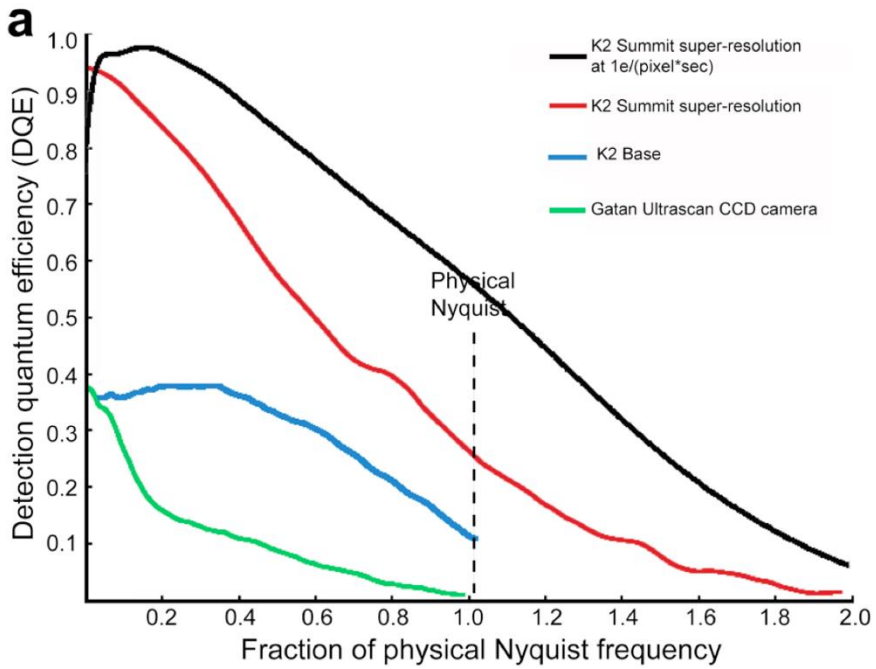
• Peltier cooling device reduces the temperature of the sensor to approximately -35°C, which reduces dark current and improves signal-to-noise
• Indirect, scintillator-based, detection reduces the DQE of a camera

Detectors – The biggest contributors to the “Resolution Revolution"



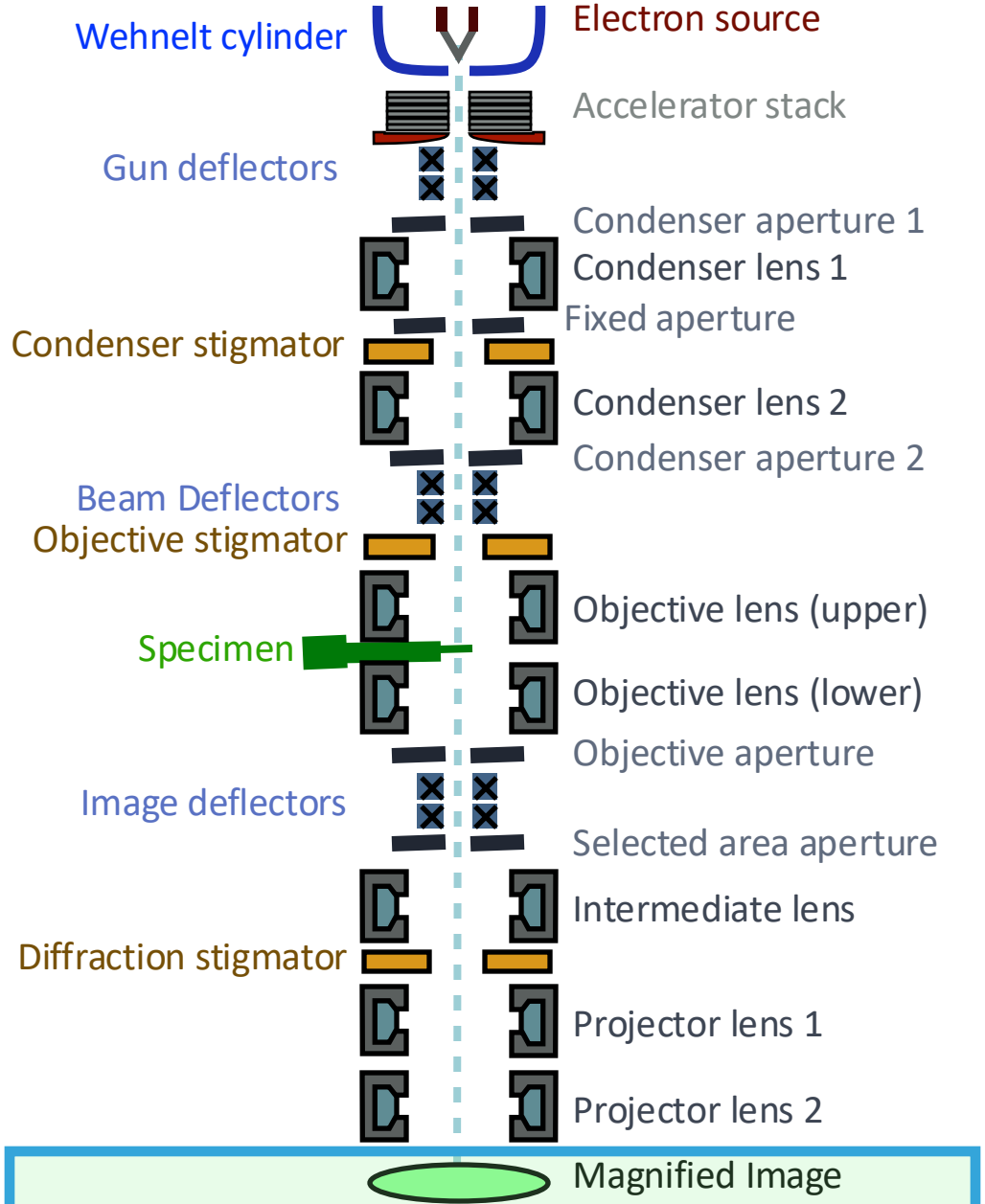
• The Detective Quantum Efficiency is a measure of the combined effects of the signal and noise performance of an imaging system, generally expressed as a function of spatial frequency.

$$DQE = \frac{SNR_{out}^2}{SNR_{in}^2}$$



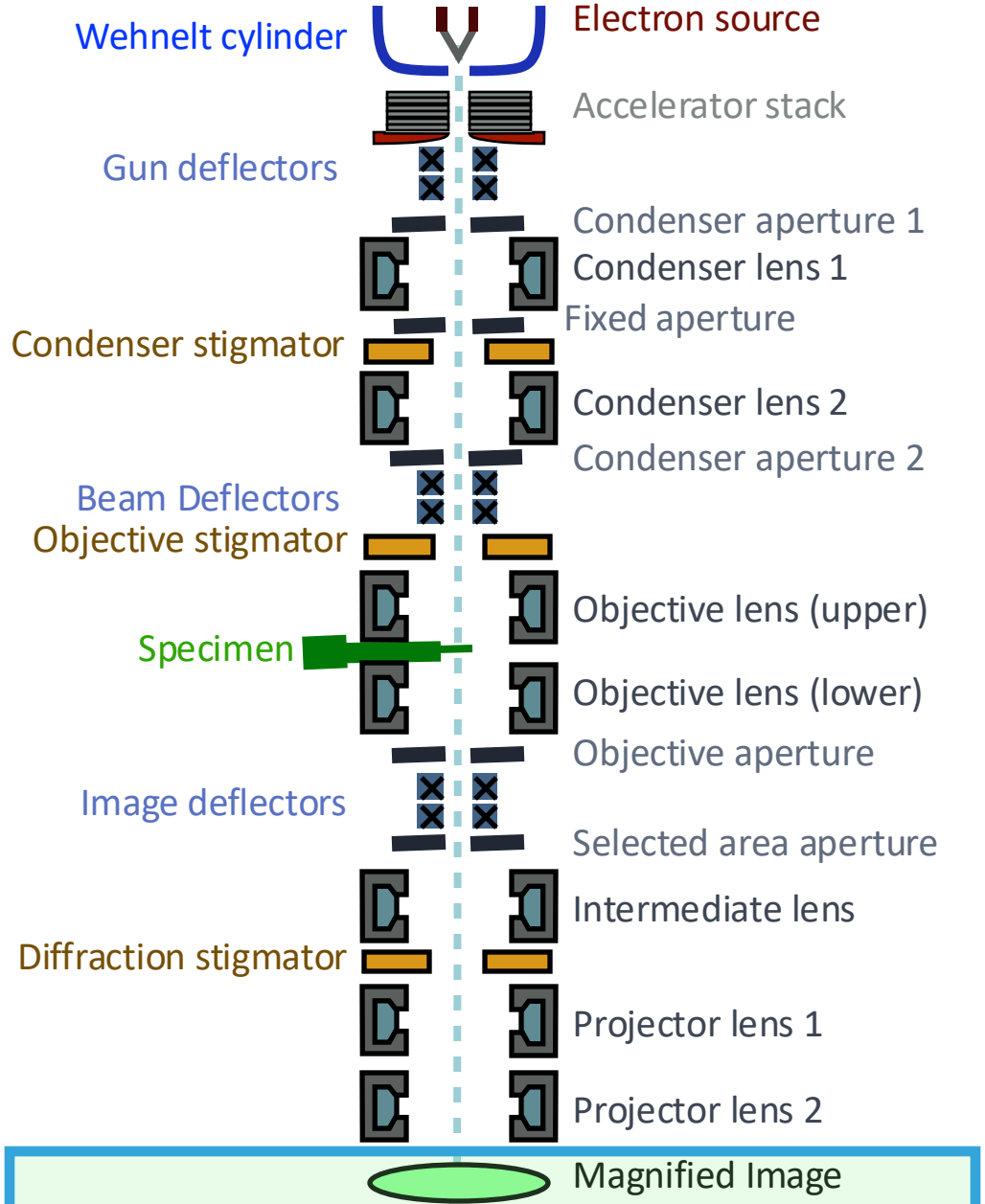
(Resolution)

Detectors – The biggest contributors to the “Resolution Revolution"



- Direct Electron Detectors based on the complementary metal oxide semiconductor (CMOS) came in early 2000s
- Very high signal-to-noise ratio (SNR) for primary electrons (>30:1) enables single electron counting.
- Excellent DQE ratios across resolutions combined with small pixel size (5-15µm)
- Radiation-hardened to withstand direct exposure to the electron beam. Resolution decreases with radiation damage.

Detectors – The biggest contributors to the “Resolution Revolution"



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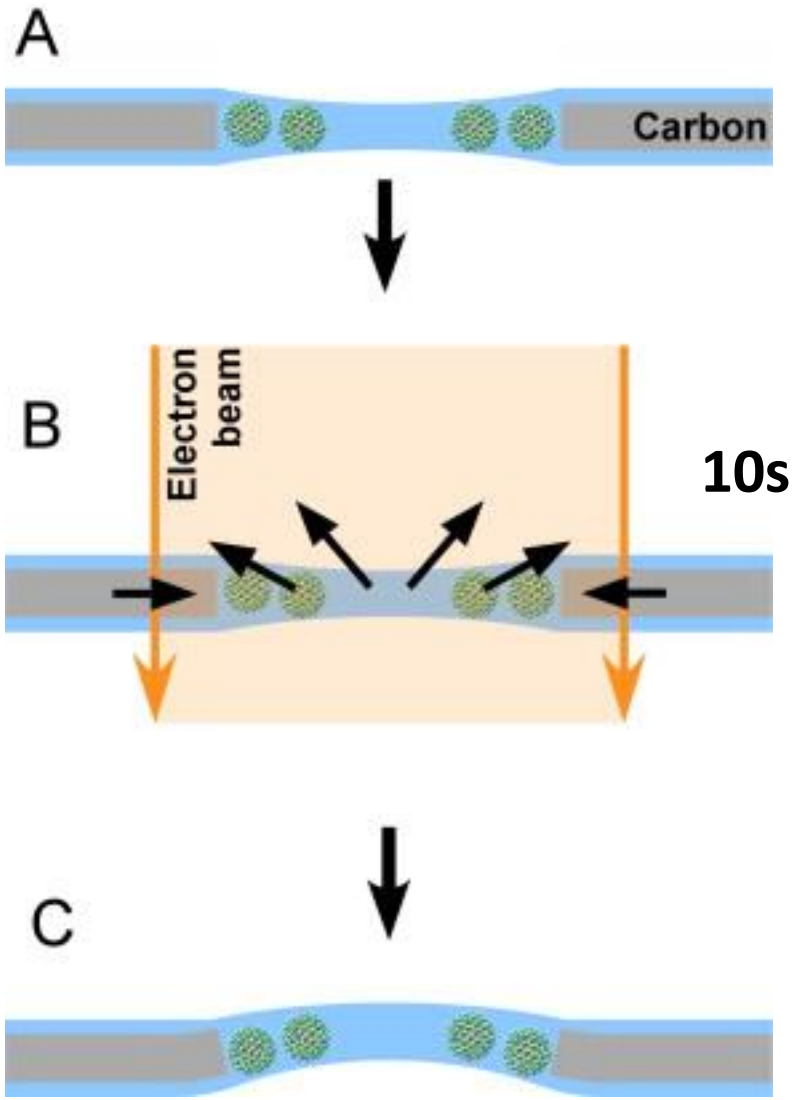
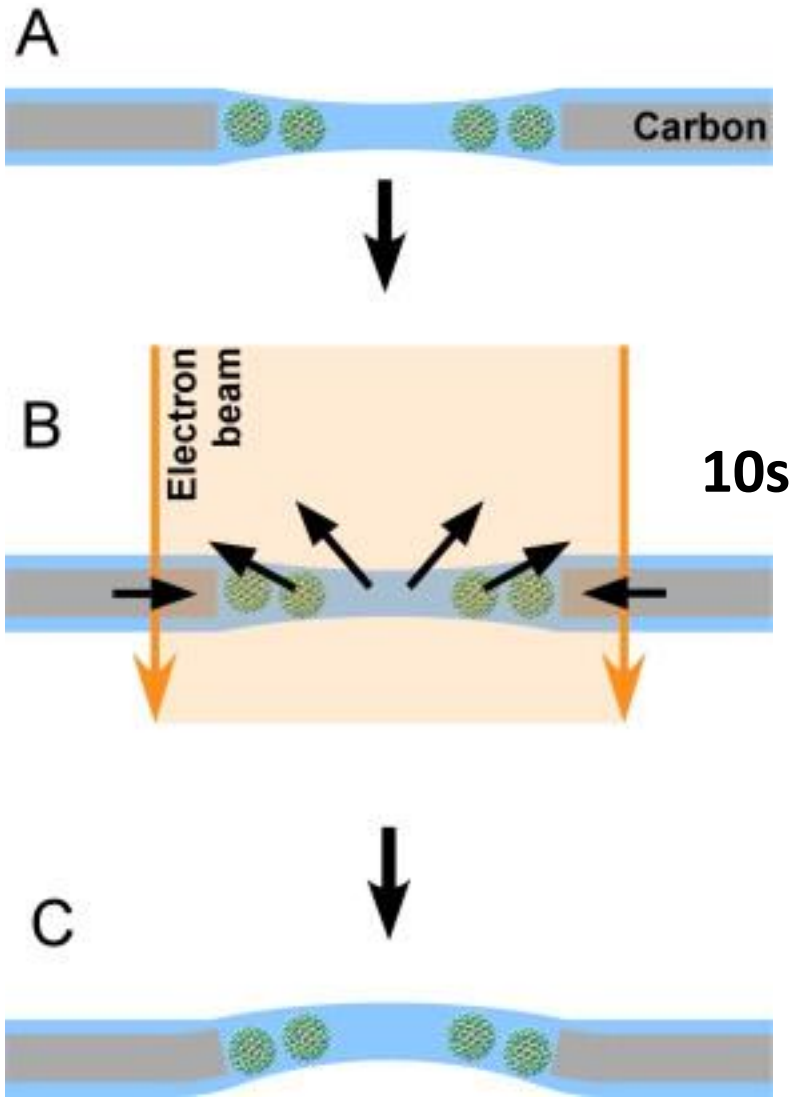
DED allow to correct for Beam-induced motion in EM



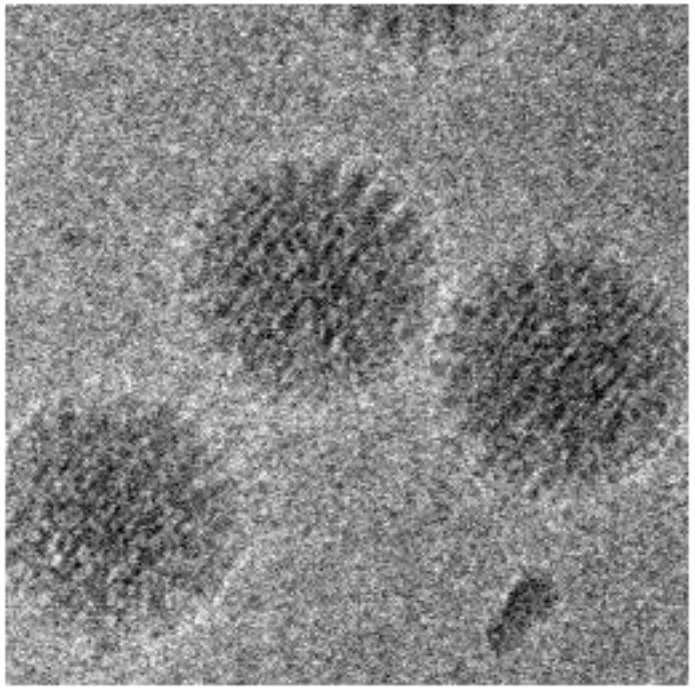
10s exposure -> 1 image

DED allow to correct for Beam-induced motion in EM

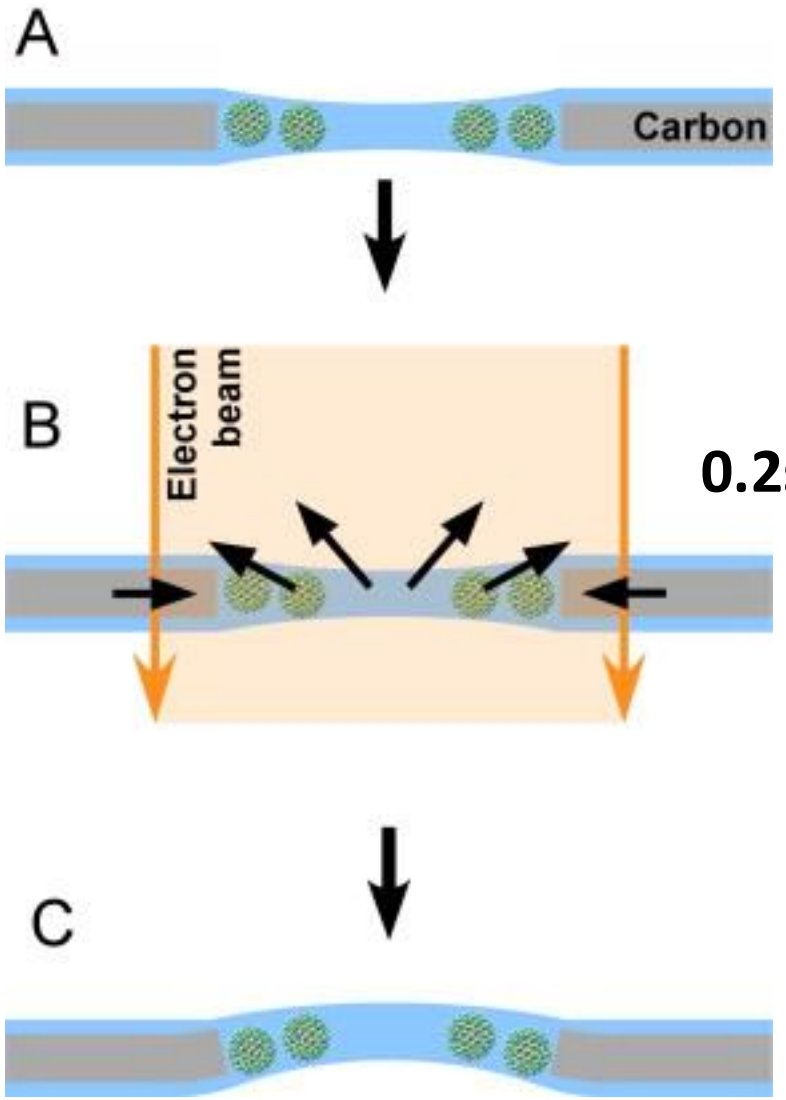


10s exposure ->

Blurry image – limited resolution

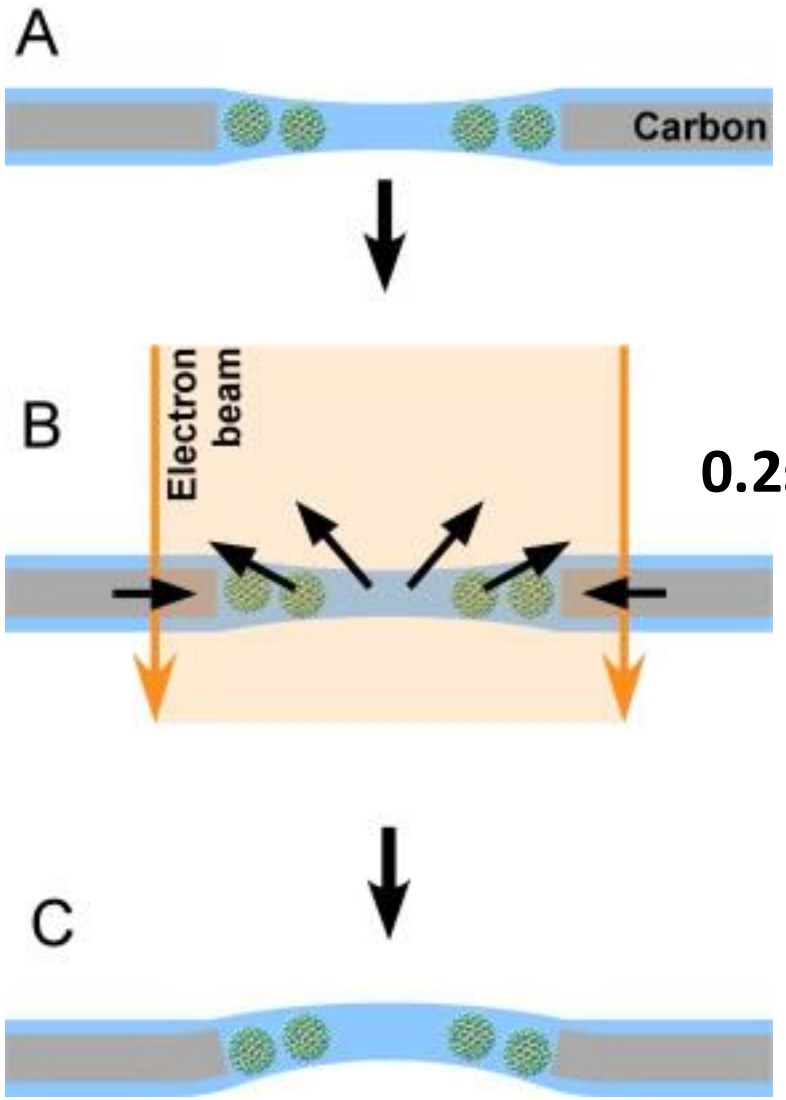


DED allow to correct for Beam-induced motion in EM



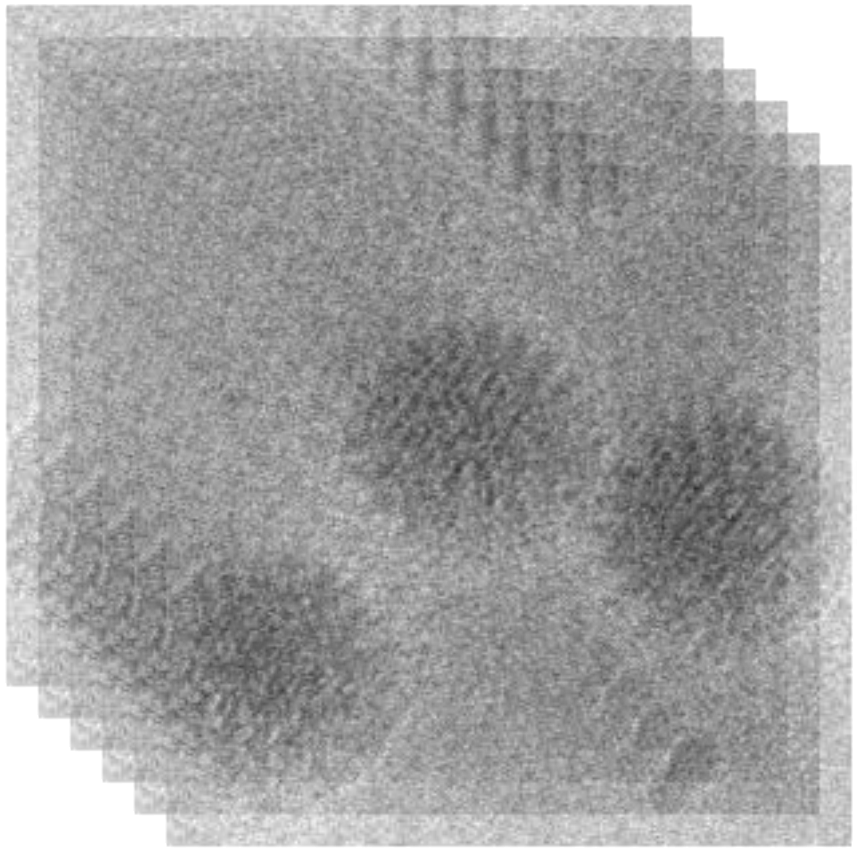
0.2s exposure -> 50 images

DED allow to correct for Beam-induced motion



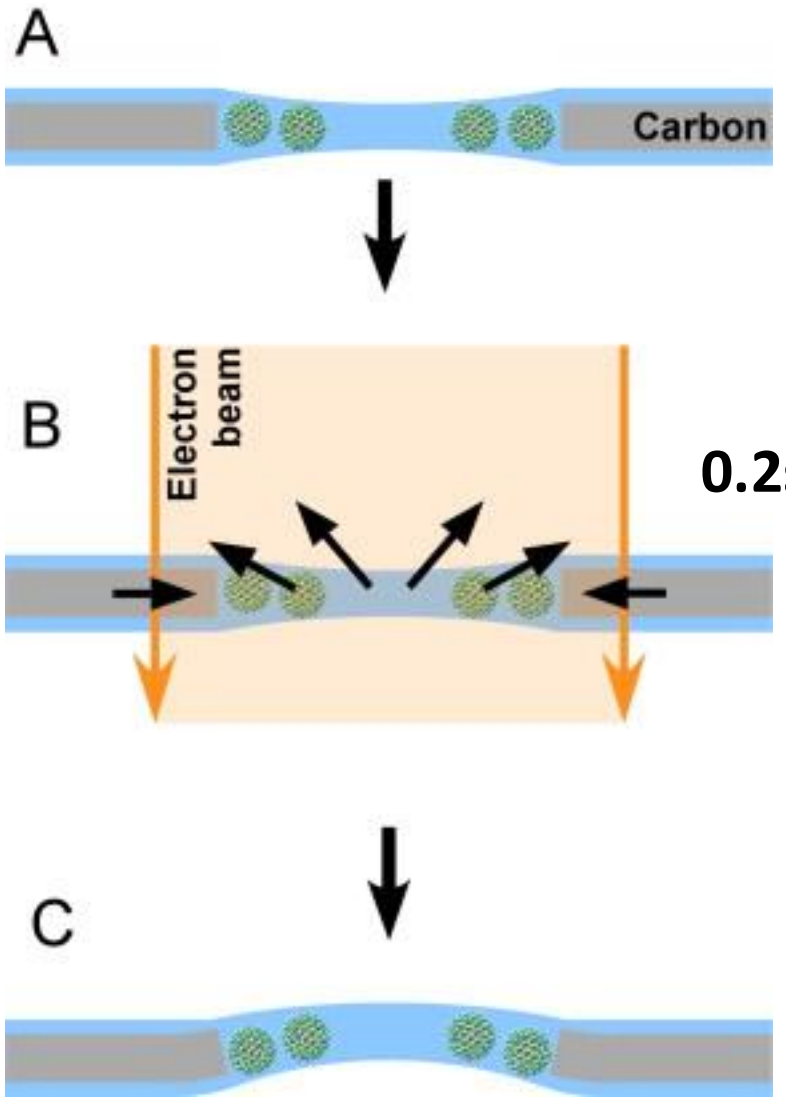
0.2s exposure ->

A stack of frames (movie)



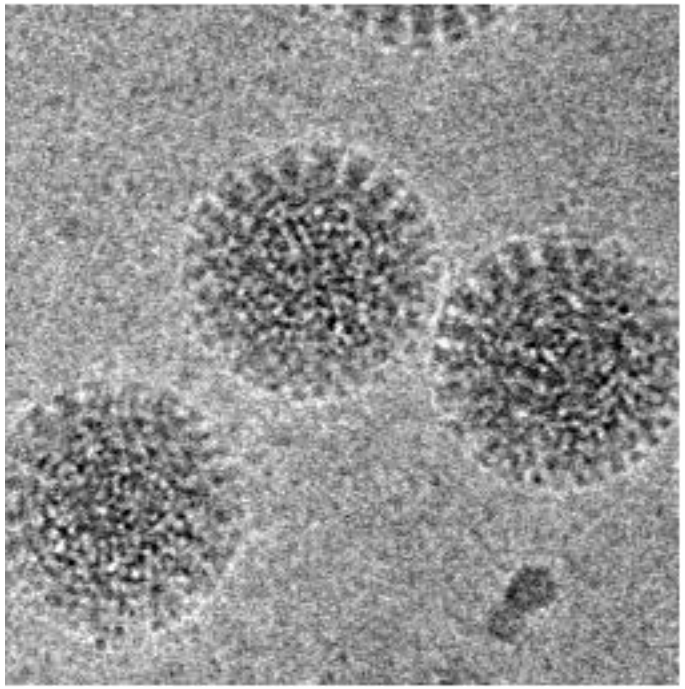
Allows to correct for beam induced motion

DED allow to correct for Beam-induced motion in EM

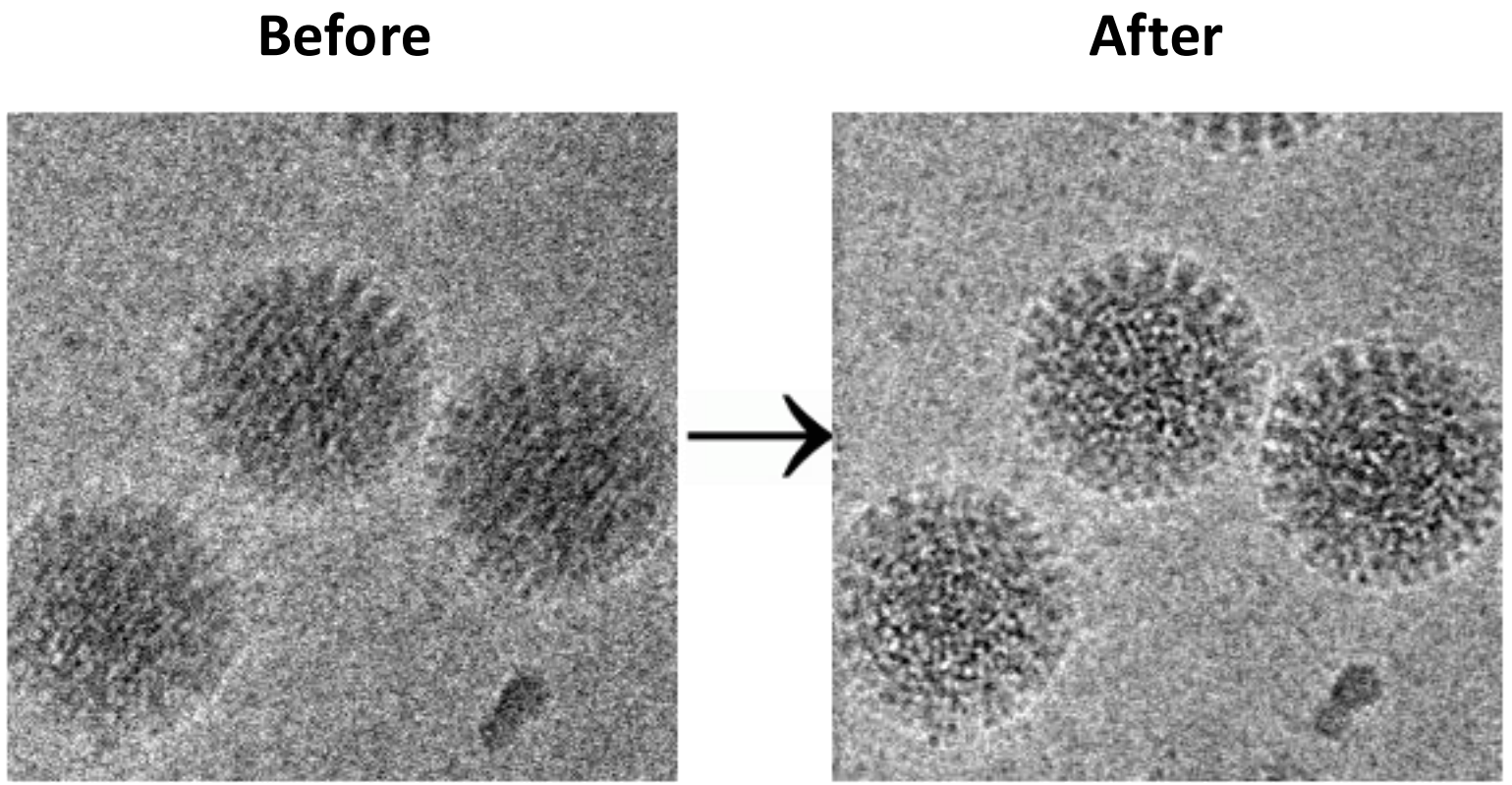


0.2s exposure ->

Sharp image – high resolution



DED allow to correct for Beam-induced motion in EM

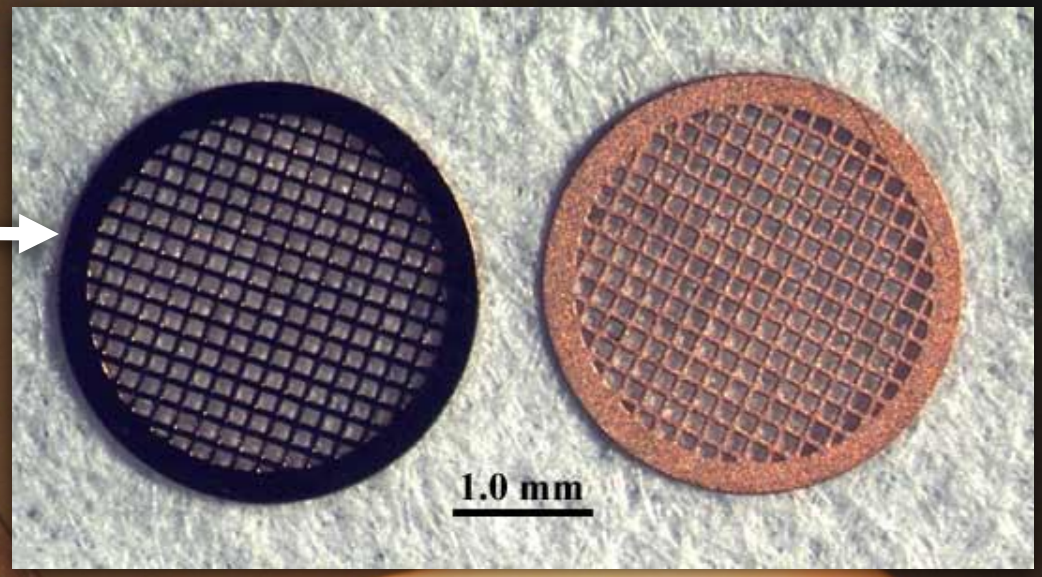


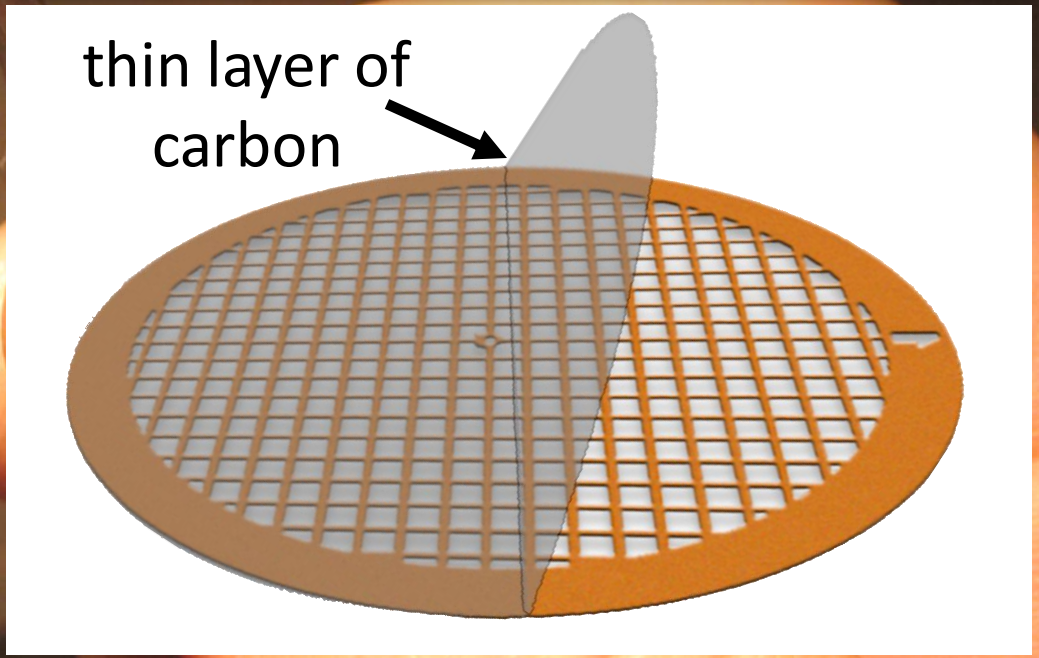
Sample preparation

EM GRIDS

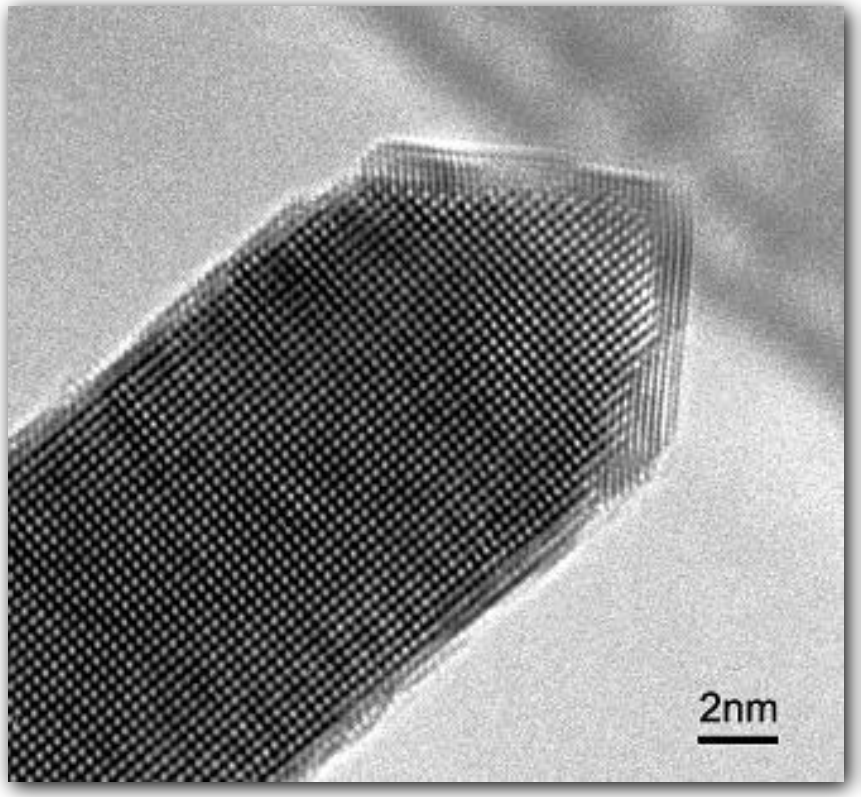
Grid materials:

Copper
Gold
Nickel
Titanium
Silicon
CuRh
Molybdenum
Aluminum
Tungsten

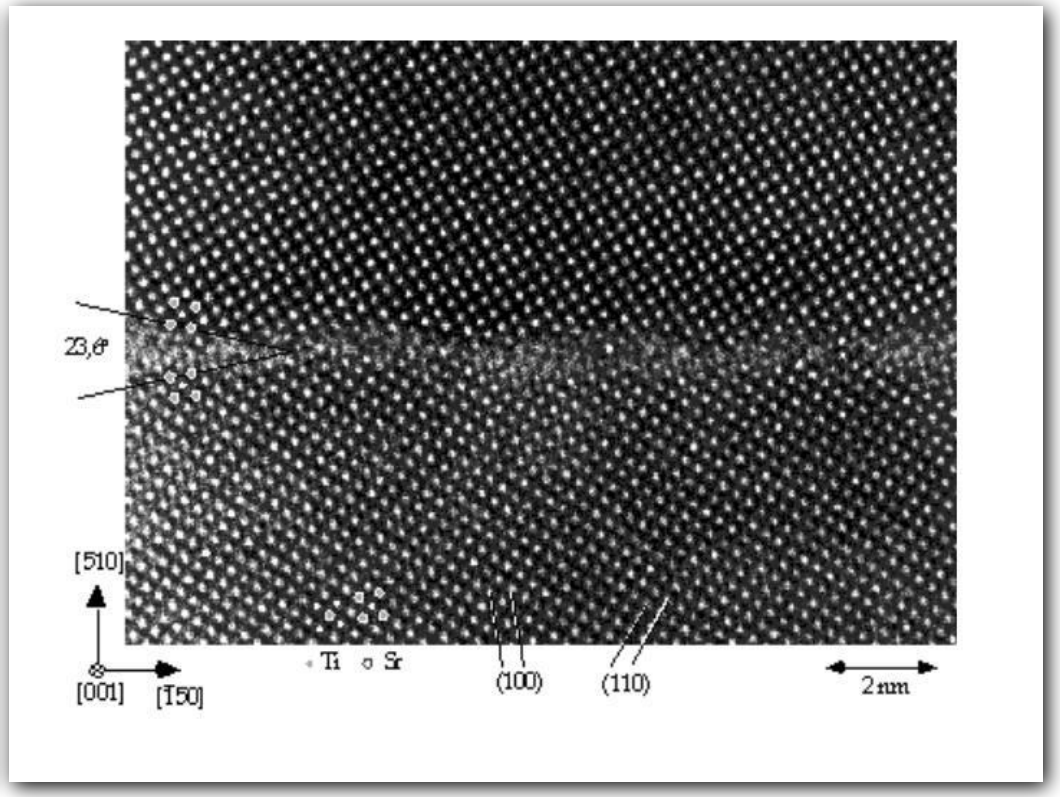




Atomic resolution in EM micrographs



RuO_2 nanorod



$SrTiO_3$ film

resolution: ~2Å

Why aren't all EM structures of biological samples at this resolution?

Preserving Biological Specimens for EM Imaging

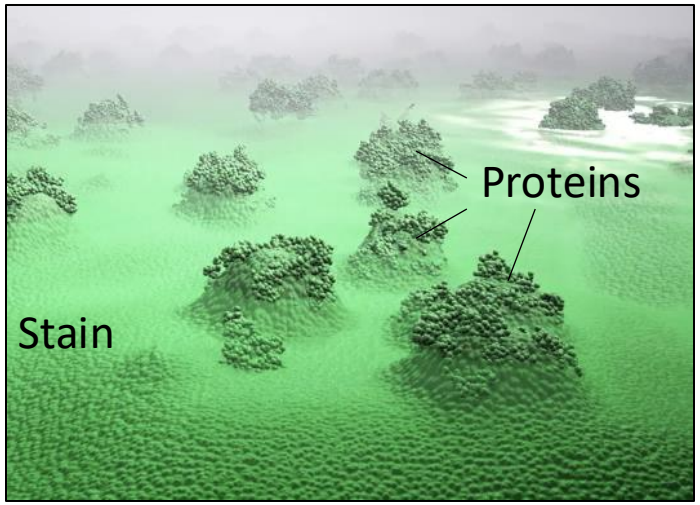
- Biological samples are about 80% water
  - Placing biological samples into the vacuum of electron microscope will cause immediate dehydration and destruction of sample
  - Need to preserve ultrastructure to withstand vacuum (e.g., by freezing or staining)
- Biological samples are susceptible to radiation damage
  - Conversion of electron beam flux to radiation dose:
    $1 Coulomb/m^2$ @100keV = 40Mrad ($4x10^7$ rad)
  - To view samples in EM, typically use $30C/m^2/sec$ (1200Mrad/sec)

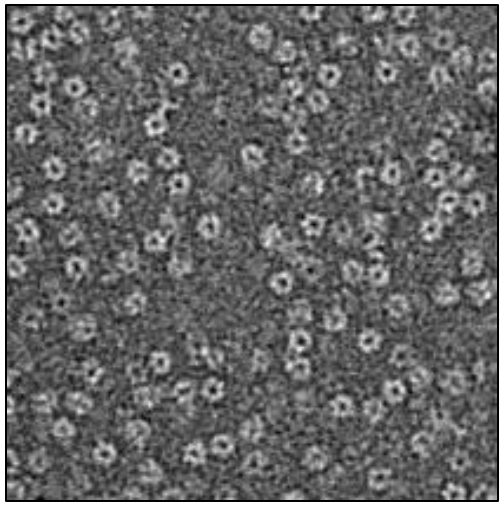
Equivalent to standing 30m from a 10Mt H-bomb explosion

Examples of methods for sample application on EM grids

• Samples are applied onto EM grids to assure optimal contrast, mechanical support while imaging and protection from radiation damage. There are 2 main approaches:

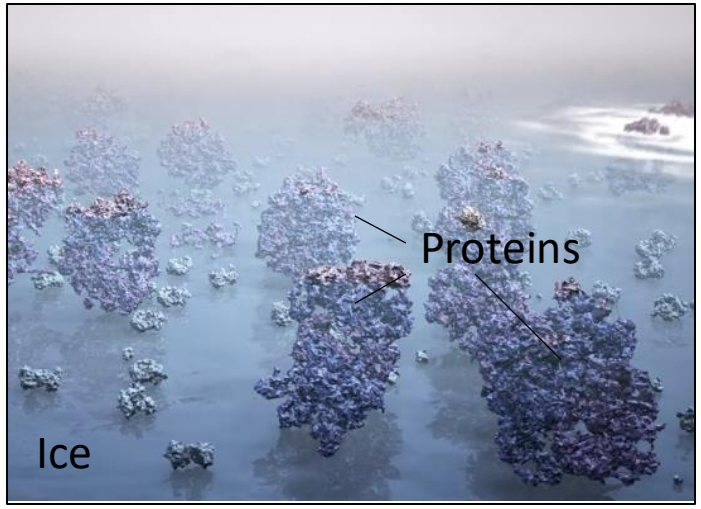
Negative staining

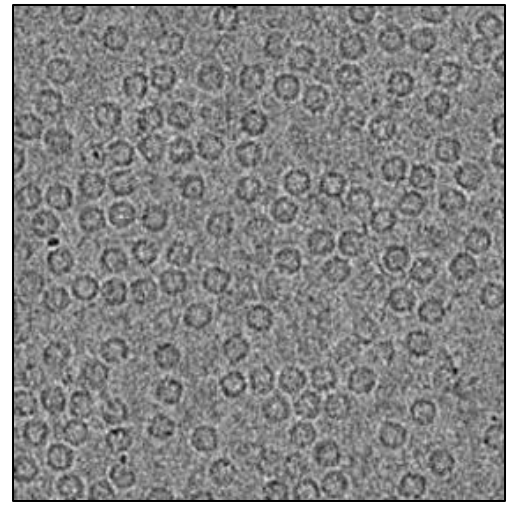




- Staining agents are heavy nuclei (uranium, tungsten)
- Straightforward and quick to prepare
- Excellent **sample preservation in a dried form**
- Contrast enhancing due to stain
- Low-resolution information only (~10Å)

Sample vitrification





- Sample vitrified in a thin layer of ice
- More challenging for preparation and handling
- Excellent **sample preservation in a frozen form**
- Contrast generated by the molecules themselves
- Can achieve atomic resolution after processing

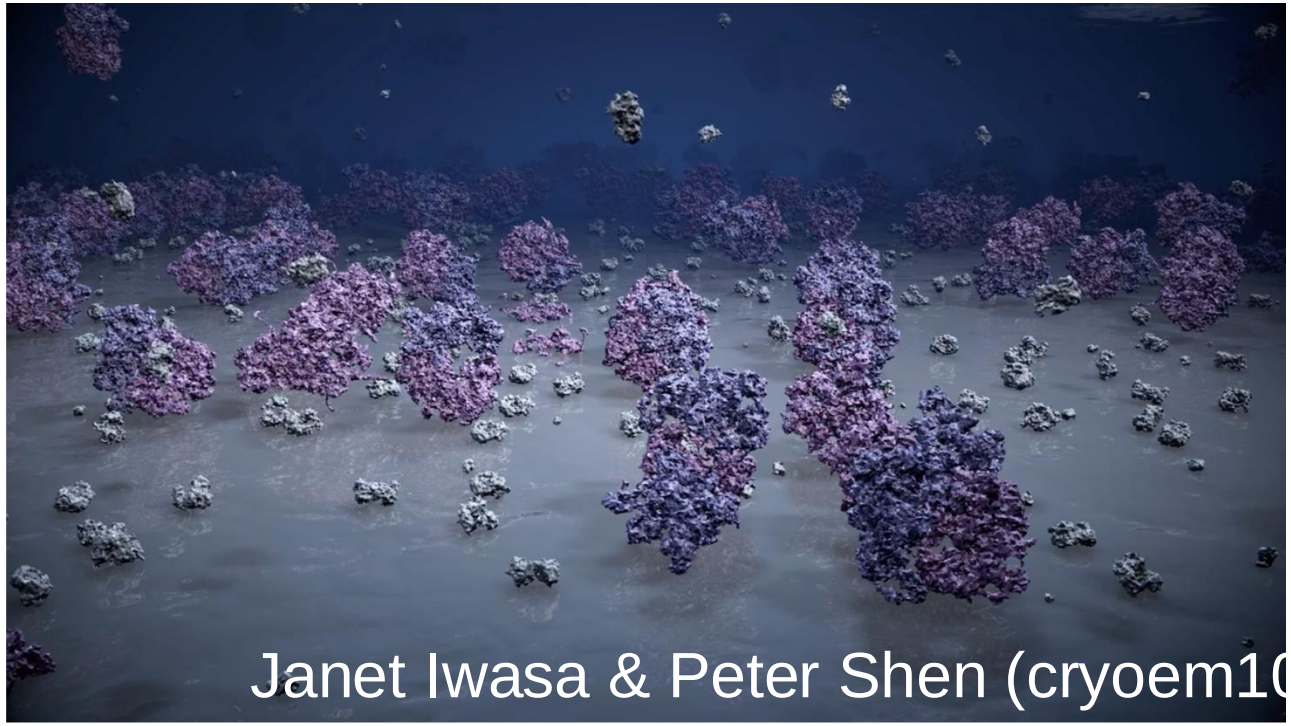
Preparing Biological Specimens for EM Imaging by freezing

- Mean free path of 300 keV electrons through biological sample ~280 nm.

Preparing Biological Specimens for EM Imaging by freezing

- Mean free path of 300 keV electrons through biological sample ~280 nm.

- A 3 µl drop is ~100,000 times too thick!

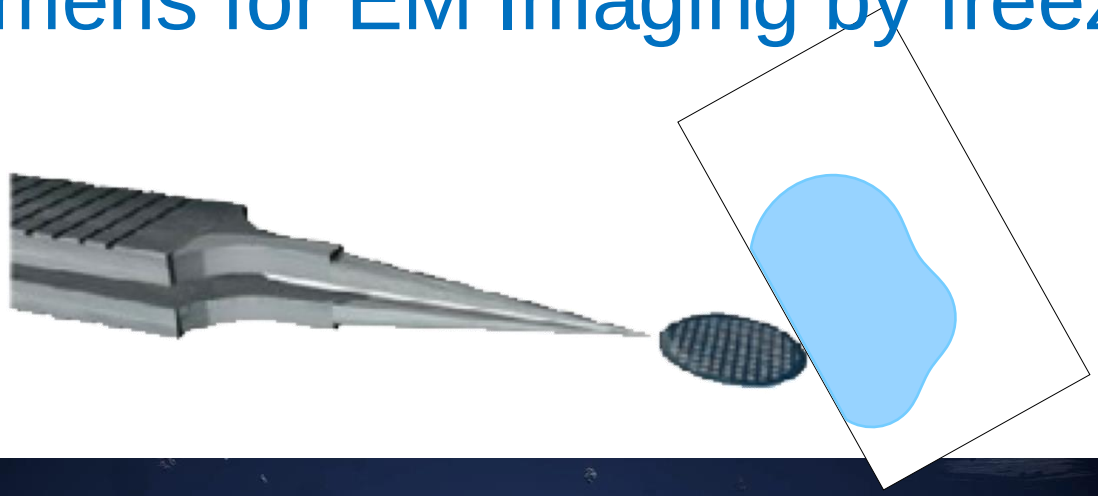


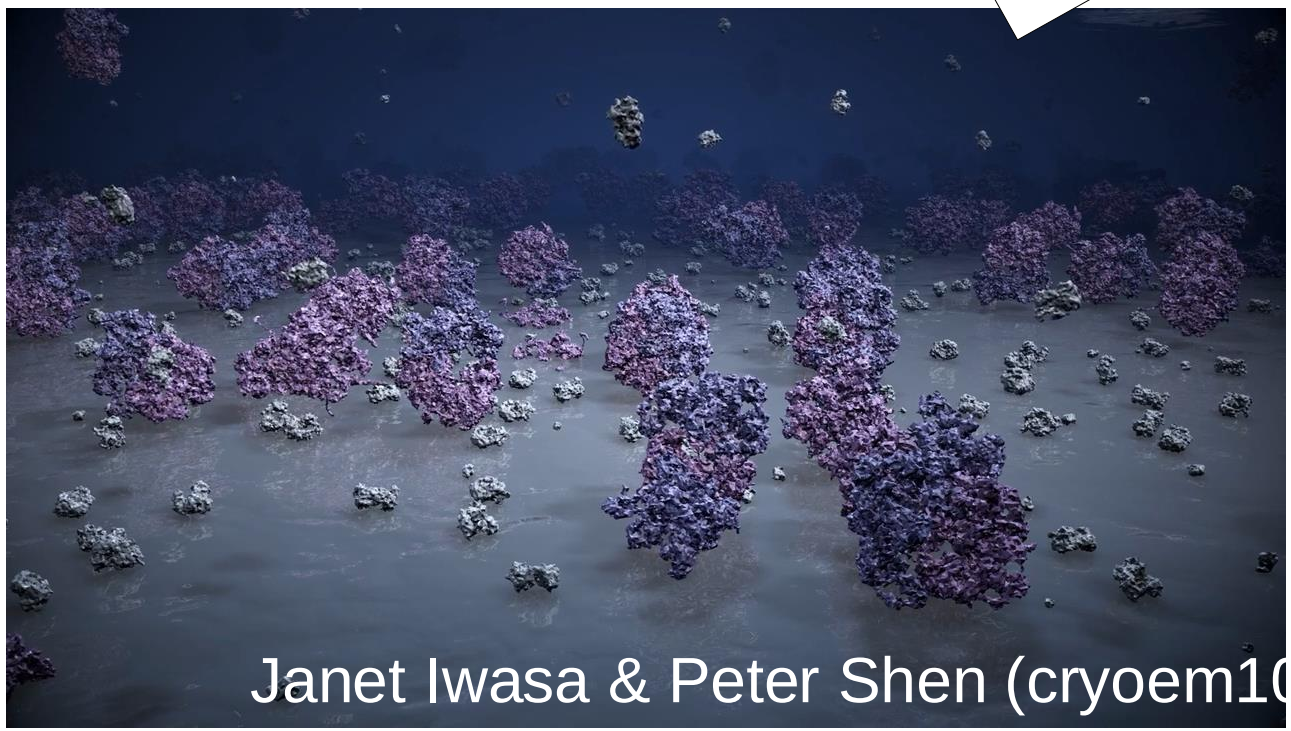
Preparing Biological Specimens for EM Imaging by freezing

- Mean free path of 300 keV electrons through biological sample ~280 nm.

- A 3 µl drop is ~100,000 times too thick!

- Excess sample can be blotted-off with a filter paper and then frozen

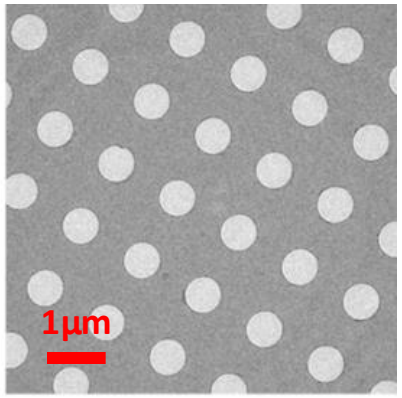




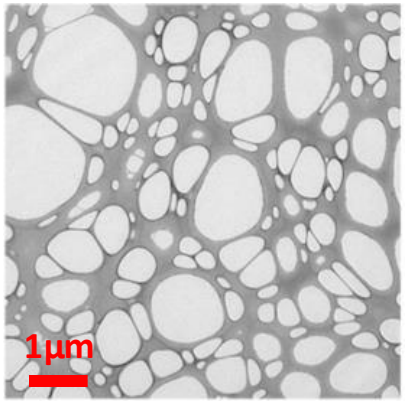
Vitrification of samples on cryoEM grids

• The carbon support film for grids is commonly perforated with holes where biomolecules are get captured in a thin layer of ice. The thickness of carbon typically varies between 10-200nm.

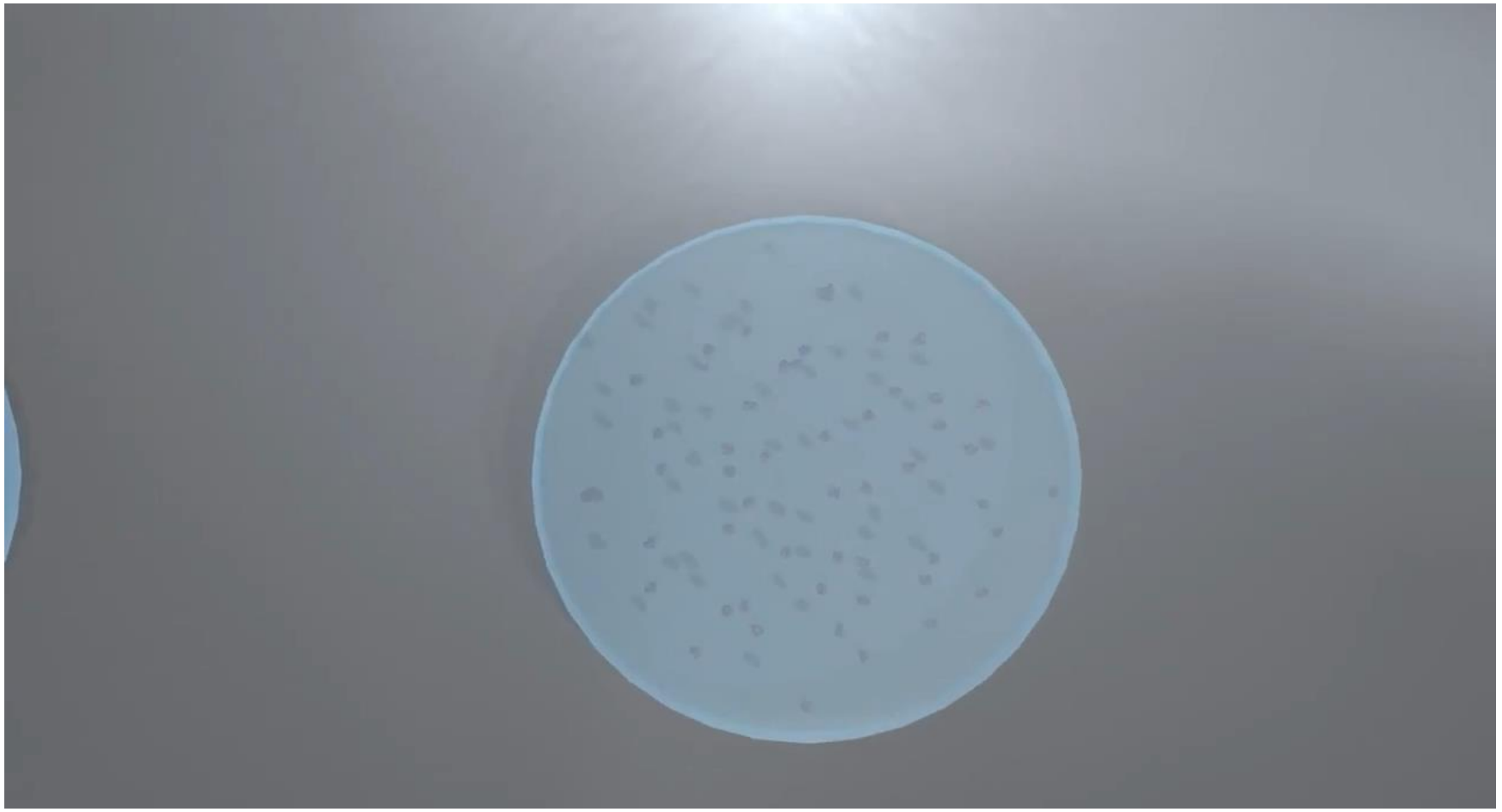
Holey carbon



Lacey carbon



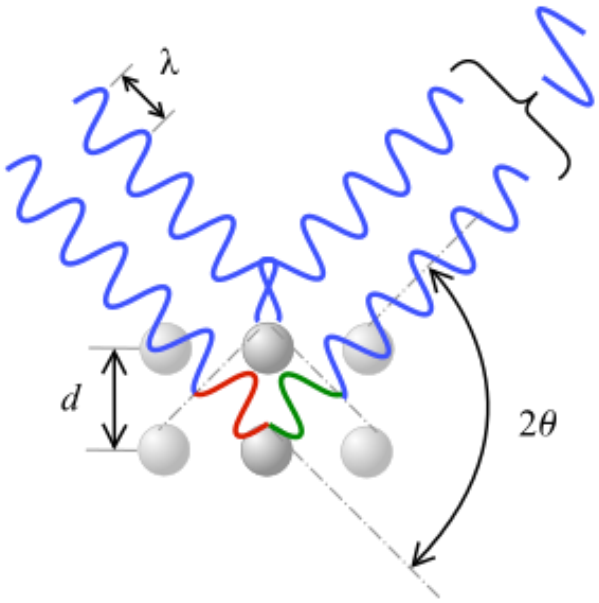
Holey carbon grid with vitrified sample



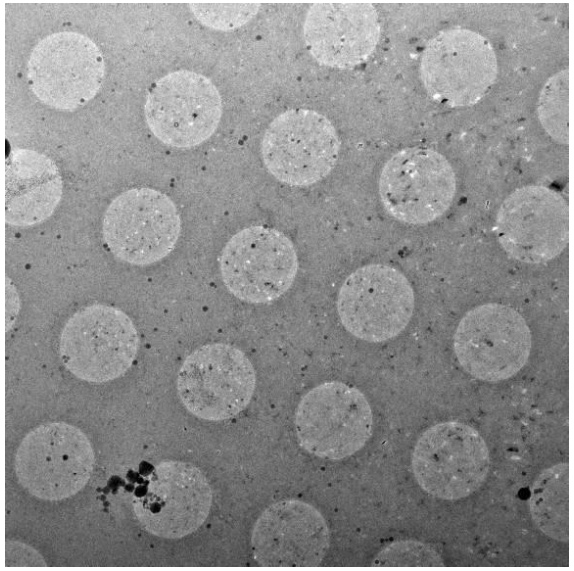
Grid freezing also assures that the layer of ice has the necessary mechanical stability

Slow freezing leads to formation of crystalline ice

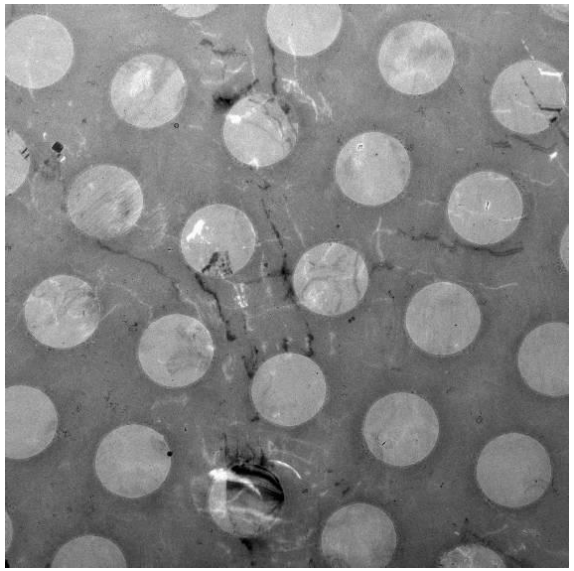
- Crystalline ice results in image defects due to electron diffraction effects
- Image defects are dependent on the type of ice

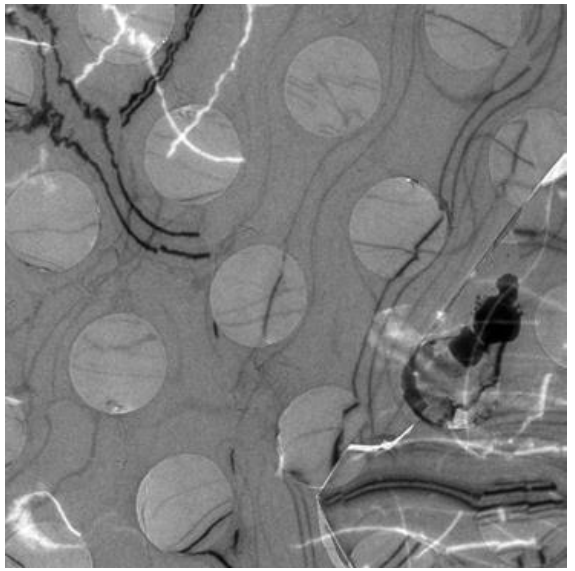


Cubic Ice



Hexagonal Ice

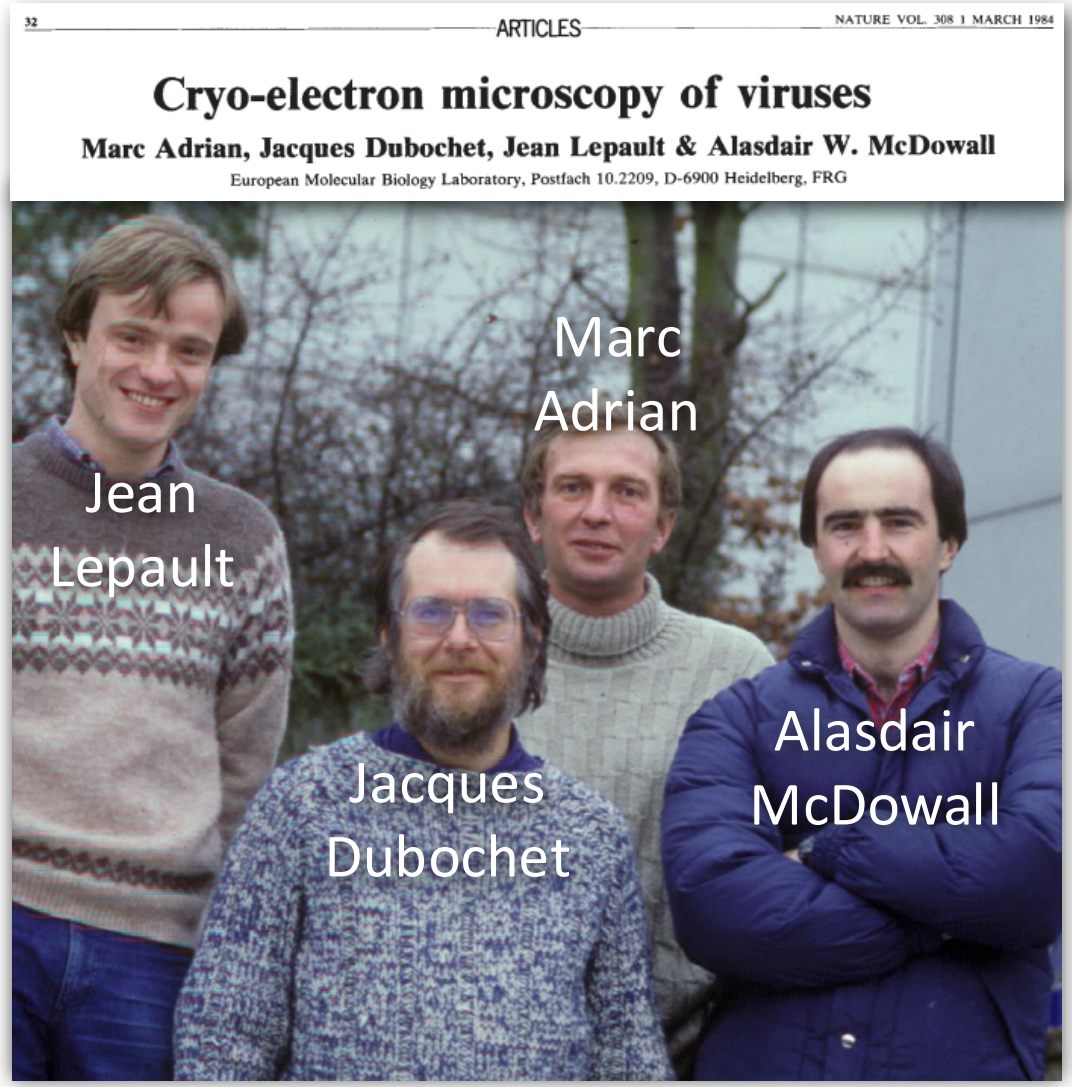




- For a few decades this was a major problem in the field until…

images courtesy Matthijn Vos, Thermo Fisher Scientific

Big breakthrough – Method for vitrification of biological specimens



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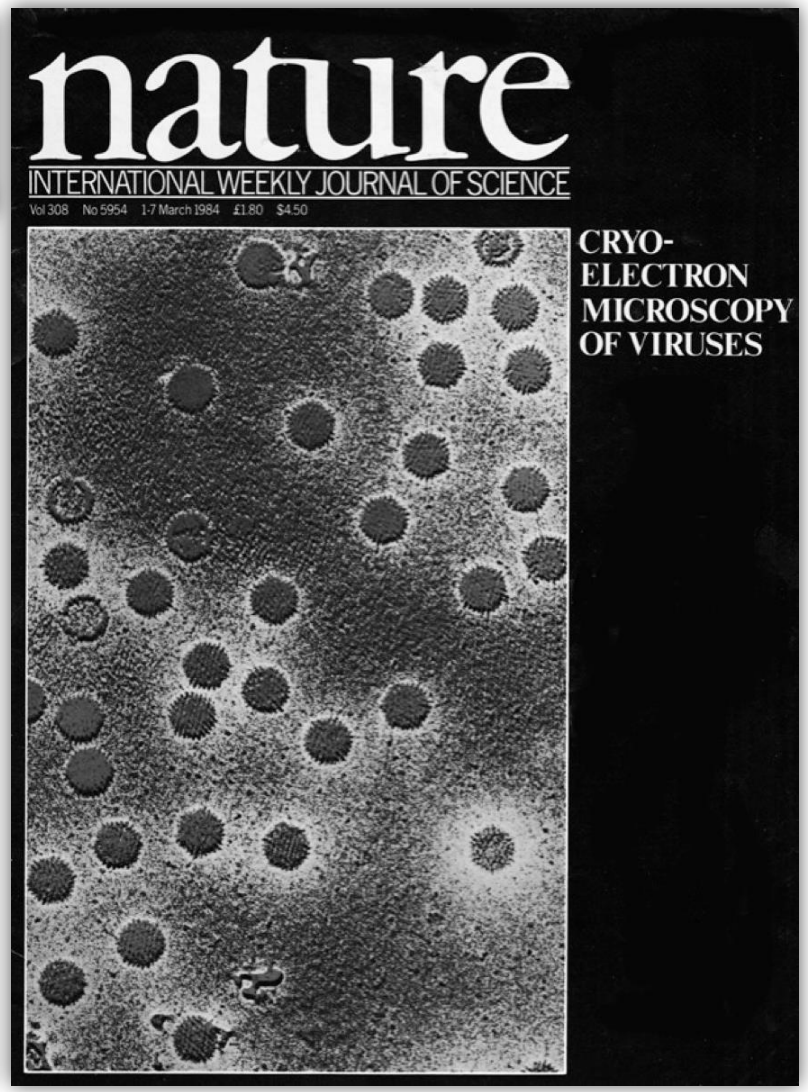
Cryo-electron microscopy of viruses

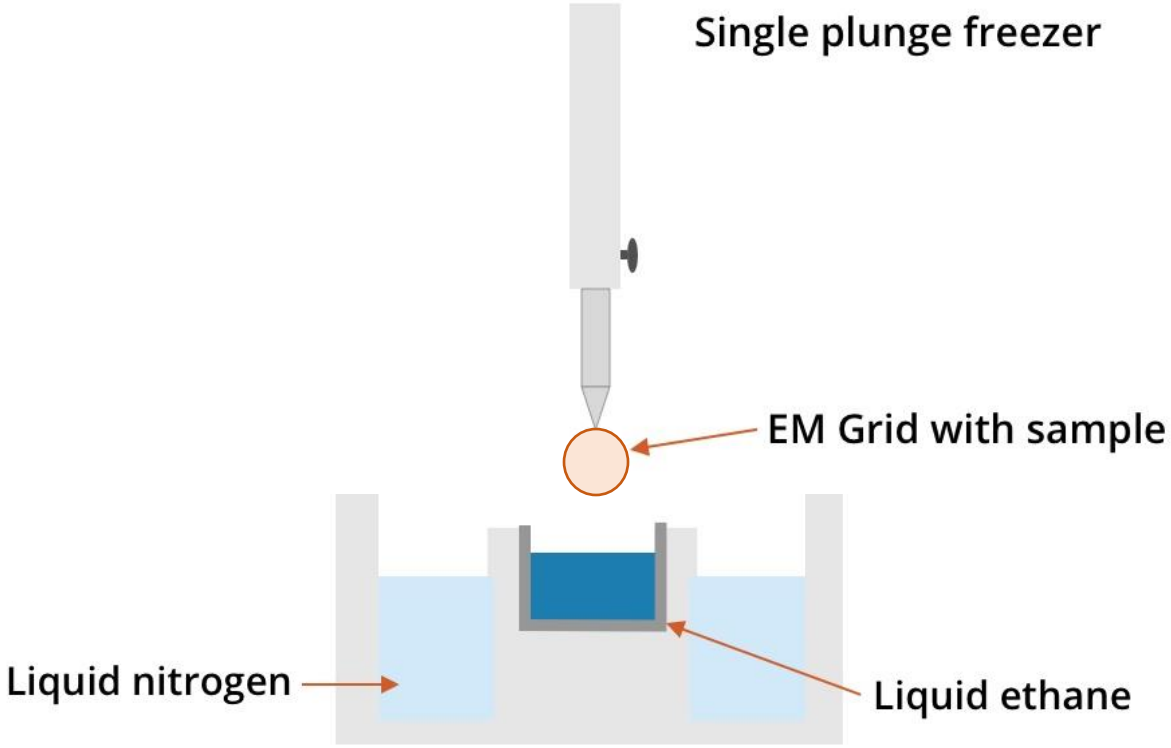
Marc Adrian, Jacques Dubochet, Jean Lepault & Alasdair W. McDowall

European Molecular Biology Laboratory, Postfach 10.2209, D-6900 Heidelberg, FRG

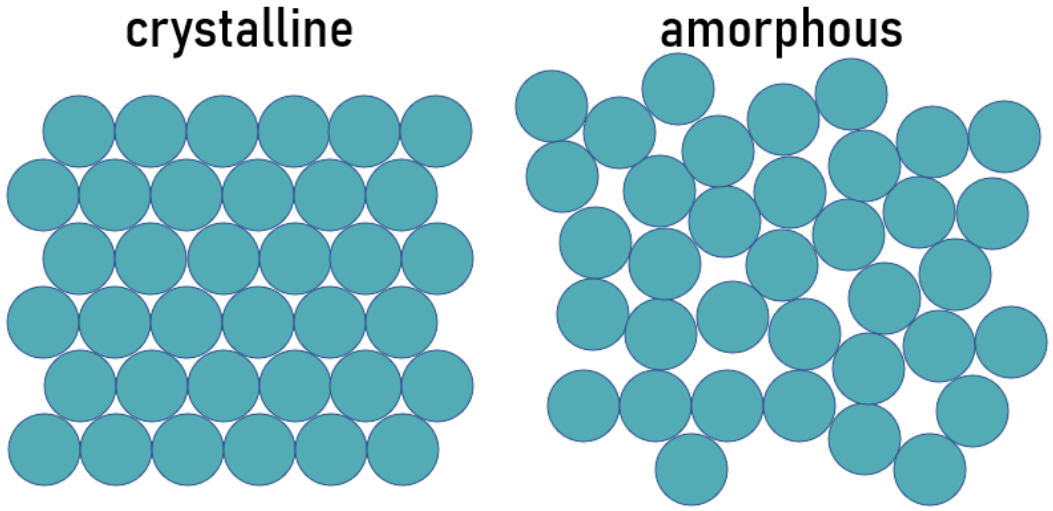


Plunge-freezing method (the “cryo” part of cryoEM)

- Ethane has ~5 times higher heat capacity (Cp) than liquid nitrogen. It takes ~5x more heat to change its temperature by 1˚C
- Ethane is liquid at temperatures just slightly above those of liquid nitrogen (its melting point is -188 °C)
- Therefore, liquid ethane is cold enough to vitrify water quickly, while not boiling off in the process.



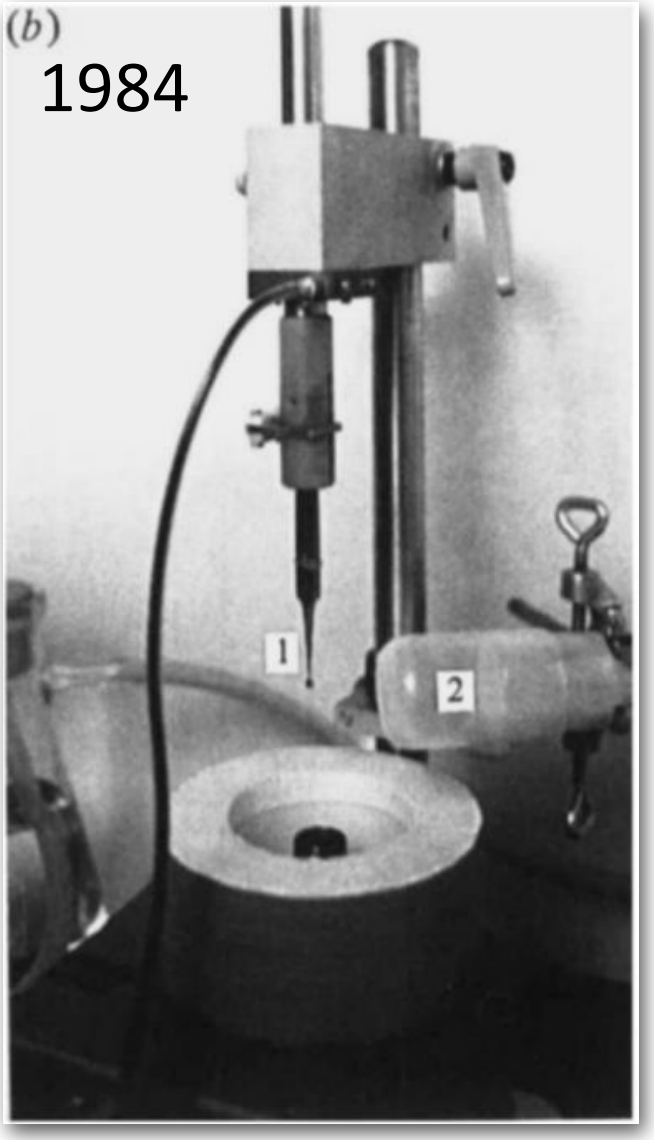
Rapid vitrification can reduce cristalline ice formation



Slow freezing

Rapid freezing

One of the original designs by Jacques Dubochet



Plunge-freezing devices – User assisted

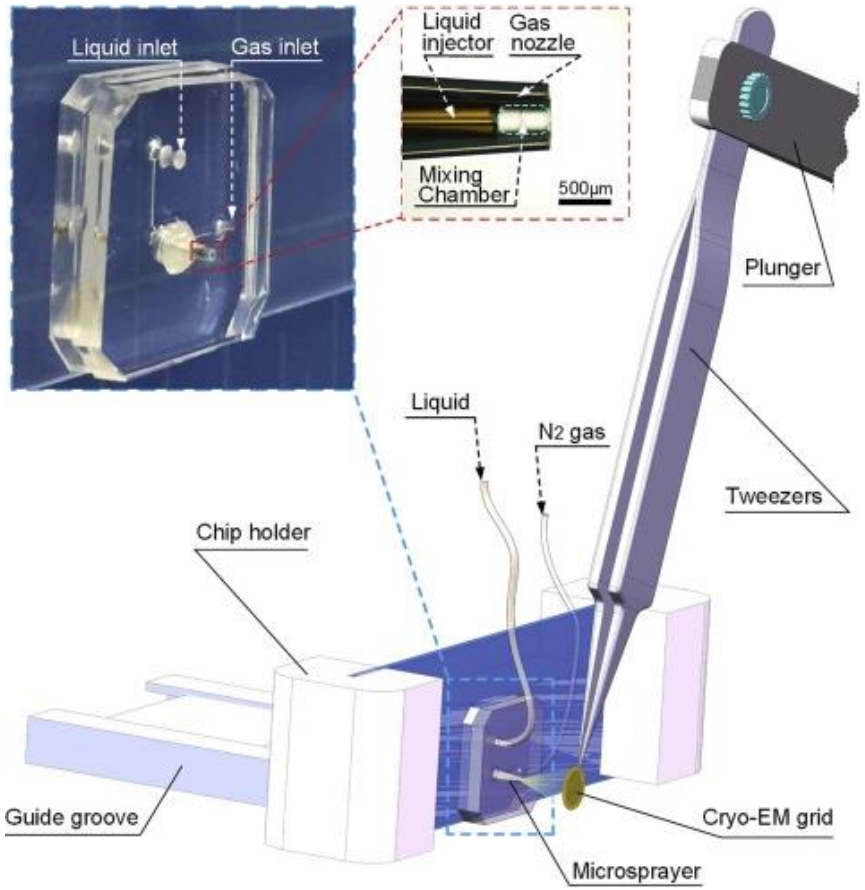
FEI Vitrobot

Gatan Cryoplunge

Leica EM GP

Allows to control the inside the device atmosphere, blotting force, blotting time

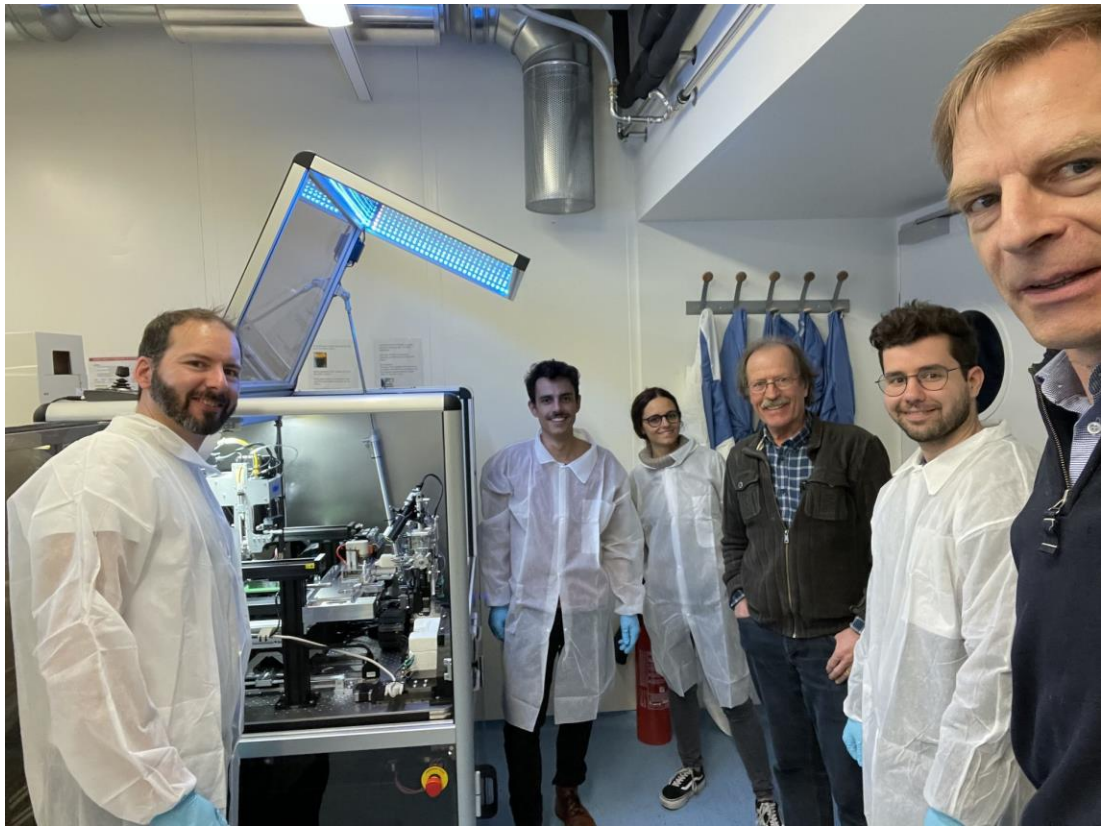
Fully-automated sample application and vitrification systems



Project Spotiton

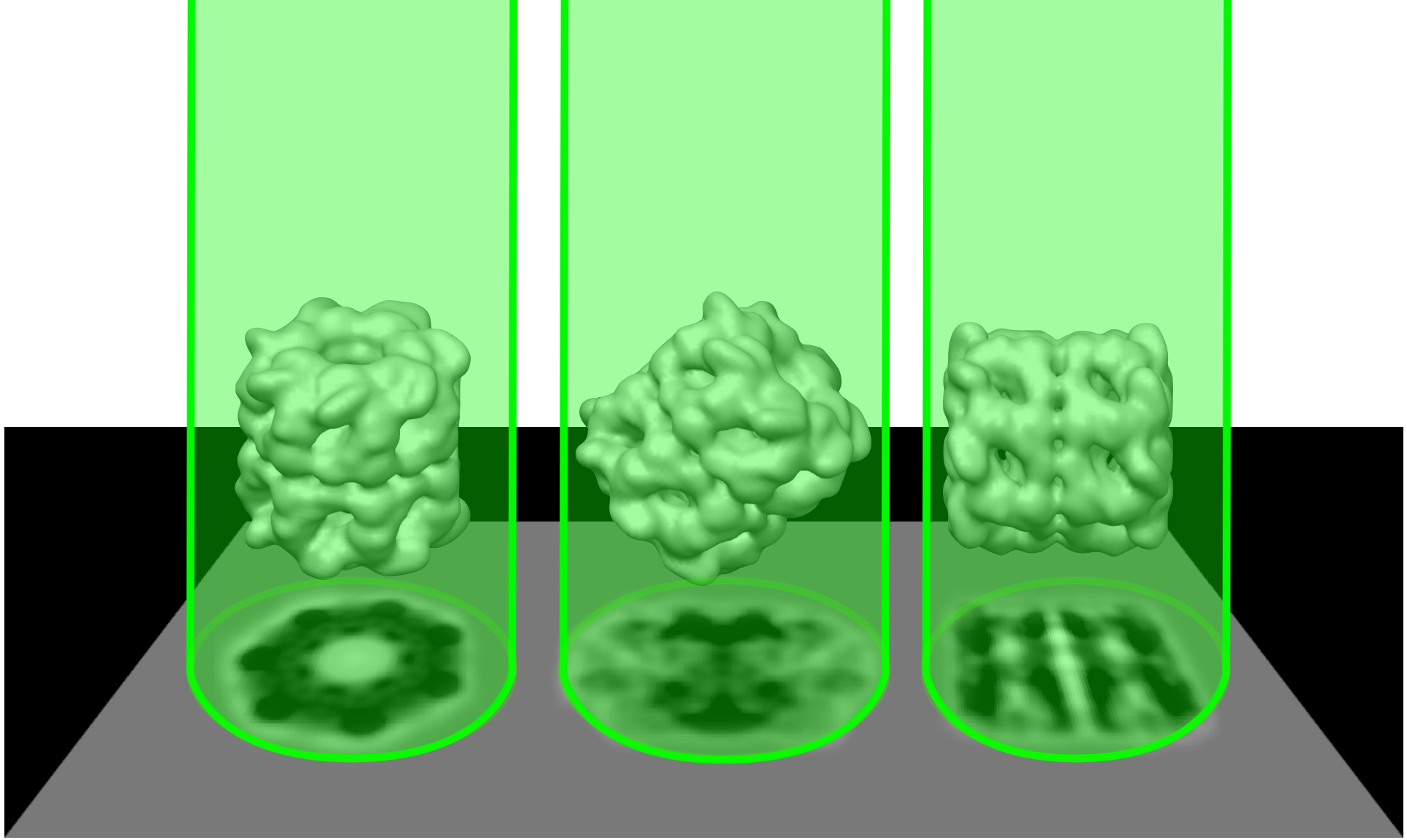
Feng et al., 2017

Stahlberg Lab (EPFL/UniL)

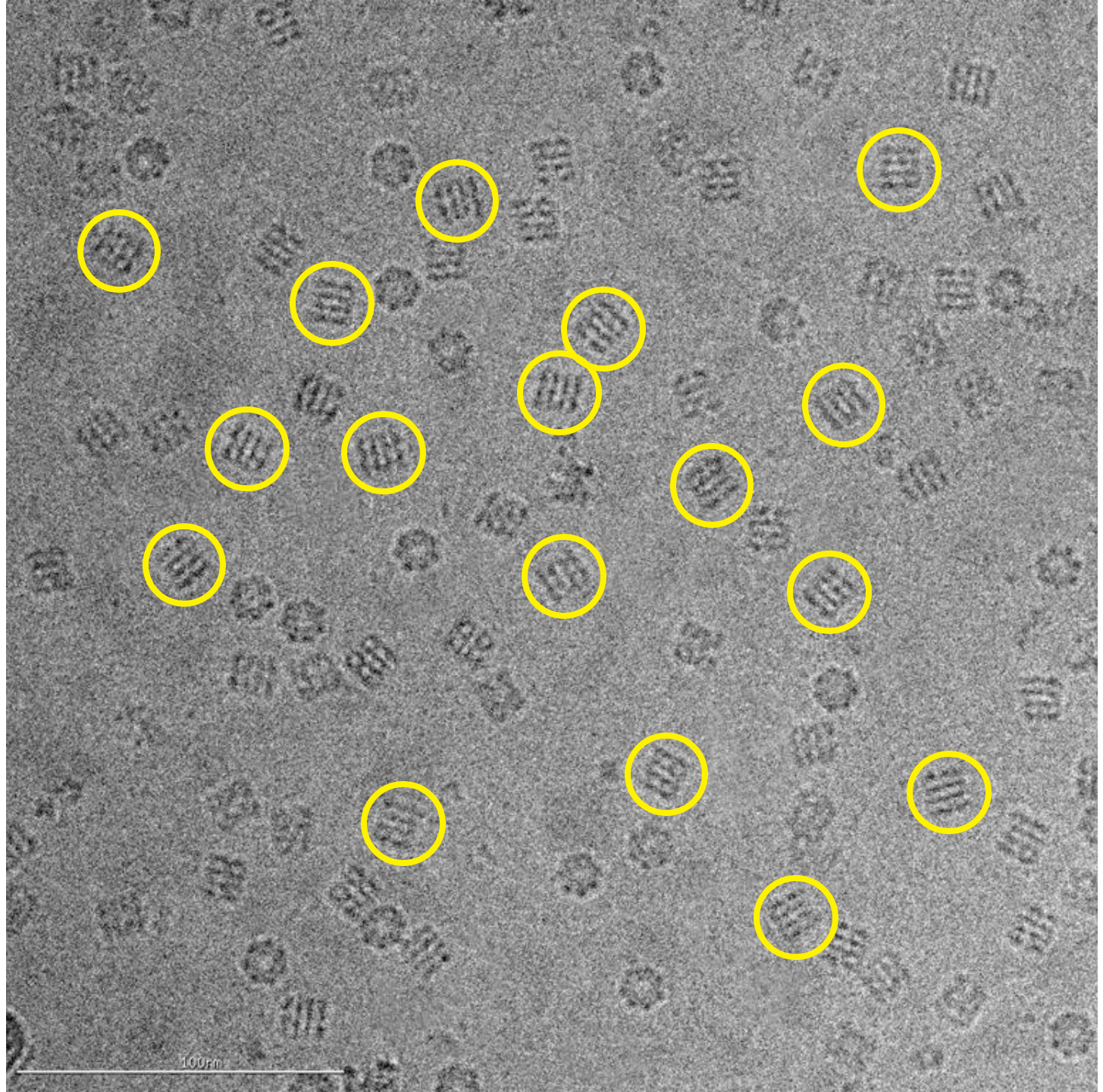


cryoWriter

Rima et al., 2022



In cryoEM the contrast is generated by the particles themselves

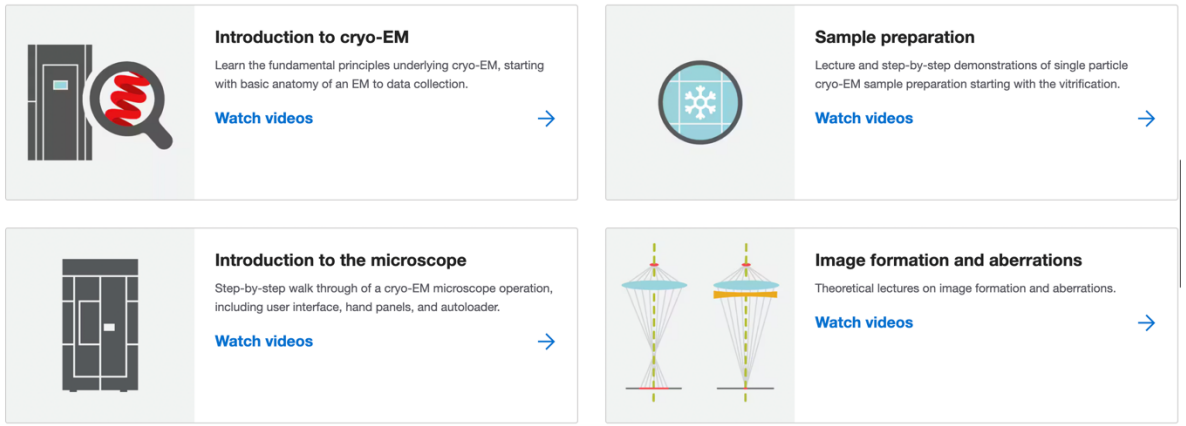

100nm

The main topics/questions from today's lecture

- How does electron microscopy compares to NMR and X-ray crystallography?
- Why are electrons advantageous over X-rays for biomolecular microscopy?
- What are the basic components of an electron microscope?
- What is the difference between scanning and transmission EM?
- How are electrons produced in a microscope? How are they focused?
- What are direct electron detectors?
- Which technological developments lead to the "resolution revolution"?
- How are biological samples applied onto electron microscopy grids?
- What is the purpose of sample vitrification?
- What is the difference between negative-stain and cryoEM imaging?

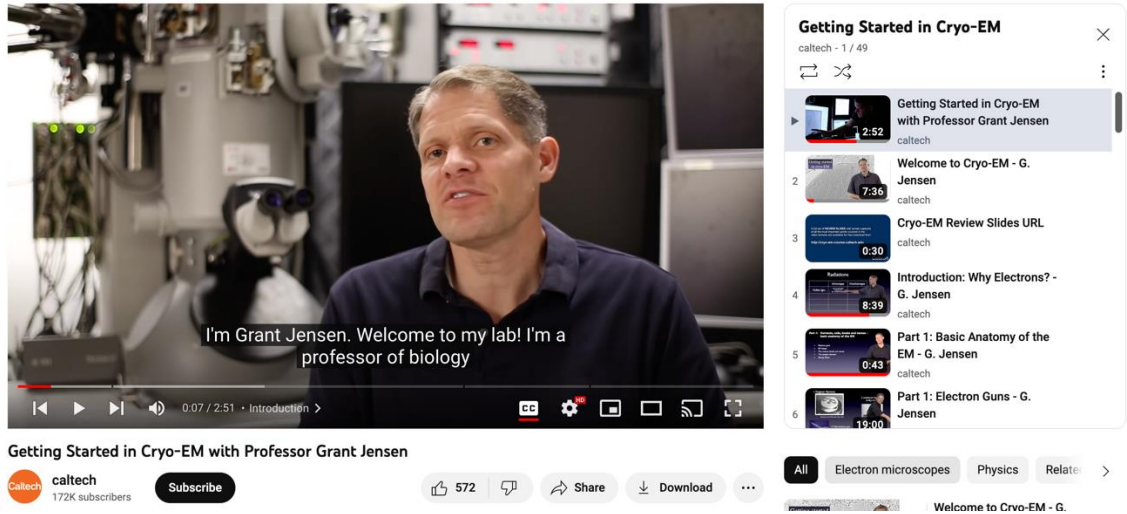
Learning resources

Cryo-EM University



https://www.thermofisher.com/ch/en/home/electron-microscopy/life-sciences/learning-center/cryo-em-university.html

Grant Jensen Lectures



https://cryo-em-course.caltech.edu/