MUNI
SCI

C8116 Immunochemical techniques

Electron microscopy

Spring term 2025

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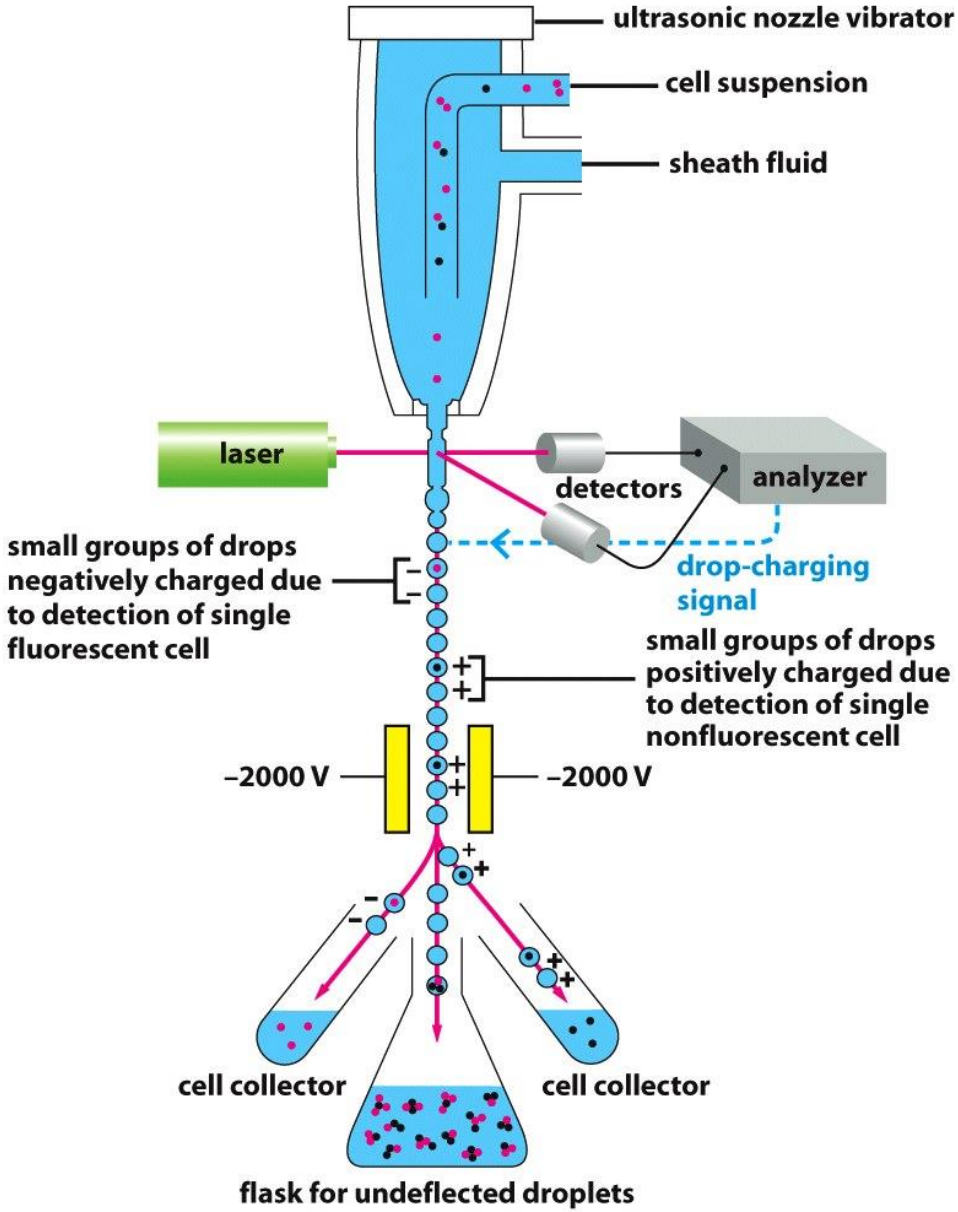
Fluorescence activated cell sorting (FACS)



Different types of luminescent nanoparticles

- Polymer nanoparticles
- Silica nanoparticles

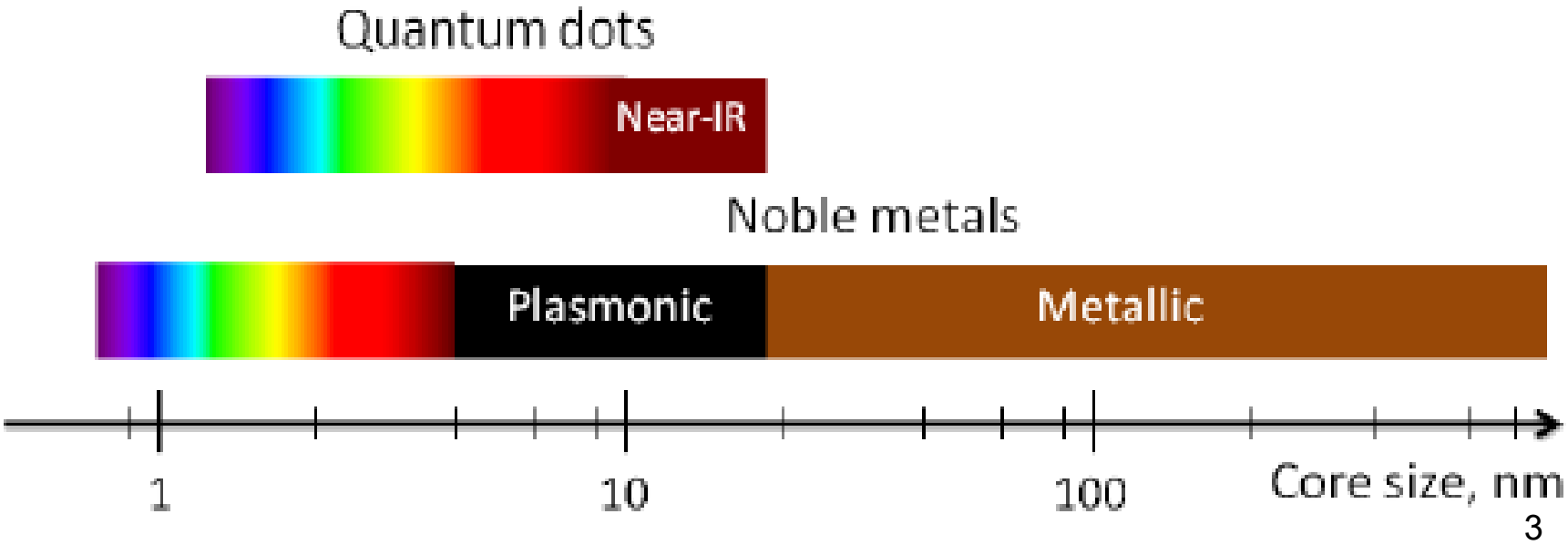
} based on organic fluorophores

- Quantum dots (QDs)
- Metallic nanoparticles

} size-dependent luminescence

- Photon-upconversion nanoparticles (UCNPs)

} based on nanomaterial properties



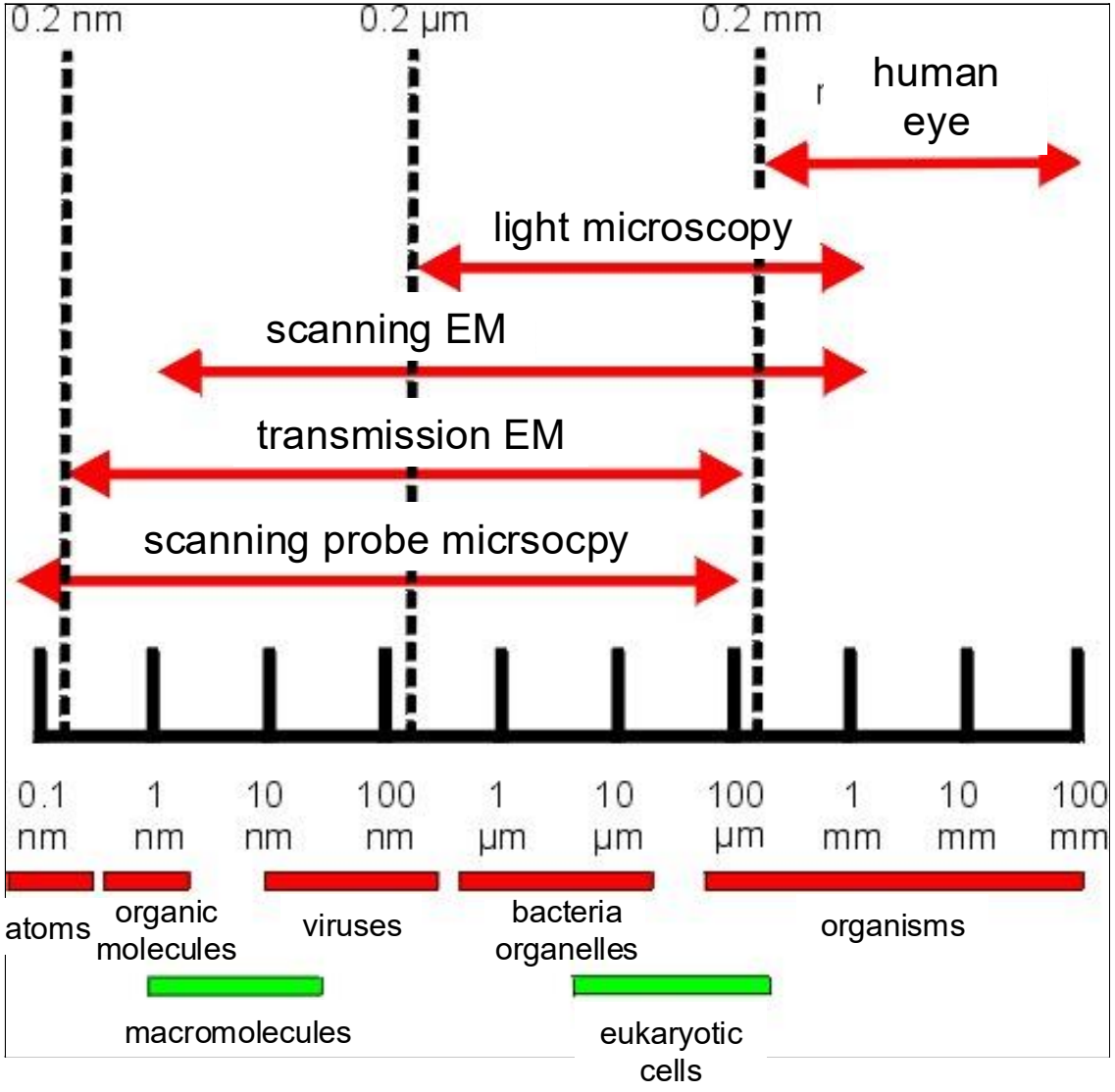
A.P. Demchenko, Methods Appl. Fluoresc. (2013) 1: 022001

Electron Microscopy (EM)

Transmission Electron Microscopy (TEM)

Scanning Electron Microscopy (SEM)

What we can “see“

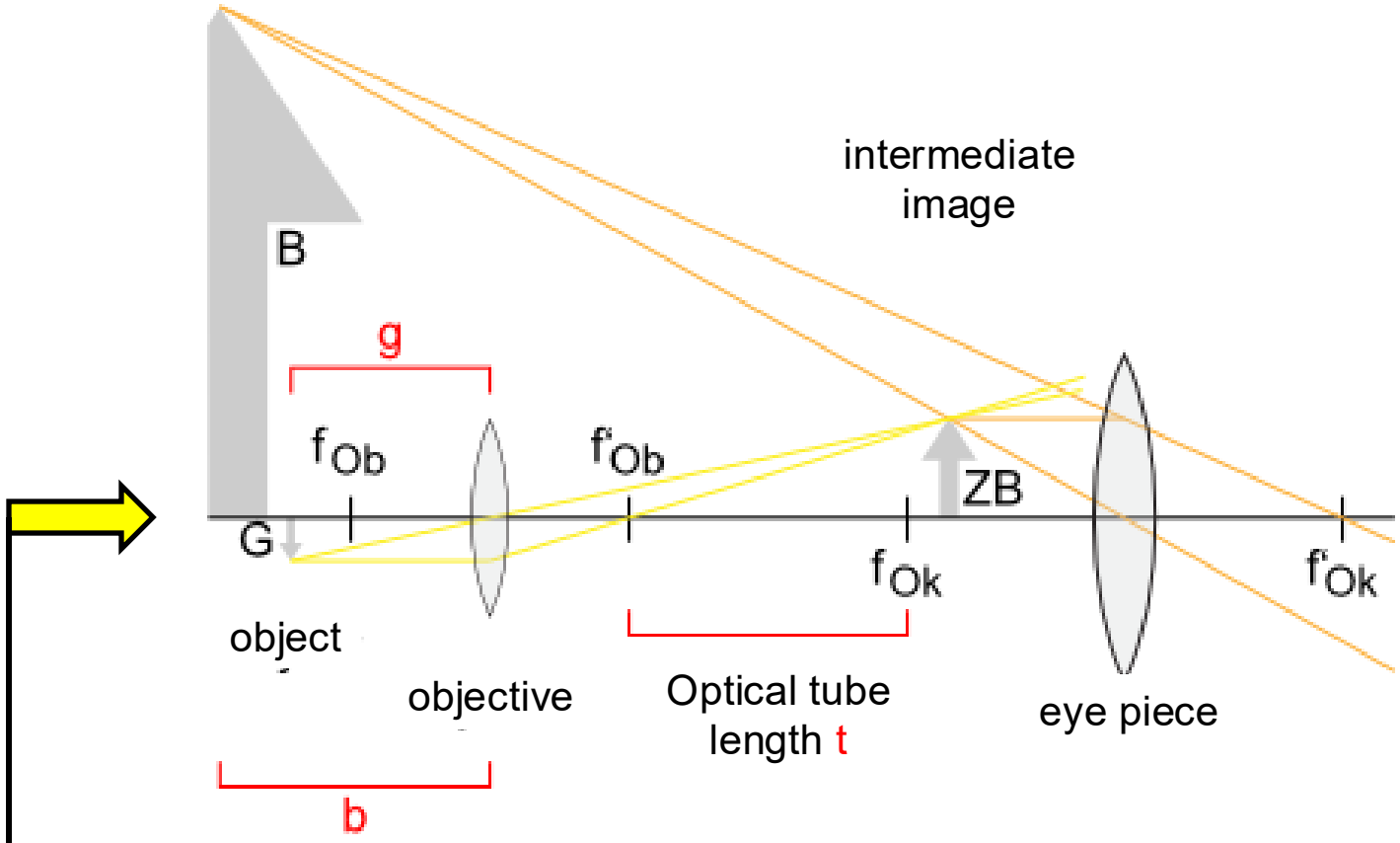


TEM: 1933
Ernst Ruska
Max Knoll
Bodo von Borries

REM: 1937
Manfred von Ardenne

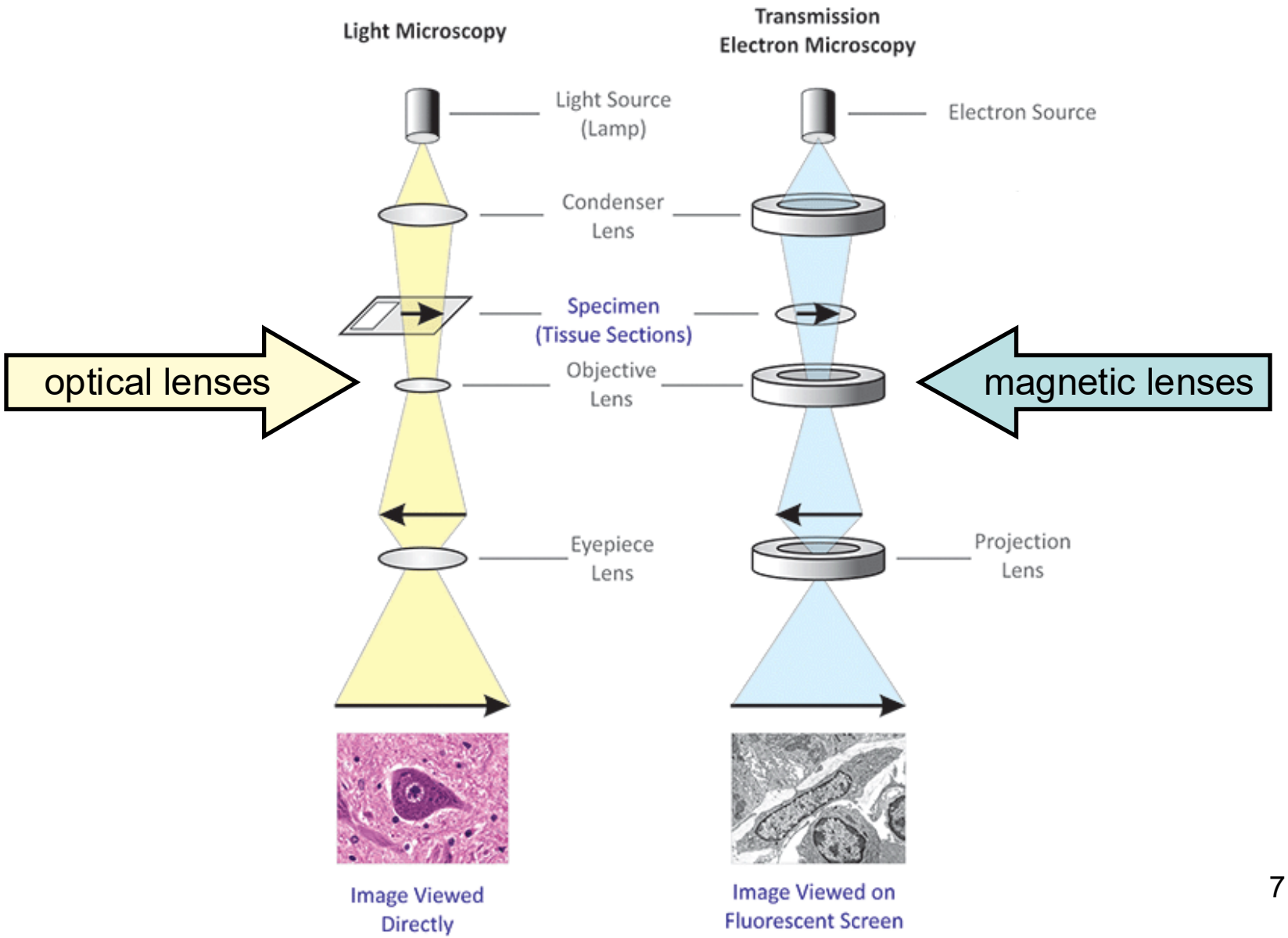
=> Why don‘t we always use electron microscopy?

Imaging beam path of light microscopy



„Transmission“: **Light passes through the object**
=> Condenser gathers light from the lamp to illuminate the object
Contrast is generated by staining the object

Imaging beam path (transmission): light ⇔ TEM



Microscopy using electrons instead of photons

Electrons have both a particle and a wave character
(Wave-particle duality, de Broglie 1924)

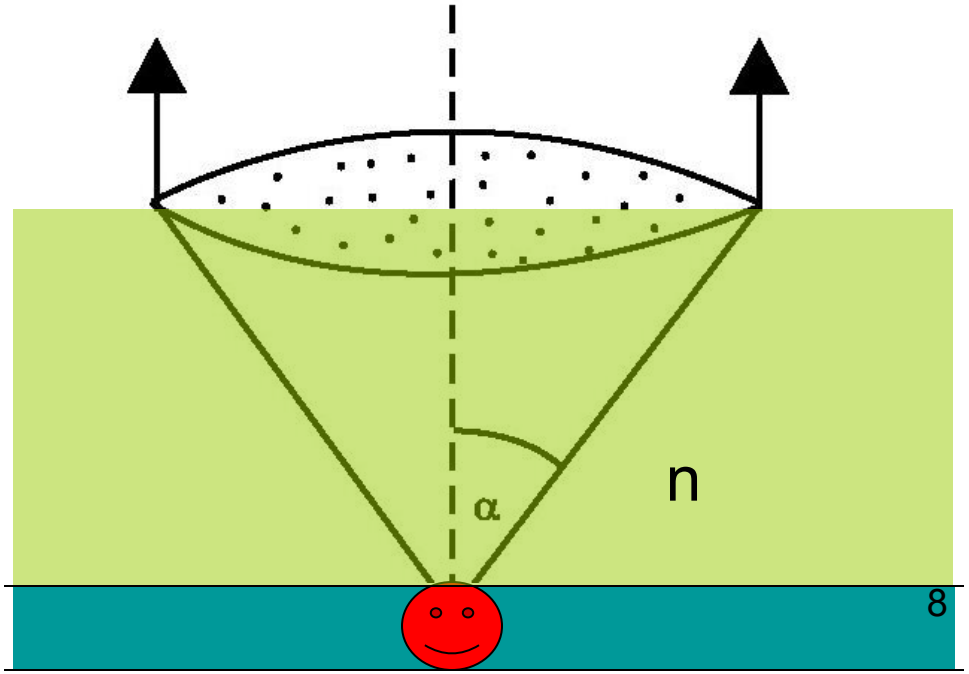
⇒ Electrons are subject to the laws of wave optics

⇒ Electrons are diffracted

⇒ Image of electron microscopy is diffraction limited according to Abbe:

$$d = \frac{\lambda}{2(n \sin \alpha)} = \frac{\lambda}{2\mathrm{NA}}$$

In electron microscopy:
Numerical Aperture (NA)
≈ 0.01



De Broglie relationship: wave-particle duality

Wave-like nature of electrons:

relativistic term

$$\lambda = \frac{h}{p} = \frac{h}{mv} = \frac{h}{\left(\frac{m_0}{\sqrt{1-\frac{v^2}{c^2}}}\right)v} = \frac{h}{m_0 v}\sqrt{1-\frac{v^2}{c^2}}$$

Photons (have no mass)

Electrons (have a mass)

λ: wavelength
h: Planck's constant
p: momentum
m: mass of electron
v: velocity of electron (variable)
m_0: rest mass of electron
c: speed of light

- Electrons are accelerated in an electric field: $E = q \cdot U$
- After leaving the acceleration field: $E = E_{kin} = ½\, m_0 \cdot v^2$
- The resulting velocity *v* is: $v = (2 \cdot q \cdot U / m_0)^{0.5}$

=> Wavelength can be controlled by the acceleration voltage (U).

De Broglie relationship: The limit of resolution

Classic calculation of the wavelength of electron beams

$$\lambda = \frac{h}{(2 \cdot m \cdot q \cdot U)^{0.5}}$$

for U = 100 kV => λ = 0.00387 nm

Calculation of wavelength of electron beams with relativistic correction

$$\lambda = \frac{h \cdot c}{(q \cdot U \cdot (2 \cdot m_0 \cdot c^2 + q \cdot U))^{0.5}}$$

for U = 100 kV => λ = 0.00369 nm

De Broglie relationship: The limit of resolution

1. Velocity of electron at 100 kV acceleration voltage

$v = (2 \cdot q \cdot U / m_0)^{0.5}$

2. De Broglie wavelength

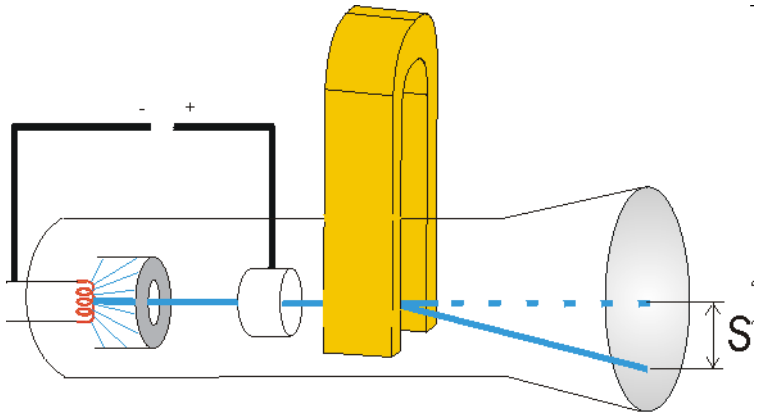
$$\lambda = \frac{h}{p} = \frac{h}{mv} = \frac{h}{\left(\frac{m_0}{\sqrt{1-\frac{v^2}{c^2}}}\right)v} = \frac{h}{m_0 v}\sqrt{1-\frac{v^2}{c^2}} = 0.0037 \text{ nm}$$

3. Resolution at NA = 0.01

$$d = \frac{\lambda}{2(n \sin \alpha)} = \frac{\lambda}{2\mathrm{NA}} = 0.185 \text{ nm}$$

TEM: instrumental features

- Gas molecules diffract electrons => optical path in EM must be evacuated (10^{-7} bar) => Vacuum technique*
- Focusing of electron beam needs different lens systems
  $\Rightarrow$ magnetic lenses (iron-wrapped coils; the magnetic field and thus the focal length can be adjusted by the coil current)



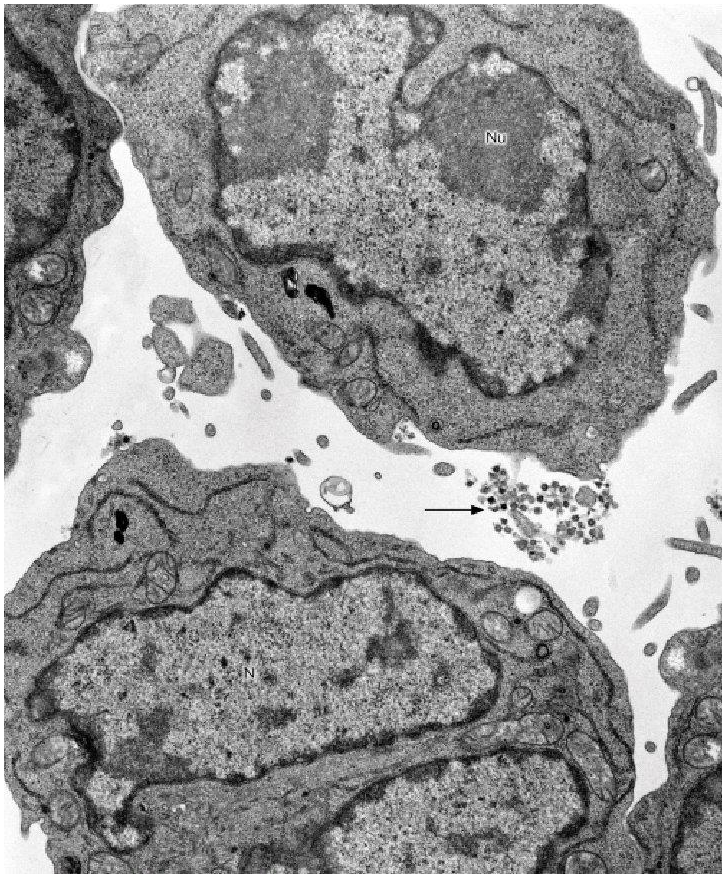
Lorentz force

- TEM and REM use different contrast mechanisms

* newer development: Environmental Scanning Electron Microscopy (ESEM)

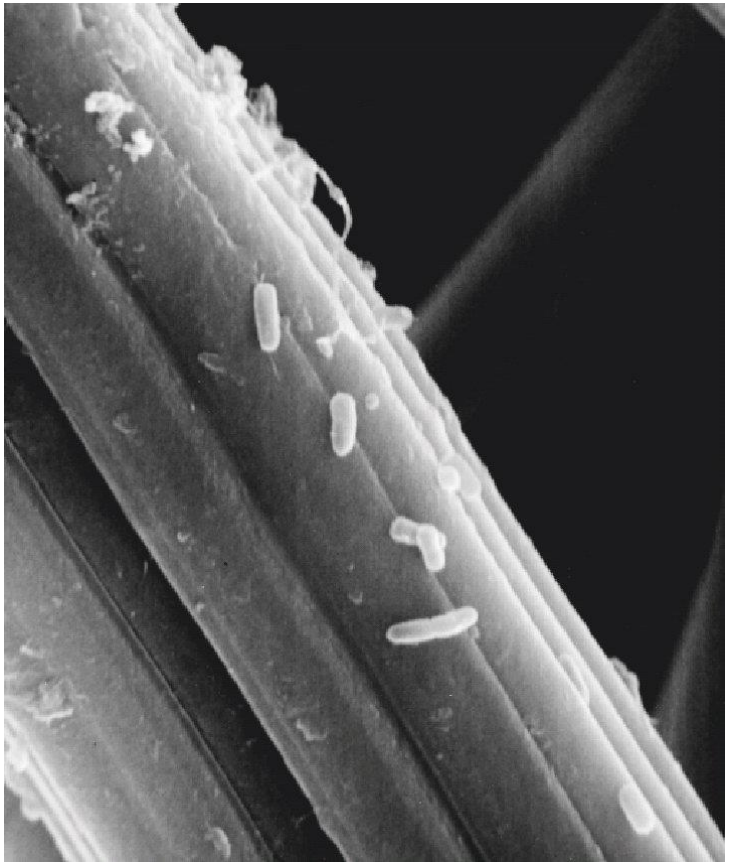
Electron microscopy: TEM ⇔ SEM

TEM



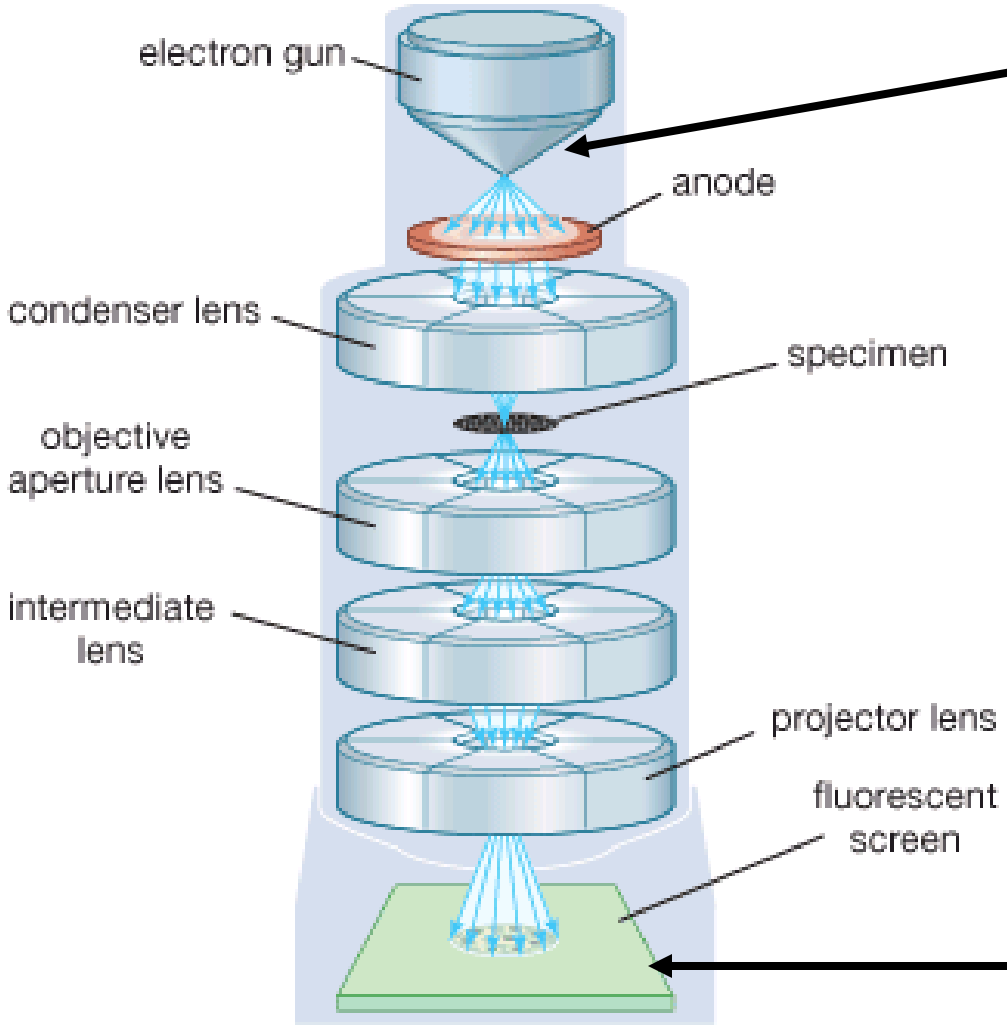
Transmission (thin samples)

SEM

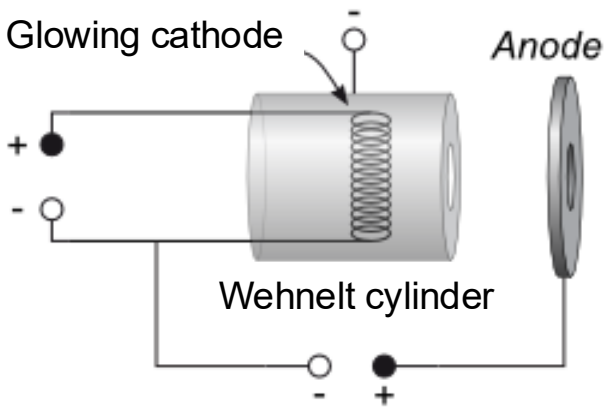


Back scattering on surface of object

TEM: beam path

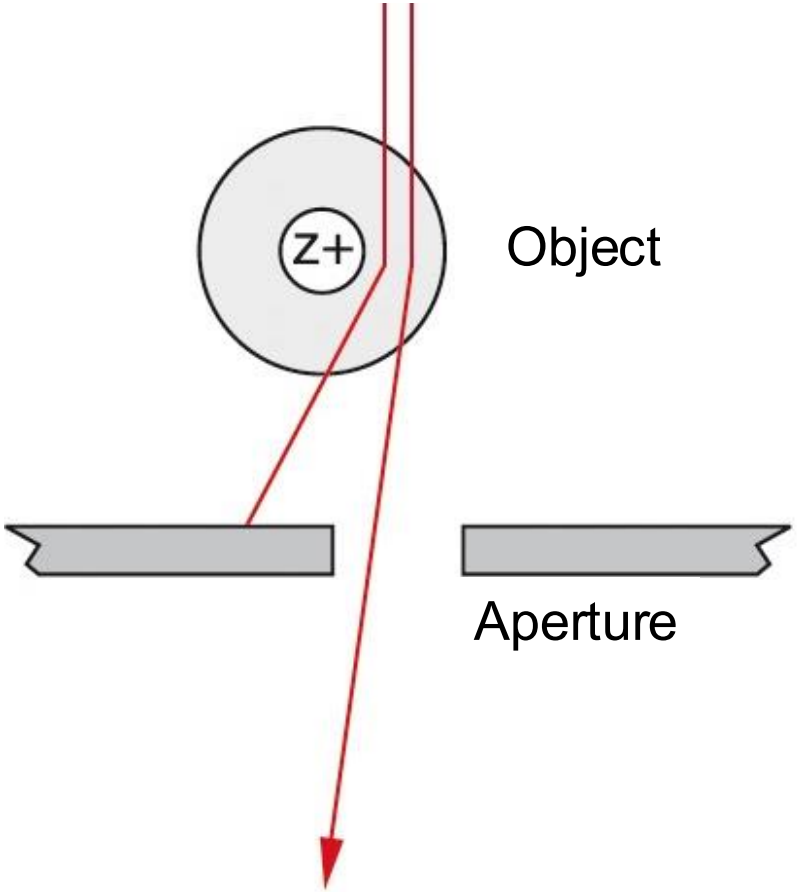


Electron source:
Field emission cathode
Wehnelt cylinder (s. below)

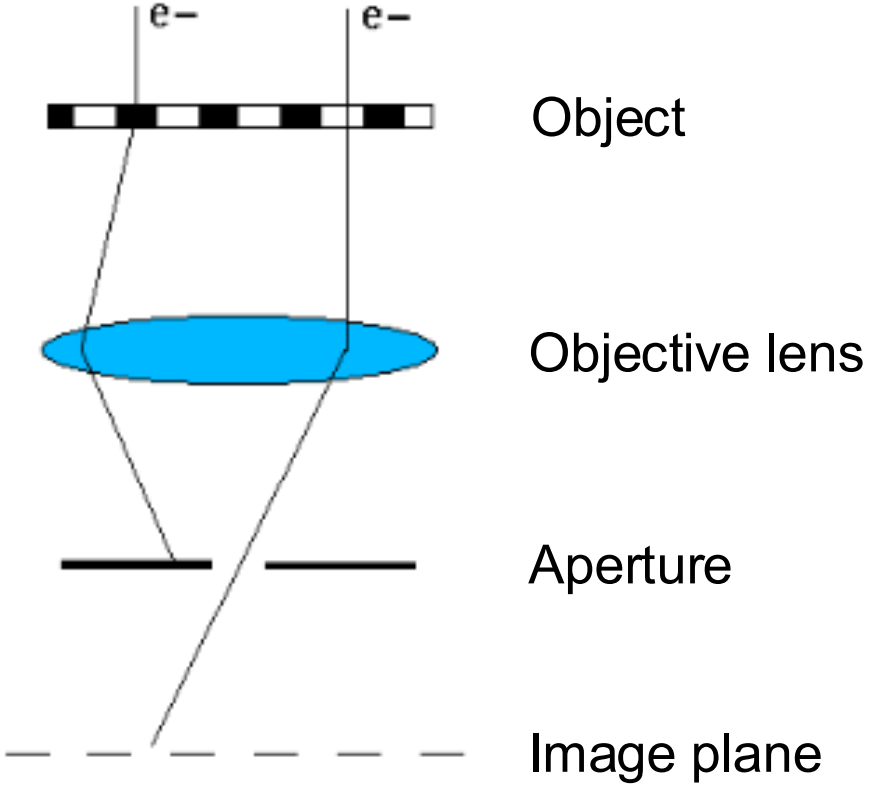


alternatively: CCD-Kamera

Generation of contrast



Diffraction of electrons depends on atomic number of the scattering nucleus
=> the larger the atomic number the larger is α (in the order of 6°).



Electron dense areas
of the sample appear dark.

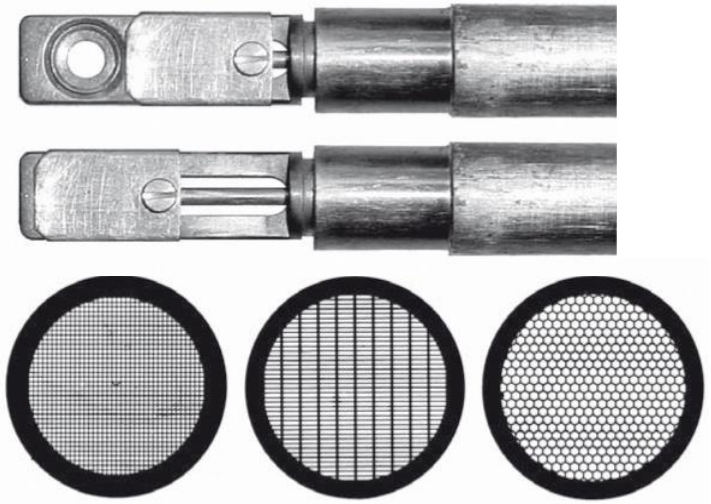
Generation of contrast

Electron beam passes through the sample (=> transmission)

- Elastic scattering of the electron beam by atomic nuclei results in contrast
- A higher atomic number of the nucleus results in stronger scattering
- Slower electrons result in stronger scattering
  => Compromise: resolution / contrast
- Electron-dense points of the object appear dark
- The large number of scattering centers requires thin samples (d < 0.1 µm)
- Negative contrast by applying heavy metal salts
  => Uranyl acetate / Osmium tetroxide (OsO_4)
- Cryo-electron microscopy: without contrast enhancement
- Immunolabeling => antibodies conjugated to gold nanoparticle labels
- Typically in wide-field mode, but STEM (scanning) is also possible

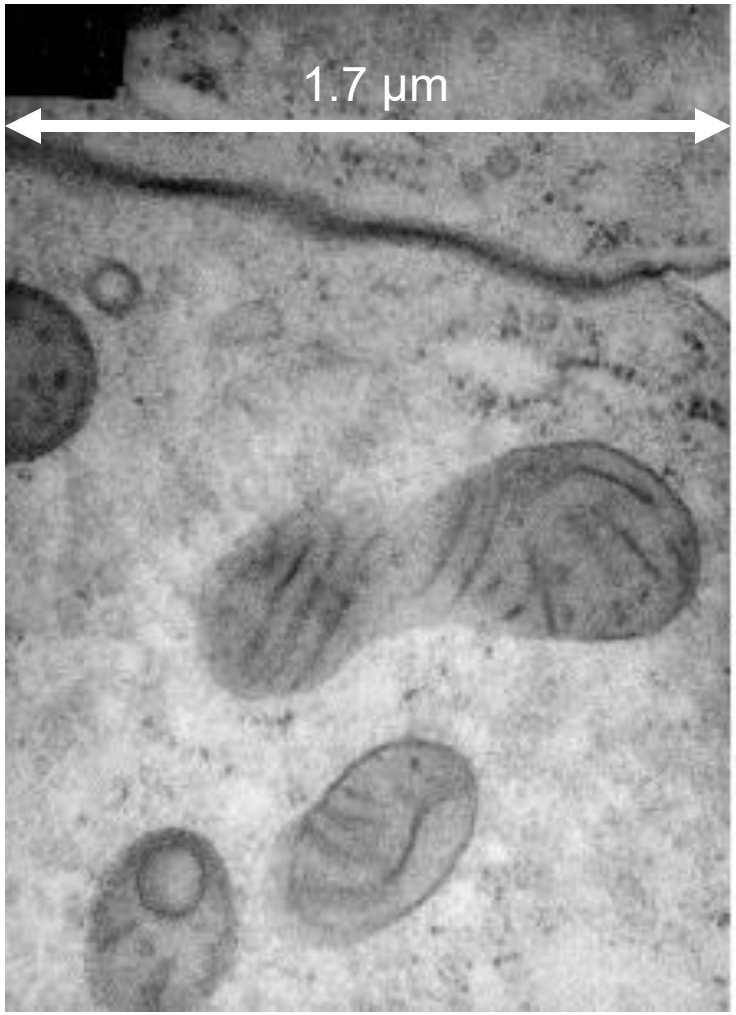
Sample preparation

- Biological samples must be chemically fixed and dried
- Adding a solution of heavy metal salts (1 - 2 %)
- Embedding in a resin or materials that polymerize in the cold
- Sample must be ultra-thin: < 100 nm
  preparation with microtome



- Grid (copper) +
  carbon layer (5-10 nm) +
  stabilized biological section
- The cross section reveals inner structures

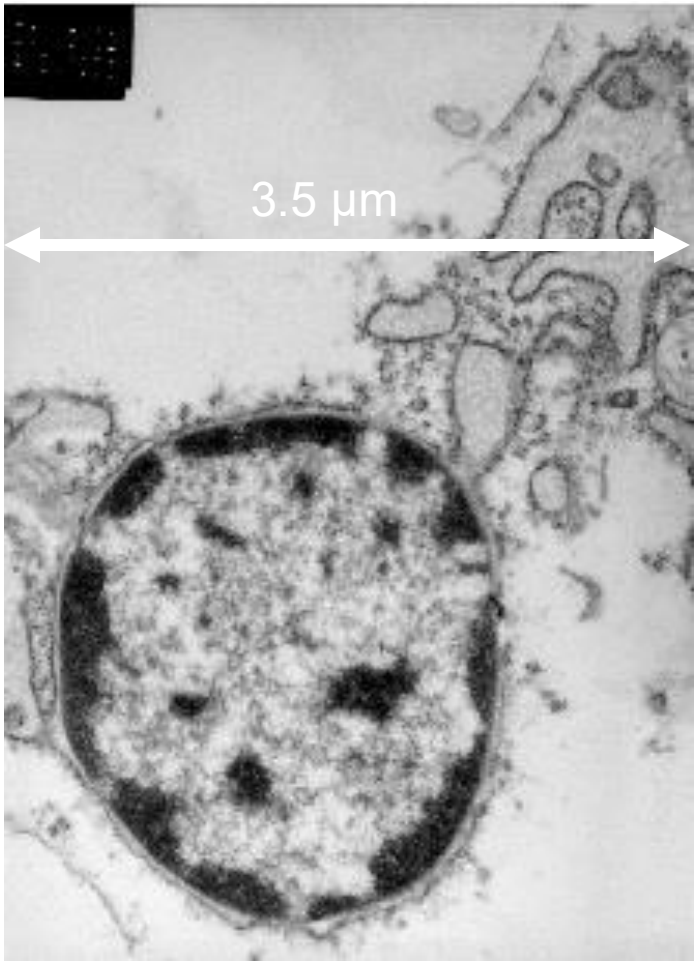
TEM images



Mitochondria of a liver cell including invagination of inner membrane (cristae).

=> Image interpretation requires experience / awareness of staining artifacts

TEM images

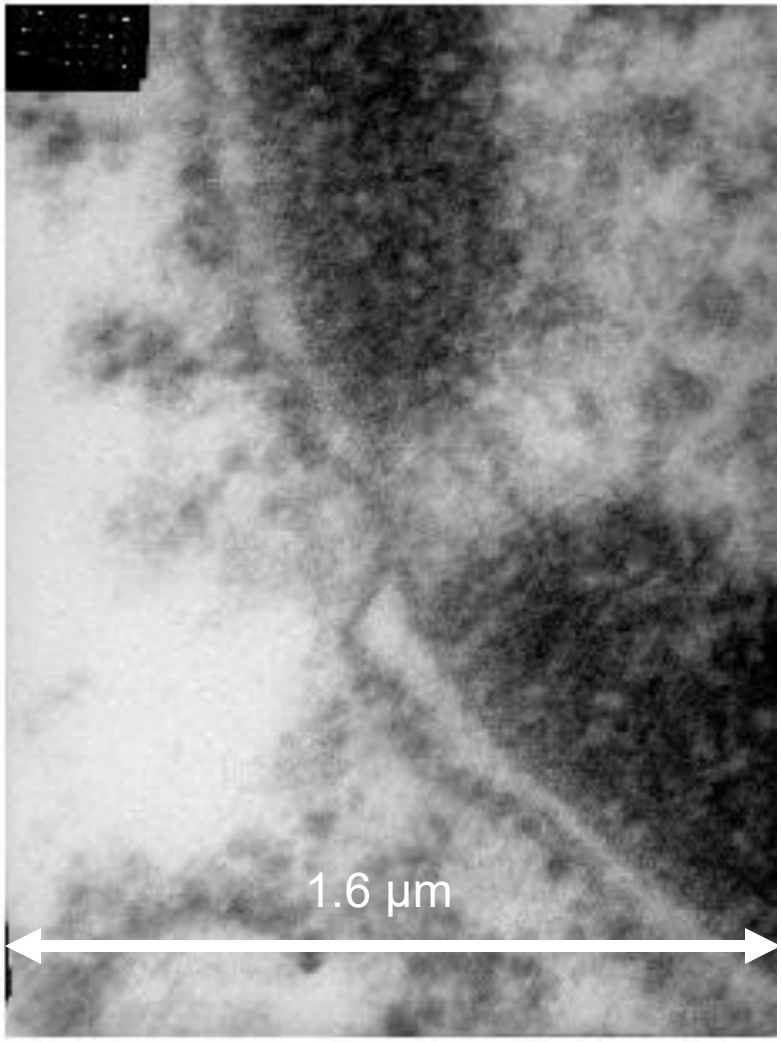


Nucleus of a kidney cell including double membrane

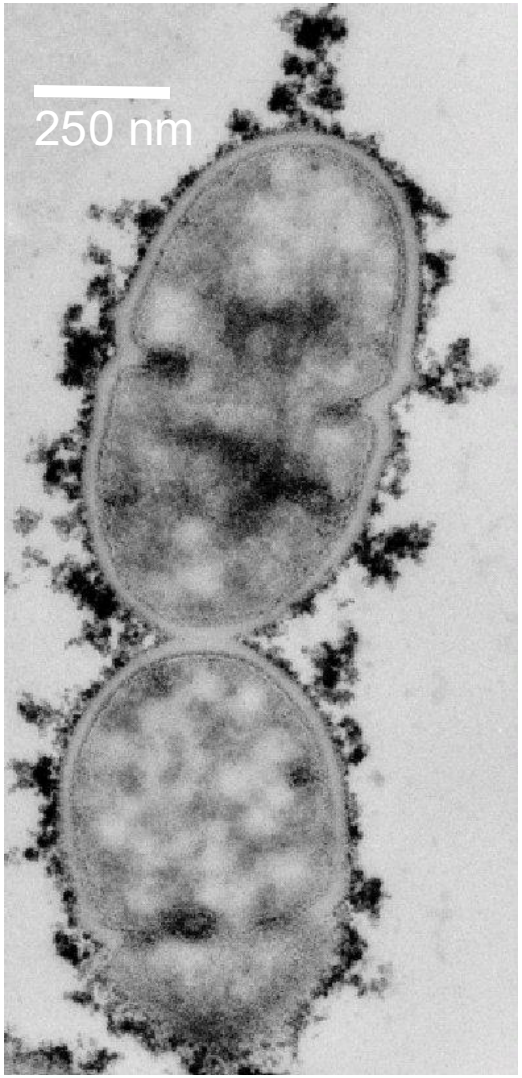
Staining of membrane by OsO_4

TEM images

Nuclear pore in detail



TEM images

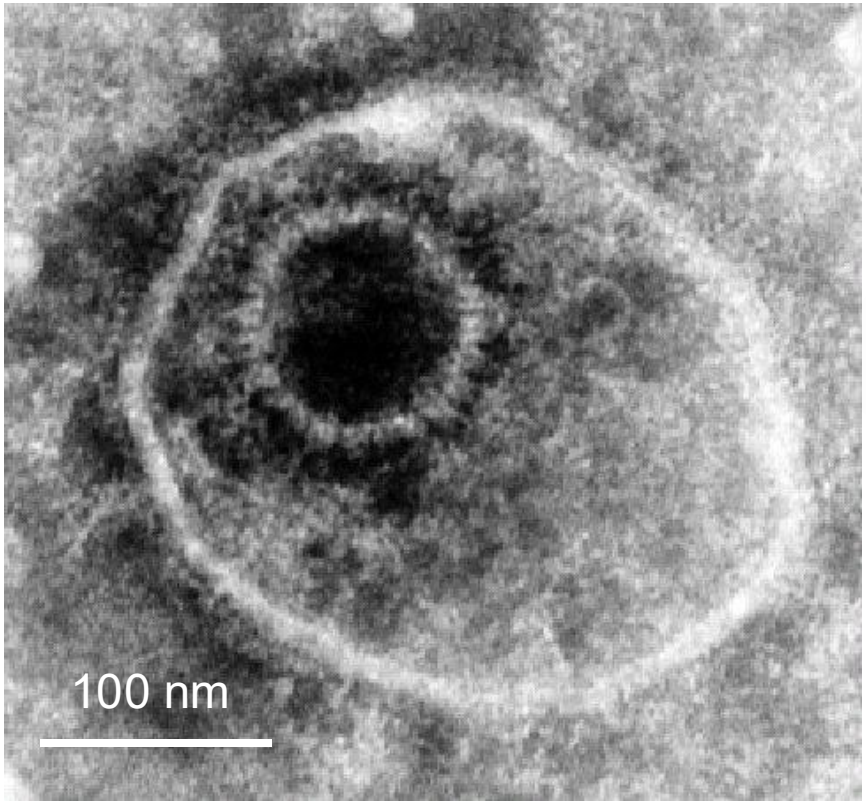


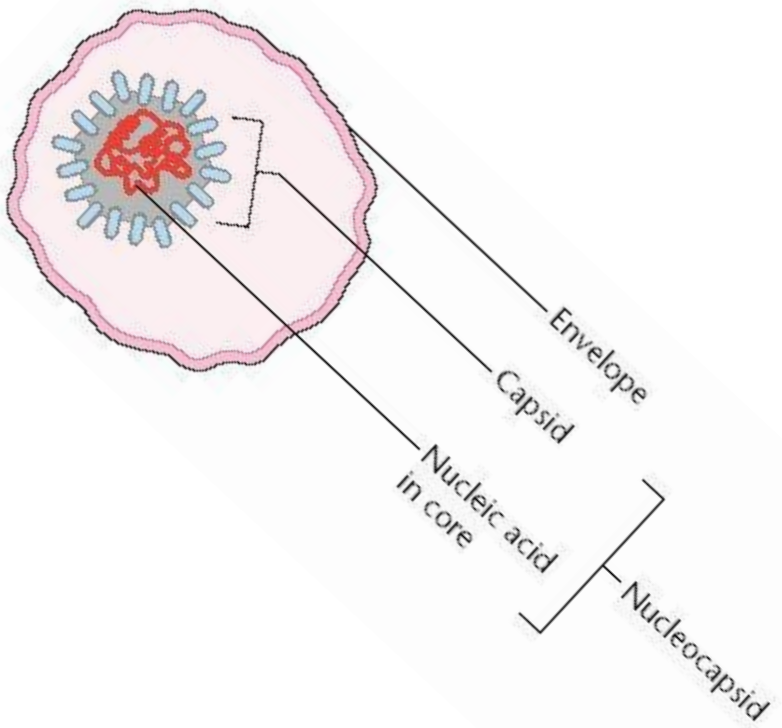
Bacteria on a tooth:

Ultra-thin cut (d < 70 nm) of streptococci

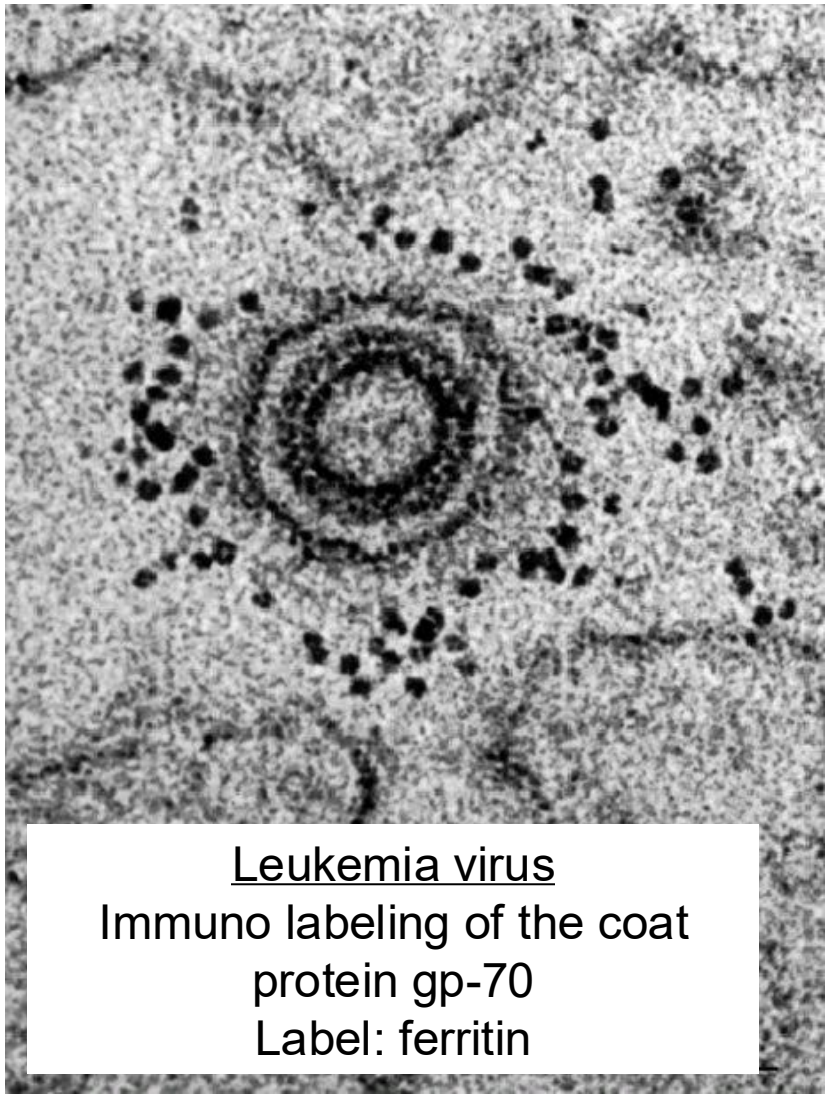
TEM images

Herpes virus



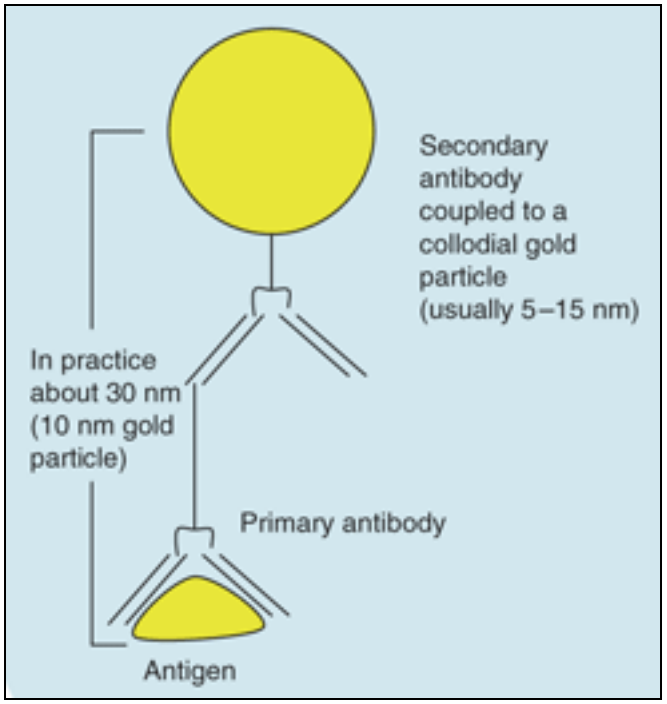


Immunolabeling



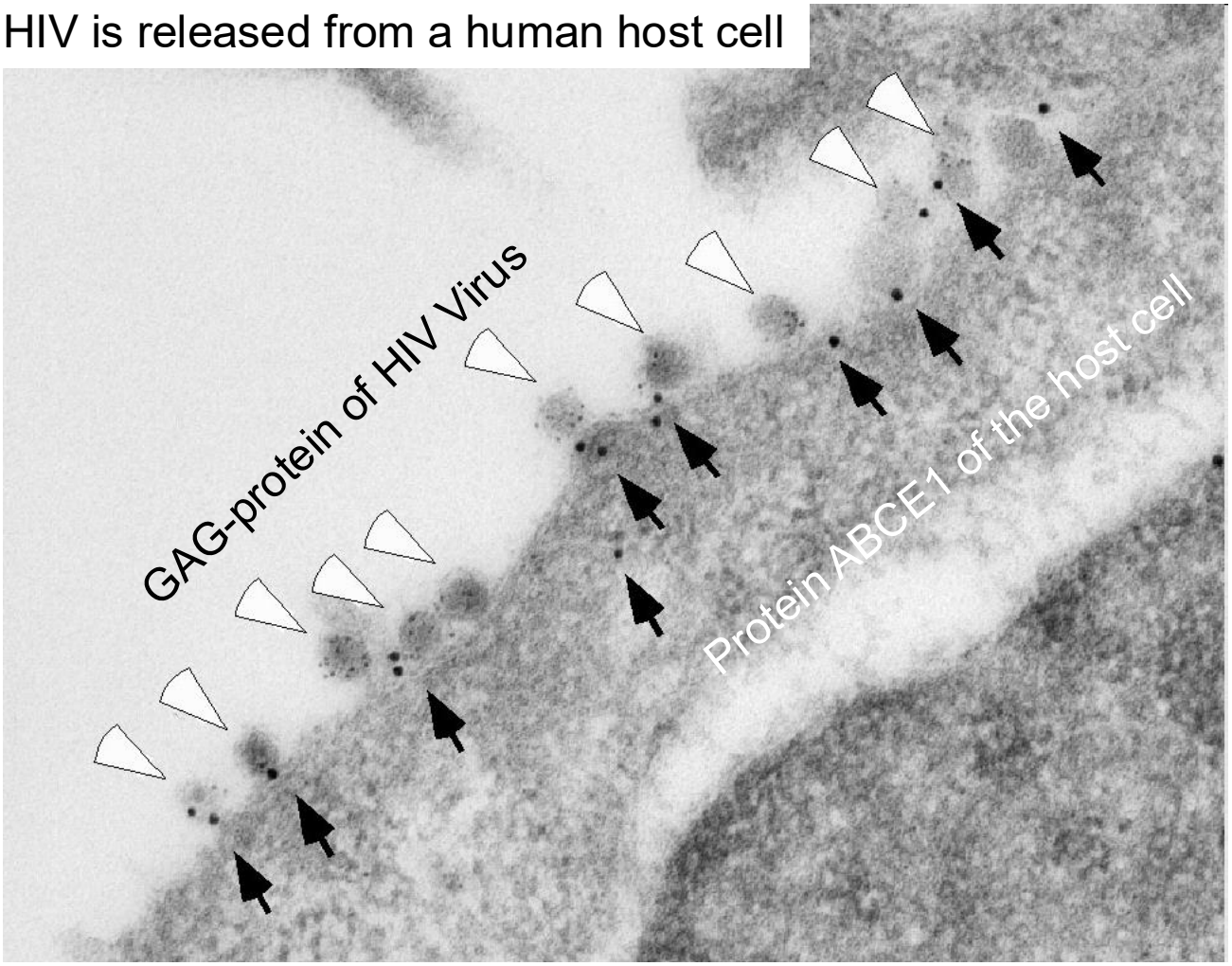
Leukemia virus
Immuno labeling of the coat protein gp-70
Label: ferritin

„Immunogold“ labeling



Double immunolabeling

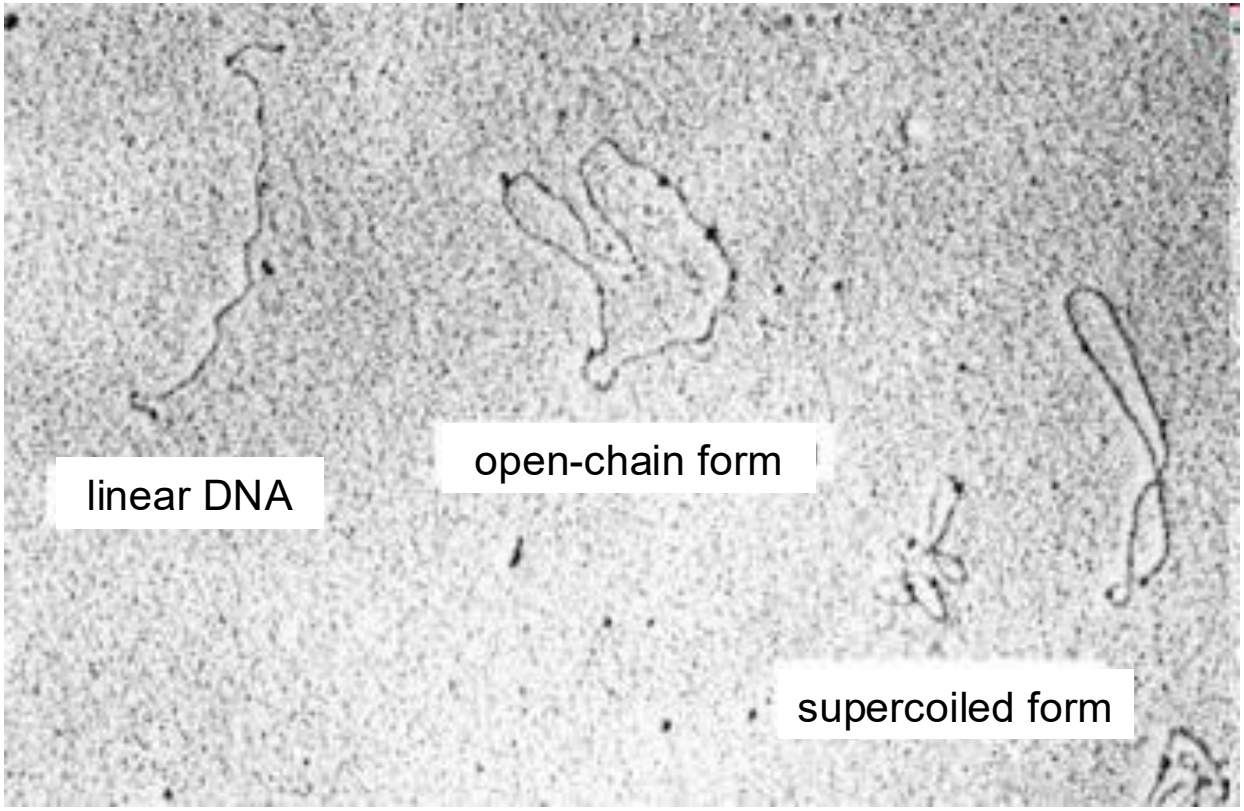
HIV is released from a human host cell



Double staining by gold nanoparticles of different diameters.

TEM images

single DNA molecules (Ø 2 nm)



Limitations of TEM

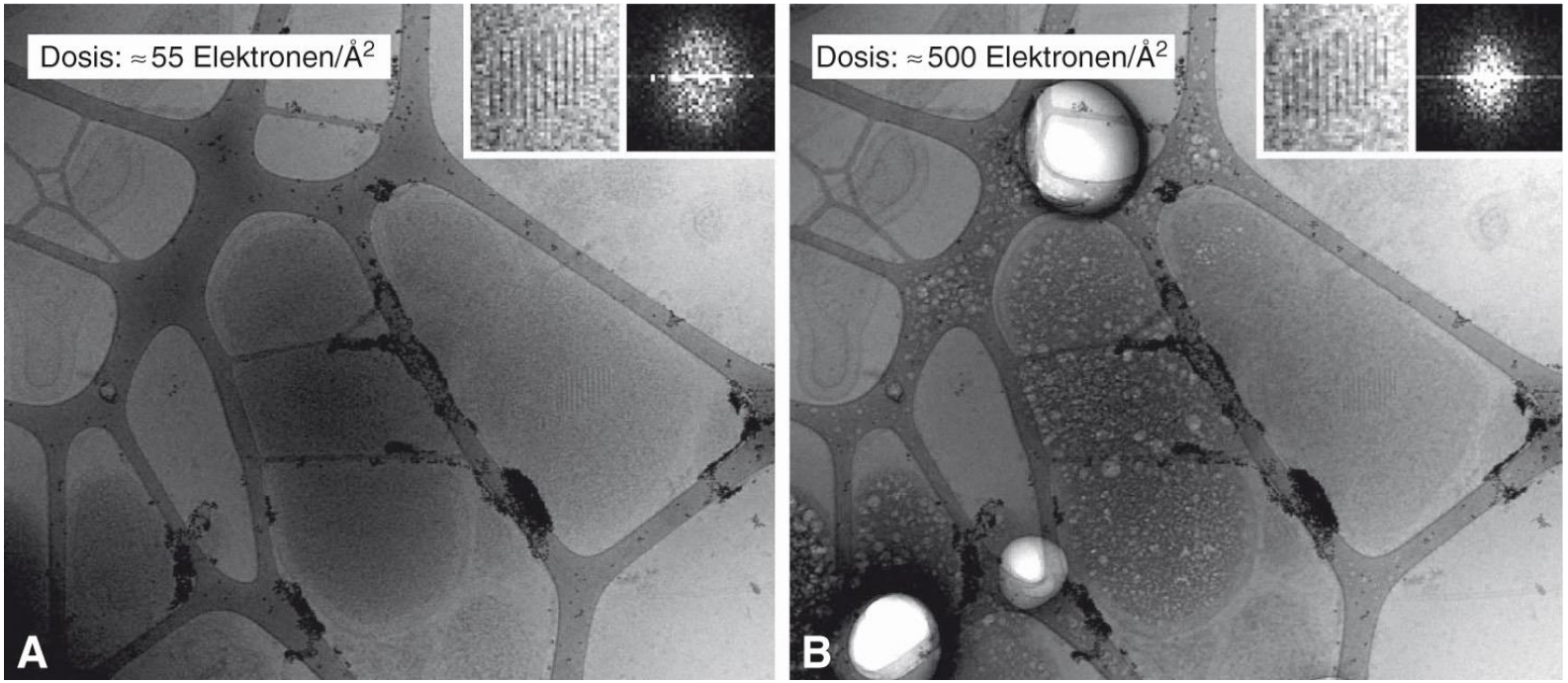


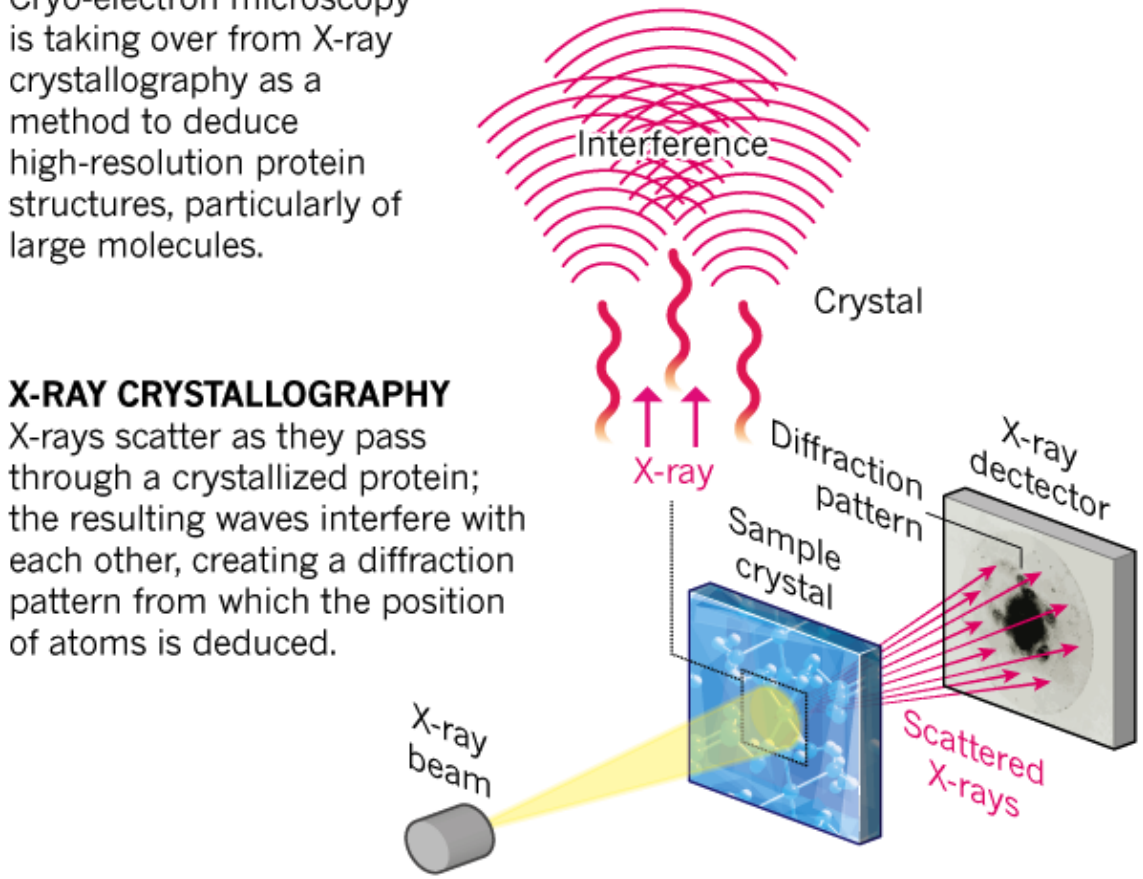
Image of Archaea
A) No irradiation damages
B) Formation of blisters by decomposition of water

Cryo-electron microscopy

- Biological samples do not need to be fixed, dried or stained => less artifacts
- Intact cells are embedded into amorphous ice => physical „fixation“ (Vitrification avoids crystallization)
- Ultra-thin cut with cryotome
- The ultra-thin cut is transferred to a porous carbon layer (5-10 nm)
- The cryogenic environment reduces damages of the electron beam
- Typically, a section of the object can only be imaged once
- Also applicable to single protein complexes
  => images of several complexes can be taken followed by image stacking
- 3D rendered images can be assembled by taking images from different observation angels (tilt)

Cryo-electron microscopy

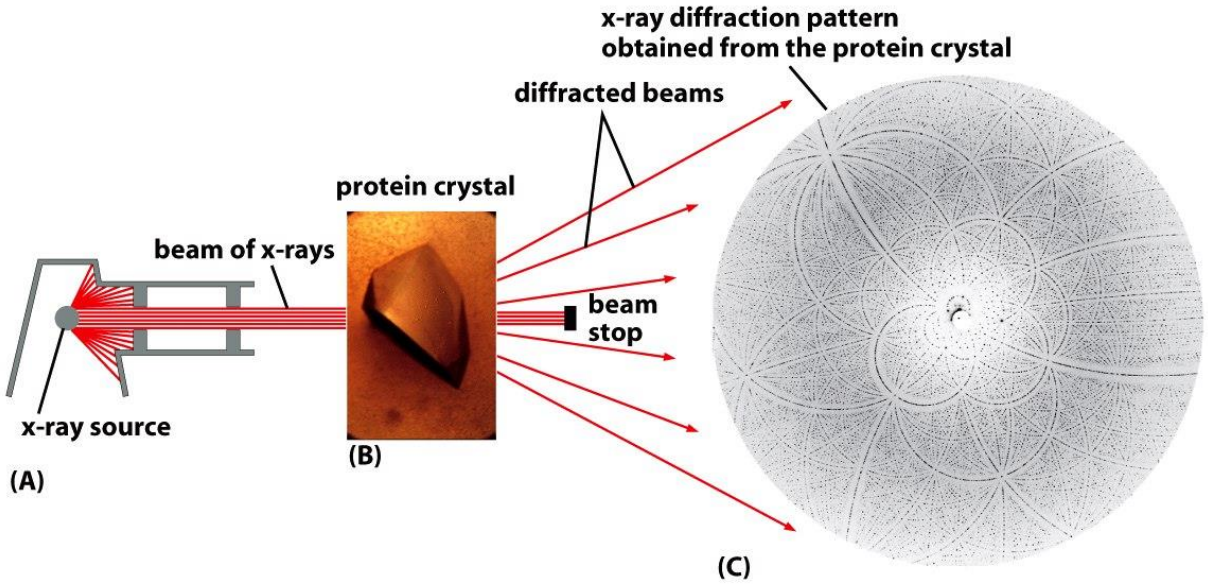
Cryo-electron microscopy is taking over from X-ray crystallography as a method to deduce high-resolution protein structures, particularly of large molecules.



X-RAY CRYSTALLOGRAPHY
X-rays scatter as they pass through a crystallized protein; the resulting waves interfere with each other, creating a diffraction pattern from which the position of atoms is deduced.

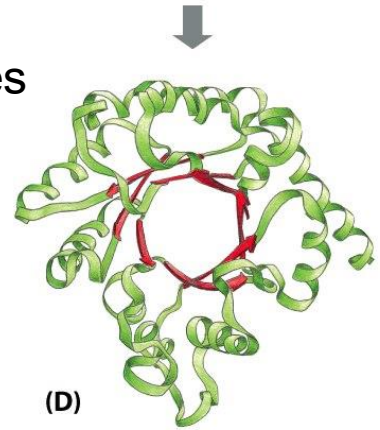
=> Protein crystallization is challenging, and in many cases not successful

Excursion: X-ray crystallography

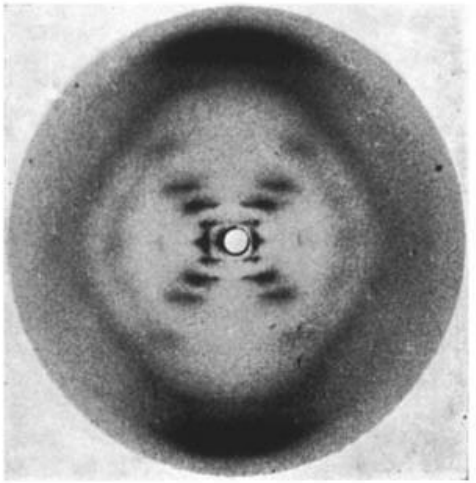


X-rays wavelength: 0.1 nm

=> Determination of protein structures with Ångström resolution



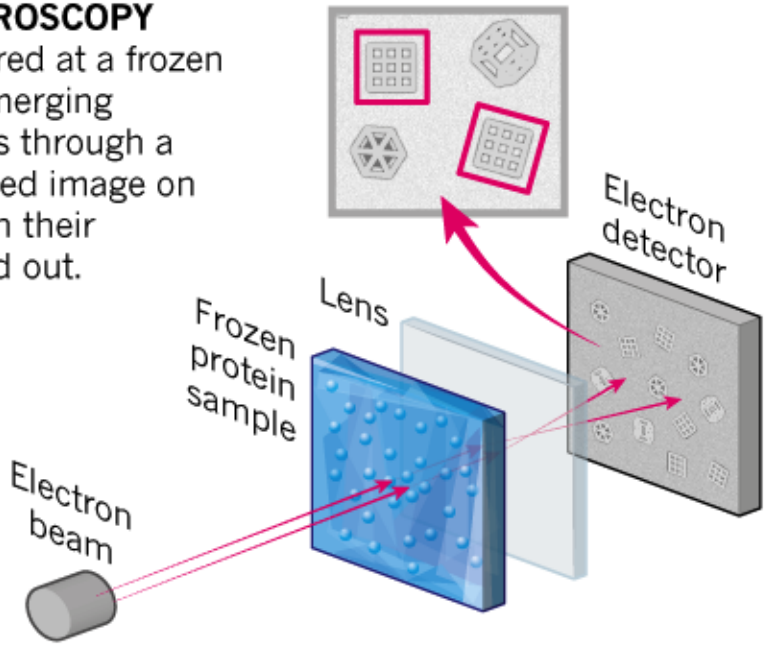
"Photo 51"



Diffraction pattern of DNA

Cryo-electron microscopy

CRYO-ELECTRON MICROSCOPY
A beam of electron is fired at a frozen protein solution. The emerging scattered electrons pass through a lens to create a magnified image on the detector, from which their structure can be worked out.

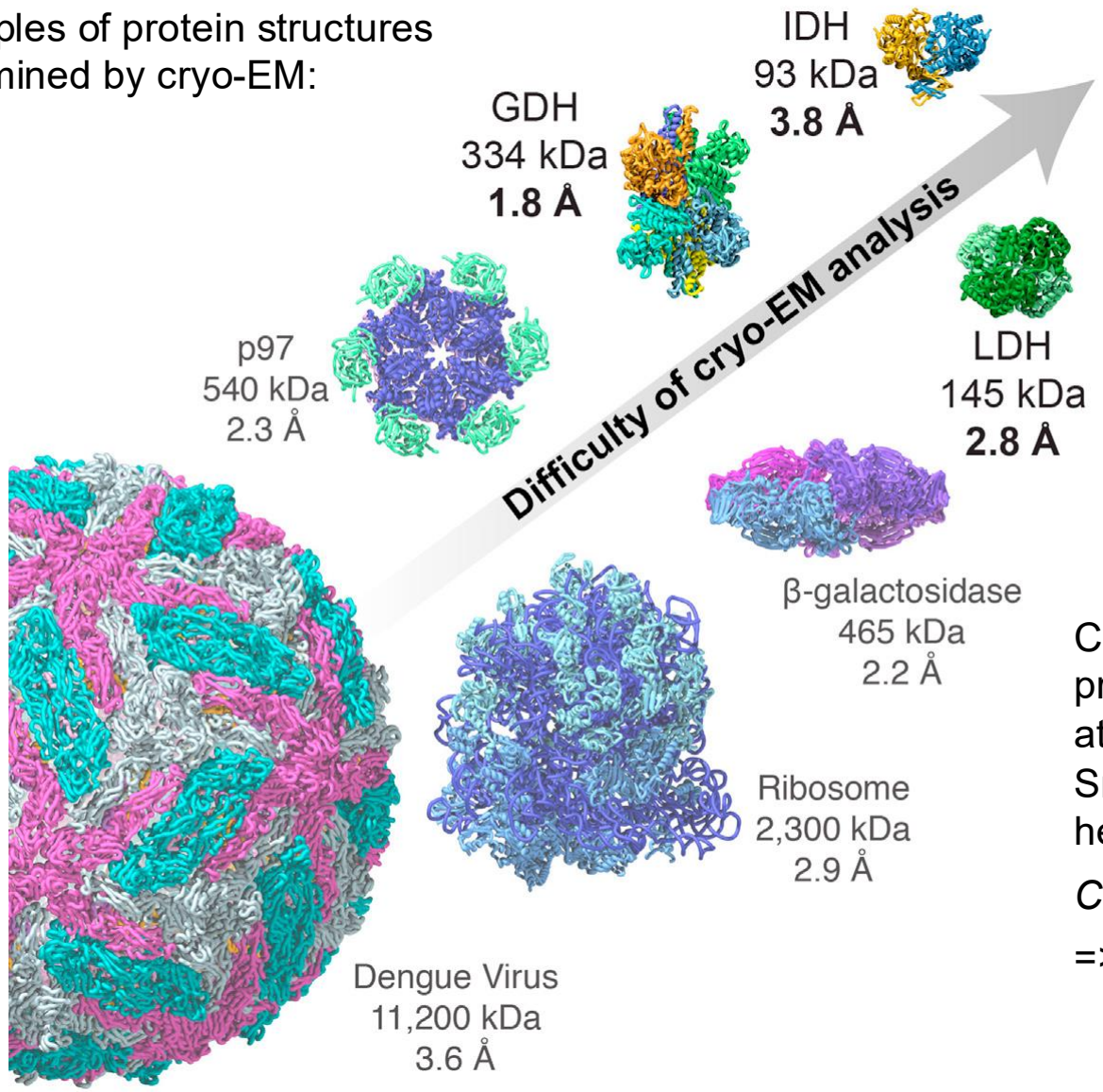


Tilting the specimen or analysis of many particles positioned at different orientations => 3D reconstruction

Nobel prize in chemistry (2017):
Jacques Dubochet
Joachim Frank
Richard Henderson

Cryo-electron microscopy

Examples of protein structures determined by cryo-EM:

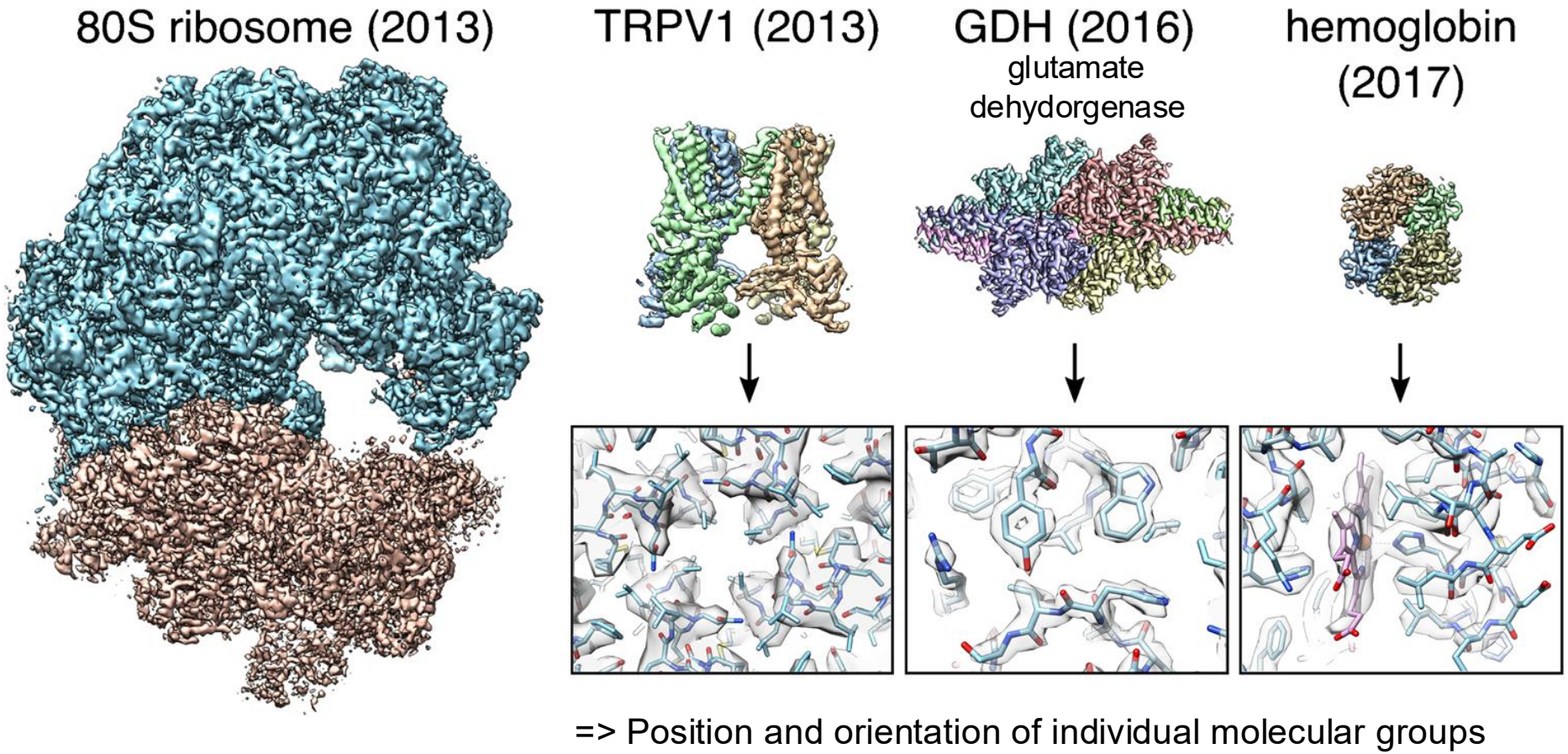


Cryo-EM can determine protein structures at near-atomic resolution.
Smallest protein until 2017: hemoglobin (64 kDa).

Cell 165, 2016, 1698-1707

=> available in the IS folder

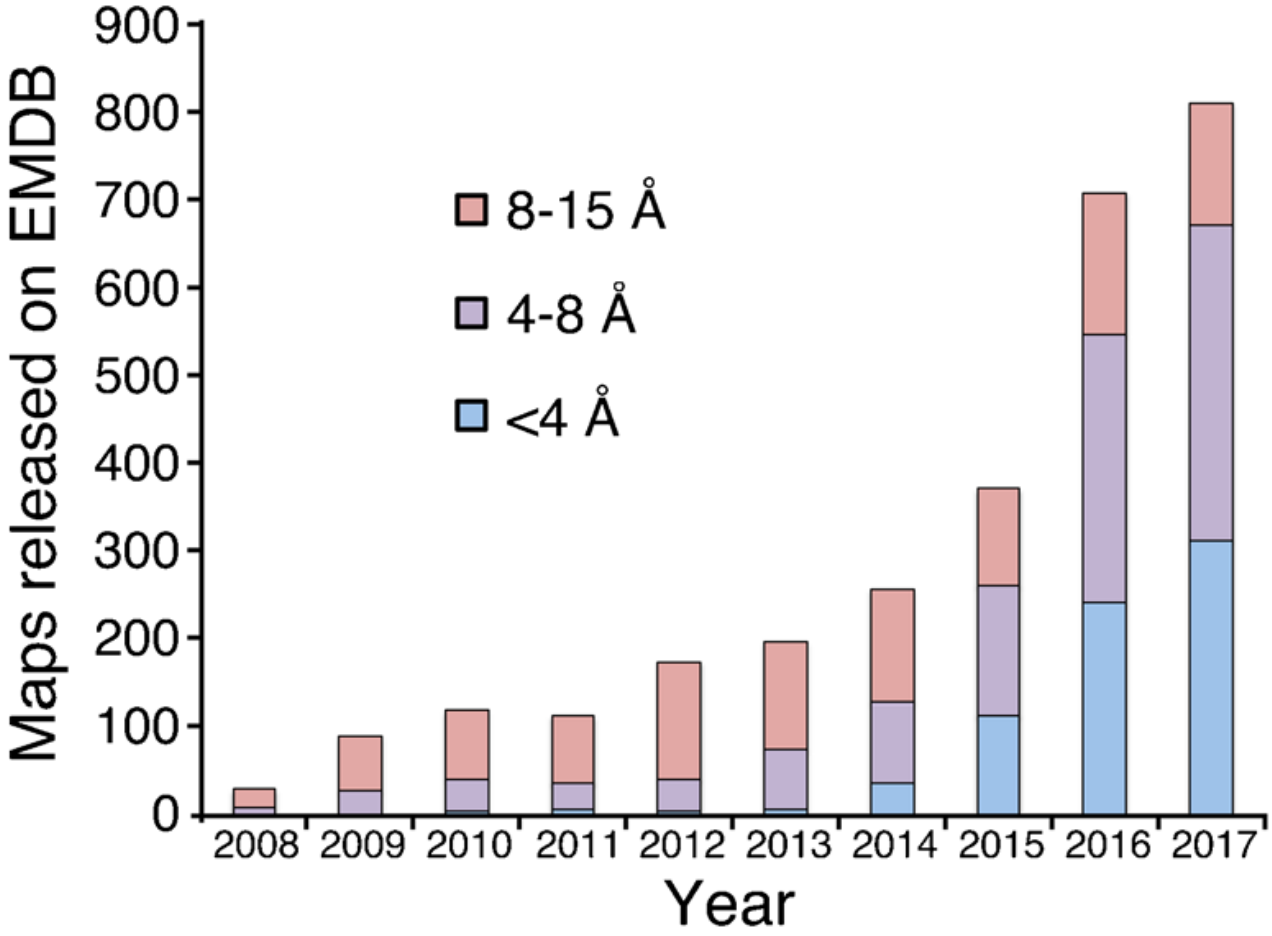
Cryo-electron microscopy

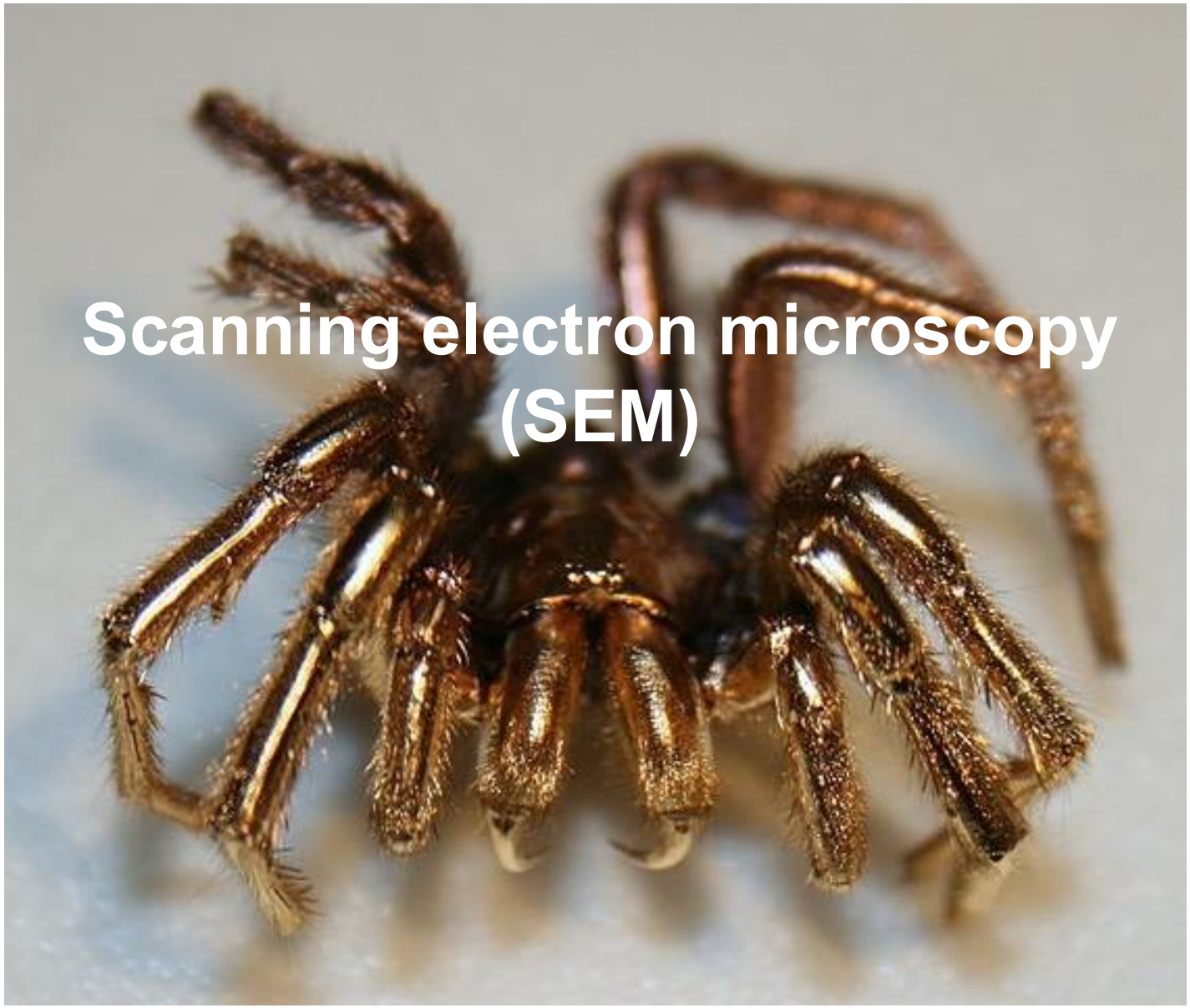


=> Position and orientation of individual molecular groups

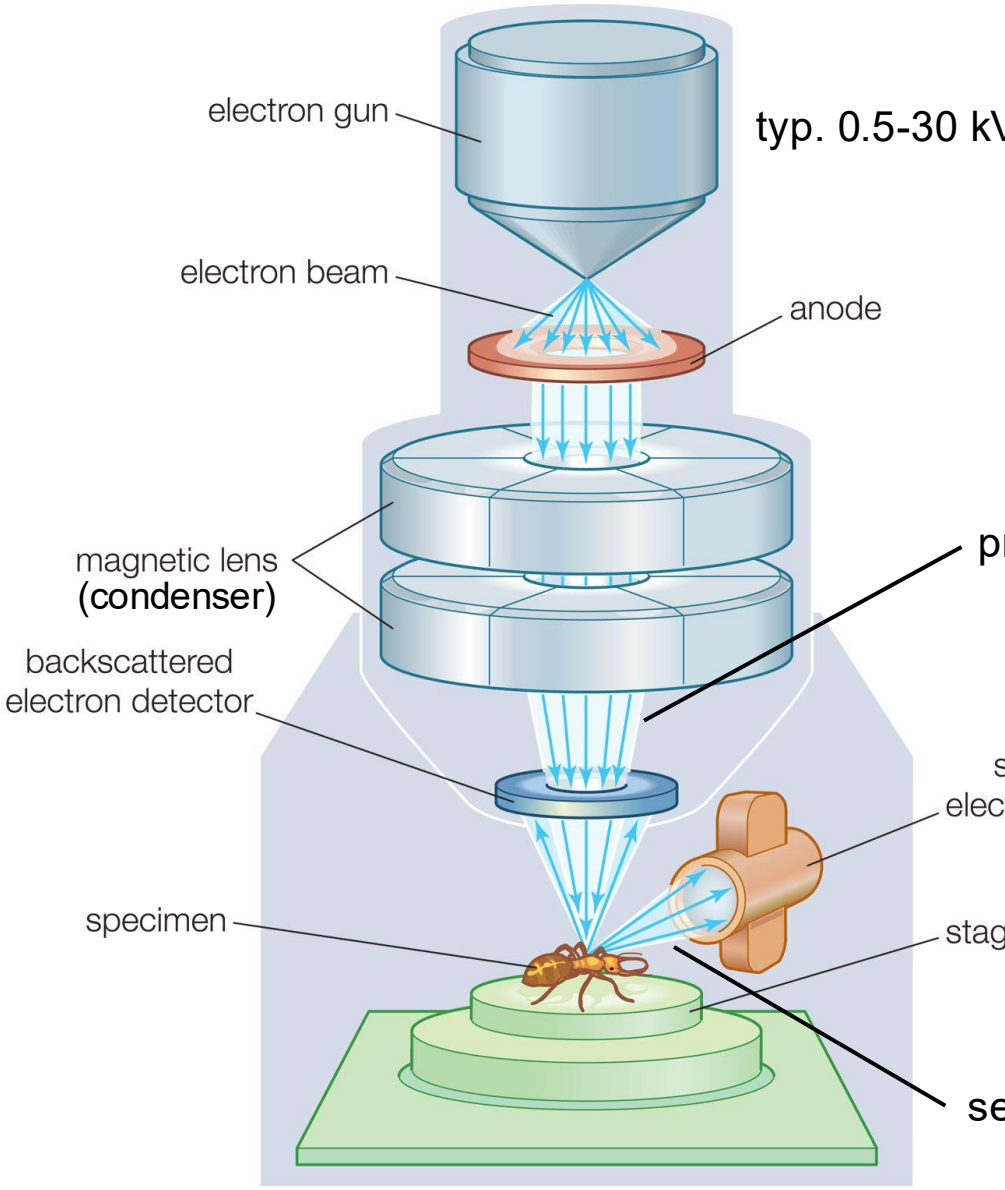
Cryo-electron microscopy

Electron microscopy data bank (EMDB)




Scanning electron microscopy
(SEM)

SEM: Beam path

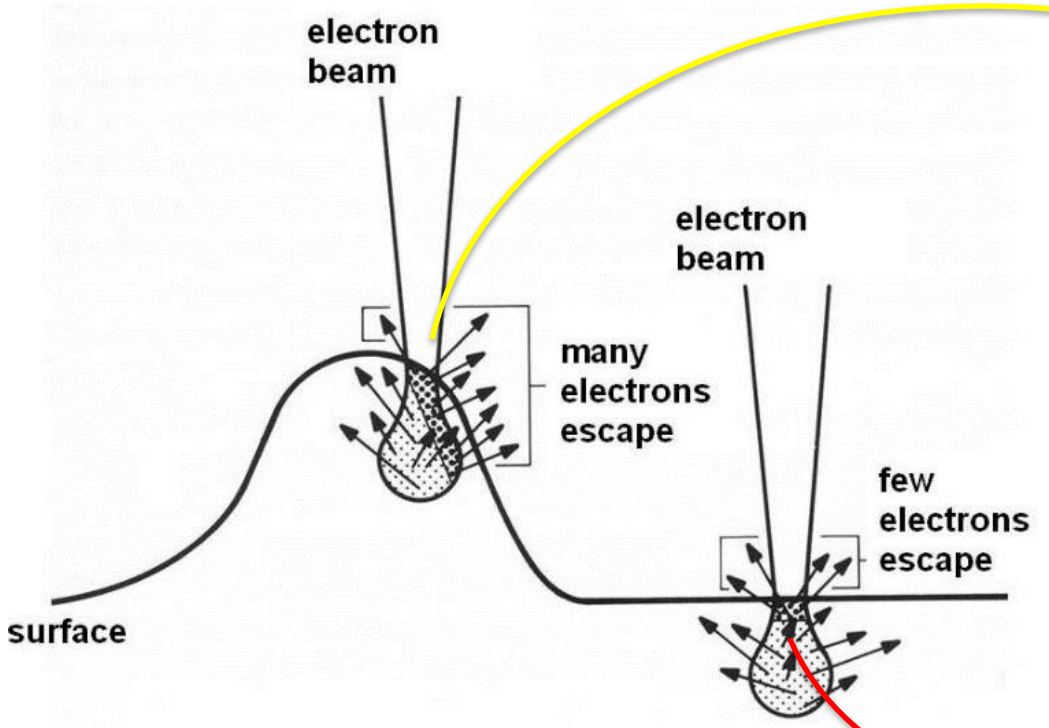


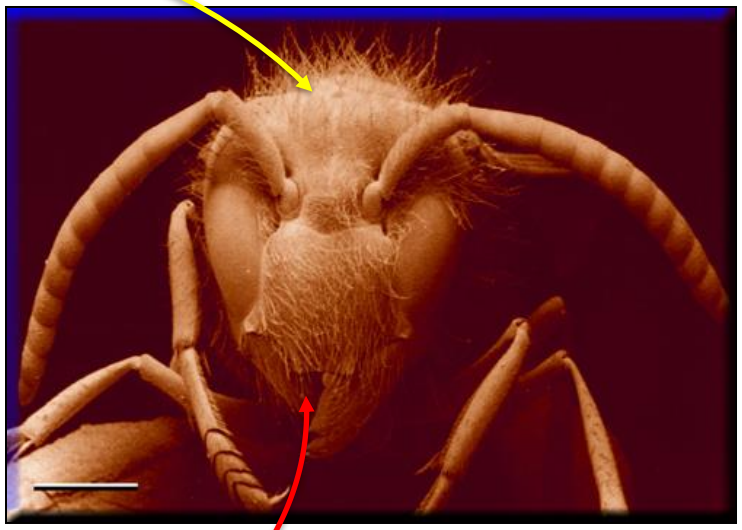
Elastic
back scattering
(information
on material)

Inelastic
scattering
(topography)

Generation of contrast

Different release of secondary electrons results in contrast



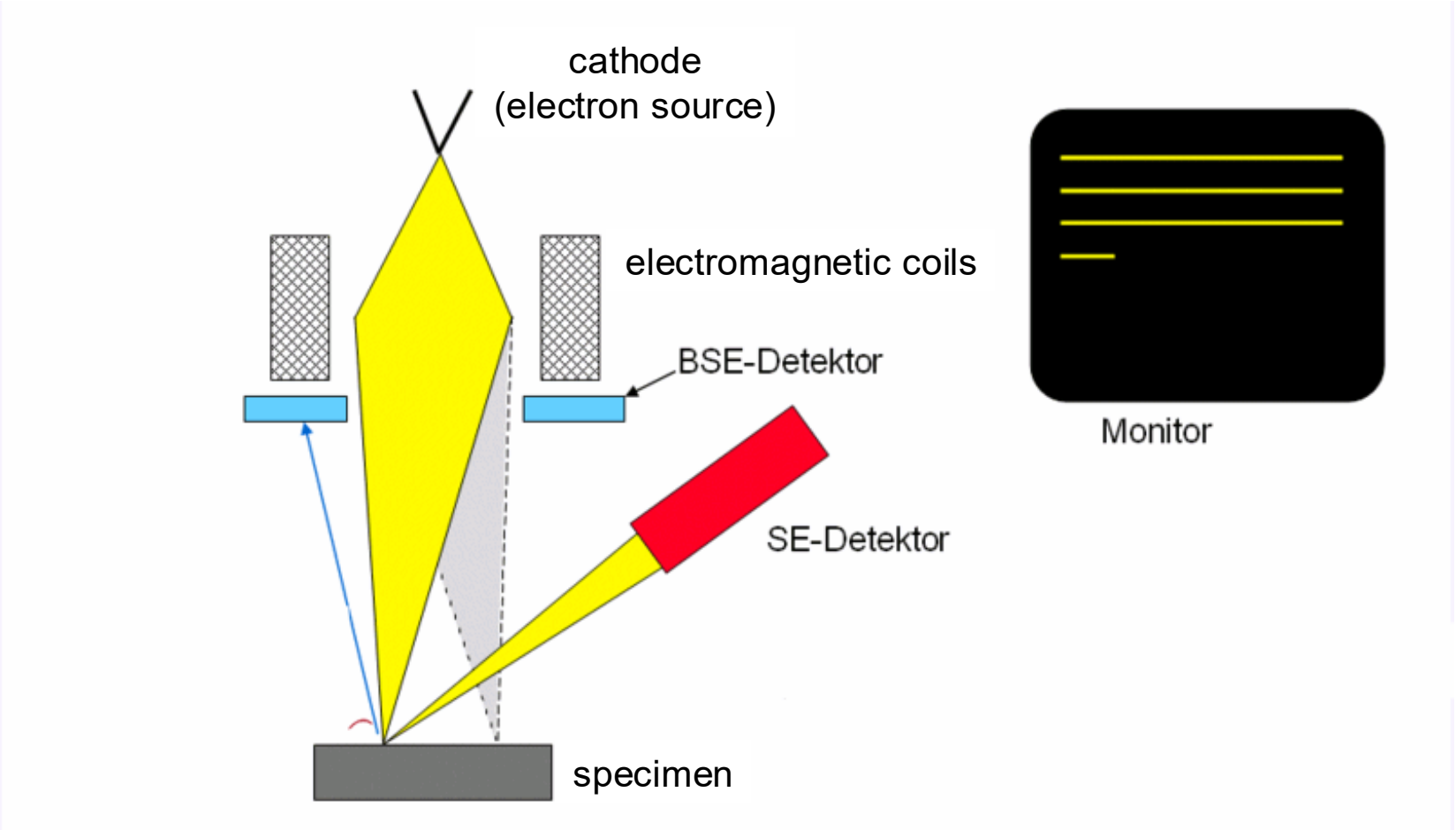


A wasp‘s head

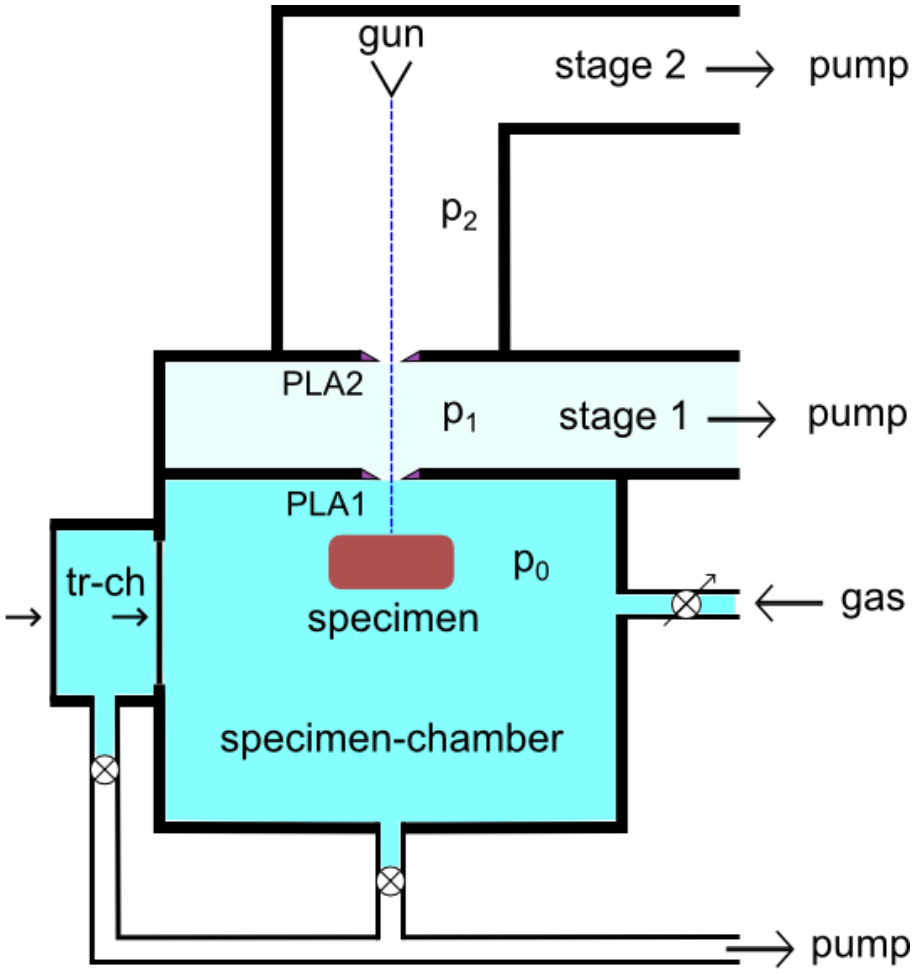
Generation of contrast

- Electrons are back-scattered on the surface of the sample or the primary electron beam releases secondary electrons from the surface
- Detector is located on the same site of the sample as the electron source
- After fixation and dehydration, the sample is coated with a thin gold film
- The efficiency of backscattering / release of secondary electrons depends on the topography
- SEM produces a high-resolution image of the surface with depth of field
- Due to the lower acceleration voltage, SEM has a lower resolution (> 10 nm) than TEM, but SEM is also applicable to larger structures

Scanning technique



Environmental scanning electron microscopy (ESEM)



Basic ESEM gas pressure stages

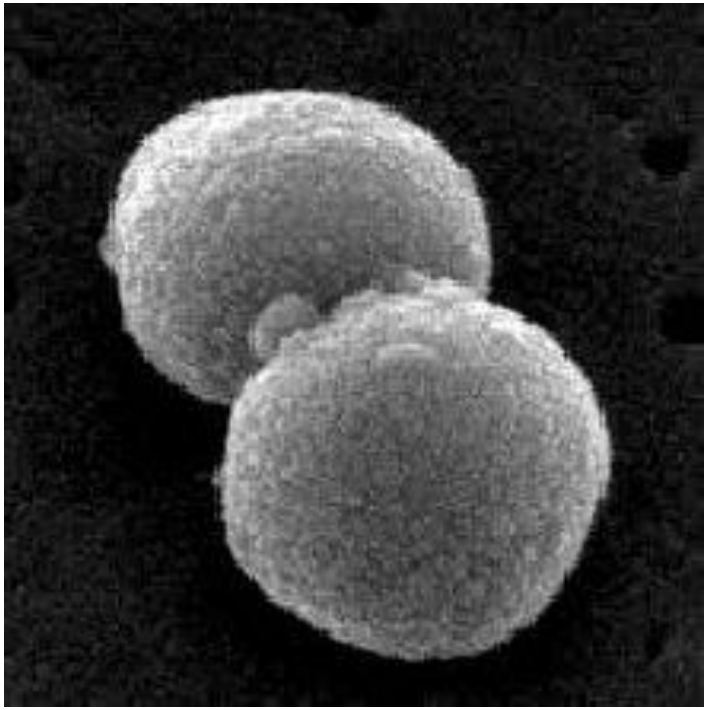
Differential pressure stages:

p_0 (ambient) >> p_1 >> p_2 (UHV, nP)

Sample preparation

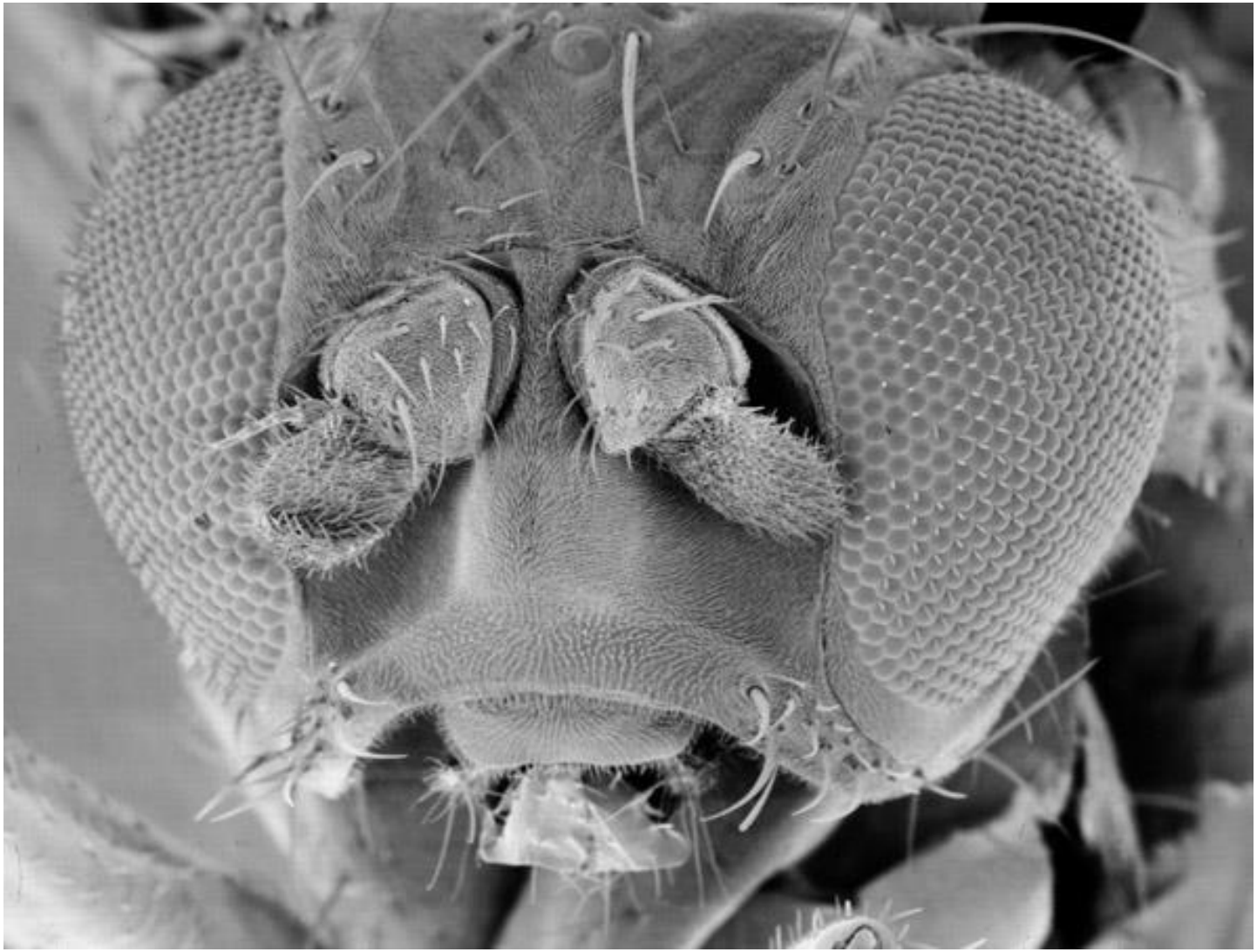
- Chemical fixation of the biological sample (glutaraldehyde, formaldehyde)
  => keeps the structure intact
- Dehydration by using increasing concentrations of alcohol
  (Water is replaced by more volatile alcohol)
- Drying at critical point: Alcohol is replaced by CO_2 at critical point
  (=> no phase transition between fluid / gaseous)
- A thin layer of a conductive metal (typically Au or Ag) is sputtered on the sample
- The sample must be grounded to avoid electric charging
- Microscopy

SEM images



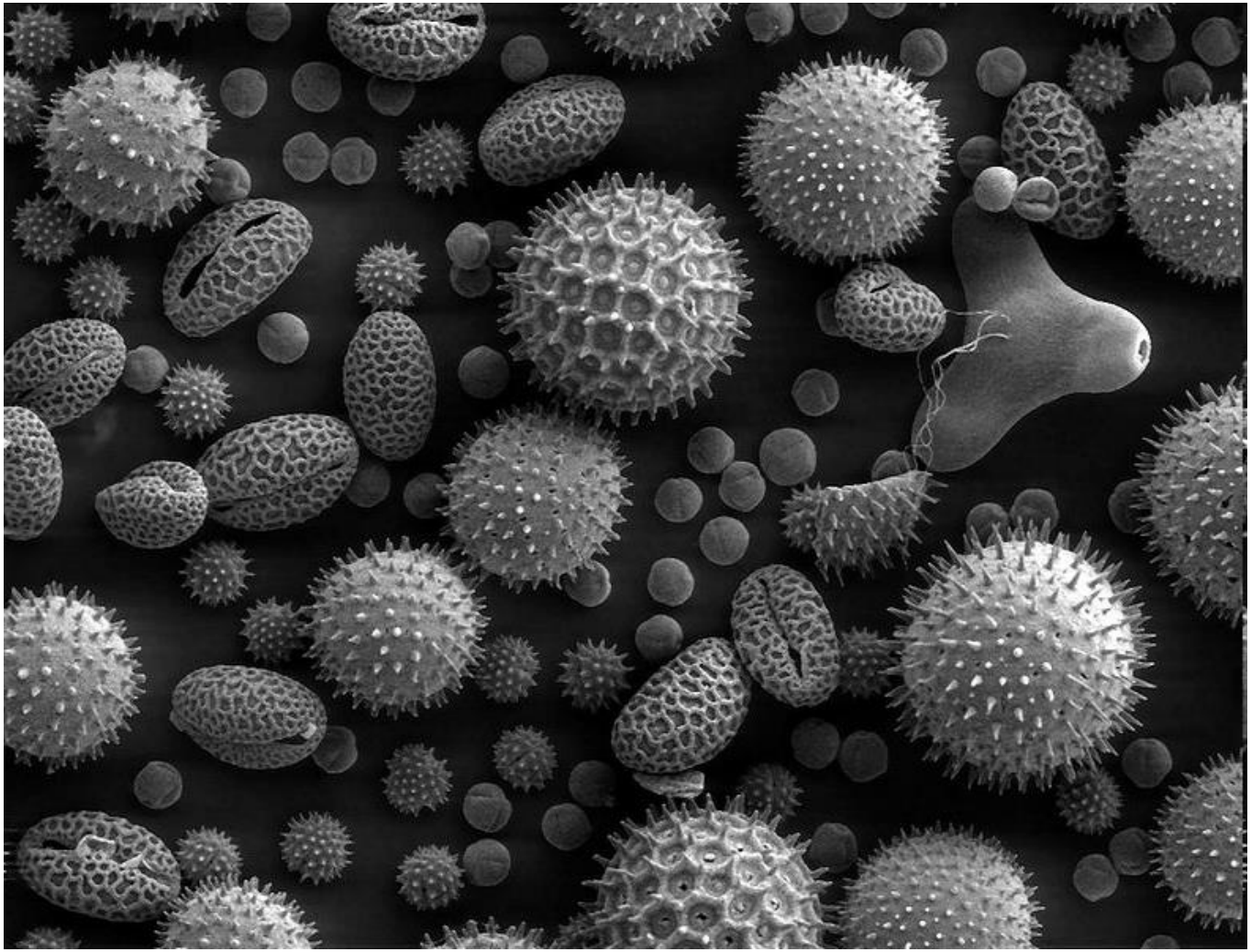
Streptococcus pneumoniae

SEM images



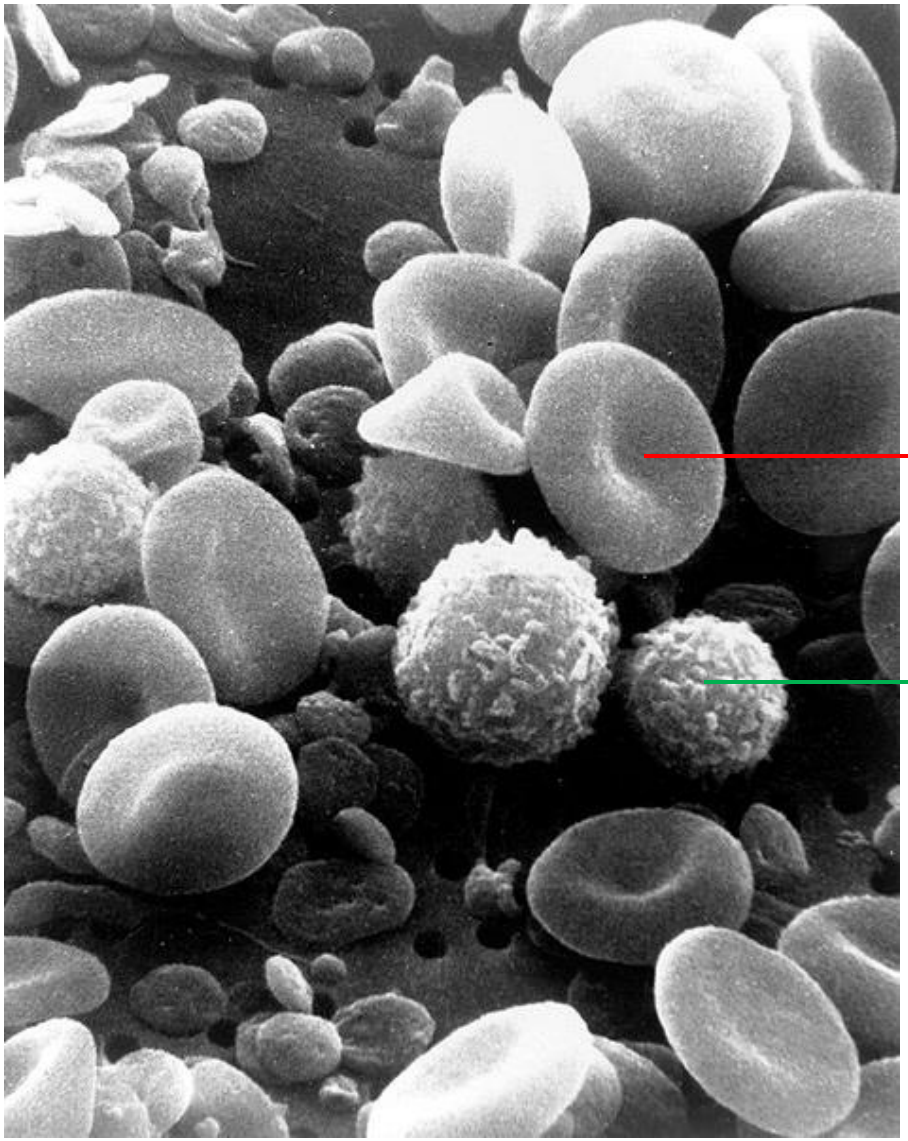
Insect‘s head

SEM images



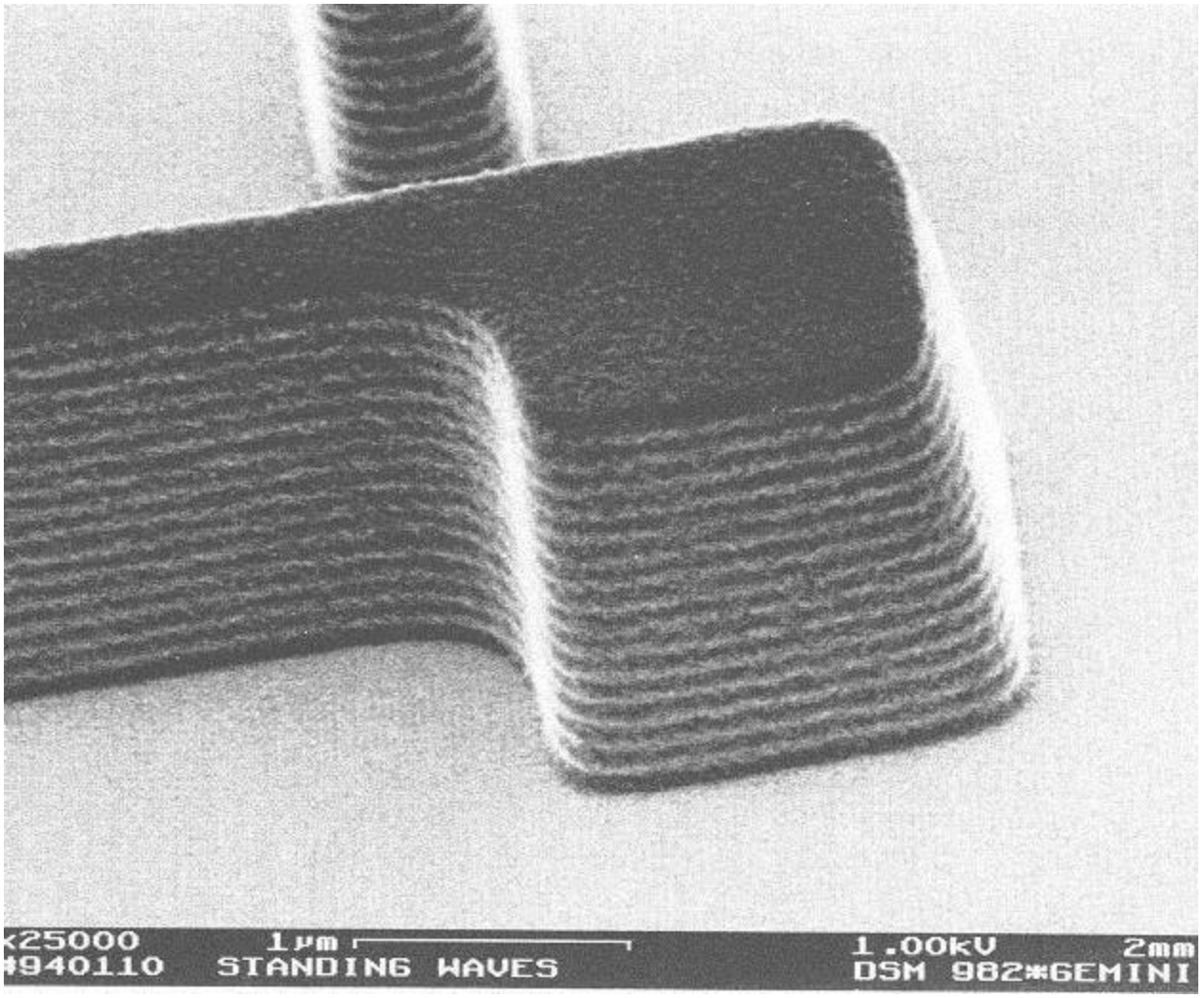
pollen grains

SEM images



blood cells

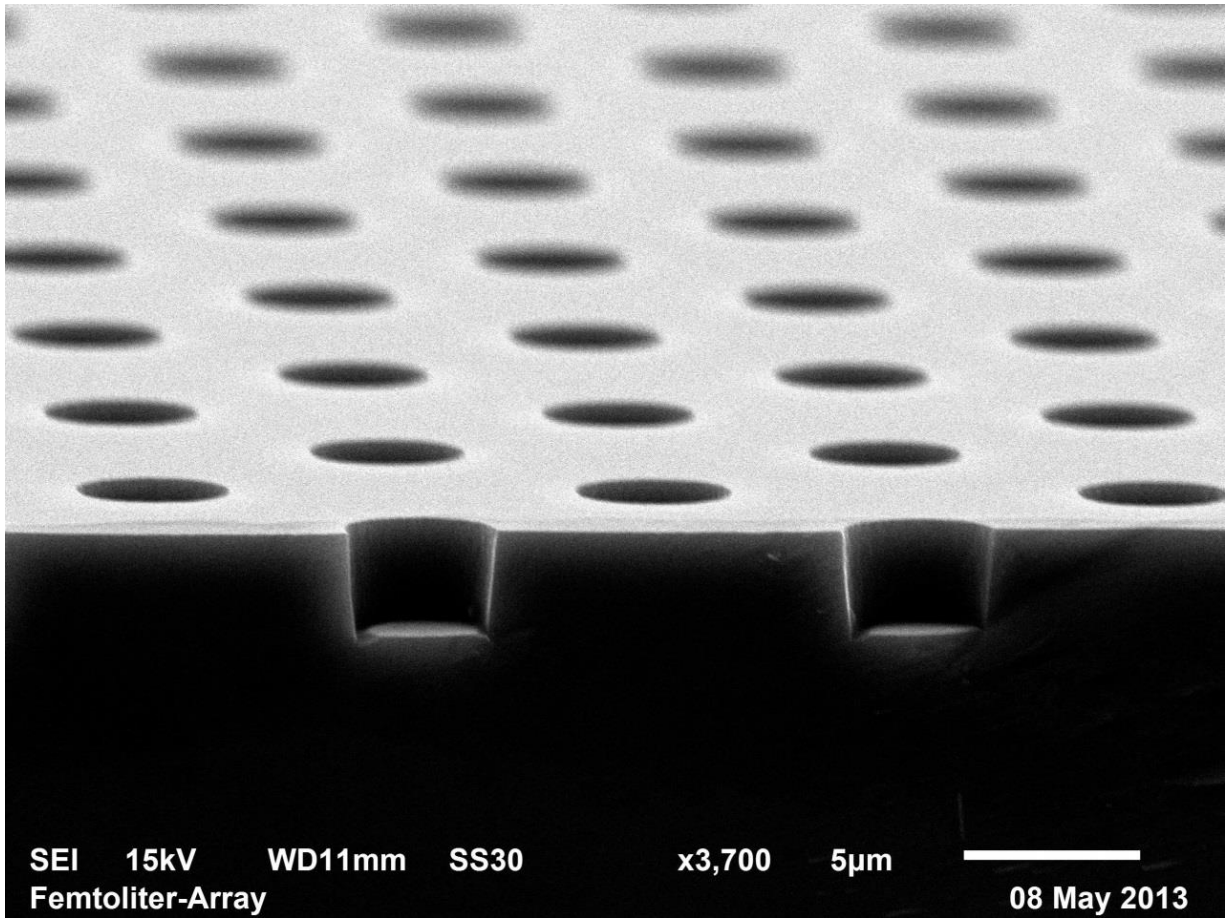
Technical samples



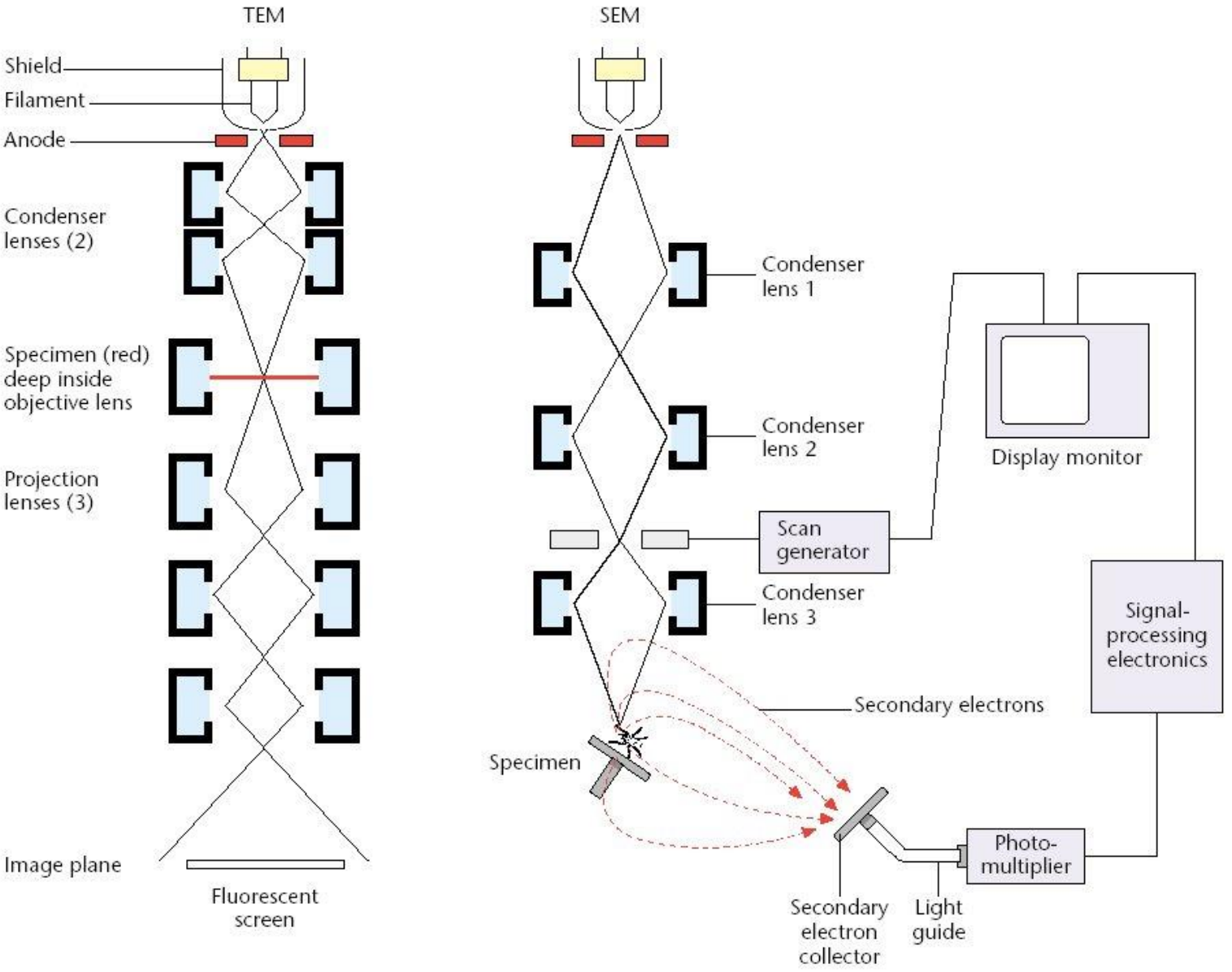
=> Additionally:
Elastic back-scattering provides information on material composition

Technical samples

Characterization of a surface structures prepared by lithography



Comparison of TEM and SEM



Inspirations for this lecture

- Textbooks
- Tero Soukka (University of Turku, Finland)
- Joachim Wegener (University of Regensburg, Germany)
- Martin Hof (Heyrovský Institute of Physical Chemistry, ASCR, Prague)

Oral exams

Preparation
=> It‘s time for your questions

Schedule of examination days

Wed, May 28th

09 - 10: Aneta Kozeleková / Veronika Krátka

10 - 11: Büşra Topçu / Dilara Karlıkaç

11 - 12: Akintunde Aremu Oluwasegun / Payal Makrand Oak

15 - 16: Martin Höchsmann / (Jana Kremzová)

Location: office 315, Institute of Biochemistry
Duration: ca. 60 min

Some questions from an old exam

Question 1:

(a) What are the advantages of fluorescence microscopy compared to electron microscopy (TEM)? Explain the reasons for these advantages.

(b) What is the equation for the limit of resolution and who described it first? Where is the limit of resolution of classic light microscopy and transmission electron microscopy and what does it depend on?

Some questions from an old exam

Question 2:

(a) Why is the background signal of conventional fluorescence microscopy too high to visualize single fluorescent molecules although the fluorophores in principle are bright enough for single molecule detection? Please explain the interfering phenomena shortly.

(b) Give the names of two approaches for super-resolution microsocopy and describe **one** of these in detail.

Some questions from an old exam

Question 3:

(a) Draw a sketch of of the beam path in a scanning electron microscope (SEM) and describe all essential elements in an SEM.

(b) What are the advantages of SEM as compared to TEM?

(c) How is the contrast generated in SEM?

(d) Why is the resolution of SEM lower compared to TEM? In what size range is the resolution limit of SEM?

Thank you for your attention