

BRIEF INTRODUCTION TO MICROSCOPY TECHNIQUES AND TO THE COURSE.

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Second Workshop / Course on Electron Microscopy

Lisboa, 29 May 2017



1590 - Two Dutch spectacle makers, Zacharias Jansen and his father Hans started experimenting by mounting two lenses in a tube, the first compound microscope.



1609 - Galileo Galilei develops a compound microscope with a convex and a concave lens.

1665 - Robert Hooke's book called Micrographia,



1674 - Anton van Leeuwenhoek used his knowledge of grinding lenses to achieve greater magnification which he utilised to make a microscope, enabling detailed observations to be made of bacteria.

1826 - Joseph Jackson Lister created an achromatic lens to eradicating the chromatic effect caused by different wavelengths of light.



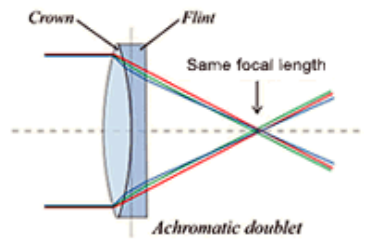
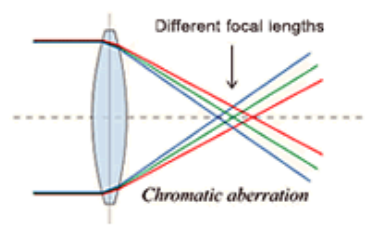
1860s - Ernst Abbe discovers the Abbe sine condition (a condition that must be fulfilled by a lens or other optical system in order for it to produce sharp images)



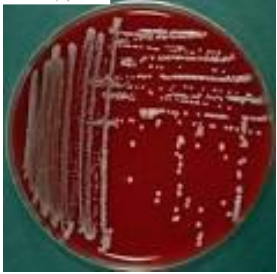
1931- Ernst Ruska at the University of Berlin, along with Max Knoll, combined these characteristics and built the first transmission electron microscope (TEM) in 1931, for which Ruska was awarded the Nobel Prize for Physics in 1986.

1965- First SEM commercially available.

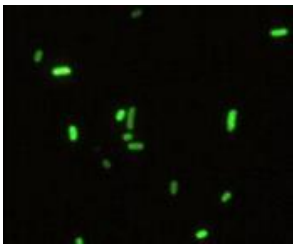
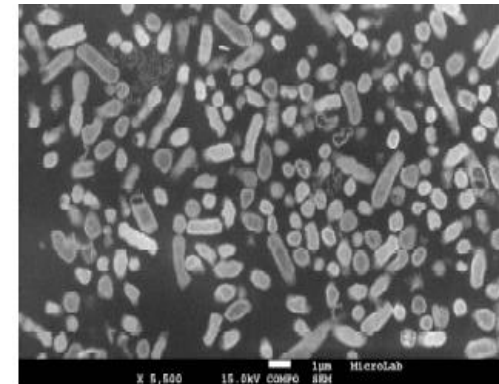
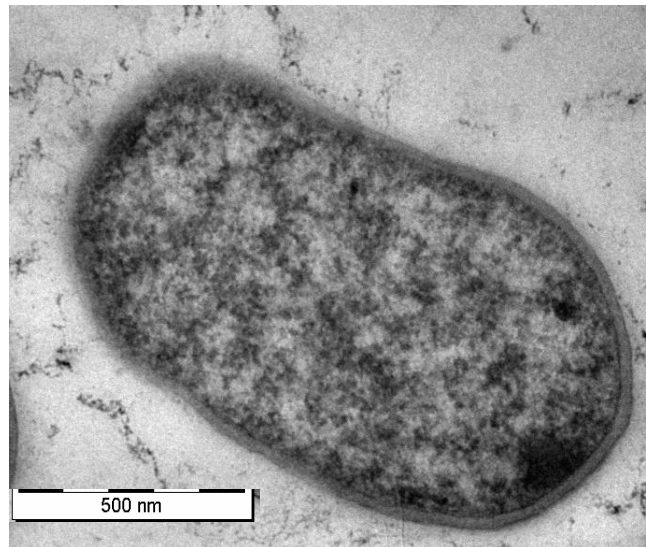
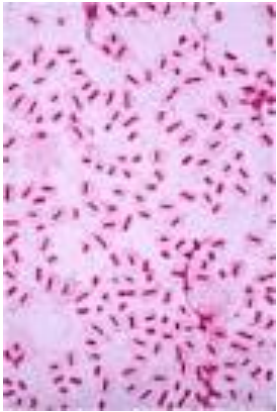
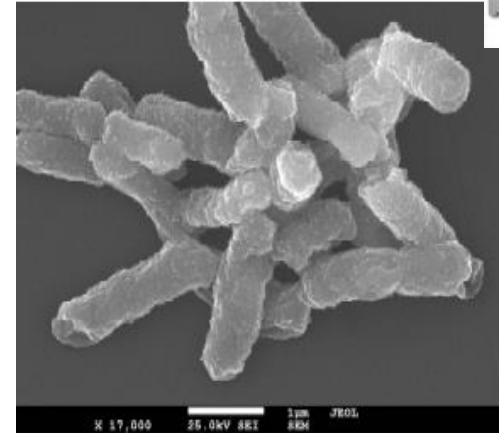
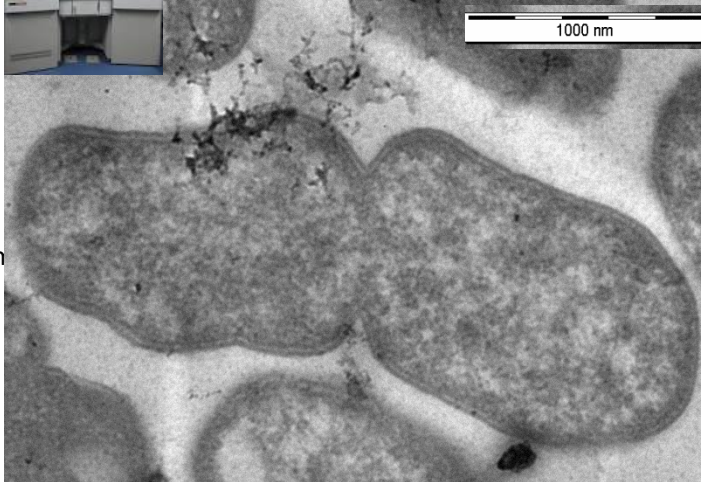
1990s- "Environmental" electron microscopes



Method / Image



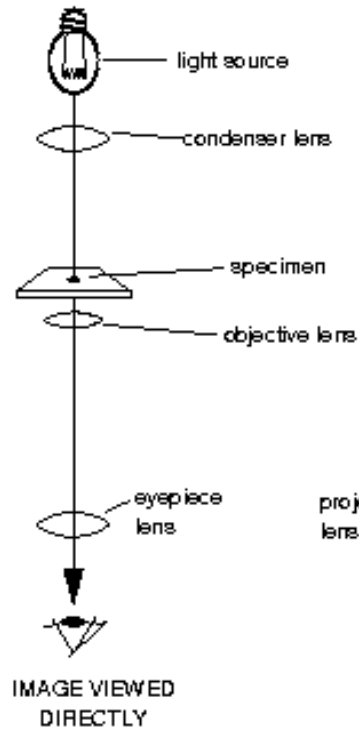
<http://klebsiella-pneumoniae.org/treatment.htm>



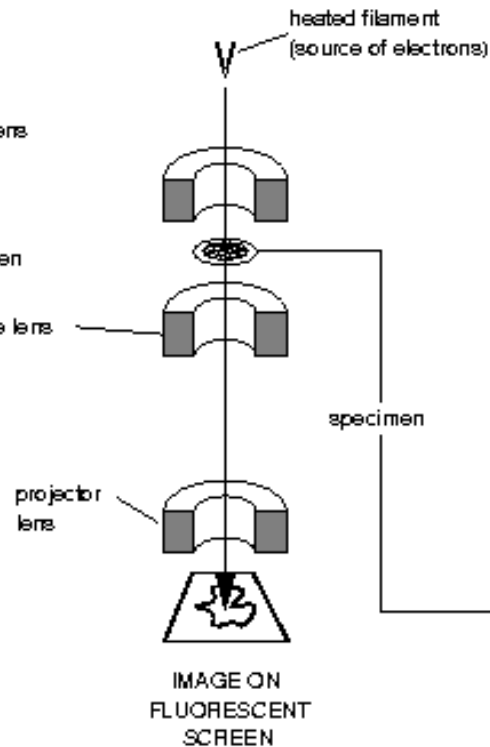
Brief introduction

Electron microscope

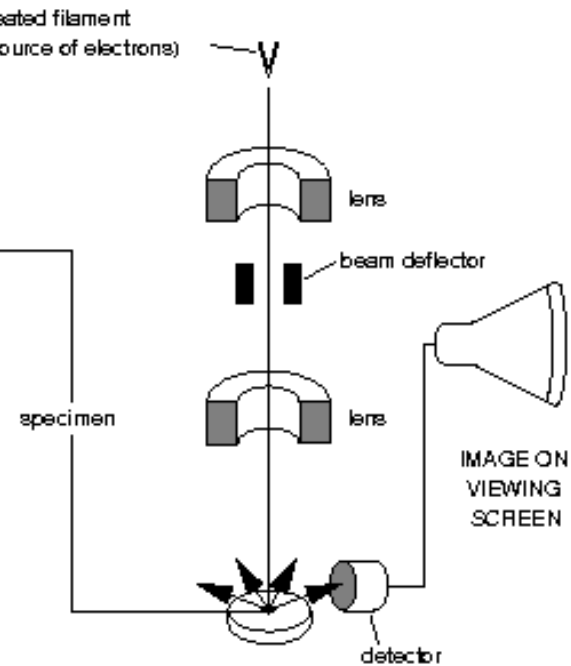
Light Microscope



Transmission (TEM)



Scanning (SEM)



Magnification ~ 2000 x

~1 500 000 x

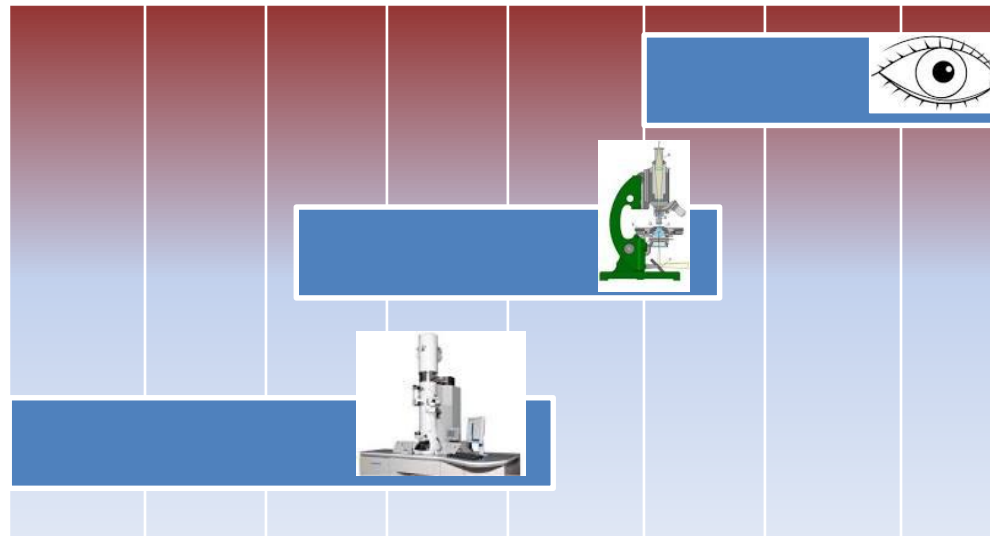
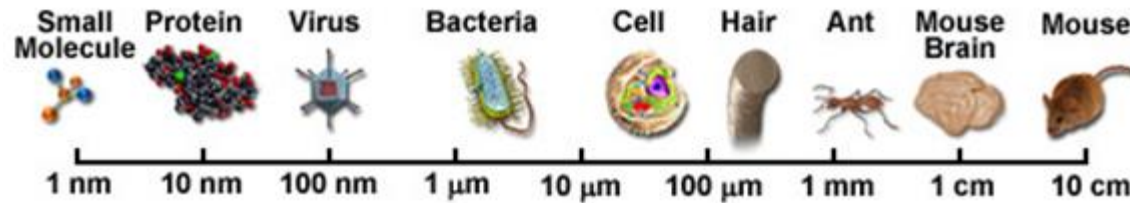
~ 1 000 000 x

Resolution 200 nm

0.1 nm

0.5 nm

Spatial Resolution of Biological Imaging Techniques



Adaptado de: <http://zeiss-campus.magnet.fsu.edu/print/superresolution/introduction-print.html>

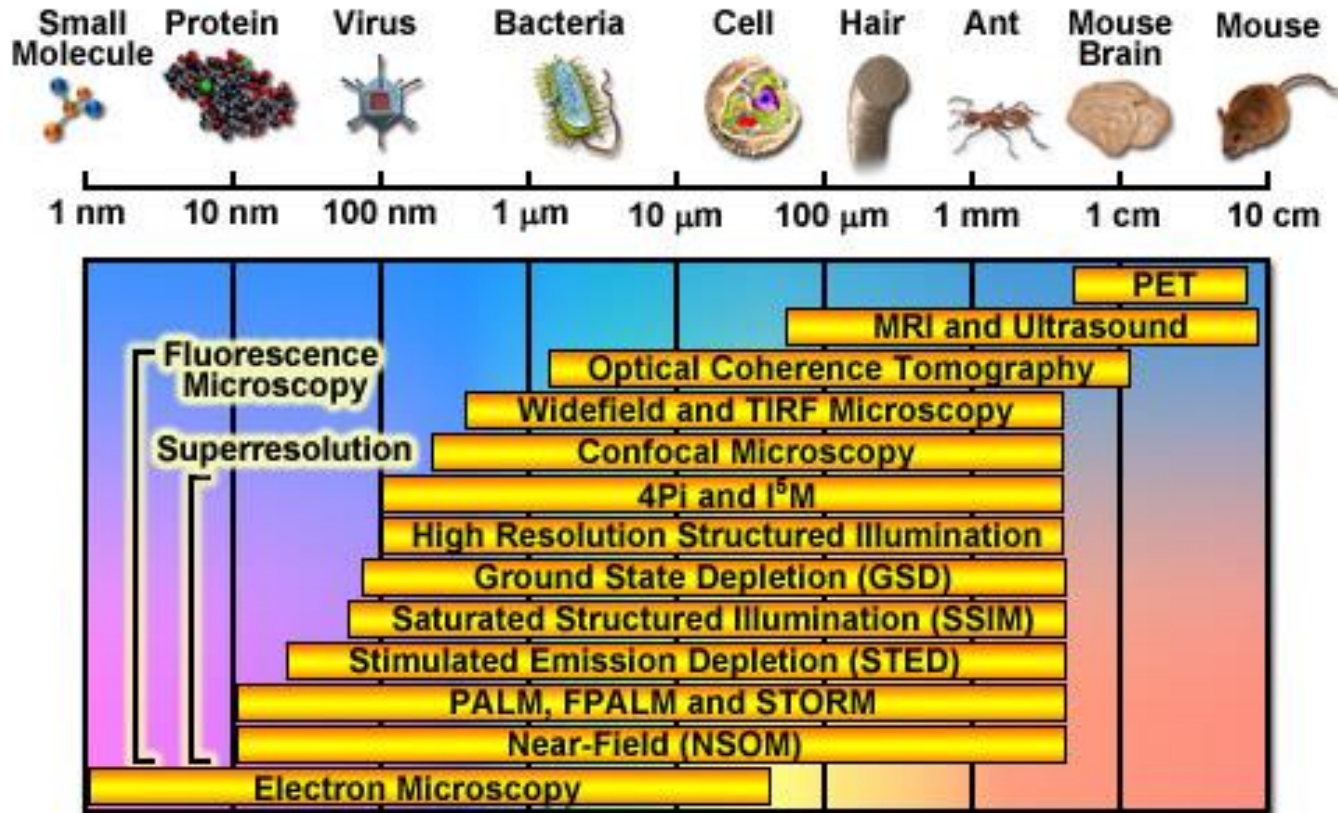
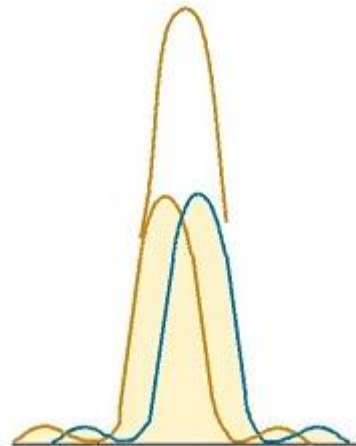
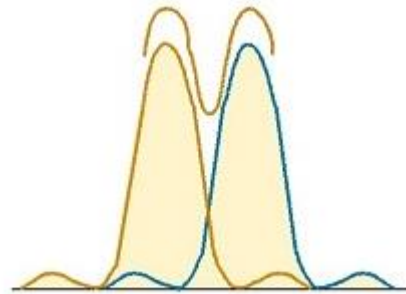
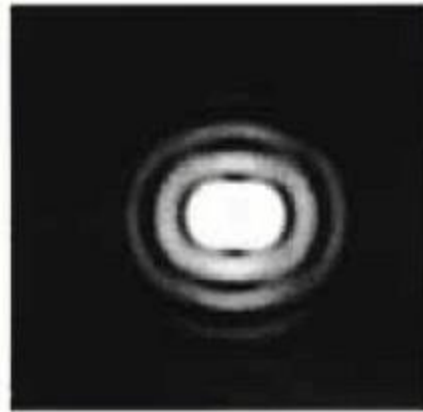
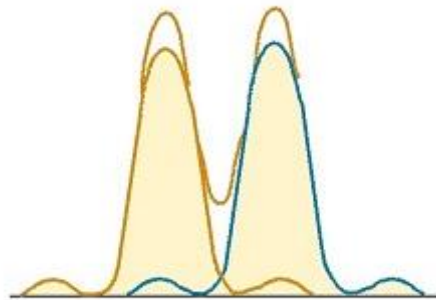
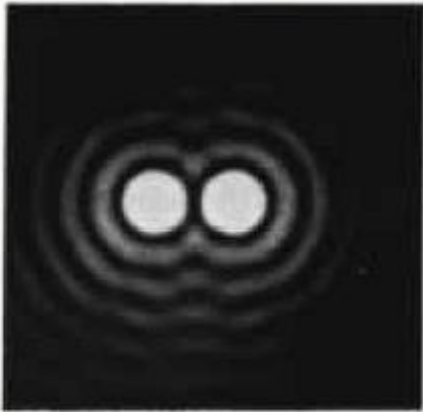


Figure 1

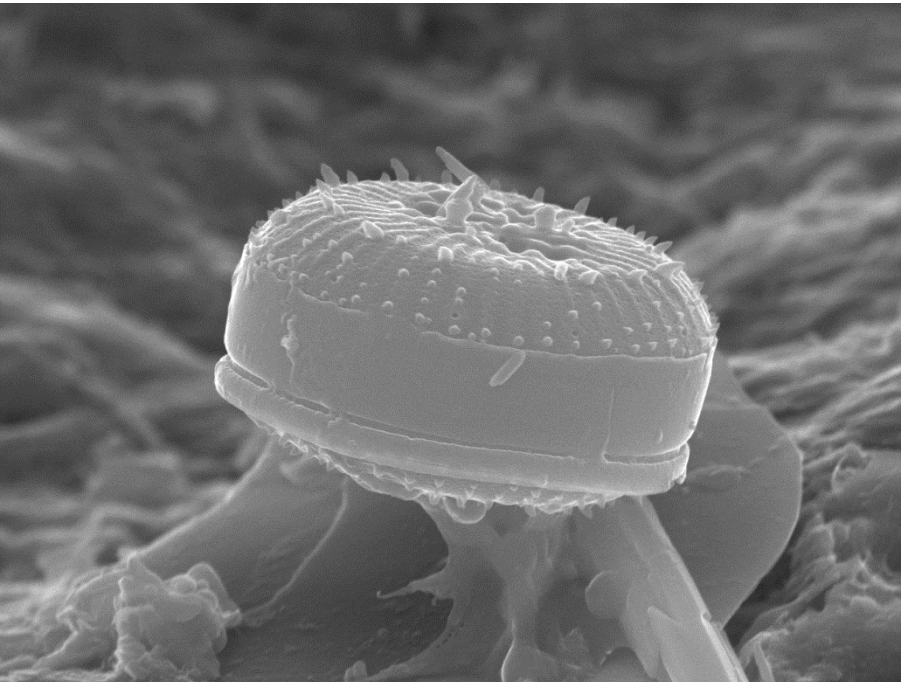


$$\rho = 0.6\lambda/(\eta\sin\alpha)$$

Light Microscope	Electron Microscope
$\lambda = 0.5 \mu\text{m}$	$\lambda \sim \sqrt{\frac{150}{V_0}} = 0.055 \text{ \AA} (@50 \text{ kV})$
$\eta = 1.5 \text{ (glass)}$	$\eta = 1.0 \text{ (Vacuum)}$
$\alpha = 70^\circ$	$\alpha = 1^\circ$
$\rho = 0.2 \mu\text{m} = 2000 \text{ \AA}$	$\rho = 0.00016 \mu\text{m} = 1.6 \text{ \AA}$

Cr terio de Rayleigh

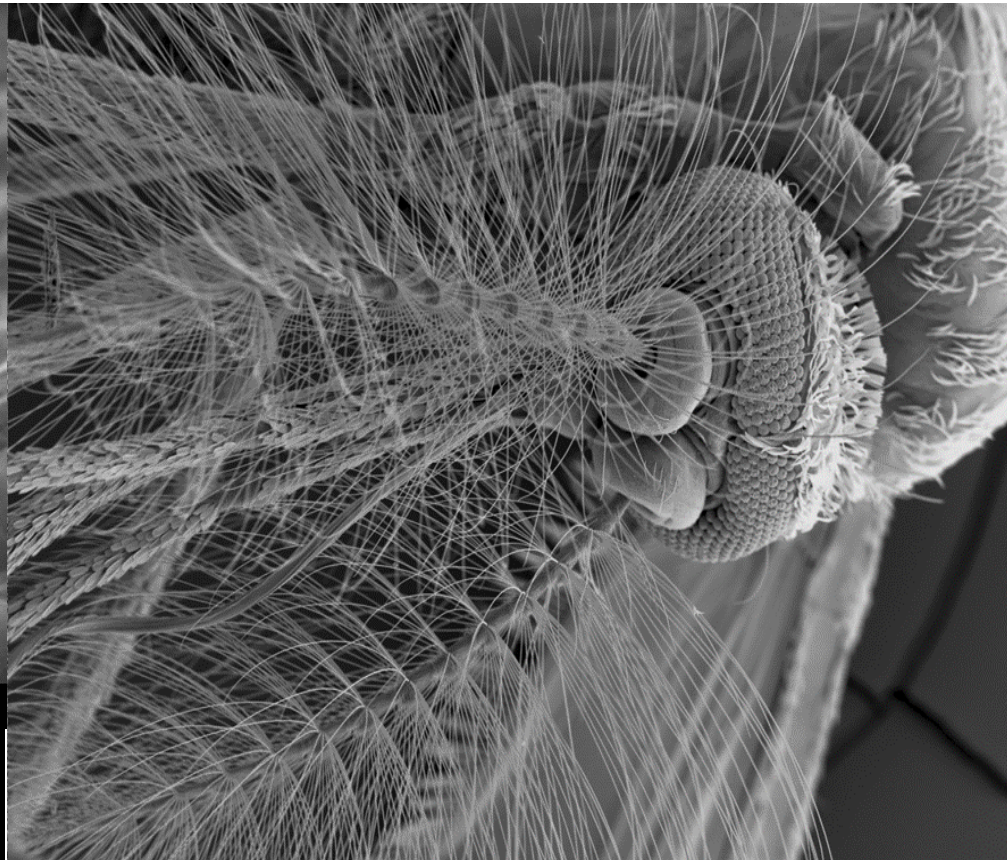
SEM – Typical Images



X 8,000

15.0kV SEI

1µm
SEM
MicroLab

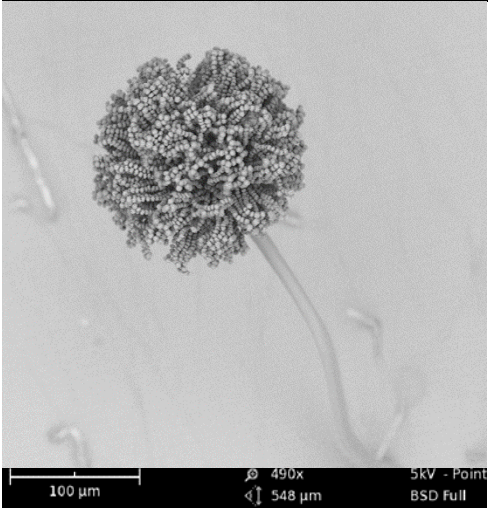
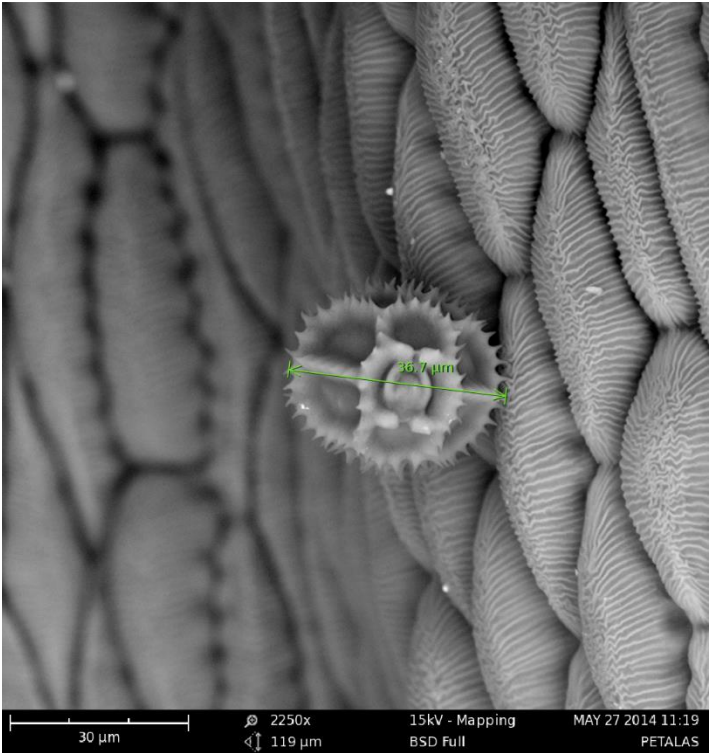


Beschleunigungsspannung	Vergrößerung	Arbeitsabstand	g_004.tif
15 kV	70 x	7.9 mm	-200 µm-



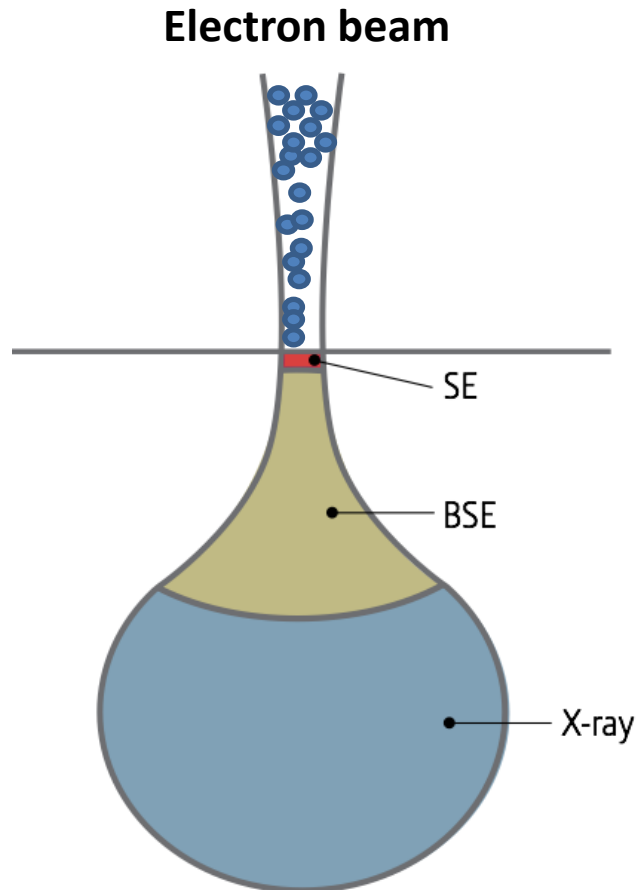
SEM: high depth of field.

SEM – Typical Images



Low Vacuum.

SEM – Beam/specimen interactions (signal types)



Of the many signal types emitted by the sample, secondary electrons (SE), backscattered electrons (BSE) and X-rays are the most used ones. A typical interaction volume has a pear-like shape and the size and the volume depends on many factors, including accelerating voltage and sample composition. **SE** have **higher spatial resolution** (since these electrons originate from a smaller volume), than **BSE** and then **X-rays**: The resolution of the signals primarily depends on the emitting diameter that in turn depends on the primary beam diameter at the sample.

Biological structures collapse under vacuum...

Fixation: Stabilization of biological material.

Post- Fixation: OsO₄.

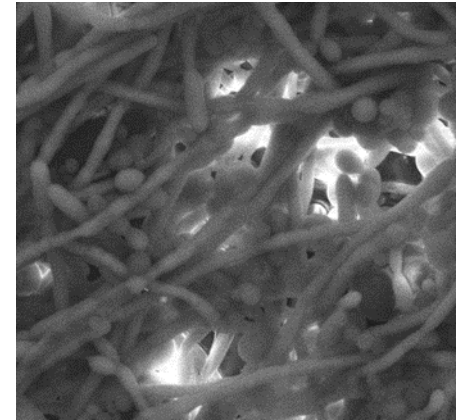
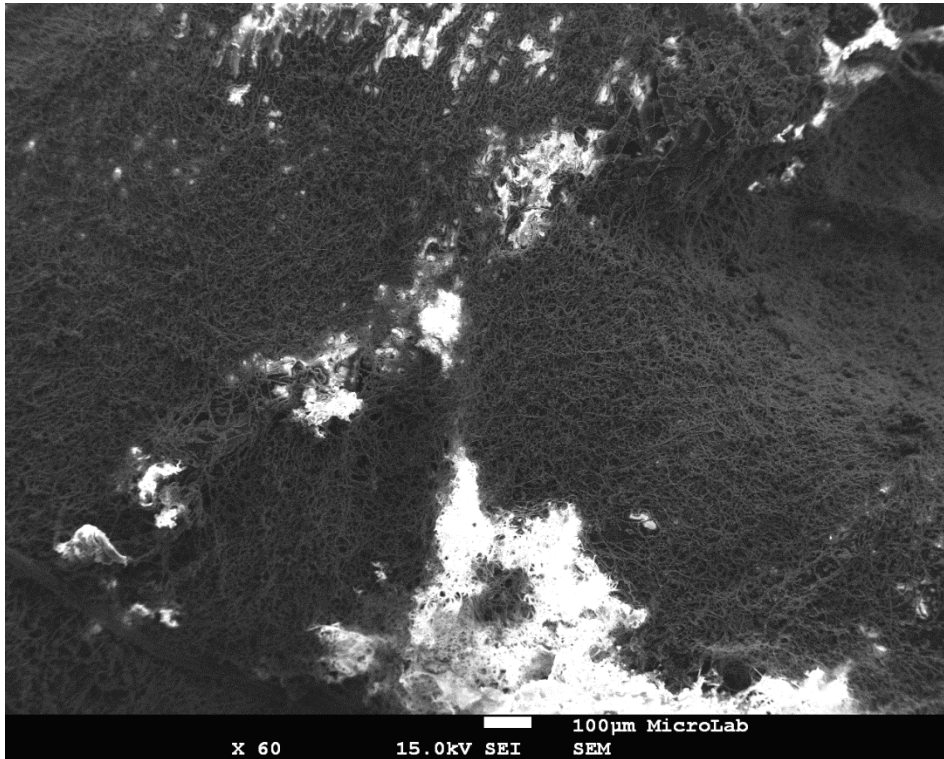
Dehydration: Substitution of water with solvent (ethanol, acetone) Usually performed with gradient of different concentrations.

Critical point drying: replace ethanol with CO₂ brought to critical point (31.1 C and 1,073 psi), becomes dense vapor phase; Gaseous CO₂ vented slowly to avoid condensation.
Or Hexametildisilizane (HMDS) drying: transition from ethanol to HMDS (reduces capillarity effects during drying), left overnight in desiccator with silica gel.

Mounting: on a stub that can be inserted in the SEM sample holder.

Coating: with conductive layer. Prevent charging effects that hinder suitable image formation.

Sample preparation - Coating



Advantages vs Disadvantages of SEM

Advantages

- high depth of field
- direct observation of the external form of real objects at high magnifications
- wide range of magnifications (below 50 x to over 100 000x)
- local chemical and crystallographic analyses

Disadvantages

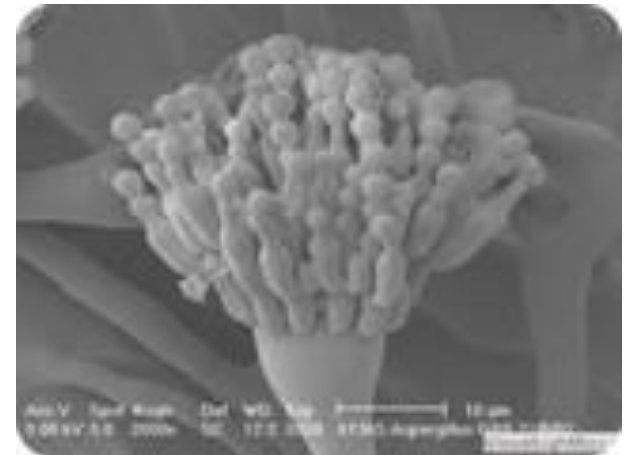
- high vacuum environment of the specimen
(difficult with porous materials, damaging for biological samples...)
- inability to show internal detail
- inability to obtain highest resolution
- conductive layer: The samples need to be conductive because otherwise they tend to charge.

Cryo-scanning electron microscope (Cryo-SEM)

A [cryo-scanning electron microscope](#) is a conventional SEM that has been fitted with specific equipment that allows samples to be viewed in the frozen state. This is particularly useful for directly viewing hydrated (wet) samples, delicate biological samples, hydrogels, food, biofilms, foams, fats, and waxes, suspensions, pharmaceuticals and nanoparticles. The sample can be snap frozen outside the machine and then inserted in its frozen state, or placed into the machine in an unfrozen state and frozen more slowly in the machine. It is imaged using either [secondary electrons](#) (SE) or [backscattered electrons](#) (BSE). Frozen samples can also be fractured or cut during preparation to reveal internal structures.



Pollen - Pyracantha



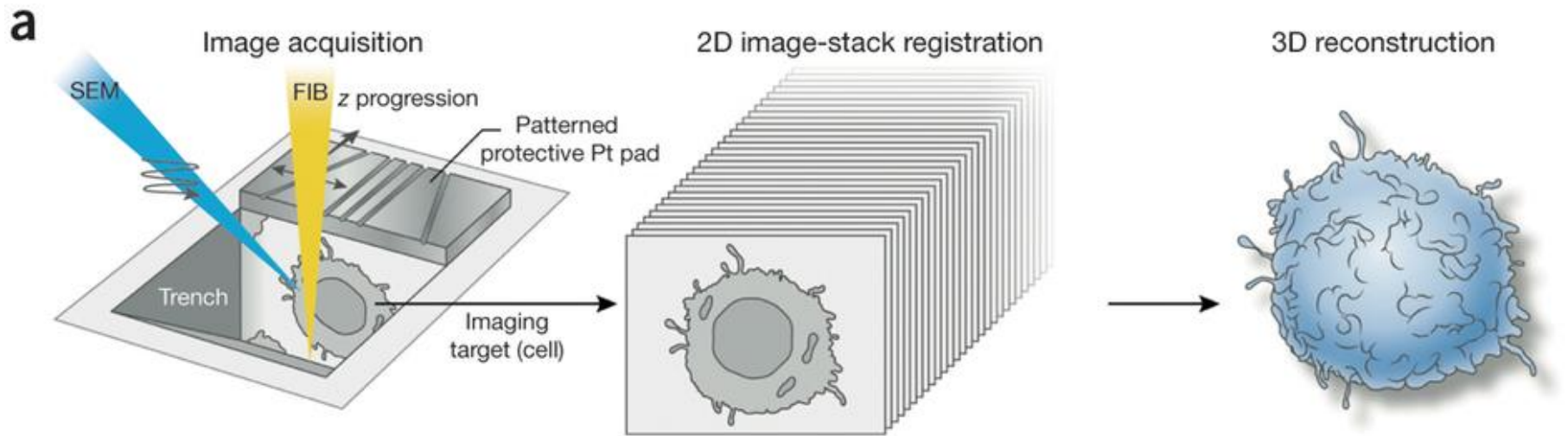
Aspergillus

Focused ion beam (FIB) technology: FIB- SEM

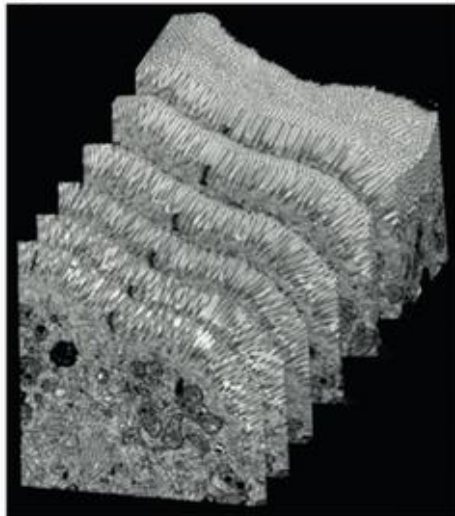
This technology involves using an **ion beam** (typically gallium ions) directed onto a hard sample. The beam is focussed to an extremely fine probe size (<10 nm) onto the surface of a specimen. The sample can be sectioned or shaped with the ion beam while it is being monitored by scanning electron microscopy (SEM). These sections can be taken off as sequential sections, each viewed in turn with the SEM mode, and this imaging information used to construct a 3D image.

When milling a region or cutting a piece out of a sample, it is important to firstly lay down a strip of metal that will stop the ion beam from eroding that region. This allows a well defined edge to be achieved on the area being excised.

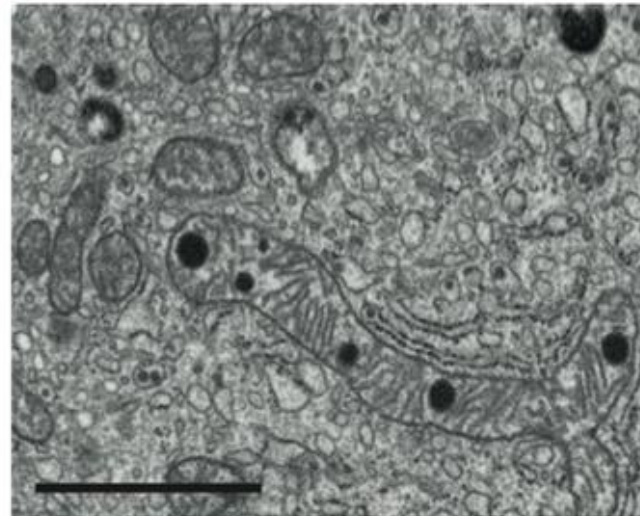
Machines can have both ion and electron columns on a single instrument (called dual beam instruments). The advantage of dual beam machines is that they allow specimens to be imaged in detail using the electron beam, without damaging the surface of the specimen with the ion beam.



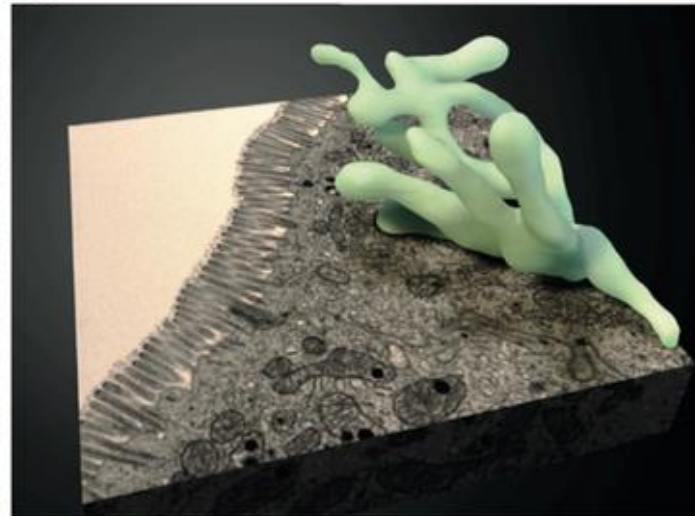
b



c

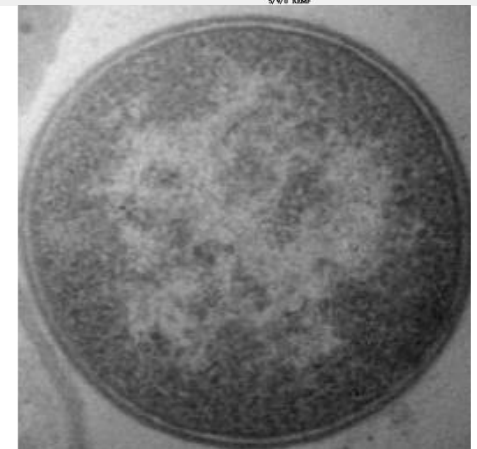
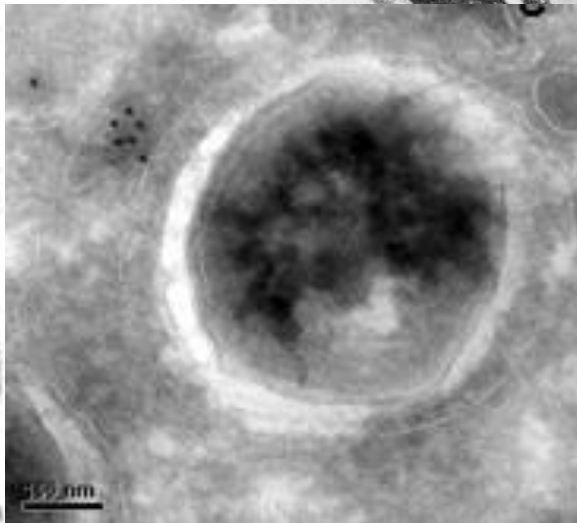
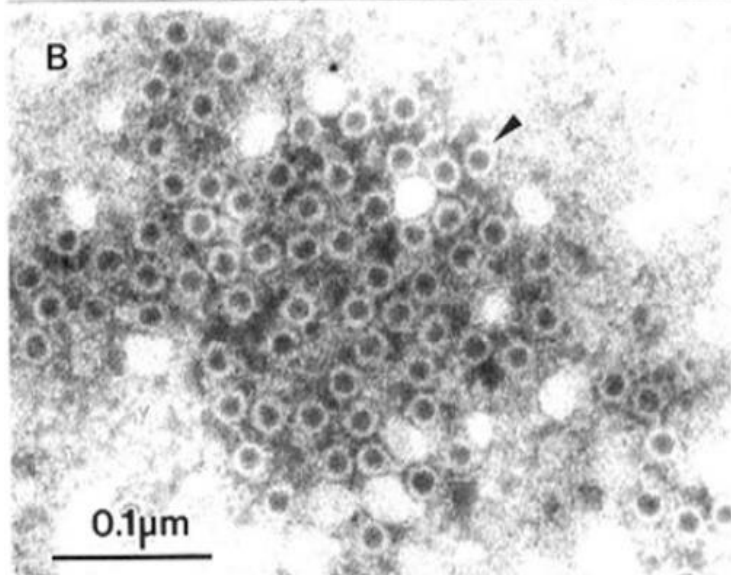
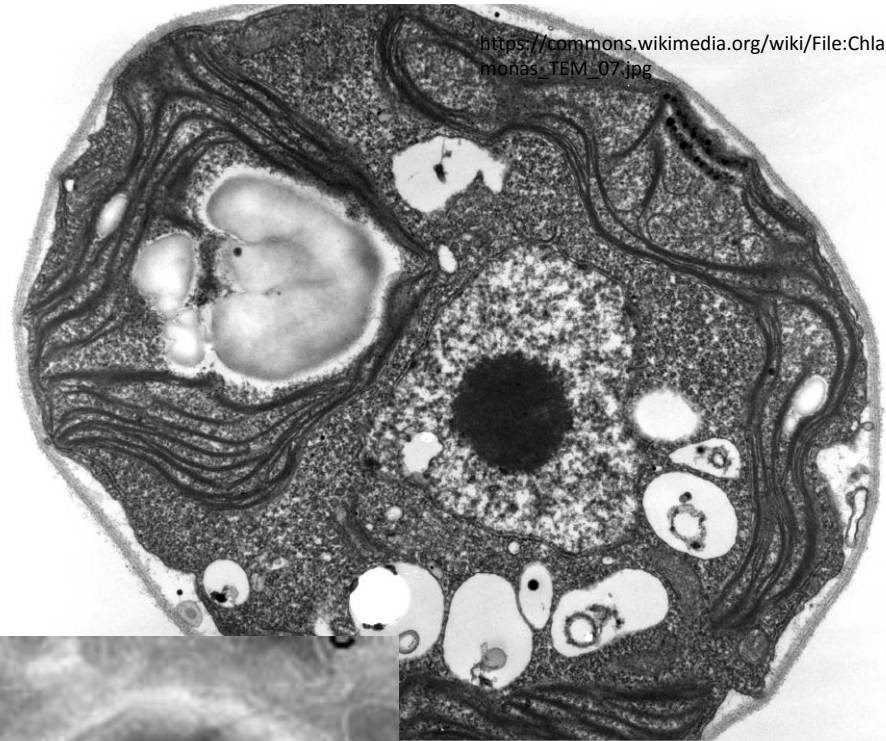


d

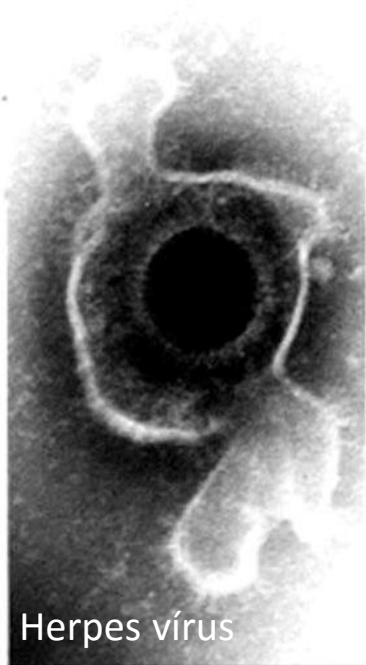
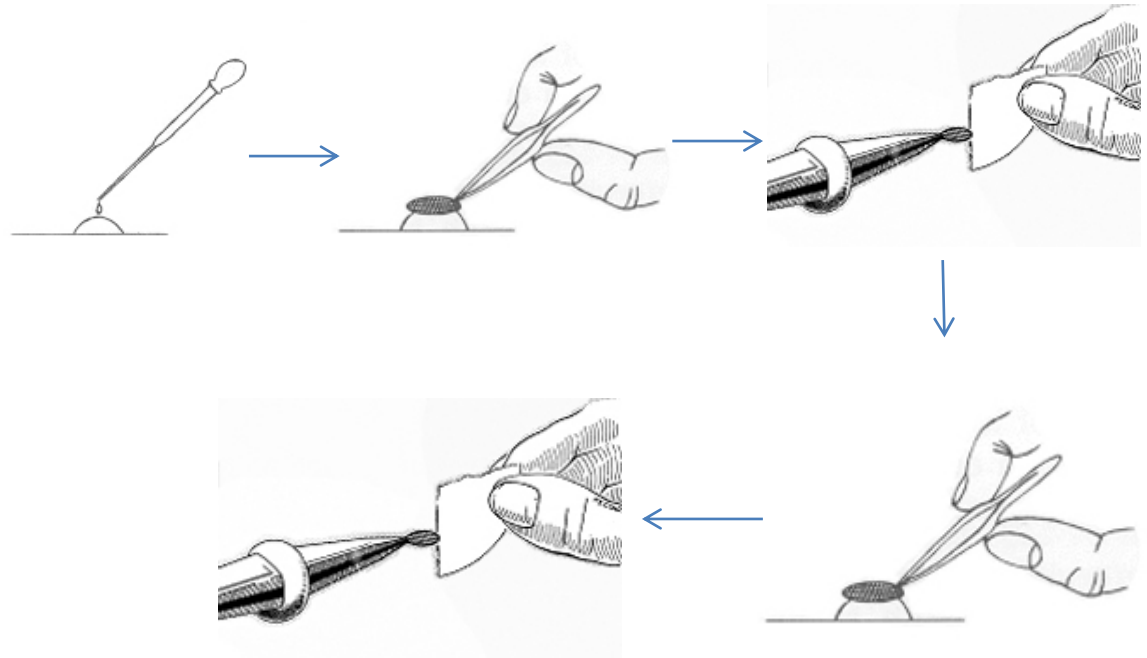
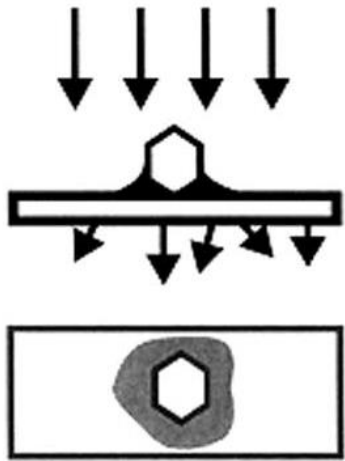


From: Focused ion beams in biology. Kedar Narayan & Sriram Subramaniam *Nature Methods* 12, 1021–1031 (2015) doi:10.1038/nmeth.3623

TEM - Images



Negative Contrast



Fast

Viruses analysis

SPIEM: Solid-phase immunoelectron microscopy

Herpes virus

Sample preparation

Fixation: Stabilization of biological material.

Post- Fixation: OsO₄.

Dehydration: Substitution of water with solvent (ethanol, acetone) Usually performed with gradient of different concentrations.

Embedding: material is gradually infiltrated with the still unpolymerized resin.

Trimming of the block of resin and ultrathin sectioning: Sections are cut using an ultramicrotome.

Picking up sections on a grid: Sections are contrasted.

TEM - Limitations

- Extensive sample preparation
- Time consuming with low throughput
- Structure of the sample might change during the process
- Small field of view may not give conclusive results of the all sample