SEM TEM

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Outline –

1. What is electron microscopy (EM) and why it is used

2. Interaction electron beam – matter and contrast mechanisms for image formation

3. Types of EM:
   - Transmission Electron Microscopy (TEM)
   - Scanning Electron Microscopy (SEM)

4. Electron Microscopy application to biological samples

5. Cryo EM – why and how for biological samples

1. What is electron microscopy (EM) and why it is used

- EM are imaging techniques using a beam of accelerated electrons as illumination source for obtaining high resolution images of biological and non-biological specimens.
- EM is used in biomedical research to investigate the detailed structure of tissues, cells, organelles and macromolecular complexes. EM images provide key information on the structural basis of cell function and of cell disease.
- EM is used in conjunction with a variety of ancillary techniques (e.g. thin sectioning, immuno-labeling, negative staining) to answer specific questions.
- The high resolution of EM images results from the use of **electrons** (which have very short wavelengths) as the source of illuminating radiation.

Louis de Broglie (Nobel Prize 1929)

postulated the **wave nature of electrons** (1924) and introduced the wave-particle duality theory of matter (any moving particle has an associated wave).

Some properties of electrons

e^- Very similar to photons: → Wave-particle duality

→ Optical properties: diffraction, chromatic & spherical aberrations, etc.

→ Resolution depends on aperture and wavelength (Diffraction limited resolution)

Abbe's equation **d = 0.5 λ/NA**

$$NA = n \cdot \sin \alpha$$

Because of the light wavelengths greater than ~400 nm, the best resolution that can be achieved by optical microscopes is about ~200 nm.

One way to beat the diffraction limit of light is to use an illumination source with a shorter wavelength than photons in visible region:

Maggiore risoluzione ➔ λ minore

Energia vs lunghezza d'onda

Fotoni energia $\in$ [6eV – 120keV]

- EUV $\lambda \in$ [10nm,200nm]
- Raggi x molli $\lambda \in$ [1nm,10nm]
- Raggi x duri $\lambda \in$ [0,01nm,1nm]

Elettroni energia $\in$ [1eV – 120keV]

$$\lambda = ?$$

Louis de Broglie relationship: $\lambda = \frac{h}{p}$ or $\lambda = \frac{h}{mv}$

where h is Planck's constant h=6.626 x 10^{-34} J s,
and p is the momentum of an electron of mass m moving with velocity v.

The velocity of the electrons is determined by the accelerating voltage, or electron potential V:

$$eV = \frac{1}{2}mv^2 \longrightarrow v = \sqrt{\frac{2eV}{m}}$$

Therefore, the wavelength of propagating electrons at a given accelerating voltage can be determined by

$$\lambda = \frac{h}{\sqrt{2meV}}$$

Since the mass of an electron is m= 9.1 x 10^{-31} kg and e = 1.6 x 10^{-19} C:

$$\lambda = \frac{6.62*10^{-34}}{\sqrt{2*9.1*10^{-31}*1.6*10^{-19}*V}} = \frac{12.25*10^{-10}}{\sqrt{V}}$$

If the microscope is operated at V=100 keV, the wavelength of electrons is calculated to be
λ = 3.88 pm (1 pm= 10-12 m= 10-3 nm), **λ** = 2.74 pm @ 200 KeV, **λ** = 2.24 pm @ 300 KeV

However, because the velocities of electrons in an electron microscope reach about 70% the speed of light, with an accelerating voltage of 200 keV, there are relativistic effects on these electrons. These effects include significant length contraction, time dilation, and an increase in mass.

By accounting for these changes,

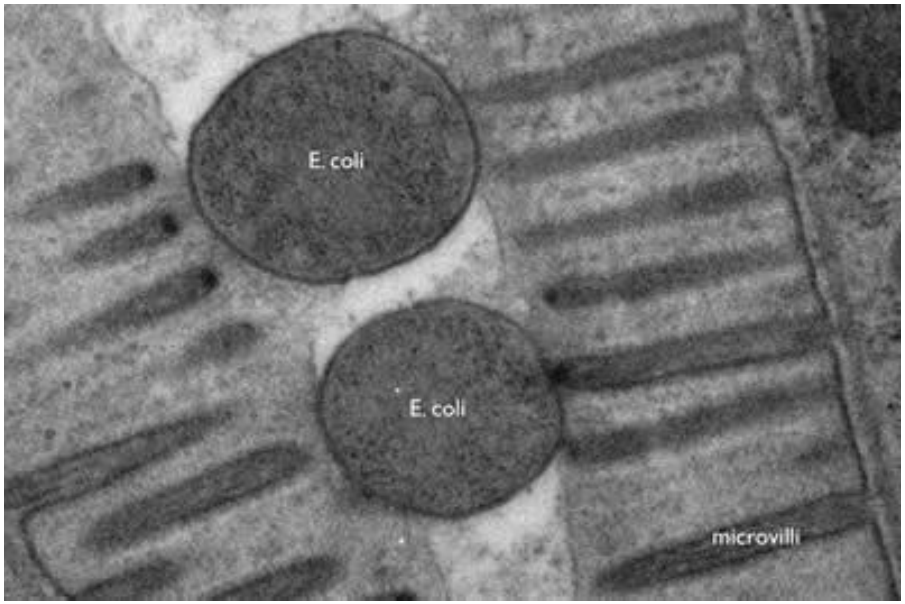
$$\lambda = \frac{12.25 * 10^{-10}}{\sqrt{V}} * \frac{1}{\sqrt{1 + \frac{eV}{2mc^2}}}$$

where c is the speed of light, which is ~3 x 10^8 m/s.
Therefore, the wavelength at 100 keV, 200 keV, and 300 keV
in electron microscopes is 3.70 pm, 2.51 pm and 1.96 pm, respectively

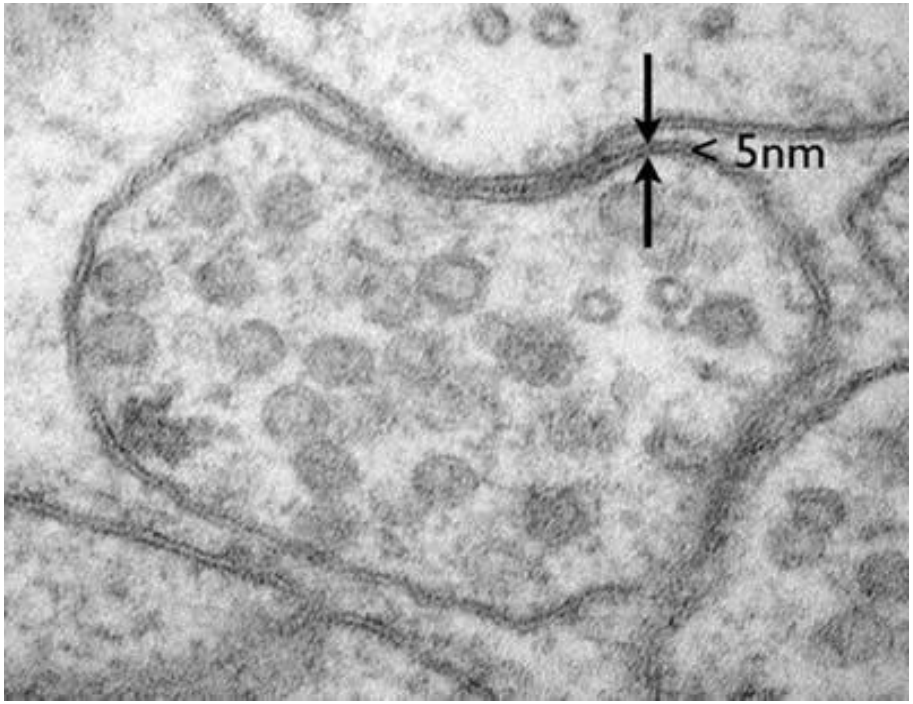
In conclusion:

The wavelength in an EM is much lower than in an optical microscope (OM).
Hence, EM can provide a much higher resolution than OM.

However, the numerical aperture NA is lower for EM than for OM.
Hence the effective resolution is of the order of nanometer and not picometer.



Examples of EM images electron microscopy can resolve subcellular structures that could not be visualized using standard fluorescences microscopy, such as the microvilli of intestinal cells or the internal structure of a bacterium (Figure 1).



EM can even resolve molecular structure in a cell, for example the plasma membrane is only about 5nm in diameter. The membrane is composed of a lipid bilayer, each layer is a single molecule thick. These fatty acids are about 20 carbons long with a hydrophilic head group. These individual lipid layers can be distinguished in an electron micrograph (Figure 2).

https://advanced-microscopy.utah.edu/education/electron-micro/index.html

Electron - material interaction with application to EM

The different types of electron scattering are the basis of most EM methods.

These interactions can be classified in two types:

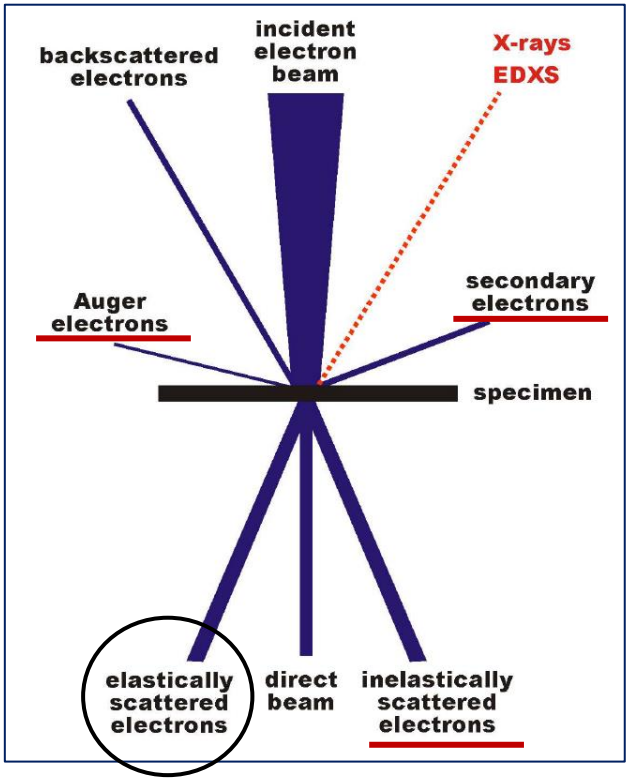
ELASTIC and INELASTIC

according to the energy transfer from the electron to the sample

Example

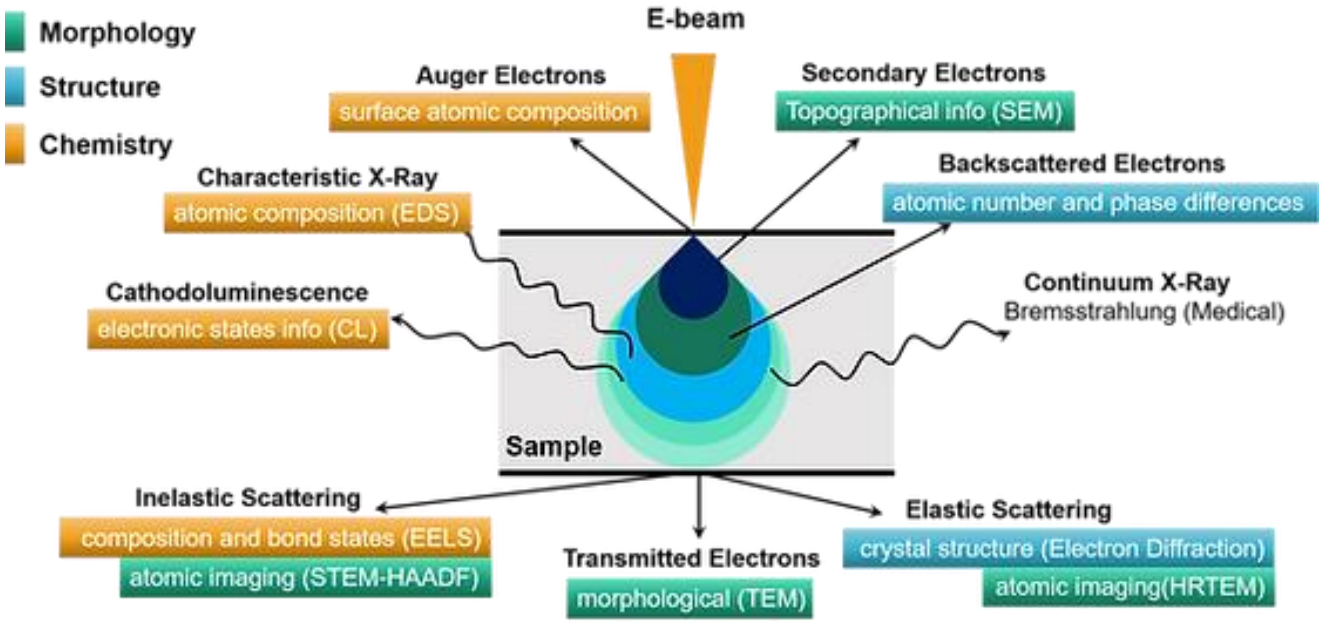
- elastic: no energy transfer: Coulomb interaction with the positive potential inside the electron cloud

- Inelastic: the energy transferred to the specimen can cause different signals as X-rays, Auger or secondary electrons, plasmons, phonons, UV or cathodoluminiscence

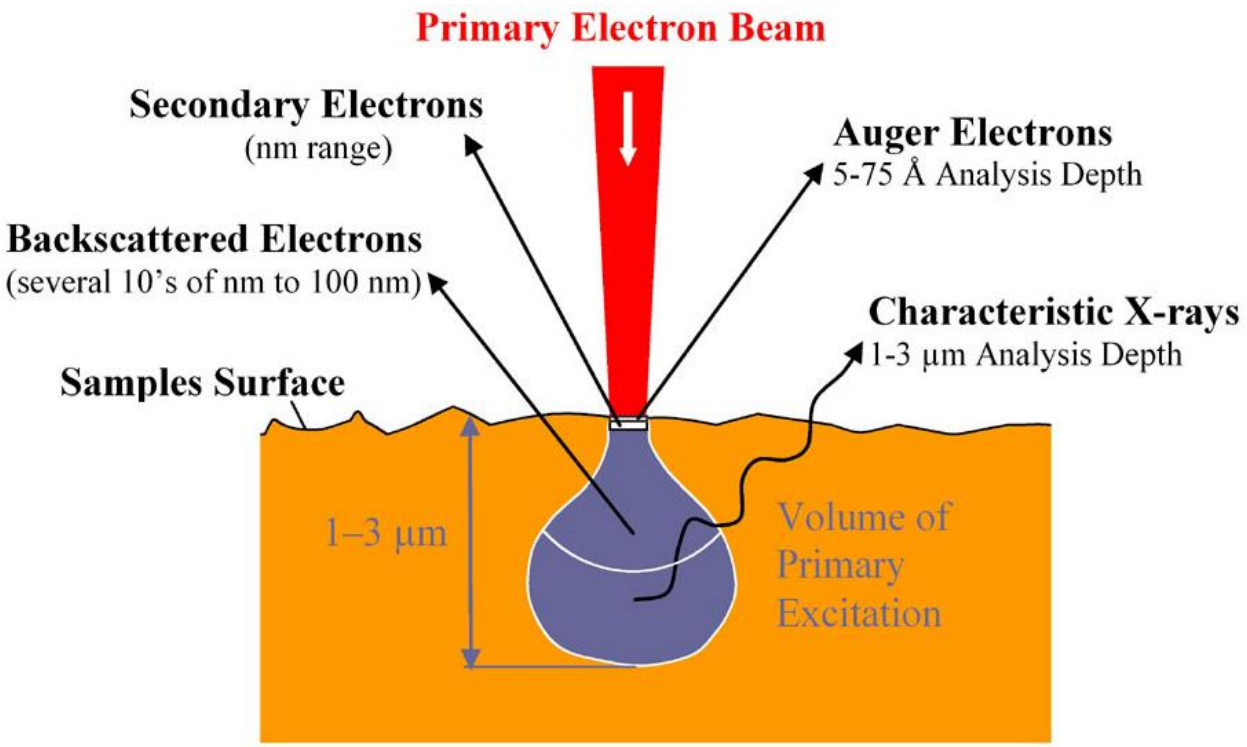


Scheme of electron-matter interactions arising from the impact of an electron beam onto a specimen. A signal below the specimen is only observable if the thickness is small enough to allow some electrons to pass through.

How can be used the emitted radiation for sample investigation?



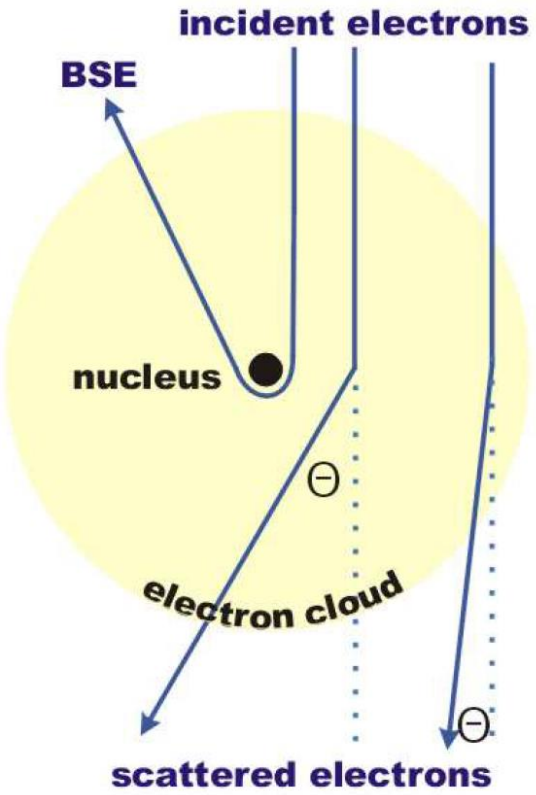
Localizzazione della provenienza dei vari segnali



A causa della bassa energia degli elettroni secondari (2-5eV) solo gli elettroni emessi in prossimità della superficie contribuiscono a formare il segnale dei secondari.

Per questa ragione gli elettroni secondari *utili* vengono emessi in prossimità del fascio prima che questo si allarghi nella pera di interazione e sono utilizzati per generare immagini ad alta risoluzione

Scattering of electrons by an atom
Elastic interaction

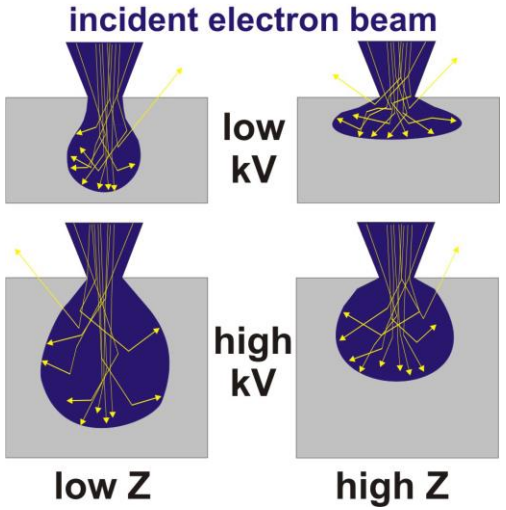


Scattering of an electron inside the electron cloud of an atom.

$$F = Q_1 Q_2 / 4\pi\varepsilon_o r^2$$

Thus, the
Coulomb force increases with increasing
atomic number Z of the respective element.

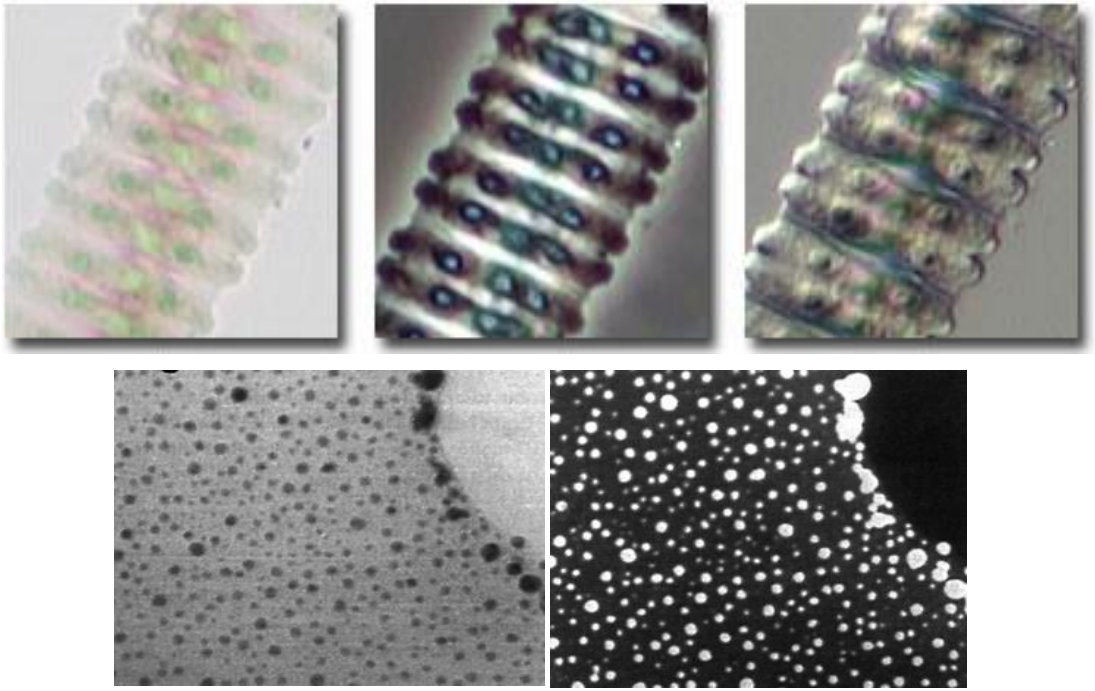
Interaction volume



Il contrasto non è una proprietà inerente al campione!

Dipende da:

1) *Interazione sonda/campione*
2) *Efficienza del sistema ottico (funzione di trasferimento del contrasto)*
3) *Efficienza rivelatore*



Necessità di conoscere gli eventi che hanno prodotto il contrasto

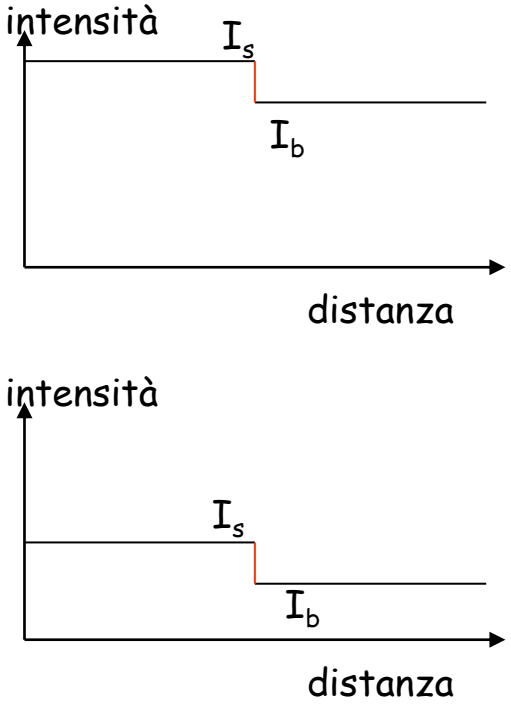
L'occhio è sensibile solo al contrasto di intensità o di lunghezza d'onda (colore)→ necessità di trasformare ogni altro meccanismo (fase, polarizzazione,…) in intensità o colore

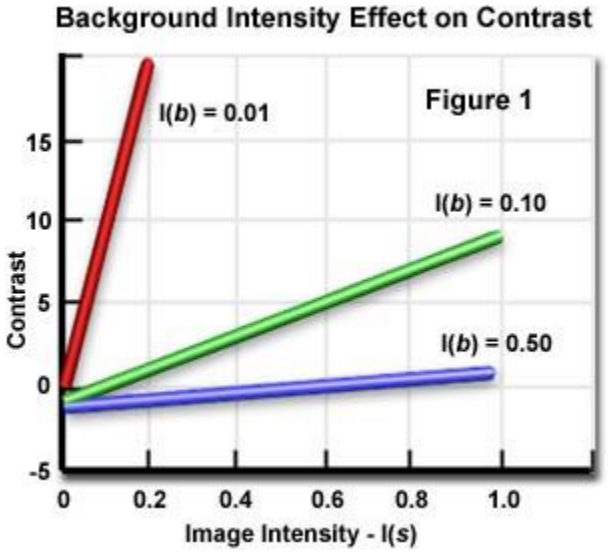
Contrasto

Per vedere qualcosa in una immagine dobbiamo avere contrasto (C) fra aree adiacenti del campione:

$$C = \frac{I_s - I_b}{I_b} = \frac{\Delta I}{I_b}$$

L'occhio umano non riesce ad apprezzare differenze di intensità inferiori al 5-10% (utilità di acquisire immagini digitali da elaborare)





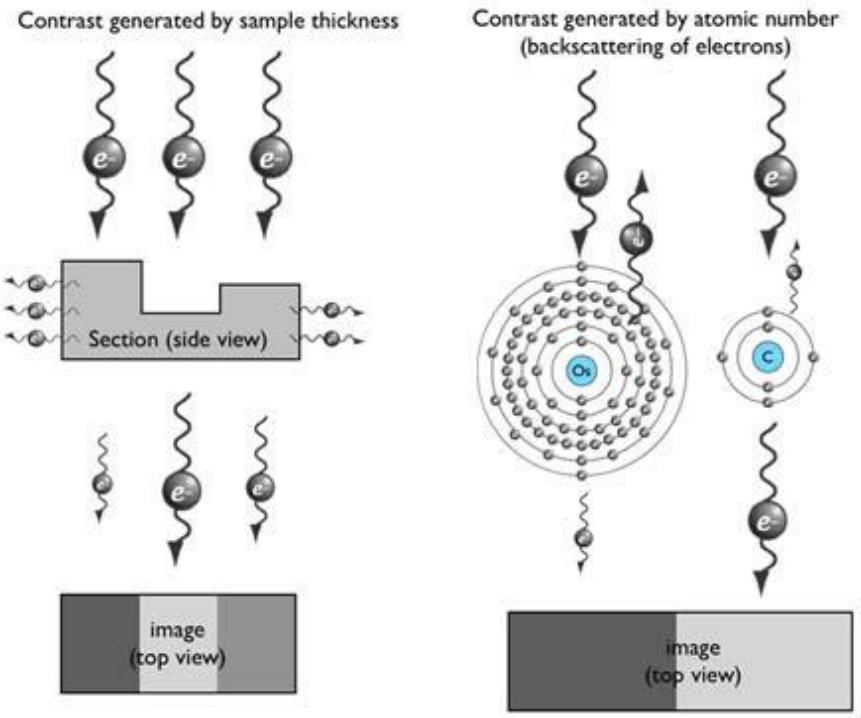
Contrast generation in EM

The simple model of elastic scattering by Coulomb interaction of electrons with the atoms in a material is sufficient to explain the basic contrast mechanisms in TEM.

The Mass – Thickness contrast mechanism

Thickness:
the number of actually occurring scattering events depends on the numbers of atoms that are lying on the path of the electron (more electrons scattered in thick samples).
Thick areas appear darker than thin areas of the same material.

Mass:
the probability that an electron is deviated from its path by an interaction with an atom increases with the number of charges that the atom carries.
Heavier elements represent more powerful scattering centers than light element; the contrast of areas in which heavy atoms are localized will appear darker than of such comprising light atoms.



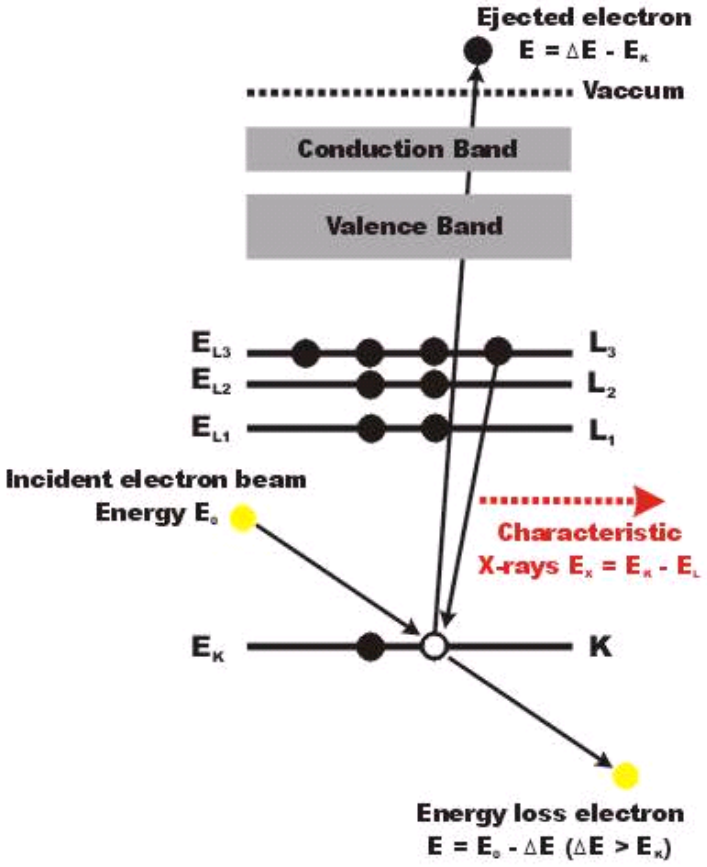
This contrast mechanism can be related to that of the optical microscopy.
Instead of light absorption (OM), it is the local scattering power that determines the contrast in TEM.

Inelastic interaction

If a part of the energy that an electron carries is transferred to the specimen, several processes might take part leading to the generation of the following signals:

1. Inner-shell ionisation
2. Braking radiation (“Bremsstrahlung”)
3. Secondary electrons
4. Phonons
5. Plasmons
6. Cathodoluminescence

all effects depend on the material, its structure and composition. That different kinds of information are obtainable from these I nteractions provides the basics for the methods of analytical EM.



Generation of a characteristic X-ray quantum. In the first step, the ionization, energy is transferred from an incident electron to an electron in an inner shell of an atom. Depending on the energy actually taken up, this electron is promoted to the lowest unoccupied level or ejected into the vacuum, leaving a vacancy in the low energy level, here the K shell. In the second step, an electron from a higher state, here the L_3 level, drops down and fills the vacancy. The surplus difference energy is emitted as an X-ray quantum.

Types of EM:

Transmission Electron Microscopy (TEM) Scanning Electron Microscopy (SEM)

•A transmission electron microscope employs an electron beam produced at high voltage to brighten the specimen and produce an image to be viewed
•The working principle of this microscope is that the electrons pass through the exhibit and create a projection image of the specimen
•TEM is typically used to view thin samples such as molecules, tissue sections etc.
•TEM is **similar to a compound light microscope** but it used to achieve a very degree of magnification thereby allowing the observation of specimens at a nanometer level.
TEM is used, among other things, to image the interior of cells, the structure of protein molecules (contrasted by metal shadowing), the organization of molecules in viruses and cytoskeletal filaments (prepared by the negative staining technique), and the arrangement of protein molecules in cell membranes (by freeze-fracture).

•An SEM creates magnified images of the specimen by probing along a rectangular area of the specimen with a focused electron beam. This process is called the raster scanning.
•It is called a scanning electron microscope because the image is formed by scanning the surface of the specimen in a raster pattern using a focused electron beam.
•SEM relies on the secondary emission of electrons from the surface of the specimen to achieve magnified image to be viewed
•The major advantage of a SEM over TEM is that it can produce detailed image of the whole organisms and surfaces of the cells
SEM has great depth of focus and provides detailed images of the surfaces of cells and whole organisms that are not possible by TEM. The image in SEM is formed by scanning a focused electron beam onto the surface of the specimen in a raster pattern. The interaction of the primary electron beam with the atoms near the surface causes the emission of particles at each point in the raster (e.g., low energy secondary electrons, high energy back scatter electrons, X-rays and even photons). These can be collected with a variety of detectors, and their relative number translated to brightness at each equivalent point on a cathode ray tube. Because the size of the raster at the specimen is much smaller than the viewing screen of the CRT, the final picture is a magnified image of the specimen. Appropriately equipped SEMs (with secondary, backscatter and X-ray detectors) can be used to study the topography and atomic composition of specimens, and also, for example, the surface distribution of immuno-labels.

Resolution of electron microscopes

The higher the energy of the electrons, the lower the wavelength, the higher the resolution

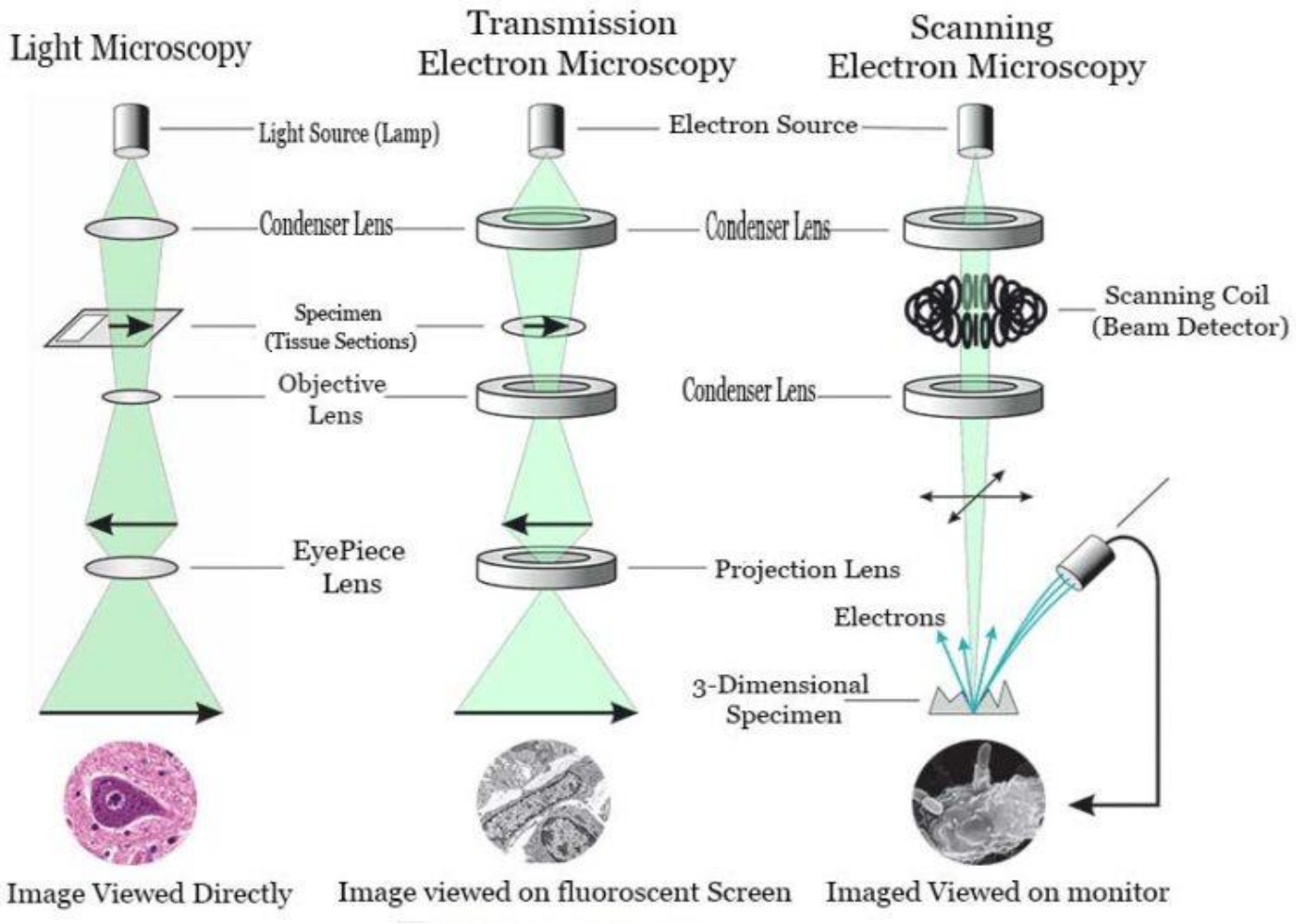
TEM: 40 – 300 kV

Effective instrument resolution TEM: ≈ 0.2 nm (300 kV)

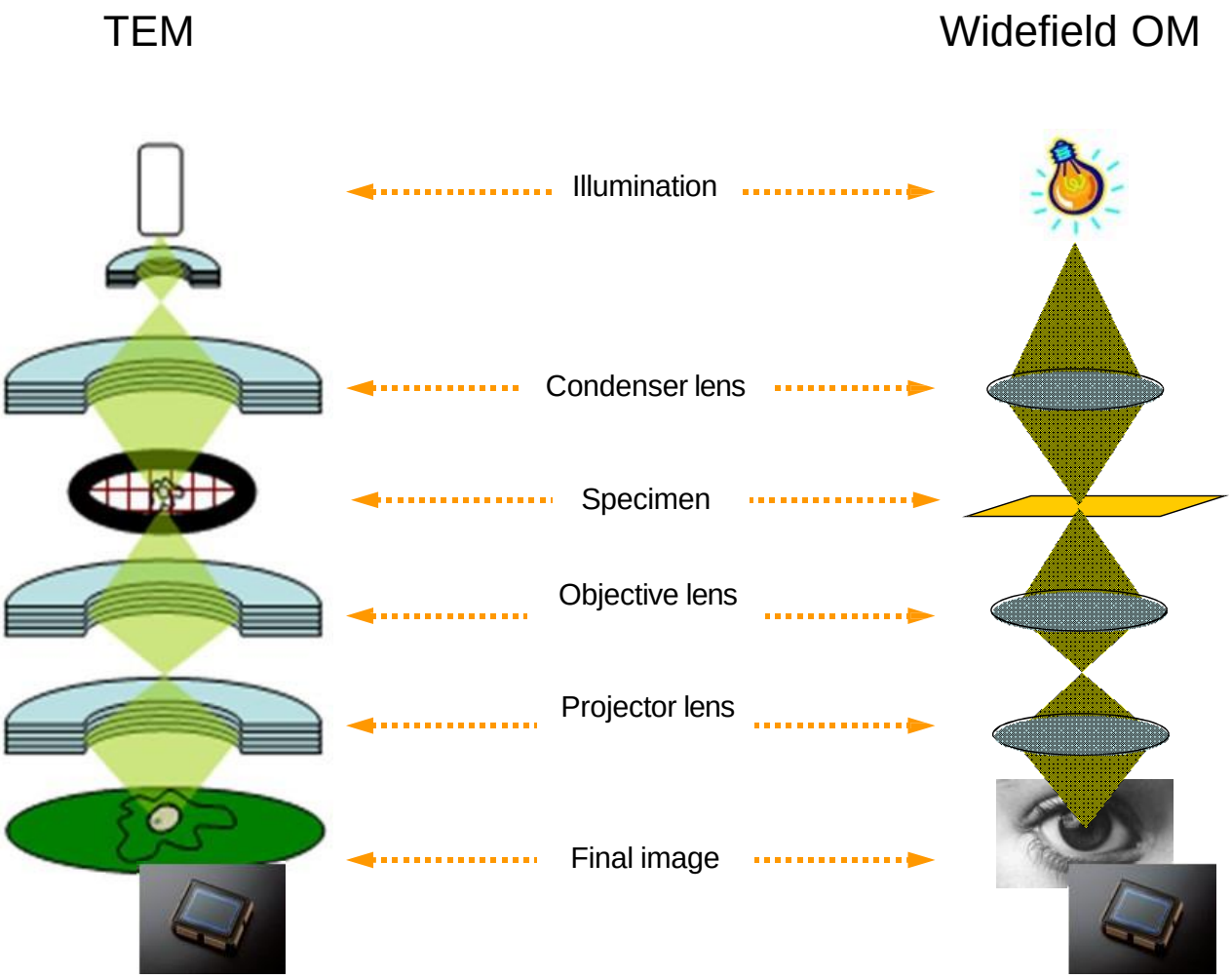
SEM: 0.5 – 30 kV

Effective instrument resolution SEM: ≈ 1 nm

Resolution of biological objects limited by **specimen preparation**:
Practical resolution: **>** 1 nm

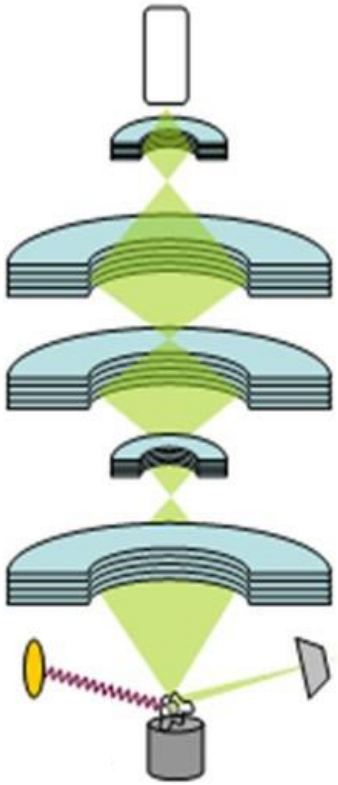

Light Microscopy
Transmission Electron Microscopy
Scanning Electron Microscopy
Light Source (Lamp)
Electron Source
Condenser Lens
Condenser Lens
Specimen (Tissue Sections)
Scanning Coil (Beam Detector)
Objective Lens
Condenser Lens
EyePiece Lens
Projection Lens
Electrons
3-Dimensional Specimen
Image Viewed Directly
Image viewed on fluoroscent Screen
Imaged Viewed on monitor

TEM vs. Widefield OM



SEM vs. Confocal OM

Scanning electron microscope



Illumination

Detector

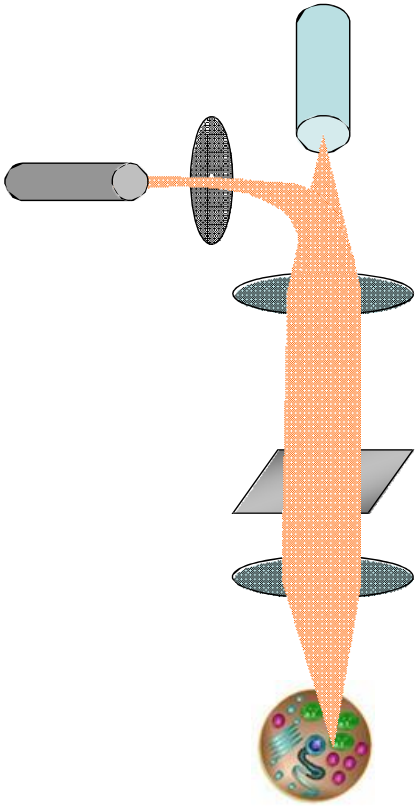
Lens system

Beam scanner

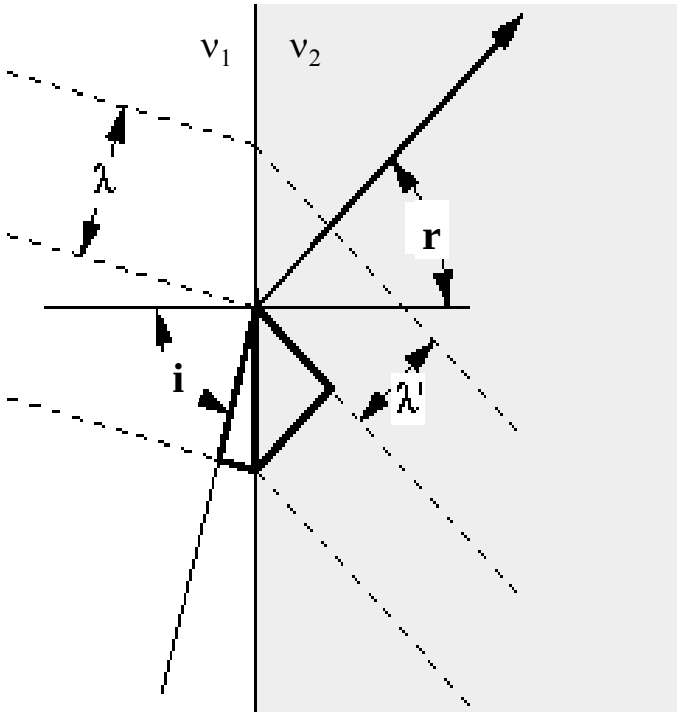
Lens system

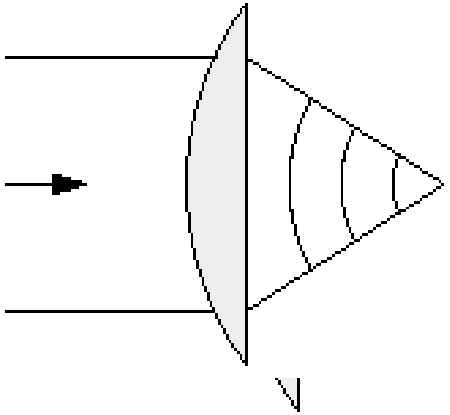
Specimen

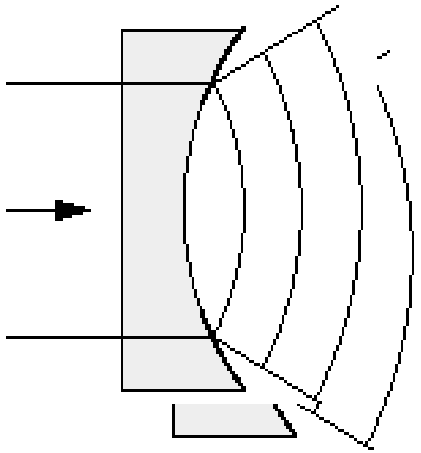
Confocal OM



Lenti per la luce







f dipende dal raggio di curvatura

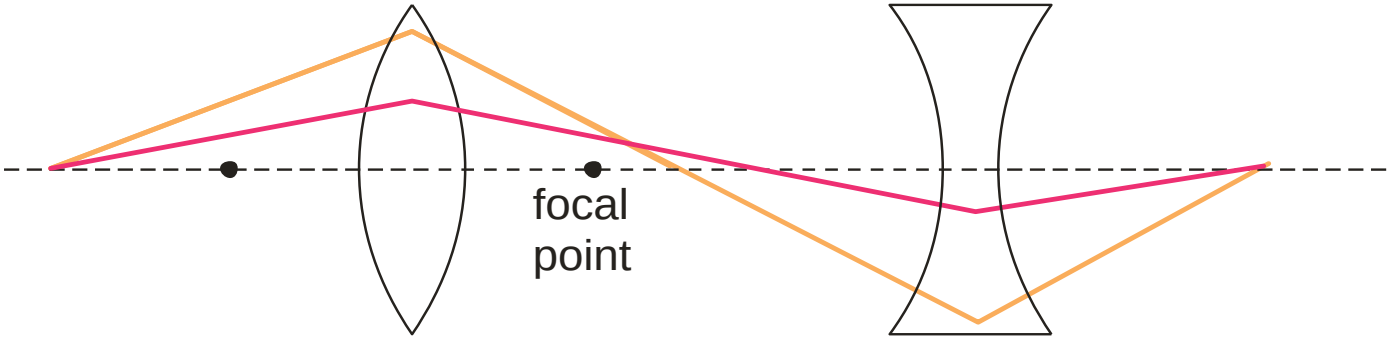
Convergenza e divergenza dei fasci è dovuta alla differenza di indice di rifrazione tra aria e vetro, cioè alla differente velocità della luce in questi due mezzi

Legge di Snell seni/senr=n_2/n_1

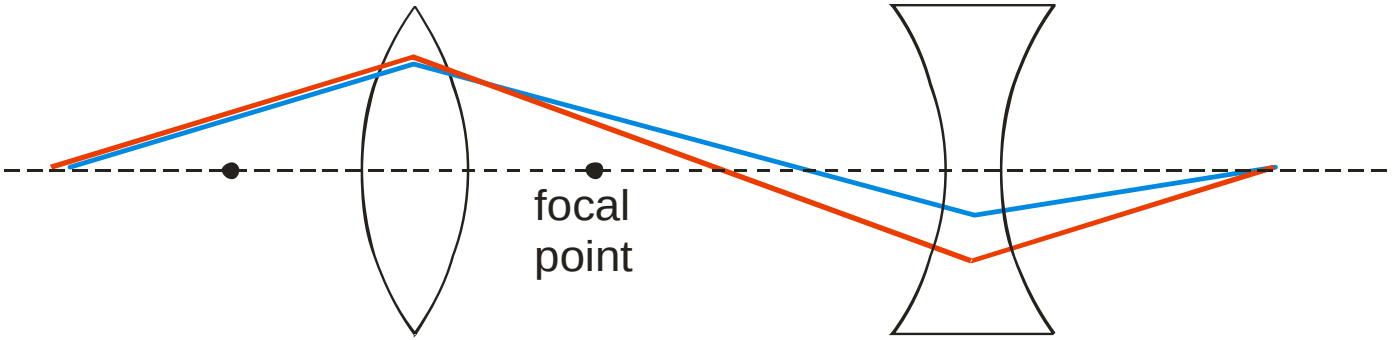
Utilizzo di lenti concave per la correzione di aberrazioni ottiche

Round **convex** lenses

Round **concave** lenses

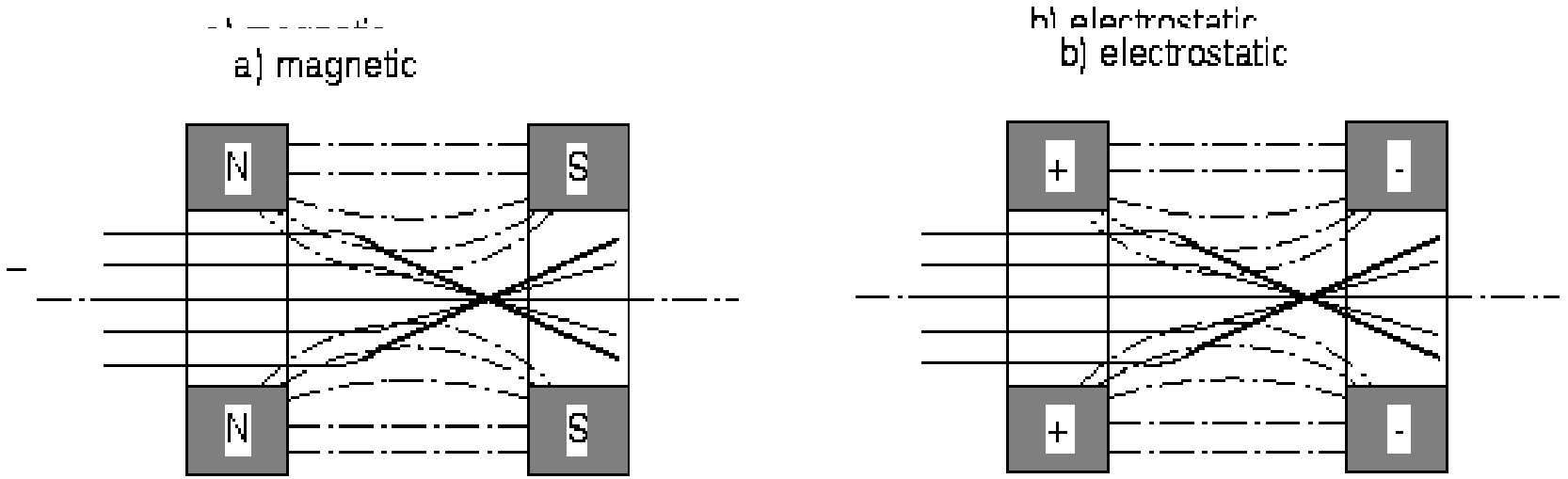


Aberrazioni sferiche



Aberrazione cromatiche

Lenti per gli elettroni

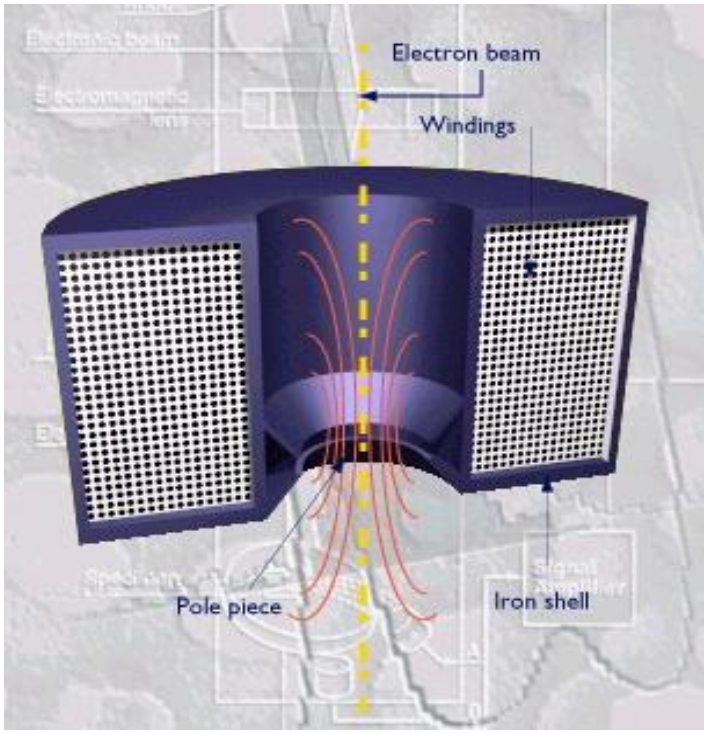


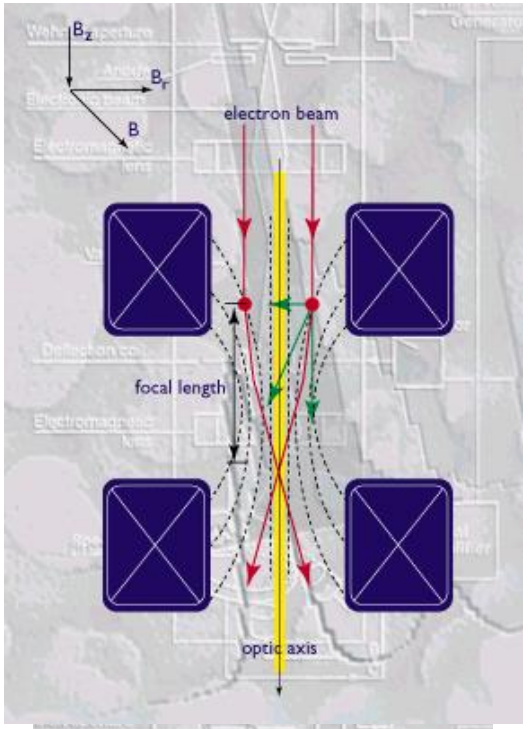
L'azione di focalizzazione è dovuta a un campo elettrico e/o magnetico che può modificare le traiettorie degli elettroni a seguito della forza di Lorentz

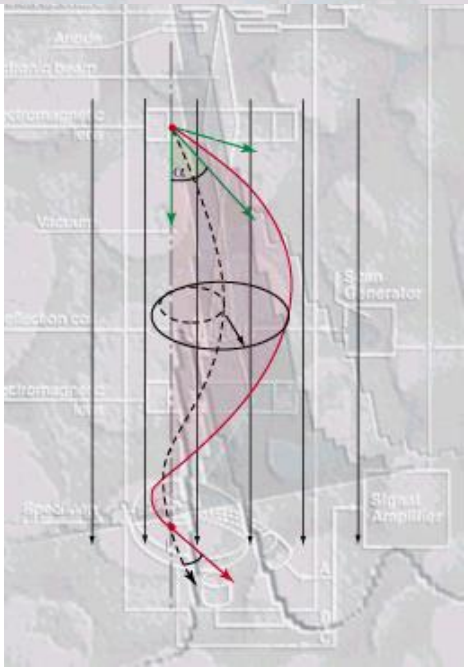
$$\mathbf{F} = e\,(\mathbf{E} + \mathbf{v} \times \mathbf{B})$$

Lenti elettromagnetiche

$$\mathbf{F} = e\,\mathbf{v} \times \mathbf{B}$$

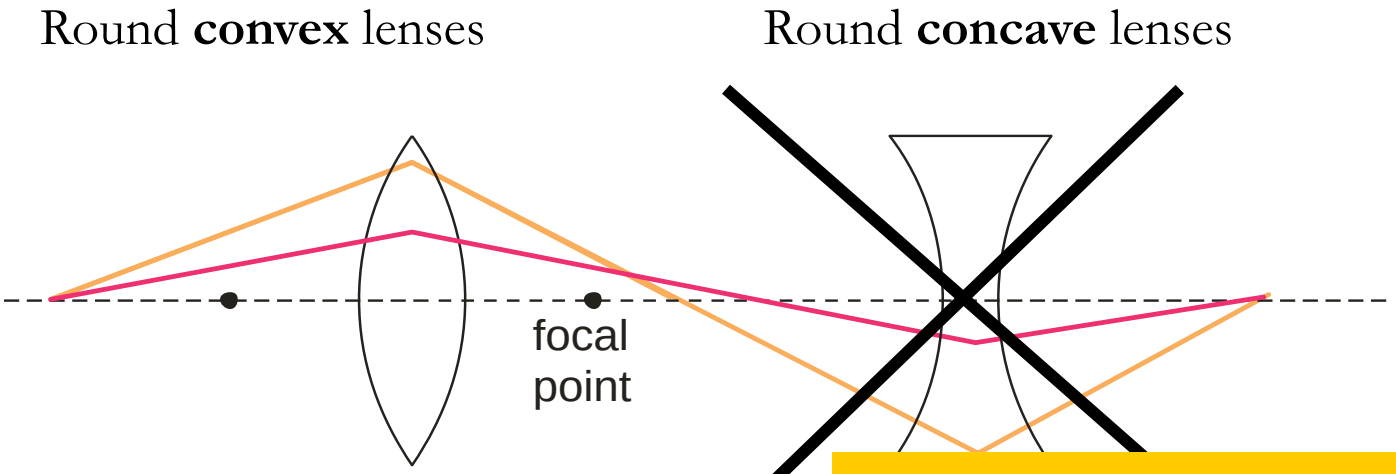






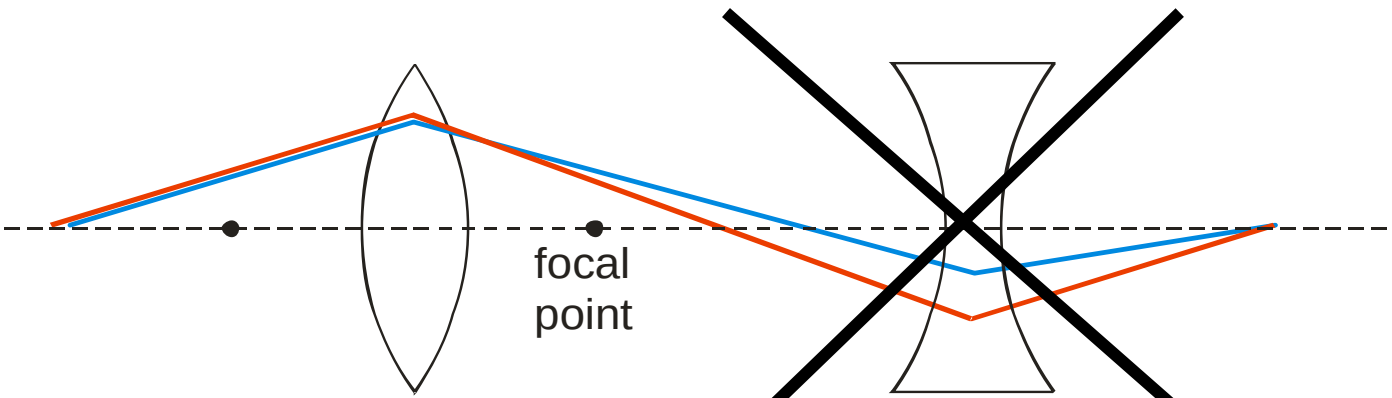
$$\frac{1}{f} = \frac{\alpha B_z^2}{E_{kin}}$$

Utilizzo di lenti concave per la correzione di aberrazioni ottiche



Aberrazioni sferiche

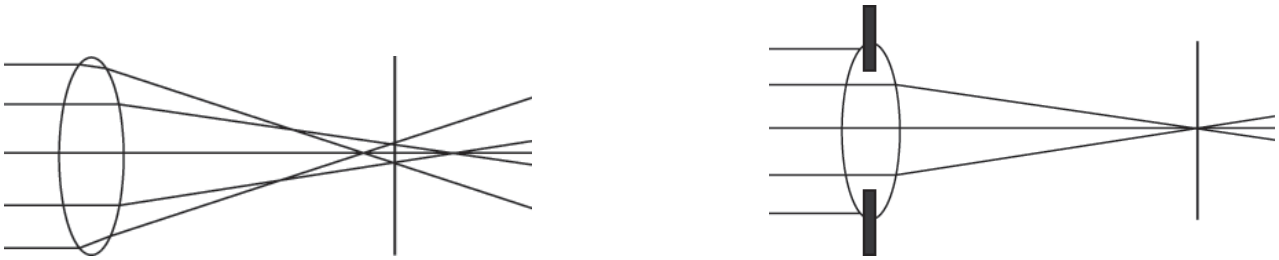
Non esistono ottiche concave per elettroni



Aberrazione cromatiche

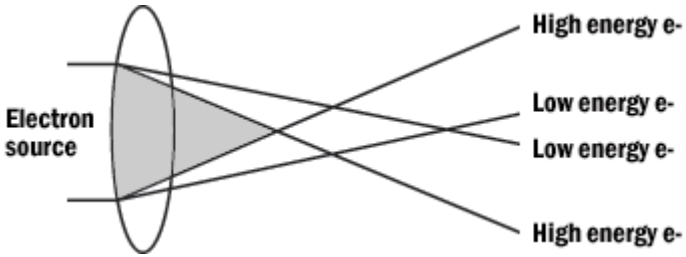
Aberrazioni nei microscopi elettronici

1. Aberrazione sferica:



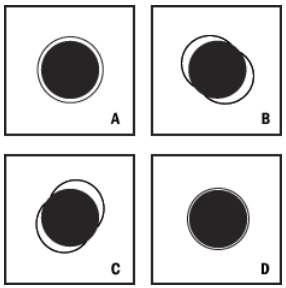
$C_s \sim f/2 \sim 0.5\text{-}3$ mm

2. Aberrazione cromatica: dovuta alla dispersione in energia ΔE degli elettroni

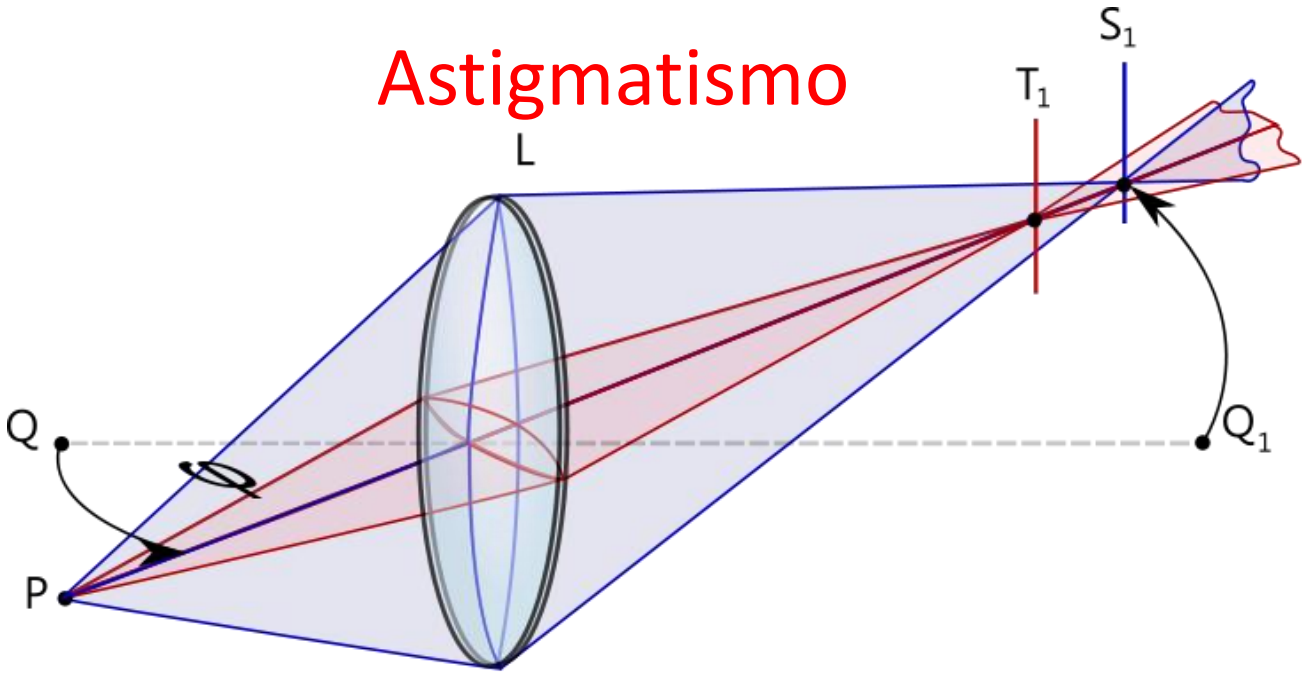


$C_c \sim 1\text{-}2$ mm

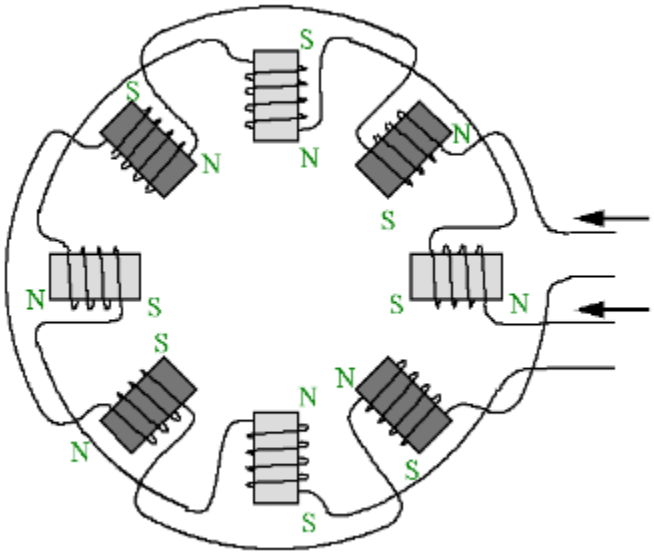
3. Astigmatismo: disuniformità del campo B (correzione con stigmatori = ottupoli)

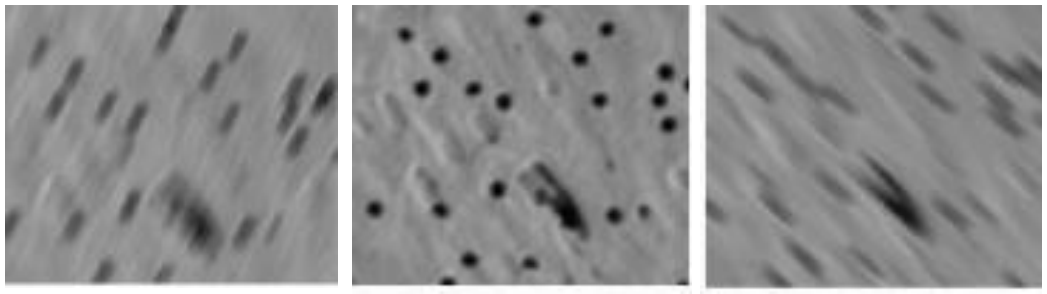


Astigmatismo

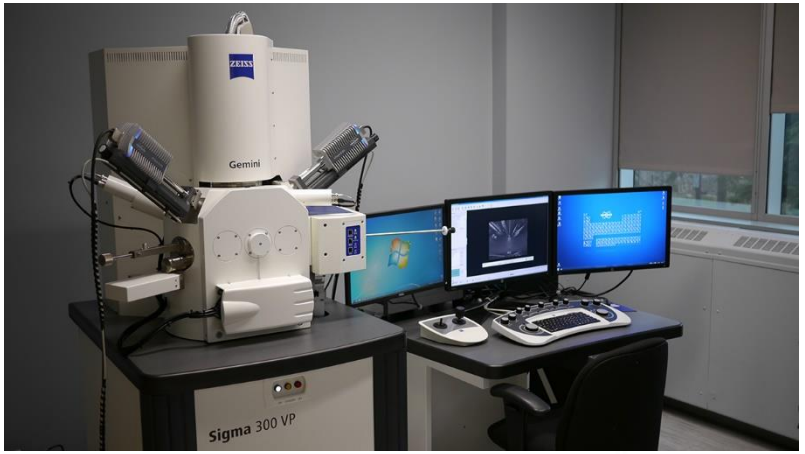


L'astigmatismo è tipico dell'EM e deriva da diversi piani focali in direzioni perpendicolari (piani sagittale e tangenziale).Può dipendere dall'allineamento del raggio ma anche dalla conducibilità del campionePuò essere corretto utilizzando uno stigmatore, un insieme di lenti magnetiche che rimodellano il raggio conferendogli una forma circolare





Vantaggi e svantaggi del SEM

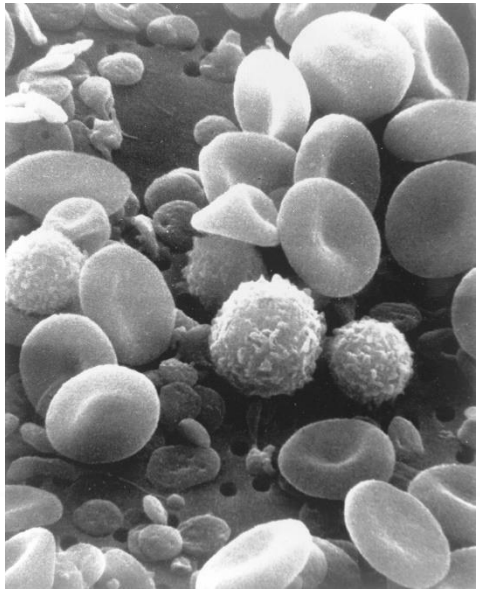


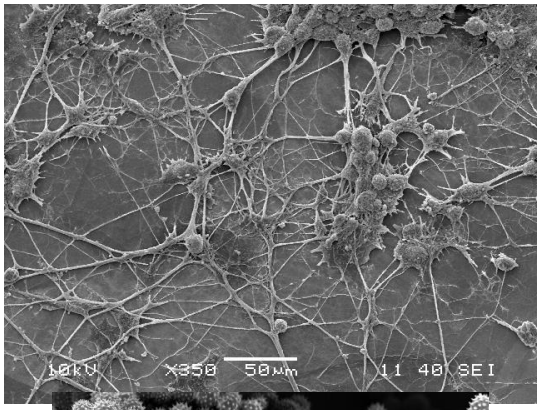
Advantages of Scanning Electron microscope:

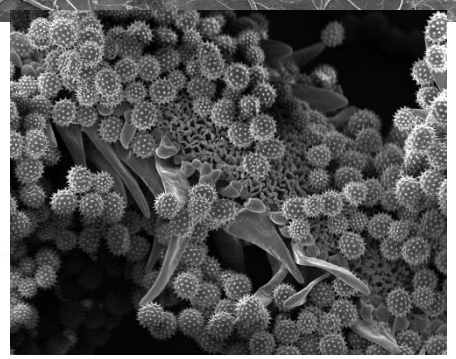
- Magnifies objects more than 500 000X
- Possible to investigate a greater field of depth
- Modern SEM allow for the generation of data in digital form
- Most SEM samples require minimal preparation actions

Disadvantages of Scanning Electron microscope:

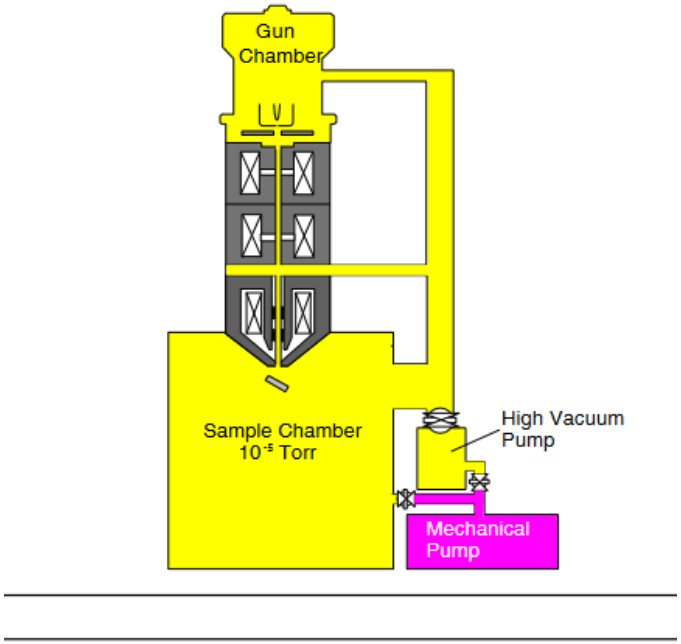
- Very large (operated in special rooms)
- Affected by magnetic fields
- Preparation of material is lengthy
- Require expertise
- Preparation may distort material
- Images are in black and white
- Expensive to purchase and operate
- SEMs are limited to solid samples

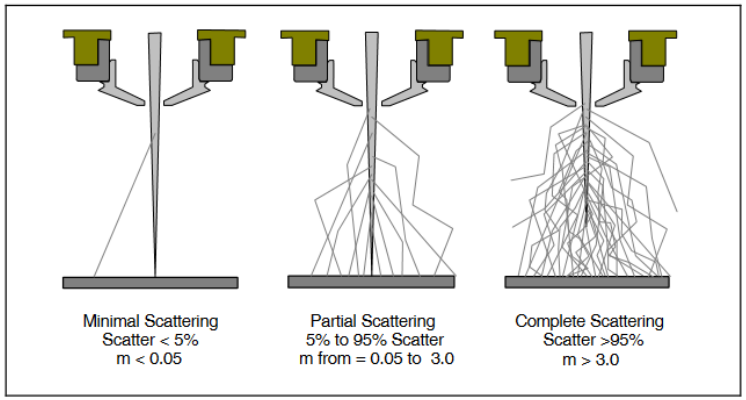


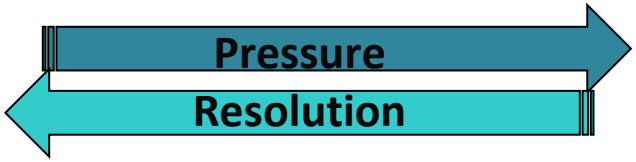


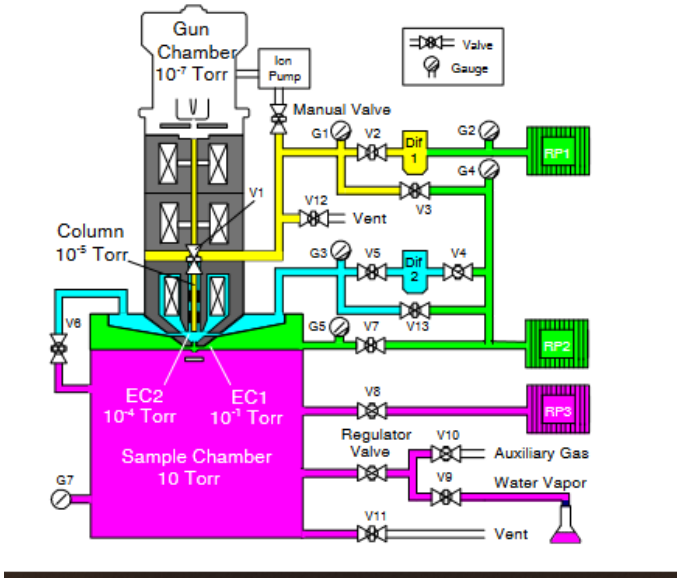


High vacuum SEM



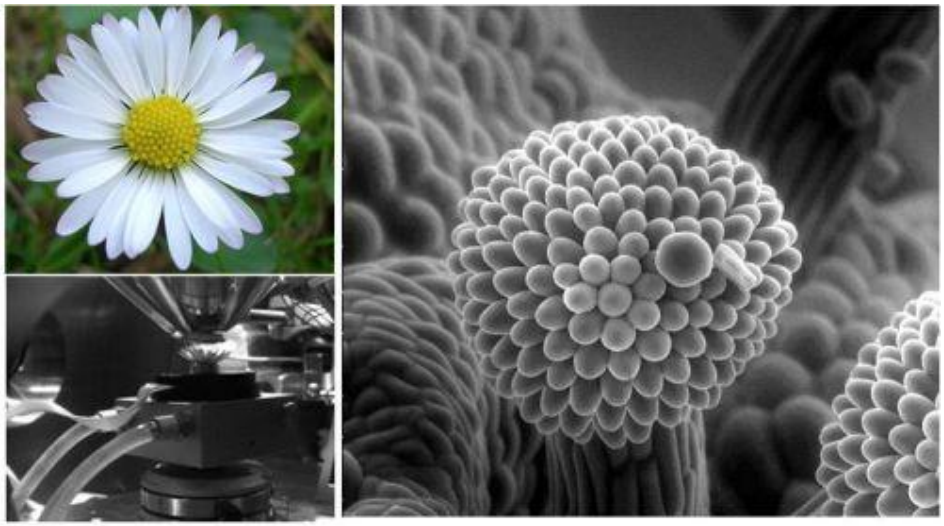


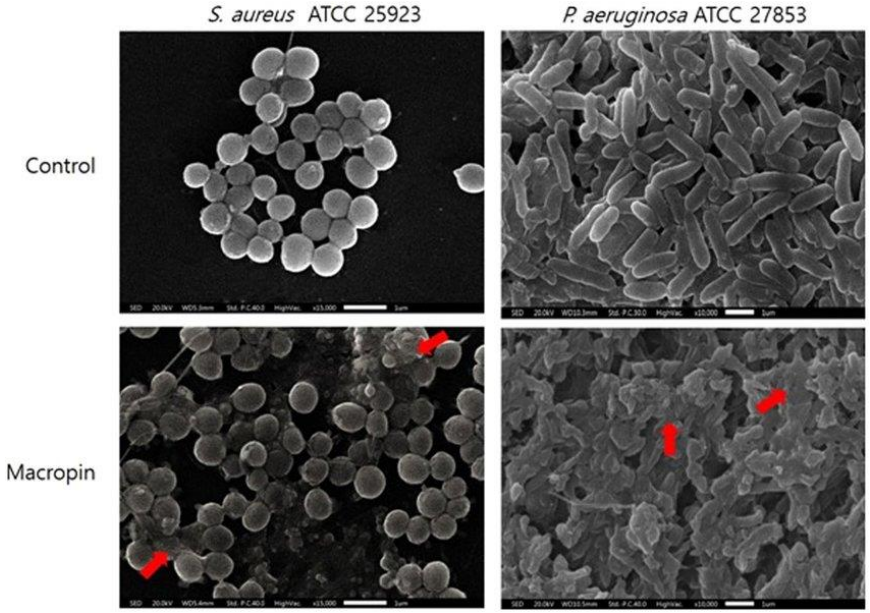




ESEM

The **environmental scanning electron microscope** or **ESEM** is a SEM that allows for the option of collecting electron micrographs of specimens that are "wet," uncoated, or both by allowing for a gaseous environment in the specimen chamber.

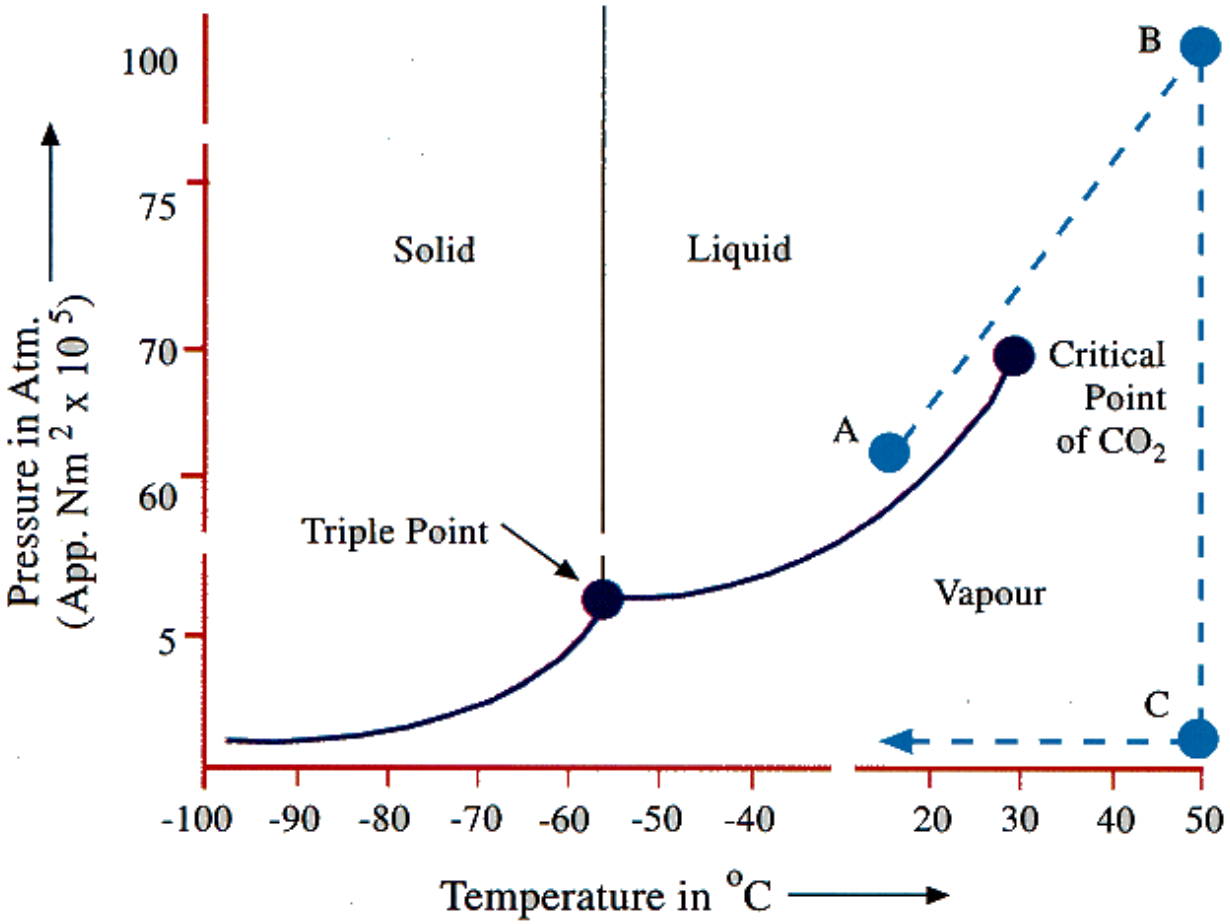




S. aureus and P. aeruginosa

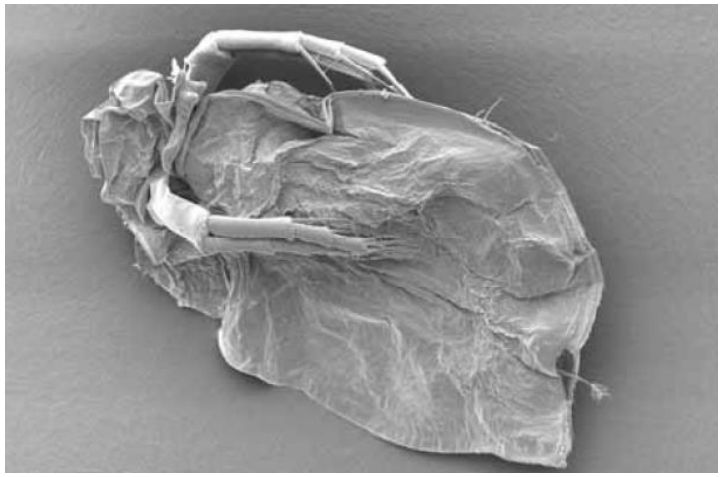
peptide treated S. aureus and P. aeruginosa

Supercritical drying

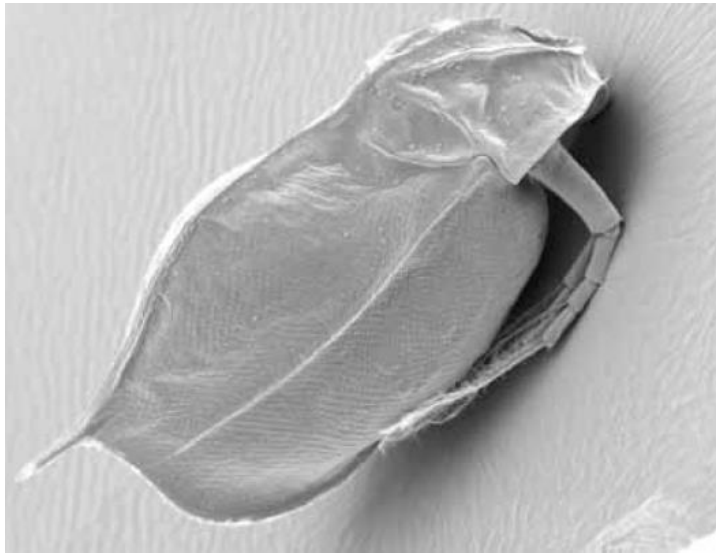


Essenziale per rimuovere l'acqua da campioni biologici senza modificarne la struttura 3D

Supercritical drying



Air dried sample (Water flea)

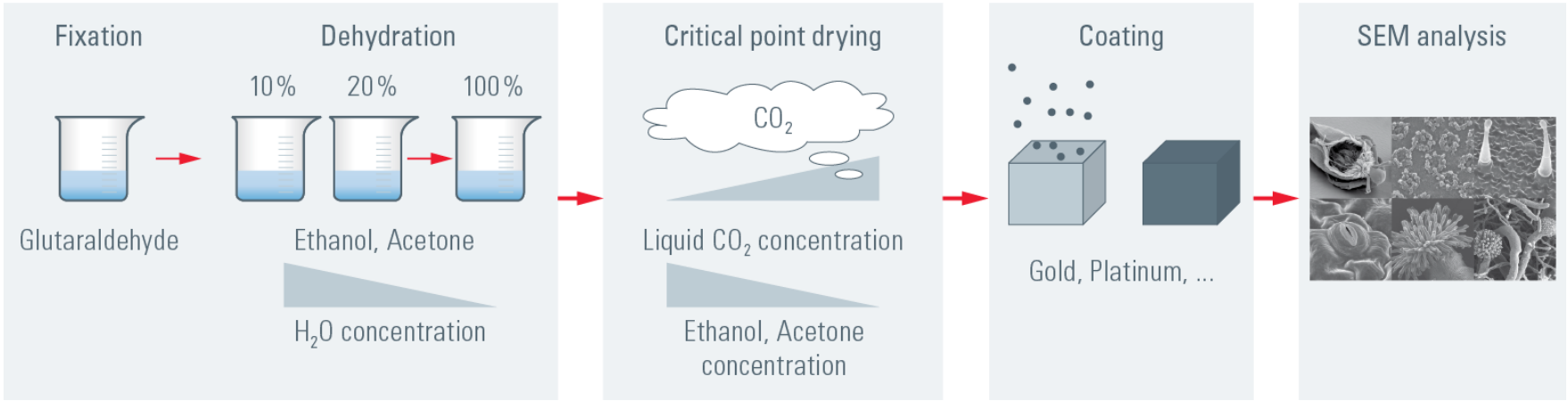


Critical point dried sample (Water flea)

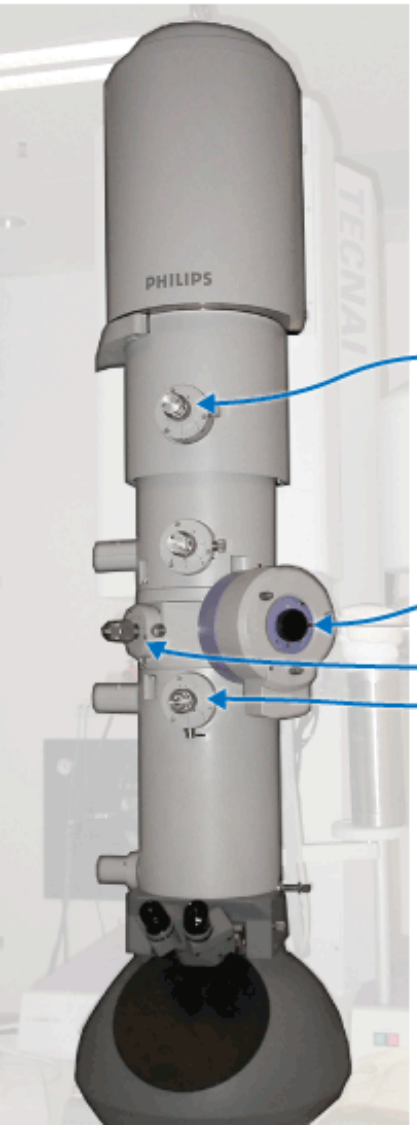
Perchè usare CO_2?

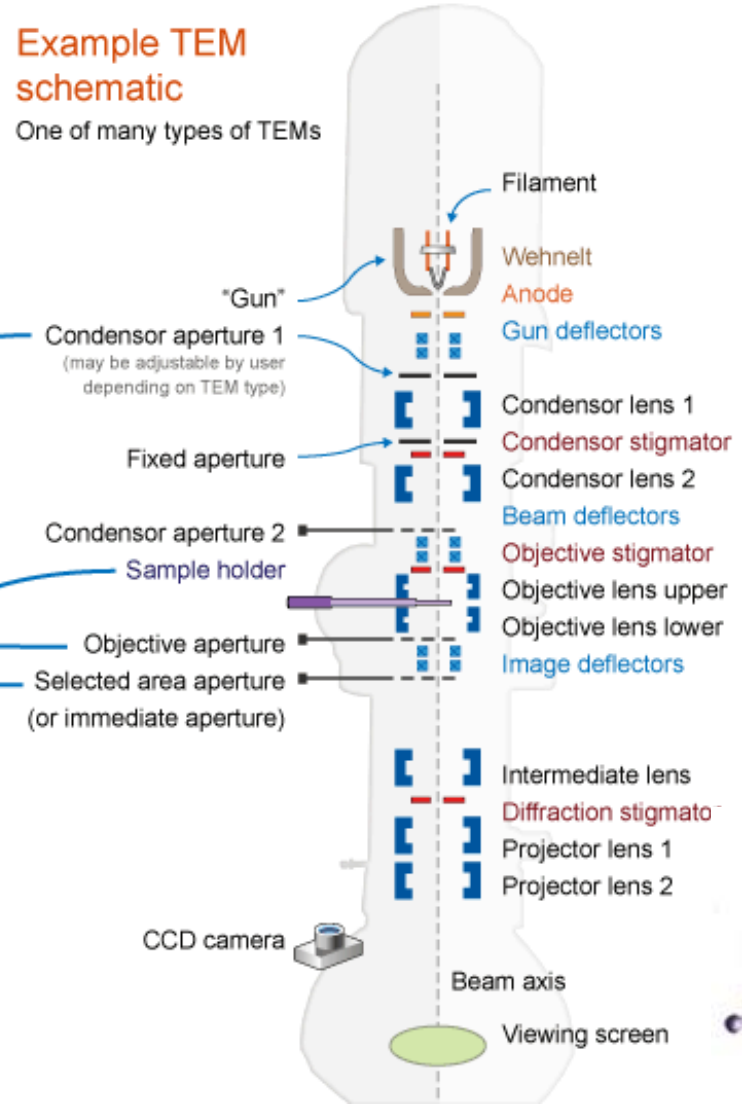
Substance	Temp. °C	P.S.I
Hydrogen	-234.5	294
Oxygen	-118	735
Nitrogen	-146	485
Carbon Dioxide	+31.1	1072
Carbon Monoxide	+141.1	528
Water	+374	3212

La CO2 non è però miscibile con l'acqua:
sono necessari degli ulteriori passaggi per la preparazione dei campioni idratati



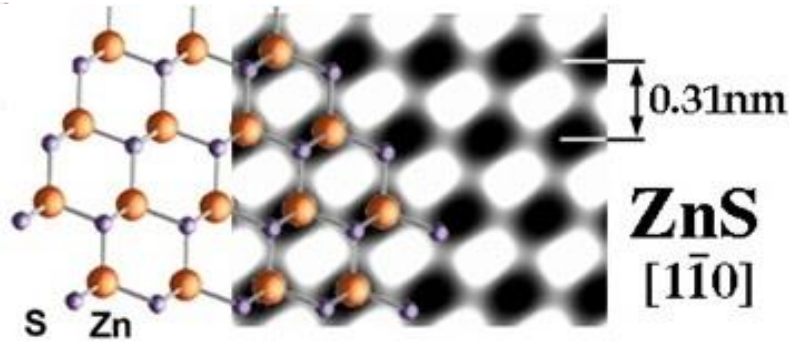
Transmission Electron Microscope (TEM)





In TEM the electron beam is not focused:
a highly parallel and monochromatic beam passes across the sample, projecting a shadow in correspondence of the denser materials.

At atomic resolution the electron pass in the voids between atoms,
A process known as channeling.

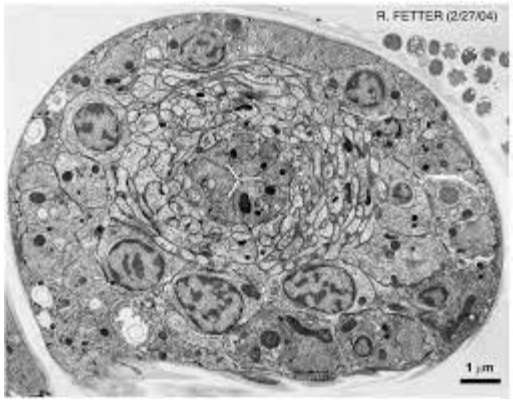


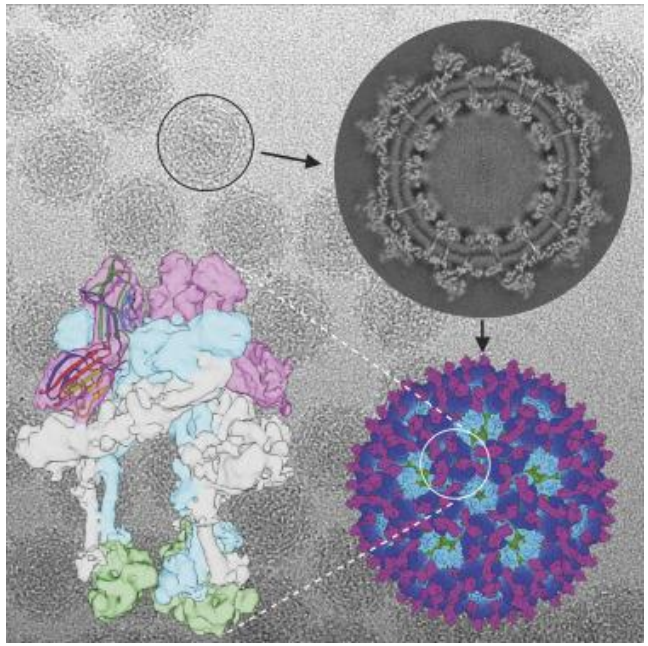
Transmission Electron Microscope (TEM)

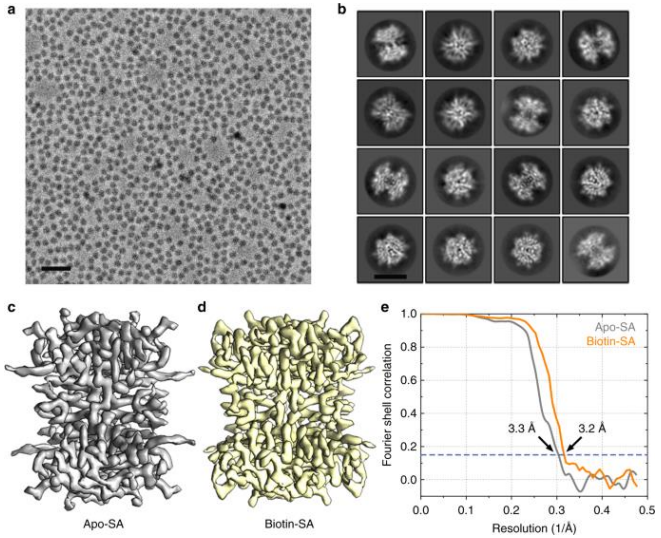
Pros/Cons

- Pros:
- Very high resolution
- Requires very little sample to test
- Quantitative and Qualitative
- Can be modified in many ways to account for different substances and requirements

- Cons:
- Tough sample prep.
- Hour consuming runs to get a few images
- Small field of view, may take several runs to find what is being studied
- Sample destruction, especially biological samples



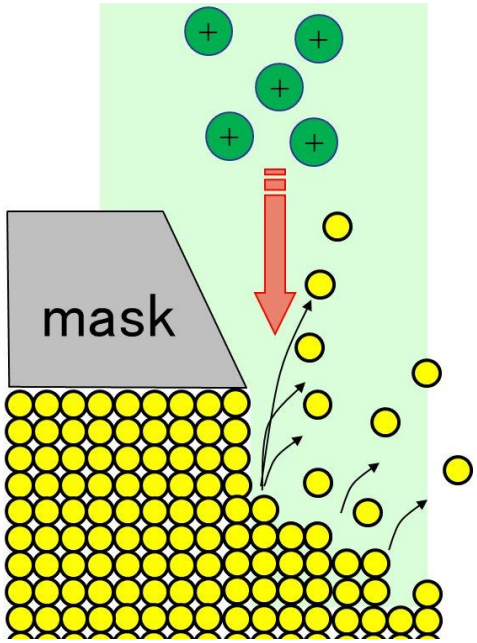




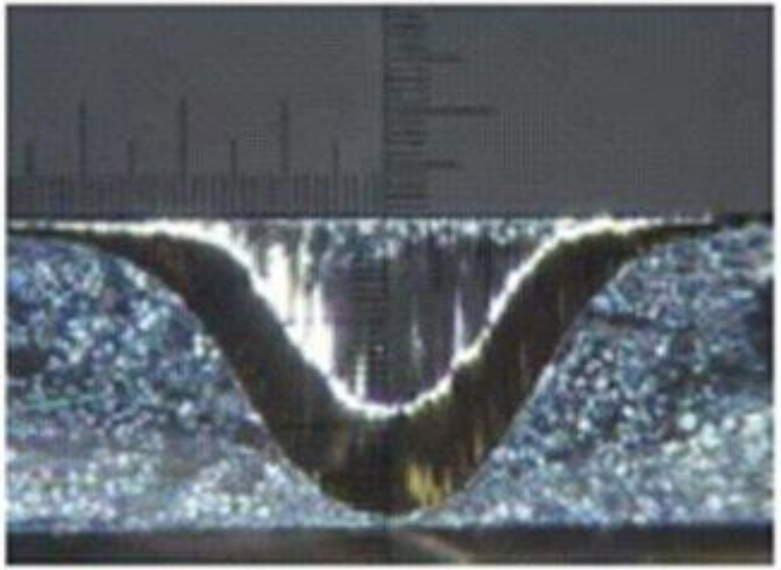
Sample preparation (TEM)

For transmission electron microscopy investigations, samples have to be trimmed down to a size of typically 3 mm in diameter and tens or hundreds of nanometer in thickness. Moreover, the sample has to withstand the vacuum condition inside the TEM.

For high resolution studies of crystalline materials, the sample needs to be prepared with respect to a certain crystallographic orientation and be thinned down to a thickness below 20 nanometer.



This is usually obtained by ion milling



But can be applied only for crystalline materials

I Procedure of sample preparation of a biological specimen

I–1 Preparing a biological sample for ultramicrotomy

Tools and chemicals listed here are necessary for preparation.

(Procedure)	(Instruments/Chemicals)
1. Extraction of tissue	• Tweezers • Glass bottles • Razor
2. Fixation	• Glutaraldehyde • Osmium tetroxide • Phosphate buffer
3. Dehydration	• Ethanol • Propylene oxide
4. Embedding	(• Disposable syringes) (• Disposable beakers) • Epoxy resin
5. Polymerization	• Embedding plate (Top photo), Embedding capsule (Middle photo) • Oven for polymerization (Bottom photo)
6. Sectioning	• Ultramicrotome (Top photo) • Grid • Diamond knife (Middle photo) (• Glass knife) (• Knife maker) (Bottom photo)
7. Staining	• Uranyl acetate • Lead citrate
8. Observation	

Fixation

Embedding plate

Embedding capsule

Oven for polymerization

Ultramicrotome

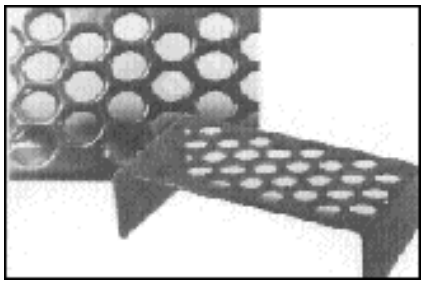
Diamond knife

Knife maker

Section Mounting

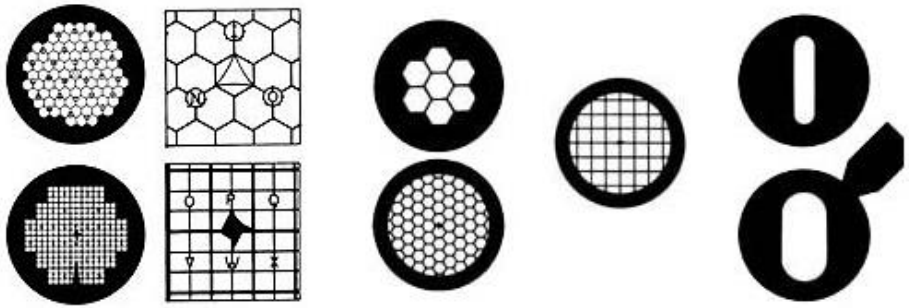
- **A 200m grid has 60% open area; a 400m grid only 40%**

- **Thin-bar grids...more fragile, more expensive.**



- **Ultrathin sections can be supported on a bare grid of no greater than 200micron.**

- **Commonly used TEM grid types:**



Contrast

Transmission Electron Microscopy:

- Contrast is produced by the adsorption of heavy metals to specimen macromolecules.

- The ability of an atom to absorb electrons is directly related to its mass.

- Since biological specimens are composed mostly of low atomic # elements (C,O,H,N), they lack endogenous contrast....thus contrast is induced by "staining" with heavy metals.

- Microscopists refer to the measure of a specimen's ability to absorb electrons as its electron density (vs electron “transparency”).

Contrast

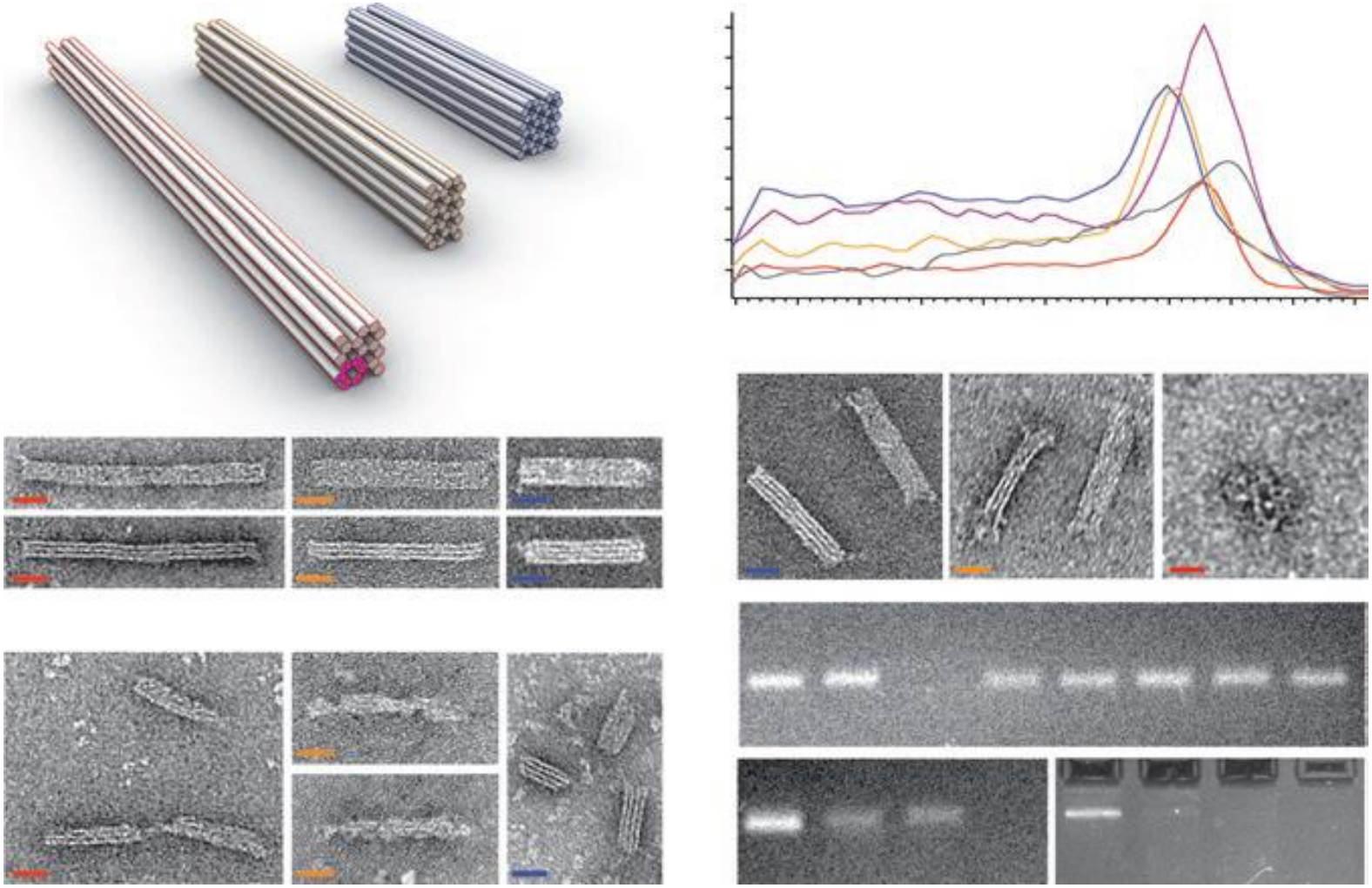
Transmission Electron Microscopy:

- Heavy metals commonly used for contrasting in TEM: uranium, lead, osmium, ruthenium, molybdenum, gold, silver.

- It is the differential adsorption of various heavy metals to tissue components that produces the electron image of biological thin-sectioned materials.

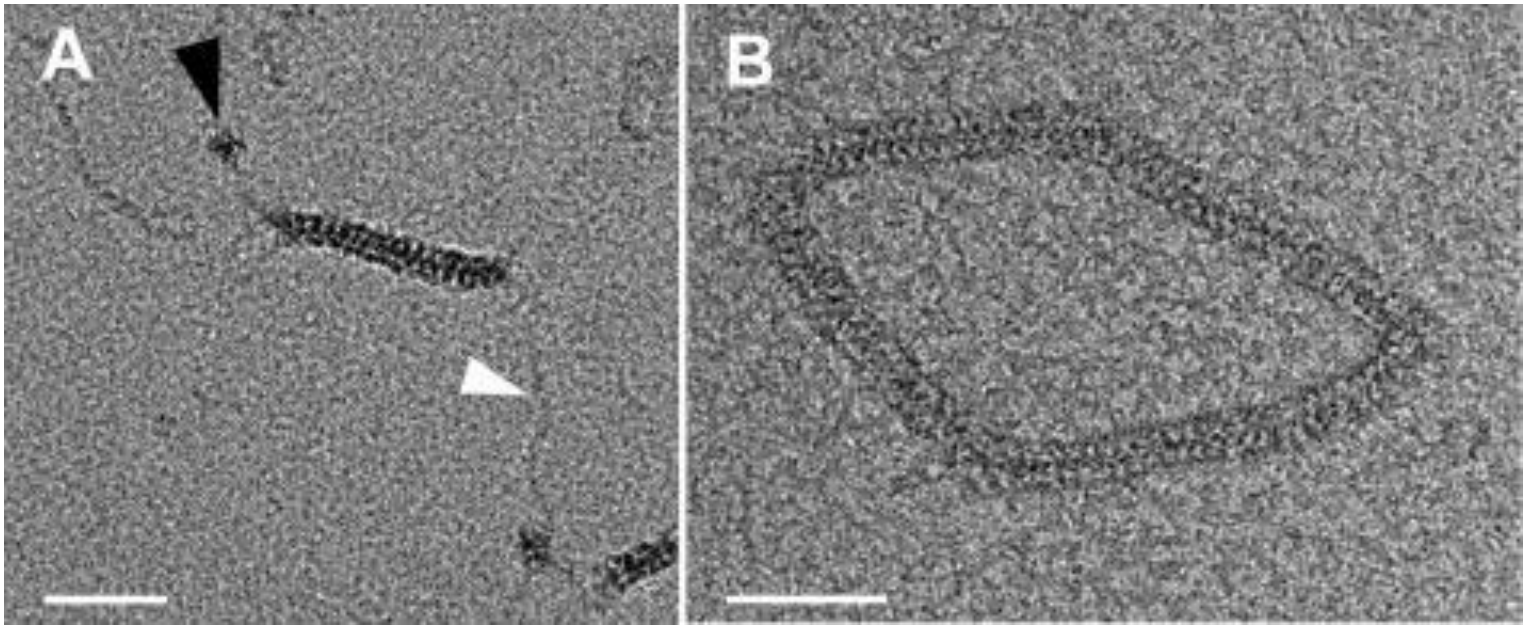
- The image may be composed of areas ranging from completely black to completely white with all ranges of grey in between.

Negative staining with DNA origami



Negative-stain TEM with 2% uranyl formate

Positive staining of protein-DNA complex



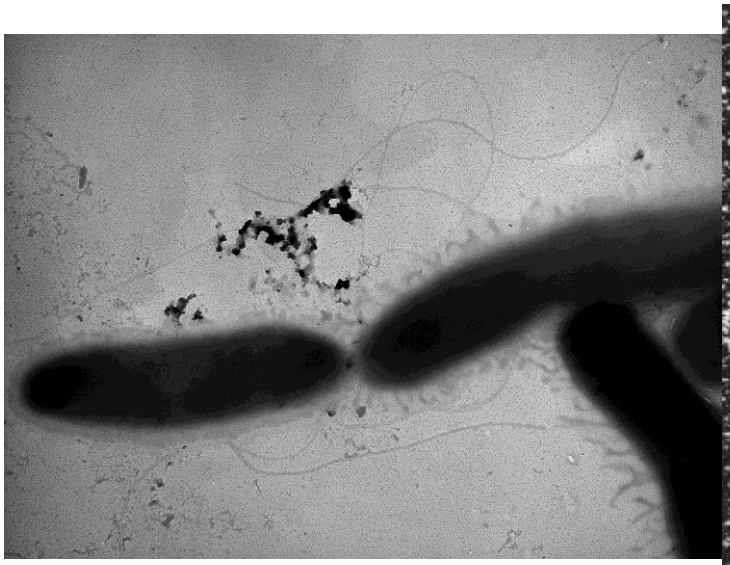
VirE2 is a large (63.5 kDa) single-stranded DNA-binding protein used by *Agrobacterium tumefaciens* infect its host

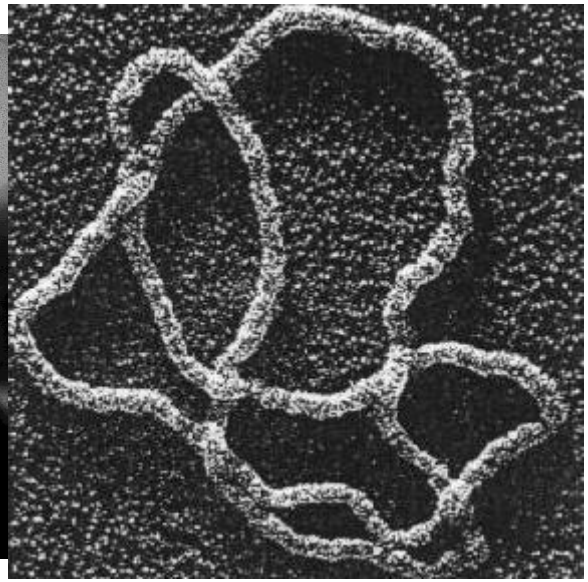
Both protein and ssDNA were positively stained with 1% uranyl acetate for 30s.

Polymerization of VirE2 on ssDNA is regulated by VirE1.

A, The helical complex and ssDNA are positively stained with uranyl acetate. In addition, naked DNA (*white arrowhead) and a VirE2-ssDNA ring (black arrowhead) can be observed. B, TEM micrograph showing a typical VirE2-ssDNA complex formed between* co-expressed VirE1–VirE2 complexes and M13 ssDNA prepared in the presence of 15% PEG 8000. *Scale bars, 50 nm*

Positive Staining



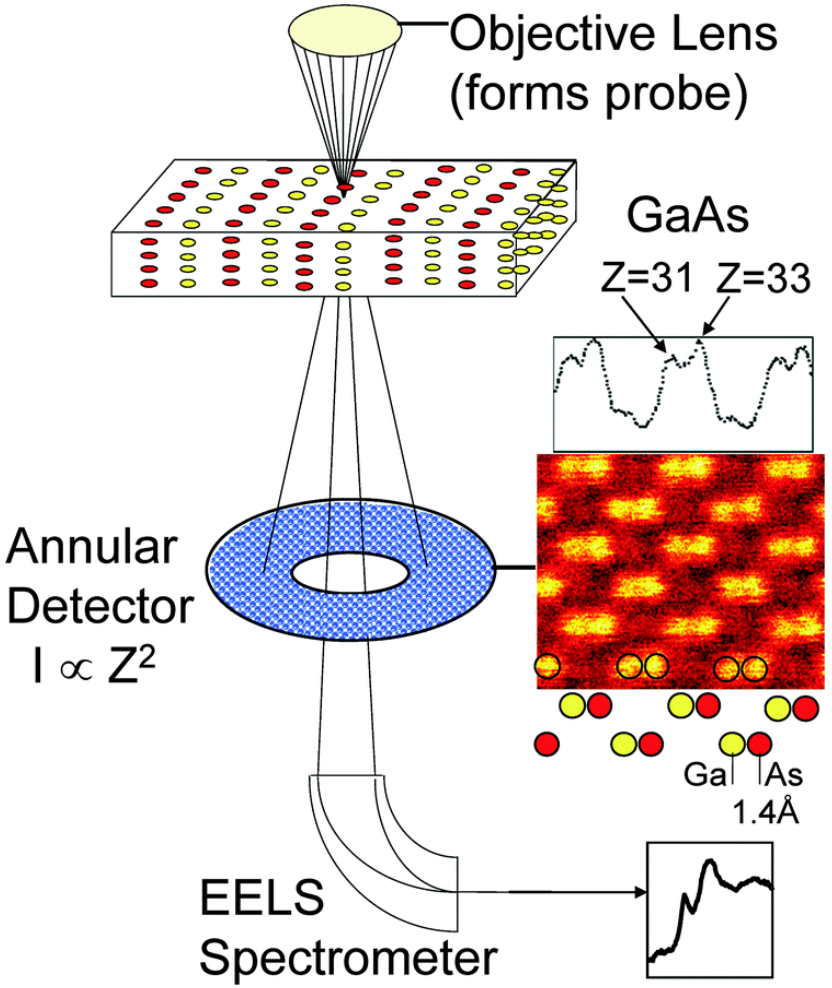


Esempi:

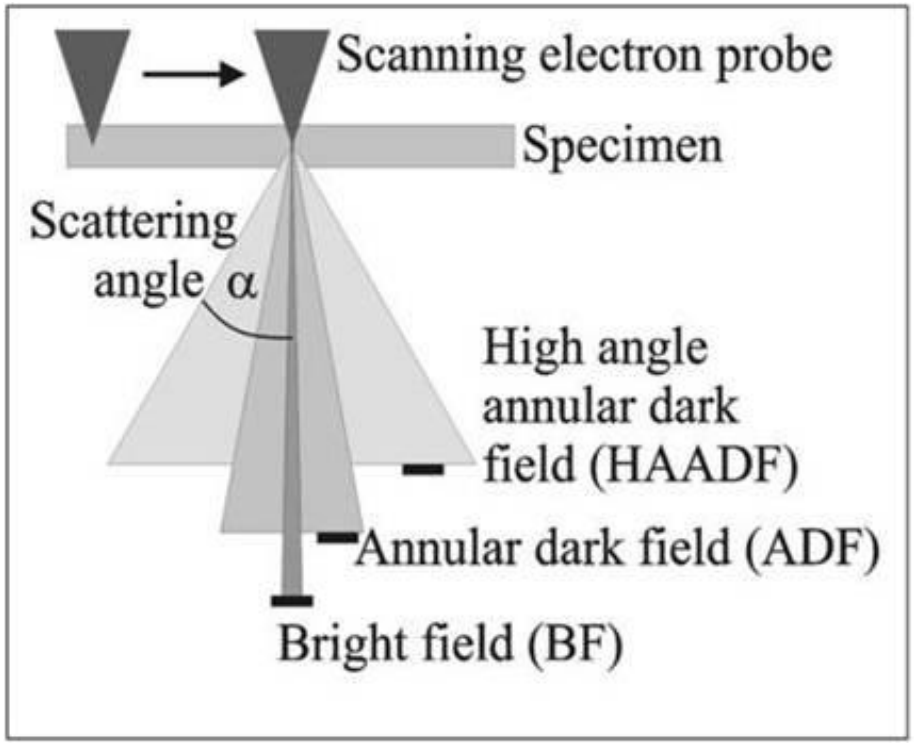
Tetrossido di rutenio (Z=44) ➔ anelli aromatici
Sali di piombo (Z=82) ➔ fosfati e DNA
Acido fosfotungstenico (Z=74) ➔ OH, COOH, NH_2

HAADF

Traditional TEM



STEM



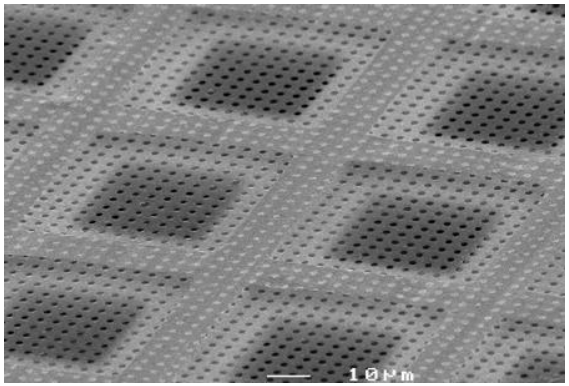
Cryo EM

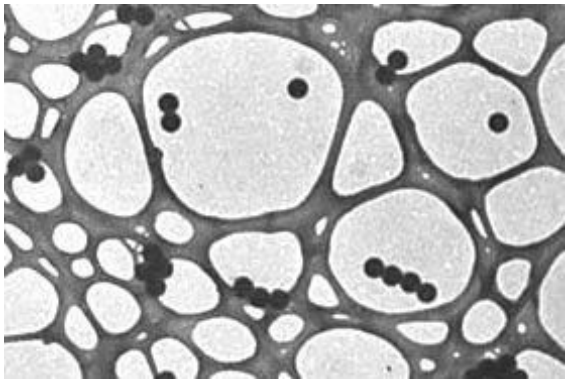
The original rationale for cryoelectron microscopy was as a means to fight radiation damage for biological specimens. The amount of radiation required to collect an image of a specimen in the electron microscope is comparable to placing the sample about 20 m away from a thermonuclear source.

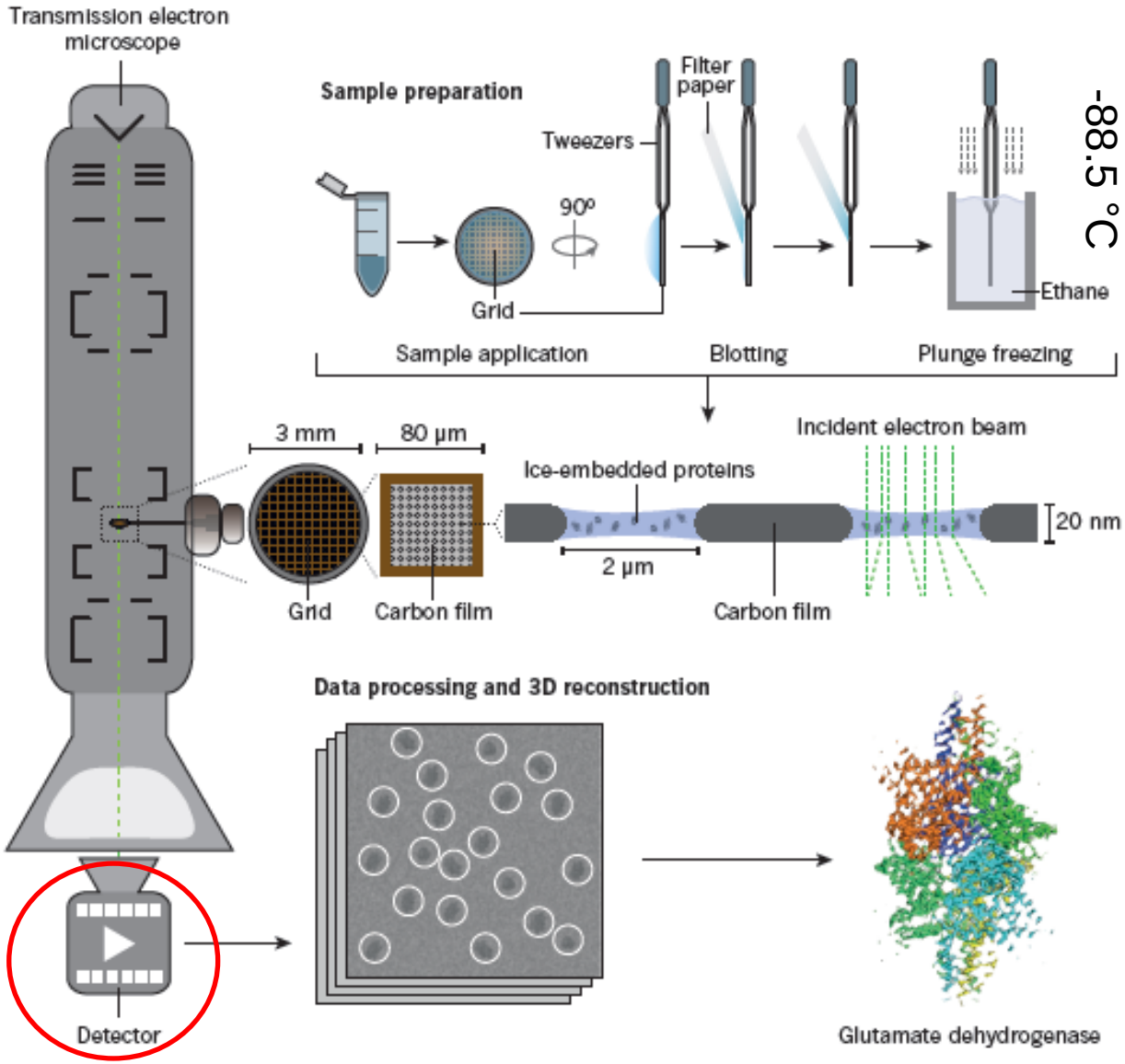
Embedding the samples in ice below the sublimation temperature was a possibility that was contemplated early on, but water tends to arrange into a crystalline lattice of lower density upon freezing and this tends to destroy the structure of anything that is embedded in it.

The solution was found in vitreous Ice. Amorphous ice consists of water molecules that are randomly arranged like the molecules of common glass, while everyday ice is a crystalline material where the molecules are regularly arranged in a hexagonal lattice

Cryo EM – sample vitrification

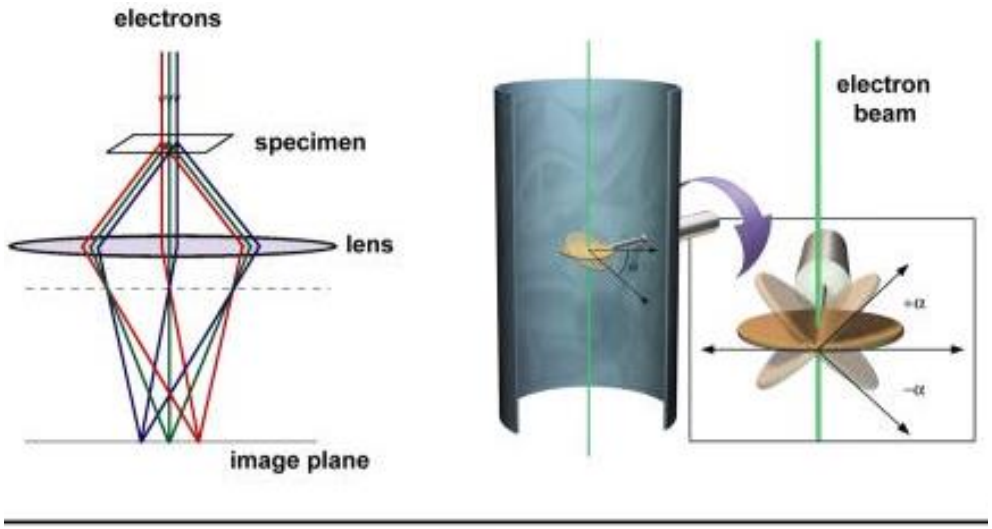


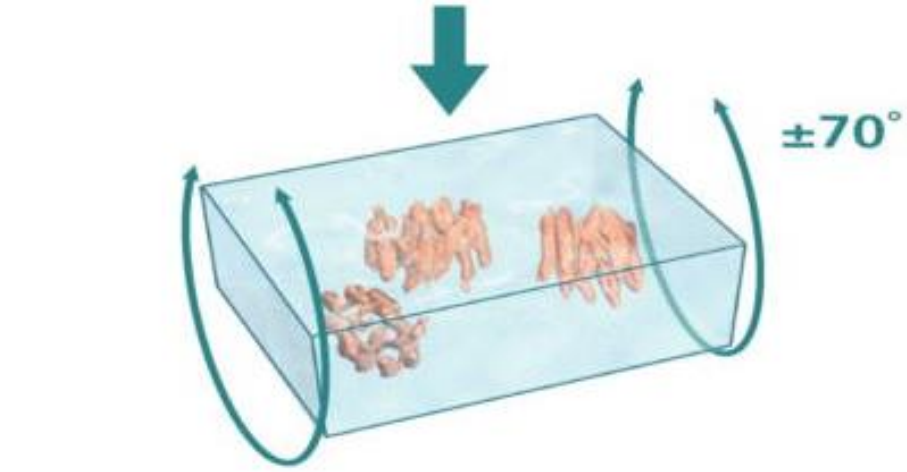




A 1.5M€ direct electron detector is a key feature of CryoEM

Cryo EM



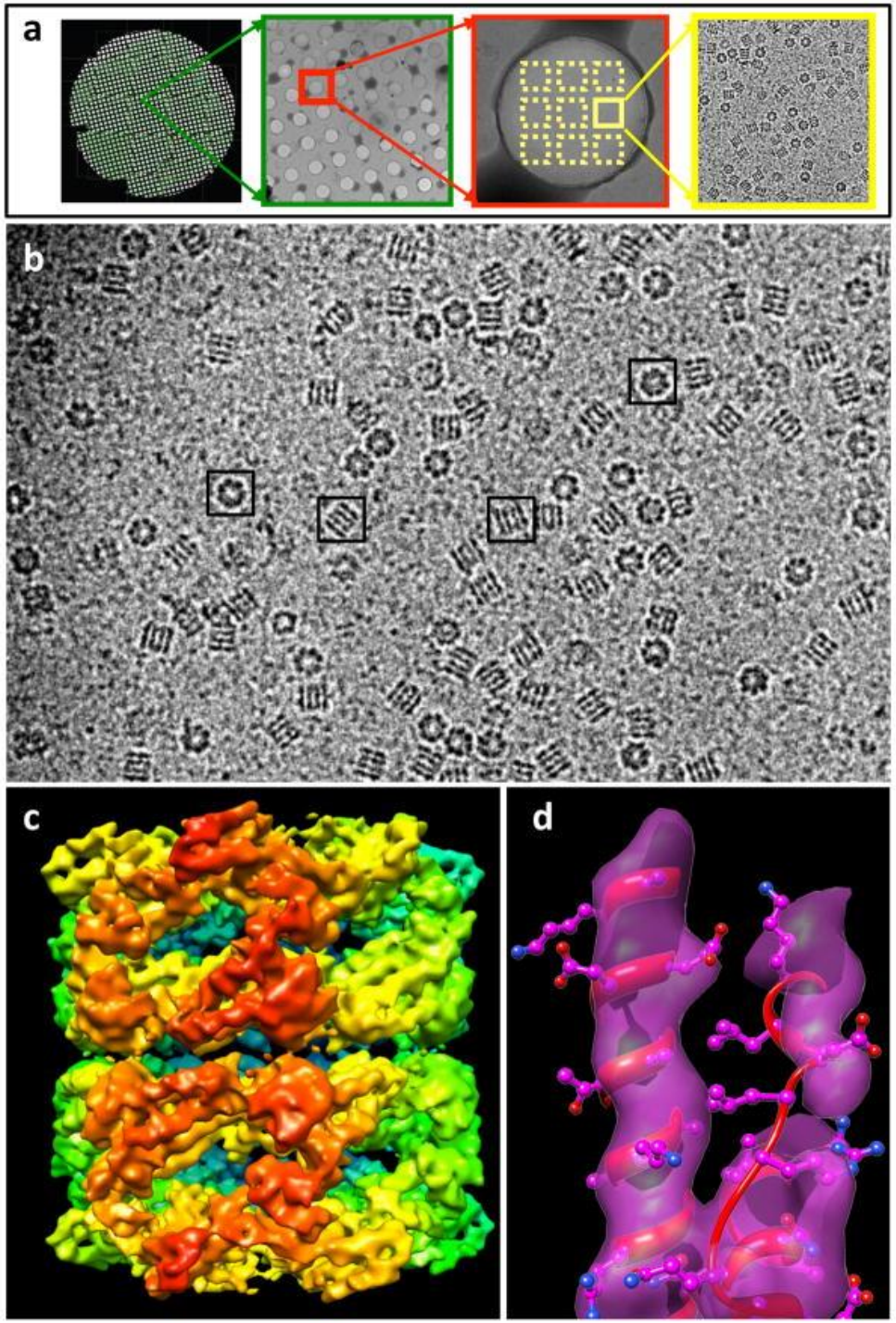


Data collection for electron tomography. As the specimen is tilted relative to the electron beam, a series of images is taken of the same field of view.

Selected projection views generated during cryo-electron tomography as a vitrified film (formed by rapidly freezing a thin aqueous suspension) is tilted relative to the electron beam.

To reconstruct the three-dimensional volume, a set of projection images is “smeared” out along the viewing directions to form back-projection profiles.

The images are combined computationally to recover the density distribution of the object



(a) Images of a cryo-EM grid at sequentially higher magnification,

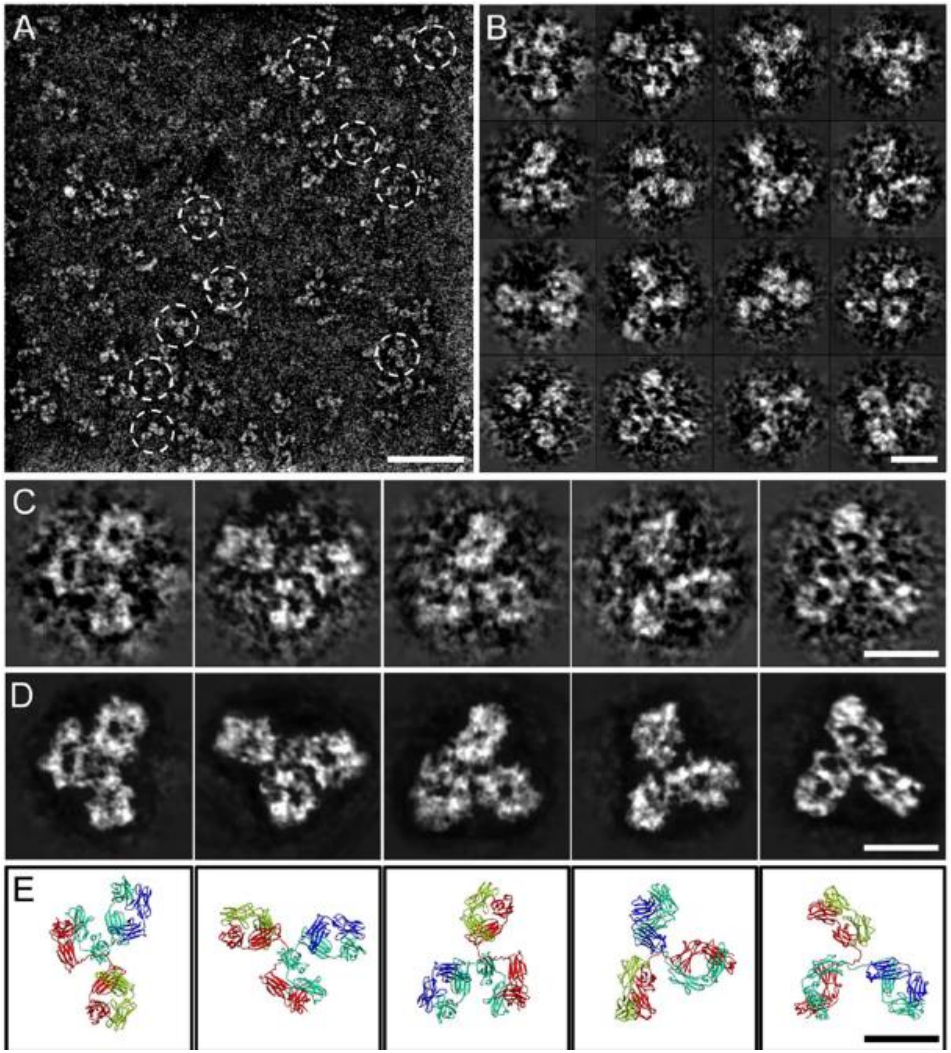
(b) Representative projection image from a frozen-hydrated specimen of purified GroEL protein complexes. Complexes with distinct orientations relative to the electron beam can be discerned as indicated in the boxed examples.

(c) 3D reconstruction using ~ **28,000** individual projection images

~ 7 Å resolution.

(d) Demonstration that the resolution achieved is adequate to visualize α-helices, illustrated by the superposition of a density map of a region of the polypeptide with the corresponding region of a GroEL structure determined by X-ray crystallography

INDIVIDUAL PARTICLE imaging



Individual particle electron tomography,

- cryo TEM
- low current to avoid damage
- proper staining
- select one particle
- take several different angle images
- reconstruct by post processing
- image with 2nm lateral resolution

The conformational variation within the same population can be observed

The Nobel Prize in Chemistry 2017

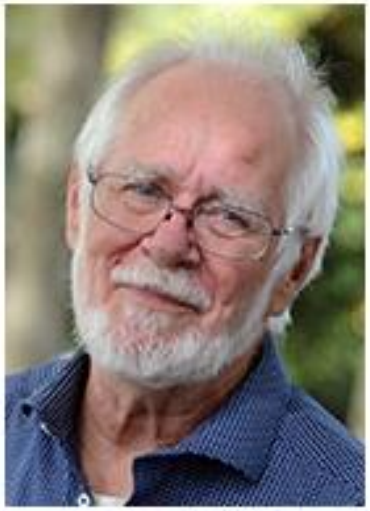


Photo: Félix Imhof © UNIL [CC BY-SA 4.0]
Jacques Dubochet
Prize share: 1/3

Photo: B. Winkowski © Columbia University Medical Center
Joachim Frank
Prize share: 1/3

Photo: MRC Laboratory of Molecular Biology
Richard Henderson
Prize share: 1/3

The Nobel Prize in Chemistry 2017 was awarded to Jacques Dubochet, Joachim Frank and Richard Henderson *"for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution"*.

The Nobel Prize in Chemistry 2017

Image analysis

Photo: B. Winkowski © Columbia University Medical Center

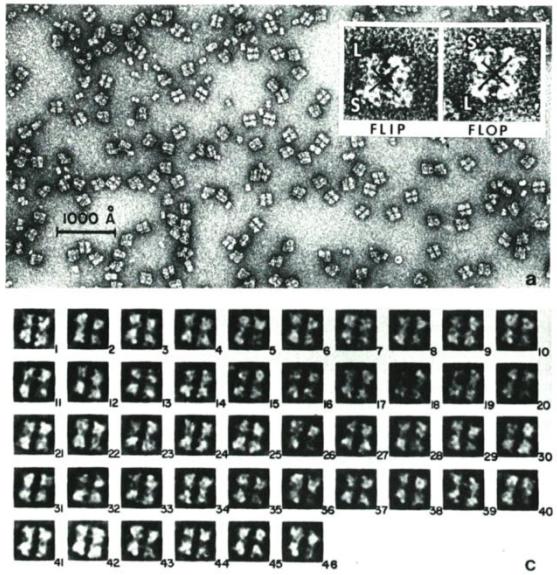
Joachim Frank

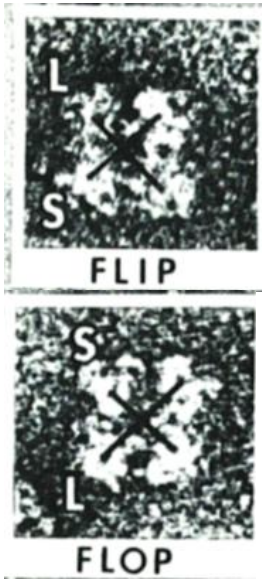
Ultramicroscopy 6, (1), (1981), pp.187–194

Use of multivariates statistics in analysing the images of biological macromolecules

"…We have developed a new technique of analysis that allows automatic classification of molecule images according to subtle differences.."

hemocyanin half-molecules from Limtdus polyphemus





The Nobel Prize in Chemistry 2017

Sample preparation

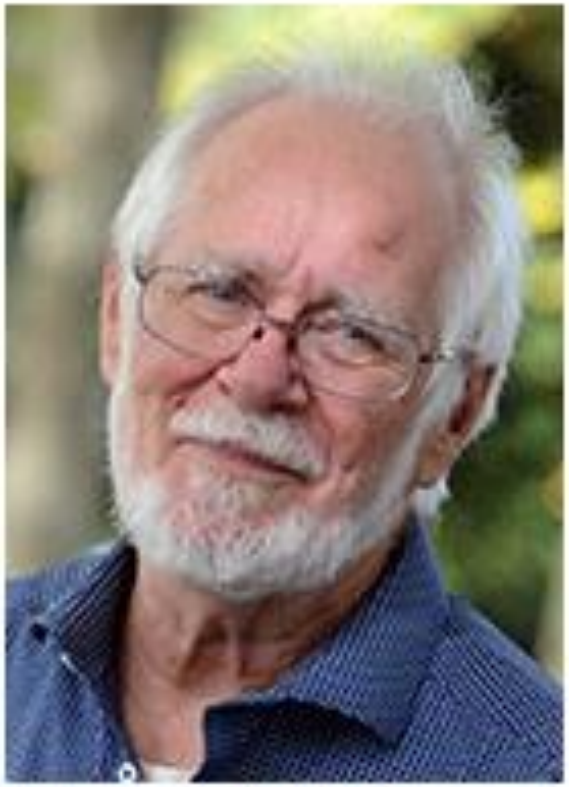


Photo: Félix Imhof © UNIL [CC BY-SA 4.0]

Jacques Dubochet

Quarterly Reviews of Biophysics 21 (2), pp. 129-228 (1988)

Cryo-electron microscopy of vitrified specimens

”… at that moment the method looked more like a laboratory game than a useful tool…”

“…Since that time, water, which was once the arch enemy of all electron microscopists, became what it always was in nature – an integral part of biological matter and a beautiful substance….”

The Nobel Prize in Chemistry 2017

TEM microscopy

Photo: MRC Laboratory of Molecular Biology

Richard Henderson

Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy	Henderson, R., Baldwin, J.M., Ceska, T.A., (...), Beckmann, E., Downing, K.H.	1990	Journal of Molecular Biology	2253
View at Publisher				
Three-dimensional model of purple membrane obtained by electron microscopy	Henderson, R., Unwin, P.N.T.	1975	Nature	1357
View at Publisher				
Structure of a β1-adrenergic G-protein-coupled receptor	Warne, T., Serrano-Vega, M.J., Baker, J.G., (...), Tate, C.G., Schertler, G.F.X.	2008	Nature	1086
View at Publisher				
Electron-crystallographic refinement of the structure of bacteriorhodopsin	Grigorieff, N., Ceska, T.A., Downing, K.H., Baldwin, J.M., Henderson, R.	1996	Journal of Molecular Biology	797
View at Publisher				
Molecular structure determination by electron microscopy of unstained crystalline specimens	Unwin, P.N.T., Henderson, R.	1975	Journal of Molecular Biology	733

The Nobel Prize in Chemistry 2017

Detector

ELSEVIER

Journal of Structural Biology

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FEI's direct electron detector developments: Embarking on a revolution in cryo-TEM

Maarten Kuijper, Gerald van Hoften, Bart Janssen, Rudolf Geurink, Sacha De Carlo, Matthijn Vos, Gijs van Duinen, Bart van Haeringen, Marc Storms

The keyplayers were:
- **DED - direct electron detector**
- **Volta phase plates**

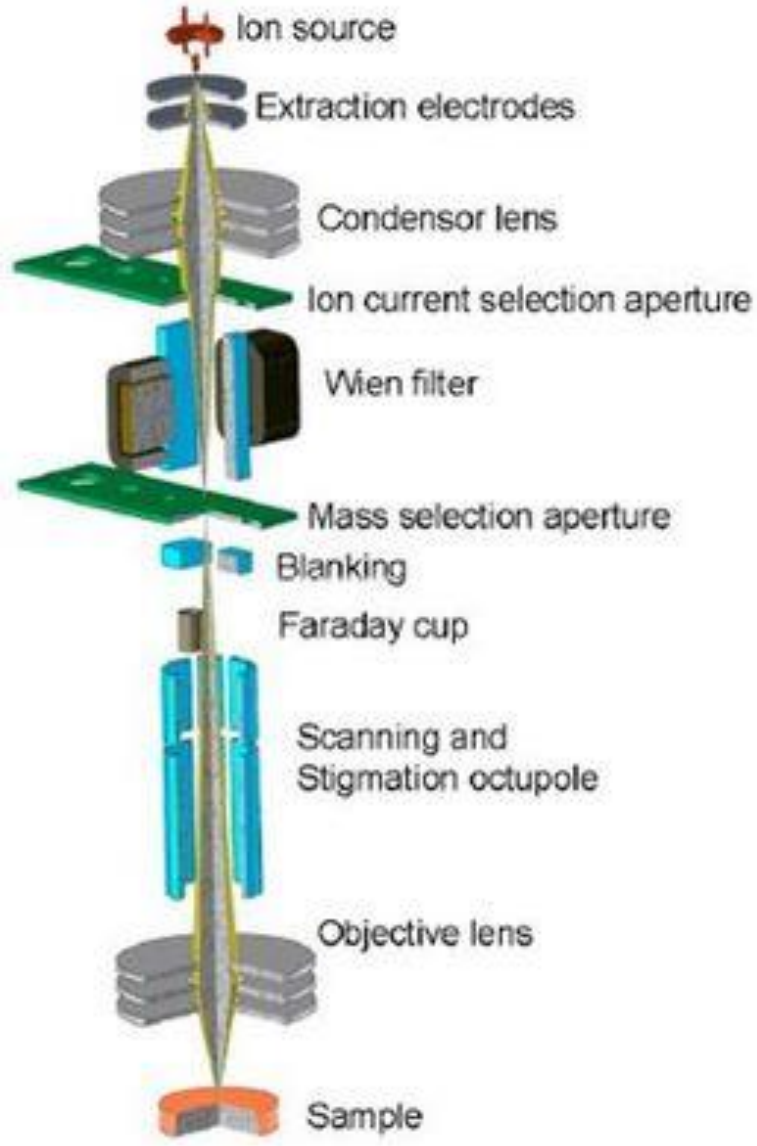
FIB

The Focused Ion Beam (FIB) is a scanning microprobe similar to a Scanning Electron Microscope (SEM).

In both case a **beam**, *a 30 keV Ga^+ ion beam in the case of the FIB*, is rastered over a surface
the secondary electron or ion intensity is displayed.

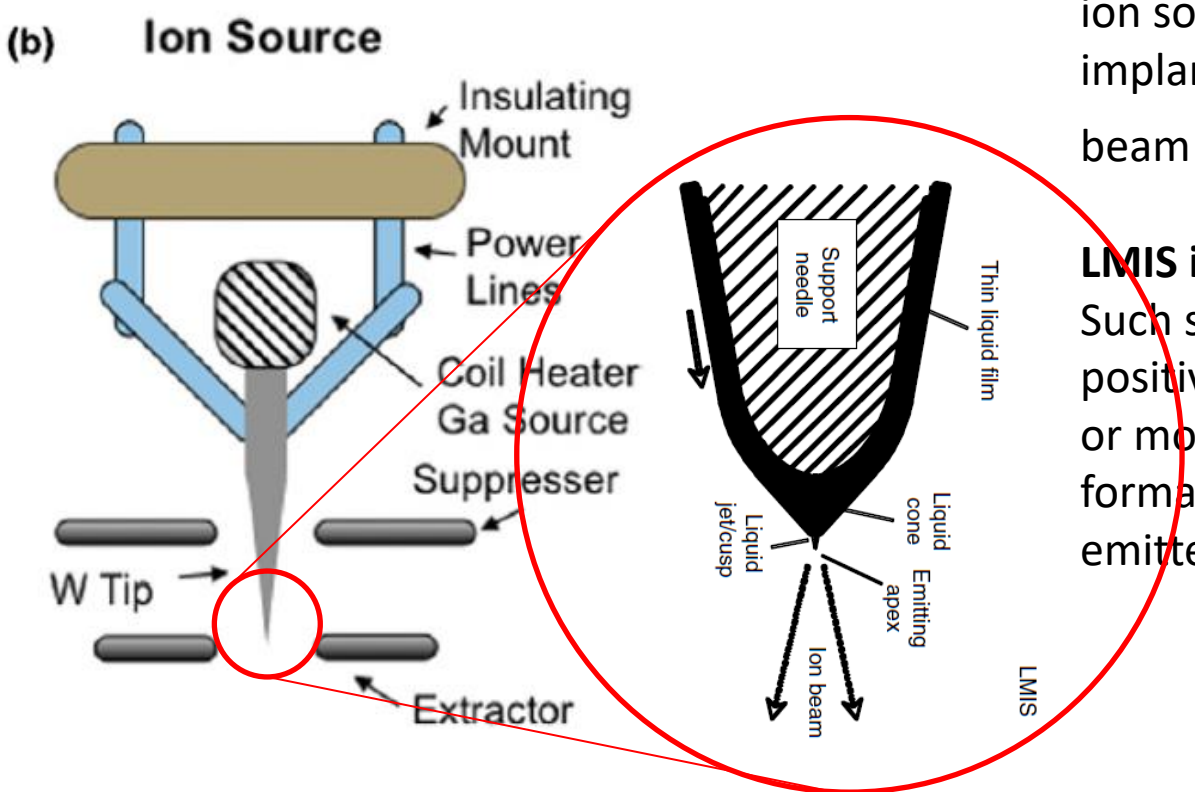
The image is produced by contrast due to differences in elements, Z contrast, crystallographic orientation, channeling contrast, or topography.

The advantage of the FIB is that since an ion beam is used material can be removed from the sample through ion milling. This allows shorts in electronic circuits to be fixed as well as features to be milled into materials for a variety of purposes.



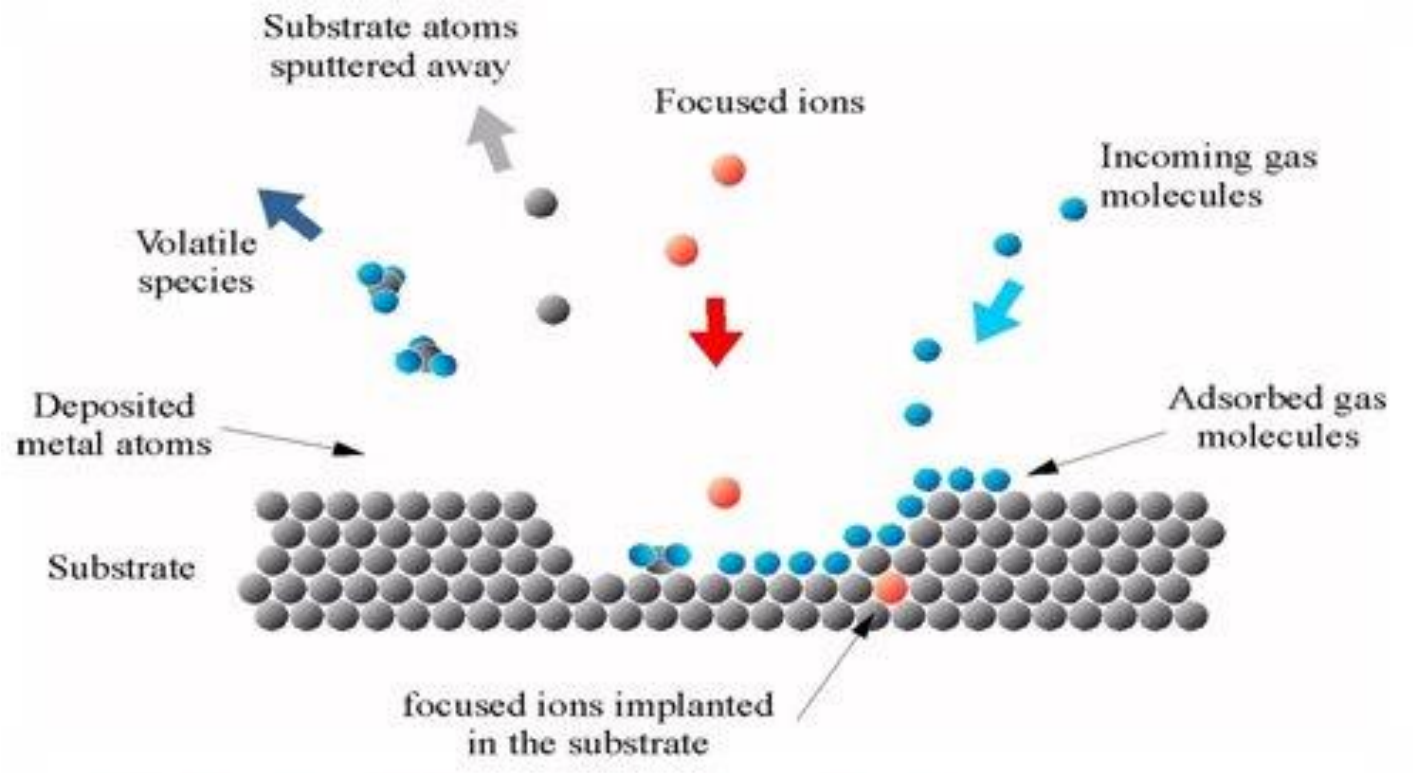
FIB

In a **Liquid metal ion source (LMIS)**, a metal (typically Gallium T_M = 30C) is heated to the liquid state and provided at the end of a capillary or a needle. Then a Taylor cone is formed under the application of a strong electric field. As the cone's tip get sharper, the electric field becomes stronger, until ions are produced by field evaporation. These ion sources are particularly used in ion implantation or in focused ion beam instruments.



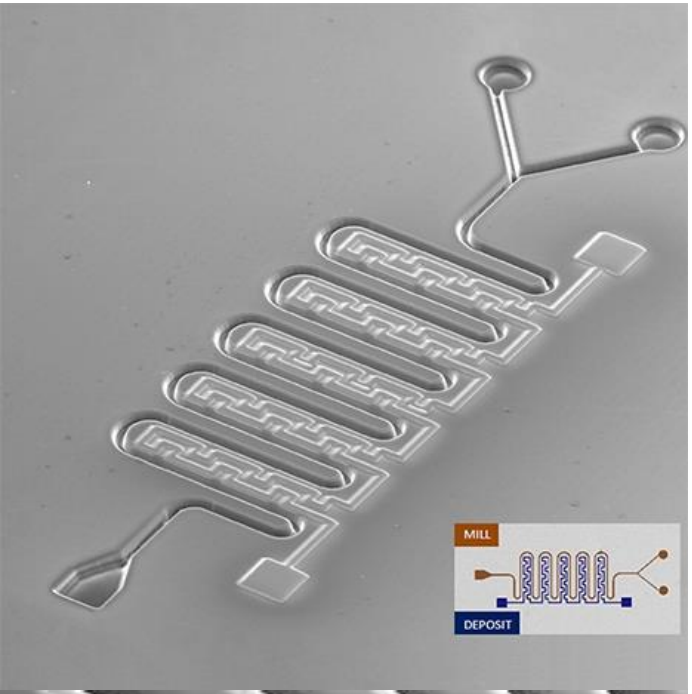
LMIS is a field ion emission source. Such sources generate high–brightness positive ion beams from neutral atoms or molecules, by field–induced ion formation at the tip of a needlelike emitter.

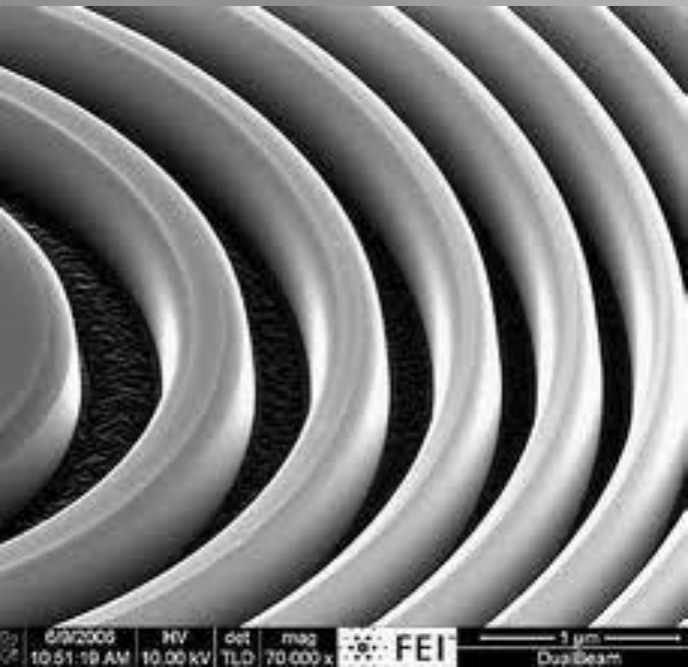
FIB - milling

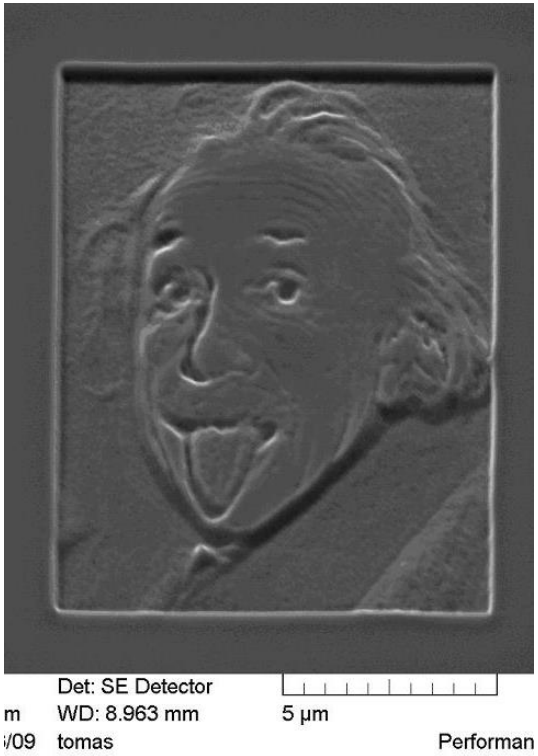


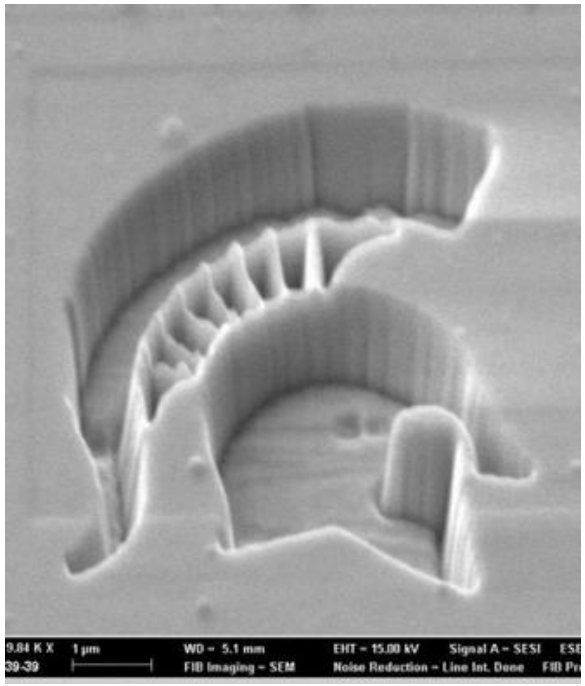
1. Adsorption of the gas molecules on the substrate
2. Interaction of the gas molecules with the substrate
   Formation of volatile and non volatile species
3. Evaporation of volatile species and sputtering of non volatile species

FIB - milling



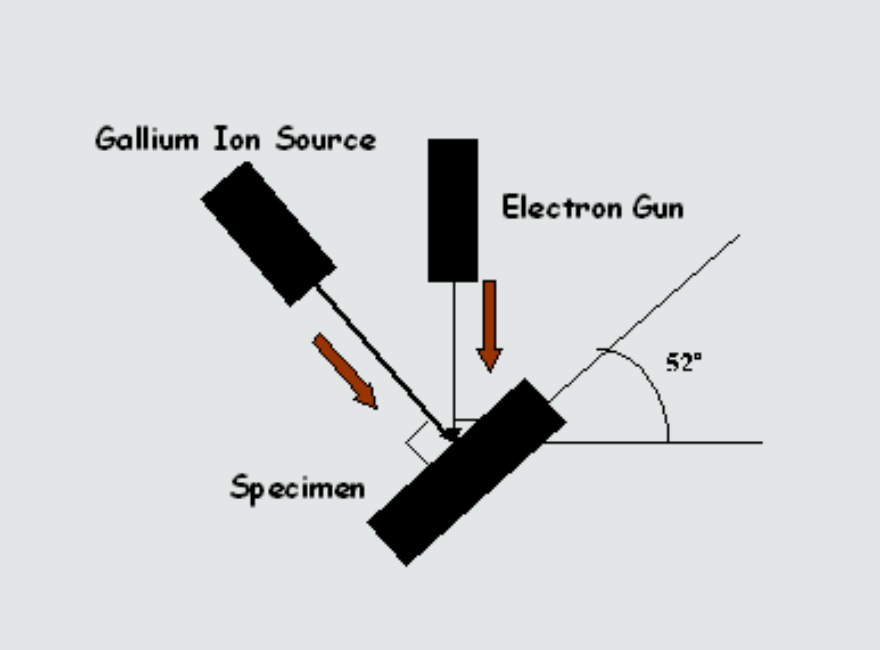


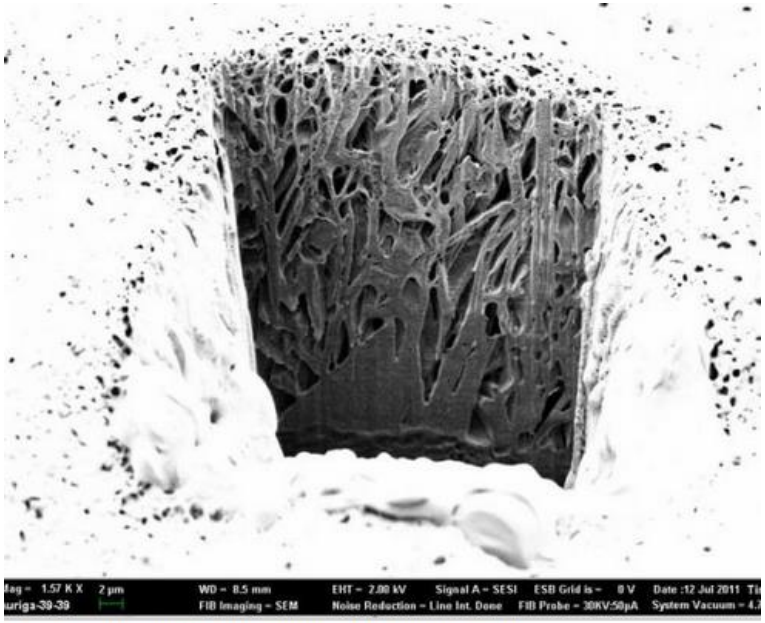




Useful (a microfluidic circuit or a zone plate)
and less useful features
can be produced

FIB – dual beam





Cross section of a mouse bone. Courtesy of Baumman, et al.

Usually a FIB column is coupled with a SEM column.
In this way the FIB column can e used to dig the sample and the SEM to image in cross section…

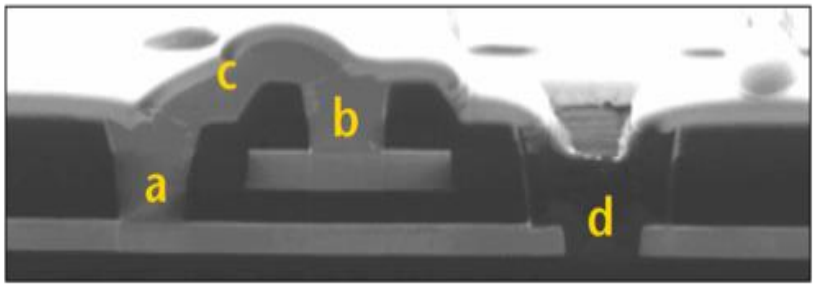
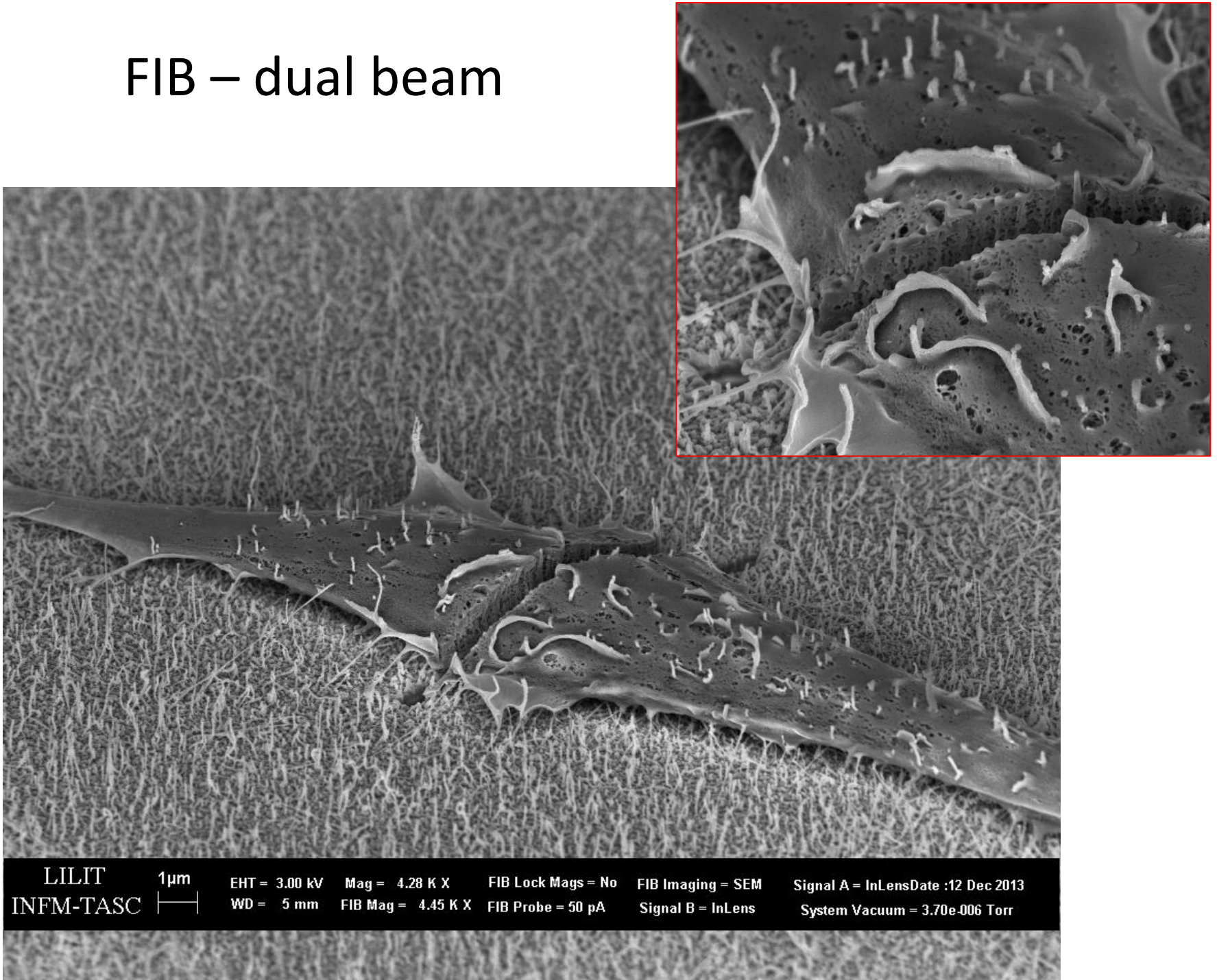
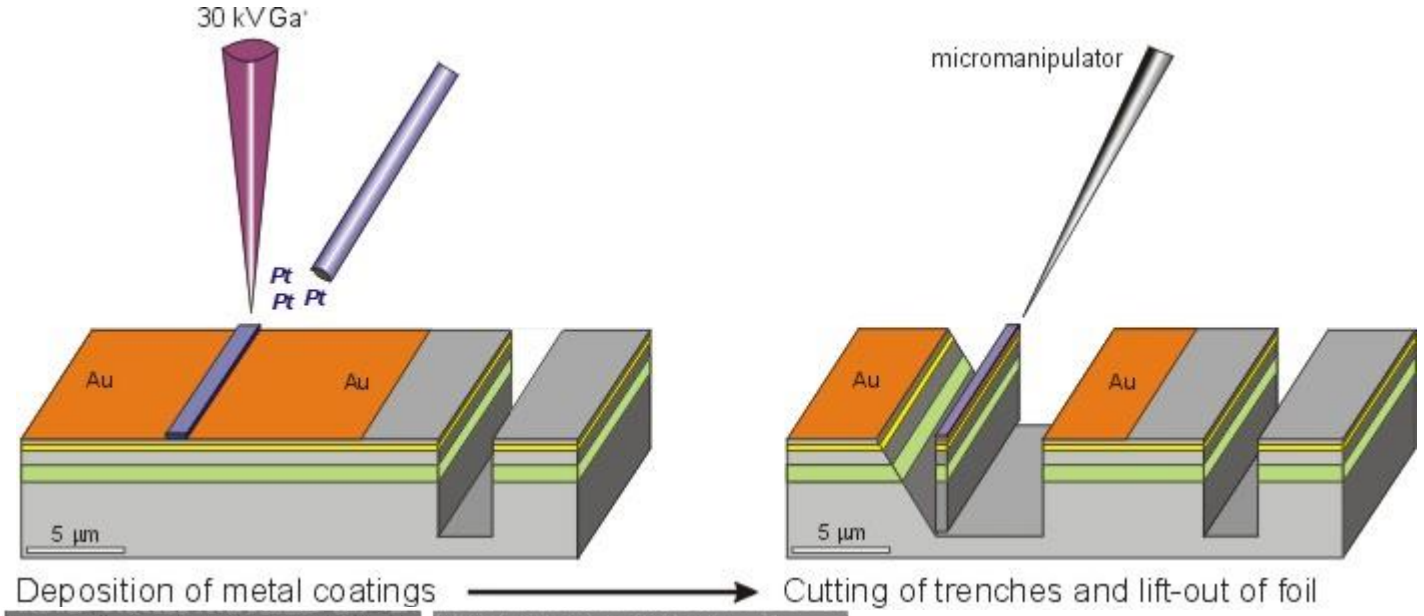


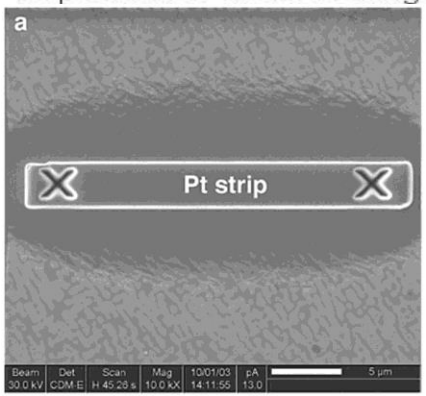
Figure 2 Cross-section view of the edit shown in figure 1 on a two metal layer IC

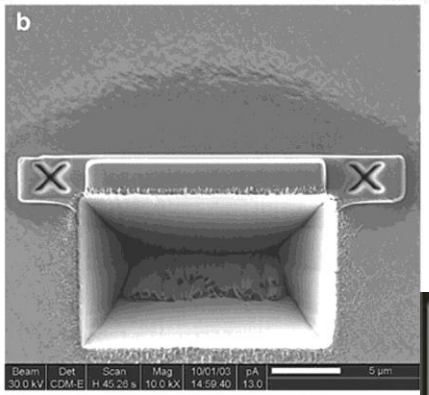
FIB – dual beam

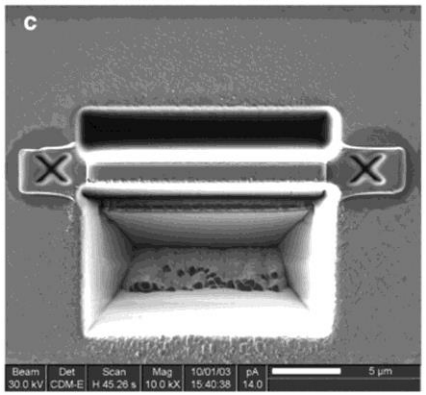


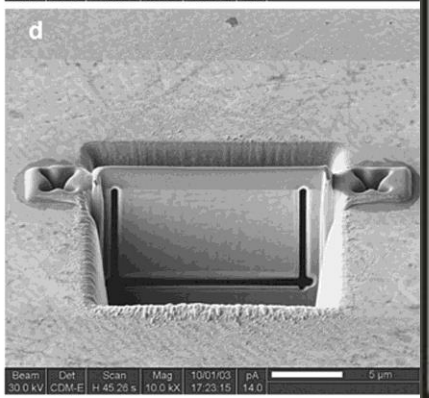
FIB – TEM preparation

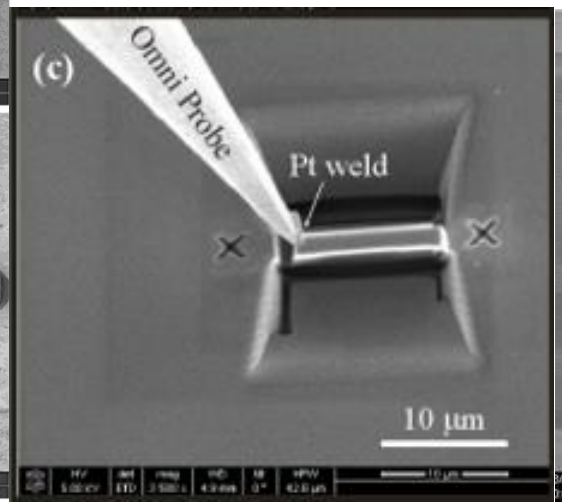


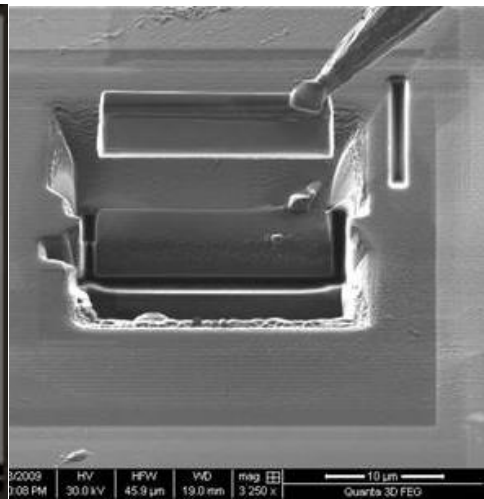




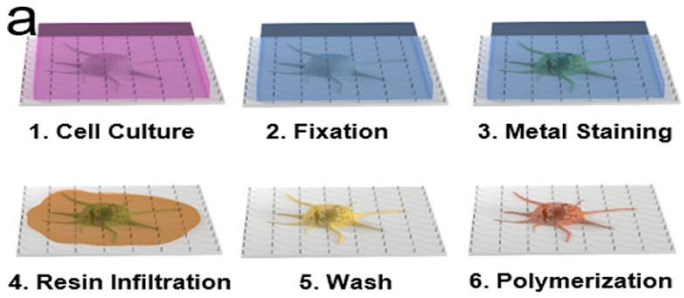


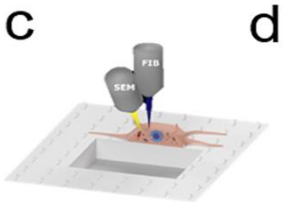


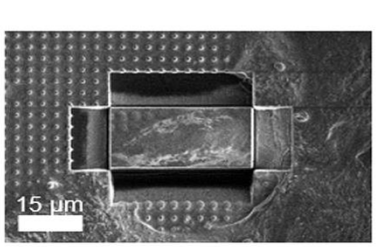


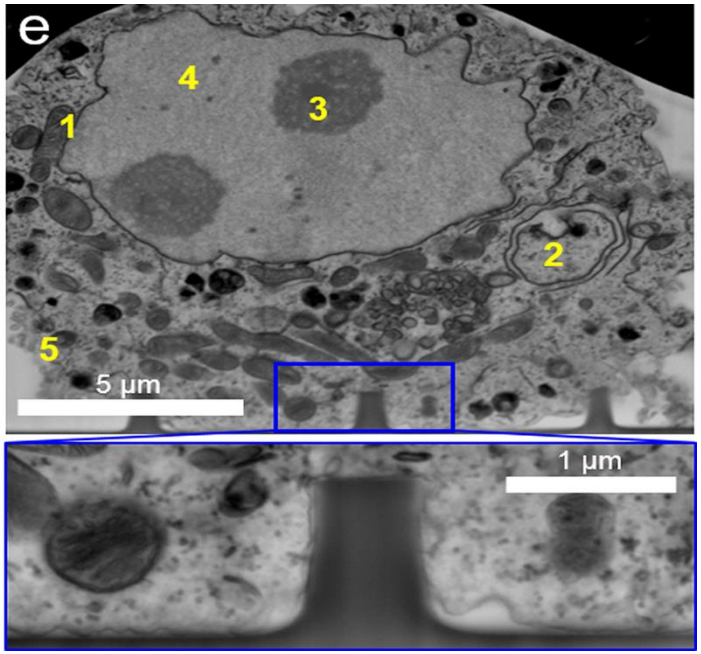


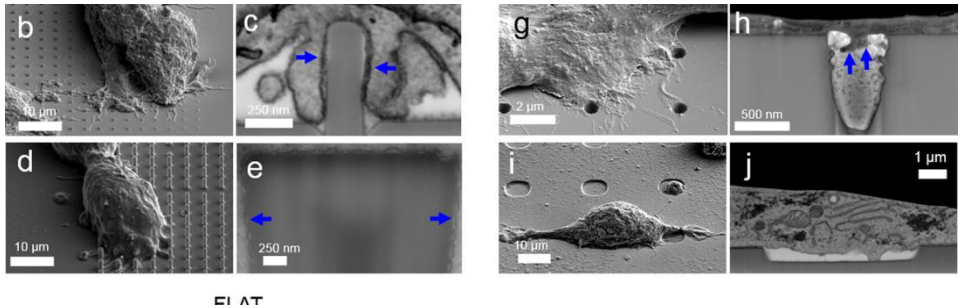
FIB – cross section on fixed cells





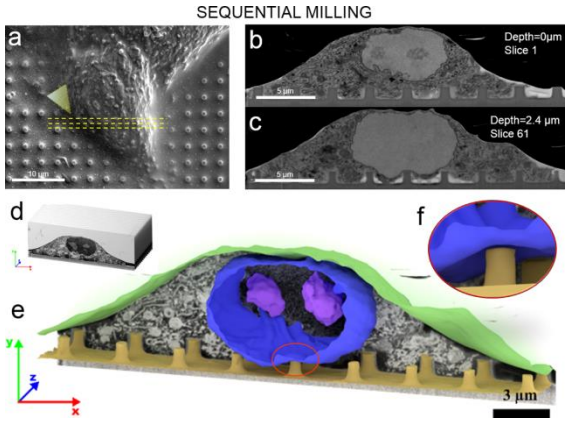






Cell membrane readily deforms inward and wraps around protruding structures, but hardly deforms outward to contour invaginating structures.

A positive membrane curvatures with a radius <200 nm trigger Clathrin-mediated endocytosis (CME).



Also the nuclear envelope is deformed upward by a nanopillar

The interface between cells and nonbiological surfaces that regulates cell attachment, chronic tissue responses, and ultimately the success of medical implants or biosensors **is strongly influenece by nanotopography**

Cryo - FIB

Features:

- sample preparation and transfer at cryotemperature (LN2 -193C)

- Cooled sample holder and cold shield (to minimiza sample contamination)

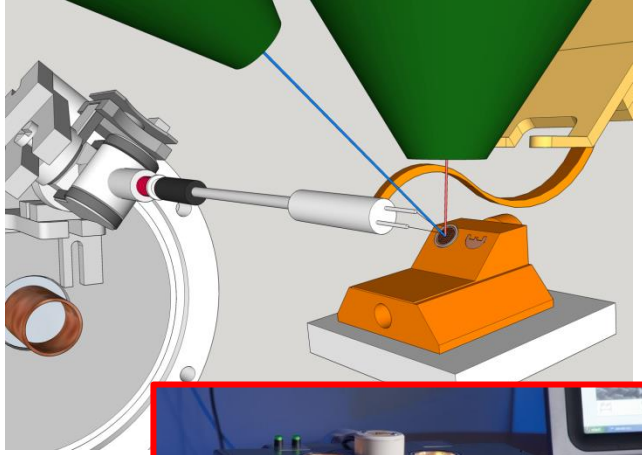
- No need for drying process

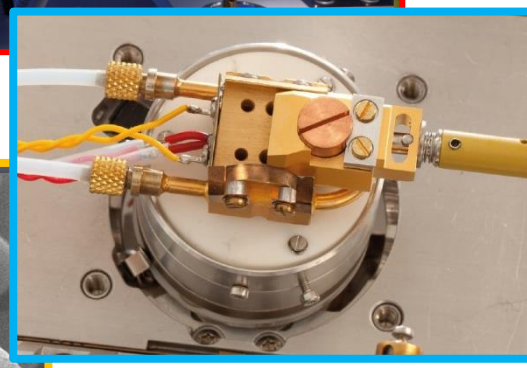
- e-beam damage reduction

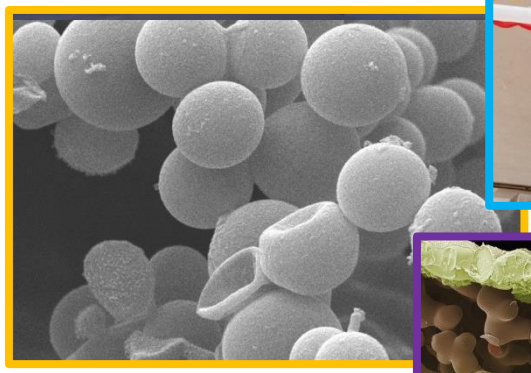
- Freeze fracture

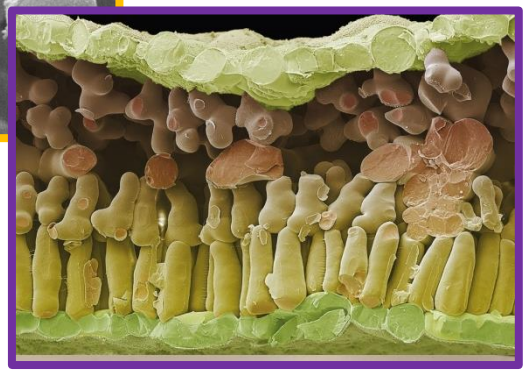
- Sectioning

- TEM slice preparation



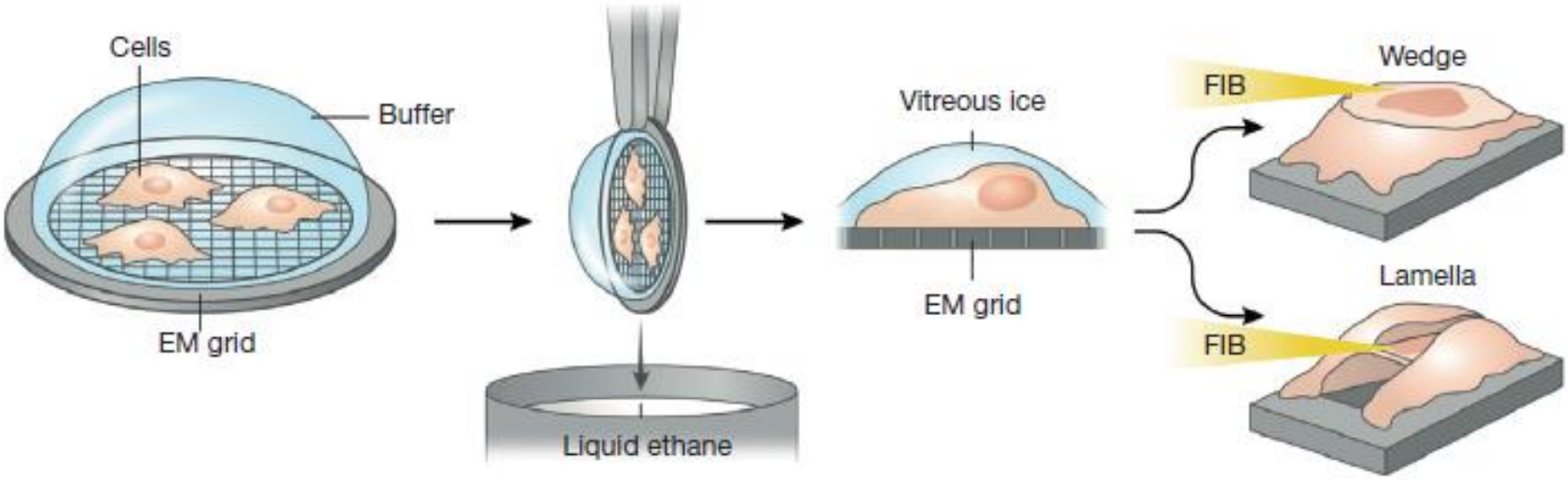




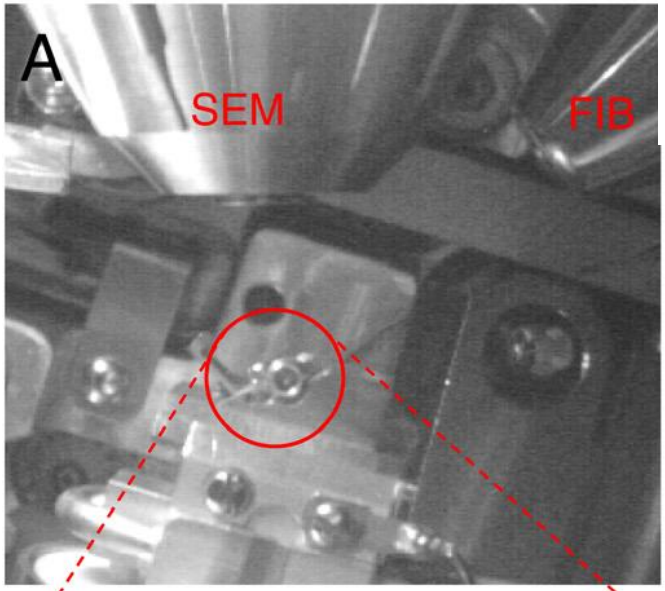


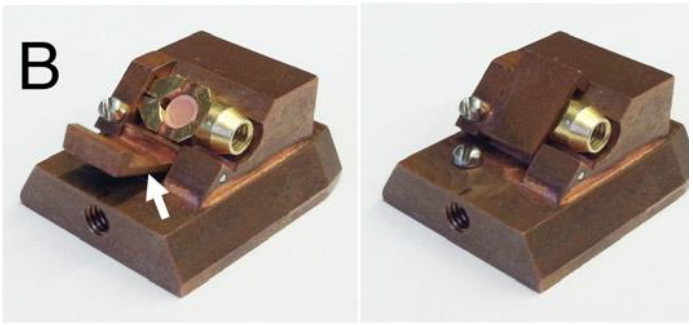
Cryo – FIB - example

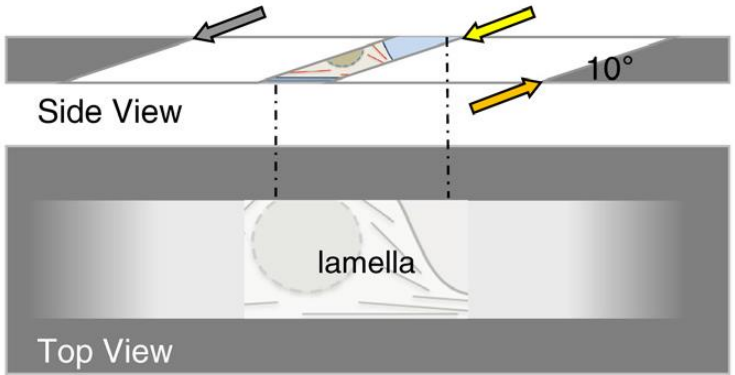
It has been known for several decades that fixing a biological sample in vitreous ice preserves it in a near-native state83. Still, there are limits on the thickness of a sample to be imaged by TEM, and this has restricted microscopy at cryogenic temperatures to studies



FIB operations, however, perform well under cryogenic conditions, and different groups have exploited this to generate TEM-ready lamellae from thick biological samples using various approaches



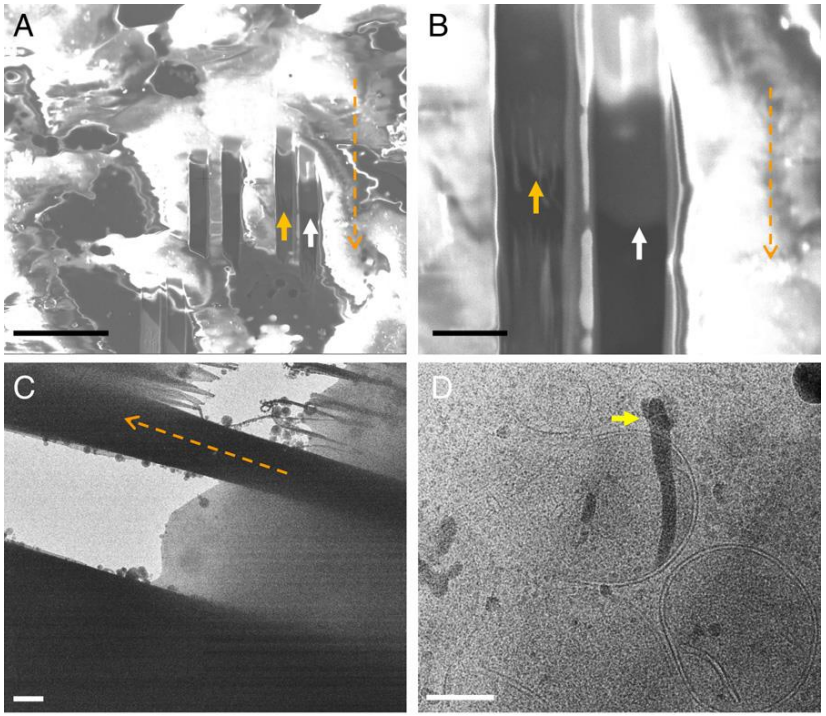




3D structure determination of native mammalian cells using cryo-FIB and cryo-electron tomography

Ke Wang [a,1], Korrinn Strunk [b,1], Gongpu Zhao [a], Jennifer L. Gray [b], Peijun Zhang [a,*]

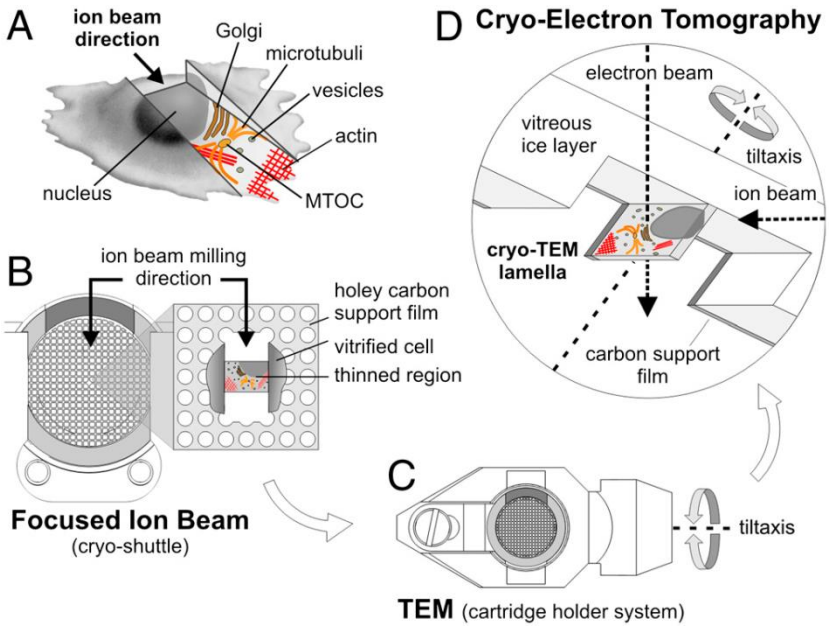
They show a simple and robust method for creating in situ, frozen-hydrated cell lamellas using a cryo-FIB, allowing *in-situ* access to any interior cellular regions of interest.

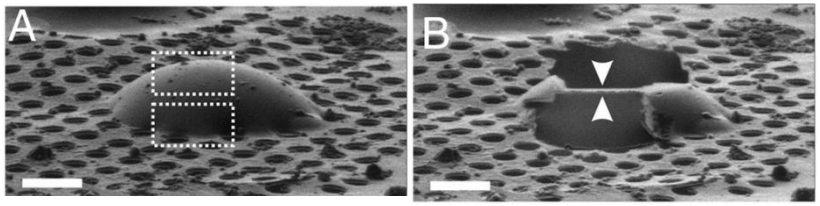


Cryo-FIB milling and cryo-ET of frozen-hydrated HeLa cells

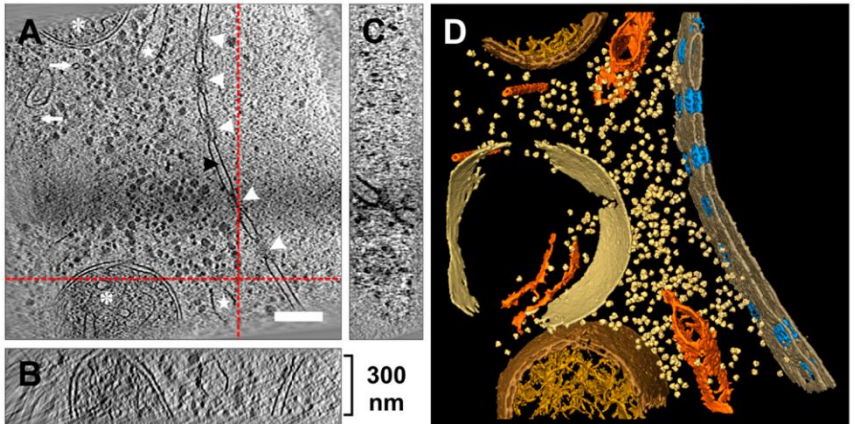
Focused ion beam micromachining of eukaryotic cells for cryoelectron tomography

Alexander Rigort[1], Felix J. B. Bäuerlein[1], Elizabeth Villa, Matthias Eibauer, Tim Laugks, Wolfgang Baumeister[2], and Jürgen M. Plitzko[2]





FIB is used for the micromachining of cells embedded in vitreous ice.
Thin lamellae are cut out of cellular volumes with geometries suitable for electron tomography.
The lamellae are left in situ during transfer to the EM supported only by the surrounding bulk ice.



Cryoelectron tomograms of D. discoideum cells.

(A) Slice through thebx; y-plane of a tomographic reconstruction showing the nuclear envelope (black arrowhead) with nuclear pore complexes (white arrowheads) separating cytoplasm from nucleoplasm

Endoplasmic reticulum (white stars), tubular mitochondria (asterisks) and microtubules (white arrows)

(B and C) x; z and y, z-planes.

The thickness of the lamella is approximately 300 nm.

(D) Surface rendered visualization, displaying nuclear envelope, endoplasmic reticulum, mitochondria, microtubules, vacuolar compartment, and ribosomes