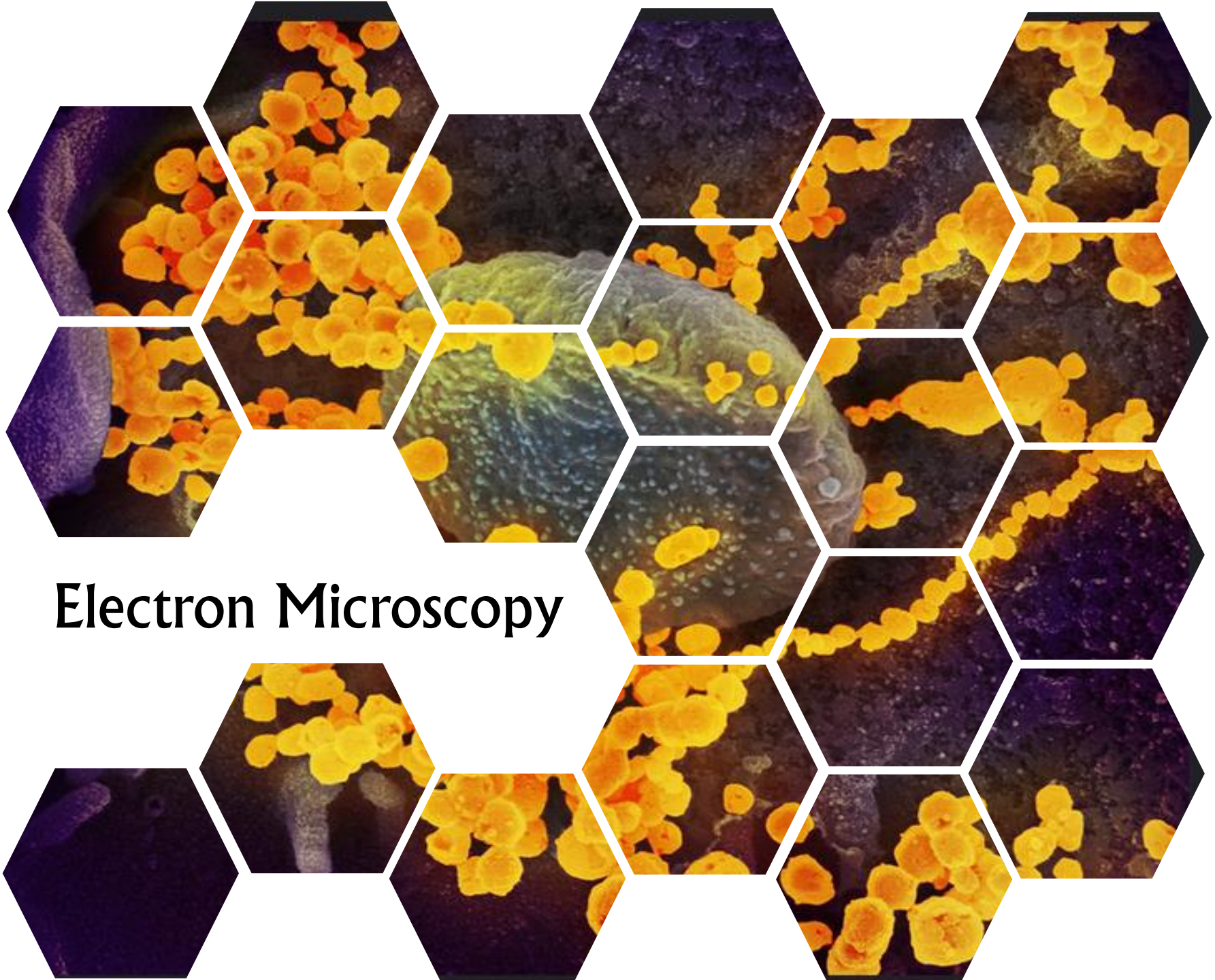
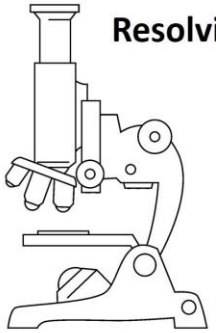
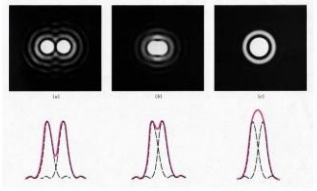


Electron Microscopy

Limit of resolution



Resolving power of Microscope



Resolution limits (diffraction)

Abbe equation: $$d = \frac{0.61\lambda}{n \sin \alpha}$$

d – resolution
λ – wavelength of particle
n – refractive index relative to free spac
α – semi-angle in radians

$\lambda \Rightarrow$ limit of resolution
(or resolving power)

- EUV $\lambda \in$ [10nm,200nm]
- Soft x rays $\lambda \in$ [1nm,10nm]
- Hard x-rays $\lambda \in$ [0,01nm,1nm]

Problems

Matter interaction: Absorbance, optics

Photons generation: complex sources

Electrons: particles and waves

1906 – J.J. Thomson was awarded the Nobel Prize, for demonstrating the existence of the electron as a particle.

1937 – G.P. Thomson was awarded the Nobel Prize for demonstrating that the electron was a wave. [Thomson and Reid (1927), Davisson and Germer (1927)]

The De Broglie equation(1924) states that any particle with p momentum is associated with a wavelength λ.

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

Where h is the Planck constant (6.63 x 10^{-34} J·s).

Why electrons?

Electrons are **charged** particles.
Their associated **wavelength** at 103 - 104 *KeV* *is* smaller than the average atomic dimension.
They strongly **interact** with matter: electrons interact with the Coulomb potential of the atoms (nuclei and electron clouds). The atomic scattering amplitude for electrons is 103 - 104 times larger than the corresponding ones for X-rays and neutrons.

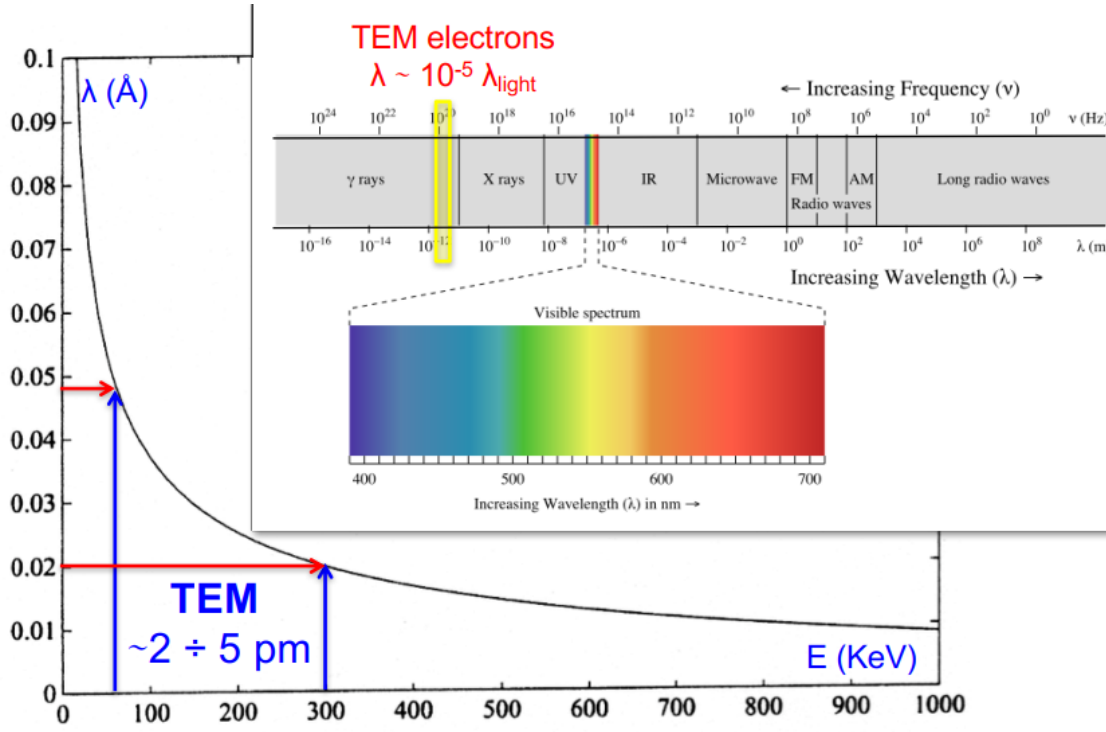
As a consequence:
• Electron trajectories can be easily deflected by electro-magnetic fields (electron lenses)
• higher resolution achievable
• multiple signals can be collected for deeper information

	X-rays	Electrons	Neutrons
(1) Charge	0	-1 e	0
(2) Rest mass	0	9.11×10^{-31} kg	1.67×10^{-27} kg
(3) Energy	10 keV	100 keV	0.03 eV
(4) Wavelength	1.5 Å	0.04 Å	1.2 Å
(5) Bragg angles	Large	1°	Large
(6) Extinction length	10 μm	0.03 μm	100 μm
(7) Absorption length	100 μm	1 μm	5 cm
(8) Width of rocking curve	5″	0.6°	0.5″
(9) Refractive index $n = 1 + \delta$	$n < 1$ $\delta \approx -1 \times 10^{-5}$	$n > 1$ $\delta \approx +1 \times 10^{-4}$	$n \lesseqgtr 1$ $\delta \approx \mp 1 \times 10^{-6}$
(10) Atomic scattering amplitudes f	10^{-3} Å	10 Å	10^{-4} Å
(11) Dependence of f on the atomic number Z	$\sim Z$	$\sim Z^{2/3}$	Nonmonotonic
(12) Anomalous dispersion	Common	—	Rare
(13) Spectral breadth	1 eV $\Delta\lambda/\lambda \approx 10^{-4}$	3 eV $\Delta\lambda/\lambda \approx 10^{-5}$	500 eV $\Delta\lambda/\lambda \approx 2$

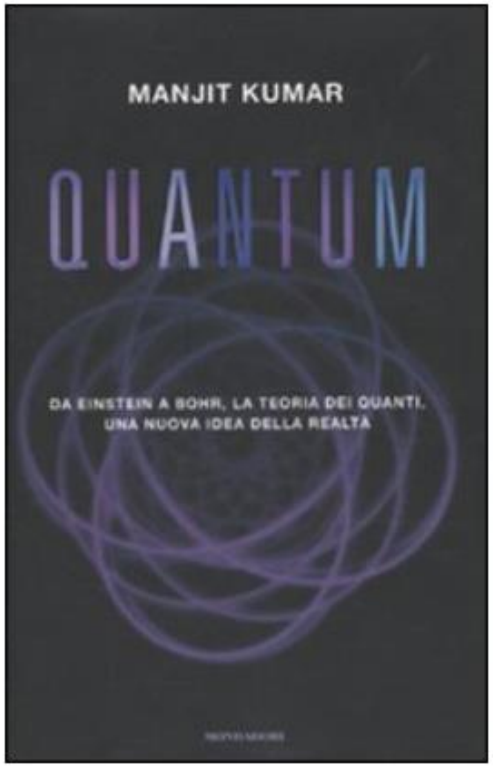
Electron wavelenght

E (kV)	λ (Å)	$(v/c)^2$
80	0.0418	0.2524
100	0.037	0.3005
200	0.0251	0.4835
500	0.0142	0.7445
1000	0.0087	0.8856
2000	0.005	0.9586
10000	0.0012	0.9976

$$\lambda = \frac{h}{\sqrt{2m_0eE\left[1+\left(eE/2m_0c^2\right)\right]}}$$



History: wave mechanics and the invention of TEM



"*In a short time Max Knoll e Ernst Ruska took advantage of the wave nature of electron to invent the electron microscope, in 1931*" (from *Quantum*, Manjit Kumar, Mondadori, 2010, p.153)

... *a posteriori* reconstruction seems reasonable, but in fact things went very differently...

History: wave mechanics and the invention of TEM

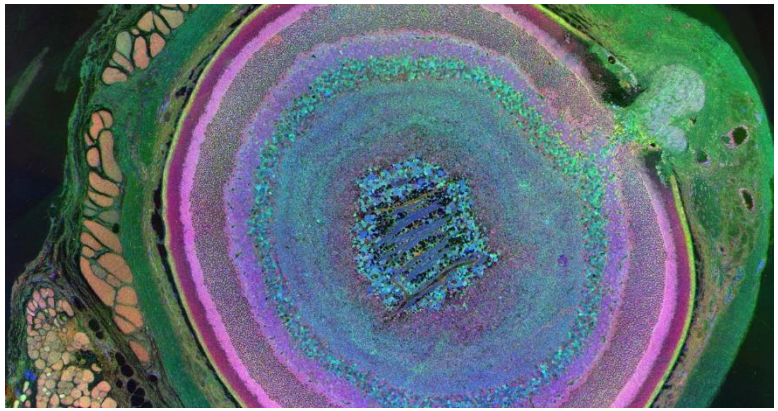
From the interview to **E. Ruska**(*), appearing in TV series "*Microscopia Elettronica*", by **P.G. Merli**, **G. Morandi**, **L. Morettini** (1976).

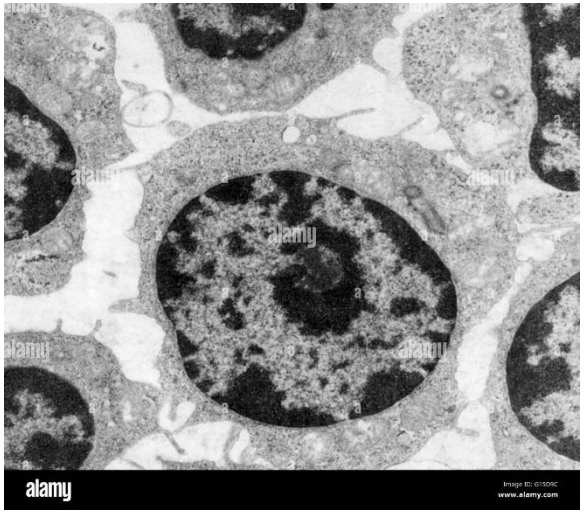
(*) Nobel laureate in physics in 1986, for the invention of the electron microscope.

"There was no influence of wave mechanics throughout the development of the electron microscope to the delivery of the first instrument with a resolving power higher than the optical one, which occurred towards the end of 1939. I and my co-workers were electrical engineers, we knew nothing of wave mechanics and during our work we were essentially guided by ideas of ray (geometrical) optics"

History: wave mechanics and the invention of TEM

Physicists who were aware of the wave theory of electron did not attempt to build an electron microscope (i.e. radiation damage in the TEM), while engineers who didn't know the de Broglie theory, actually invented it!



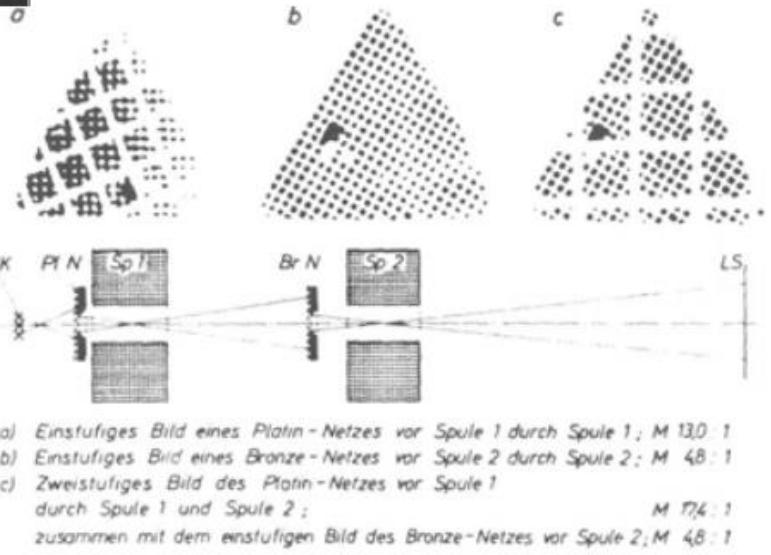


History: wave mechanics and the invention of TEM

1927 ***Hans Busch*: Electron beams can be focused in an inhomogeneous magnetic field.**

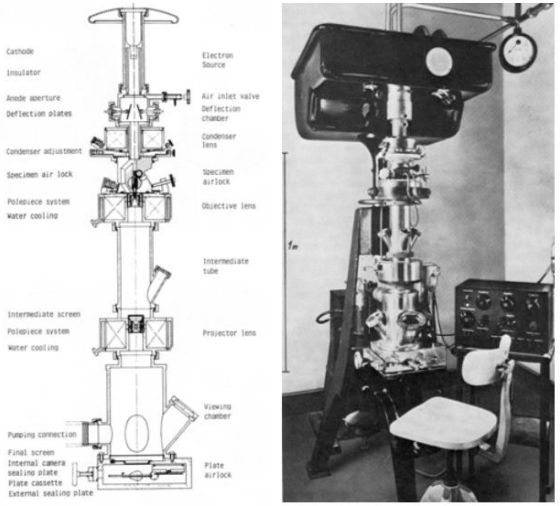
1931 ***Max Knoll* and *Ernst Ruska* built the first TEM.**

1986 **Nobel prize for *Ruska***



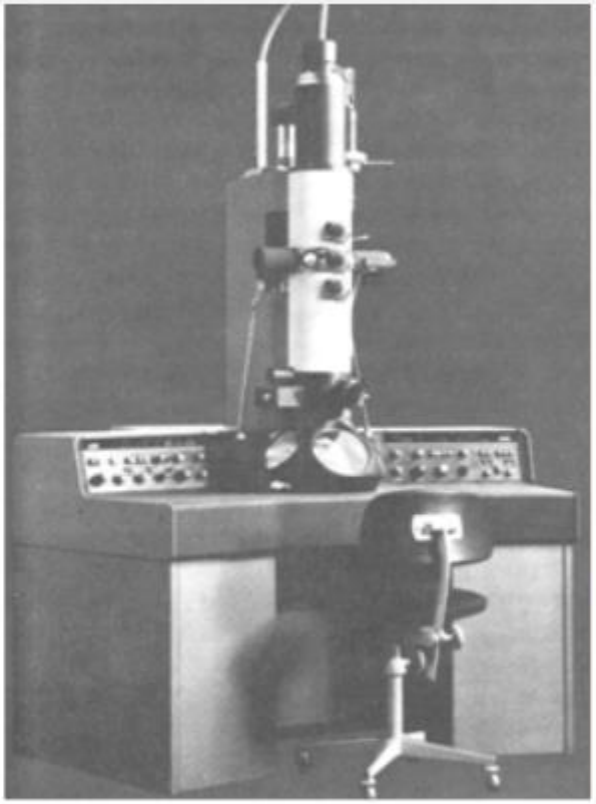
a) Einstufiges Bild eines Platin-Netzes vor Spule 1 durch Spule 1; M 13,0 : 1
b) Einstufiges Bild eines Bronze-Netzes vor Spule 2 durch Spule 2; M 4,8 : 1
c) Zweistufiges Bild des Platin-Netzes vor Spule 1 durch Spule 1 und Spule 2; M 17,4 : 1
zusammen mit dem einstufigen Bild des Bronze-Netzes vor Spule 2; M 4,8 : 1

1938 First Siemens Electron Microscope (Resolution ca. 13 nm)



History: wave mechanics and the invention of TEM

1939: first TEM serially produced by Siemens
resolution ca. 7 nm



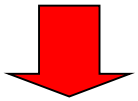
~1970: HRTEM
Philips EM400, V = 120 kV
resolution ca. 0.35 nm

~1990
Philips CM30, V = 300 kV
resolution ca. 0.2 nm

Modern electron microscope

Major differences between TEM e SEM

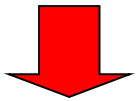
- TEM analyses small samples placed between the polar pieces of the objective lens, which can therefore be operated at very small focal lengths with (relatively) small values of the aberration coefficient



(Possible) high resolution

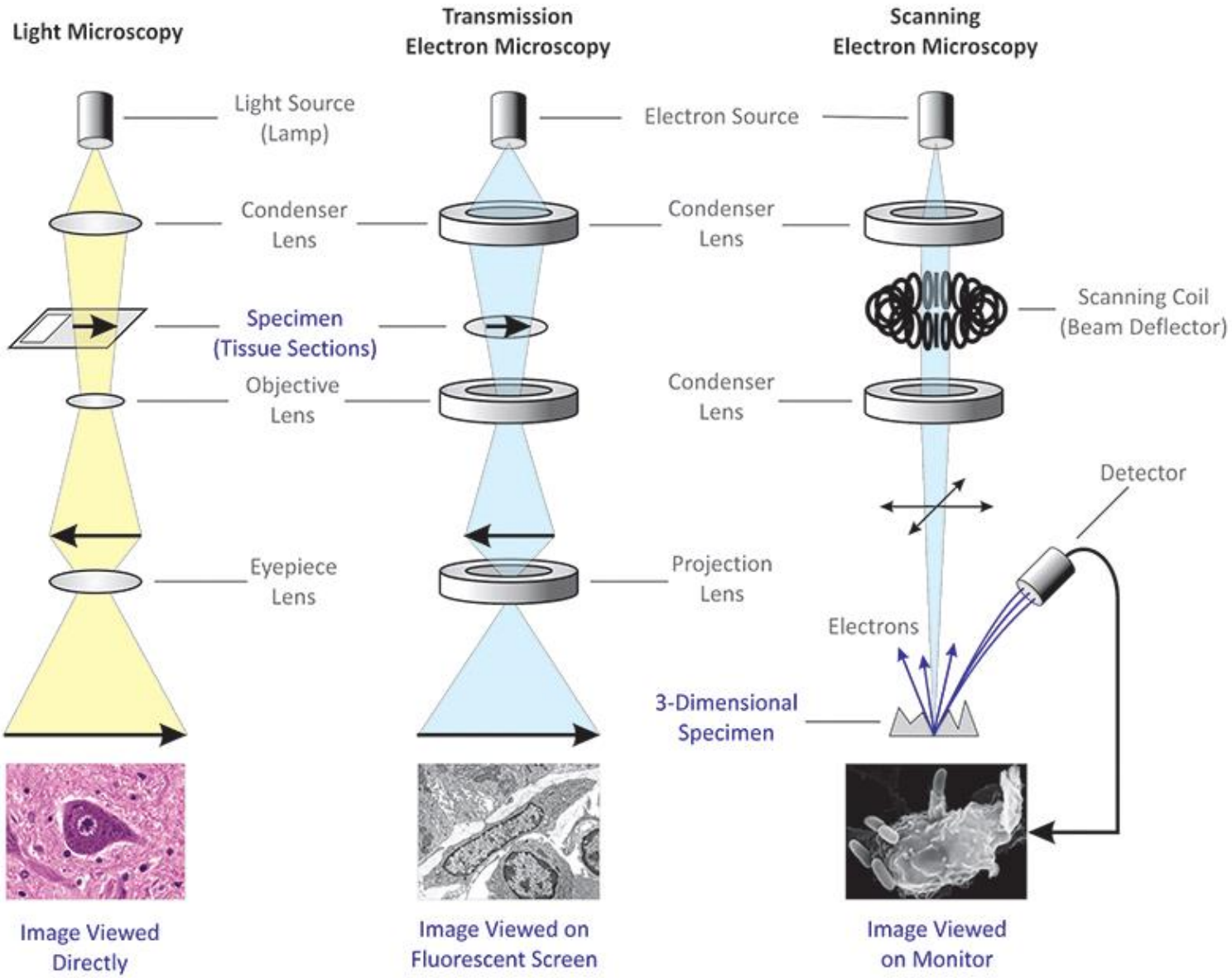
(secondary consequence: the higher the resolution, the less space there is for orienting the sample or for various accessories).

- In SEM large samples are placed outside the objective lens, which is operated at large focal lengths



Limited (lower) resolution

OM TEM and SEM: similarity



History: wave mechanics and the invention of TEM

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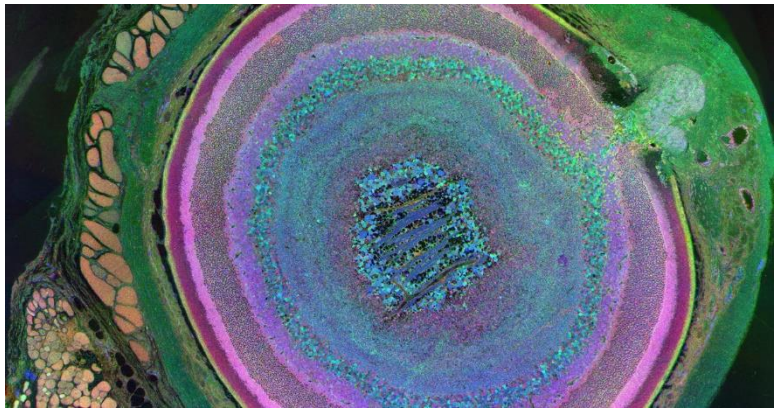
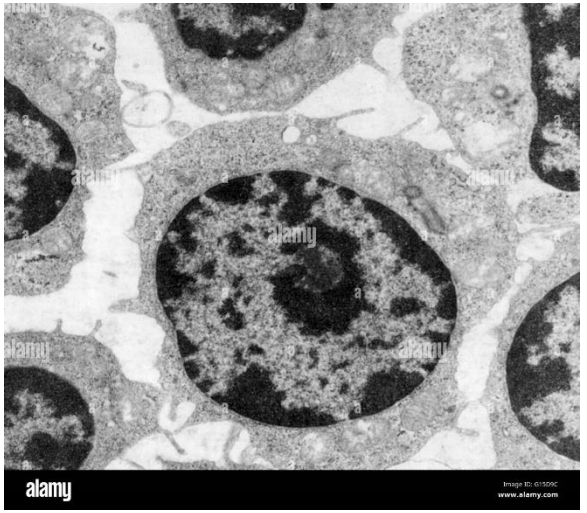
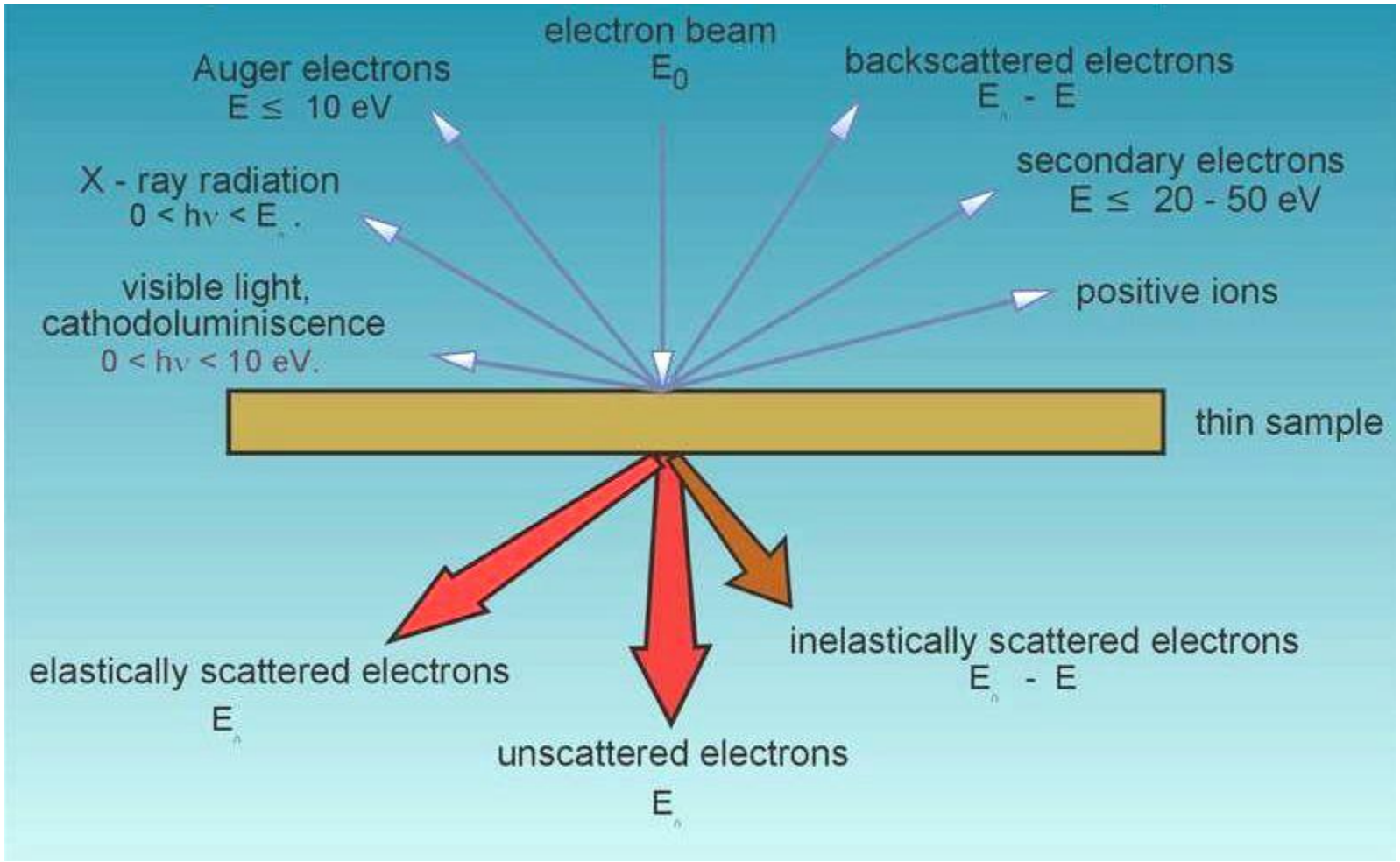




Electro-matter interaction



Scattering of electrons

Elastic scattering:

no changes on the state of the atom

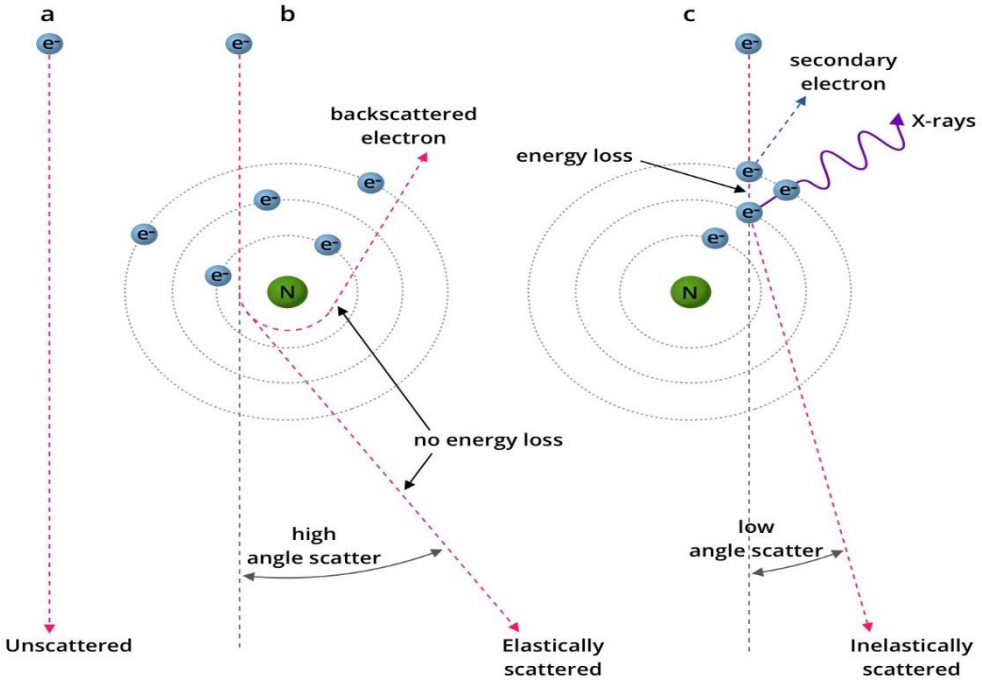
- electron is attracted only by the electric potential of the nucleus and does not interact with the electron cloud
- change in the momentum of the incident electron is permissible.
  - λ *does't change* ($\lambda\, d = \lambda\, i$)

Inelastic scattering

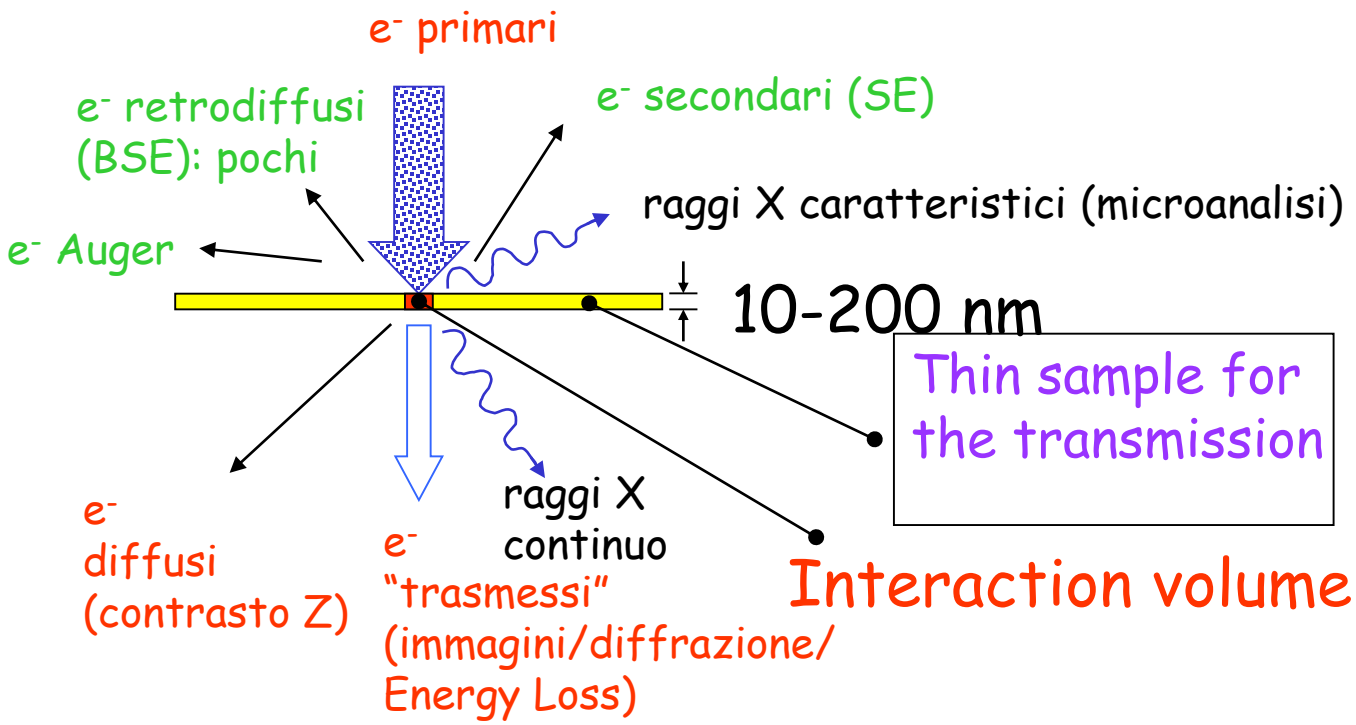
energy transfer between the incident electron and the target.

Inelastic scattering involves an energy transfer between the incident electron and the (target) atom electron cloud. This involves internal electron shells as well as valence band excitations down to the Fermi level in solids.

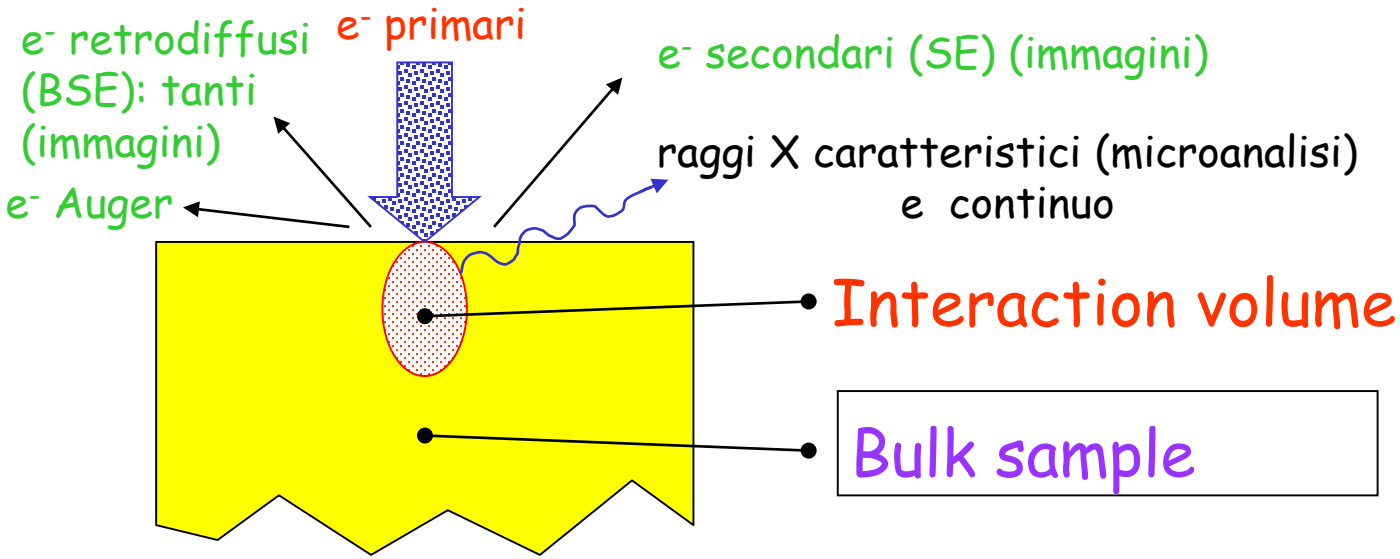
- λ *is not preserved* ($\lambda d \neq \lambda i$).



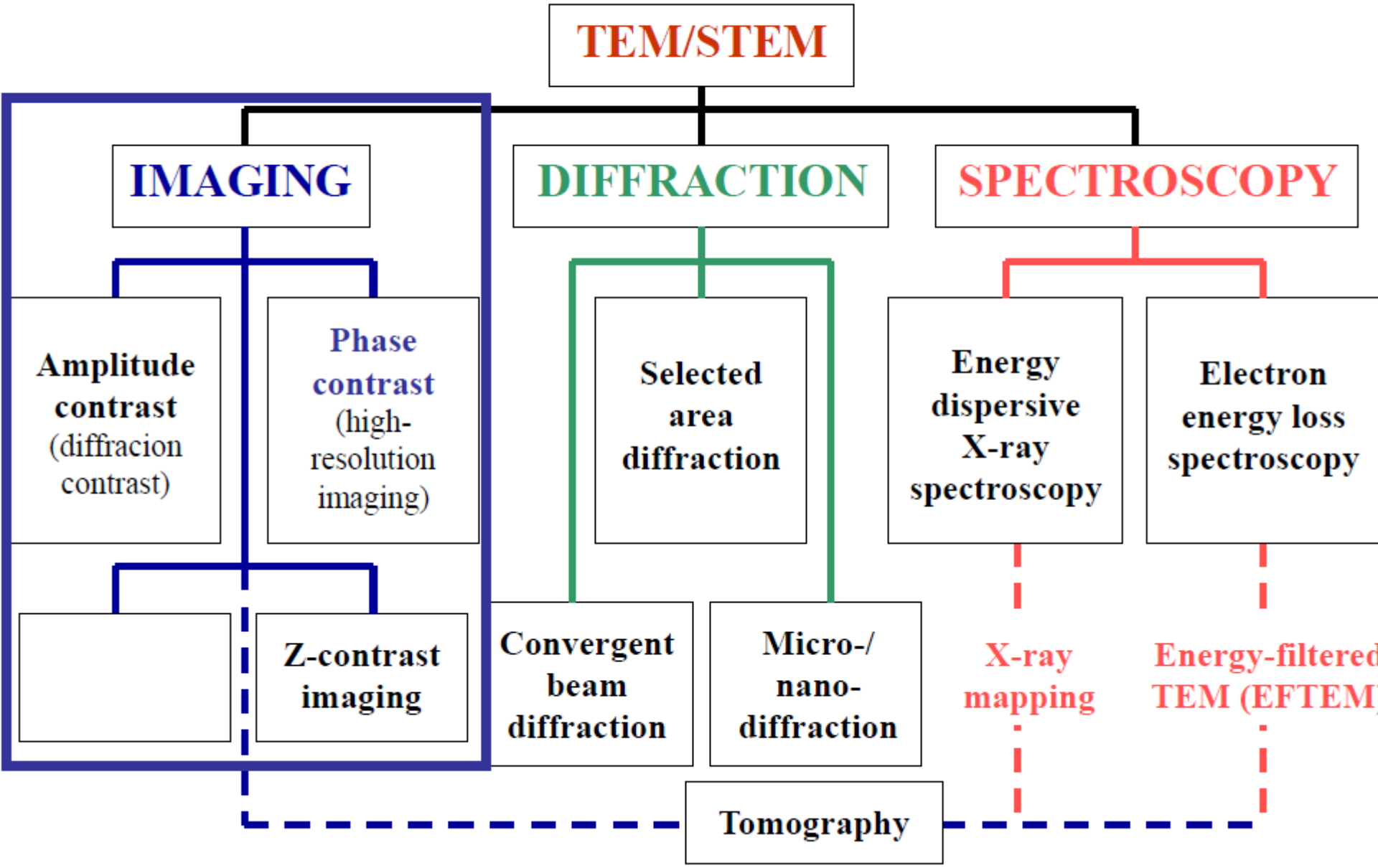
(TEM)


e⁻ primari
e⁻ retrodiffusi (BSE): pochi
e⁻ secondari (SE)
raggi X caratteristici (microanalisi)
e⁻ Auger
10-200 nm
Thin sample for the transmission
e⁻ diffusi (contrasto Z)
raggi X continuo
e⁻ "trasmessi" (immagini/diffrazione/ Energy Loss)
Interaction volume

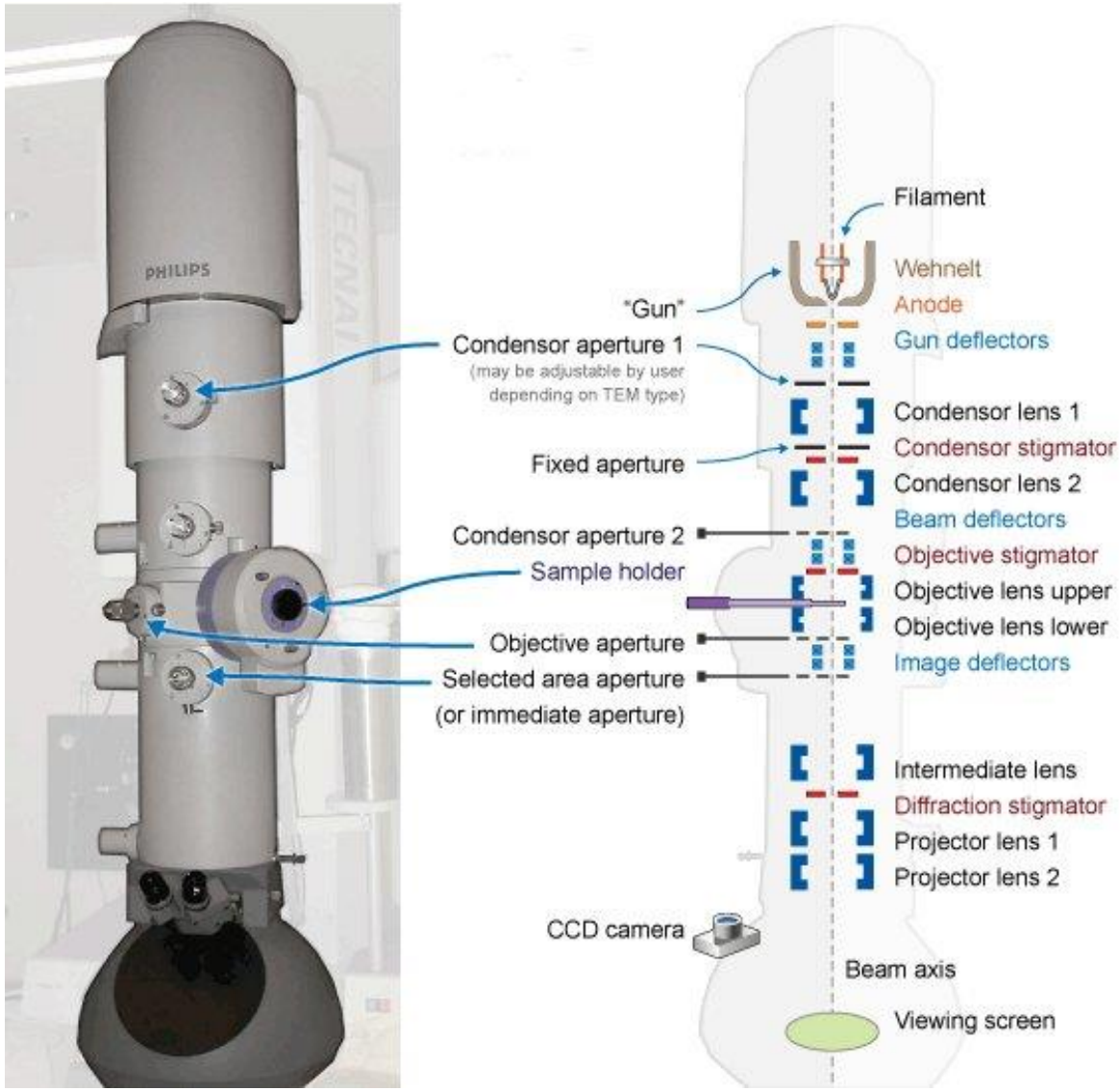
(SEM)


e⁻ retrodiffusi (BSE): tanti (immagini)
e⁻ primari
e⁻ secondari (SE) (immagini)
raggi X caratteristici (microanalisi) e continuo
e⁻ Auger
Interaction volume
Bulk sample

TEM Techniques



Inside a TEM


PHILIPS
Filament
Wehnelt
"Gun"
Anode
Condensor aperture 1
(may be adjustable by user
depending on TEM type)
Gun deflectors
Condensor lens 1
Condensor stigmator
Fixed aperture
Condensor lens 2
Beam deflectors
Condensor aperture 2
Objective stigmator
Sample holder
Objective lens upper
Objective lens lower
Objective aperture
Image deflectors
Selected area aperture
(or immediate aperture)
Intermediate lens
Diffraction stigmator
Projector lens 1
Projector lens 2
CCD camera
Beam axis
Viewing screen

Electron sources

- Mainly because of spherical aberration, we are forced to use diaphragms that limit the beam aperture (at the expense of resolution). Limiting the beam means losing current

Importance to have high internsity

- The key concept, however, is not intensity (current density) but brilliance (brightness), that is, current density per unit solid angle.

i beam corrent

d_0 diameter

α_0 divergence angle

$$\beta = \frac{i}{\pi\left(\frac{d_0}{2}\right)^2 \pi\alpha_0^2} = \frac{4i}{(4d_0\alpha_0)^2} \quad [\text{A cm}^{-2}\text{ster}^{-1}]$$

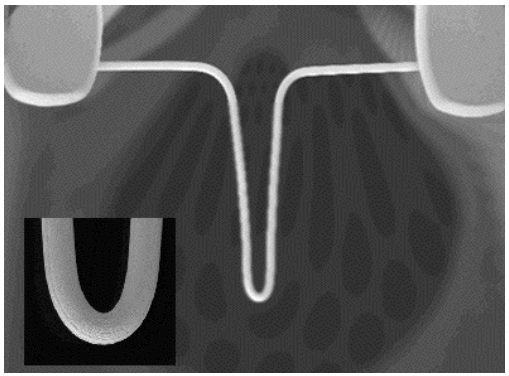
Area

Angolo solido

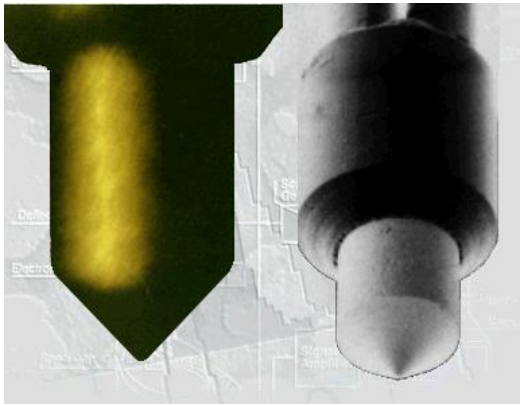
Thermoionics

$$J = AT^2 e^{-F/kT}$$

W filament



LaB_6 crystall <110>



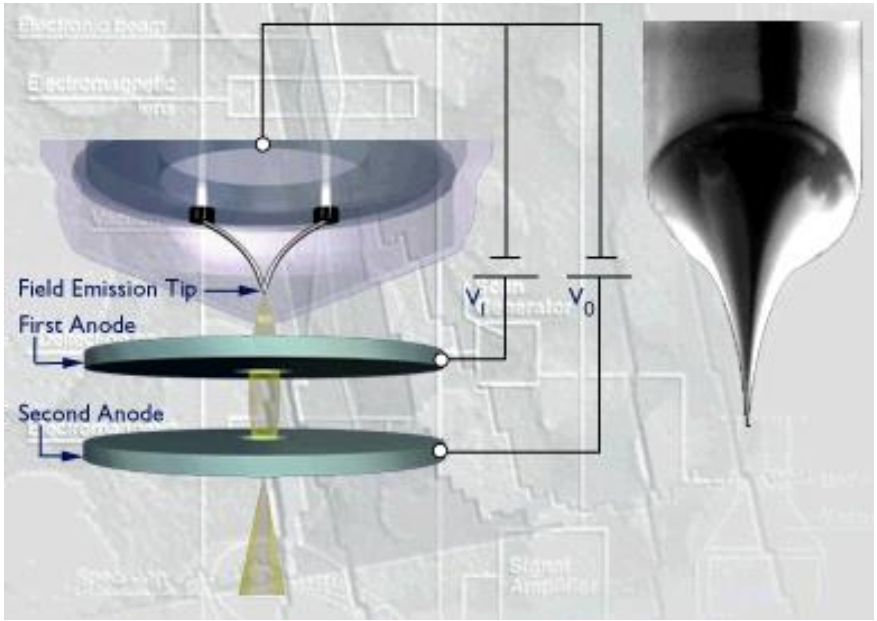
Field emission gun

Amplification of the electric field on the tips (E=V/r) → tunnel effect

Cold needs of ultra high vacuum (UHV) to avoid oxidation

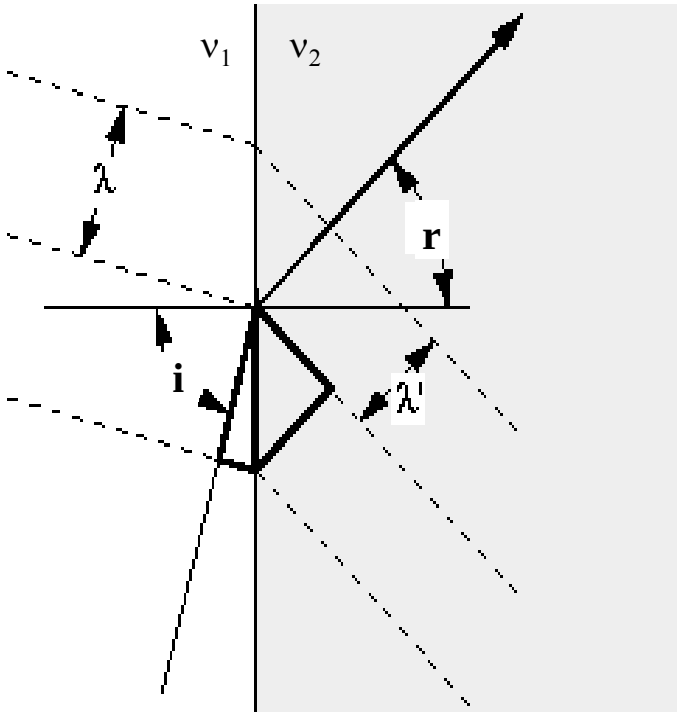
Hot (assisted emission)

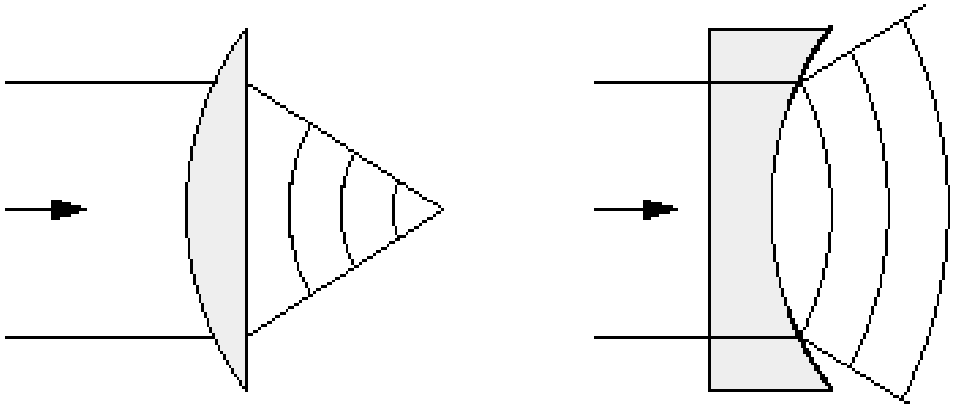
W (orientation <310>) coated with ZrO_2



	Thermoionic W	Thermoionic LaB_6	Thermal FEG ZrO-W	Cold FEG
β (200 kV) [A/cm^2 ster]	~ $5x10^5$	~ $5x10^6$	~ $5x10^8$	~ $5x10^8$
Source size [μm]	50	10	0.1-1	0.01-0.1
Energy spread [eV]	2.3	1.5	0.6-0.8	0.3-0.5
Operating Pressure [Pa]	10^{-3}	10^{-5}	10^{-7}	10^{-8}
Operating Temperature [K]	2800	1800	1800	300
Lifetime [hr]	100	500		>1000

Light lenses



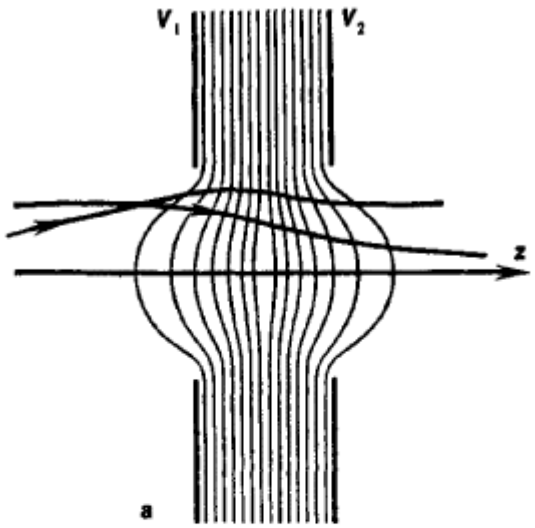


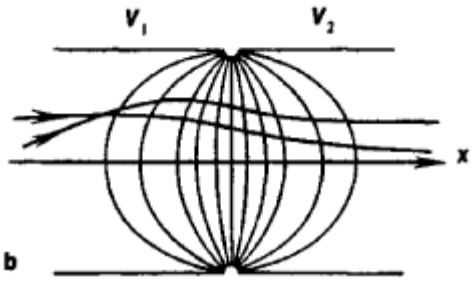
f depends on the curvature

Convergence and divergence of beams is due to the difference in refractive index between air and glass, that is, the different speed of light in these two media Snell law: seni/senr=n_2/n_1

Electrons lenses

Electrostatic lenses





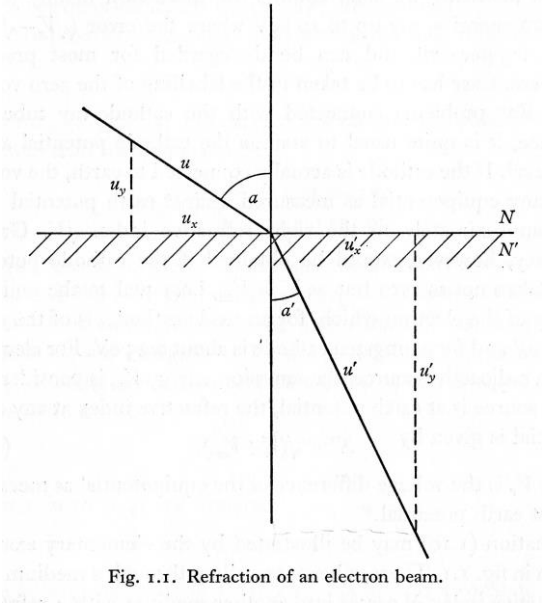
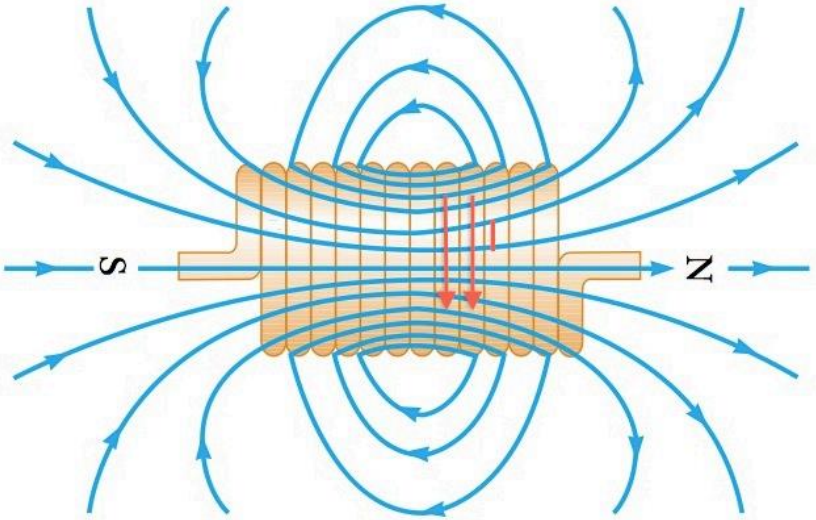


Fig. 1.1. Refraction of an electron beam.

(electro)Magnetic lenses



Electrons lenses

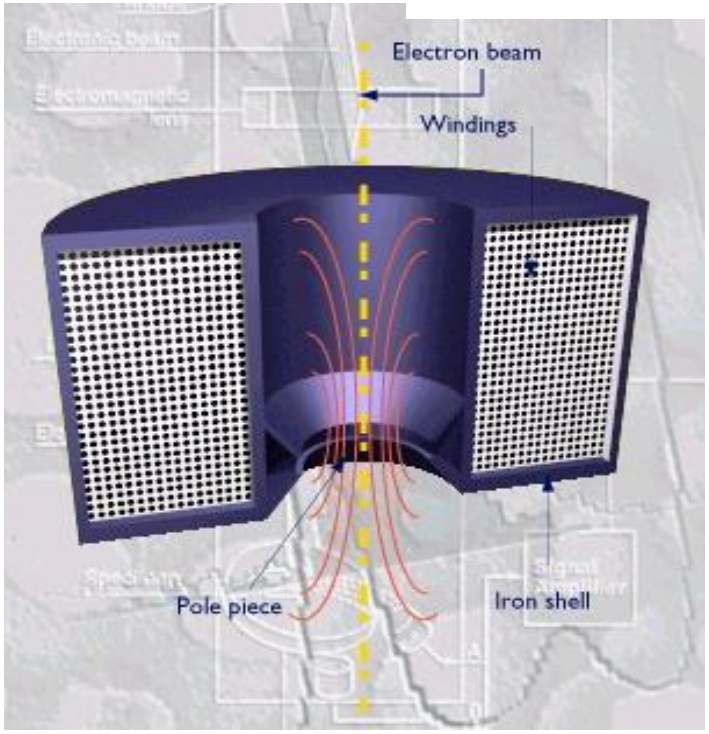
pros electrostatic lenses	pros magnetic lenses
no image rotation	less aberrations
low weight, low power consumption	no need for high voltage insulation
low sensitivity to high voltage instability	samples can be immersed into the field of a lens without perturbing the field itself
easy to focus heavy ions	

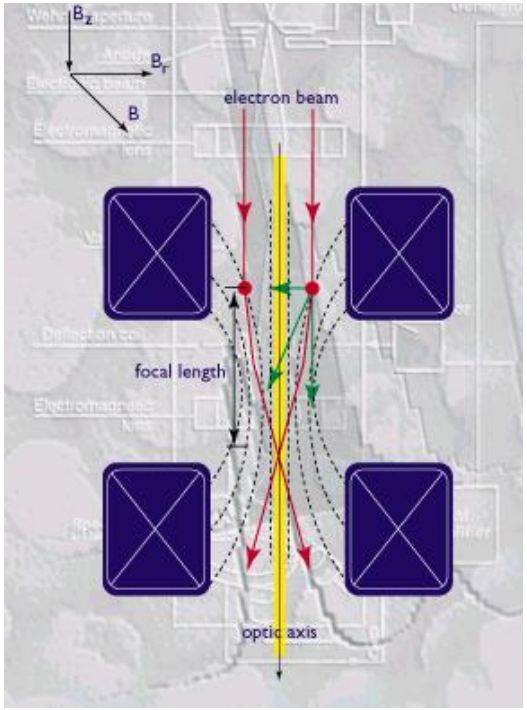
In the columns of modern electron microscopes magnetic lenses are mostly used; electrostatic lenses are used almost only in the electron source region where they have the also the function of extracting electrons from the source and accelerating them to the desired energy.

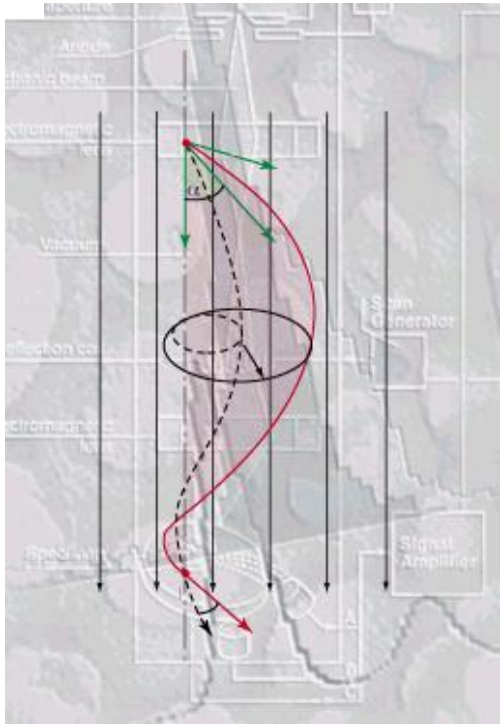
Electromagnetic lenses

Shielded axially symmetric solenoid: Fe-alloy (usually FeCo) shielding to prevent flux leakage from solenoid and concentrate magnetic field in a short region of the optical axis

Lorentz's force $\mathbf{F} = -e\,\mathbf{v} \times \mathbf{B}$



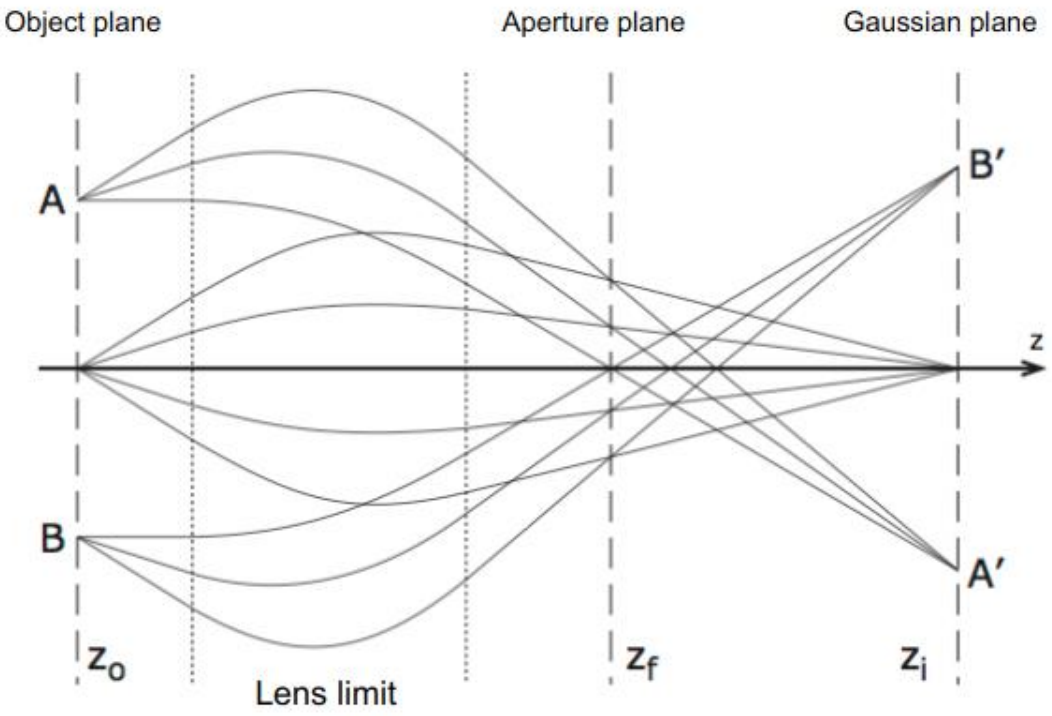




$$\frac{1}{f} = \frac{\alpha B_z^2}{E_{kin}}$$

The trajectory is a spiral with decreasing distance from the axis: the focal point f is at finite distance.

Aberrations in EM

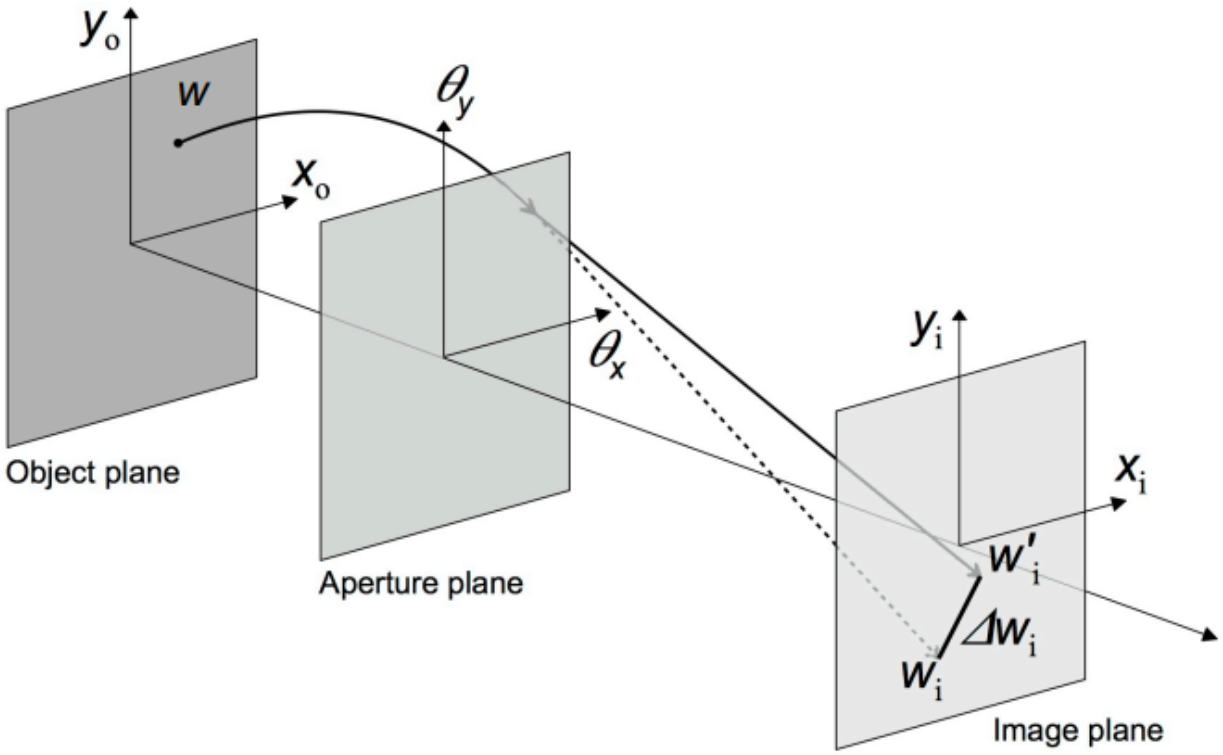


An ***ideal stigmatic optical system*** is defined as:

- All rays emerging from the same point in the object plane converge to the same point in the Gaussian image plane.
- All rays emerging with same direction from the object plane converge to individual points in the back focal plane (aperture plane).

Rotationally symmetric magnetic lenses have *unavoidable aberrations*, namely **spherical** and **chromatic aberration**, which make the optical system **non-stigmatic**

Aberrations in EM



Real optical systems are not stigmatic… they are **aberrated**!

Real optical systems deviate from the stigmatic imaging condition, they are ***aberrated***. A point $\boldsymbol{w_o}$ would be imaged on the image plane as the point $\boldsymbol{w_i}$ but it is imaged in a different position $\boldsymbol{w'_i}$.
The difference $\boldsymbol{\Delta w_i}$ between the ideal position $\boldsymbol{w_i}$ and the real one $\boldsymbol{w'_i}$ is what we call the ***image aberration.***

Aberrations in EM

Sources for aberrations can be:

- Mechanical **imperfections** and electrical **instabilities**:

  Parasitic aberrations

- Deviations of real electron trajectories from the **paraxial approximation**:

  Geometric aberrations

- Electron of the beam can have **different energies** – *or they can loose energy when interacting with the sample*:

  Chromatic aberrations

Besides these optical effects there are additional *disturbances* limiting the ideality – *and the resolution* – of an electron microscope: ***the ideal imaging system does not exist!***

Chromatic Aberration

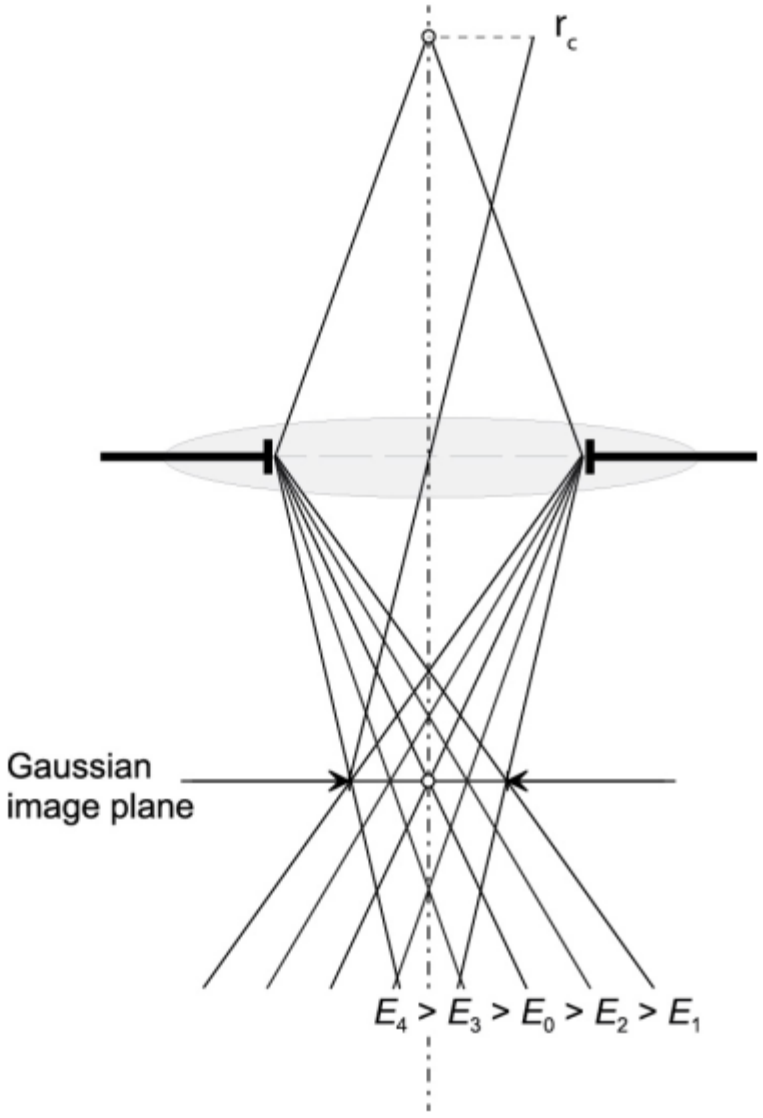
The **chromatic aberration** is due to the fact that *the focal length of a cylindrical magnetic lens changes with the energy of the electron energy*. The result is an *incoherent sum* of different images, each with a slightly different defocus.

If we consider that fluctuations of the accelerating potential are small (few microvolt), the main sources for energy differences are:

1. The energy spread of the ***source electrons***: 0.3 eV for a FEG Gun and 1 eV for thermionic ones.

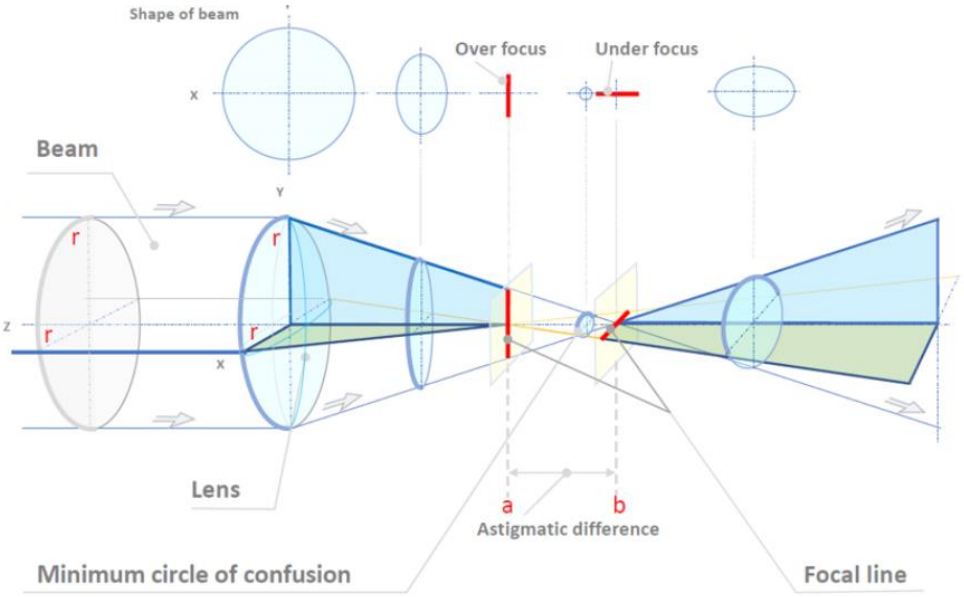
1. The energy spread induced by the ***energy loss*** due to specimen interaction.

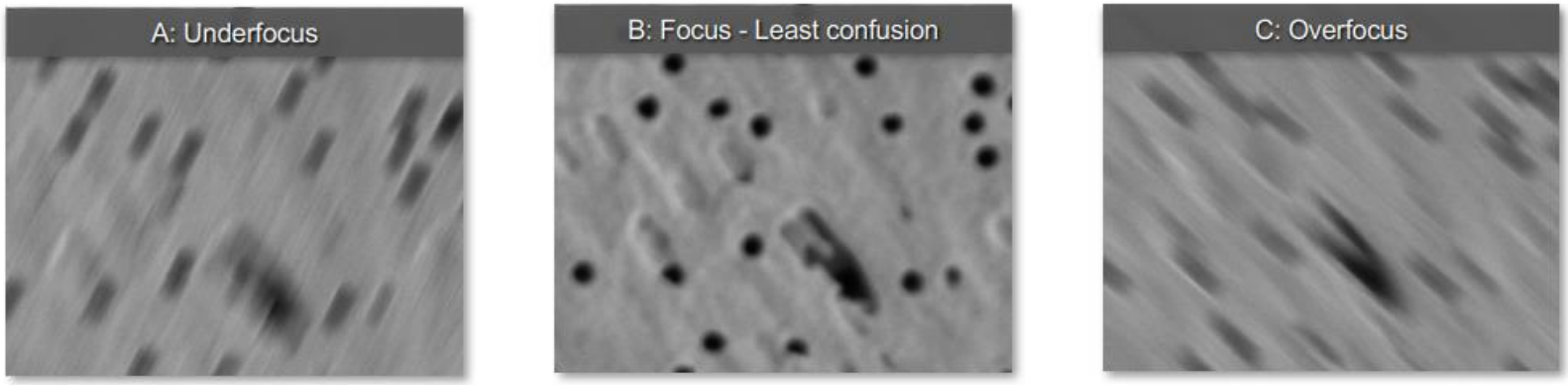
$$r_c = C_c \frac{\Delta E}{E_0}\theta\ (mm) \qquad C_c \approx [0.5-1]f$$



Astigmatism

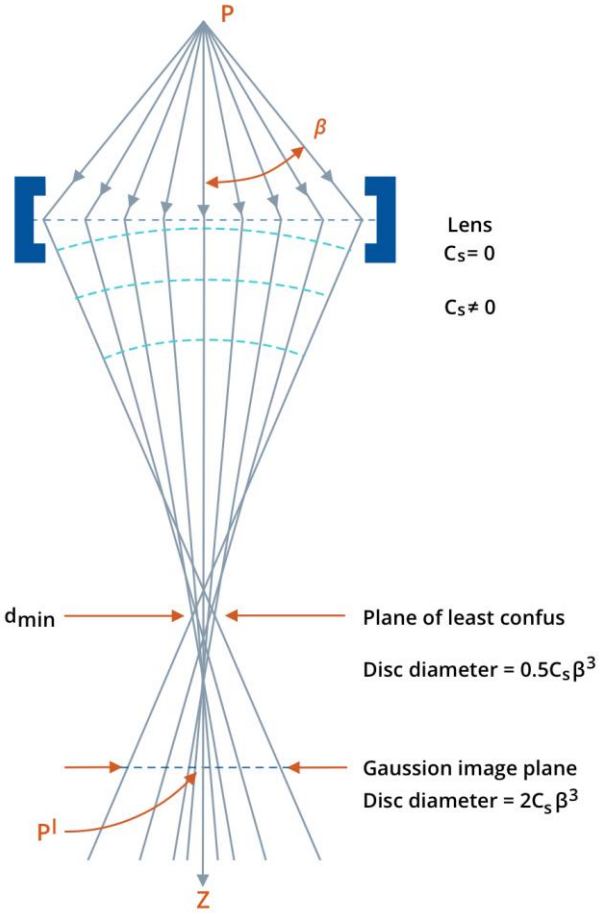
The astigmatism is an aberration which arises from the difference in the focal positions between the electron beams passing on two axes orthogonal to the optical axis when the beams pass through an electron lens.





Spherical aberration

Electrons travelling far from the optical axis feel a stronger magnetic field. Therefore lens focusing depends on the distance of the trajectory from the axis, and therefore from the angle ω. Rays far from the optical axis are focused before the Gaussian plane.



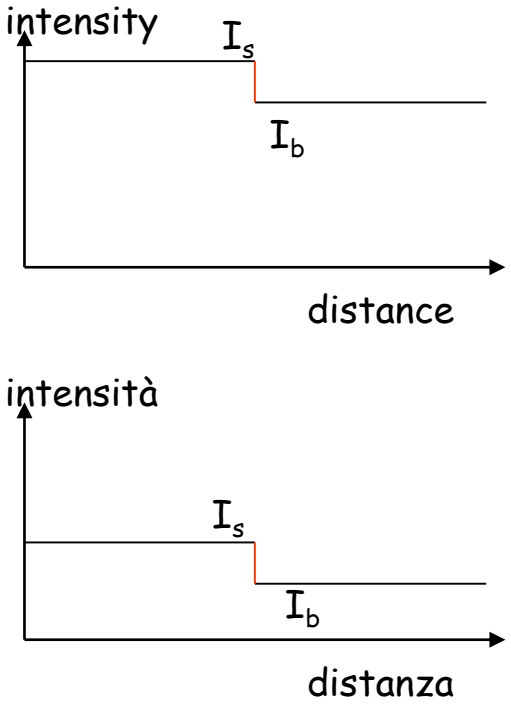
effect of the spherical aberration is to image a point into a disk.

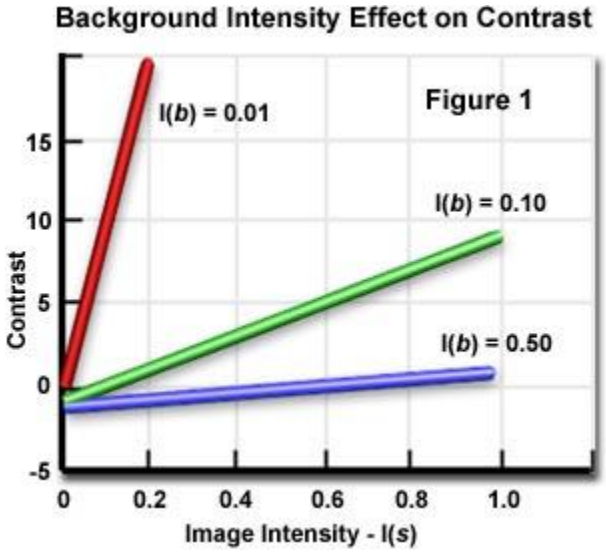
Contrast

To see something in an image we must have contrast (C) between adjacent areas of the sample:

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

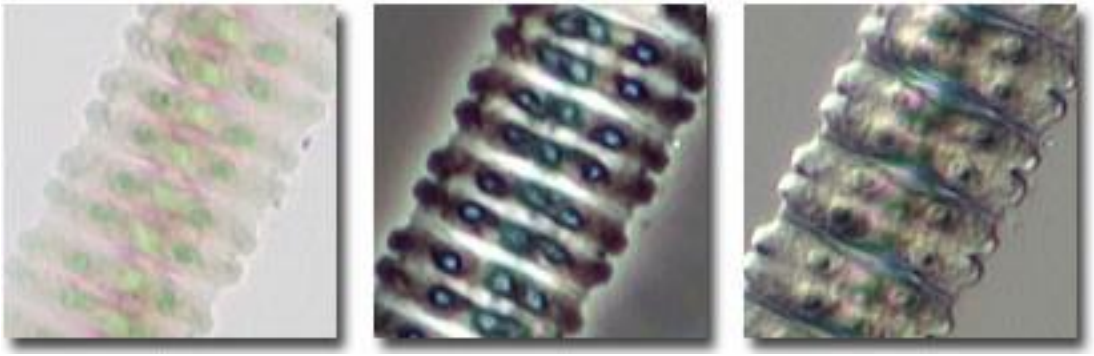
The human eye cannot appreciate intensity differences of less than 5-10% (usefulness of acquiring digital images for processing)

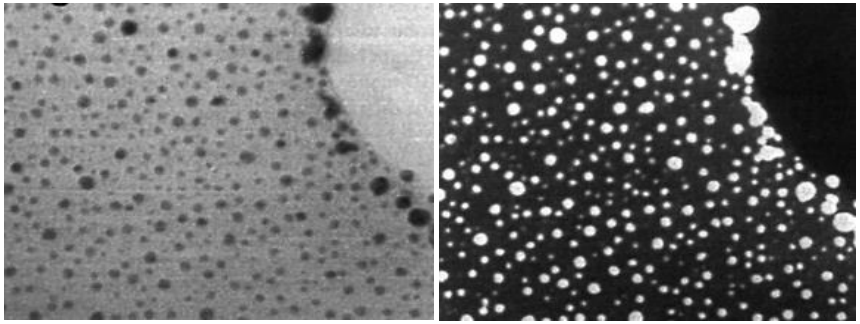




The contrast is not a property of the sample. It depends on:

1) Interaction probe/sample





The eye is sensitive only to intensity or wavelength contrast (color) → Need to transform every other mechanism (phase, polarization,…) into intensity or color

2) Efficiency of the optical system

3) Efficiency of the detector

Amplitude Contrast

Two principal types

Mass-thickness contrast

-Primary contrast source in amorphous materials

-Assume incoherent electron scattering (atoms scatter independently)

Diffraction contrast

-In crystaline materials

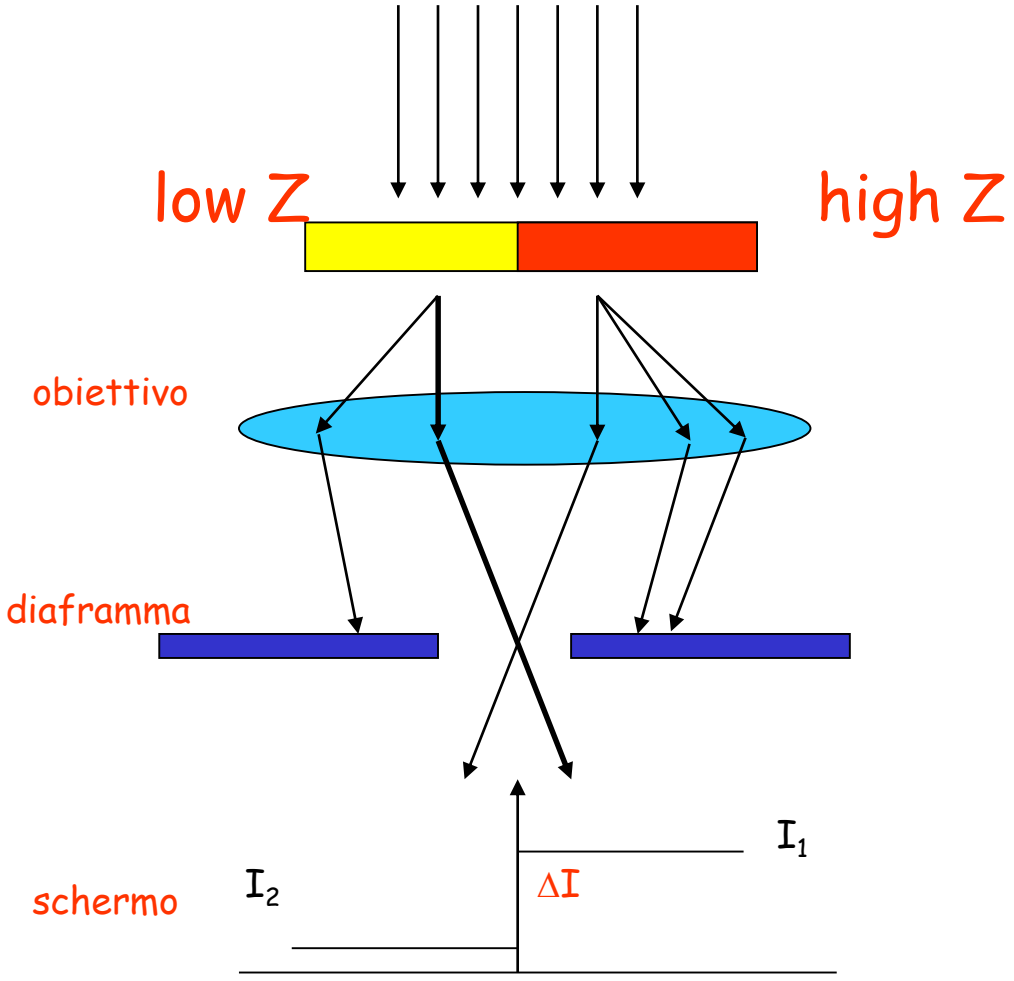
-Coherent electron scattering (atoms does not scatter independently)

Both types of contrasts are seen in BF and DF images

TEM: Contrasto di ampiezza (massa-spessore)

E' legato allo scattering incoerente elastico alla Rutherford (proporzionale a Z, alla densità ρ e allo spessore t, piccato in avanti – $\theta < 5°$) ed è presente anche in campioni amorfi.
Spessore maggiore significa scattering multiplo quindi zone a Z maggiore diffondono di più di quelle a Z minore.

Però in campioni cristallini è "concorrenziale" con la diffrazione



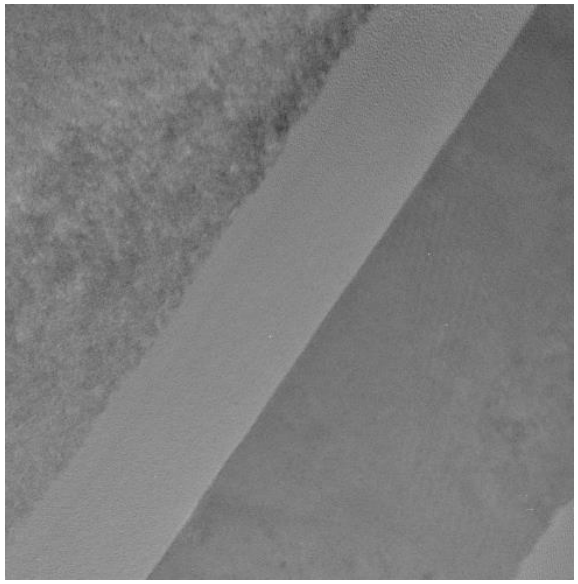
In realtà raccogliendo anche i pochi elettroni diffusi incoerentemente ad angoli > 5° si ha il cosiddetto contrasto Z (HAADF) in cui non è presente il contributo della diffrazione neanche in campioni cristallini.

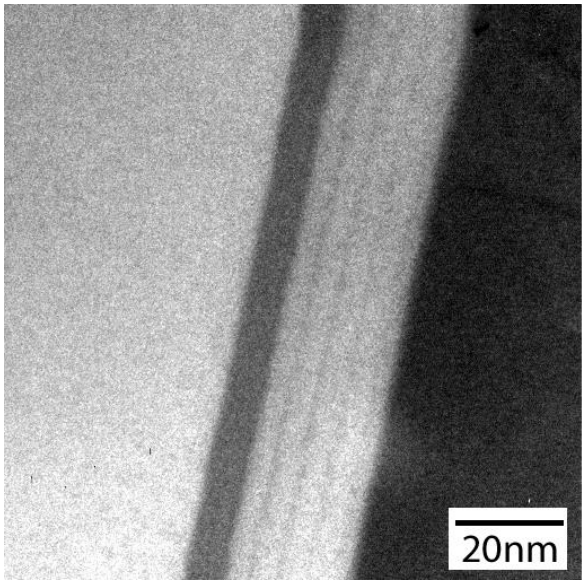
Amplitude Contrast

- Difference in intensity of two adjacent areas:

$$C = \frac{(I_2 - I_1)}{I_1} = \frac{\Delta I}{I_1}$$

The eyes can not see intensity differences < 5-10%.
However, contrast in images can be enhanced digitally.



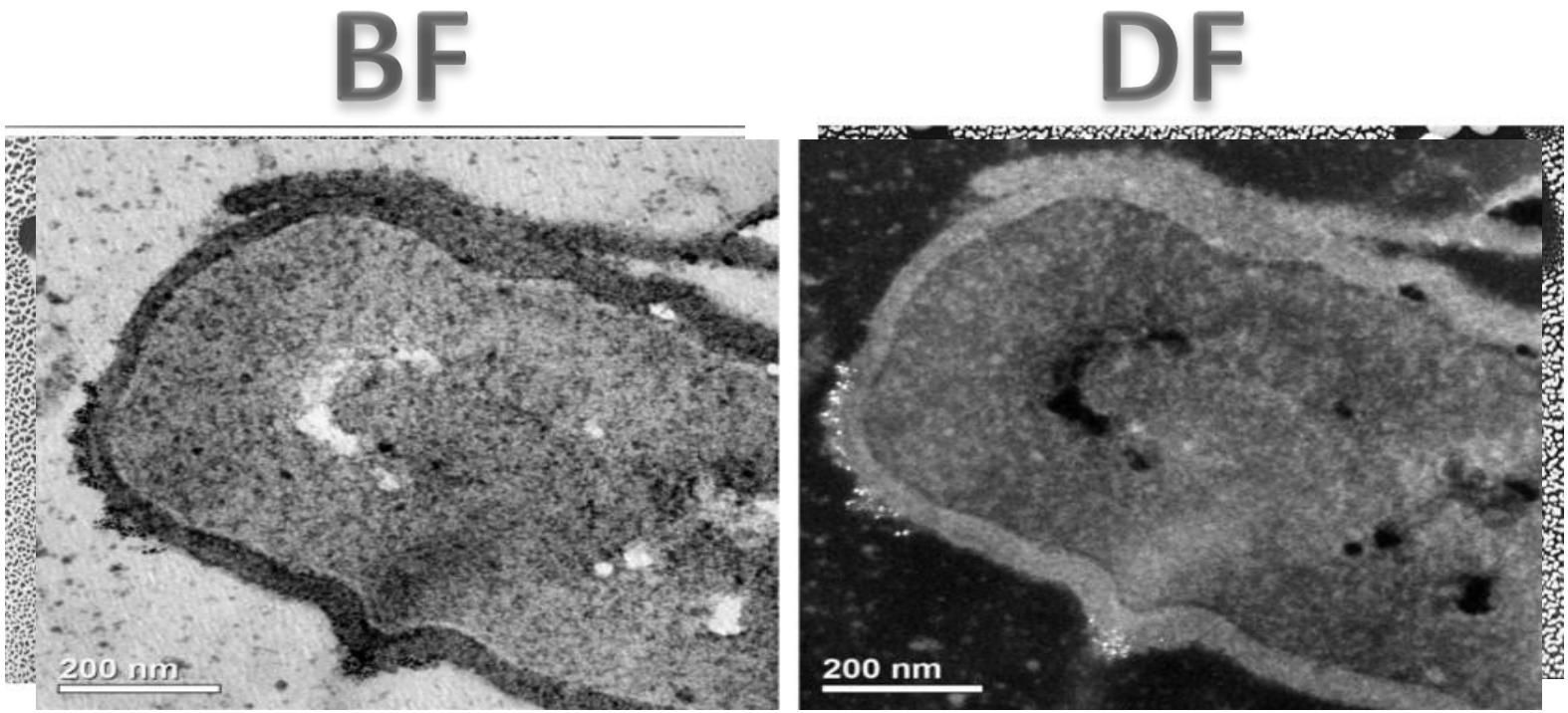


NB! It is correct to talk about strong and weak contrast, but not bright and dark contrast (dark and bright refers to density (number/unit area) of electrons hitting the screen/detector)

Example of mass-thickness contrast in TEM mode- Metal shadowing

TEM image of latex particles on an amorphous C-film.

Effect of evaporation of a heavy metal (Au or Au-Pd) thin coating at an oblique angle.



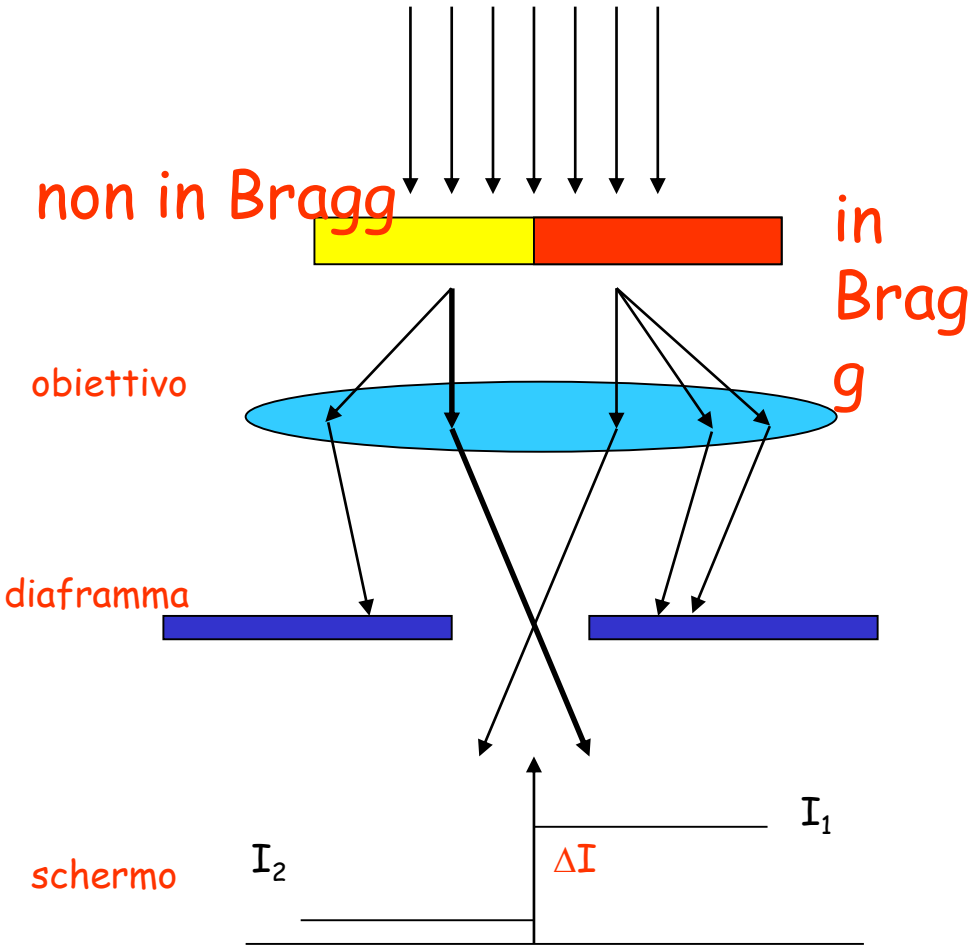
Bacillus subtilis

TEM: Contrasto di diffrazione

E' legato allo scattering coerente elastico alla Bragg ed è presente in campioni cristallini.

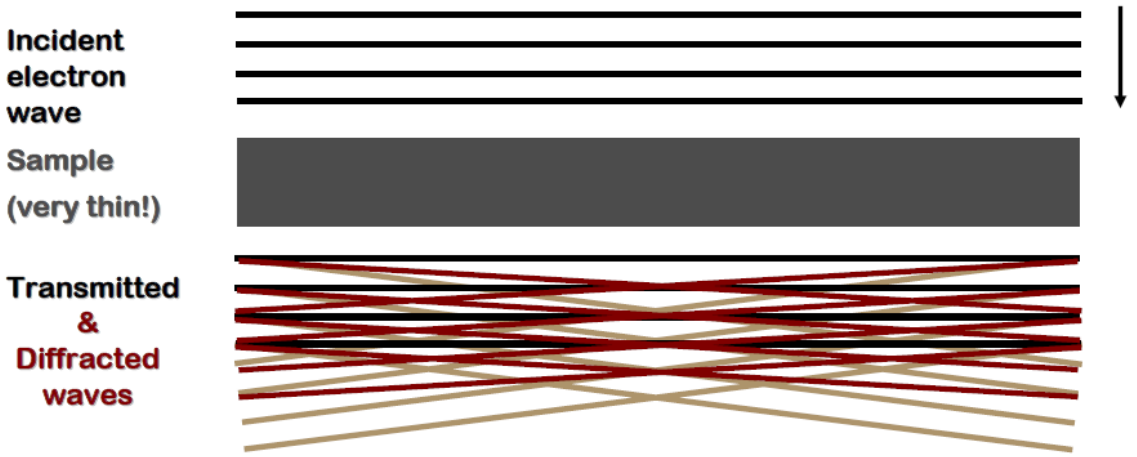
Se un dominio o una zona del campione di trova in condizione di Bragg e un altro no ho contrasto legato agli elettroni rimossi dal fascio trasmesso (isolato con un diaframma)

Per formare l'immagine posso selezionare o il fascio trasmesso (immagine in campo chiaro o bright-field - BF) o un diffratto (immagine in campo scuro o dark-field – DF)



Phase Contrast Imaging

Exploits differences in the refractive index of different materials to differentiate between structures under analysis

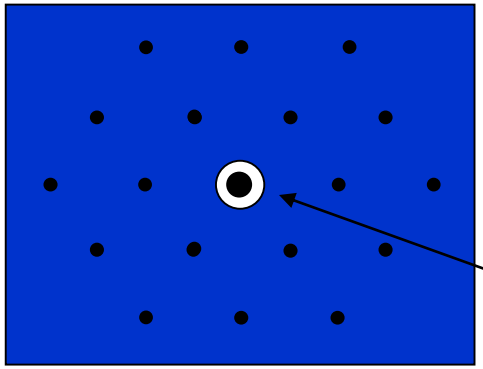


Transmitted & diffracted waves each have a different phase

Result is an interference pattern - our 'phase contrast' or HREM image

Formazione dell'immagine in campo chiaro (BF) e campo scuro (DF)

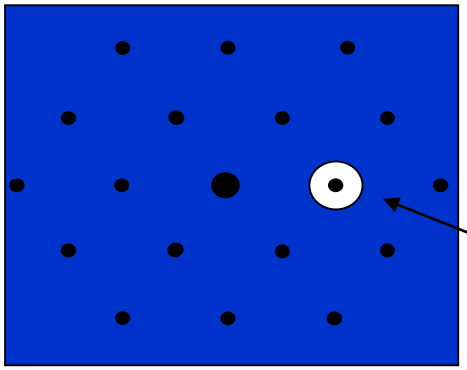
Bright-Field (BF)



Contrasto di massa–spessore e di diffrazione: le parti con Z più alto o quelle cristalline in Bragg sono più scure.

diaframma

Dark-Field (DF)



Contrasto dovuto alla diffrazione: la parte che diffrange secondo lo spot isolato è chiara, il resto è scuro

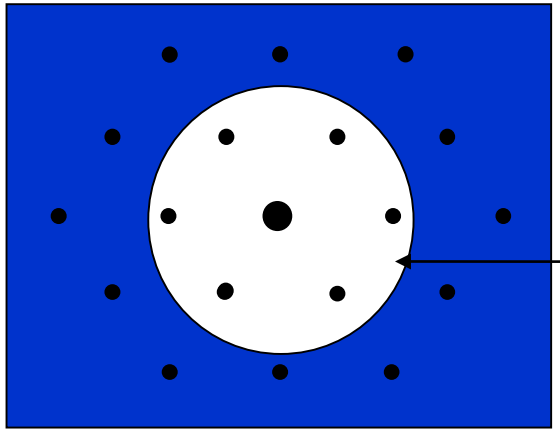
diaframma

TEM: Contrasto di fase

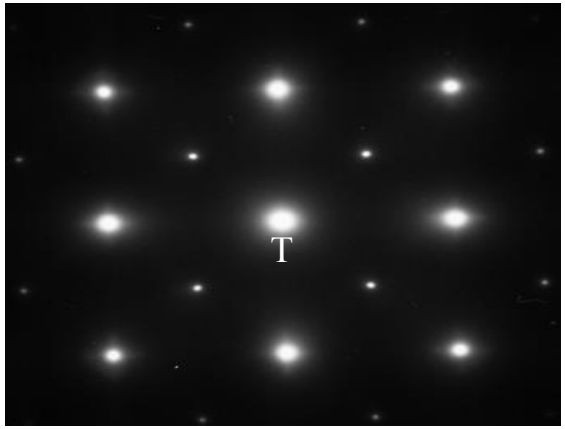
E' legato alla interferenza tra fasci diffratti i quali portano una differenza di fase fra loro da cui si ottengono informazioni sulle distanze interplanari (TEM in alta risoluzione)

Formazione dell'immagine in Alta Risoluzione (HRTEM)

Faccio interferire molti fasci diffratti



diaframma



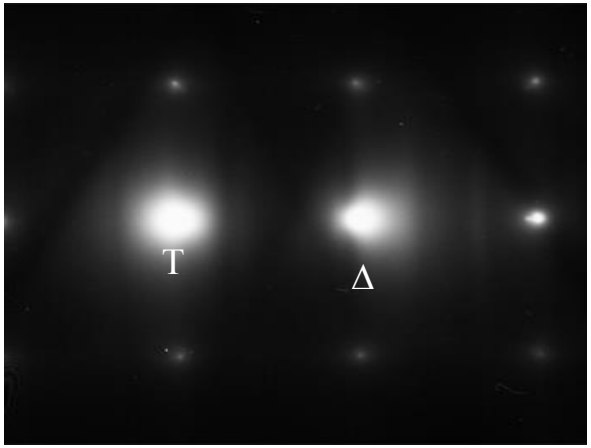
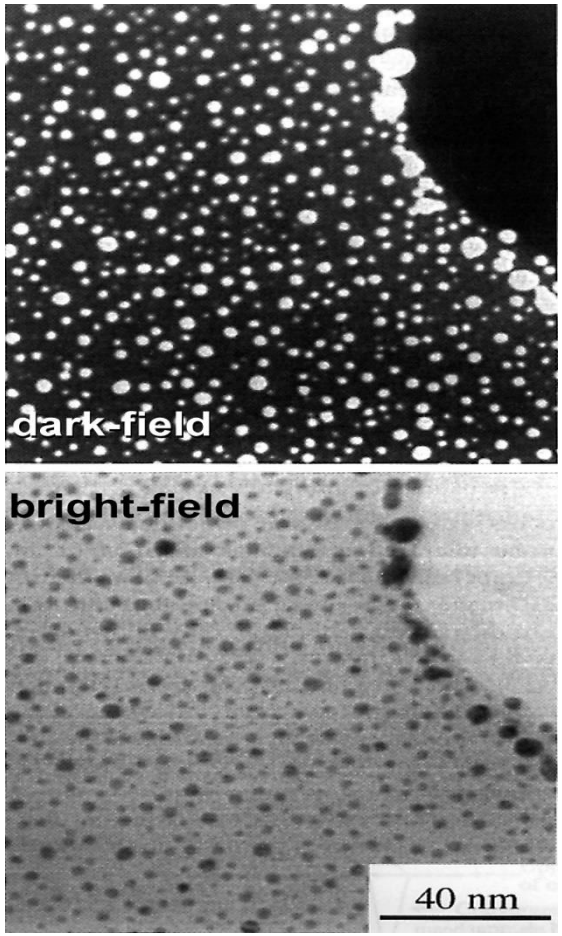


Figure di diffrazione di un monocristallo di GaAs (f.c.c. a=0.565 nm) in condizioni di asse di zona [001] e di due fasci.

Particelle di Au su C



Campo scuro
(DF)

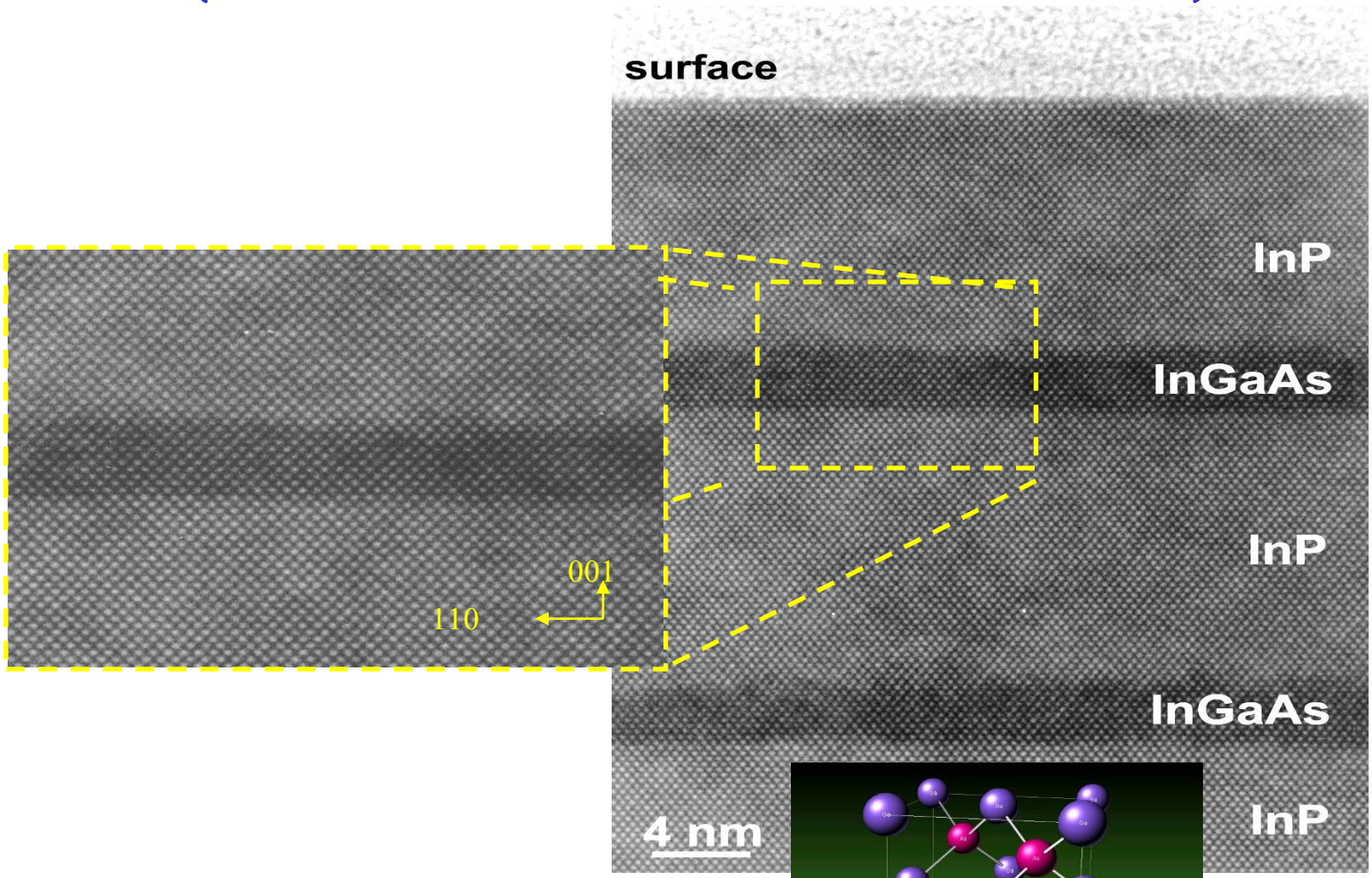
$$I_{tot} = I_{\vec{g}}$$

Campo chiaro
(BF)

$$I_{tot} = I_0$$

Fin qui abbiamo visto lo scattering degli elettroni incidenti …

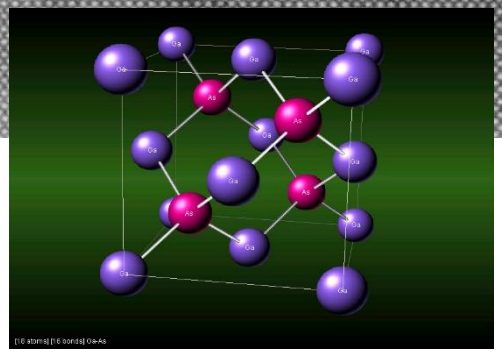
Alta Risoluzione (HREM)
(sezione trasversale di multistrato InP/InGaAs)



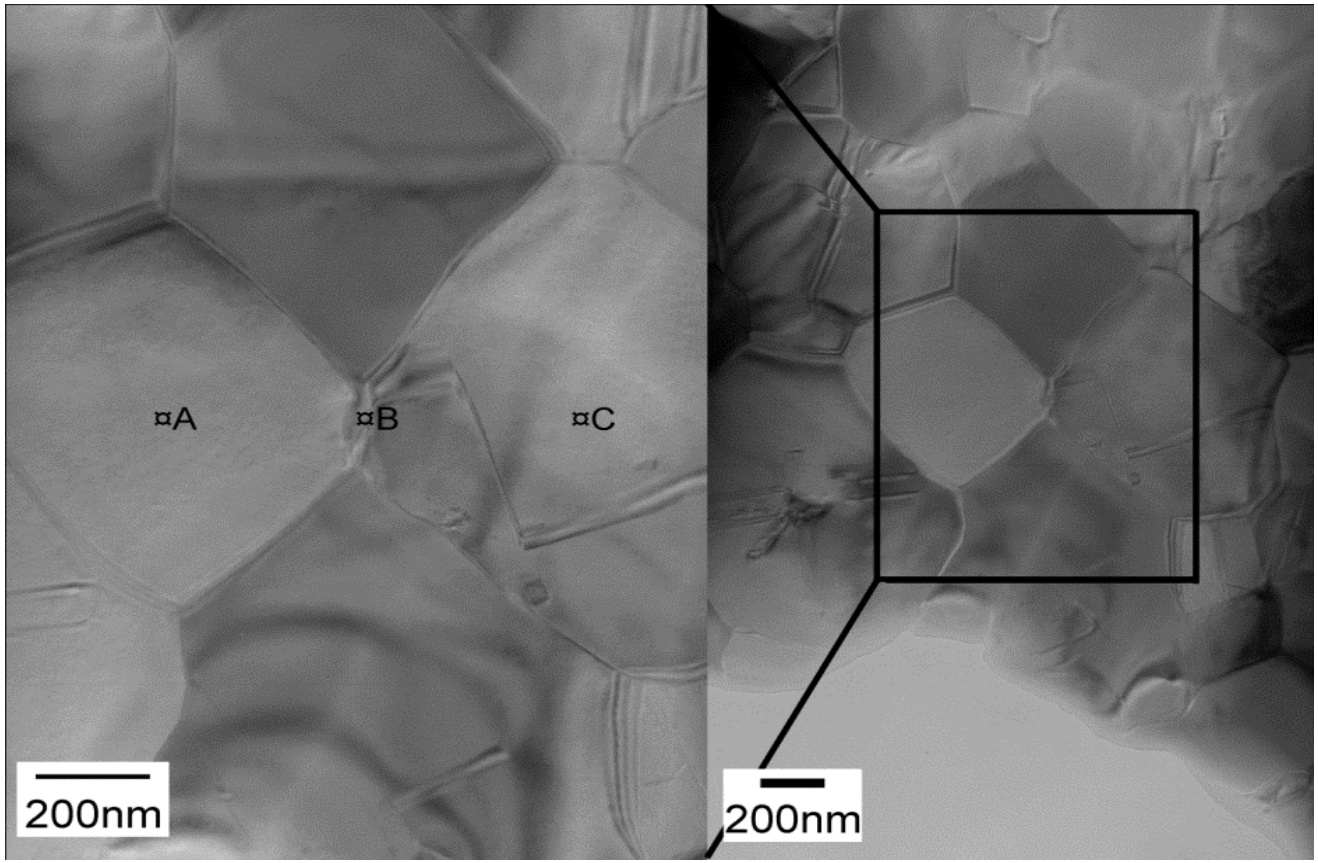
Ζινχβλενδε (χυβιχ)

ΓαΑσ: $\alpha = 0.565$ νμ

ΙνΠ: $\alpha = 0.587$ νμ



TEM diffraction contrast

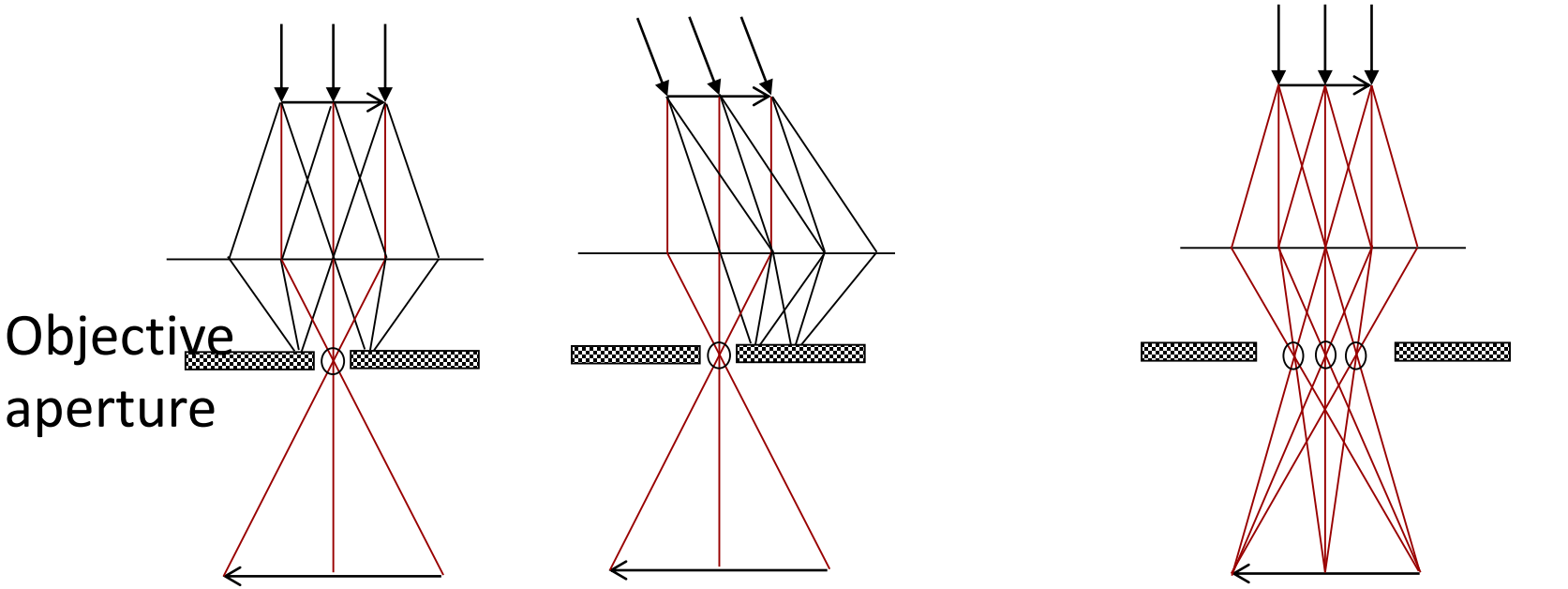


Bragg diffraction contrast:

- Coherent elastic scattering (atoms do not scatter independently)
- Crystal structure
- Orientation of the sample

Size of objective aperture

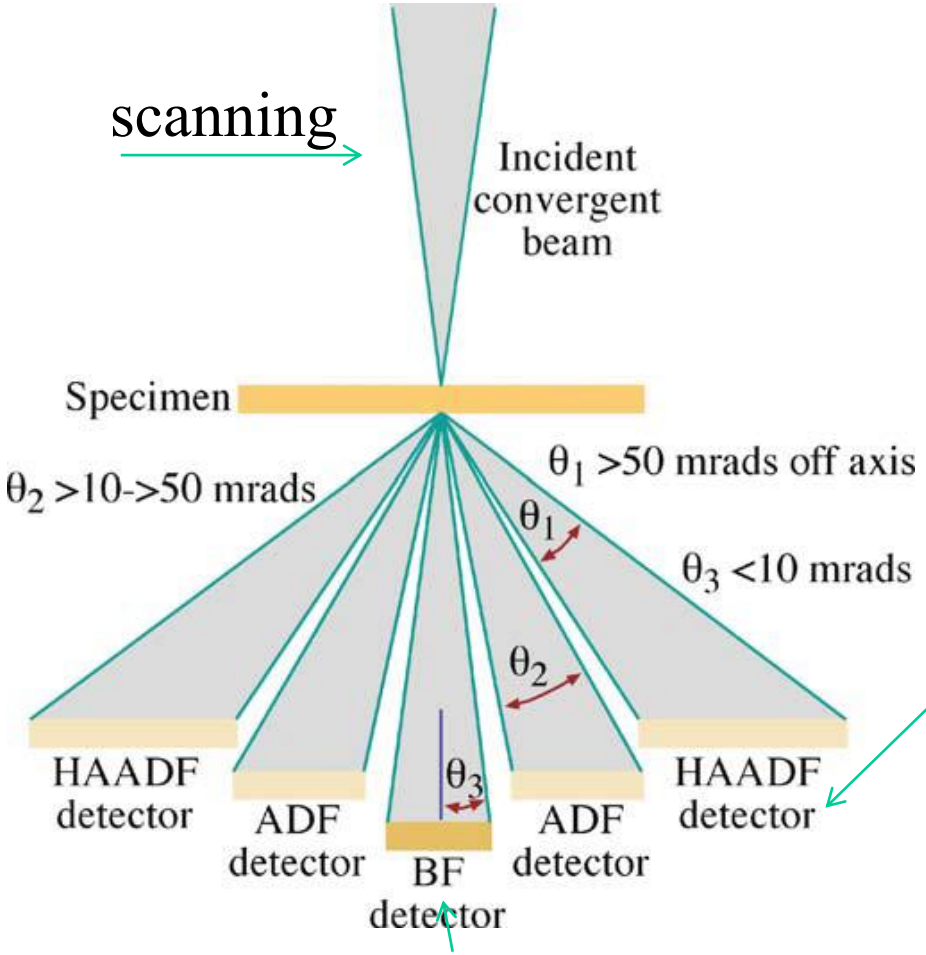
Bright Field (BF), Dark Field (DF) and High Resolution EM (HREM)C



Amplitude/Diffraction contrast

Phase contrast

BF–DF in STEM



Collects only electrons scattered more than 50 mrad (~3º).
Intensity: Z-dependent.

Collects Bragg electrons

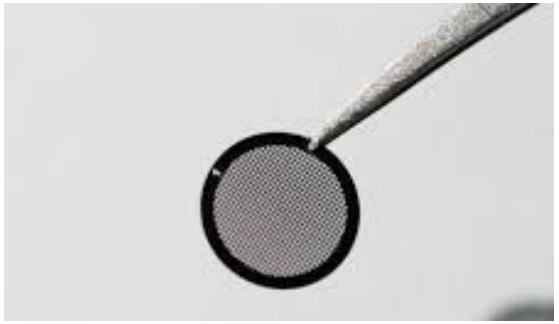
Cryo EM

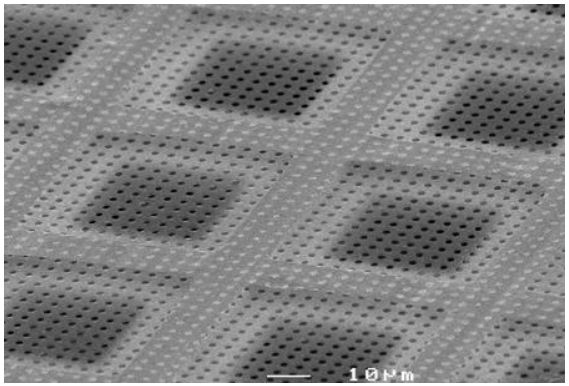
Il razionale originale della microscopia crioelettronica era quello di combattere i danni da radiazioni per i campioni biologici. La quantità di radiazioni necessaria per raccogliere l'immagine di un campione al microscopio elettronico è paragonabile al posizionamento del campione a circa 20 m di distanza da una sorgente termonucleare.

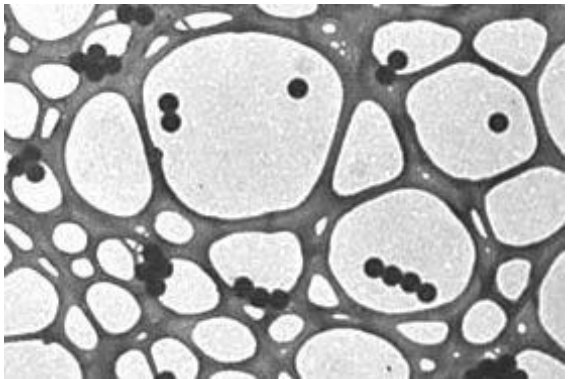
L'inclusione dei campioni nel ghiaccio al di sotto della temperatura di sublimazione è stata una possibilità contemplata all'inizio, ma l'acqua tende a disporsi in un reticolo cristallino di densità inferiore al momento del congelamento e questo tende a distruggere la struttura di qualsiasi cosa vi sia incorporata.

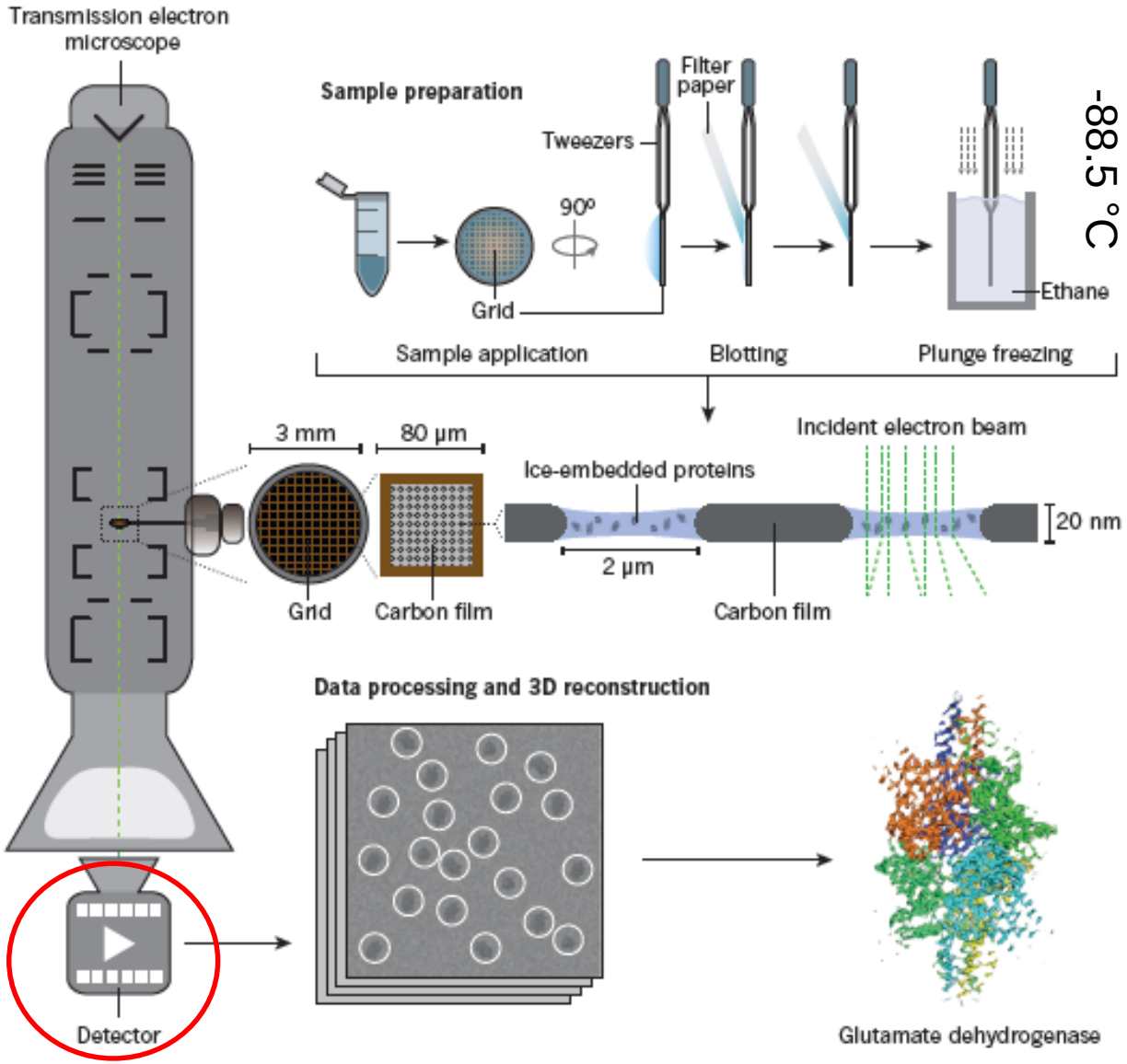
La soluzione è stata trovata nel ghiaccio amorfo. Il ghiaccio amorfo è costituito da molecole d'acqua disposte in modo casuale come le molecole di un comune vetro, mentre il ghiaccio quotidiano è un materiale cristallino in cui le molecole sono disposte regolarmente in un reticolo esagonale.

Cryo EM – sample vitrification



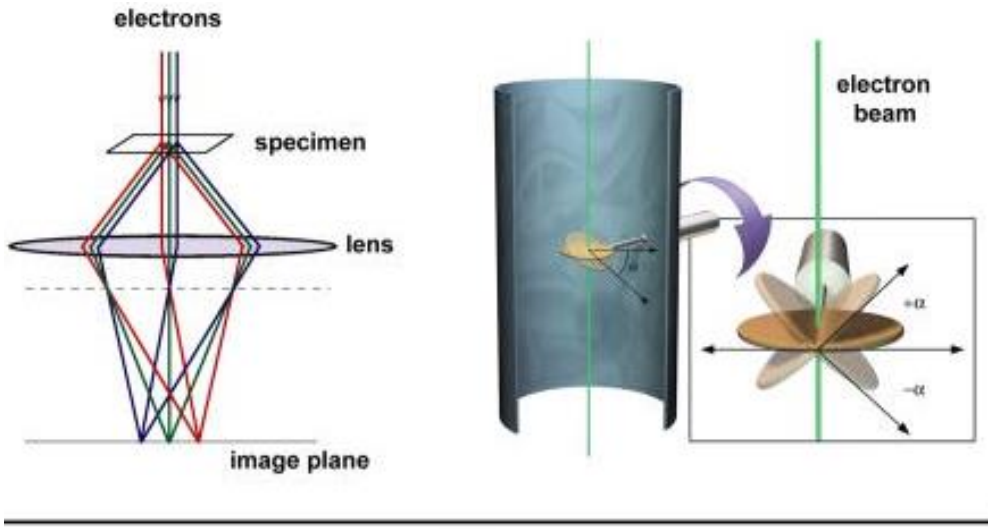


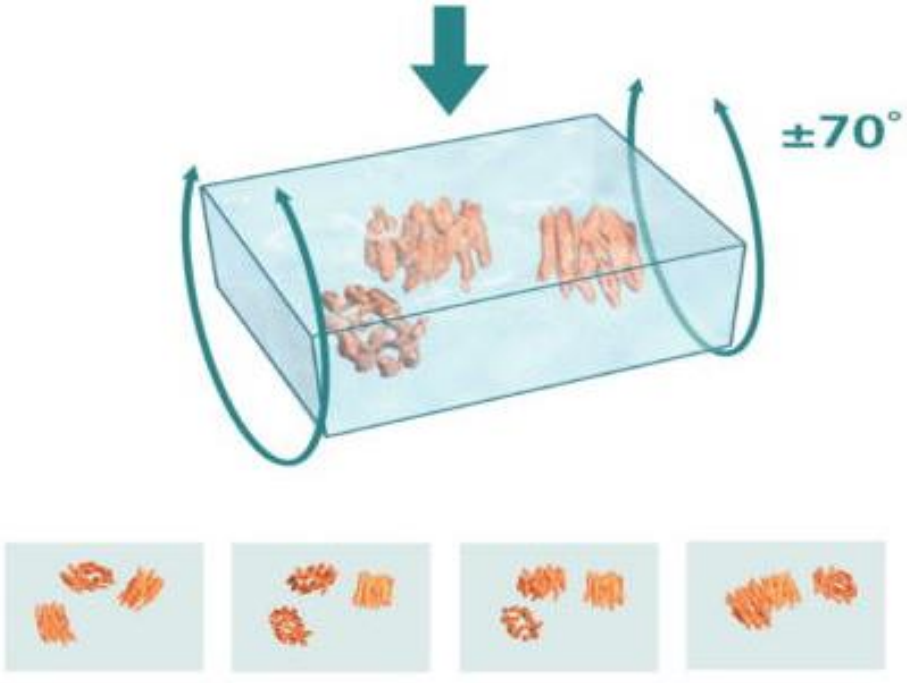




A 1.5M€ direct electron detector is a key feature of CryoEM

Cryo EM



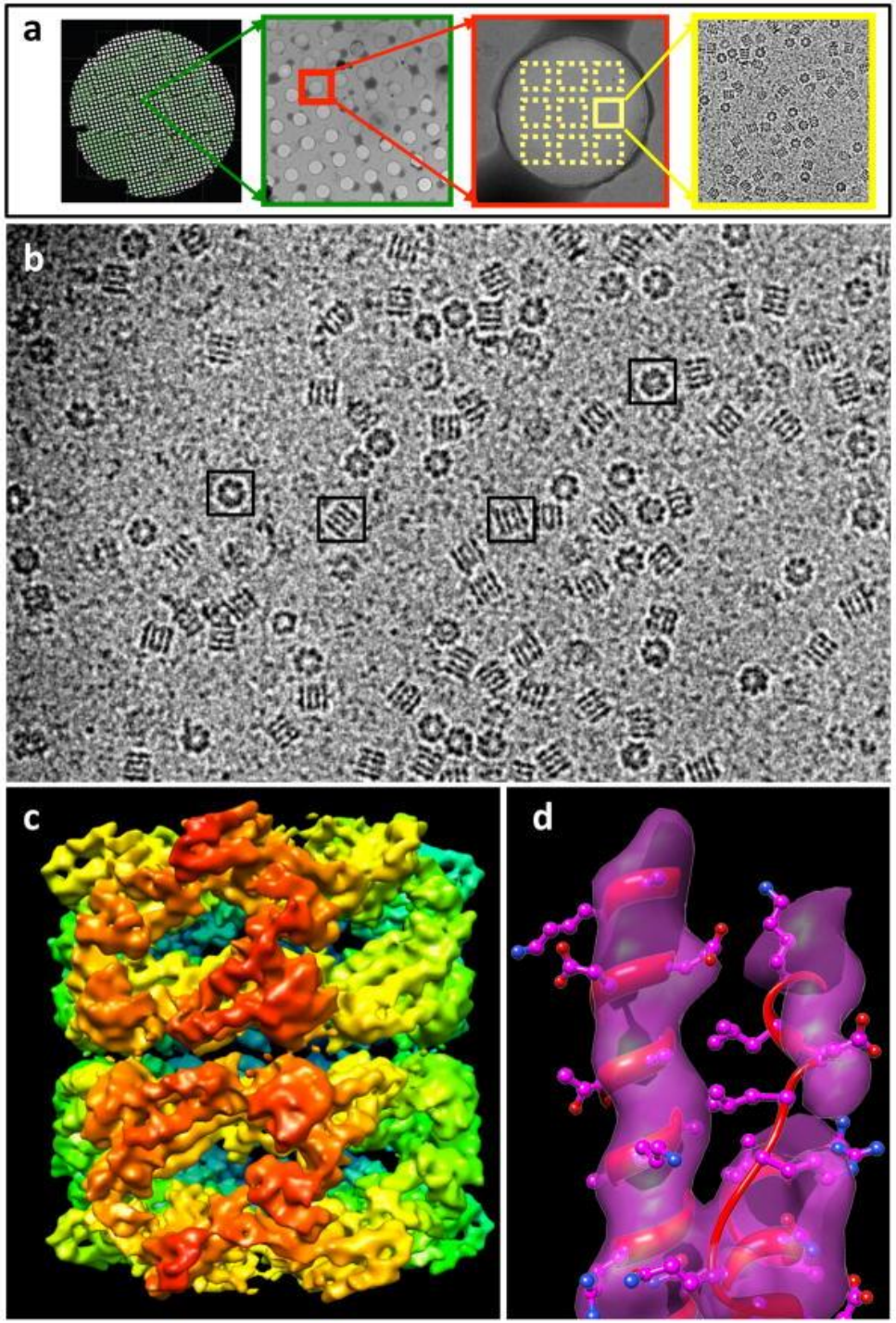


Data collection for electron tomography. As the specimen is tilted relative to the electron beam, a series of images is taken of the same field of view.

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,

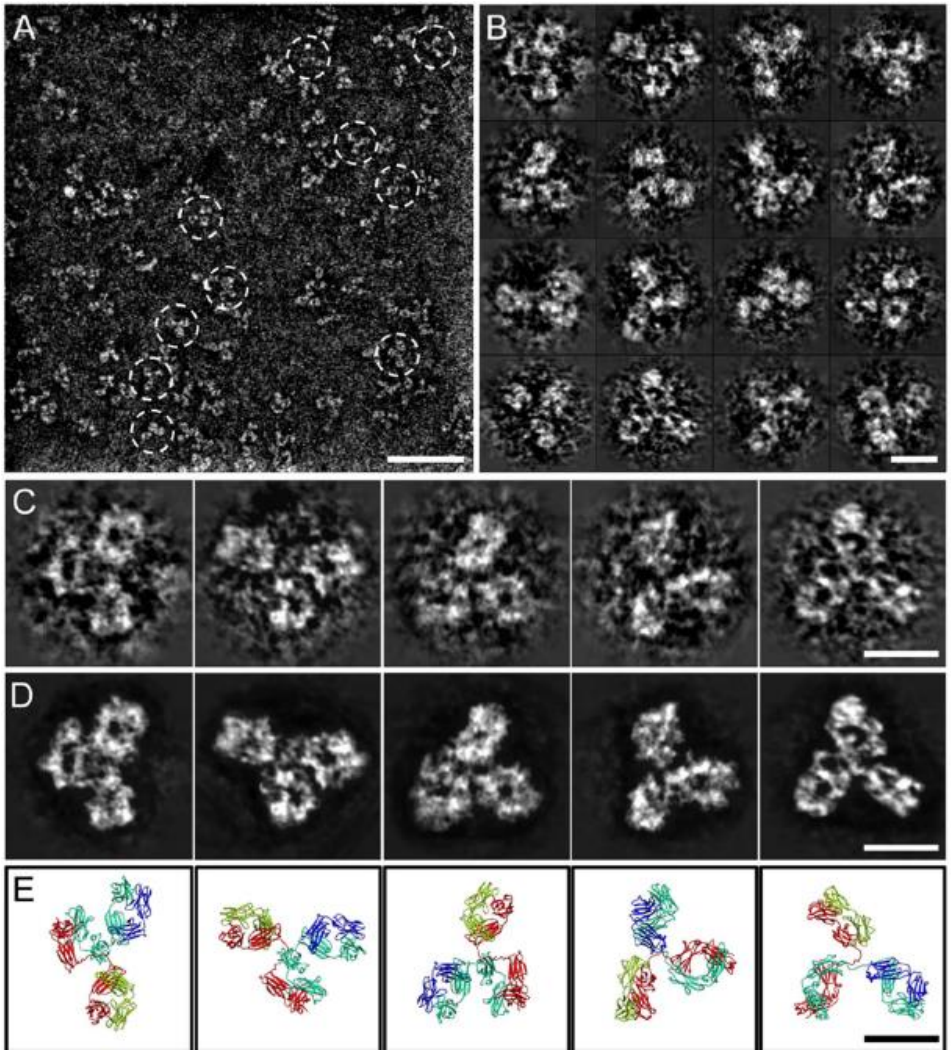
(b) Representative projection image from a frozen-hydrated specimen of purified GroEL protein complexes. Complexes with distinct orientations relative to the electron beam can be discerned as indicated in the boxed examples.

(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

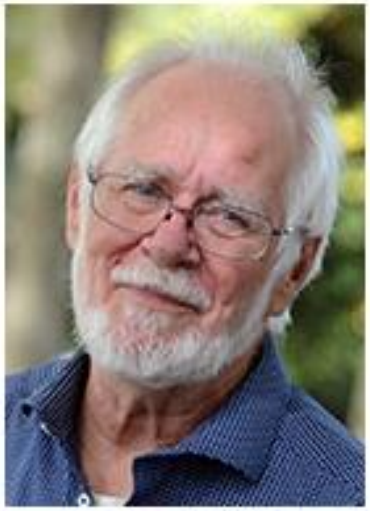


Photo: Félix Imhof © UNIL [CC BY-SA 4.0]
Jacques Dubochet
Prize share: 1/3

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

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

Sample preparation

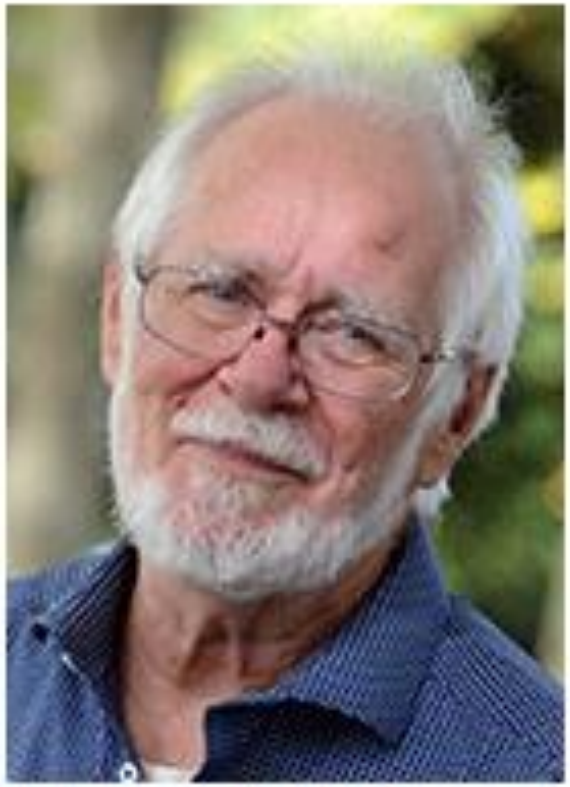


Photo: Félix Imhof © UNIL [CC BY-SA 4.0]

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 electronmicroscopists, became what it always was in nature – an integral part of biological matter and a beautiful substance….”

The Nobel Prize in Chemistry 2017

Image analysis

Photo: B. Winkowski © Columbia University Medical Center

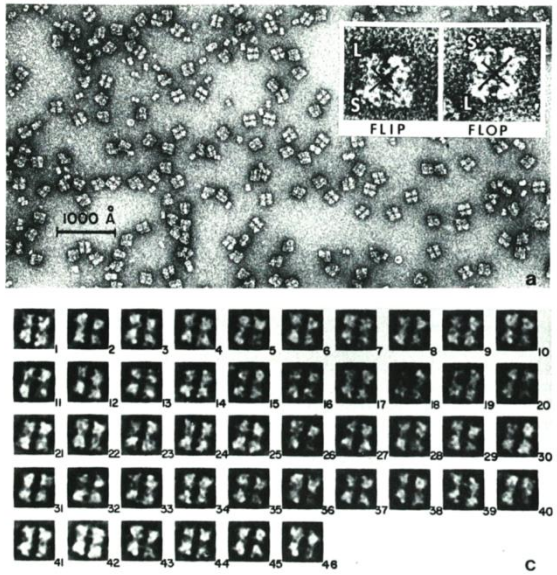
Joachim Frank

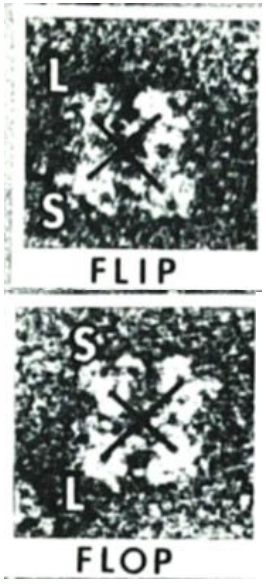
Ultramicroscopy 6, (1), (1981), pp.187–194

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

Detector

ELSEVIER

Journal of Structural Biology

Volume 192, Issue 2, November 2015, Pages 179-187

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**

Scanning electron microscopy

SEM advantages

Risoluzione ad alto ingrandimento.

La migliore risoluzione possibile in un LM è di circa 200 nm, mentre un tipico SEM ha una risoluzione migliore di 10 nm (in genere 5 nm).

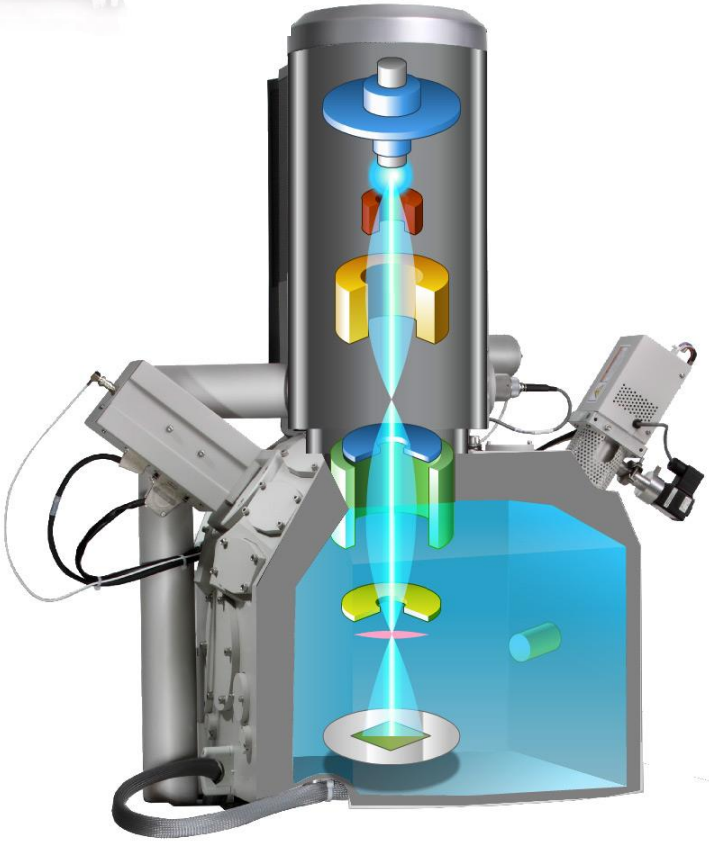
Profondità di campo

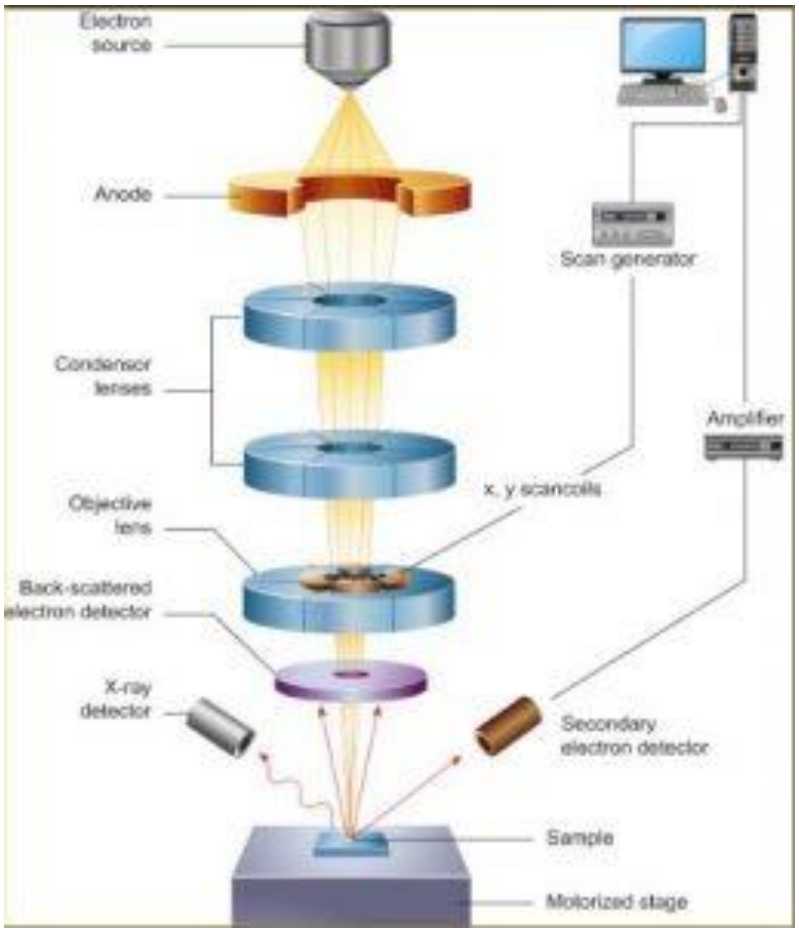
l'altezza di un campione che appare a fuoco in un'immagine - più di 300 volte la profondità di campo rispetto al LM. Ciò significa che è possibile ottenere un grande dettaglio topografico. Per molti utenti, l'aspetto tridimensionale (3D) dell'immagine del campione è la caratteristica più preziosa del SEM. Infatti tali immagini, anche a bassi ingrandimenti, possono fornire molte più informazioni su un campione rispetto a quelle disponibili con il LM.

SEM drawbacks

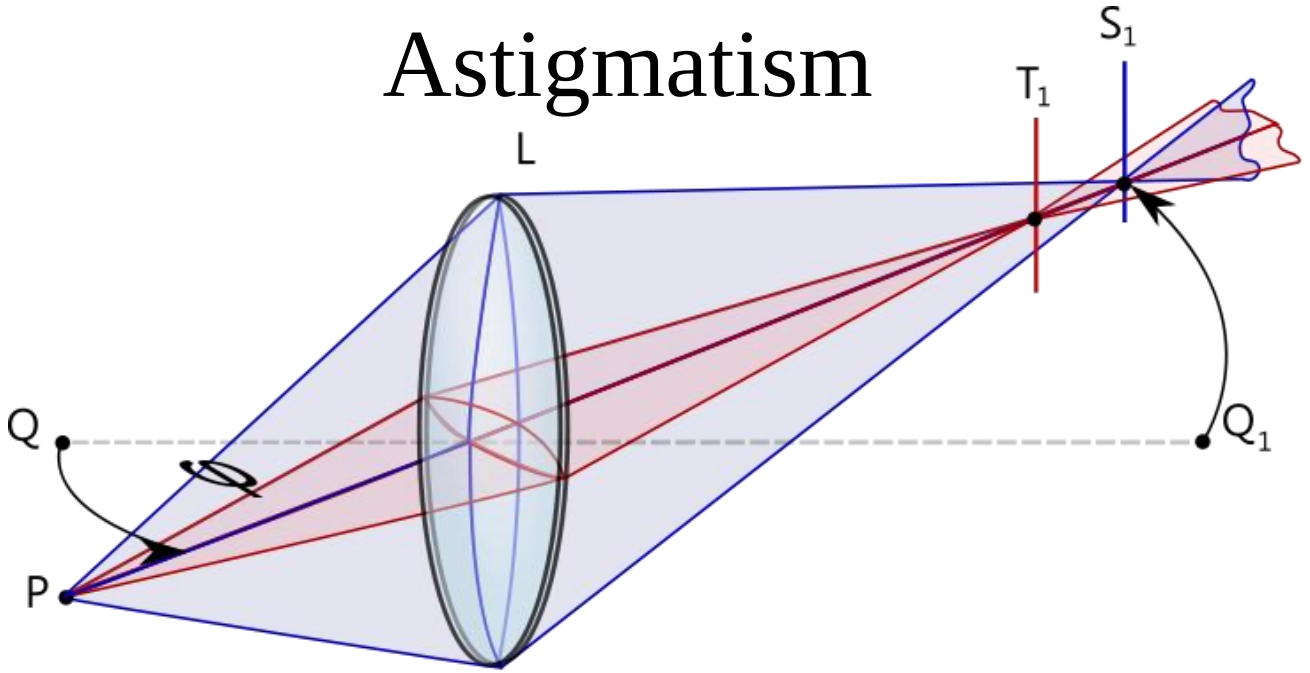
Il SEM opera sotto vuoto e in molti SEM i campioni devono essere resi conduttivi per poter essere osservati.
Questo si ottiene spesso rivestendo i campioni con uno strato molto sottile di metallo o di carbonio.

Dentro un SEM

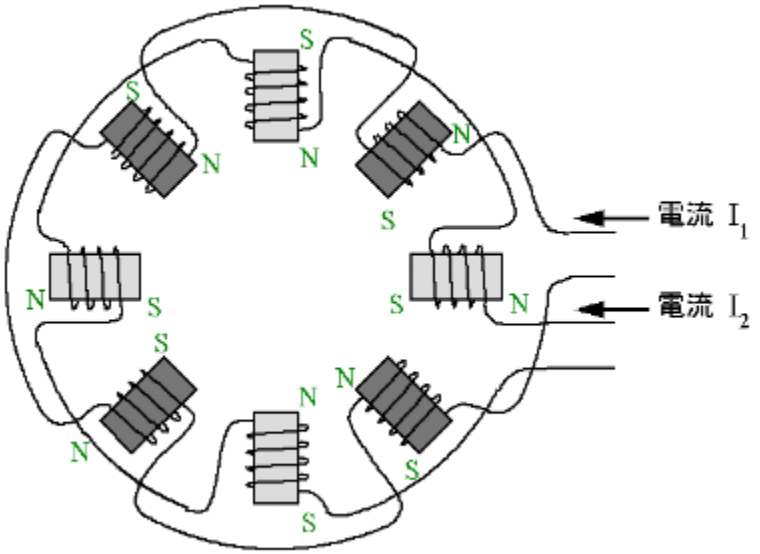


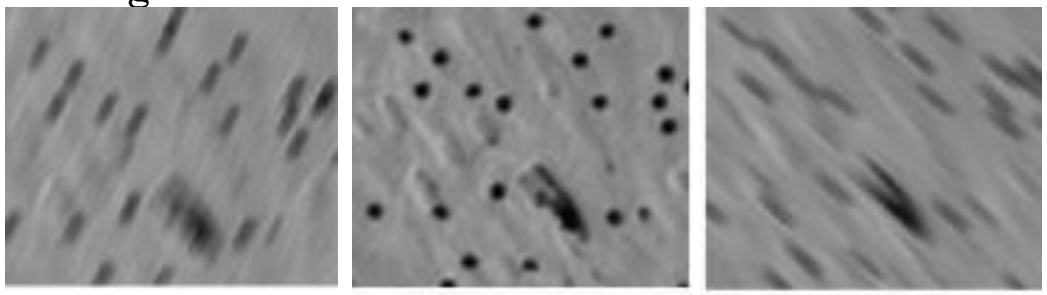


Astigmatism

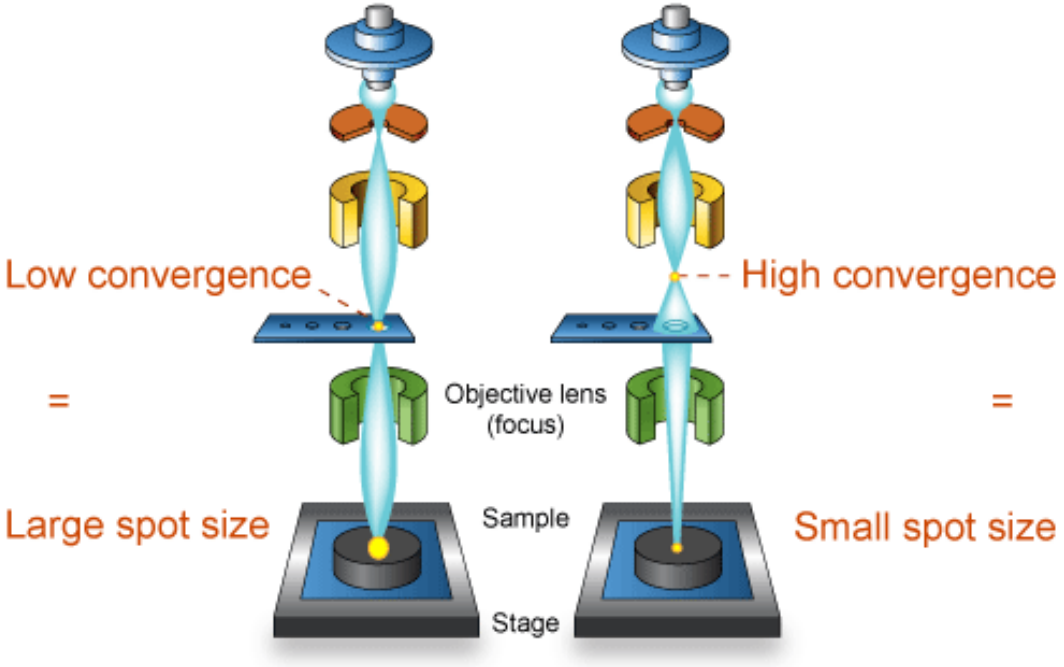


L'astigmatismo è tipico degli EM e deriva da piani focali diversi in direzioni perpendicolari (piani sagittali e tangenziali).Può dipendere dall'allineamento del fascio ma anche dalla conducibilità del campione. Può essere corretto utilizzando uno stigmatore, un insieme di lenti magnetiche che rimodellano il fascio dandogli una forma circolare.



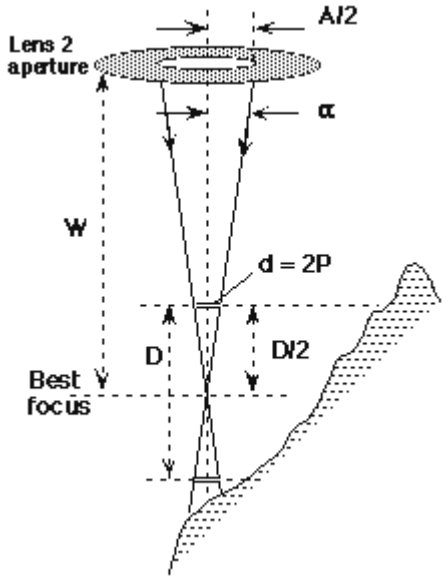


Aperture



Il diaframma impedisce agli elettroni fuori asse o fuori energia di avanzare lungo la colonna. Può anche restringere il fascio al di sotto dell'apertura, a seconda delle dimensioni del foro selezionato.

Riguardo la profondità di fuoco



$\alpha < 1°$

Mag	A = 100 µm	200 µm	400 µm	Light Micros.	
	Depth of Field (µm)				
50X	800	400	200	100	
100X	400	200	100	20	
500X	80	40	20	2	
1000X	40	20	10	0.6	
5000X	8	4	2		
10,000X	4	2	1		
1000X	80	40	20		W = 20 mm
1000X	160	80	40		W = 40 mm

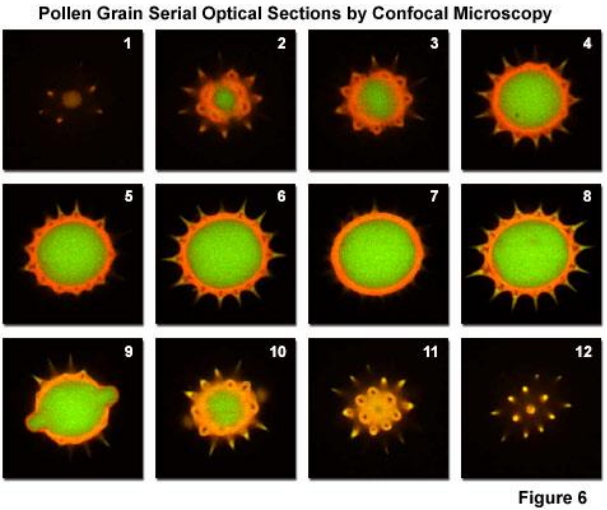
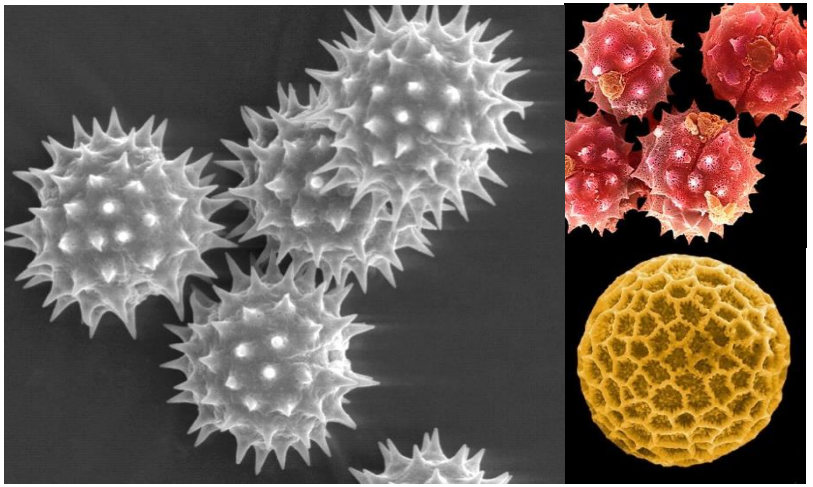


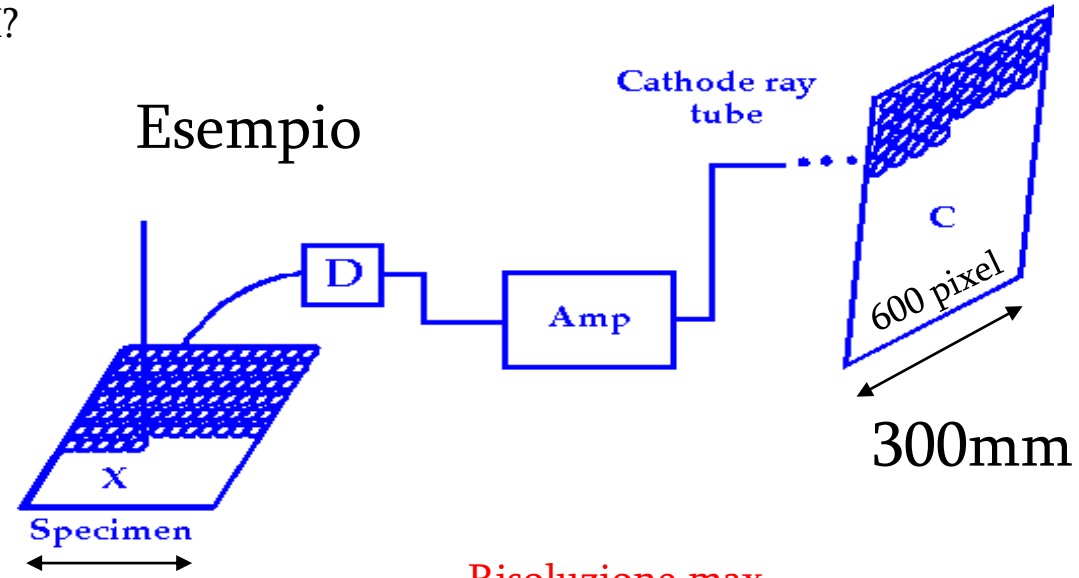
Figure 6

In a single image



A cosa è dovuto l'ingrandimento nel SEM?

Esempio



300mm

300µm

Risoluzione max

300µm/600pixel → 0.5 µm/pixel

Nessuna informazione può essere ottenuta sulla struttura interna del pixel sul campione

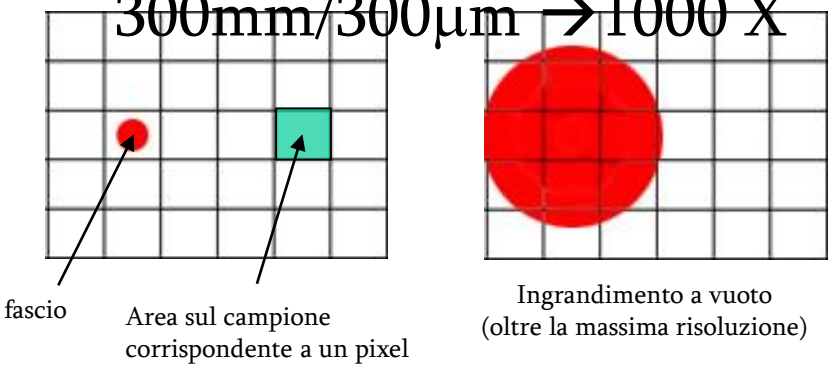
Il numero di pixel nello schermo e la dimensione dell'area scansionata definiscono la dimensione dell'area del campione corrispondente ad un pixel, cioè la risoluzione.

L'ingrandimento è il rapporto tra la lunghezza della linea sullo schermo e quella sul campione.

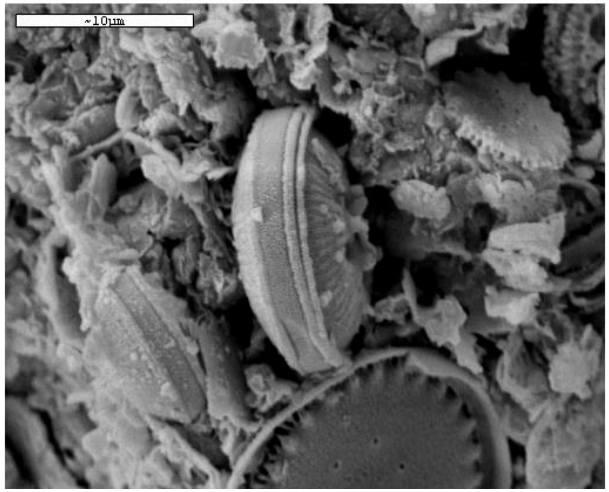
La risoluzione ha un limite inferiore nella dimensione del fascio.

Ingrandimento

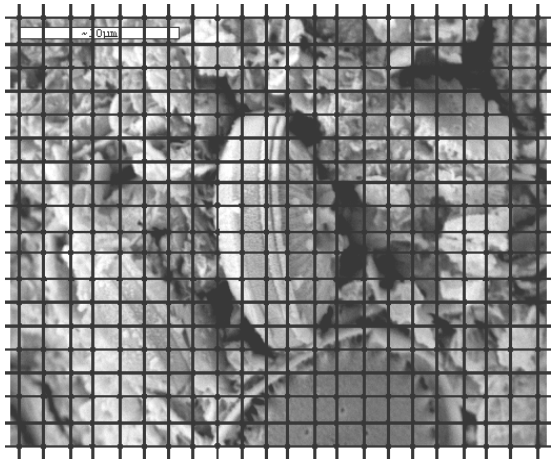
300mm/300µm →1000 X



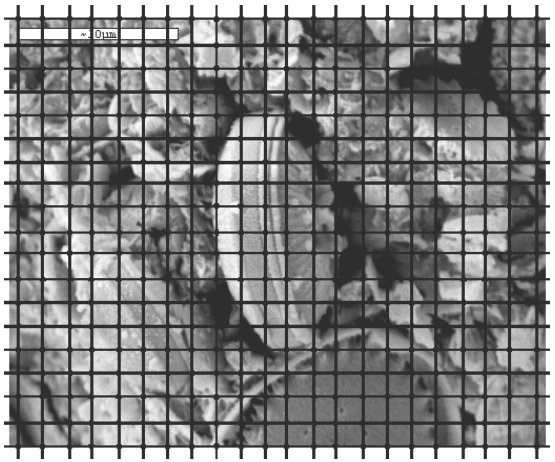
Specimen

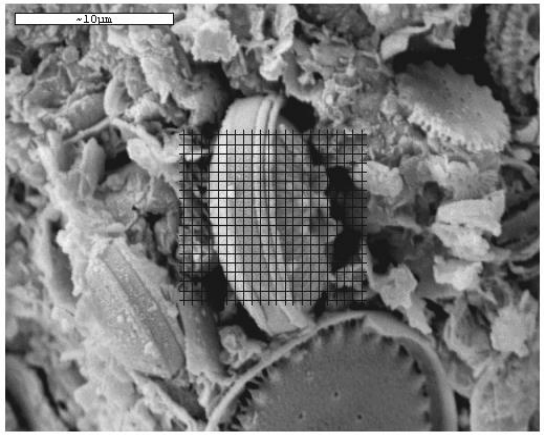


Image

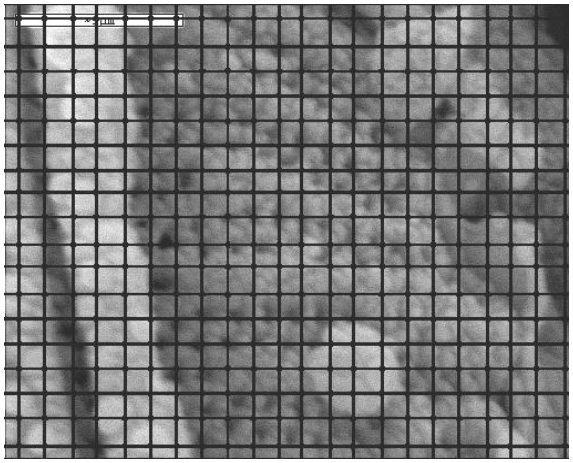


Mag=1

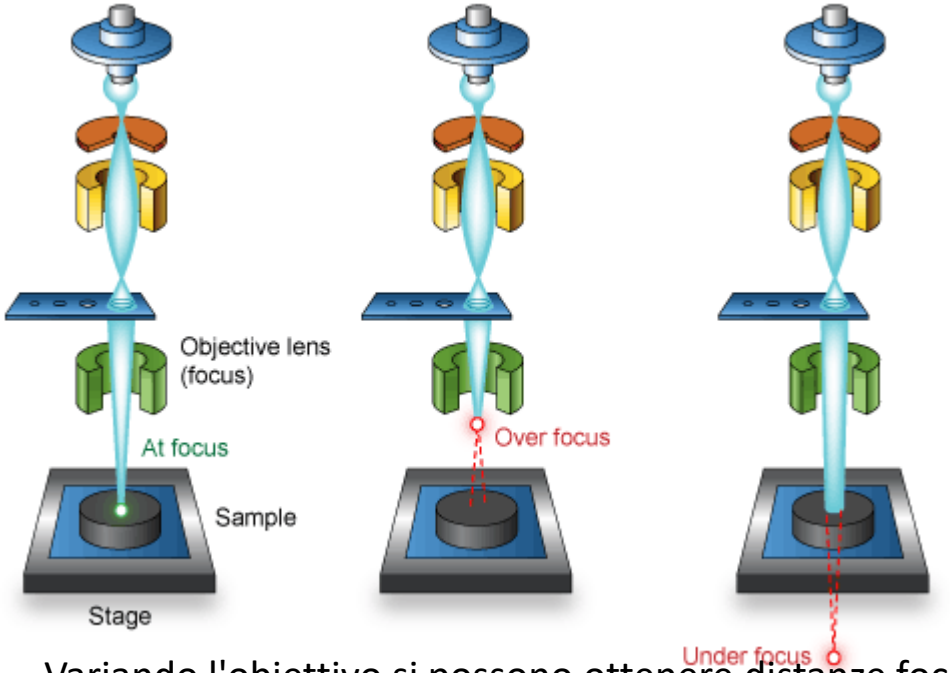


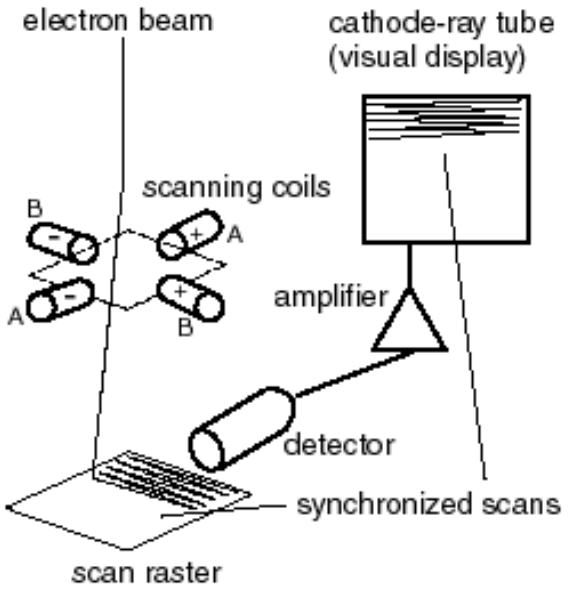


Mag=3



Focusing and scanning





Variando l'obiettivo si possono ottenere distanze focali diverse. La distanza tra l'obiettivo e il campione è la distanza di lavoro.
WD breve = risoluzione più elevata

La WD in EM può essere regolata, mentre in LM è fissa.

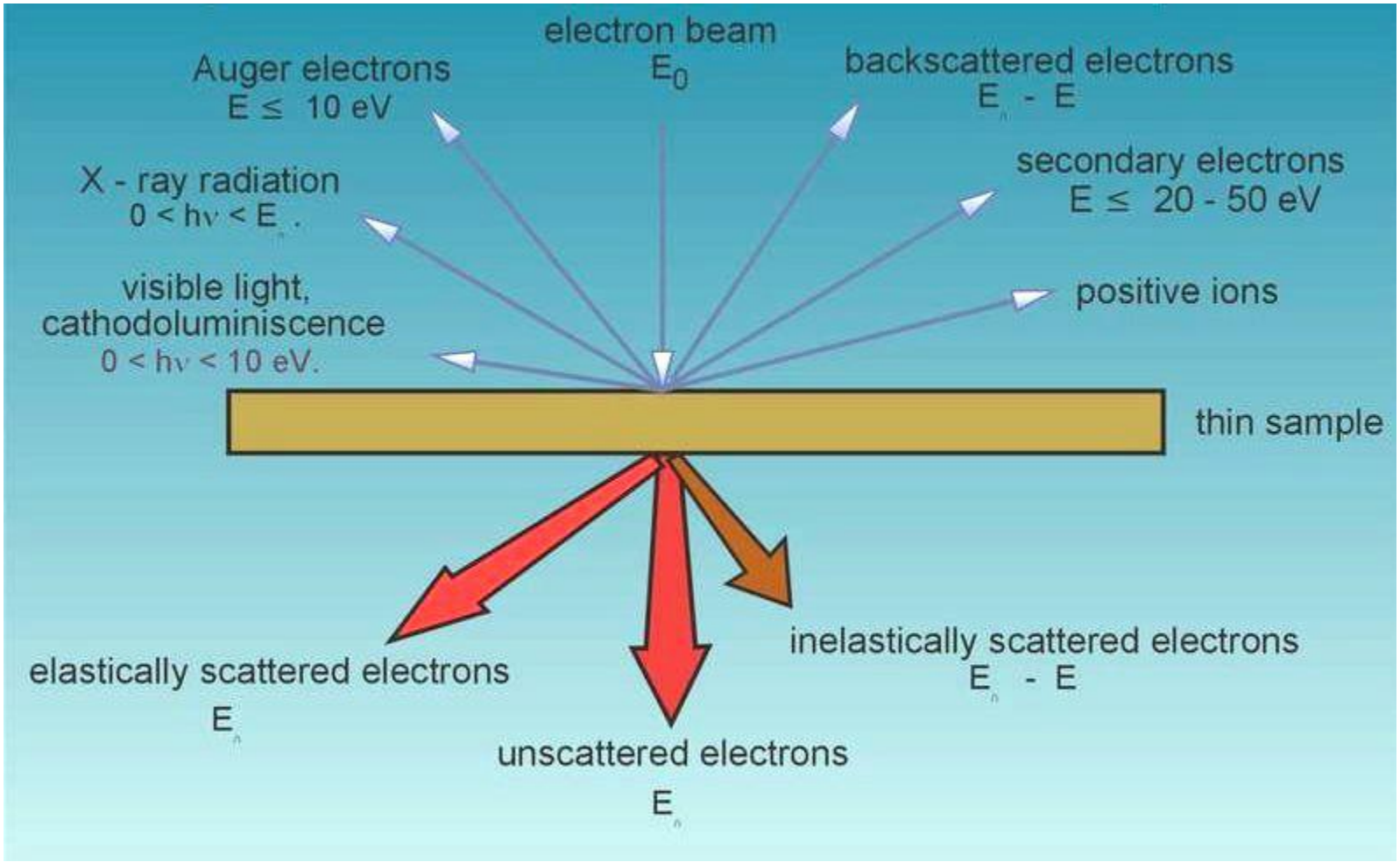
Nell'EM la NA è piccola (0.01 e $\lambda \cong 0.01$nm)
La risoluzione è di circa 1nm

La profondità di fuoco è di diversi 10 mm

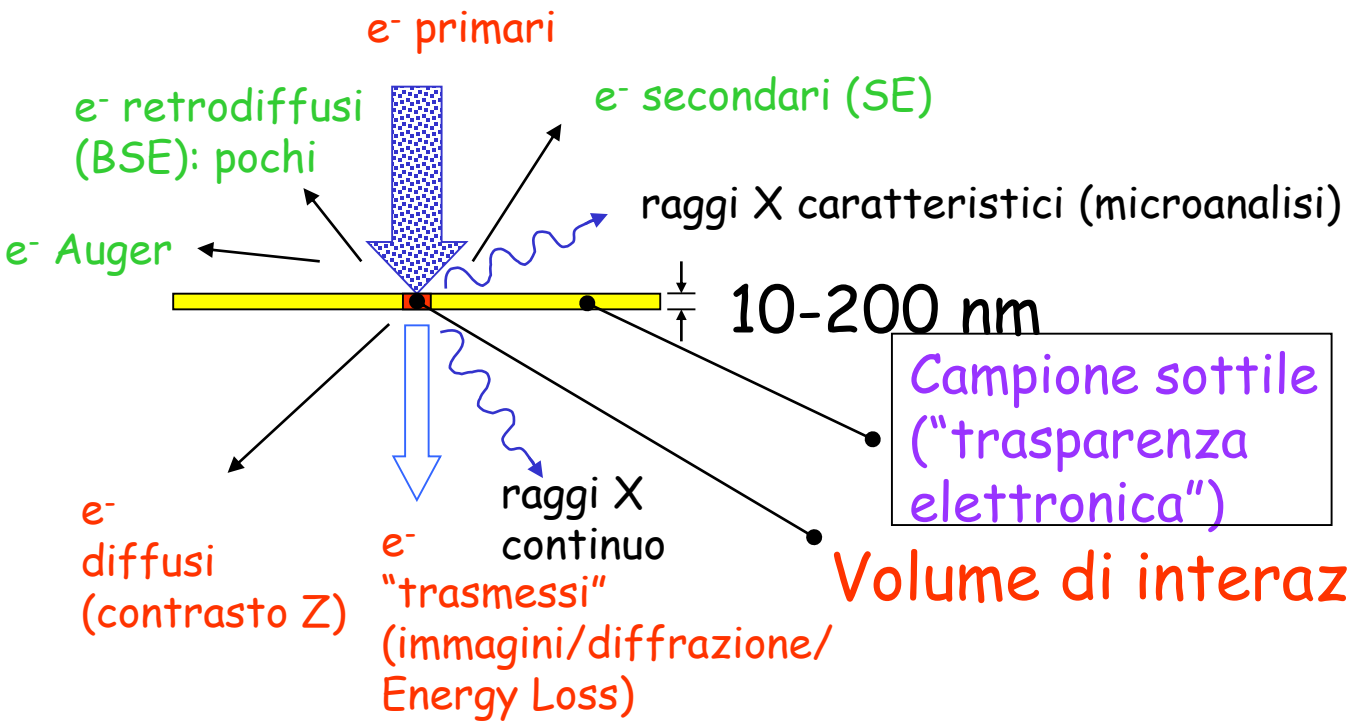
Per generare un'immagine, il fascio viene sottoposto a scansione raster sul campione e viene acquisito un segnale ....

Quale segnale possiamo acquisire?

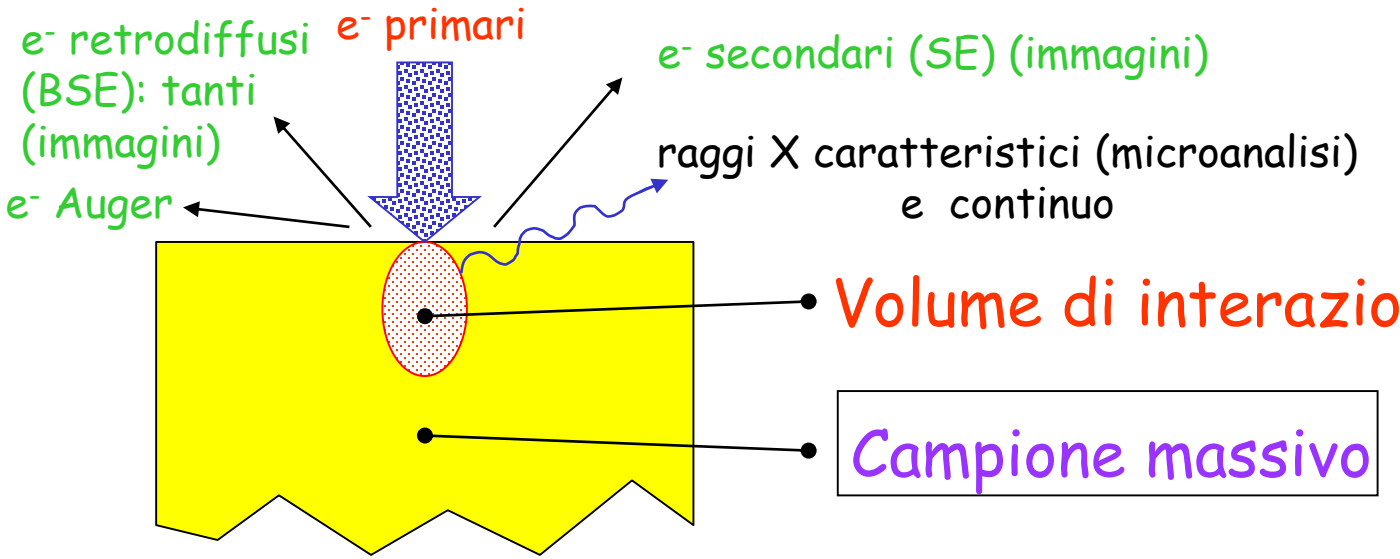
Electro-matter interaction



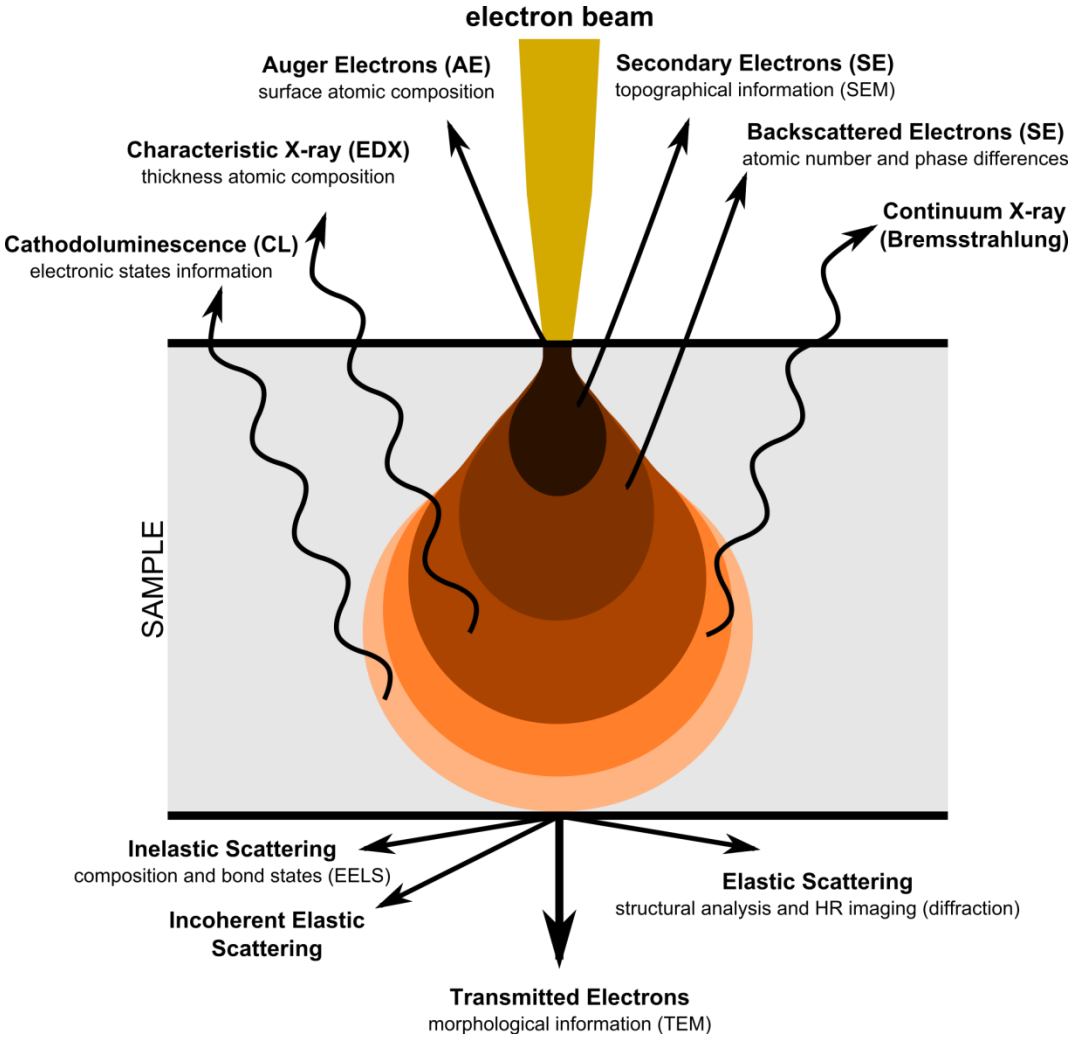
Microscopia elettronica in trasmissione (TEM)



Microscopia elettronica a scansione (SEM)



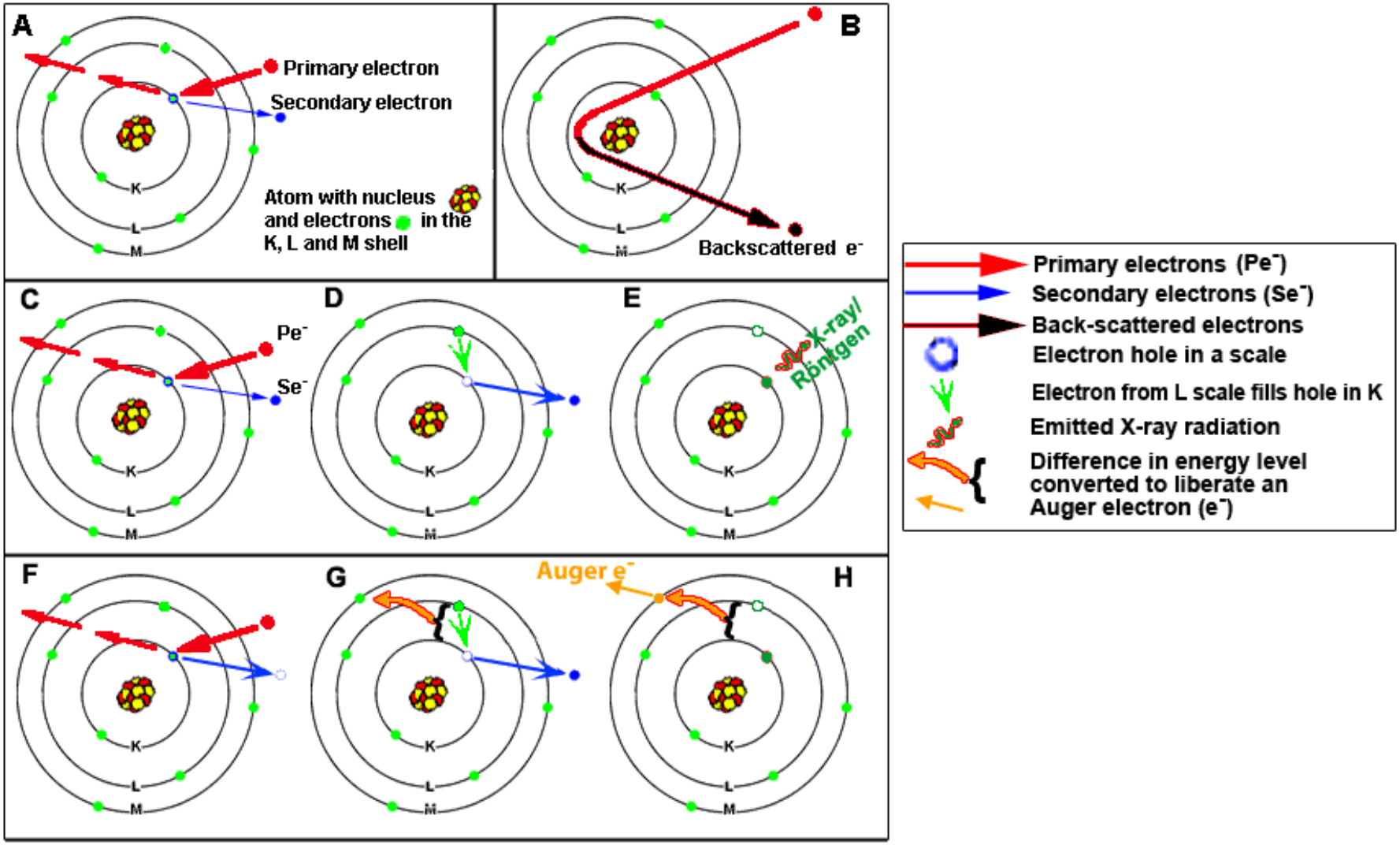
Localizzazione della provenienza dei vari segnali



A causa della bassa energia degli elettroni secondari (2-50eV) solo gli elettroni emessi in prossimità della superficie contribuiscono a formare il segnale dei secondari.

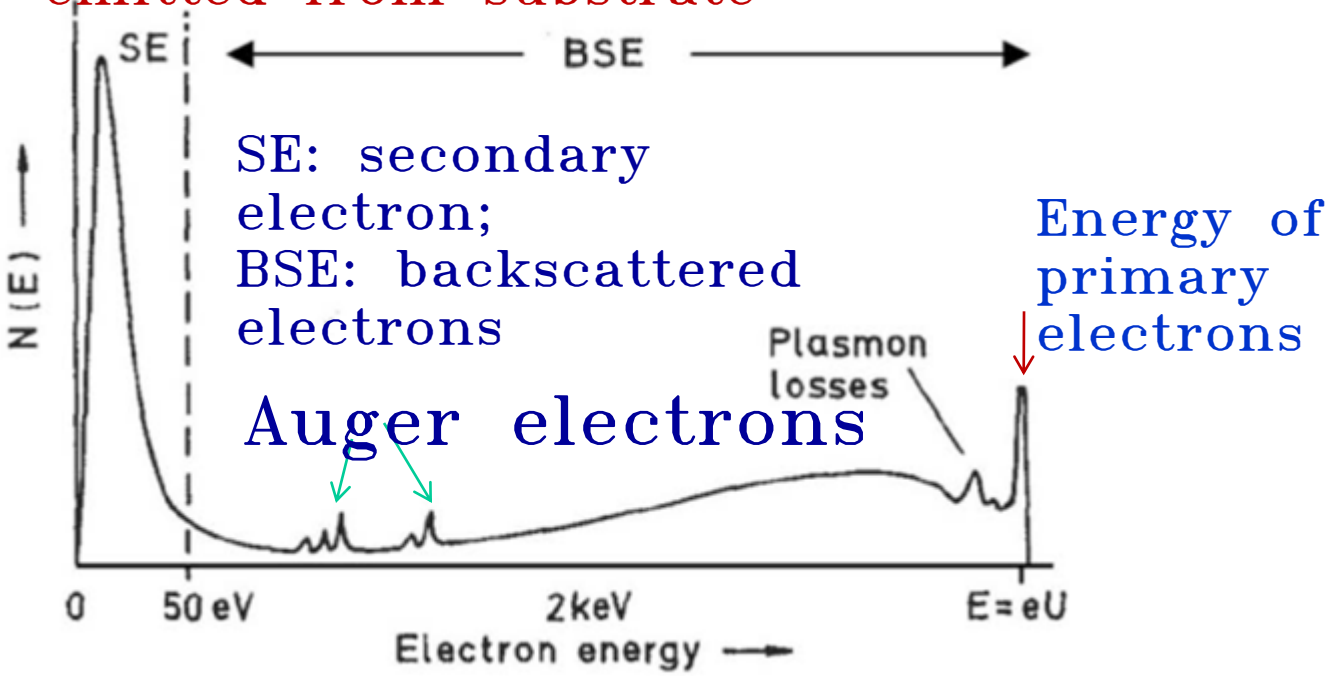
Per questa ragione gli elettroni secondari *utili* vengono emessi in prossimità del fascio prima che questo si allarghi nella pera di interazione e sono utilizzati per generare immagini ad alta risoluzione

SE vs Backscattered



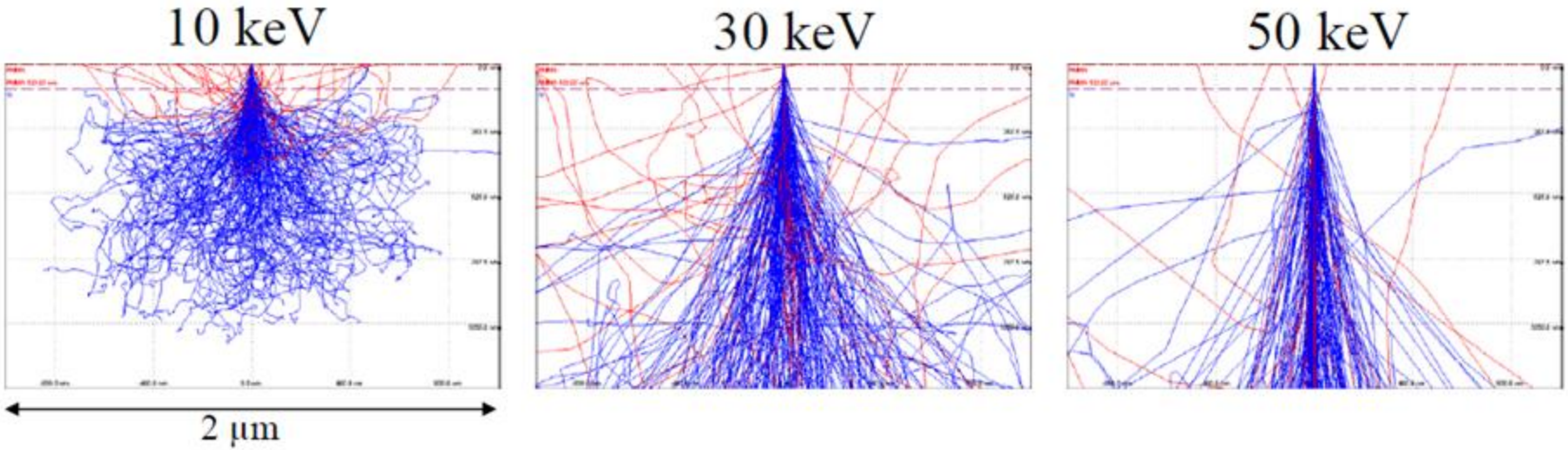
Forward/back scattering events

Energy spectrum of electrons emitted from substrate

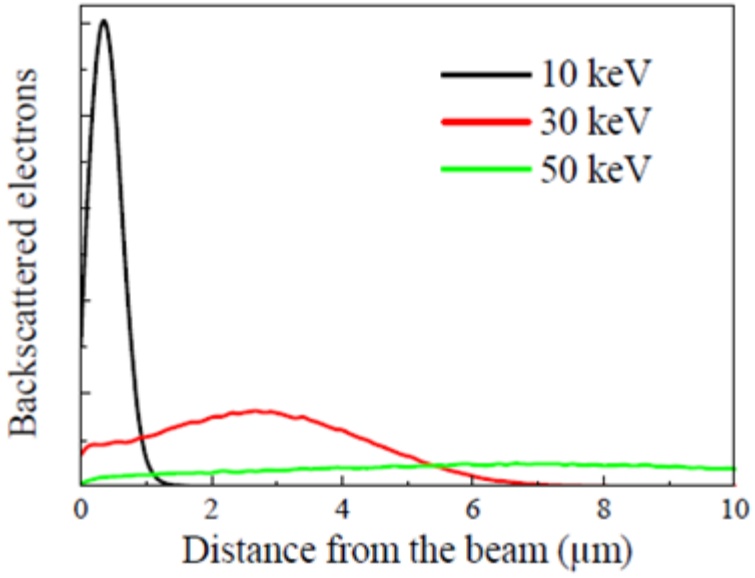


There is no clear-cut distinction of SE and BSE. Typically
SE with several eV are responsible for most (not all) resist
Such SE diffuses laterally a few nm, which is one limiting

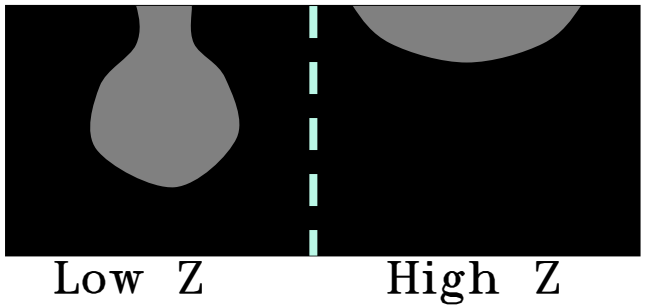
Monte-Carlo simulations of electron trajectory



Scattering probability varies as square of atomic number Z, and inversely as the incident kinetic energy.

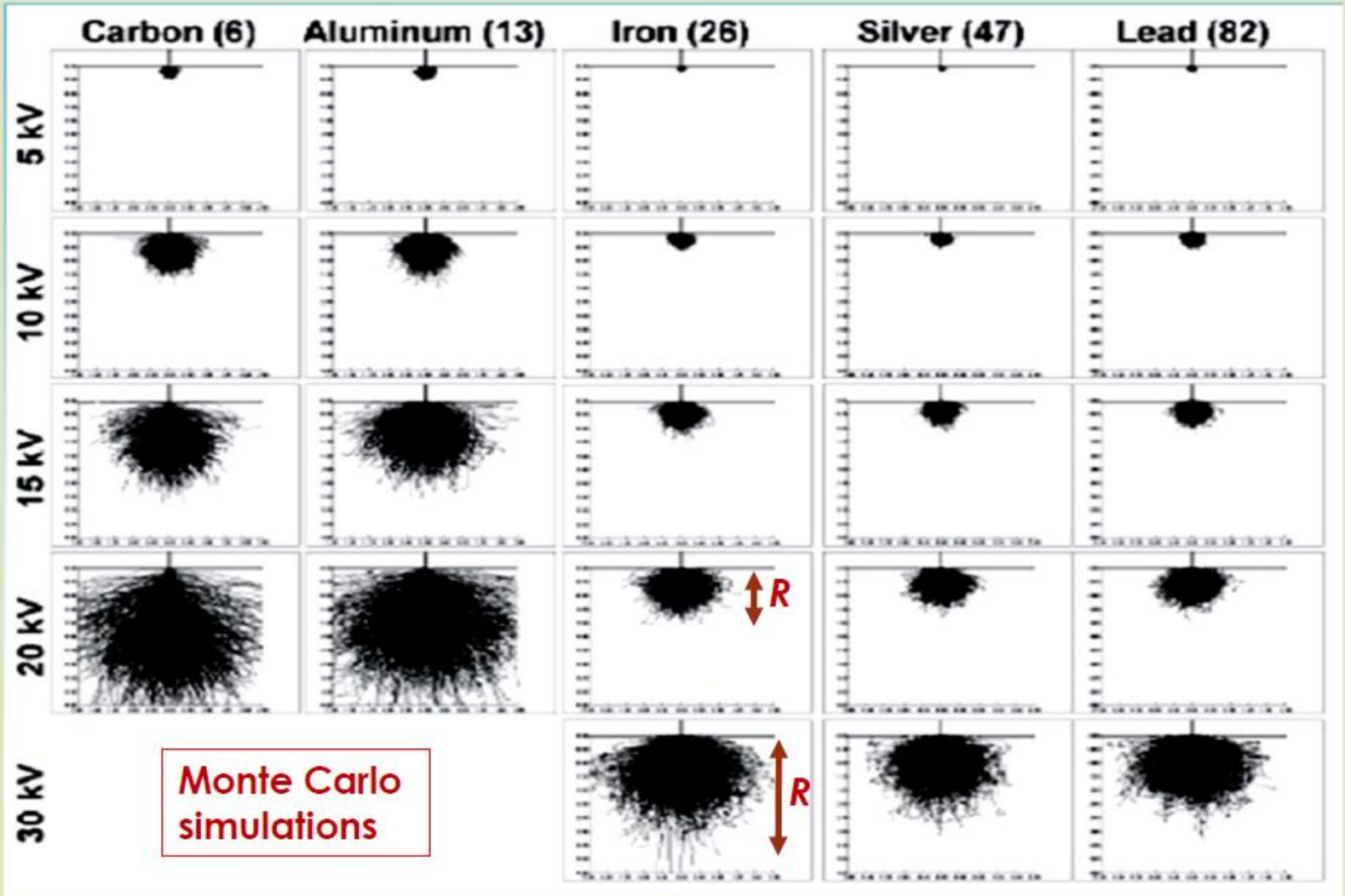


Penetration depth decrease with Z.



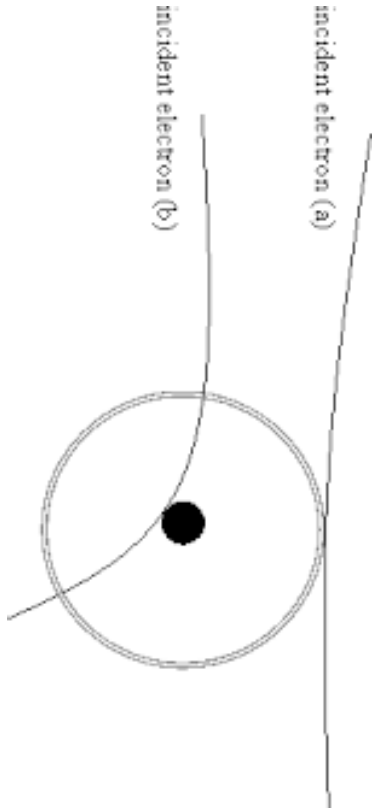
Number of backscattered electrons is not so dependent on energy, but its spatial distribution is.
Proximity effects are "diluted" (spread over larger area) at high energies.

Interaction Volume



Backscattered electrons

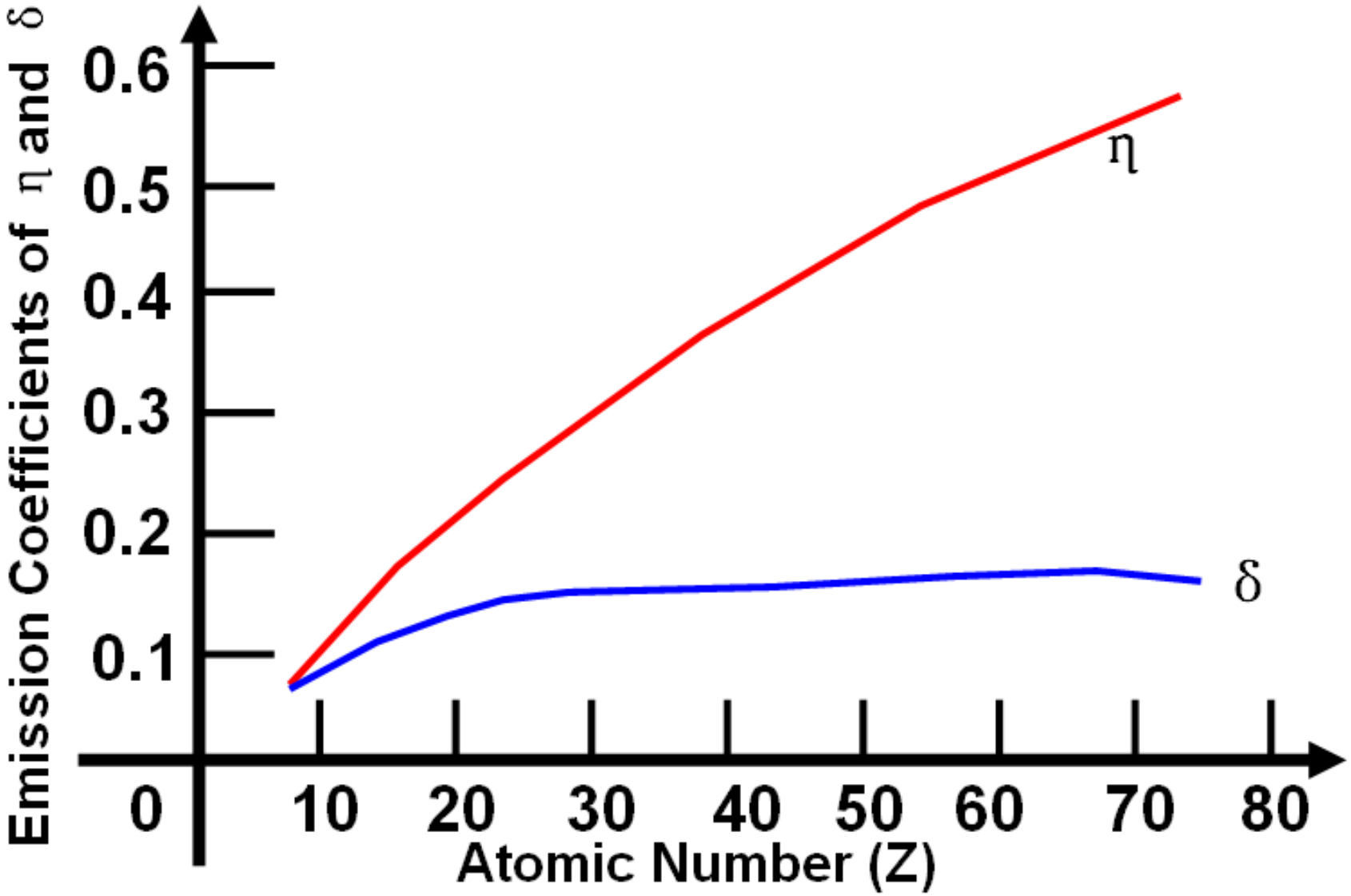
- Gli elettroni sono retrodiffusi in modo elastico dagli atomi del campione (Elastico = senza perdita/trasferimento di energia)
- Nel backscattering, la traiettoria dell'elettrone viene modificata di oltre 90 gradi rispetto alla direzione del moto. Si noti che tra gli eventi elastici multipli (che formano gli elettroni retrodiffusi), gli elettroni del fascio possono anche essere coinvolti in eventi anelastici, riducendo così l'energia dell'elettrone.
- Gli elettroni retrodiffusi hanno solitamente un'energia dell'ordine dei kV (< energia del fascio).
- Poiché gli elementi pesanti (ad alto numero atomico) retrodiffondono gli elettroni più fortemente degli elementi leggeri (a basso numero atomico) e quindi appaiono più luminosi nell'immagine, gli ESB vengono utilizzati per rilevare il contrasto tra aree con composizioni chimiche diverse.



*le interazioni **e^- - e^-** sono altamente probabili e ognuna di esse comporta un piccolo trasferimento di energia*

*L'interazione **e^- - nuclei** comporta un grande trasferimento di quantità di moto, ma è tipicamente elastica.*

Backscattered electrons

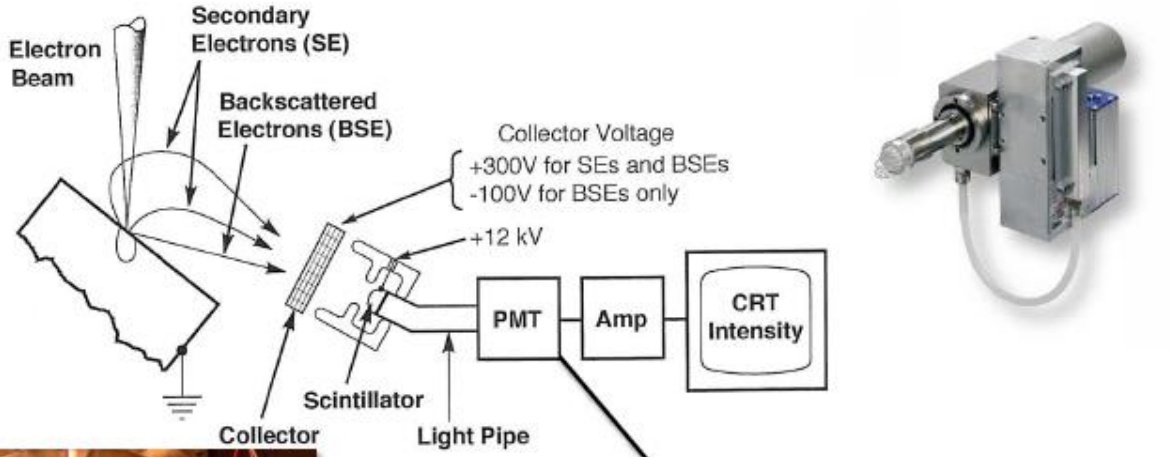


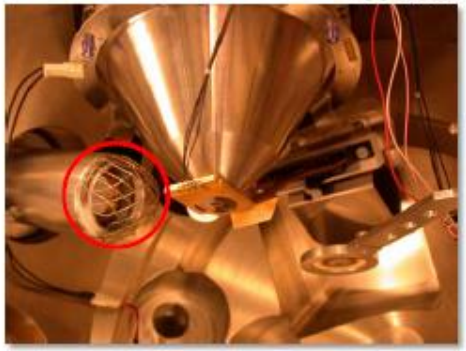
N.B. Z(number of protons)≠ A(Atomic mass) for C A=12 for Au A=197

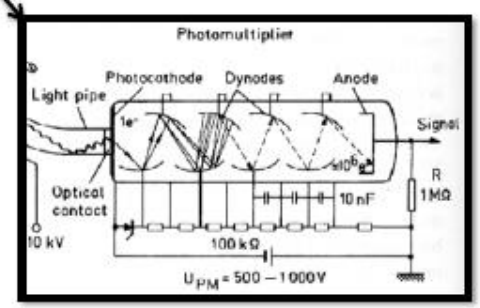
Secondary electrons

- Gli elettroni secondari prodotti dall'interazione del fascio di elettroni primari sono definiti SE1.
- Gli elettroni secondari generati dagli elettroni retrodiffusi sono definiti SE2.
- Gli elettroni secondari generati dall'interazione del fascio con la camera del campione, l'asta, ecc. sono definiti SE3.

Secondary electrons sono rivelati da un Everhart-Thornley Detector





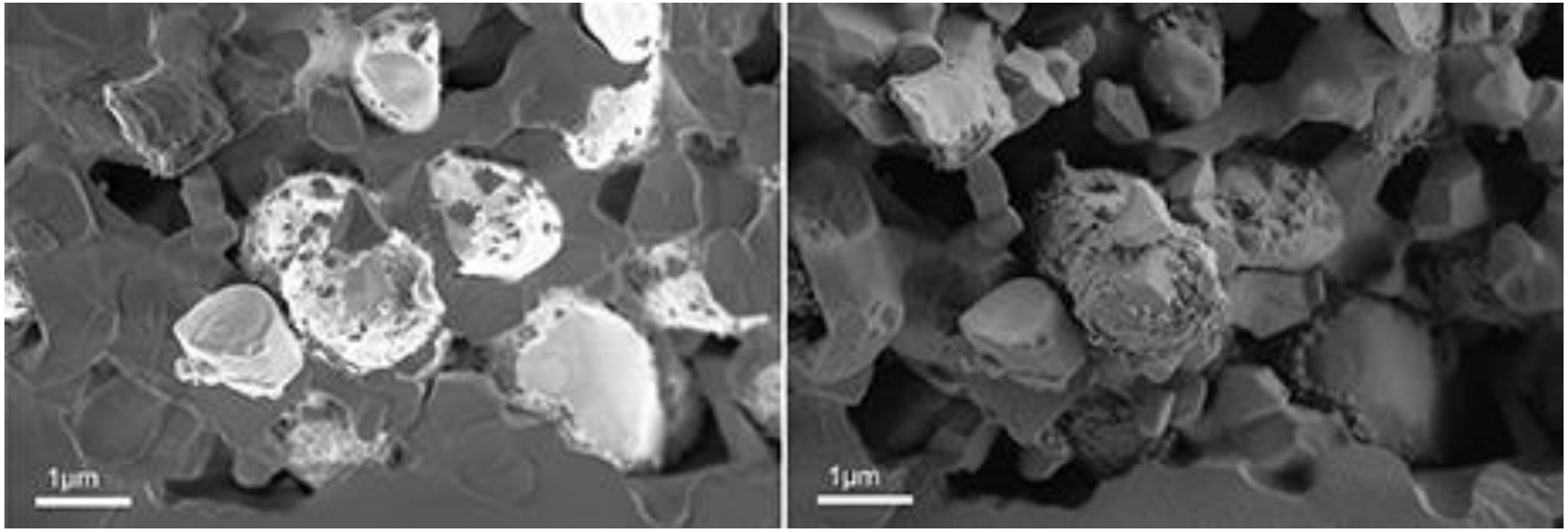


Una griglia di collettori può essere impostata per selezionare la diversa energia di SE

Elettroni liberi espulsi dall'orbitale k mediante scattering anelastico di elettroni primari Elettroni liberi espulsi dall'orbitale k mediante scattering anelastico di elettroni primari

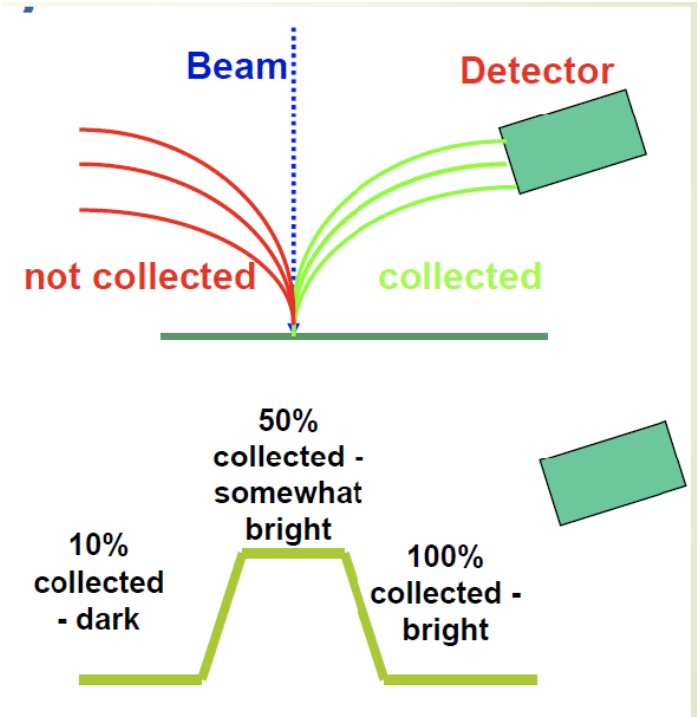
Energy < 50 eV

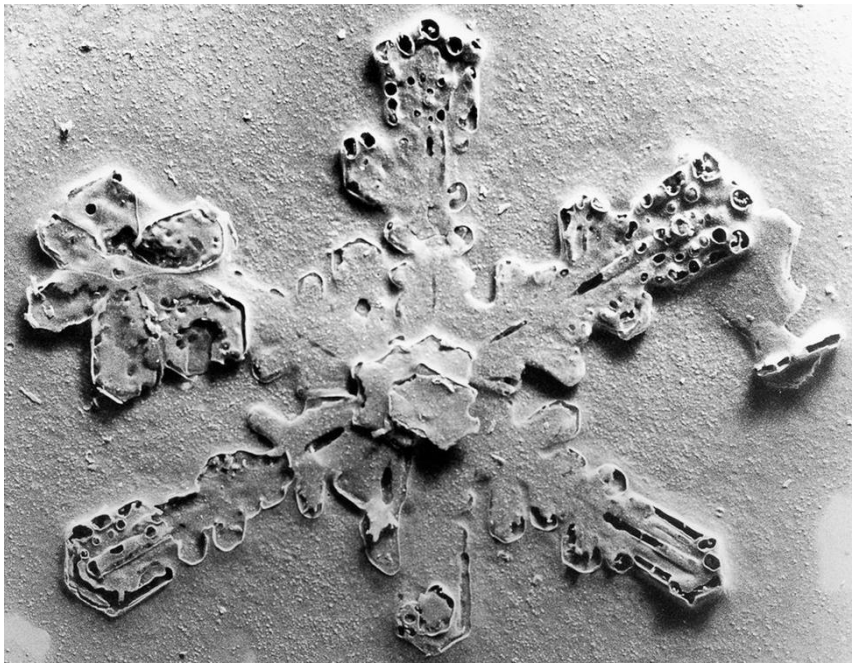
• Profondità di fuga ridotta (~2nm), informazioni solo sulla superficie del campione



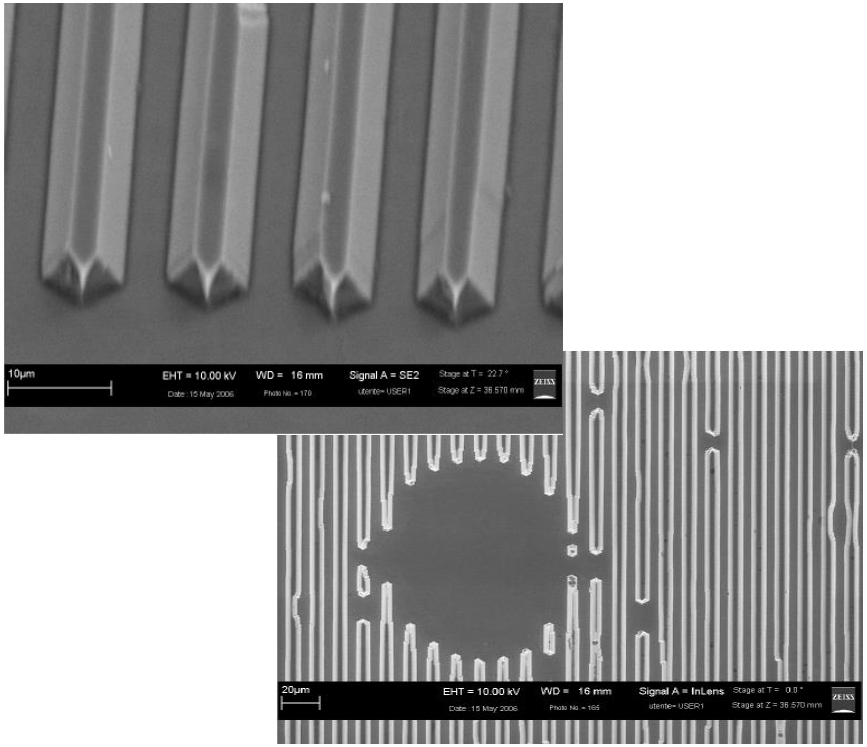
Secondary electrons

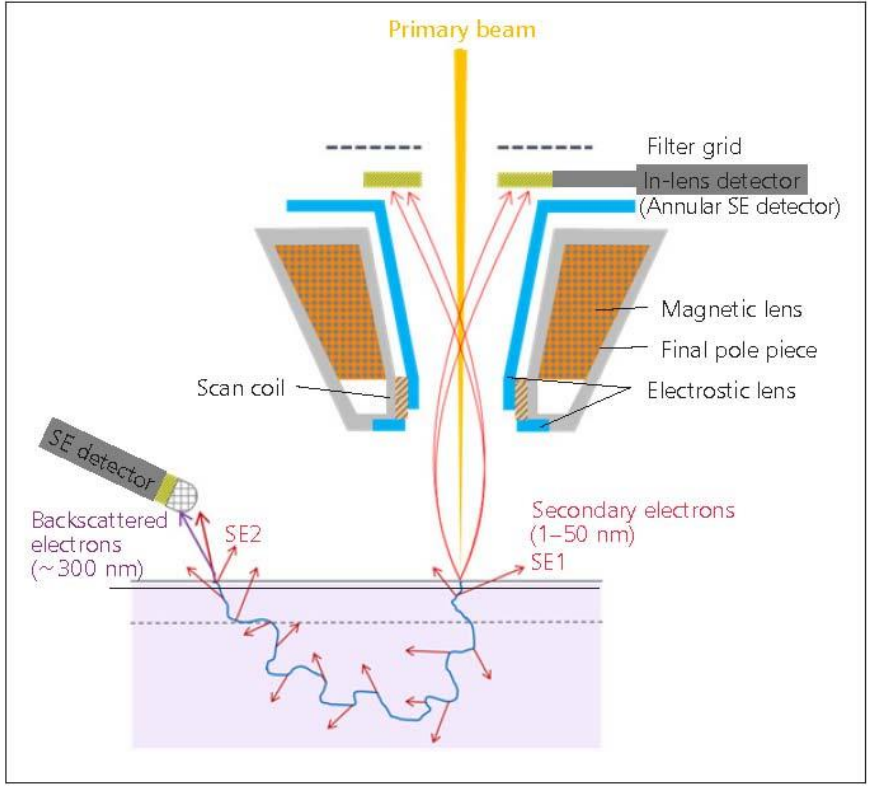
Poiché il rivelatore SE è solitamente posizionato sul lato della telecamera e gli elettroni SE sono generati dalla superficie, si genera un "effetto ombra".





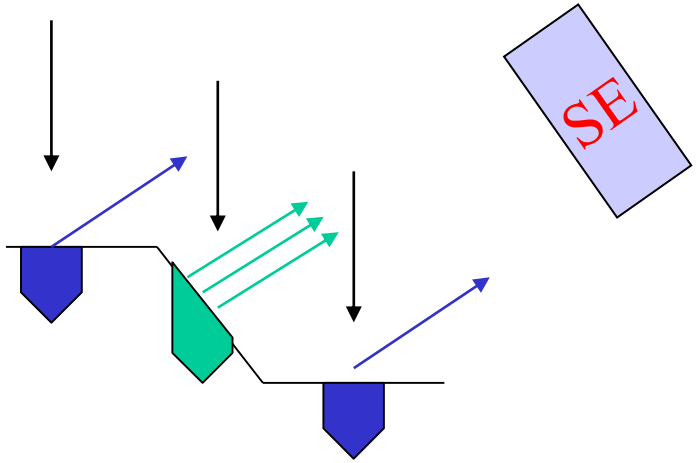
Immersion detector





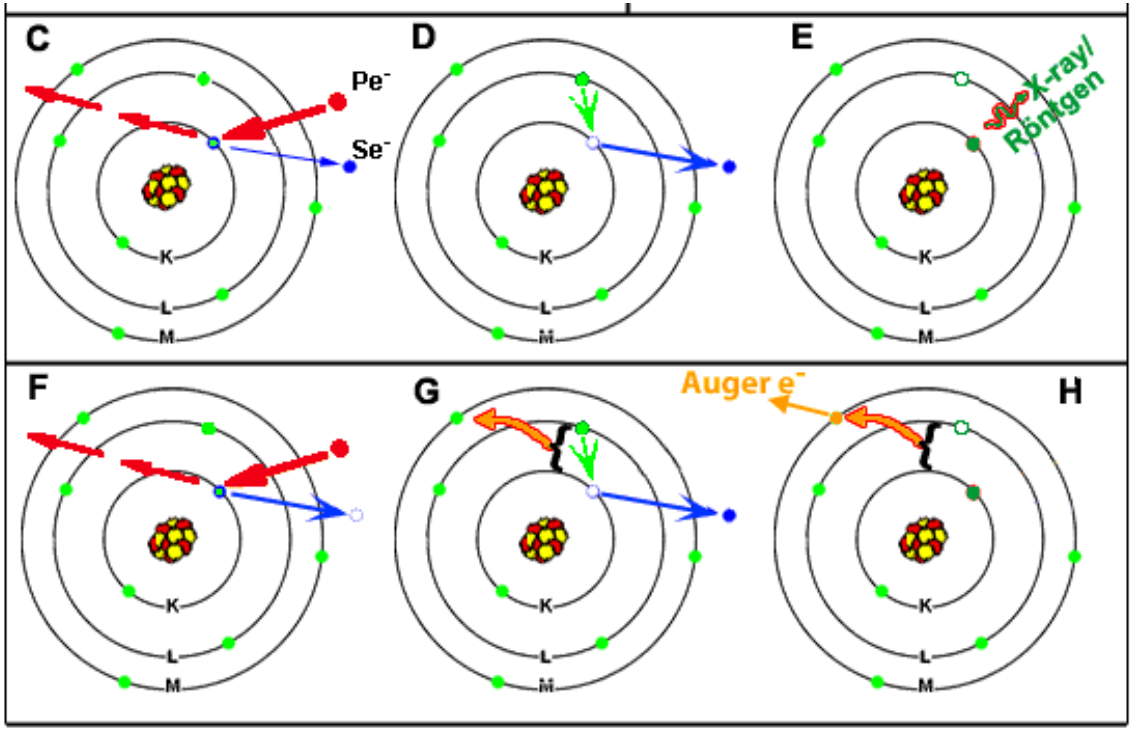
In lens

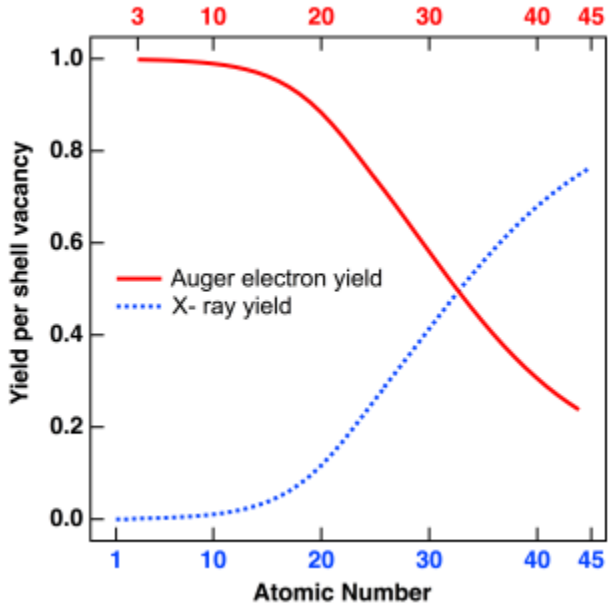
L'uso del rilevatore interno all'obiettivo: -
- fornisce un'immagine simmetrica con bordi evidenziati in tutte le direzioni.
- consente di operare a WD estremamente corti, aumentando così la NA e la risoluzione, ma diminuendo la profondità di fuoco.



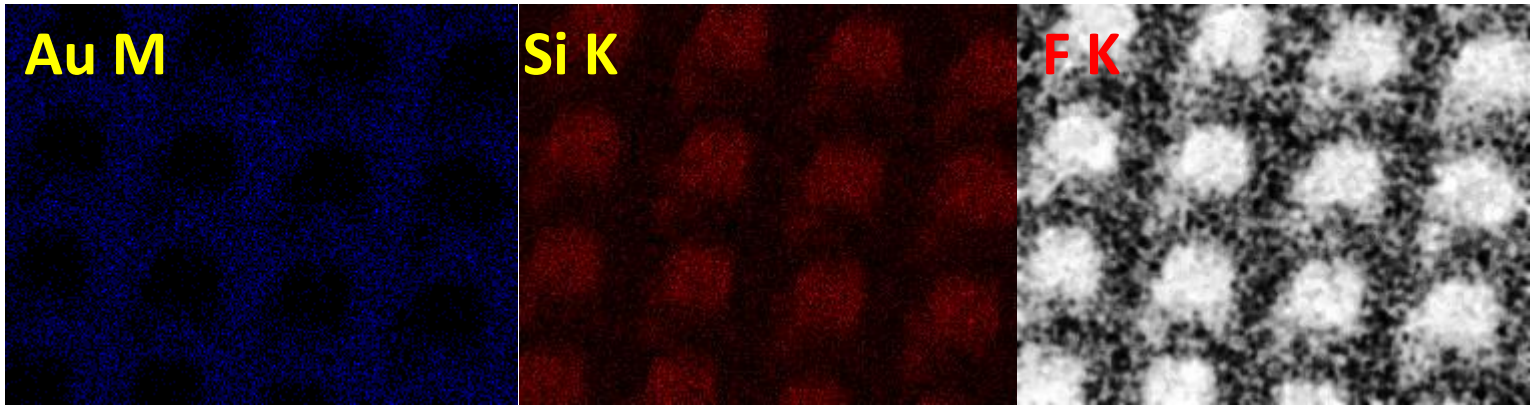
Energy-dispersive X-ray spectroscopy (EDS, EDX, or XEDS)

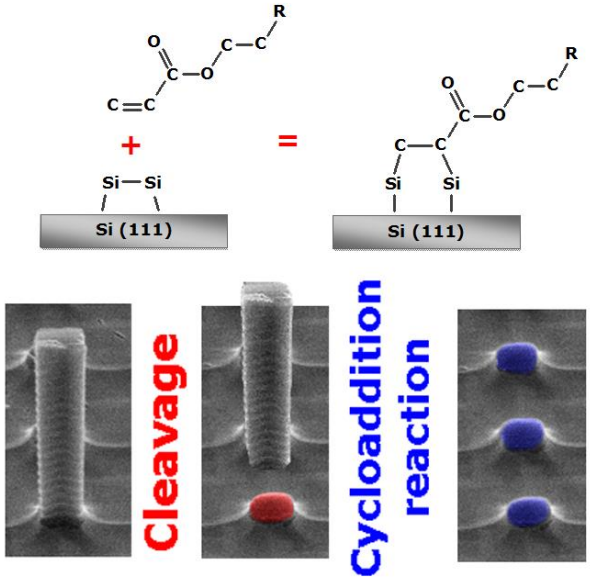
L'EDS è una tecnica analitica utilizzata per l'analisi elementare o la caratterizzazione chimica di un campione.

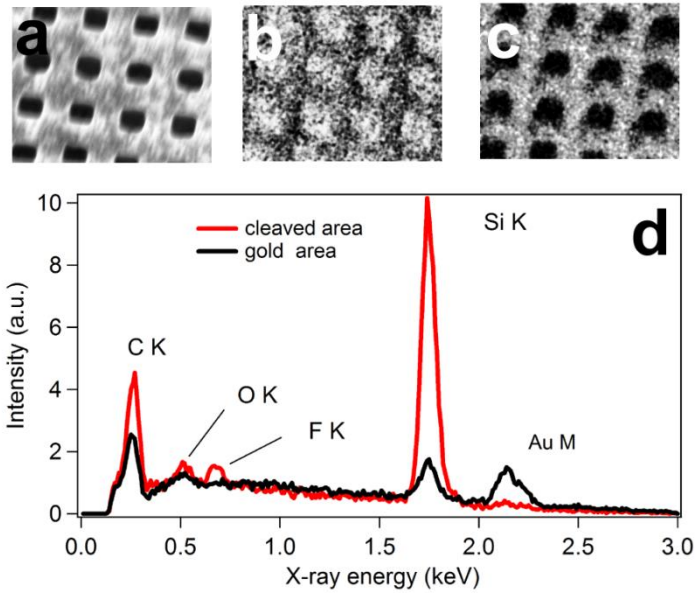




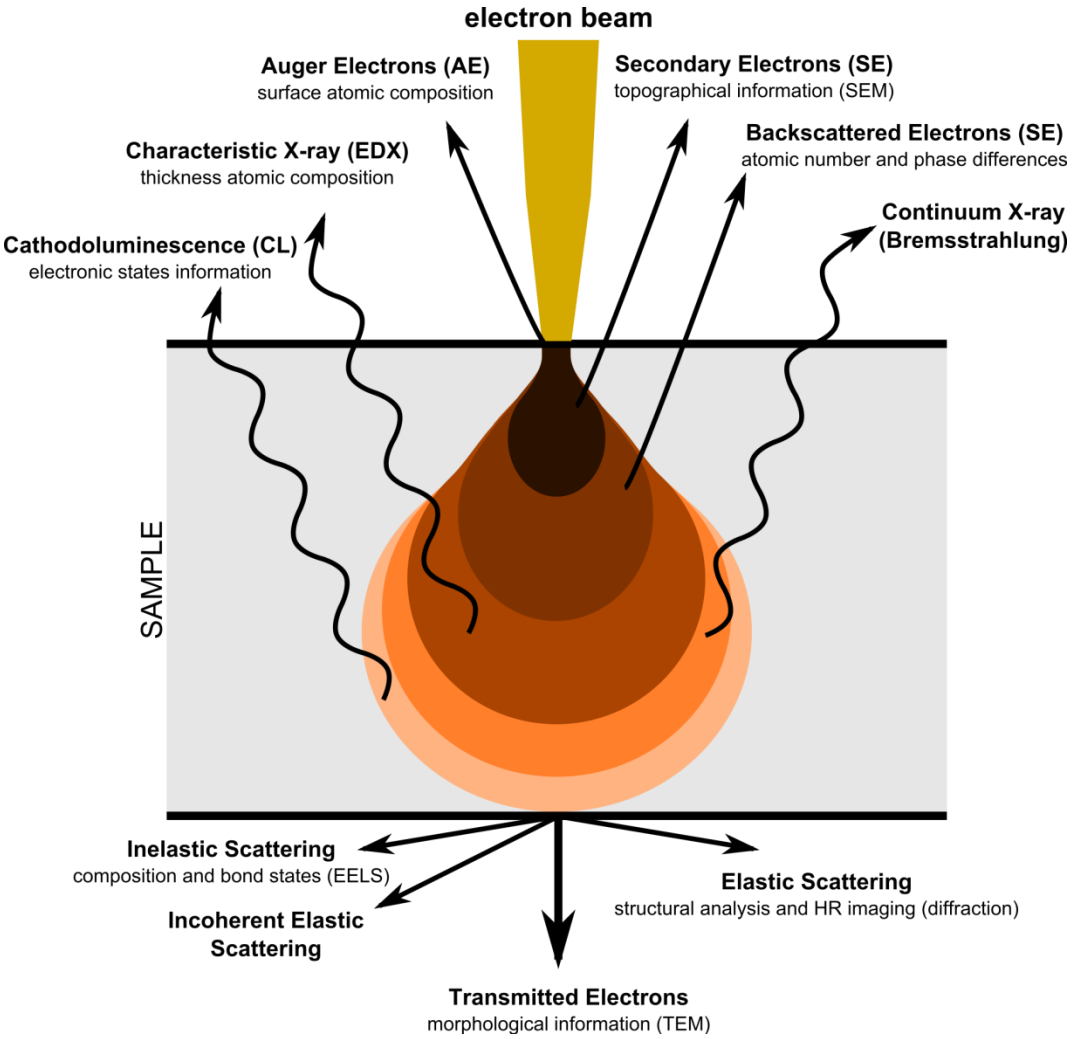
Energy-dispersive X-ray spectroscopy



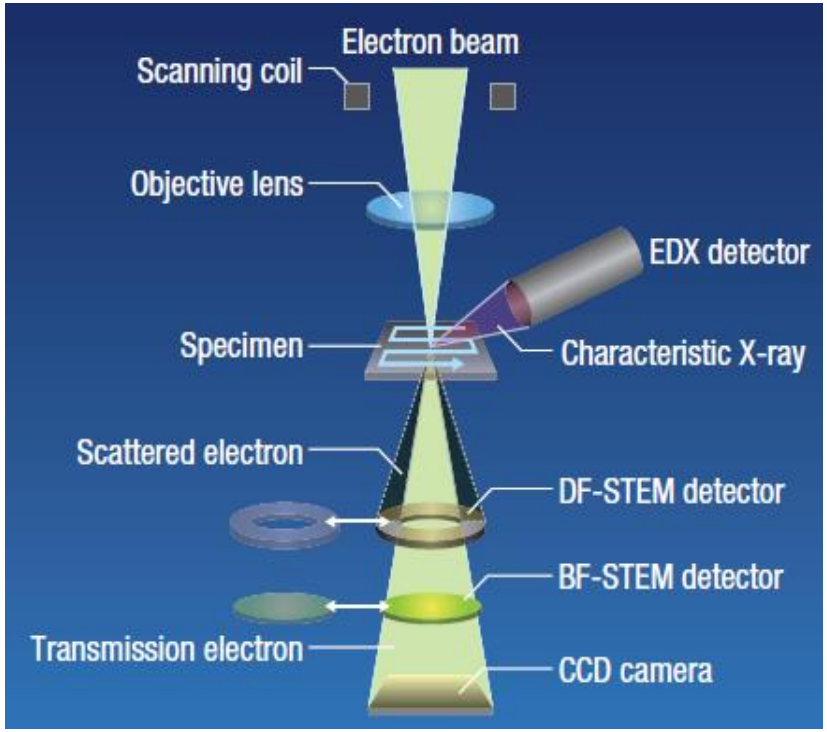


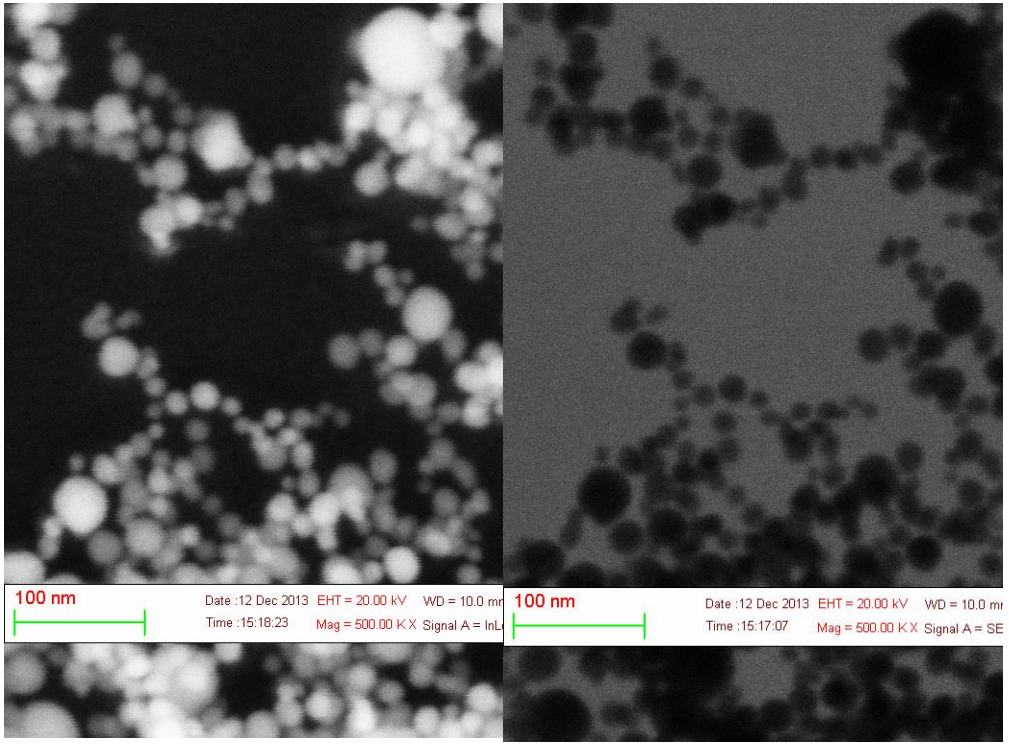


Risoluzione EDS = Risoluzione SEM ?

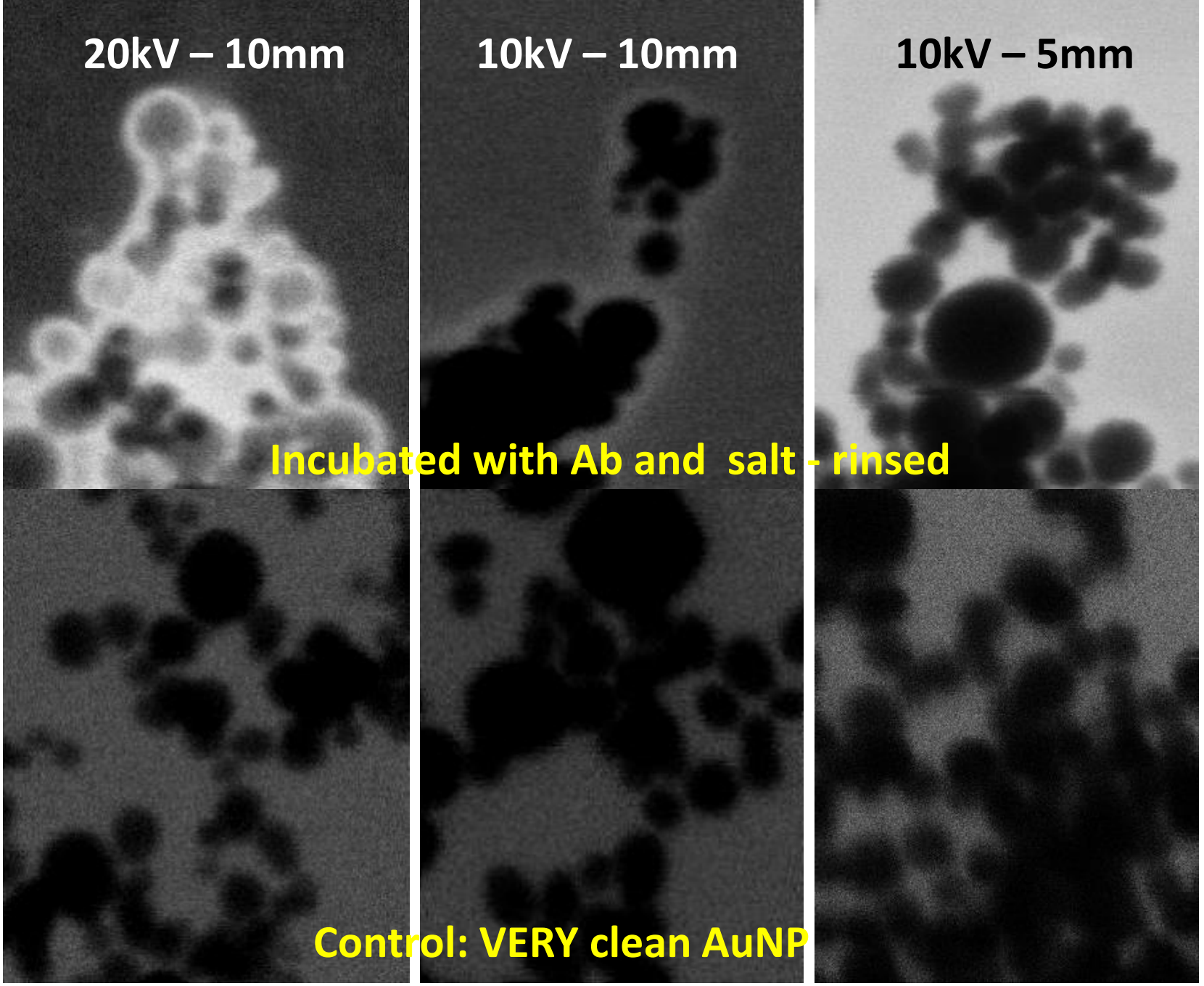


Scanning Transmission EM STEM

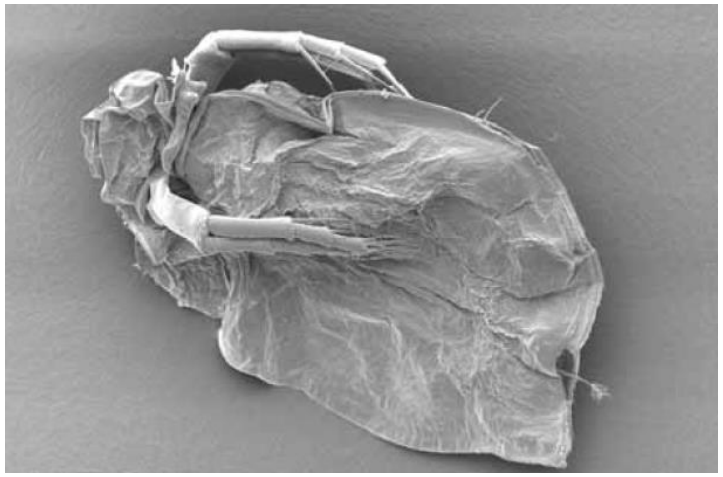




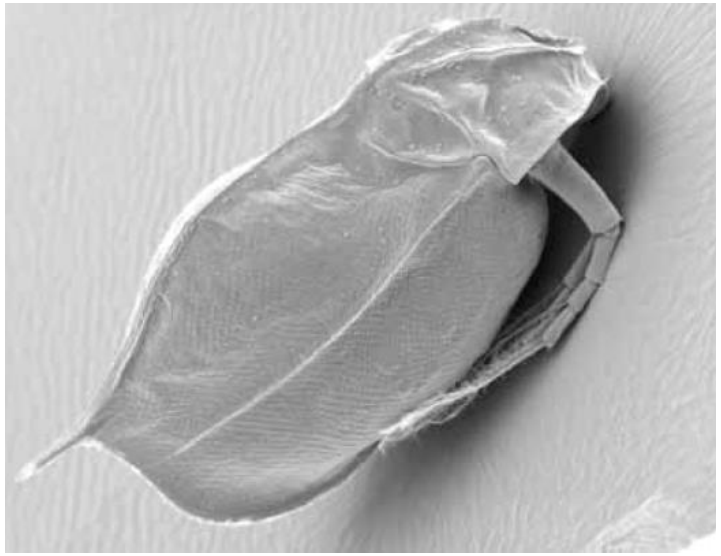
In STEM il fascio di elettroni viene scansionato sul campione e gli elettroni trasmessi vengono raccolti.A seconda della rilevazione di elettroni deviati o non deviati, si possono ottenere diversi contrasti.Il SEM può essere adattato per funzionare come microscopio STEM a bassa E.


20kV – 10mm
10kV – 10mm
10kV – 5mm
Incubated with Ab and salt - rinsed
Control: VERY clean AuNP
Transmitted electrons – STEM mode

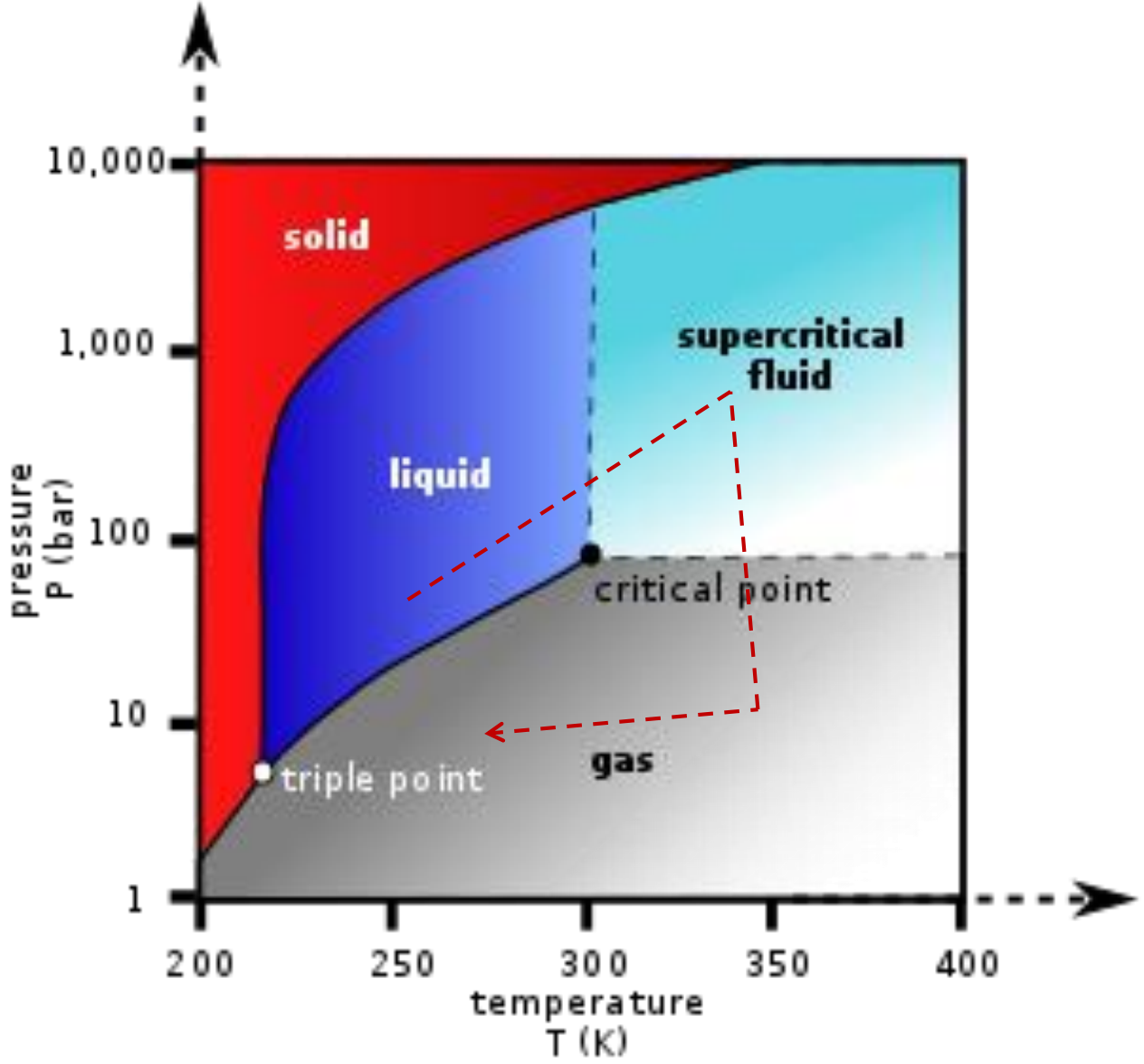
Supercritical drying



Air dried sample (Water flea)



Critical point dried sample (Water flea)



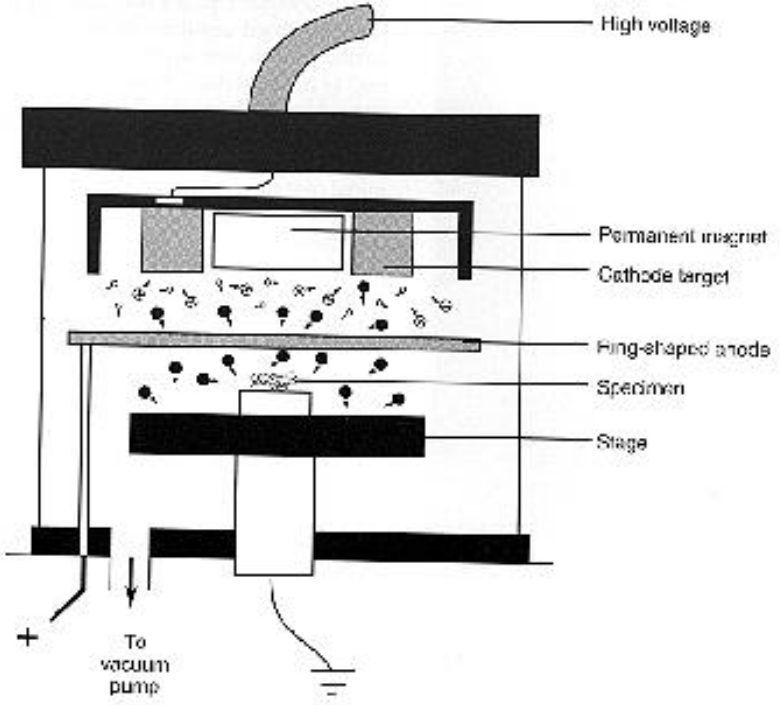
Essenziale per rimuovere l'acqua da campioni biologici senza modificarne la struttura 3D

Sputter coating

Gold, gold palladium target

-vacuum of approx. 2 millibar

-thickness 7.5 nm to 30nm



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

I-2-4 Substitution

① Only propylene oxide (PO) ····· 10 min × 2-3 times
(Change Liquid 2 or 3 times every 20minutes.)

② Mixed-solution of PO and resin

PO : Epoxy resin = 2:1	30 min.
PO : Epoxy resin = 1:1	1 hr.
PO : Epoxy resin = 1:2	1 hr.

③ Only Epoxy resin ····· 2hr. or overnight

I-2-5 Embedding (The freshly mixed resin should be used.)

Epoxy resin
[In the case of TAAB EPON 812 resin]

①EPON 812	48g
②DDSA	19g
③MNA	33g
④DMP-30	2g

Preparation of Epoxy resin

[In the case of TAAB EPON 812 resin]

①Put EPON 812 · DDSA · MNA in beaker.

*The use of disposable syringes and beakers facilitates the post washing.

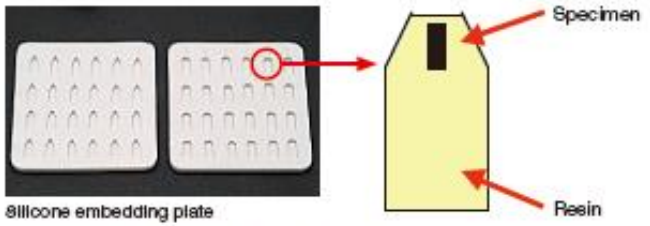
②Mix it well.

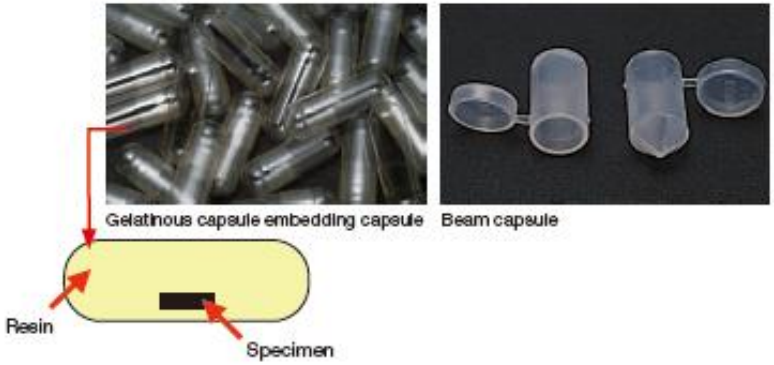
③Add DMP-30 and stir well.

*Since an added volume is small, the use of syringes for tuberculin is recommended.

The specimen is placed in a well of a silicone embedding plate. Resin is poured over the tissue. Ensure no air bubbles are trapped within the resin.

*For a specimen with various orientations, use a silicone embedding plate.



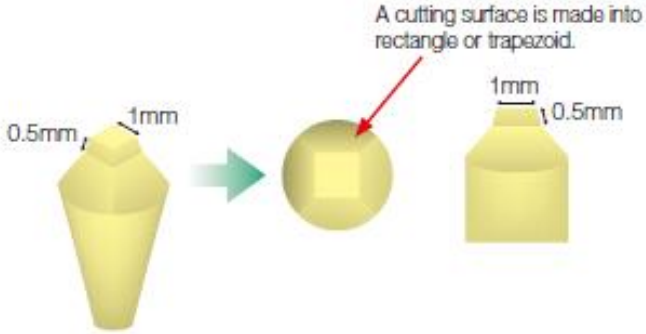


I－3 Steps in sectioning with an ultramicrotome

I-3-1 Trimming

Expose the tissue.

Trim the tip using a razor to form a pyramidal shape.



Examples of trimming

Square　Rectangle　Trapezoid

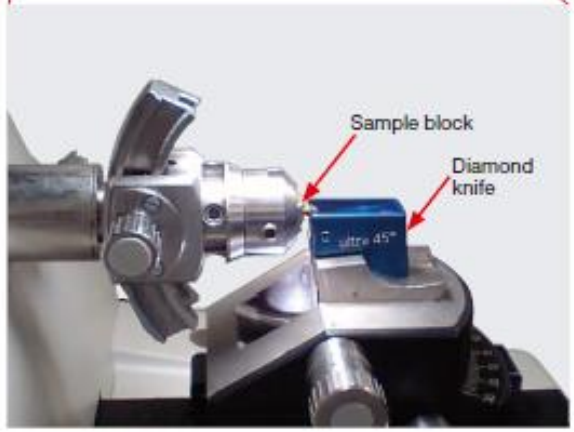
① Square : Ideal for when ultra-low magnification images are needed.
② Rectangle : Suitable when sequential sections are cut and when the block contains both hard and soft tissue.
③ Trapezoid : Ideal for keeping track of the order of sections in a ribbon.

I－3－2 Sectioning

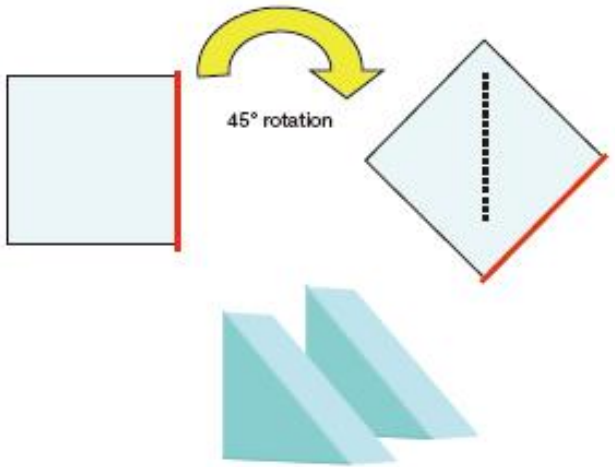
① Setting a specimen

A sample block and a knife are set on a microtome.

Ultramicrotome
(Leica EM UC6)



3) Place a 25 mm square glass block in the knifemaker with the fresh cut to the right.
4) Score the block along a diagonal line and by applying pressure fracture the glass along the score line.



Two knifes from one 25 mm square block

⑦ Diamond knife

Typical diamond knives

Ultra
Used in room temperature.
For thin sectioning.

Cryo dry
Used in low temperature.
For thin sectioning

Ultratrim
For preparation of resin block

② Making of ultrathin sections

Sectioned thin specimens float and are flattened on water in the knife boat.

A) The sections floated in the surface.

B) A actual section

③ Thickness of ultrathin section

The thickness of thin specimens may vary depending on the hardness of resin and room temperature.

It is necessary to judge the thickness by color of interference.

The interference color and thickness of an ultrathin section

The optimal thickness	Thickness
Gray	< 60 nm
Silver	60 – 90 nm
Gold	90 – 150 nm
Purple	150 – 190 nm
Blue	190 – 240 nm
Green	240 – 280 nm
Yellow	280 – 320 nm

The optimal thickness for observation in a 120 kV TEM.

④ Picking up sections

Pick up thin specimens on water using a TEM grid.

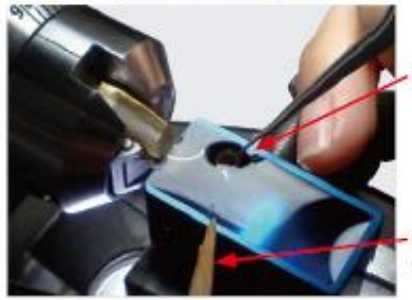
A) Press Method

Place a grid on a thin specimen with a support film side down.

Advantage : Simple and easy
Disadvantage : Thin specimen may be wrinkled
Thin specimens may be overlaid

B) Pull up Method

Place a TEM grid under thin specimens with a support film on the top.
Bring thin specimens on the grid by using an eyelash probe.

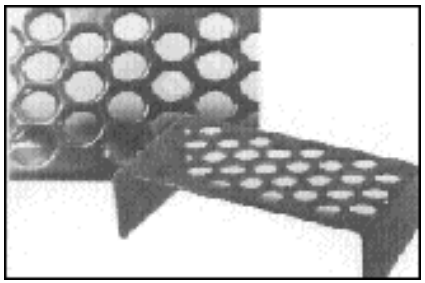


Advantage : No wrinkles on specimens
You can place specimens as you desire
Disadvantage : Skill needed

Section Mounting

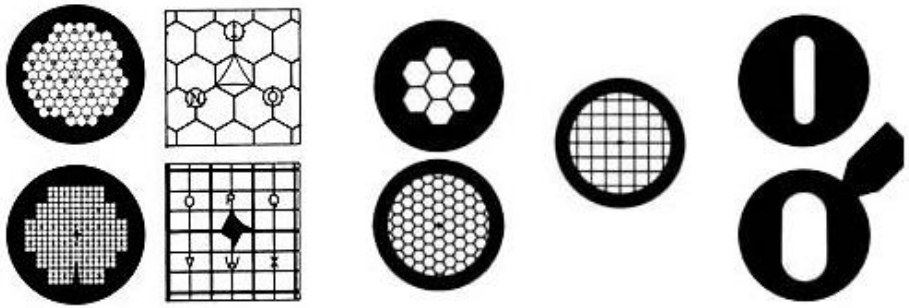
- **A 200m grid has 60% open area; a 400m grid only 40%**

- **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:**



Embedding and Sectioning

Section Thickness

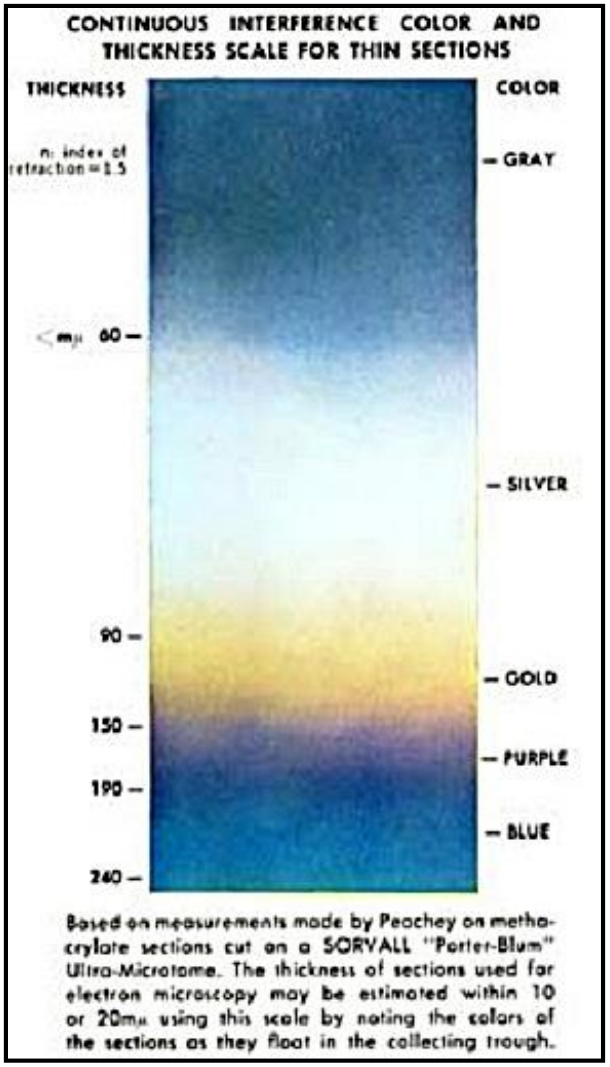
•Ideally, sections should be in the 55 - 60 nm range.

•This allows for enough stain uptake for contrast, and maximum resolution (limited in the TEM by specimen-induced chromatic aberration).

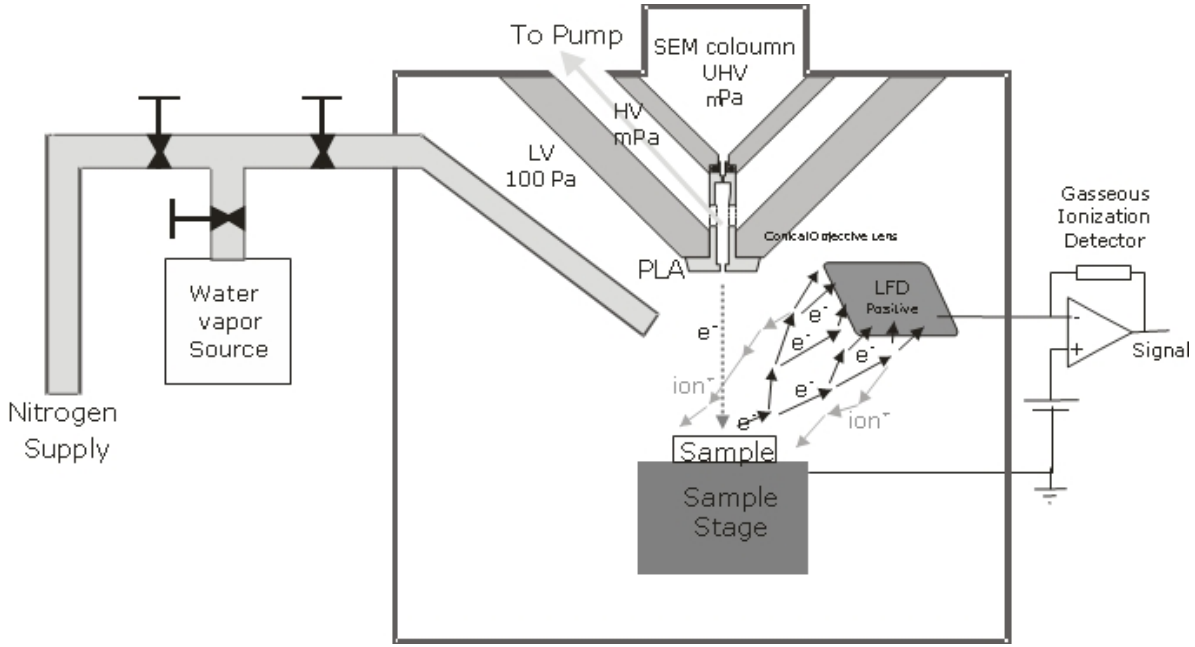
•Determined by interference colors.

•Maximum thickness should not exceed 85 - 90 nm (light gold).

•Thickness can sometimes be reduced by one color range by flattening sections - smooths out compression to a limited extent. Toluene, xylene, chloroform, heat**.**



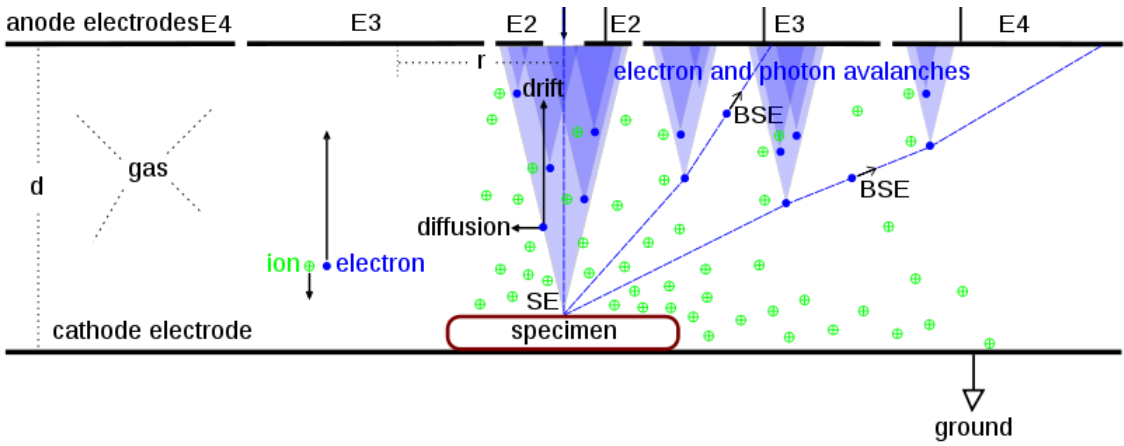
ESEM



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.

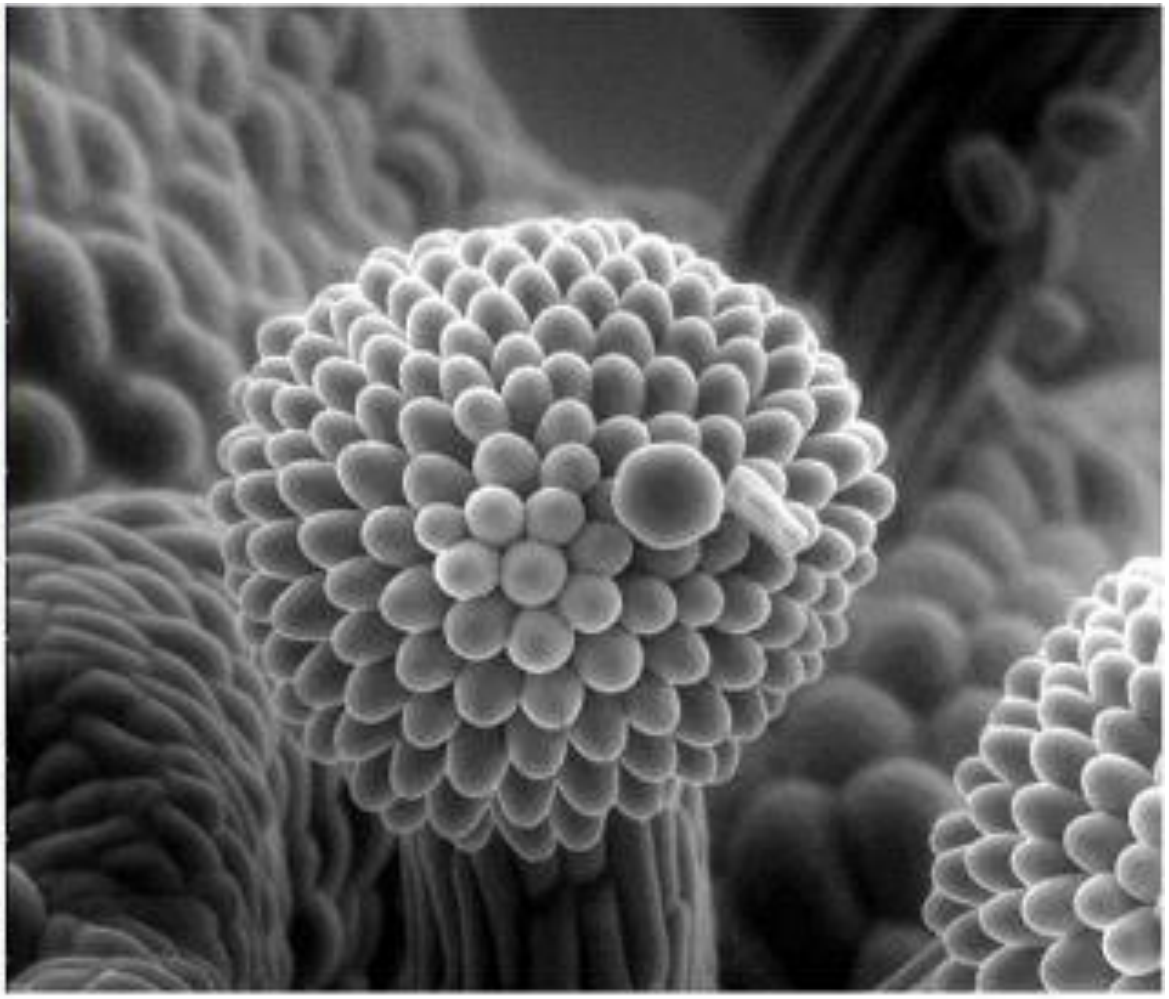
Main differences:

- differential pumping with sample pressure at tens of mbar
- electron detection (not with a ET detector because of HV), Instead gas ionization is used to monitor electron production
- sample cooling to condense vapor and keep the water content in equilibrium



Gaseous Detection Device (GDD) - principle

ESEM



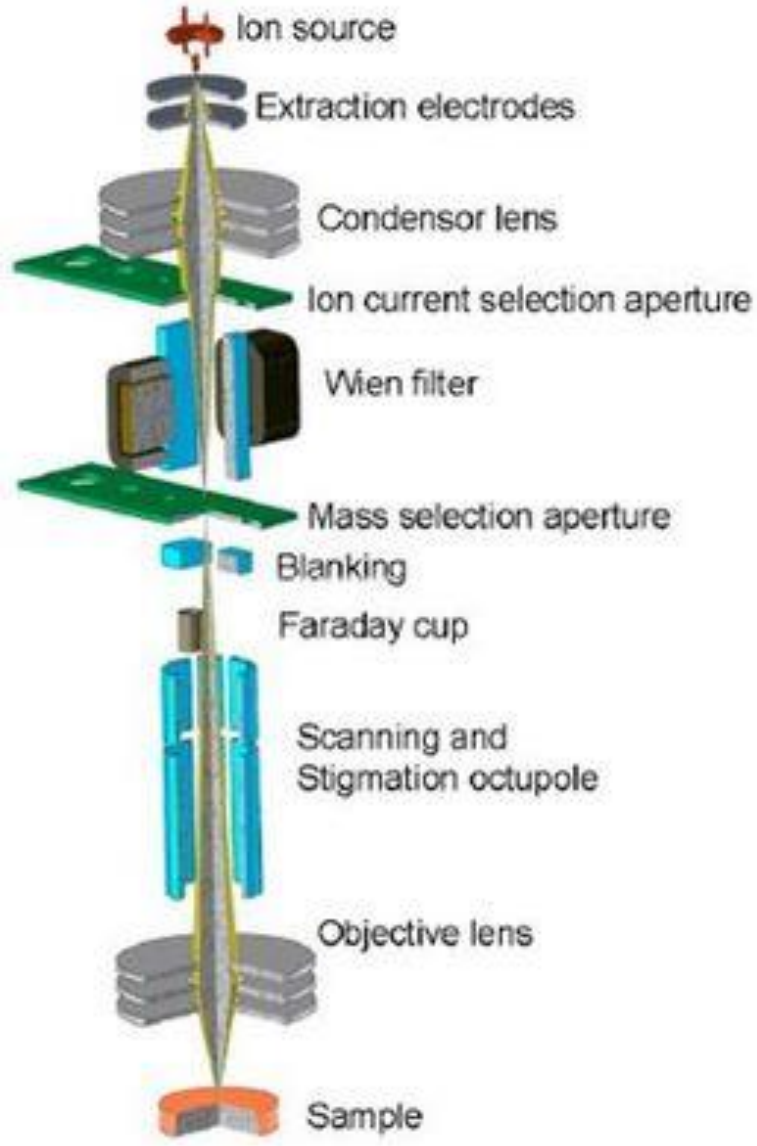
FIB

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.

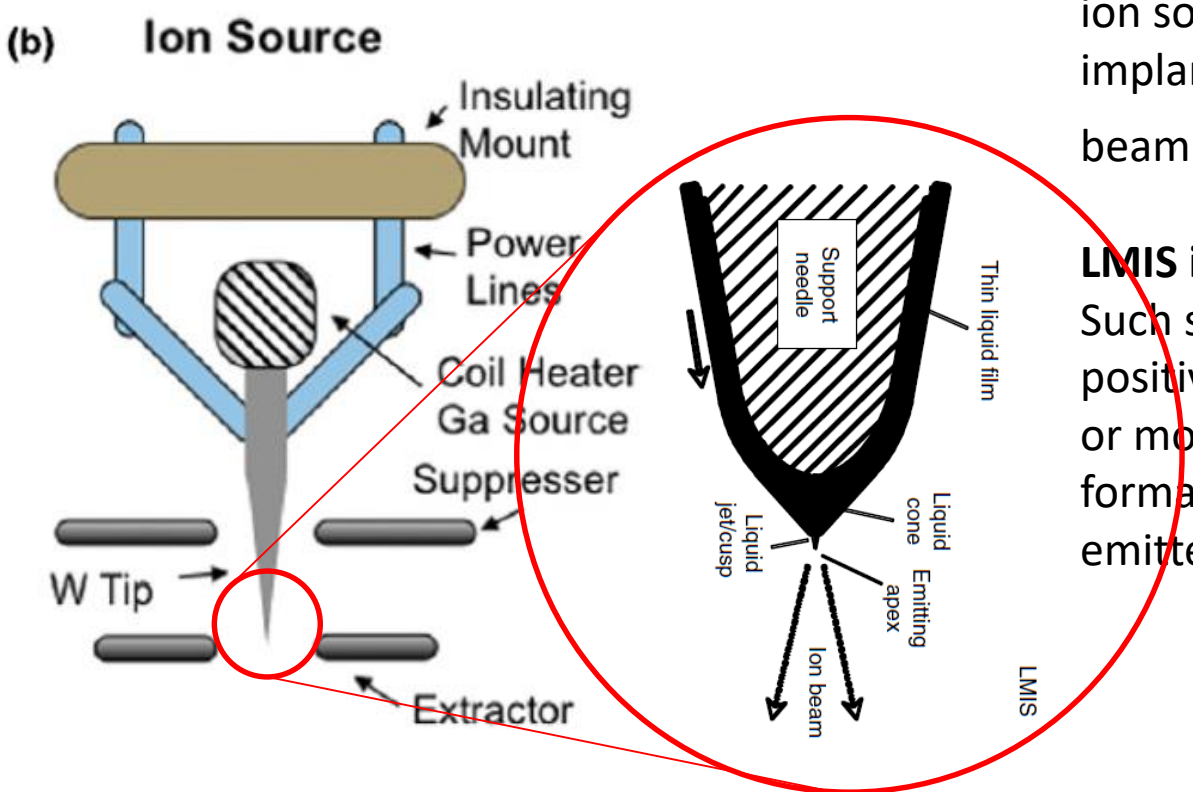
The image is produced by contrast due to differences in elements, Z contrast, crystallographic orientation, channeling contrast, or topography.

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.



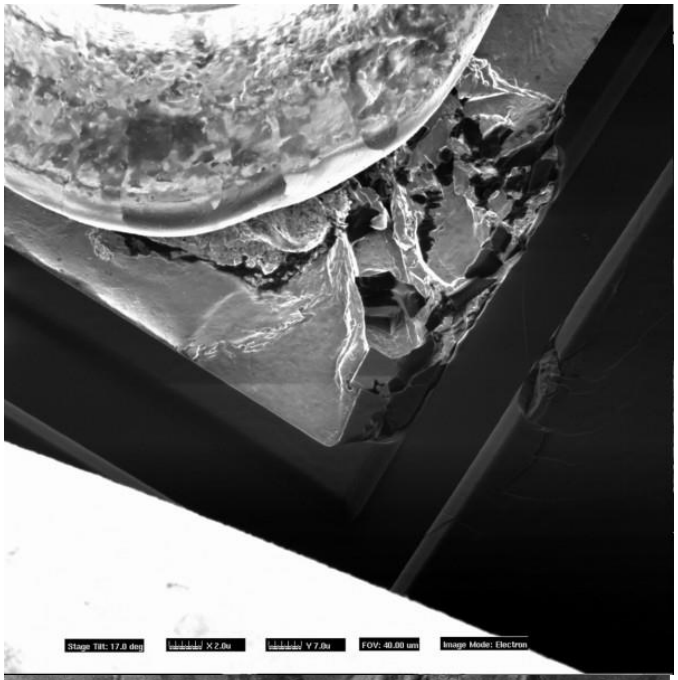
FIB

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.

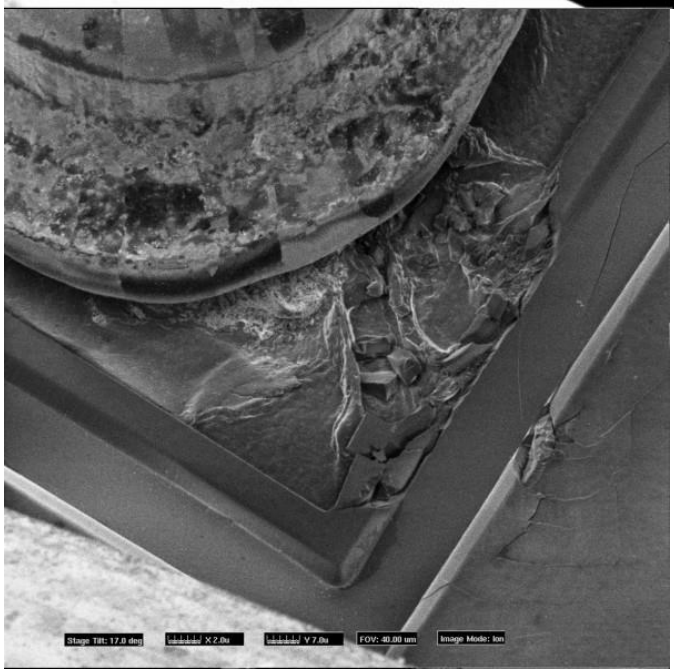


LMIS is a field ion emission source. Such sources generate high–brightness positive ion beams from neutral atoms or molecules, by field–induced ion formation at the tip of a needlelike emitter.

FIB secondary **ion** mode, which reveals additional detail in the dielectrics.



FIB secondary **electron** image of mechanical damage to a bond pad.



FIB - imaging

Ga+ primary ion beam sputters a small amount of material, which leaves the surface as either secondary ions (i+ or i-) or neutral atoms (n0).

The primary beam also produces secondary electrons (e-).

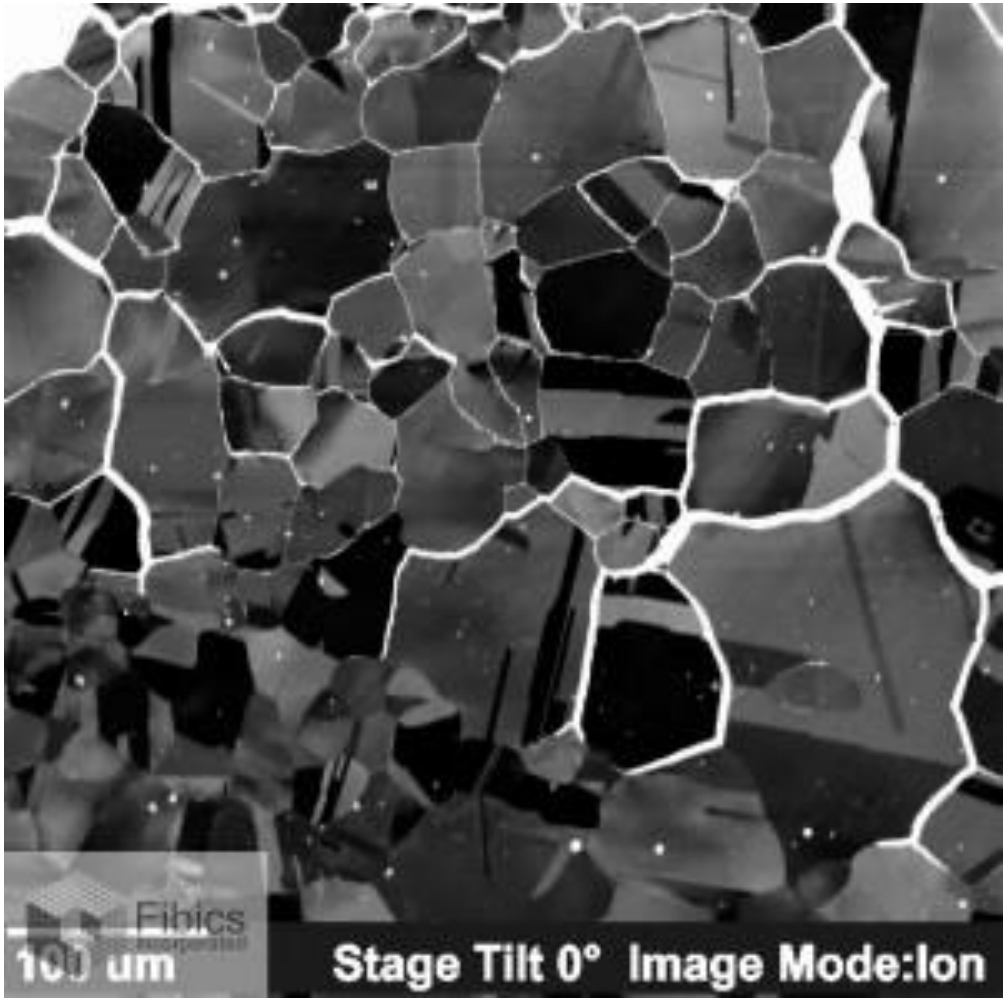
As the primary beam rasters on the sample surface, the signal from the sputtered ions or secondary electrons is collected to form an image.

Modern FIB systems, can achieve under 1 nm imaging resolution.

If the sample is non-conductive, a low energy electron flood gun can be used to provide charge neutralization

positive secondary ions image
+ low energy electron flood gun
= highly insulating samples imaging
- A conducting surface coating,

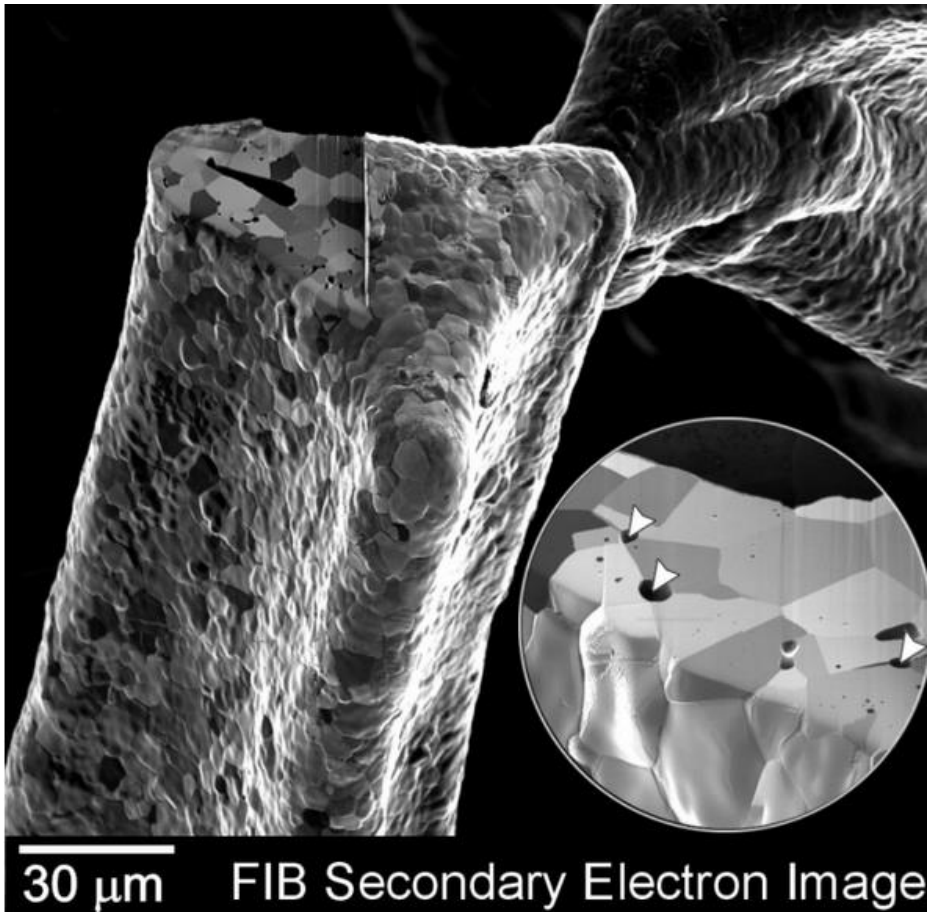
FIB imaging

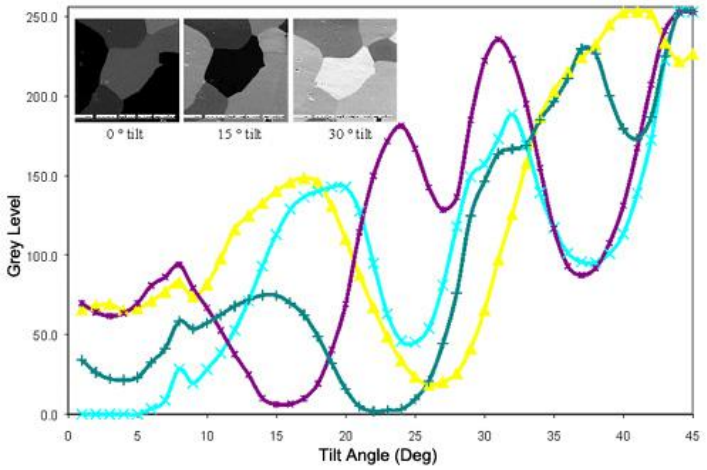


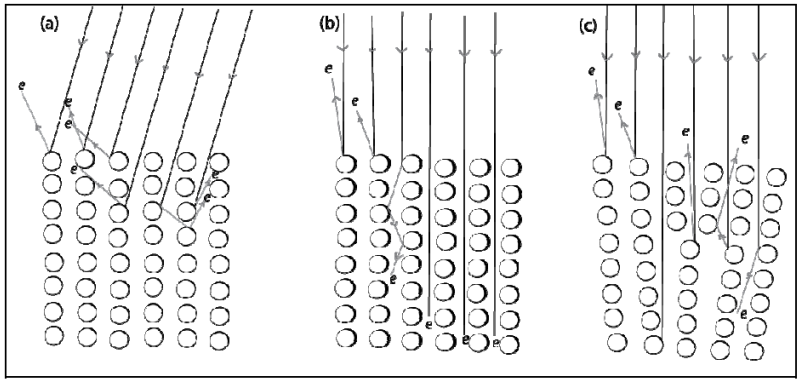
secondary ion yield of a metal is greatly enhanced in in the presence of oxygen.

The grain boundaries in this Nickel samples area corroded (oxidized) nd provide a white contrast

FIB – channeling contrast

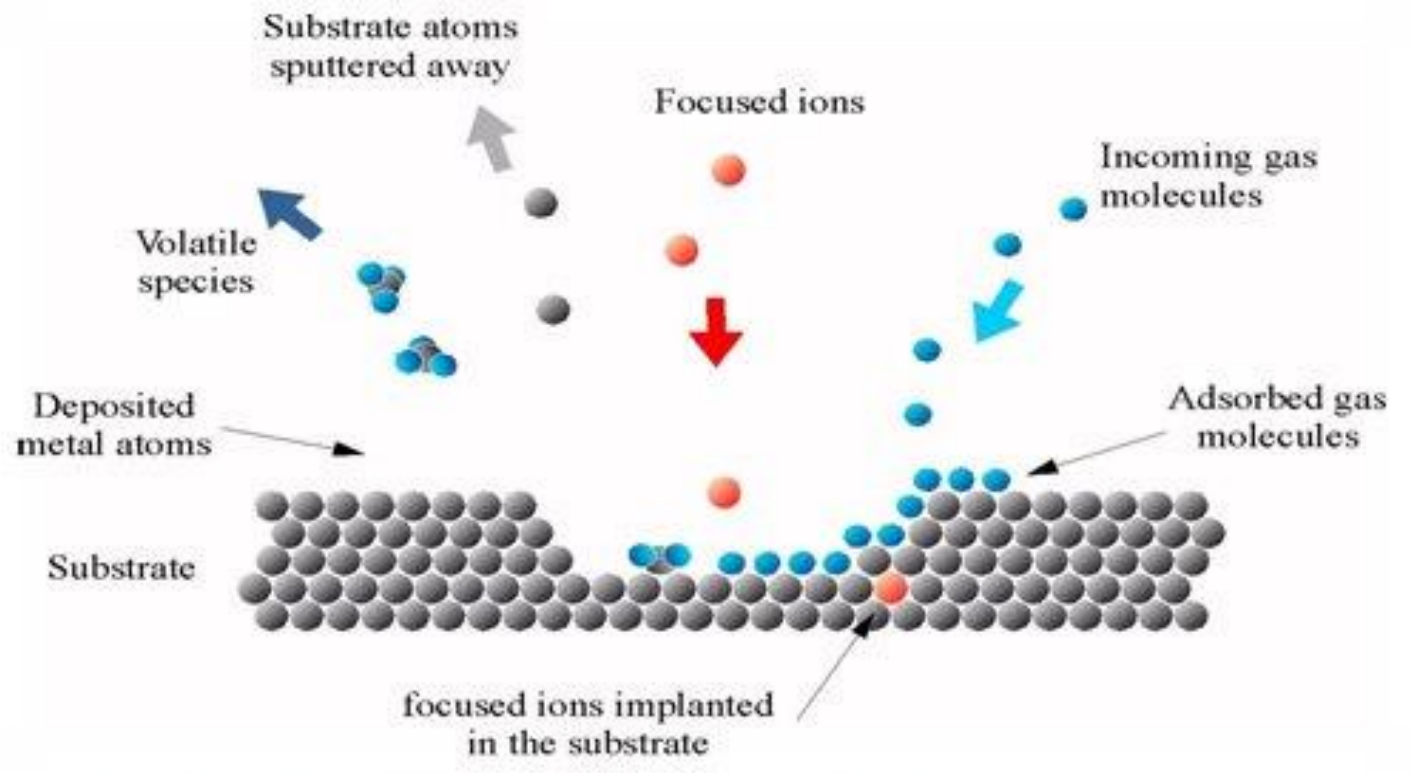






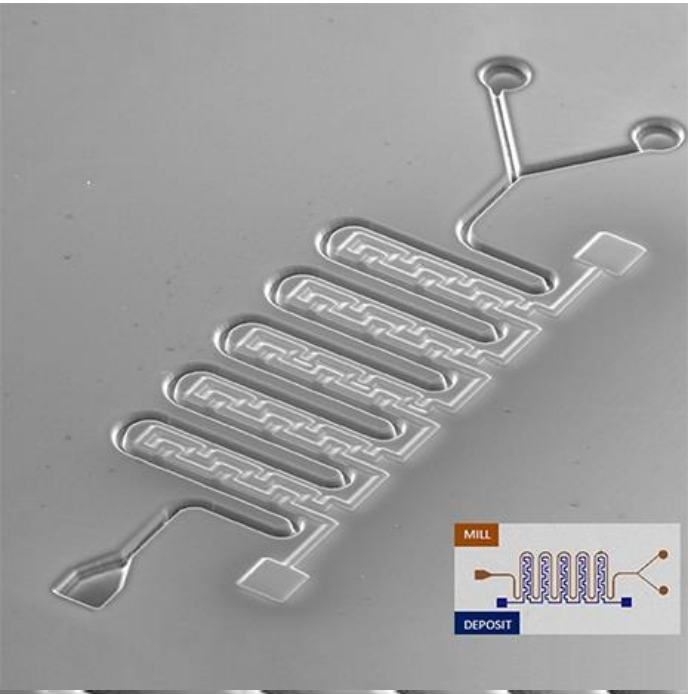
Channeling contrast induced by FIB is 4 times more intense than that produced by backscattered electrons in the SEM, and results in spectacular grain contrast, as can easily be seen in the FIB image of aluminum grains on this page

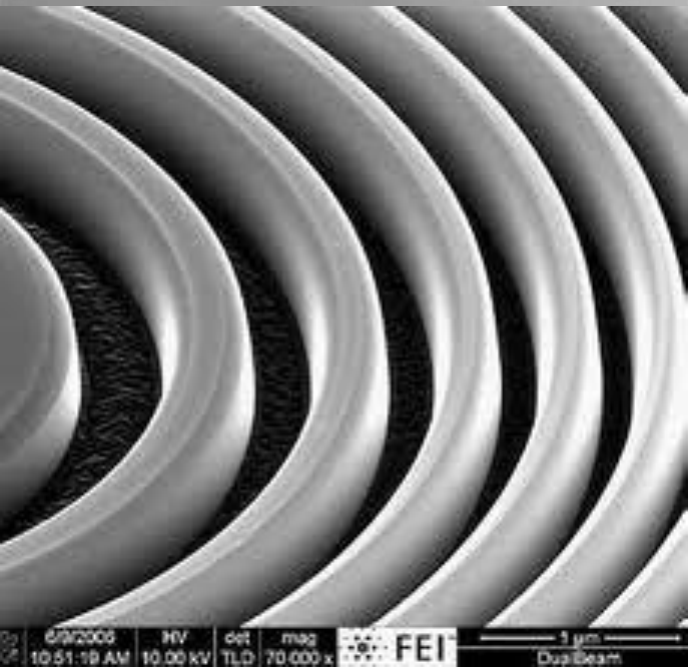
FIB - milling

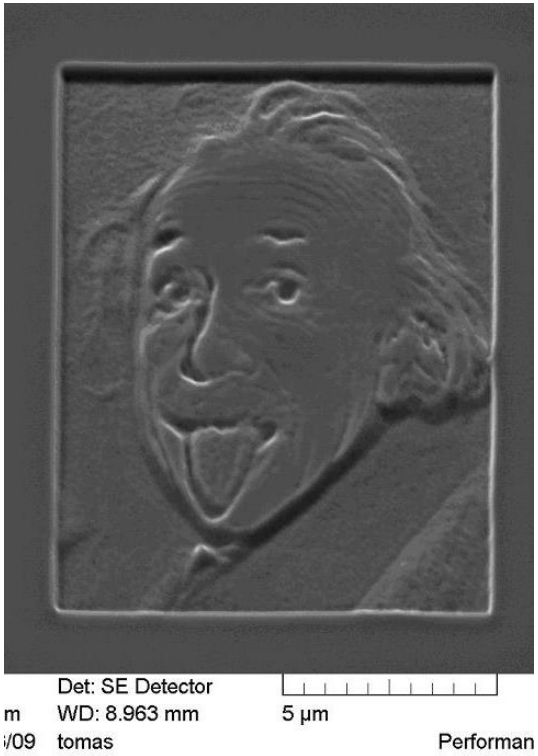


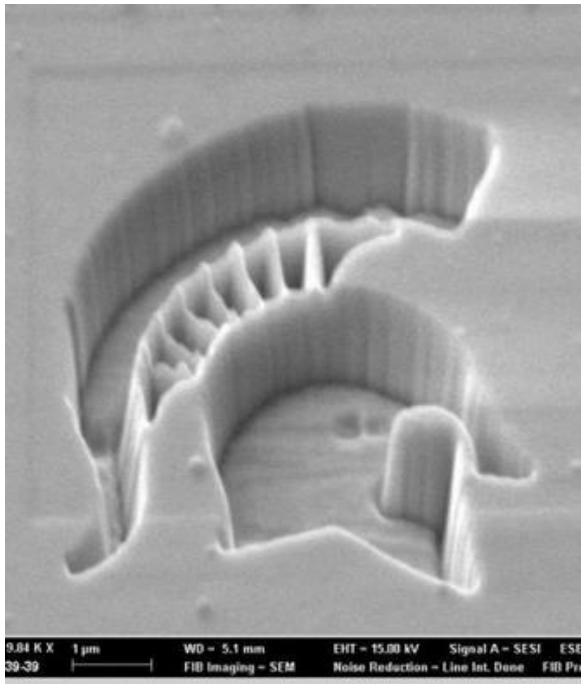
1. Adsorption of the gas molecules on the substrate
2. Interaction of the gas molecules with the substrate
   Formation of volatile and non volatile species
3. Evaporation of volatile species and sputtering of non volatile species

FIB - milling



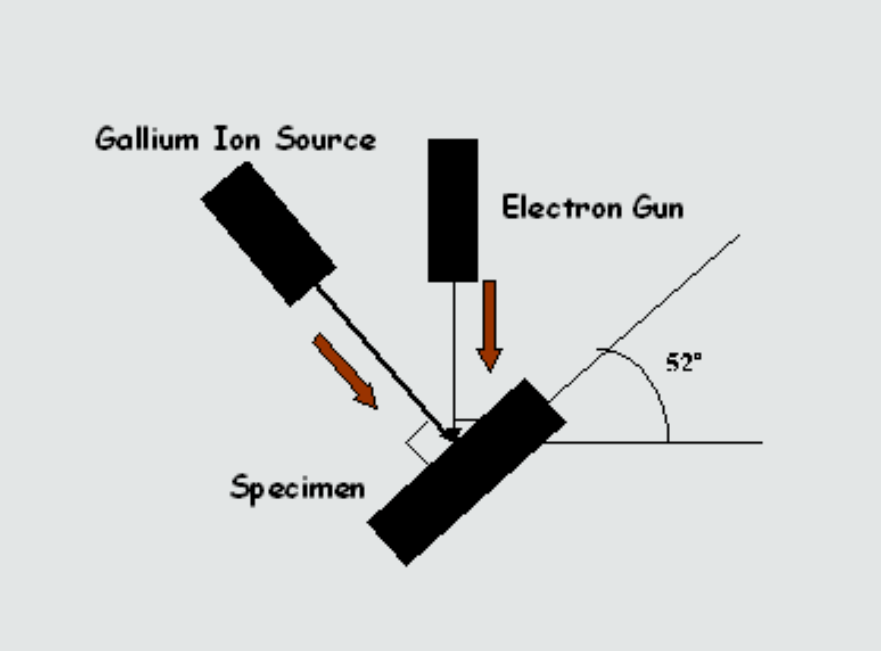


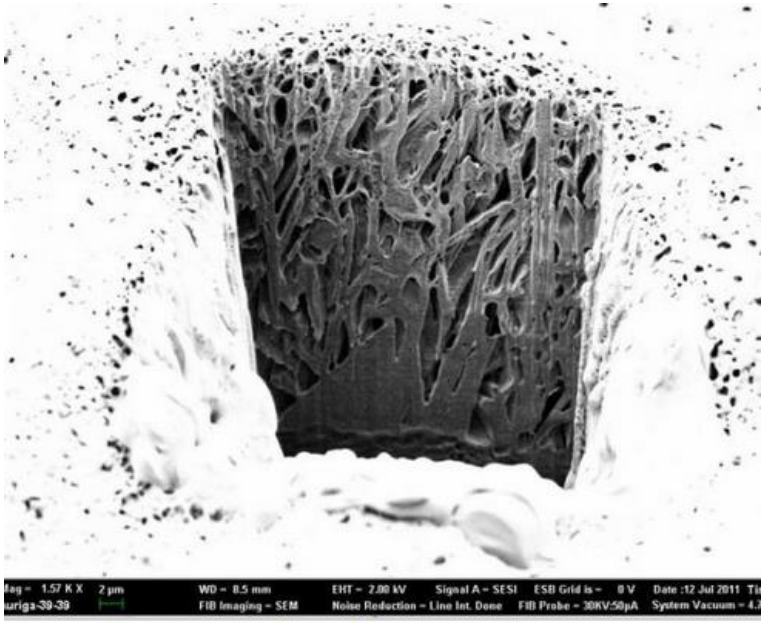




Useful (a microfluidic circuit or a zone plate)
and less useful features
can be produced

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…

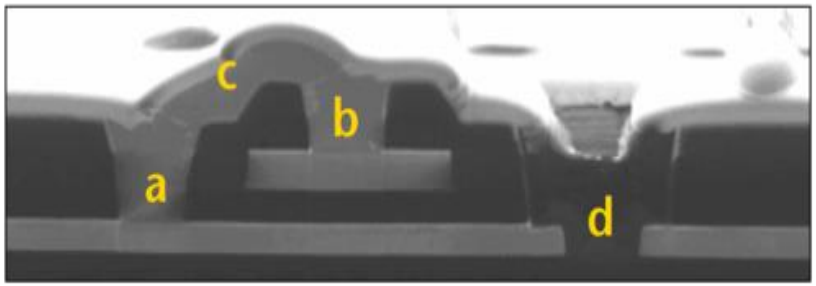
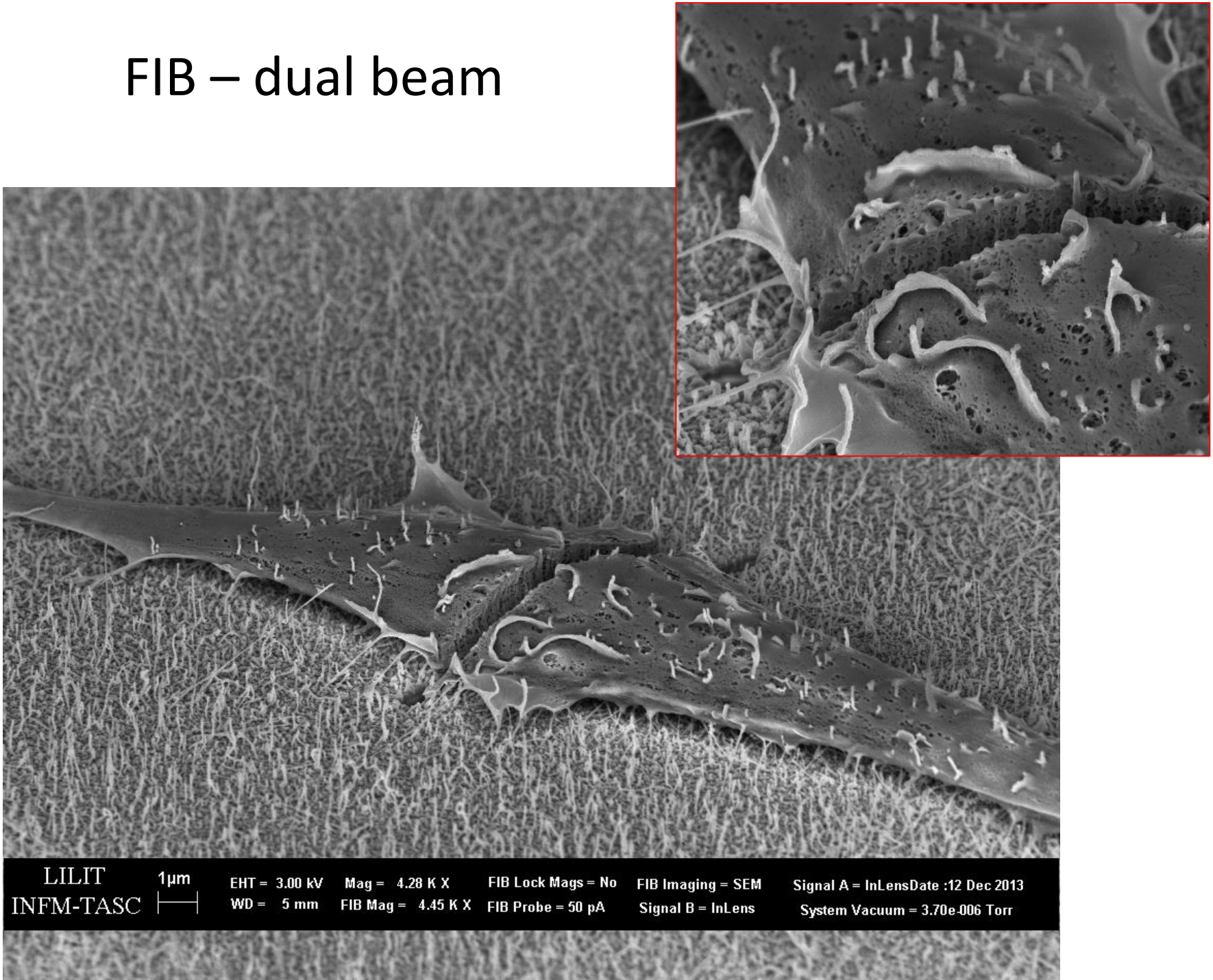
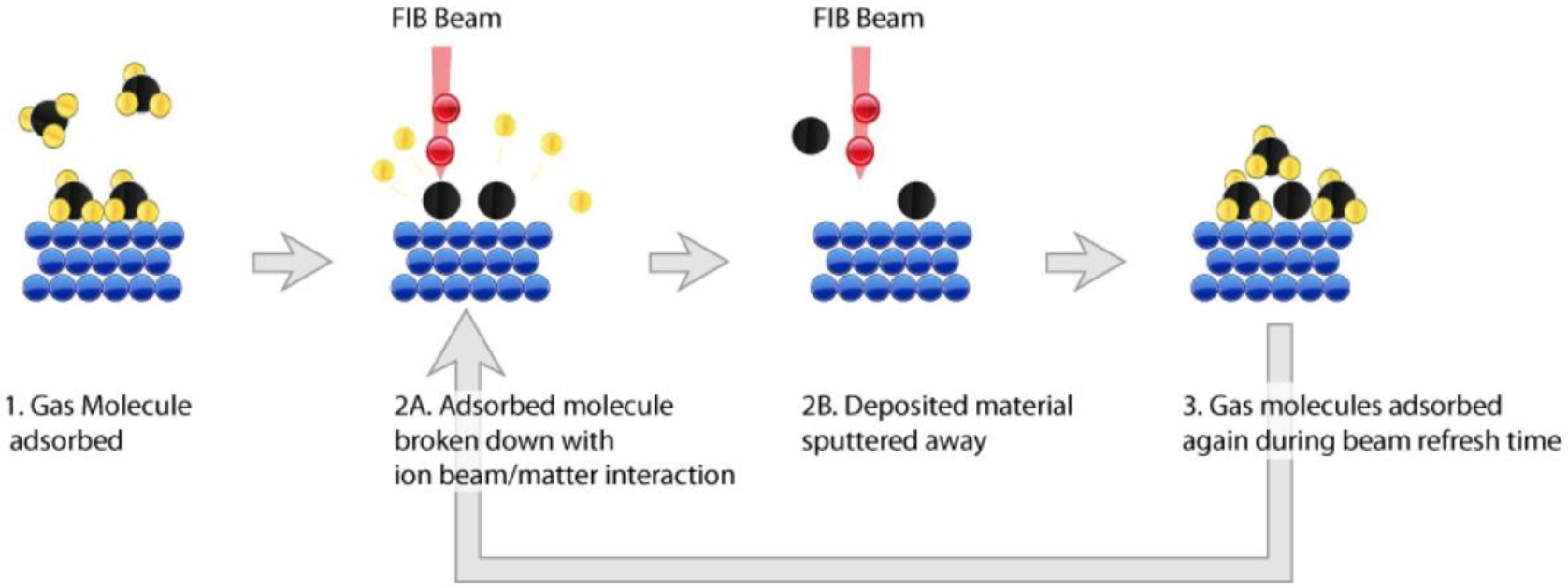


Figure 2 Cross-section view of the edit shown in figure 1 on a two metal layer IC

FIB – dual beam

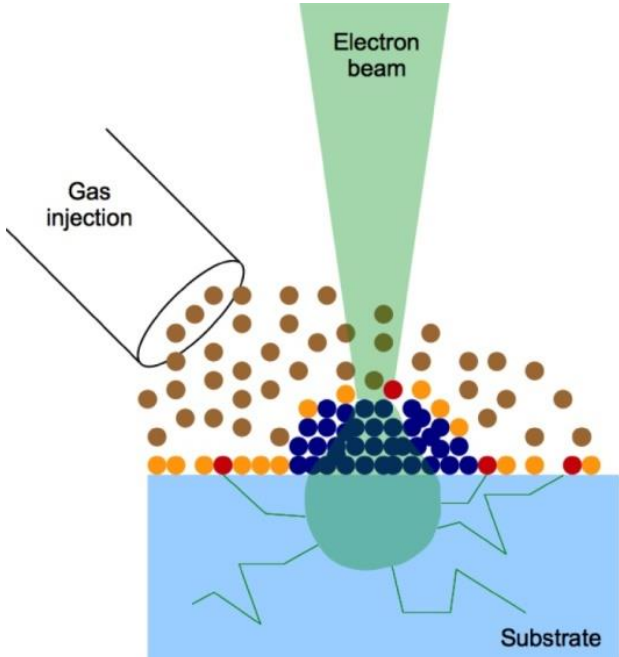


FIB induced deposition



if a gas is introduced in the vicinity of the ion beam impact point: FIB induced deposition (FID) occurs.

The gas is introduced by a nozzle which is positioned a few hundreds of microns above the area of interest. The gas is then adsorbed on the surface of the material. When the FIB beam hits the surface, secondary electrons with energy ranging from a few eV to a few hundreds of eV are generated. These secondary electrons will break chemical bounds of the adsorbed gas molecules which will separate into different components: some of which remains volatile, others will form a deposition on the surface

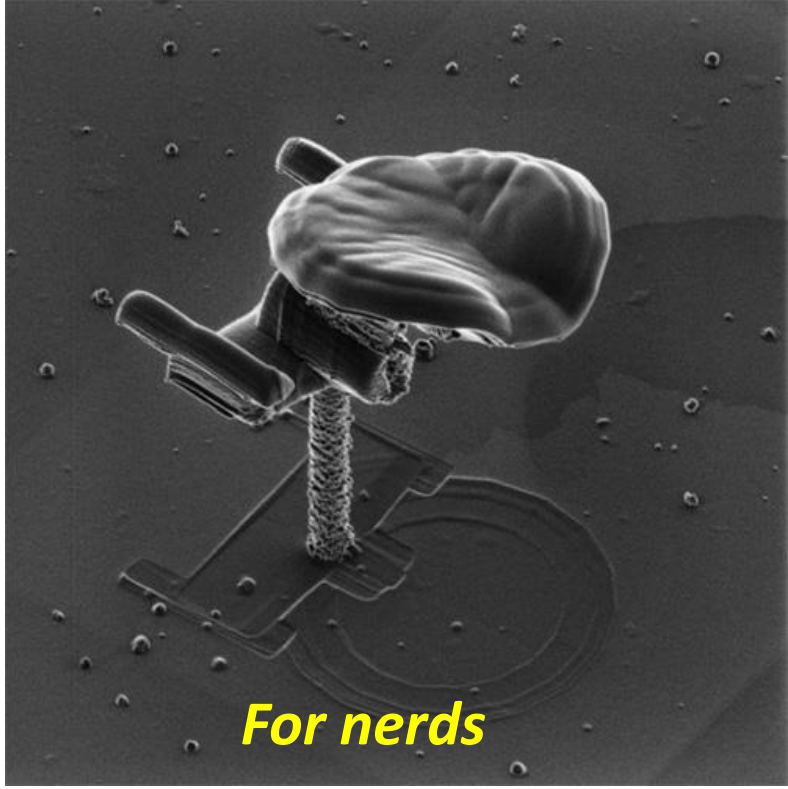


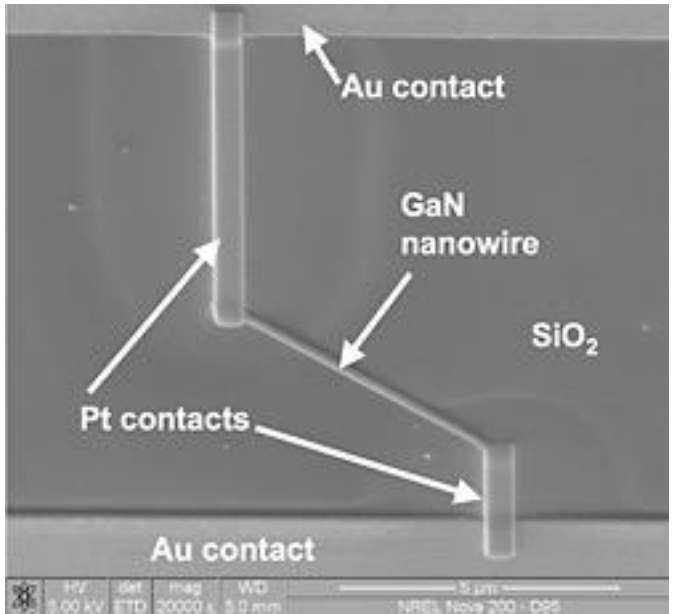
FIB induced deposition

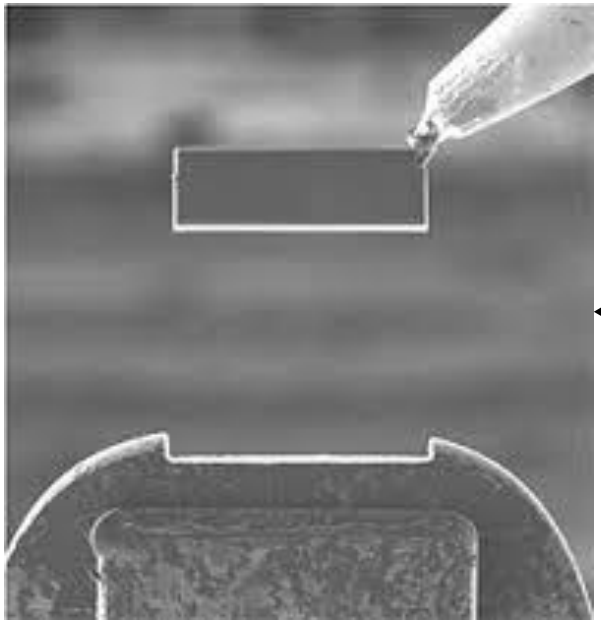
Common precursors are:

- W -Tungsten Carboxyl, $W(CO)_6$
- Al -Trimethyl Al (TMA) $Al(CH_3)_3$
- C - Naphtalene ($C_{10}H_8$)
- Fe – Iron pentacabonyl $Fe(CO)_5$
- Pt – $C_6H_{16}Pt$

(methyl cyclopetandienyl) trimethyl Pt

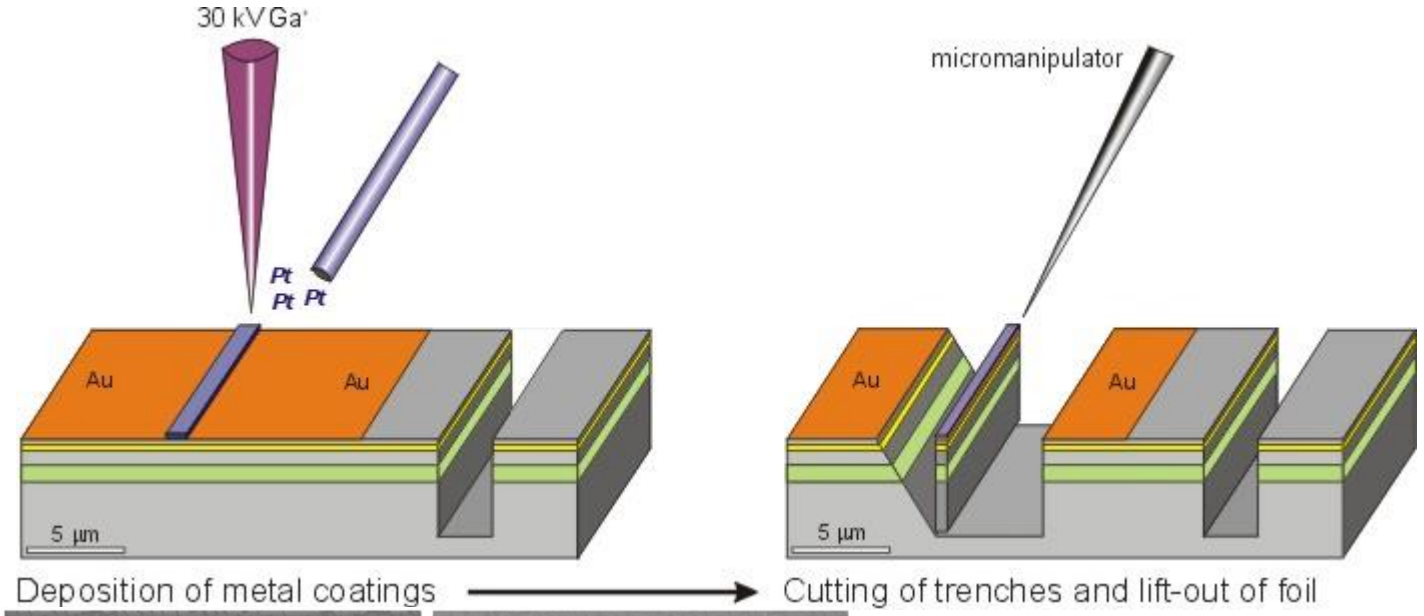


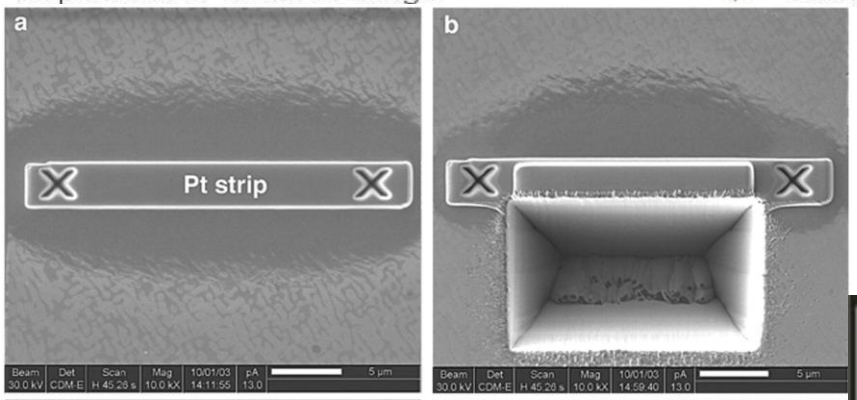


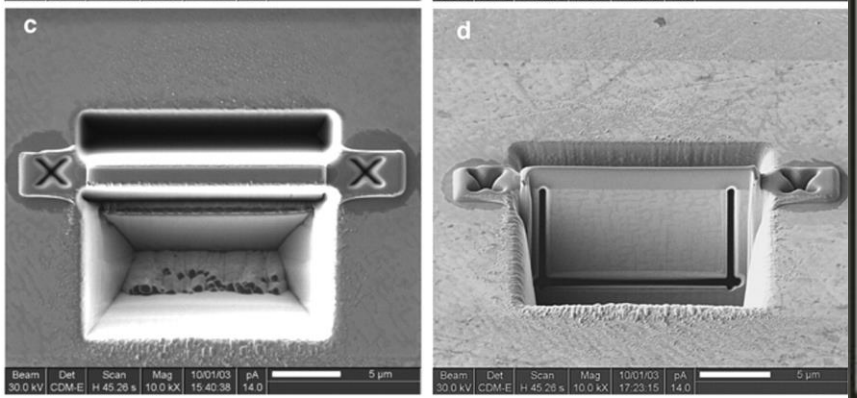


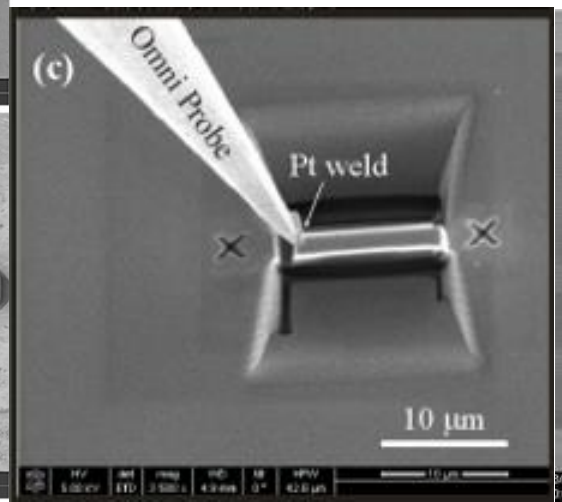
Or useful

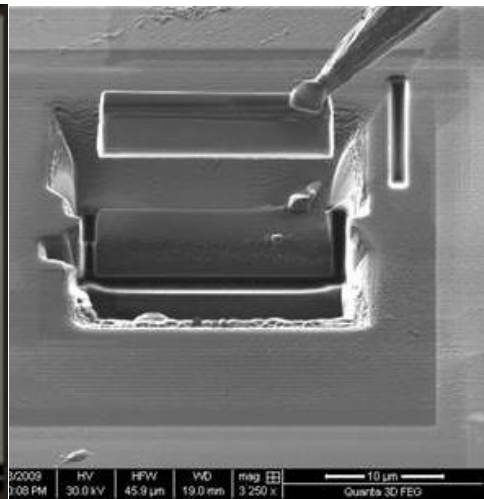
FIB – TEM preparation



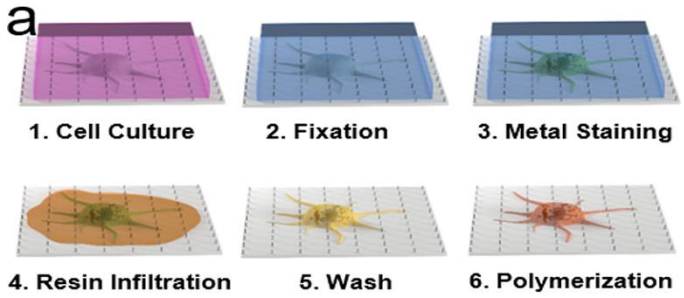


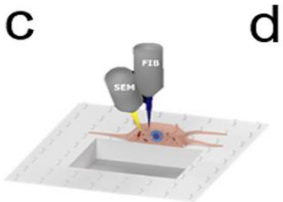


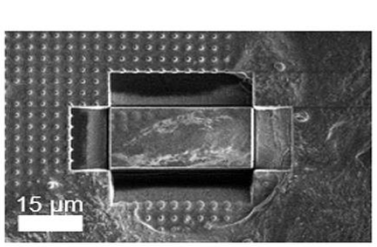


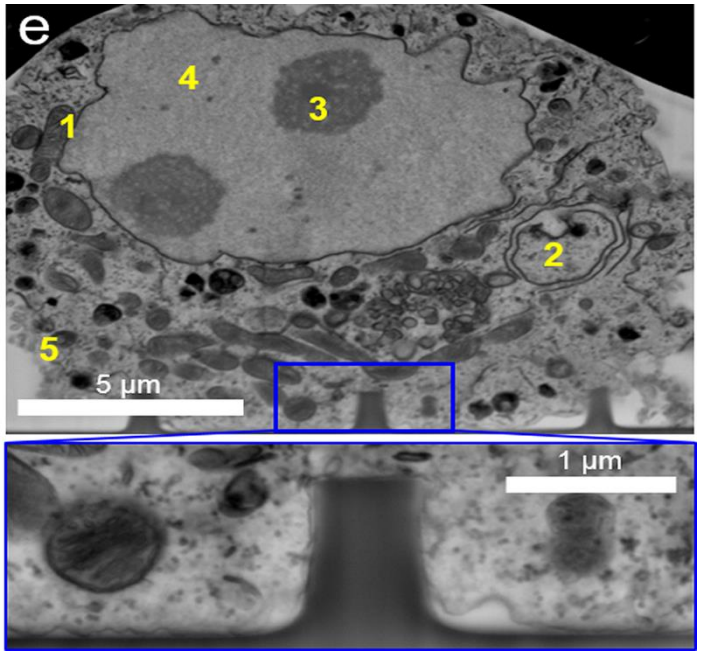


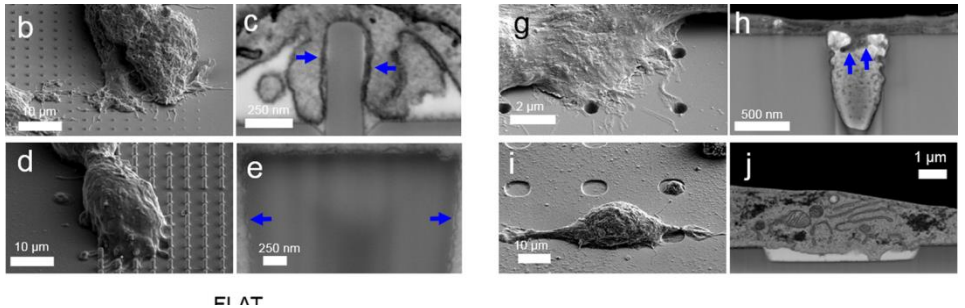
FIB – cross section on fixed cells





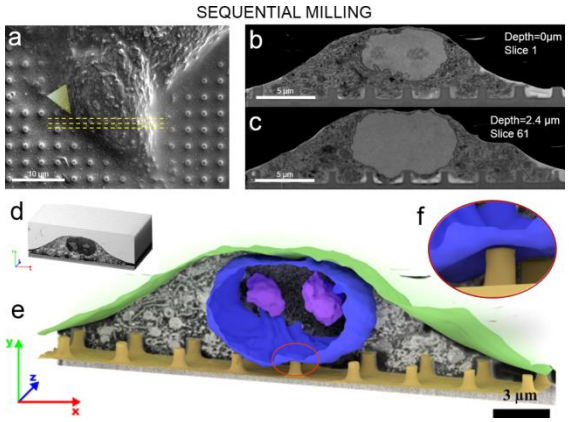






Cell membrane readily deforms inward and wraps around protruding structures, but hardly deforms outward to contour invaginating structures.

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

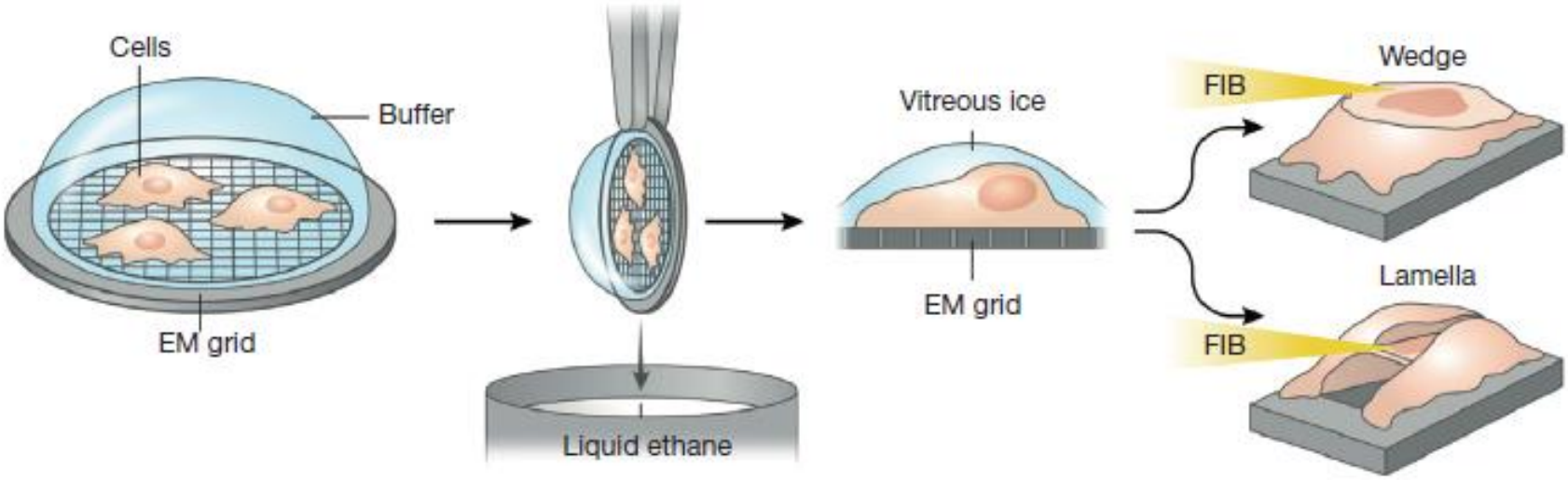


Also the nuclear envelope is deformed upward by a nanopillar

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 - example

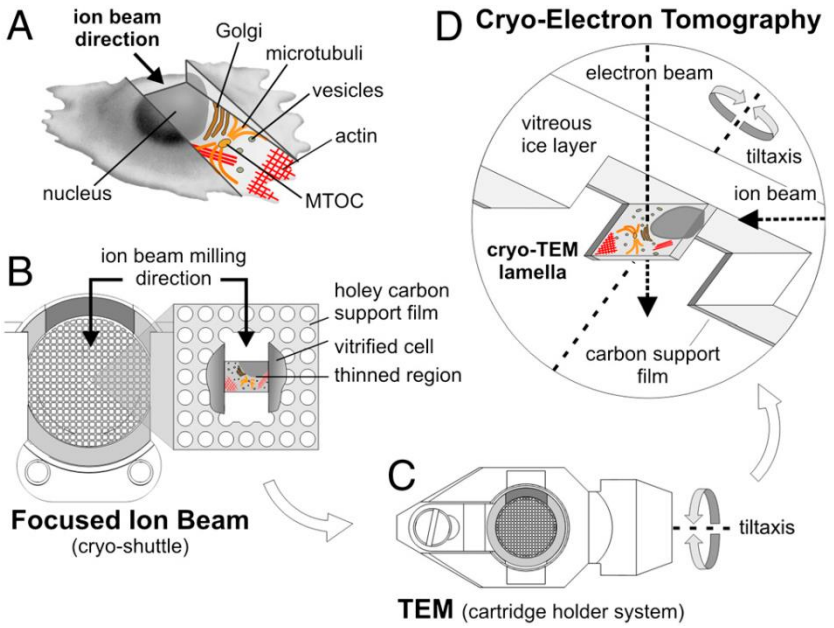
It has been known for several decades that fixing a biological sample in vitreous ice preserves it in a near-native state83. Still, there are limits on the thickness of a sample to be imaged by TEM, and this has restricted microscopy at cryogenic temperatures to studies

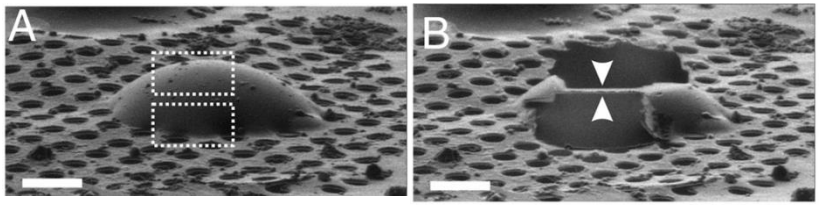


FIB operations, however, perform well under cryogenic conditions, and different groups have exploited this to generate TEM-ready lamellae from thick biological samples using various approaches

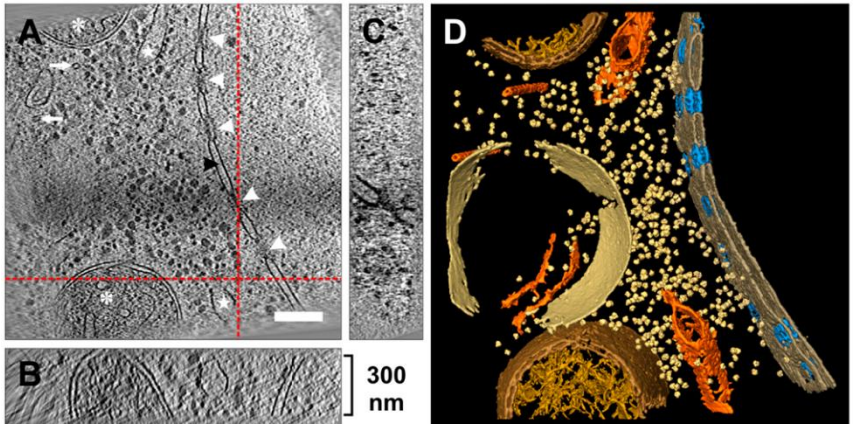
Focused ion beam micromachining of eukaryotic cells for cryoelectron tomography

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

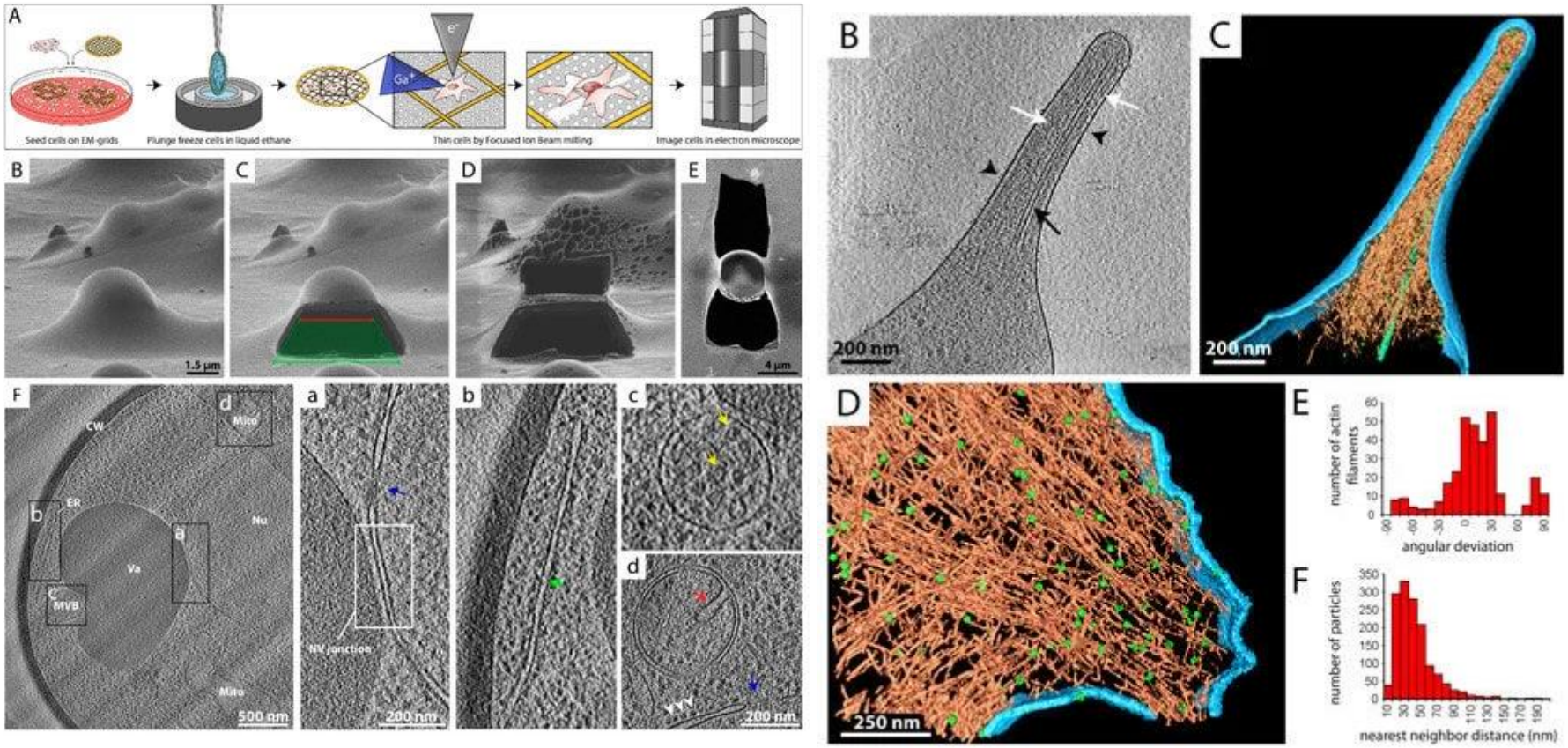
cells

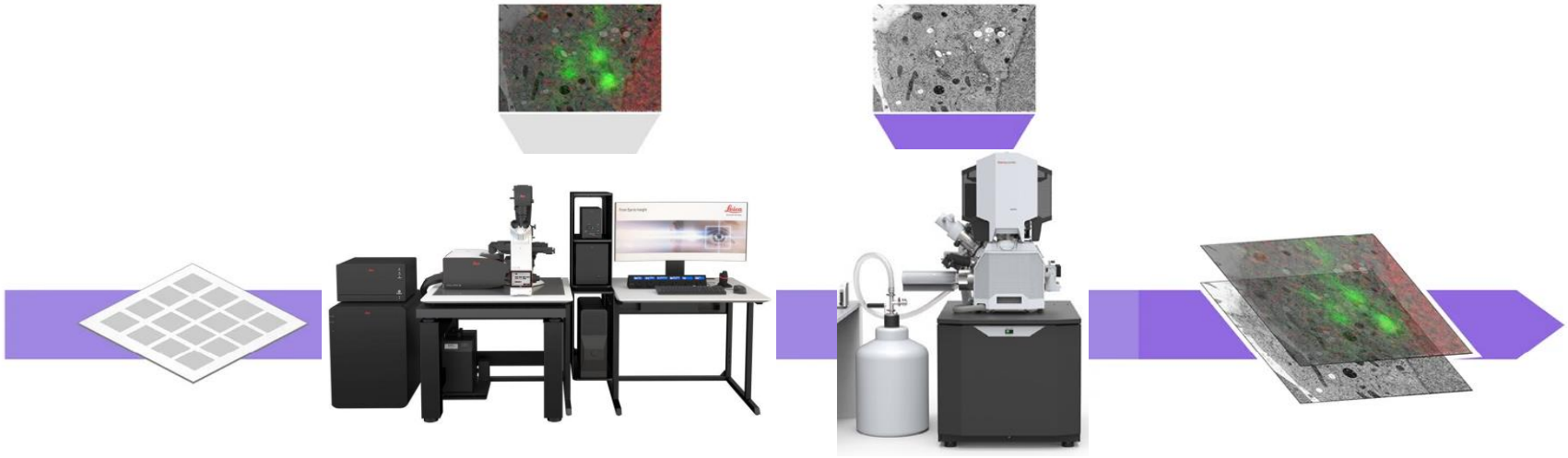
MDPI

Review

Cellular and Structural Studies of Eukaryotic Cells by Cryo-Electron Tomography

Miriam Sarah Weber [1], Matthias Wojtynek [1,2] and Ohad Medalia [1,3,*]



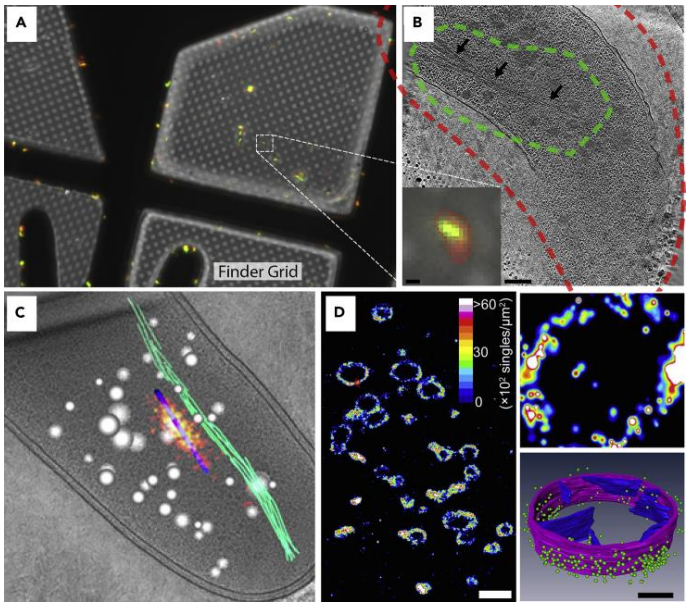


Sample preparation

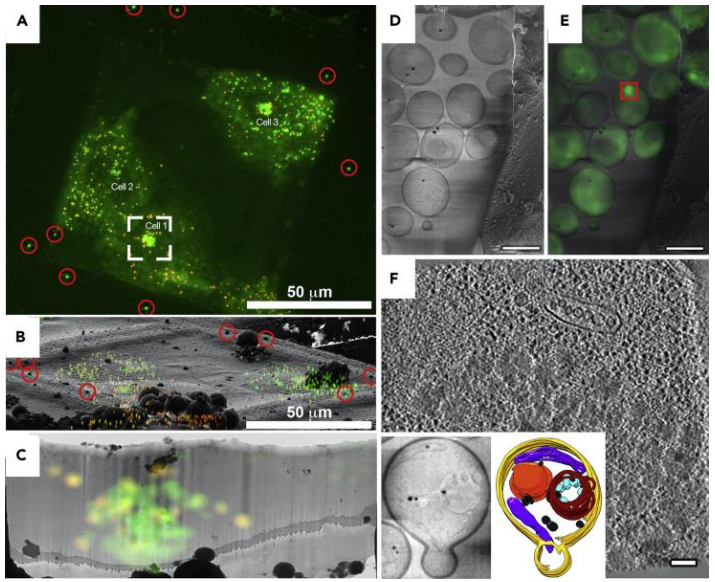
Light microscopy imaging

Electron microscopy imaging

Image correlation



CLEM microscopy workflow of V. cholerae cells



Cryo-confocal image of lipid droplets inside of cells grown on an EM grid and plunge-frozen.