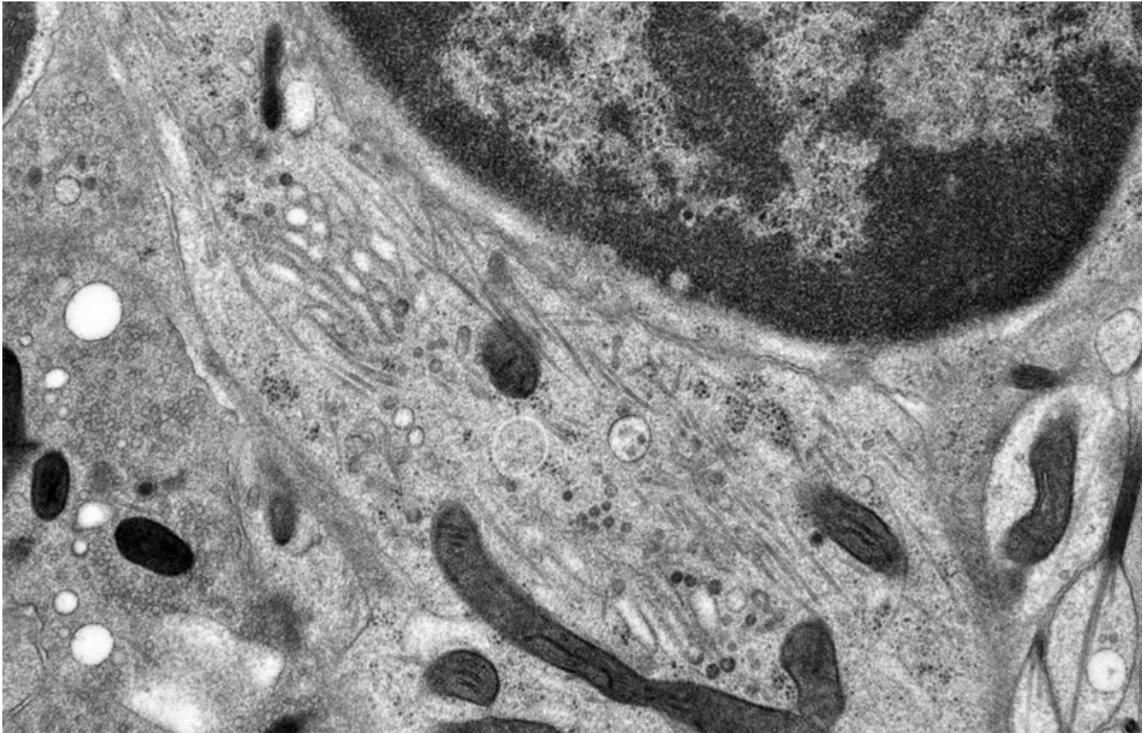


# An Introduction to Electron Microscopy Instrumentation, Imaging and Preparation



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## 1. Introduction

Microscopy enables a “direct” imaging of organisms, tissues, cells, organelles, molecular assemblies and even individual proteins. Analytical techniques as used in molecular biology provide a data set which represents cellular processes in an abstract way. Therefore, microscopy was and still is an important, complementary technique to visualize the macro and/or microscopic structure and to assign structure to function and vice versa.

Microscopy techniques, their resolution limit, scales and corresponding objects are depicted in figure 1.1. The typical size of eukaryotic cells is 10-20 micrometer and their subcellular components can not be seen by naked eyes. Additionally, cells are colorless and transparent. The organelles or other constituents have to be stained to make them visible in the light microscope. A variety of histological stains and fluorescent markers have been developed.

Imaging the ultrastructure of cellular components requires electron microscopes which have a much higher resolving power than light microscopes. The physical properties of electron microscopes (e.g. high-vacuum) demand specific preparation and staining techniques to reveal the ultrastructure of cells and tissues.

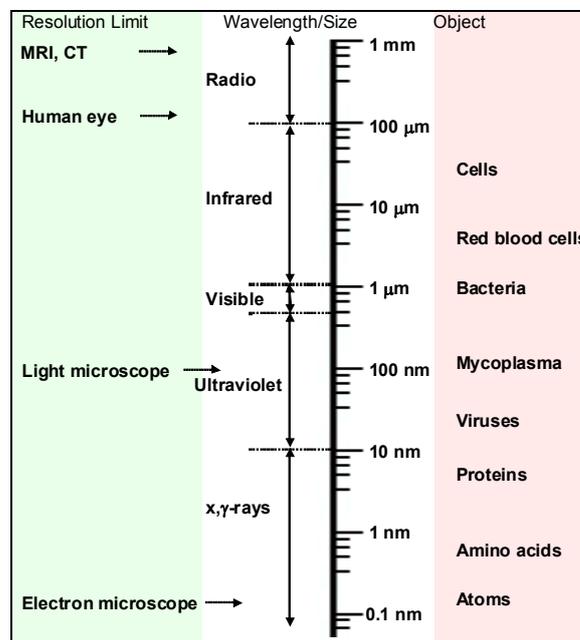


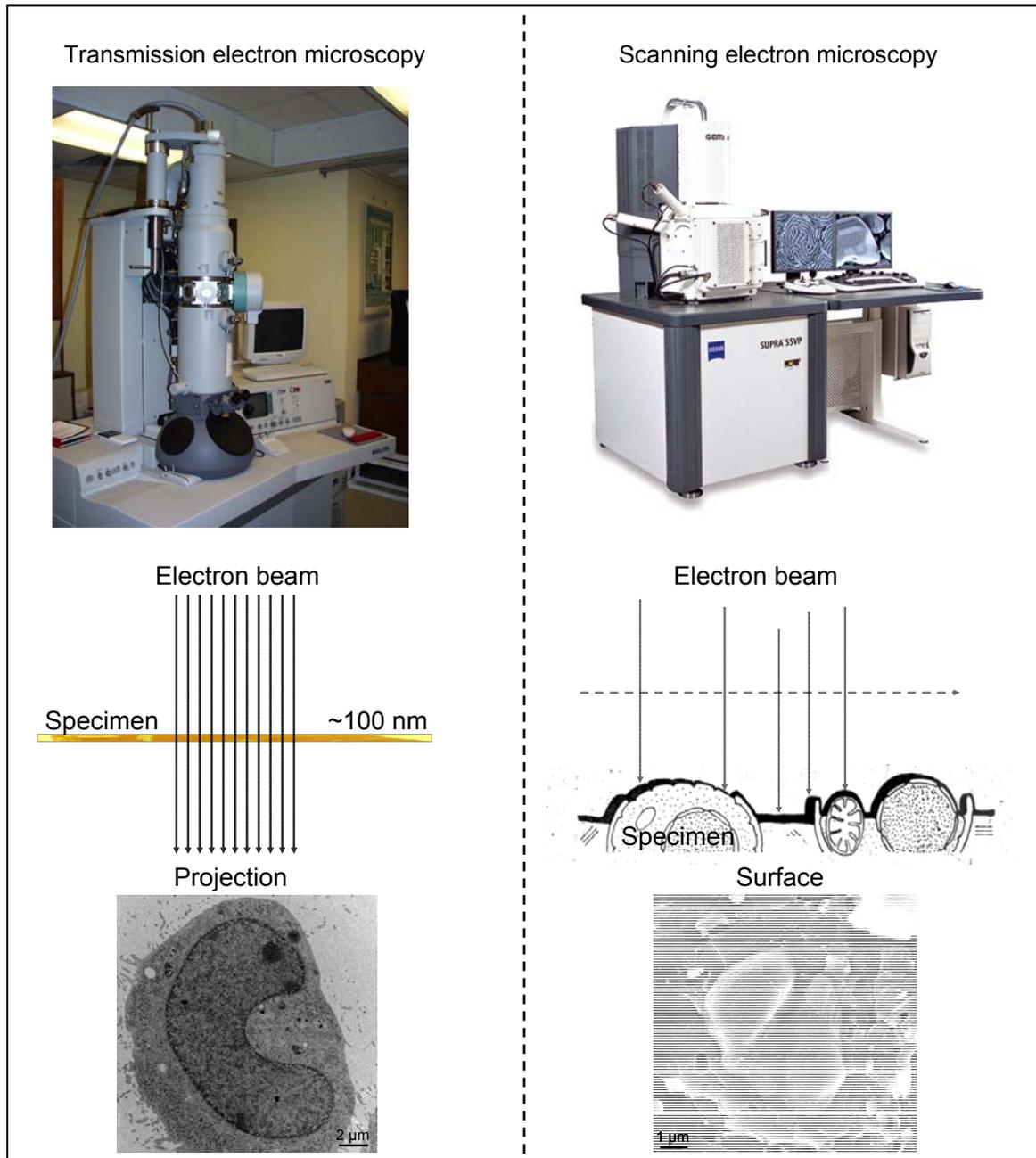
Fig. 1.1: Resolution limit of different imaging techniques, involved radiation and size of biological objects.

The limit of resolution of an optical system depends on the numerical aperture and the wavelength of light (Law of Ernst Abbé). This law holds as well for electrons, of which the speed determines their wavelength: The higher the speed of the electrons, the smaller the wavelength and the better the resolution. The practical resolution of a transmission electron microscope (TEM) run at 100 kV (acceleration voltage that determines the speed) is approximately 0.5 nm and exceeds the resolution of light microscopes by far. In scanning electron microscopes (SEM), the effective resolution is around 1 nm. The resolution of modern electron microscopes is much better than the resolution that a prepared biological specimen can provide due to preparation.

The most important and challenging part of electron microscopy of biological material is the preparation procedure. With every preparation step artifacts are introduced. Electron microscopists have put a lot of effort in the preparation of the specimens to reduce the artifacts to a minimum and to image the specimens in a more lifelike state. The image of the specimen can never be better than the entity of the introduced artifacts during preparation.

In combination with immunocytochemical methods, electron microscopy is a powerful method to label and highlight single, specific proteins enabling a correlation of the ultra structure and its function.

Note: Preparation of biological specimens for electron microscopy is often a very time-consuming process.



*Fig. 1.2: Overview of the different types of electron microscopes and their general mode of imaging. Top row: Biological transmission electron microscope on the left and a scanning electron microscope on the right. Second row: According imaging modes in transmission and scanning electron microscopy. A thin sample is transmitted by the electrons to form a projected image of the sample in TEM. A bulk sample is scanned with a focused beam of electrons in an SEM providing a surface view. Images show Hela cells, thin section of a fixed, dehydrated, plastic embedded cell culture (70 nm thickness) on the left, and freeze-fractured Hela cell imaged in the frozen state in a cryo SEM on the right.*

## 2. Principle of SEM and TEM

The overall design of an electron microscope is similar to that of a light microscope. In the electron microscope, the light is substituted with electrons and the glass lenses are substituted with electromagnetic/electrostatic lenses. The design of a transmission electron microscope and its similarity to a light microscope is depicted in figure 2.1.

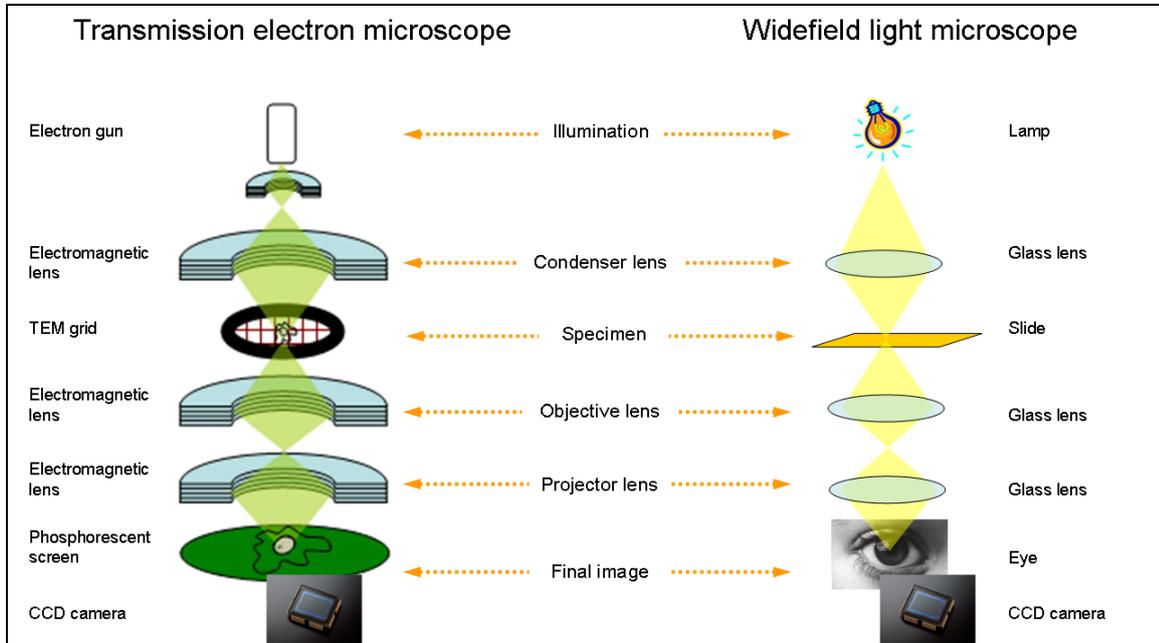


Fig. 2.1: Similarity of a transmission electron microscope with a wide field light microscope. An electron beam is formed at the tip of a heated filament. The electrons are accelerated with high voltages (60 – 1200 kV depending on the type of TEM) and are guided through the electron microscope column by electromagnetic lenses. The beam penetrates and interacts with the specimen and leads to an image. The image is monitored on a phosphorescent screen or specially designed CCD camera and recorded.

The design of a scanning electron microscope and its similarity to a confocal laser scanning microscope is depicted in figure 2.2.

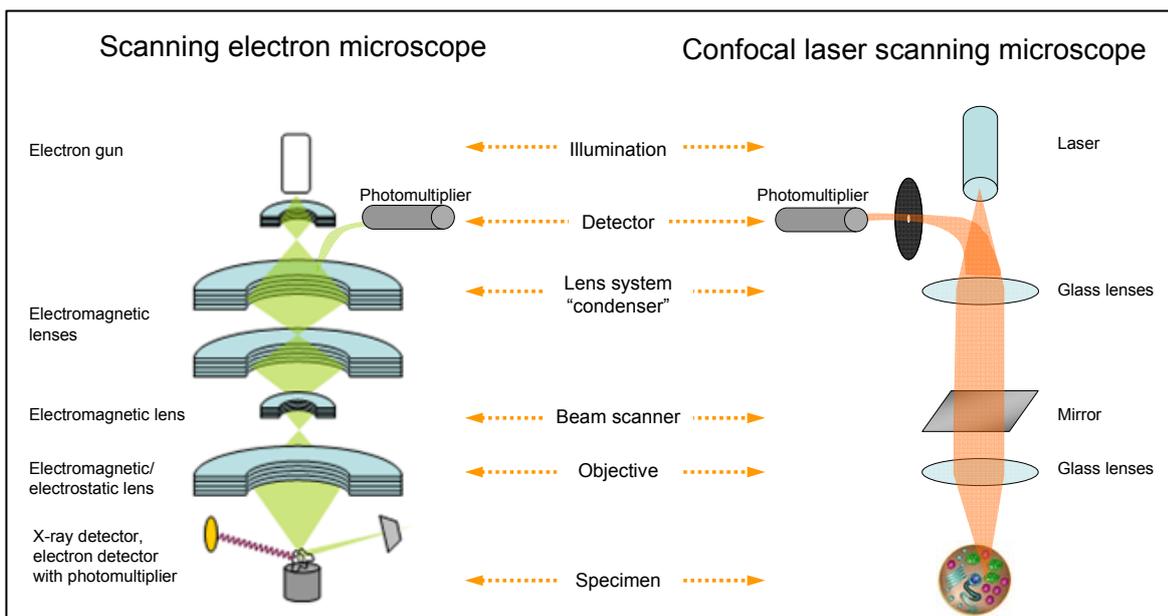


Fig 2.2: Similarity of a scanning electron microscope with a confocal laser scanning microscope. An electron beam is formed at the tip of a heated filament. The electrons are accelerated with voltages between 0.2 – 30 kV and are guided through the scanning electron microscope column by electromagnetic lenses. The beam is focused in the objective lens and a small beam

spot is moved/scanned over the specimen surface using deflector coils. The beam interacts with the specimen and emitted electrons are detected in specific detectors and amplified using photomultiplier tubes. Emitted radiation as x-rays is detected in according detectors. Every scanned pixel on the specimen represents a pixel on the screen. Important: There is no imaging device such as a CCD camera in SEM.

Electron microscopes are high-vacuum systems. In the area of the electron source a vacuum of  $10^{-7}$  to  $10^{-10}$  mbar is necessary to prevent oxidation/burning of the heated filament. The column and the area of the specimen are evacuated steadily to a vacuum of  $10^{-5}$  to  $10^{-7}$  mbar. The mean free path, referred to as the distance a molecule can fly before hitting another particle, accounts to about 50 meter at a vacuum of  $10^{-6}$  mbar. Any collision with a residual gas molecule in the system reduces the resolution and performance by scattering of electrons. The vacuum system of an electron microscope consists of a cascade of low and high-vacuum pumps. An example for a TEM is given in figure 2.3. High vacuum pumps can not directly pump against ambient pressure. Therefore, a serial setup of low and high vacuum pumps is required.

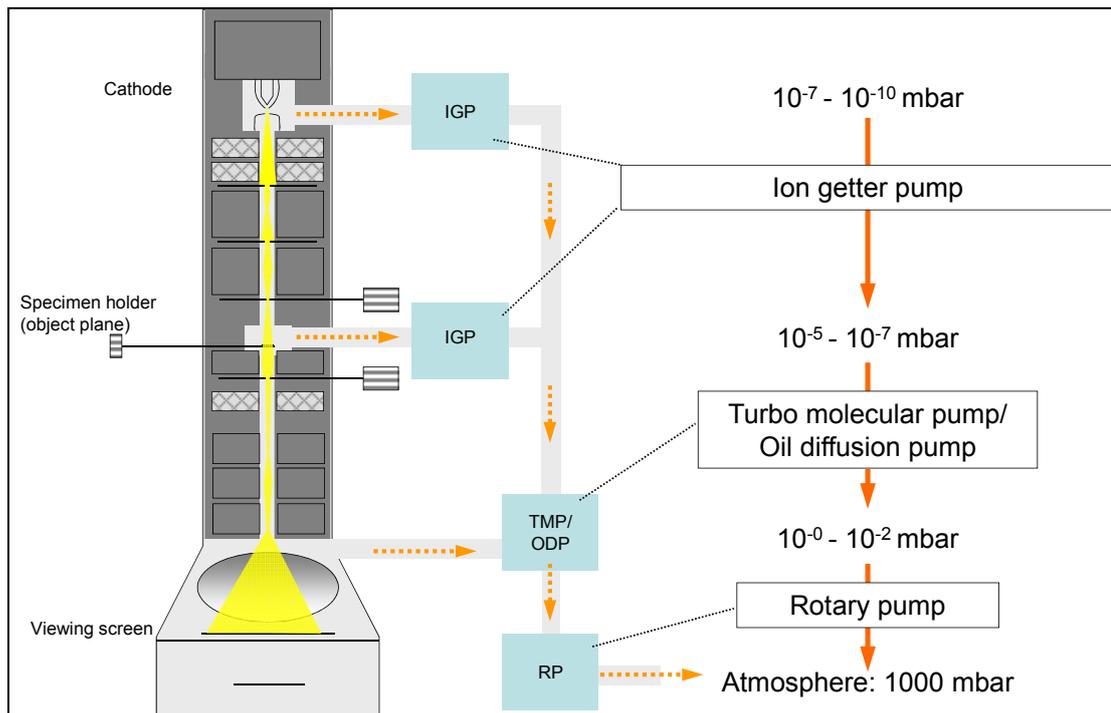


Fig 2.3: Example of a vacuum system for a TEM. RP...rotary pump, TMP...turbo molecular pump, IGP...ion getter pump, ODF...oil diffusion pump

## The electron source

Electrons can either be produced by thermionic emission or in a process called cold field emission. The material of the electron source and its properties are depicted in figure 2.4. During thermionic emission, a very fine tip of a tungsten filament, an LaB6 crystal or a ZrO/W Schottky emitter is heated by an electrical current flowing through the electron source enabling the escape of electrons. The electrons leaving the filament have a low energy and, therefore, need to be accelerated to the desired speed before entering the electron column. A high voltage between the electron source (cathode) and an anode plate is applied leading to an electrostatic field through which the electrons are guided and accelerated. During cold field emission, the electrons can escape from an extremely fine tungsten tip without heating (room temperature). The advantage of cold field emission sources is the very high yield of electrons and the very low chromatic aberration of the electrons allowing imaging at atomic resolution. These instruments are very costly and require particularly high vacuum.

	Tungsten	Thermionic LaB6	Schottky	Cold field emission
Material	W	LaB6	ZrO/W	W
Heating temp. (K)	2700	1800	1800	300
Normalized brightness				
Required vacuum (Pa)	high			Ultra high
$\Delta E$ (eV)	Chromatic aberration!			

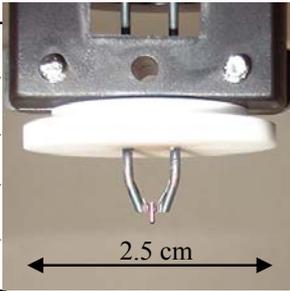


Fig. 2.4: Different types of electron sources and their properties. The image shows a Schottky emitter.

### Electromagnetic lenses

Electromagnetic lenses consist of a huge bundle of windings of insulated copper wire, a soft iron cast and pole piece (figure 2.5 A). A magnetic field is induced by the current in the winding and reaches its main strength at the pole piece of the lens. The accelerated electrons entering the magnetic field are deviated following the law of a charge passing a magnetic field. The resultant force is always perpendicular to the plane defined by the direction of the magnetic field and the direction of the electrons. In conclusion, the electrons take a circular path through the lens system (figure 2.5 B).

Depending on the strength of the magnetic field, the focal width of the lens is changed. The first lens system in an electron microscope acts as a condenser lens, which bundles the electrons, determines the overall brightness of the beam and guides it to the specimen. The electrons transmit the specimen and the objective lens forms the primary image. Focusing of the image is performed with the strength of the magnetic field in the objective lens. The final magnification is determined by the following projective lenses, a systems of two or three lenses which act in combination and project the final image on the fluorescent screen or a CCD camera (or a photosensitive film), see also figure 2.1 and 2.2 for lens set up. The circular path of the electrons leads to a rotation of the image depending on the magnification (the lens current of the projective lenses). This rotation is corrected in modern TEM by a set of corrector coils.

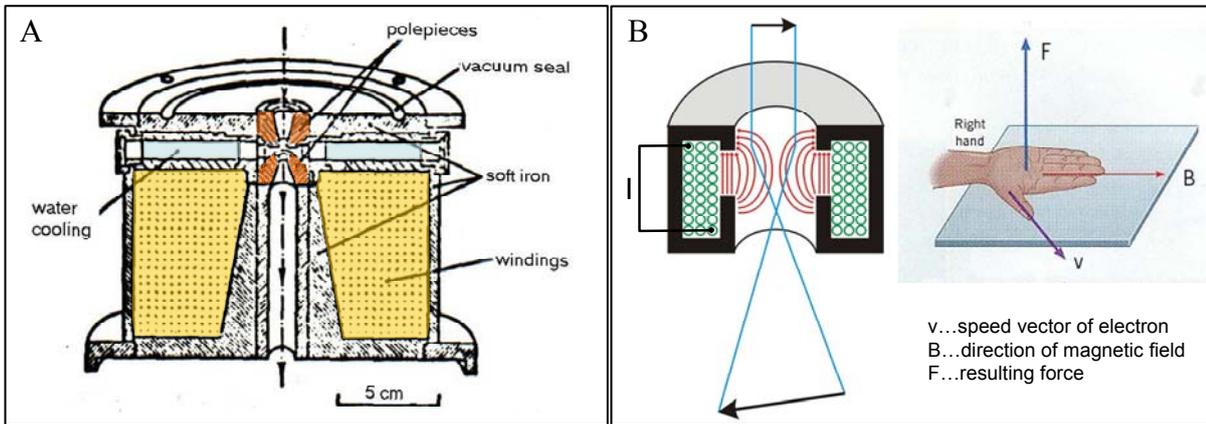


Fig. 2.5: Electromagnetic lens. A) The magnetic field is strongest in the area of the pole piece. The focal length of the lens is changed by the current through the windings. The lens is water cooled to maintain stability during operation. B) Electrons passing the magnetic field are deviated perpendicular to the plane defined by the magnetic field B and the velocity vector v. The black arrows demonstrate the rotation of the image.

Electromagnetic lenses show the same aberrations as glass lenses such as chromatic, spherical aberration, distortion and astigmatism. The most prominent aberration in electron microscopy is the axial astigmatism. The different electrons deriving from a point source do not match one and the same point in the image plain leading to a confusion of the image (figure 2.6). Astigmatism is caused by inhomogeneities of the lenses, contamination of lenses and apertures and charging of the specimen. In particular in SEM, the astigmatism must be corrected regularly, in extreme cases after every movement of the specimen. The correction is done again with a set of corrector coils.

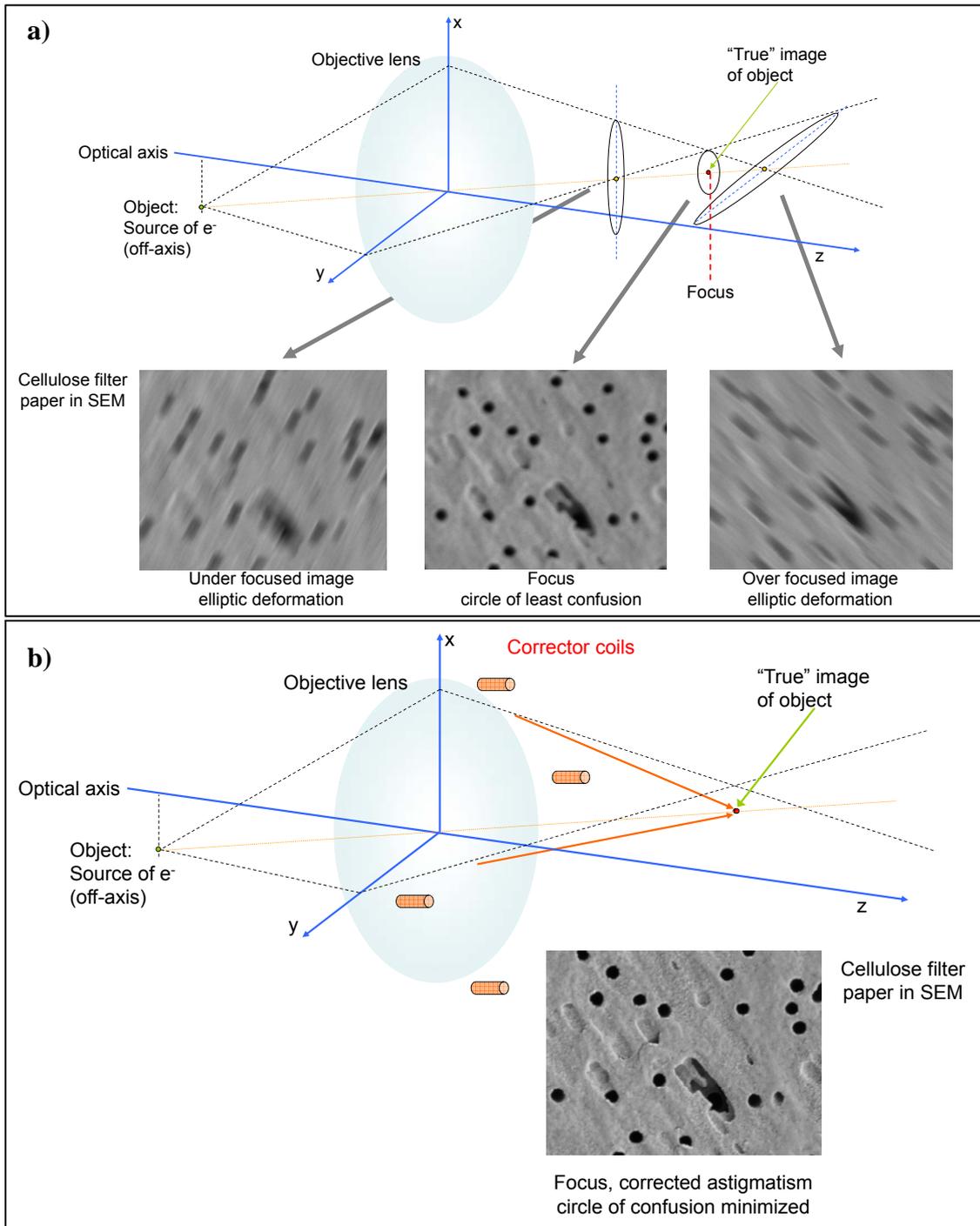


Fig. 2.6: a) Axial astigmatism in an electron optical system. In focus, a point source appears as a circle of least confusion in the image plane (focus). Underfocus and overfocus images appear as ellipses orthogonal to each other. b) Corrected astigmatism using corrector coils.

## Specimen holders and stages

In TEM, the electron column does not offer a lot of space for the specimen. Additionally, the specimen must be very thin so that the electrons can penetrate the specimen and form an image. The average thickness of a biological specimen should be around 70 nm for a TEM with an acceleration voltage for the electrons of  $\sim 100$  kV (the higher the voltage, the thicker specimens can be examined). Thin sections of the specimen are mounted on copper grids of 3 mm diameter, which are available in a wide variety of materials and mesh sizes. The grids with the sections on top are attached in a holder and introduced into the goniometer of the TEM through a vacuum lock, since the system always stays under high vacuum. The goniometer is the mechanical setup which enables highly

precise and stable control of the specimen holder during imaging. Any drift or instability results in an unsharp image, in particular at high magnifications (figure 2.7).

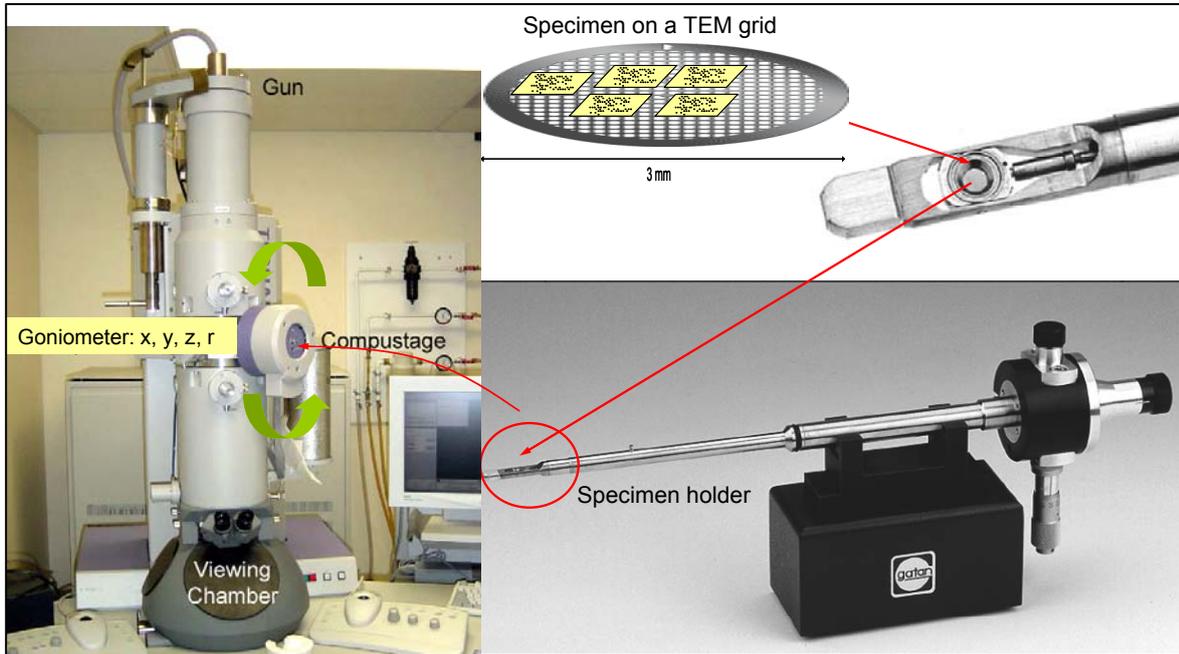


Fig. 2.7: Thin sections of a specimen on a TEM grid, holder tip and complete specimen holder, which is introduced into the goniometer of the TEM through a vacuum lock.

The specimen chamber of SEMs are large and offer much more space for the specimen. In routine SEM the specimen can be at least 10 cm in diameter and 2 cm thick, since it does not have to be permeable to electrons (bulk specimens). The specimen usually attached on a small aluminium stub (1 cm diameter) is mounted on a holder able to carry 8 stubs for example. The stub holder then is mounted on a 5 axis stage of the microscope, allowing controlled movement in x, y, z, tilting and rotation. The stub holder can be inserted in the microscope by hand requiring venting of the whole chamber or by using a vacuum lock.

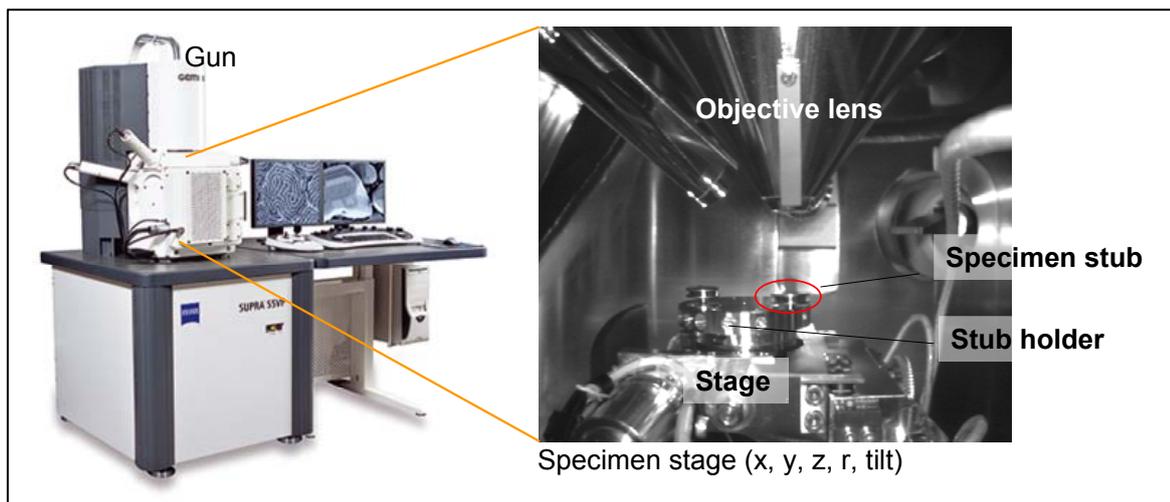


Fig. 2.8: The inside view of an SEM chamber as seen with an infrared camera mounted on the door of the instrument.

Electron microscopes are very sensitive to vibration and electromagnetic fields. The electron column is mounted on air cushions which absorb vibration. Often, they are even setup on special fundamentals which are disconnected from the rest of the building to minimize disturbance by vibration. Electromagnetic fields can be compensated with electromagnetic coils (inductors) mounted to the walls, ceiling and floor.

### 3. Electron specimen interactions

The electron beam (TEM and SEM) hits the sample and either passes the sample unaffected or interacts with it. Three different interactions of electrons with an atom can be generally discerned (Fig 3.1):

- i) An electron of the primary beam is scattered by the electrostatic interaction with the positively charged nucleus of an atom at an angle of more than  $90^\circ$ , yielding **backscattered electrons**. This type of electrons has practically the same energy as the ones of the primary beam.
- ii) An electron of the primary beam is scattered by the electrostatic interaction with the positively charged nucleus of an atom at an angle of less than  $90^\circ$ , yielding **elastically scattered electrons**. Also these electrons do not lose energy and therefore are referred to as elastically scattered electrons.
- iii) Electrons can also lose energy while interacting with the “electron cloud” of the atom. These are called inelastically scattered electrons. This interaction can lead to the following processes (see also <http://www.matter.org.uk/tem/default.htm>):

#### Inner-shell ionisation

An electron is pushed out of the electron cloud. The electron „hole“ is filled by an electron of an outer shell: Surplus energy is either emitted as **characteristic x-ray** or transferred to another electron, which is emitted (Auger electron).

#### Bremsstrahlung (continuum x-rays)

Deceleration of electrons in the Coulomb field of the nucleus

⇒ Emission of x-ray carrying the surplus energy  $\Delta E$

⇒ Uncharacteristic x-rays

#### Secondary electrons (SE)

Loosely bound electrons (e.g., in the conduction band) can easily be ejected

⇒ low energy ( $< 50$  eV)

#### Phonons

Lattice vibrations (heat) ⇒ beam damage

#### Plasmons

Oscillations of loosely bound electrons in metals

#### Cathode luminescence

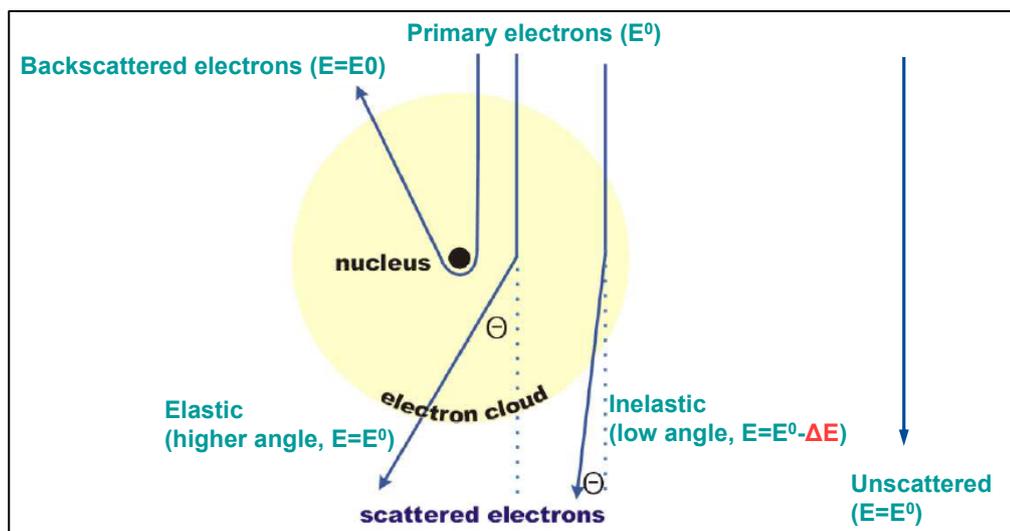


Fig. 3.1: Interaction of electrons with an atom. Note: Inelastically scattered electrons lead to a variety of processes like heating, radiation and electron emission as described in the text.

Almost all types of electron interactions can be used to retrieve information about the specimen. Depending on the kind of radiation or emitted electrons which are used for detection, different properties of the specimen such as topography, elemental composition can be deduced.

The following figure 3.2 shows the interaction of the electron beam with the specimen. In biological TEM analysis, the elastically scattered electrons are mainly used for imaging. The inelastically scattered electrons will be recorded as well with the imaging device (e.g. CCD camera). They have a slightly lower energy (a longer wavelength) than the primary electrons. In conclusion, they are deviated less strongly in the electron lenses and do not merge at the same spot as the elastically scattered electrons (chromatic aberration). They contribute to unsharpness of the image. In high-end instruments, the inelastically scattered electrons can be filtered out with an energy filter.

In SEM, mainly secondary electrons are used for imaging of biological specimens. These electrons have a very low energy (around 50 eV) compared to the energy of the primary electrons (up to 30 keV). Due to the low energy, these electrons can escape only from the surface area of the specimen (for details see chapter 5) and therefore provide information about the surface topography. Also backscattered electrons are frequently used for imaging with an according backscatter detector. The number of electrons which are backscattered from a certain spot of the specimen depends on the local elemental density of the specimen. Hence, backscattered electrons provide a “density image” and information about the elemental composition, respectively. Backscattered electrons are also more resistant to charging due to their high energy.

X-rays are always produced when matter interacts with an electron beam. The energy of a part of the emitted x-rays is specific for different atoms and can be used for elemental analysis in TEM and SEM using special detectors. However, the analysis of light elements is rather difficult and the high corrugation of biological specimen surfaces makes the interpretation of elemental maps very demanding. A lot of experience is necessary to prevent pit falls.

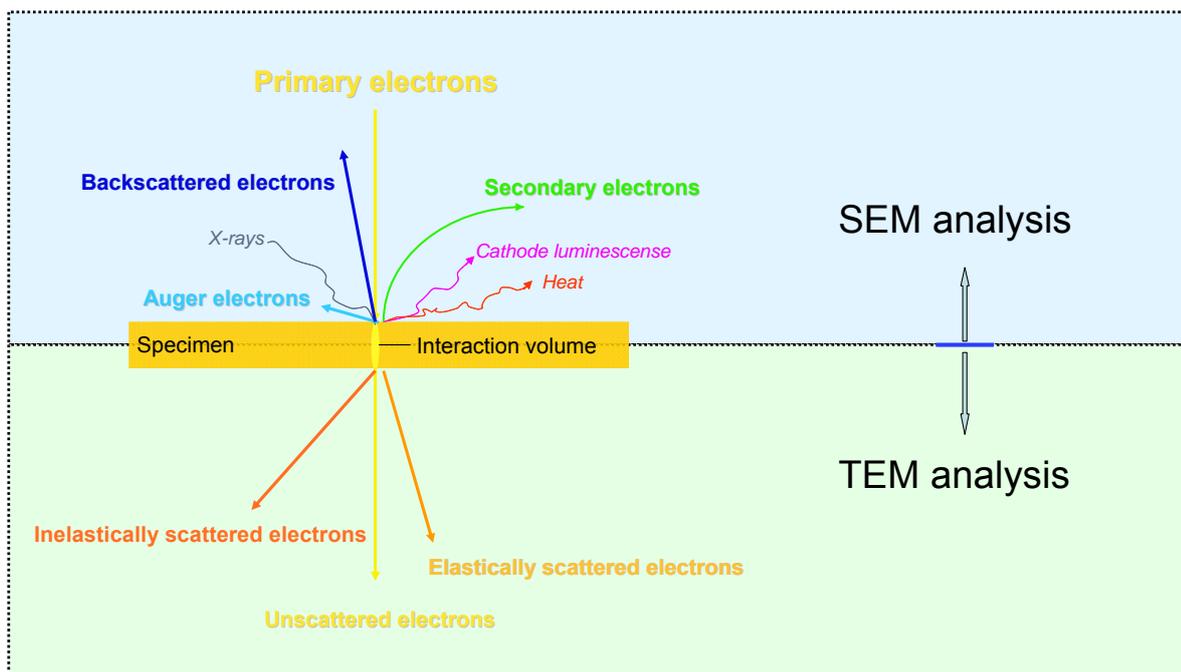


Fig 3.2: Interaction of electrons with specimen/matter, induced radiation and emission. Transmitted electrons like elastically scattered electrons are typically used for TEM imaging of biological specimens. Secondary electrons and backscattered electrons are mainly used for imaging in the SEM.

#### 4. Contrast formation and imaging in TEM

The image formation in TEM is basically the same as for regular light microscopy, since the properties of electrons are very similar to the ones of light. As for light, electrons can be observed as particles or as waves depending on the physical phenomenon to be explained (wave particle duality).

The primary electron beam passes through the thin specimen and generates non-diffracted and diffracted electromagnetic waves. The objective lens collates the diffracted and non-diffracted waves in the image plane and forms the primary image (see also lecture Bio 416 Light Microscopy by Urs Ziegler). Biological objects usually consist mainly of very light elements like C, O, H, N, S, P and lead to poor interaction with the electrons and, hence, provide almost no contrast.

Also the absorption of electrons by the specimen is very small and does not contribute to contrast formation. Absorption of electrons would result in heat damage of the specimen. At high magnifications at which a small area of the specimen is exposed to high radiation, beam damage is an issue and can easily be observed.

In conclusion, the specimen (thin section) must be treated to provide enough contrast for imaging. This is done by impregnation of the thin section with heavy metals like uranium and lead. The positively charged uranium and lead ions bind specifically to different constituents of biological matter and to a different extent. The high atomic number and density of the heavy metal ions leads to a much higher interaction and diffraction of the electrons. The main contrast formation is achieved by trapping the numerous diffracted electrons using the objective aperture located in the back-focal plane of the objective lens (figure 4.1). Spots of the sample containing more heavy metals will lead to a higher number of diffracted/scattered electrons, which will hit the objective aperture. These electrons do not reach the imaging device (CCD, screen) and will cause a “dark” or “electron dense” area in the image. Therefore, the image formation of biological specimens is based on **amplitude contrast** (more or less electrons will reach the CCD, screen), figure 4.2.

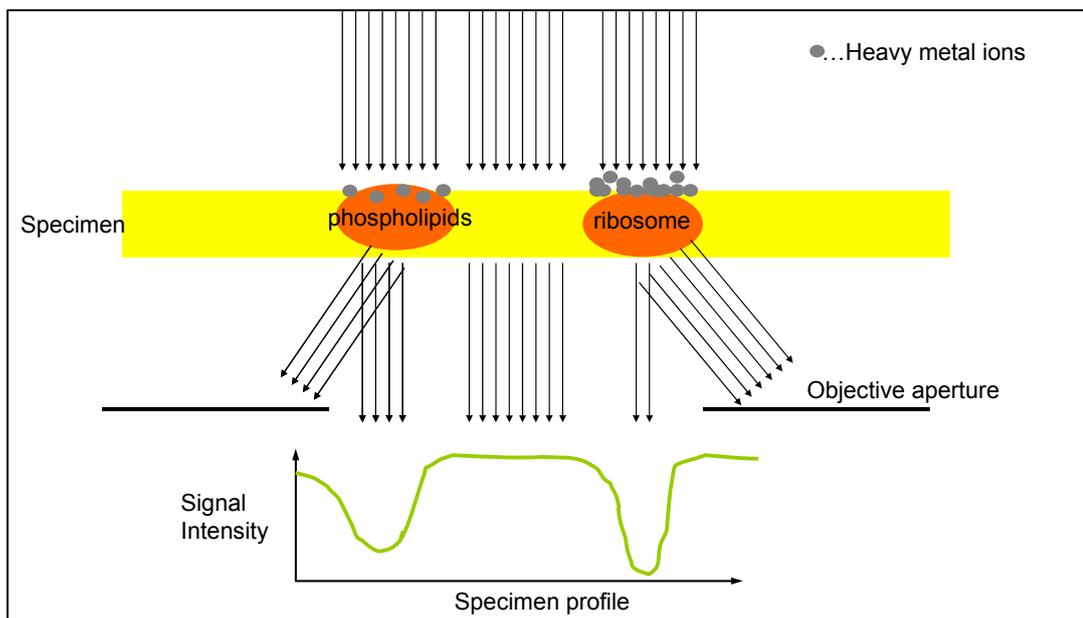


Fig. 4.1: Contrast formation in biological objects (simplified): A different number of heavy metal ions stick to the sample surface depending on the constituents, e.g. membranes (phospholipids), ribosomes, chromatin. Many diffracted/scattered electrons hit the objective aperture and do not reach the imaging device (CCD, viewing screen). Specimen spots with a lot of heavy metals will yield low signal intensity (electron dense, dark spots) whereas spots with no or few heavy metal ions yield high signal intensity (bright spots).

The difference of heavy metal stained and non-stained thin section of an alga is shown in figure 4.2.

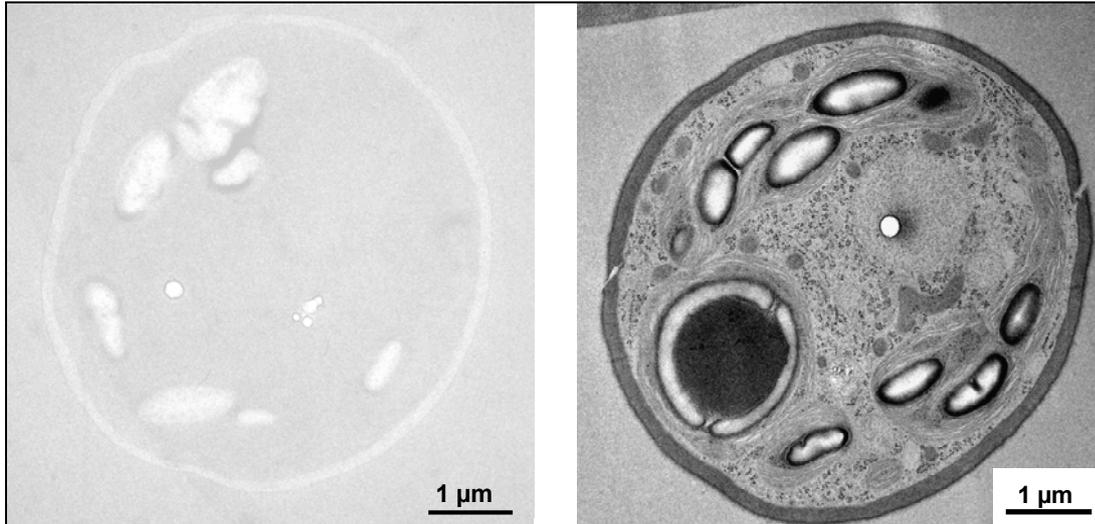


Fig. 4.2 Non-stained versus heavy metal stained thin section of an alga.

Contrast of biological specimens can be further improved by slight underfocusing. Underfocusing leads to Fresnel rings (deletion/amplification of signal), which improve the visibility of biological structures (Fig 4.3). Note: Too much underfocus or overfocus introduces artifacts and the image gets unsharp. True focus (Gaussian focus) corresponds to minimum contrast.

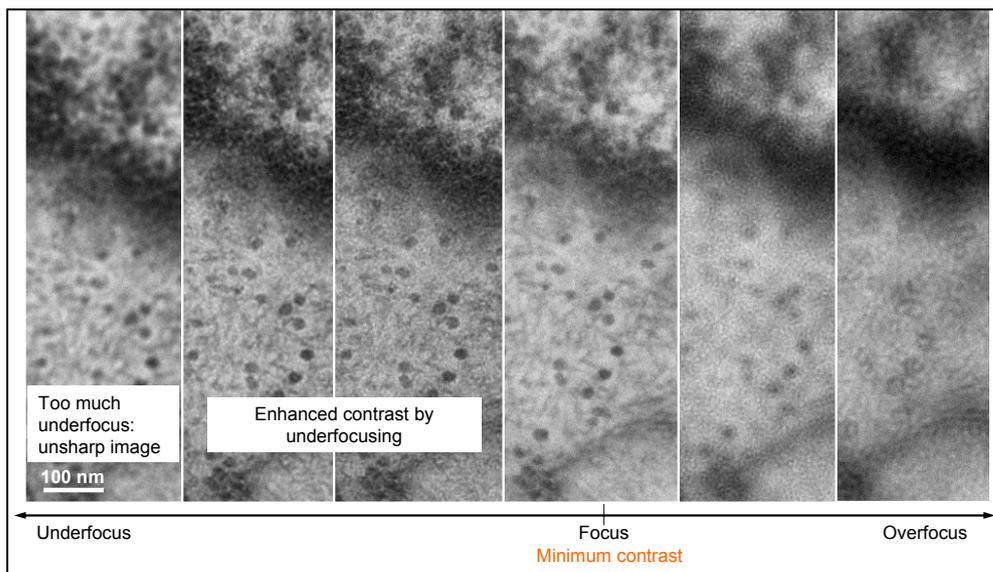


Fig. 4.3: Under-focus, over-focus and true focus of a thin section of a cell. Minimum contrast corresponds to true Gaussian focus. Too much over- or underfocus provides an unsharp image with artefacts.

In particular, thin sections of frozen soft, hydrated organic matter can not be stained by incubating them with heavy metal solutions. The only way to generate enough contrast for imaging is using underfocus. An example is displayed in figure 4.4.

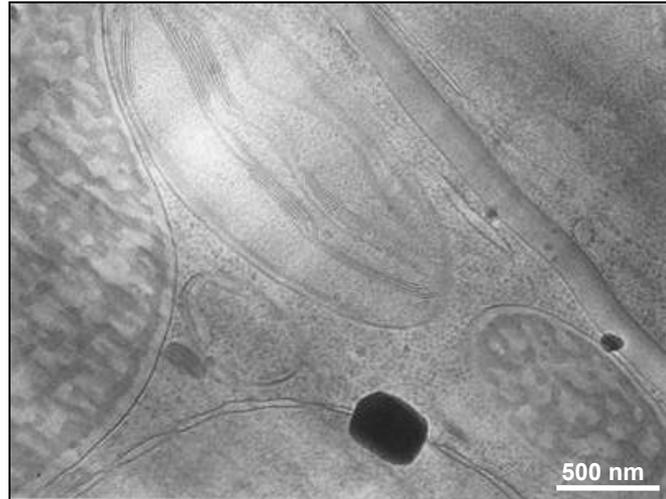


Fig. 4.4: Frozen hydrated thin section of a Golden delicious apple leaf as imaged in a cryo transmission electron microscope at  $< -150^{\circ}\text{C}$ . Contrast is tremendously enhanced by underfocusing.

The primary image is magnified or demagnified by the projective lenses arranged below the objective lens. The final image can be viewed by eye on a large screen in the microscope (approximately 20 cm diameter). The screen consists of a phosphorescent layer which starts to emit light when hit by electrons. Electron microscopy is usually performed in the dark to receive a good contrast on the viewing screen. For many decades, images were taken by exposing electron sensitive films to the electron beam and the negatives were developed by conventional film development in a dark room. Nowadays, specialized CCD cameras are used for image acquisition. CCD cameras are available which reach the resolution of a conventional chemical film (very expensive though). Standard CCD cameras for TEM have a number of  $2\text{k} \times 2\text{k}$  to  $4\text{k} \times 4\text{k}$  pixels, being sufficient for most applications for biological imaging. Pixel binning (e.g. 2, 4, 8 times) enables fast real-time viewing. Searching of the area of interest and acquisition of images can easily be performed with the CCD camera on computer screens.

The assembly of a CCD camera for TEM is explained in figure 4.5. The CCD chip cannot be directly bombarded with electrons. The chip would be damaged. Therefore, the electrons have to be primarily converted to photons with a scintillator. On top of every pixel of the CCD camera, a small fiberoptic element guides the photons from the scintillator to the pixel of the CCD. Cameras acquiring images directly with electrons have become commercially available recently (very expensive).

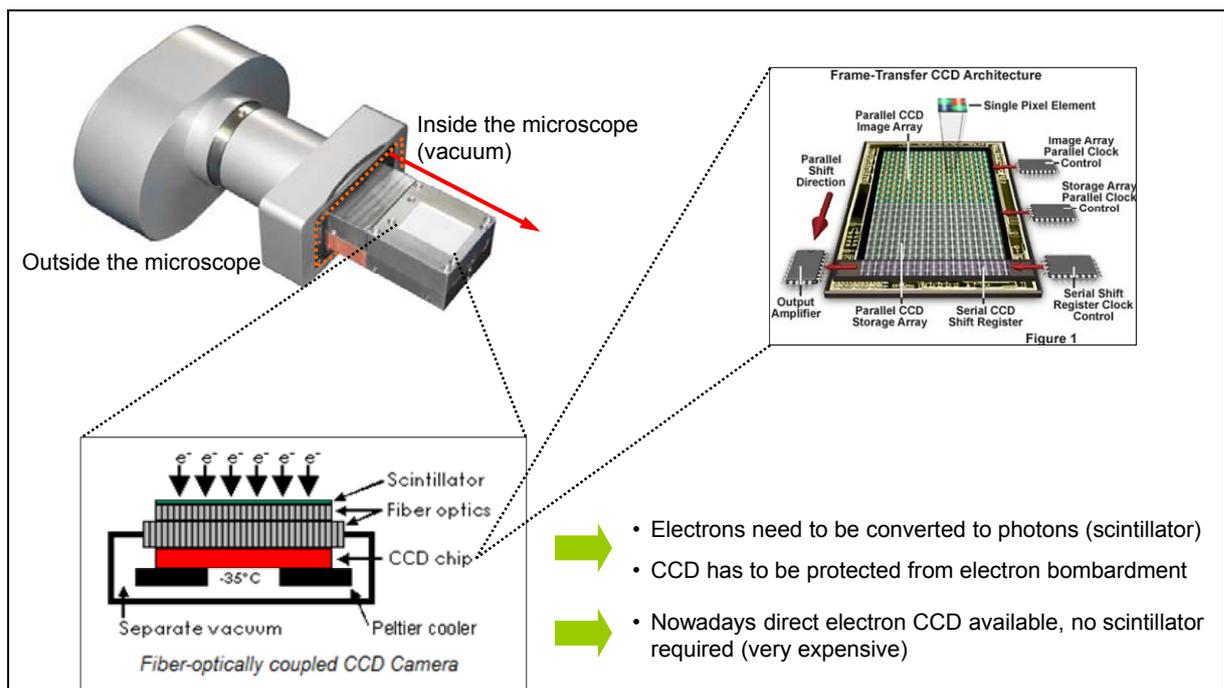


Fig. 4.5: Design of a CCD camera for electron microscopy

## 5. Contrast formation and imaging in SEM

In SEM, an electron beam with a tiny spot is guided pixel by pixel over the surface of a specimen, e.g. 1024 x 768 pixels. At every pixel, the beam stays for a defined time and generates a signal (e.g. secondary electrons) which are detected, amplified and displayed on a computer screen. The scanning of the beam is synchronized with the display of the computer screen. The signal deriving from every single pixel on the sample is simultaneously displayed on the corresponding pixel of the computer screen and finally forms the image. Note: There is no CCD camera for image acquisition in SEM! The image consists of displayed grey levels, e.g. 256 grey levels in an 8 bit image. The magnification is changed by scanning a smaller area with the same number of pixels. The pixel size therefore gets smaller and the resolution is increased.

The beam of electrons which hits a spot of the surface of the specimen interacts with a whole volume of the specimen. The interaction volume looks like a pear (figure 5.1). Secondary electrons are produced in the whole volume but can escape only from a small volume close to the surface of the specimen due to their low energy (around 50 eV). Backscattered electrons manage to escape from a larger depth since their energy is the same as the primary electrons. On their way through the sample, backscattered electrons can again interact with atoms and produce secondary electrons (called SE 2 electrons). X-rays can escape from the whole interaction volume.

The size of the interaction volume is dependent on the acceleration voltage of the primary electrons and the atomic number of the atoms close to the surface. Heavy atoms decelerate the beam more than light elements and therefore reduce the interaction volume.

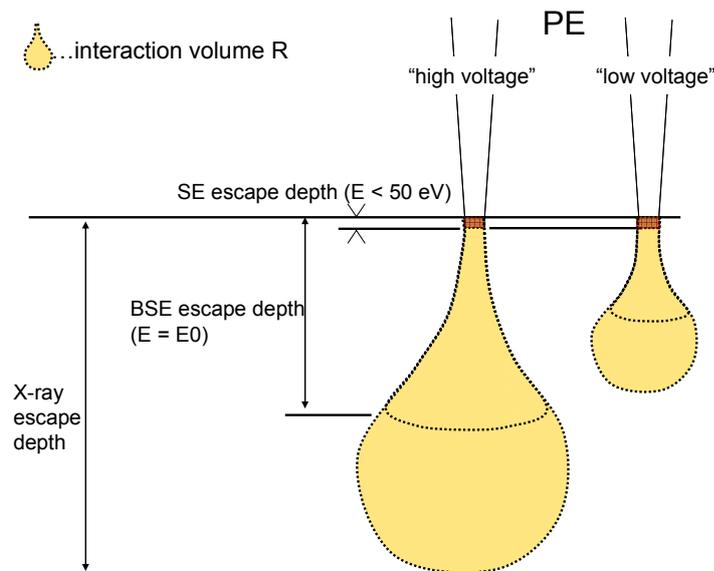


Fig. 5.1: Interaction volume of the electron beam with a bulk sample. Escape depths of SE, BSE, and x-rays are indicated. Note: The SE escape depth is independent on the energy of the primary electrons (PE).

### Imaging using secondary electrons

Secondary electrons (SE) are mainly used in SEM for imaging the surface topography of biological specimens. They are detected with an Everhart Thornley detector. The principle of detection is shown in figure 5.2. The SE are collected by a collector grid. A voltage of + 200 to 500 V is applied to the collector grid which attracts the low energy electrons. The SE then hit a scintillator which converts the electrons to photons. The photons are guided by a light conducting tube on the photomultiplier tube (figure 5.3), where the photons again are converted to electrons that are amplified finally leading to an electrical signal. The current is translated into a gray value and displayed on the screen of the monitor.

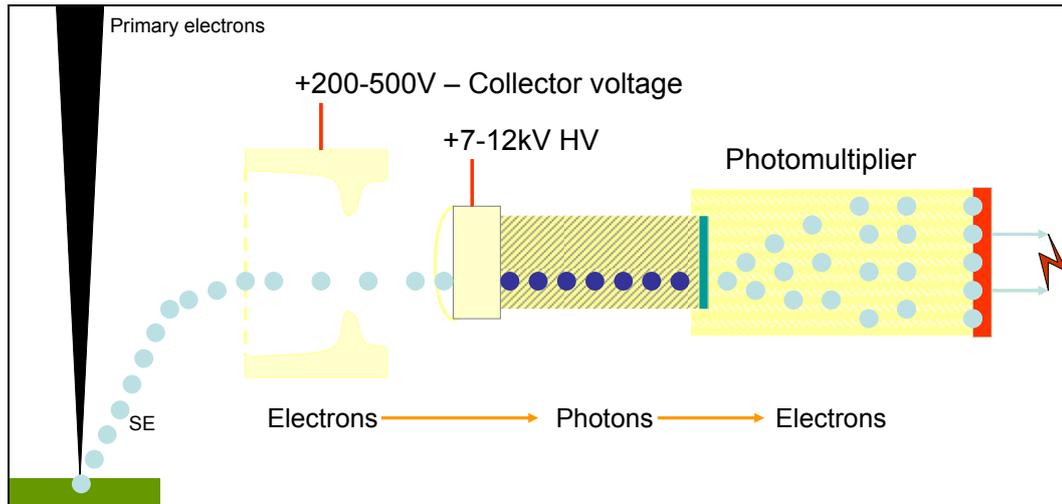


Fig. 5.2: Principle of SE detection with an Everhard-Thornley detector. Explanations see text.

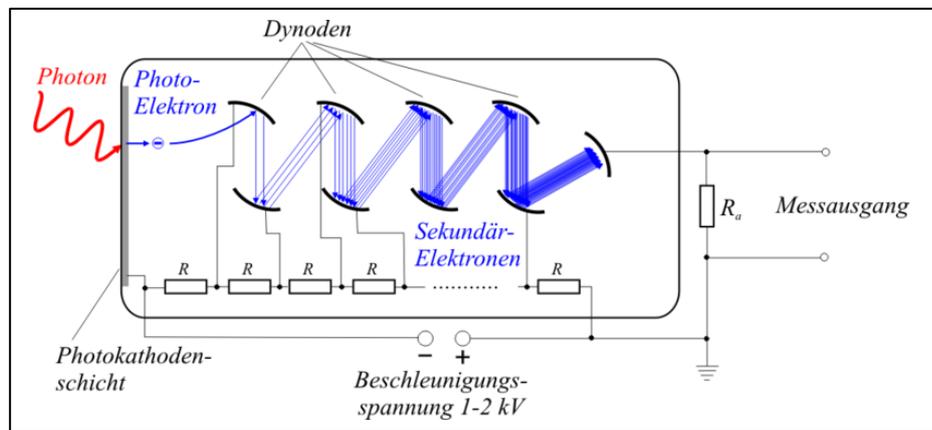


Fig. 5.3: Working principle of a photomultiplier tube. The amplification is monitored by the voltage of the photomultiplier.

The SE escape depth ( $\lambda$ ) as depicted in figure 5.1. is *independent* on the acceleration voltage of the primary electrons. However, the percentage of the SE produced in this area compared to the SE produced in the whole interaction volume  $R$  is larger at lower acceleration voltages because more electrons interact with the specimen in the uppermost layer. In conclusion, the number of the emitted secondary electrons from the SE escape depth at lower voltages is higher and provides a stronger signal. The higher the acceleration voltage, the more electrons penetrate deeply into the specimen and the produced SE cannot escape anymore.  $\lambda$  is also dependent on the density (atomic number) of the specimen. It is 10 – 100 nm for carbon, 2 – 3 nm for Chromium, and 1 – 2 nm for Platinum. Since biological specimen consist of light elements only, the interaction volume is large and the number of SE escaping the specimen is very low. Moreover, the SE escaping the specimen derive from a relatively large volume. The result is a faint, unsharp signal with very poor contrast. To receive a sharp and clear signal from the surface, biological specimens need to be coated with a thin layer of a heavy metal (few nm of Au, Pt etc.). Now, the signal is localized to the surface, many more SE are produced in the surface layer, and SE deriving from lower areas can not escape through the heavy metal layer and obscure the signal. This situation is explained in figure 5.4 and figure 5.5.

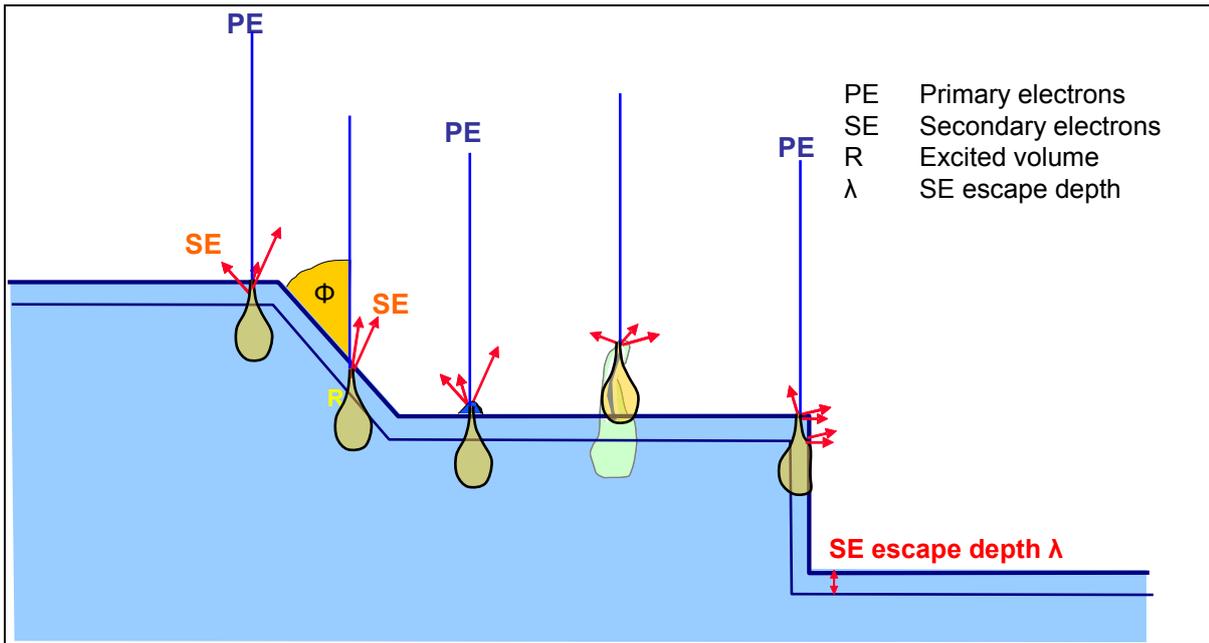


Fig. 5.4: The coating of the specimen with heavy metals localizes the signal to the surface. The yield of SE deriving directly from the spot of the primary electron beam (PE) is high. The different number of SE escaping the specimen at different spots represents the contrast. The contrast is a function of the angle between primary beam and the specimen surface when the proper acceleration voltage is selected.

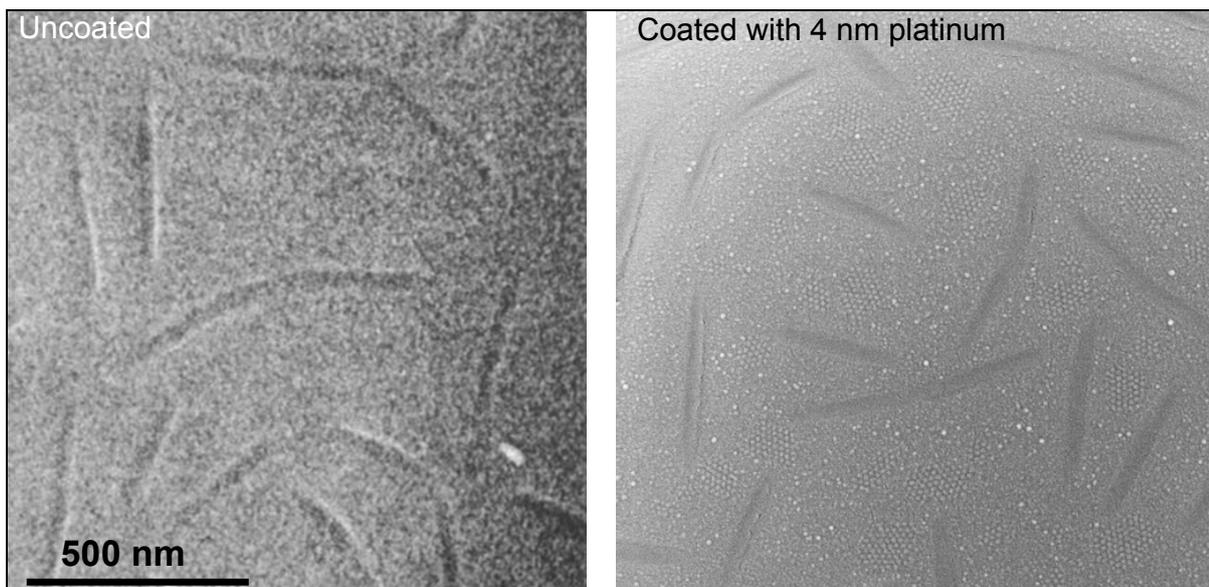


Fig. 5.5: The images represent a view on the inner surface layer of the plasma membrane of freeze-fractured yeast. Left: The surface was not coated and therefore appears noisy, unsharp and detail structures are obscured. Only invaginations of the membrane are recognizable. Right: The surface was coated with 4 nm of platinum localizing the signal to the surface providing a sharp signal at a high signal to noise ratio. Detail structures like hexagonal protein patterns are visible (bright spots are ice contaminations).

### Imaging using backscattered electrons

Backscattered electrons (BSE) are also used to image the surface of the specimens but also for detection of differences in the specimen composition. For detection of BSE, a different arrangement of the detector is applied. No collector grid is necessary, since the BSE have the same energy as the primary electron beam (much higher voltages than 50 eV of the SE). The BSE preferably are directed back towards the objective lens. Therefore, the BSE detector is located just beneath the objective lens (figure 5.6).

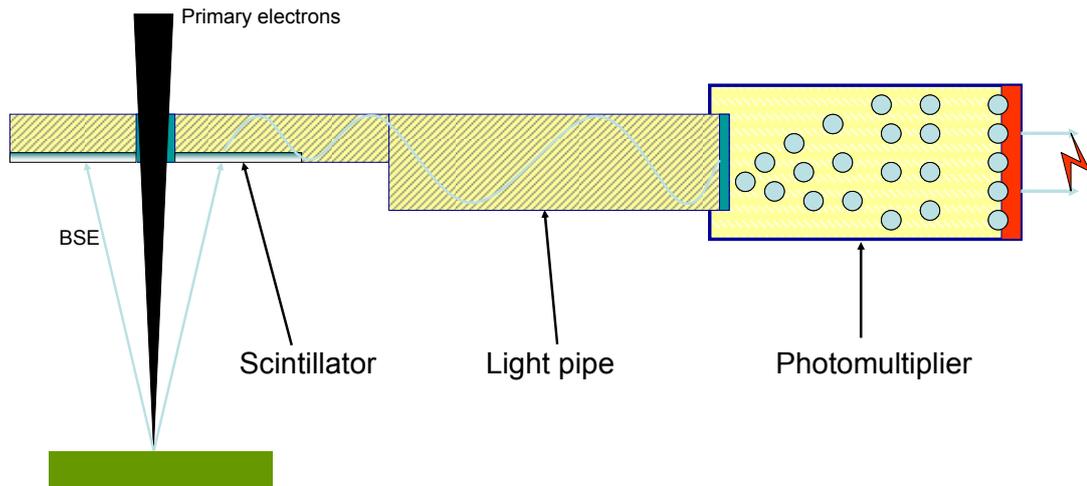


Fig. 5.6: Arrangement and working principle of a BSE detector. The scintillator is arranged circular around the primary beam just beneath the objective lens. BSE hit the scintillator and produce photons, which are guided to the photomultiplier tube. As for the SE detector, the signal is amplified, converted to a gray level and displayed on the computer screen.

Figure 5.7 is an example, how the BSE detector can be used to get additional information of the specimen, e.g. detection of gold particles on the surface of red blood cells.

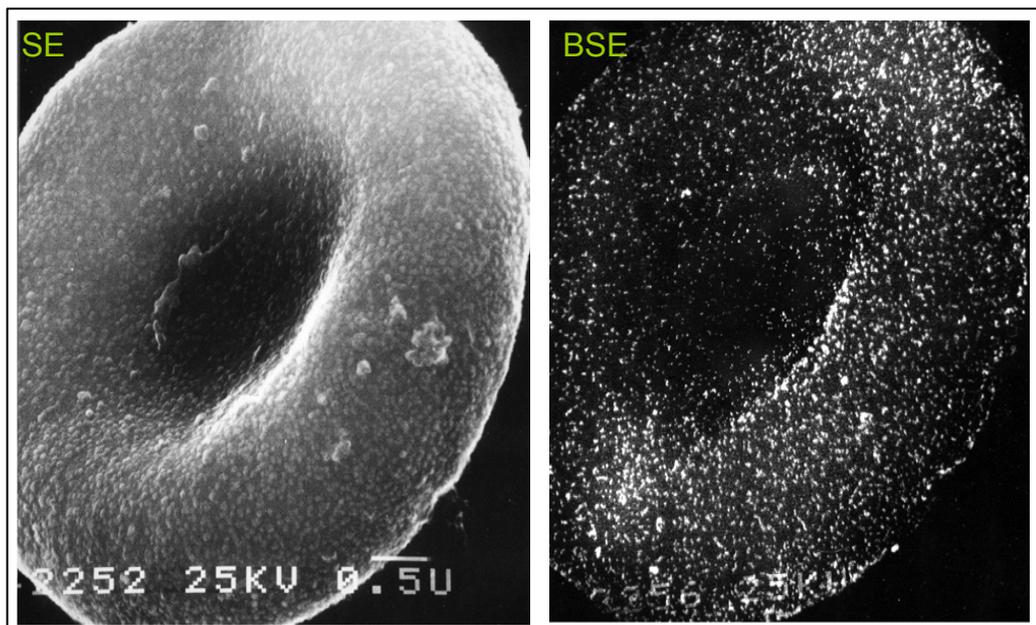


Fig. 5.7. Red blood cells labelled against surface proteins. The antibody is connected to a gold particle (heavy metal aggregation). Whereas the SE signal provides the surface topography, the BSE image distinctly shows the gold particles. The images were recorded simultaneously at 25 kV acceleration voltage. The whole sample is coated with a thin layer of heavy metal (ca. 2 nm). SE derive mainly from this layer and therefore do not clearly accentuate the gold particles, which have a size of around 20 nm. On the contrary, a lot of electrons are backscattered where the gold particles are, but only very few are backscattered from the thin metal coat.

### Elemental analysis using x-ray

X-rays produced in the specimen can be used for elemental analysis of the specimen (Fig 5.8). The x-ray energy is specific for different elements. A dedicated x-ray detector detects the x-rays deriving from every spot of the sample and assigns the according element. Elemental mapping is possible for any area of interest. In particular the interpretation of x-ray spectra is very challenging and needs a lot of experience. A highly corrugated surface of a specimen – typical biological specimen - can lead to misinterpretation. Quantitative analysis with biological specimens is usually not possible.

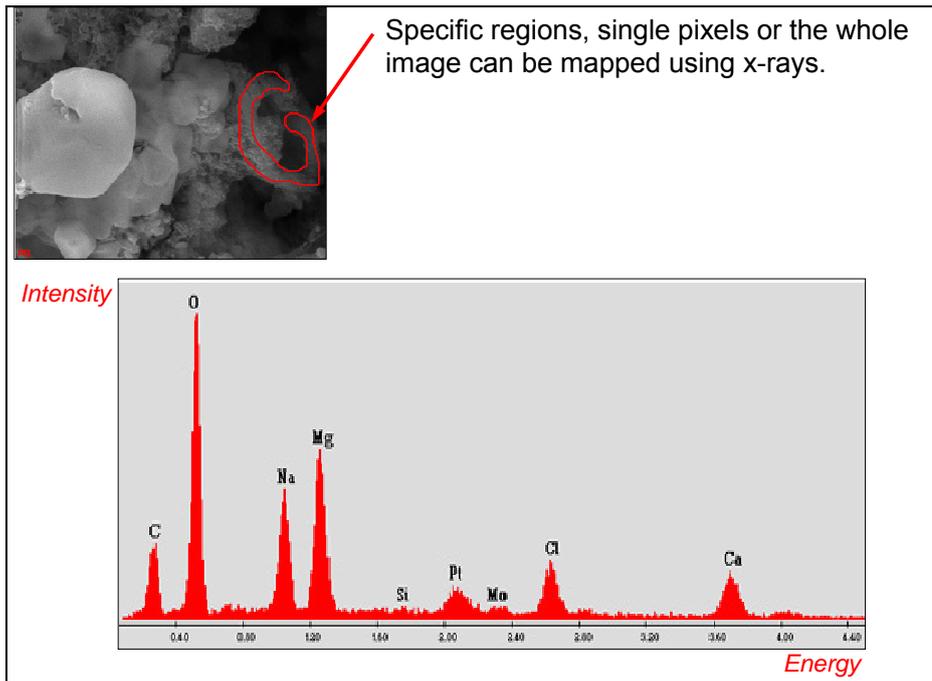


Fig 5.8: Elemental analysis using x-rays. The elemental spectrum derives from the red region in the image.

## Artefacts

Imaging in the scanning electron microscope can lead to a lot of artefacts. When the beam penetrates the specimen, charge is introduced into the material and must be dissipated in order to prevent charging. A typical charging artefact is shown in figure 5.9. Note: The coating of the specimen with a thin heavy metal layer not only locates the signal to the surface but also adds a conducting layer on non-conducting specimens.

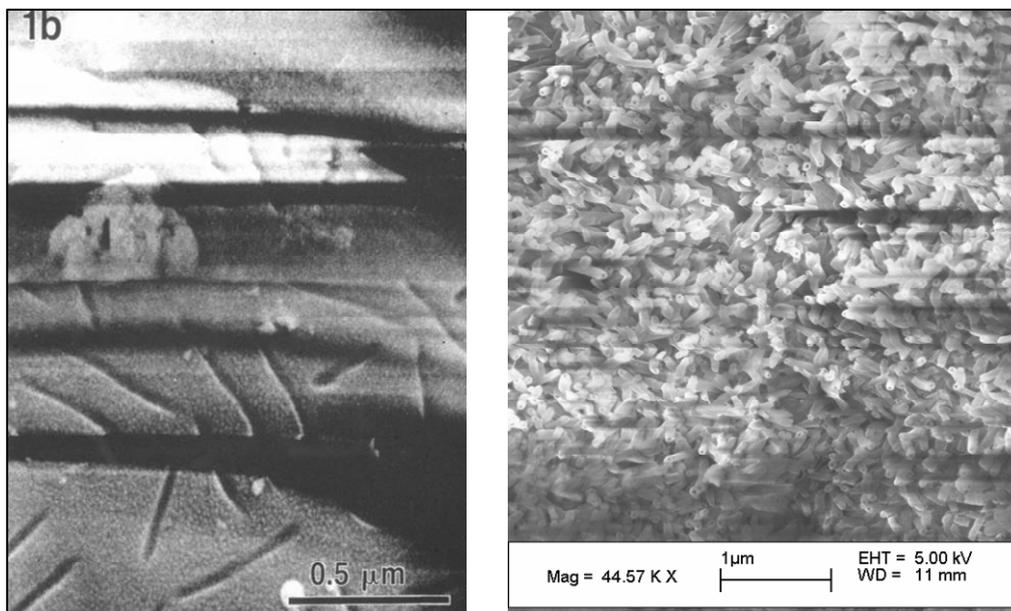
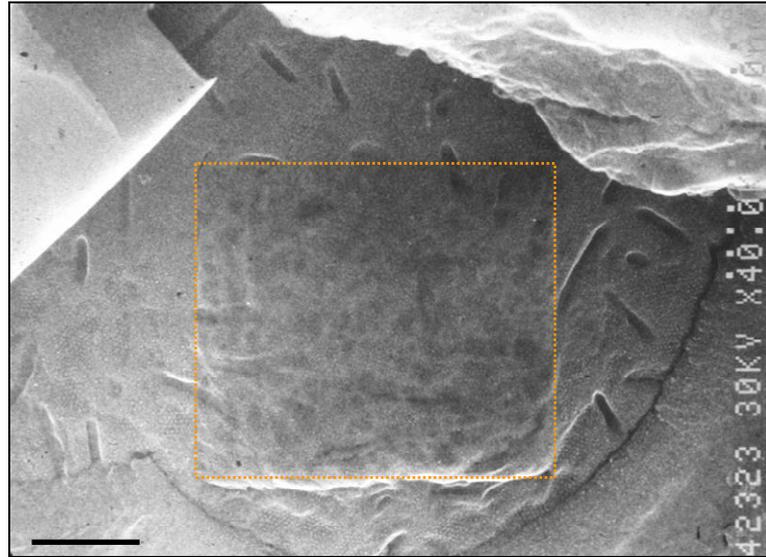


Fig 5.9: Charging artefacts on the surface of a freeze-fractured plasma membrane of yeast (left) and a waxy surface of a lotus leaf (right)

Beam damage is another artefact which often is encountered. The electron beam can locally heat the sample and destroy the surface in many different ways, figure 5.10.



*Fig. 5.10: Beam damage on the surface of a freeze-fractured, frozen-hydrated yeast surface after one scan. Scale bar 500 nm.*

## 6. Sample preparation

As we have discussed in the earlier sections, electron microscopes are run under high vacuum conditions. A high vacuum, however, is a very unfriendly environment for biological specimens like cells and tissues (or condensed organic hydrated matter in general). Electron microscopes pose the following prerequisites to the specimen for investigation.

- Resistant in the vacuum
- Electron beam resistant
- Providing contrast
- Penetrable for electrons (transmission electron microscope)

Large biological specimens (cells, tissues) do not fulfill any of these requirements and have to be treated in order to be investigated in the electron microscope. Typically, the specimen is turned into a solid state to make it suitable for vacuum and resistant in the electron beam. Moreover, it has to be cut to very thin sections of ca. 100 nm for transmission electron microscopy. Such a specimen still does not provide contrast. Biological material is basically composed of light elements like C, O, H, N, S, P, which do not interact with electrons. Selective staining or contrast enhancement with heavy metals is required to form an image as already pointed out in chapters 4 and 5 (contrast formation and imaging in TEM and SEM).

### Preparation of cells and tissues for transmission electron microscopy

Typical preparation procedures for cells or tissues for transmission electron microscopy include the steps depicted in figure 6.1.

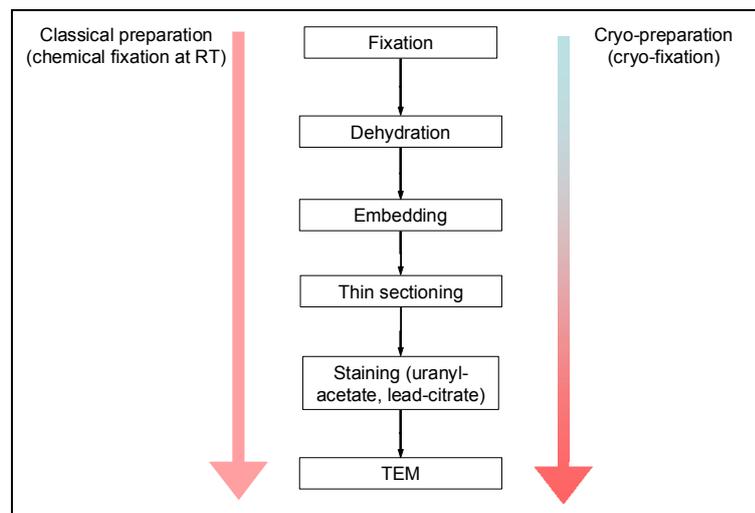
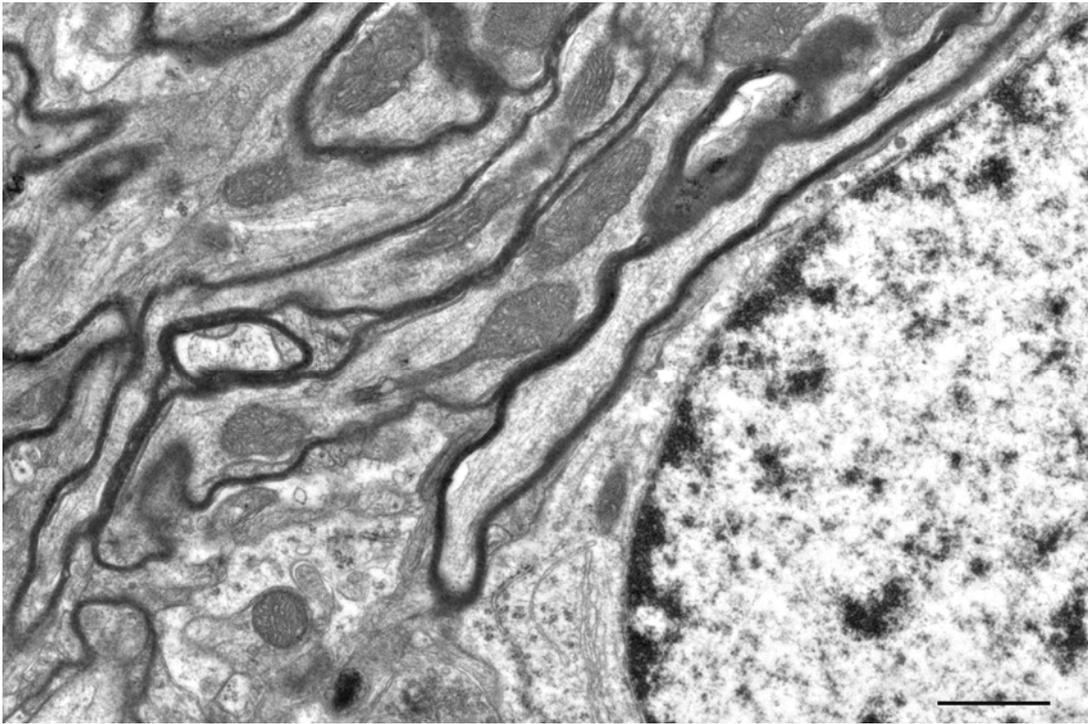


Figure 6.1: Left: Classical preparation pathway for transmission electron microscopy. The whole process is performed at room temperature. Right: Cryo-preparation pathway for transmission electron microscopy. The specimen is frozen and slowly warmed up during freeze-substitution (low temperature dehydration).

The classical preparation is performed at room temperature. During fixation, the native biological specimen is chemically stabilized with chemical fixatives like glutaraldehyde and osmium tetroxide. All biological processes are arrested during fixation. The fixed specimen is ready for dehydration with a sequence of different ethanol concentrations until it is completely dehydrated. Subsequently, the ethanol is exchanged with a monomer solution of a plastic formulation (e.g. Epon/Araldite) in a sequence of rising concentrations of plastic components dissolved in ethanol or acetone. The monomers are polymerized by heat or UV light and provide a hard plastic block containing the embedded specimen. This enables cutting of thin sections (100 nm) in an ultramicrotome. The sections are stained with uranyl-acetate and lead citrate. Uranium and lead ions selectively bind to lipids, proteins and nucleic acid in the specimen and provide a distribution of electron dense material and finally the image in the electron microscope (Figure 6.2).



*Figure 6.2: TEM micrograph of a thin section of classically prepared cerebellar slice culture of mouse. Scale bar 1  $\mu\text{m}$ .*

A lot of artifacts are introduced during classical TEM preparation. The fixation with chemicals is slow (seconds to minutes) and the specimen has enough time to react to the chemical attack and undergoes changes. The whole preparation procedure is conducted at room temperature leading to extraction and shrinkage of material during dehydration and embedding.

Cryo preparation methods were introduced and developed in order to prevent or reduce these artifacts. In a first step, the specimen is generally frozen without chemical pretreatment (pure physical process) – cryo-fixed. Freezing is very fast and can be performed within milliseconds. However, the freezing is a challenging process because of the nature of water to form ice crystals during freezing. Ice crystal segregations distort and damage the ultra structure. High-pressure freezing was developed to prevent ice crystal formation by changing the physical properties of the water at 2100 bar just before freezing. Despite this technique, satisfying freezing quality (Figure 6.3) is achieved only for biological specimens with a thickness of less than 200  $\mu\text{m}$ .

Once the specimen is frozen, it is dehydrated and fixed with chemical fixatives simultaneously at very low temperatures (e.g.,  $-90^{\circ}\text{C}$ , gradient to room temperature) in a process called freeze-substitution. The embedding in plastic, sectioning, staining of the specimen is typically performed the same way as during classical preparation.

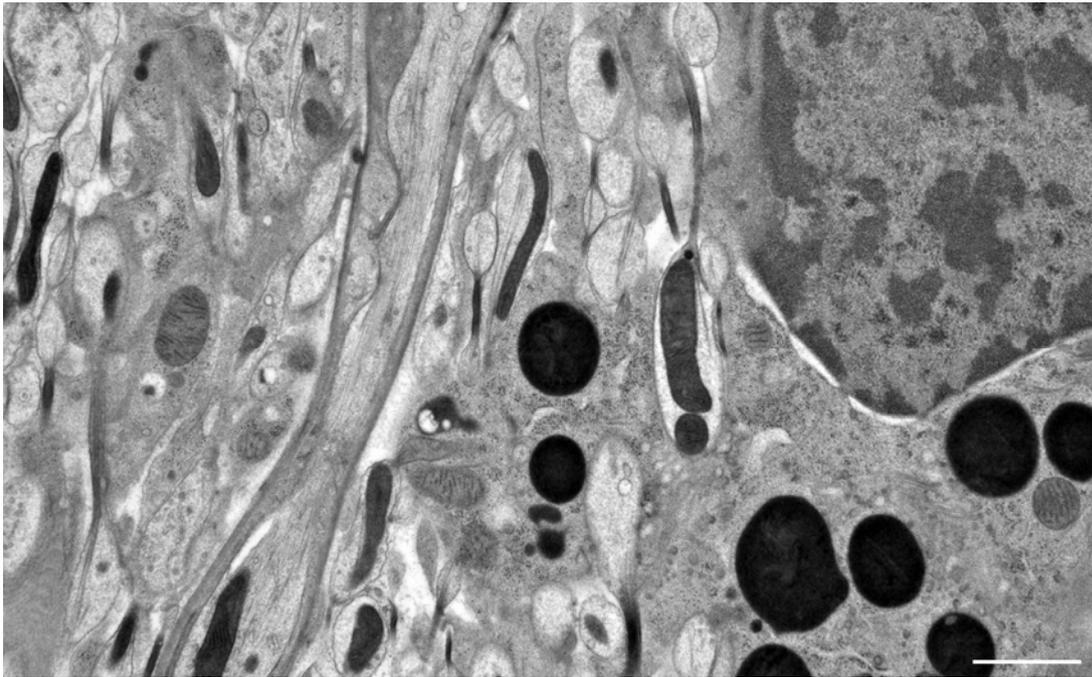


Figure 6.3: TEM micrograph of a thin section of a high-pressure frozen, freeze-substituted cerebellar slice culture of mouse. Scale bar 1  $\mu\text{m}$ .

#### Negative staining

Single particles like viruses, proteins or other small particles can be prepared in a much simpler way than cells or tissues by a process called negative staining. A few micro liters of an adequately concentrated suspension containing the particles are pipetted on a carbon coated TEM grid. The suspension is removed with a filter paper after a short incubation time (seconds to minutes) and washed with a droplet of water. The particles attach to the carbon layer of the TEM grid. Subsequently, a droplet of an aqueous heavy metal solution (tungsten, uranium ions) is added on the grid. Again, the heavy metal solution is removed with a filter paper after an incubation time of seconds or minutes and the grid is dried in the air and ready for investigation in the transmission electron microscope. The heavy metal ions aggregate around and on the surface of the particles according to their topography and provide a “negative contrast” (Figure 6.4). The whole processes of fixation, dehydration and embedding can be omitted, making this technique a powerful tool for investigation of single particles at high resolution.

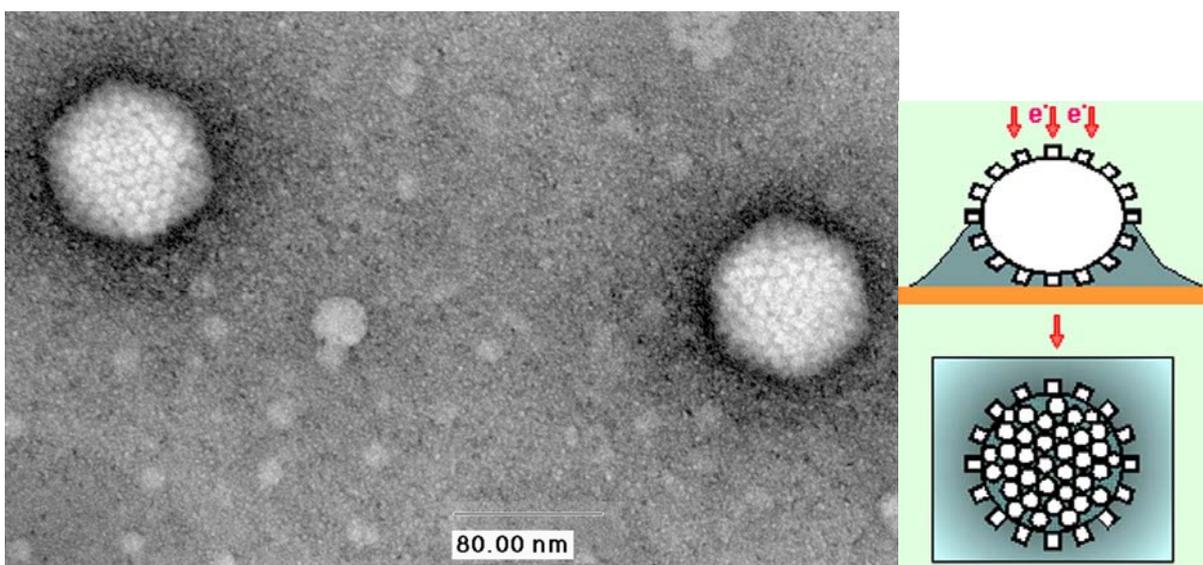


Figure 6.4: TEM micrograph of adenoviruses prepared by negative staining. The principle of negative staining and image formation is shown in the drawing on the right side.

## Preparation of cells and tissues for scanning electron microscopy

Typical preparation procedures for cells or tissues for scanning electron microscopy include the steps depicted in figure 6.5.

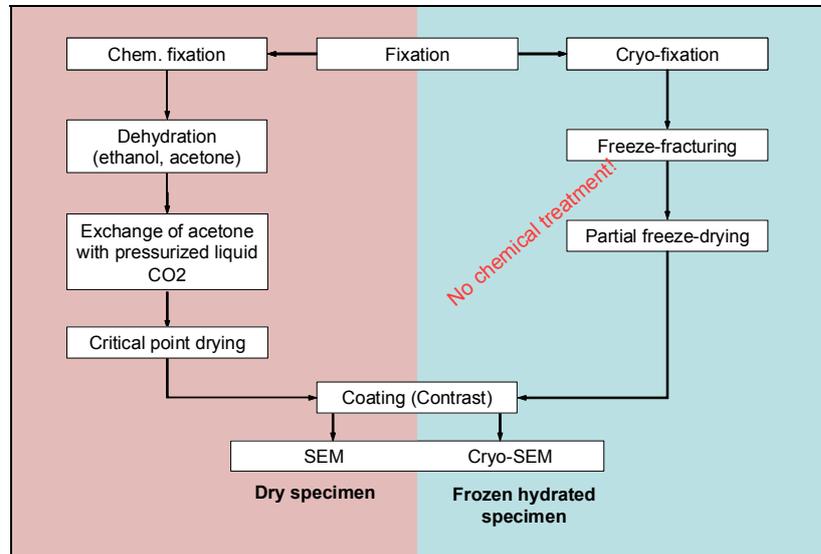


Figure 6.5: Left: Classical preparation pathway for scanning electron microscopy. The specimen is chemically fixed and dehydrated as described for classical preparation for TEM, followed by critical point drying and coating. Right: Cryo preparation pathway. The specimen is frozen, freeze-fractured, partially freeze-dried, coated and imaged in the cold state in the SEM (Cryo-SEM). The specimen always stays at low temperature and undergoes physical treatment only.

The classical preparation procedure for scanning electron microscopy involves critical point drying (left path in figure 6.5). Air drying of specimens at room temperature leads to a collapse of the sample structure caused by the high surface tension of water. Critical point drying was developed in order to prevent the destroying forces of the surface tension. The first steps including fixation and dehydration are performed the same way as described for the classical preparation for TEM. After dehydration, the ethanol is exchanged with acetone. In a dedicated device (critical point dryer) the acetone is exchanged with liquid pressurized carbon dioxide. Subsequently, the temperature and the pressure in the chamber of the critical point dryer are increased until they reach the critical point of CO<sub>2</sub> (31°C, 74 bar). The CO<sub>2</sub> in the critical state (neither gas nor liquid) is released from the chamber very slowly providing a gently dried specimen. The process is shown in figure 6.6.

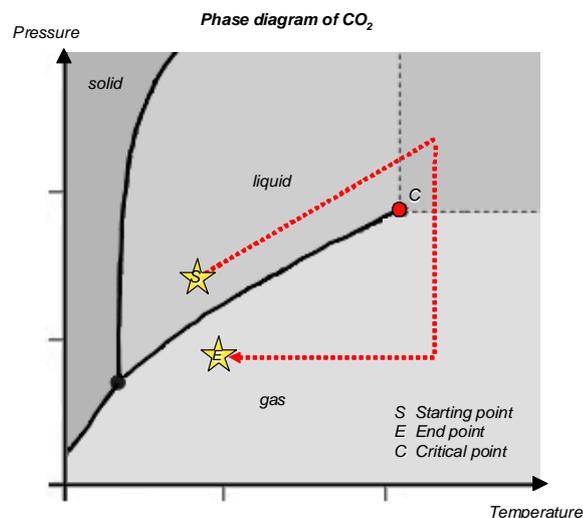


Figure 6.6: Phase diagram of carbon dioxide. The red line in the panel shows the process of critical point drying.

The critical point dried specimen is coated with a thin layer of heavy metal (e.g. 2 nm) by sputter coating or electron beam evaporation. The heavy metal layer renders the specimen conductive and localizes the signal in the scanning electron microscope to the surface providing the required yield of electrons and contrast for imaging (Figure 6.7).

Note: The critical point of water is at 374°C and 221 bar. Critical point drying is not possible directly with the aqueous specimens since it would be destroyed at the critical point conditions required for water.



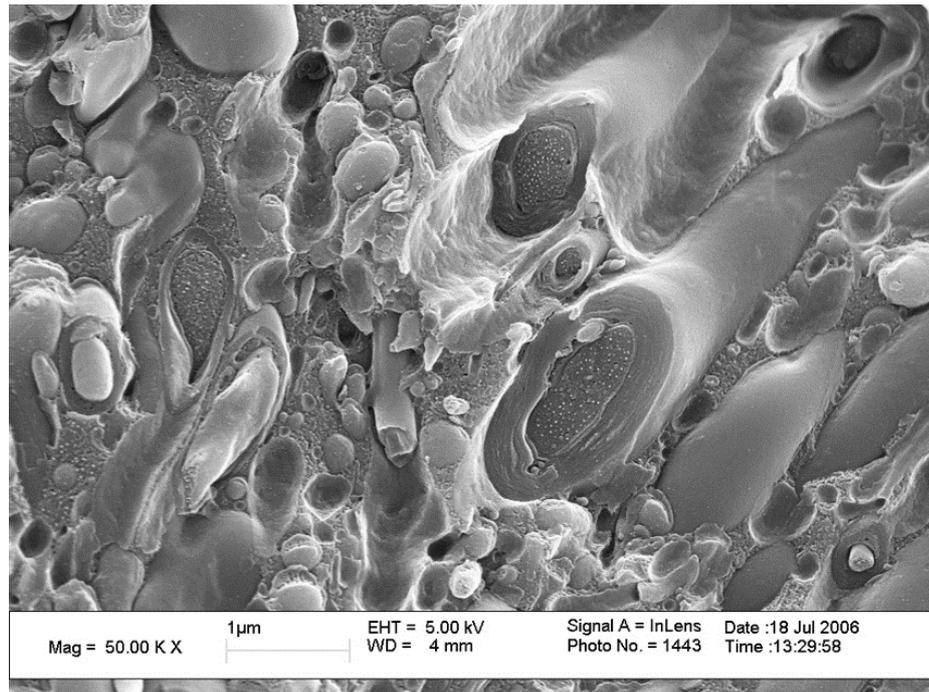
Figure 6.7: Critical point dried liver tissue, coated with platinum. Blood vessel with erythrocytes and leukocyte.

Similar artifacts as during classical TEM preparation are introduced during this process since it involves as well chemical fixation and dehydration at room temperature. Many of these artifacts can be prevented again by using cryo techniques for SEM (right path in figure 6.5). The freezing or cryo immobilization is the same as described for cryo preparation for TEM. Once the specimen is adequately frozen, it is fractured at low temperatures (e.g. -120°C) in a dedicated freeze-fracturing machine. The fracturing through the specimen is a random process providing cross-fracture planes as well as fracture planes through biological membranes (figure 6.8).



Figure 6.8: Freeze-fracturing of biological specimens. PF...plasmatic fracture face of the lipid bilayer. EF...Exoplasmatic fracture face of the lipid bilayer. Cyt...Cytosol, the cytosol is cross-fractured in the cell on the right.

After fracturing, the visibility of the ultrastructure can be enhanced by sublimation of small amounts of the frozen water (partial freeze drying). The fracture face of the specimen finally is coated with a heavy metal layer at low temperature for imaging in the cryo scanning electron microscope (figure 6.9). Note: This preparation procedure exclusively involves physical processes. The specimen is not treated chemically and stays at low temperatures through the whole process of preparation and imaging.



*Figure 6.9: Cryo-SEM image of high-pressure frozen mouse brain tissue, freeze-fractured, partially freeze dried and coated with platinum.*

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