Microscopes

A **microscope** (from the Greek: μικρός, *mikrós*, "small" and σκοπεῖν, *skopeîn*, "to look" or "see") is an instrument for viewing objects that are too small to be seen by the naked or unaided eye. The science of investigating small objects using such an instrument is called **microscopy**. The term **microscopic** means minute or very small, not visible with the eye unless aided by a microscope.

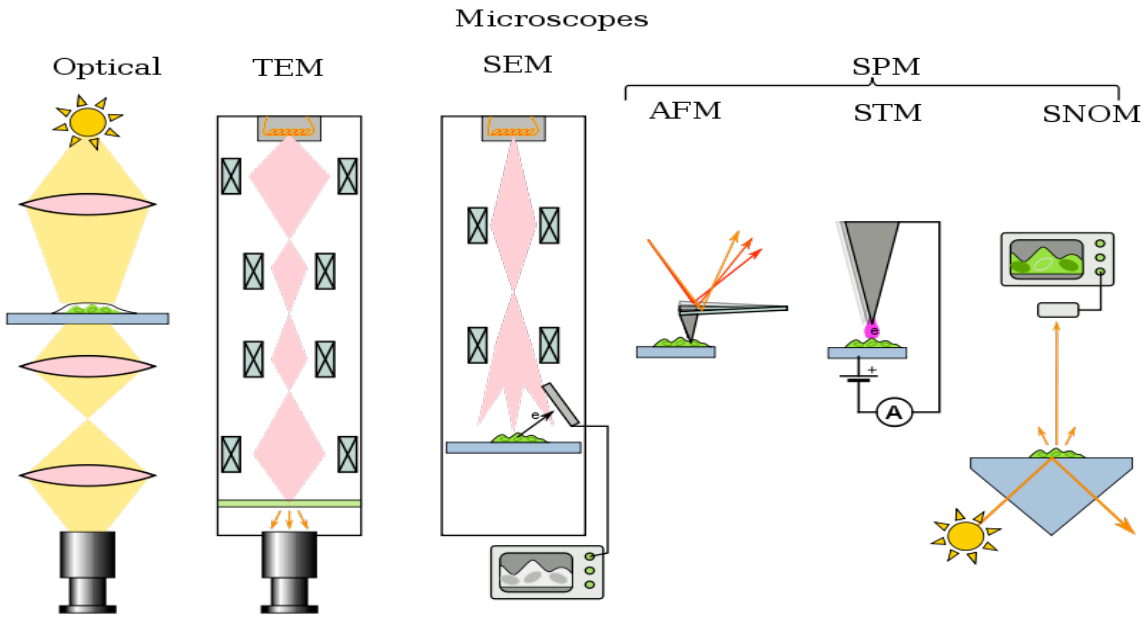
Contents

1. History of Microscopes

The first true microscope was made in 1590 in Middelburg, The Netherlands.[1] Three different eyeglass makers have been given credit for the invention: Hans Lippershey (who also developed the first real telescope); Sacharias Jansen; with the help of his father, Hans Janssen. The coining of the name "microscope" has been credited to Giovanni Faber, who gave that name to Galileo Galilei's compound microscope in 1625.[2] (Galileo had called it the "*occhiolino*" or "*little eye*".)

The most common type of microscope—and the first to be invented—is the optical microscope. This is an optical instrument containing one or more lenses that produce an enlarged image of an object placed in the focal plane of the lens(es). There are, however, many other microscope designs.

2. Types of Microscopes



Several types of microscopes

"Microscopes" can largely be separated into three classes: optical theory microscopes (Light microscope), electron microscopes (e.g.,TEM), and scanning probe microscopes (SPM). Optical microscopes are microscopes which function through the optical theory of lenses in order to magnify the image generated by the passage of a wave through the sample, or reflected by the sample. The waves used are either electromagnetic (in optical microscopes) or electron beams (in electron microscopes). The types are the Compound Light, Stereo, and the electron microscope.

3. Optical Microscope

The **optical microscope**, often referred to as the "light microscope", is a type of microscope which uses visible light and a system of lenses to magnify images of small samples. Optical microscopes are the oldest and simplest of the microscopes. However, new designs of digital microscopes are now available which use a CCD camera to examine a sample and the image is shown directly on a computer screen without the need for expensive optics such as eye-pieces. Other microscopic methods which do not use visible light include scanning electron microscopy and transmission electron microscopy.

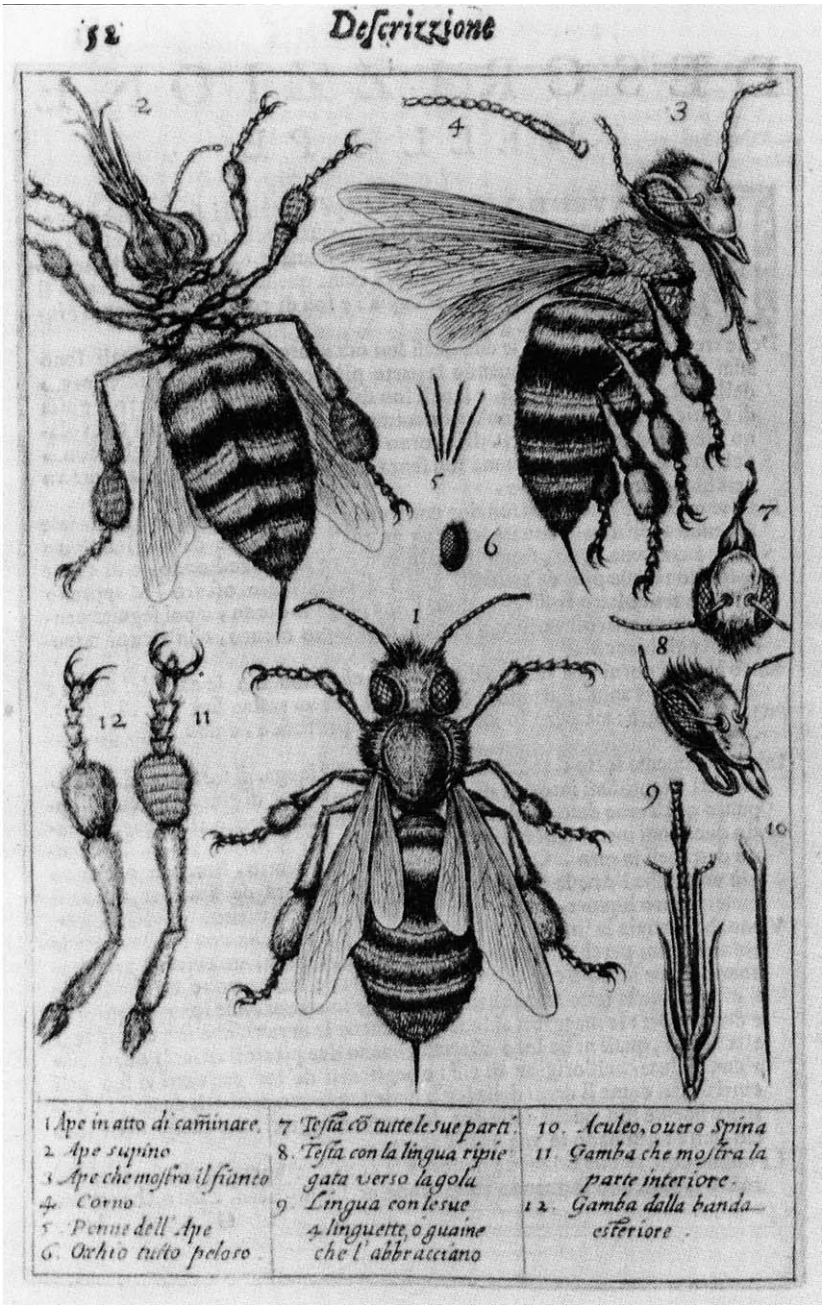
3.1 Optical configurations

There are two basic configurations of the conventional optical microscope in use, the simple (one lens) and compound (many lenses). Digital microscopes are based on an entirely different system of collecting the reflected light from a sample.

3.2 Light microscope

A *simple microscope* is a microscope that uses only one lens for magnification, and is the original light microscope. Van Leeuwenhoek's microscopes consisted of a small, single converging lens mounted on a brass plate, with a screw mechanism to hold the sample or specimen to be examined. Demonstrations by British microscopist have images from such basic instruments. Though now considered primitive, the use of a single, convex lens for viewing is still found in simple magnification devices, such as the magnifying glass, and the loupe. Light microscopes are able to view specimens in colour, an important advantage when compared with electron microscopes, especially for forensic analysis, where blood traces may be important, for example.

3.3 History of Light Microscope



The oldest published image known to have been made with a microscope: bees by Francesco Stelluti, 1630[1]

The earliest evidence of magnifying glass forming a magnified image dates back to the *Book of Optics* published by Ibn al-Haytham (Alhazen) in 1021. After the book was translated into Latin, Roger Bacon described the properties of magnifying glass in 13th-century England, followed by the development of eyeglasses in 13th-century Italy.[2]

It is difficult to say who invented the compound microscope. Dutch spectacle-makers Hans Janssen and his son Zacharias Janssen are often said to have invented the first compound microscope in 1590, but this was a declaration

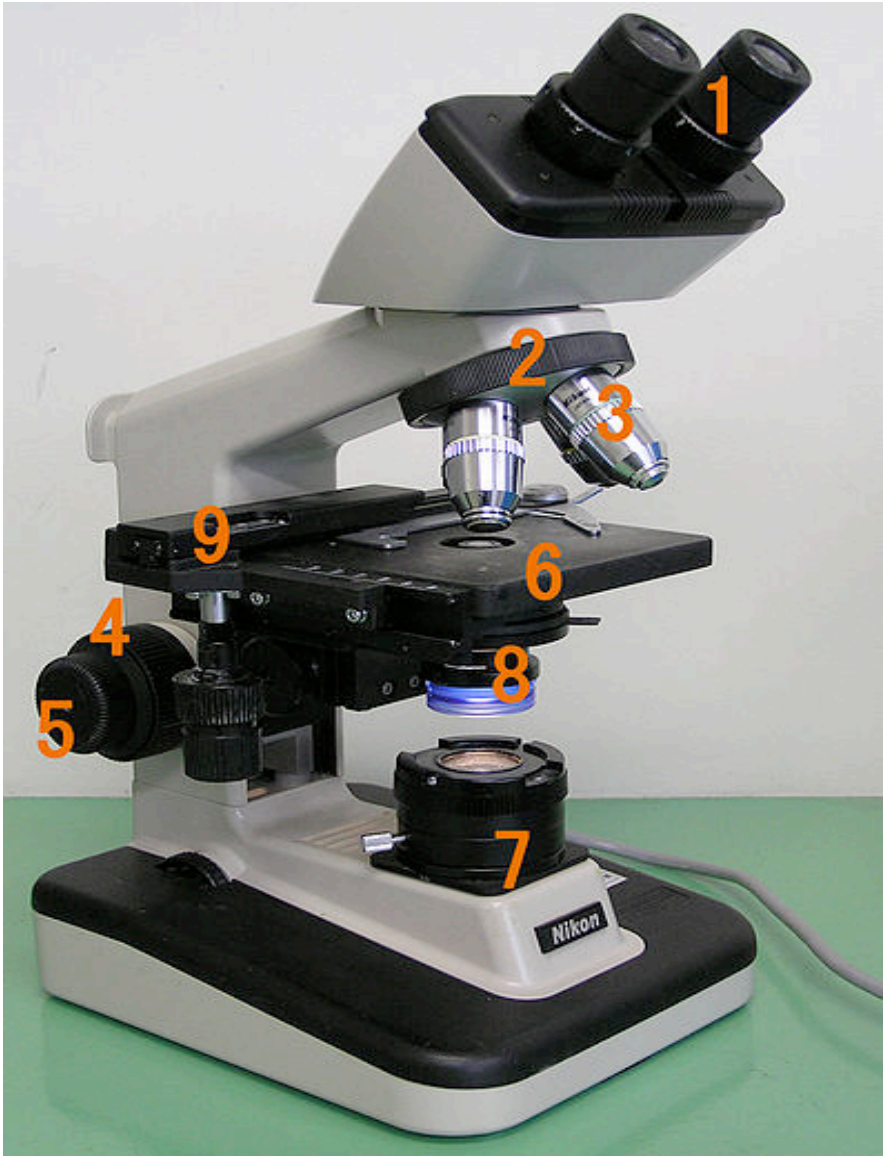
made by Zacharias Janssen himself during the mid 1600s. The date is unlikely, as it has been shown that Zacharias Janssen actually was born around 1590. Another favorite for the title of 'inventor of the microscope' was Galileo Galilei. He developed an *occhiolino* or compound microscope with a convex and a concave lens in 1609. Galileo's microscope was celebrated in the Accademia dei Lincei in 1624 and was the first such device to be given the name "microscope" a year latter by fellow Lincean Giovanni Faber. Faber coined the name from the Greek words *μικρόν* (micron) meaning "small", and *σκοπεῖν* (skopein) meaning "to look at", a name meant to be analogus with "telescope", another word coined by the Linceans[3].

Christiaan Huygens, another Dutchman, developed a simple 2-lens ocular system in the late 1600s that was achromatically corrected, and therefore a huge step forward in microscope development. The Huygens ocular is still being produced to this day, but suffers from a small field size, and other minor problems.

Anton van Leeuwenhoek (1632-1723) is credited with bringing the microscope to the attention of biologists, even though simple magnifying lenses were already being produced in the 1500s. Van Leeuwenhoek's home-made microscopes were very small simple instruments, with a single, yet strong lens. They were awkward in use, but enabled van Leeuwenhoek to see detailed images. It took about 150 years of optical development before the compound microscope was able to provide the same quality image as van Leeuwenhoek's simple microscopes, due to timely difficulties of configuring multiple lenses. Still, despite widespread claims, van Leeuwenhoek is not the inventor of the microscope.

Anton Van Leeuwenhoek's *new, improved microscope allowed people to see things no human had ever seen before.*

3.4 Components of a Light Microscope



Basic optical transmission microscope elements(1990's)
1 ocular lens, or eyepiece
2 objective turret
3 objective lenses
4 coarse adjustment knob
5 fine adjustment knob
6 object holder or stage
7 mirror or light (illuminator)
8 diaphragm and condenser

All optical microscopes share the same basic components:

The eyepiece - a cylinder containing two or more lenses to bring the image to focus for the eye. The eyepiece is inserted into the top end of the body tube. Eyepieces are interchangeable and many different eyepieces

can be inserted with different degrees of magnification. Typical magnification values for eyepieces include 5x, 10x and 2x. In some high performance microscopes, the optical configuration of the objective lens and eyepiece are matched to give the best possible optical performance. This occurs most commonly with apochromatic objectives.

- The objective lens - a cylinder containing one or more lenses, typically made of glass, to collect light from the sample. At the lower end of the microscope tube one or more objective lenses are screwed into a circular nose piece which may be rotated to select the required objective lens. Typical magnification values of objective lenses are 4x, 5x, 10x, 20x, 40x, 50x and 100x. Some high performance objective lenses may require matched eyepieces to deliver the best optical performance.
- The stage - a platform below the objective which supports the specimen being viewed. In the center of the stage is a hole through which light passes to illuminate the specimen. The stage usually has arms to hold *slides* (rectangular glass plates with typical dimensions of 25 mm by 75 mm, on which the specimen is mounted).
- The illumination source - below the stage, light is provided and controlled in a variety of ways. At its simplest, daylight is directed via a mirror. Most microscopes, however, have their own controllable light source that is focused through an optical device called a condenser, with diaphragms and filters available to manage the quality and intensity of the light.

The whole of the optical assembly is attached to a rigid arm which in turn is attached to a robust U shaped foot to provide the necessary rigidity. The arm is usually able to pivot on its joint with the foot to allow the viewing angle to be adjusted. Mounted on the arm are controls for focusing, typically a large knurled wheel to adjust coarse focus, together with a smaller knurled wheel to control fine focus.

Updated microscopes may have many more features, including reflected light (incident) illumination, fluorescence microscopy, phase contrast microscopy and differential interference contrast microscopy, spectroscopy, automation, and digital imaging.

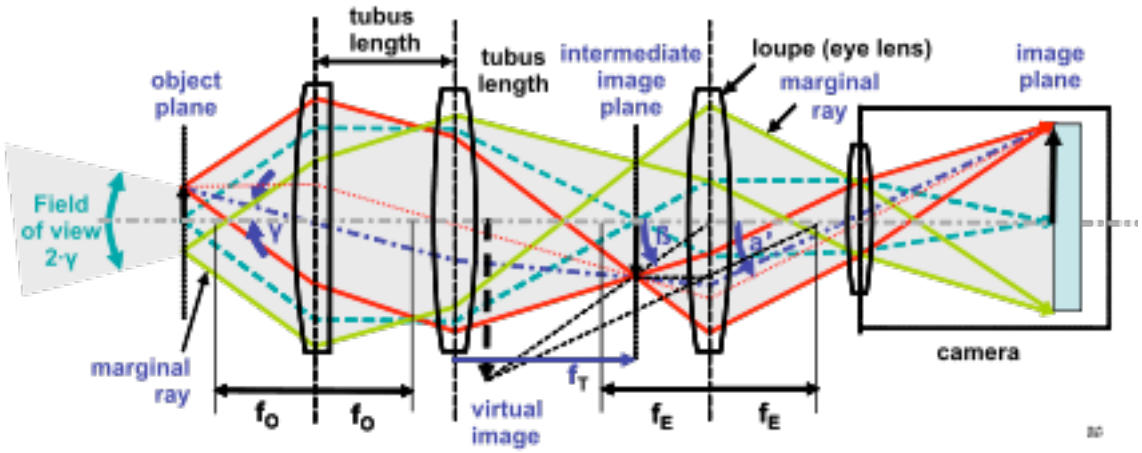
On a typical compound optical microscope, there are three objective lenses: a scanning lens (4×), low power lens (10×)and high power lens (ranging from 20 to 100×). Some microscopes have a fourth objective lens, called an oil immersion lens. To use this lens, a drop of immersion oil is placed on top of

the cover slip, and the lens is very carefully lowered until the front objective element is immersed in the oil film. Such immersion lenses are designed so that the refractive index of the oil and of the cover slip are closely matched so that the light is transmitted from the specimen to the outer face of the objective lens with minimal refraction. An oil immersion lens usually has a magnification of 50 to 100×.

The actual power or magnification of an optical microscope is the product of the powers of the ocular (eyepiece), usually about 10×, and the objective lens being used.

Compound optical microscopes can produce a magnified image of a specimen up to 1000× and, at high magnifications, are used to study thin specimens as they have a very limited depth of field.

3.5 LM Operation



Optical path in a typical microscope

The optical components of a modern microscope are very complex and for a microscope to work well, the whole optical path has to be very accurately set up and controlled. Despite this, the basic optical principles of a microscope are quite simple.

The objective lens is, at its simplest, a very high powered magnifying glass *i.e.* a lens with a very short focal length. This is brought very close to the specimen being examined so that the light from the specimen comes to a focus about 160 mm inside the microscope tube. This creates an enlarged image of the subject. This image is inverted and can be seen by removing the eyepiece and placing a piece of tracing paper over the end of the tube. By carefully focusing a brightly lit specimen, a highly enlarged image can be seen. It is this real image that is viewed by the eyepiece lens that provides further enlargement.

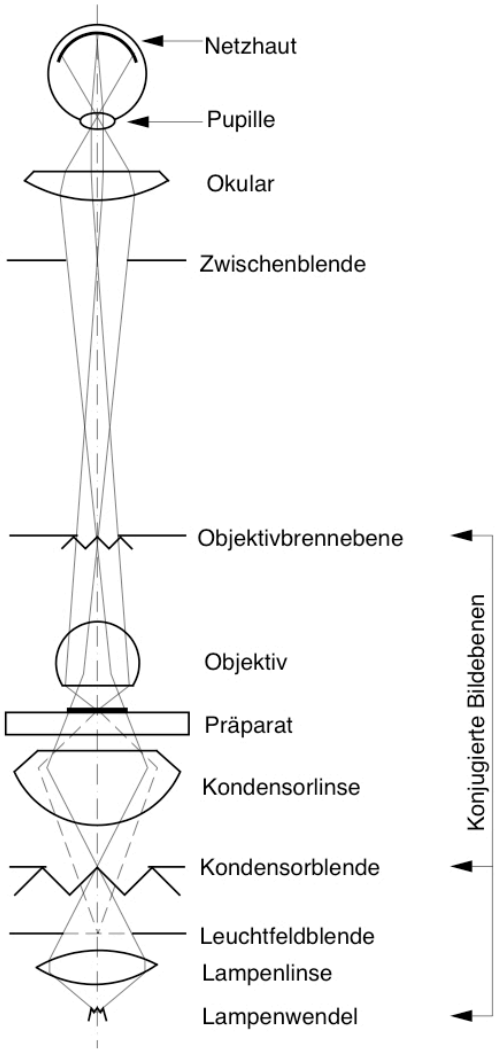
In most microscopes, the eyepiece is a compound lens, with one component lens near the front and one near the back of the eyepiece tube. This forms an air-separated couplet. In many designs, the virtual image comes to a focus between the two lenses of the eyepiece, the first lens bringing the real image to a focus and the second lens enabling the eye to focus on the virtual image.

In all microscopes the image is viewed with the eyes focused at infinity (mind that the position of the eye in the above figure is determined by the eye's focus). Headaches and tired eyes after using a microscope are usually signs that the eye is being forced to focus at a close distance rather than at infinity.

3.6 Köhler Illumination

Köhler illumination is a method of specimen illumination used in transmitted- or reflected-light microscopy[1]. It was designed by August Köhler in 1893, and overcame the limitations of previous techniques of sample illumination (ie: critical illumination). Prior to the advent of Köhler illumination, the filament of the bulb used to illuminate the sample could be visible in the sample plane. This created what is known as a filament image. Various techniques were used to remove the filament image, for example lowering the power of the light source, using an opal bulb, or placing an opal glass diffuser in front of the light source. However, all these techniques, although effective in reducing the filament image to a certain degree, had the effect of reducing the quality and uniformity of light reaching the sample. Reducing the power of the light source and introducing an opal bulb both caused a reduction in the spectrum of incident light. For transmitted-light microscopy wide spectrum white light is desirable in order to realize the maximum amount of contrast. Further, adding an opal glass diffuser will cause the light reaching the sample to be uneven. Uniformity of light is essential to avoid shadows, glare, and inadequate contrast when taking photomicrographs. Köhler illumination overcomes these limitations.

Köhler Illumination is used for Bright field microscopy, Phase contrast microscopy, Differential interference contrast microscopy, Dark field microscopy and Polarized light microscopy



3.6.1 Setting Up Köhler Illumination

1. Focus on the specimen.

2. Close the field diaphragm to its most closed state so that you can see the edges of the diaphragm (may be blurry) in the field of view.

3. Use the condenser focus knobs to bring the edges of the field diaphragm into the best focus possible.

4. Use the condenser-centering screws to center the image of the closed field diaphragm in the field of view.

5. Open the field diaphragm just enough so that its edges are just beyond the field of view.

6. Adjust the condenser diaphragm to introduce the proper amount of contrast into your sample. The amount of contrast added will depend on the sample, however too much contrast can introduce artifacts into your images.

7. Adjust the light intensity as necessary. To adjust light intensity it is best to use a neutral density filter rather than increasing or reducing the supply of power to the lightsource. Neutral density filters block all wavelengths of light equally, while changing the power to the light source will alter the balance in the spectrum of incident light giving a yellow/brown appearance to the image.

3.7 Bright field microscopy

Bright field microscopy is the simplest of all the optical microscopy illumination techniques. Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light. The most common use of the microscope involves the use of an organism mounted to a glass microscope slide.

3.7.1 Common components

Base - Supporting structure that usually contains an electrical light source or illuminator.

Objective lens(es)- Magnify the image.

Oculars - Magnify the image from the objective lens. A microscope with one ocular lens is often called a monocular, a microscope with two oculars is called a binocular.

Arm - The support structure that connects the lens systems to the base.

Body tube - Sends light to the ocular lens.

Condenser lens - Directs light to pass through the specimen.

Stage - Platform that allows mechanical movement of a microscope slide.

Adjustment knobs - Course and fine focus adjustment.

The magnification of an optical microscope is only limited by the magnifying power of the lens system. However, the limit of magnification for most light microscopes is 1000x which is set by an intrinsic property of lenses called resolving power.

3.7.2 Advantages

Simplicity of setup with only basic equipment required.

No sample preparation required, allowing viewing of live cells.

3.7.3 Limitations

Very low contrast of most biological samples.

Low apparent optical resolution due to the blur of out of focus material.

3.7.4 Enhancements

Reducing or increasing the amount of the light source via the iris diaphragm.

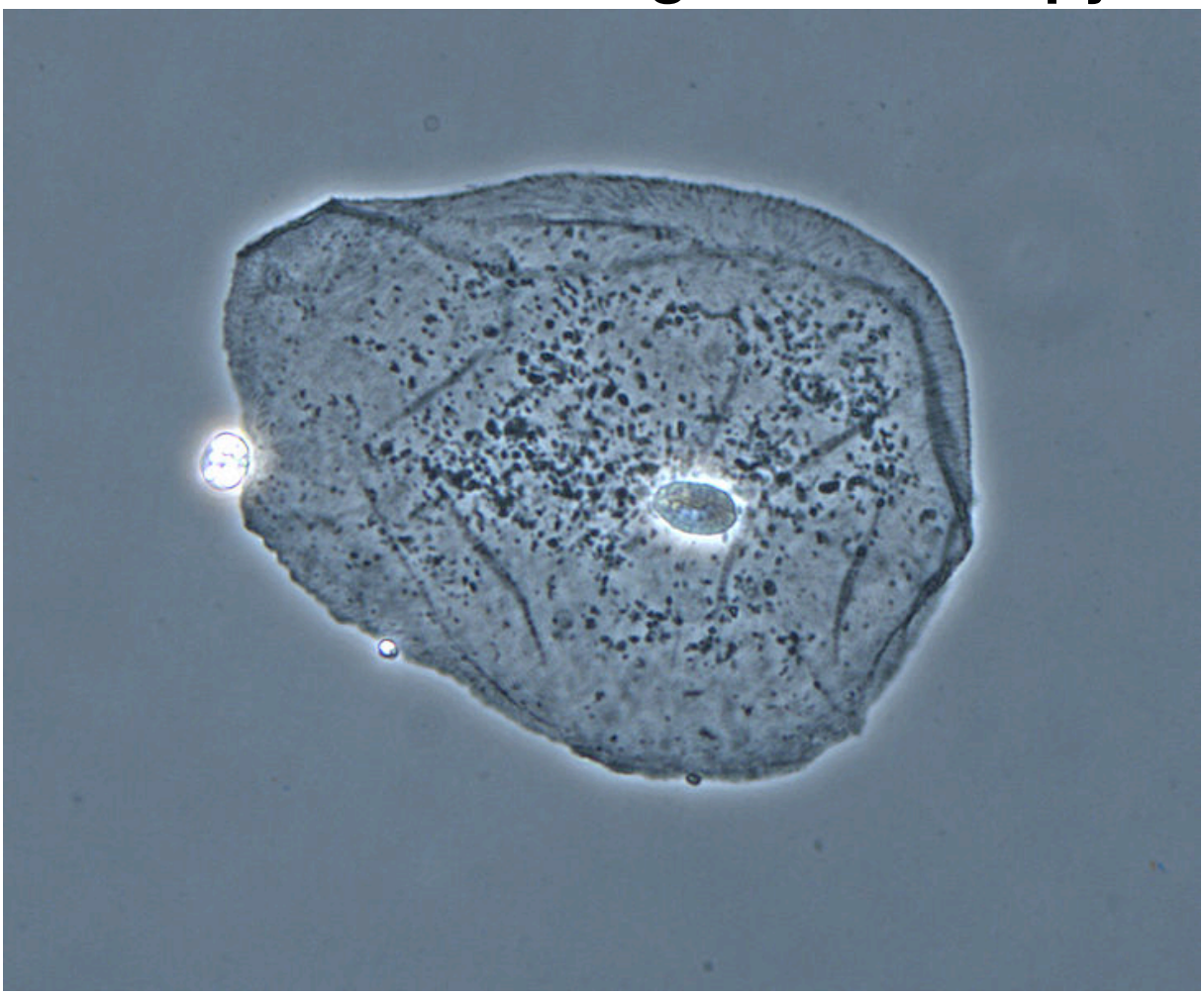
Use of an oil immersion objective lens and a special immersion oil placed on a glass cover over the specimen. Immersion oil has the same refraction

as glass and improves the resolution of the observed specimen.

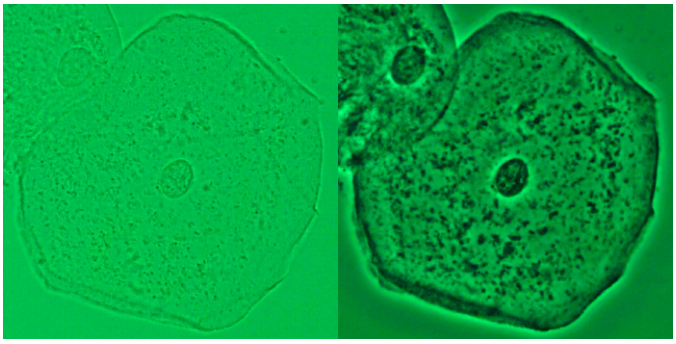
Use of sample staining methods for use in microbiology, such as simple stains (Methylene blue, Safranin, Crystal violet) and differential stains (Negative stains, flagellar stains, endospore stains).

Use of a colored (usually blue) or polarizing filter on the light source to highlight features not visible under white light. The use of filters is especially useful with mineral samples.

3.8 Phase Contrast Light Microscopy



Phase contrast image of a cheek epithelial cell



Epithelial cell in brightfield (BF) using a Plan Fluor 40x lens (NA 0.75) (left) and with phase contrast using a DL Plan Achromat 40x (NA 0.65) (right). A green interference filter is used for both images.

Phase contrast microscopy is an optical microscopy illumination technique in which small phase shifts in the light passing through a transparent specimen are converted into amplitude or contrast changes in the image.

A phase contrast microscope does not require staining to view the slide. This type of microscope made it possible to study the cell cycle.

As light travels through a medium other than vacuum, interaction with this medium causes its amplitude and phase to change in a way which depends on properties of the medium. Changes in amplitude give rise to familiar absorption of light which gives rise to colours when it is wavelength dependent. The human eye measures only the energy of light arriving on the retina, so changes in phase are not easily observed, yet often these changes in phase carry a large amount of information.

The same holds in a typical microscope, i.e., although the phase variations introduced by the sample are preserved by the instrument (at least in the limit of the perfect imaging instrument) this information is lost in the process which measures the light. In order to make phase variations observable, it is necessary to combine the light passing through the sample with a reference so that the resulting interference reveals the phase structure of the sample.

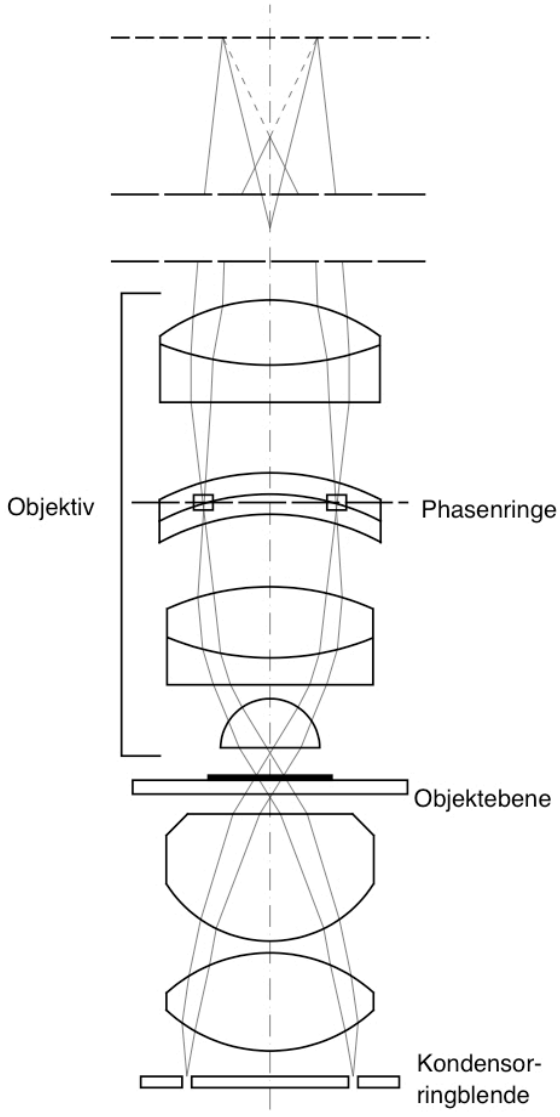
This was first realized by Frits Zernike during his study of diffraction gratings. During these studies he appreciated both that it is necessary to interfere with a reference beam, and that to maximise the contrast achieved with the technique, it is necessary to introduce a phase shift to this reference so that the no-phase-change condition gives rise to completely destructive interference.

He later realised that the same technique can be applied to optical microscopy. The necessary phase shift is introduced by rings etched accurately onto glass plates so that they introduce the required phase shift when inserted into the

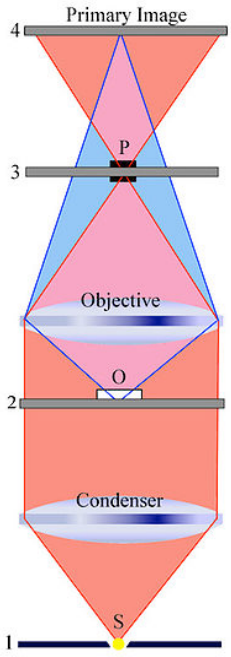
optical path of the microscope. When in use, this technique allows phase of the light passing through the object under study to be inferred from the intensity of the image produced by the microscope. This is the phase-contrast technique.

In optical microscopy many objects such as cell parts in protozoans, bacteria and sperm tails are essentially fully transparent unless stained. (Staining is a difficult and time consuming procedure which sometimes, but not always, destroys or alters the specimen.) The difference in densities and composition within the imaged objects however often give rise to changes in the phase of light passing through them, hence they are sometimes called "phase objects". Using the phase-contrast technique makes these structures visible and allows their study with the specimen still alive.

This phase contrast technique proved to be such an advancement in microscopy that Zernike was awarded the Nobel prize (physics) in 1953.



3.8.1 Explanation



1. *Condenser annulus*
2. *Object plane*
3. *Phase plate*
4. *Primary image plane*

A practical implementation of phase-contrast illumination consists of a phase ring (located in a conjugated aperture plane somewhere behind the front lens element of the objective) and a matching annular ring, which is located in the primary aperture plane (location of the condenser's aperture).

Two selected light rays, which are emitted from one point inside the lamp's filament, get focused by the field lens exactly inside the opening of the condenser annular ring. Since this location is precisely in the front focal plane of the condenser, the two light rays are then refracted in such way that they exit the condenser as parallel rays. Assuming that the two rays in question are neither refracted nor diffracted in the specimen plane (location of microscope slide), they enter the objective as parallel rays. Since all parallel rays are focused in the back focal plane of the objective, the back focal plane is a conjugated aperture plane to the condenser's front focal plane (also location of the condenser annulus). To complete the phase setup, a phase plate is positioned inside the back focal plane in such a way that it lines up nicely with the condenser annulus.

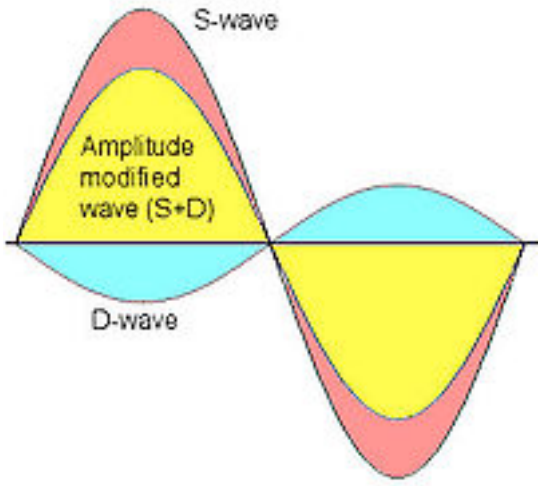
Only through correctly centering the two elements can phase contrast

illumination be established. A phase centering telescope that temporarily replaces one of the oculars is used, first to focus the phase element plane and then center the annular illumination ring with the corresponding ring of the phase plate.

An interesting variant in phase contrast design was once implemented (by the microscope maker C. Baker, London) in which the conventional annular form of the two elements was replaced by a cross-shaped transmission slit in the substage and corresponding cross-shaped phase plates in the conjugate plane in the objectives. The advantage claimed here was that only a single slit aperture was needed for all phase objective magnifications. Recentring and rotational alignment of the cross by means of the telescope was nevertheless needed for each change in magnification.

3.8.2 Technical Details

To understand how phase contrast illumination works, we study two wave fronts (see the figure to the right). This figure simplifies a few things. First, the condenser annulus is just a small aperture located in the center (see the plane labeled '1') and the phase plate is also just covering a small aperture (located in the plane labeled '3'). Second, the optical system is greatly simplified by showing only two single lenses to represent all optical elements.

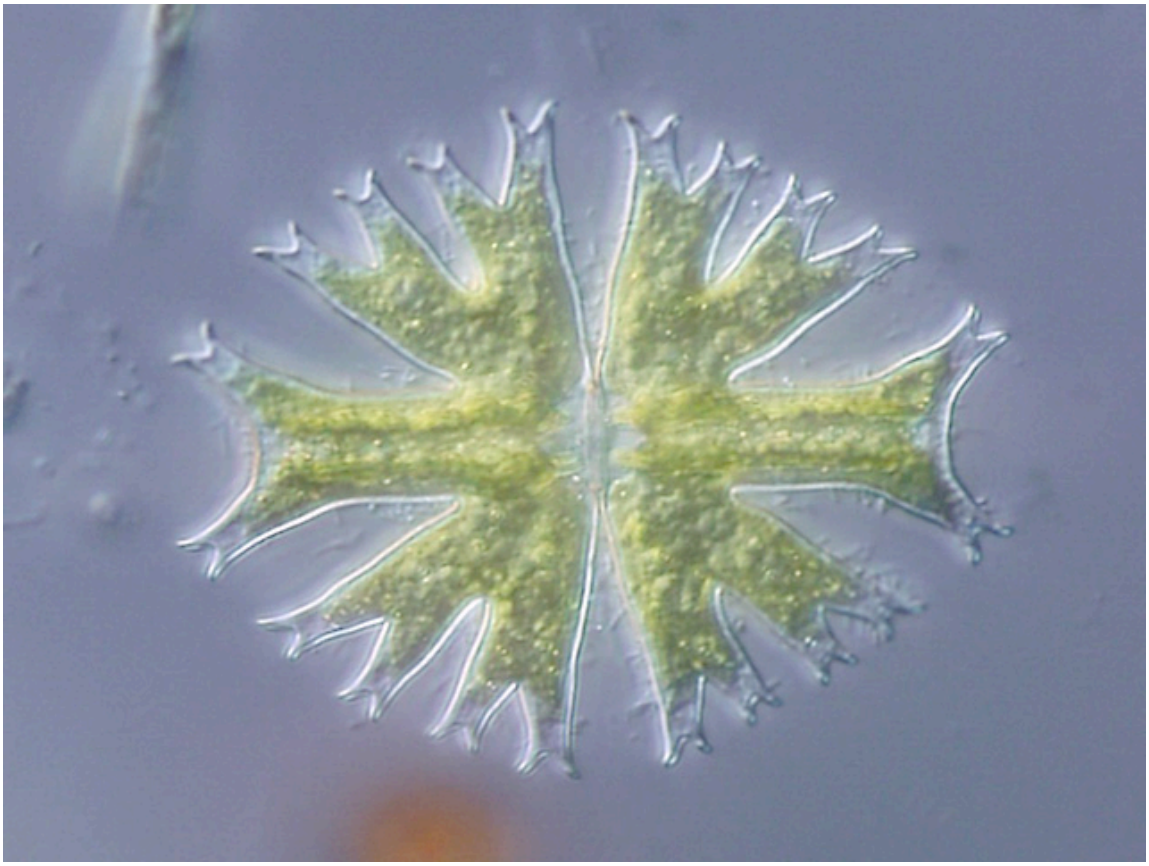


D-wave and S-wave

The plane labeled '1' is the front focal plane of the condenser. The light emanating from the small aperture 'S' is captured by the condenser and emerges as light with only parallel wavefronts from the condenser. When these plane waves (parallel wave fronts) hit the phase object 'O' (located in the object plane labeled '2'), some of this light is diffracted (and/or refracted) while

moving through the specimen. Assuming that the specimen does not significantly alter the amplitudes of the incoming wavefronts but mainly changes phase relations with respect to the "unperturbed" wavefronts, newly generated spherical wave fronts that are retarded by 90° (λ/4) emanate from 'O' (see the purple area that contains now "unperturbed" plane waves and spherical wave fronts). It is important to note that there are now two types of waves, the surround wave or S-wave and the diffracted wave or D-wave, which have a relative phase-shift of 90° (λ/4). - The objective focuses the D-wave inside the primary image plane (labeled '4'), while it focuses the S-wave inside the back focal plane (labeled '3'). The location of the phase plate 'P' has now a profound impact on the S-wave while leaving most of the D-wave "unharmed". In what is known as positive phase contrast optics, the phase plate 'P' reduces the amplitude of all light rays traveling through the phase annulus (mainly S-waves) by 70 to 90% and advances the phase by yet another 90° (λ/4). However, the phase plate leaves most of the D-waves "untouched". Hence the recombination of these two waves (D + S) in the primary image plane (labeled '4') results in a significant amplitude change at all locations where there is a now destructive interference due to a 180° (λ/2) phase shifted D-wave. The net phase shift of 180° (λ/2) results directly from the 90° (λ/4) retardation of the D-wave due to the phase object and the 90° (λ/4) phase advancement of the S-wave due to the phase plate. Without the phase plate, there would be no significant destructive interference that greatly enhances contrast. With phase contrast illumination "invisible" phase variations are hence translated into visible amplitude variations. The destructive interference is illustrated in the figure to the right. Blue and orange indicate D-wave and S-wave, respectively. The resulting wave (D + S), indicated by yellow, has a reduced amplitude.

3.9 Differential Interference Contrast Microscopy

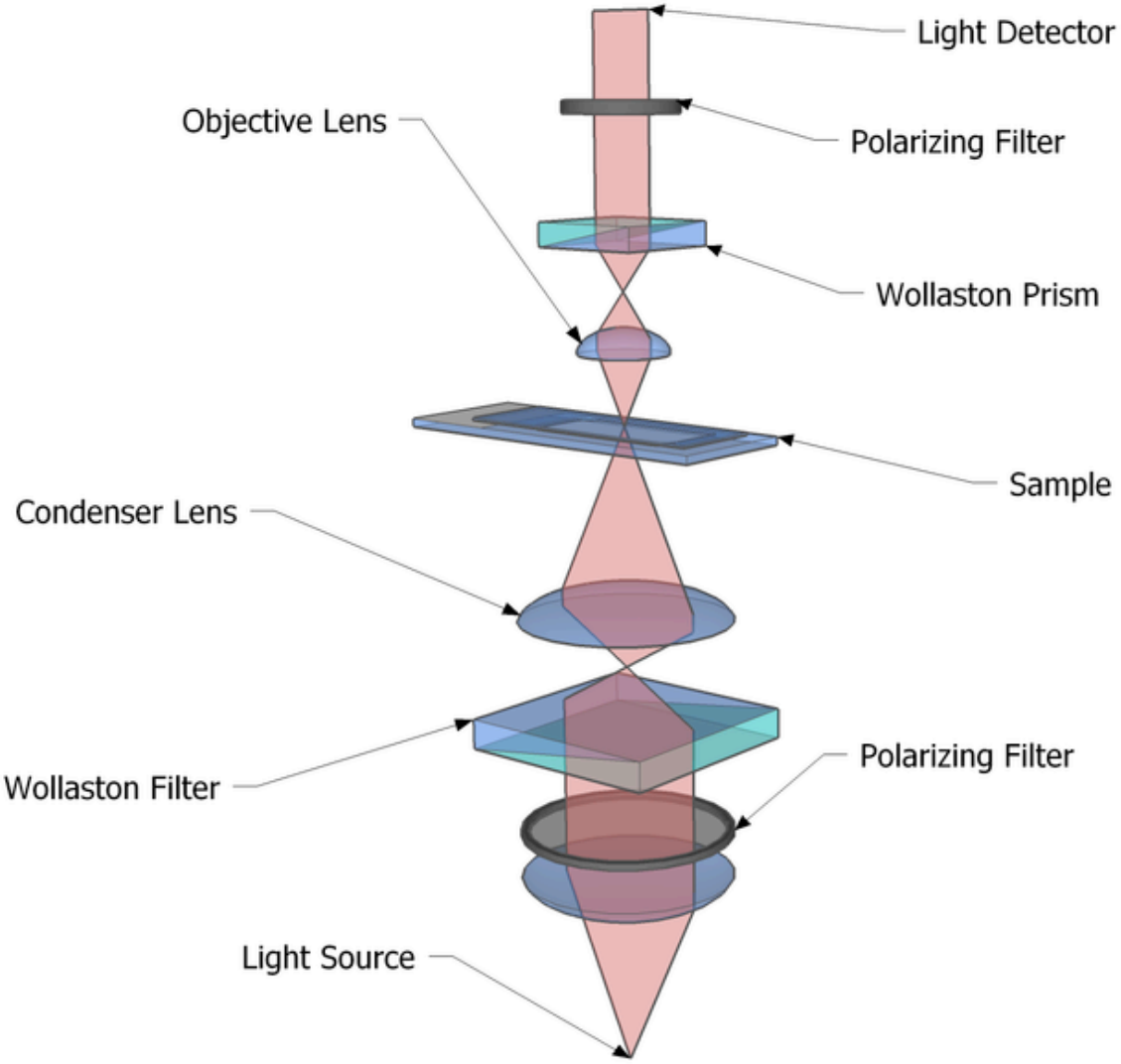


Micrasterias radiata as imaged by DIC microscopy.

Differential interference contrast microscopy (**DIC**), also known as **Nomarski Interference Contrast** (**NIC**) or **Nomarski microscopy**, is an optical microscopy illumination technique used to enhance the contrast in unstained, transparent samples. DIC works on the principle of interferometry to gain information about the optical density of the sample, to see otherwise invisible features. A relatively complex lighting scheme produces an image with the object appearing black to white on a grey background. This image is similar to that obtained by phase contrast microscopy but without the bright diffraction halo.

DIC works by separating a polarised light source into two beams which take slightly different paths through the sample. Where the length of each optical path (i.e. the product of refractive index and geometric path length) differs, the beams interfere when they are recombined. This gives the appearance of a three-dimensional physical relief corresponding to the variation of optical density of the sample, emphasising lines and edges though not providing a topographically accurate image.

3.9.1 The Light Path



The components of the basic differential interference contrast microscope setup.

1. Unpolarised light enters the microscope and is polarised at 45°.

Polarised light is required for the technique to work.

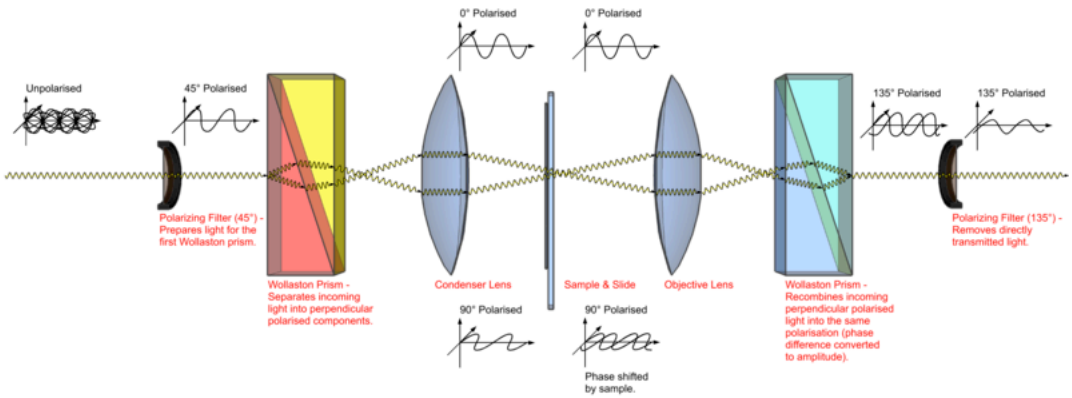
2. The polarised light enters the first Nomarski-modified Wollaston prism and is separated into two rays polarised at 90° to each other, the sampling and reference rays.

Main article: Wollaston prism

Wollaston prisms are a type of prism made of two layers of a crystalline substance, such as quartz, which, due to the variation of refractive index depending on the polarisation of the light, splits the light according to its polarisation. The Nomarski prism causes the two rays to come to a focal point outside the body of the prism, and so allows greater flexibility when setting up the microscope, as the prism can be actively focused.

3. The two rays are focused by the condenser for passage through the sample. These two rays are focused so they will pass through two adjacent points in the sample, around 0.2 µm apart.

The sample is effectively illuminated by two coherent light sources, one with 0° polarisation and the other with 90° polarisation. These two illuminations are, however, not quite aligned, with one lying slightly offset with respect to the other.



The route of light through a DIC microscope.

4. The rays travel through the different, adjacent, areas of the sample. They will experience different optical path lengths where the areas differ in refractive index or thickness. This causes a change in phase of one ray relative to the other due to the delay experienced by the wave in the more optically dense material.

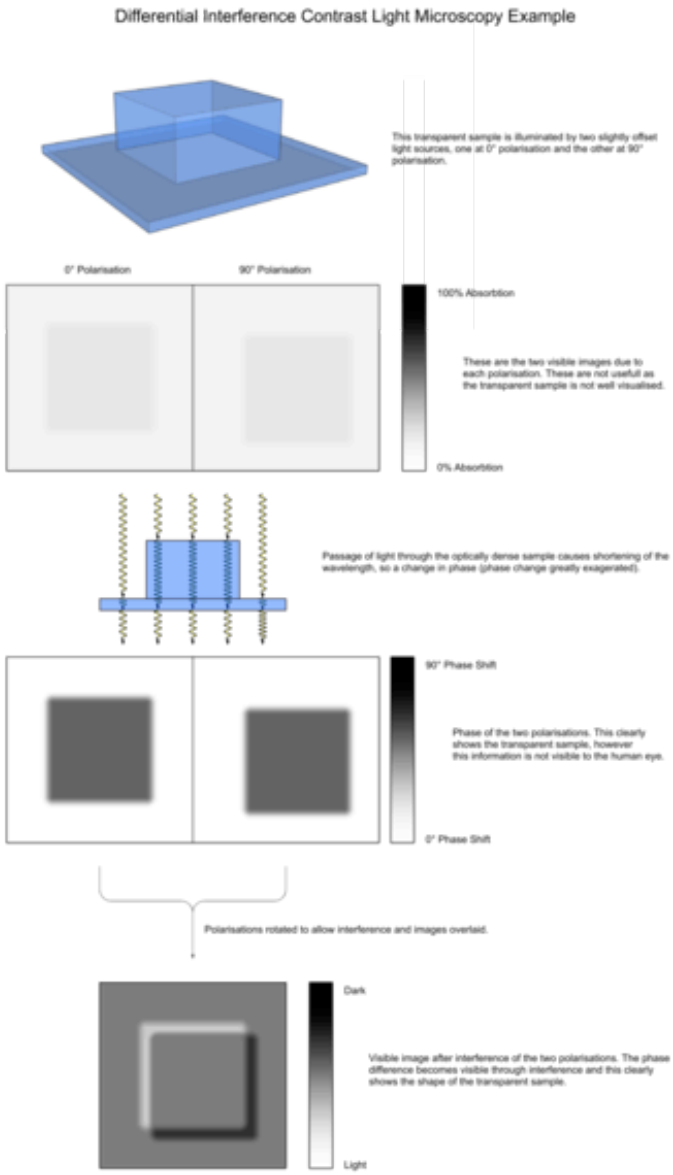
The passage of many pairs of rays through pairs of adjacent points in the sample (and their absorbance, refraction and scattering by the sample) means an image of the sample will now be carried by both the 0° and 90° polarised light. These, if looked at individually, would be bright field images of the sample, slightly offset from each other. The light also carries information about the image invisible to the human eye, the phase of the light. This is vital later. The different polarisations prevent interference between these two images at this point.

5. The rays travel through the objective lens and are focused for the second Nomarski-modified Wollaston prism.

6. The second prism recombines the two rays into one polarised at 135°. The combination of the rays leads to interference, brightening or darkening the image at that point according to the optical path difference.

This prism overlays the two bright field images and aligns their polarisations so they can interfere. However, the images do not quite line up because of the offset in illumination - this means that instead of interference occuring between 2 rays of light that passed through the same point in the specimen, interference occurs between rays of light that went through adjacent points which therefore have a slightly different phase. Because the difference in phase is due to the difference in optical path length, this recombination of light causes "optical differentiation" of the optical path length, generating the image seen.

3.9.2 The Image



An illustration of the process of image production in a DIC microscope.

The image has the appearance of a three dimensional object under very oblique illumination, causing strong light and dark shadows on the corresponding faces. The direction of apparent illumination is defined by the orientation of the Wollaston prisms.

As explained above the image is generated from two identical bright field images being overlayed slightly offset from each other (typically around 0.2µm), and the subsequent interference due to phase difference converting changes in phase (and so optical path length) to a visible change in darkness. This interference may be either constructive or destructive, giving rise to the characteristic appearance of three dimensions.

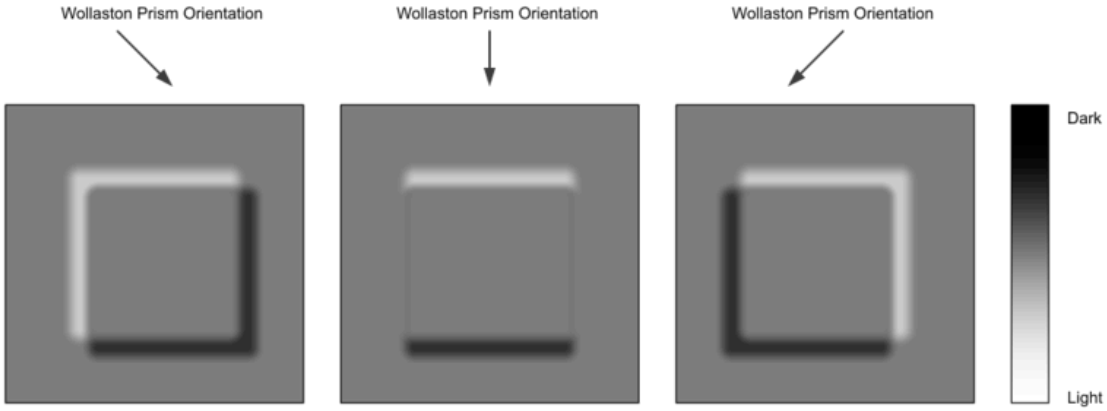
The typical phase difference giving rise to the interference is very small, very rarely being larger than 90° (a quarter of the wavelength). This is due to the similarity of refractive index of most samples and the media they are in: for example, a cell in water only has a refractive index difference of around 0.05. This small phase difference is important for the correct function of DIC, since if the phase difference at the joint between two substances is too large then the phase difference could reach 180° (half a wavelength), resulting in complete destructive interference and an anomalous dark region; if the phase difference reached 360° (a full wavelength), it would produce complete constructive interference, creating an anomalous bright region.

It is worth noting that the image can be approximated (neglecting refraction and absorption due to the sample and the resolution limit of beam separation) as the differential of optical path length with respect to position across the sample, and so the differential of the refractive index (optical density) of the sample.

3.9.3 Advantages and Disadvantages

DIC has strong advantages in uses involving live and unstained biological samples, such as a smear from a tissue culture or individual water borne single-celled organisms. Its resolution[*specify*] and clarity in conditions such as this are unrivaled among standard optical microscopy techniques.

The main limitation of DIC is its requirement for a transparent sample of fairly similar refractive index to its surroundings. DIC is unsuitable (in biology) for thick samples, such as tissue slices, and highly pigmented cells. DIC is also unsuitable for most non biological uses because of its dependence on polarisation, which many physical samples would affect.



Orientation specific imaging of a transparent cuboid in DIC.

Image quality, when used under suitable conditions, is outstanding in resolution and almost entirely free of artifacts unlike phase contrast. However analysis of DIC images must always take into account the orientation of the Wollaston prisms and the apparent lighting direction, as features parallel to this will not be visible. This is, however, easily overcome by simply rotating the sample and observing changes in the image.

3.9.4 References

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3.10 Dark Field Microscopy

Dark field microscopy (dark ground microscopy) describes microscopy methods, in both light and electron microscopy, which exclude the unscattered beam from the image. As a result, the field around the specimen (i.e. where there is no specimen to scatter the beam) is generally dark.

In optical microscopy, darkfield describes an illumination technique used to enhance the contrast in unstained samples. It works by illuminating the sample with light that will not be collected by the objective lens, and thus will not form part of the image. This produces the classic appearance of a dark, almost black, background with bright objects on it.

3.10.1 The Lightpath

The steps are illustrated in the figure where an upright microscope is used.

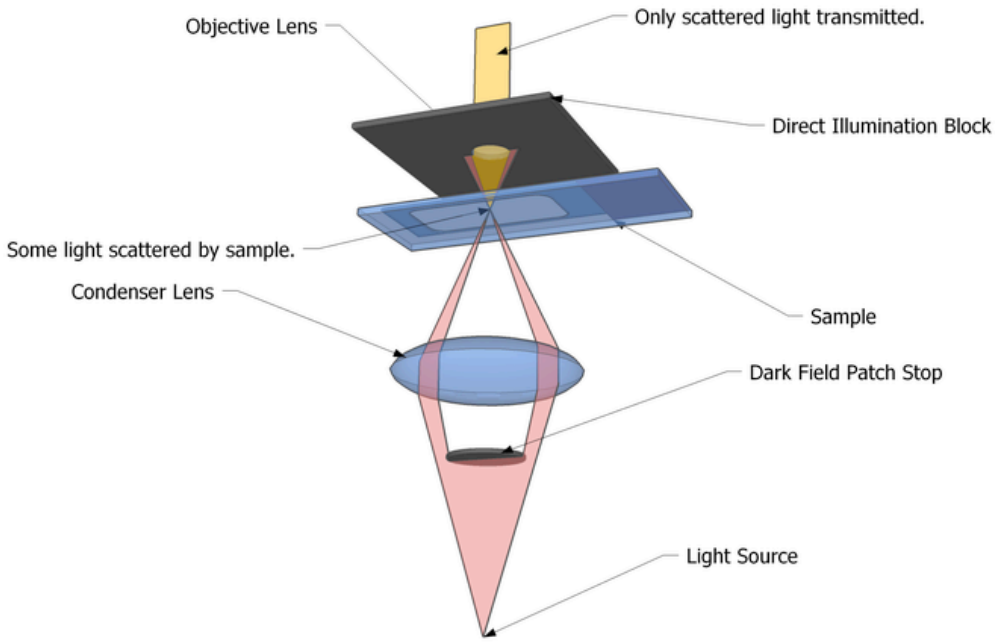


Diagram illustrating the light path through a dark field microscope.

Light enters the microscope for illumination of the sample.
A specially sized disc, the *patch stop* (see figure) blocks some light from the light source, leaving an outer ring of illumination.
The condenser lens focuses the light towards the sample.
The light enters the sample. Most is directly transmitted, while some is scattered from the sample.
The **scattered light** enters the objective lens, while the **directly transmitted light** simply misses the lens and is not collected due to a *direct illumination block* (see figure).
Only the scattered light goes on to produce the image, while the directly transmitted light is omitted.

this is the only common factor

3.10.2 Advantages and Disadvantages

Dark field microscopy produces an image with a dark background.

Dark field microscopy is a very simple yet effective technique and well suited for uses involving live and unstained biological samples, such as a smear from a tissue culture or individual water-borne single-celled organisms. Considering the simplicity of the setup, the quality of images obtained from this technique is impressive.

The main limitation of dark field microscopy is the low light levels seen in the final image. This means the sample must be very strongly illuminated, which can cause damage to the sample.

Dark field microscopy techniques are almost entirely free of artifacts, due to the nature of the process. However the interpretation of dark field images must be done with great care as common dark features of bright field microscopy images may be invisible, and vice versa.

While the dark field image may first appear to be a negative of the bright field image, different effects are visible in each. In bright field microscopy, features are visible where either a shadow is cast on the surface by the incident light, or a part of the surface is less reflective, possibly by the presence of pits or scratches. Raised features that are too smooth to cast shadows will not appear in bright field images, but the light that reflects off the sides of the feature will be visible in the dark field images.

3.10.3 Transmission Electron Microscope Applications of Dark Field

Darkfield studies in transmission electron microscopy play a powerful role in the study of crystals and crystal defects, as well as in the imaging of individual atoms.

3.10.3.1 Conventional Darkfield Imaging

Briefly, conventional darkfield imaging (P. Hirsch, A. Howie, R. Nicholson, D. W. Pashley and M. J. Whelan (1965/1977) *Electron microscopy of thin crystals* (Butterworths/Krieger, London/Malabar FL) ISBN 0-88275-376-2) involves tilting the incident illumination until a diffracted, rather than the incident, beam passes through a small objective aperture in the objective lens back focal plane. Darkfield images, under these conditions, allow one to map the diffracted intensity coming from a single collection of diffracting planes as a function of projected position on the specimen, and as a function of specimen tilt.

In single crystal specimens, single-reflection darkfield images of a specimen tilted just off the Bragg condition allow one to "light up" only those lattice defects, like dislocations or precipitates, which bend a single set of lattice planes in their neighborhood. Analysis of intensities in such images may then be used to estimate the amount of that bending. In polycrystalline specimens, on the other hand, darkfield images serve to light up only that subset of crystals which is Bragg reflecting at a given orientation.

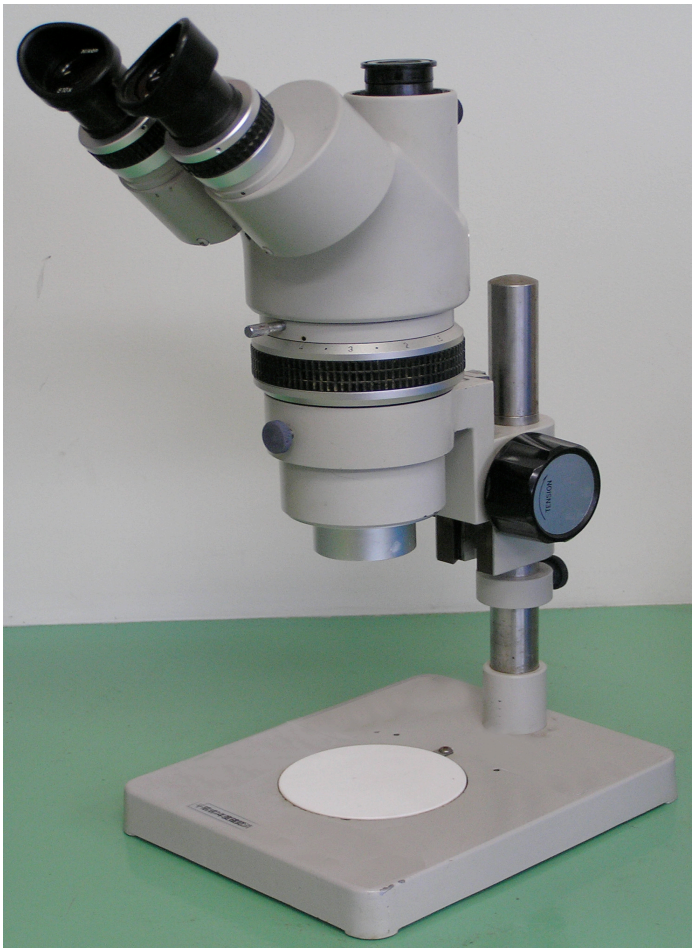
3.10.3.2 Weak Beam Imaging

Weak beam imaging involves optics similar to conventional darkfield, but use of a diffracted beam *harmonic* rather than the diffracted beam itself. Much higher resolution of strained regions around defects can be obtained in this way.

3.10.3.3 Low and High Angle Annular Darkfield Imaging

Annular darkfield imaging requires one to form images with electrons diffracted into an annular aperture centered on, but not including, the unscattered beam. For large scattering angles in a scanning transmission electron microscope, this is sometimes called Z-contrast imaging because of the enhanced scattering from high atomic number atoms.

3.11 The Stereo Microscope



Stereo microscope

The **stereo** or **dissecting microscope** is designed differently from the diagrams above, and serves a different purpose. It uses two separate optical paths with two objectives and two eyepieces to provide slightly different viewing angles to the left and right eyes. In this way it produces a three-dimensional visualization of the sample being examined.[4]

The stereo microscope is often used to study the surfaces of solid specimens or to carry out close work such as sorting, dissection, microsurgery, watch-making, small circuit board manufacture or inspection, and the like.

Unlike compound microscopes, illumination in a stereo microscope most often uses reflected (episcopic) illumination rather than transmitted (diascopic) illumination, that is, light reflected from the surface of an object rather than light transmitted through an object. Use of reflected light from the object allows examination of specimens that would be too thick or otherwise opaque for

compound microscopy. However, stereo microscopes are also capable of transmitted light illumination as well, typically by having a bulb or mirror beneath a transparent stage underneath the object, though unlike a compound microscope, transmitted illumination is not focused through a condenser in most systems.[5] Stereoscopes with specially-equipped illuminators can be used for dark field microscopy, using either reflected or transmitted light.[6]

Scientist using a stereo microscope outfitted with a digital imaging pick-up

Great working distance and depth of field here are important qualities for this type of microscope. Both qualities are inversely correlated with resolution: the higher the resolution (*i.e.* the shorter the distance at which two adjacent points can be distinguished as separate), the smaller the depth of field and working distance. A stereo microscope has a useful magnification up to 100×. The resolution is maximally in the order of an average 10× objective in a compound microscope, and often much lower.

There are two major types of magnification systems in stereo microscopes. One is fixed magnification in which primary magnification is achieved by a paired set of objective lenses with a set degree of magnification. The other is zoom or pancratic magnification, which are capable of a continuously variable degree of magnification across a set range. Zoom systems can achieve further magnification through the use of auxiliary objectives that increase total

magnification by a set factor. Also, total magnification in both fixed and zoom systems can be varied by changing eyepieces.[4]

Intermediate between fixed magnification and zoom magnification systems is a system attributed to Galileo as the "Galilean optical system" ; here an arrangement of fixed-focus convex lenses is used to provide a fixed magnification, but with the crucial distinction that the same optical components in the same spacing will, if physically inverted, result in a different, though still fixed, magnification. This allows one set of lenses to provide two different magnifications ; two sets of lenses to provide four magnifications on one turret ; three sets of lenses provide six magnifications and will still fit into one turret. Practical experience shows that such Galilean optics systems are as useful as a considerably more expensive zoom system, with the advantage of knowing the magnification in use as a set value without having to read analogue scales. (In remote locations, the robustness of the systems is also a non-trivial advantage.)

The stereo microscope should not be confused with a compound microscope equipped with double eyepieces and a binoviewer. In such a microscope both eyes see the same image, but the binocular eyepieces provide greater viewing comfort. However, the image in such a microscope is no different from that obtained with a single monocular eyepiece.

3.11.1 Digital Display with Stereo Microscopes

Recently various video dual CCD camera pickups have been fitted to stereo microscopes, allowing the images to be displayed on a high resolution LCD monitor. Software converts the two images to an integrated Anachrome 3D image, for viewing with plastic red/cyan glasses, or to the cross converged process for clear glasses and somewhat better color accuracy. The results are viewable by a group wearing the glasses.

3.12 Digital Microscopes

A digital microscope.

Low power microscopy is also possible with digital microscopes, with a camera attached directly to the USB port of a computer, so that the images are shown directly on the monitor. Often called "USB" microscopes, they offer high magnifications (up to about 200×) without the need to use eyepieces, and at very low cost. The precise magnification is determined by the working distance between the camera and the object, and good supports are needed to control the image. The images can be recorded and stored in the normal way on the computer. The camera is usually fitted with a light source, although extra sources (such as a fibre-optic light) can be used to highlight features of interest in the object. They also offer a large depth of field, a great advantage at high magnifications.

They are most useful when examining flat objects such as coins, printed circuit boards, or documents such as banknotes. However, they can be used for examining any object which can be studied in a standard stereo-microscope. Such microscopes offer the great advantage of being much less bulky than a conventional microscope, so can be used in the field, attached to a laptop computer. Although convienient, the magnifying abilities of these instruments

are often overstated; typically offering 200X magnification, this claim is based usually on 25X to 30X actual magnification PLUS the expansion of the image facilitated by the size of the available screen- so for genuine 200X magnification a ten-foot screen would be required.

3.13 Limitations of Light

At very high magnifications with transmitted light, point objects are seen as fuzzy discs surrounded by diffraction rings. These are called Airy disks. The *resolving power* of a microscope is taken as the ability to distinguish between two closely spaced Airy disks (or, in other words the ability of the microscope to reveal adjacent structural detail as distinct and separate). It is these impacts of diffraction that limit the ability to resolve fine details. The extent of and magnitude of the diffraction patterns are affected by both by the wavelength of light (λ), the refractive materials used to manufacture the objective lens and the numerical aperture (NA or AN) of the objective lens. There is therefore a finite limit beyond which it is impossible to resolve separate points in the objective field, known as the diffraction limit. Assuming that optical aberrations in the whole optical set-up are negligible, the resolution *d*, is given by:

$$d = \frac{\lambda}{2A_N}$$

Usually, a λ of 550 nm is assumed, corresponding to green light. With air as medium, the highest practical AN is 0.95, and with oil, up to 1.5. In practice the lowest value of *d* obtainable is around 0.2 micrometres or 200 nanometres.

A modern microscope with a mercury bulb for fluorescence microscopy. The microscope has a digital camera, and is attached to a computer.

Other optical microscope designs can offer an improved resolution. These include ultraviolet microscopes which use shorter wavelengths of light so the diffraction limit is lower, Vertico SMI, near field scanning optical microscopy which uses evanescent waves, and Stimulated Emission Depletion Microscopy which is used for observing **self-luminous particles** which are not diffraction limited as Abbe's theory (by Ernst Karl Abbe) is based on the fact that a non-self-luminous particle is illuminated by an external source.

Professor Stefan Hell of the Max Planck Institute for Biophysical Chemistry was awarded the 10th German Future Prize in 2006 for his development of the Stimulated Emission Depletion (STED) microscope.[7]

Several other optical microscopes have been able to see beyond the theoretical Abbe limit of 200nm. In 2005, Assistant Professor Masaru Kuno and post-graduate students Vladimir Protasenko and Katherine L. Hull of the University of Notre Dame described a single-molecule capable unit that could be constructed cheaply as a teaching tool.[8] A holographic microscope described by Courjon and Bulabois in 1979 is also capable of breaking this magnification limit, although resolution was restricted in their experimental analysis.[9]

3.14 Alternative Illuminations to Light

In order to overcome the limitations set by the diffraction limit of visible light other microscopes have been designed which use other waves.

Atomic Force Microscope (AFM)
Scanning Electron Microscope (SEM)
Scanning Tunneling Microscope (STM)
Transmission Electron Microscope (TEM)
X-ray microscope

The use of electrons and x-rays in place of light allows much higher resolution - the wavelength of the radiation is shorter so the diffraction limit is lower. To make the short-wavelength probe non-destructive, the atomic beam imaging system (atomic nanoscope) has been proposed and widely discussed in the literature, but it is not yet competitive with conventional imaging systems.

STM and AFM are scanning probe techniques using a small probe which is scanned over the sample surface. Resolution in these cases is limited by the size of the probe; micromachining techniques can produce probes with tip radii of 5-10nm.

However, all such methods use a vacuum or partial vacuum, which limits their use for live and biological samples (with the exception of ESEM). The specimen chambers needed for all such instruments also limits sample size, and sample manipulation is more difficult. Colour cannot be seen in images made by these methods, so some information is lost. They are however, essential when investigating molecular or atomic effects, such as age hardening in aluminium alloys, or the microstructure of polymers.

3.15 References

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"Introduction to Stereomicroscopy" by Paul E. Nothnagle, William Chambers, and Michael W. Davidson, *Nikon MicroscopyU*.

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4. The Fluorescence Microscope

A **fluorescence microscope** (colloquially synonymous with *epifluorescent microscope*) is a light microscope used to study properties of organic or inorganic substances using the phenomena of fluorescence and phosphorescence instead of, or in addition to, reflection and absorption.[1][2]

An inverted fluorescent microscope (Nikon TE2000). Note the orange plate that allows the user to look at the sample while protecting his eyes from the excitation UV light.

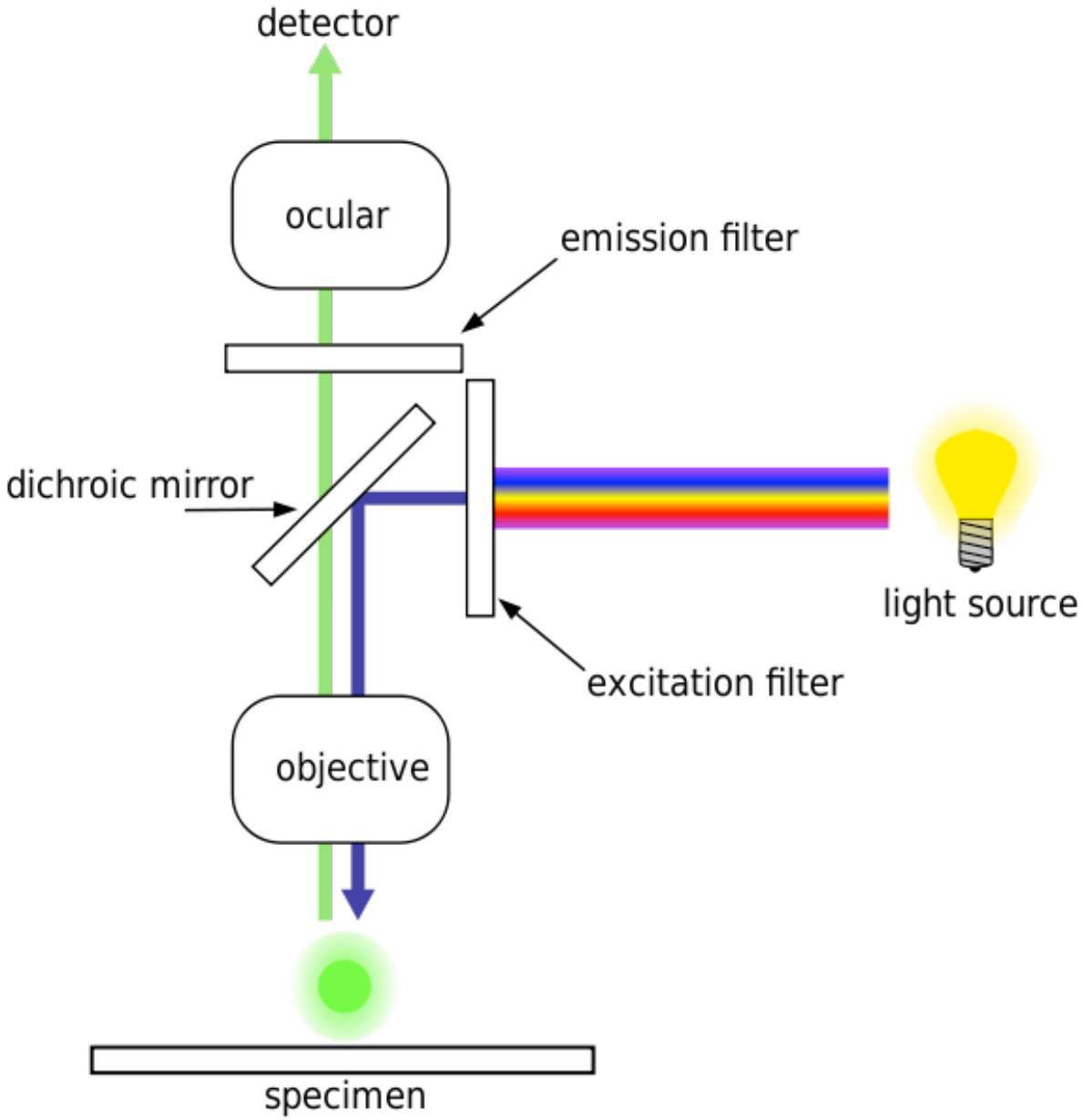
4.1 Technique

In most cases, a component of interest in the specimen is specifically labeled with a fluorescent molecule called a fluorophore (such as green fluorescent protein (GFP), fluorescein or DyLight 488).[1] The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit longer wavelengths of light (of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of an emission filter. Typical components of a fluorescence microscope are the light source (xenon arc lamp or mercury-vapor lamp), the excitation filter, the dichroic mirror (or dichromatic beamsplitter), and the emission filter (see figure below). The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen.[1] In this manner, a single fluorophore (color) is imaged at a time. Multi-color images of several fluorophores must be composed by combining several single-color images.[1]

Most fluorescence microscopes in use are epifluorescence microscopes (i.e. excitation and observation of the fluorescence are from above (*epi–*) the specimen). These microscopes have become an important part in the field of biology, opening the doors for more advanced microscope designs, such as the confocal microscope and the total internal reflection fluorescence microscope (TIRF). The Vertico SMI combining localisation microscopy with spatially modulated illumination uses standard fluorescence dyes and reaches an optical resolution below 10 nanometers (1 nanometer = 1 nm = 1×10^{-9} m).

Fluorophores lose their ability to fluoresce as they are illuminated in a process called photobleaching. Special care must be taken to prevent photobleaching through the use of more robust fluorophores, by minimizing illumination, or by introducing a scavenger system to reduce the rate of photobleaching.

4.2 **Epifluorescence Microscopy**

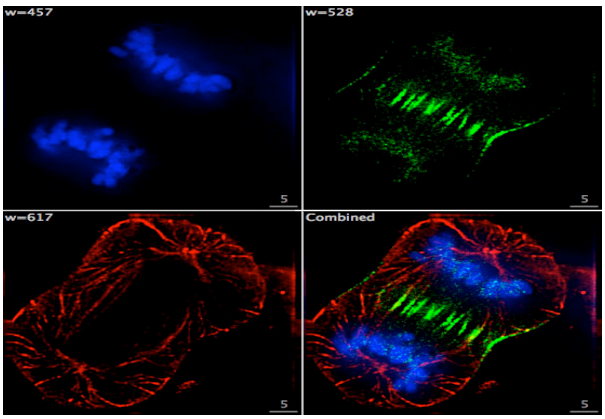


Schematic of a fluorescence microscope.

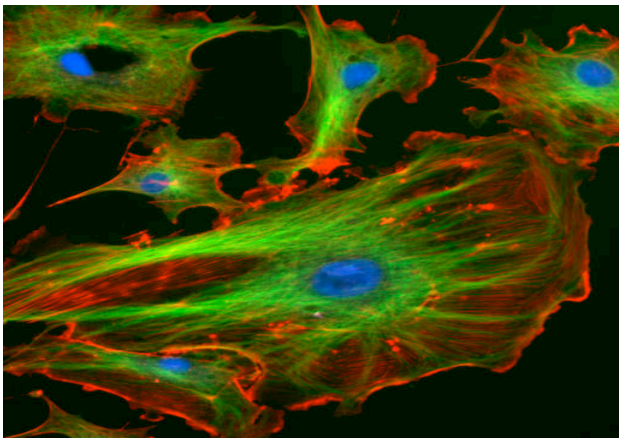
Epifluorescence microscopy is a method of fluorescence microscopy that is widely used in life sciences. The excitatory light is passed from above (or, for inverted microscopes, from below), through the objective and then onto the specimen instead of passing it first through the specimen. (In the latter case the transmitted excitatory light reaches the objective together with light emitted from the specimen). The fluorescence in the specimen gives rise to emitted light which is focused to the detector by the same objective that is used for the excitation. A filter between the objective and the detector filters out the excitation light from fluorescent light. Since most of the excitatory light is transmitted through the specimen, only reflected excitatory light reaches the

objective together with the emitted light and this method therefore gives an improved signal to noise ratio. A common use in biology is to apply fluorescent or fluorochrome stains to the specimen in order to image a protein or other molecule of interest.

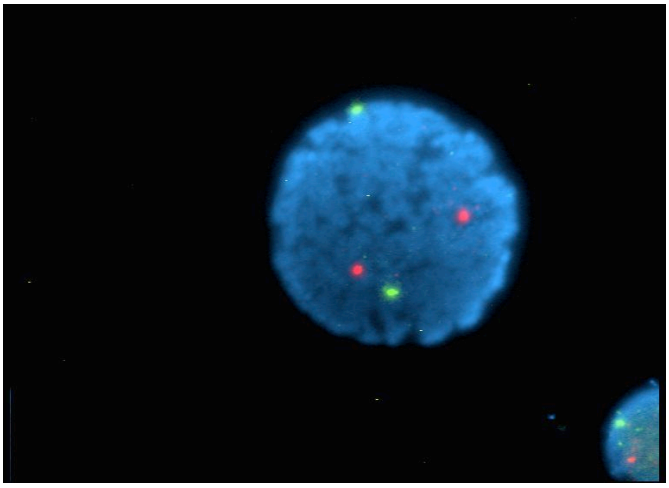
4.3 Fluorescent Image Gallery



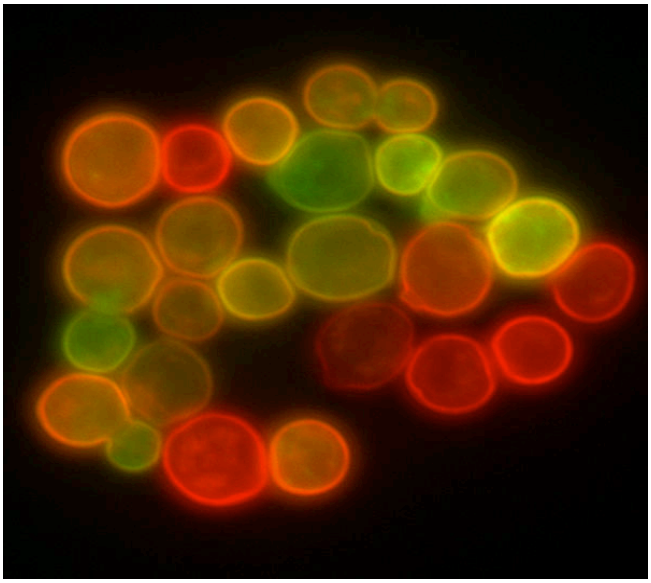
Epifluorescent imaging of the three components in a dividing human cancer cell. DNA is stained blue, a protein called INCENP is green, and the microtubules are red. Each fluorophore is imaged separately using a different combination of excitation and emission filters, and the images are captured sequentially using a digital CCD camera, then overlaid to give a complete image.



Endothelial cells under the microscope. Nuclei are stained blue with DAPI, microtubules are marked green by an antibody bound to FITC and actin filaments are labelled red with phalloidin bound to TRITC. Bovine pulmonary artery endothelial (BPAE) cells



human lymphocyte nucleus stained with DAPI with chromosome 13 (green) and 21 (red) centromere probes hybrydized (Fluorescent in situ hybridization (FISH))



Yeast cell membrane visualized by some membrane proteins fused with RFP and GFP fluorescent markers. Imposition of light from both of markers results in yellow colour.

4.4 References

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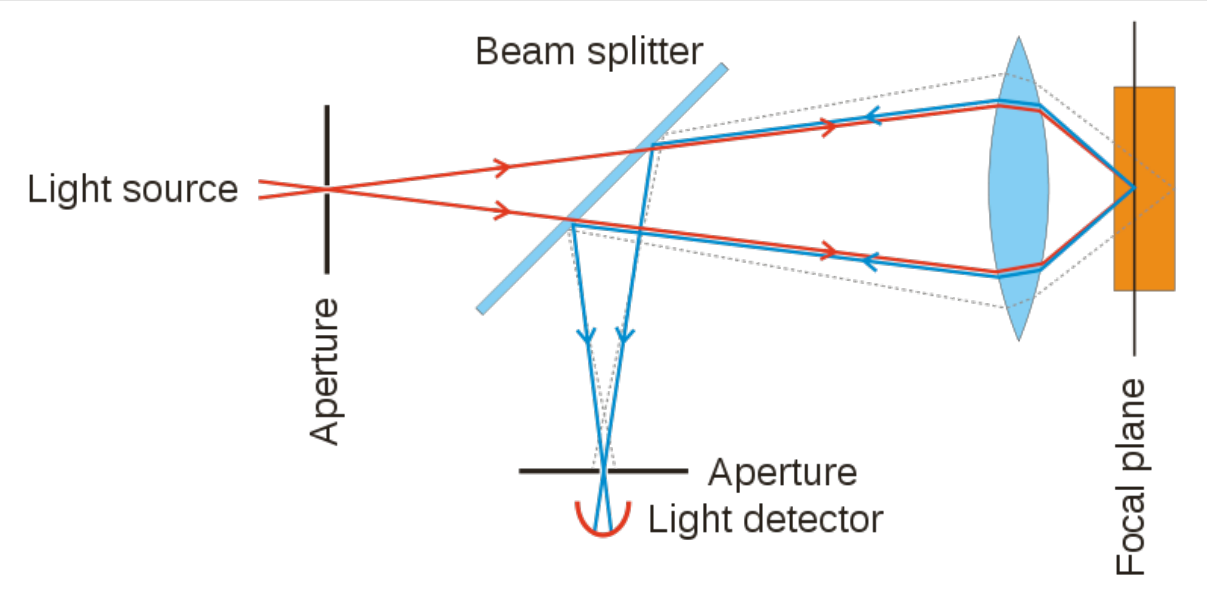
5. Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (**CLSM** or **LSCM**) is a technique for obtaining high-resolution optical images with depth selectivity.[1] The key feature of confocal microscopy is its ability to acquire in-focus images from selected depths, a process known as optical sectioning. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically-complex objects. For opaque specimens, this is useful for surface profiling, while for non-opaque specimens, interior structures can be imaged. For interior imaging, the quality of the image is greatly enhanced over simple microscopy because image information from multiple depths in the specimen is not superimposed. A conventional microscope "sees" as far into the specimen as the light can penetrate, while a confocal microscope only images one depth level at a time. In effect, the CLSM achieves a controlled and highly limited depth of focus. The principle of confocal microscopy was originally patented by Marvin Minsky in 1957,[2] but it took another thirty years and the development of lasers for CLSM to become a standard technique toward the end of the 1980s.[1] Thomas and Christoph Cremer designed in 1978 a laser scanning process which scans point-by-point the three dimensional surface of an object by means of a focused laser beam and creates the over-all picture by electronic means similar to those used in scanning electron microscopes.[3] It is this plan for the construction of a CSLM, which for the first time combined the laser scanning method with the 3D detection of biological objects labeled with fluorescent markers. During the next decade, confocal fluoresence microscopy was developed into a technically fully matured state in particular by groups working at the University of Amsterdam and the European Molecular Biology Laboratory (EMBL) in Heidelberg and their industry partners.

5.1 Image Formation

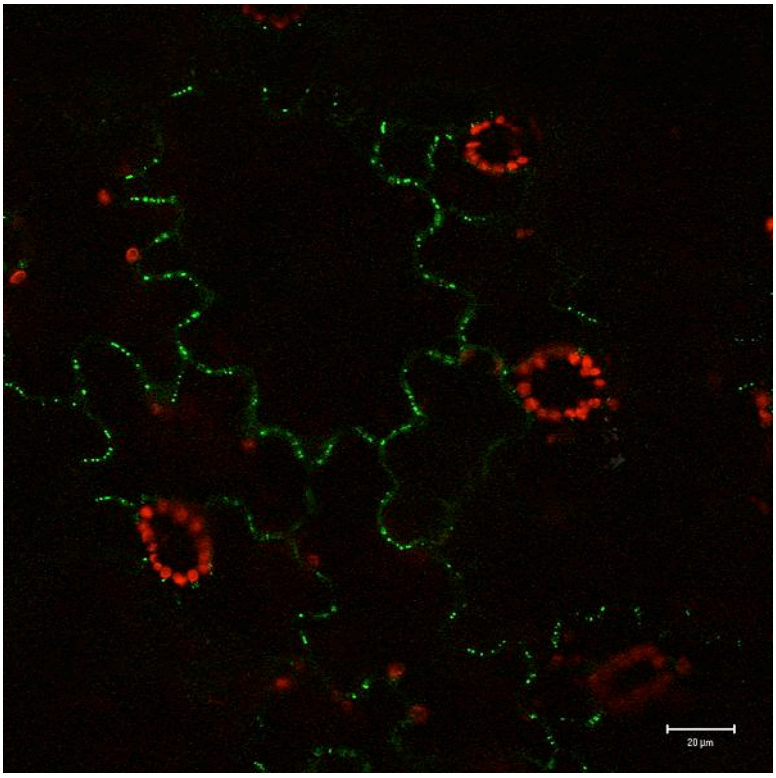


Principle of confocal microscopy.

In a confocal laser scanning microscope, a laser beam passes through a light source aperture and then is focused by an objective lens into a small (ideally diffraction limited) focal volume within or on the surface of a specimen. In biological applications especially, the specimen may be fluorescent. Scattered and reflected laser light as well as any fluorescent light from the illuminated spot is then re-collected by the objective lens. A beam splitter separates off some portion of the light into the detection apparatus, which in fluorescence confocal microscopy will also have a filter that selectively passes the fluorescent wavelengths. After passing a *pinhole*, the light intensity is detected by a photodetection device (usually a photomultiplier tube (PMT) or avalanche photodiode), transforming the light signal into an electrical one that is recorded by a computer.[4]

The detector aperture obstructs the light that is not coming from the focal point, as shown by the dotted gray line in the image. The out-of-focus light is suppressed: most of the returning light is blocked by the pinhole, which results in sharper images than those from conventional fluorescence microscopy techniques and permits one to obtain images of planes at various depths within the sample (sets of such images are also known as *z stacks*).[1]

The detected light originating from an illuminated volume element within the specimen represents one pixel in the resulting image. As the laser scans over the plane of interest, a whole image is obtained pixel-by-pixel and line-by-line, whereas the brightness of a resulting image pixel corresponds to the relative intensity of detected light. The beam is scanned across the sample in the horizontal plane by using one or more (servo controlled) oscillating mirrors. This scanning method usually has a low reaction latency and the scan speed can be varied. Slower scans provide a better signal-to-noise ratio, resulting in better contrast and higher resolution. Information can be collected from different focal planes by raising or lowering the microscope stage. The computer can generate a three-dimensional picture of a specimen by assembling a stack of these two-dimensional images from successive focal planes.[1]



An example of a GFP fusion protein.

Confocal microscopy provides the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation as well as a marginal improvement in lateral resolution.[4] Biological samples are often treated with fluorescent dyes to make selected objects visible. However, the actual dye concentration can be low to minimize the disturbance of biological systems: some instruments can track single fluorescent molecules. Also, transgenic techniques can create organisms that produce their own fluorescent chimeric molecules (such as a fusion of GFP, green fluorescent protein with the protein of interest).

5.2 Resolution Enhancement

CLSM is a scanning imaging technique in which the resolution obtained is best explained by comparing it with another scanning technique like that of the scanning electron microscope (SEM). CLSM has the advantage of not requiring a probe to be suspended nanometers from the surface, as in an AFM or STM, for example, where the image is obtained by scanning with a fine tip over a surface. The distance from the objective lens to the surface (called the *working distance*) is typically comparable to that of a conventional optical microscope. It varies with the system optical design, but working distances from hundreds of microns to several millimeters are typical.

In CLSM a specimen is illuminated by a point laser source, and each volume element

is associated with a discrete scattering or fluorescence intensity. Here, the size of the scanning volume is determined by the spot size (close to diffraction limit) of the optical system because the image of the scanning laser is not an infinitely small point but a three-dimensional diffraction pattern. The size of this diffraction pattern and the focal volume it defines is controlled by the numerical aperture of the system's objective lens and the wavelength of the laser used. This can be seen as the classical resolution limit of conventional optical microscopes using wide-field illumination. However, with confocal microscopy it is even possible to improve on the resolution limit of wide-field illumination techniques because the confocal aperture can be closed down to eliminate higher orders of the diffraction pattern. For example, if the pinhole diameter is set to 1 Airy unit then only the first order of the diffraction pattern makes it through the aperture to the detector while the higher orders are blocked, thus improving resolution at the cost of a slight decrease in brightness. In fluorescence observations, the resolution limit of confocal microscopy is often limited by the signal to noise ratio caused by the small number of photons typically available in fluorescence microscopy. One can compensate for this effect by using more sensitive photodetectors or by increasing the intensity of the illuminating laser point source. Increasing the intensity of illumination later risks excessive bleaching or other damage to the specimen of interest, especially for experiments in which comparison of fluorescence brightness is required.

5.3 Uses

CLSM is widely-used in numerous biological science disciplines, from cell biology and genetics to microbiology and developmental biology.

Clinically, CLSM is used in the evaluation of various eye diseases, and is particularly useful for imaging, qualitative analysis, and quantification of endothelial cells of the cornea.[5] It is used for localizing and identifying the presence of filamentary fungal elements in the corneal stroma in cases of keratomycosis, enabling rapid diagnosis and thereby early institution of definitive therapy. Research into CLSM techniques for endoscopic procedures is also showing promise.[6] In the pharmaceutical industry, it was recommended to follow the manufacturing process of thin film pharmaceutical forms, to control the quality and uniformity of the drug distribution.[7] CLSM is also used as the data retrieval mechanism in some 3D optical data storage systems and has helped determine the age of the Magdalen papyrus.

5.4 Related Topics

Two-photon excitation microscopy : Although they use a related technology (both are laser scanning microscopes), multiphoton fluorescence microscopes are not strictly confocal microscopes. The term *confocal* arises from the presence of a **diaphragm** in the **conjugated focal plane** (confocal). This diaphragm is usually absent in multiphoton microscopes.

Total internal reflection fluorescence microscope (TIRF)

STED microscopy

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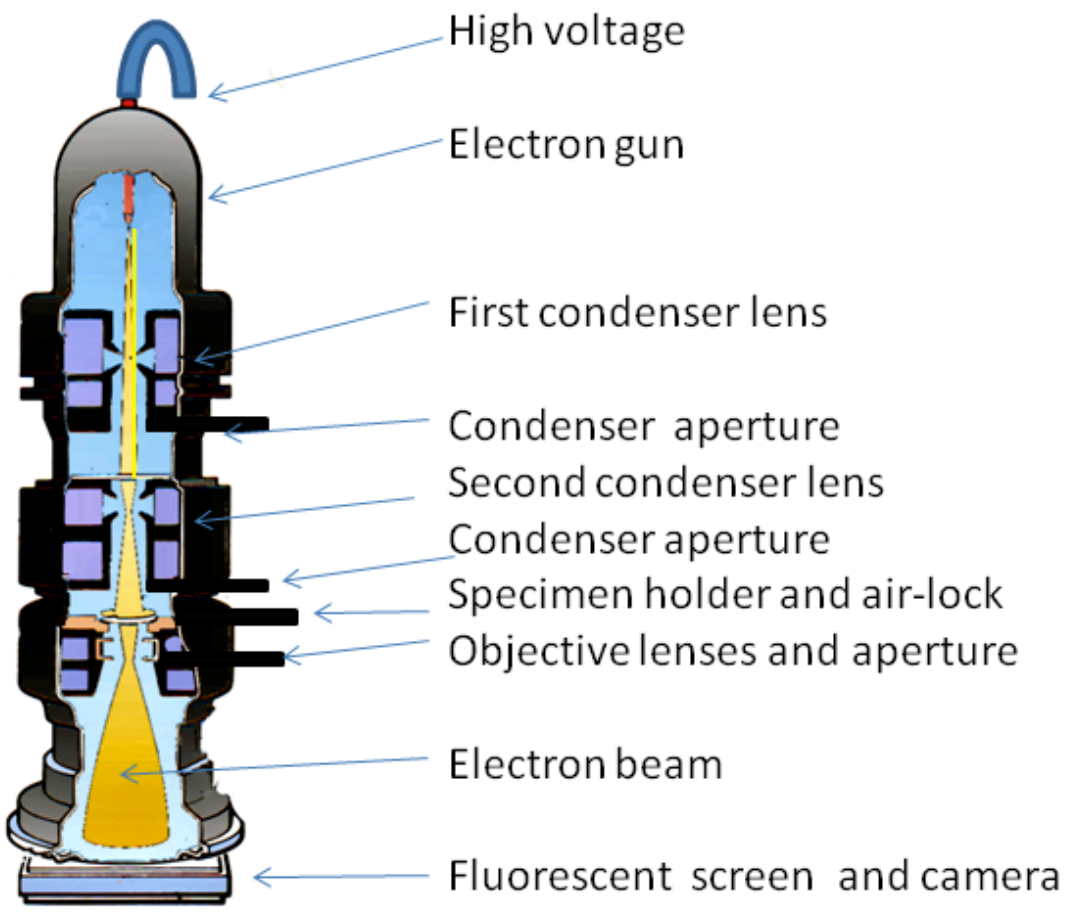
6. Electron Microscopes



Transmission Electron Microscope

Diagram of a transmission electron microscope

An **electron microscope** is a type of microscope that uses a particle beam of electrons to illuminate a specimen and create a highly-magnified image. Electron microscopes have much greater resolving power than light microscopes that use electromagnetic radiation and can obtain much higher magnifications of up to 2 million times, while the best light microscopes are limited to magnifications of 2000 times. Both electron and light microscopes have resolution limitations, imposed by the wavelength of the radiation they use. The greater resolution and magnification of the electron microscope is because the wavelength of an electron; its de Broglie wavelength is much smaller than that of a photon of visible light.

The electron microscope uses electrostatic and electromagnetic lenses in forming the image by controlling the electron beam to focus it at a specific plane relative to the specimen. This manner is similar to how a light

microscope uses glass lenses to focus light on or through a specimen to form an image.

6.1 History

Electron microscope constructed by Ernst Ruska in 1933

The first electron microscope prototype was built in 1931 by the German engineers Ernst Ruska and Max Knoll.[1] Although this initial instrument was capable of magnifying objects by only four hundred times, it demonstrated the principles of an electron microscope. Two years later, Ruska constructed an electron microscope that exceeded the resolution possible with an optical microscope.[1]

Reinhold Rudenberg, the scientific director of Siemens, had patented the electron microscope in 1931, stimulated by family illness to make the poliomyelitis virus particle visible. In 1937 Siemens began funding Ruska and Bodo von Borries to develop an electron microscope. Siemens also employed Ruska's brother Helmut to work on applications, particularly with biological specimens.[2][3]

In the same decade Manfred von Ardenne pioneered the scanning electron microscope and his universal electron microscope.

Siemens produced the first commercial Transmission Electron Microscope (TEM) in 1939, but the first practical electron microscope had been built at the University of Toronto in 1938, by Eli Franklin Burton and students Cecil Hall, James Hillier, and Albert Prebus.

Although modern electron microscopes can magnify objects up to two million times, they are still based upon Ruska's prototype. The electron microscope is an essential item of equipment in many laboratories. Researchers use them to examine biological materials (such as microorganisms and cells), a variety of

large molecules, medical biopsy samples, metals and crystalline structures and the characteristics of various surfaces. The electron microscope is also used extensively for inspection, quality assurance and failure analysis applications in industry, including, in particular, semiconductor device fabrication.

6.2 Improving Resolution

At this time the wave nature of electrons, which were considered charged matter particles, had not been fully realised until the publication of the De Broglie hypothesis in 1927. The group was unaware of this publication until 1932, where it was quickly realized that the De Broglie wavelength of electrons was many orders of magnitude smaller than that for light, theoretically allowing for imaging at atomic scales. In April 1932, Ruska suggested the construction of a new electron microscope for direct imaging of specimens inserted into the microscope, rather than simple mesh grids or images of apertures. With this device successful diffraction and normal imaging of aluminium sheet was achieved, however exceeding the magnification achievable with light microscopy had still not been successfully demonstrated. This goal was achieved in September 1933, using images of cotton fibers, which were quickly acquired before being damaged by the electron beam.

At this time, interest in the electron microscope had increased, with other groups, such as Albert Prebus and James Hillier at the University of Toronto who constructed the first TEM in north America in 1938, continually advancing TEM design.

Research continued on the electron microscope at Siemens in 1936, the aim of the research was the development improvement of TEM imaging properties, particularly with regard to biological specimens. In 1939 the first commercial electron microscope, pictured, was installed in the Physics department of I. G Farben-Werke. Further work on the electron microscope was hampered by the destruction of a new laboratory constructed at Siemens by an air-raid, as well as the death of two of the researchers, Heinz Müller and Friedrick Krause during World War II.

6.3 Further Research

After World War II, Ruska resumed work at Siemens, where he continued to develop the electron microscope, producing the first microscope with 100k

magnification. The fundamental structure of this microscope design, with multi-stage beam preparation optics, is still used in modern microscopes.

With the development of TEM, the associated technique of scanning transmission electron microscopy (STEM) was re-investigated and did not become developed until the 1970s, with Albert Crewe at the University of Chicago developing the field emission gun[9] and adding a high quality objective lens to create the modern STEM. Using this design, Crewe demonstrated the ability to image atoms using annular dark-field imaging. Crewe and coworkers at the University of Chicago developed the cold field electron emission source and built a STEM able to visualize single heavy atoms on thin carbon substrates.

6.4 Transmission Electron Microscope (TEM)

Electrons

Theoretically, the maximum resolution, *d*, that one can obtain with a light microscope has been limited by the wavelength of the photons that are being used to probe the sample, λ and the numerical aperture of the system, *NA*.

$$d = \frac{\lambda}{2\,n\,\sin\alpha} \approx \frac{\lambda}{2\,\mathrm{NA}}$$

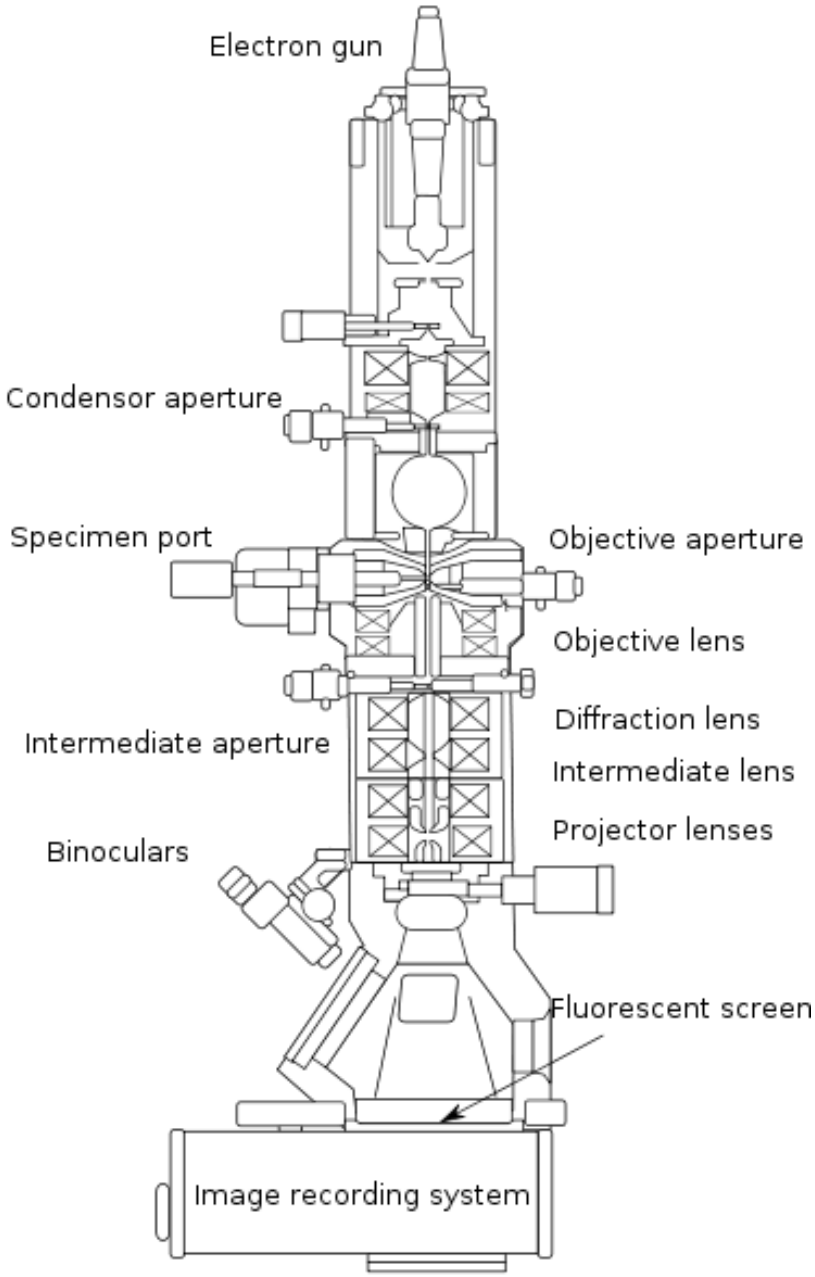
Early twentieth century scientists theorised ways of getting around the limitations of the relatively large wavelength of visible light (wavelengths of 400–700 nanometers) by using electrons. Like all matter, electrons have both wave and particle properties (as theorized by Louis-Victor de Broglie), and their wave-like properties mean that a beam of electrons can be made to behave like a beam of electromagnetic radiation. The wavelength of electrons is found by equating the de Broglie equation to the kinetic energy of an electron. An additional correction must be made to account for relativistic effects, as in a TEM an electron's velocity approaches the speed of light, *c*.

$$\lambda_e \approx \frac{h}{\sqrt{2m_0E(1+\frac{E}{2m_0c^2})}}$$

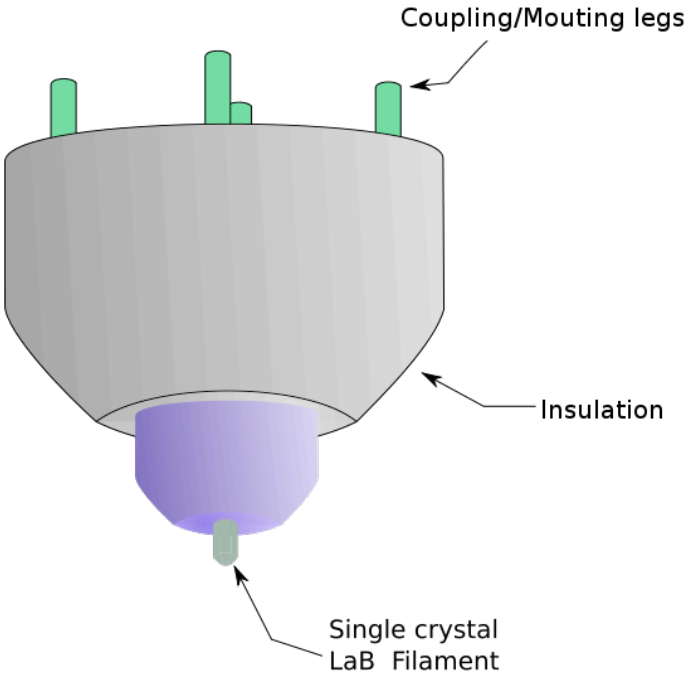
where, *h* is Planck's constant, m_0 is the rest mass of an electron and *E* is the

energy of the accelerated electron. Electrons are usually generated in an electron microscope by a process known as thermionic emission from a filament, usually tungsten, in the same manner as a light bulb, or alternatively by field electron emission.[13] The electrons are then accelerated by an electric potential (measured in volts) and focused by electrostatic and electromagnetic lenses onto the sample. The transmitted beam contains information about electron density, phase and periodicity; this beam is used to form an image.

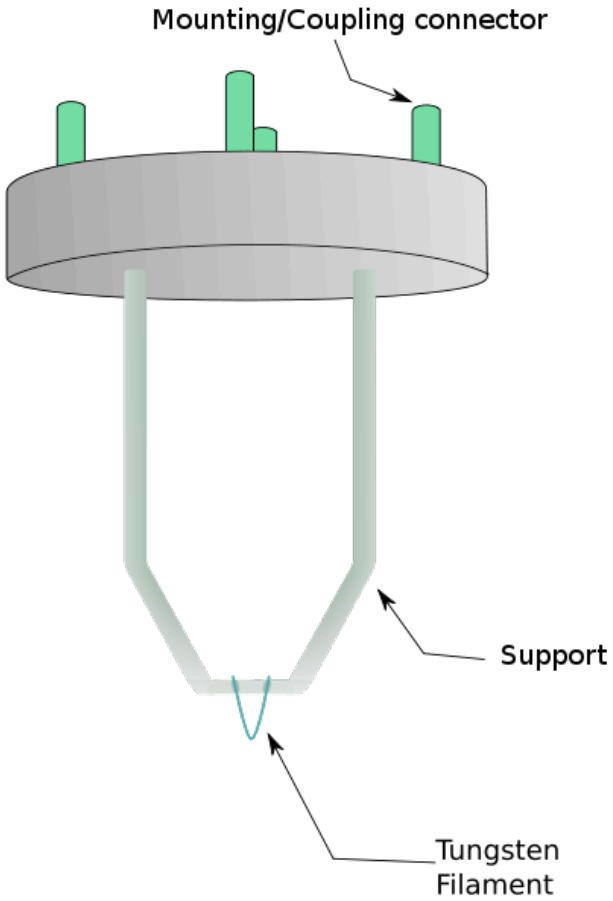
6.4.1 Source Formation



Layout of optical components in a basic TEM



Single crystal LaB_6 filament



Hairpin style tungsten filament

From the top down, the TEM consists of an emission source, which may be a tungsten filament, or a lanthanum hexaboride (LaB_6) source. For tungsten, this will be of the form of either a hairpin-style filament, or a small spike-shaped

filament. LaB_6 sources utilize small single crystals. By connecting this gun to a high voltage source (typically ~100-300 kV) the gun will, given sufficient current, begin to emit electrons either by thermionic or field electron emission into the vacuum. This extraction is usually aided by the use of a Wehnelt cylinder. Once extracted, the upper lenses of the TEM allow for the formation of the electron probe to the desired size and location for later interaction with the sample.

Manipulation of the electron beam is performed using two physical effects. The interaction of electrons with a magnetic field will cause electrons to move according to the right hand rule, thus allowing for electromagnets to manipulate the electron beam. The use of magnetic fields allows for the formation of a magnetic lens of variable focusing power, the lens shape originating due to the distribution of magnetic flux. Additionally, electrostatic fields can cause the electrons to be deflected through a constant angle. Coupling of two deflections in opposing directions with a small intermediate gap allows for the formation of a shift in the beam path, this being used in TEM for beam shifting, subsequently this is extremely important to STEM. From these two effects, as well as the use of an electron imaging system, sufficient control over the beam path is possible for TEM operation. The optical configuration of a TEM can be rapidly changed, unlike that for an optical microscope, as lenses in the beam path can be enabled, have their strength changed, or be disabled entirely simply via rapid electrical switching, the speed of which is limited by effects such as the magnetic hysteresis of the lenses.

6.4.2 Optics

The lenses of a TEM allow for beam convergence, with the angle of convergence as a variable parameter, giving the TEM the ability to change magnification simply by modifying the amount of current that flows through the coil, quadrupole or hexapole lenses. The quadrupole lens is an arrangement of electromagnetic coils at the vertices of the square, enabling the generation of a lensing magnetic fields, the hexapole configuration simply enhances the lens symmetry by using six, rather than four coils.

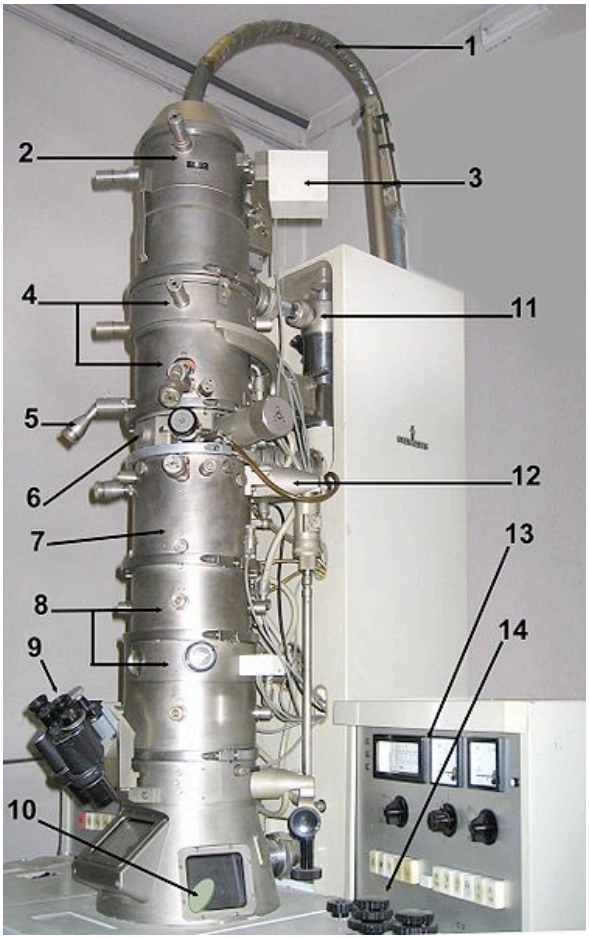
Typically a TEM consists of three stages of lensing. The stages are the condensor lenses, the objective lenses, and the projector lenses. The condensor lenses are responsible for primary beam formation, whilst the objective lenses focus the beam down onto the sample itself. The projector lenses are used to expand the beam onto the phosphor screen or other

imaging device, such as film. The magnification of the TEM is due to the ratio of the distances between the specimen and the objective lens' image plane. Additional quad or hexapole lenses allow for the correction of asymmetrical beam distortions, known as astigmatism. It is noted that TEM optical configurations differ significantly with implementation, with manufacturers using custom lens configurations, such as in spherical aberration corrected instruments, or TEMs utilising energy filtering to correct electron chromatic aberration.

6.4.3 Display

Imaging systems in a TEM consist of a phosphor screen, which may be made of fine (10-100 µm) particulate zinc sulphide, for direct observation by the operator. Optionally, an image recording system such as film based or doped YAG screen coupled CCDs. Typically these devices can be removed or inserted into the beam path by the operator as required.

6.4.1.4 Components



The electron source of the TEM is at the top, where the lensing system (4,7 and 8) focuses the beam on the specimen and then projects it onto the viewing screen (10). The beam control is on the right (13 and 14)

A TEM is composed of several components, which include a vacuum system in which the electrons travel, an electron emission source for generation of the electron stream, a series of electromagnetic lenses, as well as electrostatic plates. The latter two allow the operator to guide and manipulate the beam as required. Also required is a device to allow the insertion into, motion within, and removal of specimens from the beam path. Imaging devices are subsequently used to create an image from the electrons that exit the system.

6.4.5 Vacuum System

To increase the mean free path of the electron gas interaction, a standard TEM is evacuated to low pressures, typically on the order of 10^{-4} Pa. The need for this is twofold: first the allowance for the voltage difference between the cathode and the ground without generating an arc, and secondly to reduce the collision frequency of electrons with gas atoms to negligible levels—this effect is characterised by the mean free path. TEM components such as specimen holders and film cartridges must be routinely inserted or replaced requiring a system with the ability to re-evacuate on a regular basis. As such, TEMs are equipped with multiple pumping systems and airlocks and are not permanently vacuum sealed.

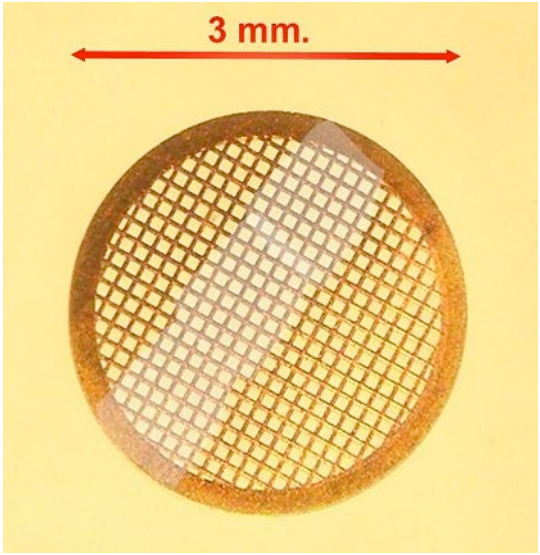
The vacuum system for evacuating a TEM to an operating pressure level consists of several stages. Initially a low or roughing vacuum is achieved with either a rotary vane pump or diaphragm pumps bringing the TEM to a sufficiently low pressure to allow the operation of a turbomolecular or diffusion pump which brings the TEM to its high vacuum level necessary for operations. To allow for the low vacuum pump to not require continuous operation, while continually operating the turbomolecular pumps, the vacuum side of a low-pressure pump may be connected to chambers which accommodate the exhaust gases from the turbomolecular pump. Sections of the TEM may be isolated by the use of gate valves, to allow for different vacuum levels in specific areas, such as a higher vacuum of 10^{-4} to 10^{-7} Pa or higher in the electron gun in high resolution or field emission TEMs.

High-voltage TEMs require ultra high vacuums on the range of 10^{-7} to 10^{-9} Pa to prevent generation of an electrical arc, particularly at the TEM cathode. As such for higher voltage TEMs a third vacuum system may operate, with the gun isolated from the main chamber either by use of gate valves or by the use

of a differential pumping aperture. The differential pumping aperture is a small hole that prevents diffusion of gas molecules into the higher vacuum gun area faster than they can be pumped out. For these very low pressures either an ion pump or a getter material is used.

Poor vacuum in a TEM can cause several problems, from deposition of gas inside the TEM onto the specimen as it is being viewed through a process known as electron beam induced deposition, or in more severe cases damage to the cathode from an electrical discharge. Vacuum problems owing to specimen sublimation are limited by the use of a cold trap to adsorb sublimated gases in the vicinity of the specimen.

6.4.6 Specimen Stage



TEM sample support mesh "grid", with ultramicrotomy sections

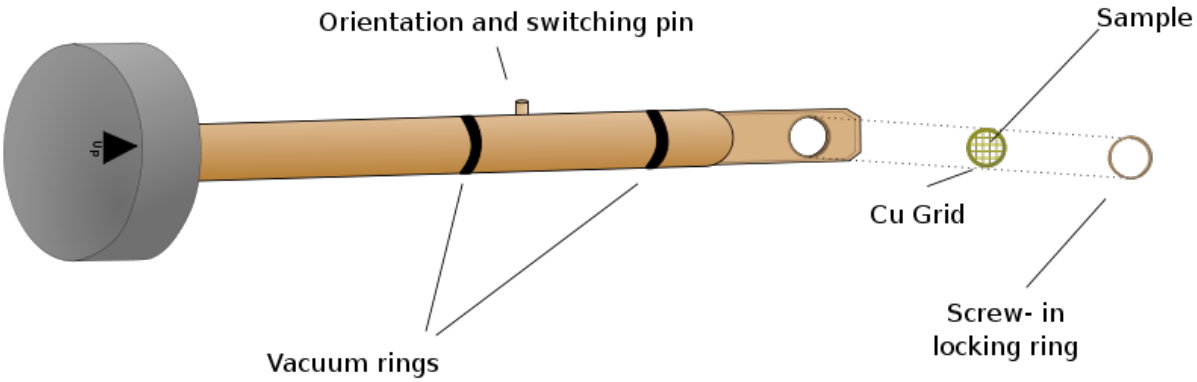
TEM specimen stage designs include airlocks to allow for insertion of the specimen holder into the vacuum with minimal increase in pressure in other areas of the microscope. The specimen holders are adapted to hold a standard size of grid upon which the sample is placed or a standard size of self-supporting specimen. Standard TEM grid sizes is a 3.05 mm diameter ring, with a thickness and mesh size ranging from a few to 100 µm. The sample is placed onto the inner meshed area having diameter of approximately 2.5 mm. Usual grid materials are copper, molybdenum, gold or platinum. This grid is placed into the sample holder which is paired with the specimen stage. A wide variety of designs of stages and holders exist, depending upon the type of experiment being performed. In addition to 3.05 mm grids, 2.3 mm grids are sometimes, if rarely, used. These grids were particularly used in the mineral sciences where a large degree of tilt can be

required and where specimen material may be extremely rare. Electron transparent specimens have a thickness around 100 nm, but this value depends on the accelerating voltage.

Once inserted into a TEM, the sample often has to be manipulated to present the region of interest to the beam, such as in single grain diffraction, in a specific orientation. To accommodate this, the TEM stage includes mechanisms for the translation of the sample in the XY plane of the sample, for Z height adjustment of the sample holder, and usually for at least 1° of rotation of the sample. Thus a TEM stage may provide four degrees of freedom for the motion of the specimen. Most modern TEMs provide the ability for two orthogonal rotation angles of movement with specialized holder designs called double-tilt sample holders. Of note however is that some stage designs, such as top-entry or vertical insertion stages once common for high resolution TEM studies, may simply only have X-Y translation available. The design criteria of TEM stages are complex, owing to the simultaneous requirements of mechanical and electron-optical constraints and have thus generated many unique implementations.

A TEM stage is required to have the ability to hold a specimen and be manipulated to bring the region of interest into the path of the electron beam. As the TEM can operate over a wide range of magnifications, the stage must simultaneously be highly resistant to mechanical drift, with drift requirements as low as a few nm/minute while being able to move several um/minute, with repositioning accuracy on the order of nanometers. Earlier designs of TEM accomplished this with a complex set of mechanical downgearing devices, allowing the operator to finely control the motion of the stage by several rotating rods. Modern devices may use electrical stage designs, using screw gearing in concert with stepper motors, providing the operator with a computer-based stage input, such as a joystick or trackball.

Two main designs for stages in a TEM exist, the side-entry and top entry version.[17] Each design must accommodate the matching holder to allow for specimen insertion without either damaging delicate TEM optics or allowing gas into TEM systems under vacuum.



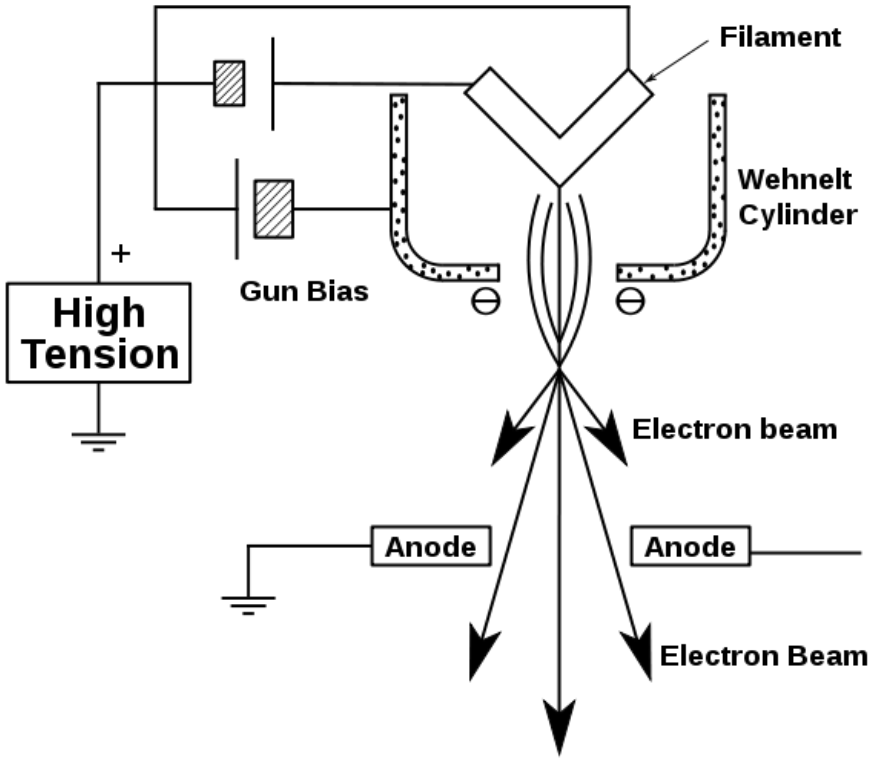
A diagram of a single axis tilt sample holder for insertion into a TEM goniometer. Titling of the holder is achieved by rotation of the entire goniometer

The most common is the side entry holder, where the specimen is placed near the tip of a long metal (brass or stainless steel) rod, with the specimen placed flat in a small bore. Along the rod are several polymer vacuum rings to allow for the formation of a vacuum seal of sufficient quality, when inserted into the stage. The stage is thus designed to accommodate the rod, placing the sample either in between or near the objective lens, dependent upon the objective design. When inserted into the stage, the side entry holder has its tip contained within the TEM vacuum, and the base is presented to atmosphere, the airlock formed by the vacuum rings.

Insertion procedures for side entry TEM holders typically involve the rotation of the sample to trigger micro switches that initiate evacuation of the airlock before the sample is inserted into the TEM column.

The second design is the top-entry holder consists of a cartridge that is several cm long with a bore drilled down the cartridge axis. The specimen is loaded into the bore, possibly utilising a small screw ring to hold the sample in place. This cartridge is inserted into an airlock with the bore perpendicular to the TEM optic axis. When sealed, the airlock is manipulated to push the cartridge such that the cartridge falls into place, where the bore hole becomes aligned with the beam axis, such that the beam travels down the cartridge bore and into the specimen. Such designs are typically unable to be tilted without blocking the beam path or interfering with the objective lens.

6.4.7 Electron Gun



Cross sectional diagram of an electron gun assembly, illustrating electron extraction

The electron gun is formed from several components: the filament, a biasing circuit, a Wehnelt cap, and an extraction anode. By connecting the filament to the negative component power supply, electrons can be "pumped" from the electron gun to the anode plate, and TEM column, thus completing the circuit. The gun is designed to create a beam of electrons exiting from the assembly at some given angle, known as the gun divergence semiangle, α. By constructing the Wehnelt cylinder such that it has a higher negative charge than the filament itself, electrons that exit the filament in a diverging manner are, under proper operation, forced into a converging pattern the minimum size of which is the gun crossover diameter.

The thermionic emission current density, *J*, can be related to the work function of the emitting material and is a Boltzmann distribution given below, where *A* is a constant, Φ is the work function and T is the temperature of the material.

$$J = AT^2 exp(-\frac{\Phi}{kT})$$

This equation shows that in order to achieve sufficient current density it is necessary to heat the emitter, taking care not to cause damage by application of excessive heat, for this reason materials with either a high melting point, such as tungsten, or those with a low work function (LaB_6) are required for the

gun filament.[*citation needed*] Furthermore both lanthanum hexaboride and tungsten thermionic sources must be heated in order to achieve thermionic emission, this can be achieved by the use of a small resistive strip. To prevent thermal shock, there is often a delay enforced in the application of current to the tip, to prevent thermal gradients from damaging the filament, the delay is usually a few seconds for LaB_6, and significantly lower for tungsten.

6.4.8 Electron Lens

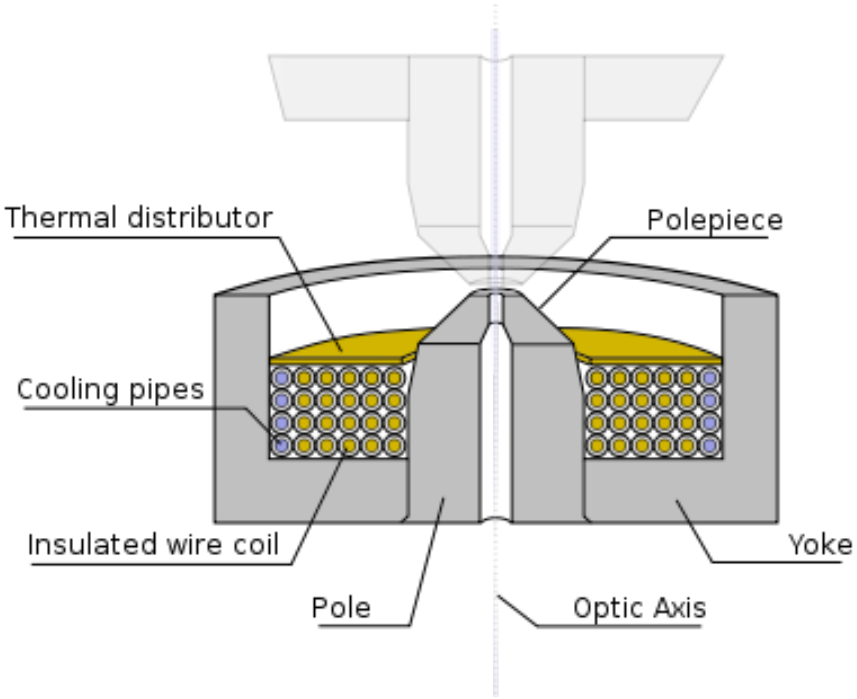


Diagram of a TEM split polepiece design lens

Electron lenses are designed to act in a manner emulating that of an optical lens, by focusing parallel rays at some constant focal length. Lenses may operate electrostatically or magnetically. The majority of electron lenses for TEM utilise electromagnetic coils to generate a convex lens. For these lenses the field produced for the lens must be radially symmetric, as deviation from the radial symmetry of the magnetic lens causes aberrations such as astigmatism, and worsens spherical and chromatic aberration. Electron lenses are manufactured from iron, iron-cobalt or nickel cobalt alloys, such as permalloy. These are selected for their magnetic properties, such as magnetic saturation, hysteresis and permeability.

The components include the yoke, the magnetic coil, the poles, the polepiece, and the external control circuitry. The polepiece must be manufactured in a very symmetrical manner, as this provides the boundary conditions for the

magnetic field that forms the lens. Imperfections in the manufacture of the polepiece can induce severe distortions in the magnetic field symmetry, which induce distortions that will ultimately limit the lenses' ability to reproduce the object plane. The exact dimensions of the gap, pole piece internal diameter and taper, as well as the overall design of the lens is often performed by finite element analysis of the magnetic field, whilst considering the thermal and electrical constraints of the design.

The coils which produce the magnetic field are located within the lens yoke. The coils can contain a variable current, but typically utilise high voltages, and therefore require significant insulation in order to prevent short-circuiting the lens components. Thermal distributors are placed to ensure the extraction of the heat generated by the energy lost to resistance of the coil windings. The windings may be water cooled, using a chilled water supply in order to facilitate the removal of the high thermal duty.

6.4.9 Apertures

Apertures are annular metallic plates, through which electrons that are further than a fixed distance from the optic axis may be excluded. These consist of a small metallic disc that is sufficiently thick to prevent electrons from passing through the disc, whilst permitting axial electrons. This permission of central electrons in a TEM cause to effects simultaneously: firstly, apertures decrease the beam intensity as electrons are filtered from the beam, which may be desired in the case of beam sensitive samples. Secondly, this filtering removes electrons that are scattered to high angles, which may be due to unwanted processes such as spherical or chromatic aberration, or due to diffraction from interaction within the sample.[23]

Apertures are either a fixed aperture within the column, such as at the condensor lens, or are a movable aperture, which can be inserted or withdrawn from the beam path, or moved in the plane perpendicular to the beam path. Aperture assemblies are mechanical devices which allow for the selection of different aperture sizes, which may be used by the operator to trade off intensity and the filtering effect of the aperture. Aperture assemblies are often equipped with micrometers to move the aperture, required during optical calibration.

6.4.10 Imaging Methods

Imaging methods in TEM utilize the information contained in the electron

waves exiting from the sample to form an image. The projector lenses allow for the correct positioning of this electron wave distribution onto the viewing system. The observed intensity of the image, I, assuming sufficiently high quality of imaging device, can be approximated as proportional to the time-average amplitude of the electron wavefunctions, where the wave which form the exit beam is denoted by Ψ.

$$I(x) = \frac{k}{t_1 - t_0} \int_{t_0}^{t_1} \Psi \Psi^* dt$$

Different imaging methods therefore attempt to modify the electron waves exiting the sample in a form that is useful to obtain information with regards to the sample, or beam itself. From the previous equation, it can be deduced that the observed image depends not only on the amplitude of beam, but also the phase of the electrons, although phase effects may often be ignored at lower magnifications. Higher resolution imaging requires thinner samples and higher energies of incident electrons. Therefore the sample can no longer be considered to be absorbing electrons, via a Beer's law effect, rather the sample can be modelled as an object that does not change the amplitude of the incoming electron wavefunction. Rather the sample modifies the phase of the incoming wave; this model is known as a pure phase object, for sufficiently thin specimens phase effects dominate the image, complicating analysis of the observed intensities. For example, to improve the contrast in the image the TEM may be operated at a slight defocus to enhance contrast, owing to convolution by the contrast transfer function of the TEM, which would normally decrease contrast if the sample was not a weak phase object.

6.4.11 Contrast Formation

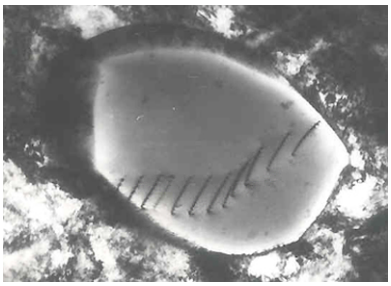
Contrast formation in the TEM depends greatly on the mode of operation. Complex imaging techniques, which utilise the unique ability to change lens strength or to deactivate a lens, allow for many operating modes. These modes may be used to discern information that is of particular interest to the investigator.

6.4.11.1 Bright Field

The most common mode of operation for a TEM is the bright field imaging mode. In this mode the contrast formation, when considered classically, is formed directly by occlusion and absorption of electrons in the sample. Thicker

regions of the sample, or regions with a higher atomic number will appear dark, whilst regions with no sample in the beam path will appear bright – hence the term "bright field". The image is in effect assumed to be a simple two dimensional projection of the sample down the optic axis, and to a first approximation may be modelled via Beer's law, more complex analyses require the modelling of the sample to include phase information.

6.4.11.2 Diffraction Contrast



Transmission Electron Micrograph of Dislocations, which are faults in the structure of the crystal lattice at the atomic scale

Samples can exhibit diffraction contrast, whereby the electron beam undergoes Bragg scattering, which in the case of a crystalline sample, disperses electrons into discrete locations in the back focal plane. By the placement of apertures in the back focal plane, i.e. the objective aperture, the desired Bragg reflections can be selected (or excluded), thus only parts of the sample that are causing the electrons to scatter to the selected reflections will end up projected onto the imaging apparatus.

If the reflections that are selected do not include the unscattered beam (which will appear up at the focal point of the lens), then the image will appear dark wherever no sample scattering to the selected peak is present, as such a region without a specimen will appear dark. This is known as a dark-field image.

Modern TEMs are often equipped with specimen holders that allow the user to tilt the specimen to a range of angles in order to obtain specific diffraction conditions, and apertures placed above the specimen allow the user to select electrons that would otherwise be diffracted in a particular direction from entering the specimen.

Applications for this method include the identification of lattice defects in crystals. By carefully selecting the orientation of the sample, it is possible not just to determine the position of defects but also to determine the type of defect present. If the sample is oriented so that one particular plane is only

slightly tilted away from the strongest diffracting angle (known as the Bragg Angle), any distortion of the crystal plane that locally tilts the plane to the Bragg angle will produce particularly strong contrast variations. However, defects that produce only displacement of atoms that do not tilt the crystal to the Bragg angle (i. e. displacements parallel to the crystal plane) will not produce strong contrast.

6.4.11.3 Electron Energy Loss

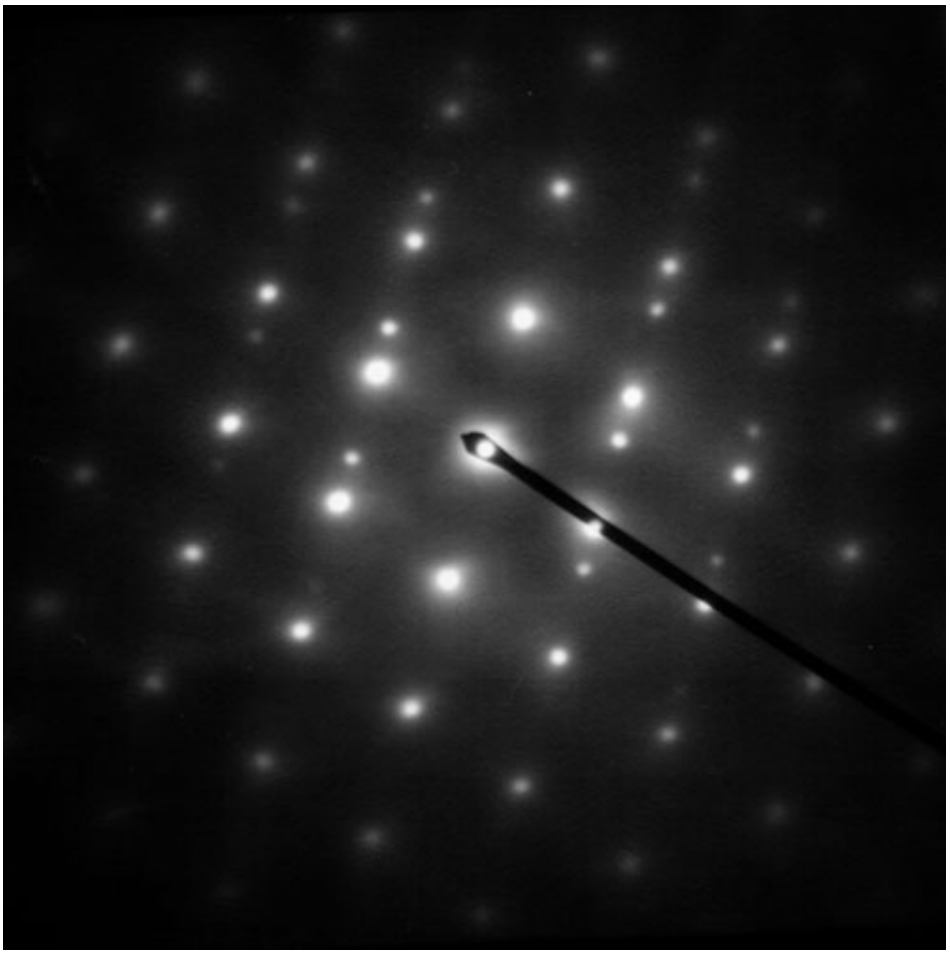
Utilizing the advanced technique of EELS, for TEMs appropriately equipped electrons can be rejected based upon their voltage (which, due to constant charge is their energy), using magnetic sector based devices known as EELS spectrometers. These devices allow for the selection of particular energy values, which can be associated with the way the electron has interacted with the sample. For example different elements in a sample result in different electron energies in the beam after the sample. This normally results in chromatic aberration – however this effect can, for example, be used to generate an image which provides information on elemental composition, based upon the atomic transition during electron-electron interaction.

EELS spectrometers can often be operated in both spectroscopic and imaging modes, allowing for isolation or rejection of elastically scattered beams. As for many images inelastic scattering will include information that may not be of interest to the investigator thus reducing observable signals of interest, EELS imaging can be used to enhance contrast in observed images, including both bright field and diffraction, by rejecting unwanted components.

6.4.11.4 Phase Contrast

Crystal structure can also be investigated by High Resolution Transmission Electron Microscopy (HRTEM), also known as phase contrast. When utilizing a Field emission source, of uniform thickness, the images are formed due to differences in phase of electron waves, which is caused by specimen interaction. Image formation is given by the complex modulus of the incoming electron beams. As such, the image is not only dependent on the number of electrons hitting the screen, making direct interpretation of phase contrast images more complex. However this effect can be used to advantage, as it can be manipulated provide more information about the sample, such as in complex phase retrieval techniques.

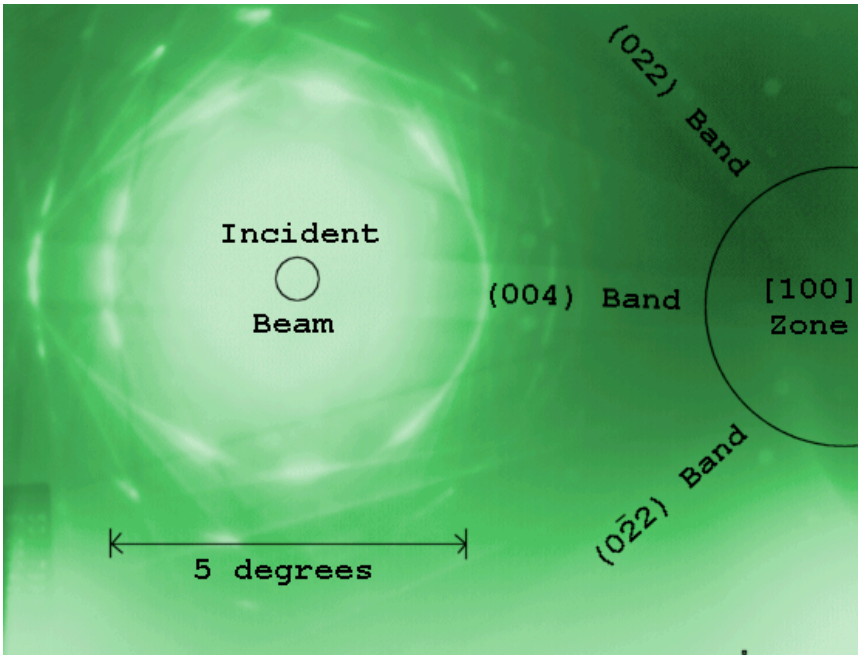
6.4.11.5 Diffraction



Crystalline diffraction pattern from a twinned grain of FCC Austenitic steel

As previously stated by adjusting the magnetic lenses such that the back focal plane of the lens rather than the imaging plane is placed on the imaging apparatus a diffraction pattern can be generated. For thin crystalline samples, this produces an image that consists of a pattern of dots in the case of a single crystal, or a series of rings in the case of a polycrystalline or amorphous solid material. For the single crystal case the diffraction pattern is dependent upon the orientation of the specimen and the structure of the sample illuminated by the electron beam. This image provides the investigator with information about the space group symmetries in the crystal and the crystal's orientation to the beam path. This is typically done without utilising any information but the position at which the diffraction spots appear and the observed image symmetries.

Diffraction patterns can have a large dynamic range, and for crystalline samples, may have intensities greater than those recordable by CCD. As such, TEMs may still be equipped with film cartridges for the purpose of obtaining these images, as the film is a single use detector.

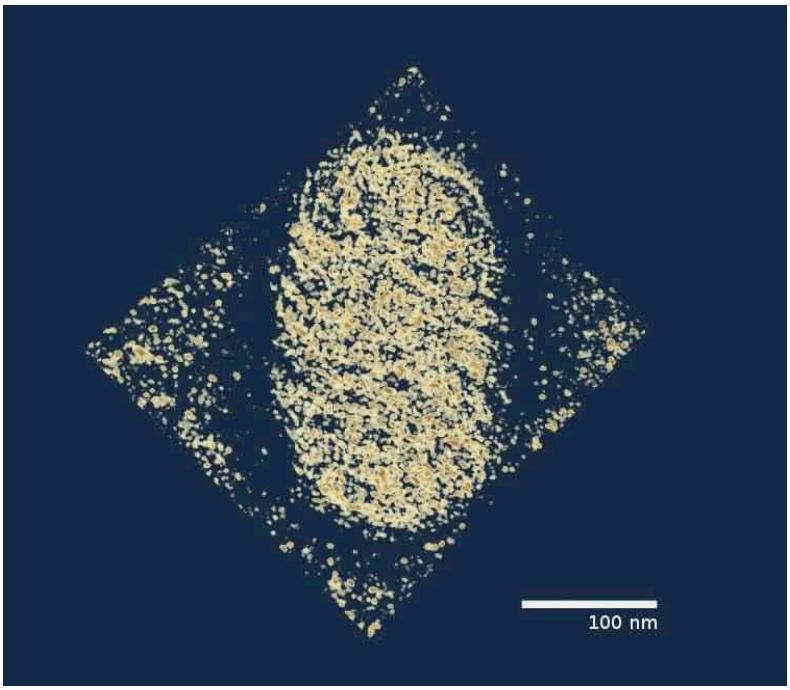


Convergent Beam Kikuchi lines from Silicon, near the [100] zone axis

Analysis of diffraction patterns beyond point-position can be complex, as the image is sensitive to a number of factors such as specimen thickness and orientation, objective lens defocus, spherical and chromatic aberration. Although quantitative interpretation of the contrast shown in lattice images is possible, it is inherently complicated and can require extensive computer simulation and analysis, such as electron multislice analysis.

More complex behaviour in the diffraction plane is also possible, with phenomena such as Kikuchi lines arising from multiple diffraction within the crystalline lattice. In convergent beam electron diffraction (CBED) where a non-parallel, i.e. converging, electron wavefront is produced by concentrating the electron beam into a fine probe at the sample surface, the interaction of the convergent beam can provide information beyond structural data such as sample thickness.

6.4.12 Three dimensional imaging



A three dimensional TEM image of a parapoxavirus

As TEM specimen holders typically allow for the rotation of a sample by a desired angle, multiple views of the same specimen can be obtained by rotating the angle of the sample along an axis perpendicular to the beam. By taking multiple images of a single TEM sample at differing angles, typically in 1° increments, a set of images known as a "tilt series" can be collected. Under purely absorption contrast conditions, this set of images can be used to construct a three-dimensional representation of the sample.

The reconstruction is accomplished by a two-step process, first images are aligned to account for errors in the positioning of a sample; such errors can occur due to vibration or mechanical drift. Alignment methods use image registration algorithms, such as autocorrelation methods to correct these errors. Secondly, using a technique known as filtered back projection, the aligned image slices can be transformed from a set of two-dimensional images, $I_j(x,y)$, to a single three-dimensional image, $I'_j(x,y,z)$. This three dimensional image is of particular interest when morphological information is required, further study can be undertaken using computer algorithms, such as isosurfaces and data slicing to analyse the data.

As TEM samples cannot typically be viewed at a full 180° rotation, the observed images typically suffer from a "missing wedge" of data, which when using Fourier based back projection methods decreases the range of resolvable frequencies in the three dimensional reconstruction. Mechanical

techniques, such as multi-axis tilting, as well as numerical techniques exist to limit the impact of this missing data on the observed specimen morphology. Variants on this method, referred to as single particle analysis, use images of multiple identical objects at different orientations to produce the image data required for three dimensional reconstruction. Assuming that objects do not have significant preferred orientations, this method does not suffer from the missing data wedge, however it assumes that the different objects imaged can be treated as if the data was generated from a single object.

6.4.13 Sample Preparation

Sample preparation in TEM can be a complex procedure. TEM specimens are required to be at most hundreds of nanometers thick, as unlike neutron or X-Ray radiation the electron beam interacts readily with the sample an effect that increases roughly with atomic number squared. High quality samples will have a thickness that is comparable to the mean free path of the electrons that travel through the samples, which may be only a few tens of nanometers. Preparation of TEM specimens is specific to the material under analysis and the desired information to obtain from the specimen. As such, many generic techniques have been used for the preparation of the required thin sections.

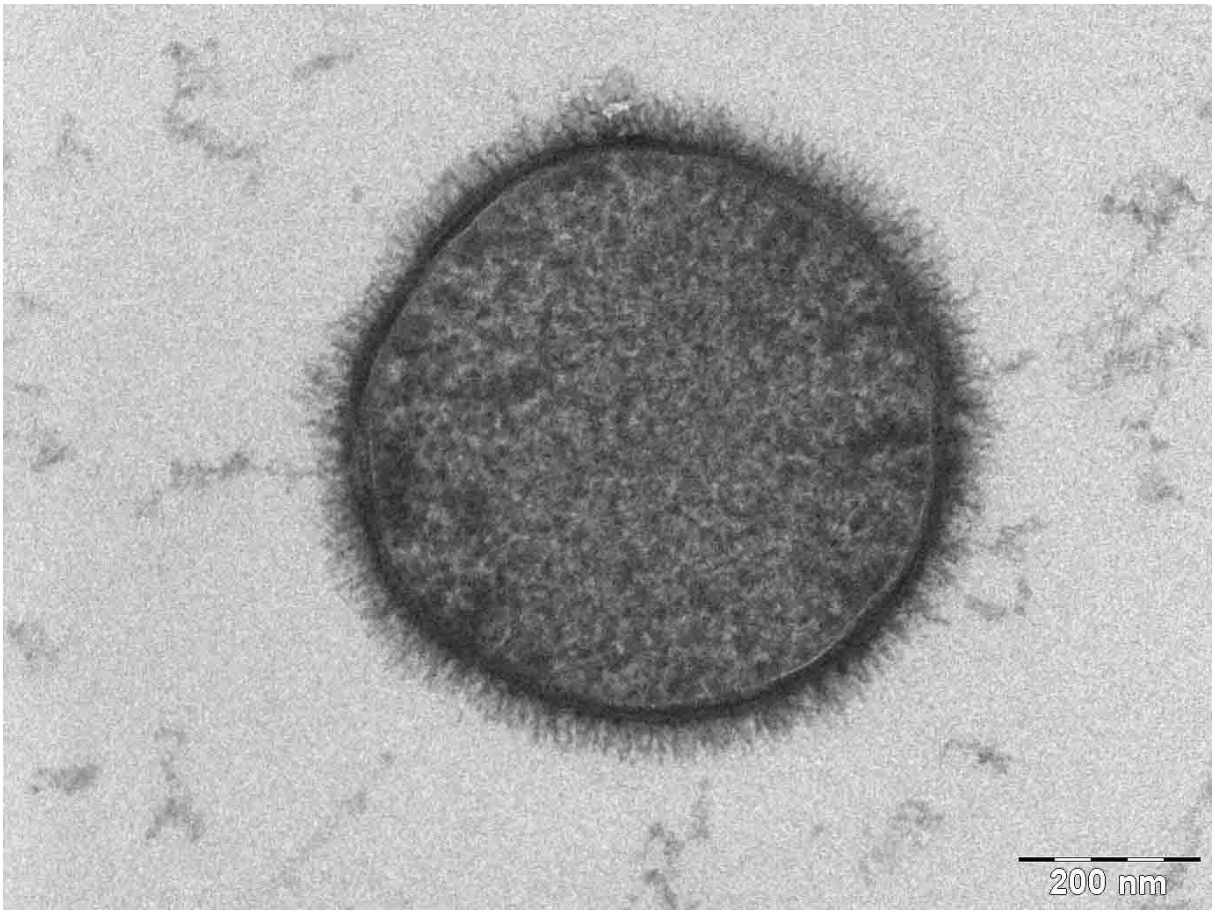
Materials that have dimensions small enough to be electron transparent, such as powders or nanotubes, can be quickly prepared by the deposition of a dilute sample containing the specimen onto support grids or films. In the biological sciences in order to withstand the instrument vacuum and facilitate handling, biological specimens can be fixated using either a negative staining material such as uranyl acetate or by plastic embedding. Alternately samples may be held at liquid nitrogen temperatures after embedding in vitreous ice. In material science and metallurgy the specimens tend to be naturally resistant to vacuum, but still must be prepared as a thin foil, or etched so some portion of the specimen is thin enough for the beam to penetrate. Constraints on the thickness of the material may be limited by the scattering cross-section of the atoms from which the material is comprised.

6.4.13.1 Tissue Sectioning

By passing samples over a glass or diamond edge, small, thin sections can be readily obtained using a semi-automated method. This method is used to obtain thin, minimally deformed samples that allow for the observation of tissue samples. Additionally inorganic samples have been studied, such as

aluminium, although this usage is limited owing to the heavy damage induced in the less soft samples. To prevent charge build-up at the sample surface, tissue samples need to be coated with a thin layer of conducting material, such as carbon, where the coating thickness is several nanometers. This may be achieved via an electric arc deposition process using a sputter coating device.

6.4.13.2 Sample Staining



A section of a cell of Bacillus subtilis, taken with a Tecnai T-12 TEM. The scale bar is 200 nm.

Details in light microscope samples can be enhanced by stains that absorb light; similarly TEM samples of biological tissues can utilize high atomic number stains to enhance contrast. The stain absorbs electrons or scatters part of the electron beam which otherwise is projected onto the imaging system. Compounds of heavy metals such as osmium, lead, or uranium may be used prior to TEM observation to selectively deposit electron dense atoms in or on the sample in desired cellular or protein regions, requiring an understanding of how heavy metals bind to biological tissues.

6.4.13.3 Mechanical Milling

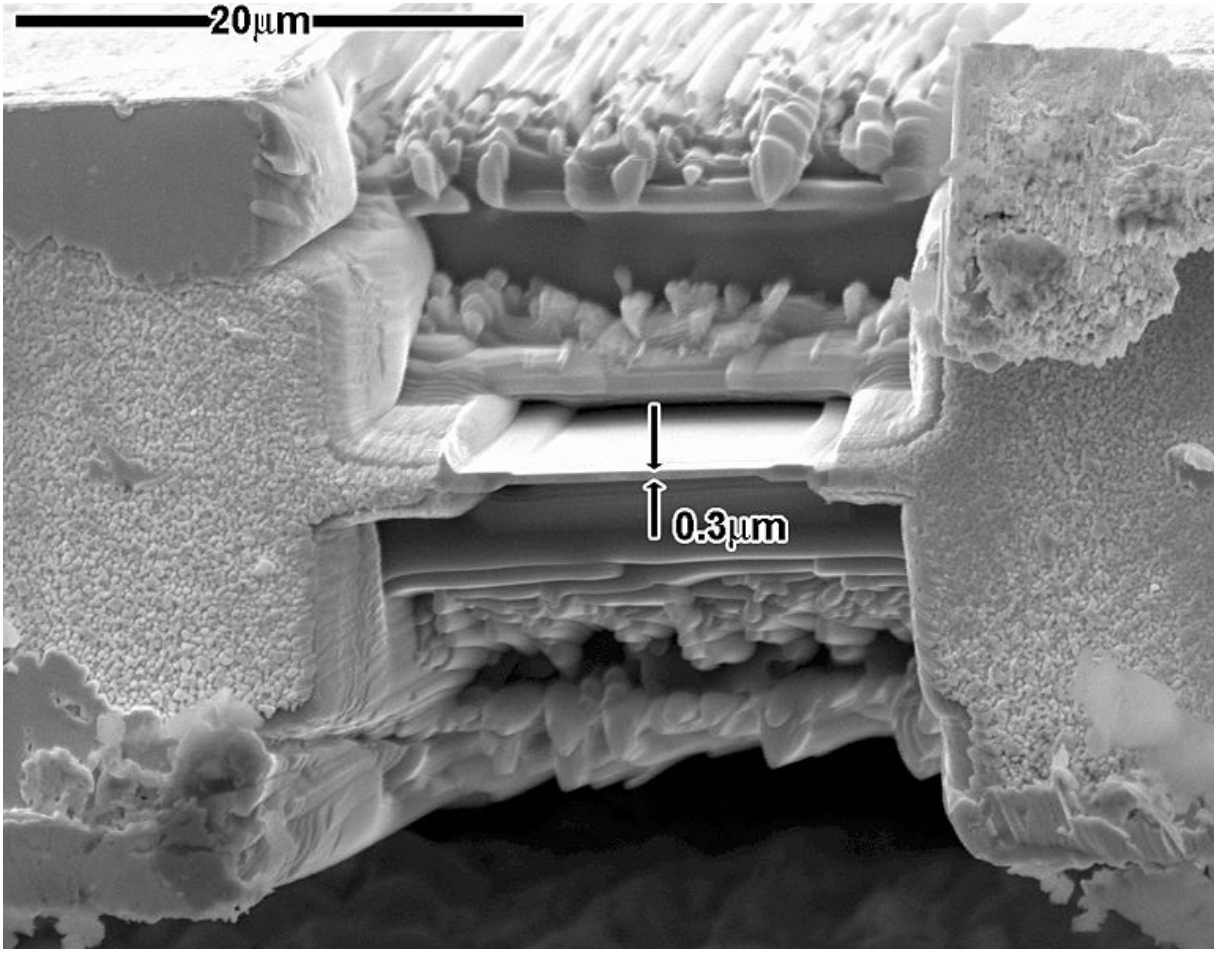
Mechanical polishing may be used to prepare samples. Polishing needs to be

done to a high quality, to ensure constant sample thickness across the region of interest. A diamond, or cubic boron nitride polishing compound may be used in the final stages of polishing to remove any scratches that may cause contrast fluctuations due to varying sample thickness. Even after careful mechanical milling, additional fine methods such as ion etching may be required to perform final stage thinning.

6.4.13.4 Chemical Etching

Certain samples may be prepared by chemical etching, particularly metallic specimens. These samples are thinned using a chemical etchant, such as an acid, to prepare the sample for TEM observation. Devices to control the thinning process may allow the operator to control either the voltage or current passing through the specimen, and may include systems to detect when the sample has been thinned to a sufficient level of optical transparency.

6.4.13.5 Ion Etching



SEM image of a thin TEM sample milled by FIB. The thin membrane shown here is suitable for TEM examination; however, at ~300-nm thick, it would not be suitable for high-resolution TEM without further milling.

Ion etching is a sputtering process that can remove very fine quantities of

material. This is used to perform a finishing polish of specimens polished by other means. Ion etching uses an inert gas passed through an electric field to generate a plasma stream that is directed to the sample surface. Acceleration energies for gases such as argon are typically a few kilovolts. The sample may be rotated to promote even polishing of the sample surface. The sputtering rate of such methods is on the order of tens of micrometers per hour, limiting the method to only extremely fine polishing.

More recently focussed ion beam methods have been used to prepare samples. FIB is a relatively new technique to prepare thin samples for TEM examination from larger specimens. Because FIB can be used to micro-machine samples very precisely, it is possible to mill very thin membranes from a specific area of interest in a sample, such as a semiconductor or metal. Unlike inert gas ion sputtering, FIB makes use of significantly more energetic gallium ions and may alter the composition or structure of the material through gallium implantation.

6.4.14 Modifications

The capabilities of the TEM can be further extended by additional stages and detectors, sometimes incorporated on the same microscope. An *electron cryomicroscope* is a TEM with a specimen holder capable of maintaining the specimen at liquid nitrogen or liquid helium temperatures. This allows imaging specimens prepared in vitreous ice, the preferred preparation technique for imaging individual molecules or macromolecular assemblies.

A TEM can be modified into a scanning transmission electron microscope (STEM) by the addition of a system that rasters the beam across the sample to form the image, combined with suitable detectors. Scanning coils are used to deflect the beam, such as by an electrostatic shift of the beam, where the beam is then collected using a current detector such as a faraday cup, which acts as a direct electron counter. By correlating the electron count to the position of the scanning beam (known as the "probe"), the transmitted component of the beam may be measured. The non-transmitted components may be obtained either by beam tilting or by the use of annular dark field detectors.

In-situ experiments may also be conducted with experiments such as in-situ reactions or material deformation testing.

Modern research TEMs may include aberration correctors, to reduce the

amount of distortion in the image. Incident beam Monochromators may also be used which reduce the energy spread of the incident electron beam to less than 0.15 eV. Major TEM makers include JEOL, Hitachi High-technologies, FEI Company (from merging with Philips Electron Optics) and Carl Zeiss.

6.4.15 Limitations

There are a number of drawbacks to the TEM technique. Many materials require extensive sample preparation to produce a sample thin enough to be electron transparent, which makes TEM analysis a relatively time consuming process with a low throughput of samples. Being almost transparent to electrons, a graphene substrate has been able to show single hydrogen atom and hydrocarbons. The structure of the sample may also be changed during the preparation process. Also the field of view is relatively small, raising the possibility that the region analysed may not be characteristic of the whole sample. There is potential that the sample may be damaged by the electron beam, particularly in the case of biological materials.

6.4.16 Resolution Limits

The limit of resolution obtainable in a TEM may be described in several ways, and is typically referred to as the information limit of the microscope. One commonly used value is a cut-off value of the contrast transfer function, a function that is usually quoted in the frequency domain to define the reproduction of spatial frequencies of objects in the object plane by the microscope optics. A cut-off frequency, q_{max}, for the transfer function may be approximated with the following equation, where C_s is the spherical aberration coefficient and λ is the electron wavelength:

$$q_{max} = \frac{1}{0.67(C_s\lambda^3)^{\frac{1}{4}}}$$

For a 200 kV microscope, with partly corrected spherical aberrations ("to the third order") and a C_s value of 1 μm, a theoretical cut-off value might be $1/q_{max}$ = 42 pm. The same microscope without a corrector would have C_s = 0.5 mm and thus a 200-pm cut-off. Practically, the spherical aberrations are suppressed in the best, "aberration-corrected" microscopes. Their resolution is however limited by electron source geometry and brightness and chromatic

aberrations in the objective lens system.

Intriguingly, the frequency domain representation of the contrast transfer function may often have an oscillatory nature, which can be tuned by adjusting the focal value of the objective lens. This oscillatory nature implies that some spatial frequencies are faithfully imaged by the microscope, whilst others are suppressed. By combining multiple images with different spatial frequencies, the use of techniques such as focal series reconstruction can be used to improve the resolution of the TEM to some extent. The contrast transfer function can, to some extent, be experimentally approximated through techniques such as Fourier transforming images of amorphous material, such as amorphous carbon.

More recently, advances in aberration corrector design have been able to reduce spherical aberrations and to achieve resolution below 0.5 Ångströms (50 pm) at magnifications above 50 million times. Improved resolution allows for the imaging of lighter atoms that scatter electrons less efficiently, such as lithium atoms in lithium battery materials. The ability to determine the position of atoms within materials has made the HRTEM an indispensable tool for nanotechnology research and development in many fields, including heterogeneous catalysis and the development of semiconductor devices for electronics and photonics.

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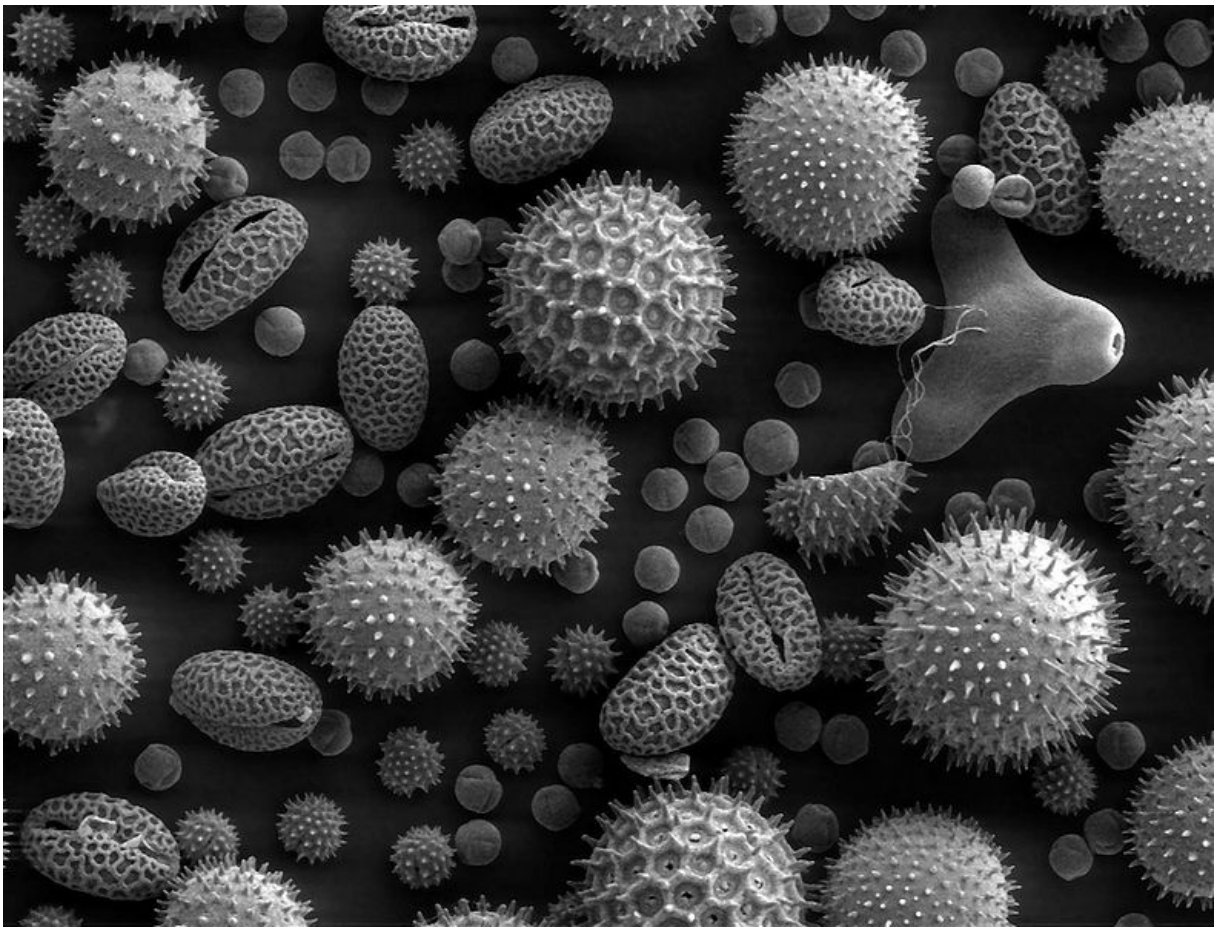
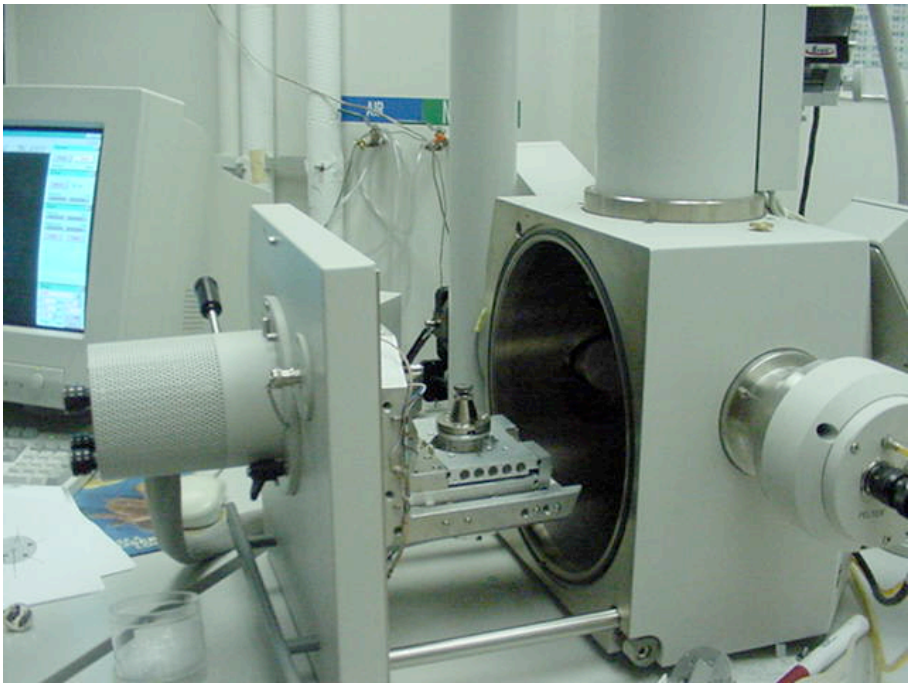
6.5 The Scanning electron microscope



These pollen grains taken on an SEM show the characteristic depth of field of SEM micrographs.



SEM opened sample chamber

The **scanning electron microscope** (**SEM**) is a type of electron microscope that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition and other properties such as electrical conductivity.

The types of signals produced by an SEM include secondary electrons, back-scattered electrons (BSE), characteristic X-rays, light (cathodoluminescence), specimen current and transmitted electrons. Secondary electron detectors are common in all SEMs, but it is rare that a single machine would have detectors for all possible signals. The signals result from interactions of the electron beam with atoms at or near the surface of the sample. In the most common or standard detection mode, secondary electron imaging or SEI, the SEM can produce very high-resolution images of a sample surface, revealing details about less than 1 to 5 nm in size. Due to the very narrow electron beam, SEM micrographs have a large depth of field yielding a characteristic three-dimensional appearance useful for understanding the surface structure of a sample. This is exemplified by the micrograph of pollen shown to the right. A wide range of magnifications is possible, from about 10 times (about equivalent to that of a powerful hand-lens) to more than 500,000 times, about 250 times the magnification limit of the best light microscopes. Back-scattered electrons (BSE) are beam electrons that are reflected from the sample by elastic scattering. BSE are often used in analytical SEM along with the spectra made from the characteristic X-rays. Because the intensity of the BSE signal is strongly related to the atomic number (Z) of the specimen, BSE images can provide information about the distribution of different elements in the sample. For the same reason, BSE imaging can image colloidal gold immuno-labels of 5 or 10 nm diameter which would otherwise be difficult or impossible to detect in secondary electron images in biological specimens. Characteristic X-rays are emitted when the electron beam removes an inner shell electron from the sample, causing a higher energy electron to fill the shell and release energy. These characteristic X-rays are used to identify the composition and measure the abundance of elements in the sample.

6.5.1 History

The first SEM image was obtained by Max Knoll, who in 1935 obtained an image of silicon steel showing electron channeling contrast.[1] Further pioneering work on the physical principles of the SEM and beam specimen interactions was performed by Manfred von Ardenne in 1937,[2][3] who produced a British patent[4] but never made a practical instrument. The SEM was further developed by Professor Sir Charles Oatley and his postgraduate student Gary Stewart and was first marketed in 1965 by the Cambridge Instrument Company as the "Stereoscan". The first instrument was delivered to DuPont.

6.5.2 Scanning Process and Image Formation

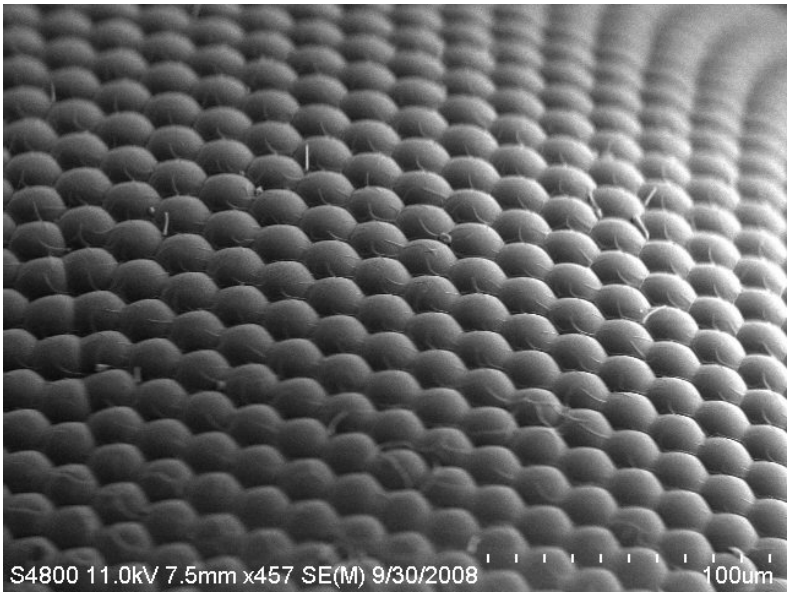
In a typical SEM, an electron beam is thermionically emitted from an electron gun fitted with a tungsten filament cathode. Tungsten is normally used in thermionic electron guns because it has the highest melting point and lowest vapour pressure of all metals, thereby allowing it to be heated for electron emission, and because of its low cost. Other types of electron emitters include lanthanum hexaboride (LaB_6) cathodes, which can be used in a standard tungsten filament SEM if the vacuum system is upgraded and field emission guns (FEG), which may be of the cold-cathode type using tungsten single crystal emitters or the thermally-assisted Schottky type, using emitters of zirconium oxide.

The electron beam, which typically has an energy ranging from a few hundred eV to 40 keV, is focused by one or two condenser lenses to a spot about 0.4 nm to 5 nm in diameter. The beam passes through pairs of scanning coils or pairs of deflector plates in the electron column, typically in the final lens, which deflect the beam in the *x* and *y* axes so that it scans in a raster fashion over a rectangular area of the sample surface.

When the primary electron beam interacts with the sample, the electrons lose energy by repeated random scattering and absorption within a teardrop-shaped volume of the specimen known as the interaction volume, which extends from less than 100 nm to around 5 μm into the surface. The size of the interaction volume depends on the electron's landing energy, the atomic number of the specimen and the specimen's density. The energy exchange between the electron beam and the sample results in the reflection of high-energy electrons by elastic scattering, emission of secondary electrons by

inelastic scattering and the emission of electromagnetic radiation, each of which can be detected by specialized detectors. The beam current absorbed by the specimen can also be detected and used to create images of the distribution of specimen current. Electronic amplifiers of various types are used to amplify the signals which are displayed as variations in brightness on a cathode ray tube. The raster scanning of the CRT display is synchronised with that of the beam on the specimen in the microscope, and the resulting image is therefore a distribution map of the intensity of the signal being emitted from the scanned area of the specimen. The image may be captured by photography from a high resolution cathode ray tube, but in modern machines is digitally captured and displayed on a computer monitor and saved to a computer's hard disc.

6.5.3 Magnification



An image of a house fly compound eye surface by using Scanning Electron Microscope at X450 magnification

Magnification in a SEM can be controlled over a range of up to 6 orders of magnitude from about 10 to 500,000 times. Unlike optical and transmission electron microscopes, image magnification in the SEM is not a function of the power of the objective lens. SEMs may have condenser and objective lenses, but their function is to focus the beam to a spot, and not to image the specimen. Provided the electron gun can generate a beam with sufficiently small diameter, a SEM could in principle work entirely without condenser or objective lenses, although it might not be very versatile or achieve very high resolution. In a SEM, as in scanning probe microscopy, magnification results from the ratio of the dimensions of the raster on the specimen and the raster

on the display device. Assuming that the display screen has a fixed size, higher magnification results from reducing the size of the raster on the specimen, and vice versa. Magnification is therefore controlled by the current supplied to the x, y scanning coils, or the voltage supplied to the x, y deflector plates, and not by objective lens power.

6.5.4 Sample Preparation

An insect coated in gold, having been prepared for viewing with a scanning electron microscope.

All samples must also be of an appropriate size to fit in the specimen chamber and are generally mounted rigidly on a specimen holder called a specimen stub. Several models of SEM can examine any part of a 6-inch (15 cm) semiconductor wafer, and some can tilt an object of that size to 45°.

For conventional imaging in the SEM, specimens must be electrically conductive, at least at the surface, and electrically grounded to prevent the accumulation of electrostatic charge at the surface. Metal objects require little special preparation for SEM except for cleaning and mounting on a specimen stub. Nonconductive specimens tend to charge when scanned by the electron beam, and especially in secondary electron imaging mode, this causes scanning faults and other image artifacts. They are therefore usually coated with an ultrathin coating of electrically-conducting material, commonly gold, deposited on the sample either by low vacuum sputter coating or by high vacuum evaporation. Conductive materials in current use for specimen coating include gold, gold/palladium alloy, platinum, osmium,[5] iridium, tungsten, chromium and graphite. Coating prevents the accumulation of static electric charge on the specimen during electron irradiation.

Two important reasons for coating, even when there is more than enough specimen conductivity to prevent charging, are to maximise signal and improve spatial resolution, especially with samples of low atomic number (Z). Broadly,

signal increases with atomic number, especially for backscattered electron imaging. The improvement in resolution arises because in low-Z materials, that is a material with low atomic number such as carbon, the electron beam can penetrate several micrometres below the surface, generating signals from an interaction volume much larger than the beam diameter and reducing spatial resolution. Coating with a high-Z material such as gold maximises secondary electron yield from within a surface layer a few nm thick, and suppresses secondary electrons generated at greater depths, so that the signal is predominantly derived from locations closer to the beam and closer to the specimen surface than would be the case in an uncoated, low-Z material. These effects are particularly, but not exclusively, relevant to biological samples.

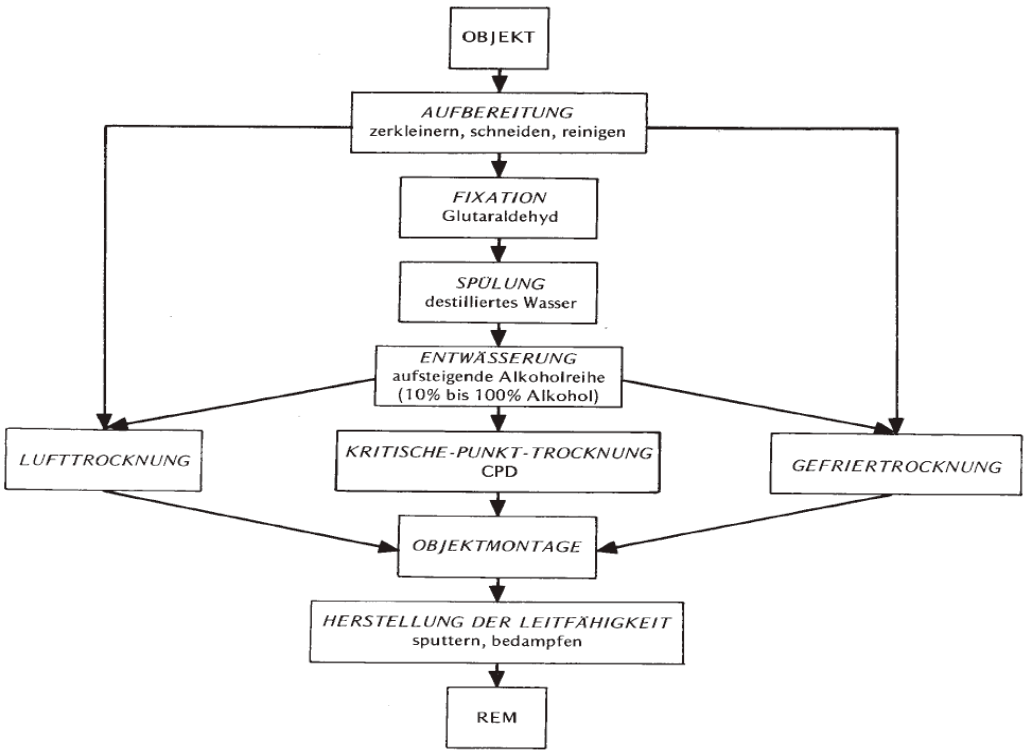
An alternative to coating for some biological samples is to increase the bulk conductivity of the material by impregnation with osmium using variants of the OTO staining method (O-osmium, T-thiocarbohydrazide, O-osmium).[6][7] Nonconducting specimens may be imaged uncoated using specialized SEM instrumentation such as the "Environmental SEM" (ESEM) or field emission gun (FEG) SEMs operated at low voltage. Environmental SEM instruments place the specimen in a relatively high pressure chamber where the working distance is short and the electron optical column is differentially pumped to keep vacuum adequately low at the electron gun. The high pressure region around the sample in the ESEM neutralizes charge and provides an amplification of the secondary electron signal. Low voltage SEM of non-conducting specimens can be operationally difficult to accomplish in a conventional SEM and is typically a research application for specimens that are sensitive to the process of applying conductive coatings. Low-voltage SEM is typically conducted in an FEG-SEM because the FEG is capable of producing high primary electron brightness even at low accelerating potentials. Operating conditions must be adjusted such that the local space charge is at or near neutral with adequate low voltage secondary electrons being available to neutralize any positively charged surface sites. This requires that the primary electron beam's potential and current be tuned to the characteristics of the sample specimen.

Embedding in a resin with further polishing to a mirror-like finish can be used for both biological and materials specimens when imaging in backscattered electrons or when doing quantitative X-ray microanalysis.

6.5.4.1 Biological Samples

For SEM, a specimen is normally required to be completely dry, since the specimen chamber is at high vacuum. Hard, dry materials such as wood, bone, feathers, dried insects or shells can be examined with little further treatment, but living cells and tissues and whole, soft-bodied organisms usually require chemical fixation to preserve and stabilize their structure. Fixation is usually performed by incubation in a solution of a buffered chemical fixative, such as glutaraldehyde, sometimes in combination with formaldehyde[8][9][10] and other fixatives,[11] and optionally followed by postfixation with osmium tetroxide.[8] The fixed tissue is then dehydrated. Because air-drying causes collapse and shrinkage, this is commonly achieved by critical point drying, which involves replacement of water in the cells with organic solvents such as ethanol or acetone, and replacement of these solvents in turn with a transitional fluid such as liquid carbon dioxide at high pressure. The carbon dioxide is finally removed while in a supercritical state, so that no gas-liquid interface is present within the sample during drying. The dry specimen is usually mounted on a specimen stub using an adhesive such as epoxy resin or electrically-conductive double-sided adhesive tape, and sputter coated with gold or gold/palladium alloy before examination in the microscope.

Beispiel einer Routine-Präparation biologischer Objekte:



If the SEM is equipped with a cold stage for cryo-microscopy, cryofixation may be used and low-temperature scanning electron microscopy performed on the cryogenically fixed specimens.[8] Cryo-fixed specimens may be cryo-fractured under vacuum in a special apparatus to reveal internal structure, sputter coated and transferred onto the SEM cryo-stage while still frozen.[12] Low-temperature scanning electron microscopy is also applicable to the imaging of temperature-sensitive materials such as ice[13][14] (see e.g. illustration at right) and fats.[15]

Freeze-fracturing, freeze-etch or freeze-and-break is a preparation method particularly useful for examining lipid membranes and their incorporated proteins in "face on" view. The preparation method reveals the proteins embedded in the lipid bilayer.

Gold has a high atomic number and sputter coating with gold produces high topographic contrast and resolution. However, the coating has a thickness of a few micrometres, and can obscure the underlying fine detail of the specimen at very high magnification. Low-vacuum SEMs with differential pumping apertures allow samples to be imaged without such coating and without the loss of natural contrast caused by the coating, but are unable to achieve the resolution attainable by conventional SEMs with coated specimens.

6.5.4.2 Materials

Back scattered electron imaging, quantitative X-ray analysis, and X-ray mapping of geological specimens and metals requires that the surfaces be ground and polished to an ultra smooth surface. Geological specimens that undergo WDS or EDS analysis are often carbon coated. Metals are not generally coated prior to imaging in the SEM because they are conductive and provide their own pathway to ground.

Fractography is the study of fractured surfaces that can be done on a light microscope or commonly, on an SEM. The fractured surface is cut to a suitable size, cleaned of any organic residues, and mounted on a specimen holder for viewing in the SEM.

Integrated circuits may be cut with a focused ion beam (FIB) or other ion beam milling instrument for viewing in the SEM. The SEM in the first case may be incorporated into the FIB.

Metals, geological specimens, and integrated circuits all may also be

chemically polished for viewing in the SEM.

Special high resolution coating techniques are required for high magnification imaging of inorganic thin films.

6.5.5 ESEM

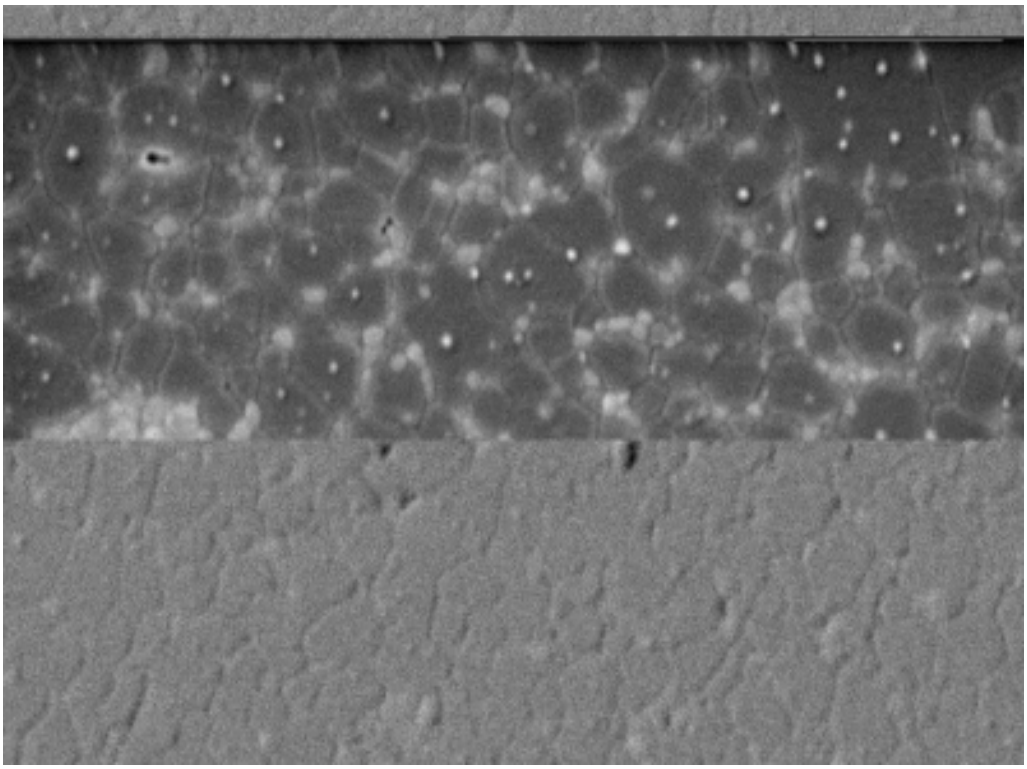
The accumulation of electric charge on the surfaces of non-metallic specimens can be avoided by using environmental SEM in which the specimen is placed in an internal chamber at higher pressure than the vacuum in the electron optical column. Positively charged ions generated by beam interactions with the gas help to neutralize the negative charge on the specimen surface. The pressure of gas in the chamber can be controlled, and the type of gas used can be varied according to need. Coating is thus unnecessary, and X-ray analysis unhindered.

6.5.6 Detection of Secondary Electrons

The most common imaging mode collects low-energy (<50 eV) secondary electrons that are ejected from the k-orbitals of the specimen atoms by inelastic scattering interactions with beam electrons. Due to their low energy, these electrons originate within a few nanometers from the sample surface.[16] The electrons are detected by an Everhart-Thornley detector[17] which is a type of scintillator-photomultiplier system. The secondary electrons are first collected by attracting them towards an electrically-biased grid at about +400 V, and then further accelerated towards a phosphor or scintillator positively biased to about +2,000 V. The accelerated secondary electrons are now sufficiently energetic to cause the scintillator to emit flashes of light (cathodoluminescence) which are conducted to a photomultiplier outside the SEM column via a light pipe and a window in the wall of the specimen chamber. The amplified electrical signal output by the photomultiplier is displayed as a two-dimensional intensity distribution that can be viewed and photographed on an analogue video display, or subjected to analog-to-digital conversion and displayed and saved as a digital image. This process relies on a raster-scanned primary beam. The brightness of the signal depends on the number of secondary electrons reaching the detector. If the beam enters the sample perpendicular to the surface, then the activated region is uniform about

the axis of the beam and a certain number of electrons "escape" from within the sample. As the angle of incidence increases, the "escape" distance of one side of the beam will decrease, and more secondary electrons will be emitted. Thus steep surfaces and edges tend to be brighter than flat surfaces, which results in images with a well-defined, three-dimensional appearance. Using this technique, image resolution less than 0.5 nm is possible.

6.5.7 Detection of Backscattered Electrons



Comparison of SEM techniques: Top: backscattered electron analysis - composition Bottom: secondary electron analysis - topography

Backscattered electrons (BSE) consist of high-energy electrons originating in the electron beam, that are reflected or back-scattered out of the specimen interaction volume by elastic scattering interactions with specimen atoms. Since heavy elements (high atomic number) backscatter electrons more strongly than light elements (low atomic number), and thus appear brighter in the image, BSE are used to detect contrast between areas with different chemical compositions.[16] The Everhart-Thornley detector, which is normally positioned to one side of the specimen, is inefficient for the detection of backscattered electrons because few such electrons are emitted in the solid angle subtended by the detector, and because the positively biased detection grid has little ability to attract the higher energy BSE electrons. Dedicated

backscattered electron detectors are positioned above the sample in a "doughnut" type arrangement, concentric with the electron beam, maximising the solid angle of collection. BSE detectors are usually either of scintillator or semiconductor types. When all parts of the detector are used to collect electrons symmetrically about the beam, atomic number contrast is produced. However, strong topographic contrast is produced by collecting back-scattered electrons from one side above the specimen using an asymmetrical, directional BSE detector; the resulting contrast appears as illumination of the topography from that side. Semiconductor detectors can be made in radial segments that can be switched in or out to control the type of contrast produced and its directionality.

Backscattered electrons can also be used to form an electron backscatter diffraction (EBSD) image that can be used to determine the crystallographic structure of the specimen.

6.5.8 Beam-injection Analysis of Semiconductors

The nature of the SEM's probe, energetic electrons, makes it uniquely suited to examining the optical and electronic properties of semiconductor materials. The high-energy electrons from the SEM beam will inject charge carriers into the semiconductor. Thus, beam electrons lose energy by promoting electrons from the valence band into the conduction band, leaving behind holes.

In a direct bandgap material, recombination of these electron-hole pairs will result in cathodoluminescence; if the sample contains an internal electric field, such as is present at a p-n junction, the SEM beam injection of carriers will cause electron beam induced current (EBIC) to flow.

Cathodoluminescence and EBIC are referred to as "beam-injection" techniques, and are very powerful probes of the optoelectronic behavior of semiconductors, particularly for studying nanoscale features and defects.

6.5.9 Cathodoluminescence

Cathodoluminescence, the emission of light when atoms excited by high-energy electrons return to their ground state, is analogous to UV-induced fluorescence, and some materials such as zinc sulfide and some fluorescent

dyes, exhibit both phenomena. Cathodoluminescence is most commonly experienced in everyday life as the light emission from the inner surface of the cathode ray tube in television sets and computer CRT monitors. In the SEM, CL detectors either collect all light emitted by the specimen, or can analyse the wavelengths emitted by the specimen and display an emission spectrum or an image of the distribution of cathodoluminescence emitted by the specimen in real colour.

6.5.10 X-ray Microanalysis

X-rays, which are also produced by the interaction of electrons with the sample, may also be detected in an SEM equipped for energy-dispersive X-ray spectroscopy or wavelength dispersive X-ray spectroscopy.

6.5.11 Resolution of the SEM

The spatial resolution of the SEM depends on the size of the electron spot, which in turn depends on both the wavelength of the electrons and the electron-optical system which produces the scanning beam. The resolution is also limited by the size of the interaction volume, or the extent to which the material interacts with the electron beam. The spot size and the interaction volume are both large compared to the distances between atoms, so the resolution of the SEM is not high enough to image individual atoms, as is possible in the shorter wavelength (i.e. higher energy) transmission electron microscope (TEM). The SEM has compensating advantages, though, including the ability to image a comparatively large area of the specimen; the ability to image bulk materials (not just thin films or foils); and the variety of analytical modes available for measuring the composition and properties of the specimen. Depending on the instrument, the resolution can fall somewhere between less than 1 nm and 20 nm. By 2009, The world's highest SEM resolution at high beam energies (0.4 nm at 30 kV) is obtained with the Hitachi S-5500. At low beam energies, the best resolution (by 2009) is achieved by the Magellan system from FEI Company (0.9 nm at 1 kV).

6.5.12 Environmental SEM

Conventional SEM requires samples to be imaged under vacuum, because a gas atmosphere rapidly spreads and attenuates electron beams. Consequently, samples that produce a significant amount of vapour, e.g. wet biological samples or oil-bearing rock need to be either dried or cryogenically frozen. Processes involving phase transitions, such as the drying of adhesives or melting of alloys, liquid transport, chemical reactions, solid-air-gas systems and living organisms in general cannot be observed.

The first commercial development of the Environmental SEM (ESEM) in the late 1980s [18][19] allowed samples to be observed in low-pressure gaseous environments (e.g. 1-50 Torr) and high relative humidity (up to 100%). This was made possible by the development of a secondary-electron detector [20][21] capable of operating in the presence of water vapour and by the use of pressure-limiting apertures with differential pumping in the path of the electron beam to separate the vacuum region (around the gun and lenses) from the sample chamber.

The first commercial ESEMs were produced by the ElectroScan Corporation in USA in 1988. ElectroScan were later taken over by Philips (now FEI Company) in 1996 [22].

ESEM is especially useful for non-metallic and biological materials because coating with carbon or gold is unnecessary. Uncoated Plastics and Elastomers can be routinely examined, as can uncoated biological samples. Coating can be difficult to reverse, may conceal small features on the surface of the sample and may reduce the value of the results obtained. X-ray analysis is difficult with a coating of a heavy metal, so carbon coatings are routinely used in conventional SEMs, but ESEM makes it possible to perform X-ray microanalysis on uncoated non-conductive specimens. ESEM may be the preferred for electron microscopy of unique samples from criminal or civil actions, where forensic analysis may need to be repeated by several different experts.

6.5.13 3D in SEM

3D data can be measured in the SEM with different methods:

- photogrametry (2 or 3 images from tilted specimen)

Photometric stereo (use of 4 images from BSE detector)

Possible applications are roughness measurement, measurement of fractal dimenstion, corrosion measurement and height step measurement.

6.5.14 Terminology

DR-SEM
Defect Review - Scanning Electron Microscopy
CD-SEM
Critical Dimension Measurement - Scanning Electron Microscopy
FIB-SEM
Focused Ion Beam - Scanning Electron Microscopy (used mainly for cutting samples)
SBFSEM
Serial Block-Face Scanning Electron Microscopy
ESEM
Environmental Scanning Electron Microscopy
LV SEM
Low Vacuum Scanning Electron Microscopy
FESEM
Field emission gun Scanning Electron Microscopy

6.5.15 References

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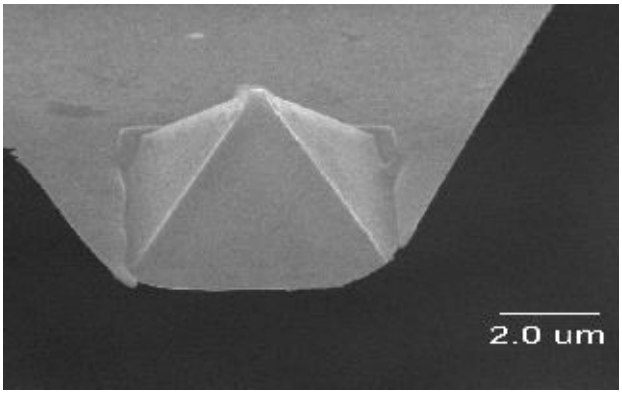
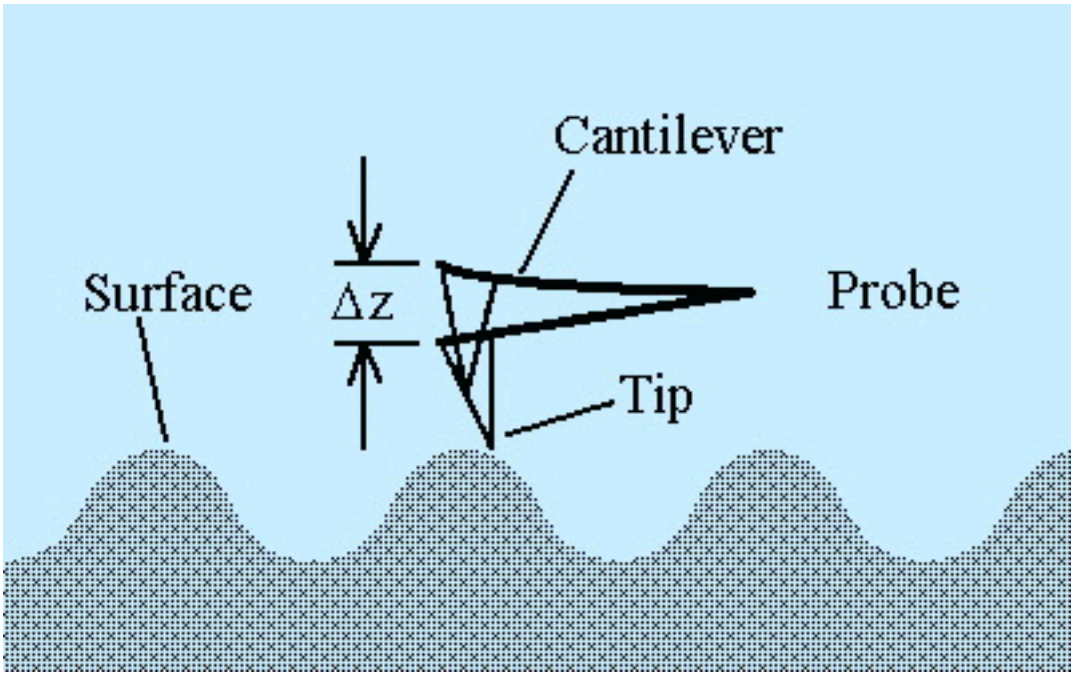
History of Electron Microscopy 1990s

7. Scanning probe microscopy



A probe used for atomic force microscopy.



How a probe tip scans over a sample (not to scale)

Scanning Probe Microscopy (SPM) is a branch of microscopy that forms images of surfaces using a physical probe that scans the specimen. An image of the surface is obtained by mechanically moving the probe in a raster scan of the specimen, line by line, and recording the probe-surface interaction as a function of position. SPM was founded with the invention of the scanning tunneling microscope in 1981.

Many scanning probe microscopes can image several interactions simultaneously. The manner of using these interactions to obtain an image is generally called a mode.

The resolution varies somewhat from technique to technique, but some probe techniques reach a rather impressive atomic resolution. They owe this largely to the ability of piezoelectric actuators to execute motions with a precision and accuracy at the atomic level or better on electronic command. One could rightly call this family of technique 'piezoelectric techniques'. The other common denominator is that the data are typically obtained as a two-dimensional grid of data points, visualized in fals

7.1 Probe Techniques

The three most common scanning probe techniques are:

Atomic Force Microscopy (AFM) measures the **interaction force** between the tip and surface. The tip may be dragged across the surface, or may vibrate as it moves. The interaction force will depend on the nature of the sample, the probe tip and the distance between them.

Scanning Tunneling Microscopy (STM) measures a **weak electrical current** flowing between tip and sample as they are held a very distance apart.

Near-Field Scanning Optical Microscopy (NSOM) scans a very **small light source** very close to the sample. Detection of this light energy forms the image. NSOM can provide resolution below that of the conventional light microscope.

There are numerous variations on these techniques. AFM may operate in several modes which differ according to the force between the tip and surface:

Mode of Operation	Force of Interaction
contact mode	strong (repulsive) - constant force or constant distance
non-contact mode	weak (attractive) - vibrating probe
intermittent contact mode	strong (repulsive) - vibrating probe
lateral force mode	frictional forces exert a torque on the scanning cantilever
magnetic force	the magnetic field of the surface is imaged
thermal scanning	the distribution of thermal conductivity is imaged

In contact mode, the tip is usually maintained at a constant force by moving the cantilever up and down as it scans. In non-contact mode or intermittent contact mode (tapping modeTM) the tip is driven up and down by an oscillator. Especially soft materials may be imaged by a magnetically-driven cantilever (MAC ModeTM). In non-contact mode, the bottom-most point of each probe cycle is in the *attractive* region of the force-distance curve. In intermittent contact mode the bottom-most point is in the *repulsive* region. Variations in the measured oscillation amplitude and phase in relation to the driver frequency are indicators of the surface-probe interaction.

To image *frictional force*, the probe is dragged along the surface, resulting in a torque on the cantilever. To image the magnetic field of the surface, a magnetically-susceptible probe is used. In other variations, the electric charge distribution on the surface or the surface capacitance is imaged. For thermal scanning microscopy (TSM) the thermal conductivity of the surface with is probed with a resistive tip that acts as a tiny resistance thermometer.

In addition to these modes, many instruments are also designed to plot the phase difference between the measured modes, for example frictional force versus contact profile. This plot is called **phase mode**.

Several types of probes with different tips are used in scanning probe microscopy.

Tip selection depends on the mode of operation and on the type of sample.

7.2 Established types of scanning probe microscopy

AFM, atomic force microscopy
- Contact AFM
- Non-contact AFM
- Dynamic contact AFM
- Tapping AFM

BEEM, ballistic electron emission microscopy
EFM, electrostatic force microscope
ESTM electrochemical scanning tunneling microscope
FMM, force modulation microscopy
KPFM, kelvin probe force microscopy
MFM, magnetic force microscopy
MRFM, magnetic resonance force microscopy
NSOM, near-field scanning optical microscopy (or SNOM, scanning near-field optical microscopy)
PFM, Piezo Force Microscopy
PSTM, photon scanning tunneling microscopy
PTMS, photothermal microspectroscopy/microscopy
SAP, scanning atom probe [1]
SECM, scanning electrochemical microscopy
SCM, scanning capacitance microscopy
SGM, scanning gate microscopy
SICM, scanning ion-conductance microscopy
SPSM spin polarized scanning tunneling microscopy
SThM, scanning thermal microscopy[1]
STM, scanning tunneling microscopy
SVM, scanning voltage microscopy
SHPM, scanning Hall probe microscopy
SXSTM synchrotron x-ray scanning tunneling microscopy

Of these techniques AFM and STM are the most commonly used followed by MFM and SNOM/NSOM.

7. 3 Probe Tips

Probe tips are normally made of platinum/iridium or gold. There are two main methods for obtaining a sharp probe tip, acid etching and cutting. The first involves dipping a wire end first into an acid bath and waiting until it has etched through the wire and the lower part drops away. The remained is then removed and the resulting tip is often one atom in diameter. An alternative and much quicker method is to take a thin wire and cut it with a pair of scissors or a scalpel. Testing the tip produced via this method on a sample with a known profile will indicate whether the tip is good or

not and a single sharp point is achieved roughly 50% of the time. It is not uncommon for this method to result in a tip with more than one peak; one can easily discern this upon scan due to a high level of ghost images.

7.4 Advantages of Scanning Probe Microscopy

The resolution of the microscopes is not limited by diffraction, but only by the size of the probe-sample interaction volume (i.e., point spread function), which can be as small as a few picometres. Hence the ability to measure small local differences in object height (like that of 135 picometre steps on <100> silicon) is unparalleled. Laterally the probe-sample interaction extends only across the tip atom or atoms involved in the interaction.

The interaction can be used to modify the sample to create small structures (nanolithography).

Unlike electron microscope methods, specimens do not require a partial vacuum but can be observed in air at standard temperature and pressure or while submerged in a liquid reaction vessel.

7.5 Disadvantages of Scanning Probe Microscopy

The detailed shape of the scanning tip is sometimes difficult to determine. Its effect on the resulting data is particularly noticeable if the specimen varies greatly in height over lateral distances of 10 nm or less.

The scanning techniques are generally slower in acquiring images, due to the scanning process. As a result, efforts are being made to greatly improve the scanning rate. Like all scanning techniques, the embedding of spatial information into a time sequence opens the door to uncertainties in metrology, say of lateral spacings and angles, which arise due to time-domain effects like specimen drift, feedback loop oscillation, and mechanical vibration.

The maximum image size is generally smaller.

Scanning probe microscopy is often not useful for examining buried solid-solid or liquid-liquid interfaces.

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8. Scanning Acoustic Microscope

A penny scanned in an acoustic microscope at 50 MHz

A **Scanning Acoustic Microscope** (**SAM**) is a device which uses focused sound to investigate, measure, or image an object (a process called Scanning Acoustic Tomography). It is commonly used in failure analysis and non-destructive evaluation. It also has applications in biological and medical research. The semiconductor industry has found the Scanning Acoustic Microscope useful in detecting voids, cracks, and delaminations within microelectronic packages.

8.1 History

The first scanning acoustic microscope was developed in 1974 by C. F. Lemons and R. A. Quate at the Microwave Laboratory of Stanford University.[1]

8.2 Principles of Operation

Scanning Acoustic Microscopy works by directing focused sound from a transducer at a small point on a target object. Sound hitting the object is either scattered, absorbed, reflected (scattered at 180°) or transmitted (scattered at 0°). Typically, either the reflected or transmitted sound is gathered and measured. Based on the measurement, a value is assigned to the location

investigated. The transducer (or object) is moved slightly and then insonified again. This process is repeated in a systematic pattern until the entire region of interest has been investigated. Often the values for each point are assembled into an image of the object. The contrast seen in the image is based either on the object's geometry or material composition. The resolution of the image is limited either by the physical scanning resolution or the width of the sound beam (which in turn is determined by the frequency of the sound).

8.3 Applications

8.3.1 Medicine and Biology

SAM can provide data on the elasticity of cells and tissues, which can give useful information on the physical forces holding structures in a particular shape and the mechanics of structures such as the cytoskeleton.[2][3] These studies are particularly valuable in investigating processes such as cell motility.[4][5]

Flaw Detection
Internal stress investigation
Elastic property characterization

8.3.2 Ultrasonic Direct Microscopy

Ultrasonic Direct Microscope (**UDM**) base on measuring the ultrasonic signal directly. The conventional sensor include a level sensor that means that the measurement instrument attitude of the signal. **ADM** technique deal with ultrasonic signal as is . That make possible to produce modulate ultrasonic signal and to check not only the main frequency, and enable wide range in order to examine little chances in to material in test.

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