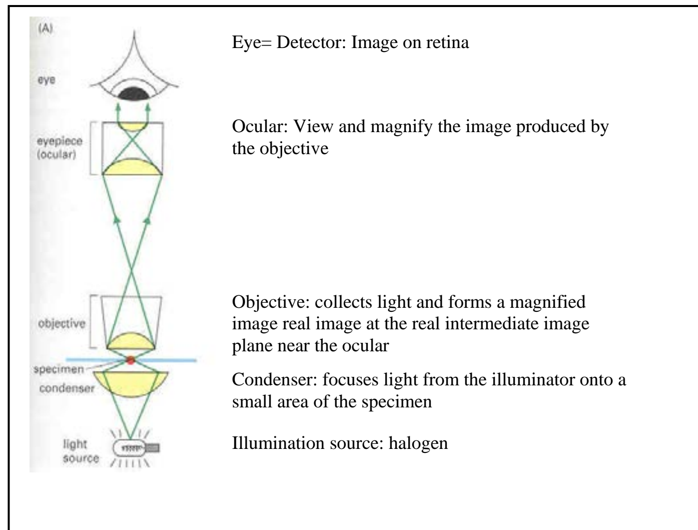
Answers to exam in course TFY4265 BIOPHYSICAL MICROMETHODS

5. December 2007

Exercise 1: Light microscopy

a) **Bright field microscopy:**

The light path and main components with functions are shown below

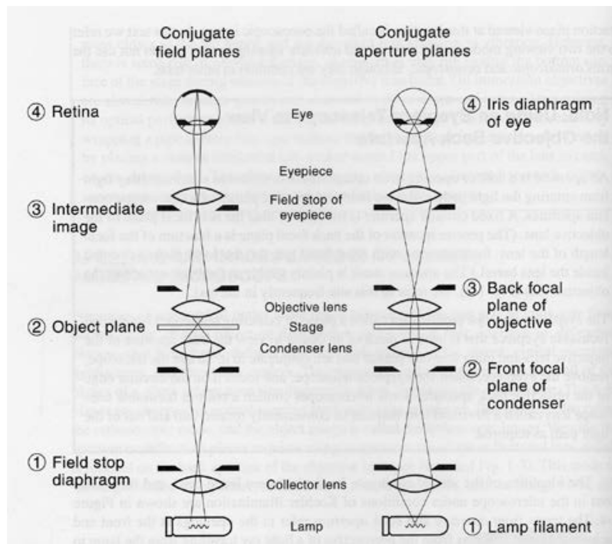


b) Köhler's illumination principle:

The principle says: A given point in the specimen has to be illuminated by every point in the light source, and a given point in the light source has to illuminate every point in the specimen. In this way optimal illumination of the sample is obtained and the best image obtained

The microscope has to be adjusted as shown below to fulfil the requirements:

- The collector lens focuses an image of the lamp filament at the front focal plane of the condenser
- Image of the field stop in the object



c) Phase contrast

The principle of phase contrast is to convert a phase difference into an amplitude difference which can be observed. When light passes through a sample a small portion of the light is diffracted due to differences in the refractive index and thickness of the sample. This phase shift is approximately $\pi/2$ or $\lambda/4$ relative to nondiffracted light. By adding another phase shift of $\pi/2$ or $\lambda/4$ by advancing the undiffracted light, destructive interference can be obtained between the diffracted and non-diffracted light. This gives positive contrast and objects of increasing density and/or higher refractive index will appear darker. Constructive interference can be obtained by retarding the nondiffracted light $\pi/2$ or $\lambda/4$ relative to diffracted light,

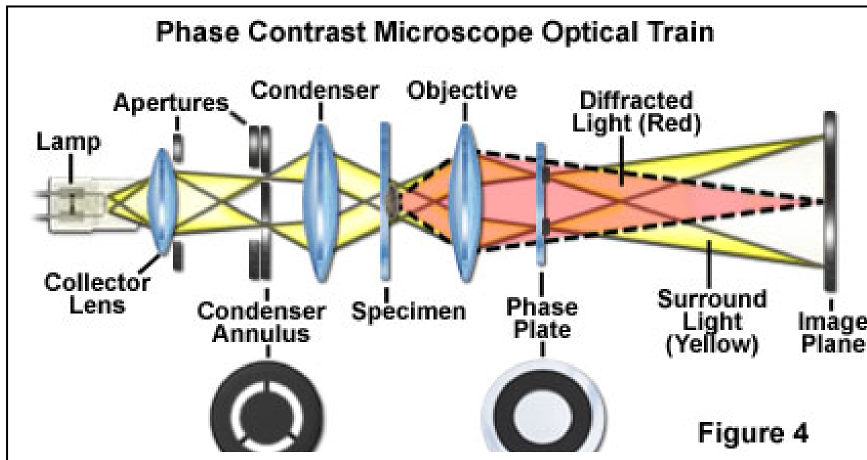
Phase contrast is obtained by inserting into the optical path:

1) A ring shaped aperture in the front focal plane of the condenser.
2) Phase ring (modulator) in the back focal plane of the objective. This partly transparent ring gives rise to a dampening of the light and a phase shift of $\pi/2$ relative to light passing outside the ring.

Light that is not diffracted through the sample will be imaged as a ring in the back focal plane of the objective, and will be advanced/retarded by the phase ring. Light that is diffracted reach the plane at various locations across the entire back aperture. Destructive/constructive interference is thus achieved between nondiffracted and diffracted light.

The amplitude of the nondiffracted light is much higher than of the diffracted light and has therefore to be attenuated by the phase ring in order to see an amplitude difference due to the interference.

Below are the light path and the ring shaped aperture and phase ring shown:

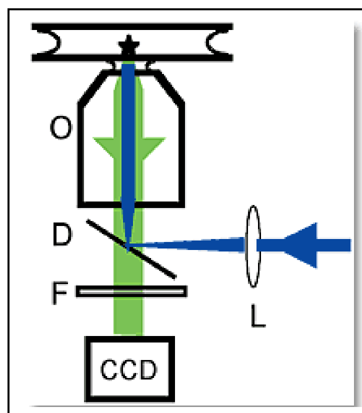


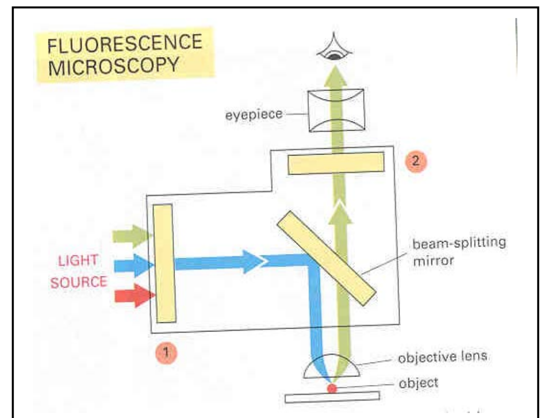
Exercise 2: Fluorescence and multiphoton microscopy

a) Epi configuration and fluorescence microscopy

The objective and condenser lens is the same lens. Incident light passes through the objective lens and emitted light from the specimen is collected by the same objective lens. In bright field microscopy the condenser and objective is two separate lens systems on different side of the specimen (see 1 a).

The incident and emitted light is separated by a dichromatic filter. A dichromatic filter reflects light under a certain wavelength and passes light above this wavelength. In addition an excitation filter has to be inserted (see figure) to make sure that the optimal wavelength for excitation is used. In front of the detector a longpass filter or a bandpass filter is placed to make sure that only the emitted fluorescence light reaches the detector.





b) Multiphoton microscopy

At high photon densities, two photons can be simultaneously absorbed (mediated by a virtual state) by combining their energies to provoke the electronic transition of a fluorophore to the excited state. Because the energy of a photon is inversely proportional to its wavelength, the two photons should have wavelengths about twice that required for single-photon excitation.

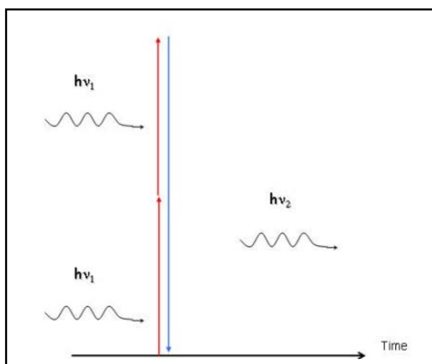
Two-photon or multiphoton excitation requires a high photon density. This is only achieved in the focal volume. Two-photon excitation is thus by itself confocal (no pinhole is requires as in conventional confocal laser scaning microscopy). The high density of photon in obtained by using high power pulsed lasers.

The main advantage of multiphoton microscopy.

- It is by itself confocal Thus light out of focus is eliminated.
- Thick samples can be imaged. This is due to: a)The penetration of light is much higher than in fluorescence microscopy as no absorption occurs outside the focal plane, b) near IR or IR light is used, c) and efficient collection of light can be done.
- Optical sectioning of thick samples can be obtained
- 3 dimensional reconstruction based on the images obtained by optical sectioning.
- Photobleaching is reduced
- UV light which may damage cells is not used.

C) Second harmonic signal

Second harmonic generation (SHG) is a nonlinear coherent process where two incident photons of frequency ω are converted into a single photon of twice the frequency 2ω. Thus there is no low of energy and the momentum is also conserved.



The criteria for a molecule to generate SHG signal is that the molecules is not centrosymmetric. A nonlinear process can be described by the equation:

$$P = \chi^{(1)}\vec{E} + \chi^{(2)} : \vec{E} \cdot \vec{E} + \chi^{(3)} : \vec{E} \cdot \vec{E} \cdot \vec{E}$$

were $\chi^{(2)}$ is the second order optical susceptibility, and has to be different from zero to obtain the SHG signal. This requires that on a molecular level til coefficient $\beta \neq 0$, where β is given by

$$\chi^{(2)} = F \sum \beta^{(n)}$$

and F = local field correction factor.

Examples of such molecules are fibrillar collagen, noncentrocymertic crystals, an interface between two media.

Exercise 3: Light-tissue interactions, laser and detectors

a) Light-tissue interactions

When light passes through a samples it is subjected to

- scattering
- absorption.

The attenuation of light intensity as a function of distance z is given by:

$$I(z) = I_o \exp -(\mu_{absoprtion} + \mu_{scattering})z$$

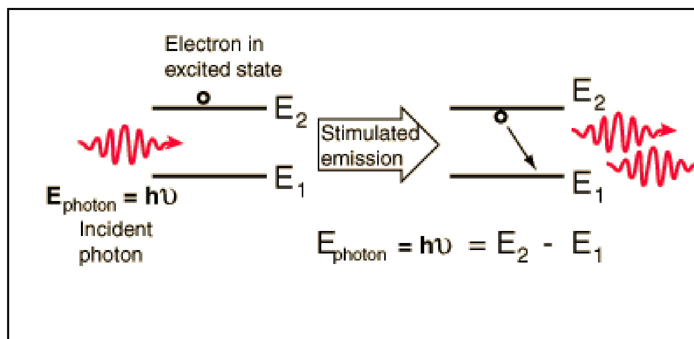
i.e.the intensity decreases exponentially with penetration distance into the sample.

b) Deexcitation

- The molecule will deexcite from S2 to S1 by internal conversion. This is a non radiative process and thus no change of energy. The transition occurs between two electronic excitation states of the same spin multiplicity. The rate constant depends on the spatial overlap between the wave functions for the initial and final conditions.
- The molecules goes from a higher vibrational excitation level in S1 to the lowest excitation level in S1 by vibrational relaxation, thus energy is released as heat.
- The molecules reaches ground state S0 by emission of a photon in the process called fluorescence. There is no change in spin quantum number of the electrons

c) Stimulated emission and lasers

Stimulated emission occurs when an excited molecule is forced to ground state by an incoming photon as shown in the figure. The excited electron reaches the ground state and a photon with the same energy as the incoming photon is emitted as shown.



Lasers =Light Amplification by Stimulated Emission is based on stimulated emission

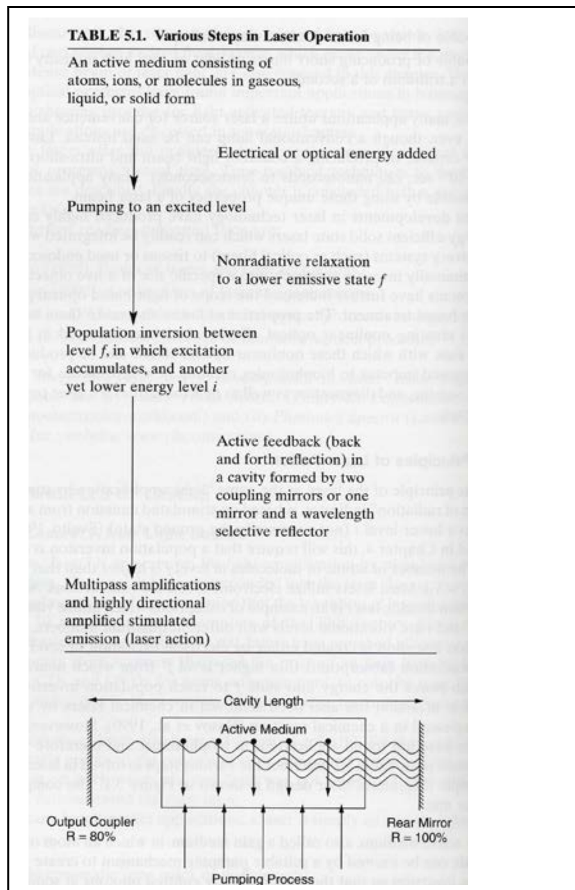


TABLE 5.1. Various Steps in Laser Operation

An active medium consisting of atoms, ions, or molecules in gaseous, liquid, or solid form

Electrical or optical energy added

Pumping to an excited level

Nonradiative relaxation to a lower emissive state *f*

Population inversion between level *f*, in which excitation accumulates, and another yet lower energy level *i*

Active feedback (back and forth reflection) in a cavity formed by two coupling mirrors or one mirror and a wavelength selective reflector

Multipass amplifications and highly directional amplified stimulated emission (laser action)

- Stimulated emission requires a population inversion, i.e. the number of excited molecules Nf is larger that the number of molecules in ground state Ni,   NF>Ni.
- This is achieved by excitation of the active medium which consists of atoms, ions, molecules in gaseous, liquid or solid state. (The various kind of active m gives various types of lasers). The active medium is excited by addition of energy either electrical or optical.
- The excited molecules reach a lower excited state f, and population inversion occurs between level f and a lower energy level i. (often ground state).
- When population inversion is achieved the electrons emitted by stimulated emission are used to stimulated new excited electrons to emission in a positive feedback process. An amplification of light by stimulated emission takes place.
- The laser cavity is limited by two mirrors which reflect light in phase, and the phase is determined by the length of the laser cavity. The light is oscillating back and forth and resonance is obtained. Depending on the wavelength of the stimulated emission and the cavity length, the waves reflected from the end mirrors will either interfere constructively or destructively.
- A proportion of the amplified light will be transmitted through the output coupler mirror at the end of the cavity.

d) Photomultiplier tubes

A photomultiplier tubes (PMT) is shown below:

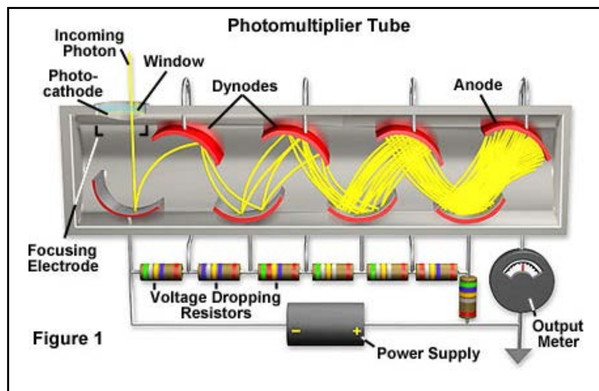


Figure 1

Incident photons hit photocathode and electrons are emitted from the cathode rear surface. These electrons are attracted to the more positive anode. Between the cathode and the anode is a train of 10-14 metal dynodes which further multiply the emission of electrons. Each electron emitted from the cathode hits the first dynode with sufficient energy to release several electrons. These released electrons are accelerated to the next dynode yielding a cascade process.

PMT is used for point detection and found in flow cytometer and confocal laser scanning microscope there a flow of single photons is detected. The benefit with PMT is that it amplifies rather weak signals. PMT is used instead of camera as a flow of photons is detected not an image frame. In confocal laser scanning microscope the flow of photons is later used to generate a 2 dimensional image.

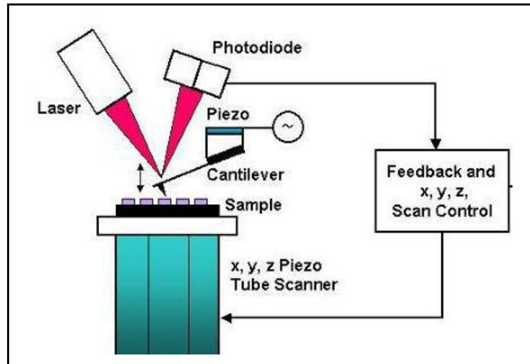
Exercise 4: Atomic force microscopy (AFM)

a) AFM principle and design

AFM operates by measuring attractive or repulsive forces between a tip and the surface of a sample. The forces measured are due to the interaction between a cloud of electrons in the sample and in the tip. This tip is very sharp and fine and it is scanning the surface of the specimen under investigation. When the tip is brought into proximity of a sample surface, forces between the tip and the sample lead to a deflection x of a cantilever holding the tip according to Hooke's law $F=-kx$ where k is the spring constant of the cantilever holding the tip.

Forces that are measured in AFM are in the nanoNewton range and include mechanical contact forces, van der Waals forces, capillary forces, chemical bonding forces, electrostatic forces, magnetic forces, etc. In addition, information about the topography of the surface under investigation, as well as its texture and viscoelastic properties, are obtained.

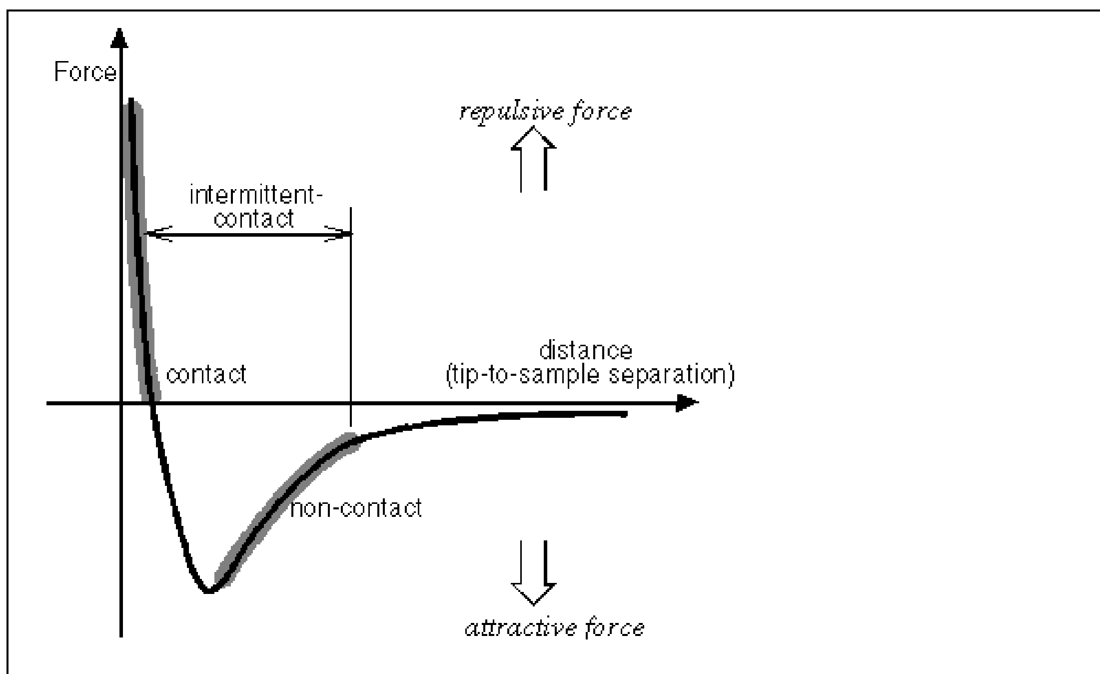
An AFM is shown in the figure below:



The main parts are: the *pizoelectic scanner*, the *tip* and tip *holder* constituting the *cantilever*, and the *detector system*, as well as the feedback system. The scanner controls the relative position of the tip relative to the specimen. The function of the tip is to transform the minute interaction forces to a signal that can be further analysed. The tip holder places the tip in an appropriate position relative to the sample and together with the scanner it allows correct mutual positioning. The tip deflection detection system transforms the mechanically induced cantilever deflection to an electronic signal. The detection system is normally based on a laser beam reflected from the back side of the cantilever combined with a detection of the position of the reflected laser by a position sensitive photodiode. Furthermore, the electronics is controlling the feedback of the system to maintain either constant probe-specimen force or constant height as the operating signal.

b) Operational modes for AFM imaging

The figure below show a typical force-distance graph of van der Waals forces as a function of distance from the samples surface. The repulsive region where there is direct contact between the tip and the sample as well as the attractive region there is an attractive force between the tip and the sample are indicated on the figure.



AFM can be operated in contact mode or oscillating mode. Oscillating mode is further grouped in non-contact and tapping mode (or intermediate contact) as shown on the figure.

In contact mode the tip is in direct contact with the sample, only separated from it by a few Å, and is in the repulsive region of the intermolecular forces. The tip is scanned over the sample and the vertical deflection of the cantilever detected. This gives information about topography and elastic properties of the sample.

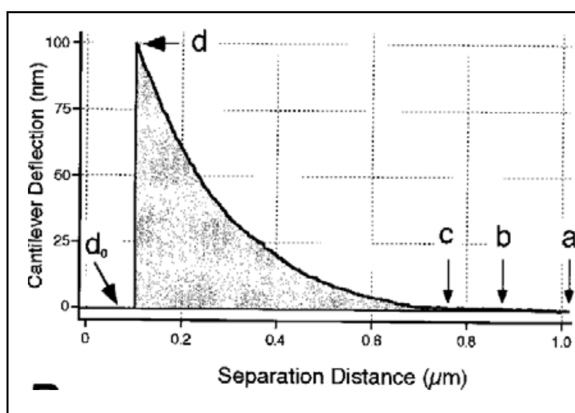
Tapping mode (also called intermittent mode) is the most common mode, and is a mode between contact and non-contact. When operated in air, the cantilever is oscillated at its resonant frequency (often hundreds of kHz) and positioned above the surface so that it only taps the surface for a very small fraction of its oscillation period. The tip is still in contact with the sample, but the very short time over which this contact occurs means that lateral forces are significantly reduced as the tip scans over the surface.

In non-contact mode the distance between the tip and the sample is several hundred Å, and is larger than the oscillation amplitude of the undamped oscillations. Due to the long distance the force is very low in the order of picoNewton. Such small forces may be difficult to measure, and stiff cantilevers with a very high spring constant are used. The tip is oscillating with a frequency close to and slightly above the resonance frequency (approximately 100-400 kHz). When the tip gets closer to the sample, changes in the resonance frequency or the vibration amplitude is detected. Non-contact mode is used to study soft and elastic samples.

Tapping mode is preferable for imaging biological samples. The forces between the tip and sample are larger than for non-contact mode, and capillary forces which are a problem for contact mode are avoided. Biological samples should be imaged in near physical conditions.

c) Force measurements with AFM

FIELD = Force integrated to equal force limit is a measure of the work carried out on the sample by the cantilever. The figure below shows the cantilever deflection as a function of distance between the sample and the tip, and the work done by the cantilever is directly proportional to the area, shown in grey, under the curve. This is the area bounded by the zero deflection line (d_o) and d at the trigger threshold. The noncontact region (*a–b*), the contact point (anywhere in b-c) and the contact region (c-d) are indicated by their corresponding arrows.



The area under the force curves w at two different positions on a surface is directly related to the ratio of the elastic constant k of the surface at these two positions.

$$\frac{w_1}{w_2} = \left(\frac{k_1}{k_2}\right)^{2/3}$$

Using this relationship the elasticity can be obtained when the work w is found from the graph.

Dynamic force spectroscopy gives information about the:
Binding force given by: Deflection of cantilever just before rupture
Elasticity of molecule: given by the shape of force jump

Exercise 5: Electron microscopy

a) Principle and design

Image formation is based on the interaction between electrons and the samples. Electrons are charged particles and are acted upon by electromagnetic forces. They are scattered by other charged particles in the sample, i.e. they are deflected from its original trajectory.

Theoretical lateral resolution in EM is given by the same criterion as in light microscopy the Rayleigh criterion The lateral resolution d is thus given by: d=0.61λ/NA, where NA is the numerical aperture. In the case of EM NA only depends on the maximum angle of incident electron rays entering the objective, as the refractive index n=1 in vacuum. The wavelength of electrons is given by de Broglie wavelength. The electrons in an EM are accelerated in an electric field with a potential V. The kinetic energy of the electrons is thus:

$$E = e \cdot V = \frac{1}{2} m_e v^2$$

and the velocity v of the electrons:

$$v = \sqrt{\frac{2e \cdot V}{m_e}}$$

The wavelength can thus be expressed by the accelerating voltage V:

$$\lambda = \frac{h}{m_e \cdot v} = \frac{h}{\sqrt{2 m_e e \cdot V}} = \frac{1.23\,\text{nm}}{\sqrt{V(\text{volt})}}$$

A typical value of the accelerating voltage in an EM is V=60kV. This gives a theoretical lateral resolution of:

$$d = \frac{0.61 \cdot 1.23\,\text{nm}}{NA\sqrt{V}} = \frac{0.61 \cdot 1.23\,\text{nm}}{NA\sqrt{60\,\text{kV}}} \approx 0.025\,\text{Å}$$

assuming that the numerical aperture of the electromagnetic lenses in an EM is of the same magnitude as for glass lenses in optical microscopes.

In light microscopy the lateral resolution is typical when assuming light of 500 nm and maximum NA=1.6 :

$$d = \frac{0.61 \cdot \lambda}{NA} = \frac{0.61 \cdot 500nm}{1.6} \approx 200nm$$

The resolution with EM is thus approx 80.000x better.

Lenses used in EM are electromagnetic lenses. Such lenses have pole pieces of iron around the coil to concentrate the magnetic field as shown in figure 12.4. The rotationally symmetric magnetic field is inhomogeneous in such a way that it is weak in the center of the gap and becomes stronger close to the bore. Electrons close to the center are less strongly deflected than those passing the lens far from the axis. The overall effect is that a beam of parallel electrons is focused into a spot. Passing a current through a single coil of wire will produce a strong magnetic field in the center of the coil. An electron beam accelerated through a vacuum will follow a helical path when it passes through such a magnetic field. After leaving the lens, the electron will focus to a point to generate an image point of the specimen.

The differences between electromagnetic and glass lenses are:
Light microscopy uses glass lenses : The refractive index changes abruptly at a surface and is constant between the surfaces. The refraction of light at surfaces separating
media of different refractive indices makes it possible to construct imaging lenses. Glass surfaces can be shaped.
EM uses electromagnetic lenses: Here, changes in the refractive index are gradual so rays are continuous curves rather than broken straight lines. Elecromagnetic lenses is always converging (electrostatic may also be diverging).
The focal length can be changed by changing the current through the coil. Focal length can not be changed for a glass lens.

The focal length f is given by:

$$f = k\frac{V}{I^2}$$

Where k is a constant depending on the coil, number of turns, geometry
V is the voltage in the vacuum tube
I is the current through the coil.
The current I is easily changed and thereby changing the focal distance.

b) TEM and SEM

Image formation:
TEM projects electrons through a very thin slice of tissue (specimen) to produce a two-dimensional image. The brightness in the image is proportional to the number of electrons that are transmitted through the specimen. Thus the bright areas are due to transmitted electrons and the dark areas are caused by scattered electrons not reaching the detector.

SEM produces an image that gives the impression of three dimensions. This microscope uses a 2 to 3 nm spot of electrons that scan the surface of the specimen to generate secondary electrons which are emitted from the surface due to excitation by the primary electron beam. The image is produced over time as the entire specimen is scanned.

The main differences between a TEM and SEM are:
Lenses system:
TEM uses two condeser lenses to focus the electron beam onto the sample whereas SEM uses 3 condeser lenses to control the electron beam spot. No other lenses are used in SEM. In TEM intermediate lenses and projector lenses are used to magnify and form the image.
Scanning:
Only SEM scans the electron beam across the specimen.

Detectors:
The detectors are different. SEM detect back scattered electrons, whereas in TEM transmitted electrons are detected.

Resolution TEM and SEM:
The theoretical resolution is given in point a. In praxis NA<<1 due to various lens aberrations. In addition to NA the resolution in TEM depends on: focal length and voltage. Sample preparation may also enhance the resolution.

In SEM the resolution depends in addition to NA on the spot size of the electron beam. A small spot size is achieved by optimally energizing all condenser lenses. Short working distance is needed. Adequate signal/nose ratio is also important.

c) Sample damage and sample preparation for
Damage: The energy of the incident electrons is sufficiently large to break covalent bonds and to form free radicals which subsequently can lead to formation of new covalent bonds. Atoms with low atomic number are in particular subjected to loss within a sample by sublimation and thereby result in loss of mass from the sample.

Staining: is necessary to increase the contrast. Biological samples consist of low molecular weight components such as hydrogen, carbon, oxygen and nitrogen, and the electrons will not be scattered sufficient by these molecules. Heavy metals are thus used to increase the contrast.

Shadowing: Heavy metals are employed in the shadowing procedure to provide good amplitude contrast of the specimen when observed in the electron microscope. The shadowing process is schematically illustrated below. There is no accumulation of heavy metal hitting the part of the specimen within the shadow, and when inspected in the electron microscope, these areas will show up as light areas (because there is no electrons scattered from these regions).

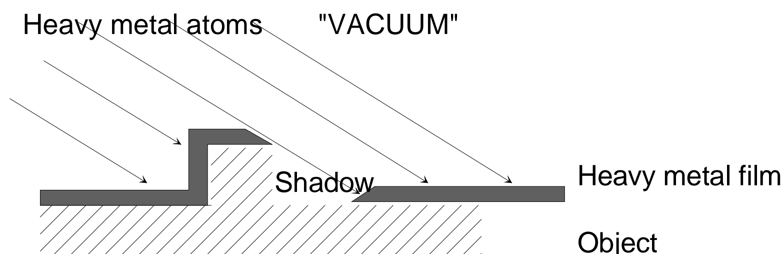


Fig. 13.7 Schematic illustration of shadow in heavy metal replication.

The three general steps in SEM sample preparation are:

1. Fixation to preserve structural details
2. Dehydration to avid collapse in vacuum
3. Staining to increase contrast

The antistatic film: Dehydrated samples are not able to conduct charge and the electrons that hit the sample may therefore accumulate charge in the sample. To avoid this, a layer of electrical conducting metal film is overlaid the specimen. The thickness of the antistatic film should be less than about 5 nm not to mask the structural details of the specimen.