NORWEGIAN UNIVERSITY OF SCIENCE AND TECHNOLOGY
DEPARTMENT OF PHYSICS

Contact during exam: Associate Professor Marit Sletmoen
Department of Physics, Realfagbygget
Phone.73593463 / 47280447

EXAM IN COURSE TFY4265 AND FY8906
BIOPHYSICAL MICROMETHODS
13. December 2010
Hours: 0900 – 1300

Permitted aids: Simple calculator according to NTNU regulations
No written books or papers

FASIT

Exercise 1

a) Figure 1 shows a schematic optical layout employed for light microscopy. Name the components depicted 1 and 2 in Figure 1 and describe their function in light microscopy.

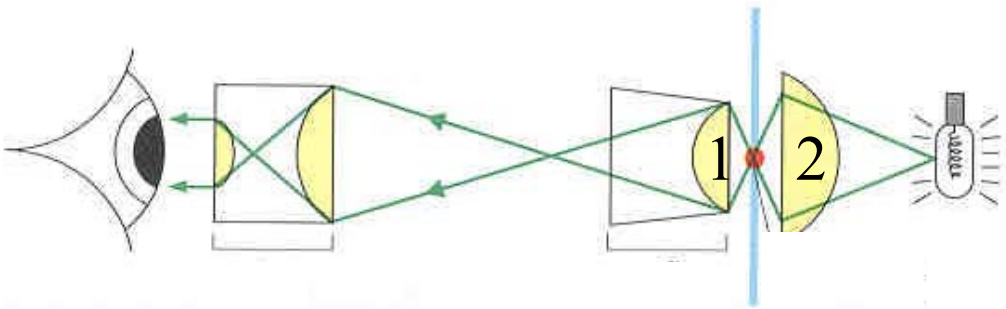


Figure 1: Schematic drawing of light path through light microscope

a)
1 = Objective: collects light and forms a magnified image real image at the real intermediate image plane near the ocular

2= Condenser: focuses light from the illuminator onto a small area of the specimen

b) Define the lateral resolution in light microscopy and describe the parameters in equation 1:

$$d = 0.61\,\lambda / NA \qquad (1)$$

b)
Lateral resolution is given by the Rayleight criterion for resolution of two closely spaced diffraction spots in the image plane. Two adjacent objects are defined as being resolved when the central diffraction spot (Airy disk) of one point coindiced with the first diffraction minimum of the other point in the image plane.

In the equation given above, d is the minimum resolved distance, λ is the wavelength, and NA is the numerical aperture of the objective lens.

c) The terms amplitude objects and phase objects are routinely employed concepts in light microscopy of biological specimens. Describe briefly what the terms amplitude and phase objects involve when employed in light microscopy, and in particular their difference.

c)

- *In the case of stained, histoligical preparations of specimens with naturally ocurring pigments, specific wavelengths are absorbed by dyes or pigments, allowing objects to appear in color when illuminated with white light.*

- *Object rays are significantly reduced in amplitude, resulting in a high-contrast image. Such objects are called amplitude objects because they directly produce amplitude differences in the image that are detected by the eye as differences in the intensity.*

- *Although most transparent biological objects do not absorb light, they do diffract light and cause a phase shift in the rays of light passing through them: thus, they are called phase objects.*

d) The optical layout employed for light microscopy schematically indicated in Figure 1 can be transformed to a phase contrast light microscope by insertion of two additional optical components. Describe briefly these two optical elements and depict the position of these two optical elemet in Figure 1. (Figure 1 is reproduced on a separate page of this exam that can be enclosed in your answer).

d)

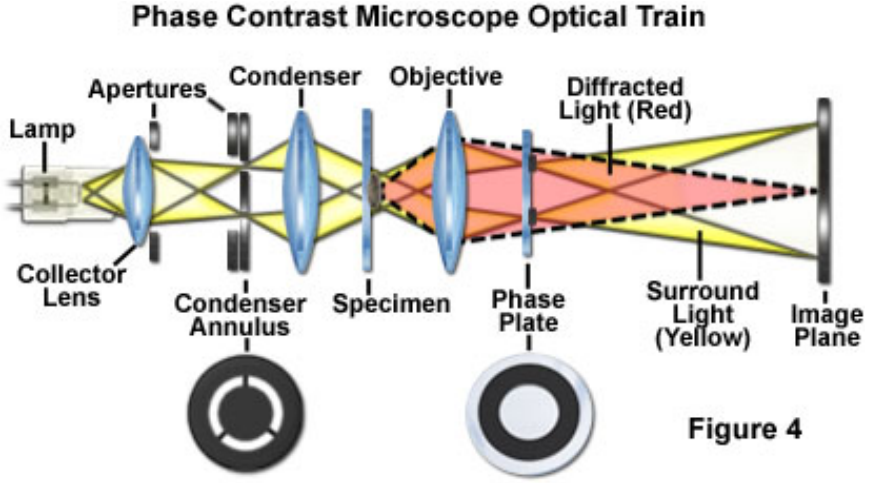


Figure 4

Light passing ring shaped aperture in condenser
Light is scattered/diffracted in the sample because of difference in refractive index, delayed λ/4
Phase ring in the objective's focal plane ⇒ light that is not scattered goes through phase ring and the phase is changed λ/4. Diffraction light through phase ring at all points. Destructive/constructive interference gives dark/bright structures in image plane

Exercise 2

a) Describe briefly the Franck Condon principle.

a) The idea underlying the Franck–Condon principle is that since electronic transitions are very fast compared with nuclear motions, vibrational levels are favored when they correspond to a minimal change in the nuclear coordinates. The Franck–Condon principle is thus the approximation that an electronic transition is most likely to occur without changes in the positions of the nuclei in the molecular entity and its environment. The quantum mechanical formulation of this principle is that the intensity of a vibronic transition is proportional to the square of the overlap integral between the vibrational wavefunctions of the two states that are involved in the transition.

b) The abreviation STED is applied for a particular high resolution optical microscopy method. What is the full name the technique abbreviated with STED?

b) Stimulated emission depletion microscopy

c) Describe briefly three essential consequtive events taking place in STED microscopy.

c)

Step 1: (Red line in figure 2A): Intensity profile for the excitation light: *The excitation light will give rise to a diffraction limited spot obtained by irradiation a small area of the sample with the use of an intense laserbeam. The minimal size of a focused light spot is limited by diffraction to about half the wavelength used. Laser-scanning microscopes can therefore not decrease the size of the excitation spot for fluorescent dyes further than approximately 200 nm.*

Step 2: (Blue line in figure 2A): doughnut-shaped de-excitation spot: *Instead of spontaneous relaxation and fluorescence emission, a molecule can also return to its ground state by stimulated emission. If an excited dye molecule is irradiated with light of similar wavelength compared to the fluorescence light, it can immediately return to the ground state and emits a photon of exactly the same wavelength and momentum of the light used. Furthermore the molecule is prevented from the spontaneous emission of a fluorescence photon after stimulated emission. Fluorescent dyes can therefore be switched off by additional irradiation of a red-shifted 'de-excitation' beam. The light originating from the spontaneous decay and from the stimulated emission can also be spectrally separated from the fluorescent light by using appropriate color filters.*

In a STED microscope the excited molecules in the outer rim of the excitation spot are switched off by stimulated emission. This is obtained by employing a second, red-shifted 'de-excitation' laser beam whose wavefront is altered so that a ring-like intensity profile is achieved. This depletion light causes almost all of the excited molecules to return to the ground state, leaving only the region of the sample very close to the center of the excitation

spot excited. Fluorescence from the remaining excited dye molecules is then detected by the microscope.

Step 3: Black line in figure 2A): remaining area allowing fluorescence: *The size of the spot where molecules are still allowed to fluoresce gets smaller with increasing intensity of the de-excitation light. This size corresponds to the achievable resolution. Therefore the resolution is controlled by the brightness of the de-excitation beam. The resolution can become much better than the diffraction-limit, indeed an arbitrary good resolution is possible provided that one can apply such a very high de-excitation beam intensity.*

STED: *By switching-off the excited molecules in a saturated manner, only a fraction of molecules in an area much smaller than the original focused excitation spot can fluoresce. Therefore structures which are smaller than the diffraction-limit can be made visible with STED.*

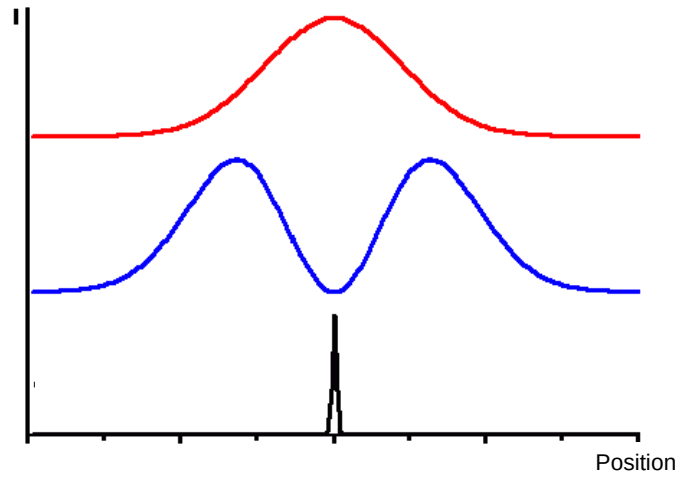


Figure 2A: Illustration of the three consecutive steps in STED microscopy

Exercise 3

AFM can be used both as an imaging technique and as a sensitive device to measure small forces between single molecules or small objects.

a) Describe how an attachment between two molecules or larger structures is exposed during a typical AFM experiment. In your explanation you should include an explanation of the equation $F = -k \Delta x_t$. Explain the three parameters in this equation and state how each of the three parameters F, k and Δx_t are determined experimentally or calculated based on experimental observations during a typical AFM experiment.

a) An attachment between two molecules is revealed through the collection of force curves. Force curves are recorded with cantilever-deflection sensor based AFMs by recording the bending of a cantilever spring as a function of the height of the fixed end of the cantilever. Hence, the data files are assumed to contain deflection (usually in voltage units) as a function of height, where height refers to the change in distance between non-flexible parts of the sample holder and the cantilever holder.

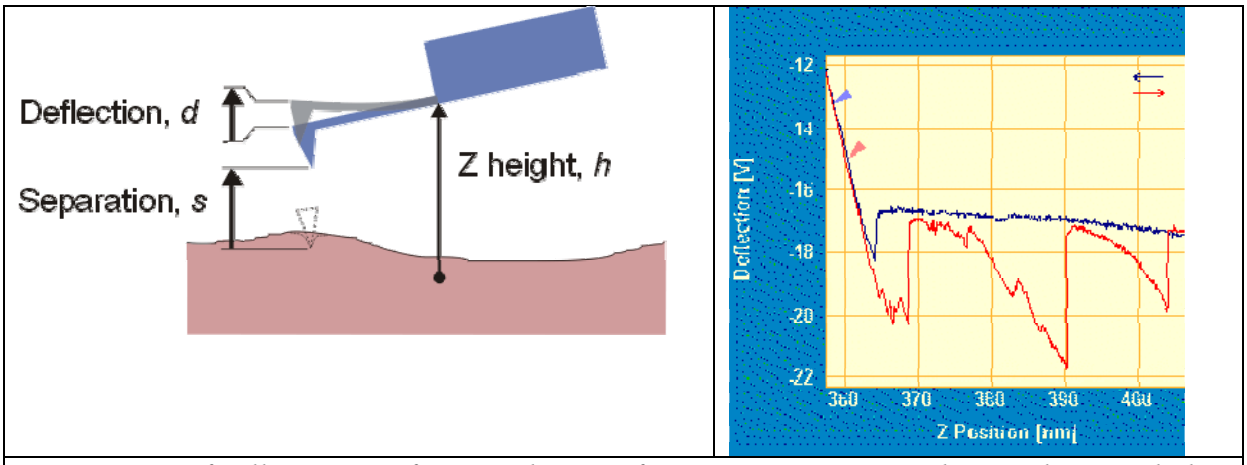


Figure 3A: Left: Illustration of essential parts of an AFM microscope: the cantilever with the tip attached to it, the sample and the ability to regulate the distance Z between sample and tip using a scanner. Right: Force curve obtained by the use of an AFM. In the force curve shown, the deflection of the cantilever is plotted as a function of z-position, i.e. the tip – sample separation distance.

The cantilever deflection is converted into units of voltage in to length units using the cantilever sensitivity factor. This factor can be measured as the slope in the contact region of the deflection vs. height curve recorded on a hard surface (e.g. a silicon surface, hard meaning with an elastic stiffness much higher that of the spring constant of the cantilever) and has the unit of Volt/nanometer.

The cantilever deflection in length units is converted in to cantilever force by multiplying the deflection with spring constant of the cantilever

Deflection[nm] = *Deflection[Volts] / Sensitivity*

Force = *Spring constant ´ Deflection[nm]*

The spring constant may be measured by the use of a second calibrated cantilever or from the cantilever's thermal vibration spectrum.

Based on the determination of the spring constant the

b) Explain the term loading rate that is used in dynamic force spectroscopy.

b) In a typical force unbinding process, the linear rise of force with time is set by the product of separation speed v_t and transducer spring constant k, which is called loading rate:

$$r_f = k\, v_t$$

Optical tweezers is another technique that can be applied for determination of minuscule forces. Figure 2 depicts schematically the the Gaussian intensity profile of light from a laser light source. The position of the polystyrene bead (grey circle) is slightly to the left of the intensity maximum of the laser light beam.

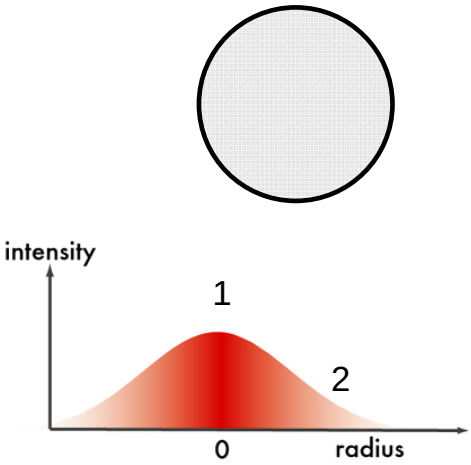


Figure 2: Schematic illustration of a polystyrene bead in a Gaussian intensity profile laser beam.

c) Assume that the polystyrene bead has a larger refractive index than the surronding medium and:

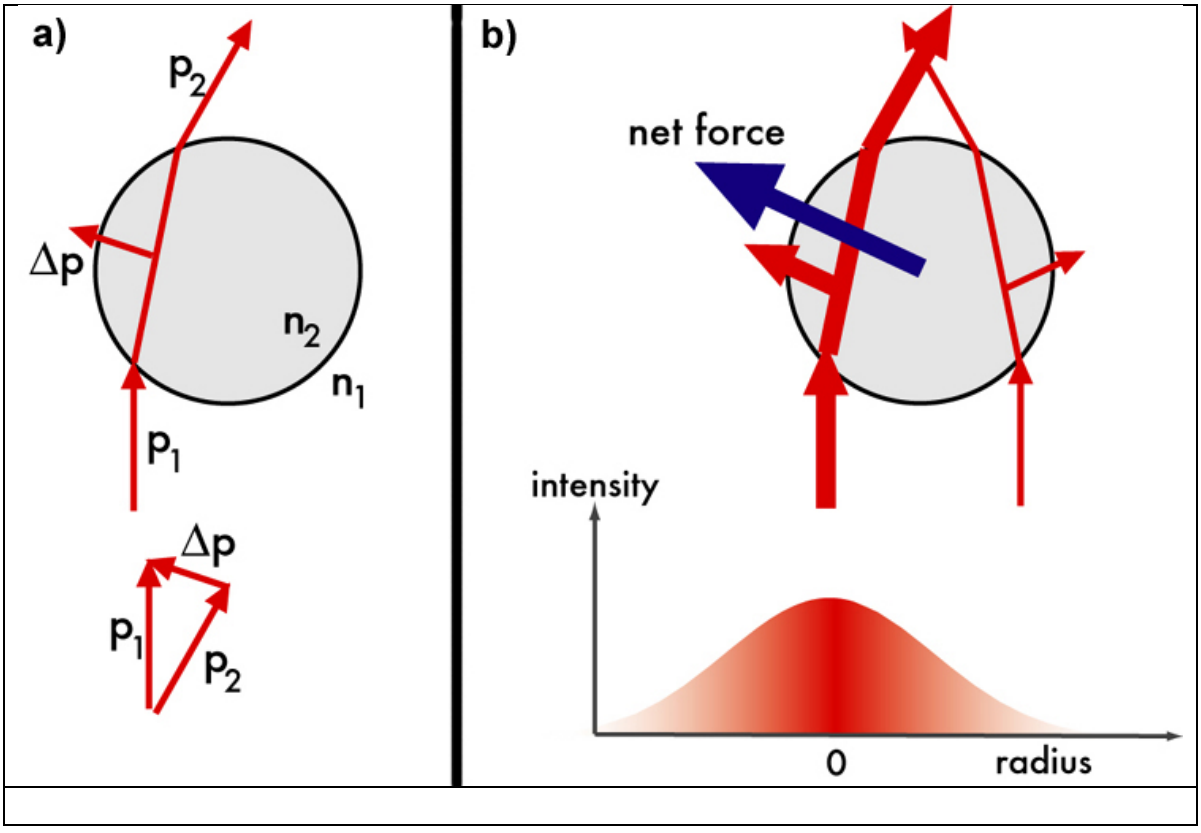
1 Indicate by arrows the propagation direction of the light rays originating from position 1 and 2 in the gaussian intensity profile of the laser (see figure 2) after being refracted at the medium-bead and bead-medium interfaces.

2 Indicate by vectors p1 and p2 the net momentum transfer to the sphere due to the light rays 1 and 2. (let the thickness of the vector indicate the amount of momentum)

3 Indicate by a separate vector the direction of the net force acting on the sphere.

Describe qualitatively what would happen if the refractive index of the bead had been less than the surrounding medium

In cases where the diameter of a trapped particle is significantly greater than the wavelength of light, the trapping phenomenon can be explained using ray optics. As shown in the figure, individual rays of light emitted from the laser will be refracted as it enters and exits the dielectric bead. As a result, the ray will exit in a direction different from which it originated. Since light has a momentum associated with it, this change in direction indicates that its momentum has changed. Due to Newton's third law, there should be an equal and opposite momentum change on the particle.



If the refractive index of the bead had been less than the surrounding medium the vector representing the net force acting on the bead will pioint away from the laser center, and the bead will thus be propelled away from the laser focus instead of being trapped.

Exercise 4

a) Describe briefly what types of electron-specimen interactions that can be used as a basis for imaging biological specimens by electron microscopy. Which type(s) of interactions are used in various types of electron microscopy? What type of information is obtained when a sample is characterized employing X-ray microanalysis, and describe briefly the fundamental process that give rise to observables.

a)

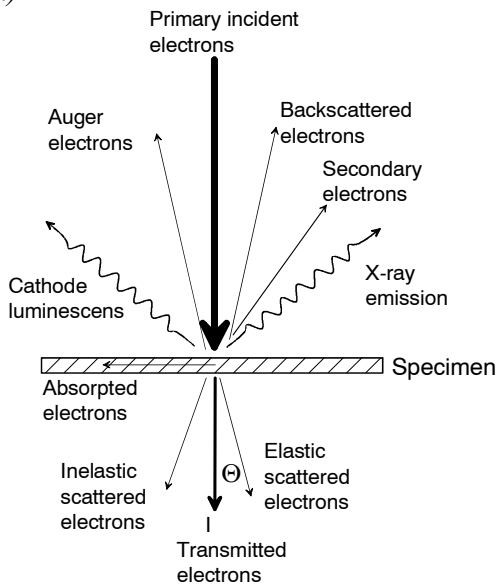


Fig. 10 Illustration of various processes in electron beam – specimen interactions.

Fig. viser ulike type vekselvirkninger (v.v.) mellom elektroner og (biologiske) prøver en kan dra nytte av i elektronmikroskopi. Disse er: (Fig. 10)
De primære v.v. er elastisk elektron spredning, uelastisk elektron spredning, og absorpsjon av elektroner. Absorpsjon av elektroner er det første steget som trengs for en rekke sekundærprosesser som: elektron emisjon, røntgen emisjon, emisjon av lys, elektrostatisk oppladning av prøve, spalting av kovalente bindinger og sublimering av materiale.
Elastisk elektronspredning er karakterisert ved at den totale kinetiske energi E_k (elektron + atom som elektronet kolliderer med) er den samme før og etter støtet. Overføring av kinetisk energi fra elektroner med E_k av den størrelseorden som er brukt i elektronmikroskopene, til atomene i prøven, kan være tilstrekkelig til å dissosiere protoner fra enkelte atom, og også atomer med lavt atomnummer fra en prøve. Dette er en viktig mekanismer for tap av materiale i biologiske prøver.
Ved uelastisk spredning vil ikke all energi bevares som E_k, men vil i tillegg føre til eksitasjon eller de-eksitasjon av systemet.

*Ved **transmisjons-, lysfeltsmikroskopi** er det de transmitterte elektronene som fanges opp ved billeddannelsen, og amplitudekontrast kommer frem ved topografiske varisjoner i hvor tap av elektroner i andre prosesser, elastisk, uelastisk og adsorberte (som første trinn til videre prosesser)elektroner.*

*Ved **transmisjons-, mørkefeltmikroskopi** er det elektroner som danner en liten vinkel med retningen til de transmitterte elektronene som fanges opp brukes som grunnlag for bildedannelsen. Dvs., det er de elastisk og uelastisk spredte elektronene som benyttes.*

*Ved **scanning elektronmikroskopi** er det enten tilbakespredte eller sekundærelektronene som danner grunnlag for bildedannelsen. Intensiteten av disse brukes som grunnlag for å modulere intensiteten på en fluorescerende skjerm på et punkt som samsvarer med instantan posisjon til den fokuserte elektronstrålen.*

*Ved **scanning-transmisjons elektronmikroskopi** er det transmitterte elektroner som brukes som grunnlag for bildedannelsen. I denne type elektronmikroskopi skyldes, som for TEM, kontrasten topografiske variasjoner i alle prosesser som fører reduksjon av intensiteten til de transmitterte elektronene. Til forskjell fra TEM, fokuseres strålen, og all registrert, transmittert intensitet brukes for å representere intensiteten til det aktuelle punkt innfallende stråle belyser på prøven. Ved scanning av elektronstrålen oppnås bildet.*

Fig. 11 viser intensitetsfordeling av emiterte elektroner i sekundærprosesser.

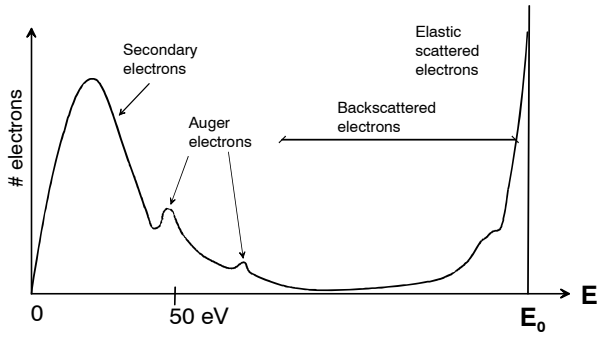


Fig. 11. Skjematisk illustrasjon av energi til elektron generert i elektron - prøve v.v.

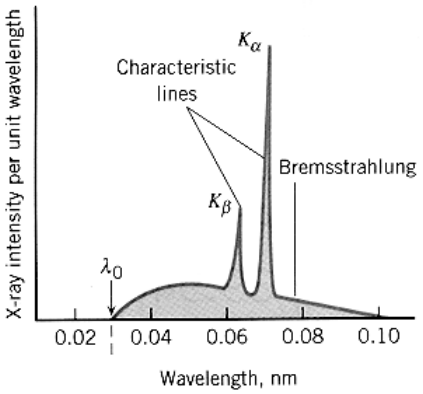
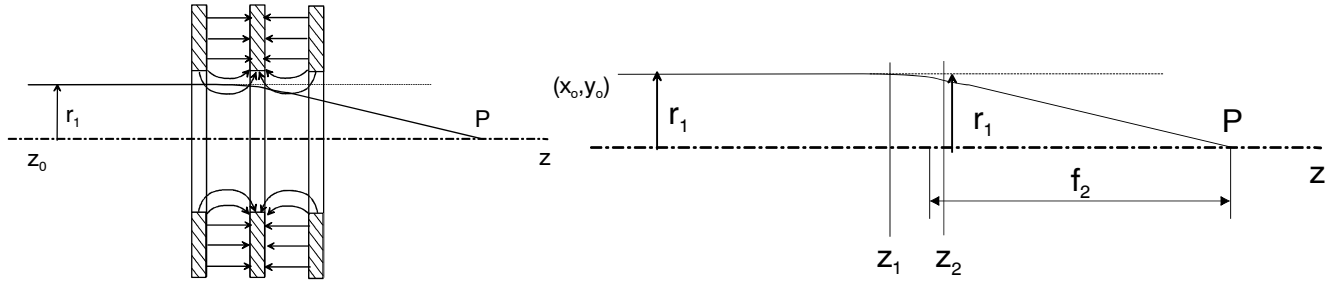


Fig. 12. Skjematisk illustrasjon av et røntgenspekter

Ved røntgen mikro-analyse, drar en nytte av følgende prinsipp. Innfallende elektroner har tilstrekkelig energi til å dissosiere sterkt bundne elektroner i orbitalene til atomene (K, L skallet, feks). Disse ledige tilstandene fylles ved overgang av elektroner fra utenforliggende orbitaler, samtidig som at den frigjorte energien sendes ut som elektromagnetisk stråling (røntgen). Kvantemekaniske utvalgsregler setter begrensninger for tillatte overganger. Totalt sett vil røntgenspekteret inneholde bremsestråling med overlagrede karakteristiske bølgelengder som reflekterer atomsammensetningen i den undersøkte prøven (Fig. 12).

b) Make a schematic drawing of a uni-potential electrostatic electron lense, and describe why such a lens design principle always will yield a focusing lens.

b) Fig.13 viser en skjematisk skisse av en unipotensial, elektrostatisk elektronlise.



a) *b)*

Fig. 13 a) Skjematisk illustrasjon av elektrostatisk unipotensiallinse, og b) parametre brukt til å beskrive avbildningsegenskaper.

Avbildingsegenskapene til elektromagnetiske linser beskrives av paraksiallikningen:

$$\frac{\partial^2 r}{\partial z^2}+\frac{\partial V(0,z)/\partial z}{2V(0,z)}\frac{\partial r}{\partial z}+\frac{\partial^2 V(0,z)/\partial z^2}{4V(0,z)}r-\frac{qB_z^2(0,z)}{8mV(0,z)}r=0 \qquad (17)$$

Her er r avstand fra (elektron)optisk akse, z avstanden langs aksen, V og B_Z er henholdsvis elektrostatisk feltstyrke og magnetisk feltstyrke i z-retning. B regnes lik 0 for det aktuelle tilfellet. Får da:

$$\frac{\partial^2 r}{\partial z^2}+\frac{\partial V(0,z)/\partial z}{2V(0,z)}\frac{\partial r}{\partial z}+\frac{\partial^2 V(0,z)/\partial z^2}{4V(0,z)}r=0 \qquad (18)$$

Nå gjelder:

$$\frac{\partial^2 r}{\partial z^2}+\frac{\partial V_0/\partial z}{2V_0}\frac{\partial r}{\partial z}=\frac{1}{\sqrt{V_0}}\frac{d}{dz}\left(\sqrt{V_0}\,r'\right) \qquad (19)$$

hvor symbolene $'=\partial/\partial z$ *og* $V_0=V(0,z)$ *er anvendt for å lette notasjonen. Innsatt i likn 18 oppnås:*

$$\sqrt{V_0}\,\frac{d}{dz}\left(\sqrt{V_0}\,r'\right)=-\frac{1}{4}\partial^2 V_0/\partial z^2\; r=0 \qquad (20)$$

Integrasjon langs elektronbanen gir (Fig. 13b):

$$\sqrt{V_0}\,r'\Big|_{z=z_2}-\sqrt{V_0}\,r'\Big|_{z=z_1}=-\frac{1}{4}\int_{z_1}^{z_2}\frac{\partial^2 V_0/\partial z^2}{\sqrt{V_0}}r\,dz \qquad (21)$$

Dersom det antas tynn linse er forholdet mellom stigningstallet til banen og avbildingsegenskapene gitt som:

$$\left.\frac{\partial r}{\partial z}\right|_{z=z_2}\cong-\frac{r_1}{f_2} \qquad (22)$$

Her er det antatt at endring i avstanden til elektronet når de går gjennom linsa, er liten: $r(z_1)\cong r(z_2)\cong r_m$*. Ut fra dette oppnås følgende uttrykk for fokalavstanden:*

$$\frac{1}{f_2}=\frac{1}{4\sqrt{V_0}}\int_{z_1}^{z_2}\frac{\partial^2 V_0/\partial z^2}{\sqrt{V_0}}dz \qquad (23)$$

Ut fra at integralet er positivt, ser en at unipotensial linsen alltid vil være konvergerende.

Attachment to exercise 1:

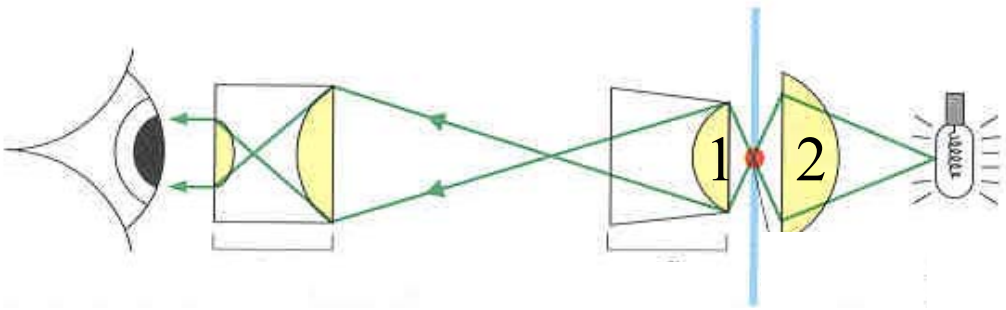


Figure 1: Schematic drawing of light path through light microscope