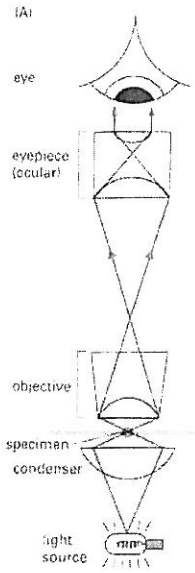
Answers to exam in course TFY4265 BIOPHYSICAL MICROMETHODS

2. December 2008

Exercise 1: Light microscopy, bright field

a) The light path and main components with functions are shown below



Eye= Detector: Image on retina

Ocular: View and magnify the image produced by the objective

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

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

Illumination source: halogen

Differential interference contrast (DIC) is based on local gradients in the optical path. The optical path length depends on the difference in refractive index n and the thickness of the sample t as $\Delta = (n_2 - n_1)t$.

In DIC microscopes some extra optical components (compared to brightfield mic) have to be inserted along the optical path of the microscope. To detect gradients in the optical path, the incident light is split into pairs of closely spaced coherent rays with a distance slightly less than the resolution (typical 100-150 nm). If the members of a ray pair traverse a phase object in a region where there is a gradient in the refractive index or thickness, or both, there will be an optical path difference between the two rays upon emergence from the object, and the gradient of the optical path is translated into a change in amplitude in the image.
Splitting the incident light beam into two adjacent rays which are perpendicular is achieved using a Wollaston prism as shown in the figure below. When the two rays are perpendicular they can not interfere with other and recombine. After emerging from the Wollaston prism, the pairs of rays are focused by the condenser onto the specimen before being collected by the objective lens.

Light rays which are undisturbed by the presence of a specimen arrive at the objective rear focal plane having the same phase relationship as when they left the condenser. The Wollaston prism located behind the objective recombines the rays and generate linearly polarized light having an electric vector vibration orientation identical to that of the polarizer transmission axis. Linearly polarized light exiting the objective prism are blocked by the second polarizer (or analyzer), which has a transmission axis oriented perpendicular to that of the polarizer (Figures below). As a result, the image background observed in the viewfield appears very dark or black, a condition referred to as *extinction.*

In the case that the paired rays have obtained a phase gradient through the specimen, when arriving at the objective lens, the phase-shifted paired rays are recombined to generate elliptically polarized light. Because a component of the elliptical wavefront is now parallel to the transmission axis of the analyzer, some portion of the light will pass through analyzer and produce plane-polarized light having a finite amplitude and ultimately being able to generate intensity in the image plane.

Differential Interference Contrast Schematic

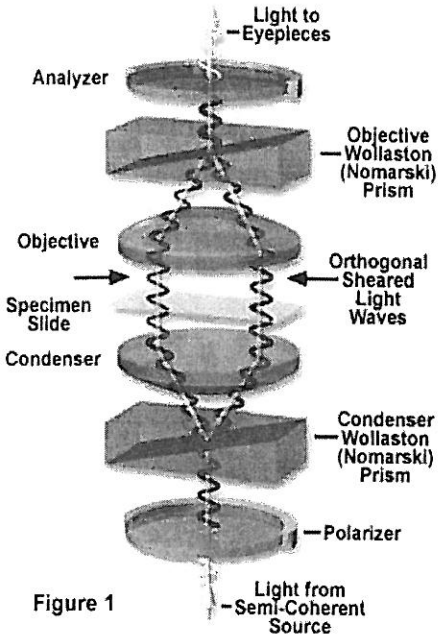


Figure 1

Analyzer: linear polarizer north-south

Wollastone 2: Combines the two rays. The rays do not interfere as E are perpendicular

Sample: Diffraction depending on refractive index and thickness of sample.

Wollastone I: separates polarized light into two rays with E perpendicular to each other. The distance between the rays approximately equal to the resolution

Polarizer: linear polarized light east-west

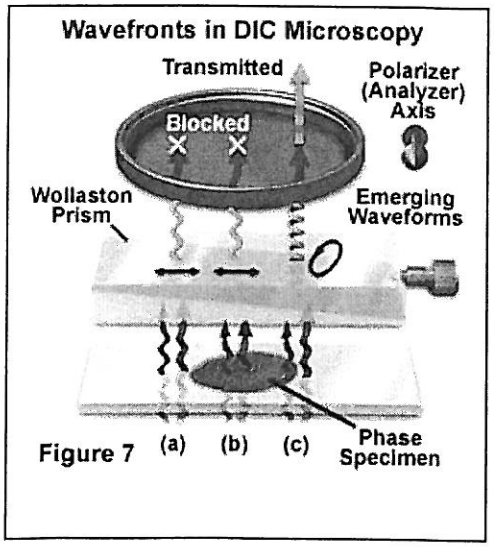


Figure 7

In analyser:

- Linearly polarised light from prism hits polarised light at a perpendicular angle and is blocked
- Elliptically polarised light passes the analyser
- interference between transmitted tight

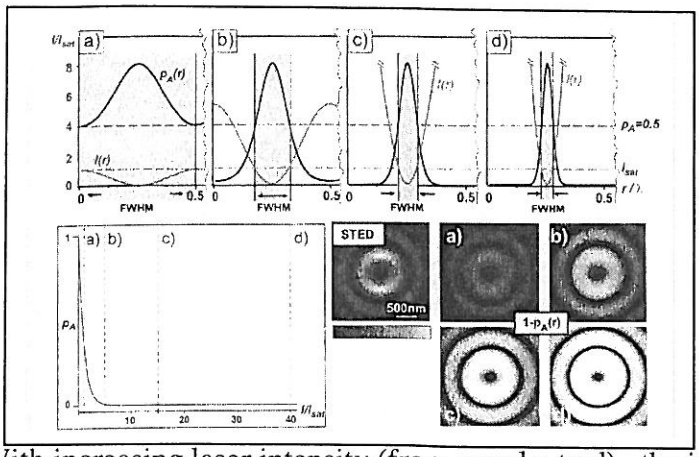
In the Wollaston prism in the objectives back focal plane the two light rays recombined:

- Light with same optical path is linearly polarised when recombined
- Light with different optical path is elliptically polarised when recombined

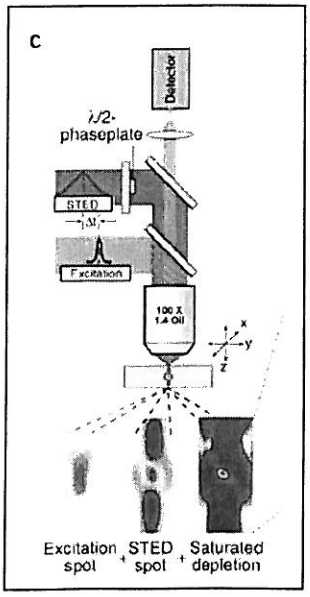
b) STED microscopy

STED microscopy is based on the idea that a population of molecules can have two sates: state A and B, one being fluorescent and the other not. There is a reversible optical transition from state A to state B which is driven by an irradiating of a spatially modulated laser beam with intensity I(r) and zero intensity in the center (doughnut like intensity profile). The laser beam induces stimulated emission depletion of the molecules in the fluorescent state everywhere except at the localization of the zero point. Thus all molecules illuminated with zero intensity remain in state A. These molecules will undergo spontaneous fluorescence and contribute to the formation of the image in STED microscopy.

The probability of finding molecules in state A after irradiation depends on the intensity of the laser illumination. The probability is given by Pa(r). The figure blow shows how depletion depends on the intensity I(r).



With increasing laser intensity (from panel a to d), the intersections of I(r) with the horizontal line marking the saturation intensity defined as Isat, more closer to the intensity maximum. Isat is the value at which 50% of the molecules are transferred to state B (non fluorescent). Thus Pa(r)=0.5 when I=Isat. With increasing intensity the points where Pa(r) drops to 0.5 move closer together, reducing FWHM (Full width at half maximum)
The intensity of the laser used for stimulated emission depletion determines the FWHM and thus the resolution, and FWHM<< λ (wavelength).



The schematic setup for a STED microscope is shown in the figure below. The dye molecule is excited by a pulse of a first laser beam within the diffraction-limited excitation volume. Before the dye fluoresces, a second laser pulse from a laser with wavelength in the red spectral region illuminates regions outside the geometric focus. Stimulated emission takes place, and if the intensity of the second laser pulse is increased beyond the saturation level, fluorescence is allowed only from confined regions where the intensity of the second beam is zero. With typical saturation intensities ranging from 1 to 100 MW/cm^2, saturation factors up to 120 may be achieved. This potentially yields a 10 fold resolution improvement over the diffraction barrier, but imperfections in the doughnut have usually limited the improvement to 5- to 7-fold.

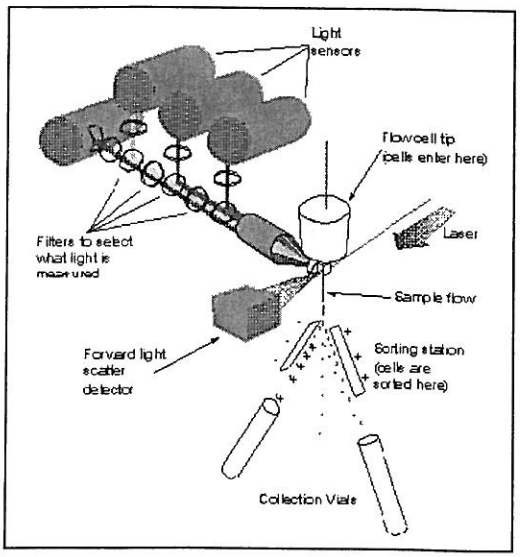
Exercise 2: Fluorescence- based techniques

a) Flow cytometry

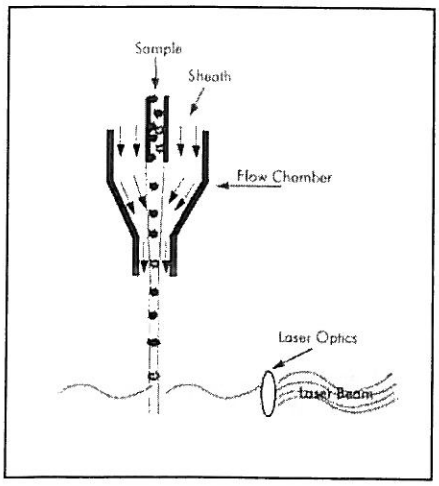
Principle: Flow cytometers (FCM) are characterized by their ability to determine properties of a distribution of a cellular population by recording two physical parameters: fluorescence and light scattering from each individual particle/cell. The cells have to be in suspension. To obtain quantitative measurements of cellular molecules/structures they have to be labelled with specific fluorochromes or fluorescent labelled antibodies in a stoechiometric manner, i.e. the amount of bound fluorochrome is proportional to the amount of the cellular molecules to be quantitated. With appropriate illumination, the amplitude of the fluorescence signal detected is proportional with the amount of the cellular molecules.

Design of a FCM:

The particles/cells flow through the focus of the light source, normally lasers. Following the excitation, there will be a fluorescence emission yielding a light pulse. The fluorescent light is detected on a photomultiplier tube (PMT). If the cells are labelled with several fluorochromes to analyze several cellular components simultaneously, the fluorescent light is separated using dichroic mirrors and filters to selectively detect the fluorescence signal within a certain wavelength band that corresponds to the emission spectrum of the employed fluorochromes.



The light scattering is another signal that is detected. Both the low-angle ("forward" scattering) and 90 ° scattering are standard signals to collect. The scattered signals are separated from the fluorescence-emitted light due to the difference in wavelength. While fluorescence light always have longer wavelength than the excitation light, there is no change in wavelength in the scattered signal (elastic scattering). The low angle scattered signal is mainly dependent on the size of the particle/cell, whereas the 90 ° scattered signal also depend on the structure and shape of the cell.

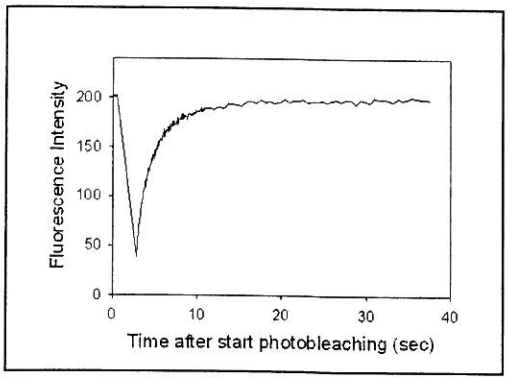


An important design parameter in FCM concerns the hydrodynamic focusing/centering of the particles in the excitation light / focus plane. It is of critical importance for exact quantitative measurements that each individual cell is illuminated to the same extent. The intensity distribution of the laser beam is Gaussian and very cell should be illuminated by the maximum laser intensity. In addition the cells should have constant velocity in the excitation beam. This is achieved using a support fluid called sheath flow that determine the overall particle velocity independent of the size of the individual particle, and at the same time position the particle within the jet in the same position. The particle suspension is injected in a central canule of a sheat fluid flow, which when forced through the orifice is centering the particles by hydrodynamic focusing se figure.

Flow cytometer may also sort cells. The flow is broken into droplets by a vibrating piezoelectric crystal on the top of the nozzle. Some of the droplets will contain a cell. The droplets pass an electrostatic field which is formed by to charged plates. The droplets containing cells that should be sorted are charged positively or negatively and will be deflected to electrodes with opposite charge. The deflected droplets are collected in tubes. Which droplets should be charged are determined based on characteristic properties of the cells.

b) Fluorescence recovery after photobleaching (FRAP)

FRAP is based on bleaching of fluorophore labelled molecules using a high-intensity laser. The bleached area is monitored using a laser with low intensity and from the recovery of fluorescence into the bleached area shown in figure below, the diffusion coefficient is obtained. An appropriate mathematic equation is fitted to the experimental recovery curve to estimate the diffusion coefficient.

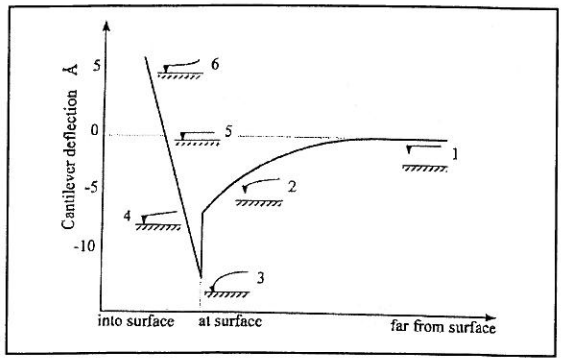


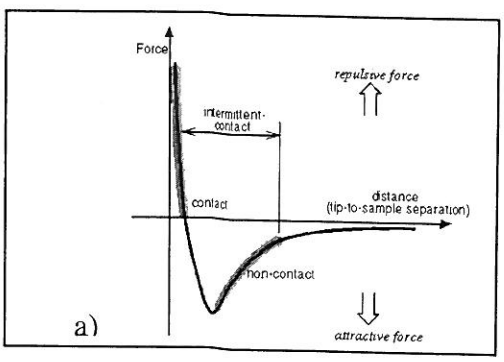
Fluorescence intensity before bleaching, during and after bleaching.

The bleaching is based on one- or two-photon excitation. Using single photon excitation and low NA objective the bleaching area may be approximated to a cylinder and the 2-dimensional diffusion coefficient obtained. Two-photon excitation on the other hand create a well defined focal volume, and bleaching occur only in the focal plane. FRAP based on two-photon excitation may therefore be used for accurate measurements of 3-dimentional diffusion.

Exercise 3: Atomic force microscopy

a) Force-distance diagram:



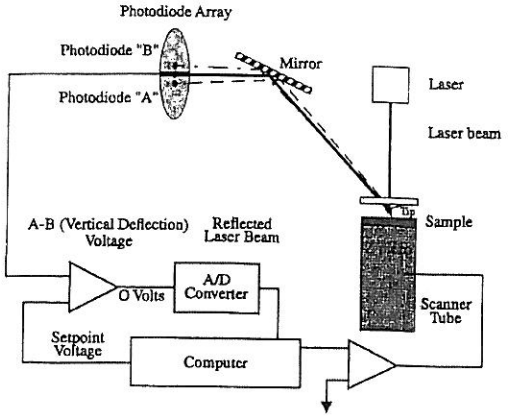


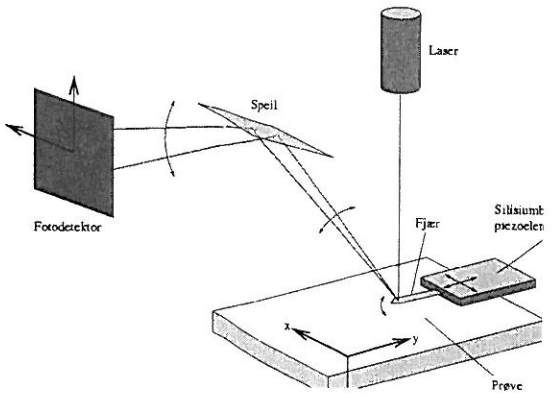
1) Tip far from sample: no force
2) Deflection increases with decreasing distance to sample
3) Sudden contact, force is changed with constant distance
4) Contact, cantilever is not deflected if sample is not easily deformed
5) Balance between attractive (force from cantilever + capillary force) and repulsive force
6) Tip even closer to sample, repulsive force – cantilever deflected opposite direction

b) **Principle for atomic force microscopy (AFM)**

The cantilever with its sharp tip is scanned over the sample/ or the samples scanned under the tip. The cantilever will be deflected due to forces in the piconewton range between the atoms in the sample and the tip. The relationship between the deflection and the forces follows Hook's law. F=-kx. The deflection is registered on a photodiode as described below. AFM gives information about the topography of the sample, elastic properties and forces.

The design of the AFM:
The AFM consist of the detection system, detecting the deflection of the cantilever; a pizoelectric crystal which the sample is placed on the top of, the cantilever with the tip; and a electric feedback system





Detection is based on the optical lever system. A laser beam hits the cantilever and the reflected laser light reaches a position sensitive photodiode. The photodiode is divided into two or fours areas to register vertical and horizontal displacement of the position of the cantilever. Deflection of the cantilever is registered vertically. Friction forces registered horizontally. A mirror may be inserted in the reflected beam path to increase the distance between the cantilever and the photodiode. Due to the long distance from the cantilever to the photodiode compared to the short distance of the cantilevers, any small deflection of the cantilever is amplified and registered as a large vertical displacement on the photodiode.
The tip: the quality of the tip determined the resolution of the image.

Constant distance between tip and sample is maintained due to the feedback system. The piezoelectical scanner controls the position of the tip relative to the sample
The deflection of the cantilever is detected as a vertical displacement of the laser beam on the photodiode, and converted into a electrical signal. The electrical signal is directed to the pizoelectical scanner, and the scanner will reposition itself in the vertical direction to maintain constant tip-sample distance. See figure.

Friction force causes a horizontal displacement and/or twisting of the cantilever. This movement is registered as a horizontal displacement of the reflected laser beam onto the photodiode. The friction forces is thus determined by the intensity in the four region of the photodiode and given by:

$$F_{friction} = \beta\left[\left(I_{upperleft} + I_{lowerleft}\right) - \left(I_{upperright} + I_{lowerright}\right)\right]$$

Excercie 4: Electron microscopy (EM)

The resolution of EM is determined by the diffraction of the electrons and given by the same equation as for light microscopy:

$$r_{airy} = \frac{0,61 \cdot \lambda}{NA}$$

The wavelength λ for electrons is according to Broglie:

$$\lambda = \frac{h}{p} = \frac{h}{m \cdot v} = \frac{h}{\sqrt{2meV}} = \frac{1,23nm\sqrt{volt}}{V}$$

$$d = \frac{0,61 \cdot \lambda}{NA} = \frac{0,75nm\sqrt{volt}}{V}$$

Typical theoretical value is 0,025Å if the numerical is NA=1 and the accelerating voltage V=60kV
However, the real resolution is 1-2 Å

The difference between theoretical and real resolution is mainly due to:

Electromagnetic lenses have extreme small solid angle so NA is small. The small angle is necessary to reduce spherical and chromatic aberration.

Contrast

The contrast is determined by the amount of scattered electrons. When electrons hit the sample they are transmitted or scattered. Bright parts of the image are due to transmitted electrons and dark part reflects that no electrons are detected due to scattering.
Atoms with high atomic number are scattering electrons more efficiently.

High contrast/high resolution: High contrast requires maximum interaction between electrons and sample, and that lens aberrations are reduced. High resolution requires collecting a large number of electrons, and high accelerating voltage. Thus one has to choose between high contrast and high resolution as explained in the table below.

High contrast	High resolution
Reduce lens aberrations by using shutters with small apertures to remove peripheral electron beams	Shutters with small apertures to remove peripheral electron beams will reduce amount of electrons forming the image and thus reduce resolution
Increase focal length to increase the depth of field	Use shortest possible focal length. Long focal length results in narrower aperture angles and a loss of resolution
Lower accelerating voltages, thereby the electrons are more affected by differences in specimen density and thickness	Higher accelerating voltages will reduce the wavelength of the electrons

b) Lenses in optical microscope and EM

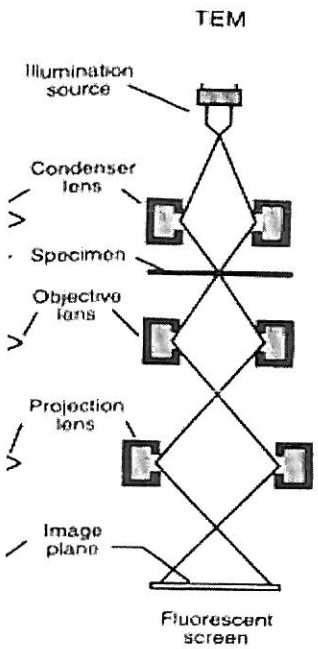
Lenses in light mic	**Lenses in EM**
Made of glass	Electromagnetic: solenoid Electrostatic: charged electrodes
Refractive index changes abruptly at the lens surface, thus rays are broken straight lines	Refractive index changes gradually, thus rays are continuous curves
Lenses convex or concave	Electromagnetic: always convex Electrostatic convex or concave

Focal distance f is given by:
$f=kv/I^2$

k= constant given by number of turns of lens coil and the geometry
V=accelerating voltage
I= current

Thus the focal distance can easily be changed by changing the current through the coil.

c) Design of a transmision electron microcope



Illumination source – electron gun. Cathode filament that release electrons and the electrons are accelerating to the anode.

Condenser lens: focuses e onto the sample. There are two condenser lenses. Lens 1 is a demagnifying lens, whereas lens 2 magnify the spot formed by lens 1. Together the two lenses determined the amount of e which hit the sample and the diameter of the lens.

Objective lens: Forms the initial image.

Intermediate lens magnify the image. The degree of magnification is determined by the current though the solenoid.

Projector lens. Most common with two lenses which magnify the image. The lenses have great depth of focus. This is important because the camera and screen are not in the same plane. Total of 4 linses to image and magnify the object.

Viewing screen and recording camera:
During microscopy the imaged viewed on a phosphorescence screen.
The image is recorded on a photographic film or a CCD camera.

Vacuum system: The EM from the electron gun to the imaging screen has to be in vacuum to avoid collision between e and gas molecules. Such collisions would change the energy and thereby the wavelength of the e.