NTNU

Department of physics

Examination paper for TFY4265 Biophysical micromethods

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Instructions

Each subquestions carries equal weight. Answer as concisely and precisely as possible. You may answer in English or Norwegian.

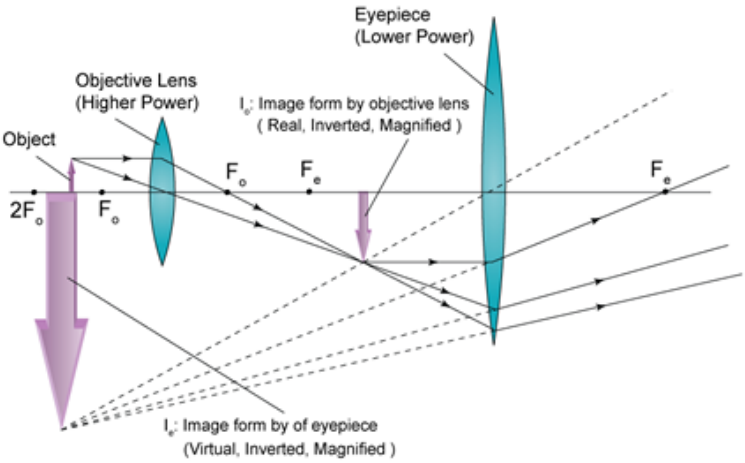
Problem 1: Optical microscopy

(a) Name three types of microscopy where the polarization of the light is important. Explain why and how the polarization is important for the function of the type of microscopy. The importance of the polarization can either be in the interaction with the sample or to somehow modify the light illuminating the sample.

> **Solution:** A nonexhaustive list:
>
> - In polarization microscopy, linearly polarized light (created with a polarizer) is used to illuminate the samle. If the sample is birefringent (refractive index depends on orientation) the polarization will become elliptical. An analyzer (orthogonal to polarizer) will then pass light which has become elliptical, indicating regions of birefringent material.
> - In DIC polarized light hits a prism of birefringent materials (Nomarski or Wollaston prism) that laterally shifts the two component polarizations relative to each other. If there are gradients in the optical thickness (product of refractive index and physical thickness) in the sample there will be a relative phase shift between the two components. A second prism recombines the two beams. If there was no variation in optical thickness, the combined beam will be linearly polarized again. However, if there was a relative phase shift the recombined beam will be elliptically polarized. An analyzer after the prism will then pass part of the ellipitical light while the linearly light is blocked creating contrast based on the variation in optical thickness.
> - In fluorescence anisotropy measurements polarized light is usede to excite the sample. If the molecules are relatively stationary the emission will also be polarized. However, if the molecules are free to move their orientation will be randomized before emission resulting in unpolarized emission. This can then be used to assess the mobility of the molecules (free or bound)

(b) Draw a sketch which illustrates the image formation in a compound microscope using geometrical optics. Include the intermediate image plane and indicate whether the images are real or virtual.

> **Solution:** Draw something like figure below. The intermediate image is real and the final magnified image is virtual.



(c) Two essential components in a phase contrast microscope are the *condensor annulus* and the *phase plate*. Explain why these two components have to be placed in conjugate planes.

Solution: If the condensor annulus and the phase plate are placed in conjugate planes the ring-shaped aperture of the condensor annulus will be imaged on the phase plate. Thus, the undiffracted light will phase through the dark, thick region of the phase plate and be attenuated. The diffracted light will pass through the transparent, thin region and thus acquire a relative phase change relative to the undiffracted light.

(d) Explain how (pulsed) STED uses two laser beams to achieve resolution which is better than the diffraction limit.

Solution: In STED, an excitation laser is first used to excite fluorophores in a diffraction limited volume. Before the molecules have time to deexcite, a second, doughnut shaped depletion laser with slightly longer wavelength will completely deexcite the molecules at the edges of the focal volume through stimulated emission. As this is a saturable process all the molecules will be driven to the ground state. Excited molecules remain in the center which can subsequently deexcite and emit normal fluorescence at wavelengths that are slightly longer than the depletion beam.

(e) Explain briefly how *fluorescence correlation spectroscopy* can be used to determine the diffusivity of fluorescent molecules in a solution.

Solution: In fluorescence correlation spectroscopy one measures the intensity variation of the fluorescence from the focal volume as fluorescent molecules diffuse in and out of the focal volume (or the fluorescence changes due to other mechanisms). The frequency of the variation will depend on how fast the molecules are diffusing. Molecules with higher diffusivity (typically smaller molecules) will lead to high frequency variations, while molecules with lower diffusivity will lead to lower frequency variations. The frequency of the variations can be quantified by an autocorrelation.

(f) Assume you have designed a nanoparticle which carries a cancer drug. The surface of the drug is covered with a molecule (ligand) that you think binds specifically to a surface receptor on cancer cells. Now you ask yourself two questions: 1) Is the nanoparticle actually binding to the receptor I believe? 2) Is the cell taking up (internalizing) the nanoparticle or is it just sticking to the cell

surface. Describe experiments where you would be able to answer these questions. Assume you can tag both the nanoparticle and the receptor with fluorophores without modifying their function.

Solution: Confocal microscopy should be able to answer if the cell is taking up the drug or if it is sticking to the surface. By testing the drug on a cancer cell-culture, the z-sectioning capability of confocal microscopy should be sufficient to determine if the cell is inside the cell or on the surface (the resolution is about 1 μm and the cell is about 10 μm). However the the resolution might not be sufficient to determine if the molecule is sticking to the receptor in question. The improved lateral resolution of STED or single molecule localization techniques (e.g. STORM) are more likely to be sufficient for this.

Problem 2: Force based techniques

(a) What is the difference between *constant force* and *constant height* imaging modes in AFM?. Discuss the advantages and disadvantages of both modes.

Solution: In constant force mode, the feedback system is continuously trying to adjust the piezoelectric stage so that the force and deflection of the cantilever is constant. This has the advantage that large variations in surface morphology can be accommodated, but scanning is typically slower as the feedback system has a finite response time.

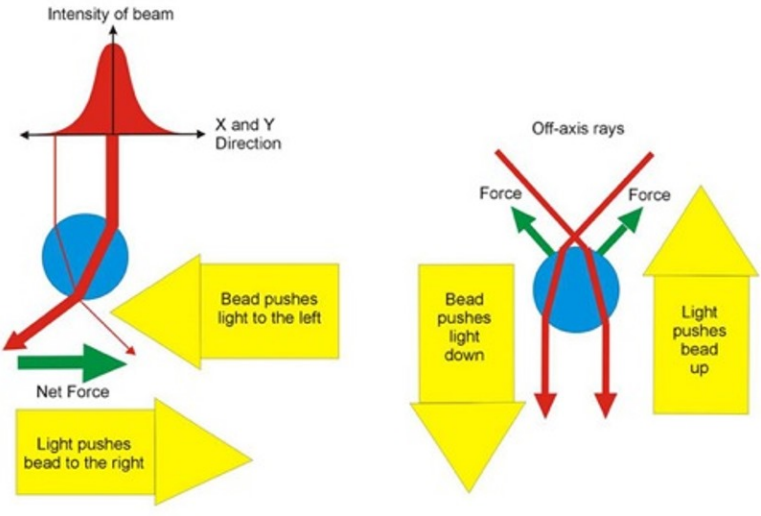
In constant height mode, the height of the stage is kept constant and the deflection of the cantilever varies according the the morphology and chemistry of the surface. In this way scanning can be fast (limited by resonances in the cantilever) but only small surface variations (given by maximum deflection of the cantilever) can be accommodated.

(b) Why is the loading rate important in dynamic force spectroscopy?

Solution: The loading rate is important due to the stochastic nature of bond rupture. The thermal energy supplied to the system to cause rupture is a random process and the length of time the the bond experiences a force in a certain interval will affect the likelihood that the bond will rupture during this force interval. A slower loading rate will thus increase the likelihood that the bond will break at a lower applied force.

(c) Explain using geometrical optics why a particle trapped with optical tweezers needs to have a higher refractive index than the surroundings.

Solution: With the refraction as indicated in the figure below we see that the particle is attracted laterally towards the center of the beam and axially towards the focus. We see that the refraction is such that the light is bent towards the surface normal when entering the particle, indicating a higher refractive index. If the refractive index was lower, the forces would be opposite and the particle pushed away.



Problem 3: Electron microscopy

(a) Explain what the difference is between *backscattered electrons* and *secondary electrons*. Explain why we use different detectors for these two interactions and how these detectors work.

Solution: Backscattered electrons are incident electrons that are scattered at high angles through the interaction with the atomic nuclei. This are highly energetic. They are typically detected by an annular detector surrounding the axis of the incident beam.

Secondary electrons are electrons ejected through ionization of the atoms by the incidence beam. They have typically a much lower energy than the backscattered electrons. A detector to the side of the beam collects these electrons by applying a voltage difference between the detector and the sample (the backscattered has such a high velocity that they experience little deflection).

Both detectors (typically) consist of a scintillator which converts electrons to photons. The photons are subsequently converted to an electrical signal by a photomultiplier tube.

(b) Outline the steps that are necessary for preparing a sample for *scanning electron microscopy*, including the rational behind each step.

Solution:

1. First the sample must be fixed as quickly as possible after extraction to avoid sample autolysis and halt biological processes. This is usually done using glutaralaldehyde and subsequent postfixing with osmium tetroxide.

2. Secondly the sample must be dehydrated as any water (or other fluid) would immediately evaporate in the high vacuum of the microscope. First the water is substituted with acetone through a series of mixtures with increasing concentration of acetone. Subsequently the fluid is removed in a critical point dryer. A high pressure chamber is used where liquid carbon dixoide is substituted for the acetone. The temperature is then increased so that the system is beyond the critical point where the abrupt phase change between liquid and gas disappears. The pressure is decreased and the gas removed.

3. The dried sample is then mounted on a conductive sample holder and sputtered with a thin layer of conductive material to make the sample conductive so that charge accumulation from the electron beam can be removed.

(c) In environmental scanning electron microscopy (ESEM) it is possible to image samples under much higher pressure than in conventional scanning electron microscopy. Explain how this is possible.

Solution: In ESEM a differential pumping system is used. The sample is placed in a chamber which has a relatively high pressure close to a small opening where the electron beam will pass through. On the other side of the of the opening a vaccum pump maintains a much lower pressure, and a high pressure gradient between the two chambers is possible due to the small opening. A second small opening is used the separate this chamber from the beam shaping column which is maintained at an even lower pressure.