ENGLISH

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DEPARTMENT OF PHYSICS

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Solution set for
EXAM IN TFY4265/FY8906
Biophysical microtechniques

December 19th, 2012
4 hours

Allowed Aids: C
Specified printed matter: Karl Rottmann, Mathematical formulas. Specified calculator allowed.

General information

There are 10 problems and each problem is worth 10 points for a total of 100 points (i.e. you have on average 24 minutes for each problem).

Problem 1- Optical microscopy

a) Optical microscopy can be severely limited by several aberrations if not corrected for. Explain what is meant by chromatic and spherical aberrations. Under which circumstances are these effects significant and what can be done to reduce them?
Refractive indicies of materials depend on wavelength, and as it is the difference in refractive index between the lens and the surroundings which determines the refraction of a lens, different wavelength will be focused differently and not coincide at the same focus. This is a problem when mulitple wavelengths are employed. Examples are standard microscopy using halogen lamp, fluorescence microsocpy with fluorophores excited with different lasers, or multiphoton microscopy requiring broadband pulses. Spherical aberration means that light passing through the lens close to the optical axis is refracted differently than light passing further away from the optical axis and therefore not coinciding in the same focus. This is especially a problem for high NA objectives which have large collection angles and appertures.

b) What are the advantages of nonlinear optical microscopy (multiphoton microscopy)? What extra components are needed to upgrade a standard confocal microscope to a nonlinear optical microscope? Describe the following interactions and include the appropriate Jablonski diagrams:

- Two-photon fluorescence
- Second harmonic generation
- Stimulated Raman scattering.

Nonlinear optical microsocpy has several advantages:

- **Increased penetration depth due to longer wavelengths**
- **Less out-of-focus photodamage and photobleaching**
- **Inherently confocal, allows for non-descanned detection**
- **Several endogenous signals may be used to give contrast**

Two photon fluorescence is the absorption of two photons, to excited the molecule into a state with twice the energy of the photons in the incoming wave. From this state the molecule relaxes to the lowest vibrational state at the excited electronic state and from there relaxes to the ground state with the emission of a fluorescence photon. Second harmonic generation is the conversion of two photons with the same energy to one single photon with twice the energy of the incoming photons. Stimulated Raman scattering is a process where photons from one beam is converted into photons with energy corresponding to another beam of light when the difference between the frequency of the beams corresponds to a Raman vibrational mode. (see fig.1)

c) What is *fluorescence resonance energy transfer* (FRET)? What are its uses? What are the spectral (emission and absorptions) requirements for the fluorophores used in FRET?
FRET is a process where energy is transferred from one molecule in an excited state to another molecule to excited this, without emitting a photon. The second molecule can then fluoresce as if excited by a photon. FRET only occurs when the molecules are very close and can thus be used to measure the confirmation of molecules by attaching fluorescent markers to different parts of the molecule. To have FRET the emission spectra of the donor must overlap to some degree with the absorption spectrum of the acceptor.

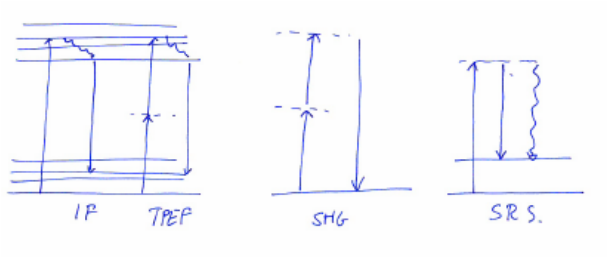

Figure 1: Nonlinear interactions

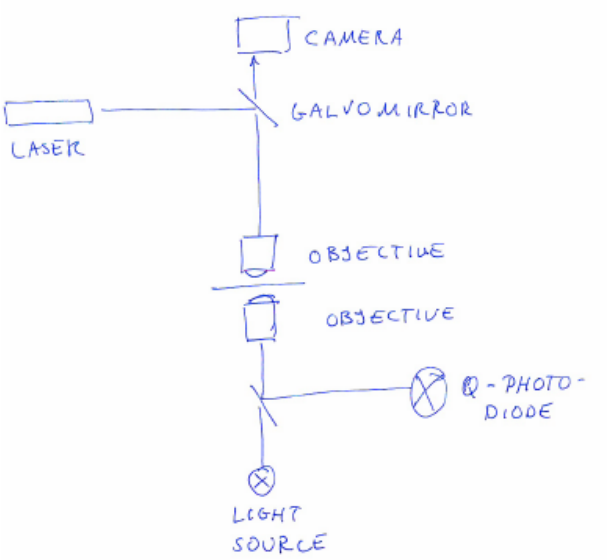

Figure 2: Optical tweezers

d) Explain what is meant by the diffraction limit in optical microscopy. How does Stimulated emission depletion (STED) microscopy overcome this limit?
When focusing light the resulting pattern in the focal plane is not a point but a diffraction pattern with a central maximum and then decaying oscillations in intensity away from the focus. The diffraction limit is defined as the distance from the central maximum to the first minimum. In STED we excited the fluorophores as in standard confocal microsocpy. However, immediately after the excitation a doughnut shaped beem is applied. This will deexcite the molecules in the doughnut, leaving only the molecules in the central spot to fluoresce.

Problem 2- Optical Tweezers

Explain how light can be used to trap a particle in three dimensions. Sketch a setup for optical tweezers that is used to measure forces, explaining what the necessary components are used for.
As light has momentum it will transfer some momentum to a particle when it scatters off that particle. By analyizing the refraction of a spherical particle one can find that the momentum transfer will cause the particle to be attracted to regions of high intensity and will therefore be trapped in the focal spot of a highly focused laser beam. For an OT setup we need a trapping laser, beam stearing (galvomirrors), a camera for visualization, a quadrant photodiode to detect motion of the trapped particle, and a high NA objective. (See fig.2)

Problem 3- Atomic force microscopy

a) What are the main components necessary to realize an atomic force microscope? What determines the resolution in atomic force microscopy?
We need a pizeoelectric stage for precision motion of the sample, a flexible cantilever with a sharp tip, and a laser and corresponding quadrant detector for detecting cantilever motion. The resolution is ultimately limited by the size and shape of the cantilever tip.

b) What characterizes contact mode vs. tapping mode? How are forces typically measured (differently) in the two modes?
In contact mode the cantilever is always very close to the sample so that the interaction between sample and tip is always strong. In this mode the interaction is typically measured using deflection of the tip. In tapping mode the tip is oscillating and is interacting with the sample in only a small part of the period of the oscillations. In this mode the interaction is typically measured by changes in the resonance frequency of the tip.

c) In dynamic force spectroscopy, a typical measurement is to find the most likely unbinding force F^* of some interaction and plot this against the loading rate r_f. Typically we plot $F^*(\ln r_\mathrm{f})$. Show how the width x_β and height E_b of an energy barrier can be found from this plot. You may have use of the following relations (Assume that t_D is a known constant. It should be evident what the other variables are from the context.):

$$
\begin{aligned}
F^* &= F_\beta \ln \frac{r_\mathrm{f}}{r_0} \\
\frac{1}{t_0} &= \frac{1}{t_\mathrm{D}} \exp\left(-\frac{E_b}{k_\mathrm{B}T}\right) \\
r_0 &= F_\beta / t_0 \\
F_\beta &= \frac{k_\mathrm{B}T}{x_\beta}
\end{aligned}
$$

The first equation can be written

$$F^* = F_\beta \ln \frac{r_\mathrm{f}}{r_0} = F_\beta \ln r_\mathrm{f} - F_\beta \ln r_0$$

We see that F_β is the slope of a graph of F^* vs the loading rate which can be read from the graph. We can then find x_β from the last equation since we know the value of $k_\mathrm{B}T$. We can also see that r_0 is equal to the intercept of the graph with the x-axis. Once we have r_0 it is straightforward algebra to find an expression for E_b from the middle to equations.

$$E_b = k_\mathrm{B}T \ln\left(\frac{F_\beta}{r_0 t_D}\right)$$

Problem 4- Electron microscopy

a) Describe the possible interactions and emitted particles (photons are also particles) when high energy electrons strike a sample in electron microscopy. Which of these signals are typically utilized in SEM and TEM?
As electrons hit a sample we can have the following interactions (see fig.3)

- **Scattered electrons which are weekly scattered during interaction with another electron which is ejected with low energy.**
- **This low energy photons are called secondary electrons**

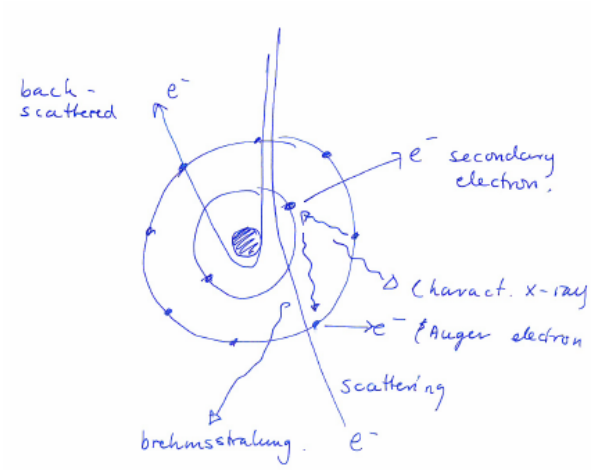

Figure 3: Electron sample interactions

- **Backscattered electrons are high energy electrons which are scattered at high angles (backwards) due to interactions with the nuclei of the sample**
- **Brehmsstralung is x-ray emitted by the primary electrons as they are scattered (accelerated)**
- **Characteristic x-ray are photons which are emitted as electrons in higher orbitals fill lower lying orbitals which are empty due to the emission of a secondary electrons.**
- **Auger electrons are electrons which are emitted through the absorption of a characteristic electron and are thus emitted with specific energies.**

Typically in TEM we measure the reduction in electron intensity due to primary scattered electrons. In SEM we typically measure the secondary electrons.

b) Describe the briefly the main steps necessary to prepare a biological sample for TEM. EM is typically performed in vacuum. How is it possible to perform electron microscopy under physiological conditions (environmental TEM)?
For TEM we first fix that sample, that stabilize the morhpology through cross-linking. There are many options here but the most common are glutaraldehyd and osmium tetroxide. Then the water must be substituted typically with alcohol or acetone. The samples are then embedded in a medium which makes it harder and therefore easier to cut in thin sections. These sections can than be stained with various heavy elements. There is a huge range of variation on this theme to optimize the fixation of specific structures.
In environmental SEM the sample is placed in a sample holder such that there is only an extremely thin layer of whatever is needed to provide physiological conditions around the sample. As the layer is very thin it is still possible to send electrons through it without too much absorption.