NTNU

Department of physics

Examination paper for TFY4265 Biophysical micromethods

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Each subquestions carries equal weight. None of the questions require lengthy answers so answer as concisely and precisely as possible.

Problem 1: Optical microscopy

(a) After excitation of N_0 molecules, the number of molecules $N(t)$ that are still excited as a function of time will follow an exponential decay, $N(t) = N_0 \exp(-t/\tau)$, where τ is the lifetime (the average time a molecule resides in the excited state). The various deexcitation processes can be described by rate constants k_i where $k_i dt$ is the probability of deexcitation through process i in a small time interval dt. We can separate the deexcitation processes into radiative (k_r) and non-radiative (k_n). Write down a justified expression for the lifetime and the quantum yield Q of a molecule in terms of k_r and k_n.

You will probably see that the lifetime depends on the rate of non-radiative decay. What practical applications can this dependence have?

Solution:

Since $k_i dt$ is the probability a single molecule will deexcite in a small time interval, the number of molecules from an ensemble $N(t)$ that will dexcite will be $k_i N(t) dt$. We can thus write down the differential equation

$$dN = -k_i N(t) dt$$

In the presence of multiple rate constants the differential equation becomes

$$dN = -(k_r + k_n) N(t) dt$$

The solution is

$$N(t) = N_0 \exp(-(k_0 + k_n)t)$$

The lifetime τ is expressed by $N(t) = N_0 \exp(-t/\tau)$. The lifetime is thus $\tau = \dfrac{1}{k_r + k_n}$.

The quantum yield is the ratio of emitted photons to the total number of deexcitations and is thus $\dfrac{k_r}{k_r + k_n}$. (Other arguments can be valid)

One application is to measure the lifetime of a molecule and use this to measure the presence of other molecules in the solution which increase the rate of non-radiative decay (quenching).

(b) Between the collector and the condensor lens in a microscope set up for Kohler illumination there are two adjustable apertures/diaphragma. What are they called? What is their function? What other planes in the optical system are conjugate to these two apertures (make a sketch to illustrate this)?

Solution:

The *aperture diaphragm* (also called *condenser aperature*) is placed in the front focal plane of the condenser. The *field diaphragm* is placed close to the collector lens. The *aperture diaphragm* controls the angle of the cone of light which illuminates the sample and can therefore control the resolution and contrast. The *field diaphragm* is used to control the area of illumination of the sample. The *aperture diaphragm* is conjugate to the light source, back focal plane of objective and the

eye pupil (for compound microscope). The *field diaphragm* is conjugate to the sample, the intermediate image plane and the retina.

(c) Compare the contrast mechanism in phase contrast microscopy and differential interference contrast (DIC) microscopy. How are they similar? How are they different?

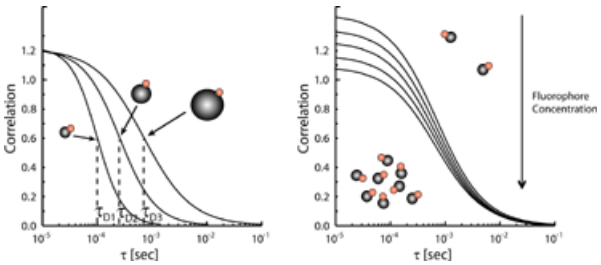
Solution:
Both phase contrast and DIC are dependent on the optical thickness (product of thickness and refractive index) of the material. In phase contrast the variation in small phase changes due to the sample are transferred to an amplitude variation by using the phase plate to damp the unscattered light (surround wave) and generate an additional phase change change to the scattered light (diffracted wave) and letting the two waves interfere. In DIC the gradient in the optical thickness is used to change the polarization state by letting the two waves with orthogonal polarization that are slightly shifted spatially experience different phase shifts that will modulate the resulting polarization when the two beams are combined.

(d) Both stimulated emission depletion (STED) and ground state depletion (GSD) microscopy are two methods for achieving superresolution imaging. How are they similar in achieving this? How are they different?

Solution:
Both STED and GSD typically use the donut shaped beam to make molecules in the donut enter a dark (off) state that cannot fluoresce so that we only get signal from the central hole in the donut. STED relies on stimulated emission from the excited state to the ground state which is then the dark state. In GSD, the donut beam drives the molecules to the triplet state which is then the dark state from which the molecules cannot fluoresce. Both these process are saturable which provides the necessariy nonlinearity for the superresolution technique to work.

(e) Fluorescence correlation spectroscopy is a method which can be used to measure the diffusivity of fluorescent molecules in a solution. Explain the main steps in the analysis of such data (you can, but are not required to, use mathematical expressions).

Solution:
First, the time dependent fluorescence intensity is measured. The variation in the signal is caused by molecules diffusing in and out of the focal volume. The signal is then autocorrelated with itself and the autocorrelation function is plotted as a function of the time shift. The autocorrelation will be high at time shifts smaller than the typical time the molecule resides in the focal volume. For longer time shifts, the time shifted signal is uncorrelated with the original. See figure below. By locating the time shift at which the autocorrelation decays, the diffusivity can be determined.



(f) What are the advantages of multiphoton microscopy?

Solution:
In multiphoton microsopy the signal is only generated in the focal point so there is no need for a confocal pinhole. This makes it possible to collect also scattered photons. Furthermore, light with longer wavelength can be used which makes it possible to image deeper into the sample. There are also a wider range of endogenous signals (e.g. SHG and CARS) which can be used to generate contrast.

Problem 2: Force based techniques

(a) In atomic force microscopy, a piezoelectric scanner is used to scan the sample under the cantilever. What is a piezoelectric material? What do we mean by hysteresis in the piezoelectric material? How do we correct for the hysteresis in a piezoelectric scanner?

Solution:
A piezoelectric material is a material which changes size (strain) due to an applied electric field across the material and also can generate a voltage due to an applied stress which changes the size (strain) of the material. Hysteresis means that a response is dependent on the past history of the applied signal, and not just the current value. E.g. for a piezoelectric material the resulting strain when increasing the voltage will be different from when the voltage is reduced. The difference in resulting strain (as well as the nonlinearity) is correcte by using different applied voltage curves when increasing and decreasing the voltage.

(b) When approaching a sample with the AFM tip there will first be an attractive force due to van der Waal forces and then repulsion due to Pauli exclusion. Draw a curve showing the interaction force as a function of distance and indicate in which regions of the curve the cantilever is operated in the three main imaging modes (contact, oscillating, tapping)

Solution:
In contact mode the AFM is operated in the repulsive region, in oscillating mode, the AFM is operated in the attractive regime and in tapping mode tha cantilever is oscilating with a larger amplitude covering both the attractive and repulsive regime.

(c) The calibration of optical tweezers is based on the theory of Brownian motion. The starting point is the equation of motion for the trapped particle in the solution (the

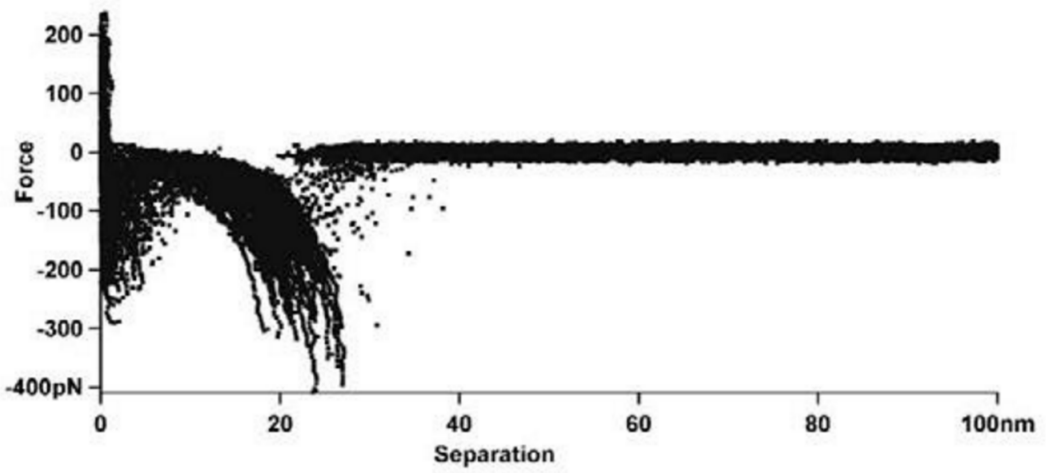


Figure 1: Approach-retract curves

Langevin equation),

$$mx''(t) + \gamma x'(t) + kx(t) = \eta(t)$$

Explain the physical origin of the terms γ, k, and η.

Solution:
γ is the viscous damping of the fluid the trapped particle is moving in. k is the spring constant of the trapping force created by the optical trap. η is the random force created by the thermal motion of the water molecules bouncing of the particle.

(d) Dynamic force spectra can be analysed using the idea of the *most likely unbinding force* f^* to say something about the potential energy of the molecular bonds. The result of the theory is that

$$f^* = f_\beta \ln(r_f) - f_\beta \ln(f_\beta k_0)$$

where f_β and k_0 are fitted parameters. Figure 1 is a large data set of multiple approach-retract curves showing unbinding events. Explain how f^* and r_f (loading rate) are determined from this data and how the parameters f_β and k_0 are typically fitted to the data using the above equation

Solution:
For each retract curve, the loading rate r_f is measured as the slope of the curve just before rupture and the unbinding as the maximum force measured before unbinding. The data are then binned depending on the apparent loading rate, and for each bin, a histogram of the various unbinding forces are plotted and the most likely rupture force f^* is determined from the peak fo the distribution. Hence, for each loading rate we have a corresponding most likely rupture force. We can then plot f^* as a function of $\ln(r_f)$ and from the equation given in the problem we see that we can find the parameters f_β and k_0 from the slope and intercept of the curve.

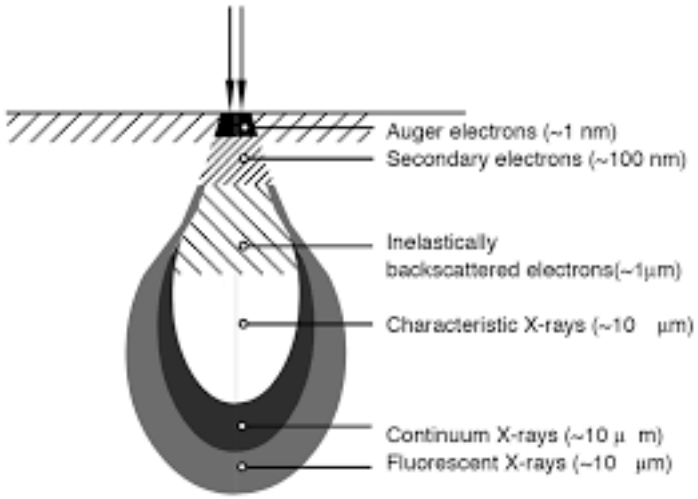
Problem 3: Electron microsocpy

(a) When the electron beam hits the sample in an electron microscope, both electrons and photons are emitted. Explain what we mean by secondary electrons, backscattered

electrons, auger electrons, bremsstrahlung, and characteristic x-rays. Also sketch the relative sizes of the regions in the sample from where we can acquire these signals.

Solution:

A secondary electron is an electron of relatively low voltage which is emitted by the electron cloud by interaction with the incident beam. Backscattered electrons are electrons from the primary beam that scatter almost elastically backwards by interaction with the nucleus. Auger electrons are electrons which are ejected when they are hit by characteristic x-rays. Characteristic x-rays are emitted when low lying orbitals that have initially been vacated by the incident electron beam are filled by outer electrons. Se figure below for the interaction volumes.



(b) In preparation of samples for scanning electron microscopy there are many essential steps. Three of these are fixation, critical point drying and coating. Why are these steps performed and how are they done?

Solution:

Fixation is usually done by immersion in solutions of glutaralaldehyd and Osmium tetroxide. These chemicals create covalent crosslinks between the biological molecules and protects them from lysis by enzymes or microbial attacks. To be able to sustain the vacuum within the SEM, the sample must be completely dry, otherwise the water would rapidly evaporate, causing structural damage to the tissue. First the water is exchanged with acteone. Then the acetone is replaced by liquid CO_2 at high pressure. The CO_2 is heated beyond the critical point where there is no clear phase transition between the gas and the liquid and the pressure is reduced so the critical liquid turns into a gas and can be removed gently from the sample. Coating means sputtering a thin layer of metal on top of the sample to make it conductive so that absorbed electrons can be removed from the sample.