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DEPARTMENT OF PHYSICS

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EXAM IN COURSE TFY4265
BIOPHYSICAL MICROMETHODS
19. December 2009
Hours: 0900 – 1300

Answers:

Exercise 1: Light microscopy

1 (credit 10/40)
a) (credit 3/40)
1. Stokes shift is the difference in wavelength / frequency between maximum absorption and emission of the same electronic transition (see fig 1). Stokes shift occurs because the molecule loses a small amount of the absorbed energy as thermal energy/vibrational relaxation before re-releasing the rest of the energy as fluorescence. Consequences:

- Fluorescence emission peak wavelength is red-shifted with respect to absorption peak wavelength.
- This shift may vary typically from 5 to more than 100 nm, depending on the electronic structure of the molecule.

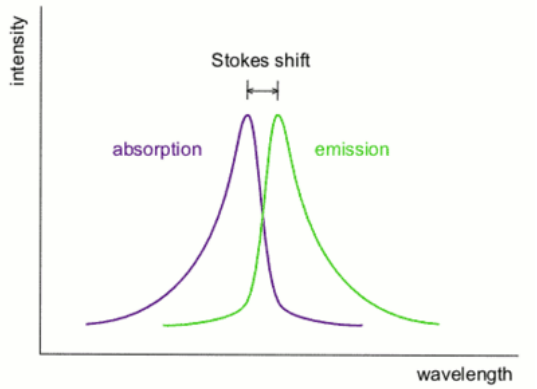


Figure 1: Stokes Shift.

2: Principle for FRET: Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule *without emission of a photon*. The efficiency of FRET is dependent on the inverse sixth power of the intermolecular separation, making it useful over distances comparable with the dimensions of biological macromolecules. Thus, FRET is an important technique for investigating a variety of biological phenomena that produce changes in molecular proximity. When FRET is used as a

contrast mechanism, colocalization of proteins and other molecules can be imaged with spatial resolution beyond the limits of conventional optical microscopy.

Requirements:

- The emissions spectrum for the donor must overlap with the excitationspectrum for the acceptor.
- Donor and accseptor must be separated by < 100 Å.

3 Advantages of Qdots over organic fluorophores for bioimaging:

- Qdots have narrow emissions; spectral crosstalks minimized
- Emission life time is longer (hundreds of ns); thus by gating can suppress shorter lifetime autofluorescence
- Qdots do not readily photobleach
- Qdots not subject to microbial attack

1 b) (credit 2,5/40)

1: 2: combined condenser lens + objective lens
3: sample
5 dichroic mirror
6 detector
7 pinhole

2: The incertion of a pinhole in an optically conjugate plane in front of the detector will eliminate out-of-focus information in specimens that are thicker than the focal plane. As only light produced by fluorescence very close to the focal plane can be detected the image resolution, particularly in the sample depth direction, is much better than that of wide-field microscopes.

1. c) (credit 2/40)

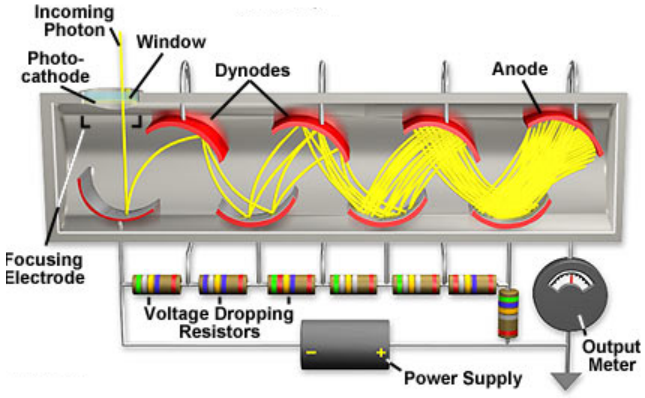


Figure 2: PMT tube

Name: Photomultiplier tube (PMT)
How it works:
A PMT amplifies weak signals by converting the detected photons into multiple electrons. Incident photons hit photocathode $\Rightarrow$ electrons emitted from the cathods rear surface hits dynode (see fig 2). Electrons accelerating in the field Vc-V1 between photocathode and first diode, etc. Typical number of diodes: 11, Low noise < 50cps. Spectral response, quantum

efficiency, sensitivity, and dark current: determined by the composition of the photocathode. (often gallium-arsenide). The PMT can be cooled to reduce dark current originating from electrons released by thermal activation.

1 d) (credit 2,5/40)
Components incerted in the light path in a DIC microscope:

Polarizer: creates linear polarized light

Wollastone prism 1: separates polarized light into two rays with E perpendicular to each other. The distance between the rays approximately equal to the resolution

Wollastone prism 2: Combines the two light rays. The rays do not interfere as E perpendicular
• Light with same optical path is linearly polarised when recombined
• Light with different optical path is elliptically polarised when recombined. Since the electric field vectors still are perpendicular, they cannot interfere with each other.

Analyzer: linear polarizer
• Axes of analyzer is perpendicular to axes of polariser and the light that is unchanged through the microscope is blocked.
• If the light has become elliptically polarised due to interactions with the sample, a component of the elliptical wavefront will be parallel to the transmission axis of the analyzer, and a portion of it will thus pass through the analyser and subsequently undergo interference to generate the DIC image.

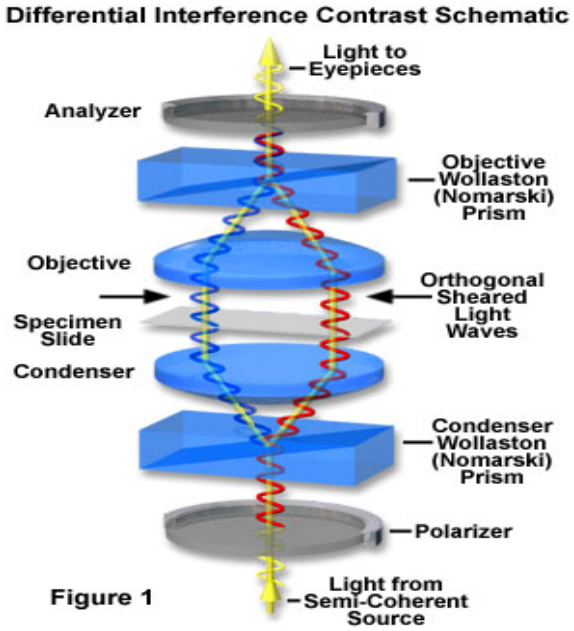


Figure 3: optical components in DIC microscope

Exercise 2: Optical tweezers (credit 10/40))
a) (credit 2/40)
Lateral trapping:

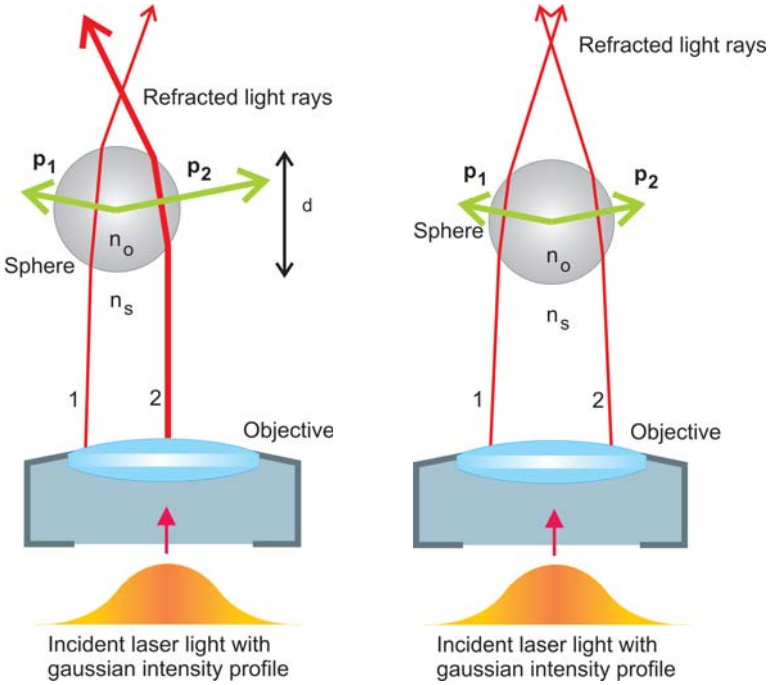
- If a ray crosses an interface separating two media of different refractive indices, the direction of propagation for the refracted rays can be determined using Snell's refraction law: ni sinθi = nt sin θt.
- Snell's law and momentum conservation is sufficient to give a basic model for optical trapping, where complications like reflections and absorption are neglected.
- Assume that a dielectric sphere of diameter d and refractive index no is immersed in a solution with a lower index of refraction ns < no.

The bead is displaced to the left of the beam center. Light ray 1 and 2 transfer a momentum of p1 and p2 to the sphere, respectively.

The net momentum transfer to the sphere is p1 + p2 and will cause acceleration to the right, toward the center of the beam where the light intensity is highest.

→ the Gaussian intensity distribution is important for an optical tweezer since it causes |p1| < |p2| and thereby ensures lateral trapping.

By symmetry, a bead displaced to the right will be pushed left, again toward the beam center as illustrated in figure 4.



1. The laser-beam enters the objective from below and is bent inward, toward a focus.
2. Light from the laser is refracted as it travels through the sphere, and the direction of propagation is changed. Because light carries momentum, this corresponds to altering
3. the momentum of the light.
4. Since there are no external forces acting, the total momentum is conserved, and the sphere will have its momentum changed by an equal and opposite amount.

Figure 4: trapping of particles using laser light

Axial trapping:

- The sphere is positioned below the laser focus (the two rays have not intersected before they refract in the sphere).

- The net momentum is p1 + p2 and points upward, toward the focal point, because the two rays have equal intensity.

- By reversing the light paths and turning the page 180 degrees, the case where the sphere is above the focus is handled similarly. From this point of view the rays intersect in a focal point before they reach the sphere, and by performing the vector calculations as before, the net force is again directed toward the focal point.

Alternative explanation if assuming that the particle is small compared to the lasers wavelength

- laserbeam = electromagnetic wave and will induce electric dipoles in the dielectric medium of the sphere. (bead = single point dipole).
- Near the lasers focal point the laser intensity gradient is steep and the electric field is non-uniform.
- Any electric dipole placed in an inhomogeneous electric field will be affected by a net force, because the force affecting the two poles will be different.
- In addition, the induced dipole will oscillate with the electric field from the laser and emit radiation.

- The net force affecting the sphere is divided into two components:
  1) Scattering Force:
- The dipole scatters laser light. This process will transfer momentum to the dipole, since emitted radiation may propagate in a direction different from absorbed radiation. The net sum of momentum transferred to the dipole per unit time is known as the scattering force.

  2) Gradient Force
  = The Lorentz force (force acting on the bead due to the EM fields).
  E^2= the mean square electric field, α =polarizability of the sphere.
- The gradient force is parallel to the local field gradient, and will draw the bead toward the focus of the laser beam since the electric field in strongest there.

b) Detection of lateral (off-axis) displacement (credit 2/40)

- An efficient method for detecting the lateral (off-axis) displacement is called back-focal-plane interferometry (BFPI) (Figure 5).

- BFPI: The condenser lens is positioned so the bead is located in the forward focal plane. The back focal plane is occupied by a quadrant photo-diode (QPD). This way the condenser directs both the scattered light from the bead and unscattered light from the laser onto the QPD where complex interference patterns are observed.

- The QPD consists of four separate photodiodes, arranged next to each other. Light incident on a diode will lead to a voltage difference over the device proportional to the intensity of the light. In this way, the intensity pattern is converted into four voltage readings, one for each diode.

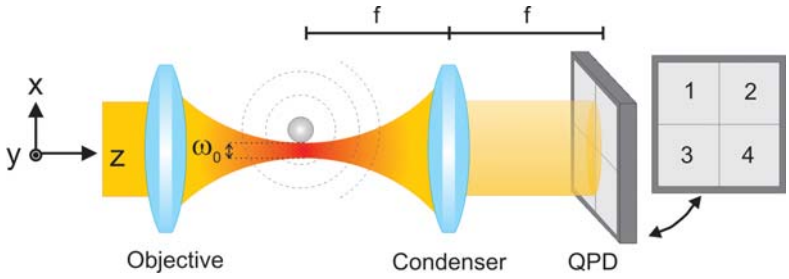


Figure 5: Illustration BFPI

c) (credit 2/40)
For a strong trap a relatively high force is required in order to induce a small displacement of the bead away from its equilibrium position in the senter of the intensity gradient of the laser beam.
The stiffness of the trap can most easily be regulated by regulating the intensity of the laser.

d) (credit 2/40)
Using a large difference in trap stiffnes, the displacement of the left bead will be very small compared to the diplacement of the right bead, and can therefore be neglected. Only the right bead moves: displacement is measured for this bead.

e) (credit 2/40)
How forces are measured using optical tweezers: Fig 6 = schematic illustration.

- The histogram of a trapped spheres position follows a Gaussian distribution.
- The potential energy U(x) of the bead in the trap can be deduced from the histogram by using the Boltzmann distribution that gives the probability density for occupying a state with energy U(x). The Gaussian position distribution is the signature of a harmonic potential (U(x) proportional to x^2). In the lateral directions, the optical trap can be characterized by two spring constants: k_x and k_y. → position data are directly proportional to the force on the sphere!

- Conclusion: the measured Qx on the quadrant photo diode (QPD) is directly proportional to both the actual displacement x and the force F acting on the sphere.
- The factor that translates detector signal Q into physical displacement x is called the detector sensitivity β.

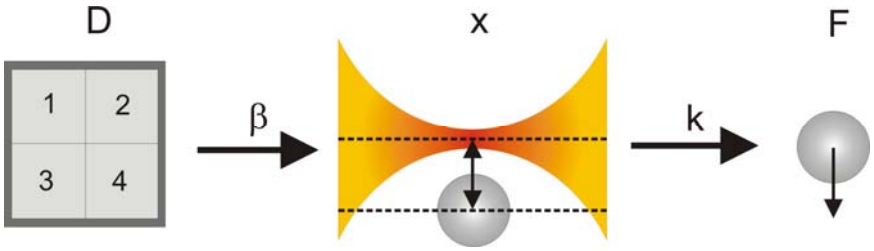


Figure 6: illustration force determination in optical tweezers.

Comparison of the force range that can be determined using optical tweezers with the force range accessible with the atomic force microscope:
Optical traps: 10^{-13}-10^{-10} N, AFM: 10^{-11}-10^{-7} N

Exercise 3: AFM (credit 10/40)

a) (credit 2/40)
Tapping mode AFM imaging:

- Distance tip – sample surface between contact and non-contact
- Tip scanned across the sample
- Attractive forces
- Only taps sample for a small fraction of the oscillating period
- Cantilever is oscillating around 90 % of resonance frequency
- Amplitude of oscillation: ten - hundreds Å
- Registers change in resonant frequency or vibration amplitude when tip is close to sample. Held constant by negative feedback loop to piezoelectric scanner

Factors limiting the lateral resolution in AFM:

- the geometry of the tip (Radius 5-50 nm).
  - Diameter of tip < structure recorded
- Elastic properties of the sample
- The size of the hight differences in the sample
- Typical value: distance between objects 10-20 nm

b) (credit 2/40)
The following expression describes the rate of escape from bound to free states under force:

$$k_{off} \approx \frac{D}{l_c l_{ts}} \exp\left[-\frac{E_b(f)}{k_B T}\right]$$

Here, k_{off} is the rate of dissociation events, $k_B T$ is the thermal energy and $D/l_c l_{ts}$ is the attempt frequency. E_b (f) is the energy difference between bound and transition states (the height of the barrier).
Application of a persistent force to the molecular interaction will tilt the energy landscape in the direction of the force, and the component of the force along the dissociation reaction coordinate will lower $E_b(f)$ (see figure 7). If assuming a sharp barrier and a deep potintial the following expression of the dissociation rate under persistent force is obtained:

$$k_{off} \approx \frac{1}{t_0} \exp\left[\frac{f}{f_\beta}\right]$$

Where $f_\beta = k_B T/x_\beta$ with x_β equal to the displacement of the unbonding transition along the reaction coordinate. This dissociation rate defines the probability that the interaction will break at a given time. Since distant barriers (large x_β) are more affected by the applied force than barriers situated closer to the binding site/energy minimum), new energy barriers in the energy landscape of the interaction may be uncovered (see figure 7).

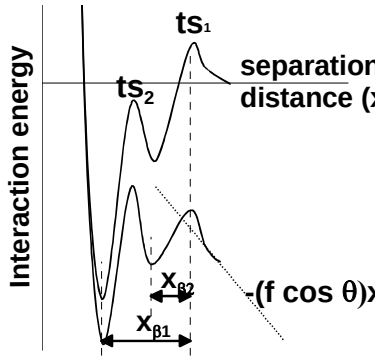
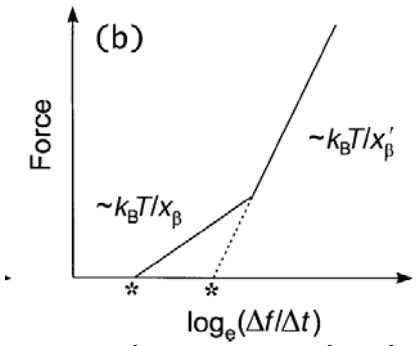


Figure 7: Energy landscapes for bound states confined by sharp activation barriers. An external force *f* is applied along a pulling direction oriented at the angle θ to the molecular co-ordinate *x*. This external force adds a mechanical potential $-(f\cos\theta)x$ that tilts the landscape and lowers the barrier. The barrier is thus lowered by force in proportion to the thermally averaged projection $x_\beta = x\cos\theta$ of the barrier along the direction of the force. The situation illustrates describes a cascade of barriers under force. At low r_f, information about $x_{\beta 1}$ is obtained. The escape from the inner barrier becomes the limiting process when the outer barrier is driven below it by $\geq k_BT$, i.e. at high forces. For large enough forces one or more of the intermediate bound states may become deeper than the fundamental bound state before unbinding has occurred. In such cases a considerable population might remain in the intermediate bound states between the new and the old limiting barriers. The escape of this population will yield a secondary maximum of the unbinding force distribution, and the parameter x_β should for such situations be interpreted as the distance $x_{\beta 2}$.

A plot of force versus the logarithm of the loading rate is named a dynaic force spectrum and should be linear of slope f_β (= k_BT/x_β) assuming the eistence of one single energy barrier. If the energy landscape of the interactions contains several energy barriers, the existence and position of these may be revealed. Figure 8 presents a schematic drawing of a dynamic force spectrum reflecting the existence of two separate energy barriers in the energy landscape of the intermolecular interaction.



Two sharp energy barriers

Figure 8: dynamic force spectrum reflecting the existence of two separate energy barriers in the energy landscape of the intermolecular interaction investigated

c) (credit 2/40)
Explain the molecular origin of the force jumps observed in the force curve.
Suggest some types of information that can be obtained when performing force-elongation (= force induced denaturation):
The saw-tooth pattern in the force - extension data of titin are characterised by a sudden fall of the force following stretching of the molecule. The increase of the force before the drop in

force reflects the elastic restoring force of the chain. As the force increases, the load on the ordered domains becomes so large that the secondary/tertiary structure of one immunoglobulin or fibronectin domain ultimately cannot tolerate the stress and unfolds. Following the unfolding event, there is a drop in the force due to the increased length of the molecule, i.e. the extra slack is partly taken up by elastic recoil throughout the chain. Further extension of the protein leads to unfolding of the next domain, and so on. This process is repeated as long as folded domains remain within the structure.

Molecular information obtained:
The separation in extension between the unfolding of the individual Ig/Fn3 domains is in the range 25 - 38 nm. The magnitudes of the force jumps are between 150 and 300 pN.

Types on information that can be obtained from such experiments:
Information concerning the existence of modular domains in the proteins and the size of these domains (length of polypeptide chain)
The stability of the modular domains (force needed to be applied inorder to induce unfolding.)

d) (credit 2/40)
The most common method used for calibration of the cantilever used in AFM:
Method based on observation of the thermally driven fluctuations of the cantilever:
The spring constant is determined by looking at the power spectrum brought on by thermal fluctuations of the cantilever using the equipartition:

$$\frac{1}{2}k\langle z^2\rangle = \frac{1}{2}k_B T$$

By measuring the thermal noise $\langle z \rangle^2$ in the position of the cantilever (by the use of a QPD), the spring constant can be derived.

e) (credit 2/40)
Q-control in AFM:

- Tapping mode operation in liquid yields an increase of the effective oscillating mass and a large increase in the damping factor due to the viscous drag.
  - The reduction of the resonance frequency is typically from 200 - 300 kHz down to tenths of kHz.
  - These changes yield a reduction in the Q-factor.

- Method to compensate for the reduction in Q: use a Q-control to increase the Q-factor to a similar value as that for the oscillation in air.

- The principle of the Q-control is to use a separate feedback loop that gives a feedback signal to the drive that is nearly in phase with the velocity term entering the differential equation of motion of the system.

Exercise 4 Multiple choice. (credit 10/40)

A The stiffness of an optical trap
□ is independent of the size of the bead size but is dependent on its material properties (i.e. refractive index).
■ depends on the bead size and shape and the laser power.
□ only depends on the laser power.

B The factor that translates detector signal Q into physical displacement x is called
■ detector sensitivity.
□ detector conversion factor.
■ β.

C In flowcytometry, the hydrodynamic focusing/centering of the particles in the excitation light / focus plane of the objective lens is obtained using:
■ a support fluid.
□ an optical lens with high NA
□ an electrostatic field

D Interlacing:
□ Interlacing is a technique used for signal readout in interline transfer CCD
■ The aim of interlacing is to provide a picture that humans observe as more stable.
□ Interlacing always occurs to a certant extent during image formation in CCD chips.

E Quantum efficiency (QE) of a CCD camera:
■ is defined as the % of the photons that form photoelectrons.
□ Is independent of the wavelength of the light.
□ Maximum in the UV range.

F Loading rate used in dynamic force spectroscopy equals:
■ Force / time
□ Force x speed
■ Spring constant (k) x speed (v_t)

G The principle of total internal reflection is used in light microscopy to:
□ control the direction of the incoming light towards an object so that one can observe using the dark field principle.
■ selectively excite and thus induce fluorescence from fluorescent molecules localised close to an interface.
□ control the direction of the light that has been reflected off a sample in order to achieve improved contrast.

H FRAP is an abbreviation used for:
■ Fluorescence recovery after photobleaching
□ Fluorescence resonance after phosphoresence
□ Fluorescence rotation after polarization
□ Fluorescence recovery after phosphoresence