Advanced Practical Course in Biophysics: FRET

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1 Introduction

Fluorescence spectroscopy and fluorescence microscopy are essential tools in biology. Biological molecules can be labelled with fluorescent molecules and thus, their localization and dynamics can be studied. One challenging task therewith is the measurement of distances on the order of nanometers – i.e., below the optical resolution of visible light; here, FRET comes into play. FRET, Förster Resonance Energy Transfer, is the process in which non-radiative energy is transferred between two molecules with inducible dipole moments. Energy is transferred from a fluorescence donor to an acceptor through dipole-dipole interactions. Since the efficiency of the energy transfer is a well-defined function of the distance (between donor and acceptor fluorophores), relative distances in the range of 1-10 nanometer can be characterized. FRET can, thus, be thought of as a "spectroscopic ruler".

2 Theoretical Background

2.1 Fluorescence Spectroscopy

Fluorescence spectroscopy analyzes the fluorescence light emitted from a sample of interest. Before considering fluorescence emission, it is useful to consider the absorption of light by a solute in solution. Many substances, such as multi-ring organic molecules, contain π-electron systems that absorb light in the visible range. This absorption process corresponds to the transition of the molecule from its ground electronic state to an excited electronic state. Vibrational states (with smaller energy differences) are superimposed onto the electronic states, and rotational states (with yet smaller energy differences) are superimposed thereon.

One can consider these vibrational levels to be quantum oscillators with corresponding potential energies of the molecule, as shown in Fig. 1. When light of the fitting frequency is absorbed, the molecule can be excited from the ground state, S_0, to any of the rotational-vibrational levels in the first excited state, S_1. Electronic transitions occur so quickly, in 10^{-16} – 10^{-15} s, that the nucleus does not have time to react, as the nucleus is much heavier than the electrons. After the electronic transition, the nucleus finds itself in a new Coulomb potential and must change its vibrational state (Franck-Condon Principle).

Fluorescence lifetimes are long (10^{-9} – 10^{-6} s) compared to vibrational relaxation times, and therefore, emission of a fluorescence photon is most likely to occur from a vibrational ground state.

Next, consider the emission of fluorescence photons from an excited molecule. A molecule in an excited electronic state, S_1, with energy E_1 may drop to a lower energy state, S_0, with energy E_0 emitting a photon of energy $E_1 - E_0$. If the process is spontaneous, the spontaneous transition rate between the state S_1 and the state S_0, which is called A_{10} , is

$$A_{10} = \frac{32\pi\nu^3}{3c^{-3}\hbar} \cdot |\langle\psi_1|\hat{\mu}|\psi_0\rangle|^2, \tag{1}$$

where μ is the transition dipole moment. Without perturbation, the rate of de-excitation from the state S_1 is

$$\frac{dn_1}{dt} = -A_{10}n_1. \tag{2}$$

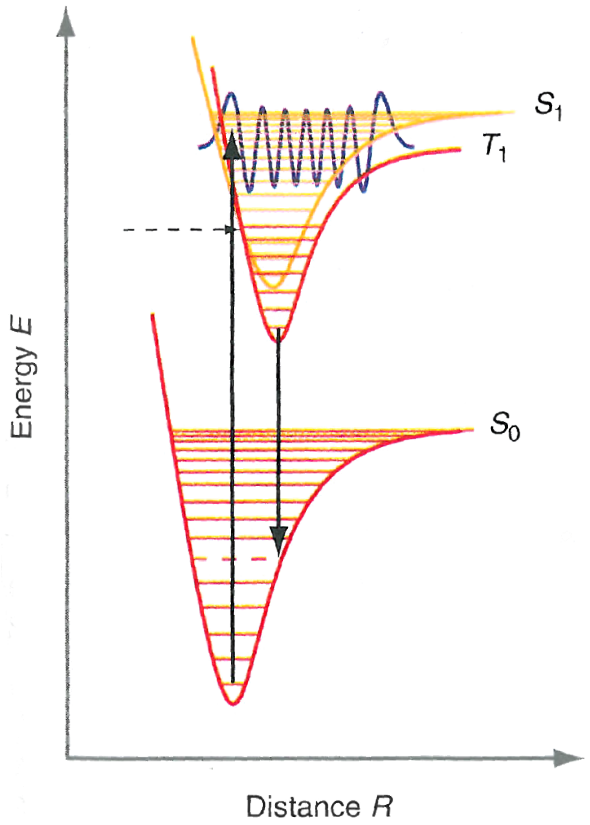

Figure 1: Franck-Condon principle. A photon with an energy corresponding to the energy difference between two electronic states is absorbed and the system transitions to an excited electronic state S_1. Vibrational levels are superimposed onto electronic levels. Initial excitation to S_1 occurs to a vibration state of maximum overlap (blue vibrational wave function). There is a finite probability of transitioning to a triplet state (T_1) if the geometry and vibrational levels in T_1 and S_1 coincide (dashed arrow). Image taken from [1].

Solving Eqn. 2, one finds

$$n_1(t) = n_1(0) \cdot e^{-A_{10}t}. \tag{3}$$

Thus, the radiative lifetime of state S_1 can be defined as

$$\tau_r = \frac{1}{A_{10}}. \tag{4}$$

Photons with an energy $E_1 - E_0$ are absorbed (Fig. 2) and the system is excited from the S_0 to the S_1 state. Due to the Franck-Condon principle, the system is in an excited electronic and in an excited vibrational state, initially. As the lifetime of the vibrational state is shorter than that of the electronic state, vibrational relaxation occurs prior to

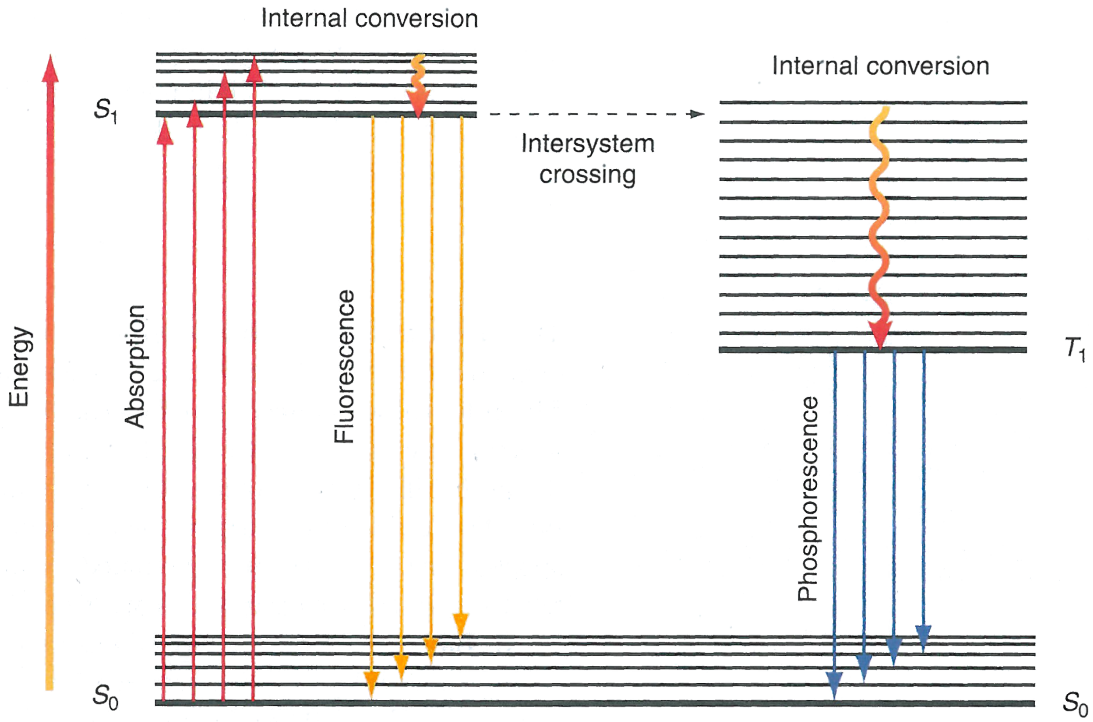

Figure 2: Jablonski scheme. The electronic ground state S_0 is shown, as well as the excited electronic state (singlet) S_1 and the excited electronic state (triplet) T_1. Image taken from [1].

the electronic transition to the ground state. Thus, the energy of the emitted photon is lower than that of the excitation photon (Stokes shift). The transition back to the ground state S_0 through emission of a photon is called fluorescence. As a result of the Stokes shift, a spectrum is obtaind that is shifted to higher wavelengths. Instead of several thin bands, corresponding to various intersystem crossings and external conversions, a spectral envelope is observed. This is due to the so-called broadening effect.

By coupling with other molecules in solution, the system can transition from a singlet state, S_1, to a triplet state, T_1; this process is called intersystem crossing. The transition from the triplet state, T_1, back to the ground state, S_0, is a forbidden transition involving a spin flip; this photon emission process is called phosphorescence. The lifetime of the T_1 state (10^{-8} to 10^{-3} s) is longer than that of the S_1 state (10^{-9} to 10^{-7} s). As the triplet state is very chemically reactive, fluorescent dye molecules are likely to undergo an irreversible chemical reaction after prolonged fluorescing light-exposure. This reaction permanently renders the dye molecules non-fluorescent and is crucial in fluorescence recovery after photobleaching (FRAP).

Non-radiative excitation energy can also be released by collision with solvent molecules or through internal vibrational modes. These processes are called internal or external conversion.

2.2 Förster Resonance Energy Transfer (FRET)

If two dye molecules are in close proximity, the dipoles of both molecules interact such that energy can be transferred from the excited molecule (donor) to the molecule in the ground state (acceptor) without radiation. If the donor's emission energy coincides with the acceptor's absorption energy, very weak coupling can permit the following resonance to take place

$$\hat{\mathrm{D}} + \mathrm{A} \xrightarrow{\mathrm{w}} \hat{\mathrm{A}} + \mathrm{D}, \tag{5}$$

where the hat denotes that the molecule is in an excited state and w is the rate of energy transfer. In practice, the donor has a somewhat higher excitation energy than that of the acceptor, and therefore the energy transfer is practically irreversible due to the Stokes shift.

The rate of energy transfer is described by Fermi's golden rule with

$$\mathrm{w} \propto |\langle \psi_i | \hat{\mathrm{V}} | \psi_f \rangle|^2, \tag{6}$$

where $|\psi_i\rangle$ is the wave functions of the initial state (donor in an excited electronic state and acceptor in the ground electronic state) and $|\psi_f\rangle$ is the wave function of the final state (donor in the ground electronic state and acceptor in the excited electronic state). This is written mathematically as

$$|\psi_i\rangle = |\hat{\mathrm{D}}\mathrm{A}\rangle \tag{7}$$

$$|\psi_f\rangle = |\mathrm{D}\hat{\mathrm{A}}\rangle. \tag{8}$$

The potential V of two coupled dipoles is given by

$$\hat{\mathrm{V}} = \frac{3}{\mathrm{r}^5}(\hat{\mu}_\mathrm{A} \cdot \vec{\mathrm{r}})(\hat{\mu}_\mathrm{D} \cdot \vec{\mathrm{r}}) - \frac{\hat{\mu}_\mathrm{A}\hat{\mu}_\mathrm{D}}{\mathrm{r}^3}, \tag{9}$$

where $\vec{\mathrm{r}}$ is the distance between the donor and acceptor, and $\hat{\mu}_\mathrm{A}$ and $\hat{\mu}_\mathrm{D}$ are the dipole moment operators. Factoring out the orientational contribution of the dipole moments,

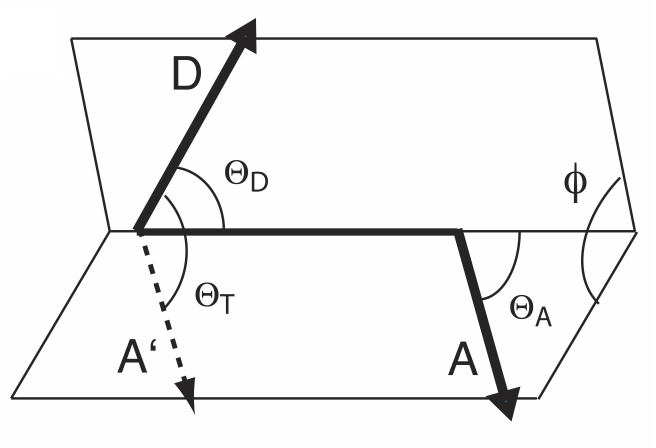

Figure 3: Orientation factor κ. Transition dipole vectors for a donor $\vec{D}$ and acceptor $\vec{A}$ are shown. $\vec{A}'$ is the in-plane transtation of $\vec{A}$. κ, as given in Eqn. 11, can take on values between 0 and 4. If isotropic reorientation occurs in a time much shorter than the donor excited state lifetime, $\kappa^2 = \frac{2}{3}$. Image taken from [2].

one ends up with

$$\mathrm{w} \propto \left| \left(\frac{\kappa}{\mathrm{r}^3} \right) \langle \psi_i | \hat{\mu}_\mathrm{A} \hat{\mu}_\mathrm{D} | \psi_f \rangle \right|^2, \tag{10}$$

where κ can be written in terms of the relative orientation between the dipole moment of the donor and acceptor molecules (Fig. 3),

$$\kappa^2 = (2 \cos\theta_D \cos\theta_A - \sin\theta_D \sin\theta_A \cos\varphi). \tag{11}$$

Because both $\hat{\mu}_\mathrm{A}$ and $\hat{\mu}_\mathrm{D}$ depend only on the coordinates of the acceptor and the donor group respectively, Eqn. 10 can be factorized to

$$\mathrm{w} \propto \left(\frac{\kappa^2}{\mathrm{r}^6} \right) |\langle D | \hat{\mu}_\mathrm{D} | \hat{D} \rangle|^2 |\langle \hat{A} | \hat{\mu}_\mathrm{A} | A \rangle|^2. \tag{12}$$

The matrix elements can be written as integrals over the absorption spectrum using the following expression

$$|\langle \hat{A} | \hat{\mu}_\mathrm{A} | A \rangle|^2 \propto \int (\varepsilon / \nu) d\nu, \tag{13}$$

where ε corresponds to the molar extinction coefficient, a proportionality constant between the fraction of light absorbed and the number of absorbing molecules.

Considering that the transition takes place at only one frequency, ν, Eqn. 13 can be

written as

$$|\langle \hat{A}|\hat{\mu}_{\mathrm{A}}|A\rangle|^2 \propto \varepsilon_A \nu^{-1}. \tag{14}$$

Using Eqn. 1 and 4, and considering that the donor undergoes an absorption process, the donor term can be rewritten as

$$|\langle D|\hat{\mu}_{\mathrm{D}}|\hat{D}\rangle|^2 \propto \nu^{-3}\mathrm{A}_{10} = \nu^{-3}\tau_r^{-1}. \tag{15}$$

Next, defining the quantum yield of the donor (the fraction of excited singlets that decay by fluorescence) as a new quantity

$$\varphi_D = \frac{\tau_D}{\tau_r}, \tag{16}$$

where τ_D is the fluorescence decay time of the donor in the absence of the acceptor, Eqn. 15 can be rewritten as follows

$$|\langle D|\hat{\mu}_{\mathrm{D}}|\hat{D}\rangle|^2 \propto \nu^{-3}\ \frac{\varphi_D}{\tau_D}. \tag{17}$$

Combining Eqn. 12, 14 and 17, the energy transfer rate can be written as

$$\mathrm{w} \propto \left(\frac{\kappa^2}{\mathrm{r}^6}\right)\frac{\varphi_D}{\tau_D}\varepsilon_A\ \nu^{-4} \tag{18}$$

If the absorption occurs over a range of frequencies, the extinction coefficient will be a function of the frequency $\varepsilon_A(\nu)$, dependent on donor fluorescence intensity. Defining $\mathrm{f}_D(\nu)$ as the fraction of donor fluorescence at frequency ν, the transfer rate becomes

$$\mathrm{w} \propto \left(\frac{\kappa^2}{\mathrm{r}^6}\right)\frac{\varphi_D}{\tau_D}\int \varepsilon_A\ (\nu)\nu^{-4}\ \mathrm{f}_D(\nu)d\nu = \left(\frac{\kappa^2}{\mathrm{r}^6}\right)\frac{\varphi_D}{\tau_D}J(\nu). \tag{19}$$

Introducing the Förster radius, R_0, and taking into account all the numerical constants, the previous equation becomes

$$\mathrm{w} = \frac{1}{\tau_D}\left(\frac{\mathrm{R}_0}{r}\right)^6, \tag{20}$$

with

$$\mathrm{R}_0 = \left(8.8\cdot 10^{-25} J n^{-4}\kappa^2\varphi_D\right)^{\frac{1}{6}}\ \mathrm{cm}, \tag{21}$$

where n is the refractive index of the solution, κ^2 is the orientation factor, φ_D is the donor's quantum yield and J is the overlap integral of the donor and acceptor

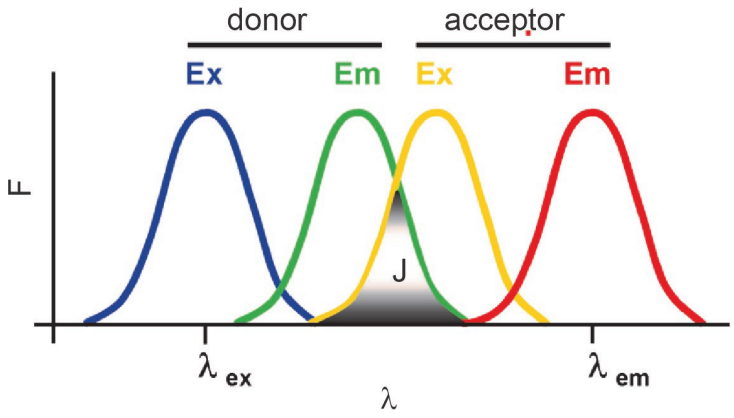

Figure 4: The overlap integral J is a measure of how well the emission spectrum of the donor and the excitation spectrum of the acceptor overlap, here highlighted in grey. The efficiency of FRET depends on this quantity.

spectra (Fig. 4). If the dye molecules are free to rotate around their points of attachment, the orientation factor κ^2 assumes its isotropic value ($\bar{\kappa^2} = \frac{2}{3}$). This assumption will be used throughout this experiment. Typical values for Förster radii are in the range of 1 - 6 nm. The overlap integral J is defined as

$$J = \int \varepsilon_A(\lambda)\lambda^4 f_D(\lambda) d\lambda, \tag{22}$$

where $f_D(\lambda)$ is the normalized donor intensity per wavelength, expressed in cm^{-1}, giving J units of $\mathrm{cm}^3\mathrm{M}^{-1}$.

If the donor is excited in the absence of the acceptor, the intensity of the fluorescence emission spectrum will be highest. If though the acceptor is present as the donor is being excited, an energy transfer takes place between the molecules. The fluorescence intensity at the donor's optimal emission wavelength will decrease and emission at the acceptor's emission-wavelength maximum will occur. The efficiency E of the energy transfer is defined as the fraction of excited donor molecules $\hat{\mathrm{D}}$ that are de-excited by energy transfer to the acceptor. This quantity can be determined as

$$\mathrm{E} = 1 - \frac{F_{D,A}}{F_D}, \tag{23}$$

where F_D and $F_{D,A}$ is the intensity maximum in the emission spectrum of the donor in the absence and presence of the acceptor, respectively. The efficiency is connected to

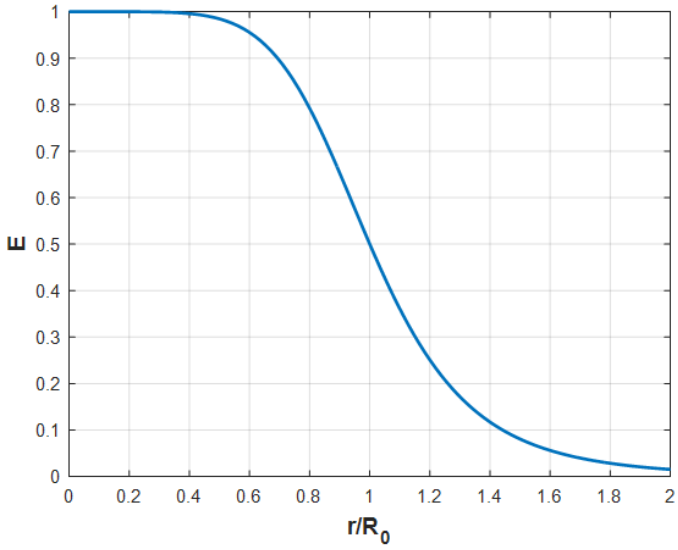

Figure 5: The FRET efficiency Edepends on the distance r between donor and acceptor molecule. At a distance equal to the Förster radius R_0, E is decreased to half of its maximum value.

the Förster radius and the distance r between the donor and acceptor molecules through

$$E = \frac{1}{1 + (\frac{r}{R_0})^6} \ . \tag{24}$$

The efficiency of the resonance energy transfer decays as the inverse of the distance r between donor and acceptor molecule to the power of six. This makes FRET very sensitive to distance, but only near R_0, where a small change in r leads to a large change in E.

3 The Experiment's Layout

This experiment is divided into two parts that both illustrate the general applications of FRET. FRET has a wide variety of applications including characterization of: the structure and conformation of proteins, receptor/ligand interactions, the distribution and transport of lipids, and the spatial distribution of proteins. Here we will focus on two applications using DNA. In the first experiment, FRET will be used as a molecular ruler to measure the length of short DNA molecules. In the second part, we will apply a method known as "molecular beacons" to determine whether a long DNA molecule contains a specific sequence of interest.

Figure 6: Double-stranded DNA. The distance between base pairs in DNA corresponds to 0.34 nm. The notation 5' to 3' at the end of single DNA strands is the convention used to indicate the directionality of the single strands. ssDNA is read from 5' to 3'.

3.1 Molecular Rulers

One important application of FRET is the measurement of distances in the range of 1-10 nm. A very exact biological ruler is DNA, where the distance between two base pairs is known to be 3.4 Å(Fig. 6). DNA forms a double-helix in which both strands run in an anti-parallel fashion. Each strand of DNA has a specific sequence of bases – a string of the letters C, G, T, and A. Base-pairing between two single-stranded DNA (ssDNA) molecules occurs only if the bases of both strands are complementary – i.e., cytosine (C) is complementary to guanine (G) and thymine (T) is complementary to adenine (A). If two ssDNA molecules which are complementary to each other are mixed in solution, they form double-stranded DNA (dsDNA).

To measure the length of a DNA molecule, two ssDNA molecules will be used. Both of the ssDNA molecules have a fluorophore attached at their 5'-end. In this experiment, two sample sets will be provided. Each set will contain three samples (Fig. 7): ssDNA with only the donor fluorophore, ssDNA with only the acceptor fluorophore and an annealed mixture of the two. The two fluorophores that will be used are Cy3 and Cy5. The abbreviation Cy stands for cyanine, a fluorescent dye that covers the light spectrum from IR to UV. In the case of Cy3, the dye fluoresces yellow-green, while Cy5 fluoresces red.

Using a fluorescence spectrometer, you will acquire the excitation and emission spectra of both the donor and acceptor. Afterwards, an emission spectrum of the annealed mixture should be taken. As the two ssDNA molecules are complementary, they will form dsDNA when mixed. The resulting dsDNA has the donor at one end and the acceptor at the other (Fig. 7). The FRET efficiency E can be measured by comparing

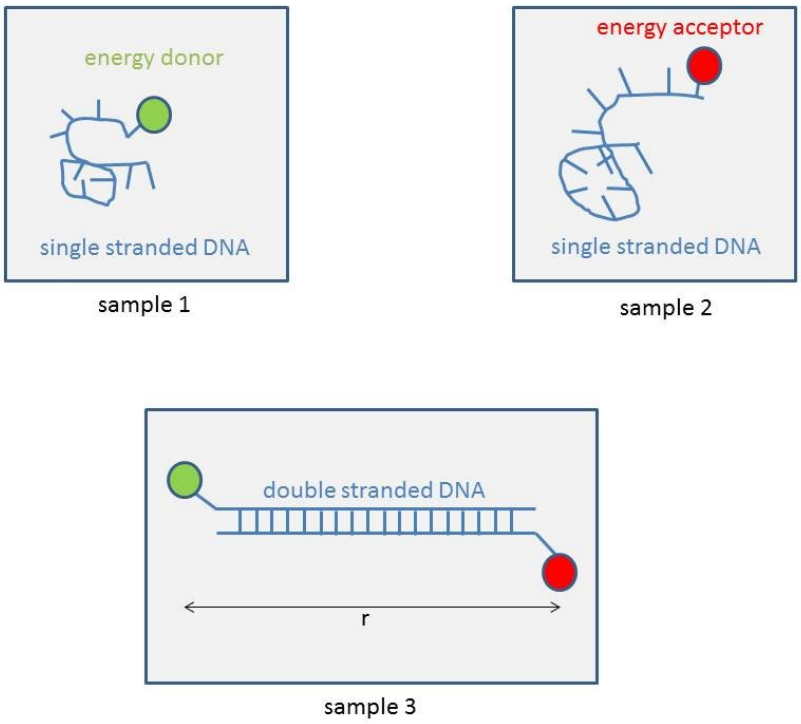

Figure 7: Experimental setup for measuring the length of a dsDNA molecule. In sample 1, ssDNA has the fluorescence donor attached to its 5' end. In sample 2, the strand complementary to sample 1 has an acceptor attached to its 5' end. Both samples are mixed in sample 3 and form dsDNA. The efficiency of FRET depends on the distance r between donor and acceptor and thus on the length of the DNA molecule.

the emission spectra of the donor in the presence and absence of the acceptor. The overlap integral, J, can be determined from the spectral overlap of the donor-emission and acceptor-excitation spectrum. With J in hand, the Förster radius, R_0, can be estimated. Using E and R_0 you will be able to determine the length of the dsDNA molecule.

3.2 Molecular Beacons

FRET is used with molecular beacons to detect specific DNA sequences (henceforth called a sequence of interest). Suppose one has isolated DNA from an organism and wants to know whether the DNA in question (henceforth called sample) contains a specific sequence A, a stem loop structure can be designed whose loop contains the complementary sequence to the sequence of interest. Stem loop structures are ssDNA molecules with lengths of at least 25 to 35 nucleotides. The stem sequences are located

at the 5' and 3' end of the ssDNA molecule, are 5-8 nucleotides long and are designed to be complementary to each other. This allows the ends to bind and the loop the form. In the center of the stem loop structure ,there is a sequence which is 15-30 nucleotides long and complementary to the sequence of interest. A key principle behind the use of stem loop structures is that the length of complementary sequence in the loop is much longer than that of the complementary stem region. In the absence of the sequence of interest, the stem loop structure is thermodynamically favored. Upon addition of a sample with the sequence of interest, double strand formation between the molecuar beacon and the sample is favored, breaking the double strand formation at the stem. This means that the state of the beacon is dependent on the presence of the sequence of interest. At the

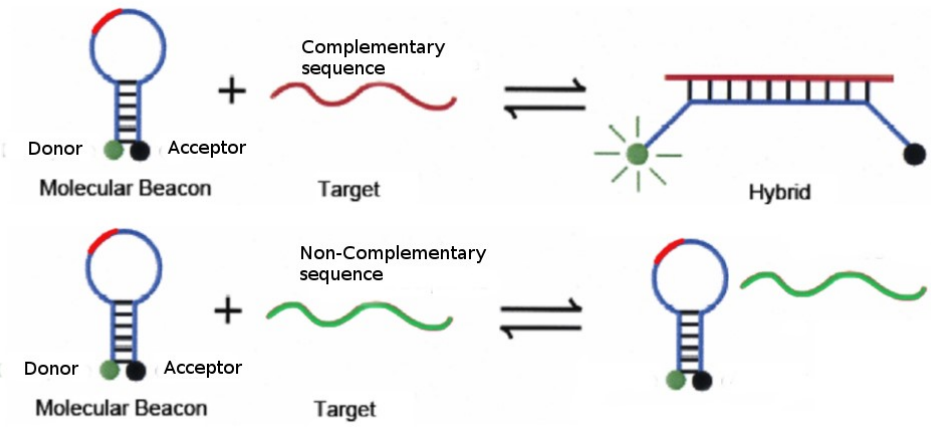

Figure 8: With the molecular beacon, if the sequence of interest (target) introduced is complementary to the loop portion of the molecular beacon (red line), the two will combine and the stem of the molecular beacon will unzip. As the donor and acceptor are separated, the hybrid will emit a fluorescence signal (upper scenario). If the target is not complementary, the two will not combine and therefore not unzip the stem of the molecular beacon. The acceptor will quench the donor and no fluorescence signal will be emitted (lower scenario).

5' and 3' ends of the molecular beacon, an energy donor and an energy acceptor are attached. With molecular beacons, the readout is usually the fluorescence intensity of the donor. In the absence of a sample with DNA complementary to the sequence of interest, the donor fluorescence is quenched. With the introduction of a sample with DNA complementary to the sequence of interest, the loop portion of the molecular beacon will bind to the complementary DNA strand. A rigid double stranded structure forms which causes the stem structure to unbind, thereby separating the donor and acceptor. The donor is no longer quenched and emits a strong fluorescence signal (Fig. 8).

Figure 9: Molecular beacon used in this experiment. The two fluorophores correspond to the donor, Texas Red, at the 5' end and the acceptor, BHQ2, at the 3' end. The first five base pairs are complementary to the last five allowing them to bind together to form the stem portion of the stem-loop structure.

The molecular beacon used in this experiment consists of a fragment with 46 bases where the first five bases are complementary to the last five bases creating the stem structure (Fig. 9). A red fluorescent dye called Texas Red is located at the 5' end, and an acceptor called BHQ2 is located at the 3' end. The difference between this acceptor and the one used in the previous section is that the energy absorbed by this acceptor is released as heat. These types of acceptors are called quenchers, because no fluorescence is seen if they are close enough to the donor.

4 Experimental set-up

Measurements will be carried out on a PerkinElmer LS 55 Fluorescence spectrometer. This fluorescence spectrometer uses a Xenon lamp and measures excitation and emission spectra between 200 - 800 nm. The light is focused by a toroidal mirror onto a monochromator. The rotation of this monochromator allows the frequency (wavelength) of the excitation wavelength to be selected or a spectrum of wavelengths to be scanned. Between the diffraction grating and the sample there is a beam splitter that reflects a portion of the incident light onto the reference photocell, providing an output that is proportional to the intensity of the incident light. Before and after the sample, filters can be inserted which allow one to polarize or attenuate incoming or outgoing light.
The fluorescence light emitted from the sample is detected under an angle of 90° to the incident light beam, to reduce the amount of excitation light that reaches the detector. The fluorescence light is focused onto another grating that selects a specific fluorescence wavelength, or scans the fluorescence light, to provide an emission spectrum (Fig. 10).

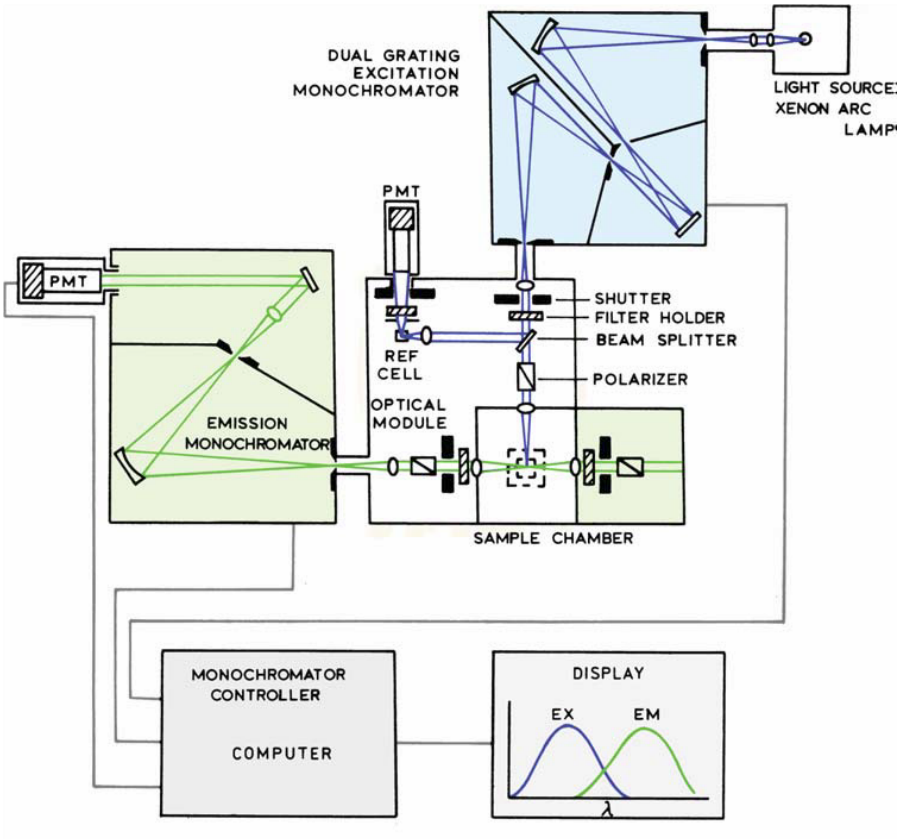

Figure 10: Sketch of a fluorescence spectrometer. The excitation light passes through a monochromator, filters, and then excites the sample. To reduce the amount of excitation light passing directly through the sample to the detector, the emission light is detected perpendicular to the direction of the excitation light. A second monochromator is located in front of the photomultiplier tube (PMT) to select out emitted light wavelengths. Image taken from [3].

5 Experimental procedure and analysis

In this section, both experiments will be introduced in more detail. All experimental steps will be explained, as well as the tasks and calculations that are done in the later analysis.
Come prepared with reference emission and excitation spectra for Cy3 and Cy5, as well as TexasRed, to know over which wavelengths to scan.

5.1 Molecuar Ruler - measuring the length of DNA molecules

In this part, you will receive 7 samples in total: 2 sets of 3 each, plus one additional reference sample containing TRIS-HCl only with which the background measurement is done. Each set contains a specific DNA strand with a) ssDNA strand with Cy5 added, b) the reverse complementary ssDNA strand with Cy3 added, c) both strands mixed and annealed such that they form a dsDNA strand with Cy3 on the one and Cy5 on the other end.

1. Pipette 100 μl of a Cy3 sample into the glass cuvette. Familiarize yourself with the slit widths, filters, and photomultiplier tube (PMT). Use fitting excitation/emission wavelengths!
    - Record spectra with different settings.
    - Keep the best settings and double check with the assistant.
2. Keeping the above settings, obtain the following spectra for both sample sets:
    - For the samples with either Cy3/Cy5: record the excitation and emission spectra of the fluorophores
    - For the mixed samples: record the emission spectrum over a wider range so to see the FRET-specific emission at the acceptors maximum emission wavelength.
    - !! Be sure to use the same PMT voltage when taking the Cy3 emission scan and the Cy3/Cy5 mixture emission scan. !!
3. Obtain background spectra using TRIS-HCl for all used settings.

Step 2 is repeated for both sets of samples.

Analysis Before starting, subtract the background from all spectra.

1. Visualize the spectra and characterize what you see
   - Plot the spectra of the different voltage/slit width settings and discuss the effect on the intensity.
   - Plot the spectra for the samples containing only one fluorophore together in one plot, roughly similar to what is shown in Fig. 4. As spectra will have different intensities, normalize them for better visualization.
   - Plot for each of the two sets the emission spectra of the donor with and without the acceptor present. How can you see from the plots that FRET took place?

2. Calculate the length of the DNA strands
   - Determine the overlap integral J (compare Fig 4) using Eqn. 22 for both datasets. The integral is transformed to a sum of the form

$$J = \sum_{\lambda=\lambda_a}^{\lambda_e} \varepsilon_A(\lambda)\lambda^4 f_D(\lambda) \cdot \Delta\lambda. \tag{25}$$

     Here, $\varepsilon_A(\lambda)$ is the acceptor's molar extinction coefficient at every wavelength. We obtain this by normalizing the excitation spectrum of our acceptor to the literature value at the maximum wavelength $\varepsilon_A(\lambda_{\max})$: First, normalize the excitation spectrum of the acceptor with respect to the highest value, then multiply by $\varepsilon_A(\lambda_{\max})$.
     $f_D(\lambda)$ is the normalized emission spectrum of the donor in units of cm^{-1} and $\Delta\lambda$ is the step size in the spectral data. (Pay attention to the units, J should be in units of cm^3/M.)
   - Determine the Förster radius R_0 for both datasets using Eqn. 21, where n is the refractive index of water, and φ_D is the donor's quantum yield. Make sure to insert J in the correct unit.
   - Determine the FRET efficiency E using Eqn. 23 for both datasets. Therefore, you need the peaks of the donor's emission spectrum with and without the acceptor present. (Estimate the error you are introducing by reading off the maximum. Perform a error calculation.)

- With all the results from above and Eqn. 24, you can now calculate the distance r and the number of nucleotides making up the strands.
- Consider the error of the obtained quantities.

3. Discuss: Compare J and R_0 to the literature values. Which measurement was more reliable? Are the distances r realistic?

5.2 Molecular beacon - detecting a sequence of interest

You will receive 5 samples of different unknown identity. The assistant will specify which combinations you are to expect, some w/wo molecular beacon, some w/wo sequence of interest and a blank TRIS-HCl reference background sample.

1. For the control Molecular Beacons sample, obtain an emission scan to determine an appropriate excitation wavelength for our molecular beacon.
2. After selecting the excitation wavelength, obtain an emission spectrum for all unknown samples.

Analysis

1. Plot the spectra of the scan, highlight the one corresponding to your wavelength of choice and discuss. What is the problem with higher/ lower wavelengths?
2. Plot the unknown sample emission spectra in one plot.
3. Identify the samples. Discuss.

The following publication will be relevant for the oral exam: [4]

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