Lab 1: Ensemble Fluorescence Basics

(Last Edit: Feb 18, 2016)

This laboratory module is divided into two sections. The first one is on organic fluorophores, and the second one is on ensemble measurement of FRET (Fluorescence Resonance Energy Transfer)

I. Organic Fluorophores[1]

In this lab you will be measuring the absorption, emission, and lifetime of cyanine dyes (Cy3, Cy5 and Cy5.5). Once you are familiar with the instruments, you will measure the absorption, emission and lifetime of three unknown samples to identify the fluorophore within the sample.

Objective:

1. Learn to take absorption spectra, emission spectra and lifetime measurement of fluorophores
2. Understand properties of organic fluorophores (resonance delocalization, cis-trans isomerism)
3. Be comfortable with pipetting as an essential basic for future labs

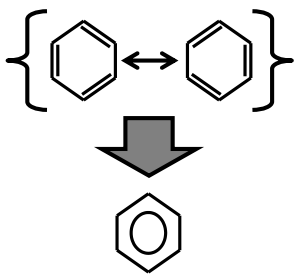
Cyanines, polyenes, and resonance delocalization

A common structure in chromophores is alternating single and double bonds (conjugated bonds), often in the form of aromatic structures. These types of structures are responsible for delocalizing the electrons over many atoms, leading to a red-shift of the electronic transitions into the optical range. This phenomenon – resonance delocalization – is the topic of our first experiment. Comparing a series of cyanine and polyene molecules, we will see that resonant structures lead to complete delocalization, while unresonant structures with conjugated bonds only have partial delocalization.

Conjugated bonds and partial delocalization

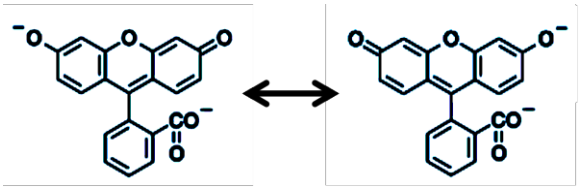
The polyenes are hydrocarbon chains with alternating single and double bonds. Recall that a double bond consists of a σ-bond localized between two atoms and a π-bond localized above and below the atoms. In conjugated structures, the neighboring π-bonds are aligned and there is some overlap that occurs. This leads to some "leakage" (i.e. partial delocalization) between neighboring double bonds.

Resonance delocalization

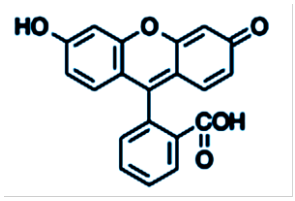


The simplest example of a resonant structure is given by the benzene molecule C_6H_6. There are two equivalent structures for benzene, as shown at the top of the picture (single and double bonds inverted). In either of these structures, as drawn, the electrons would be localized in π-bonds above and below two carbon atoms (with some "leakage" between them). However, the molecule is not actually in one state or the other. Rather, it exists in a hybrid of these states (bottom figure) in which the electrons are delocalized across all six carbon atoms equally (to imagine the corresponding molecular orbital, think of doughnuts above and below the plane of the ring).

Fluorescein and pH dependence

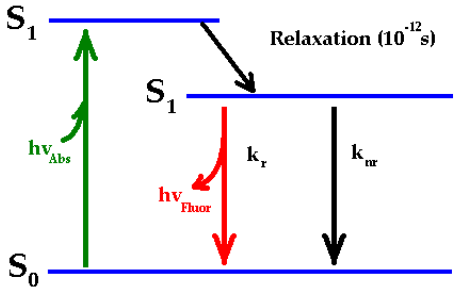


Fluorescein is a bright green fluorophore commonly used in fluorescence measurements. It has two ionizable groups, –COOH and –OH. Depending on the pH, it is found in four forms: dianion, monoanion, neutral, and cation. In its dianionic form, it is a strong absorber of visible light (ε_{490} ~90,000 L/mol-cm) and has a high fluorescence efficiency (ϕ_f > 90%). This is because its structure is highly resonant, as shown in the figure, such that the electrons are delocalized across the three rings at the top. Note that the bottom ring is not part of the delocalization because the –COOH group causes steric interference and forces the ring to rotate out of the plane of the other rings.



In its neutral form with the phenol and carboxylic groups protonated, fluorescein's emission degrades considerably and the absorption shifts toward the blue and decreases. The reason is that the molecule is no longer in complete resonance. The presence of the –OH group requires a proton to be transfered from one side to the other in order to draw equivalent resonant structures.

Fluorescence lifetime



A fluorophore excited by a photon will drop to the ground state through radiative and non-radiative decay pathways. Fluorescence lifetime is the average time a fluorophore spends in the excited state before returning to ground state. When a solution of fluorophore is excited with a pulse of light, an initial population of fluorophores (n_0) will be in the excited state. This excited state population decreases with time with a constant decay rate $k_{tot} = k_r + k_{nr}$, where k_r and k_{nr} are the radiative and non-radiative decay rate respectively. The fluorescence intensity is proportional to the excited state population, and will decay exponentially following the formula $I(t) = I_0 \exp(-t/\tau)$, where I(t) is the intensity at time t, I_0 is the initial intensity and τ is the lifetime that is the inverse of the total decay rate ($\tau = 1/k_{tot}$).

The lifetimes of organic fluorophores typically fall in the nanosecond regime. The fluorescence lifetimes of cyanine dyes are marked by large non-radiative decay rate (k_{nr} ~10x larger than k_r for Cy3) caused by cis-trans photoisomerization[2]. Excited state of cyanine dyes undergoes photoisomerization from trans to cis conformation. Once formed, the cis isomer undergoes thermal back-isomerization to the ground state. This non-radiative process reduces the lifetime and quantum yield of the dyes, and is strongly dependent on the microenvironment the dye is in. When attached to single-stranded DNA (ssDNA) or double-stranded DNA (ssDNA), cyanine dyes may show two component lifetimes indicative of multiple states arising from the DNA-dye interaction.

Experiment and Report

(1) You will each start with concentrated samples of Cy3, Cy5 and Cy5.5. Dilute each of them 400x (2 μL sample in 800 μL ddH_2O) before taking any measurement. Cy5.5 has low solubility, so be sure to shake the tube it is in well before pipetting.

(2) Measure the absorption spectra for the cyanine dyes from 450 to 750 nm *(for operating instruction and dilution protocols, see Appendix 2.1. It's important to read beforehand!)*. Record the peak absorption wavelength and the absorption in Table 1, and determine the concentration of the dyes in mol/L. To calculate concentration, use the formula A = εcL, where A is the absorption measured at the peak wavelength, ε is the extinction coefficient, c is the concentration and L is the path length. The relevant extinction coefficients at the peak wavelength are ε_{Cy3} = 150,000 L/mol-cm, ε_{Cy5} = 250,000 L/mol-cm, and $\varepsilon_{Cy5.5}$ = 250,000 L/mol-cm. The path length of the cuvette is 1 cm. If your absorption spectrum is too noisy to resolve the peak, increase the integration time (remember to take a new blank with the same integration time).

(3) ***(2 pts)*** Measure the emission spectra of the cyanine dyes *(Appendix 2.2)*. Use the values below for the emission scan and excitation wavelength. Record the peak emission wavelength in Table 1 and calculate the Stokes shift by subtracting the emission peak from the absorption peak.

	Start Scan [nm]	End Scan [nm]	Excitation [nm]	Time [s]
Cy3	540	700	510	0.2
Cy5	630	750	610	0.2
Cy5.5	670	800	650	0.2

	Abs. Max [nm]	Abs.	Em. Max [nm]	Stokes Shift [nm]	Conc. [mol/L]
Cy3					
Cy5					
Cy5.5					

Table 1. Absorption and emission peaks, stokes shifts, and concentrations of Cy3, Cy5 and Cy5.5

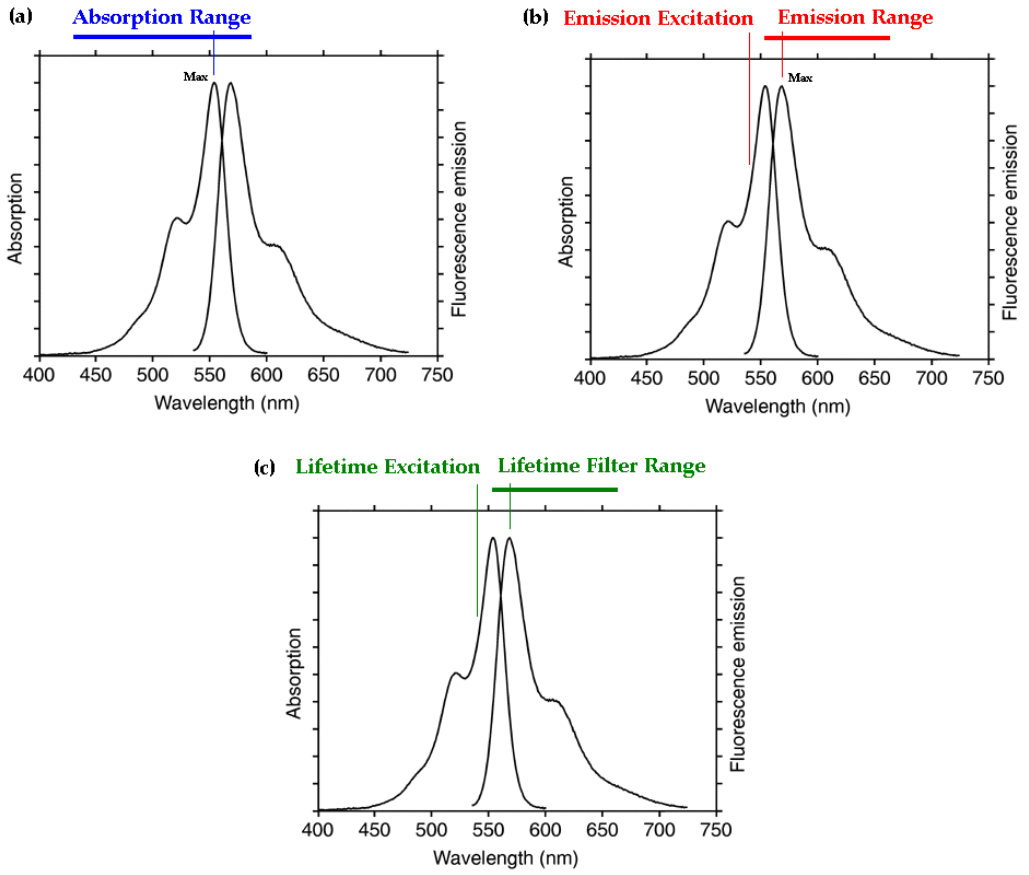
(4) ***(2 pts)*** Measure the lifetime of Cy3 attached to different substrates *(Appendix 2.3)*. Use the parameters below for each measurement. Record the lifetimes in Table 2. Each student will pick one sample and share the data with the group. Measure up to the second lifetime component.

Ref. Dye	Time Base	Frequency	Ref. Lifetime [ns]	Filter [nm]	Excitation [nm]
Erythrosin-B	1	10– 200 MHz	0.46	550 - 620	540

	τ_1 (ns)	τ_2 (ns)	Fraction$_1$	τ_{ave} (ns)
Free-Cy3				
Cy3-ssDNA				
Cy3-dsDNA				

Table 2. Lifetimes of Cy3 attached to different substrates

(5) ***(3 pts)*** Measure the absorption, emission and lifetime of one of the unknown samples given (A, B and C). Each of you will pick one unknown and share the data with the group. Together with the group, plan the parameters to be used for each measurement. First estimate the absorption max of the sample from its color (the color you see are those not absorbed by the sample). Then estimate the emission max (generally 20-30 nm greater than the absorption max). Knowing these peaks, you can estimate the parameters needed for absorption, emission and lifetime measurement. Use the figure below to help guide your decision. Summarize your plan in Table 3. Record your finding in Table 4 and use Table 5 to identify the unknown samples.



Ref. Dye	Time Base	Frequency	Ref. Lifetime [ns]	Filter [nm]	Excitation [nm]
Erythrosin-B	1	10– 200 MHz	0.46	550 - 620	540
Fluorescein	1	4 – 90 MHz	4.0	500 - 520	480

	Estimate		Absorption		Emission			Lifetime			
	Abs. Max [nm]	Em. Max [nm]	Start [nm]	End [nm]	Start [nm]	End [nm]	Excit. [nm]	Ref. Dye	Ref. τ [ns]	Filter [nm]	Excit. [nm]
A											
B											
C											

Table 3. Plan for absorption, emission and lifetime measurement of unknown samples.

	Abs. Max [nm]	Em. Max [nm]	τ (ns)	Identity
A				
B				
C				

Table 4. Results for unknown sample.

Fluorophores	Abs. Max [nm]	Em. Max [nm]	τ (ns)
Alexa Fluor 488	494	519	4.1
Bodipy FL	502	510	5.7
GFP	498	516	3.2
Acridine Orange	500	530	2.0
Rhodamine-6G	525	555	4.08
Rhodamine-B	562	583	1.68

Table 5. Absorption peak, emission peak and lifetime of a few common fluorophores

(6) ***(3 pts)*** From your experimental data, calculate the shift in absorption wavelength [nm] for the addition of the following chemical structures to a cyanine dye.

Δλ	Another double bond inserted along the chain	
Δλ	An additional pair of phenyl rings on the ends of the dye	

Annealing DNA for FRET experiment

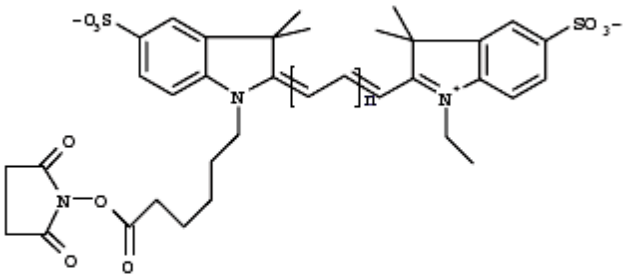
Here you will be preparing DNA samples for ensemble FRET experiment next week:

1. Mix the following single stranded DNA (ss-DNA) to make double stranded DNA. The DNAs are dissolved in annealing buffer

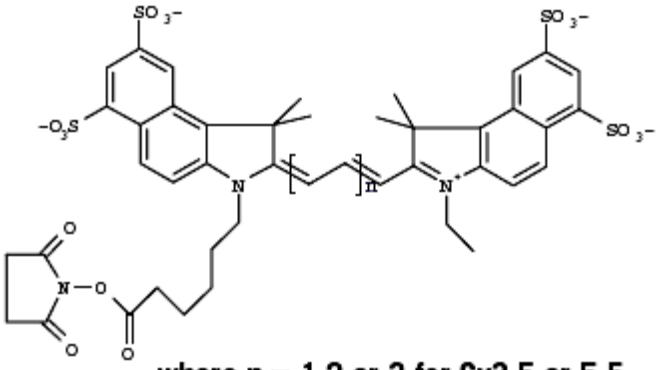
DNA	12bp-Donor	12bp-FRET	16bp-Donor	16bp-FRET
(1) Cy3-12bp-1	25 μL	25 μL		
(2) Cy5-12bp-2		35 μL		
(4) 12bp-2	35 μL			
(5) Cy3-16bp-1			25 μL	25 μL
(6) Cy5-16bp-2				35 μL
(8) 16bp-2			35 μL	

2. Using a thermal cycler, heat DNA to 95°C for 2 minutes
3. Ramp cool to 25°C over a period of 45 minutes. Keep tubes at 4°C after 45 minutes.
4. Briefly spin the tubes to draw all moistures from lid, then store at -20°C

(7) ***(4 pts)*** Identify the chemical structures in Cy3/Cy5 and Cy3.5/Cy5.5 corresponding to water solubility, chemical reactivity (bioconjugation), major shifts in absorption (strongly resonant), and minor shifts in absorption (moderately conjugated). Circle these parts in each molecule and label their identity. Hints: Water is highly polar, so what would help something dissolve in it? Delocalization of the ◎-electron system (the degree of conjugation) generally results in a red-shift of the absorption and fluorescence as well as an increased quantum yield. So identify the parts of the molecule that would cause small/large amounts of shift.



where n = 1,2 or 3 for Cy3, 5 or 7



where n = 1,2 or 3 for Cy3.5 or 5.5

(8) ***(3 pts)*** Using the experimental data in Tables 8.2 and 8.4 on the next page, draw a rough (rough, but still at least with evenly spaced ruling on the axes) sketch plotting the maximum absorption wavelength, λ^*_{abs} [nm], versus the number of π-electrons, N, in the molecule for the polyenes and cyanines with N = 4, 8, 12, and 16 (use experimental columns in Tables 8.2 and 8.4). Describe in your own words with a couple sentences the graph and how the trends relate to resonance and electron delocalization.

(9) ***(3 pts)*** Cyanine dyes have a relatively low quantum yield (~5 to 20%) compared to other fluorophores like fluorescein and rhodamines (~70 to 95%). Give an explanation of this based on what you have learned about the differences in chemical structures for these types of dyes.

Table 8.2 Symmetrical cyanine cations $(CH_3)_2\overset{\oplus}{N}{=}CH-(CH{=}CH)_k-\ddot{N}(CH_3)_2$ with $k = 0$ to $k = 6$ ($N = 4$ to $N = 16\,\pi$ electrons). Calculated and experimental absorption maxima λ_{max} and oscillator strengths f (see Section 8.3)

k	N	λ_{max}/nm		Color of solution	f	
		Calculated	Experiment		Calculated	Experiment
0	4	206	224	Colorless	0.7	
1	6	332	313	Colorless	1.0	0.9
2	8	459	416	Yellow	1.3	1.0
3	10	587	519	Red	1.6	1.2
4	12	716	625	Blue	1.7	1.5
5	14	844	735	Green	2.2	2.0
6	16	973	848	Colorless	2.4	

The color of the solution of the dye is complementary to the color of the absorbed light; e.g., if red light is absorbed the color of the solution is green. The absorption maxima are calculated according to equation (8.21), the oscillator strengths according to equation (8.29) with $\hat{n} = 1.42$ (index of refraction of the solvent dichloromethane). References see Further Reading

Table 8.4 Absorption maxima λ_{max} of polyenes with $N\,\pi$ electrons ($N/2$ Double Bonds)

Polyene	N	$\Delta E/10^{-19}$ J	$\Delta E'/10^{-19}$ J	λ_{max}/nm	
				Calculated	Experiment
Ethene	2	10.4	13.4	150	162
Butadiene	4	6.2	9.2	220	217
Hexatriene	6	4.5	7.5	270	257
Octatetraene	8	3.5	6.5	310	290
	10	2.8	5.8	340	317
	12	2.4	5.4	370	344
	14	2.1	5.1	390	368
	16	1.8	4.8	410	386
	18	1.6	4.6	430	413
	20	1.5	4.5	440	420
(β-Carotene)	22	1.4	4.4	450	453
	24	1.2	4.2	470	461
	26	1.2	4.2	470	471
	28	1.1	4.1	480	500
	30	1.0	4.0	500	504
Polyacetylene	∞	0	3.0	660	650

ΔE is the excitation energy of a corresponding π electron system with equally long bonds according to equation (8.36) with $d_0 = 140\,\text{pm}$, $\Delta E'$ is the excitation energy with correction for bond alternation according to equation (8.35). References see Further Reading.

(10) ***(3 pts)*** For the lifetime experiment, is the lifetime of Cy3 the same when conjugated to different substrates? What are the possible factors that cause these differences? You can refer to the reference: "*Fluorescence Properties and Photophysics of the Sulfoindocyanine Cy3 Linked Covalently to DNA*" for helpful hints.

References:

[1] Materials are partially adapted from PHYS 552 class by Prof. Robert Clegg

[2] Sanborn, M. E., Connolly, B. K., Gurunathan, K., Levitus, M.. Fluorescence Properties and Photophysics of the Sulfoindocyanine Cy3 Linked Covalently to DNA. J. Phys. Chem 2007

Part 2: Ensemble FRET

Introduction:

In this lab, you will be doing FRET measurements using both steady-state and time-resolved methods. You will measure the FRET efficiency and calculate the distance of two DNA samples with different lengths (12 base-pair and 16 base-pair), and analyze the FRET efficiency using the sensitized emission and lifetime method.

Background:

A. Samples:

For the lifetime experiments, we will be using 12 and 16 base-pair oligo DNA from Integrated DNA Technologies. One strand is labeled with the donor fluorophore Cy3, the other by the acceptor fluorophore Cy5. For this lab, we will use singly-labeled and hybridized versions to estimate the FRET efficiency.

12 base-pair oligo:	16 base-pair oligo:
/Cy3/CCA CTG GCT AGG	/Cy3/CCA CTG CAC TGC TAG G
+	+
/Cy5/CCT AGC CAG TGG	/Cy5 /CCT AGC AGT GCA GTG G

B. Sensitized Emission Method

There are two ways to determine the FRET efficiency using sensitized emission. One is through direct method, and the second way is the $(ratio)_A$ method. We can understand them from the figure below:

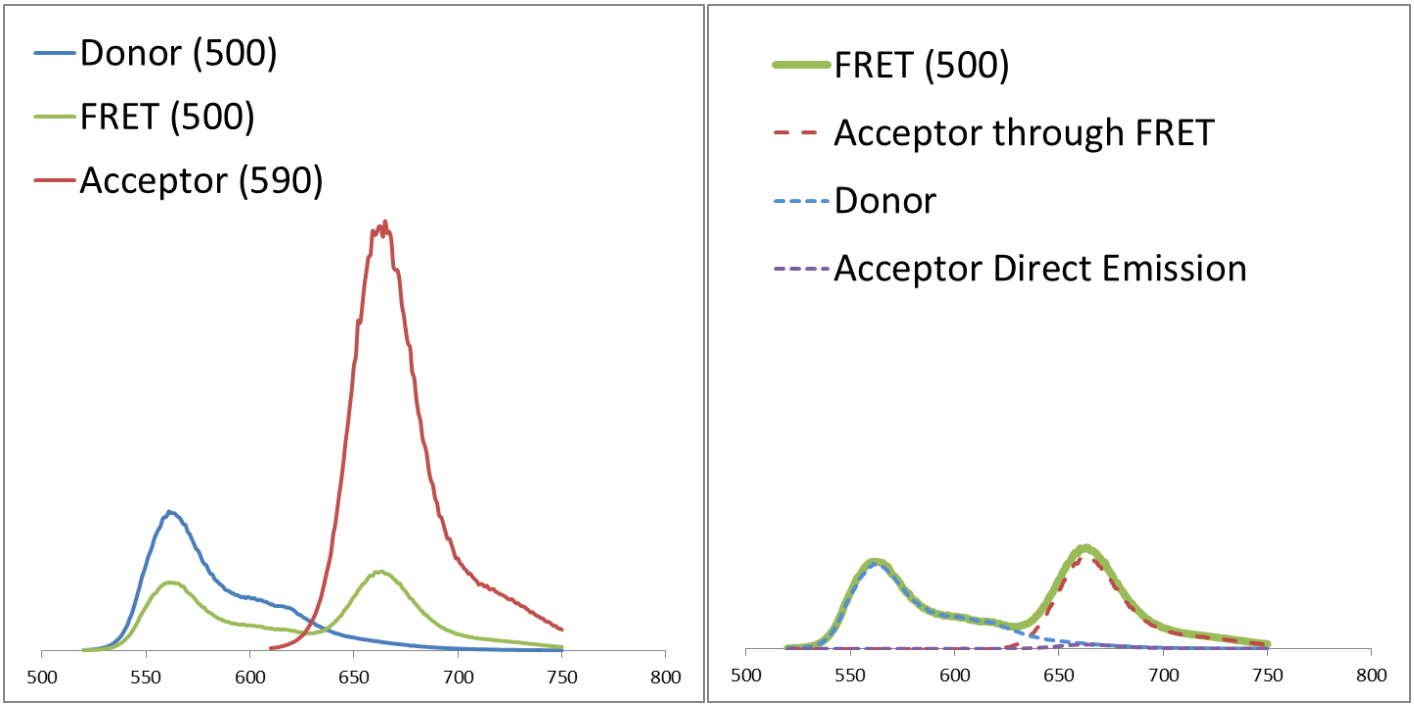


Figure 1a (left): Donor, FRET and acceptor spectra excited at 500 nm or 590 nm excitation source.
Figure 1b (right): The FRET spectra is composed of the donor quenched emission (blue), acceptor sensitized emission through FRET (red), and acceptor direct emission (purple)

Direct Method:

With the direct method to calculate FRET efficiency, we compare the area under the red and blue graph in Figure 1b. The FRET efficiency is simply the acceptor sensitized emission through FRET (red graph) divided by the total FRET emission (donor + acceptor through FRET, or red + blue graphs):

$$E_{FRET} = \frac{I_{AD}}{I_{DA} + I_{AD}}$$

where I_{AD} is the acceptor emission through FRET and I_{DA} is the donor quenched emission due to FRET. The equation above assumes that the quantum yield of the donor and acceptor are the same. If the quantum yields are different, as is the case for Cy3 and Cy5, we simply account for the different quantum yield and use the equation below:

$$E_{FRET} = \frac{I_{AD}/q_A}{I_{DA}/q_D + I_{AD}/q_A}$$

where q_A and q_D are the quantum yields of the acceptor and donor respectively.

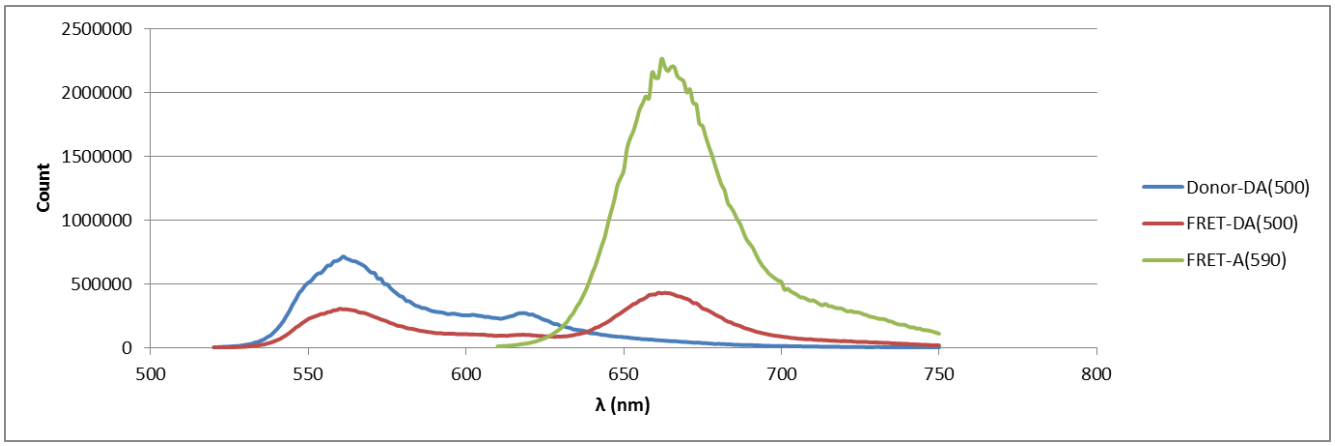
$(ratio)_A$ Method:

In the $(ratio)_A$ method, the emission of the acceptor is examined at two different excitation wavelengths to determine the FRET efficiency. More specifically, the value $(ratio)_A$ is the emission of the acceptor measured while exciting the donor divided by the emission of the acceptor undergoing direct excitation. In Figure 1, $(ratio)_A$ is the acceptor extracted emission (red + purple graphs in Figure 1b) divided by direct acceptor emission at 590 nm (red graph in Figure 1a). The FRET efficiency (E) is functionally dependent on $(ratio)_A$ and the extinction coefficients of our donor and acceptor taken at specific wavelengths. This allows for a fairly straight-forward way to determine FRET efficiencies.

To use $(ratio)_A$, we will need to measure the following spectra

1. *Donor (500)*, the emission spectrum of **donor-only sample** collected at **500 nm** excitation wavelength
2. *FRET (500)*, the emission spectrum of the **FRET sample** collected throughout at **500 nm** excitation wavelength. This will include emission from the donor because of direct excitation by light at 500 nm wavelength, as well as from the acceptor because of (a) FRET and (b) the small amount of direct excitation by light at 500 nm wavelength.
3. *FRET (590)*, the emission spectrum of the **FRET sample** collected at **590 nm** excitation wavelength. This gives us a measure of the total number of acceptors in our sample.

The three spectra are shown in the figure below:



The numerator of the equation describing $(ratio)_A$ is the emission of the acceptor taken while exciting the donor (undergoing FRET). To do this, we must subtract the donor emission from FRET(500). The donor peak in Donor(500) must be re-scaled to match the donor peak in FRET(500). The denominator of the $(ratio)_A$ is simply, FRET(590). The final equation is shown below,

$$(ratio)_A = \frac{[FRET(500)] - [N \cdot Donor(500)]}{[FRET(590)]}$$

where N = FRET-DA(500)/ Donor-DA(500). The spectra in the numerator and denominator will be integrated to calculate $(ratio)_A$. As mentioned previously, $(ratio)_A$ is functionally dependent of the FRET efficiency of the system (E) as shown below,

$$(ratio)_A = \frac{\epsilon_D(500) \cdot E + \epsilon_A(500)}{\epsilon_A(590)}$$

where $\varepsilon_D(500)$ is the extinction coefficient of Cy3 at 500 nm, $\varepsilon_A(500)$ is the extinction coefficient of Cy5 at 500 nm and $\varepsilon_A(590)$ is the extinction coefficient of Cy5 at 590 nm.

We have made some simplifying assumptions about the fraction of labeling to arrive at this simplified expression. A derivation of this will be provided with your lab report questions, but you can motivate the formula by noting that the first term in the numerator is proportional to the FRET enhanced acceptor emission (the FRET efficiency E times the amount of absorption by the donor) and the second proportional to the amount of emission by direct excitation at λ_{EX}^{D} which is proportional to the amount absorbed by the acceptor. The denominator is proportional to the amount of light absorbed by the excitation at λ_{EX}^{A} and the subsequent acceptor emission.

C. Lifetime Method

Measuring the FRET efficiency of a particular system with lifetimes typically measures the amount by which the lifetime of the donor shortens from the additional de-excitation pathway present (while undergoing FRET). In other words, the additional pathway makes it more likely for the excited donor molecule to return to the ground state, which therefore shortens the lifetime (average time spent in the excited state). The relationship between the FRET efficiency (E), the lifetime of the donor undergoing FRET (τ_{DA}) and the lifetime of the donor without the acceptor present (τ_D) is written below

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$

To derive the equation, remember that the efficiency of energy transfer is the same thing as the "quantum yield" of energy transfer. As discussed in lecture, the quantum yield of a given de-excitation pathway (like FRET) is the rate of that pathway divided by the rates of all the pathways of de-excitation available. Therefore, we can relate the FRET efficiency to the rates of the deactivation pathways as shown,

$$E = \frac{k_T}{k_T + \sum_{i \neq T} k_i}$$

In this equation, k_T is the rate of energy transfer and k_i are the rates of the other pathways present.

Next, to determine the relation between E and the lifetimes (τ_{DA} and τ_D), the rate constants in the above equations need to be related to the lifetimes. The rate constants that describe the de-excitation pathways for the molecule to leave the excited state are inversely proportional to the lifetime. This is shown below,

$$\tau_D = \frac{1}{\sum_{i \neq T} k_i}$$

$$\tau_{DA} = \frac{1}{k_T + \sum_{i \neq T} k_i}$$

With some algebra, the three previous equations can be used to derive the equation relating the FRET efficiency (E) to the lifetime of the donor undergoing FRET (τ_{DA}) and the lifetime of the donor not undergoing FRET (τ_D) as shown below.

$$E = 1 - \frac{\tau_{DA}}{\tau_D}$$

C. Fluorescent Anisotropy

We will measure the anisotropy of free Cy3, Cy3-ssDNA and Cy3-dsDNA that we measure lifetime for in the previous lab. The anisotropy value of a fluorophore is related to its rotational lifetime ϕ through the relation:

$$r = \frac{r_0}{1 + \tau/\phi}$$

Where r is the observed anisotropy, r_0 is the intrinsic anisotropy of the molecule, τ is the fluorescence lifetime and ϕ is the rotational time constant. For our purposes, we can assume that r_0 is 0.386.[1]

Experiments:

Note:

- We are using Quartz cuvettes. Orient the longest path length of the cuvette to the excitation source. Add 740 μL T-50 buffer to sample to make a total volume of 800 μL
- Please do not dispose your samples once you are done with measurement. Simply place them back to their eppendorf tubes with glass pipette once you are done with a measurement

A. Sensitized Emission Measurement

Procedure for spectrum *Donor(500)*

1. Check with your instructor that the fluorometer is calibrated.
2. Place the solution marked "12bp-Donor" in the sample holder in the position 'S'
3. In Vinci, under experiment, please select experiment, default
4. Go to Spectra > Emission, then enter the settings below
5. Use "Raw Channel" for emission measurement. This was different from last week
6. Under View > Visualization, choose "Emission" for the second channel
7. Enter the settings below and hit the green play button

	Start Scan [nm]	End Scan [nm]	Excitation [nm]	Time Base [s]	Measurement
Donor-DA(500)	520	800	500	0.4	Raw Channel
FRET-DA(500)	520	800	500	0.4	Raw Channel
FRET-A(590)	610	800	590	0.4	Raw Channel

Procedure for spectrum *FRET(500)* and *FRET(590)*

1. Place the solution marked "FRET" in the sample holder in the position 'S'
2. Enter the settings above to collect *FRET(500)* and *FRET(590)*
3. Hit the green play button

Intensity correction

The machine sometimes wrongly scales the absolute intensity. We need to correct this by recording actual and reported count for individual peaks, and scale each spectra to the actual peak intensity. For each spectra that you collect, go to 'Instrument Control' and record the intensities at excitation and emission wavelength below

Spectrum	Excitation λ (nm)	Emission λ (nm)	Reported Count	Actual Count
12bp-Donor(500)	500	562		
12bp-FRET(500)	500	562		
12bp-FRET(590)	590	662		
16bp-Donor(500)	500	562		
16bp-FRET(500)	500	562		
16bp-FRET(590)	590	662		

B. Lifetime Measurements

Procedure

1. Load 1 ml of each DNA solution (Donor12, FRET12, Donor16 and FRET16) and measure separately.
2. Use Erythrosin-B as the reference for the lifetime. The parameters are as below:

Ref. Dye	Freq	Time Base	Mod Freq	Ref. Lifetime [ns]	Filter [nm]	Excitation [nm]
Erythrosin-B	12	3 s	10–200 MHz	0.46	550 - 620	547

Please record the lifetimes for the DNA samples below and calculate the average lifetime:

	τ_1 (ns)	τ_2 (ns)	Fraction$_1$	Ave. τ (ns)
12bp-Donor				
12bp-FRET				
16bp-Donor				
16bp-FRET				

C. Fluorescent Anisotropy

Measure and record the fluorescent anisotropy of the samples below. To start polarization experiment, go to Experiment > Single Point > Polarization in Vinci. Use excitation wavelength of 545 nm and emission wavelength of 565 nm. Set 'Measurement' to 'Polarization', time base to 1 s, and the number of iterations to 10.

Calculate the rotational constant ϕ based on the lifetime we obtain in the previous lab. Experimentally, the anisotropy r can be calculated with the equation:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

Recall that we can obtain the rotational constant with the equation:

$$r = \frac{r_0}{1 + \tau/\phi}$$

where r is the observed anisotropy, r_0 is the intrinsic anisotropy of the molecule, τ is the fluorescence lifetime and ϕ is the rotational time constant. For our purposes, we can assume that r_0 is 0.386.[1]

	r	$\tau_{Average}$	ϕ
Free-Cy3			
Cy3-ssDNA			
Cy3-dsDNA			

Report Questions

A. Sensitized Emission (Direct and (ratio)$_A$ Method)

A1) ***(4 pts)*** Correct all the emission spectra you collected by dividing the emission count by the excitation count at each wavelength. Prepare a plot showing the following spectra (put all five spectra on one plot):

- *FRET (500)* spectrum
- The *renormalized* donor spectrum *N·Donor(500)*
- The extracted acceptor spectrum $F_{extracted}$
- The directly excited acceptor spectrum at 590 nm: *FRET(590)*
- The directly excited acceptor spectrum at 500 nm, *Acceptor(500).* This is inferred from *FRET(590)* spectra, and calculated using the formula: $\varepsilon_A(500)/\varepsilon_A(590)$* *FRET(590)*, where $\varepsilon_A(500)$ and $\varepsilon_A(590)$ are the extinction coefficients of acceptor at 500 nm and 590 nm respectively. The values are given below:

ε_A(500)	1,350 $cm^{-1}M^{-1}$
ε_A(590)	72,050 $cm^{-1}M^{-1}$

Clearly label each spectrum on your plot.

Below are directions to help you get the renormalized donor spectrum and the extracted acceptor spectrum.:

Using the data from the Cy3-Cy5 FRET sample, extract the acceptor emission spectrum from *FRET(500)* and plot the fluorescence emission spectra

- Locate the wavelength at 562 nm
- Normalize *Donor (500)* so that the donor emission peaks are equal for *FRET (500)* and *Donor(500)*.

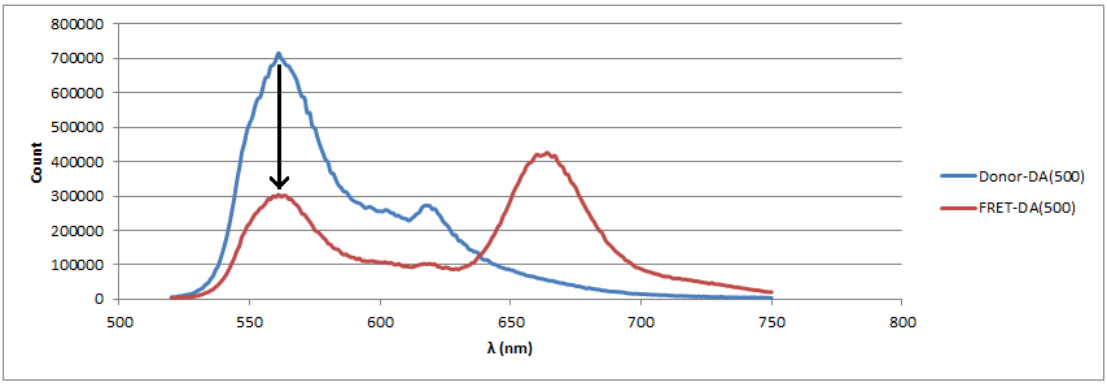


Figure: Scaling of the Donor(500) spectra to overlap FRET(500) spectra

- If your renormalized donor spectrum, *N· Donor(500),* does not overlap well with the donor peak in *FRET(500),* you may need to manually adjusts N by a small value.

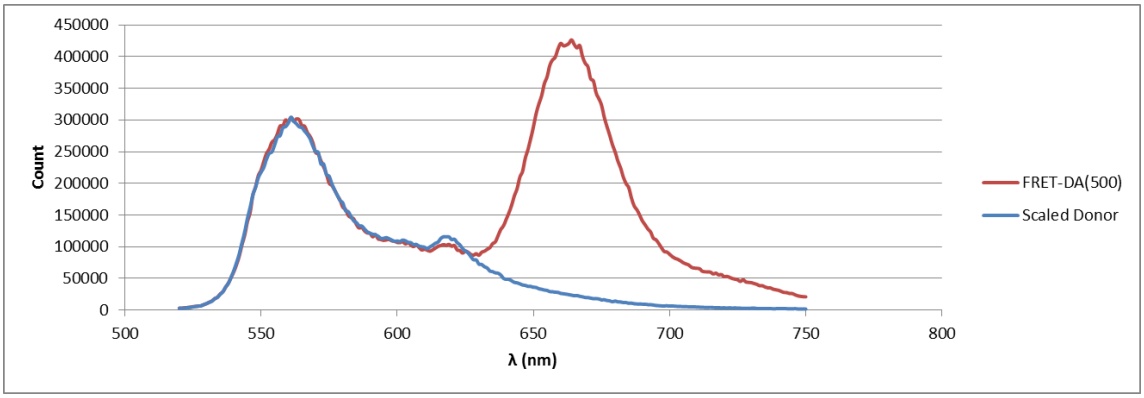


Figure: Donor(500) spectra is now normalized with respect to FRET(500) spectra

- Now calculate the acceptor emission spectrum of the doubly-labeled dsDNA sample:

$$F_{Extracted} = FRET(500) - N \cdot Donor(500)$$

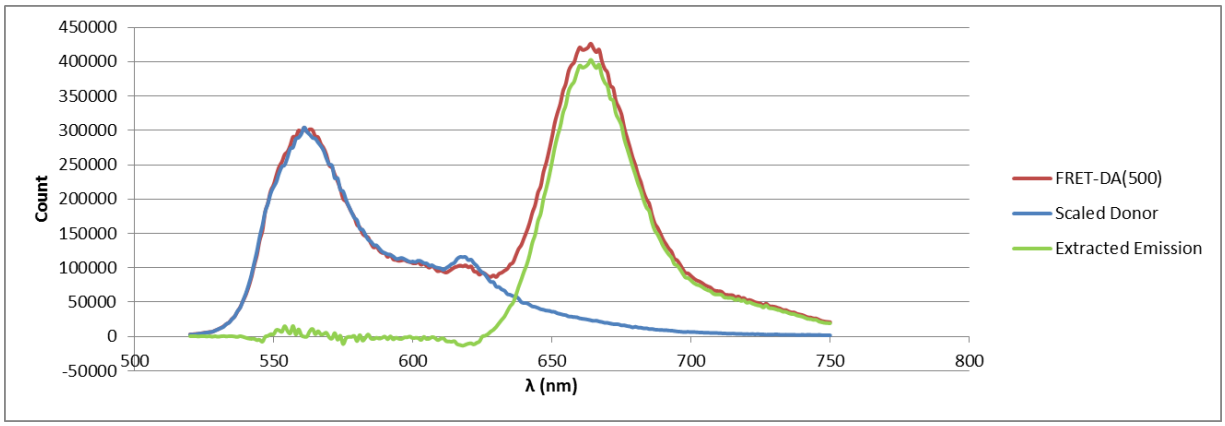


Figure: Extracted emission spectra is just taking the difference between FRET(500) and the normalized Donor(500) spectra

A2) *(3 pts)* Calculating FRET efficiency using direct method for sensitized emission:

Report your calculated values for

a) $\sum_{610-800} F_{Extracted}$

b) $\sum_{610-800} Acceptor(500)$

c) $\sum_{520-800} N \cdot Donor(500)$ (sum of renormalized donor spectrum)

d) FRET efficiency calculated with direct method

Recall that:

$$E_{FRET} = \frac{I_{AD}/q_A}{I_{DA}/q_D + I_{AD}/q_A}$$

where I_{AD} is $\sum_{610-800} F_{Extracted}$ - $\sum_{610-800} Acceptor(500)$, I_{DA} is $\sum_{520-800} N \cdot Donor(500)$, q_A is 0.21 for Cy5 and q_D is 0.13 for Cy_3.

A3) *(3 pts)* Calculating FRET efficiency using $(ratio)_A$:

Report your calculated values for

a) $\sum_{610-800} F_{Extracted}$

b) $\sum_{610-800} FRET(590)$

c) $(ratio)_A$

A couple of points to note:

$$(ratio)_A = \frac{\sum_{610-800} F_{Extracted}}{\sum_{610-800} FRET(590)} = \frac{\sum_{610-800}[FRET(500) - N \cdot Donor(500)]}{\sum_{610-800} FRET(590)}$$

The integration over the emission wavelength was not written out explicity in the experiment handout. You should do the integration for your writeup. If you need help, I can assist you in Matlab and EXCEL.

Below are the necessary extinction coefficients that you will need for the FRET efficiency calculation.

ε_D(500)	32,350 $cm^{-1}M^{-1}$
ε_A(500)	1,350 $cm^{-1}M^{-1}$
ε_A(590)	72,050 $cm^{-1}M^{-1}$

Calculate and report the FRET efficiency from your measured $(ratio)_A$.

B. Lifetime Measurement

B1) ***(3 pts)*** **Calculating the FRET efficiencies.**
Compute and report the FRET efficiency for both the FRET12 and FRET16 samples.

$$FRETEfficiency = 1 - \frac{\tau_{DA}}{\tau_D}$$

τ_D = Lifetime of unpaired donor

τ_{DA} = Lifetime of paired donor from the FRET12 (and separately FRET16). There should be two separate FRET efficiencies reported here.

B2) ***(4 pts)*** **Calculate the expected FRET efficiency**.
Please perform the steps (a and b) for both the case of the 12 base pair DNA and separately for the 16 base pair DNA (Use 56 Å for the Förster radius). FRET efficiency can be related to R / R_0, where R is the distance between the donor and the acceptor and R_0 is the Förster radius

$$E = \frac{1}{\left[1 + \left(R / R_0\right)^6\right]}$$

a) Calculate the expected FRET efficiency for a linear geometry between the donor and acceptor of N*3.4 Å + about 13 Å for the diagonal distance (since the DNA is helical) between the dyes. N is the number of base pairs in the DNA duplex (it is not the normalization factor used to extract the acceptor emission spectrum).
b) Calculate the expected FRET efficiency for a helical geometry using the following equation and parameters.

$$R = \left[\left(3.4 * N + L\right)^2 + (d \sin\theta)^2 + (a - d\cos\theta)^2\right]^{\frac{1}{2}}$$

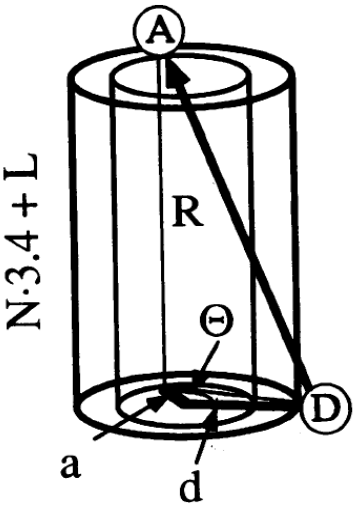


Fig. 2.

Use the following values for the parameters: L=13 Å, ϕ=227°, d=19 Å, a=13 Å. The parameters are described in the figure and legend below.

Schematic representing helical geometry of the DNA molecule. For this simple model, we assume that κ^2 remains constant for all samples, an assumption that is corroborated by the agreement between the data and the simulation. Vector **R** originates on the donor molecule and points toward the acceptor molecule; length |**R**| is the distance. Component of **R** projected along N base pairs on the helical axis is given as (N* 3.4 + L) Å. This assumes that the bases are 3.4 Å in height; distance L accounts for the fact that the projections of the molecular centers of D and A onto the DNA helical axis may not correspond with the planes of the base pairs to which they are attached. L represents the theoretical vectorial distance along the helical axis separating D and A if they would be attached to the same base pair (L can be negative). A and D can extend different perpendicular distances away from the helical axis; this is accounted for by the distances d and a, which refer to the distances of the D and A molecules, respectively. Polar angle ϕ is

the cylindrical angle between D and A for the case that $N = 1$. Distance R is given as a function of N; N is the only variable that varies independently throughout all the series of doubly labeled molecules…$\theta = N*36+\phi$

B3) ***(6 pts)*** **Error.**

1. For both the 12 and 16 base pair DNA duplexes, calculate the percent error between the FRET efficiencies measured from the lifetime method, direct sensitized emission method, and $(ratio)_A$ method compared to the expected FRET efficiencies from the linear and helical geometries.
2. FRET efficiencies have also been determined experimentally. From the reference: "Orientation dependence in fluorescent energy transfer between Cy3 and Cy5 terminally attached to double-stranded nucleic acids", what are the FRET efficiencies for Cy3 and Cy5 attached to 12 bp and 16 bp DNA? You can refer to Fig. 3 of the paper. How close is the FRET efficiencies you obtained to these experimental values?
3. What sources of error can affect the FRET efficiency you measured? What causes your FRET efficiency to deviate from the theoretical calculations using the linear and helical geometries of DNA?

C. Fluorescent Anisotropy

C1) ***(2 pts)*** From the reference "*Fluorescence Properties and Photophysics of the Sulfoindocyanine Cy3 Linked Covalently to DNA*", what is the equation relating the quantum yield and the fluorescence lifetime of a fluorophore? If a fluorophore has high quantum yield, do we expect its lifetime to be high or low?

C2) ***(2 pts)*** From the calculation of the rotational constant of Cy3 bound to no DNA, single-stranded DNA and double-stranded DNA, which one has the shortest, and longest rotational constant? Is this expected?