Lab 1: Ensemble Fluorescence Basics

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.

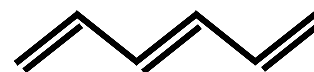
Objective:

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

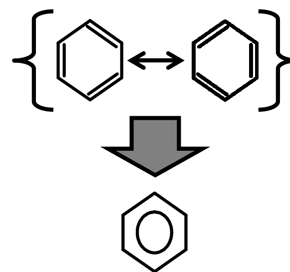
A common structure in chromophores is alternating single and double bonds (conjugated bonds), often in the form of aromatic structures. These types of structures are responsible for delocalizing the electrons over many atoms, leading to a red-shift of the electronic transitions into the optical range. 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.

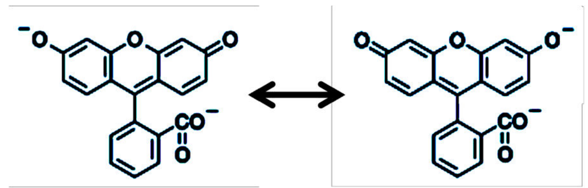
Resonance delocalization



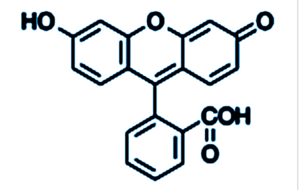
The simplest example of a resonant structure is given by the benzene molecule C_6H_6. There are two equivalent structures for benzene, as shown at the top of the picture (single and double bonds inverted). In either of these structures, as drawn, the electrons would be localized in π-bonds above and 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.

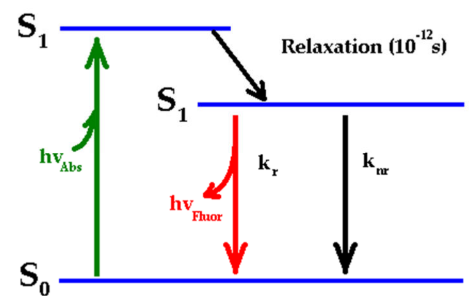


In its neutral form with the phenol and carboxylic groups protonated, fluorescein's emission degrades considerably and the absorption shifts toward the blue and decreases. The reason is that the molecule is no longer in complete resonance. The presence of the –OH group requires a proton to be transfered 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 150x (10 uL sample in 1.5 mL annealing buffer) before taking any measurement.

(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 $A = \varepsilon cL$, 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) 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 (absorption max – emission max).

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

	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

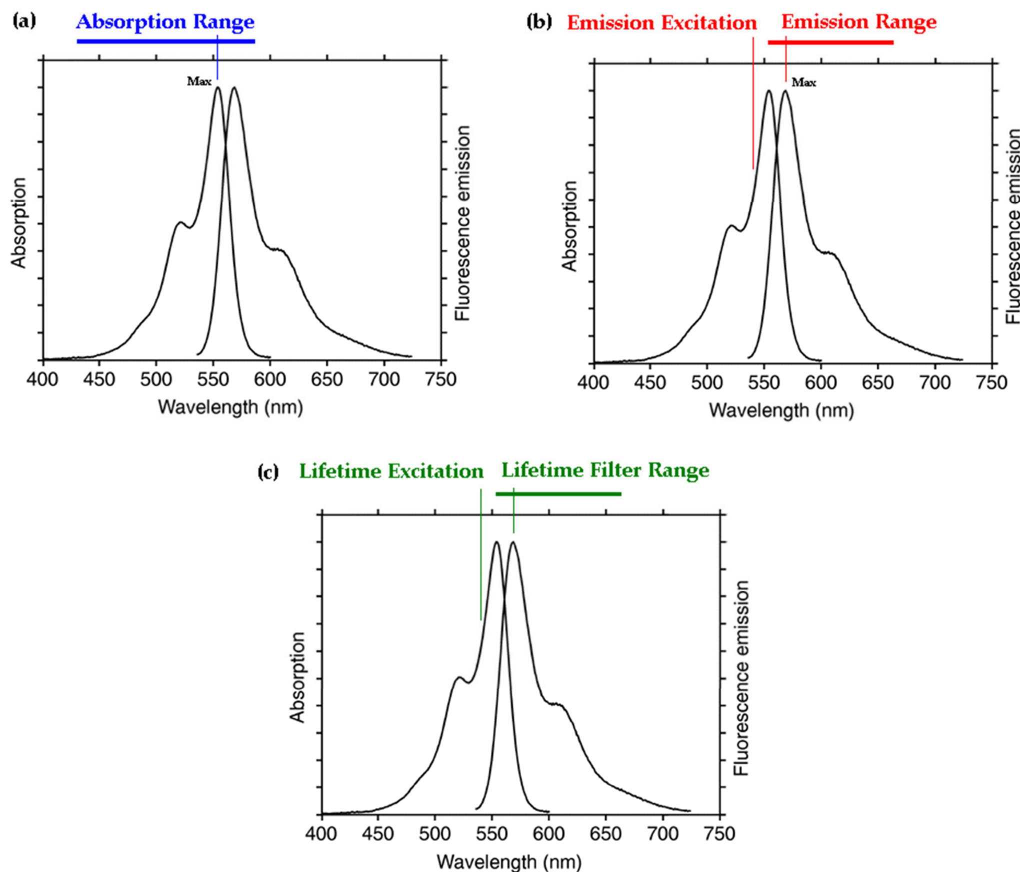
(4) Measure the lifetime of Cy3 attached to different substrates *(Appendix 2.3)*. Use the parameters below for each measurement. Record the lifetimes in Table 2. Each student will pick one sample and share the data with the group. Measure up to the second lifetime component.

Ref. Dye	**Time Base**	**Frequency**	**Ref. Lifetime [ns]**	**Filter [nm]**	**Excitation [nm]**
Erythrosin-B	1	10– 200 MHz	0.46	550 - 610	540

	τ_1 (ns)	**τ_2 (ns)**	**$Fraction_1$**
Free-Cy3			
Cy3-ssDNA			
Cy3-dsDNA			

Table 2. Lifetimes of Cy3 attached to different substrates

(5) 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 - 610	540
Fluorescein	1	4 – 90 MHz	4.0	485 - 545	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) 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

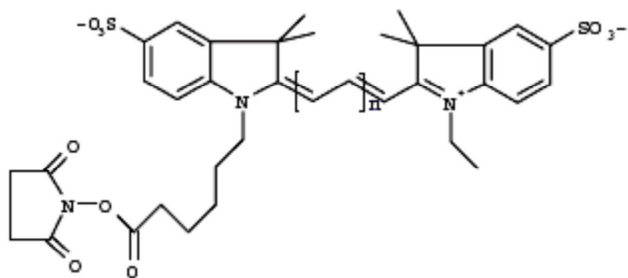
Here you will be preparing DNA samples for ensemble FRET experiment next week:

1. Mix the following single stranded DNA (ss-DNA) to make double stranded DNA

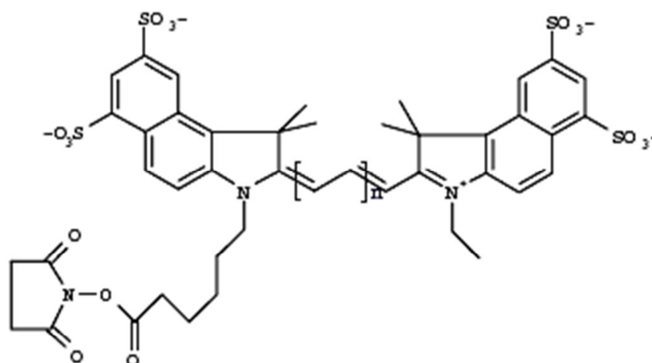
DNA	12bp-Donor	12bp-FRET	16bp-Donor	16bp-FRET
Annealing Buffer	200 μL	200 μL	200 μL	200 μL
(1) Cy3-12bp-1	4 μL	4 μL		
(2) Cy5-12bp-2		4 μL		
(4) 12bp-2	4 μL			
(5) Cy3-16bp-1			4 μL	4 μL
(6) Cy5-16bp-2				4 μL
(8) 16bp-2			4 μL	

2. Switch on heat block to 95°C
3. Incubate mixtures in 95°C for 45s
4. Turn off heat, and let DNA gradually cool to room temperature for 5 hours

(7) 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) 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) 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 $d_0 = 140$ pm, $\Delta E'$ is the excitation energy with correction for bond alternation according to equation (8.35). References see Further Reading.

(10) 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.

(11) Lab evaluation: What was the most interesting thing you learned from this lab? What are you confused about? Related to this lab, what would you like to know more about? Any helpful comments?

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