Answer Sheet. Lab 1: Ensemble Fluorescence Lab Report

Q3. Answers may vary from student to student

	Abs Max (nm)	Absorbance	Emission max (nm)	Stokes shift (nm)	Conc [mol/L]
Cy3	545	0.0285	563	18	0.00000019
Cy5	645	0.0563	665	20	2.252E-07
Cy5.5	676	0.00983	696	20	3.932E-08

Q4. Answers may vary from student to student

	τ_1 (ns)	τ_2 (ns)	Fraction$_1$	τ_{ave} (ns)
Free-Cy3	0.209	-	-	0.209
Cy3-ssDNA	2.337	0.545	0.4	1.2618
Cy3-dsDNA	0.6917	0	0.9348	0.6466

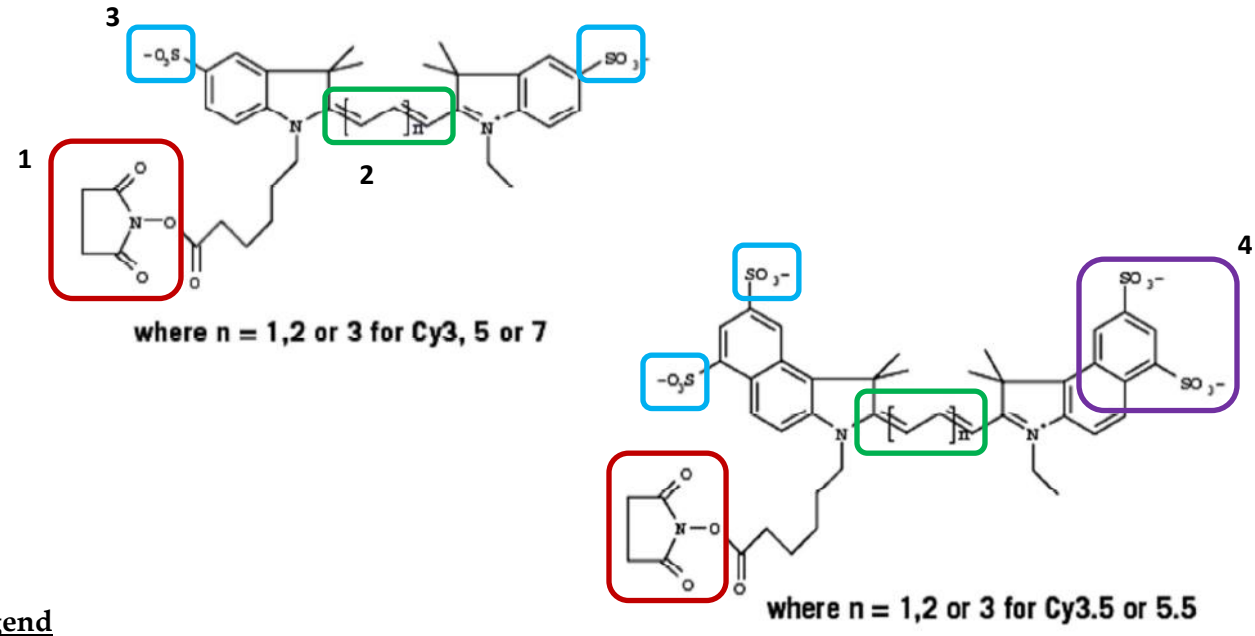
Q5. Answers may vary from student to student

	Abs max (nm)	Emission max (nm)	τ (ns)	Identity
A	491	535	1.801	*Acridine Orange*
B	557	577	1.42	*Rhodamine B*
C	468	512	5.7	*Biodipy FL*

Q6. Answers may vary from student to student

Δλ	Cy5(abs max) - Cy3 (abs max)	100
Δλ	Cy5.5(abs max) - Cy5 (abs max)	31

Q7.



Legend

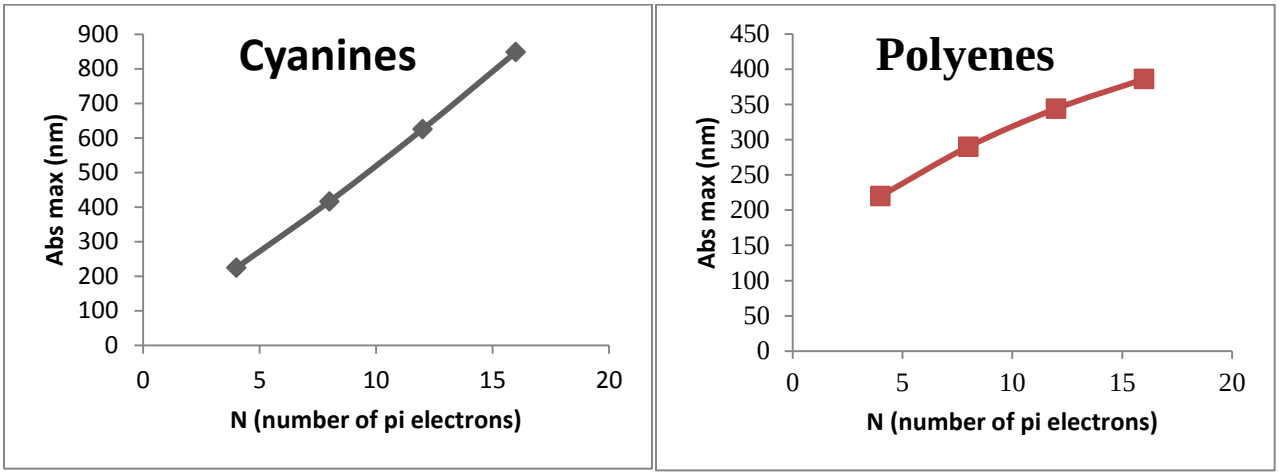
1. Red: Reactive NHS ester for bioconjugation

2. Green: Strongly resonant double bonds causes major shifts in absorption

3. Blue: Charged component helps solubility in water

4. Purple: Moderately conjugated phenyl ring causes minor absorption shift

Q8.



As the number of pi electrons increases, the absorbance maximum also increases
This means that the higher the resonance or electron delocalization a molecule has, the absorption wavelength will be more red-shifted.

Q9.

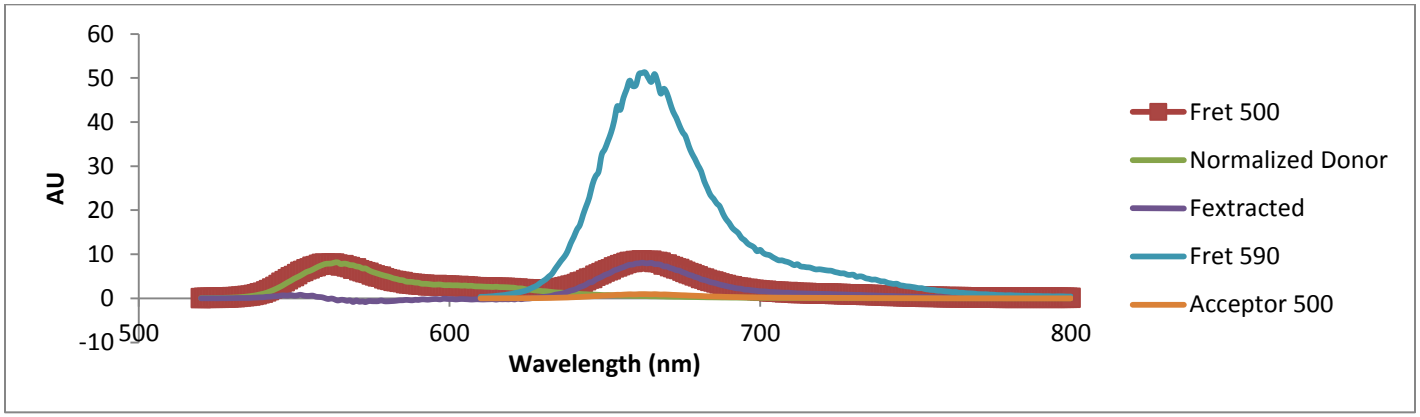
Fluorescein and rhodamine dyes have highly resonant structures which allow them to have high quantum yields. Cyanine dyes undergo trans to cis isomerization and that reduces their quantum yields due to additional relaxation pathway competing with fluorescence. Fluorescein and rhodamine are rigid, and have less pathways competing with fluorescence, allowing them to have high quantum yield.

Q10.

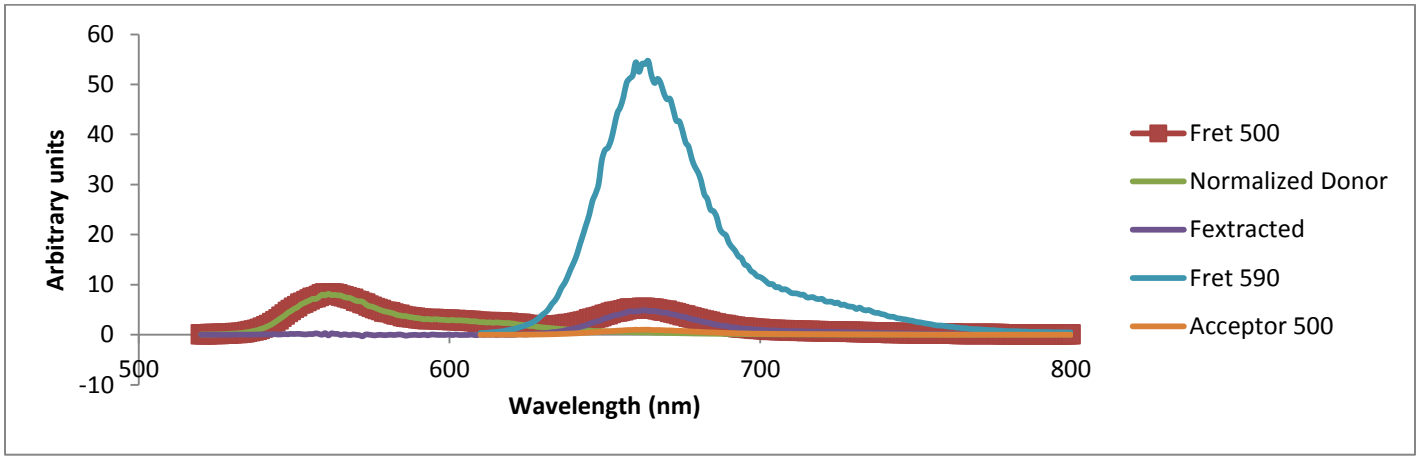
The lifetime varies when the Cy3 is attached to different substrates. Free Cy3 undergoes trans-cis isomerization leading to low quantum yield and lifetime. This is because the trans-cis isomerization provides an alternative relaxation pathway that competes with fluorescence, causing Cy3 to stay at its excited state at a shorter time, decreasing its lifetime. Interaction of Cy3 with nucleic acid stabilizes the trans isomer, allowing higher quantum yield and lifetime. Cy3 interacts more strongly with single-stranded DNA compared to double-stranded DNA, yielding higher lifetime for single-stranded than double-stranded DNA

Lab 1B: Ensemble FRET

A1. For 12 bp :



For 16 bp:



A2. For 12bp

a. 358
b. 44
c. 425
d. $E_{12bp-direct} = \frac{(358-44)/0.21}{425/0.13+(358-44)/0.21} = \frac{1495}{3269+1495} = 0.313$

For 16bp:

a. 221
b. 47
c. 420
d. $E_{16bp-direct} = \frac{(221-47)/0.21}{420/0.13+(221-47)/0.21} = \frac{829}{3230+829} = 0.204$

A3)

FRET efficiency by ratio$_A$ method

For 12 base pairs:

a. 358
b. 2356
c. $ratio_A = \frac{358}{2356} = 0.151$
d. $(ratio)_A = \frac{\epsilon_D(500)\cdot E + \epsilon_A(500)}{\epsilon_A(590)} = \frac{32{,}350\cdot E + 1{,}350}{72{,}050}$

$$\frac{32{,}350 \cdot E + 1{,}350}{72{,}050} = 0.151$$

$E_{12bp-ratioA} = 0.294$

For 16 base pairs:

a. 221
b. 2497
c. $ratio_A = \frac{221}{2497} = 0.0885$
d. $(ratio)_A = \frac{\epsilon_D(500)\cdot E + \epsilon_A(500)}{\epsilon_A(590)} = \frac{32{,}350\cdot E + 1{,}350}{72{,}050}$

$$\frac{32{,}350 \cdot E + 1{,}350}{72{,}050} = 0.0885$$

$E_{16bp-ratioA} = 0.155$

B. Lifetime measurement

Sample	Tau (ns)
12 bp donor	0.855
12bp FRET	0.411
16bp Donor	0.6476
16 bp FRET	0.4308

B1)

$$E_{12bp-Lifetime} = 1 - \frac{\tau_{DA}}{\tau_D} = 1 - \frac{0.411}{0.855} = 0.519$$

$$E_{16bp-Lifetime} = 1 - \frac{\tau_{DA}}{\tau_D} = 1 - \frac{0.4308}{0.6476} = 0.335$$

B2

For 12 base pairs

a. Linear geometry:

$$E_{Linear} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} = \frac{1}{1 + \left(\frac{53.8}{56}\right)^6} = \frac{1}{1 + (0.96)^6} = 0.559$$

b. Helical geometry:

$$R_{Helical} = (3.4 * N + L)^2 + (d \cdot sin\theta)^2 + (a - d \cdot cos\theta)^2$$

$$R_{Helical} = \sqrt{(3.4 * 12 + 13)^2 + (19 \cdot \sin(12 * 36 + 227))^2 + (13 - 19 \cdot \cos(12 * 36 + 227))^2}$$
$$= \sqrt{2894.44 + 276.15 + 14.35} = 56.4$$
$$E_{Helical} = \frac{1}{1 + \left(\frac{56.4}{56}\right)^6} = 0.489$$

For 16 base pairs

a. Linear geometry:

$$E_{Linear} = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} = \frac{1}{1 + \left(\frac{67.4}{56}\right)^6} = \frac{1}{1 + (0.96)^6} = 0.247$$

b. Helical geometry:

$$R_{Helical} = (3.4 * N + L)^2 + (d \cdot sin\theta)^2 + (a - d \cdot cos\theta)^2$$
$$R_{Helical} = \sqrt{(3.4 * 16 + 13)^2 + (19 \cdot \sin(16 * 36 + 227))^2 + (13 - 19 \cdot \cos(16 * 36 + 227))^2}.$$
$$= \sqrt{4542.76 + 355.63 + 114.15} = 70.8$$
$$E_{Helical} = \frac{1}{1 + \left(\frac{70.8}{56}\right)^6} = 0.197$$

B3)

1.

				Percent Errors (%)			
	Method	**12-bp**	**16-bp**	**12bp-linear**	**12bp-helical**	**16bp-linear**	**16bp-helical**
Experiment	**Direct**	0.313	0.204	44	36	17	-4
	Ratio$_A$	0.294	0.155	47	40	37	21
	Lifetime	0.519	0.335	7	-6	-36	-70
Theory	**Linear**	0.559	0.247				
	Helical	0.489	0.197				

2. From the paper it looks like the E for 12 bp is about 0.5 and for 16 bp it is about 0.38. These values will differ from student to student.

3. Possible sources of error include:

- R_0 is assumed to be 56 Angstrom because the orientation factor κ^2 to calculate FRET efficiency is assumed to be 2/3, but this may not be the case. Orientation factor κ^2 is 2/3 when the dyes can rotate freely, but in our case the dyes may interact with DNA, limiting its mobility
- There may also be error due to incomplete annealing of single-stranded DNA to form double-stranded DNA, or presence of excess single-stranded DNA
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C. **Fluorescence Anisotropy**

C1

$$\phi_f(Cy3) = \frac{k_f}{(k_f + k_{ic} + k_{Nr}(T,\eta))} = k_f\tau$$

If quantum yield is high, we expect the lifetime to be high

C2

	r	τ_{ave}	ϕ
Free dye	0.228	0.209	0.3
Cy3-ssdna	0.183	1.13	1.0
Cy3-dsdna	0.273	0.6917	1.7

The free dye has the shortest rotational time constant, which means it rotates the quickest. This is expected because it has the lowest mass. The dsDNA has the longest rotational time constant, which is also expected because it has the largest mass.