Duke University

Department of Electrical and Computer Engineering

Senior Thesis

Study of chromophore pair interaction using DNA self-assembly

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Abstract

The primary objective of this project was to verify dependence of Forster resonance energy transfer (FRET) on angular separation of chromophore pair. A secondary objective was to investigate the formation of exciplex, a heterodimeric compound that forms as a result of electrostatic interaction between a pair of proximal chromophores with either species in the excited state. In this study, photochemical interaction between a select pair of chromophores was studied. A method based on functionalization of chromophores on oligonucleotides was developed in order to place chromophores in a rigid conformation with respect to distance and angular separation. The method consisted of functionalizing maleimide variants of chromophores to thiolated terminus of oligonucleotides and creating a rigid structure via hybridization of complementary strands. In the close proximity that the chromophores were situated, angular separation becomes the predominant factor behind modulation of transfer efficiency. This phenomenon was verified with FRET pair studies involving three configurations, with two of the three attaching the chromophores to base pairs that were positioned orthogonal to each other. Steady-state fluorescence measurements were used to interpret the photochemical properties of the structures, while sample temperature was varied in order to investigate the changes in these properties as structure dissociates. Results show that fluorescence of the donor appear to be strongly quenched in the two orthogonal configurations (Φ_T = 0.1~0.2), contrary to the other configuration where the two chromophores are placed in close proximity (Φ_{Tmax} = ~0.6). However, the formation of exciplex could not be observed from the results obtained in this experiment, primarily due to the difficulties encountered in identifying the contribution of exciplex emission from the total emission of the system. In conclusion, angular dependence of FRET efficiency was observed to be related to the base pair separation of chromophore pair. From a high-level perspective, this investigation is a step towards synthesizing molecular logic gates using RET logic. Future directions include identifying chromophore pairs that can form exciplexes, as well as studying the effect of tether lengths to the formation of exciplexes between chromophores conjugated to a DNA nanostructure. With two or more exciplex-forming species with a common acceptor, it is possible to envision a system that selectively interacts with one donor over another, creating a molecular pass gate whose behavior is modulated entirely by electromagnetic waves.

Intorduction

Using resonance energy transfer as a mechanism for performing logical operations has been studied in the past at Duke Self-Assembled Systems Group. An important aspect of this enterprise is the characterization of chromophore interactions, which forms the basis of this project. The primary objective of this project was to verify dependence of Forster resonance energy transfer (FRET) on angular separation of chromophore pair. A secondary objective was

to investigate the formation of exciplex, a heterodimeric compound that was identified as a potential candidate for use in designing an electromagnetic-source modulated FRET pass gate.

A. Modulation of FRET Efficiency

Forster Resonance Energy Transfer (FRET) is a non-radiative energy transfer mechanism. When a donor molecule is excited by incident EM wave, it may transfer its excited state to an acceptor molecule through a nonradiative dipole-dipole interaction. In absence of an acceptor, the donor may radiate energy as the excited electron returns to a lower orbital. In case of chromophores as donors, this would result in a release of photons at their characteristic emission wavelengths. In presence of an acceptor however, the excited donor may act like an oscillating dipole and transmit energy directly to the acceptor molecule, resulting in exciting the acceptor as the donor reaches ground state (1).

A key feature of this mechanism is its operating distance. FRET takes place between molecules separated by several tens of Angstroms, but its efficiency is strongly attenuated as the separation between chromophores extend beyond a threshold distance. An inverse 6^{th} power law governs transfer efficiency between a given donor and acceptor:

$$\Phi_T = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

Represented graphically, transfer efficiency undergoes a sharp transition when $r = R_0$.

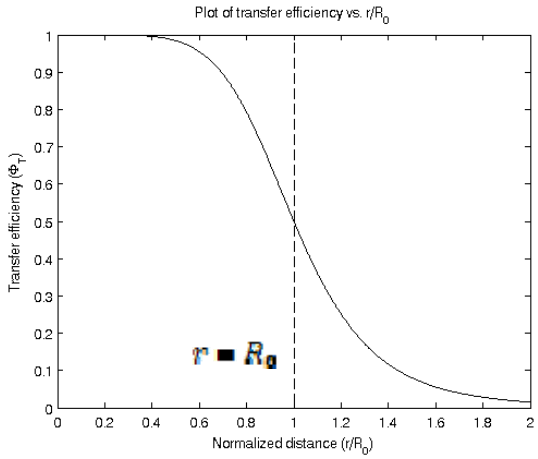


Fig.1 : Transfer efficiency as a function of normalized radius (r/R_0). Observe the sharp transition of transfer efficiency at $r=R_0$. Using this behavior, several FRET systems can be constructed in areas separated by $r >> R_0$ with minimal crosstalk.

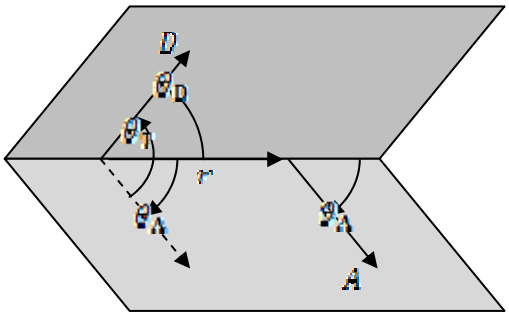
R_0, the Forster radius, is defined as the donor-acceptor pair separation at which transfer efficiency is 50%. In theory, this distance (in Angstrom) is obtained by the following equation:

$$R_0 = \sqrt[6]{\frac{9000 \cdot 10^{20} \cdot Q_D (\ln(10)) \kappa^2 J}{128 \pi^5 n^4 N_A}}$$

Here, NA represents Avogadro's number, n represents the refractive index of sample (assumed 1.4 for biochemical samples in aqueous solutions), and Q_D represents the quantum yield of donor chromophore. Note the presence of scaling factor (10^{20}) which was added in order to produce the final value of R_0 in Angstroms (2).

An interesting feature of FRET is anisotropy: due to its nature as a dipole-dipole interaction, the alignment of donor's emission dipole with acceptor's absorption dipole affects the efficiency of energy transfer. This is factored into transfer efficiency as orientation factor κ^2, which is a function of geometric parameters as shown in the following equation:

$$\kappa = \cos\theta_T - 3\cos\theta_D \cdot \cos\theta_A$$



Diag. 1: Visualization of orientation factor.

Here, θ_T is the angle between donor emission transition moment and acceptor absorption transition moment; θ_D and θ_A are the angles of donor and acceptor transition moments to the axis connecting D-A pair.

Another useful feature of FRET is the integral overlap J, which represents the overlap of excitation spectrum of the acceptor molecule with the emission spectrum of the donor molecule. The overlap integral is computed as follows:

$$J = \int_0^\infty f_D(\lambda) \cdot \varepsilon_A(\lambda) \cdot \lambda^4 d\lambda$$

The unit of extinction coefficient is in $M^{-1}cm^{-1}$, while wavelength is in nm. This results in an integration of the donor fluorescence emission and acceptor extinction coefficient, representing the overlapping area. The donor fluorescence emission in this equation is a quantity which is normalized such that its integral with respect to wavelength is unity.

B. Exciplex

Exciplex, or excited state complex, is a heterodimeric structure that forms when one of the two exciplex-forming species is in an excited state. The two exciplex-forming compounds do not bond when both molecules are in ground state, but excitation results in an electrostatic interaction that causes physical displacement of the species towards each other. This is a unique mechanism whereby electromagnetic excitation can modulate the physical alignment of molecules.

Several factors determine the formation of exciplexes. Firstly, exciplex-forming species are typically planar organic compounds capable of adopting sandwich-like orientation. Thermodynamically, exciplex formations are more favorable in systems with greater difference between ionization potential of the donor molecule and electron affinity of the acceptor, which is a condition similar to those required for ion pair formation (3). However, exciplexes are distinguished from ion pairs by the fact that they produce fluorescent emission. Exciplex is characterized by a broad and red emission profile, which is often significantly different from emission spectrum of either molecule involved in exciplex formation (4). This feature may be potentially useful in modulating FRET by using an acceptor whose absorption spectrum overlaps with exciplex emission spectrum, but not with emission spectrum of either of the two constituent molecules.

Exciplex formation has also demonstrated potential for FRET signal cascading. Recent studies have utilized their occurrence in close-proximity and confined orientation to design exciplex-based sensors. In addition to their ability to cascade FRET signals (5), they have also been found to occur between DNA-conjugated chromophores (6) (4) (7). This makes exciplex formation a potential candidate for implementing a switching mechanism in a FRET logic device.

C. FRET Pass Gate

By leveraging the effect of angular separation on transfer efficiency and modulation of overlap integral using exciplex emission, the following is proposed as a potential design for a FRET pass gate. The proposed design uses a two-way exciplex-forming system as a channel that alternates between ON/OFF states depending on the wavelength of input signal. Instead of changing the alignment of a single channel chromophore to modulate energy transfer from input to output, two potential exciplex-forming donors are used to specify the orientation of exciplex in ON or OFF state. As each exciplex-forming donor provides a fixed point of reference for channel alignment, it intends to provide precise control over the orientation of the channel chromophore.

This design utilizes anisotropic nature of FRET in order to transfer energy between specific pairs of chromophores. It also utilizes exciplex formation in order to facilitate a shift in emission wavelength of a donor chromophore. Exciplex formation has been demonstrated in multiple studies for a pyrene-pyrelene pair attached along a single-stranded DNA, and this phenomenon results in emission spectrum extending at much longer wavelengths than that of either pyrene or pyrelene (4). By exciting a donor chromophore, it is hypothesized that an exciplex would form with a flexibly attached acceptor molecule and long-wavelength emission would result.

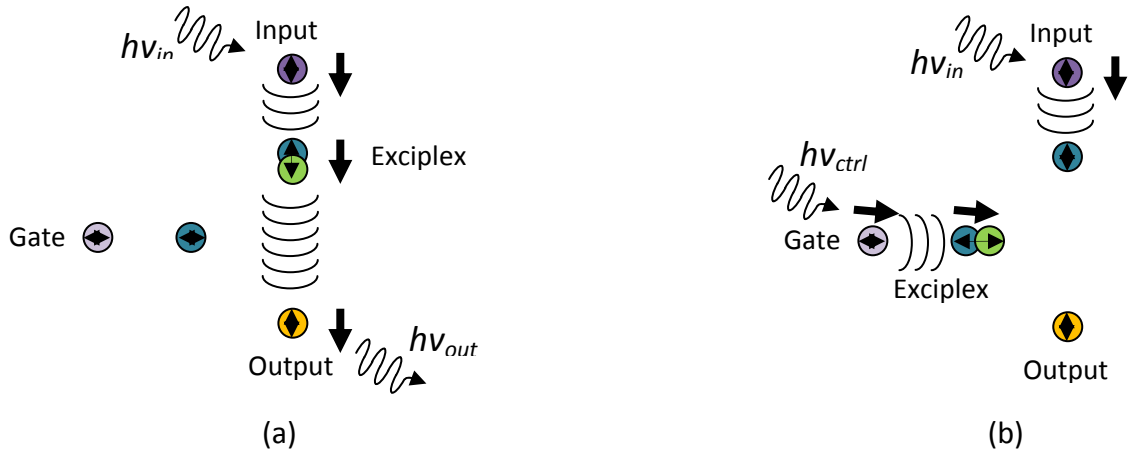


Fig. 2: Schematic of an inverting pass gate using preferential exciplex formation. (a) shows energy transfer between input and output via exciplex formed between intermediary chromophore and channel chromophore. (b) shows blocked transmission between input and output due to exciplex formation between channel chromophore and an intermediary chromophore excited by gate input.

Methods

This section is an overview of design goals, material selection and experimental design.

A. Design goals

This project aimed at exploring two aspects of chromophore interaction relevant to FRET logic. The first aspect was the study of angular dependence of transfer efficiency. Based on the principles established by Theodore Forster, angular separation between the emission transition dipole of the donor and the excitation transition dipole of the acceptor can be represented by a geometric factor κ^2, which ranges from 0 (orthogonal), 1 (parallel on a plane) to 4 (collinear). Since Forster radius is directly proportional to κ^2, theory stipulates that a misalignment of dipoles can result in a full attenuation of FRET. The second aspect was to study the formation of exciplex between donor and acceptor chromophores. Based on the knowledge that exciplex emission manifests as a redshifted, featureless peak, exciplex formation could be identified by

an uncharacteristic redshift to the acceptor emission peak when chromophores are located in close proximity.

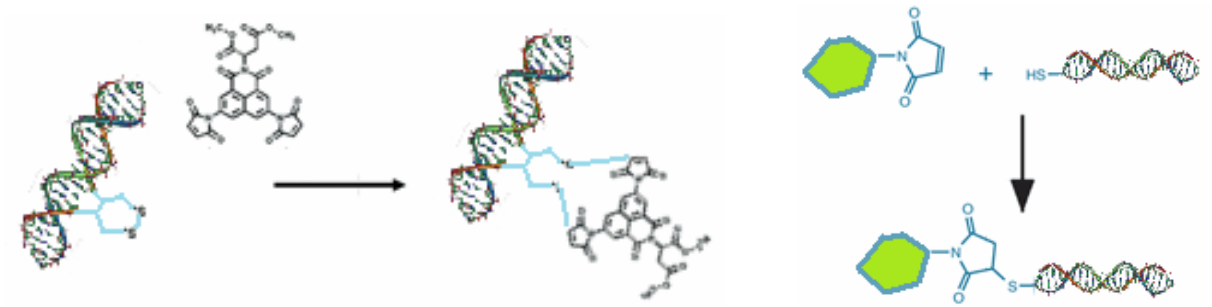
B. Material selection

a. Harness selection

A harness structure was proposed to provide a platform on which to test various chromophore interactions. It was initially designed to utilize the existing designs for DNA tiles that were immediately available in the Duke Self-Assembled Systems group. Upon further consideration, a custom 15-bp LNA-DNA hybrid was used as a harness structure. The use of LNA hybrid intended to confer a number of benefits to the harness, such as improved thermal stability and greater stiffness, resulting in an overall enhancement of structural integrity which is crucial for relating FRET signals to physical phenomenon. A potential drawback was the alteration of helix structure from a fully-hydrate B-form helix towards an A-form helix, which changes the geometric relations between the base-pairs.

b. Conjugation selection

Conjugation is an important aspect of the experiment, as the length and rigidity of the linker between the chromophore and the supporting structure influences chromophore interactions in the microenvironment, which in turn affects the observed fluorescence signal. Several linkers were considered in this experiment. Maleimide-thiol conjugate was selected on the basis of its availability and accessibility.



Diag. 2: Visualization of bismaleimide-DTPA and maleimide-thiol conjugates

i) Maleimide-thiol conjugate

Maleimide-thiol conjugates are one of the most conventionally used conjugates. The double bond in the unsaturated imide reacts readily with thiols to form a stable thiol-carbon bond, which makes it a widely adopted linker in the biotechnology industry. This type of linker has also been used in previous studies by the Duke Self-assembled Systems Group, and therefore

demonstrated its compatibility with the DNA tile structures that will eventually serve as platforms. An advantage of using this conjugate is the wide selection of chromophores that are commercially available in maleimide form. The extension of thiol linkers with carbon-carbon spacers was initially a concern, as an extended linker has been shown to result in chromophore interaction with the oligonucleotide and result in quenching (8). This problem was addressed by using the shortest spacers available (6C) from Integrated DNA Technologies (Coralville, IA), which constrains the chromophore to a position away from the helix. It is also expected that the conjugated chromophore would find itself in a rigid position rather than behaving like a free rotator, since the microenvironment around the DNA is known to be densely populated with various counterions.

ii) Bismaleimide-DTPA conjugate

Bismaleimides are unique in possessing two maleimide groups connected through a molecular unit. If made available to two thiol groups, these compounds would form a strong connection that would rigidly hold the target molecule at two points. Since the rigidity of bound chromophore is important for studying the effects of geometrical variation on resonance energy transfer, this type of conjugate was promising. The proposed design replaced one of the bases of an oligonucleotide with dithiol phosphoramidite (DTPA), a linker with two thiol groups provided by Glen Research (Sterling, VA) (9). It was found that the synthesis of bismaleimide fluorogens is an arduous process, and such chromophores are currently not commercially available (10). While interesting in theory, the use of bismaleimide-DTPA conjugate for the proposed study may require some time for these material to become widely available.

iii) Base-pair analogs

These chromophores have become available more recently than the other variants. The chromophore is attached directly to the phosphate-deoxyribose backbone of an oligonucleotide, replacing a nucleoside. This type of chromophore has a number of clear advantages in the proposed study. Firstly, the chromophores are placed in a highly confined position, sandwiched between two base-pairs and affixed directly to the backbone of the oligonucleotide. Secondly, some types of base-pair analogs can be used to replace any base positions on a given duplex strand.

A major drawback in using these chromophores has been attributed to their generally poor quantum yield, which is known to result mainly due to the quenching effect caused by neighboring base pairs (11). A related issue is the variability of quantum yield based on the types of base pairs in its vicinity (12), which limits the flexibility of using these compounds in systems that may require cascading of FRET signal across multiple chromophores. In addition, these chromophores are currently only available in small selections that generally share a similar excitation/emission profile, which limit our ability to form FRET pairs. While several studies have demonstrated synthesis of base-pair analogs (13), the process was identified as time-consuming and considered beyond the focus of this investigation (14). While base-pair analogs may be a promising solution to the goal of making chromophores geometrically fixed

with respect to the platform structure, further refinement may be required in order to increase the quantum yields as well as to expand the selection of excitation and emission profiles. A promising development with respect to quantum yield is noted in the development of base-pair analogue tC, which exhibits a quantum yield of 0.2 at a wide pH interval (15).

c. Chromophore selection

In addition to conjugation, the selection of fluorescent compound is fundamental to this experiment. In order to provide a clear signal that could be attributed directly to a donor or an acceptor, chromophores with the following properties were sought: 1) large Stokes shift; 2) minimal overlap between donor excitation spectrum and acceptor excitation spectrum; 3) minimal overlap between donor emission spectrum and acceptor emission spectrum. A large Stokes shift in the donor chromophore provides a clear separation between excitation wavelength and emission spectrum, which helps exclude scattering from fluorescence signal. A minimal overlap between donor and acceptor excitation spectrum enables the assessment of donor fluorescence signal as resulting entirely from FRET, which makes it useful for an accurate estimation of transfer efficiency. Minimizing overlap between donor emission and acceptor emission spectrum also helps differentiate between signals attributed to the donor and acceptor chromophores, which is useful when additional signals such as exciplex emissions are being sought.

In the initial phase of this project, a number of exciplex-forming chromophore pairs were identified from literature and sought for use with the harness structure. However, this plan was altered when many of the species mentioned in the list were found to be synthesized to oligonucleotides by individual laboratories, rather than being available commercially. The following list may be a useful reference for future investigations of scale that may permit synthesis of chromophore conjugates.

Donor	Acceptor	Excitation Peak (nm)	Emission Peak (nm)	Solvent
Pyrene	Dimethylaniline	345	480	Aqueous
Pyrene	Perylene	348	490	Aqueous
Anthracene	Dimethylaniline	358	460	Diethyl ether
Naphthalene	Anthracene	366	450	Aqueous
Perylene	Dimethylaniline	350	460	--
Diethyaniline	Anthracene	350	465	N-hexane
Pyrene	Ag+	334	375~385	Aqueous

Table 1: Exciplex-forming species in literature (16) (17) (18) (19) (20) (21)

i) Donor chromophore

Alexa Fluor 350, a coumarin derivative, was selected as the donor chromophore. With an excitation peak at 346nm and emission peak at 442nm, the chromophore provides a very large Stokes shift of ~100nm. The chromophore was also selected on the basis of its high water

solubility and brightness of its fluorescence in conjugated form, which were desirable properties in this experiment (22).

ii) Acceptor chromophore

Oregon Green 488, a fluorescein derivative, was selected as the acceptor chromophore. Often used in cellular imaging, this chromophore was selected on the basis of its high photostability and brightness of its fluorescence in conjugated form (23).

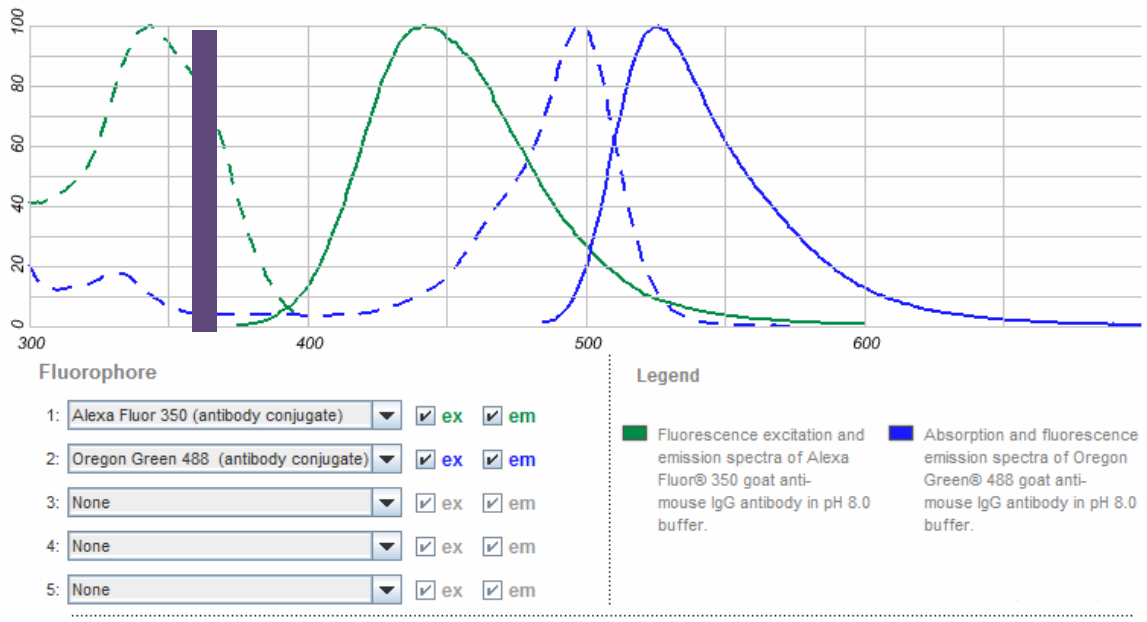


Fig. 3: Excitation spectra (dotted line) and emission spetra (solid line) of donor-acceptor pair selected for this study. (Screenshot obtained from Invitrogen Spectra Viewer) Excitation source used in the following experiment is highlighted in violet (24).

C. Experimental Procedure

a. Procedure Development

i) Conjugation

Conjugation reactions in this project were based on the procedure that was previously developed in the Duke Self-Assembled Systems Group, which itself is based on a guideline suggested by Molecular Probes, a subsidiary of Invitrogen (Carlsbad, CA) (25). The procedure initially uses a 50-fold excess of TCEP to reduce disulfide bonds on thiolated oligonucleotides, followed by the addition of maleimide chromophores as reactants in a 60-fold excess.

ii) Filtration

Filtration procedure was initially developed using exclusion matrix filter columns purchased from Sigma-Aldrich (St. Louis, MO). However, an unidentified preservative found in all columns was found to prevent hybridization of the oligonucleotides after filtration, preventing its use.

An alternative procedure was developed using Amicon YM-3 membrane filters from Millipore (Billerica, MA). A standard procedure was developed after experimenting with various configurations of buffers and operating temperatures. With increased temperatures, it was found that filtration took place more rapidly. This was attributed to dilation of pores due to increased temperature. As the total size of the harness structure (~6kDa) was very close to the molecular weight cut-off of the filter column (3kDa), temperature was kept at 10^{o}C during centrifugation. This temperature was also maintained in order to prevent the annealed structure from denaturing.

A heuristic was developed in order to determine the appropriate number of filtration iterations before a sample was considered to be purified. The fluorescence signal of filtrate was observed after successive iteration and filtration process was terminated when the signal appeared to reach steady state, which may suggest a constant dispensing of dyes as a result of conjugate-filter interactions rather than the presence of excess unconjugated dyes.

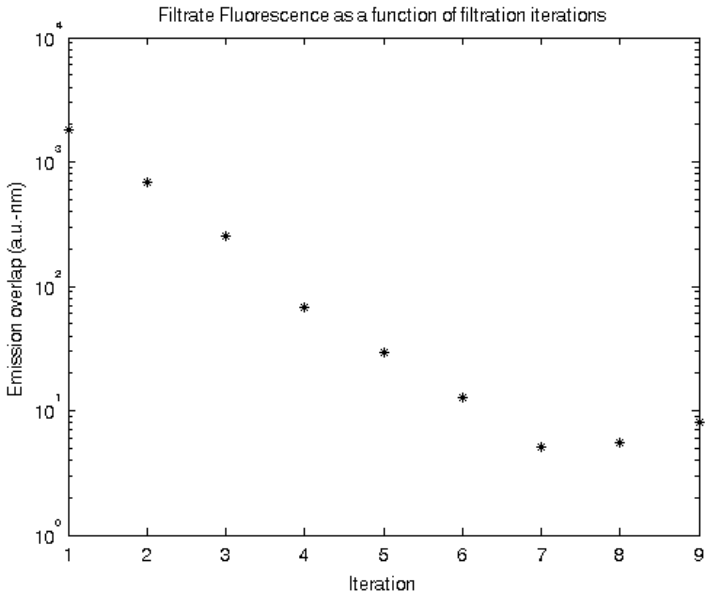


Fig. 4: Fluorescence signal integral after each filtration step. As shown here, filtrate signal reached steady state at steps 7~9, resulting in termination at step 9.

iii) Harness annealing

Annealing of conjugated harness was achieved by placing the sample in 55^{o}C for 30 minutes and cooling to 4^{o}C over 1 hour period in dark. As both of the 3' strands were below the molecular cutoff of the membrane filter, annealing of the constituent strands preceded all procedures pertaining to filtration.

iv) Buffer

The choice of buffer affected three aspects of the experiment: 1) harness/chromophore conformation based on DNA hydration, 2) chromophore fluorescence, 3) thermal stability of harness structure. It was identified through an extensive study that the presence of dimethyl sulfoxide (DMSO) from conjugation reaction affected chromophore behavior significantly, resulting in a major shift in emission profile. In order to minimize this phenomenon, DMSO was gradually removed through a filtration process that added 400uL 1xTAE buffer with Mg^{2+} prior to each iteration.

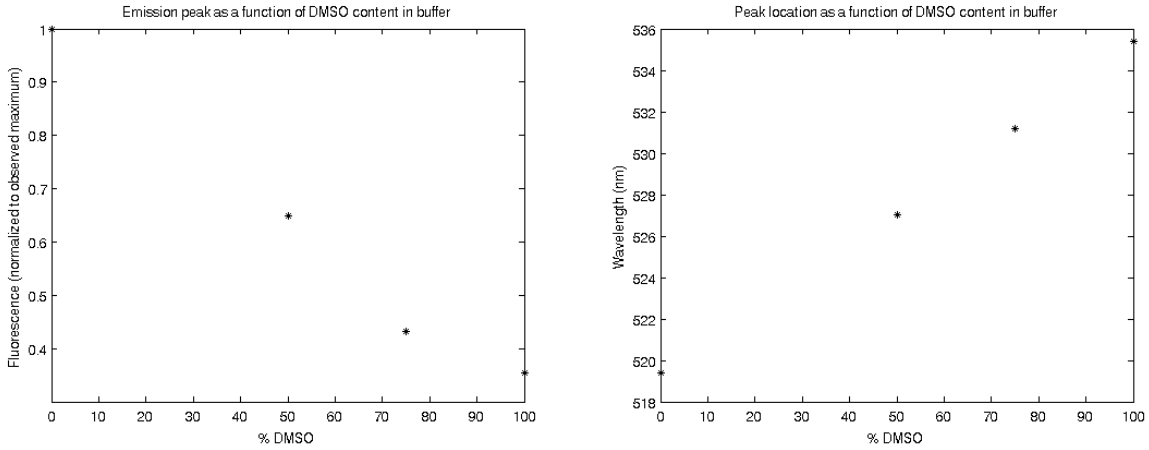


Fig. 5: *(LEFT)* Plot of peak intensity as a function of DMSO percentage in buffer (by volume). As shown, an increase in DMSO content results in significant quenching of the chromophore (Oregon Green 488). *(RIGHT)* Plot of peak wavelength shift as a function of DMSO percentage in buffer (by volume). As shown here, the increase in DMSO content also shifts the emission profile of the chromophore (Oregon Green 488) towards the red, indicating an increase in modes of energy dissipation. It is hypothesized that dye-DMSO interaction may be responsible.

v) Signal acquisition

Steady-state fluorescence measurement was performed using a custom spectrofluorometry setup with thermal control. Temperature was varied between 10°C and 55°C. Fluorescence signal was obtained with 180 seconds of acquisition time per data point. Fluorescence measurement was performed with 0.µM stock solutions of quinine sulfate in 0.5M H_2SO_4 as

reference dye (QY = 0.55 at 349nm λ_{ex}) (26).Reference signal was obtained at the lowest-

temperature point of each data set. All waveforms were normalized to the peak emission intensity of the reference waveform.

vi) Data pre-processing

Raw spectral data was smoothed using a 2nd order Savitzky-Golay filter with a span of 81 points out of a total of 2048 points spanning from 176.25nm to 878.98nm. This span width was identified by generating multiple waveforms using various span widths and identifying one that most closely resembled the profile expected from Alexa Fluor 350 emission from Invitrogen Spectra Viewer. This region is located between wavelengths of 425nm and 465nm and was chosen on the basis of its distinct feature, separation from scattering signal at 360nm, and the lack of overlap with emission profile originating from Oregon Green 488. In addition, any offset

observed in the non-emissive wavelengths (λ<325nm, λ>660nm) was subtracted from the

waveform.

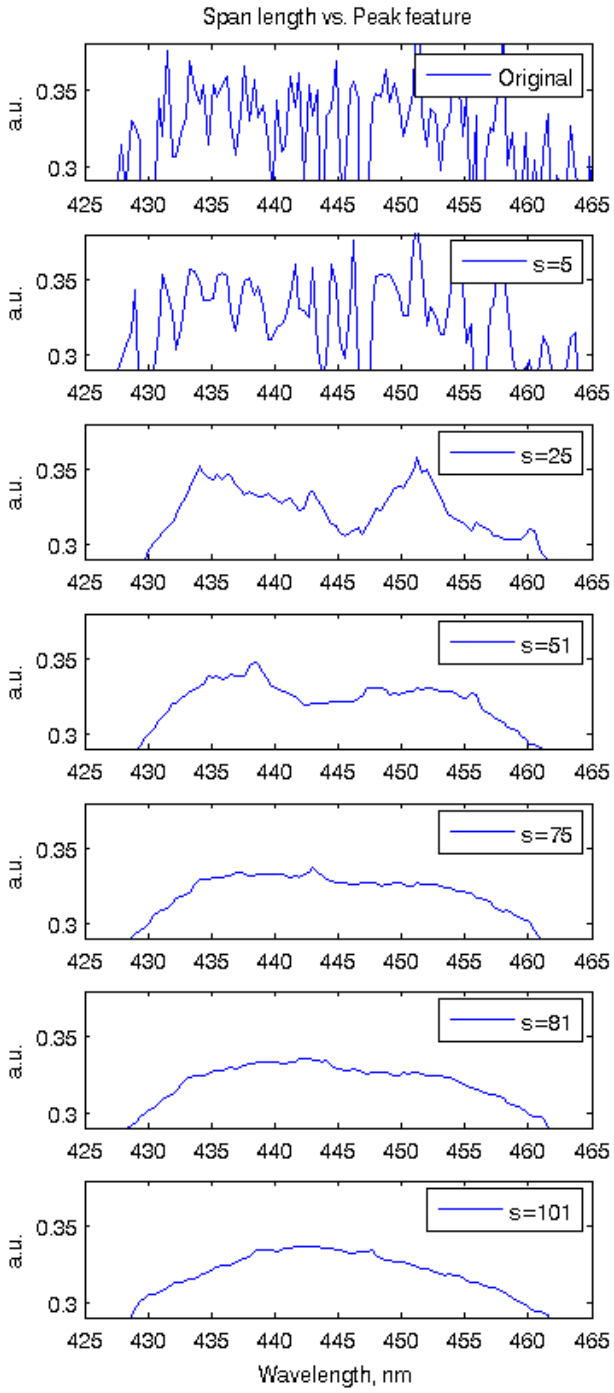


Fig.6: Smoothing of waveforms using a 2nd order Savitzky-Golay filter of varying span sizes. Span of 81 points appeared to best capture the profile of donor emission waveform.

b. Outline of Procedure

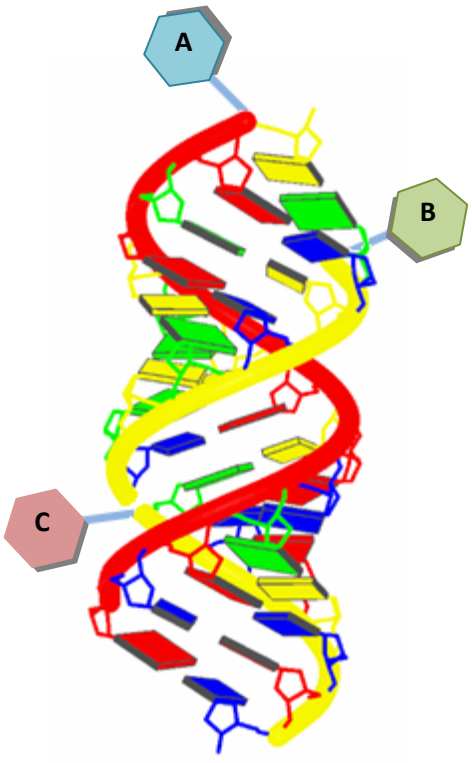
i) Materials and Test Setup

The DNA harness consisted of three strands: 1) a 5' strand 'backbone' consisting of a thiolated 5' terminus and a locked-nucleic acid (LNA) at several locations; 2) a 7-bp 3' strand consisting of a thiolated 3' terminus; 3) an 8-bp 3' strand consisting of a thiolated 3' terminus. The LNA segments were positioned proximal to nicks and terminus of the duplex in order to ensure rigidity of strand at these regions, thereby retaining expected dimensions once the strands have annealed. The use of two 3' strands provided a total of three possible sites for conjugation of chromophores, enabling study of FRET behavior in three configurations.

Strand Name	Sequence	Tm (^{o}C)
5' strand	5'thiol-6C-[A][T]AGTA[C][A][C][G]CAG[A][C]-3'	46.3
3' strand #1	5'-GTCTGCGTGTACTAT-6C-3'thiol	18.2
3' strand #2	5'-TGTACTAT-6C-3'thiol	2.7

Table 2: List of strands used to construct DNA harness structure. Square bracket represents LNA bases. Thiolated termini are indicated.

The following is a list of configurations tested, using a visual representation of the DNA harness represented as a B-DNA (Generated using 3DNA) (27).



A	**B**	**C**	**Name**
Acceptor	Donor	Vacant	Pair On Top
Acceptor	Vacant	Donor	+90^{o}
Vacant	Acceptor	Donor	-90^{o}

Table 3: Configurations to be tested using DNA harness structure. A separation of 8bp produces approximately 85.7^{o} angular separation radial from helical axis. If dipoles are assumed to be at a fixed orientation from the bases to which the chromophores are conjugated, then this separation produces a near-orthogonal orientation. As such, FRET efficiency is anticipated to be strongly attenuated.

ii) Conjugation

Alexa fluor 350 C5-maleimide and Oregon Green 488 C5-maleimide were purchased from Molecular Probes, a subsidiary of Invitrogen (Carlsbad, CA) conjugated to thiolated ssDNA strands custom ordered from Integrated DNA Technologies (Coralville, IA). ssDNA was stocked in 1xTAE buffer with Mg^{2+}. TCEP solution was added to ssDNA in 1:50 ratio (by concentration) and was incubated in dark for 30 minutes under 80^{o}C. Chromophore stocked in DMSO was added to the resulting ssDNA solution in 1:60 ratio (by concentration) and was incubated in dark for 3 hours under 80^{o}C, and then incubated in dark for 24hrs at 4^{o}C.

iii) Annealing

The resulting solution was mixed with complementary strands in 1:1:1 ratio (by concentration) and incubated in dark for 3 hours under varying temperature conditions: 1hr under a temperature ramp from 23^{o}C to 55^{o}C, 1hr at 55^{o}C, and 1hr under a temperature ramp from 55^{o}C to 4^{o}C.

iv) Filtration

Amicon YM-3 membrane filters purchased from Millipore Corp. (Billerica, MA) were used to purify annealed structures from the prepared solution. Filter was washed twice using 500uL dH2O and 500uL 1xTAE buffer with Mg^{2+} at 10^{o}C and 14000g prior to filtration steps. Each filtration step was stopped after confirming 100uL retentate volume. Each filtration step began by adding buffer solution until total volume in the membrane filter reached 500uL. First two steps in filtration used 1xTAE buffer with Mg^{2+} containing 50% DMSO by volume; subsequent steps used 1xTAE buffer with Mg^{2+}. Fluorescence signal was measured for the filtrate obtained after successive iteration of the filtration process, until successive measurements resulted in constant fluorescence intensity.

c. Data analysis

It was hypothesized that the increase in temperature introduces eventual dissociation of the DNA harness structure into its constituent parts, resulting in a separation between donors and acceptors that greatly exceeds their Forster radius as free rotators. Using this assumption, transfer efficiency was estimated from the observed data by the following equation:

$$\Phi_T = 1 - \frac{I_D}{I_{DH}}$$

The quantity I_D represents reference-normalized donor emission peak intensity, while I_{DH} represents the same quantity when T>>Tm. As fluorescence signal tended to remain unchanged for temperatures above 45^{o}C, T>45^{o}C was used as a point of reference for an approximately complete dissociation of the harness structure in sample.

In order to compare the experimental results with expected orientations of the chromophores, experimental values of transfer efficiency was compared against expected efficiency for free

rotators. Transfer efficiency was computed analytically using the formula for Forster radius and transfer efficiency. Molar extinction coefficient of acceptor was found to be 70,000 M cm^{-1} at 496nm, and was used to scale absorbance spectra obtained from Invitrogen (28). Fluorescence emission waveform of Alexa Fluor 350 was obtained using the spectrofluorometry setup, while its quantum yield was estimated using the published experimental value of 0.55, obtained by Molecular Probes (29). Geometrical parameters of the chromophores were estimated by assuming radial alignment of dipoles from the helical axis. The helix was represented as a cylindrical object resembling a B-DNA, with a diameter of 20Å and rotation of 10.5bp per turn (30). While the presence of LNA in every two bases of the 5' strand may result in some shift in conformation towards A-DNA, the resulting changes in parameters were considered minor (26Å diameter and 11bp/turn).

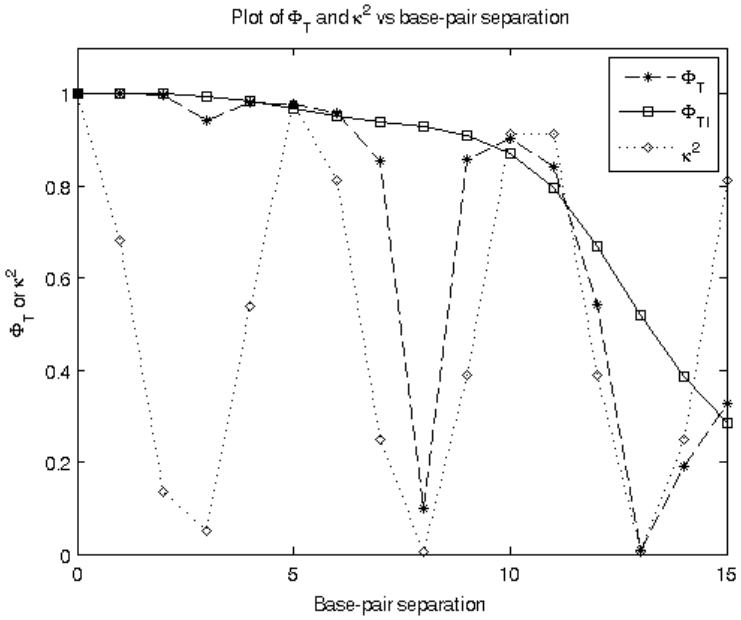


Fig. 7: FRET efficiency and orientation factor as a function of base pair separation. Note the general consistence of FRET efficiency over the distance of 15bp when isotropy of

chromophores is assumed (Φ_{TI}, solid line). This is in contrast to the pronounced attenuation at

8bp separation when radial alignment of dipoles is assumed (Φ_{T}, dashed line). This figure

suggests that for AF350-OG488 FRET pair, distance of 8bp does not result in a significant attenuation of FRET efficiency. The three harness configurations are designed to test whether FRET anisotropy is observed at this position.

Results and Conclusion

This study shows several interesting results. First, there appears to be a major anisotropy in FRET taking place for all three harness configurations tested, based on the discrepancy of measured transfer efficiency to the calculated transfer efficiency for isotropic donor-acceptor pair. Second, there is indication of intense quenching at the two configurations that were anticipated to behave like orthogonally-placed FRET pairs.

a) Transfer efficiency as a function of temperature

Analytical evaluation suggest R_0 = 45.6Å for free rotators, which would result in Φ_T=0.993 for

pair on top, Φ_T=0.949 for +90° configuration and Φ_T=0.966 for -90° configuration. Interestingly, the observed results show significantly smaller values of Φ_T in harness structure. This result is indicative of significant anisotropy in FRET between the chromophores in all 3 configurations. The similarity of results obtained for +90° and -90° configuration and their differences with the result from pair on top configuration imply that there may be a correlation between anisotropy and FRET efficiency.

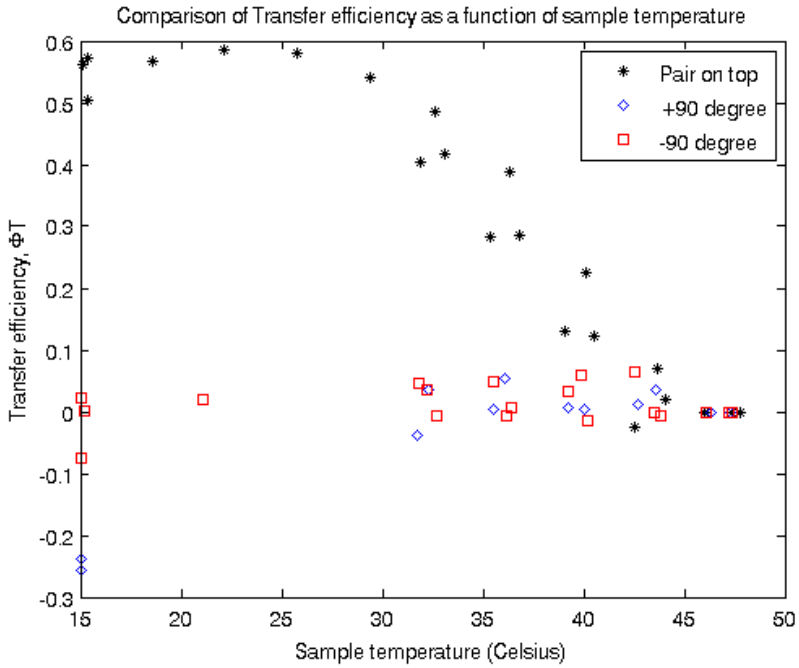


Fig. 8: FRET Efficiency for three chromophore harness configurations vs. temperature

b) Acceptor emission peak shift as a function of temperature

The purpose of this observation was to identify presence of exciplex emissions that may be hidden in the acceptor emission waveform. As it was not possible to identify any distinct emission peak that was not associated with either of the two chromophores, it was anticipated that any small deformations in acceptor emission profile may have been due to exciplex emissions that were small but present. This would have been indicated by a gradual blue-shifting of acceptor emission peak wavelength with increased temperature, as dissociation of structure results in increased separation between chromophores and reduces contact-based events such as exciplex formation taking place.

However, no clear trends could be observed from acceptor peak shift data. It seems likely that any exciplex signal that may have existed may have been below the noise level of the data acquisition device, which would mean that the observed data predominantly reflects signal fluctuations due to noise.

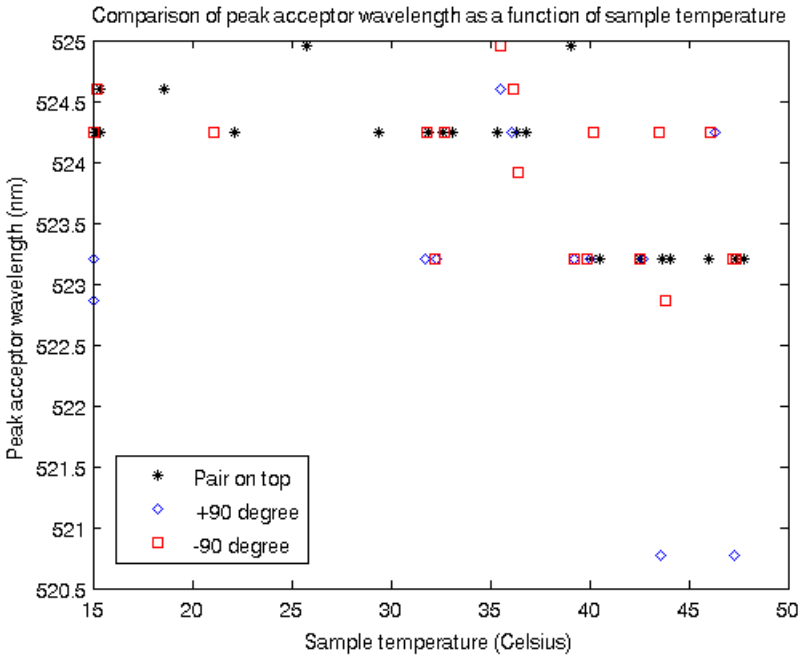


Fig. 9: Acceptor emission peak wavelength vs. temperature

Discussion

a) Interpretation of results

It is noted that +90^{o} structure yields negative transfer efficiency when structure is formed. In this case, the estimate for transfer efficiency no longer applies. The observed signal shows a decrease in both acceptor and donor fluorescence as temperature is increased, which may

suggest that there may have been an enhancement of donor fluorescence as a result of its conformation in the harness structure. The acceptor in any of the 90^{o} structure is conjugated to the central nick of the harness, which may suggest that there may be enhancement due to interaction between the bases and the chromophore via contact with the groove. Such phenomenon has been previously observed in Cy3-conjugated dsDNA, where the interaction of the dye with double helix results in conformation change of the dye and produces enhanced fluorescence (31).

The lack of clear evidence for exciplex signal highlights several issues. The first is the inability to recognize such a signal in absence of prior knowledge regarding its profile and relative magnitude with respect to fluorescence emission from independent chromophores. While the experimental setup for this investigation anticipated strong emission that could be distinctively observed in the vicinity of the red end of the acceptor emission spectrum, emission profiles suggest that this was not the case. One possible hypothesis is that the length of tethers may have been too short to enable contact between the chromophores in the appropriate orientation for exciplex formation. Considering the diameter of DNA harness (20 Angstroms assuming B-DNA analogue), it may be that the chromophores were unable to reach each other on the other side of the helix, preventing exciplex formation.

b) Future directions

Several studies may be necessary to investigate the properties of exciplex formation before its proposed use as a switching mechanism in a FRET system can be realized. An important phase would be to expand the database of exciplex-forming pairs and their affinity towards forming exciplexes. An experiment with two species of unconjugated dyes could demonstrate the formation of exciplex while overcoming the issue of noisy signal. The sample may be prepared in great excess of donor chromophores, which is expected to provide an environment favorable to exciplex occurring with the small amount of acceptors available.

Another possible study would be to vary the lengths of conjugate tethers in order to observe its effect on FRET efficiency and formation of exciplexes. Lengthening the tethers may result in greater freedom of movement of individual dyes, which may come at a cost of increased isotropy but may also result in an observable exciplex emission.

An extension of this study would be to demonstrate preferential binding of exciplex pairs using two known exciplex-forming species with a common acceptor. By exciting one donor and gradually increasing the intensity of excitation source for the second donor, a transition from one exciplex emission to another could be identified. If the two donors or exciplexes exhibit observable differences in lifetime, time-resolved fluorescence measurements may reveal when formation of one species of exciplexes begin to dominate over another.

The result of such a study could be tested on a DNA platform such as the chromophore harness used in this investigation. These investigations may provide key steps towards synthesizing FRET pass gates based on exciplex formation.

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