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Laboratory Experiment

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Evaluating the Relationship between FRET Changes and Distance Changes Using DNA Length and Restriction Enzyme Specificity

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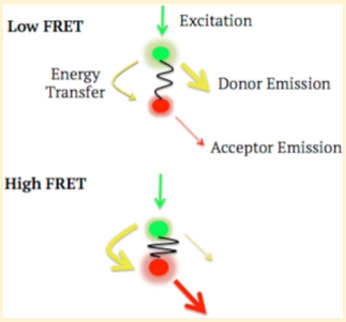
Supporting Information

ABSTRACT: FRET (Förster resonance energy transfer) involves the transfer of energy from an excited donor fluorophore to an acceptor molecule in a manner that is dependent on the distance between the two. A biochemistry laboratory experiment is described that teaches students how to use FRET to evaluate distance changes in biological molecules. Students measured the apparent FRET between donor and acceptor fluorophores located on the ends of several DNAs of unknown lengths, enabling them to order the DNAs according to size. In addition, students investigated site-specific DNA cleavage by restriction endonucleases, using loss of apparent FRET to determine which enzyme cut sites were present in each of the DNAs. After completing this experiment, students understood the inverse relationship between changes in FRET and changes in distance, and understood how changes in FRET could be used to monitor a conformational change in a molecule. As an extension to the experiment, a tutorial is included that uses the same DNAs to illustrate the ability of single-molecule FRET measurements to resolve heterogeneity in a sample, which cannot be done via more traditional ensemble measurements.



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OVERVIEW AND LEARNING OBJECTIVES

It is important to expose emerging scientists at the undergraduate level to the principles of FRET (Förster resonance energy transfer). Students continuing in biomedical research are likely to encounter FRET since it has become a widely used tool in many academic and industrial research settings to probe biochemical and biological mechanisms in vitro and in cells (see refs 1−4 for reviews of using FRET to study biological systems). The current biochemical education literature contains a handful of FRET experiments.[5−8] Some of these are geared toward more advanced physical chemistry or biophysics courses;[6,8] the experiment described here is more appropriate for a general biochemistry lab course. This experiment uses readily available, as opposed to specialized,[5] reagents. It has a high success rate (i.e., the experiment "works" for almost all students). Also distinct is the focus on demonstrating the relationship between changes in FRET and changes in distance using measurements of apparent FRET, which are straightforward to make. This approach is illustrated using two approaches: DNA length and restriction enzyme specificity. Although restriction enzyme specificity is not new to the biochemical education literature (for examples, see refs 9−12), its application to understanding concepts of FRET is unique.

The main student learning goals are to understand (1) the inverse relationship between changes in FRET and changes in distance, and (2) how changes in FRET can be used to monitor a conformational change in a biomolecule. In this experiment, double stranded DNAs of different lengths with donor and acceptor fluorophores on their 5′-ends were used as a model system. Each DNA also contained a different restriction endonuclease cut site. When the DNA was cut by its specific endonuclease, the apparent FRET was lost. The students did not know the relative lengths of the DNAs or which restriction sites were present in each DNA. The students were required to apply their knowledge of the principles of FRET to order the DNAs from shortest to longest and to identify which restriction site was present in each DNA. Hence, at the conclusion of the experiment, students understood that apparent FRET decreased as the distance between the fluorophores increased, and that changes in FRET enabled them to detect a conformational change in the DNAs (i.e., going from uncut to cut).

Achievement of these learning goals was assessed by the students' ability to (1) orally explain their experimental data to the instructor at the end of class (when they checked to see if they ordered their DNAs correctly and determined cut sites correctly), and (2) provide clear written results in their lab notebooks. This experiment developed skills in data interpretation and drawing conclusions. In addition, a tutorial is provided that complements the experiment and introduces students to single-molecule measurements (see the Supporting Information). The tutorial provides a unique pedagogical opportunity to introduce a timely and cutting-edge technology.

BACKGROUND AND THEORY

FRET is the distance-dependent radiationless transfer of energy from an excited donor fluorophore to an acceptor molecule (for

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J. Chem. Educ. XXXX, XXX, XXX−XXX

reviews on FRET theory and applications see refs 13−16). For FRET to occur, the excitation spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor. During FRET, the efficiency of transfer (E) is defined as the probability that an excited donor decays by transferring its excitation energy to the acceptor. This is dependent on the inverse sixth power of the distance between the donor and acceptor molecules (R):

$$E = R_0^6/(R_0^6 + R^6) \quad (1)$$

where R_0 is the distance at which E = 0.5 (the Förster radius); different donor/acceptor pairs have different R_0 values. FRET is sensitive to small changes in distance between the donor and acceptor, which is the primary basis for its use in investigating inter- and intramolecular changes in biomolecules. Typically, FRET occurs between donor and acceptor molecules spaced approximately 10−100 Å apart, a distance well suited for probing biological mechanisms.[1−4]

For the experiment described here, the primary objective is to demonstrate the relationship between changes in FRET and changes in distance. Therefore, changes in the apparent FRET between samples were used to evaluate differences in DNA lengths and conformation. The apparent FRET, also referred to as the proximity ratio, is a read-out for monitoring relative distance changes between donor and acceptor molecules. It is not a precise FRET efficiency, so it cannot be used as a molecular ruler to determine the exact distance between the donor and acceptor molecules.

Instructors can adapt this experiment to measure a precise FRET efficiency and calculate distances between the fluorophores. To do so, additional factors that depend on the fluorophores and the instrument used to measure FRET must be considered. These include parameters such as the quantum yield of the fluorophores, the relative orientations of the donor and acceptor molecules, direct excitation of the acceptor fluorophore by the donor laser, and bleedthrough of donor emission into the acceptor channel, which are described in more detail elsewhere.[13−16] We have previously described a biochemistry experiment that measures FRET efficiency in order to calculate distances.[5] Although successful, some students found the additional measurements and calculations to be confusing; the use of apparent FRET described here has been an effective tool to introduce biochemistry students to the concept of FRET, albeit at the expense of determining distances between fluorophores.

EXPERIMENTAL PROCEDURES

Time and Teams

This experiment was taught in a general biochemistry laboratory course. The experiment and data analysis were completed in one 5 h laboratory session, with students working in groups of three. More information about the course, reagent and equipment needs, and experimental details is found in the Instructor Notes (Supporting Information).

Design of the DNAs

The experiment used four double stranded DNAs that were 12, 15, 18, and 21 basepairs (bp) in length; one 5′-end contained a donor fluorophore (Cy3) and the other contained an acceptor fluorophore (Cy5) (Figure 1). The R_0 for Cy3 and Cy5 is ∼55 Å, making this FRET pair sensitive for detecting differences in the lengths of the DNAs used (∼40−70 Å). In addition, the DNAs each contained a recognition sequence for a different restriction endonuclease (boxed sequences in Figure 1).

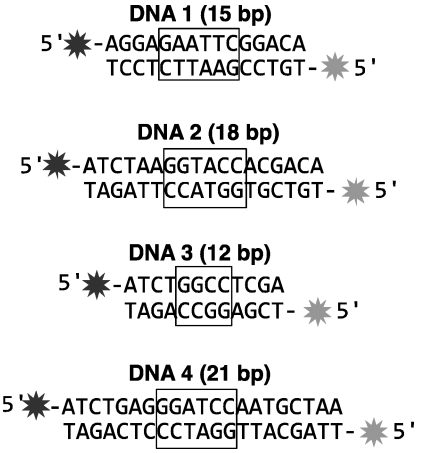

Figure 1. DNA constructs used for the FRET experiment. The dark gray star represents Cy5, the light gray star represents Cy3. Restriction enzyme cut sites are boxed; EcoRI, KpnI, HaeIII, and BamHI recognize DNAs 1−4, respectively.

Sample Assembly

The instructor prepared in advance the double stranded DNAs shown in Figure 1 (the preparation protocol is described in the Supporting Information). During class, the students assembled the samples for the experiment using the following protocol. Five master mixes were prepared in 1.7 mL Eppendorf tubes according to Table 1. Note that the sample volumes or DNA concentrations can be increased as needed to accommodate the requirements of different fluorimeters. An aliquot (30 μL) of each master mix was added to 25 individual Eppendorf tubes according to Table 2 (e.g., the DNA 1 master mix was aliquoted to tubes 1−5). Restriction endonuclease was added (1 μL of 20 units/μL enzyme) according to Table 2 and mixed well. If the protocol is adjusted such that significantly more DNA is used, the amount of restriction enzyme may need to be increased. The sample tubes were incubated at 37 °C for 30 min. A portion (25 μL) of each reaction was added to individual wells of a 384-well borosilicate microplate (Intermountain Scientific Corporation), while keeping careful track of sample numbers.

Fluorescence Measurements and Apparent FRET Calculation

The microplate was scanned for fluorescence using a Typhoon Imager 9400 (GE Biosciences). Any fluorimeter or fluorescent imaging system that will excite Cy3 and collect emission from Cy3 and Cy5 can be used. The donor fluorophore (Cy3) was excited using the 532 nm laser and emission was collected with two filters: (1) 580 nm (with 20 nm bandpass) to collect donor emission, and (2) 670 nm (with 30 nm bandpass) to collect acceptor emission. The focal plane was set +3 mm from the surface, and the PMT was set at 600 V. The donor and acceptor emissions in each sample were quantified using the .gel files from the Typhoon scan and ImageJ software (as explained in the Supporting Information).

Background donor and acceptor emissions in the five wells with no DNA (samples 21−25) were averaged. These average values were subtracted from the fluorescence in each sample well (reactions 1−20). Apparent FRET efficiency (E_{app}) was calculated using the following equation:

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J. Chem. Educ. XXXX, XXX, XXX−XXX

Table 1. Recipes for the Five Master Mixes

Sample Components	Volume of Each Component Added To Make Each DNA Master Mix, μL				
	DNA 1	DNA 2	DNA 3	DNA 4	No DNA
H_2O (double distilled)	159	159	159	159	162
Cut Smart Buffer (10×)	18	18	18	18	18
DNA 1 Stock[a]	3	–	–	–	–
DNA 2 Stock[a]	–	3	–	–	–
DNA 3 Stock[a]	–	–	3	–	–
DNA 4 Stock[a]	–	–	–	3	–

[a]DNA stocks were ∼3−5 μM. The DNA concentration does not impact the outcome, but should be high enough to ensure a strong signal.

Table 2. Distribution of Experimental Samples

Master Mix	Sample[a] Tube Numbers 1−25 by Enzyme				
	KpnI-HF	EcoRI-HF	HaeIII	BamHI-HF	none
DNA 1	1	2	3	4	5
DNA 2	6	7	8	9	10
DNA 3	11	12	13	14	15
DNA 4	16	17	18	19	20
No DNA	21	22	23	24	25

[a]Each sample received 30 μL of the indicated master mix and 1 μL of the indicated enzyme. For example, reaction 8 received the DNA 2 master mix and the HaeIII enzyme.

$$E_{app} = A/(A + D) \quad (2)$$

where A refers to the background-corrected emission from the acceptor fluorophore (Cy5), and D refers to the background-corrected emission from the donor fluorophore (Cy3).

HAZARDS

The reagents for this lab are aqueous buffers, salts, DNA, and enzymes, which pose minimal risk. Personal protective equipment is recommended when working in the laboratory.

RESULTS AND ANALYSIS

Representative student data showing apparent FRET efficiency values are displayed in Table 3, and arranged according to the sample numbers displayed in Table 2.

For each DNA (i.e. each row), four of the apparent FRET values were similar and one was notably lower. This lower value revealed that the DNA was cut by a restriction endonuclease, resulting in a separation of the donor and acceptor fluorophores. Students concluded that EcoRI-HF cut DNA 1, KpnI-HF cut DNA 2, HaeIII cut DNA 3, and BamHI-HF cut DNA 4. The tight specificity of restriction enzymes was apparent because each enzyme cut one, and only one, of the DNAs. Moreover, the apparent FRET values of the uncut samples were very similar, showing that no off-target cutting occurred, and that the enzymes themselves did not appreciably impact the fluorophores and alter the observed FRET. The apparent FRET efficiency after cutting did not decrease to zero. Since this value was calculated without correcting for direct acceptor excitation and donor bleedthrough, E was non-zero even in the absence of energy transfer. Nonetheless, the changes in apparent FRET due to cutting were stark enough to allow students to easily determine cut from uncut.

Students used the apparent FRET values to order their DNAs from smallest to largest (i.e., highest to lowest apparent FRET). The four values for each uncut DNA construct were averaged and plotted in Figure 2.

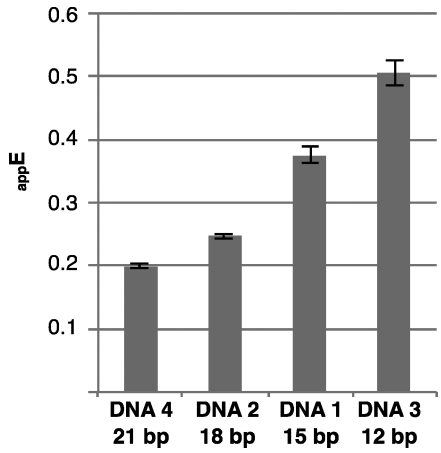

Figure 2. Average apparent FRET efficiencies for uncut DNA; the error bars are one standard deviation.

Students concluded the DNAs were ordered from longest to shortest as follows: DNA 4 > DNA 2 > DNA 1 > DNA 3. Through the use of DNAs of different lengths and cutting with different restriction enzymes, the relationship between changes in FRET and changes in distance was clearly illustrated. At the completion of the experiment, students were required to explain this principle of FRET when they checked to see if they

Table 3. Effect of Enzyme and DNA Length on Apparent FRET Values

Master Mix	Apparent FRET Value by Enzyme				
	*Kpn*I-HF	*Eco*RI-HF	*Hae*III	*Bam*HI-HF	none
DNA 1	0.384	0.102[a]	0.387	0.373	0.359
DNA 2	0.100[a]	0.249	0.249	0.249	0.240
DNA 3	0.513	0.516	0.118[a]	0.515	0.475
DNA 4	0.203	0.200	0.200	0.116[a]	0.196
No DNA	Background	Background	Background	Background	Background

[a]These data are the reactions in which an enzyme cut the DNA, increasing the fluorophore separation distance.

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J. Chem. Educ. XXXX, XXX, XXX−XXX

accurately determined the size ordering and restriction cut sites in each DNA.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00440.

A single-molecule FRET tutorial that complements the experiment; instructor notes, pre-lab preparation protocols, experimental tips, and student handouts/protocols (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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J. Chem. Educ. XXXX, XXX, XXX−XXX