Phys598 BP Spring 2016
University of Illinois at Urbana-Champaign

Lab 3: Single Molecule FRET

Location: Loomis 108 Ha lab
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Objective: In this module, we will learn about a powerful technique, **single-molecule fluorescence resonance energy transfer (smFRET)** and use it to study nucleic acids dynamics. We will also learn how to set up the optical system for performing smFRET experiments as well as different analysis approaches to interpret experimental data.

Experiment: G-quadruplex conformations and dynamics using FRET.

Advance Reading Materials

Background

- Single-Molecule Techniques: A Laboratory Manual. Cold Spring Harbor Laboratory Press ISBN 978-087969775-4, 507 pp (2008), Chapter 2, Joo, C. and T. Ha, "Single-Molecule FRET with Total Internal Reflection Microscopy."

- Roy, R., S. Hohng and T. Ha., "A practical guide to single-molecule FRET.", Nat. Methods 5, 507-516 (2008)

G-quadruplex dynamics

- Lee J.Y., Okumus B, Kim D.S., and Ha T. "Extreme conformational diversity in human telomeric DNA", Proc Natl Acad Sci. 102(52), 18938–43 (2005).

Hidden Markov Modeling-

- Rabiner, LR. 1989. A Tutorial on Hidden Markov-Models and Selected Applications In Speech Recognition. Proc. IEEE 77 (2): 257-286.

- Liu, Y; Park, J; Dahmen, KA; Chemla, YR; Ha, T. 2010. A Comparative Study of Multivariate and Univariate Hidden Markov Modelings in Time-Binned Single-Molecule FRET Data Analysis. J. Phys. Chem. B 114 (16): 5386-5403.

Introduction

Single-molecule fluorescence techniques are powerful methods widely used to study the properties of nucleic acids, proteins and their interactions. We will become familiar with the prism type TIRF microscopy which is a commonly used setup in single-molecule studies. The former method requires labeling the two interacting species in specific and unique locations and is a measure of the relative distance between the two dyes. The latter technique requires only one dye on a substrate and is based on an increase in the fluorescence intensity of this dye as a function of protein proximity. We will use these two techniques to first look at a model system for FRET, the Holliday Junction and then use FRET to record dynamics. Additionally, this module will give you an introduction to surface passivation, surface tethering, visualization of biomolecules, data acquisition and analysis of the sm fluorescence data.

Single-molecule FRET and TIR microscopy

The first technique that we will use for this training is based on Förster (or Fluorescence) Resonance Energy Transfer (FRET) and the method is total internal reflection microscopy (TIR). FRET is based on the energy transfer from an excited fluorophore (donor) to a second fluorophore (acceptor) in close vicinity through a non-radiative dipole-dipole coupling (Figure 1a).
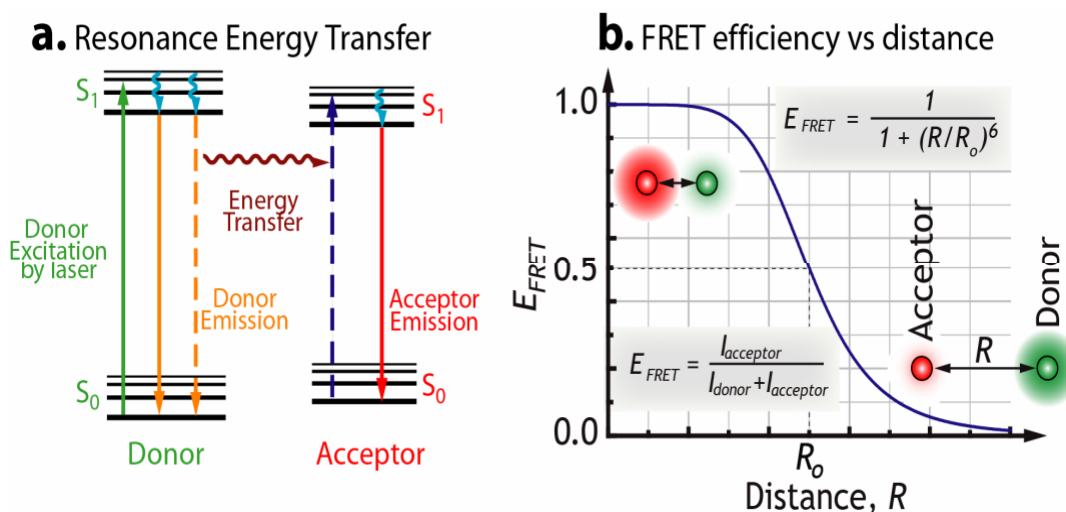

Figure 1. (a) FRET scheme in electronic states. (b) FRET efficiency and distance

The efficiency of energy transfer is given by $E = 1/(1+(R/R_o)^6)$, where R is the distance between the fluorophores and R_o is the Förster radius which is typically several nanometers. Figure 1b shows energy transfer efficiency as a function of distance between the dyes. Changes in the relative intensities of donor and acceptor provide us with a measure of distance between the two probes. We use the setup shown in figure 2, to excite the molecules and acquire data. The donor fluorophores (Cy3) are excited with a green laser ($\lambda = 532\ nm$). This wavelength is

outside the absorbance spectrum of the acceptor fluorophore (Cy5) so the only pathway for acceptor excitation is via FRET.
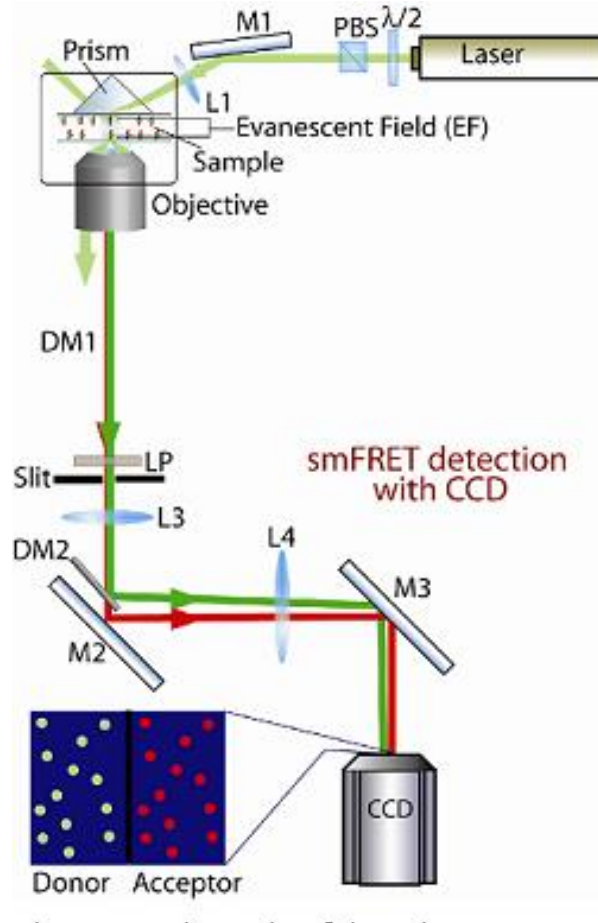
The signal which we record in our measurements is the fluorescence emission of the donor and the acceptor fluorophores. The very low penetration depth of the evanescence wave (due to TIR) excites molecules that are immobilized on the surface and thus enhances our signal to noise ratio. Donor and acceptor emission paths are separated and intensities are recorded by a sensitive EMCCD camera. Movies of an area that is approximately 35μm x 70μm containing 200-400 molecules are acquired using a homemade program. The data (intensities of donor and corresponding acceptor fluorophores spanning the movie time) are further analyzed by custom IDL and MATLAB codes. $E = \dfrac{I_A}{I_A + I_D}$ is a good approximation to calculate the experimental apparent FRET efficiency from acceptor intensity (I_A) and donor intensity (I_D). While this is not perfect for obtaining distance information between the dyes, it is a very effective way to determine relative changes in distance between the two fluorophores.

Surface Immobilization

To visualize single molecules in a TIR microscope, they must be immobilized on the slide surface at low densities so that individual molecules can be easily resolved and analyzed. We use a biotin-neutravidin-biotin linkage to immobilize DNA. DNA is biotinylated and neutravidin is 'sandwiched' in the middle as shown in Figure 5. Each neutravidin molecule has 4 binding sites for biotin. They bind to a biotin on the surface from one side and to a DNA molecule from the opposite site. There are two ways to immobilize the biotin on the surface: 1) biotinylated bovine serum albumin (BSA) can be used to adsorb to the glass surface (Figure 5a) and 2)

biotinylated PEG (See the text below; Figure 5b). In our module, we will use the second method because it is more reproducible and provides a better surface passivation.
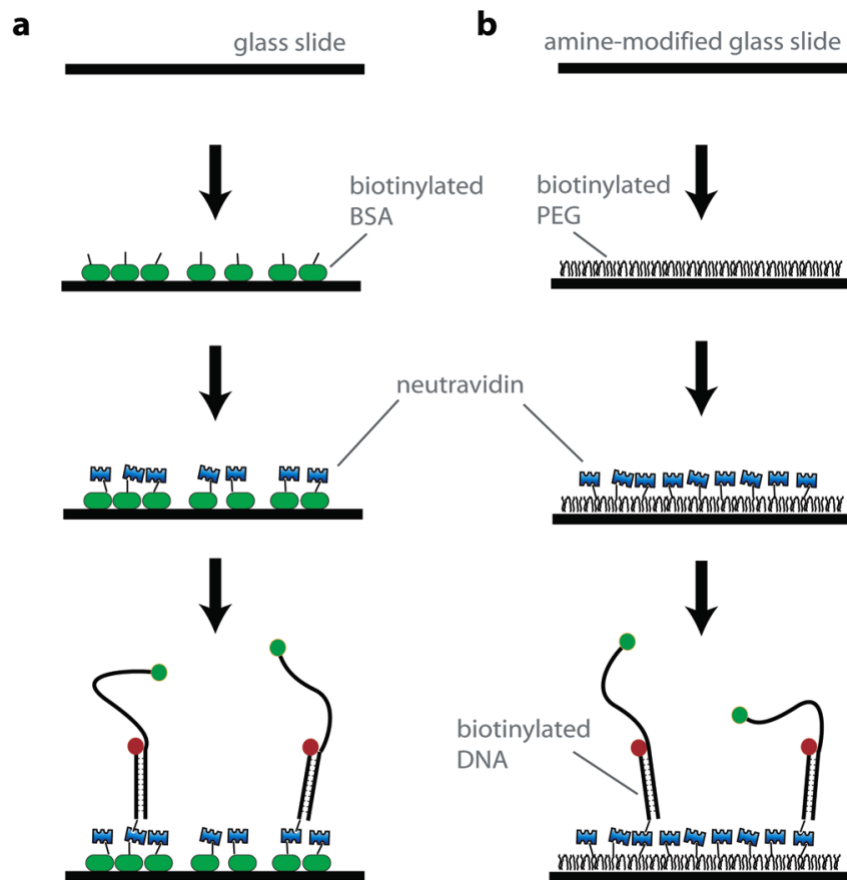

Figure 5. Surface immobilization. (a) Biotinylated-BSA surface and (b) PEG-coated surface.

Polymer-passivated surface preparation

When proteins are used in the experiments, a more rigorous surface passivation is required. In our lab we use a polyethylene glycol (PEG)-coated surface in order to eliminate nonspecific surface adsorption of proteins. The common protocol for preparing the PEGylated surface contains three steps (Selvin and Ha, 2007):
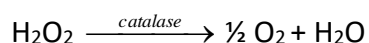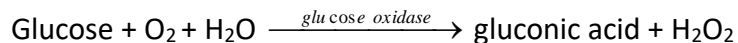
1.   Pre-cleaning and surface activation
2.   Aminosilanization of the surface
3.   PEGylation (Coating the amino-modified surface with PEG–NHS esters)

In the third step, a small fraction (~ 3%) of biotin-PEG–NHS ester (Bio-PEG-SC, Laysan Bio) is mixed with regular PEG–NHS ester (mPEG-SC, Laysan Bio) for the purpose of immobilizing biomolecules. The detailed steps are described in Selvin and Ha (2007).

Imaging Buffer:

One of the most important requirements in order to get high quality data in single molecule experiments is to keep the fluorescent dyes from reacting in the excited state to form permanently nonfluorescent compounds or entering nonfluorescing states. The first problem is called photobleaching and it happens when fluorophores permanently lose their ability to fluoresce due to photon-induced chemical damage. The amount of time it will take for this to happen will depend on the environment, the excitation energy, etc. The second problem is called blinking and it relates to the dye being trapped in a state that does not allow fluorescence, usually a triplet state. One of the most common culprits for both photobleaching and blinking is excited state oxygen. When these molecules collide with the excited state fluorophore they can permanently react with it or temporarily send it to a triplet state. These oxygen molecules are also the good guys in the sense that if a dye is in a triplet state they can also knock it out and return it to its normal singlet state. The photostability of Cy dyes is greatly improved by using the oxygen scavenger system and by reducing agents. The oxygen scavenger system consists of 0.8% w/v D-glucose, 1 mg/ml (165 U/ml) glucose oxidase, and 0.04 mg/ml (2170 U/ml) catalase.

The coupled reactions are:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\textit{glucose oxidase}} \text{gluconic acid} + H_2O_2$$
$$H_2O_2 \xrightarrow{\textit{catalase}} \tfrac{1}{2} O_2 + H_2O$$

Once the oxygen is removed, the likelihood of having excited state oxygen in solution is greatly decreased. This is both good news and bad news. Although reactive oxygen causes photobleaching, it is useful by knocking the dyes out of non-fluorescing triplet state. Therefore, a triplet state quecher is also necessary in the absence of oxygen. Previously, our lab used 140 mM β-mercapto ethanol (βME) as the triplet state quencher, but we have recently found that Trolox (Reference?) is more effective in quenching the triplet state and at much lower concentrations (1–2 mM) than βME.

Assembling the chamber:

Sample chambers are made by putting a quartz slide (1) and a glass coverslip (3) together with double-sided tape (2) and sealing with epoxy. The channel volume is about 20 µl and channel depth is about 70 µm. The holes on the quartz slide are used for the inlet and outlet of solution exchange through each channel. The holes are 0.75 mm in diameter and should be drilled in the slide prior to pegylation.
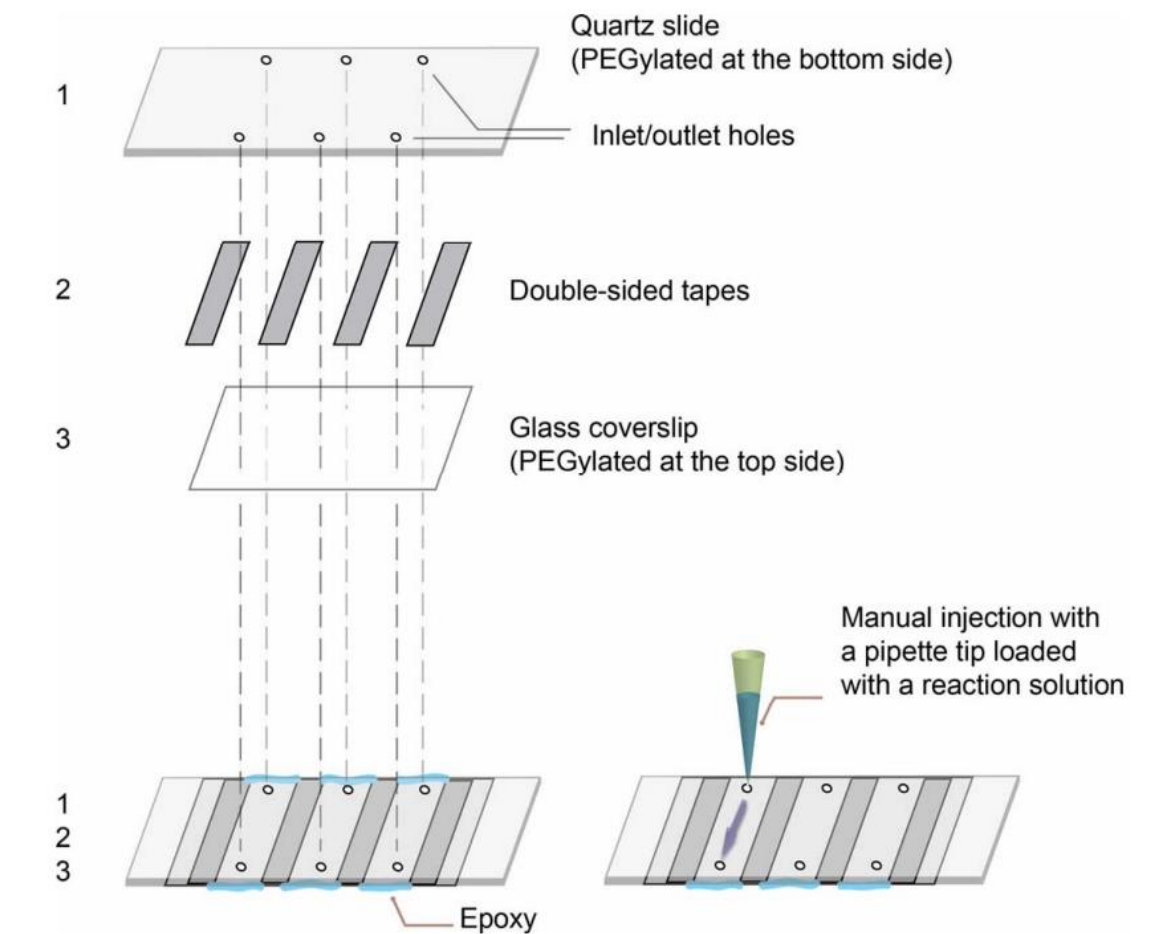

Figure 6. Assembling the flow chamber

Optical alignment protocol for setting up the TIRF microscope

Prior to performing the optical alignment, In a PowerPoint presentation, we will go over the basic principles of various optical components used for making a TIRF microscope. This will set us up well for performing the actual alignment the protocol for which is as follows:

1. Make sure you have all the components necessary for the alignment, eg. Lenses, mirrors, posts, post holders etc.
2. Set the heights of all the optical components (including the laser and the two irises) to approximately be the same height as the back port of the microscope (7.5 inches).
3. Mount the laser source and then begin alignment. Make sure all the optical components are tightened and positioned well before proceeding to the next one. This will ensure that if things are right, they stay right.
4. Place the first mirror (M1) and 2 irises along the same path of holes on the optical table. Changing the translation, rotation, and angle of the mirror let the beam roughly go pass the center of the irises.
5. Place the second mirror (M2) and ensure the light passes through the center of both irises positioned in the light path. The light is made to pass through the first iris by adjusting the first mirror, and the light is made to pass through the second iris by adjusting the second mirror. Do this iteratively until the light passes through both the irises.
6. Place the third mirror and the fourth mirror (M3 and M4) to guide the light to the back port of the microscope. Approximately direct the beam to the back port of the microscope without objective in place and let the beam go through the center of the slot of the objective turret by adjusting M3. Make the beam travel upward along a vertical path by adjusting M4.
7. Further iteratively align the beam using M3 and M4 while watching the beam spot on the TV or eyepiece. The spot is positioned to the center of TV/eyepiece by adjusting M3. Adjust M4 for a perfectly symmetrical beam profile. This is equivalent to the iteration procedure we employed with the irises. This time we ensure the beam is going straight through the objective lens.
8. Once the mirrors are placed, we can start placing lenses. A thumb rule for placing each lens is that there should be no distortion introduced to the beam path by the lens. Hence adjust the beam height, tilt and position by keeping this in mind. Do not touch or adjust mirrors at this point.
9. We plan to achieve a 10X beam expansion by using a focal length pair of f1= 5cm and f2= 50cm. The resulting magnification is f1/f2=10 and the two lenses are to be separated by f1+f2=55cm. Using the lens pair provides and expanded and collimated beam.

10. Place lens L1 of f1=5cm. The beam should have now diverged significantly. The position of L1 is not critical. Ensure that the beam is centered on the TV/eyepiece and symmetrical in shape.

11. Place L2 at a distance 55cm away from L1. Adjust L2 to make sure that the beam size remains expanded but does not diverge or converge (is collimated) beyond L2. Ensure that the beam still centered on TV/eyepiece and symmetrical in shape.

12. Finally place L3 on a translation stage at a distance from the back port which is equal to the focal length of L3 (in this case 30cm). We can do this by adjusting the lens position until we see a collimated light beam exiting the microscope. Ensure that the beam still centered on TV/eyepiece and symmetrical in shape.

13. Direct the beam towards the edge of the back port by translating L3 along x to achieve TIR. Observe a fluorescent bead sample through TV/eyepiece.
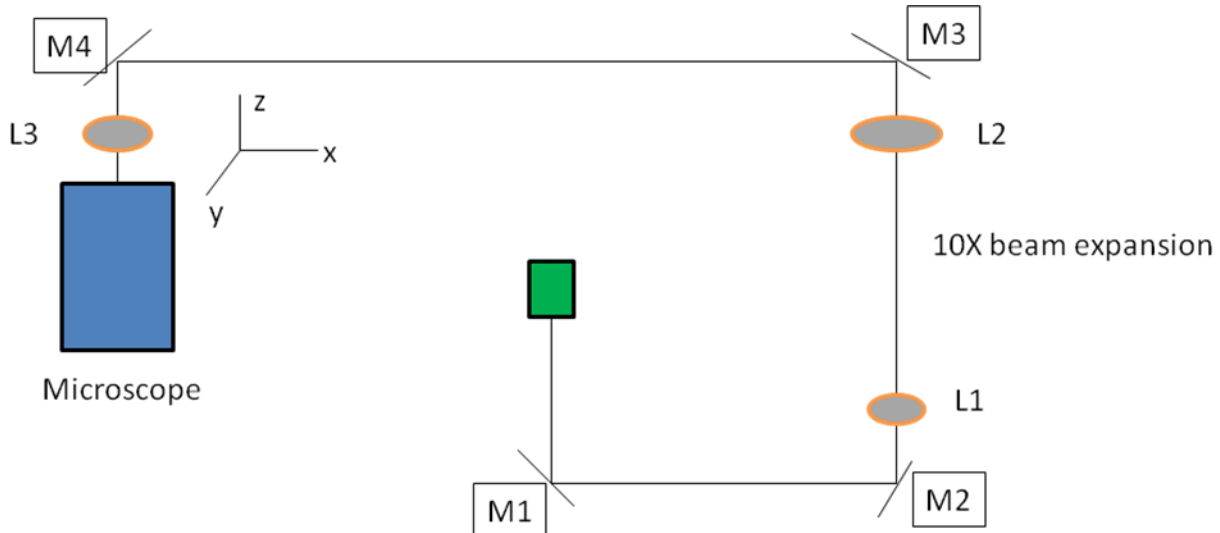
Experiment: G-quadruplex conformations and dynamics using FRET

Background

G-Quadruplexes (GQ) are DNA structures that are found in a variety of sequences, ranging from the telomere to duplex promoter sequences. In humans, the telomeric sequence consists on average of 8 to 50 telomeric repeats (TTAGGG). The telomere caps the ends of chromosomes as seen in the image below, the telomere consists of two parts: a duplex and single stranded section. For this experiment the single stranded portion is of most interest. The telomeric repeat of TTAGGG is capable of forming a stable GQ. These structures serve to protect chromosome end from deterioration and fusion. In addition, Telomeric GQ have been shown effect enzymatic function at these distal ends of the chromosome. Most importantly, these structures have been shown to effect the function of the Telomerase. 85% of cancers have unregulated telomerase. Thus, telomeric repeats have become an interesting target for the treatment of cancers.

In addition, promoter regions in the gene carry sequences rich in guanines that can also form GQ. The formation of the GQ can be stabilized by introducing small molecules (drugs) that can bind strongly and irreversibly to these structures. This has been shown to effect expression of specific genes.
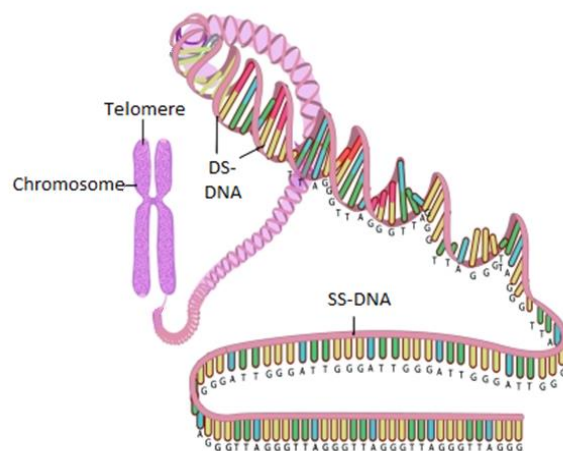
Structure

G-Quadruplexes consist of four repeats of three or more Guanine bases separated by at least one base. The Guanine bases stack on top of each other forming a GQ via Hoogsteen hydrogen bonding. GQ secondary structure is stabilized by a monovalent cations, most strongly by potassium. The cation is positioned in the middle of the Guanine tetrad. This experiment will monitor GQ formation in a variety of salt conditions
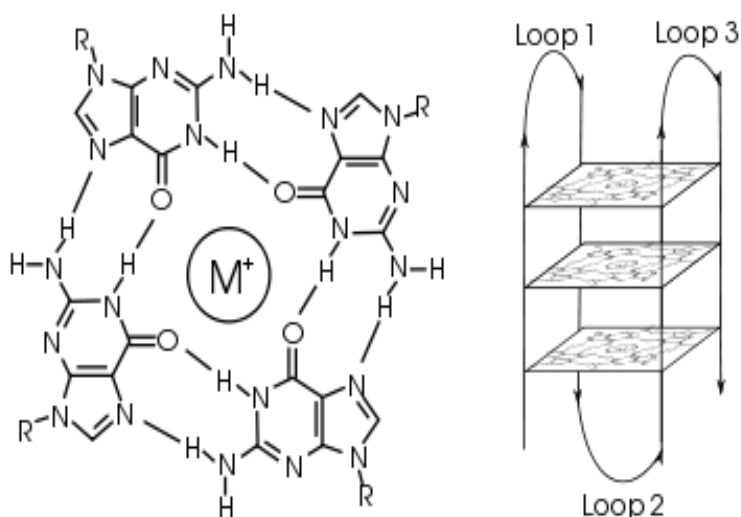
Goals: Ensemble studies are unable to differentiate between the folded and the unfolded GQ states, as well as transient protein-bound states and intermediates. With the single molecule FRET system, we can distinguish between folded, unfolded, and intermediate states of individual telomeric overhangs. Our goal in this experiment would be **to carry out a potassium**

salt titration to observe the dynamics involved in telomeric GQ formation. With increasing amount of salt concentration, we will examine the distribution of folded and unfolded DNA construct as well as the conformational dynamics using single molecule FRET experiments on a TIR microscope.

Assay: We will study the telomeric sequence (TTAGGG)$_4$ through immobilization of the substrate to a quartz surface coated with 95% m-PEG and 5% biotin-PEG. The substrate has two fluorophores (CY3 and CY5 dyes), which upon excitation can report on the folding conformation state of the single stranded overhang. Formation of the G-Quadruplex will result in high FRET as the dyes come closer, while a single strand overhang will result in low FRET.
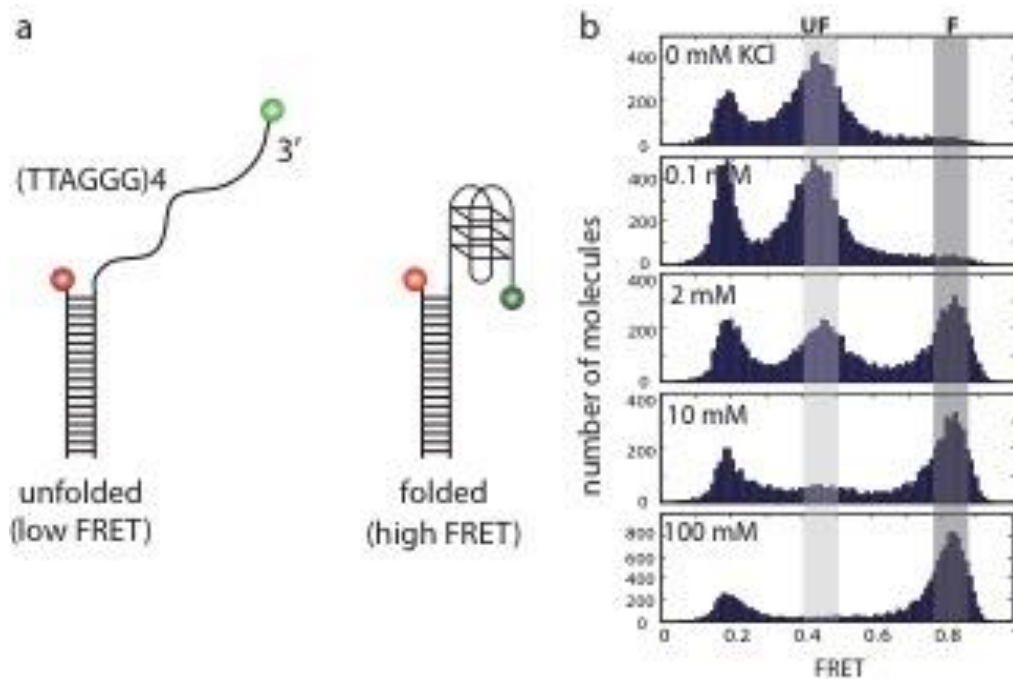
FRET DNA construct: Low FRET (unfolded) and high FRET (folded) states. Increasing the potassium concentration shifts the population from unfolded G-quadruplex to folded G-quadruplex.

Protocol:
1. Assemble the slides as instructed using the double-sided scotch tape. Then place it on the microscope
2. Add Neutravidin (20 µg/ml) to the channel, and wash with T50.
3. Add 30 ul of 100pM DNA construct to the chamber.
4. The channel is then flushed again with 100 uL of T50 to remove any unbound DNA.
5. The channel is then flushed with 200uL buffer containing a series of salt concentrations (.1mM KCl, 1mM KCl, 10mM, 100mM KCl) with 20 mM Tris pH 8.0.
   At each step, make sure the imaging buffer containing (10 mM Tris pH 8.0, 0.8% (wt/wt) dextrose monohydrate, 0.1 mg/ml glucose oxidase, 0.02 mg/ml Catalase (Sigma)[*Oxygen Scavenging System to improve the photo-stability of dyes*] and 2mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), IB)

Experimental Buffer and Solutions

Name	Component 1	Component 2	Component 3	Component 4	Component 5	Final Vol./Temp.
100 mM KCl Buffer	**Trolox**	**1M Tris**	**1M KCl**	20% Glucose Solution		
	215 uL	5 uL	25 uL	5 uL		250 uL at 22°C
10 mM KCl Buffer	**Trolox**	**1M Tris**	**1M KCl**	20% Glucose Solution		
	237.5 uL	5 uL	2.5 uL	5 uL		250 uL at 22°C
1 mM KCl Buffer	**Trolox**	**1M Tris**	**100 mM KCl** buffer from above	20% Glucose Solution		
	237.5 uL	5 uL	2.5 uL	5 uL		250 uL at 22°C
0.1 mM KCl Buffer	**Trolox**	**1M Tris**	**10 mM KCl** buffer from above	20% Glucose Solution		
	237.5 uL	5 uL	2.5 uL	5 uL		250 uL at 22°C
Neutravidin:						
Just use as it is supplied						
25 pm DNA *Flow this into the channel after excess Neutravidin has been washed off*	**10 nm DNA Stock**	**T50**				
	0.2 uL	~100 uL				250 uL at 22°C
Imaging Buffer *Flow this into the channel and start taking movies*	**Choose any of the above buffer at the needed K+ concentration**	**Gloxy** *as supplied to you*				
	200 uL	2uL				250 uL at 22°C

Analysis: The main goal of this experiment is to monitor the dynamics from low to high salt, which would symbolize the corresponding shift in the states of the Quadruplex. Through smFRET, we can get anticorrelated intensity traces of both CY3 and CY5 which can be used to calculate FRET over time and at each salt condition. We will collect the FRET values from thousands of molecules to build a histogram.

Introduction to Hidden Markov analysis
To quantify the dynamics of the G-Quadruplex, we will use Hidden Markov Modeling (HMM) to determine the time sequence of FRET states (figure x). This will enable us to determine the average transition rates between the states quickly and without bias from the experimenter. HMM assumes that the switching between the two conformations can be represented as a Markov chain. A Markov chain is a random process with discrete states (*e.g.* the number of conformations with unique FRET values) where the transition rates (*e.g.* probability per unit time that a transition from the low FRET state to the hi FRET stat occurs) depend only on the current state, not the history of states visited. This implies that the distribution of inter-event times is exponential.

The process is "hidden" because we do not directly observe the FRET state. Instead we observe the fluorescence intensity, which fluctuates due to instrumental noise and photophysical effects. The goal of HMM is to predict which state the system is in as a function of time given the observed FRET signal. In so doing the mean intensity for each state, the initial state distribution, and the transition rates between all of the states can be calculated as well. The HMM analysis alone cannot determine the correct number of states to start with. To determine the optimal number of states, the analysis is ran for a range of states, and an information criterion is used to choose the best number. Detailed knowledge of the HMM algorithms are not necessary to use the method, however interested students should read the papers listed above for an elementary introduction. The software we will use for the summer school was developed by members of the Ha group in 2006. It is available at http://bio.physics.illinois.edu/HaMMy.html.
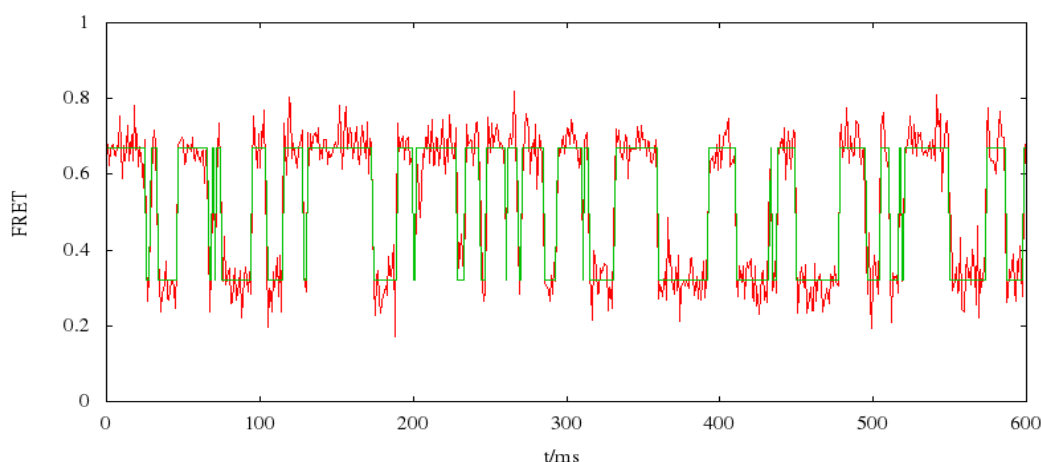
FRET trajectory for the folded and unfolded G-quadruplex conformations with state sequence from HMM.

Appendix: PEGylation protocol

Slide and coverslips cleaning

1. Required: Glass beaker, milliq, methanol, acetone, 10% Alconox, 1M KOH, razor blade, jars, quartz slides, 40 mm coverslips, flask.
2. Thaw aminosilane in dark
3. Microwave slides for 15 minutes
4. Scrub slides with
   (a) hot water
   (b) methanol
   (c) acetone
   (d) rinse with milliq
5. Sonicate quartz slides and coverslips in acetone for 30 min.
6. Rinse slides and coverslips with milliq.
7. Sonicate jars with slides/cslips in 1 M KOH for 20 min.
8. Rinse jars with milliq.

Aminosilanization

9. Required: Clean quartz slides and coverslips, Propane torch, glass tips, Acetic acid, Aminosilane, clean flask, PEG prep boxes.
10. (i) Rinse each slide/cslip with milliq
    (ii) burn with a propane torch (1 min per side for a quartz slide, 5 sec per side for a glass cslip)
11. Return the slides to the milliq filled jar.
12. Rinse slides and coverslips with milliq and then methanol and dry the jars.
13. (i) Prepare Aminosilanization mixture, mix well with glass pipette tip and (ii) replace methanol with mixture: 150 mL methanol + 7.5 mL Acetic Acid + 1.5 mL aminosilane
14. Incubate for 10 min then sonicate the jars for 1 min and incubate for another 10 min.
15. In the meantime prepare fresh pegylation buffer: 84 mg $NaHCO_3$ in 10 mL milliq.

PEG Coating

16. Required: Biotin-PEG, m-PEG, $NaHCO_3$, , PEG assembly boxes
17. Replace aminosilanization mixture in the jars with methanol and
    (a) Rinse each slide or coverslip in milliq 5 times.
    (b) Rinse in methanol 2 times.
    (c) dry with nitrogen
    (d) Place in PEG prep/assembly boxes
18. Rinse Amino-Jars with methanol, then dry in oven

19. Weigh out PEG, shake well in a 1.5 mL tube, mix thoroughly by tapping. For 5 slides, use 80 mg mPEG + 2 mg biotin-PEG + 300 mL freshly made PEG buffer
20. PEG storage:
    (a) Vacuum dry both PEG for 10 min
    (b) Fill with dry nitrogen
    (c) Seal twice with parafilm
    (d) Store in -20 C
21. (a) Make PEG buffer and add to PEG tube, (b) shake and flip thoroughly to disperse
22. Centrifuge at 10,000 rpm for 1 min
23. Apply 60 µL PEG per slide to each slide first and then place coverslip on each
24. Check for sliding after 15 min and incubate for 3 hours or more in a dark, stable place.

Disassembly and Storage

1. Disassemble and rinse each thoroughly with milliq
2. Dry with nitrogen and store each in a separate tube.
3. Keep in -20 C. Make sure when storing the PEGelated surfaces are not touching each other or other surfaces.

Questions for the lab report

1. What is TIRF microscopy? Draw a schematic of a TIRF setup and give one fundamental reason on how it helps in improving the signal to noise ratio? (2+2+1 points)

2. Write a brief note about what your experience with the optical alignment we did for the TIRF setup. Please explain the importance and use of any two major optical components (including objective)? (3+4)

3. Explain briefly the meaning of 'FRET Histogram'? How does it help in determining the heterogeneity of any biological system?   (4 points)

4. Part 1: Please elucidate the role of K^+ ions in stabilizing the G Quadruplex structures? How does it compare with other ions such as Na^+? Also attach the FRET histogram you obtained from your experiment of G Quadruplex dynamics at different potassium ion concentration. (1+1+5 points)

    Part 2: Various ligands have been reported in the scientific literature that affects the stability of G Quadruplex. Name any one such ligand and the effect it would have on the FRET histogram at different K+ concentrations at which G-Q dynamics was studied? (3 points) Reference: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2737377/

<u>Part 3</u>: What is a single molecule FRET trace? An imaging movie made during the smFRET experiments will study multiple molecules at a time but not all attainable traces will be 'single molecule FRET trace'. List any two important criteria for selecting a single molecule trace and include one representative trace from your experiment of G Quadruplex dynamics at different potassium ion concentration. (4+4 points)

5. Why do we need to passivate the surface of slides to do single molecule experiments, and how does PEG (Polyethylene glycol) help in achieving the passivation? Why is Biotin-Avidin interaction used to immobilize the biomolecules on surface for a range of single molecule experiments? Explain very briefly with the structural details of avidin.   (4+4 points)

6. Explain the utility of HaMMy (Hidden Markov Modeling) in analyzing the single molecule FRET traces to study G Quadruplex dynamics? Please include two traces with HaMMY states?   (4+4 points)