Förster Resonance Energy Transfer Investigations of the Structure of the Immunoglobulin G Class of Antibody

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Förster Resonance Energy Transfer Investigations of the Structure of the
Immunoglobulin G Class of Antibody

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Abstract

The work put forth in this thesis focuses on single molecule spectroscopic methods used to study IgG structure. Ensemble and single molecule methods were used to study model systems of 11 base pair and 16 base pair oligonucleotides. Ensemble Förster resonance energy transfer (FRET) results were analyzed using two different approaches. Single molecule FRET studies on the DNA samples were performed and compared to the ensemble results. From these experiments it was possible to determine the best ensemble analysis method to use, in conjunction with single molecule experiments, to investigate the structure of IgG in solution.

To study the relative positions of the antigen-binding fragments (Fab), the binding sites were labeled with a FRET dye pair. To do this, IgG 38C2 was reacted with a small linker molecule, or hapten, that had been conjugated with a fluorophore. Binding of the hapten to 38C2 and fluorescent probes was verified using thin layer chromatography (TLC) and UV-Vis spectroscopy. The FRET dye pair was bound to hapten molecules and used to label 38C2 (94% binding efficiency). Single molecule fluorescence measurements on the antibody indicate minimal FRET occurring between the dye pair. This suggests the distance between the two dyes is too large to be measured using single molecule FRET.

Ensemble and single molecule FRET were also used to investigate proposed conformational changes that occur within the crystallizable fragment (Fc) region of the antibody when the oligosaccharides are removed. For this experiment two surface-exposed serine residues were mutated to cysteines for selective attachment of fluorophores. Ensemble measurements were indistinguishable for the IgG samples. The single molecule FRET histogram shows a narrow, 16% efficiency of energy transfer for the glycosylated IgG and a broad distribution with a center at 53% for the aglycosylated IgG. This suggests the absence of oligosaccharide molecules facilitates a greater flexibility within the Fc region of the antibody.
Chapter 1: Introduction

The Immune System and Antibodies with a Focus on the IgG Class

The immune system consists of two major components: the innate immune response and the adaptive immune response. The innate immune response comprises the complement system and a host of leukocytes that work in a nonspecific manner. Complement proteins are serine proteases that recognize foreign matter indiscriminately and mark it for removal by leukocytes (e.g. macrophages)\(^1\). The adaptive immune system is primarily composed of T cells and B cells. Much of the recognition of foreign matter, or antigens, is done by the immunoglobulin-class of proteins also known as antibodies.

B cells differentiate into two distinct primary cell types: memory cells and plasma cells. Plasma cells are short-lived cells capable of secreting thousands of antibodies per second whereas memory B cells are long-lived, with a lifespan of up to 10 years, and retain the ability to produce antibodies if the same antigen is encountered in the future\(^1\). Most B cell types can synthesize antibodies of which there are five known classes: immunoglobulin A (IgA), IgD, IgE, IgG and IgM (Figure 1.1). The IgA class of antibodies is recognized for its ability to cross epithelial barriers and its presence is highly concentrated in the intestines and other parts of the digestive system\(^1\). IgG is the most abundant class of antibody present in blood serum and one hallmark of this protein is its ability to cross the placental barrier and provide the developing fetus with a type of antibody that it cannot synthesize on its own\(^2\). IgM antibodies are recognized as a pentamer of Ig molecules and as such are highly effective at binding serum antigen. Little is known about IgD and IgE antibodies. It is known that IgE molecules are involved in the allergic response, but the involvement of this class of antibodies in the adaptive immune response is a mystery. IgE molecules are believed to play a role in the body’s defense
against parasitic worms and other larger organisms\(^3\). IgD, though present on the surface of all developing B-lymphocytes, is the least understood of all antibodies.

![Diagram of antibody isotypes](image)

**Figure 1.1: The Five Antibody Isootypes.** All five antibody classes are shown here. It can be seen that the most common form of IgA is a dimer and IgM is a pentamer. IgG, the focus of this thesis, is shown in the upper left. Figure 1.1 was obtained from Janeway’s Immunobiology (see reference 1).

Each class or isotype has further structural and chemical behavior that distinguishes it from others. Here the focus will be on the IgG subtype of immunoglobulin proteins.

Immunoglobulin G proteins are always a dimer of dimers; the protein is composed of two identical heavy chains and two identical light chains. Both chains are subdivided into variable and constant regions. The light chains possess a single variable and a single constant domain whereas the heavy chain possesses a single variable region and three constant domains: \(C_H1\), \(C_H2\), and \(C_H3\) (Figure 1.2). The \(C_H2\) and \(C_H3\) regions together make up the Fc (fragment crystallizable) region and the variable regions (\(V_H\) and \(V_L\)) together with the constant region of the light chain, \(C_L\), and the \(C_H1\) region form the Fab (fragment antigen binding) region. The length of the heavy chains is part of what distinguishes each class of immunoglobulin molecule (IgM and IgE both have an extra constant domain)\(^1\).
Figure 1.2: Diagram of an IgG Antibody. Shown are the two heavy chains (blue and yellow) and the two light chains (red). The carbohydrate moieties are shown as blue hexagons attached to the C_H2 region. Disulfide bonds that link the two heavy chains are present in the hinge region. The Fc region encompasses the C_H2 and C_H3 regions whereas the Fab region is defined as the V_L and C_L domains together with the V_H and C_H1 domains. Figure 1.2 was obtained from Janeway’s Immunobiology (see reference 1).

As can be seen in Figure 1.2, the separate dimers are joined together by disulfide bonds at a hinge region, so named because it is believed to be highly flexible; this region is missing from the IgM and IgE antibodies which possess an additional constant domain. The last structural feature of IgG shown in Figure 1.2 is the carbohydrates joined to the protein at asparagine residue 297 in the C_H2 region of the antibody. The presence and arrangement of carbohydrate molecules is unique to each antibody isotype.

The variable region of the antibody, V_H and V_L, is responsible for binding to foreign antigen. This region of the protein is aptly named, because the body generates a great deal of diversity in each antibody molecule and this is concentrated in the variable region of the protein. There are multiple copies of each gene segment that encode for the variable region of the protein, so there is combinatorial diversity generated based on the different gene segments that combine within the individual chain and then further diversity based upon how the light and heavy chains combine (Figure 1.3). It is estimated that over 10^{11} possible combinations of antibodies can be
expressed on the surface of naïve B cells, or B cells that have yet to be conditioned through the process of somatic hypermutation\(^1\). Somatic hypermutation occurs late in the immune response and is a process where subtle mutations are introduced to antibodies that recognize a specific antigen. After this mutation, antibodies that bind their antigen with high affinity are selected over those that bind with lower affinity\(^1\). The diversity of antibodies is further expanded through this conditioning process which is discussed in more detail elsewhere\(^5-8\).

**Figure 1.3: Gene Segments Encoding Antibody Proteins.** There are two light chain gene loci (kappa and lambda) and one heavy chain locus in humans. The heavy chain possesses an additional gene segment (D) different from the light chain that adds to the combinatorial diversity of antibodies. One V and one J segment combine to code for the light chain and one V, one D and one J segment combine and code for the heavy chain. Figure 1.3 was obtained from Janeway’s Immunobiology (see reference 1).

The involvement of secreted antibody molecules in the immune response is initiated by the binding of the variable region to an epitope, i.e. the region of an antigen that is recognized by an antibody. These epitopes are typically present on the surface of a pathogen or antigen-presenting cells within the body. Three fates are possible for the bound immunoglobulin molecule: neutralization, opsonization and complement activation. Many foreign pathogens, such as viruses and some bacteria, must invade host cells by first binding to and then diffusing across the cell membrane. When antibodies coat the surface of these invaders, they can lose the ability to bind to the cell membrane; this process is referred to as neutralization. In some instances, the coat of antibody molecules serves as a point of recognition for macrophages and
other leukocytes which can then digest the invaders through phagocytosis (opsonization). Lastly, the binding of antibodies to complement proteins can activate the innate immune response.

IgG proteins are involved in all three of these processes in the immune response. IgG proteins have been shown to bind to bacterial toxins, such as those secreted by the botulism bacteria *Clostridium botulinum* and the cholera bacteria *Vibrio cholerae*\(^9\)-\(^{11}\). Synthetic antibodies have been generated as therapeutic agents to protect against the botulism toxin\(^{12}\). The inhibition of viral binding by IgG proteins has been observed in yellow fever vaccines\(^{13}\). In addition, therapeutic antibodies have been developed that can inhibit the binding of HIV-1 and the herpes simplex virus\(^{14\text{-}17}\).

IgG antibodies (and IgM) are also important precursors to the complement pathway. The complement protein C1q can bind to IgG molecules attached to the surface of a pathogen as long as there are at least two IgG proteins near one another (Figure 1.4). C1q will then recruit additional proteins that can start an enzymatic cascade that results in the coating of the pathogen surface by proteins that promote opsonization. The binding of IgG has been shown to enhance proteolytic cleavage of some complement proteins, thus speeding up the signal cascade\(^{18}\). The ability of IgG to bind complement has also been implicated in some disease states, such as certain forms of arthritis\(^{19}\). The cartoon in Figure 1.4 shows the binding of C1q to the constant region of the IgG proteins. The presence of the hinge region has also been shown to be important, if not necessary, for complement binding to occur\(^{20}\).
Figure 1.4: IgG Binding to Complement Proteins. Shown is a cartoon whereby two IgG antibodies have bound antigen expressed on the surface of a pathogen. When they are in close proximity to one another, they form a platform to which complement protein C1q can bind and recruit additional proteins in the complement pathway. Image taken from http://www.cartage.org.lb/en/themes/sciences/lifescience/generalbiology/Immunology/ComplementSystem.

As complement binding is only one pathway for the IgG-bound pathogens, the fate of many IgG complexes is dictated by another process. The Fc region is structurally distinct for each antibody isotype, which results in differential binding each antibody class to a diverse range of proteins called Fc receptors. These receptors are so specific that a single amino acid mutation in the antibody Fc region can inhibit recognition by the Fc receptor. The receptors specific to IgG are classified into two groups: inhibitory and activating. These receptors are found expressed on the surface of most lymphocytes: natural killer cells, monocytes, mast cells, eosinophils, macrophages, neutrophils and platelets. Activators are so named because they trigger a signal cascade that causes the effector cell, the cell that expresses the activator, to either phagocytose the pathogen (in the case of macrophages) or stimulate the release of granules (in the case of natural killer cells) that contain the membrane-piercing enzyme perforin which induces apoptosis in the pathogen. Inhibitory FcγRII receptors are found on B cells and are believed to prohibit further growth and differentiation when triggered. The major activating Fc receptors for IgG are the FcγRI and FcγRIII receptors while the FcγRIIb receptors are inhibitory.
In addition to the three classes of IgG receptors mentioned previously, there is an additional IgG receptor, the neonatal receptor, or FcRn. The neonatal receptor is responsible for the translocation of IgG from mother to fetus across the placental barrier during development. As the fetus cannot produce its own antibodies, these IgG molecules are the only serum antibodies present in the developing fetus. Additional studies have also shown that expression of FcRn is needed to extend the serum half-life of circulating IgG proteins from 1 day to 6-8 days. FcRn binds to the IgG antibody at a site that is distinct from other Fc receptors and the C1q binding site; these binding site locations are mapped in Figure 1.5.

**Figure 1.5: Important Regions of Binding on an IgG Antibody.** Antigen binding occurs at the interface of the V_H and V_L regions. FcγRI, FcγRII, and FcγRIII have been shown to bind at the interface of the C_H2 and hinge region while FcRn, the neonatal receptor, binds at the junction of the C_H2 and C_H3 regions of the heavy chain. The complement protein C1q binds only to the C_H2 region.

Of interest in studying the antibody-mediated immune response is an understanding of the structure of an intact immunoglobulin molecule. This would give a better understanding of the molecules, amino acids and oligosaccharides, involved in the effector response. X-ray crystallography is the preferred method of ascertaining the three-dimensional structure of proteins, but this has been notoriously difficult to use with antibodies. As the antibody is a very
large protein (~150 kDa) and proposed to be very flexible in part due to the hinge region, a fully intact IgG antibody has only been crystallized a select few times.\textsuperscript{23,24} Some information has been garnered through crystallization of the Fc region\textsuperscript{25,26} and crystallization of the Fab region when bound to antigen\textsuperscript{27-30}, but this does not give a complete picture of the antibody structure under physiological conditions. Recently, cryo-electron tomography has emerged as a lower resolution technique that can be used to image antibodies in solution\textsuperscript{31-33}. While this technique has made some argument for the flexibility of the IgG molecule, the resolution is not high enough to detect smaller conformational changes. An additional problem with this technique is that the experimental conditions cannot match a physiologically relevant environment and it does not provide dynamic information, only static images.

In an attempt to get a more complete picture, Förster Resonance Energy Transfer (FRET) was used to investigate the conformational preferences of IgG molecules in solution. FRET describes the non-radiative transfer of energy from one molecule, termed the donor, to a nearby molecule, the acceptor. The process is distance-dependent and more likely to occur if the donor and acceptor fluorophores are in close proximity (10 to 100 Å). Typically, one or both of the molecules is a fluorophore, so changes in distance between the two molecules are reflected in the relative amounts of fluorescence from the donor and acceptor. If both donor and acceptor are attached to a biomolecule, such as an IgG protein, and the fluorescence signals from both chromophores are monitored, changes in the relative amounts of fluorescence can be attributed to conformational changes in the protein. By understanding conformational changes or structural preferences of IgG, it is possible to gain a better understanding of the role of IgG in the immune response.
**Förster Resonance Energy Transfer**

FRET is a non-radiative energy transfer process that can be attributed to the weak dipole-dipole coupling of the donor and acceptor molecules\(^{34}\). Förster first proposed that the donor and acceptor molecules could be treated as point dipoles and he derived a mathematical description of the rate constant of energy transfer. Much of this derivation can now be found in physical chemistry and spectroscopy textbooks\(^{35,36}\). Starting with Fermi’s Golden Rule it is possible to derive a mathematical expression for the rate constant of energy transfer, \(k_{\text{EET}}\) (1.1).

\[
k_{\text{EET}} = \frac{2\pi}{\hbar} \int u^2 dE
\]  

(1.1)

Fermi’s Golden Rule follows from time-dependent perturbation theory in which the Hamiltonian operator can be separated into separate time-dependent and time-independent operators. In this equation, \(u\) is defined as the resonance matrix element that contains the wavefunctions of the donor and acceptor molecules. The integration is taken over the range of all possible energy values. The resonance matrix can be seen in equation (1.2) below where \(\Psi_f\) and \(\Psi_i\) represent the final and initial wavefunctions of the system, respectively, and \(V\) represents the time-independent portion of the Hamiltonian operator.

\[
u = \int \Psi_f^* V \Psi_i d\tau
\]  

(1.2)

As FRET is a result of electronic energy transitions, the Born-Oppenheimer approximation can be applied to separate the electronic and nuclear wavefunctions of the two chromophores. Because the nuclear motion is on a much slower timescale than electronic motion, the nuclear wavefunctions can be separated from the resonance integral as Franck-Condon factors.

If a multipole expansion is carried out on the electronic portion of the resonance integral to determine the interaction energy between the two dipoles, the resonance integral can then be expressed as the following relationship:
In this expression, \( \kappa \) corresponds to an orientation factor for the two dipoles; \( \mu_A \) and \( \mu_D \) correspond to the magnitude of the transition dipole moments for the acceptor and donor molecules, respectively. The factor \( n \) is the refractive index of the medium, assumed constant, and \( R \) corresponds to the distance between the two point dipoles. The integrals correspond to the Franck-Condon factors for the donor (D) and acceptor (A) (the asterisk superscripts on the D and A terms denote the electronically excited donor and acceptor chromophores as opposed to the complex conjugates of the wavefunctions).

With a more complete definition of the resonance matrix element, equation (1.3) was substituted into the expression for Fermi’s Golden Rule (1.1) in order to gain an expression that relates the rate constant of energy transfer to more easily defined terms. It was then necessary to make one further assumption that the system under study has reached thermal equilibrium. Boltzmann factors \((g)\) are then introduced to describe the population of energy states at equilibrium. The resulting expression is shown in (1.4) where the units of integration have been converted from energy to frequency.

\[
k_{EET} = \frac{\kappa^2}{\hbar^2 n^4 R^6} \int \left[ \mu_D^2 \int g \left( \int \phi_D^* \phi_D \, d\tau \right)^2 \, dE \right] \int \left[ \mu_A^2 \int g \left( \int \phi_A^* \phi_A \, d\tau \right)^2 \, dE \right] \, d\nu
\]

(1.4)

Fürster was then able to use Einstein’s expressions for absorption and emission of photons to substitute for each of the terms in brackets\(^\text{37}\). By converting to wavenumbers and simplifying the expression, the relationship reduces to (1.5)

\[
k_{EET} = \frac{9000 (\ln 10) c^4 \kappa^2}{128 n^4 \pi^5 N_A \tau_i R^6} \int \frac{f(\bar{\nu}) \varepsilon(\bar{\nu})}{\bar{\nu}^4} \, d\bar{\nu}
\]

(1.5)
where \( c \) refers to the speed of light (cm/sec), \( N_A \) is Avogadro’s number and \( \tau_i \) is the fluorescence lifetime of the donor in the absence of non-radiative processes. The integral corresponds to the spectral overlap of the normalized donor fluorescence \( f(\tilde{\nu}) \) and acceptor absorbance spectra \( \varepsilon(\tilde{\nu}) \) as a function of frequency in wavenumbers (\( \tilde{\nu} \)). This integral is often represented as \( J \) in equation (1.5). A sample of two overlaid spectra is shown in Figure 1.6.

![Figure 1.6: Spectral Overlap Integral for Alexa Fluor® 568 and 647. The absorbance and emission spectra have been normalized to show relative fluorescence and absorbance between the two dyes. The larger the overlap, the greater the \( R_0 \) value for the dye pair.](image)

Substituting in known values for the constants leads to a final expression for the rate constant (1.6).

\[
k_{EET} = \frac{8.8 \times 10^{-25} k^2}{n^4 \tau_i R^6} J \quad (1.6)
\]

Because the intrinsic fluorescence lifetime, \( \tau_i \), is not an easily measurable quantity, it is convenient to make a substitution using the observable fluorescence lifetime, \( \tau_D \). The relationship for \( \tau_D \) takes into account the presence of the additional processes of internal conversion, intersystem crossing and collisional quenching that allow a molecule to return to the electronic
ground-state without the emission of a photon. The ratio of $\tau_D/\tau_i$ relates the absorbed photons to the emitted photons of light and is defined as the fluorescence quantum yield ($\phi$). This relationship can be substituted into the previous expression to relate the rate constant of energy transfer to observable or calculable parameters.

$$k_{EET} = \frac{8.8 \times 10^{-25} k^2 \phi D}{n^4 \tau_D R^6}$$ (1.7)

This makes it possible to calculate the rate constant of FRET, but does not yet show how this phenomenon can be used to detect conformational changes in macromolecules such as proteins and DNA. Using this relationship, changes in the rates of energy transfer are distance dependent and vary as $R^{-6}$. It is possible to determine changes in distance between a FRET pair (donor and acceptor), by measuring changes in the observed rate constant of energy transfer. To do this, the efficiency of energy transfer is defined as the ratio of the rate constant of energy transfer to rate constants for all relaxation processes.

$$E = \frac{k_{EET}}{k_{EET} + k_D}$$ (1.8)

where $k_D$ is the rate constant of all additional relaxation processes and is defined as $1/\tau_D$. Using this relationship, it is possible to determine the rate constant of energy transfer and efficiency values by measuring changes in the fluorescence lifetime of a donor molecule in the presence and absence of acceptor molecules. Measuring fluorescence lifetimes has been used to detect energy transfer and conformational changes in various types of biomolecules\textsuperscript{38-40}.

Another common practice, and the one used throughout this work, requires the introduction of an additional term, $R_0$, the Förster radius. This is the characteristic distance,
unique to each FRET dye pair, at which the efficiency of energy transfer is 50%. In this instance $k_{\text{EET}}$ is equal to $k_D$. The expression for $k_D$ (i.e., $1/\tau_D$) can be substituted into (1.7) to yield (1.9).

$$R_0^6 = \frac{8.8 \times 10^{-25} \kappa^2 \phi_D}{n^4 J}$$  \hspace{1cm} (1.9)

It can be seen from this expression that the Förster distance for a given dye pair will increase with the spectral overlap of the two chromophores as well as the quantum yield of the donor.

Using this term, a new expression for the efficiency can be derived. By substituting (1.9) into equation (1.7), the following relationship is established.

$$R_0^6 = k_{\text{EET}} R^6 \tau_D$$  \hspace{1cm} (1.10)

Equation (1.10) can then be substituted into (1.8) to yield

$$E = \frac{R_0^6}{R^6 + R_0^6}$$  \hspace{1cm} (1.11)

This is probably the most commonly seen FRET equation that relates the efficiency of energy transfer to the distance between the chromophores. Because the rate constants of energy transfer and the rate constants of fluorescence are directly related to the intensity of light emitted by the chromophores, it is possible to arrive at an expression (1.12) for the efficiency that is related to the intensity of donor fluorescence ($I_D$) and the intensity of acceptor fluorescence ($I_A$).

$$E = \frac{R_0^6}{R^6 + R_0^6} = \frac{I_A}{\gamma I_D + I_A}$$  \hspace{1cm} (1.12)

The constant $\gamma$ arises due to the differing fluorescence quantum yields and detection efficiencies of the donor and acceptor chromophores. Over the distances where the assumptions in the derivation of FRET are valid (10-100 Å), these efficiency values can be used to calculate changes in distance between the FRET pair. Fluorescence intensities, and thus FRET efficiencies, can be measured using fluorometers that are readily accessible to most research.
laboratories. Because companies like Molecular Probes® provide $R_0$ and quantum yield information for their commercially available fluorophores, FRET experiments are viable means to detect conformational changes between a FRET pair attached to the surface of a biomolecule. Indeed, these experiments have been performed both on proteins and nucleic acids labeled with fluorophores since Stryer first suggested the use of this technique as a ‘spectroscopic ruler.’

In recent years, novel techniques have emerged that utilize FRET, the most prevalent of which is single molecule spectroscopy. Single molecule experiments have a distinct advantage over bulk measurements in that they can distinguish between an average FRET efficiency value and individual FRET efficiencies that could be caused by multiple conformations as shown in (Figure 1.7).

![Figure 1.7: Ensemble vs. Single Molecule Measurements](image)

Figure 1.7: Ensemble vs. Single Molecule Measurements. Ensemble measurements are only capable of determining a bulk average among a given population, whereas single molecule methods are able to distinguish between multiple subpopulations. Each FRET efficiency distribution shown has the same ensemble average, yet single molecule measurements can be used to reveal multiple subpopulations (top panel) or assess the breadth of a distribution to gain information about the flexibility of a molecule (bottom two panels).
The first single molecule FRET experiments on freely diffusing molecules were performed on DNA oligomers using confocal microscopy\textsuperscript{44,45}. Some of the most common spectroscopic techniques used now include alternating laser excitation spectroscopy (ALEX), total internal reflection fluorescence microscopy (TIRF), and single molecule FRET on freely diffusing species. These techniques have been used to study four-way DNA junctions\textsuperscript{46-48}, RNA behavior\textsuperscript{49,50}, protein folding\textsuperscript{51,52} and other biological systems.

ALEX and TIRF techniques typically use surface-tethered biomolecules. The advantage of this approach is that dynamics of a single molecule can be monitored in real time. This is an ideal approach with which to study protein folding or denaturation \textit{in vitro}. The limitation, however, is that while immobilized, the tethered portion of the protein is not free to interact with the rest of the molecule. As such, a complete picture of the native protein state may not be available. Additionally, interactions with the tether and surface coating (usually biotin and streptavidin), may affect the dynamics of the molecule under study.

The approach described in this thesis involves the examination of freely-diffusing molecules in solution. In this way, it is possible to observe a distribution of molecules under physiologically relevant conditions without the additional constraints of the tether\textsuperscript{53}. This approach has been used previously to detect subpopulations in oligonucleotide samples\textsuperscript{54}, protein folding\textsuperscript{55}, and additional conformational subpopulations present in proteins\textsuperscript{56}. Here FRET is used to look at dye-labeled oligonucleotides and to detect preferred conformations within distinct regions of the IgG antibody.

To provide a baseline of analysis for studies with the IgG antibody and to ensure the single molecule apparatus was working correctly, 11 base pair (11bp) and 16 base pair (16bp) double-stranded oligonucleotides (dsDNA) were labeled with the FRET pair Alexa Fluor\textsuperscript{®} 488
and Alexa Fluor® 594. Ensemble analysis methods, using data from a Nanodrop 3300 fluorometer, were applied and compared to ascertain the most appropriate ensemble data analysis technique. This conclusion was reached by comparing single molecule FRET efficiency values to those obtained from the two ensemble analysis approaches used. Double-stranded DNA was used as the model, because DNA FRET systems are well studied and have been shown to adopt a fairly rigid structure with a single conformation\textsuperscript{44,54,57-59}.

Once the dsDNA systems were studied thoroughly, the reactivity of catalytic amino acid residues within the Fab region of IgG antibody 38C2 were used to attach a FRET pair (Alexa Fluor® 568 and Cy5.5) to the protein. Because the amino acid residues are located in a pocket within the protein, a short hapten linker was synthesized in order to secure the fluorophores to the antibody. Once the dyes were attached to the protein, single molecule measurements were carried out to detect the presence of preferred conformations between the Fab ‘arms’ of the antibody and assess the predicted flexibility of the hinge region.

The final system discussed in this thesis is the Fc region of the antibody. Two surface serine residues in the C\textsubscript{H}2 region were mutated to cysteines in order to attach the FRET pair Alexa Fluor® 488 and Alexa Fluor® 594. The focus of this project was to detect changes in protein conformation in the presence and absence of the oligosaccharides found in the C\textsubscript{H}2 region of the antibody. Ensemble and single molecule experiments were both used to determine changes in the distances between fluorophores as a result of the aglycosylation process. Conformational differences between the glycosylated and aglycosylated forms of the antibody were then used to rationalize previously observed changes in binding affinity\textsuperscript{60-62}. 
Chapter 2: Ensemble and Single molecule FRET Studies of 11bp and 16bp dsDNA Oligomers

Introduction

FRET has been used to examine the structures of a wide variety of biological molecules. Initial applications of FRET to biological systems involved studying an ensemble of molecules, where an average structure was determined\textsuperscript{63}. More recently, single molecule FRET techniques have become prevalent, as this technique provides a histogram of the structures present, allowing conformational subpopulations to be detected\textsuperscript{44,52,54,64}.

Both ensemble and single molecule FRET techniques have been validated and refined based on studies of model systems, including polyproline peptides\textsuperscript{42,65,66} and oligomers of double-stranded DNA (dsDNA)\textsuperscript{44,57,67-69}. These systems serve as good models, as they are fairly rigid and are expected to exhibit only a single conformation, such that ensemble and single molecule FRET measurements should agree. In fact, this agreement has generally been good, particularly when the acceptor normalization method put forth by Clegg et al.\textsuperscript{67} has been employed for the ensemble experiments. This approach requires the use of two excitation wavelengths – one absorbed only by the donor chromophore and one absorbed only by the acceptor – in order to account for both incomplete labeling of the sample with dye molecules and instrumental artifacts\textsuperscript{67}.

The acceptor normalization method can be carried out using a standard commercial fluorometer. This type of an instrument often requires sample sizes (1 – 3 mL) that are larger than desirable for expensive samples, such as DNA and proteins labeled with dye molecules. The advent of UV-Vis and fluorescence instruments capable of examining microliter quantities of sample introduces the possibility of using these instruments for ensemble FRET studies of expensive or difficult to produce samples. The ThermoScientific NanoDrop 3300
fluorospectrometer (ND-3300) is one such instrument, capable of acquiring fluorescence spectra of 2 µL samples excited by one of three LED excitation sources: one centered at 365 nm, another at 470 nm, and another that spans the entire visible range. This instrument has been used primarily to quantitate DNA and protein samples; however, it has also been employed for FRET studies\textsuperscript{70-72}. A limitation of this instrument is the fact that the excitation wavelength cannot be specified precisely. This limitation removes the possibility of using the acceptor normalization approach for most cases\textsuperscript{67}. Because the white light LED of the ND-3300 covers the entire visible spectrum, and the majority of dye molecules used in FRET experiments absorb visible light, it cannot be used to reliably excite only the donor or the acceptor. Therefore, use of this instrument for ensemble FRET studies requires a different approach.

In order to determine the optimal method for FRET experiments using the ND-3300, we have examined FRET results for dye-labeled, double-stranded DNA oligomers. Two alternative analysis methods are employed that have been used previously for FRET experiments. One is referred to as the donor quenching method, in which the efficiency of energy transfer, $E_{dq}$, is calculated from the decrease of the donor fluorescence in the presence of the acceptor according to

\begin{equation}
E_{dq} = 1 - \frac{I_{DA}}{I_D}
\end{equation}

where $I_{DA}$ is the fluorescence of the donor chromophore in the presence of the acceptor and $I_D$ is the fluorescence from the donor in a sample in which the acceptor is absent. This analysis approach is often used in cases where the acceptor chromophore is a quencher molecule that does not fluoresce\textsuperscript{72}. The other approach is referred to as the ratiometric method, as the efficiency of energy transfer, $E_r$, is calculated from the ratio of the acceptor fluorescence due to energy transfer ($I_A$) to the sum of the donor ($I_D$) and acceptor fluorescence intensities using
equation (1.12). The ratiometric method is generally employed to analyze single molecule FRET data.

Single molecule FRET experiments are relatively immune to some of the issues present in ensemble studies, particularly the presence of donor-only labeled molecules in a doubly-labeled sample. The presence of molecules labeled with only a donor chromophore in a sample presumed to be labeled with both donor and acceptor chromophores leads to an artificially high donor fluorescence intensity. This causes the calculated energy transfer efficiency to be underestimated in ensemble measurements. In the experiments discussed here, this is observed as a result of DNA melting, which causes an increase in donor fluorescence because the acceptor molecule is no longer present to quench the fluorescence. In single molecule FRET experiments, donor-only contaminants or melted DNA strands will lead to a FRET efficiency of zero (zero acceptor fluorescence is observed), which can be separated from the true FRET efficiency.

The goal of this work is to compare the donor quenching and ratiometric analysis methods in terms of reproducibility and reliability. Ensemble FRET experiments in which an ND-3300 was used have employed both of these approaches. In order to assess these methods, FRET experiments were carried out using an ND-3300 on 11 base pair (11bp) and 16 base pair (16bp) double-stranded DNA samples labeled with donor and acceptor dye molecules at the 5’ ends of complementary strands. FRET efficiencies, $E_{dq}$ and $E_r$, were calculated for these samples and compared to those found from single molecule FRET studies of the same dsDNA samples. This comparison has allowed the best approach to use in FRET experiments employing Nanodrop fluorometry to be determined.
Materials and Methods

dsDNA Sample Preparation

Unlabeled and dye-labeled single-stranded DNA (ssDNA) oligos of 11 and 16 base pair lengths were purchased from Integrated DNA Technologies (IDT). The sequences of the DNA oligos examined are the same as those used by Iqbal et al. (5’CCACTGCTAGG 3’ and 5’CCACTGCACTGCTAGG 3’ for the 11 and 16 base pair strands, respectively)\(^6\)\(^8\). The donor dye is Alexa Fluor® 488 and the acceptor dye is Alexa Fluor® 594. The dyes are attached at the 5’ ends of complementary strands via a 6-carbon linker. The ssDNA complementary strands were annealed in sodium chloride-Tris-EDTA (STE) buffer (pH 7.4) at dye concentrations of 30 μM in 15 μL aliquots using an Eppendorf thermocycler (Mastercycler Pro S). In order to examine the impact of DNA melting on the FRET signal for the doubly-labeled samples, ratios of 1:1, 1:2, and 1:3 donor:acceptor strands (30 μM Alexa Fluor® 488; 30/60/90 μM Alexa Fluor® 594) were used in the annealing process. Donor-only and acceptor-only control samples were also prepared in 1:1, 1:2, and 1:3 ratios of the complementary strands. The annealing process involved heating the samples to 95 °C followed by slow cooling at a rate of 1°C per 5 minutes while holding an extra 25 minutes at 37 °C (\(T_m\) for 11bp dsDNA); the dsDNA was stored at 4°C until use later in the same day. Following this method, donor-only, acceptor-only, and doubly-labeled duplex DNA samples were obtained.

Ensemble Measurements

The annealed dsDNA was diluted using STE buffer (pH = 7.4) to a final volume of 30 μL. Absorbance spectra (1.2 μL samples) were obtained using a ThermoScientific Nanodrop 1000 spectrophotometer. The average absorbance value at 494 nm (Alexa Fluor® 488) or 591 nm (Alexa Fluor® 594) was used to determine the concentration of dye-labeled dsDNA (the absorbance at 494 nm was used to determine the concentrations of the doubly-labeled samples.
after correction for the contribution at 494 nm due to Alexa Fluor® 594). Fluorescence spectra of the same samples, with no additional dilution, were acquired using a ThermoScientific NanoDrop-3300 fluorometer. The samples were excited as described above.

**dsDNA Dissociation Constant Measurements**

Duplex DNA was annealed at higher concentrations (up to 110 μM) in order to calculate the dissociation constant, $K_d$, of 11bp and 16bp dsDNA at 25 °C. Fluorescence spectra were acquired using a ThermoScientific NanoDrop-3300 fluorometer for doubly-labeled dsDNA over a range of 6 – 75 μM as well as for Alexa Fluor® 488 and Alexa Fluor® 594-labeled ssDNA at the same concentrations. The samples were excited using the blue LED (470 ± 10 nm) with the autogain feature activated. Three measurements (2.0 μL samples) were taken of each sample.

**Single Molecule Measurements**

Single molecule measurements were carried out using a home-built apparatus based around an Olympus IX70 confocal microscope. The 488 nm line of an argon-ion laser (Spectra Physics, Model 163C) was used to excite the samples when Alexa Fluor® 488 acted as the donor. Prior to excitation, the beam passed through a 5× Galilean beam expander and a Z48810X excitation filter (Chroma Optics). The laser light was then focused 50 μm into the sample solution using an Olympus UPLANAPPO 60x, NA 1.2 water-immersion objective. The sample fluorescence was collected through the same objective and then separated from the excitation source using both Z488RDC and HQ500LP filters (Chroma Optics). The fluorescence of the donor was separated from that of the acceptor using a 565DCLP filter (Chroma Optics). The donor fluorescence was further isolated using an ET525/50nm band-pass filter (Chroma Optics) and the acceptor fluorescence was isolated using an ET565LP filter (Chroma Optics). The donor and acceptor fluorescence signals were transmitted to SPCM-AQR-14-FC avalanche photodiode detectors (PerkinElmer) using multimode fiber optic cables with a diameter of 62.5μm.
(ThorLabs Me1L01) sealed with black tape to prevent light leakage. The donor and acceptor detector outputs were transmitted to a PCI-6602 counter/timer data acquisition card (National Instruments) through a BNC 2121 connector block (National Instruments). The PCI-6602 was used to bin photon counts from the donor and acceptor channels in 1 ms increments. A home-written LabView (National Instruments) program was used to record the fluorescence data.

Single molecule samples of dsDNA were prepared by diluting the samples used for Nanodrop ensemble measurements so that the final concentration of Alexa Fluor® 488 was approximately 50 pM in STE buffer. In order to minimize DNA denaturation, 16 base pair, unlabeled-dsDNA at a concentration of 1nM was included in the buffer. To maximize the signal-to-noise ratio, single molecule measurements were taken immediately following dilution. LabTek borosilicate chambered coverglass wells, #1 thickness (Nalgene), were filled with 500 μL of the dsDNA solutions. More concentrated samples were used for alignment: 10 nM Alexa Fluor® 488 was used for coarse alignment and 1nM Alexa Fluor® 488 was used to align the stages holding the fiber optic cables. Single molecule data for each sample were collected for 5 minutes in 1 ms time bins.

**Results and Discussion**

To compare the calculated energy transfer efficiencies for dsDNA labeled with Alexa Fluor® 488 (donor, AF488) and Alexa Fluor® 594 (acceptor, AF594), fluorescence emission spectra were obtained for dsDNA of 11 and 16 base pairs in length. Energy transfer efficiencies were calculated using single molecule measurements and both the ratiometric and donor-quenching ensemble methods. For FRET, the efficiency of energy transfer, \( E \), is given by (1.11). For the FRET pair used here, Alexa Fluor® 488/594, bound to dsDNA, the calculated value of \( R_0 \) is 56 Å.
11bp and 16bp dsDNA have fairly low melting temperatures ($T_m$) due to their short length. To investigate the impact of DNA melting on the calculated FRET efficiency, donor-labeled ssDNA was annealed with acceptor-labeled ssDNA in ratios of 1:1, 1:2 and 1:3 such that the extra acceptor-labeled strand would shift the equilibrium to favor the annealed DNA construct. These samples were used both for ensemble and single molecule measurements, though extra complementary strand was not expected to have an impact on single molecule experiments as the presence of ssDNA would only contribute to the observed peak at a FRET efficiency of zero. To quantitatively assess the effect of DNA melting on the calculated FRET efficiencies for ensemble measurements, the fraction of dsDNA melted and the dissociation constant for DNA melting were determined at 25 °C for both the 11bp and 16bp DNA oligomers.

**Dissociation Constant Measurements**

The fraction of dsDNA melted ($\alpha$) and the dissociation constant ($K_d$) for DNA melting at 25 °C for the 11bp and 16bp oligomers were determined from ensemble FRET measurements according to the procedure reported by Orte et al.$^{73}$ By observing the increase in acceptor fluorescence as a result of DNA annealing, the value of $\alpha$ could be determined. This was accomplished using the relationship between the acceptor fluorescence signal and $\alpha$ shown below$^{73}$.

$$I_A = C_T I_A^{ss}(\alpha + x) + C_T I_A^{ds}(1 - \alpha) + I_A^{buffer} \quad (2.2)$$

Here all $I_A$ values refer to integrated fluorescence intensities from 650 – 751 nm; a region in which only the acceptor fluoresces to an appreciable degree. $I_A$ is the acceptor fluorescence for the sample studied; $C_T I_A^{ss}$ is the fluorescence intensity observed for a sample that is completely dissociated; $C_T I_A^{ds}$ is the fluorescence intensity of a sample that is 100% annealed. The values of $C_T I_A^{ss}$ and $C_T I_A^{ds}$ were determined from measurements of acceptor-labeled ssDNA and highly
concentrated (110 μM) dsDNA, respectively. The term $I_{\text{buffer}}^A$ accounts for any fluorescence present in the 650 – 751 nm region due to the buffer alone. $C_T$ is the total concentration of the donor-labeled ssDNA. This is also the maximum possible concentration of dsDNA for the sample (i.e., the concentration of dsDNA if 100% of the DNA is annealed). The $x$ in (2.2) allows non-equal ratios of donor and acceptor strands to be accounted for. This quantity is equal to 0, 1, and 2 for the 1:1, 1:2, and 1:3 samples, respectively.

Using the measured acceptor fluorescence signal and (2.2), the value of $\alpha$ was calculated for various DNA concentrations. Using these values of $\alpha$, the equilibrium concentrations of the ssDNA (equal to $\alpha C_T$ for each strand) and the dsDNA [equal to $(1-\alpha) C_T$] could be calculated and used to determine the dissociation constant for DNA melting according to the expression given below.

$$K_d = \frac{\alpha^2 C_T}{1-\alpha}$$  \hspace{1cm} (2.3)

The average value of $K_d$ determined for the 11bp oligomer is $1.4 \pm 0.5$ μM. For the 16bp oligomer, the average $K_d$ value is $0.8 \pm 0.5$ μM. These values were used to determine the percent of dsDNA dissociated in the DNA samples used for ensemble FRET measurements. With these percentages known, calculated ensemble FRET efficiency values could be corrected to account for the presence of ssDNA in the mixture.

**Ensemble FRET Results**

Ensemble FRET measurements carried out using the ND-3300 were analyzed using two different methods: donor-quenching and ratiometric. The fluorescence spectra analyzed appear in Figure 2.1. The efficiency of donor quenching can be calculated using the relationship detailed in equation (2.1). Fluorescence intensities were determined by integrating portions of the
emission spectra of the donor-only and doubly-labeled dsDNA. Because of spectral overlap between the donor and acceptor fluorescence, isolated regions of each spectrum were integrated: donor fluorescence was integrated over the wavelengths 499-555 nm and acceptor fluorescence was integrated from 611-751 nm.

Figure 2.1: Comparison of Emission Spectra of Donor-only (dotted line) and Doubly-labeled (solid line) dsDNA Samples. In all cases, the spectra have been normalized to the AF488 concentration and corrected for direct excitation of the acceptor. A – C: 11bp dsDNA in 1:1, 1:2, and 1:3 donor:acceptor strand ratios, respectively. D – F: 16bp dsDNA in 1:1, 1:2, 1:3 donor:acceptor strand ratios, respectively. As expected, the efficiency of energy transfer is noticeably lower for the 16-base pair DNA relative to the 11-base pair DNA, as indicated by the relative amounts of donor and acceptor fluorescence.

For the donor-quenching method, the differing concentrations of the donor-only dsDNA and doubly-labeled dsDNA samples needed to be accounted for. This was done by normalizing the integrated fluorescence intensities using the absorbance of Alexa Fluor® 488 (AF488) for each sample. Substituting the normalized fluorescence intensities of the donor, in the presence
and absence of the acceptor, into (2.1) yields the efficiency of energy transfer, $E_{dq}$. The values determined for each sample of the 11bp and 16bp dsDNA constructs appear in Table 2.1. The value of $E_{dq}$ for the 11bp 1:1 sample is significantly lower than that observed for the samples with extra acceptor strand, suggesting a significant amount of ssDNA is present in the 1:1 sample. This is not unexpected, because the 11bp dsDNA has a low melting temperature (37 °C). Using the value of $K_d$ for 11bp dsDNA, the percent of dissociated dsDNA under the experimental conditions was determined to be 31% for the 1:1 sample. The smaller difference between the $E_{dq}$ values for the 1:2 and 1:3 samples for the 11bp dsDNA suggests that most of the DNA is annealed even when there is only twice as much acceptor strand present. This observation is supported by the calculated values of the percent of dissociated dsDNA for the 1:2 and 1:3 samples, which are 11% and 6%, respectively.

**Table 2.1: FRET Efficiency Values for 11bp and 16bp dsDNA Constructs**

<table>
<thead>
<tr>
<th>Size of dsDNA construct</th>
<th>Donor:Acceptor Strand Ratio</th>
<th>n</th>
<th>$E_{dq} = 1 - \frac{I_{DA}}{I_D}$</th>
<th>$E_r = \frac{I_A}{I_A + \gamma I_D}$</th>
<th>Single Molecule (SM)</th>
<th>n (SM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11bp</td>
<td>1:1</td>
<td>3</td>
<td>0.27 ± 0.03*</td>
<td>0.343 ± 0.004*</td>
<td>0.57 ± 0.03*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>3</td>
<td>0.57 ± 0.02</td>
<td>0.54 ± 0.02</td>
<td>0.56 ± 0.02*</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>1:3</td>
<td>3</td>
<td>0.63 ± 0.01</td>
<td>0.57 ± 0.01</td>
<td>0.56 ± 0.02*</td>
<td>7</td>
</tr>
<tr>
<td>16bp</td>
<td>1:1</td>
<td>6</td>
<td>0.43 ± 0.03*</td>
<td>0.19 ± 0.02</td>
<td>0.22 ± 0.05*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1:2</td>
<td>6</td>
<td>0.23 ± 0.01</td>
<td>0.237 ± 0.005</td>
<td>0.25 ± 0.04*</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>1:3</td>
<td>6</td>
<td>0.24 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>0.25 ± 0.03*</td>
<td>7</td>
</tr>
</tbody>
</table>

*Stated uncertainties are standard deviations from multiple measurements or propagated from the uncertainties in absorbance and fluorescence intensity values; the larger uncertainty is reported. Values marked with an asterisk are statistically different from the single molecule results, as determined by t-test analysis ($p < 0.05$).

In contrast, the 16bp DNA shows a marked decrease in the FRET efficiency when the acceptor strand concentration is doubled and tripled (Table 2.1). Because the $T_m$ for 16bp dsDNA is much higher (53 °C) it was expected to be more resistant to melting. This is indicated by the calculated percent dissociation values for the 1:1, 1:2, and 1:3 16bp dsDNA samples, which are...
25%, 7%, and 4%, respectively. The larger percent dissociation of the 1:1 sample would be expected to lead to an underestimated FRET efficiency value. The fact that the calculated FRET efficiency for the 1:1 sample is higher than that observed for the 1:2 and 1:3 samples indicates that the donor quenching analysis method is subject to errors beyond those introduced by incomplete labeling of a sample.

The primary issue with this method is that it involves the comparison of fluorescence intensities from two different samples (donor-only and doubly-labeled). Fluctuations in noise and small differences in the samples can lead to significant alterations in the calculated FRET efficiencies. This is particularly true for the 1:1 16bp sample, as the decrease in the donor fluorescence for the doubly-labeled sample will not be large, due to both the small FRET efficiency expected given the distance between the dye molecules and the fact that only 75% of the sample is annealed. Previous confocal microscopy imaging studies also indicated a lack of reliability and the occasional occurrence of negative FRET efficiency values when using the donor quenching method\textsuperscript{74}. The authors of the microscopy study attribute the lack of reliability of the donor quenching method solely to its reliance on different samples\textsuperscript{74}.

This deficiency is not a factor in the ratiometric method, which was also used to determine the FRET efficiency for ensemble measurements according to the relationship shown in equation (1.12). The $\gamma$ correction factor accounts for the different fluorescence quantum yields and detection efficiencies for the donor and acceptor, as shown in (2.4):

$$\gamma = \frac{\phi_A \eta_A}{\phi_D \eta_D}$$  \hspace{1cm} (2.4)

In this expression, $\phi_D$ and $\phi_A$ are the fluorescence quantum yields of the donor and acceptor, respectively, and $\eta_D$ and $\eta_A$ represent the detection efficiencies of the donor and acceptor, respectively. For ensemble measurements, the detection efficiency is equivalent to the fraction of
the total fluorescence intensity from the dye molecule that is included in $I_A$ or $I_D$ as a result of the wavelength range selected for the integrated intensity. The detection efficiency for each dye (0.835 ± 0.002 for AF488 and 0.794 ± 0.009 for AF594) was calculated using the fluorescence from singly-labeled samples and used to correct the integrated intensity values. Experimental measurements indicate quantum yield values of 0.67 for AF488 and 0.51 for AF594 when they are attached to the dsDNA sequences used in this experiment, as opposed to the reported values of 0.92 and 0.66 for the dyes (AF488 and AF594, respectively) free in solution (Invitrogen website). The substantial difference in the quantum yield values is likely due to the quenching of fluorescence from nearby guanine residues. Because the quantum yield did not decrease for single-stranded DNA ($\phi = 0.97$) which has no guanine residues in close proximity to the fluorophore, it is likely this is the cause for the reduced quantum yield numbers observed.

Two additional correction factors are necessary to accurately determine the value of $I_A$: a “bleedthrough” factor to account for the small amount of donor fluorescence that contributes to the acceptor signal and a direct excitation value that accounts for any acceptor molecules that absorb light from the blue LED directly. To determine the bleedthrough factor in an ensemble measurement, the amount of donor fluorescence in the acceptor region was first determined using a donor-only sample. The ratio of this signal to the integrated donor signal used for $I_D$ is the bleedthrough factor, $B$. To account for this effect, $B$ is multiplied by the donor intensity of the doubly-labeled dsDNA sample and subtracted from the acceptor signal in doubly-labeled samples, as shown in (2.5). Additionally, the amount of acceptor signal occurring as a result of directly exciting the acceptor can be accounted for by integrating the fluorescence signal of an acceptor-only labeled sample of dsDNA of equal concentration (or determining the ratio of acceptor fluorescence to concentration of acceptor) and subtracting this value, $D$, from $I_A$. 
Subtracting both factors from $I_A$ in a doubly-labeled sample will produce the corrected intensity value. After applying these correction factors, equation (1.12) can be expressed as

$$E_r = \left(1 + \gamma \frac{I_D}{I_A}\right)^{-1} = \left(1 + \gamma \frac{I_D}{I_D - B I_D - D}\right)^{-1}$$

(2.5)

The FRET efficiency values, $E_r$, for the 11bp and 16bp dsDNA samples determined using the ratiometric method are shown in Table 2.1. For the 11bp dsDNA, a substantially lower value of $E_r$ is obtained for the 1:1 donor:acceptor strand ratio. This substantiates the claim that it is the significant percent dissociation (31%) due to the low $T_m$ for this sample, rather than a deficiency of either analysis method, that leads to a low energy transfer efficiency. The $E_r$ values calculated for the 1:2 and 1:3 samples agree within the stated uncertainties, indicating that decreasing the percent dissociation to approximately 10% mitigates the effect of donor-labeled ssDNA on the calculated FRET efficiency. The differences in calculated efficiencies are much less pronounced for the 16bp dsDNA samples and show only a slight increase in the presence of extra acceptor strand. This is likely due to the smaller percent dissociation of the 1:1 16bp sample (25%) relative to the 1:1 11bp sample (31%).

The average values calculated using the ratiometric method tend to be lower than those observed with the donor-quenching approach, though this is not always the case (Table 2.1). It has been suggested previously that the donor-quenching efficiency is artificially high due to an alternative quenching pathway in the presence of an acceptor molecule\(^{57}\). While this may be possible, the primary difference between these analysis methods is that the donor-quenching method relies on separate donor-only and doubly-labeled dsDNA samples whereas the ratiometric method directly compares fluorescence within only the doubly-labeled dsDNA samples.
Single molecule FRET Results

To further ascertain the best method with which to analyze ND-3300 ensemble data, single molecule experiments were performed on the dsDNA with the goal of calculating FRET efficiencies for both the 11bp and 16bp dsDNA samples. As the dsDNA sequences are rather short, they are prone to melting at the highly dilute concentrations (50 pM) used in single molecule FRET experiments on freely-diffusing molecules. Single molecule FRET (smFRET) measurements, however, can be used to distinguish between ssDNA labeled with a donor (which will have an energy transfer efficiency of zero) and dsDNA labeled with a FRET pair. As such, the calculated FRET efficiency using single molecule data serves as a point of comparison that should agree with optimally-analyzed ensemble measurements.

Single molecule FRET efficiencies were calculated using the ratiometric method. FRET events were chosen using a SUM protocol with a threshold of 50 photon counts/millisecond in both channels as detailed in previous publications\textsuperscript{44,54}. Once events were chosen, they were corrected using the amended equation (2.5). As the sensitivity of the optics and detectors is different for the single molecule measurements, $\gamma$ has a value of 1.3 using the single molecule apparatus as opposed to the value of 0.72 used in the ensemble calculations. Direct excitation and bleedthrough were also calculated in a different manner. Single molecule data obtained using acceptor-only labeled-dsDNA were used to determine background signal due to noise and direct excitation, and the average counts were subtracted from the doubly-labeled dsDNA data. Next, donor-only labeled-dsDNA was analyzed in the same manner. Donor bleedthrough percentages (into the acceptor channel) were calculated by determining the ratio of the donor to acceptor counts and averaging across all events. This factor was then used to correct the acceptor fluorescence intensity for all evaluated FRET events.
The average smFRET efficiencies obtained for the 1:1, 1:2, and 1:3 mixtures of 11bp dsDNA and 16bp dsDNA are reported in Table 2.1. These values were obtained by plotting a histogram of all calculated efficiency values and fitting this histogram with a double-Gaussian function (Figure 2.2). The values obtained for the varying ratios of donor and acceptor strands agree within the stated uncertainties, supporting the presumption that smFRET measurements are not affected by the presence of donor-only labeled sample. This validates the use of smFRET values as a standard by which the quality of ensemble FRET analysis methods can be judged. The smFRET values are in good agreement, within the stated uncertainty, with ensemble data analyzed using the ratiometric approach in the presence of extra acceptor (see Table 2.1). Only the transfer efficiency of the 1:1 11bp sample is statistically distinct (according to t-test results) from values obtained from the single molecule experiments. However, there is a larger discrepancy between the single molecule results and the ensemble results obtained using the donor-quenching analysis. In this case, the results obtained are generally better when the degree of labeling (i.e., number of molecules with both a donor and acceptor present) is maximized, but there is not a consistent effect on the energy transfer value obtained due to the presence of donor-only species. For the 1:1 ratio 11bp samples, the value of $E_{dq}$ is too low, whereas for the 16bp samples, the $E_{dq}$ value is too high. Because of this inconsistency, it can be difficult to interpret ensemble FRET results using this method if a significant portion of the sample (> 25%) is labeled with only one dye molecule.
Figure 2.2: Single Molecule FRET Efficiency Histograms for DNA Oligos Labeled with AF488 and AF594, Respectively. The peak that appears at an efficiency of zero in both plots is due to donor-only single-stranded DNA or duplex DNA in which the acceptor is photobleached. A double Gaussian curve (the solid black line) was used to fit the histograms in both cases. (A) The peak FRET efficiency for the 11-base pair sample appears at 0.570 ± 0.009; the width of the peak is 0.24 ± 0.01. (B) The peak FRET efficiency for the 16-base pair sample appears at 0.255 ± 0.003; the width of the peak is 0.197 ± 0.005.

Because the ensemble FRET efficiencies for the 1:1 samples, which have the highest percent dissociation, generally show a statistically significant difference from the smFRET values, it was investigated whether or not the observed differences can be quantitatively explained by accounting for the percent dissociation of the dsDNA sample. To do so, the values of $I_D$ and $I_A$ that appear in equation (2.1) and equation (2.5) are corrected by subtracting the fluorescence contributions from ssDNA. The corrected fluorescence intensity of the donor is given by
\[ I_D^{ss} = I_D - \alpha C_I I_D^{ss} \]  

(2.6)

All \( I_D \) values in (2.6) refer to the integrated fluorescence intensity of the donor from 499 – 555 nm. \( I_D \) is the observed donor fluorescence intensity of the sample, \( \alpha C_I I_D^{ss} \) is the donor fluorescence intensity if the entire sample is ssDNA, and \( I_D^{ds} \) is the corrected donor fluorescence intensity arising only from annealed DNA. The value of \( \alpha C_I I_D^{ss} \) was determined from donor-only labeled ssDNA samples. This correction accounted for both the percent dissociation and the increased fluorescence quantum yield of AF488 on ssDNA relative to AF488-labeled dsDNA.

The expression for the corrected fluorescence intensity of the acceptor, shown below in (2.7), is more complex as it requires corrections for bleedthrough from AF488-labeled ssDNA (\( \alpha C_I B I_D^{ss} \)), bleedthrough from AF488-labeled dsDNA [(1 – \( \alpha \))\( C_I B I_D^{ds} \)], direct excitation of AF594-labeled ssDNA (\( \alpha C_I D^{ss} \)) and direct excitation of AF594-labeled dsDNA ((1 – \( \alpha \))\( C_I D^{ds} \)).

The single- and double-stranded correction factors needed to be accounted for separately as both AF488 and AF594 exhibit different fluorescence quantum yields when bound to ssDNA vs. dsDNA. In all cases, the correction factors contain either an \( \alpha C_I \) or a (1 – \( \alpha \))\( C_I \) term to account for the concentrations of the ssDNA and dsDNA species. As seen in (2.7), by subtracting all of the corrective terms from the observed integrated acceptor fluorescence intensity from 611 – 751 nm, \( I_A \), the acceptor fluorescence intensity arising only from FRET, \( I_A^{ds, FRET} \), could be calculated.

\[ I_A^{ds, FRET} = I_A - \alpha C_I B I_D^{ss} - (1 - \alpha) C_I B I_D^{ds} - \alpha C_I D^{ss} - (1 - \alpha) C_I D^{ds} \]  

(2.7)

Substituting the corrected donor and acceptor fluorescence intensities into equations (2.1) and (2.5) resulted in corrected \( E_{dq} \) and \( E_r \) values for the 1:1 11bp and 16bp samples, respectively. These corrected values appear in Table 2.2, along with the \( E_{dq} \) and \( E_r \) values calculated without
accounting for the percent dissociation of the DNA. As expected, the corrections increase the
calculated FRET efficiencies as the artificially high donor fluorescence intensity arising from
donor-labeled ssDNA has been reduced. In general, this leads to improved agreement with
smFRET values. However, these corrections do not fully account for the differences present
between the ensemble and single molecule FRET results. The ratiometric method corrected
efficiencies give better agreement, with a 20% error for the 11bp DNA and a 14% error for the
16bp DNA (which is not a statistically significant difference). The percent errors for the
corrected donor quenching efficiencies are 35% and > 100% for the 11bp and 16bp DNA,
respectively. As FRET efficiency values for confocal microscopy instruments are known to
exhibit percent errors on the order of 20% for the most reliable methods, the fact that the
corrected ratiometric efficiencies deliver values in this range lends greater confidence to this
method as opposed to the donor quenching approach. The poor signal-to-noise present in a DNA
sample with less than 75% of the molecules labeled with both a donor and an acceptor appears to
prohibit the accurate determination of ensemble FRET efficiencies for these samples using
Nanodrop fluorometry.

**Table 2.2: Impact of Correction for ssDNA on Calculated FRET Efficiencies**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>11bp (n = 3)</th>
<th>16bp (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ (μM)</td>
<td>1.4 ± 0.5</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>0.31 ± 0.07</td>
<td>0.25 ± 0.09</td>
</tr>
<tr>
<td>$E_{dq}$</td>
<td>0.27 ± 0.03$^*$</td>
<td>0.43 ± 0.03$^*$</td>
</tr>
<tr>
<td>Corrected $E_{dq}$</td>
<td>0.37 ± 0.02$^*$</td>
<td>0.5 ± 0.1$^*$</td>
</tr>
<tr>
<td>$E_r$</td>
<td>0.343 ± 0.004</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>Corrected $E_r$</td>
<td>0.458 ± 0.008$^*$</td>
<td>0.25 ± 0.04</td>
</tr>
</tbody>
</table>

$^a$Stated uncertainties are standard deviations from multiple measurements. Values marked with an asterisk are statistically different from the single molecule results, as determined by t-test analysis ($p < 0.05$).
Conclusions

In summary, it has been shown here that it is possible to obtain and analyze ensemble FRET data using a commercially available fluorometer capable of measuring fluorescence from a 2 μL sample. Two common ensemble analysis methods were compared to single molecule results and it was demonstrated that while both donor-quenching and ratiometric approaches can be used to analyze ensemble data, the ratiometric approach is a more robust method when using the ND-3300 fluorometer. In cases when the ratiometric approach will not work (i.e., when the acceptor molecule is non-fluorescent), we have shown that careful sample preparation is needed in order to obtain meaningful results from the donor-quenching method; slight differences between donor-only and doubly-labeled samples can lead to vastly different FRET efficiencies.

In addition, it has been shown here that DNA melting impacts the calculated FRET efficiencies at the concentrations used in these experiments (10-15 μM) when using the ND-3300. For accurate ensemble results, a molar excess of at least double acceptor strand is needed, even for samples with high melting temperatures. As the $T_m$ of dsDNA is a contributing factor to the error in FRET analysis, we plan to investigate dsDNA of increased length with internal fluorophore labeling. The increased length of the DNA strand should substantially increase the value of the melting temperature and limit error due to the presence of ssDNA. It is anticipated that both the ratiometric and donor-quenching approaches will provide improved values in this case. By demonstrating the more reliable nature of the ratiometric method for ensemble FRET studies, the use of this approach is encouraged in future Nanodrop fluorometry studies.
Chapter 3: The Selective Attachment of Fluorescent Probes to Catalytic Antibodies for Förster Resonance Energy Transfer (FRET) Studies

Introduction

The system of interest in this experiment is the catalytic aldolase antibody 38C2\textsuperscript{75}. Catalytic antibodies are antibodies that are able to catalyze chemical reactions. 38C2 is referred to as an aldolase antibody because it catalyzes the aldol condensation reaction. A unique feature of this antibody is that the catalytic component is the neutral amine of a lysine residue\textsuperscript{76}. 38C2 is unusual as the amine group of lysine is usually protonated and positively charged at biological pH conditions. As such, amine reactive compounds will not react with other amino acids that make up the antibody because they are in their protonated form and are not nucleophilic (basic). Because the catalytic lysine is sequestered from water molecules, it remains unprotonated and reactive, even in aqueous solution. In theory, amine-reactive dye molecules could be selectively attached to 38C2 at the catalytic lysine residues. If the antibody can be labeled with a donor and an acceptor molecule, then FRET can be used to determine conformational changes in the arms of the antibody (Figure 3.1).
Figure 3.1: Diagram of Antibody 38C2. The image diagrams the major features of an IgG antibody. The letters ‘D’ and ‘A’ correspond to the desired locations of the donor and acceptor molecules for FRET studies (Protein image was generated from the protein data bank file 1IGT.pdb).

The experiments detailed here involve the attachment of the fluorescent probes Alexa Fluor® 568 (AF568), Alexa Fluor® 594 (AF568) and Cy5.5 to the antibody. Previous crystallography experiments have been used to determine that the catalytic lysine is located in a pocket approximately 10 Å deep\(^76\). The pocket is formed from folds in the antibody at the antigen binding site and creates a nonpolar environment that keeps the lysine in its neutral form because it is inaccessible to water molecules (Figure 3.2).
Figure 3.2: Hydrophobic Pocket of IgG 33F12. Shown here is the hydrophobic pocket located within the Fab region of IgG 33F12 which is an analog of IgG 38C2. The carboxylate form of hapten 2 is shown within the pocket along with the location of the catalytic lysine residue (H33). This figure was obtained from JACS (2009) 131-51 (see reference 74).

Because of this, a hapten molecule (Figure 3.3), a small organic linker molecule, must first be attached to the dye before it can bind to the antibody. The goals of this experiment are twofold. One is to verify that the chosen hapten will bind to antibody 38C2 and a commercially available dye of interest (AF594). The second is to perform single molecule fluorescence studies on freely diffusing antibody-dye conjugate by monitoring changes in the FRET efficiency of this system.

Figure 3.3: Hapten Molecules. The two hapten molecules used in the experiment are shown. The amine hapten (1) was used to verify that binding did occur between the antibody and the hapten molecules. The N-hydroxysuccinimidyl ester (NHS) hapten molecule (2) was reacted with fluorescent probes to provide a long chain that mitigates potential steric effects of attaching a large dye molecule to the antibody.
Both hapten molecules were chosen for their \( \beta \)-diketone functionality. This group can react with the nucleophilic lysine residue to form a stable enaminone intermediate (Figure 3.4). The enaminone has been shown to absorb weakly at 316 nm and can be used to assess hapten binding to the antibody\(^{76}\). Hapten 1 was used for verification purposes, because it can be produced in fewer synthetic steps. Hapten 2 was chosen for the FRET experiment, because the longer chain length ensures the \( \beta \)-diketone group access to the reactive lysine without steric hindrance from the fluorophore.

![Figure 3.4: Attachment of Dye to 38C2.](image)

**Figure 3.4: Attachment of Dye to 38C2.** The fluorophore is attached via the \( \beta \)-diketone hapten (2). The hapten can react with the catalytic lysine residue to form an enaminone intermediate.

For the single molecule fluorescence studies, AF568 and Cy5.5 were chosen as the FRET donor-acceptor pair; the dyes were chosen for the large \( R_0 \) value (see equation 1.9) associated with the pair (76 Å). AF568 has a relatively large quantum yield (0.69) and the two dyes show large overlap between emission (AF568) and absorbance (Cy5.5) spectra. A large \( R_0 \) value was desired as previous distance measurements suggest the Fab sites can be separated by large distances (100-150 Å)\(^{31,32}\). Presented here are the results of the binding experiment of Alexa
Fluor® 594 with the hapten linker along with the single molecule FRET study on AF568/Cy5.5-labeled 38C2.

**Materials and Methods**

**Hapten Synthesis:**

The amine hapten (1) and the N-hydroxysuccinimidyl ester (NHS)-hapten (2), (Figure 3.3), were synthesized according to the procedure detailed by Sinha et al. The starting materials, p-nitrobromobenzene and acetylacetone were reacted in the presence of lithium diisopropylamide. The resulting nitro compound was then hydrogenated to form hapten (1) or hydrogenated in the presence of glutaric anhydride to form a carboxylate hapten. The carboxylate hapten was reacted with N-hydroxysuccinimide in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to form the NHS hapten (2). The product was verified using $^1$H NMR spectroscopy and purified by column chromatography with a mixture of ethyl acetate and hexanes. A small amount of NHS-hapten (2) was stored in dry tetrahydrofuran (THF) at a concentration of 1 mg/mL at 4°C whereas the remainder was stored in the freezer at -20°C.

**Reaction of Hapten with Antibody 38C2:**

Antibody 38C2 was combined with 20 μL of amine hapten in phosphate buffered saline (PBS, pH = 7.4) such that the antibody was brought to a final volume of 200 μL at a concentration of 2 mg/mL. The reaction was allowed to incubate at room temperature overnight. The reaction mixture was placed in a Millipore Amicon Ultra Centrifugal Filter Unit with a molecular weight cutoff of 10,000 g/mol and centrifuged at 14,000 relative centrifugal force (rcf) for 4 minutes. The recovered sample was compared, via UV-Vis spectroscopy, to the solution that passed through the filter using a ThermoScientific NanoDrop 1000 UV-Visible spectrophotometer. The antibody concentration was determined using the extinction coefficient.
given by Sigma Aldrich (technical bulletin AL-207). The concentration of hapten was determined using an experimentally derived extinction coefficient in PBS of 17,200 L/mol·cm at 330 nm, not to be confused with the enaminone peak at 316 nm.

**Reaction of Dye with Hapten Molecule:**

Alexa Fluor® 568 hydrazide (Invitrogen) was stored at a concentration of 10 mg/mL in dry dimethyl formamide (DMF). Excess NHS-hapten (in THF) was added to 10 μL of the dye in a ratio of approximately 10:1 to reach a final volume of 450 μL. The reaction was incubated with constant stirring at room temperature overnight. Thin layer chromatography (TLC) was used to gauge the extent of the dye-hapten reaction. A solvent system that consisted of a 60:40 methanol:ethyl acetate ratio was observed to give the best separation of reactants from starting materials.

**Column Chromatography:**

The dye-hapten reaction product was isolated from the starting materials by filtration through a 10 cm silica gel column. Hexanes were used to equilibrate the column and wash unreacted hapten. A solution of 60:40 methanol:ethyl acetate was used to elute the dye-hapten product. The methanol:ethyl acetate solution was added until all of the dye had washed out of the column and 15 x 1 mL fractions were collected as the solution was eluted. A ThermoScientific NanoDrop 1000 UV-Visible spectrophotometer was used to analyze the fractions collected from the column and verify the dye-hapten linkage.

**Isolation of Product:**

Due to poor recovery from this column chromatography process, an extraction process was also used to separate the hapten from the hapten-dye conjugate. After the dye-hapten reaction was completed, approximately 350 μL of PBS (pH, 7.2) was added to 50 μL of the dye-hapten mixture. A precipitate, likely to be the hapten, was observed to form. The solution was
then washed with methylene chloride in order to remove any hapten remaining in the aqueous layer. Centrifugation was performed and the supernatant containing the soluble dye-hapten conjugate (along with free dye) was separated from the pellet before reacting it with the antibody.

**Antibody-Dye-Hapten Attachment:**

The dye-hapten mixture was combined with antibody in a 2:1 molar ratio and diluted to a final reaction volume of 250 µL. Reaction conditions were such that the antibody concentration was kept at 2 mg/mL. Three solutions were prepared: a solution of AF568-hapten with antibody, a solution of Cy5.5-hapten with antibody and a sample with both AF568- and Cy5.5-hapten molecules with antibody. Each reaction was incubated at room temperature overnight with constant stirring. Free dye was separated from the antibody-hapten-dye conjugates using dialysis cassettes (Pierce). The 250 µL reaction volume was diluted to 1 mL and dialyzed against PBS (pH, 7.2) for 48 hours at 4°C. The reaction mixture was filtered using a Millipore Amicon Ultra Centrifugal Filter Unit as described before. Binding was assessed by comparing the solution that passed through the filter (flowthrough) with the recovered sample using a ThermoScientific NanoDrop 1000 UV-Visible spectrophotometer.

**Single molecule Sample Preparation:**

Solutions that were 50 pM in antibody and dye were prepared for single molecule fluorescence experiments. The antibody solutions were diluted with PBS buffer prepared with fluorescence-grade or LC/MS-grade water. Control solutions included PBS buffer, a solution of antibody, a solution of AF568-bound antibody, and a solution of Cy5.5-bound antibody. The experimental sample contained doubly-labeled (AF568/Cy5.5) antibody. A 750 µL-sample of each solution was added to a LabTek borosilicate chambered coverglass (Nalgene) for use with the confocal microscope.
Single molecule measurements were obtained using the apparatus described in Chapter 2 with the following exceptions. The 543 nm line of a helium-neon laser (Thorlabs) was used to excite the samples when Alexa Fluor® 568 acted as the donor. Prior to excitation, the beam passed through a 5× Galilean beam expander and a XF1204 excitation filter (Omega Optical). The sample fluorescence was collected through the objective and then separated from the excitation source using both XF3090 and XF2018 filters (Omega Optical). The fluorescence of the donor was separated from that of the acceptor using a XF2023 filter (Omega Optical). The donor fluorescence was further isolated using a QMAX600-650 band-pass filter (Omega Optical) and the acceptor fluorescence was isolated using an XF3104 filter (Omega Optical).

**Results and Discussion**

The reaction of antibody with the amine hapten was assessed using spectroscopic techniques after the sample had been purified using the centrifugal filters. Figure 3.5 displays the spectra of the mixture both before and after centrifugation. The peak for the antibody at 280 nm is seen to increase after filtration, but the peak for the hapten at 330 nm stays approximately the same size and shifts slightly to a shorter wavelength. Because the hapten (MW = 205.26 g/mol) is smaller than the filter cutoff, it was expected to pass through the filter and any peak that appears in the 330 nm region must be a result of hapten that is bound to the antibody. If the hapten did not pass through the filter, it would be expected that the peak would increase in a fashion similar to the antibody due to the increased sample concentration. The absence of signal in the flowthrough indicates that free hapten was caught in the filtration and did not pass completely through. While this does not impede the labeling process, a method where unreacted hapten could be recovered is more desirable and may be pursued at a later date.
Figure 3.5: Hapten Binding to Antibody 38C2. Absorbance data are shown for the reaction of the amine hapten and antibody 38C2 before and after filtration. The peak at 280 nm is due to the antibody. As expected, the concentration of antibody increases after filtration. The peak at ~330 nm is caused by bound hapten as the free hapten would have passed through the 10,000 MWCO filter. The flowthrough shows no absorbance. The ratio of the peak heights (antibody:hapten) corresponds to a binding efficiency of 87.4%.

The concentration of antibody was determined to be 25.7 μM. The amine hapten concentration was determined to be 44.9 μM. The analysis was made slightly more difficult because the absorbance spectrum of the hapten overlaps with that of the enaminone absorption at 316 nm. Because of this, an experimentally derived extinction coefficient was used to determine the concentration of bound hapten. Accounting for two binding sites per antibody molecule, one in each Fab, the binding efficiency of the hapten is calculated to be 87.4%.

Alexa Fluor® 594 was combined with the NHS-hapten in order to assess the extent of reaction prior to conjugation with antibody 38C2. After incubation overnight, binding was assessed using TLC in 60:40 methanol:ethyl acetate. The products separated into four distinct bands. A band corresponding to the free hapten travelled with the solvent whereas the two trailing bands mirrored those travelled by the free dye. The presence of a fourth band at an
intermediate distance, between the dye and hapten bands, suggests the presence of the dye-hapten conjugate. Figure 3.6 is a qualitative illustration of the paths travelled by each substance.

Figure 3.6: TLC of AF594-Hapten Mixture. The free dye (top) split into several bands while the free hapten (middle) travelled with the solvent (60:40 methanol:ethyl acetate). The presence of the faint intermediate band in the lane with the reaction mixture (bottom) likely corresponds to the desired dye-hapten conjugate.

Following the TLC evidence of dye-hapten conjugate formation, the hapten was reacted with AF568 and Cy5.5 and recovered using a methylene chloride extraction. The dye-hapten conjugates were then reacted with antibody 38C2. Binding of the two dyes was assessed using UV-Visible spectroscopic methods after free dye was removed (Figure 3.7). In this figure prominent peaks are visible for the antibody (280 nm), the enaminone (316 nm), and the two dye molecules (580 nm and 680 nm). Concentrations were again calculated using absorbance values: the concentration of binding sites was determined to be 28 μM (14 μM antibody), the concentration of Alexa Fluor® 568 and Cy5.5 were calculated to be 20 μM and 6.4 μM, respectively. It is believed that the concentration of Cy5.5 is lower, because red-shifted dyes tend to be less stable and more prone to aggregation.78
The percent of binding sites labeled with dye molecules was calculated at 94%. Also of note was the concentration of hapten molecules bound to the antibody. The absorbance indicates a concentration of bound hapten equal to 33 μM calculated using the published extinction coefficient of 15,000 cm⁻¹M⁻¹ at 316 nm. This value is indicative of binding greater than 100% (120%) for hapten, not the conjugate, bound to the antibody. This is best explained due to the spectral overlap of the Alexa Fluor® 568 with that of the enaminone peak in the region of 316 nm. Additionally, antibody-bound hapten not conjugated to dye molecules is likely contributing to the absorbance within this region.

Single molecule data were acquired using donor-only, acceptor-only and doubly-labeled 38C2 samples. Three minutes of data are shown in Figure 3.8 where data were collected in millisecond time intervals. Figure 3.8 shows photon counts in both the donor (pink) and acceptor (blue) detection channels. Figure 3.8A depicts fluorescence measurements for an antibody
labeled only with AF568. As expected, there is only significant fluorescence in the donor channel. The fluorescence of the antibody solution with both donor and acceptor dyes bound (3.8B) looks very similar; photon counts in both channels are of similar number in each case.

![Figure 3.8: Single Molecule Fluorescence Data for Antibody 38C2.](image)

To verify that no FRET was occurring between dye molecules in the solution containing antibody and the dye pair, a FRET efficiency histogram was created (Figure 3.9). Events were again chosen using the SUM protocol. Typically, the histogram would be fit to a double-Gaussian distribution: one at 0% efficiency and one at a higher number corresponding to energy transfer occurring between the FRET pair. This is not observed for the doubly-labeled sample in this case; the histogram could not be fit to a double-Gaussian function. In addition, the FRET efficiency histogram was nearly identical to that of the donor-only labeled sample. This suggests the distance between the FRET pair when bound is too great for energy transfer to occur.
Figure 3.9: FRET Efficiency Histogram for Doubly-Labeled Antibody 38C2. The majority of events occur with less than 15% energy transfer efficiency for the 38C2 labeled with AF568 and Cy5.5.

There are several explanations for the observed results. The simplest explanation is that the distance between AF568 and Cy5.5 is too great when attached to the antigen binding site of antibody 38C2. If this distance is greater than 114 Å no detectable energy transfer will occur as the efficiency of energy transfer will be so small, the peak would not be resolvable from the zero efficiency peak in the histogram. One of the few crystal structures of an intact IgG molecule indicates a distance of 160 Å (80 Å per light chain) would be present between the dye pairs if the Fab-Fab angle is 180°. This distance is far too great to observe FRET with the Alexa Fluor 568/Cy5.5 dye pair. However, experimental evidence suggests that immunoglobulin G molecules are highly flexible species as both previous energy transfer studies and cryo-electron tomography experiments support this hypothesis. The cryo-electron tomography images show the antibodies in various structural conformations with the average Fab-Fab angle equal to 110 ± 30°. Considering the lower value for the average Fab-Fab angle of 80°, the approximate distance between dye molecules is 103 Å and FRET should occur with approximately 14% efficiency. While this efficiency is low, there should be an observable population of molecules that contribute to a FRET efficiency peak at 14% in our histogram.
Another explanation for the lack of observed FRET events in the AF568/Cy5.5 sample is that there are errors in the calculation of the \( R_0 \) parameter, the \( \gamma \) correction factor, or both. Typically, \( \kappa^2 \) in the \( R_0 \) equation (1.9) is assumed to have a value of \( 2/3 \). This assumption implies that the dye molecule orientation is rapidly changing on a timescale that is much smaller than the fluorescence lifetime of the excited fluorophores\(^8\). It has since been shown that for fluorophores Cy3 and Cy5 bound to DNA molecules the observed energy transfer efficiency was not as high as that predicted using a \( \kappa^2 \) value of 2/3 and a faulty assumption could result in distance estimates that are off by as much as 12 Å\(^6\). The orientation factor has also been shown to impact dyes bound to proteins, specifically a polyproline chain labeled with Alexa Fluor\(^\circledR\) 488 and Alexa Fluor\(^\circledR\) 594\(^6\). If the \( \kappa^2 \) value is less than 2/3, then the Förster radius (\( R_0 \)) would be smaller than the 76 Å calculated for the Alexa Fluor 568/647 dye pair; this could explain the lack of FRET observed during the experiment. To counteract this problem, a longer more flexible linker hapten molecule could be used as long as it did not significantly increase the distance between the fluorophore pair as this would facilitate rotational freedom of the dye molecules.

As seen in equation (1.12) the \( \gamma \) factor also plays a role in determining the efficiency of FRET between the dye pairs. This parameter accounts for differences in fluorescence quantum yield and detection efficiencies. In this experiment, the \( \gamma \) factor used was 0.57 to correct for the larger quantum yield for the donor molecule (detector efficiencies were relatively similar). This correction was calculated using reported quantum yield values (Molecular Probes, Invitrogen). However, it has been observed that the quantum yield of fluorophores changes upon labeling to nucleic acid systems. Cy3 shows a drastic increase in quantum yield from 0.03 up to 0.4 depending on the size of nucleic acid\(^8\). To correct the \( \gamma \) factor used in this experiment, it would be necessary to measure the quantum yield of each dye molecule when bound to 38C2.
Another common occurrence that has been seen to affect FRET measurements is the photobleaching of dyes caused by molecular oxygen\textsuperscript{51,83}. Photobleaching of the dye molecules ultimately prevents the observation of fluorescence and can severely hinder FRET experiments; red dyes are particularly susceptible to interactions with molecular oxygen. To counteract the effect of molecular oxygen, microfluidic devices have been created that flush the dye solutions with N\textsubscript{2} gas and remove molecular oxygen. This technique has caused substantial increases in the observed FRET events between Alexa Fluor dyes attached to DNA molecules\textsuperscript{51}. Another common technique for removing O\textsubscript{2} from solution is to introduce an oxygen scavenging system. The most common system contains the glucose oxidase enzyme, glucose and the catalase enzyme. Additionally, the vitamin E analog Trolox has been shown to enhance photostability of the fluorophores, so it is often added to the system as well\textsuperscript{83}. As the antibody solution is in aqueous PBS buffer, it is of interest to remove O\textsubscript{2} from the system and take additional measurements of the AF568/Cy5.5 system.

**Conclusions**

The results of the hapten-antibody reaction indicate that the amine hapten binds to antibody 38C2 with an 87.4\% binding efficiency when no dye was present. Additionally, the fluorophores Alexa Fluor\textsuperscript{®} 568 and Cy5.5 were shown to bind to 94\% of the possible binding sites when the NHS-hapten was used as a linker. This dye system was then studied using single molecule techniques. Noticeable energy transfer was not observed for the antibody labeled with the FRET dye pair. This likely reflects the large distance between the two Fab regions of the antibody, which cryo-electron tomography experiments suggest is, on average, 130 Å: a distance far too great to measure with the fluorophores used. However, the IgG molecules in these studies show high flexibility, so some FRET events are still expected with this dye pair. The
photobleaching by molecular oxygen and possible disparities in the $R_0$ calculation as additional reasons for the lack of observed FRET events should still be explored.
Chapter 4: Probing the Structural Effects when Oligosaccharide Molecules are Removed from the Crystallizable Fragment (Fc) Region of a Humanized IgG Antibody

Introduction

Present within the Fc region of IgG antibodies is an oligosaccharide network linked to the highly conserved asparagine 297 (N297) residue. The pattern in this network, however, is not conserved from protein to protein. It has been implicated in a number of disease states including rheumatoid arthritis, a highly prevalent autoimmune disorder\textsuperscript{84-86}. It has also been shown that the mere presence of these oligosaccharides is necessary to elicit a healthy immune response\textsuperscript{87,88}. Additional investigations have shown that binding of some Fc receptors is affected upon sugar removal, and the effect is related to the specific receptor molecule interacting with the Fc region\textsuperscript{60-62,89}. The locations of these binding interactions have been mapped previously and are diagrammed in Figure 1.5.

The immune response initiated by the FcγRI, FcγRII, and FcγRIII receptors have all been seen to drastically diminish upon removal of the oligosaccharide molecules\textsuperscript{90,91}. In contrast, the C1q binding interaction and the interaction with the neonatal receptor (FcRn) are not affected to a significant degree when the sugar molecules have been removed\textsuperscript{92}. It has been hypothesized that a conformational change occurs upon removal of the oligosaccharides which is responsible for the diminished response\textsuperscript{61}. A crystal structure of the Fc region with the sugars removed suggests an inward collapse of the C\textsubscript{H2} portion of the Fc region that might explain the change in binding\textsuperscript{93}. Further evidence in support of this hypothesis has been shown by the Georgiou group in a recent work; they took an aglycosylated IgG protein and introduced mutations in the C\textsubscript{H3} region that enhanced the native binding affinity of the activating FcγRI and FcγRIII.
receptors\textsuperscript{60,61}. This suggests that the mutation may produce a new conformation that was inaccessible when the oligosaccharides were present in the antibody.

Spurred by this evidence Förster Resonance Energy Transfer, or FRET, was used to investigate the occurrence of these conformational changes. The system under study was a humanized IgG1 antibody (hIgG), a non-human antibody with mutations present that increase sequence similarity to human proteins, mutated to contain two surface-exposed cysteine residues. The cysteine residues were selectively labeled with a FRET pair and examined using both ensemble and single molecule approaches. Measurements were then duplicated on enzymatically-aglycosylated versions of the labeled antibody. From the single molecule FRET efficiency histograms, evidence is shown for the increased flexibility of the Fc region which is likely related to the diminished binding affinity observed for aglycosylated IgG1 interacting with effector molecules.

**Materials and Methods**

**IgG Sample Labeling**

Humanized IgG was purchased from Creative Biolabs (Shirley, New York). The protein has a cysteine mutation incorporated in place of serine residue 254; the location of the cysteine residues is shown in Figure 4.1. The presence of exposed cysteine residues was verified using the QuantIt thiol quantitation kit (Invitrogen) using a BioTek Synergy H1 multimodal plate reader. The protein received was placed in a Millipore Amicon Ultra Centrifugal Filter Unit with a molecular weight cutoff of 10,000 g/mol and centrifuged at 14,000 rcf for 8-10 minutes. This process was repeated until the final protein concentration was between 5-10 mg/mL. The antibody was stored in PBS (pH, 7.4) at -80°C in 100 μL aliquots.
Labeling reactions were performed on individual aliquots. First Alexa Fluor® 594, dissolved in dry dimethylformamide (DMF) at a concentration of 10 mM, was added to the antibody such that a 10:1 ratio of dye to antibody was obtained. The reaction was allowed to proceed at room temperature in the dark for 1-2 hours. After 1-2 hours, the excess AF594 was removed using dye removal columns (ThermoScientific). The remaining dye-antibody mixture was then reacted with 10 mM Alexa Fluor® 488 (AF488) in DMF in a 2:1 ratio of dye to antibody; this mixture was allowed to react for 1 hour in the dark. The free dye was again removed using dye-removal columns. Similar labeling reactions were carried out for singly-labeled samples except only one dye was reacted with each antibody aliquot. Final concentrations of protein and dyes were obtained using the “Proteins and Labels” setting of a ThermoScientific Nanodrop 1000 spectrophotometer. The isolated antibody-dye conjugates were stored in 10 μL aliquots at -80°C.

**Aglycosylation Procedure**

Doubly-labeled hIgG aliquots (10 μL) were combined with 3 μL of PNGase (New England Biolabs), 10 μL of reaction buffer and 17 μL of Milli-Q water in accordance with the

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**Figure 4.1: Crystal Structure of IgG Fc region with Oligosaccharides Present.** The crystal structure of the Fc region shown was created from the PDB file 1FC1. Serine residue 254, the site of mutation, is highlighted in yellow.
company’s protocol. The reaction was allowed to proceed for 96 hours at room temperature in the dark. Samples were removed from the reaction after 48 and 72 hours to monitor the progress of the reaction. A control experiment was also performed where a 10 μL aliquot of dye-labeled IgG was mixed with 10 μL of reaction buffer and diluted with 20 μL of water to keep the final antibody concentrations the same. This sample was also kept at room temperature for 96 hours. At the end of the reaction, excess PNGase F was removed using a Millipore Amicon Ultra Centrifugal Filter Unit with a molecular weight cutoff of 100,000 g/mol centrifuged at 14,000 rcf for 8-10 minutes. Protein was used for single molecule measurements on the same day as the reaction was terminated.

**Ensemble Measurements**

Fluorescence spectra of the donor-only, acceptor-only and doubly-labeled humanized IgG constructs were obtained using a ThermoScientific ND-3300 fluorometer. Both glycosylated and aglycosylated antibodies were examined. The samples were excited using the blue LED (470 ± 10 nm) with the autogain feature activated. Three to five measurements (2.0 μL samples) were taken of each dye-labeled, IgG sample. This protocol was saved under the “Other Fluorophores” settings under the heading of FRET 488_594.

**Single Molecule Measurements**

Single molecule measurements were performed according the procedure described previously in Chapter 2. Measurements were performed on donor-only, acceptor-only, and doubly-labeled hIgG samples. Both glycosylated and aglycosylated antibodies were used. The PBS buffer was kept at a pH of 7.4 and crafted using LC/MS- or fluorescence-grade water. Laser power was set to 60 μW when measured immediately after the beam expander.
Results and Discussion

Labeling Reaction and Ensemble Measurements.

After labeling the humanized IgG samples, the extent of labeling was determined through UV-Vis measurements. The absorbance at 494 nm was used to determine the concentration of the donor (AF488), the absorbance at 590 nm was used to determine the concentration of the acceptor (AF594) and the absorbance at 280 nm was used to obtain the antibody concentration (Figure 4.2). All concentration data for both glycosylated and aglycosylated doubly-labeled antibody are presented in Table 4.1.

![UV-Vis Spectrum of Glycosylated IgG Labeled with AF488 and AF594](image.jpg)

Figure 4.2: UV-Vis Spectrum of Glycosylated IgG Labeled with AF488 and AF594. From the spectrum, the concentration of antibody is 5.87 μM, the concentration of AF488 is 5.99 μM and the concentration of AF594 is 3.62 μM. This corresponds to 82% of all cysteine residues labeled with a dye molecule.

The concentration of donor, acceptor and protein can be used to determine the degree of labeling for all sulphydryl groups present (Table 4.1); as an antibody contains two identical heavy chains, the concentration of available cysteine residues is twice the concentration of antibody. Using the ratio of dye concentration to sites available and probability calculations (accounting for the 9 possible combinations of donor-labeled, acceptor-labeled or empty sites) it is possible to determine the percent of doubly-labeled antibodies if the labeling at each site
occurs randomly. For the glycosylated antibody, 31.6% of the antibodies posses a donor and acceptor molecule whereas 22% of the aglycosylated antibodies will possess both fluorophores. This calculation assumes that all free dye has been removed from the system and labeling at other sites on the antibody does not occur.

Table 4.1. Degree of Labeling for Glycosylated and Aglycosylated IgG Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>[Antibody] μM</th>
<th>[AF488] μM</th>
<th>[AF594] μM</th>
<th>Degree of Labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycosylated</td>
<td>5.87</td>
<td>5.99</td>
<td>3.62</td>
<td>82%</td>
</tr>
<tr>
<td>Aglycosylated</td>
<td>4.60</td>
<td>4.12</td>
<td>2.26</td>
<td>69%</td>
</tr>
</tbody>
</table>

Ensemble fluorescence measurements were taken for donor-only, acceptor-only and doubly-labeled samples (both with and without oligosaccharides) of similar concentrations. In Figure 4.4, only normalized donor-only (light blue) and doubly-labeled (dark purple) fluorescence emission spectra are shown for emphasis. Direct excitation of acceptor molecules has also been subtracted from the spectra. The decrease in signal over the range of 499-555 nm and the increase of signal from 590-650 nm is believed to be the result of FRET between the Alexa 488 and Alexa 594 molecules.
Figure 4.3: Ensemble Fluorescence of Glycosylated and Aglycosylated IgG. The fluorescence from glycosylated antibody is shown in the top panel while fluorescence from the aglycosylated antibody is shown in the bottom panel. Donor-only samples are shown in light blue while doubly-labeled samples are shown in violet. All concentrations have been normalized to the most concentrated donor-only sample.

For the glycosylated antibody (Figure 4.3, top), the intensity of donor and acceptor fluorescence closely matches that of the aglycosylated antibody (Figure 4.3, bottom). The efficiency of energy transfer was calculated using equation (2.5) and the previously established ratiometric approach (Chapter 2). The resulting efficiencies of energy transfer are 0.18 and 0.16 for the glycosylated and aglycosylated samples, respectively. Because much of the donor fluorescence is a result of donor-only sample, 44.6% of the glycosylated antibody has at least one donor and no acceptor (47.5% of the aglycosylated sample), this signal should be subtracted prior to performing any FRET analysis. It should also be noted that the samples with 2 donor molecules must be accounted for as they will yield double the fluorescence. This amounts to a
background subtraction of 69.1% of donor fluorescence (69.1% of donor molecules are not paired with an acceptor) for the glycosylated antibody and 76.6% for the antibody with no sugar molecules. However, this correction is so large that when applied, the resultant efficiency values are nonsensical. This means the ensemble fluorescence has a signal-to-noise ratio that is too low to predict accurate distances between the fluorophores.

**Single Molecule Measurements**

To investigate this system further, single molecule fluorescence measurements were carried out on dye-labeled antibodies freely diffusing in solution. The single molecule experiments should indicate the presence of any preferred conformations as long as they have a lifetime greater than the 1 ms time resolution for the instrument. Single molecule measurements on the glycosylated antibody were used to create the FRET efficiency histogram seen in Figure 4.4 using the previously described SUM protocol and equation 1.12.

![Figure 4.4: FRET Efficiency Histogram for Glycosylated IgG.](image)

The Histogram shows a significant peak at 0.0 due to a significant donor-only contribution. Additionally, a shoulder peak is observed at a slightly higher efficiency. A double-Gaussian fit was applied and the center of this peak is at 0.16 ± 0.02 with a width of 0.18 ± 0.01.

Once even events are chosen, they are analyzed according to the modified efficiency relationship shown in equation (1.12). The gamma correction factor of 1.3 is again used for the
AF488/AF594 FRET pair. The individual events were analyzed and used to construct the histogram. The histogram was fit to a double-Gaussian function with centers at zero and 0.16 ± 0.02. The width of the peak at 0.16 is 0.18 ± 0.01. The peak at zero is expected to be due to the incomplete labeling of the immunoglobulin molecules with both a donor and acceptor pair; the presence of donor-only samples would be expected to give an energy transfer efficiency of zero.

The shoulder peak corresponds to a 16% energy transfer efficiency when the oligosaccharides are present in the antibody. This value is lower than expected for the Alexa Fluor 488 and 594 pair as the \( R_0 \) parameter is equal to 56 Å and the cysteine residues are approximately 58 Å apart. Using equation (1.11), the approximate distance between the dyes is 77.8 Å. However, this may be explained because the dyes are attached through 6-carbon linkers which would each be 8-10 Å long in an extended conformation, so the expected FRET efficiency would be lower for this scenario. If a value of 18 Å (9 Å per dye) is added to the 58 Å, the dyes would be 78 Å apart which is consistent with the observed FRET efficiency.

Single molecule measurements were also taken following enzymatic removal of the oligosaccharides with PNGase F. FRET events chosen with the same algorithm were analyzed and used to construct the histogram shown in Figure 4.6. The striking difference between this histogram and the first is the presence of a substantial number of events that are almost evenly distributed over regions of higher efficiency (0.2-1.0) than previously observed. This histogram was also fit to a double-Gaussian with a large peak centered about zero and another broad distribution with a center at 0.53 ± 0.06.
The broad range of efficiency values suggests an increased flexibility in the protein once the sugar network has been removed. Though the energy transfer efficiencies increase, the absence of a sharp peak disputes the claim that the protein collapses inwards into a more compact conformation upon removal of the oligosaccharides. As this was evidence from a crystal structure of solely an Fc region, it is understandable that a fully intact antibody may behave differently under similar circumstances. The increased flexibility agrees with the hypothesis put forth by Jung et al. They hypothesize that removal of the sugars disrupts the more rigid structure in the C_\(\text{H}2\) region, but the C_\(\text{H}2\)-C_\(\text{H}3\) interface and the C_\(\text{H}3\) regions are largely unaffected. This is the suggested reason for the diminished affinity for the FcγRI, FcγRII, and FcγRIII receptors which interact solely with the C_\(\text{H}2\) region.

Additionally the binding of C1q and the neonatal receptor, FcRn, showed similar affinity both prior to and following the enzymatic removal of the oligosaccharides. There are two possible explanations for this: either the regions of these protein-protein interactions are largely
undisturbed by the removal of the oligosaccharides or the increased flexibility does not inhibit binding of the receptor molecules. Because the dye molecules are located in the same vicinity as the site which interacts with the C1q molecule, this suggests that the increased flexibility does not interfere with binding to this protein. Likewise, because FcRn interacts with the C_H2/C_H3 interface, it is unlikely that the binding of this receptor is affected by an increase in Fc flexibility. A similar experiment to that reported here could be performed on an antibody with surface-exposed cysteines introduced in the C_H3 region to see if the flexibility of this region is impacted by the enzymatic removal of the N-linked oligosaccharides.

Conclusions

It has been shown here that mutating a surface-exposed serine to a cysteine in a humanized IgG antibody allows for site-specific labeling of a FRET dye pair. The structure of this antibody can then be observed using both ensemble and single molecule techniques. Specifically in this experiment, ensemble and single molecule measurements were performed on both glycosylated and aglycosylated antibodies labeled with Alexa Fluor® 488 and Alexa Fluor® 594. The ensemble fluorescence spectra are indistinguishable for the glycosylated system and the proteins with no sugar molecules present and were not correctable due to the degree of incomplete labeling. Calculated from the uncorrected spectra, the efficiencies of energy transfer were 0.18 and 0.16 for the glycosylated and aglycosylated forms respectively. As these ensemble measurements are limited, this suggests the absence of a global conformational change, but the data are too inconclusive to rule out any conformational differences in the two systems.

Single molecule measurements were able to distinguish a change in the distribution of signal for the two systems. The glycosylated immunoglobulin possessed a substantial population with an energy transfer efficiency of 0.16 ± 0.02 and a width of 0.18 ± 0.01. High-efficiency
events are all but missing from this distribution. This likely corresponds to the presence of fluorophores, attached with a 6-carbon linker, in a fully extended conformation while the sugars are present. Upon enzymatic removal of the oligosaccharides, the distribution of FRET events changed dramatically such that the distribution broadened, high-efficiency events are present, and there does not seem to be a preferred conformation. The change in FRET efficiencies is attributed to increased flexibility in the $C_{H2}$ region and may explain why there is diminished binding to the FcγRI, FcγRII, and FcγRIII receptors upon removal of the oligosaccharides.
Appendix: Single molecule Experimental Protocols

Preparation of PBS (phosphate buffered saline) for Single Molecule Measurements

1X Buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄

Follow the recipe given below to make a 10X solution of PBS in Milli-Q water. Adjust the pH using 1.0 N HCl or NaOH prior to dilution. Typically we prepare the buffers in media bottles; this means the solutions need not be prepared volumetrically and you may use weight boats or weighing paper to mass the salts. Use only Milli-Q water as the solvent for this buffer and try to make sure your acid/base solutions are also prepared in Milli-Q water.

1 L of 10X PBS

80 g of NaCl
2 g of KCl
14.4 g of Na₂HPO₄
2.4 g of KH₂PO₄

Dilute the 10X solution to a working solution (1X) with the fluorescence-grade water.

Check the pH again, but avoid adding any more HCl or NaOH if possible

Preparation of STE (sodium chloride, Tris and EDTA) Buffer for Single Molecule Measurements

1X Buffer: 10 mM Tris base, 50 mM NaCl, 1 mM EDTA, pH 8.0

The recipe comes from the IDT website and it is what we started using both to anneal and store the dsDNA. The recipe is shown for 500 mL of solution.

500 mL of 10X STE Buffer:

6.057 g of Tris base
14.61 g of NaCl
1.461 g of EDTA
Adjust the pH of the 10X solution prior to dilution. The pH of the diluted sample should be relatively unaffected and the reagents used to pH the solution can contaminate your fluorescence-grade water.

**Sonication Procedure for Cleaning Borosilicate Chambered Coverglass (micro well plates)**

Adapted from T.J. Ha’s cleaning protocol detailed in his review article. I took this solution and adapted it to clean the 8-chambered well-plates. The chambers are held to the glass with adhesive, and I believe the methanol and other organic solvents may have begun to dissolve the adhesive as I noticed my samples begin to leak as I was taking data.

1. Sonicate for 10 minutes in 2% Micro-90 solution.
2. Rinse thoroughly with DI water.
3. Sonicate for 10 minutes in DI water.
4. Rinse with Milli-Q water.
5. Sonicate for 10 minutes in Milli-Q water.
6. Rinse thoroughly with Milli-Q water.
7. Store in Milli-Q water (I don’t recommend storing them for very long).

Now we have microscope cover slips and silicone spacers. These are nice, because they prevent cross-contamination as only a single sample is present on each slide. Typically, the cover slips are clean enough for our experiments that they only need to be cleaned with Milli-Q water and dried before using. The silicone spacers I typically soak in soap for 5 minutes and then place them into a beaker of Milli-Q water; I am afraid the soap will leech into the spacers if left too
long. I then perform one final rinse with Milli-Q water and then wrap them in parafilm for storage.

Protocol for Annealing DNA Oligonucleotides

This procedure was taken directly from the IDT website

Original Procedure:

1. Dissolve oligos in STE buffer at a concentration of 1-10 OD\textsubscript{260} units/100 μL (OD\textsubscript{260} = optical density/absorbance at a wavelength of 260).
2. Mix two strands together in equal molar amounts. The final volume should be 100 μL.
3. Heat to 94°C and gradually cool. Slow cool by turning off the water bath and allowing the solution to equilibrate to room temperature (may take 4-5 hours so plan accordingly).
4. The product should be stored at 4°C or frozen.

Modified Procedure:

Typically, we prepare our dsDNA at a final concentration of 75 μM after annealing. This means that when ssDNA arrives from IDT (or another company if they become cheaper), it should be stored in STE buffer at a concentration of 150 μM. If there is not much ssDNA, you should be fine as long as you plan for the dsDNA concentration to be at least 10-20 μM after annealing (I am assuming that Dr. Southern will order only internally labeled DNA from now on).

To anneal the dsDNA, we no longer heat in a water bath and cool on the bench. We go to the Biochemistry lab (McGowan South room 220) and use the Thermocycler Pro S in the prep room. The login password for Dr. Southern is 159. The protocol we have developed is called
annealDNA and runs for over eight hours. It is set to heat up to 95°C and then cools at a rate of 1°C every five minutes. It should be noted the cooling stops at the $T_m$ value for 30 minutes (instead of 5) before proceeding; currently this is set to 37°C, the $T_m$ for our 11bp dsDNA. This should be changed if you do measurements with different DNA oligomers.

**Preparation of Samples for Single Molecule Measurements**

This is probably the most time-consuming part of the day. The first thing you should do, after turning on the laser, is thaw your samples and determine the concentrations of the dyes in your stock solution using the Nanodrop-1000. Next, you should plan out your dilutions. I am pretty comfortable with dilution calculations, but I still wrote them out ($M_1V_1 = M_2V_2$) 99% of the time. Typically your stock solution will have a concentration of between 5 and 15 μM. If this is the case, I would plan on doing 3 dilutions; 50 nM, 500 pM and 50 pM are generally good concentrations to aim for. I would also try to avoid using the P-200 micropipets, because they tend to be highly inaccurate. I also recommend doing all of your dilutions in Falcon tubes instead of micro-centrifuge tubes as they are easier to label. I generally color-code my dilutions, because nothing is more frustrating than spending an hour making solutions only to find out something went wrong and it is too concentrated (as it is you will probably only get workable data once every three tries or so). You only need to make between 1-5 mL of each dilution as they don’t really keep very well and you don’t need a large volume for one day’s measurements.

Once your calculations/tubes are set, you can begin your dilutions. Make the first two dilutions using the appropriate buffer in Milli-Q water and the final dilution using the buffer in LC/MS-grade water. (If your sample isn’t thermodynamically stable, then wait to do the dilutions until just before you are ready to take the measurement.) You can then place a clean
silicone spacer on a clean coverslip (see cleaning procedures); I usually rest these on an index card covered with a piece of lens tissue. You can then place 300 μL of your sample into the spacer and take it behind the curtain*. You should also make two additional slides that have 1nM and 2-5 nM alignment solutions (these are typically solutions of the donor dye). You are ready to align/take single molecule data.

*Note: if you are performing measurements on proteins, you should first place 300 μL of BSA in the spacer and let it adsorb to the surface for 5 minutes. Then dry it with nitrogen in the hood (it will look crusty, but it’s fine) and deposit your 300 μL of sample in the spacer.

**General Operation of the Apparatus and Alignment Procedures**

I have a routine that I go through every time that I take data (sadly most of it involves the labeling of tubes in the previous steps). The first thing you should do when you are taking single molecule measurements is turn the laser on when you arrive at the lab. You will have to stand on the stool to do this; try not to hit your head. There are three things to remember about this process.

1. You should turn on the surge protector first; this will turn on the cooling fan.
2. Turn on the power supply for the laser.
3. Turn the key on the key remote to the right and wait for the laser to come on. Once it has come on, flip the first and third switches (current ➔ power and standby ➔ run).

You now have some time to wait (30 minutes to an hour), so you can read a paper or start preparing your dilutions.

Once the laser is ready, you want to check the power and alignment. The laser should be directed through the neutral density filters into the prism where the desired laser line is centered on the next mirror. This will then be further isolated using an iris and directed through a 5× beam expander. This is typically where I check the power. For DNA measurements, I typically run the
laser at 100 μW after the beam expander and I usually run it at lower power (~60 μW) for the antibody samples. This can always be adjusted after. **Note: try not to nudge the beam expander, it is very top heavy and will come out of alignment fairly easily.** It is the second most annoying piece of the apparatus to align, so be careful.

The light will then be directed around the table and to the periscope: the **most** annoying part of the apparatus to align. This is also right next to the coarse/fine focus of the microscope, so be careful not to knock it with your arm. Otherwise, you shouldn’t have to worry about this on a regular basis at all. Once the laser beam passes the periscope, it will be directed into the microscope. You can then check the alignment using the bull’s-eye tube. Unscrew the microscope stage using one of the hex keys that I have conveniently stored in a pocket on the left side of the microscope. The bull’s-eye then screws into one of the empty objective slots. You can then see if the light passes through the center of both targets; use either the two mirrors before the objective or the two mirrors on the periscope to align the laser through the bull’s-eye (I prefer not having to touch the periscope if it can be avoided). When it looks good, screw everything back in.

Once you have checked the power and alignment around the table, take the two alignment solutions with you into the curtain. Grab your mag-lite, make sure the Velcro is sealed and the monitor contrast is set low (around 5). Apply a drop of Milli-Q or LC/MS water to the surface of the objective lens and then place the coverslip with the 5-10 nM solution above the center of the objective. Make sure the objective is set to eye-port, open the shutter (lever on the right side of the microscope, just under the stage) and begin turning the coarse focus (big knob) towards you slowly while looking in the eye port. You won’t be able to see anything at first, but it will start to form a fuzzy glow that gets brighter and brighter. Eventually, a bright point will
appear in the center. Stop turning the coarse focus and adjust the fine focus 50-100 μm towards you. You are finished with this solution. Wipe the objective with some lens tissue and replace the water droplet. You can then put your 1 nM alignment solution on top of the droplet (it should form a seal now).

You are now going to be using the detectors, so double-check that all the light is sealed off and the microscope is set to eye-port. Activate the LabView file “Two Counter Dark Counts”. When you do, you should see the virtual instrument (VI) appear on the screen. Click on the white arrow to run the VI. It should read zero in both channels while the detectors are off. Plug the two detectors into the surge protector and turn them on while the VI is running. You should see numbers appear in each of the channels; the values for the donor channel typically level off around 60-100 while the acceptor channel stabilizes around 500-700 counts. You can then turn the objective to the side port and you should see a spike in the counts in both channels, most notably the donor channel. For a 1 nM solution of 488, you should see several hundred thousand counts in the donor channel if your alignment is good. You may then begin aligning the fiber optic cables using the coarse/fine foci on the stages that are attached to the side port. This is iterative and you are just looking for the highest counts (this may change day-to-day depending on a number of factors, but it is typically 300,000-500,000), don’t stress out trying to get the perfect number. Once you are satisfied, turn the objective to eye-port, close the shutter, turn off and unplug the detectors, remove your sample, dry/cover the objective lens and go get your single molecule samples.

**Data Collection using LabView Software**

Place a drop of water on the objective and then put your sample slide on top again (it should still be aligned, so you will still form a seal). Put the “Two Counter Dark Counts” VI on again, turn the detectors on and switch the objective to side-port. Take a reading of the dark
counts. If either channel is above 7,000, this is usually too high to get good signal-to-noise, so you may need to remake your samples. Additionally, if your counts are below 2,000 in the donor channel, you probably have a solution that is too dilute. If you are in the range of 2-7,000, then you can turn off the “Two Counter Dark Counts” VI and open one of the “FRET Data Collection” VIs; we typically take the data using the 5-minute VI. Turn the objective back to eye-port and close the shutter.

Before you start, make sure you name your two files (the left one is the acceptor). I typically click the folder icon, create a new folder corresponding to the month/day/year, then find a previously named file and cut/paste that into the window. You still have to change the name of your folder/sample, but it is much better than forgetting some portion of the name (like the .txt). Once you have named your files, make sure the shutter is opened and the objective is back on side-port; you can then click on the arrow to start the data collection process. You will get an error message every time, but this is due to the fact that it is trying to save to a file that doesn’t exist yet (but it will create it). You can then watch the data as it is being collected on the waveform graph; typically peaks above 2-300 are really high, so you may have to redo the measurements with a more dilute sample or lower power. Once the VI finishes running, switch to eye-port, and close the shutter. Repeat this process for additional samples. If this was your last sample, then turn off the detectors, clean/cover the objective (turn the wheel until it is hidden) and turn off the laser, but keep the fan running for a good 20 minutes after you shut off the laser.

**Data Analysis Protocol using Igor**

When you are ready to begin analyzing your single molecule data, open the Igor File on the desktop called “single molecule data analysis”. Compile the macros by clicking on the
macros tab and highlighting compile. Next, load data sets from the folder you created for your single molecule data; do this by selecting the macro “load data sets from folder”.

You should start the analysis by determining the background signal in each channel. To do this, select the “background” macro and then choose either your buffer data files or your acceptor-only data files (choose them donor first). The background counts in each channel will then be presented on the screen. You will need these numbers later, so write them in your notebook. Next, you need to calculate the ‘bleedthrough’ of the donor fluorescence into the acceptor channel. Choose the “bleedthrough” macro and then select your donor-only sample. You will be prompted for a threshold to enter. Based on the amount of signal you received from the waveform graphs, you can estimate a good threshold for fluorescence (I typically go with 25 or 50 depending whether there is a lot of signal or a little). You will then get a fraction that corresponds to the ‘bleedthrough’ of the donor into the acceptor channel that you will need later.

Now, you should be able to analyze your data and create your histograms. Select the “analyze data” macro and select the appropriate donor and acceptor files. You will then be prompted to name the donor, acceptor, and clock; just name them sequentially each time you select the analysis file (d0, a0, c0; d1, a1, c1; d2, a2, c2;…). Then name your plot, enter the parameters you have previously determined and name your plot. A histogram will show up for your single molecule data. You can then fit this to a single- or double-Gaussian function using the ‘curve fitting’ tab. Remember, if you are performing a double-Gaussian fit, you have to select the ‘coefficients’ tab and put in initial guesses for each of the fit parameters. Repeat this process for each of your samples.
Determination of Fluorescence Quantum Yield

Fluorescence quantum yields are determined using the protocol put forth in the Analytical Chemistry reference. In this method, you need to have a quantum yield standard, as in other comparison methods. The quantum yield standards we typically use are fluorescein and Rhodamine 6G for dyes that absorb at lower wavelengths (AF488). For dyes that absorb at longer wavelengths (AF594, AF647), we have used Rhodamine B and cresyl violet, though I am not confident of the values obtained using cresyl violet. We focused entirely on method (1a) in this paper as (1b) has an additional correction factor and we don’t possess the setup to use method 2. For these measurements, you will be using the spectrophotometer and fluorometer in room 326 (the instrument room). There is a laminated instruction guide for using the UV-Vis and one for the fluorometer as well. It is ideal to use the same solution on both instruments, so concentrations of 0.1-10 μM are a good place to start.

Calculation of the γ Correction Factor

The γ factor is calculated by taking the ratio of quantum yields, ϕ, and detection efficiencies, η, for the FRET pair as seen in the equation below.

\[ \gamma = \frac{\phi_A \eta_A}{\phi_B \eta_D} \]

The values for quantum yield are typically reported by vendors. If you are concerned that the quantum yield is different when your dye is conjugated to a biomolecule, you may follow the previous protocol to calculate a relative quantum yield value. You can then split up the detection efficiency into two parameters, one reported by the company that makes the detectors (they are more sensitive to redder wavelengths); there is a sheet that Dr. Southern has that you can refer to for this number (α). The additional parameter (β) accounts for the different filters used.
To determine the value of $\beta$, the emission spectra must be saved (either from the Nanodrop or the company’s website) and uploaded into an Igor file. You must then download the spectra for each of the optics used in the apparatus for this dye pair. Overlay them onto a graph to make sure they are normalized (area under each curve is equal to 1). You can then determine the relative area that is allowed to pass through the collection filter (either a band pass or long pass filter). You can integrate the area in Igor using the ‘print area’ command [print area (wave, $x_1$, $x_2$)]. This fraction is equal to $\beta$ if your spectra are normalized.

References


5. Schroeder, K., Herrmann, M. & Winkler, T.H. The role of somatic hypermutation in the generation of pathogenic antibodies in SLE. *Autoimmunity* 46, 121-7 (2013).


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