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Single Molecule Spectroscopy for Studying Conformational Dynamics of Short Oligonucleotides

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Single Molecule Spectroscopy for Studying
Conformational Dynamics of Short Oligonucleotides

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Biochemistry and Molecular Biology

by

Ron Reuven Lin

2012
ABSTRACT OF THE DISSERTATION

Single Molecule Spectroscopy for Studying Conformational Dynamics of Short Oligonucleotides

by

Ron Reuven Lin

Doctor of Philosophy in Biochemistry and Molecular Biology

University of California, Los Angeles, 2012

Professor Shimon Weiss, Chair

Understanding biology at the molecular level has been driving technological advances in biological and medical science for many years. Methods for probing molecular systems are often dependent on sampling the concerted actions of large assemblies of molecules rather than for studying individual molecules operating in isolation. Most methods used in experimental biology are largely insensitive to the activity of a single molecule. Over the past twenty five years, advances in a variety of disciplines have been employed which allow researchers to use single molecule approaches to for examining biomolecules, a development which has had remarkable implications for advancing the understanding of cellular processes. Single molecule techniques have been used to resolve questions about everything from replication,
recombination, transcription and translation and protein folding, among other subjects.

The structure-function relationship in biology is central to the understanding of cellular processes, and has provided one of the most significant intellectual frameworks for understanding molecular pathways. Its success is validated by numerous studies using X-ray crystallography that now routinely allow researchers to make rational predictions explaining why and how biomolecules interact. The famous “lock and key” model for enzymatic function perhaps best exemplifies this framework. Despite their predictive power, structure-functional relationships often gloss over a basic fact of biological systems—both structure and function may in fact possess a remarkable degree of dynamism.

This dissertation summarizes my efforts to develop and refine methods for interrogating the dynamical properties of single molecules, with a particular emphasis on studying structural properties of DNA and examining protein-DNA interactions. I provide an overview of fluorescence, FRET and fluorescence lifetime spectroscopy in Chapter 1, and discuss the methods I developed using fluorescence lifetime to examine conformational fluctuations of sub-persistence length segments of DNA. In Chapter 2, I describe a microfluidic-based platform developed in the Shimon Weiss Lab for conducting single molecule FRET assays, which was used to exploit persistence length changes in DNA upon hybridization to screen context-dependent RNA polymerase transcription. Finally, in Chapter 3, I describe a new detector with improved red spectrum sensitivity, which provides a foundation for
further work applying fluorescence lifetime analysis to studying structural perturbations in DNA on nanosecond timescales.
The dissertation of Ron Reuven Lin is approved.

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2012
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## VITA

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PUBLICATIONS AND PRESENTATIONS


Chapter 1

Single Molecule Spectroscopy for Studying Conformational Dynamics of Short Oligonucleotides
1.1. Examining Dynamics in Bio-molecules

Understanding biology at the molecular level has been driving technological advances in biological and medical science for many years. Methods for probing molecular systems are often dependent on sampling the concerted actions of large assemblies of molecules rather than for studying individual molecules operating in isolation. In fact, most methods used in experimental biology are largely insensitive to the activity of a single molecule. Condensed phase spectroscopy of a single fluorescent molecule at low temperatures was first achieved in 1989 by W. E. Moerner and L. Kador, and later refined by Michael Orrit. (1) By 1993, E. Betzig had conducted near-field optical microscopy at room temperatures, paving the way for more recent advances which have expanded the single molecule repertoire to studying biomolecules. The ability to study molecules at biologically relevant temperatures has had remarkable implications for advancing the understanding of cellular processes. Single molecule techniques have been used to resolve questions about everything from replication, recombination, transcription and translation and protein folding, among other subjects. (2, 3, 4, 5, 6) Single molecule methods are useful for studying complex heterogeneous systems, a particularly salient advantage for the study of biology. Many current methods have a tendency to offer only an ensemble average of conformational or functional states, rather than insights into the distribution of states sampled by biomolecules.

The structure-function relationship in biology is central to the understanding of cellular processes, and has provided one of the most significant intellectual frameworks for understanding molecular pathways. Its success is validated by numerous studies
using X-ray crystallography that now routinely allow researchers to make rational predictions explaining why and how biomolecules interact. (7) The famous “lock and key” model for enzymatic function perhaps best exemplifies this framework. (8) Despite their predictive power, structure-functional relationships often gloss over a basic fact of biological systems—both structure and function may in fact possess a remarkable degree of dynamism.

This dissertation summarizes my efforts to develop and refine methods for interrogating the dynamical properties of single molecules, with a particular emphasis on studying structural properties of DNA and examining protein-DNA interactions. I provide an overview of fluorescence, FRET and fluorescence lifetime spectroscopy in Chapter, and discuss the methods I developed using fluorescence lifetime to examine conformational fluctuations of sub-persistence length segments of DNA. In Chapter 2, I describe a microfluidic-based platform developed in the Shimon Weiss Lab for conducting single molecule FRET assays, which was used to exploit persistence length changes in DNA upon hybridization to screen context-dependent RNA polymerase transcription. Finally, in Chapter 3, I describe a new detector with improved red spectrum sensitivity, which provides a foundation for further work applying fluorescence lifetime analysis to studying structural perturbations in DNA on nanosecond timescales.

1.2. The Structure of DNA

The challenge of solving the structure of DNA represents one of the most famous (and controversial) chapters in scientific history. In 1953, James Watson and Francis Crick postulated that DNA was a double helix, largely based on data derived from X-ray
crystallography provided by Rosalind Franklin and Maurice Wilkins. (9) Paired nucleotides form hydrogen bonds and stabilize via base stacking interactions, such that adenine and thymine pair, while guanine pairs with cytosine. This model for DNA hybridization is commonly referred to as Watson-Crick (WC) base pairing.

DNA is a linear polymer assembled from monomeric units of four nucleotides. The linear sequence of these units encodes the genetic information from which all living entities are derived. As is well known, these two polymer strands wind around one another, forming the famous double helix.

The monomeric unit of DNA is a deoxyribonucleotide, consisting of three parts: (1) a nitrogenous base (2) a pentose sugar, and (3) a phosphate. The nitrogenous base is found in two forms: six-membered ring pyrimidines and fused-ring purines shown in Figure 1.1A. The purine bases used in DNA are adenine (A) and guanine (G), while cytosine (C) and thymine (T) belong to the class of pyrimidines. In RNA, the thymine base is replaced by uracil, which differs in a methyl group. The monomeric units, called bases, join covalently in an N-glycosidic bond to the 1’ carbon of the pentose sugar, while the phosphate is esterified to the 5’ oxygen, as depicted in Figure 1.1B. The phosphate groups form a “phosphate backbone” which links successive nucleotides together with phosphodiester bonds.
Figure 1.1. (A) Nucleosides come in two forms, purines and pyrimidines, and form the basis for what is now referred to as Watson-Crick base pairs. (B) A sugar phosphate backbone forms a scaffold for anti-parallel strands of DNA, which associate via hydrogen bonds between complementary nucleotides. (C) B-form DNA is the most common structural form and consists of two phosphate backbone spirals winding around one another to form a double helical structure.

Along with recognizing WC base pairing, early crystallographic studies characterized other properties of double-stranded DNA. The right-handed B-form DNA helix, one of the dominant structural forms of DNA, winds around a central axis every 10.4-10.5 base pairs. The two opposing DNA strands coil around one another, producing a hydrophobic interior and a charged, hydrophilic phosphate backbone (Figure 1.1C).

To faithfully preserve genomic content, DNA acquires molecular and structural stability from a few factors. First, the highly selective nature of WC base pairing is stabilized by base stacking interactions, forming the sturdy double helix. Second, there are numerous repair and monitoring mechanisms that are responsible for preserving the integrity of genomic information. And third, DNA is often “packaged” in cellular contexts as chromatin, whereby long chains of DNA are packaged under torsional stress or via extensive protein-DNA interactions. These phenomena have implications for the study of DNA topology, and ultimately determine the accessibility of the genome to other
vital cellular machines. As a result, understanding the sources of DNA’s molecular and structural stability has implications for determining many aspects of DNA-protein interactions and nucleosome positioning, which is significant for determining gene expression levels.

1.2.1. Biological Function of DNA

DNA is a long term and stable storage mechanism for information. Although DNA had been chemically identified as early as 1869, it was not conclusively shown to be the conveyer of genetic information until 1944, as a result of work by Avery, McCarty and MacLeod at Rockefeller University. Their work on bacteria, in association with other studies, appeared to resolve the question of inheritability and determined that long chains of deoxyribonucleotides were responsible for transmitting heritable information from one generation to the next. (10)

1.2.2. Intermolecular Interactions of DNA

The complementarity of DNA as a result of WC base pairing provides a natural replication mechanism for the transmission of genetic information across generations. All cells that emerge from the two major branches of life, eukaryotes and prokaryotes, undergo cellular division (or mitosis) whereby cells divide into mother and daughter cells. This process is usually preceded by separation of the two strands, before synthesis of a new complementary copy can proceed. The pre-existing strands of DNA provide an easy-to-copy template for producing duplicates of the genetic code.

Not long after postulating the structure of the double helix, Francis Crick would also propose what would has become known as the “Central Dogma of Molecular Biology.”
In Eukaryotes, genetic information is transduced into agents of biological function through a flow of information. Briefly, the “Central Dogma” states that DNA is transcribed into RNA in the cell nucleus, before being exported to the cytosol where RNA is translated into a functional protein form by the ribosome. Like DNA replication, the process of transcription requires the separation of the DNA strands in order for the enzyme RNA polymerase to gain access to the gene template and transcribe DNA into RNA. (11)

Not all of the genome is transcribed by RNA polymerase and translated into protein. Instead, only specified regions of the genome—called exons—constitute genes that code for a protein. Fundamental questions have lingered regarding how exactly RNA polymerase is able to distinguish regions of the genome which are important to transcribe from regions that are not transcribed. This question has gained increasing importance due to recent discoveries that show that vast amounts of the genome appear to be transcribed into non-protein coding RNA, and non-coding RNA appears to possess biological function. Alternative splicing mechanisms have also been characterized, showing that messenger-RNA undergoes modification prior to being translated into a functional protein. Thus, through splicing, single genes can be further multiplexed into a number of proteomic variants. These discoveries are responsible for highlighting nuances in the unidirectional flow of information that long characterized the Central Dogma, and suggest there are opportunities to refine our understanding. More importantly, these phenomena present clues reinforcing the belief that the structural properties of DNA are of central importance to understanding biological regulation. (12)
1.2.3. DNA Packaging

Accessibility of regions of the genome to the replication and transcription machinery is thought to be at least partially controlled via the process of DNA packaging. The 6 billion DNA bases constituting the human genome would stretch several meters in length if they were laid end to end. However, the entirety of the human genome is easily encapsulated into the cell nucleus, a compartment in eukaryotic cells that is merely 1 micron (1 um) in diameter. This is accomplished largely by wrapping or “packaging” DNA around protein tetrameters called histones. Histones form the sub-structure of nucleosomes, which provide the structural basis for what we now know as chromosomes. While DNA is a linear polymer, the chromosome arrives at its familiar morphology as a result of several tiers of packaging, all of which allows the entire genome to be packaged efficiently inside of micron-sized cells.

1.2.4. DNA Topology

The fact that DNA is packaged and hierarchically structured has significant implications because DNA topology determines the accessibility of the genome to protein and DNA binding partners. Binding of proteins to DNA is thought to induce structural irregularities, such as loops or kinks, which provide recognition motifs for RNA polymerase and other proteins to subsequently bind and transcribe a given region of DNA. In addition to promoting transcription, such protein-DNA interactions are capable of inhibiting transcription. Therefore, it is widely thought that the structural features of DNA determine the rate and frequency with which given regions DNA are transcribed into RNA, and subsequently into proteins.
1.3. **Methods to Study Bio-molecular Structure**

Gel electrophoresis, High-pressure liquid chromatography (HPLC), and fast protein liquid chromatography (FPLC) have allowed separation and thus identification of proteins and nucleic acids present in cellular milieus. Many of these methods have long relied on selecting molecular constituents based on size or molecular weight. Molecular biology in the 1950s through 1970s made great strides in identifying and classifying molecular compounds in this way. The anomalous structure of A-tract DNA (repetitive sequence consisting of consecutive adenosine nucleotides) was discovered using acrylamide gels in 1982, (13) based on the observation that A-rich sequences of DNA migrated more slowly as compared to other segments of DNA. This observation later led to research which concluded that A-tract DNA had a characteristic “bend”. This was one of the earliest examples of conclusive evidence showing that DNA structure was base sequence specific.

Over the latter half century, a major trend in the study of molecular biology has been with regards to developing specific stains and labels to bind to a molecular target, as a means to identify and localize the target. The emergence of staining techniques to identify proteins and organelles resulted in commensurate developments in optical methods and microscopic tools used to examine them. Staining techniques relying on visualization with the naked eye or with low-magnification microscopy ultimately gave way to improved labeling techniques, spurring forth innovation and improvements in detection methods, including improvements in cameras, optics and chemical labeling techniques.
One of the earliest examples of improvements in labeling techniques came from the development of radioactive labeling and nucleic acids, which was quickly adapted to what is known as the Sanger method (or “chain termination method”) for DNA sequencing. With developments allowing the incorporation of fluorescent-labeled nucleotides, the Sanger method was rapidly adapted to a variety of inexpensive and higher-throughput techniques which culminated in the Human Genome Project. Fluorescence labeling of synthetic oligonucleotides has resulted in the development of a variety of techniques to identify protein and DNA targets in biological samples, including fluorescence in situ hybridization (FISH) and many commercial instantiations of enzyme-linked immunosorbent assay (ELISA). The advantage of fluorescence techniques as compared to radioactive techniques has long been ease-of-use, lower costs and the ability to use spectral selectivity for multiplexing fluorescence signals and allowing parallel detection of different targets.

Understanding cell biology relies on the ability to observe interactions between molecular constituents. Optical microscopy has long been a primary means by which scientists have studied interactions between molecules. For example, as staining methods have given way to recombinant methods, imaging using fluorescence proteins has allowed researchers to study an immense number of molecular targets. These methods, in no uncertain terms, have revolutionized the study of biology by equipping scientists with a general framework that can be almost universally applied to labeling and studying molecules and structures inside of a cell.
The dogma “structure yields function” has contributed to a virtuous cycle of microscopy being used to identify interesting interactions, followed by structural biologists pursuing the rational basis for such interactions. The structural biology toolkit has largely relied on the field of X-ray crystallography or, more recently, nuclear magnetic resonance (NMR). Crystallography possesses a central place in biology, since it was the methodology used by Linus Pauling to first elucidate the structural properties of proteins (sperm whale myoglobin), and then the structural properties of DNA shortly thereafter.

Despite its popularity, X-ray crystallography has significant limitations. First, as its name implies, the method requires crystallization in order to yield a structure. This requirement is both experimentally and conceptually problematic for several reasons. It is experimentally challenging inasmuch as identifying the conditions in which a protein or biomolecule may be crystallized is non-trivial. Laboratories around the world increasingly utilize high-throughput screens in the hopes of obtaining a crystallized protein or biomolecule. Such screens appear to have only nominal success at producing crystals, and so the cost in terms of expended material and manpower are significant.

From the perspective of pharmaceutical research, the application of crystallography to identify drug targets underestimates the importance of the fact that most (over half) of the popular medications administered today are known to target membrane proteins, including the well-studied G-coupled protein receptor (GPCR). Despite the obvious centrality of membrane proteins to drug development, of the thousands of proteins for which crystal structures are available, only a relatively small
number of crystal structures are available for membrane proteins. (14, 15) Membrane proteins are intrinsically resistant to crystallization, due to their amphiphilic and often-disordered structures. Their hydrophobicity renders them largely resistant to solubilization, while their function as receptor targets suggests that structural dynamism may be a critical factor in determining their function. In other words, the very pursuit of a crystal structure inherently suggests that proteins assume rigid, static structures. The unfortunate reality is that this may simply not be true for most membrane proteins, and may also not be the case for many soluble proteins as well. Dynamism itself represents an important structural feature in the function of proteins in the cellular milieu.

When a crystal structure is obtained, the conceptual difficulty of crystallography lies in the fact that the conditions often used to obtain said structures may be of biologically dubious relevance. Methods for probing dynamical biological systems are a central and increasingly urgent requirement. Our objective throughout the course of my dissertation work was to apply fluorescence-based techniques to studying structural features of biomolecules.

1.4. Fluorescence for Visualizing Biomolecules

Fluorescence has been popular as a tool for labeling and detecting molecular species. There are any number of ways in which fluorescence has been used to study biological systems, including encoding gene fusions of green fluorescent protein (GFP) using recombinant methods, useful for “tagging” a protein of interest and tracking its emission profile and localization in a cell. In addition to expressing fluorescent proteins, a variety of techniques have emerged in the past century which allows the specific
conjugation of an organic fluorescent molecule (called a fluorophore) to a molecule of interest.

Affixing fluorescent probes to DNA can be accomplished by both direct and indirect labeling of nucleic acids. Direct labeling can be described as covalent conjugation or non-covalent binding of fluorophores to nucleic acids. Indirect labeling is performed through intermediaries, often by labeling a protein or ligand, which is then bound to DNA. DNA was labeled via the phosphate group at the 5’ position as early as 1973, and by 1989, this capability was expanded to labeling interphosphate groups. Labeling of the nucleic acid base became routine by the late 1970s, and quickly found applications in DNA sequencing. In addition to the commercial applications for fluorescent-labeled DNA for gene sequencing, numerous groups have employed a variety of strategies to use fluorescence to study structural properties of DNA and other biomolecules. (17, 18, 19)

1.4.1. Single Molecule Spectroscopy

There are two basic requirements for the detection of single molecules. First, a signal must be discriminated from other sources, including from background. Second, the detection volume must be defined such that only one molecule is present at the time of observation. This is best achieved by generating a small detection volume, usually on the order of a femtoliter, which is possible using a tightly focused laser excitation, in conjunction with a detection pinhole for implementing confocal microscopy. The size of the detection volume is limited by the diffraction size of light, which at visible wavelengths is on the order of 300-600 nm. In addition to confocal microscopy, imaging
modalities have also been used to monitor samples immobilized on a surface. Total internal reflection microscopy (TIRF) uses a high-sensitivity CCD camera to observe immobilized fluorescent species, where the immobilized species need to be deposited on the surface at sufficiently low densities as to enable observation of individual molecules (Figure 1.2). (20)

Diffusion experiments and immobilized experiments provide very different kinds of information about a system in question. Diffusion experiments are characterized by the observation of brief bursts of light that provide information about the diffusion properties of the underlying molecule. Bursts are analyzed for burst intensity, duration, spectrum, and lifetime. The bursts are short, which limits the amount of information available, but they are free of potential perturbations or biases that are introduced by surface immobilization. (21, 22)

The detection of a fluorescent molecule involves the repeated cycling of the fluorescent molecule through ground and singlet electronic excited states. In order to apply fluorescence microscopy to studying biomolecules, the fluorophore needs to be affixed, or conjugated, to the molecule of interest. In some cases, conjugation of a fluorescent dye may be done non-specifically, serving as a beacon to observe the

![Figure 1.2](image-url)

**Figure 1.2.** Representative image obtained using TIRF to visualize severing of F-actin filaments by cofilin. (a) Immobilized actin filaments labeled with Cy3B on mPEG-biotin surface before addition of F-actin severing protein cofilin, and (b) after addition of cofilin. (unpublished results, in collaboration with Christine Chen/Emil Reisler Lab at UCLA)
diffusion properties of a biomolecule. However, many applications require site-specific labeling. (23)

1.4.2. **Single Molecule Spectroscopy for Studying Dynamics**

Conformational dynamics are thought to play important roles in the functional characteristics of biomolecules, including proteins, DNA and RNA. Sampling the dynamics of these biomolecules presents a set of challenges that few experimental methods can overcome. Spectroscopy is particularly well-suited for examining single-molecule dynamics, because of the availability of sensitive detectors and the specificity of fluorescence signals. Polarization anisotropy and Förster Resonance Energy Transfer (FRET) are two examples of methods that have been applied extensively in the past decade. The group of Yale Goldman has used polarization anisotropy to examine the orientations of individual macromolecules, primarily to examine cellular structural proteins like F-actin and myosin. In addition to sampling the dynamics of macromolecule orientations, they have also been successful in measuring the torsional rigidity of macromolecular complexes. (16)

1.5. **Förster Resonance Energy Transfer (FRET)**

Förster Resonance Energy Transfer (FRET) is a non-radiative energy transfer between two weakly interacting dipoles. In cases where there is spectral overlap, two fluorophores in close proximity may transfer excited electronic state energy from a “donor” organic dye molecule to an “acceptor” dye molecule in a strongly distance-dependent manner. FRET processes tend to be sensitive to small fluctuations in the distances between fluorophores, on a length-scale between 1 nm and 75 nm. First
described in 1946 by Theordor Förster, FRET provides a molecular assay that offers precise sensitivity to nanoscale structural changes that are often beyond the sensitivity of traditional experimental techniques. As such, FRET uniquely serves as a highly sensitive “meter stick” to probe nanoscale distance and structural changes in underlying biomolecules. (24, 25)

Single-pair FRET (spFRET) is not only useful for probing average distances in an ensemble experiment, but also direct observation of a distribution of distances and the time-evolution of distance changes. Such a method is useful for examining a variety of biological processes, including protein folding, biopolymer fluctuations, and enzyme kinetics. In the intervening years since the first single-molecule methods for FRET were introduced, experimentalists have used it for studying the action of ribosomes, RNA polymerase, DNA polymerase, as well as for elucidating protein folding kinetics and molecular interactions. A variety of methodologies have emerged for applying spFRET, including using immobilized samples on a surface in conjunction with total internal reflection fluorescence (TIRF) microscopy, which has been used to examine DNA repair mechanisms. In addition, spFRET with diffusing species has gained widespread acceptance due to its methodological simplicity.

The rate of Forster Resonance Energy Transfer (FRET) is often depicted as a function of the distance between the donor and acceptor fluorophores. The rate of FRET at a distance R is given by the following equation:

\[ k_{FRET} = \frac{1}{\tau_D} \left( \frac{R_0}{R} \right)^6 \]
\( \tau_D \) refers to the fluorescence decay lifetime of the donor fluorophore. \( R_0 \) is called the Forster distance, and reports on the distance at which energy transfer between two corresponding fluorophores is 50\% (Figure 1.4B). \( R_0 \) is represented in a more complete functional form as:

\[
(R_0)^6 = \frac{9000 \ln 10 \kappa^2 Q_d}{128 \pi^5 N n^4} \int_0^\infty F_d(\lambda) \varepsilon_a(\lambda) \lambda^4 d\lambda
\]

where \( Q_d \) is the quantum efficiency of the donor, \( N \) is Avogadro’s number, \( n \) is the index of refraction of the medium between the fluorophores, \( F_d \) is the normalized emission spectrum of the donor, \( \varepsilon_a \) is the extinction coefficient of the acceptor at the wavelength \( \lambda \), and \( \kappa^2 \) is the orientation factor for the interaction between the donor and acceptor. The two most problematic factors are \( n \) and \( \kappa^2 \). The index of refraction \( n \) depends on the solution and the organic matter (protein or DNA) that lies between the two fluorophores. The orientation factor is often approximated to assume that the fluorophores are freely rotating. It is possible to verify the orientation factor by performing polarization anisotropy and measuring the rotational diffusion of the tethered fluorophore. This approximation implies that \( \kappa^2 = \frac{2}{3} \).

Most generally, the first order approximation for the FRET efficiency (or the amount of transfer from donor to acceptor) is depicted by the following equation:

\[
E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}
\]
This expression shows FRET efficiency, \( E \), to be inversely related to the distance between the fluorophore dye pair, but also dependent on dye orientation, spectral overlap and a variety of other important factors which are expressed in the \( R_0 \) term.

### 1.5.1. Measuring FRET Using Fluorescence Lifetime

In principle, combining FRET with fluorescence lifetime measurements, one can achieve nanometer length-scale sensitivity in spatial resolution, and nanosecond timescale resolution on the order of the fluorescence lifetime—which is usually on the order of nanoseconds. Conventional ratiometric analysis of FRET employs binning photon arrival times with micro- or millisecond timing resolution, which implies that fast time-scale dynamics (\(<\)microseconds) are beyond the scope of observation. (25) By employing pulsed nanosecond lasers in combination with fast detectors and time-correlated single photon counting (TCSPC) hardware, it is possible to observe a photon arrival histogram, a method for measuring the photon arrival distribution as a function of a sharp excitation pulse. In this way, the fluorescence lifetime can be measured. Fluorescence lifetime has advantages as compared to intensity-derived FRET measurements. Namely, lifetime can easily be concentration and excitation intensity independent and can report on the distribution of distances sampled on the fluorescence lifetime timescale, which in most cases implies sub-microsecond dynamics are accessible. While signal from an individual burst might only yield 100 photons, it is possible to accumulate photons over many bursts to gain a complete picture of the distribution of lifetimes sampled by individual molecules passing through a confocal observation volume. (22, 26)
1.5.2. Time Correlated Single Photon Counting

Single molecule detection has been combined with time correlated single photon counting, which has been used by several groups to classify photon bursts according to lifetime. Van Orden et al. have shown that measuring burst size and intraburst fluorescence lifetime is capable of increasing the accuracy of identifying subpopulations in a sample mixture. (28) The group of Seidel has expanded on this work and now routinely combines fluorescence lifetime and anisotropy to increase the data content in single molecule fluorescence assays. (22, 29)

Figure 1.3. A setup configured for single molecule spectroscopy with TCSPC. The configuration above provides a two-channel detection, though other configurations were used for the work referenced.
The physics of fluorescence excitation and relaxation is well understood. Fluorescence is a form of luminescence that results when a material absorbs a photon, resulting in an excited state electron. The time-scale at which the electron resides in an excited state is often referred to as the “fluorescence lifetime” and is depicted in Figure 1.4A. Briefly, the fluorescence lifetime reflects the residence time of an electron in the first electronic excited state ($S_1$ in Figure 1.4A). Upon relaxation from the first excited state to the ground state, a photon is released. The emission of photons by a fluorophore is described approximately by Poissonian statistics.

![Jablonski Diagram](image)

**Figure 1.4.** (A) Jablonski diagram depicting fluorescence excitation and emission. Fluorescence results when an electron in the singlet electronic state absorbs a photon which results in its being elevated to the first excited state, $S_1$. When an electron relaxes back to the $S_0$ state, a photon is emitted. (B) FRET is a non-radiative transfer between Donor and Acceptor fluorophores that is dependent on the distance between the dye fluorophores. $R_0$ is called the “Forster Radius” and represents the distance at which 50% of the energy is transferred from donor to acceptor. A donor fluorophore is excited by an incident photon and transfers energy to the acceptor. The amount of energy transfer is related to the distance between the donor and acceptor fluorophores.
Energy transfer between donor and acceptor fluorophores results in signature changes in the fluorescence lifetime of the fluorescent probes. The acceptor fluorophore depletes the population of excited state donor electrons. As a result, the intrinsic fluorescence lifetime of the donor fluorophore is quenched when the acceptor is brought to closer proximity.

On the other hand, the fluorescence lifetime of the acceptor reports on a variety of effects. First, the acceptor lifetime reports on the timescale of energy transfer between the donor and acceptor fluorophore. It also reports on the intrinsic fluorescence lifetime of the fluorophore. The result is that the photon arrival histogram of the acceptor fluorophore reflects a convolution between the intrinsic lifetime and the distant-dependent time-scale of energy transfer. These features are evident in the photon arrival histogram, displayed below, where the donor decay (Figure 1.5, blue curve) exhibits a sharp comparatively shortened (or fast) decay with respect to the acceptor curve (Figure 1.5, green curve), which exhibits a broadened decay. The broadening evident in the acceptor channel is reporting the time scale of energy transfer between donor and acceptor, while the decay curve on longer timescales reports the intrinsic lifetime of the acceptor.
Figure 1.5. Raw photon arrival histogram with FRET. Donor emission (blue) shows a sharp mono-exponential decay, while acceptor emission (green) shows a broadened peak. The broadening evident in the acceptor emission is a result of the FRET transfer process, which involves a convolution of fluorescence excitation with energy transfer. The raw instrument response function, as measured with Erythrosin B, is shown in red.

1.6. DNA Structure and Fluorescence

Most of the information regarding the structure and dynamics of DNA has been gained previously using X-ray crystallography and Nuclear Magnetic Resonance (NMR) Spectroscopy. However, for reasons explained previously, these techniques are limited by a number of factors, and are often unable to report information in real-time. With the tools available for site-specific labeling of DNA, several studies embarked on utilizing newly fluorescence techniques to probe structural and physical characteristics of DNA.

Fluorescence spectroscopy offers an alternative method for studying the structure and dynamics of DNA that allows for observations of changes in a fluorophore’s microenvironment, with precisely the spatial and temporal resolution necessary for reaching beyond the scope of existing methods like NMR and X-ray crystallography. By
incorporating a fluorophore into DNA at a specific site, it is possible to use spectroscopic methods to examine dynamic processes of the underlying polymer. The absence of natural fluorescence from DNA and RNA molecules ensures that observations can be made without any competing background signals. Spectroscopic methods (as well as other methods) have already been used for the investigation of DNA structure, dynamics, and recognition. (28)

1.6.1. Dynamics and Structural Perturbations in DNA Structure

Most biological activity involving DNA requires structural changes in the tightly-coiled DNA found in the cell nucleus. These deformations include, but are not limited to: base flipping, strand cleavage and ligation, and local conformational changes. Many of these processes are aided by accessory proteins or are influenced by local solvent conditions. Furthermore, it is thought that DNA interacts with itself through hydrogen bonds of the duplex as well as with RNA and a number of regulatory proteins.

X-ray structures show that DNA-repair enzymes, such as the methyl transferases and human glycosyl transferase, complex with segments of damaged dsDNA and flip a base from the helical base stack. (30) A variety of pharmacological agents also are known to open base pairs, as well as bind to and intercalate DNA, breaking G-C base pairs where they bind. In addition to these local modifications to DNA structure, there are a number of local conformations that are distinct from the B-form WC double helix. For example, there are a variety of loops that can form, such as T4 loops, pseudo-dumbbell, and slipped hairpins. DNA also takes on alternative conformations, such as the triple helix or other structural forms of DNA (most commonly A-form or Z-form). (31)
An important conformation of DNA is the transcription bubble, which occurs upon binding of the enzyme RNA polymerase to a DNA template, following binding to a recognition motif or TATA box. The formation of a transcription bubble coincides with the opening of terminal base pairs, keeping only the linked strands of these two duplexes open at about 50 Å across. This allows for the construction of a complementary RNA strand during the process of transcription.

The DNA double helix is held together by two major forces: hydrogen bonding between complementary base pairs and base stacking interactions. The hydrogen bonding between base pairs is responsible for the specificity of a sequence of DNA. The base stacking interactions make a major contribution to the stability of the duplex. The bases of the double helix also play a central biological role, namely, it is the conveyor of genetic information through the generations. The complementarity of DNA strands allows for replication to occur. For replication to proceed, the two strands must be separated, and then synthesis of a new complementary strand follows. Each preexisting strand functions as a template to guide the synthesis of the complementary strand. To better understand the replication of genetic information, an understanding of DNA structure and dynamics is needed. This task demands the development of techniques and probes that detect changes in the local environment of the duplex without altering the structure of the duplex. Such probes must not disrupt the stability of the duplex, and ideally, maintain the hydrogen bonding face of a desired base.

1.6.2. Worm-like Chain
The bending force constant of DNA is a measure of the energy required to bend the helix, which is directly related to flexibility of DNA due to thermal motion. This flexibility is described by a parameter called the persistence length, $l_p$. The persistence length can roughly be considered the length over which there is some correlation between the initial and final direction of the helix. This property is often expressed mathematically in the following way:

$$l_p = \frac{B}{kT}$$

where $B$ is the bending stiffness, $k$ is the Boltzmann constant and $T$ is the temperature. At biologically relevant temperatures, the persistence length is approximated to be 50 nm or 150 bp.

A number of methods have been used to measure the bending and twisting rigidities of DNA, but some of the first significant deviations from accepted theory were reported by circularization experiments performed by Jon Widom. (36) These experiments concluded that the formation of DNA loops was orders of magnitude higher than would be predicted by a worm-like chain. Specifically, 100 bp DNA helices circularized two to four orders of magnitude faster than would be predicted by the wormlike chain model. More recently, Mathew-Fenn argued using small angle x-ray scattering that short DNA exhibited broader than expected distributions of end-to-end distances, and attributed these broad distributions to stretching fluctuations. (32)

The resistance of DNA to stretching is apparently weaker in the absence of tension. In other words, DNA stretching experiments report on the apparent structural properties of DNA under torsional stress, which may be different than physiological
conditions. The wormlike chain (WLC) model predicts that the variance increases linearly with the length of the polymer, while Mathew-Fenn reported a quadratic dependence. A quadratic increase in variance can only occur if the stretching fluctuations of neighboring base steps in a duplex are tightly correlated. (32)

The looping mechanism seems to confer advantages in terms of function (33). From the biophysical perspective, it is remarkable that loop formation appears to occur even when gene operators are located much less than a persistence length distance apart. A number of experimental methods have been utilized reproducing DNA looping in vitro, to minimize the effects of unknown factors and focus on the structural determinants. The concept of a “j factor” has been introduced to report the local effective concentration of the opposing end of a segment of DNA, which contributes to determining a probability of loop formation. (34)

The value of the j-factor depends on persistence length, the helical repeat, and the torsional rigidity of DNA. The j factor depends on the length of DNA, and the amplitude of this dependence oscillates and relates primarily on the torsional rigidity. The helical repeat unit and the bending rigidity of a segment clearly also help determine the j factor. Using this simple formulation, Vologodskii et al. reported on sequence dependent differences in the persistence length for a library of different sequences and extracted $l_p$ values between 41.7 nm and 55.3 nm. (34) An average persistence length extracted from these experiments, 48.5 nm, is in close conformity to other measurements of DNA persistence length. In summary, this work provides excellent experimental justification that there is substantial variability in the sequence-specific flexibility properties of DNA.
1.7. Experimental Aims

My experimental aim was largely to advance the use of fluorescence lifetime analysis to examine fast and short-length structural heterogeneities in DNA. This objective required overcoming a number of experimental and theoretical impediments. First, it required characterizing the limits of the experimental approach and determining the limits of probing fast-time scale fluctuations. Second, it required developing a number of theoretical techniques to minimize fluorescence cross-talk and contamination which could impair the quality of the results. The first task was to push the limits of reproducibly quantifying fluorescence lifetime in order to perform comparative measurements to screen small libraries of short oligonucleotides. Performing a fluorescence lifetime measurement entails using pulsed picosecond lasers to excite fluorescent molecules and recording photon arrivals using time-correlated single photon counting (TCSPC). A fluorescence lifetime measurement records a convolution of the laser excitation profile with the fluorescence emission characteristics of the fluorophore. Therefore, to accurately fit and quantify the rate of decay of a fluorescence lifetime, it is necessary to mathematically deconvolve the fluorescence lifetime decay from the experimentally recorded decay.

1.7.1. Methods

We developed and extended a methodology previously described for measuring an “instrument response function”, which is used to mathematically deconvolve the fluorescence decay signal. This required identifying a fluorophore that emitted in the same spectral range as the signal of interest, while also providing a fast fluorescence
decay. Erythrosin B is a water-soluble fluorophore that we used for measuring reliable, reproducible IRFs. IRF measurements were generally performed on the same day as experiments, and prepared by dissolving Erythrosin B in Phosphate Buffer Solution (PBS). The solution was diluted in PBS to a dilute concentration between 1-100 nM. Measuring the IRF using Erythrosin B requires a dilute sample to avoid photon reabsorption in the solution, which would make the IRF appear broader. A sample chamber was prepared using a silicone gasket and two Fisher coverslips, allowing us to seal the solution into a sample chamber, and preventing evaporation. Measurements proceeded at a laser excitation intensity similar to those used during the single molecule fluorescence measurement, usually between 75-125 uW. A characteristic IRF decay curve was generated, as shown.

![Figure 1.6](image_url) Characteristic instrument response functions are represented. The plot shows three repetitions of the pulsed excitation profile of the laser, combined with the fast decay of the Erythrosin B sample. These measurements were performed to extract an ideal instrument response function.

Once an IRF was produced, single molecule fluorescence experiments were conducted to collect photon arrival histograms, used to measure a fluorescence lifetime decay of short dual-labeled oligonucleotides.
In practice, the accuracy of the measured lifetime is determined by the stability of the detectors and the TCSPC system. In our experience, the IRF drift was largely dependent on the detector. As will be discussed in Chapter 3, the Single Photon Avalanche Photodiodes (SPADs) from Perkin Elmer used in many of our experiments were susceptible to temporal drift, an unwelcome feature that is reported elsewhere.

\[ I(t) = e^{-\frac{t}{\tau}} \otimes IRF \]

To account for drift, we provided a shift parameter along the time bin axis during fitting, enabling us to measure IRF lifetime decays. Imprecision in the fluorescence decay constant extracted from the IRF is primarily a result of electronics jitter that is widely reported in Perkin Elmer SPADs.

**Figure 1.7.** (A) Raw photon arrival histogram for Erythrosin B (red) and Cy3B (blue). (B) Fitted photon arrival histogram shows fitted results including distribution of errors ($\chi^2$) for Cy3B in Tris-EDTA buffer (50 mM NaCl) measurement.

In Figure 1.7A, we show IRF and raw fluorescence photon arrival histograms. After performing a deconvolution to account for the contributions to the fluorescence decay, a Simplex iterative fit that applies a Maximum Likelihood Estimator was used to extract
After establishing the procedure for collecting TCSPC data, our first objective was to determine the sensitivity of the lifetime to changes in the persistence length of DNA. A model system for examining this was to study the transition from single-stranded DNA to double-stranded DNA. Hybridization of two strands of ssDNA results in an increase in the persistence length of DNA, and such changes are easily observable using established ratiometric FRET techniques. In fact, this structural transition served as the basis for other work referenced in this dissertation in Chapter 2, which included building a microfluidic device for measuring the transcriptional activity of RNA polymerase in vitro.

Figure 1.8. Single-stranded DNA (ssDNA) is a comparably flexible polymer, when compared to double-stranded DNA (dsDNA). Upon introduction of a complementary strand, ssDNA strands hybridize which is responsible for an increase in the persistence length of the underlying polymer. Using FRET, it is possible to detect changes in the persistence length of DNA upon hybridization.

1.7.2. Results and Discussion

After acquiring data, we perform a conventional burst search analysis to isolate photons that were emitted by large molecules floating through the confocal spot of the
Photons are classified and plotted according to computed E and S values in a 2D E-S histogram, which provides a convenient means to “sort” bursts according to distinct subpopulations. In this way, we are able to distinguish between a donor-only and dual-labeled FRET subpopulations. The importance of the donor-only population is to determine the intrinsic fluorescence lifetime of the donor fluorophore in the absence of the acceptor. A molecular species with both a donor and acceptor fluorophore exhibits a donor lifetime that is comparatively quenched, relative to the donor-only lifetime. This feature was commonly used as a built-in control across all of our experiments.

Next, we analyzed the lifetime of the donor fluorophore in the presence of an acceptor to determine the hybridization state of DNA. When ssDNA hybridizes to its complement, the distance between the donor and acceptor fluorophores increase due to the underlying change in persistence length of the polymer, resulting in a reduction in the quenching of the donor’s fluorescence lifetime. We quantified this change using dual-labeled single-stranded DNA (40 bases) labeled with tetramethylrhodamine (TMR, Sigma-Aldrich, St. Louis) as the donor and ATTO647N (Atto-tech GmBh, Germany) as the acceptor. TMR’s fluorescence lifetime was fit to a single-exponential decay and residuals were calculated using a Maximum Likelihood Estimator, as described by Laurence. (35)
Figure 1.9. (A) After selecting only photons from a dual-labeled FRET subpopulation, we compare the ssDNA and dsDNA species, showing that dual-labeled ssDNA donor fluorophores are qualitatively “quenched” compared to the more rigid dsDNA species. (B) FRET sub-populations should report a comparatively quenched donor fluorophore compared to the donor-only sub-population.

To determine the sensitivity of the assay to differences in flexibility, we screened short oligonucleotides with different sequence or structural motifs which we expected would correspond to subtle differences in the underlying polymer rigidity. Among the first samples we screened were samples with short segments of mismatched bases, ostensibly resulting in formation of a “bubble.”
Figure 1.10. (A) Double-stranded DNA that is fully-hybridized and (B) DNA with n-basepair mismatches are represented as having a “kink” or bend. (C) Results from our FRET experiments suggest that fully hybridized DNA can be distinguished from DNA with basepair mismatches. Results from initial experiments suggested that the method was largely insensitive to differences in flexibility between the sequences with increasing number of base-pair mismatches, although experiments did suggest that the method was sufficient to distinguish between fully hybridized and a base-pair mismatched sample. Figure 1.10C shows that donor fluorophores in mismatched samples were measurably quenched compared to the samples that were fully hybridized.

In addition to base-pair mismatched sequences, we also considered measurements that compare short sequences of DNA which would be known to have putatively “flexible” sequences, and random sequences which would be comparatively rigid. This flexibility is largely determined by the G-C content of a DNA sequence, which forms three stabilizing hydrogen bonds compared to the two hydrogen bonds formed between
A-T bases. Future work is envisioned which would conduct a more thorough set of comparative lifetime measurements showing minute differences in flexibility in different oligonucleotide sequences, as a function of sequence and basepair mismatches.
1.8. References


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Chapter 2

High-throughput study on *in vitro* transcription by RNA Polymerase using a microfluidic platform
2.1 Introduction

In this chapter, we describe our development of a microfluidic platform to conduct rapid, high-throughput screens on in vitro transcriptional processes in response to a variety of chemical contexts. This custom-built, high-throughput and fully automated single molecule measurement system is capable of examining conformational changes of dual-labeled biomolecules with single molecule resolution and as a function of different chemical environments. In addition, our microfluidic mixing device utilizes integrated valves and pumps, which further provide high-precision control to accurately accomplish titration with picoliter resolution.

Microfabricated integrated circuits revolutionized the world by dramatically lowering the cost and increasing the accuracy of large-scale computation. It is widely recognized that microfluidic systems will enable an analogous expansion of our understanding of chemistry and biology by allowing for increasingly automated, allowing rapid, highly parallelized experimentation and analysis with the added advantage of using comparably little reagent.

A review of the development of microfluidic-based devices would be involved and beyond the scope of this work, but there are numerous challenges with regards to assembling miniaturized microfluidic devices while also addressing some of the physical properties of fluids on small spatial length scales. Research into micro- and nanofluidics has resulted in the miniaturization of fluidic control mechanisms, valves and pumps.[ref]

In the past several years, the emergence of optofluidics allows the use of computer-controlled microfluidic devices in combination with fluorescence microscopy
to study fluids. This builds on the work of Whitesides 2006; Squires and Quake 2005; Atencia and Beebe 2005; Ng et al. 2002. We developed an optofluidic system for conducting single molecule assays based on the microfluidic platform framework originally developed at the lab of Stephen Quake at Stanford University, first envisioned to automate X-ray crystallography assays.[ref]

Optofluidics involves the combination of microfluidic devices with optical microscopy. First, a microfluidic dual-layer chip is made by etching flow-chambers onto glass substrates using soft lithography and PDMS. In our setup, an optofluidic device developed is integrated with pumps to precisely control mixing of solutions, while the transparent optofluidic chamber enables laser-induced excitation and detection of light using confocal microscopy. We further employed continuous-mode alternating excitation lasers and single molecule analysis, allowing us to apply single molecule methods originally developed by Weiss in a high-throughput, automated manner to generate a high-dimensional context-dependent plot of biomolecule reaction kinetics. (ref)

2.2 Context-dependent Analysis of Biomolecular Conformation

Biological function is context-dependent, and in vitro study of biological systems are often limited to studying a predefined chemical environment which is calibrated to reproduce facets of the system’s function in vivo. It is widely recognized that many biological systems operate on the basis of sampling non-equilibrium states, and therefore developing systems for sampling non-uniform conditions would be of tremendous value (1, 2). For example, chemical and environmental stress will radically alter the properties of biomolecule structure and interaction networks in a cell (3). We developed and
characterized a methodology that can easily screen many hundreds of chemical environments to aid in the construction of a ‘phase diagram’ of biological function in the context of chemical space.

A widely-adopted readout of biomolecule conformation (or interaction) is single molecule fluorescence resonance energy transfer (smFRET), wherein a single donor-acceptor pair reports on the distance between dyes in the 1-10 nm scale (4), a technique used in an ever-growing list of systems (helicases, polymerases, and ribosomes, to name a few).

Microfluidic technology enables precise and rapid handling of small liquid volumes and thus facilitates vast chemical screening of biological molecules with minimal sample consumption (5, 6) and it is rapidly becoming a useful tool in single molecule biophysics. Microfluidic hydrodynamic focusing has been used previously for single smFRET measurements of protein folding in microfluidic environments (7-10). Groisman and co-workers used a “gradient generator” to titrate detergent and temperature for ensemble FRET measurements (11) and control oxygen levels in smFRET measurements (12). Williamson and co-workers mixed RNA in a microfluidic formulator device (13) to measure ensemble FRET and perform fluorescence correlation spectroscopy (14).

2.3 Experimental Overview

We used a microfluidic formulator to perform large scale, automated single molecule measurements across a wide range of chemical conditions. Mixing was achieved with a microfluidic mixing ring (13) wherein a peristaltic pump made of three
integrated valves is used to inject reagents of interest into a ring and a second pump circulates and mixes the reagents (Figure 2.2A). The dual-layer PDMS device consists of a control-layer which uses “push-down” valves to manipulate fluid on the flow-layer, with nominal channel width of 100 μm and height of 10 μm. Molecules of interest are detected using confocal microscopy in the Confocal Viewing Chamber. The first peristaltic-pump (Figure 2.2A) injects the contents of seven independently addressable input channels into the mixing ring with tens of picoliter precision. As reagents are pumped into the ring, the reagent valve, inlet valve and outlet valve are opened and previous contents of the ring are displaced. Calculations of final concentrations must take this volume displacement into account.

The push-down valve configuration permits sample detection directly above the glass coverslip, a benefit for single molecule spectroscopy. An observation chamber (50 μm tall x 250 μm wide) was designed into the mixing ring to allow optimal confocal detection. The ring is then flushed and prepped for a sequential measurement. Software for coordination between microfluidic device control and microscopic data acquisition was developed to enable long-term unassisted data collection. With this device we automatically titrate molecules to screen multi-dimensional chemical space for conformational and enzymological changes in biomolecules. This single molecule, microfluidic approach allows for large sampling of parameter space while avoiding ensemble averaging with high reproducibility.
2.4 Accuracy of titrations

Before conducting a concerted study of a biomolecule of interest, we sought to first validate the precision of the microfluidic system for titrating samples and ensuring uniform mixing. For each channel, a peristaltic pump was used to inject the fluorescent dye Alexa 488, while fluorescence readouts were used to calibrate the system. This approach determined that a single injection slug resulted in a 243-fold dilution of the initial solvent, as demonstrated in the calibration curves (Figure 2.1A). The mixing rate was monitored by measuring the transient response to an injected slug of fluorescence dye, shown in Figure 2.1B. These measurements demonstrate clearly that uniform mixing conditions are achieved, evidenced by a fluorescence steady state after 8000 ms.

Further controls were performed to assess the reproducibility of subsequent measurements, yielding identical results. To prevent cross-contamination, flow channels were incubated with 0.5% pluronic f-127 for 1 hour prior to measurements.

Figure 2.1. (A) Injection into the mixing ring over a large concentration range obeys a linear relationship between iterations of the on-chip peristaltic pump ("injection slugs") and resulting relative final concentration in mixing ring. This relation is obeyed for all input channels of the device used in this study (#1 through #6). This calibration was conducted with the fluorescent dye Alexa Fluor 488, where relative concentration was determined by relative fluorescence intensity after mixing (reported as relative final...
concentration [%]). (B) The transient mixing response (reported as fluorescence intensity as function of time) following an initial injection (at ~1200 ms) of dye into the mixing ring of the formulator and subsequent iterations of mixing (in the window 4000-8000 ms). Steady state of mixing is achieved at ~10,000ms (10 s).

2.5 Using a Microfluidic platform to study DNA hybridization

In this study, we use a ssDNA probe consisting of a poly(dT) sequence of 20 nucleotides, flanked by donor (5(6)-carboxytetramethylrhodamine) and acceptor (Alexa Fluor 647) dyes on 3’ and 5’ nucleotide backbone (called Poly(dT) hereafter). This simple sequence was utilized to study DNA polymer behavior and hybridization, using smFRET readouts to resolve changes in conformation due to compaction and hybridization, and to study RNA polymerase activity, employing the ssDNA probe as an aptamer for nascent RNA transcript in a run-off transcription assay. This study also had the advantage of independently showing how the compaction of single-stranded DNA influences the hybridization efficiency.
Figure 2.2. The microfluidic mixing device, a tool for high-throughput smFRET, is used to generate a hybridization landscape of a ssDNA molecule. Molecules are fed into the device and mixed into the mixing ring (A, blue arrow), the schematic of which depicts features of the control and flow layer (black and red lines, respectively) proximal to the mixing ring. (B) smFRET measurements are represented as a 2D histogram of FRET vs. Stoichiometry; in the schematic, subpopulations of interest are hybridized poly(dT) (Low FRET population marked as “ds”) or unhybridized poly(dT) (high FRET population, marked as “ss”). (C) Titration of complementary strand versus titration of [NaCl] (increasing rightward) demonstrates a pronounced shift to hybridization in the presence of ionic strength and complementary strand. (D) Quantification of hybridization efficiency (see text) of the data in (C) presented as a heat-map.

We first sampled the poly(dT) conformation as a function of ionic strength in the microfluidic device. Consecutive smFRET measurements of titrated salt concentrations were performed automatically with the microfluidic scheme. Each measurement is shown
as a scatter plot with dimensions of FRET (approximated by a ‘proximity ratio’). The proximity ratio is not representative of the precise distance between donor and acceptor dyes; rather a proximity ratio reports on the ratio of subpopulations. Therefore, we reported the proximity ratio as acceptor photons / all photons during donor excitation. The stoichiometry (‘S’, measured using alternating laser excitation spectroscopy (15)), reported on labeling efficiency of the molecules of interest, and allows filtering of subpopulations in order to eliminate singly-labeled biomolecules.

Figure 2.3. RNA Polymerase activity is measured as a function of regulation by an osmolyte, glutamate (A,C); the assay detects transcriptional activity of RNAP by hybridization of the Poly(dT) probe to newly produced RNA (B). Poly(dT) hybridizes with nascent RNA transcript as a function of RNA Polymerase concentration (increasing rightward) resulting in reduction in FRET. RNAP activity is regulated by addition of glutamate (increasing upwards). (C) Quantification of transcription efficiency (see text) of the data in (A) presented as a heat-map. The map demonstrates the dual regulatory roles of glutamate: positive regulation observed until highest concentration (top row), where relative transcript levels are reduced.

In each 2D plot, each point represents a single molecule and donor-acceptor molecules of interest are found in the center half of the S-axis (S = 0.25 – 0.75). As shown elsewhere (16), a ssDNA molecule collapses (lower end-to-end distance, higher FRET) upon ionic strength increase of NaCl (Figure 2.2D, lowest row, left to right, and
This well-established effect of ionic strength on the conformation of ssDNA is due to ionic screening and consequent reduction in persistence length, the magnitude of which is in agreement with similar FRET-based studies (16). DNA conformation was also studied with 5 different cations, in which the magnitude of each salt effect follows the order of Hofmeister series (Figure 2.4)(17).

Figure 2.4. (A) Increase in ionic strength collapses ssDNA, as reported by FRET histograms (plotted as function of [NaCl]; same data as in bottom row of Figure 1C). (B) FRET change as function of salt concentration for 5 different salts (Guandine HCl, CaCl\(_2\), MgCl\(_2\), NaCl, and KCl; derived from data as in (A)).

2.6 Estimation of DNA hybridization midpoints

This approach allowed us to study the ssDNA conformation and hybridization efficiency (to a complimentary strand) under the influence of two competing processes: rigidification due to hybridization and chain collapse due to increase in ionic strength. We constructed a matrix of 64 serial measurements, comprised of an 8-increment [NaCl] gradient and an 8-increment [complementary strand] gradient (Figure 2.2). Raw data is plotted with increasing NaCl concentrations (from 115 mM to 917 mM, left to right) along the horizontal-axis and increasing poly(dA) hybridizing strand (from 7 nM to 59 nM, bottom to top) along the vertical-axis, demonstrating the differential effects of hybridization (lower FRET) and ionic strength (higher FRET, for ssDNA). Coexistence of single-stranded and double-stranded DNA is observed in some cases (Figure 2.2D,
second and higher columns). Subpopulations were fit to 2D Gaussian distributions and the extent of hybridization is shown as the ratio, $R$, of double-stranded to total (ds- & ss-) DNA subpopulation $R = \frac{[\text{dsDNA}]}{[\text{ssDNA}]+[\text{dsDNA}]}$. At the lowest salt concentrations and complementary strand concentrations, hybridization efficiencies were negligibly low (blue squares, Figure 2.2C), and chain collapse caused by increasing ion content can be seen. At complementary strand concentrations greater than 7nM, the hybridizing effect (low FRET population) dominates the chain collapse (high FRET population) as cation concentration is increased. As can be clearly seen, increase in ionic strength not only promotes ssDNA compaction, but also promotes hybridization.

Theoretical estimates for poly(dT) hybridization midpoint agree extremely well with the observed midpoints (open black circles in Figure 2.5). We have therefore recapitulated a large two-dimensional titration to directly measure the hybridization...
landscape of ssDNA to its complement in 64 different chemical environments. Screening a large phase-space of physiochemical parameters is crucial, for example, for the successful design of biologically relevant aptamers, molecular beacons or short interfering RNA constructs. To demonstrate the higher dimensionality accessible with this technique, we perform a titration of two salts, NaCl and MgCl2, against poly(dA). The resulting 3-dimensional hybridization phase map (Figure 2.6) reveals the comparative and combinatorial effects of the monovalent and divalent salts.

Figure 2.6. A three-dimensional (4x4x4) exploration of physiochemical space (with complementary strand, monovalent and divalent salts) demonstrates the role of environment in hybridization and conformation of ssDNA. Poly(dT) was combinatorially titrated with 10dA ssDNA, MgCl2, and NaCl as indicated by the axes. The data represents a 3-dimensional landscape of the hybridization efficiency of doubly labeled 20dT ssDNA. Along with
comparing the effects of the different reagents, the additive effects of reagents can be visualized with this method. (a) Raw ALEX data showing FRET and S values for every condition. (b) Processed heatmaps, color represents hybridization ratio as in Figure 1 in the main text.

It should be emphasized that the results presented in Figure 1 could not be easily achieved using ensemble-based methods, while the single molecule assay readily resolves the various subpopulations: high-FRET ssDNA, low-FRET ssDNA, ds-DNA, and mixtures thereof. The alternative, taking an ensemble measurement (e.g. fluorimeter), is equivalent to the integral over the distribution, which results in a single, averaged number.

We extended this approach to the study of enzyme activity, exploiting the poly(dT) probe as a biosensor for productive mRNA synthesis by bacterial RNA polymerase (RNAP). Previous reports have established dependence of RNAP activity on glutamate concentration, showing that the osmolyte acts as both a positive and negative regulator of transcription, depending on the absolute glutamate concentration (18). Glutamate facilitates RNAP promoter escape, enabling productive transcription, by altering the conformation of RNAP (likely due to the Hofmeister effect). Conversely, high concentrations of the anion were reported to cause considerable decreases in transcription by inhibiting RNAP from binding to the ribosomal promoters. Our optofluidic single molecule transcription assay allows thorough exploration of the glutamate response space for RNAP activity.

A DNA template for transcription was designed such that the RNA transcript would hybridize to poly(dT) (Figure 2.3B). Maintaining a constant poly(dT) concentration (250 pM), we titrated [RNAP] in 6 increments and [potassium glutamate] in six increments (Figure 2.3A). Here, low-FRET species correspond to DNA-RNA
heteroduplexes of poly(dT) hybridized to the mRNA transcripts. The volume under the low-FRET peak therefore scales with productive mRNA synthesis (Figure 2.3A). Fitting of each subpopulation to obtain a hybridization ratio provides a metric to directly quantify RNA transcripts (e.g., ~188 pM mRNA for condition in row 4, column 5 of Figure 2A).

Although changes to glutamate concentrations affect ssDNA conformation, hybridized and unhybridized subpopulations are clearly resolved. Increases in hybridized transcript are apparent with increasing RNAP and glutamate concentrations until excessive concentrations of glutamate (514 mM) are reached (Figure 2.3A & 2.3C), consistent with previous observations of glutamate inhibition at concentrations >500 mM (18). Although the contribution of ionic strength to hybridization efficiency could in principle complicate this readout, this effect was minimal when measured directly.

In contrast with traditional approaches to transcript detection, the single molecule approach can detect very low concentrations of RNA; for instance, at 50% hybridization, we expect only ~100pM RNA transcript in only a few nanoliters. Using this approach, we are able to sample enzyme activity in a large physiochemical space. The possibilities for this type of multi-parameter inquiry may help in elucidating the complex and multi-factorial mechanisms underlying transcriptional regulation and other similarly complex enzymatic systems.

The ability to observe multi-parameter physio-chemical landscapes without having to manually dilute and load the sample on the microscope significantly simplifies the process and dramatically reduces titration/pipeting errors. Whereas typical pipetting
error can be around 0.5% or 50 nanoliters for microliter reactions, the ring injection error is $\sim10^{-4}\%$ or 0.6 picoliters (13). A possible enhancement to the approach presented here would take advantage of emerging multi-spot photon counting detectors (19).

### 2.7 Conclusions

The high-throughput elucidation of conformational landscapes may significantly enrich studies of protein regulation, interaction networks, allostery, and folding. Moreover, with regard to mRNA quantification, this novel optofluidic technique serves as a significant step towards direct, single-cell gene expression profiling.
2.8 References


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Chapter 3

Benchmark of a new red-enhanced

SPAD detector for single-molecule FRET
3.1. Introduction

Single molecule spectroscopy has relied on advanced single-photon avalanche photodiodes (SPADs) to interrogate the physical characteristics, diffusion properties and elucidate molecular interactions of biomolecules. Fluorescence microscopy coupled with these sensitive photodetectors has spawned a variety of techniques for detection, tracking and spectroscopic analysis of biomolecules like fluorescent-labeled DNA and proteins. These studies have been instrumental in understanding biological processes, including cellular processes like DNA replication and RNA transcription, among others. (1, 2, 3) Though commercially available SPADs are precise and sensitive enough to detect the incidence of a single photon, there are a number of notable limitations including low quantum efficiency in one part of the visible spectrum, poor timing resolution, and a lack of temporal stability. (4) Furthermore, some SPADs fail after exposure to strong intensity signals. In this study, we characterized a new custom-technology SPAD detector which addresses these drawbacks, and we compare the performance of a new detector with the popular, commercially available SPADs for applications in single molecule fluorescence microscopy. We perform two-color single molecule FRET experiments on dual-labeled double-stranded DNA (dsDNA) using Alternating Laser Excitation (ALEX), and compute FRET proximity values using both intensity-based and lifetime analysis. (5) Our results validate the suitability of these red-enhanced SPADs for single molecule spectroscopy, both for intensity-based and lifetime-based measurements.

3.2. Methods
FRET, or Forster Resonance Energy Transfer, is a popular technique for probing nanometer-scale changes in distance between a donor and acceptor fluorophore. FRET, a non-radiative process, reports on inter-dye distances between 1-10 nm. smFRET has been used to study a growing list of macromolecules, including oligonucleotides, polymerases and helicases, to name just a few. FRET is commonly quantified using either an intensity-based or lifetime approach. Intensity-based approaches for measuring FRET have been the most widely used, but suffer from sensitivity to concentration and from oft-overlooked requirements to characterize and calibrate instruments according to the excitation and detection parameters of a microscope setup. In equation (1), we express the proximity ratio (E) as the ratio of photons detected in the donor and acceptor channels, with $\gamma$ reporting on the relative detection and emission efficiencies of the donor and acceptor fluorophores:

$$E = \frac{F^D_A}{F^D_A + \gamma F^D_D}$$  

(1)

Lifetime FRET is not concentration-dependent, and is not subject to the same biases that intensity-derived calculations are vulnerable to (6). FRET processes result in the quenching of donor fluorescence, and so quantification of a FRET proximity ratio (E) can be achieved by measuring a fluorescence lifetime of the donor fluorophore in the absence of the acceptor and comparing this lifetime to the donor in the presence of the acceptor:

$$E = 1 - \frac{\tau_{DA}}{\tau'_D}$$  

(2)
In this study, we employed laser-induced fluorescence using two picosecond pulsed lasers (532 nm High-Q Lasers, 635 nm PicoQuant GmBH), in concert with Alternating Laser Excitation (ALEX). We studied two dual-labeled 42-basepair species of dsDNA, which share an arbitrary sequence but were prepared with fluorescent labels at different inter-dye distances (13 base pairs and 25 base pairs, respectively). Cy3B (GE Lifesciences) and ATTO647N (ATTO-TEC GmBH) were chemically attached to the DNA via amino-dT modifiers, and serve as donor and acceptor probes with a 6.5 nm Förster radius ($R_0$). Employing ALEX enables us to sort subpopulations of molecules, particularly useful for distinguishing singly or doubly-labeled samples as a result of incomplete labeling of the DNA samples. ALEX ensures high-quality and accurate quantification of a FRET proximity ratio. Intensity-based and lifetime FRET results were all obtained using time-correlated single photon counting (TCSPC) on a Becker-Hickl SPC-630 device, and analyzed using custom MATLAB routines.

Measurements were performed on a custom-built microscope which could accommodate a direct comparison between the detection efficiency, timing resolution and burst counting rate of commercially-available Perkin Elmer SPADs and the red-enhanced custom SPAD from the Politecnico di Milano. Dilute picomolar concentrations of dsDNA were studied in buffered solution (Tris-EDTA with 50 mM NaCl, pH 8.0), and experiments measured fluorescence emitted by single molecules diffusing through the confocal volume of the microscope. A post-acquisition burst analysis was performed, which distinguished “burst photons” from background signal using a “sliding window” burst analysis (7) and classified emitted photons according to whether the photons were
excited by a donor or acceptor laser excitation. In this way, photons from single molecule diffusion events can be sorted into distinct subpopulations, including singly-labeled subpopulations (constituting a donor-only subpopulation) and dual-labeled FRET subpopulations.

3.3. Results and Discussion

Our results suggest that the Politecnico solid state SPADs perform comparably for detecting single molecule bursts. We compared the results of FRET measurements on both low and high FRET samples, and quantified the detection efficiencies accordingly. The Politecnico SPADs feature a 3-fold improvement in dark counting rate (Table 1). However, the Politecnico SPADs have inferior quantum detection efficiency across the visible spectrum, which led us to expect an estimated 15% lower counting rate. Our results reflect the lower detection efficiency in the red with the Politecnico SPADs, and additional deviations may be attributed to errors in aligning the detectors. This is likely because the Politecnico SPADs have a 50 um² detection area, as compared to the 200 um² observation area on the Perkin-Elmer SPADs. It should be noted that despite this, the Politecnico SPADs were remarkable easy to align, and the results suggest that alignment issues do not impair the ability to obtain excellent correspondence between the detectors.

<table>
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<tr>
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<th>Politecnico SPADs</th>
<th>Perkin Elmer SPADs</th>
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<tbody>
<tr>
<td>Dark Counting Rate (counts/s)</td>
<td>80</td>
<td>300</td>
</tr>
<tr>
<td>Donor Photon Counting Rate</td>
<td>1170</td>
<td>1570</td>
</tr>
<tr>
<td>Acceptor Photon Counting Rate</td>
<td>1380</td>
<td>2340</td>
</tr>
</tbody>
</table>
### Table 3.1 – Dark counting and photon counting rates were measured under identical conditions (50 mM NaCl in Tris EDTA pH 8.0) for a sample of dual-labeled 42-bp dual-labeled dsDNA.

<table>
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<th>Gamma (γ) [8]</th>
<th>0.95</th>
<th>0.95</th>
</tr>
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</table>

Solution-FRET is particularly useful for studying biomolecular conformations and the dynamics of enzymatic processes. All single molecule techniques share some basic requirements, including the need for optimized excitation and high efficiency collection of the fluorescence signal, rejection of background signal and optimization of the temporal resolution of the acquisition (4). The starkest contrast between the Perkin-Elmer SPADs and red-enhanced Politecnico SPAD is the 300 ps timing resolution versus 90 ps timing resolution. To emphasize the significance of the differences in timing resolution, we measured the radiative decay of the donor and acceptor fluorophores during our smFRET experiments. As is clear in Figure 3.1, the Politecnico SPADs (left) have a noticeably sharper rise time and dramatically faster response. There is excellent correspondence between the measurements on the Perkin Elmer SPADs versus the Politecnico SPADs, including similar radiative decays for the donor-only subpopulations of burst photos, which were fit to single-exponential decays. Similar results were also obtained when measuring high and low FRET samples of dual-labeled double-stranded DNA using an intensity-based ratiometric approach.
Figure 3.1. Instrument response functions of the Politecnico SPADs versus the Perkin Elmer SPADs at varying counting rates, as measured using Erythrosin B. [11] Single molecule experiments are typically performed at counting rates equivalent to 3-10 kHz.

3.4. Intensity FRET

Though measuring FRET using the intensity-based approach is technically simpler, it is subject to a variety of biases including sensitivity to concentration that have been well described elsewhere (9). Though lifetime FRET requires specialized TCSPC hardware, it is insensitive to concentration, and therefore is of importance for examining a variety of biochemical processes.
Figure 3.2. Intensity FRET histograms relate distinct subpopulations, including donor-only and FRET (dual-labeled) subpopulations. Subpopulation selection is used for generating photon arrival histograms, below.

Figure 3.3. Photon arrival histograms were measured for low- and high-FRET samples of dual-labeled dsDNA. The high FRET samples (right panels) display marked quenching of the donor decay in the presence of the acceptor.
Figure 3.2 displays the corrected E-S histograms for measurements made using the Politecnico and Perkin Elmer SPADs. Encouragingly, the two devices report similar results. We employed intensity FRET to isolate photons that belong to specific subpopulations in our DNA measurements. In Figure 3.3, we present the photon arrival histograms which correspond to these distinct subpopulations, illustrating the influence of FRET on the radiative decays of the donor fluorophore. Across all the samples, the donor-only lifetime was consistent at 3.2 ns. The subpopulation corresponding to the donor in the presence of acceptor was qualitatively quenched relative to the donor-only decay, shown in Figure 3.3. We used a maximum likelihood estimator to fit these curves to single and double-exponentials, and extracted $\tau_{DA}$ which is used to calculate a lifetime FRET value. (10, 11) Table 3.2 reports on the correspondence between results for FRET calculated using intensity-based method and the lifetime method.

<table>
<thead>
<tr>
<th></th>
<th>Politecnico</th>
<th>Perkin Elmer</th>
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<tbody>
<tr>
<td></td>
<td>25 bp</td>
<td>13 bp</td>
</tr>
<tr>
<td>$\tau_D$ (ns)</td>
<td>3.25</td>
<td>3.10</td>
</tr>
<tr>
<td>$\tau_{DA}$ (ns)</td>
<td>2.38</td>
<td>0.65</td>
</tr>
<tr>
<td>$E_{\text{lifetime}}$</td>
<td>0.27</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Table 3.2. Results from the lifetime FRET measurements show relative agreement between the proximity ratio $E$ obtained from Politecnico SPADs and the commercially-available Perkin Elmer SPADs.

3.5. Lifetime FRET
The radiative decays of samples with high FRET can be difficult to resolve, due to the fact that donor fluorescence is quenched by the transfer of energy to the acceptor, thereby reducing the number of photons emitted in the donor detection channel. Timing resolution is limited by the width of the instrument response function on any detector, which makes fast decaying components in the donor channel difficult to quantify. The results suggest that the Politecnico SPADs have exquisite sensitivity to high FRET samples of dual-labeled double-stranded DNA. One salient observation is contrasting the high FRET decays with the fast time resolution of the Politecnico SPADs, as compared to the Perkin Elmer SPADs. The broad instrument response function dominates the width of the donor channel fluorescence decay, and illustrates the limitations of this detector for quantifying high FRET samples. The high FRET photon arrival histogram on the Politecnico SPAD is easily distinguishable, and hints at clear advantages for resolving fast decaying components.

3.6. Concluding Remarks

The previous data validates the suitability of the Politecnico SPADs for single molecule microscopy. In particular, we show that the new detectors have all of the advantages expected from traditional SPADs, in addition to enhanced timing resolution, timing stability and superior tolerance to photo-damage. We conducted smFRET on dual-labeled DNA to compare and quantify the detection properties of the two types of detectors side-by-side, concluding that the new detectors have a comparable detection efficiency, comparable burst count rate, on top of a 3-fold improvement in timing resolution. The Politecnico SPADs offer a variety of avenues for future exploration,
including development of multipixel detectors to improve data throughput. The timing resolution also suggests the ability to measure faster dynamics using lifetime FRET for monitoring short radiative decays. Therefore, future work will continue to develop the solid state SPADs for multipixel detectors, high-throughput analysis and development of new imaging modalities which incorporate single-photon detectors.
3.7. References


