ADVANCES IN SINGLE MOLECULE SPECTROSCOPY AND MICROSCOPY FOR BIOLOGICAL IMAGING AND POLYMER CHARACTERIZATION

BY

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DISSERTATION

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ABSTRACT

Single molecule fluorescence microscopy and single molecule spectroscopy provide tremendously powerful methods for studying the behavior of a wide variety of biological systems. In this way, single molecule techniques can be used to gain an increased understanding of molecular mechanisms underlying basic phenomena in biology, materials science, and soft matter. In general, these approaches allow for detailed molecular information to be obtained when compared to bulk level methods performed using macroscopic techniques. In the first part of this thesis, we use single molecule fluorescence microscopy (SMFM) to develop and characterize a new class of fluorescent probes. Using SMFM, target biomolecules are commonly labeled with single fluorescent dyes allowing for real-time observation of dynamics and transient events. However, single molecule fluorescence imaging critically relies on bright dyes for robust signal detection above a noisy cellular background. Additionally, photostable dyes are desired to allow for continuous imaging of long time scale biological processes. To address this challenge, we developed a new class of fluorescent probes for SMFM using a two primary strategies designed to increase brightness and photostability. First, we developed fluorescent dendrimer nanoconjugates (FDN) consisting of multiple individual Cy5 dye molecules conjugated to a polymer dendrimer scaffold, which allows for increases in the total brightness of the molecules. In addition, we designed a series of ‘self-healing’ dendrimers that have a photoprotective molecule, Trolox, covalently attached to the probes, thereby resulting in increased photostability. Specifically, we designed the ‘self-healing’ FDNs using two complementary synthetic strategies, termed ‘random addition’ and ‘controlled addition’ allowing for control over the average stoichiometric ratio between Trolox and Cy5, and
exact conjugation of Trolox and Cy5 with a precise one-to-one ratio. In all FDNs synthesized with the ‘self-healing’ strategy, we observe increases in probe photostability.

In the second part of this thesis, we use single molecule force spectroscopy (SMFS) to study the mechanical properties of polymer systems. Here, we specifically examine the effect of the photostabilizer Trolox on the physical properties of the biopolymer, DNA. Using this technique, we exert force on a single polymer and observe the response of the molecule (typically increased extension under a stretching force). Using this method, we are able to determine polymer physical properties such as persistence length and contour length. We use magnetic tweezers for SMFS, a commonly used technique where a paramagnetic bead is attached to a tethered polymer, allowing for an external magnetic field to pull on the polymer. By observing the bead position over time, we can extract information on the polymer physical properties. We compare how these properties change with the addition of commonly used solution additives, primarily Trolox, used in fluorescence studies in polymer physics to provide enhanced photostability. In particular, we show that the addition of Trolox into solution with DNA induces an increase in the total contour length, consistent with results from our lab on DNA stretching experiments with SMFM. In summary, my work applies a consistent theme of addressing dye photostability and the photophysical properties of fluorescent probes for single molecule techniques. We demonstrate the development of a new class of photostable probes for fluorescence microscopy, and we determine the effect of commonly used photostabilizer on the physical properties of a model polymer system, DNA. Overall, this work will help advance the techniques available in single molecule imaging by increasing our understanding of the photophysical mechanisms underlying
multi-dye conjugates and the possible physical changes to a system that can occur when using photostabilizing agents.
ACKNOWLEDGMENTS

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I would like to express my sincerest of gratitude towards my family, for listening in my stressful times, and supporting me in the ups and downs of grad school, and in always expressing an interest in my work. In particular, the pride that I can hear expressed by my parents has been an invaluable source of inspiration to continue in this path I have chosen.

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CHAPTER 1: INTRODUCTION

1.1: Thesis Overview

The primary goal of this thesis is to develop and use new techniques in fluorescence microscopy and single molecule spectroscopy to gain insight into the properties of biological systems and single biomolecules. In the first part of this thesis, we develop a new class of multichromophoric fluorescent probes for imaging, and in the second part of the thesis, we use magnetic tweezers to study the properties of single DNA molecules. These techniques provide tremendous insight into a variety of fields that benefit from the observation of single molecule phenomena. Recent methods such as super-resolution fluorescence imaging allow for determination of intricate and detailed structures of nanometer scale features that is only achievable with spatial resolutions below the diffraction limit of light. Importantly, we are able to observe molecular scale dynamic events using these techniques, thereby enabling increased insight into biological and biophysical phenomena at the molecular scale. In this way, it is often possible to extract mechanistic information determining the function or role of a molecule or cellular component.

Chapter 1 provides an outline for the thesis and places our work in the proper context in the field by providing an overview of the current state of single molecule techniques in relation to our contributions. Chapter 2 details our work in developing a new class of fluorescent probes for single molecule biomolecular imaging. In particular, we developed a new class of multichromophoric probes with covalently linked photostabilizers that are shown to exhibit increased brightness and photostability though a ‘self-healing’ dye strategy. Chapter 3 explains the use of single molecule force spectroscopy using the technique of magnetic tweezers to study polymer physical properties. Primarily, we explore the role of commonly employed solution-based
additives used as photostabilizers on the physical properties of DNA. Here, we show that one commonly used photoprotective agent in DNA fluorescence assays has a significant effect on the force-extension relation of DNA and increases the DNA contour length relative to natural B-form double stranded DNA. Finally, Chapter 4 summarizes the major results comprising this thesis and describes future work that has been inspired by these projects.

1.2: Introduction of Research

Single molecule measurements provide several advantages to studying molecular scale bioprocesses in many fields of research. In particular, single molecule techniques allow for direct observation of molecular-scale dynamic behavior and molecular sub-populations, thereby revealing additional information that can be obscured in bulk-level methods. Several sub-fields in biophysics have benefitted from these techniques, and as one example, we consider early pioneering experiments on single ion channel recordings. These early experiments showed that single biological molecules can be recorded, but in this case, the variety of biomolecules and types of experiments were severely limited to ion conductance measurements. Even in this first experiment of its kind, researchers were able to directly observe opening and closing of membranes channels by tracking ion conductance. Moreover, these results further verified the claims of single channel recording by comparing to similar results derived from bulk measurements. Additionally, these researchers were able to measure total current through a single channel and by collecting multiple independent recordings and gathering them into a histogram, they were able to determine the percentage of one, two, three or more channels transmitting current within a given time period. Single ion recordings were thoroughly analyzed for potential sources of noise that were complicating experimental analysis and after accounting for several of these, the experiment was
expanded allowing for single ion recordings with a higher single-to-noise ratio, lower temporal resolution and an increase in the types of cell membranes that could be analyzed\textsuperscript{2,3}.

For many years, single cells, organelles, and small biological structures have been imaged using optical microscopy. However, standard optical microscopy generally suffers from low contrast and diffraction-limited imaging. Contrast refers to the difference between the brightest elements of an image and its background intensity relative to the background; a low contrast reduces the ability to distinguish details in an image. This is particularly problematic with biological samples that provide low contrast due to low amounts of light absorbed in cells\textsuperscript{4}. This problem can be circumvented by adding a bright molecular probe to the cell or structure that provides extrinsic contrast, which is the basis for fluorescence microscopy. Standard fluorescence microscopy has been used for decades, though in recent years, revolutionary methods for single molecule fluorescence microscopy have been developed.

An optical absorption spectrum of a single isolated molecule was first obtained in 1989 by W. E. Moerner\textsuperscript{5} but required the use of extremely cold temperatures and immobilization in a crystal. In order to import this technique to practical biological systems, it was necessary to greatly reduce background and out-of-focus fluorescence using total internal reflection fluorescence microscopy (TIRF-M) along with improved camera technologies. These improvements enabled single molecule studies of the rotation of ATPase\textsuperscript{6}, direct observation of kinesin proteins moving along microtubules\textsuperscript{7}, and many other biological systems. Despite the nearly immediate pervasiveness of these studies, several experimental challenges remain in applying single molecule imaging to new applications. In particular, major challenges include photophysical issues including low brightness and rapid photobleaching and/or difficulties in labeling and bioconjugation of probes to target molecules of interest. Since the widespread adoption of single
molecule techniques in biology, a wide variety of fluorescent probes have been developed to help address these experiment difficulties.

Fluorescent probes that are currently used for single molecule fluorescence spectroscopy can be coarsely divided into three groups: genetically encoded fluorescent proteins, inorganic quantum dots (QDs), and small molecule organic dyes. Each of these different probes has various advantages and disadvantages, and each have been used in a multitude of studies, which will be summarized in the following sections.

**Fluorescent Proteins**

The archetypal example of a fluorescent protein is the green fluorescent protein (GFP) which was isolated from a jellyfish in 1962\(^8\). The photophysical properties of GFP were greatly improved by introducing a single point mutation (S65T) in the Tsien lab\(^9\), followed by additional attempts at protein evolution and protein discovery. Soon after this development, GFP was imaged at the single molecule level in the Moerner lab\(^10\). Since the discovery of GFP, several protein variants have been developed that span the visible spectrum and respond to pH or redox changes. A major advantage of fluorescent protein derivatives, however, is the ability to be genetically encoded in a cell, which circumvents the need for addition of exogenous fluorescent probes to a cell. Although these proteins have been used in a variety of single molecule experiments \(^{11-13}\), several FPs suffer from low brightness and low photostability, which complicates their use in detecting single molecules in live cells.

**Quantum Dots**

Quantum dots (QDs) are crystals composed of inorganic semiconductor metals that fluoresce with extremely high brightness and effectively infinite photobleaching times. QDs, however, undergo rapid photoswitching (known as blinking) between on/off states that can be mitigated through the
addition of reducing agents such as thiols or other core/shell synthetic approaches\textsuperscript{14,15}. QDs have desirable photophysical properties for single molecule fluorescence imaging such as increased brightness and photostability relative to FPs, however, they have not achieved widespread use in biological experiments for several reasons. A major drawback to using QDs in live cell imaging arises due the potential for biological perturbation due to their relatively large size (>10 nm) that is increased even further through surface passivation with neutral PEG groups or antibodies particularly for live cell imaging.

**Organic Dyes**

Small molecule organic dyes have been widely used in single molecule fluorescence due to their small size. A wide variety of synthetic small molecule probes have been developed with emission wavelengths spanning the visible spectrum, thereby providing versatility in chemical and biological imaging experiments. Due to the requirement for bioconjugation of a single dye to a target biomolecule in order to study activity, the small size (<~1 nm) of most organic dyes makes them less perturbative to the biological function of these molecules when compared with quantum dots. In addition, a large variety of organic dyes have been synthesized for various purposes, including: DNA imaging wherein fluorescence increases by as factor of ~1000x upon probe binding to DNA in solution\textsuperscript{16}, specific organelle labeling of mitochondria, lysosomes, endoplasmic reticulum and others\textsuperscript{17}, along with many other applications.

Despite the many advantages in using organic dyes for fluorescence imaging, there are two main drawbacks preventing applications involving single molecule fluorescence experiments: limited brightness and low photostability. In single molecule imaging experiments, a dim probe will result in a low signal to noise ratio, thereby hampering the localization and tracking of single molecules. Moreover, the low degrees of photostability of many types of organic dyes can severely
limit the timescales of imaging, thereby constraining the types of experiments that can be performed. In addition, many organic dyes exhibit a photophysical phenomenon commonly referred to as “blinking”, wherein a dye molecule rapidly switches between a dark state and a bright state. Taken together, short photobleaching times and dye blinking are collectively referred to as fluorophore instability, and there is a large body of work aimed at minimizing these instabilities to a level that allows for practical usage of organic dyes in biological imaging. This discussion is summarized in Figure 1.1, which shows the types of fluorophores available, relative sizes, and important advantages and disadvantages (including the FDN probes that are the focus of Chapter 2).

During fluorescence excitation, organic fluorophores are known to deviate from ideal behavior by transitioning from an excited electronic state \( S_1 \) to a triplet spin state, \( T_1 \) by intersystem crossing (Figure 1.2). The triplet state is more chemically reactive than the singlet state, so a transition to \( T_1 \) tends to increase the probability of an undesirable chemical reaction in solution causing irreversible photobleaching. In most biological experiments, a common mechanism whereby fluorophores undergo irreversible photobleaching is through reaction of the triplet state with molecular oxygen in solution. Therefore, the most common method used to delay photobleaching in fluorescence imaging experiments is removal of oxygen from the imaging solution, either enzymatically or through degassing. Although this technique delays the rate of dye photobleaching, it paradoxically worsens the blinking problem by increasing the amount of time the dye spends in the \( T_1 \) state. Blinking increases in the absence of oxygen because oxygen also serves as an efficient triplet state quencher; therefore, without oxygen present, periodic blinking of single dyes is often observed, and both of these properties shown in Figure 1.3.
To counteract the blinking issue present in many organic dyes, researchers have developed a host of “protective agents” that can be added to imaging solutions to reduce blinking. These include β-mercaptoethanol\textsuperscript{24}, cysteamine\textsuperscript{25}, N-propyl-gallate\textsuperscript{26} and others that generally work by scavenging radical oxygen species present in solution. Recently, a new class of protective agents based on redox chemistry was introduced, and these are known as reducing and oxidizing systems (ROXS). ROXS systems generally function through the cycle shown in Figure 1.4\textsuperscript{27}. Commonly used protective agents in this class include the combination of ascorbic acid and methyl viologen\textsuperscript{27} or Trolox, where the latter is known to partially degrade in solution to a quinone compound that serves as the redox partner to the reducing Trolox\textsuperscript{19,28}. To date, these compounds have shown tremendous promise in preventing fluorescence instability, but certain drawbacks are associated with them. Many protective agents are toxic or modify biological function\textsuperscript{29}, and because they are in solution and operate through a diffusive collision based mechanism, they must be present at relatively high concentrations to function effectively, which poses a problem because several of these additives are hydrophobic and exhibit poor aqueous solubility.

In this thesis, we study the effect of photostabilizing compounds on the properties of dyes and biological molecules. Our efforts are aimed at overcoming the challenges associated with the poor water solubility and potentially deleterious effects of adding high concentrations of these compounds to biological systems. In Chapter 2, we report a new class of fluorescent dyes bearing multiple dyes and direct covalent attachment of photostabilizers, thereby avoiding the need for addition of photostabilizers into solution. This chapter explores the photophysical properties of ‘self-healing’ probes. In Chapter 3, we investigate how Trolox affects the physical properties of double stranded DNA, which is a model biopolymer using in a wide array of biophysical
experiments. These results demonstrate the need for careful consideration in using redox active photoprotective groups for biological imaging.

1.3: Overall Perspective on Research

_Self-healing Fluorescent Dyes_

In our work, we develop and characterize a new class of multichromophoric fluorescent probes that exhibit self-healing photoprotective properties. Although the concept of covalently attaching redox active photoprotectants is not a completely new strategy for protection, it is a recent advance in the field of fluorescent probe development. Moreover, our work in extending this concept to multi-dye conjugated probes is a new development in the field. In 2011, the concept of ‘self-healing’ dyes was demonstrated in a study where the fluorescent dye Cy5 was covalently conjugated with three separate photoprotective groups, and a photostability parameter was calculated and compared with native Cy5 and Cy5 with the groups in solution (Figure 1.5)

This strategy was generalized to several different dye molecules with fluorescence emission spanning the visible spectrum. Our work extends this strategy to multi-dye conjugates (Chapter 2). In previous studies, ‘self-healing’ dyes generally exhibited enhanced photostability, characterized by the time before photobleaching or average on time. Prior results, however, showed only equal probe brightness between native dye and dye-protectant conjugates. Our work allows for the use of multiple dyes on the same macromolecular unit to increase the brightness while simultaneously achieving improvements in photostability. In addition, we are able to vary the ratios of Trolox to dye, and we observed enhancements in the photostability upon moving to higher ratios of Trolox:dye. Overall, we believe that this work is an important contribution to the field of self-healing fluorescent dyes and represents a step forward towards better probe technology.
**Interactions between nucleic acids and small molecules**

It is known that small molecules, termed intercalators, can directly impact the physical properties of double stranded DNA. Intercalators are small molecules that bind to DNA by stacking in between base pairs, thus it is not surprising that these interactions can alter the physical and/or mechanical properties of DNA. DNA intercalators serve a wide variety of uses including cancer chemotherapy\textsuperscript{32}, but their properties make them particularly suitable as DNA stains or dyes for visualization. YOYO-1 is a widely used DNA intercalator dye that is essentially non-fluorescent when in free solution, but the fluorescence emission intensity increases \(\sim1000x\) upon binding to DNA. A variety of studies have reported increases in the contour length of DNA of about 30-40\% with YOYO-1 binding\textsuperscript{33,34}. To our knowledge, there have been no studies reported on how photostabilizing groups that are commonly used in solution may affect the physical properties of DNA, although a recent study showed that introduction of small amounts of PEG-8000 (1-5\% by mass) into solution with intercalating dyes can reduce the change in physical properties closer to that of native DNA (Figure 1.6)\textsuperscript{35}. In our lab, we use YOYO-1 for single molecule fluorescence observation of DNA and find a qualitative increase in photoprotective properties with the inclusion of Trolox. In Chapter 3, I use single molecule force spectroscopy, in particular magnetic tweezers, to study the influence of Trolox on DNA mechanical properties, both in the presence and absence of Trolox and YOYO-1.

In summary, this thesis reports the use of single molecule fluorescence spectroscopy to study properties of a new class of probes based on a self-healing strategy with Trolox as a photoprotective group. In addition, single molecule force spectroscopy is used to study the influence of the photoprotective molecule Trolox on the mechanical properties of DNA, where it would often be used in solution to provide photostabilization. Overall, the work presented in this
thesis provides an important contribution to the field of single molecule fluorescence imaging, primarily by leveraging the tremendous effect redox active photostabilizing molecules can have on emission properties of fluorescent molecules, and additionally shows that despite the usefulness of such molecules for biological imaging, they can influence the physical properties of biomolecules such as DNA.
1.4: Figures

<table>
<thead>
<tr>
<th>Advantages</th>
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<tr>
<td>bright, stable</td>
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<td>bright, stable can be multicolor</td>
<td>requires chemical conjugation</td>
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<td>easy genetic encoding</td>
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<td>can be bright, large variety of probes</td>
<td>labeling cells can be difficult</td>
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Figure 1.1: Fluorescent probes commonly used in biological imaging: Common advantages, disadvantages, and approximate dimensions of quantum dots (shown here surface passivated with antibodies), fluorescent dendrimer nanoconjugates (new class of probes developed in our lab), fluorescent proteins (3D rendering of GFP shown) and small molecule organic dyes (Cy5 shown here). For comparison, a dye-conjugated antibody is also shown, as this structure is often used for bioconjugate labeling for cell imaging.
Figure 1.2: Photophysical origins of fluorescence phenomenon. Jablonski diagram showing excitation from the ground electronic state $S_0$ to the first electronic excited state $S_1$ by absorption of a photon of the appropriate wavelength. Spontaneous fluorescence emission results in a return back down to the ground state. In some cases, an electron in $S_1$ is able to undergo intersystem crossing (ISC) with low probability to the first triplet state $T_1$ which decays to $S_0$ much slower than fluorescence decay (microseconds or higher compared with nanoseconds).
Figure 1.3: Effect of molecular oxygen on transient fluorescence emission of single Cy5 dyes.

This experiment was performed with single Cy5 dyes tethered to a surface measured with single molecule fluorescence microscopy. (a) With oxygen present, the time before photobleaching is short, although the fluorescence intensity is stable. (b) With oxygen absent, the time before bleaching is longer, albeit with a higher variance in intensity.  

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Figure 1.4: Photophysical mechanism of reducing and oxidizing systems (ROXS). Complex Jablonski diagram showing the influence of ROXS on fluorescence. After intersystem crossing from an excited electronic state $S_1$ to $T_1$, a fluorophore is either oxidized to a radical cation and then reduced to the ground state, or otherwise reduced to a radical anion followed by oxidation. The rate constants leading to a redox cycle are higher than the triplet state decay constant $k_T$, causing the appearance of a stable and non-blinking trajectory $^{27}$. 
Figure 1.5: Photophysical characterization of ‘self-healing’ Cy5 conjugates. The red emitting dye Cy5 was conjugated to the photoprotective molecules cyclooctatetraene (COT), nitrobenzyl alcohol (NBA), and Trolox, and the average time the molecule spent in an ‘on’ fluorescent state was compared with native Cy5 and each of the molecules in solution\(^{30}\).
Figure 1.6: Increased contour length with ethidium bromide intercalation. (a) Schematic showing the increased extension of DNA by an intercalation effect of ethidium bromide. (b) Relative contour length of DNA-ethidium bromide (EtBr) complexes as a function of ligand concentration. Black circles: PBS buffer without PEG; red diamonds: PBS buffer with PEG at 1% in mass; blue triangles: PBS buffer with PEG at 5% in mass.
1.5: References


CHAPTER 2: FLUORESCENT NANOCONJUGATE DERIVATIVES WITH ENHANCED PHOTOSTABILITY FOR SINGLE MOLECULE IMAGING

2.1: Chapter Overview

Fluorescence-based imaging techniques critically rely on bright and photostable probes for precise detection of biological molecules. Recently, a new class of multichromophoric probes based on fluorescent dendrimer nanoconjugates (FDNs) was developed for single molecule fluorescence microscopy (SMFM). FDNs are generated by covalent conjugation of multiple fluorescent dyes onto macromolecular polymeric scaffolds and show marked increases in brightness and long-term photostability relative to their single organic dye constituents. Multichromophoric probes, however, are generally known to suffer from transient fluorescence emission intensities and long excursions into dark states. To overcome these issues, photostabilizers can be added to bulk solution, though some small molecule additives may exhibit poor aqueous solubility or biological toxicity. In this work, we develop enhanced FDN derivatives by covalently linking a redox-active photostabilizer (Trolox) directly onto FDN molecular scaffolds. In one approach, multiple organic dyes (Cy5) and Trolox molecules are randomly distributed on dendritic scaffolds in tunable stoichiometric amounts, and in a second approach, Cy5 dyes are covalently linked to Trolox in a precise 1:1 stoichiometry followed by covalent attachment of Cy5-Trolox conjugates onto dendrimers. In all cases, FDN-Trolox conjugates show increases in photostability, brightness, and reduced fluctuations in transient fluorescent intensity relative to FDN probes. Bulk and single molecule photophysical data for FDN probes are compared to single self-healing dye systems such

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as Cy5-Trolox, and as a proof-of-principle demonstration, we use FDN-Trolox derivatives for bulk immunofluorescence imaging. Overall, our work suggests that self-healed multichromophoric systems such as FDN-Trolox probes present a useful strategy for increasing fluorescent probe photostability.

2.2: Introduction

Single molecule fluorescence techniques allow for the direct observation of biological processes, thereby providing a window into viewing molecular-level phenomena. Fluorescence imaging with high spatial and temporal resolution critically relies on bright and photostable fluorescent probes, which provide increased sensitivity for biological imaging and molecular identification. To this end, advances in molecular probe development have opened up new vistas in single molecule fluorescence imaging, allowing for subcellular events to be visualized at unprecedented spatial resolution in the nanometer size range.\textsuperscript{1–3} Organic dyes such as cyanine family dyes (\textit{e.g.}, Cy3 or Cy5) and near-infrared emitting dyes (\textit{e.g.}, Cy7 or Alexa750) can be used as relatively non-perturbative fluorescent labels due to their small molecular-scale size.\textsuperscript{4,5} However, the vast majority of organic dyes suffers from two major drawbacks: rapid irreversible photobleaching, which limits the effective photon count from single molecules, and transient fluctuations in intensity, including intermittent bright/dark states (\textit{i.e.} blinking),\textsuperscript{6,7} which complicates the study of dynamics at the molecular level.\textsuperscript{8}

Transient conversion into dark states can be caused by several different photophysical and photochemical mechanisms. Organic dyes with internal \(\pi\)-bond conjugation such as the cyanine series have been shown to transition to short-lived (microseconds) intermittent dark states due to \textit{cis/trans} isomerization.\textsuperscript{9,10} In addition, intersystem crossing from an excited singlet (\(S_1\)) to a non-fluorescent triplet state (\(T_1\)) can also result in dark states (microseconds to milliseconds).\textsuperscript{11,12} In
some cases, even longer-lived dark states (milliseconds or longer) have been shown to occur due to oxidation/reduction reactions of dyes in excited states with redox-active partners in solution.\textsuperscript{13} Moreover, the red-emitting dye Cy5 has been shown to exhibit long-lived, reversible dark states via Michael addition with a primary thiol or phosphine, forming an adduct on the polymethine bridge and breaking $\pi$-conjugation, which has proven useful for super-resolution imaging.\textsuperscript{14–17} Nevertheless, potential damage to organic dyes resulting in irreversible photobleaching is generally associated with conversion to triplet states, which has strongly motivated the development of photostabilizers to quench triplet states and improve the performance of organic dyes in solution.\textsuperscript{18}

A general strategy to improve the photostability of organic dyes involves the addition of anti-fading reagents or photostabilizers to solution. These are designed to restore the ground singlet state by quenching triplet states or scavenging radical species to prevent chemical damage to fluorophores. In many cases, oxygen is removed from solution using coupled enzymatic systems such as glucose oxidase-catalase (GOC)\textsuperscript{19} or protocatechuic acid-protocatechuate-3,4-dioxygenase (PCA/PCD),\textsuperscript{20} which prevents the formation of reactive oxygen species (ROS) that can cause irreparable damage to organic dyes. However, oxygen serves as a triplet state quencher, so enzymatic removal of oxygen also tends to increase the conversion into dark triplet states.\textsuperscript{21,22} One of the first protective agents used in single molecule experiments to suppress photobleaching is the reducing agent $\beta$-mercaptoethanol ($\beta$ME).\textsuperscript{23} More advanced protective agents such as triplet state quenchers (TSQs) such as cyclooctatetraene (COT), nitrobenzylalcohol (NBA), or redox-active compounds such as Trolox (TX) or ascorbic acid (AA) and methyl violgen (MV) are typically added to solution as photostabilizing reagents in conjugation with oxygen scavenging systems.\textsuperscript{24–29} To this end, addition of a reducing and oxidizing system (ROXS) such as AA and
MV can be used to effectively quench triplet states and improve the photostability of organic dyes such as Cy5.30,31 However, solution-based addition of TSQs or ROXS requires relatively high concentrations in the millimolar range, which could perturb innate biological function or be toxic to living cells.32

To circumvent potential issues associated with solution-based additives, a series of “self-healing” fluorophore derivatives was recently developed by covalently linking TX, 4-nitrobenzyl alcohol (NBA), or COT directly onto the cyanine dye Cy5.33,34 For these organic dye derivatives, the close proximity of a redox-active species or TSQ increased the local concentration of photostabilizers, thereby promoting molecular collisions and effectively quenching triplet states. Self-healing dye derivatives were observed to exhibit large increases in average time spent in a bright or “on” state, along with increased bulk photostability. The mechanism of self-healing cyanine dyes was subsequently explored using single molecule methods.35,36 In the case of Cy5-TX derivatives, the quenching mechanism is thought to involve the sequential reaction of the triplet state dye with the reducing agent TX to form a radical anion dye and radical cation TX, followed by rapid oxidation of the dye with the TX radical cation. This series of redox reactions regenerates a self-healed ground state dye and TX in a “ping-pong” photocatalytic cycle. The general strategy was extended to self-healing dyes spanning the visual spectrum using a similar conjugation scheme with Cy2, Cy3, Cy3.5, Cy5, Cy5.5 and Cy7.37

Moving beyond self-healing organic dye derivatives, there is a need for development of new fluorescent probes with combined increases in photostability and brightness, thereby allowing for precise fluorescence detection of biomolecules. To this end, a series of probes known as fluorescent dendrimer nanoconjugates (FDN) was recently developed for single molecule imaging.38 FDN probes are multichromophoric organic dye conjugates based on polyamidoamine
(PAMAM) dendrimers, which are macromolecular in size (~2-5 nm) and generally smaller than inorganic quantum dots and exhibit a high degree of water solubility. Dendritic polymers provide a versatile molecular scaffold for direct conjugation of multiple fluorescent dyes (such as Cy3 or Cy5) and chemical moieties for subsequent conjugation and biomolecular labeling. In prior work, FDN probes containing 8 Cy5 dyes showed a ~4x increase in average brightness compared to single Cy5 dyes. In addition, Cy5-FDNs showed a ~6-10x increase in long-term photostability (duration of time to irreversible photobleaching) compared to a single Cy5 dye, thereby providing substantial increases in total photon output over the lifetime of an FDN probe.

Although FDN probes show enhanced brightness and long-term photostability relative to their constituent single organic dyes, FDN probes are multichromophoric systems with close spatial proximity of dyes, which can result in complex photophysics. Multichromophoric systems have been shown to exhibit transient and dynamic fluorescence emission intensities at the single molecule level, including hydrophobic, aromatic dendrimer systems containing multiple fluorescent dyes. Single FDN probes were observed to show transient fluorescence emission, with broad unstructured peaks and long excursions (hundreds of milliseconds) into dark states, which complicates the use of FDN probes in single molecule fluorescence experiments. Interestingly, addition of the redox-active photostabilizer TX into solution (in millimolar amounts) substantially decreased the transient fluctuations in the fluorescence emission trajectories of single FDN probes, which suggests that photostabilizers such as TSQs and redox-active compounds could play a key role in further enhancing the photostability of multichromophoric fluorescent probes despite the complex photophysics.

In this chapter, we report the development of multichromophoric FDN derivatives with covalent attachment of the photostabilizer TX. We synthesized two different versions of these
FDN derivatives: (1) r-FDN-TX probes containing multiple Cy5 dyes and multiple TX molecules randomly distributed on the dendrimer surface, which allows for control over the average stoichiometric loading and ratio between Cy5 and TX, albeit without precise control over the spatial distribution of Cy5 and TX molecules, and (2) c-FDN-TX probes containing Cy5-TX conjugates directly linked to the dendrimer surface, thereby yielding controlled physical spacing between Cy5 dyes and photostabilizer molecules on the dendritic scaffold. Using this approach, we generated a series of r-FDN-TX and c-FDN-TX derivatives, and we characterized the photophysical properties of these multichromophoric probes using single molecule fluorescence microscopy (SMFM). We observe that direct covalent attachment of TX to FDN probes generally stabilizes the transient fluorescence emission of single probes, and we explore the role of the spatial arrangement of dye and photostabilizer molecules on the photostability for FDN-TX probes with comparisons to single Cy5-TX conjugates. Finally, FDN-TX derivatives are used in proof-of-principle immunofluorescence imaging experiments to highlight applications to biological systems, along with specific labeling of DNA for single molecule imaging and detection.

2.3: Materials and Methods

2.3.1 Synthesis of FDN compounds

Amine-reactive Cy5-NHS ester dyes were synthesized as previously described\textsuperscript{44} and directly conjugated to generation-5 and generation-6 (G5, G6) PAMAM-amine terminated dendrimers (Figure 2.1). PAMAM dendrimers nominally contain 128 and 256 surface primary amines, respectively, allowing for facile chemical reaction with a variety of NHS-ester substituents. Dendrimers were labeled with either biotin for biotin-avidin affinity labeling or dibenzocyclooctyne (DBCO) for strain-promoted copper-free click chemistry labeling.\textsuperscript{45,46} For the
indicated samples, an NHS-activated form of Trolox (TX) was prepared and covalently conjugated to dendrimer scaffolds. Alternatively, the NHS-activated form of TX was covalently attached to Cy5 before linking to dendritic scaffolds. After each successive addition reaction and purification, the average degree of substitution is determined using MALDI-TOF mass spectrometry (Tables 2.1 and 2.2).

2.3.2 Photophysical characterization

FDN compounds were characterized using both bulk absorbance/fluorescence emission and single molecule fluorescence imaging. Absorbance spectra were obtained using a Nanodrop UV/Vis spectrophotometer, and emission spectra were obtained with a Cary Eclipse Fluorescence Spectrophotometer (Figure 2.2). Fluorescent probes are linked to glass coverslip surfaces using specific chemical linkages via biotin-avidin affinity labeling or copper free click chemistry. Glass coverslips are first functionalized with PEG/PEG-biotin followed by incubation with Neutravidin. FDN-biotin probes are subsequently incubated directly with PEG/PEG-biotin-Neutravidin surfaces, whereas FDN-DBCO probes are first conjugated with biotin-azide linkers, followed by incubation with functionalized glass surfaces. In addition to surface-based in vitro photophysical characterization, FDNs are used for immunofluorescence imaging experiments and single molecule DNA labeling and imaging. Further details on this process is provided in the following sections.

2.3.3 Single molecule fluorescence microscopy and immunofluorescence imaging.

Single organic dyes and FDN molecules were imaged using an inverted microscope (Olympus IX-71) equipped for objective-type total internal reflection fluorescence microscopy (TIRF-M) using a 100x oil-immersion objective lens (NA=1.40, U PLAN S-APO, Olympus) and an electron multiplied charge coupled device (EMCCD) camera (Andor iXon DU-897). Samples were
illuminated using a solid-state continuous wave laser (Coherent, 637 nm), with laser powers ranging from 2 to 7 mW and with camera exposure time set to 100 ms for all experiments, except for the immunofluorescence data in Figure 5, which used 50 ms for cell imaging. Emission light was separated from excitation light using a 650 nm dichroic mirror (Semrock, FF650-Di01-25x36), followed by 665 nm long-pass emission filter (Chroma, HQ665LP and a band-pass filter (Chroma, HQ700/75) to further reduce background noise in the final image.

2.3.4 Surface immobilization via PEG/PEG-biotin

For in vitro single molecule experiments, all probes (FDN or single Cy5) were specifically linked to PEGylated surfaces using the following procedure. PEGylated glass slides were prepared using a mixture of biotin-PEG-NHS ester (MW 3500 g/mol) and mPEG-NHS ester (MW 5000 g/mol), as previously described. PEGylated coverslips are then assembled into a flow cell by first attaching two strips of double sided sticky tape to a quartz microscope slide to generate a thin channel and then affixing the PEGylated slide on top of the slide, thereby forming a closed chamber (~50 µL in volume) with two openings for inflow and outflow. Once the flow cell was assembled, it is copiously washed with aqueous buffer TE50 (10 mM Tris/Tris HCl at pH 8.0, 50 mM NaCl, 1.0 mM EDTA), followed by incubation with NeutrAvidin (0.1 mg/mL), and washed again with TE50 to remove unbound NeutrAvidin. Biotin-functionalized FDN samples were incubated in a flow cell at ~100 pM for 5 minutes and washed with TE50 to remove unbound FDN. DBCO-functionalized FDNs (G5-7Cy5 and G5-r-7Cy5-18TX) were first mixed with biotin-PEG3-azide (Click Chemistry Tools) at 100 nM FDN and 2 µM biotin-PEG3-azide and then reacted for ~16 hours at 50 °C. These FDN probes are then incubated in a flow cell at ~100 pM for 5 minutes, followed by washing with TE50 to remove unbound FDN.
2.3.5 Single molecule imaging and analysis

Single molecule imaging was performed using TE50 imaging buffer (10 mM Tris/Tris HCl at pH 8.0, 50 mM NaCl, 1.0 mM EDTA) in the presence of an oxygen scavenging system to minimize photobleaching, which consisted of glucose oxidase, catalase, and glucose (0.2 mg/ml, 0.3 mg/ml, 1.0% w/w, respectively). For experiments with βME, we used a concentration of 7 mM in the imaging buffer. Image analysis was performed with custom written scripts in Matlab (R2011b, Mathworks Inc.). For in vitro single molecule experiments, a peak-finding algorithm is used to determine a subset of molecules exhibiting fluorescence emission intensity above a background threshold.

For photophysical characterization results, photon counts are obtained by fitting a 2D Gaussian function to peak intensity values, integrating around the peak and subtracting background values, using a maximum likelihood estimator (MLE) for fitting as previously described. In photobleaching decay curves, the number of photons per frame is summed until photobleaching, with >100 molecules per field of view and at least 5 fields of view per reported average. The number of fluorescent spots per frame is normalized by the initial number, and this fraction is plotted over time (averaged over >5 movies for each sample). To calculate decay constants, each sample decay curve is fit to a single exponential of the form $y = e^{-t/t_0}$, where $t_0$ is the characteristic decay time. To quantify transient stability of intensity trajectories, the root-mean-square values of fluorescence intensity (RMS), essentially the average deviation of intensity about the mean, was chosen as the metric for comparison. We first extract single molecule intensity trajectories from each fluorescent spot in the field of view. Each single molecule trajectory is then scaled to a maximum fluorescence intensity value of 1.0, and then the sum of the squared difference in transient fluorescence intensity is subtracted from the overall scaled mean. The fluorescence
intensity values for each trajectory are considered up until the time of irreversible photobleaching. This process is summarized in Eq. 1.

\[
\text{Normalized RMS} = \sqrt{\left\langle \left( \frac{I(t) - I_{\text{mean}}}{I_{\text{max}}} \right)^2 \right\rangle}
\]

Where \( \langle \ldots \rangle \) denotes an average over all time points until photobleaching, \( I(t) \) is the background corrected intensity value of a particular molecule at time \( t \), \( I_{\text{mean}} \) is the average of \( I(t) \) over all time points until photobleaching, and \( I_{\text{max}} \) is the maximum of \( I(t) \). Each peak in a given movie has a normalized RMS value calculated, these are averaged to give a single RMS value per movie, with the reported RMS values averaged over >5 movies. All error bars reported are standard deviations.

2.3.6 Antibody labeling and immunofluorescence imaging

Anti-GAPDH polyclonal antibodies (Genscript, A00191-4) were reacted with azide-PEG4-NHS ester (Click Chemistry Tools) at a molar ratio of 50:1 (antibody:PEG linker) in order to label surface-exposed lysines with azide. Unreacted azide is removed through dialysis (Thermo, Slide-A-Lyzer MINI 10,000 MWCO), followed by reacting the azide-labeled antibody with either FDN probes or Cy5 at a molar ratio of 10:1 (dye:antibody). For immunofluorescence imaging experiments, *Escherichia coli* cells (DH5α) were grown to log phase in LB media, fixed with formaldehyde (2.5% in solution), and deposited onto a poly-l-lysine coated chamber slide (Nunc). Fixed cells were then permeabilized (1 mg/mL lysozyme, 5 mM EDTA, 0.5% glucose, 0.1% Triton X-100 in TE50 buffer) and washed copiously with a blocking buffer, 2% BSA (Sigma) in PBS (Mediatech, 0.795 g/L Na₂HPO₄, 0.144 g/L KH₂PO₄, 9.0 g/L NaCl). FDN- or Cy5-labeled antibodies were incubated with the cells for 18 hours at 4 °C at a final antibody concentration of 6.6 nM, followed by rinsing with the blocking buffer to remove unreacted dye and unbound antibody. Antibody-labeled cells were then incubated with DAPI nuclear stain (0.5 μg/mL for 5
minutes), followed by rinsing with PBS (Figure 2.3). Imaging was performed on an Olympus IX-71 inverted microscope, with a 40x objective lens (NA=0.9, U PLAN S-APO, Olympus) as described above with the imaging buffer containing PBS and Gloxy. For each sample, three movies were acquired for 100 seconds at a 50 ms exposure time in different fields of view, along with images acquired with a filter set appropriate for DAPI stain (dichroic filter: Chroma-ZT488rdc, emission filter: Semrock- FF03-525/50-25) and a 488 nm laser (SpectraPhysics Excelsior, CW, DPSS) to confirm presence of DAPI-stained DNA as a cellular marker.

Photobleaching decay curves for the immunofluorescence experiments were determined by tracking fluorescence intensity in a subset of pixels above the background as a function of time. Next, a relative intensity per frame is determined by dividing by the initial number of pixels above the background.

2.3.7 DNA labeling and single molecule imaging.

In order to assay for specific conjugation and labeling of FDN-C onto DNA, we used PCR amplification to create 2 identical 1 kilobase pair DNA molecules: one with a terminal azide label, and a second without (termed W-azide and N-azide, respectively), and both with a terminal biotin on the other end to facilitate surface tethering to a coverslip for imaging. For the PCR amplification of target DNA and addition of azide and biotin groups, the following materials were used: λ-DNA as PCR template (New England Biolabs, Ipswich, MA), Taq DNA Polymerase with Thermopol Buffer (New England Biolabs, Ipswich, MA), deoxynucleotide (dNTP) solution (New England Biolabs, Ipswich, MA), custom oligonucleotide primers (Table 2.3, IDT DNA, Coralville, IA), QIAquick PCR Purification Kit (QIAGEN, Valencia, CA), SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Grand Island, NY). The amounts of materials and protocol for the PCR amplification are shown in Table 2.4. Approximate molecular weight of PCR products was confirmed via
agarose gel electrophoresis, and all products were purified using QIAquick PCR Purification Kits. Concentrations of purified products were measured using a NanoDrop UV-Vis spectrophotometer (Thermo Scientific).

To assay for the amount of specific binding with our charge modulated FDNs, we incubated the FDN-C sample with identical DNA strands except for the presence or absence of the specific azide linker under identical incubation conditions. Thus, if we detect the presence of FDNs on both samples through fluorescence microscopy, we would not have achieved specific labeling, and the charge modulation strategy would have failed. However, a successful outcome of the strategy would be indicated by fluorescence emission from the azide terminated DNA with much lower levels of emission from the non-azide terminated DNA, which is only possible if FDN-C has enough electrostatic repulsion from the DNA to only bind specifically through covalent click chemistry linkage to the azide terminated DNA.

2.3.8 Simulated TX:Cy5 ratio on random addition dendrimers.

We simulated the addition of stoichiometric amounts of Cy5 and TX to a large number of dendrimers to obtain distributions of the TX:dye ratio. Each dendrimer is represented initially as vector of $n$ zeros, with each zero indicating an unoccupied surface site ($n$=128 for G5 dendrimers), and with $N$=200,000 dendrimers per simulation. Next, each Cy5 and TX is represented as a 1 or 2 in the vector of dendrimer surface sites for computational identification. The random addition process proceeds by, first picking two random integers, one from 0 to $N$ and the other from 0 to $n$, for each Cy5 and TX to be added (indicating a specific surface site on a specific dendrimer). If a site to be added to is already occupied during the addition process, another random site is chosen. Theoretically, this process should result in Poisson distributions in the number of Cy5 and TX on the ensemble of dendrimers, and this is confirmed in Figure 2.4. In Figure 2.5 we plot the
simulated ratio of TX:Cy5 for r-5 Cy5 10 TX and r-7 Cy5 18 TX, showing an expected distribution centered about the stoichiometric mean, but with variation that would result in distributions in the photophysical quantities that describe each sample. The controlled addition samples have an intrinsic advantage of yielding a TX:Cy5 ratio of 1:1, with no variance.

2.4: Results and Discussion

FDN derivatives were synthesized using two different synthetic strategies for dye stabilization of multichromophoric systems. In one class of FDN probes, multiple Cy5 dyes and multiple TX molecules are covalently conjugated onto dendrimer surfaces with control over the average stoichiometric amount of Cy5 and TX (Figure 2.6). In essence, this method results in a random spatial distribution between Cy5 dyes and TX molecules on the dendrimer surface, and we refer to these probes as r-FDN-TX derivatives. For r-FDN-TX probes, we synthesized samples with a Cy5:TX dye ratio of 1:2.0 and 1:2.7 to assess the effect of increased TX loading on fluorescence emission and photostability. In a second class of FDN-TX probes, the photostabilizer TX is first conjugated to Cy5 dye, followed by direct conjugation of multiple Cy5-TX ‘self-healing’ probes onto the dendrimer surface in variable loadings (Figure 2.7). This second class of multichromophoric probes has a controlled (or local) physical spacing between Cy5 dyes and TX molecules, and we refer to these molecules as c-FDN-TX probes. In addition, biotin and/or DBCO is conjugated to dendrimer probes for surface immobilization or biomolecule labeling, and in some cases, surface charge groups are added to modulate electrostatics. In all cases, the average degree of chemical substitution can be controlled in a fairly narrow range through the reagent-to-dendrimer stoichiometry in a series of sequential chemical conjugation reactions. During synthesis, MALDI-TOF mass spectrometry analysis is used to determine the average degree of chemical
substitution for each reaction step (Tables 2.1 and 2.2), and bulk photophysical properties are characterized via absorption and fluorescence emission spectra (Figure 2.2). Polymeric dendrimers serve as versatile molecular scaffolds for conjugation of multiple fluorescent dyes and chemical groups, thereby allowing for the tailored design and preparation of fluorescent probes with desired properties such as surface charge, dye loading, or enhanced photophysical properties via direct linkage of photostabilizers.

Using this system, we aimed to explore the role of the Cy5:TX loading ratio and physical spacing between Cy5 and the photostabilizer TX on the photophysical properties of FDN probes. It is known that the efficiency of photoinduced redox action is strongly correlated to the distance between TX and a dye molecule. From this perspective, we sought to determine the role of dye-to-photostabilizer ratio on the photophysical behavior of multichromophoric systems using single molecule imaging (Figure 2.8). We pursued this idea using two different approaches. For one class of molecules (r-FDN-TX), we aimed to assess the impact of increasing the loading of the photostabilizer TX on Cy5 dye emission using average stoichiometric ratios. Moreover, we also probe the role of stabilization due to putative dye-dye interactions in the absence of TX compared to photostabilization due to increasing TX loadings on a multi-dye dendrimer construct. In a second class of molecules (c-FDN-TX), we aimed to determine the impact of increasing the loading of Cy5-TX heterodimers on the photophysical properties of FDN derivatives, where the local physical spacing between dye and TX is fixed. Indeed, the photophysical properties of self-healing Cy5-TX molecules have been studied previously, but it is unknown how these results map onto multichromophoric systems in terms of increased brightness and enhanced photostability.
2.4.1 Brightness characterizations

We first consider probe brightness for a selected variety of synthesized probes. Characteristic single molecule intensity plots for a handful of selected probes are shown in Figure 2.9 which includes diffraction limited images of single Cy5, single Cy5-TX, and G6-c-10(Cy5-TX) under identical illumination conditions. Here, we observe similar fluorescence intensities for single Cy5 and single Cy5-TX indicating that Trolox conjugation does not cause an appreciable change in probe brightness. However, we show a ~4-5x increase in intensity for G6-c-10(Cy5-TX) relative to single dyes, consistent with prior data on FDNs without covalent coupling of photostabilizers.\(^3^8\)

The total number of accumulated photons before irreversible photobleaching is shown for all samples in Figure 2.10, which reveals several interesting features for these fluorescent probes. In all cases, covalent attachment of TX leads to increases in total photon output compared to the parent “non-healed” version of the probe. Furthermore, we find a larger increase in total photon output for r-FDN-TX probes with a 1:2.7 ratio of Cy5:TX (relative to the parent r-FDN probe without TX) compared to r-FDN-TX probes with a 1:2.0 ratio Cy5:TX. These results suggest that increasing the loading of TX in r-FDN-TX probes increases total photon output for a given system, presumably by decreasing the physical spacing between dye and photostabilizer. Interestingly, we find an insignificant difference in the photon output for G6-3Cy5 compared to G6-8Cy5 probes, which suggests that dye-dye interactions and quenching likely play a major role only at very high dye loadings in this system. By creating “self-healed” versions of FDN probes such as G6-c-3(Cy5-TX) and G6-c-10(Cy5-TX), we observe a nearly ~2x increase in the number of accumulated photons before bleaching. Importantly, these results suggest that dye-dye quenching can be suppressed in multichromophoric systems by control over the spatial arrangement of dye and photostabilizer, leading to enhanced brightness and photon output.
2.4.2 Photostability characterizations

We next characterized the photobleaching behavior of these fluorescent probes using single molecule imaging (Figure 2.11). For these experiments, we immobilize the indicated molecule on a glass coverslip surface using specific chemical linkages and image the sample under continuous illumination. Next, we use image analysis software to localize and detect the number of fluorescent molecules as a function of time. In Figure 2.11.a, we plot the “active” fraction of fluorescent probes for the random addition samples (r-FDN) as a function of time, along with single Cy5 for comparison. Similarly, Figure 2.11.b, shows transient photobleaching behavior for the controlled addition samples (c-FDN) compared to single Cy5 and single Cy5-TX. To quantify the photobleaching behavior in the previous figures, the transient fluorescence trajectories are fit to a single exponential decay in order to obtain a characteristic decay time. In order to compare the photophysical properties of probes independent of illumination conditions, photobleaching decay times are normalized by the characteristic decay time for single Cy5 in each experiment, which effectively serves as an internal reference for these samples. Figure 2.12 shows normalized characteristic photobleaching times for all samples. We find that the characteristic decay times for G5-r-5Cy5-10TX and G5-r-7Cy5-18TX are much larger than their “non-healed” counterparts, with a larger increase in the 1:2.7 Cy5:TX sample compared to the 1:2.0 Cy5:TX sample, as expected. Moreover, the samples with the highest degrees of photostability are probes with “controlled” physical spacing between dye and Trolox, including single Cy5-TX, G6-c-3(Cy5-TX), and G6-c-10(Cy5-TX). Here, we observe nearly equal characteristic decay times for single Cy5-TX heterodimers or controlled structure c-FDN-TX probes. Based on these data, it appears that FDNs bearing multiple “self-healed” Cy5-TX probes show an increase in total photon output
(or total probe brightness), albeit with a characteristic photobleaching time similar to single self-healed Cy5-TX probes.

The observation that FDNs with controlled structures bearing multiple Cy5-TX molecules exhibit similar photobleaching behavior compared to Cy5-TX heterodimers likely indicates that Trolox dominates the photophysics due to the high local concentration of TX. For this reason, FDNs with controlled structures behave similarly to Cy5-TX heterodimers in terms of time before irreversible photobleaching. Moreover, the total photon output can be increased in FDNs by conjugating multiple Cy5-TX dimers on dendrimer scaffolds, which yields an overall increased brightness per probe. In the case of c-FDN-TX probes and Cy5-TX heterodimers, the enhancements in photostability are achieved due to the photoprotective effects of the Trolox molecule. Interestingly, in the absence of photoprotectants on FDN scaffolds, there is an apparent modest increase in photostability as evidenced by an increase in time to photobleaching for FDN probes bearing multiple dyes and without Trolox compared to Cy5. We hypothesize that dye-dye interactions could give rise to this apparent increase in photostability. In particular, it is possible that the close proximity of multiple dyes may give rise to weak excitonic coupling between dyes on the underlying dendrimer scaffold, which could explain these effects. Nevertheless, the increase in photostability provided by covalent addition of Trolox vastly improves photostability and the photophysical properties of dye-conjugated probes.

To further characterize the behavior of these probes, we studied the photobleaching behavior of a subset of samples under different solution conditions (Figure 2.13). Unless otherwise stated, the imaging buffer consists of an enzymatic oxygen removal system (glucose oxidase/catalase, Gloxy) with no reducing agent (beta-mercaptoethanol, βME) in solution. In a series of experiments, we characterized the photophysical properties of several fluorescent probes
in the presence or absence of Gloxy and βME, specifically +/-Gloxy and +/-βME. A particularly interesting result arises from the -Gloxy/-βME condition, where we observe a nearly ~2x increase in the characteristic photobleaching decay time for G6-c-10(Cy5-TX) compared to single Cy5-TX. Therefore, in the presence of oxygen, c-FDN-TX probes appear to exhibit a modest increase in photostability relative to single Cy5-TX probes. The increase in photostability for c-FDN-TX probes compared to single Cy5-TX in the presence of oxygen (-Gloxy/-BME) could arise due to modest protection from the underlying dendrimer scaffold, which could aid in preventing oxygen damage in the context of dendrimer probes.

Aside from brightness and long-time photobleaching, transient variance in fluorescence emission is a key property for single molecule imaging experiments. In particular, it is highly desirable to achieve stability in transient fluorescence emission intensity for applications such as single molecule particle tracking, colocalization, or quantitative bioassays such as single molecule protein pull down (SiMPull). In Figure 2.14 we show representative single molecule intensity trajectories for a subset of fluorescent probes. Comparing Figures 2.14.a to 2.14.b we observe much higher stability in Cy5-TX over Cy5. In Figure 2.14.c we see some level of stabilization of G5-r-5Cy5 compared with Cy5, due either to dye-dye interactions or inherent stabilization due to the polymer scaffold. Finally in Figure 2.14.d, we see higher levels of stabilization by addition of Trolox into the structure. In order to quantify local transient photostability, we calculated the normalized root-mean-square deviation from the mean (RMS) of single molecule intensity until irreversible photobleaching, such that a lower value indicates a lower variation in intensity. The results are shown in Figure 2.15 for the two classes of self-healing dyes in standard buffer conditions (+Gloxy/-βME). As expected, we observe a large increase in RMS for single Cy5 compared to single Cy5-TX conjugates, which validates this approach for
studying local transient photostability. For r-FDN-TX probes, we see increased levels of stabilization with higher loadings of Trolox, as expected (a difference of ~10% for G5-5Cy5 over G5-r-5Cy5-10TX compared with ~30% for G5-7Cy5 over G5-r-7Cy5-18TX). Moreover, the controlled structure probes G6-c-3(Cy5-TX) and G6-c-10(Cy5-TX) show only a modest stabilization in local transient fluorescence compared to the parent FDN probes without Trolox. The fairly modest enhancement in photostability for the c-FDN-TX probes can be explained in part by inherent photostabilization of dyes due to the underlying polymeric scaffold or due to dye-dye interactions, which has been observed previously. 49,50

2.4.3 Photophysical property comparisons with Trolox in solution

In addition, we compared the photophysical performance of dendrimer probes with covalently linked Trolox to dendrimer probes without covalent TX both in the presence and absence of Trolox in solution (Figure 2.16). In particular, these results include characterization of the total number of accumulated photons before photobleaching, the non-bleached fraction of molecules, and the normalized RMS fluctuation value for the FDN samples with a 1:2 dye:TX labeling ratio (including the control sample with no covalent Trolox on the dendrimer). Our results show nearly identical behavior for total number of accumulated photons and time before irreversible photobleaching for dendrimer probes with covalently linked Trolox and dendrimer probes without covalent TX but with 2 mM Trolox in solution. For these samples, we generally observe a slight decrease in transient fluctuations in fluorescence upon addition of Trolox to solution. These results demonstrate that increasing the local concentration of photoprotectants via covalent conjugation greatly improves photophysical performance, which can be advantageous for experiments wherein addition of large amounts of Trolox or photoprotectant to bulk solution is not possible.
2.4.4 Proof-of-principle biological experiments

Moving beyond in vitro photophysical characterization of FDN-Trolox derivatives using SMFM, we performed several proof-of-principle imaging experiments using these probes, including bulk immunofluorescence imaging and single molecule DNA labeling experiments. In a first set of experiments, we used FDN-Trolox probes in immunofluorescence imaging of fixed bacterial cells (Escherichia coli). Here, we targeted glyceraldehyde phosphate dehydrogenase (GAPDH), an oxidoreductase and key enzyme in glycolysis that catalyzes the conversion of glyceraldehyde 3-phosphate (GADP) to D-1,3-bisphosphoglycerate (1,3BPG) using NAD$^+$ as an electron acceptor. In these experiments, we aimed to characterize the long-term photostability of FDN derivatives for bulk immunofluorescence imaging by labeling anti-GAPDH primary antibodies with single Cy5 dye, G5-7Cy5, and G5-r-7Cy5-18TX (Figure 2.17).

Immunofluorescence imaging results are shown in Figure 2.17.a as a function of time, along with images of cellular DAPI stain in the same field of view, which properly overlays with the immunofluorescence images and serves as an internal reference for cells. Transient fluorescence intensities during imaging under constant illumination are shown in Figure 2.17.b. First, these results demonstrate that FDN-TX probes can be used to label and image antibodies using a standard immunofluorescence protocol. Characteristic photobleaching trajectories show that anti-GAPDH primary antibodies labeled with FDN-Trolox derivatives generally exhibit an enhanced photostability compared to primary Abs labeled with Cy5 or FDN probes without covalent attachment of Trolox, which is consistent with the results in Figure 2.12. Generally speaking, this increased photostability would allow additional biological or chemical information to be extracted from immunofluorescence experiments using this set of probes. To further quantify photostability in this experiment, we determined the characteristic photobleaching decay time
constants for G5-r-7Cy5-18TX and G5-7Cy5 (Figure 2.17.c), including a comparison to the in vitro photobleaching experiments shown in Figure 2.12. We observe only a very small difference between the data gleaned from both experiments, which further suggests that FDN probes can be confidently used in immunofluorescence imaging experiments.

Specific labeling of target biomolecules is highly desirable for biological imaging applications. In certain cases, however, specific labeling can be challenging due to electrostatic interactions. For example, in prior work, we labeled DNA oligonucleotides with FDNs containing terminal amine groups, which bear a nominal positive charge at neutral pH. In this case, specific labeling was accomplished by addition of high salt and a non-specific blocking agent to the reaction buffer, thereby screening electrostatic interactions between the positively charged FDN probes and negatively charged DNA, which otherwise results in non-specific binding. To circumvent these requirements and to allow for facile specific labeling in a simple buffer, we synthesized FDN samples with variable surface charge to suppress electrostatic interactions between FDN probes and target DNA. Specifically, we synthesized FDNs with terminal carboxyl groups imparting a negative surface charge at neutral pH to impart an electrostatic repulsion to DNA. Using this strategy we successfully labeled DNA with carboxyl terminated FDNs with the results shown in Figure 2.18.

2.5: Conclusions

In this work, we expand the use of “self-healing” fluorophores from single organic dyes to multi-dye dendrimer nanoconjugates, which offers a new set of bright and photostable probes for single molecule fluorescence microscopy and general immunofluorescence imaging. Using two complementary synthetic strategies, we synthesized self-healing FDN probes with either average
stoichiometric loading (or random spatial distribution) of Cy5 and Trolox molecules on the dendrimer surface, or with direct covalent attachment of multiple “self-healing” Cy5-Trolox conjugates. In the first strategy, we are able to modulate the average stoichiometric loading of Trolox (per dye), which leads to enhanced fluorescence stabilization upon increasing the Trolox to dye ratio. However, this strategy affords essentially no control over the spatial arrangement of dye or photostabilizer substituents, which precludes absolute control over the distance between fluorophore-protectant pairs. In addition, we only control the average stoichiometry per dendrimer using this synthetic method, which leads to distributions in the ratio of dye:TX (Figures 2.4 and 2.5).

In an alternative strategy, we synthesized multichromophoric dendrimers bearing Cy5-Trolox conjugates. Interestingly, these fluorescent probes emit a large number of total photons before irreversible photobleaching, though they exhibit characteristic photobleaching times comparable to single Cy5-TX molecules, implying that a single self-healed Cy5-TX conjugate achieves a maximal amount of photostabilization, albeit only with the brightness of a single dye. In particular, G6-c-10(Cy5-TX) is an FDN probe bearing multiple Cy5-TX conjugates that shows a very large photon output, which could find applications in single molecule particle tracking experiments, allowing for precise, long-term localization for a biomolecules. However, despite the advantages of multichromophoric FDN-TX systems, it should be noted that dendrimer-based probes are larger in size (~5 nm) compared to single organic dyes (~1 nm).

Prior studies have examined the role of covalently linked Trolox on single fluorophores, whereas our work extends this approach to multichromophoric systems with enhanced brightness. Our results appear to be consistent with the “ping-pong” mechanism attributed to the photoprotective action of Trolox covalently localized to a single dye. Here, the triplet state of a
dye is reduced by an adjacent Trolox molecule, followed by rapid reoxidation of the dye with the radical Trolox cation. This “self-healing” mechanism critically depends on the local proximity between the two molecular species. Indeed, our results show increased photoprotection upon increasing the dye:TX ratio on random addition FDN probes (r-FDN-TX probes). However, it is possible that a combination of photostabilization dye to TX and dye-dye interactions results in increased photoprotection in the 1:2.7 dye:TX sample. On the other hand, the controlled addition samples (c-FDN-TX) allow for independently changing dye loading while maintaining a constant TX:dye ratio and a constant (local) physical spacing between dye and Trolox. For these samples, we generally observe an increase in the total number of accumulated photons due to an increased dye loading, despite minor differences between the photostability for G6-c-3(Cy5-TX) and G6-c-10(Cy5-TX) samples.

In conclusion, we synthesized and characterized two distinct classes of “self-healing” multichromophoric dendrimers. By linking Trolox directly to an organic dye before covalent attachment to a dendrimer, we avoid distributions in the ratio of TX:dye with control over the average stoichiometric loading of Cy5-TX heterodimers on dendritic scaffolds. This synthesis scheme inherently leads to variations in photophysical properties within a batch of fluorescent probes, which precludes use in applications such as single molecule Forster resonance energy transfer (smFRET) requiring quantitative knowledge of dye number and spatial distribution. In future work, these issues can be overcome by designing a structurally-defined multi-dye probe system allowing for precise control over dye attachment in the absence of the underlying dendritic scaffold.
2.6: Tables and Figures

<table>
<thead>
<tr>
<th></th>
<th>v (Cy5)</th>
<th>w (Trolox)</th>
<th>x (biotin)</th>
<th>y (DBCO)</th>
<th>z (carboxyl)</th>
<th>Mn, Mw</th>
<th>PDI</th>
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<tr>
<td>G5-r-5Cy5</td>
<td>5</td>
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<td>0</td>
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<td>70</td>
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Table 2.1: Stoichiometry of substituents for random addition samples (r-FDN).

Mn denotes number average molecular weight, Mw denotes weight average molecular weight, and PDI (=Mw/Mn) is the polydispersity index. This table denotes the average stoichiometry of substituents on each of the random addition samples studied in Chapter 2, along with data on molecular weight averages.
<table>
<thead>
<tr>
<th></th>
<th>( i )</th>
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<th>( k )</th>
<th>( M_n, M_w )</th>
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<td>G6-3Cy5</td>
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Table 2.2: Stoichiometry of substituents for controlled addition samples (c-FDN).

Average stoichiometry of substituents on controlled addition samples is shown, along with data on molecular weight averages.
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<tr>
<th>Sample Description</th>
<th>Target length</th>
<th>Nucleic acid sequence (5’→3’)</th>
<th>Modification</th>
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<tr>
<td>W-azide-forward primer</td>
<td>951 bp</td>
<td>GACAGCGTACAGCCCCGTTCA</td>
<td>5’-azide</td>
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<tr>
<td>N-azide-forward primer</td>
<td>951 bp</td>
<td>GACAGCGTACAGCCCCGTTCA</td>
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</tr>
<tr>
<td>W-azide and N-azide-reverse primer</td>
<td>951 bp</td>
<td>TCGCGTCATTCATCCTCTCC</td>
<td>5’-biotin</td>
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<th><strong>Table 2.3: Custom oligonucleotide primers.</strong></th>
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<th>Nucleic acid sequence (5’→3’)</th>
<th>Modification</th>
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<td>951 bp</td>
<td>GACAGCGTACAGCCCCGTTCA</td>
<td>5’-azide</td>
<td></td>
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<td>N-azide-forward primer</td>
<td>951 bp</td>
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<td>--</td>
<td></td>
</tr>
<tr>
<td>W-azide and N-azide-reverse primer</td>
<td>951 bp</td>
<td>TCGCGTCATTCATCCTCTCC</td>
<td>5’-biotin</td>
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Targeted sequence was designed using VectorNTI software. Primer sequences used to design DNA scaffolds using PCR for the DNA labeling and imaging experiment (**Figure 2.18**).
<table>
<thead>
<tr>
<th>Reagents - 50 µL total volume per sample</th>
<th>PCR step</th>
<th>Temp. / Time</th>
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<tr>
<td>1X Thermopol buffer</td>
<td></td>
<td>95 °C / 30 sec</td>
</tr>
<tr>
<td>0.25 uL Taq DNA Polymerase</td>
<td></td>
<td>95 °C / 15 sec</td>
</tr>
<tr>
<td>200 nM Wazide-forward or Nazide-forward</td>
<td></td>
<td>60 °C / 30 sec</td>
</tr>
<tr>
<td>200 nM Wazide or Nazide-reverse</td>
<td>*Extension</td>
<td>68 °C / 60 sec</td>
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<tr>
<td>20 ng λ DNA</td>
<td>Final Extension</td>
<td>68 °C / 120 sec</td>
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<tr>
<td>200 uM dATP, dCTP, dGTP, dTTP</td>
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</table>

*cycle steps 30x.

**Table 2.4: PCR Protocol**: PCR protocol followed to create the identical DNA strands (except for the presence or absence of the linker) for the DNA labeling and imaging experiment (Figure 2.18).
Figure 2.1: Structure of PAMAM dendrimer. Full chemical structure of a G5 PAMAM dendrimer with 8 Cy5 molecules attached. For simplicity, the structure is shown with only dye attachment and without further modifications such as DBCO, biotin, Trolox, or carboxyl groups.
Figure 2.2: Absorbance and fluorescence emission spectra. (a) Absorbance and (b) fluorescence emission spectra for FDN compounds. No significant shift in absorbance or emission is observed for probes upon Trolox conjugation.
Figure 2.3: Antibody labeling and cellular incubation. Schematic showing the protocol for labeling antibodies against GAPDH with FDN probes, followed by incubation in fixed *E. Coli* cells for imaging on the microscope.
Figure 2.4: Simulated distributions of Cy5 and Trolox number per dendrimer. Histograms of Cy5 and Trolox per dendrimer obtained from simulations of the random addition process, along with curves of a Poisson distribution of the given mean. (a) and (b) simulated addition of r-5 Cy5 10 TX. (c) and (d) simulated addition of r-7 Cy5 18 TX.
Figure 2.5: Simulated distribution of the ratio of TX:dye over the random addition samples.

(a) Histogram of simulated TX:dye ratio for the sample r-5 Cy5 10 TX. (b) Histogram of simulated TX:dye ratio for the sample r-7 Cy5 18 TX.
Figure 2.6: Schematic chemical structure for ‘random addition’ FDNs. Chemical structure of the r-FDNs with labels indicated for stoichiometric amounts of Cy5, Trolox, biotin, DBCO and carboxylic acid groups. The values for these substituents are shown in Table 2.1.
Figure 2.7: Schematic chemical structure for ‘controlled addition’ FDNs. Chemical structure of the c-FDNs with labels indicated for stoichiometric amounts of Cy5, Cy5-Trolox conjugates, and biotin groups. The values for these substituents are shown in Table 2.2.
Figure 2.8: Schematic of single molecule fluorescence microscopy experiment. The probes are specifically linked to a glass coverslip through biotin-Neutravidin affinity binding. The surface is protected from non-specific binding through PEGylation of the surface.
Figure 2.9: Single molecule characterization of fluorescent probe brightness. Single molecule images and fluorescence intensity plots for single Cy5, single Cy5-TX, and G6-c-10(Cy5-TX), respectively (scale bar 1 μm). Illumination intensity is .5 kw/cm².
Figure 2.10: Total accumulated photons for all synthesized samples. Total accumulated photons for all samples in the presence of glucose oxidase/catalase and absence of reducing agents. For these data and subsequent bar plots, error bars are calculated as the standard deviation of experimental data, and averages are calculated over > 5 independent fields-of-view in the each sample.
Figure 2.11: Photobleaching decay curves for all samples. (a) Active fraction of molecules before irreversible bleaching for single Cy5 and the random addition samples (r-FDN and r-FDN-TX) under constant laser illumination (637 nm, 0.5 kW/cm²). (b) Active fraction of molecules before bleaching for single Cy5, single Cy5-TX, and controlled addition samples (c-FDN and c-FDN-TX) under a constant illumination (637 nm, 2 kW/cm²).
Figure 2.12: Normalized photobleaching decay constants for all samples. Time constants obtained from a single exponential fit of fluorescent probe samples from Figure 2.11(a) and (b), normalized by the characteristic time constant for single Cy5 in +Gloxy/-BME buffer conditions.
Figure 2.13: Photobleaching decay constant across variable buffer conditions. Characteristic photobleaching decay constants for selected samples in four different buffer solution conditions. +/- gloxy (where gloxy is an enzymatic oxygen removal system, so equivalent to +/- oxygen in solution) and +/- β-mercaptoethanol, a commonly used reducing agent to provide additional photostability in many fluorescence based assays.
Figure 2.14: Single molecule trajectories for selected samples. Fluorescence intensity under continuous illumination plotted for (a) single Cy5, (b) single Cy5-TX, (c) G5-r-5Cy5 and (d) G6-c-10(Cy5-TX).
Figure 2.15: Normalized root mean square error. Fluctuations for all samples characterized by the normalized root mean square error over all the traces calculated with Eq. 1.
Figure 2.16: Photophysical properties of selected probes compared with Trolox in solution.

The photophysical properties of (a) accumulated photons, (b) time before irreversible photobleaching and (c) Normalized RMS for selected ‘self-healed’ probe (G5-r-5Cy5-10TX) and its control with no Trolox (G5-r-5Cy5) compared with no Trolox in solution to 2mM Trolox in solution.
Figure 2:17: Bulk immunofluorescence imaging in *Escherichia coli* using FDN-labeled antibodies. (a) Immunofluorescence images of *E. coli* cells under identical illumination conditions as a function of time. For reference, a nuclear DAPI stain is shown. Scale bar: 10 μm. (b) Relative fluorescence intensity of immunolabeled cells shows transient photobleaching. (c) Photobleaching time constants from a single exponential fit for G5-7Cy5 and G5-r-7Cy5-18TX from *in vivo* immunofluorescence experiments and *in vitro* experiments shown in Figure 2.12.
Figure 2.18: Single molecule DNA labeling. Single molecule DNA labeling and imaging using FDN probes. (a) Schematic of DNA hybridization and localization. FDN is incubated with DNA with or without a terminal azide and immobilized on a coverslip for imaging. (b) Single molecule images and average number of localizations over multiple trajectories for each sample. Scale bar: 5 µm.
2.7: References


CHAPTER 3: CHARACTERIZING THE ROLE OF PHOTOSTABILIZERS ON THE PHYSICAL PROPERTIES OF DNA

3.1: Chapter Overview

Trolox (TX) has garnered widespread use as a photostabilizing agent in single molecule fluorescence imaging due to its ability to increase photostability of fluorescent dyes. Despite the popularity of TX in single molecule fluorescence experiments, the role of its interactions with biomolecules such as DNA has not been fully characterized. In this work, we use several different single molecule techniques to study the impact of TX on the physical properties of DNA and to probe the use of TX as a photoprotectant for fluorescently labeled DNA. Using single molecule force spectroscopy (SMFS), we found that addition of TX results in an increase in the contour length of double stranded DNA, which is a similar result observed for nucleic acid intercalating dyes such as YOYO-1. Interestingly, we also observe a reduced degree of DNA stretching in a buffer containing both YOYO-1 and TX compared to a buffer containing YOYO-1. Based on these results, we conjecture that TX and YOYO-1 competitively intercalate DNA. We also performed a series of direct single molecule fluorescence microscopy (SMFM) experiments, wherein we observe single branched and linear DNA-based polymers stretching in extensional flow generated in a microfluidic device. We also characterize single molecule stretching and relaxation dynamics of lambda phage DNA (λ-DNA) in the absence and presence of TX using SMFM. In the presence of TX, we observe a change in steady-state extension that is consistent with measurements from SMFS. Application of a TX-dependent length correction results in single polymer relaxation and

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2 This chapter is adapted from Mai DJ*, Reilly DT*, Cuculis LW, Schroeder CM. (2016). The impact of Trolox on the physical properties of DNA. In preparation. * These authors provided equal contribution
steady-state extension behaviors consistent with the existing literature. Overall, these results demonstrate the utility of TX as a photoprotectant for DNA molecules labeled with Cy5 and YOYO-1, however, our results highlight the importance of understanding the role of photoprotectants on the physical properties of DNA.

3.2: Introduction

Single molecule techniques have enabled the direct observation of molecular-level phenomena, thereby revealing unprecedented levels of detail in physical processes in biology, chemistry, and materials science.\(^1\)\(^-\)\(^6\) Recent advances in single molecule fluorescence imaging have focused on the implementation of photostabilizing agents (PAs) to increase imaging time by delaying photobleaching and improving other photophysical properties. The most common approach to enhancing photostability is the inclusion of solution additives, such as oxygen scavenging systems,\(^7\)\(^,\)\(^8\) triplet state quenchers,\(^9\)\(^,\)\(^10\) and reducing and oxidizing systems.\(^11\)\(^-\)\(^13\)

PAs offer several advantages to single molecule and general fluorescence imaging, though their effects can result in complex photophysics. A widely implemented class of PAs used in fluorescence imaging is enzymatic systems for oxygen removal, which reduce oxygen concentration in solution by approximately 3 orders of magnitude. Since molecular oxygen is the primary agent responsible for photobleaching, this strategy results in a much more photostable probe and a large increase in time before irreversible photobleaching, and very few SMFS experiments are carried out in the presence of oxygen since their introduction. Implementing this technique of oxygen removal is not without its own problems however, since oxygen serves as an effective triplet state quencher, reducing the time the fluorophore spends in the dark triplet state.
Without any quenching of the triplet state, the fluorescence process of a single molecule manifests as visually distinct ‘blinks’ or repetitive on/off cycling.

An early solution to this problem was an alternative PA, \( \beta \)-mercaptoethanol (\( \beta \)ME), which serves as a triplet state quencher and a scavenger of reactive oxygen species\(^{14} \). This reduces the blinking problem present when oxygen is removed from solution and can further extend the time before irreversible photobleaching. Recently, more advanced additives for photostability, termed redox active PAs have been introduced. These function by reducing (or oxidizing) the dye from a triplet state, and correspondingly oxidizing (or reducing) down to the ground state for sustained fluorescence. This redox process proceeds very quickly and results in stable single molecule intensity trajectories.

The photoprotectant Trolox (TX) likely operates through a redox mechanism and has garnered widespread use for its favorable effects on photophysical fluorescent properties,\(^ {12,13,15} \) but there are some apparent disadvantages to its use. It exhibits poor solubility in water, low cell membrane permeability, and has been shown to affect biological function in cells.\(^ {15} \) Furthermore, TX and other hydrophobic PAs are known to change the physical properties of cell membranes, specifically decreasing the bending stiffness of lipid bilayers.\(^ {16} \) As shown in Chapter 2, self-healing dyes represent one approach to avoid this problem\(^ {47} \), however, these materials may require complex syntheses. Furthermore, when studying single polymers with fluorescence, it can be difficult to find appropriate chemical linkers to allow self-healing dyes to be used. Moreover, there are at present no ‘self-healing’ versions of DNA intercalating dyes, which are most commonly employed in single polymer fluorescence studies. For these reasons, PAs are oftentimes employed in solution.
Despite recent widespread use of TX in single molecule imaging experiments, the impact of TX in solution on the physical properties of DNA is not fully known. DNA is a biomolecule of particular interest, not only encoding the genetic information of life, but also exhibiting interesting physical phenomena across relevant length scales spanning orders of magnitude. These scales range from nanometer-length DNA oligomers studied by single molecule fluorescence resonance energy transfer (smFRET)\textsuperscript{17} to micron-length concatemers of λ-DNA and longer genomic DNA fragments revealing hysteretic conformational behavior of polymers.\textsuperscript{18} From this perspective, DNA serves as model polymer system to study physical properties of polymers in non-equilibrium fluid flow. In this chapter, we study the dynamics of DNA under extensional stretching forces, and we examine how these physical properties can vary when PAs are added to the imaging buffer solution.

Several single polymer experiments rely on the use of fluorescently labeled double-stranded DNA.\textsuperscript{19} YOYO-1 is a dimeric cyanine dye that exhibits a greater than 1000-fold increase in fluorescence upon forming a complex with DNA.\textsuperscript{20} YOYO-1 is widely employed for single molecule fluorescence imaging of DNA,\textsuperscript{18, 21-25} despite the susceptibility of YOYO-labeled DNA molecules to photodamage.\textsuperscript{26} TX is known to reduce DNA damage in physiological systems\textsuperscript{27-29} and act as a photoprotectant for cyanine dyes (as discussed in Chapter 2).\textsuperscript{15} For these reasons, we sought to probe the use of TX as a general photoprotectant for YOYO-labeled DNA and to further examine the effect of TX on the contour length and persistence length of double stranded DNA. Moreover, we extend the use of TX to a new class of topologically complex or branched DNA-based polymers known as comb polymers,\textsuperscript{30, 31} which contain alternate cyanine dyes on the branches that are well known to benefit from the photostabilizing properties of TX.
YOYO-1 is known to impact the physical properties of DNA, specifically increasing DNA contour lengths by ~30%. The effect of YOYO-1 on the persistence length of DNA has been less clear in the literature, with results dependent on the study and technique used, and a recent paper used AFM and found no significant change. It is important to understand the role that TX has on the physical properties of DNA to further our comprehension of solution additive induced changes in the physical and mechanical properties of DNA. In addition, for the sake of consistency, understanding the effects of TX on the physical properties of DNA are important for comparison of new results to existing literature. We also note that TX is commonly employed in studies where smFRET is utilized as a high-precision technique to measure nanometer scale distances within and between nucleic acids and proteins. It is therefore critical to understand and accurately measure changes in the physical length of DNA due to TX using a high-resolution single molecule technique, such as magnetic tweezers.

In this work, we utilize single molecule force spectroscopy (SMFS) to measure the impact of TX on the physical properties of DNA, and we observe competing effects between TX and YOYO-1 on DNA contour length. Separately, we use single molecule fluorescence microscopy (SMFM) to image fluorescently labeled DNA molecules in the absence and presence of TX. We demonstrate the effectiveness of TX as a photoprotectant, as well as report a change in steady-state extension of stretched DNA molecules in flow, which is consistent with measurements from SMFS. Application of a TX-dependent length correction results in single polymer relaxation and steady-state extension behaviors consistent with those reported throughout single DNA polymer literature. Our results suggest that the molecular-level effects of TX on biological and physical systems should be carefully considered, from nucleic-acid-binding proteins to molecular-scale rulers to topologically complex polymers.
3.3: Materials and Methods

3.3.1 DNA synthetic procedure

DNA synthesis for single molecule force spectroscopy. All DNA molecules are synthesized using polymerase chain reaction (PCR) with chemically modified primers (Table 3.1). For SMFS, 10,052 bp amplicons are end-functionalized separately at the two distal termini with biotin and digoxigenin (DIG) (Table 3.2). A Vivacon 2 column with 100,000 MWCO Hydrosart Membrane (Vivaproducts) is used for excess primer removal and buffer exchange (30 mM Tris, pH 8.0, 5 mM NaCl, 2 mM EDTA).

DNA synthesis for single molecule fluorescence microscopy. All DNA molecules are again synthesized using polymerase chain reaction (PCR) with chemically modified primers (Table 3.1). For SMFM, DNA comb polymers are synthesized as previously described (Cy5-labeled 951 bp branches grafted onto 40,051 bp backbones via strain-promoted azide-alkyne cycloaddition). PCR protocols for the generation of the combs (branches and backbone synthesized separately) are indicated in Table 3.3 (branches) and Table 3.4 (backbone). DNA comb polymers are purified using gel filtration chromatography. Further detailed information of purification and synthesis of DNA comb polymers is provided below.

Following PCR, branch (BR) products are purified with Qiagen QIAquick PCR Purification Kits, and backbone (BB) products are purified with pre-rinsed Vivacon 2 columns. DNA combs are synthesized via strain-promoted azide-alkyne cycloaddition (SPAAC). Specifically, equal masses of purified BR and BB are mixed (1 μg/each per 50 μL reaction, ~40 molar excess of BR) in a reaction buffer of 250 mM Tris (pH 8.0), 1.0 M NaCl, and 10 mM EDTA. The reaction mixture is incubated at 70 °C for ~100 hours. Excess BR products are removed from DNA combs via gel filtration chromatography.
Purification of DNA combs. A Tricorn 10/300 column was packed with Sephacryl S-1000 gel filtration media and installed on an AKTAFPLC system (GE Healthcare Life Sciences). After equilibrating the column with an aqueous buffer system (200 mM NaCl, 30 mM Tris, 2 mM EDTA, filtered using 0.22 μm Millipore Stericup), SPAAC products are flowed through the column at a volumetric flow rate of 0.05 – 0.1 mL/min. The first peak to elute is identified as purified DNA comb polymer and concentrated using a Vivacon 2 column.

Materials and reagents for DNA synthesis. Materials and reagents: Bacteriophage λ-DNA (New England Biolabs, Ipswich, MA), PCR Extender System (5 PRIME, Gaithersburg, MD), Taq DNA Polymerase with Thermopol Buffer (New England Biolabs, Ipswich, MA), 5-DBCO-dUTP (Jena Bioscience GmbH, Germany), Cy5-dUTP-PCR (Jena Biosci-ence GmbH, Germany), deoxynucleotide (dNTP) solution (New England Biolabs, Ipswich, MA), custom oligonucleotide primers (IDT DNA, Coralville, IA), QIAquick PCR Purification Kit (QIAGEN, Valencia, CA), Vivacon 2 with 100,000 MWCO Hydrosart Membrane (Vivaproducts, Littleton, MA).

3.3.2 Conjugation of anti-digoxigenin to paramagnetic beads.

Anti-digoxigenin (Anti-DIG) (polyclonal from sheep, Roche) is covalently conjugated to paramagnetic beads (2.8 μm diameter, Dynabeads M-270 carboxylate acid-modified at 30 mg/mL, Invitrogen) in order facilitate antibody-antigen reaction to the DIG functionalized DNA. We used the following protocol, which is modified from the manufacturer’s instructions:

1. Rinse beads by mixing 3 mg of carboxylic acid modified beads (100 μL) at 1:1 v/v ratio into 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0). (Note: to ensure an accurate concentration of beads from the stock bottle, always vortex the bottle of paramagnetic beads before drawing out the 100 uL necessary for reaction because the beads settle to the bottom of the bottle).
2. Mix the beads by rotation for thorough mixing and pull down the beads to the bottom of the tube with an external magnet. Remove the supernatant by careful pipetting out of the tube (after pulling down with the magnet, the beads are seen as a dark brown pellet at bottom of tube). Be careful to not remove any of this when removing supernatant.

3. Repeat steps 1 & 2 three times. Add 200 uL of 25 mM MES buffer and set bead solution on ice.

4. Dissolve 50 mg each of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and \(N\)-Hydroxysuccinimide (NHS) into 1 mL of 25 mM MES buffer and set on ice (here, the concentration of each component is 50 mg/mL).

5. Pull down the beads again, remove supernatant, and add 50 uL of the EDC and 50 uL of the NHS solution to the beads. Mix thoroughly by pipetting up and down, and immediately set the solution to mix by slow rotation in a 4\(^\circ\) fridge.

6. While the reaction from step 5 is proceeding, prepare 2.5 mg/mL anti-digoxigenin in MES buffer (200 ug of antibody into 80 uL of 25 mM MES) and keep on ice. After the EDC/NHS coupling reaction is complete the beads are activated to conjugate to the amine groups of the antibody.

7. Pull down the beads after the coupling reaction is complete and rinse/clean the beads with MES buffer 3x as in steps 1 and 2. Then add 60 uL of the antibody solution to the beads, immediately followed by 40 uL of 25 mM MES.

8. Mix the solution by pipetting up and down several times followed by rotation at 4 degrees for 90 minutes to ensure conjugation of the antibody to the beads.

9. When the above reaction is completed, pull down the beads, remove the supernatant and add 200 uL of a reaction quench buffer (50 mM Tris, 200 mM NaCl, 10% v/v glycerol).
Mix by rotation for 30 minutes to ensure the reaction has been quenched (from excess primary amine in the Tris buffering reagent).

10. After the reaction is quenched, pull down the beads and remove the supernatant. Next, wash the beads with the 200 μL of the washing/storage buffer (10 mM phosphate buffer, 200 mM NaCl, 0.2% Tween 20, 1 mM EDTA, 10% glycerol, 0.0005% sodium azide).

11. Repeat the washing step with 200 μL of washing buffer 4x, removing the supernatant after each wash. After the washing, remove the supernatant and add 1 mL of the storage buffer (same as washing buffer) for a final concentration of 3 mgs beads/mL.

12. For storage, split the bead solution into 10 x 100uL aliquots and store in 4° fridge.

13. When using beads in single molecule pulling experiments, if a different buffer if desired for a particular experiment (e.g. Tris instead of phosphate) then a bead aliquot can be pulled down, supernatant consisting of storage buffer removed, then washed with the new buffer once, and added to 100 uL of the new buffer to ensure the same concentration.

3.3.3 Sample preparation for magnetic tweezers experiment

DNA is end-functionalized to enable specific binding with a single paramagnetic bead at one terminus and specific chemical linkage to a glass coverslip surface at the other terminus. Permanent magnets are mounted approximately 4-5 cm directly above the microscope objective (at furthest distance) to exert vertical pulling forces on the bead, as shown in Figure 3.1. Anti-DIG coated paramagnetic beads (Dynabeads, 2.8 μm, Invitrogen) are prepared by conjugating anti-DIG antibodies (polyclonal from sheep, Roche) via carbodiimide activation and NHS-ester conjugation to magnetic bead surfaces (Section 3.3.2). Anti-DIG-coated beads are mixed with DNA at a 1.33:1 molar ratio (antibody:DNA) to minimize multiple DNA tethers per bead (reaction mixture is 0.33
pM beads mixed with 0.25 pM DNA, both ~40 uL of volume). The bead-DNA mixtures are rotationally mixed overnight at 4 °C.

A quartz microscope slide with two drilled holes and a glass coverslip are sonicated in ethanol and 1 M potassium hydroxide prior to assembly into a microfluidic flow cell. The slide and coverslip are attached together using double-sided tape, forming a 2.5 cm-long channel between the drilled holes with a tape thickness of ~ 150 um and channel width of .25 cm, leading to a flow cell volume of ~10 uL. Epoxy is used to seal the channel and polyethylene tubing is inserted into the holes, which enable continuous buffer exchange. Following assembly, the flow cell is successively incubated with BSA-biotin (0.1 mg/mL, Sigma-Aldrich, 10 minutes), Neutravidin (0.1 mg/mL, Pierce, 10 minutes), BSA (1 mg/mL, New England Biolabs, 10 minutes), and the aforementioned bead-DNA mixture (1 hour). Following the bead-DNA incubation, the flow cell is incubated with streptavidin coated polystyrene beads (Spherotech, 7 µm, 0.5% w/v) which are diluted ~1:200 before flowing into the cell. These beads are used as fiducial and remain stationary despite the application of magnet forces. In this way, these beads aid in internal drift correction by subtracting changes in the reference bead tracked position from the magnetic bead tracked positions.

Each incubation step is preceded by a wash with magnetic tweezers (MT) buffer (30 mM Tris, 50 mM NaCl, 2 mM EDTA) to minimize non-specific binding. The flow cell is rinsed with a blocking buffer (MT buffer supplemented with 0.1% Tween-20, 0.1% ribonucleic acid from Tortula yeast, and 1% glycerol) to minimize non-specific interactions between the bead and surface at the final incubation step and subsequent washes. Buffer exchange or sample incubation through the tubing is accomplished with a syringe pump (Harvard Apparatus). Each sample incubation or buffer exchange uses ~400 uL of sample to ensure the tubing and flow cell is
completed filled. The syringe pump is set to a withdrawal setting, pulling liquid from a tube containing sample or buffer, ensuring that fresh sample or buffer enters the flow cell at every step. Finally, the syringe pump is operated at a relatively slow withdrawal rate of 40 \( \mu \text{L/min} \) to ensure that the DNA-bead constructs are not disturbed by high forces due to the flowing liquid. Experiments are carried out in variable buffer conditions as noted below: MT buffer with no additives, YOYO-1 (200 nM), TX (4 mM), or YOYO-1 and TX (200 nM and 4 mM, respectively).

### 3.3.4 Magnetic tweezers apparatus for single molecule force spectroscopy

Two permanent magnets with vertical position control are mounted above an inverted microscope (Olympus IX-81) with a 100× objective (UAPON 100XOTIRF, NA 1.49) fitted on a vertical positioner piezostage (Mad City Labs). Samples are illuminated using a red LED (ThorLabs) and observed using an electron multiplying charge coupled device (EMCCD) camera (Andor iXon 897 Ultra). The vertical bead height can be accurately determined based on the linear dependence of the width of the diffraction ring pattern as a function of the height of the bead above the focal plane. The vertical position of each magnetic bead (tethered to a DNA molecule) is calibrated by holding the samples at high force (minimal fluctuations in position) and stepping the objective position to construct a lookup table (LUT) of diffraction patterns.\(^{36}\) The radial intensity pattern at each objective height can be later compared to diffraction ring patterns during image acquisition. By referencing the table of radial intensity patterns, the height can be accurately computed based on closest intensity profile. The LUT spans a distance of 10 µm in 100 nm increments; this range exceeds the length of fully extended DNA samples in this work and enables determination of vertical bead position within 10 nm. The open-source software QITracker is used to track bead center position over time with nanometer scale precision in the lateral displacements of the bead.\(^{38}\)
3.3.5 Sample preparation for single molecule fluorescence microscopy for polymer imaging

DNA (comb DNA or linear DNA template) is fluorescently labeled by incubation with YOYO-1 (Invitrogen) at a dye-to-base-pair ratio of 1:4 in an aqueous incubation buffer (30 mM Tris, pH 8.0, 5 mM NaCl, 2 mM EDTA) for 1-2 hours in the dark. A viscous imaging buffer (55 wt% sucrose in all experiments using the DNA-comb polymers and 57 wt% sucrose for all experiments involving λ-DNA imaging, 30 mM Tris, pH 8.0, 5 mM NaCl, 2 mM EDTA and vacuum filtered with Millipore Stericup, 0.22 μm) is mixed with an oxygen scavenging system and a reducing agent to minimize photobleaching, consisting of glucose (5 mg/mL), glucose oxidase (0.3 mg/mL), catalase (0.3 mg/mL), and β-mercaptoethanol (150 mM). TX-containing buffers are mixed with TX in excess of its solubility limit (4 mM) at 4 °C overnight, followed by filtering (Pall Life Sciences, 0.22 μm) to remove excess TX prior to mixing with labeled DNA. Labeled DNA and imaging buffers are rotationally mixed for 30 minutes prior to injection into a microfluidic device. A two-layer, microfluidic cross-slot device is fabricated using polydimethylsiloxane (PDMS) via soft lithography as described before. Pressure-driven flow is used to generate a planar extensional flow field near the center of the cross slot (Eqs. 1 and 2), where $v_x$ is velocity in the $x$ direction, $v_y$ is fluid velocity in the $y$ direction, and $\dot{\varepsilon}$ is the fluid strain rate.

\[ v_x = \dot{\varepsilon}x \]  
\[ v_y = \dot{\varepsilon}y \]  

Strain rates are measured via particle imaging velocimetry using 0.84 μm fluorescent beads (Spherotech) as tracer particles. Automated feedback control is implemented to enable hydrodynamic “trapping” of single molecules near the stagnation point, as shown in Figure 3.2.
3.3.6 Optical setup for single molecule fluorescence microscopy

Single DNA polymers were imaged by excitation with a 488 nm laser (50 mW SpectraPhysics Excelsior Laser) and/or a 637 nm laser (140 mW Coherent OBIS Laser). The 488 nm optical path includes absorptive neutral density filters (Thorlabs), a 488 nm long-pass dichroic mirror (ZT488rdc, Chroma), and a long-pass emission filter (BLP01-488R-25, Semrock). The intensity at the sample was ~0.1 kW/cm². The 637 nm optical path included an absorptive neutral density filter (ThorLabs), 650 nm long-pass dichroic mirror (FF650-Di01-25x36, Semrock), and long-pass emission filter (HQ665LP, Chroma). The intensity at the sample was ~0.6 kW/cm². For simultaneous YOYO-1/Cy5 imaging, the 488 nm and 637 nm optical paths were modified to include a dual-band dichroic mirror (FF500/646-Di01-25x36, Semrock) and a 630 nm dichroic beamsplitter (MS-630LDX-1826, Photometrics). The short wavelength channel was passed through a band pass emission filter (FF01-550/88, Semrock), and the long wavelength channel was passed through band-pass (HQ700/75m, Chroma) and long-pass (HQ665LP, Chroma) emission filters.

All SMFM experiments are carried out at 22.5 °C (η = 38.5 cP) on an inverted epifluorescence microscope (Olympus IX-71) with a 100× oil-immersion objective lens (NA 1.40) and 1.6× additional zoom lens. DNA comb polymers are imaged by simultaneous excitation with a 488 nm laser (50 mW, SpectraPhysics Excelsior Laser) and a 637 nm laser (140 mW, Coherent OBIS Laser). An EMCCD camera (Andor iXon Ultra 897) is fitted with a 630 nm dichroic beamsplitter (Photometrics DV2) for independent imaging of DNA branches and backbones. Relaxation and stretching dynamics of λ-DNA are imaged by excitation with a 488 nm laser and detection with an EMCCD camera.
3.4: Results and Discussion

3.4.1 Force extension curve for DNA

Using the magnetic tweezers apparatus, force-extension curves are constructed by measuring vertical bead positions (using the look-up table method\textsuperscript{36}) and lateral fluctuations (by tracking the bead center using the QItracker software\textsuperscript{38}) over a series of magnet positions. To determine the force exerted on the bead \( F \) at a given magnet position, use the expression for a Hookean spring (Eq. 3), where \( k \) is a stiffness constant for the magnets and \( x \) is DNA extension:

\[
F = k x
\]  

The stiffness constant \( k \) is determined using the equipartition theorem in one dimension (Eq. 4), where \( \langle \delta y^2 \rangle \) describes lateral fluctuations in bead position, \( k_b \) is the Boltzmann constant, and \( T \) is absolute temperature:

\[
k \langle \delta y^2 \rangle = k_b T
\]  

We determine the force on the bead using Eq. 5, which results by combining Eqs. 3 and 4:

\[
F = \frac{x k_b T}{\langle \delta y^2 \rangle}
\]  

To increase accuracy of the force calculation, the analysis is carried out in frequency space and converted back to real space using Fourier transforms of the resulting equations.\textsuperscript{39} Figure 3.3a shows sample traces of \( \langle \delta y^2 \rangle \) over time at constant magnet positions. When the magnet is far from the sample, low forces exerted on the beads result in large positional fluctuations. As the magnet approaches the sample, higher magnetic forces result in smaller bead fluctuations. A sample curve of DNA extension with respect to magnet position is shown in Figure 3.3b. Here, it can be observed that the polymer is at (nearly) full extension when the magnet is close to surface, and as the magnet is moved to positions further from the surface, the polymer extension tends to small values (zero extension) because the vertical component of magnetic force exerted on the polymer
is lessened. Data in Figure 3.3b are determined by averaging from a video recording of one bead acquired at 50 Hz over a length of 2000-4000 frames.

With the above methods, we can determine the physical properties of single polymers using the magnetic tweezing apparatus using the following method. First, we acquire a video at 50 Hz for 2000-4000 frames and tracking the center position of the bead over time with the magnet at constant height (and therefore constant force). Next, we determine the force for the given magnet position. We can likewise find the extension (stretch) of the molecule at each frame by comparing the radial intensity profile (pattern formed by diffraction rings) with the LUT. By performing this procedure over a range of magnet positions, we can calculate a force-extension curve of the polymer (Figure 3.4). Furthermore, physical parameters such as persistence length \((\ell_p)\) and contour length \((L_c)\) are further determined from the resulting force-extension curves using a non-linear least squares fit to the Marko-Siggia expression, also known as the wormlike chain (WLC) model (Eq. 6),\(^{40}\) which is a well-established force-extension relation for semi-flexible polymers such as DNA.\(^{22, 33, 41, 42}\)

\[
F = \frac{k_BT}{\ell_p} \left[ \frac{1}{4} \left( 1 - \frac{x}{L_c} \right)^{-2} - \frac{1}{4} + \frac{x}{L_c} \right]
\]

A fit of the data is also shown in Figure 3.4, with \(\ell_p\) and \(L_c\) determined from the non-linear least squares fit. To validate the assay, we construct force-extension curves for a large number of molecules (9 molecules) and construct histograms of the collected physical properties, \(\ell_p\) and \(L_c\). These data are shown in Figure 3.5 and we find \(\ell_p = 65 \pm 4\) nm and \(L_c = 2.37 \pm 0.03\) \(\mu\)m. These values show very consistent values with essentially normal distributions. These data are consistent with previously measured values for natural DNA, despite some minor differences in the persistence length that could arise due to the sensitivity of the non-linear least squares fitting method.
3.4.2 Stretching trajectories from SMFS

In a second experiment, the total change in polymer extension from high force (high extension) to very low force (low extension) is measured under a variety of solution conditions. Here, the magnet is initially positioned near the surface with the polymer starting in a fully extended high force state, and vertical bead position is measured as the magnet is moved at a constant speed away from the surface until the force has decayed to negligible values. We note in this type of experiment, we cannot construct force extension curves, as we do not acquire quantitative force information during the experiment. However, the aim of the experiment is to determine how solution additives can modify polymer extension, so numerical data on the force is not necessary.

Figure 3.6 shows single molecule trajectories (gray) and ensemble averages (black, \(N = 8\)) of the total extension change, \(\Delta x\). Specifically, we compare MT buffer with (a) no additives, (b) YOYO, (c) TX, and (d) YOYO and TX. Single molecule trajectories are acquired at 50 Hz and smoothed with a moving average of 30 frames. We observe noticeable increases in total change in extension when YOYO and/or TX are added to the buffer system. The total changes in extension across several different experimental solution conditions are summarized in Table 1, as well as known contour lengths and various comparisons between extension changes and contour lengths.

Our experiments show that the extension of natural DNA increases by 37% in the presence of YOYO-1, which is consistent with previous findings.\(^{22,33}\) DNA extension also increases by 20% in the presence of TX. Importantly, DNA extension increases by only 26% in the presence of both YOYO-1 and TX, which is 8% less than extension caused by YOYO-1 alone.

Based on these results, we conjecture that TX acts as an intercalating agent based on the measured increase in DNA extension. Our observations reflect those in literature for other intercalating agents.\(^{32,43,44}\) Furthermore, many similarities exist between the chemical structures
of TX and known intercalating agents: planar, polycyclic aromatic systems with size on the order of a base pair.\textsuperscript{45} Our results also suggest competing effects between YOYO-1 and TX on DNA extension, which could be competitive intercalation or a depletion interaction, such as those measured between PEG and DNA intercalators.\textsuperscript{44} In either case, our results directly show that it will be essential to consider corrections for DNA contour length for quantitative experiments involving DNA stretching dynamics for using TX in SMFM.

3.4.3 Single molecule visualization of linear and branched DNA with TX

We next turn to a separate experiment and use SMFM to measure the fluorescence emission from linear and branched DNA with and without TX in solution. This experiment aims to assess the benefit of using TX to increase the photostability of fluorescently labeled DNA. We observe the expected benefits of TX-containing buffers on DNA, including enhanced photostability, shown qualitatively in Figure 3.7, along with an overall reduction of photocleavage events. Figures 3.8a and 3.8b demonstrate drastic quantitative improvements to the photostability of Cy5-labeled DNA combs in the presence of TX. Figure 3.8b suggests negligible impacts of TX on the brightness of YOYO-labeled DNA, despite the potential competition between YOYO-1 dyes and TX observed in MT experiments. These results suggest that TX-containing buffers will be advantageous in future studies of DNA with alternate dye systems to study unexplored conformational dynamics\textsuperscript{46} and complex polymer topologies.\textsuperscript{30}

3.4.4 Direct visualization of DNA stretching dynamics in the presence of TX

It is known that linear polymers undergo an abrupt coil-stretch transition in an extensional flow field. In the final experiment we performed using SMFM, we characterize the stretching dynamics of λ-DNA in the presence of YOYO+TX compared with just YOYO. Based on the fact that any changes made to the physical properties of DNA, such as a change in the contour length, will cause
a change in the relaxation time of the molecule, and the relaxation time is important for all quantitative comparisons of polymer stretching in flow, it is imperative to understand how TX affects the contour length and relaxation time. We compare the extension as a function of Weissenberg number, $Wi = \dot{\varepsilon} \tau_1$, where $\dot{\varepsilon}$ is the fluid strain rate and $\tau_1$ is the longest polymer relaxation time, with these experimental values measured by SMFM.

To measure $\tau_1$, $\lambda$-DNA molecules are stretched to at least 50% of maximum extension in flow, followed by direct observation of chain relaxation upon flow cessation. We determined $\tau_1$ by fitting the average fractional extension ($x/L_c$) over time ($t$) to a single exponential decay function (Eq. 7), where $x$ is the measured 2D projection of polymer extension and $c_1$ and $c_2$ are fitting constants.

$$\frac{x}{L_c}^2 = c_1 \exp(-t/\tau_1) + c_2$$  \hspace{1cm} (7)

Based on our single molecule force spectroscopy measurements, we define a rescaled contour length for each condition: $L_c^{YOYO} = 21.1 \mu m^{24}$ and $L_c^{YOYO+Tx} = 19.4 \mu m$ (based on Table 3.5). The fit is performed over the linear force region ($x/L < 0.30$). We observe differences in DNA relaxation times in the absence ($\tau_1 = 4.1 \pm 0.3 \ s$, $N = 15$) and presence ($\tau_1 = 3.5 \pm 0.2 \ s$, $N = 25$) of TX. After scaling for viscosity and contour length (Eq. 8, scaling exponent of 1.64), both measurements are consistent with reported relaxation times of $\lambda$-DNA at 1 cP ($\tau_1^{YOYO} = 0.068 \pm 0.005 \ s$, $\tau_1^{YOYO+Tx} = 0.066 \pm 0.004 \ s$, $\tau_{ref}^{24} = 0.058 \pm 0.068 \ s$).

$$\tau_{ref} = \tau \left( \frac{\eta_{ref}}{\eta} \right) \left( \frac{N_{ref}}{N} \right)^{1.64}$$  \hspace{1cm} (8)

Where the reference is based on unstained $\lambda$-DNA in water ($\tau_{ref} = 1 s$, $\eta_{ref} = 1 cp$, $N_{ref} = 16 \mu m$, the length of unstained $\lambda$-DNA).

**Figure 3.9a** shows the steady-state extension behavior of YOYO-1 labeled $\lambda$-DNA in the absence of TX (red circles), which agrees with previous measurements by Perkins, *et al.* (black
Addition of TX in the imaging buffer (blue triangles) results in a clear decrease in DNA extension compared to the TX-free buffer. To account for this change in extension, we normalize the data for contour length, including the change in contour length found with SMFS. Figure 3.9b indicates that plotting fractional extension ($x/L$) as a function of $Wi$ results in consistent steady-state extension behavior across all experiments.

3.5: Conclusions

In this work, we utilize two independent single molecule techniques to study the impact of TX on the physical properties of DNA. Our findings from SMFS show that the contour length of DNA is increased in the presence of TX, which suggests intercalation of TX with DNA. Our results further show competitive interactions between TX and fluorescent nucleic acid dye YOYO-1. We apply these findings to a single molecule flow experiment, in which we image YOYO-labeled DNA in buffers with and without TX. We demonstrate the effectiveness of TX as a photoprotectant for DNA comb polymers with Cy5-labeled branches. We also report a change in steady-state extension consistent with measurements from SMFS.

TX has been repeatedly shown to be a tremendously useful additive for single molecule fluorescence studies. Increasing the photostability is particularly useful in the case of studying the dynamics of single polymer molecules, where it is essential to observe a given molecule for a long enough time for it to sample its entire space of the relevant observable variable. TX has been shown to change the biological properties of cell membranes by changing the elasticity of the lipid bilayer$^{16}$, but its effect on other biomolecules or systems in general has not been well characterized.

In this chapter, we show how TX in solution increases the contour length of bare DNA and decreases the total contour length of YOYO-1 dyed DNA using single molecule force spectroscopy and single molecule fluorescence microscopy. Based on these results, we conjecture that TX
intercalates DNA and has a competitive intercalation effect with YOYO. Although TX changes the contour length of DNA, which could complicate analysis, consideration of a TX-dependent length correction results in single polymer relaxation and steady-state extension behavior consistent with those reported in prior single polymer studies. While we recommend the use of TX for enhanced photoprotection in SMFM, we also recommend careful consideration of molecular-level effects of TX on biological, chemical, and physical systems.
3.6: Tables and Figures

<table>
<thead>
<tr>
<th>Name</th>
<th>Target length</th>
<th>Nucleic acid sequence (5’ → 3’)</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-F</td>
<td>10,052 bp</td>
<td>CTGATGAGTTGTCCGTACGTCAACTGGCGTAATC</td>
<td>5’-biotin</td>
</tr>
<tr>
<td>MT-R</td>
<td>10,052 bp</td>
<td>ATACGCTGTATTCAACACCGTCAGGAACACG</td>
<td>5’-DIG</td>
</tr>
<tr>
<td>BR-F</td>
<td>951 bp</td>
<td>GACAGCGTACAGCCCGTTCA</td>
<td>5’-azide</td>
</tr>
<tr>
<td>BR-R</td>
<td>951 bp</td>
<td>TCGCGTCATTCATCCCTCTCC</td>
<td>None</td>
</tr>
<tr>
<td>BB-F</td>
<td>40,052 bp</td>
<td>CTGATGAGTTGTCCGTACGTCAACTGGCGTAATC</td>
<td>None</td>
</tr>
<tr>
<td>BB-R</td>
<td>40,052 bp</td>
<td>TAATGCAAAACTACGCGCCCTCGTATCACATGG</td>
<td>None</td>
</tr>
</tbody>
</table>

MT = magnetic tweezers, BR = branches, BB = backbones, DIG = digoxygenin

Table 3.1: Primer sequences for PCR synthesis reactions. DNA was synthesized using the indicated primer sequences. The ‘Name’ column indicates whether the magnetic tweezer setup was used (MT), and when SMFM was used, the abbreviation indicates if it was a backbone strand (BB) or a branch strand (BR). F indicated a forward primer, and R indicates the reverse primer. All custom oligonucleotides were purchased from IDT DNA (Coralville, IA).
Table 3.2: PCR amplification for Magnetic Tweezers DNA. PCR amplification was used to generate DNA strands for use in the magnetic tweezers assays, as indicated in the protocol reported in this table. This protocol is specifically used to generate DNA strands of 951 base-pair length 10,052. Following PCR, products are purified using Vivacon 2 columns.
Table 3.3: PCR amplification for SMFM of branches. PCR amplification was used to generate DNA strands for use in single molecule fluorescence microscopy for DNA observation, as indicated in the protocol reported in this table. This protocol is specifically used to generate DNA branches of 951 base-pair length (BR). Cy5 is incorporated for visualization and confirmation of branch attachment.
<table>
<thead>
<tr>
<th>BB Reaction composition</th>
<th>PCR step</th>
<th>Temp. / Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 μL per reaction</td>
<td>Initial denaturation</td>
<td>93 °C / 3 min</td>
</tr>
<tr>
<td>1X Tuning buffer with Mg²⁺</td>
<td>*Denaturation</td>
<td>93 °C / 15 sec</td>
</tr>
<tr>
<td>0.4 μL PCR Extender Polymerase Mix</td>
<td>*Anneal</td>
<td>62 °C / 30 sec</td>
</tr>
<tr>
<td>400 nM BB-F primer</td>
<td>*Extension</td>
<td>68 °C / 21 min</td>
</tr>
<tr>
<td>400 nM BB-R primer</td>
<td># cycles constant</td>
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<tr>
<td>20 ng λ-DNA</td>
<td># cycles ramping</td>
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<td>Increase per cycle</td>
<td>+ 20 sec</td>
</tr>
<tr>
<td>2.5 μM dUTP-DBCO</td>
<td></td>
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</tr>
</tbody>
</table>

Table 3.4: PCR amplification for SMFM of backbone. PCR amplification was used to generate DNA strands for use in single molecule fluorescence microscopy for DNA observation, as indicated in the protocol reported in this table. This protocol is specifically for the generation of the backbone of DNA comb polymers for a total length of 40,052 basepairs (BB).
Table 3.5: Summary of changes in polymer contour lengths from magnetic tweezer experiments. The change in contour length of DNA (10,052 bp) was obtained by analyzing vertical position data from moving magnet at constant speed from close to the surface (full extension) to far from the surface (zero extension).
Figure 3.1: Schematic of magnetic tweezers apparatus. Permanent magnets are mounted above a microscope stage exert a vertical magnetic force on DNA-paramagnetic bead conjugates specifically bound to a glass coverslip surface through biotin-avidin affinity labeling (not to scale). Lateral bead fluctuations occur due to thermal motion in solution.
Figure 3.2: Schematic of hydrodynamic trapping flow cell. Two inlet flows on opposite sides with corresponding outlet flows generate an extensional flow near the center of the flow cell, with the velocity near the center described by Eqs. 1 and 2. A stagnation point of zero velocity results at one point, with its position determined by the values of inlet and outlet flow rates. This can be used to automatically trap fluorescently labeled polymers at the stagnation point.\(^\text{48}\) (Here, the polymer depicted is a branched DNA molecule, with the backbone in green, labeled with YOYO-1 dye, and the branches in red, labeled with Cy5).
Figure 3.3: Pulling on single DNA molecules using the magnetic tweezers apparatus. (a) Lateral (y-direction) tracked position of the bead is shown (with graphs offset along y-axis for ease of viewing). As the force exerted on the bead is changed from low to high, a large variation in position changes to very small variation in position. Each individual trajectory maintains a constant magnet height over the length of recording, and the magnet is moved controlled distances to modulate the force exerted. (b) As the magnet distance above the surface increases, the DNA extension (z-position) decreases from fully extended to zero, since the force exerted is decreasing to zero. Error bars are standard deviation of these z positions of the bead over the full length of the recording. Data are taken at 50 Hz.
Figure 3.4: Force-extension curve for DNA. Physical properties of a DNA polymer molecule measured with magnetic tweezers shown through a force-extension curve (the force required to extend the polymer by different amounts). Black circles are experimental data points, blue line is the resulting fit to the WLC model by a non-linear least squares fitting routine (implemented in Matlab). The persistence length and contour length are consistent with expectations.
Figure 3.5: Histograms of polymer physical properties. (a) Persistence length and (b) contour length measured for 8 and 9 different DNA molecules, respectively, by non-linear least squares fits of force-extension data to the WLC expression. Histograms show normal distributions about the mean, validating the robustness of the magnetic tweezers apparatus for measuring polymer properties.
Figure 3.6: Single molecule stretching trajectories of DNA in various buffer conditions:

Extension of DNA molecules determined as magnet exerting the force on the paramagnetic bead is moved towards the surface at a constant velocity, going from negligible force and extension, to high force and close to full extension. Gray lines indicate each individual molecule’s trajectory, with the black line showing the ensemble average (total of 8 molecules). The following buffer conditions were tested. (a) MT buffer with no solution additives, (b) YOYO-1 (200 nM), (c) saturated TX (~4 mM), (d) YOYO-1 (200 nM) and TX (4 mM).
Figure 3.7: Direct single molecule visualization of DNA comb polymers in the presence and absence of TX. Stretched DNA combs, scale bars = 2.0 μm. Left: overlay of YOYO-labeled DNA (green) and Cy5-labeled branches (red). Intensity is seen to decrease as time progresses with continuous laser illumination, showing the process of photobleaching. The decay of intensity is slowed by the addition of TX.
Figure 3.8: Quantitative characterization of photostabilizing effects of TX on DNA combs.

(a) Intensity of Cy5-labeled branches in the absence and presence of TX. (b) Intensity of YOYO-labeled DNA in the absence and presence of TX.
Figure 3.9: Steady state extension of λ-DNA in planar extensional flow in presence and absence of TX. (a) Steady-state extension of λ-DNA labeled with YOYO-1 as a function of $Wi$ in the absence of Trolox (red circles) and presence of Trolox (blue triangles). Data from this experiment are compared to previous results of Perkins, et al. (black squares).\(^{24}\) (b) Steady-state fractional extension ($x/L$) as a function of $Wi$ in the absence ($L_{YOYO} = 21.1 \, \mu m$) and presence of Trolox ($L_{YOYO+Tx} = 19.4 \, \mu m$). In part (b), the y-axis is scaled by the different contour lengths between the two conditions.
3.7: References


CHAPTER 4: CONCLUSIONS AND FUTURE WORK

4.1: Conclusions and Future Work

Single molecule techniques represent a growing paradigm in biophysics, materials science, soft matter and an increasing number of fields, where a fundamental description of molecular interactions and dynamics are becoming an integral part of a mechanistic understanding of the fields. The overarching goal in this thesis is to expand the use of single molecule techniques in two main applications, with a common theme of implementing the novel photostabilizing group Trolox as a way of increasing the photostability of fluorescent dyes and understanding the effects it can produce.

In Chapter 2, we create a new class of probes, fluorescent dendrimer nanoconjugates (FDNs) with specific enhancements designed to increase their utility for single molecule fluorescence imaging. The FDN probes were contrasted by employing a multi-dye system conjugated on a dendritic scaffold, allowing for attachment of dyes, specific and orthogonal linkers, as well as dye photoprotective units in later designs, termed ‘self-healing’ FDNs. One of the primary problems associated with the FDN probes at first was a low stability of probe intensity over time. This is a common problem even in many single dyes systems, and in the multi-dye dendrimers, we observed unstructured, widely variable single dye intensity trajectories which would limit the usefulness of our probes for many applications, where stable, sustained fluorescence in necessary, such as single particle tracking, or single molecule FRET.

Due to this drawback in probe performance, we designed ‘self-healing’ FDNs, using a strategy of conjugating a photostabilizing group, Trolox, directly onto the scaffold, either adjacent to or directly conjugated to the dyes. We proceeded to image these conjugates, immobilized on a microfluidic flow cell surface, and found improvements in photophysical parameters such as
photon count and time before irreversible photobleaching. We additionally used ‘self-healing’ FDNs in proof-of-principle biological imaging experiments and confirmed identical increases in the photostability when compared to the in vitro imaging experiments. Trolox is commonly added to imaging buffer solutions to improve photophysical properties of the dyes, but it suffers from poor water solubility and has been shown to impair biological function, so avoiding adding it to solution provides by conjugation of Trolox to the fluorophore offers distinct advantages for biological imaging. Specifically, the one biological function that was shown to be modified was in the lipid bilayer comprising the cell membrane, specifically in measuring the free energy change between monomer and dimer formation of a particular ion conducting channel through the membrane. However, it was unknown if other biological molecules had impaired function when Trolox was present in solution.

In Chapter 3, we address an important question of whether Trolox present in solution can have additional effects on the function of biomolecules, specifically DNA. To address the question, we used two complementary techniques. Magnetic tweezers, an experiment for single molecule force spectroscopy that uses magnetic fields and external magnets to pull on single polymer molecules, along with using single molecule fluorescence microscopy. We found that the contour length, essentially the full length of the molecule while uncoiled, is increased by having bare DNA in solution with TX and decreases when DNA is incubated with TX along with the fluorescent dye YOYO-1, which is the dye present for single molecule observation of the molecule. Additionally, YOYO-1 alone is known (and is shown in our data) to increase the contour length to an even greater degree than TX alone does.

This led us to conjecture that TX intercalates in the grooves of DNA, as the mechanism for the increase in contour length we observed. Additionally, it is known that YOYO-1 functions as
an intercalating dye as well, and thus the decrease in contour length when both molecules are present compared with just YOYO-1 is seen as competitive intercalation effect. Since TX alone does not cause as much of an increase in contour length with intercalation as does YOYO-1 alone, when both are present, TX outcompetes with YOYO-1 for some fraction of spots on the DNA molecule, leading to an overall decrease in the length.

Trolox has been widely reported and shown to be a tremendously useful molecule for enhancing the photophysical properties of single fluorescent dyes. In this thesis, we show how it improves photostability in a newly designed class of probes and increases the ease of single molecule microscopy for both biological imaging applications and for observation of single polymer dynamics.

The potential implications of this research are wide ranging. By developing a new class of extremely stable and bright fluorescent dyes in Chapter 2, there should be new types of biological problems that can be studied and addressed with single molecule fluorescence microscopy. Particularly in applications that benefit from very large increases in the brightness, such as super-resolution microscopy due to a better resolution with a higher photon count, or single particle tracking in with a very noisy background, such as in a cell or other environments with a large amount of background fluorescence, a very bright probe would be advantageous, along with stabilized intensity trajectories, which result from the self-healing aspect of the probes, for tracking applications.

Likewise, this research will help advance any fields that use DNA imaging with fluorescence. For instance, DNA is widely used as a model polymer system and observing it with fluorescence microscopy allows extrapolation to polymer dynamics and properties. Including the photostabilizing agent, Trolox, in solution with the DNA molecules is advantageous for imaging
purposes, but any changes or effects it may have on the properties of DNA must be understood before it can be used. Our work indeed finds that the contour length of DNA is modified by having Trolox in solution with DNA, and this must be accounted for in analyzing the results of an experiment or in applying scaling relations that depend on the contour length, as detailed in Chapter 3.

Some future experiments are inspired by the work presented in this thesis. In Chapter 2, we created self-healing dyes using the redox active photostabilizing molecule Trolox, which has been widely used as a solution based additive for increasing the photostability of fluorescent dyes. However, there are other photostabilizing agents that operate based on a similar mechanism, two examples of which are cyclooctotraene (COT) and nitrobenzyl alcohol (NBA). In particular, it has been noted that these photostabilizing agents can in some circumstances operate most effectively when combined rather than used separately.

An interesting experiment considering the previously stated fact would be to create a new class of self-healing FDNs with all three of these redox active photostabilizing molecules attached onto the molecular scaffold. Since they have been shown to act in a way such that they can supplement each other’s effectiveness in increasing photostability, we might expect very stable conjugates for single molecule fluorescence microscopy. This would additionally lead to a wide variety of different types of probes when we consider that they could all be attached on the dendrimer using the ‘random addition’ strategy, or there are several different ways they could be attached using the ‘controlled addition’ strategy. This would be particularly interesting if we developed a synthetic procedure for attaching multiple photostabilizing agents onto one Cy5 molecule, instead of being limited to a one to one ratio.
In Chapter 3, it was noted that the first experimental evidence of Trolox modifying biological function was in a study where the properties of the lipid bilayer comprising the cell membrane are changed when Trolox is in solution around the membrane. In fact, in this study, they found that including all three commonly used redox active photostabilizing agents: TX, COT and NBA had effects on the cell membrane. Interestingly, it was noted that TX and NBA increased the value of the bilayer property they were measured in the study (free energy change between monomer and dimer formation of ion conducting channels through the membrane), whereas COT decreased this value. Then, combining all three of these agents, they found that the total change in bilayer property was less, or closer to native function with all three agents present than with any single one of them present. It would thus be very interesting to measure how the contour length of DNA is affected by combinations of the photostabilizing agents TX, COT and NBA.
4.2: References
